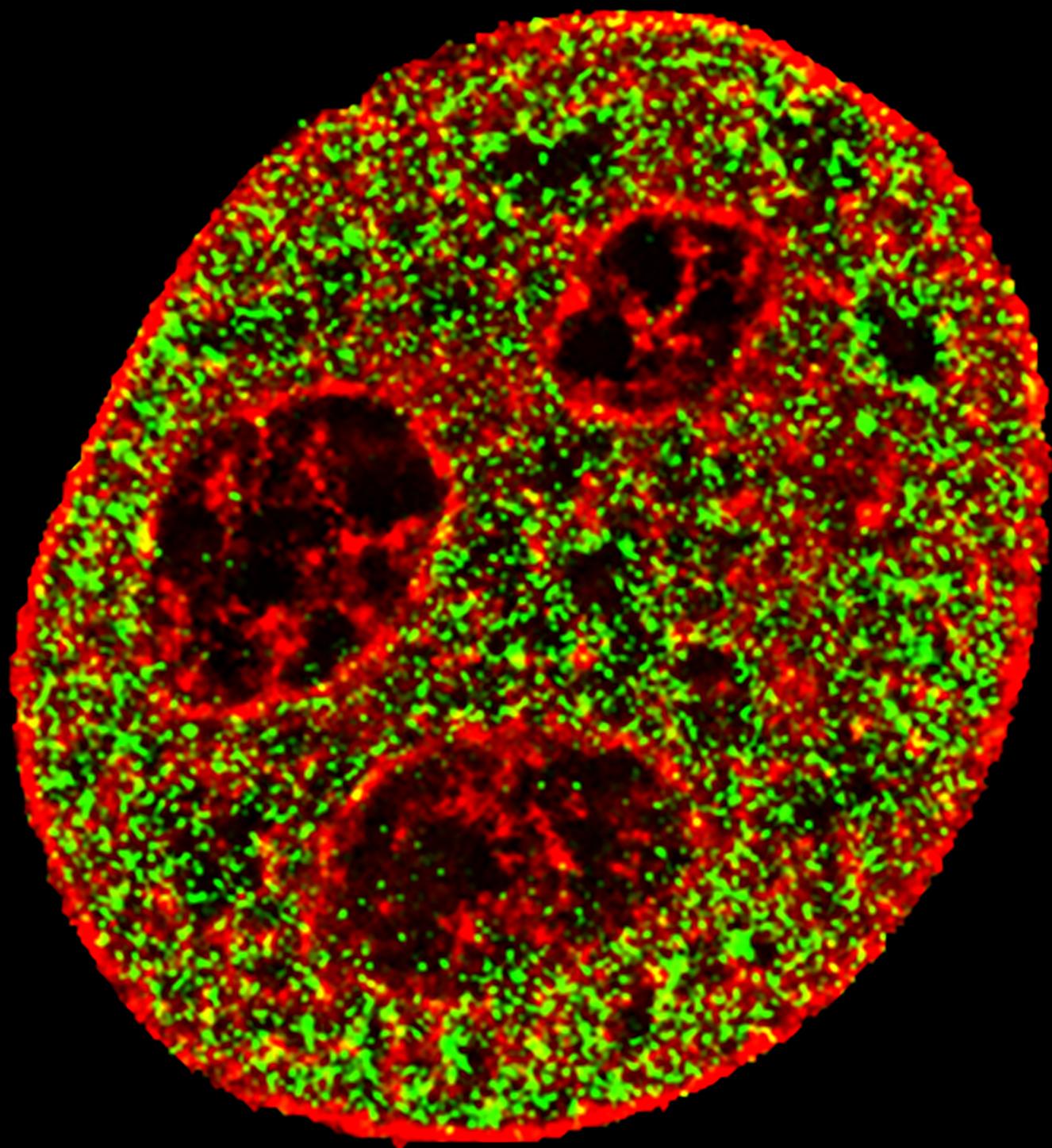


Reprint Compendium

# Bio-protocol Selections | 2020

## Cell Imaging



# Free access to more than 4000 high-quality protocols

- Contributed by 10,000+ scientists
- Each validated in at least one primary publication
- >91% reproducibility (2018 survey of Bio-protocol users)
- ~1000 videos of key procedural steps

## **Foreword**

We are pleased to launch Bio-protocol first series of reprint collections, which consist of most widely used protocols published in 2018 and 2019, and reprint them as “Protocol Selections” highlighting a given research area or application. In this series, it is Protocol Selections focusing on cancer research.

Established in 2011 by a group of Stanford scientists, Bio-protocol’s mission is to improve research reproducibility and usability through the publication of high quality step-by-step peer-reviewed life science protocols. One primary method for Bio-protocol to publish this content is to invite contributions from authors that have published methods in brief that are used in results-oriented literature (called “Original research article” in the Protocol Selections) but not described in sufficient detail for others to replicate. Our survey carried out in 2018 showed that over 91% of users (2166 users) who tried their downloaded bio-protocols were able to successfully reproduce the experiment. This users’ feedback indicates that indeed most of bio-protocols (if not all) are highly reproducible.

In this reprint collection, we have selected 28 of the most-used cell imaging protocols based on published dates (published in last 2 years) as well as some metrics such as view number, download number and citations. We have to admit that the measurement of the “most-used” protocols is not absolutely accurate, in particular, given the fact that it would take time to cite a relatively new published protocol. That said, we believe this Protocol Selections well represents a collection of high-quality protocols contributed by outstanding researchers in the community.

Hopefully, you will find this collection intriguing and visit [www.bio-protocol.org](http://www.bio-protocol.org) to check out the entire collection of protocols. Please feel free to email us ([eb@bio-protocol.org](mailto:eb@bio-protocol.org)) your feedback. Also, look forward to your contribution of protocols to Bio-protocol in the future.

The Bio-protocol Editorial Team

<i>Original research article:</i> <i>Nat Plants</i> 3: 17082	<b>Electron Tomography to Study the Three-dimensional Structure of Plasmodesmata in Plant Tissues—from High Pressure Freezing Preparation to Ultrathin Section Collection</b>	<b>9</b>	<i>Original research article:</i> <i>eLife</i> 5:e19048	<b>Real-time Analysis of Auxin Response, Cell Wall pH and Elongation in <i>Arabidopsis thaliana</i> Hypocotyls</b>	<b>34</b>
	William J. Nicolas, Emmanuelle Bayer and Lysiane Brocard		Lanxin Li, S. F. abriel Krens, Matyáš Fendrych and Jiří Friml		
<i>Original research article:</i> <i>Sci Rep.</i> 7(1):5061	<b>Fluorescent Measurement of Synaptic Activity Using FM Dyes in Dissociated Hippocampal Cultured Neurons</b>	<b>44</b>	<i>Original research article:</i> <i>EMBO Mol Med.</i> 8(5):569-585.	<b>Measuring Mitochondrial ROS in Mammalian Cells with a Genetically Encoded Protein Sensor</b>	<b>62</b>
	Roman M. Lazarenko, Claire E. DelBove and Qi Zhang		Xin Zhang, Christine Silvia ibhardt, Sabrina Cappello, Katharina Maria Zimmermann, Adina Vultur and Ivan Bogeski		
<i>Original research article:</i> <i>eLife</i> 6:e27451	<b>Characterising Maturation of GFP and mCherry of Genomically Integrated Fusions in <i>Saccharomyces cerevisiae</i></b>	<b>73</b>	<i>Original research article:</i> <i>RNA</i> 23(10): 1582-1591.	<b>Terminal Deoxynucleotidyl Transferase Mediated Production of Labeled Probes for Single-molecule FISH or RNA Capture</b>	<b>83</b>
	Sviatlana Shashkova, Adam JM Wollman, Stefan Hohmann and Mark C Leake		Imre aspar, Frank Wippich and Anne Ephrussi		
<i>Original research article:</i> <i>Immunity</i> . 46(4): 609-620.	<b>Imaging Cytokine Concentration Fields Using PlaneView Imaging Devices</b>	<b>103</b>	<i>Original research article:</i> <i>J Neurosci.</i> 37(20): 5099-5110.	<b>Intracellular and Mitochondrial Reactive Oxygen Species Measurement in Primary Cultured Neurons</b>	<b>115</b>
	Alon Oyler-Yaniv and Oleg Krichevsky		Seung Hyun Baek, Yoonsuk Cho, Jeongmi Lee, Bo Youn Choi, Yuri Choi, Jin Su Park, Harkkyun Kim, Jaehoon Sul, Eunae Kim, Jae Hyung Park and Dong-yu Jo		

*Original research article:* *Immunity.* 47(3):498-509.

- 130** **Quantification of Extracellular Double-stranded RNA Uptake and Subcellular Localization Using Flow Cytometry and Confocal Microscopy**  
Tan A Nguyen, Lachlan Whitehead and Ken C Pang

*Original research article:* *eLife* 7:e32579  
**Detection and Differentiation of Multiple Viral RNAs Using Branched DNA FISH Coupled to Confocal Microscopy and Flow Cytometry**

- 149** Nicholas van Buuren and Karla Kirkegaard

*Original research article:* *Nature.* 551(7682):629-33.  
**Staining the Germline in Live *Caenorhabditis elegans*: Overcoming Challenges by Applying a Fluorescent-dye Feeding Strategy**  
K. Adam Bohnert

*Original research article:* *eLife* 7:e29312  
**Quantification of Mouse Hematopoietic Progenitors' Formation Using Time-lapse Microscopy and Image Analysis**

- 201** Isabelle Bergiers, Christian Tischer, Özge Vargel Bölükbaşı and Christophe Lancrin

*Original research article:* *Curr Biol.* 26(3): 362-370.  
**Quantification of Starch in Guard Cells of *Arabidopsis thaliana***  
Sabrina Flütsch, Luca Distefano and Diana Santelia

*Original research article:* *Proc Natl Acad Sci.* 114(46):9873-82  
**Single-molecule Fluorescence in situ Hybridization (smFISH) for RNA Detection in Adherent Animal Cells**  
Al Haimovich and Jeffrey E. Ernst

*Original research article:* *Nucleic Acids Res.* 46(2): 748-764  
**Microirradiation for Precise, Double-strand Break Induction *in vivo* in *Caenorhabditis elegans***  
Kailey E. Harrell, Emily Koury and Sarit Smolikove

*Original research article:* *Cell Rep.* 24(4): 873-82  
**Imaging Higher-order Chromatin Structures in Single Cells Using Stochastic Optical Reconstruction Microscopy**  
Jianquan Xu and Yang Liu

*Original research article:* *J Virol.* 92(16): e00477-18

**On-demand Labeling of SNAP-tagged  
Viral Protein for Pulse-Chase Imaging,  
Quench-Pulse-Chase Imaging, and  
Nanoscopy-based Inspection of Cell**

**233**

**Lysates**

Roland Remenyi, Raymond Li and  
Mark Harris

*Original research article:* *eLife* 7:e34843

**Straight Channel Microfluidic Chips for  
the Study of Platelet Adhesion under  
Flow**

**266**

Alexander Dupuy, Lining Arnold Ju and  
Freda H Passam

*Original research article:* *Dev Cell.* 43(3): 290-304

**Visualization of Plant Cell Wall**

**297**

**Epitopes Using Immunogold Labeling  
for Electron Microscopy**

Mateusz Majda

*Original research article:* *eLife* 7:e36316

**Quantitative Plasmodesmata**

**Permeability Assay for Pavement Cells of  
*Arabidopsis* Leaves**

**316**

Min Diao, Qiannan Wang and Shanjin  
Huang

*Original research article:* *eLife* 7:e37812

**Simultaneous Fluorescent Recordings  
of Extracellular ATP and Intracellular  
Calcium in Mammalian Cells**

**324**

Nicholas Mikolajewicz and Svetlana V  
Komarova

*Original research article:* *PLoS Pathog.* 14(9): e1007279

**Hypochlorous Acid Staining with R19-S  
in the *Drosophila* Intestine upon  
Ingestion of Opportunistic Bacteria**

**335**

Salma Hachfi, Olivia Benguettat and Armel  
allet

*J Biol Chem.* 293(50):

*Original research article:* 19330-19343

**Lipid-exchange Rate Assay for Lipid  
Droplet Fusion in Live Cells**

**345**

Jia Wang, Boon Tin Chua, Peng Li and  
Feng-Jung Chen

*Original research article:* *Nat Immunol.* 20(2): 163-172

**Fibroblast Gap-closure**

**Assay-Microscopy-based *in vitro***

**Assay Measuring the Migration of**

**369**

**Murine Fibroblasts**

Agnieszka P. Looney and Mallar Bhattacharya

*Original research article:* *Nat Commun.* 2016(7): 12742.

**Conjugation of Fab' Fragments with**

**Fluorescent Dyes for Single-molecule**

**Tracking on Live Cells**

**374**

I-Ting Teng, Xiangning Bu, and Inhee Chung

*Original research article:* *Nat Immunol.* 10(1):1178

**Time-lapse Imaging of Alveologenesis  
in Mouse Precision-cut Lung Slices**

**389**

Khondoker M. Akram, Laura L. Yates,  
Róisín Mongey, Stephen Rothery, David  
C. A. aboriau, Jeremy Sanderson,  
Matthew Hind, Mark riffiths and  
Charlotte H. Dean

*Original research article:* *Proc Natl Acad Sci.* 115(51):12961-6

**Imaging VIPER-labeled Cellular Proteins  
by Correlative Light and Electron  
Microscopy**

**412**

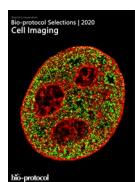
Julia K. Doh, Young Hwan Chang, Caroline  
A. Enns, Claudia S. López and Kimberly E.  
Beatty

*Original research article:* *eLife.* 8:e45239

**441**

**Opto-magnetic Selection and Isolation  
of Single Cells**

Loïc Binan, Joannie Roy and Santiago  
Costantino



**On the Cover:**

Image from protocol "Imaging Higher-order Chromatin Structures in Single Cells Using Stochastic Optical Reconstruction Microscopy"

Authors

Australia		United Kingdom	
Alexander Dupuy	Adina Vultur	Jeremy Sanderson	Harwell Campus
Freda H Passam	Christine Silvia Gibhardt	Charlotte H. Dean	
Lining Arnold Ju	Ivan Bogeski	David C. A. Gaboriau	
Ken C Pang	Sabrina Cappello	Laura L.Yates	Imperial College London
Lachlan Whitehead	Xin Zhang	Mark Griffiths	
Tan A Nguyen		Matthew Hind	
Jiří Friml		Róisín Mongey	
Lanxin Li		Stephen Rothery	
Matyáš Fendrych		Khondoker M. Akram	University of Sheffield
S. F. Gabriel Krens		Adam JM Wollman	
Joannie Roy	Christophe Lancrin	Mark C Leake	University of York
Loïc Binan	Isabelle Bergiers	Sviatlana Shashkova	
Santiago Costantino	Özge Vargel Bölkbaşı	Mark Harris	University of Leeds
Nicholas Mikolajewicz		Raymond Li	
Svetlana V. Komarova			
Austria		Italy	
Jiří Friml	Alon Oyler-Yaniv	Christophe Lancrin	
Lanxin Li	Oleg Krichevsky	Isabelle Bergiers	Epigenetics and Neurobiology Unit Monterotondo
Matyáš Fendrych		Özge Vargel Bölkbaşı	
S. F. Gabriel Krens			
Canada		Philippines	
Boon Tin Chua	Roland Remenyi	The Clinical and Translational Research Institute	
Feng-Jung Chen			
China		Republic of Korea	
Jia Wang	Bo Youn Choi	Bo Youn Choi	
Min Diao	Dong-Gyu Jo	Dong-Gyu Jo	
Peng Li	Eunae Kim	Eunae Kim	
Qiannan Wang	Harkkyun Kim	Harkkyun Kim	
Shanjin Huang	Jae Hyung Parki	Jae Hyung Parki	
Armel Gallet	Jaehoon Sul	Jaehoon Sul	
Olivia Benguettat	Jeongmi Lee	Jeongmi Lee	
Salma Hachfi	Jin Su Park	Jin Su Park	
Emmanuelle Bayer	Seung Hyun Baek	Seung Hyun Baek	
Lysiane Brocard	Yoonsuk Cho	Yoonsuk Cho	
William J. Nicolas	Yuri Choi	Yuri Choi	
France		Sweden	
Christian Tischer	Stefan Hohmann	University of Gothenburg	
Anne Ephrussi			
Lachlan Whitehead			
Frank Wippich			
Mateusz Majda	Diana Santelia	Diana Santelia	
Katharina Maria Zimmermann	Luca Distefano	Luca Distefano	
	Sabrina Flütsch	Sabrina Flütsch	
Germany		Switzerland	
United States		United States	
		K. Adam Bohnert	Louisiana State University
		Caroline A. Enns	
		Claudia S. López	Oregon Health & Science University
		Julia K. Doh	
		Kimberly E. Beatty	
		Young Hwan Chang	
		Inhee Chung	School of Medicine and Health Sciences
		I-Ting Teng	
		Xiangning Bu	
		Agnieszka P. Looney	University of California, San Francisco
		Mallar Bhattacharya	
		Emily Koury	
		Kailey E. Harrell	University of Iowa
		Sarit Smolikove	
		Claire E. DelBove	Vanderbilt University
		Qi Zhang	
		Roman M. Lazarenko	

**Editors**

Amey Redkar	<i>University of Cordoba, Spain</i>	Andrea Puhar	<i>Umea University, Sweden</i>	Arsalan Daudi	<i>University of California, San Francisco, USA</i>
Chao Jiang	<i>Zhejiang University, China</i>	David Paul	<i>MRC Laboratory of Molecular Biology, UK</i>	Dennis J Nürnberg	<i>Imperial College London, UK</i>
Gal Haimovich	<i>Weizmann Institute of Science, Israel</i>	Imre Gáspár	<i>Institute of molecular biotechnology, Austria</i>	Ivan Zanoni	<i>Harvard Medical School, USA</i>
Khyati Hitesh Shah	<i>Sutro BioPharma, USA</i>	Longping Victor Tse	<i>University of North Carolina at Chapel Hill, USA</i>	Meenal Sinha	<i>University of California, San Francisco, USA</i>
Neelanjan Bose	<i>Emery Pharma, USA</i>	Nicoletta Cordani	<i>University of Milano-Bicocca, Italy</i>	Ralph Thomas Boettcher	<i>Max Planck Institute for Biochemistry, Germany</i>
Samantha E. R. Dundon	<i>Yale University, USA</i>	Scott A M McAdam	<i>Purdue University, USA</i>	Tie Liu	<i>University of Florida, USA</i>
Vamseedhar Rayaprolu	<i>La Jolla Institute for Allergy and Immunology, USA</i>	Xi Feng	<i>University of California, San Francisco, USA</i>	Zinan Zhou	<i>Boston Children's Hospital, USA</i>

**Reviewers**

Aleksandr Gavrin	<i>Sainsbury Laboratory, University of Cambridge, UK</i>	Alexandros Alexandratos	<i>National and Kapodistrian University of Athens, Greece</i>	Anita Umesh	<i>Illumina, USA</i>
Anne-Marie Caroline Overstreet	<i>Indiana University School of Medicine, USA</i>	Carmelo Bellardita	<i>University of Copenhagen, Denmark</i>	Elena A. Ostrakhovitch	<i>Frontiers in Bioscience Research Institute in Aging and Cancer, USA</i>
Elizabeth V. Clarke	<i>University of Wyoming, USA</i>	Francesca Angileri	<i>Centre Léon Bérard - Center for Drug Discovery and Developement (C3D), France</i>	Gongjun Shi	<i>North Dakota State University, USA</i>
Henrique Borges da Silva	<i>University of Minnesota, USA</i>	Jeremy Charles Welsch	<i>University of Michigan, Medical School, USA</i>	Joshua S Titlow	<i>University of Oxford, USA</i>
Juan Facundo Rodriguez Ayala	<i>Instituto de Nanociencia y Nanotecnología, Argentina</i>	Karthik Krishnamurthy	<i>Thomas Jefferson University, USA</i>	Kristin L. Shingler	<i>University of Minnesota School of Dentistry, USA</i>
Kristofor Kenneth Ellestad	<i>University of Alberta, Canada</i>	Laura Campisi	<i>Icahn School of Medicine at Mount Sinai, USA</i>	Lili Wang	<i>Icahn School of Medicine at Mount Sinai, USA</i>
Linlin Sun	<i>New York University School of Medicine, USA</i>	Livia Ulicna	<i>Stanford University, USA</i>	Lokesh Kalekar	<i>eGenesis Inc, USA</i>
Lu Han	<i>Palo Alto Veterans Institute for Research, USA</i>	Mansi Arora	<i>The Ohio State University, USA</i>	Marco Di Gioia	<i>Harvard Medical School, USA</i>
Maria Victoria Martin	<i>Instituto de Investigaciones en Biodiversidad y Biotecnología. INBIOTEC-CONICET y FIBA, Argentina</i>	Martin V Kolev	<i>GSK Research and Development, USA</i>	Paula clarisa Ellenberg	<i>University of melbourne, Australia</i>
Rajesh D Gunage	<i>HMS-BCH, USA</i>	Samantha E. R. Dundon	<i>Yale University, USA</i>	Sandeep Dave	<i>Texas A&amp;M University, USA</i>
Smita Nair	<i>Indiana University, USA</i>	Shalini Low-Nam	<i>Purdue University, USA</i>	Sijie Wei	<i>Stanford University, USA</i>
Surabhi Sonam	<i>Institut Jacques Monod, France</i>	Swati Jalgaonkar	<i>University of Pune, India</i>	Takashi Nishina	<i>Toho University, Japan</i>
Tchern Lenn	<i>Queen Mary University of London and Imperial College London, UK</i>	Tegan M. Haslam	<i>University of Göttingen, Germany</i>	Timothy Notton	<i>Autonomous Therapeutics, USA</i>
Trinadh Venkata Satish Tammana	<i>Radboud University Medical Center, Netherlands</i>	Vikash Verma	<i>University of Massachusetts, USA</i>	Vishal S Parekh	<i>Medical school of university of michigan, USA</i>
Wenrong He	<i>Salk Institute for Biological Studies, USA</i>	Woojong Lee	<i>UW-Madison, USA</i>	Xuecai Ge	<i>University of California, Merced, USA</i>
Yan Cheng	<i>Huazhong Agricultural University, China</i>	Ying Zhen	<i>Westlake University, China</i>	Zhongyi Li	<i>Commonwealth Scientific and Industrial Research Organization, Australia</i>

## Electron Tomography to Study the Three-dimensional Structure of Plasmodesmata in Plant Tissues—from High Pressure Freezing Preparation to Ultrathin Section Collection

William J. Nicolas<sup>1,\*</sup>, Emmanuelle Bayer<sup>1,\*</sup> and Lysiane Brocard<sup>2,\*</sup>

<sup>1</sup>Laboratory of Membrane Biogenesis, UMR5200 CNRS, University of Bordeaux, Villenave d'Ornon, France; <sup>2</sup>Bordeaux Imaging Centre, Plant Imaging Platform, UMS 3420, INRA-CNRS-INSERM University of Bordeaux, Villenave d'Ornon, France

\*For correspondence: [william.nicolas.1@u-bordeaux.fr](mailto:wilhelm.nicolas.1@u-bordeaux.fr); [emmanuelle.bayer@u-bordeaux.fr](mailto:emmanuelle.bayer@u-bordeaux.fr);  
[lysiane.brocard@inra.fr](mailto:lysiane.brocard@inra.fr)



**[Abstract]** Plasmodesmata (PD) are nanometric (~20 nm wide) membrane lined pores encased in the cell walls of the adjacent plant cells. They allow the cells to exchange all types of molecules ranging from nutrients like sugar, hormones, to RNAs and various proteins. Unfortunately, they are also hijacked by phyto-viruses, enabling them to spread from cell-to-cell and then systematically throughout the whole plant. Their central position in plant biology makes it crucial to understand their physiology and especially link their function to their structure. Over the past 50 years, electron microscopists have observed them and attempted to ultrastructurally characterize them. They laid the foundation of what is known about these pores (Tilney *et al.*, 1991; Ding *et al.*, 1992; Oparka and Roberts, 2001; Nicolas *et al.*, 2017a).

Despite the explosion of three-dimensional electron microscopy (3D-EM), PD ultrastructure remained recalcitrant to such technique. The first technical difficulty is to process them in such a way where they are as close to their native state as possible. Secondly, plant samples reveal themselves as being difficult to process due to the poor staining/fixating reagents penetration rates, their increased size, their high water content and the presence of an acidic vacuole. On top of this, their very unique position in the cell wall and their nanometric size make them difficult to conveniently stain in order to see the inner-workings of these pores.

Here we describe in detail the protocol used in Nicolas *et al.* (2017b) to image PD in fine detail and produce high-resolution tomograms.

**Keywords:** Plasmodesmata, Plant, Cell wall, Electron tomography, Electron microscopy, Cryofixation

**[Background]** High Pressure Freezing (HPF) relies on the vitrification of the water present in the sample. By cooling down the sample at a high enough freezing rate ( $10^4$ - $10^5$  °C/sec), its contained water molecules cannot reorganize in a crystal-fashion and remain vitrified in an amorphous state (see Dubochet [2007] for further reading on the physics behind water crystallization). The sample is then said to be ‘cryoimmobilized’ or ‘vitrified’. This is generally achieved by the use of liquid nitrogen (-195 °C) or liquid ethane (-188 °C). At ambient pressure, this phenomenon can only be achieved on a few microns (< 5 microns), however by raising the pressure to approximately 2,000 bars (~2,000 atmospheres), this depth can reach at least 200 microns (up to 500 microns in certain conditions). This allows the vitrification of thick biological tissues without any osmotic artefacts.

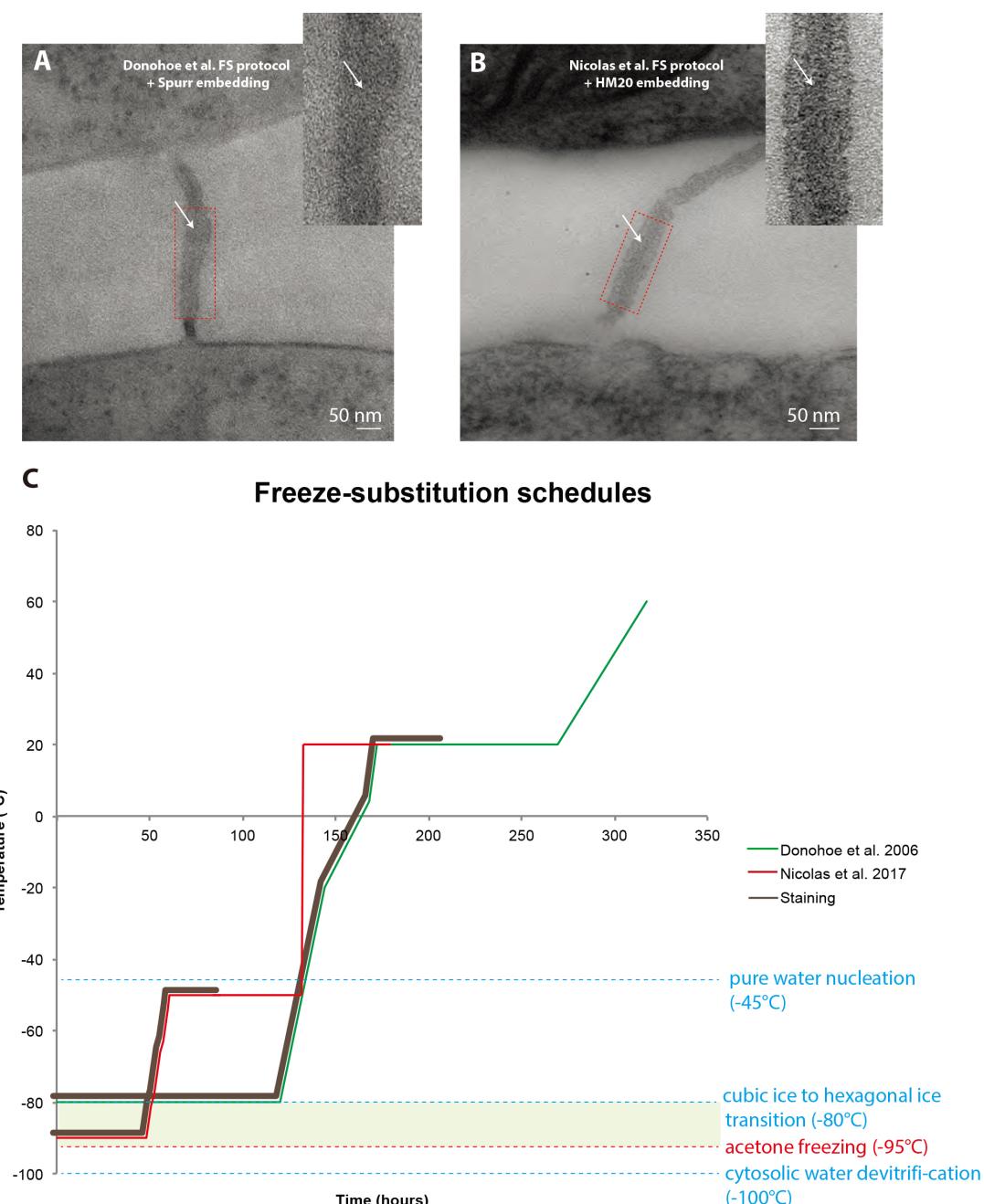
Classically, HPF is followed by Freeze Substitution (FS). This step comprises staining, dehydration and resin embedding of the sample. The good success of the FS is critical for the final sub-cellular preservation state of the sample and will be dependent upon the staining agents used, their concentration and also the temperature kinetic followed during the different stages of FS. These parameters are key for adequate fixation and cross-linking of cellular components, the proper staining (not too little nor too much) and embedding, and heavily impact the final resolution reached. Although this protocol is a good starting point, depending on the samples (tissues, developmental stage, plant species) and organelles of interest, it will need to be adapted to the reader's specific task.

Despite published FS protocols custom tailored for plant samples have yielded spectacular results over the past decades (Donohoe *et al.*, 2006; Kang *et al.*, 2011), in our hands, it was not suitable for the study of PD ultrastructure for two reasons: i) The epoxy resin used in such protocols is highly electron-scattering and generates a bad signal to noise ratio when imaged with a 120 kV electron microscope, subsequently preventing us from clearly imaging the internal details of the nanometric pores (Figure 1A). ii) The elements inside the pores are so tightly packed in such a small space (< 10 nm) that heavy staining activity during FS prevents clear imaging of these elements (Figure 1A).

In this context, we opted for HPF followed by a modified FS course to prepare our samples (Figure 1C and Table 1). This led to the production of high resolution tomograms, enabling the appreciation of PD ultrastructure in all dimensions of space. We hope our readers find this protocol useful and will eventually improve it for better visualization of nanometric membrane details in thick plant samples.

**Table 1. Comparative cryofixation procedures between Donohoe *et al.* (2006) and Nicolas *et al.* (2017b).** Left table recapitulates dehydration + fixation steps and right table the resin-embedding step. Cells marked in blue represent temperatures below 0 °C and cells marked in orange represent temperatures above 0 °C.

		Dehydration + fixation (acetone+cryomix)		Resin embedding			
		Duration (h)	Temperature (°C)	Duration (h)	Temperature (°C)	UV	Type
Nicolas <i>et al.</i> 2017	48		-90	48	-50	yes	HM20 Lowicryl
	7		-50	24	20	yes	
Donohoe <i>et al.</i> 2006	120 (5 days)		-80	144 (6 days)	96	no	Eponate 12 Epoxy
	24		-20	48	60	no	
	24		4				



**Figure 1. Comparison between freeze-substitution protocols.** A. Root tip plasmodesma micrograph acquired from a 90 nm thick section prepared with the regular cryosubstitution protocol (Donohoe *et al.*, 2006). The central element (white arrows) is barely visible, the membrane bilayers are not as lightly stained as in (B) and the overall signal-to-noise ratio is low. B. 2D micrograph of a plasmodesma situated in the root tip acquired at a 0° tilt from a 180 nm thick section prepared with our improved cryosubstitution protocol. Despite the thickness of the section, details in the vicinity of the pore are discernible, notably the central element (white arrows). Dense material can be seen in the cytoplasmic sleeve space. Insets show a close-up view of the red-boxed region ns. C. This temperature versus time curve depicts the commonly used freeze substitution schedule and the one we developed. Brown sections of the curve correspond to when the sample is submerged in

the cryosubstitution cocktail containing the highly reactive staining agents. Green zone corresponds to the ideal area for the frozen hydrated samples, below which hexagonal ice cannot form and acetone won't freeze at atmospheric pressure (between -95 °C and -80 °C). In Donohoe *et al.*, 2006 (green curve) the samples remain in the cocktail for a prolonged amount of time, until the temperature reaches 20 °C. In our protocol (red curve, Nicolas *et al.*, 2017b) the cocktail is removed from the sample very early on in the process when the temperature is at -50 °C.

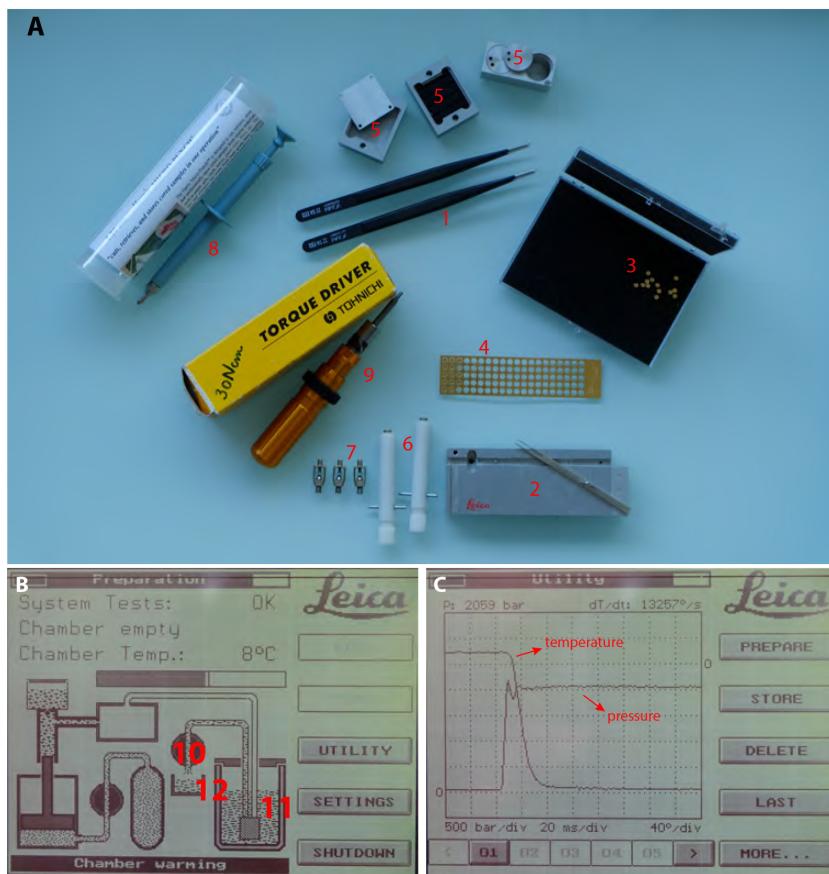
## **Materials and Reagents**

### A. Plant material

1. Square plastic culture plates for *Arabidopsis* seedlings vertical culture (VWR, catalog number: 391-0444)
2. MS medium + vitamins (Duchefa Biochemie, catalog number: M0222.0050) for seedlings and cell cultures
3. Sucrose (Sigma-Aldrich, catalog number: 84100)
4. 2-(N-morpholino)ethanesulfonic acid (MES) (Euromedex, catalog number: EU0033-A)
5. Naphthaleneacetic acid (NAA) stock solution (10 mg/ml aliquoted at -20 °C) (Sigma-Aldrich, catalog number: N064025G)
6. Kinetin hormone stock solution (1 mg/ml aliquoted at -20 °C) (Sigma-Aldrich, catalog number: K3253)
7. 1 N KOH (for pH)
8. MES (Euromedex)
9. Plant agar (Duchefa Biochemie, catalog number: P1001.1000)
10. Culture mediums for cells and seedlings (see Recipe 1)
  - a. Murashige and Skoog medium for liquid cultured cells
  - b. Murashige and Skoog medium for seedlings

### B. High pressure freezing (Figure 2A)

1. Aclar sheet, 51 µm thick (Electron Microscopy Sciences, catalog number: 50426)
2. Cryotubes (VWR, catalog number: 479-1207)
3. Eppendorf 1.5 ml tubes (SARSTEDT, catalog number: 72.708)
4. 15 ml Falcon tube (SARSTEDT, catalog number: 62.553.542)
5. Tooth picks
6. BSA fraction V (Sigma-Aldrich, old catalog number: 85040C, now catalog number: 05482)
7. Liquid MS medium
8. MethylCycloHexane (MCH) (Merck Schuchardt OHG 85662 Hohenbrunn, Germany)
9. BSA solution for cryoprotection during HPF (see Recipe 2)

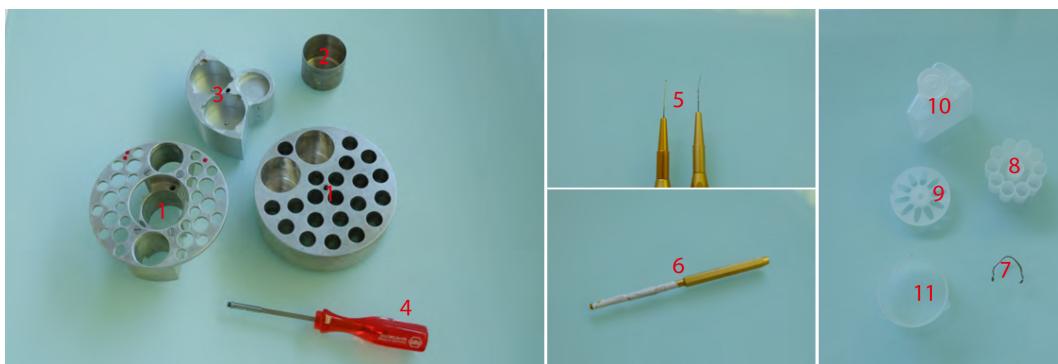


**Figure 2. High Pressure Freezing procedure.** A. 1. Insulated tweezers for manipulation in liquid nitrogen bath. 2. Leica loading system (see Video 1 for loading procedure) with its associated platelet holder rod. 3. 200 µm deep, 1.5 mm diameter, copper platelets. 4. 100 µm deep, 1.5 mm membrane carriers, copper membrane carriers. 5. Sample transfer metal containers. 6. Pod holder. 7. Pods. 8. Harris puncher for production of the 1.5 mm diameter acclar disks to be optionally laid at the bottom of the 200 µm deep platelets to facilitate the later separation of the sample. 9. Torque screwdriver for tightening the membrane carrier in the pods (#3, 4 and 5 respectively). B. View of the EMPACT1 touchscreen displaying the status of the machine. (10) is the valve controlling liquid nitrogen flux from the main tank (11) to the liquid nitrogen bath (12). C. View of the display after processing a sample. Curve dropping down is temperature and curve rising up is pressure.

C. Freeze substitution (Figure 3)

1. Screw top 2 ml tubes (SARDTEDT, catalog number: 72.693)
2. Disposable regular pipets 1.5, 2 and 3 ml and FS specific 1 ml with thin tips (Ratiolab, catalog number: 2600155)
3. Wheaton glass sample vials with snap-cap for cryomix preparation (DWK Life Sciences, WHEATON, catalog number: 225536.)
4. Personal cartridge half mask 6100 (Honeywell International, catalog number: 1029471)

5. Plastic coffins or pills Leica molds (AFS2 consumable, Leica, catalog numbers: 16707155 and 16707157 respectively)
6. Plastic solvent containers with screw tops (Leica, catalog number: 16707158)
7. Tinfoil
8. Uranyl acetate powder (Merck, catalog number: 8473)
9. Pure methanol
10. Glutaraldehyde 10% EM-grade anhydrous solutions in acetone (Electron Microscopy Sciences, catalog number: 16530)
11. Ultra pure 100% ethanol and acetone (VWR, catalog numbers: 83813.440 and 20066.558, respectively)
12. Osmium tetroxide vials 0.1 g (Electron Microscopy Sciences, catalog number: 19134)
13. Nail polish (color does not matter)
14. Cryosubstitution Uranyl-acetate stock solution (20%) (see Recipe 3)
15. Cryosubstitution mix (see Recipe 4)



**Figure 3. Freeze substitution procedure.** 1. AFS tube holders. The one on the right being made according to Heinz Schwarzes blueprints ([heinz.schwarz@tuebingen.mpg.de](mailto:heinz.schwarz@tuebingen.mpg.de)). 2. Metal cups for holding cryomix tubes. 3. Metal socket for mould containers for resin polymerization. 4. Screwdriver used to transfer tube holders 1 and 2 and sample transfer containers shown in Figure 1 (#5). 5. EMS micro-needle and Ted Pella Inc. ultra-micro-needle tools. 6. Ted Pella Inc. micro-tool adapter. 7. Hand-made tool holder. 8. Leica plastic ‘pill’ mould. 9. Leica plastic ‘coffin’ mould. 10. Plastic solvent container with screw top. 11. Leica plastic mould container.

#### D. Resin embedding

1. HM20 resin (Electron Microscopy Sciences, catalog number: 14345)
2. HM20% solutions (see Recipe 5)

#### E. Ultramicrotomy

1. Crystallizing dish (Fisher Scientific, catalog number: 11766582)  
*Manufacturer: DWK Life Sciences, DURAN, catalog number: 213133408.*

2. Fast absorbent paper filters (Whatman, Filter papers 41) (GE Healthcare, catalog number: 1441-070)
3. Glass wand (Fisher Scientific, catalog number: 12441627)  
*Manufacturer: MBL, catalog number: SRF380.*
4. Grids with different meshes (200 L/inch and 300 L/inch Delta Microscopy) and slot grids (EMS Formvar Carbon Film 2 x 0.5 mm copper grids lot, Electron Microscopy Sciences, catalog number: EMS200-Cu)
5. Paraffin films (Bemis, Parafilm 'M')
6. Pasteur pipet (VWR, catalog number: 612-1720)
7. 0.2 µm filter
8. Syringe
9. 0.5 ml Eppendorf tubes
10. 5 nm colloidal gold solution (BBI solution, catalog number: EM-GC5, [www.bbisolutions.com](http://www.bbisolutions.com))
11. Solid parlodion (Electron Microscopy Sciences, catalog number: 19220)
12. Isoamyl-acetate (Sigma-Aldrich, catalog number: W205532)
13. Toluidine blue powder (Sigma-Aldrich, catalog number: T3260)
14. Sodium borate (Sigma-Aldrich, catalog number: B3545)
15. Ultrapure (MilliQ) water
16. 2% Parlodion solution for grid filming (see Recipe 6)
17. Toluidine blue solution (see Recipe 7) for screening block sections on table top microscope during resin block milling
18. Fiducial marker solution (see Recipe 8)

## **Equipment**

### A. Plant material

1. 200 ml glass flasks for liquid cell culture (Dutscher, catalog number: 232522)
2. Flask shaker to keep liquid cultured cells moving (Digital Shaker, Southwest Science)
3. Green room for *in vitro* cultivation of seedlings and liquid cultured cells (see Recipe 1 for growth conditions)

### B. High pressure freezing (Figure 2A)

1. Liquid nitrogen, liquid nitrogen container and adapted personal protection gear
2. Air compressor (JUN-AIR)
3. Leica EMPACT1 machine (Leica Microsystems)
4. Leica loading system
5. Pod holders
6. Pods
7. Puncher for aclar disks (Harri's Micro Punch 1.20 mm)

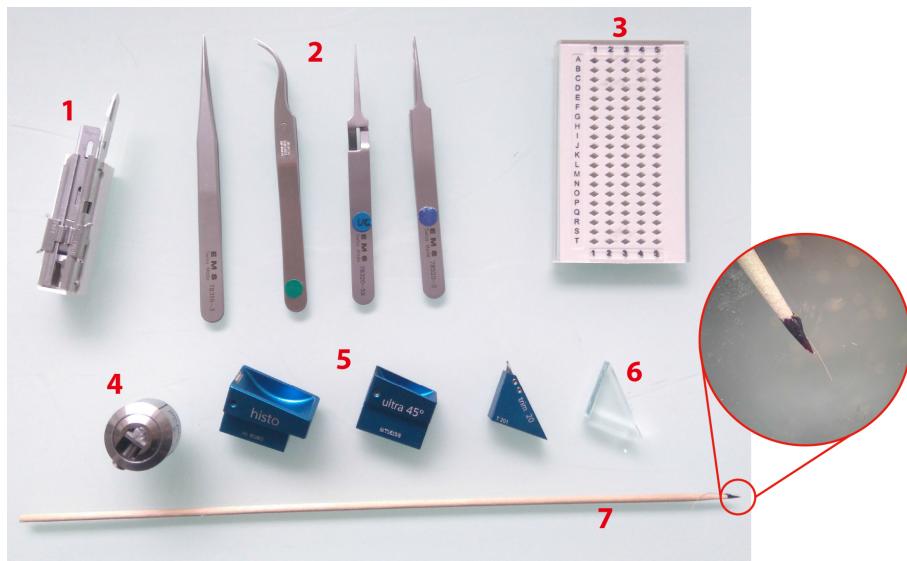
8. Regular biomolecular pipets from 2 µl to 1,000 µl range
9. 200 ml flask
10. Binocular for root dissection (Nikon, model: SMZ-10A)
11. Heating surface for quick drying of Pods and pod holders during the session
12. Insulated tweezers for manipulation in N<sub>2</sub> (VOMM Germany, 22 SA ESD)
13. Little metal scooper for sampling liquid cultured cells
14. Membrane carriers (100 µm or 200 µm deep) (Leica Microsystems, catalog numbers: 16707898 and 16706898 respectively)
15. Metal containers for frozen sample transfer and associated screw driver for lifting procedures (provided with EMPACT1)
16. Torque screwdriver (TOHNICHI, Torque driver RTD60CN) set to 30Ncm

**C. Freeze Substitution and resin embedding (Figure 3)**

1. Leica EM Freeze Substitution Processor (FSP) equipped with UV light (Leica Microsystems)
2. Leica Automatic Freeze Substitution 2 (EM AFS2) machine (Leica Microsystems, model: Leica EM AFS2)
3. Hand made tool holders (out of paper clips) for better disposal of the various tools in AFS2 well
4. AFS tube holders Metal cups
5. Metal socket for mould containers
6. Microtools for separating the membrane carriers from frozen samples and disposing samples in the moulds correctly (micro-needle #1 0.025 mm, Electron Microscopy Sciences, catalog number: 62091-01 and tungsten 5 µm ultra-micro needle, Ted Pella, catalog number: 13625)
7. Ventilated hood

**D. Ultramicrotomy (Figure 4)**

1. Petri dishes of various sizes (Dutscher, catalog numbers: 068515, 068516, 068517)
2. Various EM grade precision tweezers (EMS style 2, 5, 5X, style 7 <https://www.emsdiasum.com/> <https://www.dumonttweezers.com>)
3. Block holders
4. Carbon coater (optional) (SPI-MODULE Carbon Coater)
5. Diamond knives (DIATOME Trim20 dry, Ultra wet and HISTO wet)
6. ELMO Glow discharger (optional) (CORDOUAN Technologies)
7. Glass knife maker (LKB BROMMA, model: 7800 Knifemaker)
8. Glass knives for first milling steps to reach the sample
9. Grid carriers for grid storage
10. Leica Ultracut UC7 (Leica Microsystems, model: Leica Ultracut UC7)
11. Razor blades for coarse block preparation
12. Table top microscope (Olympus, model: CX-41)



**Figure 4. Ultramicrotomy procedure.** 1. Sharp razor blades. 2. Tweezers (shapes n°3, 7, 5 and 5X, EMS). 3. Grid holders. 4. Block holders (sold with ultramicrotome, Leica Microsystems). 5. Diamond knives Histo, ultra 45° for section collection and trim 20 dry for precise trimming (from left to right). 6. Glass knife for coarse trimming. 7. Cat whisker mounted on a wooden stick for section gathering during on-grid collection step. Circular inset shows an enlarged view of the cat whisker mounted on the stick using nail polish.

## Software

1. Tecnai imaging and analysis (TIA) software (<https://www.fei.com/software/>)  
TEM User Interface was used in conjunction with Tecnai Imaging and Analysis (TIA) software to control and acquire micrographies with the EM (<https://www.fei.com/software/>)
2. Xplore3D (FEI) was used for automated tilt series acquisitions (<https://www.fei.com/software/>)
3. ImageJ (<https://imagej.nih.gov/ij/download.html>) with the Input/Output plugin (<http://www.cmbi.fr/en/download/softwares/input-output.html>) were used to readout the .mrc tilt series files and tomograms
4. IMOD suite (<http://bio3d.colorado.edu/imod/>)

Note: All processing was done using the IMOD suite (Kremer et al., 1996) (<http://bio3d.colorado.edu/imod/>), from the alignment of the raw tilt series to tomogram reconstruction, segmentation and data analysis.

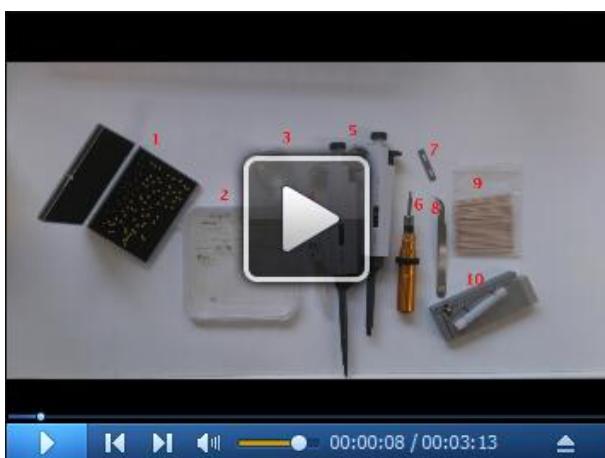
## **Procedure**

*Note: This procedure involves the manipulation of dangerous compounds, namely liquid nitrogen, acetone, osmium tetroxide, glutaraldehyde, uranyl-acetate, Lowicryl resin. If you are not familiar with how to handle such compounds, please refer to the official guidelines and our Notes 1 and 2.*

### A. High pressure freezing

Steps A1 through A13 should be carried out as fast as possible to limit deterioration of sampled live tissue. In our case, this period is less than a minute. A thorough description on how to use the EMPACT1 is provided in user manual and also in the proof of concept paper (Studer *et al.*, 2001).

1. Turn on Leica EMPACT and air compressor hooked up to it.
2. Fill up the chamber with MCH and evacuate air in the circuit by gently pushing on the vertical rod.
3. Fill in liquid nitrogen tank up to ¾ (keep liquid nitrogen source handy for eventual mid-way top-ups during session).
4. Switch on the EMPACT1 and wait until working pressure reaches between 4.8 and 5 bars.
5. On the touchscreen tap on the corresponding valve to allow filling of the bath with liquid nitrogen (Figure 2B).
6. Load an empty pod and tap on *prepare* → *lock* → *start* to shoot in order to clear out all the air out of the system.
7. Charge membrane carrier on the Leica loading system (Figure 2A, #2 and Video 1).



**Video 1. Loading the biological sample in the HPF pods.** (1) 200 µm deep membrane carriers (Leica Microsystems); (2) Vertically grown *Arabidopsis* seedlings; (3) Becker filled liquid MS medium; (4) 20% BSA diluted in liquid MS medium; (5) 0.5-2 µl and 20-200 µl range pipets (Sartorius); (6) Torque screwdriver set to 30Ncm (TOHNICHI, Torque driver RTD60CN); (7) Disposable razor blades; (8) Style 7 EMS tweezers; (9) Disposable tooth picks; (10) Leica loading system (with rod), pods, pod holder.

8. If using the 200  $\mu\text{m}$  membrane carriers with a hole on the bottom, wedge an acclar disk generated with the puncher. Disks can be generated in batches the day before cryofixation, incubated in 95% ethanol and let to evaporate.
9. With a 0.5-2  $\mu\text{l}$  pipet, fill the carrier with 20% BSA (Recipe 2) in MS (Murashige and Skoog medium). It must form a clean dome in the socket without bleeding on the edges and with no air bubbles (Video 1).
10. Handling of biological material: i) Roots are quickly dissected under binocular in MS medium on a glass slide with a sharp razor blade and then carefully picked up with a toothpick. ii) Cells are aliquoted in a 1.5 ml Eppendorf tube from the main 200 ml flask and are let to sediment down for a few minutes. Then, the cells are picked up by scratching from the green cell pellet visible at the bottom with a toothpick or a little metal spoon shaped scooper, Cells are then carefully placed in a membrane carrier. Every 2 to 3 HPF trials ( $\sim 3$  min time frame when two experimenters are implicated), replace the cells in the Eppendorf tube by fresh cells.
11. Dispose the sample in the BSA bubble in the membrane carrier. It is best when the sample is completely submerged by the BSA (Video 1).
12. Push the membrane carrier in the pod positioned in its special socket on the Leica loading system (Video 1).
13. Screw the sapphire tight on the carrier using the TORX screwdriver (set on 2.5 cN m $^{-1}$ ) (Video 1).
14. Screw the white pod holder (Video 1).
15. Load it in the Leica EMPACT machine and the loading stage and push the piston towards the left (Video 2).



**Video 2. Loading the pod containing the sample in the HPF machine**

16. On the touch screen tap on *prepare*  $\rightarrow$  *lock*  $\rightarrow$  *start*. The system automatically flushes out the pod holder, making the pod loaded with the sample fall in a liquid N<sub>2</sub> bath.  
At this stage the sample must never come out of the liquid N<sub>2</sub>, risking sudden devitrification and the waste of the sample (Video 2).

17. Check the freezing rate and pressure rise on the touchscreen. It should look like in Figure 2C, where maximum pressure is reached ~20 msec before reaching final temperature.
18. Place pod holder in the specially designed socket bathing in the liquid nitrogen (Video 2).
19. Unscrew the pod holder (Video 2).
20. Carefully release the clamping from the sapphire by unscrewing and then gently tug out the membrane carrier by tapping the pod against the bottom of the bath. Use insulated tweezers to manipulate the frozen membrane carrier (Video 2).
21. Place the membrane carrier in the sample carrier dedicated to this usage and keep in liquid N<sub>2</sub> (Video 2).
22. When all samples are ready to be transferred for subsequent steps, place metal lids on the metal containers and quickly transfer the containers from the EMPACT bath to an intermediate liquid nitrogen container. Then transfer to AFS2 machine, already running, filled up with liquid N<sub>2</sub> and pre-cooled to -90 °C.

#### B. Freeze substitution

During the freeze substitution, it is important to always maintain the frozen hydrated samples in the cold, below -80 °C to avoid too much hexagonal ice formation (Dubochet, 2007). Therefore, never pick up the tubes out of the well and always use instruments, containers and FS solutions that have been precooled in the well for a good 15-20 min.

Because Steps B7 to B9 are tedious, we would recommend at the beginning to devote a full day to it.

1. Under a vented hood, dispatch cryomix (see Recipe 4) immediately after having added osmium into the screw top tubes (numbered with a Sharpie and wrapped in scotch tape to avoid washing out of the ink) and then dip the tubes in liquid nitrogen to freeze the cryomix (less volatile when frozen).
2. Insert frozen cryomix containing tubes in the AFS well, inside the metal cups.
3. While cryomix is still solid, carefully dispatch the frozen sample containing membrane carriers inside the cryomix tubes (maximum of 2 carriers/tube).
4. During the thawing process of the cryomix from the -195 °C to -90 °C transition, the carriers will sink in the mix and freeze-substitution will start.
5. Set the AFS as following:

Temperature	Duration
-90 °C	48 h
+3 °C/h	13 h
-50 °C	Stand-by

6. (OPTIONAL but highly encouraged) At day +1 after the start of the FS, open the cryomix tubes and redispouse the carriers at the bottom in such a way that the carriers do not lay on top of each other in order to maximise the exchanges between the mix and the samples. Also, a gentle shake can be given to the tubes.

7. After completion of the -90 °C to -50 °C transition, the machine is on hold at -50 °C.
8. Wash thoroughly every tube 3 times with ultra-pure acetone and then 3 times with ultrapure ethanol (Note 3).
9. During the last wash, transfer the samples into a plastic mould container containing 100% ethanol.
10. Carefully separate the samples from the membrane carriers in a plastic mould container using the microtools (Figure 3, #5 and #6). Use them to scrape the rim of the carriers to loosen the frozen BSA. If using 200 µm deep carriers (Leica Microsystems), the hole at the bottom can be used to push out the frozen sample by fitting the ultra-micro needle tool (Figure 3, #5, right one).
11. Prior to the Transfer of the detached sample in the moulds with a 3 ml pipette, fill the moulds with cold ultrapure ethanol and let them cool down to -50 °C (Note 4).

*Note: During Steps B3 to B8, cryotubes should always be closed in between the baths in order to avoid sublimation of residual osmium tetroxide.*

#### C. Resin embedding

1. When all samples are in place in the adequate moulds, gently pipet out the excess ethanol from the moulds (see Note 3 for pipetting technique) and replace with 25% HM20 (see Recipe 5).
2. The following incubation kinetic is just a guideline. Adapt according to your work days:

Mix	Temperature	Duration
HM20 25%	-50 °C	2 h
HM20 50%	-50 °C	2 h
HM20 75%	-50 °C	Over-night (~15 h)
HM20 100%	-50 °C	2 h
HM20 100%	-50 °C	2 h

3. On the last 100% HM20 bath, place the mould containers in the metal piece (Figure 3, #3) that has been cooled down to -50 °C before hand.
4. Mount the FSP module and set the AFS as following:

Mix	Temperature	Duration
HM20 100% (+ FSP)	-50 °C	8 h
HM20 100% (+ FSP + UV)	-50 °C	24 h
HM20 100% (+ FSP + UV)	+20 °C	24 h

5. Take out hardened resin blocks from their casts by either slicing the casts carefully with razor blades or, if using the pill-shape moulds, the blocks can be punched out using a hexagonal screwdriver placed on the bottom (Video 3).



**Video 3. Puncturing out the pill-shaped blocks.** It requires a Wiha 353/SW 3.0x75 screwdriver for puncturing.

#### D. Ultramicrotomy

##### 1. Grid filming

Because we embed our samples in HM20 Lowicryl, a more fragile resin than EPON, the sections need to be deposited on grids that have been coated with parlodion or formvar.

Formvar-coated slot grids were purchased and useful because no grid bars can potentially block the imaging in high tilts. However, they are more fragile and very sensitive to shifts induced by resin retraction. For parlodion-filmed mesh grids, they are home-made as follows (Video 4):

- a. Fill a crystalizing dish, placed in a large Petri dish, with distilled water to the top.
- b. Rack the top of the crystalizing dish with a glass wand to flatten out the surface of the water.
- c. Pipet the parlodion solution with a Pasteur pipet and place the pipet vertically over the crystalizing dish. Let a drop fall on the surface of the water.
- d. Let the parlodion spread at the surface of the water.
- e. With clamps, remove this first film (used to eliminate potential dust and particles on the water surface).
- f. Repeat Steps D1b and D1c.
- g. Carefully place the grids on the floating parlodion film (opaque side face down on the film). Use the mesh desired (200 mesh/ $\mu\text{m}^2$  is optimal for better overviews and tilt series acquisition).
- h. With tweezers, gently place a Whatman type 41 absorbent paper (or equivalent absorption speed) on the floating grids and let it soak.
- i. Carefully remove excess parlodion film on the sides of the absorbent paper, either by tearing it away or folding it on top of the floating paper. This is to prevent excess film to fold onto the grids, resulting in a double layer of film on the filmed grids.

- j. To avoid rippling and/or sliding of the film, firmly grip the absorbent paper by the sides with 2 tweezers type 5X or like (which close automatically) and transfer the absorbent paper by flipping it over into a glass/plastic Petri dish.
- k. Let the Petri dish open for a day under ventilated hood, in order to let the filmed grids dry out.

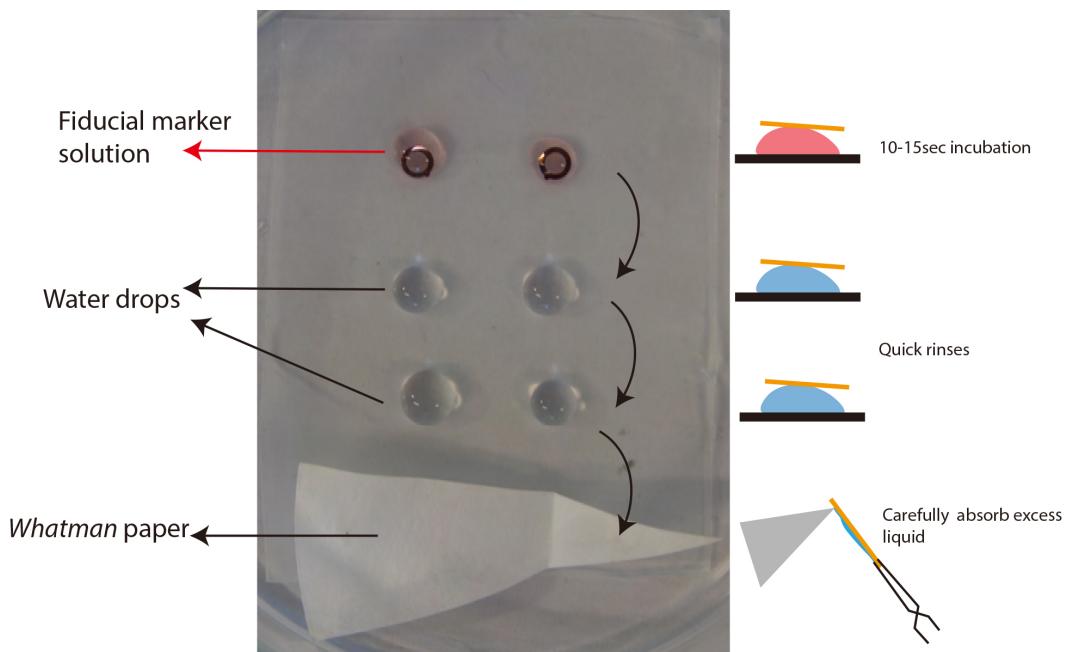
**Quality check:** Under the binocular of the ultramicrotome, pick up a filmed grid and by reflection look for a homogenous purple glare on the grid. Under the TEM, the grids may display a few little holes, which is fine. However, when a lot of holes occur, the parlodion solution may be contaminated with water and may need to be replaced.



**Video 4. Grid filming procedure.** 1- Glass Pasteur pipet and its pump; 2- Petri dish; 3- Glass wand; 4- Large Petri dish lid; 5- Whatman filter paper type 41; 6- Crystallizer; 7- EM grids. 8- 2% parlodion solution; 9- Type 5X tweezers.

2. Section collection
  - a. Since the aim of this protocol is more focused on how to process the samples as this is the critical factor for the final images, we will not go into details on how to properly use an ultramicrotome, prepare the block-face for cutting etc. Instead please refer to Hagler (2007). The following is some comments for properly performing these steps.
    - b. For the study of PD, 90 nm or 180 nm thick sections were collected with the Leica Ultracut UC7 (Leica Microsystems).
    - c. Producing serial sections can be useful for two main reasons: i) with one grid, more objects of interest, ii) serial tomography can be performed where the same structure is reconstructed on serial sections, making it possible to recover 3D volumes of structures a lot bigger than the section thickness (Kang *et al.*, 2011).
    - d. To do so, the block face needs to be reasonably small enough so that multiple sections can be placed on a single grid. Carving the block face in a trapeze shape will allow the sections to stick together as they are generated, creating a contiguous ribbon of serial sections that can be easily deposited on a single grid simultaneously.

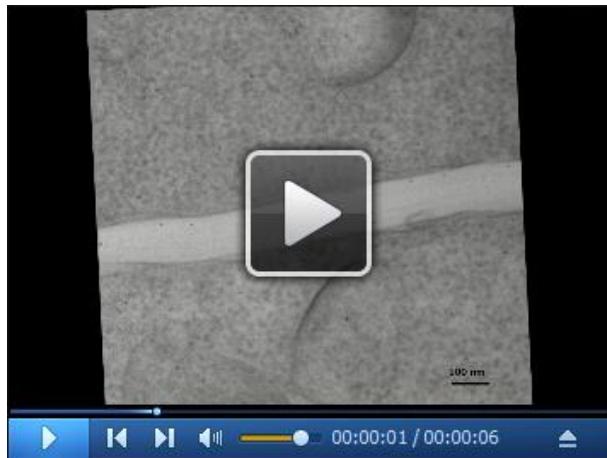
- e. Although it is usually not a problem when using non-carbonated parlodion-filmed copper grids, if sections are repelled by the grids due to static charge during the collection step, grids can be glow-discharged prior to section collection.
  - f. Depending on the EM voltage (more voltage equals more kinetic energy of the electrons, and therefore a better ability to image thick samples well) and the size of the structures of interest, thickness of the sections can vary from 90 nm to 180 nm. Finer sections, closer to 90 nm in thickness will yield slightly better x, y resolution, especially in medium range EM voltages as in our case. 180 nm and greater will allow better volume recovery at the expense of x, y resolution when imaged at 120 kV. In the case of PD study, simple ones have a diameter range from 20 to 40 nm thick, so 90 nm thick sections are appropriate. However, it has to be noted that PD can be of various lengths and sizes, especially when it comes to branched complex PD. Therefore they can extend to several hundreds of nm across the cell wall in all directions. In this case, increasing the section thickness grants better chances to fully recover the volume of the pores.
3. Coating grids with fiducial markers (gold particles)
- Gold particles are used for the subsequent alignment steps required prior to tomogram reconstruction. Because pure colloidal gold tends to aggregate due to its inherent negative charge, it is often used diluted with BSA, making the spread of the gold more homogenous across the section.
- a. On a sheet of paraffin film, lay drops of gold solution (see Recipe 8) and water (Figure 5).
  - b. Gently lay the grids on the gold drops for 20 sec approximately.
  - c. (Optional) Gently pipet up and down the mixture to favor good contact between the grid and the gold.
  - d. Sequentially lay the grids for a few seconds on two water drops to remove excess gold solution.
  - e. Absorb the remaining liquid by approaching absorbent paper (Whatman paper Grade 5) on the side of the grid.
  - f. Repeat Steps D3a to D3e on the other side of the grid in order to have gold particles on both sides.
  - g. Let the grids dry prior to introduction in the EM (damages the ionic pump system otherwise).



**Figure 5. Laying the fiducial markers on the grids.** Typical setting for fiducial marker deposition on grid prior to tomography. Grids are first laid on top of the red drops (fiducial marker + BSA solution) for 15-20 sec then dragged consecutively in the water drops and carefully dried by the rim of the grid with absorbent paper. This process needs to be repeated on both sides of the grids.

### Data analysis

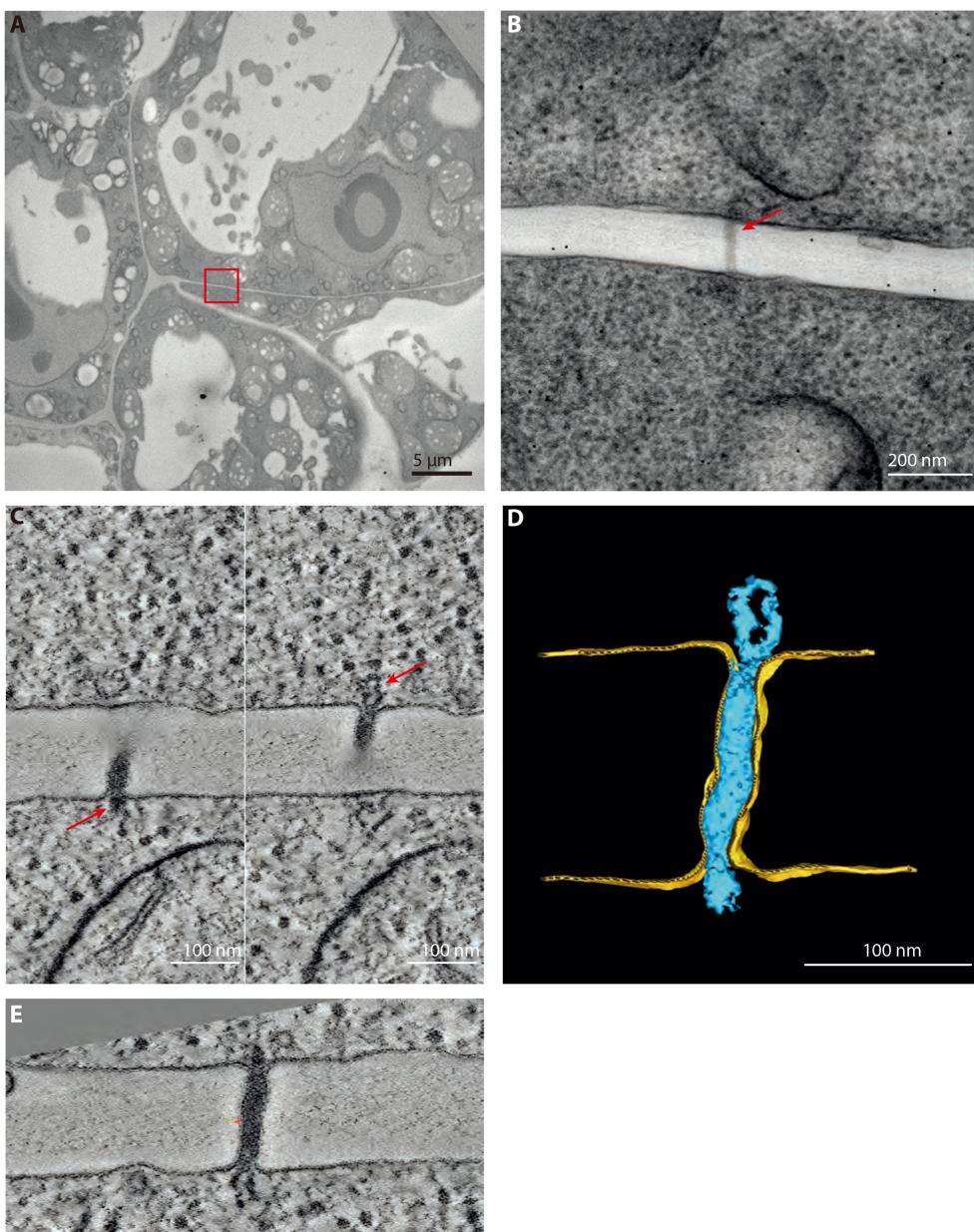
1. With this high-pressure freezing–freeze substitution protocol, morphological study of nanometric details inside the cells is rendered possible. Combined with the use of Lowicryl HM-20 resin, more fragile but more electro-lucent and able to polymerize at low temperature (with UV light), this protocol allows efficient tomographic reconstruction of details around 10 nm in dimension. Low magnification images show well-preserved clumps of cells (Figure 6A). At the interfaces between these cells, PD are seen bridging the cells (Figure 6B). Tilt series acquisition of a typical type I pore (Video 5) renders tomograms where the whole volume of the pore can be appreciated (Figures 6C-6E and Video 6).



**Video 5. Tilt series of type I PD.** Full range tilt series ( $-65^\circ$  to  $+65^\circ$ ) of the type I plasmodesma (in the centre) found in 4 days old cultured cells, processed with this protocol and shown in Figure 5. Dense black spots are the fiducial markers used for alignment with *Etomo*.



**Video 6. Tomogram of type I PD.** Reconstructed tomogram and its manual segmentation of the type I plasmodesma showed in Figure 5.



**Figure 6. Representative data obtained after HPF and FS.** A. Overview of 4 days old cells processed with the procedure described in this article and used in (Nicolas *et al.*, 2017b). B.  $0^\circ$  tilt of the tilt series of a type I plasmodesma (red arrow) found in the red boxed region in (A). Section thickness: 180 nm. C. 0.56 nm thick tomographic slices of the plasmodesma showed in (B). Left and right panel show the two extremities of the pore where the ER can be seen entering the pore (red arrows). D. Segmentation, realized with IMOD, of the same plasmodesma. E. Resliced tomographic slice of the plasmodesma in (C) to have the whole length on one plane. Done with the slicer tool of 3DMOD.

2. This protocol has also succeeded in delivering clear snapshots of plant organelles such as lipid droplets and Golgi apparatuses (Wattelet-Boyer *et al.*, 2016; Brocard *et al.*, 2017)
3. Biological replicates and independent experiments:

PD counting and PD tomography were done on at least two biological replicates, three if possible. For cultured cells, one biological replicate constituted one independent high pressure freezing session on a particular date. Indeed, because the cultured cells were usually sampled in the same flask, we did not consider different blocks from the same HPF as biological replicates.

For roots, each seedling was considered an individual, therefore were considered biological replicates. Nevertheless, data from roots was also collected on multiple HPF session.

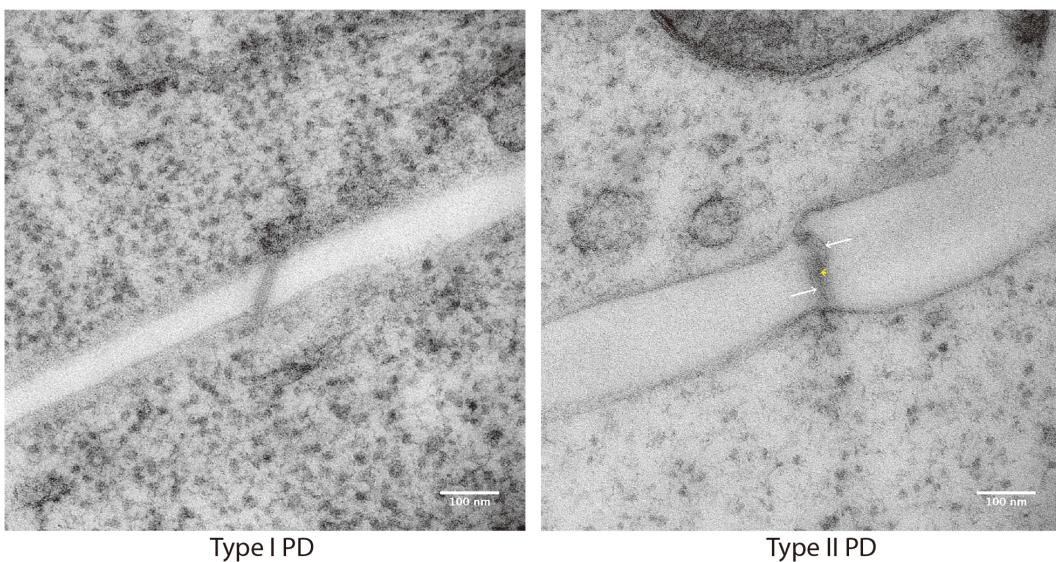
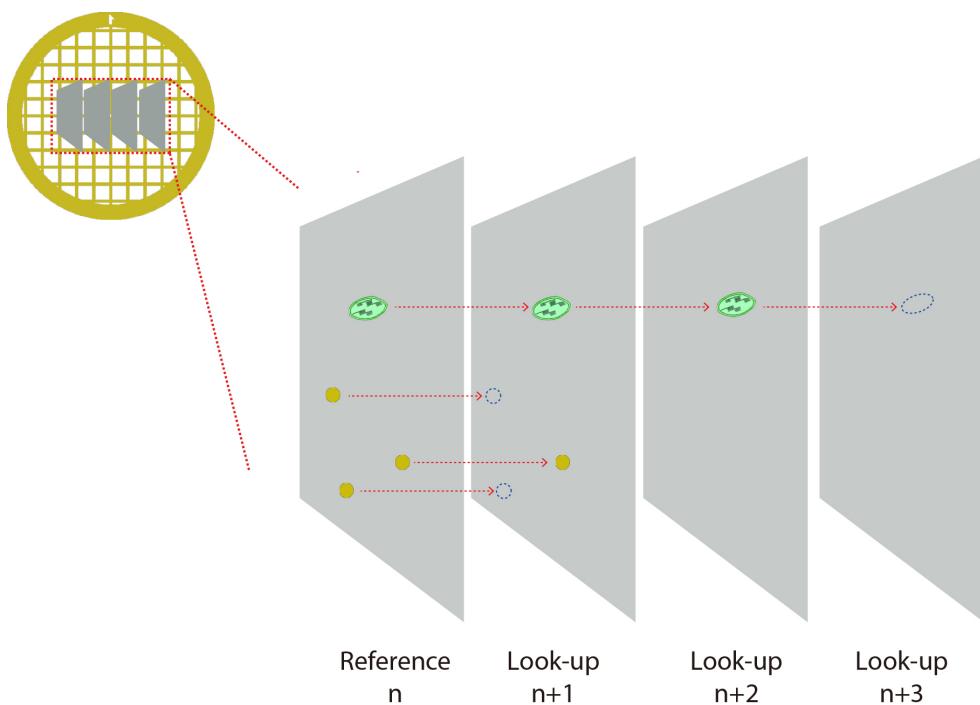
4. PD screening method:

In (Nicolas *et al.*, 2017b), counting type I versus type II pores followed basic principles of stereology (Lucocq, 1993). These are: i) avoid double counting the pores and ii) establishing a precise rule on when a PD can be included in data or not. It is important to stress out that this general method can be applied to counting other features on PD (simple versus branched for example) and also other organelles.

i) When counting on serial sections, there is an inherent risk of double counting structures that span on multiple sections. This risk increases with the size of the structures of interest. Accordingly, a precise counting protocol adapted to the size of the object of interest is required. First, the ‘chance’ of encountering a given structure of interest on multiple contiguous sections is assessed. The structures are spotted on a ‘reference’ section ( $n$ ), then we assess if these same structures are still present on a ‘look-up’ section ( $n + 1$ ). If the structure is not there anymore, it is said to be ‘resolved’ (Figure 6). The fraction of resolved features can then be computed for section  $n + 1$ ,  $n + 2$  etc., depending on the size of object of interest relative to the section thickness.

In the case of PD, which are at the most 40 nm wide, they are largely inferior to section thickness. Hence, at the  $n + 1$  section in cultured cells (Nicolas *et al.*, 2017b Figure 3g), 80% of the pores screened were resolved at section  $n + 1$ . Therefore, by counting every 2 sections ( $n$ ,  $n + 2$ ,  $n + 4$  etc.), we can reasonably avoid the double counting of PD.

ii) PD were counted as ‘valid’ and included in the data when both of the plasma membranes were visible and the PD connected at least one of the two adjacent cells (Figure 7).



**Figure 7. PD counting on serial sections.** A. Cartoon depicts 4 serial sections on a grid. Structures of interest are spotted on the reference section  $n$  and then followed on look-up sections  $n + 1$ ,  $n + 2$  etc. When structure cannot be seen anymore, it is said to be resolved. (B-C) Microographies of 90 nm thick sections of a type I PD (left) and type II (PD) typically collected for quantification. Membranes of the pores are visible, and they connect with both sides. In the latter, heterogeneous densities inside the pore allow the visualization of cytoplasmic sleeve space (white arrows). Although not as resolute as tomography, the central element (desmotubule) can be seen spanning the pore in the center.

## **Notes**

### 1. Liquid nitrogen manipulation

Because liquid nitrogen can burn, always wear adapted eye/face protection, hand protection and a lab coat during manipulation of liquid nitrogen containers.

Because this gas can cause asphyxiation, always ensure the room is properly ventilated.

### 2. Cryosubstitution reagents and resin

Glutaraldehyde and uranyl acetate solutions must be stored at -20 °C in a separate container. Osmium tetroxide should be stored apart, preferably under a vented hood and always manipulated with special lab-gloves (SHIELDskin Orange nitrile 250). When cryomix is being manipulated outside a ventilated hood, *i.e.*, in the AFS during extraction of the cryomix, experimenter (and others present in the same room) should have complete protection on, including lab-gloves, lab-coat, cartridge mask (SPERIAN half mask and T48-ABEK1 P3 dual cartridges) and protective eyewear to avoid any contact with osmium tetroxide fumes.

HM20 and any other kind of resin should also be manipulated with gloves and cartridge mask as well. For convenience, we wear complete protective gear during all steps from starting of the FS to resin polymerization.

Overall, all these compounds, including acetone, should be manipulated using lab-gloves at all time. Gloves should be changed every 20 min and changed immediately when contaminated.

### 3. Bath changing advice during cryosubstitution

Changing baths during FS must be done with care and patience. Gently pipet out the previous medium with the thin tips 1 ml pipets (prevents accidental sucking in of the sample). During this step avoid pipetting in and out; turbulences may detach the samples from the carriers. Always leave remaining medium at the bottom to prevent the samples from air-drying. Then add the next medium gently with a regular plastic pipet (either size) by letting it drip on the plastic tube wall.

### 4. Unambiguous tracing of samples in moulds

Keep precise track of what sample/condition is in which mould to avoid any ambiguity. The moulds can be marked with colored nail polish with multiple shapes/dots to distinguish them one-another.

## **Recipes**

### 1. Culture media for cells and seedlings

#### a. Murashige and Skoog medium for liquid cultured cells (1 L)

Murashige and Skoog medium + vitamins (Duchefa Biochemi): 4.41g

Sucrose: 30 g

2-(N-morpholino)ethanesulfonic acid (MES) (Euromedex): 0.5 g

NAA: 50 µl

Kinetin stock solution: 50 µl

Adjust pH with 1 N KOH solution to 5.8

Autoclave flasks 110 °C for 30 min

*Note: Cells are cultivated under constant light (20 µE/m/sec) and constant agitation at 22 °C.*

b. Murashige and Skoog medium for seedlings (1 L)

Murashige and Skoog medium + vits (Duchefa Biochemi): 4.4 g

MES (Euromedex): 0.5 g

Plant agar (Duchefa Biochemi): 7 g

Adjust pH with 1 N KOH solution to 5.8

Autoclave flasks 110 °C for 30 min

*Note: Seedlings are cultivated in vertically placed plates in order for the root to stay on the surface of medium in a greenhouse at 22 °C set on a long day photoperiod (16 h, 100 µE/m/sec).*

2. BSA solution for cryoprotection during HPF (2 ml)

Weigh 0.4 g of BSA and put it in a 15 ml Falcon tube

Complete at the 2 ml mark with liquid MS medium for cells (see Recipe 1)

Vortex thoroughly until BSA is dissolved entirely

Store at -20 °C

3. Cryosubstitution Uranyl-acetate stock solution (20%, 500 µl)

Weigh 0.1g of uranyl acetate powder (gloves and dust mask required) and put in 2 ml screwtop tube

Add pure methanol (under ventilated hood) up to 500 µl

Wrap the tube in tinfoil and store in the dark, at -20 °C in dedicated cryosubstitution box

4. Cryosubstitution mix

Compound (init. Conc.)	Volume or amount	Final concentration
Glutaraldehyde 10%	0.25 ml	0.5%
Uranyl acetate 20%	0.025 ml	0.1%
Pure acetone	4.725 ml	-
OsO <sub>4</sub> (put in last)	0.1 g	2%
Final volume	5 ml	-

5. HM20% solutions at different concentrations (4 ml)

Make HM20 solutions in the FS Leica plastic solvent containers (Figure 3, #10). Make the solutions in advance and pre-cool in AFS well at -50 °C before use

HM20 concentration	HM20 volume (ml)	Pure ethanol (ml)
25%	1	3
50%	2	2
75%	3	1
100%	4	0

6. 2% Parlodion solution for grid filming (20 ml)  
Weigh 0.4 g of solid parlodion (Electron Microscopy Sciences)  
Add isoamyl-acetate (store in a vented solvent cupboard and keep away from humidity) up to 20 ml  
Agitate until the solid parlodion is dissolved  
Wrap the flask in tinfoil and seal the lid with Parafilm
7. Toluidine blue solution  
Make **mother solution**: 1 g of Toluidine blue powder (Sigma-Aldrich) and 1 g of sodium borate (Sigma-Aldrich) in 20 ml of distilled water  
Working solution is diluted 25 times in distilled water
8. Fiducial marker solution  
Make a solution of 0.5% BSA in MilliQ water  
Filtrate solution with a 0.2 µm filter connected to a syringe  
Mix colloidal gold solution in 0.5% BSA (1:1 ratio)  
Aliquot in 0.5 ml Eppendorf tubes and store at -20 °C

### **Acknowledgments**

This work was supported by the Region Aquitaine (to E.M.B) and PEPS (Initial Support for Exploratory Projects to E.M.B) and the National Agency of Research (Grant ANR-14-CE19-0006-01 to E.M.B). All sample preparation and imaging was done on the Pôle Imagerie du Végétale, appended to the Bordeaux Imaging Centre (<http://www.bic.u-bordeaux.fr/>). The Region Aquitaine also supported the acquisition of the electron microscope (grant No. 2011 13 04 007 PFM). Thanks to Clément Chambaud that assisted in making the explanatory videos. The authors declare having no conflicts of interests.

### **References**

1. Brocard, L., Immel, F., Coulon, D., Esnay, N., Tophile, K., Pascal, S., Claverol, S., Fouillen, L., Bessoule, J. J. and Brehelin, C. (2017). [Proteomic analysis of lipid droplets from \*Arabidopsis\* aging leaves brings new insight into their biogenesis and functions](#). *Front Plant Sci* 8: 894.
2. Ding, B., Turgeon, R. and Parthasarathy, M. V. (1992). [Substructure of freeze-substituted plasmodesmata](#). *Protoplasma* 169: 28-41.
3. Donohoe, B. S., Mogelsvang, S. and Staehelin, L. A. (2006). [Electron tomography of ER, Golgi and related membrane systems](#). *Methods* 39(2): 154-162.
4. Dubochet, J. (2007). [The physics of rapid cooling and its implications for cryoimmobilization of cells](#). *Methods Cell Biol* 79: 7-21.
5. Hagler, H. K. (2007). [Ultramicrotomy for biological electron microscopy](#). In: Kuo, J. (Ed.). *Electron Microscopy: Methods and Protocols*. *Humana Press* pp: 67-96.

6. Kang, B. H., Nielsen, E., Preuss, M. L., Mastronarde, D. and Staehelin, L. A. (2011). [Electron tomography of RabA4b- and PI-4K \$\beta\$ 1-labeled trans Golgi network compartments in \*Arabidopsis\*](#). *Traffic* 12(3): 313-329.
7. Kremer, J. R., Mastronarde, D. N. and McIntosh, J. R. (1996). [Computer visualization of three-dimensional image data using IMOD](#). *J Struct Biol* 116(1): 71-76.
8. Lucocq, J. (1993). [Unbiased 3-D quantitation of ultrastructure in cell biology](#). *Trends Cell Biol* 3(10): 354-358.
9. Nicolas, W. J., Grison, M. S. and Bayer, E. M. (2017a). [Shaping intercellular channels of plasmodesmata: the structure-to-function missing link](#). *J Exp Bot*.
10. Nicolas, W. J., Grison, M. S., Trepout, S., Gaston, A., Fouche, M., Cordelieres, F. P., Oparka, K., Tilsner, J., Brocard, L. and Bayer, E. M. (2017b). [Architecture and permeability of post-cytokinesis plasmodesmata lacking cytoplasmic sleeves](#). *Nat Plants* 3: 17082.
11. Oparka, K. J. and Roberts, A. G. (2001). [Plasmodesmata. A not so open-and-shut case](#). *Plant Physiol* 125(1): 123-126.
12. Studer, D., Graber, W., Al-Amoudi, A. and Eggli, P. (2001). [A new approach for cryofixation by high-pressure freezing](#). *J Microsc* 203(Pt 3): 285-294.
13. Tilney, L. G., Cooke, T. J., Connelly, P. S. and Tilney, M. S. (1991). [The structure of plasmodesmata as revealed by plasmolysis, detergent extraction, and protease digestion](#). *J Cell Biol* 112(4): 739-747.
14. Wattelet-Boyer, V., Brocard, L., Jonsson, K., Esnay, N., Joubes, J., Domergue, F., Mongrand, S., Raikhel, N., Bhalerao, R. P., Moreau, P. and Boutte, Y. (2016). [Enrichment of hydroxylated C24- and C26-acyl-chain sphingolipids mediates PIN2 apical sorting at trans-Golgi network subdomains](#). *Nat Commun* 7: 12788.

## Real-time Analysis of Auxin Response, Cell Wall pH and Elongation in *Arabidopsis thaliana* Hypocotyls

Lanxin Li, S. F. Gabriel Krens, Matyáš Fendrych\* and Jiří Friml

Institute of Science and Technology (IST) Austria, Klosterneuburg, Austria

\*For correspondence: [fendryc1@natur.cuni.cz](mailto:fendryc1@natur.cuni.cz)



**[Abstract]** The rapid auxin-triggered growth of the *Arabidopsis* hypocotyls involves the nuclear TIR1/AFB-Aux/IAA signaling and is accompanied by acidification of the apoplast and cell walls (Fendrych *et al.*, 2016). Here, we describe in detail the method for analysis of the elongation and the TIR1/AFB-Aux/IAA-dependent auxin response in hypocotyl segments as well as the determination of relative values of the cell wall pH.

**Keywords:** Auxin signaling, Cell wall pH, Cell elongation, Hypocotyl, Live-cell imaging

**[Background]** Phytohormone auxin induces rapid growth in *Arabidopsis thaliana* hypocotyls. This process requires the TIR1/AFB-Aux/IAA auxin co-receptor. Auxin promotes the binding of TIR1/AFB and Aux/IAA, which leads to ubiquitination and degradation of the latter, and results in transcription of auxin-responsive genes. This protocol focuses on measuring the growth, auxin signaling and cell wall acidification in *Arabidopsis thaliana* etiolated hypocotyls. This protocol is based on the previous work of Schenck *et al.*, 2010, Takahashi *et al.*, 2012, Fraas *et al.*, 2014 and Spartz *et al.*, 2014; but unlike the published work, we describe the procedures that enable measuring a larger spectrum of processes occurring during growth of hypocotyls; from the macroscopically visible organ elongation, cell wall pH monitored by confocal microscopy to the real-time nuclear auxin signaling visualized by luciferase bioluminescence.

### **Materials and Reagents**

1. Aluminum foil
2. Razor blades (Gillette Wilkinson<sup>TM</sup> Sword)
3. Cellophane foil 80 mm diameter (AA Packaging, catalog number: 325 P cellulose film)
4. Black filter paper 90 mm diameter (MACHEREY-NAGEL, catalog number: 409009)
5. Falcon 60 x 15 mm dishes (Corning, catalog number: 353004)
6. 12-well tissue culture plates (TPP Techno Plastic Products, catalog number: 92412)
7. 2-well Lab-Tek<sup>TM</sup> chambered #1.0 borosilicate cover glass (Thermo Fisher Scientific, catalog number: 155380)
8. *Arabidopsis thaliana* seeds: Col-0, apo-pHusion apoplastic pH marker line (Gjetting *et al.*, 2012), auxin responsive promoter driving the expression of the firefly luciferase enzyme marker DR5::LUC (Moreno-Risueno *et al.*, 2010)

9. Household bleach (sodium hypochlorite 4.7%)
10. 37% hydrochloric acid (Sigma-Aldrich, catalog number: 435570)
11. MES (Duchefa Biochemie, catalog number: M1503.0100)
12. Sucrose (Sigma-Aldrich, catalog number: 84097-1KG)
13. Potassium hydroxide (KOH) (Merck, catalog number: 105021)
14. Agar, plant cell culture tested (Alfa Aesar, catalog number: H26724)
15. Potassium chloride (KCl) (Sigma-Aldrich, catalog number: P9541-500G)
16. Phytigel (Sigma-Aldrich, catalog number: P8169)
17. 10 mM 3-Indoleacetic acid (IAA) (Sigma-Aldrich, catalog number: I2886-5G) dissolved in ethanol
18. 1 mM D-luciferin (Duchefa Biochemie, catalog number: L1349.0100) dissolved in 1x PBS
19. Chlorine gas (see Recipe 1)
20. Half-strength MS agar media (AM+) (see Recipe 2)
21. Depletion medium (DM) (see Recipe 3)

## Equipment

1. Forceps Dumont #5
2. Binocular dissecting microscope Leica EZ4 (Leica Microsystems, model: Leica EZ4)
3. Flatbed scanner Epson Perfection V370 Photo (Epson, model: V370 Photo)
4. For the bioluminescence dark box:  
Lumazine Manual Stage Dark Box (Photometric, model: LMZ-DRK-BOX)  
Evolve EMCCD camera (Photometric, model: Evolve® 512, catalog number: EVO-512-M-FW-16-AC-RP)  
17 mm fixed lens/0.95 (Edmund Optics, model: 59-832)  
125 mm lens (Thorlabs, model: LA1384-A)
5. Zeiss 700 LSM confocal microscope (ZEISS, model: LSM 700) with a 20x/0.8 Plan-Apochromat M27 objective

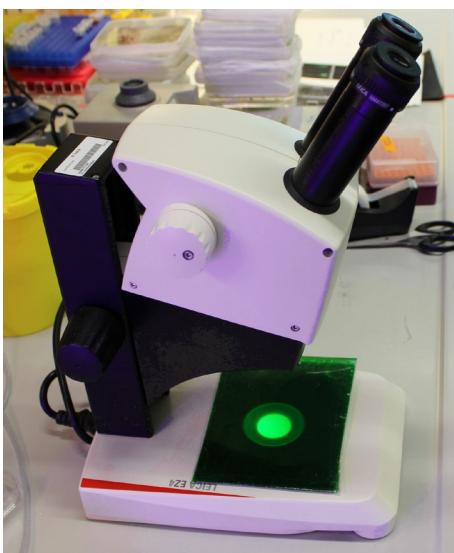
## Software

1. Microsoft Excel program (<https://products.office.com/en/excel>)
2. Fiji program (<http://fiji.sc/>)
3. MATLAB program (<https://www.mathworks.com/products/matlab.html>)
4. AutoIt program (<https://www.autoitscript.com/site/autoit/>)

## **Procedure**

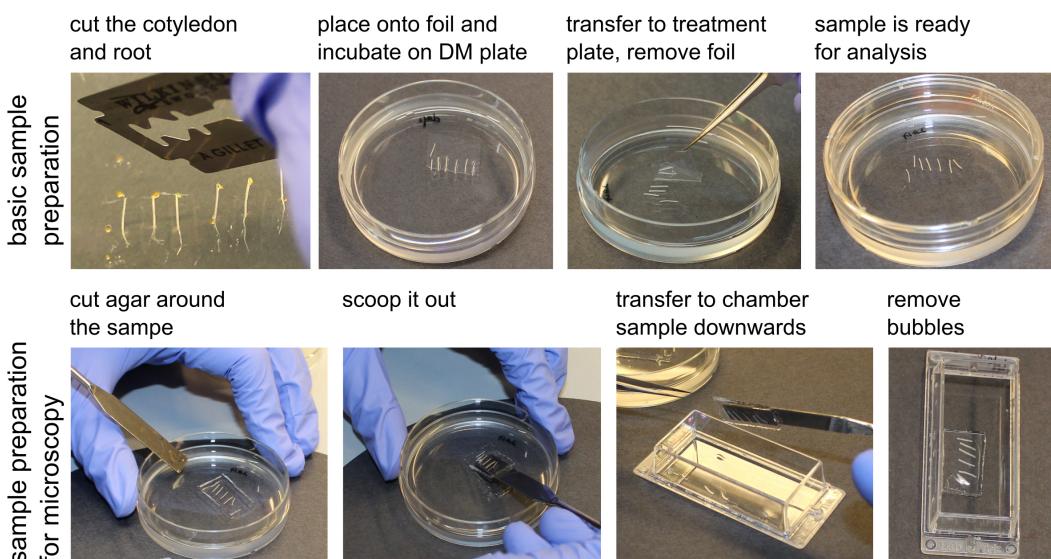
### A. Hypocotyl elongation measurement

1. Surface sterilize Col-0 seeds (or any other genotype of your interest) by chlorine gas (Recipe 1) overnight. Plate the sterilized seeds on the AM+ medium (Recipe 2). Stratify for two days at 4 °C in the dark, then place vertically under light to cultivate for around 6 h in a growth room at 21 °C. Wrap with aluminum foil and grow for another 66 h vertically at 21 °C.
2. Prepare a depletion plate with 5 ml depletion medium (DM, Recipe 3) in a Falcon 60 x 15 mm dish. After solidification, place cellophane foil onto the surface. Damp the cellophane foil with liquid depletion medium solution.
3. Place a dissecting microscope in a dark room and cover the illumination with a green filter made of 8 layers of green office foil (Figure 1).



**Figure 1. Binocular dissecting microscope with a green filter**

4. Uncover the Petri dishes with seedlings and select the seedlings with similar hypocotyl length excluding the longest and shortest ones. Decapitate the seedlings right below the apical hook and before the shoot-root junction to get a hypocotyl segment by cutting them on the surface of the agar using a very sharp razor blade. Prepare 6-8 segments for each treatment. Using sharp forceps, transfer the segments onto the cellophane foil in the depletion plate without squeezing them, the sample preparation procedure is depicted in Figure 2. Keep in darkness for 30-60 min.



**Figure 2. The sample preparation procedure**

5. Afterwards, transfer the segments by flipping the cellophane foil onto a treatment plate with the depletion medium supplemented with the desired treatment (Figure 2), in our case 10  $\mu$ M 3-Indoleacetic acid (IAA) and the mock control (Ethanol equivalent).
  6. Immediately place the treatment plates on a flatbed scanner, imaging through the layer of the phytagel. A wet black filter paper is placed into the lid of the dish to improve the contrast of the image. Scan the samples in the 8-bit grayscale and at 2,400 dpi every 10 min automatically using the Autolt program (see [Supplemental file 1](#)).
- B. Measuring the TIR1/AFB-Aux/IAA dependent response using the DR5::LUC marker line
1. Prepare the 6-10 decapitalized segments of DR5::LUC marker line for each treatment, as described before, on depletion medium. Add around 50  $\mu$ l of 1 mM D-luciferin dissolved in 1x PBS and immerse the segments entirely for 30 min.
  2. Prepare the treatment solution (DM with desired drugs)—in our case, DM + mock or DM + 10  $\mu$ M IAA. Pour 3 ml medium into each well of a 12 wells tissue culture plate and let the medium solidify; four wells can be imaged simultaneously.
- Note: There is no D-luciferin in the treatment solution, the substrate originates from Step B1. This is sufficient for approx. 5-h imaging. Alternatively, one can put D-luciferin in the treatment solution for a longer time of imaging.*
3. Using the cellophane foil, transfer the segments onto the surface of the treatment medium and remove the foil.
  4. Immediately image in a dark box (Figure 3) with a Photometric Evolve<sup>®</sup> EMCCD camera equipped with a 17 mm fixed lens/0.95 and an additional 125 mm lens. Set the multiplier EMCCD gain to 150 and the exposure time to 110 sec, and image every 2 min.



**Figure 3. Dark box with a Photometric Evolve EMCCD camera**

- C. Imaging the apoplastic pH using apo-pHusion apoplastic pH marker line
1. Prepare the decapitated hypocotyl of the apo-pHusion apoplastic pH marker line as above.
  2. Prepare 5 ml of DM medium with or without 10  $\mu$ M IAA.
  3. Transfer 5 hypocotyl segments onto the surface of the agar with the treatment. Cut out a piece of the agar with the segments using a spatula. Place the agar with the segments into the Lab-Tek<sup>TM</sup> chambered cover glass so that the segments are placed between the cover glass and the agar. When using the 2-well chambered glass, a treatment and a control sample can be imaged simultaneously.
  4. Alternatively, the treatment can be very carefully pipetted to the hypocotyl segments during imaging. Then ~50  $\mu$ l DM with the treatment can be used, but one must be extremely careful not to move the sample during imaging.
  5. Using a confocal microscope with a 20x/0.8 Plan-Apochromat M27 objective, set the position of each segment using the position manager so that the apical region of the hypocotyl segment is imaged. Image 5 z-sections, z-thickness matched to the pinhole size, of each hypocotyl segment.
  6. Set the microscope for simultaneous imaging of GFP and RFP by exciting using 488 and 555 nm diode lasers, and splitting the emitted light with a short pass 550 nm and long pass 560 nm filters, 16 bits per pixel. Image all positions every 5 min.

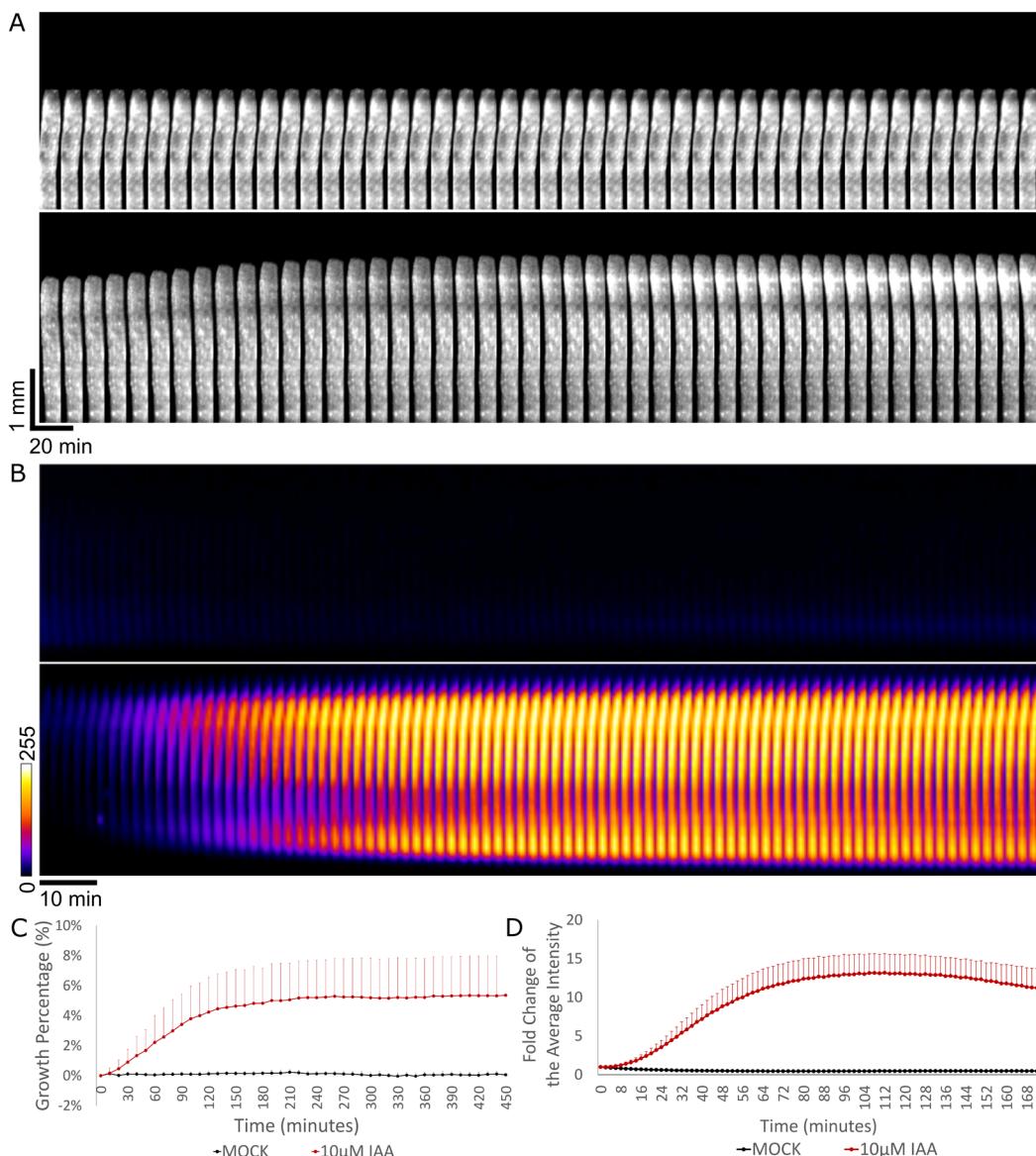
## **Data analysis**

### A. Hypocotyl elongation image analysis

1. To achieve unbiased measurement, we created a Fiji macro (see [Supplemental file 1](#)) for analyzing the length of the segment at each time point. The macro firstly creates the time lapse of the image sequence captured from the scanner, then allows you to manually create a rectangle ROI for each segment, followed by automatically thresholding each ROI and measuring the Feret's diameter, the maximum caliper, as the length of the segment. The macro eventually generates '.txt' file for each ROI or hypocotyl, including the Feret's diameter of that hypocotyl in each time point.
2. Copy and paste the result into Excel, set the initial length of the segment as 100%, and calculate the length of the hypocotyl at each time point to obtain a growth curve (Figure 4C). Besides, growth can be visualized by creating a montage-kymograph of individual hypocotyl segment in Fiji (Figure 4A).

### B. Analysis of the bioluminescence intensity

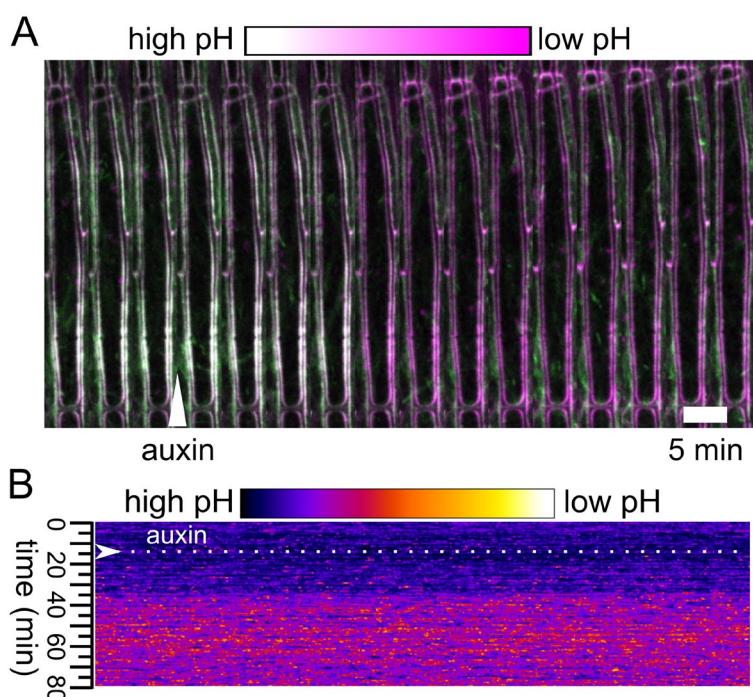
1. Analyze the image sequence in Fiji (Schindelin *et al.*, 2012). Manually outline all the segments via Polygon selection, at their brightest time frame, and add them into the region of interest (ROI) manager, followed by multi-measuring the mean grey value. This gives the average intensity of each segment at each time point.
2. Copy and paste the result into Excel. Take the initial intensity of the segment as 100%, and analyze the average of the luminescence intensity of each hypocotyl at each time point, to get an intensity curve in time. Additionally, one can visualize the growth and the luminescence intensity by creating a montage-kymograph in Fiji (Figures 4B and 4D).



**Figure 4. Hypocotyl segment growth and DR5::LUC intensity measurements.** A. The kymograph of the hypocotyl segment of Col-0 under mock treatment (upper row) and 10  $\mu$ M IAA treatment (lower row) from 0 to 460 min; time interval of 10 min. The kymograph was done by making montage of the growing part of one representative sample in Fiji. (Note that to visualize the growth better, the upper half part of the hypocotyl where growth takes place was used for making montage). Vertical and horizontal scale bars represent 1 mm and 20 min, respectively. B. The kymograph of the luminescence intensity in the mock-treated DR5::LUC hypocotyls (upper row) and 10  $\mu$ M IAA-treated (lower row) from 0 to 172 min; time interval of 2 min. The ‘FIRE’ look-up table was applied in Fiji. Scale bar is 10 min. C. Quantification of the growth of Col-0 hypocotyls treated with mock or 10  $\mu$ M IAA from 0 to 460 min. The growth is expressed as the percentage of the original segment length. D. Quantification of the luminescence intensity in the DR5::LUC hypocotyls treated with mock or 10  $\mu$ M IAA. The Fold change is the average intensity of the hypocotyls normalized by the intensity at timepoint 0.

### C. Image analysis of the cell wall pH

1. Analyze the apoplastic pH using Fiji. We use the SUM projections of the z-stacks (Figure 5A). Set the threshold of the apoplast region using the RFP channel so that only the cell wall signal is selected. Create the selection using the ‘create selection’ command and measure intensity in GFP and RFP channels. Analyze the intensity ratios in Excel program. We analyze the apoplastic pH change in relative values the lower the GFP intensity, the lower the apoplastic pH is (Gjetting *et al.*, 2012).
2. Alternatively to Step C1, the apoplastic pH can be visualized and measured using the AreaKymo MATLAB® script, Figure 5B ([Supplemental file 1](#)). The AreaKymo script essentially does the same procedure as described in Step C1, but does so automatically without the user input, allowing for rapid processing of several hypocotyls at a time. The user first merges several hypocotyl SUM projection time series into one ‘tif.’ series using the Fiji program (‘combine stacks’ command) and converts them into 16-bit ‘tif.’ images (MATLAB does not handle well the 32-bit images that the SUM projection command creates). Then the user should find a threshold of the RFP channel that is optimal for selecting just the apoplast signal. In MATLAB, the AreaKymo script is run, the combined time series is selected, and the user specifies the value of the threshold for the RFP channel and the desired width of the rectangle that will represent the individual timeframe. The script outputs the visual representation of apoplastic pH and also the values in the form of a series of boxplots.



**Figure 5. Analysis of the apoplast pH.** A. The apoplast pH after application of auxin to a hypocotyl expressing the apo-pHusion sensor. Auxin application is indicated with an arrowhead, GFP is shown in green while RFP in magenta. B. The output of the AreaKymo MATLAB® script.

Time is progressing from top to bottom; ratio of the two fluorophores is shown using the 'FIRE' look-up-table.

### **Notes**

During all steps where the hypocotyls are manipulated (cut and transferred to new plates) it is crucial to be extremely gentle with the tissue, not squeeze it but rather scoop it using sharp forceps. The tissue needs to be protected from drying; plates must be kept closed whenever possible to prevent excessive evaporation.

### **Recipes**

1. Chlorine gas sterilization

100 ml household bleach

4.5 ml 37% HCl

2. Half-strength MS agar media (AM+)

Half Murashige and Skoog Basal Salts

1% sucrose

Adjust pH to 5.8 by KOH

0.8% agar, plant cell tested

MiliQ water as solvent

3. Depletion medium (DM)

10 mM KCl

1 mM MES

Adjust pH to 6 by KOH

1.5% phytigel

MiliQ water as solvent

*Note: The phytigel brings better transparency than normal agar, contributing to the better quality of the scanning.*

### **Acknowledgments**

This protocol was adapted from Fendrych *et al.*, 2016. This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie Grant Agreement No. 665385, and Austrian Science Fund (FWF) [M 2128-B21].

The authors declare no conflict of interests.

## References

1. Fendrych, M., Leung, J. and Friml, J. (2016). [TIR1/AFB-Aux/IAA auxin perception mediates rapid cell wall acidification and growth of \*Arabidopsis\* hypocotyls](#). *Elife* 5.
2. Fraas, S., Niehoff, V., and Lüthen, H. (2014). [A high-throughput imaging auxanometer for roots and hypocotyls of \*Arabidopsis\* using a 2D skeletonizing algorithm](#). *Physiol Plant* 151: 112-118.
3. Gjetting, K. S., Ytting, C. K., Schulz, A. and Fuglsang, A. T. (2012). [Live imaging of intra- and extracellular pH in plants using pHusion, a novel genetically encoded biosensor](#). *J Exp Bot* 63(8): 3207-3218.
4. Moreno-Risueno, M. A., Van Norman, J. M., Moreno, A., Zhang, J., Ahnert, S. E. and Benfey, P. N. (2010). [Oscillating gene expression determines competence for periodic \*Arabidopsis\* root branching](#). *Science* 329(5997): 1306-1311.
5. Schenck, D., Christian, M., Jones, A., and Lüthen, H. (2010). [Rapid auxin-induced cell expansion and gene expression: a four-decade-old question revisited](#). *Plant Physiol* 152: 1183-5.
6. Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J. Y., White, D. J., Hartenstein, V., Eliceiri, K., Tomancak, P. and Cardona, A. (2012). [Fiji: an open-source platform for biological-image analysis](#). *Nat Methods* 9(7): 676-682.
7. Spartz, A.K., Ren, H., Park, M.Y., Grandt, K.N., Lee, S.H., Murphy, A.S., Sussman, M.R., Overvoorde, P.J., and Gray, W.M. (2014). [SAUR inhibition of PP2C-D phosphatases activates plasma membrane H<sup>+</sup>-ATPases to promote cell expansion in \*Arabidopsis\*](#). *Plant Cell* 26: 2129-2142.
8. Takahashi, K., Hayashi, K., and Kinoshita, T. (2012). [Auxin activates the plasma membrane H<sup>+</sup>-ATPase by phosphorylation during hypocotyl elongation in \*Arabidopsis\*](#). *Plant Physiol* 159: 632-41.

## Fluorescent Measurement of Synaptic Activity Using FM Dyes in Dissociated Hippocampal Cultured Neurons

Roman M. Lazarenko, Claire E. DelBove and Qi Zhang\*

Department of Pharmacology, Vanderbilt University, Nashville, USA

\*For correspondence: [qi.zhang@vanderbilt.edu](mailto:qi.zhang@vanderbilt.edu)



**[Abstract]** Release and recycling of synaptic vesicles are essential for neurotransmission and synaptic plasticity. To gain mechanistic understanding of these processes, direct measurements of vesicle release and retrieval is indispensable. Styryl dyes like FM1-43 and FM4-64 have been widely used for this purpose and their loading and unloading are reliable measurements for synaptic vesicle release and retrieval in cultured neurons. This protocol describes in detail the procedure of using styryl dyes to label and measure synaptic vesicle uptake and release in cultured rat hippocampal neurons. We also include a brief description of hippocampal culture. In the end, we briefly discuss the commonality and difference among FM dye, pH-sensitive fluorescent proteins and quantum dots in terms of measuring synaptic vesicle behavior.

**Keywords:** FM1-43, Synaptic transmission, Fluorescence live imaging, Dissociated hippocampal culture

**[Background]** Synaptic vesicles are indispensable for neurotransmission since they are the only organelle responsible for neurotransmitter release in chemical synapses. Their amount, release probability, fusion kinetics and recycling routes define synaptic transmission and neuronal communication. Various tools have been developed to probe synaptic vesicles, including electrophysiological recording of postsynaptic neurons, capacitance measurement of membrane trafficking, amperometry of oxidizable transmitters, electron microscope imaging of fixed synapses, and fluorescence imaging of vesicular labels in live neurons. Among all existing methods, the last is the only one that not only yields both spatial and temporal information about individual synapses but also provides high throughput (*i.e.*, more data points from single synapses of different neurons). Various fluorescent probes based on different targeting and reporting mechanisms have been developed. Styryl dye (*i.e.*, FM dyes including FM1-43, FM4-64, FM5-95), invented more than twenty years ago, remains a reliable and convenient tool. Due to its moderate affinity to lipid membrane and its lipid-sensitive emission, it can be readily loaded into recycled synaptic vesicles and released when those vesicles are exocytosed. Using more sensitive photodetectors like EMCCD, FM dyes can report single vesicle release events. Here, we provide a relatively complete description of FM-based imaging of synaptic vesicle release in primary cultures of rodent hippocampal neurons. In addition, we also discuss the commonality and the distinction between FM dyes and other fluorescent vesicle labels.

## **Materials and Reagents**

1. Pasteur pipette 9 in, cotton-plugged (Fisher Scientific, catalog number: 13-678-8B)
2. Pasteur pipette 5.75 in (Fisher Scientific, catalog number: 13-678-20A)
3. 24-well plates (Corning, Costar®, catalog number: 3524)
4. Kimwipes (KCWW, Kimberly-Clark, catalog number: 34155)
5. Round 12 mm-Ø glass coverslips #0 (Thermo Fisher Scientific, special order, 0.085 mm thick)
6. Aluminum foil (WebstaurantStore Food Service Equipment and Supply Company, Choice, catalog number: 12224X1HD)
7. Platinum wires (Alfa Aesar, catalog number: 10286)
8. 24 x 40 coverslips (Fisher Scientific, special order), all 0.085 mm thick (*i.e.*, size 0)
9. Parafilm PM-996 (Bemis, catalog number: PM996)
10. 30-mm Ø Petri dish (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 153066)
11. 15 ml conical tubes (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 339650)
12. 0.2 µm filter (Corning, catalog number: 431218)
13. 50 ml conical tubes (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 339652)
14. 1.7 ml tubes (Corning, Axygen®, catalog number: MCT-175-C)
15. Neonatal rat hippocampal neurons (P0-P3)
16. Trypsin-EDTA (trypsin: 0.025%/EDTA: 0.01%) (Thermo Fisher Scientific, Gibco™, catalog number: R001100)
17. Minimum Essential Medium (MEM, 1x) (Thermo Fisher Scientific, Gibco™, catalog number: 51200038)
18. Glucose (Thermo Fisher Scientific, Gibco™, catalog number: 15023021)
19. Sodium bicarbonate (NaHCO<sub>3</sub>) (Sigma-Aldrich, catalog number: S5761)
20. Transferrin (Sigma-Aldrich, catalog number: T1428)
21. L-glutamine (Thermo Fisher Scientific, catalog number: 25030081)
22. Insulin (Sigma-Aldrich, catalog number: I5500)
23. Fetal bovine serum (Thermo Fisher Scientific, Gibco™, catalog number: 26140079)
24. Matrigel (Corning, catalog number: 354234)
25. 70% EtOH (Decon Labs, catalog number: 2401)
26. Ara-C (Cytarabine) (Sigma-Aldrich, catalog number: C1768)
27. B27 supplement (Thermo Fisher Scientific, Gibco™, catalog number: 17504044)
28. Vacuum grease (Dow Corning, catalog number: 1597418)
29. FM1-43 (Biotium, catalog number: 70022)
30. 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide disodium salt (NBQX) (Abcam, catalog number: ab120046)
31. D-(*–*)-2-Amino-5-phosphonopentanoic acid (D-AP5) (Abcam, catalog number: ab120003)
32. N-2-hydroxyethyl piperazine-n-2 ethanesulphonic acid (HEPES) (Sigma-Aldrich, catalog number: H4034)

33. DNase (Sigma-Aldrich, catalog number: D5025)
34. 10 mM HCl (diluted with ddi water 1:100 from 1 N solution) (Sigma-Aldrich, catalog number: H9892)
35. Sodium chloride (NaCl) (Fisher Scientific, catalog number: S641-212)
36. Potassium chloride (KCl) (Sigma-Aldrich, catalog number: P5405)
37. Magnesium chloride hexahydrate ( $MgCl_2 \cdot 6H_2O$ ) (Fisher Scientific, catalog number: BP214)
38. Calcium chloride dihydrate ( $CaCl_2 \cdot 2H_2O$ ) (Acros Organics, catalog number: 207780010)
39. Matrigel stock solution (see Recipes)
40. Hanks solution (see Recipes)
41. Hanks + 20 (H + 20) (see Recipes)
42. Dissociation solution (see Recipes)
43. DNase solution (see Recipes)
44. 2 mM Ara-C stock (see Recipes)
45. Plating medium (see Recipes)
46. Ara-C solution (see Recipes)
47. Extracellular bath Tyrode's saline (see Recipes)
48. 90 K (High Potassium Tyrode's) (see Recipes)

## Equipment

1. Bunsen burner (Sigma-Aldrich, catalog number: Z270326)
2. 250 ml beaker (Fisher Scientific, Fisherbrand, catalog number: FB100250)
3. Pyrex beaker (Corning, PYREX®, catalog number: 1000-100)
4. Inverted microscope (20x lens, Mercury arc lamp, filters for EGFP) (Nikon Instruments, model: Eclipse Ti-E)
5. Electric field stimulation chamber (two platinum wires glued to the sides of RC-26G perfusion chamber) (Warner Instruments, model: RC-26G)
6. PH-1 platform (Warner Instruments, model: PH-1)
7. Six Channel Perfusion Control Valve System (Warner Instruments, model: VC-6)
8. Inline heater (e.g., Warner instruments, catalog number: 64-0104)
9. TC-344B Dual Channel Temperature Controller (Warner Instruments, model: TC-344B)
10. Prior Lumen 200 Illuminator (Prior Scientific, model: Lumen 200)
11. Cell culture hood, CO<sub>2</sub> incubator
12. Set of dissection tools (Fine Science Tools)
13. Stimulus Isolator SD9 (Grass Instruments, model: SD9)
14. Digidata 1440A (Molecular Devices, model: Digidata 1440A)
15. Dissection microscope (Nikon Instruments)
16. Centrifuge (Eppendorf, model: 5702 R)
17. Computer with time-lapse imaging system

18. Computer with Clampex software
19. Andor iXon Ultra EMCCD (Andor)
20. Vibration Isolation table (Newport)

### Software

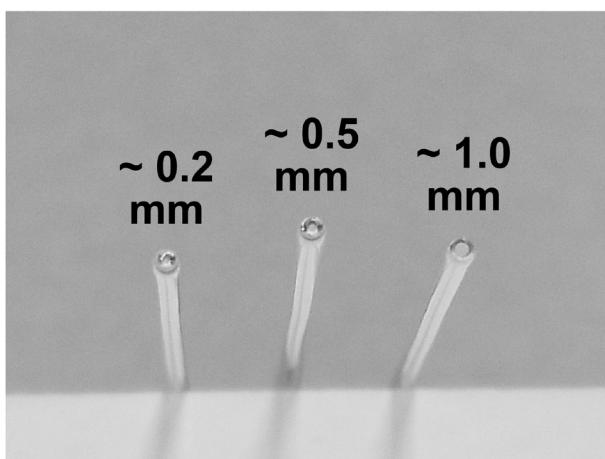
1. Micro-Manager (Schneider *et al.*, 2012) or Fiji (Schindelin *et al.*, 2012)
2. Clampex (Molecular Devices)
3. Microsoft Excel (Microsoft)

### Procedure

#### A. Primary culture of rat hippocampal neurons

*Note: Preparation of neonatal neuronal culture is technically easier than embryonic culture.*

Primary cultures of dissociated postnatal rat hippocampal cells are prepared as previously described (Liu and Tsien, 1995). Briefly, rat hippocampi (CA1-CA3) are normally dissected from P0 to P1 Sprague-Dawley rats and dissociated into a single-cell suspension with a 10-min incubation in trypsin-EDTA (trypsin: 0.025%/EDTA: 0.01%, Thermo Fisher Scientific) followed by gentle trituration using three glass pipettes of decreasing diameters (~1 mm, 0.5 mm, and 0.2 mm), sequentially (Figure 1). Pipettes were obtained by fire-polishing the tips on a Bunsen burner. Soft borosilicate glass melts and shrinks easily if exposed to fire. For the best results, pipette tips are positioned vertically to the flame and are being twisted clockwise and counterclockwise for even heating and melting. Longer flaming makes the smaller diameter tips. It is also important to make sure that the smallest tips are not fully sealed, which sometimes happens. Ideally, the smallest diameter pipette lets cell suspension through slowly and ensures single cell dissociation.



**Figure 1. Example of fire-polished Pasteur pipette tips of different diameters used for dissociation of hippocampi**

1. Dissociated cells are recovered by centrifugation ( $200 \times g$ , 5 min) at  $4^{\circ}\text{C}$  and re-suspended in plating medium composed of Minimal Essential Medium (MEM, Thermo Fisher Scientific) with (in mM) 27 glucose, 2.4 NaHCO<sub>3</sub>, 0.00125 transferrin (Sigma-Aldrich), 2 L-glutamine (Thermo Fisher Scientific), 0.0043 insulin (Sigma-Aldrich) and 10%/vol fetal bovine serum (FBS, Gibco).
2. 100  $\mu\text{l}$  of cell suspension is seeded onto round 12 mm-Ø glass coverslips (200-300 cells/mm<sup>2</sup>) pre-coated with Matrigel (Corning) placed in 24-well plates (Fisher Scientific).

*Note: Use of Matrigel as a neuronal substrate vs. commonly used poly-L-lysine substantially improves cell culture quality. 100  $\mu\text{l}$  of Matrigel is dispensed onto the surface of coverslip 1-24 h before use and is aspirated before the cell suspension is plated. Also, no antibiotics should be used in order to avoid unwanted effects on neuronal properties.*

3. Dissection tools and glassware used for cell culture are being cleaned with no detergents in order to prevent their detrimental impact on neuronal membranes. After use, tools are washed with deionized water; blood remains are removed with Kimwipes. For sterilization before the dissection, tools are incubated with 70% EtOH in a 250 ml beaker (Fisherbrand) for 30 min. To protect fine dissection tools from mechanical damage due to bumping to the bottom of the beaker—we cover the bottom with 2-3 Kimwipes. The coverslips we use are 12 mm #0 (Thermo Fisher Scientific, special order, 0.085 mm thick). Coverslips are autoclaved in a Pyrex beaker covered with 3 layers of aluminum foil for 30 min before use.

*Note: No any other special treatment of coverslips is needed.*

4. Cells are allowed to adhere to the substrate for 30-60 min before the addition of 1 ml plating medium. After 1-2 days in culture, an additional 1 ml medium containing (in mM) 27 glucose, 2.4 NaHCO<sub>3</sub>, 0.00125 transferrin, 0.5 L-glutamine, 0.002 Ara-C, 1%/vol B27 supplement (Thermo Fisher Scientific) and 5%/vol FBS is added. Ara-C (1  $\mu\text{M}$ ) in the culture media efficiently prevents astroglial proliferation.
5. Experiments are performed between DIV 12 and 18 (when synaptic transmission is well established).

## B. Imaging setup

1. For cell culture on coverslips, an inverted microscope is preferred due to the ability to use an oil-immersion objective with high N.A. high magnification. An XYZ motorized stage is preferred but not necessary.
2. An RC-26G chamber (Warner Instruments) is modified with 2 platinum wires attached to the sides of the chamber for delivering electric field stimulation. The chamber is bottom-sealed with a 24 x 40 mm size 0 cover glass (0.085 mm thick) using vacuum grease and clamped on a PH-1 platform (Warner Instruments) placed on a microscope stage (Scientifica). We use a Nikon Eclipse Ti inverted microscope with a 20x Plan Apo VC objective (N.A. 0.75) for large areas or a 100x Apo VC objective (N.A. 1.40) for a detailed view of synaptic boutons.

*Note: The summative thickness of two #0 coverslips (one 24 x 40 coverslip, Fisher Scientific special order) which seals the bottom of the chamber and one 12 mm circular coverslip with*

*cells) is equal to the thickness of one #1.5 standard coverslip (0.17 mm), for which most of the objective lenses are optimized and corrected. This eliminates the need to seal coverslips with cells directly to the bottom of the imaging chamber with vacuum grease.*

3. Solution exchange is achieved via gravity perfusion controlled by a VC-6 valve control system and a 6-channel manifold (Warner Instruments) with a constant rate of ~50  $\mu\text{l/sec}$  which allows a complete exchange of the bath solution in the recording chamber within 30 sec.
4. All experiments are performed at room temperature, but physiological temperature can also easily be achieved by using an inline heater (e.g., 64-0104, Warner Instruments) and a heated stage (e.g., PH-1 stage, Warner Instruments). Both can be set to 34-37 °C (e.g., controlled through TC-344B Dual Channel Temperature Controller, Warner Instruments). To avoid heating induced stage drift, preheating for at least 1 h is recommended. Autofocus such as Nikon Perfect Focus is also helpful.
5. An RC-26G with attached stimulation wires is clamped in PH-1 and a home-made water-tight insert for the Scientifica stage.

*Note: It is advisable to have a leak-proof system for live imaging to minimize possible damage to the microscope. PH-1 is bolted to the stage insert. Laboratory Parafilm PM-996 is used as a seal.*

6. Excitation light sources can be an arc lamp or a laser launched either through a liquid light guide or a laser launcher box. We use a Prior Lumen 200 Illuminator connected to the microscope via a liquid light guide.
7. Fluorescence excitation/dichroic/emission filter combination corresponding to selected FM dyes can be found online (e.g., <http://www.chroma.com>). Filters are either installed in the filter cubes inside the microscope housing, or separately within the light path. For example, FM1-43 imaging is done using a fluorescence filter set: Ex. 460/50; DIC: 495LP; Em: 510/25BP. All optical filters and dichroic mirrors are purchased from Chroma or Semrock.
8. Image acquisition and synchronized perfusion are controlled via Micro-Manager and Clampex software. The acquisition settings including excitation power, fluorescence filter set (excitation, dichroic and emission filters), exposure time, camera gain and frame rate are all kept the same among different samples. Images are taken at 0.1 Hz rate. Baseline fluorescence is captured during 1 min before electrical/chemical stimulation/depolarization of neurons or for at least 3 frames.
9. Imaging with Andor EMCCD. 1) Set Andor temperature to -80 °C; 2) Set Andor readout mode to 5 MHz (instead of 17 MHz); 3) Set Andor vertical speed to 0.5, instead of 3.3: these settings help to increase the dynamic range for imaging fluorescence intensity.

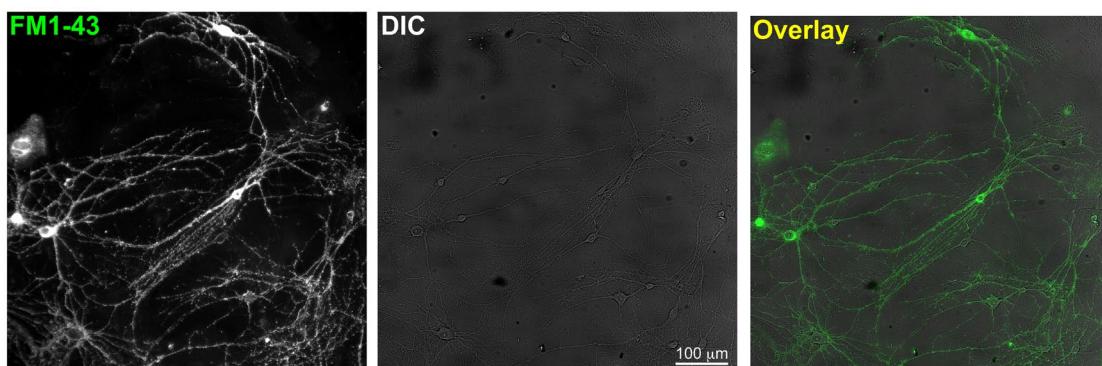
### C. Imaging procedure

1. Synaptically mature primary rat hippocampal neurons (DIV 12-18) are incubated with 10  $\mu\text{M}$  FM1-43 (i.e., SynaptoGreen C4, Biotium) for 0.5 h at 37 °C in a 5% CO<sub>2</sub> incubator to load FM1-43 into synaptic vesicles through spontaneous exo-/endocytosis. For dye loading, 10  $\mu\text{l}$

sterile 1 mM stock solution in ddH<sub>2</sub>O mixed thoroughly with 990 µl of the conditioned plating medium from the neuronal culture will be added to and dispensed into a new well of the 24-well plate. Then, a coverslip with neurons is transferred to the well containing 10 µM FM1-43 by means of burner sterilized forceps.

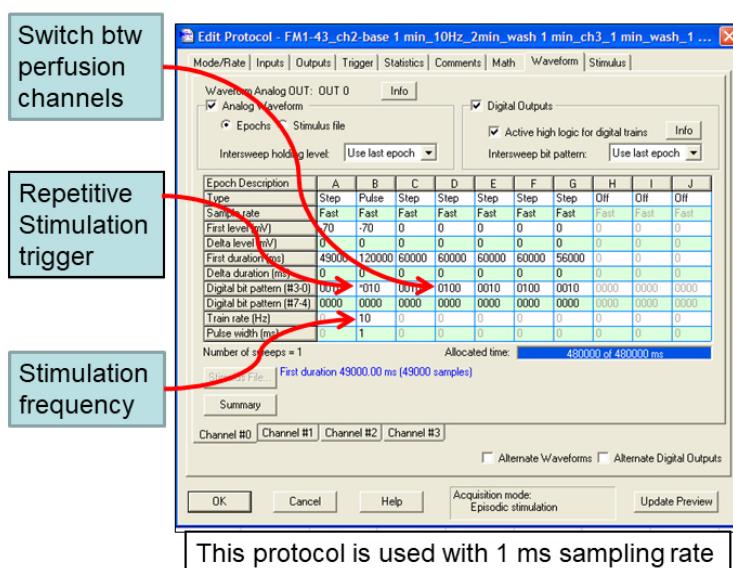
2. A more common loading procedure is acute loading by stimulation. For example, FM1-43 dye is loaded in the presence of high K<sup>+</sup> saline, which promotes massive evoked exo-/endocytosis.
3. For that, in a laminar flow culture hood, use burner sterilized forceps to transfer one coverslip of primary rat hippocampal culture from the 24-well plates to a 30-mm Ø Petri dish containing 3 ml of normal Tyrode's solution. Bring the coverslips to the microscope. To prevent dye loss due to spontaneous neuronal activity, Glutamate receptor blockers (10 µM NBQX and 20 µM D-AP5) are added at least 1 min before imaging and are present in the bath throughout the whole imaging experiment.
4. Transfer the coverslips to the imaging chamber preloaded with normal Tyrode's solution with perfusion speed of 0.05 ml/sec.
5. Stop perfusion by closing both inlet and outlet (*i.e.*, vacuum), and remove ~90-95% solution from the chamber.
6. Dropwise add 500 µl high K<sup>+</sup> solution containing 10 µM FM1-43 (*i.e.*, SynaptoGreen C4, Biotium) to the imaging chamber and mix it gently.
7. Incubate the cells for 2 min with the dye and turn on the perfusion for 5-min washout of surface FM dyes with normal Tyrode's solution. Healthy looking neurons with moderate FM1-43 labeling are identified at 20x magnification (as in Figure 2).

*Note: The two different loading procedures have their own pros and cons. If possible side effects due to membrane depolarization in high K<sup>+</sup> are to be avoided it is advisable to use the first approach (30 min at 37 °C). Acute loading helps to achieve more directed plasma membrane targeting.*



**Figure 2. Sample zoom-out images which show optimal cell density in DIC and overlay with FM1-43 fluorescence**

8. To induce synaptic vesicle release, either electric field stimulation or high K<sup>+</sup> perfusion is used. The rate of evoked FM1-43 loss from presynaptic terminals is measured.
9. The neuronal culture is exposed to electrical field stimulation at 10 Hz with 1 msec 70 V pulses generated by Grass SD9 stimulus isolator for 2 min or high-potassium (90 K) 1 min perfusions.
10. Stimulation control is interfaced through Clampex software (Molecular Devices). An example of Clampex protocol that controls timing of electric field stimulation and switch between perfusion channels is shown in Figure 3.



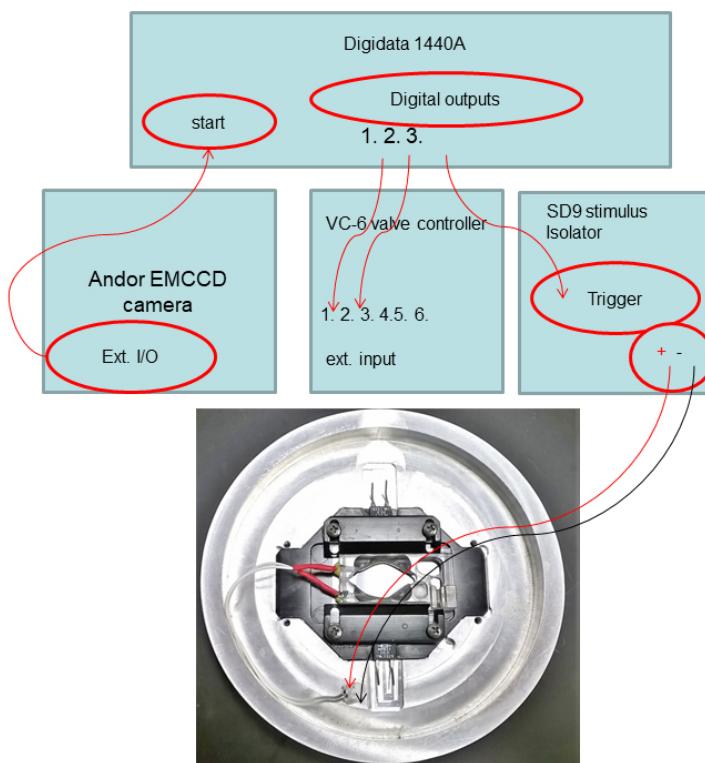
**Figure 3. Example of Clampex protocol that can be used to control timing of electric field stimulation and to switch between solution perfusion channels.** Sampling rate is set at 1 msec.

11. SD9 is triggered with a 5 V TTL pulse from #4 digital output port on 1440A digidata (Molecular Devices). SD9 settings are shown in Figure 4. 70 V output pulses are delivered from positive and negative ports on SD9 to the platinum wires (Alfa Aesar 0.5 mm dia), super-glued to the sides of the RC-26G chamber. Stimulation conditions were optimized in whole-cell patch clamp recordings on current-clamped neurons and are sufficient to reliably evoke action potentials without obvious detrimental effects. In the example Clampex protocol (Figure 2), electric field stimulation is encoded as a 10 Hz, pulse train (Epoch B), which is 120,000 msec (120 sec = 2 min) long. Repetitive triggering of the stimulator through digital output #4 is encoded by the star (\*) in digital bit pattern.



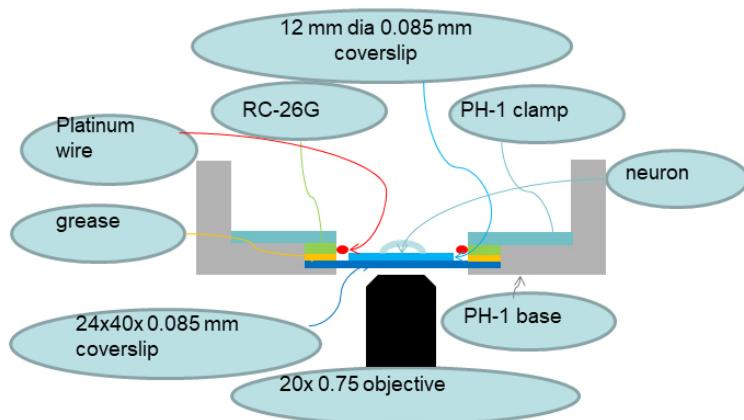
**Figure 4. Grass instruments SD9 stimulus isolator settings.** Frequency–0, Delay–0, duration–10 x 0.1, Volts–7 x 10, output–mono, polarity–normal.

12. Digital control of perfusion is achieved through a VC-6 valve controller. Individual perfusion channels are set to external trigger mode, and open or close in response to TTL pulse sent from the Digidata 1440A digital output.
13. The Andor iXon Ultra EMCCD camera's External Input/Output 'Fire' port is connected to the Start port on the Digidata 1440A, which allows it to trigger Clampex protocols via the Digitizer Start Input immediately when the imaging acquisition begins. The schematic diagram of controller/devices interconnections is depicted in Figure 5.



**Figure 5. Diagram of controller/devices interconnections**

14. Orientation of coverslips on the microscope is shown in Figure 6.
15. Baseline fluorescence is captured for 1 min (or at least 3 frames) before electrical/chemical stimulation/depolarization of neurons.

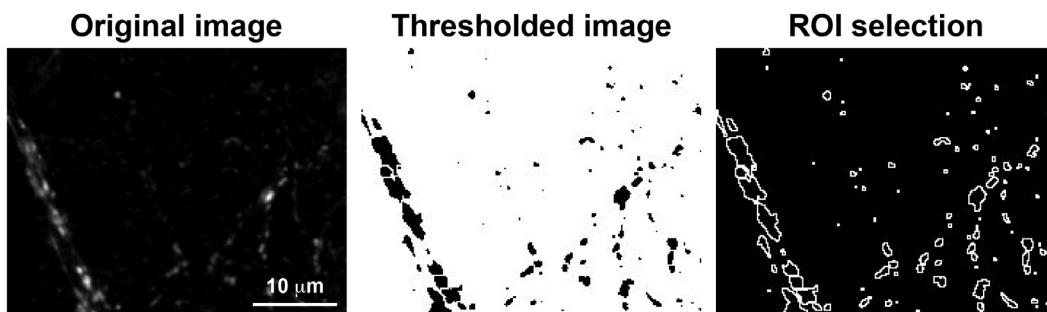


**Figure 6. Diagram of coverslips' orientation on the microscope**

#### D. Imaging analysis

##### ***Imaging analysis in ImageJ/Fiji:***

1. Open ImageJ or Fiji (not Micro-Manager).
2. File → Import image sequence.
3. Image → Duplicate first (not a stack) and use it for ROI selection.
4. If want to select/save/analyze a portion of image, e.g., area in focus: select it, right click, duplicate.
5. If you want to smoothen the image: Process → Filters → Mean (e.g., average 4-5 pixel values).
6. Process → FFT → Bandpass Filter, large structures—down to 40 px, small—down to 3, Suppress stripes: None, 5% tolerance of direction, Autoscale after filtering, Saturate image.
7. Having duplicated the active image, open Image → Adjust → Threshold, Dark background-B&W.
8. Set lower threshold level, click set, apply: the program assigns values in a range from 0 to 255 for the 8-bit image.
9. Analyze → Analyze Particles: size 2-200 pixels (good range to cover synaptic structures). An example of threshold-defined and size-restricted ROIs is shown in Figure 7.



**Figure 7. Example of the threshold based selection of FM1-43 labeled ROIs.** Image on the right depicts outlined ROIs determined based on the threshold and particle size.

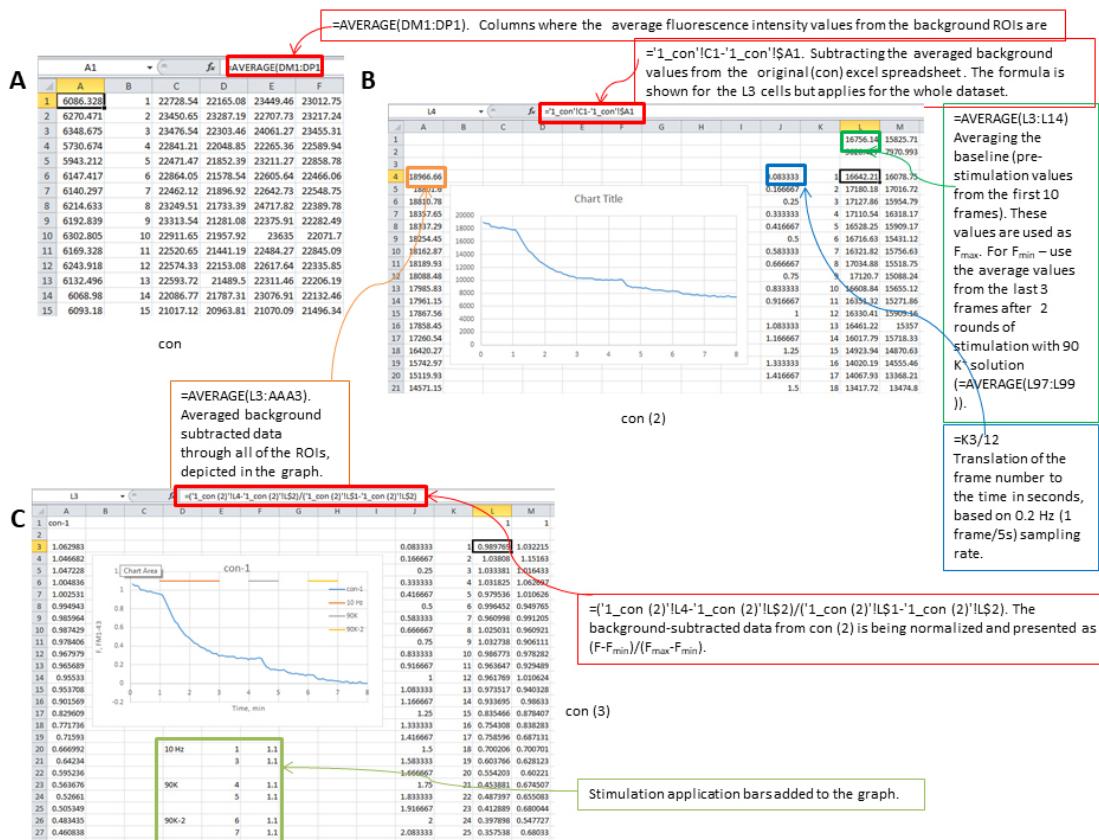
10. Image → Adjust → Brightness/Contrast: drag brightness to Max to be able to find background spots with almost no signal.
11. Select 4 ROIs for background: One by one select background regions and click Add on the ROI manager.
12. On the ROI manager, go to More → Save ROIs and save in the root folder, not in the stack.
13. Go to original image stack, open ROI manager, uncheck, check back, show all.
14. ROI manager, select More → Multi measure to get the intensity values for each ROI through the whole stack of images. Copy values **to Excel**.

*Notes:*

- a. *To correct for lateral drifting in a stack of images, have your stack open with the 1st frame active, to which you want to align subsequent frames, then go to: Plugins → Registration → StackReg → Translation. After correction, the new stack will have blank areas along the edges where drifting happened. To exclude those areas from analysis—just select the area you want to keep, right-click, duplicate and save the stack as a new image sequence in a new folder.*
- b. *StackReg is built into Fiji (if the Fiji installation does not have it, go to Help → Update, click 'Manage update sites', add <http://sites.imagej.net/BIG-EPFL/> and it will be automatically installed at next update), but can be added to ImageJ as well. It requires TurboReg.*  
<http://bigwww.epfl.ch/thevenaz/turboreg/>  
<http://bigwww.epfl.ch/thevenaz/stackreg/>
- c. *MultiStackReg (available from <http://bradbisse.net/downloads.html>, requires StackReg and TurboReg) can be used to save the transformations and apply them to another stack. This is extremely useful if another channel that has a lower signal is imaged simultaneously and is difficult to automatically correct.*

### Data analysis in Excel:

15. Insert 1 column at the beginning of the data set to be able to calculate average background values (from the last 4 columns that represent background ROIs-regions of interest).
- Note: Columns of data contain fluorescence intensity values of individual ROIs over frame number, plotted in rows. Frame numbers can easily be translated into time points from the imaging frequency if need be. If the frequency is 0.1 Hz, interval between frames is 10 sec.*
16. Make a copy of the entire spreadsheet and subtract averaged background values (in column 1) from individual intensity values.
  17. Make a second copy of the spreadsheet with background values subtracted. Insert 2 rows on top of the data set. Calculate average values of the first 3 rows (baseline,  $F_{max}$ ) and last 3 rows (after 2-nd bout in 90 K,  $F_{min}$ ).
  18. Copy the spreadsheet one more time and normalize data as  $(F - F_{min})/(F_{max} - F_{min})$  for individual ROI. Visual representation of Excel analysis can be found in Figure 8.

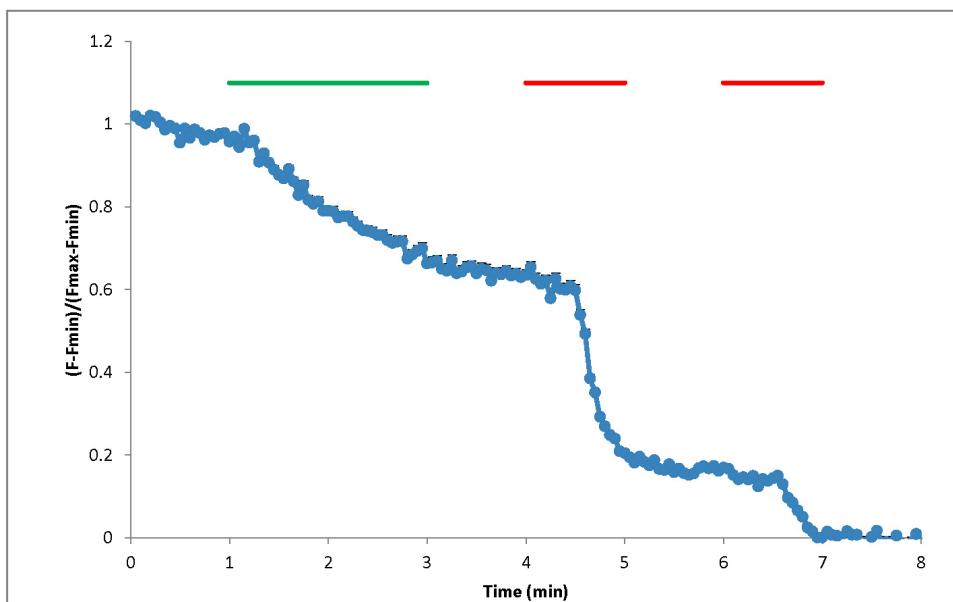


**Figure 8. Screenshots from Excel analysis.** A. Background averaging column is added to the dataset, then a copy of the spreadsheet is made (con (2)). B. In the con (2) spreadsheet background is subtracted, frame number is translated to time,  $F_{max}$  and  $F_{min}$  values are calculated, graph panel is added for visual control of the FM1-43 fluorescence changes, then the spreadsheet is copied one more time (con (3)). C. In con (3) spreadsheet the data from con

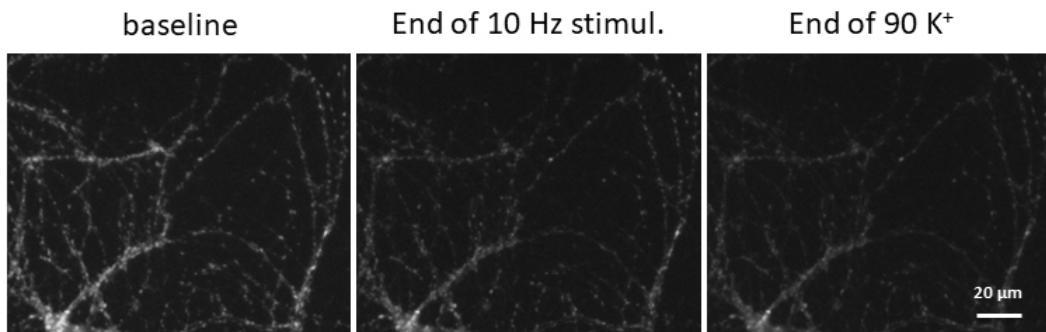
(2) is being normalized and presented as  $(F - F_{\min})/(F_{\max} - F_{\min})$  and application bars are being added to the graph.

19. Calculate average values across multiple ROIs over time. An example of the resultant graph is shown in Figure 9. Sample images at baseline, end of electric field stimulation and end of second bout in 90 K<sup>+</sup> are shown in Figure 10.

*Note: Different Fields of View (FOVs) contain different numbers of threshold-detected ROIs. To eliminate over-influence of FOVs with high ROI number on the averaged data, a set of randomly selected ROIs which is equal or smaller than the number of ROIs of the least populated FOV can be used for averaging. To randomize ROIs in excel—insert a new row on top of the ROI data set. In this new row, select cells, aligned to the columns with ROIs. Type in =rand(), then press Ctrl+Enter. Having the row of randomized values selected, go to Menu-Data-Sort-Expand the selection-Options-Sort left to right. Sort data by row1 (randomized values).*



**Figure 9. Example of normalized FM1-43 fluorescence in response to 2 min 10 Hz electric field stimulation (green bar) or 1 min high potassium (90 K) chemical stimulation (red)**



**Figure 10. Example images at baseline, end of electric field stimulation and end of second bout in 90 K<sup>+</sup>**

### Data analysis

Initial image analysis is done in ImageJ. Regions of interest (ROIs) are selected by using the same fluorescence intensity threshold across different samples. Average intensity from every ROI and average background intensities from four cell-free regions in every image stack are exported to Excel. The FM1-43 signal in every ROI is calculated as  $(F - F_{\min})/(F_{\max} - F_{\min})$ , in which  $F_{\max}$  is the average of the first 3 frames at a baseline before stimulation, and  $F_{\min}$  is the average of the last 3 frames after multiple bouts with 90 K stimulation. All fluorescence intensity values are background subtracted.

To determine the minimum number of ROIs for FM1-43 destaining, a power analysis is performed using G\*Power (Faul *et al.*, 2007). An effect size of 25% is estimated with the error probability set to 0.05, power to 0.95 and an expected standard deviation of 40% is chosen based on FM destaining experiments performed in the lab. A sample size of 53 is needed to achieve significance with a two-tailed Student's *t*-test. All image processing is performed in ImageJ. All experiments are performed in two to three different batches of cell cultures. All values presented are mean  $\pm$  SEM. For calculating statistical significance, the Student's *t*-test is used for 2-group comparison, and one-way analysis of variance (ANOVA) followed by the Tukey-Kramer method as post-hoc analysis is used for comparing three or more groups.

### Notes

#### A. Reproducibility and variability

In our tests, we observed a moderate variability in FM loading and unloading in untreated control. This variation mostly originates from the variations of cell culture such as days *in vitro* and cell density. Therefore, reducing such variables can significantly improve experimental consistency. In addition, we notice that unhealthy neurons often have excessive FM staining, which we have used as a criterion to exclude damaged synapses.

## B. The comparison between FM and other synaptic vesicle labels

Synaptic vesicles are so essential in neurobiology that many methodologies such as electrophysiology, amperometry, electron microscopy and fluorescence imaging have been developed over the past fifty years to study them. Fluorescence imaging of fluorophores that selectively target synaptic vesicles has the advantage of directness, ability to dynamically monitor synaptic events in real time with high throughput and high spatial resolution (Kavalali and Jorgensen, 2014). FM dyes are the most popular fluorescent labels (Gaffield and Betz, 2007). Other probes include fluorescently tagged antibodies recognizing vesicle luminal epitopes, vesicular proteins whose luminal domain is tagged with pH-sensitive fluorescent protein (Syn-pHFP) (Afuwape and Kavalali, 2016) and quantum dots (Qdots) with surface affinity to vesicular membranes (Zhang *et al.*, 2009) or conjugated to antibodies recognizing vesicle luminal epitopes (Park *et al.*, 2012). Below, we will make a concise comparison among those fluorescent labels in terms of operational difference.

### 1. Targeting difference

FMs are styryl dyes that randomly insert into cell membranes. With stimulation-induced vesicle release and compensatory retrieval, FM dyes are believed to end up mostly in recycled synaptic vesicles, partially in endosomes and lysosomes, and a little in Golgi and endoplasmic reticulum (ER). Qdots with vesicular membrane affinity and the vesicle-targeting antibodies conjugated to fluorophores including Qdots have similar targeting outcomes with a lower possibility of being targeted to Golgi and ER. Shortening loading time is the most effective way to reduce non-synaptic vesicle targeting. Differently, Syn-pHFPs are transgenically expressed and distributed to all cell membranes the original proteins reside in, which includes vesicles and the cell surface. Generally, it is believed that Syn-pHFPs label all synaptic vesicles, releasable and non-releasable.

### 2. Signal difference

Since FM dyes are prone to washout when surfaced, the signal reporting vesicle release is a decrease of their fluorescence. In contrast, Syn-pHFPs report exocytosis as an increase of fluorescence because exocytosis causes deacidification of the vesicular lumen, and their fluorescence decrease reports endocytosis and vesicle re-acidification.

### 3. Normalization difference

Different synaptic boutons contain different numbers of vesicles; therefore, normalization is important to obtain a measurement of population vesicle behavior. For FM dyes and other externally added probes, the most common and simplest way to normalize the response is to define the fluorescence intensity prior to stimulation as 100% with the intensity after exhaustive stimulation as 0. For genetically expressed Syn-pHFP, the most common normalization is to use a high concentration of NH<sub>4</sub>Cl solution to de-acidify all cytoplasmic compartments to completely unquench Syn-pHFP and achieve the maximum 100% fluorescence intensity. To determine minimal Syn-pHFP intensity (0%), cells are normally perfused with low pH-solution (e.g., pH 5.5 Tyrode's). Clearly, both pH manipulations have a possible impact on synaptic

vesicle behavior. Notably, our recent paper demonstrated that even a moderate concentration of NH<sub>4</sub>Cl (*i.e.*, 5 mM) can change synaptic vesicle release and retrieval over an extended period of time (Lazarenko *et al.*, 2017).

## **Recipes**

1. Matrigel stock solution
  - a. Make **Matrigel stock solution**

Thaw 10 ml bottle on ice in a cold room for 24-48 h, then aliquot in 1 ml aliquots and freeze.  
If you try to thaw it at a higher temperature, it will turn into sludge
  - b. Make **working Matrigel** (*i.e.*, what is called Matrigel in the protocol)
    - i. Thaw 1 ml aliquot on ice for several hours and mix with 49 ml MEM
    - ii. Matrigel is thick and viscous, so you will probably need to flush the 1 ml aliquot with MEM to get the whole thing out
    - iii. Shake the MEM + Matrigel to mix and let it sit overnight in a cold room to dissolve completely before using
    - iv. Aliquot it into four 15 ml conical tubes with 12.5 ml in each
    - v. Cover all tubes with aluminum foil to protect from light
    - vi. Do not use an aliquot older than one month, and do not filter Matrigel
2. Hanks solution

Hanks solution is directly made from Sigma-Aldrich H2837 powdered HBSS, or directly ordered from Invitrogen  
0.2 µm filter sterilize and aliquot in 50 ml tubes
3. Hanks + 20 (H + 20)

400 ml 1x Hanks + 100 ml fetal bovine serum (20% FBS)  
0.2 µm filter sterilized and aliquoted in 50 ml tubes
4. Dissociation solution (500 ml)

Hank's salt solution +12 mM MgSO<sub>4</sub>·6H<sub>2</sub>O
5. DNase solution

Dissolve entire 375 kU in 5 ml sterilized Milli-Q water and aliquot in 40 µl aliquots
6. 2 mM Ara-C stock (500 µl aliquots)
  - a. 9.7 mg Ara-C (usually stored in refrigerator or cold room) to 20 ml of deionized water in a 50-ml conical tube
  - b. Vortex well
  - c. Filter (0.2 µm) into 50 ml conical tube
  - d. Aliquot into 1.7 ml tubes, 500 µl each
  - e. Store at -20 °C

## 7. Plating medium

500 ml MEM

2.5 g glucose

100 mg NaHCO<sub>3</sub>

50 mg transferrin

Mix well with stir bar on lab bench

*Note: You can also make 1 L solution and divide it for plating and Ara-C.*

50 ml FBS (~10%)

5 ml 0.2 M L-glutamine

1 ml insulin stock (12.5 mg/ml insulin in 10 mM HCl)

0.2 µm filter sterilize and aliquot in 50 ml tubes in culture hood

## 8. Ara-C solution

500 ml MEM

2.5 g glucose

100 mg NaHCO<sub>3</sub>

50 mg transferrin

Mix well with stir bar on bench

1.25 ml of 0.2 M L-glutamine

10 ml B27 supplement

500 µl Ara-C stock (2 mM, in Milli-Q water), for the final concentration 1 µM

25 ml FBS (5%)

0.2 µm filter sterilize and aliquot in 50 ml tubes

## 9. Extracellular bath Tyrode's saline

150 mM NaCl

4 mM KCl

2 mM MgCl<sub>2</sub>2 mM CaCl<sub>2</sub>

10 mM N-2 hydroxyethyl piperazine-n-2 ethanesulfphonic acid (HEPES)

10 mM glucose

pH 7.35

## 10. 90 K (High Potassium Tyrode's)

64 mM NaCl

90 mM KCl

2 mM MgCl<sub>2</sub>2 mM CaCl<sub>2</sub>

10 mM N-2 hydroxyethyl piperazine-n-2 ethanesulphonic acid (HEPES)

10 mM glucose

pH 7.35

## **Acknowledgments**

The methods were adapted from (Lazarenko *et al.*, 2017). Techniques were also adapted from all of the references cited. This work was supported by NIH Grant OD008761, NS094738 and DA025143 to Q.Z. We have no conflicts of interest or competing interests to declare.

## **References**

1. Afuwape, O. A. and Kavalali, E. T. (2016). [Imaging synaptic vesicle exocytosis-endocytosis with pH-sensitive fluorescent proteins](#). *Methods Mol Biol* 1474: 187-200.
2. Faul, F., Erdfelder, E., Lang, A. G. and Buchner, A. (2007). [G\\*Power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences](#). *Behav Res Methods* 39(2): 175-191.
3. Gaffield, M. A. and Betz, W. J. (2006). [Imaging synaptic vesicle exocytosis and endocytosis with FM dyes](#). *Nat Protoc* 1(6): 2916-2921.
4. Kavalali, E. T. and Jorgensen, E. M. (2014). [Visualizing presynaptic function](#). *Nat Neurosci* 17(1): 10-16.
5. Lazarenko, R. M., DelBove, C. E., Strothman, C. E. and Zhang, Q. (2017). [Ammonium chloride alters neuronal excitability and synaptic vesicle release](#). *Sci Rep* 7(1): 5061.
6. Liu, G. and Tsien, R. W. (1995). [Synaptic transmission at single visualized hippocampal boutons](#). *Neuropharmacology* 34(11): 1407-1421.
7. Park, H., Li, Y. and Tsien, R. W. (2012). [Influence of synaptic vesicle position on release probability and exocytotic fusion mode](#). *Science* 335(6074): 1362-1366.
8. Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J. Y., White, D. J., Hartenstein, V., Eliceiri, K., Tomancak, P. and Cardona, A. (2012). [Fiji: an open-source platform for biological-image analysis](#). *Nat Methods* 9(7): 676-682.
9. Schneider, C. A., Rasband, W. S. and Eliceiri, K. W. (2012). [NIH Image to ImageJ: 25 years of image analysis](#). *Nat Methods* 9(7): 671-675.
10. Zhang, Q., Li, Y. and Tsien, R. W. (2009). [The dynamic control of kiss-and-run and vesicular reuse probed with single nanoparticles](#). *Science* 323(5920): 1448-1453.

## Measuring Mitochondrial ROS in Mammalian Cells with a Genetically Encoded Protein Sensor

Xin Zhang<sup>1</sup>, Christine Silvia Gibhardt<sup>1</sup>, Sabrina Cappello<sup>1</sup>, Katharina Maria Zimmermann<sup>2</sup>, Adina Vultur<sup>1</sup> and Ivan Bogeski<sup>1,\*</sup>

<sup>1</sup>Molecular Physiology, Institute of Cardiovascular Physiology, University Medical Center, University of Göttingen, Göttingen, Germany; <sup>2</sup>Department of Biophysics, CIPMM, School of Medicine, Saarland University, 66421 Homburg, Germany

\*For correspondence: [ivan.bogeski@med.uni-goettingen.de](mailto:ivan.bogeski@med.uni-goettingen.de)



**[Abstract]** Reactive oxygen species (ROS) are not only known for their toxic effects on cells, but they also play an important role as second messengers. As such, they control a variety of cellular functions such as proliferation, metabolism, differentiation and apoptosis. Thus, ROS are involved in the regulation of multiple physiological and pathophysiological processes. It is now apparent that there are transient and local changes in ROS in the cell; in so-called ‘microdomains’ or in specific cellular compartments, which affect signaling events. These ROS hotspots need to be studied in more depth to understand their function and regulation. Therefore, it is necessary to identify and quantify redox signals in single cells with high spatial and temporal resolution. Genetically encoded fluorescence-based protein sensors provide such necessary tools to examine redox-signaling processes. A big advantage of these sensors is the possibility to target them specifically. Mitochondria are essential for energy metabolism and are one of the major sources of ROS in mammalian cells. Therefore, the evaluation of redox potential and ROS production in these organelles is of great interest. Herein, we provide a protocol for the real-time visualization of mitochondrial hydrogen peroxide ( $H_2O_2$ ) using the  $H_2O_2$ -specific ratiometric sensor mitoHyPer in adherent mammalian cells.

**Keywords:** Mitochondrial ROS, Protein sensor, Fluorescence microscopy, Real-time imaging, Mammalian cell, HyPer, roGFP-Orp1

**[Background]** ROS are produced as by-products of mitochondrial respiration, through the leakage of electrons from the electron transfer chain. These ROS are considered toxic and cause the oxidation of lipids, proteins, and lead to mitochondrial DNA damage (Ralph *et al.*, 2010; Bogeski and Niemeyer, 2014; Cierlitzta *et al.*, 2015; Gibhardt *et al.*, 2016). While mitochondria serve as a hub of metabolism, bioenergetics, and cell death, the emerging role of mitochondrial ROS as second messengers in regulating other cellular functions is also increasingly accepted (Chandel, 2015; Reczek and Chandel, 2015; Shadel and Horvath, 2015; Wilems *et al.*, 2015). To monitor mitochondrial ROS with high spatial and temporal resolution remains challenging due to the short half-life of ROS and the limitation of available probes (Kuznetsov *et al.*, 2011; Norcross *et al.*, 2017). The primary reactive species of mitochondrial origin are superoxide anion, hydroxyl radical, singlet oxygen, and hydrogen peroxide (Gibhardt *et al.*, 2016; Idelchik *et al.*, 2017). Hydrogen peroxide ( $H_2O_2$ ) is one of the most stable ROS and is thus an attractive tracking tool for examining the cellular redox state.

During the past decade, several groups designed genetically encoded protein sensors to specifically detect H<sub>2</sub>O<sub>2</sub> (Belousov *et al.*, 2006; Gutscher *et al.*, 2009). The specificity, reversibility, and sensitivity of these protein sensors make them suitable for real-time visualization of H<sub>2</sub>O<sub>2</sub> under a broad range of physiological conditions and stimulations.

The HyPer and roGFP2-Orp1 sensors are advantageous in particular and can be used in various cell systems (Ermakova *et al.*, 2014; Hernandez-Barrera *et al.*, 2013; Gibhardt *et al.*, 2016). The HyPer sensor is a combination of a circular permuted yellow fluorescent protein (cpYFP), which is inserted in the regulatory domain of the bacterial H<sub>2</sub>O<sub>2</sub> sensing protein OxyR. The oxidation of cysteine199 found on OxyR initiates conformational changes in HyPer. In a reduced state HyPer has two excitation peaks at 420 nm and 500 nm, and one emission peak at 516 nm. Following oxidation, the peak at 420 nm decreases and the peak at 500 nm increases, thus allowing ratiometric measurement of H<sub>2</sub>O<sub>2</sub> (Bilan and Belousov, 2017). Given that pH fluctuations can also affect the signal from HyPer probes, a mutation at cysteine 199 was introduced to generate a probe named SypHer for monitoring pH, which has the same pH sensitivity but does not react to oxidation (Matlashov *et al.*, 2015; Poburko *et al.*, 2011). The roGFP probe is based on an engineered GFP containing two cysteine residues capable of forming a disulfide bond (Morgan *et al.*, 2011). It has two excitation maxima at 400 and 490 nm with the emission around 510 nm; the ratio of these two excitation maxima depends on the state of the disulfide bond. The development of roGFP probes now provides important alternative tools aimed at detecting H<sub>2</sub>O<sub>2</sub> or the potential of the glutathione redox pair (Gutscher *et al.*, 2008; Kasozi *et al.*, 2013; Habich and Riemer, 2017; Lismont *et al.*, 2017; Müller *et al.*, 2017).

Here we describe a detailed protocol for the real-time imaging and monitoring of mitochondrial H<sub>2</sub>O<sub>2</sub> with the mitoHyPer sensor. The approach can be performed on different cellular systems with a basic understanding of real-time imaging and fluorescence microscopy; the data analysis procedure depends on the software available.

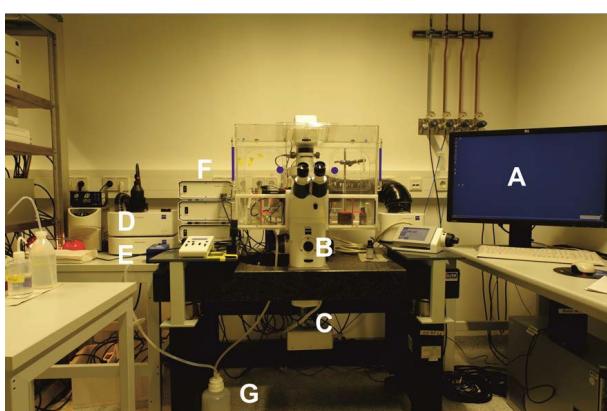
## **Materials and Reagents**

1. Round glass coverslips 25 mm No. 1.5 (Kindler/ORSA tec®, [Round cover glasses](#))
2. 6-well plates (Corning, Costar®, catalog number: 3516)
3. Falcon tubes (15 ml) (VWR, Corning, catalog number: 62406-200)
4. Serological pipettes (Corning, Costar®, catalog number: 4488)
5. Plasmids
  - mitoHyPer (Evrogen, catalog number: FP942)
  - mitoSypHer (Addgene, catalog number: 48251)
6. Cell growth medium (specific to the cells used in the experiment)
7. Fetal bovine serum (FBS) (Thermo Fisher Scientific, Gibco™, catalog number: 10270106)
8. Fugene® HD (Promega, catalog number: E2312)
9. Opti-MEM™ (Thermo Fisher Scientific, Gibco™, catalog number: 51985-026)
10. Baysilone paste (VWR, GE Bayer Silicines, catalog number: 291-1210)

11. Accutase (Sigma-Aldrich, catalog number: A6964) or Trypsin (Thermo Fisher Scientific, Gibco™, catalog number: 25300062)
12. 1x DPBS, no calcium, no magnesium (Thermo Fisher Scientific, Gibco™, catalog number: 14190-094)
13. 1,4-Dithiothreitol (DTT) (Sigma-Aldrich, catalog number: D0632)
14. Hydrogen peroxide solution 30% (w/w) in H<sub>2</sub>O, contains stabilizer (Sigma-Aldrich, catalog number: H1009)
15. Stimulants and inhibitors (these are experiment-dependent)
16. Sodium chloride (NaCl) (Sigma-Aldrich, catalog number: S9888)
17. Potassium chloride (KCl) (VWR, AnalaR NORMAPUR®, catalog number: 26764.298)
18. Calcium chloride dihydrate (CaCl<sub>2</sub>·2H<sub>2</sub>O) (Merck, catalog number: 102382)
19. Magnesium chloride (MgCl<sub>2</sub>) (Merck, catalog number: 105833025)
20. D(+)-Glucose anhydrous (Merck, catalog number: 108337)
21. EGTA (Sigma-Aldrich, catalog number: E4378)
22. 1 M HEPES (Sigma-Aldrich, catalog number: H7523)
23. Ringer buffer (0.25 mM Ca<sup>2+</sup>, pH 7.4) (see Recipes)

## Equipment

1. Z™ Series COULTER COUNTER® Cell (Beckman Coulter, model: 6605699) and Particle Counter Z1 (Beckman Coulter or any other counting device)
2. Incubator with humidity and gas control for cell culture
3. Zeiss Axio Observer.Z1 (Carl Zeiss, model: Axio Observer.Z1) setup (Figure 1) (Incubation System S includes Temp Module S, CO<sub>2</sub> Module S, O<sub>2</sub> Module S, Heating Module S)
4. Tweezers (e.g., style Dumont Nr. 7)
5. Imaging chamber and ring insert (self-made) and perfusion system (Figure 2)

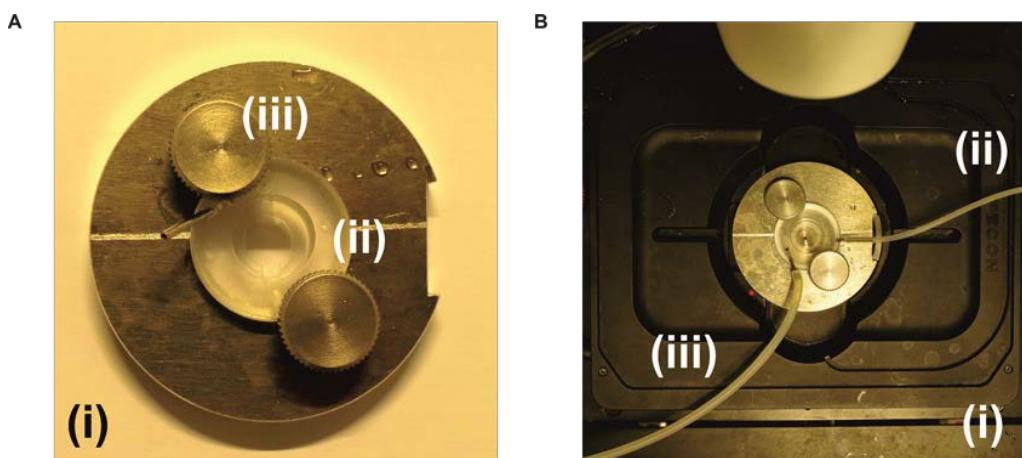


**Figure 1.** Zeiss Cell Observer.Z1 setup with temperature, CO<sub>2</sub> controlling unit, gas chamber and perfusion system. A. Analysis computer; B. Cell Observer.Z1 with 40x oil objective and corresponding filter sets; C. Evolve 512 x 512 EM-CCD camera; D. CO<sub>2</sub> supply

unit; E. Pecon XL S1 incubator and control modules; F. LED Colibri with corresponding modules. G. Pump and perfusion system.

**Notes:**

- a. For HyPer measurements, the CFP/YFP filters are essential, but a multiband filter cube with the same property is also a functional option.
- b. For HyPer experiments, we used the LED light source with the wavelength at 505 nm and 420 nm and corresponding beam splitters.



**Figure 2. Imaging chamber and mount module with temperature control.** A. The self-made imaging chamber (i) with a perfusion chamber plastic insert (ii) that is fixed with knobs (iii). The coverslip with the cells is attached to the lower part of the plastic insert and a small 12 mm coverslip is attached to the upper part of the plastic insert in order to create a small perfusion channel for the measurement. B. The imaging chamber attached to the perfusion system and to the stage of the microscope (i). The perfusion tube (ii) is attached to a syringe in order to add the solutions during the measurement, while the second perfusion tube (iii) is attached to a suction pump system to remove the waste liquid.

### Software

1. Axiovision 4.6v (Zeiss) with a license for fast acquisition function and measurement analysis or similar

### Procedure

#### A. Day 1: Cell culture and seeding

This protocol is exemplary for adherent cells, which can be transfected with reagents such as Fugene® HD. For cell lines which are difficult to transfect, we recommend an alternative transfection method (e.g., nucleofection by electroporation). If stable cells expressing the desired

sensors are available, they can also be used for the imaging experiment as described in this protocol.

1. Culture the cells with their corresponding growth medium until they reach a confluence of around 70%. Remove the growth medium, wash the cells once with 5 ml DPBS and detach the cells by incubating them with 1 ml trypsin or 1 ml accutase (as used for their normal cultivation) at room temperature.
2. Suspend the cells in growth medium and dilute 100 µl of the suspension with DPBS to a ratio of 1:100 in a total volume of 10 ml. Determine the concentration of cells in the dilution with a Z1 cell counter or hemocytometer.
3. Place autoclaved glass coverslips into a 6-well plate and seed 400,000 cells (in this example HEK293 cells) for each well in 2 ml of growth medium. Place the plate in a humidified cell culture incubator (37 °C, 5% CO<sub>2</sub>) and incubate overnight.

#### B. Day 2: Transfection

1. Remove the Opti-MEM medium and Fugene® HD solutions from the freezer and equilibrate both at room temperature for several minutes.
2. Mix 100 µl of Opti-MEM medium with 4 to 10 µl of Fugene® HD solution (according to the manufacturer's protocol), add the suggested amount of plasmid DNA (1 µg/µl endotoxin-free stock solution) to the mixture (1 µg/well is recommended, but the optimal amount can vary and depends on the cell type and plasmid). Pipette the mix up and down 15 times.

*Note: The optimal transfection conditions e.g., cell density, DNA amount, DNA:Fugene® HD ratio might need optimization for the cell line of choice.*

3. Wait for 15 min at room temperature, then add 100 µl of the transfection mixture to each well.

*Notes:*

- a. *If your cell growth medium contains antibiotics, it is advisable to change this before the transfection to growth medium without antibiotics, because they might reduce the transfection efficiency; otherwise, it is not necessary to change the growth medium before the transfection mixture is added.*
  - b. *Since mitoHyPer and mitoSypHer have the same spectrum features, they should be transfected separately (in different wells).*
4. The cells are incubated in a humidified cell culture incubator (37 °C, 5% CO<sub>2</sub>). Change the medium in the transfected wells after 6 h with fresh cell growth medium. Keep the cells in the incubator until ready for imaging (37 °C, 5% CO<sub>2</sub>), for about 24-48 h.

#### C. Day 3 or 4: Imaging

Imaging is performed with a Zeiss Cell Observer.Z1 setup with temperature, CO<sub>2</sub> controlling unit, gas chamber and perfusion system (Figure 1).

1. Gently remove a cell-covered coverslip with a pair of delicate tweezers (avoiding the scrapping of cells in the central imaging area of the coverslip). Add Baysilone-paste on the edge of the

bottom of the perfusion chamber plastic insert (self-made) and attach it to the coverslip (cells-facing-up). Fix a 12 mm coverslip with Baysilone-paste on the upper part of the plastic insert in order to create a small perfusion channel. Then fix the plastic insert (holding the coverslips) with the knobs and place the assembled chamber into the metal imaging chamber (see Figure 2A).

*Note: If simple experiments are performed (e.g., analyzing the resting levels with subsequent addition of saturating H<sub>2</sub>O<sub>2</sub>) it might be sufficient to use standard imaging chambers or glass-bottom plates and stimulate the signal changes by addition of the agents carefully with a pipette. However, for more precise experiments involving multiple additions or washing-out experiments, a perfusion system is recommended.*

2. Place the imaging chamber on the microscope stage and attach the solution containing perfusion tubes (to avoid air in the system), on opposite sides of the chamber (as shown in Figure 2B). Perfuse gently with 2 ml Ringer solution (see Recipes) to wash away detached cells. Wait for 5 min to reach a CO<sub>2</sub>(5 %) and temperature (37 °C) equilibrium before additional handling.

*Note: CO<sub>2</sub> and temperature are controlled and monitored by the imaging system with the corresponding controlling units. Our perfusion system has on one side a syringe to apply the Ringer solution by hand and on the other side a suction pump to remove the waste.*

3. Using a 40x objective, search for a proper field of view that allows you to assess separate and well attached cells. Set the LED strength in order to get a proper signal, but not too high to avoid photobleaching of the sensor. Optimize the exposure time to obtain good image quality (signal over background) and keep the ratio of exposure time for both channels (420 nm vs. 505 nm) as a constant for all experiments. This part of the procedure will require some time to optimize, based on the cell types used and on the equipment available, since the light source and camera can vary.
4. Start the experiment by measuring the resting level of H<sub>2</sub>O<sub>2</sub> in the cells every 1 sec for at least 10 sec, then add stimulating substances through the perfusion system and record until the signal stabilizes (or according to the stimulation protocol). The frame number per minute and total imaging time should be optimized to achieve proper temporal resolution but also to avoid photobleaching.

*Note: The stimulating substances leading to the production of ROS from mitochondria vary depending on the scientific question and the cell type. For other scientific questions, only the resting redox level (e.g., the physiological H<sub>2</sub>O<sub>2</sub> concentration under normal conditions) might be of interest.*

5. At the end of each measurement, a single dose of saturating H<sub>2</sub>O<sub>2</sub> (e.g., 1 mM) should be added as a positive control and to determine the maximal intensity of the sensor (this might be needed for calibrating the system). To detect the fluorescence intensity of a fully reduced sensor (which will indicate if the sensor is already oxidized during resting conditions and

provide the minimum value for calibration), we advise adding a reducing agent (e.g., 2 mM DTT) at the end of the experiment.

6. Perform the same imaging procedure with the mitoSypHer sensor as an imaging control, since the HyPer sensor can be affected by changes in pH.

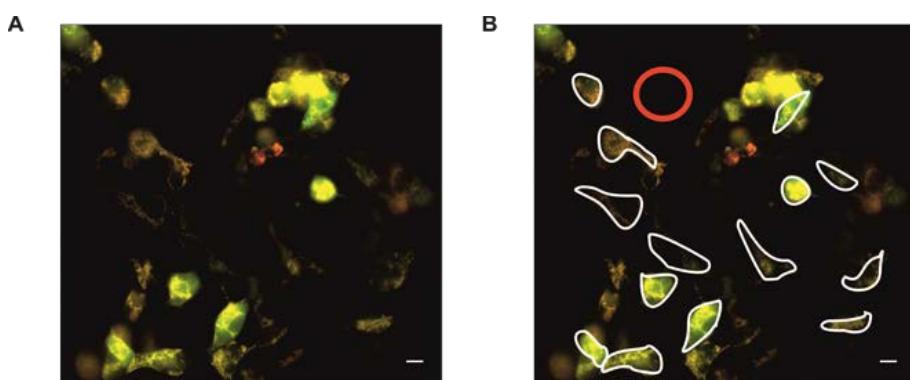
*Note: If the signal (or ratio change) obtained during the experiment with the HyPer sensor is due to oxidation, you will not see any changes in the SypHer signal during the same experimental conditions. If the pH influences your results, you will see also changes in the ratio of the SypHer sensor. Since HyPer is a pH-dependent sensor, this control is mandatory in order to discuss the data regarding redox changes.*

## Data analysis

Analysis with the Axiovision software

1. Background correction

The background correction should be performed by subtracting intensity values in a background ROI from a target (cell-based) ROI (Figure 3).



**Figure 3. Analysis example of HEK293 cells expressing mitoHyPer (also see Figure 4). A.** Merged image (420 nm green, 505 nm red); **B.** exemplary presentation of analysis. The red circle represents the background ROI in a cell free region, while the borders of some cells are marked with white freehand drawing ROI for analysis.

2. Ratiometric analysis

The ratio kinetic curve is generated with the equation:

$$\text{Ratio} = \frac{\text{Fluorescence Intensity}_{500}-\text{background}}{\text{Fluorescence Intensity}_{420}-\text{background}}$$

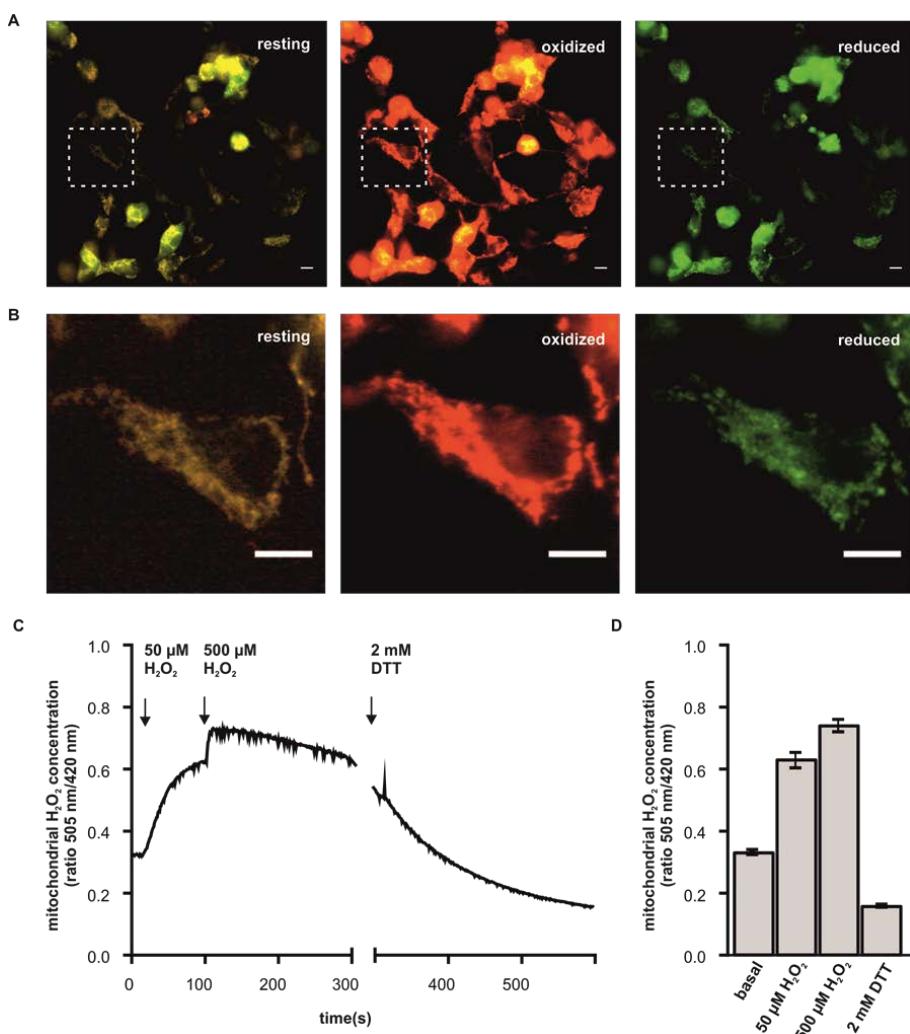
using the Axiovision software.

*Note: The data analysis can be performed with different softwares from other suppliers. The basic calculation for the HyPer ratio can also be performed using an open source software such as ImageJ (<https://imagej.nih.gov>).*

The data are usually presented as mean  $\pm$  SEM (or SD), and tested for significance with two-sided Student's *t*-test. For each condition, at least three experiments should be performed with proper replicates.

3. Below are representative images (A) with a magnification of a single cell (B), a graphed summary (C) and the statistical analysis (D) of what is expected from HEK293 cells following  $H_2O_2$  and DTT addition (Figure 4).

*Note: If the probe is fully oxidized during the measurement and could not respond to saturating  $H_2O_2$  at the end of the experiment, the result should be excluded from analysis. Since the pH is monitored with the SypHer probe, any experiment with significant fluctuations in pH should be excluded from analysis.*



**Figure 4. Exemplary ROS measurement of HEK293 cells expressing the mitochondrial  $H_2O_2$  sensor mitoHyPer.** HEK293 cells were transfected with the mitoHyPer sensor using a Fugene<sup>®</sup> HD-based solution, 48 h prior to imaging. The cells were first titrated with 50  $\mu M$  and

500  $\mu$ M H<sub>2</sub>O<sub>2</sub> for oxidation of the probe. Following the washout of H<sub>2</sub>O<sub>2</sub>, the cells were titrated with 2 mM DTT for the reduction of the probe. The change in fluorescence intensity ratio is represented as merged images (420 nm green, 505 nm red). As shown in (A), the addition of H<sub>2</sub>O<sub>2</sub> caused oxidation of the probe and increased the signal ratio, while the addition of DTT reduced the signal ratio. The indicated region in (A) is shown magnified in (B). Since mitochondrial H<sub>2</sub>O<sub>2</sub> is generated during a cell's resting state, the probe can be partially oxidized by the constitutively generated ROS and can be reduced by membrane-permeant reducing agents such as DTT. The time course corresponding to the images in (A) and (B) is shown in (C) and the statistical analysis (mean  $\pm$  SEM, n = 17) in (D). Scale bars = 10  $\mu$ m.

## Recipes

1. Ringer buffer (0.25 mM Ca<sup>2+</sup>, pH 7.4)  
155 mM NaCl  
4.5 mM KCl  
10 mM glucose  
5 mM HEPES  
2.75 mM MgCl<sub>2</sub>  
0.25 mM CaCl<sub>2</sub>

## Acknowledgements

This work was supported by the German Research Foundation (DFG) through SFB1190 project 17, SFB1027 project C4 and BO3643/3-2 research grant (all to IB). The authors declare no conflicts of interest or competing financial interests.

## References

1. Belousov, V. V., Fradkov, A. F., Lukyanov, K. A., Staroverov, D. B., Shakhbazov, K. S., Terskikh, A. V. and Lukyanov, S. (2006). [Genetically encoded fluorescent indicator for intracellular hydrogen peroxide](#). *Nat Methods* 3(4): 281-286.
2. Bilan, D. S. and Belousov, V. V. (2017). [New tools for redox biology: From imaging to manipulation](#). *Free Radic Biol Med* 109: 167-188.
3. Bogeski, I. and Niemeyer, B. A. (2014). [Redox regulation of ion channels](#). *Antioxid Redox Signal* 21(6): 859-862.
4. Chandel, N. S. (2015). [Evolution of mitochondria as signaling organelles](#). *Cell Metab* 22(2): 204-206.
5. Cierlitz, M., Chauvistre, H., Bogeski, I., Zhang, X., Hauschild, A., Herlyn, M., Schadendorf, D., Vogt, T. and Roesch, A. (2015). [Mitochondrial oxidative stress as a novel therapeutic target to](#)

- [overcome intrinsic drug resistance in melanoma cell subpopulations.](#) *Exp Dermatol* 24(2): 155-157.
6. Gibhardt, C. S., Zimmermann, K. M., Zhang, X., Belousov, V. V. and Bogeski, I. (2016). [Imaging calcium and redox signals using genetically encoded fluorescent indicators.](#) *Cell Calcium* 60(2): 55-64.
7. Ermakova, Y. G., Bilan, D. S., Matlashov, M. E., Mishina, N. M., Markvicheva, K. N., Subach, O. M., Subach, F. V., Bogeski, I., Hoth, M., Enikolopov, G. and Belousov, V. V. (2014). [Red fluorescent genetically encoded indicator for intracellular hydrogen peroxide.](#) *Nat Commun* 5: 5222.
8. Gutscher, M., Pauleau, A. L., Marty, L., Brach, T., Wabnitz, G. H., Samstag, Y., Meyer, A. J. and Dick, T. P. (2008). [Real-time imaging of the intracellular glutathione redox potential.](#) *Nat Methods* 5(6): 553-559.
9. Gutscher, M., Sobotta, M. C., Wabnitz, G. H., Ballikaya, S., Meyer, A. J., Samstag, Y. and Dick, T. P. (2009). [Proximity-based protein thiol oxidation by H<sub>2</sub>O<sub>2</sub>-scavenging peroxidases.](#) *J Biol Chem* 284(46): 31532-31540.
10. Habich, M. and Riemer, J. (2017). [Detection of cysteine redox states in mitochondrial proteins in intact mammalian cells.](#) *Methods Mol Biol* 1567: 105-138.
11. Hernandez-Barrera, A., Quinto, C., Johnson, E.A., Wu, H.M., Cheung, A.Y., and Cardenas, L. (2013). [Using hyper as a molecular probe to visualize hydrogen peroxide in living plant cells: a method with virtually unlimited potential in plant biology.](#) *Methods Enzymol* 527, 275-290.
12. Idelchik, M., Begley, U., Begley, T. J. and Melendez, J. A. (2017). [Mitochondrial ROS control of cancer.](#) *Semin Cancer Biol.*
13. Kasozi, D., Mohring, F., Rahlf, S., Meyer, A. J. and Becker, K. (2013). [Real-time imaging of the intracellular glutathione redox potential in the malaria parasite \*Plasmodium falciparum\*.](#) *PLoS Pathog* 9(12): e1003782.
14. Kuznetsov, A. V., Kehrer, I., Kozlov, A. V., Haller, M., Redl, H., Hermann, M., Grimm, M. and Troppmair, J. (2011). [Mitochondrial ROS production under cellular stress: comparison of different detection methods.](#) *Anal Bioanal Chem* 400(8): 2383-2390.
15. Lismont, C., Walton, P. A. and Fransen, M. (2017). [Quantitative monitoring of subcellular redox dynamics in living mammalian cells using RoGFP2-based probes.](#) *Methods Mol Biol* 1595: 151-164.
16. Matlashov, M. E., Boganova, Y. A., Ermakova, G. V., Mishina, N. M., Ermakova, Y. G., Nikitin, E. S., Balaban, P. M., Okabe, S., Lukyanov, S., Enikolopov, G., Zaraisky, A. G. and Belousov, V. V. (2015). [Fluorescent ratiometric pH indicator SypHer2: Applications in neuroscience and regenerative biology.](#) *Biochim Biophys Acta* 1850(11): 2318-2328.
17. Morgan, B., Sobotta, M. C. and Dick, T. P. (2011). [Measuring E\(GSH\) and H<sub>2</sub>O<sub>2</sub> with roGFP2-based redox probes.](#) *Free Radic Biol Med* 51(11): 1943-1951.

18. Müller, A., Schneider, J. F., Degrossoli, A., Lupilova, N., Dick, T. P. and Leichert, L. I. (2017). [Systematic \*in vitro\* assessment of responses of roGFP2-based probes to physiologically relevant oxidant species.](#) *Free Radic Biol Med* 106: 329-338.
19. Norcross, S., Trull, K. J., Snaider, J., Doan, S., Tat, K., Huang, L. and Tantama, M. (2017). [Extending roGFP emission via forster-type resonance energy transfer relay enables simultaneous dual compartment ratiometric redox imaging in live cells.](#) *ACS Sens* 2(11): 1721-1729.
20. Poburko, D., Santo-Domingo, J. and Demaurex, N. (2011). [Dynamic regulation of the mitochondrial proton gradient during cytosolic calcium elevations.](#) *J Biol Chem* 286(13): 11672-11684.
21. Ralph, S. J., Rodriguez-Enriquez, S., Neuzil, J., Saavedra, E. and Moreno-Sanchez, R. (2010). [The causes of cancer revisited: "mitochondrial malignancy" and ROS-induced oncogenic transformation - why mitochondria are targets for cancer therapy.](#) *Mol Aspects Med* 31(2): 145-170.
22. Reczek, C. R. and Chandel, N. S. (2015). [ROS-dependent signal transduction.](#) *Curr Opin Cell Biol* 33: 8-13.
23. Shadel, G. S. and Horvath, T. L. (2015). [Mitochondrial ROS signaling in organismal homeostasis.](#) *Cell* 163(3): 560-569.
24. Willems, P. H., Rossignol, R., Dieteren, C. E., Murphy, M. P. and Koopman, W. J. (2015). [Redox homeostasis and mitochondrial dynamics.](#) *Cell Metab* 22(2): 207-218.

A blue rectangular banner with the Bio-protocol logo at the top left. The logo consists of the word "bio-protocol" in a white, lowercase, sans-serif font, with a small green leaf icon above the letter "i". Below the main text, the tagline "Improve Research Reproducibility" is written in a smaller, italicized, white font.

**Free access to ~4000 high-quality protocols**

- Contributed by 10,000+ scientists (including Nobel Laureates)
- Validated in a primary research paper
- >91% reproducibility (survey of 2165 Bio-protocol users)
- ~1000 videos of key procedural steps

Sign up at: [www.bio-protocol.org](http://www.bio-protocol.org)

## Characterising Maturation of GFP and mCherry of Genomically Integrated Fusions in *Saccharomyces cerevisiae*

Sviatlana Shashkova<sup>1, 2, #</sup>, Adam JM Wollman<sup>1, #</sup>, Stefan Hohmann<sup>2, 3</sup>, Mark C Leake<sup>1, \*</sup>

<sup>1</sup>Biological Physical Science Institute, Departments of Physics and Biology, University of York, York, UK;

<sup>2</sup>Department of Chemistry and Molecular Biology, University of Gothenburg, Göteborg, Sweden;

<sup>3</sup>Department of Biology and Biological Engineering, Chalmers University of Technology, Göteborg, Sweden

#Contributed equally to this work

\*For correspondence: [mark.leake@york.ac.uk](mailto:mark.leake@york.ac.uk)



**[Abstract]** Single-molecule fluorescence microscopy enables unrivaled sub-cellular quantitation of genomically encoded fusions of native proteins with fluorescent protein reporters. Fluorescent proteins must undergo *in vivo* maturation after expression before they become photoactive. Maturation effects must be quantified during single-molecule analysis. Here we present a method to characterise maturation of GFP and mCherry genetic protein fusions in budding yeast *Saccharomyces cerevisiae*.

**Keywords:** Single-molecule, Fluorescence, Fluorescent protein maturation, Protein fusion, GFP, mCherry, Yeast

**[Background]** Single-molecule fluorescence microscopy enables sensitive quantification of molecular stoichiometry, mobility and copy number, not only on a cell-by-cell basis but also precisely to individual sub-cellular compartments (Leake, 2012; Wollman and Leake, 2015; Shashkova *et al.*, 2017). The technique relies on endogenously expressed fluorescent protein fusions of the wild type protein of interest such that there is one-to-one labelling. However, all fluorescent proteins have an *in vivo* maturation time varying from a few minutes to several tens of minutes before entering a bright fluorescing state (Badrinarayanan *et al.*, 2012). It is therefore of upmost importance to measure any maturation effects and quantify if there is any immature ‘dark fraction’ of labelled protein. These measurements are also particularly relevant to fluorescence recovery after photobleaching (FRAP). FRAP can be used to study molecular turnover in living cells (Beattie *et al.*, 2017). FRAP is based on photobleaching of a cell region where a fluorescently labelled component is localized, followed by quantification of any fluorescence recovery in that region over time. The measured relation between the fluorescence intensity as a function of time following an initial photobleach can be used to determine molecular mobility and kinetics parameters, such as the rate of dissociation of a particular fluorescent component from a molecular complex (Leake *et al.*, 2006). Therefore, any ‘new’ fluorescence coming from fluorescent protein maturation might affect this apparent result. We present here a protocol to characterise the maturation of Mig1-GFP and Nrd1-mCherry fusion proteins in living yeast *Saccharomyces cerevisiae* cells used in our single-molecule studies (Wollman *et al.*, 2017).

We blocked protein translation in living cells by adding cycloheximide (Hartwell *et al.*, 1970), and then

measured any cellular fluorescence recovery after cells were completely photobleached by continuous illumination. Such fluorescence recovery is then used as a metric for newly matured GFP and/or mCherry in the cell. Our results are broadly consistent with *in vivo* maturation of GFP and mCherry reported previously (Badrinarayanan *et al.*, 2012; Khmelinskii *et al.*, 2012), but since maturation kinetics may be dependent on cell type and the specific extracellular microenvironment, it is important to quantify these maturation effects under the same experimental conditions used for the *in vivo* microscopy on the actual fusion strains of interest.

## **Materials and Reagents**

1. Sterile pipette tips, 1 ml, 200 µl, 10 µl (STARLAB, catalog numbers: S1111-6801, S1111-0806, S1111-3800)
2. 14 ml conical tubes (Corning, Falcon®, catalog number: 352059)
3. Petri dishes 92 mm diameter (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 172931)
4. Microscopy slides (Fisher Scientific, catalog number: FB58622)
5. Cover slips (Scientific Laboratory Supplies, catalog number: MIC3124)
6. Yeast *S. cerevisiae* YSH2348 with Mig1-GFP and Nrd1-mCherry genetically integrated protein fusions, *MATa MIG1-GFP-HIS3 NRD1-mCherry-hphNT1 MET LYS* (Hohmann lab, University of Gothenburg, Sweden)
7. D(+)-Glucose (VWR, catalog number: 101176K)
8. Bacto-yeast extract (BD, Bacto™, catalog number: 212750)
9. Peptone from meat (Merck, catalog number: 1072241000)
10. Agar-agar (Merck, catalog number: 1016141000)
11. MilliQ water
12. Yeast nitrogen base without amino acids, without (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (Sigma-Aldrich, catalog number: Y1251)
13. Ammonium sulfate, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (Merck, catalog number: 1012171000)
14. Complete supplement mixture (ForMedium, catalog number: DCS0019)
15. Cycloheximide (Sigma-Aldrich, catalog number: C7698)
16. EtOH (VWR, catalog number: VWRC20821.330)
17. Glucose 50% w/v (see Recipes)
18. YPD agar with 4% glucose (see Recipes)
19. YPD liquid medium with 4% glucose (see Recipes)
20. YNB (Yeast Nitrogen Base) liquid medium without glucose (see Recipes)
21. YNB (Yeast Nitrogen Base) liquid medium with 4% glucose (see Recipes)
22. 100 mg/ml cycloheximide solution in EtOH (see Recipes)

## Equipment

1. Pipettes (STARLAB, model: ErgoOne® Single-Channel Pipette, 2-20 µl, 20-200 µl and 100-1,000 µl)
2. pH-meter (Scientific & Chemical Supplies, catalog number: PHM975050)
3. Two timers (Fisher Scientific, catalog number: 15177414)
4. Autoclave (Getinge, model: 400/500LS-E Series Steam Sterilizers (533LS-E))
5. Magnetic stirrer (Chemtech Scientific, model: C-MAG HS7)
6. 30 °C incubator (Eppendorf, New Brunswick Scientific™, model: Innova® 4000)
7. Spectrophotometer (Biochrom, model: WPA S800)
8. Centrifuge (Eppendorf, model: 5810 R)
9. Mercury-arc excitation fluorescence microscope Zeiss Axiovert 200M (Carl Zeiss, model: Axiovert 200 M) with an AxioCamMR3 camera with separate filter sets: 38HE for GFP and 43HE for mCherry excitation; Plan-Apochromat 1.40-numerical-aperture oil immersion, 100x objective

## Software

1. AxioVision
2. ImageJ 1.50g
3. Excel
4. MATLAB 2017a

## Procedure

### A. Cell preparation

1. Streak cells from a frozen stock, using a sterile pipette tip on a freshly-prepared YPD agar plate (see Recipes), and incubate at 30 °C for at least 24 h.
2. Set an overnight culture in a 14 ml tube by inoculating 3 ml of YPD with cells grown on a YPD plate. Single colonies are not needed for genetically integrated strains. Incubate at 30 °C, 180 rpm.
3. In the morning exchange the YPD medium (see Recipes) to YNB medium (see Recipes) supplemented with 4% glucose:
  - a. Pellet the cells by centrifugation at 1,000 x g for 3 min, remove the supernatant.
  - b. Resuspend the cells in 3 ml of YNB medium without any carbon source.
  - c. Pellet the cells by centrifugation at 1,000 x g for 3 min, remove the supernatant.
  - d. Suspend the cells in 3 ml of YNB supplemented with 4% glucose and incubate at 30 °C, 180 rpm, for ~4 h.

- e. Wash the culture by centrifugation ( $1,000 \times g$ , 3 min) and re-suspend in 2 ml of YNB with 4% glucose. Incubate at  $30^{\circ}\text{C}$ , 180 rpm, for about 10 min.
- f. Add 2  $\mu\text{l}$  of 100 mg/ml cycloheximide solution (see Recipes) to the final concentration of 100  $\mu\text{g}/\text{ml}$ . Incubate for 1 h at room temperature, without shaking, protect from light.
- g. Place 5  $\mu\text{l}$  of the culture on a microscope slide and cover with a 22 x 22 mm coverslip. Avoid any air under the coverslip.

## B. Data acquisition

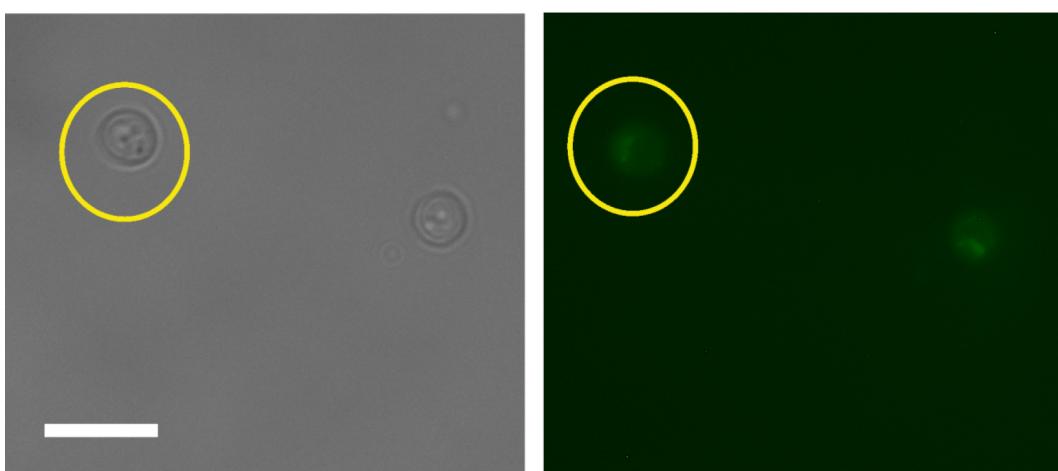
1. Place the sample under the microscope, coverslip on the objective and find a region of interest containing 5-10 cells (100x magnification) which appear stationary and firmly anchored to the glass surface.
2. Optimize exposure times for *in vivo* imaging of both GFP and mCherry fluorescent proteins to be able to detect a clear signal without saturating the detector. Under our microscope: GFP exposure time—22 sec, mCherry—7 sec.
3. Find another region with 5-10 cells positioned far away from the previous one to avoid any potential bleaching from previous illumination exposure.
4. Take a brightfield and a fluorescence image, by pressing the ‘snap’ button, with both channels using chosen exposure times, opening the mercury lamp shutter for only the length of exposure.
5. Photobleach GFP or mCherry by continuous illumination of the appropriate wavelength until the region appears completely dark. Continue for 1 min longer. With our settings the total exposure time is: 3 min 40 sec for GFP and 4 min for mCherry. Immediately after, begin timing and acquire a picture of the bleached fluorescent protein with an appropriate channel and a brightfield image. This is denoted time point 0 min.
6. Continue acquiring both fluorescent and brightfield pictures at the following time points after bleaching: 7.5, 15, 25, 30, 40, 60, 90 and 120 min.
7. As simultaneous photobleaching of GFP and mCherry is not possible under this microscope, the time points were staggered for GFP and mCherry as listed in Table 1.

**Table 1. Order of photobleaching and data acquisition using two channels**

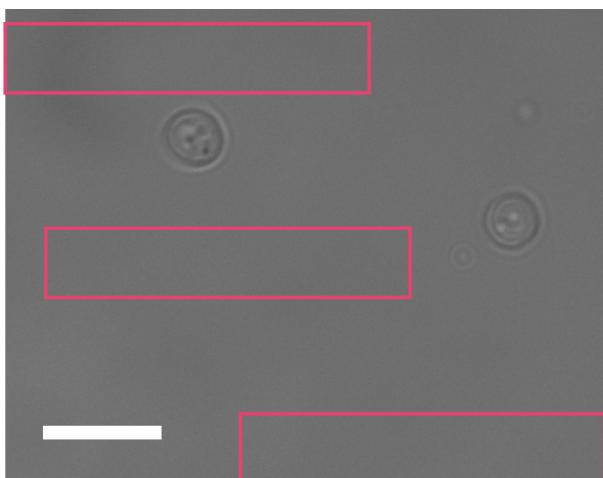
	GFP	mCherry
1	Bleaching for 3 min 40 sec	
2	Timer start, Time point 0 min	
3	Time point 7.5 min	
4		Bleaching for 4 min
5		Timer start, Time point 0 min
6	Time point 15 min	
7		Time point 7.5 min
8	Time point 25 min	
9		Time point 15 min
10	Time point 30 min	
11		Time point 25 min
12		Time point 30 min
13	Time point 40 min	
14		Time point 40 min
15	Time point 60 min	
16		Time point 60 min
17	Time point 90 min	
18		Time point 90 min
19	Time point 120 min	
20		Time point 120 min

### C. Data analysis

1. Images are converted into open standard tiff files from zvi by AxioVision software.
2. Further analysis is performed using ImageJ.
  - a. Open the first unbleached brightfield image.
  - b. By choosing an 'oval' selection tool, define a region of interest (ROI), an area around a cell as shown in Figure 1. It does not matter how much of non-cell area is included as every cell will be background-correct during the analysis.

**Figure 1. Selection of the region of interest for cell measurements.** Scale bar = 20  $\mu$ m.

- c. Open a fluorescence image of the same set, and define the same area of the same cell by simultaneously choosing 'Shift' and 'E' keys on the keyboard (Selection → Restore).
- d. From the menu bar select: Analyze → set measurements. Pick 'area' (represents a number of pixels,  $N$ ) and 'integrated density' (sum intensity for the cell,  $S_{cell}$ ). Press 'OK'.
- e. To obtain numeric values press 'Ctrl' + 'M' (Analyse → Measure). Record the result in Excel.
- f. Repeat throughout the entire data set for both channels keeping the same ROI.
- g. Repeat the entire procedure for all cells.
- h. Background correction: Choose random background areas around cells (Figure 2) and obtain numerical results for sum intensity ( $S_{bg}$ ).



**Figure 2. Selection of the region of interest for the background measurements.** Scale bar = 20  $\mu\text{m}$ .

- i. Find the average ( $S_{Abg}$ ) and multiply by the number of pixels from cell ( $N$ ) measurements. This is the intensity of the background represented within the cell area ( $I_{bg}$ ).

$$S_{Abg} = \langle S_{bg} \rangle; \\ I_{bg} = S_{Abg} \times N;$$

- j. Subtraction of the average background sum intensity ( $I_{bg}$ ) from the total intensity of the cell ( $S_{cell}$ ) represents  $I_{cell}$ , the cellular fluorescence intensity with background correction.

$$I_{cell} = S_{cell} - I_{bg}.$$

- k. The average of fluorescence intensity of all cells analysed within the data set gives the final value of the fluorescence intensity ( $I_{final}$ ) with appropriate estimation of SD and/or SE.

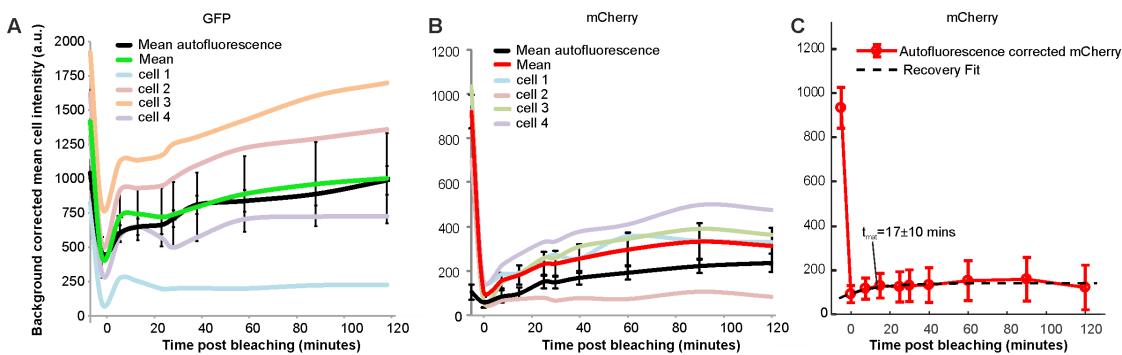
$$I_{final} = \langle I_{cell} \rangle.$$

- I. Plot the final fluorescence intensity ( $I_{final}$ ) vs. experimental time (Figures 3A and 3B) for fluorescently labelled cells and wild type autofluorescent cells. Any signal above autofluorescence is due to fluorescent protein maturation. For GFP (Figure 3A), no maturation was detected so it can be assumed that all of the fluorescent protein was mature in the cells and there is no ‘dark’ fraction. For mCherry (Figure 3B), some fluorescence recovery was measured. The following steps outline quantification of the maturation time and dark fraction.
- m. Subtract the autofluorescence from the mean fluorescent protein intensity at each time point (Figure 3C).
- n. Export the intensity and time values after the bleach by copying and pasting into two new variables in MATLAB, called x (for the time values ) and y (intensity values):
  - i. Right click on the Workspace → New. Name it x or y. Press ‘Enter’ on the keyboard.
  - ii. Double click on this new variable opens a table in the Editor where values of time (for x) or intensity (for y) can be pasted.
- o. Open the curve fitting toolbox from the Apps menu.
- p. Select x for ‘X data’ and y for ‘Y data’.
- q. Choose custom equation and type:

$$I_{rec} * \left( 1 - \exp \left( -\frac{x}{t_{mat}} \right) \right) + I_{bleach}$$

where,  $I_{bleach}$  is the remaining intensity after the bleach,  $I_{rec}$  is the recovered intensity above  $I_{bleach}$  and  $t_{mat}$  is the maturation time.

- r. If ‘Auto fit’ is ticked, fitting will be automatic.
- s. If the fit has not converged correctly, adjust the ‘Start point’ parameters in ‘Fit Options’ to reasonable estimates from the data i.e., y at x = 0 for  $I_{bleach}$  and y at x = end minus  $I_{bleach}$  for  $I_{rec}$ .
- t. If the fit has converged record the fit and goodness of fit parameters from the ‘Results’ panel. For Figure 3C,  $I_{rec} = 4.3 \times 10^4 \pm 3 \times 10^4$  counts,  $I_{bleach} = 5.2 \times 10^4 \pm 2.5 \times 10^4$  counts,  $t_{mat} = 17 \pm 10$  min with  $R^2 = 0.7$ .
- u. To calculate the proportion dark, immature protein; divide  $I_{rec}$  by the initial, autofluorescence corrected pre-bleach intensity. Here give ~5%.



**Figure 3. Characterisation of GFP and mCherry maturation times *in vivo*.** GFP maturation within genetically integrated protein fusion (A). Maturation of a genetically integrated mCherry fusion (B) and its' exponential recovery fit (C). Time normalized to bleach time at  $t = 0$ .

### Data analysis

Data was analyzed as outlined in section C of the Procedure. Statistical methods are outlined in Wollman *et al.* (2017) but also briefly outlined here. In imaging experiments, each cell can be defined as a biological replicate sampled from the cell population. Sample sizes of  $\sim 10$  cells were used to generate reasonable estimates of fluorescent protein maturation and are similar to previous studies (Badrinarayanan *et al.*, 2012). Technical replicates are not possible with irreversible photobleaching however noise is characterized by the autofluorescent of wild type control cell measurements.

### Notes

Autofluorescence is calculated as indicated in the protocol above but using a wild type yeast strain (*i.e.*, without any fluorescent proteins present).

### Recipes

1. Glucose 50% w/v  
Weigh 500 g of glucose  
Bring up to 1 L with MilliQ water  
Dissolve by using magnetic stirrer with heating  
Autoclave for 20 min at 121 °C
2. YPD agar with 4% glucose  
Mix yeast extract 5 g, Bacto-peptone (peptone from meat) 10 g and agar 10 g  
Bring up to 460 ml with MilliQ water  
Autoclave for 20 min at 121 °C  
Add 40 ml of glucose 50% w/v  
Cast plates: approximately 25 ml of the medium per plate

- Let them solidify, store upside down at 4 °C
3. YPD liquid medium with 4% glucose  
Mix yeast extract 5 g and Bacto-peptone (peptone from meat) 10 g  
Bring up to 460 ml with MilliQ water  
Autoclave for 20 min at 121 °C  
Add 40 ml of glucose 50% w/v
  4. YNB (Yeast Nitrogen Base) liquid medium without glucose  
Mix yeast nitrogen base without amino acids, without  $(\text{NH}_4)_2\text{SO}_4$  1.7 g, complete supplement 0.79 g,  $(\text{NH}_4)_2\text{SO}_4$  5 g  
Dissolve in 900 ml of MilliQ water, adjust pH 5.8-6.0 using NaOH  
Bring up to 1,000 ml with MilliQ water  
Autoclave for 20 min at 121 °C
  5. YNB (Yeast Nitrogen Base) liquid medium with 4% glucose  
Mix yeast nitrogen base without amino acids, without  $(\text{NH}_4)_2\text{SO}_4$  1.7 g, complete supplement 0.79 g,  $(\text{NH}_4)_2\text{SO}_4$  5 g  
Dissolve in 900 ml of MilliQ water, set pH 5.8-6.0 using NaOH  
Bring up to 920 ml with MilliQ water  
Autoclave for 20 min at 121 °C  
Add 80 ml of glucose 50% w/v
  6. 100 mg/ml cycloheximide solution in EtOH  
Weigh 0.5 g of cycloheximide and dissolve in 5 ml of absolute EtOH  
Aliquot and store at -20 °C

### **Acknowledgments**

This work was supported by the Biological Physical Sciences Institute, Royal Society, MRC (grant MR/K01580X/1), BBSRC (grant BB/N006453/1), the European Commission via Marie Curie-Network for Initial Training ISOLATE (Grant agreement No.: 289995), and the Royal Society Newton International Fellowship (NF160208). This protocol was adapted from Wollman *et al.* (2017).

Conflict of interests: Authors declare no conflict of interest.

### **References**

1. Badrinarayanan, A., Reyes-Lamothe, R., Uphoff, S., Leake, M. C. and Sherratt, D. J. (2012). [In vivo architecture and action of bacterial structural maintenance of chromosome proteins](#). *Science* 338(6106): 528-531.

2. Beattie, T. R., Kapadia, N., Nicolas, E., Uphoff, S., Wollman, A. J., Leake, M. C. and Reyes-Lamothe, R. (2017). [Frequent exchange of the DNA polymerase during bacterial chromosome replication](#). *Elife* 6.
3. Hartwell, L. H., Culotti, J. and Reid, B. (1970). [Genetic control of the cell-division cycle in yeast. I. Detection of mutants](#). *Proc Natl Acad Sci U S A* 66(2): 352-359.
4. Khmelinskii, A., Keller, P. J., Bartosik, A., Meurer, M., Barry, J. D., Mardin, B. R., Kaufmann, A., Trautmann, S., Wachsmuth, M., Pereira, G., Huber, W., Schiebel, E. and Knop, M. (2012). [Tandem fluorescent protein timers for \*in vivo\* analysis of protein dynamics](#). *Nat Biotechnol* 30(7): 708-714.
5. Leake, M. C. (2012). [The physics of life: one molecule at a time](#). *Philos Trans R Soc Lond B Biol Sci* 368(1611): 20120248.
6. Leake, M. C., Chandler, J. H., Wadhams, G. H., Bai, F., Berry, R. M. and Armitage, J. P. (2006). [Stoichiometry and turnover in single, functioning membrane protein complexes](#). *Nature* 443(7109): 355-358.
7. Shashkova, S., Wollman, A. J. M., Leake, M. C. and Hohmann, S. (2017). [The yeast Mig1 transcriptional repressor is dephosphorylated by glucose-dependent and -independent mechanisms](#). *FEMS Microbiol Lett* 364(14).
8. Wollman, A. J. and Leake, M. C. (2015). [Millisecond single-molecule localization microscopy combined with convolution analysis and automated image segmentation to determine protein concentrations in complexly structured, functional cells, one cell at a time](#). *Faraday Discuss* 184: 401-424.
9. Wollman, A. J., Shashkova, S., Hedlund, E. G., Friemann, R., Hohmann, S. and Leake, M. C. (2017). [Transcription factor clusters regulate genes in eukaryotic cells](#). *Elife* 6: e27451.

## Terminal Deoxynucleotidyl Transferase Mediated Production of Labeled Probes for Single-molecule FISH or RNA Capture

Imre Gaspar\*, Frank Wippich and Anne Ephrussi\*

Developmental Biology Unit, European Molecular Biology Laboratory (EMBL), Heidelberg, Meyerhofstrasse 1, 69117 Germany

\*For correspondence: [imre.gaspar@embl.de](mailto:imre.gaspar@embl.de); [anne.ephrussi@embl.de](mailto:anne.ephrussi@embl.de)



**[Abstract]** Arrays of short, singly-labeled ssDNA oligonucleotides enable *in situ* hybridization with single molecule sensitivity and efficient transcript specific RNA capture. Here, we describe a simple, enzymatic protocol that can be carried out using basic laboratory equipment to convert arrays of PCR oligos into smFISH and RAP probesets in a quantitative, cost-efficient and flexible way.

**Keywords:** Terminal deoxynucleotidyl transferase, Labelled terminator nucleotide, Probe production, Single molecule FISH (smFISH), RNA capture, RNA affinity purification (RAP)

**[Background]** The use of multiple, singly-labeled, short oligonucleotides of synthetic origin has vastly improved the detection of specific transcripts with high specificity and single molecule sensitivity (Femino *et al.*, 1998; Raj *et al.*, 2008). Such probe molecules have improved penetration and require milder hybridization conditions than the classically used long nucleic acid probes, resulting in better preservation of the structure of the specimen (e.g., Little *et al.*, 2015, Gaspar *et al.*, 2017a). Since in this design multiple oligonucleotides—typically 24–96—target different portions of the same transcript, there occurs an accumulation of signal on the specific target molecules over the aspecific background, as opposed to the equal signal produced by long multiply labeled probes (Raj *et al.*, 2008). Moreover, as the labeling of the individual short probes is quantitative—as opposed to the stochastic labelling of the long probes—the signal intensity directly and linearly correlates with the transcript copy number at a given spot, allowing precise recording/counting of the target RNA molecules (Raj *et al.*, 2008, Little *et al.*, 2015). Until now, the production of smFISH probe arrays has depended on chemical synthesis and labeling that rendered such single molecule FISH application inflexible and costly. Here, we describe an effective and cost-efficient enzymatic three-pot probe production ( $3P^3$ ) assay that makes use of terminal deoxynucleotidyl transferase (TdT) and custom labeled terminator nucleotides to convert any custom-assembled array of cheap PCR oligos into smFISH probes bearing fluorescent or non-fluorescent labels of the experimenter's choice (Gaspar *et al.*, 2017b). These enzymatically produced  $3P^3$  probes are chemically nearly identical to smFISH probes from other sources. Thus the same protocols—optimized for a given specimen under study—can be used to perform single molecule FISH (reviewed in Gaspar and Ephrussi, 2015) and RNA capture analyses (see e.g., Gaspar *et al.*, 2017a and Khong *et al.*, 2017).

## **Materials and Reagents**

1. 1.5 ml Eppendorf tube (e.g., Sigma-Aldrich, catalog number: Z336769)
2. 0.2 ml thin-walled PCR tube (e.g., Corning, catalog number: 6571)
3. 2 cm thick adhesive tape (Tesa)
4. Glass slides (e.g., VWR, catalog number: 631-0411) and coverslips (e.g., 22 x 22 x 0.17 mm, Marienfeld-Superior, catalog number: 0107052) for sample preparation
5. 15 ml tubes (e.g., Corning, Falcon®, catalog number: 352097)
6. 0.22 µm filter (e.g., Corning, catalog number: 431227)
7. 3 cm wide foldback paperclips (e.g., Staples, catalog number: WW-9130156)
8. Amine reactive labels (tested and working):
  - a. BDP-FL-NHS (Lumiprobe, catalog number: 11420)
  - b. Atto-tec Atto488-NHS (Atto-tec, catalog number: AD 488-31), Atto532-NHS (Atto-tec, catalog number: AD 532-31), Atto565-NHS (Atto-tec, catalog number: AD 565-31) and Atto633-NHS (Atto-tec, catalog number: AD 633-31)
  - c. AlexaFluor488-NHS (Thermo Fisher Scientific, Invitrogen™, catalog number: A20000)
  - d. Abberior STAR 470SXP-NHS (Abberior, catalog number: 1-0101-008-3) and Abberior STAR RED-NHS (Abberior, catalog number: 1-0101-011-3)
  - e. biotin-NHS (Sigma-Aldrich, catalog number: H1759)
9. Anhydrous DMSO (e.g., Sigma-Aldrich, catalog number: 276855)
10. Optional: silica gel (e.g., Merck, catalog number: 1.01969.1000) (see Note 1)
11. Amino-11-ddUTP (Lumiprobe, catalog number: 15040) or 5-propargylamino-ddUTP (Jena Biosciences, catalog number: NU-1619)
12. 1 M NaHCO<sub>3</sub>, pH 8.4 (e.g., Sigma-Aldrich, catalog number: S5761)
13. A custom designed target specific array of non-overlapping ssDNA oligonucleotides (desalting purification is sufficient, see Software section for the design)
14. 20 U/µl Terminal deoxynucleotidyl transferase (TdT) with 5x TdT buffer (Thermo Fisher Scientific, Thermo Scientific™, catalog number: EP0161)
15. 1-3 M Na-acetate, pH 5.5 (e.g., Sigma-Aldrich, catalog number: S2889)
16. 5 mg/ml linear acrylamide (e.g., Thermo Fisher Scientific, Invitrogen™, catalog number: AM9520)
17. Ethanol (e.g., Merck, EMD Millipore, catalog number: 1.00983)
  - a. 100% ethanol, -20 °C
  - b. 80% ethanol, 4 °C
  - c. 70% ethanol, RT
18. Nuclease free ddH<sub>2</sub>O (e.g., New England Biolabs, catalog number: B1500S)
19. 40% Acrylamide/Bis solution, 29:1 (e.g., Bio-Rad Laboratories, catalog number: 1610146)
20. Urea (e.g., Sigma-Aldrich, catalog number: U5378)

21. *N,N,N',N'-Tetramethylethylenediamine* (TEMED) (e.g., Sigma-Aldrich, catalog number: T9281)
22. 10% (w/v) ammonium persulfate (APS) (e.g., Sigma-Aldrich, catalog number: A3678)
23. 6x gel loading dye (e.g., New England Biolabs, catalog number: B7021S)
24. SYBR-GOLD (e.g., Thermo Fisher Scientific, Invitrogen<sup>TM</sup>, catalog number: S11494)
25. Optional: colorimetric Biotin Assay Kit (e.g., Sigma-Aldrich, catalog number: MAK171) (see Note 8)
26. 20 mg/ml Proteinase-K (e.g., Thermo Fisher Scientific, Invitrogen<sup>TM</sup>, catalog number: AM2546)
27. Mounting medium
  - a. VectaShield (Vector Laboratories, catalog number: H-1000)
  - b. 80% TDE (see Recipes)
28. Pierce<sup>®</sup> Avidin agarose (Thermo Fisher Scientific, Thermo Scientific<sup>TM</sup>, catalog number: 20219)
29. Dynabeads<sup>®</sup> MyOne<sup>TM</sup> C1 (Thermo Fisher Scientific, Invitrogen<sup>TM</sup>, catalog number: 65001)
30. Quick-RNA<sup>TM</sup> MicroPrep Kit (Zymo Research, catalog number: R1050)
31. Tris-HCl pH 7.0 (e.g., Sigma-Aldrich, Roche Diagnostics, catalog number: 10812846001)
32. Colorimetric Biotin Assay Kit (Sigma-Aldrich, catalog number: MAK171)
33. Tris base (e.g., Sigma-Aldrich, catalog number: T1503)
34. Ethylenediaminetetraacetic acid (EDTA) (e.g., Sigma-Aldrich, catalog number: E5391)
35. Sodium chloride (NaCl) (e.g., Merck, catalog number: 106404)
36. Potassium chloride (KCl) (e.g., Merck, catalog number: 104936)
37. Potassium dihydrogen phosphate dihydrate ( $\text{KH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ ) (e.g., Merck, catalog number: 104873)
38. Sodium phosphate dibasic ( $\text{Na}_2\text{HPO}_4$ ) (e.g., Merck, catalog number: 106342)
39. EM-grade paraformaldehyde (e.g., Electron Microscopy Sciences, catalog number: 15710)
40. Triton X-100 (e.g., Sigma-Aldrich, catalog number: X100)
41. Boric acid (e.g., Merck, catalog number: 100165)
42. Ethylene carbonate (e.g., Sigma-Aldrich, catalog number: E26258)
43. 50 mg/ml heparin (e.g., Sigma-Aldrich, catalog number: H3393)
44. 10 mg/ml salmon sperm DNA (e.g., Sigma-Aldrich, catalog number: D7656)
45. 2,2'-Thiodiethanol (Sigma-Aldrich, catalog number: 166782)
46. 20% (v/v) SDS (e.g., Sigma-Aldrich, catalog number: 05030)
47. PMSF (e.g., Sigma-Aldrich, catalog number: P7626)
48. cOmplete<sup>®</sup> mini EDTA-free protease inhibitor (Roche Diagnostics, catalog number: 11836170001)
49. RiboLock RNase Inhibitor (Thermo Fisher Scientific, Thermo Scientific<sup>TM</sup>, catalog number: EO0381)
50. Sodium citrate (e.g., Sigma-Aldrich, catalog number: S1804)
51. TE buffer (see Recipes)
52. 1x PBS (see Recipes)
53. Fixative (see Recipes)

54. PBT (see Recipes)
55. 1.5x PAGE loading buffer (see Recipes)
56. 1x and 10x TBE (see Recipes)
57. 15% PA - 8 M Urea stock (see Recipes)
58. 20x SSC buffer (see Recipes)
59. 2x full-HYBEC (see Recipes)
60. 2x wash-HYBEC (see Recipes)
61. Lysis buffer (see Recipes)
62. Capturing hybridization buffer (see Recipes)
63. Low salt wash buffer (see Recipes)
64. High salt wash buffer (see Recipes)
65. Elution buffer (see Recipes)

## **Equipment**

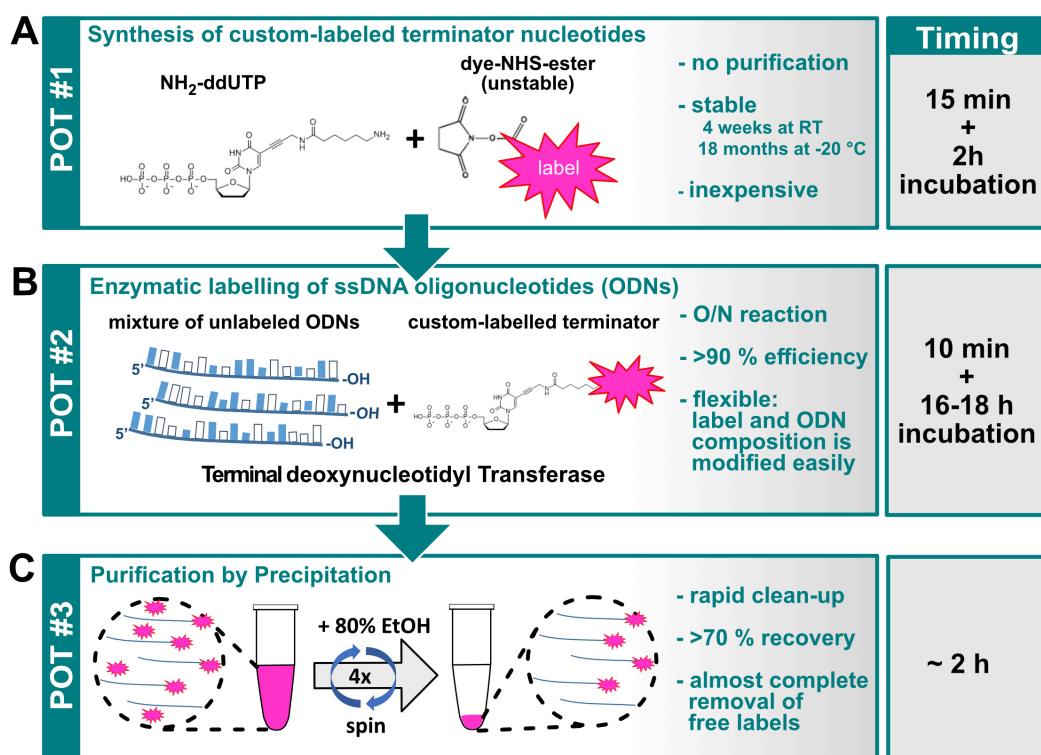
1. Optional: inert gas (e.g., Argon) glove-box (e.g., Inert Technology, model: PureLab HE 2GB) (see Note 1)
2. PCR machine with programmable hot-lid (e.g., Bio-Rad Laboratories, catalog number: 1851148)
3. -20 °C freezer
4. Refrigerated table-top centrifuge (e.g., Eppendorf, catalog number: 5426000018)
5. Erlenmeyer flask
6. Handcast PAGE system including a 1 mm spacer plate (e.g., Bio-Rad Laboratories, catalog number: 1653311), a short plate (e.g., Bio-Rad Laboratories, catalog number: 1653308) and a 15-well comb (e.g., Bio-Rad Laboratories, catalog number: 4560016)
7. Vertical Electrophoresis Cell (e.g., Bio-Rad Laboratories, catalog number: 1658005)
8. Electrophoresis power supply (e.g., Bio-Rad Laboratories, catalog number: 1645050)
9. Gel documentation system with filters to image fluorescence of SYBR-GOLD and the fluorescent dye used for labeling (e.g., Bio-Rad Laboratories, catalog number: 17001402)
10. P2, P200 and P1000 pipettes
11. Rocking thermoblock (e.g., Eppendorf, model: ThermoMixer® C, catalog number: 5382000015)
12. Microscope for imaging (we use a Leica SP8 (Leica, model: Leica TCS SP8) equipped with a 63x NA=1.4 oil immersion objective and two HyD detectors)
13. Tissue grinder (e.g., DWK Life Sciences, Kimble, catalog numbers: 8853000015 or 8853000040)
14. Rotator (e.g., Cole-Parmer, Stuart, model: Rotator SB3)
15. Magnetic rack (e.g., New England Biolabs, catalog number: S1507S)
16. Moisture free chamber (see Note 1)

17. UV/VIS spectrophotometer (e.g., Thermo Fisher Scientific, Thermo Scientific™, model: NanoDrop™ 8000, catalog number: ND-8000-GL)
18. Nutator (e.g., Labnet International, model: S0500)

### Software

1. A probe designer algorithm, e.g., the Stellaris™ Probe Designer (<https://www.biosearchtech.com/support/tools/design-software/stellaris-probe-designer>, registration required) or the provided *smFISHprobe\_finder.R* script ([Supplementary File 1](#), see Notes 2 and 17)
2. MS Office Excel to run the interactive *probe\_calculator.xls* sheet ([Supplementary File 2](#))
3. ImageJ/FIJI (<https://imagej.nih.gov/ij/>) with the xsPT plugin ([https://github.com/Xaft/xs/blob/master/\\_xs.jar](https://github.com/Xaft/xs/blob/master/_xs.jar))
4. Optional: deconvolution software, e.g., Huygens Essentials (<https://svi.nl/Huygens-Essential>) or DeconvolutionLab2 (Sage et al., 2017; <http://bigwww.epfl.ch/deconvolution/deconvolutionlab2/>)
5. R (preferentially with RStudio) for data analysis
6. *smFISH\_analysis.R* to analyze the sensitivity and specificity of smFISH ([Supplementary File 3](#))

### Procedure



**Figure 1. Graphical overview of the three-pot probe production assay.** A. Conjugation of the label to the NH<sub>2</sub>-ddUTP terminator nucleotide; B. TdT mediated labeling of ssDNA oligonucleotides; C. Purification of labeled ssDNA molecules (probes).

A. Preparing the dye conjugated ddUTP (First pot, Figure 1A)

1. Reconstitute the dye-NHS ester to 40 mM final concentration in anhydrous DMSO in a moisture free environment. (see Note 1)
2. In a clean 1.5 ml Eppendorf tube, aliquot X µl of 20 mM Amino-11-ddUTP or 5-propargylamino-ddUTP. (see Note 3)
3. Add 0.2X µl of 1 M NaHCO<sub>3</sub> (pH = 8.4).
4. Finally, add X µl of 40 mM dye-NHS ester (see Note 4). Mix well and incubate at RT for 2 h sealed from light.
5. After the incubation, add 1.8X µl dH<sub>2</sub>O to expand the reaction volume to 4X µl. This results in a 5 mM stock of dye conjugated ddUTP (see Note 5). The resulting labeled nucleotide is stable for over a year when stored at -20 °C.

B. Production of labeled ssDNA oligonucleotides (second pot, Figure 1B)

1. Prepare an equimolar mixture of all different ssDNA oligos that should be labeled together, *i.e.*, that target the same transcript. The total concentration of oligos should not be lower than 100 µM. (see Note 6)
2. To label 1 nmol of oligonucleotide mixture, prepare the following labeling mixture in a 0.2 ml thin-walled PCR tube:

Amount	Final concentration
1 nmol oligo-mixture ( <i>e.g.</i> , 4 µl of 250 µM stock)	66.67 µM
3 µl 5x TdT reaction buffer (with Co <sup>2+</sup> )	1x
3-5 nmol dye-ddUTP ( <i>e.g.</i> , 0.6-1.0 µl of 5 mM stock)	200-330 nM
1x standard amount of TdT	0.4-0.8 U/µl
dH <sub>2</sub> O	to 15 µl

Please use the interactive *probe\_calculator.xls* Excel sheet ([Supplementary File 2](#)) to get the reaction composition adjusted to the dye-ddUTP used for labeling.

3. Incubate at 37 °C O/N (16-18 h) in a PCR machine with the hot-lid set to 37 °C.

C. Purification and spectroscopic analysis of the labeled oligonucleotide mixture (third pot, Figure 1C)

1. After the O/N incubation, add 60 µl of 1 M Na-acetate (pH = 5.5), 125 µl of dH<sub>2</sub>O and 1.5 µg linear acrylamide to the reaction mixture.

2. Transfer the entire 200  $\mu$ l into a clean 1.5 ml Eppendorf tube. Add 800  $\mu$ l 100% ethanol prechilled at -20 °C. Invert the tube a couple of times and place it into the -20 °C freezer for about 15-20 min.
3. In the meantime, cool the table-top centrifuge to 4 °C.
4. After the -20 °C incubation, spin the oligonucleotide mixture at 16,000  $\times g$  for 20 min at 4 °C.
5. Remove the supernatant and add 1 ml 80 % ethanol prechilled at 4 °C. Vortex until the pellet dissociates from the bottom of the tube.
6. Spin at 16,000  $\times g$  for 5 min at 4 °C.
7. Wash away the pellet from the wall of the tube with 1 ml 80% ethanol and transfer the entire volume including the floating pellet into a clean 1.5 ml Eppendorf tube. Repeat Steps C5 and C6 two more times.
8. Remove the supernatant from the last wash and let the pellet dry on air. (see Note 7)
9. Resuspend the dried pellet in 15-50  $\mu$ l nuclease-free dH<sub>2</sub>O.
10. Measure the absorbance of the labeled oligonucleotide mix at 260 nm and at the dye absorption maximum (e.g., 570 nm for Atto565).
11. In order to calculate the concentration and the degree-of-labeling (DOL), measure the absorbance of the unlabeled, undiluted oligonucleotide mix at 260 nm. (see Note 8)
12. Calculate the molar extinction coefficient ( $\epsilon_{\text{oligo}}$ ) of the oligonucleotide mixture by dividing the measured OD<sub>260 nm</sub> value with the concentration of the mixture (in M). Increase this value by 9,000 mol<sup>-1</sup> cm<sup>-1</sup> to correct for the UTP added to the 3' of the ssDNA molecules.
13. Calculate the concentration of the labeled oligo by dividing the dye-corrected OD<sub>260 nm</sub> absorption by  $\epsilon_{\text{oligo}} \cdot \text{Coligo} = (\text{OD}_{260 \text{ nm}} - \text{Cf}_{260 \text{ nm}} \times \text{OD}_{\text{dye}}) / \epsilon_{\text{oligo}}$  (see Note 9).
14. Calculate the concentration of the dye as follows:  $c_{\text{dye}} = \text{OD}_{\text{dye}} / \epsilon_{\text{dye}}$  (provided by the dye manufacturer).
15. Typically, only a small fraction of the dye molecules is present as contaminants (i.e., free from ssDNA), therefore the DOL is estimated as follows:  $\text{DOL} = c_{\text{dye}} / c_{\text{oligo}}$ .
16. The fraction of recovered oligonucleotides is assessed by taking the ratio of the recovered and initial amounts of the oligo (recovery% =  $c_{\text{oligo}} \times V_{\text{resuspension}} / n_{\text{initial}}$ ).
17. The measured OD values can be entered into the interactive *probe\_calculator.xls* Excel sheet ([Supplementary File 2](#)) to obtain the concentration and the DOL of the labeled oligonucleotide mixture.
18. Store the labeled probes at -20 °C.

#### D. Trouble-shooting of 3P<sup>3</sup> probe production

Labeled oligo mixtures with  $0.9 < \text{DOL} \leq 1.0$  are considered good quality products that can be used in smFISH applications.

1. The most typical cause of DOLs lower than 0.9 is the composition of the oligonucleotide mixture. We currently lack an understanding of which property(s) of the mixture—e.g., formation of intra- and intermolecular hybrids in the mixture— influence the labeling efficiency. However, if

DOL < 0.9 is obtained, we recommend ‘splitting’ the oligo mixture into two-three non-overlapping fractions, *i.e.*, preparing two-three mixtures of the ssDNA oligos present in the original mixture. Individual labeling of these mixtures can help in identifying any molecules that behave extraordinarily in the labeling reaction. In most cases, we found that this ‘splitting’ almost completely alleviates the low labeling efficiency problem, *i.e.*, the DOL of each of the split mixtures increases above 0.9.

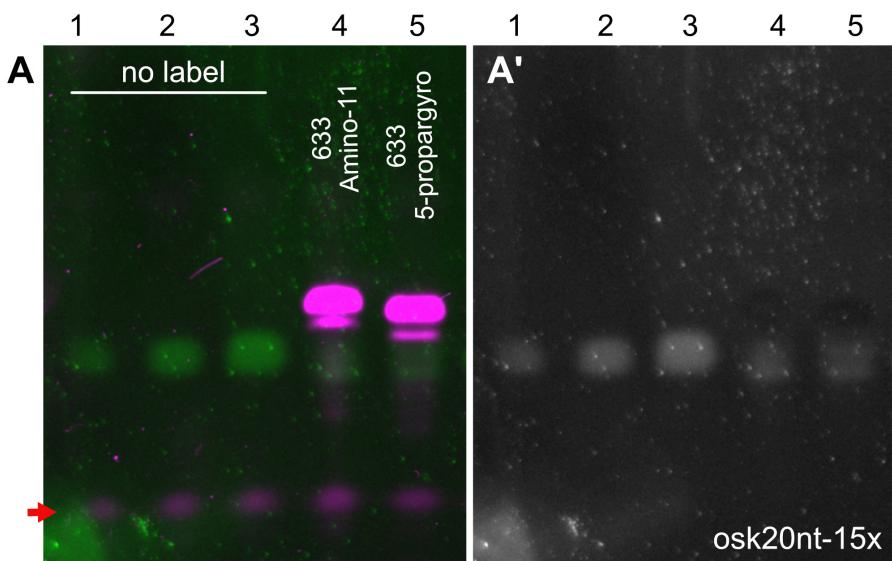
2. Another solution we found was to re-label the already labeled oligonucleotide mixture, *i.e.*, start over the labeling protocol from Step B2 using the labeled ssDNA molecules as input. (REF)
3. The second most frequent reason for low DOL is the non-accurate (lower than actual) measurement of the molar extinction coefficient of the unlabeled oligo mixture. This can be noticed that when calculating the fraction of recovered oligonucleotides (recovery %) one obtains a value higher than one. In such cases, we recommend re-measuring the OD<sub>260 nm</sub> of the unlabeled oligo mixture at different concentrations (*e.g.*, at 50, 100 and 200 μM) and estimating the  $\epsilon_{\text{oligo}}$  by taking the slope of the line fitted to the data points.
4. We found that terminal transferase activity of TdT varies from batch to batch. This may result in improper labeling (DOL < 0.9) when using 1x standard amount of the enzyme. We recommend to redefine the standard amount when starting a new aliquot of TdT, by setting up several labeling reactions of a single ssDNA oligo with Atto633-ddUTP (Procedures B and C) using increasing concentrations of TdT—*e.g.*, 1x, 2x and 3x of the standard amount found in the recipe generator. Use the smallest concentration of the enzyme that yields DOL > 0.9 as the new standard amount.
5. DOL > 1.0 may also indicate an imprecisely determined  $\epsilon_{\text{oligo}}$ . This would appear as a huge loss of the oligos (recovery < 50%). Try the same solution as in Step D3.
6. If the  $\epsilon_{\text{oligo}}$  is appropriate, DOL > 1.0 indicates free dye contamination. In such cases it is advised to measure the DOL by PAGE (Procedure E). If the DOL determined by densitometry is ~1.0 and the difference of the two DOLs is small (max. 0.05–0.1), the free dye contamination is considered harmless in the downstream applications. If it is higher, re-purification of the oligonucleotide mixture by another method (*e.g.*, by size exclusion chromatography) is recommended. We observed such high free dye contamination when using Abberior470SX-, Atto488- and AlexaFluor488-ddUTP (Gaspar *et al.*, 2017b).

#### E. PAGE analysis of the labeled oligonucleotides

Gel electrophoresis provides a simple means to confirm the DOL estimated from spectroscopy data and to calculate DOL in case of non-fluorescent modifications (*e.g.*, biotinylation). PAGE analysis also allows the quality control of most of the labeled ddUTP analogs, *i.e.*, to determine whether there are unconjugated ddUTP molecules in the dye conjugated ddUTP stock (produced in Procedure A) that will result in reduced DOL of oligonucleotides.

**IMPORTANT:** Only oligo-mixtures containing ssDNA molecules of identical length (*e.g.*, only 20mers) should be analyzed by PAGE.

1. Wipe the glass plates for gel casting clean with 70% ethanol, assemble the cassette and seal with 20 mm wide transparent tape, leaving the top (where the comb will be inserted) open. Put two fold-back clamps on the two sides of the cassette such that the clamps clamp above the spacer between the two glass plates.
2. To cast a 10 x 8 cm acrylamide gel, add 8 ml of 15% PA - 8 M urea stock (see Recipes), 40 µl 10% APS and 5 µl TEMED into an Erlenmeyer flask. Mix well by swirling the flask and pour the mixture between the two plates of the assembled cassette. Fill to the top.
3. Insert the comb, place the cassette horizontally and wait until the gel polymerizes (15-20 min).
4. Remove the clamps, the tape and the comb. Rinse the outside of the cassette with dH<sub>2</sub>O to remove gel pieces polymerized on the outer surface and assemble the PAGE chamber.
5. Fill with 1x TBE buffer (see Recipes) and pre-run the gel for 30 min with 2.5 mA/cm current (20 mA for an 8 cm long gel).
6. In the meantime, prepare 5 µl of each sample by mixing 3 µl 1.5x PAGE loading buffer (see Recipes) and 2 µl oligonucleotide mixture containing 15-60 pmoles of labeled oligo. As size marker use a dilution row of the unlabeled oligonucleotide mixture (e.g., 1.5, 3 and 6 pmoles), no boiling is necessary.
7. After the pre-run (E5), rinse all wells on the gel with 1x TBE using a P200 pipette to remove the accumulated urea that would prevent the loading.
8. Load the samples and run the gel until the xylene cyanol (blue) and the bromophenol blue (purple) markers (from the 6x gel loading dye) reach about the one-third and two-third of the gel length.
9. Image the fluorescently labeled pool of molecules on a gel-imager with appropriate filter sets to excite and detect the incorporated fluorescent dyes.
10. Incubate the gel with SYBR-GOLD (or similar RNA/ssDNA dye) diluted 1:10,000 in 1x TBE for 10-15 min.
11. Re-image the gel to detect both the non-modified and modified pools of ssDNA.
12. Due to the addition of a bulky terminator nucleotide, labeled oligonucleotides run slower and thus they are well separated from their non-modified peers during PAGE. The amount of non-modified oligos can be measured by comparing the corresponding SYBR-GOLD fluorescence intensity to that of the dilution row of the unlabeled oligonucleotide mixture used as loading control (Figures 2A and 2A').
13. The presence of unconjugated ddUTP will result in production of unlabeled, ddUTP-terminated oligonucleotides. On gel, they appear as an intermediate band migrating between the unlabeled and labeled, fluorescent pool of oligos.

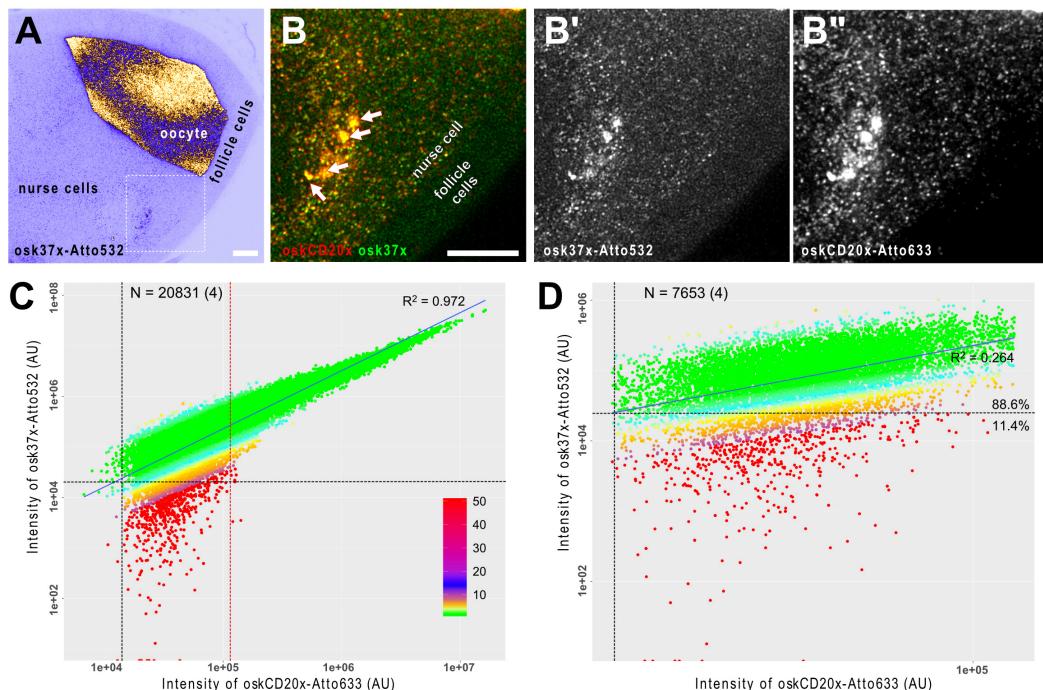


**Figure 2. PAGE analysis of the labeled oligonucleotide mixtures.** Labelling of the osk20nt-15x (Gaspar *et al.*, 2017b) probe mixture with Atto633-amino-11-ddUTP (lane 4) or Atto633-5-propargylamino-ddUTP (lane 5). The fluorescently labeled ssDNA species (magenta, A) migrate slower in the gel than the unlabeled oligonucleotides (green, A, gray, A'). Note that the probe molecules labeled with far-red fluorescence are not visible in the SYBR-GOLD channel, possibly because of a very efficient energy transfer from SYBR GOLD to Atto633 that quenches the SYBR GOLD fluorescence. While this is the case with other far-red dyes also (e.g., Abberior-RED), when there is no quenching dye present (e.g., biotinylated probes) or when the FRET fluorescence can be detected by the SYBR GOLD imaging setup (e.g., in case of Atto565), the labeled probes appear in the SYBR GOLD channel (Gaspar *et al.*, 2017b). 1.5, 3, 6, 20 and 20 pmol ssDNA mixture was loaded to lanes 1-5, respectively. After a labeling reaction with either of the terminator nucleotides, only low amounts (< 1.5 pmol) of unlabeled oligonucleotides were left in the mixtures (lanes 4 and 5A and 5A'), indicating near-quantitative labeling with both terminator nucleotides. The red arrow indicates fluorescence of the bromophenol blue dye.

#### F. Single molecule FISH in *Drosophila* ovaries

1. Dissect ovaries into a 1.5 ml Eppendorf tube containing 300-500  $\mu$ l fixative (for ovary dissection, please refer to Gaspar and Ephrussi, 2017).
2. Fix for 20 min by nutating the dissected material.
3. Remove the fixative and rinse the ovaries with 1 ml PBT (see Recipes).
4. Wash ovaries in 1 ml fresh PBT for 10 min while nutating.
5. Replace PBT and add Proteinase-K to 2  $\mu$ g/ml final concentration. Nutate for 5 min at room temperature (RT). (see Note 10)
6. Preheat 0.5 ml 0.05 v/v % SDS in PBS (see Recipes) to 95 °C.

7. After the 5 min Proteinase-K digestion, remove the PBT and immediately apply the preheated SDS/PBS to the ovaries. Incubate them for 5 min at 95 °C.
8. Add 1 ml RT PBS to the ovaries to cool the solution.
9. Replace the wash solution with 200  $\mu$ l 2x full-HYBEC (see Recipes).
10. Transfer the tube to a rocking thermoblock set to 37-42 °C and shake at 1,000 RPM for 10 min.
11. In the meantime, prepare the probe solution (5-12.5 nM/individual probe) in 50  $\mu$ l of 2x full-HYBEC. Transfer the probe solution to the thermoblock and allow it to warm up (2-3 min). (see Note 11)
12. After the 10 min incubation of the ovaries, apply the probe solution by mixing it into the 200  $\mu$ l 2x full-HYBEC already on the specimen.
13. Incubate for 1.5-3 h at 37-42 °C while rocking at 1,000 RPM.
14. 10-15 min before the end of the incubation, prewarm two times 1 ml 2x wash-HYBEC (see Recipes) to the hybridization temperature.
15. Remove the hybridization solution—it may be kept at 4 °C for another hybridization—and wash with 1 ml prewarmed 2x wash-HYBEC for 2 x 15 min.
16. Wash once with 1 ml RT PBT for another 15 min.
17. Thoroughly remove the PBT and apply mounting medium, e.g., 80% TDE (see Recipes) or VectaShield. (see Note 12)
18. After allowing the mounting medium to soak for at least one hour, mount the ovaries onto glass slides and image them using a high NA (> 1.1 NA) objective (Figure 3A).



**Figure 3. smFISH analysis of *oskar* mRNA in developing *Drosophila* egg-chambers.** A. *oskar* mRNA is produced in the transcriptionally active nurse cells (left) and it is transported into

the oocyte, where it localizes eventually at the posterior pole (on the right). The female germ-line cells (nurse cells + oocyte) are encapsulated by a layer of somatic epithelium, the follicle cells that do not express *oskar* mRNA. B. Close-up of the boxed region in A. *oskar* mRNA was detected by two different probe sets osk37x-Atto532 (green, osk3UTR#1-15 + oskCD#1-22) and oskCD20x-633 (red, oskCD#23-42, Gaspar *et al.*, 2017b). There is a high degree of co-localization of the two puncta-like signal in the nurse cell compartment (B) and no such strong accumulations of the probes is detected in the follicle cells (B-B"). Arrows indicate multiple transcriptional loci inside the polyploid nucleus of the nurse cell (B). Scale bars represent 10  $\mu$ m. C and D. Correlation of the signal intensities of the two channels. C. When all objects across the egg chamber are considered, a strong linear correlation ( $R^2 = 0.972$ ) is established, indicating that the RNA content of the detected objects is variable. By fitting multiple Gaussian functions to the signal intensity distribution (Little *et al.*, 2015; Gaspar *et al.*, 2017b), it is possible to filter the smFISH objects containing a single copy of *oskar* RNA. D. In this regime, the correlation of the signal intensities is low to moderate ( $R^2 = 0.264$ ), however, vast majority of the objects can be detected in both channels—e.g., 88.6% of the objects analyzed in the Atto633 channel are also detectable in the Atto532 channel also. Vertical and horizontal black dashed lines represent the detection thresholds for the Atto633 and Atto532 channels, respectively. Red dashed line shows the boundary between objects with single and multiple copies of RNA (C). Colours represent the relative intensity difference between the two channels (see panel C for key). The number of smFISH objects (number of experiments) is indicated in C and D.

#### G. RNA capture from ovarian lysate

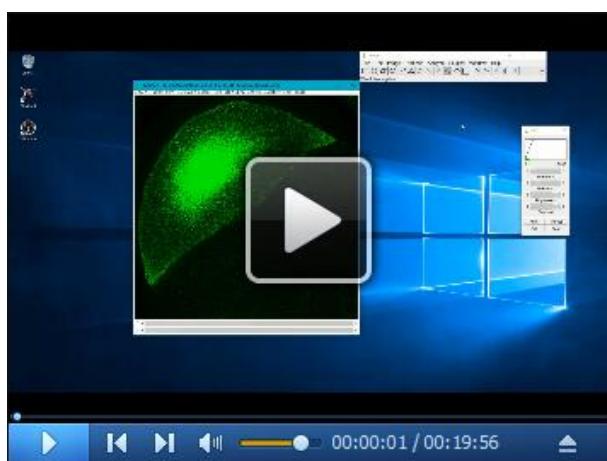
1. Isolate ovaries from well-fed 2-3 day old *Drosophila melanogaster* in PBS (see Note 13).
2. Remove PBS and resuspend with 3 volumes lysis buffer.
3. Mechanically homogenize using a tissue grinder.
4. Transfer to fresh 15 ml tubes and clear the lysate by centrifugation (5 min at 140  $\times g$ ).
5. Transfer the supernatant to a fresh tube and dilute with 2 volumes capturing hybridization buffer (see Recipes).
6. Preclear by adding 1:50 v/v Pierce® Avidin Agarose prewashed with a 1:2 mix of lysis buffer and capturing hybridization buffer.
7. Keep on a rotator for 30 min at room temperature.
8. Remove Avidin agarose by centrifugation (5 min at 140  $\times g$ ).
9. Split the precleared lysate (keep 0.1% as input sample) and supplement it with 0.25  $\mu$ g (see Note 14) of targeting and non-targeting control biotinylated DNA probes per ml of ovaries and incubate at 37 °C for 2 h on a rotator.
10. Pre-wash 3.75  $\mu$ l magnetic Streptavidin beads per ml lysate with a 1:2 mix of lysis buffer and capturing hybridization buffer.
11. Add magnetic Streptavidin beads to lysate and keep rotating for 1 h at 37 °C.

12. Collect the beads using a magnetic rack.
13. Wash beads three times for 5 min at 37 °C with low salt wash buffer (see Recipes).
14. Wash beads two times for 5 min at 37 °C with high salt wash buffer (see Recipes).
15. Wash beads three times for 5 min at 37 °C with low salt wash buffer.
16. Elute the RNA from the beads by adding elution buffer (see Recipes) and boil at 95 °C for 5 min. Use approx. 0.75 µl TE buffer per µl Streptavidin bead slurry used in Step H10.
17. Extract the RNA using an RNA extraction kit and determine the amount of captured RNA of targeting and non-targeting probes by qRT-PCR or Northern blot.

### **Data analysis**

#### A. Determining specificity and sensitivity of smFISH

1. The rationale behind using an array of singly labeled probes to detect target transcripts is the local increase of signal on the specific RNA molecule—due to specific hybridization of the entire array—relative to the background resulting from e.g., random aspecific binding of individual probe molecules (Femino *et al.*, 1998; Raj *et al.*, 2008). Consequently, most of the aspecificity appears as background that can be filtered out (e.g., by the xsPT plugin during Step 7 of this section). In extreme cases, however, the probes may accumulate in specific subcellular structures/organelles, mainly in the nucleolus. If this is observed, we recommend increasing the stringency of the hybridization and the subsequent two wash steps (Steps 10-15 of Procedure F) by increasing the temperature and/or decreasing the salt concentration in the corresponding buffers. Decreasing the probe concentration and increasing the relative amount of the organic solvent (e.g., ethylene carbonate) could be also tried out, in this order (see Notes 16 and 18).
2. To test whether the given smFISH setup—including the probe set and the applied protocol—is detecting all of the target molecules and only the target molecules, a two color smFISH reaction against the transcript of interest can be performed (Figures 3B-3B" and Video 1).



**Video 1. A short tutorial (~20 min) on how to use ImageJ and R to establish single molecule sensitivity of an smFISH experiment.** A further tutorial on using the xsPT plug-in is available in Gaspar *et al.*, 2017.

3. Split the unlabeled ssDNA oligonucleotides into two non-overlapping arrays and label them with two spectrally distinguishable fluorophores—e.g., Atto532 and Atto633—as described in Procedures B and C. (see Note 6)
4. Carry out an smFISH experiment—e.g., as described in Procedure F—using the two sets of probes simultaneously.
5. Acquire images of both fluorophores while avoiding cross-talk of the two channels—e.g., by performing sequential scan during confocal scanning microscopy.
6. Optional: perform deconvolution on the resulting images to enhance signal to noise ratio and facilitate downstream analysis.
7. Analyze the overlap of the two signals e.g., by using the xsPT plugin of ImageJ (Gaspar and Ephrussi, 2017).
8. xsPT segments one of the channels (for more details, see Gaspar and Ephrussi, 2017) and performs tracking through an image sequence, e.g., a Z-stack allowing to recognize objects in 3D.
9. Set the parameters of the plugin to detect all—or at least most of—the objects in the reference channel (for details, please refer Video S4 and S5 of Gaspar and Ephrussi, 2017). In the tracking part, set a maximum displacement of 1 px (the center of an object is not expected to shift much between the slices), set ‘Prefer short steps’ and uncheck ‘Manual tracking’ (*i.e.*, perform automated tracking). Let the plugin run.
10. RNPs—and other structures smaller than the diffraction limit—are expected to be observed in at least three consecutive slices when the image is four-fold over-sampled according to the modified Nyquist criterion (z-step size is typically between 180–300 nm). Remove trajectories that are shorter than three frames (‘Min. trajectory = 3’ and press ‘Filter trajectories’). You may further filter the recognized objects by selecting a region of interest in the image and pressing ‘Filter trajectories’.
11. Save the filtered trajectories. The integrated signal intensities of both the reference and any other channels will be stored in the resulting .csv file. Four of such .csv files ([Supplementary Files 4–7](#)) are provided as examples. These—and any user generated files—can be analyzed using the *smFISH\_analysis.R* script ([Supplementary File 3](#)).
12. Determine a detection threshold for the reference channel (e.g., the minimum or the lowest 0.1th percentile of the integrated signal intensity).
13. Repeat Steps A9–A12 using the other channel as the reference. This analysis results in independent measurements of the detection threshold for both channels.
14. Test what fraction of the objects of the non-reference channel has higher signal intensity than the corresponding detection threshold, which was determined in the parallel analysis. If this

fraction is over 80% for both channels, the smFISH analysis is considered to be at single molecule sensitivity (low false negative detection) and highly specific (low false positive detection rate) (Figure 3D).

15. Lower than 80% co-detection of the same transcripts with the two probe sets—in extreme cases no overlap of the two signals or no signal at all—indicate insufficient sensitivity that is usually due to too high stringency during the hybridization and the two subsequent washes (Steps 10-15 of Procedure F). To overcome this problem, we recommend decreasing the temperature, increasing the salt concentration in the corresponding buffers as well as the probe concentration and decreasing the relative amount of the organic solvent during hybridization (try implementing these changes sequentially, in the listed order) (see Notes 16 and 18).
16. Plotting the signal intensities of the reference and the non-reference channel can give insight into the RNA copy number of the objects (Figure 3C). In case of single RNA molecules, the two signals are not expected to correlate well as they are primarily governed by stochastic events (e.g., the hybridization efficiency of the probes, Figure 3D). If there are more than one copy of target transcripts in the objects, these stochastic events tend to average out yielding an improved linear relationship between the signals of the two channels which get stronger and stronger with the increase of the signal intensity.

#### B. Determining the efficiency and specificity of RNA capture

1. After RNA extraction, set up reverse transcription of the 0.1% input sample and eluted sample from the non-targeting and specific capture (see Note 15).
2. From the cDNA, make a dilution series of the ‘input’ (e.g., 0.1%, 0.01%, 0.001% and 0.0001%) and dilute ‘captured’ sample to at least ten-fold. Set up triplicates of qRT-PCR reactions to amplify the target transcript and other, abundant non-target RNAs (e.g., the 18S ribosomal RNA) from the ‘input’ dilutions and the eluted ‘capture’ samples using appropriate PCR primer pairs.
3. Calculate the amount of ‘captured’ RNA by comparing it to the regression of the diluted ‘input’ sample. For more details, see Gaspar *et al.*, 2017b.

#### Notes

1. You may use an argon-filled chamber. Another, simple means to obtain moisture free environment is to fill 50-100 g of silica gel into an about 20 x 20 x 20 cm polystyrene or plastic box with a lid.
2. The *smFISHprobe\_finder.R* script designs target specific antisense ssDNA oligonucleotides such that the terminal uracil nucleotide added during the labeling will become part of the hybrid. Any large gaps (> the double of *minLength*) between such probes are further scanned for potential probes that do not satisfy this terminal U rule but satisfy the other search criteria. Alternatively, the probe finder script can be used without the terminal U rule (lines 93-96 of

- smFISHprobe\_finder.R* script). Of note, we have found no adversary effect of a non-complimentary uracil nucleotide during hybridization at the 3' end of the produced probes (data not shown).
3. We found no practical differences between Amino-11-ddUTP or 5-propargylamino-ddUTP regarding dye conjugation and enzymatic incorporation of the resulting terminator nucleotide (Figure 2).
  4. We found that BDP-FL-NHS ester is quite hydrophobic and requires higher organic solvent concentration. When using this dye, add 1.8x DMSO before slowly titrating in the dye in Step A4 and skip Step A5. If precipitation occurs add more DMSO and calculate the final concentration accordingly.
  5. Originally, we added Tris-HCl, pH 7.5 to a 10 mM final concentration to quench the remaining NHS ester activity. However, we found no adverse effects of omitting this quenching step on the downstream enzymatic reaction.
  6. Although all oligonucleotides targeting the same transcript could be combined in one master-mix, we recommend preparing at least two non-overlapping mixtures, e.g., by combining every other oligonucleotides, by splitting them into 5' and 3' halves or grouping them by oligonucleotide length. Label these mixtures separately—this will facilitate troubleshooting, i.e., when the low degree of labeling is observed (Procedure E) and also allows benchmarking the smFISH performance (Procedure G). We typically order the oligos resuspended in H<sub>2</sub>O to 250 μM concentration.
  7. To facilitate drying, make sure that all residual ethanol is removed from the walls of the tube by a quick pulse of centrifugation after removing the bulk of the supernatant.
  8. To determine the degree of biotinylation, Colorimetric Biotin Assay Kit based on color loss of 2-(4-hydroxyphenylazo)-benzoic acid could be used. However, we found that incorporation of biotin-ddUTP is always 100% (see Figure 1 of Gaspar *et al.*, 2017b) and therefore we do not routinely determine the degree of biotinylation.
  9. The organic dyes usually have also some absorption at 260 nm. The correction factor at 260 nm ( $cf_{260\text{ nm}}$ ) provided by the dye manufacturer specifies this absorbance as the fraction of the maximal absorption of the dye. This  $cf_{260\text{ nm}}$  and the absorption of the dye at maximum allows for the correction of the OD<sub>260 nm</sub> value when calculating the oligonucleotide concentration.
  10. Steps 5-8 of Procedure F (smFISH) will result in a limited proteolysis and denaturation of proteins and RNA secondary structures and thus they may facilitate target recognition of the probes. However, if the fluorescence of fluorescent proteins (e.g., Emerald-GFP, as in Gaspar *et al.*, 2017a) is to be preserved these steps should be omitted. In case of *oskar* mRNA, we found a minuscule effect of omitting these steps on the hybridization results.
  11. The final probe concentration we use is between 1-2.5 nM/probe. We find that lower concentrations may impair the sensitivity of the reaction while higher concentrations—although rarely they may cause staining artifacts—do not affect the quality of the hybridization but result in unnecessary waste of material.

12. We found that 80% TDE boosts the brightness of GFP and red fluorescent proteins without affecting the signal of Atto565 and Atto633. However, the green and yellow fluorescent dye are substantially dimmer in this mounting medium and thus we recommend using e.g., VectaShield.
13. If high amounts of starting material are required due to low abundance of target RNA, we use a method for mass-isolation of ovaries (similar to Jambor *et al.*, 2015).
14. The probe concentration should be adjusted depending on the amount of RNA present in your sample. We estimated the amount of the desired target RNA by qRT-PCR or Northern blot together with known standards of target RNA.
15. Make sure not to oversaturate the RT reaction. The maximum volume is determined by the concentration of the 'input' sample.
16. We recommend changing only one parameter at a time. It is a good practice to include the original value and two-three modified values of the parameter in these optimization steps. We find that once the listed parameters are optimized for the specimen, they are an excellent starting point for all smFISH reactions performed with yet uncharacterized probe sets, and in 95% of the cases those parameters provide good quality results.
17. However, 'aspecificity' that arises from (partial) complementarity of a large fraction of the probe array to another transcript(s) may result in signal indistinguishable from that produced by the specific hybrids. This phenomenon can be only tested in control samples that lack the RNA-to-be-detected (RNA null mutants or efficient RNAi knock-downs). Since these control samples are not readily available in most cases, we recommend BLAST-ing the probe sequences against the host transcriptome during the design phase (before starting Procedure B) to rule out such cryptic targets.
18. If an already tested and proven-to-work probe set results in such low sensitivity hybridization, the cause is likely nuclease contamination in one of the buffers. In such a case, it is recommended to replace all buffers used for the hybridization.

## Recipes

1. TE buffer
  - 10 mM Tris base
  - 1 mM EDTA
  - Adjust pH to 8.0
2. 1x PBS
  - 137 mM NaCl
  - 2.7 mM KCl
  - 10 mM Na<sub>2</sub>HPO<sub>4</sub>
  - 1.8 mM KH<sub>2</sub>PO<sub>4</sub>
  - Adjust pH to 7.4
  - Store at RT

3. Fixative
  - 10 ml 16% (v/v) EM-grade paraformaldehyde
  - 30 ml 1x PBS
  - Sterile filter with 0.22 µm filter
  - Store at 4 °C
4. PBT
  - 0.1% Triton X-100 in 1x PBS
  - Store at RT
5. 1.5x PAGE loading buffer
  - ¾ 8 M urea in 1x PBS
  - ¼ 6x gel loading dye
  - Store at RT
6. 10x TBE
  - 1 M Tris base
  - 1 M boric acid
  - 20 mM EDTA
  - Store at RT
7. 15% PA - 8 M urea stock (200 ml)
  - 75 ml 40% (v/v) Acrylamide/Bis solution
  - 20 ml 10x TBE
  - 96 g urea
  - Top up to 200 ml with dH<sub>2</sub>O
  - Stir and heat (max 100 °C) until the urea completely dissolves
  - Store at RT isolated from light
8. 20x SSC
  - 3 M NaCl
  - 300 mM Na-citrate
  - pH 7.0
  - Store at RT
9. 2x wash-HYBEC
  - 2x SSC
  - 15% (v/v) ethylene carbonate
  - 1 mM EDTA
  - 0.1% Triton X-100
  - Store at RT
10. 2x full-HYBEC
  - 2x SSC
  - 15% (v/v) ethylene carbonate
  - 1 mM EDTA

50 µg/ml heparin

100 µg/ml salmon sperm ssDNA

0.1% Triton X-100

Store at RT

11. 80% TDE

80% (v/v) 2,2'-thiodiethanol (Sigma-Aldrich)

20% (v/v) 1x PBS

Mix well and store at RT

12. Lysis buffer

50 mM Tris-HCl pH 7.0

10 mM EDTA

1% (v/v) SDS

Fresh 1 mM PMSF

Fresh cComplete® mini EDTA-free protease inhibitor\*

1:2,000 fresh RiboLock RNase Inhibitor

13. Capturing hybridization buffer

50 mM Tris-HCl pH 7.0

750 mM NaCl

1 mM EDTA

1% (v/v) SDS

15% (v/v) ethylene carbonate

Fresh 1 mM PMSF

Fresh cComplete® mini EDTA-free protease inhibitor\*

1:2,000 fresh RiboLock RNase Inhibitor

14. Low salt wash buffer

2x SSC

0.5% (v/v) SDS

Fresh 1 mM PMSF

Fresh cComplete® mini EDTA-free protease inhibitor\*

15. High salt wash buffer

750 mM NaCl

30 mM sodium citrate pH 7.0

0.5% (v/v) SDS

Fresh 1 mM PMSF

Fresh cComplete® mini EDTA-free protease inhibitor\*

16. Elution buffer

10 mM Tris-HCl pH 7.0

1 mM EDTA

Store at RT

\*Note: Add according the manufacturer's instructions.

## **Acknowledgments**

This work was funded by the EMBL. This protocol is adapted from Gaspar *et al.*, 2017b. The authors declare no conflicts of competing interests.

## **References**

1. Femino, A. M., Fay, F. S., Fogarty, K. and Singer, R. H. (1998). [Visualization of single RNA transcripts \*in situ\*](#). *Science* 280(5363): 585-590.
2. Gaspar, I. and Ephrussi, A. (2015). [Strength in numbers: quantitative single-molecule RNA detection assays](#). *Wiley Interdiscip Rev Dev Biol* 4(2): 135-150.
3. Gaspar, I. and Ephrussi, A. (2017). [Ex vivo ooplasmic extract from developing \*Drosophila\* oocytes for quantitative TIRF microscopy analysis](#). *Bio-protocol* 7(13).
4. Gaspar, I., Sysoev, V., Komissarov, A. and Ephrussi, A. (2017a). [An RNA-binding atypical tropomyosin recruits kinesin-1 dynamically to oskar mRNPs](#). *EMBO J* 36(3): 319-333.
5. Gaspar, I., Wippich, F. and Ephrussi, A. (2017b). [Enzymatic production of single-molecule FISH and RNA capture probes](#). *RNA* 23(10): 1582-1591.
6. Jambor, H., Surendranath, V., Kalinka, A. T., Mejstrik, P., Saalfeld, S. and Tomancak, P. (2015). [Systematic imaging reveals features and changing localization of mRNAs in \*Drosophila\* development](#). *eLife* 4: e05003
7. Khong, A., Matheny, T., Jain, S., Mitchell, S. F., Wheeler, J. R. and Parker, R. (2017). [The stress granule transcriptome reveals principles of mRNA accumulation in stress granules](#). *Mol Cell* 68(4): 808-820 e805.
8. Little, S. C., Sinsimer, K. S., Lee, J. J., Wieschaus, E. F. and Gavis, E. R. (2015). [Independent and coordinate trafficking of single \*Drosophila\* germ plasm mRNAs](#). *Nat Cell Biol* 17(5): 558-568.
9. Raj, A., van den Bogaard, P., Rifkin, S. A., van Oudenaarden, A. and Tyagi, S. (2008). [Imaging individual mRNA molecules using multiple singly labeled probes](#). *Nat Methods* 5(10): 877-879.
10. Sage, D., Donati, L., Soulez, F., Fortun, D., Schmit, G., Seitz, A., Guiet, R., Vonesch, C. and Unser, M. (2017). [DeconvolutionLab2: An open-source software for deconvolution microscopy methods-image processing for biologists](#). *Methods* 115: 28-41.

## Imaging Cytokine Concentration Fields Using PlaneView Imaging Devices

Alon Oyler-Yaniv<sup>1,\*</sup> and Oleg Krichevsky<sup>1, 2,\*</sup>

<sup>1</sup>Physics Department, Ben-Gurion University of the Negev, Beer-Sheva, Israel; <sup>2</sup>Ilse Kats Center for Nanoscience, Ben-Gurion University of the Negev, Beer-Sheva, Israel

\*For correspondence: [alonyan@ucla.edu](mailto:alonyan@ucla.edu); [okrichev@bgu.ac.il](mailto:okrichev@bgu.ac.il)



**[Abstract]** We describe here a method to visualize concentration fields of cytokines around cytokine-secreting cells. The main challenge is that physiological cytokine concentrations can be very low, in the pico-molar range. Since it is currently impossible to measure such concentrations directly, we rely on cell's response to the cytokines—the phosphorylation of a transcription factor—that can be visualized through antibody staining. Our devices aim at mimicking conditions in dense tissues, such as lymph nodes. A small number of secreting cells is deposited on a polylysine-coated glass and covered by multiple layers of cytokine-consuming. The cells are left to communicate for 1 h, after which the top layers are removed and the bottom layer of cells is antibody labeled for the response to cytokines. Then a cross-section of cytokine fields can be visualized by standard fluorescence microscopy. This manuscript summarized our method to quantify the extent of cytokine-mediated cell-to-cell communications in dense collection of cells *in vitro*.

**Keywords:** Cytokine concentration, Cytokine niches, Imaging of cytokine fields

**[Background]** The mammalian immune system has evolved to identify and limit the spread of potential pathogens while minimizing collateral tissue damage caused by the immune system itself. To achieve this, immune cells rely on a network of cytokine mediators that enable cell-to-cell communications and broadcast information about the magnitude and nature of the pathogenic insult. Vast arrays of different cytokines bind strongly to their cognate receptors, often with characteristic binding affinities in the nano- or pico-molar range. Immunological niches are generated via cytokine communications. For example, in both the bone marrow and the thymus, secretion of Interleukin-7 (IL-7) by stromal cells supports the survival of proliferating B and T cell progenitors, respectively (Tokoyoda *et al.*, 2004; Alves *et al.*, 2009). The size of the cytokine niche controls the number of maturing progenitors, thereby keeping the blood cell compartments in equilibrium (Böyum, 1968; Weist *et al.*, 2015).

We aim to collect information about the spatial and temporal dynamics of cytokines and how these two parameters influence the immune response. This is an area of immunology that is currently under-studied. Many assays test the effects of cytokines in tissue-culture dishes, where media is well-mixed, leading to homogeneous fields of growth and differentiation factors. The intricate and highly specialized architecture of the secondary lymphoid organs sets up niches where cells sense stimuli such as pathogen components and cytokines, proliferate, mature, differentiate, and die. Cytokine concentration gradients are formed within these niches such that some cells have greater or lesser access to cytokines than others (Liu *et al.*, 2015). Measuring how far cytokines spread from their

source, and the gradients they form, is key to unravelling the mechanism of the phenotypic heterogeneity of immune cells in differentiation, proliferation, and death (Feinerman *et al.*, 2010; Busse *et al.*, 2010; Höfer *et al.*, 2012; Müller *et al.*, 2012; Thurley *et al.*, 2015).

Due to the typically low concentrations (pM range) of free cytokines *in vivo*, direct measurement of cytokine fields is difficult at best and maybe impossible. However, due to their high sensitivity to cytokine and graded, concentration-dependent response, the signaling levels of cells in response to cytokines can itself be used as a bio-sensor for cytokine concentrations (Oyler-Yaniv *et al.*, 2017).

In this protocol, we describe how to directly image the signaling response generated around a cytokine producer *in vitro*, in conditions that mimic *in vivo* conditions: high cell density and no convection. Our method is general and can be applied to any cell type and any diffusible stimulus, and only depends on the existence of a specific antibody to target the downstream signaling molecule of interest and/or of live cell reporters.

### **Materials and Reagents**

1. Pipette tips (USA Scientific, catalog numbers: 1111-1806, 1111-3800)
2. CELLSTAR Filter Cap Cell Culture Flasks T75 (Greiner Bio One International, catalog number: 658175)
3. 15 ml tube
4. Glass slides (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 4951PLUS4)
5. Coverslips (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 25X25-1)
6. Silicone rubber compound (PDMS) (Momentive, catalog number: RTV615)
7. PVP-treated PCTE Membranes, 13 mm diameter, 400 nm pore (Sterlitech, catalog number: PCT0413100)
8. B16-F10 melanoma cells (ATCC, catalog number: CRL-6475)
9. Mouse CD4 (L3T4) MicroBeads (Miltenyi Biotec, catalog number: 130-049-201)
10. Phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, catalog number: P1585-1MG)
11. Ionomycin calcium salt (Sigma-Aldrich, catalog number: I0634-1MG)
12. Ficoll-paque plus (GE Healthcare, catalog number: 17144003)
13. Recombinant mouse IL-2 (Thermo Fisher Scientific, eBioscience™, catalog number: 14-8021-64)
14. Recombinant human IL-2 (gift from Dr. Kendall A. Smith, Cornell University)
15. Trypsin/EDTA solution (Thermo Fisher Scientific, Gibco™, catalog number: R001100)
16. Phosphate buffered saline (Sigma-Aldrich, catalog number: P4417)
17. Glycine (Sigma-Aldrich, catalog number: 50046)
18. Ovalbumin peptide SIINFEKL (Sigma-Aldrich, catalog number: S7951-1MG)
19. Cell Trace Far-Red (DDAO-SE) (Thermo Fisher Scientific, Invitrogen™, catalog number: C34564)
20. Poly-L-lysine solution (Sigma-Aldrich, catalog number: P8920-100ML)

21. Paraformaldehyde solution, 4% in PBS (Alfa Aesar, Affymetrix, catalog number: J19943)
22. Methanol (Sigma-Aldrich, catalog number: MX0490-4)
23. Fluoromount Aqueous Mounting Medium (Sigma-Aldrich, catalog number: F4680-25ML)
24. Triton 100-X (MP Biomedicals, catalog number: 0230022101-1I)
25.  $\alpha$ -CD4, Alexa700, Pacific Blue (BD Bioscience clone RM4-5, BD, catalog numbers: 557956, 558107)
26.  $\alpha$ -IL-2R $\alpha$ , PE (Miltenyi Biotec clone 7D4, Miltenyi Biotec, catalog number: 130-102-593)
27. Primary antibody rabbit  $\alpha$ -phospho-STAT5 (pY694) (Cell Signaling clone C71E5, Cell Signaling Technology, catalog number: 9314S)
28. Primary antibody rabbit  $\alpha$ -phospho-STAT1 (pY701) (Cell Signaling clone 58D6, Cell Signaling Technology, catalog numbers: 9167L)
29. Secondary polyclonal antibody  $\alpha$ -rabbit IgG, Alexa 488 (Jackson ImmunoResearch, catalog number: 711-176-152)
30. RPMI 1640 media with L-glutamine (Biological Industries, catalog number: 01-100-1A)
31. Heat-inactivated fetal bovine serum (Biological Industries, catalog number: 04-127-1A)
32. HEPES buffer (Biological Industries, catalog number: 03-025-1B)
33. Non-essential amino acids (Biological Industries, catalog number: 01-340-1B)
34. Sodium pyruvate (Biological Industries, catalog number: 03-042-1B)
35. Penicillin-streptomycin solution (Biological Industries, catalog number: 03-031-5B)
36.  $\beta$ -Mercaptoethanol (Sigma-Aldrich, catalog number: M3148-25ML)
37. Complete RPMI (see Recipes)

## Equipment

1. Pipettes
2. Heraeus centrifuge with microplate swinging rotor (Thermo Fisher Scientific, Thermo Scientific™, model: Heraeus™ Biofuge™ Stratos™)
3. Zeiss Axiovert 200M microscope (ZEISS, model: Axiovert 200M)

## Software

1. MATLAB, Mathworks Inc.
2. LabVIEW, National Instruments

## **Procedure**

The protocols described here were developed for imaging of cytokine signaling in dense clusters of cytokine consuming cells that are simulated *in vivo* conditions in secondary lymphoid organs or other solid tissue (Oyler-Yaniv et al., 2017).

### A. Cell culture

1. OT-I and C57BL/6 primary cells are harvested from the lymph nodes and spleen, and mechanically separated into single-cell suspension. CD4<sup>+</sup> T-cells are isolated using Mouse CD4 (L3T4) MicroBeads. Primary cells and B16-F10 melanoma cells (ATCC CRL-6475) are maintained in complete RPMI (Recipe 1) throughout the procedure. C57BL/6 T cells are activated using 10 ng/ml PMA and 500 ng/ml Ionomycin and cultured in 30 ml of media, in a T75 flask for 3 days.
2. Dead cells are removed using Ficoll-paque plus density gradient media following standard protocols (Böyum, 1968) and subsequently cultured at 10<sup>6</sup> cells/ml in RPMI supplemented with 2 nM recombinant human IL-2 for an additional day. IL-2 secretion experiments are performed on day 4 of culture.
3. B16-F10 cells are maintained in 30 ml of complete RPMI (Recipe 1) in a T75 flask and passaged every 3 days using trypsin/EDTA solution and recultured to 10% confluence (roughly 10<sup>6</sup> cells at the start of each passage). Cells are never used beyond passage 7.

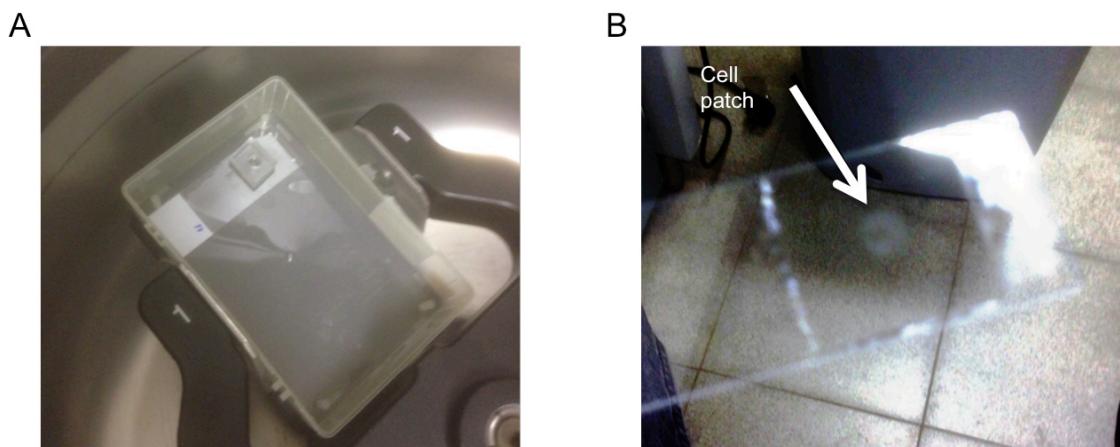
*Note: B16-F10 melanoma cells express the IFNy receptor but do not produce IFNy. They also express high levels of MHC-I and therefore serve as antigen presenting cells for TCR recognition.*

### B. Preparation of cells for imaging

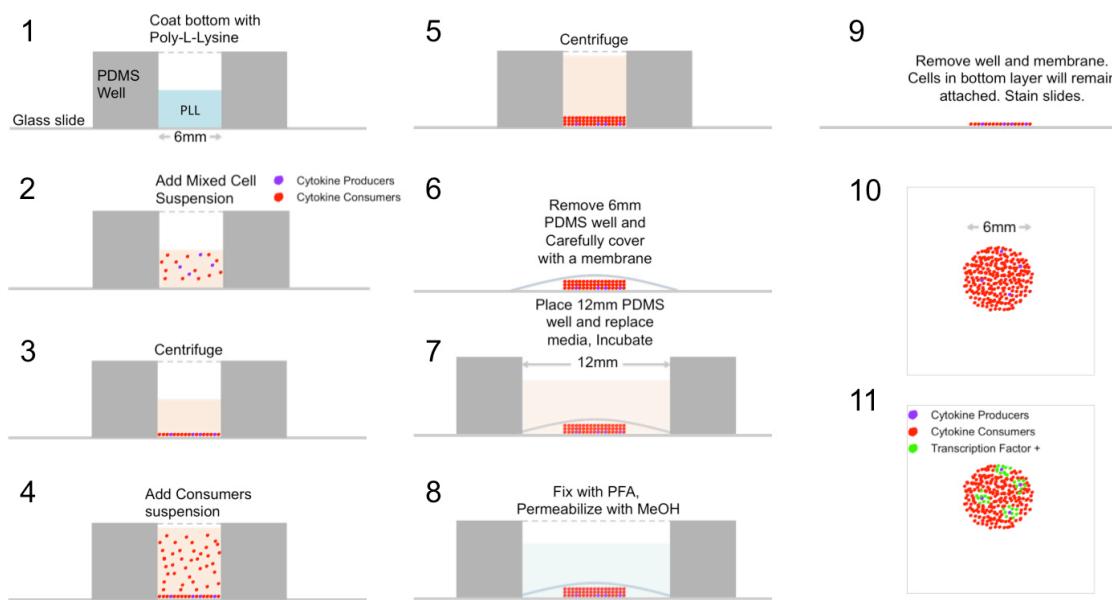
1. To remove receptor-bound cytokines, wash 5 × 10<sup>7</sup> cultured T-cells (consumer cells) in 10 ml PBS, then expose 0.1 M glycine in PBS (pH 4.0) for 1 min on ice, followed by 3 washes each with 10 ml RPMI, and rest for 1 h at in a tube containing RPMI in a 37 °C incubator.
2. To generate CD4 depleted, naïve splenocytes (inert cells), harvest lymph nodes and spleen from a C57BL/6 mouse. Separate tissue into single-cell suspension. Deplete the cell suspension of CD4<sup>+</sup> cells using Mouse CD4 (L3T4) MicroBeads.
3. Mix cultured consumer cells with inert cells at different ratios.
4. Re-administrate the previously activated T-cells cultures with 5 ng/ml PMA and 500 ng/ml Ionomycin to generate IL-2 producing cells. This reactivation leads to a rapid and extensive production of IL-2 by the T-cells. These cells are labeled with DDAO-SE by using the manufacturer's protocol at 1 µM for subsequent identification. Only the cytokine producers are labeled at this stage!
5. Pulse 10<sup>7</sup> B16-F10 cells with 1 nM of the SIINFEKL peptide in 10 ml of RPMI for 1 h under constant rotation in a 15 ml tube, in an incubator. OT-I Cells are labeled with DDAO-SE by using the manufacturer's protocol at 1 µM for subsequent identification.

### C. General description: Fabrication of imaging devices

1. Coat glass slides with poly-L-lysine (PLL) by submerging them in 0.01% PLL, diluted in H<sub>2</sub>O, for 40 min at 37 °C. Wash slides by submerging them in a large volume of H<sub>2</sub>O and allow them to dry at room temperature for 1 h. Place a small (6 mm) hollow cylinder made of PDMS on the slide, the PDMS rapidly attaches to the slide creating a small well.
2. To create a tightly packed cell pellet, cell suspensions are added into the PDMS well, the slide is put inside a pipette box cover and centrifuged for 1 min at 800 x g to allow cells to stick to the glass slide (Figure 1A). As more cells accumulate on the device, they form a 3 dimensional layered structure. Depending on cell dimensions, each layer will contain ~1-2 x 10<sup>5</sup> individual cells. Moreover, different layers composed of different cell preparations can be added sequentially, creating a stratified structure that can mimic specific *in vivo* morphologies.
3. After cell deposition, the PDMS well is removed and a 13 mm diameter semipermeable hydrophilic membrane is dipped in media and carefully placed on top of the cells. This membrane protects the cells from moving due to changing reagents, and prevents convection flows from distorting the concentration fields. The cells and membrane are then covered by a larger PDMS well (12 mm diameter) to allow for the confinement of reagents around the cells. At the end of the experiment, the cells are fixed for 20 min in 200 µl 37 °C 4% PFA and permeabilized by using 200 µl ice cold 90% MeOH for 10 min. After incubation and fixation, cells that are in contact with the glass slide remain permanently bound to it, preserving their spatial distribution. The PDMS well and the membrane are then removed using forceps, the slides are stained using standard immunofluorescence protocols, and finally are mounted on a coverslip using Fluoromount (Figure 1B). The process is illustrated in Figure 2.



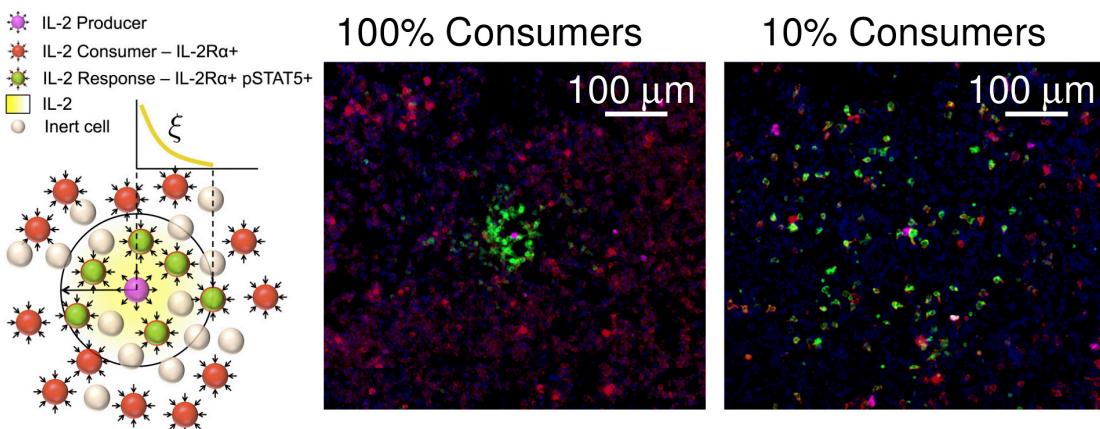
**Figure 1. Preparation of slides for imaging.** A. PlaneView device after centrifugation; B. Cell patch after staining and mounting.



**Figure 2. Graphical protocol of device preparation.** Preparation of cells for imaging is done by first coating a glass slide with poly-L-lysine (1). Then, a cell suspension containing a small fraction of cytokine producing cells is deposited in a monolayer by centrifugation (2, 3). 10 layers of cells containing no producers is deposited on top to form a 3 dimensional structure (4, 5). The cells are covered with a semipermeable membrane (6), incubated for 1 h (7), and fixed *in situ* (8). Cells are then permeabilized and stained (9, 10).

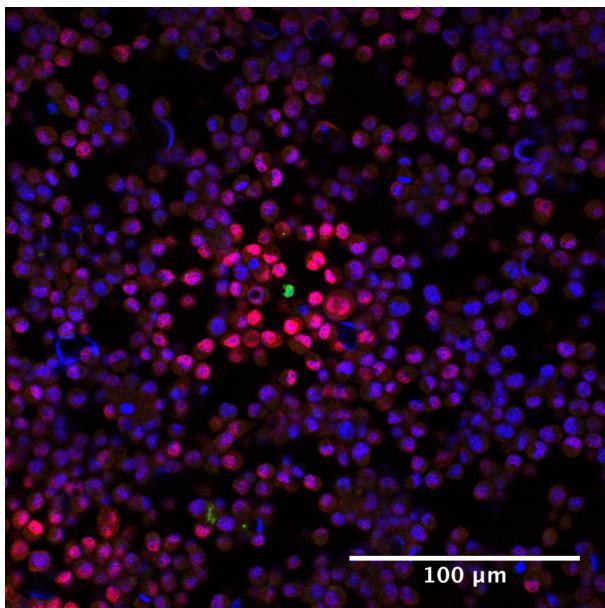
#### D. *In vitro* imaging of cytokine concentration fields

1. For measuring IL-2 concentration fields (Figure 3),  $2 \times 10^5$  IL-2 consuming T cells, or a combination of 10% consuming T cells ( $2 \times 10^4$ ) and 90% inert cells ( $1.8 \times 10^5$ ) are mixed with 0.1% IL-2 producing T cells (200 cells), each in a total volume of 20  $\mu$ l, and deposited in a monolayer. Then, 10 more layers ( $2 \times 10^6$ ) of cells containing no producers and either a 1:0 or a 1:9 ratio of consumers to inert cells, respectively, each in a volume of 180  $\mu$ l, are added on top, forming a three dimensional strata with the producing cells dispersed on the bottom (Figure 2). A semipermeable membrane is placed on the cells to preserve their positions during further processing.



**Figure 3. pSTAT5 distribution around IL-2 producers.** Immunofluorescence staining of cell preparations containing either 100% IL-2Ra+ consuming cells or 10% consuming cells and 90% IL-2Ra- inert cells, and a small number (< 0.01%) of IL-2 producing T cells in a PlaneView imaging device. Blue: DAPI, Red: IL-2Ra, Green: pSTAT5, Magenta: DDAO-SE.

2. For measuring IFNy concentration fields (Figure 4),  $10^5$  SIINFEKL pulsed B16-F10 melanoma cells are mixed with 0.2% OT-I T cells (200 cells) and deposited in a monolayer. Then, 10 more layers of B16-F10 cells ( $10^6$ ) are added on top. A semipermeable membrane is placed on the cells to preserve their positions during further processing.

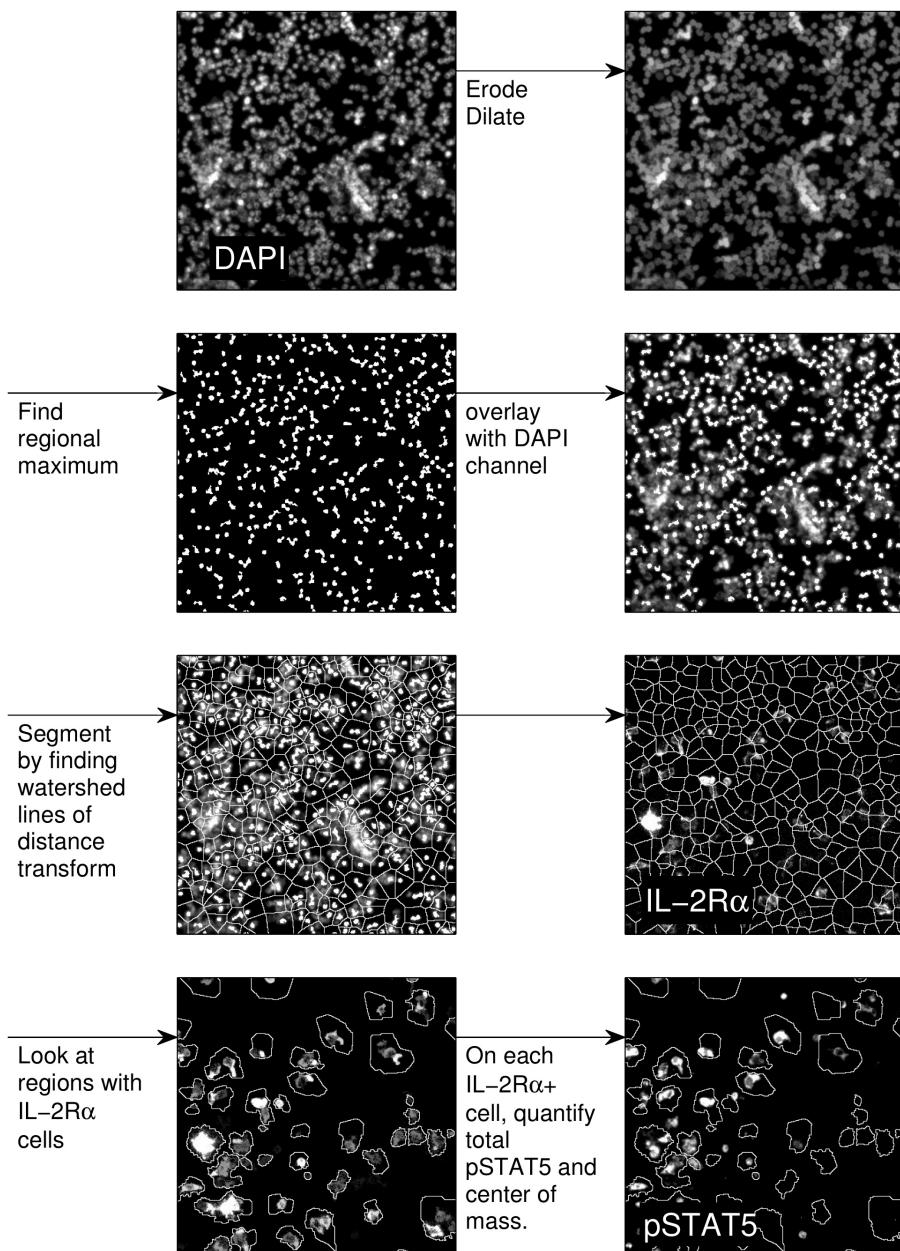


**Figure 4. pSTAT1 distribution around IFNy producer.** Immunofluorescence staining of cell preparation containing SIINFEKL pulsed B16-10A cells spiked with a small amount of OT-I cells was prepared as described in Procedure D. Blue: DAPI, Red: pSTAT1, Green: DDAO-SE.

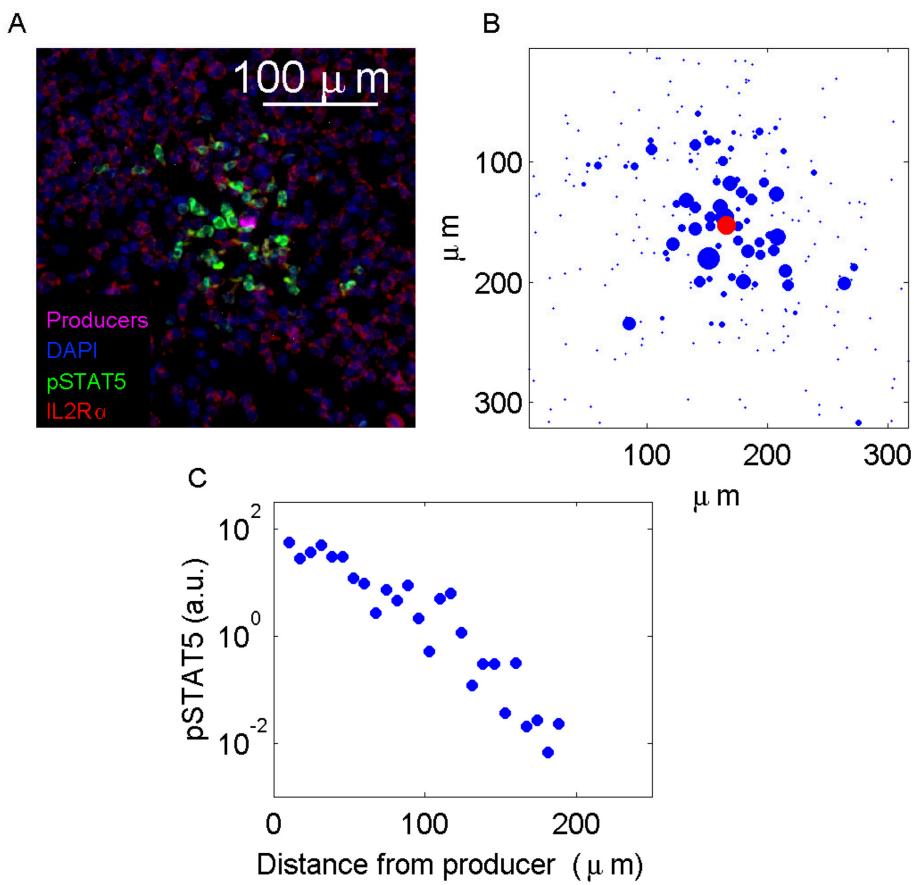
3. To control for background transcription factor phosphorylation and cytokine specificity, devices are loaded with consuming cell cultures containing no producers. The cells are then covered with either fresh media or media containing 10 nM of cytokine to serve as negative and positive controls, respectively. These samples showed effectively no signal for the negative control and bright, uniform, signal for the positive control (Oyler-Yaniv *et al.*, 2017).
4. The system is incubated at 37 °C for 1 h. After that, media is carefully aspirated off the devices and the cells are fixed for 20 min at 37 °C in 4% PFA. PFA is then removed and the cells are permeabilized by using 200 µl ice cold 90% MeOH for 10 min to allow for intracellular immunostaining. Special attention should be given during this stage to minimally disrupt the cell pellets.
5. After fixation and permeabilization, the PDMS well and the membrane are removed using forceps. At this stage, the cells would be tightly bound to the glass slide and standard immunostaining protocols can be used. Nonspecific antibody binding is blocked by a 1 h incubation in 5% FBS and 0.3% Triton X-100 in PBS at room temperature. Primary antibodies (Rabbit anti-pSTAT5 1:200) are applied in a moist chamber for 1 h at room temperature. Fluorophore-conjugated antibodies (Goat anti-Rabbit Alexa 488, Rat anti-IL-2R $\alpha$  R-PE, 1:300) are applied for 1 h at room temperature. Cells are then briefly stained with DAPI and a coverslip is mounted using Fluoromount (Figure 1B) for fluorescent imaging.

### **Data analysis**

Images are processed by segmenting individual cells (Gonzalez and Woods, 2007), then determining whether each cell is a consumer based on IL-2R $\alpha$  expression. On each consumer, the total level of pSTAT5 expression and the center-of-mass is calculated and logged. An example of the procedure is shown in Figure 5. Profiles of pSTAT5 expression on individual cells as a function of distance from the nearest producer are then generated (Figure 6).



**Figure 5. Image processing procedure**



**Figure 6. Analysis of pSTAT5 distributions.** A. A sample containing 100% IL-2 consuming T cells spiked with a small amount of IL-2 producing cells was prepared as described in Procedure D. B. Image reconstruction based on total pSTAT5 and center of mass per cell. The radius of each circle is proportional to the pSTAT5 level. C. pSTAT5 profile as a function of the distance from the cytokine producer.

## Recipes

### 11. Complete RPMI

RPMI 1640 media supplemented with:

Heat-inactivated 10% fetal bovine serum

2 mM L-glutamine

10 mM HEPES

0.1 mM non-essential amino acids

1 mM sodium pyruvate

100  $\mu\text{g}/\text{ml}$  of penicillin

100  $\mu\text{g}/\text{ml}$  of streptomycin

50  $\mu\text{M}$   $\beta$ -mercaptoethanol

## Acknowledgments

This work has been done in collaboration with Grégoire Altan-Bonnet (NIH) whom we would like to thank for the overall support and, in particular, for the critical reading of the manuscript. We are also grateful to U.S.-Israel Binational Science Foundation (grant #2012327 to G. Altan-Bonnet and O.K.) for funding. The protocol has been adapted from Oyler-Yaniv *et al.*, 2017.

The Authors declare no conflicts of interest or competing interests.

## References

1. Alves, N. L., Richard-Le Goff, O., Huntington, N. D., Sousa, A. P., Ribeiro, V. S., Bordack, A., Vives, F. L., Peduto, L., Chidgey, A., Cumano, A., Boyd, R., Eberl, G. and Di Santo, J. P. (2009). [Characterization of the thymic IL-7 niche \*in vivo\*.](#) *Proc Natl Acad Sci U S A* 106(5): 1512-1517.
2. Böyum, A. (1968). [Isolation of mononuclear cells and granulocytes from human blood. Isolation of monuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g.](#) *Scand J Clin Lab Invest Suppl* 97: 77-89.
3. Busse, D., de la Rosa, M., Hobiger, K., Thurley, K., Flossdorf, M., Scheffold, A. and Hofer, T. (2010). [Competing feedback loops shape IL-2 signaling between helper and regulatory T lymphocytes in cellular microenvironments.](#) *Proc Natl Acad Sci U S A* 107(7): 3058-3063.
4. Feinerman, O., Jentsch, G., Tkach, K. E., Coward, J. W., Hathorn, M. M., Sneddon, M. W., Emonet, T., Smith, K. A. and Altan-Bonnet, G. (2010). [Single-cell quantification of IL-2 response by effector and regulatory T cells reveals critical plasticity in immune response.](#) *Mol Syst Biol* 6: 437.
5. Gonzalez, R. C. and Woods, R. E. (2007). Digital Image Processing (3rd Edition).
6. Höfer, T. O. Krichevsky, O. and Altan-Bonnet, G. (2012). [Competition for IL-2 between regulatory and effector T cells to chisel immune responses.](#) *Front Immunol* 3: 268.
7. Liu, Z., Gerner, M. Y., Van Panhuys, N., Levine, A. G., Rudensky, A. Y. and Germain, R. N. (2015). [Immune homeostasis enforced by co-localized effector and regulatory T cells.](#) *Nature* 528(7581): 225-230.
8. Müller, A. J., Filipe-Santos, O., Eberl, G., Aebsicher, T., Spath, G. F. and Bousso, P. (2012). [CD4<sup>+</sup> T cells rely on a cytokine gradient to control intracellular pathogens beyond sites of antigen presentation.](#) *Immunity* 37(1): 147-157.
9. Oyler-Yaniv, A., Oyler-Yaniv, J., Whitlock, B. M., Liu, Z., Germain, R. N., Huse, M., Altan-Bonnet, G. and Krichevsky, O. (2017). [A tunable diffusion-consumption mechanism of cytokine propagation enables plasticity in cell-to-cell communication in the immune system.](#) *Immunity* 46(4): 609-620.
10. Thurley, K., Gerecht, D., Friedmann, E. and Hofer, T. (2015). [Three-dimensional gradients of cytokine signaling between T cells.](#) *PLoS Comput Biol* 11(4): e1004206.

11. Tokoyoda, K., Egawa, T., Sugiyama, T., Choi, B. I. and Nagasawa, T. (2004). [Cellular niches controlling B lymphocyte behavior within bone marrow during development.](#) *Immunity* 20(6): 707-718.
12. Weist, B. M., Kurd, N., Boussier, J., Chan, S. W. and Robey, E. A. (2015). [Thymic regulatory T cell niche size is dictated by limiting IL-2 from antigen-bearing dendritic cells and feedback competition.](#) *Nat Immunol* 16(6): 635-641.

## Intracellular and Mitochondrial Reactive Oxygen Species Measurement in Primary Cultured Neurons

Seung Hyun Baek<sup>1</sup>, Yoonsuk Cho<sup>1</sup>, Jeongmi Lee<sup>1</sup>, Bo Youn Choi<sup>1</sup>, Yuri Choi<sup>1</sup>, Jin Su Park<sup>1</sup>, Harkkyun Kim<sup>1</sup>, Jaehoon Sul<sup>1</sup>, Eunae Kim<sup>1, 2</sup>, Jae Hyung Park<sup>2</sup> and Dong-Gyu Jo<sup>1,\*</sup>

<sup>1</sup>School of Pharmacy, Sungkyunkwan University, Suwon, Republic of Korea; <sup>2</sup>School of Chemical Engineering, College of Engineering, Sungkyunkwan University, Suwon, Republic of Korea

\*For correspondence: [jodg@skku.edu](mailto:jodg@skku.edu)



**[Abstract]** Reactive oxygen species (ROS) are chemically reactive oxygen containing molecules. ROS consist of radical oxygen species including superoxide anion ( $O_2^{-\cdot}$ ) and hydroxyl radical ( $\cdot OH$ ) and non-radical oxygen species such as hydrogen peroxide ( $H_2O_2$ ), singlet oxygen ( $O_2^{\cdot}$ ). ROS are generated by mitochondrial oxidative phosphorylation, environmental stresses including UV or heat exposure, and cellular responses to xenobiotics (Ray *et al.*, 2012). Excessive ROS production over cellular antioxidant capacity induces oxidative stress which results in harmful effects such as cell and tissue damage. Sufficient evidence suggests that oxidative stresses are involved in cancers, cardiovascular disease, and neurodegenerative diseases including Alzheimer's disease and Parkinson disease (Waris and Ahsan, 2006). Though excessive level of ROS triggers detrimental effects, ROS also have been implicated to regulate cellular processes. Since ROS function is context dependent, measurement of ROS level is important to understand cellular processes (Finkel, 2011). This protocol describes how to detect intracellular and mitochondrial ROS in live cells using popular chemical fluorescent dyes.

**Keywords:** Reactive oxygen species (ROS), Intracellular ROS, MitoSOX, CM-H<sub>2</sub>DCFDA, Primary neuron

**[Background]** ROS are important to maintain homeostasis in our bodies (Brieger *et al.*, 2012). Many diseases such as cancer, neurodegenerative disease, cardiovascular disease, and diabetics are associated with ROS (Datta *et al.*, 2000). DNA damage caused by ROS is a major cause of accelerating carcinogenesis process, and therapeutic agents targeting ROS have been actively developed (Trachootham *et al.*, 2009). In circulatory system, abnormal oxidative stress increases the production of ROS, leading to various cardiovascular diseases (Forstermann, 2008). Signaling related to diabetes is sensitive to ROS, and these signaling abnormalities induced by abnormal levels ROS cause diabetes complications (Baek *et al.*, 2017). Controlling the ROS levels in the brain is one of the most important activities because abnormal levels of ROS can cause diverse brain diseases. Amyloid beta, known as an important factor in Alzheimer's disease, causes excessive ROS generation in the brain, neuronal damage (Singh *et al.*, 2011), and eventually dementia (Polidori, 2004). Activated microglia produced by ROS which secretes a variety of cytokines result in neuronal death (Heneka *et al.*, 2014).

ROS are generated by small part of oxygen consumed in mitochondria. A principal species of ROS produced in mitochondria is superoxide anion and it is the byproduct of the electron transport chain (Batandier *et al.*, 2002). In order to detect superoxide in mitochondria, MitoSOX red, a mitochondria superoxide indicator, is used. Due to the positive charge on triphenylphosphonium group, MitoSOX red can effectively penetrate phospholipid bilayer, and accumulate into the matrix of mitochondria. Furthermore, hydroethidine of MitoSOX red allows researchers to discriminate the fluorescent signal generated by superoxide-mediated oxidative products from other non-specific signals (Robinson *et al.*, 2006; Baek *et al.*, 2017).

CM-H<sub>2</sub>DCFDA is a chloromethyl derivative of H<sub>2</sub>DCFDA (2,7 -dichlorodihydrofluorescein diacetate), a fluorogenic dye that measures hydroxyl, peroxy and other ROS activity within the cell and can be used to detect the intracellular formation of ROS (Kirkland *et al.*, 2007). Once the fluorescent probe of CM-H<sub>2</sub>DCFDA permeates cell membrane, intracellular esterases hydrolyze its acetyl groups and it can be retained in the cell. CM-H<sub>2</sub>DCFDA is more sensitive to oxidation by H<sub>2</sub>O<sub>2</sub> than superoxide (O<sub>2</sub><sup>•-</sup>) (Fowler *et al.*, 2017). CM-H<sub>2</sub>DCFDA is widely used in physiological and pathophysiological studies including virus infection (Nykky *et al.*, 2014), cancer (Khatri *et al.*, 2015; Liu *et al.*, 2017), and neurodegenerative diseases (Ng *et al.*, 2014). Using CM-H<sub>2</sub>DCFDA, we can detect intracellular ROS level by flow cytometry/fluorescence measurement and the localization of ROS producing organelle with confocal microscopy (Forkink *et al.*, 2010).

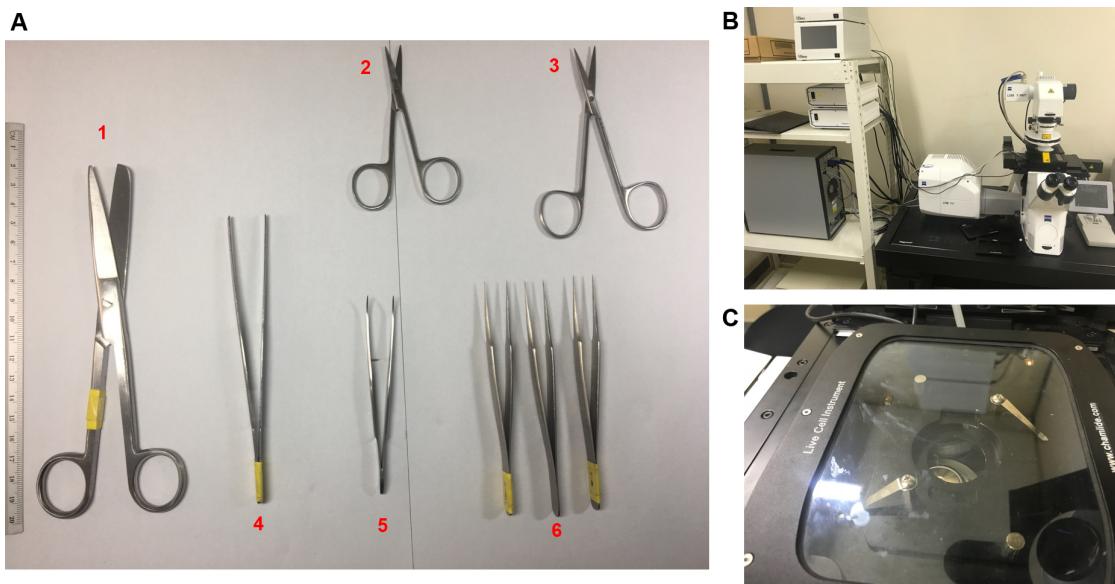
## **Materials and Reagents**

1. Glass bottom cell culture dish type 35 mm and dimension 20 mm (Nest Scientific, catalog number: 801001)
2. Cover glasses thickness No. 1 circular size 18 mm Ø (MARIENFELD, catalog number: 0111580)
3. Petri dish, 100 mm Polyesterene aseptic non-tissue culture treated (SPL Life Sciences, catalog number: 10095)
4. 15 ml conical tube (SPL Life Sciences, catalog number: 50015)
5. 10 ml Serological pipettes (SPL Life Sciences, catalog number: 91010)
6. 50 ml conical tube (SPL Life Sciences, catalog number: 50050)
7. Cell strainer 70 µm (Corning, Falcon®, catalog number: 352350)
8. Pregnant female Sprague Dawley rats (E17-E18 days gestation, Orient Korea)
9. Poly-D-lysine hydrobromide (Sigma-Aldrich, catalog number: P6407-5mg)
10. Phosphate buffered saline powder, pH 7.4, for preparing 1 L solutions (Sigma-Aldrich, catalog number: P3813)
11. CM-H<sub>2</sub>DCFDA (Thermo Fisher Scientific, Invitrogen™, catalog number: C6827)
12. Dimethyl Sulfoxide(DMSO) (Merck, catalog number: 317275)
13. MitoSOX™ Red Mitochondrial Superoxide Indicator, for live-cell imaging (Thermo Fisher Scientific, Invitrogen™, catalog number: M36008)

14. Phosphate buffered saline (PBS) powder, pH 7.4, for preparing 1 L solutions, suitable for cell culture (Sigma-Aldrich, catalog number: P3813)
15. Trypsin (2.5%), no phenol red (Thermo Fisher Scientific, Gibco™, catalog number: 15090046)
16. Fetal bovine serum (FBS) (Thermo Fisher Scientific, Gibco™, catalog number: 10082147)
17. Neurobasal Medium® (Thermo Fisher Scientific, Gibco™, catalog number: 21103049)
18. B-27™ Supplement (50x), serum free (Thermo Fisher Scientific, Gibco™, catalog number: 17504044)
19. DMEM High Glucose (4.5 g/L), with L-Glutamine, with Sodium Pyruvate (Capricorn Scientific, catalog number: DMEM-HPA)
20. Penicillin/Streptomycin (100x) (PS) (Capricorn Scientific, catalog number: PS-B)
21. Amyloid beta peptide 1-42 Human (ANYGEN, catalog number: AGP-8338)
22. CM-H<sub>2</sub>DCFDA solution (see Recipes)
23. MitoSOX™ Red solution (see Recipes)
24. Poly-D-lysine hydrobromide solution (see Recipes)
25. Prep medium (see Recipes)
26. Culture medium (see Recipes)
27. Maintain culture medium (see Recipes)

## Equipment

1. Haemacytometers (MARIENFELD, catalog number: 0630010)
2. Original Portable Pipet-Aid® Pipette Controller (Drummond Scientific, catalog number: 4-000-100)
3. Dressing Scissors (Surgimax Instruments, catalog number: 85-112-12) (Figure 1A 1)
4. Dissecting Scissors (Surgimax Instruments, catalog number: 85-127-10) (Figure 1A 2)
5. Dissecting Scissors (Surgimax Instruments, catalog number: 63-175-11) (Figure 1A 3)
6. Spring Dressing Forceps Sharp (Surgimax Instruments, catalog number: 85-076-11) (Figure 1A 4)
7. Spring Dressing Forceps Blunt (Surgimax Instruments, catalog number: 85-073-15) (Figure 1A 5)
8. Multi Purpose Forceps Pointed (Surgimax Instruments, catalog number: 05-177-11) (Figure 1A 6)
9. Clean bench (HANBAEK Scientific Technology, catalog number: HB-402)
10. Cell culture CO<sub>2</sub> incubator (ARA, catalog number: APR150)
11. Water-bath (Grant Instruments, JB Academy, catalog number: JBA18)
12. Centrifuge (Hanil Scientific, catalog number: Combi 514R)
13. Confocal microscope with live cell imaging system (Carl Zeiss, model: LSM700) (Figures 1B and 1C)



**Figure 1. Equipment for the experiment.** A. Surgery instruments; B. Confocal microscope (LSM700) with live cell imaging system; C. Live cell chamber.

## Software

### A. For measure

1. ZEN black version (ZEISS confocal microscope LSM700 software)

*Note: This is default program provided with ZEISS confocal microscope.*

### B. For analysis

1. ZEN Blue edition (ZEISS confocal microscope LSM700 software; [SR-DIP software for ZEN blue edition](#))
2. ImageJ ([ImageJ](#) is an open source image processing program)

## Procedure

In this protocol, we introduce two types of ROS measurement in primary neuronal cells under oxidative stress.

1. In advance, a 35 mm plate for live cell imaging, is coated with 2 ml poly-D-lysine solution at 4 °C for 24 h.

*Optional: Instead of using 35 mm dish for ROS measurement, the following method is possible.*

*Place the sterile cover glasses slip flat into each well of a 6-well plate. Add 2 ml of Poly-D-lysine solution to each well and coat at 4 °C for 24 h.*

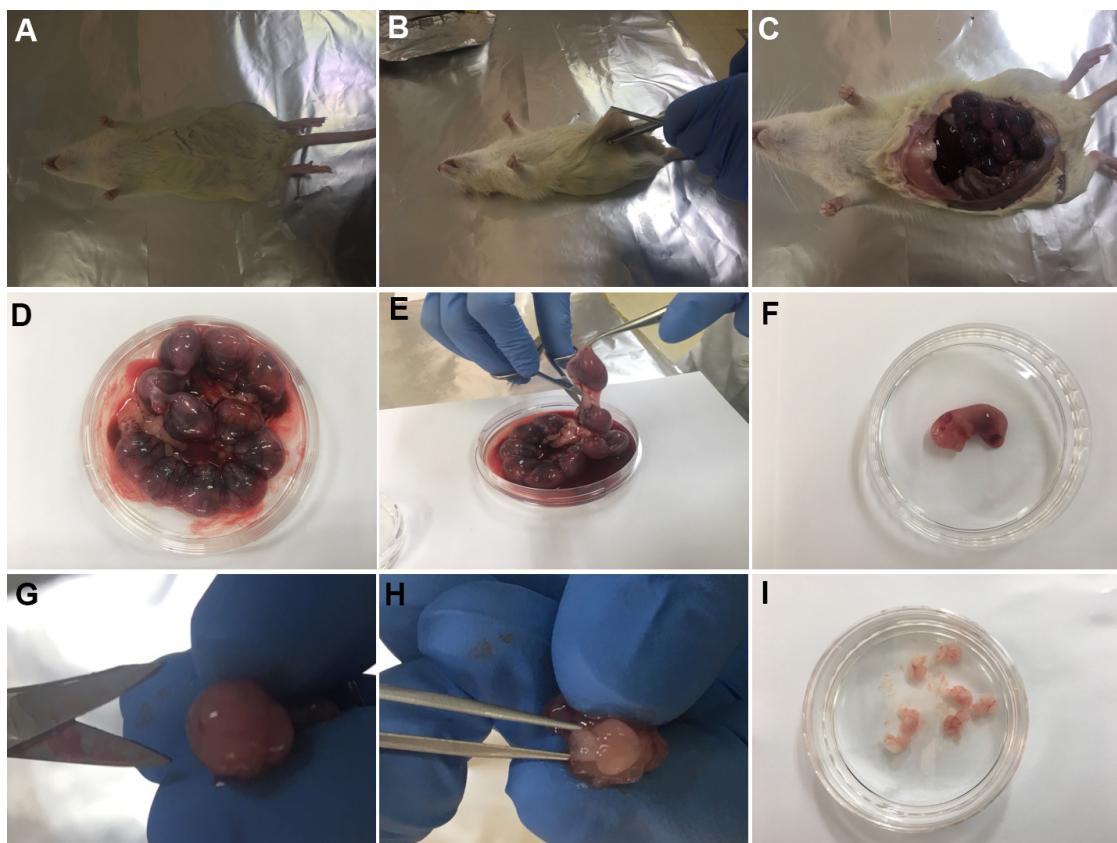
2. Remove the poly-D-lysine solution and wash twice with cold PBS.
3. Dry the coated plate on a bench while E17 rat embryo is being prepared.

4. Carefully take out the E17~E18 embryos from the Sprague Dawley rat using Dressing Scissors (Equipment 3) and Spring Dressing Forceps Blunt (Equipment 7). Place them in a Petri dish filled with cold Prep medium (Figures 2A-2D).

*Note: In this experiment, the pregnant Sprague Dawley rats were anesthetized with CO<sub>2</sub> and euthanized using CO<sub>2</sub> after embryos extraction. All experimental procedures were conducted after approval of Institutional Animal Care and Use Committee of Sungkyunkwan University.*

5. Using Dissecting Scissors (Equipment 5) and Spring Dressing Forceps Sharp (Equipment 6), carefully take out individual embryo from uterus and embryonic sack (Figures 2E-2F).
6. Using Dissecting Scissors (Equipment 4) and Multi Purpose Forceps Pointed (Equipment 8), excise the scalp and skull of the E17-E18 embryos and pull out the whole brain. Place the extracted brain in Prep medium (Figures 2G-2I).

*Note: For proper cell conditions, this process (Steps 4-6) should be finished within 15-20 min.*



**Figure 2. The process of embryo extraction in pregnant Sprague Dawley rat (E17-E18) (Steps 4-6)**

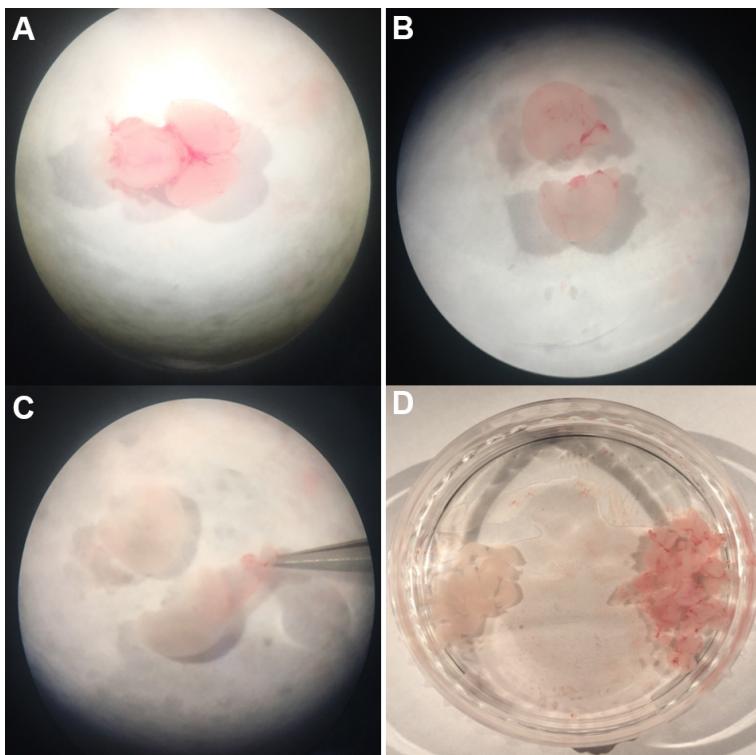
7. After taking out the embryos brain, place it in the prep medium as shown in Figure 3A.
8. Dissect the cerebral cortex from the whole brain as Figure 3B.

*Note: When separating the cortex from the embryo's brain, you must be careful not to separate other parts together. When you progress from Step 7 to Step 8 (Figure 3A to Figure 3B), insert the micro forceps between the inner side of the cortex and the outer part of the striatum, and then cut cortex from embryo's brain. As you see in Figure 3A, the cortex is on the surface as it envelops other parts of the brain. There is a striatum on the inner side just below the cortex.*

9. Remove meninges and blood vessels outside the cerebral cortex (Figure 3C). Place the cerebral cortex in Prep medium as Figure 3D.

*Notes:*

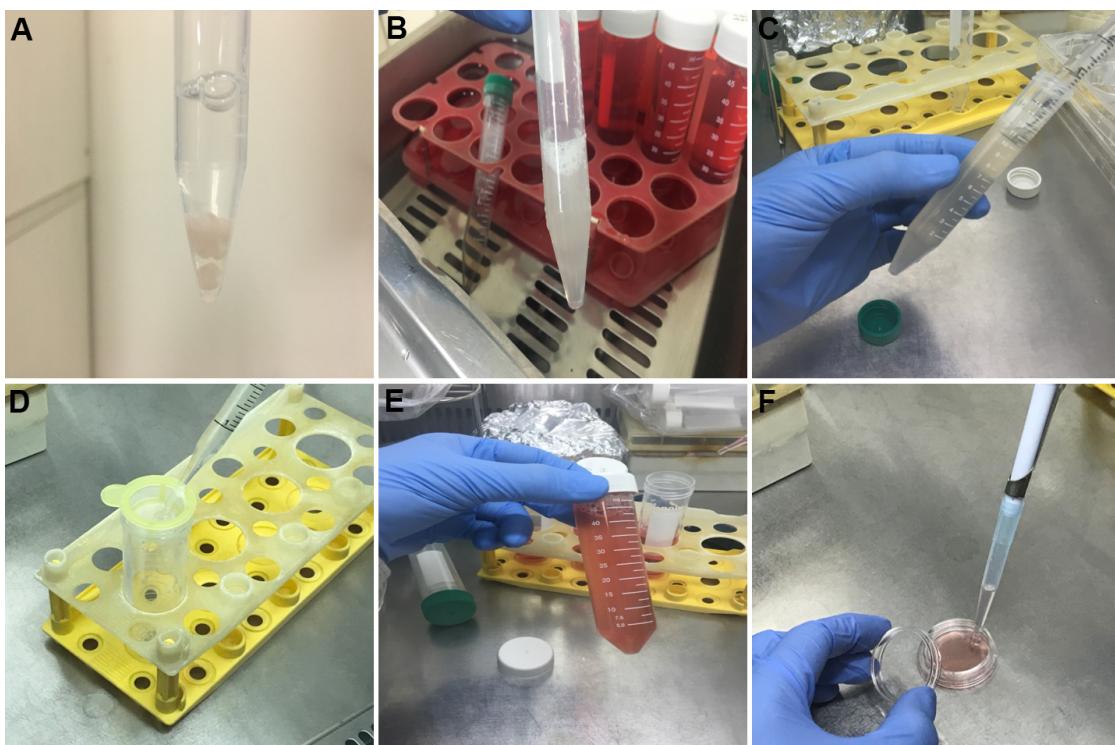
- After removing the meninges and blood vessels as shown in Figure 3, remove the hippocampus part that is attached to the inner side of the cortex. You can use the cortex part for cortical neuron cell culture or you can conduct hippocampal neuron cell culture with the hippocampus part.*
- For proper cell conditions, this process (Steps 7-9) should be done within 20-25 min.*



**Figure 3. The process of extracting cortex region from the extracted whole brain (Steps 7-9)**

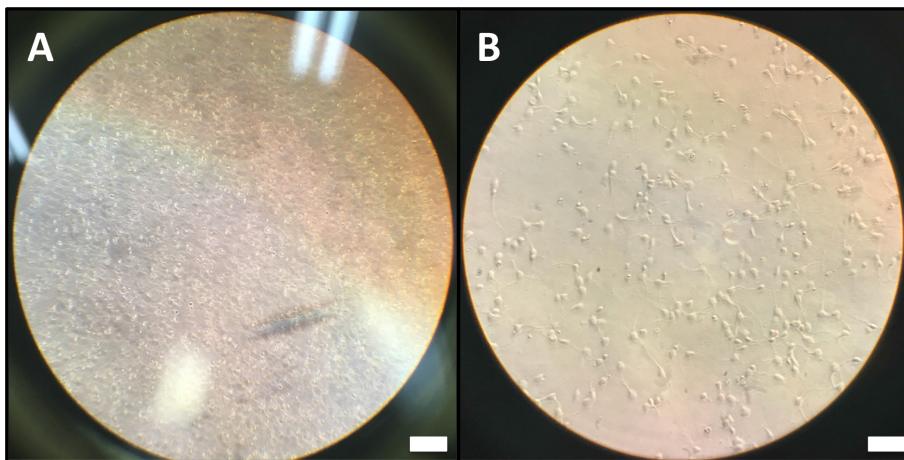
10. Transfer the cerebral cortex to a conical tube (15 ml size) filled with 2 ml of Prep medium and add 300  $\mu$ l of trypsin solution to make final concentration about 10% (Figure 4A).
11. Place the conical tube in a 37 °C incubator or water bath and gently tapping it periodically (Figure 4B).
12. After about 15 min, carefully suspend the cells using a 10 ml disposable pipette (Figure 4C).

13. Put 400  $\mu$ l of FBS in the tubes to 10% concentration and carefully suspend the cells again.
14. Filter the cells through a 70  $\mu$ m nylon cell strainer to obtain single cell suspension (Figure 4D).
15. Seed the cells at  $1 \times 10^6$  cells in a 35 mm dish after diluting the cells with 2 ml Culture medium and incubate the cells in an incubator at 37 °C for 18 h (Figures 4E and 4F).
16. Replace Culture medium in the dish with 2 ml of Maintain Culture medium.  
*Note: If cells are maintained in the Culture medium for too long, other brain cell types including microglia and astrocyte are likely to grow up. Therefore, Culture medium should be replaced with Maintain culture medium 12-18 h after the cell seeding.*
17. Replace the half of the media in the 35 mm dish with the new 1 ml Maintain culture medium every other day.



**Figure 4. The Process of making single cells of brain tissue and preparing for cell seeding (Steps 10-17)**

18. Grow the cells for at least 7 days after the seeding for experiments (Figure 5).  
*Note: When you seed the cells, various brain cells (neuron, microglia, astrocytes) are present in the medium. However, after replacement with the Maintain culture medium, only the neuron cells remain specifically because all other types of cells except for the neuron cell are removed by the B-27 constituting the Maintain culture medium.*



**Figure 5. Cell morphology at the 1st day (A) and after 8 days (B).** Scale bars, A = 500  $\mu\text{m}$ ; B = 200  $\mu\text{m}$ .

19. Incubate the cells in ROS inducing conditions for about 24 h.

*Note: 5  $\mu\text{M}$  of oligomeric  $\text{A}\beta_{1-42}$  was added to induce ROS in this protocol, but it may vary depending on your experimental conditions.  $\text{A}\beta_{1-42}$  is already known to induce ROS (Andrey et al., 2004, Shelat et al., 2008).*

20. And then treat with 1  $\mu\text{l}$  of CM-H<sub>2</sub>DCFDA solution (5 mM) or MitoSOX™ Red solution (5 mM) for 20 min.

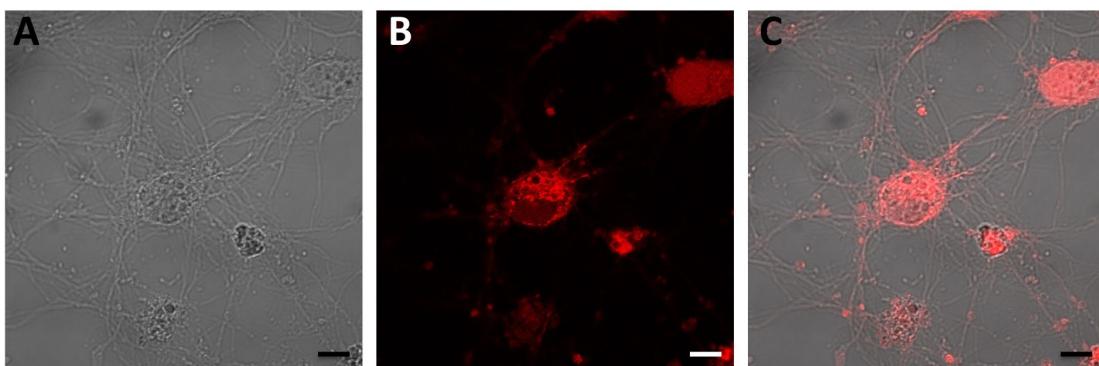
*Note: MitoSOX™ is a specific indicator for mitochondrial superoxide and CM-H<sub>2</sub>DCFDA is more sensitive to oxidation by H<sub>2</sub>O<sub>2</sub> than superoxide (O<sub>2</sub><sup>-</sup>). Therefore, even if two chemicals are processed at the same time, they are labeled with different ROS. However, it is difficult to distinguish exactly two types of ROS and analyze fluorescence in the confocal image. We recommend preparing samples separately and conduct each experiment.*

*Optional: This section provides another option for preparing samples to capture live cell images. If you have cultured cells on a coverslip, carefully put the coverslip into a new 35 mm live cell imaging dish. Put the side of coverslip that cells are attached to the bottom surface.*

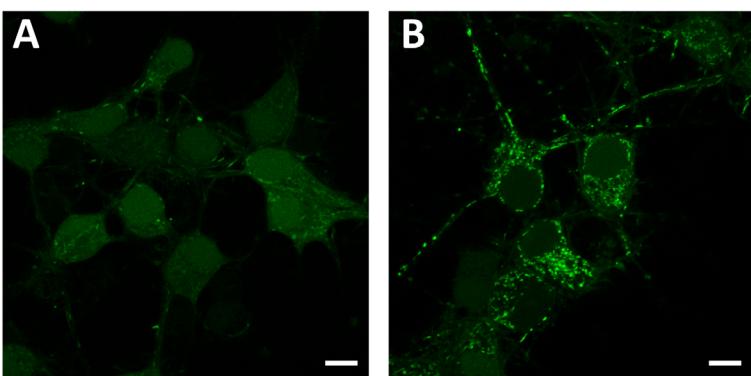
21. Replace with the new 2 ml of Maintain culture medium.

*Note: Pre-set the condition for taking live cell imaging; 37 °C and 5% CO<sub>2</sub> is required because we want to keep the growing conditions for primary neuron cells.*

22. Using confocal microscope, measure the fluorescence mediated by MitoSOX™ (510/580 nm, see Figure 6) or CM-H<sub>2</sub>DCFDA (495/520 nm, see Figure 7). Since the fluorescence will not last long, it is recommended to measure within 30 min.



**Figure 6. Detection of superoxide in rat primary neuronal cells' mitochondria with MitoSOX™ Red.** Oxidation of MitoSOX™ Red reagent by superoxide produces red fluorescence. A. Bright field rat primary cell images; B. Red fluorescence generated by superoxide; C. Merged image. Scale bars = 50 µm.



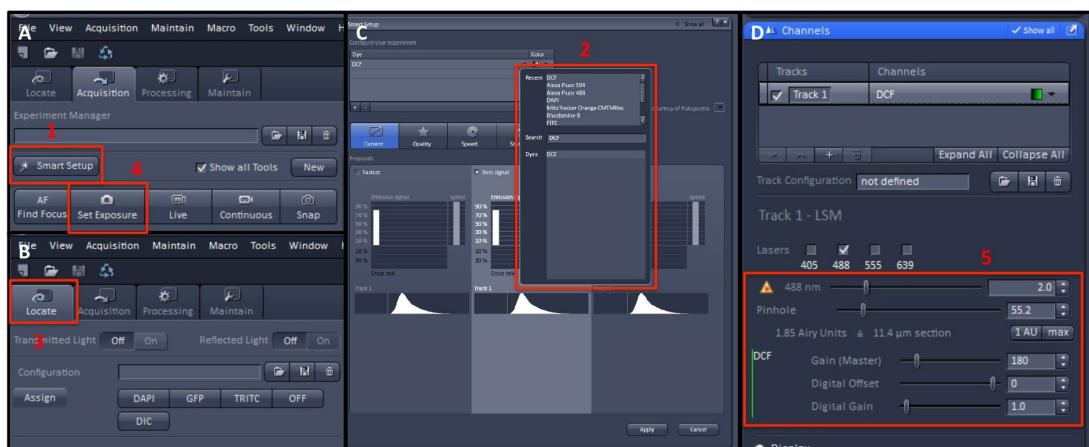
**Figure 7. Measurement of ROS in intracellular compartment induced by Aβ in rat primary cells using CM-H<sub>2</sub>DCFDA.** Green fluorescence represents intracellular ROS level of Aβ or vehicle treated sample. A. Vehicle-treated sample; B. 5 µM of Aβ treated sample. Scale bars = 50 µm.

#### *Confocal image measurement setting*

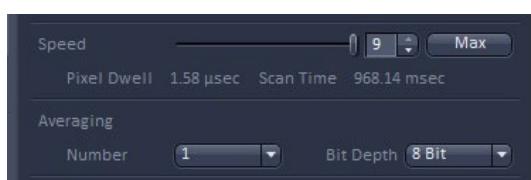
You can follow the processes in Figure 8 to measure the level of ROS using confocal microscope:

- Run the Zen black edition.
- Click the 'Smart Setup' button in 'Acquisition' tab to choose the appropriate excitation/emission wavelengths of the dye (MitoSOX™ or CM-H<sub>2</sub>DCFDA) (Figure 8 A1).
- Set the appropriate excitation/emission wavelengths of the dye and choose the image colors in the 'Search' tab (Figure 8C 2).
- Click the 'Best signal' button.
- Put the sample on the live cell imaging chamber (Figure 1C).
- Click the 'Locate' tab and adjust the focus of confocal microscope (Figure 8B 3).
- After setting the focus of the microscope, click the 'Set Exposure' button in 'Acquisition' tab.

- Note: ‘Set Exposure’ automatically adjusts the detector Gain value (Figure 8A).*
- h. Click the ‘Live’ button (Figure 8A).  
*Note: ‘Live’ performs constant scanning of real-time image.*
  - i. To get clearer and more accurate images in Live conditions, adjust each value of ‘Gain’, ‘Digital offset’, ‘Digital Gain’, ‘Pinhole’, and laser power (Figure 8D 5). You can also adjust ‘Speed’ and ‘Averaging’ to acquire better images.  
*Note: In this experiment, ‘Speed’ was set to 7 and ‘Averaging’ number was set to 8. (Figure 9)*
  - j. Click the ‘Snap’ button to acquire the image (Figure 8A).  
*Note: All samples should be measured under the same conditions. The measurement method was based on the instructions of the confocal equipment.*



**Figure 8. Flow chart of measuring method of ZEN black version of image measurement program**

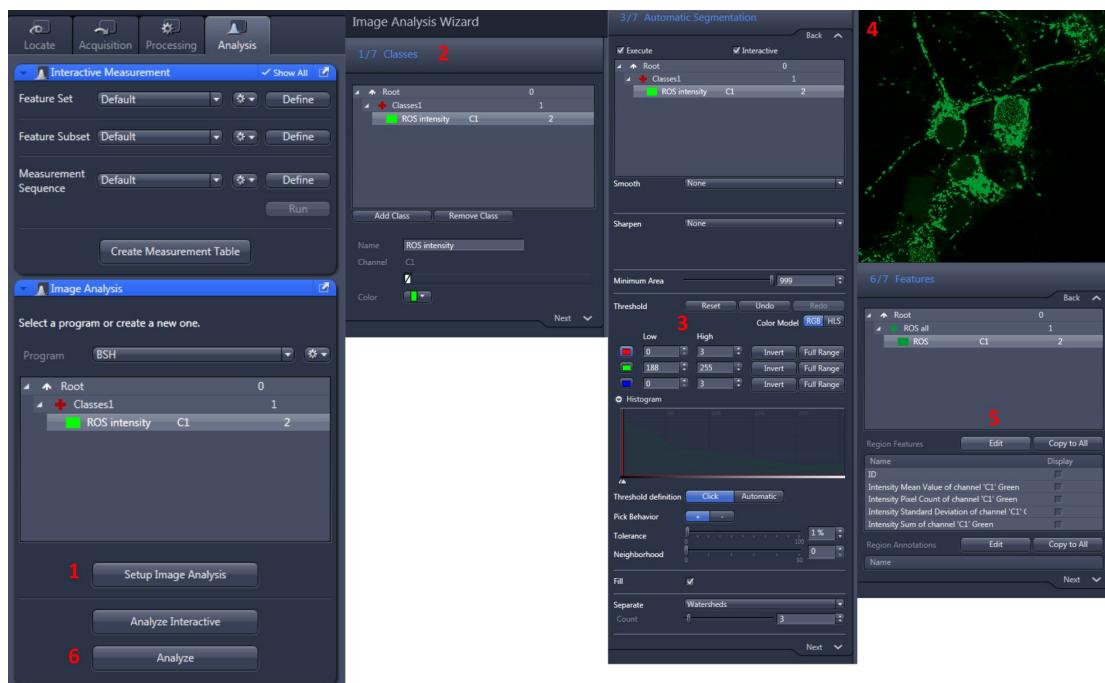


**Figure 9. The tab to control speed and averaging in ZEN black version**

## Data analysis

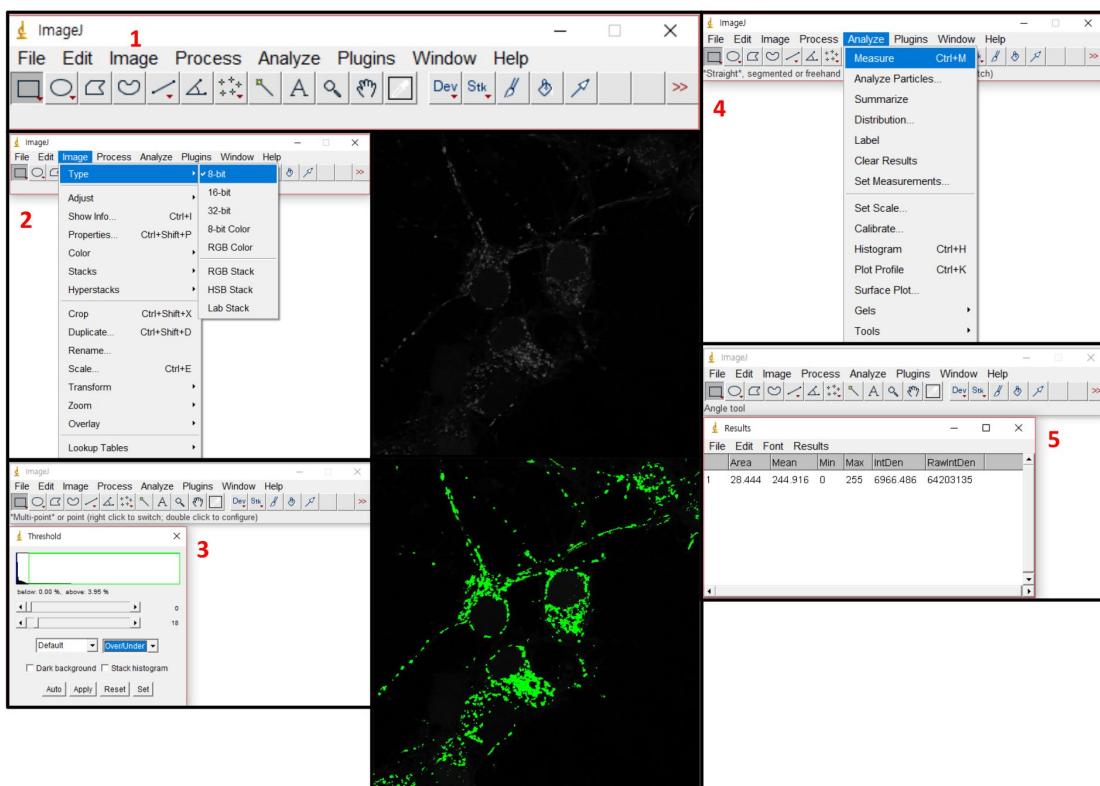
The confocal image that measure the ROS can be used for statistical analysis by quantifying the intensity of fluorescence. This protocol offers two methods.

1. Measurement method using basic confocal drive program (see Figure 10).



**Figure 10. Analysis flowchart of ZEN blue version.** Run Zen blue version, an analysis tool provided by Zeiss confocal equipment. Open the image you want to analyze and click the 'Analysis' tab. Press the 'Setup Image Analysis' button to set the analysis method (see Figure 10-1). Enter the proper analytical condition in order. In particular, set the appropriate threshold value (When you click on the area where CM-H<sub>2</sub>DCFDA emits fluorescence, the default value is automatically set) in the 3rd step (The analysis setting value must be set up based on the positive control. The settings of all images to be analyzed should be applied equally). Identify the area you want to measure as shown in Figure 10-4. Set the results (Fluorescence mean, Standard deviation, Fluorescence dot number, etc.) you want to obtain and press the 'Finish' button. Finally, check the value by pressing the 'Analysis' button.

## 2. Measurement method using ImageJ (see Figure 11)



**Figure 11. Analysis flowchart of ImageJ.** To measure the intensity of fluorescence using ImageJ, several preliminary steps are required. After opening the image you want to analyze, you have to separate the fluorescent area that you want to measure (In flowchart 3, set the range to be measured while adjusting the threshold value. see Figure 11-2, 11-3). Click on the Set Measurements tab as shown in flowchart 4 to set the results you want to obtain. Click the Measure tab and acquire the results.

### Notes

1. Each experimental group should be treated with MitoSOX™ or CM-H<sub>2</sub>DCFDA twenty minutes before measuring the fluorescence. You should not treat MitoSOX™ and CM-H<sub>2</sub>DCFDA in the experimental samples at the same time (Step 19).
2. If the cell growing conditions are not maintained, value of ROS measurement will be inaccurate. So, at least 10 minutes of stabilization time should be given before taking confocal images (Step 21).
3. When culturing primary neurons, delaying the medium replacement after cell seeding results in a decrease in the percentage of neurons in the cultured cells (Step 16).

### Recipes

1. CM-H<sub>2</sub>DCFDA solution (5 mM)  
Dissolve 50 µg CM-H<sub>2</sub>DCFDA (50 µg/1 vial) in 17 µl DMSO

2. MitoSOX™ Red solution (5 mM)  
Dissolve 50 µg MitoSOX™ Red (50 µg/1 vial) in 13 µl DMSO
3. Poly-D-lysine hydrobromide solution  
Poly-D-lysine hydrobromide (5 mg/vial)  
50 ml sterile Ultra pure water
4. Prep medium  
PBS 45 ml  
5 ml PS
5. Culture medium  
500 ml DMEM  
50 ml FBS  
5 ml PS
6. Maintain culture medium  
50 ml Neurobasal media  
1 ml B-27 supplement  
500 µl PS

### **Acknowledgments**

This research was supported by grants (2012R1A5A2A28671860, 2017M3C7A1048268) funded by the Basic Science Research Program through the National Research Foundation of Korea (NRF), the Ministry of Education, Science and Technology, Republic of Korea. The authors state that they have no conflict of interest to declare.

### **Reference**

1. Andrey, A. Y., Canevari, L. and Duchen, M. R. (2004). [β-amyloid peptides induce mitochondrial dysfunction and oxidative stress in astrocytes and death of neurons through activation of NADPH oxidase.](#) *J Neurosci* 24(2): 565-575.
2. Baek, S. H., Park, S. J., Jeong, J. I., Kim, S. H., Han, J., Kyung, J. W., Baik, S. H., Choi, Y., Choi, B. Y., Park, J. S., Bahn, G., Shin, J. H., Jo, D. S., Lee, J. Y., Jang, C. G., Arumugam, T. V., Kim, J., Han, J. W., Koh, J. Y., Cho, D. H. and Jo, D. G. (2017). [Inhibition of Drp1 ameliorates synaptic depression, Aβ deposition, and cognitive impairment in an Alzheimer's disease model.](#) *J Neurosci* 37(20): 5099-5110.
3. Batandier, C., Fontaine, E., Keriel, C. and Leverve, X. M. (2002). [Determination of mitochondrial reactive oxygen species: methodological aspects.](#) *J Cell Mol Med* 6(2): 175-187.
4. Brieger, K., Schiavone, S., Miller, F. J., Jr. and Krause, K. H. (2012). [Reactive oxygen species: from health to disease.](#) *Swiss Med Wkly* 142: w13659.

5. Datta, K., Sinha, S. and Chattopadhyay, P. (2000). [Reactive oxygen species in health and disease](#). *Natl Med J India* 13(6): 304-310.
6. Finkel, T. (2011). [Signal transduction by reactive oxygen species](#). *J Cell Biol* 194(1): 7-15.
7. Forstermann, U. (2008). [Oxidative stress in vascular disease: causes, defense mechanisms and potential therapies](#). *Nat Clin Pract Cardiovasc Med* 5(6): 338-349.
8. Fowler, T. L., Fisher, M. M., Bailey, A. M., Bednarz, B. P. and Kimple, R. J. (2017). [Biological characterization of a novel \*in vitro\* cell irradiator](#). *PLoS One* 12(12): e0189494.
9. Forkink, M., Smeitink, J. A., Brock, R., Willems, P. H. and Koopman, W. J. (2010). [Detection and manipulation of mitochondrial reactive oxygen species in mammalian cells](#). *Biochim Biophys Acta* 1797(6-7): 1034-1044.
10. Heneka, M. T., Kummer, M. P. and Latz, E. (2014). [Innate immune activation in neurodegenerative disease](#). *Nat Rev Immunol* 14(7): 463-477.
11. Kirkland, R. A., Saavedra, G. M. and Franklin, J. L. (2007). [Rapid activation of antioxidant defenses by nerve growth factor suppresses reactive oxygen species during neuronal apoptosis: evidence for a role in cytochrome c redistribution](#). *J Neurosci* 27(42): 11315-11326.
12. Khatri, R., Shah, P., Guha, R., Rassool, F. V., Tomkinson, A. E., Brodie, A. and Jaiswal, A. K. (2015). [Aromatase inhibitor-mediated downregulation of INrf2 \(Keap1\) leads to increased Nrf2 and resistance in breast cancer](#). *Mol Cancer Ther* 14(7): 1728-1737.
13. Liu, Y. H., Weng, Y. P., Lin, H. Y., Tang, S. W., Chen, C. J., Liang, C. J., Ku, C. Y. and Lin, J. Y. (2017). [Aqueous extract of \*Polygonum bistorta\* modulates proteostasis by ROS-induced ER stress in human hepatoma cells](#). *Sci Rep* 7: 41437.
14. Nykky, J., Vuento, M. and Gilbert, L. (2014). [Role of mitochondria in parvovirus pathology](#). *PLoS One* 9(1): e86124.
15. Ng, L. F., Gruber, J., Cheah, I. K., Goo, C. K., Cheong, W. F., Shui, G., Sit, K. P., Wenk, M. R. and Halliwell, B. (2014). [The mitochondria-targeted antioxidant MitoQ extends lifespan and improves healthspan of a transgenic \*Caenorhabditis elegans\* model of Alzheimer disease](#). *Free Radic Biol Med* 71: 390-401.
16. Polidori, M. C. (2004). [Oxidative stress and risk factors for Alzheimer's disease: clues to prevention and therapy](#). *J Alzheimers Dis* 6(2): 185-191.
17. Ray, P. D., Huang, B. W. and Tsuji, Y. (2012). [Reactive oxygen species \(ROS\) homeostasis and redox regulation in cellular signaling](#). *Cell Signal* 24(5): 981-990.
18. Robinson, K. M., Janes, M. S., Pehar, M., Monette, J. S., Ross, M. F., Hagen, T. M., Murphy, M. P. and Beckman, J. S. (2006). [Selective fluorescent imaging of superoxide \*in vivo\* using ethidium-based probes](#). *Proc Natl Acad Sci U S A* 103(41): 15038-15043.
19. Singh, D. K., Winocour, P. and Farrington, K. (2011). [Oxidative stress in early diabetic nephropathy: fueling the fire](#). *Nat Rev Endocrinol* 7(3): 176-184.
20. Shelat, P. B., Chalmoniuk, M., Wang, J. H., Strosznajder, J. B., Lee, J. C., Sun, A. Y., Simonyi, A. and Sun, G. Y. (2008). [Amyloid beta peptide and NMDA induce ROS from NADPH oxidase](#)

- [and AA release from cytosolic phospholipase A2 in cortical neurons.](#) *J Neurochem* 106(1): 45-55.
21. Trachootham, D., Alexandre, J. and Huang, P. (2009). [Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach?](#) *Nat Rev Drug Discov* 8(7): 579-591.
22. Waris, G. and Ahsan, H. (2006). [Reactive oxygen species: role in the development of cancer and various chronic conditions.](#) *J Carcinog* 5: 14.

## Quantification of Extracellular Double-stranded RNA Uptake and Subcellular Localization Using Flow Cytometry and Confocal Microscopy

Tan A Nguyen<sup>1, 2</sup>, Lachlan Whitehead<sup>1, 2</sup> and Ken C Pang<sup>1, 3, 4, 5, \*</sup>

<sup>1</sup>The Walter and Eliza Hall Institute of Medical Research, Parkville, VIC, Australia; <sup>2</sup>Department of Medical Biology, University of Melbourne, Parkville, VIC, Australia; <sup>3</sup>Murdoch Children's Research Institute, Parkville, VIC, Australia; <sup>4</sup>Department of Paediatrics, University of Melbourne, Parkville, VIC, Australia; <sup>5</sup>Department of Psychiatry, University of Melbourne, Parkville, VIC, Australia

\*For correspondence: [ken.pang@mcri.edu.au](mailto:ken.pang@mcri.edu.au)



**[Abstract]** Double-stranded RNA is a potent pathogen-associated molecular pattern (PAMP) produced as a by-product of viral replication and a well-known hallmark of viral infection. Viral dsRNAs can be released from infected cells into the extracellular space and internalized by neighboring cells via endocytosis. Mammals possess multiple pattern recognition receptors (PRRs) capable of detecting viral dsRNAs such as endosomal toll-like receptor 3 (TLR3) and cytosolic RIG-I-like receptors (RLRs) which lead to the production of type I interferons (IFNs). Thus, intracellular localization of viral dsRNA can provide insight into the downstream signaling pathways leading to innate immune activation. Here, we describe a quantitative method for measuring extracellular dsRNA uptake and visualizing subcellular localization of internalized dsRNA via flow cytometry and confocal microscopy respectively.

**Keywords:** Double-stranded RNA, Endosomes, Lysosomes, Viruses, Poly(I:C), TLR3, RIG-I, MDA-5, Confocal microscopy, Flow cytometry

**[Background]** Double-stranded RNAs (dsRNAs) are a common by-product of viral replication and are potent activators of antiviral immunity via the production of type I interferon (IFN) and other pro-inflammatory cytokines (Nellimarla and Mossman, 2014). Viral dsRNAs are sensed within endosomes by TLR3 (Matsumoto *et al.*, 2003) or in the cytosol by the RIG-I-like receptors (RLRs), RIG-I and MDA-5 (Kato *et al.*, 2006). During lytic infections, these dsRNAs can be released into the extracellular space where they bind surface receptors on neighboring cells, such as class A scavenger receptors (SR-A) and Raftlin, and are subsequently internalized via clathrin-mediated endocytosis (Itoh *et al.*, 2008; DeWitte-Orr *et al.*, 2010; Watanabe *et al.*, 2011; Dansako *et al.*, 2013).

In our previous study, we found out that the protein SID1 transmembrane family member 2 (SIDT2) localizes to late endosomes and lysosomes and that loss of SIDT2 leads to subcellular accumulation of the synthetic dsRNA analog, poly(I:C), while not affecting initial endocytosis-mediated internalization (Nguyen *et al.*, 2017). To do so, we developed and utilized flow cytometry and confocal microscopy-based approaches to quantitatively measure poly(I:C) uptake and subcellular localization respectively *in vitro*. In this protocol, we describe a further refinement of these assays to allow for high-throughput assessment of internalization and subcellular localization of different dsRNAs. These

methods allow for further dissection of dsRNA trafficking during viral infection and the downstream effects of these dsRNAs on innate immune signaling.

### **Materials and Reagents**

1. 8 well microscope slide (ibidi, catalog number: 80826)
2. 24 well tissue culture plate (Corning, Falcon®, catalog number: 353047)
3. Sterile filtered pipette tips (0.5 µl to 1,000 µl) (Corning, Axygen, catalogue numbers: TF-300-L-R-S, TF-20-L-R-S, TF-200-L-R-S and TF-1000-L-R-S)
4. 10 cm culture dishes (Corning, Falcon®, catalog number: 353003)
5. 10 ml centrifuge tubes (SARSTEDT, catalog number: 62.9924.284)
6. 1.5 ml microcentrifuge tubes (Sigma-Aldrich, catalog number: EP0030120086)
7. 1.2 ml Micro Titertube (Thermo Fisher Scientific, Quality Scientific Plastics, catalog number: 845-Q)
8. Serological pipettes, individually wrapped, 10 ml (Corning, Falcon®, catalog number: 356551)
9. Mammalian cell line of interest, here: mouse embryonic fibroblasts (see Note 1)
10. 70% (v/v) ethanol (Chem Supply, catalog number: EA043)
11. Dulbecco's modified Eagle medium (DMEM) or other suitable complete growth medium for culture of cell line of interest
12. Fetal bovine serum (FBS) (Sigma-Aldrich, catalog number: F9423)
13. Phosphate-buffered saline (PBS) (sterile) (Thermo Fisher Scientific, Gibco™, catalog number: 14190250)
14. Penicillin/streptomycin solution (Sigma-Aldrich, catalog number: P4333)
15. Poly(I:C)-fluorescein (InvivoGen, catalog number: tlrl-picf)
16. Poly(I:C)-rhodamine (InvivoGen, catalog number: tlrl-picr)
17. dsRNA specific monoclonal antibody (J2, SCICONS English and Scientific Consulting, catalog number: 10010200)
18. RNase A enzyme (Sigma-Aldrich, catalog number: R4875)
19. 1x trypsin-EDTA solution (Sigma-Aldrich, catalog number: 59430C)
20. Paraformaldehyde (PFA) powder (Sigma-Aldrich, catalog number: 158127)
21. Tween 20 (Sigma-Aldrich, catalog number: P1379)
22. DAPI (4',6-Diamidino-2'-phenylindole dihydrochloride) powder (Sigma-Aldrich, catalog number: D9542)
23. Immersol™ Immersion Oil (Carl Zeiss, catalog number: 4449620000000)
24. Complete growth medium (see Recipes)
25. 10% FBS/PBS (see Recipes)
26. 4% paraformaldehyde (w/v) (see Recipes)
27. Permeabilization buffer (see Recipes)
28. DAPI solution (see Recipes)

## Equipment

1. Pipetting aid (Thermo Fisher Scientific, catalog number: 9531)
2. Micropipettes from 0.5  $\mu$ l to 1 ml (Mettler-Toledo International, Rainin, model: Pipet-Lite<sup>TM</sup> XLS+)
3. Hemocytometer
4. Class II biological safety cabinet/tissue culture hood
5. Humidified CO<sub>2</sub> incubator (95% air, 5% CO<sub>2</sub>, 37 °C)
6. Inverted light microscope (phase contrast)
7. 37 °C water bath
8. Vacuum aspiration system with glass Pasteur pipettes
9. Table top centrifuge equipped with a swing-out rotor for 10 ml conical tubes
10. Microcentrifuge
11. LSRFortessa X20 (BD, BD Biosciences, model: LSRFortessa<sup>TM</sup> X-20) or equivalent flow cytometer
12. LSM 780 confocal laser scanning microscope (ZEISS, model: LSM 780) or equivalent microscope

## Software

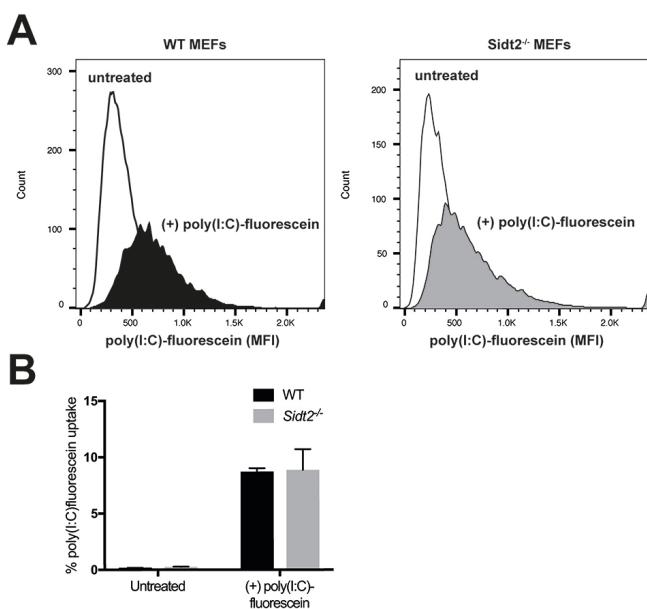
1. FIJI/ImageJ
2. Zeiss ZEN package
3. Microsoft Excel
4. FlowJo
5. GraphPad Prism 7

## Procedure

### A. Cell culture and maintenance

1. Perform all cell culture-based work in a class II biological safety cabinet/tissue culture hood. Ensure work surface and materials are sterilized using 70% (v/v) ethanol.
2. Grow mammalian cells of choice in complete growth medium using 10 cm cell culture dishes or cell culture flasks in a humidified CO<sub>2</sub> incubator (95% air, 5% CO<sub>2</sub>, 37 °C). Here, we use mouse embryonic fibroblasts (MEFs) derived from C57BL/6 mice and MEFs lacking the dsRNA transporter, SIDT2, as our gene of interest (see Note 2).
3. Maintain cells using standard cell culture procedures or as recommended by the supplier. Here, split MEFs approximately every 3-4 days or before cells reach 100% confluency using standard cell culture procedures (see Note 3).

4. One day prior to stimulation, split the cells as above and determine cell number using a hemocytometer or other appropriate methods under a light microscope.
- B. Quantification of poly(I:C) uptake by flow cytometry
  1. Seed  $1 \times 10^4$  cells in a 24 well plate in triplicate for the following conditions in a total volume of 500  $\mu\text{l}$  of complete growth medium (cells may be seeded for additional conditions as required):
    - a. Unstimulated
    - b. Stimulated with poly(I:C) (see Note 4)
  2. Let cells adhere and rest overnight in a humidified CO<sub>2</sub> incubator (95% air, 5% CO<sub>2</sub>, 37 °C).
  3. The next morning, remove cells from the incubator and add 1  $\mu\text{g}/\text{ml}$  of fluorescein-poly(I:C) to the cell culture medium (see Note 5).
  4. Stimulate cells in an incubator at 37 °C, 5% CO<sub>2</sub> for 24 h.
  5. After 24 h of stimulation, carefully aspirate complete growth medium from each well with a glass Pasteur pipette using a vacuum aspiration system.
  6. Wash cells with 1 ml of cold 1x PBS using a P1000 pipette. Carefully add PBS on the side of the well to avoid dislodging cells.
  7. Repeat Step B6 for a total of 3 washes.
  8. Harvest cells by adding 500  $\mu\text{l}$  of Trypsin to each well and incubate cells at 37 °C, 5% CO<sub>2</sub> for 5 min.
  9. Add 500  $\mu\text{l}$  of complete growth medium to each well.
  10. Gently resuspend cells by pipetting up and down 3-5 times.
  11. Transfer resuspended cells to new individual 1.5 ml microcentrifuge tubes.
  12. Centrifuge cells in a microcentrifuge for 3 min at 500  $\times g$  and resuspend the pellet in 500  $\mu\text{l}$  PBS supplemented with 10% FBS.
  13. Add 10  $\mu\text{l}$  of RNase A enzyme (5 mg/ml stock) to each well at a final concentration of 100  $\mu\text{g}/\text{ml}$  (see Note 6).
  14. Incubate cells at 37 °C and 5% CO<sub>2</sub> for 30 min.
  15. Centrifuge cells in a microcentrifuge for 3 min at 500  $\times g$ .
  16. Carefully aspirate RNase A solution from each well.
  17. Resuspend cells in 1 ml cold PBS.
  18. Repeat Step B15 for a total of 3-5 washes.
  19. Resuspend cells in 200  $\mu\text{l}$  of 10% FBS/PBS.
  20. Transfer cells to individual 1.2 ml Micro Titertubes and leave on ice.
  21. Analyze the incorporated fluorescence of cells of both genotypes and using flow cytometry. Compare the histograms and corresponding mean fluorescence intensities (MFI) between fluorescein-poly(I:C)-stimulated cells and unstimulated cells (Figure 1).



**Figure 1. Analysis of poly(I:C)-fluorescein uptake by flow cytometry.** The endocytic activity of WT and *Sidt2<sup>-/-</sup>* MEFs was assessed by measuring the uptake of fluorescein-conjugated poly(I:C) following 24 h stimulation. A. Representative histograms for the mean fluorescence intensities (MFI) for each genotype are shown. B. Mean percentage of poly(I:C)-fluorescein positive cells. n = 3 technical replicates and errors bars represent  $\pm$  SEM. Loss of SIDT2 did not affect poly(I:C) uptake.

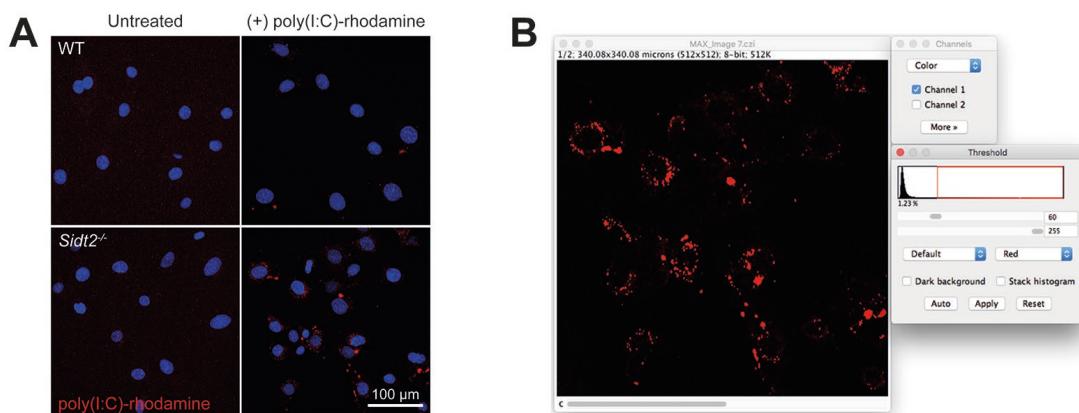
#### C. Confocal analysis of poly(I:C) subcellular localization

1. Seed  $5 \times 10^3$  cells in an 8 well chamber slide for the following conditions in a total volume of 200  $\mu$ l of complete growth medium (cells may be seeded for additional conditions as required):
  - a. Unstimulated
  - b. Stimulated with poly(I:C)
2. The next morning, remove cells from the incubator and add 1  $\mu$ g/ml of rhodamine-poly(I:C) to the cell culture medium (see Note 5).
3. Incubate cells at 37 °C, 5% CO<sub>2</sub> for 24 h.
4. Carefully aspirate and discard complete growth medium from each well with a P200 pipette.
5. Wash cells with 200 ml of cold 1x PBS using a P1000 pipette.
6. Repeat Step C5 for a total of 3 washes.
7. After washes, add 200  $\mu$ l PBS supplemented with 10% FBS to each well.
8. Add 10  $\mu$ l of RNase A enzyme (5 mg/ml stock) to each well at a final concentration of 100  $\mu$ g/ml (see Note 6).
9. Incubate cells at 37 °C and 5% CO<sub>2</sub> for 30 min.
10. Carefully aspirate RNase A solution from each well.
11. Wash cells with 200  $\mu$ l cold PBS.
12. Repeat Step C11 for a total of 3 washes.

13. Fix cells with 200 µl of 4% PFA for 10 min on ice.
14. Wash cells with 200 µl cold PBS.
15. Repeat Step C14 for a total of 3 washes.
16. Stain cells with 100 ng/ml DAPI solution in a final volume of 200 µl per well for 10 min at room temperature (see Note 7).
17. Wash cells with 200 µl cold PBS.
18. Repeat Step C17 for a total of 3 washes.
19. After the final wash, add 200 µl of PBS to each well.
20. Slides are now ready for imaging or can be stored at 4 °C for up to 2 weeks.
21. Acquire images using a ZEISS LSM 780 confocal microscope (an equivalent microscope can be used) with a 25x oil immersion objective lens.
22. Acquire Z-stack of 0.25 µm slices from the top to the bottom of the cells (Figure 2A).
23. Save images as .lsm files and separate each condition into individual folders (e.g., WT untreated, WT pIC treated, KO untreated, KO pIC treated).
24. Drag and drop all folders into a single folder (see Note 8).
25. Images are now ready for analysis using FIJI/ImageJ software (see below).

### **Data analysis**

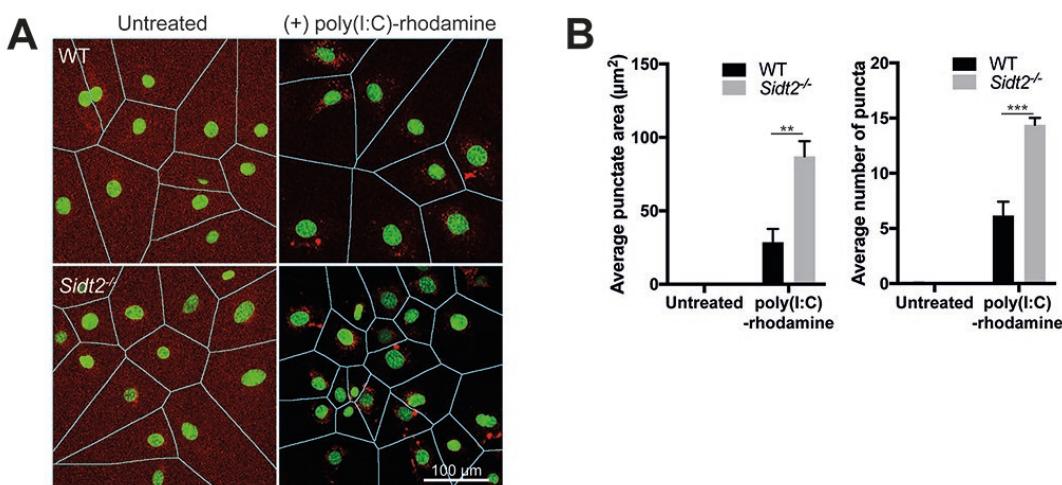
- A. Determine appropriate threshold value for images
  1. Open an image file that contains cells with representative poly(I:C)-rhodamine or alternative dsRNA signal intensity in FIJI/ImageJ.
  2. Acquire maximum projection image (Navigate to toolbar on the top left corner of the screen → Image → Stacks → Z Project → Projection type: Max intensity → Okay).
  3. Select channel corresponding to poly(I:C)-rhodamine by adjusting the C slider on the bottom of the maximum projection image window.
  4. From the toolbar, select Image → Adjust → Threshold...
  5. Set bottom slider to 255.
  6. Adjust top slider until threshold (red) is able to distinguish between each individual punctate endosome (Figure 2B). Here, we used a threshold of 60.
  7. Note down threshold value.



**Figure 2. Analysis of poly(I:C)-rhodamine subcellular localization by confocal microscopy.** A. WT and *Sidt2*<sup>-/-</sup> MEFs were treated with rhodamine-conjugated poly(I:C) for 24 h and assessed via confocal microscopy. Representative maximum projection images are shown, red = poly(I:C), blue = DAPI. B. Snapshot from FIJI/ImageJ showing selection of threshold value step.

#### B. Subcellular localization quantification

1. Download FIJI macro file from: [https://bitbucket.org/DrLachie/rna\\_subcell](https://bitbucket.org/DrLachie/rna_subcell).
2. Open macro file in FIJI/ImageJ.
3. Change 'GreenThreshold' to the threshold value determined in the above section (e.g., '60').
4. Click 'Run'.
5. Open output\ folder and check the accuracy of cell segmentation (Figure 3A) and individual cell by cell quantification of the punctate area, intensity and number of puncta determined by macro.
6. Open results.xls file located in top folder using Microsoft Excel.
7. Calculate and Graph results for the average punctate area, intensity and number of puncta in Prism GraphPad or equivalent software (Figure 3B) (see Note 9).



**Figure 3. Quantification of poly(I:C)-rhodamine subcellular localization using FIJI/ImageJ macro.** A. Representative output image following macro quantification showing segmentation of cells using DAPI to delineate between individual cells. Note that poly(I:C) localizes in the cytoplasm and not in the nucleus. B. Comparison of the average percentage punctate area, average intensity and average number of puncta between WT and *Sidt2<sup>-/-</sup>* MEFs. Data are plotted as mean  $\pm$  SEM and at least 5 representative fields of view each containing 5-22 cells were analyzed per condition. \*\* $P$  < 0.01, \*\*\* $P$  < 0.001.

### Notes

1. Other adherent cell lines of interest can also be used such as bone marrow-derived macrophages, HEK293T and NIH3T3, etc. We have also used non-adherent cells such as DC2.4 and bone marrow-derived dendritic cells by treating suspension cells in 1.5 ml microcentrifuge tubes and subsequently mounting fixed cells onto a microscope slide via a cytopspin. However, we find that this method leads to a loss of cell morphology and therefore recommend the use of adherent cells if possible.
2. We have previously reported that the loss of SIDT2 leads to accumulation of dsRNA within endosomes (Nguyen *et al.*, 2017). Here, *Sidt2<sup>-/-</sup>* MEFs were used to assess endosomal subcellular localization compared to WT MEFs. Cells lacking or overexpressing gene of interest can be used in place of *Sidt2<sup>-/-</sup>* MEFs as desired.
3. Here, we pre-warm 1x PBS, 1x Trypsin solution and complete growth medium to 37 °C using a water bath. Old medium is removed from the cell culture dish using a Pasteur pipette by vacuum aspiration. Cells are subsequently washed with 10 ml of 1x PBS before the addition of 3 ml (per 10 cm dish) of Trypsin solution and incubate for 3-5 min at 37 °C. Detached cells are resuspended in 10 ml/10 cm dish by pipetting 2-3 times up and down and transfer to a 10 ml centrifuge tube. Cells are pelleted in a tabletop centrifuge at 500  $\times$  g, 5 min at RT and resuspended in 10 ml of fresh complete growth medium. For cell maintenance, add 1 ml of cell

suspension to a new 10 cm cell culture dish with 10 ml of complete growth medium and incubate in a humidified CO<sub>2</sub> incubator (95% air, 5% CO<sub>2</sub>, 37 °C).

4. Here, rhodamine or fluorescein conjugated poly(I:C) (InvivoGen) was used to stimulate cells as it is commercially available and readily accessible. Alternate fluorescently-tagged ligands can also be used as required. We have also successfully detected viral dsRNA via immunofluorescence staining using a dsRNA specific monoclonal antibody (J2, English and Scientific Consulting).
5. The concentration of poly(I:C) and length of stimulation should be optimized according to cell line of interest. We found that MEFs do not efficiently internalize poly(I:C) and therefore require a 24 h stimulation time. However, bone marrow-derived dendritic cells and macrophages require much shorter stimulation times (1 to 3 h) for sufficient uptake.
6. Treating cells with RNase will degrade any surface-bound dsRNA while retaining internalized dsRNA. Here, we used RNase A which is able to cleave both ssRNA and dsRNA at low salt concentrations. Alternatively, RNase III can be used to specifically cleave dsRNA.
7. It is important to stain cells with DAPI in the presence of detergent to permeabilize the cell membrane and allow DAPI access to nuclear DNA. We use 0.1% Tween to permeabilize cells; however, alternative detergents can be used such as Saponin or Triton-X.
8. The FIJI macro used for image analysis requires two layers of folders in order to proceed with analysis.
9. Here, we demonstrate that loss of SIDT2 results in endosomal accumulation of poly(I:C), consistent with our previous findings (Nguyen *et al.*, 2017). In that study, we also performed transient transfection and immunofluorescence staining of various endosomal markers – EEA-1 (early endosomes), RAB-7 (late endosomes) and LAMP-1 (lysosomes) – in order to precisely determine the subcellular localization of poly(I:C) within *Sidt2*<sup>-/-</sup> cells.

## Recipes

1. Complete growth medium  
Dulbecco's modified Eagle medium supplemented with:  
10% fetal bovine serum  
100 U/ml penicillin  
100 µg/ml streptomycin  
Filter sterilize, store at 4 °C
2. 10% FBS/PBS  
450 ml Sterile Phosphate Buffered Saline  
50 ml fetal bovine serum  
Store at 4 °C

3. 4% paraformaldehyde (w/v)
  - a. Dissolve 4 g of PFA powder in 90 ml PBS and heat to 65 °C while stirring. If PFA does not dissolve, add drops of 1 M NaOH until the solution becomes clear.
  - b. Bring to 100 ml with PBS. Cool and filter. Aliquot and store at -20 °C. Thaw aliquots as needed and use immediately.
4. Permeabilization buffer  
10 ml PBS  
10 µl of Tween 20
5. DAPI solution
  - a. Dissolve stock solution in sterile dH<sub>2</sub>O at a final concentration of 1 mg/ml
  - b. Dilute stock solution to a final concentration of 1 µg/ml in permeabilization buffer. Use immediately

### **Acknowledgments**

We thank the members of the Wicks and Masters labs, WEHI for helpful discussions. This protocol was adapted from Nguyen *et al.* (2017) *Immunity* 47(3):498-509.e6. DOI: 10.1016/j.jimmuni.2017.08.007. This work was supported by Australian NHMRC (ID 520574 and 1064591), Royal Australasian College of Physicians, Menzies Foundation, CASS Foundation (SM13-4846 and SM14- 5566), and Reid Family Trust. The authors declare no conflict of interest.

### **References**

1. Dansako, H., Yamane, D., Welsch, C., McGivern, D. R., Hu, F., Kato, N. and Lemon, S. M. (2013). [Class A scavenger receptor 1 \(MSR1\) restricts hepatitis C virus replication by mediating toll-like receptor 3 recognition of viral RNAs produced in neighboring cells.](#) *PLoS Pathog* 9(5): e1003345.
2. DeWitte-Orr, S. J., Collins, S. E., Bauer, C. M. T., Bowdish, D. M., Mossman, K. L. (2010). [An accessory to the 'Trinity': SR-As are essential pathogen sensors of extracellular dsRNA, mediating entry and leading to subsequent type I IFN responses.](#) *PLoS Pathog* 6: e1000829.
3. Itoh, K., Watanabe, A., Funami, K., Seya, T. and Matsumoto, M. (2008). [The clathrin-mediated endocytic pathway participates in dsRNA-induced IFN-β production.](#) *J Immunol* 181(8): 5522-5529.
4. Kato, H., Takeuchi, O., Sato, S., Yoneyama, M., Yamamoto, M., Matsui, K., Uematsu, S., Jung, A., Kawai, T., Ishii, K. J., Yamaguchi, O., Otsu, K., Tsujimura, T., Koh, C. S., Reis e Sousa, C., Matsuura, Y., Fujita, T. and Akira, S. (2006). [Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses.](#) *Nature* 441(7089): 101-105.

5. Matsumoto, M., Funami, K., Tanabe, M., Oshiumi, H., Shingai, M., Seto, Y., Yamamoto, A. and Seya, T. (2003). [Subcellular localization of Toll-like receptor 3 in human dendritic cells](#). *J Immunol* 171(6): 3154-3162.
6. Nellimarla, S. and Mossman, K. L. (2014). [Extracellular dsRNA: its function and mechanism of cellular uptake](#). *J Interferon Cytokine Res* 34(6): 419-426.
7. Nguyen, T. A., Smith, B. R. C., Tate, M. D., Belz, G. T., Barrios, M. H., Elgass, K. D., Weisman, A. S., Baker, P. J., Preston, S. P., Whitehead, L., Garnham, A., Lundie, R. J., Smyth, G. K., Pellegrini, M., O'Keeffe, M., Wicks, I. P., Masters, S. L., Hunter, C. P. and Pang, K. C. (2017). [SIDT2 transports extracellular dsRNA into the cytoplasm for innate immune recognition](#). *Immunity* 47(3): 498-509 e496.
8. Watanabe, A., Tatematsu, M., Saeki, K., Shibata, S., Shime, H., Yoshimura, A., Obuse, C., Seya, T. and Matsumoto, M. (2011). [Raftlin is involved in the nucleocapture complex to induce poly\(I:C\)-mediated TLR3 activation](#). *J Biol Chem* 286(12): 10702-10711.



The banner features the Bio-Protocol logo at the top left, followed by the tagline "Improve Research Reproducibility". Below this, a large heading reads "Free access to ~4000 high-quality protocols". A bulleted list highlights the platform's features: "Contributed by 10,000+ scientists (including Nobel Laureates)", "Validated in a primary research paper", ">91% reproducibility (survey of 2165 Bio-protocol users)", and "~1000 videos of key procedural steps". At the bottom, a call-to-action button says "Sign up at: www.bio-protocol.org".

- Contributed by 10,000+ scientists (including Nobel Laureates)
- Validated in a primary research paper
- >91% reproducibility (survey of 2165 Bio-protocol users)
- ~1000 videos of key procedural steps

Sign up at: [www.bio-protocol.org](http://www.bio-protocol.org)

## Quantification of Starch in Guard Cells of *Arabidopsis thaliana*

Sabrina Flütsch, Luca Distefano and Diana Santelia\*

Department of Plant and Microbial Biology, University of Zürich, Zürich, Switzerland

\*For correspondence: [dsantelia@botinst.uzh.ch](mailto:dsantelia@botinst.uzh.ch)



**[Abstract]** In this protocol, we describe how to quantify starch in guard cells of *Arabidopsis thaliana* using the fluorophore propidium iodide and confocal laser scanning microscopy. This simple method enables monitoring, with unprecedented resolution, the dynamics of starch in guard cells.

**Keywords:** Starch, Guard cells, *Arabidopsis*, Stomatal opening, Propidium iodide

**[Background]** Starch is a complex polymer of glucose and represents the most abundant form in which plants store carbohydrate. Starch serves different functions, according to the cell types from which it is derived, and the external environmental conditions. In guard cells, which border the stomatal pores that control water and carbon dioxide exchange with the environment, starch can be mobilized within minutes upon transition to light, helping to generate organic acids and sugars to increase guard cell turgor and promote stomatal opening. In mesophyll cells, starch typically accumulates gradually during the day and is degraded at night to support metabolism (Santelia and Lunn, 2017).

Because guard cells comprise only a minor fraction of the total leaf, it is difficult to measure starch quantitatively using conventional methods. Up until now, starch accumulation in guard cells has been mostly visualized by iodine staining. This technique can determine the presence/absence of starch but does not provide accurate, quantitative information.

Here, we describe a fluorescence-based imaging method to quantify starch in guard cells of *Arabidopsis thaliana*. This technique is based on the covalent labeling of cell wall material and other glucan substrates, including starch, with the fluorescent pseudo-Schiff reagent propidium iodide (PS-PI). Isolated epidermal peels are treated with periodic acid to oxidize the hydroxyl groups of the glucose units to aldehyde and ketone groups. The aldehyde groups (-CHO) can then react covalently with propidium iodide, resulting in samples with highly fluorescent glucans that are well suited for confocal laser scanning microscopy. The area of single starch granules within guard cell chloroplasts can be determined using digital imaging. We applied this method to assess the dynamics of starch content in guard cells of intact *Arabidopsis* leaves over the diurnal cycle, and to determine the impact of fusicoccin (a chemical activator of the proton pump) on starch amounts in guard cells of isolated epidermal peels fragments floating in stomatal opening buffer (Horrer *et al.*, 2016).

Our protocol is an adaptation of a previous mPS-PI staining technique (Truernit *et al.*, 2008). The original method was developed to image entire plant organs for three-dimensional reconstruction of their cellular organization. The main difference between the two methods is the incubation time with the propidium iodide solution, which is 1-2 h for entire plant organs, and only about 20-40 min for epidermal peels.

The technique described here is simple, accurate and highly reproducible. By facilitating the detailed quantification of starch amounts in guard cells, this method will increase the number of questions we will be able to answer about any aspect of guard cell starch metabolism.

### **Materials and reagents**

1. Pipette tips (SARSTEDT)
2. 12-well plate (Greiner Bio One International, catalog number: 665180)
3. Falcon tubes
4. Parafilm M (Bemis, catalog number: PM996)
5. Microscope slides (Thermo Fisher Scientific, Menzel-Gläser)
6. Coverslips (Thermo Fisher Scientific, Menzel-Gläser)
7. Kimtech Science precision wipes (KCWW, Kimberly-Clark, catalog number: 75512)
8. Square Petri dish (Greiner Bio One International, catalog number: 688102)
9. *Arabidopsis thaliana* ecotype Col-0
10. Methanol (Carl Roth, catalog number: 8388.4)
11. Ethanol (Reuss Chemie, catalog number: RC-A15-A)
12. Acetic acid
13. Periodic acid (Sigma-Aldrich, catalog number: P7875)
14. Sodium metabisulfite (Sigma-Aldrich, catalog number: S9000)
15. Hydrochloric acid (Carl Roth, catalog number: 4625.1)
16. Propidium iodide (Sigma-Aldrich, catalog number: 81845)
17. Chloral hydrate (Sigma-Aldrich, catalog number: 15307-R)
18. Glycerol (Carl Roth, catalog number: 3783.1)
19. Gum arabic (Carl Roth, catalog number: 4159.3)
20. Fixative solution (see Recipes)
21. Schiff Reagent (see Recipes)
22. Chloral hydrate solution (see Recipes)
23. Hoyer's solution (see Recipes)

### **Equipment**

1. Glass beaker
2. Precision tweezers (RubisTech, catalog number: 5-SA RT)
3. Pipettes (Gilson)
4. Fume hood (Renggli AG)
5. Refrigerator (Liebherr)
6. Oven (Ehret)
7. Green light LED lamp (In-house built)

8. Confocal laser scanning microscope (Leica Microsystems, model: Leica TCS SP5)  
*Note: This product has been discontinued. Any confocal laser scanning microscope can be used.*
9. Computer

## **Software**

1. ImageJ (NIH USA, version 1.8, <https://imagej.nih.gov/ij/>)

## **Procedure**

### A. Epidermal peel harvest from *Arabidopsis thaliana*

1. Label a 12-well plate according to the defined time points and genotypes, and add 1 ml of fixative solution (see Recipes) into each labeled well.
2. Collect epidermal peels from the abaxial side of the 5<sup>th</sup> or 6<sup>th</sup> leaf using precision tweezers (Figure 1A; Video 1). Usually, 4 replicates from 4 individual plants are harvested per time point and genotype. One well contains 4 epidermal peels. See Notes 1-3 for further details.



**Video 1. Collection of epidermal peels from the abaxial side of an *Arabidopsis* leaf.** This video shows the procedure for collecting epidermal peels from *Arabidopsis* leaves that are clean from mesophyll cells contamination.

3. Incubate the 12-well plate containing the collected epidermal peels for at least 12 h at 4 °C in the dark. The samples can be stored in fixative solution up to 4 weeks at 4 °C. The epidermal peels are fragile and require careful handling from now onwards. See Note 4.

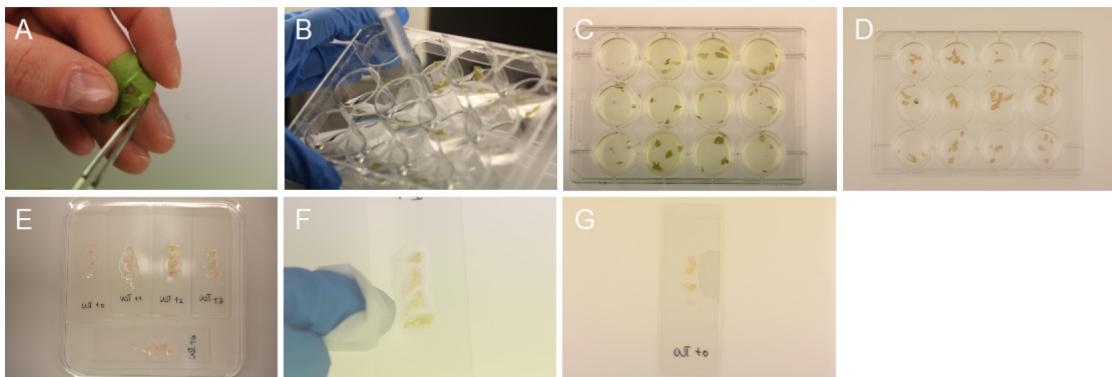
### B. mPS-PI staining

1. Perform Steps B2-B8 in a fume hood.

2. Remove the fixative solution from the wells using a P1000 pipette (Figure 1B). Collect the waste in a glass beaker and dispose it into the halogenated liquid waste at the end of the protocol.
3. Wash the samples by adding 1 ml of dH<sub>2</sub>O and slowly shaking the plate on the bench with circular movements. Remove the dH<sub>2</sub>O.
4. Destain the epidermal peels by adding 1 ml of 80% ethanol and incubating at 65 °C for 5-15 min (Figure 1C). Remove the ethanol and repeat washing step (Step B3).
5. Incubate the samples at room temperature (20 °C, RT) for 1 h in 1 ml of fixative solution. Remove the fixative solution and repeat washing step (Step B3).
6. Add 1 ml of 1% periodic acid solution to the epidermal peels and incubate the plate for 40 min at RT. Make sure to cover the peels fully with the solution. See Note 5. Remove the periodic acid solution and repeat washing step (Step B3). Proceed carefully as peels are extremely fragile after this step.
7. Stain the epidermal peels by adding 500 µl Schiff reagent (see Recipes) and 50 µl propidium iodide solution (1 mg·ml<sup>-1</sup>) for 20-40 min at RT. Make sure to cover the plant tissues fully with the solution. The samples should appear pinkish after this step (Figure 1D). Remove the propidium iodide solution and collect it separately in a Falcon tube. Propidium iodide waste should be treated like ethidium bromide waste.
8. Destain the samples in 1 ml of dH<sub>2</sub>O for 20-30 min at RT.

#### C. Microscope slide preparation

1. Label microscope slides according to the defined time points and genotypes.
2. Perform Steps C3-C4 and C6-C7 in a fume hood.
3. Add 70 µl of chloral hydrate solution (see Recipes) onto every microscope slide. Be careful to distribute the solution at the center of the slide to avoid leaking from the borders of the slide.
4. Carefully transfer the stained epidermal peels onto the microscope slide containing the chloral hydrate solution using precision tweezers. Place all four replicates next to each other (as shown in Figure 1E). Peels should be as flat as possible on the slide. Be careful; the peels will rupture easily.
5. Without placing a coverslip, transfer the microscope slides into square Petri dishes and incubate them at RT in the dark for 24 h.
6. Remove as much chloral hydrate solution as possible from the borders of the samples using Kimtech wipes (Figure 1F). Do not touch the samples with the wipes.
7. Place 2-3 drops of Hoyer's solution (see Recipes) onto the epidermal peels and add a coverslip. Apply gentle pressure on the coverslip to assure that the peels are flat and even on the microscope slide.
8. Transfer the slides back to the square Petri dishes and store them at RT in the dark for at least 3 days to allow the mountant to set (Figure 1G). See Note 6. The slides can be stored for several months in the dark.



**Figure 1. Harvest and staining of epidermal peels.** A. Epidermal peel from the abaxial side of the leaf; B. Removal of solutions; C. Destaining of epidermal peels; D. Pinkish plant tissue; E. Microscope slide containing chloral hydrate solution and epidermal peels; F. Removal of chloral hydrate solution; G. Final slide.

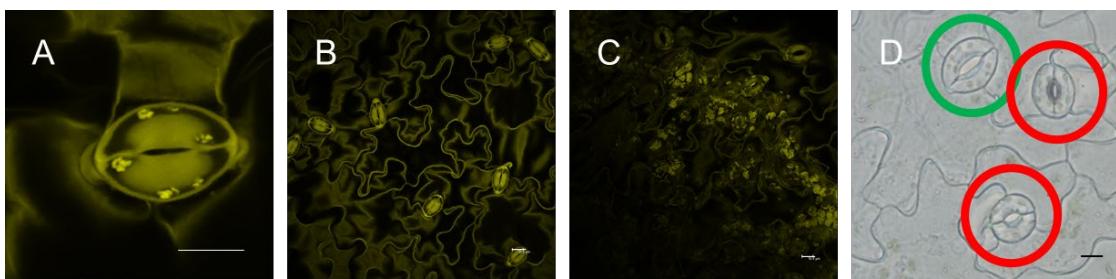
#### D. Imaging

1. Image acquisition is achieved with a Confocal Laser Scanning Microscope.
2. Set microscope as shown in Table 1.

**Table 1. Standard microscope settings for guard cell starch imaging**

Microscope settings	Standard values
Argon laser	5%
Objective	63x, glycerol
Excitation	488 nm
Detector	HyD3
Emission filter	610-640 nm
Format	1,024 x 1,024 pixel
Zoom	6x (Figure 2A)

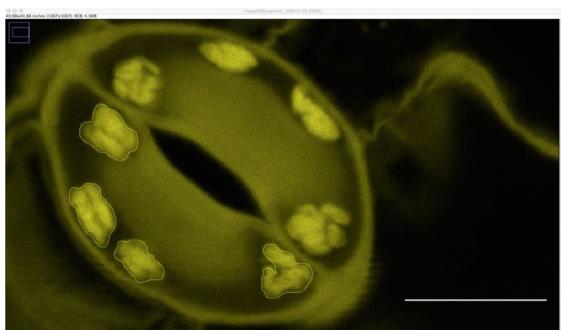
3. Acquire 20 images of individual stomata (Figure 2A; 5 images per replicate) per microscope slide. Acquire pictures of stomata only from mesophyll-free parts (Figures 2B and 2C) of the epidermal peels. Image stomata always with the same zoom factor (6x, Figure 2A) for data comparison. See Note 7.



**Figure 2. Imaging of guard cell starch granules.** A. Single stomata (scale bar = 10  $\mu\text{m}$ ); B. Mesophyll cell-free epidermal peel (scale bar = 10  $\mu\text{m}$ ); C. Mesophyll cell contamination of epidermal peel (scale bar = 10  $\mu\text{m}$ ); D. Fully (green circle) and not fully (red circle) developed stomata (scale bar = 10  $\mu\text{m}$ ).

### Data analysis

Starch granule area (in  $\mu\text{m}^2$ ) is determined using ImageJ version 1.8 (NIH USA, <http://rsbweb.nih.gov/ij/>) by encircling the granule area (Figure 3). A suitable scale is drawn onto the first picture using the software provided by the microscope manufacturer. Guard cell starch granule area is measured for each guard cell individually, totally collecting at least 40 values per time point and genotype. Starch content can then be expressed as an average of these values. Repeat the same experimental setup at least 3 times on independent plant material to obtain a final data set.



**Figure 3. Outlined guard cell starch granule area in ImageJ.** Scale bar = 5  $\mu\text{m}$ .

### Notes

1. The harvest time of epidermal peels per time point and genotype should not be longer than 1-2 min. In case of harvest of multiple genotypes, harvest one replicate per genotype. Harvest the additional replicates following the same sequence to ensure similar treatment of all genotypes.
2. In case of harvest in the dark (e.g., during the night), any green light source can be used to collect the epidermal peels.
3. To obtain a suitable epidermal peel for guard cell starch staining, it is important that a large fraction of the epidermal peel is mesophyll-free.

4. Make sure that the peels do not dry out while they are stored after the harvest and during the staining procedure.
5. Be careful in handling periodic acid as this compound is toxic.
6. The microscope slides are ready for imaging as soon as the coverslip is not movable anymore.
7. Acquire images only for fully developed stomata (Figure 2D).

## **Recipes**

1. Fixative solution

50% methanol

10% acetic acid

Make up to 500 ml with dH<sub>2</sub>O

*Note: Fixative solution can be stored for long-term usage at 4 °C.*

2. Schiff reagent

1.9 g sodium metabisulfite

3 ml 5 N HCl

97 ml dH<sub>2</sub>O

*Note: Store the Schiff reagent at 4 °C for up to 2 months.*

3. Chloral hydrate solution

40 g chloral hydrate

10 ml glycerol

20 ml dH<sub>2</sub>O

*Note: Store the chloral hydrate solution at 4 °C for long-term usage.*

4. Hoyer's solution

30 g gum arabic

200 g chloral hydrate

20 g glycerol

50 ml dH<sub>2</sub>O

*Note: Gum arabic is added to the water in a beaker with a magnet bar, which is placed on a shaker in a fume hood overnight to dissolve it. Chloral hydrate is then added in tiny amounts into the beaker using a funnel in a fume hood. Let it dissolve with shaking. Lastly, the glycerol is added. Allow the solution to set for a couple of days before use. Store the solution at RT in the dark. The solution stays turbid. Do not shake it.*

## **Acknowledgments**

This work was supported by the Swiss National Science Foundation (SNSF-Grant 31003A\_166539) and the ETH Zürich. The authors would like to acknowledge Mario Coiro for initial help in setting up the pseudo-Schiff propidium iodide staining method; Daniel Horrer and Diana Pazmino for adapting

the method to epidermal peels. This protocol is adapted from Truernit *et al.* (2008). The authors declare no conflicts of interests.

## **References**

1. Horrer, D., Flutsch, S., Pazmino, D., Matthews, J. S., Thalmann, M., Nigro, A., Leonhardt, N., Lawson, T. and Santelia, D. (2016). [Blue light induces a distinct starch degradation pathway in guard cells for stomatal opening](#). *Curr Biol* 26(3): 362-370.
2. Santelia, D. and Lunn, J. E. (2017). [Transitory starch metabolism in guard cells: unique features for a unique function](#). *Plant Physiol* 174(2): 539-549.
3. Truernit, E., Bauby, H., Dubreucq, B., Grandjean, O., Runions, J., Barthelemy, J. and Palauqui, J. C. (2008). [High-resolution whole-mount imaging of three-dimensional tissue organization and gene expression enables the study of phloem development and structure in \*Arabidopsis\*](#). *Plant Cell* 20(6): 1494-1503.

## Detection and Differentiation of Multiple Viral RNAs Using Branched DNA FISH Coupled to Confocal Microscopy and Flow Cytometry

Nicholas van Buuren and Karla Kirkegaard\*

Department of Genetics, Stanford University School of Medicine, Stanford, CA, 94305, USA

\*For correspondence: [karlak@stanford.edu](mailto:karlak@stanford.edu)



**[Abstract]** Due to the exceptionally high mutation rates of RNA-dependent RNA polymerases, infectious RNA viruses generate extensive sequence diversity, leading to some of the lowest barriers to the development of antiviral drug resistance in the microbial world. We have previously discovered that higher barriers to the development of drug resistance can be achieved through dominant suppression of drug-resistant viruses by their drug-susceptible parents. We have explored the existence of dominant drug targets in poliovirus, dengue virus and hepatitis C virus (HCV). The low replication capacity of HCV required the development of novel strategies for identifying cells co-infected with drug-susceptible and drug-resistant strains. To monitor co-infected cell populations, we generated codon-altered versions of the JFH1 strain of HCV. Then, we could differentiate the codon-altered and wild-type strains using a novel type of RNA fluorescent *in situ* hybridization (FISH) coupled with flow cytometry or confocal microscopy. Both of these techniques can be used in conjunction with standard antibody-protein detection methods. Here, we describe a detailed protocol for both RNA FISH flow cytometry and confocal microscopy.

**Keywords:** RNA flow cytometry, RNA FISH, Branched DNAs, HCV, Drug Resistance, Genetic selection, Viral evolution

**[Background]** The barriers to development of antiviral drug resistance vary greatly depending on the compound used and the host or viral target chosen. RNA viruses have particularly low genetic barriers to the development of drug resistance as their polymerases have error rates as high as  $10^{-4}$  to  $10^{-5}$  misincorporations per nucleotide synthesized. This leads to exceptionally high genetic variability amongst progeny. However, the high level of diversity observed in RNA virus progeny does not always lead to high rates of genetic selection for progeny with increased fitness. This is often due to genetic dominance of drug-susceptible viruses that are present in the same cell as newly synthesized drug-resistant variants. Drug-resistant viral RNA must first be amplified and translated in its cell of origin, making newly synthesized drug-resistant viruses susceptible to dominant suppression by their drug-susceptible parents and cousins. We have coined the term “dominant drug targets” to describe viral targets with higher barriers to the development of antiviral drug resistance due to genetic dominance of drug-susceptible viruses. Study of genetic interactions and physical location of distinct viral genomes in the same cell required the development of the new technology described here.

To identify dominant drug targets for which tool antiviral compounds are available, we first generated drug-resistant viruses and built the mutations into an infectious cDNA clone. To test whether

drug-resistant or drug-susceptible viruses were genetically dominant, we generated cells co-infected with drug-susceptible and drug-resistant viruses and then monitored selection from within them. In studies using poliovirus (Crowder and Kirkegaard, 2005; Tanner *et al.*, 2014) and Dengue virus (Mateo *et al.*, 2015) we were able to generate sufficiently high-titer virus stocks to perform coinfections at high multiplicities of infection and thus ensure that all cells in our cultures were coinfecte. Recently, we expanded tests for dominance to hepatitis C virus (HCV) (van Buuren *et al.*, 2018), for which high-titer stocks are often difficult to obtain, especially for drug-resistant variants that have reduced fitness. Therefore, when we co-infected Huh7.5.1 cells with two strains of HCV at multiplicities of infection of less than 1 PFU/cell, we generated four cell populations: co-infected cells, two types of singly infected cells and a significant population of uninfected cells. We needed to differentiate co-infected cells from the two types of singly infected cells and learn about genetic selection while doing so. To accomplish this, we were early adopters of the branched DNA (bDNA) technology originally developed by Affymetrix (now Thermo Fisher Scientific). This technology uses tiered DNA oligos to build a network of up to 8,000 fluorophores on each target RNA. This unique type of RNA fluorescent *in situ* hybridization (FISH) can be coupled with protein detection using standard antibody conjugation and detected using confocal microscopy (ViewRNA® Cell Plus Assay) and flow cytometry (PrimeFlow™ RNA Assay).

These bDNA FISH techniques first generate a series of target probes that bind the RNA of interest at adjacent sequences, but leave 3' extensions of unique sequence to bind the pre-amplifier DNA that is complementary to two different probes. Cooperative binding of the pre-amplifier DNA to two probes increases the signal-to-noise ratio because any individual mistargeted probe cannot be amplified. Typically, twenty pairs of target probes are designed to bind the RNA of interest; this requires roughly 1,000 nucleotides of sequence space. Each of the twenty pre-amplifier DNAs is then bound by a series of amplifier DNAs, and then subsequently by a series of oligonucleotide-conjugated fluorophores. This process leads to the labeling of each individual target RNA with up to 8,000 fluorophores, sufficient to visualize individual RNAs by confocal microscopy. The PrimeFlow RNA Assay and ViewRNA Cell Plus Assay kits allow for simultaneous detection of three target RNAs. The available fluorophores for PrimeFlow are Alexa Fluor® 488, Alexa Fluor® 647 and Alexa Fluor® 750 and for ViewRNA are Alexa Fluor® 488, Alexa Fluor® 546 and Alexa Fluor® 647.

To apply this technology to dominant drug targeting in HCV, we needed to generate a strain of HCV with sufficient dissimilarity in its RNA sequence that we could differentiate it from wild-type viral RNA. To accomplish this, we generated three codon-altered versions of the JFH1 strain of HCV. Codon optimization algorithms available through GeneArt (Thermo Fisher Scientific) were used to design three approximately 1,000-nucleotide regions of the JFH1 genome that had altered codon usage but retained the same protein sequence. These codon-altered JFH1 strains all contained 200-250 synonymous mutations over the 1,000-nucleotide regions. Of these three strains, two demonstrated decreased fitness, likely due to disruption of RNA secondary structures required for viral replication (Pirakitikulr *et al.*, 2016). The third strain, however, displayed growth kinetics that mimicked wild-type virus and could be used in co-infection experiments and differentiated from wild-type JFH1 using both RNA FISH and flow cytometry.

## **Materials and Reagents**

1. Pipette tips (with or without filter tips)
2. Micro slides (VWR, catalog number: 48311-702)
3. Micro cover glass (VWR, catalog number: 48380-046)
4. GenePulser cuvettes, 4 mm (Bio-Rad Laboratories, catalog number: 1652088)
5. BD FACS tubes (BD Falcon, catalog number: 352054)
6. 12-well cell culture dish (e.g., Corning, Costar, catalog number: 3513)
7. 10 cm tissue culture dish (e.g., Corning, catalog number: 430167)
8. T150 tissue culture flask (e.g., Corning, catalog number: 430825)
9. 15 ml conical centrifuge tube (e.g., AccuFlow, catalog number: EK-4020)
10. 500 ml Rapid-Flow Filter Unit, 0.2 µm (Thermo Fisher Scientific, catalog number: 566-0020)
11. Huh7.5.1 cells (Gift from Dr. Michael Gale Jr., University of Washington)
12. PrimeFlow™ RNA Assay Kit (Thermo Fisher Scientific, catalog number: 88-18005-210) contains:
  - a. Flow Cytometry Staining Buffer
  - b. Fixation Buffer 1
  - c. Permeabilization Buffer with RNase Inhibitors
  - d. Fixation Buffer 2
  - e. Wash Buffer
  - f. Target Probe Diluent
  - g. PreAmp Mix
  - h. Amp Mix
  - i. Label Probe Diluent
  - j. 100x Label Probes
13. ViewRNA® Cell Plus Assay Kit (Thermo Fisher Scientific, catalog number: 88-19000) contains:
  - a. Fixation/Permeabilization Buffer
  - b. Blocking/Antibody Diluent
  - c. Fixative
  - d. Probe Set Diluent
  - e. Amplifier Diluent along with Pre-Amplifiers and Amplifiers
  - f. Label Probe Diluent and Label Probes
  - g. Wash Buffer
  - h. PBS
  - i. DAPI
14. Target Probes (Thermo Fisher Scientific)
  - a. Wild-type JFH1 (VF1-14301)
  - b. Codon altered JFH1 (VF4-6000723)
15. Permafluor Mounting Reagent (Thermo Fisher Scientific, catalog number: TA-030-FM)

16. 0.05% Trypsin-EDTA (Thermo Fisher Scientific, Gibco, catalog number: 25300-054)
17. XbaI and CutSmart Buffer (New England Biolabs, catalog number: R0145L)
18. MEGAscript T7 Kit (Thermo Fisher Scientific, Invitrogen, catalog number: AMB1334-5)
19. Trizol® Reagent (Thermo Fisher Scientific, Ambion, catalog number: 15596018)
20. QIAquick PCR Purification Kit (QIAGEN, catalog number: 28106)
21. Human AB Serum (Omega, catalog number: HS-20)
22. Pen/Strep (Thermo Fisher Scientific, catalog number: 15140-122)
23. Glutamax (Thermo Fisher Scientific, catalog number: 35050-061)
24. Non-essential amino acids (Thermo Fisher Scientific, catalog number: 11140-050)
25. DMEM (GE Healthcare, Hyclone, catalog number: SH30243.01)
26. Fetal bovine serum (Omega, catalog number: FB-22)
27. KCl
28. CaCl<sub>2</sub>
29. K<sub>2</sub>HPO<sub>4</sub>
30. HEPES
31. EDTA
32. MgCl<sub>2</sub>
33. Human serum media (see Recipes)
34. 10% FBS media (see Recipes)
35. CytoMix (see Recipes)

## **Equipment**

1. Pipettes (with or without filter tips)
2. Ultrafine forceps (e.g., Excelta, catalog number: 5-SN)
3. Modified BD FACScan (Scanford) or LSRII Flow Cytometer
4. Bio-Rad GenePulser XCell
5. Biosafety Cabinet (BSC)
6. Incubator (VWR, model: Model 1565)
7. Heat Block (e.g., Anodized Aluminum, see Figure 2)
8. Leica SP8 Confocal Microscope (Leica Microsystems, model: Leica TCS SP8)
9. Sorvall Centrifuge (e.g., Thermo Fisher Scientific, model: Legend RT plus)
10. Heracell 150i CO<sub>2</sub> Incubator (Thermo Fisher Scientific, model: Heracell™ 150i)
11. -20 °C Freezer
12. Refrigerator

## **Software**

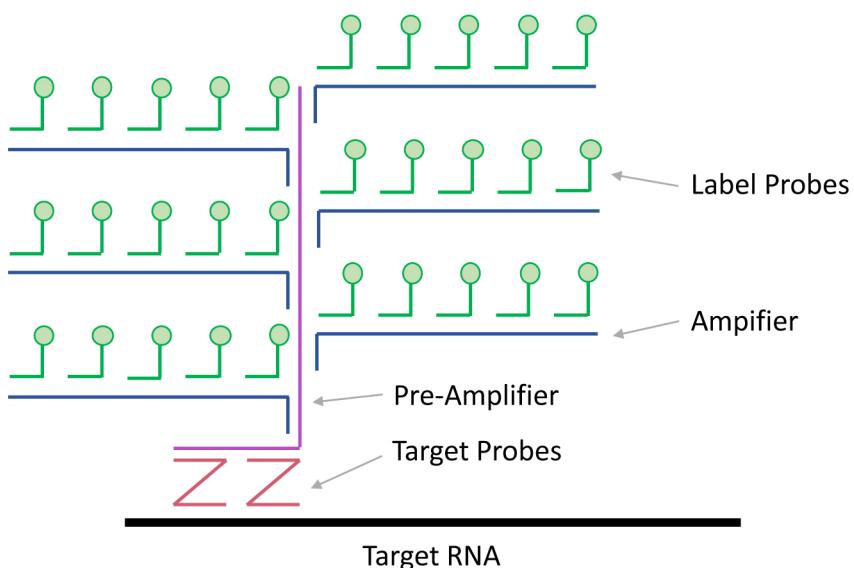
1. FlowJo® v10.0

2. Velocity v6.0 (PerkinElmer)
3. Adobe Photoshop vCS4
4. GraphPad Prism v7.0
5. Microsoft Excel v16.0

## **Procedure**

### A. Construction of codon altered sequences

1. Roughly 1,000 nucleotides of RNA sequence are required to support the hybridization of twenty bDNA trees and 8,000 fluorophores. Targets that contain less than the full complement of bDNAs can still be detected by flow cytometry but require higher copy numbers to achieve the same resolution.
2. For viral RNAs, when possible, scan the literature for any structural information available to determine which areas of the genome are the least likely to contain essential RNA secondary structures. If possible, also choose a region that has convenient cut sites for insertion of your codon-altered sequence. We chose to clone three codon-altered regions of the JFH1 genome because we anticipated decreased viability from some of the codon-altered strains.
3. GeneArt is a product offered through Thermo Fisher Scientific and can be used to synthesize genes up to 9,000 bp in length (<https://www.thermofisher.com/us/en/home/life-science/cloning/gene-synthesis/geneart-gene-synthesis.html>). The GeneArt homepage offers several tools, including the gene optimizer tool. Use the gene optimizer algorithms to design codon-altered sequences with wild-type viral RNA sequence as your template. We submitted three regions of JFH1 that were all roughly 1,000 nucleotides in length and flanked by convenient cut sites. The optimizer tool was able to alter nearly 25% of nucleotides in all three cases.
4. The newly synthesized sequence will arrive incorporated into a plasmid with a defined antibiotic-resistance marker. At this time, your codon-altered gene fragment can be subcloned into a plasmid that encodes the viral genome using restriction digestion and ligation.
5. Target Probes that differentiated viral RNA sequences were designed and manufactured by Affymetrix (now Thermo Fisher Scientific) for use with both the ViewRNA and PrimeFlow platforms (Figure 1).



**Figure 1. Detection of target RNAs using branched DNA technology.** Branched DNA technology for RNA detection can be coupled with confocal microscopy or flow cytometry. Target RNAs are first bound by pairs of Target Probes. Typically, twenty sets of target probe pairs are designed per target RNA. The Pre-Amplifier DNA only binds target probe pairs that are bound to target RNAs in the correct orientation; this greatly limits the signal to noise ratio. Pre-Amplifier DNAs are then bound by Amplifier DNAs and subsequently by Label Probes. This process results in the labeling of target RNAs by up to 8,000 fluorophores.

#### B. Collection of codon-altered JFH1 virus stocks

1. The pJFH1 plasmid encodes the full-length genome of the JFH1 strain of HCV. The wild-type plasmid and all codon-altered versions contain an XbaI cut site at the 3' end of the genome. Digest 5 µg of plasmid DNA with 20 U of XbaI in the CutSmart Buffer provided in a final reaction volume of 25 µl. Incubate digestions at 37 °C for 2 h.
2. Purify linearized DNA using the QIAquick PCR Purification Kit, as per manufacturer's protocol.
3. Using 1 µg of linearized plasmid as your template, perform *in vitro* transcription with the MEGAscript T7 kit to make full-length genomic viral RNA. Incubate *in vitro* transcription reaction at 37 °C for 6 h. The temperature and duration of this incubation can be altered for optimal yield of individual transcripts.
4. Isolate synthesized viral RNA using Trizol as per the manufacturer's protocol. Resuspend vRNA pellet in 50 µl of RNase-free water.
5. Seed 10<sup>7</sup> Huh7.5.1 cells into a 10 cm tissue culture plate and incubate overnight.
6. To electroporate 10 µg vRNA into 10<sup>7</sup> Huh7.5.1 cells to produce continuous HCV cultures:
  - a. Wash Huh7.5.1 cells with 5 ml PBS.
  - b. Add 2 ml of Trypsin and incubate at 37 °C for 5 min.
  - c. Add 5 ml of 10% FBS media and harvest cell suspension into a 15 ml conical tube.

- d. Centrifuge cells at  $400 \times g$  for 4 min.
  - e. Resuspend cell pellet in 5 ml of PBS.
  - f. Centrifuge cells at  $400 \times g$  for 4 min.
  - g. Resuspend cell pellet with 5 ml of CytoMix. Cytomix recipe can be found below under "Recipes".
  - h. Centrifuge cells at  $400 \times g$  for 4 min.
  - i. Resuspend cell pellet in 400  $\mu l$  of CytoMix and transfer to a 4 mm GenePulser cuvette.
  - j. Mix 10  $\mu g$  of viral RNA into cell suspension inside cuvette and gently pipet up and down to mix.
  - k. Electroporate RNA-cell mixture using the Bio-Rad GenePulser XCell. Settings set to 950  $\mu F$  capacitance, 270 V,  $\infty$  resistance and 4 mm cuvette size.
  - l. Allow cells to rest at room temperature for 10 min.
  - m. Transfer electroporated cells to a fresh 10 cm culture dish with 10 ml of 10% FBS media (see Recipes).
7. Culture electroporated cells for up to two weeks in 10% FBS media, passaging every 3-4 days as required. As you passage, expand the culture. Typically, cultures of  $10^7$  electroporated cells are expanded into either five or ten T150 flasks. This gives HCV time to spread and generates a culture with a higher percentage of cells that are infected and productively synthesizing progeny virus. Further expansion of cells to larger capacity can be done if needed.
  8. Convert JFH1 cultures to Human Serum Media (Steenbergen *et al.*, 2013). Growth of HCV in human serum has two benefits. First, Huh7.5.1 cells differentiate and cease cell division, therefore trypsinization and biweekly passage are no longer required. Instead, virus containing cell supernatants can simply be collected biweekly and directly replaced with fresh medium. Second, growth in Human Serum Media increases viral yield by 10 to 100-fold.
- C. Simultaneous infection with two HCV strains and detection of co-infected cells with Prime-Flow.
1. The description of this protocol has been adapted from the PrimeFlow Assay user's manual.
  2. Huh7.5.1 cells are seeded into 12-well plates at a density of  $10^5$  cells per well using 1 ml of 10% FBS media.
  3. In our hands, JFH1 cultured in human serum media can produce viral titers of  $10^5$ - $10^6$  focus forming units (FFU) per ml. Infect Huh7.5.1 cells at a multiplicity of infection of one virus particle per cell with both wild-type and codon-altered JFH1. This often equates to roughly 1-2 ml of each virus preparation. A total volume of 4 ml can be used carefully in 12-well plates.
  4. Incubate infected cells in a CO<sub>2</sub> incubator at 37 °C for 4-6 h. Following initial incubation, remove virus-containing media by aspiration. Replace media with fresh 10% FBS media and incubate infected cells for 72 h.
  5. Replace 10% FBS media with fresh 10% FBS media that either contains antiviral drugs or vehicle and incubate infected cells for 24-36 h.
  6. Aspirate off media containing antivirals or vehicle and wash cells with 1 ml PBS.

7. Harvest infected cells by treating cells with 0.5 ml trypsin and incubating at 37 °C with CO<sub>2</sub> for 5 min.
8. Inhibit trypsin by adding 1 ml of 10% FBS media to each well. Harvest all cells and transfer to one of the 1.5 ml microfuge tubes supplied in the PrimeFlow Assay kit.
9. Spin cells at 400 x g for 5 min.
10. Aspirate off media and trypsin, being careful not to lose any cells. This is achieved by only aspirating down to the 100 µl marker on the side of the Eppendorf tube. Wash cells with 1 ml of Flow Cytometry Staining Buffer. Vortex and spin at 400 x g for 5 min.
11. Aspirate Flow Cytometry Staining Buffer and fix cells using 1 ml of Fixation Buffer 1 at 4 °C for 30 min.
12. Spin cells at 800 x g for 5 min.
13. Resuspend cells in 1 ml of Permeabilization Buffer. Spin cells at 800 x g for 5 min. Repeat wash with Permeabilization Buffer 3 x.
14. Aspirate final Permeabilization Buffer wash and resuspend cells in 1 ml of Fixation Buffer 2. Incubate cells in the dark at room temperature for 60 min.
15. Spin cells at 800 x g for 5 min and resuspend in 1 ml of Wash Buffer.
16. Repeat wash step.
17. Dilute Target Probes in Target Probe Diluent at 1:20.
18. Resuspend cells in 100 µl of the Target Probe mixture. Incubate at 40 ± 1 °C for 2 h. We use a heat block in our 40 °C incubator to increase heat conduction to the tubes and protect from large fluctuation in heat (Figure 2). This incubation can be extended from 2 h to overnight. Longer incubations periods allowed for all amplification steps, flow cytometry and data analysis to be completed the following day.



**Figure 2.  $40 \pm 1$  °C incubator setup.** Two heat blocks are stored in the incubator to regulate the temperature of RNA FISH flow cytometry samples. A thermometer is kept inside to confirm the digital temperature readings.

19. Wash cells by adding 1 ml of Wash Buffer, vortex, and spin at  $800 \times g$  for 5 min.
  20. Repeat wash step.
  21. Resuspend cells in 100  $\mu l$  of PreAmp Mix. Incubate at  $40 \pm 1$  °C for 1.5 h.
  22. Wash cells by adding 1 ml of Wash Buffer, vortex, and spin at  $800 \times g$  for 5 min.
  23. Repeat wash step.
  24. Resuspend cells in 100  $\mu l$  of Amp Mix. Incubate at  $40 \pm 1$  °C for 1.5 h.
  25. Wash cells by adding 1 ml of Wash Buffer, vortex, and spin at  $800 \times g$  for 5 min.
  26. Repeat wash step.
  27. Prepare Label Probe mix by diluting Label Probes into the Label Probe Diluent at 1:100.
  28. Resuspend cells in 100  $\mu l$  of Label Probe mix. Incubate at  $40 \pm 1$  °C for 1 h.
  29. Wash cells by adding 1 ml of Wash Buffer, vortex, and spin at  $800 \times g$  for 5 min.
  30. Repeat wash step.
  31. Aspirate Wash Buffer leaving 100  $\mu l$  of residual liquid to resuspend stained cells. Resuspend cells by pipetting up and down and transfer to a labeled BD FACS tube containing 250  $\mu l$  of PBS.
  32. Analyze cells using a flow cytometer and FlowJo software (details below).
- D. Quantitation of RNA-protein colocalization using confocal microscopy.
1. Huh7.5.1 cells are plated on Micro Cover Glass inside 12-well tissue culture plates at a density of  $10^5$  cells per well one day prior to infection (Figure 3).



**Figure 3. Reagents setup for confocal microscopy.** Huh7.5.1 cells are plated onto Micro Cover Glass within a 12-well tissue culture plate. These cells are infected following a 24 h incubation to allow cell adherence to the glass. Following infection, the cells are fixed, stained for protein and RNA using the ViewRNA Cell Plus Assay kit, all within the 12-well plate. The Micro Cover Glass is then carefully transferred to a Microslide spotted with PermaFluor/DAPI using fine forceps.

2. Co-infect cells with wild type JFH1 and codon altered JFH1 at a multiplicity of infection equal to one virus per cell.
3. At 6 h post infection, aspirate inoculum and replace with 1 ml of 10% FBS media.
4. At 24 h post infection, wash cells 2 x with 1 ml of PBS.
5. Add 400  $\mu$ l of Fixation/Permeabilization Buffer to each well and incubate for 30 min at room temperature.
6. Wash cells 3 x each with 800  $\mu$ l of PBS.
7. Overlay cells with 400  $\mu$ l of Blocking/Antibody Diluent and incubate at room temperature for 20 min.
8. Dilute primary antibody in 400  $\mu$ l Blocking/Antibody Diluent as required. Overlay cells with antibody mixture and incubate at room temperature for 1 h.
9. Wash cells three times with PBS.
10. Dilute secondary antibody in 400  $\mu$ l Blocking/Antibody Diluent as required. We use anti-mouse AlexaFluor-647 diluted at 1:200 for our experiment with HCV. Overlay cells with antibody mixture and incubate at room temperature for 1 h.
11. Wash cells 3 x with PBS.
12. Add 400  $\mu$ l of Fixation Solution to each well and incubate in the dark at room temperature for 1 h.
13. Wash cells 3 x with PBS.
14. Dilute Target Probes 1:100 in Target Probe Diluent.
15. After the final wash, overlay cells with 400  $\mu$ l of Target Probe mixture. Incubate at  $40 \pm 1$  °C for 2 h.
16. Wash cells 3 x with 800  $\mu$ l Wash Buffer at room temperature.
17. Dilute Pre-Amplifiers 1:25 in Amplifier Diluent.
18. After the final wash, overlay cells with 400  $\mu$ l of Pre-Amplifier mixture and incubate at  $40 \pm 1$  °C for 1 h.
19. Wash cells 3 x with 800  $\mu$ l Wash Buffer at room temperature.
20. Dilute Amplifiers 1:25 in Amplifier Diluent.
21. After the final wash, overlay cells with 400  $\mu$ l of Amplifier mixture and incubate at  $40 \pm 1$  °C for 1 h.
22. Wash cells 3 x with 800  $\mu$ l Wash Buffer at room temperature.
23. Dilute Label Probes 1:100 in Label Probe Diluent.
24. After the final wash, overlay cells with 400  $\mu$ l of Label Probe mixture and incubate at  $40 \pm 1$  °C for 1 h.
25. Wash cells 3 x with 800  $\mu$ l Wash Buffer at room temperature.
26. Dilute DAPI 1:100 in Permafluor mounting reagent.
27. Spot 12.5  $\mu$ l of Permafluor/DAPI mixture onto a Micro Slide.
28. Using forceps, carefully remove stained Micro Cover Glass from the 12-well dish, dab on a

Kimwipe to remove excess Wash Buffer and place “cells down” onto the drop of Permafluor/DAPI. Allow to harden for at least 4 h.

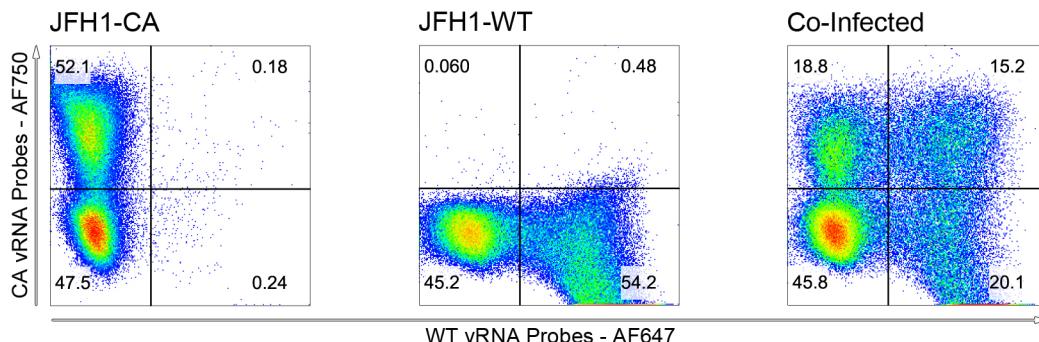
29. Visualize cells using a confocal microscope. We use a Leica SP8 Confocal Microscope fitted with a White Light Laser.

## Data analysis

### **Flow cytometry**

We analyze all flow cytometry data using FlowJo software. Data are exported from the flow cytometer as individual .fcs files for each sample as well as a .wsp file for the entire experiment. We use FlowJo to open the .wsp file and can then access all .fcs files in the same analysis window. Once files are open in FlowJo data analysis proceeds as follows:

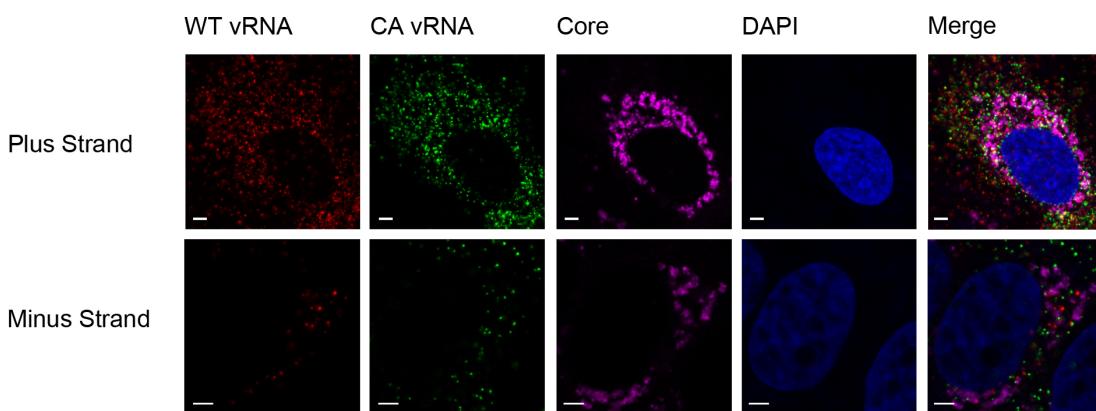
1. Open your first sample and select forward scatter versus side scatter to view cells collected. Draw a gate around the healthy cells only so that any debris or dead cells are not included in your analysis.
2. Within your healthy cell subgate, plot the two viral RNA fluorophores against one another. In our case this was typically Alexa Fluor 488 versus Alexa Fluor 750 which did not require compensation. If you are working with Alexa Fluor 657 and Alexa Fluor 750 you will need to run the compensation algorithm within FlowJo before further analysis.
3. Once data are plotted and compensated, if needed, reset the axes to biexponential (Biex) which minimizes the uninfected cells and emphasizes the viral RNA-positive populations for clearer resolution.
4. Draw quadrants that divide uninfected cells from the two singly infected cells and coinfecte cells.
5. The percentages from each population will be used to determine the dominance relationships between viral species. In the absence of drug, four cell populations will be visible. In the presence of drug, the cells singly infected with drug-susceptible virus will become uninfected and shift into the lower left quadrant. The singly infected drug-resistant virus will persist. The genetic outcome of the co-infected cells will determine their fate (Figure 4).



**Figure 4. Identification of co-infected cells by PrimeFlow RNA FISH.** Huh7.5.1 cells were infected with JFH1-CA, JFH1-WT or co-infected at multiplicities of infection equal to one virus per cell with each virus. Infected cells were incubated for 72 h before labeling viral RNAs using PrimeFlow. Analysis and compensation was performed using FlowJo.

#### Confocal microscopy

1. The Leica SP8 creates a file containing all images as a .lif file. The individual channels are exported as individual .tif files for image processing and figure construction (Figure 5).



**Figure 5. Analysis of viral RNA-protein colocalization using ViewRNA Cell Plus.** Huh7.5.1 cells were co-infected with JFH1-WT and JFH1-CA on Micro Cover Glass for 72 h. Cells were stained for HCV core protein and both viral RNAs using the ViewRNA Cell Plus Assay. Quantification of colocalization was performed using Volocity software. Scale bars are 2.5  $\mu\text{m}$  in length.

2. The .lif file can also be opened using Volocity software created by PerkinElmer.
3. Volocity has a spot-counting algorithm to determine how many puncta exist within each channel. We define a single punctum as larger than  $0.1 \mu\text{m}^2$  and smaller than  $0.25 \mu\text{m}^2$ , and ask Volocity to break larger spots into individual units. Confirm that your size range is appropriate by giving a few cells an eye test. Does the number of puncta counted appear to be the same number that you can count by eye? You may need to adjust your maximum and minimum punctum sizes based on this test.
4. We then ask Volocity to determine colocalization by counting how many spots on our Red channel shared at least  $0.05 \mu\text{m}^2$  of “Mutual Space” with puncta the Green channel. The result is plotted as the total number of puncta that share mutual space between channels versus the total number of puncta in each channel.
5. Determining colocalization between RNA and protein requires a separate algorithm as the proteins often not localize into discrete countable puncta. We therefore ask Volocity to determine how many of the viral RNA puncta “Touch” anywhere within the protein signal. We then graph the number of RNA puncta that touched protein versus the total number of RNA

puncta in each cell.

## **Notes**

1. We prefer to use the swinging bucket Sorvall centrifuge for all spins for flow cytometry as the cell pellet accumulates at the bottom of the PrimeFlow assay kit-supplied microfuge tubes, which limits cell loss during the multiple-step procedure. However, it is possible to complete the protocol and limit cell loss using a traditional bench top, fixed-angle centrifuge, with careful supernatant removal.
2. Simultaneous analysis of the Alexa Fluor 647 and Alexa Fluor 750 channels requires a high degree of compensation. To identify double-positive cells unambiguously, use Alexa Fluor 488 in combination with either of the other two channels.
3. As PrimeFlow is often coupled with antibody staining, it should be noted that not all fluorophores survive the RNA staining protocol. Specifically, all PerCP fluorophores will be inactivated by this technique and should be avoided in panel design.
4. Coverglass slips are very delicate and can easily break as they are being lifted out of the 12-well plate and placed onto the microslide. Using anything other than fine forceps makes this challenging. New students in our lab are encouraged to practice this technique using blank Micro Cover Glass in PBS prior to attempting a real experiment.

## **Recipes**

1. Human serum media  
2% human AB serum  
1x Pen/Strep  
1x glutamax  
1x non-essential amino acids  
DMEM
2. 10% FBS media  
10% fetal bovine serum  
1x Pen/Strep  
1x glutamax  
1x non-essential amino acids  
DMEM
3. CytoMix  
120 mM KCl  
0.15 mM CaCl<sub>2</sub>  
10 mM K<sub>2</sub>HPO<sub>4</sub>  
25 mM HEPES

2 mM EDTA  
5 mM MgCl<sub>2</sub>  
Adjust pH to 7.6  
Filter through a 0.2 µm Rapid-Flow Filter Unit

### **Acknowledgments**

We thank Drs. Yury Goltsev and Garry Nolan for advice on fluorescent cell sorting-based visualization of RNA, Affymetrix for the design and manufacturing of custom viral RNA probes, and Drs. Michael Gale Jr. and Ralf Bartenschlager for the generous donation of reagents.

This work was supported by funding to KK from NIH U19AI109662 (Jeffrey Glenn, P.I.), an NIH Directors Pioneer Award and the Alison and Steve Krausz Innovation Fund. NvB was supported by the Canadian Institutes for Health Research NC RTP-HepC training program and the American Liver Foundation. The Cell Sciences Imaging Facility used for confocal microscopy was supported by ARRA award number 1S10OD010580 from the NCRR. The contents of this manuscript are solely the responsibility of the authors and do not necessarily represent the official views of the NCRR or the National Institutes of Health.

### **Competing interests**

The authors have no conflicts of interest or competing interests.

### **References**

1. Crowder, S. and Kirkegaard, K. (2005). [Trans-dominant inhibition of RNA viral replication can slow growth of drug-resistant viruses](#). *Nat Genet* 37(7): 701-709.
2. Mateo, R., Nagamine, C. M. and Kirkegaard, K. (2015). [Suppression of drug resistance in dengue virus](#). *MBio* 6(6): e01960-01915.
3. Pirakitkulr, N., Kohlway, A., Lindenbach, B. D. and Pyle, A. M. (2016). [The coding region of the HCV genome contains a network of regulatory RNA structures](#). *Mol Cell* 62(1): 111-120.
4. Steenbergen, R. H., Joyce, M. A., Thomas, B. S., Jones, D., Law, J., Russell, R., Houghton, M. and Tyrrell, D. L. (2013). [Human serum leads to differentiation of human hepatoma cells, restoration of very-low-density lipoprotein secretion, and a 1000-fold increase in HCV Japanese fulminant hepatitis type 1 titers](#). *Hepatology* 58(6): 1907-1917.
5. Tanner, E. J., Liu, H. M., Oberste, M. S., Pallansch, M., Collett, M. S. and Kirkegaard, K. (2014). [Dominant drug targets suppress the emergence of antiviral resistance](#). *Elife* 3: e03803.
6. van Buuren, N., Tellinghuisen, T. L., Richardson, C. D. and Kirkegaard, K. (2018). [Transmission genetics of drug-resistant hepatitis C virus](#). *Elife* 7: e32579.

## Single-molecule Fluorescence *in situ* Hybridization (smFISH) for RNA Detection in Adherent Animal Cells

Gal Haimovich\* and Jeffrey E. Gerst

Dept. of Molecular Genetics, Weizmann Institute of Science, Rehovot 7610001, Israel

\*For correspondence: [gal.haimovich@weizmann.ac.il](mailto:gal.haimovich@weizmann.ac.il)



**[Abstract]** Transcription and RNA decay play critical roles in the process of gene expression and the ability to accurately measure cellular mRNA levels is essential for understanding this regulation. Here, we describe a single-molecule fluorescent *in situ* hybridization (smFISH) method (as performed in Haimovich *et al.*, 2017) that detects single RNA molecules in individual cells. This technique employs multiple single-stranded, fluorescent labeled, short DNA probes that hybridize to target RNAs in fixed cells, allowing for both the quantification and localization of cytoplasmic and nuclear RNAs at the single-cell level and single-molecule resolution. Analyzing smFISH data provides absolute quantitative data of the number of cytoplasmic (“mature”) mRNAs, the number of nascent RNA molecules at distinct transcription sites, and the spatial localization of these RNAs in the cytoplasm and/or nucleoplasm.

**Keywords:** mRNA, Transcription, Fluorescence *in situ* hybridization, Single molecule resolution, Fluorescence microscopy, Adherent cells

**[Background]** Regulation of gene expression is one of the key determinants of cell fate and behavior. A major parameter of gene expression is mRNA level, which is determined by the rates of transcription and degradation. Therefore, measuring mRNA levels, as well as transcription and decay rates for particular transcripts (or all transcripts) has been the focus of numerous research projects.

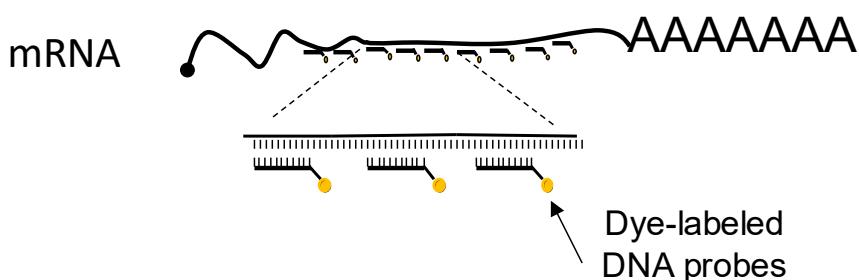
Common molecular biology techniques, such as reverse transcription-PCR (RT-PCR), Northern blot analysis or RNA sequencing (RNA-Seq), typically require RNA extraction from the entire cell population. However, the results provide only a relative measure of mRNA content for the entire cell population, with a loss of single cell information. Single-cell RNA-Seq can provide more insight on the cell-to-cell variability of transcript levels. However, the current lower limit of detection is ~10 molecules/cell for a given RNA transcript (Svensson *et al.*, 2017). RNA localization studies have shown that the spatial distribution of RNA in the cell can play a pivotal role in its function (Buxbaum *et al.*, 2015), but the above-described methods lose that information in the process.

Single-molecule Fluorescence *in situ* Hybridization (smFISH) overcomes these limitations. In this method, the cells are first fixed and permeabilized. Then the cells are hybridized with a set of probes consisting of multiple short fluorescently labeled DNA oligonucleotides, which tile the length of the mRNA (Figure 1). The multiplicity of probes on a single RNA molecule increases the signal-to-noise ratio and allows for their detection by microscopy as diffraction-limited spots of similar intensity and dimensions. A 3D Gaussian fitting algorithm is used in image analysis tools to detect the spots in the images. smFISH can detect as little as a single RNA molecule and as much as several thousands.

Importantly, smFISH provides spatial information of RNA localization in the cell. Although this protocol uses the example of mRNA, smFISH can be used to detect and quantify many types of RNA molecules, for example long non-coding RNAs (lncRNA) (Cabili *et al.*, 2015), viral RNA genomes (Chou *et al.*, 2013), ribosomal RNA (Buxbaum *et al.*, 2014) and more.

There are two major disadvantages to smFISH. First, since the cells are fixed, smFISH cannot be used for temporal analysis of gene expression in the same cell (*i.e.*, live imaging). Second, due to fluorophore limitations (*i.e.*, only a small number of colors can be used for microscopy), smFISH is currently limited to study only 1-4 genes in a single experiment. However, multiple variations of smFISH exist leading to signal enhancement, increased resolution and/or multiplexing, and ultimately the simultaneous detection of transcripts from tens to hundreds of genes (reviewed at Buxbaum *et al.*, 2015; Pichon *et al.*, 2018). smFISH can be used in any organism, in cell culture and in tissue slices. Although the basic protocol concepts are similar, specialized protocols (which are abundant in the literature) are required for each sample type. Here we provide a detailed protocol for smFISH in adherent animal cells. smFISH originated in the lab of Prof. Robert H. Singer, which initially used a few (~5) 50-mer multiple-labeled probes (which were synthesized in-lab) for detection (Femino *et al.*, 1998). Prof. Arjun Raj improved the method (Raj *et al.*, 2008) by using a larger number of shorter single-label oligos (20-mer) that tile the entire length of the RNA. These protocols are available at their respective lab websites (*e.g.*, [Singer lab](#) and [Raj lab](#)). However, these protocols are outdated (*e.g.*, in regards to reagents and types of probes), and are lacking in details. There are published method papers for smFISH, but surprisingly only a few on adherent cells (*e.g.*, Lee *et al.*, 2016). Furthermore, many labs that use smFISH routinely develop in-house software for smFISH analysis. This is inefficient, confusing, and not very user-friendly to biologists that lack programming background.

This protocol was originally developed at the Singer lab (*e.g.*, Haimovich *et al.*, 2017) and it is presented here with minor modifications made at the Gerst lab. It is partially based on the Raj protocol and the Stellaris® RNA FISH protocol (see [Biosearch technologies website](#)). A major difference from other protocols is that we recommend use of the FISH-quant program (Mueller *et al.*, 2013; Tsanov *et al.*, 2016), which is user-friendly, and hope it will be used to standardize smFISH analysis.



**Figure 1. A scheme depicting the main principle of smFISH: multiple fluorescently labeled probes tile the length of the mRNA**

## **Materials and Reagents**

1. Pipette tips
2. Microscope Glass slides 25 x 75 mm x 1 mm thick (e.g., Thermo Scientific, catalog number: 421-004T or equivalent)
3. Glass coverslips, round, 18 mm, #1 (e.g., Thermo Scientific, catalog numbers: 11709875 or equivalent)
4. 1.7 ml plastic tubes
5. 15 ml plastic tubes
6. Nuclease-free Barrier tips (10 µl, 200 µl, 1,000 µl)
7. Hybridization chamber (e.g., closed plastic box, 15 cm tissue culture dish, Petri dish)
8. Parafilm (Bemis, catalog number: PM996)
9. Kimwipes (e.g., KCWW, Kimberly-Clark, catalog number: 34120 or equivalent)
10. 12-well plates (e.g., Costar, catalog number: 3513 or equivalent)
11. Aluminum foil
12. Adherent cells of interest (e.g., mouse embryonic fibroblasts [MEFs], Gastric carcinoma NCI-N87 cells)
13. Suitable culture media and supplements (e.g., DMEM supplemented with 10% FBS and penicillin/streptavidin)
14. (Optional) Extracellular matrix substrate, e.g., Fibronectin (Sigma-Aldrich, catalog number: F1141-5mg)
15. 70% ethanol
16. Sterile PBS x1 pH 7.4, no calcium, no magnesium (e.g., Thermo Fisher Scientific, Gibco<sup>TM</sup>, catalog number: 10010-015 or equivalent)
17. 10x PBS, no calcium, no magnesium (e.g., Thermo Fisher Scientific, Gibco<sup>TM</sup>, catalog number: 14200-067 or equivalent)
18. MgCl<sub>2</sub> (e.g., Sigma-Aldrich, catalog number: M8266-100G or equivalent)
19. Glycine (e.g., Sigma-Aldrich, catalog number: G8898-500G or equivalent)
20. 32% paraformaldehyde (PFA) (Electron Microscopy Sciences)
21. Surfact-Amps<sup>TM</sup> X-100 (Triton X-100) 10% solution (Thermo Scientific, catalog number: 28314)  
*Note: This high-purity Triton X-100 gives the best results, but other Triton X-100 products will provide satisfactory results.*
22. 20x Saline-sodium citrate (SSC) buffer (e.g., Sigma-Aldrich, catalog number: S6639-1L or equivalent)
23. Formamide (Sigma-Aldrich, catalog number: 47671-250ml or equivalent) (keep at 4 °C)
24. Dextran sulfate (Sigma-Aldrich, catalog number: D6001 or equivalent)
25. *E. coli* tRNA (100 mg) (Roche, catalog number: 10109541001) (keep at -20 °C)
26. Bovine serum albumin (BSA) (20 mg/ml) (Roche, catalog number: 10711454001) (keep at -20 °C)

27. Vanadyl ribonucleoside complex (VRC) 200 mM (e.g., Sigma-Aldrich, catalog number: 94742-1 ml or equivalent) (keep at -20 °C)
28. Nuclease-free water
29. DAPI (nuclear stain) (e.g., Sigma-Aldrich, catalog number: D9542-1mg or equivalent)
30. Fluorescent oligo probe set (e.g., Stellaris probes against human HER2-Quasar570 (Bioresearch technologies, DesignReady catalog number: VSMF-2102-5) (see Procedure A for design and production of probes) (keep at -20 °C)
31. Anti-fade reagent (e.g., ProLong anti-fade series from Thermo scientific)
32. (Optional) High-quality nail polish (e.g., Electron Microscopy Sciences, catalog number: 72180)
33. Immersion oil 1.518, suitable for the microscope/objective
34. PBSM buffer (see Recipes)
35. Fixation buffer (see Recipes)
36. Quenching buffer (see Recipes)
37. Permeabilization buffer (see Recipes)
38. Pre-hybridization (Pre-hyb) buffer (see Recipes)
39. Hybridization buffer (see Recipes) (keep at -20 °C)
40. Hybridization chamber (see Recipes)
41. DAPI stain solution (see Recipes) (keep at 4 °C)

## Equipment

1. Pipet aid (recommended: S1 pipet filler, Thermo Fisher Scientific, catalog number: 9501)
2. Tweezer, straight, pointed, stainless steel tip (e.g., Ideal-Tek, catalog number: 4 SA or equivalent)
3. (Optional) Vacuum trap
4. Chemical (fume) hood
5. Biological hood/biosafety cabinet (for cell culture work)
6. Cell culture incubator suitable for cell culture of your choice (e.g., 37 °C, 5% CO<sub>2</sub>)
7. 37 °C incubator (e.g., an incubator that is used to culture bacterial plates)
8. Cardboard tray for slides (e.g., Thermo Fisher Scientific, catalog number: 12-587-10)
9. Wide-field fluorescent microscope (e.g., Olympus, model: BX-61; Nikon, model: Eclipse Ti-E inverted fluorescence microscope or Zeiss, model: AxioObserver Z1) equipped with the following:
  - a. Fluorescent light source [e.g., Illuminator HXP 120 V light source (Carl Zeiss, model: Illuminator HXP 120 V) or X-cite 120 PC lamp (Excelitas Technologies, X-Cite® 120PC)]
  - b. Filter sets suitable for the fluorophores used + DAPI (blue) filter
  - c. Automated motorized stage for sub-micron movement in X, Y, and Z axes [e.g., MS 2000 XYZ automated stage (ASI, model: MS 2000) or motorized XYZ scanning stage, 130x100 PIEZO (Zeiss, catalog number: 432027-9001-000)]

- d. Plan-Apo 100x (preferred) or 63x oil immersion objective with high NA (1.35 NA or more)
  - e. CCD or sCMOS high-resolution digital camera [e.g., Flash 4 sCMOS (Hamamatsu) or Pixis 1024 CCD camera (Photometrics)]
  - f. Software suitable to control the microscope (according to manufacturer) for automated imaging of multiple channels, multiple z-stacks and multiple fields (e.g., MetaMorph, ZEN2, µmanager)

10. Computer capable of image processing (strong CPU, at least 32 GB RAM)

11. Computer for data storage

Data storage on computer or external drive to allow for storage of 10's of GBs and up to TB's of cumulative image data.

## Software



## Procedure

#### A. Design and labeling of oligonucleotide probes

1. Probes are 18-22 mer DNA oligonucleotides that are fluorescently labeled with a fluorescent organic dye at one or both ends. The most common dyes used are the cyanine (Cy), Alexa, and Atto dye series. To design the probes, first obtain the RNA sequence of interest.

*Note: For good signal-to-noise (SNR) ratio that will allow detection of the FISH spots over the background, it is recommended to use at least 25 probes (best = ~48 probes) per transcript, which means that a short transcript (i.e., < ~500 nt) may not be suitable for this version of smFISH. Alternative methods such as smiFISH (Tsanov et al., 2016), RNAscope (Wang et al., 2012) or clampFISH (Rouhanifard et al., 2018; preprint), which enhance the FISH signal, might be more suitable for short RNAs.*
  2. We recommend using the Stellaris probe design web tool. Insert the sense strand sequence and choose the required parameters (i.e., organism, specificity level, number of probes, probe length, and minimal spacing). We recommend choosing the highest level of specificity, i.e., 5, and 48 probes of 20 nt with 2 nt spacing as default. If there are < 25 probes, parameters can be changed until you are satisfied.

3. There are multiple protocols to label the probes. The simplest solution, which we recommend for consistency and ease, is to order the Stellaris RNA FISH probes from Biosearch technologies (<https://www.biostarsearchtech.com/>). However, these are relatively expensive. One alternative is to order DNA oligos with amine ends and label with an amine reactive dye (Singer, 1998). *Note that this protocol is for 50 nt oligos, but can be utilized for 20 nt oligos.* This method works well, but is still relatively expensive. A cheaper option is to enzymatically label the probes (Gáspár et al., 2017 and 2018).
4. The oligo probe set should be suspended in nuclease-free water (for Stellaris probes, prepare a 25 µM solution) and kept at -20 °C in the dark. Probes can be divided into aliquots of 10-20 µl to avoid multiple freeze-thaw cycles (although in our experience we did not detect any noticeable deterioration after multiple freeze-thaw cycles).

## B. Cell culture

### *General comments:*

1. *Work in a bio-safety cabinet (biological hood) for sterile cell culture work.*
2. *This protocol is designed for adherent cells. For non-adherent cells, it is required to add additional steps to adhere the cells to the coverslip. Users who plan such steps need to consider these points:*
  - a. *How to adhere the cells to the glass (e.g., by poly-lysine coating)?*
  - b. *Decide whether to adhere the cells to the glass before fixation or after the final wash step. Each case could necessitate different protocol steps (e.g., either to perform the washes on coverslips or in tubes, or perform fixation either before or after adherence) and might even yield different results.*
  - c. *Calibrate the number of cells per coverslip.*
3. *If possible, it is recommended to add an additional cell line as a negative control for the FISH probes used (e.g., knockout cells, cells of a different species that express the RNA of interest, but with a nucleotide sequence that has low homology, etc.). This is helpful both for calibrating the FISH signals for the specific probes, as well as for FISH spot analysis. It is preferable to verify that the knockout cell line does not express the RNA of interest. We note that truncated RNAs might be expressed from knockout cells and these can be detected by FISH.*
  1. Pre-warm 1x PBS and culture media to 37 °C.
  2. (Optional) If coating the coverslips, prepare a coating solution (e.g., dilute fibronectin 1:100 in 1x PBS).
  3. For each sample, place a coverslip in a well of 12-well plate.
  4. Wash briefly with 1 ml of 70% ethanol. Aspirate ethanol.
  5. Wash briefly with 1 ml sterile 1x PBS.

6. (Optional) Add 1 ml of coating solution and incubate as required (e.g., for fibronectin, incubate for 10-20 min in a cell culture incubator). Remove coating solution and wash with 1 ml PBS (x1).
7. Place 1 ml culture media per well.
8. Seed adherent cells of choice.
9. Culture the cells in a cell culture incubator for required time of the experiment; do not let the culture become confluent. Aim for a maximum of ~80%-90% confluence at the time of fixation.

### C. FISH

#### *General comments:*

- a. *To avoid RNase contamination of samples, wear gloves, use barrier tips and avoid working on surfaces where there is regular use of RNases (e.g., from plasmid prep kits).*
  - b. *For safety, work with PFA and formamide solutions should be performed in a chemical (fume) hood.*
  - c. *All steps except “hybridization” are performed while the coverslips remain in the well, with 1 ml of solution added per well.*
  - d. *It is recommended to pipet liquids on the wall of the well and not directly onto the cells. For cells with delicate structures (e.g., dendrites, membrane nanotubes), it is recommended to use a pipet aid at the slowest setting and not to use vacuum aspirator.*
  - e. *There is no need to shake the 12-well plate during wash steps.*
1. Wash cells with PBSM (3 quick rinses).
  2. Fix cells by incubating with fixation buffer (prepared fresh) for 10 min (not longer, see Note 1) at room temperature (RT).
  3. Wash with quenching buffer, 10 min at RT.
  4. Wash with PBSM for 10 min at RT. Repeat this step. Cells can be left overnight at 4 °C at this point.
  5. Permeabilize cells by incubating with permeabilization buffer for 10 min (not longer, see Note 1) at RT.
  6. Wash with PBSM for 10 min at RT. Repeat this step.
  7. Incubate with Pre-hyb buffer for 30 min at RT.
  8. While waiting (Step C7), mix the pre-made hybridization buffer with the probes, and prepare the hybridization chamber (see Recipes and Note 2).
  9. Place 45 µl of hybridization buffer at each intended coverslip position in the hybridization chamber. There is no need to remove large bubbles, but avoid small foam-like bubbles.
  10. With the tweezers, gently lift each coverslip from the well, remove excess liquid by touching the edge on a Kimwipe and place the coverslips with the cells facing down on the hybridization buffer (see Video 1 and Note 3).



**Video 1. Transferring coverslips from 12-well plate to hybridization chamber**

11. Seal the hybridization chamber with Parafilm, wrap with aluminum foil, and place in a 37 °C incubator for 3 h to overnight.
12. Prepare a new (or same) 12-well plate with Pre-hyb buffer.
13. Use the tweezers to transfer the coverslips back to the 12-well plate, cells facing up.
14. Cover the plate with aluminum foil and incubate in the 37 °C incubator for 15 min.
15. Wash again in Pre-hyb buffer, 15 min at 37 °C.
16. Quick rinse with 2x SSC at RT (3 quick rinses).
17. Stain in DAPI stain solution (pre-warmed to RT) for 1 min at RT.
18. Wash for 5 min with 2x SSC.
19. During this final wash step, prepare microscope slides in the cardboard tray:
  - a. Label the slide(s).
  - b. Just before lifting the coverslips, add 20 µl of Pro-Long anti-fade solution for each cover slip (there can be two per slide). Remove any air bubbles. See also Note 4.
20. Use tweezers to lift coverslips, remove excess liquid and place cell-side facing down, on the Pro-Long anti-fade drop.
21. Let dry at RT in the dark for at least several hours (for best images, wait > 24 h).
22. (Optional) For long-term storage, seal with nail polish around the edges of the coverslip after the Pro-Long dries.
23. Slides can be stored at RT for several days (at least, we have not checked longer than a week). Keep at -20 °C for long-term storage (months to years).

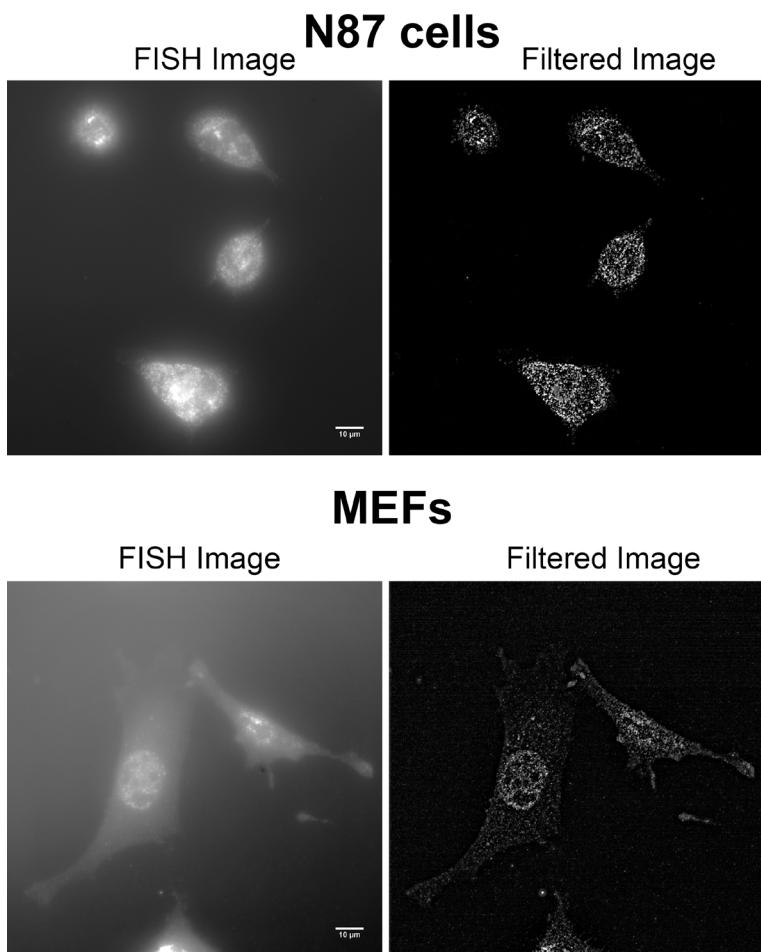
#### D. Imaging

*Important: All slides from the same experiment should be imaged using the same exact conditions.*

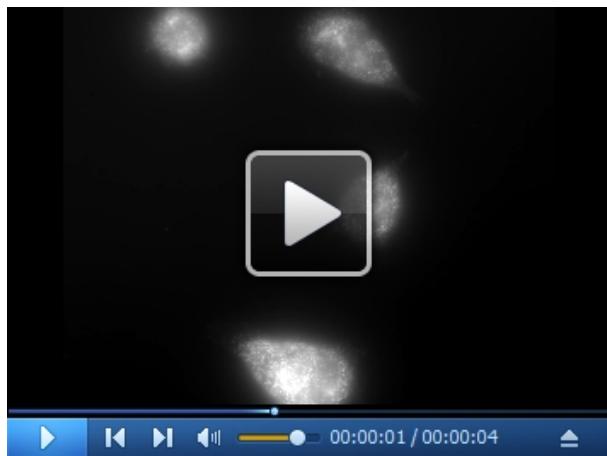
1. Imaging can be performed on any wide-field microscope, as detailed in the Equipment section.
2. Do not use a confocal microscope for smFISH imaging. The higher light intensity of the lasers can cause rapid bleaching of the FISH signal. Since the smFISH signal is relatively weak and requires long exposure times to collect enough light, photobleaching limits the total amount of

light collected. This is particularly problematic when taking multiple z sections in order to create a 3D image stack.

3. Images should be taken at the relevant channels with descending color order [e.g., Cy5 (670 nm, far-red), Cy3 (570 nm, yellow-orange), Alexa488 (520, green), and DAPI (460 nm, blue)]. This is particularly important when imaging with DAPI, since we have noticed that in some cell types (e.g., MEFs, HEK293T cells), imaging through the DAPI channel may cause the appearance of granular autofluorescence in other channels. This was infrequent (*i.e.*, it did not happen every time) but it was reproduced using several microscopes.
4. Exposure time: For FISH, it is recommended to use maximum (100%) power of the light source and an exposure time of 1-3 s for each FISH channel. For DAPI, use a low power setting and very short exposure time (e.g., 30% power, 20-40 ms).
5. For FISH spot detection, more photons (*i.e.*, longer exposure time) means better detection, but also increased background fluorescence. Therefore, the user should adjust the time and exposure parameters accordingly.
6. Z-sections: In order to detect RNA spots throughout the volume of the cell, multiple z-sections should be collected. It is recommended to use 0.2-0.3  $\mu\text{m}$  steps and collect at least 30 sections (*i.e.*, for flat cells, like fibroblasts) or more (e.g., for HEK293T or N87 cells we use 41 z-sections). When imaging, choose the option to image all z-sections in one channel before switching to the next channel.
7. Binning: For better resolution, use pixel binning 1 x 1. However, sometimes for the benefit of signal enhancement over resolution, a 2 x 2 binning may be used.
8. For examples of FISH images and z-stacks, see Figure 2 and Videos 2-5.



**Figure 2. Examples of unfiltered and filtered FISH images produced by FISH-quant.** smFISH on human NCI-N87 gastric carcinoma cells (top row) and immortalized MEFs (bottom row) were performed using Stellaris probes against human HER2 mRNA. Imaging was performed on a Zeiss AxioObserver Z1 DuoLink dual camera imaging system equipped with Illuminator HXP 120 V light source, PlanApo 100x 1.4 NA oil immersion objective and Hamamatsu Flash 4 sCMOS cameras. For both cell types, 41 steps of 0.2  $\mu\text{m}$  z-stack images were taken using a motorized XYZ scanning stage 130 x 100 PIEZO, and ZEN2 software at 0.0645  $\mu\text{m}/\text{pixel}$ . Images show a maximum projection of middle z-sections. Note that the brightness of the MEFs FISH image (lower left) was increased compared to that of N87, to allow better visualization of the cells. See Videos 2-5 for the full z-stack of each image. Scale bars = 10  $\mu\text{m}$ .



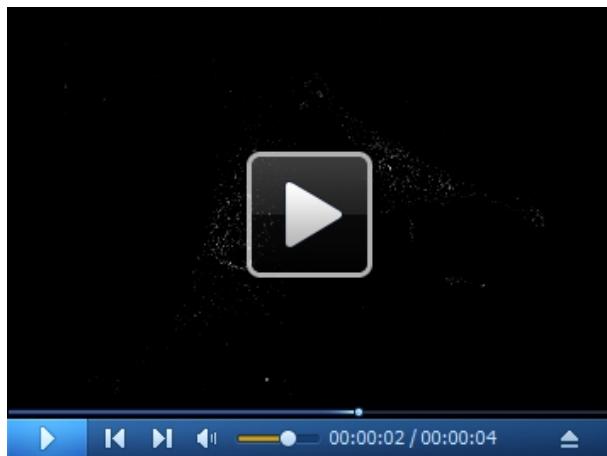
**Video 2.** 3D FISH image of N87 cell with human HER-Q570 probes



**Video 3.** 3D filtered image of the cell in Video 2



**Video 4.** 3D FISH image of MEF cell with human HER-Q570 probes



**Video 5. 3D filtered image of the cell in Video 4**

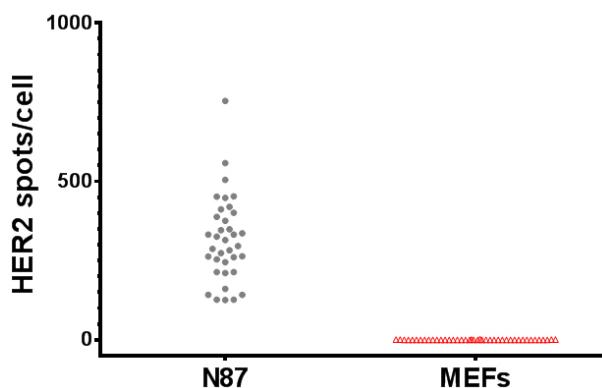
### Data analysis

FISH spot analysis is performed by programs that fit the diffraction-limited spots using a 3D Gaussian fitting algorithm. Many labs produce their own software/scripts. We recommend FISH-quant (Mueller *et al.*, 2013; Tsanov *et al.*, 2016) since it is user-friendly and can perform multiple analyses (*i.e.*, mature mRNA, transcription site analysis, co-localization). The images for FISH-quant need to be in TIFF format as a separate file of multiple z-sections obtained for each channel. If the microscope does not save files in TIFF format, convert the images by using another program (*e.g.*, FIJI). Follow the instructions for FISH-quant to perform analysis.

Briefly:

1. Install MATLAB and then install FISH-quant.
2. In the main interface of FISH-quant:
  - a. Choose the folders (main folder, images folder, outlines folder, results folder).
  - b. Insert the experimental parameters: XY pixel size (based on camera and binning), Z pixel size, refractive index of the oil, NA of the objective, excitation and emission wavelengths of the FISH probe fluorophore.
  - c. Under Tools → outline designer: draw outlines for cells, nuclei and transcription sites (TS) (hint: use the automatic “detect nucleus” and “TS auto detect” based on the DAPI and TS/FISH staining, respectively).
  - d. Upload outline of image.
  - e. Filter the background (try different modes and parameters for best filtering). For examples of filtered images and z-stacks, see Figure 2 and Videos 3 and 5.
  - f. Perform pre-detection according to instructions.
  - g. Fit spots using the Gaussian algorithm.
  - h. Use thresholding parameters to achieve the best results.
  - i. Save the detection settings.

3. From Tools → Batch processing it is possible to perform analysis of multiple images. The thresholding parameters can be modified after batch analysis to fine-tune the results. It is recommended to set the parameters so that a negative control will give close to 0 spots.
4. Use Tools → Spot inspector to eliminate obvious false positives (e.g., areas of high autofluorescence).
5. The data from FISH-quant is saved as a ‘.txt’ file. The data can be copied and pasted to Excel for further analysis (e.g., Figure 3).



**Figure 3. FISH-quant analysis of human HER2 mRNA expression level in human N87 cancer cells and MEFs.** Each circle/triangle represents the number of spots scored for a single cell.

### Notes

1. The most critical incubation times to keep are at the fixation and permeabilization steps. For all other wash steps, samples can be left for longer incubation times. From our experience, 30 min for quenching/wash and up to 60 min for Pre-hyb did not have any adverse effects on the FISH. Shorter incubation times (e.g., 5 min) or fewer washes (e.g., once instead of twice after fixation/permeabilization) had a small, but distinct, adverse effect. Shortening post-hybridization wash times will lead to a significant increase in the background signal. The incubation times in this protocol have worked well for many cell types (including MEFs, many cancer cell lines, mouse primary hippocampal neurons, and others). However, incubation times may require optimization by users to suit their own cell lines.
2. There is no need to dim the lights when working with the fluorophore, but it is recommended to minimize light exposure until the addition of the anti-fade solution. The room should be darkened during imaging to minimize autofluorescence and to avoid background light contamination.
3. Use gentle force to grab and lift the coverslips with the tweezers, since coverslips can break easily. If the coverslip breaks to two large pieces, it is still salvageable and the experiment can continue (make sure to separate the two pieces so they do not stick one on top of the other).

Furthermore, if the tweezers do not hold the coverslips firmly, the coverslips might fall to the bench. Again, the coverslip is still salvageable, but the user needs to guess the correct side of the cells.

4. There are other commercially available anti-fade solutions, as well as lab-made anti-fade solutions that can be used (e.g., by using glucose oxidase). However, we have no experience with those, and these might not be suitable for FISH or long-term storage.

## **Recipes**

1. PBSM buffer (500 ml)

50 ml 10x PBS

2.5 ml MgCl<sub>2</sub> 1 M

475 ml nuclease-free water

Store at RT

2. Fixation buffer (8 ml)

7 ml PBSM

1 ml PFA 32%

Prepare fresh

3. Quenching buffer (10 ml)

9.5 ml PBSM

0.5 ml 2 M glycine

Prepare fresh as it tends to get microorganism contaminations

***Prepare stock solutions in advance:***

- a. PBSM buffer (Recipe 1)

- b. 2 M glycine stock solution in water, filter sterilize (store at RT)

4. Permeabilization buffer (100 ml)

10 ml 10x PBS

1 ml Surfact-Amps™ X-100 10%

89 ml nuclease-free water

Store at RT

5. Pre-hybridization (Pre-hyb) buffer (10 ml)

1 ml 20x SSC

1 ml Formamide (warm to room temperature before use)

8 ml nuclease-free water

Prepare fresh

## 6. Hybridization buffer

Reagent	For 1 ml final volume (store at -20 °C)	Final Concentration
Formamide	0.1 ml	10%
Competitor (10 mg/ml <i>E. coli</i> tRNA)	0.1 ml	1 mg/ml
20% Dextran Sulfate	0.5 ml	10%
20 mg/ml BSA	10 µl	0.2 mg/ml
20x SSC	0.1 ml	2x
VRC 200 mM	10 µl	2 mM
Nuclease-free water	0.18 ml	
<b>Total Volume</b>	<b>1 ml</b>	

Add probes fresh to hybridization buffer. It is recommended to calibrate probe concentration when using probes for the first time (default: 250 nM or 10 ng/sample).

**Prepare stock solutions in advance:**

- 1) 10 mg/ml *E. coli* tRNA solution in nuclease-free water. Store at -20 °C.
- 2) 20% Dextran sulfate in water (viscous, takes 30-60 min to dissolve completely). Store at RT.

## 7. Hybridization chamber

- a. Plastic box or plastic dish (e.g., Petri dish, 15 cm tissue culture dish). The size of the chamber should accommodate all the coverslips allowing for at least a few millimeters between the coverslips
- b. Place parafilm on the bottom of the chamber. Avoid wrinkles where the coverslips will be placed
- c. (Optional) Draw a grid (each square should fit a single coverslip) and label the squares
- d. Take the cap of a 15 ml conical tube and place it at the edge of the chamber. Fill the cap with 1 ml of water or buffer (this is required to maintain humidity in the chamber)
- e. Prepare a piece of parafilm to seal the chamber after coverslips are placed
- f. Prepare aluminum foil to cover the chamber to protect from light
- g. The plastic chamber can be re-used multiple times

## 8. DAPI stain solution (200 ml)

20 ml 20x SSC

10 µl of 10 mg/ml DAPI (final concentration: 0.5 µg/ml)

180 ml nuclease-free water

Store at 4 °C in the dark

**Prepare DAPI stock solution in advance:**

10 mg/ml DAPI solution in water. Store at -20 °C in the dark

**Acknowledgments**

G.H. is a recipient of the Koshland Foundation and McDonald-Leapman Grant Senior Post-doctoral fellowship. This work was funded by grants from the Joel and Mady Dukler Fund for Cancer Research (WIS), a Proof-of-Principle Grant from the Moross Integrated Cancer Center, Weizmann Institute, and US-Israel Binational Science Foundation-National Science Foundation (#2015846) to J.E.G.

### **Competing interests**

The authors declare that there are no conflicts of interest or competing interests.

### **References**

1. Buxbaum, A. R., Haimovich, G. and Singer, R. H. (2015). [In the right place at the right time: visualizing and understanding mRNA localization](#). *Nat Rev Mol Cell Biol* 16(2): 95-109.
2. Buxbaum A. R., Wu B. and Singer R. H. (2014). [Single β-actin mRNA detection in neurons reveals a mechanism for regulating its translatability](#). *Science* 343(6169):419-422.
3. Cabili, M. N., Dunagin, M. C., McClanahan, P. D., Biaesch, A., Padovan-Merhar, O., Regev, A., Rinn, J. L. and Raj, A. (2015). [Localization and abundance analysis of human lncRNAs at single-cell and single-molecule resolution](#). *Genome Biol* 16: 20.
4. Chou, Y. Y., Heaton, N. S., Gao, Q., Palese, P., Singer, R. H. and Lionnet, T. (2013). [Colocalization of different influenza viral RNA segments in the cytoplasm before viral budding as shown by single-molecule sensitivity FISH analysis](#). *PLoS Pathog* 9(5): e1003358.
5. Femino, A. M., Fay, F. S., Fogarty, K. and Singer, R. H. (1998). [Visualization of single RNA transcripts in situ](#). *Science* 280(5363): 585-590.
6. Gáspár, I., Wippich, F. and Ephrussi, A. (2017). [Enzymatic production of single-molecule FISH and RNA capture probes](#). *RNA* 23(10): 1582-1591.
7. Gáspár, I., Wippich, F. and Ephrussi, A. (2018). [Terminal deoxynucleotidyl transferase mediated production of labeled probes for single-molecule FISH or RNA capture](#). *Bio-protocol* 8(5): e2750.
8. Haimovich, G., Ecker, C. M., Dunagin, M. C., Eggan, E., Raj, A., Gerst, J. E. and Singer, R. H. (2017). [Intercellular mRNA trafficking via membrane nanotube-like extensions in mammalian cells](#). *Proc Natl Acad Sci U S A* 114(46): E9873-E9882.
9. Lee, C., Roberts, S. E. and Gladfelter, A. S. (2016). [Quantitative spatial analysis of transcripts in multinucleate cells using single-molecule FISH](#). *Methods* 98: 124-133.
10. Mueller, F., Senecal, A., Tantale, K., Marie-Nelly, H., Ly, N., Collin, O., Basyuk, E., Bertrand, E., Darzacq, X. and Zimmer, C. (2013). [FISH-quant: automatic counting of transcripts in 3D FISH images](#). *Nat Methods* 10(4): 277-278.

11. Pichon, X., Lagha, M., Mueller, F., and Bertrand, E. (2018). [A growing toolbox to image gene expression in single cells: sensitive approaches for demanding challenges.](#) *Mol Cell* 71(3): 468-480.
12. Raj, A., van den Bogaard, P., Rifkin, S. A., van Oudenaarden, A. and Tyagi, S. (2008). [Imaging individual mRNA molecules using multiple singly labeled probes.](#) *Nat Methods* 5(10): 877-879.
13. Rouhanifard, S. H., Mellis, I. A., Dunagin, M., Bayatpour, S., Symmons, O., Cote, A. and Raj, A. (2018). [Exponential fluorescent amplification of individual RNAs using clampFISH probes.](#) *bioRxiv* 222794.
14. Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J. Y., White, D. J., Hartenstein, V., Eliceiri, K., Tomancak, P. and Cardona, A. (2012). [Fiji: an open-source platform for biological-image analysis.](#) *Nat Methods* 9(7): 676-682.
15. Singer, R. H. (1998). Preparation of probes for *in situ* hybridization. [https://www.einstein.yu.edu/uploadedFiles/LABS/robert-singer-lab/probe\\_prep.pdf](https://www.einstein.yu.edu/uploadedFiles/LABS/robert-singer-lab/probe_prep.pdf).
16. Svensson, V., Natarajan, K. N., Ly, L. H., Miragaia, R. J., Labalette, C., Macaulay, I. C., Cvejic, A. and Teichmann, S. A. (2017). [Power analysis of single-cell RNA-sequencing experiments.](#) *Nat Methods* 14(4): 381-387.
17. Tsanov, N., Samacoits, A., Chouaib, R., Traboulsi, A. M., Gostan, T., Weber, C., Zimmer, C., Zibara, K., Walter, T., Peter, M., Bertrand, E. and Mueller, F. (2016). [smiFISH and FISH-quant - a flexible single RNA detection approach with super-resolution capability.](#) *Nucleic Acids Res* 44(22): e165.
18. Wang, F., Flanagan, J., Su, N., Wang, L. C., Bui, S., Nielson, A., Wu, X., Vo, H. T., Ma, X. J. and Luo, Y. (2012). [RNAscope: a novel \*in situ\* RNA analysis platform for formalin-fixed, paraffin-embedded tissues.](#) *J Mol Diagn* 14(1): 22-29.

## Staining the Germline in Live *Caenorhabditis elegans*: Overcoming Challenges by Applying a Fluorescent-dye Feeding Strategy

K. Adam Bohnert\*

Department of Biological Sciences, Louisiana State University, Baton Rouge, Louisiana, USA

\*For correspondence: [bohnerta@lsu.edu](mailto:bohnerta@lsu.edu)



**[Abstract]** *C. elegans* provides a tractable model organism for studying germline cell biology. Microscopy experiments are relatively facile, as this worm is transparent and germline development can be observed in real-time using DIC microscopy and/or fluorescent transgenes. Despite these many tools, robust staining techniques for imaging germ cells in live worms have been more elusive, due to the tough outer cuticle of the worm, which impairs staining efficiency. This limitation has restricted the spectrum of probes that can be used to investigate reproductive cell biology in *C. elegans*. Building on previous approaches, I recently applied a fluorescent-dye feeding strategy to reproducibly label organelles and monitor physiological changes in germlines of living *C. elegans*. In this approach, fluorescent dyes are initially introduced into the agar plates and bacterial lawns on which worms are subsequently cultured. After worms are grown on the dyed plates, oocytes show staining patterns consistent with verified transgenic markers. Thus, this approach offers an effective solution for labeling difficult-to-stain tissues in live worms, and establishes an entry point for incorporating new probes and sensors into analyses of *C. elegans* germline biology.

**Keywords:** *C. elegans*, Germline, Reproduction, Fluorescence microscopy, Staining, Sensors

**[Background]** Animal reproduction is one of the most fascinating topics in biology. Understanding how germ (reproductive) cells are able to give rise to offspring with each new generation has intrigued scientists, as well as the general public, for centuries. With recent advances in imaging techniques and tools, researchers have been able to observe oocytes (female germ cells) and sperm (male germ cells) in extraordinary detail, revealing fundamental mechanisms that allow germ cells to execute their specialized functions. Model organism research, in particular, has afforded the opportunity to combine live-animal imaging with genetic analyses to tease apart relevant biological pathways important for germ cell regulation. The nematode *C. elegans*, which exists as a hermaphrodite that continually self-fertilizes throughout young-adulthood (Klass, 1977), presents a powerful experimental system for studying important questions in reproductive biology. Recently, I utilized the *C. elegans* germline to explore how signs of aging are reversed from one generation to the next (Bohnert and Kenyon, 2017). These studies required the use of dyes to track various aspects of oocyte physiology, including lysosomal acidity and mitochondrial membrane potential. Unfortunately, dyes incubated with *C. elegans* in liquid culture were inefficient at penetrating the worm's outer cuticle, which is highly-impervious (Page and Johnstone, 2007) and precludes robust staining. To circumvent this obstacle, I applied a dye-feeding approach (Hermann *et al.*, 2005; Schaheen *et al.*, 2006) in an attempt

to stain and image intact, full germlines of live *C. elegans*. When worms were grown on agar plates and food (*E. coli* OP50) to which dyes had previously been added, the germline, in addition to other adult tissues, could be clearly and accurately stained (Bohnert and Kenyon, 2017). Here, I describe this feeding-based strategy for staining the germline of live *C. elegans*, which can in principle be applied to other dyes that have difficulty penetrating the worm cuticle.

## **Materials and Reagents**

1. Graduated cylinders (Nalgene; Fisher, catalog number: 02-540-270)
2. Plastic beakers (Nalgene; Fisher, catalog number: 02-591-10H)
3. 15 ml Falcon tubes (Fisher, catalog number: 14-959-70C)
4. 15 ml bacterial culture test tubes (Fisher, catalog number: 14-956-9C)
5. 1.5 ml microcentrifuge tubes (Fisher, catalog number: 05-408-129)
6. Weighing boats (Fisher, catalog number: 08-732-112)
7. Pipet tips (Fisher, catalog numbers: 02-707-401, 02-707-415, and 02-707-436)
8. Serological pipets (Fisher, catalog number: 13-676-10J and 13-676-10K)
9. 35-mm Petri dishes (Lab Sciences, catalog number: 9333-35NV)
10. 100-mm Petri dishes (Fisher, catalog number: FB0875712)
11. Rubbermaid plastic container (Amazon, catalog number: B00YQAGNGK)
12. Foil (any household variety works)
13. Empty 1 mm gel cassette (Thermo Fisher, catalog number: NC2010)
14. Razor blade (Fisher, catalog number: 18-100-970)
15. Microscope slides (Fisher, catalog number: 12-518-100B)
16. Microscope slide coverslips (Fisher, catalog number: 12-541B)
17. *C. elegans* strains; for wild-type, use Bristol N2 (*Caenorhabditis* Genetics Center)
18. *E. coli* OP50 strain (*Caenorhabditis* Genetics Center)
19. Sodium chloride, NaCl (Fisher, catalog number: S271-10)
20. Peptone (Fisher, catalog number: BP1420-500)
21. Agar (Fisher, catalog number: BP1423-500)
22. Calcium chloride dihydrate, CaCl<sub>2</sub>·2H<sub>2</sub>O (Fisher, catalog number: C79-500)
23. Cholesterol (Alfa Aesar, catalog number: A11470-18)
24. Ethanol (Fisher, catalog number: BP2818-500)
25. Magnesium sulfate, MgSO<sub>4</sub> (Alfa Aesar, catalog number: 33337-36)
26. Potassium phosphate monobasic, KH<sub>2</sub>PO<sub>4</sub> (Fisher, catalog number: P285-500)
27. Potassium phosphate dibasic, K<sub>2</sub>HPO<sub>4</sub> (Fisher, catalog number: P288-500)
28. Sodium hydroxide, NaOH (Fisher, catalog number: S318-1)
29. Household bleach (Chlorox)
30. LB Broth, Miller (Fisher, catalog number: BP1426-2)
31. Agarose (Fisher, catalog number: BP1356-100)

32. Levamisole hydrochloride (Acros Organics, catalog number: 187870100)
33. Carbenicillin (Fisher, catalog number: BP2648-1)
34. IPTG (Fisher, catalog number: BP1755-10)
35. Dyes

*Note: The following dyes have been tested and verified to work with this protocol, but others may be suitable as well.*

- a. LysoTracker Red DND-99 (Life Technologies, catalog number: L7528)
  - b. MitoTracker Deep Red FM (Life Technologies, catalog number: M22426)
  - c. DiOC<sub>6</sub>(3) (Life Technologies, catalog number: D273)
36. 1 M CaCl<sub>2</sub> (see Recipes)
  37. 5 mg/ml cholesterol (see Recipes)
  38. 1 M MgSO<sub>4</sub> (see Recipes)
  39. 1 M KPO<sub>4</sub> pH 6.0 (see Recipes)
  40. 100 mg/ml carbenicillin (see Recipes)
  41. 1 M IPTG (see Recipes)
  42. NG agar (see Recipes)
  43. Dye stock solutions (see Recipes)
  44. LB medium (see Recipes)
  45. Worm bleaching solution (see Recipes)
  46. Agarose pads (see Recipes)
  47. Levamisole solution (see Recipes)

## Equipment

*Note: The following types of equipment will be needed for this protocol. Though the exact models are not absolutely necessary, I have included information on the specific units that my lab and I have used.*

1. Glass flasks (Kimble; Fisher, catalog number: 02-543-21)
2. Glass beakers (Kimble; Fisher, catalog number: 02-555-2)
3. Glass bottles (Pyrex; Fisher, catalog numbers: 06-414-1B, 06-414-1C, and 06-414-1D)
4. Stir bar (Fisher, catalog number: 14-513-82)
5. Pipets (Gilson; Fisher, catalog number: F167900G)
6. Motorized pipet controller (Fisher, catalog number: FB14955202)
7. Microwave (any household variety works)
8. Autoclave (Steris, model: AMSCO 3000 series)
9. Hotplate stirrer (Thermo Fisher, catalog number: 88880004)
10. Shaker (Amerex Instruments, GYROMAX, model: 737R)
11. Tabletop centrifuge (Eppendorf, model: 5804R)

12. Vortex (Thermo Fisher, catalog number: 88880017TS)
13. Hood
14. Tube revolver (Thermo Fisher, catalog number: 88881001)
15. Bacteria incubator (Fisher, catalog number: 15-103-0518)
16. Worm incubator (Thermo Fisher, catalog number: PR205745R)
17. Confocal microscope

We have used various microscope systems to image stained worms. In principle, any confocal system with appropriate filter sets for the selected dye(s) is suitable. I recommend using a 40X objective to image individual germlines. Examples of microscope systems that my lab and I have used include:

- a. Leica, model: TCS SP8 with white light laser
- b. Nikon, model: CSU-Series

## **Software**

### 1. Imaging software

This will depend on the microscope system that is used. For example, the Leica TCS SP8 is equipped with LAS X software (<https://www.leica-microsystems.com/products/microscope-software/details/product/leica-las-x-ls/>), whereas Nikon confocal microscopes have NIS Elements software (<https://www.nikoninstruments.com/Products/Software>). As with the microscope system, many different types of imaging software are in principle compatible with this protocol.

### 2. ImageJ

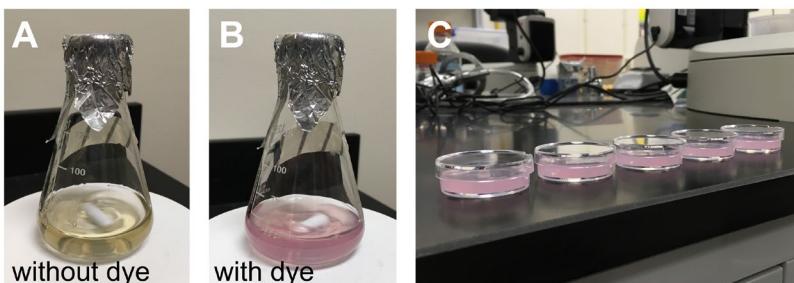
ImageJ can be used for image analysis (NIH; <https://imagej.nih.gov/ij/>).

## **Procedure**

21. Prepare 25 ml of NG agar in a flask per a standard recipe (see below) (Stiernagle, 2006).  
Include a stir bar.
22. Autoclave NG agar using a liquid cycle set at 121 °C and 15 psi pressure for at least 30 min.
23. Cool the NG agar. Add the post-autoclave ingredients from sterile stocks (see Recipes below) and stir on a hot-plate (Figure 1A).
24. Once all ingredients have been mixed, add the dye. Tested dyes have been verified to work at a final concentration of 2 µM. Different concentrations may work for other dyes; when trying new dyes, it is a good idea to compare test plates of various dye concentrations for staining efficiency. Using less dye allows more plates to be made from the same starting amount of dye, which is cost-effective. However, a lower dye concentration also results in weaker staining, so the trade-off must be assessed for each dye.
25. After the dye is added to the medium, stir on a hot-plate until the dye is evenly distributed

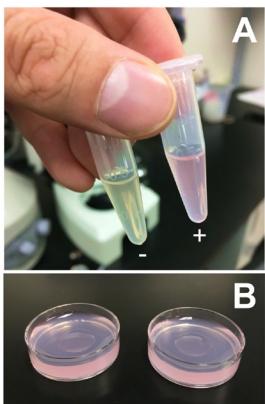
(Figure 1B).

26. Pipet 5 ml of the dyed medium into 35-mm Petri dishes. You will be able to make 5 plates in total (Figure 1C).



**Figure 1. Production of NG agar plates for staining of live *C. elegans*.** A. NG medium after autoclaving but before addition of LysoTracker Red DND-99. B. NG medium after addition of LysoTracker Red DND-99. Note the pink color. C. 35-mm Petri dishes each with 5 ml LysoTracker-containing NG agar.

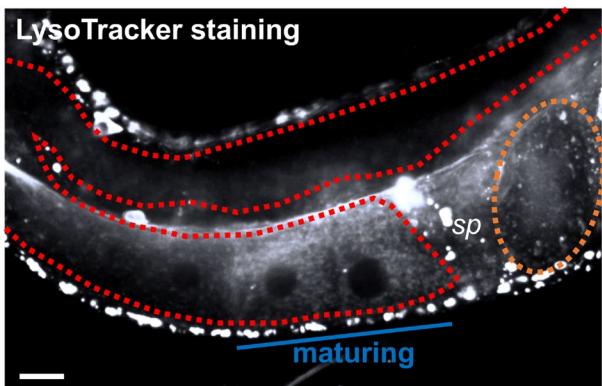
27. Put plates in a foiled container and dry at room temperature for at least 2 days. If plates are needed sooner, they can be dried in a hood (with the light off).
28. Streak out *E. coli* OP50 on to an LB agar plate without antibiotics. It is important to streak to single colonies to ensure purity. Grow at 37 °C overnight.
29. The next day, check the LB agar plate for OP50 colonies and possible contaminants. If OP50 colonies, but no contaminants, have grown, pick a single OP50 colony and inoculate a 5 ml LB liquid culture. Incubate with shaking at 37 °C overnight.
30. Overnight growth will produce a saturated OP50 culture ( $\sim 10^8\text{-}10^9$  CPU/ml). Transfer 500  $\mu$ l of the saturated OP50 culture to a microcentrifuge tube and add the same dye that was previously added to the NG agar plates (Figure 2A). Use the same final concentration of the dye as before.
31. Aliquot 100  $\mu$ l of the dyed OP50 culture on to the center of the dyed NG agar plates (Figure 2B). Keep plates in a foiled container at room temperature for at least 2 days. Bacteria will form a lawn. Plates can be maintained in a covered container for up to 1 week at room temperature, and are suitable for use as long as they do not dry out and crack.



**Figure 2. Preparation of dyed bacteria.** A. OP50 aliquots with (+) or without (-) LysoTracker Red DND-99. B. Two LysoTracker plates with 100  $\mu$ l OP50 spotted in the center.

32. For most robust staining, transfer worms to the bacterial lawns as eggs or L1 larvae. *C. elegans* eggs can be isolated by bleaching. For bleaching, gravid adult worms should be collected. Rinse plates with M9 buffer to collect the worms, and transfer the worms into a 15 ml Falcon tube. Centrifuge at 200  $\times g$  for 30 sec to pellet the worms. Remove the M9 buffer, and add 5 ml of fresh worm bleaching solution. Vortex for 5 min to dissolve the animals and release the eggs. Add 5 ml M9 buffer and invert the tube to mix. Pellet the released eggs at 200  $\times g$  for 30 sec, and wash the eggs three times with 5 ml M9 buffer. Plate the eggs directly, or nutate the eggs overnight in M9 buffer to allow the worms to develop into L1 larvae before plating. Either approach is suitable for this application. Introduce about 50 eggs/animals per plate; if more worms are included, the bacterial food source may be exhausted before worms reach adulthood.
33. Put the foiled container with the worm plates into an appropriate incubator. For standard staining, incubation at 20 °C is suitable. When expression of germline transgenes is also desired, incubate at 25 °C to prevent germline silencing (Merritt *et al.*, 2010). Allow the worms to develop into young adults (from the embryo stage, this will take around 3 days, depending on the incubation temperature).
34. Make 1 mm-thick 4% agarose pads that can be used to mount the worms for microscopy. First, separate the two halves of an empty 1 mm gel cassette. Then, melt 1 g of agarose in 25 ml H<sub>2</sub>O in a glass beaker or flask. Before the agarose solidifies, pour the agarose on to the flat inside surface of one of the cassette halves, and compress the agarose to 1 mm thickness using the other cassette half. Once the agarose solidifies, pull the two halves of the cassette apart; a 1 mm-thick sheet of agarose should stick to one half of the cassette. Use a razor to cut the agarose into small square pads.
35. Place an agarose pad onto a microscope slide and add a drop of 2 mM levamisole (a paralyzing agent used to immobilize worms). Transfer young-adult worms to the levamisole drop and place a coverslip on top.
36. Secure the slide to the stage of a confocal microscope. Use appropriate filter sets to image the

fluorophore of interest (Figure 3).



**Figure 3. Example staining of lysosomes in the *C. elegans* germline.** A gonad arm is outlined in red, and an embryo is outlined in orange. LysoTracker-positive puncta develop in maturing oocytes as they approach sperm (sp). Note that other tissues are also stained. Scale bar = 10  $\mu$ m.

### Data analysis

Staining patterns in the germline can be observed upon image acquisition (Figure 3). Fluorescence intensities can be quantified using ImageJ. In general, three independent replicates should be performed for quantification. Statistical analysis of staining patterns/intensities among experimental groups can be performed using unpaired *t*-tests, with significance defined as  $P < 0.05$ . Detailed information on data analysis and raw data for different types of experiments can be found in the original research article (Bohnert and Kenyon, 2017), which is freely available on PubMed Central (PMCID: PMC5936623).

### Notes

It is important to note that tissues other than the germline will also be stained (Figure 3). Because worms are being fed the dye, the intestine will tend to fluoresce brightly when in focus. It is easiest to image full gonad arms in live animals when proximal and distal regions of the germline are on the same z-plane and are situated on top of the intestine.

This staining approach can be applied to label the germlines of animals treated with RNAi. In this case, the dye would need to be added to RNAi plates (NG agar supplemented with 100  $\mu$ g/ml carbenicillin and 1 mM IPTG) and to the relevant bacterial strains (*i.e.*, those expressing RNAi clones). When gene knockdown is desired in late development or adulthood, worms should first be grown on normal NG plates containing the dye, and then moved to dyed RNAi plates at the appropriate stage.

## Recipes

1. 1 M CaCl<sub>2</sub>  
Dissolve 147.0 g CaCl<sub>2</sub>·2H<sub>2</sub>O in 1 L H<sub>2</sub>O, and autoclave using a liquid cycle set at 121 °C and 15 psi pressure for at least 30 min
  2. 5 mg/ml cholesterol  
Dissolve 0.25 g cholesterol in 50 ml 200-proof ethanol, and filter-sterilize
  3. 1 M MgSO<sub>4</sub>  
Dissolve 120.4 g MgSO<sub>4</sub> in 1 L H<sub>2</sub>O, and autoclave using a liquid cycle set at 121 °C and 15 psi pressure for at least 30 min
  4. 1 M KPO<sub>4</sub> pH 6.0  
Dissolve 108.3 g KH<sub>2</sub>PO<sub>4</sub> and 35.6 g K<sub>2</sub>HPO<sub>4</sub> in 1 L H<sub>2</sub>O, and autoclave using a liquid cycle set at 121 °C and 15 psi pressure for at least 30 min
  5. 100 mg/ml carbenicillin  
Dissolve 1 g carbenicillin in 10 ml H<sub>2</sub>O, and filter-sterilize
  6. 1 M IPTG  
Dissolve 2.38 g IPTG in 10 ml H<sub>2</sub>O, and filter-sterilize
  7. NG agar
    - a. Mix 75 mg NaCl, 62.5 mg peptone, and 425 mg agar in 25 ml H<sub>2</sub>O
    - b. Autoclave using a liquid cycle set at 121 °C and 15 psi pressure for at least 30 min
    - c. Cool at room temperature until the flask can be comfortably touched
    - d. Add 25 µl 1 M CaCl<sub>2</sub>, 25 µl 5 mg/ml cholesterol, 25 µl 1 M MgSO<sub>4</sub>, and 625 µl 1 M KPO<sub>4</sub> pH 6.0
    - e. Then, proceed to adding the desired dyeFor RNAi plates, also add 25 µl 100 mg/ml carbenicillin and 25 µl 1 M IPTG after autoclaving.
  8. Dye stock solutions  
Make a 1 mM stock solution of each dye in DMSO (or other recommended solvent).
- Notes:**
- a. *These stock solutions can be used at 1:500 to give a 2 µM final concentration. As noted in the Procedure, different concentrations may be suitable for other dyes that have yet to be verified.*
  - b. *Stocks should be protected from light as much as possible (for example, tubes can be wrapped in foil and maintained within boxes). Recommended storage of liquid stocks is -20 °C.*
9. LB medium
    - a. Dissolve 25 g LB Broth (Miller) in 1 L H<sub>2</sub>O, and autoclave using a liquid cycle set at 121 °C and 15 psi pressure for at least 30 min
    - b. To make LB agar, add 8 g agar per 400 ml LB before autoclaving
    - c. LB agar can be poured into 100-mm Petri dishes

**10. Worm bleaching solution**

Mix 3.5 ml H<sub>2</sub>O, 1 ml household bleach, and 0.5 ml 5 N NaOH (Stiernagle, 2006)

To make 5 N NaOH: dissolve 10 g NaOH in 50 ml H<sub>2</sub>O

**11. Agarose pads**

- a. Add 1 g agarose to 25 ml H<sub>2</sub>O in a beaker
- b. Microwave to dissolve the agarose
- c. Pour agarose on to a clean, inside surface of an old protein-gel cassette and compress to ~1 mm in thickness using the cassette's other half
- d. Cut agarose squares roughly 1.25 cm x 1.25 cm using a razor (the exact size and geometry of the pads is not a strict requirement)

**12. Levamisole solution**

- a. Make a 1 M stock solution by dissolving 0.24 g levamisole hydrochloride in 1 ml H<sub>2</sub>O
- b. Dilute 1:500 in H<sub>2</sub>O to make a 2 mM working solution

**Acknowledgments**

The study (Bohnert and Kenyon, 2017) on which this full protocol is based was originally performed in Cynthia Kenyon's lab at UCSF and Calico Life Sciences. I thank Cynthia for her mentorship, generosity, and creative insight, and members of the Kenyon laboratory for helpful comments in designing these experiments. I also thank Maria Ingaramo and Andy York for sharing microscopy advice and expertise. While in the Kenyon lab, I was an Honorary Fellow of the Jane Coffin Childs Memorial Fund. I am now an Assistant Professor at Louisiana State University. I thank my current lab members and Alyssa Johnson for thought-provoking discussions and input on this protocol.

**Competing interests**

I declare no conflicts of interest or competing interests.

**References**

1. Bohnert, K. A. and Kenyon, C. (2017). [A lysosomal switch triggers proteostasis renewal in the immortal \*C. elegans\* germ lineage](#). *Nature* 551(7682): 629-633.
2. Hermann, G. J., Schroeder, L. K., Hieb, C. A., Kershner, A. M., Rabbitts, B. M., Fonarev, P., Grant, B. D. and Priess, J. R. (2005). [Genetic analysis of lysosomal trafficking in \*Caenorhabditis elegans\*](#). *Mol Biol Cell* 16(7): 3273-3288.
3. Klass, M. R. (1977). [Aging in the nematode \*Caenorhabditis elegans\*: major biological and environmental factors influencing life span](#). *Mech Ageing Dev* 6(6): 413-429.
4. Merritt, C., Gallo, C. M., Rasoloson, D., and Seydoux, G. (2010). [Transgenic solutions for the germline](#). *WormBook*

5. Page, A. and Johnstone, I. L. (2007). [The cuticle](#). *WormBook*
6. Schaheen, L., Dang, H. and Fares, H. (2006). [Basis of lethality in \*C. elegans\* lacking CUP-5, the mucolipidosis type IV orthologue](#). *Dev Biol* 293(2): 382-391.
7. Stiernagle, T. (2006). [Maintenance of \*C. elegans\*](#). *WormBook*

A blue rectangular banner with the Bio-protocol logo at the top left. The logo consists of the word "bio-protocol" in a white serif font, with a small green leaf icon preceding the "b". Below the main text, the tagline "Improve Research Reproducibility" is written in a smaller, italicized white font. To the right of the logo, the text "Free access to ~4000 high-quality protocols" is displayed in a large, bold, white sans-serif font. Below this, a bulleted list of features is shown in white text:

- Contributed by 10,000+ scientists (including Nobel Laureates)
- Validated in a primary research paper
- >91% reproducibility (survey of 2165 Bio-protocol users)
- ~1000 videos of key procedural steps

At the bottom left of the banner, there is a blue rounded rectangle containing the text "Sign up at: [www.bio-protocol.org](http://www.bio-protocol.org)" in white.

**Microirradiation for Precise, Double-strand Break Induction *in vivo* in *Caenorhabditis elegans***

Kailey E. Harrell, Emily Koury and Sarit Smolikove\*

Department of Biology, University of Iowa, Iowa City, USA

\*For correspondence: [sarit-smolikove@uiowa.edu](mailto:sarit-smolikove@uiowa.edu)

**[Abstract]** DNA double-strand breaks (DSBs) are toxic lesions that every cell must accurately repair in order to survive. The repair of DSBs is an integral part of a cell life cycle and can lead to lethality if repaired incorrectly. Laser microirradiation is an established technique which has been used in yeast, mammalian cell culture, and *Drosophila* cell culture to study the regulation of DSB repair. Up to our studies, this method has not been adapted for use in a whole, live, multicellular organism to study this repair *in vivo*. We have recently shown that this system can be used for study of the recruitment of vital repair proteins to microirradiation-induced breaks in the transparent nematode *Caenorhabditis elegans*. With the integration of microirradiation and imaging technology, we can precisely induce DSBs in target nuclei and study the recruitment of fluorescently tagged repair proteins from the time of damage induction. Whole, live worms are plated and immobilized for targeting of nuclei, and immediately following induction the targeted region can be imaged for up to an hour and a half post-microirradiation. This method is the first that allows for study of DNA repair protein kinetics *in vivo* in an intact organism, which can be adapted in numerous ways to allow for study of repair kinetics in various aspects of the repair process.

**Keywords:** Microirradiation, Double-strand breaks, Protein recruitment, DSB repair, Live imaging, *C. elegans*

**[Background]** DNA double-strand breaks (DSBs) are one of the most toxic forms of DNA damage and can be induced exogenously (e.g., UV damage) or endogenously (e.g., SPO-11-induced meiotic DSBs). Studying the recruitment of proteins to the sites of DSBs provides valuable information regarding how the process, and specific proteins involved, are regulated. Microirradiation has been extensively used in cell culture as a means of studying DNA repair (Aten *et al.*, 2004; Kong *et al.*, 2009). This method, coupled with fluorescently-labeled proteins and time-lapse imaging, has provided critical information regarding the regulation of proteins in specific cellular contexts. However, this incredibly tractable method had not been adapted for use in a whole, live, multicellular organism.

In our paper, we describe a laser microirradiation method which applies this technology to intact, live worms (Koury *et al.*, 2018). With the use of a UVA 365 nm pulsed laser, and fluorescently tagged repair proteins RPA-1 and RAD-51, we were able to precisely induce DSBs in specific regions of the *C. elegans* germline to study DSB repair kinetics in meiotic tissue *in vivo*. RPA-1 and RAD-51 are ssDNA binding proteins that are essential for DSB repair using homologous recombination. The transparent nature of worms allows for live imaging of fluorescent proteins without any form of dissection or gonad extrusion, and no pre-sensitization of the worms is required for efficient damage induction. Not only is

this method straight-forward, but it can be adapted for, and applied to, numerous questions in the field of DSB repair and regulation in both meiotic and somatic tissues.

### **Materials and Reagents**

*Note: We indicated our vendors and cat numbers for lab reagents such as tips, tubes, and chemicals, however, any vendor will likely be suitable and sufficient.*

1. 0.2-20  $\mu$ l Pipette tips (VWR, catalog number: 89079-438)
2. Microscope slides, 1" x 3" x 0.4" (Surgipath-Leica, catalog number: 3800240)
3. Petri dishes, any 60 x 15 mm plate with ventilation ribs (Kord-Valmark, catalog number: 2901)
4. Glass bottom dishes: 35 mm dish with 14 mm glass microwell (No. 1.5 coverglass, MatTek, catalog number: P35G-0.170-14-C)
5. Parafilm, 4" x 125' (Parafilm M, catalog number: PM996)
6. Pasteur pipet, 5  $\frac{3}{4}$ " Flint Glass (Fisher Scientific, catalog number: 50-930-565)
7. General-Purpose Laboratory Labeling Tape,  $\frac{3}{4}$ " (VWR, catalog number: 89098-004)
8. 12 mm tube (any type of tube with a 12 mm diameter would work; VWR, catalog number: 20170-579)
9. Worm pick, hand-made by attaching platinum wire to a Pasteur pipette using fire
10. 0.1- $\mu$ m polystyrene beads (Polybead, Polysciences, catalog number: 00876, store at 4 °C)
11. Dissection blade (Stainless Steel Surgical Blades, 4-311)
12. *Escherichia coli* OP50 (from the Caenorhabditis Genetics Center)
13. Agarose (any brand of Agarose that is typically used for gel electrophoresis, e.g., VWR, catalog number: 9012-36-6)
14. Immersion oil, 100x/1.4 NA (Leica Microsystems, catalog number: 11513859)
15. Sodium chloride (NaCl) (RPI, catalog number: 7647-14-5)
16. Peptone (RPI, catalog number: 73049-73-7)
17. Cholesterol (Sigma-Aldrich, catalog number: 57-88-5)
18. Calcium chloride ( $\text{CaCl}_2$ ) (Sigma-Aldrich, catalog number: 10035-04-8)
19. Magnesium sulfate ( $\text{MgSO}_4$ ) (Sigma-Aldrich, catalog number: 7487-88-9)
20. Agar (RPI, catalog number: 9002-18-0)
21. Potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ) (RPI, catalog number: 7778-77-0)
22. Sodium phosphate dibasic ( $\text{Na}_2\text{HPO}_4$ ) (Sigma-Aldrich, catalog number: 7558-80-7)
23. Ethanol
24. Nematode growth medium (NGM) agar plate (see Recipes)
25. M9 buffer (see Recipes)
26. 10% agarose pads (see Recipes)

## Equipment

1. Microscope suitable for live imaging (such as Leica DMi8) with Andor MicroPoint 365 nm pulsed laser (Leica, model: Leica DMi8)
2. Humidity chamber (box with wet paper towels and elevated surface)
3. Microwave (any brand should work, we use Panasonic Inverter, model: NN-S543BFR)
4. Stereo microscope (Leica Microsystems, model: KL200 LED)
5. 20 °C incubator (Nor-Lake Scientific Refrigerated Incubator, LRI201WWW/0)
6. 37 °C incubator (Precision Scientific Thelco Incubator, model: 31483)
7. Autoclave
8. 4 °C refrigerator

## Software

1. MetaMorph Software (Molecular Devices, LLC, version 7.8.12.0)
2. Fiji (Fiji is just ImageJ) (Free to download at [imagej.net/Fiji](http://imagej.net/Fiji)) (Schindelin et al., 2012)
3. GraphPad Prism v6 (GraphPad Software, La Jolla California USA, [www.graphpad.com/](http://www.graphpad.com/))

## Procedure

### A. Worm preparation

Worm husbandry instruction can be found in WormBook (Stiernagle, 2006).

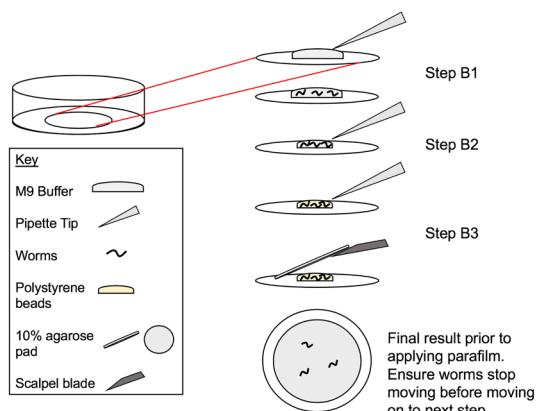
1. Pour 10 ml of NGM agar media (see Recipes) into 60 x 15 mm Petri dishes and allow to set for 2 days before seeding with *E. coli* OP50.
2. Seed with OP50 from a fresh starter and incubate at 37 °C overnight. Culture *C. elegans* strains to be used for live imaging experiments on these plates and maintain at 20 °C.
3. Pick L4 stage worms of the desired strain the day before the planned live imaging experiment so that worms are one-day old adults for the experiment. We move worms using a pick and identify the L4 stage by the shape of the vulva. However other methods such as synchronization by bleaching can be used (Porta-de-la-Riva et al., 2012).

### B. Live Imaging Sample Preparation

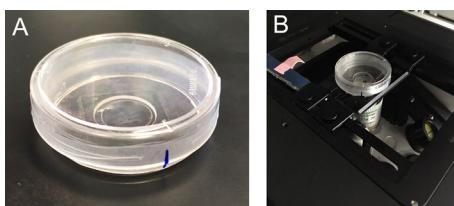
See Figure 1 for a visual representation of the following 3 steps.

1. Place 6 µl of M9 buffer in the center of the glass bottom dish to prepare the live imaging sample. Pick the desired number of worms and place into the drop of M9, taking care to transfer as little bacteria as possible.  
*Note: We typically place a maximum of 5 worms in the M9 buffer due to the limited number of stages we can save on the Leica DMi8.*

2. Gently remove as much of the M9 buffer as possible without drying out the worm and taking care not to suck up the worm into the pipette tip.
3. Place 0.75  $\mu$ l of polystyrene beads from stock onto the worm. Gently place a small, circular cut-out of the 10% agarose pad onto the worm(s), taking care not to drag the worms in the process, that will cover most of the exposed coverslip. We use a 12 mm tube to cut these circles. 10% agarose pad preparation is detailed under Recipe 3.  
*Note: This step is best done quickly to avoid desiccation of the worm(s). We use a dissection blade to lift and place the agarose pad.*
4. Observe the worms under a dissection microscope. Once worms have stopped moving underneath the pad (typically ~3 min), place the lid on top of the glass bottom dish and parafilm the lid onto the Petri dish (this prevents drying out of the agarose pad and the worm). Draw a vertical mark on the parafilm to use as a reference for setting of coordinates on the microscope (Figure 2).



**Figure 1. Live imaging sample preparation.** Steps B1-B3 from Section B of Procedures illustrating the steps for proper live imaging preparation prior to applying parafilm to the glass-bottom dish. Steps B1-B3 refer to the specific step in the procedures that the image is illustrating.



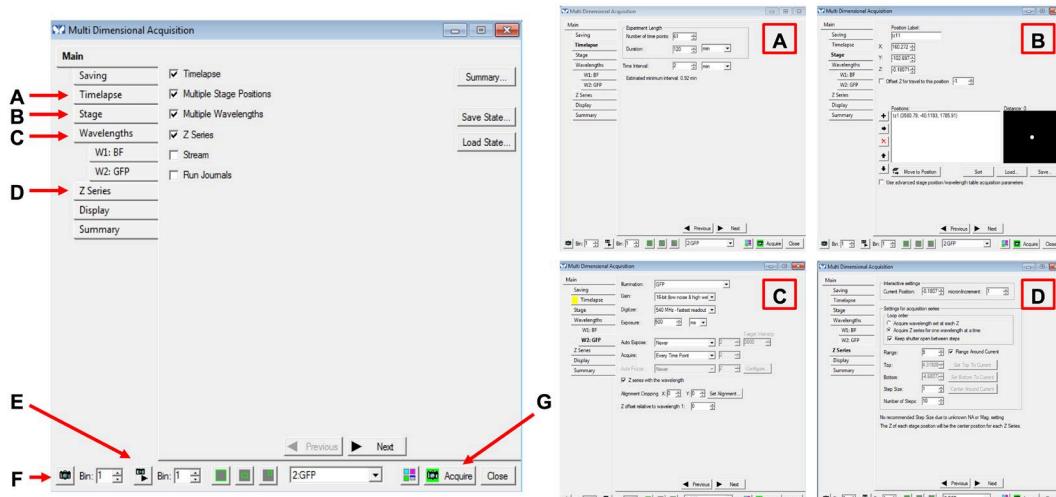
**Figure 2. Glass bottom dish set-up.** A. Glass bottom dish ready for imaging. B. Glass bottom dish on the microscope.

#### C. MetaMorph imaging setup and parameters

1. Imaging should be done in a room that maintains constant temperature at 20-23 °C and in the dark.

2. Turn on the Leica Inverted Microscope, as well as the Andor MicroPoint software. Ensure that the scope is fully on before opening the MetaMorph software.
3. Place the glass bottom dish on the scope and align your vertical reference mark on the parafilm to a location on the scope platform so that any XY-coordinates saved will be the same after the objective change (Figure 2B). Locate the worms using the 20x objective and save their XY-coordinates on the Leica Inverted Microscope. Remove the glass bottom dish before changing objectives.
4. Set the objective to 100x and place a small drop of 100x/1.4 NA immersion oil directly onto the objective lens. Ensure that magnification in the MetaMorph toolbar is set to 100x. Otherwise the microirradiation technology will not work. This will automatically raise the objective. Lower it before replacing the glass bottom dish on the platform.
5. Place glass bottom dish back onto the scope platform, using your vertical reference mark on the parafilm to orient the glass bottom dish exactly where it was before, ensuring that your saved XY-coordinates will be the same for all of your worms.
6. In MetaMorph, open up Apps > Multi Dimensional Acquisition (MDA). This will open a window that allows you to set the parameters for your time-lapse imaging (Figure 3).
7. Under the “Wavelengths” tab, specify the number of wavelengths for the experiment (e.g., 2 wavelengths, one for brightfield (BF) and one for GFP). A corresponding tab for each of these wavelengths will open and under each specify the exposure times. We use 20 ms for BF and 500 ms for GFP. Check the box for “Z series with the wavelength” to ensure that the Z series you set for the experiment will be acquired all the way through the set range in that specific wavelength (Figure 3C).
8. Set intensity of the light source to 5%.
9. To visualize the worm on the computer within the MetaMorph program, hit the button with the small camera and play icon (Figure 3E). This will pull up a live feed from the microscope which you can then use to set the appropriate stages.
10. Under the “Stages” tab of the MDA window set the stage, or location, of the area of the worm to target in X, Y, and Z planes. These stages can be saved to be revisited for adjustments, and these will be the stages that are automatically moved to and imaged during the acquisition period (Figure 3B).
11. Under the “Z Series” tab, specify the number of steps to be acquired around the central Z plane specified when setting the stage (the middle of the nucleus, in our case). The number of steps and the size of each step through the Z plane can be set under this tab (Figure 3D).
12. Under the “Timelapse” tab set the time interval and duration of experiment/number of time points (e.g., every 2 min for 60 min; Figure 3A). Be sure to take into account the number of stages you have and how many Z planes are acquired at each stage and allow for enough time between each interval acquisition for each stage to be acquired. It takes time to go through the planes at each step, and even more time if multiple wavelengths are selected.

13. Specify where the output from the program will be saved in the “Saving” tab in the MDA window.



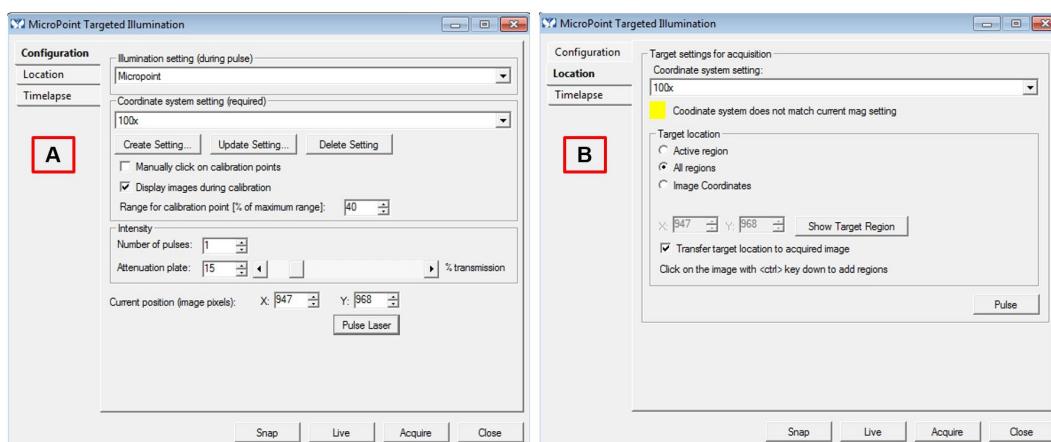
**Figure 3. Multi Dimensional Acquisition.** Screenshot of the Main configuration for movie acquisition (left) with corresponding tabs expanded (right).

#### D. Microirradiation

1. In MetaMorph, open up Devices > MicroPoint Targeted Illumination. This is used for microirradiation of the nuclei and can be set at various attenuations according to the type of DNA damage desired (e.g., Koury *et al.* [2018] uses 15% attenuation, however, microirradiation-induced foci can be observed with as low as 5%, although in reduced numbers).
2. For Koury *et al.* (2018) the following parameters were set:
  - a. “Configuration” tab (Figure 4A)
    - i. Illumination setting (during pulse): Micropoint
    - ii. Coordinate system setting (required): 100x
    - iii. Display images during calibration (box checked)
    - iv. Range for calibration point (% of maximum range): 40%
    - v. Number of pulses: 1
    - vi. Attenuation plate: 15% of transmission
  - b. “Location” tab (Figure 4B)
    - i. Coordinate system setting: 100x
    - ii. Target location: All regions
    - iii. Transfer target location to acquired image (box checked)
  - c. “Timelapse” tab
    - i. # of points: 6
    - ii. Interval: 1
    - iii. Units: microseconds
    - iv. Acquire to stack with current settings

3. In the MDA window, once all desired parameters have been set, hit “Acquire” (Figure 3G).
4. After the first time point is recorded, immediately pause the program. Visit each saved stage, take a snapshot (camera icon in the toolbar), and then use the circle selection tool from the toolbar to target selected nuclei. Hit pulse (“Location” tab, Figure 4B) after nuclei selection for each stage. Select nuclei and pulse for each stage before moving on to the next. Resume time lapse imaging and allow program to run through desired time frame.

*Note: Perform this step as quickly as possible to ensure that all stages were microirradiated at around the same time.*



**Figure 4. MicroPoint Targeted Illumination.** Screenshot of the “Configuration” (A) and “Location” (B) tabs for the MicroPoint Targeted Illumination parameters. Settings for microirradiation power and location of damage induction are configured in these two windows.

#### E. Recovery (optional)

Worms can only be viably maintained on the glass bottom dish for up to 2 h. Many experiments require analysis in later time points or analysis by other methods (e.g., dissection for immunofluorescence staining-based experiments). In this case, you will need to recover the worms.

1. Remove the parafilm from the glass bottom dishes to recover worms after microirradiation. Place 350 µl of M9 over the agarose pad.
  2. Gently lift the edge of the pad to allow M9 to wash underneath and lift the pad off of the worm(s).
- Note: We use the dissection blade mentioned in the glass bottom dish Preparation procedure to lift the agarose pad at this step.*
3. Use a glass Pasteur pipet to transfer the worm(s) to an NGM plate, to be used for any follow-up experiments.

#### Data analysis

#### A. Image export

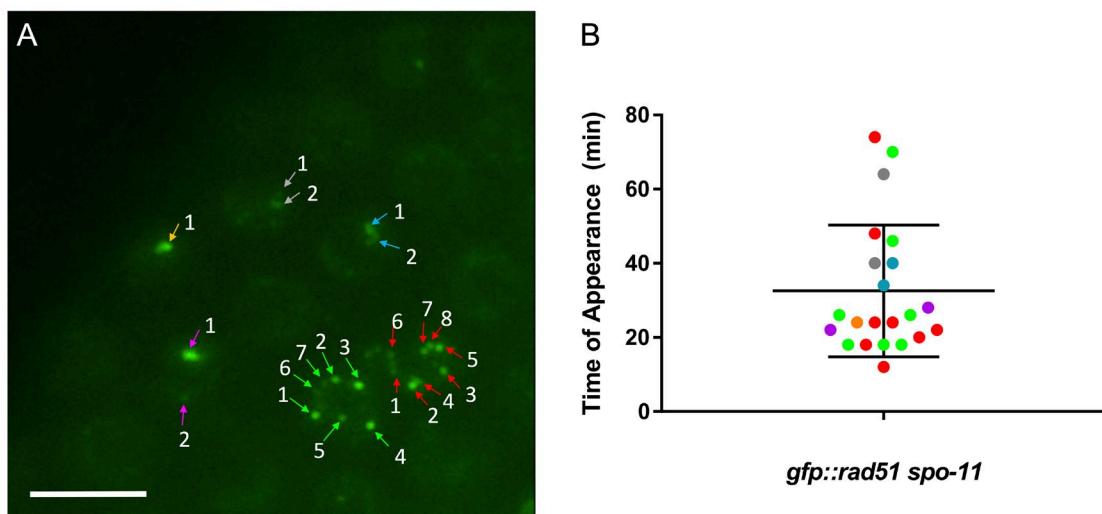
1. In MetaMorph, open Apps > Review Multi Dimensional Acquisition. Under this window highlight the fluorescent channel you wish to review. Number of time points and which Z planes to include in the exported file can be specified here.
2. Once desired time points and Z planes are chosen, hit the button “Create Rotation” under the “Z Projection” tab. Hit “Create” in the second window prompt. Save the selection as a TIFF for further analysis in Fiji (File > Save). See Video 1 for the resulting file.



**Video 1. GFP::RAD-51 in *spo-11* background.** Example output of the *gfp::rad-51; spo-11* strain before (first frame) and after microirradiation (all following frames). Green = GFP::RAD-51. A sample analysis of this same movie is presented in Figure 5.

#### B. Statistical analyses

Following manual quantification of foci in Fiji-ImageJ (see Figure 5), perform an unpaired nonparametric test (we used the Mann-Whitney test). If multiple comparisons are made in one experiment, the Kruskal-Wallis test should be performed to determine if any of the groups are significantly different than the others before performing pair-wise comparisons. Statistics can be performed with the GraphPad Prism software.



**Figure 5. Example data set.** A. Following data export from MetaMorph, images such as the one above can be analyzed in Fiji-ImageJ. Numbers indicate order of appearance of foci in each nucleus. See Video 1 for full video. Scale bar = 6  $\mu$ m. B. Quantification of time of appearance of each focus (in minutes) in all 6 microirradiated nuclei for the strain *gfp::rad-51 spo-11*. Colors correspond to the nucleus that the measurement was taken from in A. Error bars are average  $\pm$  SD (SEM can also be used). For more examples of quantified data output, see Koury *et al.* (2018).

## Notes

1. Limit exposure of the worms to the GFP channel when setting stages. Over-exposure can bleach the worms and your desired fluorescently-tagged protein if exposed for too long. Try to stay in bright field as much as possible. We typically see bleaching around an hour and a half into imaging with exposure to GFP occurring every 2 min.
2. Up to five worms can be imaged at the same time on the same glass bottom dishes at 2-min intervals, however any more than 5 worms, or at intervals less than 2 min, will likely result in bleaching of the worms before the end of the desired time interval. For shorter intervals, you may need to use less worms.

## Recipes

*Note:* For more detail see Stiernagle, (2006).

1. Nematode growth medium (NGM) agar plates
  - 3 g NaCl
  - 2.5 g Peptone
  - 1 ml of 5 mg/ml Cholesterol in ethanol
  - 1 ml 1 M CaCl<sub>2</sub>
  - 1 ml 1 M MgSO<sub>4</sub>
  - 20 g Agar

Add distilled H<sub>2</sub>O up to 1 L

Mix and autoclave (50 min at 250 °F) to sterilize

Let cool down to a little above room temperature before adding 25 ml 1 M KH<sub>2</sub>PO<sub>4</sub>

Ensure mix is at pH 6

Pour into Petri dishes and store at 4 °C

## 2. M9 buffer

Add all the following ingredients together in 1 L vessel

3 g KH<sub>2</sub>PO<sub>4</sub>

6 g Na<sub>2</sub>HPO<sub>4</sub>

5 g NaCl

1 ml of 1 M MgSO<sub>4</sub>

Add water up to 1 L

Sterilize by autoclaving (50 min at 250 °F) and store at room temperature

## 3. 10% agarose pads

20 ml M9 buffer

2 g agarose

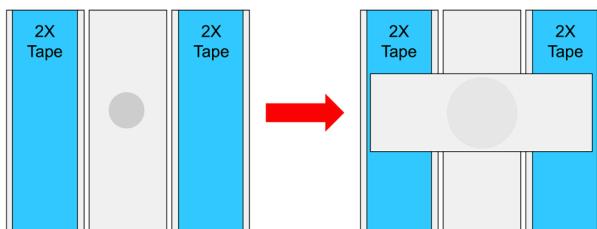
a. Stir 2 g of agarose into 20 ml of M9 to create a 10% agarose + M9 solution

b. Microwave the solution, stirring every 5 s, until the solution is clear and runny. Stir continuously before using for pad making to avoid any solidification. This will also prevent excessive accumulation of bubbles

c. Place two microscope slides with two layers of lab tape on top of each on either side of a clean microscope slide that does not have tape. Place a ½" glob of the agarose solution on the clear slide and immediately flatten with another clear microscope slide crosswise to the orientation of the bottom slide (see Figure 6)

*Note: Move quickly to avoid cooling of the agarose solution.*

d. Store in a humidity chamber to avoid drying out



**Figure 6. Agarose pad preparation.** Illustration of the pressing of heated agarose to form agarose pads for live imaging sample preparation as detailed in Recipe 3.

## Acknowledgments

This work was funded by the National Institutes of Health (NIH) [R01GM112657 to S.S.]. We thank Dylan Cooke for critically reading this manuscript. We are grateful to Yizhi Yin who helped in the initial stages of protocol development.

### **Competing interests**

The authors have no conflicts of interest or competing interests.

### **References**

1. Aten, J. A., Stap, J., Krawczyk, P. M., van Oven, C. H., Hoebe, R. A., Essers, J. and Kanaar, R. (2004). [Dynamics of DNA double-strand breaks revealed by clustering of damaged chromosome domains](#). *Science* 303(5654): 92-95.
2. Kong, X., Mohanty, S. K., Stephens, J., Heale, J. T., Gomez-Godinez, V., Shi, L. Z., Kim, J. S., Yokomori, K. and Berns, M. W. (2009). [Comparative analysis of different laser systems to study cellular responses to DNA damage in mammalian cells](#). *Nucleic Acids Res* 37(9): e68.
3. Koury, E., Harrell, K. and Smolikove, S. (2018). [Differential RPA-1 and RAD-51 recruitment in vivo throughout the \*C. elegans\* germline, as revealed by laser microirradiation](#). *Nucleic Acids Res* 46(2): 748-764.s
4. Porta-de-la-Riva, M., Fontrodona, L., Villanueva, A. and Ceron, J. (2012). [Basic \*Caenorhabditis elegans\* methods: synchronization and observation](#). *J Vis Exp*(64): e4019.
5. Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J. Y., White, D. J., Hartenstein, V., Eliceiri, K., Tomancak, P. and Cardona, A. (2012). [Fiji: an open-source platform for biological-image analysis](#). *Nat Methods* 9(7): 676-682.
6. Stiernagle, T. (2006). [Maintenance of \*C. elegans\*](#). *WormBook*: 1-11.

## Quantification of Mouse Hematopoietic Progenitors' Formation Using Time-lapse Microscopy and Image Analysis

Isabelle Bergiers<sup>1,\*</sup>, Christian Tischer<sup>2</sup>, Özge Vargel Böyükbaş<sup>1,\$</sup> and Christophe Lanclin<sup>1,\*</sup>

<sup>1</sup>European Molecular Biology Laboratory, EMBL Rome, Epigenetics and Neurobiology Unit Monterotondo, Italy; <sup>2</sup>European Molecular Biology Laboratory, EMBL Heidelberg, Advanced Light Microscopy Facility, Heidelberg, Germany; <sup>\$</sup>Current address: Department of Stem Cell and Regenerative Biology, Harvard University, Cambridge, MA, USA

\*For correspondence: [isabelle.bergiers@embl.it](mailto:isabelle.bergiers@embl.it); [christophe.lanclin@embl.it](mailto:christophe.lanclin@embl.it)



**[Abstract]** *In vitro* differentiation of mouse embryonic stem cells (mESCs) towards blood cells constitutes a well-established system to study the endothelial-to-hematopoietic transition (EHT) at the onset of blood development. Assessing the emergence of small non-adherent round blood cells in the culture without disturbing it is essential to evaluate the progression of EHT and also to test conditions potentially enhancing or repressing this process. Here, we describe how to quantify the formation of mouse hematopoietic progenitors during EHT in normal conditions or following over-expression of eight essential transcription factors using time-lapse microscopy and image analysis.

**Keywords:** Hematopoietic progenitors, Embryonic stem cells, Differentiation, Inducible cell line, Hemangioblast culture, Microscopy, Time-lapse imaging, Round cell counting

**[Background]** The first hematopoietic stem and progenitor cells (HSPCs) emerge from endothelial cells in the large arteries of the mouse embryo (de Bruijn *et al.*, 2000; Zovein *et al.*, 2008; Chen *et al.*, 2009;). This evolutionarily conserved event is called endothelial to hematopoietic transition (EHT). As a result of EHT, endothelial cells lose their specific markers, start to express hematopoietic genes, gain round morphology and eventually detach from the endothelial layer (Boisset *et al.*, 2010; Kissa and Herbomel, 2010; Rybtsov *et al.*, 2014). These specific series of events are used to detect EHT activity and monitor the progress of hematopoietic progenitor formation. The widely used method is to quantify endothelial and hematopoietic marker expressing cells. This can be done by gene expression analysis at the single-cell level (Bergiers *et al.*, 2018). Also, protein expression change can be used to assess EHT progression by flow cytometry analysis of endothelial markers such as VE-Cadherin, CD31 and hematopoietic markers such as CD41, CD45, CD43. For spatial information, the same markers can be used for immunofluorescent staining on the fixed tissue. However, all of these techniques require harvesting of the examined cells and therefore represent a single time-point. Besides, the switch between endothelial and hematopoietic gene expressions occurs gradually and in a heterogeneous way so that a marker analysis of a specific time-point is not enough to follow the transition. To complement the gene/protein expression based detection methods, there is a need of a protocol to monitor morphological changes and quantify the round hematopoietic cell progenitors arising through EHT.

To study the hematopoietic cell formation dynamics *in vitro*, researchers developed embryonic stem cell differentiation protocol (Keller *et al.*, 1993; Kennedy *et al.*, 1997; Sroczynska *et al.*, 2009). The protocol follows the developmental stages of hematopoiesis and recapitulates EHT *in vitro* (Choi *et al.*, 1998; Nishikawa *et al.*, 1998; Palis *et al.*, 1999). Briefly, mouse embryonic stem cells are cultured to form embryoid bodies containing the *in vitro* equivalent of hemangioblast, or blast colony forming cells (BL-CFCs), which is then isolated by cell sorting. Those cells grow and give rise to smooth muscle, endothelial and hematopoietic cells (Keller *et al.*, 1993; Faloon *et al.*, 2000). Time-lapse imaging capturing the BL-CFC culture allows us to visualize cells transitioning into hematopoietic progenitors in real-time. Unlike the endothelial and smooth muscle cells, the cells undergoing EHT become round and bud-off from the endothelial cell layer (Lancrin *et al.*, 2009). Here, by combining *in vitro* time-lapse imaging of an adherent BL-CFC culture with automatic image analysis we introduce a simple and efficient method to quantify those round cells during a culture period, which gives a direct measure of the number of cells undergoing EHT. This protocol enables us to easily test novel parameters affecting EHT rate such as over-expression of certain transcription factors (Bergiers *et al.*, 2018) or testing pathway inhibiting small molecules in the culture media (Vargel *et al.*, 2016). Below, we describe the details of the time-lapse microscopy of BL-CFC culture and image analysis to assess the number of cells underwent EHT.

### **Materials and Reagents**

1. Haemacytometre cover slips (Roth, catalog number: L189.1)
2. BD Falcon Conical Tubes, Polypropylene, 15 ml, high-clarity, dome-seal screw cap (BD Biosciences, catalog number: 352096)
3. BD Falcon Conical Tubes, Polypropylene, 50 ml, high-clarity, flat-top screw cap (BD Biosciences, catalog number: 352070)
4. Costar® 6-well cell culture multiple well plate, flat bottom, with lid (Corning, catalog number: 3506)
5. Millex-GP Syringe Filter Unit, 0.22 µm, polyethersulfone, 33 mm, gamma sterilized (Merck Millipore, catalog number: SLGP033RS)
6. Multi®-safety microcentrifuge tubes, SafeSeal® Tubes (Carl Roth, catalog number: 7080.1)
7. Stericup GP 0.2 µm/150 ml (Merck-Millipore, catalog number: SCGPU01RE)
8. Stericup GP 0.2 µm/500 ml (Merck-Millipore, catalog number: SCGPU05RE)
9. TipOne® 10 µl Graduated Filter Tip (Sterile), Refill (Starlab, catalog number: S1121-2710)
10. TipOne® 1,000 µl Graduated Filter Tip (Sterile), Refill (Starlab, catalog number: S1122-1730)
11. TipOne® 20 µl Graduated Filter Tip (Sterile), Refill (Starlab, catalog number: S1120-1710)
12. TipOne® 200 µl Graduated Filter Tip (Sterile), Refill (Starlab, catalog number: S1120-8710)
13. i8TFs mESC line (Bergiers *et al.*, 2018)
14. D4T endothelial cells (Choi *et al.*, 1998)
15. Ascorbic acid (Sigma, catalog number: A4544)

16. Bovine Serum Albumin (BSA) (Sigma, catalog number: A9418)
17. Doxycycline (Sigma, catalog number: D9891)
18. Fetal bovine serum (FBS) (PAA, catalog number: A15-102)
19. Gelatin (BDH, catalog number: 440454B)
20. IL-6 (R and D, catalog number: 406 ML)
21. IMDM (Lonza, catalog number: BE12-726F)
22. L-glutamine (Gibco, catalog number: 25030-024)
23. Monothioglycerol (MTG) (Sigma, catalog number: M6145)
24. Oxoid™ Phosphate Buffered Saline (PBS) Tablets (Thermo Scientific, catalog number: BR0014G)
25. Transferrin (Roche, catalog number: 10652202001)
26. VEGF (R and D, catalog number: 293-VE)
27. Distilled Water (Thermo Scientific, Gibco™, catalog number: 15230188)
28. 0.1% gelatin solution (see Recipes)
29. 10 mg/ml doxycycline stock solution (see Recipes)
30. 5 mg/ml ascorbic acid stock solution (see Recipes)
31. D4T endothelial cell supernatant (see Recipes)
32. PBS + 0.1% BSA solution (see Recipes)
33. 10 µg/ml VEGF stock solution (see Recipes)
34. 10 µg/ml IL6 stock solution (see Recipes)
35. Conditioned IMDM (see Recipes)
36. IMDM + 20% FBS (see Recipes)
37. MTG dilution (see Recipes)
38. BL-CFC culture medium (see Recipes)

## Equipment

1. Falcon® 10 ml Serological Pipet, Polystyrene, 0.1 Increments, Individually Packed, Sterile (Corning, catalog number: 357551)
2. Falcon® 2 ml Aspirating Pipet, Polystyrene, without Graduations, Individually Wrapped, Sterile (Corning, catalog number: 357558)
3. Falcon® 5 ml Serological Pipet, Polystyrene, 0.1 Increments, Individually Packed, Sterile (Corning, catalog number: 357543)
4. 37 °C, 5% CO<sub>2</sub> cell culture incubator (Thermo Scientific, model: Series II Water Jacket)
5. Biosafety cabinet (Tissue culture hood) (Thermo Scientific, model: MSC Advantage)
6. Brightfield inverted Microscope (Leica, model: DMIL LED Inverted)
7. Centrifuge (Eppendorf, model: 5810 R)
8. Computer (Dell Precision T3500)
9. IncuCyte HD (Essen Biosciences)

10. Neubauer counting chamber improved (Roth, catalog number: T729.1)
11. Pipetman P10, 1 to 10  $\mu\text{l}$  (Gilson, catalog number: F144802)
12. Pipetman Starter Kit (P20, P200, P1000) (Gilson, catalog number: F167300)
13. Pipette controller, PIPETBOY acu 2 (VWR, catalog number: 612-0928)
14. Standard -20 °C freezer (LIEBHERR, model: LCv 4010 MediLine)
15. Standard -80 °C freezer (Heraeus, model: HFU586 Top Freeze)
16. Standard fridge (LIEBHERR, model: LKUexv 1610 MediLine)
17. Vacuum pump BVC control (Vacuubrand, catalog number: 20727200)

## **Software**

1. CellProfiler (Kamentsky *et al.*, 2011; [www.cellprofiler.org](http://www.cellprofiler.org))
2. Fiji (Schindelin *et al.*, 2012; <http://fiji.sc/Fiji>)
3. IncuCyte software (Essen Biosciences, 2011A Rev2)

## **Procedure**

Prior to the start of the BL-CFC culture, ESCs are differentiated according to previously described protocols (Sroczynska *et al.*, 2009). Flk1 $^{+}$  cells are isolated using magnetic sorting following the manufacturer's instructions (Miltenyi Biotech). The procedure described below has been optimized for adherent BL-CFC culture allowing the use of time-lapse microscopy. The quantities and volumes listed were further adapted for the comparison of two culture conditions: with or without doxycycline.

*Note: Before starting, prepare working solutions as described in "Recipes". Careful, BL-CFC culture medium should be prepared fresh.*

### **Day 0**

1. Put 1 ml/well of 0.1% gelatin solution (see Recipes) into eight wells of Costar® 6-well cell culture plates (one plate of four wells for each line) and leave at room temperature (RT) for at least 20 min.
2. For each line, introduce 0.425 million Flk1 $^{+}$  cells obtained after embryoid body differentiation of ESCs and counted using a Neubauer counting chamber (Sroczynska *et al.*, 2009) into a 15-ml Falcon tube containing 5 ml of IMDM + 20% FBS.
3. Centrifuge at 290  $\times g$  for 5 min.
4. For each line, remove the supernatant using an aspirating pipet connected to the vacuum pump BVC control, gently tap the bottom of the tube to loosen the cell pellet and resuspend into 8.5 ml of BL-CFC culture medium.

*Note: Always use individual pipets and tips for each cell line to avoid cross-contamination.*

5. Aspirate the gelatin from the pre-coated Costar® 6-well cell culture plate wells using an

aspirating pipet.

6. Resuspend and evenly distribute 2 ml/well of cell suspension into four gelatin-coated wells for each cell lines.

*Note: This means that 0.1 million Flk1<sup>+</sup> cells are added per well.*

7. Agitate the plates parallel to the bench doing vertical and horizontal small jolting movements.

*Note: Careful not to do circular movements which would result in the formation of a vortex and, consequently, in all the cells going to the center of the well.*

8. Place the plate into a 37 °C, 5% CO<sub>2</sub> cell culture incubator for 24 h.

9. Keep the BL-CFC culture medium in the fridge for the doxycycline dilution on the next day.

*Note: The differentiation rate can be affected by cell confluence. Make sure the cells are equally spread across the surface of the wells to insure that the emergence of round blood cells will occur at the same rate everywhere in the wells.*

## Day 1

1. Prepare 0.2 µg/µl doxycycline dilution by adding 4 µl of 10 mg/ml doxycycline stock solution (see Recipes) to 196 µl of BL-CFC culture medium kept in the fridge from the day before. Mix well.

2. Take out the culture plates from the incubator and check under the microscope that the wells are all similar to each other.

3. Add 10 µl of 0.2 µg/µl doxycycline dilution to each +dox condition well (two wells for each line at a final doxycycline concentration of 1 µg/ml). Add the doxycycline directly in the center of the wells and mix directly after by agitating the culture plates carefully.

4. Add 10 µl of the BL-CFC culture medium to each -dox condition well as control (two wells for each line).

5. Put back the two plates inside the incubator and place them carefully into the IncuCyte HD microscope.

*Note: Don't write on top of the wells and avoid splashes of the medium on the lid to prevent malfunctioning of the IncuCyte HD as it takes pictures from the top.*

6. While the lid condensation is going away, set up the IncuCyte HD device using the IncuCyte software on the computer:

- a. Connect to the device and log in.

- b. Click on "Schedule Upcoming Scans" in the Task List panel on the left (see Figure 1 A).

- c. In the Drawer Setup window, select an empty vessel on the screen, right-click on the location where you inserted your first plate and click on "New" (see Figure 1).

- d. Keep this vessel selected and select the following settings in the Scan Setup tab, on the left side of the drawer map (see Figure 1 B):

Tray Type: Microplates

Vessel Type: 6-well Corning

Scan Type: Phase Contrast

**Scan Pattern:** Select the appropriate scan pattern or create one clicking on “Edit Scan Patterns” on the bottom left of the window (see Figure 1 C). Select 16 images/well for each 6-well.

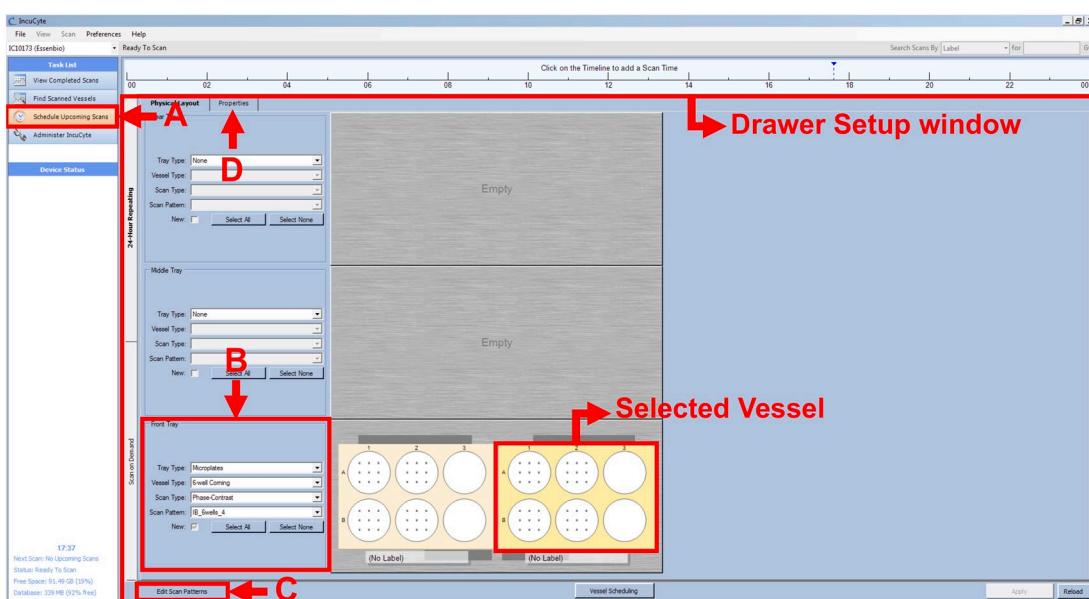
*Note: The IncuCyte can take up to 121 images per well in a 6-well plate format. However, by choosing this option, we would have increased the time of imaging considerably, therefore leading to time points spaced by at least two hours instead of the 15 min needed to capture the morphological changes occurring during the BL-CFC culture.*

- With the vessel selected, go to the Properties tab (see Figure 1 D) and fill the following fields (see Figure 2 A):

**Label:** Name of your experiment/vessel type

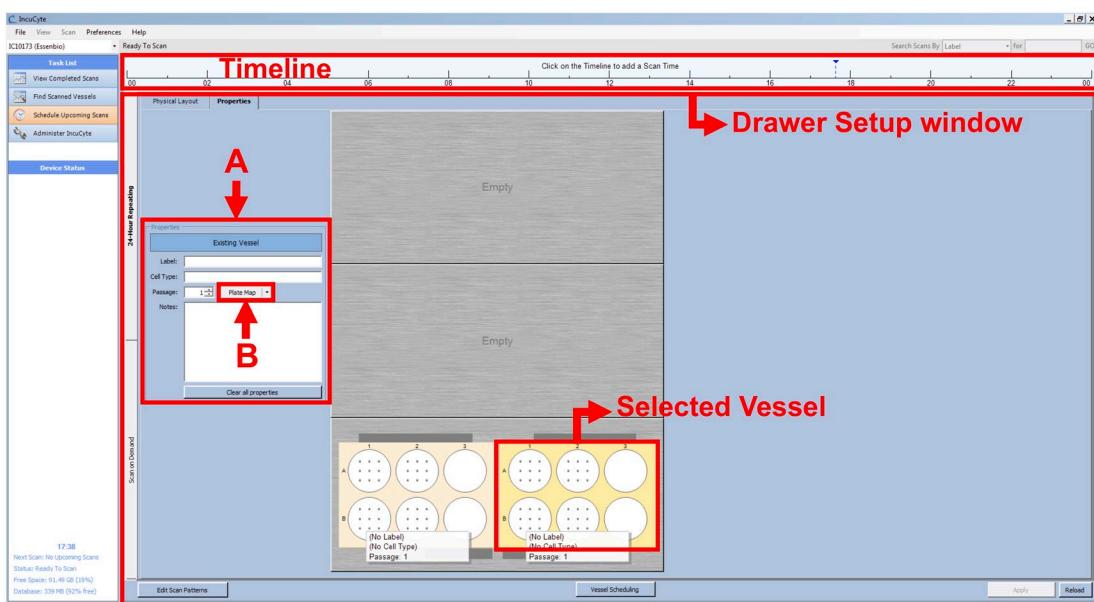
**Cell type:** Exact name of the cell line/cell type used

**Passage:** Number of passages

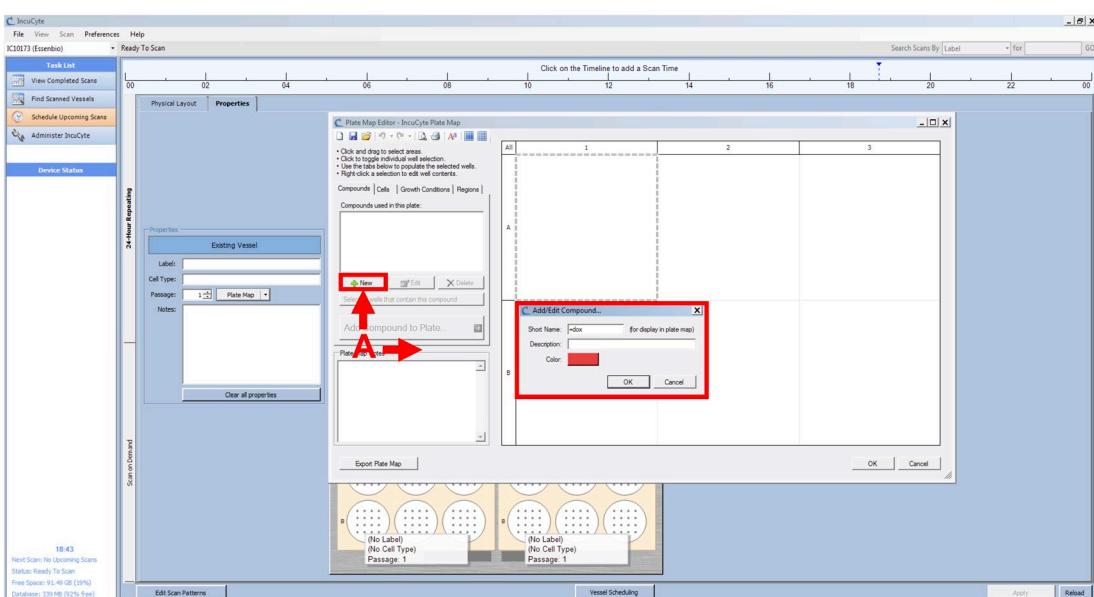


**Figure 1. IncuCyte setup**

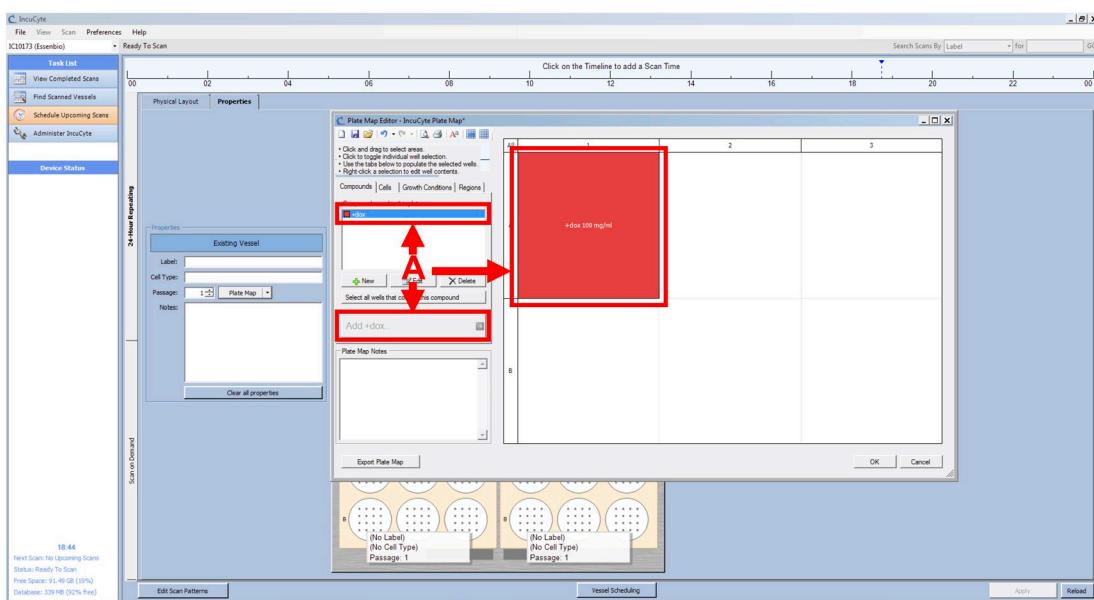
- Click on “Plate Map” (see Figure 2 B) and in the new window set up the plate map of your experiment (see Figure 3):
  - Add a new compound and name it “+dox” (see Figure 3 A).
  - Select the +dox compound, the wells that you treated with doxycycline and click on “Add +dox” (see Figure 4 A).
  - Select the concentration and click on OK.
  - Click on OK.



**Figure 2.** IncuCyte Properties setup



**Figure 3.** IncuCyte Plate Map initial setup

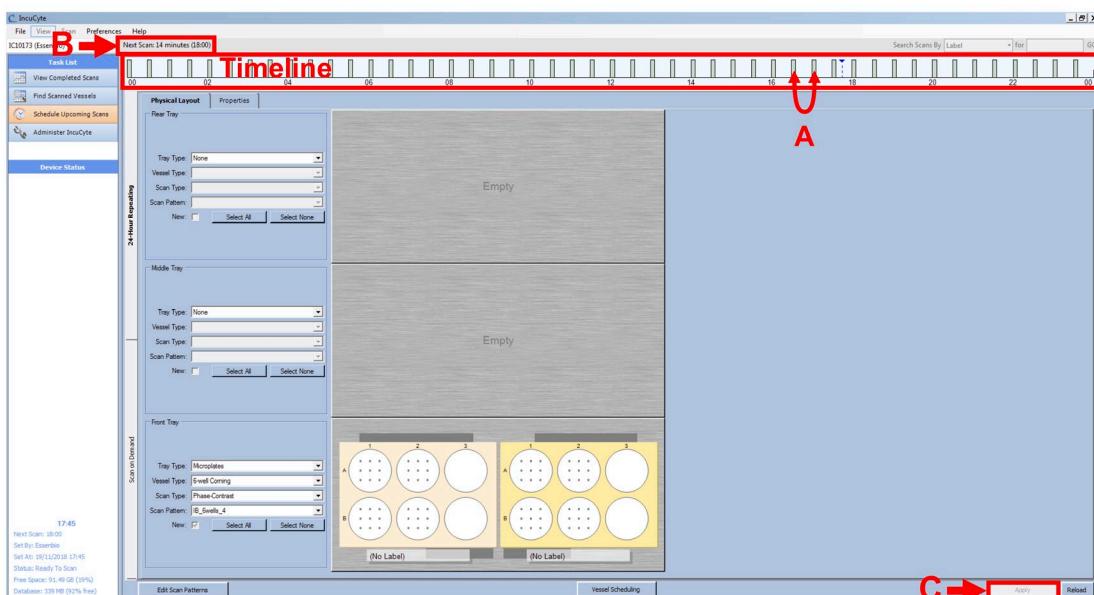


**Figure 4.** IncuCyte Plate Map setup

- g. Repeat the setup of the vessel (from Day1 Steps 6c-6f) for your second plate.
- h. Right-click on the upper timeline (see Figure 2) and click on “Set intervals”.
- i. Set intervals every 15 min starting from time 0, for a total of 24 h.

*Note: Even with a total of 24 h, the Incucyte device will scan your vessels every 15 min until you manually remove your vessels from the software.*

- j. Check the grey bars to make sure they are not overlapping (see Figure 5 A), check that IncuCyte device is not scanning (see Figure 5 B) and click on “Apply” (see Figure 5 C).



**Figure 5.** Set Intervals

*Note: When the plate is put in the incubator, the difference of temperature between the room and the incubator generates water condensation on the lid (as an example, see Figure 6 A). Wait at least 30 min before initiating the imaging so that the microscope can focus properly on the cells.*

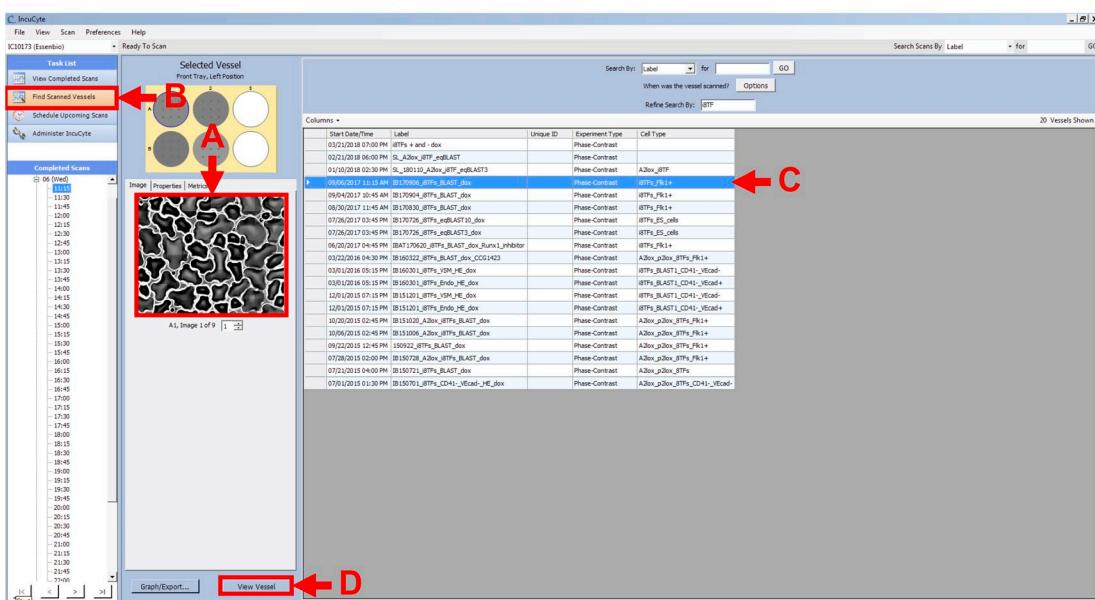
- Come back after 15 min to check that everything is working and switch off the computer if you wish.

### Day 3

- After 48-h treatment, click on “Schedule Upcoming Scans” in the Task List panel of the IncuCyte software.
- Right-click on the upper timeline and select “Delete Intervals”.
- Click on “Apply” to stop the scanning by IncuCyte.
- Take out your plates and proceed with flow cytometry analysis according to standard protocols.

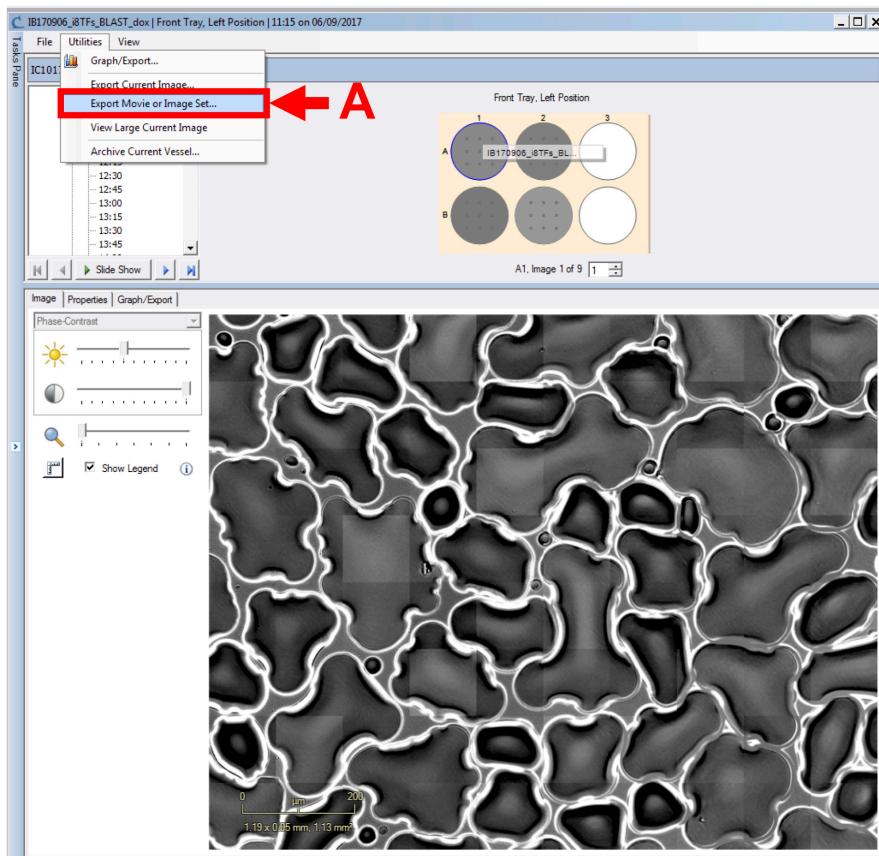
### Data analysis

- Export your images from the IncuCyte software:
  - Click on “Find Scanned Vessels” in the Task List panel of the IncuCyte software (see Figure 6 B).
  - Select one of your cell line vessels (see Figure 6 C) and click on “View Vessel” button in the lower right-hand corner of the screen (see Figure 6 D).



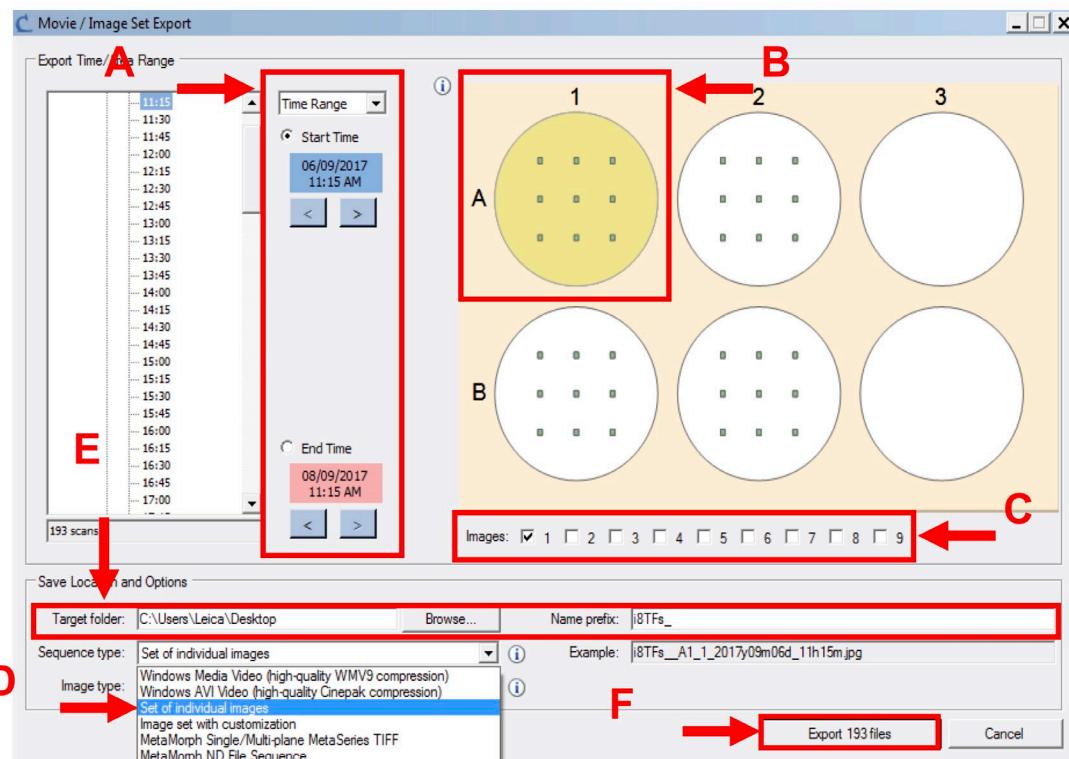
**Figure 6. View Vessels**

- c. Check that the scans are fine (no dust, condensation, etc.).
- d. Selecting “Export Movie or Image Set...” from the “Utilities” pull down menu (see Figure 7 A).



**Figure 7. Export image set**

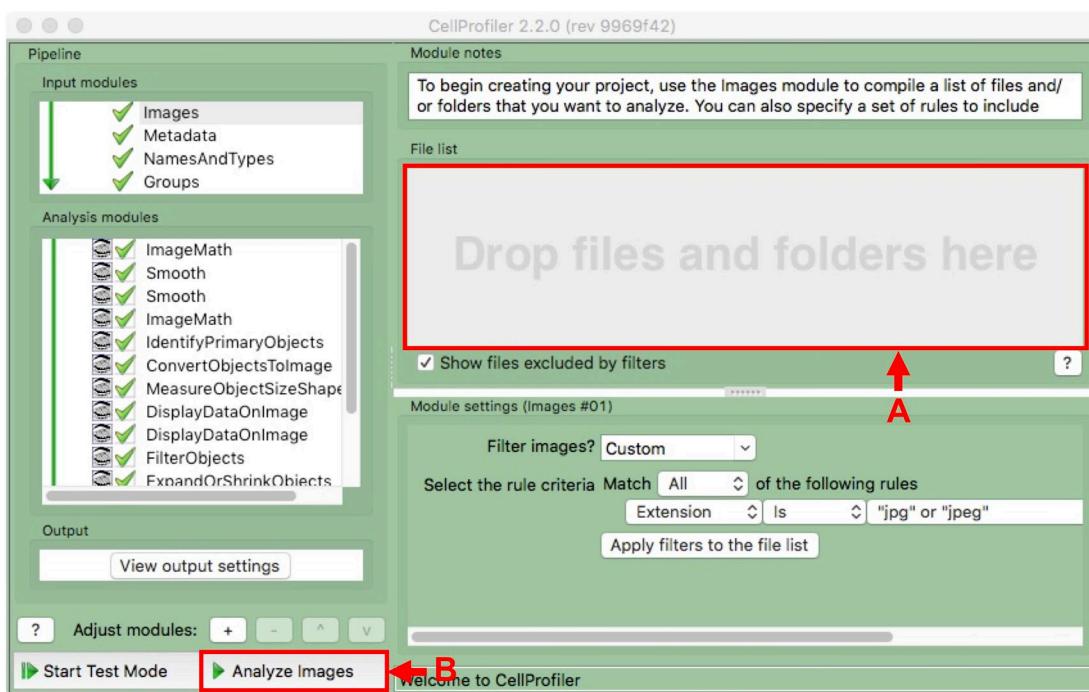
- e. In the new window, select the appropriate time frame (see Figure 8 A), the wells corresponding to one condition (see Figure 8 B), all images (see Figure 8 C), select the sequence type labeled “set of individual images” (see Figure 8 D) and export phase-contrast original image (.JPEG).
  - f. Specify the destination folder and the prefix of the files that will be generated (see Figure 8 E).
- Note: Create one folder per condition per cell line.*
- g. Click on “Export ... files” (see Figure 8 F).



**Figure 8. Final step of export image set**

- h. Repeat Steps 1a-1h for all the vessels and conditions to be analyzed (see [Supplemental File 1: Example dataset of IncuCyte images for the i8TF cell line](#) for an example dataset).
2. Analyze your images using CellProfiler software to get the number of round cells for each time-point, cell line and condition:
  - a. Install CellProfiler2.2.0, which can be downloaded from here: [http://cellprofiler.org/previous\\_releases/](http://cellprofiler.org/previous_releases/)  
*Note: You need Java as well: <http://cellprofiler.org/releases/>.*
  - b. Open CellProfiler and load the CellProfiler pipeline (see [Supplemental File 2: CellProfiler pipeline for round cell counting](#)) using *File > Import > Pipeline from File...* in the main menu of CellProfiler.
  - c. Drag and drop the whole IncuCyte export destination folder into the *File list* area of the *Images* module (see Figure 9 A).
  - d. Click on “Analyze images” on the bottom left to start processing (see Figure 9 B).

*Note: The pipeline has been developed to automatically generate an output folder named as the input folder with “-- analyzed” appended. The output folder will be generated in the folder containing the input folder. If you want to modify this, simply change the Output File Location to Default Output Folder in the SaveImages and ExportToSpreadsheet Analysis modules, and change the output folder name by selecting “View output settings” in the Output panel of the main window.*



**Figure 9. Image module of CellProfiler**

- e. Use the Browser to access the output “image.txt” file in the output folder (as an example, see [Supplemental File 3: CellProfiler output files for the example dataset](#)) and open it with Excel or in R to visualize the results as graphs. In the output .txt file, the *Count\_RoundCells* column contains the round cell numbers. The *ImageNumber* column contains the order in which the images were analyzed by CellProfiler. The *Metadata\_condition* column contains the prefix given while exporting the images from the IncuCyte software. The *Metadata\_day* column contains the day in which the images were taken. The *Metadata\_filename* column contains the file names of the images analyzed such as exported by the IncuCyte software. The *Metadata\_foldername* column corresponds to the input folder. The *Metadata\_position* column contains the position of the images inside the well. The *Metadata\_time* column contains the time in which the images were taken. The *Metadata\_well* column contains the name of the well from which the images were taken. Each row corresponds to one image.
- f. See Figures 10 and 11, and [Supplemental File 3: CellProfiler output files for the example dataset](#) for the analysis output of the example dataset.

**Notes:**

- i. The *Metadata extraction* described above relies on the IncuCyte file naming scheme. If this naming scheme is changing, e.g., due to version updates by IncuCyte, or because you are using a different microscope, it will not work. In such cases please contact us and we will help you adapting the *Metadata extraction* inside CellProfiler.
- ii. In the output folder, CellProfiler also generates copies of the individual images with all counted round cells marked by a yellow dot (Figure 10).

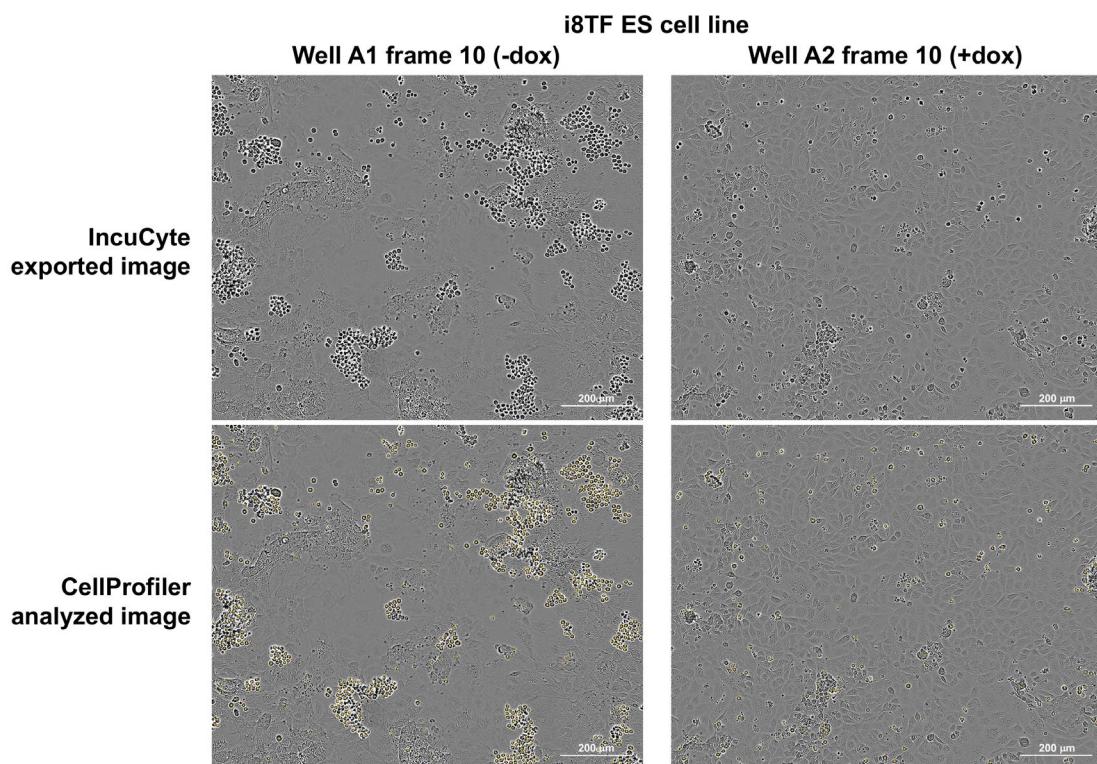


Figure 10. IncuCyte images before and after CellProfiler analysis

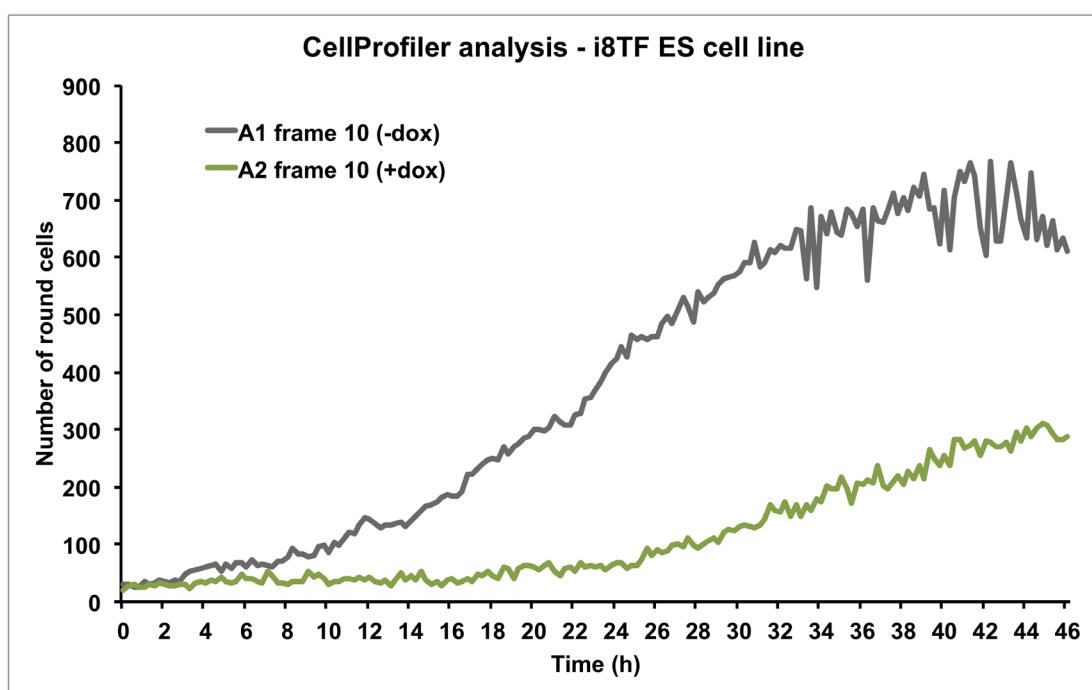


Figure 11. Graph showing the number of round cells over time as calculated by CellProfiler

3. Generate movies from your images using Fiji software to illustrate differences between round cell emergence rates and highlight other morphological variations:
  - a. Install Fiji software, which can be downloaded from here: <http://fiji.sc/Fiji>.
  - b. Open Fiji.
  - c. In your Browser, sort the IncuCyte exported files by Name, select all the files corresponding to the images taken from one frame of one condition for one cell line. By clicking on the last image, drag and drop all the files in Fiji. Let Fiji open the files without changing the order of the opened windows.
  - d. To create a stack, select *Image > Stacks > Images to Stack* and then click on OK in the new window.
  - e. Check the proper alignment of the stack slices by clicking on the *play* icon on the bottom left of the image. If the movie is readable, go directly to Step 3h.
  - f. To align the slices, select *Plugins > Image Stabilizer*. In the new window, insert the following values:

Transformation: Translation

Maximum Pyramid Levels: 1

Template Update Coefficient (0-1): 0.90

Maximum Iterations: 200

Error Tolerance: 0.0000001

Select *Output to a New Stack* and click on OK.
  - g. Check the slice alignment of the stabilized stack by clicking on the *play* icon on the bottom left of the image. If the movie is readable, go to Step 3h otherwise repeat Step 3f until all the slices are properly aligned (up to 3-4 times).
  - h. Save the movie by selecting *File > Save As > AVI....* In the new window, select JPEG Compression and 10 fps as Frame Rate.
  - i. See Videos 1 and 2 based on the example dataset.



**Video 1. BL-CFC culture in normal conditions.** The video is a 48-h-time-lapse microscopy

analysis of BL-CFC culture in absence of doxycycline.

**Video 2. BL-CFC culture following over-expression of eight key transcription factors.**

The video is a 48-h-time-lapse microscopy analysis of BL-CFC culture in presence of doxycycline, *i.e.*, following the over-expression of eight key transcription factors (Runx1, Cbfb, Gata2, Tal1, Fli1, Lyl1, Erg and Lmo2).

**Recipes**

*Note: Prepare solutions 1-9 in advance.*

1. 0.1% gelatin solution (stored at 4 °C)

0.2 g of gelatin

200 ml of PBS

*Note: Dissolve the powder, sterile filter with Stericup GP 0.2 µm/500 ml, aliquot and store at 4 °C.*

2. 10 mg/ml doxycycline stock solution (stored at -20 °C)

10 mg of doxycycline

1 ml of sterile distilled water

*Note: Dissolve the powder, aliquot and store at -20 °C. Always freshly thaw an aliquot.*

3. 5 mg/ml ascorbic acid stock solution (stored at -20 °C)

0.5 g of ascorbic acid

100 ml of distilled and sterile water

*Note: Dissolve the powder, sterile filter with a Stericup GP 0.2 µm/150ml, aliquot and store at -20 °C. Always freshly thaw an aliquot.*

4. D4T endothelial cell supernatant (stored at -20 °C)

Prepare D4T endothelial cell supernatant following the procedure described by Choi and colleagues (Choi *et al.*, 1998).

*Note: Prepare, aliquot and store at -20 °C. Once thawed, can be stored at 4 °C and used up to one month after.*

5. PBS + 0.1% BSA solution  
0.05 g of BSA  
50 ml of PBS  
*Note: Dissolve, sterile filter using Millex-GP Syringe Filter, aliquot and store at -20 °C.*
6. 10 µg/ml VEGF stock solution (stored at -80 °C)  
Dissolve a 10 µg vial in 1 ml of sterile PBS + 0.1% BSA solution  
*Note: Dissolve, aliquot and store at -80 °C. Once thawed, can be stored at 4 °C and used up to one month after.*
7. 10 µg/ml IL6 stock solution (stored at -80 °C)  
Dissolve a 10 µg vial in 1 ml of sterile PBS + 0.1% BSA solution  
*Note: Dissolve, aliquot and store at -80 °C. Once thawed, can be stored at 4 °C and used up to one month after.*
8. Conditioned IMDM (stored at 4 °C)  
1 bottle of IMDM  
5 ml of L-glutamine  
5 ml of Penicillin-streptomycin
9. IMDM + 20% FBS (stored at 4 °C)  
30 ml of FBS  
120 ml of conditioned IMDM  
*Note: Sterile filter using Stericup™ 150 ml bottle.*
10. MTG dilution (to be prepared fresh)  
13 µl of MTG  
1 ml of conditioned IMDM  
*Note: Careful, MTG is viscous. Mix well after dilution.*
11. BL-CFC culture medium (to be prepared fresh)  
14.49 ml of conditioned IMDM  
2 ml of FBS  
0.2 ml of L-glutamine  
0.12 ml of Transferrin  
0.06 ml of MTG dilution  
0.1 ml of 5 mg/ml acid ascorbic stock solution  
3 ml of D4T endothelial cell supernatant  
0.01 ml of 10 µg/ml VEGF stock solution  
0.02 ml of 10 µg/ml IL6 stock solution  
*Note: Prepare fresh in a 50 ml Falcon tube and sterile filter using Millex-GP Syringe Filter.*

### Acknowledgments

The EMBL Interdisciplinary Postdocs (EIPOD) Initiative (Post-doc fellowship) funded Isabelle

Bergiers. The European Molecular Biology Laboratory has funded this work.

### **Competing interests**

The authors do not have any conflicts of interests or competing interests.

### **References**

1. Bergiers, I., Andrews, T., Vargel Bolukbasi, O., Buness, A., Janosz, E., Lopez-Anguita, N., Ganter, K., Kosim, K., Celen, C., Itir Percin, G., Collier, P., Baying, B., Benes, V., Hemberg, M. and Lancrin, C. (2018). [Single-cell transcriptomics reveals a new dynamical function of transcription factors during embryonic hematopoiesis](#). *Elife* 7: e29312.
2. Boisset, J. C., van Cappellen, W., Andrieu-Soler, C., Galjart, N., Dzierzak, E. and Robin, C. (2010). [In vivo imaging of haematopoietic cells emerging from the mouse aortic endothelium](#). *Nature* 464(7285): 116-120.
3. Chen, M. J., Yokomizo, T., Zeigler, B. M., Dzierzak, E. and Speck, N. A. (2009). [Runx1 is required for the endothelial to haematopoietic cell transition but not thereafter](#). *Nature* 457(7231): 887-891.
4. Choi, K., Kennedy, M., Kazarov, A., Papadimitriou, J. C. and Keller, G. (1998). [A common precursor for hematopoietic and endothelial cells](#). *Development* 125(4): 725-732.
5. de Bruijn, M. F., Speck, N. A., Peeters, M. C. and Dzierzak, E. (2000). [Definitive hematopoietic stem cells first develop within the major arterial regions of the mouse embryo](#). *EMBO J* 19(11): 2465-2474.
6. Faloon, P., Arentson, E., Kazarov, A., Deng, C. X., Porcher, C., Orkin, S. and Choi, K. (2000). [Basic fibroblast growth factor positively regulates hematopoietic development](#). *Development* 127(9): 1931-1941.
7. Kamentsky, L., Jones, T.R., Fraser, A., Bray, M., Logan, D., Madden, K., Ljosa, V., Rueden, C., Harris, G.B., Eliceiri, K., Carpenter, A.E. (2011). [Improved structure, function, and compatibility for CellProfiler: modular high-throughput image analysis software](#). *Bioinformatics* 27(8):1179-1180.
8. Keller, G., Kennedy, M., Papayannopoulou, T. and Wiles, M. V. (1993). [Hematopoietic commitment during embryonic stem cell differentiation in culture](#). *Mol Cell Biol* 13(1): 473-486.
9. Kennedy, M., Firpo, M., Choi, K., Wall, C., Robertson, S., Kabrun, N. and Keller, G. (1997). [A common precursor for primitive erythropoiesis and definitive haematopoiesis](#). *Nature* 386(6624): 488-493.
10. Kiss, K. and Herbomel, P. (2010). [Blood stem cells emerge from aortic endothelium by a novel type of cell transition](#). *Nature* 464(7285): 112-115.

11. Lancrin, C., Sroczynska, P., Stephenson, C., Allen, T., Kouskoff, V. and Lacaud, G. (2009). [The haemangioblast generates haematopoietic cells through a haemogenic endothelium stage.](#) *Nature* 457(7231): 892-895.
12. Nishikawa, S. I., Nishikawa, S., Hirashima, M., Matsuyoshi, N. and Kodama, H. (1998). [Progressive lineage analysis by cell sorting and culture identifies FLK1+VE-cadherin+ cells at a diverging point of endothelial and hemopoietic lineages.](#) *Development* 125(9): 1747-1757.
13. Palis, J., Robertson, S., Kennedy, M., Wall, C. and Keller, G. (1999). [Development of erythroid and myeloid progenitors in the yolk sac and embryo proper of the mouse.](#) *Development* 126(22): 5073-5084.
14. Rybtsov, S., Batsivari, A., Bilotkach, K., Paruzina, D., Senserrick, J., Nerushev, O. and Medvinsky, A. (2014). [Tracing the origin of the HSC hierarchy reveals an SCF-dependent, IL-3-independent CD43<sup>-</sup> embryonic precursor.](#) *Stem Cell Reports* 3(3): 489-501.
15. Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.Y., White, D.J., Hartenstein, V., Eliceiri, K., Tomancak, P., Cardona, A. (2012). [Fiji: an open-source platform for biological-image analysis.](#) *Nat Methods* 9(7):676-682.
16. Sroczynska, P., Lancrin, C., Pearson, S., Kouskoff, V. and Lacaud, G. (2009). [In vitro differentiation of mouse embryonic stem cells as a model of early hematopoietic development.](#) *Methods Mol Biol* 538: 317-334.
17. Vargel, O., Zhang, Y., Kosim, K., Ganter, K., Foehr, S., Mardenborough, Y., Shvartsman, M., Enright, A. J., Krijgsveld, J. and Lancrin, C. (2016). [Activation of the TGF \$\beta\$  pathway impairs endothelial to haematopoietic transition.](#) *Sci Rep* 6: 21518.
18. Zovein, A. C., Hofmann, J. J., Lynch, M., French, W. J., Turlo, K. A., Yang, Y., Becker, M. S., Zanetta, L., Dejana, E., Gasson, J. C., Tallquist, M. D. and Iruela-Arispe, M. L. (2008). [Fate tracing reveals the endothelial origin of hematopoietic stem cells.](#) *Cell Stem Cell* 3(6): 625-636.

## Imaging Higher-order Chromatin Structures in Single Cells Using Stochastic Optical Reconstruction Microscopy

Jianquan Xu and Yang Liu\*

Biomedical Optical Imaging Laboratory, Departments of Medicine and Bioengineering, University of Pittsburgh, Pittsburgh, PA 15213, USA

\*For correspondence: [liuy@pitt.edu](mailto:liuy@pitt.edu)



**[Abstract]** Higher-order chromatin organization shaped by epigenetic modifications influence the chromatin environment and subsequently regulate gene expression. Direct visualization of the higher-order chromatin structure at their epigenomic states is of great importance for understanding chromatin compaction and its subsequent effect on gene expression and various cellular processes. With the recent advances in super-resolution microscopy, the higher-order chromatin structure can now be directly visualized *in situ* down to the scale of ~30 nm. This protocol provides detailed description of super-resolution imaging of higher-order chromatin structure using stochastic optical reconstruction microscopy (STORM). We discussed fluorescence staining methods of DNA and histone proteins and crucial technical factors to obtain high-quality super-resolution images.

**Keywords:** Higher-order chromatin, Epigenetic, Histone modification, Super-resolution, STORM

**[Background]** Recent advances in super-resolution imaging technique provide new potentials to observe the biological structures at the molecular scale. In particular, single-molecule localization based super-resolution technique such as stochastic optical reconstruction microscopy (STORM) has become a valuable tool to directly observe higher-order chromatin structure *in situ* down to ~20-30 nm resolution (Xu *et al.*, 2018). Although many detailed protocols have been devoted for STORM imaging in general, few have been focused on imaging higher-order chromatin structures. Chromatin has densely packed structure, and the nucleus of mammalian cells tends to be thicker than other membrane-based proteins. Such samples tend to give higher background and present more overlapping fluorescent emitters. Therefore, the proper optimization of nuclear staining for STORM imaging is critical to obtain high-quality super-resolution images of chromatin structure. A compromise in any of staining steps may lead to significant image artifacts and degradation in image resolution. Here we provide detailed protocols for STORM-based super-resolution imaging of higher-order chromatin structure marked by either DNA and histone proteins. We also provide detailed procedures on the conjugation of the fluorophores with secondary antibodies, for single-color and two-color STORM imaging. The protocol presented here is optimized for STORM imaging, we believe that this protocol can also be extended to most high-resolution fluorescence imaging of chromatin structures or other proteins of interest.

## **Materials and Reagents**

1. Microcentrifuge tube
2. Glass-bottom dish (World Precision Instruments, catalog number: FD3510)
3. NAP-5 size-exclusion columns (GE Healthcare, catalog number: 17-0853-02)
4. MCF-10A cells
5. DMEM/F12 (Invitrogen, catalog number: 11039-021)
6. Horse serum (Invitrogen, catalog number: 16050-122)
7. Pen/Strep (100x solution) (Invitrogen, catalog number: 15070-063)
8. EGF (Peprotech, 1 mg) (Resuspend at 100 µg/ml in sterile ddH<sub>2</sub>O. Store aliquots at -20 °C)
9. Hydrocortisone (Sigma-Aldrich, catalog number: H0888) (Resuspend at 1 mg/ml in 200-proof ethanol and store aliquots at -20 °C)
10. Cholera toxin (Sigma-Aldrich, catalog number: C-8052) (Resuspend at 1 mg/ml in sterile ddH<sub>2</sub>O and allow to reconstitute for about 10 min. Store aliquots at 4 °C)
11. Insulin (Sigma-Aldrich, catalog number: I-1882) (Resuspend at 10 mg/ml in sterile ddH<sub>2</sub>O containing 1% glacial acetic acid. Shake solution and allow 10-15 min to reconstitute. Store aliquots at -20 °C)
12. FluoSpheres™ Carboxylate-Modified Microspheres, 0.1 µm, yellow-green fluorescent (505/515) (Thermo Fisher Scientific, catalog number: F8803)
13. TetraSpeck™ Microspheres, 0.1 µm, fluorescent blue/green/orange/dark red (Thermo Fisher Scientific, catalog number: T7279)
14. Phosphate buffered saline (PBS) (Lonza)
15. Triton X-100 (Sigma-Aldrich, catalog number: T8787)
16. Bovine serum albumin (BSA) (Sigma-Aldrich, catalog number: A9647)
17. Paraformaldehyde (Sigma-Aldrich, catalog number: P6148)
18. Primary antibody
  - Rabbit anti H3K4me3 antibody (EMD Millipore, catalog number: 07-473)
  - Mouse anti H3K9ac antibody (Abcam, catalog number: ab12179)
19. Secondary antibodies
  - Donkey anti-rabbit antibody (Jackson ImmunoResearch, catalog number: 711-005-152)
  - Donkey anti-mouse antibody (Jackson ImmunoResearch, catalog number: 715-005-151)
20. Alexa 405 carboxylic acid succinimidyl ester (Thermo Fisher Scientific, catalog number: A30000)
21. Alexa 647 carboxylic acid succinimidyl ester (Thermo Fisher Scientific, catalog number: A20006)
22. Cy2 and Cy3B reactive dye (GE Healthcare, catalog numbers: PA22000, PA63101)
23. Dimethylsulfoxide (DMSO; anhydrous)
24. Click-iT™ EdU Alexa Fluor™ 647 Imaging Kit (Thermo Fisher Scientific, catalog number:

C10340)

25. Glucose (Sigma-Aldrich, catalog number: G7021)
26. Glucose oxidase from Aspergillus niger-Type VII (Sigma-Aldrich, catalog number: G2133)
27. Catalase from bovine liver-lyophilized powder (Sigma-Aldrich, catalog number: C40)
28. 1 M Tris, pH 8.0
29. NaCl (Sigma-Aldrich, catalog number: S9888)
30. 2-mercaptoethanol ( $\beta$ -ME) (Sigma-Aldrich, catalog number: 63698)
31. Cysteamine (MEA) (Sigma-Aldrich, catalog number: 30070)
32. NaHCO<sub>3</sub> (Sigma-Aldrich, catalog number: S5761)
33. Culture medium for MCF-10A (see Recipes)
34. Washing buffer (see Recipes)
35. Blocking buffer (see Recipes)
36. STORM Imaging buffer (see Recipes)

### **Equipment**

1. Olympus IX71 inverted microscope frame with an oil-immersion objective (100x, NA = 1.4, UPLSAPO 100XO; Olympus, model: IX71)
2. sCMOS camera (pco.edge 4.2, PCO-TECH)  
Two-color dSTORM images were acquired on a custom system built upon an Olympus IX71 inverted microscope frame with an oil-immersion objective and an sCMOS camera attached on the side camera port. The 0.5x adaptor was used, such that each pixel on the camera corresponds to 130 nm on the sample plane (the pixel size of approximately 80-160 nm is acceptable).
3. Objective nanopositioner (Mad City Labs, model: Nano-F100S)
4. N-STORM system (Nikon Instruments)
5. NanoDrop 2000 microspectrophotometer (Thermo Fisher Scientific, model: NanoDrop™ 2000)
6. Rocking platform

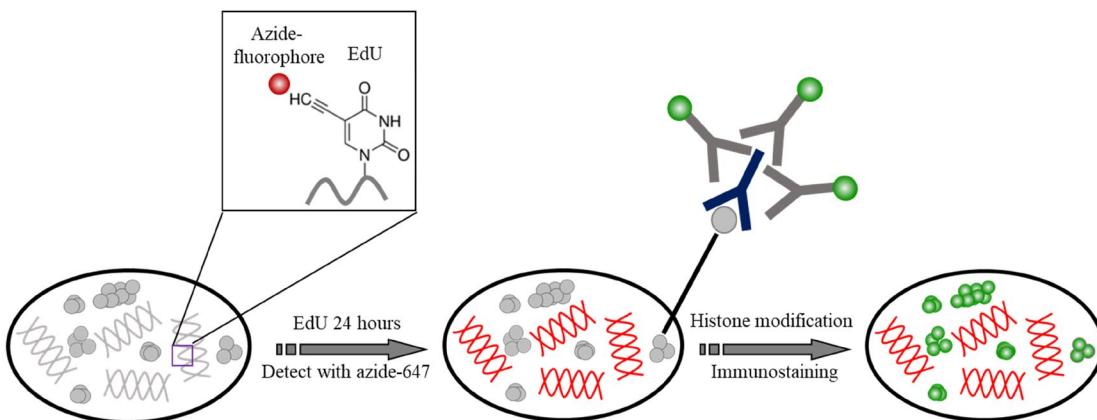
### **Software**

1. ImageJ (National Institutes of Health, <https://imagej.nih.gov/ij/download.html>)
2. ThunderSTORM plug in Ovesný et al. (2014) (downloadable at <https://github.com/zitmen/thunderstorm>)
3. Labview (National Instrument)

### **Procedure**

#### A. Conjugation of photo-switchable fluorophores to secondary antibody

1. Dissolve Alexa 405 (1.0 mg tube) and Alexa 647 (tube) in 100  $\mu$ l DMSO, divide into 50 aliquots containing 0.02 mg for each. Dissolve Cy2 (1 pack) and Cy3B (1 pack) in 20  $\mu$ l DMSO and divide into 10 aliquots. For long-term storage, lyophilize and store at -20 °C under dry conditions.
  2. Take one tube of each dye above (lyophilized powder), add 40  $\mu$ l DMSO to Alexa 647 tube (0.5  $\mu$ g/ $\mu$ l), add 10  $\mu$ l DMSO to Alexa 405 (2  $\mu$ g/ $\mu$ l), Cy2 and Cy3B tubes (10  $\mu$ g/ $\mu$ l), dissolve properly.
  3. Prepare fresh 0.5 M NaHCO<sub>3</sub> solution by dissolving 0.021 g NaHCO<sub>3</sub> into 5 ml H<sub>2</sub>O.
  4. To conjugate secondary antibodies with single fluorophore Alexa 647 or Cy3B for dSTORM imaging, thoroughly mix 40  $\mu$ l donkey anti-rabbit/mouse antibody, 10  $\mu$ l of 0.5 M NaHCO<sub>3</sub>, and 2  $\mu$ l Alexa 647 dilution from Step A2 and incubate for 30 min at room temperature, protected from light. Similarly, mix 40  $\mu$ l donkey anti-rabbit/mouse antibody, 10  $\mu$ l NaHCO<sub>3</sub>, and 1  $\mu$ l Cy3B dilution from Step A2, and incubate under the same conditions.
  5. To conjugate secondary antibodies with dye pairs: Mix 40  $\mu$ l donkey anti-rabbit/mouse antibody, 10  $\mu$ l NaHCO<sub>3</sub>, 5  $\mu$ l Cy2 dilution from Step A2, and 1  $\mu$ l Alexa 647 dilution from Step A2, and incubate at room temperature for 30 min, protected from light. Similarly, mix 40  $\mu$ l donkey anti-rabbit/mouse antibody, 10  $\mu$ l NaHCO<sub>3</sub>, 5  $\mu$ l Alexa 405 dilution from Step A2, and 1  $\mu$ l Alexa 647 dilution from Step A2, and incubate under the same conditions.
  6. During the reaction, wash the NAP-5 size-exclusion columns (see Materials and Reagents) three times by 1,000  $\mu$ l PBS. When the reaction is complete, add 150  $\mu$ l PBS to bring the reaction volume to 200  $\mu$ l.
  7. Add the entire reaction solution to the columns and allow the liquid to be completely absorbed into the column. Add 550  $\mu$ l PBS to wash, then add another 300  $\mu$ l PBS and collect the fluorophore-conjugated antibody in a microcentrifuge tube.
  8. Measure the absorbance of the fluorophore-conjugated secondary antibody using a NanoDrop 2000 Spectrophotometer to calculate the conjugation efficiency. Measure the absorbance of antibody at 280 nm, Alexa 405 at 401 nm, Cy2 at 489 nm, Cy3B at 559 nm, and Alexa 647 at 650 nm. Store the labeled antibody fraction at 4 °C. For long-term storage, aliquot and store at -20 °C.
- B. Two-color staining of DNA and histone modifications (see Figure 1. Procedure takes example of DNA and H3K4me3)



**Figure 1. The schematic of the staining of DNA and histone modifications.** DNA was incorporated with EdU and detected with Azide Alexa 647. Histone modification was then immunolabeled with Cy3B.

1. Plate MCF-10A cells on glass bottom dish at confluence 50%-70%, for the WPI (FD3510) dishes used in this protocol, place 200  $\mu$ l cell suspension (~50 cells/ $\mu$ l) in the well. Incubate overnight.
2. Add EdU to the cells at final concentration 1  $\mu$ M, total volume 200  $\mu$ l, incubate for 24 h.  
*Note: EdU concentration needs to be optimized for different samples or experimental conditions. We fully labeled DNA, by incubation throughout a complete cell cycle. For STORM imaging, we suggest using lower concentration (0.5-2  $\mu$ M) compared to the manufacturer's recommendation of 10  $\mu$ M. Over-labeling may cause extremely high blinking density and high background at the STORM imaging condition, and result in poor image resolution and artifacts.*
3. Fix cells with 4% PFA for 15 min, wash 3 times with PBS.  
*Note: Other commonly used alcohol fixative can also be used, such as methanol/ethanol (1:1) or methanol/acetone (1:1). Our test showed comparable higher-order chromatin structures compared to those from 4% PFA (Similar cluster size and density. Please refer to Xu et al., 2018).*
4. Permeabilize with 0.2% Triton X-100 in PBS for 10 min, wash 3 times with PBS. This step is not required if alcohol fixative was used.
5. Prepare Click-iT Plus reaction cocktails as follows. For a total volume of 500  $\mu$ l, cocktails contain 440  $\mu$ l 1x Click-iT reaction buffer, 10  $\mu$ l copper protectant, 1.2  $\mu$ l Alexa 647 picolyl azide, and 50  $\mu$ l reaction buffer additive. All components were provided by the manufacturer's imaging kits.  
*Note: Similar to EdU-based labeling, the concentration of Alexa 647 picolyl azide may also need adjustment to achieve the optimized labeling density for dSTORM imaging.*
6. Add Click-iT Plus reaction cocktails to cells and incubate for 30 min, protect from light. Wash 3 times with washing buffer.
7. Incubate cells with 150  $\mu$ l rabbit anti-H3K4me3 primary antibody diluted in blocking buffer

- (1:600) at 4 °C overnight.
8. Wash cells 3 times with washing buffer, 5 min per wash.
  9. Incubate with Cy3B conjugated donkey anti-rabbit secondary antibody diluted in blocking buffer for 2 h at room temperature, protected from light.

*Note: Two-color dSTORM imaging needs simultaneous labeling of two fluorophores at two distinct wavelengths. Alexa 647 and Cy5 are often the first choice as they perform the best blinking properties in the red/near-infrared channel; for the second channel, based on our experience, Cy3B and CF568 perform better blinking behavior compared to other dyes with the similar wavelength range.*

10. Wash cells 3 times with washing buffer, 5 min per wash.
11. Postfix with 4% PFA for 10 min, wash with PBS.
12. Deposit FluoSpheres™ Carboxylate-Modified Microspheres (Materials and Reagents #12) to the culture dish for drift correction. Dilute fluorescent beads at 1:500,000 in PBS, add 200 µl beads solution to the dish, deposit for 30 min, then switch to PBS. Samples are ready for imaging.

*Note: Concentration and deposition time of the beads are adjustable, to ensure at least 5 fluorescent beads in the field of view for drift correction (Principles were discussed previously, Ma et al., [2017]).*

- C. Two-color staining of different histone modifications (H3K9ac and H3K4me3)
1. Plate MCF-10A cells on glass bottom dish at confluence 50%, incubate overnight.
  2. Fix cells with 4% PFA for 15 mi, wash 3 times with PBS.
  3. Permeabilize with 0.2% Triton X-100 for 10 min, wash 3 times with blocking buffer.
  4. Incubate cells with two primary antibodies (Mouse anti H3K9ac antibody, rabbit anti H3K4me3 antibody) simultaneously diluted in blocking buffer at 4 °C overnight.
- Note: High efficiency and specificity of the primary antibody are critical for high-quality super-resolution imaging. We recommend testing ChIP grade or Knockout (KO) validated antibodies for high specificity (low background) and sufficient localization number (~ > 20 localizations per cluster), prior to biological experiments.*
5. Wash cells 3 times with washing buffer, 5 min per wash.
  6. Incubate with secondary antibodies (dye pair Alexa 405-647 conjugated donkey anti-mouse secondary antibody, Cy2-Alexa 647 conjugated donkey anti-rabbit secondary antibody) diluted in blocking buffer for 2 h at room temperature; Protect from light.
  7. Wash cells 3 times with washing buffer, 5 min per wash.
  8. Postfix with 4% PFA for 10 min, wash with PBS.
  9. Deposit fluorescent beads to the culture dish for drift correction. Dilute fluorescent beads at 1:500,000, deposit for 30 min, then switch to PBS. The samples are ready for imaging.
- Note: If a commercial N-STORM system is used (e.g., N-STORM, Nikon), it is equipped with the focus lock system for drift correction, this step may not be necessary.*

#### D. STORM imaging

1. Prepare STORM imaging buffer freshly.

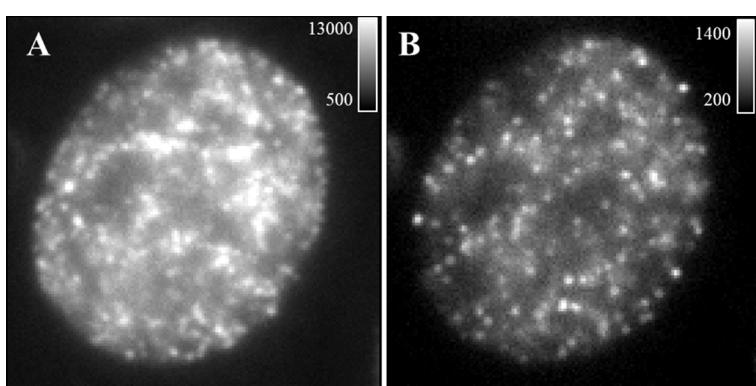
*Note: For dSTORM imaging, β-ME based buffer is preferred; for two-color STORM using dye pairs, MEA based buffer is preferred. See Recipes section below.*

2. Add STORM imaging buffer to the sample and put the sample on the microscope stage.
3. Use lower laser power (~1 mW) to identify the imaging objects, adjust focal plane to find the clearest nuclear periphery.
4. Increase the laser power to maximum (~2-10 kW·cm<sup>-2</sup> power density) to turn “off” fluorescent molecules and trigger photo-switching (see Video 1).

*Note: If the blinking is too dense (significant overlapping emitters and high background), bleach the sample for 30 sec-3 min before data acquisition. If after bleaching for several minutes, the blinking is still too dense, sample re-preparation with lower labeling density may be needed. See Figure 2.*



**Video 1. Imaging set up and real-time data acquisition of STORM imaging**



**Figure 2. Representative single frame of raw data.** A. An example of a single-frame image of the raw data with insufficient bleaching (dense emitters and high background). B. An example of a single frame of the raw data after bleaching for 2 min with acceptable emitter

density and background. Calibration bar shows photon counts.

## 5. Data acquisition

### a. Two-color dSTORM imaging

Continuous illumination with 642 nm and 561 nm lasers is used in the two-color dSTORM imaging. The two channels are imaged sequentially at an exposure time of 20 ms for 30,000 frames using 642 nm excitation, followed by 30,000 frames using 561 nm excitation. One hundred nm yellow-green fluorescent beads are used as fiduciary markers on the coverslip to correct for 3D system drift every 200 frames as previously described (Ma *et al.*, 2017; Xu *et al.*, 2017). Low power (1 to 100  $\mu$ W) of 405 nm can be added during data acquisition to facilitate the blinking if the “on” molecule in each frame becomes very sparse.

#### Notes:

- i. *For drift correction, at the beginning of the experiment, move the position of the objective nanopositioner to the focal plane where at least 4-5 fiducial markers (e.g., fluorescent beads) are visible; then record a set of 2D images of the fiducial markers at different axial positions around the focal planes set by the objective nanopositioner control software written in Labview; the initial position of fiducial markers is determined using the method described in our previous publication (Ma *et al.*, 2017). During data acquisition, for every 200 frames (~4 s), the axial position of the objective was moved to the initial position of the fiducial markers (with excitation wavelength of 488 nm) determined at the beginning of the experiment via objective nanopositioner, and take a 2D image of the fiducial markers and the patterns of point spread function from the individual fiducial markers are used to track the axial position of the samples; and adjust the focal plane of the samples accordingly as described in detail in our previous publication (Ma *et al.*, 2017). The entire process is controlled using the software written in Labview.*
- ii. *If a commercial STORM system is used (e.g., N-STORM, Nikon), it should be equipped with the focus lock for drift correction, based on the reflection between coverslip and mounting media. If there is a large mismatch of the refractive index between the coverslip ( $n = 1.515$ ) and the mounting media (if aqueous medium,  $n = \sim 1.34$ ), it will work well.*
- iii. *For two-color dSTORM imaging using Alexa 647 and Cy3B, it is preferred to image the Alexa 647 channel first, since the 561-nm laser will strongly bleach the Alexa 647 dye if Cy3B is imaged first.*

### b. Two-color STORM imaging based on dye pairs

For two-color STORM imaging, 405-nm and 488-nm lasers are used as activation lasers, and 647-nm laser is used as imaging laser. The sample is periodically illuminated using a sequence of activation-imaging laser pulse cycles. In each cycle, one of the activation lasers is turned on for one frame (activation frames), followed by three continuous frames

of 647-nm imaging laser (imaging frames), acquire ~10,000 cycles for each color (a total of 40,000 frames). The imaging frame immediately after the activation pulse (imaging frame 1) is recognized as a controlled activation event, and used for the image reconstruction.

Take an example of two-color STORM imaging using dye pairs Alexa 405-647 and Cy2-Alexa 647. Two activator lasers (405 nm and 488 nm) are pulsed on: when the 405-nm laser is pulsed on (at low power) to turn on the Alexa 405-647 channel, the imaging laser (647 nm) is turned on (at maximum power) for image acquisition in Color 1 channel; when the 488-nm laser is pulsed on (at low power) to turn on the Cy2-Alexa 647 channel, the 647-nm imaging laser is turned on for image acquisition in Color 2 channel.

*Note: It is important to adjust the power of activator laser (405 nm and 488nm) to optimize the “on-off” switch and emitter density of the imaging frames. When the activator laser is on, appropriate density of blinking should be seen; when the activator laser is off, very few molecules should be at the “on” state. See Figure 3B.*

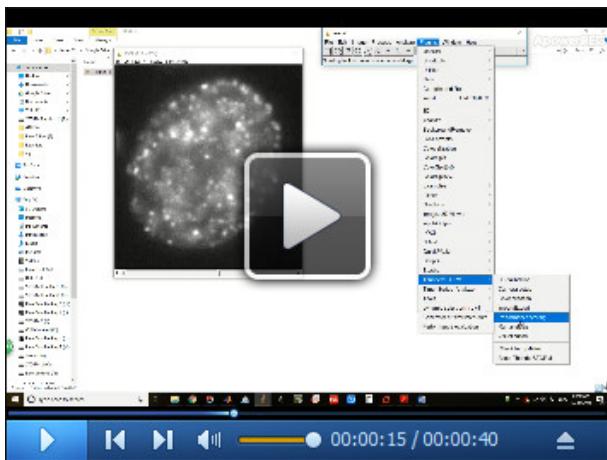
## **Data analysis**

### A. Image reconstruction of dSTORM

A final STORM image is reconstructed from the accumulated positions by Gaussian fitting of single fluorescent emitters (“on” molecules) recorded at each frame. Image reconstruction software is generally provided by the commercial STORM system (e.g., N-STORM). A lot of open-source image reconstruction software is available (Henriques *et al.*, 2010; Wolter *et al.*, 2012; Min *et al.*, 2014; Ovesný *et al.*, 2014). We recommend ThunderSTORM (Ovesný *et al.*, 2014). Briefly as follows:

1. Import the entire image sequence of raw data into ImageJ.
2. Set camera parameters (e.g., pixel size, photoelectrons per A/D count and base level).
3. Open “Run Analysis” in ThunderSTORM plugin and set corresponding parameters (e.g., Image filtering, localization methods) and the suggestions on how to set parameters have been described in detail in Xu *et al.* (2017).
4. After all the parameters are set up, click “OK” to start the image reconstruction (see Video 2 using 100 frames as an example. The actual image reconstruction often needs 10,000-40,000 frames).

*Note: Correction for chromatic aberration is crucial for super-resolution imaging. For two-color dSTORM, to correct the chromatic aberration across different color channels, multi-color fluorescence beads (TetraSpeck microspheres, 0.1-mm diameter, blue/green/orange/dark red fluorescence) that are largely uniformly distributed across the entire field of view can be used to generate the transform map (Sigal *et al.*, 2015).*

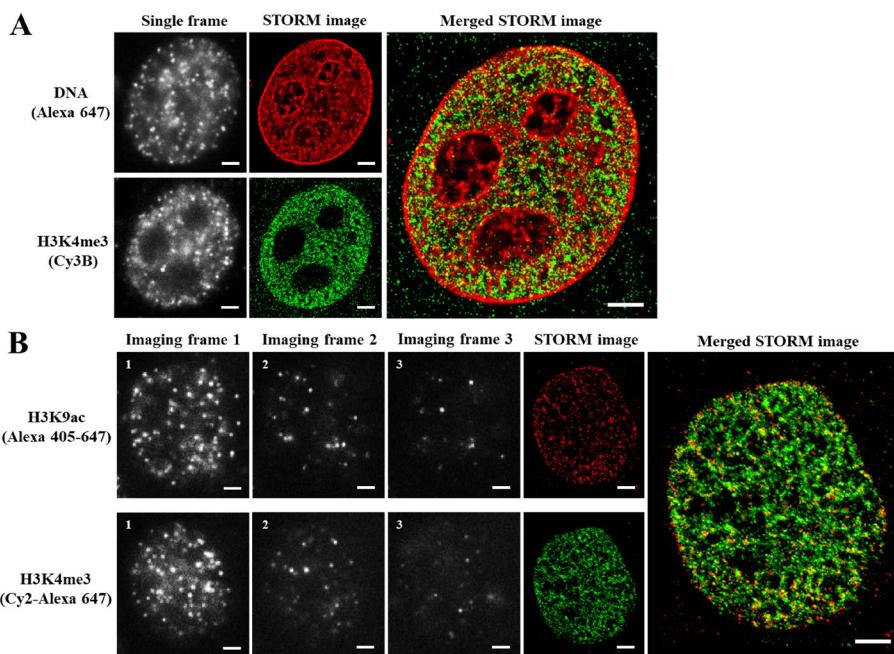


**Video 2. STORM image reconstruction by ThunderSTORM ImageJ plugin**

B. Image reconstruction of STORM imaging based on dye pairs

If dye pairs are used in STORM imaging, for each channel, only the imaging frames immediately after each activation pulse (imaging frame 1 in Figure 3B) are used for the image reconstruction following the same approach as dSTORM described above. The second and third frames (imaging frames 1 and 2 in Figure 3B) following each activation pulses were considered as non-specific activation signals, which are moved by a cross-talk subtraction algorithm described in Bates *et al.*, 2007.

*Note: For two-color STORM imaging using dye pairs, chromatic correction is not needed since the same imaging fluorophore was used for two color channels. As the correction for chromatic aberration is rarely perfect, in cases where the accurate localization of two targets is crucial to answering the biological question, dye-pair based two-color STORM free of chromatic aberration can be advantageous.*



**Figure 3. Representative two-color STORM imaging (raw data and STORM) based on dSTORM or dye pair.** A. Representative raw images and two-color dSTORM images of DNA (EdU) and H3K4me3 labeled with Alexa 647 and Cy3B, respectively. B. Representative raw images (imaging frames 1-3 for one activation-imaging cycle) and two-color STORM images of H3K9ac and H3K4me3 based on dye-pairs. H3K9ac was labeled with Alexa 405-647 and H3K4me3 were labeled with Cy2-Alexa 647. Scale bar: 2  $\mu$ m.

## Recipes

1. Culture medium for MCF-10A  
DMEM/F12  
5% horse serum  
10 mg/ml insulin  
20 ng/ml EGF  
0.5 mg/ml hydrocortisone  
100 ng/ml cholera toxin
2. Washing buffer  
0.2% BSA + 0.05% Triton X-100 in PBS
3. Blocking buffer  
3% BSA + 0.05% Triton X-100 in PBS
4. STORM Imaging buffer  
10% (w/v) glucose  
0.56 mg/ml glucose oxidase  
0.17 mg/ml catalase  
10 mM NaCl

50 mM Tris (pH 8.0)

For dSTORM imaging, use 0.14 M 2-mercaptoethanol; for two-color imaging with dye pair, use 0.1 M mercaptoethylamine (MEA)

### **Acknowledgments**

We acknowledge the funding support from National Institute of Health Grant Number R01CA185363 and R33CA225494. Our protocols were adapted based on the previously published protocols (Bates *et al.*, 2007 and 2013; van de Linde *et al.*, 2011).

### **Competing interests**

The authors declare no competing interests.

### **References**

1. Bates, M., Huang, B., Dempsey, G. T. and Zhuang, X. (2007). [Multicolor super-resolution imaging with photo-switchable fluorescent probes](#). *Science* 317(5845): 1749-1753.
2. Bates, M., Jones, S. A. and Zhuang, X. (2013). [Preparation of photoswitchable labeled antibodies for STORM imaging](#). *Cold Spring Harb Protoc* 2013(6): 540-541.
3. Henriques, R., Lelek, M., Fornasiero, E. F., Valtorta, F., Zimmer, C. and Mhlanga, M. M. (2010). [QuickPALM: 3D real-time photoactivation nanoscopy image processing in ImageJ](#). *Nat Methods* 7(5): 339-340.
4. Ma, H., Xu, J., Jin, J., Huang, Y. and Liu, Y. (2017). [A simple marker-assisted 3D nanometer drift correction method for superresolution microscopy](#). *Biophys J* 112(10): 2196-2208.
5. Min, J., Vonesch, C., Kirshner, H., Carlini, L., Olivier, N., Holden, S., Manley, S., Ye, J. C. and Unser, M. (2014). [FALCON: fast and unbiased reconstruction of high-density super-resolution microscopy data](#). *Sci Rep* 4: 4577.
6. Ovesný, M., Křížek, P., Borkovec, J., Švindrych, Z. and Hagen, G. M. (2014). [ThunderSTORM: a comprehensive ImageJ plug-in for PALM and STORM data analysis and super-resolution imaging](#). *Bioinformatics* 30(16): 2389-2390.
7. Sigal, Y. M., Speer, C. M., Babcock, H. P. and Zhuang, X. (2015). [Mapping synaptic input fields of neurons with super-resolution imaging](#). *Cell* 163(2): 493-505.
8. van de Linde, S., Loschberger, A., Klein, T., Heidbreder, M., Wolter, S., Heilemann, M. and Sauer, M. (2011). [Direct stochastic optical reconstruction microscopy with standard fluorescent probes](#). *Nat Protoc* 6(7): 991-1009.
9. Wolter, S., Loschberger, A., Holm, T., Aufmkolk, S., Dabauvalle, M. C., van de Linde, S. and Sauer, M. (2012). [rapidSTORM: accurate, fast open-source software for localization microscopy](#). *Nat Methods* 9(11): 1040-1041.

10. Xu, J., Ma, H. and Liu, Y. (2017). [Stochastic Optical Reconstruction Microscopy \(STORM\)](#). *Curr Protoc Cytom* 81(1): 12.46.11-12.46.27.
11. Xu, J., Ma, H., Jin, J., Uttam, S., Fu, R., Huang, Y. and Liu, Y. (2018). [Super-resolution imaging of higher-order chromatin structures at different epigenomic states in single mammalian cells](#). *Cell Rep* 24(4): 873-882.

## On-demand Labeling of SNAP-tagged Viral Protein for Pulse-Chase Imaging, Quench-Pulse-Chase Imaging, and Nanoscopy-based Inspection of Cell Lysates

Roland Remenyi<sup>1, \$, \*</sup>, Raymond Li<sup>1</sup> and Mark Harris<sup>1, 2</sup>

<sup>1</sup>School of Molecular and Cellular Biology, Faculty of Biological Sciences, University of Leeds, Leeds, United Kingdom; <sup>2</sup>Astbury Centre for Structural Molecular Biology, University of Leeds, Leeds, United Kingdom; <sup>\$</sup>Current address: Biomedical Research Unit, The Clinical and Translational Research Institute, The Medical City, Pasig City, Philippines

\*For correspondence: [rgr.themedicalcity.ph@outlook.com](mailto:rgr.themedicalcity.ph@outlook.com)



**[Abstract]** Advanced labeling technologies allow researchers to study protein turnover inside intact cells and to track the labeled protein in downstream applications. In the context of a viral infection, the combination of imaging and fluorescent labeling of viral proteins sheds light on their biological activity and interaction with the host cell. Initial approaches have fused fluorescent proteins such as green fluorescent protein (GFP) to the viral protein-of-interest. In contrast, self-labeling enzyme tags such as the commercial SNAP-tag, a modified version of human O<sup>6</sup>-alkylguanine-DNA-alkyltransferase, covalently link synthetic ligands, which users can add on demand. The first two protocols presented here build on previously published protocols for fluorescent labeling in pulse-chase and quench-pulse-chase experiments; the combination of fluorescent labeling with advanced light microscopy visualizes the dynamic turnover of the SNAP-tagged viral protein in intact mammalian cells. A third protocol also outlines how to inspect cellular lysates microscopically for detergent-resistant assemblies of the labeled viral protein. These protocols showcase the flexibility of the SNAP-based labeling system for tracking a viral protein-of-interest in live cells, intact fixed cells, and cell lysates. Moreover, the protocols employ recently developed commercial microscopes (e.g., Airyscan microscopy) that balance resolution, speed, phototoxicity, photobleaching, and ease-of-use.

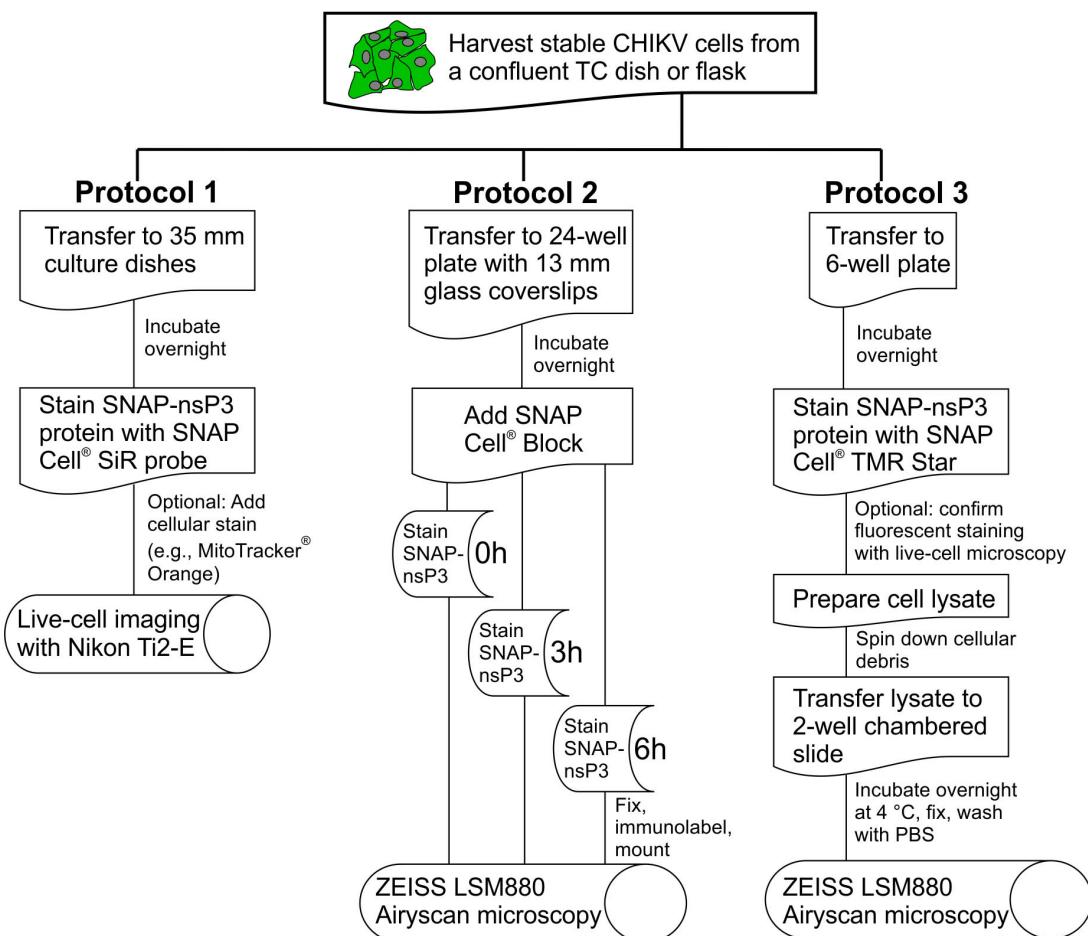
**Keywords:** Fusion proteins, Stress granules, RNPs, Site-specific protein labeling, Chemical labeling, Cell biology, Bioimaging, Live cell imaging, Chikungunya, CHIKV, Alphavirus, Arbovirus, nsP3

**[Background]** To better understand the role of a viral protein during the infectious life cycle, we have adapted existing strategies that facilitate the determination of intracellular protein location, the assessment of protein dynamics in intact cells, and the continued tracking of the viral protein after lysis of the host cell. The first two protocols build on previously published protocols for on-demand SNAP-labeling and the analysis of protein turnover (Bodor *et al.*, 2012). In previous work, our laboratory has fused a viral protein-of-interest, nonstructural protein 3 (nsP3) of Chikungunya virus (CHIKV), to the SNAP-tag, a modified form of a 20-kDa monomeric DNA repair enzyme (Remenyi *et al.*, 2017 and 2018). During SNAP-labeling, the addition of a synthetic O<sup>6</sup>-benzylguanine (BG) derivative results in a covalent bond between a reactive cysteine residue in the SNAP-tag and the BG-probe (Keppler *et al.*, 2004a and 2004b).

We have also combined SNAP-labeling with a protocol for inspecting cell lysates via light microscopy, which enables visualization of detergent-resistant protein assemblies. Similar approaches have allowed researchers to detect stable granular assemblies of GFP-tagged stress-granule proteins in cell lysates (Jain *et al.*, 2016; Wheeler *et al.*, 2017). Stress granules are assemblies of RNA and protein (RNPs), which form under conditions of cellular stress (Kedersha *et al.*, 2005). It is now possible to isolate the more stable stress granule core from both yeast and mammalian cells (Jain *et al.*, 2016). SNAP-labeling offers an alternative way of tracking tagged viral proteins that may be present in similar subcellular assemblies. Hence, Protocol 3 may not only be useful to study the biochemical nature of viral proteins but also to track any cellular protein that resides in non-membranous organelles such as RNPs and stress granules. For example, integration of the SNAP-tag into the development of cell lines that produce fluorescently tagged stress granules (Kedersha *et al.*, 2008) could increase experimental flexibility during dynamic and quantitative imaging of these cellular sub-compartments.

Our three protocols also take advantage of recently developed commercial imaging systems for multi-color fluorescence microscopy. We analyze labeled samples in Protocol 1 with live-cell imaging (Figure 1) and thus recommend following general procedures for controlling temperature, reducing phototoxicity, limiting photobleaching, and maintaining cell viability (Frigault *et al.*, 2009). The chosen 2018 Nikon Ti-E2 system allowed us to image a large field-of-view, lessen focus drift with a proprietary Perfect Focus System (PFS), and record multiple positions with the motorized stage. Moreover, illumination with an LED and light exposures not exceeding 1 s allowed for gentler imaging compared to the typical imaging setup of a laser scanning confocal microscope.

For the analysis of labeled protein in Protocols 2 and 3, we chose a confocal imaging setup with proprietary ZEISS Airyscan technology (Figure 1), which is a commercial version of the ‘image scanning microscopy’ approach (Muller and Enderlein, 2010; Sheppard *et al.*, 2013). Airyscan microscopy represents one of the recent innovations in fluorescence super-resolution microscopy, also referred to as nanoscopy (Li *et al.*, 2018). The Airyscan technology improves system resolution with an improved detector design, which features a 32-channel detector array (Huff, 2015). With a new 2D super-resolution mode for Airyscan, this detection approach can now enhance resolution 2-fold while lowering the required fluorescence intensity to obtain high-quality images (Huff *et al.*, 2017). The Airyscan system allowed us to stain our samples with fluorescent dyes from the SNAP product range (e.g., SNAP-Cell® 647-SiR and SNAP-Cell® TMR-Star) and detect them even at low levels during the recovery phase of the quench-pulse-chase protocol. Increased sensitivity also helped in the nanoscopy-based inspection of cellular lysate in Protocol 3 and allowed the visualization of granular structures made up of the SNAP-tagged viral protein. The combination of SNAP-based labeling and innovative detection via Airyscan has the potential to further bioimaging with higher resolution, sensitivity, and user-friendliness.



**Figure 1. Overview of experimental protocols.** This flowchart outlines the essential steps in Protocols 1, 2, and 3. For the definition of 'stable CHIKV cells', see Protocol 1, Procedure section.

### Protocol 1: Pulse-chase experiments for long-term imaging with Nikon Ti2-E

#### Materials and Reagents

1. 10-cm Petri dish (Corning, catalog number: 430167)
2. Micropipette tips, serological pipettes, pipette aids, and microtubes for liquid handling
  - a. TipOne® 1,000 µl XL (catalog number: S1122-1830)
  - b. TipOne® 200 µl (catalog number: S1120-8810)
  - c. TipOne® 20 µl filter tips (catalog number: S1120-1810) or SARSTEDT pipette tip 10 µl (catalog number: 70.1130.600)
  - d. Fisherbrand™ 5 ml serological pipets (catalog number: 13-676-10H)
  - e. Fisherbrand™ 10 ml serological pipets (catalog number: 13-676-10J)
  - f. Fisherbrand™ 25 ml serological pipets (catalog number: 13-676-10K)
  - g. Drummond Pipet-Aid XL (catalog number: 4-000-205)
  - h. Microtube 1.5 ml (SARSTEDT, catalog number: 72.690.001)

3. Nunc 35-mm glass bottom (#1.5 or 0.16-0.19 mm thickness) dishes with 27 mm viewing area (Thermo Fisher Scientific, catalog number: 150682)

**Notes:**

- a. *Only open packages inside a biosafety cabinet and reseal any remaining dishes to avoid contamination.*
- b. *Use any dish or slide format that is compatible with an inverted microscope. Match the thickness of the glass bottom with the suggested thickness found on the microscope's objective (typically #1.5 or 0.16-0.19 mm thickness). We prefer the dishes listed above because of their large viewing area. The cell lines used in this protocol can grow on glass substrates, but surface treatment (e.g., Poly-Lysine coating) may be necessary for other cell lines.*

4. HuH-7 cell line ([Japanese Collection of Research Bioresources \[JCBR\]](#), catalog number: JCBR 0403)

*Note: HuH-7 is a well-differentiated hepatocyte-derived cellular carcinoma cell line. It was originally taken from a liver tumor in a Japanese male in 1982 (Nakabayashi et al., 1982). The cells used in the creation of this protocol were obtained from John McLauchlan (Centre for Virus Research, Glasgow).*

5. A plasmid that encodes SNAP<sup>f</sup>, a SNAP-tag protein (NEB, catalog number: N9183S; or as part of the SNAP-Cell<sup>®</sup> Starter Kit, NEB, catalog number: E9100S)

6. For initial validation: plasmid that encodes pSNAP<sup>f</sup>-Cox8A control plasmid (also part of the SNAP-Cell<sup>®</sup> Starter Kit, NEB, catalog number: E9100S)

7. Trypsin EDTA solution (Sigma, catalog number: T3924-500ML)

8. Specific reagents for our cell line (for basic tissue culture), which we derived from the hepatoma cell line HuH-7

- a. Dulbeccos modified Eagles medium (Sigma, catalog number: D6429-500ML)

- b. 100% Fetal Calf Serum (Gibco, catalog number: 10500-064)

- c. Gibco MEM Nonessential amino acids solution (100x), store at 4 °C up to 24 months from the date of manufacture (Thermo Fisher Scientific, catalog number: 11140050)

- d. Gibco HEPES (N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid) buffer (100x), store at 4 °C up to 24 months from the date of manufacture (Thermo Fisher Scientific, catalog number: 15630130)

9. FluoroBrite DMEM, store at 4 °C up to 24 months from the date of manufacture (Thermo Fisher Scientific, catalog number: A1896701)

10. Cell-permeable SNAP-Cell<sup>®</sup> 647-SiR (New England Biolabs, catalog number: S9102S)

**Notes:**

- a. *Package contains 30 nmol of the substrate. Resuspend with 50 µl of sterile dimethyl sulfoxide (DMSO, Fisher Bioreagents<sup>TM</sup>, catalog number: BP231-100) to make up a stock solution, which can be stored at -20 °C. We have used stock solutions that have been stored for up to 3 years. However, the manufacturer's recommended shelf-life is three*

months dissolved in DMSO and two years dry.

- b. NEB also offers cell-impermeable SNAP probes ('Cell Surface' Probes); use these probes for studies of viral proteins that accumulate at the surface of host cells.
- 11. Optional: Red fluorescent or orange fluorescent cellular dye (e.g., MitoTracker™ Orange CMTMRos, Thermo Fisher Scientific, catalog number: M7510)  
*Note: To prepare a stock solution, dissolve lyophilized MitoTracker™ probe in DMSO to a final concentration of 1 mM and store frozen and protected from light.*
- 12. Molecular Probes Invitrogen ProLong Live Antifade Reagent, for live cell imaging (Thermo Fisher Scientific, catalog number: P36975)  
*Notes:*
  - a. Store at 2-8 °C for short-term storage and ≤ -20 °C for long-term storage.
  - b. According to the manufacturer's instructions, use the product within 30 days when stored at 2-8 °C. When stored at ≤ -20 °C, the product is stable for at least six months with up to four freeze-thaw cycles.
- 13. Glutamax-I (Thermo Fisher Scientific, catalog number: 35050061)
- 14. Live cell imaging solution (see Recipes)
- 15. Tissue culture medium containing serum (see Recipes)

## **Equipment**

- 1. Air-displacement micropipettes
  - a. Starlab ErgoOne® 100-1,000 µl Single-Channel Pipette (catalog number: S7110-1000) or Gilson F123602 PIPETMAN Classic Pipet P1000 (Fisher Scientific, catalog number: 10387322)
  - b. Starlab ErgoOne® 20-200 µl Single-Channel Pipette (catalog number: S7100-2200) or Gilson F123615 PIPETMAN Classic Pipet P100 (Fisher Scientific, catalog number: 10442412)
  - c. Starlab ErgoOne® 2-20 µl Single-Channel Pipette (catalog number: S7100-0220) or Starlab ErgoOne® 0.1-2.5 µl Single-Channel Pipette (catalog number: S7100-0125) or Gilson F144801 PIPETMAN Classic Pipet P2 (Fisher Scientific, catalog number: 10635313) or Gilson F123600 PIPETMAN Classic Pipet P20 (Fisher Scientific, catalog number: 10082012)
- 2. Equipment for basic cell culture techniques and aseptic procedures, i.e.,
  - a. Biosafety cabinet (e.g., Thermo Scientific Holten Safe 2010 Model 1.2, catalog number: 8207071100)
  - b. Humidified incubator set to 37 °C (Panasonic Incusafe, catalog number: MCO-20AIC)  
*Note: Any manufacturer and model of a biosafety cabinet will do, but the chosen biosafety cabinet needs to be appropriate for the containment of cells and viruses. When handling*

infectious viruses, use the proper facilities, practices, and procedures. Refer to local and international guidelines for laboratory biosafety.

3. -20 °C freezer (Labcold, catalog number: RLCF1520)
4. Water bath (e.g., Clifton, catalog number: NE2-8D; however, any water bath set to 37 °C may be used)
5. Table-top microcentrifuge, 4 °C to room temperature (RT), max speed  $\geq$  12,000  $\times$  g (Eppendorf, catalog number: 5424R)

*Note: For spinning down precipitate that may form during extended storage of SNAP probes.*

6. Live-cell imaging microscope for long-term imaging. For example, a Nikon Ti2-E inverted widefield microscope (for a similar setup, contact your local Nikon representative to create a customized order for the microscope system)
  - a. Nikon Ti2-E inverted microscope stand
  - b. Motorized stage (standard Ti2 encoded motorized XY stage)
  - c. Lumencor Spectra X LED light source
  - d. CFI Plan Apo Lambda 60x oil/1.4 NA objective
  - e. Photometric Prime 95B sCMOS monochrome camera
  - f. Semrock 32 mm filter (Green) for Nikon Ti2-E: GFP-4050B Filter cube with Ex 466/40 single-band bandpass filter (product code: FF01-466/40-25), DM495 dichroic beamsplitter (product code: FF495-Di03-25x36), BA525/50 single-band bandpass filter (product code: FF03-525/50-25)
  - g. Semrock 32 mm filter (Red) for Nikon Ti2-E: Cy3-4040C Filter cube with Ex 531/40 single-band bandpass filter (product code: FF01-531/40-25), Dichroic Mirror DM 562 (product code: FF562-Di03-25x36, Barrier Filter: BA 593/40 (product code: FF01-593/40-25)
  - h. Semrock 32 mm filter (Far-Red) for Nikon Ti2-E: Cy5-4040C Filter cube with Ex 628/40 single-band bandpass filter (product ID: FF02-628/40-25), Single Band Emitter (product ID: FF01-692/40-25), Single Band Dichroic (product ID: FF660-Di02-25x36)
  - i. Okolab stage top incubator, catalog number: H301-NIKON-NZ100/200/500-N. Set to 37 °C with 5% CO<sub>2</sub> (any manufacturer and model that can be mounted on a Nikon Ti2 motorized stage will do)
  - j. Perfect Focus System (PFS)

*Notes:*

- i. *The above list is not exhaustive but rather lists the essential components that have been useful in the creation of Protocol 1. Consult with your local Nikon representative for the remaining components of a customized microscope system.*
- ii. *We prefer to image cells in open tissue-culture dishes with a coverslip-like glass bottom, which necessitates an inverted microscope stand.*
- iii. *As Protocol 1 is a powerful approach for long-term imaging of the labeled protein, it is important to keep cells in focus and correct for focus drift caused by thermal and*

*mechanical conditions. This live-cell imaging system included Nikon's fourth-generation Perfect Focus System (PFS), which monitors the partial reflection of a low-power infrared laser beam on the interface between the dish's glass bottom and liquid media above. This feature provides continuous and real-time focus drift correction.*

- iv. *A large (25 mm x 25 mm) field of view (FOV) enables increased data throughput and capture of additional cells that would be outside the normal FOV.*
- v. *We preferred an LED light source over laser-based illumination. LED illumination limited photobleaching and phototoxicity; 'gentle' imaging was essential during acquisition of five-dimensional datasets (5-D: multi-color 3-D Z-stacks over time).*
- vi. *A motorized microscope stage and the ability to record multiple positions during one experiment further increased the throughput of the imaging system.*

## **Software**

### **1. Nikon NIS Elements AR imaging software**

Nikon NIS Elements AR imaging software controls all components of the microscope. Use NIS Elements to set-up parameters for acquisition of 5-D datasets. Users can also create rendered videos within NIS Elements and save individual frames of 5-D data. For our widefield images, we also used a Richardson-Lucy algorithm (set to 10 iterations) to deconvolve datasets within the Elements software.

## **Procedure**

A published labeling procedure forms the basis of the protocol described here (Bodor *et al.*, 2012). We build on this protocol by also describing a live-cell imaging setup that is suitable for long-term examination of protein turnover in five dimensions (*i.e.*, 3-D multi-color fluorescence microscopy over time).

*Note: Carry out all liquid handling steps that involve live cells inside the biosafety cabinet. Only use sterile pipettors, serological pipets, micropipettors, microtubes, and tips.*

### **1. Production of stable CHIKV cells**

We have previously used the HuH-7 hepatoma cell line to derive cells that stably harbor a modified CHIKV replicon; we designed this replicon to encode a fusion protein of CHIKV nsP3 and the SNAP-tag (Remenyi *et al.*, 2018). We will refer to the cells from this secondary cell line as 'stable CHIKV cells' in the rest of the protocol. These cells also constitutively express the green fluorescent protein ZsGreen.

- a. Use standard molecular cloning methods to generate N-, C-terminal, or internal SNAP-tag fusions. In our experimental system, we inserted the SNAP-tag within the C-terminal region of CHIKV nsP3 and added a flexible linker of Glycine amino acids at the N-terminal and

- C-terminal junctions (Remenyi *et al.*, 2017). A plasmid that encodes SNAP<sub>r</sub><sup>®</sup>, a SNAP-tag protein, is available from NEB (N9183S; or as part of the SNAP-Cell<sup>®</sup> Starter Kit, E9100S).
- b. After the SNAP-sequence has been inserted into a viral genome, evaluate whether modified viruses or replicons remain viable and whether the tagged protein can carry out the same biological function as the untagged protein. The methods for verification will vary depending on the virus and the protein-of-interest.
  - c. Maintain cells in preferred tissue-culture format: we routinely passage our stable CHIKV cells in 10-cm Petri dishes.
  - d. We have developed this protocol with the stable CHIKV cell line, which we derived from HuH-7 cells. Other cell lines that support prolonged replication of non-cytotoxic CHIKV replicons include the C2C12 (mouse myoblast) cell line (Remenyi *et al.*, 2018) and BHK-21 (baby hamster kidney) cell line (Utt *et al.*, 2015). However, we have not yet validated Protocols 1-3 in these cell lines.
2. Detach stable CHIKV cells from the growth surface by adding enough trypsin to cover the cells that are attached to the surface of the respective culturing vessel (e.g., 1-2 ml of trypsin for 10-cm dishes) and seed into a 35-mm dish with coverslip bottom. Prepare a second and third dish containing positive and negative control.
    - a. As a negative control, we seed naïve cells (known not to express SNAP-tag protein) in a second dish.
    - b. NEB's SNAP-Cell Starter Kit also contains a positive control plasmid (pSNAP<sub>r</sub>-Cox8A Control Plasmid), which can be transfected into cells to produce SNAP-tagged cytochrome c oxidase with a well-characterized mitochondrial localization. Thus, the third dish may contain cells transfected with pSNAP<sub>r</sub>-Cox8A control plasmid or any plasmid encoding a SNAP-tagged protein with well-characterized subcellular localization.

**Notes:**

- i. *For beginners, we recommend handling only three dishes at a time (one dish for the sample, one dish for the negative control, and one dish for the positive control) during the labeling phase of this protocol. Advanced users requiring higher throughput may consider 35-mm imaging dishes with four compartments (e.g., ibidi μ-Dish 35 mm Quad, catalog number 80416) instead of using individual dishes. Carry out simultaneous experiments (e.g., two sample conditions, one negative control and one positive control) in the subdivisions of the dish.*
- ii. *The exact amount of trypsin needed to dissociate adherent cells is dependent on the cell type and age of cells.*
- iii. *We adjust the cell seeding density depending on the length of our desired chase period and the duplication time of the cell line we use for the experiment.*
- iv. *We typically aim for 60% to 80% confluence. For example, assuming a doubling time of 24 h and a desired chase period of 24 h, we would seed our SNAP-tagged cell line to 20% confluence, stain with SNAP-reagents the following day (= pulse, at 40% confluency) and*

*image during a 24-h chase (allowing cells to reach 80% confluence during live-cell imaging).*

3. Incubate cells under standard growth conditions (i.e., 37 °C at 5% CO<sub>2</sub>) overnight.
4. On the next day, take the frozen stock solution of SNAP-Cell® 647 SiR from the -20 °C freezer and thaw at room temperature.
5. In a 1.5-ml microtube, dilute thawed SNAP-Cell® 647 SiR; perform a 1:1,000 (final concentration of 0.6 μM) dilution in complete cell culture media. Final volume should be at least 0.6 ml to cover the dish area above the coverslip. Users can increase the volume to 1 ml to reduce the risk of drying out the cells. Vortex briefly (for ≥ 5 s) or pipet the diluted labeling solution up and down (ten times).
  - a. SNAP-Cell® probes are cell-permeable. Our protocols label a fusion protein of CHIKV nsP3 and the SNAP-tag. This fusion protein localizes to intracellular compartments, and hence we only stain with cell-permeable probes.
  - b. Other probes from NEB include SNAP-Cell® 505 (green fluorescent), SNAP-Cell® Oregon Green (green fluorescent), and SNAP-Cell® TMR-Star (red fluorescent). Optimize final concentrations for different SNAP-tagged proteins and cell lines; factors like protein abundance and non-specific binding may vary depending on the tagged protein or cell line.
  - c. According to the manufacturer, optimal substrate concentrations range from 1 to 20 μM, with best results usually obtained at concentrations between 1 and 5 μM. We have found that even 0.6 μM of SNAP-Cell® SiR provided sufficient staining. We do not prepare more media than we expect to consume within one hour. Always include a negative control (naïve cells known not to express SNAP-tag protein) when optimizing labeling conditions.

*Note: Recipe for three dishes (using 0.6 ml in Step 7, scale accordingly for more dishes):*

*1998 μl Complete Cell Culture Media; 2 μl SNAP-Cell® 647 SiR.*

6. Spin the diluted labeling solution for 5 min at maximum speed (≥ 12,000 × g) to remove possible insoluble fluorescent debris. Take care not to disturb the pellet when removing supernatant (which may be invisible).
7. Replace the medium on stable CHIKV cells with 0.6-1 ml of SNAP-tag labeling medium (pre-heated to 37 °C in a water bath). Incubate for 15 min at 37 °C, 5% CO<sub>2</sub>.

*Note: At this step, the benzyl group on the SNAP-Cell® 647 SiR substrate will covalently link to the SNAP-tag and release guanine. We found that a 15-min incubation gave us optimal labeling. According to the manufacturer, optimal reaction times range from 5 to 30 min, respectively, depending on experimental conditions and expression levels of the SNAP-tagged protein.*

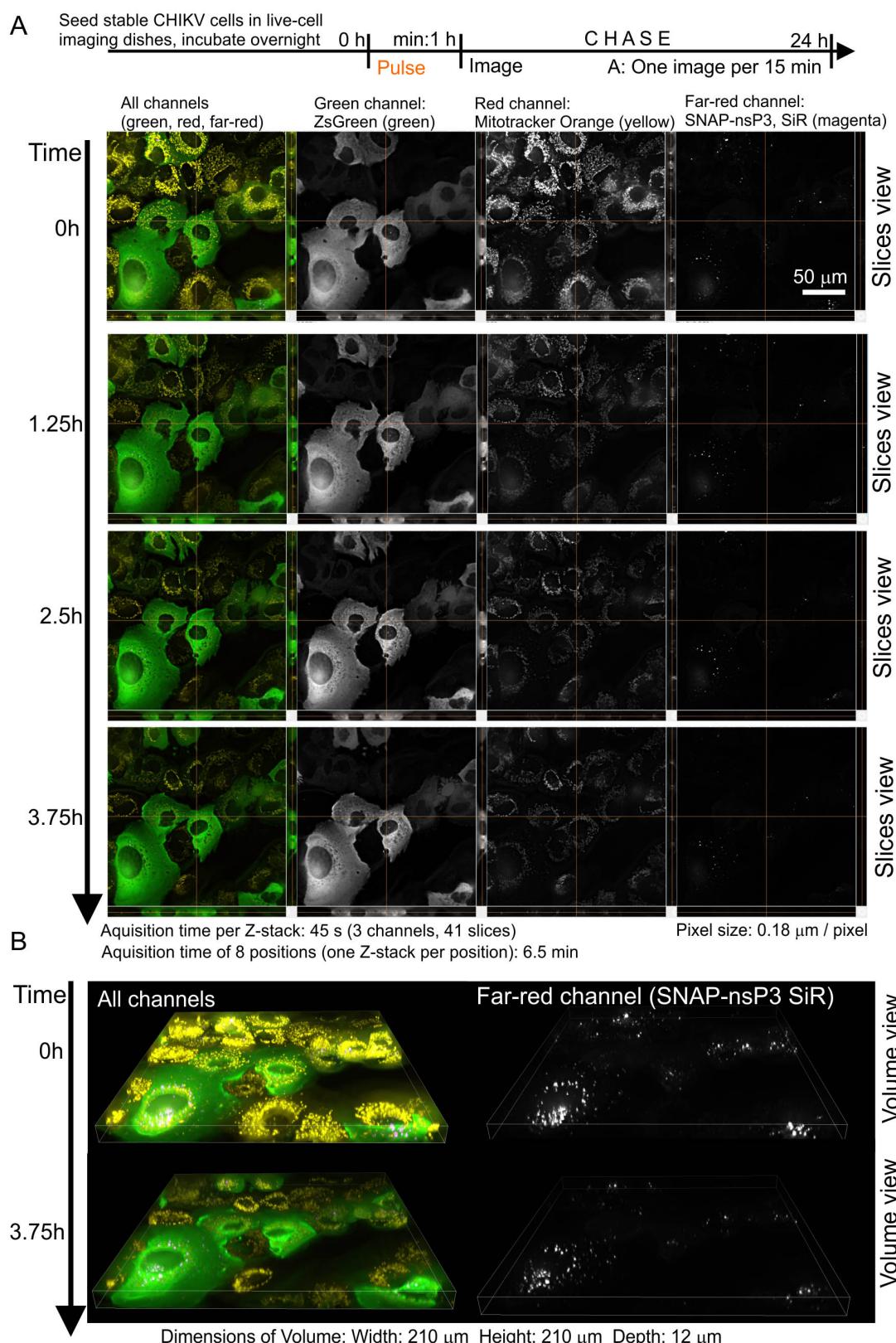
8. During this 15-min incubation period, prepare a cold water bath containing ice and water. Remove a frozen aliquot of ProLong Live reagent from the -20 °C freezer and thaw the aliquot in the cold water bath. Do not exceed 37 °C while thawing or using the reagent. We keep the reagent in the ice bath until Step 10.

9. Wash the cells three times each with 2 ml of tissue culture medium containing serum (pre-heated to 37 °C).
10. Replace the regular cell-culture media with 2 ml of fresh live cell imaging solution, consisting of FluoroBrite DMEM, supplemented with fetal bovine serum (at a final concentration of 10%), HEPES, Glutamax-I, Nonessential Amino Acids, and ProLong Live Antifade Reagent (see Recipes).
11. Place cells back into the incubator after the final wash. Incubate for another 30 min, 37 °C at 5% CO<sub>2</sub>.

**Notes:**

- a. *The primary purpose of this step is to reduce the background staining of the SNAP reagent. The background staining of some SNAP probes, such as SNAP Cell® TMR-Star can be problematic in some cell lines whereas less background staining is observed in others (Cole, 2014). If the background staining is an issue, we recommend reducing the labeling time, concentration of probe, or increasing the number of washes. Step 10 also starts the incubation with ProLong Live reagent.*
  - b. *The manufacturer's instructions of the ProLong Live reagent suggest incubating cells in the dark for 15 min to 2 h.*
  - c. *In our experience, by the time the sample dish reaches the microscope, final imaging settings have been set up, and the actual image acquisition starts, the total incubation period of cells with media containing ProLong Live will be at least 1.5 h (~40 min for processing three dishes in Steps 10-12, ~20 min for transferring dishes from tissue culture facility to microscope, and ~30 min for setting up imaging conditions).*
12. Wash cells as described in Step 9 with regular cell-culture media. Replace media with 2 ml of live cell imaging media (made up in Step 10).  
*Note: We supplement the live cell imaging media with ProLong Live solution at this step if live-cell imaging does not exceed 24 h. The manufacturer does not recommend leaving ProLong Live solution on live cells for more than 24 h. We also calculate the chase period from the completion of this step, since it marks the last time point at which the fluorescent substrate can label SNAP-tagged proteins.*
  13. Optional: Stain cells with red fluorescent or orange fluorescent cellular dye (e.g., MitoTracker™ Orange CMTMRos).
    - a. Dilute 1 mM MitoTracker™ stock solution to the final working concentration (25-500 nM) in 'live cell imaging buffer'.
    - b. Remove media from dishes and add pre-warmed (37 °C) staining solution containing MitoTracker™ probe.
    - c. Return dishes to the humidified incubator and incubate for 15-45 min, at 37 °C with 5% CO<sub>2</sub>.
    - d. After incubation period is complete, replace staining solution with fresh pre-warmed 'live cell imaging buffer'.

14. Transfer the three dishes (sample, positive control, and negative control) to microscope area for live-cell imaging with Nikon Ti2-E system. The live-cell imaging setup for SNAP-tagged cells is similar to standard configurations for live-cell fluorescence imaging.
  - a. We recommend [Nikon's resource on 'live cell imaging'](#) for an introduction on the appropriate microscope setup for timelapse imaging.
  - b. For additional resources, contact your local Nikon representative for NIS Elements Training handouts on 'Advanced Acquisition' modes (*i.e.*, Multi-channel, Multi-point, Timelapse, and Z-stack)
  - c. For alternative live-cell imaging setups, refer to Bodor *et al.* (2012).
  - d. We used a widefield imaging setup for extended imaging of the same field-of-view. We obtained high-quality results with a Nikon Ti2-E system.
  - e. Several factors determined our preference for this system, namely (i) the Ti2-E is equipped with a unique perfect focus system (PFS) that automatically corrects focus drift in real time during a prolonged period of imaging (ii) imaging with an LED light source allows for gentler imaging compared to laser-based confocal systems (iii) multipoint Z-stacks can be acquired quickly as a result of faster device movement and image acquisition (iv) quick acquisition reduces overall light exposure and subsequent phototoxicity (v) the Ti2-E provides a large field of view (FOV), which captures a large amount of cells within one FOV, and (vi) multi-point acquisitions further increase the throughput of the system.
15. Image cells with the preferred imaging system
  - a. SNAP Cell® 647-SiR should have an excitation maximum at 645 nm and an emission maximum at 661 nm.
  - b. With the Nikon Ti2-E inverted microscope, we use standard filter settings for the Cy5 dye. Stable CHIKV cells also endogenously express the green fluorescent ZsGreen reporter protein, which has an excitation maximum of 493 nm and an emission peak at 505 nm (image with a standard GFP filter set).
  - c. The advantage of using the far-red SNAP Cell® 647-SiR is that additional labeling with a red fluorescent cellular dye (*e.g.*, MitoTracker™ Orange) and imaging with filter settings for Cy3 dye is possible. Figure 2 shows representative images from a timelapse series, in which we set the microscope to take Z-stacks every 15 min for a total of 24 h.



**Figure 2. Combination of 5-D imaging and pulse-chase experiments.** A. We only show selected frames from a multi-position timelapse series, in which the microscope acquired Z-stacks every 15 min at eight positions. In this setup, each Z-stack (composed of 41 slices)

was completed within 45 s, whereas it took 6.5 min to obtain eight positions. The images on the left display all channels with pseudo-colors (green: ZsGreen, yellow: MitoTracker™ Orange, magenta: SNAP-nsP3). Note that granular structures labeled at 0 h were still present at 3.75 h. This continued presence indicated that these structures remained stable for hours. B. Selected frames from the same timelapse series, this time presented in volume view. The images on the left display all channels with pseudo-colors (green: ZsGreen, yellow: MitoTracker™ Orange, magenta: SNAP-nsP3).

## **Recipes**

1. Live cell imaging solution (enough to add 2 ml of solution to three 35-mm dishes in Steps 10, 12, and optional Step 13)  
2.7 ml Fetal Calf Serum  
270 µl HEPES  
270 µl Glutamax-I  
270 µl MEM Nonessential Amino Acids  
270-540 µl ProLong Live Antifade Reagent  
22.96-23.22 ml FluoroBrite DMEM  
The total volume of live cell imaging solution: 27 ml
2. Tissue culture medium containing serum  
500 ml Dulbeccos modified Eagles medium  
56.5 ml Fetal Calf Serum  
5.6 ml MEM Nonessential amino acids solution  
5.6 ml HEPES

## **Protocol 2: Quench-pulse-chase experiments paired with ZEISS LSM 880 Airyscan Microscopy**

### **Materials and Reagents**

1. Micropipette tips, serological pipettes, pipette aids, and microtubes for liquid handling
  - a. TipOne® 1,000 µl XL filter tips (catalog number: S1122-1830)
  - b. TipOne® 200 µl filter tips (catalog number: S1120-8810)
  - c. TipOne® 20 µl filter tips (catalog number: S1120-1810) or SARSTEDT pipette tip 10 µl (catalog number: 70.1130.600)
  - d. Fisherbrand™ 5 ml serological pipets (catalog number: 13-676-10H)
  - e. Fisherbrand™ 10 ml serological pipets (catalog number: 13-676-10J)
  - f. Fisherbrand™ 25 ml serological pipets (catalog number: 13-676-10K)
  - g. Drummond Pipet-Aid XL (catalog number: 4-000-205)
  - h. Microtube 1.5 ml (SARSTEDT, catalog number: 72.690.001)

- i. 15 ml Centrifuge Tubes, Conical, Sterile (Starlab, catalog number: E1415-0200)
2. Glass slide (Academy, catalog number: N/A142)
3. Filter paper (Whatman®, catalog number: 1001025)
4. 24-well plates (CytoOne®, catalog number: CC7682-7524)
5. Clean, sterile 13 mm coverslips (# 1.5) (Academy, catalog number: NPS16/1818)
6. Parafilm® M (Bemis, catalog number: PM-996)
7. Aluminum foil (Caterwrap, catalog number: AKL-300-030M)
8. 10-cm dishes
9. Stable CHIKV cells (see Protocol 1)
10. Specific reagents for our cell line (for basic tissue culture), which we derived from the hepatoma cell line HuH-7:
  - a. Dulbeccos modified Eagles medium (Sigma, catalog number: D6429-500ML)
  - b. 100% Fetal Calf Serum (Gibco, catalog number: 10500-064)
  - c. Gibco MEM Non-essential amino acids solution (100x), store at 4 °C up to 24 months from the date of manufacture (Thermo Fisher Scientific, catalog number: 11140050)
  - d. Gibco HEPES (N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid) buffer (100x), store at 4 °C up to 24 months from the date of manufacture (Thermo Fisher Scientific, catalog number: 15630130)
11. Trypsin EDTA (Sigma, catalog number: T3924-500ML)
12. PBS, Phosphate Buffered Saline (VWR Lifescience, catalog number: E404-100TABS)
13. Antibody for immunolabeling of the SNAP-tagged protein

*Note: We use a rabbit antibody produced in-house that detects CHIKV nsP3 (Remenyi et al., 2017 and 2018).*
14. Goat anti-rabbit IgG (H+L) Cross-Absorbed Secondary Antibody, DyLight® 405 (Thermo Fisher Scientific, Invitrogen, catalog number: 35551)

*Note: The combination of Dylight® 405, ZsGreen, and TMR-Star provides effective color separation and sensitivity for three-color imaging.*
15. SNAP-Cell® TMR-Star (New England Biolabs, catalog number: S9105S; or as part of the SNAP-Cell® Starter Kit, catalog number: E9100S)

*Note: Prepare stock solution as described for SNAP-Cell® 647-SiR.*
16. SNAP-Cell® Block (bromothenylpteridine, BTP, New England Biolabs, catalog number: S9106S; or as part of the SNAP-Cell® Starter Kit, catalog number: E9100S). Storage: -20 °C for at least three years dry or three months as a stock solution dissolved in DMSO
17. Fixative, 4% Formaldehyde in phosphate buffered saline (PBS), pH 6.9

*Note: For the detailed protocol how to prepare the fixative, see:*

[https://www.rndsystems.com/resources/protocols/protocol-making-4-formaldehyde-solution-pb\\_s](https://www.rndsystems.com/resources/protocols/protocol-making-4-formaldehyde-solution-pb_s)

*Briefly, prepare the fixative from Paraformaldehyde powder (Fisher Chemical, catalog number: T353-500). Dissolve paraformaldehyde powder in 1x sterile PBS (made from tablets) (VWR Lifescience, catalog number: E404-100TABS). Adjust the pH to 6.9 with diluted*

*Hydrochloric Acid (HCl) (Fisher Chemical, catalog number: H/1100/PB17). For long-term storage, keep 15-ml aliquots at -20 °C. When needed, thaw aliquots (vortex thoroughly to remove any precipitation) and use for each experiment. Store any remaining solution for up to 1 month at 4 °C.*

18. Mounting Medium (ProLong Diamond Antifade Mountant, catalog number: P36965)  
*Note: We use ProLong Diamond because it protects both fluorescent dyes (in our setup: SiR + DyLight® 405) and fluorescent proteins (in our setup: ZsGreen) from fading.*
19. Complete Cell Culture Media (see Recipes)

## **Equipment**

1. Micropipettes
  - a. Starlab ErgoOne® 100-1,000 µl Single-Channel Pipette (catalog number: S7110-1000) or Gilson F123602 PIPETMAN Classic Pipet P1000 (Fisher Scientific, catalog number: 10387322)
  - b. Starlab ErgoOne® 20-200 µl Single-Channel Pipette (catalog number: S7100-2200) or Gilson F123615 PIPETMAN Classic Pipet P100 (Fisher Scientific, catalog number: 10442412)
  - c. Starlab ErgoOne® 2-20 µl Single-Channel Pipette (catalog number: S7100-0220) or Starlab ErgoOne® 0.1-2.5 µl Single-Channel Pipette (catalog number: S7100-0125) or Gilson F144801 PIPETMAN Classic Pipet P2 (Fisher Scientific, catalog number: 10635313) or Gilson F123600 PIPETMAN Classic Pipet P20 (Fisher Scientific, catalog number: 10082012)
2. Metal tweezers with fine tips for lifting and handling glass coverslips (EMS, catalog number: 78316-1)
3. Equipment for basic cell culture techniques and aseptic procedures, *i.e.*,
  - a. Biosafety cabinet (*e.g.*, Thermo Scientific Holten Safe 2010 Model 1.2, catalog number: 8207071100)
  - b. Humidified incubator set to 37 °C (Panasonic Incusafe, catalog number: MCO-20AIC)  
*Note: Any manufacturer and model of a biosafety cabinet will do, but the chosen biosafety cabinet needs to be appropriate for the containment of cells and viruses. When handling infectious viruses, use the proper facilities, practices, and procedures. Refer to local and international guidelines for laboratory biosafety.*
4. -20 °C freezer (Labcold, catalog number: RLCF1520)
5. Water bath (*e.g.*, Clifton, catalog number: NE2-8D; however, any water bath set to 37 °C may be used)
6. Table-top microcentrifuge, 4 °C to room temperature (RT), max speed ≥ 12,000 x g (Eppendorf, model: 5424R)  
*Note: For spinning down precipitate that may form during extended storage of SNAP probes.*

7. Safety glasses (any manufacturer or model that protects users from formaldehyde splashes will do)
8. Confocal laser scanning microscope. For example, a customized ZEISS LSM 880 system that includes the following essential components (for a similar setup, contact your local ZEISS representative for exact ordering information as product codes may differ from customer to customer and country to country):
  - a. Axio Imager Z2 stand, motorized (upright system), product ID: 430000-9902-000
  - b. Motorized Stage, Scanning Stage 130x85 STEP, product ID: 432033-9902-00
  - c. Mounting Frame 160x116 f/ Slides 76x26, product ID: 432315-0000-000
  - d. Scan module LSM 880, product ID: 000000-1994-956
  - e. Support f/scan module LSM (Imager Tube), product ID: 000000-1265-660
  - f. Stepper motor control f, 2 Axes SMC2009, product ID: 432929-9011-000
  - g. Real-time controller standard, product ID: 000000-2031-918
  - h. Objective C PApo 63x/1.4 Oil DIC UV-IR, product ID: 421782-9900-799
  - i. 488 Vis Laser, Laser Argon Multiline 25 mW, product ID: 000000-2086-081
  - j. 561 Vis Laser, Laser 561nm for LSM 710, product ID: 00000-1410-117
  - k. 633 Vis Laser, Laser Rack LSM 880 incl. 633 Laser, product ID: 000000-2085-478
  - l. Airyscan SR module GaAsP for LSM, product ID: 000000-2058-580
  - m. Emission filter BP495-550 + LP570 for Airyscan, product ID: 000000-2070-488
  - n. Emission filter BP570-620 + LP645 for Airyscan, product ID: 000000-2070-489
  - o. Double BP 420-480 + BP495-620 for Airyscan, product ID: 000000-2095-049
  - p. Double BP 465-505 + LP525 for Airyscan, product ID: 000000-2095-051
  - q. Double BP 420-480 + LP605 for Airyscan, product ID: 000000-2095-052
  - r. User PC advanced Z55A highend, product ID: 000000-2142-968
  - s. 'Airyscan Fast' illumination module upgrade for 1x LSM 880 system

**Notes:**

- i. *The above list is not exhaustive but only lists essential components that we have found useful for our protocols. Consult with ZEISS about additional components to complete the microscope system (e.g., joystick for stage, hardware license keys, beam splitter, switching mirror, and nosepiece).*
- ii. *Follow the manufacturer's recommendations for applying all necessary Airyscan settings within the ZEN microscope software. Our LSM 880 Airyscan microscope could provide a maximum lateral resolution of 140 nm and axial resolution of 400 nm for a fluorophore emitting at 480 nm (images processed with ZEN Black software). The resolution can increase even further to 120 nm XY and 350 nm Z resolution with the use of ZEN Blue software.*
- iii. *Our original system (LSM 880 with Airyscan) was retroactively upgraded to an 'Airyscan Fast' system, whereas today's systems can be customized with an 'Airyscan Fast' module from the start. The 'Airyscan Fast' illumination system allows simultaneous illumination of 4*

*pixels simultaneously to allow very fast and gentle imaging of samples, including the option to scan at super-resolution with 1.5x resolution improvement in XY & Z, imaging at up to 19 images/second with 512 x 512 pixels, 27 images per second at 480 x 480, and 6 images per second at 1024 x 1024.*

## **Software**

1. ZEISS ZEN software (ZEN 2.3 system HWL for FAST Airyscan)

*Note: ZEISS ZEN software drives all components of the LSM 880 system, including setup of Airyscan imaging. ZEN software can also acquire and process raw super-resolution image datasets; save processed image files as ‘.czi’ files, which can be exported to bioimaging analysis software (e.g., Icy software). Saving in this file format ensures the preservation of all the metadata associated with each imaging experiment.*

2. Icy bioimaging software

*Note: Bioimaging analysis software. We recommend the free software [Icy](#) to visualize, annotate and quantify bioimaging datasets, which can be imported from ZEISS ZEN software packages (de Chaumont et al., 2012). We found that Icy has an intuitive user interface.*

## **Procedure**

We use a published labeling protocol (Bodor et al., 2012) to analyze ‘new’ (i.e., freshly translated) pools of the SNAP-tagged viral protein (SNAP-nsP3). By combining the labeling approach with a sensitive detection method, ZEISS LSM 880 Airyscan microscopy, we can visualize the re-emergence of ‘new’ protein and the intracellular sites where ‘new’ proteins accumulate. In the first step (called ‘quench’), a nonfluorescent SNAP-substrate covalently binds to the pool of SNAP-nsP3 present at the onset of an experiment (Figure 3, diagram). After a given amount of time (chase), a second, fluorescent substrate (pulse) labels the cells as described in Protocol 1 (Figure 3, diagram). The pulse only stains the protein pool synthesized during the chase period. Thus, ZEISS LSM 880 Airyscan microscopy will only make this ‘new’ (i.e., freshly translated) pool visible. The total pool of SNAP-nsP3 (pulsed pool + quench pool) can be stained with a standard immunofluorescence assay approach, which reveals the quenched pool that would otherwise remain undetected (Figure 3).

*Note: Carry out all liquid handling steps that involve live cells or formaldehyde-containing wells inside the biosafety cabinet. Only use sterile pipettors, pipets, micropipettors, microtubes, and tips when working with live cells. Wear safety glasses when handling formaldehyde solutions.*

1. Place sterile coverslips in separate wells of a 24-well plate.

*Notes:*

- a. Adjust the total number of wells according to the number of 'post-chase' timepoints. Also include a positive control (e.g., a well without any SNAP Cell® Block added) and negative control (e.g., a well without any chase period).
  - b. In this protocol: two post-chase timepoints (3 h, 6 h) and two controls (four wells in total).
2. Detach stable CHIKV cells from growth surface by adding enough trypsin solution (pre-heated to 37 °C in a water bath) to cover the cells that are attached to the surface of the respective culturing vessel (e.g., 1-2 ml of trypsin solution for 10-cm dishes) and seed stable CHIKV cells in wells from Step 1.
- Seeding density will again depend on the total duration of the experiment and the doubling time of cells. We aim for about ~80% confluence at the time of fixation of the 0-h, 3-h, and 6-h samples. Thus, we also seed fewer cells in the 3-h and 6-h wells to account for the longer incubation times compared to the 0-h well (10-15% less in 3-h well and 20-30% less in 6-h well).
3. Incubate at 37 °C, 5% CO<sub>2</sub> overnight.
  4. On the next day, take the frozen stock solution of SNAP Cell® Block from the -20 °C freezer and thaw at room temperature. In a 1.5-ml microtube, dilute SNAP Cell® Block to a final concentration of 2 µM in complete media. Vortex briefly (for ≥ 5 s). Prepare > 200 µl per coverslip. Use diluted reagent within the hour.
  5. Replace media on cells with 200 µl of the SNAP Cell® Block diluted solution (pre-heated to 37 °C in a water bath) per well (three wells in total). Do not change media on the fourth well (this will serve as the 'positive control'). Incubate for 30 min.
  6. Wash cells three times with 1 ml of complete media (pre-heated to 37 °C in a water bath; this washes away any free substrate, which would interfere with downstream applications). Remove media after the last wash step.
  7. Add 1 ml of complete media (pre-heated to 37 °C in a water bath) to each well and place cells into a tissue-culture incubator for 30 min.
  8. Wash cells as in Step 6. To prevent the cells from drying out, do not remove the wash media after the last wash step.
  9. Prepare a new 24-well plate and add 1 ml of 4% formaldehyde fixative to two of the wells.
  10. Use forceps to transfer one of the 'quenched' coverslips and the 'unblocked' coverslip to the new plate and submerge in formaldehyde (make sure the side with the layer of cells remains up). The fixed cells on the 'quenched' coverslip will serve as the 'no chase' control. The cells on the 'unblocked' coverslip will serve as a positive control for SNAP-reagent staining.
  11. Place the original plate, which contains the remaining coverslips, back into the tissue-culture incubator. Incubate cells for the desired chase period of 3 h.
  12. Place the new plate containing fixed coverslips at 4 °C for storage until all coverslips for all chase periods have been collected. Seal edges of the plate during storage to avoid excessive evaporation (strips of Parafilm M work well).

*Note: If the quench was indeed complete, no labeling should occur during the ‘pulse’ period. If Airyscan microscopy can still detect unquenched SNAP-tagged protein, this indicates that the available SNAP Cell® Block reagent did not fully quench the pre-existing pool. Repeat experiments with an increased concentration of SNAP Cell® Block or with a prolonged incubation time until experimental conditions lead to complete quenching of SNAP-tagged protein.*

13. After three hours, transfer one coverslip to formaldehyde-containing well as described in Step 10 (remove the plate from 4 °C storage and add 1 ml of 4% formaldehyde to a third well). The fixed cells on this coverslip will serve as the ‘3-h chase’ sample.
14. Place the original plate, which contains the remaining coverslip, back into the tissue culture incubator. Incubate cells for another three hours. Place plate that holds fixed ‘no chase’ and ‘3-h chase’ coverslips back to 4 °C for storage.
15. Repeat Steps 13 and 14 (transfer coverslips into a fourth, formaldehyde-containing well). The fixed cells on this last coverslip will serve as the ‘6-h chase’ sample. Incubate for 30 min at room temperature or for 1-2 h at 4 °C to complete the fixation process of ‘6-h chase’ sample.
16. In the biosafety cabinet, wash ‘No chase’, ‘No quench’, ‘3-h chase’, and ‘6-h chase’ controls three times with PBS to remove the formaldehyde fixative.

*Note: At this point, users also have the option to handle 24-well plates outside a biosafety cabinet as the cells have already undergone chemical fixation. However, we still prefer to carry out Steps 17, 19, and 20 inside a biosafety cabinet to avoid the contamination of stock solutions and cell culture media.*

17. Take the frozen stock solution of SNAP Cell® TMR-Star from -20 °C freezer and thaw at room temperature. In a 1.5-ml microtube, dilute SNAP-Cell® TMR-Star; perform a 1:600 (final concentration of 1 µM) dilution in complete cell culture media. Prepare at least 200 µl per well to cover the entire coverslip. Vortex briefly (for ≥ 5 s) or pipet the diluted labeling solution up and down (ten times).

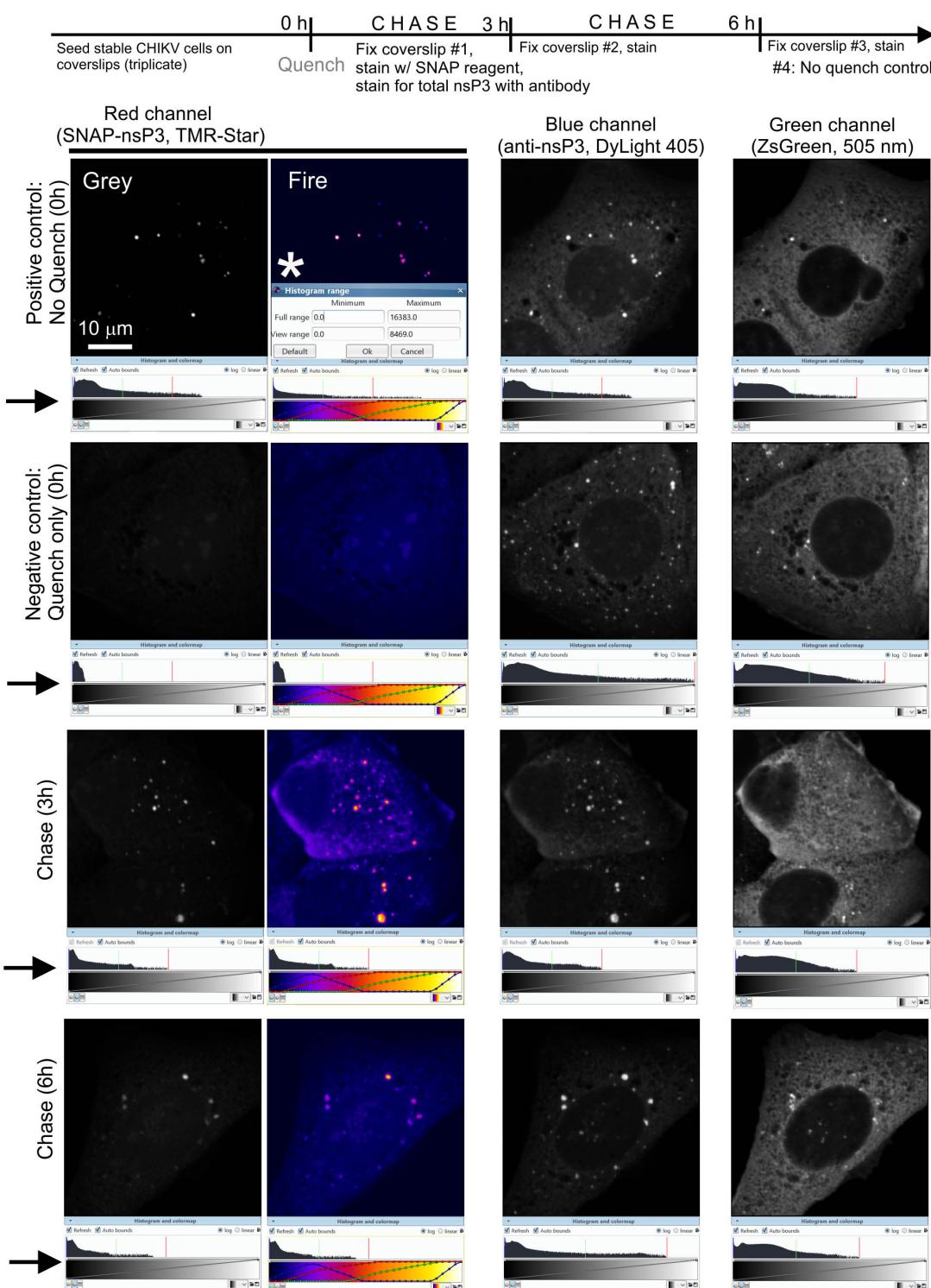
*Notes:*

- a. *This protocol should also be compatible with SNAP-Cell® 647 SiR. Use the same labeling conditions as described in Protocol 1 (i.e., 1:1,000 dilution, 15 min incubation).*
- b. *Recipe for four coverslips in 24-well plate, scale according to the number of wells:*  
*998.4 µl Complete Cell Culture Media*  
*1.6 µl SNAP-Cell® TMR-Star*

18. Spin diluted labeling solution for 5 min at maximum speed ( $\geq 12,000 \times g$ ) to remove possible insoluble fluorescent debris. Take care not to disturb the pellet when removing supernatant (which may be invisible).
19. Replace the medium on stable CHIKV cells with 200 µl of SNAP-tag labeling medium. Incubate for 15 min at 37 °C, 5% CO<sub>2</sub>. Protect samples from excessive light exposure during Steps 20-23 (e.g., wrap 24-well plate in aluminum foil).

*Note: We found that a 15-min incubation gave us optimal labeling. According to the manufacturer, optimal reaction times range from 5 to 30 min, respectively, depending on experimental conditions and expression levels of the SNAP-tagged protein.*

20. Wash the cells three times each with 1 ml of tissue culture medium containing serum (pre-heated to 37 °C).
21. Use a preferred immunofluorescence assay protocol to stain the pool of total tagged protein. At this point, users can handle 24-well plates outside the biosafety cabinet—critical reagents in our case: primary antibodies from rabbit antiserum against nsP3 and dye-conjugated secondary antibodies (anti-rabbit DyLight® 405).
22. Mount the immunolabeled coverslips in preferred mounting medium (e.g., ProLong Diamond Antifade Mountant):
  - a. Place a droplet of mountant on a glass slide, remove immunostained coverslips from 24-well plate using forceps, and slowly lower coverslip (cell side down) onto the droplet.
  - b. Use tissue or filter paper to wipe off any excess mountant. We usually fit two to three coverslips on a standard glass slide.
23. Allow mountant to cure at room temperature and in the dark (overnight or longer). We store cured slides at 4 °C.
24. Image with ZEISS LSM 880 Airyscan microscopy
  - a. SNAP Cell® TMR-Star should have an excitation maximum at 554 nm and an emission maximum at 580 nm. Set up three-color imaging with a blue, green, and red channel. Refer to ZEN software manuals for set-up of ZEISS LSM 880 Airyscan microscopy.
  - b. Upon completion of image acquisition and processing, we use Icy bioimaging software to open saved and processed Airyscan data, which we saved in the .czi file format. Figure 3 shows representative images from a three-color imaging experiment, including screenshots of the Icy histogram viewer for each channel.



**Figure 3. Airyscan microscopy after chase period reveals pools of unblocked viral protein.** Representative images from a quench-pulse-chase experiment. Note that the co-distribution of the total nsP3 pool (stained with anti-nsP3 antibody) and the new pool of nsP3 (stained with TMR-Star) suggested a lack of spatial separation of old and new pools of SNAP-nsP3. We set up the microscope for three-color imaging in the blue, green, and red channels. We adjusted image contrast within the Icy platform by dragging the adjustable

bounds of the histogram viewer (marked by arrows), which enhanced the contrast in the selected channel without altering the data. We chose a viewing range that provided the best contrast for SNAP-nsP3 channel at the 3-h and 6-h timepoints. We also used the same viewing range to display the data from the positive and negative controls ('Histogram range' window, marked by asterisk \*, view range minimum of 0 and maximum of 8469.0 pixel intensity values). Note that by applying the colormap 'Fire' (within Icy software) we could better display low-intensity granular structures.

## **Recipes**

1. Complete Cell Culture Media
  - 500 ml Dulbeccos modified Eagles medium
  - 56.5 ml Fetal Calf Serum
  - 5.6 ml MEM Nonessential amino acids solution
  - 5.6 ml HEPES

## **Protocol 3: Tracking SNAP-tagged viral protein assemblies in cell lysates with ZEISS LSM 880 Airyscan microscopy**

### **Materials and Reagents**

1. Micropipette tips, serological pipettes, pipette aids, and microtubes for liquid handling:
  - a. TipOne® 1,000 µl XL filter tips (catalog number: S1122-1830)
  - b. TipOne® 200 µl filter tips (catalog number: S1120-8810)
  - c. TipOne® 20 µl filter tips (catalog number: S1120-1810) or SARSTEDT pipette tip 10 µl (catalog number: 70.1130.600)
  - d. Fisherbrand™ 5 ml serological pipets (catalog number: 13-676-10H)
  - e. Fisherbrand™ 10 ml serological pipets (catalog number: 13-676-10J)
  - f. Fisherbrand™ 25 ml serological pipets (catalog number: 13-676-10K)
  - g. Drummond Pipet-Aid XL (catalog number: 4-000-205)
  - h. 1.5-ml Microtube (SARSTEDT, catalog number: 72.690.001)
  - i. 15 ml Centrifuge Tubes, Conical, Sterile (Starlab, catalog number: E1415-0200)
2. Parafilm® M (Bermis, catalog number: PM996)
3. Aluminum foil (Caterwrap, catalog number: AKL-300-030M)
4. Moistened paper (e.g., cut sheets of blot absorbent filter paper, Biorad, catalog number: 1703965)
5. 10-cm Petri dish (Corning, catalog number: 430167)
6. Ibidi 2-well µ-slide, an all-in-one chamber slide with polymer coverslip and ibiTreat surface for optimal cell adhesion (Ibidi, catalog number: 80286)

*Note: The IbiTreat surface modification makes the polymer coverslip surface hydrophilic. Alternative approaches to increase the adhesiveness of coverslip surfaces (i.e., Poly-Lysine treatment) and coated glass coverslips may be used (Wheeler et al., 2017).*

7. Greiner CELLSTAR multiwall culture plates, 6-well (Merck/Sigma Aldrich, catalog number: Greiner 657160)
8. Stable CHIKV cells (see Protocol 1)
9. Trypsin EDTA (Sigma, catalog number: T3924-500ML)
10. Materials and reagents for basic tissue culture (see Protocol 1 and 2)
11. SNAP-Cell® TMR-Star (New England Biolabs, catalog number: S9105S; or as part of the SNAP-Cell® Starter Kit, catalog number: E9100S), Prepare stock solution as described for SNAP-Cell® 647-SiR
12. PBS, Phosphate Buffered Saline (VWR Lifescience, catalog number: E404-100TABS)
13. 4% Formaldehyde (see Protocol 2 Step 15)
14. Triton X-100 (Sigma, catalog number: T924-500ml)
15. KCl (Fisher Chemical, catalog number: P/4240/53)
16. NaCl (Fisher Chemical, catalog number: S/3160/60)
17. Magnesium chloride ( $MgCl_2$ ) hexahydrate 99.0-101.0%, VWR Chemicals, catalog number: 25108.260)
18. Glycerol (Fisher Chemical, catalog number: G/0650/17)
19. Piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES) (Sigma, catalog number: P1851-100G)
20. Leupeptin, hemisulfate salt ≥ 85% by HPLC (Sigma, catalog number: L8511-5MG)
21. Pepstatin A (Sigma, catalog number: P5318-5MG)
22. Aprotinin, from bovine lung (Sigma, catalog number: A6279-5ML)
23. AEBSF (Pefabloc, Sigma, catalog number: 76307-100MG)
24. NaOH (Fisher Chemical, catalog number: S/4920/60)
25. Home-made Glasgow Lysis Buffer (GLB) containing protease inhibitors (see Recipes)
  - a. 1x GLB, combine the stock solutions, Glycerol, Triton-X, and protease inhibitors (see Recipes)
  - b. This protocol uses our laboratory's preferred lysis buffer. We have not tried other lysis buffers that use NP-40 or SDS as detergents. However, note that 0.5% NP-40 is a component of the stress granule lysis buffer used to prepare cell lysates for stress granule core isolation (Wheeler et al., 2017).
26. Stock solutions for GLB (see Recipes)
27. Stock solutions of protease inhibitors (see Recipes)
28. 1x GLB (see Recipes)
29. Tissue culture medium containing serum (see Recipes)

*Note: For #14-24, alternatives of equal purity are also suitable.*

## Equipment

1. Micropipettes
  - a. Starlab ErgoOne® 100-1,000 µl Single-Channel Pipette (catalog number: S7110-1000) or Gilson F123602 PIPETMAN Classic Pipet P1000 (Fisher Scientific, catalog number: 10387322)
  - b. Starlab ErgoOne® 20-200 µl Single-Channel Pipette (catalog number: S7100-2200) or Gilson F123615 PIPETMAN Classic Pipet P100 (Fisher Scientific, catalog number: 10442412)
  - c. Starlab ErgoOne® 2-20 µl Single-Channel Pipette (catalog number: S7100-0220) or Starlab ErgoOne® 0.1-2.5 µl Single-Channel Pipette (catalog number: S7100-0125) or Gilson F144801 PIPETMAN Classic Pipet P2 (Fisher Scientific, catalog number: 10635313) or Gilson F123600 PIPETMAN Classic Pipet P20 (Fisher Scientific, catalog number: 10082012)
2. Cell scrapers (Fisher Scientific, catalog number: 08-100-241)
3. Equipment for basic cell culture techniques and aseptic procedures, *i.e.*,
  - a. Biosafety cabinet (*e.g.*, Thermo Scientific Holten Safe 2010 Model 1.2, catalog number: 8207071100)
  - b. Humidified incubator set to 37 °C (Panasonic Incusafe, catalog number: MCO-20AIC)  
*Note: Any manufacturer and model of a biosafety cabinet will do, but the chosen biosafety cabinet needs to be appropriate for the containment of cells and viruses. When handling infectious viruses, use proper facilities, practices, and procedures. Refer to local and international guidelines for laboratory biosafety.*
4. -20 °C freezer (Labcold, catalog number: RLCF1520)
5. Water bath (*e.g.*, Clifton, catalog number: NE2-8D; however, any type of water bath set to 37 °C is appropriate)
6. Table-top microcentrifuge, 4 °C to room temperature (RT), max speed ≥ 12,000 x g (Eppendorf, 5424R)  
*Note: For spinning down precipitate that may form during extended storage of SNAP probes.*
7. Fume hood (any manufacturer or model that will protect users from formaldehyde fumes will do)
8. Confocal laser scanning microscope. For example, a customized ZEISS LSM 880 system that includes the following essential components (for a similar setup, contact your local ZEISS representative for exact ordering information as product codes may differ from customer to customer and country to country):
  - a. Axio Imager Z2 stand, motorized (upright system), product ID: 430000-9902-000
  - b. Motorized Stage, Scanning Stage 130x85 STEP, product ID: 432033-9902-00
  - c. Mounting Frame 160x116 f/ Slides 76x26, product ID: 432315-0000-000
  - d. Scan module LSM 880, product ID: 000000-1994-956

- e. Support f/scan module LSM (Imager Tube), product ID: 000000-1265-660
- f. Stepper motor control f, 2 Axes SMC2009, product ID: 432929-9011-000
- g. Real-time controller standard, product ID: 000000-2031-918
- h. Objective C PApo 63x/1.4 Oil DIC UV-IR, product ID: 421782-9900-799
- i. 488 Vis Laser, Laser Argon Multiline 25 mW, product ID: 000000-2086-081
- j. 561 Vis Laser, Laser 561nm for LSM 710, product ID: 00000-1410-117
- k. 633 Vis Laser, Laser Rack LSM 880 incl. 633 Laser, product ID: 000000-2085-478
- l. Airyscan SR module GaAsP for LSM, product ID: 000000-2058-580
- m. Emission filter BP495-550 + LP570 for Airyscan, product ID: 000000-2070-488
- n. Emission filter BP570-620 + LP645 for Airyscan, product ID: 000000-2070-489
- o. Double BP 420-480 + BP495-620 for Airyscan, product ID: 000000-2095-049
- p. Double BP 465-505 + LP525 for Airyscan, product ID: 000000-2095-051
- q. Double BP 420-480 + LP605 for Airyscan, product ID: 000000-2095-052
- r. User PC advanced Z55A highend, product ID: 000000-2142-968
- s. Transmitted light detector T-PMT, product ID: 000000-2014-999
- t. 'Airyscan Fast' illumination module upgrade for 1x LSM 880 system

**Notes:**

- i. *The above list is not exhaustive but only lists essential components that we have found useful for our protocols. Consult with ZEISS about additional components to complete the microscope system (e.g., joystick for stage, hardware license keys, beam splitter, switching mirror and nosepiece).*
- ii. *Follow the manufacturer's recommendations for applying all necessary Airyscan settings within the ZEN microscope software. Our Airyscan microscope could provide a maximum lateral resolution of 140 nm and axial resolution of 400 nm for a fluorophore emitting at 480 nm (images processed with ZEN Black software). Airyscan resolution can increase even further to 120 nm XY and 350 nm Z resolution with the use of ZEN Blue software.*
- iii. *Our original system (LSM 880 with Airyscan) was retroactively upgraded to an 'Airyscan Fast' system, whereas today's systems can be customized with an 'Airyscan Fast' module from the start. The 'Airyscan Fast' illumination system allows simultaneous illumination of 4 pixels simultaneously to allow very fast and gentle imaging of samples, including the option to scan at super resolution with 1.5x resolution improvement in XY & Z, imaging at up to 19 images/second with 512 x 512 pixels, 27 images per second at 480 x 480, and 6 images per second at 1024 x 1024.*

**Software**

1. ZEISS ZEN software (ZEN 2.3 system HWL for FAST Airyscan)

*Note: ZEISS ZEN software drives all components of the LSM 880 system, including setup of Airyscan imaging. ZEN software can also acquire and process raw super-resolution image*

datasets; save processed image files as ‘.czi’ files, which can be exported to bioimaging analysis software (e.g., Icy software). Saving in this file format ensures the preservation of all the metadata associated with each imaging experiment.

## 2. Icy bioimaging software

*Note: Bioimaging analysis software. We recommend the free software [Icy](#) to visualize, annotate and quantify bioimaging datasets, which can be imported from ZEISS ZEN software packages (de Chaumont et al., 2012). We found that Icy has an intuitive user interface.*

## Procedure

This protocol uses fluorescence light microscopy to reveal stable assemblies of SNAP-nsP3 protein that persist in cell lysates. It is partly based on an isolation protocol (Wheeler et al., 2017) that has been used to determine the proteome and substructure of stress granules (Jain et al., 2016). Our protocol adds additional flexibility in labeling (by using the SNAP labeling system) and detection (by using the sensitive ZEISS LSM 880 Airyscan confocal imaging system).

*Note: Carry out all liquid handling steps that involve live cells inside the biosafety cabinet. Only use sterile pipettors, pipets, micropipettors, microtubes, and tips. After cell lysis, users can handle samples outside the biosafety cabinet. Wear safety glasses when handling formaldehyde solutions.*

1. Detach stable CHIKV cells with trypsin and seed in at least one well of a 6-well microtiter plate. Also, detach and seed naïve cells, which do not express SNAP-tagged proteins, in at least one well; these cells will serve as a negative control.
2. Incubate cells under standard growth conditions (*i.e.*, 37 °C at 5% CO<sub>2</sub>) overnight.
3. On the next day, take frozen SNAP Cell® TMR-Star from -20 °C freezer and thaw at room temperature. In a 1.5-ml microtube, dilute SNAP-Cell® TMR-Star 1:600 (final concentration of 1 μM) dilution in complete cell culture media. Prepare at least 1 ml per well to cover the entire area of the well. Vortex briefly (for ≥ 5 s) or pipet the diluted labeling solution up and down (ten times). We have only validated this protocol with the SNAP-Cell® TMR-Star reagent. However, the protocol should also be compatible with SNAP-Cell® 647 SiR. Use the same labeling conditions as described in Protocol 1 (*i.e.*, 1:1,000 dilution, 15 min incubation).

*Note: Recipe for two dishes, scale accordingly for additional dishes:*

1996.8 μl Complete Cell Culture Media

3.2 μl SNAP-Cell® TMR-Star

4. Spin diluted labeling solution for 5 min at maximum speed (≥ 12,000 × g) to remove possible insoluble fluorescent debris. Take care not to disturb the pellet when removing supernatant (which may be invisible).
5. Replace the medium on stable CHIKV cells with 1 ml of SNAP-tag labeling medium (pre-heated to 37 °C in a water bath). Also, replace medium in the ‘negative control’ well. Incubate for 15 min at 37 °C, 5% CO<sub>2</sub>.

*Note: We found that a 15-min incubation gave us optimal labeling. According to the manufacturer, optimal reaction times range from 5 to 30 min, respectively, depending on experimental conditions and expression levels of the SNAP-tagged protein.*

6. Wash the cells three times each with 2 ml of tissue culture medium containing serum (pre-heated to 37 °C in a water bath). Do not remove media after the final wash.
7. Place cells back into the humidified incubator for another 45-60 min.

We use an extended wash-out period (compared to Protocol 1) to reduce the non-specific background-binding further. Moreover, we do not add ProLong Gold Live in this protocol, as live-cell imaging is only limited to an optional quality-control-step in Step 8.

8. Optional: Confirm fluorescent staining of SNAP-nsP3 with microscopy
  - a. A basic widefield fluorescent microscope equipped with a 10x or 20x objective is sufficient to evaluate the quality of SNAP-labeling through the microscope eyepiece. If documentation of staining quality is needed, take pictures with a connected camera. Also, confirm the absence of staining in the well that contains negative control.
  - b. We recommend this step when testing labeling conditions for the first time. Perform imaging quickly and proceed to Step 9 as soon as possible to limit the deterioration of cell health. If this is not logistically possible, we recommend proceeding to Step 9 directly.
9. Replace complete cell media with 1 ml PBS and use separate cell scrapers to detach cells from growth area in each well. Transfer the resulting cell suspension to a 1.5 ml microtube. Pellet cells at 1,500 x g, 3 min at room temperature. Remove supernatant.  
*Optional pause point: We freeze pellets at ≤ -20 °C if we want to carry out lysis at a later time.*
10. Lyse pellets by adding 300 µl of ice-cold Glasgow Lysis buffer containing protease inhibitors (see Recipes). Ensure complete re-suspension of pellet through repeated pipetting, flicking the tube, or vortexing. If pellet came from the freezer, thaw on ice for 5 min before adding lysis buffer.
11. Vortex Lysates for 30 s. Place on ice for 30 s. We return samples to ice in between vortexing cycles to prevent an extended incubation period at room temperature. Alternatively, lysates can be vortexed in a cold room.
12. Repeat Step 11 three times.
13. Spin at 850 x g for 5 min at 4 °C to remove remaining cellular debris.

*Note: We do not further purify assemblies of SNAP-nsP3 after this step but instead use the crude supernatant in the microscopic analysis. It would be interesting to test whether subsequent centrifugation at 18,000 x g can pellet assemblies of SNAP-nsP3; this step is essential in the isolation of a pure population of stress granule cores (Wheeler et al., 2017). After this step, users can handle cell lysates outside a biosafety cabinet.*

14. Transfer the entire volume of supernatant to a two-well Ibidi chambered plastic slide. Discard pelleted cellular debris.

*Note: We have noticed that the supernatant may appear turbid at this step. Proteins that are components of RNPs are known to undergo liquid-liquid phase transitions, and turbidity is due*

to the formation of small protein-rich droplets (Mollieix et al., 2015). However, we have not yet tested whether purified SNAP-nsP3 can form similar droplets in solution.

15. Incubate overnight at 4 °C in a humidified environment in the dark.
  - a. To obtain humidified conditions, we cover the Ibidi slide with the supplied plastic cover and place the slide in a 10-cm Petri dish that also contains moistened paper (e.g., cut sheets of Western blotting filter).
  - b. We further seal dishes with Parafilm M and wrap them in aluminum foil to prevent light exposure. We incubate overnight to give assemblies of SNAP-nsP3 enough time to settle to the bottom of the chamber.
  - c. Future experiments may determine the minimum duration for deposition of SNAP-nsP3 to the bottom of the chambered slide; this information would be useful for reducing the overall time required to complete Protocol 3.

16. In a fume hood, add 1 ml of 4% formaldehyde to wells containing cell lysates. Incubate for one hour at room temperature.

*Note: Slowly dispense formaldehyde with a micropipette. We add this step to fix assemblies of SNAP-nsP3; fixation reduces the likelihood that the assemblies detach from the well surface during subsequent washing steps.*

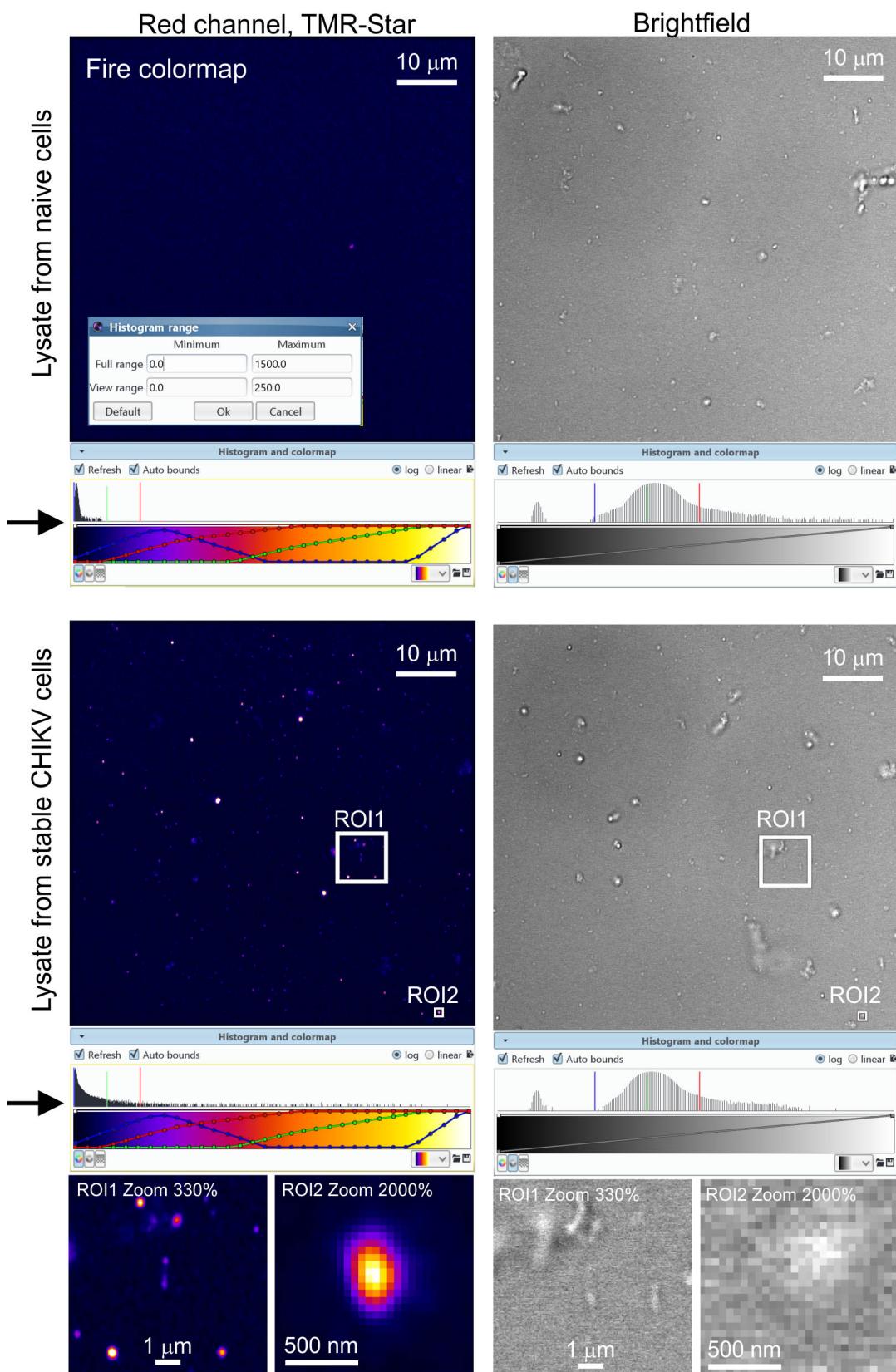
17. Wash wells three times with PBS. Ensure that wells do not dry out in between washes.

18. Transfer slide to microscopy system of choice:

As described in Protocol 2, we use the ZEISS LSM 880 imaging system operated in Airyscan mode. Use the appropriate filter settings for TMR-Star, which should have an excitation maximum at 554 nm and an emission maximum at 580 nm. Also, acquire an image in brightfield mode. This image can provide an additional record of the examined biostructure.

19. We recommend taking at least three images for each experiment:

We acquire images with the 63x objective. Export processed Airyscan files to Icy bioimaging software. Adjust the image contrast within the Icy software by dragging the boundaries of the viewing range in the software's histogram viewer (see Figure 4, arrows). We prefer viewing the SNAP-nsP3 channel with the 'Fire' colormap. Use Icy's controls to zoom into regions-of-interest. See Figure 4 for representative images.



**Figure 4. Analysis of cell lysates with Airyscan microscopy.** We processed samples according to Protocol 3 and detected granular structures containing TMR-Star-labelled

SNAP-nsP3. We chose the ‘Fire’ colormap in Icy to display signals from the TMR-Star staining. We also adjusted image contrast by dragging the adjustable bounds of the histogram viewer (marked by arrows). We acquired images at a zoom factor of 1 with a 63x objective. During image processing, we also magnified regions-of-interest (ROI) by increasing the digital zoom within the Icy software (*i.e.*, by a factor of 3.3 for ROI1 and 20 for ROI2). Lastly, we also acquired a brightfield channel to provide a reference and reveal all contrast-producing structures present in the lysate.

## Recipes

### 1. Home-made Glasgow Lysis Buffer (GLB) with protease inhibitors, 5 ml volume

Reagent	Stock Solution	Storage conditions (Stock)	Final Concentration or Percentage	Volume to add from Stock
Triton X-100	100% Triton X-100	Room temp.*	1%	50 µl
Glycerol	100% Glycerol	Room temp.	10%	500 µl
Piperazine- <i>N,N'</i> -bis (2-ethanesulfonic acid) [PIPES]-NaOH, pH 7.2	0.5 M PIPES (Adjust pH to 7.2 with 3 M NaOH)	4 °C	10 mM	100 µl
KCl	2.5 M KCl	4 °C	120 mM	240 µl
NaCl	5 M NaCl	4 °C	30 mM	30 µl
MgCl <sub>2</sub>	1 M MgCl <sub>2</sub>	4 °C	5 mM	25 µl
Leupeptin	1 mg/ml Leupeptin	-20 °C	1 µg/ml	5 µl
Pepstatin A	1 mg/ml Pepstatin A	-20 °C	1 µg/ml	5 µl
Aprotinin	2 mg/ml Aprotinin	4 °C	2 µg/ml	5 µl
AEBSF (Pefabloc)	100 mM AEBSF	4 °C	0.2 mM	10 µl
Distilled Water	100% Distilled Water	Room temp.	-	Adjust the total volume to 5 ml with Distilled Water
NaOH	3 M NaOH	Room temp.	-	Add NaOH until pH reaches 7.2

\*temp.: temperature

**2. Stock solutions for GLB**

Add the appropriate amount of solids to distilled H<sub>2</sub>O to make stock solutions of PIPES (adjust pH to 7.2 with 3 M NaOH), KCl, NaCl, MgCl<sub>2</sub> at concentration listed in the table. Store these stock solutions at 4 °C.

**3. Stock solutions of protease inhibitors, store in the following manner:**

Leupeptin 1 mg/ml at -20 °C

Pepstatin A 1 mg/ml at -20 °C

Aprotinin 2 mg/ml at 4 °C

AEBSF (Pefabloc) 100 mM at 4 °C

**4. 1x GLB**

Combine the stock solutions, Glycerol, Triton-X, and protease inhibitors according to the table.

Adjust volume to 5 ml with distilled H<sub>2</sub>O and pH to 7.2 with 3 M NaOH

*Note: Use fresh 1x GLB for each experiment.*

**5. Tissue culture medium containing serum**

500 ml Dulbeccos modified Eagles medium

56.5 ml Fetal Calf Serum

5.6 ml MEM Nonessential amino acids solution

5.6 ml HEPES

**Data analysis**

We consider the unprocessed image files that microscope users save in either the Nikon NIS Elements AR software (.nd2 file format) or ZEISS ZEN software (.czi file format) to be 'raw' data. Thus, data analysis in these protocols was for qualitative purposes and made up exclusively of image visualization and digital processing of acquired images (e.g., adjusting brightness, contrast, and pseudo-colors) in either Nikon NIS Elements AR software or Icy bioimaging software. Although it is also possible to extract quantitative data from these images, our initial method development, which focused on the application of new labeling and imaging methods, did not include these types of bioimage-informatics approaches. Future studies may benefit greatly from incorporating rigorous bioimage-informatics techniques to the protocol described here.

**Acknowledgments**

A Wellcome Trust Investigator Award funded this work (WT 096670, awarded to Mark Harris). Purchase of shared equipment was made possible by a Wellcome Trust Multi-user equipment award (Zeiss LSM 880 instrument, WT104918MA, 'Multifunctional imaging of living cells for biomedical sciences'). The funders had no role in study design, data collection, and interpretation, or the decision to submit the work for publication. We thank Dr. Sally Boxall and the Bio-imaging Facility within the Faculty of Biological Sciences of the University of Leeds for access and help with

Airyscan microscopes. The authors acknowledge Gina Gamble and Dr. Kate Lewis for their help using the Nikon Ti2-E Inverted microscope. We recognize the previous study by Bodor *et al.* (2012) which described the pulse-chase and quench-pulse-chase approaches. We are grateful to Mark B. Carascal and Dr. Samuel Ko for comments during the revision of the manuscript.

### **Competing interests**

The authors declare that no conflicts of interest or competing interests exist.

### **References**

1. Bodor, D. L., Rodriguez, M. G., Moreno, N. and Jansen, L. E. (2012). [Analysis of protein turnover by quantitative SNAP-based pulse-chase imaging](#). *Curr Protoc Cell Biol* Chapter 8: Unit8.8.
2. Cole, N. B. (2014). [Site-specific protein labeling with SNAP-tags](#). *Curr Protoc Protein Sci* 73: Unit 30.1.
3. de Chaumont, F., Dallongeville, S., Chenouard, N., Herve, N., Pop, S., Provoost, T., Meas-Yedid, V., Pankajakshan, P., Lecomte, T., Le Montagner, Y., Lagache, T., Dufour, A. and Olivo-Marin, J. C. (2012). [Icy: an open bioimage informatics platform for extended reproducible research](#). *Nat Methods* 9(7): 690-696.
4. Frigault, M. M., Lacoste, J., Swift, J. L. and Brown, C. M. (2009). [Live-cell microscopy—tips and tools](#). *J Cell Sci* 122(Pt 6): 753-767.
5. Huff, J., Bergter, A., Birkenbeil, J., Kleppe, I., Engelmann, R. and Krzic, U. (2017). [The new 2D Superresolution mode for ZEISS Airyscan](#). *Nat Methods* 14: 1223.
6. Huff, J. (2015). [The Airyscan detector from ZEISS: confocal imaging with improved signal-to-noise ratio and super-resolution](#). *Nat Methods* 12: 1205.
7. Jain, S., Wheeler, J. R., Walters, R. W., Agrawal, A., Barsic, A. and Parker, R. (2016). [ATPase-modulated stress granules contain a diverse proteome and substructure](#). *Cell* 164(3): 487-498.
8. Kedersha, N., Stoecklin, G., Ayodele, M., Yacono, P., Lykke-Andersen, J., Fritzler, M. J., Scheuner, D., Kaufman, R. J., Golan, D. E. and Anderson, P. (2005). [Stress granules and processing bodies are dynamically linked sites of mRNP remodeling](#). *J Cell Biol* 169(6): 871-884.
9. Kedersha, N., Tisdale, S., Hickman, T. and Anderson, P. (2008). [Real-time and quantitative imaging of mammalian stress granules and processing bodies](#). *Methods Enzymol* 448: 521-552.
10. Keppler, A., Kindermann, M., Gendreizig, S., Pick, H., Vogel, H. and Johnsson, K. (2004a). [Labeling of fusion proteins of O<sup>6</sup>-alkylguanine-DNA alkyltransferase with small molecules \*in vivo\* and \*in vitro\*](#). *Methods* 32(4): 437-444.

11. Keppler, A., Pick, H., Arrivoli, C., Vogel, H. and Johnsson, K. (2004b). [Labeling of fusion proteins with synthetic fluorophores in live cells](#). *Proc Natl Acad Sci U S A* 101(27): 9955-9959.
12. Li, C., Kuang, C. and Liu, X. (2018). [Prospects for fluorescence nanoscopy](#). *ACS Nano* 12(5): 4081-4085.
13. Molliex, A., Temirov, J., Lee, J., Coughlin, M., Kanagaraj, A. P., Kim, H. J., Mittag, T. and Taylor, J. P. (2015). [Phase separation by low complexity domains promotes stress granule assembly and drives pathological fibrillization](#). *Cell* 163(1): 123-133.
14. Muller, C. B. and Enderlein, J. (2010). [Image scanning microscopy](#). *Phys Rev Lett* 104(19): 198101.
15. Nakabayashi, H., Taketa, K., Miyano, K., Yamane, T., Sato, J. (1982). [Growth of human hepatoma cells lines with differentiated functions in chemically defined medium](#). *Cancer Res* 42(9): 3858-63.
16. Remenyi, R., Gao, Y., Hughes, R. E., Curd, A., Zothner, C., Peckham, M., Merits, A. and Harris, M. (2018). [Persistent replication of a chikungunya virus replicon in human cells is associated with presence of stable cytoplasmic granules containing nonstructural protein 3](#). *J Virol* 92(16).
17. Remenyi, R., Roberts, G. C., Zothner, C., Merits, A. and Harris, M. (2017). [SNAP-tagged chikungunya virus replicons improve visualisation of non-structural protein 3 by fluorescence microscopy](#). *Sci Rep* 7(1): 5682.
18. Sheppard, C. J., Mehta, S. B. and Heintzmann, R. (2013). [Superresolution by image scanning microscopy using pixel reassignment](#). *Opt Lett* 38(15): 2889-2892.
19. Utt, A., Das, P. K., Varjak, M., Lulla, V., Lulla, A., Merits, A. (2015). [Mutations conferring a noncytotoxic phenotype on chikungunya virus replicons compromise enzymatic properties of nonstructural protein 2](#). *J Virol* 89(6): 3145-3162.
20. Wheeler, J. R., Jain, S., Khong, A. and Parker, R. (2017). [Isolation of yeast and mammalian stress granule cores](#). *Methods* 126: 12-17.

## Straight Channel Microfluidic Chips for the Study of Platelet Adhesion under Flow

Alexander Dupuy<sup>1, 2</sup>, Lining Arnold Ju<sup>1, 2</sup> and Freda H Passam<sup>1, 2, \*</sup>

<sup>1</sup>Heart Research Institute, Newtown, NSW 2042, Australia; <sup>2</sup>University of Sydney, Camperdown, NSW 2006, Australia

\*For correspondence: [reda.passam@sydney.edu.au](mailto:reda.passam@sydney.edu.au)



**[Abstract]** Microfluidic devices have become an integral method of cardiovascular research as they enable the study of shear force in biological processes, such as platelet function and thrombus formation. Furthermore, microfluidic chips offer the benefits of *ex vivo* testing of platelet adhesion using small amounts of blood or purified platelets. Microfluidic chips comprise flow channels of varying dimensions and geometries which are connected to a syringe pump. The pump draws blood or platelet suspensions through the channel(s) allowing for imaging of platelet adhesion and thrombus formation by fluorescence microscopy. The chips can be fabricated from various blood-compatible materials. The current protocol uses commercial plastic or in-house polydimethylsiloxane (PDMS) chips. Commercial biochips offer the advantage of standardization whereas in-house chips offer the advantage of decreased cost and flexibility in design. Microfluidic devices are a powerful tool to study the biorheology of platelets and other cell types with the potential of a diagnostic and monitoring tool for cardiovascular diseases.

**Keywords:** Microfluidics, Biochip, Platelet, Thrombosis, Biorheology

**[Background]** Platelets primary function is to maintain blood in circulation by sealing off any breach of vessel integrity which would otherwise lead to exsanguination. For this function, platelets have evolved specialized cell surface receptors to allow adhesion to the endothelium and subendothelium at varying shear rates, ranging from  $200\text{ s}^{-1}$  in venous circulation to  $> 1 \times 10^4\text{ s}^{-1}$  in stenotic arteries (Chatzizisis *et al.*, 2008). Shear stress is implicated in the development of atherosclerotic lesions (Chatzizisis *et al.*, 2008). Shear rate and stress impact protein expression (Morigi *et al.*, 1995) and function (Yago *et al.*, 2004; Ju *et al.*, 2013 and 2015). Hence, the effect of flow on cell function must be included in vascular research.

Flow chambers, introduced in 1973 by Baumgartner (Baumgartner, 1973), incorporate the parameters of shear rate, which is the velocity gradient throughout a moving fluid, and shear stress, which is the force experienced by the wall of a conductance vessel due to the friction force of a moving fluid. The application of microfluidics has enabled significant discoveries in platelet biology. For example, by perfusing blood under controlled shear rates over adhesive proteins, it was found that platelets utilize their receptor glycoprotein Ib alpha (GPIba) to adhere to von Willebrand factor (vWF), whereas, they use their receptor integrin alpha IIb beta 3 ( $\alpha_{IIb}\beta_3$ ) to adhere to fibrinogen (Ruggeri, 2009; Ju *et al.*, 2018; Passam *et al.*, 2018). Under low-intermediate shear rates ( $< 1 \times 10^3\text{ s}^{-1}$ ), typically found in veins and large arteries, platelet adhesion is predominately mediated by integrin  $\alpha_{IIb}\beta_3$ . Under high

shear rates ( $1 \times 10^3$ - $1 \times 10^4$  s $^{-1}$ ), typically found in arterial microcirculation and in arterial stenosis, platelet adhesion is predominantly vWF-dependent (Reininger *et al.*, 2006; Ruggeri *et al.*, 2006; Jackson, 2007). Because of the effect of fluid dynamics on cell function and protein expression, microfluidics is a powerful tool to study cells in circulation.

Commercial microfluidic devices have been developed. The rheology of biological fluids passing through these devices has been well characterized (Lane *et al.*, 2012). However, these devices are limited by high cost and inflexible geometry designs. Recent studies in the field have utilized polydimethylsiloxane (PDMS) elastomers to fabricate microfluidic flow chambers, or ‘biochips’, with typical channel geometries of 10-1,000  $\mu\text{m}$  in x and y-axes and 10-200  $\mu\text{m}$  in the z-axis. PDMS is a transparent and elastic material which has been widely used to create microfluidic channels with various geometries such as stenosis (Nesbitt *et al.*, 2009; Tovar-Lopez *et al.*, 2013), bifurcation (Tsai *et al.*, 2012), aneurysm (Mannino *et al.*, 2015), and spiral channels for two-dimensional cell sorting (Hou *et al.*, 2016). In contrast to the conventional flow chamber that usually requires milli-liter sample volumes, the microfluidic biochip only requires micro-liter samples, making it a perfect analytical tool for small volumes (e.g., pediatric or rare samples and mouse studies). In addition, the biochips described in this protocol are reusable, making them an economic and versatile choice for microfluidic studies.

Using microfluidics, we have recently shown that a member of the thiol isomerase family, endoplasmic reticulum protein 5 (ERp5) regulates platelet adhesion to fibrinogen in a shear-dependent manner (Passam *et al.*, 2018). The current protocol describes the fabrication and assembly of an in-house straight-channel biochip. The protocol also describes the application of straight-channel commercial and in-house chips for the study of platelet adhesion under controlled shear conditions.

## **Materials and Reagents**

1. 2 ml glass Luer lock syringe (Tömopal, catalog number: 140-1502)
2. 24 mm x 50 mm x 0.17 mm #1 borosilicate rectangular coverslips (Thermo Fisher, catalog number: MENCS24501GP)
3. 150 mm x 20 mm Petri dish (SARSTEDT, catalog number: 82.1184.500)
4. Medical grade Tygon tubing, 0.8 mm ID and 1.6 mm OD (Watson-Marlow)
5. 20 ml Syringe Luer Lok Tip (BD, catalog number: 302830)
6. Silicon wafer, P-type boron dope, 4 inches (Research and Prototype Foundry, University of Sydney Nano Institute)
7. 365 nm UV lamp (Research and Prototype Foundry, University of Sydney Nano Institute)
8. 1.5 ml transfer pipettes (Thermo Fisher, catalog number: 282TS)
9. Connecta<sup>TM</sup> 3-way stopcock, 2 female 1 rotating male Luer lock connector (BD, catalog number: 394995)
10. Venous blood collection set (BD, Vacutainer Safety-Lok Blood Collection Set with Pre-Attached Holder, 21 G, catalog number: 368654)
11. Scotch tape

12. Whole Blood Tube w/ Acid Citrate Dextrose (ACD) Sol A (BD, catalog number: 364606)
13. Acid Citrate Dextrose (store at 4 °C, shelf-life: 6 months) (Sigma-Aldrich, catalog number: C3821-50ML)
14. Bovine serum albumin (store dessicated albumin at 4 °C, shelf-life: 6 months) (Sigma-Aldrich, catalog number: A8531-1VL)
15. Butan-1-ol (Ajax Finechem, catalog number: AJA107-2.5LGL)
16. Calcein AM (store at -20 °C, shelf-life: 6 months) (Thermo Fisher, catalog number: C1430)
17. Rat anti-mouse glycoprotein Ib antibody conjugated to Dylight fluor 488 (Emfret Analytics, catalog number: X488)
18. DMSO, store in the dark at room temperature (Life Technologies, catalog number: D12345)
19. Extran® MA 02, store at room temperature (Merck, catalog number: 1075532500)
20. Fibrinogen, 4 mg/ml (store at -80 °C, shelf-life: 12 months) (Haematologic Technologies, catalog number: HCl-0150R)
21. Von Willebrand Factor (vWF), 0.3 mg/ml (store at -80 °C, shelf-life: 12 months) (Haematologic Technologies, catalog number: HCVWF-0190)
22. Photoresist SU-8 2000 (MicroChem, catalog number: SU-8 2050)
23. Photoresist Developer Microposit Thinner Type P (Rohm and Haas)
24. Isopropanol (IPA) (Sigma-Aldrich, catalog number: W292907)
25. Edge bead remover (MicroChem, EBR PG)
26. ddH<sub>2</sub>O
27. Sylgard(R) 184 Silicone Elastomer, store at room temperature (polydimethyl siloxane; PDMS) (Dow Corning, catalog number: 1317318)
28. Prostaglandin E1 (PGE1), 5 mg/ml (14 mM) in 100% ethanol (store at -80 °C, shelf-life: 6 months) (Sigma-Aldrich, catalog number: P5515-1MG)
29. HEPES (Sigma-Aldrich, catalog number: H3375-100G)
30. NaCl (Sigma-Aldrich, catalog number: S7653-250G)
31. KCl (Sigma-Aldrich, catalog number: P933-500G)
32. Na<sub>2</sub>HPO<sub>4</sub> (Sigma-Aldrich, catalog number: RES20908-A702X)
33. KH<sub>2</sub>PO<sub>4</sub> (Sigma-Aldrich, catalog number: P0662-500G)
34. NaHCO<sub>3</sub> (Sigma-Aldrich, catalog number: S5761-500G)
35. MgCl<sub>2</sub> (Sigma-Aldrich, catalog number: M8266-100G)
36. CaCl<sub>2</sub> (Sigma-Aldrich, catalog number: C5670-100G)
37. D-glucose (Sigma-Aldrich, catalog number: G8270-100G)
38. 1x PBS (Phosphate Buffered Saline) (store at room temperature, shelf-life: 6 months) (see Recipes)
39. 10% Extran® (store at room temperature, shelf-life: 1 year) (see Recipes)
40. Blocking buffer (store at 4 °C, shelf-life: 2 weeks) (see Recipes)
41. HEPES-Tyrode's buffer with glucose, store HEPES-Tyrode's without glucose at room temperature for 6 months, add glucose fresh each time (see Recipes)

## Equipment

### A. For in-house chips

1. Centrifuge (Eppendorf, catalog number: 5810000017)
2. Heidelberg tabletop maskless aligner (Heidelberg Instruments, catalog number: MLA100)
3. Pipettes (Eppendorf, catalog number: 3120000917)
4. 1 and 6 mm Harris Uni-Core Biopsy punch (World Precision Instruments, catalog numbers: 501907, 501910)
5. Water bath (Ratek instruments, catalog number: WB20)
6. Nalgene® vacuum desiccator (Sigma-Aldrich, catalog number: D2797-1EA)
7. Heratherm Gravity convection oven (Thermo Fisher, catalog number: 51028112)
8. Hotplate (SAWATEC, catalog number: HP-150)

*Note: All hot plates used for this process are an integral part of our Ritetrack Equipment, which also contains the coater/developer modules used for this project.*

9. PHD ULTRA™ Programmable Infuse/Withdraw syringe pump (Harvard Apparatus, catalog number: 70-3007)
10. Sonicator (Thermoline, catalog number: UB-405)
11. Tabletop centrifuge (Thermo Fisher, catalog number: 75002415)
12. Olympus fluorescent microscope IX81 60x oil immersion objective NA 1.35

### B. For commercial chips

1. Inlet and Outlet pins (Cellix, catalog number: SS-P-B1IC-B1OC-PACK200)
2. Tygon tubing for BiochipConnect (Cellix, catalog number: TUBING-TYGON-BIC-B1OCROLL100FT)
3. Vena8 Fluoro+™ biochips (Cellix, catalog number: V8CF-400-100-02P10)
4. Mirus Evo Nanopump (Cellix)
5. AxioObserver A1 Inverted Epi-Fluorescence microscope (Zeiss, Germany)
6. ExiBlu CCD camera (Q imaging, Canada)

### C. For platelet separation

1. Sysmex KX21 Hematology Analyzer (Sysmex America, Inc. Lincolnshire, Illinois, USA)

## Software

### A. For in-house chips

1. Fiji/ImageJ 1.52h (<https://fiji.sc/>)
2. GraphPad Prism (GraphPad software, <https://www.graphpad.com/scientific-software/prism/>)

## B. For commercial chips

1. VenaFlux 2.3 imaging software
2. ImagePro Premier 64-bit software image analysis
3. GraphPad Prism (GraphPad software, <https://www.graphpad.com/scientific-software/prism/>)

*Note: The VenaFlux 2.3 imaging software and ImagePro Premier 64-bit image analysis software are pre-installed in the PC of the Venaflux platform (Cellix Ltd., Unit 1, Longmile Business Park, Longmile Road, Dublin 12, Ireland, [info@wearecellix.com](mailto:info@wearecellix.com)).*

## Procedure

### A. Fabrication of PDMS biochips

The microfluidic biochips can be fabricated with PDMS (Sylgard 184 kit) casted from a master mould on a silicon wafer by photolithography (Qin *et al.*, 2010). Most universities and institutes have photolithography and clean room facilities for the fabrication of the master mould. In this protocol, the photolithography is conducted at [the Research and Prototype Foundry at University of Sydney Nano Institute](#). It is possible to perform photolithography without a foundry. The equipment required would include: 1. Air plasma system, 2. Oven for PDMS curing, 3. Desiccator for PDMS degassing, 4. Programmable spin coater, 5. UV lamp–LED exposure, 6. Programmable hot plate. We refer interested readers to: [Microfluidic device design, fabrication, and testing protocols](#) or [Elveflow](#).

1. Dehydrate a 4 in. silicon wafer at 200 °C for 20 min, then apply an adhesion promoter for 30 s at 120 °C.
2. Spin coat the wafer with SU-8 2050 (high contrast, epoxy-based) photoresist using a spread cycle of 70 and 22  $\times$  g for 30 s and a development cycle of 1,000 and 0.5  $\times$  g for 30 s in order to achieve a desired film thickness in the z-axis (*e.g.*, 50  $\mu$ m in this protocol).
3. Conduct a cycle of edge bead removal for 30 s using edge bead removal solvent.
4. Directly write the pattern to the SU-8 film using a dose of 365 nm UV light at 100 mJ/cm<sup>2</sup>.
5. Crosslink the film pattern by baking on a hotplate and ramping the temperature at 5 °C/min, starting at 23 °C and holding at 90 °C to dry out the solvents. The ramping profile is achieved by proximity, plus vacuum contact bake steps, for a total duration of 805 s.
6. Allow the film to cool on the hotplate to room temperature.

*Note: Keep the film on the hotplate to avoid thermal stress.*

7. Develop unexposed SU-8 photoresist using fresh developer solution for 3.5 min in a rocker.
8. Rinse the wafer with IPA.
9. Dry the wafer using pressurized nitrogen.
10. Hard-bake the wafer at 150 °C for 30 min.

**Note:** Alternatively, follow [the Permanent Epoxy Negative Photoresist Processing Guidline](#) from MicroChem.

**Note:** The following Steps (A11-A17) can be performed in the research lab (these steps do not require a clean room). (Figure 1)

11. Transfer the silicon wafer into a 150 mm Petri dish, SU-8 side facing upwards, and secure with scotch tape.
12. Mix the Sylgard 184 kit PDMS base with the kit curing agent at a ratio of 10:1 by weight. For example, to prepare 198 g of PDMS, mix 180 g of the PDMS base with 18 g of the curing agent.

**Notes:**

- a. Due to the viscosity of the base and curing agent, it is easier to prepare the kit by weight. It is important that the base and curing agent are mixed completely to prevent inconsistencies in the curing of the final product. Mix thoroughly until no visible streaks can be seen. This process will create a large amount of small bubbles that need to be removed by degassing.
- b. Once the curing agent is added to the PDMS base, the PDMS will slowly begin to cure and harden at room temperature. This will be noticeable after 4 h. After 16 h, the PDMS will become too viscous to practically work with.

13. Pour the PDMS mixture over the pre-made mold, creating a 4-5 mm thick film.
14. Place the PDMS and mold in a Nalgene® vacuum desiccator and degas for 30 min.

**Note:** The amount of time required to degas the mixture is dependent on the volume and surface area of PDMS. The mixture must be degassed completely, otherwise bubbles will form during the curing process which will change the morphology of the device. For 200 g of PDMS placed in a 100 mm diameter Petri dish, 2 h is sufficient to completely degas the mixture.

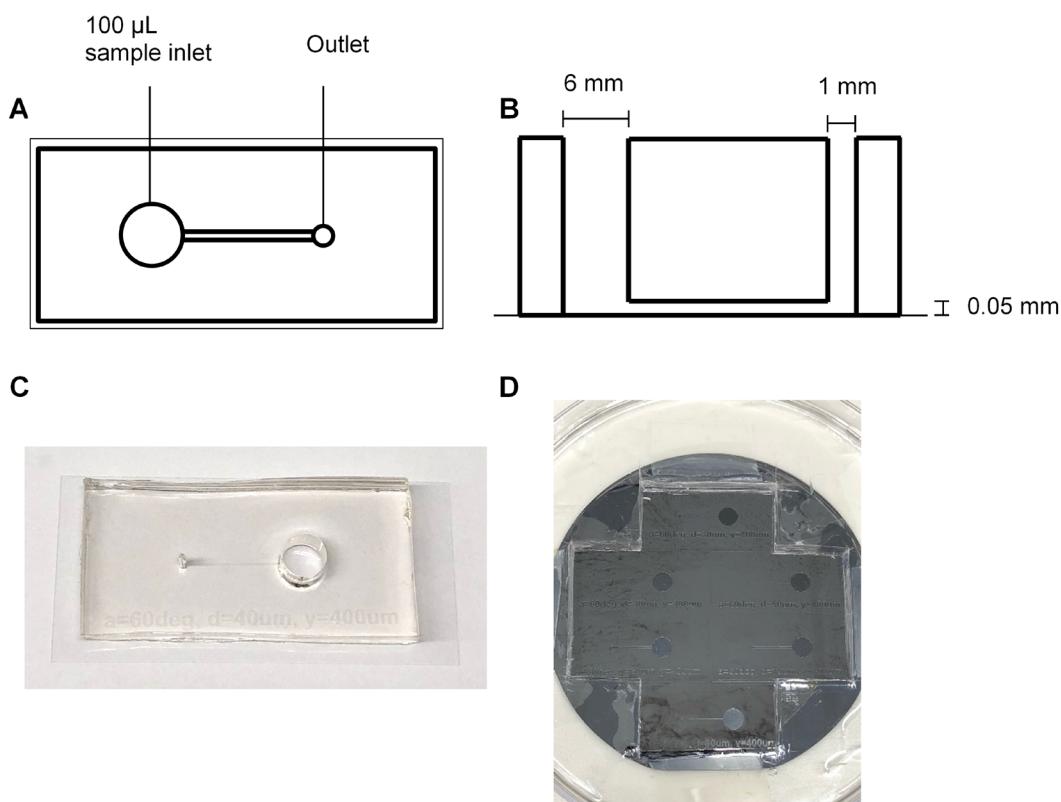
15. Bake (cure) the PDMS mixture and mold in the oven at 80 °C for 4 h.
16. Cut out the cured PDMS chips from the mold gently and carefully.

**Notes:**

- a. For the design used in this protocol, the biochip can be cut from the mold and the remaining PDMS can be left on the mold to reduce the amount of PDMS required for fabrication in the future.
- b. The typical PDMS cut-out block has a length of 4 cm, width of 2 cm and height of 5 mm. Avoid larger PDMS cut-outs as these can bend the coverslip and obscure the plane of focus during image acquisition.
- c. Take care not to damage or crack the mold whilst cutting out the biochip. Gradually cut out the PDMS by making consecutive, linear cuts into the PDMS and gently lift the PDMS off from the silicon wafer.

17. With the channel side facing upward, use a 6 mm diameter biopsy punch to cut out a hole at one end of the channel. Punch another hole at the opposite end of the channel using a 1 mm diameter biopsy punch.

*Note: The 6 mm hole is used as a well to hold the sample for the microfluidics experiment once the biochip is placed on a coverslip. The 1 mm hole is used to connect the channel to the syringe pump.*



**Figure 1. Fabrication of PDMS biochips.** The PDMS biochip produced in this protocol features a 6 mm diameter inlet (well) which can hold up to 100  $\mu\text{L}$  of sample, and a 1 mm diameter outlet which connects the channel to the pump. The channel has a length of 12 mm, width of 0.4 mm and a height of 0.05 mm (A, B, and C). The manufactured silicon wafer mask contains indentations which form the shape of the channel (D).

## B. Cleaning the biochip

Dust and other impurities can affect the coating of coverslips with fibrinogen and obstruct the field of view under the microscope. Dust may also interfere with the adhesion of PDMS to the coverslip and the adhesion of platelets to the coated coverslip. The following is a gentle cleaning procedure to remove dust and impurities from the surface of PDMS and coverslips.

1. Place the biochip in 400 ml of 10% extran and sonicate for 15 min.
2. Blow dry the biochip completely using compressed air.

*Note: Make sure the biochip is completely dry before proceeding. Remnants from small droplets can compromise the contact between the biochip and the coverslip leading to leakage during a microfluidics experiment.*

3. Place the biochip in 100% Butan-1-ol and sonicate for 30 min.

*Note: The PDMS will swell slightly and show indentations after the Butan-1-ol treatment. This is normal and the PDMS will return to its original shape and size once sonicated in dH<sub>2</sub>O.*

4. Blow dry the biochip completely using compressed air. Place the biochip in 10% extran and sonicate for 10 min.
5. Blow dry the device using compressed air. Place the biochip in dH<sub>2</sub>O and sonicate for 10 min.
6. Blow dry the device using compressed air.
7. With the channel side facing down, place the biochip on a #1 coverslip. Press the PDMS down on the coverslip with the palm of your hand and smooth out any bubbles with your finger.

*Notes:*

- a. *A good seal needs to be made between the PDMS and the coverslip to prevent leakage of cells from the sides of the channel.*
- b. *The coverslip should be cleaned to remove any dust and smudges that may interfere with contact between the PDMS and the coverslip. The coverslip can be cleaned using Steps B1 and B5.*
- a. *The PDMS can be reused after the microfluidics experiment. Clean the PDMS again by following Procedure B. Store the PDMS in a sealed container away from light. The PDMS can be reused until it is permanently bent and does not stick properly to a coverslip. In our experience, the PDMS can be reused at least 10 times.*
- b. *The size of the coverslip used depends on the size of the biochip. Choose a coverslip size that is closest to the area of the biochip.*

### C. Coating the channel of the biochip

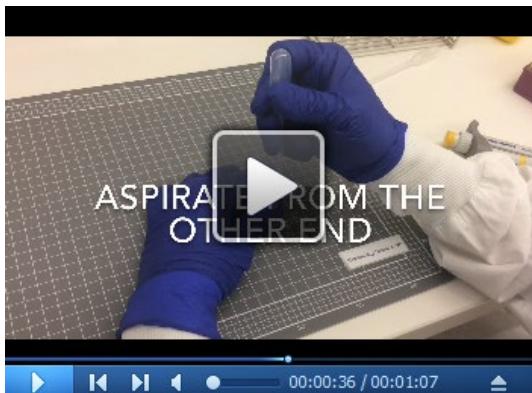
The steps involved in the coating of the biochip (Section C) can be found in Video 1.

1. Place 100 µl of fibrinogen (40 µg/ml final fibrinogen concentration in PBS, pH 7.4) in the 6 mm inlet of the biochip.
2. Using a 1.5 ml transfer pipette, draw the fibrinogen through the channel and into the pipette.

*Notes:*

- a. *A good seal between the transfer pipette and the 1 mm outlet must be made, otherwise the fibrinogen will not transverse the channel properly. Bubbles in the pipette indicate that good suction was not achieved.*
- b. *Do not drain the inlet or the channel completely. Draw up enough of the fibrinogen solution until you are certain that the channel has been filled with the fibrinogen solution. To remove the pipette from the outlet, apply positive pressure on the transfer pipette until the fluid stops drawing into the pipette, then remove the pipette.*

- c. To check that the channel has been filled with the coating substrate (fibrinogen), rotate the biochip back and forth under light. Due to the refractive index mismatch between air and PDMS, an empty channel will be slightly less transparent than a channel filled with fluid, leading to a shimmering of the channel under light.
3. Incubate the biochip for 2 h at 25 °C.  
*Note: The incubation period and temperature will depend on the coating substrate used to coat the channel. However, the longer the channel is left incubating, the greater the risk of the channel leaking.*
4. Remove the fibrinogen in the inlet well, replace with 100 µl of Tyrode's buffer or PBS and repeat Step D2 to wash the channel.
5. Remove the Tyrode's buffer or PBS from the inlet well and replace with 100 µl of blocking buffer. Repeat Step D2 to block the channel with blocking buffer. Incubate for 30 min at 25 °C.
6. Remove the blocking buffer from the inlet well, replace with 100 µl of Tyrode's buffer or PBS and repeat Step D2 to wash the channel.  
*Note: Once washed, keep the Tyrode's buffer or PBS in the channel until image acquisition. This will prevent the formation of air bubbles in the channel while flowing cells through the biochip.*



**Video 1. Coating of the in-house PDMS biochip**

#### D. Isolation of platelets from whole blood

##### **Human platelets**

The procedure of drawing human blood by venipuncture is determined by the Institution's Ethics and Protocols for human blood sampling.

1. Draw 8 ml venous blood by venipuncture into an ACD tube.
2. Centrifuge at 200 x g for 20 min, no brake, and separate the platelet-rich plasma (supernatant).
3. Allow platelet-rich plasma to rest in the water bath at 37 °C for 30 min.
4. Add PGE1 to the platelet rich plasma (1 µM final concentration) immediately prior to centrifugation.
5. Centrifuge at 800 x g for 20 min, no brake, and discard the platelet-poor plasma (supernatant).

6. Resuspend the platelet pellet in HEPES-Tyrodes' buffer with glucose. Perform a platelet count on the hematology analyzer and adjust the platelet concentration to  $3 \times 10^5/\mu\text{l}$  (Nesbitt *et al.*, 2009). Five minutes before perfusing through the microfluidic channel, add calcein to a final concentration of  $1 \mu\text{g}/\text{ml}$  and keep the platelets in the dark until use. Use the labeled platelets within an hour as the calcein is effluxed out of the cell and cell fluorescence will be lost.

### **Mouse platelets**

The procedure of drawing mouse blood by venipuncture is determined by the Institution's Animal Ethics and local protocols.

1. Draw 1 ml of blood by cardiac puncture after  $\text{CO}_2$  euthanization or by venipuncture of the inferior vena cava under anesthesia.
2. Centrifuge the blood on a tabletop centrifuge at  $400 \times g$  for 5 min.
3. Collect the platelet-rich plasma (supernatant) and 1/8 of the upper red blood cell layer.
4. Add 200  $\mu\text{l}$  of HEPES-Tyrodes' buffer with glucose to the red blood cell layer and gently mix. Centrifuge the red blood cell layer at  $400 \times g$  for 5 min.
5. Collect the supernatant and 1/8 of the upper red blood cell layer and pool with the platelet-rich plasma from Step 3.
6. Centrifuge the pooled platelet-rich plasma at  $200 \times g$  for 6 min.
7. Collect the platelet-rich plasma (supernatant) without disturbing the red blood cell layer.
8. Add 200  $\mu\text{l}$  of HEPES-Tyrodes' buffer with glucose to the red blood cell layer and mix. Centrifuge the red blood cell layer at  $200 \times g$  for 6 min.
9. Collect the supernatant without disturbing the red blood cell layer and pool with the platelet-rich plasma from Step 7.
10. Allow the platelet-rich plasma to rest for 10 min at room temperature. At the end of the incubation, add PGE1 (0.5  $\mu\text{M}$  final concentration) and centrifuge the plasma at  $500 \times g$  for 10 min.
11. Discard the platelet-poor plasma (supernatant) and resuspend the pellet in HEPES-Tyrode's buffer with glucose. Perform a platelet count on the hematology analyzer and adjust the platelet concentration to  $300 \times 10^3/\mu\text{l}$ .
12. Prior to performing a microfluidics assay, incubate the platelets with anti-GPIb 488 antibody (3  $\mu\text{g}/\text{ml}$  final concentration) for 10 min at room temperature, in the dark.

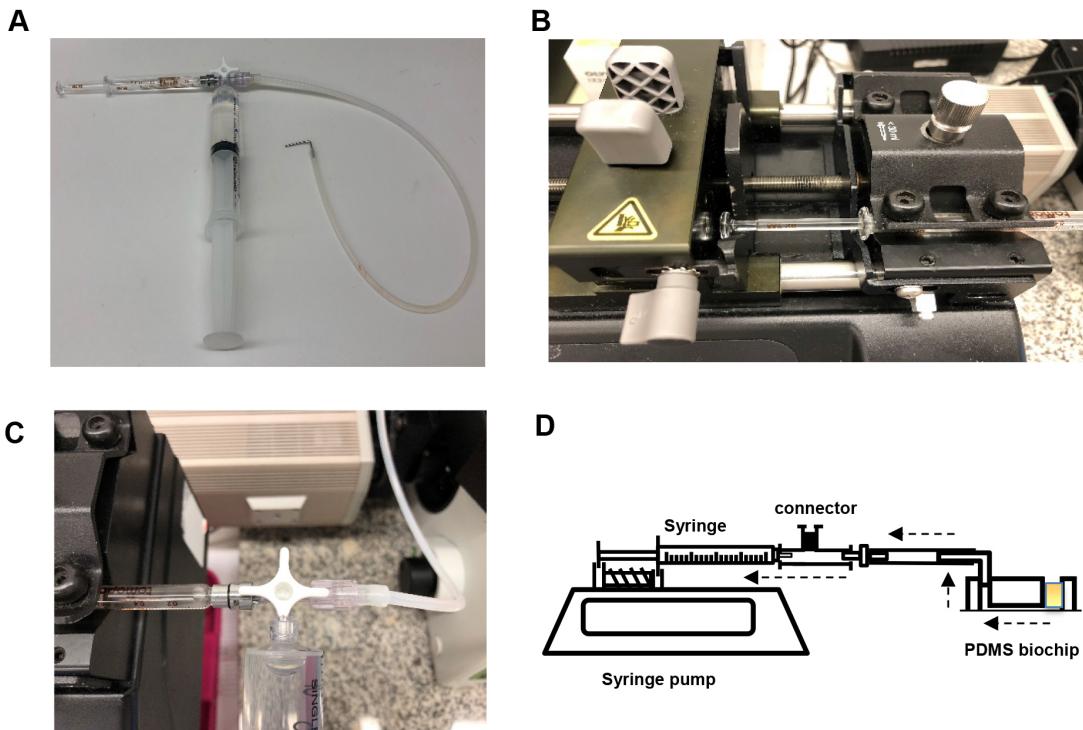
### **Notes:**

*For both human and mouse platelets:*

- a. All centrifugation steps are at room temperature.
- b. Platelet suspensions cannot be stored at  $4^\circ\text{C}$  at any step.
- c. Platelets should be used within 4 h of collection by venipuncture.
- d. Perfusion assays are performed in the dark.

### **E. Image acquisition**

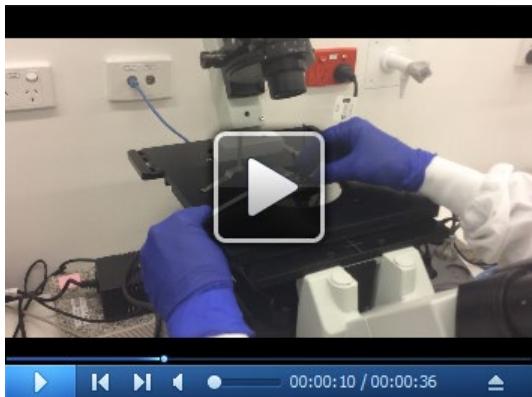
1. Assemble the syringe pump as shown below (Figure 2). Connect the 2 ml Luer lock glass syringe to the horizontal facing female Luer lock on the 3-way connector. Slip the silicone tubing onto the male Luer lock and screw the rotating lock over the tubing. Connect a water-filled 20 ml plastic Luer lock syringe to the final female Luer lock and place the glass syringe on the PHD syringe pump. Secure the glass syringe in place.



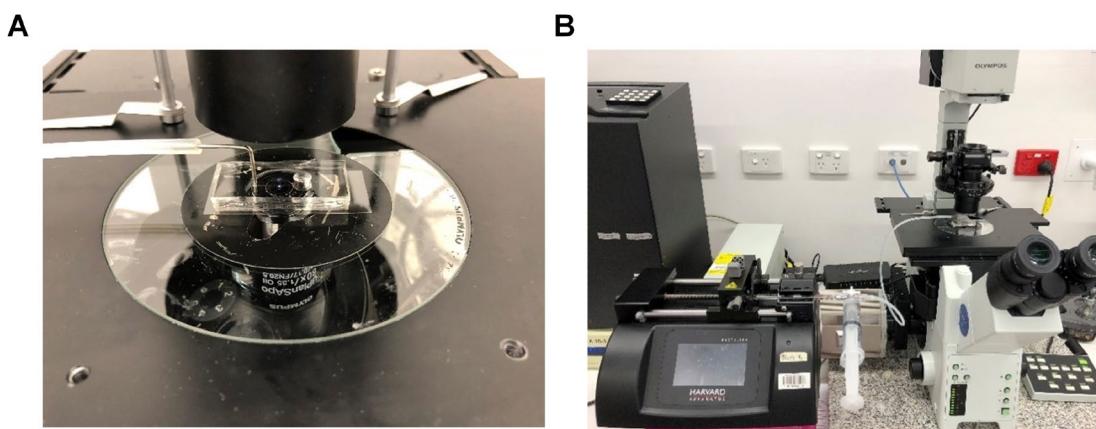
**Figure 2. Setup of the syringe pump.** A. Assembly of the 2 ml glass syringe, plastic 20 ml syringe filled with water (to flush the tubing after each experiment) and tubing (with a 1 mm diameter metal adapter) on a 3-way stopcock connector. B. Assembly of glass syringe on the pump. C. Rotation of the stopcock to enable flow from tubing to glass syringe. D. Schematic of the microfluidic chip set-up. Dashed arrows indicate the direction of flow. The yellow square in the chip represents the platelet suspension.

2. Bend a 30 mm length piece of 1 mm diameter steel tubing in half at a 90° angle and insert the other end into medical grade Tygon tubing. Insert the metal tubing into the 1 mm hole outlet of the biochip (Figure 3). Secure the biochip onto the stage of the microscope and bring the plane of the coverslip into focus (Figure 4).

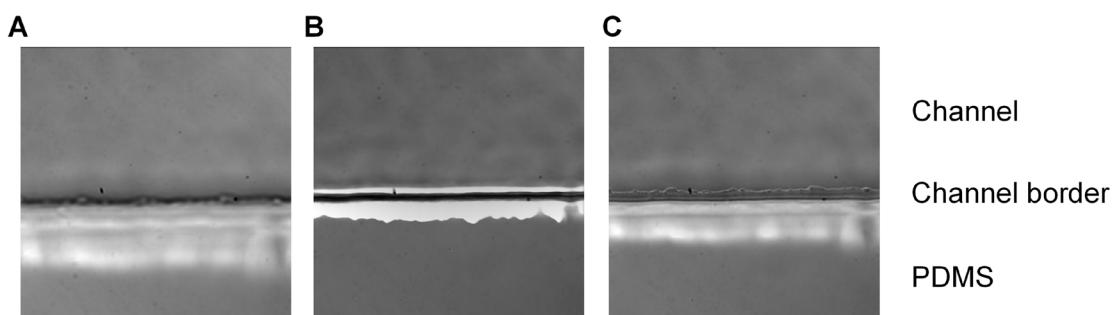
*Note: The steps involved in the assembly of the biochip to the microscope (Figures 2-3) can be found in Video 2.*



**Video 2. Assembly of the in-house PDMS biochip onto a microscope stage**



**Figure 3. Final assembly of the microfluidics system.** A. The 1 mm diameter metal tubing is connected to the 1 mm hole of the PDMS biochip. B. The system is then assembled to an inverted microscope.



**Figure 4. Planes of focus.** Imaging of the biochip placed under an Olympus fluorescent microscope using a 60x objective. The plane of focus is displayed below the coverslip (A), above the coverslip (B) and on the coverslip (C). When the coverslip is in focus, the jagged edge of the PDMS (an artifact of the fabrication process) can be seen within the channel.

3. Empty the inlet well and start the acquisition. Acquire a time-stack for both the DIC channel and fluorescent channel (excitation: 488 nm, emission: 520 nm) for ve+ stained platelets (Figure 5), setting the acquisition parameters to take images at regular time intervals.
4. Place 100  $\mu$ l of  $3 \times 10^5$   $\mu$ l washed platelets in the inlet well of the biochip.
5. Start the syringe pump and draw fluid through the channel at a rate of 5  $\mu$ l/min ( $500 \text{ s}^{-1}$ ) for 5 min. Alternatively, to expose the platelets to other shear rate and stress levels, use the simplified formulas (1, 2) below to calculate the flow rate needed. A table has been provided as a quick reference to calculate shear rates from flow rates for the chip used in this protocol (Table 1).

$$Q = Wh^2\dot{\gamma} \times 10000 \quad (1)$$

where,

$Q$  = flow rate ( $\mu$ l/min)

$W$  = width of the channel (cm)

$h$  = height of the channel (cm)

$\dot{\gamma}$  = desired shear rate ( $\text{s}^{-1}$ )

$$\text{Wall shear stress } \tau = \frac{6\mu Q}{Wh^2} \quad (2)$$

where,

$W$  = width of the channel (cm)

$h$  = height of the channel (cm)

$\mu$  = viscosity ( $\text{Pa}\cdot\text{s}$ )

$\tau$  = Wall shear stress (Pa)

At 37 °C

$\mu$  (Washed platelets in HEPES-Tyrode's buffer with glucose) =  $0.71 \times 10^{-3}$  Pa·s

$\mu$  (Whole blood) =  $3.8 \times 10^{-3}$  Pa·s = 0.038 poise

$\mu$  (Water) =  $0.695 \times 10^{-3}$  Pa·s

1 centipoise = 1 mPa·s = 0.001 Pa·s

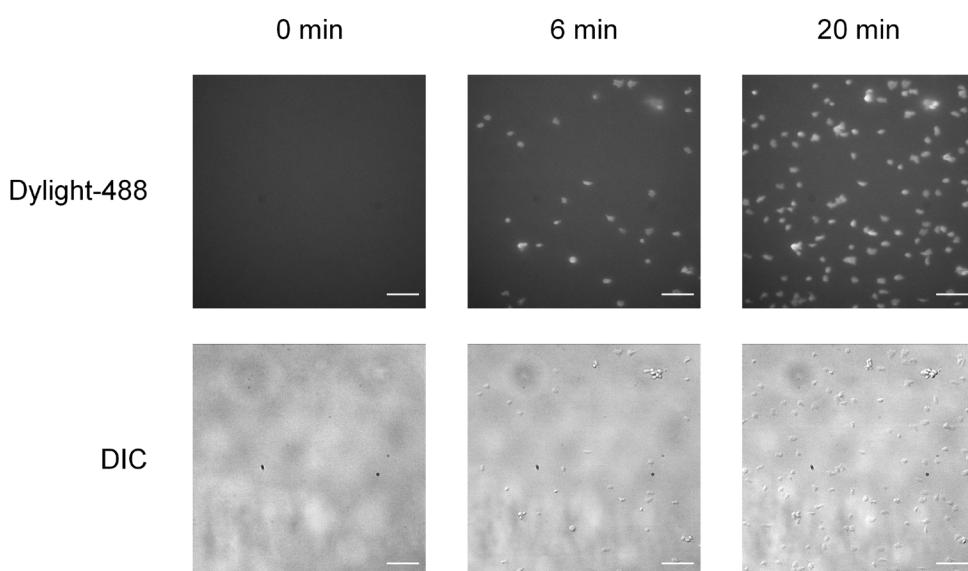
1 Pa = 10 dyn/cm<sup>2</sup> = 1 Newton/m<sup>2</sup>

Shear Stress (Pa) = shear rate ( $\text{cm}/\text{s}$  / cm) × viscosity (Pa·s)

$$\text{Shear rate} = \frac{\text{shear stress}}{\text{viscosity}} = \frac{\text{Pa}}{\text{Pa}\cdot\text{s}} = \text{s}^{-1}$$

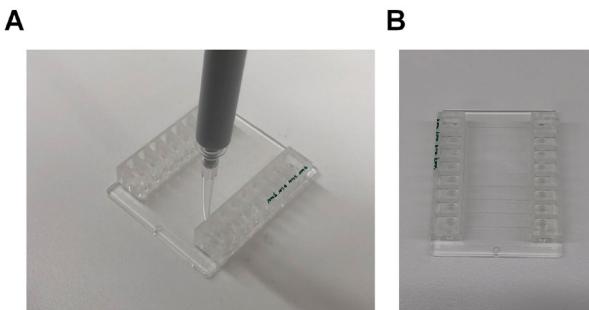
**Table 1. The flow rate of the platelet suspension required to achieve the desired shear rate and shear stress in the straight channel PDMS chip**

Shear rate $\gamma$ ( $s^{-1}$ )	Shear stress $\tau$ (Pa)	Flow rate Q ( $\mu l/min$ )
150	0.1065	1.5
300	0.213	3
600	0.426	6
1,200	0.852	12
1,800	1.278	18
5,000	3.55	50
10,000	7.1	100

**Figure 5. Adhesion of mouse platelets on fibrinogen under flow.** Perfusion of platelets, labeled with anti-GPIb-488 antibody, on PDMS channels coated with fibrinogen (40  $\mu g/ml$ ) at a shear rate of 500  $s^{-1}$ . Time course of adherent platelets as captured by epi-fluorescence (top row) and DIC (bottom row) microscopy at 0, 6, and 20 min. Scale bars = 20  $\mu m$ .**F. Platelet adhesion using Vena8 Fluoro+ biochips**

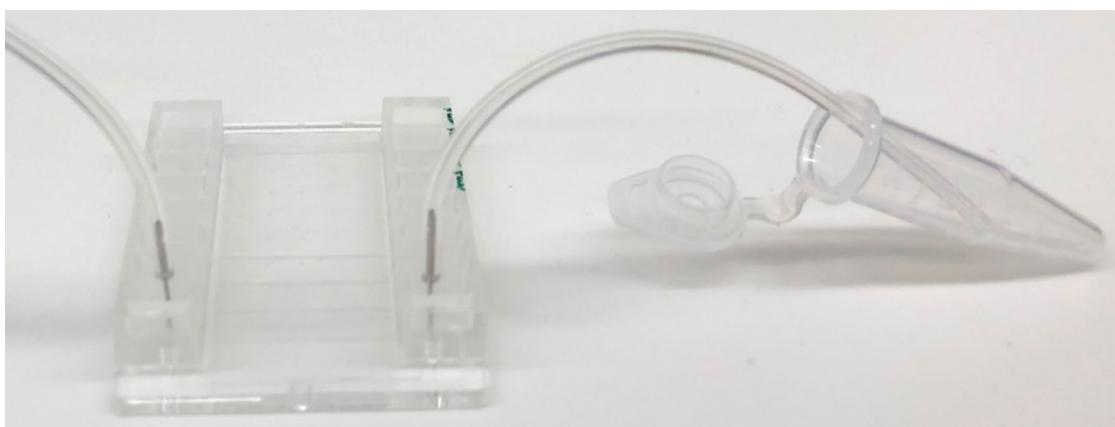
Vena8 Fluoro+ biochips are commercial biochips manufactured by Cellix™. They are 8 channel biochips with a coverslip of 0.17 mm in width. The dimension of each channel is 0.1 mm in height, 0.4 mm in width and 25 mm length. Each channel on the biochip has a capacity of 8 microliters.

37. Aspirate 10  $\mu l$  fibrinogen (40  $\mu g/ml$  in PBS pH 7.4) or vWF (100  $\mu g/ml$  in PBS, pH 7.4) using a 20  $\mu l$  pipette.
38. Place the tip of the pipette into one opening of the channel of a Vena8 Fluoro+ biochip (Figure 6). Depress the plunger of the pipette to fill the channel with the coating substrate.



**Figure 6. Coating of Vena8 Fluoro+ channels.** A. A 10  $\mu\text{l}$  pipette is placed into the opening of a channel and the channel was filled with the coating substrate. Once filled, the channel will be transparent. B. The first 4 channels of a biochip (marked green) are filled with coating substrate. The other 4 channels are empty, appearing grey.

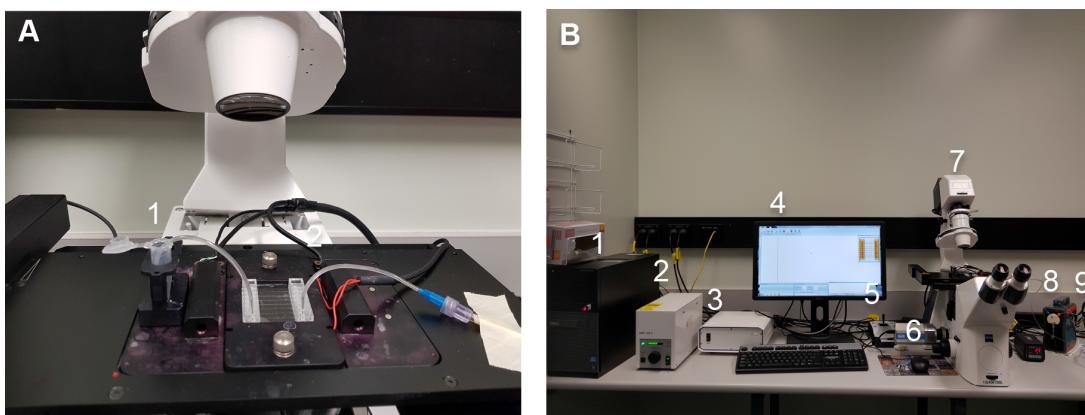
39. Place the biochip in a container with a tissue moistened with dH<sub>2</sub>O and close the container. Incubate the biochip overnight at 4 °C.
40. Remove the coating substrate from the reservoir.
41. Aspirate 10  $\mu\text{l}$  of blocking buffer with a 10  $\mu\text{l}$  pipette and fill the channel with the blocking buffer. Incubate the channel for 1 h at room temperature.
42. Aspirate 10  $\mu\text{l}$  of PBS with a 10  $\mu\text{l}$  pipette and fill the channel to wash. Repeat Step F6 once.
43. Connect the tubing to the biochip using the outlet pins.
44. Submerge the tubing of one end of the channel in an Eppendorf tube filled with the washed platelets (Figure 7). Connect the tubing of the other end of the channel to the Mirus Evo Nanopump. Initiate the pump to draw the platelet suspension through the channel of the biochips. A table for the flow rates required to achieve the desired shear rate can be found at [Cellix website](#) under the technical specification. Alternatively, use the dimensions of the channel and the Formulas 1 and 2 in Procedure E to calculate the flow rate.



**Figure 7. Setup of the Vena8 Fluoro+ biochip.** An Eppendorf tube is used as a reservoir to hold the washed platelets. The other end of the channel is connected to tubing, which is attached to the Mirus Evo Nanopump.

### G. Image acquisition

1. Assemble the syringe pump as shown below (Figure 8A): connect the inlet pin (1) (which is submerged in the Eppendorf containing platelet solution or whole blood) to the entry on one of the 8 channels on the chip. Connect the outlet pin (2) to the outlet of the channel and to the tubing (which is connected to the pump).



**Figure 8. Assembly of the Cellix microfluidics system.** A. Insertion of the Vena8 Fluoro+ biochip into the temperature-controlled stage holder. The inlet pin (1) is connected to the entry of one of the 8 channels on the chip. The outlet pin (2) is connected to the outlet of the channel and to the tubing leading to the pump. B. Setup of the Cellix microfluidics system. (1) Hard drive; (2) Lamp; (3) Motor control; (4) Screen display; (5) Stage control; (6) ExiBlu CCD camera; (7) Epifluorescence microscope; (8) Temperature control; (9) Mirus Evo Nanopump.

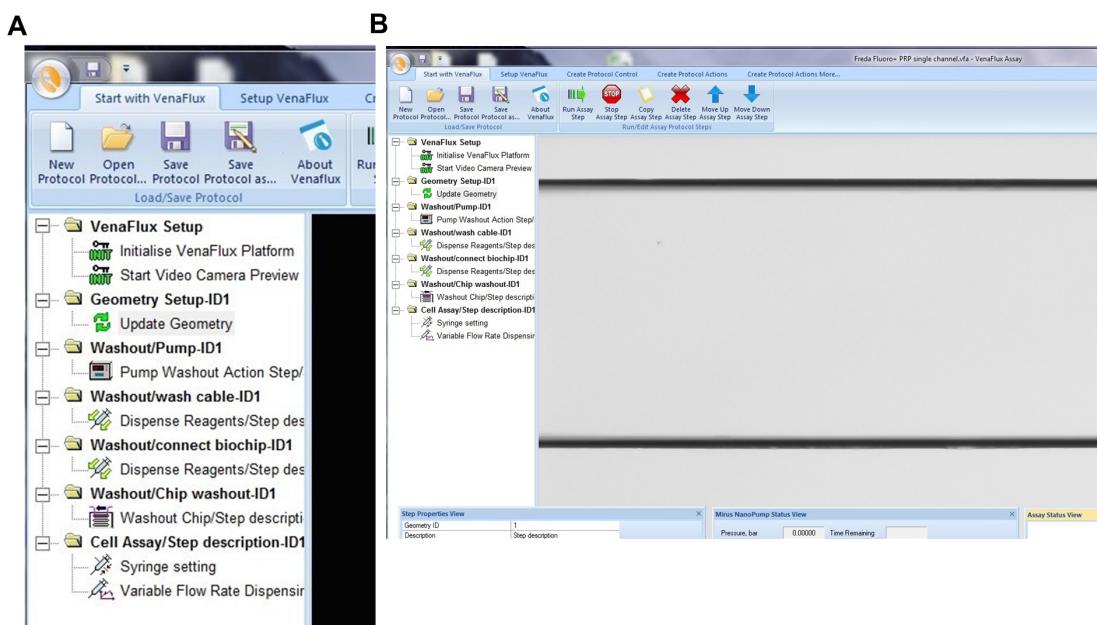
2. Switch the “Power on” button of the PC Harddrive (1), lamp (2), motor control (3), screen display (4), stage control (5), ExiBlu CCD camera (6), Epifluorescence microscope (7), temperature control (8), Mirus Evo Nanopump (9) (Figure 8B). Initiate the VenaFlux Assayx64 software installed on the PC. This will bring up the Venoflux interface.
3. Click on “new protocol” or “open protocol” (if you have already set your parameters). Both options will bring up the same screen page (Figure 9A) with a list of commands. Each command is activated by right click of the mouse, which should be followed in the order displayed. The sequence of commands is: 1. VenFlux Setup, 2. Initialize VenFlux platform, 3. Start Video camera preview, 4. Geometry set up, 5. Update geometry, 6. Washout pump, 7. Washout cable, 8. Wash/connect biochip, 9. Washout chip, 10. Cell assay (Figure 9A). After the video camera preview has been initiated the channel will be displayed on the screen (Figure 9B).

#### Notes:

- a. For steps “washout pump” and “washout cable”, the outlet pin is removed. The pump flows the connected pump solution (water or PBS) into the cable whose free end should be placed into a waste collector.
- b. For step “wash/connect biochip”, the outlet pin is connected to the cable but not to the

*outlet channel of the biochip. The needle of the outlet pin is held above the outlet position of the channel. When you right-click “wash/connect biochip”, the pump will deliver a squirt of 100 µl pump solution into the outlet well to avoid air entering into the channel when the needle is inserted into the outlet. This step can be omitted if you have already included some fluid in the well by pipetting.*

- c. *For the step “washout chip”, the outlet pin has to be inserted into the outlet of the channel. The default setting is to deliver 40 µl of pump fluid at 1 µl/sec to wash out the channel.*
- d. *For the step “cell assay”, enter the parameters for flow by adjusting the shear units, the acquisition time and capture delay (if required). After all steps have been entered, right click on “cell assay” to initiate the pump and acquire data. To stop the assay, click on “stop assay”.*
- e. *To view the channel on the screen, switch the microscope connection to the camera. Switch to eye piece for fine focus of the channel.*
- f. *The steps involved in data acquisition (Figures 9-11) can be found in video webinars by Cellix, Ltd: Cellix Webinar: [VenaFlux Platform Technical Presentation](#); Cellix Webinar: [Cellix Biochips with standard Syringe Pumps for Perfusion Assays](#).*

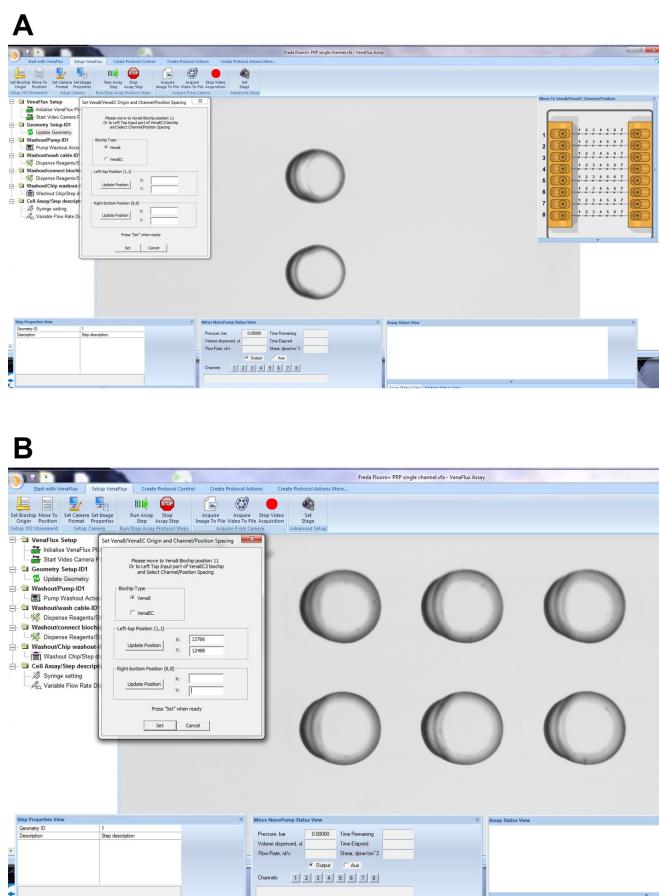


**Figure 9. Initiating the VenaFlux Protocol.** A. On the “Start with VenaFlux page” each command is activated by right click which should be followed in the order displayed. B. After the video camera preview has been initiated the channel will be displayed on the screen. The 2 horizontal black lines are the borders of the channel (20x magnification).

4. The Vena8 Fluoro+ biochip has embedded markings to assist with the mapping of the biochip to the VenaFlux assay x 64 software. Each channel 1-8 has marked positions above channels 2, 3...6 which can be visualized under the microscope with 2, 3...6 dots respectively. On the

dialogue screen of the VenaFlux assay x 64, at the top of the screen, click the option “Set up Venaflux”. This will bring another dialogue box displaying the x, y coordinates named “Set Vena8/VenaEC origin and channel/position spacing”. Click on Vena8 for biochip type. Using the joystick move the stage to focus on channel 1, position 2 (Figure 10A). Click “update position” on the left-top position. Using the joystick move the stage to channel 8, position 6 (Figure 10B). Click “update position” on the right-bottom position. Then press set. Coming back to the “Start with VenaFlux” page, you can click on the map of the biochip displayed at the right upper corner of the screen and this will automatically move the stage to the position.

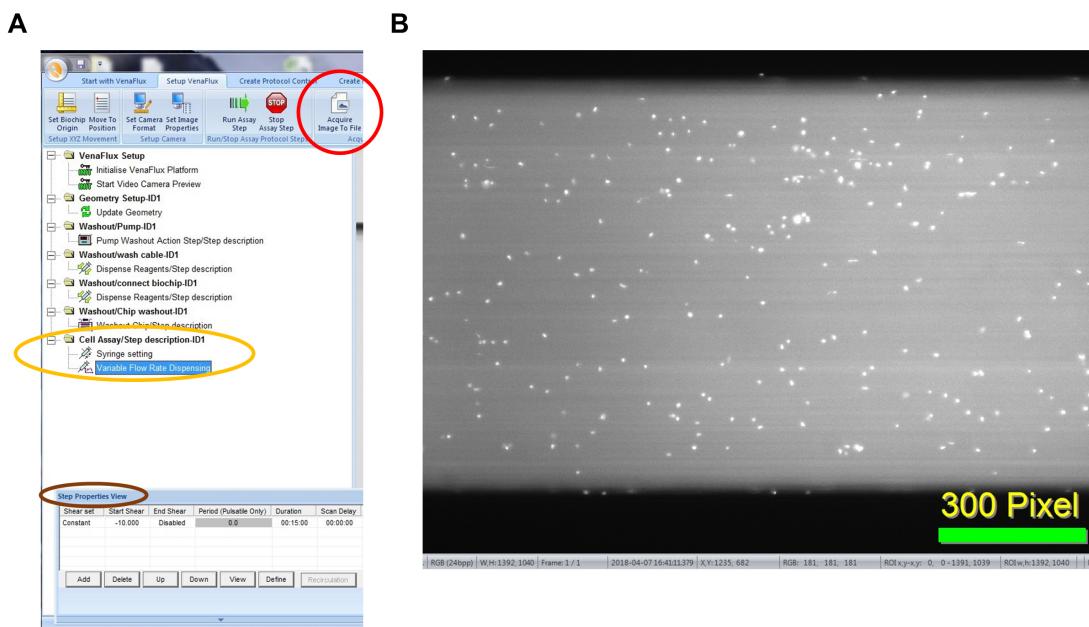
*Note: There are no dots on the biochip for positions 1 and 8. The mapping of the Vena8 chip is based on positions 2 and 6, therefore the range of acquisition is smaller than the biochip’s dimensions.*



**Figure 10. Mapping the Vena8 biochip.** A. On the “Setup VenaFlux” page, move the stage to focus on channel 1, position 2 and click “update position” on the left-top position. B. Move the stage to channel 8, position 6. Click “update position” on the right-bottom position. Then press set.

- To start the assay, enter the desired parameters for flow by adjusting the shear units, the acquisition time and capture delay (Figure 11A). After all the steps have been performed, click

on “start assay” to initiate the pump and data acquisition. To stop the assay, click on “stop assay”. To acquire an image, click on “acquire image to file” on the “Setup VenaFlux page”. This will save the image at the pre-specified location as a bitmap image (Figure 11B). To acquire a video, click on “acquire video to file”. The analysis of the images and image stacks of the videos can be performed by ImageJ as described below or using the ImagePro Premier 64-bit software. We prefer ImageJ for our analysis as it is flexible and can include adjustable macros.



**Figure 11. Initiating the VenaFlux Assay.** A. Under “Cell assay” (circled in yellow), right click on the “variable flow rate dispensing” option and adjust the flow parameters on the “step properties view” icon (circled in brown). B. Click on “acquire image” (circled in red in panel A) to capture the image as bitmap file. Displayed is an example of an image captured of human platelets labeled with calcein 1 µg/ml (white dots) and perfused on a Vena8 channel coated with vWF 100 µg/ml.

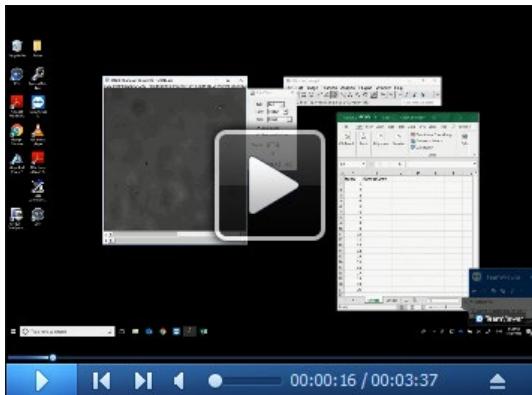
## Data analysis

### A. PDMS biochips

Two methods of manual analysis are described for the analysis of platelet adhesion over time: platelet counting and analysis by fluorescence intensity. This protocol describes the use of Fiji/ImageJ on PC for analysis, however the process is the same for Mac users.

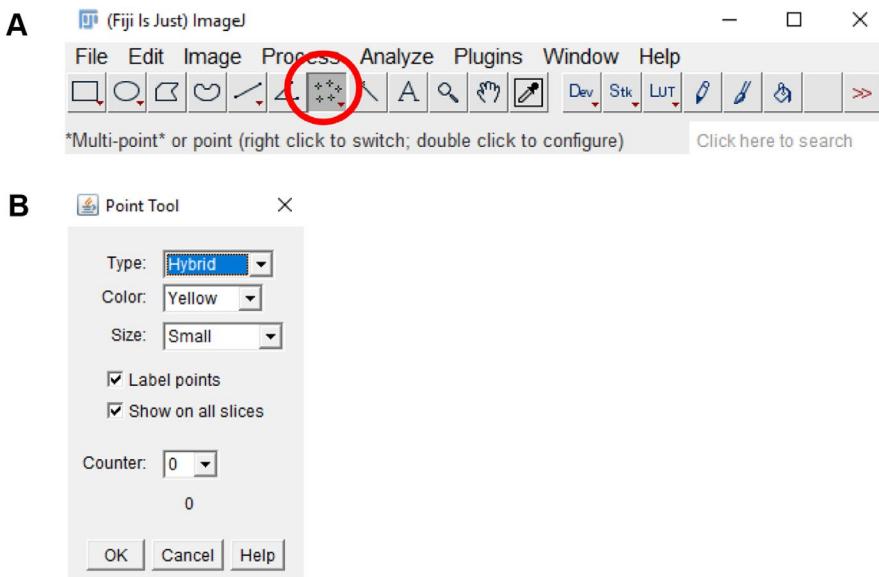
#### **Platelet adhesion analysis by counting**

The steps involved in platelet count and platelet sum fluorescence data analysis (Figures 13-14) can be found in Video 3.



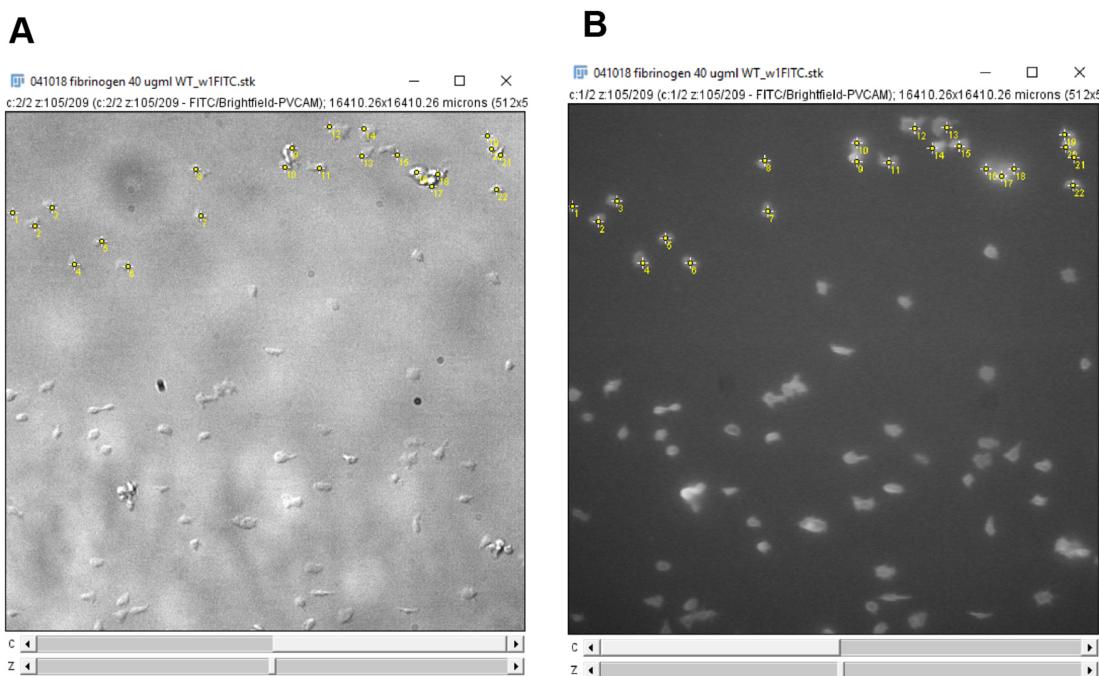
### Video 3. ImageJ analysis of platelet adhesion by counting

1. Open Fiji/ImageJ version 1.52h.
2. Click on the “Multi-point” tool on the tool bar. Double click on the “Multi-point” tool and on the “Point tool” window, check the “Label points” and “Show on all slices” checkbox (Figure 12).



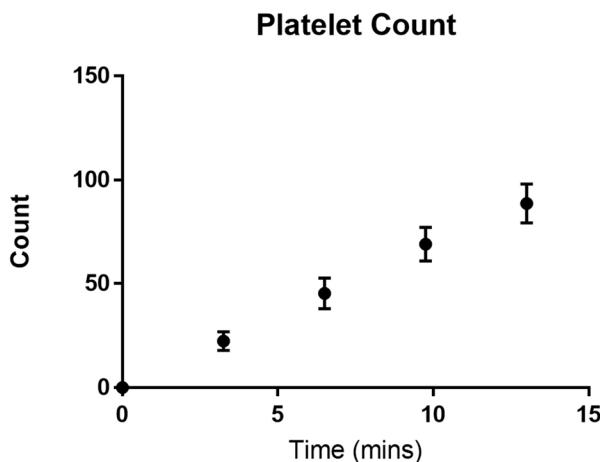
**Figure 12. ImageJ multi-point tool.** A. The multi-point tool is located on the toolbar of the ImageJ window (circled in red). B. Double-clicking the multi-point tool will open the point tool window. This window can be used to count placed markers and to adjust the display options of markers placed on the image.

3. Click on every platelet in the field of view to count the number of adhered platelets (Figure 13). Verify the cell type by ensuring that the platelets are ve+ stained on the 488 nm channel.



**Figure 13. Counting platelets using the ImageJ multi-point tool.** A. Platelets are counted on the DIC channel. B. The platelets are verified using the fluorescence channel to check for anti-GPIb-488 staining. Clicking on an image, with the multi-point tool selected, will leave a marker with a number on the image. Holding “alt” and then clicking on a marker will remove the marker. Each subsequent click or removal will increase or decrease the number on the marker by one respectively. Use this feature to count the number of platelets on the image. Alternatively, after clicking on each platelet on the image, use the counter on the point tool window (Figure 12) to determine the number of platelets counted.

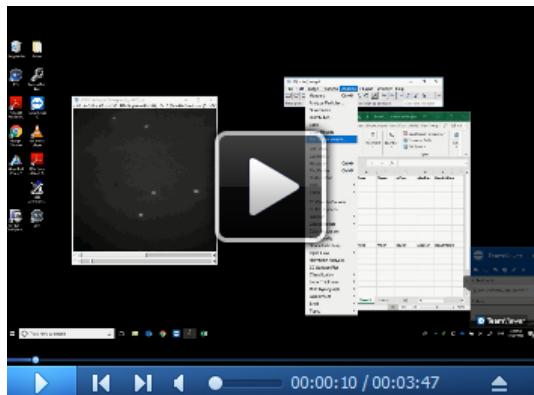
4. Enter the platelet count onto GraphPad prism or Microsoft Excel.
5. Move to the next frame on the image stack, click on additional platelets that have adhered and de-select platelets no longer present as described in step 3. Record the platelet counts (Figure 13) and repeat for each frame in the image stack.
6. Plot the platelet count against time (Figure 14).



**Figure 14. Number of platelets adhered to a fibrinogen channel over time.** Wild type C57BL/6 mouse platelets were washed and flowed across PDMS biochips coated with fibrinogen 40 µg/ml at a shear rate of 500 s<sup>-1</sup>. The platelet count in the field of view was determined every 3 min. The total number of adherent platelets was plotted against time using GraphPad prism. Dots represent the mean platelet count at the specific time point and the error bars represent the standard error of mean from n = 3 experiments.

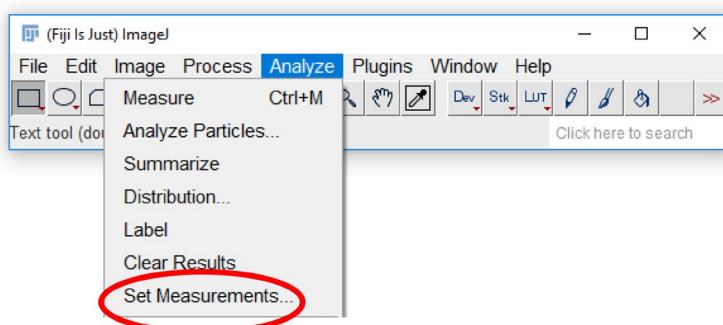
#### **Platelet adhesion analysis by fluorescence intensity analysis**

The steps involved in platelet sum fluorescence data analysis (Figures 15-22) can be found in Video 4.



**Video 4. ImageJ analysis of platelet adhesion by fluorescence intensity**

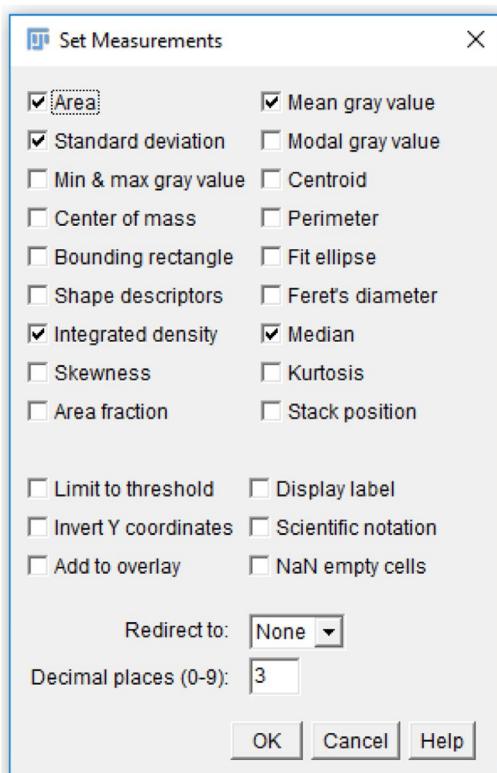
1. Open Fiji/ImageJ.
2. Click the “Analyze” tab and select “Set Measurements” (Figure 15).



**Figure 15. Accessing analysis parameters.** The parameters that will be displayed on a measurement readout can be selected from the set measurements menu. To access the menu, select the “analyze” tab (highlighted in blue), and click “Set measurements...” (circled in red).

3. Check the “Area”, “Mean gray value”, “Integrated density”, and “Median” checkboxes (Figure 16).

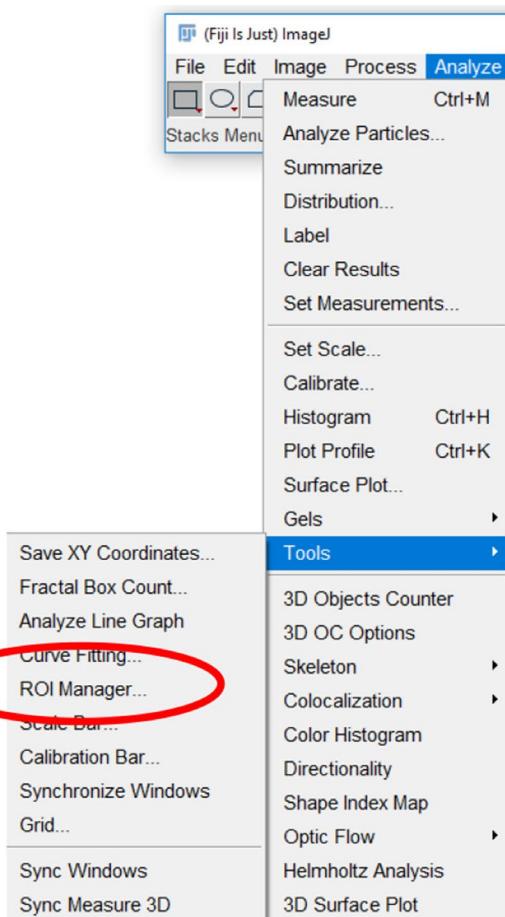
*Note: Each of these measurements provides a description of the platelets. For single platelets, “Area” describes the area taken up by the region of interest drawn around platelets. “Mean gray value” and “Median” are both methods of quantifying the mean fluorescence intensity of a single platelet or platelet aggregate. “Integrated density” quantifies the total intensity for a selected area.*



**Figure 16. Analysis parameters used for measuring platelet fluorescence intensity.**

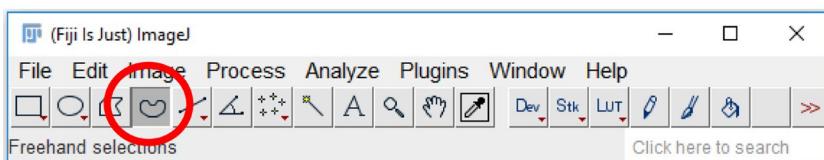
Various parameters can be selected for measuring fluorescence in a region of interest. For the analysis of platelet fluorescence intensity in this protocol, we have selected “Area”, “Standard deviation”, “Mean gray value”, and “Integrated density”.

- Click the “Analyze” tab, click “Tools”, and select “ROI manager...” (Figure 17).



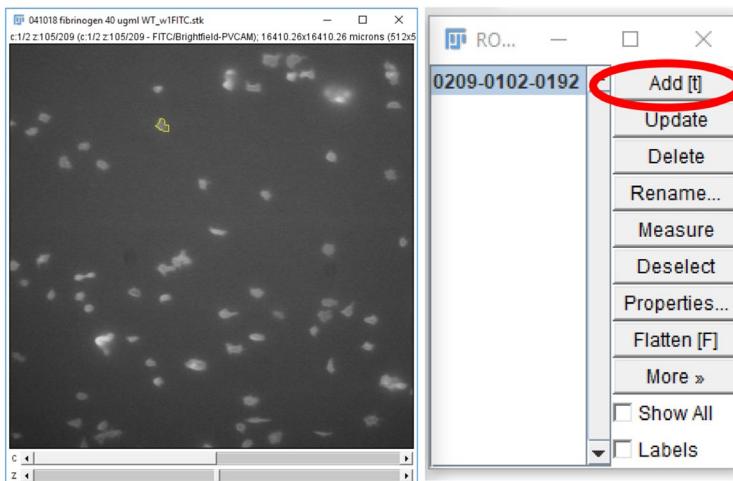
**Figure 17. Region of Interest (ROI) manager on ImageJ.** The ROI manager can be accessed by selecting “Analyze” and then “Tools” (highlighted in blue) and clicking on “ROI manager...” (circled in red).

- Click on the “Freehand selections” tool on the tool bar (Figure 18).



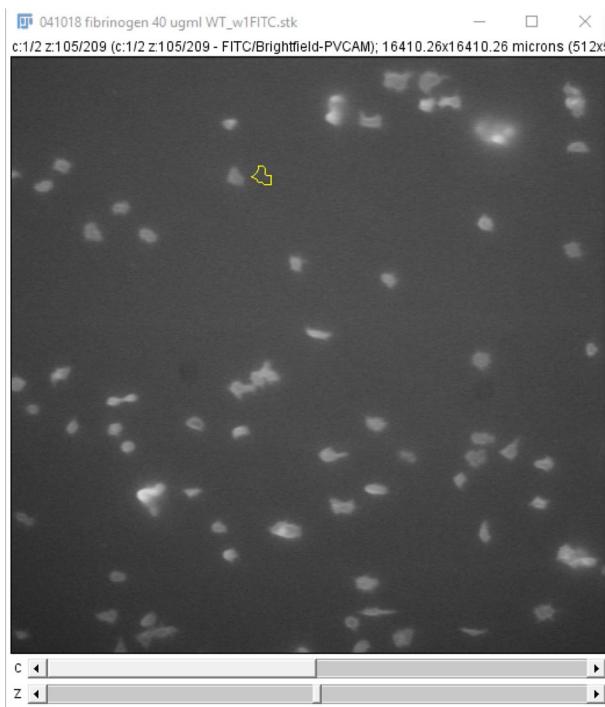
**Figure 18. Freehand selections tool on ImageJ.** The freehand selection tool (circled in red) can be used to trace a region of interest around a platelet or platelet aggregate. The region of interest can then be stored on the ROI manager, and the parameters, set in Step B3, can be measured.

6. For each frame on the time stack, on the 488 nm channel, circle a platelet or platelet aggregate and click “Add” on the ROI manager window (Figure 19).



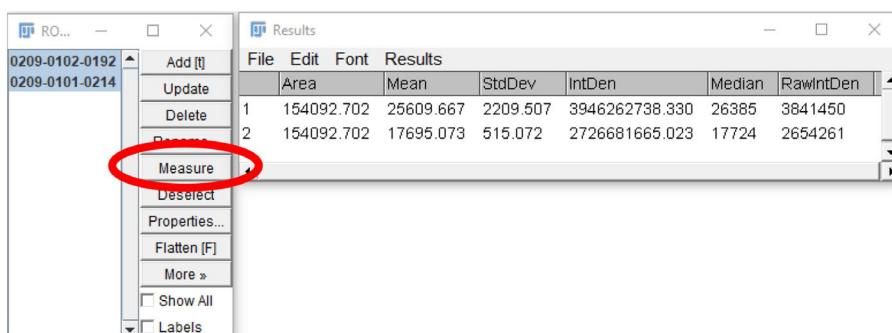
**Figure 19. Storing regions of interest on the ROI manager.** A region of interest, drawn using the freehand selections tool, can be stored on the ROI manager either by pressing “t” on the keyboard or clicking “Add” on the ROI manager (circled in red).

7. Click and drag the region of interest to an area without platelets near the original position (Figure 20) and click “Add” on the ROI manager window. This measures the background fluorescence.



**Figure 20. “Background” region of interest.** The region of interest drawn in Step B6 can be used for selecting a new region of interest to subtract background fluorescence intensity measurements. Click and drag the region of interest away from the platelet/platelet aggregate and add the new area to the ROI manager.

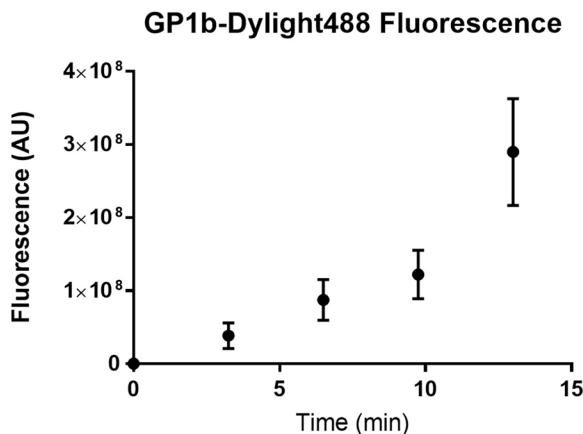
8. Select both regions on the ROI manager window and click “Measure” (Figure 21).



**Figure 21. Measurement of regions of interests.** After the desired regions of interest are selected (highlighted in blue), and “Measure” is clicked (circled in red), a new window will open, showing the measurements of the parameters selected in Step B3.

9. Subtract the Mean/Median/RawIntDen of the background fluorescence measurement from the corresponding platelet measurement. Enter the data onto GraphPad prism or Microsoft Excel.
10. Repeat steps 5-9 for each platelet and platelet aggregate in the frame, sum the fluorescence, and record the value. Repeat for all frames in the image stack.

11. Plot the fluorescence intensity over time on GraphPad prism or Microsoft Excel (Figure 22).



**Figure 22. Fluorescence of platelets adhered to a fibrinogen channel over time.** Wild type C57BL/6 mouse platelets were washed and flowed across PDMS biochips coated with fibrinogen 40 µg/ml at a shear rate of 500 s<sup>-1</sup>. The sum of platelet fluorescence in the field of view was determined every 3 min. The sum of platelet fluorescence was plotted against time using GraphPad prism. Dots represent the mean sum of fluorescence at the specific time point and the error bars represent the standard error of mean from n = 3 experiments.

#### **Statistical analysis of the change in platelet adhesion over time**

1. Open GraphPad Prism.
2. Select the “grouped” tab.
3. Check the “enter \_\_\_\_\_ replicate values in side-by-side columns”, entering the maximum number of replicates performed in the experiment.
4. In the column titles, enter the time points that are to be analyzed. Make sure that the time between each point is equal.
5. Enter the experimental data under the corresponding time points on the first row.
6. Click “Analyze” and under the “Column analyses” drop down menu, select “One-way ANOVA (and non-parametric)”.
7. Choose analysis parameters based on the desired analysis:
  - a. For data that have a Gaussian distribution, under the “Assume Gaussian distribution?” section, click “Yes, use ANOVA” option (Notes 1 and 2).
  - b. For data that do not have a Gaussian distribution, e.g., data that are skewed in distribution, under the “Assume Gaussian distribution?” section, click “No, use nonparametric test” (Notes 1 and 2).
8. Select “Ok”, and under the “Results” drop-down menu to the left of the page, select the analysis for the data and check the P-value. A P-value < 0.05 indicates statistical significance.

#### **Statistical analysis of treatment effect on platelet adhesion at a specific time point**

1. Open GraphPad Prism.

2. Select the “grouped” tab.
3. Check the “enter \_\_\_\_\_ replicate values in side-by-side columns”, entering the maximum number of replicates performed in the experiment.
4. In the column titles, enter the titles of the treatments that are to be analyzed.
5. Enter the data of the treatments at a specific time point in the first row.
6. Click “Analyze” and under the “Column analyses” drop-down menu, select “t-test (and nonparametric tests)”.
7. Choose the analysis parameters based on the desired analysis:
  - a. For data that have a Gaussian distribution, under the “Assume Gaussian distribution?” section, click “Yes, use parametric test” option (Notes 1 and 2).
  - b. For data that do not have a Gaussian distribution, e.g., data that are skewed in distribution, under the “Assume Gaussian distribution?” section, click “No, use nonparametric test” (Notes 1 and 2).

## B. Commercial biochips

Images are captured using the accompanying VenaFlux 2.3 imaging software and videos are captured at a rate of 1 frame per 5 s (30 frames). Images are analyzed at positions 2, 4 and 6 (located at 6, 14 and 22 mm from the entry site of the platelet suspension) of the channels. These positions are representative of flow nearest, mid-way and furthest from the entry of the platelet suspension into the channel. The platelet count and sum of platelet fluorescence are measured at 3-minute intervals using Image J or the ImagePro Premier 64-bit software <http://www.mediacy.com/imagepro>. Data are exported into Excel or GraphPad Prism for statistical analysis.

## Recipes

1. 1x PBS  
137 mM NaCl  
2.7 mM KCl  
10 mM Na<sub>2</sub>HPO<sub>4</sub>  
1.8 mM KH<sub>2</sub>PO<sub>4</sub>  
pH 7.4

*Note: Prepare in advance and store at room temperature for up to 1 year.*

2. 10% Extran  
40 ml Extran® MA 02  
360 ml ddH<sub>2</sub>O  
pH 7.0

*Note: Prepare fresh extran 10% dilution before use and discard after use.*

3. Blocking buffer

2% bovine serum albumin

1x phosphate buffered saline

pH 7.4

*Note: Prepare albumin 2% solution in advance and store in aliquots at -20 °C until use for up to 1 year. Avoid freeze-thawing aliquots more than 2 times.*

4. HEPES-Tyrode's buffer with glucose

20 mM HEPES

134 mM NaCl

0.34 mM Na<sub>2</sub>HPO<sub>4</sub>

2.9 mM KCl

12 mM NaHCO<sub>3</sub>

1 mM MgCl<sub>2</sub>

1 mM CaCl<sub>2</sub>

5 mM D-glucose, added prior to washing platelets

pH 7.4

*Note: Prepare the HEPES-Tyrode's buffer in advance (without the D-glucose) and store at room temperature for up to 1 year. Add the D-Glucose to an aliquot of the HEPES-Tyrode's buffer and use within 24 h.*

### **Acknowledgments**

We thank Ethel Ilagan, Fangyuan Zhou, Shaun P. Jackson and his lab for helpful discussion. This work was supported by the University of Sydney Cardiovascular Initiative Catalyst Grant for Precision CV Medicine (LAJ, FHP), National Heart Foundation of Australia Postdoctoral Fellowship 101285 (LAJ), CSANZ-BAYER Young Investigator Research Grants (LAJ), The Royal College of Pathologists of Australasia Kanematsu Research Award (LAJ, FHP).

### **Competing interests**

The authors have no conflicting interests to declare.

### **References**

1. Baumgartner, H. R. (1973). [The role of blood flow in platelet adhesion, fibrin deposition, and formation of mural thrombi](#). *Microvasc Res* 5(2): 167-179.
2. Chatzizisis, Y. S., Jonas, M., Coskun, A. U., Beigel, R., Stone, B. V., Maynard, C., Gerrity, R. G., Daley, W., Rogers, C., Edelman, E. R., Feldman, C. L. and Stone, P. H. (2008). [Prediction of the localization of high-risk coronary atherosclerotic plaques on the basis of low endothelial shear stress: an intravascular ultrasound and histopathology natural history study](#). *Circulation* 117(8): 993-1002.

3. Hou, H. W., Petchakup, C., Tay, H. M., Tam, Z. Y., Dalan, R., Chew, D. E., Li, K. H. and Boehm, B. O. (2016). [Rapid and label-free microfluidic neutrophil purification and phenotyping in diabetes mellitus](#). *Sci Rep* 6: 29410.
4. Jackson, S. P. (2007). [The growing complexity of platelet aggregation](#). *Blood* 109(12): 5087-5095.
5. Ju, L., Dong, J. F., Cruz, M. A. and Zhu, C. (2013). [The N-terminal flanking region of the A1 domain regulates the force-dependent binding of von Willebrand factor to platelet glycoprotein Ibα](#). *J Biol Chem* 288(45): 32289-32301.
6. Ju, L., Chen, Y., Zhou, F., Lu, H., Cruz, M. A. and Zhu, C. (2015). [Von Willebrand factor-A1 domain binds platelet glycoprotein Ibalpha in multiple states with distinctive force-dependent dissociation kinetics](#). *Thromb Res* 136(3): 606-612.
7. Ju, L., McFadyen, J. D., Al-Daher, S., Alwis, I., Chen, Y., Tonnesen, L. L., Maiocchi, S., Coulter, B., Calkin, A. C., Felner, E. I., Cohen, N., Yuan, Y., Schoenwaelder, S. M., Cooper, M. E., Zhu, C. and Jackson, S. P. (2018). [Compression force sensing regulates integrin α<sub>IIb</sub>β<sub>3</sub> adhesive function on diabetic platelets](#). *Nat Commun* 9(1): 1087.
8. Lane, W. O., Jantzen, A. E., Carlon, T. A., Jamolkowski, R. M., Grenet, J. E., Ley, M. M., Haseltine, J. M., Galinat, L. J., Lin, F. H., Allen, J. D., Truskey, G. A. and Achneck, H. E. (2012). [Parallel-plate flow chamber and continuous flow circuit to evaluate endothelial progenitor cells under laminar flow shear stress](#). *J Vis Exp* (59) pii: 3349.
9. Mannino, R. G., Myers, D. R., Ahn, B., Wang, Y., Margo, R., Gole, H., Lin, A. S., Guldberg, R. E., Giddens, D. P., Timmins, L. H. and Lam, W. A. (2015). [Do-it-yourself \*in vitro\* vasculature that recapitulates \*in vivo\* geometries for investigating endothelial-blood cell interactions](#). *Sci Rep* 5: 12401.
10. Morigi, M., Zoja, C., Figliuzzi, M., Foppolo, M., Micheletti, G., Bontempelli, M., Saronni, M., Remuzzi, G. and Remuzzi, A. (1995). [Fluid shear stress modulates surface expression of adhesion molecules by endothelial cells](#). *Blood* 85(7): 1696-1703.
11. Nesbitt, W. S., Westein, E., Tovar-Lopez, F. J., Tolouei, E., Mitchell, A., Fu, J., Carberry, J., Fouras, A. and Jackson, S. P. (2009). [A shear gradient-dependent platelet aggregation mechanism drives thrombus formation](#). *Nat Med* 15(6): 665-673.
12. Passam, F., Chiu, J., Ju, L., Pijning, A., Jahan, Z., Mor-Cohen, R., Yeheskel, A., Kolsek, K., Tharichen, L., Aponte-Santamaria, C., Grater, F. and Hogg, P. J. (2018). [Mechano-redox control of integrin de-adhesion](#). *Elife* 7: e34843.
13. Qin, D., Xia, Y. and Whitesides, G. M. (2010). [Soft lithography for micro- and nanoscale patterning](#). *Nat Protoc* 5(3): 491-502.
14. Reininger, A. J., Heijnen, H. F., Schumann, H., Specht, H. M., Schramm, W. and Ruggeri, Z. M. (2006). [Mechanism of platelet adhesion to von Willebrand factor and microparticle formation under high shear stress](#). *Blood* 107(9): 3537-3545.
15. Ruggeri, Z. M. (2009). [Platelet adhesion under flow](#). *Microcirculation* 16(1): 58-83.
16. Ruggeri, Z. M., Orje, J. N., Habermann, R., Federici, A. B. and Reininger, A. J. (2006).

- [Activation-independent platelet adhesion and aggregation under elevated shear stress.](#) *Blood* 108(6): 1903-1910.
17. Tovar-Lopez, F. J., Rosengarten, G., Nasabi, M., Sivan, V., Khoshmanesh, K., Jackson, S. P., Mitchell, A. and Nesbitt, W. S. (2013). [An investigation on platelet transport during thrombus formation at micro-scale stenosis.](#) *PLoS One* 8(10): e74123.
18. Tsai, M., Kita, A., Leach, J., Rounsevell, R., Huang, J. N., Moake, J., Ware, R. E., Fletcher, D. A. and Lam, W. A. (2012). [In vitro modeling of the microvascular occlusion and thrombosis that occur in hematologic diseases using microfluidic technology.](#) *J Clin Invest* 122(1): 408-418.
19. Yago, T., Wu, J., Wey, C. D., Klopocki, A. G., Zhu, C. and McEver, R. P. (2004). [Catch bonds govern adhesion through L-selectin at threshold shear.](#) *J Cell Biol* 166(6): 913-923.

**bio-protocol**  
*Improve Research Reproducibility*

**Free access to ~4000 high-quality protocols**

- Contributed by 10,000+ scientists (including Nobel Laureates)
- Validated in a primary research paper
- >91% reproducibility ( survey of 2165 Bio-protocol users)
- ~1000 videos of key procedural steps

Sign up at: [www.bio-protocol.org](http://www.bio-protocol.org)

## Visualization of Plant Cell Wall Epitopes Using Immunogold Labeling for Electron Microscopy

Mateusz Majda<sup>\$,\*</sup>

Umeå Plant Science Centre (UPSC), Department of Forest Genetics and Plant Physiology, Swedish University of Agricultural Sciences, 901 83 Umeå, Sweden; <sup>\$</sup>Current address: Department of Comparative Development and Genetics, Max Planck, Institute for Plant Breeding Research, Carl-von-Linné-Weg 10, 50829 Köln, Germany

\*For correspondence: [Mateusz.Majda@slu.se](mailto:Mateusz.Majda@slu.se)



**[Abstract]** Plant cell walls consist of different polysaccharides and structural proteins, which form a rigid layer located outside of the plasma membrane. The wall is also a very dynamic cell composite, which is characterized by complex polysaccharide interactions and various modifications during cell development. The visualization of cell wall components *in situ* is very challenging due to the small size of cell wall composites (nanometer scale), large diversity of the wall polysaccharides and their complex interactions. This protocol describes immunogold labeling of different cell wall epitopes for high-resolution transmission electron microscopy (TEM). It provides a detailed procedure for collection and preparation of plant material, ultra-thin sectioning, specimen labeling and contrasting. An immunolabeling procedure workflow was optimized to obtain high efficiency of carbohydrates labeling for high-resolution TEM. This method was applied to study plant cell wall characteristics in various plant tissues but could also be applied for other cell components in plant and animal tissues.

**Keywords:** Cell walls, Cell wall epitopes, Polysaccharides, Immunogold labeling, Transmission electron microscopy

**[Background]** Plant biomass is mainly composed of cell walls, which are widely used as an energy source in our daily life (Loqué *et al.*, 2015). At the microscopic scale, cell walls consist of cellulose microfibrils embedded in complex matrix polysaccharides (hemicelluloses, pectins) and structural proteins. Cellulose microfibrils (CMFs) are the largest wall polymers with a radius of 3-5 nm and many micrometers long (Cosgrove, 2005). The orientation of CMFs determines the direction of growth and cell anisotropy (Baskin, 2005), but the CMFs also interact with other wall components all together modifying the wall properties (reviewed in Majda, 2018; Majda and Robert, 2018). The study of cell wall composition has a long history, going back to when different chemicals were applied to bind to the wall composites; however, many of them had a wide range of targets (Wallace and Anderson, 2012; Voiniciuc *et al.*, 2018) and could be observed only via light microscope resolution. In contrast, the immunogold labeling is characterized by a high specificity of antibodies and high-resolution imaging, which can precisely localize the wall epitopes across the wall matrix (e.g., Majda *et al.*, 2017). Despite electron microscopy being a relatively old method, it is not broadly used for plant cell walls. The reason could be that it is time-consuming, requires long training and extensive preparation time. In this protocol, I will walk you through all the steps concerning sample preparation, specificity for all the reagents and

troubleshooting.

### **Materials and Reagents**

1. Adhesion slides, Polysine, 25 x 75 x 1.0 mm (VWR, catalog number: 631-0107)
2. Aluminum foil
3. Centrifuge tubes 15 ml (PluriSelect, catalog number: 05-00002-01)
4. Centrifuge tubes 50 ml (sterile) (PluriSelect, catalog number: 05-00001-01)
5. Compressed air in the can (e.g., air duster PRF 4-44)
6. Disposable pH indicator paper (universal indicator paper) (Johnson, catalog number: 101.3C)
7. Disposable plastic Pasteur pipettes (BRAND, catalog number: 747750)
8. Double-edged razor blades (Personna, catalog number: 171930)
9. Embedding capsules (8 mm flat, polypropylene capsules) (TAAB, catalog number: C095)
10. Filter papers (circles, 150 mm Ø), (Whatman, catalog number: 1001150)
11. Glass vials with plastic snap-cap (ca. 41 x 24 mm) (Karl Hecht, catalog number: 2783/3), or clear glass vials with snap-cap (closed top, PE transparent, 18 mm, 500 ml 20 x 40 mm) (VWR, catalog number: 548-0555)
12. Grids for transmission electron microscopy, e.g., grid size 100 mesh x 250 µm pitch, nickel or copper (TAAB, maxtaform HF4, catalog number: GM021N; Sigma-Aldrich, catalog number: G1528; Agar Scientific, catalog number: G2500C)
13. Light-duty 3-Ply tissue wipers (VWR, catalog number: 82003824-CS)
14. Metal needle or dissection needle (VWR, catalog number: 10806-330)
15. Microcentrifuge tubes 1.5 ml (Sigma-Aldrich, catalog number: Z606340)
16. Microscope slides, 25 x 75 x 1.0 mm (VWR, catalog number: 48300-025 or Thermo Scientific, catalog number: 10144633CF)
17. Paper tags
18. Parafilm (Sigma-Aldrich, catalog number: P7543)
19. Pencil
20. Pipette tips
21. Plastic Petri dishes (Fisherbrand, catalog number: S33580A)
22. Protective gloves (Honeywell, catalog number: Dermatril 740)
23. Round silicone rubber (TAAB, catalog number: G082)
24. Single edge razor blades with aluminum spine (VWR, catalog number: 233-0156)
25. Slide labels (Agar Scientific) or one side adhesive paper
26. Square Petri dish (120 mm) (Corning, catalog number: BP124-05)
27. Transparent tape (e.g., scotch)
28. Waste containers
29. Agar, plant agar (Duchefa Biochemie, catalog number: 9002-18-0)
30. Bovine Serum Albumin (BSA), lyophilized powder ≥ 96% (agarose gel electrophoresis)

- (Sigma-Aldrich, catalog number: 9048-46-8)
31. Disodium phosphate ( $\text{Na}_2\text{HPO}_4$ ) (store in ambient temperature: room temperature, approx. 21 °C) (Sigma-Aldrich, catalog number: 7558-79-4)
  32. Distilled water
  33. Ethanol laboratory reagent, absolute, ≥ 99.5% (flammable, stored in designated place) (Sigma-Aldrich, catalog number: 64-17-5)
  34. Formaldehyde (FA) (10 ml ampule of 16% methanol-free FA) (health hazards, store closed in ambient temperature, or open in cold room/refrigerator 4 °C) (Thermo Scientific, catalog number: 28908) or paraformaldehyde (PFA) for histology  $(\text{CH}_2\text{O})_n$ , (store in cold room/refrigerator 4 °C) (J.T. Baker, catalog number: S898-07)
  35. Formvar solution (1% formvar in dichloroethane, for microscopy) (Sigma-Aldrich, catalog number: 63148-64-1)
  36. Glutaraldehyde (GA) (grade I, 1 ml ampule of 25% GA in  $\text{H}_2\text{O}$ , specially purified for use as an electron microscopy fixative, linear formula:  $\text{OHC}(\text{CH}_2)_3\text{CHO}$  (health hazards, store closed in ambient temperature, or open in cold room/refrigerator 4 °C) (Sigma-Aldrich, catalog number: 111-30-8)
  37. Hydrogen chloride (HCl) (Sigma-Aldrich, catalog number: 7647-01-0)
  38. LR white resin medium grade–catalyzed (health hazards, store in cold room/refrigerator 4 °C) (TAAB, catalog number: L012)
  39. Monopotassium phosphate ( $\text{KH}_2\text{PO}_4$ ) (Sigma-Aldrich, catalog number: 7778-77-0)
  40. Monosodium phosphate ( $\text{NaH}_2\text{PO}_4$ ) (store in ambient temperature) (Sigma-Aldrich, catalog number: 7558-80-7)
  41. Murashige and Skoog basal medium (MS) (Sigma-Aldrich, catalog number: M5519)
  42. Potassium chloride (KCl) (Sigma-Aldrich, catalog number: 7447-40-7)
  43. Primary antibodies (PlantProbes: [www.plantprobes.net](http://www.plantprobes.net) or the University of Georgia: [www.ccrc.uga.edu](http://www.ccrc.uga.edu))
  44. Secondary antibodies, e.g., EM Goat anti-Rat IgG (H+L): 10 nm Gold (BBI Solutions, catalog number: 014990) or EM Goat anti-Mouse IgG (H+L) 10 nm Gold (BBI Solutions, catalog number: EM.GMHL10)
  45. Sodium chloride ( $\text{NaCl}$ ) (Sigma-Aldrich, catalog number: 7647-14-5)
  46. Sodium hydroxide ( $\text{NaOH}$ ) (Sigma-Aldrich, catalog number: 1310-73-2)
  47. Sucrose (Sigma-Aldrich, catalog number: 57-50-1)
  48. Toluidine blue for microscopy (Sigma-Aldrich, catalog number: 6586-04-5)
  49. TopVision low melting point agarose (LMP agarose) (store in ambient temperature) (Thermo Scientific, catalog number: R0801)
  50. Tween 20 (Sigma-Aldrich, catalog number: P1379)
  51. Uranyl acetate (UA) (health hazardous, store in ambient temperature) (VWR, catalog number: 541-09-3)
  52. Half strength MS basal medium (see Recipes)

53. Paraformaldehyde-glutaraldehyde (4% PFA and 0.05% GA) fixative solution (see Recipes)
54. Phosphate buffer (PB), 0.1 M solution (pH = 7.2) (see Recipes)
55. Low melting point (LMP) agarose 1% solution (see Recipes)
56. Different ethanol concentrations 10%-95% (see Recipes)
57. Different LRW resin concentrations 10%-75% (see Recipes)
58. Toluidine blue solution (see Recipes)
59. Blocking Reagent (BR) 1% (see Recipes)
60. Antibodies solutions (see Recipes)
61. Phosphate Buffered Saline (PBS), 0.1 M solution (pH = 7.2) (see Recipes)
62. Uranyl acetate solution (5%) (see Recipes)

## **Equipment**

1. Analytical balance
2. Autoclave (YPO, model: D66161)
3. Centrifuge (Marshall Scientific, Eppendorf, model: 5417C)
4. Conical flask
5. Desiccator (Thermo Fisher, model: 5311-0250)
6. Diagonal cutting pliers *e.g.*, stanley diagonal cutting pliers, 5 (Robosource, catalog number: 15-11)
7. Diamond knife, *e.g.*, ultra 45° (Diatome)
8. Flat beaker or crystallizer (with a wide diameter)
9. Forceps
10. Freezer (-20 °C)
11. Fume hood
12. Glass baker (50 ml)
13. Glass knife strips (Agar Scientific, catalog number: AGG336)
14. Glass knifemakers for histology knives (LKB, catalog number: LKB 7801B; or Agar Scientific, catalog number: AGL4158)
15. Grid storage box (LKB/Leica catalog number: G133, or TAAB Gilder G062)
16. Lab oven/incubator (60 °C)
17. Light microscope
18. Magnetic hotplate stirrer with magnetic stir bar
19. Metal 1.5 ml Eppendorf rack
20. Microtome (Reichert Ultracut)
21. Microwave oven
22. pH meter
23. Pipettes
24. Protective clothes and mask

25. Refrigerator (4 °C)
26. Rotary shaker or orbital shaker (IKA KS 130 Basic)
27. Slide drying rack or slide staining jar (e.g., DWK Life Sciences Wheaton)
28. Small bench clamp workshop
29. Thin painting brush
30. Transmission Electron Microscope (JEOL, model: JEM-1230)
31. Tweezers: negative-action style: thin curved tips (Dumont, catalog number: 0203-N7-PO) or thin tips (Dumont, catalog number: 0302-N0-PO-1)
32. Tweezers: straight with Geneva pattern, thin tips (Dumont, catalog number: 0103-0-PO)
33. Vortex mixer (Vortex-Genie 2)
34. Warming plate, or slide drying hotplate (Agar Scientific, model: AGL4384) or spirit lamp burner

## **Procedure**

### A. Plant material fixation

In this section, I describe the procedure for plant material preparation and fixation. This protocol was developed for *Arabidopsis thaliana* leaves, but it could also be applied to other organisms and tissues such as roots, shoots and woody tissues in tree species.

1. Sterilize the seeds before sowing
  - a. Place a small number of seeds into 1.5 ml Eppendorf tubes (approx. 5% of the Eppendorf tube volume).
  - b. Add 1 ml of 70% ethanol with Tween 20 for 2 min.
  - c. Replace 70% ethanol and Tween 20 with 1 ml of 95% ethanol for 1 min.
  - d. Remove the ethanol and wait for the seeds to dry.

*Note: Perform the seeds sterilization under a sterile fume hood.*

2. Grow *Arabidopsis* seedlings for 2 weeks on vertical agar plates (Recipe 1) in the chamber with long day condition (16 h) (temperature 20 °C and 18 °C at day and night, respectively).

*Note: To synchronize the growth, vernalize the seeds by keeping the plates in a cold room/refrigerator at 4 °C for 2-3 days (in darkness).*

3. Harvest plant material and place it directly in the vials filled with 3-5 µl of cold paraformaldehyde-glutaraldehyde (PFA-GA) fixation solution (Recipe 2).

*Note: Remember to harvest the same leaf number from each plant (counting from the bottom to the top: cotyledons, leaf 1, leaf 2, leaf 3, leaf 4, meristem). To allow the fixative to penetrate well, cut small squares (~2 mm<sup>2</sup> max). in the middle part of the leaf. Harvest at least 10 leaves from different plants. Conduct all the fixation steps under the fume hood with protective gloves and clothes (for handling restrictions see Safety Data Sheet SDS provided by the retailer). Mark the vials by writing the names with a permanent marker and sticking a transparent tape on these labels.*

4. Vacuum samples in a desiccator at ambient temperature until plant pieces will sink (approx. 4

h).

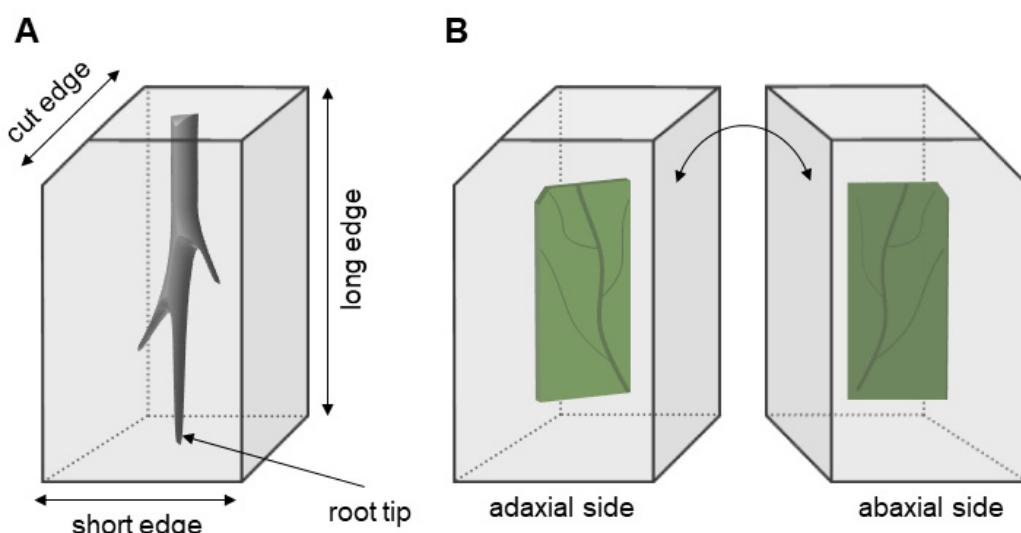
*Note: It might happen that some of the samples are floating after vacuuming, which indicates that samples more likely contain oxygen. Try to collect samples, which are at the bottom of the vials.*

5. Place the vials on a rotary or orbital shaker and leave the samples mixing for a couple of hours.
6. Keep the samples in a cold room/refrigerator (4 °C) overnight.
7. Discard PFA-GA fixative.
8. Wash the samples with phosphate buffer (PB) (Recipe 3) (twice for 30 min each).

*Note: Discard PFA-GA fixative and first PB washing by using disposable plastic Pasteur pipettes in the assigned waste bottle. Wash by adding at least 5 ml of PB or distilled water (the more PB and distilled water, the better the washing). Conduct all washing steps slowly mixing on rotary or orbital shaker under a fume hood.*

#### B. Embedding plant material in low melting point (LMP) agarose

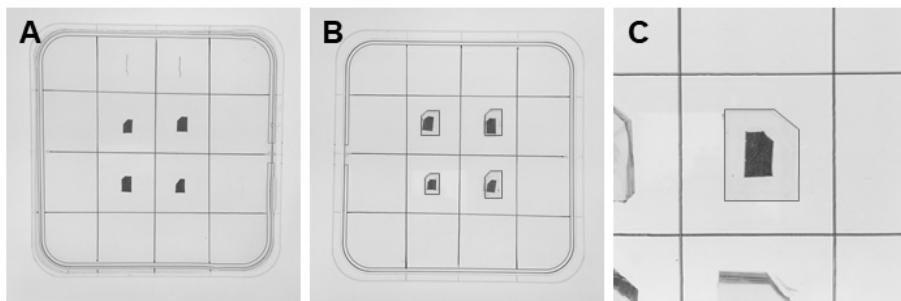
Embedding small plant pieces in LMP agarose is the best way to orient the sample for sectioning (e.g., cross or longitudinal). It marks the direction of shoot/root tip (Figure 1A) or the localization of abaxial and adaxial leaf sides (Figure 1B). Embedding also facilitates the handling and protection of samples.



**Figure 1. Orienting the plant pieces in low melting point (LMP) agarose.** A-B. Samples embedded in cuboid-shaped agarose blocks presenting one cut edge to mark the orientation of the sample (e.g., top left in Figure 1A). A. Top part of the root is marked by a cut edge. B. The adaxial side of the leaf is in the front when the cut edge is on the top left.

1. Wash the samples with distilled water (twice for 30 min each).
2. Pour a thin (3-5 mm thick) layer of agarose (Recipe 4) on a square plastic Petri dish and wait a few minutes until agarose cools down (but should not solidify).

3. Place the plant material on agarose by using tweezers (many pieces on the same plate but keep distance between the samples approx. 2-3 cm<sup>2</sup>).  
*Note: Handle the plant specimens with care not to damage the tissues.*
  4. Add more agarose ( $\pm$  3 mm thick layer) to cover the plant specimens (wait until agarose solidifies) (Figure 2A).
  5. Cut off small cubes of agarose with the embedded specimens (one specimen in one cube) (Figure 2B).
- Note: The size of agarose cubes should not be bigger than the diameter of embedding capsules (8 mm Ø).*
6. Cut one of the corners in the agarose cube to orient the sample (Figures 1, 2B and 2C).
  7. Move the blocks to vials filled with distilled water, then discard the water before proceeding to the next step.



**Figure 2. Embedding the plant material in agarose.** A. Square Petri dish filled with a layer of 3 mm thick agarose. B. Small cuboid-shaped agarose blocks with cut one of the edges (all the leaf pieces are seen from the adaxial side). C. Magnified agar cube with the abaxial side of the leaf in the front. In this case, big plant pieces were used for visualization, but they should be  $\pm$  5 times smaller.

#### C. Dehydrating specimens embedded in agarose blocks

Here I describe a common method to remove water from samples and to enable better penetration of resin. Time intervals can be adjusted; however, longer intervals give better quality embedding. Ideally, the dehydration process would be carried over the span of two days, but all dehydration steps can also be carried out within the same day (depends on the sample size).

1. Dehydrate the samples in a graded ethanol series as below (Recipe 5):

10% ethanol (twice for 30 min each)  
20% ethanol (twice for 30 min each)  
30% ethanol (twice for 30 min each)  
50% ethanol (twice for 30 min each)

*Note: The amount of ethanol added varies according to the size and number of the samples embedded in agarose. Make sure that agarose blocks are completely covered by ethanol.*

2. Leave the samples in 50% ethanol in ambient temperature (on the bench) overnight.

*Note: Close the vials with plastic snap-cap to prevent ethanol evaporation.*

- Continue to dehydrate the samples in a graded ethanol series as below:

70% ethanol (twice for 30 min each)

80% ethanol (twice for 30 min each)

90% ethanol (twice for 30 min each)

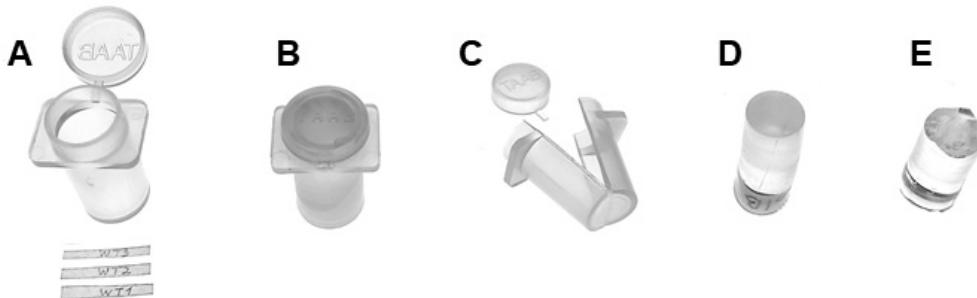
95% ethanol (three times for 30 min each)

99.5% ethanol (three times for 30 min each)

*Note: Carry all dehydration steps on rotary or orbiter shaker. The vials should be closed with plastic snap-caps. Some intermediate ethanol dilutions can be omitted (e.g., 20% and 80% ethanol), but it might affect the quality of embedding. The volume of alcohol in the last step should be the same for all vials.*

#### D. Resin embedding

In this step, alcohol is replaced with viscous and low soluble LR white (LRW) resin. All these steps must be carried gradually with vigorous mixing of the samples on an orbital shaker. Perform all these steps in cold room/refrigerator (4 °C) to prevent resin polymerization.



**Figure 3. Embedding the plant material in resin.** A. Embedding capsule and paper tags. B. Capsule after resin polymerization. C. Capsule cut along to remove the resin block. D. Resin block alone. E. Trimmed resin block into a trapezoid shape.

- Slowly add a few drops of resin to the vials with a known volume of 99.5% ethanol until the resin content will reach 10% (v/v) of the overall volume.

*Note: All steps, including the exchange of the resin, must be carried out using protective gloves and clothes under the fume hood. Keep the vials closed with the plastic snap-caps mixing in the orbital shaker in cold room/refrigerator (4 °C). Always discard the resin to designated waste.*

- Leave the vials in the orbital shaker to mix samples overnight.
- Exchange resin 10% in alcohol with a new resin in a graded resin series as below (Recipe 6):  
10% resin (approx. 5 h)  
25% resin (approx. 5 h)

50% resin overnight

75% resin (approx. 5 h)

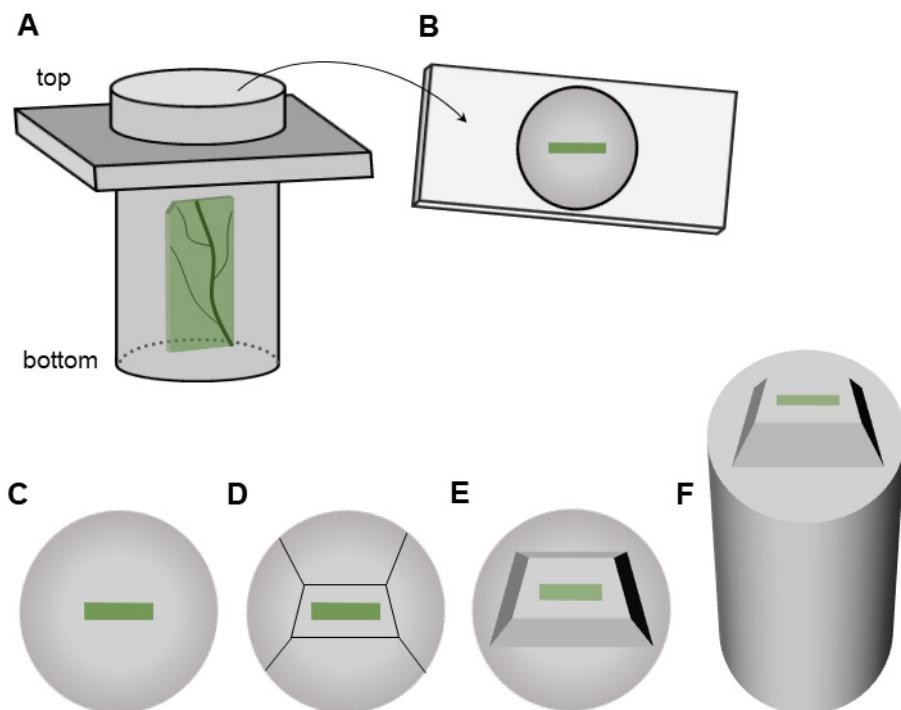
100% resin overnight

100% (fresh) resin overnight

*Note: Some intermediate resin dilutions can be omitted (e.g., 25% and 75%), but it might affect the quality of embedding.*

#### E. Closing samples in the embedding capsules and resin polymerization

This is the last step of the embedding procedure, in which you must pay attention to properly orientate the samples (Figure 1). The agarose blocks should lie flat in the middle of the bottom part of the capsule (Figures 4A and 4B), which need to be oriented in a parallel direction towards the knife edge (see Procedure G, Figure 6). Handle the capsules with care to prevent the slipping of the samples close to the corners, which could cause some issues in trimming the sample and sectioning.



**Figure 4. Trimming the plant specimen prior sectioning.** A-B. A capsule with a piece of the leaf embedded in resin (A). The sample should be in the middle of the bottom part of the capsule (B). C-E. Resin with specimen alone seen from the bottom: resin before trimming (C), the direction of trimming (D), trimmed specimen (E). F. Trimmed resin block seen from the side.

1. Place the capsule (Figure 3A) on a rack and fill half of the capsule with resin.
2. Put the sample in the capsule, orient the sample well and fill-up the capsule with fresh resin.
3. Cut small paper tags, label them with a pencil and place them in the top of the capsules.

*Note: The capsule should be filled with resin so that, by closing it, no space for air bubbles is left. Use a pencil to tag your samples instead of a pen or a marker, which can be washed out by the resin.*

4. Place the capsules in the incubator/oven (60 °C) for 24 h (or until it becomes solid) to polymerize the resin.

*Note: Remember to check the temperature of the oven (60 °C). A temperature that is too low can affect the polymerization (resin will not polymerize equally), while a temperature that is too high or an excessively long incubation can lead to cracking or breaking of the resin.*

5. Samples are ready when the resin becomes stiff (Figure 3B).

*Note: To know if the resin solidified, indent a metal needle to the resin surface.*

#### F. Preparing the grids for transmission electron microscope (TEM)

The samples for the TEM are mounted on a small grid, which is then inserted inside the TEM. The grid must be coated with formvar, which is an adhesive layer holding the sections. While preparing the grids, make sure that microscope slides, tweezers and laboratory glassware are clean (use tissue wipers and compressed air to clean the dust on the surfaces).

1. Add approx. 50 ml of formvar solution to a small beaker (Figure 5A).
2. Grab the microscope slide in a vertical position (holding a dull side) and vigorously dip approx. half of the slide in the formvar solution (no more than approx. 5 s in the solution) (Figure 5B).

*Note: The formvar will form a thin film on the side.*

3. Dry the slide with a film in a vertical position on a drying rack for 3-10 min.

*Note: Do not touch any part of the slide, which was in contact with the formvar solution.*

4. Prepare glassware with a wide diameter ( $\geq 10$  cm) and fill it up with distilled water. Place the grids on a round silicone rubber or filter paper (Figure 5C).
5. Scratch the slide edges with a sharp razor blade and blow moist air on the slide.
6. Dip the slide vertically (90° or  $\geq 45^\circ$ ) in the distilled water bath.

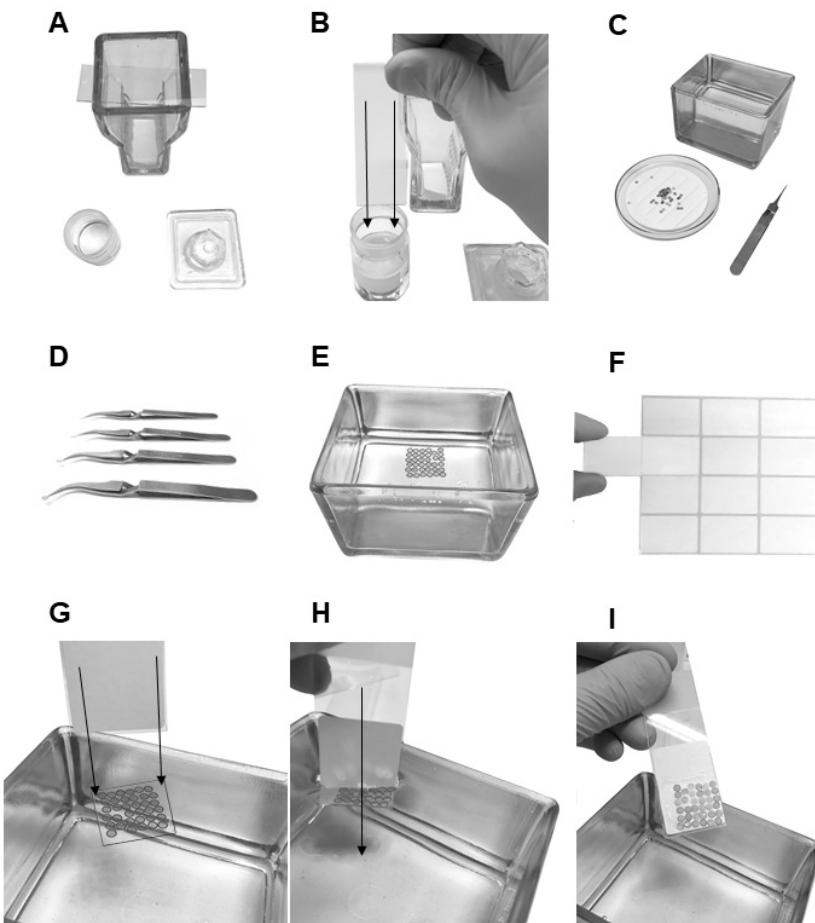
*Note: You will see the film detaching from the glass and then floating on the water surface.*

7. Using fine tweezers, put the grids onto a floating film with the dull side in contact with formvar (put as many grids as possible) (Figures 5D and 5E).

*Note: Each grid has two sides: a dull side and a shiny side (Figure 7A). Sections will be mounted on the formvar-coated dull side (this side should be in contact with buffers, antibodies, distilled water, contrasting stain) (Figure 7B), and the shiny side will be in contact with filter paper during the drying of the samples (it is up to you which side you want to coat with formvar and put the sections on but remember to be consistent).*

8. Prepare a new slide covered by one side adhesive paper or slide label (Figure 5F).
9. Grab that slide in vertical position and touch one of the edges of the floating film (Figure 5G).
10. Dip the slide into the water, which will cause adhesion of the film with grids to the slide (Figures 5H and 5I).
11. Leave the slide with grids covered with formvar until dry.

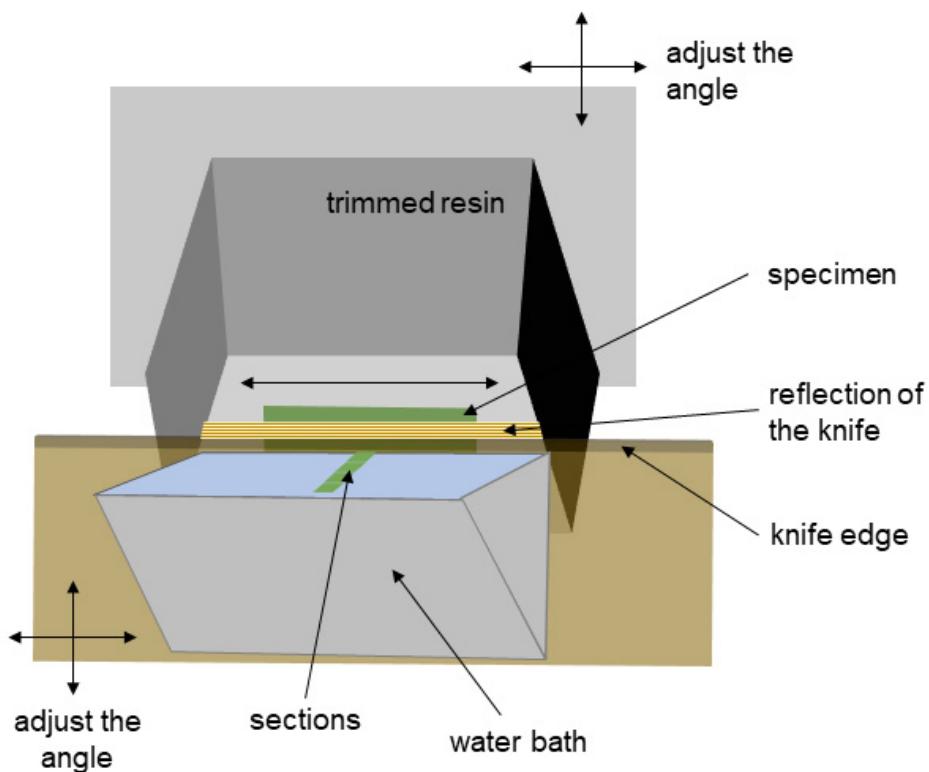
Note: Store the slides in a closed box or plastic Petri dish in cold room/refrigerator (4 °C).



**Figure 5. Preparing the grids for TEM.** A. Baker filled with formvar solution and a slide on the slide staining jar. B. Dipping of the microscope slide in formvar solution. C. Glassware filled up with distilled water, Petri dish with round silicone rubber and grids on it, tweezers. D. Tweezers holding the outer ring of the grids. E. Grids on a floating formvar film with the dull side of the film. F. Sticking a slide label to the slide. G. Slide in vertically before touching the edges of the floating film. H. Dipping the slide in water causing the adhesion of film with grids to the slide. I. Slide with formvar and grids on it.

#### G. Ultrathin sectioning for electron microscopy

The most important thing in the preparation of the sections is to properly orient the sample. Remember to regularly control the sections quality. Make sure that the cells/tissue/organ is oriented in parallel to the knife (Figure 6) and if they are not, then correct the angle between the sample and the knife edge.



**Figure 6. Ultrathin sectioning for electron microscopy.** Begin with orienting the specimen in parallel to the knife edge. When resin is close to the knife, a reflection of the knife will be seen on the surface of the resin. This reflection should be parallel to the sample (if properly oriented). With sectioning progression, make sure that the cells/tissue/organ are oriented in parallel to the knife (remember to correct the angle between the sample and the knife edge).

1. Immobilize the capsules using a small bench clamp workshop.
2. Remove the capsules using stiff single edge razor blades and diagonal cutting pliers (Figures 3C and 3D).
3. Trim the bottom of the sample with a stiff single edge razor blade and then use thin double-edged razor blades for more precise cutting (Figures 3E and 4C-4F).  
*Note: The bottom of the sample, which will be in parallel to the knife edge (see Procedure E), should be trimmed in a trapezoid shape with the major (longer) base on the bottom and minor (shorter) base on the top (Figures 3E, 4D-4F and 6). The size of the trapezoid should not exceed the length of the diamond knife ( $\leq 2$  mm).*
4. Mount the resin block on an ultramicrotome holder with the bottom of the sample oriented towards the knife.
5. Orient the resin block with the sample in the way that the trimmed bottom of the sample (Figure 4A) will be parallel to the glass knife edge (Figure 6).  
*Note: When the sample is close to the knife you will be able to see the reflection of the knife on the surface of the resin (this reflection should be parallel).*
6. Cut a few sections using a glass knife.

*Note: The sections can be more than 1 µm thick. The purpose of this point is to see how the sections look like and to orient the specimen properly.*

7. Place a drop of distilled water on a regular or a polylysine-coated slide and put a few sections on that drop.

*Note: Use a wet painting brush (the thinner, the better) to collect the sections.*

8. Dry sections on the slide (see Equipment 30).
9. Put a drop of toluidine blue stain (Recipe 7) onto the sections for a minute and wash under tap water.
10. Observe the sections under the light microscope.

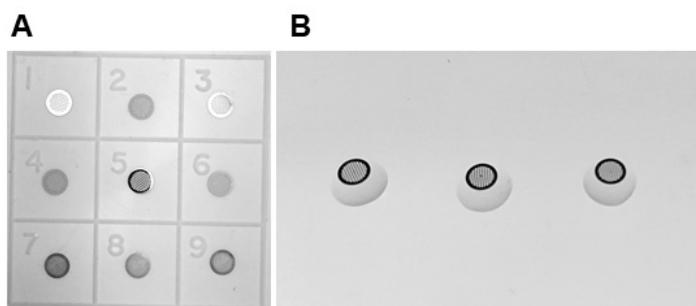
*Note: If the sample is oriented in the proper way, you can begin ultra-thin sectioning. If it is not oriented properly (cells are cut askew), adjust the sample position and repeat sectioning (Steps G5-G10).*

11. Mount a diamond knife onto the ultramicrotome and fill the bath with distilled water.
12. Orient the resin block with the sample in an analogical way as above (see Step G5).
13. Begin ultrathin sectioning (70 nm thin sections) until you see the ribbon of sections floating on the water.
14. Using the forceps dip the grid under the water (the dull side with formvar on the top) and collect some sections.
15. Leave the grids with section on the filter paper or on the round silicon rubber until dry (Figure 7A, numbers 7-9), store in the grid storage box.

*Note: Remember to place the shiny side on the bottom, and dull side with the section on the top.*

#### H. Immunogold labeling

Here I describe how to perform the labeling of cell wall epitopes. In this process, primary antibodies bind to specific cell wall epitopes, which are then recognized by secondary antibodies coupled with gold particles for visualization in TEM. The immunolocalization procedure relies on moving the grids over a series of small droplets containing different solutions, antibodies and stain for contrasting, all are placed on the parafilm (Figures 7 and 8). Remember to include controls such as wild type, untreated plants as well as secondary antibody alone, which should not give any labeling.



**Figure 7. Grids for electron microscopy.** A. Grids with two sides: a dull side and a shiny side.

Grids with the shiny side correspond to numbers 1, 3, 5. Grids with the dull side correspond to numbers 2, 4, 6, 7-9. Sections are mounted on the formvar-coated dull side (numbers 7-9). B. The dull side is in contact with droplets. Usually, the shiny side must be in contact with the filter paper while drying the samples (not shown).

**A**

	BR	primary antibody	PBS	secondary antibody	distilled water
WT					
Mutant (M)					
control (WT)					
Control (M)					

parafilm

**B**

	BR	primary antibody	PBS	secondary antibody	distilled water
WT					
Mutant (M)					
control (WT)					
control (M)					

parafilm

**Figure 8. Immunolocalization procedure.** The labeling relies on moving the grids over a series of small droplets containing different solutions (blocking reagent [BR], primary antibodies, PBS buffer, secondary antibody, distilled water) all placed on the parafilm.

1. Place a piece of parafilm on a clean table.

*Note: Use 70% ethanol to clean the table. The side of parafilm covered by paper is clean and should be on top (where droplets of different solutions will be placed). Avoid touching the*

parafilm with hands to not contaminate it. Add a few drops of water below the parafilm and stick the corners of the parafilm with a tape to stabilize it and keep flat.

2. Place a few droplets of BR (depending on the number of grids) in a row on the parafilm (for BR see Recipe 8) (Figure 8A).

3. Place the grids on droplets of BR and leave it for 15 min at ambient temperature (the dull side of the grid containing the section should be in contact with the droplet) (Figures 7B and 8B).

*Note: Always transfer the grids with clean tweezers. It is recommended to use a set of tweezers (negative-action style). To avoid cross-contamination between antibodies, before touching the grids, always clean tweezers with 70% ethanol, rinse in distilled water and dry with a clean tissue wiper before contact with a grid. Always handle the grids with care. The tweezers should be in contact only with the external ring of the grid.*

4. Put the droplets of primary antibody solutions (Recipe 9) in the second row (use BR instead of primary antibodies for the controls, which will be treated only with secondary antibodies).

5. Take the grid out of BR droplet, touch the edge of the grid with a clean filter paper (to absorb the reagent) and transfer the grid onto a droplet with primary antibody.

*Note: From the circles of the filter paper, cut small triangles, use the sharp tip to touch the grid edge.*

6. Incubate with primary antibody for 45-60 min at ambient temperature.

*Note: During the incubation in primary and secondary antibodies make sure that the solution will not dry out. To prevent drying, cover the samples with e.g., a large glass Petri dish.*

7. Put droplets of PBS (Recipe 10) in the four next rows.

8. Take the grid out of the primary antibody droplet, touch the edge of the grid with a clean filter paper (to absorb the reagent), and transfer the grid onto a droplet with PBS.

*Note: Rinse the grids four times in PBS. Leave the grids for 5 min on each droplet. Before transferring to the next PBS droplet, always absorb previous PBS with a filter paper.*

9. Put the droplets of the secondary antibody solution (Recipe 10) into the next row.

10. Transfer the grids in an analogical way to above (Steps H5 and H8).

11. Incubate with the corresponding secondary antibody for 60 min at ambient temperature.

12. Put the droplets of distilled water into the four next rows.

13. Transfer the grids in an analogical way to above (Steps H5 and H8).

*Note: Rinse the grids four times in distilled water. Leave the grids for 5 min on each droplet. Before transferring to the next water droplet, always absorb previous water with a filter paper.*

14. Take out the grid from the distilled water droplet, touch the edge of the grid with a clean filter paper (to absorb the reagent) and transfer the grid on a filter paper or on a silicon rubber (the dull side with the section should be on top).

15. Leave the grids for at least 60 min or until dry (cover with a large Petri plate to prevent contamination).

16. Put a droplet of uranyl acetate (UA) (Recipe 11) on a new piece of the parafilm.

*Note: Briefly spin at maximum speed before use.*

17. Transfer the grid onto a droplet of uranyl acetate (UA) and leave it for 10 min at ambient temperature in darkness (UA is light sensitive).
18. Take out the grid from UA droplet, touch the edge of the grid with a clean filter paper (to absorb the reagent) and transfer the grid onto the distilled water droplet.
19. Holding the grids with negative-action style tweezers rinse each grid under tap water for 30 s, then place the grids onto the filter paper or silicon rubber, wait until dry (the dull side with the section should be on the top) (Figure 7A, points 7-9) and store in a grid storage box.

*Note: All the steps involving the use of UA must be carried with special attention by using protective gloves and clothes under the fume hood. All UA waste must be placed in assigned waste bins. Avoid too long incubation in UA as it can give too high contrast.*

## **Recipes**

1. Half strength MS basal medium
  - a. Add 2.2 g of MS
  - b. Add 10 g of sucrose (1% v/v)
  - c. Add distilled water to a final volume of 1 L
  - d. Mix the solution on a magnetic stirrer with a magnetic stir bar
  - e. Adjust pH to 5.6 by decreasing pH with hydrogen chloride (HCl) or increasing pH with sodium hydroxide (NaOH)
  - f. Add 7 g of plant agar (0.7% v/v)
  - g. Autoclave the medium
2. Paraformaldehyde-glutaraldehyde (4% PFA and 0.05% GA) fixative solution
  - a. Add 4 g of paraformaldehyde (PFA) powder to a conical flask with 100 ml of 0.1 M phosphate buffer (4% of PFA for total concentration)
  - b. Add a magnetic stir bar, cover with aluminum foil to prevent evaporation and place the flask on a magnetic hotplate stirrer (90-100 °C)
  - c. Add a few drops of sodium hydroxide solution (NaOH) to speed up dissolving the powder (use 0.1 M concentration of NaOH or lower) and then leave it mixing until the powder dissolves completely (it can take a couple of hours)
  - d. Adjust pH to 7.2 by decreasing pH with hydrogen chloride (HCl) or increasing pH with sodium hydroxide (NaOH)
  - e. Add 200 µl of 25% glutaraldehyde (GA) (0.05% of GA for total concentration)
  - f. Store the fixative solution in a designated (ventilated) refrigerator (for a short storage) or freeze the fixative in -20 °C (for long storage)

*Note: PFA and GA are highly toxic. Conduct all the work under the fume hood, with protective gloves, clothes and mask. Discard the fixative into designated waste. It is recommended to prepare fresh fixative or to use already prepared PFA solution. To not generate additional waste, it is recommended to calculate the exact amount of fixative which is required (see Step A1). To*

*not contaminate the pH meter, it is recommended to use a disposable pH indicator paper instead.*

3. Phosphate buffer (PB), 0.1 M solution (pH = 7.2)
  - a. Prepare a stock solution of 0.1 M disodium phosphate ( $\text{Na}_2\text{HPO}_4$ ) and another solution of 0.1 M monosodium phosphate ( $\text{NaH}_2\text{PO}_4$ ) by dissolving these salts in distilled water
  - b. Adjust phosphate buffer pH to 7.2 by mixing 68.4 ml of disodium phosphate solution ( $\text{Na}_2\text{HPO}_4$ ) with 31.6 ml of monosodium phosphate solution ( $\text{NaH}_2\text{PO}_4$ ) (Sambrook *et al.*, 1982)

*Note: It is recommended to prepare fresh stock concentrations because salt might precipitate. Confirm the pH with a pH meter measurement. Autoclave phosphate buffer solution before storing in ambient temperature.*

4. Low melting point (LMP) agarose 1% solution
  - a. Dissolve 1 g of LMP agarose in 100 ml of distilled water
  - b. Warm it up in a microwave until boiling
  - c. Wait to cool it down before contact with the plant material
5. Different ethanol concentrations 10%-95%
  - a. Mix ethanol absolute  $\geq$  99.5% with distilled water to obtain different concentrations
  - b. Prepare around 100 ml of each concentration and keep it in closed bottles
6. Different LWR resin concentrations 10%-75%  
Mix LWR resin with ethanol absolute  $\geq$  99.5% to obtain different concentrations
7. Toluidine blue solution
  - a. Dissolve 0.5 g of toluidine blue powder in 50 ml of 70% ethanol.
  - b. Prepare 1% sodium hydroxide (NaOH) solution by dissolving 0.5 g of NaOH in 50 ml of distilled water
  - c. Mix toluidine blue solution with 1% NaOH in ratio 1:5
  - d. Adjust pH of NaOH to 2.5 by decreasing pH with hydrogen chloride (HCl) or increasing pH with sodium hydroxide (NaOH)
8. Blocking Reagent (BR) 1%  
Dissolve 1 g of Bovine Serum Albumin (BSA) in 100 ml of PBS (for 1% of BR for total concentration)
9. Antibodies solutions  
Primary antibodies are diluted in BR (1:10) and secondary antibodies are diluted in BR (1:50)
10. Phosphate Buffered Saline (PBS), 0.1 M solution (pH = 7.2)  
8 g sodium chloride (NaCl)  
0.2 g potassium chloride (KCl)  
1.15 g disodium phosphate ( $\text{Na}_2\text{PO}_4$ )  
0.2 g monopotassium phosphate ( $\text{KH}_2\text{PO}_4$ )  
Refill with distilled water up to 1 L  
Adjust pH to 7.2 by decreasing it with hydrogen chloride (HCl) or increasing it with sodium

hydroxide (NaOH)

*Note: Autoclave phosphate buffer solution before storing in ambient temperature.*

#### 11. Uranyl acetate solution (5%)

- a. Add 0.5 g of UA to 5 ml of double distilled water in a 15 ml tube
- b. Adjust the pH to 3.5 by decreasing it with hydrogen chloride (HCl) or increasing it with sodium hydroxide (NaOH)
- c. Fill the tube with double distilled water up to 10 ml and energetically shake the content
- d. Filter the solution through a filter paper or spin the solution in the centrifuge at maximum speed for 10 min and transfer the liquid part to a new tube
- e. Keep UA solution in the dark

*Note: Prepare UA solution under the fume hood with special attention using protective gloves and clothes. All UA waste must be placed in assigned waste bins. To not contaminate the pH meter, it is recommended to use a disposable pH indicator paper instead.*

#### **Acknowledgments**

I would like to greatly acknowledge Stéphanie Robert and Richard S. Smith for comments on the manuscript and support of the work. I acknowledge Nicola Trozzi for proofreading the manuscript. I would like to thank Lenore Johansson, Kjell Olofsson and Janusz Kubrakiewicz for sharing their experience. This work was performed at Umeå Core Facility Electron Microscopy at Umeå University. The work was supported by the Swedish Research Council Vetenskapsrådet (grant nos. VR2012-2343 and VR2016-00768), Vinnova (Verket för Innovationssystem) and ERA-CAPS. This protocol was adapted from the published study (Majda *et al.*, 2017).

#### **Competing interests**

I declare no conflicts of interest or competing interests.

#### **References**

1. Baskin, T. I. (2005). [Anisotropic expansion of the plant cell wall](#). *Annu Rev Cell Dev Biol*(21): 203-222.
2. Cosgrove, D. J. (2005). [Growth of the plant cell wall](#). *Nat Rev Mol Cell Biol* 6(11): 850-861.
3. Loqué, D., Scheller, H. V. and Pauly, M. (2015). [Engineering of plant cell walls for enhanced biofuel production](#). *Curr Opin Plant Biol* 25: 151-161.
4. Majda, M. (2018). [Role of the cell wall in cell shape acquisition](#). Swedish University of Agricultural Sciences, Umeå, Sweden. Acta Universitatis agriculturae Sueciae. 10: 1652-6880. ISBN 978-9-17-760160-9.
5. Majda, M. and Robert, S. (2018). [The role of auxin in cell wall expansion](#). *Int J Mol Sci* 19(4):

E951.

6. Majda, M., Grones, P., Sintorn, I. M., Vain, T., Milani, P., Krupinski, P., Zagorska-Marek, B., Viotti, C., Jonsson, H., Mellerowicz, E. J., Hamant, O. and Robert, S. (2017). [Mechanochemical polarization of contiguous cell walls shapes plant pavement cells.](#) *Dev Cell* 43(3): 290-304 e294.
7. Sambrook, J., Fritsch, E. F. and Maniatis, T. (1982). Molecular cloning: A laboratory manual. 2nd edition. Cold Spring Harbor Laboratory.
8. Voiniciuc, C., Pauly, M. and Usadel, B. (2018). [Monitoring polysaccharide dynamics in the plant cell wall.](#) *Plant Physiol* 176(4): 2590-2600.
9. Wallace, I. S. and Anderson, C. T. (2012). [Small molecule probes for plant cell wall polysaccharide imaging.](#) *Front Plant Sci* 3: 89.

**Quantitative Plasmodesmata Permeability Assay for Pavement Cells of *Arabidopsis* Leaves**Min Diao<sup>1, 2, #, \*</sup>, Qiannan Wang<sup>1, #</sup> and Shanjin Huang<sup>1, \*</sup>

<sup>1</sup>Center for Plant Biology, School of Life Sciences, Tsinghua University, Beijing 100084, China; <sup>2</sup>iHuman Institute, Shanghai Tech University, 393 Middle Huaxia Road, Shanghai 201210, China

\*For correspondence: [diaomin@shanghaitech.edu.cn](mailto:diaomin@shanghaitech.edu.cn); [sjhuang@tsinghua.edu.cn](mailto:sjhuang@tsinghua.edu.cn)

#Contributed equally to this work



**[Abstract]** Plasmodesmata (PD) are intercellular channels between walled plant cells that enable the transportation of materials between adjacent cells, which are important for plant growth and development. The permeability of PD must be tightly regulated. Assays to determine the permeability of PD are crucial for related studies on the regulation of PD development and permeability. Here we describe an assay for the determination of PD permeability via the observation and quantification of GFP diffusion and cell-to-cell transport of CMV MP-GFP in *Arabidopsis* leaves.

**Keywords:** Plasmodesmata, PD permeability, GFP, CMV MP-GFP, Particle bombardment

**[Background]** Plasmodesmata (PD) are plant-specific channels between cells, which are important for plant growth and development as well as the interaction between plants and the surrounding environment (Maule *et al.*, 2011; Lee, 2015; Cheval and Faulkner, 2018). Water and small molecules can pass through PD freely. Other macromolecules, e.g., proteins, RNA, and some pathogens, can also move via PD. However, the mobility of those molecules between adjacent cells is tightly regulated, although the mechanism underlying this transport is not well understood (Sevilem *et al.*, 2015). Currently, there are several approaches reported for measuring the permeability of PD in plant tissues. One of the methods is to visualize the diffusion of fluorescent dextrans or other fluorescent probes from the targeted cells into neighboring cells (Ding *et al.*, 1996). This approach allows the researchers to select fluorescent dextrans of different sizes which will facilitate the study of PD permeability. In addition, this method also allows researchers to perform the coinjection of fluorescent dextrans with special drugs or proteins that allows the colleagues to assay the effect of those drugs or proteins on the permeability of PD. However, this method requires a rigorous experimental system and a highly experienced operator to perform the experiments. The second method is Drop-AND-See (DANS) which uses the 5(6)-carboxy fluorescein diacetate (CFDA) for the rapid assessment of PD permeability (Cui *et al.*, 2015). DANS is a simple and rapid approach, but it is impossible to trace the spread of CFDA at the single cell resolution. The researchers in this field also used the green fluorescent protein (GFP) (Crawford and Zambryski, 2001; Liarzi and Epel, 2005) and *Cucumber mosaic virus* (CMV) Movement Protein (MP)-GFP (CMV MP-GFP) (Iglesias and Meins, 2000) as probes to assay the permeability of PD. The expression of GFP or CMV MP-GFP was achieved by delivering plasmids expressing them into plant cells via particle bombardment. This method enables the researchers to visualize the diffusion of GFP from targeted cells into adjacent cells or cell-to-cell movement of CMV MP-GFP. Here,

we introduce the detailed method of GFP diffusion and cell-to-cell movement of CMV MP-GFP, which is adapted from our recently published paper (Diao *et al.*, 2018).

### **Materials and Reagents**

1. 9 cm glass dish (NORMAX, catalog number: 5058546)
2. Pipette tips (USA Scientific, catalog numbers: 1112-1720, 1110-1200)
3. Eppendorf tubes (Fisher Scientific, Fisherbrand<sup>TM</sup>, catalog number: 05-408-129)
4. Macrocarriers (Bio-Rad, catalog number: 1652335)
5. 1,100 psi rupture discs (Bio-Rad, catalog number: 1652329)
6. Stopping screens (Bio-Rad, catalog number: 1652336)
7. Coverslip (THOMAS SCIENTIFIC, catalog number: 6672A46)
8. *Arabidopsis thaliana* plants
9. Tungsten M10 or M17 microcarriers (Bio-Rad, catalog numbers: 1652266 or 1652267)
10. TIANprep Midi Plasmid Kit (TIANGEN, catalog number: DP103-03)
11. Spermidine (Sigma-Aldrich, catalog number: S2626)
12. Calcium chloride dihydrate ( $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ) (Sigma-Aldrich, catalog number: C7902)
13. Plasmid pdGN (Lee *et al.*, 2005)
14. Plasmid CMVMP-pdGN (Diao *et al.*, 2018)
15. Bio-Rad 1.0  $\mu\text{m}$  Gold Microcarriers (Bio-Rad, catalog number: 165-2263)
16. Ethanol (AMRESCO, catalog number: E193)
17. 50% sterile glycerin (Sigma-Aldrich, catalog number: G5516)
18.  $\text{KNO}_3$  (Sigma-Aldrich, catalog number: P6083)
19.  $\text{NH}_4\text{NO}_3$  (AMRESCO, catalog number: 94629)
20.  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (Sigma-Aldrich, catalog number: M1880)
21.  $\text{KH}_2\text{PO}_4$  (Sigma-Aldrich, catalog number: P5655)
22.  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$  (Macklin, catalog number: M813652)
23.  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  (Macklin, catalog number: Z820817)
24.  $\text{H}_3\text{BO}_4$  (Sigma-Aldrich, catalog number: B6768)
25. KI (Sigma-Aldrich, catalog number: V900056)
26.  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$  (Sigma-Aldrich, catalog number: M1651)
27.  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (Macklin, catalog number: C805354)
28.  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  (Aladdin, catalog number: C8396-01)
29. Glycine (Sigma-Aldrich, catalog number: V900144)
30. VB1 (Sigma-Aldrich, catalog number: V-014)
31. VB6 (Sigma-Aldrich, catalog number: V-018)
32. Nicotinic Acid (Sigma-Aldrich, catalog number: V900424)
33. Inositol (Sigma-Aldrich, catalog number: I5125)
34.  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  (Macklin, catalog number: I809845)

35. Na<sub>2</sub>-EDTA·2H<sub>2</sub>O (AMRESCO, catalog number: 0105)
36. Sucrose (Sigma-Aldrich, catalog number: V900116)
37. Agar (Sigma-Aldrich, catalog number: A1296)
38. MS medium (see Recipes)
39. Gold suspension solution (see Recipes)

## **Equipment**

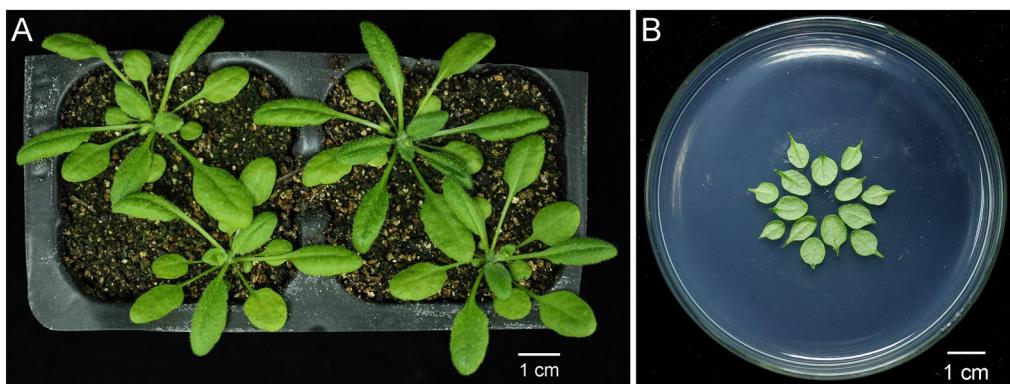
1. Ophthalmic scissors
2. Ophthalmic tweezers
3. Centrifuge (Eppendorf, model: 5417R)
4. PDS-1000/He™ Biostatic Particle Delivery System (Bio-Rad, model: 1652257)
5. Plant LED Incubator
6. Zeiss LSM 510 META or an equivalent confocal microscope
7. Vortex (Corning, model: Corning® LSE™ Vortex Mixer)
8. NanoDrop 2000 (Gene Company Limited)
9. -20 °C freezer

## **Software**

1. ImageJ (<https://imagej.nih.gov/ij/> version 1.51)
2. IBM SPSS Statistics (version 25)

## **Procedure**

1. Prepare Petri dish containing 20 ml of MS medium with 0.8% agar.
2. *Arabidopsis* plants grown under the 16 h/8 h light/dark cycle for 3-4 weeks (Figure 1A). Cut rosette leaves of *Arabidopsis* plants that had not yet bolted by ophthalmic scissors and place in a Petri dish with the abaxial side facing upward as shown in Figure 1B.  
*Note: Cells of young leaves work better than old leaves. Leaves from different genotypes should be placed on MS medium equally divided (Figure 1B).*



**Figure 1. Preparation of the leaves of 4-week-old *Arabidopsis* plants for particle bombardment.** A. *Arabidopsis* plants after growing under the 16 h/8 h light/dark cycle for 4 weeks. Scale bar = 1 cm. B. Rosette leaves that had not yet bolted were cut from *Arabidopsis* plants shown in (A) by ophthalmic scissors and placed concentrically with abaxial side facing upward on the surface of solid Murashige & Skoog medium. Scale bar = 1 cm.

3. Prepare pdGN plasmid from *E-coli* (Lee et al., 2005). Isolated the plasmid using A TIANprep Midi Plasmid Kit. The concentration of plasmid should be at least 1  $\mu$ g/ $\mu$ l.

*Note: The concentration of the plasmid is important for the next step. The pdGN-35S:HDEL-mCherry plasmid should also be prepared when we need to indicate the cells targeted by particle bombardment (Batoko et al., 2000).*

4. Prepare bullets of biolistic bombardment. Vortex 10  $\mu$ l gold suspension solution (Recipe 2) in a microcentrifuge tube with 2  $\mu$ g plasmid DNA for 1 min. Add 20  $\mu$ l fresh 0.1 M spermidine and vortex again for 1 min. Add 50  $\mu$ l 2.5 M CaCl<sub>2</sub> and continue vortexing for another 3 min. Allow the mixture to settle for 5 min on ice and centrifuge the mixture for 10 s, at 2,348  $\times g$ . Discard the supernatant. Wash the pellet with 1 ml of 70% ethanol and then with 1 ml of 100% ethanol. Mix and centrifuge gently. Finally, resuspend the pellet in 15  $\mu$ l 100% ethanol.

*Notes:*

- a. The maximum total volume of plasmid DNA should be  $\leq$  10  $\mu$ l. When both pDGN plasmids and CMV MP-pDGN plasmids need to be expressed simultaneously, 2  $\mu$ l of each and gold particles are mixed together in a single test tube.
  - b. The 1 M spermidine stock can be stored at -20 °C for at least 3 months.
5. Vortex the mixture gently and transfer it to a macrocarrier using a pipette with a 10-200  $\mu$ l tip. Air dry until the ethanol has evaporated, assemble all of the parts necessary for performing a particle bombardment according to the manufacturer's instructions (Figure 2). Set the pressure to 1,100 psi and use 1,550 psi rupture discs. Place the MS plate with *Arabidopsis* leaves 9 cm below the macrocarrier (level 3) in the bombardment chamber (Figure 2). Set the vacuum level in the bombardment chamber to 28 psi and bombard.



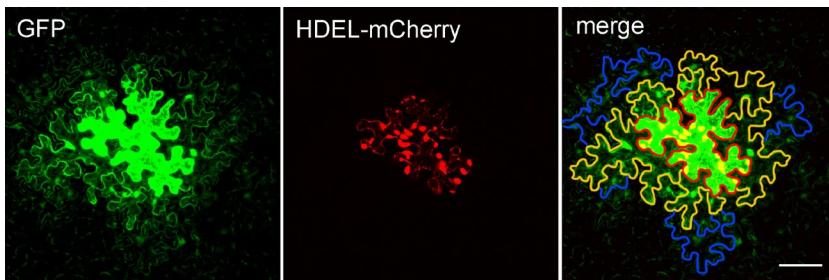
**Figure 2. Side view of PDS-1000/He™ Biolistic Particle Delivery System.** Before performing particle bombardment, the Petri dish containing *Arabidopsis* leaves was moved into the PDS-1000/He™ Biolistic Particle Delivery System (left). Part 1 and part 2 of the system were zoomed in at right. a is 1,100 psi rupture disk. b is microparticles carrier disk, which carries 1.0 µm Gold Microcarriers. c is stopping screen.

6. Incubate the bombarded leaves for 24 h or 48 h at 23 °C in the dark.
7. Mount the bombarded leaves on a glass slide with a coverslip to image with a Zeiss LSM 510 META or an equivalent confocal microscope. The GFP signal is excited by a 488 nm argon laser and emission is captured in the range of 505–545 nm; the mCherry signal is excited by a 543 nm HeNe laser and the emission is captured in the range of 590–625 nm. Use a 20x/0.25 Fluar objective lens to scan the GFP cell clusters. Twenty percent laser intensity is used to image every fluorescent cell clearly.

*Note: Acquire at least 60 images from each genotype for statistical analysis.*

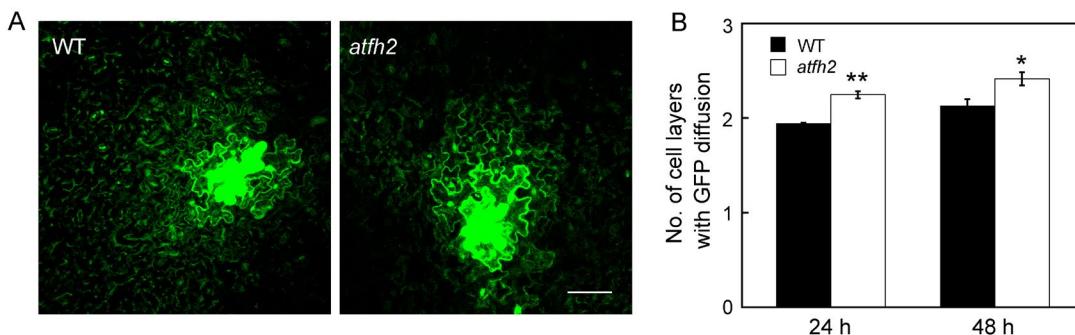
### Data analysis

1. Open each image in ImageJ and find the cell expressing GFP (generally the cell with strongest fluorescence) which is defined as layer 0 (the edge of the cell is marked with red line in Figure 3). GFP protein diffuses from the layer 0 cell into other cells around it via PD. The cells that share a common cell wall with layer 0 cells were defined as layer 1 (the edge of cells is marked with orange lines in Figure 3). Cells that share a common cell wall with layer 1 cells, but not with layer 0 cells, were defined as layer 2 cells (the edge of cells is marked with blue lines marked in Figure 3). Cells that expressed GFP but showed no diffusion were not counted to avoid the situation in which damage was caused by the bombardment.



**Figure 3. *Arabidopsis* leaf epidermal cells expressing *pdGN-35:GFP* and *pdGN-35S:HDEL-mCherry*.** HDEL-mCherry is non-mobile, and was used to indicate the bombarded cell. The edge of the layer 0 cell is marked by a red line. The edges of the layer 1 cells and layer 2 cells are marked by orange and blue lines, respectively. Scale bar = 50  $\mu$ m.

2. Count the number of cell layers in the cell clusters. As the bombardment will cause damage for cells or induce the non-uniform expression of GFP that prevent the subsequent analysis, we normally capture at least 60 images from more than 6 plants per set but only pick data from three plants with healthy cells and uniform expression of GFP for the statistical analysis. Repeat the experiment at least three times.
3. The IBM SPSS Statistics (version 25) software was used to perform the statistical analysis. First, the normality of the datasets was assessed by Shapiro-Wilk tests (Shapiro and Wilk, 1965) the data were not normally distributed by Shapiro-Wilk tests, the Mann-Whitney *U*-test (Fay and Proschan, 2010) was applied for the subsequent statistical analyses.



**Figure 4. AtFH2 redundantly regulate PD permeability.** A. Images of GFP diffusion in leaf epidermal pavement cells of WT and *atfh2* plants. Scale bar = 50  $\mu$ m. B. Quantification of the number of GFP diffusion layers in leaves of WT and *atfh2* plants. Values represent mean  $\pm$  SE. \* $P$  < 0.05, and \*\* $P$  < 0.01 by Mann-Whitney *U*-test.

## Recipes

1. MS medium (1 L)  
76 g/L KNO<sub>3</sub>

66 g/L NH<sub>4</sub>NO<sub>3</sub>  
14.8 g/L MgSO<sub>4</sub>·7H<sub>2</sub>O  
6.8 g/L KH<sub>2</sub>PO<sub>4</sub>  
13.28 g/L CaCl<sub>2</sub>  
1.69 g/L MnSO<sub>4</sub>·H<sub>2</sub>O  
8.6 g/L ZnSO<sub>4</sub>·7H<sub>2</sub>O  
6.3 g/L H<sub>3</sub>BO<sub>4</sub>  
0.83 g/L KI  
0.25 g/L Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O  
0.025 g/L CuSO<sub>4</sub>·5H<sub>2</sub>O  
0.025 g/L CoCl<sub>2</sub>·6H<sub>2</sub>O  
0.2 g/L Glycine  
0.1 g/L VB1  
0.05 g/L VB6  
0.05 g/L Nicotinic Acid  
10 g/L Inositol  
2.78 g/L FeSO<sub>4</sub>·7H<sub>2</sub>O  
3.73 g/L Na<sub>2</sub>·EDTA·2H<sub>2</sub>O  
10 g/L Sucrose  
6 g/L Agar

## 2. Gold suspension solution

- a. Place 30 mg of gold microcarriers into a 1 ml microcentrifuge tube
- b. Add 1 ml of 70% ethanol and vortex for 3 min
- c. Allow the mixture to settle for 15 min and centrifuge the mixture for 5 s
- d. Remove the supernatant and add 1 ml sterile water and repeat the previous step two times
- e. Remove the supernatant and add 500 µl 50% sterile glycerin
- f. Vortex the mixture gently and store at -20 °C

## Acknowledgments

This work was supported by grants from national natural science foundation of China (31471266; 31671390).

## Competing interests

The authors declared that they have no conflicts of interest to this work.

## References

1. Batoko, H., Zheng, H. Q., Hawes, C. and Moore, I. (2000). [A Rab1 GTPase is required for transport between the endoplasmic reticulum and golgi apparatus and for normal golgi movement in plants.](#) *Plant Cell* 12(11): 2201-2218.
2. Cheval, C. and Faulkner, C. (2018). [Plasmodesmal regulation during plant-pathogen interactions.](#) *New Phytol* 217(1): 62-67.
3. Crawford, K. M. and Zambryski, P. C. (2001). [Non-targeted and targeted protein movement through plasmodesmata in leaves in different developmental and physiological states.](#) *Plant Physiol* 125(4): 1802-1812.
4. Cui, W., Wang, X. and Lee, J. Y. (2015). [Drop-ANd-See: a simple, real-time, and noninvasive technique for assaying plasmodesmal permeability.](#) *Methods Mol Biol* 1217: 149-156.
5. Diao, M., Ren, S., Wang, Q., Qian, L., Shen, J., Liu, Y. and Huang, S. (2018). [Arabidopsis formin 2 regulates cell-to-cell trafficking by capping and stabilizing actin filaments at plasmodesmata.](#) *Elife* 7: e36316.
6. Ding, B., Kwon, M.-O. and Warnberg, L. (1996). [Evidence that actin filaments are involved in controlling the permeability of plasmodesmata in tobacco mesophyll.](#) *Plant J* 10(1): 157-164.
7. Fay, M. P. and Proschan, M. A. (2010). [Wilcoxon-Mann-Whitney or t-test? On assumptions for hypothesis tests and multiple interpretations of decision rules.](#) *Stat Surv* 4: 1-39.
8. Iglesias, V. A. and Meins, F., Jr. (2000). [Movement of plant viruses is delayed in a  \$\beta\$ -1,3-glucanase-deficient mutant showing a reduced plasmodesmatal size exclusion limit and enhanced callose deposition.](#) *Plant J* 21(2): 157-166.
9. Lee, J. Y. (2015). [Plasmodesmata: a signaling hub at the cellular boundary.](#) *Curr Opin Plant Biol* 27:133-140.
10. Lee, J. Y., Taoka, K., Yoo, B. C., Ben-Nissan, G., Kim, D. J. and Lucas, W. J. (2005). [Plasmodesmal-associated protein kinase in tobacco and \*Arabidopsis\* recognizes a subset of non-cell-autonomous proteins.](#) *Plant Cell* 17(10): 2817-2831.
11. Liarzi, O. and Epel, B. L. (2005). [Development of a quantitative tool for measuring changes in the coefficient of conductivity of plasmodesmata induced by developmental, biotic, and abiotic signals.](#) *Protoplasma* 225(1-2): 67-76.
12. Maule, A. J., Benitez-Alfonso, Y. and Faulkner, C. (2011). [Plasmodesmata - membrane tunnels with attitude.](#) *Curr Opin Plant Biol* 14(6): 683-690.
13. Sevilem, I., Yadav, S. R. and Helariutta, Y. (2015). [Plasmodesmata: channels for intercellular signaling during plant growth and development.](#) *Methods Mol Biol* 1217: 3-24.
14. Shapiro, S. S. and Wilk, M. B. (1965). [An analysis of variance test for normality \(complete samples\).](#) *Biometrika* 52(3/4): 591-611.

## Simultaneous Fluorescent Recordings of Extracellular ATP and Intracellular Calcium in Mammalian Cells

Nicholas Mikolajewicz<sup>1, 2</sup> and Svetlana V Komarova<sup>1, 2, \*</sup>

<sup>1</sup>Faculty of Dentistry, McGill University, Montreal, Canada; <sup>2</sup>Shriners Hospital for Children—Canada, Montreal, Canada

\*For correspondence: [svetlana.komarova@mcgill.ca](mailto:svetlana.komarova@mcgill.ca)



**[Abstract]** Extracellular ATP is a potent signaling molecule that stimulates intracellular calcium responses through purinergic (P2) receptors in mammalian cells. While extracellular ATP and intracellular calcium can be measured separately, simultaneous monitoring can offer additional insights into P2 receptor physiology. This protocol takes advantage of the overlapping fluorescence spectra between the ATP-detection substrate luciferin and calcium indicator dye Fura2. Mammalian cells are loaded with Fura2-AM and live-cell recordings are acquired in the presence of a luciferin-luciferase imaging solution. This protocol allows to study stimulus-induced ATP release and directly relate changes in extracellular ATP concentration to observed calcium responses.

**Keywords:** Fluorescence, Fura2, Extracellular ATP, Intracellular calcium, Luciferin, P2 receptors, Mammalian cells

**[Background]** ATP is a potent extracellular signaling molecule that is released in response to a variety of stress-related stimuli, including mechanical stimulation or injury (Mikolajewicz *et al.*, 2018a). Extracellular ATP is an autocrine and paracrine signal that acts through a number of purinergic (P2) receptors, which consist of two sub-families, P2X and P2Y receptors, and are omnipresent in virtually all mammalian cells (Burnstock and Verkhratsky, 2009). P2X receptors consist of 7 subtypes (P2X<sub>1-7</sub>) of ligand-gated cation channels that permit calcium influx upon stimulation. P2Y receptors include 8 subtypes of G-protein coupled receptors (P2Y<sub>1-2, 4, 6, 11-14</sub>), several which can induce release of calcium from intracellular calcium stores through inositol triphosphate.

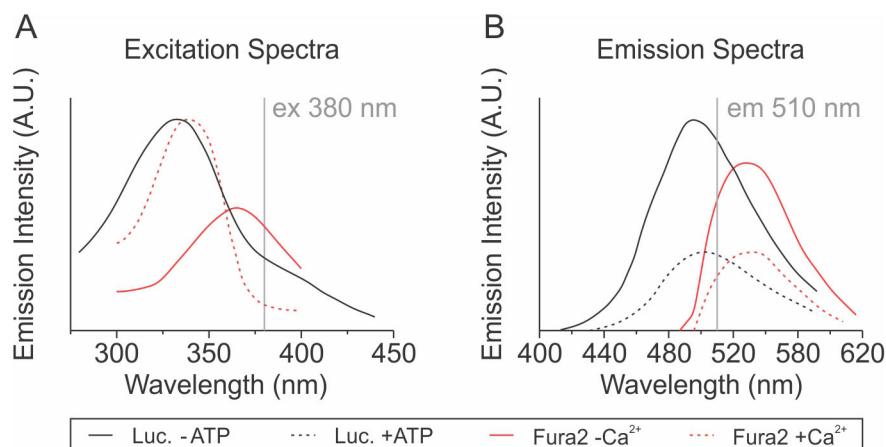
The luciferin-luciferase bioluminescence assay is the gold standard for measuring ATP concentrations (Seminario-Vidal *et al.*, 2009). In the presence of ATP, luciferase oxidizes luciferin and produces bioluminescent light proportionally to the amount of ATP present (Table1). While sensitive and robust, bioluminescence method is difficult to employ in microscopy applications and requires specialized equipment due to the low signal intensity. Luciferin is also a cell-impermeable fluorescent molecule, a property that was originally explored by Sorensen and Novak (2001) to develop a fluorescence method to measure extracellular ATP. This method is compatible with standard fluorescence microscopy and relies on the detection of a decrease in luciferin fluorescence emission intensity when it is depleted in the presence of ATP (Table 1).

**Table 1. Bioluminescence and fluorescence properties of luciferin-luciferase reaction.** In the presence of ATP, luciferin is oxidized by luciferase to generate oxyluciferin thereby resulting in production of bioluminescence and loss of fluorescence proportional to the concentration of ATP.

Reactants	Products
Luciferin + O <sub>2</sub> + ATP	$\xrightarrow{+ \text{luciferase}}$
↑ fluorescence	↓ fluorescence
↓ bioluminescence	↑ bioluminescence

Calcium indicator dyes are used to monitor changes in cytosolic free calcium ( $[Ca^{2+}]_i$ ) in live cells (Paredes *et al.*, 2008). While there is a wide selection of indicator dyes with varying properties (e.g., affinity, brightness, spectral range), Fura2-acetoxymethyl ester (Fura2-AM) is among the most commonly used (Grynkiewicz *et al.*, 1985). When added to a cell culture, Fura2-AM accumulates intracellularly, where the acetoxymethyl ester group is removed by cellular esterases, producing an active dye entrapped within the cell. Fura2 is a ratiometric dye that changes its excitation spectrum upon binding  $Ca^{2+}$ . When  $Ca^{2+}$ -bound Fura2 is excited at 340 nm, emission at ~510 nm is proportional to  $[Ca^{2+}]_i$ . Conversely, when  $Ca^{2+}$ -unbound Fura2 is excited at 380 nm, emission at ~510 nm is inversely proportional to  $[Ca^{2+}]_i$ . Thus, measuring fluorescence at a single emission of 510 nm, with alternating excitation with 340 and 380 nm, provides a ratiometric measure of  $[Ca^{2+}]_i$  that is independent of dye loading and environmental artifacts.

Since the excitation and emission spectra of luciferin overlap with Fura2 (Figure 1), and the dyes are compartmentalized in the extracellular (luciferin) and intracellular (Fura2) spaces, this overlap in fluorescence spectra can be used to simultaneously measure extracellular ATP concentrations and changes in intracellular  $[Ca^{2+}]_i$ , as reported in our prior work (Mikolajewicz *et al.*, 2018b) and presented in detail below.



**Figure 1. Fluorescent characteristics of D-luciferin and Fura2.** A. Fluorescence excitation spectra for D-luciferin [Luc., em 550 nm (Goda *et al.*, 2015)] and Fura2 in the presence and

absence of  $\text{Ca}^{2+}$  [em 500 nm (Grynkiewicz *et al.*, 1985)]. B. Fluorescence emission spectra of luciferin (Sorensen and Novak, 2001) and Fura2 (Grynkiewicz *et al.*, 1985) in the presence (+) and absence (-) of ATP and  $\text{Ca}^{2+}$ , respectively.

## **Materials and Reagents**

1. BMP2-transfected C2C12 myoblast cells (courtesy of Dr. M. Murshed, McGill University)  
*Note: Any adherent mammalian cell line can be used for this protocol.*
2. Pipette tips
3. 35 mm glass-bottom dish (MatTek Corporation, catalog number: P35G-0-14-C)
4. 48-well glass-bottom culture plates (MatTek Corporation, catalog number: P48G-1.5-6-F)
5. Sterile disposable bottle top filters with polyethersulfone (PES) membrane, 0.2  $\mu\text{m}$  pore size (Thermo Fisher Scientific, Nalgene Rapid-Flow, catalog number: 595-4520)
6. Pyruvate kinase from rabbit muscle (Sigma-Aldrich, catalog number: P9136)
7. Fura2-AM, cell permeant (Thermo Fisher Scientific, Invitrogen, catalog number: F1221)
8. D-Luciferin, potassium salt (Thermo Fisher Scientific, Invitrogen, catalog number: L2916)
9. Adenosine 5'-triphosphate magnesium salt, ATP (Sigma-Aldrich, catalog number: A9187)
10. Luciferase from Photinus pyralis (Sigma-Aldrich, catalog number: L9420)
11. Sodium chloride, NaCl (Thermo Fisher Scientific, Fisher Chemical, catalog number: S671-3)
12. Potassium chloride, KCl (Thermo Fisher Scientific, ACROS Organics, catalog number: 7447-40-7)
13. Sodium phosphate dibasic anhydrous,  $\text{Na}_2\text{HPO}_4$  (Thermo Fisher Scientific, Fisher Chemical, catalog number: 7558-79-4)
14. Potassium dihydrogen phosphate,  $\text{KH}_2\text{PO}_4$  (Thermo Fisher Scientific, Fisher Chemical, catalog number: 7758-11-4)
15. Magnesium chloride hexahydrate,  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  (Thermo Fisher Scientific, Fisher Chemical, catalog number: 7786-30-3)
16. Calcium chloride dihydrate,  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  (Thermo Fisher Scientific, Fisher Chemical, catalog number: 10035-04-8)
17. D-(+)-glucose (Sigma-Aldrich, catalog number: G7528)
18. HEPES, 1 M (Wisent Bioproducts, catalog number: 330-050-EL)
19. Phosphoenolpyruvic acid monopotassium salt (Sigma-Aldrich, catalog number: 860077)
20. Fura2 stock solution, 1,000x (see Recipes)
21. Luciferin stock solution, 5,000x (see Recipes)
22. Luciferase stock solution, 500x (see Recipes)
23. Physiological solution (PS), pH 7.4 (see Recipes)
24. Fura2-staining solution, 1x (see Recipes)
25. Luciferin imaging solution with luciferase (see Recipes)
26. Luciferin imaging solution without luciferase (see Recipes)

27. ATP solutions, 100x (see Recipes)
28. Phosphate-buffered saline (PBS), pH 7.4 (see Recipes)

## Equipment

1. Pipettes
2. Benchtop pH meter (Thermo Fisher Scientific, Mettler Toledo FE20 FiveEasy, catalog number: 10526655)
3. Levy counting chamber (VWR, Hausser Scientific, catalog number: 15170 208)
4. Benchtop Centrifuge (Precision, Durafuge 300)
5. Laminar flow biological hood
6. Water-jacketed CO<sub>2</sub> incubator (Thermo Fisher Scientific, Forma Series II)
7. Vortex (Fisher Scientific, Vortex Genie 2, catalog number: 12-812)
8. Fluorescence inverted microscope (Nikon, Eclipse TE2000-U)
9. Fura2 Shemrock BrightLine Filter set (FURA2-A-000)
10. Oil immersion UV-corrected 40x lens (Nikon Plan Fluor 40x/1.30 Oil Objective)
11. UV Lamp excitation lamp (Sutter Ozone free 175 Watt xenon bulb Model # 0661176)
12. High speed wavelength switcher (Lambda DG-4, Quorum Technologies)

## Software

1. Excel (Microsoft)
2. Volocity (Improvision)
3. *Optional:* ImageJ (NIH)

*Note.* ImageJ is freely accessible image analysis software that can be used as alternative to Volocity to define regions of interest (ROI) and extract data from image stacks. See Data Analysis section.

## Procedure

### A. Fura2 loading

1. 2-3 days prior to experiment, plate cells in uncoated 35 mm glass-bottom dish (or uncoated 48 mm glass-bottom culture plates) and allow cells to grow to sub-confluence in CO<sub>2</sub> incubator (5% CO<sub>2</sub>, 37 °C).
2. On the day of the experiment, aspirate medium and add 1 ml Fura2 staining solution (solution is at room temperature when added). Incubate cells at room temperature for 30 min in the dark.
3. Aspirate Fura2-staining solution, and wash cells twice with physiological solution. Add imaging solution with luciferase (990 µl/35 ml dish or 297 µl/well in 48-well plate) and allow cells to acclimatize for 10 min at room temperature on the bench (in light-limiting conditions) prior to

imaging.

#### B. Instrument preparation

1. Turn on the UV lamp to warm up 10-20 min prior to imaging.
2. When ready to image, add drop of oil (Carl Zeiss™ Immersol™ Immersion Oil, Fisher Scientific, catalog number: 12-070-397) onto 40x objective lens and position glass bottom dish/plate on top of lens. Bring cells into focus and ensure cell and cell-free regions are visible within the field-of-view.
3. Open imaging software (e.g., Volocity) and enter the following imaging parameters:

**Sampling rate:** 2 timepoints per second

**Exposure time:** 50-200 ms

*Note: Find minimal exposure time required to obtain low-noise images. Longer exposures may result in photobleaching effects.*

**Excitation/emission:** 380 nm/510 nm

*Optional: If automated shutter is available (e.g., Lambda DG-4), close shutter between exposures to minimize photobleaching effects.*

#### C. Imaging

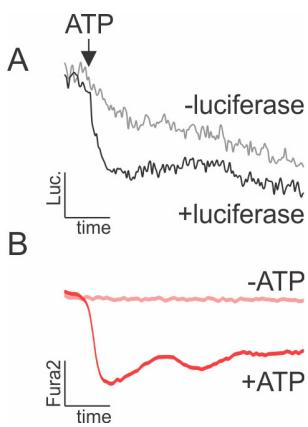
1. For all imaging trials, record 10-15 s of baseline, apply stimulation and record for additional 100-120 s. Stimulation can be mechanical, for example using a glass micropipette as described in our prior work (Mikolajewicz *et al.*, 2018b), or can be a biochemical stimulus carefully added at 1% volume at 100x of the desired final concentration.

*Note: For representative fluorescent images of Fura2-loaded cells bathed in luciferin imaging solution, refer to our prior work (Mikolajewicz *et al.*, 2018b). Also see Sorensen and Novak, (2001) for images of cells bathed in luciferin-imaging solution.*

2. To control for effects of drugs/solutions on D-luciferin signal, acquire recordings of cell-free wells containing luciferase-free imaging solution following stimulation with vehicle alone versus vehicle + drugs.
3. To control for effects of drugs/solutions on luciferase activity, acquire recordings of cell-free wells containing luciferase-supplemented imaging solution following stimulation with ATP alone versus ATP + drugs.
4. To control for effects of mechanical agitation resulting from the application of drugs, add vehicle (e.g., physiological solution) to wells containing Fura2-loaded cells bathed in luciferase-supplemented imaging solution.
5. To establish ATP calibration curve for luciferin recordings, carefully add 1% volume of 100x ATP solution (e.g., 3 µl of 100 µM ATP added to 297 µl imaging solution in 48-well plate to achieve 1 µM ATP stimulation) into the well containing imaging solution, in presence and absence of luciferase (positive and negative controls shown in Figure 2).

**Notes:**

- a. Triplicate wells are recommended for each concentration.
  - b. Concentrations ranging from 0.1-1,000  $\mu$ M ATP are recommended.
  - c. Calibration curve can be established in the absence or presence of fura2-loaded cells. The latter is recommended to better mimic experimental conditions and control for the influence of any cell-derived factors that may influence luciferase activity. Measuring Fura2-responses in the same wells also provides additional information about the range of ATP concentrations that Fura2-loaded cells will respond to.
6. The application protocols should be the same for experimental recordings, controls and calibration.



**Figure 2. Effect of ATP on D-luciferin and Fura2 fluorescence (ex 380/em 510).** A. Fluorescence of luciferin in the presence and absence of luciferase following addition of 1  $\mu$ M ATP. B. Fluorescence of Fura2-loaded cells stimulated by 1  $\mu$ M ATP (+ATP) or vehicle (-ATP).

## Data analysis

### A. Data extraction

1. For each recorded image stack, define intracellular and extracellular regions of interest (ROI) manually and extract time-series data into a spreadsheet.

#### Notes:

- a. Intracellular ROIs (Fura2) are used for monitoring intracellular calcium responses and are manually selected to include the entire area of the cell.
- b. Extracellular ROIs (luciferin) are used to measure extracellular ATP concentrations and are selected ~15  $\mu$ m from the cell's edge. Smaller ROIs provide greater spatial resolution but are typically noisier. We recommend ROI selections of ~15 $^2$   $\mu$ m $^2$ . Avoid selecting extracellular ROIs immediately adjacent to the cell surface as these areas may exhibit "shadows" due to non-linear optics.
- c. To extract data in Velocity, draw an ROI using ROI tools and select multiple ROIs while holding 'shift' key. To extract tabular data from selections, select 'Make Measurement Item'

from ‘Measurements’ menu, specify ‘All timepoints’ option and press ‘OK’. A new measurement item will appear in the Volocity library which can then be exported to in a tab-delimited format using ‘Export’ in the ‘file’ tab.

- d. *Optional: ImageJ is a freely accessible image analysis software which can be used for data extraction (as an alternative to Volocity). Online resources are available detailing how to select ROIs and extract data from image stacks in ImageJ.*
2. Import luciferin and Fura2 data into separate Excel spreadsheets.

## B. ATP analysis

1. To estimate extracellular ATP concentrations, determine the ATP calibration curve by calculating the absolute percentage change in average luciferin fluorescence  $L_i^j$  pre and post stimulation:

$$\Delta_{luc}(\%) = \left| \frac{L_{post}^{(+luc)}}{L_{pre}^{(+luc)}} - \frac{L_{post}^{(-luc)}}{L_{pre}^{(-luc)}} \right| \times 100\%$$

where  $i$  specifies pre- or post-ATP application and  $j$  indicates the presence (+luc) or absence (-luc) of luciferase in the imaging solution.

### Notes:

- a.  $L_{post}^{(-luc)} / L_{pre}^{(-luc)}$  is the negative control.
  - b.  $L_{pre}^j$  is the baseline signal (prior to stimulation) which can be either (1) the average signal intensity in the first 10 s of the recording or (2) the maximal intensity observed prior to stimulation.
  - c.  $L_{post}^j$  is the post-stimulation signal which is typically taken as the average intensity 10-20 s post-stimulation.
2. Plot  $\Delta_{luc}$  as a function of ATP concentration ( $\log_{10}$  scale) and obtain equation for the line of best fit.
- Note: We used an exponential curve ( $\Delta_{luc} = ae^{b \cdot \log_{10}([ATP])}$ ) to fit the relationship between ATP concentration [ATP] (Molar) and  $\Delta_{luc}(\%)$ . Parameter estimates:  $a = 60$  and  $b = 0.55$ , with a goodness of fit  $R^2 = 0.998$ .*
3. Use  $\Delta_{luc}(\%)$  from experimental conditions and relate to extracellular ATP concentration using calibration curve (Figure 3).

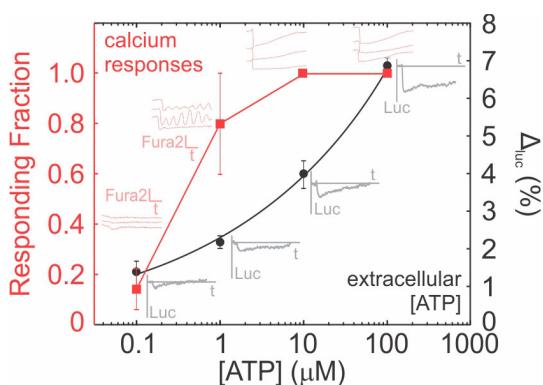
## C. Calcium analysis

1. For Fura2 recordings, quantitative analysis is limited since only one excitation wavelength is recorded, however the presence or absence of a calcium response can be discerned and related to local ATP concentrations.

*Note: If low extracellular ATP concentrations are expected (< 0.1  $\mu$ M), a dose-dependency*

curve for Fura2 is recommended to determine the lowest ATP concentration that can stimulate a discernable response (Figure 3—red curve).

2. Plot Fura2 recordings as a function of time and determine (1) the number of responding cells, i.e., those that exhibit a  $> 4$  standard deviation (STD) decline in Fura2 signal from baseline (Lopez-Ayon et al., 2014) and (2) the total number of cells imaged in the field of view.
3. Calculate the responding fraction of cells as  $\text{Responding fraction} = \frac{\text{responding cells}}{\text{total cells}}$  (Figure 3).



**Figure 3. ATP dose-response curves for Fura2 and D-luciferin.** Representative traces for intracellular Fura2 emission in individual Fura2-loaded cells (*light red traces*) and changes in extracellular luciferin (*gray traces*) are shown. Luc: luciferin emission (380 em/510 ex), Fura2: Fura2 emission (380 em/510 ex), t: time,  $\Delta\text{Luc}$ : Percentage change in luciferin emission. Step-by-step analysis of the raw data used to generate this figure is provided as separate excel spreadsheets ([fura2 analysis.xlsx](#) and [luciferin analysis.xlsx](#)).

## Notes

1. If alternative imaging solution is used, ensure it is phenol red-free to avoid interference.
2. The protocol described here details how to image extracellular ATP and intracellular calcium simultaneously, however each can be done independently with appropriate amendments to the protocol.
3. Due to calcium-chelating properties of Fura2, Fura2-loaded cells will begin to lose viability over time. It is recommended that all cell imaging be completed within 30-60 min after Fura2-loading.
4. Ensure any treatments used in imaging experiments are assessed to determine whether they interfere with Fura2 or luciferin signal.
5. It may be necessary to adjust luciferin and luciferase concentrations for optimal results. Sorensen and Novak (2001) obtained satisfactory results using up to 1.67 mM D-luciferin and 25-188 μg/mL luciferase in a bicarbonate-free Ringer solution instead of the physiological solution described here (Sorensen and Novak, 2001).
6. A limitation of the current protocol is difficulty in achieving an instantaneously homogenous

solution upon addition of drugs. The simplest solution is to plan for a larger sample size to negate noise that arises from inhomogeneous addition of solutions. Alternatively, a perfusion system may be adopted to deliver drugs, however, changes in ATP concentrations may be more difficult to detect due to the washout effect of the perfusion system. Nonetheless, in certain studies, such as those examining the effects of fluid flow on vascular calcium responses and ATP release, this may be a feasible alternative.

## **Recipes**

1. Fura2 stock solution, 1,000x  
1 µg/µl Fura2-AM in DMSO  
Store at -20 °C, protect from light
2. Luciferin stock solution, 5,000x  
30 mM D-luciferin in physiological solution  
Store at -80 °C, protect from light
3. Luciferase stock solution, 500x  
1 mg/ml firefly luciferase in physiological solution  
Store at -80 °C, protect from light
4. Physiological solution (PS), pH 7.4  
130 mM NaCl  
5 mM KCl  
1 mM MgCl<sub>2</sub>  
1 mM CaCl<sub>2</sub>  
10 mM glucose  
20 mM HEPES  
Sterilized by 0.2 µm vacuum filtration  
Store at 4 °C
5. Fura2-staining solution, 1x  
Fura2 stock solution (1:1,000 dilution)  
Physiological solution  
Prepare fresh, protect from light
6. Luciferin imaging solution with luciferase  
2 µl 30 mM D-luciferin stock solution (1:5,000 dilution)  
40 µl of 1 mg/ml firefly luciferase stock solution (1:250 dilution)  
10 ml physiological solution  
Prepare fresh, protect from light
7. Luciferin imaging solution without luciferase  
2 µl 30 mM D-luciferin stock solution (1:5,000 dilution)  
10 ml physiological solution

- Prepare fresh, protect from light
8. ATP solutions  
10  $\mu$ M-100 mM ATP in luciferin imaging solution without luciferase (1x)  
Prepare fresh, protect from light
- Notes:*
- For ATP calibration curve, prepare 10 mM ATP solution and serially dilute in luciferin imaging solution to obtain lower concentrations (i.e., 1 in 10 dilution to obtain 1 mM ATP, etc.).*
  - ATP solutions often contain ADP (Mikolajewicz et al., 2019); we advise to prepare homogenous ATP stock solution by enzymatically converting contaminating ADP to ATP. This is accomplished by adding 5  $\mu$ l 20 U/ml pyruvate kinase and 5  $\mu$ l 100  $\mu$ M phosphoenolpyruvate (PEP) into ATP stock solution, incubating for 30 min at 37 °C, then heat-inactivating for 5 min at 95 °C.*
9. Phosphate-buffered saline (PBS), pH 7.4  
140 mM NaCl  
3 mM KCl  
10 mM Na<sub>2</sub>HPO<sub>4</sub>  
2 mM KH<sub>2</sub>PO<sub>4</sub>  
Sterilize by autoclaving, store at room temperature

### **Acknowledgments**

This work was supported by Natural Sciences and Engineering Research Council (NSERC, RGPIN-288253) and Canadian Institutes for Health Research (CIHR MOP-77643). NM was supported by the Faculty of Dentistry, McGill University and le Réseau de Recherche en Santé Buccodentaire et Osseuse (RSBO).

### **Competing interests**

The research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

### **References**

- Burnstock, G. and Verkhratsky, A. (2009). [Evolutionary origins of the purinergic signalling system](#). *Acta Physiol (Oxf)* 195(4): 415-447.
- Goda, K., Hatta-Ohashi, Y., Akiyoshi, R., Sugiyama, T., Sakai, I., Takahashi, T. and Suzuki, H. (2015). [Combining fluorescence and bioluminescence microscopy](#). *Microsc Res Tech* 78(8): 715-722.

3. Grynkiewicz, G., Poenie, M. and Tsien, R. Y. (1985). [A new generation of  \$\text{Ca}^{2+}\$  indicators with greatly improved fluorescence properties.](#) *J Biol Chem* 260(6): 3440-3450.
4. Lopez-Ayon, G. M., Liu, H. Y., Xing, S., Maria, O. M., LeDue, J. M., Bourque, H., Grutter, P. and Komarova, S. V. (2014). [Local membrane deformation and micro-injury lead to qualitatively different responses in osteoblasts.](#) *F1000Res* 3: 162.
5. Mikolajewicz, N., Mohammed, A., Morris, M. and Komarova, S. V. (2018a). [Mechanically stimulated ATP release from mammalian cells: systematic review and meta-analysis.](#) *J Cell Sci* 131(22).
6. Mikolajewicz, N., Zimmermann, E. A., Willie, B. M. and Komarova, S. V. (2018b). [Mechanically stimulated ATP release from murine bone cells is regulated by a balance of injury and repair.](#) *Elife* 7: e37812.
7. Mikolajewicz, N., Sehayek, S., Wiseman, P. W. and Komarova, S. V. (2019). [Transmission of mechanical information by purinergic signaling.](#) *Biophys J* 116(10): 9654.
8. Paredes, R. M., Etzler, J. C., Watts, L. T., Zheng, W. and Lechleiter, J. D. (2008). [Chemical calcium indicators.](#) *Methods* 46(3): 143-151.
9. Seminario-Vidal, L., Lazarowski, E. R., and Okada, S. F. (2009). [Assessment of extracellular ATP concentrations.](#) *Methods Mol Biol* 574: 25-36.
10. Sorensen, C. E. and Novak, I. (2001). [Visualization of ATP release in pancreatic acini in response to cholinergic stimulus. Use of fluorescent probes and confocal microscopy.](#) *J Biol Chem* 276(35): 32925-32932.

## Hypochlorous Acid Staining with R19-S in the *Drosophila* Intestine upon Ingestion of Opportunistic Bacteria

Salma Hachfi<sup>1, 2</sup>, Olivia Benguettat<sup>1, 2</sup> and Armel Gallet<sup>1, 2, \*</sup>

<sup>1</sup>Université Côte d'Azur, CNRS, INRA, ISA, France; <sup>2</sup>Institut Sophia Agrobiotech, UMR CNRS 7254/INRA 1355/UNS, 400 route des Chappes, BP 167, 06903 Sophia Antipolis Cedex, France

\*For correspondence: [gallet@unice.fr](mailto:gallet@unice.fr)



**[Abstract]** The intestine is endowed with an innate immune system that is required to fight any exogenous bacteria that are swallowed along with the food. The first line of defense that is mounted by the gut epithelium is the release of immune Reactive Oxygen Species (ROS), such as hypochlorous acid (HOCl), into the lumen. HOCl is produced within 1.5 h of bacterial ingestion and is very labile once released. Therefore, to monitor HOCl production upon ingestion of allochthonous bacteria, one needs a detection system that can quickly and efficiently detect HOCl production in the intestine. While most of the ROS-sensitive probes available in the market detect all kinds of ROS without any distinction, the R19-S fluorescent probe has been developed to specifically detect HOCl. Here, we describe a protocol to monitor HOCl production using this probe in the gut lumen of adult *Drosophila* upon ingestion of the opportunistic bacteria *Bacillus thuringiensis*.

**Keywords:** Intestine, Innate immune system, Reactive Oxygen Species, HOCl, Opportunistic bacteria, *Drosophila melanogaster*

**[Background]** The evolutionarily conserved innate immune system is the first line of defense against pathogenic bacteria. In the gut, the local innate immune system first produces Reactive Oxygen Species (ROS) to damage bacterial wall and slow down the proliferation of ingested bacteria (Kim and Lee, 2014). Then, the visceral mesoderm triggers spasmodic contractions to favor bacterial eviction (Benguettat *et al.*, 2018). The third line of defense is the production of antimicrobial peptides that kill the bacteria (Xia *et al.*, 2017). Finally, the gut is able to regenerate damaged epithelial cells to maintain gut integrity, avoiding bacterial entry into the internal milieu (Bonfini *et al.*, 2016). In *Drosophila*, the immune ROS are produced by the enterocytes of the anterior midgut in a DUOX-dependent manner between 0.5 and 1.5 h after bacterial ingestion. The main immune ROS to be released in the gut lumen is hypochlorous acid (HOCl) (Lee *et al.*, 2013; Benguettat *et al.*, 2018). Therefore, HOCl monitoring is a good proxy for the gut innate immune response to a microbe. However, HOCl is a very labile compound that quickly disappears once it is released into the lumen. Here, we describe a protocol that we designed to efficiently detect HOCl production in the gut lumen of adult *Drosophila* upon ingestion of low doses of the Gram-positive *Bacillus thuringiensis kurstaki* (*Btk*) opportunistic bacteria. This protocol can equally be used to detect HOCl upon ingestion of any other pathogens and it also can be used to measure HOCl production in the gut of *Drosophila* larvae.

## **Materials and Reagents**

### A. *Drosophila* rearing

1. 6 oz *Drosophila* stock bottles (Genesee Scientific, catalog number: 32-130)
2. Cotton balls for stock bottles (Genesee Scientific, catalog number: 51-102B)
3. CantonS flies (Bloomington *Drosophila* Stock Center, catalog number: 64349) ([flystocks.bio.indiana.edu](http://flystocks.bio.indiana.edu))
4. Agar (VWR, BDH®, catalog number: 20768-361)
5. Sugar (Carrefour or any other supermarket)
6. Cornflour (AB, Celnat-NaturDis)
7. Yeast (Biospringer, catalog number: BA10/0-PW)
8. Tegosept (Apex, Fly Food preservative, Genesee Scientific, catalog number: 20-258)
9. Standard nutrient medium for *Drosophila* (see Recipes)

### B. Bacterial culture

1. Petri dishes (Thermo Scientific-Nunc™, catalog number: 263991)
2. Sterile tips (Sigma Aldrich, catalog number: CLS4138)
3. 15 ml tubes (Corning, Falcon®, catalog number: 352096)
4. Graduated cylinder (Azlon, catalog number: 11940985)
5. *Bacillus thuringiensis* var. *kurstaki* (*Btk*) strain identified under the code 4D22 at the Bacillus Genetic Stock Center (<http://www.bpsc.org/>) and described by Gonzalez *et al.*, 1982
6. Luria broth powder (Conda, catalog number: 1551)
7. Agar bacteriological (Euromedex, catalog number: 1330)
8. LB medium (see Recipes)
9. LB-agar medium (see Recipes)

### C. Inoculation

1. Cotton balls for 25 mm narrow vials (Genesee Scientific, catalog number: 51-101)
2. Spectrophotometry cuvettes (Ratiolab, catalog number: 2712120)
3. 2 ml microtubes (Paul Boettger, catalog number: 02-043)
4. 20 mm filter disks (3 MM Chr chromatography paper; GE Healthcare, catalog number: 3030-917)
5. 50 ml tubes (Fisher Scientific, catalog number: 10788561)
6. 25 mm narrow *Drosophila* vials (Genesee Scientific, catalog number: 32-109RL)
7. Sucrose (Euromedex, catalog number: 200-301-B)
8. 5% sucrose (see Recipes)
9. 1 mM R19-S (Futurechem, FC-8001, Seoul, South Korea) (see Recipes)

**D. Dissection**

1. 10x PBS (Euromedex, catalog number: ET330)
2. 1x phosphate-buffered saline (PBS) (see Recipes)

**E. Assay**

1. 1.5 ml microtubes (Paul Bottger, catalog number: 02-063)
2. 12-well black microplates (VWR international, catalog number: 734-2324)
3. Aluminum foil (available at any supermarket)
4. Formaldehyde 16% (Thermo Scientific, catalog number: 28908)
5. Vectashield/DAPI (Sigma, catalog number: F6057)
6. 4% Formaldehyde (see Recipes)

**Equipment****A. *Drosophila* rearing**

Refrigerated oven at a constant temperature of 25 °C and with a 12 h/12 h light/dark cycle (Fisher Scientific, catalog number: 11857552). Humidity must be maintained between 40% and 70%.

**B. Bacterial culture**

1. 500 ml sterile flask
2. 30 °C shaking incubator (Infors, model: AK 82)

**C. Inoculation**

1. Spectrophotometer (Aqualabo, Secomam, model: Prim Light & Advanced)
2. CO<sub>2</sub>-anesthesia system (INJECT+MATIC sleeper)

**D. Dissection**

1. Dumont forceps #5 (Fine Science Tools, catalog numbers: 11251-20 and 11252-20)
2. Watch glass (Steriplan Petri dishes, DWK Life Sciences, catalog number: 237554008)
3. Stereomicroscope (Leica Microsystems, model: Leica M60)

**E. Assay**

Fluorescent microscope (Zeiss Axio Imager Z1 Apotome) equipped with a camera (AxioCam MRm) and a Rhodamine 43 fluorescent filter

**Software**

1. ZEN 2012 (Zeiss)
2. ImageJ (<https://imagej.nih.gov/ij/>)

3. Kyplot (<http://kyenslab.com/en/index.html>)
4. Excel (Microsoft)

## **Procedure**

### A. *Drosophila* rearing

1. Canton S flies (Bloomington) are reared on standard medium for *Drosophila melanogaster* (see Recipes) at 25 °C.
2. To obtain synchronized mated females, remove adult flies from rearing bottles and wait for the emergence of new flies for one day. Then, transfer the newly emerged flies (males and females) to new bottles for five more days at 25 °C before inoculation. In our experiments, five to six-day-old mated females were used.

*Note: If you want to work only on virgin females, once new flies have hatched, remove the males immediately and place the virgin females in a separated vial until they reach the correct age.*

### B. Bacterial culture

In the experiments presented below, flies were inoculated with the *Btk* bacterial strain (see Materials and Reagents). A solution of 5% sucrose serves as the negative control (Ctrl).

1. Spread bacteria from the stock onto LB agar Petri dishes and grow overnight at 30 °C.
2. Pick a single colony using a sterile tip and inoculate 250 ml of LB in a 500 ml sterile flask, and incubate overnight at 30 °C with shaking at 180 rpm.

*Note: The growth of *Btk* is very efficient. There is no need for a starter culture.*

### C. Inoculation

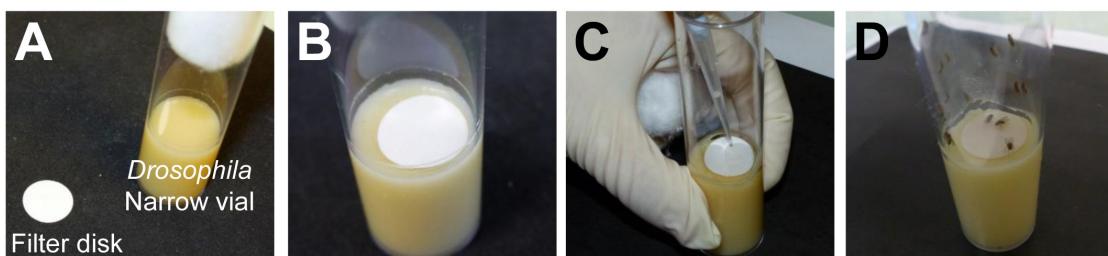
1. On the day of infection, separate 5 to 6-day-old females from males and put into a vial without medium for a 2 h starvation period at 25 °C. This ensures the synchronization of food intake when they are introduced to Medium with bacteria.
2. During this time, measure the optic density (OD) of the overnight bacterial culture. If necessary, dilute the overnight culture to get an OD between 0.2 and 0.8.
3. Preparation of the bacterial solution: the required OD to inoculate flies with 10<sup>8</sup> Colony Forming Unit (CFU) of *Btk* per *Drosophila* is presented in Table 1.

**Table 1. Bacterial solution**

Bacterium	Volume to place on the media	Required OD	CFU/vial (for 10 flies/vial)	CFU/fly
<i>Btk</i>	25 µl	400	1 × 10 <sup>9</sup>	1 × 10 <sup>8</sup>

*Notes:*

- a. The OD of the overnight culture will be obviously too low and you must concentrate it. Pellet the bacterial culture by spinning at 2,500 x g for 15 min at room temperature. Resuspend the pellet with the required volume of 5% sucrose to get the correct CFU according to the formula  $fOD \times fV = iOD \times iV$  ( $fOD$  = final OD;  $iOD$  = initial OD,  $fV$  = final Volume;  $iV$  = initial Volume). For example, to obtain an OD 400 ( $fOD$ ) of *Btk* bacteria from an overnight culture at OD 2 ( $iOD$ ), spin 25 ml of the bacterial culture ( $iV$ ) and resuspend the pellet in a volume of 5% sucrose that is 200x less than the initial volume of LB, and thus  $fV = 125 \mu\text{l}$ .
- b. One can inoculate flies with any other bacteria. However, depending on the type of bacteria, the OD that is required to yield the desired CFU will be probably different.
4. Dilute the 1 mM stock solution of R19-S (see Recipes) to 100  $\mu\text{M}$  in sucrose 5%. Keep the dilution in the dark.
5. Dilute the bacterial solution (Table 1) at a 1:1 ratio with the 100  $\mu\text{M}$  R19-S probe solution. This mixture will constitute the inoculation solution.
- a. The **negative control batch** is prepared by combining 25  $\mu\text{l}$  of the 100  $\mu\text{M}$  R19-S solution and 25  $\mu\text{l}$  of 5% sucrose.
  - b. The ***Btk* batch** is prepared by combining 25  $\mu\text{l}$  of 100  $\mu\text{M}$  R19-S and 25  $\mu\text{l}$  of the *Btk* solution (OD<sub>400</sub>). The concentration of bacteria used is  $1 \times 10^8$  CFU/5 cm<sup>2</sup>/fly.
- Note: Preparation of the R19-S mixture must be done under dark conditions.*
6. Place a 20 mm filter disk on the top of the medium inside the *Drosophila* narrow vials (Figures 1A and 1B).
7. Deposit the inoculation solution on the filter disk (Figure 1C).
8. Transfer 10 starved flies into one *Drosophila* narrow vial and allow the *Drosophila* to feed for 30 min on the inoculation solution (Figure 1D).
- Note: For the 30 min time point, skip Step C9 below and dissect flies as described in Procedure D below.*
9. Remove flies from vials and put them into fresh vials with filter disks only soaked with 25  $\mu\text{l}$  of 100  $\mu\text{M}$  R19-S solution + 25  $\mu\text{l}$  of 5% sucrose. Let the flies feed until the time of dissection (1 h and 1.5 h).



**Figure 1. Inoculation procedure.** A. Cut filter disks with a diameter of 20 mm. B. Place on the top of the medium within the vial. C. Deposit the inoculation solution on the filter disk. D. Introduce the flies.

#### D. Dissection (Video 1)

At the desired time points: 0.5 h, 1 h and 1.5 h start dissection.

1. Before starting, the forceps and dissecting watch glasses have to be rinsed with a 70% ethanol solution.
2. Ten females per condition are anesthetized with CO<sub>2</sub> using the *Drosophila*-Sleeper.
3. Place one fly in a watch glass pre-filled with 1 ml 1x PBS (see Recipes).
4. Using forceps, pull the head away. Hold the fly gently and make an incision in the abdomen at the thorax/abdomen boundary. Gently separate the abdomen from the thorax. You should see the intestine still attached to both the thorax and the abdomen.
5. Then, the intestine is carefully stretched.
6. Cut the gut at the boundaries between foregut and hindgut. Remove the Malpighian tubules and/or the ovaries if still attached to the midgut at the midgut/hindgut boundary.

*Note: If possible, dissection must be carried out in less than 30 s/intestine to avoid fading of the R19-S fluorescence.*



**Video 1. Dissection procedure**

#### E. Sample preparation and image capture

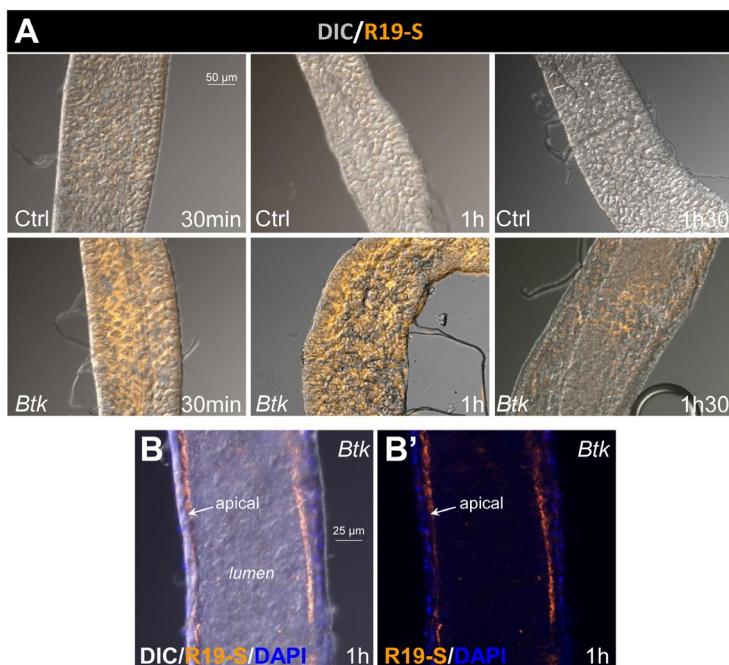
1. Fix *Drosophila* guts in 500 µl of 4% formaldehyde in PBS (see Recipes) in 12-well plates at room temperature without agitation for 50 min in darkness.

*Note: Cover the plate with aluminum foil to keep intestine samples in the dark.*

2. Rinse twice with PBS (2 x 5 min).
3. For each experiment, mount guts in Vectashield/DAPI and immediately observe the samples using a fluorescence microscope (Zeiss Axioplan Z1 with Apotome 2 microscope). R19-S fluorescence is visible within the "Rhodamine" emission range (excitation at 515 nm). A

differential interference contrast (DIC) image is also taken to merge with the fluorescence image.

- Take the pictures in the anterior part of midguts because HOCl is produced there. Below, in Figure 2, we present results for two conditions ( $H_2O$  and *Btk*). For each condition, we performed 3 (at 30 min and 1 h) or 4 (at 1.5 h) independent experiments.



**Figure 2. Image capture.** A. Apotome image capture with the Rhodamine filter (orange) to visualize R19-S fluorescence and with the differential interference contrast (DIC) channel to outline intestine contours. Images were captured in the anterior part of the midgut. Ctrl: 5% sucrose negative control batch. *Btk*: *Btk* batch. B-B. Cross section of anterior midgut at the level of the lumen. R19-S fluorescence (orange) is localized at the apical surface of the epithelium facing the lumen. Blue (DAPI) marks the epithelial nuclei. B incorporates the DIC image for orientation, whereas B' does not.

### Data analysis

- For each condition, analyze at least 3 independent experiments that have been performed on different days.
- At least 20 intestines per condition have to be analyzable when you combine the three independent experiments. If this is not the case, you must carry out a supplementary experiment to reach the threshold of 20 intestines.
- The percentage of R19-S-positive intestines is calculated for each experiment independently. An intestine is considered positive for R19-S labeling when at least 10% of the anterior midgut

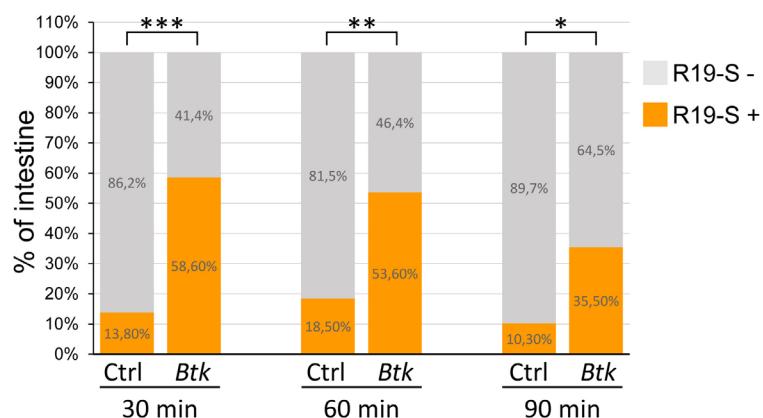
displays a fluorescent labeling outlining the enterocyte membranes. Scattered cells are not taken into account.

*Note: For each experiment, the number of R19-S-positive intestines under control conditions (fed with 5% sucrose) must not exceed 25% of the intestines. This would indicate that the flies underwent an unexpected stress before or during the experiments. Therefore, if more than 25% of the intestines are R19-S positive in the control experiment, discard all the experiments (control and bacteria-infected batch).*

4. Pool the 3 (or more) independent experiments for each condition and compare as presented in the Excel figure below (Figure 3).  
5. Statistical analysis

If  $n < 30$  intestines, perform a non-parametric Kruskal and Wallis test using Kplot software.

If  $n \geq 30$  intestines, perform a parametric *t*-test using Excel or Kplot software.



**Figure 3. Percentage of R19-S positive midgut.** R19-S-positive midguts are represented in orange and R19-S-negative midgut in grey. Data were acquired 30, 60 and 90 min post-inoculation. Ctrl: Negative control batch. Btk: Btk batch. Since  $27 < n < 31$ , we used a non-parametric Kruskal and Wallis test.

## Recipes

1. Standard nutrient medium for *Drosophila melanogaster*

*Note: All the reagents are prepared with distilled water.*

8 g/L agar

25 g/L sugar

80 g/L cornflour

20 g/L yeast

6 g/L tegosept (stock solution at 100 g/L in 95% ethanol. Store at 4 °C)

2. LB medium

a. Weigh out 25 g of Luria broth medium powder

b. Adjust to 1 L with distilled water in a graduated cylinder

- c. Adjust the pH to 7.2 if necessary
  - d. Autoclave
  3. LB-agar medium
    - a. Weigh out 25 g of Luria broth medium powder
    - b. Adjust to 1 L with distilled water in a graduated cylinder
    - c. Adjust the pH to 7.2 if necessary
    - d. Add 15 g agar powder
    - e. Autoclave
  4. 5% sucrose
    - a. Weigh out 2.5 g of sucrose in a 50 ml tube
    - b. Add 50 ml sterile distilled water and vortex
- Note: Make the solution just before use.*
5. 1x phosphate-buffered saline (PBS)
    - a. Add 100 ml of 10x PBS solution to 900 ml of distilled water in a graduated cylinder
    - b. Autoclave
  6. 4% formaldehyde  
Add 750 µl of 1x PBS solution to 250 µl of 16% formaldehyde
  7. 1 mM R19-S
    - a. Prepare the stock solution at 1mM from the commercial powder in 100% acetonitrile
    - b. Store at 4 °C in the dark. Make aliquots if necessary

### **Acknowledgments**

SH has been supported by the French government, through the UCA<sup>JEDI</sup> Investments in the Future project managed by the National Research Agency (ANR; reference number ANR-15-IDEX-01). OB was supported by INRA and AG by the CNRS. The procedure presented here was adapted from that of Lee and colleagues from the Won-Jae Lee Lab.

### **Competing interests**

The authors have no conflicts of interest or competing interests.

### **References**

1. Benguettat, O., Jneid, R., Soltys, J., Loudhaief, R., Brun-Barale, A., Osman, D. and Gallet, A. (2018). [The DH31/CGRP enteroendocrine peptide triggers intestinal contractions favoring the elimination of opportunistic bacteria.](#) *PLoS Pathog* 14(9): e1007279.
2. Bonfini, A., Liu, X. and Buchon, N. (2016). [From pathogens to microbiota: How \*Drosophila\* intestinal stem cells react to gut microbes.](#) S0145-0305X(0116)30032-30035.

3. Gonzalez, J. M., Jr., Brown, B. J. and Carlton, B. C. (1982). [Transfer of \*Bacillus thuringiensis\* plasmids coding for delta-endotoxin among strains of \*B. thuringiensis\* and \*B. cereus\*.](#) *Proc Natl Acad Sci U S A* 79(22): 6951-6955.
4. Kim, S. H. and Lee, W. J. (2014). [Role of DUOX in gut inflammation: lessons from \*Drosophila\* model of gut-microbiota interactions.](#) *Front Infect Microbiol* 3: 116.
5. Lee, K. A., Kim, S. H., Kim, E. K., Ha, E. M., You, H., Kim, B., Kim, M. J., Kwon, Y., Ryu, J. H. and Lee, W. J. (2013). [Bacterial-derived uracil as a modulator of mucosal immunity and gut-microbe homeostasis in \*Drosophila\*.](#) *Cell* 153(4): 797-811.
6. Xia, X., Cheng, L., Zhang, S., Wang, L. and Hu, J. (2017). [The role of natural antimicrobial peptides during infection and chronic inflammation.](#) *Antonie Van Leeuwenhoek* 30(10): 017-0929.

## Lipid-exchange Rate Assay for Lipid Droplet Fusion in Live Cells

Jia Wang<sup>1</sup>, Boon Tin Chua<sup>2</sup>, Peng Li<sup>1</sup> and Feng-Jung Chen<sup>2, 3, 4, \*</sup>

<sup>1</sup>State Key Laboratory of Membrane Biology and Tsinghua-Peking Center for Life Sciences, Beijing Advanced Innovation Center for Structural Biology, School of Life Sciences, Tsinghua University, Beijing 100084, China; <sup>2</sup>Institute of Metabolism and Integrative Biology, Fudan University, Shanghai 200438, China; <sup>3</sup>Human Phenome Institute, Fudan University, Shanghai 201203, China; <sup>4</sup>Zhongshan Hospital, Fudan University, Shanghai, 200032, China

\*For correspondence: [derrick\\_chen@fudan.edu.cn](mailto:derrick_chen@fudan.edu.cn)



**[Abstract]** Lipid droplets (LDs) are central organelles in maintaining lipid homeostasis. Defective LD growth often results in the development of metabolic disorders. LD fusion and growth mediated by cell death-inducing DNA fragmentation factor alpha (DFFA)-like effector (CIDE) family proteins are crucial for various biological processes including unilocular LD formation in the adipocytes, lipid storage in the liver, milk lipid secretion in the mammary epithelia cells, and lipid secretion in the skin sebocytes. Previous methodology by Gong *et al.* (2011) first reported a lipid-exchange rate assay to evaluate the fusion ability of each LD pair in the cells mediated by CIDE family proteins and their regulators, but photobleaching issue remains a problem and a detailed procedure was not provided. Here, we provide an improved and detailed protocol for the lipid-exchange rate measurement. The three key steps for this assay are cell preparation, image acquisition, and data analysis. The images of the fluorescence recovery are acquired after photobleaching followed by the measurement of the intensity changes in the LD pair. The difference in fluorescent intensity is used to obtain the lipid exchange rate between the LDs. The accuracy and repetitiveness of the calculated exchange rates are assured with three-cycle of photobleaching process and the linear criteria in data fitting. With this quantitative assay, we are able to identify the functional roles of the key proteins and the effects of their mutants on LD fusion.

**Keywords:** Lipid metabolism, Lipid droplet fusion, Lipid-exchange rate, Fluorescence recovery after photobleaching, Lipid droplet size

**[Background]** Lipid droplets (LDs) play a key role in maintaining lipid homeostasis (Farese and Walther, 2009; Yang *et al.*, 2012). Defective LD maturation and growth are closely associated with the development of metabolic diseases such as obesity, fatty liver disease, cardiovascular disease, and type II diabetes (Krahmer *et al.*, 2013; Rosen and Spiegelman, 2014; Gluchowski *et al.*, 2017). LDs are dynamic organelles which budded from the endoplasmic reticulum (ER) (Gross *et al.*, 2011; Choudhary *et al.*, 2015) and continue to grow via triglyceride synthesis and lipid transfer from the ER (Fujimoto *et al.*, 2007; Wilfling *et al.*, 2013; Xu *et al.*, 2018) or LD fusion (Gong *et al.*, 2011). LD-associated cell death-inducing DNA fragmentation factor alpha-like effector (CIDE) family proteins including CIDEA, CIDEB, and CIDEC/Fsp27 (Gao *et al.*, 2017) are crucial regulators in the lipid homeostasis by governing atypical LD fusion and growth for lipid storage. Previously, we reported that CIDEC mediates

LD fusion through directional lipid transfer from small (donor) to large (acceptor) LDs (Gong *et al.*, 2011). The enrichment of CIDE family proteins at the LD-LD contact site (LDCS) and the formation of the fusion pore are two essential steps for lipid exchange and transfer to occur. To evaluate the fusion ability of each LD pair in cells mediated by the CIDE family proteins and their regulators such as Perilipin1 (Sun *et al.*, 2013a) or Rab8a (Wu *et al.*, 2014), lipid-exchange rate assay was first proposed and performed as previously described (Gong *et al.*, 2011; Sun *et al.*, 2013b). However, the analytic process reported in the previous methodology neither eliminated the photobleaching effect upon laser exposure nor provided a detailed procedure to ensure experimental reproducibility and accuracy.

Here, we detailed the protocol of our renewed lipid-exchange rate assay used in Wang *et al.* (2018). The three key steps in the assay including cell preparation, fluorescence recovery after photobleaching (FRAP) image acquisition, and data analysis using mean of intensities (MOI) and BODIPY-C12-stained size measurement of LDs were reported. Upon performing a three-cycle photobleaching process and the pre-estimation of exchange rate based on the linear criteria in the data analysis, we can ensure the accuracy and repetitiveness of the fitted exchange rates by using our proposed equation underlying molecular thermodynamics in the Theory section. This lipid-exchange rate assay is also applicable for the evaluation of a nanometer size channel connecting two vesicles when the micrometer volumes of the vesicles are measured in advance.

## **Materials and Reagents**

### **A. Materials**

1. Pipette tips (Corning, Axygen, catalog numbers: T-1000-B, T-200-Y, T-300)
2. 100 mm culture dish (Thermo Fisher, Nunc, catalog number: 150462)
3. 35 mm glass bottom culture dish (Thermo Fisher, catalog number: 150682)
4. 1.5 ml Eppendorf tube (Corning, Axygen, catalog number: MCT-150-C)
5. 50 ml, 15 ml centrifuge tubes (Thermo Fisher, catalog numbers: 339652, 339650)
6. Gene Pulser (Bio-Rad, catalog number: 165-2086)

### **B. Biological materials**

1. 3T3-L1 pre-adipocyte (ATCC, catalog number: CL-173)
2. Cidec-GFPN1 plasmid (Wang *et al.*, 2018; available from the corresponding author upon request)

*Note: The full-length Cidec gene (NCBI accession number: NM\_178373) was inserted into pEGFPN1 plasmid at the Xhol and EcoRI restriction enzyme sites.*

### **C. Reagents**

1. BODIPY 558/568 C12 (Thermo Fisher, Molecular Probes, catalog number: D3835)
2. Sodium oleate (Sigma-Aldrich, catalog number: O7501)
3. Dulbecco's modified Eagle's medium (DMEM), high glucose, with L-Glutamine and Phenol Red

- (Gibco, catalog number: 11965084)
4. Fetal bovine serum (FBS) (Gibco, catalog number: 16140071)
  5. Penicillin-streptomycin Mixed Solution (P/S) (Gibco, catalog number: 15140122)
  6. Electroporation buffer (Bio-Rad, catalog number: 1652676)
  7. Trypsin-EDTA (Thermo Fisher, Life Technologies, catalog number: 25200-072)
  8. NaCl (Sigma-Aldrich, catalog number: S3014)
  9. KCl (Sigma-Aldrich, catalog number: P9541)
  10. Na<sub>2</sub>HPO<sub>4</sub> (Sigma-Aldrich, catalog number: S5136)
  11. KH<sub>2</sub>PO<sub>4</sub> (Sigma-Aldrich, catalog number: P9791)
  12. CaCl<sub>2</sub> (Sigma-Aldrich, catalog number: C5670)
  13. MgCl<sub>2</sub> (Sigma-Aldrich, catalog number: M4880)
  14. Phosphate-buffered saline (PBS) (see Recipes)

## **Equipment**

1. Pipettes (Gilson, models: PIPETMAN P10, P20, P200, P1000)
2. Cell counting chamber (Easybio, catalog number: BE6138)
3. 37 °C, 5% CO<sub>2</sub> cell culture incubator (NuAire, model: NU-49SOE)
4. Biosafety cabinet (NuAire, model: NU-425-400E)
5. Centrifuge (Cence, China, model: TDZ5-WS)
6. Electroporation (Lonza, model: Amaxa Nucleofactor II)
7. Confocal fluorescent microscope (Nikon Instruments, model: Nikon A1+ Confocal Microscope)
8. Live cell station (Oko laboratory, model: A1 Confocal)
9. Computer (Lenovo, model: ThinkStation P510)

## **Software**

1. NIS-element analysis (Nikon,  
<https://www.microscope.healthcare.nikon.com/products/software>)
2. Fiji (NIH software, <http://fiji.sc/Fiji>)
3. Open-source plugin-based image analysis software based on ImageJ (<https://imagej.nih.gov/ij/>)
4. LabVIEW 8.5 with a plug-in installation of NI Vision 8.6 module (National Instruments, <http://www.ni.com/en-us/support/downloads/software-products/download.labview.html>)
5. Custom-made LabVIEW modules:
  - a. 1\_Exchange rate assay.llb (applied in Step B of Data analysis)
  - b. 2\_Check a fitting region.vi (applied in Step C of Data analysis)
  - c. 3\_Calculation of exchange rate.vi (applied in Step D of Data analysis)
6. Prism 5 (GraphPad Inc.)

## Procedure

**Theory:** For the estimation of LD fusion ability, we measure a lipid-exchange rate as an alternative indicator of potential pore size between two contacted LDs. During LD fusion, the lipids constantly exchange between the two LDs. For the measurement of the lipid-exchange rates between the LDs in live cells, the small (donor) LD of each LD pair pre-stained with BODIPY-C12 was photobleached. The fluorescence-labeled lipids in the large (acceptor) LD diffused into the small LD, and the photobleached lipids went into the large LD. The images of the small and large (acceptor) LDs, before and after photobleaching, were recorded and their fluorescent intensities were normalized to the initial fluorescent intensity of the large LD at time 0 s after photobleaching, giving rise to the initial intensity ratio of 1 for the large LD. The ratio of fluorescent intensity of the photobleached small LD to the large LD at time 0 s is denoted as  $G_0$ . At any time after photobleaching, the time-dependent ratios of the fluorescent intensities of the large and small LDs are denoted as  $G_1(t)$  and  $G_2(t)$ , respectively. Therefore, a time-invariable exchange rate ( $\phi_e$ ) of the neutral lipid molecules between an LD pair is calculated from the measurement of the fluorescence recovering rate in the LD pair according to the ordinary differential equation as follows:

$$\phi_e = d \ln \left[ \frac{G_1(t) - G_2(t)}{1 - G_0} \right] / (-b \cdot dt)$$
$$b = \left( \frac{1}{V_1} + \frac{1}{V_2} \right)$$

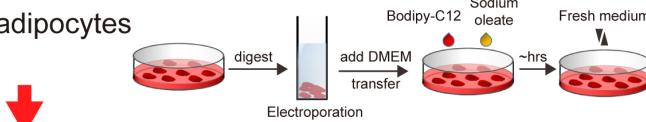
where  $V_1$  and  $V_2$  represent the volumes of the large and small LDs, respectively. The unit of the lipid-exchange rate is  $\mu\text{m}^3/\text{s}$ . The detailed equation derivation is shown in [Supplementary Information](#).

### A. Overall procedure

The procedure for the lipid-exchange rate assay of LDs in live cells is shown in Figure 1.

**Cell preparation**

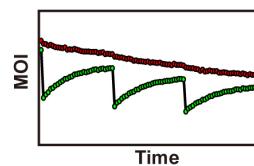
culture 3T3-L1 pre-adipocytes  
and transfection

**Image acquisition**

The small LD of a LD pair stained  
by Bodipy-C12 was photobleached  
and recorded over time

**Data analysis**

Exchange rate was calculated from the mean  
of intensity (MOI) and size measurement of  
the LD pair



**Figure 1. Schematics of LD lipid-exchange rate measurement in live cells**

**B. Cell preparation**

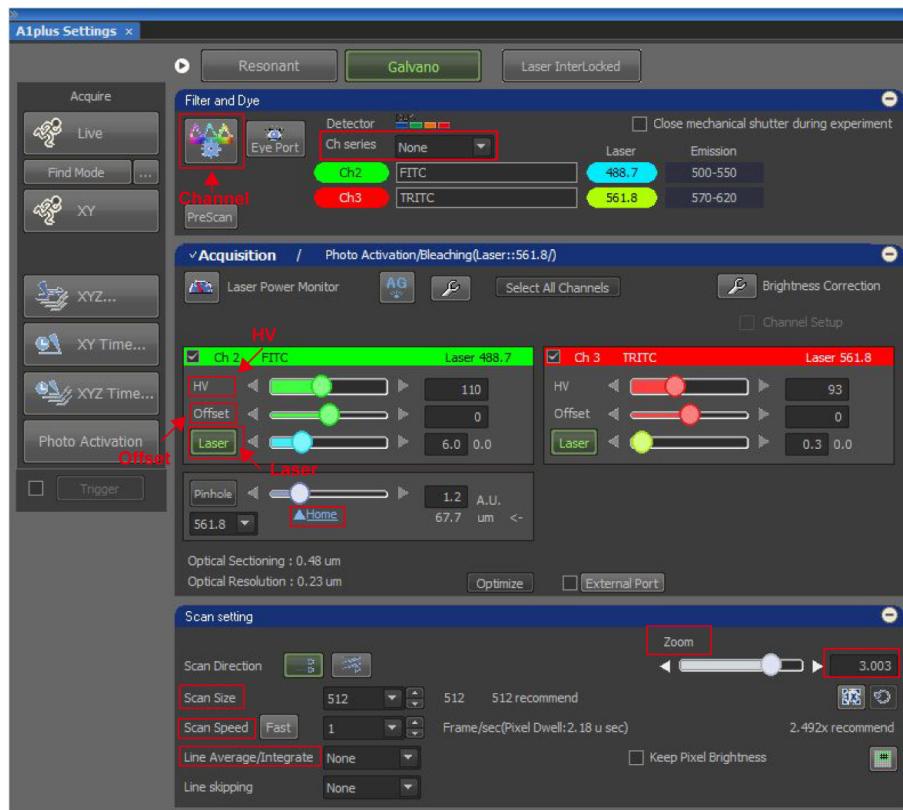
1. Culture 3T3-L1 pre-adipocytes in 10 cm plastic dish in 10 ml DMEM supplemented with 10% FBS, 100 µg/ml streptomycin and 100 U/ml penicillin.
2. At 80% confluence, trypsinize the cells using 2 ml 0.25% trypsin solution. Terminate the process using 5 ml DMEM. Transfer the cells with DMEM into a 15 ml tube and centrifuge at 150 x g for 3 min, discard the supernatant.
3. Resuspend the cell using 1 ml PBS.
4. Count cells using a cell counting chamber.
5. Centrifuge at 150 x g for 3 min, discard the supernatant.
6. Resuspend the cells with appropriate electroporation buffer to reach a cell density of 2-3 x 10<sup>6</sup>/ml.
7. Aspirate 100 µl of cells, mix with 0.5-1 µg plasmid (Cidec-GFP) in a 1.5 ml Eppendorf tube.
8. Transfer the cell-DNA mix into a genepulser, place the genepulser into Nucleofector II, choose the program A-033, which is optimized for 3T3-L1 pre-adipocytes transfection, execute the electroporation step.
9. Add 100 µl of DMEM into the genepulser, aspirate out the mixture into a 15 ml centrifuge tube with 2 ml DMEM medium.
10. Add BODIPY-C12 558/568 into the medium to a final concentration of 1 µg/ml.
11. Add sodium oleate solution into the medium to a final concentration of 200 µM to provide lipids for LD formation and growth in cells.
12. After the addition of the above reagents, aspirate the mixture into a 35 mm glass bottom culture dish and incubate for 18-20 h in an incubator at 37 °C, 5% CO<sub>2</sub>.
13. Change the culture medium with 2 ml fresh DMEM for two reasons: 1) for the removal of BODIPY-C12 dye from the medium to eliminate background fluorescence; 2) for the removal of sodium oleate to reduce the influence of triglycerides synthesis on LD fusion. Next, place the

culture dish with the cells in the incubator for 1 h before FRAP experiments to help the cells accommodate to the changed medium.

### C. Fluorescence recovery after photobleaching (FRAP) image acquisition

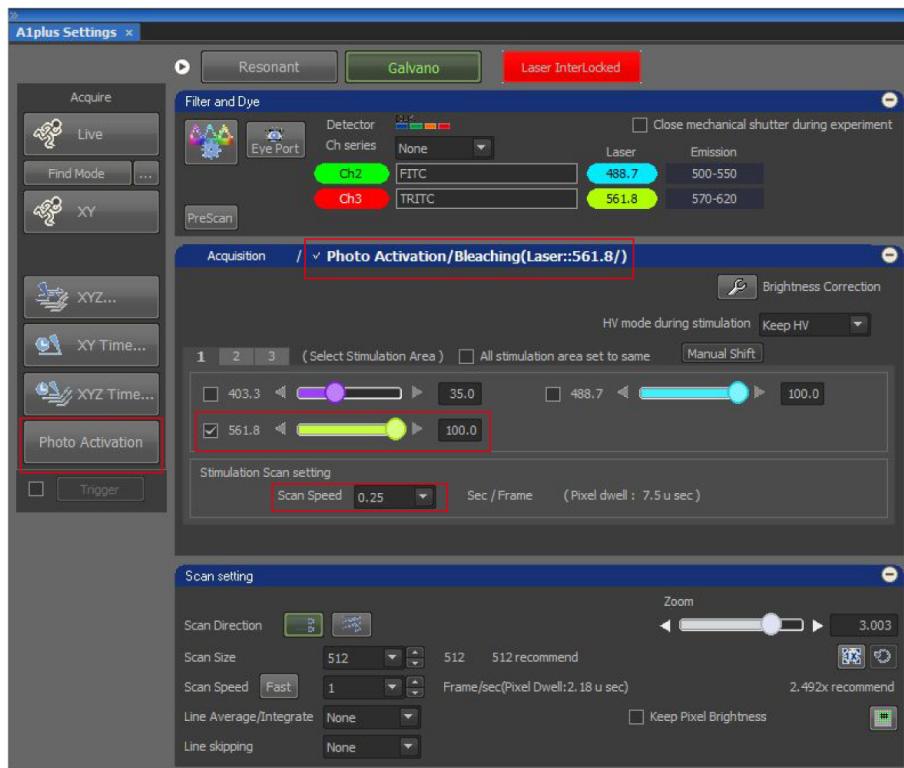
The module of FRAP is conventional in most commercial confocal microscopes. Here, we used a Nikon A1 confocal microscope to perform FRAP measurements as an example. This following procedures can also be used for FRAP experiments when using the other confocal microscopes.

1. Turn on the live cell station, set the temperature to 37 °C, switch on the 5% CO<sub>2</sub>, add an appropriate amount of double distilled water in a heating trough.
2. Turn on the confocal microscope, laser generator, microscope controller, mercury lamp, electric automatic stage, z-axis piezoelectric stage, and computer workstation. Turn on the perfect focus system (PFS). Launch the Nis-element software. Choose the 100x oil-immersion objective (Numerical aperture 1.45). Add objective oil dropwise onto the objective, place the 35 mm glass bottom culture dish onto the sample holder, and adjust the height of the objective to immerse the culture dish in the oil. Allow the cells to accommodate for 1 h if possible, or at least 0.5 h to ensure the stability of the following measurement.
3. Set the parameters of the microscope as shown in Figure 2. Click on the channel button, select “Fluorescein isothiocyanate (FITC)” and “Tetramethylrhodamine (TRITC)” channels. Choose “none” in Channel series. To capture the fluorescent images, fine settings of the optimized laser power and the gain and sensitivity of detector are critical. There are several commercial microscopes but their definitions of the setting parameters are different. Here, we used Nikon A1 confocal microscope as an example to show our settings, in which “Laser power” indicates the level of real laser power, “HV” indicates the gain of detector (brightness of images), and “Offset” indicates the sensitivity of detector (contrast of images). Thus, set the FITC laser power to “6.0”, HV “60-120”, off set “0”; the TRITC laser “0.3”, HV “60-120”, off set “0”. Set pinhole as home under the wavelength of 561 nm. Set scan size to “512 x 512 pixels”, scan speed “1 frame/s”, zoom “3.0”, and line average “none”.



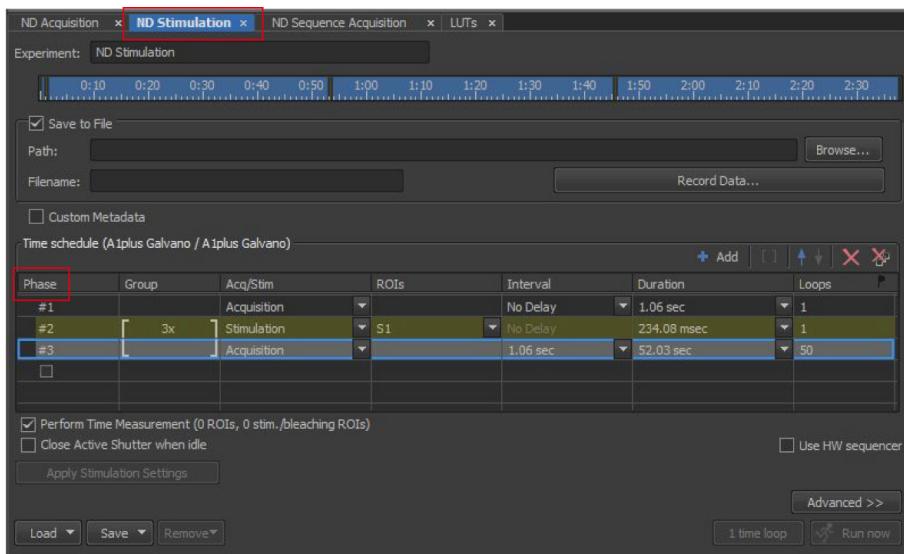
**Figure 2. NIS-element settings of images acquisition.** The image shows the settings of the laser channel, laser power, HV, Offset, pinhole size, scan size, scan speed, and other detailed parameters for image acquisition.

4. Set the photobleaching parameters:
  - a. Switch on the photoactivation interface as shown in Figure 3. Set the photobleaching laser as the wavelength of 561 nm, power of 100%. Set the stimulation scanning speed as 0.25 sec/frame.



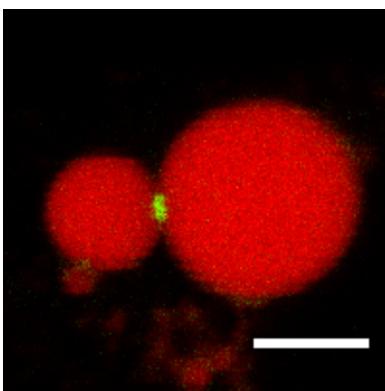
**Figure 3. NIS-element settings of photobleaching.** The image shows the settings of laser power and scan speed for photobleaching.

- b. Go to the N-dimensional (ND) Stimulation Interface as shown in Figure 4, in which we could set the XYZ locations of a few images captured and the time schedule options. Create three phases corresponding to the three-steps in time-sequential operations. In Phase 1, choose “Acquisition” in Acq/Stim, “No delay” in Interval, a duration of 1.06 s and 1 loop to capture one image, which shows the initial fluorescent intensities of an LD pair before photobleaching. In Phase 2 and Phase 3, set 3x in Group option. In addition, in Phase 2, set “Stimulation” in Acq/Sim, “S1” in ROIs and 1 loop to perform photobleaching. The stimulation duration will be automatically set according to the setting of stimulation scanning speed in Figure 3. In Phase 3, choose “Acquisition” in Acq/Stim, “1.06 s” in time Interval, and 30 (fast exchange rate) or 50 (slow exchange rate) loops to capture time-lapse images with a time interval of 1.06 s, which record the change in the fluorescent intensities of the LD pair after photobleaching. Subsequently, the duration is automatically calculated. Choose “Perform Time Measurement”. Check “Save to File”, input the path and name the file.



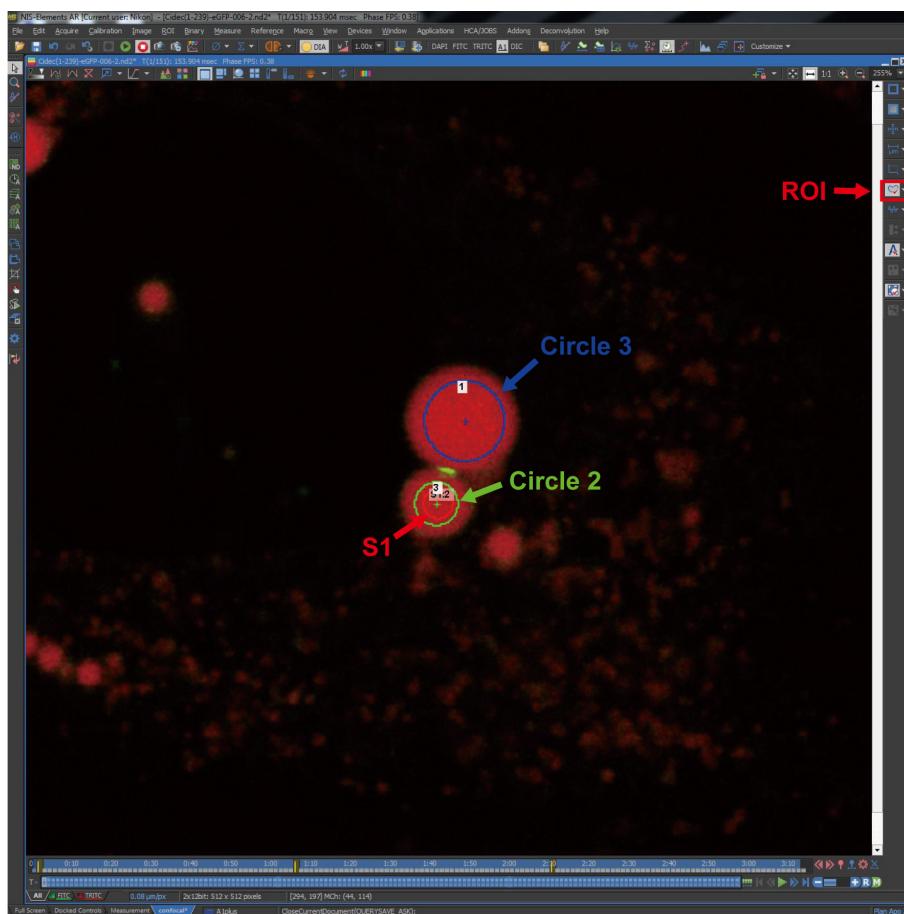
**Figure 4. NIS-element settings of ND Stimulation.** The image shows three phases corresponding to the three-step time-sequential operations in the ND stimulation setting.

- c. Click on the button “Time measurement” in the menu to stick the time measurement interface in front of all interfaces. The interface is used to monitor the change in fluorescent signals of the selected regions-of-interest over time.
5. Find target LD pairs:
  - a. Click on the FITC or GFP button in the interface of the NIS-element software and switch to the microscope control mode. Using the mercury lamp illumination, select an LD pair with GFP signal enriched at the LD-LD contact site (LDCS) as shown in Figure 5. Adjust the focus plane of the objective to get a clear image of the LD pair. The diameters of the selected donor LDs are preferred to be in the range of 3-6  $\mu\text{m}$  to reduce variation.



**Figure 5. An example image of a suitable LDCS.** The image shows the red signal of BODIPY-C12 stained LD pairs and the green signal of Cidec-GFP enriched at the LDCS. Scale bar, 5  $\mu\text{m}$ .

- b. Switch to the computer control mode by clicking on the button “A1”. Click on the button “Laser interlocked” to stimulate the laser. Click on the button “Live” to preview the LD pair. Move the cursor on the interface of the software to the LDCS, right click on the mouse, choose “move the point to the center of the image” to place the LD pair at the center of the vision. Scroll the mouse to adjust the focus plane of the objective once the LD pair went out of focus. Adjust HV in GFP and TRITC channels to achieve the highest fluorescent intensity without overexposure as the photograph taken by detector. Press the button “PFS” on the microscope front panel to lock the imaging plane during the acquisition process.
- c. Click on the ROI button as shown in Figure 6, select the round circle, draw a circle covering about 70% area of the donor LD at its center, and denote as S1 (stimulation ROI). The reason for photobleaching donor LDs is described in the Notes section below. Next, draw another circle at the center of the donor LD that covers about 70% area of the donor LD; Draw a third circle at the center of the acceptor LD that covers about 70% area of the acceptor LD. The reason for selecting 70% area is dependent on our experience with the optimal HV value and bleaching laser power used. It is possible to adjust the percentage area on case by case basis. In addition, the ROIs selection here was only used to preview the real-time fluorescent intensities of the BODIPY-C12 dyes in LDs and to pre-check the fluorescence recovery data.



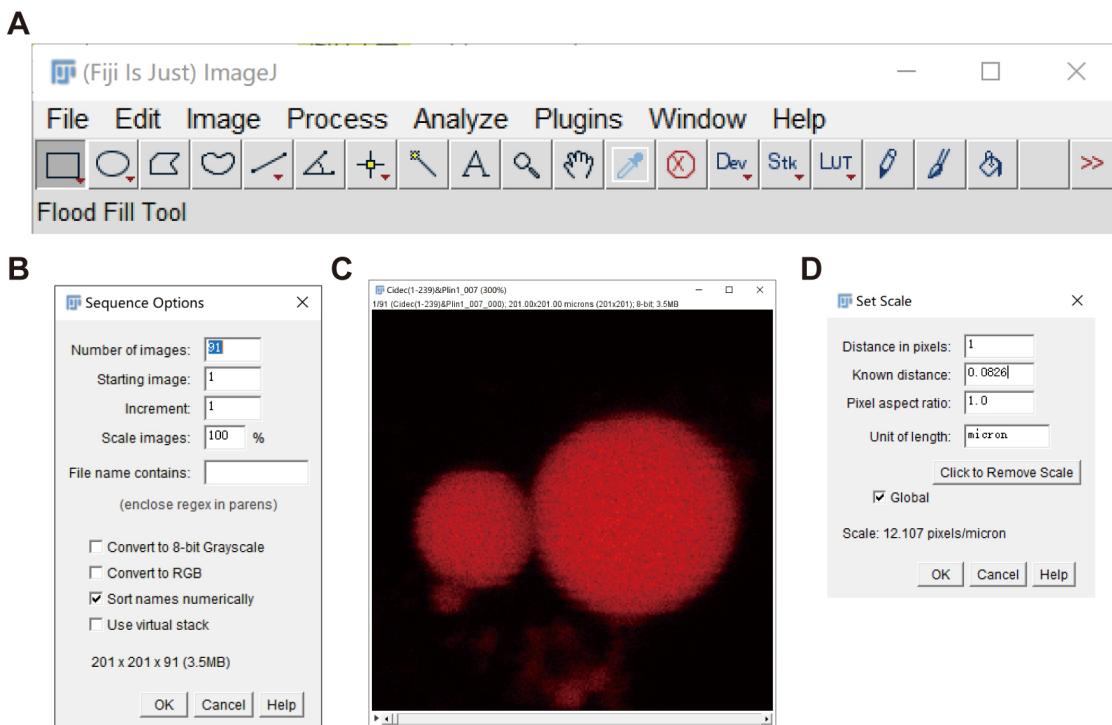
**Figure 6. ROI settings.** The image shows the ROIs and the schematic diagram of circles on LDs. S1, a photobleaching region on the donor LD of an LD pair; Circle 2, the monitoring ROI on the photobleaching donor LD; Circle 3, the monitoring ROI on the acceptor LD.

6. Execute the photobleaching process. Before the process, make sure the LD pair is ready on the imaging plane and all the parameters are set correctly. Click on the button “run now” in the ND stimulation interface and allow the program to complete.
7. Look for next LD pairs and repeat Steps C5 and C6 to acquire FRAP data for a series of LD pairs.

### Data analysis

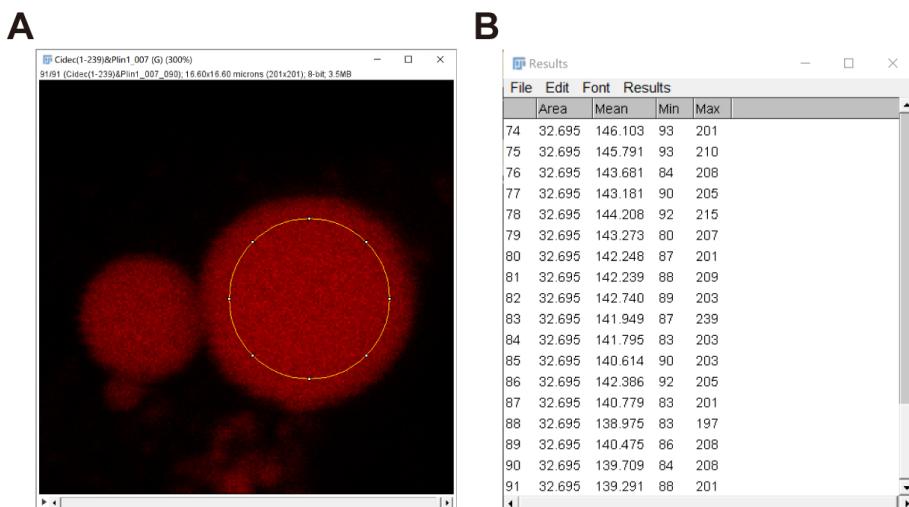
Below, we will demonstrate two alternative methods to analyze the FRAP image data. Specifically, the first method is a partial manual measurement, using Fiji software. The second method is a totally automatic measurement, using custom-built codes in LabVIEW program. After testing on cases of LD pairs ranged from 3 to 6  $\mu\text{m}$  in diameter, the final FRAP results measured using both the above methods are almost alike. But, in view of the other to-be-measured LD pairs maybe of different sizes and fluorescent intensities, as well as the potential limitation of the custom-built LabVIEW program to handle any condition of FRAP data, we also provide the manual method to process the most difficult cases. For example, for donor LDs with a diameter smaller than 1  $\mu\text{m}$ . However, if possible, we recommend the automatic method.

- A. Mean of intensity (MOI) and size measurement of BODIPY-C12 stained LDs by manual
  1. Open the Fiji software.
  2. Go to “File” → “Import” → “Image Sequence”, to open a series of time-sequential images ([8-bit TIF format files](#), the data in Figure 7 is used as an example).



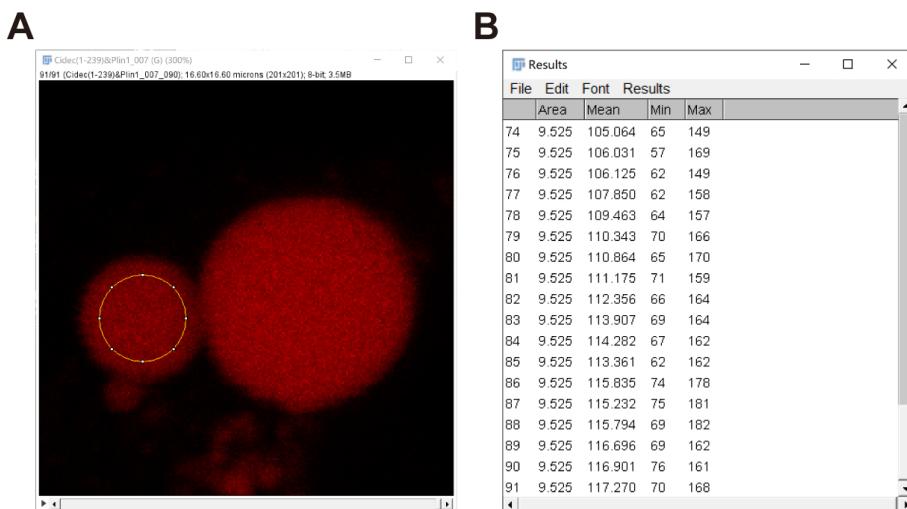
**Figure 7. Import a series of 8-bit TIF format images.** A. The menu of the Fiji software. B. The sequence options of the imported image sequence. C. A representative set of time-sequential images input. D. The scale of the input images.

3. To set the scale bar of the images, go to “Analysis” → “Set Scale”. Here the calibrated scale is 12.107 pixels/ $\mu\text{m}$  as shown in Figure 7D.
4. To obtain a series of “Mean gray values (Mean)” information of a large LD over time.
  - a. Select at least 70% of large LD area with “Oval” tool.
  - b. Go to “Analyze” → “Measure”, to get “Area”, “Mean gray value (Mean)”, “Min gray value (Min)”, and “Max gray value (Max)” information of the large LD.
  - c. Press on the button “▶” as shown in the lower right of Figure 7C or “>” on the keyboard to go to the next frame of the sequential images.
  - d. Click the center of the selected circle and move the circle to the center of the large LD in the next frame with drag-and-drop.
  - e. Repeat steps b-d to obtain a series of information of the large LD to the last frame (Figure 8).



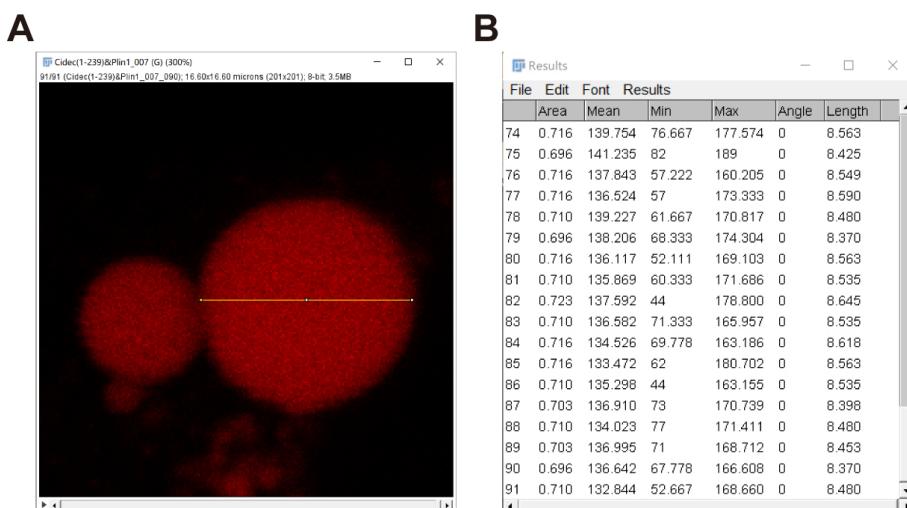
**Figure 8. “Mean gray values (Mean)” data of a large LD in a series of time-lapse fluorescent images quantified by Fiji.** Here, the total number of frames analyzed in this image is 91 with a time interval of 1.12 s.

5. Save the data set of the large LD as an Excel file.
6. To obtain a series of “Mean” information of a small LD over time.
  - a. Press on the back button “◀” as shown in the lower left of Figure 9A or “<” on the keyboard to return to the first frame of the sequential images (for example press 90 times for the sequential set of 91 images).
  - b. Re-select at least 70% of the small LD area with “Oval” tool.
  - c. Go to “Analyze” → “Measure”, to get “Area”, “Mean gray value (Mean)”, “Min gray value (Min)”, and “Max gray value (Max)” information of the small LD.
  - d. Press on the button “▶” or “>” to go to the next frame of the sequential images.
  - e. Click the center of the selected circle and move the circle to the center of the small LD in the next frame with drag-and-drop.
  - f. Repeat steps c-e to get a series of information of the small LD to the last frame (Figure 9).



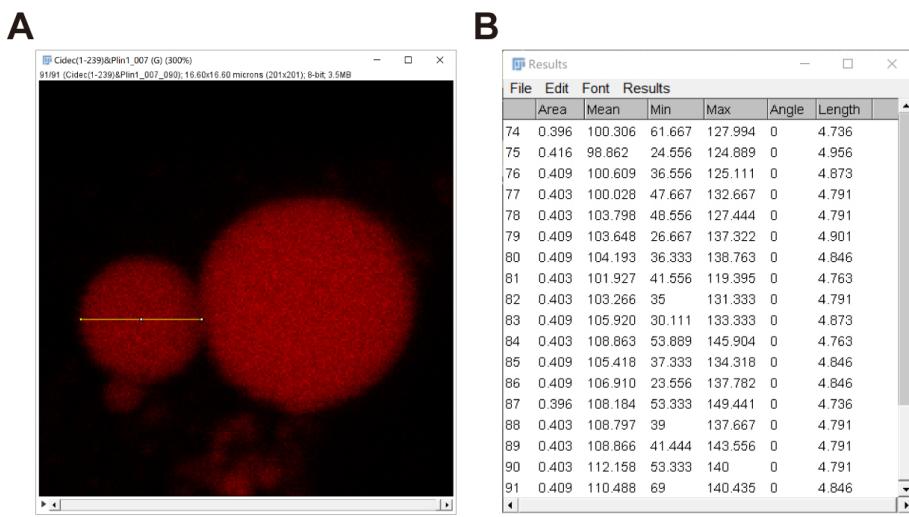
**Figure 9.** “Mean gray values (Mean)” data of a small LD in a series of time-lapse fluorescent images quantified by Fiji

7. Save the data set of the small LD as an Excel file.
8. To obtain a series of “Size” information in the region of the large LD over time.
  - a. Press on the back button “◀” or “<” to return to the first frame of the sequential images (for example press 90 times for the sequential set of 91 images).
  - b. Measure the size of the large LD with the “Line” tool.
  - c. Go to “Analyze” → “Measure”, to get “Area”, “Mean gray value (Mean)”, “Min gray value (Min)”, “Max gray value (Max)”, “Angle”, and “Length” information of the large LD. For the following analytical process, only “Length” information is required.
  - d. Press on the button “▶” or “>” to go to the next frame of the sequential images.
  - e. Repeat steps b-d to get a series of the size information of the large LD to the last frame (Figure 10).



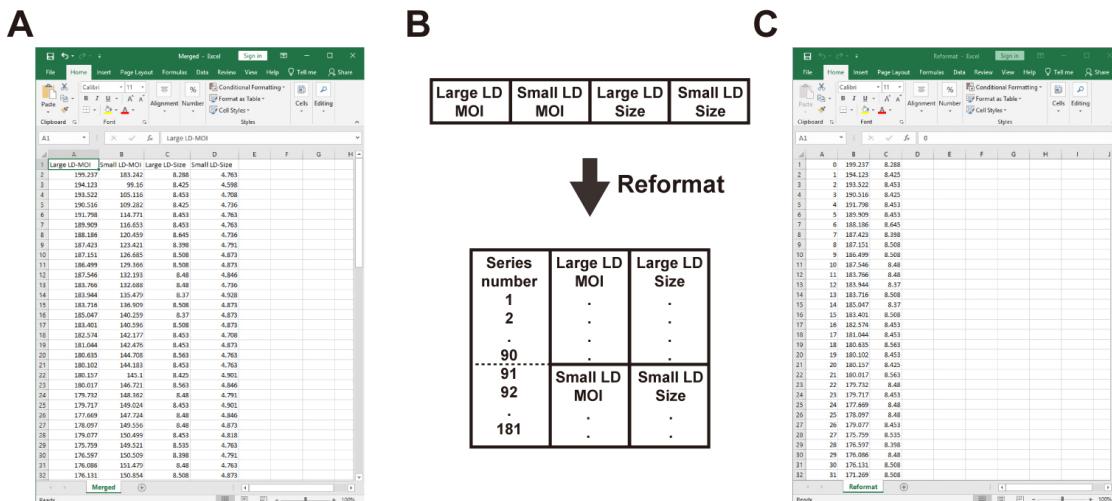
**Figure 10.** “Size” data of a large LD in a series of time-lapse fluorescent images quantified by Fiji

9. Save the data set of the large LD as an Excel file.
10. To obtain a series of “Size” information of the small LD over time.
  - a. Press on the back button “◀” or “<” to return to the first frame of the sequential images (for example press 90 times for the sequential set of 91 images).
  - b. Measure the size of the small LD with the “Line” tool to obtain a series of the size information of the small LD until the last frame by repeating the same steps 8b-8e for the large LD measurement (Figure 11).



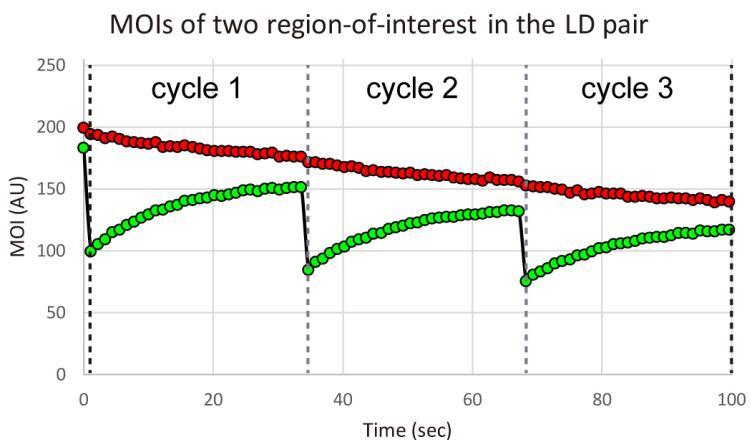
**Figure 11. “Size” data of a small LD in a series of time-lapse fluorescent images quantified by Fiji**

11. Save the data set of the small LD as an Excel file.
12. To get the plot of time-sequential MOIs over time, only “Mean” value from the intensity measurement and the “Length” values from the size measurement are required. Next, reformat the data sets as a new Excel file as shown in Figure 12. The data is now ready for the subsequent analysis in Step C of Data analysis.



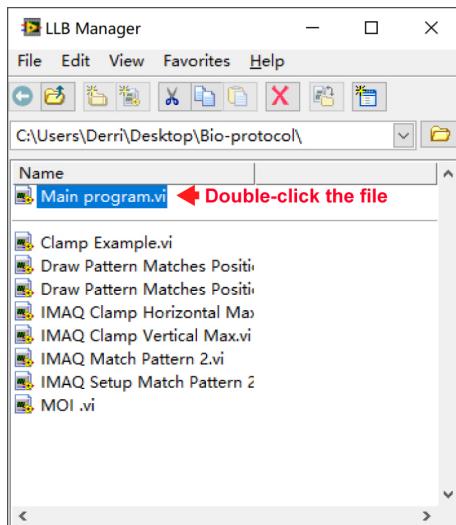
**Figure 12.** Merge and reformat the data sets in a new Excel file

13. Plot the sequential MOIs of the two regions-of-interest of the LD pair over time in the Excel software (Figure 13).

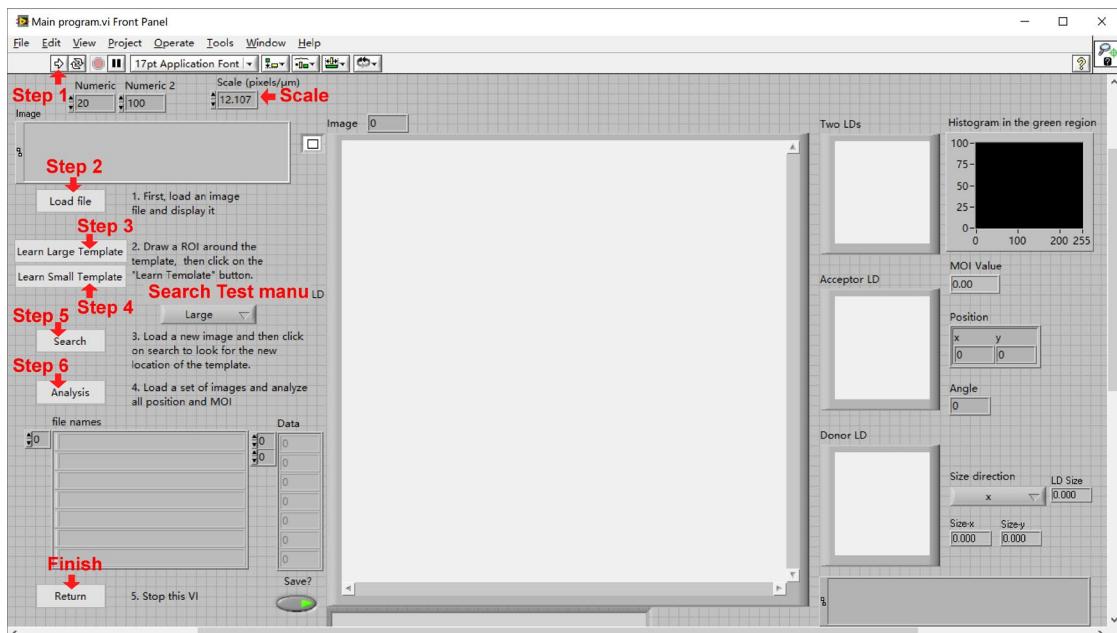


**Figure 13.** Plot of sequential MOIs over time

- MOI and size measurements of BODIPY-C12 stained LDs using an automatic identification program
  - Open the LabVIEW software (here using the version of LabVIEW 8.5 with a plug-in installation of NI Vision 8.6 module).
  - Double-click the LabVIEW sub-lvb file “1\_Exchange rate assay.lvb” to open the main module “Main program.vi” as shown in Figure 14. The interface of the sub-VI program is shown in Figure 15.



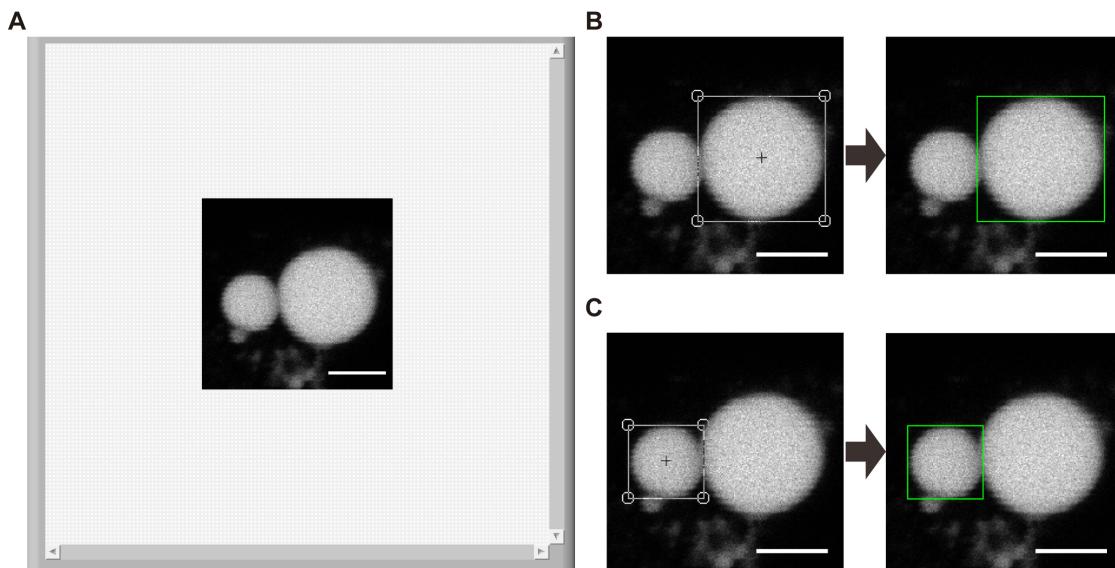
**Figure 14.** The dialog box of LLB manager



**Figure 15.** The interface of the main module “Main program.vi”. Red asterisks indicate a set of regular steps in a standard procedure. Follow the steps to obtain the series of information of each LD pair over time.

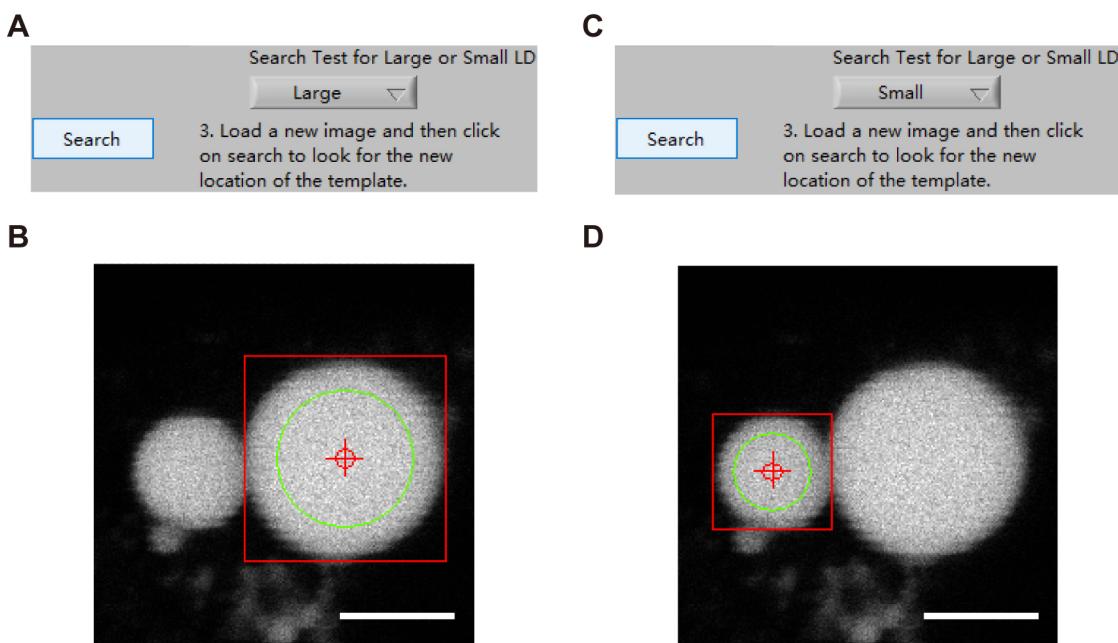
3. To obtain a series of “Mean” information of the large and small LDs over time.
  - a. Set the scale of input images marked by “Scale” in Figure 15 (here using the value of 12.107 pixels/ $\mu\text{m}$ ).
  - b. Run the sub-VI program by pressing on the button marked by “Step 1” in Figure 15 or alternatively press on a combination keyboard button “Ctrl + R.”
  - c. Press on “Load file” marked by “Step 2” in Figure 15 to open a series of time-sequential images (8-bit TIF format files, here using the data of Figure 16A as example).

- d. Select the whole large LD area with a default “Square” tool by drag-and-drop directly on the image as shown in Figure 16B and press on “Learn Large Template” marked by “Step 3” in Figure 15 to record the template image of the large LD.
- e. Repeat step d for the small LD: select the whole small LD area with “Square” tool as shown in Figure 16C and press on “Learn Small Template” marked by “Step 4” in Figure 15 to record the template image of the small LD.



**Figure 16. Loading an image set and recording the template images of large and small LDs.** A. A set of time-sequential images of an LD pair were loaded into the computer memory and showed accordingly on the interface of the main program. B. A template image of a large LD was selected with “Square” tool and recorded into the computer memory as a reference for the subsequent identification of the large LD in the next frames. C. A template image of a small LD was selected with “Square” tool and recorded into the computer memory as a reference for the subsequent identification of the small LD in the next frames. Scale bars, 5  $\mu\text{m}$ .

- f. Select “Large” or “Small” in the Search Test menu and press on the button “Search” marked by “Step 5” in Figure 15 to confirm the accurate identification of the selected large or small LDs (Here showing Figure 17 as an example).

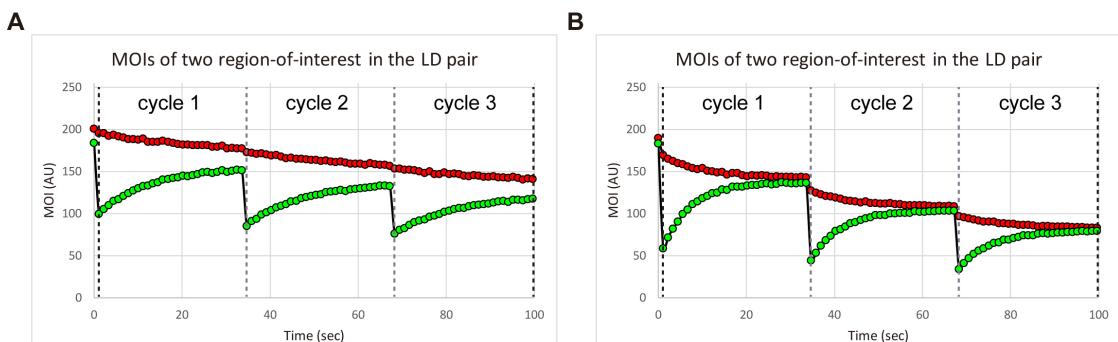


**Figure 17. Confirmation of the identified LD size.** Select “Large” (A) or “Small” (C) to check that the identification of the selected large (B) or small (D) LD is accurate. Scale bars, 5  $\mu\text{m}$ .

- g. Select “Large” in the Search Test menu and press on the button “Analysis” marked by “Step 6” in Figure 15 to get a series of “Frame”, “Mean gray value (Mean)”, “Min gray value (Min)”, and “Size” information of the large LD over time automatically.
- h. Select “Small” in the Search Test menu and press on the button “Analysis” again to get a series of “Frame”, “Mean gray value (Mean)”, “Min gray value (Min)”, and “Size” information of the small LD over time automatically.
- i. Press on the button “Load file” marked by “Step 2” in Figure 15 to input the next set of [time-sequential images](#) and repeat steps d-h to get the series of information of the next LD pairs over time. Or press on the button “Return” marked by “Finish” in Figure 15 after the completion of the MOI measurement of the LD pairs.

*Note: After pressing either the “Load file” or “Return” button, the series of Mean and Size information of the larger and smaller LDs will be saved as an Excel file automatically. The generated Excel file is in ready format for the subsequent analysis in Step C of Data analysis.*

4. Check the data sets of the LD pairs saved previously as an Excel file (here using the two sets of data as example).
5. Plot the sequential MOIs of the two regions-of-interest in the individual LD pairs over time in the Excel software (Figure 18).
6. Create a folder name “MOI&Size” and copy the Excel data set into the folder for the subsequent exchange rate analysis. Transfer the folder with these data into the same folder where there are LabVIEW sub-VIs “2\_Check a fitting region.vi” and “3\_Calculation of exchange rates.vi” files.

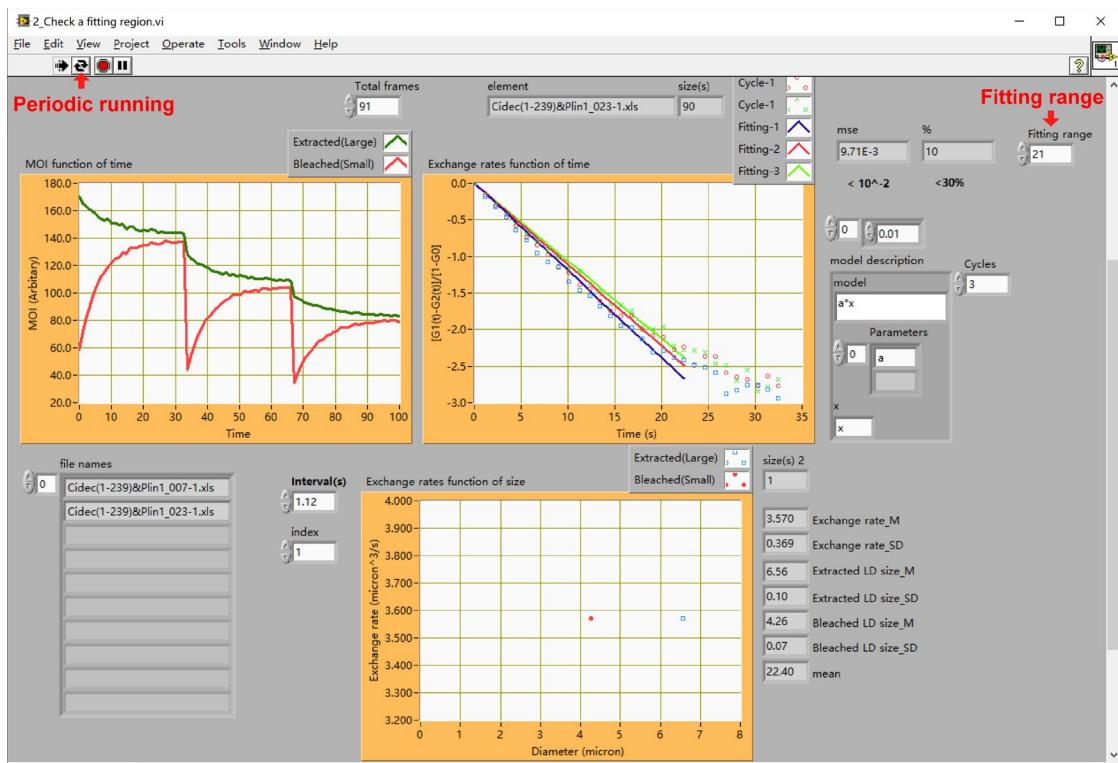


**Figure 18. Plot of sequential MOIs over time.** Data shown in (A) are the same data shown in Figure 13. B. In contrast, the MOIs were obtained using another set of time-sequential images as mentioned in Step B3i of Data analysis.

#### C. Pre-estimation of the exchange rate underlying linear criteria

After the imaging process and obtaining “MOI” and “Length” information generated in Step A or B of Data analysis, the Excel files are now ready for the following analysis. Here, we show the pre-estimation process of the exchange rate.

1. Open the LabVIEW software.
2. Open the LabVIEW sub-VI “2\_Check a fitting region.vi” file. The interface of the sub-VI program is shown in Figure 19.

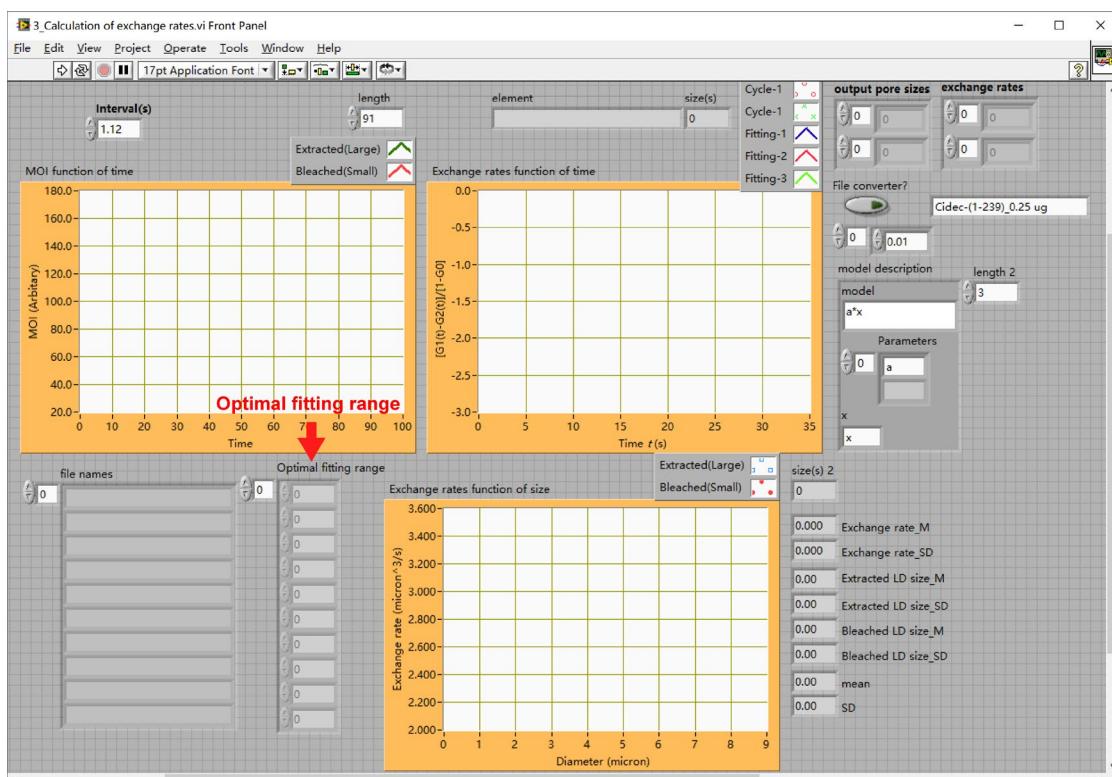


**Figure 19. The interface of sub-VI “2\_Check a fitting region.vi”.** Here using the data from Figure 18B as an example.

3. Obtain the optimal fitting range of an LD pair's exchange rate.
  - a. Run the sub-VI program by pressing on the button marked by “Periodic running” in Figure 19.
  - b. Adjust and choose the maximum value of the fitting range marked by “Fitting range” in Figure 19 to ensure the linearity (MSE) and repetitiveness (%) of the adjusted fitted lines satisfy the experienced optimal values ( $MSE < 0.01$  and  $\% < 30\%$ ). We define the two upper limit values as linear criteria.
  - c. Record each optimal fitting range for each set of LD pair measured.

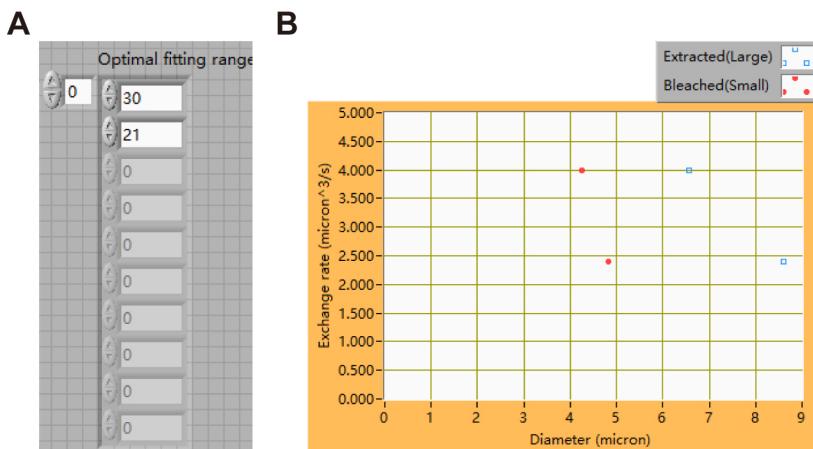
#### D. Calculation of the exchange rates between individual fused LD pairs

1. Open the LabVIEW sub-VI “3\_Calculation of exchange rates.vi” file. The interface of the sub-VI program is shown in Figure 20.



**Figure 20. The interface of sub-VI “3\_Calculation of exchange rates.vi”**

2. Input all the optimal fitting ranges in the table marked by “Optimal fitting range” as shown in Figure 21A.
3. Run the sub-VI program by pressing on “Ctrl + R” to obtain the mean  $\pm$  SD values of the exchange rates. Exchange rates were plotted against large or small LD sizes as shown in Figure 21B.



- e. Dilute PBS stock with deionized H<sub>2</sub>O to 1x PBS

### **Acknowledgments**

We thank the members of P. Li Laboratory at Tsinghua University for their helpful discussions and Jinyu Wang at SLSTU-Nikon Biological Imaging Center for imaging support. This work was supported by grants from National Basic Research Program Grants 2018YFA0506900 (to P.L.) and National Natural Science Foundation of China Grants 91857103 (to F.J.C), 31430040, 31690103, and 31621063 (to P.L.). This protocol was adapted from our previous publication in the Journal of Biological Chemistry (Wang *et al.*, 2018).

### **Competing interests**

The authors declare no conflicts of interest or competing interests.

### **References**

1. Choudhary, V., Ojha, N., Golden, A. and Prinz, W. A. (2015). [A conserved family of proteins facilitates nascent lipid droplet budding from the ER](#). *J Cell Biol* 211(2): 261-271.
2. Farese, R. V., Jr. and Walther, T. C. (2009). [Lipid droplets finally get a little R-E-S-P-E-C-T](#). *Cell* 139(5): 855-860.
3. Fujimoto, Y., Itabe, H., Kinoshita, T., Homma, K. J., Onoduka, J., Mori, M., Yamaguchi, S., Makita, M., Higashi, Y., Yamashita, A. and Takano, T. (2007). [Involvement of ACSL in local synthesis of neutral lipids in cytoplasmic lipid droplets in human hepatocyte HuH7](#). *J Lipid Res* 48(6): 1280-1292.
4. Gao, G., Chen, F. J., Zhou, L., Su, L., Xu, D., Xu, L. and Li, P. (2017). [Control of lipid droplet fusion and growth by CIDE family proteins](#). *Biochim Biophys Acta Mol Cell Biol Lipids* 1862(10 Pt B): 1197-1204.
5. Gluchowski, N. L., Becuwe, M., Walther, T. C. and Farese, R. V., Jr. (2017). [Lipid droplets and liver disease: from basic biology to clinical implications](#). *Nat Rev Gastroenterol Hepatol* 14(6): 343-355.
6. Gong, J., Sun, Z., Wu, L., Xu, W., Schieber, N., Xu, D., Shui, G., Yang, H., Parton, R. G. and Li, P. (2011). [Fsp27 promotes lipid droplet growth by lipid exchange and transfer at lipid droplet contact sites](#). *J Cell Biol* 195(6): 953-963.
7. Gross, D. A., Zhan, C. and Silver, D. L. (2011). [Direct binding of triglyceride to fat storage-inducing transmembrane proteins 1 and 2 is important for lipid droplet formation](#). *Proc Natl Acad Sci U S A* 108(49): 19581-19586.
8. Krahmer, N., Farese, R. V., Jr. and Walther, T. C. (2013). [Balancing the fat: lipid droplets and human disease](#). *EMBO Mol Med* 5(7): 973-983.

9. Rosen, E.D. and Spiegelman, B.M. (2014). [What we talk about when we talk about fat.](#) *Cell* 156(1-2): 20-44.
10. Sun, Z., Gong, J., Wu, H., Xu, W., Wu, L., Xu, D., Gao, J., Wu, J. W., Yang, H., Yang, M. and Li, P. (2013a). [Perilipin1 promotes unilocular lipid droplet formation through the activation of Fsp27 in adipocytes.](#) *Nat Commun* 4: 1594.
11. Sun, Z., Gong, J., Wu, L. and Li, P. (2013b). [Imaging lipid droplet fusion and growth.](#) *Methods Cell Biol* 116: 253-268.
12. Wang, J., Yan, C., Xu, C., Chua, B. T., Li, P. and Chen, F. J. (2018). [Polybasic RKKR motif in the linker region of lipid droplet \(LD\)-associated protein CIDEC inhibits LD fusion activity by interacting with acidic phospholipids.](#) *J Biol Chem* 293(50): 19330-19343.
13. Wilfling, F., Wang, H., Haas, J. T., Krahmer, N., Gould, T. J., Uchida, A., Cheng, J. X., Graham, M., Christiano, R., Frohlich, F., Liu, X., Buhman, K. K., Coleman, R. A., Bewersdorf, J., Farese, R. V., Jr. and Walther, T. C. (2013). [Triacylglycerol synthesis enzymes mediate lipid droplet growth by relocating from the ER to lipid droplets.](#) *Dev Cell* 24(4): 384-399.
14. Wu, L., Xu, D., Zhou, L., Xie, B., Yu, L., Yang, H., Huang, L., Ye, J., Deng, H., Yuan, Y. A., Chen, S. and Li, P. (2014). [Rab8a-AS160-MSS4 regulatory circuit controls lipid droplet fusion and growth.](#) *Dev Cell* 30(4): 378-393.
15. Xu, D., Li, Y., Wu, L., Li, Y., Zhao, D., Yu, J., Huang, T., Ferguson, C., Parton, R. G., Yang, H. and Li, P. (2018). [Rab18 promotes lipid droplet \(LD\) growth by tethering the ER to LDs through SNARE and NRZ interactions.](#) *J Cell Biol* 217(3): 975-995.
16. Yang, H., Galea, A., Sytnyk, V. and Crossley, M. (2012). [Controlling the size of lipid droplets: lipid and protein factors.](#) *Curr Opin Cell Biol* 24(4): 509-516.

## Fibroblast Gap-closure Assay-Microscopy-based *in vitro* Assay Measuring the Migration of Murine Fibroblasts

Agnieszka P. Looney\* and Mallar Bhattacharya

Division of Pulmonary, Critical Care, Allergy and Sleep Medicine, Department of Medicine, University of California, San Francisco; CA 94158, USA

\*For correspondence: [agalooney@gmail.com](mailto:agalooney@gmail.com)



**[Abstract]** Pulmonary fibrosis is characterized by pathological scarring of the lung. Similar to other fibrotic diseases, scar formation is driven by excessive extracellular matrix deposition by activated, proliferative, and migratory fibroblasts.

Currently, the two most widely used chemotaxis and cell migration assays are the scratch assay and the transmembrane invasion assay. Here we present a gap closure assay that employs commercially available cell lines, equipment and reagents and is time efficient as well as straightforward. The protocol uses an Oris pro cell migration assay 96-well plate with a dissolvable plug in the center of each well to create a cell free area at the time of seeding. Cell repopulation of the empty zone is captured via light microscopy at different time points and quantified with free image analysis software. The clear advantages of this assay in comparison to similar protocols are the use of uncomplicated cell culture methods and the ability to image the experiment throughout.

**Keywords:** Fibroblast, Migration, Fibrosis, Gap closure, Chemotaxis

**[Background]** Few treatments for fibrosing diseases exist because of an incompletely understood and complex etiology (Rockey *et al.*, 2015). Current efforts to develop therapies for organ fibrosis have focused on pathologic fibroblasts, also known as myofibroblasts (Blackwell *et al.*, 2014). In addition to secretion of matrix proteins such as collagen, which comprise scar, a hallmark of pathologic fibroblasts is their increased proliferative and migratory capacity (Kendall and Feghali-Bostwick, 2014). A number of studies have shown that innate immune cells interact with and mediate fibroblast activation (Desai *et al.*, 2018). Thus, we investigated the effects of lung macrophages on fibroblasts in a recent study, where we found a novel population of macrophages that localizes to fibrotic scar in murine lung (Aran *et al.*, 2019). These macrophages highly expressed PDGF-AA, a secreted factor known for promoting fibroblast migration and proliferation. Thus, we treated mouse 3T3 embryonic fibroblasts with conditioned media from cultured mouse lung macrophages, with and without PDGF-AA blocking antibody, and measured fibroblast gap closure.

Here we describe the protocol for the assay, which should be useful for other investigators studying paracrine signaling between adjacent cellular lineages. Since the method presented employs an established, adherent cell line, it may be easily adapted to other cell types and treatments in fields where cell migration is an important pathological characteristic, such as cancer or wound healing. Importantly, fibroblasts and the extracellular matrix have been recently recognized as fundamental

players in the tumor microenvironment and as such are of the outmost interest in oncology research (Bu *et al.*, 2019).

Compared with other methods for investigating chemotaxis (Justus *et al.*, 2014), such as the scratch assay, our approach does not introduce mechanical stress to the cells, which can potentially activate fibroblasts and obscure results. It also does not require optimization of cell culture as required for the transwell/chamber invasion assay, and data from the same well may be collected at multiple timepoints while cells are visualized in real time. Our assay can be easily scaled up to a high throughput format for drug screening purposes. One limitation of the assay is that it does not address directional cell migration. Also, our protocol measures both migration and proliferation of fibroblast cells; a proliferation assay should be conducted if further distinction is needed.

### **Materials and Reagents**

1. Oris pro cell migration assay plate, 96-well (Platypus Technologies, catalog number: PROCMA1), stored at room temperature
2. 3T3 cells (ATCC, catalog number: CRL-1658), stored in liquid nitrogen
3. DMEM (Corning, catalog number: 10-101-CV), stored at 4 °C
4. Fetal Bovine Serum (HyClone, catalog number: SH3039603LR), stored at 4 °C
5. Antibiotic-antimycotic solution (Corning, catalog number: 30-004-Cl), stored at 4 °C
6. Trypsin 0.25% (Corning, catalog number: 25053CL), stored at 4 °C
7. PDGF-AA antibody (Millipore, catalog number: 07-1436), stored at -20 °C
8. Cell culture media (see Recipes)
9. Fully supplemented media (see Recipes)
10. Serum free media (see Recipes)

### **Equipment**

1. Microscope  
Zeiss Axio Observer D1 (Carl Zeiss) equipped with a Yocogawa spinning wheel coupled to a photometrics EMCCD camera (Evolve 512 delta)

### **Software**

1. Zeiss Zen Blue (Carl Zeiss)
2. Fiji/ImageJ (<https://fiji.sc/>)
3. Prism (<https://www.graphpad.com/scientific-software/prism/>)

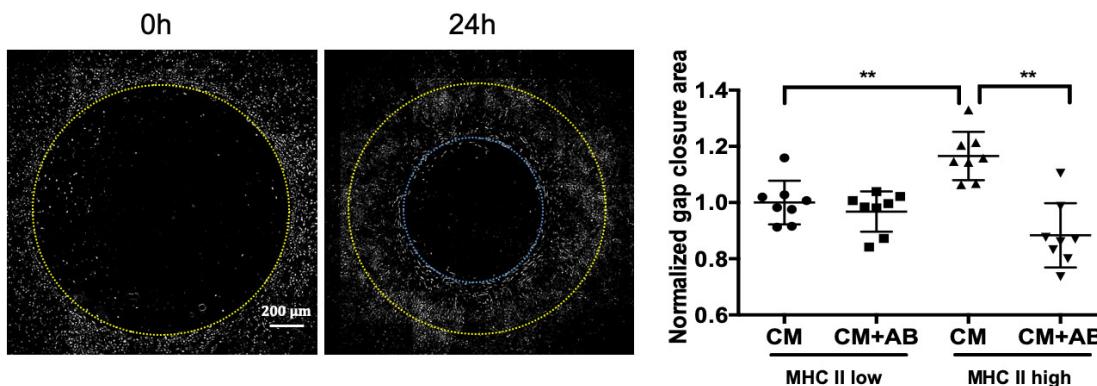
## **Procedure**

1. Establish healthy culture of 3T3 cells in a fully supplemented media (10% serum) according to instructions from ATCC.
2. On the day of the experiment, trypsinize the cells, count and re-suspend in a pre-warmed serum-free media (100  $\mu$ l per well). Add approximately  $5 \times 10^4$  cells per well (100% confluent) to a migration assay plate; this step may need optimization depending on cell size and doubling time. Plan to have 3 wells per condition. Let the cells to adhere for 3 h. Note that the plaque in the center of the well dissolves within 30 min; therefore, it is suitable only for rapidly adherent cells. For example, we found that freshly sorted primary mouse lung fibroblasts needed a longer time to adhere, and we failed to obtain consistent results from primary cells.
3. Using the light microscope (phase contrast for unlabeled cells), verify the cell attachment and the presence of a circular cell-free zone in each well. Exclude the wells that have an excessive amount of cells in a cell-free zone or have uneven distribution of cells in cell growth area (approximately 5% of wells). Take the 0 h timepoint picture of each well, remembering to number or name the wells and images. We recommend using a microscope with a built-in incubator chamber to avoid additional stress to the cells.
4. Add 100  $\mu$ l of pre-warmed media with antibodies/drug or conditioned media. Make sure that all of the wells have the same serum conditions. For example, our conditioned media from mouse macrophages contained 10% serum; we therefore used 10% serum media for blocking antibody and all control wells.
5. Acquire images at 24 h and, if needed, at later timepoints for each well. We found that at 48 h the gaps in majority of wells were completely closed and the most significant difference of the gap closure occurred during the first 24 h. It is possible that shorter timepoints might be necessary for other cell types, or when using conditions with potent mitogens.

## **Data analysis**

We used Zeiss microscope software Zen Blue to stitch the phase-contrast images of the wells acquired at 20x magnification. Fiji (ImageJ) was used for all further image processing and data analysis. As seen in Figure 1, inverting the colors of the pictures greatly improved clarity of the cell-free zone border. We manually selected the cell-free zones using the circular selection tool for 0 h timepoint images and the circular or manually drawn (if the area was not circular) selection tool for 24 h timepoint images. We took individual pictures of wells at time 0 h, because we noticed slight size variability of the cell free zones between the wells. Next, the area of selection was measured by implementing the commands in Fiji: analyze > measure > compute area. We calculated the migration area as a difference between area at time 0 h and 24 h for each well, averaged the area for triplicate wells, and then normalized the data (*i.e.*, we divided all data points

from all groups by a mean of the ‘baseline’ group). We used two-sided Wilcoxon rank-sum test as the statistical test.



**Figure 1. Representative images and quantification of fibroblast gap closure assay.** Pictures of 3T3 fibroblasts were taken at time 0 h and 24 h. Colors in the image were inverted for clarity, the yellow circle indicates the border of the cell free zone at time 0 h and blue circle at time 24 h. Representative data reproduced from Aran *et al.*, 2019; 3T3 fibroblasts were incubated with conditioned media (CM) from lung macrophages sorted by MHCII expression, with and without PDGF-AA blocking antibody (AB). Wilcoxon test two-sided P values are presented. \*\* $P < 0.01$ .

## Recipes

1. Cell culture media  
DMEM  
Fetal Bovine Serum  
Antibiotic-antimycotic solution  
Trypsin  
Prepare cell culture media in sterile conditions
2. Serum free media  
DMEM  
1% antibiotic-antimycotic
3. Fully supplemented media  
DMEM  
10% serum  
1% antibiotic-antimycotic

## Acknowledgments

This work was supported by a UCSF Marcus Award and a National Institutes of Health grant (HL131560) to Mallar Bhattacharya.

Our assay uses commercially available Oris Pro Migration Assay (<https://www.platypustech.com/cell-migration>), which has been optimized and modified for this protocol.

### **Competing interests**

The authors declare no competing financial interest.

### **References**

1. Aran, D., Looney, A. P., Liu, L., Wu, E., Fong, V., Hsu, A., Chak, S., Naikawadi, R. P., Wolters, P. J., Abate, A. R., Butte, A. J. and Bhattacharya, M. (2019). [Reference-based analysis of lung single-cell sequencing reveals a transitional profibrotic macrophage](#). *Nat Immunol* 20(2): 163-172.
2. Blackwell, T. S., Tager, A. M., Borok, Z., Moore, B. B., Schwartz, D. A., Anstrom, K. J., Bar-Joseph, Z., Bitterman, P., Blackburn, M. R., Bradford, W., Brown, K. K., Chapman, H. A., Collard, H. R., Cosgrove, G. P., Deterding, R., Doyle, R., Flaherty, K. R., Garcia, C. K., Hagoood, J. S., Henke, C. A., Herzog, E., Hogaboam, C. M., Horowitz, J. C., King, T. E., Jr., Loyd, J. E., Lawson, W. E., Marsh, C. B., Noble, P. W., Noth, I., Sheppard, D., Olsson, J., Ortiz, L. A., O'Riordan, T. G., Oury, T. D., Raghu, G., Roman, J., Sime, P. J., Sisson, T. H., Tschumperlin, D., Violette, S. M., Weaver, T. E., Wells, R. G., White, E. S., Kaminski, N., Martinez, F. J., Wynn, T. A., Thannickal, V. J. and Eu, J. P. (2014). [Future directions in idiopathic pulmonary fibrosis research. An NHLBI workshop report](#). *Am J Respir Crit Care Med* 189(2): 214-222.
3. Bu, L., Baba, H., Yoshida, N., Miyake, K., Yasuda, T., Uchihara, T., Tan, P. and Ishimoto, T. (2019). [Biological heterogeneity and versatility of cancer-associated fibroblasts in the tumor microenvironment](#). *Oncogene* 38(25): 4887-4901.
4. Desai, O., Winkler, J., Minasyan, M. and Herzog, E. L. (2018). [The role of immune and inflammatory cells in idiopathic pulmonary fibrosis](#). *Front Med (Lausanne)* 5: 43.
5. Justus, C. R., Leffler, N., Ruiz-Echevarria, M. and Yang, L. V. (2014). [In vitro cell migration and invasion assays](#). *J Vis Exp*(88). doi: 10.3791/51046.
6. Kendall, R. T. and Feghali-Bostwick, C. A. (2014). [Fibroblasts in fibrosis: novel roles and mediators](#). *Front Pharmacol* 5: 123.
7. Rockey, D. C., Bell, P. D. and Hill, J. A. (2015). [Fibrosis--a common pathway to organ injury and failure](#). *N Engl J Med* 373(1): 96.

## Conjugation of Fab' Fragments with Fluorescent Dyes for Single-molecule Tracking on Live Cells

I-Ting Teng, Xiangning Bu, and Inhee Chung\*

Department of Anatomy and Cell Biology, George Washington University, School of Medicine and Health Sciences, Washington, District of Columbia, USA

\*For correspondence: [inheec@gwu.edu](mailto:inheec@gwu.edu)



**[Abstract]** Our understanding of the regulation and functions of cell-surface proteins has progressed rapidly with the advent of advanced optical imaging techniques. In particular, single-molecule tracking (SMT) using bright fluorophores conjugated to antibodies and wide-field microscopy methods such as total internal reflection fluorescence microscopy have become valuable tools to discern how endogenous proteins control cell biology. Yet, some technical challenges remain; in SMT, these revolve around the characteristics of the labeling reagent. A good reagent should have neutrality (in terms of not affecting the target protein's functions), tagging specificity, and a bright fluorescence signal. In addition, a long shelf-life is desirable due to the time and monetary costs associated with reagent preparation. Semiconductor-based quantum dots (Qdots) or Janelia Fluor (JF) dyes are bright and photostable, and are thus excellent candidates for SMT tagging. Neutral, high-affinity antibodies can selectively bind to target proteins. However, the bivalence of antibodies can cause simultaneous binding to two proteins, and this bridging effect can alter protein functions and behaviors. Bivalence can be avoided using monovalent Fab fragments generated by enzymatic digestion of neutral antibodies. However, conjugation of a Fab with a dye using the chemical cross-linking agent SMCC (succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate) requires reduction of the interchain disulfide bond within the Fab fragment, which can decrease the structural stability of the Fab and weaken its antigen-binding capability. To overcome this problem, we perform limited reduction of F(ab')<sub>2</sub> to generate Fab' fragments using a weak reducer, cysteamine, which yields free sulfhydryl groups in the hinge region, while the interchain disulfide bond in Fab' is intact. Here, we describe a method that generates Fab' with high yield from two isoforms of IgG and conjugates the Fab' fragments with Qdots. This conjugation scheme can be applied easily to other types of dyes with similar chemical characteristics.

**Keywords:** Single-molecule tracking, Live-cell imaging, Antibody, Quantum dots (Qdots), Janelia Fluor (JF) dyes, Fab', Fab, Conjugation, Cysteamine, Pepsin

**[Background]** Cell-surface protein functions are tightly regulated in their native environment. Gaining a comprehensive understanding of their functions necessitates monitoring their interactions with various cell membrane components, such as other proteins and lipids, and cytoskeletal machinery and cellular organelles below the membranes. Optical tools enabling live-cell based imaging at a molecular level (Joo *et al.*, 2008; Lord *et al.*, 2010; Chung, 2017) include conventional methods such as confocal

microscopy and total internal reflection fluorescence microscopy (TIRFM) combined with specific modalities such as Förster resonance energy transfer (FRET) (Sekar and Periasamy, 2003), single-molecule tracking (SMT) (Moerner, 2012), and fluorescence correlation spectroscopy (FCS) (Kim *et al.*, 2007), and cutting-edge super-resolution microscopy, such as photoactivated localization microscopy (PALM) (Betzig *et al.*, 2006), stochastic optical reconstruction microscopy (STORM) (Rust *et al.*, 2006), stimulated emission depletion (STED) microscopy (Hein *et al.*, 2008), structured illumination microscopy (SIM) (Gustafsson, 2000 and Gustafsson *et al.*, 2009), and lattice light sheet (LLS) microscopy (Chen *et al.*, 2014). Using these tools for live-cell imaging to monitor endogenous proteins requires bright fluorophore-coupled reagents that specifically bind to target proteins. To this end, bright dyes such as semiconductor quantum dots (Qdots) (Dahan *et al.*, 2003; Chung and Bawendi, 2004; Lidke *et al.*, 2004; Chung *et al.*, 2010; Bien-Ly *et al.*, 2014; Chung and Mellman, 2015; Chung *et al.*, 2016) and Janelia Fluor (JF) dyes (Grimm *et al.*, 2015 and 2017) conjugated to high-affinity and non-perturbing antibody-based reagents are widely used. However, an antibody can bind to two target proteins simultaneously. This problem is typically circumvented by digesting antibodies to Fab fragments using proteolytic enzymes such as papain, which cleaves at the hinge region of immunoglobulins (Chung *et al.*, 2010; Bien-Ly *et al.*, 2014; Chung and Mellman, 2015; Chung *et al.*, 2016). Conjugation of Fab with fluorescent dyes relies on a thiol-maleimide reaction. This reaction, however, can destabilize Fab when the interchain disulfide bond within a Fab is reduced, which elicits loss of binding capability to target proteins within a relatively short period of time (a few weeks at best). Consequently, laboratories must frequently regenerate the conjugates, imposing higher cost and hours lost. Thus, we use Fab' fragments containing free sulfhydryl groups in the hinge region (Selis *et al.*, 2016), which can be used for a succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC)-based conjugation reaction without reducing the interchain disulfide bond within Fab'. To this end, we perform two-step reactions, in which IgG is digested into F(ab')2 by pepsin and Fab' is generated by limited reduction of F(ab')2 using cysteamine. In this protocol, we showcase a method that generates Fab' fragments from two different types of antibodies and subsequently conjugates one type of Fab' with quantum dots (Qdots) to monitor EGFR on live cells using SMT. We believe this conjugation scheme will most likely improve the overall yield and stability of the tagging reagents for various types of live-cell imaging of endogenous proteins.

## **Materials and Reagents**

1. Pipette tips (Olympus, catalog numbers: 24-120RL, 24-150RL, 24-165RL)
2. Sterile pipette tips (Olympus, catalog numbers: 24-401, 24-404, 24-412, 24-430)
3. Sterile serological pipets (Olympus, catalog numbers: 12-102, 12-104)
4. 15 ml centrifuge tubes (Olympus, catalog number: 28-101)
5. 50 ml centrifuge tubes (Fisher Scientific, catalog number: 14-955-239)
6. 1.5 ml microcentrifuge tubes (Olympus, catalog number: 24-281)
7. 1.5 ml Protein LoBind Tubes (Eppendorf, catalog number: 022431081)

8. 15 ml Protein LoBind Conical Tubes (Eppendorf, catalog number: 0030122216)
9. Adjustable-volume pipettes (Eppendorf, catalog number: 2231300008)
10. Pierce disposable columns (Thermo Scientific, catalog number: 29920)
11. NAP-5 desalting columns (GE Healthcare, catalog number: 17-0853-01)
12.  $\mu$ -Dish 35 mm, high glass bottom (Ibidi, catalog number: 81158)
13. Treated cell culture flasks (Thermo Scientific, catalog number: 12-556-010)
14. Pierce Protein Concentrators PES, 30K MWCO, 0.5 ml (Thermo Scientific, catalog number: 88502)
15. Pepsin (Sigma Aldrich, catalog number: P6887-250MG)
16. Mouse IgG1 Isotype Control (Invitrogen, catalog number: 02-6100);  $\alpha$ EGFR (rat IgG2a) antibody (Abcam, catalog number: ab231)
17. MDA-MB-468 breast cancer cell line (ATCC, catalog number: HTB-132)
18. BCA protein assay kit (Thermo Scientific, catalog number: 23225)
19. Superdex G200 (GE Healthcare, catalog number: 17-1043-01)
20. Sulfo-SMCC [sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate], No-Weigh Format (Thermo Scientific, catalog number: A39268)
21. DMSO (Dimethylsulfoxide) (Thermo Scientific, catalog number: 20684)
22. Qdot 605 ITK Amino (PEG) Quantum Dots (amino-PEG-Qdot605, Invitrogen, catalog number: Q21501MP) or Qdot 565 ITK Amino (PEG) Quantum Dots (amino-PEG-QDot565, Invitrogen, catalog number: Q21531MP)
23. HEPES, 1 M solution, pH 7.3, molecular biology grade, ultrapure (Thermo Scientific, catalog number: J16924AE)
24. Sodium chloride, 5 M (Lonza, catalog number: 51202)
25. Cysteamine (Sigma-Aldrich, catalog number: M9768-5G)
26. Dye labeled marker, CAL Fluor Red 610 T10 (LGC Biosearch Technologies, catalog number: RD-5082-5)
27. Glycerol (Thermo Scientific, catalog number: J16374AP)
28. Dulbeccos PBS (GenClone, catalog number: 25 -508)
29. Trypsin EDTA (Corning, catalog number: 25-052-CV)
30. Pierce F(ab')2 Preparation Kit (Thermo Scientific, catalog number: 44988, see Note 1), include:
  - a. Zeba Spin Desalting Columns (Thermo Scientific, catalog number: 89889)
  - b. NAb Protein A Plus Spin Columns (Thermo Scientific, catalog number: 89956)
  - c. PBS (Thermo Scientific, catalog number: 1890535)
31. Acetate buffer, pH 4.0 (Fisher Scientific, catalog number: 50-255-309)
32. EDTA (Fisher Scientific, catalog number: 03-500-506)
33. Cell growth medium (RPMI 1640 with 10% fetal bovine serum and 1% penicillin-streptomycin)
  - a. RPMI 1640, with L-Glutamine, 2000 mg/L D-Glucose (GenClone, catalog number: 25-506)
  - b. Fetal bovine serum (FBS), heat-inactivated, U.S. Origin (GenClone, catalog number: 25-514H)

- c. Penicillin-streptomycin mixture (Lonza, catalog number: 17-602E)
- 34. Acetate digestion buffer, pH 4.0 (see Recipes)
- 35. Exchange buffer, pH 7.2 (see Recipes)

## **Equipment**

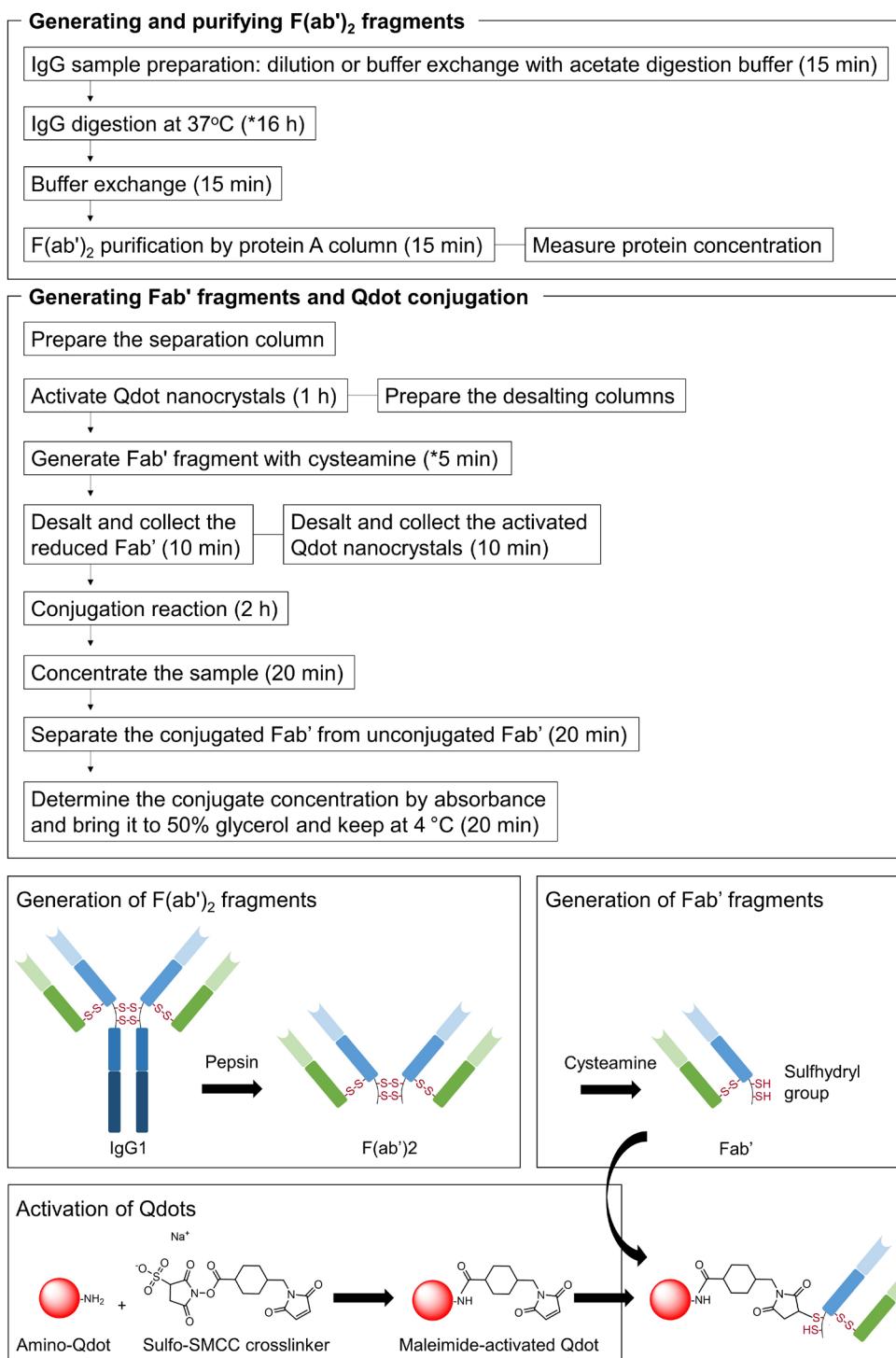
- 1. Eppendorf easypet 3 (Eppendorf, catalog number: 4430000018)
- 2. pH meter (Fisherbrand, Accumet, model: 15)
- 3. Vortex mixer (VWR, model: Analog Vortex Mixer)
- 4. Centrifuge (Eppendorf, model: 5810R)
- 5. Thermo mixer (Thermo Scientific, model: 13687720)
- 6. End-over-end mixer (Argos Technologies, RotoFlex, model: R2000)
- 7. CO<sub>2</sub> incubator air jacket TC (VWR, catalog number: 10810-902)
- 8. Biosafety cabinet (LabConco, model: A2)
- 9. Nikon Eclipse TE2000 inverted microscope with TIRF illuminator and a 100x/1.49NA Plan Apo objective (Nikon, model: Eclipse TE2000-E)
- 10. iXon back-illuminated EMCCD camera (Andor Technology, catalog number: DU-888E-C00-#BV-500)
- 11. 488 nm line of solid-state lasers (Andor Technology)

## **Software**

- 1. ImageJ 1.52i with Java 1.8.0\_172

## **Procedure**

This procedure describes generation of Fab' fragments from IgG and their subsequent conjugation to fluorescent dyes (here, we use Qdot), as summarized in Figure 1. We optimized the digestion and reduction schemes using a mouse IgG1, and then applied these procedures (see Note 8) to further optimize the conditions for conjugating Fab' fragments of an αEGFR antibody (rat IgG2a) with Qdots to perform SMT.



**Figure 1. Overview of the procedure with cartoon schematic.** The reaction conditions may vary between different antibodies (see Notes 5 and 11).

#### A. Preparation of acetate digestion buffer and pepsin solution

1. Prepare acetate digestion buffer (Recipe 1).
2. Prepare a 10 mg/ml pepsin solution in acetate digestion buffer (see Notes 2 and 3).

**B. IgG sample preparation (see Note 4)**

1. Twist off the bottom closure of a Zeba Spin Desalting Column and loosen cap. Place the column in a 15 ml centrifuge tube.
2. Centrifuge the column at 1,000  $\times$  g for 2 min to remove storage solution. Discard the flow-through.
3. Add 1 ml of acetate digestion buffer to the column. Centrifuge at 1,000  $\times$  g for 2 min and remove the flow-through. Repeat this step three times.
4. Place the equilibrated column in a 15 ml Protein LoBind Conical Tube. Remove the cap and slowly add 0.5 ml of antibody sample to the center of the resin bed in the column. Be careful not to disturb the resin bed.
5. Replace the cap and centrifuge at 1,000  $\times$  g for 2 min. Collect the IgG sample. Figure 2 shows that the total IgG amount was slightly decreased (Lane 2) relative to the original amount (Lane 1) from this buffer exchange step.

**C. Pepsin digestion of IgG into F(ab')2**

1. Transfer 0.5 ml of the prepared IgG sample to a 1.5 ml Protein LoBind Conical Tube.
2. For 1 mg/ml IgG, add 2.5  $\mu$ l of 10 mg/ml pepsin solution to the tube. Antibody/pepsin w/w ratio is 20:1.
3. Let the digestion reaction proceed for 16 h on a thermo mixer at 37 °C with agitation at 1,000 rpm. This condition can vary for different IgG molecules (see Note 5). The band of ~100 kDa in lane 3 in Figure 2 shows F(ab')2.

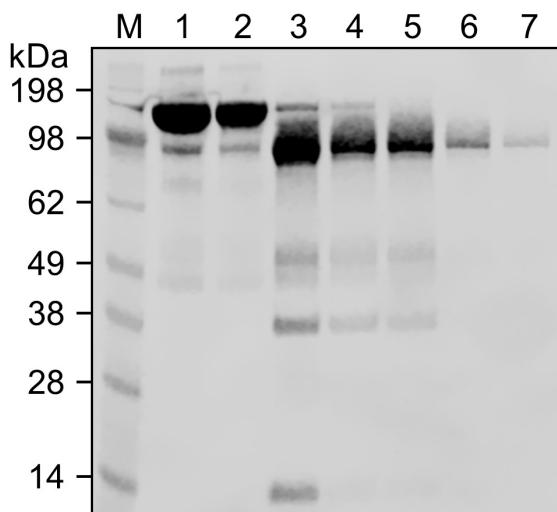
**D. Termination of the digestion reaction (see Note 6)**

1. Twist off the bottom closure of a Zeba Spin Desalting Column and loosen the cap. Place the column in a 15 ml centrifuge tube.
2. Centrifuge the column at 1,000  $\times$  g for 2 min to remove storage solution.
3. Add 1 ml of PBS (pH 7.4) to the column. Centrifuge at 1,000  $\times$  g for 2 min and discard the flow-through. Repeat this step three times.
4. Place the equilibrated column in a 15 ml Protein LoBind Conical Tube. Remove the cap and slowly apply 0.5 ml of the digested IgG sample to the center of the resin bed. Be careful not to disturb the resin bed. Pepsin digestion of IgG will be terminated at this step.
5. Replace the cap and centrifuge at 1,000  $\times$  g for 2 min to collect the flow-through. See Lane 4 in Figure 2.

**E. Purification of F(ab')2 sample**

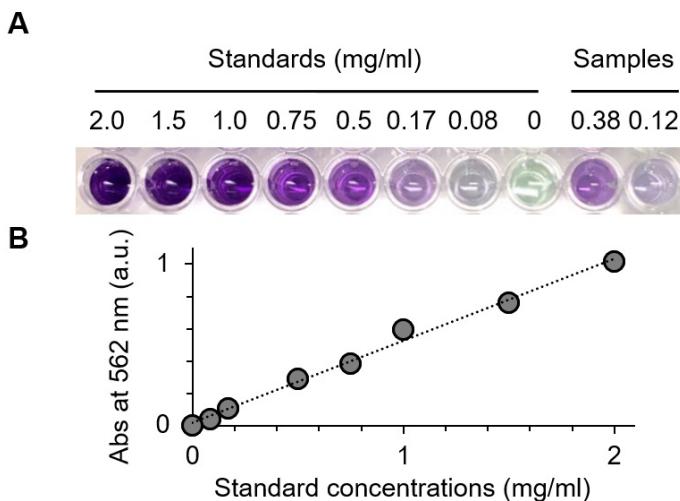
1. Allow the NAb Protein A Plus Spin Column and PBS to come to room temperature. Set the centrifuge speed to 1,000  $\times$  g.
2. Loosen the top cap on the NAb Protein A Plus Spin Column and snap off the bottom closure. Place the column in a 15 ml centrifuge tube and centrifuge for 1 min to remove storage solution.

3. Disperse the resin by adding 2 ml of PBS. For a 0.5 ml sample, use half of the resin.
4. Equilibrate the resin in the column with 2 ml of PBS. Centrifuge for 1 min and discard the flow-through. Repeat this step once.
5. Cap the bottom of the column with the included rubber cap. Apply the digestion mixture to the column and cap the top tightly. Resuspend the resin and sample by inversion. Incubate at room temperature on an end-over-end mixer for 15 min.
6. Loosen the top cap and remove the bottom cap. Place the column in a 15 ml Protein LoBind Conical Tube and centrifuge for 1 min. Save the flow-through (this fraction contains F(ab')2). See Lane 5 in Figure 2.
7. For optimal recovery, wash the column with 0.5 ml PBS. Centrifuge for 1 min and collect the flow-through. Repeat and collect two wash fractions containing additional F(ab')2 (see Lanes 6 and 7 in Figure 2 and Notes 7 and 8).
8. Measure protein concentration using BCA protein assay.
  - a. Prepare the BCA reagents and a serial dilution of the protein standard following the manufacturer's instruction. Mix 200  $\mu$ l of the BCA reagents with 10  $\mu$ l of either the standards or the samples collected above.
  - b. After incubation at 37 °C for 30 min, the color of the mixture will turn from pale green to purple in response to the protein concentration. See Figure 3.
  - c. Measure the absorbance at 562 nm with a spectrophotometer or a plate reader to estimate the protein concentration by comparison to the standard curve.



**Figure 2. SDS-PAGE analysis of samples from each step before and after pepsin digestion of mouse IgG1.** Lane 1: The whole IgG1 sample that was originally stored in PBS (pH 7.4). Lane 2: The IgG1 sample after a buffer exchange to bring it to the acetate digestion buffer (pH 4.0). Lane 3: The sample resulting from a 16 h digestion on a thermo shaker at 37 °C (a strong band at ~100 kDa position indicates F(ab')2). Lane 4: The sample after a buffer exchange to bring it to PBS (pH 7.4) to terminate the reaction. Lane 5: The flow-through from a

Protein A column after a 15-min incubation at room temperature. Lanes 6 and 7: Two consecutive portions collected from washing the protein A column to maximize the output.

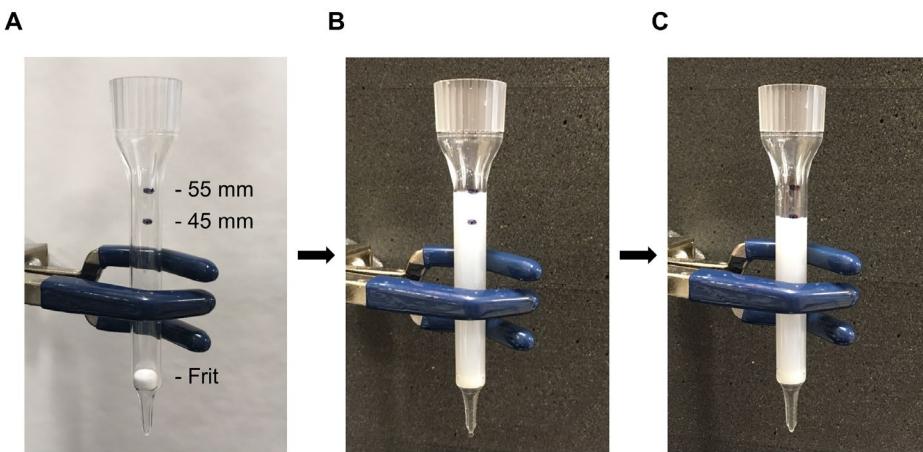


**Figure 3. BCA assay for determining the protein concentrations after purification.** A. The degree of green-to-purple color conversion of BCA solutions is proportional to the protein concentration. B. A standard curve of absorbance (at 562 nm) versus known protein concentrations ( $y = 0.507x + 0.0188$ ) is used to determine the concentrations of F(ab)2 samples collected from the Protein A column flow-through.

#### F. Preparation of the separation column for Fab'-Qdot conjugate

*Note: This segment is a modification of the Qdot® Antibody Conjugation protocol by Invitrogen.*

1. Suspend the separation medium (Superdex G200) in the bottle by gentle shaking or vortexing. Ensure the medium is fully suspended before starting column preparation with Pierce disposable columns.
2. Mark the column with two lines, one at 45 mm above the top of the frit, and a second at 55 mm above the frit (Figure 4A).
3. Wet the frit with pure water before loading the media. After ensuring that the separation medium is a uniform suspension, load media into the column with a 1 ml pipette to the second line at the 55 mm mark (Figure 4B) and let it settle into a packed gel bed that is ~45 mm high (Figure 4C).
4. Gently add 0.5 ml distilled water to the top of the gel while maintaining a level bed surface.
5. As the solvent level drops to near the top of the settled gel bed, fill the column with PBS, and allow the PBS level to drip down to just above the top of the gel bed. Repeat this two more times.
6. Replace the bottom and the top caps when the PBS level from the last fill drops to 2 to 3 mm above the top of the settled gel bed.



**Figure 4. Preparation of the separation column.** A. The image shows two marks (45 and 55 mm) above the frit. B. The gel suspension fills the column to the upper mark (55 mm). C. The gel settles to the lower mark (45 mm).

G. Activation of Qdot nanocrystals (see Note 9)

1. Prepare a freshly dissolved 20 mM solution of sulfo-SMCC in DMSO. To do this, dissolve 2 mg Pierce No-Weigh Sulfo-SMCC in 229  $\mu$ l of DMSO.
2. Add 1.75  $\mu$ l of 20 mM solution of sulfo-SMCC in DMSO to 62.5  $\mu$ l of an 8  $\mu$ M stock solution of amino-PEG-QDot605. Vortex briefly.
3. Incubate for 1 h at room temperature with agitation at 500 rpm to activate the Qdots. Avoid light.
4. Prepare desalting columns while the activation step is proceeding.

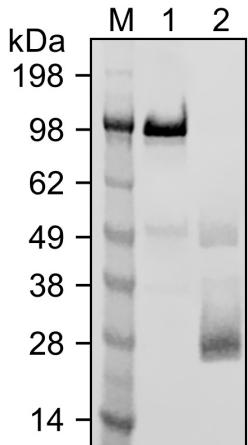
H. Equilibration of desalting columns

1. Prepare exchange buffer (see Recipe 2).
2. Label the NAP-5 desalting columns. Mark one with “reduced Fab” and the other with “activated Qdot”.
3. Remove the top and bottom caps from both columns and allow the storage liquid in the columns to drain. Just as the liquid in each column is approaching the top of the column gel bed, begin adding exchange buffer.
4. Equilibrate each column with 10 ml of exchange buffer.
5. While there is still exchange buffer visible above the gel bed in each column, cap the bottom of each column and set that aside until the F(ab')<sup>2</sup> reduction and activation of Qdot are completed.

I. Generation of Fab' fragments

1. Prepare 100  $\mu$ g of F(ab')<sup>2</sup> in 300  $\mu$ l of PBS by dilution or concentration, as necessary.
2. When the Qdot activation is almost finished, incubate the F(ab')<sup>2</sup> sample with cysteamine (5 mM) and EDTA (2 mM) at 10 °C for 5 min, mixing them at 500 rpm (see Note 8 and 10). The bands in Lane 2 of Figure 5 represent Fab' (50 kDa) and further reduced fragments (25 kDa); the latter fragments are unlikely to retain antigen binding capacity. To maximize Fab' yield, we

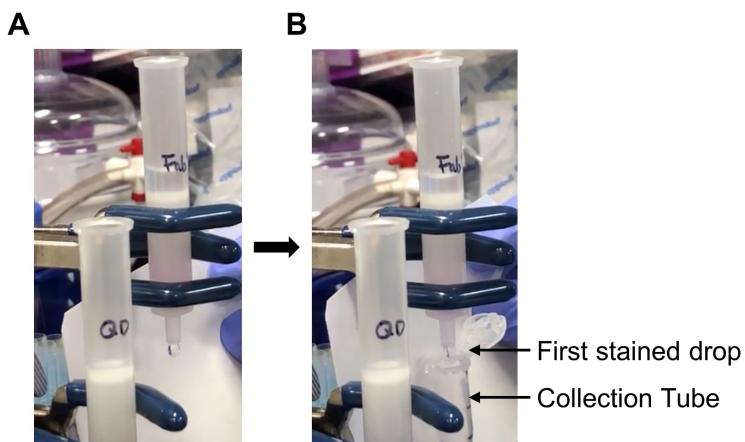
recommend testing various reduction conditions (see Notes 11 and 12) for different F(ab')2 samples using small aliquots (~5-10  $\mu$ l each lane) for gel analysis.



**Figure 5. SDS-PAGE analysis of F(ab')2 reduction to Fab' with cysteamine.** Lane 1: The F(ab')2 sample from Lane 5 in Figure 2. Lane 2: The sample after a reduction with 5 mM of cysteamine for 5 min at pH 7 and 10 °C (see Note 11), before buffer exchange. The band at ~50 kDa indicates Fab'.

J. Desalting and collection of the reduced Fab' fragment

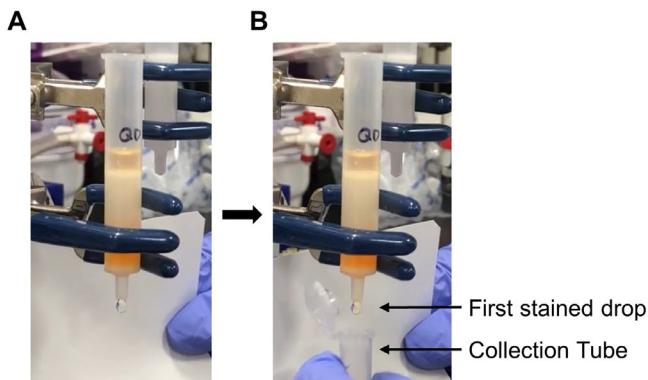
1. Add 40  $\mu$ l of distilled water to one vial of the dye-labeled marker and mix. This makes enough dye-labeled marker for two conjugation reactions. Store at 2-6 °C when not in use.
2. Add 500  $\mu$ l of water to a 1.5 ml microcentrifuge tube and mark the outside of the tube at the meniscus. Add another 500  $\mu$ l of water and make a second mark on the outside of the tube corresponding to a 1,000  $\mu$ l volume. Discard the water. This tube is used to collect the reduced Fab' in Step J7 and the activated Qdot nanocrystals in Step K4.
3. When the F(ab')2 reduction is completed (Step I2), add 20  $\mu$ l of dye-labeled marker (prepared in Step J1) to the reduced Fab'.
4. Uncap the desalting column labeled "reduced Fab" and allow the remaining exchange buffer to enter the gel bed. Immediately following this, add the reduced mixture (prepared in Step J3) to the top of the gel bed.
5. Allow the reduced Fab' mixture to completely enter the gel.
6. Add 1 ml of exchange buffer to the top of the gel bed to elute the Fab'.
7. Begin collecting reduced Fab' into a centrifugation tube (marked in Step J2) when the first colored drop elutes (see Figure 6); collect no more than 500  $\mu$ l (to the lower marked line).



**Figure 6. Collection of the reduced Fab' fragment from the desalting column.** A. One milliliter of exchange buffer was added to the desalting column after the mixture of the reduced Fab' with the dye marker entered the column completely. B. The first stained drop is being collected in the tube marked in Step J2.

K. Desalting and collecting the activated Qdot nanocrystals

1. Uncap the desalting column labeled “activated Qdot”. Allow remaining exchange buffer to enter the gel bed. Immediately after this, add the activated Qdot nanocrystals (from Step G3) to the top of the gel bed.
2. Allow the activated Qdot nanocrystals mixture to completely enter the gel bed.
3. Add 1 ml of exchange buffer to the top of the gel bed to elute the Qdot nanocrystals.
4. When the first drop of colored material elutes from the column, begin collecting directly into the centrifugation tube containing the reduced and desalted Fab'. See Figure 7.
5. Stop collecting when the final volume reaches 1 ml (up to the top line marked in Step J2; 500  $\mu$ l of activated Qdot nanocrystals).
6. Mix briefly.



**Figure 7. Collection of the activated Qdot from the desalting column.** A. One milliliter of exchange buffer was added to the desalting column after the activated Qdots completely entered the gel. B. The first stained drop is being collected in a tube.

#### L. Conjugation reaction

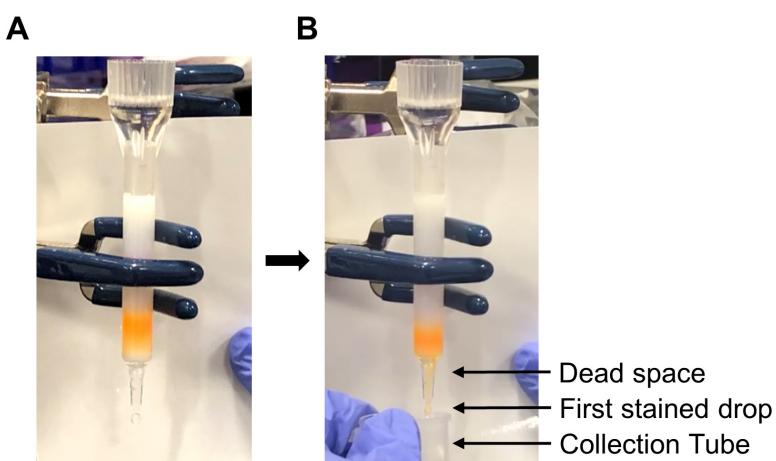
Allow the reduced Fab' and activated Qdot nanocrystals to react for 2 h at room temperature. Avoid light.

#### M. Concentrating the sample

1. Split the volume (from Step L1) into two protein concentrators.
2. Concentrate each volume to ~20  $\mu$ l by centrifuging at 4,000  $\times g$  for 15 min. If the volume is > 20  $\mu$ l after the centrifugation, continue centrifuging for another 5 min.

#### N. Separation of the Qdot-conjugated Fab' from unconjugated Fab'

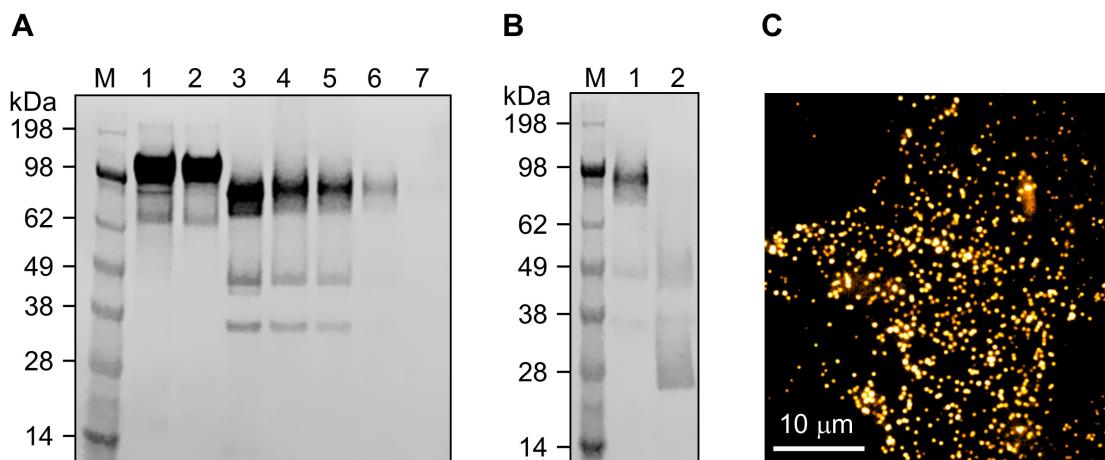
1. Uncap the separation column (from Step F6) and allow the remaining PBS to enter the gel bed by gravity. Immediately following this, add the concentrated conjugate reaction solution combined from the two protein concentrators (~40  $\mu$ l total volume) to the top of the column. Avoid disturbing the gel bed.
2. Allow the conjugate reaction solution to enter the gel and then gently add 50  $\mu$ l PBS. Let this buffer run into the gel bed.
3. Gently fill the reservoir above the column with PBS and allow the sample to elute by gravity. Visually monitor the “dead space” between the frit and the column tip.
4. When color appears in the “dead space,” collect only the first 8-10 drops of colored solution in a centrifugation tube. See Figure 8.
5. Determine the Qdot concentration by absorbance measurements at the first absorption peak of the Qdot.
6. Bring the final conjugate pool to 50% v/v of glycerol and store at 4 °C.



**Figure 8. Collection of the Qdot-conjugated Fab' from the separation column.** A. The separation column is filled with PBS after the conjugation mixture completely enters the column. B. The collection of the eluate begins when the color appears in the dead space.

#### O. Single-molecule tracking of EGFR on live cells using $\alpha$ EGFR Fab'-Qdot605 conjugates

1. Digest  $\alpha$ EGFR antibodies into F(ab')2 using pepsin [20:1 antibody/pepsin (w/w ratio)] in acetate digestion buffer (pH 4.0) at 37 °C for 16 h with agitation, followed by a Protein A column purification (Figure 9A).
2. Reduce the resulting F(ab')2 to Fab' with 5 mM cysteamine at 10 °C for 5 min (Figure 9B).
3. Conjugate Fab' with activated Qdot605 for generating  $\alpha$ EGFR Fab'-Qdot605 conjugates.
4. Allow 2 nM conjugates in the full growth medium to bind to target proteins (EGFR) for 10 min at room temperature on live cells (MDA-MB-468 breast cancer cell line that overexpresses EGFR) plated on glass bottom dishes. Wash three times with full growth medium (See Note 13).
5. Perform single-molecule tracking (SMT) (we followed the methods described in Chung and Mellman, 2015; Chung, 2017) with light excitation at 488 nm using the TIRFM on an inverted microscope with a 100x/1.49NA Plan Apo objective. Image acquisition was done at ~11 Hz using an EMCCD camera. One snapshot image of individually labeled Qdots that was rendered using ImageJ is shown in Figure 9C.
6. Estimate the binding specificity of the conjugates by determining the density of bound conjugates after saturating the epitope binding with original antibody (~100 times the Kd will suffice), relative to the density without the saturation. The binding specificity of the Fab'-QD conjugates was ~89%.



**Figure 9. Generation of Fab'-Qdot conjugates for SMT using  $\alpha$ EGFR antibodies (IgG2a).** A and B. An  $\alpha$ EGFR IgG2a was used to demonstrate the applicability of the Fab'-Qdot605 conjugates in SMT on live cells. SDS-PAGE analyses of the pepsin digestion (A) and cysteamine reduction (B) of  $\alpha$ EGFR rat IgG2a. (B) The Fab' throughput of the IgG from the reduction showed similar dependencies to changes of temperature, cysteamine concentration, and pH to those for the mouse IgG1 F(ab')2 shown in Figure 5 (see Note 11). C. One snapshot (10.72 Hz) SMT image (488 nm illumination, 100x oil objective) by a total internal reflection fluorescence microscope (TIRFM) after labeling EGFR with the  $\alpha$ EGFR Fab'-Qdot605 conjugates on ~ 60% confluent MDA-MB-468 cells.

## **Notes**

1. Procedures A-E are modified from the protocol for Pierce F(ab')2 Preparation Kit (Thermo Scientific, catalog number: 44988). Zeba Spin Desalting Columns, PBS, and NAb Protein A Plus Spin Columns used in this protocol can be found in this kit.
2. Pepsin solution should be prepared freshly for each reaction.
3. Pepsin will be irreversibly denatured in higher pH buffer. Never prepare the pepsin solution in neutral buffer.
4. When using concentrated IgG samples, simply dilute the samples to desired concentration in acetate digestion buffer and skip Procedure B.
5. Pepsin digestion varies for different IgG molecules. A time course (1-5 and 16 h) test using small aliquots of desired IgG before the preparation of F(ab')2 is recommended. If the digestion time is set to be 16 h as proposed in Procedure C, start this reaction at the end of a day and stop the reaction the next morning.
6. The purpose of changing the acetate buffer to PBS after the pepsin digestion is to terminate the reaction and to prepare the sample for Protein A purification (for removal of undigested IgG) since the Protein A column is ineffective at acidic pH.
7. Protein A column can be regenerated by following the manufacturer's instructions using IgG elution buffer (pH 2.8).
8. The digestion/reduction products can be verified using non-reducing SDS polyacrylamide gel electrophoresis as shown in Figures 2, 5, and 9, as necessary.
9. This conjugation scheme can be applied directly to a maleimide linked dye, where active maleimide can react with sulphydryl groups within the Fab'.
10. Cysteamine is sensitive to air and moisture. Store the original bottle in a vacuum desiccator. Prepare cysteamine solution right before use.
11. The reduction yield by cysteamine may vary depending on different F(ab')2 samples. A pilot test on a range of pH (5, 6, and 7), temperature (4 °C, 10 °C, 22 °C, and 37 °C), and cysteamine concentration (0.5-20 mM) is highly recommended.
12. HPLC or FPLC can be used to further purify the reduced sample for Fab'.
13. The incubation time and concentration of the conjugates can vary depending on experiments, affinity of the Fab', etc., We typically vary these in the ranges of 2-10 min (incubation time) and 0.5 to 10 nM (conjugate concentration).

## **Recipes**

1. Acetate digestion buffer, pH 4.0
  - 0.1 M acetate buffer
  - 0.01 M EDTA
2. Exchange buffer, pH 7.2

50 mM HEPES

150 mM NaCl

### **Acknowledgments**

We thank the members of the Chung Laboratory for useful discussion and Jore Kotryna Vismante for photographing the procedure. This work was supported by the GW Cancer Center and Katzen Research Cancer Research Pilot Award. The procedure introduced here was modified from past single-molecule tracking studies (Chung *et al.*, 2010; Bien-Ly *et al.*, 2014; Chung *et al.*, 2016).

### **Competing interests**

The authors declare no financial or non-financial competing interests related to this work.

### **References**

1. Betzig, E., Patterson, G. H., Sougrat, R., Lindwasser, O. W., Olenych, S., Bonifacino, J. S., Davidson, M. W., Lippincott-Schwartz, J. and Hess, H. F. (2006). [Imaging intracellular fluorescent proteins at nanometer resolution](#). *Science* 313(5793): 1642-1645.
2. Bien-Ly, N., Yu, Y. J., Bumbaca, D., Elstrott, J., Boswell, C. A., Zhang, Y., Luk, W., Lu, Y., Dennis, M. S., Weimer, R. M., Chung, I. and Watts, R. J. (2014). [Transferrin receptor \(TfR\) trafficking determines brain uptake of TfR antibody affinity variants](#). *J Exp Med* 211(2): 233-244.
3. Chen, B. C., Legant, W. R., Wang, K., Shao, L., Milkie, D. E., Davidson, M. W., Janetopoulos, C., Wu, X. S., Hammer, J. A., 3rd, Liu, Z., English, B. P., Mimori-Kiyosue, Y., Romero, D. P., Ritter, A. T., Lippincott-Schwartz, J., Fritz-Laylin, L., Mullins, R. D., Mitchell, D. M., Bembeneck, J. N., Reymann, A. C., Bohme, R., Grill, S. W., Wang, J. T., Seydoux, G., Tulu, U. S., Kiehart, D. P. and Betzig, E. (2014). [Lattice light-sheet microscopy: imaging molecules to embryos at high spatiotemporal resolution](#). *Science* 346(6208): 1257998.
4. Chung, I. (2017). [Optical measurement of receptor tyrosine kinase oligomerization on live cells](#). *Biochim Biophys Acta Biomembr* 1859 (9 Pt A): 1436-1444.
5. Chung, I., Akita, R., Vandlen, R., Toomre, D., Schlessinger, J. and Mellman, I. (2010). [Spatial control of EGF receptor activation by reversible dimerization on living cells](#). *Nature* 464(7289): 783-787.
6. Chung, I. and Bawendi, M. G. (2004). [Relationship between single quantum-dot intermittency and fluorescence intensity decays from collections of dots](#). *Physical Review B* 70(16): 165304.
7. Chung, I. and Mellman, I. (2015). [Single-molecule optical methods analyzing receptor tyrosine kinase activation in living cells](#). *Methods Mol Biol* 1233: 35-44.

8. Chung, I., Reichelt, M., Shao, L., Akita, R. W., Koeppen, H., Rangell, L., Schaefer, G., Mellman, I. and Sliwkowski, M. X. (2016). [High cell-surface density of HER2 deforms cell membranes.](#) *Nat Commun* 7: 12742.
9. Dahan, M., Levi, S., Luccardini, C., Rostaing, P., Riveau, B. and Triller, A. (2003). [Diffusion dynamics of glycine receptors revealed by single-quantum dot tracking.](#) *Science* 302(5644): 442-445.
10. Grimm, J. B., Brown, T. A., English, B. P., Lionnet, T. and Lavis, L. D. (2017). [Synthesis of Janelia Fluor HaloTag and SNAP-Tag ligands and their use in cellular imaging experiments.](#) *Methods Mol Biol* 1663: 179-188.
11. Grimm, J. B., English, B. P., Chen, J., Slaughter, J. P., Zhang, Z., Revyakin, A., Patel, R., Macklin, J. J., Normanno, D., Singer, R. H., Lionnet, T. and Lavis, L. D. (2015). [A general method to improve fluorophores for live-cell and single-molecule microscopy.](#) *Nat Methods* 12(3): 244-250, 243 p following 250.
12. Gustafsson, M. G. (2000). [Surpassing the lateral resolution limit by a factor of two using structured illumination microscopy.](#) *J Microsc* 198(Pt 2): 82-87.
13. Gustafsson, M. G., Kner, P., Chhun, B. B. and Griffis E. R. (2009). [Structured-illumination microscopy of living cells.](#) *Abstracts of Papers of the American Chemical Society* 238.
14. Hein, B., Willig, K. I. and Hell, S. W. (2008). [Stimulated emission depletion \(STED\) nanoscopy of a fluorescent protein-labeled organelle inside a living cell.](#) *Proc Natl Acad Sci U S A* 105(38): 14271-14276.
15. Joo, C., Balci, H., Ishitsuka, Y., Buranachai, C. and Ha, T. (2008). [Advances in single-molecule fluorescence methods for molecular biology.](#) *Annu Rev Biochem* 77: 51-76.
16. Kim, S. A., Heinze, K. G. and Schwille, P. (2007). [Fluorescence correlation spectroscopy in living cells.](#) *Nat Methods* 4(11): 963-973.
17. Lidke, D. S., Nagy, P., Heintzmann, R., Arndt-Jovin, D. J., Post, J. N., Grecco, H. E., Jares-Erijman, E. A. and Jovin, T. M. (2004). [Quantum dot ligands provide new insights into erbB/HER receptor-mediated signal transduction.](#) *Nat Biotechnol* 22(2): 198-203.
18. Lord, S. J., Lee, H. L. and Moerner, W. E. (2010). [Single-molecule spectroscopy and imaging of biomolecules in living cells.](#) *Anal Chem* 82(6): 2192-2203.
19. Moerner, W. E. (2012). [Microscopy beyond the diffraction limit using actively controlled single molecules.](#) *J Microsc* 246(3): 213-220.
20. Rust, M. J., Bates, M. and Zhuang, X. (2006). [Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy \(STORM\).](#) *Nat Methods* 3(10): 793-795.
21. Sekar, R. B. and Periasamy, A. (2003). [Fluorescence resonance energy transfer \(FRET\) microscopy imaging of live cell protein localizations.](#) *J Cell Biol* 160(5): 629-633.
22. Selis, F., Foca, G., Sandomenico, A., Marra, C., Di Mauro, C., Saccani Jotti, G., Scaramuzza, S., Politano, A., Sanna, R., Ruvo, M. and Tonon, G. (2016). [Pegylated trastuzumab fragments acquire an increased \*in vivo\* stability but show a largely reduced affinity for the target antigen.](#) *Int J Mol Sci* 17(4): 491.

## Time-lapse Imaging of Alveogenesis in Mouse Precision-cut Lung Slices

Khondoker M. Akram<sup>1,\*</sup>, Laura L. Yates<sup>2</sup>, Róisín Mongey<sup>2</sup>, Stephen Rothery<sup>2,3</sup>, David C. A. Gaboriau<sup>2,3</sup>, Jeremy Sanderson<sup>4</sup>, Matthew Hind<sup>2,5</sup>, Mark Griffiths<sup>2,6</sup> and Charlotte H. Dean<sup>2,4,\*</sup>

<sup>1</sup>Department of Oncology & Metabolism, University of Sheffield, Sheffield, UK; <sup>2</sup>National Heart and Lung Institute, Imperial College London, London, UK; <sup>3</sup>Facility for Imaging by Light Microscopy, NHLI, Faculty of Medicine, Imperial College London, London, UK; <sup>4</sup>MRC Harwell Institute, Harwell Campus, Oxfordshire, UK; <sup>5</sup>National Institute for Health Research (NIHR) Respiratory Biomedical Research Unit at the Royal Brompton & Harefield NHS Foundation Trust and Imperial College, London, UK; <sup>6</sup>Peri-Operative Medicine Department, St Bartholomew's Hospital, London, UK

\*For correspondence: [k.m.akram@sheffield.ac.uk](mailto:k.m.akram@sheffield.ac.uk); [c.dean@imperial.ac.uk](mailto:c.dean@imperial.ac.uk)



**[Abstract]** Alveoli are the gas-exchange units of lung. The process of alveolar development, alveogenesis, is regulated by a complex network of signaling pathways that act on various cell types including alveolar type I and II epithelial cells, fibroblasts and the vascular endothelium. Dysregulated alveogenesis results in bronchopulmonary dysplasia in neonates and in adults, disrupted alveolar regeneration is associated with chronic lung diseases including COPD and pulmonary fibrosis. Therefore, visualizing alveogenesis is critical to understand lung homeostasis and for the development of effective therapies for incurable lung diseases. We have developed a technique to visualize alveogenesis in real-time using a combination of widefield microscopy and image deconvolution of precision-cut lung slices. Here, we describe this live imaging technique in step-by-step detail. This time-lapse imaging technique can be used to capture the dynamics of individual cells within tissue slices over a long time period (up to 16 h), with minimal loss of fluorescence or cell toxicity.

**Keywords:** Imaging alveogenesis, Time-lapse imaging, Deconvolution software, Precision-cut lung slices, Lung development

**[Background]** Prenatal and postnatal lung development is classified into several distinct stages beginning with budding from the foregut endoderm followed by branching morphogenesis, sacculation and alveogenesis within surrounding lung mesenchyme. This developmental process is tightly regulated by a well-orchestrated signaling programme and cellular components (Kotton and Morrisey, 2014; Akram *et al.*, 2016). The major function of the lungs is gas exchange, which occurs via diffusion (Herriges and Morrisey, 2014). This diffusion takes place between the thin cellular layers of alveolar epithelium and capillary endothelium (Roth-Kleiner and Post, 2005). Dysregulated alveogenesis is linked with a number of neonatal and infant diseases, including bronchopulmonary dysplasia (BPD) and pulmonary hypoplasia (Kreiger *et al.*, 2006; Hilgendorff *et al.*, 2014). In adults, alveolar damage is a component of several chronic lung diseases such as chronic obstructive pulmonary disease (COPD) and idiopathic pulmonary fibrosis (IPF). Currently, there are no curative treatments for these diseases other than lung transplantation (Warburton *et al.*, 2006; Madurga *et al.*, 2013; McGowan, 2014) and

there is an unmet need to understand the mechanisms of alveogenesis in order to develop effective treatments.

In mice, sacculation begins around embryonic day 17.5 and is followed by alveogenesis, which begins within the first few days of postnatal life and is mostly completed in first month of life (Herriges and Morrisey, 2014). However, the most active phase of alveogenesis occurs in first two weeks of postnatal life with the majority of alveoli formed by postnatal day (P) 21 (Hind *et al.*, 2002; Snoeck, 2015).

Current understanding, based on static imaging experiments, is that alveogenesis occurs through repeated septation events that sub-divide primary air sacs to increase the number and surface area of alveoli (Amy *et al.*, 1977; Mund *et al.*, 2008). Real-time visualization of alveogenesis is challenging due to their location deep inside the body and the relatively slow duration of this process. A recent study used both *ex vivo* and *in vivo* live-imaging to study the sacculation stage of mouse lung development, immediately prior to alveogenesis, but these techniques are not suitable for imaging postnatal lungs (Poobalasingam *et al.*, 2017; Li *et al.*, 2018).

Precision cut lung slices (PCLS) contain intact alveoli and are increasingly used to study lung biology and disease pathogenesis (Meng *et al.*, 2008; Sanderson, 2011; Thornton *et al.*, 2012). Time-lapse imaging of PCLS has been used to show dynamic interactions of mesenchymal cells and macrophages with the extracellular matrix in adult normal and fibrotic mouse lungs, as well as in PCLS of human lungs (Burgstaller *et al.*, 2015). In addition, quantifiable *ex vivo* alveogenesis has been demonstrated in early postnatal mouse PCLS culture (Pieretti *et al.*, 2014). Using a combination of widefield microscopy and image deconvolution on postnatal mouse PCLS we have developed a method to capture the morphological mechanisms of alveogenesis in real-time (Akram *et al.*, 2019). Here we describe the detailed protocol for real-time live imaging of postnatal alveogenesis.

## **Materials and Reagents**

1. 50 ml centrifuge tubes (Thermo Fisher, catalog number: 338652)
2. Metallic spatula (Fisher Scientific, catalog number: 11523482)
3. Glass coverslips (Thermo Fisher, catalog number: 102260)
4. Probe Point (Blunt) needles, 25 G, 19 mm (0.75 inch) (Harvard Apparatus, catalog number: 725461) for P3 mice; Monoject blunt needles with Aluminum Hub, 23 G, 1 inch (Harvard Apparatus, catalog number: 722349) for P7 mice, and 21 G (Custom made from 21 G syringe needle) for P14 and adult mice (all from Harvard Apparatus UK)
5. 24-well plate (Corning® Costar® TC-Treated Multiple Well Plates) (Sigma-Aldrich, CLS3527-100EA)
6. 96-well plate (Thermo Fisher, catalog number: 249952)
7. Ibidi 24-well μ-plate (Uncoated) (Ibidi, catalog number: 82401)
8. Transwell (0.4 μm pore, 12 mm, polyester membrane) (Corning, catalog number: 3460)
9. Microscope slides, SuperFrost®, Menzel Gläser (VWR, catalog number: 631-1318)

10. Non-sterile silk black braided suture spool, 22.9 m, Size 5-0 (Harvard Apparatus UK, catalog number: 517607)
11. BD Micro-Fine+ 29 G, 1 ml Insulin Syringes (MediSupplies, catalog number: PMC3743)
12. Syringes, 5 ml (VWR International, catalog number: SART16644-E)
13. Thermo Scientific™ Nunc™ Cell Culture/Petri Dishes, 100 mm (Fisher Scientific, catalog number: 10508921)
14. Swann-Morton Surgical scalpels, No.22 (MediSupplies, catalog number: PMC0105)
15. Wet ice and ice box
16. C57BL/6 male and female mice from Charles River Laboratories
17. EpCAM-FITC (CD326) monoclonal antibody (eBioscience, catalog number: 11-5791-80; Clone G8.8)
18. Alexa-647 conjugated PECAM antibody (CD31-Alexa 647) (Biolegend, catalog number: 102416; Clone 390)
19. Laboratory tissue (Blue roll)
20. Metal flat washer (weight 1.66 g) (M8-5/16<sup>th</sup> inches diameter) (B&Q, UK)
21. Absolute ethanol (Sigma-Aldrich, catalog number: 34852-M)
22. Pentobarbitone (Pentoject, Animalcare, catalog number: XVD 132)
23. Low-melting-point agarose (Sigma-Aldrich, catalog number: A9414)
24. Hanks Balanced Salt Solution (HBSS) (1x) (Life Technologies, catalog number: 14025 -050)
25. HEPES 1 M (Life Technologies, Gibco, catalog number: 15630080)
26. Phosphate Buffered Saline (PBS) (Life Technologies, Gibco, catalog number: 20012068)
27. Dulbeccos Modified Eagle Medium (DMEM) (Life Technologies, Gibco, catalog number: 31966-021), also referred to as DMEM basal media" in the Recipes
28. Penicillin-Streptomycin (10,000 U/ml) (Life Technologies, Gibco, catalog number: 15140122)
29. MTT reagent (Thiazolyl Blue Tetrazolium Bromide) (Sigma-Aldrich, catalog number: M2128)
30. Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, catalog number: 276855)
31. 10% neutral buffered formalin (Sigma-Aldrich, catalog number: HT501128)
32. DAPI (Stock concentration 10 mg/ml) (Sigma-Aldrich, catalog number: D9542)
33. LIVE/DEAD® Viability/Cytotoxicity Kit (Thermo Fisher Scientific, catalog number: L3224)
34. Methanol (Sigma-Aldrich, catalog number: 34860)
35. ProLong® Gold Antifade Mountant (Thermo Fisher Scientific, catalog number: P36930)
36. Silicon rhodamine far-red fluorophore-conjugated DNA minor groove binder bisbenzimide (SiR-DNA) (tebu-bio Ltd, catalog number: SC007)
37. Phenol red-free DMEM with HEPES (Life Technologies, catalog number: 21063029), also referred to as Phenol red -free DMEM with HEPES basal media" in the Recipes
38. Bovine Serum Albumin (BSA) (Sigma-Aldrich, catalog number: A7030)
39. Triton X-100 (Sigma-Aldrich, catalog number: X100)
40. (Optional) para-Nitroblebbistatin (Cayman Chemical Company, catalog number: 13891)
41. (Optional) Cytochalasin D (Sigma-Aldrich, catalog number: C8273)

42. HBSS/HEPES ice cold buffer (see Recipes)
43. Agarose solution (see Recipes)
44. SF-DMEM (see Recipes)
45. Image media (see Recipes)
46. MTT working solution (see Recipes)
47. 70% ethanol (see Recipes)
48. 70% methanol (see Recipes)

## **Equipment**

1. Curved dissecting forceps, 10 cm, Serr/C (World Precision Instruments Ltd, catalog number: 15915)
2. Fine tip dissecting scissors, 10 cm, straight (World Precision Instruments Ltd, catalog number: 14393)
3. Surgical scissors, 14 cm, straight (World Precision Instruments Ltd, catalog number: 14192)
4. Spring scissors, 12 cm straight, 12 mm extra-fine blades (World Precision Instruments Ltd, catalog number: 14125)
5. Stainless steel blades for vibratome (Campden Instruments LTD, catalog number: 7550-1-SS)
6. Automated vibratome (Compressstome® VF-300-0Z; Precisionary Instruments LLC, USA)
7. Incubator (Humidified, 37 °C, 5% CO<sub>2</sub>)
8. -20 °C freezer
9. GFP filter, excitation 450-490 nm, emission 500-550 nm (for EpCAM-FITC)
10. Cy-5 filter, excitation 625-655 nm, emission 665-715 nm (for SiR-DNA and PECAM)
11. Plate reader (Tecan; Sunrise™, INSTSUN-1)
12. Zeiss Axio Observer inverted widefield microscope, with Lumencor Spectra X LED light source and Hamamatsu Flash 4.0 camera (Zeiss, Germany)
13. Zeiss LSM-510 inverted confocal microscope (Zeiss, Germany, model: LSM 510)
14. Leica DM2500 widefield microscope (Leica Microsystems, model: Leica DM2500)

## **Software**

1. Zen2 acquisition software, blue version (Zeiss, Germany)
2. ZEN 2009 (black edition) software (Zeiss, Germany)
3. FIJI (ImageJ, version 2.0)
4. Icy open source bioimaging analysis software (Version 1.9.8.0; created by the Quantitative Image Analysis Unit at Institut Pasteur, Paris, France)
5. Huygens deconvolution software (Scientific Volume Imaging, SVI, Essential version 17.10)
6. NIS-Elements (Version 4.50, Nikon Instruments, UK)
7. GraphPad Prism version 5

8. Microsoft Excel (Microsoft Office 2011 version)
9. Microsoft PowerPoint (Microsoft Office 2016 version)

## **Procedure**

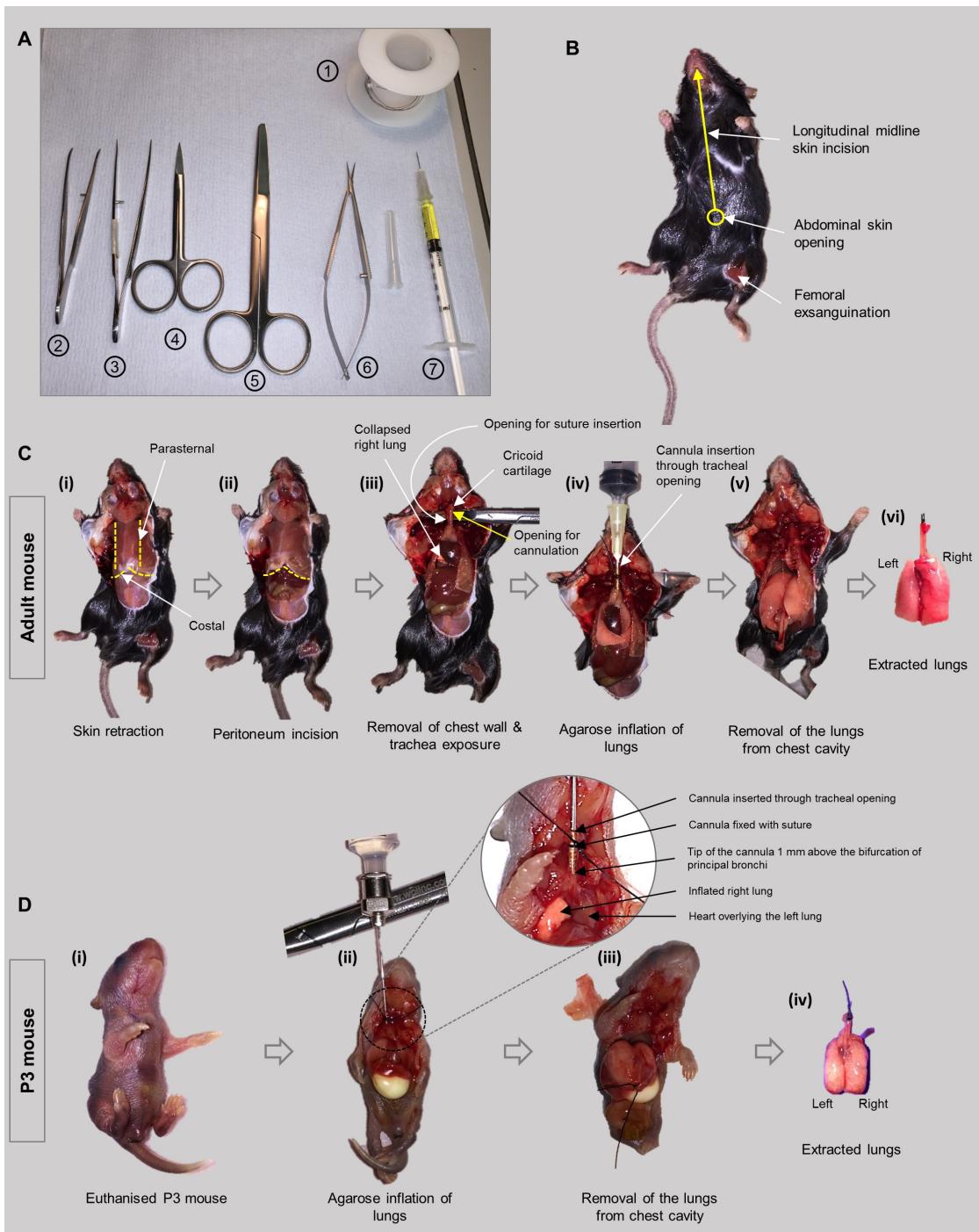
### A. Producing precision-cut lung slices

1. Mouse dissection and lung harvesting
  - a. Humanely kill postnatal day 3 (P3), P7, P14 or adult (6-8 weeks) C57BL/6 mice by intraperitoneal injection of pentobarbital (50 mg/kg body weight). Inject 25 µl of pentobarbital for P3, 50 µl for P7, 75 µl for P14 and 100 µl for adult mice using 29 G, 1 ml Insulin Syringes (Figure 1A7). Mice will die in 5-10 min after injection. Confirm death by exsanguination of femoral artery.
  - b. Place the mouse on a dissection tray in supine position. Spray 70% ethanol over the anterior aspect of the abdomen, chest and neck.
  - c. Using curved forceps (Figure 1A2) to pinch the skin from the anterior abdominal wall and gently pull it up, cut a small piece of skin leaving the inner peritoneum intact.
  - d. Using a pair of fine scissors (Figure 1A4) make a longitudinal incision along the midline of anterior abdomen and chest wall and extend it up to the chin (Figure 1B).
  - e. Using two pairs of curved forceps, retract the skin on both sides to expose the anterior aspects of the abdomen, chest wall and neck. (Figure 1Ci) (Video 1).
  - f. Using curved forceps carefully lift the peritoneal wall at the upper part of the abdomen, just below the costal margin and make a small opening using the tip of the fine scissors.
  - g. Extend the opening to the right and left using fine scissors. This will expose the diaphragm.
  - h. Gently lift the lower end of the sternum and make an opening on the upper part of diaphragm using the tip of the fine scissors. Then extend the opening to both side of the diaphragm along the inner surface of the costal margins (Figure 1C i, ii, yellow dotted line). At this stage you will be able to see pale pink colored right and left collapsed lungs (Figure 1C iii, arrow).
  - i. Lift the chest wall by holding the lower end of the sternum with curved forceps and carefully make a parasternal incision (to the left and right) to separate anterior chest wall from the body (Figure 1C i, iii, yellow dotted line) (Video 1).
  - j. Using curved forceps and fine scissors, remove muscle and fascia from the anterior and lateral aspects of the neck wall.
  - k. Using curved forceps, make an opening between the posterior aspect of the trachea and the anterior wall of the esophagus at the midpoint of the neck (Video 1).
  - l. Insert a piece of sterile suture through the opening and place it perpendicular to the trachea at its midpoint (Figure 1).

- m. Make a small opening in the anterior wall of the trachea just below the cricoid cartilage using fine tipped straight spring scissors (Figure 1A, (6), 1C (iii) yellow arrow, Figure 1D (ii) inset).
  - n. Carefully insert a rigid metallic cannula 25 G for P3, 23 G for P7 and 21 G for P14 and adult mice through the trachea up to a millimeter above the bifurcation of the principal bronchi and fix in place with a suture (Figure 1C (iv) and 1D (ii) inset). The suture was tied, with moderate tightness, around the trachea including cannula inside, by applying two knots.
  - o. After cannulation, inflate the lungs by gently injecting 1.5% low-melting-point agarose prepared with HBSS/HEPES buffer warmed to 37 °C (see Recipes). Use a 5 ml syringe to instill the agarose and keep the syringe attached to the cannula until the agarose solidifies. Inflate both lungs keeping them *in situ* within the chest cavity using 0.2 ml for P3, 0.275 ml for P7, 0.35 ml for P14 and 1 ml for adult lungs. These volumes enable the lungs to be fully inflated without hyper- or sub-optimal inflation (Figures 1C and 1D).
  - p. After inflation, place a piece of double layered laboratory blue roll over the exposed chest wall and put some wet ice on to the blue roll to solidify the agarose.
- Note: Depending on mouse age, this will take 1 to 2 min.*
- q. Remove the cannula by gently pulling it out from the trachea and tighten the suture knot.
  - r. Excise the trachea from its upper (mouth) end. Lift the trachea by holding the free ends of the suture and gently remove the entire heart and lungs from the chest cavity (Figures 1C and 1D).
  - s. Immerse the whole heart and lungs in ice-cold serum-free DMEM (SF-DMEM) in a 50 ml centrifuge tube, secure the cap and store on wet ice until slicing. The tissue can be kept on ice for 2-3 h.



**Video 1. Mouse dissection: from skin incision to trachea mobilization.** (All animal maintenance and procedures were carried out according to the requirements of the Animal (Scientific Procedures) Act 1986. Animal work was approved by the South Kensington and St. Mary's AWERB committee, Imperial College London.)



**Figure 1. Mouse dissection and lung harvesting workflow.** A. Dissection equipment: 1. Suture, 2. Curved forceps, 3. Fine tip forceps, 4. Fine tip dissecting scissors, 5. Surgical scissors, 6. Spring scissors, 7. Pentobarbital in 1 ml syringe attached with hypodermic needle. B. Skin incision line. C. Adult mouse dissection, agarose inflation and harvesting of lungs. D. Postnatal day 3 (P3) mouse dissection, agarose inflation and harvesting of lungs.

## 2. Precision-cut lung slicing

- a. Prepare the automated vibratome: Sterilize the buffer tray of the vibratome thoroughly by spraying with 70% ethanol and leave to dry (Figure 2A). Attach vibratome blade to the blade holder using super glue and leave to dry. Sterilize specimen holder and specimen tube using 70% ethanol spray and air dry. Place the syringe chilling block in -20 °C freezer for 15-30 min to cool it down prior to use (Figure 2B).
- b. Have ready approximately 30 ml of 1.5% agarose gel made with HBSS/HEPES at 37 °C temp. Prepare 200 ml of sterile ice cold 1x HBSS/HEPES buffer and keep on ice. Prepare a 24-well plate with 1 ml ice cold, sterile SF-DMEM per well and keep on ice.
- c. In a 10 cm diameter Petri dish, separate the left lung lobe from the left bronchus using a scalpel blade (Figures 2C and 2D).
- d. Excise a tiny section of tissue from the basal end of the lung using the scalpel blade (Figure 2D; outline of the tissue excised is marked with dotted line).

*Note: This helps the lobe to sit on the specimen holder vertically.*

- e. Place the lung lobe on a piece of clean laboratory blue roll to soak up excess media (Figure 2D).
- f. Place a tiny drop of super glue onto the cutting end of the specimen holder (Figure 2Ei) and gently place the cut end of the lung lobe onto the glue drop, hold it vertically with curved forceps until the lung lobe tightly adheres to the specimen holder (Figure 2Eii).

*Note: It takes 15-30 s to attach the lung lobe onto the specimen holder.*

- g. Insert the specimen holder into the metal specimen tube, hold it vertically keeping the lung lobe inside the tube and facing vertically up (Figure 2Eiii).

- h. Using a 5 ml syringe, fill the specimen tube with 37 °C warm 1.5% agarose so that the entire lobe is submerged in agarose (Figure 2Eiii).

- i. Place the cold syringe chilling block (Figure 2B) around the specimen tube and hold it in place until the agarose tissue block solidifies (Figure 2Eiv).

*Note: It takes about a minute to solidify the agarose.*

- j. Insert the specimen tube into the buffer tray and align blade and cutting end of the agarose tissue block.

- k. Fill the buffer tray with ice cold HBSS/HEPES buffer. Set the desired thickness of tissue slices to 300 µm. Set the cutting speed to number 5 and cutting oscillation frequency to 5. Operate the vibratome in ‘continuous mode’.

- l. Start collecting transverse precision-cut lung slices (PCLS). Remove the lung slices from the first 1/3<sup>rd</sup> of the lung lobe from the buffer tray using a small metallic spatula. Collect the slices from the middle 1/3<sup>rd</sup> of the lobe and place them in the wells of a 24-well plate containing ice cold SF-DMEM (Figure 2F). The middle third of the lung lobe provides almost equal sized slices. Take 1 lung slice in each well in 1 ml media. (Using this technique, a P3 left lung provides 12 slices and an adult lung provides approximately 36 slices.)

*Note: It is important to use equal sized slices for imaging and metabolic assays, the remaining slices can be used for additional assays, e.g., immunostaining, protein or RNA extraction.*

- m. Incubate PCLS at 37 °C in the presence of 5% CO<sub>2</sub> for 2 h and then wash twice with warm SF-DMEM to remove excess agarose from around the tissue in sterile conditions, under the flow hood.

*Note: Some agarose will remain in the airspaces within the lungs and this is important to retain lung architecture during culture.*

- n. Incubate the PCLS for a further 1 h in SF-DMEM at 37 °C in the presence of 5% CO<sub>2</sub>. From this point the PCLS are ready for live imaging and further experimentation.

*Note: Use the samples immediately for imaging (and migration analysis); but slices can be kept in culture overnight for other experimentation.*

## B. Live cell imaging of lung alveologenesis

### 1. Label-free, bright-field live imaging

To visualize gross structural changes in lung slices over time, live bright-field imaging can be performed as follows:

- a. Use phenol red-free DMEM and ibidi 24-well μ-plate for live imaging.
- b. Prepare transwells for imaging: using scissors cut and remove the rim of the transwell so that the transwell filter comes into contact with the lung slice at the bottom of the well (Figure 2G). Wash the transwells with HBSS (warm) to remove debris and place them in unused wells of ibidi 24-well μ-plate without buffer.
- c. Add 50 μl of warm (37 °C) phenol red-free SF-DMEM to the center of a well of the ibidi μ-plate.
- d. Using a thin sterile metallic spatula take a PCLS from Step A2n above and carefully place at the center of the well of ibidi μ-plate. Make sure that the lung slice is placed in the center and is flat and not folded.
- e. Then gently place the modified transwell on top of the PCLS.
- f. Add 500 μl phenol red-free SF-DMEM to the upper chamber and 300 μl to the bottom chamber of the transwell.
- g. To keep the PCLS in place, put a 1.66 g metal flat washer with a central hole on top of the transwell housing (Figures 2H and 2I).

*Note: This weight puts light pressure on tissue slice through the permeable transwell membrane, which holds the slice in place without damaging the tissue. The metal flat washer weight should not exceed 3.32 g.*

- h. Incubate the PCLS at 37 °C in presence of 5% CO<sub>2</sub> and 95% air (~21% O<sub>2</sub>) in a humidified incubator for 2 h. This incubation step allows the lung slice to settle down prior to image acquisition.

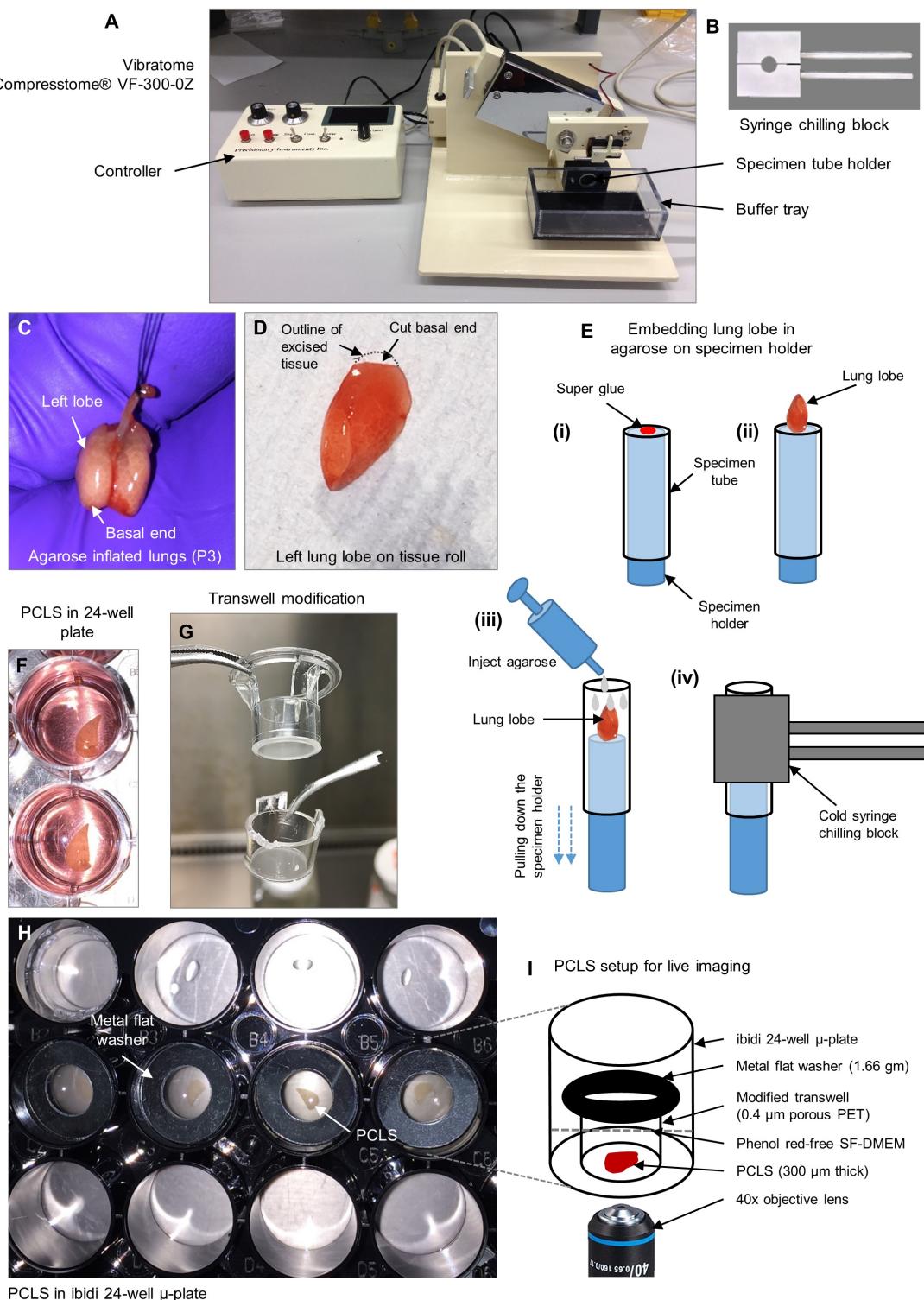
*Note: Similar sized PCLS should be allocated for MTT viability assay after live images, as shown in Figure 3.*

- i. At the same time, humidify and pre-equilibrate the incubator chamber of an inverted Zeiss Axio Observer widefield epifluorescence microscope (or similar) for 30 min to 1 h with the following conditions: 37 °C, 5% CO<sub>2</sub> and room air oxygen levels, approx. 21%.
- j. After incubation and pre-equilibration transfer the ibidi 24-well plate containing PCLS to the microscope incubator and maintain the incubator chamber conditions as above throughout the entire time-lapse duration.
- k. Start capturing time-lapse images using a long working distance 40x (0.7 NA, air) objective lens under bright-light (Figure 2l).
- l. For bright-field imaging, find the best focused plane at the region of interest and keeping this point as the center select 30 z-slices towards the upper surface of the slice and 30 slices towards the bottom surface of the slice with 1 µm step-gap between the slices (a total of 60 slices in the middle 60 µm z-axis of tissue).
- m. Set the time-lapse image capture interval to every 15 min for 12-19 h.

*Note: Four images per hour (15 min interval) reduces light exposure, hence minimizes fluorophore bleaching but produces smooth time-lapse video with 10-12fps (see, Supplementary Movie 2 in Akram et al, 2019).*

- n. Select 4 separate fields of interest within alveolar regions, avoiding airways, from the four quadrants of a PCLS.

*Note: The 2 h incubation and pre-equilibration of the microscope incubator are crucially important to avoid the plane of focus drifting during time-lapse image acquisition. Image 4 separate PCLS per experiment.*



**Figure 2. Precision-cut lung slicing and live imaging setup.** A. Lung slicer: Vibratome, Compresstome® VF-300-0Z. B. Syringe chilling block for solidifying agarose. C. Agarose inflated lungs harvested from a P3 mouse. D. Separated left lung lobe on laboratory tissue roll. E. Schematic diagram showing agarose embedding of left lung lobe on specimen holder for making PCLS. F. Precision-cut lung slices are in 24-well plate. G. Modification of transwell for stabilizing lung slices on well plate. H. Precision-cut lung slices are set in ibidi 24-well  $\mu$ -plate

using modified transwells and metal flat washer (black rim). I. Schematic showing the relative position and alignment of metal flat washer, transwell, lung slice, and 40x long working distance air objective lens on an inverted microscope for live imaging.

## 2. Live fluorescence imaging of immunostained PCLS

### **Dual staining of PCLS for live imaging**

- a. To visualize and track epithelial cells within the PCLS, dual stain cells with FITC-conjugated EpCAM antibody (EpCAM-FITC) and SiR-DNA. (EpCAM selectively labels epithelial cell membranes. SiR-DNA is silicon rhodamine far-red fluorophore-conjugated DNA minor groove binder bisbenzimidide and stains the nuclei of cells).
- b. Incubate PCLS (from Step A2n) for 1 h at 37 °C with EpCAM-FITC antibody diluted 1:200 and SiR-DNA diluted 1:300 in DMEM (without shaking). Use 500 µl of antibody/SF-DMEM media for each well per PCLS.
- c. To visualize epithelial and endothelial cells within the alveolar region, incubate PCLS for 1 h at 37 °C with dual Alexa-647 conjugated PECAM antibody and EpCAM-FITC antibody both at 1:200 in 500 µl SF-DMEM per sample per well.
- d. After incubation, wash the PCLS three times with warm SF-DMEM.

### **Time-lapse fluorescence imaging of PCLS**

- e. Image media: Prepare image media by adding EpCAM-FITC antibody at 1:500 and SiR-DNA at 1:1000 for epithelial cells or PECAM at 1:500 along with EpCAM-FITC (1:500) for dual-labeling of epithelial/endothelial cells in 800 µl phenol red-free SF-DMEM for each PCLS per well.

*Note: SiR-DNA at 1:1000 dilution can be used as a nuclear marker for cells that are dual labeled with epithelial or endothelial markers.*

- f. Place a 50 µl drop of image media at the center of a well in an ibidi 24-well µ-plate and carefully place a dual stained PCLS onto the image media using a thin metallic spatula.
- g. Place a modified transwell on top of the PCLS, add 500 µl of image media to the upper chamber and 300 µl image media to the bottom chamber and finally stabilize the transwell by placing a flat metal washer onto the transwell as described above (Figures 2H and 2I).
- h. Incubate the PCLS at 37 °C in presence of 5% CO<sub>2</sub> and 95% air in a humidified incubator for 2 h. This incubation step intensifies the staining and allows the lung slice to settle down prior to image acquisition.
- i. Pre-equilibrate the microscope incubator chamber as described for bright-field imaging (as Step B1i).
- j. After incubation and pre-equilibration transfer the 24-well plate to an inverted Zeiss Axio Observer widefield epifluorescence microscope incubator and maintain the incubator conditions as above throughout the time-lapse duration.

- k. Start recording time-lapse images using a long working distance 40x (0.7 NA, air) objective lens using a GFP filter, excitation 450-490 nm, emission 500-550 nm (for EpCAM-FITC) and Cy-5, excitation 625-655 nm, emission 665-715 nm (for SiR-DNA and PECAM) from 4 fields of alveolar regions per slice for 8-19 h at 15 min intervals. Capture 11 images from the middle 11 µm thickness of tissue along the z-axis with a 1 µm step-gap between each slice to make a z-stack from each PCLS.

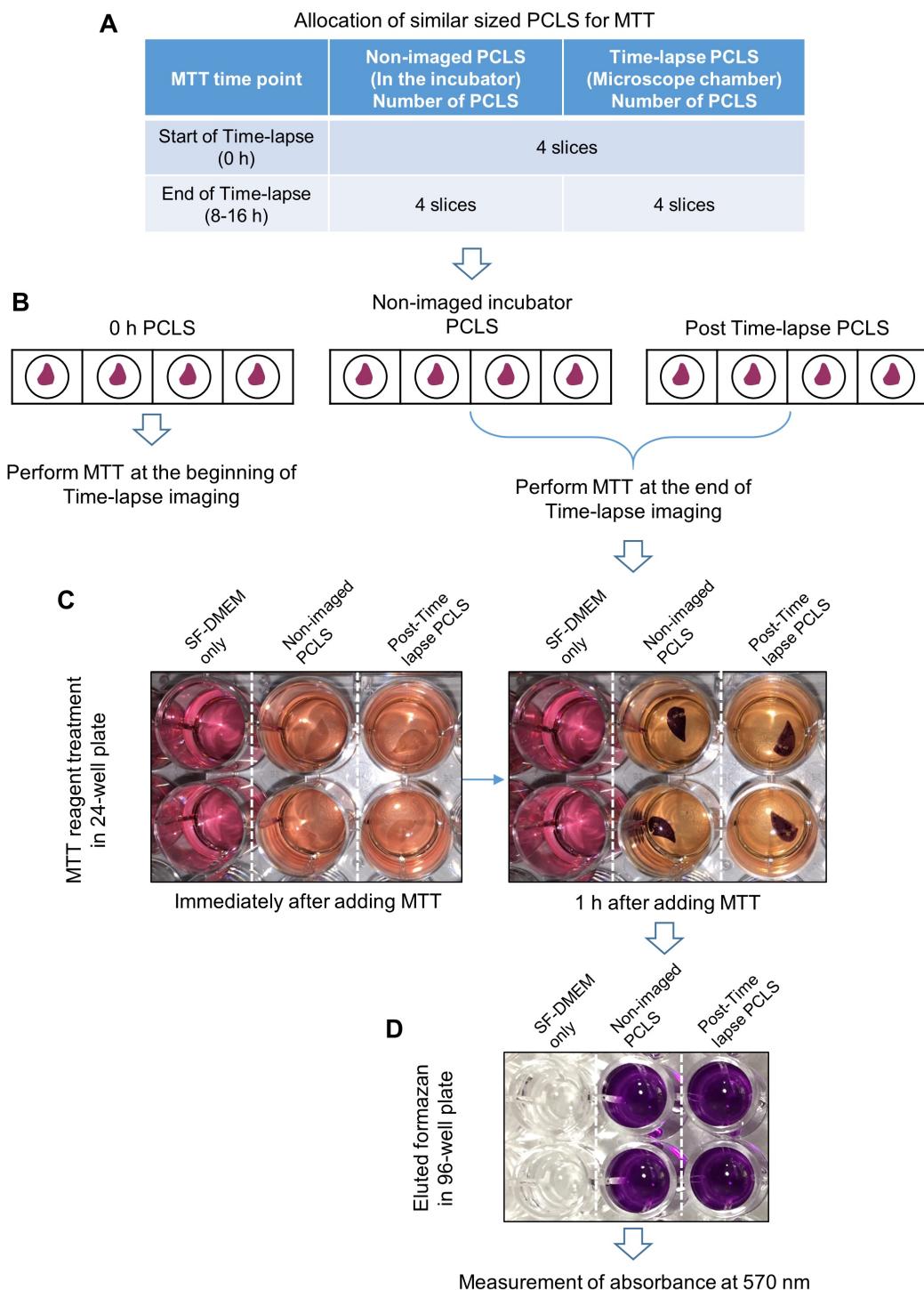
Notes:

- i. *Perform time-lapse imaging on four PCLS at a time per experiment. Do not capture bright-field images and fluorescent images from the same PCLS within an experiment. Exposure of bright light significantly bleaches the intensity of fluorescent staining. Do not use a UV or DAPI filter (excitation 358 nm and emission 463 nm) for live imaging of PCLS as this causes significant cell death. Zen2 acquisition software, blue version was used for both brightfield and fluorescence live imaging.*
- ii. *During live cell imaging, cellular dynamics or other behaviors can be manipulated by adding various factors such as cytokines or small molecules inhibitors into the image media. The resulting cell responses can be recorded in real-time. For example, we recently added the actin cytoskeleton modifiers Blebbistatin and Cytochalasin-D to PCLS and assessed their effects on cell migration using this live-PCLS imaging technique (Akram et al., 2019).*
- iii. *To ensure the viability of PCLS, perform a viability assay on PCLS after every time-lapse live imaging experiment. For bright-field imaging on unlabeled PCLS perform Live/Dead staining and for fluorescence imaging on dual stained PCLS, perform an MTT assay.*
- iv. *Duration of experiment p: From mouse dissection to the start of time-lapse imaging takes 7-8 h.*

### C. Cell viability assays on PCLS after live cell imaging experiments

1. MTT assay
  - a. Place four PCLS that have undergone live imaging into an ibidi 24-well µ-plate, 1 slice per well. Place four similar sized, non-imaged PCLS that have been kept in an incubator with 37 °C, 5% CO<sub>2</sub> for the same duration as the imaged slices in a conventional 24-well plate, 1 slice per well in phenol red-free SF-DMEM. Four similar-sized slices were selected to perform MTT assay to determine cell viability at the start of time-lapse imaging experiments (Figure 3).
  - b. To assess metabolic activity within cells at the initiation of live imaging (0 h control), add 500 µl of 10% MTT solution (Stock Con. 5 mg/ml) made with SF-DMEM to each well.  
*Note: This MTT needs to be done before starting live imaging (Figure 3B).*
  - c. Incubate the PCLS at 37 °C in presence of 5% CO<sub>2</sub> for 1 h (Figure 3C).
  - d. Remove the MTT media from the wells.

- e. Solubilize the formazan crystals formed within the viable cells by adding an equal volume of DMSO (500 µl) and incubating at 37 °C for 10 min.
- f. Take 200 µl of eluted formazan solution from each PCLS and place into individual wells of a 96-well plate (Figure 3D).
- g. Measure the absorbance (OD) at 570 nm and correct at 690 nm using a plate reader.
- h. To assess metabolic activity within post-time lapse PCLS, carefully remove the transwells after completion of time-lapse imaging. If PCLS adhere to the under surface of the transwell PET membrane, flush with warm HBSS using a pipette, to detach it.
- i. Wash the PCLS with warm HBSS once.
- j. Then perform MTT assay on PCLS undergone time-lapse imaging and non-imaged PCLS cultured in a conventional incubator in the same way as described for initial time-point control PCLS above.
- k. Compare the initial time-point (0 h) and post-time lapse (Imaged and non-imaged PCLS) OD values to evaluate cell viability after time-lapse imaging (For examples, see Figure 1 j in reference Akram *et al*, 2019).



**Figure 3. MTT assay workflow.** A. Allocation of PCLS for MTT assay. B. Plating of PCLS in 24-well plates for treating with MTT solution. C. PCLS are treated with MTT solution. D. Eluted formazan in wells of a 96-well plate.

## 2. Live/Dead assay

- a. Perform cell viability assay on PCLS using LIVE/DEAD® Viability/Cytotoxicity Kit.

- b. Place four PCLS into an ibidi 24-well  $\mu$ -plate for time-lapse imaging and four PCLS into a conventional incubator in a 24-well plate to culture for the same duration as the time-lapse without imaging, in 500  $\mu$ l phenol red-free SF-DMEM. Place four PCLS into an ordinary 24-well plate to perform Live/Dead assay to evaluate viability at the initial time-point (0 h).
- c. For the non-imaged slices initial time-point (0 h), remove phenol red-free SF-DMEM and incubate PCLS with 2  $\mu$ M Calcein AM and 2  $\mu$ M Ethidium homodimer-1 (EthD-1) in 250  $\mu$ l warm HBSS for 30 min at 37 °C, 1 PCLS per well.
- d. Wash the PCLS two times with HBSS and fix with 10% neutral buffered formalin for 30 min at room temperature (RT). Wash twice with HBSS and store the slices in HBSS at 4 °C prior to imaging.
- e. At the end of time-lapse imaging, remove transwells from each well as described for MTT assay and wash once with HBSS. Then perform Live/Dead assay on both time-lapse imaged and non-imaged PCLS, *i.e.*, those cultured in conventional incubator for same duration as time-lapse, as above.
- f. As a positive control for dead cells, treat PCLS with 70% methanol for 30 min at RT and incubate with Calcein AM and EthD-1 as described above.
- g. Mount the PCLS on glass slides with a drop of ProLong® Gold Antifade Mountant and cover with a glass coverslip. Allow 1 h for mountant to set at room temperature.
- h. Capture images using a Zeiss LSM-510 confocal microscope with a 20x 0.8 NA air objective and ZEN 2009 (black edition) software (or equivalent).

#### D. Cell tracking for cell migration quantification using Icy software

1. Open the Icy software and import an EpCAM-FITC, SiR-DNA labeled raw time-lapse image sequence file.
2. In the FITC channel, select the best focused plane, *i.e.*, a single slice from the z-stack of the image sequence (Figure 4B).
3. Select the desired time duration you wish to conduct cell tracking for (*e.g.*, 8–16 h).
4. Use the Spot detection and Tracking tools (under the Detection/Tracking tab) to detect EpCAM positive epithelial cells and track them throughout the pre-defined time-lapse duration.
5. To detect EpCAM-FITC positive cells, set the Spot detector parameters as follows on full frame image: (a) Detect bright spot over dark background option, (b) Size of spots 7 and 13 pixels with 100% sensitivity, (c) Filtering parameter with 100–200 (adjust accordingly to correctly spot the individual cell), (d) Enable swimming pool option under the Output tab. Then Click 'Start detection'. Check that the detector is identifying individual cells (which will be marked by red circle). If necessary correct spot detection by altering the Pixel size and Filtering parameters.
6. After optimization of spot detection, click 'Run Tracking' to quantify the cell migration. Quantify cell migration on X-Y axis only.

*Note:* 3D X-Y-Z axis migration quantification does not work accurately under this setting.

7. After tracking, check every cell migration trail in each image field by rewinding the video sequence on Tracking mode a few times to see if there is any off-target tracking. If there is any off-target tracking, deselect these trails. Off-target tracking can occur when a trail is falsely generated without the presence of a migrating cell (Figure 4B).

*Note: This manual checking is crucial as under this setting Icy often tracks off-target objects. For cell tracking do not perform batch processing, it does not work under this setting. Track cells of each field from each PCLS individually slice by slice and by adjusting the detection parameters with post-tracking off-target screening for each video sequence.*

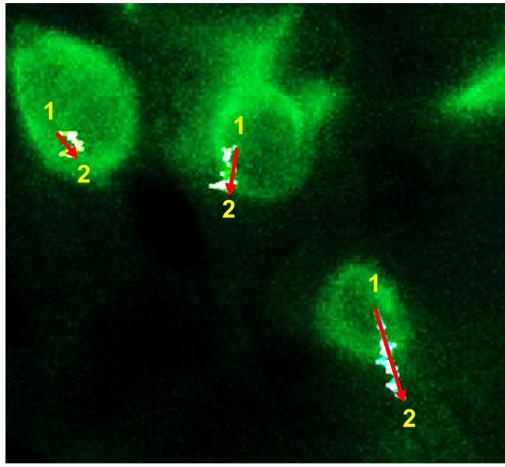
8. After completion of tracking add ‘Motion profiler processor’ to the ‘Track processor’ option under Track manager. Use Real unit option ( $\mu\text{m}/\text{s}$ ) for migration and speed quantification from Track manager (Figure 4A).
9. Export data as Excel file and the cell tracking trace as image file (Figures 4A and 4C).

*Note: For an example of generated cell tracking data, and cell migration data, see Figures 2 h, i, j in the reference Akram et al, 2019.*

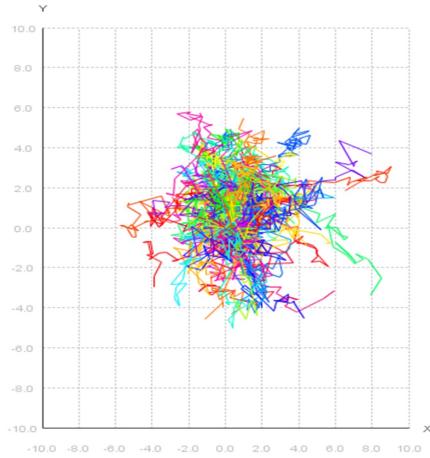
**A** Raw tracking data from PCLS generated by Icy Track Manager

Track #	Start (sec)	End (sec)	Duration (sec)	Total disp. ( $\mu\text{m}$ )	Net disp. ( $\mu\text{m}$ )	Linearity (%)	Search radius ( $\mu\text{m}$ )	Min. disp. ( $\mu\text{m}$ )	Max. disp. ( $\mu\text{m}$ )	Avg. disp. ( $\mu\text{m}$ )
0	0	35119.95079	35119.95079	26.20123654	7.970668236	0.304209621	8.677481336	0	1.903863667	0.671826578
1	0	8104.604028	8104.604028	3.373595174	0.4646013066	0.137542604	1.735843965	0	1.573578132	0.374843908
2	0	19811.25429	19811.25429	13.36337406	6.623777876	0.495666577	7.852193063	0	1.702004332	0.607426094
3	0	2701.534676	2701.534676	2.699838612	1.062654829	0.393599389	1.849825406	0.678547166	1.218609324	0.899946204
4	0	57632.73976	57632.73976	35.42465347	0.550443674	0.015538435	5.02823729	0.042673563	1.86683364	0.553512021
5	0	2701.534676	2701.534676	0.625056848	0.535504869	0.056729864	0.535504869	0	0.341335908	0.208352283
6	0	31517.90455	31517.90455	17.90347204	6.168968654	0.344568285	6.972168065	0	1.65885636	0.511527773
7	0	25214.32364	25214.32364	17.30381385	3.649085188	0.21088329	5.969618408	0.123188475	1.558778192	0.617993352
8	0	25214.32364	25214.32364	14.68911037	9.227136749	0.628161714	9.227136749	0	1.752608886	0.524611085
9	0	40523.02014	40523.02014	26.83045556	4.872970318	0.181620856	5.563616349	0	1.887064907	0.596232346
10	0	9005.11587	9005.11587	6.646068757	4.51769898	0.679755074	4.51769896	0.203104818	1.156167949	0.664606876
11	0	38721.99702	38721.99702	23.04243289	7.10545459	0.308363905	8.697476383	0	1.722035819	0.535870532
12	0	27015.34676	27015.34676	13.23004643	4.570772626	0.345484247	4.570772626	0.107220822	1.414172307	0.441001548
13	0	57632.73976	57632.73976	29.58503195	6.493759868	0.219494773	7.084983383	0.022155376	1.150017738	0.462266124
14	0	57632.73976	57632.73976	37.18877155	2.604747504	0.070041235	5.794749124	0.033241951	1.807997089	0.581074555
15	0	27015.34676	27015.34676	16.48518422	5.009527469	0.303880588	5.987549555	0	1.671819528	0.549506141
16	0	45025.57794	45025.57794	26.66925432	7.098925801	0.266183888	7.287350019	0.0909098047	1.151671909	0.533385086
17	0	16209.20806	16209.20806	9.134546323	3.390459219	0.371168868	3.832283762	0.087667844	1.144406594	0.507474796
18	0	8104.604028	8104.604028	3.467275496	2.076077511	0.598763356	2.076077511	0.03474825	1.17021666	0.385252833
19	0	23413.30053	23413.30053	19.22487924	5.689343808	0.295936517	6.795428465	0.07027675	1.744740278	0.739418432
20	0	57632.73976	57632.73976	28.33367513	5.489711931	0.193752201	7.310488065	0	1.152684948	0.442713674
21	0	57632.73976	57632.73976	38.88462362	5.812100931	0.149470418	7.153161156	0.049457979	1.897228322	0.607572244
22	0	41423.5317	41423.5317	30.2755919	1.849601123	0.061092154	3.734777679	0.197272005	1.872613416	0.658165041

**B** Cell migration trails tracked by Icy Track Manager



**C** Cell tracking trace produced by Icy Track Manager



**Figure 4. Cell tracking by Icy.** A. Raw cell tracking data from P3 PCLS generated by Icy Track Manager (22 out of 118 cell tracking data presented). B. Cell migration trails of 3 EpCAM-FITC positive cells from P3 PCLS tracked by Icy Track Manager showing 3 cells migrated from position 1 to position 2 in 16 h (zoomed raw image). Red arrows indicate the linear ‘Net cell migration’. C. Cell tracking trace produced from 1 full-frame visual field from a P3 PCLS by Icy Track Manager.

E. Image deconvolution and movie generation

1. Image deconvolution using Huygens deconvolution software

*Note: The raw fluorescent images obtained by widefield microscopy from 300  $\mu\text{m}$  thick PCLS are hazy and unsuitable for individual cell observations due to the limitations of widefield imaging, and light diffraction caused by sample thickness. Deconvolution software uses an algorithm to eliminate out-of-focus light and produce sharper images. (See Figure 1 i and Supplementary movie 2 in reference Akram et al., 2019).*

- a. Upload the widefield z-stack of time-lapse fluorescent images to the Huygens deconvolution software (Step B2k). Each z-stack consists of 11 separate z-slices per sample, with a 1  $\mu\text{m}$  step between slices.

*Note: The optimum step-gap between slices is dependent on the objective lens and requirements of the deconvolution software. For Huygens deconvolution software for 40x objective lens optimum step-gap is no more than 1  $\mu\text{m}$ . This configuration may vary for other deconvolution software packages. Optimizing the step-gap is crucial to generate confocal-like, deconvolved images.*

- b. Deconvolve the image sequence using the built-in automated CMLE algorithm with the following parameters: (a) Maximum iterations 50, (b) Signal to noise ratio 40, (c) Quality threshold 0.01, (d) Iteration mode Optimized.
  - c. Save the deconvolved time-lapse image sequence as a TIFF z-stack image sequence file for 2D and 3D time-lapse video generation.
2. 2D and 3D and bright-field video generation
- a. For 2D video generation, import deconvolved, time-lapse z-stacks into Icy software (Step E1c). Select the best-focused single z-plane, adjust the brightness and contrast and crop the area of interest. Save the file as .avi file format.
  - b. For 3D video reconstruction, import deconvolved z-stack time-lapse files into NIS-Elements and align to correct for X-Y drift (Step E1c). Generate 3D videos using the 'Volume View' and 'Movie Maker' modules of NIS-Elements.
  - c. For 2D brightfield video generation, import raw image z-tack files into Fiji (ImageJ) software. Select the best-focused single z-plane, adjust brightness and contrast. Save the file as .avi video file.
  - Note: Brightfield images do not need deconvolution.*
  - d. For annotation on 2D fluorescent and brightfield videos, insert the .avi video files on Microsoft PowerPoint (MS Office 2016) as video file, resize the slide size to match with the video window dimensions, annotate the video accordingly using Text and Shape tools. Export the PowerPoint file as high resolution .mp4 movie file. (See supplementary movies in Akram *et al.*, 2019).

## Data analysis

### A. Quantification of cell behaviors

1. To quantify cellular events, such as cell clustering, hollowing, septation and cell extension during post-natal alveogenesis in PCLS carefully examine deconvolved and brightfield time-lapse video files (using 40x objective) on screen. Record the number of times each event is observed in a single field of view from a video file. Examine videos from at least 4 different fields from different PCLS and from 3 independent experiments.

2. Present data as the frequency of each type of cellular behavior as the total number of observations of that behavior per field. (For examples of results, see Results section, pages 4-7 in Akram *et al.*, 2019)

#### B. Quantification of cell migration

1. Track manager in Icy produces a range of cell migration information from the tracking data including: Net cell migration, Total displacement, migration speed (Figure 4A).
2. To quantify and compare epithelial cell migration between different postnatal age groups (e.g., P3, P7, P14 and Adult) or different treatment conditions determine (a) Net cell migration and (b) the net distance traveled by individual cells within a specified time-period. Net cell migration is the mean value calculated to present how much linear distance in the X-Y axis (Initial point A to end point B) a cell migrates. A mean value of migrated distances of the total number of cells from each field is presented as mean net cell migration. (For example of results, see Figure 2 h, i, j in Akram *et al.*, 2019).  
*Note: For migration analysis use 'Net Displacement' not 'Total Displacement' data from cell tracking record (Figure 4A, yellow highlight).*
3. There are two groups of epithelial cells present in the lung slices: one group of cells are sessile, and other group of cells are highly motile. To differentiate these two groups and to compare them between different postnatal age groups and treatment conditions, determine the net distance traveled by individual cells. Rank them and present as percent or proportion of cells that migrate the ranked distances (See Figure 2 h-j and Figure 6 k-n in Akram *et al.*, 2019).

#### Recipes

1. HBSS/HEPES ice cold buffer  
1x HBSS  
1% HEPES 1 M
2. Agarose solution  
1.5 g low-melting point agarose powder  
100 ml HBSS/HEPES buffer  
Dissolve agarose in HBSS/HEPES buffer in microwave. Prepare 30 ml agarose for processing  
1 lung
3. SF-DMEM  
DMEM basal media  
1% Penicillin-Streptomycin
4. Image media  
Phenol red-free DMEM with HEPES basal media  
1% Penicillin-Streptomycin  
EpCAM-FITC antibody 1:500

- PECAM antibody 1:500 or SiR-DNA 1:1,000
5. MTT working solution
  - DMEM basal media
  - 10% MTT stock solution (Stock Con. 5 mg/ml in DMSO)  
*Note: Stock solution is stable for 1 year when stored at -20 °C in a dark container.*
  6. 70% ethanol
  - 70 ml of absolute ethanol
  - 30 ml distilled water
  7. 70% methanol
  - 70 ml methanol
  - 30 ml PBS

### **Acknowledgments**

This project was funded by a Leverhulme Trust grant to CHD (RPG-2015-226). The Facility for Imaging by Light Microscopy (FILM) at Imperial College London is part-supported by funding from the Wellcome Trust (grant 104931/Z/14/Z) and BBSRC (grant BB/L015129/1). This protocol is adapted from the method published in Akram *et al.*, 2019.

### **Competing interests**

The authors declare that they have no conflict of interest.

### **Ethics**

All animal maintenance and procedures were carried out according to the requirements of the Animal (Scientific Procedures) Act 1986. Animal work was approved by the South Kensington and St. Mary's AWERB committee, Imperial College London.

### **References**

1. Akram, K. M., Patel, N., Spiteri, M. A. and Forsyth, N. R. (2016). [Lung regeneration: endogenous and exogenous stem cell mediated therapeutic approaches](#). *Int J Mol Sci* 17(1).
2. Akram, K. M., Yates, L. L., Mongey, R., Rothery, S., Gaboriau, D. C. A., Sanderson, J., Hind, M., Griffiths, M. and Dean, C. H. (2019). [Live imaging of alveologenesis in precision-cut lung slices reveals dynamic epithelial cell behaviour](#). *Nat Commun* 10(1): 1178.
3. Amy, R. W., Bowes, D., Burri, P. H., Haines, J. and Thurlbeck, W. M. (1977). [Postnatal growth of the mouse lung](#). *J Anat* 124(Pt 1): 131-151.

4. Burgstaller, G., Vierkotten, S., Lindner, M., Konigshoff, M. and Eickelberg, O. (2015). [Multidimensional immunolabeling and 4D time-lapse imaging of vital ex vivo lung tissue](#). *Am J Physiol Lung Cell Mol Physiol* 309(4): L323-332.
5. Herriges, M. and Morrisey, E. E. (2014). [Lung development: orchestrating the generation and regeneration of a complex organ](#). *Development* 141(3): 502-513.
6. Hilgendorff, A., Reiss, I., Ehrhardt, H., Eickelberg, O. and Alvira, C. M. (2014). [Chronic lung disease in the preterm infant. Lessons learned from animal models](#). *Am J Respir Cell Mol Biol* 50(2): 233-245.
7. Hind, M., Corcoran, J. and Maden, M. (2002). [Alveolar proliferation, retinoid synthesizing enzymes, and endogenous retinoids in the postnatal mouse lung. Different roles for Aldh-1 and Raldh-2](#). *Am J Respir Cell Mol Biol* 26(1): 67-73.
8. Kotton, D. N. and Morrisey, E. E. (2014). [Lung regeneration: mechanisms, applications and emerging stem cell populations](#). *Nat Med* 20(8): 822-832.
9. Kreiger, P. A., Ruchelli, E. D., Mahboubi, S., Hedrick, H., Scott Adzick, N. and Russo, P. A. (2006). [Fetal pulmonary malformations: defining histopathology](#). *Am J Surg Pathol* 30(5): 643-649.
10. Li, J., Wang, Z., Chu, Q., Jiang, K., Li, J. and Tang, N. (2018). [The strength of mechanical forces determines the differentiation of alveolar epithelial cells](#). *Dev Cell* 44(3): 297-312 e5.
11. Madurga, A., Mizikova, I., Ruiz-Camp, J. and Morty, R. E. (2013). [Recent advances in late lung development and the pathogenesis of bronchopulmonary dysplasia](#). *Am J Physiol Lung Cell Mol Physiol* 305(12): L893-905.
12. McGowan, S. E. (2014). [Paracrine cellular and extracellular matrix interactions with mesenchymal progenitors during pulmonary alveolar septation](#). *Birth Defects Res A Clin Mol Teratol* 100(3): 227-239.
13. Meng, Q. J., McMaster, A., Beesley, S., Lu, W. Q., Gibbs, J., Parks, D., Collins, J., Farrow, S., Donn, R., Ray, D. and Loudon, A. (2008). [Ligand modulation of REV-ERB \$\alpha\$  function resets the peripheral circadian clock in a phasic manner](#). *J Cell Sci* 121(Pt 21): 3629-3635.
14. Mund, S. I., Stampanoni, M. and Schittny, J. C. (2008). [Developmental alveolarization of the mouse lung](#). *Dev Dyn* 237(8): 2108-2116.
15. Pieretti, A. C., Ahmed, A. M., Roberts, J. D., Jr. and Kelleher, C. M. (2014). [A novel in vitro model to study alveologenesis](#). *Am J Respir Cell Mol Biol* 50(2): 459-469.
16. Poobalasingam, T., Salman, D., Li, H., Alcada, J. and Dean, C. H. (2017). [Imaging the lung: the old ways and the new](#). *Histol Histopathol* 32(4): 325-337.
17. Roth-Kleiner, M. and Post, M. (2005). [Similarities and dissimilarities of branching and septation during lung development](#). *Pediatr Pulmonol* 40(2): 113-134.
18. Sanderson, M. J. (2011). [Exploring lung physiology in health and disease with lung slices](#). *Pulm Pharmacol Ther* 24(5): 452-465.
19. Snoeck, H. W. (2015). [Modeling human lung development and disease using pluripotent stem cells](#). *Development* 142(1): 13-16.

20. Thornton, E. E., Krummel, M. F. and Looney, M. R. (2012). [Live imaging of the lung](#). *Curr Protoc Cytom* Chapter 12: Unit12 28.
21. Warburton, D., Gauldie, J., Bellusci, S. and Shi, W. (2006). [Lung development and susceptibility to chronic obstructive pulmonary disease](#). *Proc Am Thorac Soc* 3(8): 668-672.

## Imaging VIPER-labeled Cellular Proteins by Correlative Light and Electron Microscopy

Julia K. Doh<sup>1</sup>, Young Hwan Chang<sup>1, 2</sup>, Caroline A. Enns<sup>4</sup>, Claudia S. López<sup>1, 2, 3, \*</sup>

and Kimberly E. Beatty<sup>1, 2, \*</sup>

<sup>1</sup>Department of Biomedical Engineering, Oregon Health & Science University, Portland, Oregon 97239, USA; <sup>2</sup>OHSU Center for Spatial Systems Biomedicine, Oregon Health & Science University, Portland, Oregon 97239, USA; <sup>3</sup>Multiscale Microscopy Core, Oregon Health & Science University, Portland, Oregon 97239, USA; <sup>4</sup>Department of Cell, Developmental, and Cancer Biology, Oregon Health & Science University, Portland, Oregon 97239, USA

\*For correspondence: [beattyk@ohsu.edu](mailto:beattyk@ohsu.edu); [lopezcl@ohsu.edu](mailto:lopezcl@ohsu.edu)



**[Abstract]** Advances in fluorescence microscopy (FM), electron microscopy (EM), and correlative light and EM (CLEM) offer unprecedented opportunities for studying diverse proteins and nanostructures involved in fundamental cell biology. It is now possible to visualize and quantify the spatial organization of cellular proteins and other macromolecules by FM, EM, and CLEM. However, tagging and tracking cellular proteins across size scales is restricted by the scarcity of methods for attaching appropriate reporter chemistries to target proteins. Namely, there are few genetic tags compatible with EM. To overcome these issues we developed Versatile Interacting Peptide (VIP) tags, genetically-encoded peptide tags that can be used to image proteins by fluorescence and EM. VIPER, a VIP tag, can be used to label cellular proteins with bright, photo-stable fluorophores for FM or electron-dense nanoparticles for EM. In this Bio-Protocol, we provide an instructional guide for implementing VIPER for imaging a cell-surface receptor by CLEM. This protocol is complemented by two other Bio-Protocols outlining the use of VIPER (Doh *et al.*, 2019a and 2019b).

**Keywords:** Protein tag, Electron microscopy, Fluorescence, Microscopy, Cell biology, Peptide, CLEM, Quantum dot

**[Background]** Multiple protein targets can be imaged at once by fluorescence microscopy (FM), electron microscopy (EM), or correlative light and EM (CLEM) (Giepmans *et al.*, 2005; Lucas *et al.*, 2012; Philimonenko *et al.*, 2014; Johnson *et al.*, 2015; Kim *et al.*, 2015). FM enables multi-color microscopy in both living and fixed cells, and acquiring data can be relatively fast and easy. However, EM offers better resolution for imaging nanoscale features, including cell receptors, membrane boundaries, neuronal connections (Hildebrand *et al.*, 2017), chromatin organization (Ou *et al.*, 2017), or the endocytic machinery (Sochacki *et al.*, 2017). We anticipate an increased reliance on multi-color, cross-platform imaging for investigating proteins associated with normal cell function and human diseases (Megason and Fraser, 2007; Lichtman *et al.*, 2008; Milne and Subramaniam, 2009; Muller and Heilemann, 2013; Plaza *et al.*, 2014; Kremer *et al.*, 2015; Lucocq *et al.*, 2015; Karreman *et al.*, 2016; Laine *et al.*, 2016; Romero-Brey and Bartenschlager, 2017). Currently, most high-resolution imaging studies obtain protein-specific contrast with immunolabeling, which has known shortcomings

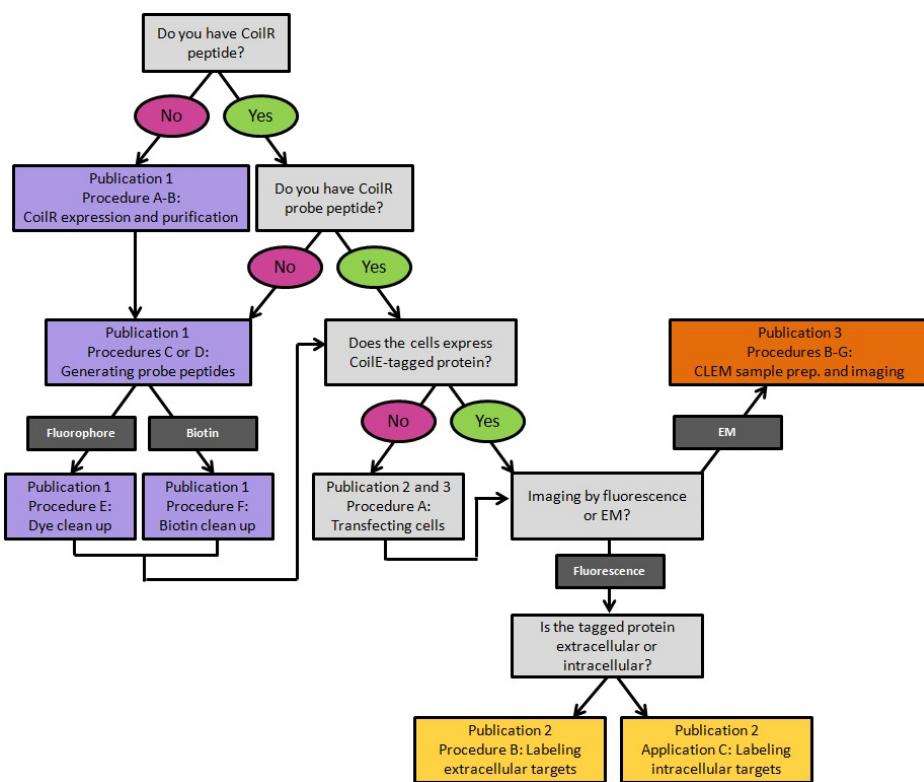
(Berglund *et al.*, 2008; Bordeaux *et al.*, 2010; Baker, 2015; Bradbury and Pluckthun, 2015).

The central obstacle that has limited progress in multi-scale microscopy is the shortage of genetic tags for labeling proteins. There are a number of protein tags available for imaging proteins by FM, including fluorescent proteins and self-labeling enzyme tags (Sunbul and Yin, 2009; Hinner and Johnsson, 2010). However, there are few genetic tags for EM or CLEM (Ellisman *et al.*, 2012). Most EM tags rely on the oxidation of diaminobenzidine (DAB) to form a polymer that is stained with osmium tetroxide to generate contrast. Examples include APEX (Martell *et al.*, 2012; Lam *et al.*, 2015), miniSOG (Shu *et al.*, 2011), the tetracysteine tag (Gaietta *et al.*, 2002), and others (Kuipers *et al.*, 2015; Liss *et al.*, 2015). Among the DAB-reliant EM tags, miniSOG, FLIPPER (Kuipers *et al.*, 2015), and the tetracysteine tag are compatible with CLEM. However, DAB staining is finicky, and it can be difficult to localize the stain sufficiently to resolve targets. Further progress in multi-scale microscopy will require new methods for labeling proteins with EM- and CLEM-compatible reporters, such as quantum dots (Qdots) (Giepmans *et al.*, 2005) or FluoroNanogold<sup>TM</sup> particles (Takizawa *et al.*, 2015).

We recently described a new class of tags for multiscale microscopy called Versatile Interacting Peptide (VIP) tags (Tane *et al.*, 2017; Doh *et al.*, 2018). VIP tags use heterodimerizing coiled-coil peptides to label proteins. One coil is expressed as a fusion to the protein of interest. This coil has a partner, the probe peptide, that is conjugated to a reporter molecule to deliver protein-specific contrast. The probe peptide can be conjugated to a number of reporters, such as fluorophores, small molecules (*e.g.*, biotin), or nanoparticles.

In this Bio-Protocol, we outline the use of VIPER to image a transmembrane receptor by CLEM. VIPER is a VIP tag comprised of CoilE tag and CoilR probe peptide. In Procedure A, we describe the plating and transfection of cells to express a CoilE-tagged receptor: transferrin receptor 1 (TfR1-CoilE). Procedure B describes how to label a cell receptor with CoilR-biotin for subsequent detection with streptavidin-Qdot655. In Procedure C we have an illustrated guide on how to mount ITO coverslips to a slide holder for correlative fluorescence imaging. Procedure D describes image acquisition with a commercially available CLEM microscope, the FEI CorrSight<sup>TM</sup>. In Procedures E and F we describe methods for preparing samples for EM. Procedure G details the acquisition of SEM micrographs on a Helios Nanolab<sup>TM</sup> 660 EM. We additionally developed a quantitative image analysis pipeline for automated image segmentation on high magnification SEM images (see the Data Analysis section). This program runs in Matlab and reports the number of nanoparticles within a field of view in SEM micrographs.

This protocol is published in tandem with two supporting publications detailing the use of VIPER (Figure 1). Doh *et al.* (2019a) details how to generate CoilR probe peptides, including biotinylated CoilR. Doh *et al.* (2019b) describes imaging VIPER-labeled proteins in cells by FM.



**Figure 1. A decision tree for implementing VIPER for labeling cellular proteins.** Procedures are color-coded by the publication in which they appear. Publication 1: Doh *et al.*, 2019a; Publication 2: Doh *et al.*, 2019b; Publication 3: this article.

## Materials and Reagents

Note: “\*\* indicates a brand that is critical to the success of the experiment.

### Materials

1. Aluminum coverslip holder  
*Note: We used a custom machined aluminum plate with a hole for a 22 x 22 mm coverslip. This plate is 76 x 26 x 1.5 mm with a 12 mm diameter hole in the center.*
2. \*Indium tin-oxide 22 x 22 mm coverslips (2SPI, catalog number: 06486-AB)
3. Tape (Scotch® Magic™ Tape)
4. Desiccator cabinet (Thermo Scientific Nalgene™, catalog number: 53170070)
5. Desiccant (Drierite™, catalog number: D1085)
6. Kimwipes (Kimtech, catalog number: 34120)
7. \*Conductive silver paint “Leitsilber” (Ted Pella, catalog number: 16035)
8. \*SEM pin stub specimen mount (Ted Pella, catalog number: 16144)
9. \*Carbon thread (Leica, catalog number: 16771511116)
10. LDPE 500 ml squeeze wash bottle (Thermo Scientific, catalog number: 24010500)
11. Transfer bulb pipette (VWR, catalog number: 16001-182)

## Reagents

1. CHO TRVb cells (courtesy of Prof. Timothy McGraw, Cornell University, Ithaca, New York) (McGraw *et al.*, 1987)
2. Ham's F-12 Medium (Life Technologies, Gibco<sup>TM</sup>, catalog number: 11765062)
3. Dulbecco's phosphate-buffered saline without calcium or magnesium; DPBS (Gibco<sup>TM</sup>, catalog number: 14190144)
4. Trypsin-EDTA (0.25%) (Life Technologies, Gibco<sup>TM</sup>, catalog number: 25200056)
5. Fetal bovine serum (FBS) (GE, Hyclone<sup>TM</sup>, catalog number: SH30910.03)
6. \*Lipofectamine<sup>TM</sup> 2000 (Thermo Scientific, catalog number: 11668019)
7. Opti-MEM (Life Technologies, Gibco<sup>TM</sup>, catalog number: 31985070)
8. \*Streptavidin-Qdot<sup>TM</sup> 655 conjugate (Invitrogen, catalog number: Q10121MP)
9. Anhydrous ethanol (Decon Labs, catalog number: 2716)
10. 20% (v/v) paraformaldehyde (PFA) stock (Electron Microscopy Sciences, catalog number: 15713S)
11. Live Cell Block solution (see Recipes)
12. Qdot Block solution (see Recipes)
13. Qdot Labeling solution (see Recipes)

## Equipment

1. Hemocytometer (Hausser Scientific, catalog number: 1475)
2. Humidified CO<sub>2</sub> Incubator (New Brunswick Galaxy 170S, catalog number: C0170S-120-0000)
3. Tissue culture hood (Thermo Scientific, model: 1300 Series A2)
4. Tissue culture inverted light microscope (Carl Zeiss, Zeiss Primovert)
5. Fine point tweezers (Ted Pella Dumostar Biology, catalog number: 525-PS)
6. Diamond-tipped scribe (Ted Pella, catalog number: 54468)
7. Stainless steel crinkle washers (Tousimis Washers, catalog number: 8767-01)
8. Orbital Shaker (Stovall Belly Dancer<sup>TM</sup>, catalog number: BDRAA1158)
9. Spinning disk confocal fluorescence microscope (FEI CorrSight<sup>TM</sup>)
10. 63x objective lens (Carl Zeiss, 1.4 NA Plan-Apochromat M27, catalog number: 420780-9900-000)
11. 5x objective lens (Carl Zeiss, 0.16 NA EC Plan-Neofluar M27, catalog number: 420330-9901-000)
12. Scanning electron microscope (FEI Helios Nanolab<sup>TM</sup> 660 SEM)
13. Critical point dryer (Leica EM CPD300)
14. High Vacuum Flash Carbon Coating Machine (Leica EM ACE600)

## Software

1. FEI MAPS (FEI version 2.1.38.1199 and version 3.0)
2. FEI Helios Nanolab™ XT Microscope Control (FEI version 5.5.1 and version 10.1.7)
3. Matlab (Mathworks Software Version R2017b)

## Procedures

### A. Transfected cell lines to express a VIPER-tagged protein

This protocol describes the transfection of a tissue culture cell line to express a VIPER-tagged transmembrane receptor (*i.e.*, TfR1-CoilE). We used the CHO TRVb cell line because it does not express TfR1 or transferrin receptor 2 (McGraw *et al.*, 1987). After transfection, all TfR1 receptor will encode the C-terminal CoilE tag on the extracellular domain.

We recommend optimizing plating density and transfection conditions for each cell line. Refer to Thermo Fisher's Protocol Pub No. MAN0007824 Rev 1.0 (Reference 23) for more information on transfection with Lipofectamine 2000. In our protocol, we recommend passaging cells on Day 1, transfecting cells on Day 2, and VIPER labeling cells on Day 3 (see Procedure B).

#### **Recommendations for Protocol A:**

1. Use sterile technique and work within a tissue culture (*laminar flow*) hood when working with live cells.
  2. When transfecting cells with a vector encoding a CoilE-tagged protein (*e.g.*, pcDNA3.1\_TfR1-CoilE), we also recommend transfecting cells with an untagged protein to compare labeling specificity (*e.g.*, pcDNA3.1\_TfR1). Untransfected cells also serve as a control for labeling specificity.
  3. The sequence for TfR1-CoilE can be found in the Sequences ([Supplemental file](#)).
- 
1. **Day 1:** Passage cells and plate onto indium tin oxide (ITO) coverslips
    - a. Before starting, visually inspect cells on a tissue culture microscope to confirm that cells are adherent, healthy, and 80-90% confluent.
    - b. Place single 22 x 22 mm ITO coverslips into each well of a sterile 6-well polystyrene tissue culture plate.
      - i. The ITO coverslips are provided in a small box with the conductive side oriented face up. Transfer the coverslip to the 6-well plate without flipping the coverslip over. Maintain this orientation during transfection, labeling, and processing.
      - ii. Pre-coating coverslips with poly-L-lysine or other cell-surface treatments may be necessary to adhere cells to glass. CHO TRVb cells adhere to glass without additional support.
    - c. Seed 1 x 10<sup>6</sup> cells per well (for CHO TRVb). Grow cells in Ham's F12 medium supplemented with 5% FBS. Do not include antibiotics in the media.

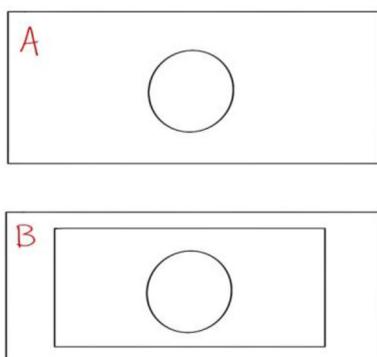
- i. For other cell lines, seed at a density that will result in 80-90% confluence after 24 h.
  2. Incubate cells overnight in a humidified tissue culture incubator (37 °C with 5% CO<sub>2</sub>).
  3. **Day 2: Prepare the transfection mixture**
    - a. Dilute vector DNA into Opti-MEM:
      - i. For a 6-well plate, use 2 µg DNA in 100 µl of Opti-MEM per well.
      - ii. The DNA quality is paramount for a high transfection efficiency. Quantify DNA quality by UV spectroscopy and verify that the 260/280 nm ratio falls between 1.8 and 2.0.
      - iii. Keep the volume of DNA to less than 10% of the total volume in the transfection mixture.
    - b. Dilute Lipofectamine 2000 into Opti-MEM and incubate for 5 min: For a 6-well plate, use 4 µg Lipofectamine2000 in 100 µl of Opti-MEM per well.
    - c. Combine equal volumes of the Lipofectamine2000 and DNA solutions and mix by pipetting. The final ratio of DNA to Lipofectamine2000 will be 1:2. Incubate the mixture for at least 30 min at room temperature.
  4. Transfect cells:
    - a. Aspirate media from cells.
    - b. Add Opti-MEM to wells. For 6-well dishes, use 800 µl per well.
    - c. Add 200 µl of the transfection mixture to each well (1:5 dilution). The total volume of fluid in each well should be 1 ml.
    - d. Gently mix the solution by pipetting up and down or by rocking the plate.
    - e. Incubate cells in the transfection mixture for 4 h in a tissue culture incubator (37 °C, 5% CO<sub>2</sub>).
      - i. This time frame is recommended for transfecting CHO TRVb cells.
      - ii. Monitor cells by viewing on a TC microscope. Cell size and shape should appear unchanged during transfection. If cells contract or detach, then adjust the transfection conditions.
    - f. Aspirate to remove the transfection media and wash once with complete media (e.g., media with FBS).
    - g. Add complete media and grow in a tissue culture incubator (37 °C, 5% CO<sub>2</sub>) for at least 24 h. Cells will be ready for use between 24 and 48 h after transfection.
- B. Labeling VIPER-tagged receptors with Qdots on ITO coverslips
- Protocol B describes a method for labeling a transmembrane receptor (TfR1-CoilE) with a biotinylated probe peptide (CoilR-biotin). Biotinylated receptor is subsequently detected with a streptavidin-Qdot655 conjugate. The biotinylated receptor could be labeled with other streptavidin conjugates, such as streptavidin-gold. We counter-stained with fluorescent transferrin (Tf-AF488), the ligand of TfR1, to enable the rapid identification of transfected cells. Lastly, live cells were cooled to 4 °C to pause endocytosis during labeling of the cell-surface receptor.

1. **Day 3:** Visually inspect each well on a tissue culture microscope to confirm that transfected cells are adherent, healthy, and 80-90% confluent.
2. Remove media and add 500  $\mu$ l of Live Cell Block Solution (Recipe 1) to each well. Return to the tissue culture incubator for 30 min.
  - a. Use a pipette or transfer bulb pipette to aspirate media from wells, taking care to always leave enough media to keep the cells and coverslips hydrated.
3. While the cells are in Live Cell Block Solution, prepare the CoilR labeling solution.
  - a. Dilute CoilR-biotin probe peptide and 50  $\mu$ g/ml Tf-AF488 into pre-chilled Ham's F12 media without serum. Prepare 500  $\mu$ l of the labeling solution per well.
  - b. We recommend testing a range of probe peptide concentrations to obtain optimal signal to noise (e.g., 100 nM to 500 nM). For CHO TRVb cells expressing TfR1-CoilE, we recommend 100 nM CoilR-biotin in Ham's F12 media.
  - c. Other labeling reagents can be added at this time.
- Note: Qdots have spectral overlap with Hoechst 33342, so we do not use this nuclear stain for these studies.*
4. Remove Block Solution 1 from each well.
5. Add 500  $\mu$ l of the labeling solution to each well. Label cells for 30 min at 4 °C, protected from light.
  - a. Labeling is done at 4 °C in pre-chilled media to restrict endocytosis of VIPER-labeled receptors. Accessibility of the receptors on the cell surface will be especially critical for the subsequent SEM detection of CoilR-biotin with streptavidin-Qdots.
6. Wash each well three times with ice-cold DPBS.
7. Fix the cells using ice-cold 4% v/v PFA in DPBS. Incubate cells in fixative for 15 min at 4 °C.
8. Wash each well twice with DPBS to remove fixative.
9. Block the ITO coverslips with 800  $\mu$ l Qdot Block Solution (Recipe 2) for 1 h at room temperature.
10. While cells are in Qdot Block Solution, prepare the Qdot labeling solution:
  - a. The Qdots are supplied by Invitrogen as a 1  $\mu$ M stock solution. Before using, centrifuge (17,000 x g, 5 min) the streptavidin-Qdot655 conjugate to remove aggregated Qdots from solution.
  - b. A range of concentrations should be tested to ensure optimal labeling.
  - c. We used 10 nM streptavidin-Qdot655 in Qdot Labeling Solution (Recipe 3) for CHO TRVb transfected with TfR1-CoilE.
  - d. Prepare 500  $\mu$ l of labeling solution per well (e.g., 3 ml of solution for 6 samples).
11. Remove Qdot Block Solution from each well.
12. Add 500  $\mu$ l of the streptavidin-Qdot655 labeling solution to each well and label for 1 h at room temperature. Protect samples from light during this step.
13. After Qdot labeling, wash three times with DPBS.

14. Image and map ITO coverslips by confocal FM (e.g., with an FEI CorrSight<sup>TM</sup>) before processing samples for CLEM (Procedures C and D).

#### C. Mounting ITO coverslips for CLEM imaging

This procedure describes the methods for handling ITO coverslips prepared in Procedure B. This procedure uses a custom-machined aluminum slide to hold the ITO coverslips during imaging. This slide was created by Ingo Gestmann (FEI). The dimensions of the slide are 76 x 26 x 1.5 mm. The hole is 12 mm in diameter, which is compatible with the 22 x 22 mm ITO coverslips. There are likely commercially-available coverslip holders, but we have not tested any. The custom-machined aluminum slide (Figure 2) has a piece of coverglass glued with epoxy to one side (Side B). The other side remains open, with a circular chamber (Side A).



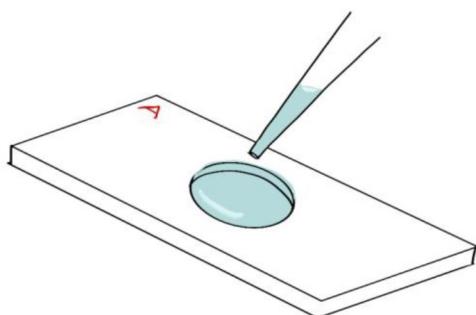
**Figure 2. Diagram of the aluminum coverslip holder.** Side A shows a circular hole. Side B has a rectangular piece of coverglass glued to cover the circular hole. This set-up allows the user to create an enclosed chamber of buffer by taping an ITO coverslip to side A.

1. Using a diamond-tipped scribe, scratch a small “F” into the center of the ITO coverslip to create a fiducial marker. Make strokes roughly 1 cm in length. A larger fiducial generates better correlation later on during SEM, but it should still be fully visible through the hole in the aluminum slide (see Figure 3). The coverslip can be stabilized during this process by holding it down with fine point tweezers.
  - a. For multiple samples, use the scribe to make additional marks to enable quick differentiation of your samples. An example scheme would be:
    - i. Sample 1: “F” only; no notch,
    - ii. Sample 2: notch below “F”,
    - iii. Sample 3: notch above “F”,
    - iv. Sample 4: notch to the left of “F”,
    - v. Sample 5: notch to the right of “F”,
    - vi. Sample 6: notch below and above “F”.



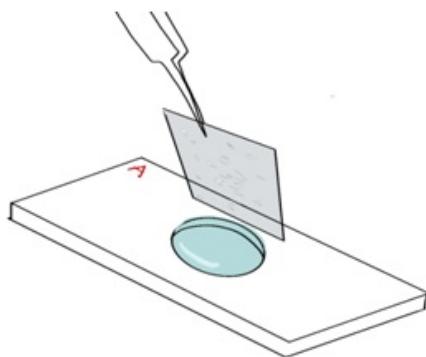
**Figure 3. An “F” carved into the center of a 22 x 22 mm ITO coverslip.** Note the size of the “F” relative to the size of the coverslip.

2. Place the aluminum slide on the laboratory bench with side A facing up.
3. Pipette DPBS into the circular chamber drop-wise until the chamber is full. Add one more drop to create a raised meniscus (Figure 4).



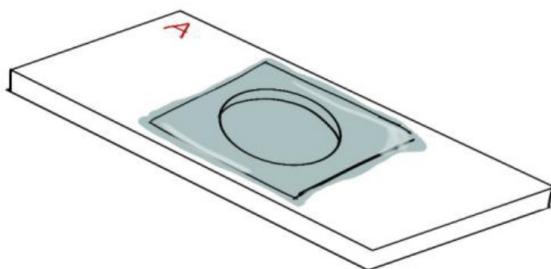
**Figure 4. The aluminum coverslip holder with the circular chamber filled with buffer**

4. Remove the labeled ITO coverslip from the 6-well plate using fine point tweezers.
5. Allow the excess DPBS to drip off but do not let the coverslip dry out. The ITO coverslip should be wet for the next step.
6. Position one edge of the wet ITO coverslip against the aluminum coverslip holder with the cells facing down, towards the surface of Side A (Figure 5).



**Figure 5. Positioning the ITO coverslip on the aluminum coverslip holder.** The side of the ITO coverslip with the adhered cells is positioned facing towards the chamber.

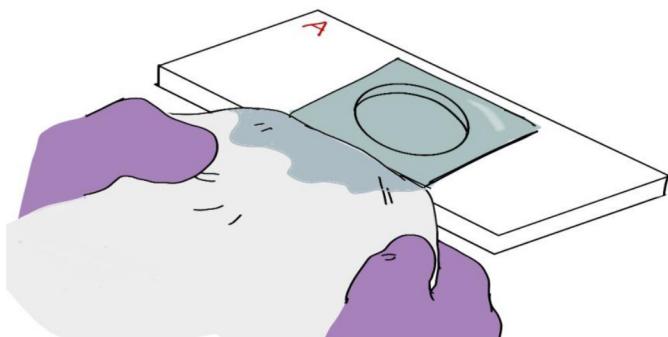
7. Use tweezers to carefully lower the ITO coverslip onto Side A. The ITO coverslip should be floating on a thin layer of fluid (Figure 6). Do this quickly to avoid trapping air in the chamber.
  - a. There should be no bubbles in the chamber between the ITO coverslip and the glass on Side B of the slide. Air bubbles will dry out the cells. Small bubbles are permissible if they do not contact the ITO coverslip when the slide is inverted.
  - b. If a large air bubble is trapped under the ITO coverslip, use tweezers to lift the ITO coverslip at a shallow angle before dropping the ITO coverslip onto the DPBS again.
  - c. The coverslip can also be nudged gently off of the slide with tweezers to free the bubble from the side of the coverslip.
  - d. The position of the coverslip can be adjusted at this point as long as it is free-floating.
  - e. Make sure you can see the entire fiducial marker ("F") through the hole of the chamber.



**Figure 6. The ITO coverslip floating on a thin layer of DPBS over the circular chamber on Side A of the aluminum coverslip holder**

8. Blot excess fluid from the slide. Fold a Kimwipe in half and touch the folded edge to the side of the coverslip to blot excess fluid (Figure 7). Repeat for all four sides until dry. If there is fluid on top of the coverslip you can lay a piece of tissue on top of the coverslip to blot the remainder. Only do this step after blotting the sides.
  - a. This will "seal" the coverslip to the slide. Moving or re-positioning the ITO coverslip at this point could damage the cells.

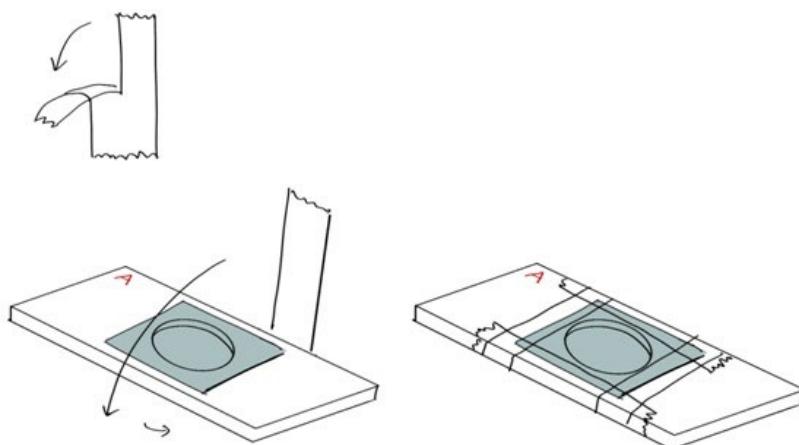
- b. If there is a bubble in the circular chamber or the ITO coverslip is poorly positioned after this step, DPBS can be added gently to the sides of the coverslip. Wait for the DPBS to flow underneath the coverslip and then lift it off the slide.



**Figure 7. Removing excess DPBS from the aluminum coverslip holder by blotting with a Kimwipe**

9. Tear a strip of clear tape (e.g., 3M Scotch™ tape) in half and secure the coverslip to the slide by taping at the sides. Repeat for all sides (Figure 8).

*Note: Avoid applying an excess of tape because it will need to be removed after fluorescence imaging.*



**Figure 8. Attaching the coverslip to the aluminum coverslip holder using clear tape**

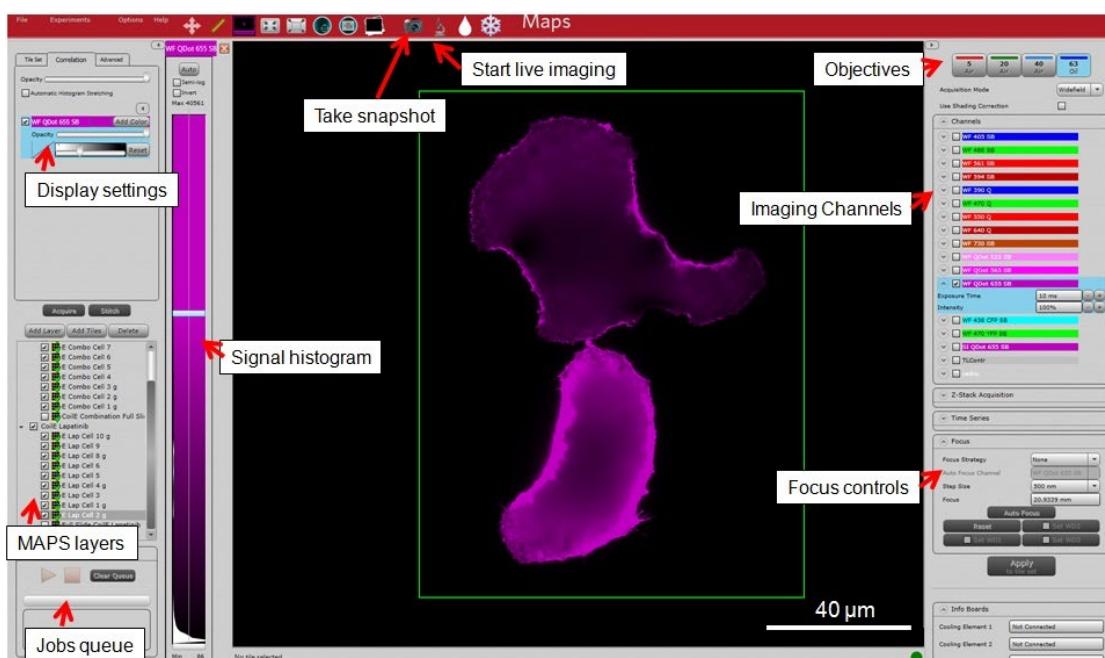
10. Repeat this procedure for each ITO coverslip.
11. Image the prepared samples on an inverted confocal fluorescence microscope. We imaged an FEI CorrSight™ in the following Procedure D.

*Note: We recommend imaging soon after preparing the samples because some evaporation may occur.*

#### D. Fluorescence imaging and mapping cells on an FEI CorrSight™ System

This procedure describes using the FEI CorrSight™ imaging system to collect micrographs of labeled cells. Samples prepared in Procedures A-C are first imaged by confocal FM to identify fluorescent cells. Confocal FM is used to image VIPER fluorescence (Qdot655) and Tf-AF488 on the cell surface. During FM imaging, the FEI MAPS software enables the user to select and “map” cells for subsequent high-resolution imaging by scanning EM (SEM). Figure 9 provides an overview of the FEI MAPS software.

*Note: Once the slide is placed on the stage and imaging commences, the slide must remain in place until it is fully mapped. Even small adjustment to the slide placement will shift the slide and make the map inaccurate, which makes it difficult to re-locate cells for SEM imaging.*



**Figure 9. Annotated screen capture of the FEI MAPS 2.1.38 software**

1. Place immersion oil on the 63x objective lens. Then switch to the 5x objective lens. This can be controlled in the panel labeled “Objectives”. Refer to Figure 9 for software controls from this point on. Additional information can be found in the FEI CorrSight™ user manual (Reference 9).

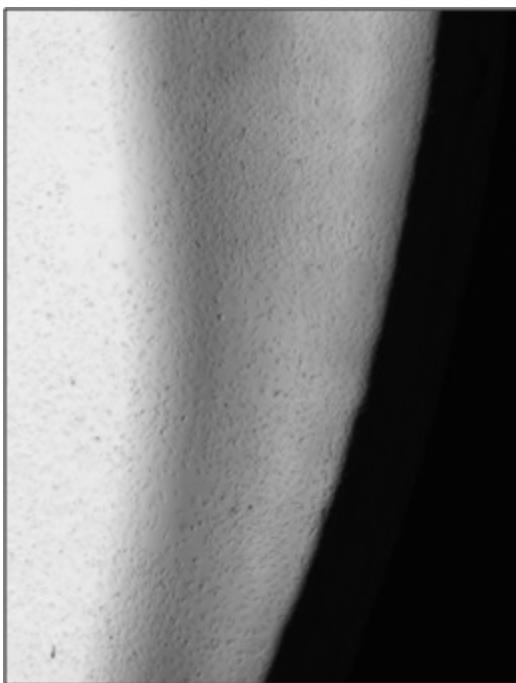
*Note: This is a critical step. Removing the slide after 5x imaging to load oil on the 63x objective will offset the mapping. This will make re-locating cells difficult by SEM.*

2. Place the aluminum coverslip holder on the microscope stage with the ITO coverslip (Side A) facing downwards towards the objective.
3. Turn off all the fluorescence channels using the panel labeled “Imaging Channels” by unchecking all the boxes in that panel. Image the sample using transmitted light.
  - a. In the panel labeled “Imaging Channels,” turn on the “TLControl” (transmitted light) channel by checking its box.
  - b. In the panel labeled “Focus Controls,” set the focus to 20 mm.

- c. Start live imaging by clicking the button labeled “Live Imaging”.
- d. Adjust the focus with scroll wheel of the mouse until the cells and the fiducial marker (“F”) are in focus. You can adjust the step size of the focusing in the panel labeled “Focus controls.”

*Note: The focal plane will vary across the coverslip. Therefore, it is important is to keep the fiducial “F” in focus as you move the stage.*

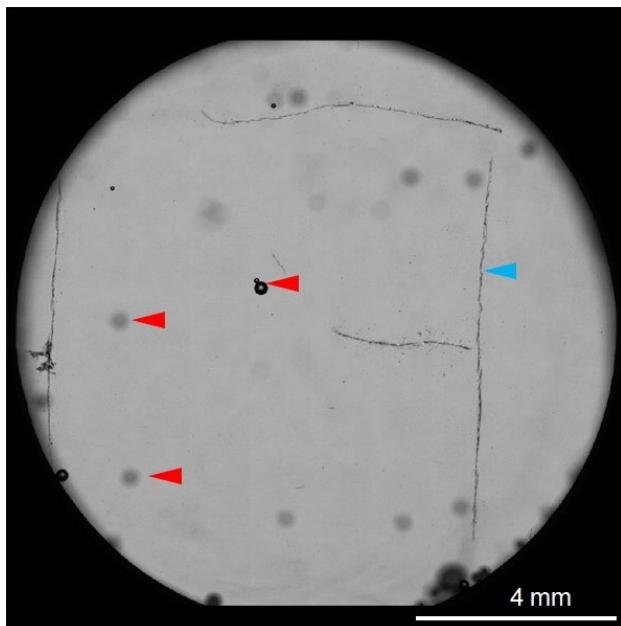
4. Drive the stage to the edge of the circular hole in the aluminum coverslip holder.
    - a. To do this, left click on the live image where you want the stage to move.
    - b. Once you have located an edge, take a snapshot (Figure 10) by clicking the button labeled “Take snapshot”. The “Take snapshot” function loads the data into an aggregate layer called “PreviewImages” in the layers panel.
- Note: This is not a viable place to store image data. However, these preview snapshots allow you to reference the edges of the circular chamber. These boundaries are needed in order to draw the tile-set that will capture the whole ITO coverslip.*
- c. Continue taking snapshots until you can determine of the boundaries of the circular chamber. This can generally be done with 4 snapshots placed at the top, bottom, left, and right of the circle.



**Figure 10. A snapshot of the ITO coverslip (light gray) showing the boundary of the circular chamber in the aluminum coverslip holder (black)**

5. Left click and drag over the slide hole (using the snapshots of the hole boundaries to guide you) and right-click “Add tiles here”. This will create a tile array to image the entire slide, capturing the fiducial F.

6. Execute the acquisition in the “Jobs queue” panel to generate a stitched tile image of the coverslip by transmitted light (Figure 11).



**Figure 11.** A stitched tile image showing the ITO coverslip (light gray) mounted on the aluminum coverslip holder (black). Note that the entire “F” fiducial, denoted by a blue arrowhead, was captured and in focus. Small air bubbles can also be seen, with a few denoted by a red arrowhead.

7. Prepare to image cells by FM.
  - Check box on the “WF 488 SB” channel in the “Imaging Channels” panel. This setting excites Tf-AF488 at 488 nm and collects emission through a 525/50 nm filter.
  - Check box on the “WF Qdot 655 SB” channel. This setting excites Qdot655 at 405 nm and collects emission through a 690/50 nm filter.
  - You can modify the intensity of each channel in the “Imaging Channels” panel by clicking on the channel name. Leave the intensity at 100% and set the exposure time appropriately by imaging live in these channels.
    - “Good” acquisition settings generate signal that occupies more than 50% of the dynamic range of the 16-bit detector, without saturated signal in any pixels. This can be observed in the panel labeled “Signal histogram.”
  - The settings may require some adjustment after switching to the 63x objective lens.
8. Cells can now be imaged and mapped at 63x magnification.
  - During image acquisition use the 5x objective to locate cells by low magnification. Then switch to the 63x objective oil lens to collect high-resolution images. When a cell is “mapped”, the fluorescence image is acquired and the image is automatically saved with the coordinate location.

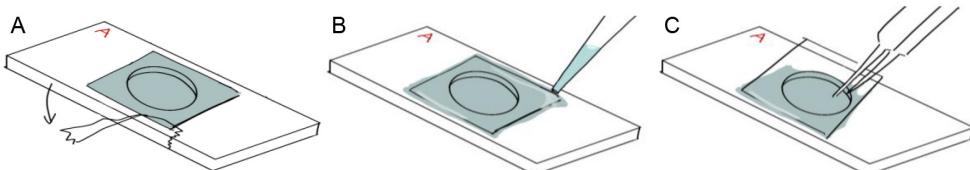
- b. We recommend selecting cells for imaging based on their Tf-AF488 signal to avoid biasing cell selection towards the brightest Qdot655 signal (*i.e.*, the brightest VIPER-labeled cells).
  - c. We recommend taking 5x fluorescence images of the slide. These images are helpful for locating the mapped cells by SEM. This is especially true for samples with high cell density, where finding individual cells based on size and shape alone is difficult.
  - d. Repeated switching between 5x and 63x can create air bubbles in the immersion oil. These do not affect imaging at 63x, but the bubbles will appear in the 5x images.
9. To map cells, locate a desired cell at 63x magnification using the live-imaging function. Left click and drag a single tile over the cell location and right-click “Add tiles here”. This will create a tile to image the cell and register its location.
  10. Execute the acquisition in the “Jobs queue panel”.

*Note: The FEI MAPS acquisition software will remember the absolute position of cells imaged at 63x relative to the full size of the coverslip mapped at 5x magnification.*

11. Create a new layer for each cell. You can group samples by creating Layer groups. For each layer, make the names descriptive (*e.g.*, Cell 1 VIPER Qdot slide A). The names will become the folder names where data is stored.

*Note: All of the individual images collected by the FEI MAPS software are automatically named “Tile\_000\_0001”. Therefore, descriptive layer names are important.*

12. After imaging and mapping, remove the slide from the FEI CorrSight™ microscope.
13. Clean the slide thoroughly with lens paper to remove immersion oil. Then clean with lens paper using 70% ethanol.
14. Remove the tape gently and return the ITO coverslip to DPBS in a 6-well plate for further processing (Figure 12).
  - a. The ITO coverslip should be oriented with cells facing upwards in the 6-well plate.
  - b. The tape can be scored with a razor blade to help remove it from the aluminum slide.
  - c. If the sample is “stuck” on the aluminum slide after tape removal, apply a few drops of DPBS around the edges of the slide and wait for the fluid to move under the coverslip, lifting it from the slide. Forcing the coverslip off with tweezers can break the coverslip or damage the cells by sliding them against the aluminum slide.



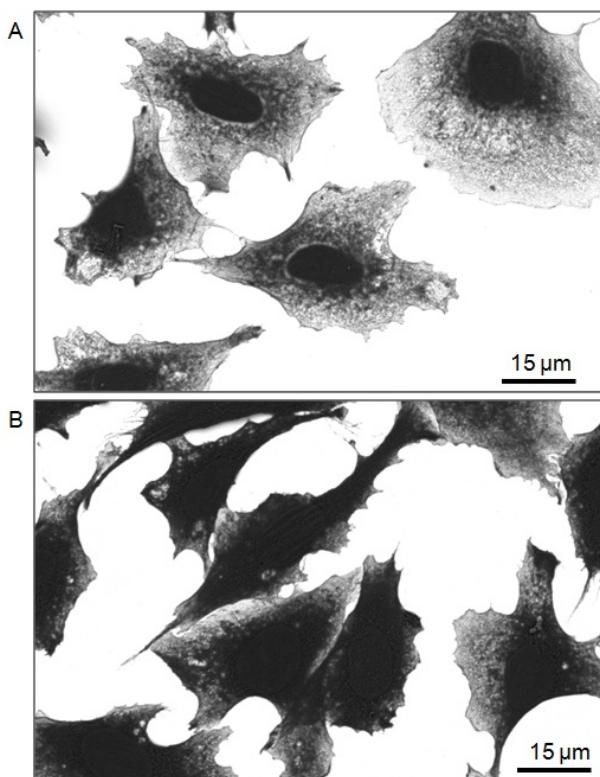
**Figure 12. Removing the ITO coverslip on the aluminum coverslip holder.** A. Remove the tape by pulling towards the side, instead of by pulling up, to minimize force applied to coverslip. B. Add fluid to the edges of the ITO coverslip and wait for it to penetrate underneath the coverslip. C. Use fine point tweezers to gently lift the coverslip off from the edges.

## E. Processing ITO coverslips for SEM imaging

This procedure describes the methods used to dehydrate samples for SEM imaging on the Helios Nanolab™ 660. The methods continue from the last step of Procedure D, after samples have been imaged by FM and ITO coverslips are placed in a 6-well plate.

1. Prepare solutions of ethanol that will be used to dehydrate samples.
  - a. Dilutions should be prepared with anhydrous ethanol and sterile DI water.
  - b. Prepare 10-15 ml stocks of: 25%, 50%, 75%, and 90% (v/v) ethanol. Undiluted ("100% ethanol") will also be used for dehydrating samples.

*Note: For SEM imaging, it is critical to fully remove water from the cells when dehydrating because residual water can cause cell breakage and deformation (Figure 13). Using a fresh bottle of anhydrous ethanol every time minimizes this risk.*



**Figure 13. Micrographs of dehydrated CHO TRVb cells processed for SEM imaging.** SEM micrographs were acquired in backscattered mode on an FEI Helios Nanolab™ 660 SEM at 3,500x magnification (horizontal field width: 119 μm). The ITO coverslip appears bright white in this acquisition mode. A. Micrographs of damaged cells that were dehydrated with an ethanol gradient prepared using an old bottle of ethanol (opened and reused over several months). Cells are light gray and have a lace-like appearance with pronounced nuclei. The white ITO substrate can be seen from behind cells that are damaged, resulting in cells that are light gray around the nucleus. B. Micrographs of well-preserved cells that were dehydrated with an ethanol gradient prepared using a new bottle of anhydrous ethanol. In contrast to cells in A,

these cells are intact, appear dark gray, and are more raised.

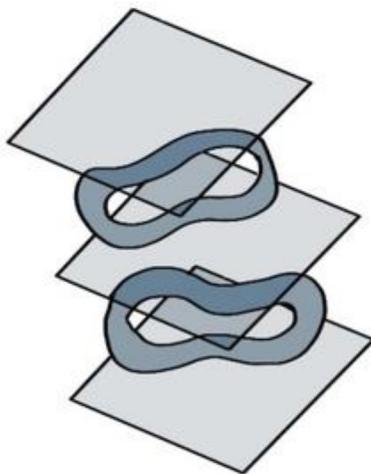
2. Withdraw most of the DPBS from the ITO coverslips using a micropipette or transfer pipette. Leave a small layer of fluid over the cells. If the samples dry out in air, the cells may break or appear damaged by SEM. To minimize this risk, never let the ITO coverslips go dry. This is especially important when using high percentages of ethanol, which evaporates quickly.
3. Wash the ITO coverslips with DI water (1.5 ml/well) to remove salts. Wash 5 min at room temperature with gentle agitation.

*Note: Gentle agitation can be achieved by hand or on a rocking stage.*

4. Withdraw most of the water from the wells, again working to ensure that the coverslips remain wet.
5. Add 25% (v/v) ethanol (1.5 ml/well) to the slides to start the dehydration gradient. Incubate for 5 min at room temperature with gentle agitation.
6. Withdraw most of most of the 25% (v/v) ethanol from the wells, leaving enough solution so that the slides stay covered.
7. Repeat Steps E5 and E6 with: 50%, 75%, 90%, and 100% ethanol.

*Note: This ethanol step-gradient dehydrates the cells slowly to minimize cell shrinking and structural damage during dehydration.*

8. Incubate coverslips a second time in 100% ethanol to ensure that all water is removed from the samples.
9. Coverslips are now ready to be loaded into the critical point dryer.
10. Use a critical point dryer (Leica EM CPD300) to dehydrate samples.
  - a. Open the sample chamber and load the ITO coverslips.
    - i. Using fine point tweezers, transfer the first layer of coverslips with the cells facing up.
    - ii. ITO coverslips can be stacked on top of each other through the use of stainless steel crinkle washers. Place a washer gently on top of the ITO coverslip, and then add the next coverslip (Figure 14). The washers are large enough that the cells of interest should be in the hole of the washer and not in contact with the washer itself. A total of 4 layers of coverslips can fit inside the sample chamber.



**Figure 14. Using stainless steel crinkle washers to separate ITO coverslips during dehydration.** The washers prevent the coverslips from adhering to each other during the final dehydration steps.

- b. Use the critical point dryer to dehydrate the samples. On the Leica system, we programmed the following dehydration conditions:

Stirring: 50%

Automatic exchanging: on,

Speed of CO<sub>2</sub> injection: slow,

Fillers: 1 stage (fillers are solid plastic pieces that are placed in the chamber to take up volume and minimize the drying volume),

Delay of CO<sub>2</sub> injection: 120 s,

Speed of CO<sub>2</sub> exchange: 1 (out of 10),

Number of CO<sub>2</sub> exchange cycles: 25,

Heating for CO<sub>2</sub> gassing out: slow,

Speed of CO<sub>2</sub> gassing out: slow.

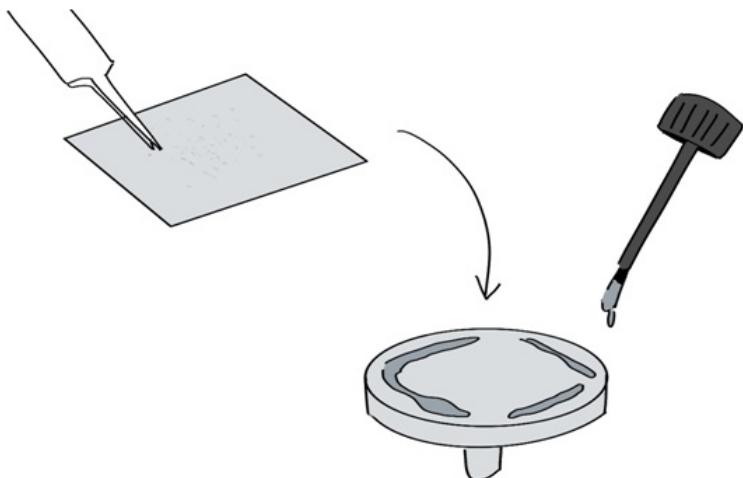
11. Dried samples are now ready for mounting to pin stub specimen mounts (Procedure F).

#### F. Mounting coverslips and carbon-coating for SEM imaging

This procedure describes methods used to mount dehydrated ITO coverslips on SEM pin stub specimen mounts (Ted Pella), referred to herein as “SEM mounting pins” or “pins”. It also describes the method used to carbon coat the samples. This procedure starts with dehydrated samples prepared in Procedure E.

1. Brush on a small amount of conductive silver paint around the edges of an SEM mounting pin.
  - a. Apply the paint near to where the contours of the ITO coverslip will be when it is placed on the pin.
  - b. The silver paint must fully off-gas and dry before it is placed under vacuum in the coating machine. Gluing coverslips by the edges rather than the center makes the drying process

- go faster in Step F3.
- c. The SEM mounting pin can be labeled with the sample identity by writing on the underside.
  2. While the silver paint is wet, place the ITO coverslip with the cells facing up on the SEM mounting pin using fine point tweezers (Figure 15).

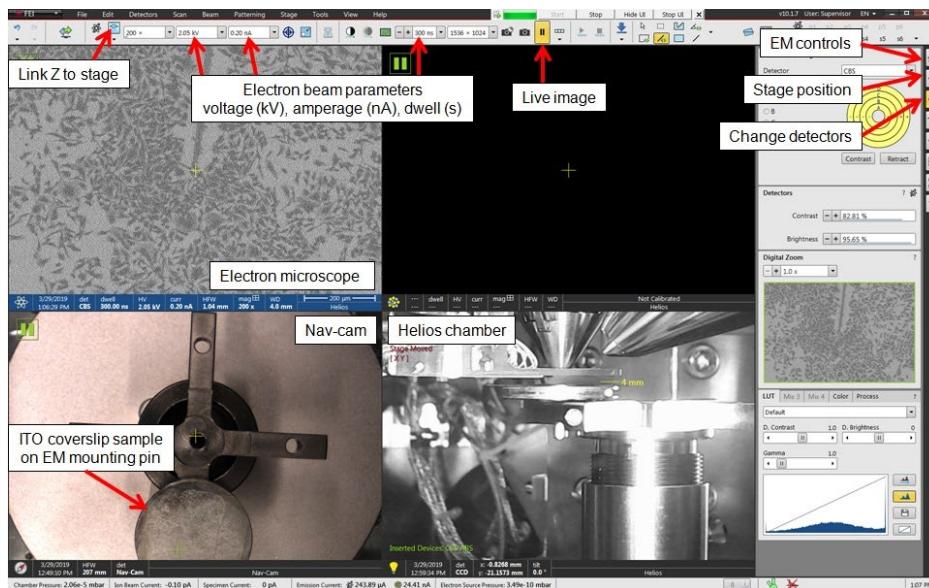


**Figure 15. Gluing the ITO coverslip to the SEM mounting pin using conductive silver paint.** The paint is applied sparsely to the edges of the SEM mounting pin.

3. Store the pin-mounted ITO coverslips under desiccation and dry the silver paint overnight.  
*Note: We store pin-mounted coverslips in a desiccator cabinet with desiccant (i.e., Drierite™).*
4. After the silver paint has dried, transfer samples to a high vacuum flash coating machine (Leica EM ACE600):
  - a. Vent the chamber of the coating apparatus. When the chamber reaches atmospheric pressure (100 mbar), open the door.
  - b. Place samples inside the chamber by seating the pins in the carousel holder. Do not overlap samples. Close the door.
  - c. Load the carbon thread into the coating unit above the sample chamber.
  - d. Place the coating unit back in the coating machine.
5. Coat the samples under vacuum:
  - a. Run program “Pulse sgh coater”. Use these settings:  
Coat thickness: 10 nm,  
Sample height: 3 nm,  
Tilt: 0 degrees.
6. Once the carbon coating is finished and the sample chamber is vented, the samples can be removed and are ready for imaging by EM (Procedure G).  
*Note: SEM mounting pins can be reused by gently prying off the ITO coverslip and washing the pins with 100% ethanol. Let pins fully dry before adding silver paint.*

## G. Operating the FEI Helios Nanolab™ 660 SEM instrument for SEM imaging

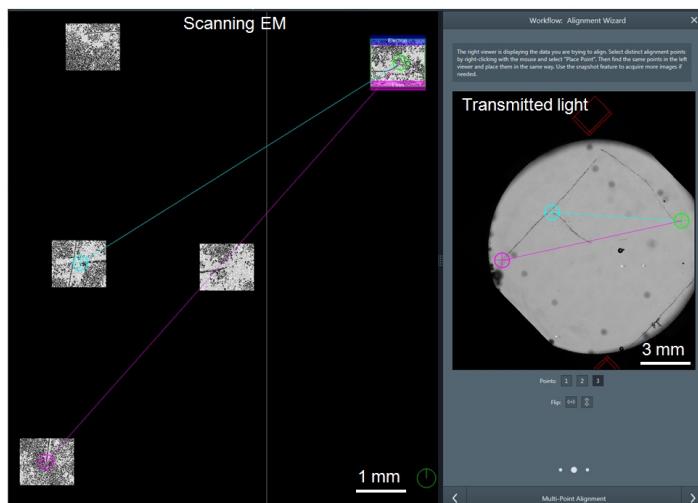
This procedure uses samples that were prepared through the end of Procedure F. These samples should contain cells on ITO coverslips that were mapped, dehydrated, glued to SEM mounting pins, and carbon coated.



**Figure 16. Annotated screen capture of the FEI Helios Nanolab™ xT Microscope Control software (version 10.1.7)**

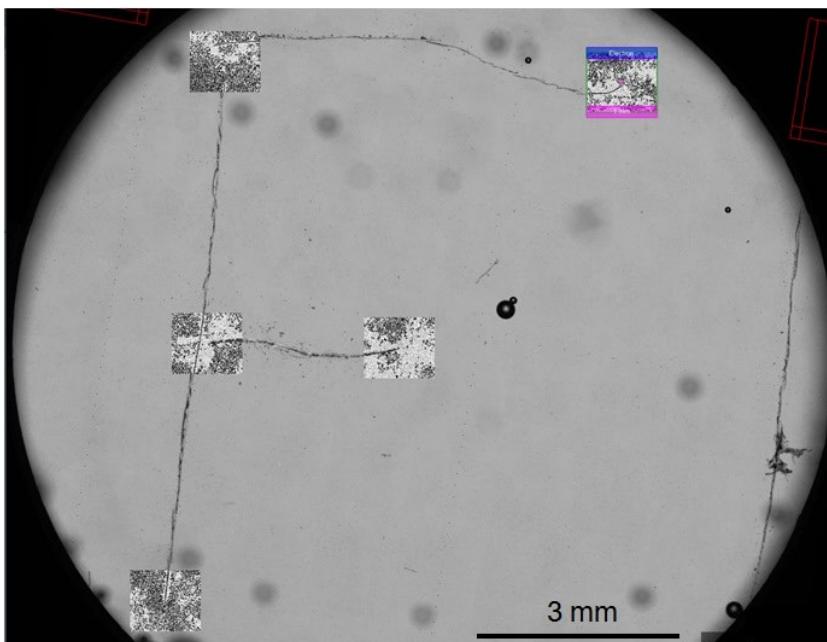
1. Refer to Figure 16 for an annotated screen capture of the software controls. Additional information can be found in the FEI Helios Nanolab™ 660 user manual (Reference 10).
2. Vent the sample chamber of the FEI Helios Nanolab™ 660 SEM. A button labeled “Vent” is found in the panel labeled “EM controls.”
3. Once it is fully vented the chamber can be opened and the samples can be loaded onto the stage (up to 2 due to the size of the pins used). You will know when the chamber is fully vented when the SEM makes a hissing sound. You will also not be able to physically open the chamber door until it is fully vented.
4. Fasten the samples to the sample holder using the screw driver.
  - a. Swing out the camera head (the “Nav-cam”) and take a photo of the stage with the samples loaded. This is done by pressing the single button on the camera head.
  - b. Retract the Nav-cam head and close the chamber.
  - c. Pump down the chamber to reach optimal vacuum. This is done by pressing the button labeled “Pump” found in “EM controls”.
5. Open the FEI MAPS 3.0 software and load the MAPS files from the previous FEI CorrSight™ mapping session.
6. Prepare for operation of the SEM. The SEM is set to acquire on the Everhart-Thornley Detector (ETD) by default.

- a. Once the chamber is under vacuum, turn on the electron beam in “EM controls”. Set the beam voltage to 3.0 kV and 0.2 nA. These are conditions that balance sample preservation with image quality.
  - b. Using the Nav-cam photograph of the stage (bottom left, Figure 16), drive the microscope to the sample by double left-clicking on the sample.
  - c. Zoom in on a feature at > 2,000x magnification and focus the microscope using the controller dashboard attached to the SEM. Click “Link Z to stage” in the software.
  - d. Set the stage height to 4 mm in the panel labeled “Stage position” in preparation for Step G7.
7. Switch to the circular backscatter (CBS) detector in the panel labeled “Change detectors”. The live camera view of the chamber should show a metal detector arm (the CBS detector) swing under the electron gun.
- Note: Inappropriate stage height can damage your sample and the detector arm (see Step G6d).*
8. Use the Alignment Wizard to globally align the SEM to the coverslip light image collected in Procedure D (Figure 11 and Figure 17).
    - a. Left click on “Global Alignment” in the FEI MAPS software.
    - b. Drive the SEM to different landmarks of the “F” fiducial marker.
    - c. Take snapshots by right-clicking the center marker in the FEI MAPS software and select “Snapshot here”. The snapshots will load into FEI MAPS.
    - d. Use the global alignment panel to match the features of the fiducial “F” by SEM to the “F” on the transmitted light image of the ITO coverslip.
    - e. Run the alignment command. If done correctly, the SEM images collected should now align perfectly with the “F” on the transmitted light image collected in Procedure D (Figure 18).



**Figure 17. Aligning the FEI Helios Nanolab™ 660 SEM stage to the map generated by the FEI CorrSight™ using the Global Alignment tool.** Backscatter SEM snapshots of the fiducial marker “F” are aligned to the transmitted light image of the same “F” using a 3-point alignment.

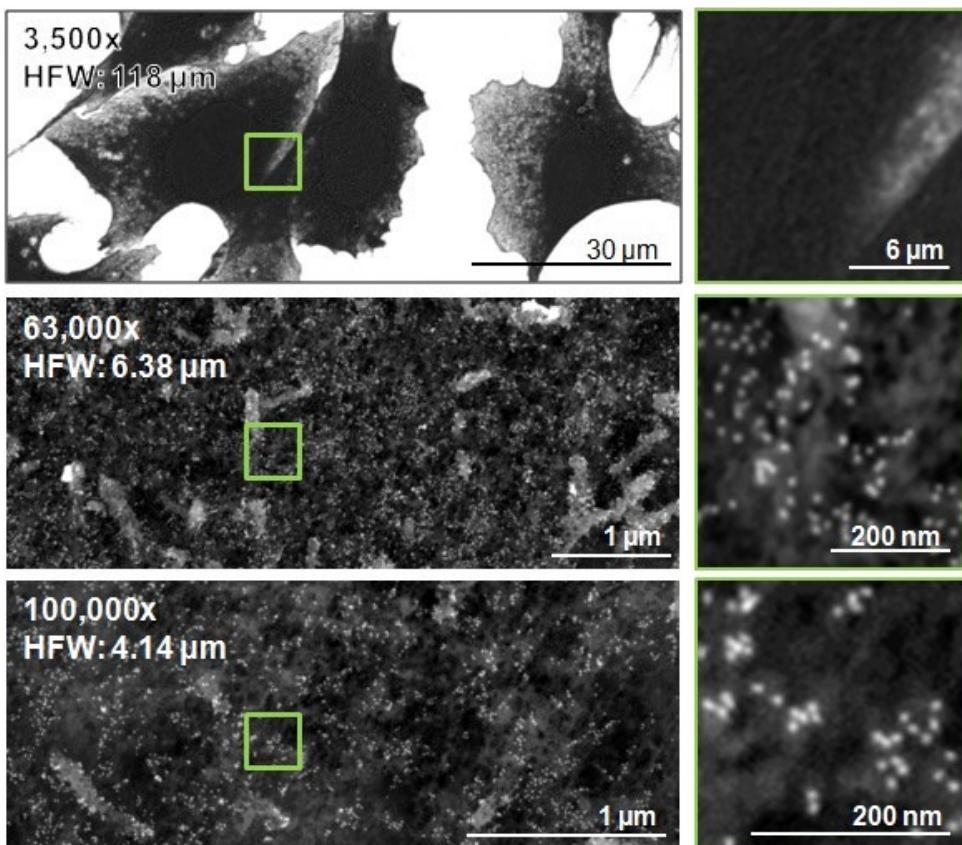
The user manually matches three landmarks on the “F” between the SEM and transmitted light image. The location marked by a green circle in the SEM is the same location as the green circle in the transmitted light view. The same process is repeated for the blue and pink circles.



**Figure 18. The FEI Helios Nanolab™ 660 SEM snapshots of the fiducial marker “F” aligned with the transmitted light image from the FEI CorrSight™.** After SEM image alignment with light-based images generated by the FEI CorrSight™, the FEI Helios Nanolab™ 660 SEM is now aligned with the FEI MAPS software. The user can now select anywhere on the micrographs collected by FM to drive the SEM stage to approximately the same location. Then the same cell can be imaged at high magnification by SEM.

9. After completing the global alignment, any cell from a prior fluorescent image can be selected in FEI MAPS and the SEM will drive to the location of the cell for high magnification SEM imaging.
  - a. Due to the difficulty of aligning high magnification images, the cell will likely be off from the position selected by 10-100 microns.
  - b. Use the shape of the cells or the 5x fluorescent images to locate the exact cell you want to image by SEM.
10. Image at 3,500x magnification (horizontal field width: 119  $\mu\text{m}$ ) to capture the entire cell in a single field-of-view.
  - a. Similar to FEI CorrSight™ operation, create a new layer for each cell and each set of images. Samples can be grouped by creating Layer groups. Make layer names descriptive because they will be the folder names where your data is kept. All of the individual images collected by the FEI MAPS software are automatically named “Tile\_000\_0001” so descriptive layer names are important.

11. Image at 65,000x (horizontal field width: 6.37  $\mu\text{m}$ ) or 100,000x (horizontal field width: 4.14  $\mu\text{m}$ ) magnification to capture the cell surface and to resolve individual Qdots.
  - a. The electron beam is destructive and will ablate cells (*i.e.*, damage cells and introduce holes). While adjusting the imaging conditions (*i.e.*, focus, stigmatism), examine an area adjacent to the area containing the cell that will be imaged. This will help preserve cell morphology for SEM imaging at 65,000x or higher magnification. See Figure 19 for micrographs acquired at 3,500x, 63,000x, and 100,000x magnifications.
  - b. Optionally, quantify the Qdots in the SEM micrographs as described in “Data analysis.”
12. Acquire multiple images per cell, and multiple cells per condition. We recommend at least 2 images per cell and at least 3 cells.
  - a. Transient transfection produces cell-cell variability in protein expression. Therefore, it is important to include replicates and image multiple cells to compensate for this variability.
  - b. For quantification of Qdots in SEM micrographs, we imaged 6 cells per condition with 2 images per cell, resulting in 12 images per condition.



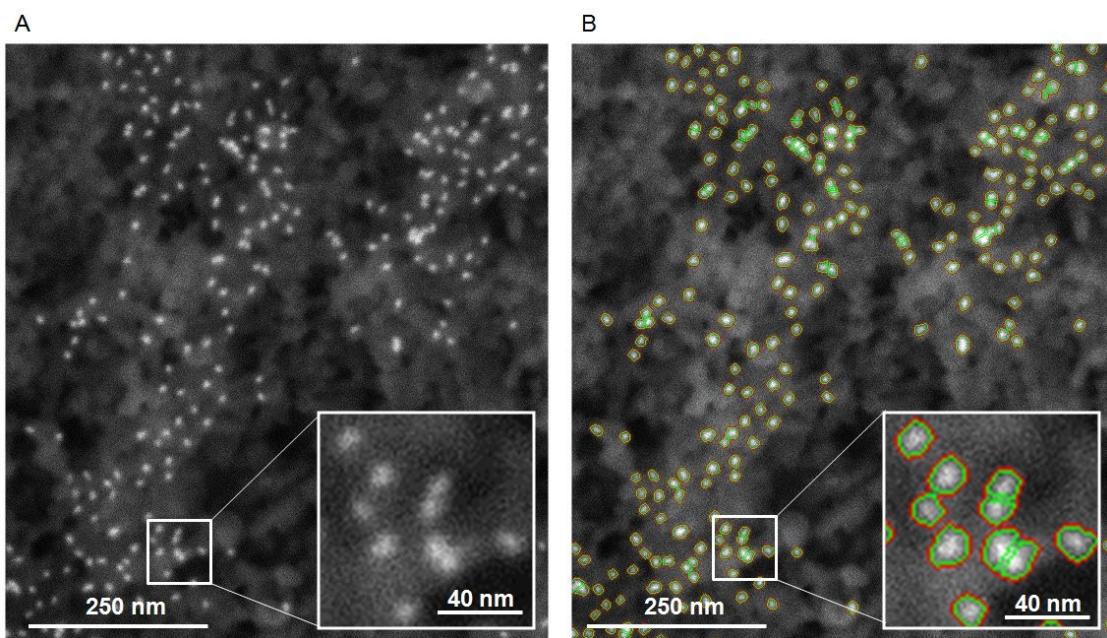
**Figure 19. SEM micrographs of cells imaged at 3,500x (horizontal field width: 118  $\mu\text{m}$ ), 63,000x (horizontal field width: 6.38  $\mu\text{m}$ ) and 100,000x magnification (horizontal field width: 4.14  $\mu\text{m}$ ).** 5x digital magnification of each capture is shown in the right column, highlighting the area outlined by a green box in the corresponding micrograph. Qdots are visible in the high-magnification micrographs as light gray circular particles.

## Data analysis

### Quantification of particles in SEM images

VIPER labeling with Qdots is stoichiometric, meaning that there will be one Qdot per VIPER tag. This feature enables labeled proteins to be quantified by algorithmic segmentation and counting of the Qdots. We recommend acquiring SEM images at high magnification (65,000x or 100,000x) for optimal particle detection and segmentation.

We developed a custom pipeline in Matlab (ver. R2017b) for automated detection and quantitation of particles in backscattered SEM images. We first detected bright objects of interest on a dark background by using morphological top-hat filtering. The object segmentation was performed using a succession of mathematical morphology operations. Briefly, automatic intensity thresholding was performed to detect Qdots. Clustered objects were separated using a seeded watershed transformation from the ultimate eroded results. Next, we counted the segmented single particles ( $n = 1$ ), dimers ( $n = 2$ ), and multimers ( $n > 2$ ). A representative micrograph before and after segmentation is provided in Figure 20.



**Figure 20. Algorithmic segmentation and automated counting of Qdots on the cell surface.** A. The original, unprocessed SEM micrograph acquired at 100,000x magnification is shown. Qdots appear as bright white dots on the cell surface, which appears dark gray. B. The micrograph in A was processed in Matlab to generate a counting mask. The red mask (red outline) indicates the objects detected by morphological top-hat filtering. The green mask (green outline) indicates the objects detected and separated using watershed transformation. Insets show a 3x magnification of the region indicated.

After the automated image processing, we visually inspected the segmentation to refine parameters and exclude objects falsely annotated as Qdots. False-annotations were rare, but typically result from irregular background, intensity variations, background artifacts, or errors in segmentation overlooked by the automated procedure described above.

The parameter sets were optimized to quantify Qdot655 in SEM micrographs acquired at 65,000x-100,000x magnification on an FEI Helios Nanolab™ 660 SEM instrument using a CBS detector. These parameters may need to be adjusted to identify particles depending on the image quality, resolution, or type of particle (e.g., 10 nM gold or Qdot555). The "VIPER\_object\_detection.m" code requires two input parameters related to: (i) intensity (*i.e.*, "int\_threshold" in VIPER\_object\_detection.m) and (ii) object size ("obj\_size", "obj\_size\_filter" in VIPER\_object\_detection.m). The intensity-related parameter should be changed if there are variations in intensity across the images. The object size parameter ("obj\_size") will need to be adjusted if the detection particles are a different physical size (*e.g.*, large gold particles) or if the resolution varies (*i.e.*, pixel size). Also, the code includes filtering options for removing small objects. The "obj\_size\_filter" can be adjusted to remove undersized annotations. These are the parameters that we used for our analysis of Qdot655 particles:

```
int_threshold = 60; % intensity parameter;  
obj_size = 10; % object size parameter;  
obj_size_filter = 5; % filter for small size object.
```

Here is a brief tutorial that describes how to run our custom analysis pipeline to detect and count particles (*i.e.*, Qdots) and to count the segmented particles:

1. Copy the file [VIPER\\_object\\_detection.m](#) into the desired computer directory.
2. Within the same computer directory, make a folder titled ./img/ .
3. Copy SEM images (tiff file format) under ./img/ .
4. Run [VIPER\\_object\\_detection.m](#) in the command window in Matlab.
5. The program will start segmentation and count the objects.
6. The output file will provide the following information:
  - qdot\_count.csv: number of objects (single Qdots, dimers, multimers,  $N_{total} \geq N_1 + N_2 \times 2 + N_{more} \times 3$ ),
  - [filename]-PTmask.mat: object mask,
  - [filename]-Overlay.png: overlaid image with boundaries (red: objects, green: separated objects).

## **Recipes**

1. Live Cell Block solution  
10% (v/v) FBS, 6% (w/v) BSA in Ham's F12 media
2. Qdot Block solution  
10% FBS and 6% BSA in DPBS

3. Qdot Labeling solution  
6% (w/v) BSA in DPBS

### **Acknowledgments**

This work was funded by the OHSU School of Medicine and the National Institutes of Health (R01 GM122854). JKD was partially funded by the Portland Chapter of Achievement Rewards for College Scientists (ARCS). The protocols described herein were originally described in Doh *et al.* (2018). CHO TRVb cells were graciously provided by Prof. Timothy McGraw. EM and CLEM experiments were performed at the Multiscale Microscopy Core at OHSU and supported by the Advanced Multiscale Microscopy Shared Resource at the OHSU Knight Cancer Institute (NIH P30 CA069533). The aluminum coverslip holder was fabricated by Ingo Gestmann (FEI).

### **Competing interests**

The authors declare no financial or non-financial competing interests. An international patent application is pending on the VIP technology (PCT/US17/60609).

### **References**

1. Baker, M. (2015). [Reproducibility crisis: Blame it on the antibodies](#). *Nature* 521(7552): 274-276.
2. Berglund, L., Bjorling, E., Oksvold, P., Fagerberg, L., Asplund, A., Szigyarto, C. A., Persson, A., Ottosson, J., Wernerus, H., Nilsson, P., Lundberg, E., Sivertsson, A., Navani, S., Wester, K., Kampf, C., Hofer, S., Ponten, F. and Uhlen, M. (2008). [A gene-centric Human Protein Atlas for expression profiles based on antibodies](#). *Mol Cell Proteomics* 7(10): 2019-2027.
3. Bordeaux, J., Welsh, A., Agarwal, S., Killam, E., Baquero, M., Hanna, J., Anagnostou, V. and Rimm, D. (2010). [Antibody validation](#). *Biotechniques* 48(3): 197-209.
4. Bradbury, A. and Pluckthun, A. (2015). [Reproducibility: Standardize antibodies used in research](#). *Nature* 518(7537): 27-29.
5. Doh, J. K., Enns, C. A. and Beatty, K. E. (2019a). [Implementing VIPER for imaging cellular proteins by fluorescence microscopy](#). *Bio-protocol* 9(21): e3413.
6. Doh, J. K., Tobin, S. J. and Beatty, K. E. (2019b). [Generation of CoilR probe peptides for VIPER-labeling of cellular proteins](#). *Bio-protocol* 9(21): e3412.
7. Doh, J. K., White, J. D., Zane, H. K., Chang, Y. H., López, C. S., Enns, C. A. and Beatty, K. E. (2018). [VIPER is a genetically encoded peptide tag for fluorescence and electron microscopy](#). *Proc. Natl Acad Sci U.S.A.* 115(51): 12961-12966.
8. Ellisman, M. H., Deerinck, T. J., Shu, X. and Sosinsky, G. E. (2012). [Coeerlative light and electron microscopy](#). In *Methods in cell biology*. Academic Press 111:139-155.
9. FEI CorrSight™ user manual. FEI. (Accessed 12-Oct, 2019, at [http://www.fei.co.jp/\\_documents/CorrSightDatasheet.pdf](http://www.fei.co.jp/_documents/CorrSightDatasheet.pdf).)

10. FEI Helios Nanolab™ 660 user manual. FEI. (Accessed 12-Oct, 2019, at <https://engineering.unl.edu/downloads/files/SOP-Helios660FIB-2019.pdf>)
11. Gaietta, G., Deerinck, T. J., Adams, S. R., Bouwer, J., Tour, O., Laird, D. W., Sosinsky, G. E., Tsien, R. Y. and Ellisman, M. H. (2002). [Multicolor and electron microscopic imaging of connexin trafficking](#). *Science* 296(5567): 503-507.
12. Giepmans, B. N. , Deerinck, T. J., Smarr, B. L., Jones, Y. Z. and Ellisman, M. H.(2005). [Correlated light and electron microscopic imaging of multiple endogenous proteins using Quantum dots](#). *Nat Meth*2(10): 743-749 .
13. Hildebrand, D. G. C., Cicconet, M., Torres, R. M., Choi, W., Quan, T. M., Moon, J., Wetzel, A. W., Scott Champion, A., Graham, B. J., Randlett, O., Plummer, G. S., Portugues, R., Bianco, I. H., Saalfeld, S., Baden, A. D., Lillianey, K., Burns, R., Vogelstein, J. T., Schier, A. F., Lee, W. A., Jeong, W. K., Lichtman, J. W. and Engert, F. (2017). [Whole-brain serial-section electron microscopy in larval zebrafish](#). *Nature* 545(7654): 345-349.
14. Hinner, M. J. and Johnsson, K. (2010). [How to obtain labeled proteins and what to do with them](#). *Curr Opin Biotechnol* 21(6): 766-776.
15. Johnson, E., Seiradake, E., Jones, E. Y., Davis, I., Grunewald, K., Kaufmann, R. (2015). [Correlative in-resin super-resolution and electron microscopy using standard fluorescent proteins](#). *Sci Rep*5: 9583.
16. Karreman, M. A., Mercier, L., Schieber, N. L., Solecki, G., Allio, G., Winkler, F., Ruthensteiner, B., Goetz, J. G. and Schwab, Y. (2016). [Fast and precise targeting of single tumor cells \*in vivo\* by multimodal correlative microscopy](#). *J Cell Sci* 129(2): 444-456.
17. Kim, D., Deerinck, T. J., Sigal, Y. M., Babcock, H. P., Ellisman, M. H. and Zhuang, X. (2015). [Correlative stochastic optical reconstruction microscopy and electron microscopy](#). *PLoS One* 10(4): e0124581.
18. Kremer, A., Lippens, S., Bartunkova, S., Asselbergh, B., Blanpain, C., Fendrych, M., Goossens, A., Holt, M., Janssens, S., Krols, M., Larsimont, J. C., Mc Guire, C., Nowack, M. K., Saelens, X., Schertel, A., Schepens, B., Slezak, M., Timmerman, V., Theunis, C., R, V. A. N. B., Visser, Y. and Guerin, C. J. (2015). [Developing 3D SEM in a broad biological context](#). *J Microsc* 259(2): 80-96.
19. Kuipers, J., van Ham, T. J., Kalicharan, R. D., Veenstra-Algra, A., Sjollema, K. A., Dijk, F., Schnell, U., Giepmans, B. N.(2015). [FLIPPER, a combinatorial probe for correlated live imaging and electron microscopy, allows identification and quantitative analysis of various cells and organelles](#). *Cell Tissue Res* 360(1): 61-70.
20. Laine, R. F., Kaminski Schierle, G. S., van de Linde, S. and Kaminski, C. F. (2016). [From single-molecule spectroscopy to super-resolution imaging of the neuron: a review](#). *Methods Appl Fluoresc* 4(2): 022004.
21. Lam, S. S., Martell, J. D., Kamer, K. J., Deerinck, T. J., Ellisman, M. H., Mootha, V. K. and Ting, A. Y. (2015). [Directed evolution of APEX2 for electron microscopy and proximity labeling](#). *Nat Methods* 12(1): 51-54.

22. Lichtman, J. W., Livet, J. and Sanes, J. R. (2008). [A technicolour approach to the connectome.](#) *Nat Rev Neurosci* 9(6): 417-422.
23. Lipofectamine® 2000 Reagent Protocol 2013. (2013). (Accessed 12-Oct, 2019, at [tools.thermofisher.com/content/sfs/manuals/Lipofectamine\\_2000\\_Reag\\_protocol.pdf](https://tools.thermofisher.com/content/sfs/manuals/Lipofectamine_2000_Reag_protocol.pdf).)
24. Liss, V., Barlag, B., Nietschke, M. and Hensel, M. (2015). [Self-labelling enzymes as universal tags for fluorescence microscopy, super-resolution microscopy and electron microscopy.](#) *Sci Rep* 5: 17740.
25. Lucas, M. S., Gunthert, M., Gasser, P., Lucas, F. and Wepf, R. (2012). [Bridging microscopes: 3D correlative light and scanning electron microscopy of complex biological structures.](#) *Methods Cell Biol* 111: 325-356.
26. Lucocq, J. M., Mayhew, T. M., Schwab, Y., Steyer, A. M. and Hacker, C. (2015). [Systems biology in 3D space--enter the morphome.](#) *Trends Cell Biol* 25(2): 59-64.
27. Martell, J. D., Deerinck, T. J., Sancak, Y., Poulos, T. L., Mootha, V. K., Sosinsky, G. E., Ellisman, M. H. and Ting, A. Y. (2012). [Engineered ascorbate peroxidase as a genetically encoded reporter for electron microscopy.](#) *Nat Biotech* 30(11): 1143-1148.
28. McGraw, T. E., Greenfield, L. and Maxfield, F. R. (1987). [Functional expression of the human transferrin receptor cDNA in Chinese hamster ovary cells deficient in endogenous transferrin receptor.](#) *J Cell Biol* 105(1): 207-214.
29. Megason, S. G. and Fraser, S. E. (2007). [Imaging in systems biology.](#) *Cell* 130(5): 784-795.
30. Milne, J. L. and Subramaniam, S. (2009). [Cryo-electron tomography of bacteria: progress, challenges and future prospects.](#) *Nat Rev Microbiol* 7(9): 666-675.
31. Muller, B. and Heilemann, M. (2013). [Shedding new light on viruses: super-resolution microscopy for studying human immunodeficiency virus.](#) *Trends Microbiol* 21(10): 522-533.
32. Ou, H. D., Phan, S., Deerinck, T. J., Thor, A., Ellisman, M. H. and O'Shea, C. C. (2017). [ChromEMT: Visualizing 3D chromatin structure and compaction in interphase and mitotic cells.](#) *Science* 357(6349).
33. Philimonenko, V. V., Philimonenko, A. A., Sloufova, I., Hraby, M., Novotny, F., Halbhuber, Z., Krivjanska, M., Nebesarova, J., Slouf, M. and Hozak, P. (2014). [Simultaneous detection of multiple targets for ultrastructural immunocytochemistry.](#) *Histochem Cell Biol* 141(3): 229-239.
34. Plaza, S. M., Scheffer, L. K. and Chklovskii, D. B. (2014). [Toward large-scale connectome reconstructions.](#) *Curr Opin Neurobiol* 25: 201-210.
35. Romero-Brey, I. and Bartenschlager, R. (2017). [Viral infection at high magnification: 3D electron microscopy methods to analyze the architecture of infected cells.](#) *Viruses* 7(12): 6316-6345
36. Shu, X., Lev-Ram, V., Deerinck, T. J., Qi, Y., Ramko, E. B., Davidson, M. W., Jin, Y., Ellisman, M. H. and Tsien, R. Y. (2011). [A genetically encoded tag for correlated light and electron microscopy of intact cells, tissues, and organisms.](#) *PLoS Biol* 9(4): e1001041.
37. Sochacki, K. A., Dickey, A. M., Strub, M. P. and Taraska, J. W. (2017). [Endocytic proteins are partitioned at the edge of the clathrin lattice in mammalian cells.](#) *Nat Cell Biol* 19(4): 352-361.

38. Sunbul, M. and Yin, J. (2009). [Site specific protein labeling by enzymatic posttranslational modification.](#) *Org Biomol Chem* 7(17): 3361-3371.
39. Takizawa, T., Powell, R. D., Hainfeld, J. F. and Robinson, J. M. (2015). [FluoroNanogold: an important probe for correlative microscopy.](#) *J Chem Biol* 8(4): 129-142.
40. Tane, H. K., Doh, J. K., Enns, C. A. and Beatty, K. E. (2017). [Versatile interacting peptide \(VIP\) tags for labeling proteins with bright chemical reporters.](#) *Chembiochem* 18(5): 470-474.

## Opto-magnetic Selection and Isolation of Single Cells

Loïc Binan<sup>1, 2, \*</sup>, Joannie Roy<sup>1</sup> and Santiago Costantino<sup>1, 2</sup>

<sup>1</sup>Research center, Maisonneuve-Rosemont Hospital, Montreal, Canada; <sup>2</sup>Department of ophthalmology, University of Montreal, Montreal, Canada

\*For correspondence: [loic.binan@polytechnique.org](mailto:loic.binan@polytechnique.org)



**[Abstract]** Capturing single cells from large heterogenous populations based solely on observable traits is necessary for many cell biology applications and remains a major technical challenge. The protocol we present allows the isolation of viable and metabolically active cells selected for their shape, migration speed, contact to other cells, or intracellular protein localization. We previously introduced a method termed Cell Labeling via Photobleaching (CLaP) for the efficient tagging of cells chosen for visual criteria. Here we describe a new protocol for capturing such cells using ferromagnetic beads termed single-cell magneto-optical capture (scMOCa). This technology is especially useful when the number of target cells represents an extremely low fraction of the total population (potentially one single cell), a situation in which conventional sorting techniques like fluorescent or magnetic activated cell sorting (F/MACS) cannot provide satisfactory results in terms of capture efficiency and specificity. scMOCa uses the lasers of a confocal microscope to photobleach and crosslink biotin-4-fluorescein molecules to cell membranes. Streptavidin coated magnetic beads then adhere to biotin moieties and a magnet allows the capture of illuminated cells. By precisely controlling liquid volumes and spacing between the different parts of a simple setup, high cell selectivity and capture efficacy can be achieved. scMOCa allows visual selection and isolation of any number of cells in a microscopy field and captured cells remain viable to generate new colonies of chosen phenotypes for downstream analyses.

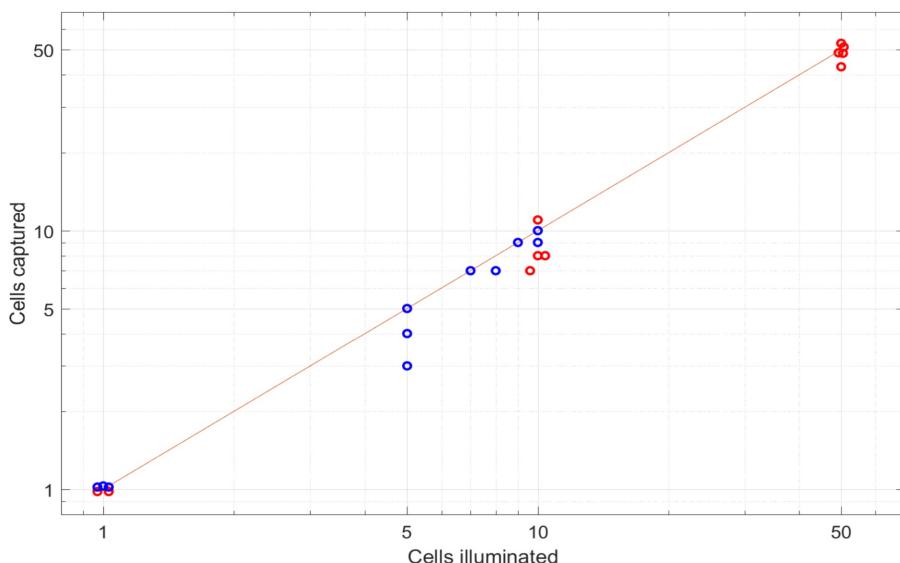
**Keywords:** Cell sorting, Rare cells, Magnetic, Laser, Marker-free

**[Background]** Wide-spread cell sorting techniques rely on the use of highly specific biochemical labels, such as antibodies and fusion proteins, which can be functionalized to allow FACS or MACS (Yan *et al.*, 2009; Kuka and Ashwell, 2013). scMOCa represents a novel alternative which is advantageous when cell selection is based on visual traits such as cell migration (Kurosaka and Kashina, 2008), cell morphology (Prasad and Alizadeh, 2019), intracellular protein organization or cell-to-cell contacts (Garcia *et al.*, 2018) which lack specific molecular markers. For example, cell movement allows embryonic and tumor development, wound healing and immune responses. Morphology reflects different stages of cell growth, differentiation and disease. Cell-to-cell contacts or distance to sources of chemical cues are involved in chemokinesis, differentiation, neural function, and immune responses. Finally, fluorescent protein fusions reveal interesting molecular behavior, such as protein relocalization within cells upon various stimuli. Using smMOCa, these cells can easily be isolated for their extensive characterization. Other approaches that allow the identification of cells without knowledge of a specific marker are based on photoactivated fluorescent proteins (Patterson and Lippincott-Schwartz, 2002;

Lovatt *et al.*, 2014). Once tagged, these cells may still need to be isolated, which can be done using cytometry approaches. Unfortunately, FACS or MACS cannot sort these cells since they are sensitive to the total signal intensity rather than its localization. Some rare label free techniques are based on size selection but have very limited applications (Khojah *et al.*, 2017; Zhao *et al.*, 2017). New technologies based on microfluidics and droplets encapsulation reach extremely low capture rates (less than 10%) (Salomon *et al.*, 2019). A microfluidic chip can only capture a given number of cells that depends on the size of the chip (for instance 96 capture sites, or 384...) which is necessarily smaller than the total number of cells in the sample (which may reach millions). Because most cells are lost, chances to extract a cell population that is already rare in the original population is low.

Finally, some situations involve cells for which there is a marker, but that are too rare to be efficiently extracted with FACS or MACS (Leary, 2000). For instance, rare mutated metastatic or transfected cells. scMOCA is optimized for low cell numbers and as such is an ideal technique in these situations.

Our protocol allows manual selection of single cells one by one based on imaging, regardless of their biochemistry. A sample can be imaged or filmed with a microscope to identify fast migrating cells, or cells with different expression patterns (such as the localization of a 53bp1-GFP [Binan *et al.*, 2019]). Chosen cells are then tagged by crosslinking biotin on their membrane using photobleaching: a laser is used to photobleach biotin-4-fluorescein in the close proximity of the membrane of the cell of interest. Upon photobleaching, a reactive free radical is created, which will bind to the cell membrane, hence biotinyling the cell of interest (Binan *et al.*, 2016; Binan *et al.*, 2019). Because this reaction does not depend on the surface chemistry of the cell, any cell type can be labeled. Using scMOCA, as few as one single cell can be extracted as shown in Figure 1 to generate highly pure samples.



**Figure 1. Number of cells captured as a function of the number of cells tagged.** scMOCA allows high capture rates, even when low numbers of cells are tagged. Red dots represent experiments done on glass, blue dots represent experiments done on plastic substrates. (Originally published in Binan *et al.*, [2019]. [Creative Commons Attribution License](#)).

## **Materials and Reagents**

1. N35 Neodymium Disc Magnet 3/8"x1/16" Rare Earth Disc Magnets, CMS magnetics, store away from hard drives and cell phones as it may damage them
2. Nails from a hardware store. Nails should be made of iron (not steel) and have a 1 mm diameter head. This nail will channel magnetic field lines to the center of the collection chamber
3. Coverslip N1 (VWR, catalog number: 4804-455 on US website)
4. Pipette tips (10, 200 and 1,000  $\mu$ l)
5. 50 ml conical-bottom centrifuge tubes with Flat cap (VWR, catalog number: 89401-562 on US website)
6. Petri dishes
7. Disposable biopsy punches, 5 mm (Integra<sup>TM</sup> miltex<sup>®</sup>, catalog number: 21909-142)
8. Soft-Jet<sup>®</sup> 3-Part Dispensible Syringes, Air-Tite (VWR, catalog number: 89215-238)
9. Millex-GS Syringe Filter Unit, 0.22  $\mu$ m (Sigma-Aldrich, catalog number: SLGSV255F)
10. polystyrene (Sigma-Aldrich, catalog number: P5606-400EA)
11. Biotin-4-fluorescein (b4f) (Sigma-Aldrich, catalog number: B9431-5 mg), store at 4 °C
12. Dynabeads<sup>TM</sup> M270 streptavidin (Thermo Fisher scientific, catalog number: 65305), store at 4 °C
13. Trypsin-EDTA (0.25%), phenol red (ThermoFisher scientific, catalog number: 25200114), store at -20 °C
14. Polydimethyl siloxane (PDMS), Dow SYLGARD<sup>TM</sup> 184 Silicone Encapsulant Clear 0.5 kg Kit, Ellsworth adhesive, 184 SIL ELAST KIT 0.5KG, store at room temperature
15. Gelatin from porcine skin (Sigma-Aldrich, catalog number: G2500-100G) or Poly-L-Ornithine Solution (0.01%) (Sigma-Aldrich, catalog number: A-004-C) or Collagen Type I, rat tail (Sigma-Aldrich, catalog number: 08-115)
16. Phosphate Buffered Saline, purchased or homemade, pH 7.4
17. Phosphate Buffered Saline (containing Ca<sup>2+</sup> and Mg<sup>2+</sup>), purchased or homemade, pH 7.4
18. Conditioned medium (see Recipes)

## **Equipment**

1. Lego bricks:
  - a. 2x 4211428
  - b. 8x 4211393
  - c. 2x 4211406
  - d. 1x 324526
  - e. 2x 4210963
  - f. 1x 4211394
  - g. 2x 4211452

2. Vacuum chamber
3. Cell culture hood with UV light
4. Confocal microscope

A confocal microscope (inverted), or a similar set-up in which visible lasers can be pointed at specific locations in a microscopy field of view.

## **Procedure**

### **A. Preparing culture chamber**

Preparing small chambers takes 2 days, therefore cell culture can be started at the same time in 10 cm dishes. These cells will then be used to fill the chambers, and the sorting can be done 24 h later.

1. In a 50 ml tube, mix SYLGARD base and curing agent (1/10) as per manufacturer instructions (see number 13 in Materials and Reagents section) and vortex.
2. Pour 10 ml of the solution in a 10 cm dish.
3. Place the dish to degas overnight in a vacuum chamber.
4. Wait for two days for the polymer to be well cured.
5. Using a biopsy punch, cut a 5 mm diameter chamber in a block of polymer of approximately 10 mm x 10 mm. Because the polymer is soft, it is easier to first cut the 5 mm chamber, then make a cut around it to separate it from the block of polymer.
6. Place chamber on coverslip and press it to generate good adhesion. PDMS naturally adheres to glass or plastic.
7. On the day the cells are plated, coat the chamber substrate with a solution of choice for improved cell adhesion (gelatin, poly-ornithine or collagen). Usually, a 1/10 dilution of the coating agent in PBS containing Ca<sup>2+</sup> and Mg<sup>2+</sup> for an hour at 37 °C, followed by 3 rinses in PBS works well.

*Note: We recommend testing different coating solutions for each new cell type used and selecting the one that provides the best outcome in terms of sample purity and sorting efficacy. When working with highly adherent cells, coating may not be necessary. Sensitive cells often require a collagen coating. Nevertheless, this option generates collagen fibers in which both negative and positive cells may be trapped after cell suspension, hence reducing the selectivity of the technique. For this reason, we found that gelatin and poly-ornithine provide good intermediate solutions in most cases. As an example, we used a gelatin coating for sorting embryonic stem cells, but a collagen coating for HUVECS and MDA-MB-231 and no coating at all for U2OS cells.*

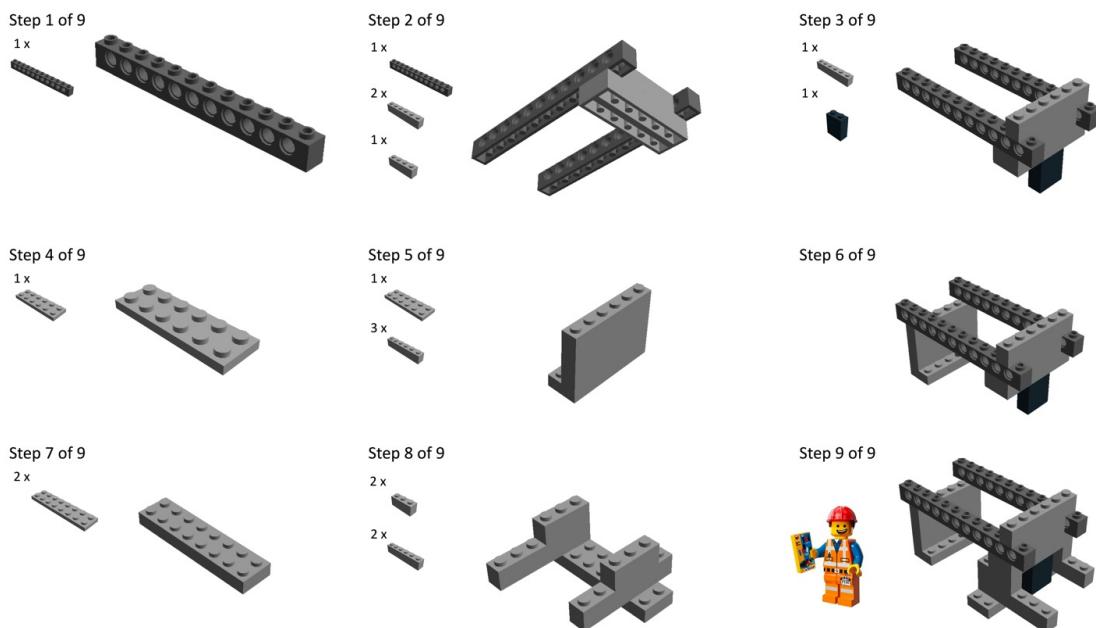
8. Place under UV in cell culture hood for sterilization.

### **B. Building Chamber holder**

Build chamber holder using Lego bricks. This structure will be used to maintain the collection

chamber and the magnets at appropriate distances from the donor chamber.

1. We provide building instructions in Figure 2 and the setup is visible in Video 1.



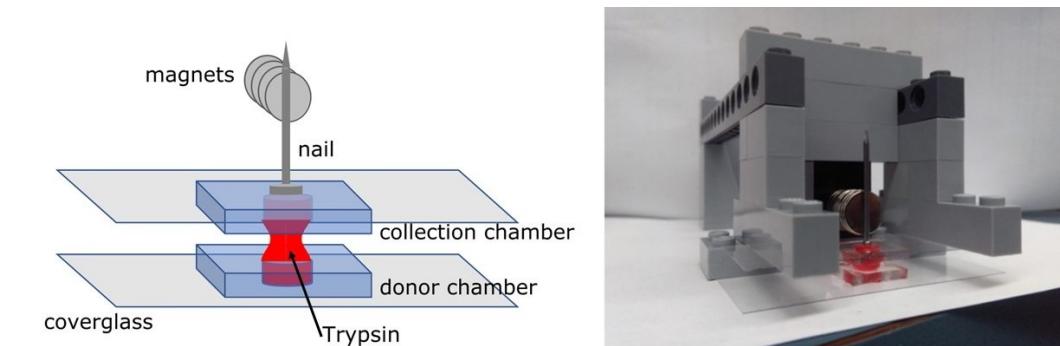
**Figure 2. Instructions to build the structure with Lego bricks** (Originally published in Binan et al., [2019]. [Creative Commons Attribution License](#))



**Video 1. Assembly and use of the setup**

2. If the setup is built without Lego bricks, important parameters that must be kept constant are the distance between chambers (6 mm from the bottom of the donor chamber to the bottom of the collection chamber) and the ability to maintain 10 magnets close to a nail which head is centered in the collection chamber as shown Video 1.
3. The coverslip with the collection chamber will be inserted just above the long bricks from Step 7 in Figure 2. The long transversal bars from Step 8 in Figure 2 will be used to pinch it in position.

4. The double height, black brick in Step 3 in Figure 2 (number 16.d from the Material and Reagents list above) is hollow and two magnets are inserted inside. They allow holding the rest of the pile of magnets on the side of the brick, as visible in Figure 3 and Video 1.



**Figure 3. Position of the chambers in the setup** (Originally published in Binan *et al.*, [2019].  
[Creative Commons Attribution License](#))

#### C. Conditioned medium preparation

When preparing chambers, the day prior to the experiment, keep medium from the culture and filter it through a 0.22 µm filter to remove debris (with syringe and syringe filters). This medium is rich in secreted factors and helps grow cells at very low density, improving sorted cell survival.

#### D. Laser alignment

In order to precisely tag single cells, it is key to locate the exact location at which the laser points. If the experiment is performed on a microscope that allows pointing at a given location on the image and to bleach cells, this step is not necessary and can be skipped. On the other hand, on home-made optical systems without this functionality, or if cells of interest are identified with bright field or epifluorescence imaging, precise recording of the laser position is required.

1. Prepare a fluorescent coverslip: place a drop of fluorescein (or b4f) stock solution between two coverslips and let it dry.
2. Once dried, observe this fluorescent slide and adjust focus using an inverted microscope.
3. Immobilize the blue laser from the confocal microscope and illuminate the slide for a few seconds.
4. Without moving the slide/stage, observe again in fluorescence and record the position of the bleached spot (we used a simple pen mark on the computer screen, but one must then be careful not to move the position of the windows on the screen afterwards). This dark photobleached area is the precise location that will be illuminated in the sample.

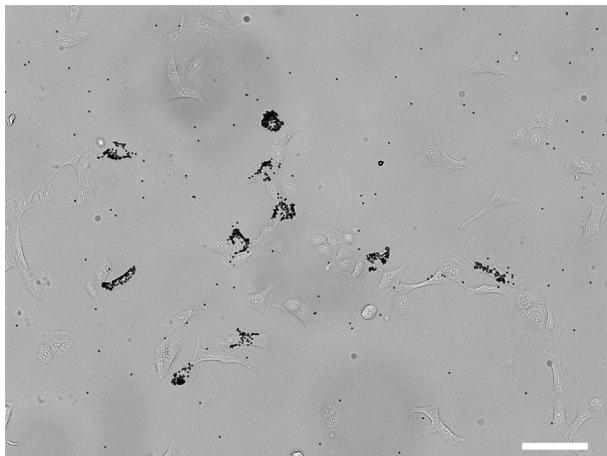
#### E. Laser-assisted membrane biotinylation and bead attachment

1. Seed cells 24 h in advance in culture chambers prepared in Procedure A.
2. On the day of the experiment, add biotin-4-fluorescein to medium to reach 0.04 mg/ml.

3. Observe sample and choose the cells to capture. It is easier (but not necessary) to use the same objective to select cells of interest and to label them by photobleaching. We generally used a 10x, 0.4NA objective, unless the observation of the features of interest required higher magnification. In this particular case, a 60x can be used to identify cells that will be captured. Then the objective can be replaced by a 10x, 0.4NA to label the cells with the usual power settings. Using a relatively low NA increases the chances of successfully tagging the cells by reducing the importance of adjusting the focus for each cell of interest.
4. Depending on the setup used, either point the laser to the cell of choice and illuminate for 2 s, or move the sample such that one of the chosen cells is positioned in the laser illumination mark obtained in Sorting Procedure D and open the shutter to irradiate the cell for 2 s. Laser power needs to be optimized depending on cell type and experimental setup, with ideal power varying from 30  $\mu$ W to 400  $\mu$ W using a 0.4NA objective. The ideal power is the lowest power that allows efficient labeling of the cells of interest. It is only dependent on the setup used and should not vary from a cell type to another. Repeat this step for each cell that needs to be captured.
5. Rinse thoroughly in PBS.

The medium has to be well rinsed as remaining free biotin-4-fluorescein will block binding sites on the beads. It is key that the chamber never dries, which can happen very fast due to its extremely small total volume. In our hands, dipping the culture chamber in a PBS beaker several times provides excellent results whereas pipetting tends to detach some cells, and the tip may scratch the bottom of these small chambers.

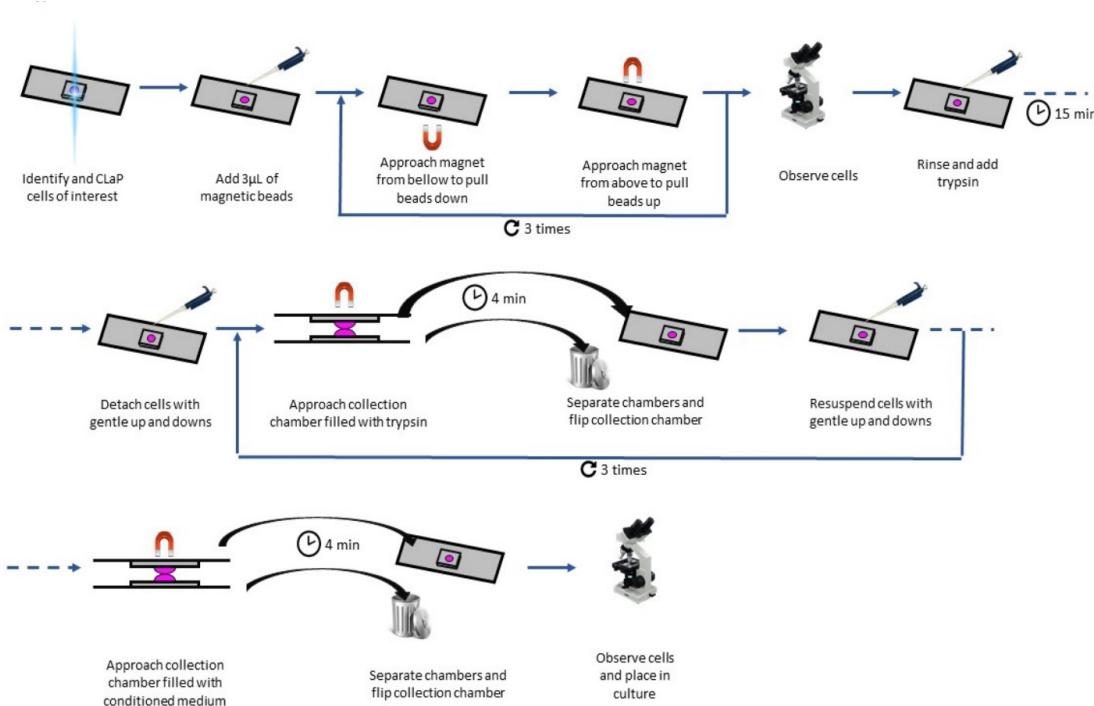
6. Replace liquid in the chamber with medium containing 3  $\mu$ l of beads washed in PBS as per manufacturer instructions, without further dilution.
7. Use a magnet to pull beads down towards the cells.  
When strongly adherent cells are used, the magnet can be used to pull beads across the whole area to ensure maximal attachment of the beads. When sensitive cells are manipulated, beads can be pulled down with the magnet, then resuspended either by pipetting the solution up and down a few times or by placing the magnets above the sample, then pulled back down with the magnets three times. The ideal strategy has to be determined for each different cell type and culture substrate as it is dependent on adhesion strength.
8. Rinse again in PBS. Three repeats of a 30 s immersion in a beaker of PBS is still the ideal solution here.
9. Observe under a bright field microscope to verify that beads decorate the cells of choice. Expected result is shown in Figure 4.



**Figure 4. Bright field image of 10 tagged U2Os cells.** Scale bar: 100  $\mu\text{m}$ .

#### F. Cell sorting

1. Replace liquid with trypsin-EDTA (0.25%) in the donor chamber. Wait for the cells to become round and detach. Perform a few ups-and-downs with a pipette to carefully detach all cells.
2. Place a collection chamber (an identical cell culture chamber, without cells) in the sorting setup, fill it with a drop of trypsin/PBS/medium (see details below) and place it above the donor chamber in a symmetric position, to bring both liquid drops to merge as shown in Figure 3 and Video 1. Chamber dimensions are designed to allow surface tension forces to maintain the drop of liquid inside the chamber without falling, even when the chamber is upside down.
3. Wait 4 min for positive cells to gather in the collection chamber while negative cells settle in the donor chamber.
4. Flip the setup, remove collection chamber and verify with a microscope that cells were captured.
5. Gently pipette the solution up and down a few times to resuspend the cells and repeat the procedure up to 4 times to reach a pure sample. The number of repeats depends on how sticky the cells are, and on the density of the original cell culture. These sorting steps are depicted in Figure 5. When working with resistant cells, all repeats may be performed in trypsin. When working with more sensitive cells, they may be done in PBS. One must be careful to not let the cells re-adhere in any of the intermediate chambers by thoroughly pipetting the solution up-and-down to efficiently resuspend them between each step. For each intermediate sorting, the collection chamber should not be coated but rather made of bare glass to avoid undesired cell adhesion to the substrate. The final step should be performed in a coated chamber, filled with medium. Because collected cells usually end up being at very low density in the collection chamber, their viability may be reduced. To solve this issue, the medium used for the last sorting step, and for following medium changes in this chamber, should be conditioned medium (see Procedure C).



**Figure 5. Detailed schematic of the sorting procedure** (Originally published in Binan *et al.*, [2019]. [Creative Commons Attribution License](#))

## Notes

1. Before its first use on any setup and with any new biological system, the procedure's parameters should be optimized. The adequate laser power that permits efficient cell labeling without killing has to be determined for each experiment and is highly dependent on the alignment of the laser with the microscope objective but should not vary with the cell type. The ideal laser power is the lowest power that allows efficient cell labeling on the setup used. We usually use around 50 µW.
2. The appropriate surface preparation for the culture chambers depends on the cell type. Most sensitive cell types may require collagen coating, but this option makes following steps more difficult. When cells are more resistant, one may prefer to choose gelatin or poly-ornithine, or even bare glass when working with resistant cells.
3. A frequent cause of failure is inefficient rinsing. The concentration of biotin-4-fluorescein (b4f) is extremely high and therefore the three conventional rinses in PBS are often not sufficient to remove all traces of free b4f. This is detrimental to the binding of magnetic beads to tagged cells because any free floating b4f will block binding sites. Observation of the sample after incubation with beads allows troubleshooting this issue: any beads remaining in the chamber should not be fluorescent in the fluorescein channel. Beads only become fluorescent if free b4f binds to streptavidin.
4. Because losing as few as one cell may be very detrimental to this experiment, rinsing should

be well optimized. In our hands, the best solution was obtained by immersing the culture chamber in large volumes (10 to 30 ml) of PBS.

5. A few parameters are key to successful captures and should not be modified: the chamber must be 5 mm in diameter. Thus, surface tension prevents the liquid drop from falling when the chamber is upside down and reduces the distance from the edge to the center of the chamber, where the cells will be attracted by the magnets. The distance between the two chambers substrates should be kept at 6 mm, which is close enough for the magnets to efficiently pull up positive cells, but also far enough to maintain negative cells far from the collection chamber.

## **Recipes**

1. Conditioned medium

When passaging cells, the day prior to the experiment, keep old medium and filter it through 0.22 µm filter to remove debris (with syringe and syringe filters). This medium is rich in secreted factors and helps culture cells at very low density.

## **Acknowledgments**

This work was supported by grants from the Natural Science and Engineering Research Council of Canada, Genome Canada/Génome Québec and Canadian Cancer Society, Fonds de Recherche du Québec-Nature et Technologies. S.C. holds salary awards from the Fonds de Recherche du Québec-Santé. This protocol is derived from the paper Opto-magnetic capture of individual cells based on their phenotype (Binan *et al.*, 2019).

## **Competing interests**

The authors declare no competing financial interests.

## **References**

1. Binan, L., Belanger, F., Uriarte, M., Lemay, J. F., Pelletier De Koninck, J. C., Roy, J., Affar, E. B., Drobetsky, E., Wurtele, H. and Costantino, S. (2019). [Opto-magnetic capture of individual cells based on visual phenotypes](#). *Elife* 8. pii: e45239.
2. Binan, L., Mazzaferri, J., Choquet, K., Lorenzo, L. E., Wang, Y. C., Affar, E. B., De Koninck, Y., Ragoussis, J., Kleinman, C. L. and Costantino, S. (2016). [Live single-cell laser tag](#). *Nat Commun* 7: 11636.
3. Garcia, M. A., Nelson, W. J. and Chavez, N. (2018). [Cell-Cell junctions organize structural and signaling networks](#). *Cold Spring Harb Perspect Biol* 10(4).
4. Khojah, R., Stoutamore, R. and Di Carlo, D. (2017). [Size-tunable microvortex capture of rare](#)

- cells. *Lab Chip* 17(15): 2542-2549.
5. Kuka, M. and Ashwell, J. D. (2013). A method for high purity sorting of rare cell subsets applied to TDC. *J Immunol Methods* 400-401: 111-116.
  6. Kurosaka, S. and Kashina, A. (2008). Cell biology of embryonic migration. *Birth Defects Res C Embryo Today* 84(2): 102-122.
  7. Leary, J. F. (2000). Rare-event detection and sorting of rare cells. In *Emerging Tools for Single - Cell Analysis* pp: 49-72.
  8. Lovatt, D., Ruble, B. K., Lee, J., Dueck, H., Kim, T. K., Fisher, S., Francis, C., Spaethling, J. M., Wolf, J. A., Grady, M. S., Ulyanova, A. V., Yeldell, S. B., Griepenburg, J. C., Buckley, P. T., Kim, J., Sul, J. Y., Dmochowski, I. J. and Eberwine, J. (2014). Transcriptome in vivo analysis (TIVA) of spatially defined single cells in live tissue. *Nat Methods* 11(2): 190-196.
  9. Patterson, G. H. and Lippincott-Schwartz, J. (2002). A photoactivatable GFP for selective photolabeling of proteins and cells. *Science* 297(5588): 1873-1877.
  10. Prasad, A. and Alizadeh, E. (2019). Cell form and function: Interpreting and controlling the shape of adherent cells. *Trends Biotechnol* 37(4): 347-357.
  11. Salomon, R., Kaczorowski, D., Valdes-Mora, F., Nordon, R. E., Neild, A., Farbehi, N., Bartonicek, N. and Gallego-Ortega, D. (2019). Droplet-based single cell RNAseq tools: a practical guide. *Lab Chip* 19(10): 1706-1727.
  12. Yan, H., Ding, C. G., Tian, P. X., Ge, G. Q., Jin, Z. K., Jia, L. N., Ding, X. M., Pan, X. M. and Xue, W. J. (2009). Magnetic cell sorting and flow cytometry sorting methods for the isolation and function analysis of mouse CD4+ CD25+ Treg cells. *J Zhejiang Univ Sci B* 10(12): 928-932.
  13. Zhao, W., Cheng, R., Lim, S. H., Miller, J. R., Zhang, W., Tang, W., Xie, J. and Mao, L. (2017). Biocompatible and label-free separation of cancer cells from cell culture lines from white blood cells in ferrofluids. *Lab Chip* 17(13): 2243-2255.