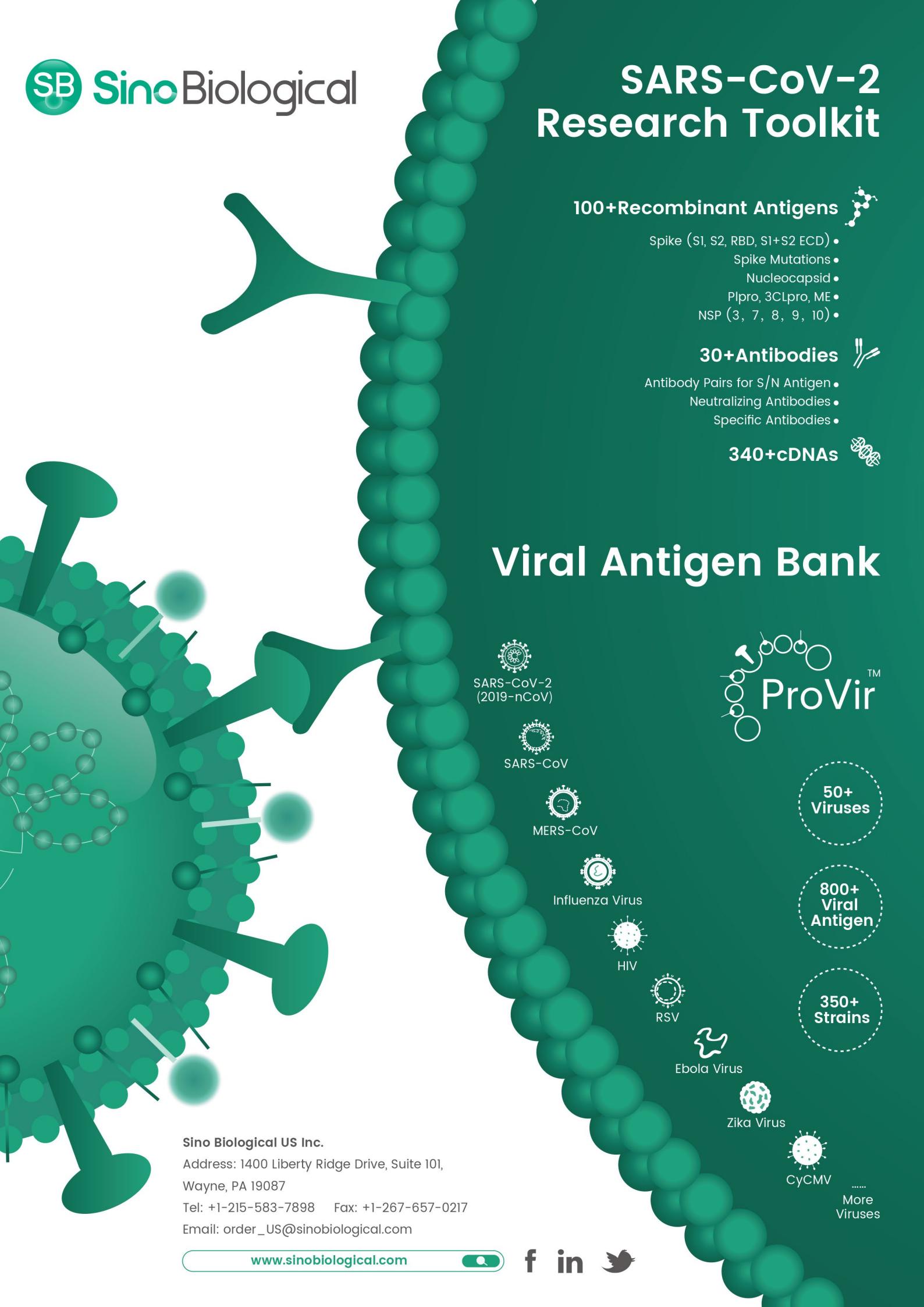


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## **Foreword**

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 16 of the most-used cancer research 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.

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*Original research article:* eLife 6:e26509

**Quantitative Live-cell Reporter Assay for  
Noncanonical Wnt Activity**

7

Edith P. Karuna, Michael W. Susman and  
Hsin-Yi Henry Ho

*Original research article:* Nucleic Acids Res. 45(12):e116

**Detection and Analysis of Circular RNAs  
by RT-PCR**

Amaresh C Panda and Myriam orospe

*Original research article:* Proc Natl Acad Sci. 114(8):1928-33

**Centromere Chromosome Orientation**

26

**Fluorescent in situ Hybridization  
(Cen-CO-FISH) Detects Sister Chromatid  
Exchange at the Centromere in Human  
Cells**

Simona iunta

*Original research article:* Nucleic Acids Res. 45(12):e116

**3D Co-culture System of  
Tumor-associated Macrophages and  
Ovarian Cancer Cells**

40

Lingli Long, Mingzhu Yin, and Wang Min

*Original research article:* Proc Natl Acad Sci. 114(8):1928-33

**Generation of Luciferase-expressing  
Tumor Cell Lines**

50

Todd V. Brennan, Liwen Lin, Xiaopei  
Huang and Yiping Yang

*Original research article:* J Biol Chem. 292(51):21102-16

**Measurement of Oxygen Consumption  
Rate (OCR) and Extracellular  
Acidification Rate (ECAR) in Culture  
Cells for Assessment of the Energy  
Metabolism**

Birte Plitzko and Sandra Loesgen

*Original research article:* Immunity 47(5):875-89

**Isolation of Microvascular  
Endothelial Cells**

76

Kenneth C.P. Cheung and Federica M.  
Marelli-Berg

*Original research article:* Oncogene. 36(18):2515-28

*Original research article:* J Clin Invest. 128(1):500-16

**Cell Synchronization by Double  
Thymidine Block**

106

uo Chen and Xingming Deng

**An *in vitro* Co-culture System for the  
Activation of CD40 by  
Membrane-presented CD40 Ligand  
versus Soluble Agonist**

Khalidah Ibraheem, Christopher J.  
Dunnill, Myria Ioannou, Albasir  
Mohamed, Balid Albarbar and Nikolaos  
T. eorgopoulos

*Original research article:* Clin Cancer Res. 22(7):1813-24

**Qualitative *in vivo* bioluminescence imaging**

Devbarna Sinha, Zalitha Pieterse and Pritinder Kaur

*Original research article:* Cancer Res. 76(1):35-42

**Adoptive Transfer of Monocytes Sorted from Bone Marrow**

Damya Laoui, Eva Van Overmeire, Chloé Abels, Jiri Keirsse and Jo A Van Inderachter

*Original research article:* Mol. Cell. 72(1):84-98

**Measuring Protein Synthesis during Cell Cycle by Azidohomoalanine (AHA) Labeling and Flow Cytometric Analysis**

Koshi Imami and Tomoharu Yasuda

*Original research article:* Cancer Cell. 34(5): 823-39

**Isolation and Quantification of Metabolite Levels in Murine Tumor Interstitial Fluid by LC/MS**

Hai Wang and Xiang H.-F. Zhang

*Original research article:* eLife 7: e32490

**Zebrafish Embryo Xenograft and Metastasis Assay**

Ilkka Paatero, Sanni Alve, Silvia Ramolelli, Johanna Ivaska and Päivi M. Ojala

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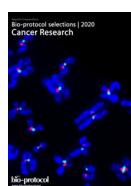
**Calvarial Bone Implantation and *in vivo* Imaging of Tumor Cells in Mice**

Kyoko Hashimoto, Shingo Sato, Hiroki Ochi, Shu Takeda and Mitsuru Futakuchi

*Original research article:* J Cell Biol. 217(4): 1521-36

**SIRF: A Single-cell Assay for *in situ* Protein Interaction with Nascent DNA Replication Forks**

Sunetra Roy and Katharina Schlacher



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Image from protocol "Centromere Chromosome Orientation Fluorescent *in situ* Hybridization (Cen-CO-FISH) Detects Sister Chromatid Exchange at the Centromere in Human Cells"

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## Quantitative Live-cell Reporter Assay for Noncanonical Wnt Activity

Edith P. Karuna<sup>1</sup>, Michael W. Susman<sup>2</sup> and Hsin-Yi Henry Ho<sup>1,\*</sup>

<sup>1</sup>Department of Cell Biology and Human Anatomy, University of California, Davis School of Medicine, Davis, California, USA; <sup>2</sup>Department of Neurobiology, Harvard Medical School, Boston, Massachusetts, USA

\*For correspondence: [hyhho@ucdavis.edu](mailto:hyhho@ucdavis.edu)



**[Abstract]** Noncanonical Wnt signaling functions independently of the  $\beta$ -catenin pathway to control diverse developmental processes, and dysfunction of the pathway contributes to a number of human pathological conditions, including birth defects and metastatic cancer. Progress in the field, however, has been hampered by the scarcity of functional assays for measuring noncanonical Wnt signaling activity. We recently described the Wnt5a-Ror-Kif26b (WRK) reporter assay, which directly monitors a post-transcriptional regulatory event in noncanonical Wnt signaling. In this protocol, we describe the generation of the stable GFP-Kif26b reporter cell line and a quantitative reporter assay for detecting and measuring Wnt5a signaling activities in live cells via flow cytometry.

**Keywords:** Noncanonical Wnt reporter, Wnt5a signaling, Kif26b, Regulated degradation, Flow cytometry

**[Background]** Historically, transcriptional reporter assays have facilitated the delineation of major signaling pathways. In particular,  $\beta$ -catenin-dependent luciferase- or GFP-based transcriptional reporters have been instrumental in elucidating the molecular mechanisms of the canonical Wnt/ $\beta$ -catenin pathway (Korinek *et al.*, 1997; Fuerer and Nusse, 2010). Although a number of noncanonical Wnt signaling reporters based on JNK-dependent transcription have been described, it remains unclear whether these transcriptional responses are primary or secondary to noncanonical Wnt signaling (Veeman *et al.*, 2003, Nishita *et al.*, 2010, Ohkawara and Niehrs, 2011). Also, a reporter for real-time detection of non-transcriptional Wnt5a-Ror signaling events has not been available. The Wnt5a-Ror-Kif26b (WRK) reporter assay, which directly monitors a non-transcriptional Wnt5a-Ror signaling event, adds to the current repertoire of molecular tools for studying noncanonical Wnt signaling (Ho *et al.*, 2012; Susman *et al.*, 2017).

As described in our recent publication, Wnt5a-Ror signaling modulates the steady-state protein level of the kinesin superfamily member Kif26b by inducing its ubiquitin- and proteasome-dependent degradation (Susman *et al.*, 2017). This reporter assay enables further identification and mechanism-based analysis of other Wnt5a-Ror signaling components, most of which remain unknown or relatively unexplored. In addition, the WRK assay may also facilitate the screening of pharmacological agents in Wnt5a-Ror related diseases such as certain cancers and developmental disorders.

This protocol describes the generation of the stable GFP-Kif26b reporter cell line and a quantitative method of detecting Wnt5a signaling levels in live GFP-Kif26b reporter cells via flow cytometry.

## **Materials and Reagents**

1. Pipette tips (USA Scientific, catalog numbers: 1122-1832, 1120-8812, 1123-1812, 1121-3812)
2. 10-cm tissue culture dish (Corning, Falcon®, catalog number: 353003)
3. 1.5 ml microcentrifuge tubes (Denville Scientific, catalog number: C2170)  
*Note: Autoclave before use.*
4. 6-well plate
5. 48-well tissue culture plate (Corning, Costar®, catalog number: 3548)
6. 5 ml round-bottom tubes with 35 µm cell strainer snap cap (Corning, Falcon®, catalog number: 352235)
7. NIH/3T3 Flp-In cells (Thermo Fisher Scientific, Invitrogen™, catalog number: R76107)
8. pCAG-GFP (available upon request), or any GFP plasmid suitable for mammalian expression
9. pEF5-FRT-GFP-Kif26b (Addgene, catalog number: 102862) reporter construct
10. pOG44 Flp-Recombinase expression vector (Thermo Fisher Scientific, Invitrogen™, catalog number: V600520)
11. Recombinant Wnt5a (R&D Systems, catalog number: 654-WN-010)
12. Genjet *In Vitro* Transfection Reagent for NIH/3T3 cells (SigmaGen Laboratories, catalog number: SL100488, 3T3)
13. Hygromycin B (50 mg/ml solution) (Corning, Mediatech, catalog number: 30-240-CR)
14. Poly-D-lysine (Sigma-Aldrich, catalog number: P6407-10X5MG)
15. Wnt-C59 (Cellagen Technology, catalog number: C7641-2s)
16. Trypsin EDTA (Corning, Mediatech, catalog number: 25-052-CI)
17. Dulbecco's modified Eagle's medium (DMEM) (Corning, Mediatech, catalog number: 15-017-CV)
18. Fetal bovine serum (FBS) (Thermo Fisher Scientific, Gibco™, catalog number: 16000069)  
*Note: The FBS is used directly without heat-inactivation.*
19. Glutamine (100x solution, 200 mM) (Corning, Mediatech, catalog number: 25-005-CI)
20. Penicillin-streptomycin (100x solution, 100 IU/ml) (Corning, Mediatech, catalog number: 30-002-CI)
21. Bovine serum albumin (BSA) (Fisher Scientific, catalog number: BP1600-1)
22. CHAPS detergent (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 28300)
23. Phosphate buffered saline (PBS) (GE Healthcare, catalog number: SH30256.01)
24. Growth media (see Recipes)
25. Wnt control buffer (see Recipes)
26. Cell resuspension buffer for flow cytometry (see Recipes)

## Equipment

1. Pipetters (e.g., Eppendorf, model: Research<sup>®</sup> plus)
2. 37 °C, 5% CO<sub>2</sub> incubator (e.g., Heracell by Thermo Fisher Scientific)
3. Centrifuge with cooling capabilities (e.g., Thermo Fisher Scientific, Thermo Scientific<sup>TM</sup>, model: Sorvall<sup>TM</sup> Legend<sup>TM</sup> Micro 21R)
4. Fluorescent microscope with 488 nm light source (e.g., Thermo Fisher Scientific, model: EVOS<sup>®</sup>)
5. Flow cytometer with 488 nm laser (e.g., BD, model: FACScan)

## Software

1. FlowJo software (FlowJo, LLC; <https://www.flowjo.com/>)

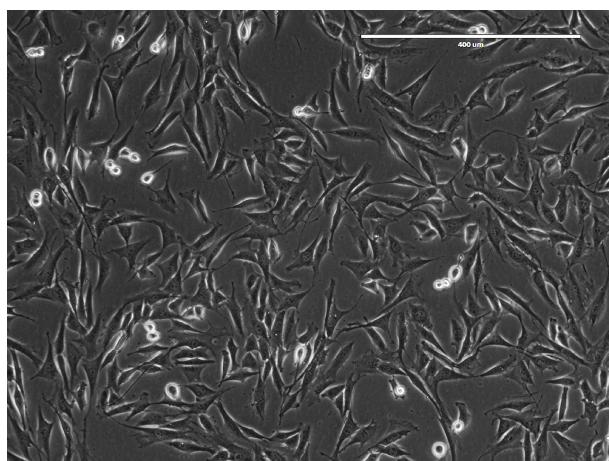
## Procedure

### A. Generation of stable reporter cell lines using the Flp-In NIH/3T3 cell line

#### 1. Cell plating for transfection

Seed cells at 1.62 M cells/plate in a 10-cm plate in 10 ml of growth media. Culture the cells at 37 °C until they reach 80% confluence (about 18-24 h, Figure 1).

*Note: Prepare 1 plate of cells for each reporter construct, plus 1 additional plate for the pCAG-GFP, which serves as both a negative control for the Flp-In and a reference for transfection efficiency.*



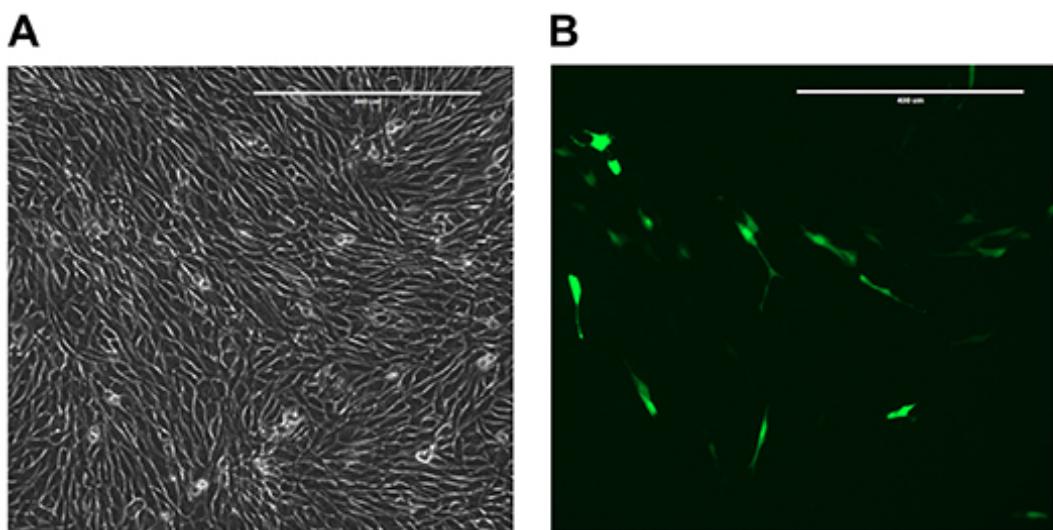
**Figure 1. Confluence (80%) at the time of transfection.** Phase contrast, 10x magnification.  
Scale bar represents 400 μm.

## 2. Transfection

- a. One hour before transfection, remove media from cells and replace with 6 ml fresh growth media.
- b. Dilute DNA: In a 1.5 ml microcentrifuge tube, add 1.35 µg pEF5-FRT-GFP-Kif26b and 12.15 µg pOG44 to 675 µl of serum-free media (plain DMEM). In parallel, for the GFP control plate, prepare a tube of 675 µl serum-free media with 13.5 µg of pCAG-GFP but no pOG44. Mix well by pipetting.  
*Note: Total mass of transfected DNA is 13.5 µg. Transfect with a 1:10 molar ratio of reporter plasmid to flp recombinase; adjust masses according to the size of the plasmid.*
- c. Dilute the GenJet transfection reagent: for each plate, prepare a separate 1.5 ml microcentrifuge tube of 40.5 µl GenJet transfection reagent in 675 µl of serum-free media (plain DMEM). Mix well by pipetting.
- d. Add each tube of diluted GenJet solution all at once to each respective DNA solution.

*Note: The GenJet solution must be added to the DNA solution, not the reverse. Vortex gently for 4 sec to mix.*

- e. Incubate the transfection mixes for 15 min at room temperature. Do not let the incubation proceed for more than 20 min.
- f. Add the transfection mixes drop-wise to their respective plates of cells.
- g. Gently rock the plates to mix well and return the plates to the incubator.
- h. After 12-18 h, check transfection efficiency by visualizing the GFP control plate under a fluorescent microscope (Figure 2). Remove transfection media and replace with 10 ml of growth media.

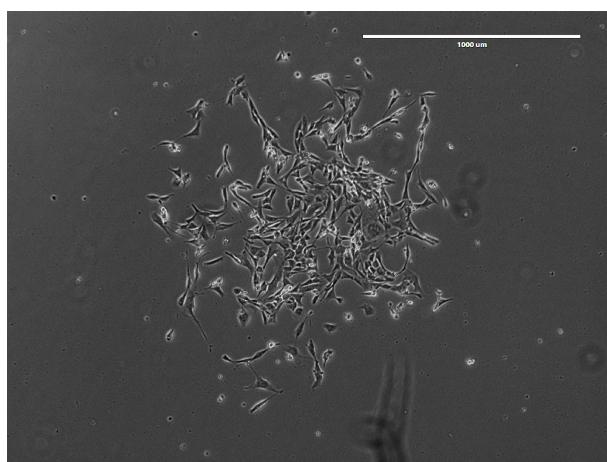


**Figure 2. GFP control plate 12-18 h after transfection.** A. Phase contrast channel, 10x magnification. Scale bar represents 400 µm. B. GFP channel, 10x magnification. Scale bar represents 400 µm.

### 3. Antibiotic selection

- a. Two days after transfection, split each 10-cm plate into 4 x 10-cm plates in growth media to avoid overcrowding cells during selection (do not use selection antibiotics during the split).
- b. After cells adhere to the plate, remove media and replace with fresh growth media containing 200 µg/ml hygromycin B. Replace with fresh hygromycin media every 3-4 days. Selection should take about 7-10 days. Between 6-20 colonies per plate is typically expected (Figure 3).

*Note: A kill curve was conducted to determine that 200 µg/ml hygromycin B is optimal for NIH/3T3 Flp-In cells. The optimal selection concentration may vary slightly depending on the source of hygromycin B and cell lines.*



**Figure 3. A representative colony at 7 days post-hygromycin B selection.** Phase contrast, 4x magnification. Scale bar represents 1,000 µm.

- c. Cells may be pooled from 1 or 2 10-cm plates into a single well of a 6-well plate and passaged in growth media without selection antibiotics.

*Note: This step is only performed for the reporter constructs. The GFP control plate, which should yield no colonies, is discarded.*

### B. Wnt5a stimulation assay

Experimental design: For a basic Wnt5a stimulation, include one condition for stimulation (+Wnt5a, where Wnt5a-containing media is added) and one condition for control (-Wnt5a, where control buffer-containing media is added) for each reporter cell line. The experiment setup will vary depending on your application of the assay; see Data analysis section for details on other types of stimulations.

1. Seed reporter cells at 0.09 million/well in the poly-D-lysine-coated 48-well plate in 400 µl growth media per well. Cells should be about 90% confluent.

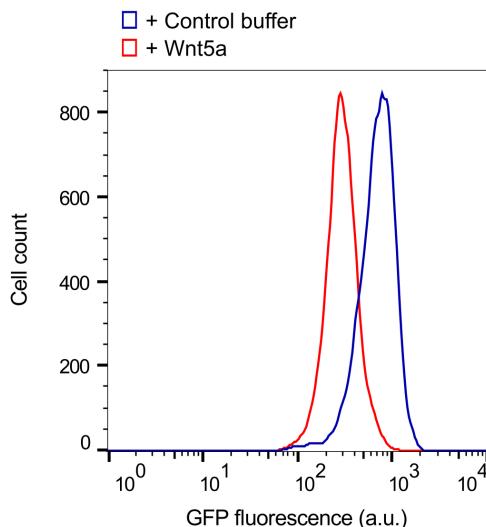
**Notes:**

- a. *Plate coating is done by adding 200 µl of a poly-D-lysine solution (0.1 mg/ml in water; sterile filtered) to each well of a 48-well plate, incubating at room temperature for 15 min, removing the poly-D-lysine solution, and washing the wells with 400 µl of water three times. Air dry the plate completely (with the lid removed) before plating cells. Coated plates can also be stored at room temperature for future use.*
  - b. *For quantification, we typically plate cells in triplicate wells for each experimental condition.*
2. The next day, gently remove media and replace with 400 µl growth media containing 10 nM Wnt-C59. Wnt-C59 inhibits the processing and secretion of endogenous Wnts. Allow cells to reach 100% confluence in Wnt-C59-containing media (generally one day). Cells should be as confluent as possible on the day of Wnt5a stimulation.
- Note: If the monolayer of cells retract or peel off, repeat cell plating. Retracted cells do not signal well.*
3. To stimulate cells with Wnt5a, gently remove media and replace with media containing 10 nM Wnt-C59 and the respective concentration of Wnt5a. For mock stimulation, use media containing Wnt-C59 and Wnt control buffer. If other drugs are used in conjunction with Wnt5a, pretreatment of the drug (typically for 1 h) may be necessary before addition of Wnt5a- and drug-containing media. Avoid disturbing the cell monolayer during media change.
  4. Incubate cells with Wnt5a at 37 °C for 6 h.
  5. To harvest cells for flow cytometry analysis, dissociate the cells with 100 µl trypsin per well at 37 °C for 3-5 min. Neutralize the trypsin with 500 µl of growth media and transfer the cell suspensions to 1.5 ml microcentrifuge tubes.
  6. Centrifuge cells at 12,000 x g at 4 °C for 3 min to pellet the cells.
  7. Remove the supernatant from each sample. Avoid disturbing the pellet.
  8. Resuspend the pellets at room temperature in 100-150 µl flow cytometer buffer. Mix by pipetting until the sample is homogenously resuspended and strain the cell suspension into a round-bottom tube through the strainer cap.
  9. Analyze the cells using a flow cytometer. We routinely use the Becton Dickinson FACScan and analyze 30,000 cells per sample.
  10. Analyze data files in software (e.g., FlowJo). See next section for details.

**Data analysis**

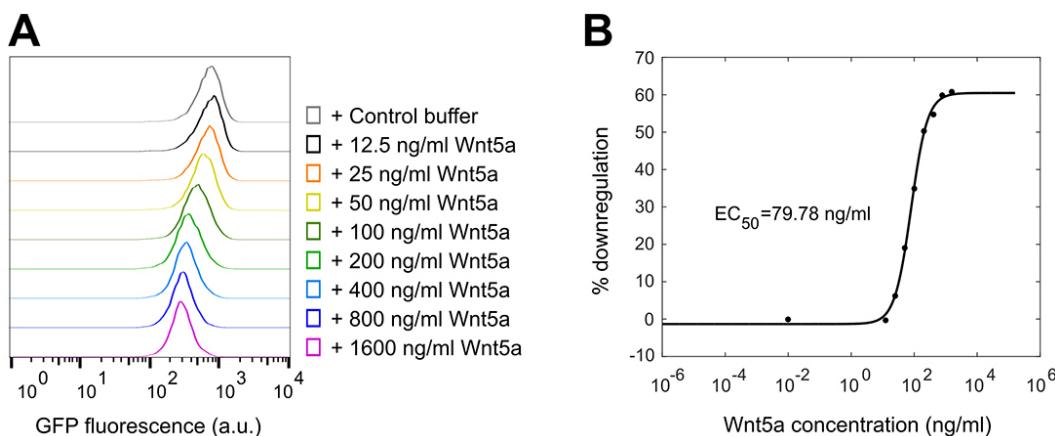
1. For general data analysis, gate the live cell population via side scatter and forward scatter parameters in the flow cytometry software to exclude dead cells. The wild-type NIH/3T3 Flp-In parent cell line (*i.e.*, untransfected) is used as a reference for autofluorescence; however, we do not typically gate the cell population based on the GFP signal to ensure that the entire live cell population is included in the reporter analysis. Generate a raw histogram of GFP fluorescence vs. cell count for the live gated population. Overlay the histograms from each

sample to be compared to obtain the difference in median fluorescence between each sample population (Figure 4). This difference in medians is expressed as a percentage: [(Control median - stimulated median)/control median] x 100 (labeled as ‘% downregulation’ in Figure 5B). Multiple histograms may be overlaid for comparison or reference (Figure 5A, Figure 7A).



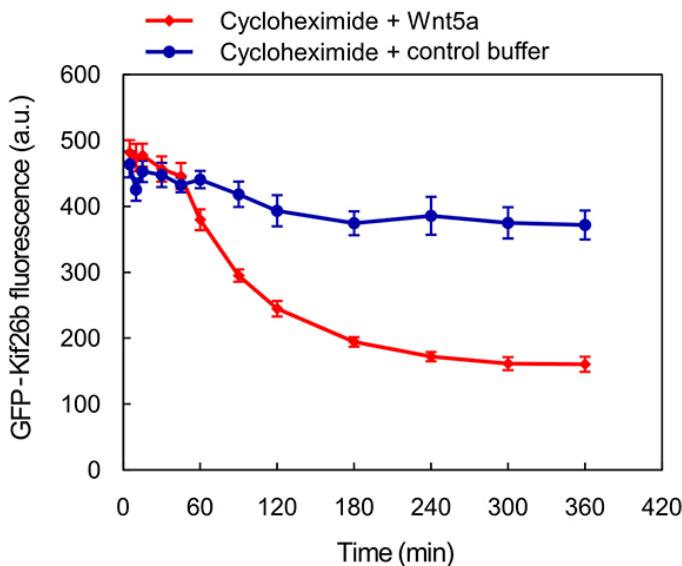
**Figure 4. Basic analysis using the WRK reporter assay.** Overlaid histograms from one set of samples showing the downregulation of GFP-Kif26b fluorescence in the WRK reporter cell line after Wnt5a stimulation (0.2 µg/ml Wnt5a) for 6 h.

- For a dose-response analysis, we analyze a minimum of six samples with varying concentrations of the Wnt5a ligand or small molecule inhibitors, including a 0 dose point. The medians may be plotted against the concentrations to generate the dose-response curve (Figure 5B). For inhibitors, we typically vary the drug concentration in the presence of a fixed concentration of Wnt5a to determine the dose-response relationship.



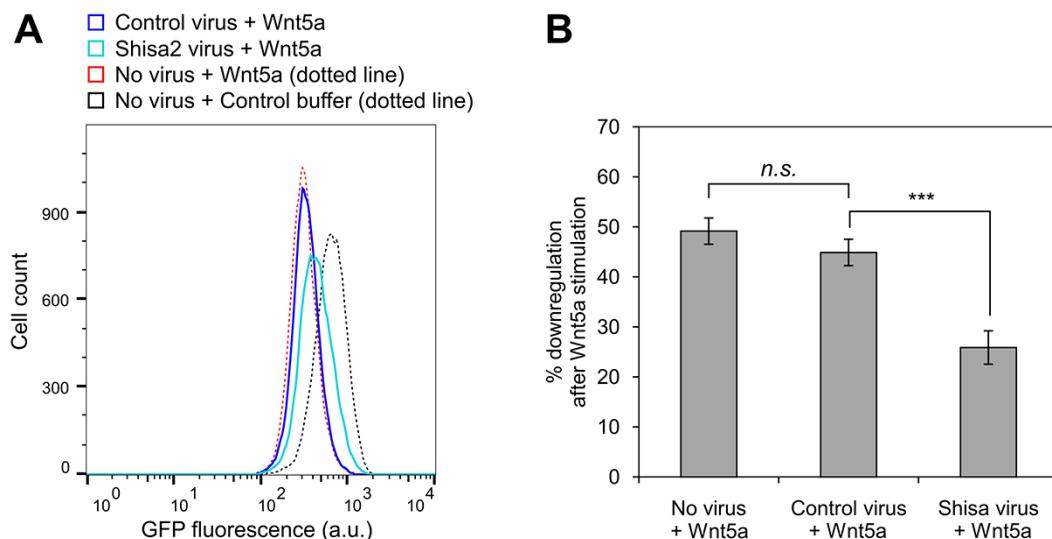
**Figure 5. Example of a dose-response analysis using the WRK reporter assay.** Raw histograms (A) and the resulting dose-response curve (B) showing GFP-Kif26b downregulation as a function of Wnt5a concentration in the WRK reporter assay.

3. For a time course experiment, such as the Kif26b stability analysis shown in Figure 6, we stimulate samples with Wnt5a at regular time intervals until the end of the experiment, when all samples are harvested at once. The medians are plotted against the duration of stimulation.



**Figure 6. Example of a time course experiment using the WRK reporter assay.** The kinetics of GFP-Kif26b turnover in the absence or presence of Wnt5a stimulation, as measured in the WRK reporter assay. Cycloheximide was used to block new protein synthesis in the reporter cells.

4. For statistical analysis during quantification, we use a minimum of three biological replicates (cells plated and treated with Wnt5a and/or inhibitors in concurrent cultures). To assess the difference between two sets of data, we perform a two-tailed, unpaired Student's *t*-test (Figure 7B). We include error bars for each set of replicates representing the standard error of the mean, which we generate by calculating the standard deviation of the medians of the replicates and dividing that number by the square root of *N*, where *N* is the number of replicates (Figure 6, Figure 7B).



**Figure 7. Example of a pathway analysis experiment using the WRK reporter assay.**

Partial blocking of Wnt5a-induced reporter activity after ectopic Shisa2 expression via lentiviral transduction. A. Representative overlaid histograms show the effect of ectopic Shisa2 expression on Wnt5a-induced downregulation of GFP-Kif26b in the WRK reporter line. Shisa2 is an antagonist of the Frizzled family of Wnt receptor (Yamamoto *et al.*, 2005). The effect of Wnt5a or control buffer treatment on the WRK reporter line is included as a reference. B. Quantification of the results shown in panel (A). *t*-tests were performed for the following comparisons: Control virus vs. no virus,  $P = 0.0957$  (not significant); control virus vs. Shisa2 virus,  $P < 0.001$  (significant).

## Notes

Wnt5a signaling as detected by this assay appears to be highly sensitive to cell density. Signaling activity occurs best when cells are as confluent as possible, and activity decreases drastically when cells are less than 100% confluent. Some optimization may be required to determine the most optimal plating conditions for specific cell types and applications.

## Recipes

1. Growth medium  
DMEM supplemented with:  
10% FBS  
1x glutamine (2 mM)  
1x penicillin-streptomycin (1 IU/ml)
2. Wnt control buffer  
1x PBS supplemented with:  
0.1% bovine serum albumin

- 0.5% (w/v) CHAPS
3. Cell resuspension buffer for flow cytometry  
1x PBS supplemented with 0.5% FBS

### Acknowledgments

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## Detection and Analysis of Circular RNAs by RT-PCR

Amaresh C Panda<sup>1,\*</sup> and Myriam Gorospe<sup>2</sup>

<sup>1</sup>Genetic & Autoimmune Diseases, Institute of Life Sciences, Bhubaneswar, Odisha, India; <sup>2</sup>Laboratory of Genetics and Genomics, National Institute on Aging, National Institutes of Health, Biomedical Research Center, Baltimore, Maryland, USA

\*For correspondence: [amarchpanda@gmail.com](mailto:amarchpanda@gmail.com)



**[Abstract]** Gene expression in eukaryotic cells is tightly regulated at the transcriptional and posttranscriptional levels. Posttranscriptional processes, including pre-mRNA splicing, mRNA export, mRNA turnover, and mRNA translation, are controlled by RNA-binding proteins (RBPs) and noncoding (nc)RNAs. The vast family of ncRNAs comprises diverse regulatory RNAs, such as microRNAs and long noncoding (lnc)RNAs, but also the poorly explored class of circular (circ)RNAs. Although first discovered more than three decades ago by electron microscopy, only the advent of high-throughput RNA-sequencing (RNA-seq) and the development of innovative bioinformatic pipelines have begun to allow the systematic identification of circRNAs (Szabo and Salzman, 2016; Panda *et al.*, 2017b; Panda *et al.*, 2017c). However, the validation of true circRNAs identified by RNA sequencing requires other molecular biology techniques including reverse transcription (RT) followed by conventional or quantitative (q) polymerase chain reaction (PCR), and Northern blot analysis (Jeck and Sharpless, 2014). RT-qPCR analysis of circular RNAs using divergent primers has been widely used for the detection, validation, and sometimes quantification of circRNAs (Abdelmohsen *et al.*, 2015 and 2017; Panda *et al.*, 2017b). As detailed here, divergent primers designed to span the circRNA backsplice junction sequence can specifically amplify the circRNAs and not the counterpart linear RNA. In sum, RT-PCR analysis using divergent primers allows direct detection and quantification of circRNAs.

**Keywords:** Circular RNA, Backsplice junction, Divergent primer, RT-PCR, RNase R

**[Background]** CircRNAs are covalently closed, single-stranded RNAs lacking 5' or 3' ends. Although their genesis is poorly understood, they can arise from pre-mRNAs by a process called backsplicing (Panda *et al.*, 2017d; Jeck *et al.*, 2013). CircRNAs have been reported to be abundant, ubiquitously expressed, and conserved across species (Jeck *et al.*, 2013). A number of studies have established that circRNAs can regulate gene expression by acting as competitors of pre-mRNA splicing, as decoys for microRNAs, as sponges for RBPs, and possibly also as substrates for translation (Panda *et al.*, 2017d). In recent years, more than one hundred thousand circRNAs have been reported bioinformatically from high-throughput RNA sequencing (RNA-seq) (Glazar *et al.*, 2014). Unfortunately, there is little overlap among different bioinformatic pipelines and there is no 'gold standard' method to validate the accuracy of circRNAs identified by different bioinformatic tools (Szabo and Salzman, 2016). However, RT-PCR has been widely used for validation of circRNAs identified by RNA-seq. This protocol describes the design of divergent primers which face away from each other on the linear RNA,

so that they can only amplify the circRNAs, and not the linear RNAs with the same sequence. The PCR amplicon for the detection of circRNAs using divergent primers spans the backsplice junction of circRNAs. This method has been successfully used in several studies for the detection and quantification of circRNAs.

## **Materials and Reagents**

1. Standard pipette tips with a volume capacity of 10 µl, 20 µl, 200 µl, and 1 ml
2. Nuclease-free 1.7-ml microcentrifuge tubes (Denville Scientific, catalog number: C2171)
3. ThermoGrid™ rigid strip 0.2-ml PCR tubes [(Denville Scientific, catalog number: C18064 (1000859))]
4. MicroAmp® optical 384-well reaction plate (Thermo Fisher Scientific, Applied Biosystems™, catalog number: 4309849)
5. Optical adhesive film (Thermo Fisher Scientific, Applied Biosystems™, catalog number: 4311971)
6. Dulbecco's phosphate-buffered saline (DPBS) (Thermo Fisher Scientific, Gibco™, catalog number: 14040-133)
7. Total RNA isolation-miRNeasy Mini Kit (QIAGEN, catalog number: 217004)
8. (Optional) TRIzol reagent (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 15596018)
9. Nuclease-free water (Thermo Fisher Scientific, Invitrogen™, catalog number: AM9930)
10. RiboLock RNase inhibitor (40 U/µl) (Thermo Fisher Scientific, Thermo Scientific™, catalog number: EO0381)
11. RNase R (Lucigen, Epicentre, catalog number: RNR07250)
12. dNTP mix (10 mM each) (Thermo Fisher Scientific, Thermo Scientific™, catalog number: R0193)
13. Random primers (150 ng/µl) (Sigma-Aldrich, Roche Diagnostics, catalog number: 11034731001)
14. Maxima reverse transcriptase (Thermo Fisher Scientific, Thermo Scientific™, catalog number: EP0741)
15. KAPA SYBR® FAST ABI prism 2x qPCR master mix (Kapa Biosystems, catalog number: KK4605), or SYBR Green from other vendors
16. QIAquick Gel Extraction Kit (QIAGEN, catalog number: 28704)
17. TBE Buffer, 10x, Molecular Biology Grade (Sigma-Aldrich, catalog number: 574795-1L)
18. 1 Kb Plus DNA Ladder (Thermo Fisher Scientific, Invitrogen™, catalog number: 10787018)
19. UltraPure™ Agarose (Thermo Fisher Scientific, Invitrogen™, catalog number: 16500500)
20. Ethidium bromide solution (Sigma-Aldrich, catalog number: E1510-10ML)
21. 2% agarose gel (see Recipes)

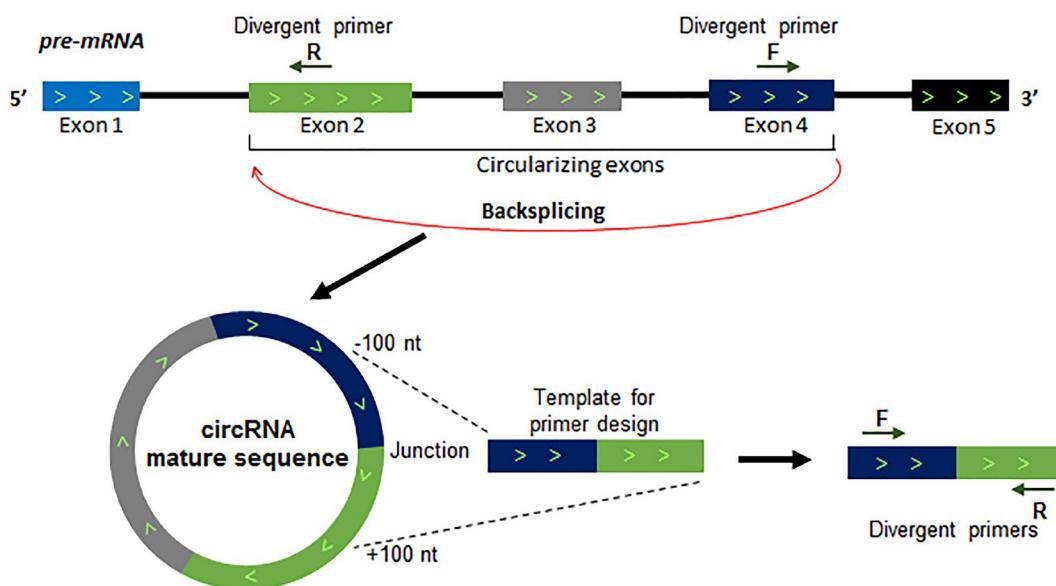
## Equipment

1. Manual Pipettes set of 2 µl, 20 µl, 200 µl and 1,000 µl (Mettler-Toledo, Rainin, catalog number: 17014393, 17014392, 17014391, and 17014382)
2. Cell scraper (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 179707PK)
3. Vortex mixer (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 88880018)
4. UV transilluminator
5. Refrigerated centrifuge (Eppendorf, model: 5430 R)
6. NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific, Thermo Scientific™, model: NanoDrop One™, catalog number: ND-ONE-W)
7. PCR strip tube rotor, mini centrifuge C1201 [Denville Scientific, catalog number: C1201-S (1000806)]
8. Eppendorf® Thermomixer® C (Eppendorf, model: Thermomixer® C, catalog number: 5382000015)
9. Veriti® 96-well thermal cycler (Thermo Fisher Scientific, Applied Biosystems™, model: Veriti™ 96-Well, catalog number: 4375786)
10. Owl™ EasyCast™ B1 Mini Gel Electrophoresis Systems (Thermo Fisher Scientific, Thermo Scientific™, model: Owl™ EasyCast™ B1, catalog number: B1)
11. Gel imaging system (ProteinSimple, catalog number: FluorChem E system)
12. MPS 1000 mini plate spinner (Next Day Science, catalog number: C1000)
13. QuantStudio 5 Real-Time PCR System, 384-well (Thermo Fisher Scientific, Applied Biosystems™, model: QuantStudio™ 5, catalog number: A28140)

## Procedure

### A. Divergent primer design

1. Get the mature sequence of circular RNA from the UCSC genome browser (<https://genome.ucsc.edu/>) using the genomic coordinates (Note 4).
2. As shown in Figure 1, make the PCR amplicon template by joining the 100 nt sequence from the 3' end to 100 nt sequence at the 5' end of the circRNA (Note 5).



**Figure 1. Schematic illustration of circRNA biogenesis from backsplicing of pre-mRNA (top) and schematic representation of the design of divergent primers using the circRNA junction as template for PCR amplification (bottom)**

3. Use the above PCR amplicon template sequence to design PCR primers using the Primer 3 webtool (<http://bioinfo.ut.ee/primer3/>) or NCBI primer-BLAST (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>).
4. Make sure the PCR amplicon is between 120-200 nt long (Note 6).
5. If you know the CircBase ID of your circRNA (Glazar *et al.*, 2014), you may design divergent primers using the CircInteractome webtool (Dudekula *et al.*, 2016) ([http://circinteractome.ipr.nia.nih.gov/Divergent\\_Primers/divergent\\_primers.html](http://circinteractome.ipr.nia.nih.gov/Divergent_Primers/divergent_primers.html)).

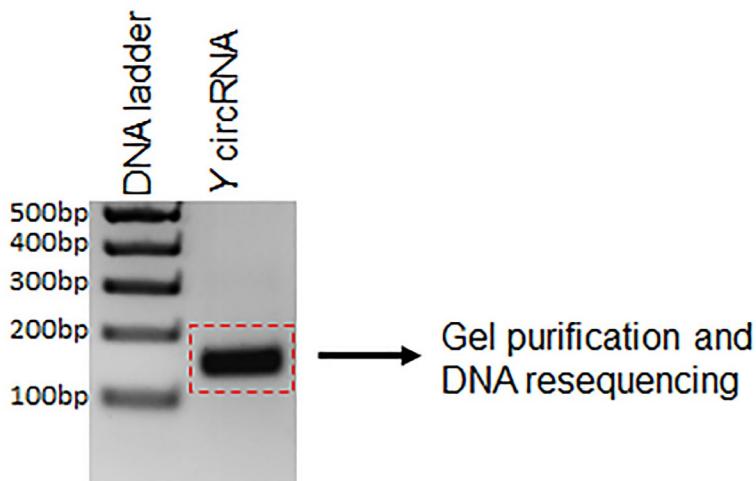
#### B. Total RNA isolation

1. Take ~2 million cultured cells and remove the culture media.
2. Wash the cells three times with cold PBS at 4 °C.
3. Immediately scrape the cells and transfer them to a 1.7-ml tube using cold DPBS to rinse the plate.
4. Collect the cell pellet by centrifugation at 500 x g for 5 min at 4 °C.
5. Immediately add 700 µl of QIAzol Lysis Reagent provided in the miRNeasy Kit and disrupt the cell pellet by pipetting.
6. Prepare the total RNA using the miRNeasy Kit following the manufacturer's instructions (Note 1).
7. The RNA in nuclease-free water can be stored for 6 months at -20 °C or -80 °C, or used immediately for RNase R digestion and cDNA synthesis.

- C. Degradation of linear RNA by digestion with RNase R and cDNA synthesis
1. Measure RNA concentration with a NanoDrop spectrophotometer.
  2. Prepare an RNase R digestion reaction containing 2 µg of prepared RNA, 1 µl RiboLock, 2 µl 10x RNase R reaction buffer, and 1 µl of RNase R; adjust the volume to 20 µl with nuclease-free water (Note 2).
  3. Prepare a control reaction exactly the same as the RNase R reaction but without RNase R.
  4. Incubate the reactions at 37 °C for 30 min and immediately proceed to RNA isolation.
  5. Prepare the RNA from the RNase R and control treated samples using miRNeasy Kit following the protocol provided by the manufacturer and elute in 40 µl of nuclease-free water.
  6. Prepare the cDNA synthesis reaction containing 12 µl of prepared RNA, 1 µl RiboLock, 1 µl dNTP mix, 1 µl random primers, 4 µl 5x RT buffer, and 1 µl Maxima reverse transcriptase (Note 2).
  7. Prepare No-RT reaction containing everything except the Maxima reverse transcriptase (Note 3).
  8. Mix the reaction gently and centrifuge for 10 sec to settle the reaction at the bottom of the tube.
  9. Incubate the reaction at 25 °C for 10 min followed by 30 min incubation at 50 °C for cDNA synthesis.
  10. Inactivate the reverse transcriptase by incubating the reaction at 85 °C for 5 min.
  11. The prepared cDNA can be stored at -20 °C or used immediately for PCR analysis.

D. PCR and circRNA sequencing

1. Prepare the forward and reverse divergent primer mix at a final concentration of 1 µM in nuclease-free water for the circRNA.
2. Prepare the PCR reactions containing 25 µl of 2x SYBR Green mix, 0.1 µl cDNA, 12.5 µl divergent primer mix, and adjust the volume to 50 µl with nuclease-free water (Notes 2 and 10).
3. Prepare another reaction same as above with 0.1 µl of no-RT instead of cDNA.
4. Mix the reaction by tapping the tube with finger and centrifuge for a few seconds to settle the reactions at the bottom of the tube.
5. Perform the PCR on a thermal cycler with a cycle setup of 3 min at 95 °C and 35 cycles of 5 sec at 95 °C plus 5 sec at 60 °C.
6. Prepare ethidium bromide-containing 2% agarose gel (see Recipes) in 1x TBE buffer and resolve the whole 50 µl PCR product at 100 V until the loading dye reaches 3/4 of the gel.
7. Visualize the PCR products on an ultraviolet transilluminator to confirm the size of the PCR product amplified (Note 7).
8. Purify the PCR product from the agarose gel using the QIAquick Gel Extraction Kit following the manufacturer's instructions (Figure 2).
9. Quantify the PCR product concentration in the prepared DNA sample.
10. Sequence the amplified PCR products with forward or reverse primers to find the backsplice junction sequence.



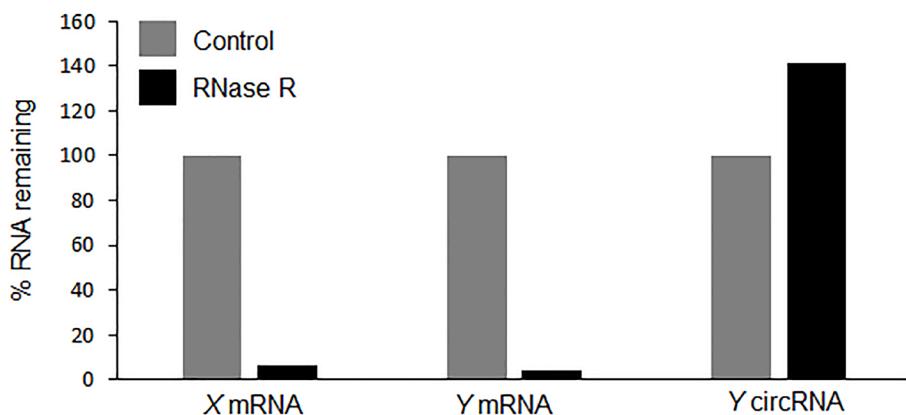
**Figure 2. Example circRNA PCR product resolved and visualized on ethidium bromide-stained agarose gel.** The PCR product is submitted for DNA sequencing after gel purification.

#### E. Quantitative PCR (qPCR) analysis of circRNA

1. Prepare forward and reverse primer mixes for target mRNAs and circRNAs at a final concentration of 1  $\mu$ M in nuclease-free water.
2. Prepare the qPCR reactions in a 384-well plate containing 10  $\mu$ l of 2x SYBR Green mix, 0.1  $\mu$ l cDNA, and 5  $\mu$ l primer mix. Adjust the volume to 20  $\mu$ l with nuclease-free water (Notes 2 and 11).
3. Vortex the reaction plate for few seconds after sealing the plate with optical adhesive film.
4. Spin the plate for a few seconds to settle the reactions at the bottom of the wells.
5. Set up the qPCR reaction cycle for 2 min at 95 °C and 40 cycles of 2 sec at 95 °C and 10 sec at 60 °C on QuantStudio 5 Real-Time PCR System (Note 2).
6. The percentage (%) RNA left after RNase R treatment using the delta CT method as described in Table 1 (Notes 8 and 9) (Figure 3).

**Table 1. % of RNA left after RNase R treatment relative to control.** The example CT values for linear and circRNAs in control and RNase R-treated samples, and calculation of RNA left after RNase R treatment.

Target RNA	Hypothetical CT values		% of RNA in Control	% of RNA in RNase R
	Control	RNase R	$2^{\Delta CT} (\text{Control-Control}) * 100$	$2^{\Delta CT} (\text{Control-RNase R}) * 100$
X mRNA	22	26	100	6.25
Y mRNA	25	29.5	100	4.41
Y circRNA	27	26.5	100	141.42



**Figure 3. Hypothetical qPCR data showing the resistance of circRNA to RNase R treatment as calculated in Table 1.** The qPCR results showing the levels of circRNAs and linear RNAs in RNase R (black) treated sample compared with the control treatment (grey).

### Data analysis

To validate the existence of a circRNA, Sanger sequencing is to be performed on the PCR product amplified with the divergent primers (Figure 2). The PCR product sequence should match exactly the expected circRNA junction sequence as predicted from the RNA-seq (Panda *et al.*, 2017a and 2017c). However, this analysis does not inform on whether the backsplice junction sequence is coming from a scrambled exon linear transcript or a real backsplice junction. To study this possibility, RNA is digested with RNase R, a 5' to 3' exonuclease known to degrade linear RNAs. As shown in Figure 3, following RNase R treatment, the linear X mRNA and Y mRNA are depleted to a level lower than 10%, while Y circRNA was not degraded (Table 1). The fact that Y circRNA level did not show depletion while the counterpart linear Y mRNA depleted to a minimal level with RNase R treatment supports the notion that RNase R degrades linear RNAs specifically leading to enrichment of circRNA population (Figure 3) (Panda *et al.*, 2017c).

### Notes

1. Total RNA can also be prepared with the TRIzol reagent (Thermo Fisher Scientific) or any other total RNA isolation kit.
2. To avoid contamination, a PCR workstation may be used to prepare the reaction mixtures for RNase R treatment, cDNA synthesis, RT-PCR, and qPCR.
3. A 'No-RT' cDNA reaction serves as a negative control for cDNA synthesis and only low-level background should be amplified in the RT-PCR using specific primer sets.
4. The mature sequence of circRNA can be obtained by joining the exon sequence present between the backsplice site coordinates in the genome.

5. If the circular RNA is shorter than 200 nt, then the mature circRNA sequence can be divided into two halves and the template for primer design can be generated by joining the 3' half to the 5' end of 5' half.
6. The PCR amplicon of the divergent primers should span the circRNA backsplice junction; care should be taken that no primer overlap with the junction sequence.
7. The PCR product on agarose gels should show a single product of the expected size and the 'No-RT' reaction should not amplify a product.
8. The dissociation curve analysis for each primer set should show a single peak.
9. CT values should represent the average of triplicate reactions. No normalization is needed for the qPCR analysis. The actual level of linear RNA depletion in RNase R treatment can be estimated by normalizing it to the level of counterpart circRNA.
10. Any *Taq* polymerase in place of SYBR green mix can be used for this PCR reaction.
11. As pipetting 0.1 µl of cDNA is difficult and error-prone, a master mix of cDNA and water is prepared depending on the final number of reactions required for each cDNA sample. Alternatively, the 20 µl prepared cDNA can be diluted to 1 ml with nuclease-free water and 5 µl of the diluted cDNA can be used in the qPCR reaction.

### **Recipes**

1. 2% agarose gel  
2 g of agarose in 100 ml of 1x TBE  
Ethidium bromide (EtBr) at a final concentration of approximately 0.2 µg/ml

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This protocol was adapted from the previously published papers (Dudekula *et al.*, 2016 and Panda *et al.*, 2017b). The protocol was tested and optimized by different researchers in the Gorospe laboratory, National Institute on Aging, NIH (Panda *et al.*, 2017c and Abdelmohsen *et al.*, 2017). The authors have no conflicts of interest or competing interests to declare.

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## Centromere Chromosome Orientation Fluorescent *in situ* Hybridization (Cen-CO-FISH) Detects Sister Chromatid Exchange at the Centromere in Human Cells

Simona Giunta\*

Laboratory of Chromosome and Cell Biology, the Rockefeller University, New York, NY, USA

\*For Correspondence: [sgiunta@rockefeller.edu](mailto:sgiunta@rockefeller.edu) or [simona.giunta@cantab.net](mailto:simona.giunta@cantab.net)



**[Abstract]** Human centromeres are composed of large tandem arrays of repetitive alpha satellite DNA, which are often sites of aberrant rearrangement in cancers (Mitelman *et al.*, 1997; Padilla-Nash *et al.*, 2001). To date, annotation of the human centromere repetitive sequences remains incomplete, greatly hindering in-depth functional studies of these regions essential for chromosome segregation. In order to monitor sister chromatid exchange happening at the centromere (C-SCE) due to recombination and mutagenic events, I have applied the Chromosome-Orientation Fluorescence *in situ* Hybridization (CO-FISH) technique to centromeres (Cen-CO-FISH) in human cells. This hybridization-based method involves (1) the incorporation of nucleotide analogs through a single round of replication, (2) enzymatic digestion of the newly synthesized DNA strand and (3) subsequent hybridization of single-stranded probes, in absence of a denaturation step. The resulting signal allows to differentially label each sister chromatid based on the 5'-3' directionality of the DNA and to score aberrant staining patterns indicative of C-SCE. The Cen-CO-FISH method applied to human centromeres revealed that human centromeres indeed undergo recombination in cycling cells resulting in C-SCE, and centromere instability is enhanced in cancer cell lines and primary cells undergoing senescence (Giunta and Funabiki, 2017). Here, I present the detailed protocol of the preparation, experimental procedure and data acquisition for the Cen-CO-FISH method in human cells. It also includes a conceptual overview of the technique, with examples of representative images and scoring guidelines. The Cen-CO-FISH represents a valuable tool to facilitate exploration of centromere repeats.

**Keywords:** Centromere, Fluorescence *in situ* hybridization, CO-FISH, Alpha satellite, Repetitive DNA, Genome stability, Recombination, Sister chromatid exchange

**[Background]** The human genome project was marked completed in 2003, yet it omitted over 10% of the human repetitive DNA (de Koning *et al.*, 2011), including the centromere. The human centromere is a highly specialized genomic locus (Choo, 1997) playing a critical role during chromosome segregation where it serves as the site of kinetochore assembly to allow interaction with microtubules and sister chromatids separation during cell division (Cheeseman, 2014). Human centromeres are made of characteristic repetitive DNA sequences called alpha-satellites, whose linear assembly remains largely absent from the reference genomes. Here, I present the application of the Cen-CO-FISH technique to label human centromere and monitor recombination events resulting in crossover. Introduced by Bailey and colleagues over 20 years ago (Bailey *et al.*, 1996), the CO-FISH method has been widely applied to detect recombination, fragility, replication timing, fusion and inversions at telomeres repeats, as well

as to monitor mitotic segregation patterns and non-random sister chromatid segregation (Bailey *et al.*, 2010). The application of this methodology to centromere, hereby called Cen-CO-FISH method, has revealed that the centromere-specific histone variant CENP-A, and CENP-A associated proteins CENP-C and CENP-T/W, work to prevent centromere instability and this functionality is compromised in cancer cell lines and in primary cells approaching replicative senescence that display higher number of C-SCE (Giunta and Funabiki, 2017). Cen-CO-FISH was used to assess centromere instability in cancer and during cellular senescence in human cells (Giunta and Funabiki, 2017) and it has been previously applied to study recombination (Jaco *et al.*, 2008; de La Fuente *et al.*, 2015) and sister chromatid separation patterns in mouse cells (Falconer *et al.*, 2010). The wide application potentials of this methodology spans from quantitative detection of alpha satellite repeats, centromere recombination resulting in C-SCE, fragility, replication timing, fusion and inversions, as well as to monitor mitotic segregation patterns and non-random sister chromatids segregation (Bailey *et al.*, 2010; Yadlapalli and Yamashita, 2013). Cen-CO-FISH fills the gaps in the missing genetic information that have cast a shadow over the centromere and other repetitive regions, bringing new light into the possibilities for functional exploration of these important loci of our genome.

## **Materials and Reagents**

1. 6 or 10 cm Petri dish (Corning, Falcon®, catalog numbers: 353002 or 353003)
2. 15 ml Falcon tube (Corning, Falcon®, catalog number: 352097)
3. Frosted slides (Superfrost Plus; Fisher Scientific, catalog number: 12-550-15)
4. Coverslips (24 x 60 mm) (Fisher Scientific, catalog number: 12-545-M)
5. Paper towel
6. Glass Pasteur pipette (Fisher Scientific, catalog number: 13-678-20A)
7. Gloves and lab coat
8. Human cells of interest and appropriate medium

*Note: This protocol is for adherent cells, changes can be made for use for non-adherent cultures.*

9. 5'-Bromodeoxyuridine (BrdU) (MP Biomedicals, catalog number: 100166)

*Note: Prepare 10 mM stock solution in double distilled water (1,000x); make aliquots and store at -20 °C.*

10. 5'-Bromodeoxycytidine (BrdC) (Sigma-Aldrich, catalog number: B5002)

*Note: Prepare 10 mM stock solution in double distilled water (1,000x); make aliquots and store at -20 °C.*

11. Colcemid (Roche Diagnostics, catalog number: 10295892001; already diluted 10 µg/ml)—Store at 4 °C

12. Phosphate-buffered saline (PBS)

13. Trypsin-EDTA (Thermo Fisher Scientific, Gibco™, catalog number: 25300)

14. Fetal bovine serum (Atlanta Biologicals)

15. Potassium chloride (KCl) (Fisher scientific, catalog number: P217-500)
16. RNase A (Sigma-Aldrich, catalog number: R5000)  
*Note: Prepare stock solution 50 mg/ml in 10 mM Tris-HCl pH 7.2. Aliquot and store at -20 °C*
17. Hoechst 33258 (Thermo Fisher Scientific, Invitrogen™, catalog number: H3569)  
*Note: Make a 10 µg/ml solution in double distilled water and store at 4 °C away from light.*
18. Exonuclease III and buffer (Promega, catalog number: M1811)—Keep at -20 °C
19. DAPI (Sigma-Aldrich, catalog number: D9542)—0.5 mg/ml stock in water. Keep at 4 °C in the dark for one year
20. ProLong Gold Anti-fade Reagent (Thermo Fisher Scientific, Invitrogen™, catalog number: P36934)
21. Nail varnish (Sally Hansen, Transparent Harder)
22. Methanol (Fisher Scientific, catalog number: A452-4)
23. Glacial acetic acid (Fisher Scientific, catalog number: A38C-212)
24. Ethanol 100% (Decon, catalog number: 2716), 90% and 70%
25. Blocking reagent (Roche Diagnostics, catalog number: 11096176001)
26. Maleic acid (Sigma-Aldrich, catalog number: M0375)
27. Sodium chloride (NaCl) (Merck, catalog number: SX0420-5)
28. Sodium hydroxide (NaOH) (Fisher Scientific, catalog number: S318-500)
29. Tris-HCl pH 7.2 (Sigma-Aldrich, CAS number: 1185-53-1)
30. Formamide (Fisher Scientific, catalog number: BP228; use deionized for hybridization)
31. Bovine serum albumin (BSA)
32. Tween-20 (Hoefer, CAS number: GR128-500)
33. Sodium citrate (Sigma-Aldrich, CAS number: 6132-04-3)
34. Magnesium chloride ( $MgCl_2$ ) (Fisher Scientific, catalog number: AC41341-5000)  
*Manufacturer: Acros Organics, catalog number: 413410025.*
35. Dithiothreitol (DTT) (Thermo Fisher Scientific, Thermo Scientific™, catalog number: R0861)
36. Hypotonic solution (see Recipes)
37. Fixative solution (see Recipes)
38. Ethanol dilution (see Recipes)
39. Blocking solution (see Recipes)
40. Hybridization solution (see Recipes)
41. Hybridization wash #1 (see Recipes)
42. Hybridization wash #2 (see Recipes)
43. Peptide nucleic acid (PNA) probes (custom probes from PNABio) (see Recipes)
44. Sodium chloride and sodium citrate buffer (SSC, see Recipes)

## Equipment

1. Pipettes (Gilson)
2. Centrifuge (Eppendorf, model: 5810 R)
3. Coplin Jars (Scienceware, Sigma-Aldrich, catalog number: S5641)
4. Heating block (VWR)
5. Water bath (Fisher Scientific, model: Isotemp™ 210)
6. Stratalinker with 365-nm UV light bulbs (Spectralinker XL-1000 1800 UV irradiator) (Spectronics Corporation, model: XL-1000)
7. Slides plastic tray—to fit into the Stratalinker
8. Hybridization chamber (see text for more details)
9. Orbital shaker
10. Imaging equipment:
  - a. DeltaVision Image Restoration microscope system (Applied Precision/GE Healthcare)
  - b. Olympus IX-70 microscope (Olympus, model: IX70)
  - c. 100x/1.40 UPLSAPO objective lens
  - d. CoolSnap QE CCD camera (Photometrics)

## Software

1. SoftWoRx (Sold by Applied Precision)
2. Metamorph 7.8 (Sold by Universal Imaging)
3. Prism 5 (Sold by GraphPad)

## Procedure

- A. Incorporation of BrdU:C and metaphase chromosome spread preparation
1. Cells are seeded into a 6 or 10 cm dish at least two days before harvesting, to be about 70% confluent at the time of harvesting. In a 10 cm dish, seed ~1 million cells.
  2. Cells are then labeled for 16-20 h by incubating with 5'-Bromodeoxyuridine (BrdU) and 5'-Bromodeoxycytidine (BrdC). Thaw a 1,000x aliquot of BrdU and BrdC and mix 3:1 to obtain 7.5 mM BrdU + 2.5 mM BrdC. Add to the cells medium at a 1:1,000 dilution and incubate the cells overnight.

*Note: Adjust the time of BrdU:C labeling according to whether your cells grow slower/faster to avoid double labeling or incompletely labeled cells. You are aiming for cells to incorporate BrdU and BrdC throughout S-phase and to prevent them to exit from mitosis after replication. Typically, HeLa cells are incubated with BrdU:C for 14-16 h before adding colcemid; to validate the time of BrdU:C incubation one can synchronize cells with thymidine at G1/S, release and monitor exit from mitosis by flow cytometry. Cells that exit mitosis 15 h post-thymidine release should be*

treated with BrdU:C for 16–17 h before addition of colcemid to ensure only cells that started labeling before S-phase are trapped by the colcemid block.

3. Add colcemid to a final concentration of 0.1 µg/ml (1:100 dilution of 10 µg/ml stock) to accumulate mitotic cells for about 2 h before harvesting.

*Note: Avoid leaving cells into colcemid for over 3 h, because long exposure to colcemid will lead to very compacted chromosome morphology.*

4. Harvest floating cells by saving the supernatant in a 15 ml Falcon tube, wash the dish once with PBS and collect the PBS wash as well into the same tube. Trypsinize for 5 min, block with medium containing fetal bovine serum, and collect the rest of the cells in the same tube.

5. Pellet cells for 5 min at 175 x g. Remove supernatant and resuspend cells in 10 ml of pre-warmed 75 mM KCl solution. Gently pipette up and down to resuspend the pellet.

6. Incubate the cells in the KCl hypotonic solution at 37 °C for 30 min (slowly invert the tube every 10 min to keep the cells suspended). Before pelleting the cells, adding 200 µl of fixative solution and mixing by slowly inverting the tubes twice helps the metaphases to remain intact during centrifugation, because cells are extremely fragile after the hypotonic treatment. Pellet the cells for 5 min at 175 x g.

7. Aspirate most supernatant, leaving about 200 µl of KCl to resuspend cells by tapping the tube or slowly pipetting up and down with a P1000.

8. Fix the resuspended pellet by adding 10 ml of freshly made ice-cold MeOH-acetic acid (3:1) fixative solution dropwise (prepare it 30 min before use, keep at -20 °C) while continuously vortexing the tube on a Vortex mixer at the lowest speed setting (#1).

*Note: It is important to resuspend the pellet before addition of the fixative, and to add the fixative one drop at a time while continuously vortexing the cells at low speed for at least the first 2 ml. The remaining 8 ml can be added all at once.*

9. Mix by inverting the tube a few times.

10. Cells can now be kept at 4 °C for several months, stored away from light.

11. Once you are ready to drop the cells, spin the tube down at 380 x g for 5 min. Aspirate the fixative leaving about 0.5–2 ml depending on how many cells are in each tube. Thoroughly resuspend cells in the remaining fixative.

12. If you are dropping onto Superfrost Plus slides, go directly to Step A13. If using regular slides, wash in soapy water and rinse 3 times with Milli-Q water before use. Keep slides in Milli-Q water in the fridge for an hour and dry well right before use.

13. Humidify the slide you are about to ‘drop’ on by hovering it over a hot wet towel placed on a heating block, until it steams up. You can also achieve this by ‘breathing’ onto the slide.

*Note: Creating conditions of humidity, for instance by having wet paper towels onto an adjacent 90 °C block that continues to release vapor, and humidifying the air immediately adjacent to where slides are dropped, or using a hybridization hood with set humidity and temperature, helps with the quality of the spreads.*

14. Using a glass Pasteur pipette, pipette up and down and aspirate the cell suspension. Drop several drops of cell suspension onto different places on the slide from about 20 cm away. You should aim to cover all parts of the slide with each drop.

*Note on safety: Gloves and lab coat should be used to protect from possible splashes. The entire metaphase spread procedure can be performed in a laboratory chemical fume hood for additional protection.*

15. Immediately after dropping, rest the slide for about 2-3 min onto a 42 °C block with a wet paper towel covering the block, cells side up.

*Note: Factors that influence the spreading are: humidity, temperature, distance of dropping cells onto the slide and drying time.*

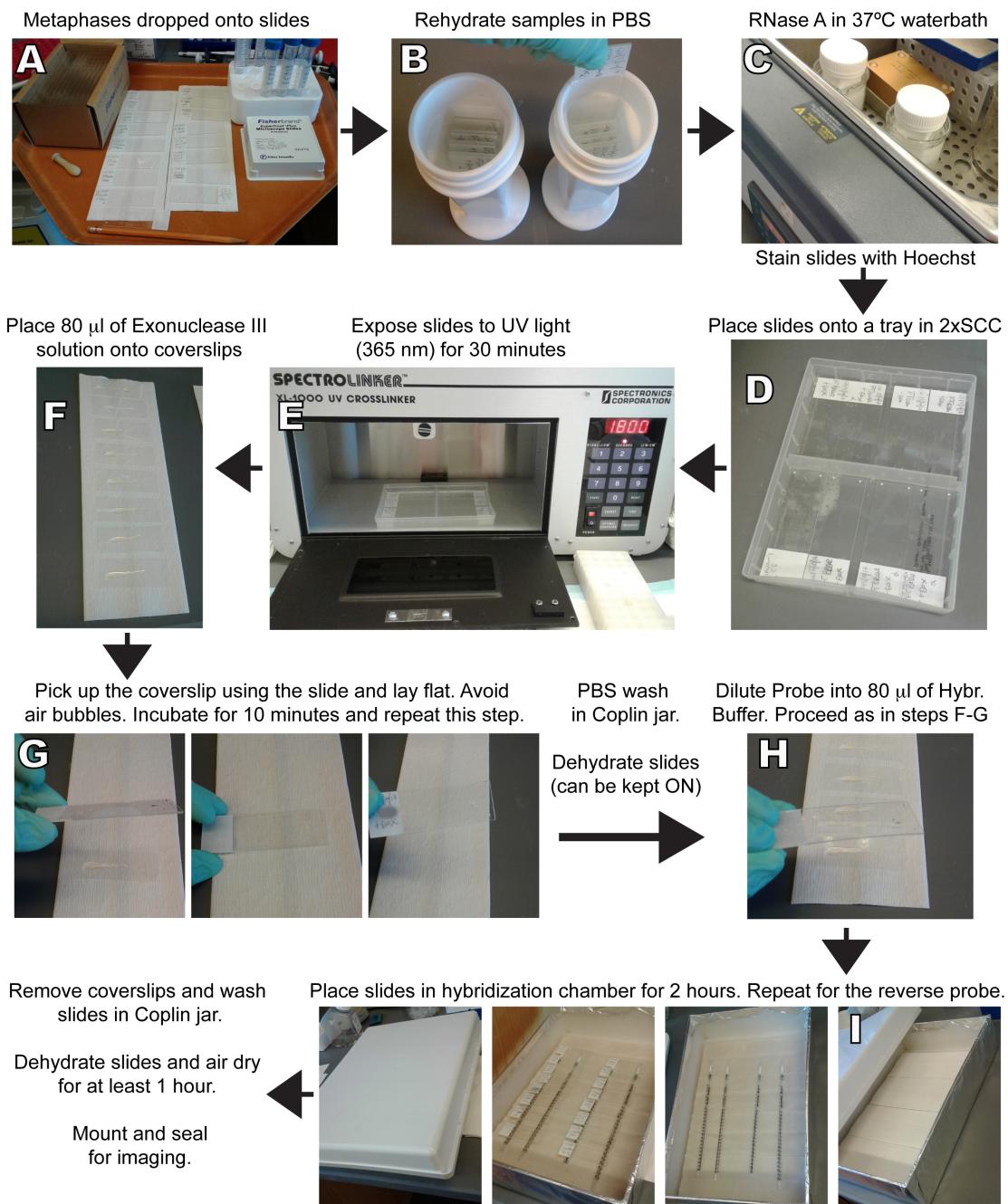
16. Air-dry slides overnight at room temperature away from light. You can keep any cells left into the falcon tube stored in fresh fixative at 4 °C for up to one year.

#### B. Enzymatic digestion of BrdU:C labeled, newly synthesized strand

1. Dropped slides are inserted in a Coplin jar and rehydrated in PBS (pH 7.0-7.5) for 5 min (about 35 ml of solution is needed for each jar).
2. Treat slides with 0.5 mg/ml RNase A (in PBS, DNase free) for 10 min in a 37 °C water bath.
3. Stain slides with 1 µg/ml Hoechst 33258 (Sigma-Aldrich) diluted into 2x SSC for 15 min at room temperature with slight agitation.
4. Place slides in a shallow plastic tray and add just enough 2x SSC buffer to cover the slides. Expose the slides to 365 nm UV light at room temperature for 1,800 sec (30 min; equivalent to 5,400 J/m<sup>2</sup>) in a Stratalinker 1800.
5. Digest the BrdU:C labeled UV nicked DNA strand with 80 µl of 10 U/µl Exonuclease III (Promega) into buffer supplied by the manufacturer diluted to 1x (50 mM Tris-HCl, 5 mM MgCl<sub>2</sub>, 5 mM DTT, pH 8.0). Add the 80 µl solution onto a 24 x 60 coverslip that will make contact with the entire surface of the cells on the slide. Let it adhere to the slide, then place the slide cells side up for 10 min, away from light. Avoid air bubbles.
6. Remove the coverslips and repeat the Exonuclease III digestion step with fresh solution for another 10 min.
7. Wash slides in PBS for 5 min and dehydrate successively in 70%, 90%, 100% ethanol series for 2 min each.
8. Air dry slides and store at room temperature in the dark (can be left overnight).

#### C. Fluorescent *in situ* hybridization

1. Place 80 µl of hybridization mix onto coverslips and pick up the coverslip with the slide, then put the slide into the hybridization chamber (see Figures 1G-1I for instructions on how to make a hybridization chamber and for a visual illustration of these steps). Avoid air bubbles.



**Figure 1. Representative images of the Cen-CO-FISH procedure at different stages of the protocol.** A. Cells are harvested, hypotonically swollen and fixed overnight. Samples can be stored at 4 °C at this stage. Cells are then dropped onto glass slides to let the metaphases spread. Slides can be stored in the dark at room temperature (RT) before proceeding to Step B. B. Rehydrate the slides into PBS in a Coplin jar for 5 min. C. Remove PBS and add RNase A solution into the jar, incubate in a 37 °C water bath for 10 min. Remove RNase solution and incubate with Hoechst in 2x SSC for 15 min at RT. D. Place slides into plastic try and cover with just enough 2x SSC. E. Place tray into Stratalinker oven with 365 nm UV bulbs and expose for 30 min (1,800 sec). F. Prepare 80 μl of Exonuclease III solution for each slide and add the solution to a 24 x 60 coverslip that will make contact with the entire surface of the slide. G.

Immediately after UV exposure, pick up the coverslip with Exonuclease III solution as shown. Invert the slide, adjust the coverslip to make sure it is central, the liquid is well distributed and there are no air bubbles. Incubate cells side up for 10 min at RT and then repeat Steps F-G one more time for an additional Exonuclease incubation. Wash in PBS and dehydrate in ethanol series. Store slides at RT in the dark (overnight). H. Prepare one of the probes (1:1,000-1:5,000) into hybridization solution, heat for 10 min at 60 °C before adding 80 µl onto the coverslips. Proceed as in Steps F-G shown. I. Place slides into a Hybridization chamber and incubate for 2 h at RT. To make a chamber, take a box, fill it with paper towels and wet throughout until all towels are humid. Do not put the slides directly on top of the wet paper. Use pipettes or other forms of support to raise the coverslips over the wet paper. Place slides cells side up and close the box away from light. Upon opening the box after 2 h, you should see a small amount of condensation on the lid. Wash with Hybridization buffer #1 and repeat Step H with the second, reverse complement probe (you can invert the order of the hybridization between forward and reverse complement probes, it should yield identical results). Wash in Hybridization buffer #1 and #2, including the DAPI step, dehydrate and air dry for at least one hour. Mount and seal before imaging.

2. Hybridize with the Forward PNA probe at a 1:1,000 dilution (heated at 60 °C for 10 min right before use) in the dark for 2 h in the hybridization chamber.
3. Rinse in Wash #1 for 30 sec. Leave slides draining vertically for 10 sec.
4. Hybridize with the Reverse Complement PNA probe at a 1:1,000 dilution (heated at 60 °C for 10 min right before use) in the dark for 2 h.  
*Note: Inverting the order of the probes will not affect the hybridization or quality of the signal.*
5. Wash slides in Wash #1 for 15 min 2 times on an orbital shaker.
6. Wash slides in Wash #2 for 5 min 3 times. To the second wash, add 1:500 DAPI from 0.5 mg/ml stock.
7. Dehydrate in 70%, 90%, 100% ethanol series for 3 min each.
8. Air dry slides for about 1 h.
9. Mount slides using Prolong Gold embedding medium (avoid bubbles) and seal using nail varnish. Slides are ready for imaging.
10. Slides can be stored up to 1 week at 4 °C or at -20 °C for longer storage.

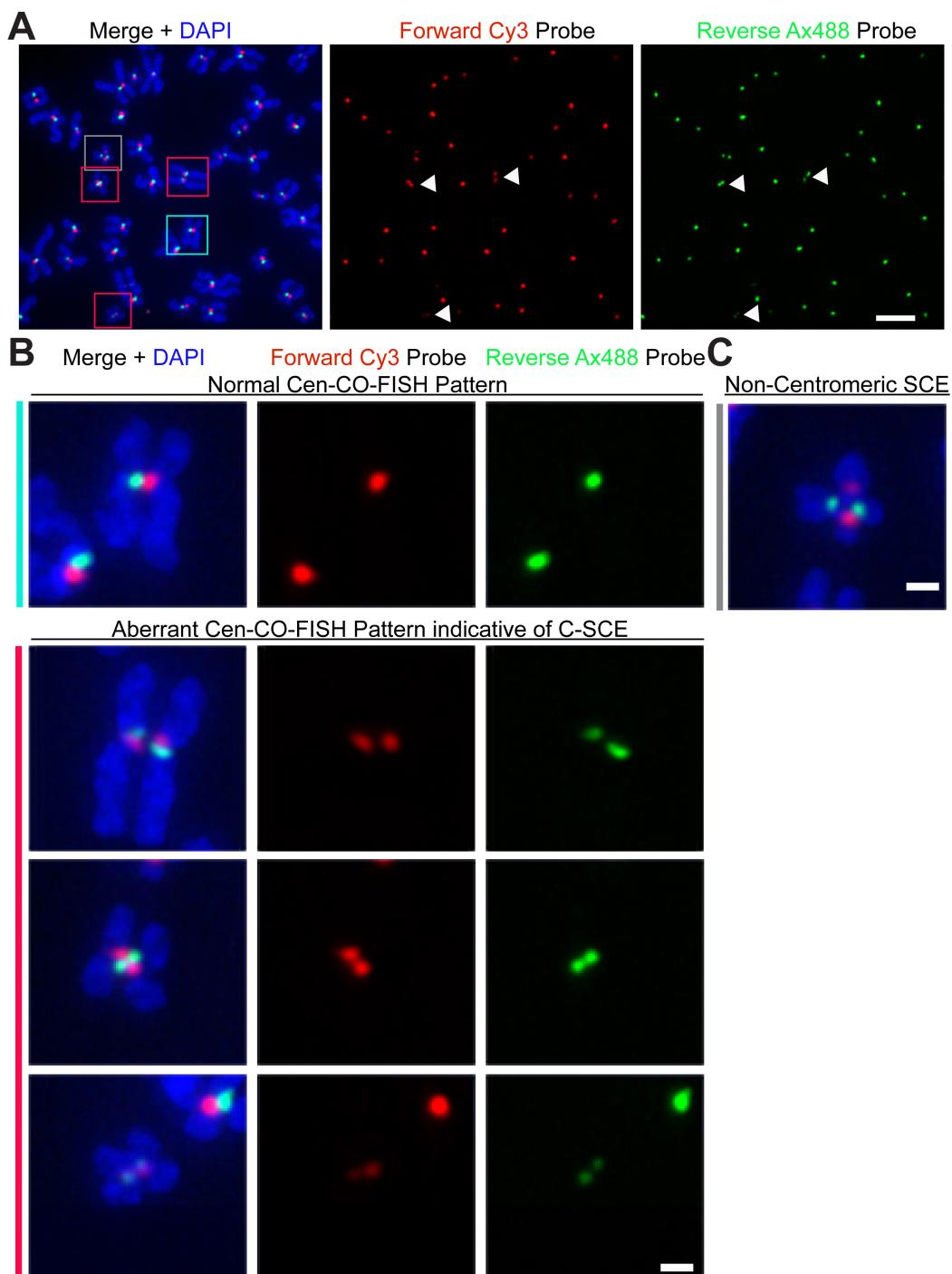
## **Data analysis**

### **Image acquisition and processing**

1. Cells are imaged using a DeltaVision Image Restoration microscope system (Applied Precision/GE Healthcare), mounted on an Olympus IX-70 microscope and fitted with a 100x/1.40 UPLSAPO objective lens and a CoolSnap QE CCD camera (Photometrics).
2. 15 or more metaphase spreads for each experiment are imaged to yield statistically significant and representative results. When imaging the cells, care should be taken to select the best spreads where chromosomes are nicely separated (Figure 2A) and approximately 46 chromosomes are present in the case of karyotypically stable diploid cells. Typical exposure times for the DAPI is 0.3 sec, for the TRITC channel (red probe) is 0.1 sec and for the FITC (green probe) is 0.5 sec.
3. The image is acquired as a z-stack containing about 15-30 0.2 µm sections. Acquired images are deconvolved using SoftWoRx (Applied Precision) and exported to Metamorph (Universal Imaging) for analysis as maximum projections, as shown in the examples in Figures 2A and 2B.

### **Representative images and scoring**

1. Following the Cen-CO-FISH protocol outlined, high-quality metaphase spreads are obtained stained with a specific centromere marker, as shown in Figure 2A. The forward probe, labeled in red, and the reverse strand probe, labeled in green, should not overlap but be adjacent to each other, labeling one individual sister chromatid of each chromosome (Figure 2B). Quantifications are performed by counting the number of aberrant Cen-CO-FISH patterns indicative of C-SCE in each metaphase spreads (Figure 2A, colored boxes). Examples of normal (top panel, teal box; Figure 2B) and aberrant Cen-CO-FISH pattern indicative of C-SCE are shown (bottom panels, red boxes; Figure 2B). In high chromosomal instability (CIN) lines or some cancer cell lines, a small number of aberrant patterns can be found resulting from fusion events occurring outside of the centromere region, as indicated by the presence of DAPI separating the two pairs of centromere signal, and should be excluded from the quantification of C-SCE (grey box; Figure 2C). The number of C-SCE can be presented as the percentage of aberrant centromeres over the total number of labeled centromere pairs for each metaphase and plotted in a scatter plot using Prism, as previously shown (Giunta and Funabiki, 2017).



**Figure 2. Detecting recombination events at human centromere using the Cen-CO-FISH method.** A. HeLa cells were stained with Cen-CO-FISH using forward (red) and reverse (green) probes raised against the CENP-B box sequence, present in all chromosomes in the metaphase spread shown in A. Individual channels allow the visual identification of aberrant Cen-CO-FISH staining patterns, often present as a ‘double dot’ for the red channel and the same for the green channel, as indicated by the white arrows. Color combined image merged with DAPI is also shown. Boxes are enlarged in Figure 2B. Scale bar = 5  $\mu$ m. B. Enlarged boxes from Figure 2A, left panel, showing a representative image for a normal Cen-CO-FISH

staining pattern (marked in teal), and three panels below showing aberrant patterns (marked in red) indicative of C-SCE. Scale bar = 1  $\mu$ m. C. Examples of an aberrant staining pattern resulting from recombination outside the centromere sequence (marked in grey), thus excluded from quantification of centromere recombination and C-SCE. Scale bar = 1  $\mu$ m.

2. Cen-CO-FISH is a reliable, reproducible and sensitive labeling technique to visualize repetitive genomic regions. The technique relies on two crucial steps: repetitiveness of the locus and incorporation of BrdU and BrdC into the newly synthesized strand. The probes used for the Cen-CO-FISH hybridization are fluorescently-labeled PNA 17 or 18-mer. Designing the probes to be aimed at sequences that are repeated enables the amplification of the fluorescence intensity. Centromere alpha satellite repeats spans from 0.5-5 Mb with sequences common to each human chromosomes (Schueler *et al.*, 2001; Fukagawa, 2004), yielding a robust Cen-CO-FISH signal for visualization, acquisition using conventional microscopy, and functional analyses. The reliance of the technique on labeling of the newly synthesized strand, which will subsequently be enzymatically digested, signify that this method can only be applied to cycling cells. However, this also represents an advantage for this method, where the yielded patterns are indicative of recombination events having happened during a single prior cell cycle. Thus, Cen-CO-FISH provides a bird's-eye view on these dark genomic regions while giving specific temporal resolution and sequence orientation information to examine the behavior of the centromere repeats. Previous studies have reported the application of the Cen-CO-FISH technique to monitor centromere recombination in mouse cells (Bailey *et al.*, 1996; Jaco *et al.*, 2008; Falconer *et al.*, 2010) indicating that the technique can be successfully applied to tissue culture cells from different species using specifically designed probes.

## Recipes

1. Hypotonic solution

75 mM KCl

Prewarm the required amount in a 37 °C water bath before each use

2. Fixative solution

3 parts methanol

1 part glacial acetic acid

Must be made fresh each time

Use ice-cold

3. Ethanol dilution

70% and 90% in double distilled water

4. Blocking solution

Dissolve the blocking reagent (Roche) into maleic acid buffer to make a 10% stock:

100 mM maleic acid

150 mM NaCl

Adjust pH to 7.5 with NaOH and store at 4 °C

Shake vigorously before each use

5. Hybridization solution

10 mM Tris-HCl pH 7.2

70% formamide

0.5% blocking reagent (from 10% stock)

6. Hybridization wash #1

10 mM Tris-HCl pH 7.2

70% formamide (Sigma-Aldrich)

0.1% BSA (dissolve in double distilled water before adding formamide)

7. Hybridization wash #2

0.1 M Tris-HCl pH 7.2

0.15 M NaCl

0.1% Tween-20

For the DAPI wash, add 1:500 DAPI using the 0.5 mg/ml stock

8. Peptide nucleic acid (PNA) probes (custom probes from PNABio)

Set #1 against the alpha satellite:

F3003 CENT-Cy3 (Cy3–OO–AAACTAGACAGAACGATT)

Reverse complement CENT-RC-488 (Alexa488–O–AATGCTTCTGTCTAGTTT)

Set #2 against the CENP-B box:

F3002 CENP-B Cy3 (ATTCGTTGGAAACGGGA)

Reverse complement CENP-B-RC-488 (TCCCGTTCCAACGAAT)

Notes:

- a. *Lyophilized probes were diluted to 50 µM in double distilled water based on manufacturer's instructions. Aliquot and store at -80 °C to avoid multiple freeze-thawing.*
  - b. *For hybridization, thaw and spin the probes, and dilute into hybridization solution at a 1:1,000-1:5,000 dilution (adjust according to the signal obtained). Heat at 60 °C for 10 min before use.*
9. Sodium chloride and sodium citrate buffer (SSC; 20x)  
NaCl (3 M), sodium citrate (300 mM) dissolved in distilled water  
Adjust pH to 7.0 and autoclave  
Working dilution is 2x

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Competing interests statement: The author declares no competing interests.

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## 3D Co-culture System of Tumor-associated Macrophages and Ovarian Cancer Cells

Lingli Long<sup>1</sup>, Mingzhu Yin<sup>2</sup>, and Wang Min<sup>1, 2, \*</sup>

<sup>1</sup>Center for Translational Medicine, The First Affiliated Hospital, Sun Yat-sen University, 58 Zhongshan Rd II, Guangzhou 510080, China; <sup>2</sup>Department of Pathology and the Vascular Biology and Therapeutics Program, Yale University School of Medicine, New Haven, CT06520, USA

\*For correspondence: [wang.min@yale.edu](mailto:wang.min@yale.edu)



**[Abstract]** Ovarian cancer is fairly unique in that ovarian carcinoma cells can detach and spread directly through peritoneal cavity. It has been unclear, however, how detached cancer cells survive in the peritoneum and form spheroid structure. We have recently reported that there is a strong correlation between Tumor-associated macrophages (TAMs)-associated spheroid and clinical pathology of ovarian cancer, and that TAMs promote spheroid formation and tumor growth at early stages of transcoelomic metastasis in orthotopic mouse models. We have established an *in vitro* spheroid formation assay using a 3D co-culture system in which mouse GFP<sup>+</sup>F4/80<sup>+</sup>CD206<sup>+</sup> TAMs isolated from spheroids of ovarian cancer-bearing donor tomato<sup>lysM-cre</sup> mice were mixed with ID8 cells (TAM:ID8 at a ratio of 1:10) in medium containing 2% Matrigel and seeded onto the 24-well plate precoated with Matrigel. As transcoelomic metastasis is also associated with many other cancers such as pancreatic and colon cancers, TAM-mediated spheroid formation assay would provide a useful approach to define the molecular mechanism and therapeutic targets for ovarian cancer and other transcoelomic metastasis cancers.

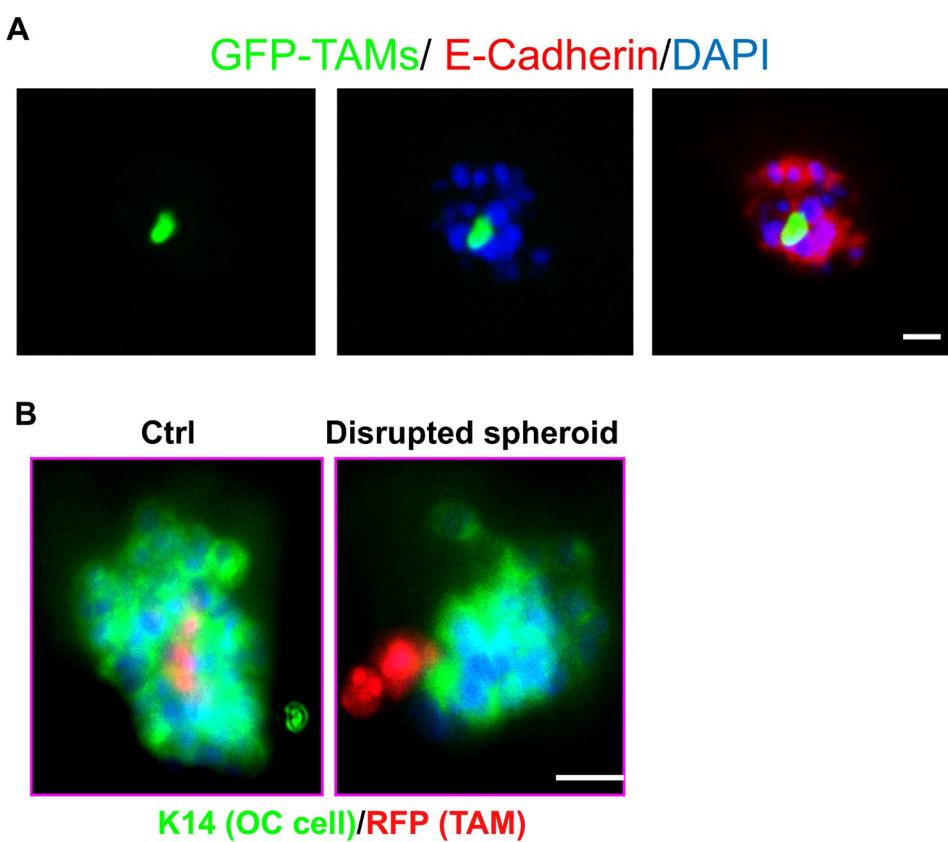
**Keywords:** Ovarian cancer, Tumor-associated macrophage, 3D co-culture system, Spheroid formation, Transcoelomic metastasis

**[Background]** Ovarian cancer (OC) is the second most common gynecological cancer and the leading cause of death in the United States (Jemal *et al.*, 2009; Siegel *et al.*, 2012). The major reason for the poor prognosis of OC is intraperitoneal and pelvic extensive implantation metastasis, which is usually unable to be removed completely by surgery. The most widely ascribed explanation for the phenomenon of peritoneal metastasis is that tumor cells become detached from the primary tumor after extension into the peritoneal surface and are transported throughout the peritoneal cavity by peritoneal fluid before seeding intraperitoneally. It has been suggested that the process of transcoelomic metastasis could be divided into several steps: 1) cell detachment, survival and resistance of anoikis; 2) evasion of immunological surveillance; 3) epithelial-mesenchymal transition; 4) spheroid formation; 5) ascites formation; and 6) peritoneal implantation (Tan *et al.*, 2006; Peart *et al.*, 2015; Rafehi *et al.*, 2016). However, it remains unclear how free detached tumor cells survive in transcoelomic environment and form spheroids at initial steps of transcoelomic metastasis. Our recent study reveals that TAMs play an essential role in the survival and proliferation of free cells detached from the primary

tumor in transcoelomic environment and spheroid formation at early stages of transcoelomic metastasis (Yin et al., 2016).

One critical method in this study is an *in vitro* spheroid formation assay using a 3D co-culture system to determine how TAMs facilitate spheroid formation. In this assay, mouse GFP<sup>+</sup>F4/80<sup>+</sup>CD206<sup>+</sup> TAMs isolated from spheroids of ovarian cancer-bearing donor tomato<sup>lysM-cre</sup> mice were mixed with ID8 cells (TAM:ID8 at a ratio of 1:10) in medium containing 2% Matrigel and seeded onto the 24-well plate precoated with Matrigel. Similarly, we use human CD14<sup>+</sup> TAMs isolated from OC patients and human ovarian cancer SKOV3 cells. In this model, we detect spheroid formation at 48 h of co-culture (Figure 1).

Here, we summarize our detailed protocols for 3D spheroid formation assay.



**Figure 1. TAMs and OC cells *in vitro* 3D co-culture system were showed by Immunofluorescence.** A. TAMs and OC cells form spheroids in an *in vitro* 3D co-culture system. GFP<sup>+</sup>F4/80<sup>+</sup>CD206<sup>+</sup> TAMs isolated from spheroids of ovarian cancer-bearing donor tomato<sup>lysM-cre</sup> mice and ID8 cells were co-cultured in the Matrigel-precoated 24-well plate for 48 h. The spheroids were subjected to immunofluorescent staining for E-cadherin for tumor cells. Images for GFP<sup>+</sup> TAMs, E-Cadherin<sup>+</sup> OC cells and DAPI for all cells in the spheroids are shown. B. Human TAMs were isolated and infected with lentivirus expressing RFP. RFP-expressing TAMs were incubated with SKOV3 human ovarian cancer cells followed by 3D co-culture for 72 h. Spheroids were immunostained with keratin-14. Scale bars = 10  $\mu$ m.

## **Materials and Reagents**

1. Pipette tips
2. 15 cm Petri dish
3. 10 ml serological pipette
4. 50 ml sterile conical tube (Corning, Falcon®, catalog number: 352070)
5. 18-gauge needle
6. 10 ml syringe
7. Falcon™ cell strainers, 100 µm (Corning, Falcon®, catalog number: 352360)
8. 1.5 ml microcentrifuge tubes
9. Greiner cellstar multiwall culture plates (24 wells, Greiner Bio One International, catalog number: 662102)
10. Cell lines: ID8 ovarian cancer cell line (Yin *et al.*, 2016) was a gift from Jack Lawler and Carmelo Nucera at Beth Israel Deaconess Medical Center (Harvard Medical School, Boston, Massachusetts, USA).  
*Note: ID8 cells are mouse epithelial OC line derived from C57BL/6 background; Passage under 30 and less than 1 week culture before injections.*
11. C57BL/6 mice, female, age: 8 weeks
12. Tomato reporter transgenic mice: ID8 OC cells were labeled by stably expressing mCherry fluorescence protein while LysMCre mice crossed to the tomato reporter *mT/mG*
13. Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher Scientific, Gibco™, catalog number: 10567014)
14. D-glucose
15. Fetal bovine serum (FBS) (Thermo Fisher Scientific, Gibco™, catalog number: 26140079)
16. Penicillin-streptomycin (Thermo Fisher Scientific, Gibco™, catalog number: 15140)
17. 0.25% Trypsin-EDTA (Thermo Fisher Scientific, Gibco™, catalog number: 25200056)
18. Ketamine
19. Bovine serum albumin (BSA) (Thermo Fisher Scientific, Thermo Scientific™, catalog number: B14)
20. Collagenase (Thermo Fisher Scientific, Gibco™, catalog number: 17104019)
21. F4/80 monoclonal antibody, APC conjugate for flow cytometry (Thermo Fisher Scientific, Invitrogen, catalog number: MF48005)
22. PE anti-mouse CD206 (MMR) antibody (BioLegend, catalog number: 141705)
23. DAPI (Vector Laboratories, catalog number: H-1200)
24. Anti-mouse E-Cadherin antibody (BD, Pharmingen™, catalog number: 610404)
25. Donkey anti-mouse (Alexa Flour 594) (Thermo Fisher Scientific, Invitrogen™, catalog number: A-21203)
26. Live cell tracker CMFDA (Thermo Fisher Scientific, Invitrogen™, catalog number: C2925)
27. EGFP (abm, catalog number: LV011-a)

28. Na<sub>2</sub>HPO<sub>4</sub>
29. NaCl
30. KH<sub>2</sub>PO<sub>4</sub>
31. KCl
32. Corning Matrigel (Basement membrane matrix, Corning, catalog number: 356234)
33. Tween 20 (Sigma-Aldrich, catalog number: P7949-500ML)
34. Paraformaldehyde (Sigma-Aldrich, catalog number: 16005)
35. PBS (see Recipes)
36. PBST (see Recipes)
37. AC buffer (0.5% BSA) (see Recipes)
38. 2% Matrigel (see Recipes)
39. 3.7% paraformaldehyde (see Recipes)

## **Equipment**

1. Safety cabinet
2. Pipette-aid
3. Centrifuge with swinging-bucket rotor and adaptors for 50-ml conical tubes
4. Water bath set at 37 °C
5. Humidified cell culture incubator set to 37 °C and 5% CO<sub>2</sub>
6. Zeiss Axiovert 200 fluorescence microscope (Carl Zeiss, model: Axiovert 200)
7. Upright microscope with 10x objective
8. Cell sorter and the scale (BD, model: FACSAria™ II); sorting at a rate of 80,000 cell/h

## **Software**

1. Quantitation of the average distance between the geometrical center of nuclei of adjacent cells can be measured using Openlab3 software (Improvision, Lexington, MA) or other commercially available image analysis software
2. SAS software (version 9.1.4, SAS Institute, Cary, NC)

## **Procedure**

*Note: All animal studies were approved by the Institutional Animal Care and Use Committee of Yale University.*

### A. Macrophage isolation and labeling

1. Cross Lysozyme Cre mice with tomato reporter C57BL/6 background mice and isolate EGFP-positive macrophages from peritoneal cavity by FACS sorting (Yin *et al.*, 2016).
2. As a positive control for spheroids, ID8 cells ( $1 \times 10^6/\text{ml}$ ) are injected into the abdominal cavity of Lysozyme Cre C57BL/6 mice (Age: 8 weeks) in 100  $\mu\text{l}$  of DMEM. Sacrifice the mice after nine weeks according to procedures approved by Yale's Institutional Animal Care and Use Committee, and analyze and sort the primary mouse tumor-associated macrophages (TAMs-GFP) using FACS(Yin *et al.*, 2016).

### B. Preparation of ID8 cell cultures

1. Expand the ID8 mouse ovarian cancer cells (Number of starting cells:  $1 \times 10^6$  ) in DMEM (4.5 g/L D-glucose, 10% FBS, 100 IU penicillin and 100  $\mu\text{g}/\text{ml}$  streptomycin) on a 15 cm Petri dish and culture ID8 cells until reaching 70-80% confluence.
  - a. Preparation: Prepare a safety cabinet. Switch on and allow the cabinet to reach working airflow pressure for approximately 15 min before use.
  - b. Warm medium. To avoid unnecessary stress to the cells, pre-equilibrate the medium in the incubator. If you are going to passage adherent cells, also thaw and warm an aliquot of trypsin.
  - c. Checking cells: Cells should be checked microscopically daily to ensure they are healthy and growing as expected. Attached cells should be mainly attached to the bottom of the Petri dish, round and plump or elongated in shape and refracting light around their membrane.
2. Upon reaching 70-80% confluence, aspirate the culture medium from the dish and add 10 ml of fresh DMEM.
3. Harvest the ID8 cells by washing them from the Petri dish with the DMEM (10 ml) using a pipette-aid and 10 ml serological pipette.
4. Transfer the ID8 cell suspension from the plate to a 50 ml conical tube, and pellet the cells by centrifugation at  $200 \times g$  for 4 min (room temperature).
5. Suspend the ID8 cell in DMEM and count the cells and prepare a suspension of the ID8 in DMEM at a density of  $1 \times 10^6$  cells per ml and ID8 cells should be kept on ice for injection.

### C. ID8 tumor implantation and metastasis model

1. Pathogen-free C57BL/6 background mice are purchased from THE JACKSON LABORATORY.
2. ID8 cells ( $1 \times 10^6/\text{ml}$  in 100  $\mu\text{l}$  of DMEM) will be injected into the abdominal cavity of C57BL/6 background mice.

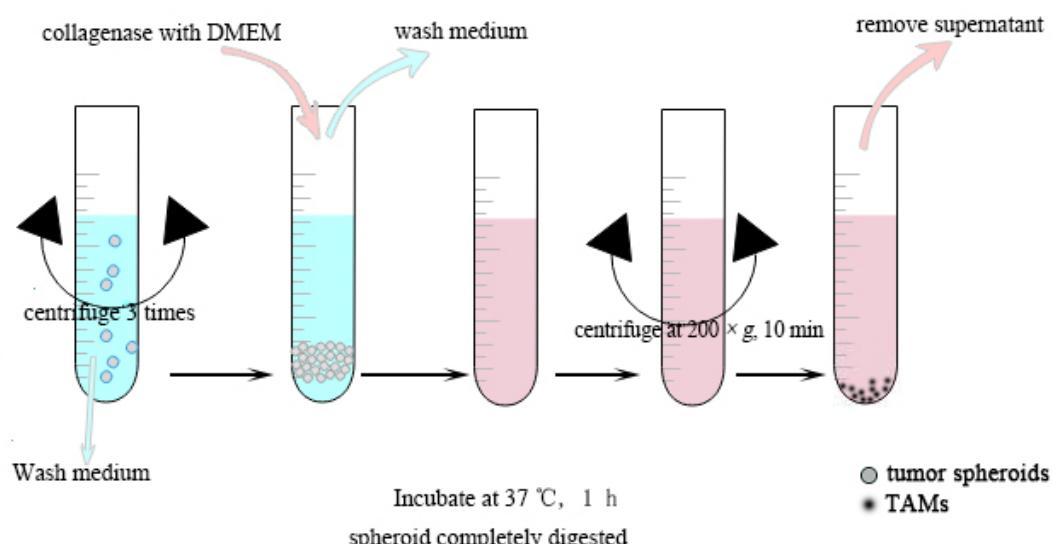
3. Mice body weight gains will be measured weekly.
4. The mice will be sacrificed at nine weeks prior to obvious signs of distress, pain or death.
5. Ascitic fluid volume and tumor weight will be measured, and the tumor spheroids will be analyzed by histology and FACS.

Ascitic fluid may begin to build up within 8-10 weeks following the injection of the ID8 cells. Tap the fluid when the mouse is noticeably large, but before the mouse has difficulty moving. Carefully withdraw as much fluid as possible with an 18-gauge needle attached to 10 ml syringe.

*Note: Sedating the mouse will make the collection of the ascitics fluid easier: ketamine 2 ng/mouse.*

#### D. Collect TAMs from tumor spheroids (Figure 3)

### Collect TAMs from tumor spheroids

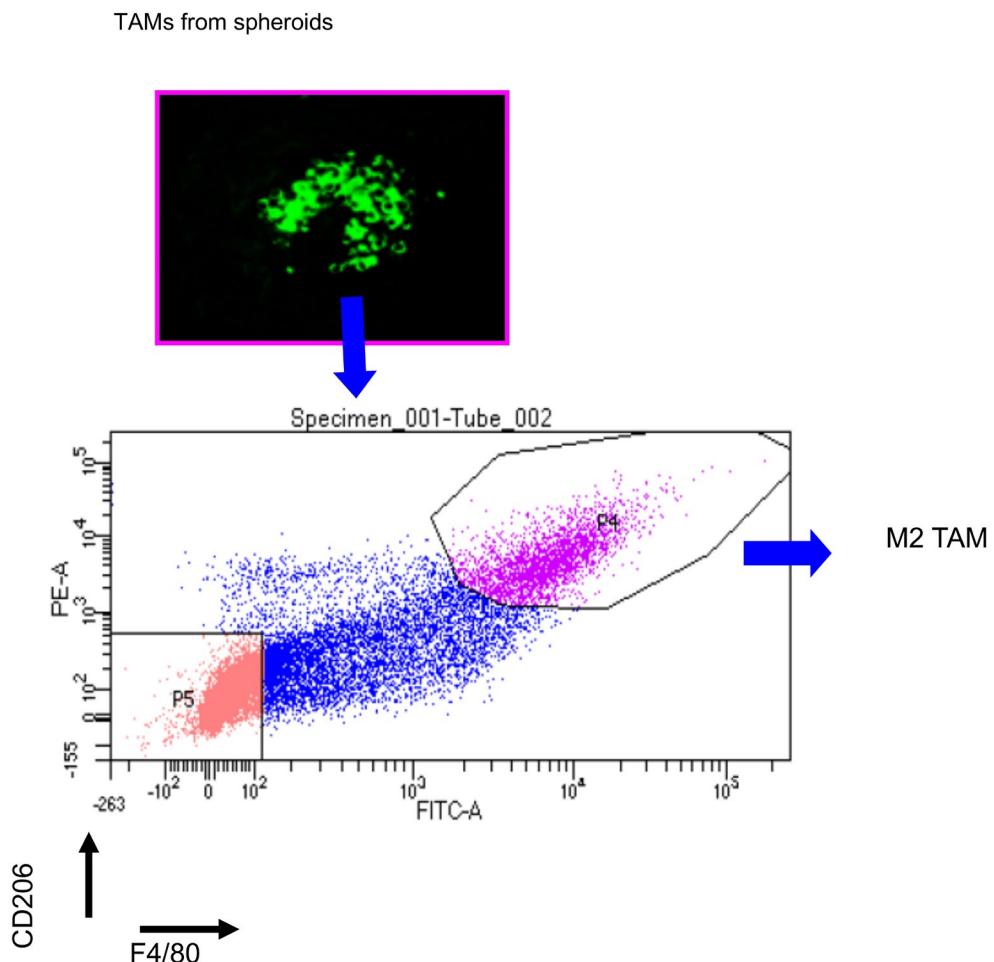


**Figure 3. The steps of collecting TAMs from spheroids**

1. Collect tumor spheroids from 3 ml ascitic fluid by using Falcon™ cell strainers.
2. Transfer the tumor spheroids to a 50 ml conical centrifuge tube, add wash medium (0.5% BSA with PBS 10 ml) for 2 min and discard it, and then add wash medium, repeat this washing step 3 times.
3. At the end of the washing cells, centrifuge the cell suspension at 200  $\times$  g for 3 min (room temperature).
4. Discard the wash medium and add 10 ml 500 U/ml collagenase in DMEM solution to the tube containing the tumor spheroids.
5. Incubate the tube at 37 °C for 1 h, and swirl the tube vigorously every 15 min. At the end of the incubation period, spheroid should be almost completely digested and no longer visible.

*Note: If spheroids are still visible after 1 h, continue incubation, checking every 15 min, until spheroids are no longer visible (do not exceed 2.5 h).*

6. At the end of the collagenase digestion, centrifuge the cell suspension at 200  $\times$  g for 10 min (room temperature).
  7. Remove the supernatant from the tube carefully without dislodging the pellet.
  8. Add 2 ml FACS buffer (0.5% BSA with PBS) to the 50 ml tube and suspend the cell pellet, and then transfer the suspension to a FACS tube through filters (Corning Falcon 5 ml round bottom tube with cell strainer snap cap, A 35  $\mu$ m nylon mesh).
- Note: It is necessary to obtain a single cell suspension.*
9. Centrifuge the cell suspension again at 200  $\times$  g for 10 min (room temperature).
  10. Remove the supernatant from the tube carefully without dislodging the pellet.
  11. Resuspend the pellet in 500  $\mu$ l FACS buffer.
  12. Add 2.5  $\mu$ l F4/80 (APC, 0.2 mg/ml) and 5  $\mu$ l CD206 (PE, 0.2mg/ml) antibody to stain TAMs.
  13. Sort GFP $^{+}$  F4/80 $^{+}$  and CD206 $^{+}$  TAMs through FACS analysis (Figure 2).



**Figure 2. FACS sorting of M2 TAMs.** Spheroids were collected from ovarian cancer-bearing donor mice and dispersed to single cells. Cells labeled with anti-F4/80 and anti-CD206 were isolated by FACS sorting on FACSAriaII (BD Biosciences).

#### E. Precoat 24-well plate with Matrigel

A 3D culture model was examined to determine the suitable cell culture system for our study.

1. In the 3D-base model, a 24-well plate is precoated with Matrigel as basement membrane by adding 160  $\mu$ l of Matrigel to each well.
2. Incubate at 37 °C for 30 min to let the Matrigel solidify.
3. Plate the cells (**TAMs from step D7, ID8 cells or SKOV-3**) onto the gel in 1 ml regular medium containing 2% Matrigel (3D-base and embedded, **containing laminin as a major component, collagen type IV, heparin sulfate proteoglycan, entactin, and other minor components**).

#### F. 3D co-culture system of TAM and ID8 cells

1. Isolate mouse GFP<sup>+</sup> F4/80<sup>+</sup> and CD206<sup>+</sup> TAMs by FACS sorting from spheroids of ovarian cancer-bearing donor tomato<sup>lysM-cre</sup> mice.
2. Precoat the 24-well plates with Matrigel as described above.
3. Seed the mixtures of TAMs and ID8 cells (at a ratio of 1:10 but with a fixed total cell number as 40,000 cells/well) directly onto the Matrigel-precoated 24-well plate with 300  $\mu$ l DMEMs in each well.
4. Incubate the cells were incubated at 37 °C for up to 48 h to allow the aggregates spheroids to form.

*Note: The wells without cells but containing medium are used as negative control.*

#### G. E-Cadherin staining in ID8 cells by immunofluorescent method

1. Gently wash the wells 2 times each with 500  $\mu$ l PBS to remove DMEM.
2. After PBS removal, add 300  $\mu$ l of buffered paraformaldehyde (3.7% paraformaldehyde, 10 nM, pH 7.4) for 10 min.
3. Wash the wells 2 times with PBS for removing the PFA and add 100  $\mu$ l E-Cadherin antibody (1:200 with 1% BSA) to incubate at 4 °C overnight (staining protocol see Yin *et al.*, 2016).
4. On the second day wash spheroids 3 times with 500  $\mu$ l PBST for about 15 min/time.
5. Add 100  $\mu$ l PE anti-mouse second antibody (1:500 with 1% BSA) into the 24-well plate with spheroids and incubate for 1-h at RT.
6. Capture fluorescent microscopic images by using Openlab 3 software (Improvision, Lexington, MA) under Zeiss Axiovert 200 fluorescence microscope (Carl Zeiss MicroImaging; Thornwood, NY). The captured images are used for analyzing the cell morphology.

#### Data analysis

1. Spheroids were visualized directly under a fluorescence microscope or were subjected to Line were subjected to immunostaining with anti-CD68, anti-K14 and DAPI followed by confocal imaging (see Figure 1).
2. The number (per well) and size (area) of spheroids at 48 h were quantified.

3. Statistical analyses: The differences of results of spheroid formation were analyzed by Student's *t*-test. Statistical analyses in this study were performed using SAS software (version 9.1.4, SAS Institute, Cary, NC). All statistical tests were two-tailed, and *P*-values less than 0.05 were considered statistically significant. All data are presented as means ± SEM, n = 5, \*, *P* < 0.05; \*\*, *P* < 0.01; \*\*\*, *P* < 0.001.

### **Notes**

1. For Matrigel preparation, freeze pipette tips to better handle Matrigel (which should be used at 4 °C).
2. Alternatively, mouse peritoneal macrophages from C57BL/6 background mice were isolated followed by pre-staining with live cell tracker CMFDA (Qin *et al.*, 2014) or by transduction with lentivirus expressing EGFP as we have shown for vascular endothelial cells by FACS sorting (Zhou *et al.*, 2016).
3. Euthanasia: Mice were euthanized with an overdose of ≥ 100 mg/kg bw pentobarbital and exsanguinated.
4. SKOV3 human ovarian cancer cells: (human ovarian adenocarcinoma cell line) were obtained from ATCC. SKOV3 cells are resistant to tumor necrosis factor and several cytotoxic drugs, including diphtheria toxin, cisplatinum, and adriamycin.

### **Recipes**

1. PBS  
8 mM Na<sub>2</sub>HPO<sub>4</sub>  
136 mM NaCl  
2 mM KH<sub>2</sub>PO<sub>4</sub>  
2.6 mM KCl
2. PBST  
500 ml 1x PBS  
1 ml Tween-20
3. AC buffer (0.5% BSA)  
Add 50 mg BSA into 10 ml PBS
4. Regular medium  
Add 50 ml fetal bovine serum and 5 ml penicillin and streptomycin into 500 ml DMEM medium
5. 2% Matrigel  
Add 200 µl Matrigel into 10 ml regular medium

*Note:* 2% Matrigel contains 8-12 mg/ml protein which includes laminin (major component), collagen type IV, heparin sulfate proteoglycan, entactin, and other minor components. Addition of Collagen type IV to the gel increases polymerization.

6. 3.7% paraformaldehyde  
Add 37 mg paraformaldehyde into 1 ml PBS

### **Acknowledgments**

Conflict of interest disclosure: None.

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## Generation of Luciferase-expressing Tumor Cell Lines

Todd V. Brennan<sup>1</sup>, Liwen Lin<sup>1</sup>, Xiaopei Huang<sup>2</sup> and Yiping Yang<sup>2, 3, \*</sup>

<sup>1</sup>Department of Surgery, Duke University Medical Center, Durham, NC, USA; <sup>2</sup>Department of Medicine, Duke University Medical Center, Durham, NC, USA; <sup>3</sup>Department of Immunology, Duke University Medical Center, Durham, NC, USA

\*For correspondence: [yang0029@mc.duke.edu](mailto:yang0029@mc.duke.edu)



**[Abstract]** Murine tumor models have been critical to advances in our knowledge of tumor physiology and for the development of effective tumor therapies. Essential to these studies is the ability to both track tumor development and quantify tumor burden *in vivo*. For this purpose, the introduction of genes that confer tumors with bioluminescent properties has been a critical advance for oncologic studies in rodents. Methods of introducing bioluminescent genes, such as firefly luciferase, by viral transduction has allowed for the production of tumor cell lines that can be followed *in vivo* longitudinally over long periods of time. Here we describe methods for the production of stable luciferase expressing tumor cell lines by lentiviral transduction.

**Keywords:** Lentivirus, Tumor, Lymphoma, Leukemia, Luciferase, GFP, Mouse, Methods

**[Background]** Paramount to tracking cells *in vivo* is the ability to detect them externally by minimally invasive methods. Enzymatic bioluminescence using luciferase derived from the firefly (*Photinus pyralis*) is a widely used method for image-based cell tracking *in vivo*. Bioluminescence has been used for a variety of *in vivo* application including the noninvasive imaging of reporter gene expression (Herschman, 2004), studying circadian rhythms (Southern and Millar, 2005), imaging cerebral strokes (Vandepitte *et al.*, 2014), and for tracking genetically engineered T cells (Costa *et al.*, 2001; Cheadle *et al.*, 2010). Perhaps the field where bioluminescent cell lines have been most applicable is oncology where they have been instrumental for the monitoring tumor growth (Jenkins *et al.*, 2005; Brennan *et al.*, 2016; Byrne *et al.*, 2016) and tumor metastasis (Rosol *et al.*, 2003; Simmons *et al.*, 2015) in mouse models. While some subcutaneously implanted tumors can be detected by palpation and measured with calipers, these methods are not effective for monitoring metastases or tracking tumors that disseminate widely, such as hematological malignancies that commonly grow in the bone marrow, lymph nodes and spleen.

Firefly luciferase oxides luciferin in the presence of molecular oxygen, magnesium and adenosine triphosphate to produce yellow-green light at 560 nm (Wilson and Hastings, 1998; Fraga, 2008). Benefits of luciferase bioluminescence in cell tracking include penetration of tissue for non-invasive monitoring and the re-usability of the enzymatic marker. Another advantage of luciferases is that most cells are not luminescent such that high signal-to-noise ratios can be achieved. A limitation bioluminescence is photon attenuation caused by intervening tissues, such as skin, bone, or hair.

Firefly luciferase is a single polypeptide specified by the *luc* gene that can be readily cloned into vectors used in gene delivery. Transient expression by plasmid transfection or non-integrating virus transduction limits the time over which cell tracking can be performed. This is especially problematic for oncology studies that may last several months. The ability of retroviruses to integrate into the genome is a key attribute that favors their use in producing stable cell lines. However, some oncoretroviruses, such as the Moloney murine leukemia virus can be limited by transgene silencing over time (Jähner *et al.*, 1982). Further, retroviral vectors require cell division for genomic integration and can be inefficient at transducing highly differentiated cells such as neurons, dendritic cells, or resting lymphocytes.

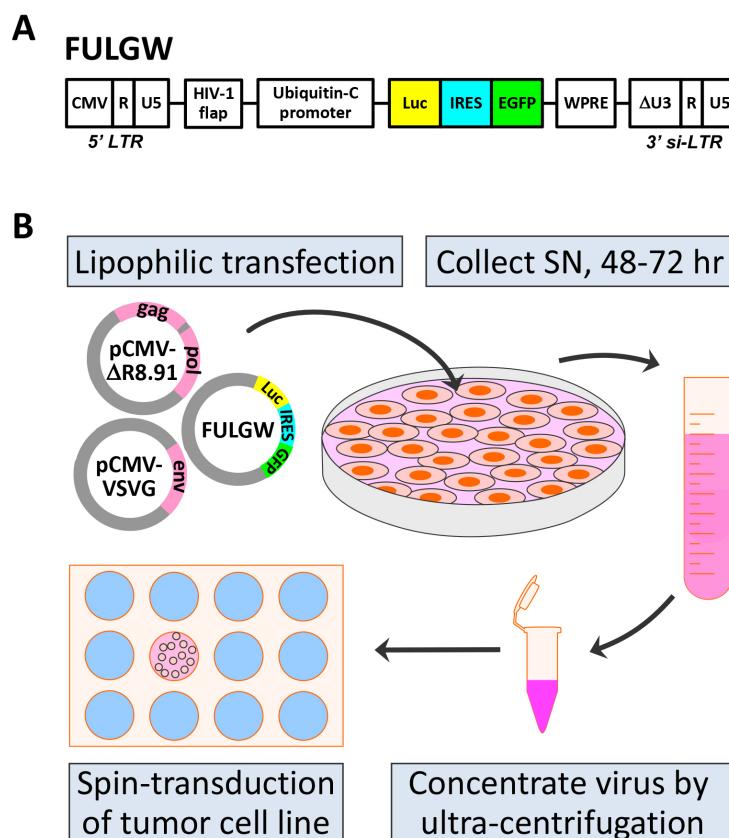
For the purpose of making luciferase expressing cell lines, lentiviral retroviral vectors derived from human immunodeficiency virus-1 (HIV-1) are highly effective. An advantage of lentiviral vectors over other retroviral vectors, is their ability to integrate into the genome of non-dividing cells. This property makes them suitable gene delivery vehicles for targeting highly differentiated cells, such as neurons, dendritic cells and lymphocytes (Naldini *et al.*, 1996). Lentiviruses also deliver very stable genomic integration and long-term transgene expression, to the extent that they have been used to make transgenic mice following embryo transduction (Lois *et al.*, 2002).

In order to track hematologic tumor cells in an *in vivo* murine leukemia model, we made the FULGW lentiviral vector that co-expresses firefly luciferase (Luc) and enhanced green fluorescent protein (EGFP) for the purpose of B-cell lymphoma (A20) cell line transduction and stable clone production. The FULGW vector is based on a self-inactivating vector previously described by Miyoshi *et al.* (1998) that had been engineered to express the GFP reporter gene behind the human ubiquitin-C promoter by Lois *et al.* (2002), making the FUGW vector. To make FULGW, we replaced the EGFP sequence of FUGW with a Luc-IRES-EGFP sequence from rKat.Luc2.IRES.EGFP, previously developed by Cheadle *et al.* (2010).

FULGW contains unique elements that enhance gene integration and expression (Figure 1A). It encodes the human immunodeficiency virus-1 (HIV-1) flap element, giving it karyotropic properties that permit efficient genomic integration in non-replicating cells (Zennou *et al.*, 2000). It contains the wood-chuck hepatitis virus posttranscriptional regulatory element (WPRE) that increases gene expression by transcript stabilization (Zufferey *et al.*, 1999). In addition, it includes a 3' self-inactivating long terminal repeat (3' si-LTR) that contributes to maintaining it as a replication deficient virus. The 3' si-LTR was developed by the deletion of a 133 bp region in the U3 region ( $\Delta$ U3) of the 3' LTR that renders the 5' LTR of the integrated provirus transcriptionally inactive (Miyoshi *et al.*, 1998).

Virus production is performed by the co-transfection of HEK-293T cells with the lentiviral plasmid (FULGW) and the two packaging plasmids, pCMV- $\Delta$ R8.91 and pCMV-VSVG (Figure 1B). HEK-293T cells are a human embryonic kidney cell line that stably expresses the CMV large T antigen, which greatly increases gene expression by the CMV promoter, generating robust virus production. pCMV $\Delta$ R8.91 is an HIV-1 Gag and Polymerase (Pol) expression plasmid that was modified from the dR8.9 vector by deletion of four accessory HIV-1 genes, Vif, Vpr, Vpu, and Nef (Zufferey *et al.*, 1997). pCMV-VSVG expresses the pan-tropic envelop (Env) protein derived from the vesicular stomatitis virus glycoprotein (VSVG) (Stewart *et al.*, 2003). [*Note:* Both FULGW and pCMV- $\Delta$ R8.91 are large plasmids

and best grown in chemically competent *recA1*-deficient *E. coli* with high transformation efficiency such as One Shot TOP10 *E. coli* (Invitrogen) grown at 30 °C for 24–28 h]. Using the FULGW lentiviral vector packaged with these helper plasmids, we have produced multiple types of tumor cell lines on various genetic backgrounds that stably express luciferase and GFP for use in oncologic studies (Table 1).



**Figure 1. Production of FULGW lentivirus and transduction of tumor cell lines.** A. Diagram of key regions of the FULGW vector including the Luc-IRES-EGFP transgene. Transgene expression is driven by the human ubiquitin-C promoter. CMV (cytomegalovirus promoter), U5 (LTR unique 5' region), R (LTR repeat region), HIV-1 flap (human immunodeficiency virus-1 flap element), Luc (firefly luciferase), IRES (intra-ribosomal element sequence), EGFP (enhanced green fluorescent protein), WPRE (wood-chuck hepatitis virus posttranscriptional regulatory element), si-LTR (self-inactivating LTR). B. FULGW is packaged and pseudotyped by lipophilic co-transfecting with pCMV-ΔR8.91 and pCMV-VSVG. Virus rich culture supernatant (SN) is collected at 48 and 72 h and virus is concentrated by ultracentrifugation. The concentrated virus is used to transduce tumor cell lines by spin-transduction in the presence of polybrene. Illustrated schematics make use of Motifolio templates ([www.motifolio.com/](http://www.motifolio.com/)).

**Table 1. Luciferase-GFP expressing tumor cell lines produced by FULGW transduction**

Cell line	Description (Mouse strain)
C1498	Myeloid leukemia (C57BL/6)
EL-4	T-cell lymphoma (C57BL/6)
RMA	T-cell lymphoma (C57BL/6)
RMA/S	T-cell lymphoma, MHC-I deficient (C57BL/6)
A20	B-cell lymphoma (BALB/c)
P815	Mastocytoma (DBA/2)
CT26	Colorectal carcinoma (BALB/c)
Yac-1	T-cell lymphoma, MHC-1 deficient (A/Sn)
MB49	Uroepithelial carcinoma (C57BL/6)

### **Materials and Reagents**

1. Pipette tips (USA Scientific, catalog numbers: 1110-3000, 1110-1000, 1111-2021)
2. T75 flask (Corning, Falcon®, catalog number: 353136)
3. 100 mm TC-treated Tissue Culture Dish (Corning, Falcon®, catalog number: 353003)
4. Sterile syringe 0.45 µm filter (VWR, catalog number: 28145-505)
5. Beckman ultra-clear 25 x 89 mm tubes (Beckman Coulter, catalog number: 344060)
6. Centricon Plus-70 unit (Merck, catalog number: UFC710008)
7. 15 ml Falcon tubes (Corning, Falcon®, catalog number: 352099)
8. 1.5 ml Eppendorf tubes (USA Scientific, catalog number: 1615-5500)
9. 12-well plates (Corning, catalog number: 3513)
10. 24-well plates (Corning, Costar®, catalog number: 3526)
11. 96-well plates (Greiner Bio One International, catalog number: 650185)
12. Sterile 500 ml 0.22 µm filter system (Corning, catalog number: 430758)
13. 29 ga. needles attached to 0.5 ml syringe (Terumo Medical Corporation, Elkton, MD, USA)
14. BALB/c mice (THE JACKSON LABORATORY, catalog number: 000651)
15. HEK 293T (ATCC, catalog number: CRL-3216)
16. A20 B-cell lymphoma (ATCC, catalog number: TIB-208)
17. pCMV-VSVG plasmid (Addgene, catalog number: 8454)
18. pCMVΔR8.91 plasmid (Lifescience Market, catalog number: PVT2323)
19. pFULGW (Lentiviral luciferase-IRES-GFP plasmid, Available on request)
20. Lipofectamine 2000 (Thermo Fisher Scientific, Invitrogen™, catalog number: 11668027)
21. Opti-MEM (Thermo Fisher Scientific, Gibco™, catalog number: 11058021)
22. Dulbecco's phosphate buffered saline (DPBS) (Corning, catalog number: 21-031-CM)
23. Trypsin EDTA (Thermo Fisher Scientific, Gibco™, catalog number: 25200056)
24. Polybrene (Sigma-Aldrich, catalog number: TR-1003-G)
25. Propidium iodide (Sigma-Aldrich, catalog number: P4864-10ML)
26. Bright-Glo™ Luciferase Assay system (Promega, catalog number: E2610)
27. D-Luciferin, potassium salt (Gold Bio, catalog number: LUCK-1G)

28. Isoflurane; Abbott Laboratories (Abbott Park, Illinois, USA)
29. Fetal bovine serum (Corning, catalog number: 35-010-CV)
30. DMEM–high glucose (Sigma-Aldrich, catalog number: D6429-500ML)
31. L-Glutamine (Thermo Fisher Scientific, Gibco<sup>TM</sup>, catalog number: 25030081)
32. Gelatin, 2% in H<sub>2</sub>O, tissue culture grade (Sigma-Aldrich, catalog number: G1393)
33. RPMI (Thermo Fisher Scientific, Gibco<sup>TM</sup>, catalog number: 11875093)
34. Penicillin/streptomycin (Thermo Fisher Scientific, Gibco<sup>TM</sup>, catalog number: 15140122)
35. D10 growth media (see Recipes)
36. 2% gelatin (see Recipes)
37. D-Luciferin stock solution (see Recipes)

### **Equipment**

1. Pipettes (Mettler-Toledo, Rainin, catalog numbers: 17008653, 17008650, 17008649; Thermo Fisher Scientific, Thermo Scientific<sup>TM</sup>, catalog number: 4641070N)
2. Tissue culture hood
3. Tissue culture incubator (Eppendorf, New Brunswick, model: Galaxy<sup>®</sup> 170 S)
4. Fluorescent inverted microscope with GFP filter (Leica Microsystems, model: Leica DM IL LED)
5. FACS-Canto flow cytometer (BD Biosciences)
6. Microcentrifuge (Eppendorf, model: 5424)
7. Table-top centrifuge (Eppendorf, model: 5810)
8. Ultracentrifuge (Beckman Coulter, model: L8-80M) equipped with an SW-28 rotor (Beckman Coulter, model: SW 28)
9. Balance (VWR, catalog number: 10204-990)
10. Xenogen IVIS Imaging System (Perkin Elmer, Hopkinton, MA, USA)
11. Tabletop Laboratory Animal Anesthesia System (VetEquip, catalog number: 901806)
12. Autoclave

### **Software**

1. Acquisition software (CellQuest, BD Biosciences)
2. Analysis software (FlowJo v9.3, TreeStar)
3. Living Image Software (Caliper Life Sciences, Hopkinton, MA, USA)
4. Graphing software (GraphPad Prism v7.0c, La Jolla, CA, USA)

## Procedure

### A. Lentiviral plasmid transfection and virus production

1. Prepare tissue culture plates by coating them with 2% gelatin (see Recipes). Briefly rinse each 10-cm plate with 10 ml of the 2% gelatin solution and let dry in a hood.

*Note: The same 10 ml of gelatin solution can be reused to coat several plates.*

2. Plate 293T cells on the gelatin-coated tissue culture plates 24 h before transfection at 6-7.5 x 10<sup>6</sup> cells per 10 cm plate in 10 ml D10 growth media (see Recipes).

*Note: We prepare 4 plates for each lentivirus preparation.*

3. Transfect 293T cells with lentivirus (e.g., pFULGW) and packaging plasmids (pCMV-ΔR8.91, pCMV-VSVG).

*Notes:*

- a. *Lentiviruses are classified as Biosafety Level 2 (BSL-2) organisms due to their ability to infect primary human cells and experiments need to be conducted in appropriate facilities.*
- b. *Transfection efficiency can be checked by imaging GFP expression using an inverted fluorescent microscope.*

- a. For each plate, prepare 60 µl of Lipofectamine in 1.5 ml Opti-MEM.
- b. For each plate, also prepare 13.3 µg FULGW, 10 µg pCMV-ΔR8.91, and 6.7 µg pCMV-VSVG in 1.5 ml Opti-MEM.
- c. Mix the Lipofectamine and plasmid containing Opti-MEM together and incubate for 25 min at room temperature.
- d. Remove 2 ml of media from each plate (now 8 ml total).
- e. Add 3 ml of the Lipofectamine/plasmid mix to each plate in a dropwise manner, evenly distributing the mix over the plate.

4. Incubate for 6 h at 37 °C, aspirate the media and add 10 ml of fresh D10.
5. Harvest SN (the culture media) at 48 h and store at 4 °C. Add another 10 ml fresh D10 media and harvest again at 72 h following transfection. Combine the SNs and clear by centrifugation at 2,000 rpm (805 x g) for 5' in a table-top centrifuge. Then filter SN through a 0.45 µm filter that has been pre-wetted with 10 ml D10.
6. Concentrate the virus.

*Note: Use proper containment when working with lentiviruses as per BSL-2 and institutional guidelines.*

- a. Ultra-centrifugation method

- i. Put the SN in Beckman ultra-clear 25 x 89 mm tubes (each holds up to 35 ml) and weigh on a balance to the hundredth of a gram to insure they are of equal weight such that the rotor will be balanced.
- ii. Spin at 25,000 rpm (112,000 x g) for 90 min at 4 °C in an SW-28 rotor.

- iii. Discard supernatant by inverting the tube. Maintain the tube in the inverted position in the tissue culture hood and aspirate the remaining fluid from the walls of the tube to dry it completely without disturbing the viral pellet.

*Note: The pellet will be small and can be difficult to visualize.*

- iv. Add 200  $\mu$ l of serum-free media directly to the viral pellet and allow it to resuspend for 12 h at 4 °C. Gently pipette to improve resuspension.

b. Filtration method

- i. Place SN in Centricon Plus-70 unit (Millipore, Bedford, MA). Each unit holds approximately 60 ml.
- ii. Centrifuge at 3,000 rpm (1811  $\times$  g) at 15 °C for 2-2.5 h using a table-top centrifuge.
- iii. Discard flow through.
- iv. Invert unit and spin at 1,500 rpm (453  $\times$  g) at 15 °C for 3 min to collect the viral concentrate.

7. Aliquot 20  $\mu$ l of virus per Eppendorf tube and store at -80 °C.

*Note: Aliquoting the virus helps to avoid freeze-thaw cycles, each of which will decrease the MOI by about 50%.*

B. Titer lentivirus

1. One day prior, plate 293T cells in 24-well plates at 250,000 cells/well in 0.5 ml D10.
2. Transfect cells by adding the equivalent of 10, 3, 1, 0.3, 0.1, and 0  $\mu$ l of viral concentrate per well of each prep.
3. After 2 days, harvest cells by rinsing with 100  $\mu$ l of PBS and then adding 100  $\mu$ l per well of trypsin-EDTA and incubating for 5 min at 37 °C.
4. FACS cells and determine % GFP positive.
5. Calculate:

$$\text{Titer} = \frac{(\# \text{ cells plated}) \times (\% \text{ GFP positive})}{(\text{mL of viral positive})}$$

C. Cell line transduction

1. Mix 5-10 MOI of lentivirus in serum-free media with polybrene (6-8  $\mu$ g/ml) and use it to replace the media of your cell line in a 12- or 24-well tissue culture plate. Spin transfect at 32 °C for 3-4 h at 1,000  $\times$  g (~2,300 rpm on a table-top centrifuge).
2. Incubate at 37 °C for another 3 h, then replace the media with fresh culture media.
3. Repeat viral transduction the next day if starting with lower MOI.
4. Grow for 2 days and check transduction efficiency with FACS or with a fluorescent microscope. GFP expression is heterogeneous in cells 2 days following transduction with FULGW and will decrease over time in culture (Figure 2A).

**Notes:**

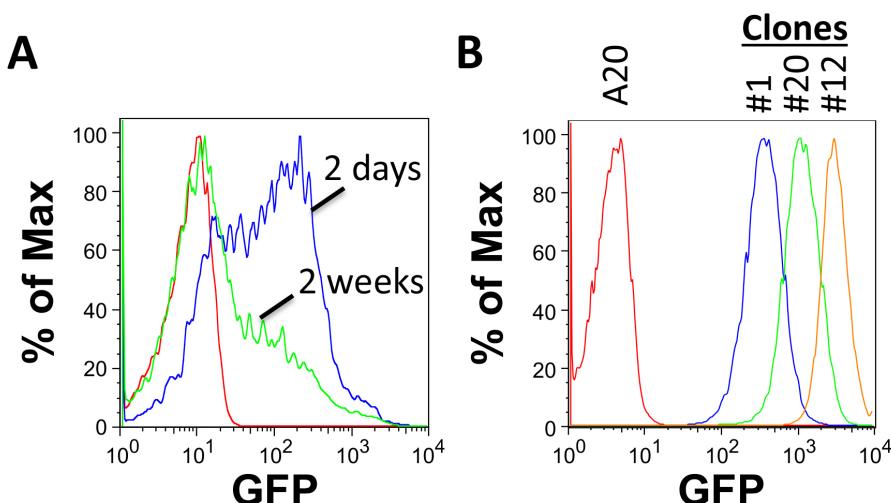
- a. This decrease likely is due to the competition of untransduced clones or transgene loss where viral integration did not occur.
- b. When testing by FACS, add 0.5 µg/ml of propidium iodide to the FACS solution to exclude dead cells.

**D. Isolate luciferase expressing clones**

1. Plate transduced cells into round-bottom 96-well plates with the goal of obtaining one positive cell per well. The most efficient method to accomplish this is to FACS sort individual GFP-expressing cells 2-3 days after transduction into wells containing 100 µl of growth media. Alternatively, single-cell clones can be obtained by methods of limiting dilution. For example, by preparing plates with 100 µl of growth media containing ~5 cells/ml.

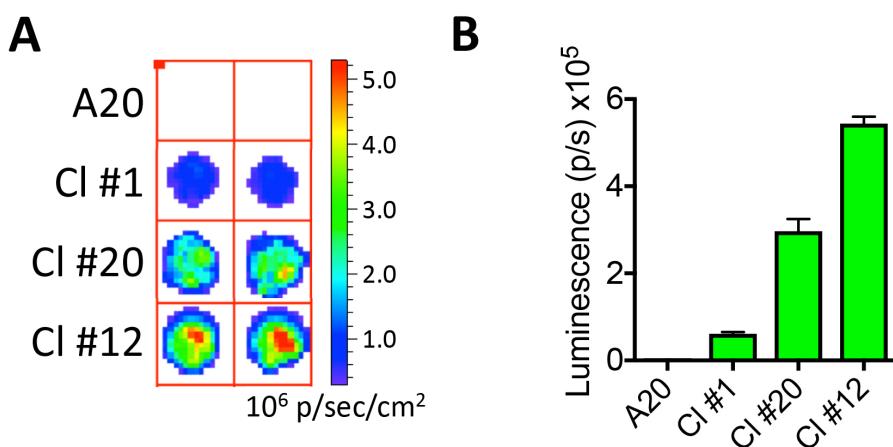
*Note: Plate multiple plates to ensure the development of sufficient clones.*

2. Allow cell clones to grow for ~2 weeks, giving them another 100 µl of fresh culture media at 1 week. Observe for clone growth by simply looking for wells with media that is becoming yellow, or by holding the plate up to a light and looking for colonies in the bottom of the wells.
3. Transfer the clones to 24-well plates and expand over a few days.
4. Test for GFP expression by FACS analysis. Clones derived from single cells will have a homogenous, narrow range of GFP expression. Select clones with different levels of expression for further testing (Figure 2B).



**Figure 2. Assessing tumor cell line transduction by FACS.** A. GFP expression in A20 cells 2 days and 2 weeks following transduction with FULGW. B. Isolated A20-Luc/GFP clones (#1, 12 and 20) demonstrate stable and homogenous GFP expression.

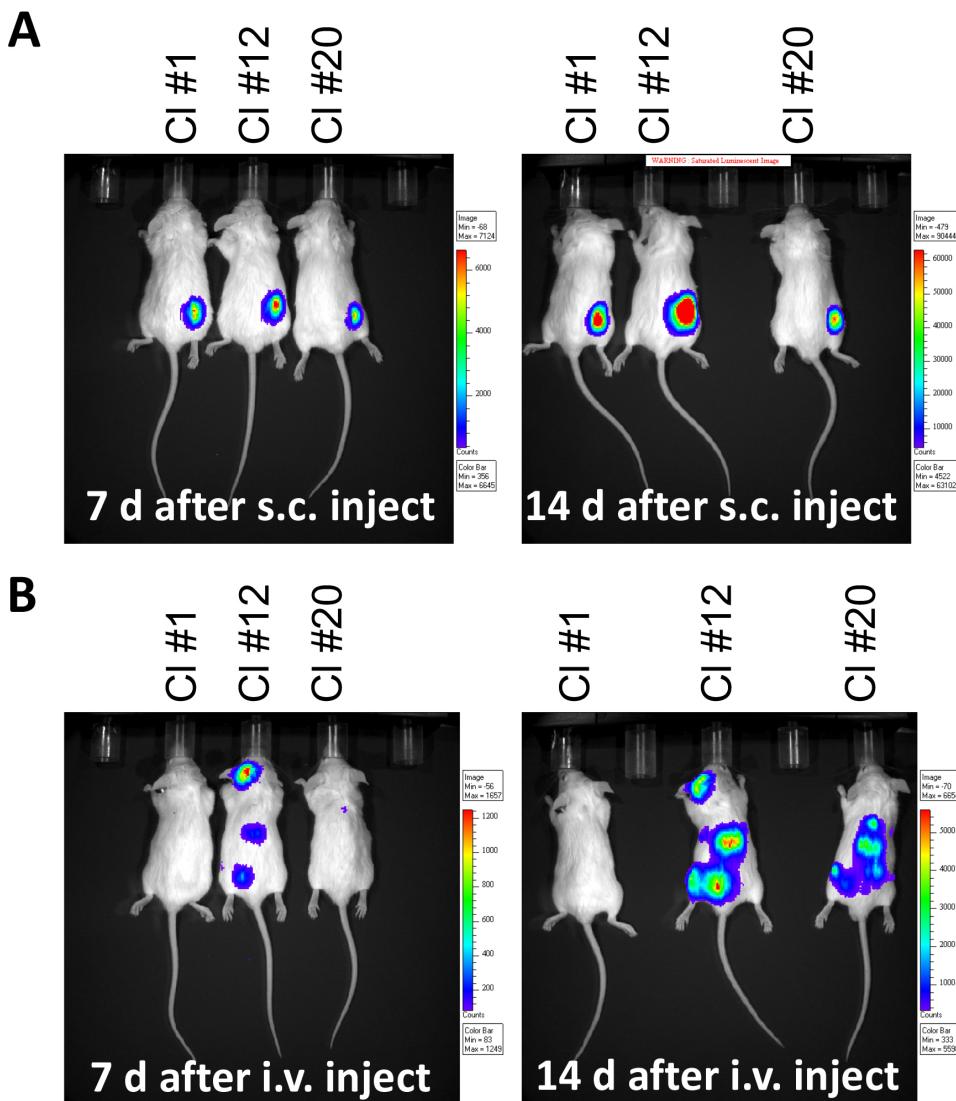
5. Test for luciferase activity.
  - a. Place 10,000 cells in round-bottom 96-well tissue culture plates.
  - b. To each well add a volume of Bright-Glo™ Reagent equal to the volume of culture medium in the well, and mix. For 96-well plates, typically 100  $\mu$ l of reagent is added to cells in 100  $\mu$ l of culture medium.
  - c. Wait at least 2 min to allow cell lysis, then measure luminescence in a luminometer or an IVIS system (Figures 3A and 3B).
6. Select clones and retest for GFP and luciferase expression after two weeks of culture to insure stable integration of the transgenes.
7. Freeze and store multiple aliquots of the cell line in liquid nitrogen for future use.



**Figure 3. Confirmation of luciferase expression.** A. Imaging A20 and A20-Luc/GFP B-cell lymphoma clones #1, 20, and 12 (10,000 cells from each clone are plated in a round-bottom 96-well plate) and tested for luciferase activity using an IVIS imaging system following the addition of D-Luciferin. B. Luminescence for each clone is quantified in photons per second (p/sec).

- E. Image luciferase cell lines *in vivo*
1. It is important to determine whether cell lines will grow efficiently *in vivo*.
  2. Administer  $0.5\text{--}1 \times 10^6$  Luc/GFP tumors cells either subcutaneously or intravenously into mice of the same genetic background (e.g., BALB/c for A20 cells).  
*Note: It may be necessary to precondition mice with 4 Gy of total body irradiation 4 h prior to tumor administration to permit tumor growth in some strains.*
  3. Quantify tumor burden by measuring luciferase activity by IVIS (Figure 4).
    - a. Inject mice with D-Luciferin (150 mg/kg, i.p.) and anesthetize by exposure to 4% isoflurane in an anesthetic chamber.
    - b. Once sedated, transfer the mice onto the pre-warmed stage inside the IVIS imaging system specimen chamber with continuous exposure to 2% isoflurane flowing into the nose-cone to sustain sedation.

- c. Image mice and measure photon flux approximately 10 min following injection of the substrate.



**Figure 4. Monitoring tumor burden in mouse leukemia model.** Luminescence imaged by IVIS at 7 and 14 days following, A. subcutaneous (s.c.) injection of  $1 \times 10^6$  A20-gfp/luc clones on the right flank, or B. after intravenous (i.v.) injection of  $5 \times 10^5$  A20-gfp/luc clones by tail vein injection.

4. Euthanize mice when primary tumors reach  $\sim 1 \text{ cm}^3$  (estimate from palpation), if animals lose significant weight ( $> 20\%$ ), develop hind-limb paralysis or become moribund, whichever comes first according to the method approved by your Institutional Animal Care and Use Committee (IACUC). For intravenously injected leukemia models, where tumor burden is difficult to determine by palpation, we typically euthanize animals when their measured luminescence reaches  $10^7 \text{ p/sec}$ .

5. As a control, obtain a baseline measurement of luminescence in an untreated mouse following D-Luciferin injection.

### **Data analysis**

IVIS data is collected in regions of interest and can be exported to Excel spreadsheet for analysis. The significance of differences between treatment groups can be determined by unpaired Student's *t*-test.

### **Recipes**

1. D10 growth media  
Add 50 ml of heat-inactivated fetal bovine serum (FBS) to 450 ml DMEM supplemented with glutamine and glucose
2. 2% gelatin  
Dissolve 1 mg gelatin in 500 ml H<sub>2</sub>O and autoclave at 121 °C, 15 psi for 30 min
3. D-Luciferin stock solution  
Dilute to 15 mg/ml in 1x Dulbecco's phosphate buffered saline (DPBS) (Life Technologies) and sterile-filtered (0.22 µm)

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## Measurement of Oxygen Consumption Rate (OCR) and Extracellular Acidification Rate (ECAR) in Culture Cells for Assessment of the Energy Metabolism

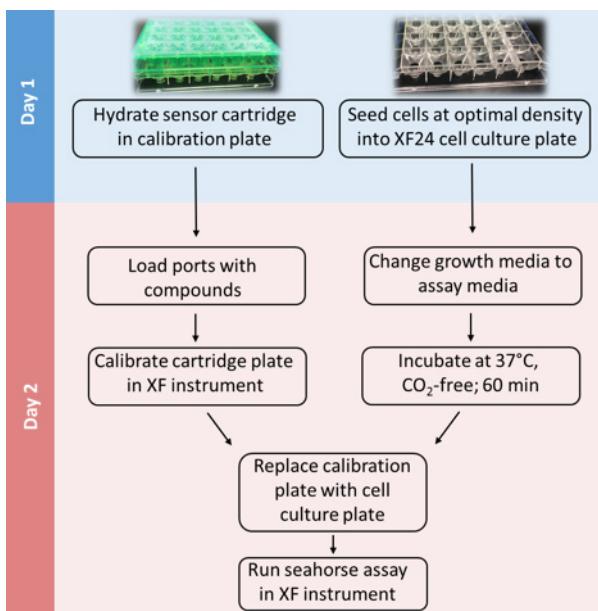
Birte Plitzko\* and Sandra Loesgen\*

Department of Chemistry, Oregon State University, Corvallis, Oregon, USA

\*For correspondence: [birte.plitzko@yahoo.de](mailto:birte.plitzko@yahoo.de); [sandra.loesgen@oregonstate.edu](mailto:sandra.loesgen@oregonstate.edu)



**[Abstract]** Mammalian cells generate ATP by mitochondrial (oxidative phosphorylation) and non-mitochondrial (glycolysis) metabolism. Cancer cells are known to reprogram their metabolism using different strategies to meet energetic and anabolic needs (Koppenol *et al.*, 2011; Zheng, 2012). Additionally, each cancer tissue has its own individual metabolic features. Mitochondria not only play a key role in energy metabolism but also in cell cycle regulation of cells. Therefore, mitochondria have emerged as a potential target for anticancer therapy since they are structurally and functionally different from their non-cancerous counterparts (DSouza *et al.*, 2011). We detail a protocol for measurement of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) measurements in living cells, utilizing the Seahorse XF24 Extracellular Flux Analyzer (Figure 1). The Seahorse XF24 Extracellular Flux Analyzer continuously measures oxygen concentration and proton flux in the cell supernatant over time (Wu *et al.*, 2007). These measurements are converted in OCR and ECAR values and enable a direct quantification of mitochondrial respiration and glycolysis. With this protocol, we sought to assess basal mitochondrial function and mitochondrial stress of three different cancer cell lines in response to the cytotoxic test lead compound mensacarcin in order to investigate its mechanism of action. Cells were plated in XF24 cell culture plates and maintained for 24 h. Prior to analysis, the culture media was replaced with unbuffered DMEM pH 7.4 and cells were then allowed to equilibrate in a non-CO<sub>2</sub> incubator immediately before metabolic flux analysis using the Seahorse XF to allow for precise measurements of Milli-pH unit changes. OCR and ECAR were measured under basal conditions and after injection of compounds through drug injection ports. With the described protocol we assess the basic energy metabolism profiles of the three cell lines as well as key parameters of mitochondrial function in response to our test compound and by sequential addition of mitochondria perturbing agents oligomycin, FCCP and rotenone/antimycin A.



**Figure 1. Overview of seahorse experiment**

**Keywords:** Bioenergetics, Seahorse XF, Mitochondrial metabolism, Glycolysis, Mitochondrial respiration

**[Background]** Natural products are small molecules that are isolated from natural sources. Over the last century, these molecules have been instrumental in treating human diseases, especially inspired chemotherapeutics. Metabolites like taxol, vincristine, and doxorubicin have revolutionized how we treat malign cancers and other natural products, for example rapamycin, oligomycin, and baflomycin, are used as molecular probes and enable molecular studies of biochemical and cellular processes in the laboratory. While studying the mechanism of action of the cytotoxic natural product mensacarcin, we found that a fluorescently labeled mensacarcin probe localizes to a great extent in mitochondria (Plitzko *et al.*, 2017). To investigate if mensacarcin's cytotoxic properties might be derived from interference with mitochondrial function, we sought to examine mensacarcin's effects on cellular bioenergetics. Using a Seahorse Extracellular Flux Analyzer, we monitored cellular oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) in real time as measures of mitochondrial respiration and glycolysis, respectively (Wu *et al.*, 2007; Serill *et al.*, 2015). The Seahorse XF24 Extracellular Flux Analyzer allows continuous direct quantification of mitochondrial respiration and glycolysis of living cells. The instrument uses a sensor cartridge in a 24-well plate format with each sensor being equipped with two embedded fluorophores: one which is quenched by oxygen (O<sub>2</sub>) and the other that is sensitive to change in pH. During measurements, the sensor cartridge is lowered 200 µm above the cell monolayer, forming a micro-chamber of about 2 µl. The Seahorse instrument contains fiber optic bundles that emit light, excite the fluorophores, and then measures the change in the fluorophore's emission. The very small test volume formed by the transient micro chamber allows for sensitive, precise, and nondestructive measurements of parameters in real time. Changes in oxygen concentration and pH are automatically calculated and reported as Oxygen Consumption Rate

(OCR) and Extra Cellular Acidification Rate (ECAR). Once a measurement is completed, the sensors lift which allows the larger medium volume above to mix with the medium in the transient micro chamber, restoring values to baseline. The sensor cartridge contains ports that allow sequential addition of up to four compounds per well during the assay measurements.

With the described protocol we assessed the energy metabolism of three cell lines (HCT-116, SK-Mel-28, and SK-Mel-5) (Figure 6). Addition of mensacarcin was found to have pronounced effect on the basal OCR of melanoma cells and no increasing effect on ECAR. An increase in glycolysis is often observed as a compensatory response. Mitochondria are essential for the energy metabolism of cells and have a key role in apoptotic cell death. Alteration of the mitochondrial respiration or the equilibrium between the pro-apoptotic and anti-apoptotic proteins can induce mitochondrial failure. To gain insights into the induced mitochondrial impairment in melanoma cells, we assessed key parameters of mitochondrial respiration by consecutively exposing cells to well described mitochondria perturbing reagents. Following addition of our test compound mensacarcin, we sequentially added oligomycin, FCCP, and lastly rotenone and antimycin A (Figure 5). Oligomycin inhibits ATP synthase and reduces OCR, FCCP uncouples oxygen consumption from ATP production and raises OCR to a maximal value, and antimycin A and rotenone target the electron transport chain and reduce OCR to a minimal value. The mitochondria stress test protocol provides information on basal respiration, ATP-linked respiration, proton leak, maximal respiration capacity, and non-mitochondrial respiration of cells. Therefore, this assay can be used to provide insight on the mechanism of action of compounds that directly target mitochondrial respiration.

Traditional measurements of mitochondrial function or glycolysis require an oxygen electrode, or kits and dyes that utilize colorimetric or fluorimetric detection (Li and Graham, 2012; TeSlaa and Teitel, 2014). Most of these methods are invasive and cumbersome methods that only allow low sample throughput. In contrast, the Seahorse analyzer assay with its sensor cartridge system enables measurement of mitochondrial respiration and glycolysis in real time and in a non-invasive manner that does not require any dyes or labels. Cellular energy metabolism research is highly topical in all fields of mammalian cell biology. The following protocol was developed for researchers in cancer biology but with approaches that suit studies of energy metabolism in all mammalian cell systems.

## **Materials and Reagents**

1. CELLSTAR® Tissue Culture Plates, 96-well (Greiner Bio One International, catalog number: 655180)
2. Sterile racked pipette tips (1 ml and 200 µl) (VWR, catalog numbers: 613-0738; 613-0742)
3. Sterile basins (Corning, Costar®, catalog number: 4870)
4. Sterile reagent tubes (15 and 50 ml) (VWR, catalog numbers: 89039-668; 89039-662)
5. Sterile Serological pipettes (5, 10, 25, 50 ml) (Fisher Scientific, catalog numbers: 13-678-11, 13-678-11D, 13-678-11E, 13-678-11F)
6. Glass bottles (500 ml) (Fisher Scientific, catalog number: FB8001000)

7. HCT-116, SK-Mel-5 and SK-Mel-28 cells (ATCC, catalog numbers: CCL-247, HTB-70, HTB-72)
8. Seahorse XF24 FluxPak (including sensor cartridges, tissue culture plates, calibrant solution and calibration plates) (Agilent Technologies, Santa Clara, CA)
9. Trypsin/EDTA (0.25%/2.21 mM) (Corning, catalog number: 25-053-CI)
10. 1x Ca<sup>2+</sup>/Mg<sup>2+</sup>-free DPBS (Thermo Fisher Scientific, Gibco™, catalog number: 14190250)
11. Liquid Dulbecco's modified Eagle's medium (DMEM) (Corning, catalog number: 10-013)
12. Fetal bovine serum (FBS) (Atlanta Biologicals, catalog number: S11150)
13. Penicillin/streptomycin solution 100x (Corning, catalog number: 30-002-CI)
14. Powder Dulbecco's modified Eagle's medium (DMEM) without Na<sub>2</sub>HCO<sub>3</sub>, without HEPES (Corning, catalog number: 50-013)
15. Sodium hydroxide (NaOH) (VWR, catalog number: 97064-476)
16. Oligomycin (Merck, catalog number: 495455-10MG)
17. DMSO (VWR, catalog number: BDH1115-1LP)
18. FCCP (Cayman Chemical, catalog number: 15218)
19. Rotenone (Cayman Chemical, catalog number: 13995)
20. Antimycin A (Enzo Life Sciences, catalog number: ALX-380-075-M005)
21. Culture media (10% (v/v) FBS) (see Recipes)
22. Assay media (see Recipes)
23. NaOH (1 M) (see Recipes)
24. Oligomycin (10 µM) (see Recipes)
25. FCCP (5 µM) (see Recipes)
26. Rotenone (5 µM)/antimycin A (5 µM) (see Recipes)

## Equipment

1. Hemacytometer (Hausser Scientific, catalog number: 1490)
2. Biological Safety Cabinet Class II, Type A2 (NuAire, model: NU-425-400ES)
3. Seahorse XF Extracellular Flux Analyzer (Agilent Technologies, Santa Clara, CA)
4. Pipet-Lite Pipette XLS STD 20 XLS (Mettler Toledo, Rainin, model: SL-2XLS+)
5. Pipet-Lite Pipette XLS STD 200 (Mettler Toledo, Rainin, model: SL-200XLS+)
6. Pipet-Lite Pipette XLS 1000 (Mettler Toledo, Rainin, model: SL-1000XLS+)
7. Multichannel Pipet-Lite Pipette XLS 8-CH 1200 (Mettler Toledo, Rainin, model: L8-1200XLS+)
8. Multichannel Pipet-Lite Pipette XLS 8-CH 200 (Mettler Toledo, Rainin, model: L8-200XLS+)
9. Aspirator pump
10. Humidified non-CO<sub>2</sub> incubator (XF Prep Station; Agilent Technologies, Santa Clara, CA)
11. Shallow water bath (Thermo Fisher Scientific, Thermo Scientific™, model: Precision 180)
12. Pipette controller (BrandTech Scientific, model: Accu-Jet® Pro, catalog number: 26330)
13. Humidified, 37 °C, 5% CO<sub>2</sub> incubator (Eppendorf, model: Galaxy® 170 R)
14. -20 °C biomedical freezer (Sanyo, model: MDF-U731M)

15. Autoclave (Consolidated Sterilizer Systems, model: SSR-3A, ADVPB)
16. Inverted light microscope (Nikon Instruments, model: Eclipse TS100)
17. pH-meter with semi-micro electrode (Thermo Fisher Scientific, Thermo Scientific™, model: Orion Star™ A211, with ROSS 8103BN electrode: (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 8103BN)

## **Software**

1. Seahorse Bioscience XF24 software
2. Excel (Microsoft)
3. GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA)

## **Procedure**

### A. Optimization of seeding density

In an initial experiment, the optimal seeding density is required for each cell type. Typically, the cell density ranges from 10,000 to 60,000 cells per well and can vary widely among cell lines. A first point of orientation can be the cell number that gives confluence of approx. 95% overnight in a 96-well cell culture plate as the seeding surface is comparable to the seahorse culture plate. The seeding number should give a confluent and healthy and consistent monolayer on the day of the assay.

1. HCT-116, SK-Mel-5 and SK-Mel-28 cells were seeded in a Seahorse XF24 cell culture plate at various concentrations ranging from 10,000 to 30,000 cells/well with a two-step seeding technique as described below in Procedure B (Figure 2). Seeding cells in triplicates is recommended.

	1	2	3	4	5	6
A		SK-Mel-5 30K	SK-Mel-5 30K	SK-Mel-5 30K	SK-Mel-28 25K	SK-Mel-28 25K
B	SK-Mel-5 10K	SK-Mel-5 20K	SK-Mel-5 30K		SK-Mel-28 25K	SK-Mel-28 25K
C	SK-Mel-5 10K	SK-Mel-5 20K		SK-Mel-28 10K	SK-Mel-28 20K	SK-Mel-28 20K
D	SK-Mel-5 10K	SK-Mel-5 20K	SK-Mel-28 10K	SK-Mel-28 10K	SK-Mel-28 20K	

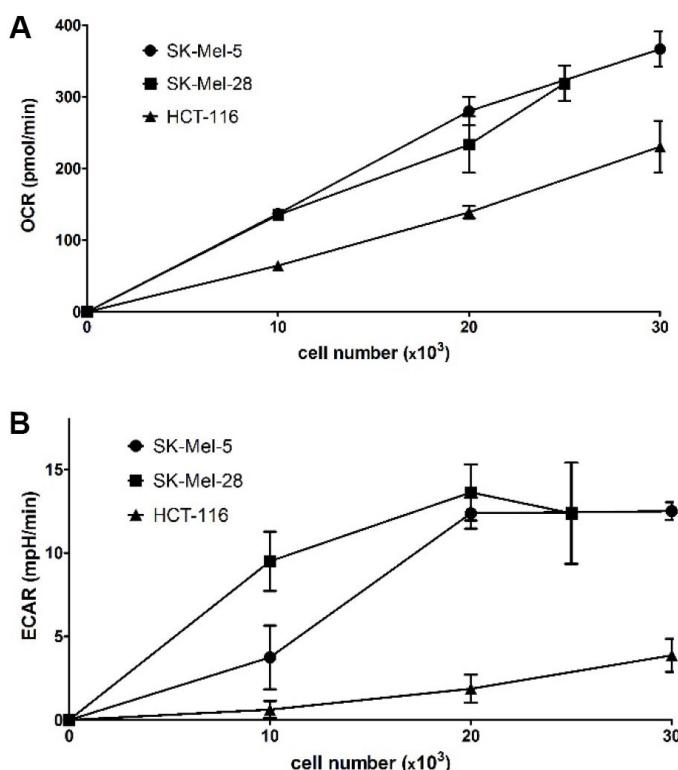
**Figure 2. Plate layout for cell density evaluation.** Shown here is the exemplary seeding layout for the SK-Mel-5 and SK-Mel-28 cell lines (seeding density for HCT-116 cells was evaluated on a second plate; not shown).

- Cells were then assayed in the XF24 instrument as described in Procedure E (without loading compounds into ports) using Table 1 commands.

**Table 1. Protocol commands for cell density evaluation**

Command	Time (min)
Calibrate	
Equilibrate	
Loop Start	8x
Mix	3.00
Wait	2.00
Measure	3.00
End	

As seen in Figure 3, a linear increase of OCR values with increasing cell density was observed in all three cell lines. ECAR values begin to level off at 20,000 cells/well for SK-Mel-28 and SK-Mel-5 while being much lower and steadily increasing for HCT-116. Thus, a seeding number of 20,000 cells/well for SK-Mel-28 and SK-Mel-5 and of 35,000 cells/well for HCT-116 were chosen to ensure being within the linear response range while having high reading values to observe increases as well as decreases in OCR and ECAR.

**Figure 3. Optimization of assay conditions: evaluation of OCR and ECAR depending on the seeding density of three different cell lines**

**B. Seeding cells into Seahorse XF24 tissue culture plate (Day 1)**

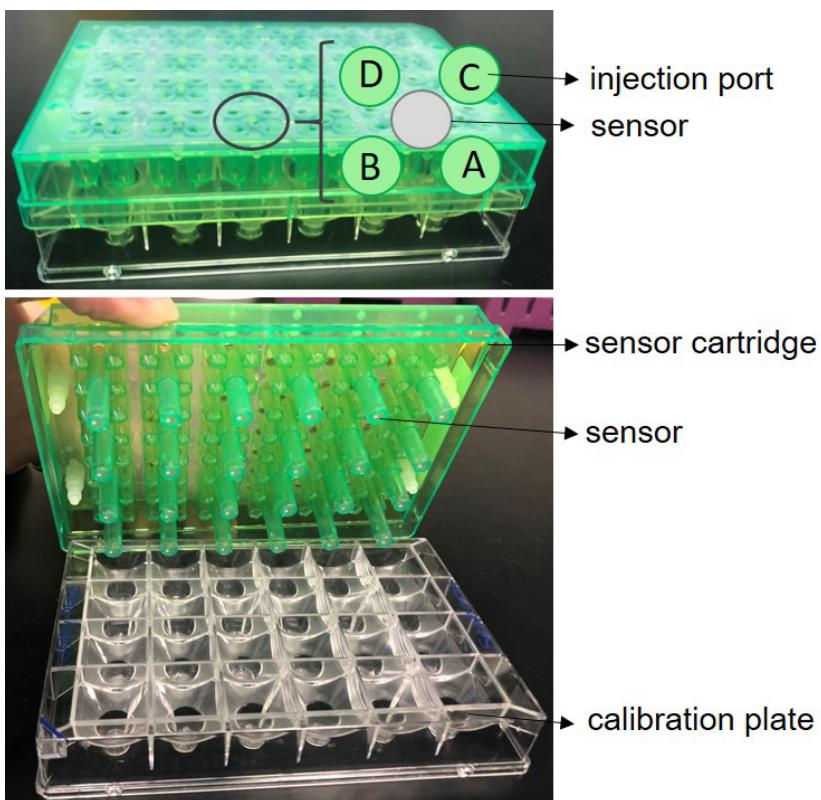
*Note: The seeding and growing of cells are performed with good sterile cell culture technique.* A two-step seeding method is used to obtain a consistent even monolayer which is vital to obtain consistent and accurate data:

1. Pre-warm culture media, trypsin solution and DPBS to 37 °C.
2. For adherent cells, wash cells with DPBS, and add trypsin and wait until cells begin to detach. Add culture media with serum to deactivate trypsin and pipette up and down to create a uniform cell suspension. Count cells with a hemocytometer and resuspend cells in growth media to the desired final concentration to seed in 100 µl.
3. Plate 100 µl cell suspension into a Seahorse XF24 tissue culture plate. Put media only (no cells) in the background correction wells (A1, B4, C3, D6).
4. Let the culture plate sit for 1 h in the bio-hood without moving it around (in order to let cells settle evenly).
5. Place the culture plate into an incubator (37 °C, 5% CO<sub>2</sub>) for 4 h.
6. Carefully add 150 µl growth media (final volume in well 250 µl). Hold the pipette tip at an angle and add to the well side to not destroy even layer of newly attached cells.
7. Let cells grow overnight at 37 °C, 5% CO<sub>2</sub>.

*Note: The following steps are performed without sterile technique, but caution to keep the cells and equipment as clean as possible.*

**C. Hydrate sensors (Day 1)**

1. Open XF 24 FluxPak and take out the sensor cartridge (green) and calibration plate (clear) (Figure 4).



**Figure 4. Seahorse XF 24 sensor cartridge.** A. The sensor cartridge sitting on top of a calibration plate with injection ports shown. B. Bottom side of the sensor plate which shows sensors with embedded fluorophores.

2. Place the sensor cartridge (sensors up) next to the calibration plate (be careful not to touch sensors).
  3. Fill each well of the calibration plate with 1 ml of Seahorse XF Calibrant.
  4. Lower the sensor cartridge onto the calibrant plate submerging the sensors in calibrant (be careful not to touch walls with sensors).
  5. Place in a non-CO<sub>2</sub> 37 °C incubator overnight. To prevent evaporation of the XF Calibrant, verify that the incubator is properly humidified.
- D. Stabilization of instrument (Day 1)
1. Turn on an XF24 Analyzer, open Seahorse Bioscience software and log in.
  2. Write the assay template. When planning and writing the assay protocol be careful not to create a protocol that is longer than cells can manage without CO<sub>2</sub> in unbuffered media. Depending on cell type this is 2-3 h. If in doubt, a cell viability assay can be performed after the seahorse assay.
  3. Leave the XF24 Analyzer on overnight with XF24 software running and logged in to ensure equilibration to 37 °C.

**E. Seahorse assay (Day 2)**

1. Check on the confluence of cells. Evenly spacing of cells is needed, without large cell clumps or blank patches, as this could impair the accuracy of data.
2. Pre-warm assay media to 37 °C.
3. Pre-warm compounds and adjust to pH 7.4 with NaOH (1 M) if necessary.
4. Perform media exchange in a Seahorse XF24 tissue culture plate:
  - a. Remove 150 µl growth media with a multichannel pipet.
  - b. Add 1 ml assay media with a multichannel pipette.
  - c. Remove 1 ml with a multichannel pipette.
  - d. Add 475 µl assay media with a multichannel pipette (575 µl final volume).
  - e. Place the cell plate into a CO<sub>2</sub>-free incubator for approx. 60 min.
5. Load cartridge with desired compounds:
  - a. Pre-warm compounds to 37 °C.
  - b. Load 50-100 µl of compound into appropriate port of cartridge (for mitochondrial stress test: 64 µl into port A, 71 µl port B, 79 µl port C, 88 µl port D). (see Note 1) Load equivalent amounts of assay media into equivalent port for background wells (see Note 2).
  - c. Place back into the incubator (non-CO<sub>2</sub>) for 10 min to allow heating up to 37 °C again. Handle carefully, carry only by holding onto the calibration plate. Move as less as possible.
6. Calibration and running seahorse assay:
  - a. Load assay template in Seahorse XF24 software.
  - b. Press green 'START' button.
  - c. Make sure to load the correct protocol, the correct save directory and saving name.
  - d. Press 'START'.
  - e. Load sensor cartridge with calibration plate into instrument tray (the notch goes in the front, left corner. Make sure that the plate sits correctly and flat, between all 8 tabs)
  - f. Follow the instructions on the screen in order to calibrate and equilibrate sensors.
  - g. Once equilibration step is done, remove the calibration plate and replace with cell culture plate.

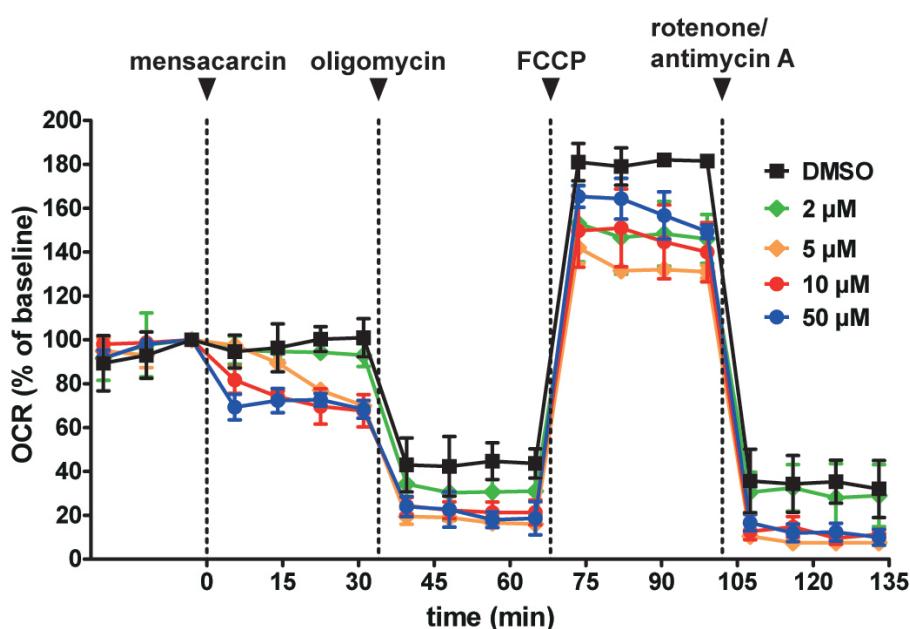
## F. Protocol commands (mitochondria stress test, Table 2, Figure 5)

**Table 2. Protocol commands for mitochondrial stress test**

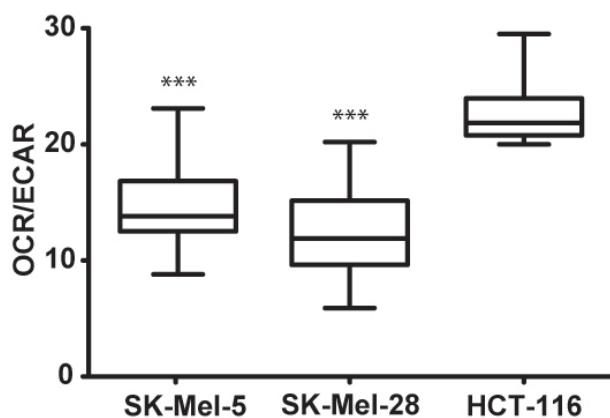
Command	Time (min)	Port
Calibrate		
Equilibrate		
Loop Start	4x	
Mix	3.00	
Wait	2.00	
Measure	3.00	
Loop End		
Inject		A (test compound)
Loop Start	4x	
Mix	3.00	
Wait	2.00	
Measure	3.00	
Loop End		
Inject		B (oligomycin)
Loop Start	4x	
Mix	3.00	
Wait	2.00	
Measure	3.00	
Loop End		
Inject		C (FCCP)
Loop Start	4x	
Mix	3.00	
Wait	2.00	
Measure	3.00	
Loop End		
Inject		D (rotenone and antimycin A)
Loop Start	4x	
Mix	3.00	
Wait	2.00	
Measure	3.00	
Loop End		

**Data analysis**

Results were initially reviewed using the seahorse XF data viewer which automatically saves data as MS Excel (.xls) file. Graphic and statistical analyses were carried out using GraphPad Prism. The significance of observed differences of the basal bioenergetics of cell lines was evaluated by the non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison post hoc test. In all cases,  $P < 0.05$  was considered to be significant. Experimental values are reported as mean  $\pm$  standard deviation (Figure 5) or in a box plot (Figure 6).



**Figure 5. Mitochondrial stress test.** OCR was measured after mensacarin was injected (black arrow) in different concentrations to SK-Mel-28 cells followed by consecutive injections of oligomycin (1  $\mu$ M), FCCP (0.5  $\mu$ M), and antimycin A (0.5  $\mu$ M)/rotenone (0.5  $\mu$ M) ( $n = 3$ ).



**Figure 6. Basal bioenergetic state of SK-Mel-28, SK-Mel-5 and HCT-116 cells.** The basal energy metabolism of each cell line was assessed by analyzing OCR/ECAR ratios. OCR and ECAR were acquired with the same protocol as described above but without the injection of compounds. The protocol commands consisted of one loop with 8 measurements. Several separate assays were performed ( $n = 25$ ).

## Notes

1. Pipet into ports with angle, do not touch the bottom, do not tap to prevent leakage. The liquid is only held by capillary forces.
2. It is mandatory to load ports for the background wells with assay media that contains the same concentration of DMSO as the compounds to account for any DMSO effects on cells.

3. Once injected into the wells, compounds are diluted 1:10. This will give a final concentration of 1  $\mu\text{M}$  oligomycin and 0.5  $\mu\text{M}$  FCCP, rotenone and antimycin A, respectively, in the cell culture well.

## **Recipes**

1. Culture media (10% (v/v) FBS)

*Note: Work under sterile conditions in a laminar flow hood.*

- a. Open liquid DMEM bottle
- b. Take out 55 ml with a sterile Serological pipette and discard the liquid
- c. Add 50 ml FBS with a sterile Serological pipette
- d. Add 5 ml penicillin/streptomycin solution
- e. Store at 4 °C

2. Assay media (sterile, unbuffered, 250 ml)

*Note: Work under sterile conditions in a laminar flow hood.*

- a. Autoclave 250 ml ultrapure H<sub>2</sub>O in a glass bottle
- b. Dissolve 3.34 g powder DMEM without NaHCO<sub>3</sub> and without HEPES in 250 ml autoclaved H<sub>2</sub>O
- c. Warm to 37 °C
- d. Adjust to pH 7.40 with NaOH (1 M)
- e. Store at 4 °C

3. NaOH (1 M)

Dissolve 4 g NaOH pellets in 100 ml autoclaved H<sub>2</sub>O

4. Oligomycin (10  $\mu\text{M}$ )

- a. Prepare freshly on the day of seahorse assay (day 2) (see Note 1)
- b. Prepare 1 mM solution in 1 ml DMSO: Dissolve 0.7911 mg oligomycin in DMSO
- c. Dilute to 10  $\mu\text{M}$  in assay media (1% DMSO): Pipet 20  $\mu\text{l}$  of 1 mM oligomycin into 1,980  $\mu\text{l}$  assay media
- d. Warm to 37 °C and adjust to pH 7.4 with NaOH (1 M) if necessary

5. FCCP (5  $\mu\text{M}$ )

- a. Prepare freshly on the day of seahorse assay (day 2) (see Note 1)
- b. Prepare 50 mM solution in DMSO: Dissolve 2.54 mg FCCP in 200  $\mu\text{l}$  DMSO
- c. Dilute to 500  $\mu\text{M}$ : Pipet 10  $\mu\text{l}$  of 50 mM FCCP into 990  $\mu\text{l}$  DMSO
- d. Dilute to 5  $\mu\text{M}$  in assay media (1% DMSO): Pipet 20  $\mu\text{l}$  of 500  $\mu\text{M}$  FCCP into 1,980  $\mu\text{l}$  assay media
- e. Warm to 37 °C and adjust to pH 7.4 with NaOH (1 M) if necessary

6. Rotenone (5  $\mu\text{M}$ )/antimycin A (5  $\mu\text{M}$ )

- a. Prepare freshly on the day of seahorse assay (day 2) (see Note 1)

- b. Prepare 50 mM solution in DMSO: Solve 3.94 mg rotenone and 5.49 mg antimycin A in 200 µl DMSO
- c. Dilute to 1 mM: Pipet 10 µl of 50 mM rotenone/antimycin A into 490 µl DMSO
- d. Dilute to 5 µM in assay media (0.5% DMSO): Pipet 20 µl of 1 mM rotenone/antimycin A into 1,980 µl assay media
- e. Warm to 37 °C and adjust to pH 7.4 with NaOH (1 M) if necessary

### **Acknowledgments**

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## Isolation of Microvascular Endothelial Cells

Kenneth C.P. Cheung\* and Federica M. Marelli-Berg

William Harvey Research Institute, Barts and The London School of Medicine and Dentistry, Queen Mary University of London, London EC1M 6BQ, United Kingdom

\*For correspondence: [c.cheung@qmul.ac.uk](mailto:c.cheung@qmul.ac.uk)



**[Abstract]** The vascular endothelium is essential to normal vascular homeostasis. Its dysfunction participates in various cardiovascular disorders. Murine endothelial cell culture is an important tool for cardiovascular disease research. This protocol demonstrates a quick, efficient method for the isolation of microvascular endothelial cells from murine tissues without any special equipment. To isolate endothelial cells, the lung or heart were mechanically minced and enzymatically digested with collagenase and trypsin. The single cell suspension obtained was then incubated with an anti-CD31, anti-CD105 antibody and with biotinylated isolectin B-4. The endothelial cells were harvested using magnetic bead separation with rat anti-mouse Ig- and streptavidin-conjugated microbeads. Endothelial cells were expanded and collected for subsequent analyses. The morphological and phenotypic features of these cultures remained stable over 10 passages in culture. There was no overgrowth of contaminating cells of non-endothelial origin at any stage.

**Keywords:** Primary culture, Endothelial cells, Tight junctions, CD31, Pecam1

**[Background]** Microvascular endothelial cells play a central role in the development of immune responses by regulating leukocyte recirculation and as antigen presenting cells to T lymphocytes. The wellbeing of the endothelium is essential to vascular homeostasis. The dysfunctional endothelium participates in various cardiovascular disorders, including atherosclerosis, vasculitis and ischemia/reperfusion injuries (Cid *et al.*, 2004; Wang *et al.*, 2007). Therefore, *in vitro* endothelial cell cultures are important tools for studying vascular physiology and disease pathology. However, the isolation of primary murine endothelial cells is considered particularly difficult because most protocols described have involved the perfusion of organs or large vessels with digesting enzymes and time-consuming purification process (Gumkowski *et al.*, 1987).

The purpose of this protocol is to provide a simple method to isolate and expand endothelial cells from the lung/heart without using any special equipment. Using this method, we previously complemented *in vivo* studies demonstrating the importance of CD31 signaling in endothelial cells cytoprotection (Cheung *et al.*, 2015).

## **Materials and Reagents**

### A. Materials

1. Pipette tips
2. Multiwell plate (cell culture grade) (Greiner Bio One International, catalog number: 662160)
3. 50 ml centrifuge tubes (cell culture grade) (Greiner Bio One International, catalog number: 210261)
4. 10 ml disposable pipette (Greiner Bio One International, catalog number: 607160)
5. Cell strainers (100 µm, Corning, catalog number: 352360; 70 µm, Corning, catalog number: 352350)
6. Scalpel
7. miniMACS separation unit (Miltenyi Biotec, catalog number: 130-042-102)

*Note: Magnetic cell sorting of labeled EC was performed using a miniMACS separation unit (Miltenyi Biotec, Bisley, Surrey, UK) including two magnets. Labeled cells were incubated with MACS magnetic goat anti-rat IgG (H+L) (Miltenyi Biotec) MicroBeads and streptavidin (Miltenyi Biotec) MicroBeads and then separated using a high gradient magnetic separation column (MS<sup>+</sup> columns, Miltenyi Biotec) placed on the separation unit, according to the manufacturer's instructions.*

8. High gradient magnetic separation column (MS<sup>+</sup> columns) (Miltenyi Biotec, catalog number: 130-042-201)

### B. Animals

Mice (Balb/c, age 6 weeks up to 1 year from Charles River, UK or the in-house breeding facility)

### C. Reagents

1. Ice
2. Isoflurane
3. Phosphate buffered saline solution (PBS, Gibco)
4. Collagenase type II (Thermo Fisher Scientific, Gibco<sup>TM</sup>, catalog number: 17101015)
5. EC media
6. DNasel solution
7. 0.125% trypsin in 0.2% EDTA (Life Technologies)
8. Dako mounting media (Dako)
9. MicroBeads and streptavidin (Miltenyi Biotec, catalog number: 130-048-101)
10. Antibodies

- a. Biotinylated isolectin B4 (purchased from Vector Laboratories, Peterborough, UK)

*Note: The anti-CD40 mAb 3/23 (rat IgG2a) (Van Den Berg et al., 1996) was a kind gift from Dr. G. Klaus (National Institute for Medical Research, London, UK).*

- b. Rat IgG2a (clone R35-95, BD, Pharmingen<sup>TM</sup>, catalog number: 553927)

- c. Hamster IgGs (BD, CompBead™, catalog number: 552845)
- d. Mouse IgG1 (TdT Cocktail Control, Harlan Sera-Lab, Oxon, UK, Thermo Fisher Scientific, catalog number: 31903)

*Note: The above mAbs 16b and 16c were used as isotype-matched control antibodies in staining experiments: rat IgG2a (clone R35-95); hamster IgGs. To block Fc receptors, mouse IgG2a and mouse IgG1 (TdT Cocktail Control, Harlan Sera-Lab, Oxon, UK) were used.*

- e. MACS magnetic goat anti-rat IgG (H+L) (Miltenyi Biotec, catalog number: 130-048-501)
- f. Rat IgG<sub>2b</sub> anti-mouse CD16/CD32 monoclonal antibody (BD, catalog number: 553141)
- g. Secondary antibody conjugated rhodamine red-X (Molecular Probes)

## 11. FACS

*Note: The following antibodies were purchased from Pharmingen (La Jolla, CA).*

- a. CD31 (PECAM-1, clone MEC 13.3, rat IgG2a, k) (BD, Pharmingen™, catalog number: 550274)
- b. CD105 (Endoglin, clone MJ7/18, rat IgG2a) (BD, Pharmingen™, catalog number: 550546)

## 12. Immuno Fluorescence Staining

PECAM-1(MEC 13.3) (Santa Cruz Biotechnology, catalog number: sc-18916)

13. Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher Scientific, Gibco™, catalog number: 41966-052)

14. Glutamine (Thermo Fisher Scientific, Gibco™, catalog number: 25030)

15. 10,000 U/ml Penicillin-Streptomycin (Thermo Fisher Scientific, Gibco™, catalog number: 15140122)

16. Sodium pyruvate (Thermo Fisher Scientific, Gibco™, catalog number: 11360039)

17. HEPES (Thermo Fisher Scientific, Gibco™, catalog number: 15630056)

18. 1% non-essential amino acids (Thermo Fisher Scientific, Gibco™, catalog number: 11140050)

19. 2-mercaptoethanol (Thermo Fisher Scientific, Gibco™, catalog number: 31350010)

20. Heat-inactivated fetal calf serum (FCS; Globepharm, Esher, UK)

21. EC growth supplement (Sigma-Aldrich, catalog number: E0760)

22. 2% gelatin (type B from bovine skin, Sigma-Aldrich, catalog number: G7765) coated tissue culture flasks (Nunc, Life Technologies, Paisley, UK)

23. Working medium (see Recipes)

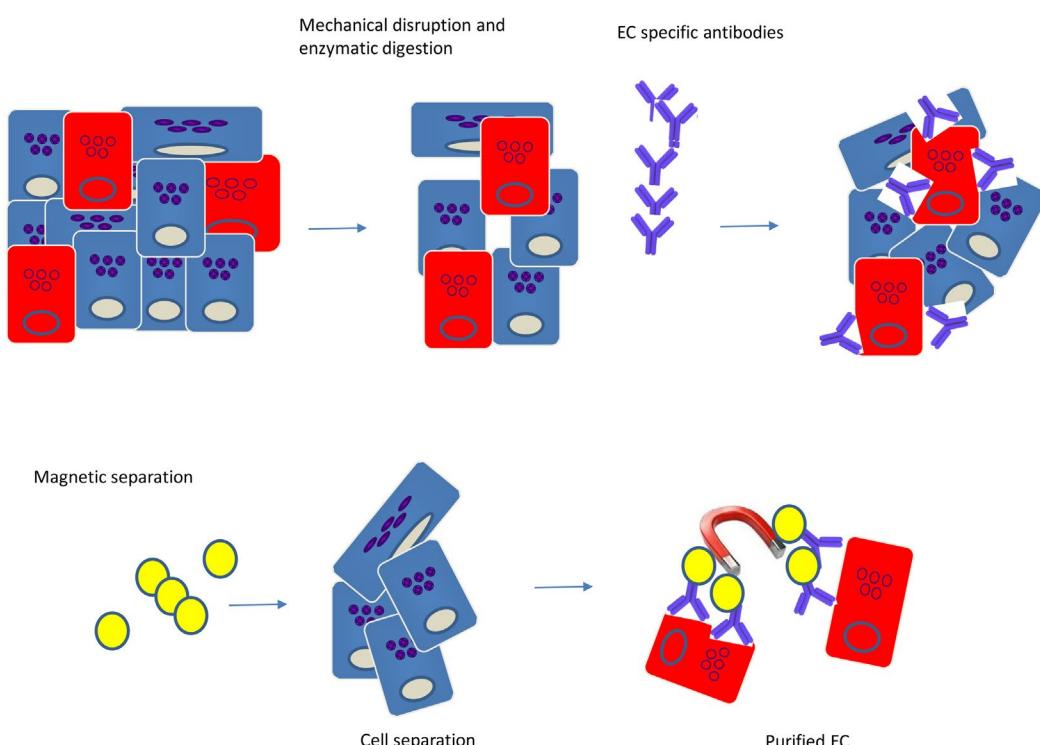
## Equipment

1. Pipettes
2. Sterile beakers 100-150 ml (sterilize at 180 °C)
3. Laminar flow work bench
4. Tweezers (sterilize at 180 °C)
5. Scissors (sterilize at 180 °C)
6. Shaker

7. Water bath
8. Centrifuge (Hettich Instruments, model: UNIVERSAL 320 R)
9. Fixed-angle rotor (Hettich Instruments, catalog number: 1620A)
10. EPICS Profile Cytometer (Coulter Electronics, Luton, UK)
11. Fluorescence microscopy (Zeiss epi-fluorescent microscope)

## Procedure

*Note: For the EC purification described, tissue from no more than one to two animals is required (see Figure 1 for diagram).*



**Figure 1. Isolation of endothelial cells (ECs) from tissues by immunomagnetic separation.** Tissues consist of diverse cell types and matrix components, and in general, the endothelial content constitutes 1-2% of the total tissue mass. Tissues are mechanically disrupted and enzymatically digested to create a single-cell suspension. Endothelial-specific Abs are added to the single-cell suspension to label the EC. ECs are separated from the other components by using magnetic beads, resulting in highly pure population of ECs.

1. Euthanize mice according to your local animal regulations. We use an overdose of isoflurane, which leads to breathing arrest within one minute.
2. Working as steriley as possible remove murine organs such as heart, lung, and liver using autoclaved instruments and rinse in PBS to remove blood.
3. In a Petri dish, using sterile crossed scalpels, dissect tissue into 2-mm<sup>3</sup> blocks.

4. Wash twice in PBS by low-speed centrifugation ( $210 \times g$ , 1 min).
5. Incubate diced tissue in a solution of collagenase (0.5 mg/ml) for 1 h at  $37^{\circ}\text{C}$  in a humid incubator.

*Note: We use Gibco® Collagenase Type II because compared to other collagenase preparations it has a higher clostripain activity and is well-suited for the digestion of heart, bone, thyroid, cartilage, and liver tissues. N.B. it is isolated from Clostridium histolyticum and packaged as a lyophilized, non-sterile powder for research use in cell or tissue dissociation and organ perfusions. Gibco® Collagenase Type II activity is guaranteed to be greater than 125 units/mg.*

6. Subsequently, add 75  $\mu\text{l}$  DNase I solution per 10 ml cell suspension and incubate for another 30 min in a  $37^{\circ}\text{C}$  water bath with continuous agitation.
7. Pass digested tissue through a cell strainer to remove undigested blocks.
8. Rinse the cell strainer twice with PBS supplemented with 2.5% FCS to collect any remaining cell.
9. Incubate for a further 10 min in 0.25% trypsin (1 ml of trypsin for every 100 mg of tissue) to obtain single cell suspension.
10. Wash once in 500  $\mu\text{l}$  PBS supplemented with 2.5% FCS.
11. Incubate for 30 min at  $4^{\circ}\text{C}$  with murine immunoglobulins to block Fc receptors.

*Note: Mouse BD Fc Block is a purified rat IgG<sub>2b</sub> anti-mouse CD16/CD32 monoclonal antibody.*

12. Wash twice in cold PBS supplemented with 2.5% FCS.
13. Incubate for 45 min at  $4^{\circ}\text{C}$  with rat anti-mouse CD31, rat anti-mouse CD105 and biotinylated isolectin B4.
14. Wash twice in cold 500  $\mu\text{l}$  PBS supplemented with 0.5% FCS and count cells.
15. Resuspend pellet and incubate with PBS 0.5% FCS (200  $\mu\text{l}/\text{L}$ ,  $2.5 \times 10^7$  cells), rat anti-mouse Ig (25  $\mu\text{l}/\text{L}$ ,  $2.5 \times 10^7$  cells)- and streptavidin-conjugated microbeads (25  $\mu\text{l}/\text{L}$ ,  $2.5 \times 10^7$  cells) for 15 min at  $4^{\circ}\text{C}$  (total volume 250  $\mu\text{l}$ ). Meanwhile, load columns onto the separation unit (use one column every  $1-2.5 \times 10^7$  cells) and wash each column with 500  $\mu\text{l}$  PBS 0.5% FCS as per manufacturer's instructions.
16. Load each column with 250  $\mu\text{l}$  cell suspension. The magnetically labeled cells are retained in the column(s) while non-labeled cells pass through. After the cell suspension has flowed through the column, wash the column twice with 500  $\mu\text{l}$  PBS 0.5% FCS. For detailed procedure, please refer to [the video](#) on the manufacturer's website.
17. Unload the column(s) from the magnet and elute the magnetically retained cells with PBS 0.5% FCS using the plunger provided.
18. Wash the eluted cells and centrifuge at  $200 \times g$  for 5 min. Resuspend in EC medium ( $10^5$  cells/ml) and plate out in dishes/plates (Table 1) pre-coating with gelatin. The phenotype and morphology of these cultures remain stable over 10-15 passages in culture, and no overgrowth of contaminating cells of non-endothelial origin is observed at any stage. You can get about 80% of yield.

*Note: Coat P100 dishes with gelatin, let sit in an incubator(37 °C) for at least 30 min, wash in PBS and leave to dry.*

**Table 1. Passaging Seeding Density and Volume**

	Surface area (cm <sup>2</sup> )	Seeding density	Cells at confluence	Growth medium (ml)
6-well	9	0.3 x 10 <sup>6</sup>	1.2 x 10 <sup>6</sup>	3-5
T-25	25	0.7 x 10 <sup>6</sup>	2.8 x 10 <sup>6</sup>	3-5

19. After overnight culture on 5% CO<sub>2</sub>, remove non-adherent cells and replace medium with 75 µg/ml EC growth supplement.
20. At confluence, detach EC with either trypsin-EDTA or cell dissociation solution and characterize.

*Note: To preserve the features of EC physiology and gene expression, the tissue specimen should preferably be processed immediately after resection. It is advisable to have ready-to-use aliquots of the work solutions in storage.*

### Data analysis

#### A. FACS analysis

ECs were detached from the culture flasks with trypsin/EDTA (Life Technologies), washed and resuspended in phosphate buffered saline solution (PBS, Gibco) containing 1% FCS (Globepharm). Cells (10<sup>6</sup> cells/ml) were then incubated with the indicated monoclonal antibody for 30 min at 4 °C. Cells were then washed twice in cold PBS with 1% FCS and incubated for a further 30 min at 4 °C with the appropriate FITC conjugated secondary antibody. After two additional washes, the cells were analyzed using an EPICS Profile Cytometer (Coulter Electronics, Luton, UK).

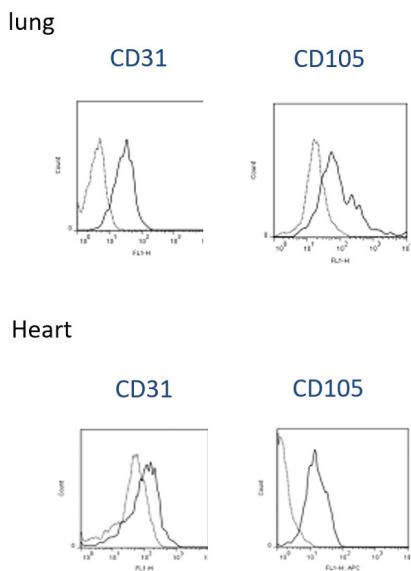
#### B. Immunocytochemistry

Primary antibody diluted in 3% bovine serum albumin was applied overnight at 4 °C, followed by incubation with a secondary antibody conjugated rhodamine red-X (Molecular Probes) for 1 h at room temperature. Nuclei were labeled with Hoechst 33258 diluted in 3% bovine serum albumin for 15 min at room temperature. All cells were mounted using Dako mounting media (Dako), and fluorescence images were captured using fluorescence microscopy (Zeiss epi-fluorescent microscope).

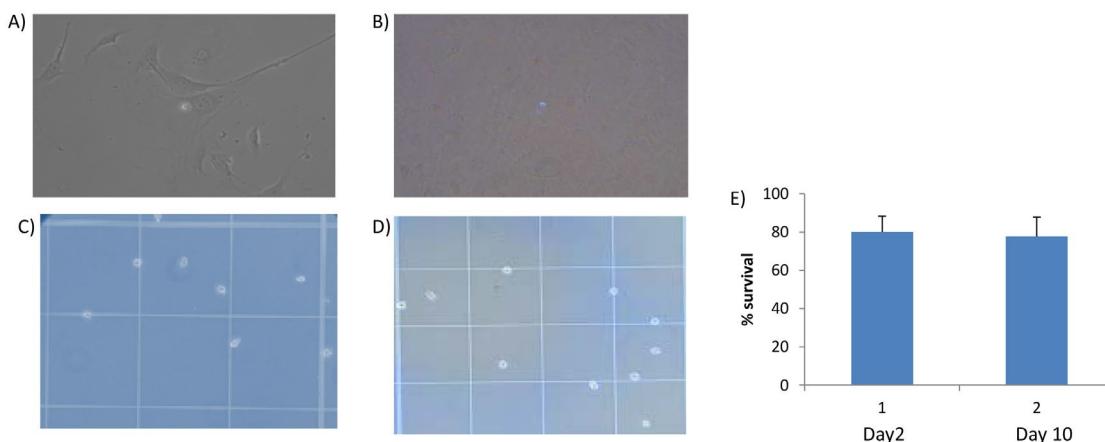
#### C. Results

1. The surface molecule expression pattern of primary cultures EC express CD31, and CD105 was determined by flow cytometry. As demonstrated, isolated ECs show high-level expression of CD31 and CD105 in both heart and lung after isolation (Figure 2). In addition, murine EC grew in

several clusters formed monolayers and demonstrated spindle-shaped and cobblestone-like appearances after ten days as demonstrate under bright field microscopy Figures 3A and 3B. We further investigated the availability of these cells using dye exclusion trypan assay and found that the viability remains high, reaches over 80% after the second passage as shown in Figures 3C and 3D. Figure 3E shows statistical analysis of percentage cell survival in day 2 and day 10 EC seeding.

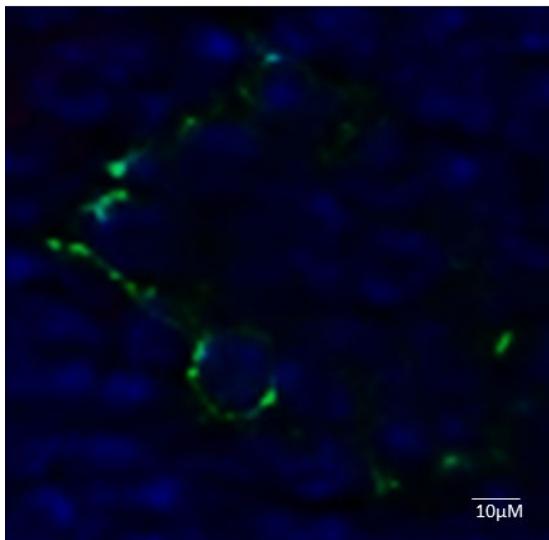


**Figure 2. Phenotypic characterization of EC purified from murine lung and heart.** EC isolates from murine lung (passage 1) were stained with the antibodies specific for the surface molecules indicated within each graph. Isotype-matched irrelevant antibodies were used as a control.



**Figure 3. Bright field images of primary murine lung and heart endothelial cell after isolation.** A and B. Isolated ECs were subjected to light microscopy analysis for morphology day 2 (A) and day 7 (B). C, D and E. Trypan blue counting indicates 80% of the cells are surviving on day 2 and 10.

2. In this protocol, the cultured cells retain their morphological and functional key characteristics of *in vivo* ECs. We were also able to show the immunofluorescent staining positive cells for CD31 (Figure 4).



**Figure 4. Immunofluorescent characterizations of isolated endothelial cells (ECs).** Cells were spun onto microscope slides and subjected to immunofluorescence staining against the indicated antigens. PECAM-1 (MEC 13.3) (green) with 4,6-diamidino-2-phenylindole (DAPI) (blue).

3. The significance applications of primary cultured mouse endothelial cells  
The protocol provides a great opportunity to study the endothelial-specific activities of targeted molecules. The ability to yield high numbers of mouse ECs makes it very useful in cardiovascular research. This method also reduces cost and improves the potential of studying EC-based therapy in murine models through engraftment of endothelial cells.

#### D. Conclusion

Here we follow this simple and quick method to generate primary EC lines. This can be used for multiple passages for vascular research.

#### E. Statistical analysis

Results are expressed as mean  $\pm$  SD or SEM, as indicated. The Student's *t*-test and ANOVA were used. All reported *P* values are two-sided. A *P*-value of less than 0.05 was regarded as significant.

## Recipes

### 1. EC medium

Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Paisley, Scotland)  
2 mM glutamine (Gibco)  
100 U/ml penicillin (Gibco)  
100 µg/ml streptomycin (Gibco)  
1 mM sodium pyruvate (Gibco)  
20 mM HEPES (Gibco)  
1% non-essential amino acids (Gibco)  
50 mM 2-mercaptoethanol (Gibco)  
Freshly added 20% heat-inactivated foetal calf serum (FCS; Globepharm, Esher, UK)  
75 µg/ml EC growth supplement (Sigma, Poole, UK)  
Prepared in 2% gelatin (type B from bovine skin, Sigma) coated tissue culture flasks (Nunc, Life Technologies, Paisley, UK)

## Acknowledgments

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## An *in vitro* Co-culture System for the Activation of CD40 by Membrane-presented CD40 Ligand *versus* Soluble Agonist

Khalidah Ibraheem<sup>1</sup>, Christopher J. Dunnill<sup>1</sup>, Myria Ioannou<sup>1</sup>, Albasir Mohamed<sup>1, \$</sup>,  
Balid Albarbar<sup>1</sup> and Nikolaos T. Georgopoulos<sup>1, \*</sup>

<sup>1</sup>Department of Biological Sciences, School of Applied Sciences, University of Huddersfield, Huddersfield, United Kingdom; <sup>\$</sup>Current address: Department of Medical Laboratory Sciences, Sebha University, Tripoli, Libya.

\*For correspondence: [N.Georgopoulos@hud.ac.uk](mailto:N.Georgopoulos@hud.ac.uk)



**[Abstract]** One fundamental property of the TNF receptor (TNFR) family relates to how ‘signal quality’ (the extent of receptor ligation or cross-linking) influences the outcome of receptor ligation, for instance the induction of death in tumour cells. It is unequivocal that membrane-presented ligand (delivered to target cells via cell-surface presentation by co-culture with ligand-expressing third-party cells) induces a greater extent of carcinoma cell death *in vitro* in comparison to non-cross-linked agonists (agonistic antibodies and/or recombinant ligands). The CD40 receptor epitomises this fundamental property of TNF receptor-ligand interactions, as the extent of CD40 cross-linking dictates cell fate. Membrane-presented CD40 ligand (mCD40L), but not soluble agonists (e.g., agonistic anti-CD40 antibody), induces high level of pro-inflammatory cytokine secretion and causes extensive cell death (apoptosis) in malignant (but not normal) epithelial cells. In this article, we describe a co-culture system for the activation of CD40 by mCD40L and subsequent detection of various features of apoptosis (including cell membrane permeabilisation, DNA fragmentation, caspase activation) as well as detection of intracellular mediators of cell death (including adaptor proteins, pro-apoptotic kinases and reactive oxygen species, ROS).

**Keywords:** TNF receptors (TNFRs), CD40, Receptor ligation, Membrane-presented ligand, Soluble agonist, Co-culture, *In vitro*, Cell death, Apoptosis, Caspase activation, DNA fragmentation, Immunoblotting

**[Background]** The role of the TNFRs and their ligands in regulating cell proliferation or death in lymphoid tissues as well as in epithelial (and particularly carcinoma) cells has been under extensive research, as their ability to induce cell death (mainly via apoptosis) represents a promising target for cancer therapy. Importantly, however, there is a clear difference in the ability of TNFR agonists to trigger cell death when presented in soluble *versus* membrane-bound form. Soluble agonists often demonstrate relatively low cytotoxic potency when administrated as a sole treatment, whereas membrane-presented ligands appear to be superior (Albarbar *et al.*, 2015).

In this context, CD40 represents the most prominent TNFR family member. The receptor is expressed on a variety of epithelial cells and the effect of CD40 activation is exquisitely contextual (Young and Eliopoulos, 2004). Most importantly, the ability of CD40 to induce cytostasis or cell death

(apoptosis) is highly dependent on the ‘quality’ of receptor engagement (degree of receptor cross-linking). Soluble CD40 agonists (recombinant soluble CD40L or agonistic antibody) are only cytostatic or weakly pro-apoptotic and only rendered pro-apoptotic by pharmacological intervention (Bugajska *et al.*, 2002). By contrast, membrane-presented CD40L (mCD40L) is highly pro-apoptotic and induces extensive apoptosis in carcinoma cells, when presented to target carcinoma cells on the surface of third-party cells (Georgopoulos *et al.*, 2006 and 2007) or by mCD40L-expressing, naturally-activated immunocytes (Hill *et al.*, 2008).

The ability of mCD40L (but not soluble agonists) to efficiently kill malignant cells, and in a tumour cell-specific fashion, reflects the two most remarkable properties of the CD40-mCD40L dyad and our recent studies have deciphered these two fundamental properties of CD40. We utilised a co-culture system that involved culture of target, carcinoma (or normal) cells with growth-arrested, third-party, effector cells engineered to express the CD40L on their surface, in order to achieve presentation of mCD40L. This allowed us to study the ability of mCD40L to induce a number of different morphological and biochemical features of apoptosis, as well as define the intracellular mediators of cell death (Dunnill *et al.*, 2017). Here, we provide a detailed protocol for the preparation of the co-culture system for mCD40L delivery to epithelial target cells (in comparison to soluble agonist, *i.e.*, agonistic anti-CD40 antibody) and methodologies to assess mCD40L-induced apoptosis and detection of its intracellular mediators.

## **Materials and Reagents**

### **A. Materials**

1. T75 tissue culture flasks with vent (SARSTEDT, catalog number: 83.1813.002)
2. T25 tissue culture flasks with vent (SARSTEDT, catalog number: 83.1810.002)
3. 96 well Nunc, white, flat bottom tissue culture multi-well plates (Thermo Fisher Scientific, catalog number: 136101)
4. 96 well, flat bottom, Costar transparent tissue culture plates (Corning, catalog number: 3595)
5. 96 well ELISA microplates (Greiner Bio One International, catalog number: 655101)
6. 24 well plates (Corning, catalog number: 3526)
7. 6 well plates (Corning, catalog number: 3516)
8. Tissue culture dishes, 10 cm diameter, Nunclon with lid (Thermo Fisher Scientific, catalog number: 150350)
9. Bijou tubes 5 ml sterile (x2,000) (SARSTEDT, catalog number: 60.9921.532)
10. Cryopure tubes, 2.0 ml white cap (SARSTEDT, catalog number: 72.380)
11. 5 ml serological pipettes (SARSTEDT, catalog number: 86.1253.001)
12. 10 ml serological pipette (SARSTEDT, catalog number: 86.1254.001)
13. 25 ml serological pipettes (SARSTEDT, catalog number: 86.1685.001)
14. 120 ml sterile container graduated (x250) (SARSTEDT, catalog number: 75.9922.420)
15. 250 ml Sterilin™ containers (Thermo Fisher Scientific, catalog number: 190A)

16. 30 ml Sterilin™ universals (Thermo Fisher Scientific, catalog number: 128AFS)
17. 2 ml aspiration pipette individually wrapped sterile Non Pyrogenic (SARSTEDT, catalog number: 86.1252.011)
18. 5.0 ml TipOne® Repeat Dispenser Tip (Sterile) (STARLAB, catalog number: S4761-0500)
19. 1.25 ml TipOne Repeat Dispenser Tip (Sterile), Ind. Wrapped (STARLAB, catalog number: S4786-0125)
20. Cell scrapers (Fisher Scientific, catalog number: FB55199)
21. 20 ml disposable sterile syringe (BD, catalog number: 300296)
22. 10 ml disposable sterile syringe (BD, catalog number: 302188)
23. 1.5 ml tubes (SARSTEDT, catalog number: 72.690.001)
24. 0.5 ml tubes (5,000) (SARSTEDT, catalog number: 72.699)
25. 0.2 µm syringe filter sterile (SARSTEDT, catalog number: 83.1826.102)
26. Syringe filter 0.4 µm (x50) (GE Healthcare, Whatman, catalog number: 6896-2504)
27. Haemocytometer (Fisher Scientific, catalog number: MNK-420-010N)
28. Haemocytometer cover slips (Fisher Scientific, catalog number: MNK-504-030M)
29. X2500 Countess Chamber Slides (2,500) (Thermo Fisher Scientific, catalog number: C10314)
30. Blue loose pipette tips (1,000 µl) (SARSTEDT, catalog number: 70.762)
31. 200 µl yellow pipette tips (SARSTEDT, catalog number: 70.760.002)
32. Neutral pipette tips (10 µl) (SARSTEDT, catalog number: 70.1130)

## B. Cells

1. Human bladder carcinoma-derived EJ cells and colorectal carcinoma-derived HCT116 cells (obtained from the ATCC)  
*Note: They were cultured in a 1:1 (v/v) mixture of DMEM and RPMI 1640 containing 5% FBS, referred to as 'DR/5%' medium.*
2. Normal human urothelial (NHU) cells  
*Note: They were established and cultured in complete KSF as described (Bugajska et al., 2002).*
3. 3T3neo and 3T3CD40L fibroblasts – stably transfected NIH3T3 derivatives generated as previously described (Bugajska et al., 2002)  
*Note: They were cultured in DMEM supplemented with 10% FBS and containing 0.5 mg/ml G418, with omission of antibiotic in co-culture experiments (Georgopoulos et al., 2006).*

## C. Reagents

1. Purified water (double distilled ddH<sub>2</sub>O)
2. D-PBS, 10x, no calcium, no magnesium (Thermo Fisher Scientific, catalog number: 14200067)
3. Mikrozid AF liquid, 10 L canister (LAVABIS, catalog number: SF000301)
4. G418, 100 mg/ml solution (InvivoGen, supplied by Source BioScience LifeSciences, catalog number: ant-gn-1)

5. Keratinocyte Serum Free Medium (KSF) and supplements (Thermo Fisher Scientific, Gibco™, catalog number: 17005075)
6. RPMI-1640 Medium, with sodium bicarbonate, without L-glutamine (Sigma-Aldrich, catalog number: R0883-6X500ML)
7. Dulbecco's Modified Eagle's Medium (DMEM) (high glucose) with sodium bicarbonate, without L-glutamine (Sigma-Aldrich, catalog number: D6546-6X500ML)
8. Hanks Balanced Salt Solution (HBSS) (Sigma-Aldrich, catalog number: H9394-6X500ML)
9. L-Glutamine solution (Sigma-Aldrich, catalog number: G7513-100ML)
10. Fetal bovine serum (FBS) (qualified fetal bovine serum, 500 ml) (Sigma-Aldrich, catalog number: F7524-500ML)
11. Ethylenediaminetetraacetic acid (EDTA) (Santa Cruz Biotechnology, catalog number: sc-29092)
12. Trypsin-EDTA (Sigma-Aldrich, catalog number: T4174)
13. Dimethyl Sulphoxide (DMSO) (Sigma-Aldrich, catalog number: D2650-100ML)
14. Mr. Frosty™ Freezing Container (Thermo Fisher Scientific, catalog number: 5100-0001)
15. Isopropanol (Fisher Scientific, catalog number: A415-4)
16. MycoProbe™ Mycoplasma detection assay (R&D Systems, catalog number: CUL001B)
17. Mitomycin C (10 mg) (Santa Cruz Biotechnology, catalog number: sc-3514B)
18. CK18 (cytokeratin 18) monoclonal antibody clone CY-90 (Sigma-Aldrich, catalog number: C8541-.2ML)
19. Goat anti Rabbit IgG IRDYE800 secondary antibody (tebu-bio, catalog number: 039611-132-122)
20. Goat anti-Mouse IgG Alexa Fluor® 680 secondary antibody (Thermo Fisher Scientific, Invitrogen™, catalog number: A-21057)
21. TRAF-3 antibody (Santa Cruz Biotechnology, catalog number: sc-949)
22. Phospho-ASK1 (Thr845) antibody (Cell Signalling Technology, catalog number: 3765)
23. Agonistic anti-CD40 mAb G28-5 (used at 10 µg/ml), purified from culture supernatants of the HB-9110 hybridoma line (purchased from the ATCC)
24. Affinity-purified human serum protein-adsorbed goat anti-mouse IgG (X-linker) (used at 5 µg/ml) (Sigma-Aldrich, catalog number: M8645)
25. Staurosporine from *Streptomyces* sp. (Sigma-Aldrich, catalog number: S4400-.1MG)
26. Docetaxel (Sigma-Aldrich, catalog number: 01885-5MG-F)
27. CellTiter 96® AQ<sub>ueous</sub> One Solution Assay (5,000 assays) (Promega, catalog number: G3581)
28. Cellular DNA Fragmentation ELISA kit (for up to 500 tests) (Roche Diagnostics, catalog number: 11585045001)
29. Cytotox-Glo™ cytotoxicity assay (5 x 10 ml) (Promega, catalog number: G9291)
30. Sensolyte® Homogeneous AFC Caspase-3/7 Assay kit (Cambridge Bioscience, catalog number: ANA71114)

31. CM-H2DCFDA (chloromethyl derivative of 5-(and-6)-chloromethyl-2,7 -dichlorodihydrofluorescein diacetate) (Thermo Fisher Scientific, catalog number: C6827)
32. Glycerol (Fisher Scientific, catalog number: 10795711)
33. Sodium dodecyl sulphate (SDS) (Thermo Fisher Scientific, Invitrogen™, catalog number: NP0002)
34. Tris-HCl, powder, 1 KG (Melford, catalog number: T1513)
35. Sodium fluoride (Acros Organics, catalog number: 424325000)
36. Sodium pyrophosphate tetrabasic (Sigma-Aldrich, catalog number: P8010-500G)
37. Sodium orthovanadate (Sigma-Aldrich, catalog number: S6508-10G)
38. Protease inhibitor (PI) cocktail (Merck, catalog number: 535140-1)
39. PI cocktail (New England Biolabs, catalog number: 5872S)
40. AQUAGUARD-1 solution for disinfection of water baths and CO<sub>2</sub> incubators (Biological Industries, catalog number: 01-867-1B) (see Recipes)
41. 1x PBS (see Recipes)
42. PBS/EDTA solution (see Recipes)
43. Freezing medium (see Recipes)
44. FACS buffer (see Recipes)
45. 70% ethanol (see Recipes)—Ethanol, Absolute (Fisher Scientific, catalogue number: E/0650DF/P17)
46. DR medium (see Recipes)
47. SSB buffer (see Recipes)
48. Lysis buffer (see Recipes)

## Equipment

1. Gilson Pipettes
  - P1000 (200-1,000 µl)
  - P200 (50-200 µl)
  - P20 (2-20 µl)
  - P10 (1-10 µl)
2. Gilson REPETMAN electronic pipette 0.1-50 ml (Gilson, catalog number: F164503)
3. Water bath (Memmert)
4. Boiling water bath (Grant)
5. -80 °C freezer
6. Sparkfree Refrigerator and Freezer (Labcold)
7. Electrophoresis Power Supply
8. Refrigerated centrifuge (PRISM R)
9. Universal 320 benchtop centrifuge (Hettich Zentrifugen)

10. Vortex mixer
11. Ultrasonic Homogenizer Sonicator
12. NuAire CellGard ES Biological Safety Cabinet (TripleRed)
13. Iso Class 5 Nuaire Autoflow IR direct heat CO<sub>2</sub> incubator with a HEPA filtration system at 37 °C and 5% CO<sub>2</sub> (TripleRed)
14. Odyssey™ Infra-red Imaging system (Li-Cor)
15. Guava EasyCyte™ Flow Cytometer (Millipore)
16. Countess II Automated Cell Counter (Thermo Fisher Scientific, catalog number: AMQAX1000)
17. EVOS™ XL Core Imaging System (Fisher Scientific)
18. FLUOstar OPTIMA (BMG Labtech)

### **Software**

1. MARS software (BMG Labtech), Version 2.0
2. Guava EasyCyte flow cytometry software (Millipore), Guavasoft Version 2.6
3. Image Studio Life, Version 4.0
4. Adobe Photoshop CS, Version 8.0

### **Procedure**

#### A. General cell culture

1. Cell maintenance and passage
  - a. Cells were routinely maintained at 37 °C in a 5% CO<sub>2</sub> humidified atmosphere provided by autoclaved ddH<sub>2</sub>O, supplemented with 1% AQUAGUARD-1 (see Recipes). All class II cabinet surfaces as well as CO<sub>2</sub> incubators were routinely disinfected with either Mikrozid (weekly) or 70% ethanol (daily) solution.
  - b. Cultured cells were routinely observed by phase contrast microscopy. All cell lines were maintained in T75 flasks with 12-14 ml medium or T25 flasks in 4-5 ml medium. All cell lines were sub-cultured every 2-3 days or when ~80-95% confluent. Epithelial cells were routinely passaged at 1:10 ratios and effector (3T3Neo and 3T3CD40L) cells at 1:6 ratios.
  - c. For routine passaging, cells were collected by washing with a solution of 0.1% (w/v) EDTA in phosphate buffered saline (EDTA/PBS) (see Recipes) for 5 min and then addition of Trypsin-EDTA solution, until cells detached from the culture flasks. Trypsin was inactivated by the addition of the respective serum-supplemented culture medium when cells were re-suspended, followed by centrifugation to aspirate medium and resuspension in fresh culture medium.
  - d. Cultured cells were medium-changed every two days, unless otherwise stated. 3T3 cells were harvested and passaged as with carcinoma cells with the exemption of a very short EDTA/PBS treatment, as extended periods risked rapid cell detachment.

## 2. Cryopreservation and recovery

- a. For long-term storage, following growth and appropriate passage, cell ‘banks’ and ‘sub-banks’ were cryo-preserved in liquid nitrogen as described below.
- b. For cryopreservation of cell lines, cultures were collected by routine passaging and precipitated by centrifugation. The cell pellet was re-suspended in Freezing medium (see Recipes) at a cell density not less than  $1 \times 10^6$  cells/ml.
- c. Cells were aliquoted in a total of 1-1.5 ml to polypropylene cryovials and then transferred to an ice-cold ‘Mr Frosty freezing container’ containing 250 ml of isopropanol to control the cooling rate to 1 °C per minute. Cells were then placed within a -80 °C freezer for 4-6 h prior to transfer to liquid nitrogen.
- d. Cells were recovered by thawing rapidly at 37 °C, before 5-10 ml of pre-warmed growth medium was added. Cells were centrifuged at  $210 \times g$  for 5 min and then seeded to tissue culture flasks as required (T25 flasks for routine maintenance and T75 flasks for preparation of co-cultures).

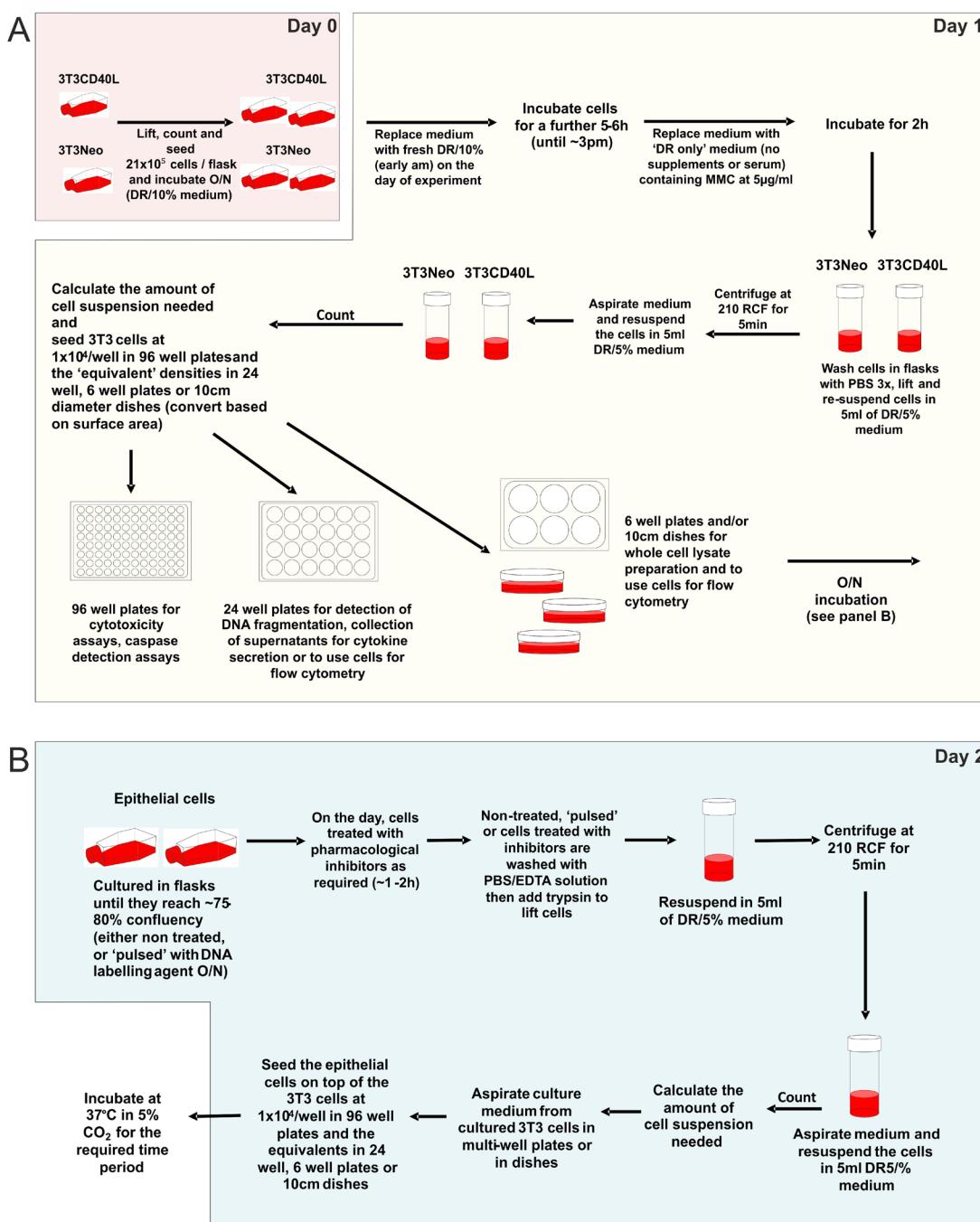
### Notes:

- i. *We recommend that experiments be carried out for cultures maintained for a maximum of 10-15 passages. To avoid any artefacts due to possible culture-associated genetic drift, new cell cultures should be established following that number of passages. The usage of cell ‘banks’ and ‘sub-banks’ allows all experimentation to be performed with consistent results.*
- ii. *For cell lines maintained in culture medium supplemented with FBS, the serum batch used was previously tested ('batch-testing') to ensure it was optimal for cell growth. Cell growth/proliferation was tested using the CellTiter 96® AQ<sub>ueous</sub> One Solution Assay.*

## 3. Other

All cell lines were routinely tested to confirm absence of Mycoplasma contamination using the MycoProbe™ Mycoplasma detection assay (see Reagents).

## B. Co-culture of effector (3T3Neo and 3T3CD40L) and target (epithelial) cells (Figure 1)



**Figure 1. Step-by-step guide for the preparation of co-cultures for the delivery of mCD40L to target epithelial cells.** Effector (3T3) cells are prepared for co-cultures ('Day 0' and 'Day 1') as detailed (A) and seeded in appropriate multi-well formats (dependent on the readout/assay). Thereafter, target epithelial cells are prepared ('Day 2') and seeded on top of the effector cells (B) to set up the co-cultures. Following completion of the procedure, the co-cultures are incubated for the time period required in order to detect biochemical markers of apoptosis or intracellular signaling mediators of cell death (as indicated in A).

1. Preparation of effector and target cells ('Day 0')
  - a. The day before establishing the co-cultures, the effector cells (3T3Neo and 3T3CD40L) should be in exponential growth phase and no more than 85% confluent.
  - b. Passage 3T3Neo and 3T3CD40L effector cells and seed in T75 flasks at a density of 21 x 10<sup>5</sup> cells per flask and incubate overnight (O/N).
  - c. In parallel ensure that target cells (epithelial cells, e.g., HCT116 and EJ) are passaged appropriately in order to ensure they are in exponential growth phase (the 'splitting' ratio depends on the growth characteristics of the cell line).
  - d. The following day, i.e., the day when co-culture experiments are initiated ('Day 1' – see below), replenish the culture medium for all cultures (i.e., medium-change 3T3s and target epithelial cells).

*Note: Ensure this takes place first thing am (particularly for 3T3 cells, before the next step is performed).*
2. Growth-arresting of effector 3T3 cells by Mitomycin C (MMC) treatment ('Day 1')
  - a. Approximately 5-6 h (i.e., mid-afternoon) after the effector 3T3 cells have been medium-changed, remove the medium from 3T3Neo and 3T3CD40L flasks and add 9 ml per T75 flask of DR only medium (without supplements) containing 5 µg/ml of Mitomycin C (MMC), and incubate for 2 h at 37 °C. Figure 2A shows representative cultures of 3T3 cells before and after MMC treatment.
  - b. Aspirate the medium and wash the cells with PBS 3-times to remove any traces of MMC. Add 0.8 ml of trypsin-EDTA to each flask and incubate for 1-2 min until cells have detached, then inactivate with complete culture medium. Centrifuge at 210 x g for 5 min, aspirate the supernatant and resuspend in complete culture medium.
  - c. Count cells (3T3Neo and 3T3CD40L) and calculate the required volume of cell suspension. Add 100 µl containing 1 x 10<sup>4</sup> cells per well in 96 well plates (for cytotoxicity assays), 6 x 10<sup>4</sup> cells per well in 24 well plates (for supernatant collection), 3 x 10<sup>5</sup> cells per well in 6 well plates, or 30 x 10<sup>5</sup> cells per well in 10 cm diameter dishes (for cultures to be used for flow cytometry experiments and preparation of whole cell lysates).

*Note: The number of cells and minimal volume per well for different types of multi-well plates is based on a conversion according to the differences in culture surface area.*
  - d. Once the effector 3T3 cells have been seeded, incubate plates O/N at 37 °C (Figure 1A).

*Note: For DNA fragmentation assays, the epithelial cells can be pre-pulsed with DNA labelling agent O/N (see below).*
3. Seeding of target (epithelial cells) onto 3T3 effector cells (co-culture) ('Day 2')
  - a. The following day (first thing in the morning), the target cells are washed with EDTA/PBS (following medium removal), then 0.8 ml of trypsin-EDTA are added per flask and cells are incubated at 37 °C for 2-3 min until cells have detached. The trypsin is inactivated with addition of complete culture medium.

*Note: If necessary, target cells can be pre-treated with any pharmacological inhibitors (for functional experiments) as required, before trypsinisation and counting.*

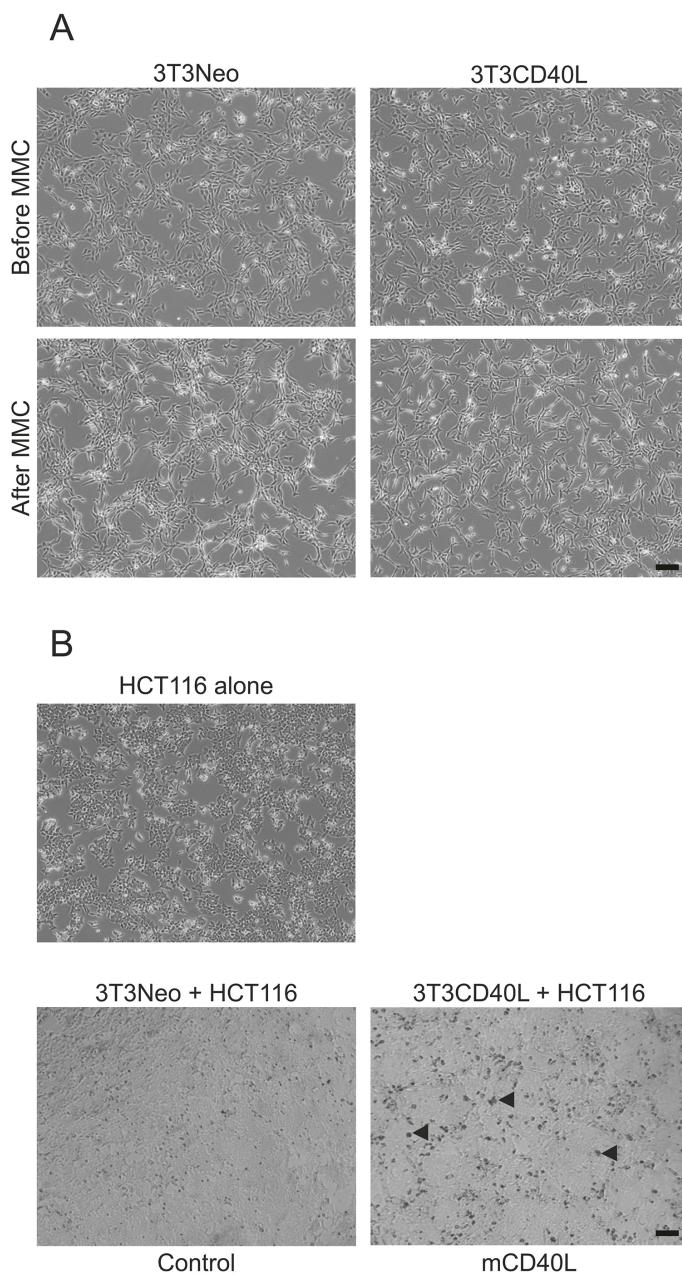
- b. Centrifuge at  $210 \times g$  for 5 min and then aspirate the supernatant and resuspend in complete culture medium. Then, count the cells and calculate the required volume of cell suspension.
- c. Remove the culture medium from each well or culture dishes containing the effector cells (3T3s) from the previous day (Step B2c above) using a small yellow tip and gentle aspiration (*it helps to ensure the multi-well plate is tilted and the tip touches just the edge at the bottom of the well to avoid removal of adhered cells*).
- d. Add 100  $\mu\text{l}$  of suspension containing  $1 \times 10^4$  cells per well of epithelial cells on top of the 3T3 effectors (or the equivalent volume and number of cells based on the conversion described in Step B2 above).

*Note: By seeding the above-stated number of epithelial cells, a 1:1 (effector:target) ratio is utilised (e.g., HCT116 cells); however in some cases a 1:0.8 ratio might be optimal (e.g., EJ cells).*

- e. The co-cultures are now established and multi-well plates (or dishes) are incubated at 37 °C (Figure 1B).
- f. Incubation period varies depending on the assay or procedure to be performed (e.g., cell death assays, collection of supernatants or to lyse the cells). Figure 2B provides an example of representative co-cultures at 72 h.

*Notes:*

- i. *The same steps are followed for the co-culture of normal epithelial cells with 3T3 effectors, however for such co-cultures normal (NHU) cells are seeded in their own (serum-free, KSF medium).*
- ii. *For functional inhibition experiments, co-cultures can take place in the continued presence of any pharmacological inhibitor, as required.*



**Figure 2. Images of effector (3T3Neo and 3T3CD40L) cells alone or in co-culture with epithelial (HCT116) cells.** A. Representative phase contrast images of 3T3Neo and 3T3CD40L effector cells before and after MMC treatment, which is carried out in order to mediate 3T3 cell growth arrest. The images confirm how 3T3 cells remain viable and healthy following treatment. B. Phase contrast microscopy images of representative co-cultures of growth-arrested 3T3Neo and 3T3CD40L effector cells with the carcinoma cell line HCT116 for 72 h. Co-culture of HCT116 cells with 3T3CD40L fibroblasts represents delivery of 'mCD40L', whilst co-culture with 3T3Neo serves as 'Control'. Note how cytotoxicity is evident by the presence of dark, phase-non-bright apoptotic cells (representative dead cells, or aggregates of such cells, are indicated by solid triangles). Scale bars = 100  $\mu$ m.

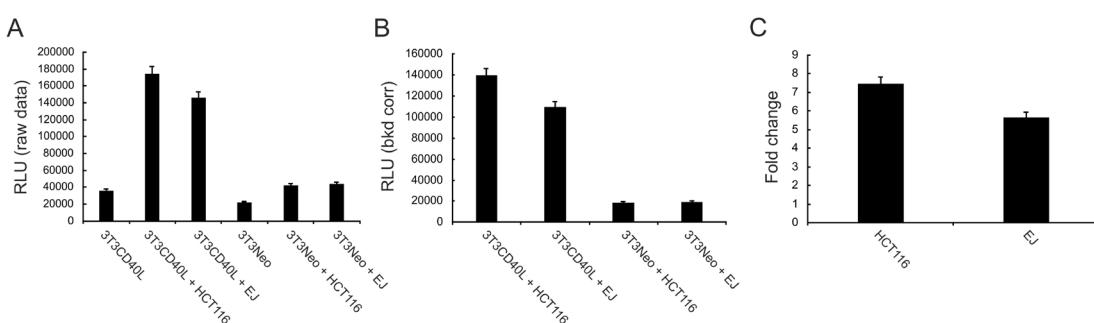
### C. Activation of CD40 ligation by soluble agonist

- In order to compare the effects of mCD40L to those of soluble agonists, epithelial cells seeded at the same densities described above, are treated routinely with the well-characterized agonistic anti-CD40 mAb G28-5 (Bugajska *et al.*, 2002).
- Add G28-5 monoclonal antibody (mAb) during seeding of the epithelial cells at a concentration of 10 µg/ml (mAb was purified from culture supernatants of the HB-9110 hybridoma line).
- To increase the cross-linking capacity of the mAb, crosslink the antibody with the affinity-purified human serum protein-adsorbed goat anti-mouse IgG (X-linker) used at 2.5 µg/ml. Add the X-linker to the cultures 30 min following addition of the G28-5 mAb.

*Note: The differences in the pro-apoptotic capabilities of soluble agonists versus mCD40L have been presented and detailed elsewhere (Dunnill *et al.*, 2017).*

### D. Detection of biochemical markers of cell death (apoptosis)

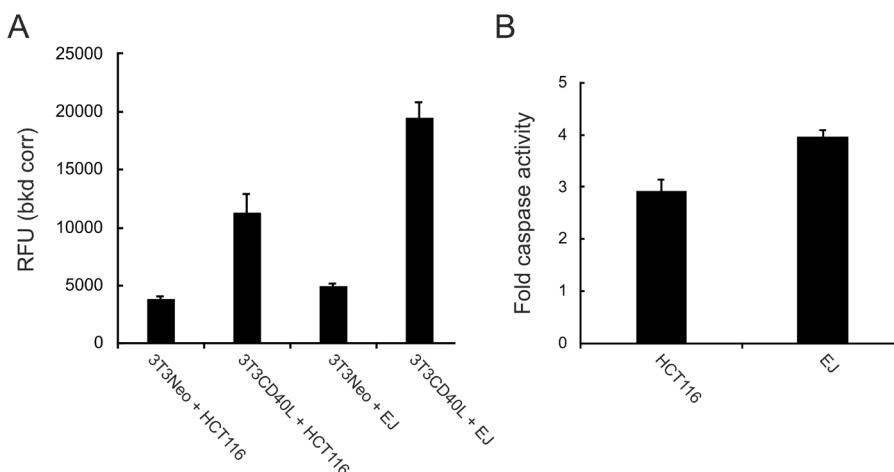
- Loss of cell membrane integrity (permeabilisation)
  - Seed carcinoma cells on top of growth-arrested 3T3s in 96 well white, tissue culture-treated plates (as detailed above) and incubate at 37 °C for 48 h (or 72 h).
  - Add 50 µl of Cytotox-Glo™ substrate (see Reagents) and incubate for 10 min at room temperature in the dark (therefore the final volume is 150 µl/well).
  - Use a FLUOstar OPTIMA (BMG Labtech) plate reader to measure luminescence (RLU).
  - Acquire data using MARS software (BMG Labtech) and analyse by Microsoft Excel appropriately to correct for background associated with 3T3 fibroblasts (as detailed in the Data analysis section); representative data are provided in Figure 3.



**Figure 3. Detection of loss of plasma membrane integrity as a marker of cell death.** 3T3Neo and 3T3CD40L fibroblasts were co-cultured with HCT116 and EJ tumour cells as detailed in the main text for 48 h. Loss of membrane integrity was detected using the Cytotox-Glo reagent. Raw data (relative luminescence units, RLU) for all cultures (co- and mono-cultures) are shown in panel A. Data for co-cultures only, obtained following pairwise subtraction of 3T3Neo (Control) and 3T3CD40L (mCD40L) culture RLU readings from the RLU readings obtained for the respective co-cultures ('bkgd corr'), are presented in panel B (see also Data analysis). Finally, data are expressed as fold change (*i.e.*, 'mCD40L RLU'/'Control RLU' for each co-culture) in panel C.

## 2. Effector caspase activation

- a. Seed carcinoma cells on top of growth-arrested 3T3s in 96 well white, tissue culture-treated plates (as detailed above) and incubate at 37 °C for 48 h or 72 h (the latter time-point often provides more intense caspase activity).
- b. Top up all wells with 50 µl of medium, then add 50 µl of SensoLyte® Homogenous AFC Caspase-3/7 substrate (see Reagents), so the total volume is 200 µl/well.
- c. Incubate the plates at room temperature (RT) overnight in the dark, then measure fluorescence (RFU) using a FLUOstar OPTIMA (BMG Labtech) plate reader, using Excitation/Emission 355 nm/520 nm filters, respectively.
- d. Acquire data using MARS software (BMG Labtech) and analyse using Microsoft Excel appropriately in order to correct for background related to 3T3 fibroblasts (as detailed in the Data analysis section); representative data are provided in Figure 4.

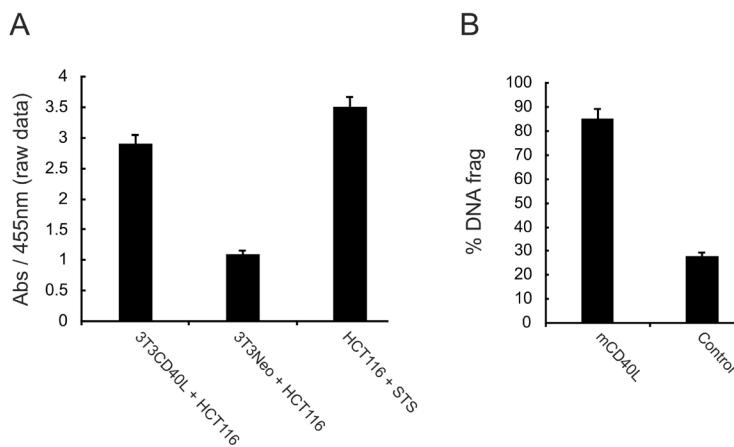


**Figure 4. Detection of effector caspase activation.** 3T3Neo and 3T3CD40L fibroblasts were co-cultured with HCT116 and EJ tumour cells as in Figure 3 for 72 h. Effector caspase 3/7 activity was detected using the Sensolyte caspase 3/7 reagent. Relative fluorescence unit (RFU) data for co-cultures were obtained following pairwise subtraction of 3T3Neo (Control) and 3T3CD40L (mCD40L) mono-culture RFU readings from the RFU readings obtained for the respective co-cultures ('bkgd corr') and shown in panel A. Data are also expressed as fold caspase activity (*i.e.*, 'mCD40L RFU'/'Control RFU' for each co-culture) in panel B.

## 3. DNA fragmentation

- a. For this assay, and as shown in Figure 1B, on the day when 3T3 fibroblasts are treated with MMC and seeded, the epithelial cells can be treated ('pulsed') with DNA labelling agent BrdU at a concentration of 10 µM (according to the manufacturer's instructions, see Reagents) overnight (O/N).
- b. The next day, epithelial cells are washed once with EDTA/PBS, lifted by trypsinisation, counted and then added as a 200 µl suspension on top of the growth-arrested 3T3 cells

- (following medium removal from the wells and as described in the co-culture protocol above). Cell cultures are then incubated for 48 h (or 72 h).
- c. As positive control and for the purposes of calculating % DNA fragmentation (below), treat carcinoma cells alone with 0.5  $\mu$ M of Staurosporine (STS) (or, alternatively, 3  $\mu$ g/ml Doxorubicin) for 48 h.
  - d. The day before DNA fragmentation is measured, prepare ('coat') ELISA plates for the detection of fragmented DNA according to the manufacturer's instructions.
  - e. On the day of the experiment, collect supernatants from co-cultures and add 100  $\mu$ l of culture supernatant to the appropriate wells in the coated 96 well ELISA plates; perform ELISA as described by the manufacturer and when complete, incubate the 96 well ELISA plate(s) O/N at 4 °C.
  - f. On the following day, complete the ELISA assay (as described in the manufacturer's instructions) and using a FLUOstar OPTIMA (BMG Labtech) plate reader measure absorbance (Abs) at 455 nm.
  - g. Acquire data using MARS software (BMG Labtech) and analyse by Microsoft Excel.
  - h. Calculate % DNA fragmentation as follows: % apoptotic cells = (Abs of co-cultured cells) x 100/(Abs of target cells treated with STS); representative data are provided in Figure 5.



**Figure 5. Detection of DNA fragmentation.** 3T3Neo and 3T3CD40L fibroblasts were co-cultured with DNA labelling agent (BrdU)-pulsed HCT116 cells for 48 h and DNA fragmentation was detected using an ELISA assay as detailed in the main text. Raw absorbance readings (at 455 nm wavelength) are presented in panel A for both co-cultures, whilst treatment of HCT116 cells with staurosporine (STS) at 0.5  $\mu$ M served as a positive control for maximal DNA fragmentation. Values obtained for co-cultures were expressed as % DNA fragmentation (% DNA frag) relative to STS (panel B), calculated as detailed in the appropriate section in the main text.

E. Detection of intracellular mediators of cell death

1. Reactive oxygen species

**Measurement of fluorescence by spectrophotometry**

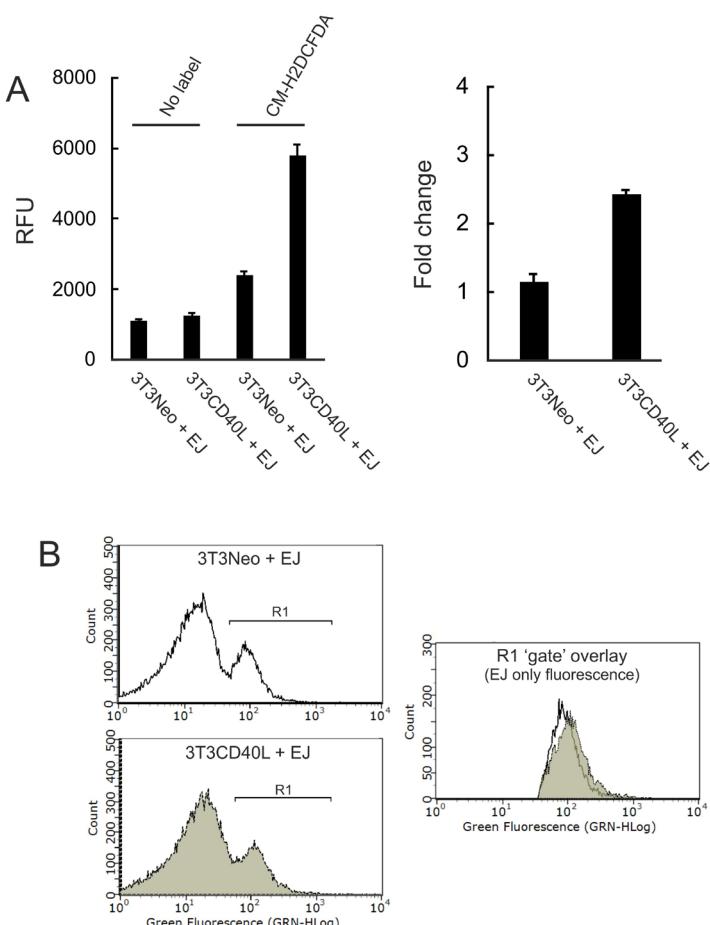
- a. Epithelial cells are co-cultured with 3T3Neo and 3T3CD40L cells as described above (in 96 well plates). Notably, however, when such co-cultures are performed, 3T3s are not growth-arrested with MMC to avoid high background fluorescence readings due to interaction with CM-H2DCFDA.

*Note: The duration of the experiments is short and therefore any growth of 3T3 cells does not affect overall cell viability in such short-term co-cultures.*

- b. Incubate co-cultures for the required period of time (from 30 min to 3 h).
- c. Wash cells with PBS to remove any culture medium then add 100 µl/well of pre-warmed (at 37 °C) PBS containing 1 µM of CM-H2DCFDA and incubate for 30 min at 37 °C in 5% CO<sub>2</sub>. After treatment, remove the CM-H2DCFDA reagent and add 100 µl of pre-warmed (at 37 °C) PBS.
- d. Measure fluorescence on a FLUOstar OPTIMA (BMG Labtech) plate reader at Excitation 485 nm/Emission 520 nm.
- e. Acquire data using MARS software (BMG Labtech) and analyse using Microsoft Excel appropriately in order to correct for background related to 3T3 fibroblasts (as detailed in the Data analysis section); representative data are provided in Figure 6A.

**Measurement of fluorescence by flow cytometry**

- a. Treat (label) the required number of epithelial cells (e.g., EJ) with pre-warmed PBS containing 1 µM of H2DCFDA and incubate for 30 min at 37 °C in 5% CO<sub>2</sub>. Epithelial cells can be labelled whilst adherent or in suspension.
- b. Co-culture CM-H2DCFDA labelled epithelial cells with 3T3Neo and 3T3CD40L effectors as described above in 6 well plates for 3 h.
- c. Following co-culture, add 200 µl of trypsin to lift cells, and when the cells have detached, inactivate by adding 500 µl of PBS containing 5% FBS.
- d. Collect cells in 1.5 ml sample tubes and centrifuge for 10 min at 210 x g at 4 °C.
- e. Discard the supernatant, resuspend in cold PBS (or FACS buffer) and measure fluorescence by flow cytometry using a Guava EasyCyte instrument. Analyse data post-acquisition using Guava EasyCyte software; Figure 6B provides an example of such analysis and illustrates the 'gating' strategy followed to measure ROS induction in epithelial cells only (and to exclude any 3T3 cell associated auto-fluorescence).



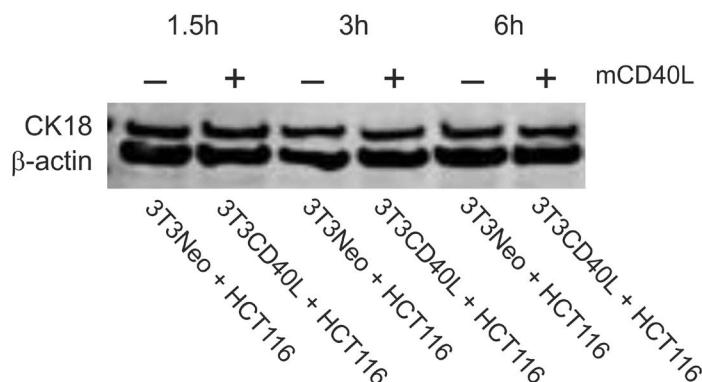
**Figure 6. Detection of ROS by spectrophotometry (A) and flow cytometry (B) analysis.** A. 3T3Neo and 3T3CD40L fibroblasts were seeded in 96 well plates before being co-cultured with EJ cells for 30 min. Cell cultured were labelled with CM-H2DCFDA as described in the main text. RFU data were obtained following pairwise background subtraction from 3T3Neo and 3T3CD40L alone mono-cultures. Unlabelled co-cultures (No label) served as negative controls (left). Data are also presented as fold change in RFU readings relative to controls (right). B. 3T3Neo and 3T3CD40L fibroblasts were seeded in 24 well plates before being co-cultured with EJ cells for 3 h (EJ were labelled with CM-H2DCFDA before co-culture). Green fluorescence intensity histograms provided a pattern of fluorescence indicative of two populations ('peaks'), 3T3 and epithelial cells, which were unlabelled and labelled with CM-H2DCFDA, respectively. Appropriate 'gating' on the epithelial cell population (right peak) was performed as indicated (gate R1) for 3T3Neo/EJ (upper histogram) and 3T3CD40L/EJ co-cultures (lower histogram). Data from both were overlaid (right panel) following gating, in order to identify changes in fluorescence intensity in EJ cells only. The shift in intensity (grey *versus* empty) indicates ROS induction mediated by mCD40L.

*Note:* For both spectrophotometric and flow cytometric detection of ROS, 3T3 cells were not growth-arrested with MMC to ensure lack of significant background fluorescence following labelling with CM-H2DCFDA.

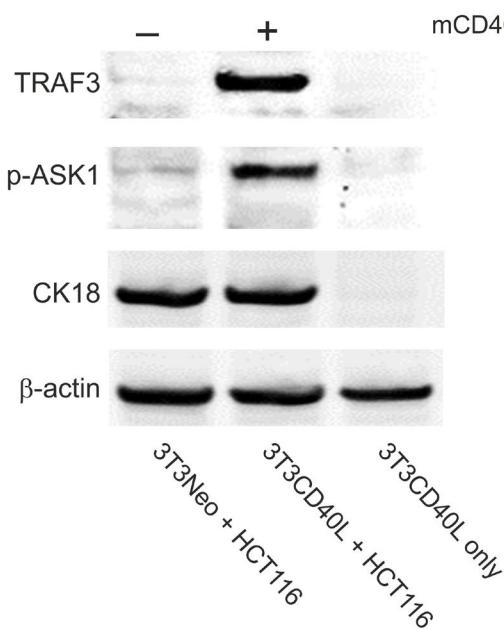
2. Adaptor proteins and pro-apoptotic kinases (immunoblotting)
  - a. Seed 3T3Neo and 3T3CD40L cells following treatment with MMC (above) in 10 cm diameter dishes at  $30 \times 10^5$  cells/dish in 10 ml of DR 5%, or in 6 well plates at  $3 \times 10^5$ /well in 2 ml of medium (prepare duplicate dishes or wells for every condition). Incubate dishes/plates at 37 °C O/N.
  - b. Following O/N attachment, remove supernatants and add 10 ml of target cell suspension in DR/5% medium containing  $30 \times 10^5$  cells/dish or 2 ml containing  $3 \times 10^5$  cells/well in 6 well plates.
  - c. Incubate the dishes/plates at 37 °C and 5% CO<sub>2</sub> for the required time period (can vary from 1.5 h to 36 h).
  - d. Upon completion of the incubation period, prepare lysates by adding 150 µl of complete lysis buffer/dish or 70 µl/well for 6-well plates (see Recipes for lysis buffer preparation) and quantify protein concentration in all lysates as described (Dunnill et al., 2017).
  - e. Perform SDS-Polyacrylamide gel Electrophoresis (SDS-PAGE) and immunoblotting for 40 µg of protein lysate for the detection of intracellular proteins expression in target cells; representative data are shown in Figure 7B.

*Note: Although expression of β-actin can be used to demonstrate overall equal loading, because lysates are prepared from 3T3neo/3T3CD40L and epithelial cell co-cultures (and thus are a mixture of cell types – epithelial and non-epithelial cells), it is essential to ensure that sample loading is confirmed (and adjusted if necessary) for epithelial cells; this can be carried out according to reactivity with human-specific anti-cytokeratin antibodies e.g., anti-CK8 or anti-CK18 – see Figure 7A for an example and elsewhere (Dunnill et al., 2017).*

A



B



**Figure 7. Detection of intracellular signalling proteins activated by mCD40L using immunoblotting.** HCT116 cells were co-cultured with 3T3Neo ('-') and 3T3CD40L ('mCD40L') fibroblasts for the indicated time periods and whole cell protein lysates prepared as described in the main text. Forty microgram of total protein were analysed by SDS-PAGE and immunoblotting. β-actin expression demonstrated overall equal loading, whilst correct loading for epithelial cell lysate was confirmed according to reactivity with human-specific anti-CK18 antibody (see main text) (panel A). Representative experiments for detection of TRAF3 and phospho-ASK1 are shown in panel B. Note how the inclusion of lysate from 3T3CD40L culture alone confirmed the specificity of the antibodies for human (epithelial) cell protein detection.

### **Data analysis**

1. Analysis was carried using the software listed above for the generation of raw data and images. Data was analysed on Excel and images were processed using Adobe Photoshop CS.
2. To calculate cell death or measure ROS induction, it was essential to ensure that any background ‘noise’ caused due to the presence of effector 3T3 cells was accounted for by subtraction from the readings of co-cultured cells. Therefore, background fluorescence or luminescence readings were subtracted pairwise as appropriately; *i.e.*, RFU or RLU readings for 3T3CD40L alone cultures were subtracted from 3T3CD40L + HCT116 co-culture readings (and similarly for 3T3Neo and 3T3Neo + HCT116 co-culture readings). The exemption was DNA fragmentation, as this was unnecessary due to the pre-labelling of target epithelial cells only. Moreover, in all experiments blank controls were included as appropriate.
3. Mean values and standard deviation (SD) were used as descriptive statistics. Two-tailed, paired or non-paired Student’s *t*-tests, and where appropriate ANOVA, were used for evaluation of statistical significance. For all experiments a minimum of 5-6 technical replicates were included and experiments were performed in triplicate ( $n = 3$ ).

### **Notes**

1. In order to ensure that all cells used for the preparation of the co-cultures are healthy, it is important to work reasonably fast and ensure that cells are provided with adequate CO<sub>2</sub> and are kept at 37 °C when not being used.
2. All reagents used for the detection of markers of apoptosis (and unless otherwise stated by the manufacturer) must be thawed and brought to room temperature adequately before addition to cells.

### **Recipes**

1. AQUAGUARD-1 solution  
10 ml AQUAGUARD-1  
1,000 ml ddH<sub>2</sub>O
2. 1x PBS  
900 ml ddH<sub>2</sub>O  
100 ml 10x D-PBS
3. PBS/EDTA  
1 g Ethylenediaminetetraacetic acid (EDTA)  
1,000 ml Phosphate-buffered saline (PBS) (1x)
4. Freezing medium  
10% (v/v) FBS

- 10% (v/v) DMSO
- 80% (v/v) standard culture medium
5. 70% ethanol
- 700 ml ethanol
- 300 ml ddH<sub>2</sub>O
6. DR medium
- 250 ml DMEM
- 250 ml RPMI
- Supplement either 5% FBS and 1% L-Glutamine for maintenance of carcinoma cells or 10% FBS and 1% L-Glutamine for the maintenance of 3T3 cells
7. FACS buffer
- 9 ml 1x PBS
- 1 ml FBS
8. SSB buffer
- |                                 |                                      |
|---------------------------------|--------------------------------------|
| Glycerol                        | 10 ml                                |
| Sodium dodecyl sulphate (SDS)   | 1 g                                  |
| Tris-HCl                        | 6.25 ml (stock solution 1 M, pH 6.8) |
| Sodium fluoride (NaF)           | 0.42 g                               |
| Sodium pyrophosphate tetrabasic | 0.446 g                              |
| Sodium orthovanadate            | 0.0184 g                             |
- Make up to a final volume of 50 ml using ddH<sub>2</sub>O
- Note: The solution requires stirring whilst being heated at 50 °C.*
9. Lysis buffer (for preparation of cell lysates)
- 1 ml SSB buffer
- 13 µl DTT solution (2 mg/ml in PBS)
- 2 µl protease inhibitor (PI) cocktail (VWR) or 10 µl PI cocktail (New England Biolabs)

### Acknowledgments

The co-culture protocol is a refined and further optimised version based on our previously published study (Dunnill *et al.*, 2017). KI was supported by a Ph.D. scholarship from the Iraqi Ministry of Higher Education and Scientific Research. AM and BA were supported by overseas Ph.D. scholarships from the Libyan Ministry of Higher Education. MI is a Ph.D. student currently being supported by the Jacquie Roeder Research Fund from the Laura Crane Youth Cancer Trust. All authors declare no conflicts of interest.

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## Cell Synchronization by Double Thymidine Block

Guo Chen and Xingming Deng\*

Department of Radiation Oncology, Emory University School of Medicine and Winship Cancer Institute of Emory University, Atlanta, USA

\*For correspondence: [xdeng4@emory.edu](mailto:xdeng4@emory.edu)



**[Abstract]** Cell synchronization is widely used in studying mechanisms involves in regulation of cell cycle progression. Through synchronization, cells at distinct cell cycle stage could be obtained. Thymidine is a DNA synthesis inhibitor that can arrest cell at G1/S boundary, prior to DNA replication. Here, we present the protocol to synchronize cells at G1/S boundary by using double thymidine block. After release into normal medium, cell population at distinct cell cycle phase could be collected at different time points.

**Keywords:** Cell synchronization, Cell cycle, Thymidine, DNA synthesis, DNA replication

**[Background]** Cell cycle and cell division lie at the heart of cell biology. To build multicellular organism, cell duplication is necessary to generate specialized cells, which can execute particular function. The normal cell cycle is composed of interphase (G1, S and G2 phase) and mitotic (M) phase (Rodríguez-Ubreva *et al.*, 2010; Léger *et al.*, 2016). During interphase, the genetic materials are duplicated and make everything ready for mitosis. Whereas, during mitotic phase, the duplicated chromosomes are segregated and distributed into daughter cells (Sakaue-Sawano *et al.*, 2008).

To precisely preserve genetic information, cell cycle progression must be tightly regulated. Cyclin/CDK complexes control the cell cycle progression through rapidly promoting activities at their respective stages, and are quickly inactivated when their stages are completed (Graña and Reddy, 1995).

Cell synchronization is particularly useful for investigating a cell-cycle regulated event. Using different methods, cells could be synchronized at different cell cycle stage. Treatment of nocodazole, which is an inhibitor of microtubule formation, could synchronize cells at G2/M phase (Ho *et al.*, 2001), while, hydroxyurea, a dNTP synthesis inhibitor, synchronize cells at early S phase (Koç *et al.*, 2004). As an Inhibitor of DNA synthesis (Schvartzman *et al.*, 1984), thymidine can arrest cell at G1/S boundary. Here, we describe a detail method to synchronize cells at G1/S boundary by thymidine (Chen *et al.*, 2018).

### Materials and Reagents

1. 10 cm culture dish (Corning, catalog number: 430167)
2. Gloves (VWR International, catalog number: 82026)
3. Protective clothing (VWR International, catalog number: 414004-444)
4. Eyewear (VWR International, catalog number: 89187-984)

5. Human tumor cell lines: H1299 (ATCC, catalog number: ATCC® CRL-5803™)
6. Dulbecco's Modified Eagle's Medium (DMEM) (high glucose with L-glutamine) (Corning, catalog number: 10-013-CV)
7. Phosphate-Buffered Saline (PBS) (Corning, catalog number: 21-040-CV)
8. Fetal bovine serum (FBS) (ATLANTA BIOLOGICALS, catalog number: S11150)
9. Thymidine (Sigma-Aldrich, catalog number: T9250)
10. Propidium Iodide (PI) (Thermo Fisher Scientific, catalog number: P3566)
11. Antibodies
  - a. Anti-Cyclin A (Abcam, catalog number: ab38)
  - b. Anti-Cyclin D (Santa Cruz Biotechnology, catalog number: sc-753)
  - c. Anti-β-Actin (Santa Cruz Biotechnology, catalog number: sc-58673)
12. Tris-HCl, pH 8.0 (Thermo Fisher Scientific, catalog number: 15568025)
13. NaCl (Sigma-Aldrich, catalog number: S9888)
14. NP-40 (Abcam, catalog number: ab142227)
15. EDTA (Thermo Fisher Scientific, catalog number: 15576028)
16. β-Mercaptoethanol (Sigma-Aldrich, catalog number: M6250)
17. EBC cell lysis buffer (see Recipes)
18. Electrophoresis running buffer (see Recipes)
19. Transfer buffer (see Recipes)

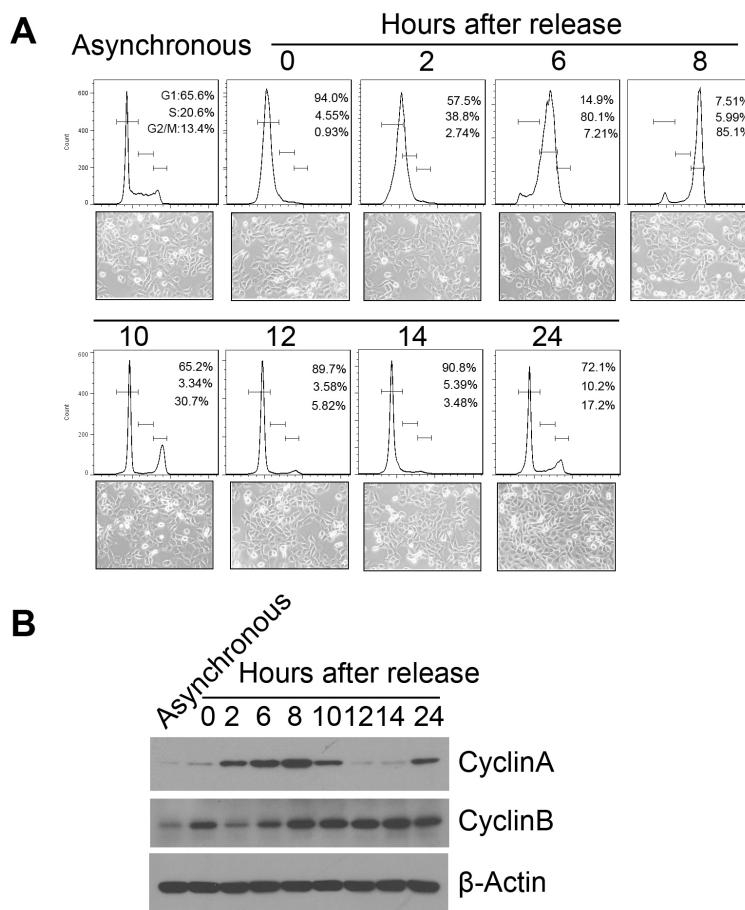
## Equipment

1. Cell culture incubator (VWR International, model: 98000-368)
2. Flow cytometry system (BD, model: FACSLyric)
3. X-RAY Film processor (Konica Minolta Healthcare Americas, model: SRX-101A)

## Procedure

1. Plate H1299 cells at 20-30% confluence in a 10 cm culture dish ( $2 \times 10^6$ - $3 \times 10^6$  cells per dish) containing 10 ml of Roswell Park Memorial Institute (RPMI) 1640 Medium supplemented with 10% Fetal Bovine Serum (FBS).
2. Incubate cells at 37 °C overnight.
3. Add thymidine to a final concentration of 2 mM.
4. Culture cells in a tissue culture incubator at 37 °C for 18 h.
5. Remove thymidine by washing cells through addition of 10 ml pre-warmed 1x PBS and discard PBS.
6. Add 10 ml of pre-warmed fresh medium and incubate for 9 h in a tissue culture incubator at 37 °C.
7. Add second round of thymidine to a final concentration of 2 mM.

8. Culture cells at the tissue culture incubator for another 18 h at 37 °C.
9. Cells are now in G1/S boundary.
10. Release cells by washing with pre-warmed 1x PBS and incubating cells in pre-warmed fresh media. Cells are collected at 0, 2, 6, 8, 10, 12, 14, 24 h for analysis of cell cycle by DNA staining using PI, or analysis of protein by Western blot using cyclin A, cyclin D and β-Actin antibodies (Figure 1).



**Figure 1. G1/S phase synchronized H1299 cells enter into normal cell cycle progression after release into fresh medium.** A. Cell cycle profiles at indicated time points after release following double thymidine block. B. Expression levels of Cyclin A, Cyclin B and β-actin in cells at indicated time points after release.

### Data analysis

Cell cycle was analyzed by flow cytometry with Flowjo software (Figure 1A). Cyclin A, Cyclin B and β-actin were detected by Western blotting (Figure 1B). Data are the representative of three independent experiments.

## **Notes**

1. Dissolve thymidine in PBS and make 100 mM stock solution.
2. The time points for distinct cell cycle phase are dependent on the cell cycle progression time of different cell lines.
3. Propidium Iodide (PI) is a mutagen. Gloves, protective clothing, and eyewear should be worn.

## **Recipes**

1. EBC cell lysis buffer
  - 50 mM Tris-HCl pH 7.6-8.0
  - 120 mM NaCl
  - 0.5% NP-40
  - 1 mM EDTA
  - 1 mM Na<sub>3</sub>VO<sub>4</sub>
  - 50 mM NaF
  - 1 mM β-Mercaptoethanol
2. Electrophoresis running buffer
  - 25 mM Tris-HCl pH 8.3
  - 192 mM glycine
  - 0.1% SDS
3. Transfer buffer
  - 25 mM Tris-HCl pH 8.3
  - 192 mM glycine
  - 10% methanol
4. Cell culture medium
  - Roswell Park Memorial Institute (RPMI) 1640 Medium
  - 10% Fetal Bovine Serum (FBS)

## **Acknowledgments**

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**Competing interests**

The authors have declared that no conflict of interest exists.

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## Qualitative *in vivo* Bioluminescence Imaging

Devbarna Sinha<sup>1</sup>, Zalitha Pieterse<sup>2</sup> and Pritinder Kaur<sup>1, 2, \*</sup>

<sup>1</sup>Epithelial Stem Cell Biology Laboratory, Peter MacCallum Cancer Centre, Melbourne, Victoria, Australia; <sup>2</sup>School of Pharmacy & Biomedical Sciences, Curtin Health Innovation Research Institute, Curtin University, Perth, Australia

\*For correspondence: [Pritinder.kaur@curtin.edu.au](mailto:Pritinder.kaur@curtin.edu.au)

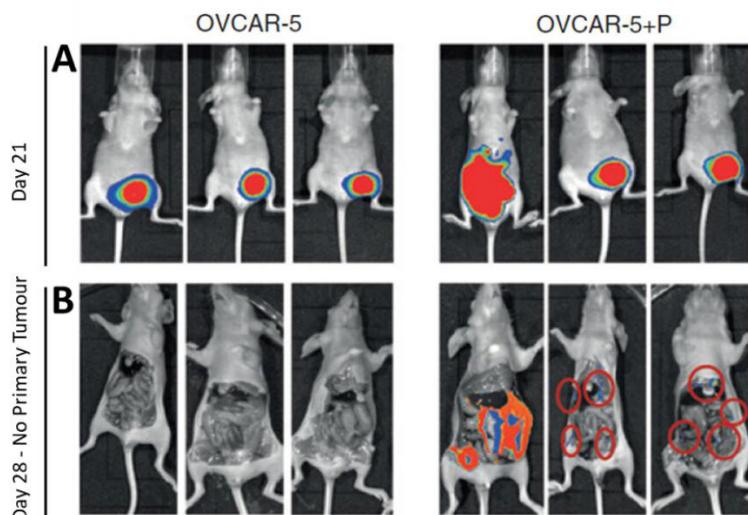


**[Abstract]** Bioluminescence imaging (BLI) technology is an advanced method of carrying out molecular imaging on live laboratory animals *in vivo*. This powerful technique is widely-used in studying a variety of biological processes, and it has been an ideal tool in exploring tumor growth and metastatic spread in real-time. This technique ensures the optimal use of laboratory animal resources, particularly the ethical principle of reduction in animal use, given its non-invasive nature, ensuring that ongoing biological processes can be studied over time in the same animal, without the need to euthanize groups of mice at specific time points. In this protocol, the luciferase imaging technique was developed to study the effect of co-inoculating pericytes (contractile,  $\alpha$ SMA<sup>+</sup> mesenchymal stem cell-like cells, located abluminally in microvessels) on the growth and metastatic spread of ovarian cancers using an aggressive ovarian cancer cell line—OVCAR-5—as an example.

**Keywords:** Bioluminescence, Tumor imaging, Luciferase Imaging, Metastasis, Ovarian cancer

**[Background]** The principle of bioluminescence imaging (BLI) is based on the light-emitting properties of a relatively simple biochemical process, *i.e.*, luciferase-mediated oxidation of the molecular substrate luciferin to produce light. In cancer research, BLI is a popular tool (Contag *et al.*, 2000) used to study the metastatic spread of luciferase-transduced cancer cells in live animals *in vivo*. Most animal tissues have little to no baseline bioluminescent properties, which ensures that there is a very high signal to noise ratio in BLI experiments. Nevertheless, it is always pragmatic to ensure that BLI experiments are conducted with non-substrate injected rodents as a negative control group. A few crucial factors are key to ensuring best practice in performing BLI experiments on rodents for the detection of luciferase-tagged cancer cells. Firstly, BLI is a powerful tool that allows easy detection of luciferase expressing-cells by imaging emitted bioluminescence under anesthesia (Figure 1). However, this technique is essentially qualitative and attempts to quantitate BLI signal can be misleading given that the strength of signal is dependent on several factors including duration of exposure, anesthetic technique, time elapsed after injecting luciferin, *etc.* Moreover, published evidence indicates that light emission (*i.e.*, quantity of photons) is not directly related to luciferase activity (Rice *et al.*, 2001). Secondly, the location or depth of the tissue of interest, particularly its distance from the skin's surface, and the size of the metastatic cell mass are important considerations in planning a BLI experiment (Weissleder, 2001). This is because photon loss occurs as the signal travels through the tissue mass—consequently, luciferase-tagged cells/tissues that are closer to the skin's surface tend to appear brighter as do larger metastases.

Micrometastases can be detected but may require sacrificing the animal and imaging the organs directly after skin removal in place of intact animals. Notwithstanding these and other challenges, BLI is an efficient and powerful, non-invasive technique for studying biological processes *in vivo*.



**Figure 1. Imaging primary subcutaneous OVCAR-5 tumors and metastases.** A. GFP-luciferase labeled OVCAR-5 cells, xenografted alone (OVCAR-5) or co-injected with pericytes (OVCAR-5+P) generated tumors that were imaged at regular intervals. The increased BLI signal observed in images of pericyte co-injected xenografts show that pericytes promote OVCAR-5 tumor growth rate and induce metastases compared to the control group. B. At day 28 the BLI signal from the primary tumor is saturated, and the mice were sacrificed and the primary tumors (along with the abdominal skin excised to permit clearer imaging of the metastatic nodules—metastatic nodules marked with red rings).

## **Materials and Reagents**

### A. Materials

1. Pipette tips (Interpath Services, catalog numbers: 39770, 39730)
2. Hamilton® syringe (Hamilton, catalog number: 80366)
3. Tissue culture plasticware (6-well plates BD; 2, 5 and 10 ml pipettes SARSTEDT)
4. Steritop-GP polyethersulfone with low binding PES membrane (0.22 µm pore size) (Merck, Millipore, catalog number: SCGPS05RE)
5. Pasteur pipette (Biologix, catalog number: 30-0138A1)

### B. Biological materials

1. Lentiviral vector pFUGW-Pol2-ffLuc2-eGFP (Addgene, catalog number: 71394)
2. OVCAR-5 cell line was obtained from NCI, and authenticated using short tandem repeat markers to confirm cell identity against the Genome Project Database (Wellcome Trust Sanger

Institute)

3. HIV-1 packaging vector pCMV-deltaR8.2 (Addgene, catalog number: 8455), a kind gift from Dr. Cameron Johnstone, Anderson Lab, Peter MacCallum Cancer Centre, Melbourne
4. Packaging cell line HEK293T (ATCC, catalog number: CRL-3216), a kind gift from Dr. Cameron Johnstone, Anderson Lab, Peter MacCallum Cancer Centre, Melbourne
5. Mice: 6-8 weeks old female athymic nude Balb/c mice were obtained from Walter Eliza Hall Institute, housed in a pathogen-free 12 h light–dark environment, fed ad libitum were used for tumorigenicity assays. This age range of mice is optimal for good tumor take rates which decline if older mice (e.g., 10 weeks old) are used.

#### C. Reagents

1. Bovine serum albumin (Sigma-Aldrich, catalog number: A9418-500G)
2. DAPI:4', 6'-Diamidino-2-Phenylindole Dihydrochloride (Sigma-Aldrich, catalog number: D9542-5MG)
3. DMEM (Thermo Fisher Scientific, Gibco<sup>TM</sup>, catalog number: 11965092)
4. Diflucan (Fluconazole) (Sigma-Aldrich, catalog number: F8929)
5. D-Luciferin: Sodium salt 4,5-Dihydro-2-(6-hydroxy-2-benzothiazolyl)-4-thiazolecarboxylic acid sodium salt (Gold Biotechnology, catalog number: LUCNA-1G)
6. FuGENE 6 (Roche Diagnostics, Mannheim, Germany)
7. Endothelial basal media (EBM<sup>TM</sup>-2) (Lonza, catalog number: CC-3156)
8. Endothelial growth media (EGM<sup>TM</sup>-2) Singlequots<sup>TM</sup> (Lonza, catalog number: CC-4147)
9. Fetal Calf Serum (FCS) (Thermo Fisher Scientific, Gibco<sup>TM</sup>, catalog number: 10099141)
10. Forthane/Isoflurane (Sigma-Aldrich, catalog number: 792632)
11. HEPES pH 7.4 (Sigma-Aldrich, catalog number: H0887-100ML)
12. Matrigel<sup>TM</sup> (standard) (BD, catalog number: 356234)
13. PBS without Ca<sup>2+</sup> and Mg<sup>2+</sup> (GE Healthcare, Hyclone, catalog number: SH30256.FS)
14. Penicillin-Streptomycin (Thermo Fisher Scientific, Gibco<sup>TM</sup>, catalog number: 15140122)
15. Polybrene (Sigma-Aldrich, catalog number: 107689)
16. Potassium chloride (Sigma-Aldrich, catalog number: P5405)
17. Potassium dihydrogen phosphate (Sigma-Aldrich, catalog number: P5655)
18. RMPI-1640 (Thermo Fisher Scientific, Invitrogen<sup>TM</sup>, catalog number: 11875)
19. Sodium bicarbonate (Sigma-Aldrich, catalog number: S5761)
20. Sodium chloride (Astral Scientific, catalog number: AMX190)
21. Sodium hydrogen phosphate (Sigma-Aldrich, catalog number: S5136)
22. Trypsin 0.05% (Thermo Fisher Scientific, Gibco<sup>TM</sup>, catalog number: 25300120)
23. Trypan blue (Thermo Fisher Scientific, Gibco<sup>TM</sup>, catalog number: 15250061)
24. Phosphate buffered Saline (PBS) (see Recipes)
25. RPMI-1640 media (see Recipes)

## Equipment

1. Pipettes (Corning, catalog numbers: 4487, 4488, 4489)
2. Hemocytometer (ProSciTech, catalog number: SVZ2NI0U)
3. Cell culture incubator (NuAire, model: NU-5510/E)
4. Centrifuge (Beckman Coulter, model: Allegra X-12)
5. Electronic calipers (Fisher Scientific, catalog number: 14-648-17)
6. SW28 Rotor (Beckman Coulter, catalog number: 342207)
7. Ultracentrifuge (Beckman Coulter, model: Optima<sup>TM</sup> XE-100)
8. Fluorescent Microscope (Nikon Instruments, model: Nikon A1<sup>+</sup> Confocal Microscope)
9. Xenogen Realtime Imaging System (IVIS Lumina II)
10. Becton Dickinson Biosciences FACS Diva<sup>TM</sup> cell sorter

## Software

1. Prism 6 (GraphPad Inc.)
2. Photoshop CS 6.0 (Adobe Inc.)
3. Metamorph (Molecular devices)
4. ImageJ (NIH software)

## Procedure

In the protocol below we describe luciferase imaging to measure the metastatic spread of OVCAR-5 cells after the establishment of primary tumors following subcutaneous injection in immunocompromised mice. However, other cell types are equally amenable to this approach, and we have used this protocol to track OVCAR-8 cells for metastatic spread and monitor the persistence and survival of pericytes *in vivo*, when co-injected with OVCAR cells (Sinha *et al.*, 2016).

### A. Lentiviral vector

All work with lentiviruses was performed with due regard to biosafety concerns in line with guidelines set down by the Office of the Gene Technology Regulator of the Government of Australia and with Institutional approval (#09/2006). The replication defective 3<sup>rd</sup> generation lentiviral vector pFUGW-Pol2-ffLuc2-eGFP (Addgene plasmid #71394) expressing green fluorescent protein (GFP) from jellyfish *Aequorea victoria* and the firefly luciferase gene (Day *et al.*, 2009) is used to transduce OVCAR-5 cells in this protocol, by co-transfection of the HIV-1 packaging vector and VSV-G envelope glycoprotein (Sinha *et al.*, 2016).

### B. Lentiviral production

The packaging cell line HEK293T is used to produce lentivirus for transduction of OVCAR-5 cells (Sinha *et al.*, 2016). Briefly, HEK293T cells are plated at 2 × 10<sup>5</sup> cells per 60 mm plate in 3 ml

DMEM containing 10% FCS, and allowed to adhere and grow overnight at 37 °C in a humidified incubator containing 5% (v/v) CO<sub>2</sub>. HEK293T cells are then transfected using FuGENE 6 (Roche Diagnostics, Mannheim, Germany) at a FuGENE ( $\mu$ l): pFUGW-Pol2-ffLuc2-eGFP plasmid DNA ( $\mu$ g) ratio of 3:1 as follows:

1. Add an appropriate volume of FuGENE 6 to serum free media and incubate for 5 min at room temperature.
2. Determine the concentration of plasmid DNA using 260 nm absorption; then add it to the FuGENE/media mix at the appropriate concentration, and incubate for 15 min at room temperature.
3. Add the FuGENE/DNA/media mixture dropwise to the plated cells at 100  $\mu$ l per 60 mm plate. Incubate the plate for 24-48 h at 37 °C.
4. Collect viral supernatant from HEK293 cells, place it in a sterile tube and concentrate by spinning at 1,000  $\times g$  at 25 °C for 90 min in an ultracentrifuge using an SW28 rotor; aspirating the media and resuspending the viral pellet in 1 ml of RPMI-1640 media.

#### C. Lentiviral transduction

1. One day prior to infection, plate OVCAR-5 cells into 6-well plates at 1  $\times$  10<sup>5</sup> cells per well in RPMI-1640 (Sinha *et al.*, 2016).
2. For each well of OVCAR-5 cells to be transduced, add 2.5  $\mu$ l of 10 mg/ml polybrene to 2 ml of media, and add to 500  $\mu$ l of viral supernatant. This is the infection cocktail.
3. Overlay OVCAR-5 cells with infection cocktail for 12 h at 37 °C with 5% CO<sub>2</sub>, aspirate virus-containing medium and replace with fresh medium.
4. Assess transduction efficiency of OVCAR-5 cells using fluorescence microscopy, as judged by the number of GFP positive cells.
5. Propagate transduced cells and expand in culture for up to two passages monitoring GFP transduction efficiency by fluorescence microscopy, prior to sorting for enrichment for GFP-positive cells. To sort, trypsinize cells and resuspend in blocking buffer (2% [v/v] FCS and 2% BSA in PBS without Ca<sup>2+</sup> and Mg<sup>2+</sup>) containing DAPI at a final concentration of 2  $\mu$ g/ml. Viable (DAPI negative) GFP<sup>+</sup> cells are sorted and collected on a BD FACS Diva™ sorter and expanded prior to use in luciferase imaging experiments. Lentiviral transduction efficiencies are extremely high and transduction efficiencies of 85-90% were routinely achieved in our laboratory even with primary cells such as pericytes.

#### D. GFP-luciferase<sup>+</sup> OVCAR-5 cell culture and preparation for injection into mice

1. Culture GFP-luciferase<sup>+</sup> OVCAR-5 cells to 80-90% confluency.
2. Aspirate culture media using sterile Pasteur pipette.
3. Wash cell monolayer with a small volume of pre-warmed PBS without Ca<sup>2+</sup> and Mg<sup>2+</sup>.
4. Add an appropriate volume of 0.05% trypsin at 37 °C and incubate for 1-2 min.
5. Tap plate gently to dislodge adherent cells.

6. Quench trypsin activity with an equal volume of media containing FCS.
7. Transfer cell suspension to centrifuge tube and spin at 400  $\times$  g for 5 min at 4 °C.
8. Discard supernatant.
9. Perform cell count using trypan blue stain and hemocytometer.
10. Resuspend 8  $\times$  10<sup>5</sup>-8  $\times$  10<sup>6</sup> cells in 100  $\mu$ l of a PBS/Matrigel™ (5  $\mu$ g/ml) mixture per mouse on ice.

#### E. Animal preparation

1. Inject immunocompromised mice with 100  $\mu$ l cell suspension subcutaneously under the front flank using a Hamilton® syringe with a 26 G needle. (See video clip of procedure at <https://youtube.com/watch?v=nrOTzLiWC8U>)
2. Keep mice under daily observation after injection to ensure full recovery, looking for signs of stress.
3. Monitor tumor volume by taking metric measurements using electronic calipers.

*Note: OVCAR-5 tumors were monitored every alternate day. Modify protocol depending on the tumor growth rate of cell lines in use.*

#### F. Luciferase Imaging

1. Inject mice subcutaneously on the non-tumor bearing flank with 150  $\mu$ g per gram of body weight in 100  $\mu$ l sterile PBS of D-luciferin, the substrate for the luciferase enzyme.

*Note: Acquire imaging within 10-12 min of injecting with D-Luciferin.*

2. Allow mice free movement for 6-8 min.
3. Anesthetize mice with 2.5% Forthane (commercial isoflurane anesthetic) in oxygen.  
*Note: Take care not to anesthetize for longer than 3 min.*
4. Place anesthetized mice to inhalation cones in induction chamber of Xenogen Real-Time Imaging System on the heated platform.
5. Close chamber door securely.
6. Acquire images at the same exposure length and time interval for all experimental groups within 10-12 min of luciferin injection prior to signal decay.

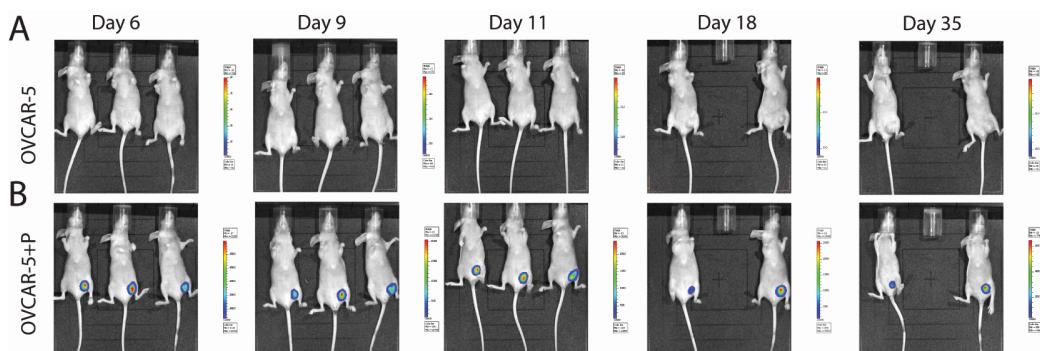
#### G. End point

1. Repeat above luciferase imaging (Steps F1-F6) every 7 days to continue to track metastatic spread.
2. At the experimental end point, the luminescent signal from large primary tumors is saturated and can block the signal from the smaller metastatic nodules. In order to overcome this, inject mice with D-luciferin and allow free movement for 6-8 min, euthanize the animals and surgically excise the primary tumors. Open the peritoneal cavity surgically, remove skin tissue over the anatomical sites to be imaged and proceed with BLI, adjusting exposure length to ensure signals from smaller metastatic nodules are captured.

*Note: All experiments were performed in compliance with Institutional ethics committee guidelines which stipulate that animals should not be subjected to undue distress—experimental animals were monitored daily for signs of distress and were sacrificed when tumors reached a volume of 1,500 mm<sup>3</sup> (where tumor volume = [width]<sup>2</sup> x length x 0.5) even if this occurs before the planned end point of the experiment. No experiments were taken beyond Day 35 after injection.*

### **Data analysis**

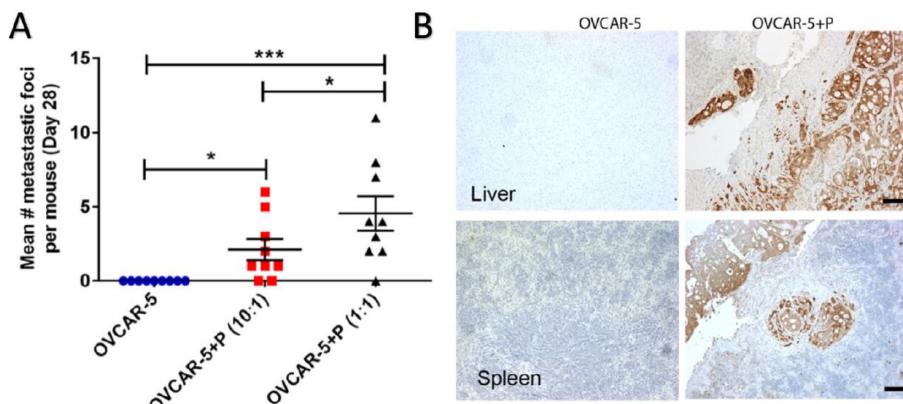
1. Metastatic spread detected by BLI is a qualitative assay permitting the investigator to determine whether particular experimental conditions (in this case co-injection with pericytes) leads to metastasis or not. It is also possible to compare the time of onset of metastases under specific experimental conditions—in this case establishing that co-injection of OVCAR-5 cells with pericytes, leads to earlier onset of metastases than OVCAR-5 cells alone, by imaging at different time points. However, to establish that OVCAR-5 cells consistently yield metastatic nodules when co-injected with pericytes at specific time points, 10 mice per experimental group were imaged at several time points and each experiment replicated twice. BLI can also be used to track the persistence of non-tumor cells—as shown in Figure 2, by transducing pericytes with the GFP-luciferase lentivirus and co-injecting with untagged OVCAR-5 cells.



**Figure 2. Tracking pericytes in OVCAR-5 xenografts *in vivo*** (Sinha et al., 2016). BLI images of xenografts generated by unlabeled OVCAR-5 cells alone (A); or OVCAR-5 cells co-injected with GFP-luciferase labeled pericytes (B). Imaging was conducted in 3 mice per group per time point, in 2 replicate experiments. Unlabeled OVCAR-5 tumors acted as a negative control, giving no signal despite luciferin injection (A). Sequential BLI of OVCAR-5 tumors co-injected with GFP-luciferase labeled pericytes displaying a positive BLI signal from Day 6 to Day 35, suggesting the persistence of pericytes in xenografts (B).

2. BLI can be used to quantitate the number of metastatic nodules obtained with OVCAR-5 cells alone versus OVCAR-5 cells co-injected with pericytes but is best determined at the endpoint of the experiment after sacrificing the animals and enumerating the number of nodules detected by BLI from 10 mice per group from two independent experiments. Data are then expressed as

mean number  $\pm$  SEM of metastatic nodules per mouse and statistical analysis for differences between experimental groups performed using one-way ANOVA (Figure 3). In addition, metastases must be verified independently to confirm their OVCAR-5 cell origin by an independent means—thus each metastatic nodule is harvested, processed for histological analysis via paraffin sections that are immunostained for GFP expression using an antibody to GFP.



**Figure 3. Quantification and immunohistochemical verification of metastatic nodules** (Sinha *et al.*, 2016). A. The number of metastatic nodules obtained under varying experimental conditions was counted at the experimental endpoint, revealing that OVCAR-5 cells do not yield metastases at Day 28 whereas co-injection with pericytes does. Increasing the ratio of pericytes: tumor cells leads to an increase in metastatic nodules (compare OVCAR-5: pericyte ratio of 10:1 versus 1:1). B. GFP staining of GFP-luciferase<sup>+</sup>OVCAR-5 tumors generated from OVCAR-5 cells alone or with pericyte co-injection (OVCAR-5+P) in the liver and spleen verifying that the metastatic nodules detected by BLI are derived from OVCAR-5 cells and not attributed to spurious signal.

### Notes

1. The absolute amount of BLI signal detected is dependent on the number of GFP-luciferase<sup>+</sup> cells being imaged and the duration of exposure after luciferin injection. Thus, imaging smaller tumors at early stages of tumor development or the lower number of pericytes can be enhanced by increasing the duration of exposure after luciferin injection. However, care has to be taken to ensure that the same exposure is used across all experimental groups of animals compared within an experiment.
2. Detection of metastatic nodules by BLI is limited by their size and depth of location within the body—the deeper their location from the imaged surface, the lower their chance of detection. Low signal from small metastatic nodules can also be obliterated by the strong signal from the primary tumor. Thus, while BLI is a good indicator of the presence or absence of metastasis, it is advisable to use the endpoint of experiments to establish the full extent and number of

metastases. At this stage, animals are sacrificed, the primary tumor excised and the skin removed over the region to be imaged for metastases.

3. Use of different surgical tools to excise primary tumors from each experimental animal is advisable due to the possibility of transferring luciferase positive cells from one animal to the next, giving rise to false positive BLI readings. This also makes it critical to verify that each “metastatic nodule” detected by BLI is indeed derived from GFP-luciferase labeled tumor cells by harvesting the BLI positive metastasis, processing for histological sectioning and immunohistochemical analyses after staining for GFP as shown in Figure 3B.

## **Recipes**

1. Phosphate buffered Saline (PBS)
  - a. Dissolve 1 g KCl; 1 g KH<sub>2</sub>PO<sub>4</sub>; 5.75 g Na<sub>2</sub>HPO<sub>4</sub> and 40 g NaCl in approximately 2 L of MillQ H<sub>2</sub>O
  - b. Make volume up to 5 L with MilliQ H<sub>2</sub>O and stir well until all ingredients have dissolved
  - c. Measure pH and adjust to pH 7.0-7.5. Ensure osmolarity is 270 ± 13 mM
  - d. Filter sterilize using a 20 L pressure tank and Sartobran filter
  - e. Store at 4 °C
2. RPMI-1640 media
  - a. Supplement RPMI-1640 with 25 mM HEPES, 1% penicillin-streptomycin, 1.5% Diflucan and 10% (v/v) heat inactivated FCS
  - b. To heat-inactivate FCS, incubate FCS at 56 °C for 45 min
  - c. Filter sterilize the RPMI-1640 medium using a 20 L pressure tank and Sartobran filter

*Note: In this study, RPMI-1640 is used to culture the OVCAR-5 cell line.*

## **Acknowledgments**

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### **Competing interests**

The authors have no conflicts of interest or competing interests to declare.

### **Ethics**

All animal experimentation was conducted with approval from the Peter MacCallum Animal Research Ethics Committee—AEEC (#E394 and #E504) within reference to guidelines of ethical experimentation on animals laid down by the National Health & Medical Research Council of Australia.

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## Zebrafish Embryo Xenograft and Metastasis Assay

Ilkka Paatero<sup>1,\*</sup>, Sanni Alve<sup>3</sup>, Silvia Gramolelli<sup>3</sup>,

Johanna Ivaska<sup>1, 2</sup> and Päivi M. Ojala<sup>3, 4, 5,\*</sup>

<sup>1</sup>Turku Centre for Biotechnology, University of Turku and Åbo Akademi University, Turku, Finland;

<sup>2</sup>Department of Biochemistry, University of Turku, Turku, Finland; <sup>3</sup>Research Programs Unit, Translational Cancer Biology, University of Helsinki, Helsinki, Finland; <sup>4</sup>Foundation for the Finnish Cancer Institute, Helsinki, Finland; <sup>5</sup>Section of Virology, Division of Infectious Diseases, Department of Medicine, Imperial College London, London, United Kingdom

\*For correspondence: [ilkka.paatero@utu.fi](mailto:ilkka.paatero@utu.fi); [p.ojala@imperial.ac.uk](mailto:p.ojala@imperial.ac.uk)



**[Abstract]** Xenograft models, and in particular the mouse xenograft model, where human cancer cells are transplanted into immunocompromised mice, have been used extensively in cancer studies. Although these models have contributed enormously to our understanding of cancer biology, the zebrafish xenograft model offers several advantages over the mouse model. Zebrafish embryos can be easily cultured in large quantities, are small and easy to handle, making it possible to use a high number of embryos for each experimental condition. Young embryos lack an efficient immune system. Therefore the injected cancer cells are not rejected, and the formation of primary tumors and micrometastases is rapid. Transparency of the embryos enables imaging of primary tumors and metastases in an intact and living embryo. Here we describe a method where GFP expressing tumor cells are injected into pericardial space of zebrafish embryos. At four days post-injection, the embryos are imaged and the formation of primary tumor and distant micrometastases are analyzed.

**Keywords:** Zebrafish, Embryo, Cancer, Xenograft, Melanoma, Micrometastases, Protocol

**[Background]** Zebrafish (*Danio rerio*) is a small fresh water fish that has gained popularity as a model organism not only in developmental biology, but also increasingly in biomedicine. One of the rapidly growing fields, is the use of zebrafish as a model for cancer biology research. Zebrafish mate efficiently and reliably, and can produce lots of offspring that are small, transparent, develop externally and can be cultured easily in e.g., multi-well plates (White *et al.*, 2013). Interestingly, human tumor cells can be implanted into zebrafish embryo and many of these xenografts are able to faithfully recapitulate their malignant behavior by growing, invading and metastasizing in the embryo (Lee *et al.*, 2005; Nicoli *et al.*, 2007; Chapman *et al.*, 2014). Zebrafish has also become a highly tractable model system for molecular studies on vascular development, angiogenesis and lymphangiogenesis and helped us to improve our understanding of vascular disease in humans (Hogan and Schulte-Merker, 2017). The possibility to visualize both blood and lymphatic vasculature using live imaging techniques makes zebrafish suitable for investigating tumor cell dissemination and metastasis from the primary site (Hogan and Schulte-Merker, 2017). As compared to mouse xenografts, the zebrafish embryo xenograft experiments require lower costs, smaller numbers of tumor cells and are faster to carry out.

Due to small size of the embryos, the transplantation needs to be performed using specific microinjection equipment. This consists of a fluorescence stereomicroscope, micromanipulator and a microinjector. Here, the xenotransplantation protocol is described using a GFP-expressing melanoma cell line WM852 (Pekkonen *et al.*, 2018), but similar approaches can be used with a number of other cell lines (Veinotte *et al.*, 2014). The use of cells labeled with a fluorescent protein is preferred, as this enables straight-forward quantitation of the tumor growth and direct detection of tumor cells in the embryos. Here, we have utilized mounting of the embryos inside agarose gel to allow precise transplantation of the cells into the pericardial cavity of the embryos. Other anatomical sites for injections such as yolk sac, perivitelline space, vasculature and hindbrain ventricle can be also used (Veinotte *et al.*, 2014) and the choice of transplantation site depends on the experimental question and the used cell line. As the pericardial cavity is optically clear (in contrast to yolk sac), it enables a good view on the transplanted tumor cells, and the pericardial space is also easily accessible allowing reliable transplantation of tumor cells. Moreover, at the embryonic and larval stages used here, the pericardial cavity is not a site of prominent angiogenesis (in contrast to often used perivitelline space injections) and hence in this model, the cancer cells need to first invade locally before being able to reach the vascular network.

### **Materials and Reagents**

1. Glass capillaries (World Precision Instruments, catalog number: TW100-4)
2. PAP-pen (Ted Pella, catalog number: 22311)
3. Pasteur glass pipettes (VWR, catalog number: 612-1702)
4. Humidor made from large Petri dishes (145 mm) (Greiner Bio One International, catalog number: 639160) (Add wetted paper towel on the bottom of the dish to prevent mounted embryos from drying while injecting them.)
5. 10 cm Petri dishes (Greiner Bio One International, catalog number: 633185)
6. 10 cm cell-culture dishes (Greiner Bio One International, catalog number: 664160)
7. Normal glass microscopy slide (VWR, catalog number: 631-1551)
8. Paper towel
9. Pipette tips (fitting for 100-1,000 µl, 20-200 µl, 2-50 µl and 0.5-10 µl pipettes)
10. FACS tubes with 40 µm mesh cap (BD, catalog number: 352235)
11. 15 ml tubes (Greiner Bio One International, catalog number: 188271)
12. 12-well plate (Greiner Bio One International, catalog number: 665180)
13. Zebrafish (*e.g.*, casper strain (*roy*-/-; *mitfa*-/-)
14. Tricaine (MS-222 or Ethyl 3-aminobenzoate methanesulfonate) (Sigma-Aldrich, catalog number: A5040)
15. Low-melting-point agarose (Sigma-Aldrich, catalog number: A9414-10G)
16. Polyvinylpyrrolidone K 60 Solution, 45% (PVP) (Sigma-Aldrich, catalog number: 81430-500ML)
17. Phosphate buffered saline (PBS) (Biowest, catalog number: L0615-500)

18. N-phenyl-thiourea (PTU) (Sigma-Aldrich, catalog number: P7629)
19. Tris base (Sigma-Aldrich, catalog number: T1503)
20. WM852 (RRID:CVCL\_6804) transduced with p-lenti6 GFP lentivirus
21. Trypsin-EDTA solution (Lonza, catalog number: 17-161E)
22. Dulbecco's Modified Eagle Media (DMEM) (Sigma-Aldrich, catalog number: D6546)
23. Pen-Strep solution (Sigma-Aldrich, catalog number: P0781)
24. Fetal Calf Serum (FCS) (Thermo Fisher Scientific, Gibco<sup>TM</sup>, catalog number: 10270106)
25. NaCl (VWR, catalog number: 27810.295)
26. KCl (VWR, catalog number: 26764.260)
27. CaCl<sub>2</sub>·2H<sub>2</sub>O (Honeywell, Fluka<sup>TM</sup>, catalog number: 31307-500G)
28. MgSO<sub>4</sub>·6H<sub>2</sub>O (Fisher Scientific, catalog number: 15640520)  
*Manufacturer: Honeywell, Fluka<sup>TM</sup>, catalog number: M0250-500G.*
29. 60x E3 stock solution (see Recipes)
30. 1x E3 (see Recipes)
31. E3 with 0.2 mM PTU (1x E3 + 0.2 mM PTU) (see Recipes)
32. 20x Tricaine stock solution (4 g/L) (see Recipes)
33. 1 M Tris-HCl, pH 9.0 (see Recipes)
34. 1,000x PTU stock solution (0.2 M) (see Recipes)

## Equipment

1. Pipettes (100-1000 ul, 20-200 ul, 2-50 ul, 0.5-10 ul)
2. Zebrafish housing system (e.g., AQUA SCHWARZ, model: Stand-alone unit V30)
3. Mating boxes (e.g., AQUA SCHWARZ, model: SpawningBox 3, catalog number: AS 006-0642)
4. Micropipette puller (NARISHIGE, catalog number: PB-7).
5. Fluorescence stereomicroscope (e.g., ZEISS, model: SteREO Lumar. V12)
6. Micromanipulator (Eppendorf, model: InjectMan® NI 2, catalog number: 5181 000.017)
7. Microinjector, CellTramVario (Eppendorf, catalog number: 5176 000.033)
8. CEDEX XS Cell counter (also Bürker chamber is perfectly adequate)
9. Incubator, 33 °C (no CO<sub>2</sub>!!)
10. Forceps, Dumont No. 5 or similar (Sigma-Aldrich, catalog number: F6521)
11. Pipette pump (Fisher Scientific, Fisherbrand<sup>TM</sup>, catalog number: 15239805)
12. Glass-tubing cutter (Sigma-Aldrich, catalog number: Z150770)
13. Heating-block (Eppendorf, model: Thermomixer 5355)
14. Vortex mixer (Scientific Instruments, model: Vortex-Genie 2)
15. Centrifuge (Eppendorf, model: 5804)

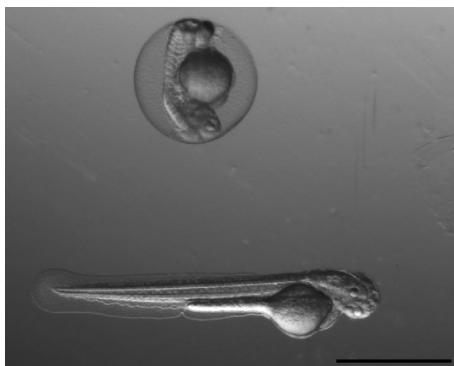
## Software

1. ImageJ/FIJI (<https://imagej.nih.gov/ij/download.html>; <https://fiji.sc/>)
2. GraphPad Prism 6.0

## Procedure

### A. Preparing the embryos

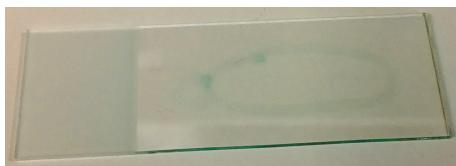
1. Set-up mating two days before the injection day (Day 1). Three males and three females in a 3 L mating box are usually enough.
2. Collect embryos in the morning around 10:00 AM (Day 0) (The zebrafish mate when the lights turn on).
3. Select healthy fertilized embryos and place on separate 10 cm dishes—50 embryos/dish in 25 ml of 1x E3 + 0.2 mM PTU (see Recipes). Place in a 33 °C incubator (no CO<sub>2</sub>!!) (Day 0).
4. On the injection day (Day 2), dissect the embryos out from the chorion membrane with fine sharp forceps (e.g., Dumont No. 5), if necessary. Many of the 2 dpf embryos may have hatched spontaneously (Figure 1).



**Figure 1. An unhatched embryo encased by chorion membrane (above) and a hatched embryo (below).** Scale bar: 1 mm.

5. Anesthetize the embryos with 200 mg/L Tricaine (MS-222, 4 g/L stock solution) (see Recipes) for 5 min.
6. Melt aliquots of 0.7% low-melting point agarose (see Recipes) at 95 °C heating block. Let the block cool down to 37 °C before the next step.  
*Note: Low-melting point agarose stays liquid at 37 °C.*
7. Add 53 µl of 4 g/L Tricaine-stock solution into 1 ml of 0.7% low-melting point agarose (final concentration of agarose is 0.66%). Vortex briefly, and spin down. Place the tube back to 37 °C.
8. Prepare mounting slide. Take a normal glass microscopy slide and wipe it clean. Draw a bigger ellipse with a PAP-pen (for embryos) and a small circle (for 10 µl of cells) (Figure 2). Allow

PAP-smear to dry for a couple of minutes. These hydrophobic linings will help to keep liquids in the correct place on the glass slide.



**Figure 2. Glass microscope slide with PAP-pen smears (faint greenish color)**

9. Add 15 dechorionated and anesthetized embryos in the middle of the larger circle (Figure 3).



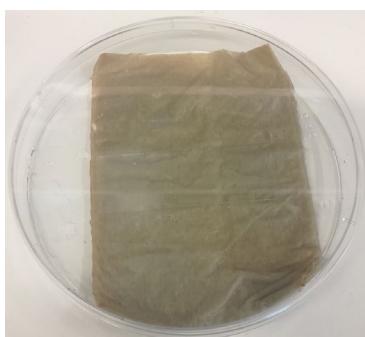
**Figure 3. Embryos in a droplet of E3 on the microscope slide**

10. Add 200  $\mu\text{l}$  of low-melting-point agarose and align the embryos gently using a gentle orientation tool (e.g., a pipette tip with a bit of attached nylon line) (Figure 4).



**Figure 4. Aligned embryos inside the agarose on a microscope slide.** Orientation tool made of two pipette tips and a nylon line is in the front.

11. Allow the agarose to solidify. (The embryos stay alive within the agarose.) Store mounted embryos in a large (150 mm) humidified Petri dish (Figure 5), until ready to inject.



**Figure 5. A humidified Petri dish.** Wetted paper towel is placed on the bottom of the Petri

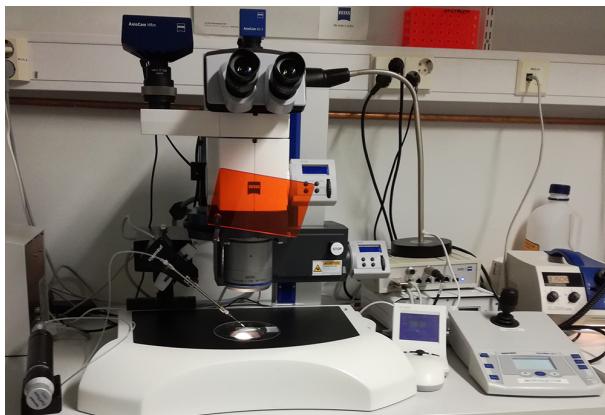
dish and covered with a lid. To prevent drying, the microscope slides with mounted embryos are placed in this chamber.

#### B. Preparing the cells

1. Use a 10 cm plate of GFP expressing tumor cells (50-90% confluent)/treatment.
2. Wash cells 2 times with PBS.
3. Aspirate PBS, add 3 ml of Trypsin-EDTA solution.
4. Incubate for 5 min at 37 °C, or until cells have detached.
5. Collect detached cells into a 15 ml Falcon tube and add 5 ml of DMEM + 10% FCS.
6. Pellet cells (180 x g), 3 min and resuspend in 10 ml of PBS.
7. Pellet cells (180 x g), 3 min and resuspend in 10 ml of PBS.
8. Pellet cells (180 x g), 3 min and resuspend in 1 ml of PBS.
9. Optional step, if using a cell line prone to clumping:
  - a. Pipet the cell suspension into a FACS tube through a 40 µm mesh cap (BD Falcon).
  - b. Pellet cells with a centrifuge for 3 min (180 x g).
10. Aspirate all liquid and resuspend the cells in 20 µl of 2% PVP/PBS injection solution. (Addition of PVP is optional, but helps to maintain stable cell suspension during injections.)
11. Count cell density (2 µl of cell suspension, 198 µl of PBS) using CEDEX XS cell counter.
12. Dilute into a final concentration of 3-10 × 10<sup>7</sup> cells/ml. In 4 nl injection, this is 120-400 cells.
13. Store cell suspensions on ice until ready to inject.

#### C. Injection of tumor cells into embryos

1. Place an empty glass capillary needle (self-made capillary without filament, e.g., TW100-4, WPI) in CellTram injector. (Figure 6)



**Figure 6. Microinjection station**

2. Break a large enough opening so that cells can be easily expelled (> 25 µm, a larger tip helps to get more cells in embryo but generates more damage to the embryo and is harder to get through the skin of the embryo) (Video 1).

**Video 1. Breaking the capillary needle**

3. Gently rotate the oil outwards, until all air bubbles are out and oil comes out from the tip of the needle (Video 2).

**Video 2. Expelling the air bubbles**

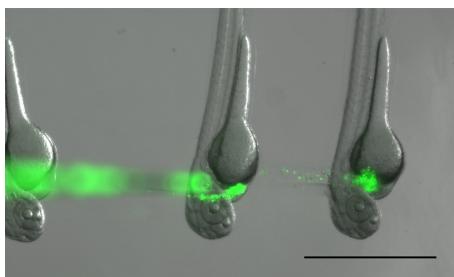
4. Gently touch the side of the tip with a paper tissue to absorb the excess oil.

*Note: The glass capillary is extremely fragile!*

5. Add 10  $\mu\text{l}$  of cell suspension in the smaller circle on the slide. Go to the injection work station.
6. Move the needle into cell suspension and draw some cell suspension into the needle (Video 3). Monitor under a stereomicroscope that some liquid and cells actually go into the needle and it doesn't get stuck.

**Video 3. Filling the capillary needle**

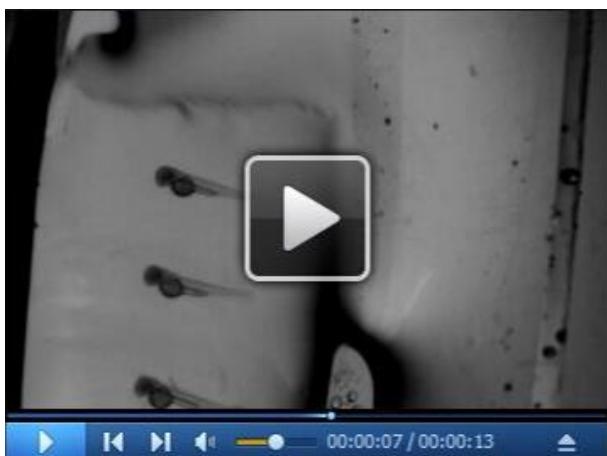
7. Next, inject the embryos (Video 4). Move the needle through the skin of the embryo. Try to hit an empty space around the heart (pericardial cavity). Expel a little bit of cell suspension into the cavity (one fine notch with CellTram). Look that some cells actually went into the embryo and then move the needle out (Figure 7).

**Video 4. Injection of tumor cells into pericardiac space of the embryos****Figure 7. Embryos right after injection with GFP labeled tumor cells.** Often some tumor cells escape from the injection site when the needle is withdrawn. Scale bar: 1 mm.

8. Move to the next embryo. Continue until all embryos are injected. In one session easily > 50 embryos can be injected.
9. After injections, discard the needle carefully according to your institutional guidelines.
10. Break the gel using fine forceps (Video 5) and by flushing the gel pieces into a dish with 1x E3-medium (Video 6) (see Recipes). Pipette up and down with a large bore Pasteur pipette to release the embryos from the gel (Video 7). Move the released embryos to a new Petri dish with fresh 1x E3 + 0.2 mM PTU (see Recipes).



**Video 5. Breaking the gel with forceps**



**Video 6. Releasing the gel pieces from the microscope slide**

**Video 7. Releasing the embryos from the gel**

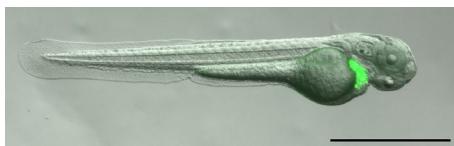
11. Add antibiotics (1:100 Pen-strep) and incubate 50 embryos/dish at 33 °C until transplanted embryos are selected for the experiment.

**Notes:**

- a. *Culturing the embryos at a higher temperature (33 °C) than usually (28.5 °C) facilitates the growth of human tumor cells in this model.*
- b. *Without a separate animal experiment license, the experiment has to be ended at latest when the embryos are 5 days old !!! Consult your Animal Experiment Board for details.*

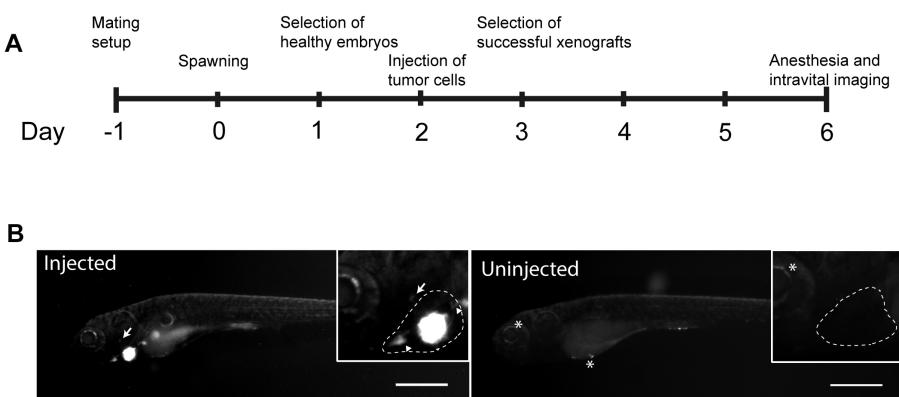
**D. Imaging**

1. At 1-day post-injection (1 dpi) anesthetize embryos with 200 mg/L Tricaine and select successfully transplanted and healthy embryos (Figure 8) under a stereomicroscope into the experiment.



**Figure 8. A healthy transplanted embryo.** Scale bar: 1 mm.

2. Place 1 embryo/well in a 12-well plate with 2 ml of E3 + Tricaine, orient gently with an orientation tool and image both on GFP and bright field channels. After all the embryos have been imaged, aspirate excess medium out carefully and place 2 ml of fresh 1x E3 + 0.2 mM PTU medium in the wells. (Do not let the embryos dry!)
3. At 4 days post-injection anesthetize the embryos with tricaine again. Image embryos again using both GFP and bright field channels. (Figure 9)



**Figure 9. Timeline of the experiment and examples of the results.** A. Time line of zebrafish xenograft experiments. B. Intravital fluorescence microscopy images of six dpf zebrafish embryos taken four days post injection (4 dpi). Fluorescence in GFP channel is shown. Scale bar, 500  $\mu$ m. Inset shows magnification of the primary tumor. Tumor cells invading outside pericardial space are marked with an arrow, invading cells in the pericardial cavity with a triangle and unspecific fluorescence in eye and yolk sac with an asterisk (\*). The outline of the pericardial cavity is depicted with dashed line. Figure and text reproduced and modified from Pekkonen *et al.* (2018).

### Data analysis

1. In the image analyses, use ImageJ/FIJI software.
2. Subtract background (subtract background > rolling ball radius 25) in the GFP channel.
3. Outline the primary tumor area using a segmented line tool. Measure fluorescence intensity and shape (circularity) of the primary tumor.
4. Count manually the cells that have invaded outside the pericardial cavity. Adjust contrast/brightness so that you can see individual cells. These are much dimmer than the bright primary tumor.  
*Note: Autofluorescence is often observed in the lens and in the yolk.*
5. For statistical analyses, use GraphPad Prism 6.0 software (other statistical software is ok too). Perform non-parametric Mann-Whitney (2 groups) or Kruskal-Wallis test (for > 2 groups).
6. For examples of the results, please see Figure 9b above and more detailed in Figure 7 in (Pekkonen *et al.*, 2018).

### Notes

1. For handling the embryos, a glass Pasteur pipette with a pipette pump works the best. The tip needs to be expanded by cutting it with a glass cutter for safe transport of embryos. Also, plastic Pasteur pipettes can be used.
2. Phenylthiourea (PTU) can be used to prevent pigmentation in non-albino zebrafish strains.

PTU is neurotoxic → wear gloves and handle stock solution in chemical hood!

3. Xenograft assay also works with many other, but not all, cell types, melanoma cell line WM852 is used here as an example.
4. Sometimes the size of the implanted tumors may vary, if this turns out to be a problem, the relative tumor growth can be calculated and the number of invading cells normalized to the size of the primary tumor.
5. Due to technical and biological variability, a fairly large number of embryos (> 25) need to be xenografted and analyzed to gain robust results. This can be achieved by combining data from multiple independent experiments.
6. If one desires to implant a very large number of embryos (> 200/day), it is recommended to perform the experiment working as a pair. One researcher injects the embryos and the other one mounts and dismounts the xenografted embryos.
7. Embryos are fragile and can break easily. Avoid forceful handling and touching of embryos with sharp objects (other than the capillary needle).
8. Capillary needle is extremely sharp and filled with cancer cells. Caution is required so that the researcher doesn't inject him/herself.
9. During low-resolution imaging with stereomicroscope, the mounting of anesthetized embryos is not necessary. If one desires to perform higher resolution imaging e.g., using confocal microscope, the mounting of embryos using low-melting point agarose on glass-bottom dishes is required.

## Recipes

1. 60x E3 stock solution (1 L) (Nüsslein-Volhard *et al.*, 2002)

17.2 g NaCl

0.76 g KCl

2.9 g CaCl<sub>2</sub>·2H<sub>2</sub>O

4.9 g MgSO<sub>4</sub>·7H<sub>2</sub>O

2. 1x E3

5 mM NaCl

0.17 mM KCl

0.33 mM CaCl<sub>2</sub>·2H<sub>2</sub>O

0.33 mM MgSO<sub>4</sub>·7H<sub>2</sub>O

Make by adding 16.67 ml of 60x stock solution into a 1 L vessel and fill up to 1 L with MilliQ water

3. E3 with 0.2 mM PTU (1x E3 + 0.2 mM PTU)

Add 1 ml of 1,000x PTU stock to 1,000 ml of E3

4. 20x Tricaine stock solution (4 g/L)

Make by adding 4.0 g of Tricaine into a 1 L vessel and fill up to 1 L with 1x E3 medium

- To adjust the pH to 7, add approximately 5 ml of Tris-HCl, pH 9.0
5. 1 M Tris-HCl, pH 9.0  
Make by adding 12.14 g of Tris into 100 ml vessel  
Add 100 ml of MilliQ water and adjust the pH to 9.0 using HCl and/or NaOH
  6. 1,000x PTU stock solution (0.2 M)  
3.044 g of PTU in 100 ml of ethanol  
*Note: This solution is toxic, store in an air-tight bottle.*
  7. 0.7% low-melting point agarose
    - a. Add 0.7 g of low-melting point agarose powder into 100 ml of 1x E3 medium
    - b. Heat in microwave until agarose has melted and the solution is completely clear
    - c. Aliquot 1 ml of solution into 1.5 ml microcentrifuge tubes and store at -20 °C until used

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These procedures have been evolved from the number of earlier work dealing with zebrafish embryo xenografting (Lee *et al.*, 2005; Nicoli *et al.*, 2007; Teng *et al.*, 2013; White *et al.*, 2013; Chapman *et al.*, 2014; Veinotte *et al.*, 2014; Xie *et al.*, 2015; Yen *et al.*, 2014).

### **Competing interests**

The authors have no conflicts of interest or competing interests.

### **Ethics**

Experimentation with zebrafish was performed under license ESAVI/9339/04.10.07/2016 issued by national Animal Experimentation Board (Regional State Administrative Agency for Southern Finland).

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## Adoptive Transfer of Monocytes Sorted from Bone Marrow

Damya Laoui<sup>1, 2, \*</sup>, Eva Van Overmeire<sup>1, 2</sup>, Chloé Abels<sup>1, 2</sup>,  
Jiri Keirsse<sup>1, 2</sup> and Jo A Van Ginderachter<sup>1, 2</sup>

<sup>1</sup>Myeloid Cell Immunology Lab, VIB Center for Inflammation Research, Brussels, Belgium; <sup>2</sup>Lab of Cellular and Molecular Immunology, Vrije Universiteit Brussel, Brussels, Belgium

\*For correspondence: [dlaoui@vub.be](mailto:dlaoui@vub.be)



**[Abstract]** Inflammatory Ly6C<sup>hi</sup> monocytes can give rise to distinct mononuclear myeloid cells in the tumor microenvironment, such as monocytic myeloid-derived suppressor cells (Mo-MDSC), immature macrophages, M2-like tumor-associated macrophages (TAMs), M1-like TAMs or monocyte-derived dendritic cells (Mo-DCs). This protocol describes a method to assess the fate and recruitment of inflammatory Ly6C<sup>hi</sup> monocytes in the tumor microenvironment.

**Keywords:** Adoptive transfer, Monocytes, Pre-cDCs, Tumor-associated macrophages, Tumor-associated dendritic cells, Cell sorting

**[Background]** Tumors are heterogeneous microenvironments where complex interactions take place between neoplastic cells and infiltrating inflammatory cells, such as tumor-associated macrophages (TAMs) and tumor-associated dendritic cells (TADCs). The relevance of tumor-infiltrating mononuclear myeloid cells is underscored by clinical studies showing a correlation between their abundance and poor prognosis (Bolli *et al.*, 2007). The origin of TAMs and TADCs has been a matter of debate, since several levels of complexity result in considerable TAM and TADC heterogeneity (Movahedi *et al.*, 2010; Laoui *et al.*, 2014; Laoui *et al.*, 2016; Van Overmeire *et al.*, 2016; Kiss *et al.*, 2018). Here, we describe a valuable method to adoptively transfer bone-marrow derived monocytes permitting the assessment of their recruitment and fate in tumors.

### **Materials and Reagents**

1. Polyester filters cut in 10 x 10 cm squares, thread diameter 70 µm (SpecturmLabs, catalog number: 146490)
2. 10 ml syringes (Omnifix, catalog number: 473203)
3. 1 ml syringes (Greiner, catalog number: 470203)
4. 27 G needles (BD Bioscience, catalog number: 300635)
5. 25 G needles (BD Biosciences, catalog number: 300400)
6. 19 G needles (BD Biosciences, catalog number: 301500)
7. Falcon standard tissue culture dish (Fisher Scientific, catalog number: 353003)
8. BD Falcon 50 ml polypropylene tubes (BD Biosciences, catalog number: 2070)
9. BD Falcon 15 ml polypropylene tubes (BD Biosciences, catalog number: 2096)

10. BD Falcon 5 ml polypropylene round-bottom tube (BD Biosciences, catalog number: 352063)
11. 70 µm sterile nylon gauze
12. LS columns (Miltenyi, catalog number: 130-042-401)
13. Naive mice: Age preferably between 6 and 12 weeks, strain can vary depending on the experiment/project (in this example we used C57BL/6 mice)
14. Ethanol absolute analaR Normapur ACS (VWR Chemicals, catalog number: 84857360)
15. RPMI-1640 medium (RPMI) (Life Technologies, catalog number: 52400-041)
16. Fetal calf serum (FCS) (Life Technologies, Gibco, catalog number: DE14-801F)
17. L-glutamine (Life Technologies, catalog number: 25030-024)
18. Penicillin-streptomycin (Life Technologies, catalog number: 15140-130)
19. Ammonium chloride (NH<sub>4</sub>Cl) (Merck KGaA, catalog number: 1011450500)
20. Potassium bicarbonate (KHCO<sub>3</sub>) (Merck KGaA, catalog number: 104852)
21. EDTA (Duchefa Biochemie, catalog number: E0511.1000)
22. Hank's buffered salt solution (HBSS) (Life Technologies, Gibco, catalog number: 14175129)
23. Anti-CD11b microbeads (Miltenyi, catalog number: 130-049-601)
24. Purified CD16/CD32 (FcBlock) (clone 2.4G2) (BD Biosciences, catalog number: 553142)
25. PE-Cy7-conjugated anti-CD11b antibody (clone M1/70) (BD Biosciences, catalog number: 552850)
26. AF647-conjugated anti-Ly6C antibody (clone ER-MP20) (Serotec, catalog number: MCA2389A647)
27. PerCP-Cy5.5-conjugated anti-I-A/I-E (MHC-II) antibody (clone M5/114.15.2) (BioLegend, catalog number: 107626)
28. FITC-conjugated anti-Ly6G antibody (clone 1A8) (BD Biosciences, catalog number: 551460)
29. APC-Cy7-conjugated anti CD45 (clone 30-F11) (BioLegend, catalog number: 103116)
30. CellTrace Violet (Thermo Fisher Scientific, Molecular probes™, catalog number: C34557)
31. Trypan blue (Life Technologies, Gibco, catalog number: 15250061)
32. DMSO
33. 70% ethanol (see Recipes)
34. Complete medium (see Recipes)
35. Erythrocyte lysis buffer (see Recipes)
36. MACS buffer (see Recipes)
37. Sorting buffer (see Recipes)
38. Violet tracer (see Recipes)

## Equipment

1. Sterile culture hood, PSM Optimale 18 (ADS)
2. Surgical scissors and forceps
3. 37 °C, 5% CO<sub>2</sub> cell culture incubator (Binder, VWR)

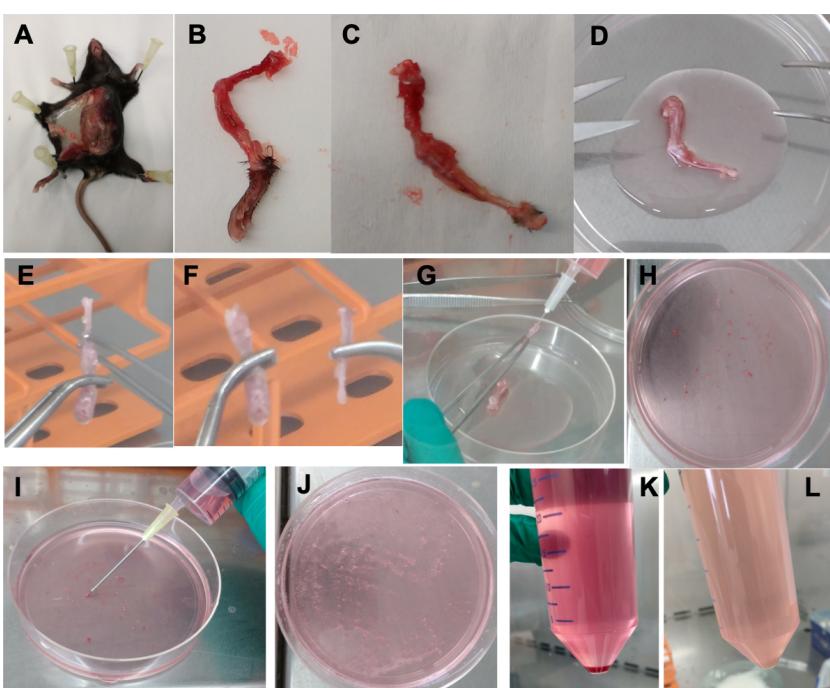
4. Pipettes (Gilson)
5. Centrifuges 5810 R (Eppendorf, model: 5810 R)
6. Shaker KS 260 Basic (IKA, model: KS 260 basic)
7. Microscope Eclipse TS100 (Nikon, model: Eclipse TS100)
8. MidiMACS™ Separator and MultiStand (Miltenyi, catalog number: 130-042-301)
9. Multicolor FACS Sorter-FACS Aria II (BD Biosciences Aria flow cytometer)

## **Procedure**

### A. Preparation of a bone-marrow single cell suspension

1. Sacrifice a naive mouse and restrain it by pinning its paws into a foam surface using syringe needles. Disinfect the skin of the mouse with 70% ethanol (see Recipe 1). Make a parallel incision from the base of the tail up to the neck along the mouse's abdomen and to the paws without puncturing the peritoneum. Gently pull back the skin and pin it to the foam surface to expose the hind limb (Figure 1A).
2. Cut the hind limb free from the skin and the body by cutting in the pelvis just behind the femur-pelvis joint. Keep the femur and tibia whole. Try to remove as much excess tissue (muscles, fibers...) surrounding the bone as possible using scissors or with a scalpel (Figure 1B). Do this procedure gently in order to avoid breakage of the bones.
3. Gently pull the hind paw from the limb by moving it back and forwards (Figure 1C).
4. Clean the bone by submerging it in 70% ethanol and store the bone in 5 ml complete medium (see Recipe 2) in a 50 ml Falcon tube on ice.
5. Repeat this action with the second hind limb.
6. Detach the tibia from the femur and cut the fibula and patella away and put the bones in a Falcon standard tissue culture dish (Figures 1D, 1E and 1F).
7. Insert a 27 G needle attached to a 10 ml syringe containing complete medium in the femur and gently flush the femur with 10 ml complete medium (Figures 1G and 1H). Before flushing, the tibia cut the 'white' bone that was adjacent to the paw away and insert the needle in the bone at the other side. If any resistance is perceived when flushing the bones, cut a small fraction of the bone from the end and reinsert the needle in the remaining bone. The flushing is complete when all the red bone marrow is in the plate, and red tissue can no longer be seen in the bone.
8. Homogenize the bone-marrow by passing (aspirating and pressing) the medium containing bone-marrow two times through the 19 G needle (Figures 1I and 1J).
9. Filter the bone-marrow suspensions through a 70 µm sterile nylon gauze into a sterile 50 ml conical tube and wash the culture plate and the gauze with 10 ml complete medium.
10. Centrifuge the 50 ml tubes at 450  $\times$  g for 6 min at 4 °C and discard the supernatants (Figure 1K).
11. Remove the red blood cells by resuspending the pellet in 5 ml erythrocyte lysis buffer (see Recipe 3) and leave at room temperature for 2 min.

12. Neutralize by adding 15 ml complete medium, and transfer the suspension to a new 50 ml tube through a 70  $\mu$ m sterile nylon gauze.
13. Centrifuge the 50 ml tubes at 450  $\times g$  for 6 min at 4 °C and discard the supernatants (Figure 1L).
14. Count the living cells using trypan blue and resuspend the cells in MACS buffer (see Recipe 4) at a concentration of 10<sup>8</sup> cells/ml. From the total bone marrow, in general, 10% to 15 % normally would constitute monocytes (varies slightly with age, mouse strain, and animal house type), which can be enriched/purified as explained below.



**Figure 1. Bone-marrow single-cell preparation.** A. Naive mouse. B. Hind limb with paw. C. Hind limb without paw. D. Cleaned hind limb without paw. E. Detaching of the tibia from the femur. F. Femur (left) and tibia (right). G. Flushing the bones. H. Bone marrow right after flushing. I. Homogenization of the bone marrow. J. Homogenized bone-marrow single cell suspension. K. Cell pellet before erythrocyte lysis buffer. L. Cell pellet after erythrocyte lysis buffer.

- B. Purification of Ly6C<sup>high</sup> monocytes from the bone marrow
1. Add a 5  $\mu$ l aliquot of anti-CD11b magnetic microbeads per 10<sup>7</sup> cells and incubate for 20 min at 4 °C on an orbital shaker at 50 rpm.
  2. Wash by adding 10 ml MACS buffer, centrifuge at 450  $\times g$  for 6 min at 4 °C and discard the supernatants.
  3. Place an LS column in a MidiMACS™ Separator attached to a magnetic MultiStand and wash it by putting 3 ml MACS buffer on the top. The liquid passes the column by gravity.

4. Resuspend the pelleted cells in 1 ml MACS buffer and pipette the labeled cell suspension on top of the LS separation column. When the cell suspension has passed through the column, wash the column by adding 3 x 3 ml MACS buffer.
5. Remove the LS column from the separator, add 5 ml MACS buffer on top of the column and immediately flush the column by firmly pressing the provided plunger on the column to wash the magnetically labeled cells out in a sterile 15 ml tube.
6. Incubate the CD11b<sup>+</sup> cell suspension with rat anti-mouse CD16/CD32 (10 µg per 10<sup>7</sup> cells) on ice-cold water for 20 min, in order to block the Fc receptors present on the cells' surface.
7. Incubate the cell suspension with fluorescently labeled antibodies of interest (1 µg per 10<sup>7</sup> cells) for another 20 min on ice-cold water, protected from exposure to light.
8. Wash by adding 10 ml MACS buffer, centrifuge at 450 x g for 6 min at 4 °C and discard the supernatants.
9. Meanwhile, precoat 5 ml polypropylene round-bottom tubes and 15 ml tubes with heat-inactivated fetal calf serum, add respectively 1 ml or 2 ml heat-inactivated fetal calf serum. Shake the tubes gently by hand so that the heat-inactivated fetal calf serum covers the whole surface of the tube, and discard the excess of heat-inactivated fetal calf serum. This will prevent the cells to stick to the tubes and hence enhance the recovery of cells.
10. Resuspend the pellet in 1 ml sorting buffer (see Recipe 5) per 10<sup>7</sup> cells and transfer into a sterile 5 ml polypropylene round-bottom tube precoated with heat-inactivated fetal calf serum.
11. Ly6C<sup>high</sup> monocytes can be sorted on a BD FACS Aria as CD45<sup>pos</sup> CD11b<sup>pos</sup> Ly6G<sup>neg</sup> Ly6C<sup>high</sup> MHC-II<sup>neg</sup> cells (Laoui *et al.*, 2016; Van Overmeire *et al.*, 2016).
12. Collect the sorted monocytes in 15 ml tubes precoated with heat-inactivated fetal calf serum containing 3 ml complete medium.

#### C. Labeling of the monocytes for *in vivo* tracking

1. Option I: Bone-marrow donor mice are wild-type mice (Laoui *et al.*, 2016). Ideally, the donor and recipient differ in their CD45 allele (CD45.1 vs. CD45.2).
  - a. Centrifuge the 15 ml tubes containing the sorted monocytes at 450 x g for 10 min at 4 °C and discard the supernatant.
  - b. Resuspend the cell pellet in HBSS at a concentration of 10<sup>6</sup>/ml and add 1 µl of CellTrace (see Recipe 6) to stain 1 ml cells (hence a 1:1,000 dilution). It is important that the labeling happens in a protein-free medium. Incubate the cell suspension for 20 min at 37 °C, protected from exposure to light.
  - c. Wash by adding 10 ml HBSS, centrifuge at 450 x g for 6 min at 4 °C and discard the supernatant.
  - d. Resuspend the cell pellet in HBSS at a concentration of 5 x 10<sup>6</sup>/ml and keep on ice till entering the animal facility.
  - e. Inject the recipient CD45.2 mice with 200 µl intravenously in the tail vein using a 25 G needle and a 1 ml syringe (Video 1).



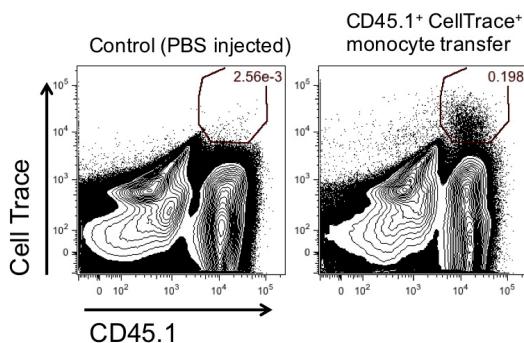
**Video 1. Intravenously tail vein injection.** This video was made at Vrije Universiteit Brussels according to guidelines from the Belgian Council for Laboratory Animal Sciences and were approved by the Ethical Committee for Animal Experiments of the Vrije universiteit Brussels (license 15-220-2).

- f. The progeny of the monocytes can be traced as from one day, until 10 days after inoculation (ideally 48 to 72 h). The monocyte-derived cells can be traced in the Pacific Blue-channel (405 nm) by flow cytometry. In addition, CD45.1 and CD45.2 can be used as a complementary staining.
2. Option II: Bone-marrow donor mice are Ubiquitin-GFP mice (Van Overmeire et al., 2016).
  - a. Centrifuge the 15 ml tubes containing the sorted monocytes at  $450 \times g$  for 10 min at  $4^{\circ}\text{C}$  and discard the supernatant.
  - b. Resuspend the cell pellet in HBSS at a concentration of  $5 \times 10^6/\text{ml}$  and keep on ice till entering the animal facility.
  - c. Inject the recipient CD45.2 mice with 200  $\mu\text{l}$  of cells intravenously in the tail vein using a 25 G needle and a 1 ml syringe (Video 1).
  - d. The progeny of the monocytes can be traced as from one day, until 10 days after inoculation (ideally 48 to 72 h). The monocyte-derived cells can be traced in the FITC-channel by flow cytometry.

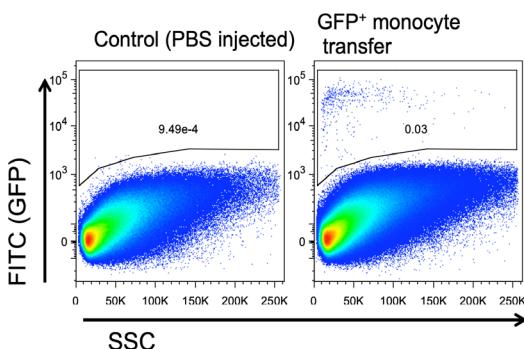
### Data analysis

Flow cytometry is commonly used to visualize the transferred monocytes in the tumors of the recipient mice. As the transferred monocytes are diluted systemically in the recipient mice, only few cells will reach the tumors or other organs of interest and differentiate *in situ* into macrophages or dendritic cells (Figures 2 and 3). Hence, it is important to acquire many events by flow cytometry. After gating on the transferred CellTrace<sup>+</sup> or GFP<sup>+</sup> cells, the fate of their progeny can be

determined using additional markers specific for monocytic myeloid-derived suppressor cells (Mo-MDSC), immature macrophages, M2-like tumor-associated macrophages (TAM), M1-like TAM or monocyte-derived dendritic cells (Laoui *et al.*, 2016; Van Overmeire *et al.*, 2016). For these type of experiments, two independent repeats containing each  $n \geq 3$  are generally accepted as many donor mice can be needed for acquiring enough cell in the receptor mice.



**Figure 2. Adoptive transfer of CellTrace-labeled CD45.1<sup>+</sup> monocytes in CD45.2 recipient mice.** One million CellTrace-labeled CD45.1<sup>+</sup> monocytes were adoptively transferred to 11-day old LLC tumor-bearing mice. Two days after CellTrace-labeled CD45.1<sup>+</sup> monocytes transfer, mice were sacrificed and tumors were collected. Graphs show the percentage of GFP<sup>+</sup>Ly6C<sup>hi</sup> monocytes present in total tumors.



**Figure 3. Adoptive transfer of GFP<sup>+</sup> monocytes in CD45.2 recipient mice.** One million GFP<sup>+</sup> monocytes were adoptively transferred to 11-day old LLC tumor-bearing mice. Four hours after GFP<sup>+</sup> monocyte transfer, mice were sacrificed, and tumors were collected. Graphs show the percentage of GFP<sup>+</sup>Ly6C<sup>hi</sup> monocytes present in total tumors.

## Recipes

1. 70% ethanol (for 100 ml)  
70 ml 99.9% ethanol (VWR Chemicals)  
30 ml demineralized water
2. Complete medium

- Roswell Park Memorial Institute (RPMI)-1640  
10% (v/v) heat-inactivated fetal calf serum (FCS)  
 $300 \mu\text{g}\cdot\text{ml}^{-1}$  L-glutamine  
 $100 \text{ U}\cdot\text{ml}^{-1}$  penicillin  
 $100 \mu\text{g}\cdot\text{ml}^{-1}$  streptomycin
3. Erythrocyte lysis buffer  
8.29 g·L<sup>-1</sup> NH<sub>4</sub>Cl  
1 g·L<sup>-1</sup> KHCO<sub>3</sub>  
37.2 mg·L<sup>-1</sup> EDTA  
Bring at pH 7.2
4. MACS buffer  
Hank's buffered salt solution  
0.5% (v/v) heat-inactivated fetal calf serum  
2 mM EDTA
5. Sorting buffer  
Hank's buffered salt solution  
0.5% (v/v) heat-inactivated FCS  
5 mM EDTA
6. Violet tracer  
Resuspend CellTrace in 20 µl DMSO (provided)

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### **Competing interests**

The authors declare no competing financial interests.

### **Ethics**

All procedures followed the guidelines of the Belgian Council for Laboratory Animal Science and were approved by the Ethical Committee for Animal Experiments of the Vrije Universiteit Brussel (licenses 11-220-3 and 15-220-2).

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## Calvarial Bone Implantation and *in vivo* Imaging of Tumor Cells in Mice

Kyoko Hashimoto<sup>1, #</sup>, Shingo Sato<sup>1, 2, #, \*</sup>, Hiroki Ochi<sup>1</sup>, Shu Takeda<sup>3</sup> and Mitsuru Futakuchi<sup>4</sup>

<sup>1</sup>Department of Physiology and Cell Biology, Tokyo Medical and Dental University (TMDU), Graduate School, Tokyo 113-8519, Japan; <sup>2</sup>Department of Orthopaedic Surgery, Tokyo Medical and Dental University (TMDU), Graduate School, Tokyo 113-8519, Japan; <sup>3</sup>Division of Endocrinology, Toranomon Hospital Endocrine Center, Tokyo 105-8470, Japan; <sup>4</sup>Department of Pathology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, 852-8523, Japan

\*For correspondence: [satoshin.phy2@tmd.ac.jp](mailto:satoshin.phy2@tmd.ac.jp)



#Contributed equally to this work

**[Abstract]** Bone is one of common metastasis sites for many types of cancer. In bone metastatic microenvironment, tumor-bone interactions play a significant role in the regulation of osteolytic or osteoblastic bone metastasis. In order to investigate the direct interaction between tumor cells and bone tissue, it is essential to generate appropriate animal models that mimic the behavior of tumor cells in bone metastatic lesions. Calvarial implantation model (bone invasion model) is a newly-established animal model that accurately recapitulates the behavior of tumor cells in the tumor-bone microenvironment. The surgical technique for tumor cell implantation is simpler than intracardiac, intra-arterial, or intraosseous injection techniques. This model can be useful for the identification of key factors driving tumor-induced osteolytic or osteoblastic changes.

**Keywords:** Tumor microenvironment, Tumor-bone interaction, Tumor cell implantation, Calvaria implantation model, Osteolytic bone metastasis, Osteoblastic bone metastasis

**[Background]** Bone is one of frequent metastasis sites for many types of cancer, including prostate, breast, and lung cancer. Based on their radiographic appearance, bone metastatic lesions are mainly classified into two types: osteolytic or osteoblastic lesions. These lesions are induced by an imbalance between bone formation and bone resorption in the tumor-bone microenvironment. In bone metastatic lesions, tumor cells interact with many types of cells, including osteoblasts, osteoclasts, or mesenchymal stem cells. According to previous reports, the increased bone-resorbing or bone-forming activity is mediated by many paracrine factors secreted by metastasized tumor cells. However, the detailed mechanisms responsible for the osteolytic or osteoblastic phenotype remain to be fully elucidated. To investigate the molecular mechanisms underlying how metastasized tumor cells can induce osteolytic or osteoblastic phenotypes, it is essential to generate animal models that mimic the behavior of tumor cells in bone metastatic lesions.

Calvarial implantation model (bone invasion model) was originally established by Mitsuru Futakuchi and his colleagues [Lynch *et al.*, 2005]. This model accurately recapitulates the behavior of various tumor cells (e.g., rat prostate cancer cells [Lynch *et al.*, 2005], murine breast cancer cells [Wilson *et al.*, 2008], etc.) in bone metastatic microenvironment. In addition, surgical technique required for the

implantation of tumor cells is simpler than intracardiac, intra-arterial or intraosseous injection techniques. On the other hand, this model has a couple of limitations. It does not represent the metastasis process from primary organs to bone. Besides, calvaria is not a typical metastasis site for most of cancers.

This model is especially useful in identifying key factors driving tumor-induced osteolytic or osteoblastic changes. Using this model, Futakuchi and his colleagues has demonstrated an important role of MMP-7, cathepsin G, or transforming growth factor  $\beta$  (TGF $\beta$ ) signaling in tumor-induced osteolysis or tumor growth in bone metastatic microenvironment (Lynch *et al.*, 2005; Sato *et al.*, 2008; Wilson *et al.*, 2008; Futakuchi *et al.*, 2009). In another study, the effect of bisphosphonate or RANKL-targeting therapy was evaluated using this model (Hikosaka *et al.*, 2006; Futakuchi *et al.*, 2018). Hashimoto *et al.* also used this model to investigate the tumor-stromal interactions by cancer-secreted microRNAs (Hashimoto *et al.*, 2018).

### **Materials and Reagents**

1. Pipette tips
2. 1 ml tuberculin syringe (TERUMO, catalog number: SS-01T)
3. 21 G needle (TERUMO, catalog number: NN-2138R)
4. 23 G needle (TERUMO, catalog number: NN-2325R)
5. 15 ml or 50 ml tube
6. Immunodeficient mice (8-10-week-old BALB/c nu/nu or NOD-SCID mice)
7. Tumor cells (e.g., 4T1, Cl66, and Cl66M2 murine breast cancer cells [Wilson *et al.*, 2008], rat prostate cancer cells [Lynch *et al.*, 2005], MDA-MB-231-Luc human breast cancer cells [Cell Biolabs, catalog number: AKR-231], etc.)  
*Note: Luciferase stably expressing tumor cells (e.g., MDA-MB-231-Luc cells) were used for IVIS in vivo imaging system.*
8. Anesthetic agents  
A combination anesthetic was prepared with 0.3 mg/kg of medetomidine (ZENOAQ), 4.0 mg/kg of midazolam (Sandoz K.K.), and 5.0 mg/kg of butorphanol (Meiji Seika Pharma Co., Ltd.)
9. PBS
10. Matrigel Matrix Basement (Corning, catalog number: 354234, storage temperature -20 °C)
11. D-luciferin (Summit Pharmaceuticals International, catalog number: XFL-1, storage temperature -20 °C)
12. 2.5 g/L Trypsin/1 mmol/L EDTA solution (NACALAI TESQUE, catalog number: 32777-15)

### **Equipment**

1. Pipettes
2. Centrifuge (TOMY, EX-125)

3. IVIS *In Vivo* Imaging System (PerkinElmer, IVIS Lumina III)
4. Micro-computed tomography (Scan: Rigaku, R\_mCT2; Analysis: Ratoc, TRI/FCS-BON)

## **Procedure**

### A. Tumor cell preparation

1. Culture tumor cells according to the indicated culture method.

*Note: 4T1, Cl66, and Cl66M2 murine breast cancer cells, MDA-MB-231-Luc human breast cancer cells, and rat prostate cancer cells were used in the author's previous studies.*

2. Aspirate the culture media and add PBS to rinse the cells.

3. Aspirate PBS and add 0.25% trypsin/EDTA solution.

*Note: Ensure the solution covers the entire surface of the cells by tilting the culture plate.*

4. Incubate the cells at 37 °C for a few minutes in a humidified 5% CO<sub>2</sub> environment.

5. Add the culture media to neutralize the trypsin, detach the adherent cells by pipetting the media back and forth, and make a single cell suspension.

6. Transfer the cell suspension to a 15 ml or 50 ml tube and centrifuge it at 500 x g for 5 min at 4 °C.

7. Aspirate the supernatant, resuspend the cell pellet in cold PBS, and count the cell number.

8. Centrifuge the cell suspension again and resuspend the cell pellet with cold Matrigel at 3 x 10<sup>6</sup> cells per 100 µl Matrigel.

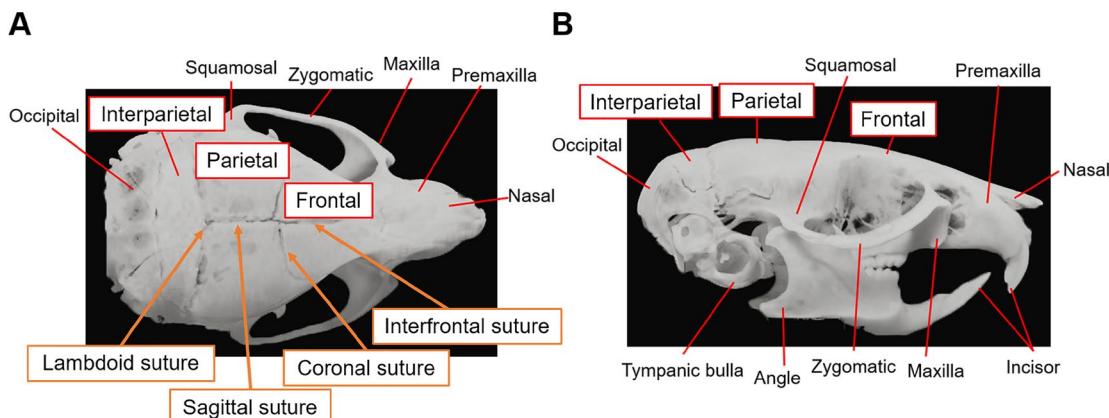
*Note: Matrigel is a liquid at 4 °C. It polymerizes into a solid gel when maintained at 24-37 °C.*

9. Keep the resuspended cells on ice until the cells are implanted.

### B. Tumor cell implantation on mouse calvarial bone (Figure 2)

#### Anatomy of mouse calvarial bone

Mouse calvarial bone is mainly composed of frontal, parietal and interparietal bones (Figures 1A and 1B). There are two major midline sutures: interfrontal suture between the paired frontal bones and sagittal suture between the paired parietal bones. In addition, there are two major transverse sutures: coronal sutures between the frontal and parietal bones and lambdoid sutures between the parietal and interparietal bones (Figure 1A).

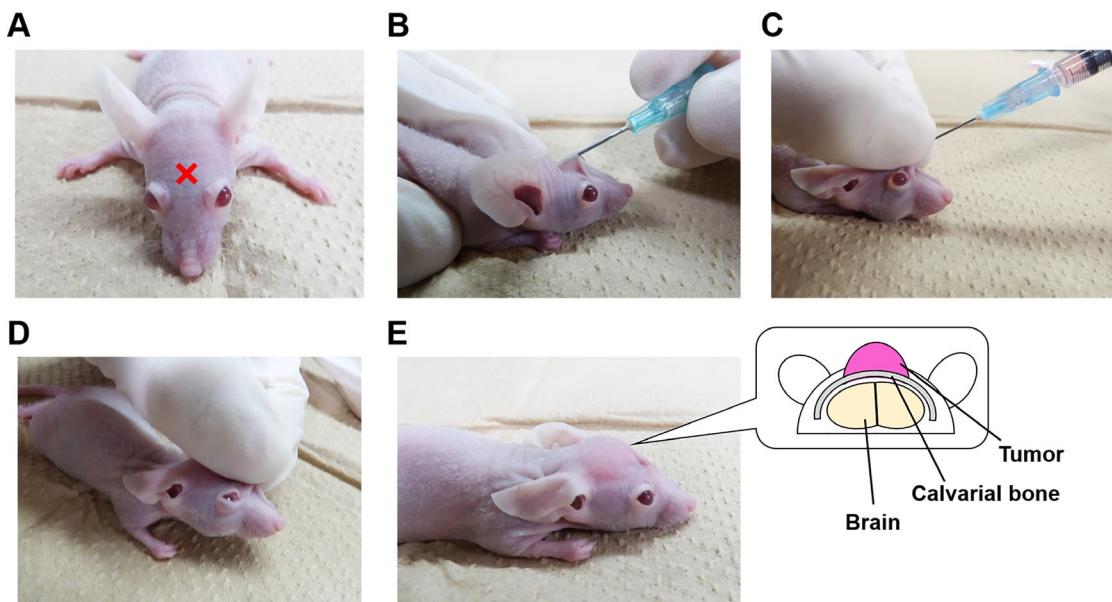


**Figure 1. Three-dimensional μCT reconstructions of mouse calvarial bone.** A. Top view. B. Lateral view.

1. Prepare 8-10-week-old BALB/c-nu/nu or NOD-SCID mice for the experiment.
2. Anesthetize a mouse by intraperitoneal injection of anesthetic agents as previously reported (Kawai *et al.*, 2011).
3. Insert a 21 G needle into the forehead skin (Figure 2A, X: Needle insertion point) and scratch the periosteum of calvarial bone with the tip of the needle in order to expose the surface of bone (Figure 2B).

*Note: Complete exposure of bone surface is important for tumor implantation.*

4. Transfer the cell suspension into a 1 ml syringe and attach a 23 G needle.  
*Note: Ensure the cells are homogeneously suspended by pipetting up and down before the transfer.*
5. Insert the injection needle from the same skin hole and inject 100  $\mu$ l ( $3 \times 10^6$  cells) of the cell suspension over the calvarial bone (Figure 2C).
6. After pulling the needle out, pinch the injection hole with fingers for about 30 sec until Matrigel polymerizes into a solid gel (Figures 2D and 2E).  
*Note: Take care not to let the cell suspension leak through the hole.*
7. Place the injected mouse on a heating pad and monitor it until it completely becomes awake.



**Figure 2. Cancer cell implantation on mouse calvarial bone**

C. After implantation

1. Monitor tumor growth regularly.
2. Measure tumor size at the implanted site weekly with a vernier caliper.
3. Perform IVIS *in vivo* imaging and micro-computed tomography ( $\mu$ CT) scanning weekly or every 2 weeks and check the size of tumor, osteolytic lesions or osteoblastic lesions (see Data analysis).
4. Sacrifice the implanted mouse at approximately 3-6 weeks (based on  $\mu$ CT analysis) after the implantation and harvest the resulting tumor for further analyses (e.g., histological analysis and gene expression analysis).

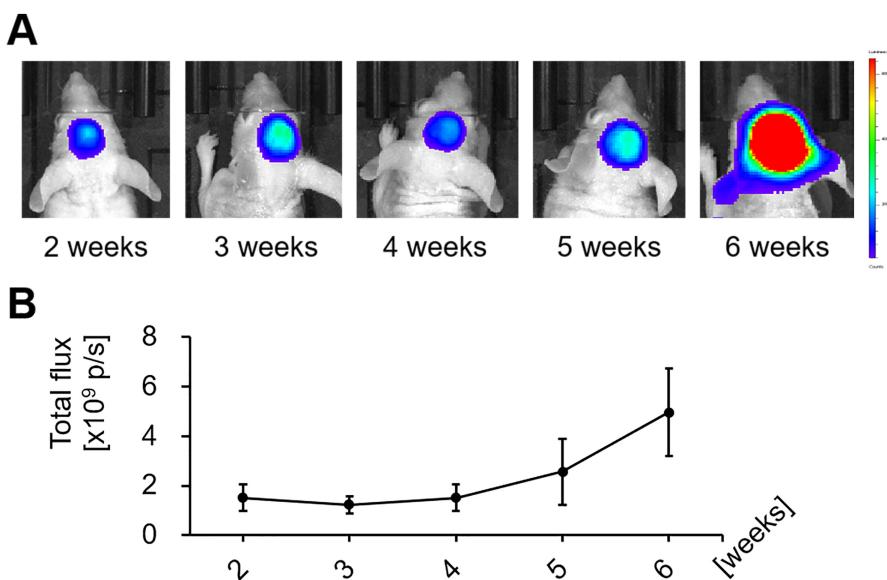
*Note: At early time points, little or no change of the calvarial bone is observed. Osteolytic or osteoblastic lesions were visually identified at approximately 2-4 weeks (depend on implanted tumor cells) after the tumor cells are implanted.*

### Data analysis

#### Imaging tumor growth and bone lesions

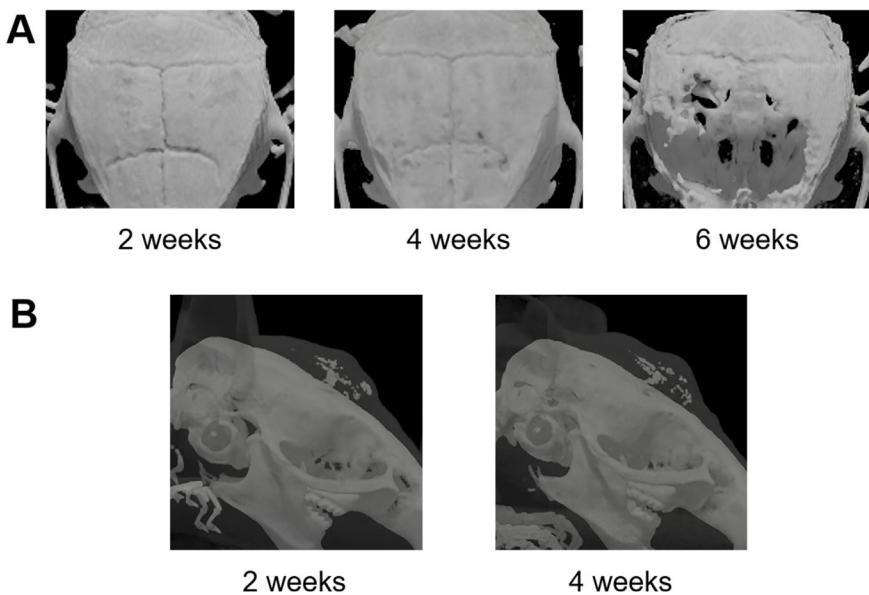
1. IVIS imaging system: Luciferin is a common bioluminescent reporter used for *in vivo* imaging of the expression of luciferase. IVIS imaging system is recommended to be performed weekly after the implantation of luciferase stably expressing tumor cells (e.g., MDA-MB-231-Luc cells, etc.). Before the imaging, 300  $\mu$ l (4.5 mg) of D-luciferin (15 mg/ml) is injected intraperitoneally into an implanted mouse under anesthetic condition. Images are obtained 10 min after the injection using IVIS Lumina III. The same mouse was investigated at different time points. If the tumor cells are successfully implanted, the bioluminescent intensity is increased gradually after the implantation (Figures 3A and 3B). Sometimes, implanted tumor cells (e.g., LNCaP and

C4-2 human prostate cancer cells, etc.) fail to survive. The precise reason for the failure still remains unclear. Generally, the more cells that were implanted, the more cells tend to survive.



**Figure 3. IVIS *in vivo* imaging of implanted MDA-MB-231-Luc cells.** A. IVIS images. B. Bioluminescent intensity of photons (Total flux [p/s]) emitted from the implanted tumor cells. The data are the means  $\pm$  SEMs ( $n = 5$ ).

2. Micro-computed tomography ( $\mu$ CT) analysis:  $\mu$ CT analysis is recommended to be performed weekly or every 2 weeks after the tumor cells are implanted.  $\mu$ CT scanning was conducted under anesthetic condition using R\_mCT2 (Rigaku) with an isometric resolution of 40  $\mu$ m, and the images were reconstructed using TRI/FCS-BON (Ratoc). In the case that the osteolytic phenotype-inducing tumor cells (e.g., MDA-MB-231 cells) are implanted, osteolytic lesions can be observed at 4-6 weeks after the implantation. Bone absorption areas become larger gradually (Figure 4A). On the other hand, in the case that the osteoblastic phenotype-inducing tumor cells (e.g., rat prostate cancer cells [Lynch *et al.*, 2005], hsa-miR-940-expressing MDA-MB-231 breast cancer cells [Hashimoto *et al.*, 2018], etc.) are implanted, osteoblastic lesions can appear at 10-14 days after the implantation (Figure 4B).



**Figure 4. Three-dimensional μCT reconstructions of the implanted site. A. Osteolytic lesions. B. Osteoblastic lesions.**

### **Acknowledgments**

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### **Competing interests**

All authors declare no conflicts of interest.

### **Ethics**

All animal experiments regarding calvarial bone implantation were performed with approval from the Animal Study Committee of University of Nebraska Medical Center, Nagoya City University, Nagasaki University, and Tokyo Medical and Dental University and conformed to their relevant guidelines and legislation.

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## Measuring Protein Synthesis during Cell Cycle by Azidohomoalanine (AHA)

### Labeling and Flow Cytometric Analysis

Koshi Imami<sup>1</sup>, \* and Tomoharu Yasuda<sup>2</sup>

<sup>1</sup>Department of Molecular and Cellular BioAnalysis, Kyoto University, Kyoto, Japan; <sup>2</sup>Department of Molecular and Cellular Biology, Kyushu University, Fukuoka, Japan

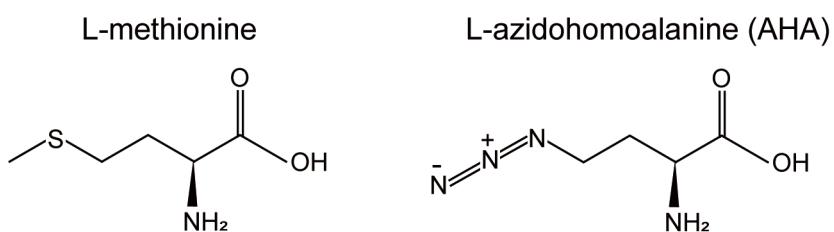
\*For correspondence: [imami.koshi.3z@kyoto-u.ac.jp](mailto:imami.koshi.3z@kyoto-u.ac.jp)



**[Abstract]** Protein synthesis is one of the most fundamental biological processes to maintain cellular proteostasis. Azidohomoalanine (AHA) is a non-radioactive and “clickable” amino acid analog of methionine which can be incorporated into newly synthesized proteins. Thus, AHA-labeled nascent proteins can be detected and quantified through fluorescent labeling by "click" chemistry. Here we describe a protocol to measure protein synthesis by AHA labeling and flow cytometry. Taking advantage of gating different cell populations, we provide a typical example of the flow cytometric-based analysis of protein synthesis during the cell cycle. While we used mouse B cells in this protocol this method can be readily applied to any cell types and organisms.

**Keywords:** Protein synthesis, Translation, AHA, Click chemistry, Flow cytometry, Cell cycle, Mitosis, Non-radioactive amino acid

**[Background]** Traditionally measurement of protein synthesis is performed by pulse labeling of translation products using radiolabeled amino acids such as [<sup>35</sup>S]methionine and [<sup>35</sup>S]cysteine but the use of radioactive materials is the major disadvantage. Recent advance in bio-orthogonal chemical reporters such as azides and alkynes allows us to quantitatively monitor and track biomolecules (proteins, lipids or nucleic acids) through click chemistry (Dieterich, 2010). Azidohomoalanine (AHA) (Figure 1) is a methionine analog that contains an azide moiety and is incorporated into newly synthesized proteins. Thus, nascent proteins can be detected through "click" reaction between AHA (azido) and a fluorescent alkyne (Dieterich, 2010).



**Figure 1. Chemical structures of L-methionine and L-azidohomoalanine (AHA)**

Measuring protein synthesis during the cell cycle in mammalian cells has been challenging. To do this, cell synchronization in specific phases of the cell cycle is necessary but the use of drugs such as nocodazole was shown to affect translation (Coldwell *et al.*, 2013; Shuda *et al.*, 2015). To measure

protein synthesis without drug treatment, here we used metabolic pulse labeling with AHA in a flow cytometric assay (Kiick *et al.*, 2002; Shuda *et al.*, 2015). In combination with AHA, we used anti-phospho-Histone H3 (Ser10) antibody as a mitotic marker to monitor mitotic translation in mouse B lymphoma cells (19DN) (Sander *et al.*, 2015).

## **Materials and Reagents**

1. Pipette tips (Eppendorf, epT.I.P.S.)
2. 6-well plate (Thermo Fisher Scientific, BioLite 6-well Multidish, catalog number: 130184)
3. 96-well U- or V-bottom cell culture plate (Corning, catalog number: 6902D09 or 6928A17)
4. 5 ml round bottom polystyrene FACS tube (Falcon, catalog number: 38007)
5. L-Azidohomoalanine (AHA) (Anaspec, catalog number: AS-63669, 500 mM stock solution in deionized water, keep at -20 °C up to at least a few months)
6. Methionine-free medium such as Dulbecco's Modified Eagle's Medium–high glucose With 4,500 mg/L glucose and sodium bicarbonate, without L-methionine, L-cystine and L-glutamine, liquid, sterile-filtered, suitable for cell culture (Merk, catalog number: D0422)
7. Fetal bovine serum, South America origin, dialyzed, sterile filtered (Pan Biotech, catalog number: P30-2102)
8. L-Methionine (Merck, catalog number: M9625, make 300 mM stock solution in deionized water, keep at -20 °C up to at least a few months)
9. L-Cystine dihydrochloride (Merck, catalog number: C2526, prepare fresh 200 mM (1,000x for DMEM) solution in deionized water)
10. GlutaMAX (Thermo Fisher Scientific, catalog number: 35050061, 200 mM stock solution)
11. D-PBS (1x), no calcium chloride, no magnesium chloride, sterile-filtered (Thermo Scientific Fisher, catalog number: 14190144)
12. Alexa Fluor488 alkyne (Thermo Fisher Scientific, catalog number: A10267)
13. Phospho-Histone H3 (Ser10) (D2C8) XP Rabbit mAb (Alexa Fluor 647 Conjugate) (Cell Signaling Technology, catalog number: 3458S)
14. (+)-Sodium L-ascorbate (Merck, catalog number: A7631)
15. Copper(II)-Sulphate (CuSO<sub>4</sub>) (Baseclick, catalog number: BCMI-004-50)
16. Paraformaldehyde (PFA) (Roth, 335)
17. Saponin (Sigma-Aldrich, catalog number: 47036)
18. (Optional) Triton X-100 (Sigma-Aldrich, catalog number: T9284)
19. Bovine serum albumin (BSA) (Roth, 8076)
20. DMSO (Merck, catalog number: D8418)
21. EDTA (Merck, catalog number: EDS)
22. NaN<sub>3</sub> (Merck, catalog number: 71289)
23. Cycloheximide (Santa Cruz Biotechnology, catalog number: sc-3508)
24. Cell culture medium for AHA labeling (w/o methionine or AHA) (see Recipes)

25. Washing solution (see Recipes)
26. Fixing solution (see Recipes)
27. Permeabilization solution (see Recipes)
28. FACS buffer (see Recipes)

## **Equipment**

1. (Optional) Multi channel pipette (Eppendorf, catalog number: 3122 000.043 [10-100 µl])
2. CO<sub>2</sub> Incubator (Thermo Fisher Scientific, catalog number: 51026331)
3. Refrigerated centrifuge (Eppendorf, models: 5418 R [fixed angle for tubes], 5804 R [swing out for 96 well plates])
4. Flow cytometer (FACS Aria instrument) (BD Biosciences)

## **Software**

1. Analysis Software: FlowJo

## **Procedure**

### A. AHA pulse labeling

1. Seed cells in a 6-well plate containing normal cell culture medium so that cells should be 50%-60% confluent next day. Mouse B cells (in suspension) were used in this study but any cell types can be used.
2. Next day, starve cells in the methionine-free medium for 30 min.  
*Note: This step may be omitted to avoid cellular stress.*
3. Start pulse labeling by adding 1 mM AHA or methionine (negative control) to the methionine-free medium and incubate cells for at least 10 min in a 37 °C CO<sub>2</sub> Incubator. Cycloheximide treatment (100 µg/ml) can be used as a negative control as well.
4. Wash cells with ice-cold PBS. Spin down cells at 500 x g, 4 °C for 3 min and remove the supernatant. Repeat this step once more. For adherent cells such as HEK293, 1 mM EDTA in PBS can be used to harvest cells.

*Note: From this step, we used 96-well U- or V-bottom cell culture plate for subsequent steps to increase the throughput. For subsequent steps, use 100-200 µl solution to suspend cells after spinning.*

5. Fix cells in fixing solution (4% PFA in PBS) for 15 min at RT. Spin down cells and remove the supernatant.
6. Permeabilize cells with 1% BSA with 0.2% saponin (or 0.25% Triton X-100) in PBS for 15 min. Spin down cells at 500 x g, at RT for 3 min and remove the supernatant.

**B. Click reaction**

1. Prepare the click reaction solution (see Recipe 5).
2. Incubate samples with 100  $\mu$ l of the click solution for 30 min at RT. Protect from light.
3. Wash cells once with 1% BSA with 0.2% saponin in PBS. Spin down cells and remove the supernatant. In case of Triton X-100 is used for permeabilization, wash cells with 1% BSA in PBS.

**C. Immunolabeling of a mitotic marker, histone H3 phospho Ser10**

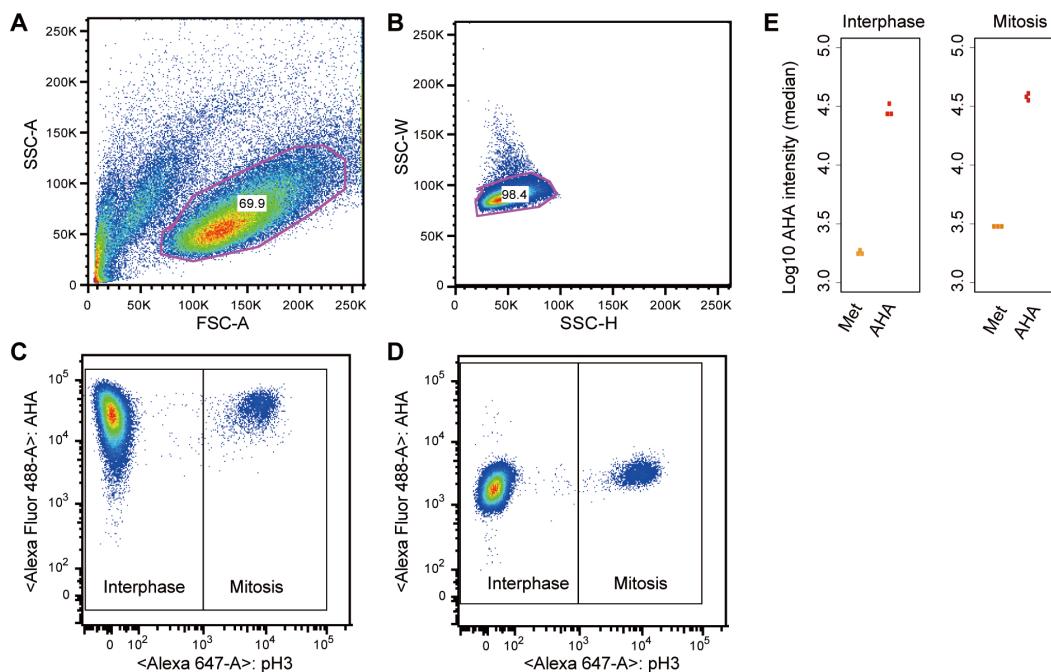
1. Incubate samples with anti-phospho histone pH3 antibody (1:100, v/v) in 100  $\mu$ l 1% BSA in PBS for 60 min. Protect from light.
2. Wash cells twice with 1% BSA in PBS. Spin down cells and remove the supernatant.
3. Resuspend in FACS buffer (1% FBS, 1 mM EDTA, 0.05%  $\text{NaN}_3$  in PBS) and transfer cells into a 5 ml round bottom polystyrene FACS tube.

**D. Flow cytometric analysis**

1. Gate the main cell population by the forward (FSC-A) and side scatter profile (SSC-A) to exclude cellular debris and dead cells (Figure 2A). Gate further the singlet subset by SSC-H and SSC-W (Figure 2B).
2. For the subset of cells gated in the Step D1, record AHA-incorporation (y-axis, alexa488) and the mitotic marker (x-axis, alexa647) for at least 10,000 events (Figure 2C). Quantify AHA intensities (y-axis) in interphase and mitotic cells (x-axis) that are separated based on fluorescence intensity of the mitotic marker (see Step D4).

*Note: Prepare single color (stained with the Alexa Fluor488-AHA or the Alexa Fluor 647 conjugated antibody) and no color controls for setting FACS parameters and compensation on the flow cytometer. Each compensation control is acquired on the flow cytometer to determine compensation values for each fluorochrome combination. This protocol does not cover detailed instructions for FACS parameter setup. For assistance, please contact your FACS core facility.*

3. Check if the negative control sample (methionine-labeled cells) shows the low level of AHA-incorporation (Figure 2D).
4. Quantify the AHA intensities for individual cells (e.g., median intensity) in interphase and mitosis using FlowJo. To do this, open FlowJo and load the files (.fcs) into a workspace. Double-click on a file in the workspace and a plot like Figure 2A will appear. Select subsets for further analyses based on the forward (FSC-A) and side scatter profile (SSC-A) as described in Step D1. Select corresponding populations of interphase and mitosis (see Figure 2C) and click on “Workspace” → “Statistics” → “Median” → “AHA”, which calculates median intensities of AHA signal. Examples of data analysis are shown in Figure 2E.



**Figure 2. Monitoring global protein synthesis in interphase and mitosis.** A and B. Gating strategy. C. The main cell population was further analyzed by dual staining for phospho-H3 S10 and AHA-labeled proteins. Global protein synthesis was monitored by AHA incorporation (y-axis) in interphase and mitosis (based on H3 pS10 staining, x-axis). D. As a negative control, methionine incorporation into proteins instead of AHA was also monitored. E. Examples of quantitative outputs: each dot represents the median AHA intensity calculated from the interphase or mitotic cell population in an independent experiment. The results from three independent experiments are shown.

## Recipes

1. Cell culture medium for AHA labeling (w/o methionine or AHA)  
500 ml of DMEM without methionine, cysteine, glutamine  
50 ml of dialyzed serum (normal serum can be used in case use of dialyzed serum affects cell growth.)

*Notes:*

- a. *As long as methionine-free medium is used, the use of normal serum instead of dialyzed serum will not significantly affect pulse labeling results.*
- b. *For Cysteine and GlutaMAX, follow the defined concentration of the medium you use.*
- c. *Directly add methionine or AHA to the medium just before pulse labeling.*

2. Washing solution  
1% BSA in PBS
3. Fixing solution  
4% PFA in PBS

**4. Permeabilization solution**

1% BSA with 0.2% saponin (or 0.25% Triton X-100) in PBS

**5. Click reaction solution**

Reagent	Stock solution	Final conc.	Volume (per ml)
1x PBS	-	-	879 µl
Alexa Fluor488 alkyne	1 mM in DMSO (keep at -20 °C up to at least 6 months)	1 µM	1 µl
(+)-Sodium L-ascorbate	100 mM in PBS (prepare fresh on the day of experiment)	10 mM	100 µl
CuSO <sub>4</sub>	100 mM in deionized water (keep at RT up to at least 6 months)	2 mM	20 µl

**6. FACS buffer**

1% FBS

1 mM EDTA

0.05% NaN<sub>3</sub> in PBS

**Notes**

You can perform washing, fixing, click reaction and etc. in a 96-well U- or V-bottom cell culture plate to increase the throughput. You can sort the AHA-labeled cells and the sorted cells can be used for many applications including western blot analysis and quantitative analysis using mass spectrometry.

**Acknowledgments**

We would like to thank Shuda *et al.* for describing the original protocol (Shuda *et al.*, 2015). We adapted and modified the protocol for this study. We would also like to thank Matthias Selbach (Max Delbrück Center for Molecular Medicine) for critical reading of the manuscript and for his advice.

**Competing interests**

The authors declare no conflicts of interest or competing interests.

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## SIRF: A Single-cell Assay for *in situ* Protein Interaction with Nascent DNA Replication Forks

Sunetra Roy and Katharina Schlacher\*

Department of Cancer Biology, University of Texas MD Anderson Cancer Center, Houston, TX, USA

\*For correspondence: [KSchlacher@mdanderson.org](mailto:KSchlacher@mdanderson.org)



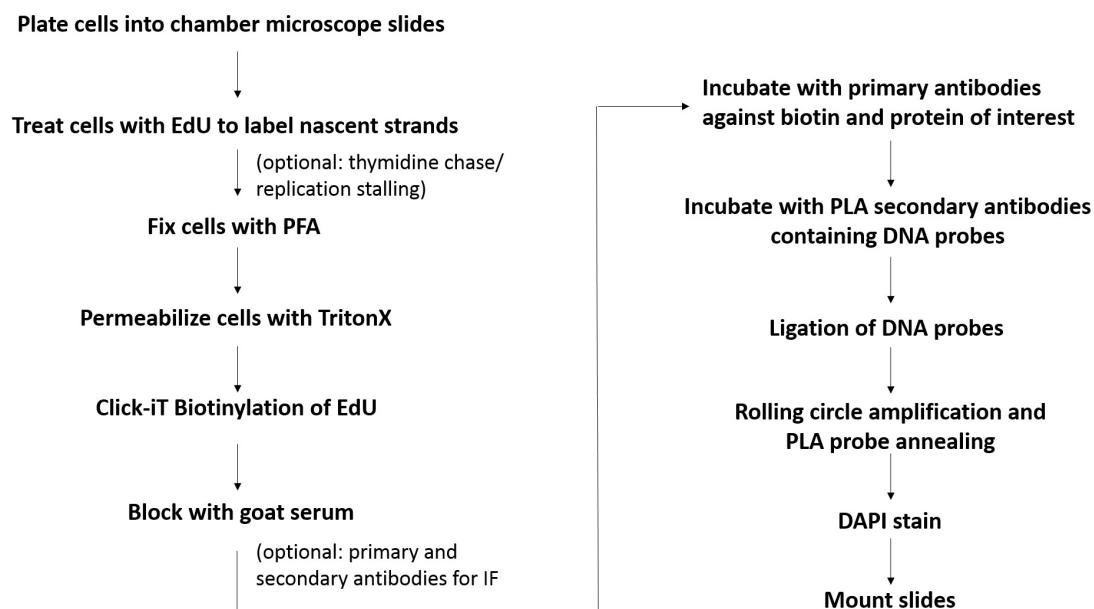
**[Abstract]** The duplication of DNA is a fundamental process that is required for the transfer of the genetic information from parent to daughter cells. Aberrant DNA replication processes are associated with diverse disease phenotypes, including developmental defects, ageing disorders, blood disorders such as Fanconi Anemia, increased inflammation and cancer. Therefore, the development of tools to study proteins associated with error-free DNA replication processes is of paramount importance. So far, methods to study proteins associated with nascent replication forks relied on conventional immunofluorescence and immunoprecipitation assays of 5'-ethylene-2'-deoxyuridine (EdU) labeled DNA (iPOND). While greatly informative and important, these methods lack specificities for nascent fork interactions (e.g., IF) or assay an average change of millions of cells without single-cell resolution (e.g., iPOND). The assay system described here combines proximity ligation assay (PLA) with EdU coupled click-iT chemistry, which we termed “*in situ* Protein Interaction with Nascent DNA Replication Forks (SIRF)”. This method enables sensitive and quantitative analysis of protein interactions with nascent DNA replication forks with single-cell resolution, and can further be paired with conventional immunofluorescence marker analysis for added multi-parameter analysis.

**Keywords:** SIRF, DNA replication, Stalled replication forks, Genome instability, Proximity ligation assay, Fork protection, iPOND

**[Background]** Defects in high fidelity DNA replication are associated with severe biological outcomes. These amongst others include developmental abnormalities, premature aging, hematological disorder and cancer (Loeb and Monnat, 2008; Magdalou *et al.*, 2014). To avoid DNA replication instability, cells have evolved intricate mechanisms to ensure error-free propagation of its genetic material. There is an ever-growing list of disease-suppressing proteins that actively ensure DNA replication stability and function at all stages of DNA replication processes. A comprehensive understanding of the functions of DNA replication stability proteins is imperative to allow development of effective and specific agents targeting disease outcomes.

Historically, visualization of proteins localized at or near DNA replication forks was advanced by the advent of nucleoside analogs, such as 5-bromo-2'-deoxyuridine (BrdU) that is incorporated into newly replicating DNA and further detected with fluorescent-labeled antibodies (Gonchoroff *et al.*, 1986; Leif *et al.*, 2004). Immunofluorescence (IF) assays examining co-localization of proteins with the BrdU-label indicates that the protein is present in close vicinity to actively replicating DNA (Sengupta *et al.*, 2003). This method provided critical information on replication processes. However, it is an indirect method that lacks the resolution that would be required to unequivocally distinguish protein-DNA interactions

from proteins located near DNA. Another ground-breaking procedure was iPOND (standing for isolation of proteins on nascent DNA) that allows to detect and isolate proteins on newly replicated DNA (Petermann *et al.*, 2010; Sirbu *et al.*, 2011 and 2013; Dungrawala *et al.*, 2015). In this procedure, nascent DNA is labeled with the thymidine analog EdU, which is then biotinylated using click-IT chemistry (Moses and Moorhouse, 2007). The newly synthesized biotinylated DNA is immuno-precipitated with streptavidin-coated agarose or magnetic beads. This isolates proteins that bind to newly replicated DNA, which either can be detected by Western blotting or by mass spectrometry. While this method revolutionized the DNA replication field by enabling to directly study protein interactions with replication forks, iPOND detects the average of thousands of replication forks and cells. iPOND lacks the single-cell level resolution that is achieved by IF, which can provide valuable additional parameter information such as stage of the S-phase, or cell identity. Moreover, the technique is labor-intensive and requires copious amounts of starting material (~100,000,000 cells per condition). The assay system described here termed *in situ* protein interaction with nascent DNA replication forks (SIRF) (Roy *et al.*, 2018a and 2018b) combines EdU-biotin Click-chemistry with proximity ligation assay (PLA) to overcome these challenges. The PLA technology developed by Soderberg *et al.* for single-molecule protein-protein interaction studies, utilizes antibodies with oligonucleotides conjugates, that can be ligated into a circular DNA molecule when two antibodies are in close proximity to each other (< 40 nm) (Soderberg *et al.*, 2008). Once ligated, the DNA circle can be amplified by rolling circle DNA polymerase. After annealing of fluorescent DNA probes that are complementary to the DNA circle, this procedure results in a highly amplified fluorescent signal, so that even single molecule interactions are readily detectable by conventional immunofluorescence microscopy. In SIRF, PLA is used with antibodies against biotin (detecting biotinylated EdU) and a protein of interest, which results in a PLA signal only if the protein is located within 40 nm or less from the newly synthesized DNA (Figure 1). The SIRF method requires little amounts of cells (~100-1,000), preserves the single-cell resolution as seen with IF, and can be readily and accurately quantified. Moreover, the single-cell resolution provides additional valuable information including cell morphology, cell-cycle stage (early or late S-phase), spatial orientation of protein signal in the nucleus, which can be associated with changes in replication fork-protein dynamics. The SIRF assay furthermore can be combined with conventional IF for additional cell-biomarker analysis, which amongst others allows to examine replication proteins in a heterogeneous cell population that can be differentiated by lineage specific markers. In this case, primary and secondary antibody staining for IF is performed prior to the PLA reaction to avoid interference of PLA secondary antibodies from cross-reacting with IF primary antibodies. The SIRF assay can be used to study protein dynamics at ongoing and stalled DNA replication forks, as well as chromatin maturation at newly replicated DNA regions (Petruk *et al.*, 2017, Roy *et al.*, 2018a and 2018b).



**Figure 1. Flowchart for the SIRF assay.** Shown is the basic workflow and the major steps involved in SIRF assay.

### **Materials and Reagents**

1. 8-well chamber microscope slides (Thermo Scientific, Nunc, catalog number: 177402)
2. Plastic cover slips (Electron Microscopy Sciences, catalog number: 72261-50)
3. Glass cover slips (Fisher Scientific, catalog number: 12-548-5M)
4. Kimwipes (Fisher Scientific, catalog number: NC9855580)
5. Aluminum foil (Fisher Scientific, catalog number: 01-213-105)
6. Paper towel (Envision, catalog number: 23304)
7. 0.22 µm bottletop filter (Corning, catalog number: 430758)
8. Thymidine (Sigma-Aldrich, catalog number: T1895)
9. Rabbit anti-biotin antibody (Cell Signaling, catalog number: 5597S)
10. Mouse anti-biotin antibody (Sigma-Aldrich, catalog number: B7653)
11. Rabbit anti-RPA antibody (Abcam, catalog number: ab79398)
12. Goat serum (Sigma-Aldrich, catalog number: G9023)
13. 5'-ethylene-2'-deoxyuridine (EdU) (Invitrogen, catalog number: A10044)
14. Hydroxyurea (HU) (Sigma-Aldrich, catalog number: H8627)
15. Paraformaldehyde 32% solution, EM grade (EMS, catalog number: 15714)
16. Triton X-100 (Sigma-Aldrich, catalog number: T8787)
17. Biotin Azide (Invitrogen, catalog number: B10184)
18. Alexa Fluor 488 azide (Invitrogen, catalog number: A10266)
19. Copper sulfate solution (Fluka Analytical, catalog number: 35185)
20. Sodium ascorbate (Sigma-Aldrich, catalog number: 11140)

21. Phosphate buffered saline (PBS) (Sigma-Aldrich, catalog number: P4417)
22. Duolink® Mouse plus PLA probe (Sigma-Aldrich, catalog number: DUO92001-100RXN)
23. Duolink® Rabbit minus PLA probe (Sigma-Aldrich, catalog number: DUO92005-100RXN)
24. Duolink® PLA detection reagent red (Sigma-Aldrich, catalog number: DUO92008-100RXN)
25. Duolink® Duolink *In Situ* Wash Buffers (Sigma-Aldrich, catalog number: DUO82049-20L)
26. 4', 6-diamidino-2-phenylindole (DAPI) (Life Technologies, catalog number: 62248)
27. Prolong Gold antifade reagent (Invitrogen, catalog number: P36934)
28. Tween 20 (Sigma-Aldrich, catalog number P7949)
29. Hydrochloric acid (Fisher Scientific, catalog number: A144-212)
30. Tris base (Fisher Scientific, catalog number: BP152-1)
31. IMDM (Gibco, catalog number: 12440-053)
32. FBS (Gemini Bio-products, catalog number: 100-106)
33. Sodium Chloride (Fisher Scientific, S271-3)
34. Fixation solution (see Recipes)
35. Permeabilization solution (see Recipes)
36. Blocking Buffer (see Recipes)
37. Wash Buffer A (see Recipes)
38. Wash Buffer B (see Recipes)

## **Equipment**

1. Autoflow IR water jacketed CO<sub>2</sub> incubator (NUAIRE, model: NU-4750)
2. Nikon eclipse Ti-U inverted microscope (Nikon, model: Ti-U)
3. Coplin jar (Thermo Scientific™ E94)
4. Fine curved forceps (Fine Science Tools, Dumont #7, catalog number: 11271-30)
5. Slide box (VWR, catalog number: 82003-414)
6. Vortexer (VWR analog vortex mixer, catalog number: 10153-838)
7. 4 °C refrigerator (BSI, model: SCGP21OW1AREF)
8. -20 °C freezer (BSI, model: ABT-2020MB)

## **Software**

1. Duolink® Analysis tool  
(Sigma-Aldrich, <http://www.Sigma-aldrich.com/catalog/product/sigma/duo90806?Lang=en&ion=US>)
2. NIS-elements (Nikon, <https://www.nikoninstruments.com/Products/Software>)
3. Microsoft Excel (Microsoft, <https://products.office.com/en-us/excel>)
4. ImageJ (ImageJ, <https://imagej.net/Welcome>)
5. GraphPad Prism (GraphPad, <https://www.graphpad.com/scientific-software/prism/>)

## **Procedure**

### A. Cell labeling

1. Plate  $1 \times 10^4$ - $2 \times 10^4$  log-phase growing cells in 300  $\mu\text{l}$  growth medium for each well of an eight-chamber glass microscope slide on the day before the experiment, such that the cells are 50-60% confluent the day of cell labeling. We typically use adherent cells, for e.g. HAP-1 cells that grow in IMDM medium supplemented with 10% FBS.

*Note: We have not used this procedure with suspension cells. However, in principle it should be possible to use suspension cells as they can be treated with EdU in solution, deposited on microscopic slides using a cytocentrifuge, and then fixed to proceed with the assay.*

2. The next day, gently aspirate growth medium from wells and add 200  $\mu\text{l}$  pre-warmed growth medium containing EdU (125  $\mu\text{M}$ ). Incubate the slide at 37 °C in a tissue culture incubator for 8 min before fixation. This condition allows to detect proteins at unperturbed newly replicated DNA, ‘E only’.

*Note: Equilibrate growth media to 37 °C prior to treating cells to ensure uninterrupted replication of cells during treatments. Be quick with washes and treatments, so as to limit exposure to room temperature that may disturb replication.*

3. (Optional) For replication stalling conditions, aspirate the EdU-media after 8 min, wash wells swiftly but gently twice with PBS, aspirate and add growth medium containing hydroxyurea (HU) or any replication stalling agent of choice for a desired amount of time, typically 1-5 h at 37 °C. This condition allows detection of proteins at stalled forks, ‘E-HU’.

*Note: We typically use 200  $\mu\text{M}$  or 4 mM HU for replication stalling conditions. Lower concentrations of HU represent a transient fork stalling condition while high concentrations can cause DNA breakage (Roy et al, 2018b). One hour HU treatment would enable visualization of early replication stalling events, while 4 h HU (or longer) treatment enables visualization of later events.*

4. (Optional) The EdU treatment (Step A2) is chased with thymidine. This allows to differentiate proteins bound to newly synthesized DNA from proteins associated with ongoing DNA replication forks; Proteins that travel with the DNA replication fork will have moved on during incubations with thymidine and will no longer be in proximity to the original EdU label, which makes the PLA reaction unproductive. For a thymidine chase, aspirate the EdU-containing media after 8 min, wash wells twice with PBS and add back growth medium containing thymidine (100  $\mu\text{M}$ ). Incubate slides at 37 °C for 4 h. This condition allows detection of proteins that are bound to replicated DNA and not progressing with the active replication fork, ‘E-Thy’. Similarly, the absence of a signal under this condition combined with the presence of a signal under the “E only” condition defines proteins traveling with ongoing DNA replication forks.

5. Always include a well with no EdU treatments as a negative control, ‘No EdU’. The EdU-EdU SIRF (see notes) serves as a positive control.

*Note: It is advisable to perform all the conditions including the optional ones, to distinguish*

between proteins at a regular unperturbed replication fork (*E* only), versus at stalled replication forks (*E-HU*) or a chromatin bound protein that is in close proximity to a replication fork (*E-Thy*). The *E-Thy* condition distinguishes between proteins bound DNA itself from proteins bound to active replication forks.

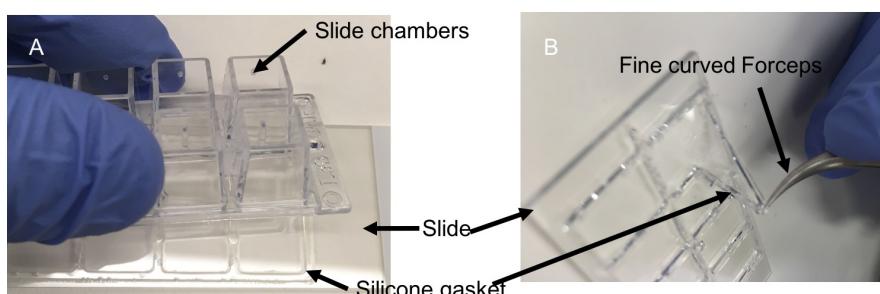
#### B. Cell fixation

1. Gently aspirate growth medium, and carefully add 200  $\mu$ l of fixation solution to each well (2% PFA diluted in PBS [pH 7.4]). Incubate at room temperature (RT) for 15 min without disturbing the slide. Fixation time may vary depending on cell type.

*Note: Handle PFA with caution inside a biochemical safety cabinet. Discard PFA waste according to institutional biosafety guidelines.*

2. After fixation, discard of PFA and wash wells with PBS (pH 7.4) twice for 5 min. Dis-assemble chambers from chamber slide (Figure 2), ensuring that the wells do not dry out during disassembly and carefully place slides in Coplin jar containing 60 ml PBS.

*Note: Fixed slides can be stored at 4 °C for up to one week.*



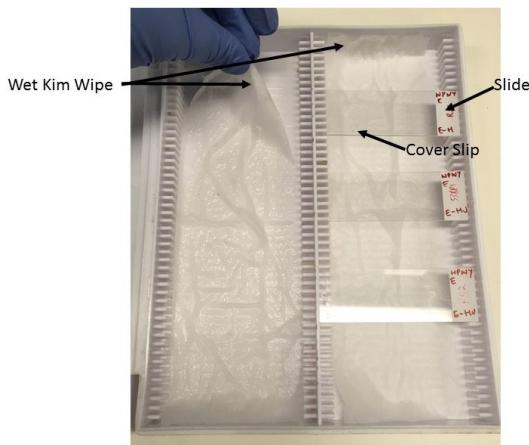
**Figure 2. Slide chamber dis-assembly.** A. Dis-assembly of slide chambers. B. Removal of silicone gasket (demarcating the chambers) using fine curved forceps. Apply caution to avoid scratches on the glass wells with forceps.

#### C. Cell permeabilization

1. Permeabilize cells by placing slides in Coplin jar containing 60 ml permeabilization solution (0.25% Triton X-100 in PBS) for 15 min at RT.
2. Wash slides in Coplin jar with PBS (pH 7.4) twice for 5 min each.

#### D. Click-iT reaction

1. Prepare humid slide chamber by placing wet kimwipes inside a slide box at RT and equilibrate for 5 min (Figure 3).

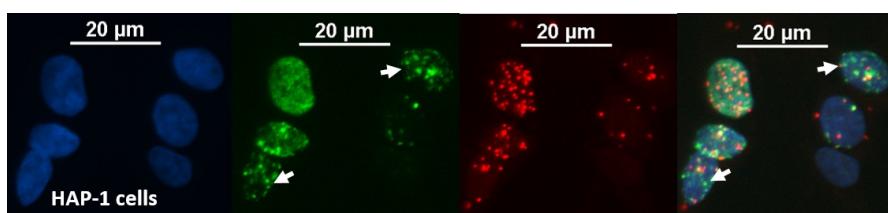


**Figure 3. Humid slide chamber.** Preparation of humid slide chamber by placing folded paper wipes (Kimwipes) wetted with distilled water at the bottom of a slide box. Slides are laid flat, facing up during antibody and PLA solution incubations and are covered with plastic cover slips during respective incubations.

2. Prepare fresh click-iT reaction cocktail by combining 2 mM copper sulfate, 10  $\mu$ M biotin-azide and 100 mM sodium ascorbate in PBS in said order and mix well by vortexing.

**Notes:**

- Make 1 M sodium ascorbate fresh, prior to use. Limit light exposure to biotin azide aliquots.*
- A mixture of Alexa 488 azide and biotin azide (1:10, total 10  $\mu$ M) can be used instead of biotin azide alone, during click reaction. This enables normalization of PLA signal to the EdU signal and determination of the cell-cycle phase, such as early, mid and late S-phase (Figure 4).*



**Figure 4. Representative image to distinguish SIRF signals at various stages of the cell cycle.** The above panel shows an example of RAD51 SIRF (red channel) in HAP-1 cells treated with 125  $\mu$ M EdU for 8 min and 200  $\mu$ M HU for 4 h. The arrows indicate cells in late S-phase based on EdU patterning. Note that the green signal for Alexa 488 azide co-click enables distinction of late S-phase versus early S-phase cells. Scale bars = 20  $\mu$ m.

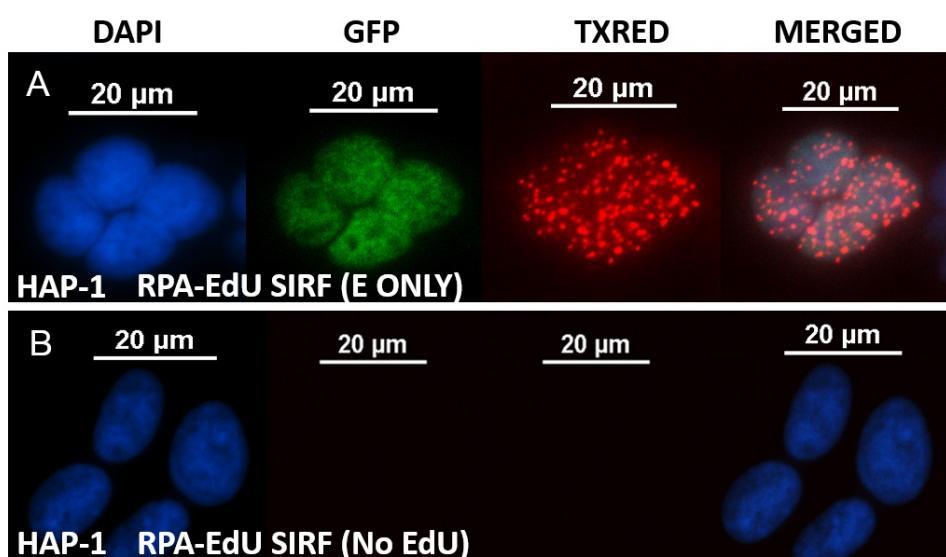
3. Place slides in the slide box facing up (Figure 3), and add 30  $\mu$ l/well click-iT reaction cocktail. Cover slides with new plastic cover slips, taking care to avoid air bubbles.
4. Close lid of slide box, which allows for humid chamber conditions, and incubate at RT for 1 h.

## E. Blocking and primary antibody incubation

1. Remove plastic cover slips and wash slides in Coplin jar containing PBS (pH 7.4) for 5 min at RT.
2. Place slides back in humid chamber and add 30  $\mu$ l/well blocking buffer (10% goat serum and 0.1% Triton X-100 in PBS). Cover slides with new plastic cover slips, taking care to avoid and discourage air bubbles.
3. Close slide box and incubate at RT for 1 h.
4. Dilute primary antibodies of interest in blocking buffer. Different dilutions may need to be tested for optimal signals. Use a complementary anti-biotin antibody in conjunction with an antibody against the protein of interest. For example, shown in Figure 5, is the use of a mouse anti-biotin (1:100, Sigma) and rabbit anti-RPA antibody pair (see Notes). When using a mouse anti-protein of interest antibody, chose a rabbit anti-biotin (1:200, Cell Signaling).
5. Take off plastic cover slips from slides and gently tap off the blocking buffer.
6. Add 30-40  $\mu$ l primary antibody mixture per well and apply new plastic cover slips, avoiding and discouraging air bubbles.
7. Place slides inside the humid chamber and incubate at 4 °C overnight.

## F. Proximity ligation assay (PLA)

1. Wash slides in Coplin jar containing 60 ml Duolink wash buffer A (10 mM Tris-HCl pH 7.5, 150 mM NaCl and 0.05% Tween 20) three times for 5 min each.
2. Prepare Duolink *In Situ* PLA probes anti-plus and anti-minus against the respective primary antibodies by diluting them 1:5 in blocking buffer. For example, prepare anti-mouse plus and anti-rabbit minus in the example given in Figure 5 when using rabbit anti-RPA and mouse anti-biotin.



**Figure 5. Representative RPA-SIRF image in HAP-1 cells.** A. Representative images of an RPA-SIRF assay in HAP-1 cells that have been treated with 125  $\mu$ M EdU for 8 min. The cells

were co-clicked with biotin-azide and Alexa 488-azide (10:1, total 10  $\mu$ M). The GFP channel shows the EdU-Alexa 488 signal of S-phase cells. PLA signals are visualized in TXRED channel. The PLA signal can be normalized to the Alexa 488 signal to account for PLA signal differences depending on EdU content of the cells. B. Representative images of RPA-SIRF with no EdU condition (negative control). Scale bars = 20  $\mu$ m.

3. Tap off excess wash buffer and place slides in pre-warmed (37 °C) humid chamber. Swiftly add 30  $\mu$ l/well diluted PLA probes, taking care to prevent wells from drying out.
4. Place new plastic cover slips on the slides and incubate humid slide chamber at 37 °C for 1 h.
5. Wash slides again in Coplin jar containing 60 ml Duolink wash buffer A, three times for 5 min each.
6. Prepare ligation mix by diluting Duolink ligation stock buffer (1:5) and Duolink ligase enzyme (1:40) in autoclaved water. Vortex the ligation stock buffer during and after thawing, making sure to dissolve any precipitate. Keep ligase enzyme in a freezing block at -20 °C and add to ligation buffer.
7. Tap off excess wash buffer and place slides back in humid slide chamber, add 30  $\mu$ l/well ligation mix taking care to prevent slides from drying out.
8. Put new plastic cover slips on the slides and incubate humid slide chamber at 37 °C for 30 min.
9. Wash slides again in Coplin jar containing 60 ml Duolink wash buffer A, two times for 2 min each.
10. Prepare amplification mix by diluting Duolink amplification stock (1:5) and Duolink rolling circle polymerase enzyme (1:80) in autoclaved water. Limit the exposure of the amplification stock to light, e.g., by preparing the solution in an amber light-protected tube, or in a tube covered with aluminum foil. Keep polymerase enzyme in a freezing block at -20 °C.
11. Tap off excess wash buffer and place slides back in humid slide chamber. Add 30  $\mu$ l/well amplification mix, taking care to prevent slides from drying out.
12. Cover with a new plastic cover slip and incubate humid slide chamber at 37 °C for exactly 100 min.
13. Wash slides in Coplin jar containing 60 ml Duolink wash buffer B (200 mM Tris-HCl pH 7.5 and 100 mM NaCl), three times for 10 min each in the dark.
14. Wash slides in Coplin jar containing 60 ml 0.01x diluted Duolink wash buffer B for 1 min in the dark. Proceed to DAPI staining.

#### G. DAPI staining and mounting

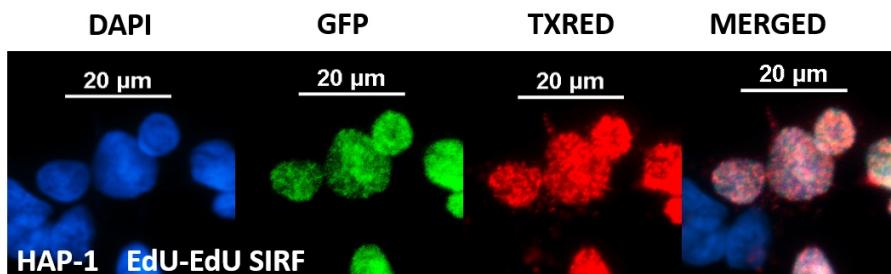
1. Prepare DAPI solution (1:1000, 1  $\mu$ g/ml end concentration) in PBS. Protect from light.
2. Tap off excess wash buffer B and place slides back in humid slide chamber. Add 30  $\mu$ l/well DAPI solution, taking care to prevent slides from drying out.
3. Cover with new plastic cover slips and incubate humid slide chamber at RT for 5 min.
4. Wash slides in coplin jar containing PBS two times for 5 min each.

5. Tap off excess PBS from slides and place them on a paper towel for mounting.
6. Add 20  $\mu$ l of Prolong Gold antifade reagent to each well of the slide and mount with glass cover slips (1.5 mm). Avoid air bubbles while mounting and wipe off excess mounting reagent from the edges of the cover slip. Keep slides in a dry slide box at RT overnight to cure and avoid exposure to light.

*Note: Store slides at -20 °C, if not imaged within 2 days.*

### **Data analysis**

1. Image slides using fluorescent microscope (such as Nikon Eclipse Ti) at a magnification of 40x/0.95 numerical aperture (N.A.) (20x/0.75 N.A. may also be sufficient for larger cells). PLA signals are captured in the TXRED channel (Ex 594 nm, Em 624 nm, as recommended by Sigma Duolink for Duolink detection reagent Red). The GFP filter is used to image cells when co-clicked with Alexa 488-azide (Ex 495 nm, Em 519 nm). DAPI filter is used to visualize cell nuclei (Ex 358 nm, Em 461 nm). The merged image file is used for subsequent analysis (Figure 5). Typically, 3-5 image fields in different areas of the well are obtained for a total of minimally 100-200 cells per condition.
2. If the PLA signals are discrete and well dispersed, the Duolink quantification tool or the microscope intrinsic quantitation software, such as Nikon NIS-elements software, can be used to determine the number of signals per nuclei. Alternatively, when signals are very abundant and indistinguishable by the software (e.g., Figure 6) the mean fluorescent intensity of the TXRED channel is measured using ImageJ software or the software of the microscope such as Nikon NIS-elements software. Differences in EdU levels will alter the efficiency of the PLA between protein of interest and biotinylated EdU. Therefore, the PLA signals per cell should be normalized either individually to the total EdU signal per cell (as measured by Alexa 488-EdU signal in green channel) or to the median PLA signals measured in the EdU-EdU SIRF condition.



**Figure 6. Representative EdU SIRF image in HAP-1 cells.** The above panel shows an example of EdU-EdU SIRF in HAP-1 cells treated with 125  $\mu$ M EdU for 8 min. The PLA signals are too abundant to be hand counted. Mean TXRED intensity in this case can be measured for PLA quantification. The green channel represents Alexa 488-EdU signal. Scale bars = 20  $\mu$ m.

3. The preferred method to normalize SIRF signals to EdU content is to divide the number of SIRF signals per nucleus by the total GFP intensity per nucleus obtained by Alexa 488 for GFP+ cells (# SIRF signals per nucleus/sum GFP intensity of that given cell). Note that this condition should be compared to the identical SIRF assay without Alexa 488-azide co-click, to ensure that the overall PLA-signal abundance remains unchanged by the co-click.
4. Alternatively, an EdU-EdU SIRF can be performed by using mouse anti-biotin and rabbit anti-biotin antibodies (see Notes). The mean TXRED intensity in this case is an indicator of EdU content of the cell. The number of SIRF signals per nucleus is then divided by the median EdU-EdU TXRED intensity per condition (# SIRF signals per nucleus/median TXRED intensity of all cells per condition).

*Note: Exclude cells that are EdU-Alexa488 negative when calculating the median intensity.*

5. If the data follows a non-normal distribution as measured by the DAgostino & Pearson normality test, a Mann-Whitney *t*-test is used to determine the statistical significance of the differences in SIRF signals between conditions or cell lines (Roy *et al.*, 2018a).

## **Notes**

Optional: perform an additional PLA reaction solely with mouse anti-biotin and rabbit anti-biotin antibodies for identical experimental conditions on a separate slide. This “EdU-EdU” SIRF can be used for normalizing the PLA signals with the EdU content (Figure 6), as an alternative to normalization against EdU-Alexa 488 intensities when Alexa 488-azide is co-clicked with the biotin-azide (Figure 5).

The fixation time that we used is optimized for HAP-1, MCF10a, MEF, MCF7, HCT116, U2OS, H1299 cells. The amount and duration of fixation may need to be optimized for different cell lines.

## **Recipes**

1. Fixation solution
  - a. Add 10 ml of 32% PFA solution stock (EMS) in 150 ml of PBS pH 7.4 to make 2% PFA, mix well
  - b. Store leftover diluted PFA in air-tight bottles, protected from light at either RT (for up to a week) or 4 °C for up to a month
2. Permeabilization solution
  - a. Add 12.5 ml 10% Triton X-100 solution in 487.5 ml PBS (pH 7.4) to make 0.25% Triton X-100 solution
  - b. Mix well and store at RT
3. Blocking Buffer
  - a. Add 1 ml goat serum and 100 µl Triton X-100 (10%) solution in 8.9 ml sterile PBS

- b. Mix well and store at 4 °C for up to one week
4. Wash Buffer A
  - a. Dissolve 8.8 g NaCl, 1.2 g Tris base and 0.5 ml Tween 20 in 800 ml autoclaved water
  - b. Adjust pH to 7.4 using HCl
  - c. Add autoclaved water to 1,000 ml (final concentrations 0.01 M Tris, 0.15 M NaCl and 0.05% Tween 20)
  - d. Filter the solution (0.22 µm filter) and store at 4 °C. Bring the solutions to room temperature before use
5. Wash Buffer B
  - a. Dissolve 5.84 g NaCl, 4.24 g Tris base and 26 g Tris-HCl in 500 ml autoclaved water
  - b. Adjust pH to 7.5 using HCl
  - c. Add autoclaved water to 1,000 ml (final concentrations 0.2 M Tris and 0.1 M NaCl)
  - d. Filter the solution (0.22 µm filter) and store at 4 °C. Bring the solutions to room temperature before use
6. EdU stock solution  
Add 2 ml DMSO directly into the vial containing 50 mg EdU to make 100 mM stocks. Dissolve powder by vortexing. Store aliquots of stock solution at -20 °C protected from light

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### **Competing interests**

The authors declare no conflicts of interest.

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## Isolation and Quantification of Metabolite Levels in Murine Tumor Interstitial Fluid by LC/MS

Mark R Sullivan<sup>1</sup>, \$, Caroline A Lewis<sup>2</sup> and Alexander Muir<sup>3</sup>, \*

<sup>1</sup>Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Cambridge, USA; <sup>2</sup>Whitehead Institute for Biomedical Research, Massachusetts Institute of Technology, Cambridge, USA; <sup>3</sup>Ben May Department for Cancer Research, University of Chicago, Chicago, USA; \$Current address: Department of Immunology and Infectious Diseases, Harvard T.H. Chan School of Public Health, Boston, MA, USA

\*For correspondence: [amuir@uchicago.edu](mailto:amuir@uchicago.edu)



**[Abstract]** Cancer is a disease characterized by altered metabolism, and there has been renewed interest in understanding the metabolism of tumors. Even though nutrient availability is a critical determinant of tumor metabolism, there has been little systematic study of the nutrients directly available to cancer cells in the tumor microenvironment. Previous work characterizing the metabolites present in the tumor interstitial fluid has been restricted to the measurement of a small number of nutrients such as glucose and lactate in a limited number of samples. Here we adapt a centrifugation-based method of tumor interstitial fluid isolation readily applicable to a number of sample types and a mass spectrometry-based method for the absolute quantitation of many metabolites in interstitial fluid samples. In this method, tumor interstitial fluid (TIF) is analyzed by liquid chromatography-mass spectrometry (LC/MS) using both isotope dilution and external standard calibration to derive absolute concentrations of targeted metabolites present in interstitial fluid. The use of isotope dilution allows for accurate absolute quantitation of metabolites, as other methods of quantitation are inadequate for determining nutrient concentrations in biological fluids due to matrix effects that alter the apparent concentration of metabolites depending on the composition of the fluid in which they are contained. This method therefore can be applied to measure the absolute concentrations of many metabolites in interstitial fluid from diverse tumor types, as well as most other biological fluids, allowing for characterization of nutrient levels in the microenvironment of solid tumors.

**Keywords:** Nutrients, Metabolomics, Microenvironment, Interstitial fluid, Cancer metabolism, Mass spectrometry

**[Background]** Cell division requires the duplication of the biomass of the mother cell prior to division. As a result, growing cells must be able to utilize the nutrients available in their environments to synthesize the macromolecules required to divide. To sustain cancerous proliferation, tumors often exhibit altered metabolism (DeBerardinis and Chandel, 2016). In many cases, tumor metabolism is driven by cell-intrinsic processes such as oncogenic activation (Cairns *et al.*, 2011; Nagarajan *et al.*, 2016). However, recent work has highlighted the importance of cell-extrinsic factors in dictating cancer cell metabolism (Anastasiou, 2017; Bi *et al.*, 2018; Muir *et al.*, 2018). The importance of the extracellular environment in shaping cancer metabolism is perhaps unsurprising, as the nutrient

environment in which a cancer cell exists constrains which metabolic reactions are possible within that cell. Since cell-extrinsic metabolite levels can play a role in determining the behavior of tumor cells, it is critical to examine tumor cell metabolism under physiological conditions. However, our understanding of the metabolic composition of the tumor microenvironment is lacking.

The nutrient environment that a cancer cell has access to is predominantly composed of interstitial fluid (Wiig and Swartz, 2012). Understanding the nutrient content of tumor interstitial fluid would provide insight into the metabolic constraints imposed upon tumor cells by their environment. There exist multiple methodologies for isolating interstitial fluid from normal organs and from tumors (Wiig *et al.*, 2010). However, early attempts to measure the nutrient content of interstitial fluid were limited by their inability to measure multiple metabolites, and consequently our knowledge of nutrient availability in tumors is restricted to a few metabolites in a limited number of animal tumor models (Burgess and Sylven, 1962; Gullino *et al.*, 1964). The advent of mass spectrometry has allowed for detection of many metabolites simultaneously. However, despite technological advances, metabolomics studies are complicated by the fact that components present in biological fluids can suppress or enhance the detection of specific metabolites. These discrepancies in detection of metabolites between different biological fluids are termed “matrix effects,” and are a major confounding factor in comparing metabolite concentrations between different biological fluids and in accurately quantitating metabolites in those fluids (Panuwet *et al.*, 2016; Sullivan *et al.*, 2019).

Here we demonstrate a method for centrifugation-based isolation of tumor interstitial fluid and the subsequent absolute quantitation of numerous metabolites within that fluid using stable isotope dilution, a technique in which stable isotope-labeled internal standards for metabolites of interest are added to experimental samples. These stable isotope internal standards are subject to the same matrix effects as the corresponding metabolite in the sample and can be distinguished by their increased mass compared to the metabolites in the sample. To measure many metabolites simultaneously, we first quantitate the concentrations of  $^{13}\text{C}$  metabolites from an extract of polar metabolites from yeast that are cultured with  $^{13}\text{C}$  isotopically labeled glucose as the sole carbon source. This quantitated yeast extract is then used as an internal standard that allows for reliable quantification of targeted metabolites in biological samples while minimizing systematic error from matrix effects. This approach provides a robust method to quantitate polar metabolites in biological fluids and complements similar existing isotope dilution based methods, such as the commercially available Biocrates AbsoluteIDQ kits (Gieger *et al.*, 2008) that primarily quantify non-polar lipids in biological samples.

The absolute quantitation of metabolite levels enabled by this protocol can allow for direct comparison of interstitial fluid composition in diverse tumor types, providing the opportunity to systematically interrogate nutrient availability in animal models of diverse cancers and human tumor samples. Further, the absolute quantification of metabolite levels in interstitial fluid allows for the generation of tissue culture media that mimics physiological conditions found in a tumor, thus expanding the range of *in vitro*/ *ex vivo* experiments that can be carried out under physiological nutrient conditions. Most broadly, this protocol provides a method to absolutely quantify many metabolites simultaneously in complex biological fluids, which can be used to study the metabolic composition of

any biological material.

### **Materials and Reagents**

1. 50 ml conical tube (Falcon, catalog number: 14 959 49A)
2. Lab tape (Thermo Fisher, catalog number: 15-901-20H)
3. EDTA-coated plasma collection tubes (Sarstedt, catalog number: 41.1395.105)
4. 1 ml 25G TB syringe (Becton Dickinson, catalog number: 309625)
5. 20 µM mesh nylon filter (Spectrum Labs, catalog number: 148134)
6. Whatman paper (Thermo Fisher, catalog number: 88600)
7. Petri dishes (Corning, catalog number: 07 202 011)
8. Eppendorf tubes (Corning, catalog number: 14 222 168)
9. LC/MS sample vials (Thermo Fisher, catalog number: C4000-11)
10. LC/MS vial caps (Thermo Fisher, catalog number: C5000-54B)
11. Glassware (bottles, graduated cylinders, conical flasks) reserved for LCMS (not washed by central glass washing services, as this can introduce surfactant and other contaminants into your system)
12. Pipettes (Gilson, catalog number: F167380)
13. Mouse surgical kit (Thermo Fisher, catalog number: 14516249)
14. Blood bank saline (Azer Scientific, catalog number: 16005-092)
15. 70% Ethanol (Thermo Fisher, catalog number: 04-355-305)
16. Acetonitrile LC/MS Optima 4 L (Fisher Scientific, catalog number: A955-4)
17. Methanol LC/MS Optima 4 L (Fisher Scientific, catalog number: A456-4)
18. Pierce formic acid (99%, LC/MS grade, Life Technologies, catalog number: 28905)
19. Water LC/MS Optima 4 L (Fisher Scientific, catalog number: W64)
20. Ammonium carbonate (Sigma-Aldrich, catalog number: 379999)
21. Ammonium hydroxide solution (28%, Sigma-Aldrich, catalog number: 338818)
22. Metabolomics amino acid standard mix (Cambridge Isotope Laboratories, Inc., catalog number: MSK-A2-1.2)
23. <sup>13</sup>C isotopically labeled yeast metabolite extract (Cambridge Isotope Laboratories, Inc., catalog number: ISO1)
24. <sup>13</sup>C<sub>3</sub> lactate (Sigma-Aldrich, catalog number: 485926)
25. <sup>13</sup>C<sub>3</sub> glycerol (Cambridge Isotope Laboratory, catalog number: CLM-1510)
26. <sup>13</sup>C<sub>6</sub> <sup>15</sup>N<sub>2</sub> cystine (Cambridge Isotope Laboratory, catalog number: CNLM-4244)
27. <sup>2</sup>H<sub>9</sub> choline (Cambridge Isotope Laboratory, catalog number: DLM-549)
28. <sup>13</sup>C<sub>4</sub> 3-hydroxybutyrate (Cambridge Isotope Laboratory, catalog number: CLM-3853)
29. <sup>13</sup>C<sub>6</sub> glucose (Cambridge Isotope Laboratory, catalog number: CLM-1396)
30. <sup>13</sup>C<sub>2</sub> <sup>15</sup>N taurine (Cambridge Isotope Laboratory, catalog number: CNLM-10253)
31. <sup>2</sup>H<sub>3</sub> creatinine (Cambridge Isotope Laboratory, catalog number: DLM-3653)

32.  $^{13}\text{C}_5$  hypoxanthine (Cambridge Isotope Laboratory, catalog number: CLM-8042)
33.  $^{13}\text{C}_3$  serine (Cambridge Isotope Laboratory, catalog number: CLM-1574)
34.  $^{13}\text{C}_2$  glycine (Cambridge Isotope Laboratory, catalog number: CLM-1017)
35. Chemical standard library pool 1

<b>Metabolite name</b>	<b>Manufacturer</b>	<b>Part#</b>
Alanine	Sigma	A7469
Arginine	Sigma	A6969
Asparagine	Sigma	A7094
Aspartate	Sigma	A7219
Carnitine	Sigma	C0283
Citrulline	Sigma	C7629
Cystine	Sigma	C7602
Glutamate	Sigma	G8415
Glutamine	Sigma	G3126
Glycine	Sigma	G7126
Histidine	Sigma	53319
Hydroxyproline	Sigma	H54409
Isoleucine	Sigma	I7403
Leucine	Sigma	L8912
Lysine	Sigma	L8662
Methionine	Sigma	M5308
Ornithine	Sigma	75469
Phenylalanine	Sigma	P5482
Proline	Sigma	P5607
Serine	Sigma	S4311
Taurine	Sigma	T0625
Threonine	Sigma	T8441
Tryptophan	Sigma	T8941
Tyrosine	Sigma	T8566
Valine	Sigma	V0513
Lactate	Sigma	L7022
Glucose	Sigma	G7528

36. Chemical standard library pool 2

<b>Metabolite name</b>	<b>Manufacturer</b>	<b>Part#</b>
2-hydroxybutyric acid	Sigma	220116
2-aminobutyric acid	Sigma	162663-25G
AMP	Sigma	A1752
Argininosuccinate	Sigma	A5707-50MG
Betaine	Sigma	61962

Biotin	Sigma	B4639
Carnosine	Sigma	C9625-5G
Choline	Sigma	C7017
CMP	Sigma	C1006
Creatine	Sigma	C0780-50G
Cytidine	Sigma	C4654
dTMP	Sigma	T7004-100MG
Fructose	Sigma	F0127
Glucose-1-phosphate	Sigma	G6750
Glutathione	Sigma	G4251
GMP	Sigma	G8377
IMP	Sigma	57510-5G
O-phosphoethanolamine	Sigma	P0503-1G
Pyridoxal	Sigma	P9130-500MG
Thiamine	Sigma	T4625
trans-Urocanate	Cayman	16228
UMP	Sigma	U6375-1G
Xanthine	Sigma	X7375-10G

37. Chemical standard library pool 3

Metabolite name	Manufacturer	Part#
3-hydroxybutyric acid	Sigma	H6501
Acetylalanine	Sigma	A4625-1G
Acetylaspartate	Sigma	00920-5G
Acetylcarnitine	Sigma	A6706
Acetylglutamine	Sigma	A9125-25G
ADP	Sigma	A5285
Allantoin	Sigma	05670-25G
CDP	Abcam	ab146214-100 mg
CDP-choline	Alfa	J64161-10 g
Coenzyme A	Sigma	C4282
Creatinine	Sigma	C4255-10MG
gamma-aminobutyric acid	Sigma	A2129-10G
GDP	Sigma	G7127
Glutathione disulfide	Sigma	G4376
Glycerate	Sigma	367494
Hypoxanthine	Sigma	H9377
myo-Inositol	Sigma	I5125
NAD+	Sigma	N1511
p-aminobenzoate	Sigma	A9878

Phosphocholine	Sigma	P0378-5G
Sorbitol	Sigma	W302902
UDP	Sigma	94330-100MG
UDP-glucose	Sigma	U4625-100MG

38. Chemical standard library pool 4

Metabolite name	Manufacturer	Part#
Phenylacetylglutamine	Cayman	16724-25mg
Acetylglutamate	Sigma	855642
Acetylglycine	Sigma	A16300-5G
Acetylmethionine	Sigma	01310-5G
Asymmetric dimethylarginine	Cayman	80230
ATP	Sigma	A2383
CTP	Sigma	C1506
dATP	Sigma	D6500
dCTP	Sigma	D4635
Deoxycytidine	Sigma	D3897
Folic acid	Sigma	F8758
GTP	Sigma	G8877
Hypotaurine	Sigma	H1384-100MG
Methionine sulfoxide	Sigma	M1126-1G
Methylthioadenosine	Sigma	D5011-25MG
Phosphocreatine	Sigma	P7936-1G
Pyridoxine	Sigma	P9755
Ribose-5-phosphate	Sigma	83875
SAH	Sigma	A9384-25MG
Thymidine	Sigma	T9250
Trimethyllysine	Sigma	T1660-25MG
Uridine	Sigma	U3003
UTP	Sigma	U6625

39. Chemical standard library pool 5

Metabolite name	Manufacturer	Part#
3-phosphoglycerate	Sigma	P8877
cis-aconitic acid	Sigma	A3412-1G
Citrate	Mallinckrodt	754
DHAP	Sigma	51269
Fructose-1,6-bisphosphate	Sigma	F6803
Fumarate	Sigma	240745
Glucose-6-phosphate	Sigma	G7879
Glycerol-3-phosphate	Cayman	20729-100 mg

Guanidinoacetate	Sigma	G11608
Kynurenine	Sigma	K8625
Malate	Sigma	2288
NADP+	Sigma	N0505
Niacinamide	Sigma	72340
2-oxoglutarate	Sigma	75890-25G
Phosphoenolpyruvate	Sigma	P3637
Pyruvate	Sigma	P5280
Succinate	Sigma	S3674
Uracil	Sigma	U0750

40. Chemical standard library pool 6

Metabolite name	Manufacturer	Part#
3-hydroxyisobutyric acid	Adipogen	CDX-H0085-M250
2-hydroxyglutarate	Sigma	H8378
Aminoadipate	Sigma	A0637
beta-alanine	Sigma	14064
Carbamoylaspartate	Alfa	A17166-10 g
Cystathionine	Cayman	16061-50 mg
Cysteic acid	Santa Cruz	sc-485621
FAD	Sigma	F6625
Glycerophosphocholine	Sigma	G5291-50MG
Inosine	Sigma	I4125
Orotate	Sigma	O2875
Pantothenate	Sigma	P5155
Phosphoserine	Fluka	79710
Riboflavin	Sigma	R9504
UDP-GlcNAc	Sigma	U4375
Uric acid	Sigma	U2625-25G

41. Chemical standard library pool 7

Metabolite name	Manufacturer	Part#
Itaconic acid	Sigma	I29204
Homocysteine	TCI	H0159
2-oxobutyric acid	Sigma	K401
2-hydroxybutyric acid	Sigma	220116
Ascorbate	Sigma	A7506
Sarcosine	Sigma	131776-100G
Dimethylglycine	Sigma	D1156-5G
N6-acetyllysine	Sigma	A4021-1G
Pipecolate	Sigma	P45850-25G

Indolelactate	Sigma	I5508-250MG-A
Picolinate	Sigma	P42800-5G
3-methyl-2-oxobutyrate	Sigma	198994-5G
3-methyl-2-oxopentanoic acid	Sigma	198978-5G
Formyl-methionine	Sigma	F3377-250MG
2-aminobutyric acid	Sigma	A2536-1G
Homocitrulline	Santa Cruz	sc-269298-100 mg
gamma-glutamyl-alanine	Sigma	483834-500MG
Mannose	Sigma	M6020-25G
Cysteine-glycine (dipeptide)	Sigma	C0166-100MG
42. Mobile Phase A (see Recipes)		
43. Mobile Phase B and Needle Wash (see Recipes)		
44. Rear Seal Wash (see Recipes)		
45. 80% methanol containing $^{13}\text{C}$ - $^{15}\text{N}$ labeled amino acid mix (see Recipes)		
46. Extraction Buffer with isotopically labeled internal standards (EB) (see Recipes)		
47. Chemical standard library preparation (see Recipes)		

## Equipment

1. Sorvall Legend X1R Refrigerated Centrifuge (Thermo Fisher, catalog number: 75004260)
2. Sorvall Legend Micro 21 Refrigerated Centrifuge (Thermo Fisher, catalog number: 75002447)
3. Mixer Mill (Retsch, catalog number: MM301)
4. 50 ml Mixing Jar (Retsch, catalog number: 01.462.0216)
5. 5 mM Grinding Balls (Retsch, catalog number: 05.368.0034)
6. LP Vortex Mixer (Thermo Fisher, catalog number: 11676331)
7. Analytical balance (Mettler Toledo, catalog number: AL54)
8. Dionex Ultimate 3000 UHPLC equipped with RS Binary Pump, RS column oven and RS autosampler (Thermo Fisher Scientific, San Jose, CA)
9. QExactive hybrid quadrupole-Orbitrap benchtop mass spectrometer equipped with an Ion Max ion source and HESI-II probe (Thermo Fisher Scientific, San Jose, CA)
10. SeQuant® ZIC®-pHILIC 5  $\mu\text{m}$  150 x 2.1 mm polymeric analytical PEEK HPLC column (Millipore Sigma, catalog number: 1504600001)
11. SeQuant® ZIC®-pHILIC 5  $\mu\text{m}$  20 x 2.1 mm PEEK Guard Kit with coupler (3 pc) (Millipore Sigma, catalog number: 15043800001)

## Software

1. Thermo Scientific Xcalibur 4.1 SP1 (Thermo Fisher Scientific)
2. Microsoft Excel

## **Procedure**

### A. Study design

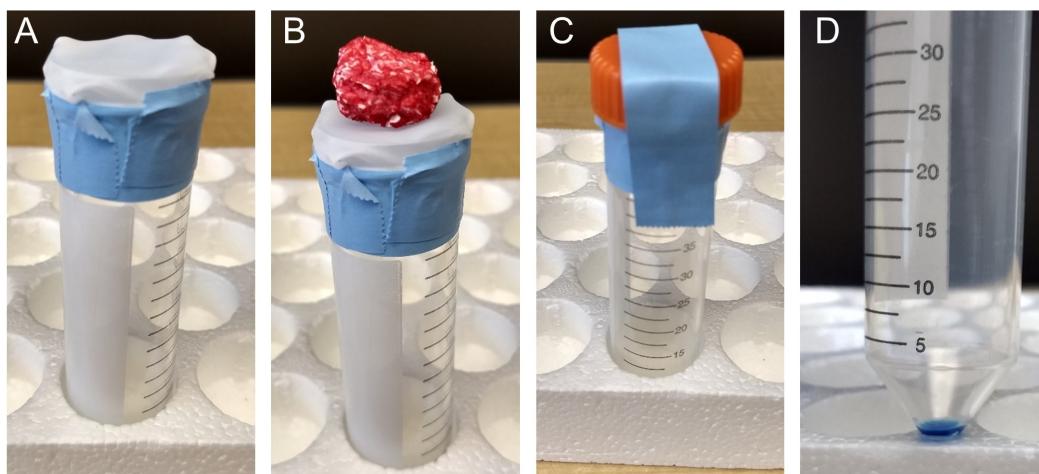
1. How many biological replicates are recommended?
  - a. The number of biological replicates needed for studies will depend on the variability between samples. For animal studies, where a large number of variables can be controlled (i.e., tumor genetics, tumor size, animal genetics, animal diet, time of interstitial fluid isolation), variability will likely be smaller than for human samples. Additionally, the number of replicates required will depend on the intended purpose of the experiment to be performed. For example, if the intended purpose is to determine if there is a nutritional difference in the interstitial fluid between two tumor types, it is important to determine an effect size between the groups in addition to variability between samples in order to estimate sample sizes needed. We recommend generating pilot data or utilizing previously published data on TIF composition differences (Sullivan *et al.*, 2019) to estimate effect sizes and utilizing power analysis software in Metaboanalyst (Chong *et al.*, 2018) or other statistical analysis software to estimate the number of biological replicates needed.
  - b. Additionally, when determining the number of biological replicates needed, it is important to note that not every tumor will necessarily yield interstitial fluid. In our experience, roughly 75% of murine pancreatic adenocarcinomas yielded tumor interstitial fluid (TIF) with volumes ranging from 5 to 180 µl of fluid (Sullivan *et al.*, 2019). Therefore, additional samples may be required to achieve the required number of replicates determined from power analysis.
2. We recommend harvesting TIF at the same time from all animals involved in a study. Circadian rhythm and food intake can alter plasma metabolite levels and therefore TIF metabolite levels, introducing additional variability (Dallmann *et al.*, 2012; Abbondante *et al.*, 2016). If TIF must be harvested from animals on different days, we recommend harvesting TIF at the same time during the day.
3. We recommend two people work together to isolate TIF and cardiac blood to increase the speed of TIF harvest to prevent alterations in TIF composition due to prolonged periods of ischemia occurring between euthanasia and TIF harvest. In our own experiments, dissection was completed in ~2 min. and we found limited evidence of ischemia altering tumor metabolite levels (Sullivan *et al.*, 2019).
4. We have successfully isolated TIF using the protocol described in Procedure B from multiple genetically engineered and implantation mouse models of breast, lung, prostate, pancreas and melanoma cancers and others have isolated TIF from murine models of melanoma and breast cancer (Ho *et al.*, 2015; Eil *et al.*, 2016; Spinelli *et al.*, 2017; Zhang *et al.*, 2017) using similar protocols. Additionally, similar protocols have been used to isolate TIF from renal (Siska *et al.*, 2017) and ovarian carcinomas (Haslene-Hox *et al.*, 2011). Thus, we anticipate the TIF isolation

protocol can be used successfully for a variety of tumor types, of mouse and human origin.

5. The analysis described in Procedure C uses 7 separate chemical standard pools as described in (Sullivan *et al.*, 2019) that enable the quantification of 149 metabolites commonly measured in biofluids (Lawton *et al.*, 2008; Evans *et al.*, 2009; Mazzone *et al.*, 2016; Cantor *et al.*, 2017). However, depending on experimental goals, the full analysis utilizing all 7 standard pools may not be required. Subsets of the chemical standard pools can be used that cover analytes of interest if the full analysis is not needed. Note though that individual metabolites in the standard pools provided in this protocol have been carefully selected so as to avoid metabolites with the same m/z (isomeric and isobaric species) being in the same pool. In addition, metabolites that could be generated by in-source fragmentation from larger metabolites have been separated.
6. The description of the liquid chromatography-mass spectrometry analysis in this protocol (Procedure D) is a rough guideline for experienced operators of such instruments to perform the analysis described. Successful mass spectrometry analysis of the samples will require a trained UHPLC and Thermo Scientific hybrid quadrupole-Oribtrap mass spectrometer operator.

#### B. Isolation of TIF and plasma from tumor bearing animals

1. Prepare a TIF isolation tube (Figure 1 A).
  - a. Take a nylon filter and place it over the top of a 50 ml conical tube.
  - b. Tape the filter down using lab tape. Make sure the filter is affixed somewhat loosely to the top of the conical tube, such that the tumor can push the filter down slightly into the tube.



**Figure 1. Collecting TIF using nylon mesh filters attached to conical tubes.** A. The filter is loosely affixed to the top of the conical tube using laboratory tape, so that the filter will sag slightly into the conical tube when a sample is placed on top of the filter. B. A sample on top of the filter and conical tube. C. After adding the sample to the filter, the lid of the conical tube is placed on top, but not screwed onto the conical. Instead, it is taped using laboratory tape in place. D. ~30 µl of tumor interstitial fluid (colored blue to here for contrast) collected in the conical tube after centrifugation.

2. Prepare materials in advance to allow for rapid mouse dissection.
  - a. Put pre-chilled (4 °C) saline (~25 ml) into a Petri dish for washing the tumor.
  - b. Make a ~4 cm square piece of Whatman paper for drying the tumors after the saline rinse.
  - c. Have a 25G TB syringe ready for cardiac blood collection.
  - d. Label and pre-chill one EDTA-coated plasma collection tubes on ice for 10 min prior to mouse dissection.
3. Euthanize the mouse by cervical dislocation.
4. Spray around the incision site with 70% ethanol to prevent contamination of samples with hair.
5. Quickly and cleanly dissect the tumor away from the animal. The exact procedure for the dissection will depend on the anatomical location of the tumor. One person should continue with the following steps while the other person can start on plasma isolation (Step B6).
  - a. Place the tumor into the saline containing Petri dish to rinse the tumor.
  - b. Blot the tumor dry on Whatman paper. Take care at this step to ensure all saline is blotted from the tumor, so that saline does not contaminate the TIF.

If concerned about saline contamination during TIF isolation, we suggest the following additional step: add a non-natural metabolite such as norvaline to the saline. Continue with the remainder of the protocol as normal. Subsequently, when analyzing TIF samples, determine if the non-natural metabolite is present in the TIF sample. If the non-natural metabolite is detected this indicates saline contamination, whereas if the metabolite is not detected saline contamination is unlikely.
  - c. Optional: Weigh the tumor if this information is needed. Tumor volume can also be determined by caliper measurements if needed.
  - d. Place the tumor on top of the nylon mesh filter that is affixed to the conical tube (Figure 1B). Place the 50 ml conical lid over the tumor and tape the lid in place (Figure 1C).
  - e. Centrifuge the tumor at 4 °C for 10 min at 106 x g using a refrigerated clinical centrifuge (e.g., Sorvall Legend X1R).
  - f. Remove the 50 ml conical tube. If the isolation worked, there will be 10-50 µl of fluid in the bottom of the conical tube (Figure 1D). Keep the tube on ice.

*Note: Human tumors have been found to yield 5-150 µl of fluid per gram of tumor (Haslene-Hox et al., 2011). Similarly, we isolated 10-50 µl of fluid from murine pancreatic tumors weighing ~0.3-2.5 g (Sullivan et al., 2019). There is not an exact correlation between tumor size and TIF volume, as many factors likely influence interstitial volume. However, larger tumors are more likely to yield TIF in larger volumes. Tumors with large fluid filled cysts can give hundreds of µl of fluid per gram of tumor. Disregard these from analysis as it is unclear if the cystic fluid is representative of interstitial fluid.*
  - g. Remove the isolate fluid to a fresh Eppendorf tube. The fluid can be extracted directly for LC/MS analysis or frozen and stored at -80 °C for future analysis.

We have analyzed TIF samples both before and after 2 months of storage at -80 °C

(avoiding freeze-thaw cycles) and detected similar metabolite concentrations after storage (Sullivan *et al.*, 2019). Thus, TIF samples can be stored for at least 2 months without freeze-thaw cycles prior to analysis.

- h. The tumor from which TIF was isolated can be used for additional analysis using other appropriate protocols.

*Note: We have successfully used tumors from which TIF was isolated for immunohistochemical, immunoblotting and flow cytometric analyses.*

6. Use the 25G TB syringe to isolate blood from the mouse heart by cardiac puncture. Cardiac puncture can be a difficult technique to perform. Detailed protocols with video documentation of cardiac puncture blood isolation have been previously published (Schroeder, 2019). If unfamiliar with this technique, we recommend utilizing these resources for more detailed information on isolating blood in this manner.

- a. Dissect open the thoracic cavity.  
b. Insert the syringe into the ventricle.  
c. Slowly withdraw blood to prevent collapse of the heart.  
d. Remove the needle from the syringe to prevent cell lysis when expelling the cells from the syringe.  
e. Expel the blood into the EDTA-coated plasma collection tube.  
f. Keep plasma on ice.  
g. Spin the EDTA-coated plasma collection tubes at 845  $\times$  g in benchtop centrifuge (e.g., Sorvall Legend Micro 21) for 15 min at 4 °C.  
h. Remove the plasma from the pelleted blood cells and put into fresh Eppendorf tube.  
i. Extract this plasma directly for LC/MS analysis or freeze and store at -80 °C for future analysis.

*Note: Previous studies have found that plasma samples can be stored at -80 °C (without freeze-thaw cycles) for up to 30 months without significant alterations in the levels of many metabolites (Stevens *et al.*, 2019). Thus, plasma samples can be stored for many months prior to analysis.*

#### C. Extraction of metabolites from TIF and plasma

1. Prepare libraries of pooled chemical standards that include the metabolites to be quantified. See Recipes section and Tables 5-11 for details on how libraries were compounded in (Sullivan *et al.*, 2019).
2. Prepare metabolite extraction buffer (EB) (Recipe 5) with appropriate isotopically labeled internal standards. See Recipes section for details on making EB with isotopic standards as described in (Sullivan *et al.*, 2019).

*Note: Make enough EB for the number of samples and standards you have plus an additional 10%, so as not to run out of EB before extracting metabolites from every sample. 45 µl of EB is needed for each sample and standard pool dilution. Isotopically labeled metabolite standards in*

*the EB are not indefinitely stable. Prepare EB fresh prior to each experiment.*

3. Prepare dilutions of chemical standard libraries in HPLC grade water. The highest concentration should be 5 mM.
  4. Next, make dilution series of each standard pool in HPLC grade water as listed below in Step C5. Keep these on ice prior to extraction. Note that multiple dilutions of the chemical standard libraries are required for construction of standard curves relating known metabolite concentration to LC/MS response, which is necessary for downstream analysis of metabolite concentrations. Below we recommend a scheme to generate 8-point standard curves covering physiological concentrations of metabolites, but variations are possible.
  5. The 5 mM pool will not be always in solution for each pool, therefore vortex vigorously and immediately pipette from this mixture in order to prevent error from settling particles:
    - a. Take 20 µl of 5 mM stock and dilute into 80 µl HPLC grade water to yield a 1 mM solution.
    - b. Take 30 µl of 1 mM stock and dilute into 70 µl HPLC grade water to yield a 300 µM solution.
    - c. Take 33.33 µl of 300 µM stock and dilute into 66.67 µl HPLC grade water to yield a 100 µM solution.
    - d. Take 30 µl of 100 µM stock and dilute into 70 µl HPLC grade water to yield a 30 µM solution.
    - e. Take 33.33 µl of 30 µM stock and dilute into 66.67 µl HPLC grade water to yield a 10 µM solution.
    - f. Take 30 µl of 10 µM stock and dilute into 70 µl HPLC grade water to yield a 3 µM solution.
    - g. Take 33.33 µl of 3 µM stock and dilute into 66.67 µl HPLC grade water to yield a 1 µM solution.
  6. Thaw the TIF and plasma samples on ice.
  7. Add 5 µl of each TIF sample, plasma sample and chemical standard library dilution (Recipe 6) to a fresh Eppendorf tube. Keep on ice.
  8. Add 45 µl of EB to each sample/standard. Keep on ice.
  9. Vortex all the samples for 10 min at maximum speed at 4 °C.
  10. Spin down all samples for 10 min at 21,000 x g at 4 °C.
  11. Take 20 µl of the mixtures from the Eppendorf tube and add to an LC/MS sample vial. Cap the vial.
- Note: A minimum of 15 µl is needed in the LC/MS vial to ensure correct and accurate injection by the autosampler. The vials described in Materials and Reagents contain fused inserts. Vials without inserts will require larger volumes.*
12. Freeze the remaining sample in the Eppendorf tubes and store at -80 °C. This sample can be run later if the initial LC/MS is not successful.
- D. Liquid chromatography-mass spectrometry analysis of extracted metabolites
1. Start off with a clean system (use appropriate LC cleaning methods in place in your lab).
  2. Calculate the amount of Mobile Phase A (Recipe 1) required and prepare fresh on the day of

analysis. Store this for no more than one week.

*Note: Depending on your system, you will use ~2 ml per injection and require an additional 50-100 ml in the bottle. Do not forget to make enough Mobile Phase A for additional injection types such as solvent blanks and system suitability tests that must be run in addition to the samples.*

3. Calculate the amount of Mobile Phase B (Recipe 2) required and prepare more if needed. Depending on your system, you will need ~2 ml per injection plus an additional 50-100 ml in the bottle.
4. Check the level of rear seal wash (Recipe 3) and top up if needed.
5. If using an UHPLC system that has a separate needle wash, fill this with acetonitrile.
6. Connect a SeQuant® ZIC®-pHILIC 5 µm 150 x 2.1 mm analytical column to the Guard column using the connector supplied in the Guard kit.
7. Connect the column and guard to your UHPLC system using standard techniques.
8. Set the column oven temperature to 25 °C.
9. Set the autosampler temperature to 4 °C.
10. Set initial conditions: set the flow rate to 0.150 ml/min with 80% B. Record initial pressure value.

*Note: ZIC-pHILIC columns cannot tolerate such high back pressures and injection volumes as typical reverse phase columns. Keep an eye on the back pressure and do not let it exceed the maximum pressure recommended by the manufacturer. It is good practice to set a maximum pressure in your method that is below that set by the manufacturer to avoid damage to the column.*

11. Equilibrate the column with starting conditions (80% B) for 30 min prior to running anything on the system.
12. Check the mass calibration on the mass spectrometer. If the mass has not been calibrated within the last week, or if it fails the mass check, recalibrate using the standard calibration mixes recommended by the manufacturer. In addition, perform a custom low-mass calibration by spiking glycine and aspartate into the calibration mix, or as recommended by the manufacturer.
13. Ensure your entire LCMS system performance is acceptable by running system suitability tests, such as injecting a mixture of amino acids onto your column and into the MS. Check for signal intensity as well as peak shape and separation.
14. Use the conditions shown in Table 1 below for the UHPLC gradient:

**Table 1. LC parameters**

Time (min)	Flow rate (ml/min)	%B
0.00	0.150	80

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20.0	0.150	20
20.5	0.150	80
28.0	0.150	80

---

15. Operate the mass spectrometer in full-scan, polarity-switching mode, with a scan range of 70-1000 m/z. Include an additional narrow-range scan from 220 to 700 m/z in negative mode to improve detection of nucleotides. Use the parameters shown below in Tables 2 and 3 for the MS:

**Table 2. MS source parameters**

---

Parameter	Setting
Spray voltage	3.0 kV (pos); 3.1 kV (neg)
Heated capillary	275 °C
HESI probe	350 °C
Sheath gas	40 units
Aux gas	15 units
Sweep gas	1 unit

---

**Table 3. MS scan parameters**

---

Parameter	Setting
Resolution	70,000
AGC target	1E6
Max IT	20 ms

---

16. Note that in this particular study, we had previously collected MS/MS data for each metabolite being quantified, and used this to confirm retention times using a library of chemical standards. If adding new metabolites to your standard pools, collect MS/MS data on the standard itself, as well as on a pooled biological sample to help confirm peak identification.
17. Write your sequence (sample run order) using Thermo Scientific Xcalibur Sequence Setup View.

*Note: Given the extremely low volume of samples used in this method, the sequence differs from typical sequences which will include column conditioning injections, as well as quality control pooled samples. As multiple standard curves are run and each sample includes <sup>13</sup>C-labeled internal standards, we chose to forgo using precious sample to create QC pools and instead determine linearity and consistency of metabolite detection using the standard curves and the <sup>13</sup>C internal standards.*

- a. Start off by injecting several water blanks to ensure system is clean from carry over and contaminants.
  - b. Include solvent blanks, using the 75/25/0.1 acetonitrile/methanol/formic acid mix that was used to make the extraction mix.
  - c. Follow with a system suitability test (SST) injection. We use 80% methanol containing <sup>13</sup>C-<sup>15</sup>N labeled amino acid mix (Recipe 4).
  - d. Add the samples to your sequence and follow with the standard curves, starting with the lowest concentration and working up to the highest concentration for each curve. Separate each curve with solvent blank and check for carry-over.
  - e. Insert additional SST injections every 8-10 samples. These will be used as QCs to ensure no loss of signal over time.
  - f. Set the injection volume to 2 µl for each injection type.
  - g. Set the instrument method to the appropriate method.
  - h. Save the sequence file.
  - i. Randomize sample running order to decrease the chance of signal loss over time. Export the sequence as a .csv file. Open in Microsoft Excel, cut and paste the samples into a new tab, leaving behind the blanks, STTs and standard curves. Add an additional column and use the = rand () function to create random numbers for each of the samples. Now sort the samples from smallest to largest using the random number values. Cut and paste back into the previous sequence containing the blanks, SSTs and the standard curves. Save the .csv file with “random” in the file name. Import the new randomized sequence back into Xcalibur Sequence View and save using “random” in the file name.
  - j. Place your sample vials in the autosampler in the vial positions according to the sequence. Use the *non-randomized* sequence to check vial positions for your samples.
  - k. Ensure the solvent blank vials and SST vials contain enough volume for multiple injections.
18. Run the sequence.

*Note: For examples of expected outputs from the LC/MS analysis of TIF and plasma samples, LC/MS data from (Sullivan et al., 2019) is available at <https://www.metabolomicsworkbench.org/data/DRCCMetadata.php?Mode=Project&ProjectID=PR000750>.*

## Data analysis

A. Identify metabolite peaks. This protocol will describe peak identification in Thermo Scientific Xcalibur, but could be adapted with any other peak identification method.

1. Generate a processing method file that will be used to identify peaks for each metabolite of interest:

- a. Create a new processing method.
- b. Load a .raw file containing LC/MS data derived from an external standard sample that contains the metabolite of interest as well as a  $^{13}\text{C}$  internal standard for that metabolite.  
*Note: Typically using an external standard sample that is in the middle of the standard curve works best; sometimes high concentrations points on the standard curve have poor quality peaks.*
- c. Calculate the exact mass of the metabolite of interest.

Notes:

- i. *Exact mass is determined by summing the masses of the most abundant isotopes of each element in a compound. For instance, the exact mass of CO<sub>2</sub> would be the summed masses of a carbon-12 atom (12.000) + two oxygen-16 atoms (15.995 + 15.995) = 43.990. This exact mass of a compound will differ from its molecular weight; the molecular weight of an element is derived by averaging the masses of each of the isotopes of that element, weighted by the abundance of each isotope in nature.*
- ii. *If using Thermo Scientific Xcalibur, calculate the exact mass using the Isotope Simulation tab in the QualBrowser module. Enter the chemical formula, ensure the “adduct” check box is unchecked and select “New”. Ensure that the software global settings are set to a mass tolerance of 5 ppm and mass precision is set to 5 decimal places. Ideally, the peak integration software that you are using should calculate this for you.*
- d. Calculate the mass to charge ratio (m/z) of the metabolite of interest in positive and negative ionization mode.

Notes:

- i. *For analysis of small polar metabolites, the most common ions will be those that have gained or lost a single proton and most molecules will have a charge state of 1.*
- ii. *If using Thermo Scientific Xcalibur, calculate the m/z using the Isotope Simulator in the QualBrowser module by entering the formula, checking the “adduct” box and selecting a charge of either +1 or -1, depending on whether you are calculating the m/z in positive or negative mode, respectively.*
- iii. *There are a variety of online tools available that will provide exact mass information, as well as calculate m/z for a variety of different adducts. The most comprehensive is the Metlin data base (Gujas et al., 2018):*

[https://metlin.scripps.edu/landing\\_page.php?pgcontent=mainPage.](https://metlin.scripps.edu/landing_page.php?pgcontent=mainPage)

- e. In the processing method, select either positive ionization mode or negative ionization mode depending on whether you will be searching for a positively charged ion or a negatively charged ion.

*Note: Some metabolites are more easily detected in positive or negative mode. A list of recommendations for which mode to use for a variety of metabolites is located in Supplementary File 1 of (Sullivan et al., 2019). If no recommendations are available, empirically determine which method gives better detection by trying both.*

- f. Identify and validate the retention time of each metabolite:
    - i. Search for the exact mass of the ion of interest.
    - ii. Note the retention time of any peaks that match the exact mass of the ion of interest within 5 ppm.
    - iii. Open a .raw file of a different external standard sample with the metabolite of interest at a lower concentration.
      - 1) Note which peaks that match the exact mass of the ion of interest decrease in area.
      - 2) Repeat with each of the external standard samples that contain the metabolite of interest, checking which peak areas track with the expected amount of the metabolite.
      - 3) Refer to MS/MS data to confirm peak identification.
    - iv. Open a .raw file that does not contain the metabolite of interest. Ensure that any candidate peaks are not present in this .raw file.
    - v. Search for the exact mass of the <sup>13</sup>C labeled version of the ion of interest.

*Note: This peak should be approximately the same area in all samples.*
    - vi. Check that the retention time of the <sup>13</sup>C labeled standard peak exactly matches that of the candidate peak.
    - vii. Repeat for all metabolites of interest.
  - g. Assign <sup>13</sup>C labeled standards as internal standards for their corresponding <sup>12</sup>C metabolites. For metabolites with no <sup>13</sup>C internal standard, assign a <sup>13</sup>C metabolite with a similar retention time as the internal standard.
2. Use the processing method to pick and validate peaks for all metabolites in all LC/MS data files (both experimental samples and external standards):
    - a. Once all peaks have been automatically picked, manually inspect every peak for each metabolite and for each sample to ensure that all peaks have been correctly identified. Some common examples of errors that occur with automatic peak picking algorithms:
      - i. Incorrect peak was picked: this can occur for isobaric compounds with similar retention times, such as leucine and isoleucine.
      - ii. Peak was not fully picked from baseline to baseline.
      - iii. Peak was picked but overlaps with a second peak: this occasionally happens where

biological samples have an overlapping peak that was not present in the external standards. If this is the case, this metabolite should not be quantitated using this LC/MS method and an alternative method of chromatographic separation should be identified.

- b. Export the ratio of peak areas for the sample versus the  $^{13}\text{C}$  internal standard to Microsoft Excel or your data processing software of choice.
  
- B. Determine the relationship between relative peak area and concentration of metabolite in external standards.
  1. Calculate the exact concentration of each metabolite in each point on the external standard curve based on the amount that was weighed out.
  2. Generate a graph of metabolite concentration in each external standard sample versus the relative peak area of the metabolite.
  3. Check if the relative peak area of the metabolite increases linearly with concentration:
    - a. Fit a linear regression to the graph.
    - b. The  $R^2$  value for the linear regression should be  $\geq 0.995$ .Metabolites often respond non-linearly at high concentrations. If the standard curve has points that are much higher than the concentrations present in experimental samples, the highest points on the standard curve can be removed. Just ensure that the relative peak areas for all samples fall within the linear range of the standard curve.
  - c. Non-linear metabolites should be excluded from quantitative analysis, as this lack of linearity will prevent accurate quantitation by isotope dilution.

- C. Determine the concentrations of the internal standards that were added to all samples.

Solve for the concentration of  $^{13}\text{C}$  internal standard present in each external standard sample using the following relationship:

$$\frac{\text{actual concentration } ^{12}\text{C metabolite}}{\text{actual concentration } ^{13}\text{C metabolite}} = \frac{\text{relative peak area } ^{12}\text{C metabolite}}{\text{relative peak area } ^{13}\text{C metabolite}}$$

*Note: This relationship can be used to calculate the concentration of the  $^{13}\text{C}$  internal standard in each of the external standard samples; the same concentration should be present in each. To derive the most accurate value for the concentration of the  $^{13}\text{C}$  internal standard, average the concentrations derived from the external standard points most similar in concentration to the experimental samples.*

- D. Calculate the concentration of each metabolite in the experimental samples by isotope dilution. Solve for the concentration of the  $^{12}\text{C}$  metabolite using the same relationship defined in step C.

- E. Calculate the semi-quantitative concentration of all other analytes using the external standard curves. These values are considered semi-quantitative as they are subject to matrix effects arising from the biological samples being compared to external standards dissolved in water. These matrix effects can be substantial (Sullivan *et al.*, 2019).
1. Calculate the slope and intercept of the linear regression calculated in step B3a.
  2. Use this slope and intercept to calculate the semi-quantitative value approximating the concentration of the metabolite.
  3. Manually evaluate the concentrations derived from this calculation:
    - a. Check that the value of each metabolite is zero in the external standard samples that do not contain that metabolite.
    - b. Any data that shows a negative concentration should be removed.
- F. Perform statistical analysis of the data using Metaboanalyst (<https://www.metaboanalyst.ca/MetaboAnalyst/faces/home.xhtml>) (Chong *et al.*, 2018) or another program for statistical analysis.
1. Auto-scale the data (mean-center and divide by the standard deviation of each concentration).
  2. To broadly compare if there are differences in the metabolites present in two sample types, perform principal component analysis or hierarchical clustering.
  3. To identify specific metabolites that differ in concentration between sample types, generate a volcano plot in which a raw *P*-value of 0.01 and a fold change of 1.5 are used to identify significantly altered metabolites.

## Recipes

1. Mobile Phase A  
20 mM ammonium carbonate  
0.1% ammonium hydroxide (pH 9.4-9.6)  
Optima LC/MS water
2. Mobile Phase B and Needle Wash  
Optima LC/MS acetonitrile
3. Rear Seal Wash  
10% Optima LC/MS methanol  
Optima LC/MS water
4. 80% methanol (containing  $^{13}\text{C}$ - $^{15}\text{N}$  amino acid standard mix) (200 ml)  
160 ml Optima LC/MS methanol  
40 ml Optima LC/MS water  
40  $\mu\text{l}$  Metabolomics Amino Acid Standard Mix
5. Extraction Buffer with isotopically labeled internal standards (EB) (Table 4)

*Notes:*

- a. This is the recipe containing isotopically labeled internal standards for analysis of metabolites as in (Sullivan et al., 2019). If analysis of other metabolites using stable isotope internal standards is desired, purchase or synthesize the desired isotopically labeled metabolite and add it to the Extraction Buffer. Quantification with isotopically labeled standards performs best when the abundance of the isotopically labeled metabolite is similar to the unlabeled metabolite to be quantified. Therefore, when adding isotopically labeled internal standards, add the isotope such that it will be at roughly a similar abundance as the unlabeled metabolite when the sample is diluted in the Extraction Buffer.
- b. This recipe is for 180 samples at 45  $\mu$ l per sample for a total of 8,100  $\mu$ l in volume. Adjust the volumes accordingly as needed for the number of samples you intend to analyze. After adding all components, vortex briefly to ensure EB is well mixed, and store on ice while in use. Make fresh prior to each experiment. Remember to include extra 75/25/0.1 acetonitrile/methanol/formic acid for use as solvent blanks in your calculations.

**Table 4. Extraction Buffer (EB) composition**

Component	Volume added	Final concentration
HPLC grade acetonitrile	5771.25 $\mu$ l	71.25%
HPLC grade methanol	1923.75 $\mu$ l	23.75%
HPLC grade formic acid	15.39 $\mu$ l	1.9%
~15 mg of isotopically labeled yeast extract (ISO1) dissolved in 1.5 ml of HPLC grade water.	405 $\mu$ l	5%
<i>Note: After adding water to the yeast extract, dissolve the yeast extract by vortexing and/or rocking the yeast extract and water at 4 °C for approximately 30 min. Solution can be stored at -80 °C although some metabolites will degrade over time (see manufacturer's instructions).</i>		
2 mM solution of $^2\text{H}_9$ choline prepared in HPLC grade water (stored at -20 °C)	4.03 $\mu$ l	1 $\mu$ M
50 mM solution of $^{13}\text{C}_4$ 3-hydroxybutyrate prepared in HPLC grade water (stored at -20 °C)	0.81 $\mu$ l	5 $\mu$ M
200 $\mu$ M solution of $^{13}\text{C}_6$ $^{15}\text{N}_2$ cystine prepared in HPLC grade water (stored at -20 °C)	81 $\mu$ l	2 $\mu$ M
100 mM solution of $^{13}\text{C}_3$ lactate prepared in HPLC grade water (stored at -20 °C)	16.2 $\mu$ l	200 $\mu$ M
57.3 mM solution of $^{13}\text{C}_6$ glucose prepared in HPLC grade water (stored at -20 °C)	7.05 $\mu$ l	50 $\mu$ M
100 mM solution of $^{13}\text{C}_3$ serine prepared in HPLC grade water (stored at -20 °C)	1.62 $\mu$ l	20 $\mu$ M
750 mM solution of $^{13}\text{C}_2$ glycine prepared in HPLC grade water (stored at -20 °C)	1.62 $\mu$ l	150 $\mu$ M

2 mM solution of $^{13}\text{C}_5$ hypoxanthine prepared in HPLC grade water (stored at -20 °C)	2.02 µl	0.5 µM
200 mM solution of $^{13}\text{C}_2\ ^{15}\text{N}$ taurine prepared in HPLC grade water (stored at -20 °C)	2.02 µl	50 µM
60 mM solution of $^{13}\text{C}_3$ glycerol prepared in HPLC grade water (stored at -20 °C)	2.02 µl	15 µM
4 mM solution of $^2\text{H}_3$ creatinine prepared in HPLC grade water (stored at -20 °C)	2.02 µl	1 µM

## 6. Chemical standard library preparation

Below are recipes for the preparation of chemical standard libraries described in (Sullivan *et al.*, 2019). To prepare these chemical libraries, purchase the chemicals from the listed supplier and weigh them out as indicated, placing each metabolite into a 50 ml mixing mill jar. Mix the combined metabolites using a Mixer Mill MM301 with five 5 mm diameter stainless steel grinding balls. Perform 6 cycles of 1 min mixing at 25 Hz followed by 3 min resting. Store the now mixed chemical standard library powder stocks at -20 °C prior to use. For use, resuspend each mixed chemical library in HPLC grade water at 5 mM concentration as indicated for each library below.

Custom chemical standard libraries can be produced by acquiring desired pure chemical standards and mixing the pure chemical standards in equimolar amounts. When generating libraries, it is important to ensure that each library will not contain metabolites that have the same exact mass, as it is not then possible to determine the correct retention time for both metabolites when compounded into the same library. Consider putting these metabolites into separate pooled libraries (Tables 5-11).

**Table 5. Chemical standard library pool 1**

Metabolite name	Molecular weight of metabolite	Molecular weight of chemical standard	Amount to weigh (mg)
Alanine	89.09	89.09	429.99
Arginine	174.2	210.66	1016.75
Asparagine	132.12	150.13	724.60
Aspartate	133.11	133.11	642.45
Carnitine	161.199	197.66	954.00
Citrulline	175.2	175.2	845.60
Cystine	240.3	240.3	1159.80
Glutamate	147.13	147.13	710.12
Glutamine	146.14	146.14	705.34
Glycine	75.066	75.066	362.30
Histidine	155.1546	155.1546	748.85

Hydroxyproline	131.13	131.13	632.89
Isoleucine	131.1729	131.1729	633.10
Leucine	131.17	131.1729	633.10
Lysine	146.19	182.65	881.56
Methionine	149.21	149.21	720.16
Ornithine	132.16	168.62	813.84
Phenylalanine	165.19	165.19	797.28
Proline	115.13	115.13	555.67
Serine	105.09	105.09	507.21
Taurine	125.15	125.15	604.03
Threonine	119.119	119.119	574.92
Tryptophan	204.225	204.225	985.69
Tyrosine	181.19	181.19	874.51
Valine	117.151	117.151	565.42

**Table 5. Continued**

Metabolite name	Molecular weight of metabolite	Molecular weight of chemical standard	Amount to weigh (mg)
Lactate	90.09	112.06	540.85
Glucose	180.1559	180.1559	869.52

*Note: Dissolve this pool at 20.19 mg/ml for 5 mM solution.*

**Table 6. Chemical standard library pool 2**

Metabolite name	Molecular weight of metabolite	Molecular weight of chemical standard	Amount to weigh (mg)
2-hydroxybutyric acid	104.1	126.09	12.61
2-aminobutyric acid	103.12	103.12	10.31
AMP	347.2212	347.22	34.72
Argininosuccinate	290.273	334.24	33.42
Betaine	117.1463	117.15	11.71
Biotin	244.31	244.31	24.43
Carnosine	226.2324	226.23	22.62
Choline	104.1708	139.62	13.96
CMP	323.1965	367.16	36.72
Creatine	131.133	131.13	13.11
Cytidine	243.2166	243.22	24.32
dTMP	320.1926	366.17	36.62
Fructose	180.16	180.16	18.02

Glucose-1-phosphate	260.135	336.32	33.63
Glutathione	307.3235	307.32	30.73
GMP	363.22	407.18	40.72
IMP	348.206	392.17	39.22
O-phosphoethanolamine	141.063	141.06	14.11
Pyridoxal	167.16	203.62	20.36
Thiamine	265.35	337.23	33.72
trans-Urocanate	137.118	137.12	13.71
UMP	324.1813	368.15	36.82
Xanthine	152.11	152.11	15.21

*Note: Dissolve this pool at 28.54 mg/ml for 5 mM solution.*

**Table 7. Chemical standard library pool 3**

Metabolite name	Molecular weight of metabolite	Molecular weight of chemical standard	Amount to weigh (mg)
3-hydroxybutyric acid	104.1045	126.09	252.18
Acetylalanine	131.1299	131.13	262.26
Acetylaspartate	175.139	175.14	350.28
Acetyl carnitine	203.2356	239.70	479.40
Acetylglutamine	188.183	188.18	376.36
ADP	427.203	501.32	1002.64
Allantoin	158.121	158.12	316.24
CDP	403.177	403.20	806.40
CDP-choline	489.332	510.31	1020.62
Coenzyme A	767.535	767.53	1535.06
Creatinine	113.12	113.12	226.24
gamma-aminobutyric acid	103.12	103.12	206.24
GDP	443.201	443.20	886.40
Glutathione disulfide	612.631	612.63	1225.26
Glycerate	106.0773	286.25	572.50
Hypoxanthine	136.1115	136.11	272.22
myo-Inositol	180.16	180.16	360.32
NAD+	663.43	663.43	1326.86
p-aminobenzoate	137.138	137.14	274.28
Phosphocholine	184.152	329.73	659.46
Sorbitol	182.17	182.17	364.34
UDP	404.1612	448.12	896.24
UDP-glucose	566.302	610.27	1220.54

*Note: Dissolve this pool at 37.23 mg/ml for 5 mM solution.*

**Table 8. Chemical standard library pool 4**

Metabolite name	Molecular weight of metabolite	Molecular weight of chemical standard	Amount to weigh (mg)
Phenylacetylglutamine	264.3	264.3	17.17
Acetylglutamate	189.1659	189.1659	12.29
Acetylglycine	117.1033	117.1033	7.61
Acetylmethionine	191.245	191.245	12.43
Asymmetric dimethylarginine	202.25	275.2	17.88

**Table 8. Continued**

Metabolite name	Molecular weight of metabolite	Molecular weight of chemical standard	Amount to weigh (mg)
ATP	507.18	551.14	35.82
CTP	483.1563	527.12	34.26
dATP	491.2	535.15	34.78
dCTP	467.2	511.12	33.22
Deoxycytidine	227.2172	227.2172	14.76
Folic acid	441.3975	441.3975	28.69
GTP	523.2	523.18	34.00
Hypotaurine	109.1475	109.1475	7.09
Methionine sulfoxide	165.21	165.21	10.73
Methylthioadenosine	297.3335	297.3335	19.32
Phosphocreatine	211.114	255.08	16.58
Pyridoxine	169.18	205.64	13.36
Ribose-5-phosphate	230.11	310.1	20.15
SAH	384.4	384.41	24.98
Thymidine	242.2286	242.2286	15.74
Trimethyllysine	189.279	224.73	14.60
Uridine	244.2014	244.2014	15.87
UTP	484.1411	559.09	36.34

*Note: Dissolve this pool at 36.75 mg/ml for 5 mM solution.*

**Table 9. Chemical standard library pool 5**

<b>Metabolite name</b>	<b>Molecular weight of metabolite</b>	<b>Molecular weight of chemical standard</b>	<b>Amount to weigh (mg)</b>
3-phosphoglycerate	186.06	230.02	57.51
cis-aconitic acid	174.108	174.11	43.53
Citrate	192.124	294.10	73.53
DHAP	170.06	180.19	45.05
Fructose-1,6-bisphosphate	340.1157	406.06	101.52
Fumarate	116.07	116.07	29.02
Glucose-6-phosphate	260.135	282.12	70.53
Glycerol-3-phosphate	172.0737	370.40	92.60
Guanidinoacetate	117.1066	117.11	29.28
Kynurenine	208.2139	208.21	52.05
Malate	134.0874	134.09	33.52

**Table 9. Continued**

<b>Metabolite name</b>	<b>Molecular weight of metabolite</b>	<b>Molecular weight of chemical standard</b>	<b>Amount to weigh (mg)</b>
NADP+	744.413	765.39	191.35
Niacinamide	122.12	122.12	30.53
2-oxoglutarate	146.11	146.11	36.53
Phosphoenolpyruvate	168.042	267.22	66.81
Pyruvate	88.06	110.04	27.51
Succinate	118.09	118.09	29.52
Uracil	112.0868	112.09	28.02

*Note: Dissolve this pool at 20.77 mg/ml for 5 mM solution.*

**Table 10. Chemical standard library pool 6**

<b>Metabolite name</b>	<b>Molecular weight of metabolite</b>	<b>Molecular weight of chemical standard</b>	<b>Amount to weigh (mg)</b>
3-hydroxyisobutyric acid	104.1045	126.09	22.07
2-hydroxyglutarate	148.114	192.10	33.62
Aminoadipate	161.156	161.16	28.20
beta-alanine	89.093	89.09	15.59
Carbamoylaspartate	176.128	176.13	30.82
Cystathionine	222.263	222.26	38.90
Cysteic acid	169.16	169.16	29.60

FAD	785.5497	829.51	145.16
Glycerophosphocholine	258.231	257.22	45.01
Inosine	268.229	268.23	46.94
Orotate	156.1	194.19	33.98
Pantothenate	219.23	238.27	41.70
Phosphoserine	185.07	185.07	32.39
Riboflavin	376.369	376.37	65.86
UDP-GlcNAc	607.3537	651.32	113.98
Uric acid	168.1103	168.11	29.42

*Note: Dissolve this pool at 21.52 mg/ml for 5 mM solution.*

**Table 11. Chemical standard library pool 7**

Metabolite name	Molecular weight of metabolite	Molecular weight of chemical standard	Amount to weigh (mg)
Itaconic acid	130.0987	130.10	52.04
Homocysteine	135.185	135.19	54.07
2-oxobutyric acid	102.0886	102.09	40.84
2-hydroxybutyric acid	104.1045	126.09	50.44
Ascorbate	176.1241	198.11	79.24
Sarcosine	89.0932	89.09	35.64
Dimethylglycine	103.1198	103.12	41.25
N6-acetyllysine	188.2242	188.22	75.29
Pipecolate	129.157	129.16	51.66
Indolelactate	205.2099	205.21	82.08
Picolinate	123.1094	123.11	49.24
3-methyl-2-oxobutyrate	116.1152	138.10	55.24
3-methyl-2-oxopentanoic acid	130.1418	152.12	60.85
Formyl-methionine	177.221	177.22	70.89
2-aminobutyric acid	103.1198	103.12	41.25
Homocitrulline	189.2123	189.21	75.68
gamma-glutamyl-alanine	217.2224	218.21	87.28
Mannose	180.16	180.16	72.06
Cysteine-glycine (dipeptide)	178.21	178.21	71.28

*Note: Dissolve this pool at 14.33 mg/ml for 5 mM solution.*

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### **Competing interests**

The authors report no competing interests. CAL is a paid consultant for ReviveMed.

### **Ethics**

This study was performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals. All animal experiments were performed using protocols (#1115-110-18) that were approved by the MIT Committee on Animal Care (IACUC). All surgeries were performed using isoflurane anesthesia administered by vaporizer and every effort was made to minimize suffering.

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