

Reprint Compendium

Bio-protocol Selections 2022

Developmental Biology

Foreword

We are pleased to launch the 2022 *Bio-protocol* series of reprint collections, comprising some of the most used protocols published in 2021 in several research areas. This collection focuses on Developmental Biology.

Established in 2011 by a group of Stanford scientists, Bio-protocol aims to improve research reproducibility and usability through the publication of high quality step-by-step peer-reviewed life science protocols. *Bio-protocol* invites contributions from authors who have published methods in brief, as part of other research articles, and who might want to provide more detailed versions to facilitate use by others.

A survey carried out in 2018 showed that, of more than 2300 users who had followed a protocol published in *Bio-protocol*, 91% (2166 users) were able to successfully reproduce the method they tried.

In this reprint collection, we have selected 21 of the most viewed, downloaded, and cited research protocols related to Developmental Biology that were published in *Bio-protocol* in 2021.

Hopefully, you will find this collection intriguing and visit <http://www.bio-protocol.org> to check out the entire archive of protocols. Please feel free to email us (eb@bio-protocol.org) with feedback, and please consider contributing a protocol to Bio-protocol in the future.

The Bio-protocol Editorial Team

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On the Cover:

Image from protocol “**A Workflow for High-pressure Freezing and Freeze Substitution of the *Caenorhabditis elegans* Embryo for Ultrastructural Analysis by Conventional and Volume Electron Microscopy**”

Optimized Immunostaining of Embryonic and Early Postnatal Mouse Brain Sections

Kawssar Harb^{1,2}, Michele Bertacchi¹ and Michèle Studer^{1,*}

¹Université Côte d'Azur, CNRS, Inserm, iBV, France

²Neuronal Translational Control Group, Center for Molecular Neurobiology (ZMNH), University Medical center Hamburg-Eppendorf (UKE), Falkenried 94, 20251, Hamburg, Germany

*For correspondence: michele.studer@unice.fr

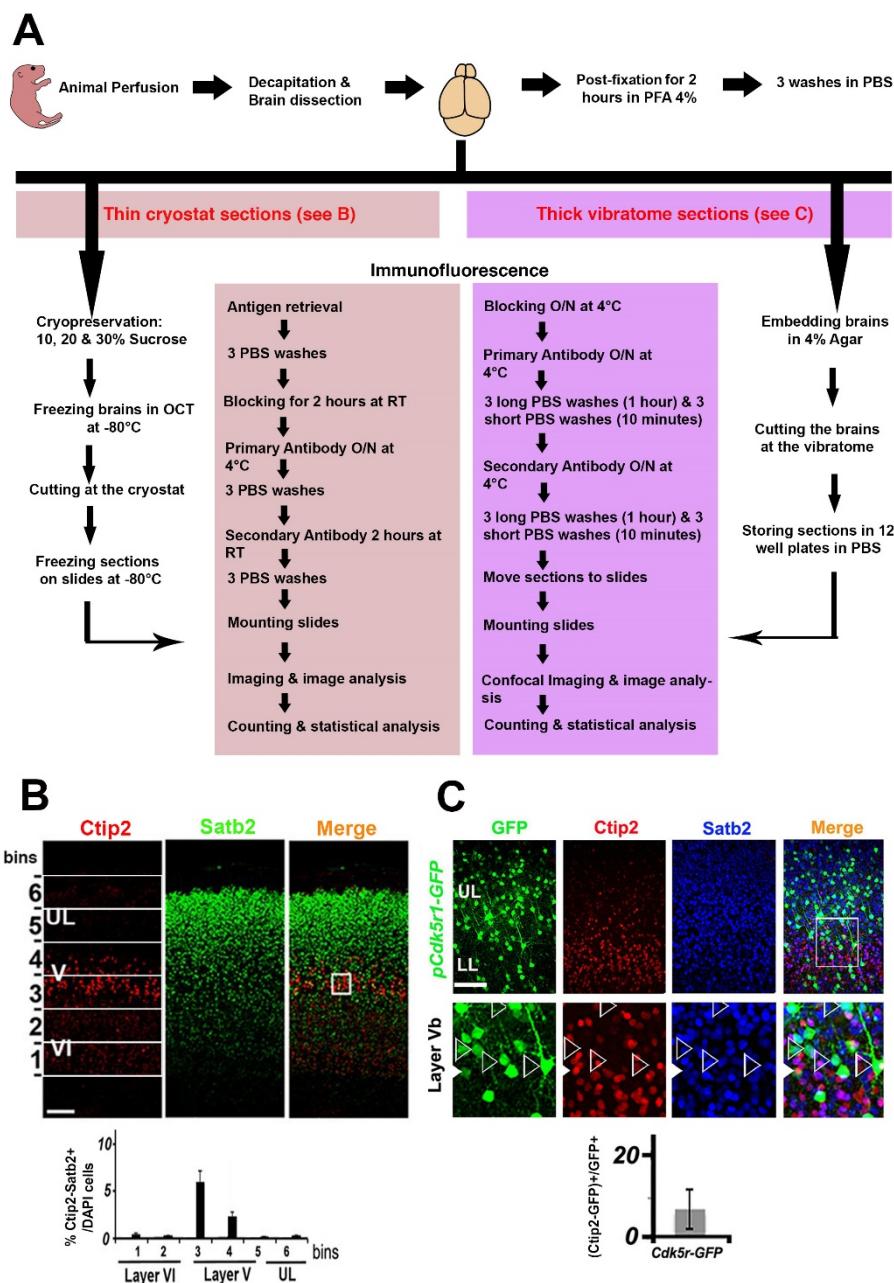
Abstract

The mammalian neocortex, the outer layer of the cerebrum and most recently evolved brain region, is characterized by its unique areal and laminar organization. Distinct cortical layers and areas can be identified by the protein expression of graded transcription factors and molecular determinants that define the identity of different projection neurons. Thus, specific detection and visualization of protein expression is crucial for assessing the identity of neocortical neurons and, more broadly, for understanding early and late developmental mechanisms and function of this complex system. Several immunostaining/immunofluorescence methods exist to detect protein expression. Published protocols vary with regard to subtle details, which may impact the final outcome of the immunofluorescence. Here, we provide a detailed protocol, suitable for both thin cryostat sections and thick vibratome sections, which has successfully worked for a wide range of antibodies directed against key molecular players of neocortical development. Ranging from early technical steps of brains collection down to image analysis and statistics, we include every detail concerning sample inclusion and sectioning, slide storage and optimal antibody dilutions aimed at reducing non-specific background. Routinely used in the lab, our background-optimized immunostaining protocol allows efficient detection of area- and layer- specific molecular determinants of distinct neocortical projection neurons.

Keywords: Mouse brain, Neocortex, Immunofluorescence, Cryostat section, Vibratome section, Protein expression, Imaging

This protocol was validated in: eLife (2016), DOI: 10.7554/eLife.09531

Graphical Abstract:



Workflow chart for the optimized immunostaining protocol of mouse brain sections.

A. A flow chart for different steps of the optimized immunostaining protocol on both thin cryostat and thick vibratome sections. B. Example for immunostaining against Satb2 and Ctip2 on a thin coronal section (20 µm) at the level of the somatosensory cortex. The first column to the left shows the binning system where 6 bins can be overlaid on the image. On the bottom, an example of counting analysis showing the percentage of marker-positive cells normalized to the total number of DAPI or Hoechst-positive cells. C. Example for immunostaining against Satb2 and Ctip2 on a GFP+ thick vibratome section (200 µm). Images are taken at low magnification (10x, left) and high magnification (40x, right). The graph shows a counting of the percentage of Ctip2-positive neurons normalized to the total number of GFP-electroporated neurons on high-magnification images. Images on B and C are modified from Harb *et al.* (2016).

Cite as: Harb, K. et al. (2021). Optimized Immunostaining of Embryonic and Early Postnatal Mouse Brain Sections. Bio-protocol 11(1): e3868. DOI: 10.21769/BioProtoc.3868.

Background

The mammalian neocortex is characterized by its radial subdivision into six distinct neuronal layers. In addition, during embryonic development and with further refinement postnatally, the neocortex gets organized tangentially into distinct primary areas dedicated to the elaboration and analysis of both motor outputs and sensory inputs, a developmental process termed “arealization”. These areas within the neocortex are positioned along the antero-posterior and medio-lateral axes by graded expression of key nuclear transcription factors. The basic neocortical structure of the six layers adapts within each area, as layers are populated with specific neuronal populations, which are defined by the expression of key molecular determinants driving the area-specific acquisition of their identity, morphology and connectivity. Thus, detection of selected protein of interest in neocortical neurons is crucial for investigating the tangential and radial expression of these determinants, which in turn is critical for exploring developmental and functional mechanisms occurring in this complex structure. Widely used in biology labs to determine the tissue and cellular localization of a protein of interest, the immunohistochemistry (or immunofluorescence) is a technical procedure that assesses the presence of a specific protein or antigen by the use of a specific primary antibody, followed by a secondary antibody coupled with a fluorochrome, thus allowing indirect visualisation and examination under a microscope. Many immunostaining protocols are available in literature; however, even subtle changes in the whole procedure can affect the final outcome. Here, we present a protocol that worked in our hands for most of the antibodies aimed at detecting key determinants of neocortical development in thick vibratome or thin cryostat sections. We added exhaustive details regarding all steps, from animal sample collection until image analysis, which can help beginners in the field to easily use the protocol. Optimized for the detection of nuclear determinants in neocortical neurons of embryonic or early postnatal mouse neocortex, the protocol is easily adaptable to a wide variety of biological samples, such as other neural or non-neural tissues.

Materials and Reagents

For animal perfusion:

1. 12-well plates (Falcon, catalog number: 353043)
2. Needles, syringes
 - a. Butterfly needles:
Winged Infusion Set: 25 G 0.50 × 19mm + 30 cm tubing (Braun, catalog number: 4056370)
Winged Infusion Set: 21 G 0.80 × 19mm + 30 cm tubing (Braun, catalog number: 4056337)
 - b. Hypodermic needles:
18 G (Terumo Neolus, catalog number: NN-1838R)
21 G (Terumo Neolus, catalog number: NN-2138R)
25 G (Terumo Neolus, catalog number: NN-2516R)
30 G (BD Microlance 3, catalog number: 304000)
 - c. Syringes without needle:
20 mL (Terumo, catalog number: SS+20ES1)
50 mL (Terumo, catalog number: SS+50ES1)
 - d. Syringes with needle:
U-100 Insulin syringe, 0.5 mL 0.33 mm (29G) (Fisher Scientific, BD Medical, catalog number: 324892)
3. Ketamine
4. Xylazine
5. Paraformaldehyde (PFA) (Sigma-Aldrich, catalog number: P6148-1KG, Lot: MKCD5277)
6. Phosphate Buffer Saline (PBS) (Gibco, catalog number: 14190-094) or homemade PBS (Recipe below)
7. Sodium chloride (VWR, AnalaR Normapur, catalog number: 27810.295)
8. Potassium chloride (Merck Millipore, catalog number: 1.04936.1000)
9. Sodium phosphate dibasic heptahydrate (Sigma-Aldrich, catalog number: S9390-2.5KG)
10. Potassium dihydrogen phosphate (Merck Millipore, catalog number: 1.04873.1000)

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11. 4% PFA (see Recipes)
12. 1× PBS (see Recipes)

Tissue sample fixation and washing:

1. 12-well plates (Falcon, catalog number: 353043)
2. Paraformaldehyde (PFA) (Sigma-Aldrich, catalog number: P6148-1KG, Lot #MKCD5277)
3. Phosphate Buffer Saline (PBS) (Gibco, catalog number: 14190-094) or homemade PBS (Recipe below)
4. Sodium Azide (Sigma-Aldrich, catalog number: S8032-100G, Lot #0001453190)
5. 4% PFA (see Recipes)
6. 1× PBS (see Recipes)

Embedding in agar for thick vibratome sections:

1. 12-well plates (Falcon, catalog number: 353043)
2. Select Agar ≥ 99.5% (GC) (Sigma-Aldrich, catalog number: S5054-250G, Lot #MKCB2702V)
3. Phosphate Buffer Saline (PBS) (Gibco, catalog number: 14190-094) or homemade PBS (Recipe below)
4. 4% Agar (see Recipes)

Cryopreservation and Freezing for thin cryostat sections:

1. 6 cm Petri dish
2. Optimal cutting medium (OCT) (Leica Tissue Freezing Medium, catalog number: 14020108926)
3. Dry ice
4. Sucrose (Sigma-Aldrich, catalog number: S9378-1KG, Lot #BCBS5325V)
5. Phosphate Buffer Saline (PBS) (Gibco, catalog number: 14190-094) or homemade PBS (Recipe below)
6. 1× PBS (see Recipes)
7. 10, 20 and 30% sucrose (see Recipes)

Vibratome sectioning (thick sections):

1. 12-well plates (Falcon, catalog number: 353043)
2. Blades (Gillette "Bleue Extra")
3. Phosphate Buffer Saline (PBS) (Gibco, catalog number: 14190-094) or homemade PBS (Recipe below)
4. Glue (Henkel, Loctite superglue-3)
5. 1× PBS (see Recipes)

Immunofluorescence on thick vibratome sections:

1. 12-well plates (Falcon, catalog number: 353043)
2. Slides (Thermo Scientific, Superfrost Plus: J1800AMNZ, Lot #0180)
3. Cover slips (Dia path, catalog number: 061061)
4. Nail polish
5. Phosphate Buffer Saline (PBS) (Gibco, catalog number: 14190-094) or homemade PBS (Recipe below)
6. Goat serum (GS) (Thermo Fisher scientific, Gibco, catalog number: 16210-064, Lot #16711329)
7. Newborn calf serum (NBCS) (Thermo Fisher scientific, Gibco, catalog number: 16010-167)
8. Bovine serum albumin (BSA) (Sigma-Aldrich, catalog number: A-4503 or A7906-50G, Lot #SLBX7550)
9. Triton (Sigma-Aldrich, catalog number: T8787-250ML, Lot #SLBV4122)
10. N-propyl Gallate (Sigma-Aldrich, catalog number: P3130-100G, Lot #SLBD6728V)
11. Glycerol (VWR Chemicals, catalog number: 24388.295, Batch: 14E050030)
12. Hoechst 33342, Trihydrochloride (Invitrogen, catalog number: H3570, Lot #1116437)
13. Primary antibodies, example:
 - a. Nr2f1/COUP-TFI (Abcam, catalog number: ab181137 or R&D, catalog number: H8132)
 - b. Ctip2 (Abcam, catalog number: ab18465)
 - c. Satb2 (Abcam, catalog number: ab51502)

14. Secondary antibodies, Example: Alexa Fluor 488, 555, 594 and 647 anti-mouse or anti-rabbit IgG conjugates (Thermo Fisher scientific)
15. Blocking Solution (see Recipes)
16. Antibody Solution (see Recipes)
17. Mounting medium (see Recipes)
18. Antibody Solution for vibratome thick sections (see Recipes)

Cryostat sectioning (thin sections):

1. Slides (Thermo Scientific, Superfrost Plus: J1800AMNZ, Lot #0180)
2. Blades (Thermo Fisher scientific, Edge-Rite: 4280L)
3. Optimal cutting medium (OCT) (Leica Tissue Freezing Medium, 14020108926)

Immunofluorescence on thin cryostat sections:

1. Coverslips (Dia Path, catalog number: 061061)
2. Phosphate Buffer Saline (PBS) (Gibco, catalog number: 14190-094) or homemade PBS (Recipe below)
3. Distilled water
4. Trisodium Citrate Dihydrate (Sigma-Aldrich, catalog number: S1804-1KG, Lot #BCBF5684V)
5. Citric acid (Sigma-Aldrich, catalog number: C-0759, Lot #34H1314)
6. Goat serum (GS) (Thermo Fisher scientific, Gibco, catalog number: 16210-064, Lot #16711329)
7. New-born calf serum (NBCS) (Thermo Fisher scientific, Gibco, catalog number: 16010-167)
8. Triton (Sigma-Aldrich, catalog number: T8787-250ML, Lot #SLBV4122)
9. N-propyl Gallate (Sigma-Aldrich, catalog number: P3130-100G; Lot #SLBD6728V)
10. Glycerol (VWR Chemicals, catalog number: 24388.295; Batch 14E050030)
11. Hoechst 33342, Trihydrochloride (Invitrogen, catalog number: H3570; Lot #1116437)
12. Primary antibodies, example:
 - a. Nr2f1/COUP-TFI (Abcam, catalog number: ab181137 or R&D, catalog number: H8132)
 - b. Ctip2 (Abcam, catalog number: ab18465)
 - c. Satb2 (Abcam, catalog number: ab51502)
 - d. Sox5 (Abcam, catalog number: ab94396)
 - e. GFP (Thermo Fisher scientific, catalog number: A-11122; or: Abcam, catalog number: ab13970)
 - f. Ski (Santa Cruz, catalog number: sc-9140)
 - g. For other antibodies, see “Materials and Methods” in Harb *et al.* (2016).
13. Secondary antibodies, Example: Alexa Fluor 488, 555, 594 and 647 anti-mouse or anti-rabbit IgG conjugates (Thermo Fisher Scientific)
14. Unmasking Solution (see Recipes)
15. Blocking Solution (see Recipes)
16. Antibody Solution (see Recipes)

Equipment

1. Scissors, forceps, dissection and tissue handling tools
 - a. Sharp scissors (Holtex, model: IC12411)
 - b. Fine scissors (Fine Science Tool, catalog number: 14060-11)
 - c. 45° angled tip forceps (Fine Science Tool, model: Dumont #5, catalog number: 11251-35)
 - d. Straight tip forceps (Fine Science Tool, Dumont Mini Forceps – Style 5)
 - e. Double-ended micro spatula (Fine Science Tool, catalog number: 10091-12)
 - f. Perforated spoon (WPI, catalog number: 501997)
2. Microwave
3. Embedding moulds:

- Peel-A-Way® embedding moulds (Sigma-Aldrich, catalog number: E6032-1CS)
Peel-A-Way® Disposable Embedding Moulds (VWR Polysciences, catalog number: 18646A-1)
4. Brush
 5. Boxes for storage (Dutscher, catalog number: 037516)
 6. Glass (Leica, Insert Glass 70 mm, catalog number: 14047742497)
 7. Boxes for boiling (Tissue-Tek® Staining Dish, Sakura, catalog number: 4456)
 8. Vertical boxes for slides wash (Fisher Scientific, Fisherbrand 5-place slide mailer: HS15986)
 9. Shaker (Stuart 3D gyratory rocker, catalog number: SSL3)
 10. Horizontal Microscope slide boxes (Kartell Labware, #276 [98 mm × 83 mm], #277 [230 mm × 97 mm] and #278 [230 mm × 180 mm])
 11. pH meter (Fisher Scientific, model: accumet AE150)
 12. Cryostat (Leica, model: CM3050S)
 13. Vibratome (Leica, model: VT1000S)
 14. Microscope (Leica, model: DM6000B +CTR6000)
 15. Confocal microscope (Zeiss, model: LSM 710, 2012)
 16. Water bath (Fisher Scientific, catalog number: Polystat 24, +5L/8662F)

Software

1. Adobe Photoshop CS (CS5 Extended, Version 12.0 x32)
2. Microsoft Excel (2005 Version)
3. GraphPad Prism (Version 7.00)
4. Zen Software for confocal images (Zen Black, Version 11 SP3 HF88.1.8.484)
5. Leica Microsystem CMS GmbH LASX (Version: 3.3.3.16958)

Procedure

A. Mouse Brain Collection and Fixation

1. Anesthetize the mouse with an intra-peritoneal injection of Ketamine/Xylazine mix (100 mg/kg Ketamine mixed with 10 mg/kg Xylazine in isotonic saline solution; contact local animal ethical committee for drug use and animal procedures). In our case, all mouse experiments were conducted in accordance with relevant national and international guidelines (European Union rules; 2010/63/UE), and have been approved by the local ethical committee in France (CIEPAL NCE/2014-209 and NCE/2019-548).
2. For postnatal/adult mice, perfuse them intracardially with 4% PFA; for embryonic stages, recover embryos on ice-cold PBS from the uterus of the anesthetized mother.
3. Decapitate adults/embryos and dissect the brains; for early stage embryos (embryonic age < 15.5 days), brain dissection is not mandatory, but highly recommended to reduce Background signal on skin and other tissues surrounding the brain.
4. Keep brains in ice-cold PBS in 12-well plates during and after collection.
5. Post-fix brains for 2 h in 4% PFA at 4°C (gentle agitation on shaker).
6. Wash the brains 3 times for 10 min each in PBS at 4°C (on shaker).
7. Store brains at 4°C until further treatment (few days); for long-term storage (few weeks), supplement PBS with 0.05% Sodium Azide.

B. Embedding in Agar for vibratome sectioning (thick sections: 50-200 µm)

1. Prepare a solution of 4% agar in PBS.

2. Boil in the microwave until homogenization.
3. Keep the 4% agar in a water bath at 50°C.
4. Add 4% agar to a 12-well plate.
5. Remove the brain from PBS with perforated spoon and dry it on a paper towel.
6. Quickly immerge the brain in agar and correctly position it with forceps before agar polymerization.
7. Store the brain at 4°C for short term or immediately proceed with sectioning.

C. Cutting vibratome thick sections

1. Trim the agar surrounding the brain and fix it on the vibratome with glue.
2. Add ice cold PBS to the vibratome and keep it cold by adding ice around the mould for cutting.
3. Cut 50-200 µm thick sections.
4. Use a brush to gently collect the sections from the vibratome before moving them to a 12-well plate; keep the plate on ice-cold PBS.
5. Create series of sections representing the whole cortex or brain by distributing similar sections on subsequent wells.
6. You can add up to 3-4 sections in each well.

D. Immunostaining on vibratome sections (50-200 µm)

1. Remove the PBS by carefully aspirating it with a pipette tip, without touching the sections (repeat for following passages).
2. Incubate the sections in Blocking solution (10% GS, 3% BSA, 0.3% Triton) over-night (O/N) at 4°C by adding 1 mL for each well.
3. The following day add 500 µl of primary antibodies by diluting them to the appropriate concentration with the antibody solution (3% GS, 3% BSA, 0.3% Triton).
4. Incubate the sections with primary antibodies O/N at 4 °C.
5. The day after, wash the sections 3 times for 1 h each in PBS, followed by 3 short washes for 10 min each in PBS (all washes are performed at room temperature (RT), on shaker).
6. Prepare secondary antibodies by diluting them to 1/500 in the antibody solution (3% GS, 3% BSA, 0.3% Triton).
7. Incubate the sections with 500 µL of diluted secondary antibodies O/N at 4°C by shaking.
8. The following day wash the sections in PBS as described in Step D5.
9. Incubate the sections in 1 µg/mL Hoechst in PBS for 1 h.
10. Carefully move the sections using a brush from the wells to a slide covered with PBS.
11. Aspirate the PBS with a pipette tip and let the sections dry.
12. Add 3 drops of mounting solution (80% glycerol, 2% N-propyl gallate, 1 µg/mL Hoechst) to each slide.
13. Cover the slides with cover slips and seal the edges with nail polish.
14. Leave the nail polish to dry for 5 min at RT.
15. Store the slides until imaging at -20°C.

E. Cryopreservation and Freezing brains for thin sectioning at the cryostat (thin sections: 10-20 µm)

1. Prepare 10%, 20% and 30% sucrose in PBS.
2. Put the brains (dissected and post-fixed as detailed in Procedure A) in 10% sucrose, keep at 4°C with gentle shaking. Wait to equilibrate (usually 8-12 h) until they sink to the bottom of the wells.
3. Repeat Step E2 with sucrose 20% and then 30%. Increasing sucrose concentration in a gradual way is key for preserving optimal histology.
4. Remove all sucrose from the brains by shaking them with a Pasteur pipette in OCT in a 6 cm Petri dish.
5. Transfer the brains in OCT in Peel-A-Way® boxes.

6. Snap freeze them on dry ice.
7. Store them at -80°C until sectioning.

F. Cutting sections at the cryostat (10-20 µm)

1. Equilibrate the brains at -20°C for 30 min at the cryostat.
2. Cut 20 µm thin sections for postnatal brains and 12-16 µm for embryonic brains.
3. During cutting, collect series of sections on cryostat slides.
4. Let the slides dry O/N at RT.
5. Store them at -80°C until use.

G. Immunostaining on cryostat sections

1. Defreeze the sections for 1 h at RT.
2. Put the slides on a vertical slide box for boiling.
3. If your antibody needs unmasking, boil the slides for 15 s in an unmasking solution (0.1 M sodium citrate, pH 6).
4. Boil them a second time for 1 s with fresh unmasking solution (0.1 M sodium citrate, pH 6).
5. Cool the sections on ice for 10 min.
6. Move the slides to a vertical slide box for washing
7. Wash the sections 3 times in PBS for 10 min each at RT (on shaker).
8. Create a humidified chamber by adding water or PBS in a plastic box where the slides can lay horizontally.
9. Lay your slides horizontally and avoid the sections to dry by immediately adding the blocking solution (10% GS, 0.3% Triton in PBS).
10. Block the sections in blocking solution for 1 h at RT by adding 1 mL for each slide.
11. Prepare 200-300 µL of primary antibodies for each slide by diluting them to the appropriate concentration with the antibody solution (3% GS, 0.3% Triton in PBS).
12. Add 200-300 µL of the diluted primary antibodies to the slides and cover them with cover slips to homogeneously distribute the antibody.
13. Incubate the sections with primary antibodies (ON at 4°C).
14. The following day move the slides to the vertical slide box containing PBS.
15. Wash the sections 3 times for 10 min each in PBS at RT while shaking.
16. Prepare 200-300 µL of secondary antibodies per slide by diluting them to 1/300 in the antibody solution (3% GS, 0.3% Triton).
17. Lay the slides horizontally again in the humidified chamber.
18. Add 200-300 µL of the diluted secondary antibodies to the slides and cover them with cover slips to homogeneously distribute the antibody.
19. Incubate the sections for 2 h at RT with diluted secondary antibodies.
20. Move the slides to the vertical slide box containing PBS.
21. Wash the sections 3 times for 10 min each in PBS at RT while shaking.
22. Lay the slides horizontally after taking away extra PBS.
23. Add 3 drops of mounting solution (80% glycerol, 2% N-propyl gallate, 1 µg/mL Hoechst) to each slide.
24. Cover the slides with cover slips and seal the edges with nail polish.
25. Leave the nail polish dry for 5 min at RT.
26. Store the slides at -20°C until imaging.

H. Imaging

1. Take images of your area and layer of interest with the appropriate magnification
 - a. For thin sections:
Acquire plane images with a 10×/20× magnification using a fluorescent optical microscope.

- b. For thick sections: Acquire Z-stack images using a confocal microscope.
Acquire Z-stack images using a confocal microscope.
2. Use low (10 \times) and high magnification (40 \times) images of your layer of interest.

Data analysis

A. Data analysis for thin cryostat sections (coronal cutting plane)

1. Process the 1 plane images acquired with 10 \times /20 \times magnification using Adobe Photoshop.
2. For quantification of specific markers based on their layer position, use a binning system in Adobe Photoshop by overlaying a grid with 6-8 bins on the neocortical area of interest, from the marginal zone until the glial surface. Six bins are typically used for embryonic or postnatal day (P) 0 brains, while 8 bins can be used from P7.
3. Count the cells expressing the protein of interest using Photoshop counting tool or an automated counting Software like ImageJ.
4. Normalize the number of marker-positive cells to the total number of cells (quantified by counting Hoechst⁺ nuclei) in each bin.
5. Count at least 3 sections per animal and use at least 3 animals of each genotype for reliable statistical analysis.
6. To avoid variability occurring between litters, the best option is to use littermate controls to compare the expression of specific genes/proteins of interest in different genetic Backgrounds.
7. Use two-tailed Student's *t*-test on Microsoft Excel or on GraphPad Prism for statistical analysis of two independent data groups. For comparing multiple (> 2) groups, use analysis of variance (ANOVA) or other appropriate statistical tests.

B. Data analysis for thick vibratome sections

1. Process the Z-Stack images using the correspondent Software of your confocal microscope (for example, Zen lite) and make a maximum intensity projection image.
2. Count the cells expressing the protein of interest and normalize them to the total number of cells or to another marker depending on the purpose.
3. Count at least 3 sections per animal and use at least 3 animals of each genotype for statistical analysis.
4. Use littermate controls to compare the expression of specific genes/proteins of interest in different genetic Backgrounds (example: wild-type *versus* knock-out) or after specific treatments, depending on your scientific question.
5. Use two-tailed Student's *t*-test on Microsoft Excel or on GraphPad Prism for statistical analysis of two independent data groups. For comparing multiple (> 2) groups, use analysis of variance (ANOVA) or other appropriate statistical tests.

Notes

1. To minimize subjective bias, sample identity (e.g., genotypes) can be randomized by associating an identification number to each sample before processing.
2. Fixed embryos/brains/sections with clearly damaged tissue must be excluded from any further analysis/processing.
3. Long O/N incubation with Blocking solution BEFORE addition of antibodies, together with long and repeated PBS washes, are crucial for Background reduction and signal specificity, as they contribute to mask non-specific epitope sites and to remove excess antibodies, respectively.

4. *Troubleshooting 1:* Poor tissue fixation can result from perfusion with low-quality PFA. Avoid repeated thaw-freeze cycles by freezing PFA aliquots of appropriate volumes, and by using in one-two weeks after thawing.
5. *Troubleshooting 2:* Poor histology commonly results from inefficient tissue dehydration prior OCT embedding. Be sure to cryopreserve with long incubation in increasingly concentrated sucrose solutions (see Steps E1-E3).
6. *Troubleshooting 3:* In our experience, poor adhesion of cryostat tissue sections on glass slides can happen when using expired slides. Always check the expiring date of SuperFrost slides for optimal adhesion.
7. *Troubleshooting 4:* For correct Background removal, be sure to use a normal serum from the same species as the one in which the secondary antibody was generated. The use of the incorrect serum can result in non-specific signal.
8. *Tips 1:* Many of the antibodies we used for studying cortical development detect nuclear transcription factors. The easiest way to check for the specificity of the staining, is to check for proper localization of the signal inside the cell nucleus.
9. *Tips 2:* When performing multiple co-staining, use secondary antibodies associated to fluorochromes that do not overlap on the light spectrum. This largely depends on your microscope settings; check technical specifications of your microscope lasers/filters. As an example, we experienced some signal overlap when using Alexa Fluor 594 (Rhodamine) together with Alexa Fluor 647 (Far red/Cy5) on our Leica Microscope; better results (no signal overlap) were obtained by replacing Alexa Fluor 594 with Alexa Fluor 555 (Texas Red).

Recipes

1. 4% PFA

To prepare 1 L of PFA 4%:

- a. Dissolve 40 g of Paraformaldehyde powder (Sigma-Aldrich) in 1× PBS to reach 1 L
- b. Heat while stirring under the chemical hood on a temperature of approximately 60°C
- c. When solution is transparent, let cool down then filter with Whatman paper to remove undissolved particles
- d. Freeze PFA aliquots in 50 mL tubes at -20°C

2. 1× PBS

- a. Add the following chemicals to 1 L of distilled water

8 g Sodium chloride
200 mg Potassium chloride
1.44 g Sodium phosphate dibasic heptahydrate
240 mg Potassium dihydrogen phosphate

- b. Adjust pH to 7.4 with HCl
- c. Autoclave it
- d. Store it at RT

3. Unmasking Solution

- a. Prepare 1 M Trisodium Citrate Dihydrate solution in distilled water (store at 4°C)
- b. Prepare 1 M citric acid solution in distilled water (store at 4°C)

To prepare 1 L of unmasking solution, add 85 mL of sodium citrate 1 M to 800 mL of distilled water.

- a. Measure pH while stirring
- b. Adjust to pH 6 using 1 M citric acid solution
- c. Bring the volume to 1 L with distilled water
- d. Filter your solution
- e. Store it at 4°C

4. Agar 4%

For each brain, you need to use approximately 5 mL of Agar 4% in a 12-well plate, to prepare 100 mL of Agar 4%:

- a. Dissolve 4 g of Select Agar ≥99.5% (GC) in 100 mL of PBS
- b. Boil in the microwave to obtain a clear Agar solution with no aggregates
- c. Incubate the agar solution in the water bath at 50°C until mounting all the brains

5. 10, 20 and 30% Sucrose

To prepare 50 ml of sucrose 10, 20 and 30%:

- a. Add respectively 5 g, 10 g and 15 g of sucrose and reach 50 mL with PBS 1×
- b. Vortex the solutions vigorously until fully dissolving the sucrose
- c. Store the solutions at 4°C; for long term storage, freeze them at -20°C

6. Blocking Solution for cryo-sections

50 mL for immunostaining on thin cryo-sections:

- a. Add 5 mL Goat serum or NBCS (in case one of your primary antibody is made in goat)
- b. Add 150 µL of Triton
- c. Reach 50 mL by adding PBS
- d. Vortex until fully homogenizing the solution
- e. Store it at 4°C (for few days)

7. Antibody Solution for thin cryo-sections

50 mL for immunostaining on thin cryo-sections:

- a. Add 3 mL Goat serum (or NBCS, in case one of your primary antibody is made in goat)
- b. Add 150 µL of Triton
- c. Reach 50 mL by adding PBS
- d. Vortex until fully homogenizing the solution
- e. Store it at 4°C (for few days)

8. Blocking Solution for vibratome sections

50 mL for immunostaining on vibratome thick sections:

- a. Add 5 mL Goat serum or NBCS (in case one of your primary antibodies is made in goat or sheep)
- b. Add 150 µL of Triton 100
- c. Add 1.5 g of BSA
- d. Reach 50 mL by adding PBS
- e. Vortex until fully homogenizing the solution
- f. Store it at 4°C (up to two weeks)

9. Antibody Solution for vibratome thick sections

50 mL for immunostaining on vibratome thick sections:

- a. Add 3 mL Goat serum or NBCS (in case one of your primary antibody is made in goat)
- b. Add 150 µL of Triton 100
- c. Add 1.5 g of BSA
- d. Reach 50 mL by adding PBS
- e. Vortex until fully homogenizing the solution
- f. Store it at 4°C (up to two weeks)

10. Mounting medium

For 50 mL

- a. Add 40 mL glycerol
- b. Add 1 g N-propyl gallate

- c. Reach 50 mL with distilled water
- d. Boil in the microwave until homogenizing the solution
- e. Add 5 µL of Hoechst 33342, Trihydrochloride
- f. Store at 4°C covered with aluminium foil to protect from light

Acknowledgments

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Competing interests

The authors declare no competing interests.

Ethics

All animal procedures were conducted in strict accordance with relevant national and international guidelines and regulations (European Union rules; 2010/63/UE; and: Guide for the Care and Use of Laboratory Animals of the French Ministry of Research). All animal experiments were approved by the local ethical committee (CIEPAL registration numbers: NCE/2014-209 and NCE/2019-548). Standard approved housing conditions consisted in a 12 h light-dark cycle and housing with the recommended environmental enrichment (wooden cubes, cotton pad, igloo) with food and water *ad libidum*, three animals per cage max.

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Extraction and Quantification of Sphingolipids from Hemiptera Insects by Ultra-Performance Liquid Chromatography Coupled to Tandem Mass Spectrometry

Ni Wang¹, Xiaoxiao Shi¹, Chao Zhang¹, Wenwu Zhou^{1,2, *} and Zengrong Zhu^{1,2, *}

¹Institute of Insect Sciences, Key Laboratory of Biology of Crop Pathogens and Insects of Zhejiang Province, Key Laboratory of Molecular Biology of Crop Pathogens and Insects, Ministry of Agriculture, State Key Laboratory of Rice Biology, Hangzhou 310058, China

²Hainan Research Institute, Zhejiang University, Sanya 572000, China

*For correspondence: zrzhuz@zju.edu.cn; wenwuzhou@zju.edu.cn

Abstract

Sphingolipids are major structural components of endomembranes and have also been described as an intracellular second messenger involved in various biological functions in all eukaryotes and a few prokaryotes. Ceramides (Cer), the central molecules of sphingolipids, have been depicted in cell growth arrest, cell differentiation, and apoptosis. With the development of lipidomics, the identification of ceramides has been analyzed in many species, mostly in model insects. However, there is still a lack of research in non-model organisms. Here we describe a relatively simple and sensitive method for the extraction, identification, and quantification of ceramides in Hemiptera Insects (brown planthopper), followed by Ultra-Performance Liquid Chromatography coupled to tandem mass spectrometry (UPLC-MS/MS). C18 is used as the separation column for quantitative detection and analysis on the triple quadrupole liquid mass spectrometer. In this protocol, the standard curve method is adopted to confirm the more accurate quantification of ceramides based on the optional detection conditions.

Keywords: Hemiptera Insects, *Nilaparvata lugens* Stål, Sphingolipids, Ceramides, UPLC-MS/MS, Extraction, Identification, Quantification, Standard curve

This protocol was validated in: Sci Rep (2018), DOI: 10.1038/s41598-018-19219-y

Background

Sphingolipids are the second largest group of membrane lipids in living organisms and play an important role in many aspects of cell structure, metabolism, and regulation (Lahiri and Futerman, 2007). At first, it was thought that sphingolipids were a complex family of structurally related molecules, but more and more studies have shown that sphingolipids are involved in numerous cellular processes (Mao and Obeid, 2008). Ceramides (Cer) are essential bioactive lipids implicated in various cell biological processes ranging from cell growth regulation to cell death and senescence (Futerman and Hannun, 2004; Hannun and Obeid, 2008) through influencing of multiple signaling pathways. Although the physiological roles of ceramides are widely reported, few studies have described the extraction, identification, and quantification, thus, analysis of ceramides has gained significant interest in investigating the physiological functions of sphingolipid metabolism in Hemiptera Insects.

Currently, various methods have been described for this purpose, such as Diacylglycerol (DAG) Kinase assay (Preiss *et al.*, 1987), Thin-layer chromatography (TLC) (Gorska *et al.*, 2002), Gas chromatography mass spectrum (GC-MS) (Tseng *et al.*, 2003), High-performance liquid chromatography (HPLC) (Yano *et al.*, 1998; Dobrzyn and Gorski, 2002). In the beginning, DAG kinase assay was commonly used for Cer quantitation, but the specificity has been questioned (Watts *et al.*, 1997). Thin layer chromatography was the method of choice, but the resolution, sensibility, and separation were limited, resulting in inefficient separation of similar molecules (Bielawski *et al.*, 2010). Despite the high sensitivity of chromatographic analysis, this method had some limitations, such as the need for standard substances and derivatization (Dobrzyn *et al.*, 2004). High-performance liquid chromatography (HPLC) was introduced to obtain ceramides separation with higher resolution, but complex samples like tissue extracts, therefore, produced many unspecific signals that did not provide any information concerning the metabolism of molecular species by HPLC (Yano *et al.*, 1998; Bode and Graler, 2012).

Given the ultra performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS), it is more efficient, rapid, and sensitive, improving the separation condition of extremely complex samples and reducing matrix interference (Cutignano *et al.*, 2010). Therefore, the current choice method is the analysis of ceramides by Ultra Performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS). This protocol provides a relatively rapid and reproducible method. Moreover, this method can be used to profile ceramides from the extraction of the plant sample. It has evolved as the method of choice to detect sphingolipids metabolites due to its high sensitivity and superior specificity. The content of ceramide is determined and a quantitative system of sphingolipids in Hemiptera Insects is established, which lay a foundation for understanding the metabolic process and elucidating the biological function of each component. This method was described and used successfully to extract other sphingolipids in previously published studies (Bielawski *et al.*, 2010; Shi *et al.*, 2018 and 2019).

Materials and Reagents

1. 1.5 mL Eppendorf tubes (Axygen, catalog number: MCT-150-C)
2. 1.0 mm Ceramic Beads (Nalgene, catalog number: 150010C)
3. 2 mL Micro tube (Sarstedt, catalog number: 72.609)
4. Pipette tips (Axygen, catalog numbers: T-300, T-200-Y, T-1000-B)
5. Glass Centrifuge Tubes (VWR International, catalog number: 734-4240)
6. Nitrogen gas (>99% Purity) (any brand will suffice)
7. Isopropyl alcohol (Sangon Biotech, catalog number: A503069)
8. Ethyl acetate (Sangon Biotech, catalog number: A507048)
9. Liquid nitrogen (any brand will suffice)
10. HPLC-grade methanol (Sigma-Aldrich, catalog number: 34806)
11. Formic acid (Sangon Biotech, catalog number: A503066)
12. MilliQ Water (Millipore, catalog number: Direct-Q3)
13. Standards (see Table 1)

Note: All Ceramides standards list is shown in Table 1.

14. Solvent extraction solution A (see Recipes)
15. Solvent extraction solution B (see Recipes)
16. Mobile phase A (see Recipes)
17. Mobile phase B (see Recipes)
18. Internal standard (Avanti company) (see Recipes)

Table 1. Example of ceramides standards list

Compound name	Standard	Source
Cer(d18:1/14:0)	Std	Avanti Lipids Polar, catalog number: 860514
Cer(d18:1/16:0)	Std	Avanti Lipids Polar, catalog number: 860516
Cer(d18:1/18:1)	Std	Avanti Lipids Polar, catalog number: 860519
Cer(d18:1/18:0)	Std	Avanti Lipids Polar, catalog number: 860518
Cer(d18:1/20:0)	Std	Avanti Lipids Polar, catalog number: 860520
Cer(d18:1/22:0)	Std	Avanti Lipids Polar, catalog number: 860501
Cer(d18:1/24:1)	Std	Avanti Lipids Polar, catalog number: 860525
Cer(d18:1/24:0)	Std	Avanti Lipids Polar, catalog number: 860524
Cer(d18:0/14:0)	Std	Avanti Lipids Polar, catalog number: 860632
Cer(d18:0/16:0)	Std	Avanti Lipids Polar, catalog number: 860634
Cer(d18:0/18:1)	Std	Avanti Lipids Polar, catalog number: 860624
Cer(d18:0/18:0)	Std	Avanti Lipids Polar, catalog number: 860627
Cer(d18:0/24:1)	Std	Avanti Lipids Polar, catalog number: 860629
Cer(d18:0/24:0)	Std	Avanti Lipids Polar, catalog number: 860628
Cer(d18:0/12:0)	IS	Avanti Lipids Polar, catalog number: 860635

Std: Standard; IS: Internal standard

Equipment

1. Nitrogen evaporator N-EVAP (Organomation, model: HGC-24A)
2. Centrifuge (Eppendorf, model: 5430R)
3. Autoclave (SANYO, model: MLS-3780)
4. UHPLC-Q-TOF-MS/MS system (AB SCIEX, Framingham, MA, USA)
5. UHPLC column (Zorbax sb-C8, 2.1 × 150 mm, 3.5 µm; Agilent, Palo Alto, CA, USA)
6. Ivory PTFE/red silicone rubber septa (Agilent Technologies, catalog number: 5182-0731)
7. 2 mL amber screw vial with patch USP 1 expansion (HAMAG Technologies, catalog number: HM-0716H)
8. Blue open-topped polypropylene cap and white PTFE/red Scilicone septa (HAMAG Technologies, catalog number: HM-0722)
9. 250 µL clear glass pulled conical-bottom (HAMAG Technologies, catalog number: HM-2085)
10. Analytical balance (METTLER TOLEDO, model: XS105)
11. Tissue homogenizer (MP Biomedicals, USA, FastPrep-24)
12. Oven (Bluepard, model: BPG-9040A)
13. Vortexer (Germany, IKA, model: vortex 2)
14. -80°C freezer

Software

1. PeakView (AB Sciex)
2. Excel Software (Microsoft office 2010)
3. Data Processing Software (DPS)

Procedure

A. Insect samples collection

The laboratory strain of *N. lugens* (brown planthopper) used in this study originated from a field population in the Huajiachi campus of Zhejiang University, Hangzhou, China. The BPHs (brown planthopper) were reared on susceptible rice seedlings cv.Taichung Native 1 (TN1) at $27 \pm 1^\circ\text{C}$, 70% relative humidity and a 16:8 h light:dark photoperiod.

About 2 g fresh weight of insects was determined and collected at different development stages (e.g., eggs, first-fifth instar nymphs, female and male adults) in labeled 2 mL tissue grinding tubes. Samples were then stored at -80°C after quickly freezing in liquid nitrogen. The sample was set for three biological repeats.

B. Total sphingolipids extraction (Figure 1)

Sphingolipids were extracted from insects according to Bielawski's method (Bielawski *et al.*, 2010). Details were prepared as follows:

Note: All following steps were performed at room temperature if not stated otherwise.

1. The samples were taken out from the -80°C freezer and dissolved the melted samples in 1 mL solvent extraction solution A (see Recipe 1), to which 10 μL Internal standard had been added (see Recipe 5).
2. Samples were next grounded twice in tissue homogenizer with grinding beads, each time for 20 s.

Note: In the case of plant material, clean and pre-cooled mortars and pestles were used to grind the samples in liquid nitrogen, and the powder was then transferred to a 15 mL glass tube. The sample was homogenized into a fine powder and that powder was kept frozen at every homogenization step. The purpose of the gridding bead is to facilitate insect tissue grinding, so it is okay just to cover the bottom layer for the amount of gridding beads per mass of sample and total volume.

3. Powdered tissue was vortexed vigorously for 5 min and centrifuged for 5 min at $1,000 \times g$.
4. After centrifugation, the solvent from the upper lipid-containing phase was transferred to a 15 mL sterilized glass bottle.

Note: The vial label was protected with a clear tape to avoid being wiped off by the solution.

5. The extraction was repeated by adding 1 mL solvent extraction solution A (see Recipe 1) to the remaining aqueous phase, and the organic layers were combined and concentrated under a stream of nitrogen gas.
6. The dried lipid fractions were re-suspended into 500 μL solvent extraction solution B (see Recipe 2), and immediately sufficient liquid was transferred to glass vials and capped tightly.
7. The solvent was transferred into a labeled 1.5 mL microcentrifuge tube, centrifuge the samples at $12,000 \times g$ for 5 min at room temperature.

Note: The tube was capped tightly to avoid leakage of liquid during centrifugation.

8. The reconstitution solution was finally transferred to a mass spectrometer flask with an internal cannula and stored at -20°C for machine test.

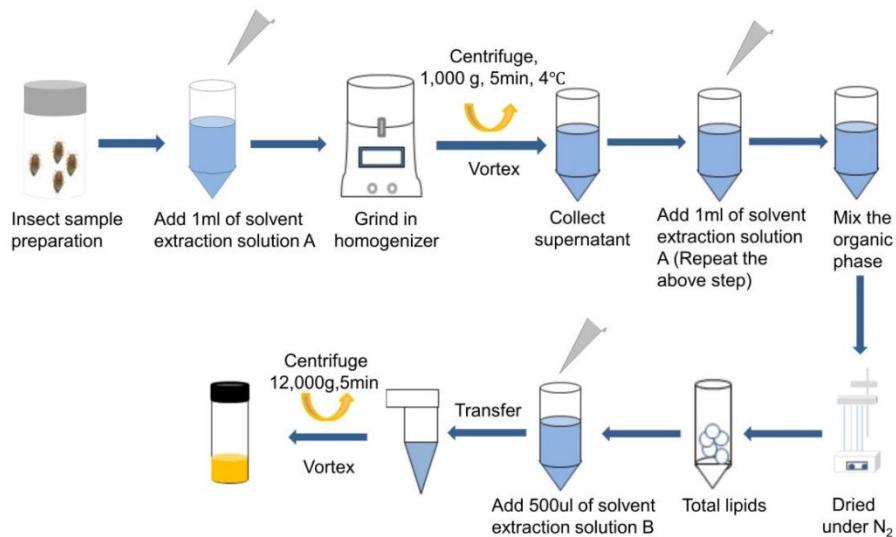


Figure 1. Workflow of sphingolipids extraction from Insect sample

C. Sphingolipids detection and identification

Sphingolipids were analysed on an AB Series 5600+quadrupole Time-of-Flight (Q-TOF) Premier mass spectrometer combined with a Waters Acquity Ultra Performance liquid chromatography.

1. HPLC-program
 - a. Solvent A (see Recipe 3)
 - b. Solvent B (see Recipe 4)
 - c. Constant flow at a rate of 0.3 mL/min stated by solvent A was running in a Waters UPLC (Waters Corp, Milford, MA, USA) coupled with an AB Triple TOF 5600 plus System (AB SCIEX, Framingham, MA, USA).
 - d. The changes in gradient were comprised of an increase in solvent B (methanol) from 80 to 99% over 20 min and then from 99 to 100% over 15 min, followed by a reduction back to 80% over 1 min. The percentage of solvent B was then held at 80% for the last 9 min.
2. The reversed-phase analytical column (Zorbax sb-C8, 2.1 × 150 mm, 3.5 μm; Agilent, Palo Alto, CA, USA) was used to separate ceramides.
3. 10 μL of the samples were applied to the column.
4. The column was kept at 35°C during the whole procedure.
5. The mass spectrum was acquired with an electrospray ionization (ESI) ion source in the positive ionization mode and following settings (Table 2).

Table 2. The instrument settings for sphingolipids analysis

Ion source voltage	5,500
Ion source heater temperature	600°C
Ion source gas 1	50 psi
Ion source gas 2	50 psi
Curtain Gas	30 psi
Ion release delay	67
Ion release width	25
UV detector	245 nm
Declustering potential	100 V
Collision energy	10 V
MS/MS Capture collision energy	40±20 V

Ion release delay	67
Scan range	100-1,500 m/z

D. Sphingolipids profiling parameters for detection

The C18 column was used as the separation column on the Aligent 6460+ triple four-pole liquid mass spectrometer to explore the optimal detection conditions of each standard sample. The profiling parameters include precursor ion/targeted ion, retention time, fragmentor, and collision energy. Cer (d18:0/12:0) was used as the internal standard. The scanning parameters for each lipid class were listed in Table 3.

Table 3. Scanning parameters for sphingolipids detection

Compound name	Standard	Mass of ion	Fragmentor	Collision energy	R _T [min]	Precursor ion m/z
Cer(d18:1/14:0)	Std	510.81	55	20	14.97	264.4
Cer(d18:1/16:0)	Std	538.91	60	20	16.55	264.4
Cer(d18:1/18:1)	Std	564.95	55	20	17.07	264.4
Cer(d18:1/18:0)	Std	566.96	60	4	17.93	548.6
Cer(d18:1/20:0)	Std	595.01	55	24	19.26	264.4
Cer(d18:1/22:0)	Std	623.01	55	24	20.39	264.4
Cer(d18:1/24:1)	Std	649.01	55	28	20.61	264.4
Cer(d18:1/24:0)	Std	651.01	223	20	20.94	632.7
Cer(d18:0/14:0)	Std	512.00	80	28	14.99	266.4
Cer(d18:0/16:0)	Std	541.01	60	16	17.04	522.6
Cer(d18:0/18:1)	Std	567.01	60	4	17.93	548.7
Cer(d18:0/18:0)	Std	568.98	182	16	18.40	550.6
Cer(d18:0/24:1)	Std	651.01	60	20	20.94	632.7
Cer(d18:0/24:0)	Std	653.13	213	20	21.75	634.7
Cer(d18:0/12:0)	IS	482.01	60	20	13.28	264.4

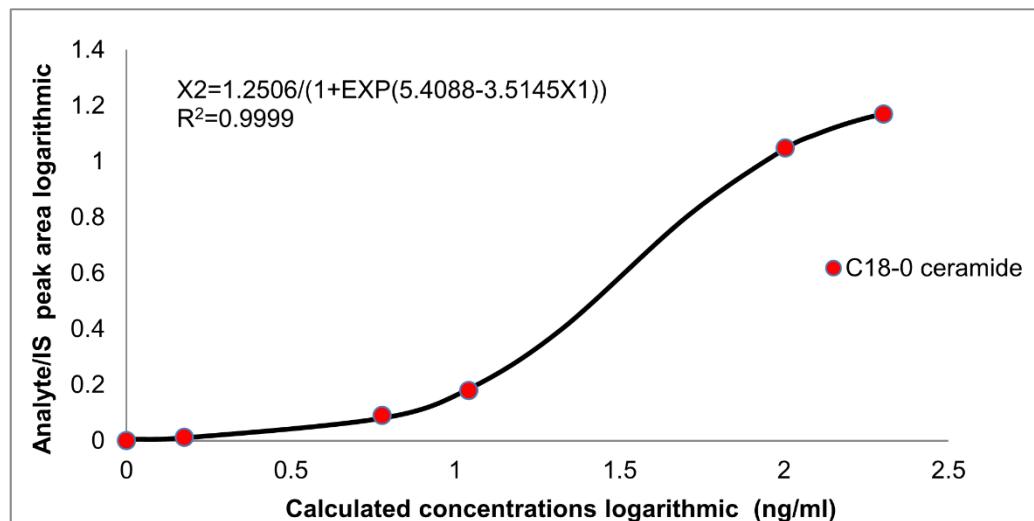
Method optimization notes: For samples containing large amounts of low volatility compounds, the HPLC-program can be made more stringent by increasing the rate flow and by increasing the sampling time. HPLC-program can be adjusted as appropriate for specific analytes and columns by altering holding times. For a few analytes which degrade at high temperatures, it may be desirable to reduce Ion source heater temperatures to 550-580 °C. The timed MS parameters may be used to reduce or eliminate signal from very abundant peaks or contaminants. Those sphingolipids that have different chain lengths, branching, or unsaturation will fragment to yield ions of different m/z. Thus, different precursor ion scans would be necessary to determine the corresponding molecular species. Furthermore, the scan range and collision energy will vary depending on the size and substitution of the various subspecies. All such method adjustments should only be undertaken with the assistance of an experienced MS user.

Data analysis

1. A standard curve with standards from 0.5 ng/mL to 200 ng/mL was generated for quantitative analysis. Curves consisted of triplicates of one blank sample and five calibration points at a concentration ranging from 0.5 ng/mL to 200 ng/mL (Table 4). The amount of each lipid species was calculated according to the sample peak area compared with the normalized internal standard peak area. We used the fitting curve to show the relationship between the concentration and peak area, followed by the fitting curve equation. Three or more biological repeats were recommended (Figure 2).

Table 4. The data points of ceramide standard curve

Concentration (ng/mL)	0	0.5	5	10	100	200
Volume of C18-0 ceramide	0	0.0270	0.2308	0.5122	10.1798	13.7741

**Figure 2. Example of ceramide standard curve.**

The following ceramides amounts were used: 0 ng/mL, 0.5 ng/mL, 5 ng/mL, 10 ng/mL, 100 ng/mL, 200 ng/mL. The x-coordinate is the concentration and the y-coordinate is the Analyte/IS peak area. For the generation of the standard curve, we converted the horizontal and vertical to logarithmic form.

- C18 sphingoid bases are the major backbone of most sphingolipids in mammals (Järne *et al.*, 2018). So we chose the C18-0 as a representative to describe the standard curve (Figure 2). For calibration line measurement, equal amounts and 100 ng Cer (d18:0/12:0) as internal standard were added into different calibration points. The calibration lines from 0.5 ng/mL to 200 ng/mL were converted to a fitted curve, with r values consistently greater than 0.9999 during validation (Table 5). The logistic curve in DPS (Data Processing) Software was used to fit the equation.

Table 5. The fitting value of ceramide standard curve

A	B	C	D	E
Concentration	C18-0 ceramide	Concentration ($\log_{10}(A+1)$)	C18-0 ceramide ($\log_{10}(B+1)$)	Fitted
0	0.0000	0.0000	0.0000	0.0056
0.5	0.0270	0.1761	0.0116	0.0103
5	0.2308	0.7782	0.0902	0.0807
10	0.5122	1.0414	0.1796	0.1853
20	/	1.3222	0.0000	0.3980
50	/	1.7076	0.0000	0.8053
100	10.1798	2.0043	1.0480	1.0467
125	/	2.1004	0.0000	1.0980
150	/	2.1790	0.0000	1.1313
175	/	2.2455	0.0000	1.1543
200	13.7741	2.3032	1.1695	1.1703

3. For acquisition, the multiple reaction monitoring (MRM) mode and the Software PeakView were used. The internal standard with the mass transition 482.00 m/z → 264.40 m/z, the retention time of the internal standard is 13.19 min. The Cer (d18:1/16:0) standard with the mass transition 538.91 m/z → 264.40 m/z, the retention time of the Cer (d18:1/16:0) standard is 16.45 min. Since the retention times and compound-specific ionization and fragmentation values are highly dependent on the used instrumentation, the given values list in Table 2 may be used as a reference but should be individually determined for different instrument setups (Figure 3; Figure 4).

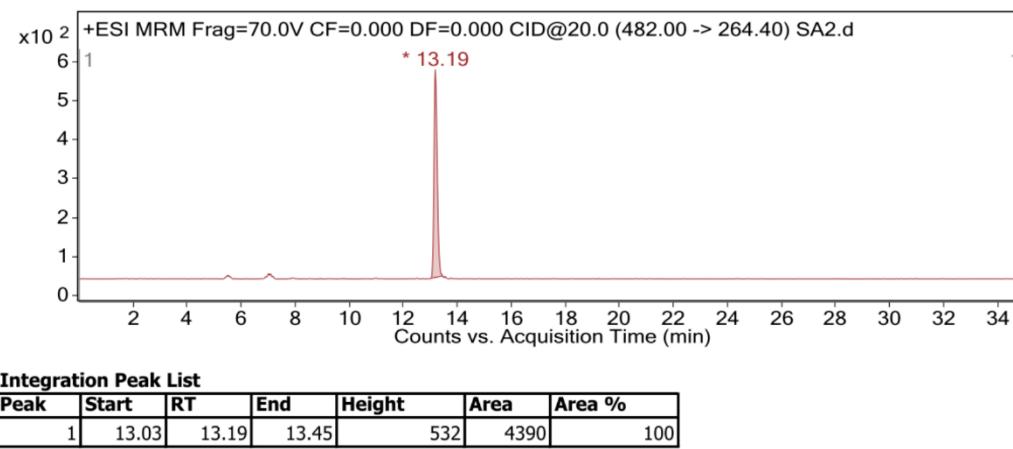


Figure 3. Example of internal standard mass spectrometry acquired with ESI ion source in positive mode from *Nilaparvata lugens*.

Representative signal of internal standard Cer (d18:0/12:0) was plotted. Retention time of internal standard was slightly different from the table 2 list (13.19 min vs. 13.28 min).

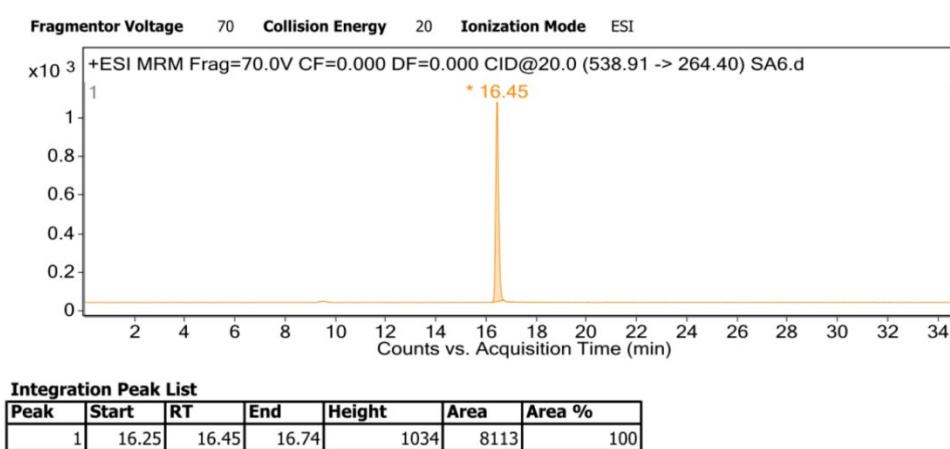


Figure 4. Example of Cer (d18:1/16:0) standard mass spectrometry acquired with ESI ion source in positive mode from *Nilaparvata lugens*.

Representative signal of Cer (d18:1/16:0) was plotted. Retention time of internal standard was slightly different from the table 2 list (16.45 min vs. 16.55 min).

4. Different ceramides were identified by comparing MS/MS ions of analysts with those of sphingolipid standards in ChemSpider base (<http://www.chemspider.com/>) through the Software PeakView ([Http://scie.com.cn/products/Software/peakview-Software](http://scie.com.cn/products/Software/peakview-Software)). The maximum allowed error for the reliability was set to ± 10 ppm.

Recipes

1. Solvent extraction solution A

Ethyl acetate:isopropanol:water, 60:30:10 (vol/vol/vol)

2. Solvent extraction solution B

Methanol:0.1% formic acid, 9:1 (vol/vol)

3. Solution A

MQ water containing 0.1% formic acid

4. Solution B

100% Methanol

5. Internal standard

100 µg/mL Cer (d18:0/12:0) dissolved in HPLC-grade MeOH

Acknowledgments

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Competing interests

The authors declare that no competing financial interest.

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Molecular and Phenotypic Characterization Following RNAi Mediated Knockdown in *Drosophila*

Saurabh Jayesh Kumar Mehta^{1, #, \$}, Pradyumna A. Joshi^{1, #} and Ram Kumar Mishra^{1, *}

¹Department of Biological Sciences, Indian Institute of Science Education and Research (IISER) Bhopal, Bhopal, India

\$Present address: Department of Genetics, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA

*For correspondence: rkmishra@iiserb.ac.in

#Contributed equally to this work

Abstract

Loss of function studies shed significant light on the involvement of a gene or gene product in different cellular processes. Short hairpin RNA (shRNA) mediated RNA interference (RNAi) is a classical yet straightforward technique frequently used to knock down a gene for assessing its function. Similar perturbations in gene expression can be achieved by siRNA, microRNA, or CRISPR-Cas9 methods also. In *Drosophila* genetics, the UAS-GAL4 system is utilized to express RNAi and make ubiquitous and tissue-specific knockdowns possible. The UAS-GAL4 system borrows genetic components of *S. cerevisiae*, hence rule out the possibility of accidental expression of the system. In particular, this technique uses a target-specific shRNA, and the expression of the same is governed by the upstream activating sequence (UAS). Controlled expression of GAL4, regulated by specific promoters, can drive the interfering RNA expression ubiquitously or in a tissue-specific manner. The knockdown efficiency is measured by RNA isolation and semiquantitative RT-PCR reaction followed by agarose gel electrophoresis. We have employed immunostaining procedure also to assess knockdown efficiency.

RNAi provides researchers with an option to decrease the gene product levels (equivalent to hypomorph condition) and study the outcomes. UAS-GAL4 based RNAi method provides spatio-temporal regulation of gene expression and helps deduce the function of a gene required during early developmental stages also.

Keywords: *dElys*, Nucleoporins, RNAi, UAS-Gal4, *Drosophila melanogaster*, Semiquantitative PCR, Nuclear pores

This protocol was validated in: J Biol Chem (2020), DOI: 10.1074/jbc.RA119.009451

Background

Drosophila melanogaster (fruit fly) is a versatile model organism frequently used in research laboratories. Fruit flies are easy to handle, propagate, and maintain. Moreover, the elaborate yet short life span with high fecundity is an added advantage of *Drosophila*. The facile nature of *Drosophila* genetics tools helps develop a comprehensive understanding of a gene function. Since 60% of the *Drosophila* genes are homologous to human genes, and other advantages mentioned earlier, *Drosophila* is an obvious model organism of choice to study *in vivo* gene functions.

The UAS-GAL4 system was used for *in vivo* activation of transcription of genes. Moreover, it was suggested that antisense RNAs could be expressed using the UAS-GAL4 system to achieve significant inhibition of gene expression (Brand and Perrimon, 1993). For using the UAS-GAL4 system in *Drosophila*, transgenic fly lines carrying the upstream activating sequence (UAS) capable of regulating RNAi expression are generated. Genome-scale shRNA-dependent RNAi resource was generated for stage-specific effective knockdown of *Drosophila* genes (Ni *et al.*, 2011). RNAi lines are publicly available from Vienna Drosophila Resource Center (VDRC, maintaining GD and KK RNAi lines) and Drosophila RNAi Screening Center (TRiP and shRNA lines were generated at DRSC and are currently maintained and available from Bloomington Drosophila Stock Center, BDSC). The detailed methodology for the generation of different RNAi lines can be found at the VDRC stock center (<https://stockcenter.vdrc.at/control/main>) and DRSC (<https://fgr.hms.harvard.edu/>) websites.

When the RNAi fly line is crossed with the GAL4 driver fly line, the progenies can have both components of the UAS-GAL4 system expressed. The upstream promoter guides GAL4 expression, and wherever the GAL4 is expressed, it binds to the UAS sequence with high affinity, allowing expression of the RNAi component (Figure 1). In the absence of GAL4 expression, the transgenic RNAi flies behave like wild-type due to lack of dsRNA expression. This method allows the knockdown of the desired gene in a spatiotemporal manner. Classically, X-ray mutagenesis, and P-element mobilization have been used to achieve the loss of gene expression. Further, the EMS mutagenesis screens have been utilized to induce point mutation, occasionally leading to truncations affecting gene expression. The RNAi mediated knockdown is rather facile and less labor-intensive.

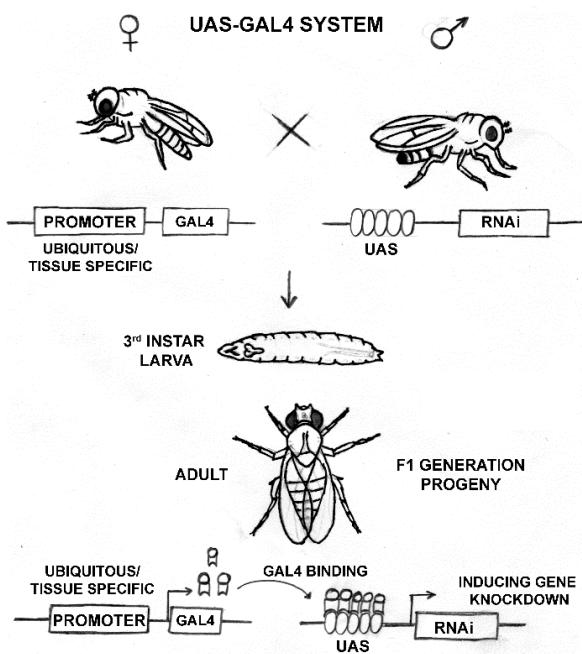


Figure 1. Schematic diagram detailing the functioning of the UAS-GAL4 system in *Drosophila*

Due to varying GAL4 expression degrees in different tissues, a gradient in the knockdown can be seen in different tissues. Besides, temperature-dependent regulation on the GAL4 function provides an advantage of regulating knockdown levels. Together, these handles on gene expression regulation using the UAS-GAL4 system provide

more information regarding the functions of our gene of interest. Importantly, this method provides a controlled spatiotemporal regulation on RNAi mediated knockdown and can help study functions of developmentally essential genes, genes with housekeeping functions, and mutations in genes associated with harmful consequences. This method also helps in the functional characterization of a new gene like *dElys* (Mehta *et al.*, 2020).

A typical qRT-PCR or semiquantitative PCR validates the reduction in gene expression levels upon RNAi mediated knockdown. The knockdown efficiencies can be further assessed by detecting the desired gene product levels by immunostaining and Western blotting methods. We have utilized the RNAi mediated knockdown in salivary glands and assessed its effectiveness using immunostaining or assessing mRNA levels by semiquantitative PCR. We successfully depleted nucleoporins in eyes and wings using the RNAi-mediated knockdown method and reported the importance of nucleoporins in tissue and organism development.

Materials and Reagents

1. 1.5 mL microcentrifuge tubes (Genaxy, catalog number: GEN-MT-150-C-S)
2. Barrier tips
 - a. 0.5-10 μ L tips (Axygen Scientific, catalog number: TF-300-L-R-S)
 - b. 1-20 μ L tips (Axygen Scientific, catalog number: TF-20-L-R-S)
 - c. 1-200 μ L tips (Axygen Scientific, catalog number: TF-200-L-R-S)
 - d. 100-1,000 μ L tips (Axygen Scientific, catalog number: TF-1000-L-R-S)
3. Glass vials and bottles
4. SYLGARD 184 Silicone elastomer (DOW, catalog number: 1673921)
5. Micropipette tips (2, 20, 200, 1,000 μ L)
 - a. 0.5-10 μ L tips (Axygen Scientific, catalog number: T-300-L-R-S)
 - b. 1-200 μ L tips (Axygen Scientific, catalog number: TR-222-C-L-R-S)
 - c. 100-1,000 μ L tips (Axygen Scientific, catalog number: T-1000-C-L-R-S)
6. 3 cm dish (Eppendorf, catalog number: 0030700112)
7. Food bottles
8. CO₂ pads
9. Cotton plugs
10. Corn flour
8. Table sugar
9. Yeast extract (HiMedia, catalog number: RM027)
10. Agar (Merck, catalog number: A5306)
11. Dextrose (HiMedia, catalog number: GRM077)
12. Methyl-4-Hydroxybenzoate (Sigma-Aldrich, catalog number: H5501)
13. Ortho-Phosphoric Acid (Merck, catalog number: 100573)
14. Propionic Acid (HiMedia, catalog number: GRM3658)
15. Ethanol (Changshu Hongsheng Fine Chemical CHN01)
16. Liquid Nitrogen
17. RNA isolation kit (Favorgen, catalog number: FATRK001)
18. RNaseZAP (Thermo Scientific, catalog number: AM9780)
19. DNase (Thermo Scientific, catalog number: EN0523)
20. Diethyl pyrocarbonate (HiMedia, catalog number: MB076)
21. Ethanol (MP Biomedicals, catalog number: 180077)
22. Formamide (Sigma-Aldrich, catalog number: F7503)
23. 6 \times DNA loading dye purple (New England Biolabs, catalog number: B7024S)
24. cDNA synthesis kit (iScript, Bio-Rad, catalog number: 1708891)
25. Agarose (Invitrogen, catalog number: 16500500)
26. Ethidium bromide (EtBr) (MP Biomedicals, catalog number: 193993)
27. Custom Primers (Integrated DNA Technology)

28. G9-Taq DNA polymerase (GCC Biotech, catalog number: G7115A)
29. dNTPs solution mix (New England Biolabs, catalog number: N0447L)
30. Sodium Chloride (Sigma-Aldrich, catalog number: S3014)
31. Potassium Chloride (Sigma-Aldrich, catalog number: P5405)
32. di-Sodium hydrogen phosphate heptahydrate (Merck, catalog number: 1065751000)
33. Potassium dihydrogen phosphate (Sigma-Aldrich, catalog number: P9791)
34. Tris-base (Sigma-Aldrich, catalog number: T6066)
35. Glacial acetic acid (Merck EMPLURA, catalog number: 1.93402.2521)
36. Carbon conductive tape (TED PELLA Inc. catalog number: 16073-1)
37. Aluminum stub (TED PELLA Inc. catalog number: 16111-9)
38. Glutaraldehyde (Sigma-Aldrich, catalog number: G5882)
39. Gold target (Quorum Technology inc, Catalog number: SC502-314A)
40. EDTA (Merck EMPARTA, catalog number: 1.93312.1021)
41. Paraformaldehyde (Sigma-Aldrich, catalog number: 158127)
42. Triton X-100 (Sigma-Aldrich, catalog number: X100)
43. Glass Slide (Borosil, catalog number: 9100P02)
44. Coverslips (Borosil, catalog number: 9115S01)
45. Bovine serum albumin (Merck, catalog number: 621650500501730)
46. Fluoroshield with DAPI (Sigma-Aldrich, catalog number: F6057)
47. mAb414 (Biolegend 902901) - mAb414 recognizes FG-repeat rich four distinct nucleoporins of nuclear pores
48. Anti-*dElys* (Mehta *et al.*, 2020)
49. Alexa Fluor-488 (Invitrogen, catalog number: A-11029)
50. Alexa Fluor-568 (Invitrogen, catalog number: A-11036)
51. Coverslip sealant (Transparent nail polish)
52. Diethyl ether (Merck, catalog number: 107026)
53. Phosphate Buffer Saline (1×) (see Recipes)
54. PBS-T (see Recipes)
55. Fly food ingredients (see Recipes)
56. RNA loading dye (see Recipes)
57. DEPC treated water (see Recipes)
58. TAE Buffer (50×) (see Recipes)
59. 4% Paraformaldehyde (see Recipes)

Fly stocks

Control Wild type (*w¹¹⁸*)

Driver lines (in house generated combinations)

1. +/++; *Actin5C-GAL4/CyO-GFP; UAS-Dicer/UAS-Dicer*
2. +/++; *wingless-GAL4/wingless-GAL4; UAS-Dicer/UAS-Dicer*
3. +/++; *eyeless-GAL4/eyeless-GAL4; UAS-Dicer/UAS-Dicer*

RNAi lines

1. +/++; v103547/v103547; UAS-Dicer/UAS-Dicer (*dElys* RNAi KK line combined with UAS-Dicer, in house generation)
2. *dElys* RNAi KK line (VDRC, VDRC ID: 103547)
3. *Nup160* RNAi GD line (VDRC, VDRC ID: 21937)
4. *Sec13* RNAi KK line (VDRC, VDRC ID: 110428)
5. *Nup107* RNAi GD line (VDRC, VDRC ID: 22407)

Equipment

1. Micropipette (Nichiryo, Gilson, Corning)
2. BOD Incubators
3. Millipore water purification unit
4. Genova Nanodrop (Bibby Scientific, model: 737501)
5. Forceps Dumont 5 (Fine Science Tools, catalog number: 11295-10)
6. Forceps Dumont 55 (Fine Science Tools, catalog number: 11295-51)
7. Scissors (Fine Science Tools, catalog number: 91500-09)
8. Leica DM2500
9. Fluorescent stereomicroscope (Leica, model: M205 FA)
10. Confocal Laser Scanning Microscope (Zeiss, model: LSM780)
11. Stereomicroscope (Leica, model: S6E)
12. Scanning Electron microscope (Zeiss, model: Gemini II Ultra plus)
12. Tabletop centrifuge (Eppendorf, model: 5424, Thermo Scientific MicroCL 21R)
13. PCR machine (Applied Biosystems, model: 2720 Thermal cycler)
14. Water bath (Grant-Bio, model: PSU-10i)
15. Gel running apparatus (CBS Scientific, model: GCMGU-202T)
16. Power packs (CBS Scientific, model: EPS 300)
17. UVP MultiDoc-It Digital Imaging System (UVP, catalog number: 97-0192-02)

Software

1. ZEN 3.2 Blue edition (Zeiss)
2. ImageJ/Fiji (Free, NIH)

Procedure

The UAS-GAL4 dependent and RNAi mediated knockdown of genes in *Drosophila* requires several steps. It includes steps like rearing the organism, setting up genetic crosses, harvesting larvae of desired genetic combination, RNA isolation and knockdown assessment, dissections, immunostaining, and phenotypic analysis. Mentioned below is the general scheme of tissue preparation and processing (Figure 2).

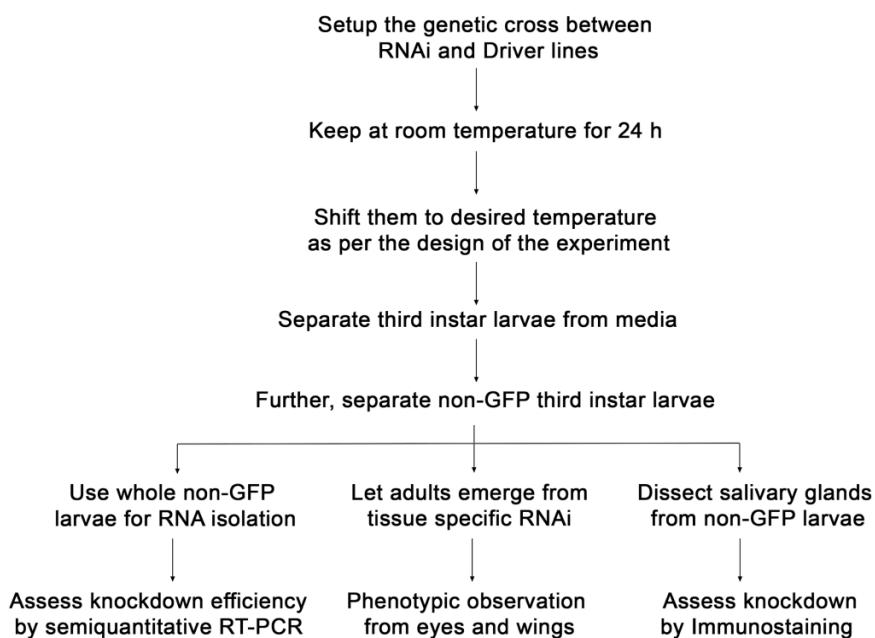


Figure 2. A flow chart with details of the protocol

A. Genetic crosses for RNAi mediated knockdown

1. Grow $+/+$; *Actin5C-GAL4/CyO-GFP*; UAS-Dicer/UAS-Dicer flies in large numbers in vials containing fly media.
2. Collect virgin females having the GAL4 driver in a separate media vial.
3. Grow RNAi and wild type (w^{1118}) flies in separate media vials to get sufficient male flies.
4. Place 6-7 virgin driver females and 2-3 UAS-RNAi males of the same age (3-4 days old) inside a fresh media vial to establish a mating cross.
5. For the control cross, use w^{1118} males instead of UAS-RNAi males and set up the cross with virgin females as in Step A4.
6. Incubate vials with flies for the genetic cross at room temperature for 24 h.
7. According to the experimental design, shift them to 28°C, 25°C, or 18°C.

Note: Incubation at a higher temperature allows higher GAL4 activity inducing increased RNAi expression.

8. Keep crosses in an incubator set at the desired temperature until the third instar larvae are visible.
9. Remove adult flies and put 2-3 mL water or 2% sucrose solution to harvest larvae from the media's top layer.

Note: Steps A8 to A13 are in Figure 3.

10. Using a paintbrush, mix the upper layer of food and water and collect it in a clean plastic dish.
11. Separate third instar larvae in a clean distilled water/phosphate buffer saline containing plate.
12. Wash collected larvae 2-3 times with water to clean them.
13. Segregate non-GFP larvae ($+/+$; *Actin5C/RNAi*; UAS-Dicer $+$), indicating the desired genetic combination of Driver and RNAi from GFP ($+/+$; RNAi/CyoGFP; UAS-Dicer $+$) larvae for subsequent processing.
14. For the control, use non-GFP larvae from the control cross as described in Step A5.
15. Dissect the salivary gland or the head complex of *Actin5C-GAL4* driven RNAi from the non-GFP third instar larvae in PBS.

Note: Refer to Figure 3 below and Steps 1-5 mentioned in Part-C for details of salivary gland dissection.

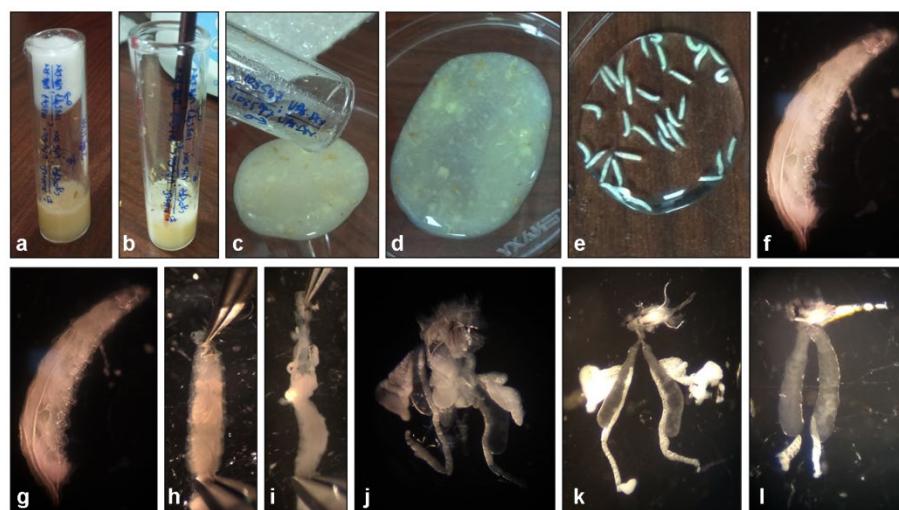


Figure 3. Steps in the isolation of Salivary glands from *Drosophila* third instar larva.

Upper panels (a-f), Steps in larva separation and cleaning; (a) Larvae and Pupae in food vial, (b) Squirting water and suspending the top layer of food and larva with a brush, (c) Decanting suspension in a petri-plate, (d) Fly food and third instar larva suspension, (e) Cleaned larva collected in distilled water, (f) A third instar larva. Lower panels (g-l) Steps in larval dissection and salivary gland isolation (g) A third instar larva, (h) Holding a third instar larva with forceps, (i) Pulled out salivary glands and abdominal parts of the third instar larva, (j) Head complex having the brain, imaginal disc, and salivary gland (k) Salivary gland with associated fat bodies, (l) Dissected and cleaned up pair of salivary glands with an attached mouth hook.

B. RNA isolation

The most critical step of this method is RNA isolation. RNAi mediated knockdown reduces the specific RNA levels to affect the gene function. RNA digesting enzyme, RNaseA is a very stable and active protein; thus, taking proper precaution and using RNase inactivating solution like RNaseZAP is necessary.

1. Prepare DEPC treated water for use (see Recipes).
2. Make sure to use barrier tips, RNase-free microcentrifuge tube (MCT), and glassware.
3. Put larvae inside a clean RNase-free MCT, flash freeze, and store for RNA isolation.
4. Dissect sufficient salivary glands from larvae and flash freeze glands inside liquid nitrogen.
5. Crush the salivary gland/larva using RNase free pestle and follow the manufacturer's protocol for RNA extraction given in the kit.
6. Crush flash-frozen tissues directly using a pestle or first add lysis solution from the RNA isolation kit and then crush the tissue or larva in the solution.
7. If adding the lysis solution before crushing, add half of the lysis buffer's final volume, and properly crush the tissue/larva. Subsequently, add the remaining lysis buffer.
8. Check the purity of RNA using a UV-VIS spectrophotometer/nanodrop. $A_{260/280}$ and $A_{260/230}$ ratio equal to 2.0-2.2 suggests pure RNA. Lower values of these ratios indicate the presence of contaminants like proteins, carbohydrates, or phenol.
9. In an MCT, heat the purified RNA sample at 65°C for 10 min in a 25% formamide containing RNA loading dye.
10. Keep RNA sample tubes on ice for 2 min and load immediately on the EtBr containing 1% agarose gel made in Tris-Acetate EDTA (TAE) buffer and run the gel to check the integrity of the isolated RNA.

11. Prepare cDNA as per instruction given with the BioRad cDNA synthesis kit and store at -20°C until further use.
12. A typical cDNA synthesis reaction in 20 µL final volume contains 1 µg purified RNA, 1× iScript reaction mixture, 1 µL of iScript reverse transcriptase enzyme.
13. Reaction steps include 5 min of priming at 25°C, 20 min of reverse transcription at 46°C, and 1 min of inactivation at 95°C.
14. Use gene-specific primers (200 nM) on prepared cDNA and run a typical PCR reaction (see Table 1 for primer sequences).

Note: We used a standard annealing temperature (T_a) of 55°C for all semiquantitative PCR.

15. Use *RpL49* primers as an internal control gene in the PCR reaction (see Table 1 for primer sequences).

Table 1. List of primers used for assessing the knockdown levels

Sl#	Primer Name	Sequence (5'→3')
1	Nup107F	CTGGGAGCACAAGGTAAAGG
2	Nup107R	GATCTCAGGAATGCAGATGGAG
3	Nup160F	CCAGTGGCTGATAAACTCCTAC
4	Nup160R	AGAGCTGTGGTTGCTATG
5	Sec13F	TCACATTGTGGAAGGAGAACAC
6	Sec13R	GTTGGACAGGTTCGAGCTATG
7	RpL49F	CGTTTACTGCGGCGAGAT
8	RpL49R	GTGTATTCCGACCACGTTACA

16. Load 50% of PCR product on EtBr containing 1% Agarose gel to assess the extent of knockdown (Figure 4).

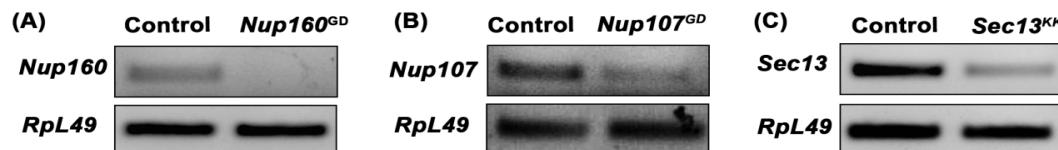


Figure 4. Semiquantitative PCR based assessment of knockdown.

cDNAs prepared from Control and nucleoporin knockdown used in a PCR reaction. Upper panels show levels of *Nup160* (A), *Nup107* (B), and *Sec13* (C) from control and knockdown tissues. *RpL49* served as an internal loading control between control and knockdown samples.

C. Dissection and Staining of Salivary gland

Salivary gland is post-mitotic tissue and carries large nuclei. It possesses polytene chromosomes, which serve as excellent model tissue in several cytological studies and gene expression analyses. The loss of nucleoporins and consequent perturbations in nuclear pore assembly and functions can be studied using immunostaining. *dElys* was knocked down in the salivary glands using RNAi. Subsequent steps in the dissection of salivary glands in immunostaining are the following.

1. Dissect out salivary glands in PBS on a silicone elastomer plate from 20 third instar larvae.

Note: Replace PBS solution after every 2-3 dissections.

2. Grab the larva approximately at the middle portion of its size using forceps.
3. Use Dumont 5 forceps to grab the mouth-hook and pull out the head complex.
4. Simultaneously remove the gut region and other carcass tissues using Dumont 55 forceps.
5. Use pointed Dumont 5 forceps to grab the head complex by mouth-hook all this while.
6. Clean the dissected salivary gland by removing the brain, imaginal discs, and fat tissue and ensure that the mouth-hook remains attached.
7. After dissecting each salivary gland, immediately transfer it to an MCT containing 1 mL of 4% paraformaldehyde.
8. After all the salivary glands are dissected and collected, keep the MCT on an orbital shaker with gentle agitation for 40 min at room temperature.

Note: Fixation should not go beyond 1 h; otherwise, the tissue becomes fragile, and staining is affected.

9. Remove paraformaldehyde and wash tissues three times with PBS-T for 10 min each.

Note: Do all the wash steps carefully to avoid the loss of salivary glands. It applies to all subsequent steps.

10. Dissecting the salivary gland with the mouth hook attached to it gives an additional advantage that we can easily see the salivary gland, and thus their loss will be restricted.
11. Block salivary glands in 3% normal goat serum containing PBS-T or 3% Bovine Serum Albumin (BSA) in PBS-T.
12. Keep at room temperature for 1 h on an orbital shaker with 110 rotations per minute (rpm).

Note: Perform all incubations/wash on an orbital shaker set at 110 rpm.

13. Add 200 µL of anti-*d*Elys and mAb414 primary antibodies containing solution (1:800 dilution of each of the two antibodies prepared in PBS-T).
14. Incubate overnight at 4°C on a shaker.
15. Wash samples three times with 200 µL of PBS-T for 10 min each at room temperature (25°C).
16. Add 200 µL of Alexa Fluor-488 or Alexa Fluor-568 conjugated secondary antibodies dilution (1:800 dilution prepared in PBS-T) and incubate at room temperature (25°C) for 1.5-2.0 h.
17. Wash samples three times with 200 µL of PBS-T, 10 min each at room temperature (25°C).
18. Put 15 µL of Fluoroshield with DAPI mounting medium.
19. Pick all salivary glands using a cut 200 µL pipette tip.

Note: Cut the sample inlet end of the fine 200 µL pipette tip to avoid any tissue damage.

20. Place salivary glands on a previously cleaned slide and spread the tissue gently using Dumont 55 forceps.

Note: Do not damage the tissue while spreading. Use the mouth hook to grab the tissue, only if required.

21. Carefully place a clean coverslip on the tissue.
22. Avoid trapping air bubbles between the slide and coverslip.
23. Gently remove the excess mounting medium using tissue paper and seal the coverslip edges with transparent nail polish.
24. Observe processed tissue under a fluorescent Leica microscope.
25. On Zeiss LSM780, use the control slide to adjust settings and focus in a field with a clear view of cells.
26. Scan the field having salivary gland nuclei under 63× objective in a Confocal Laser Scanning Microscope LSM780.
27. Capture images from different visual fields of the control slide (Figure 5).
28. Reuse the same settings for the knockdown samples also.

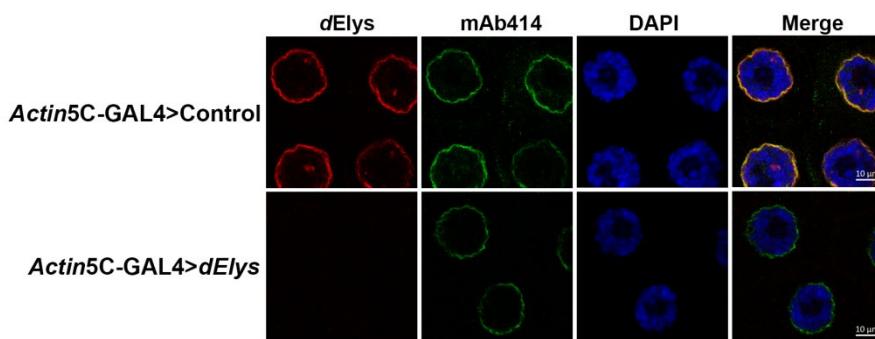


Figure 5. RNAi mediated *dElys* knockdown depleted *dElys* signals from salivary gland nuclei.

The salivary glands dissected from Control RNAi (upper panels) and *dElys* RNAi (lower panels) third instar larvae and stained with *dElys* (red, first panels), mAb414 (green, second panels), DAPI (blue, third panels). The fourth panels represent the merged images. Scale bars =10 μ m.

D. Wing and Eye specific knockdown

1. Set up crosses between *nup160* RNAi and +/+; *Ey*-GAL4/*Ey*-GAL4; UAS-Dicer/UAS-Dicer (for eye-specific knockdown) or *nup160* RNAi and +/+; *wg*-GAL4/*Wg*-GAL4; UAS-Dicer/UAS-Dicer (for wing-specific knockdown).
2. Transfer crosses to a lower temperature (18°C). The lower temperature reduces the GAL4 activity.
3. Reduced GAL4 activity at 18°C causes a reduction in knockdown levels and allows adult fly emergence.

E. Dissection and visualization of eyes and wings

1. Visualization of eyes

Drosophila compound eyes are complex yet pertinent tissue to observe growth defects upon RNAi mediated knockdown. A definite transcriptional paradigm regulates the arrangement of each ommatidium in the compound eye during development. Any perturbation in the ommatidium arrangement, their smooth morphology, and the presence of a bristle with each ommatidium due to RNAi mediated knockdown serves to indicate the importance of the gene function. After knocking down the *Nup160* using RNAi, process wings in the following manner. If the gene of interest induces embryonic to larval lethality, eye-specific knockdown helps assess the gene function. After knocking down the *Nup160* using RNAi, process compound eyes in the following manner.

- a. Collect adult flies in an empty vial.
- b. Apply 100-200 μ L of diethyl ether on cotton and plug the fly containing vial to anesthetize them.
- c. Orient them properly (compound eye facing up) under Stereomicroscope Leica S6E.
- d. Fix the position of the fly using carbon conductive tape for steady observation of eyes.
- e. Observe eye morphology using Leica fluorescent stereomicroscope M205 FA at $\times 123$ magnification (left panels in Figure 6A).

2. SEM analysis of eye phenotype

- a. Anesthetize the flies using diethyl ether. Flash freeze them and separate heads from the body.
- b. Fix head tissues with 2.5% Glutaraldehyde in PBS for 2 h at 4°C.
- c. Wash with PBS and 4% sucrose solution.
- d. Dehydrate tissue using a series of graded ethanol wash (single wash with 25%, 50%, 75% ethanol, and twice with 100% ethanol) for 2 h each at room temperature.
- e. Keep inside a lab desiccator for critical point drying.
- f. Mount dried tissue on aluminum stubs with carbon-conductive tape.
- g. Coat the flies with gold particles in a sputter coating apparatus.

- h. Image using Zeiss Gemini II FESEM with In lens detector (middle and right panels in Figure 6A).
3. Visualization of wings

Drosophila adult wings serve as an excellent tissue to observe the developmental defects induced by perturbation in a gene function. The changes in bristles, wing veins, and notched wings are distinct phenotypes seen with the knockdown of genes. Wing imaginal discs are present in the third instar larva. If the gene of interest induces post-larval lethality, wing imaginal discs are routinely analyzed. After knocking down the *Nup160* using RNAi, process wings for visualization in the following manner.

 - a. Follow Steps E1a and E1b of the visualization of eyes procedure.
 - b. Cut the wings with scissors and place them on a clean slide.
 - c. Put coverslip on top of the wing tissue and seal.
 - d. Observe under upright light microscope Leica DM2500 at 10 \times magnification (panels in Figure 6B).

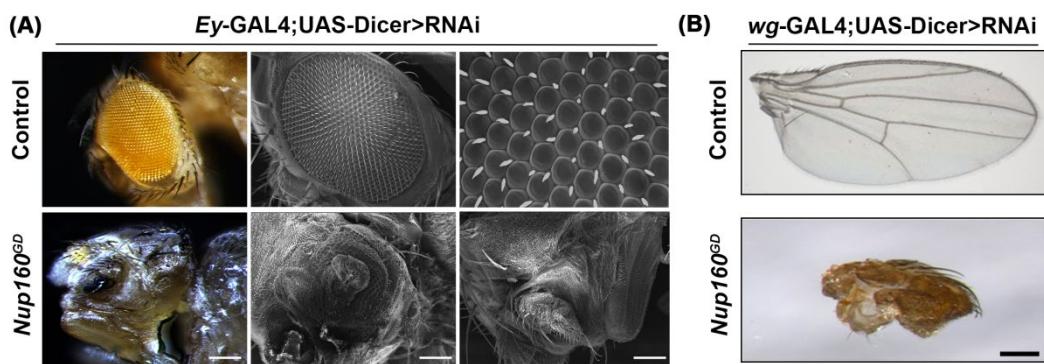


Figure 6. Knockdown of *Nup160* in eyes and wings induces developmental defects.

(A) Eye specific knockdown of *Nup160* using *Ey*-GAL4 and visualization of the compound eye under bright field light microscope and SEM. (B) Wing specific knockdown of *Nup160* using *Wg*-GAL4 and visualization of wings under bright field light microscope. The control knockdown is for comparison. Scale bars (A) are 200 μ m in the left panels, 20 μ m in the middle panels, and 2 μ m in the right panels. The scale bar in (B) is 200 μ m.

Data analysis

We have utilized RNAi mediated knockdown of *dElys* in *Drosophila* and have explored the functional significance of *dElys* in *Drosophila* development. Observations made regarding *dElys* function highlight that *dElys* is important for embryonic development, nuclear pore assembly, nucleocytoplasmic shuttling of the developmentally regulated molecule, Dorsal. The genetic crosses between flies carrying RNAi against *dElys*, *Nup160*, *Nup107*, and *Sec13*, and promoter-specific GAL4 sequences produced desired progenies where both RNAi and UAS-GAL4 were present in the same fly. Progenies were selected at the third instar larva stage based on the absence of GFP expression (non-GFP larva). In salivary gland specific analysis, we dissected paired salivary glands from non-GFP larvae (Figure 3). Use of *Actin5C*-GAL4 for knockdown of genes of interest, *Nup160*, *Nup107*, and *Sec13* successfully achieved significant gene expression knockdown. The semiquantitative RT-PCR analysis established that these genes are efficiently depleted (Figure 4).

We achieved a knockdown ranging from 50-80% using RNAi for different genes. Determination of *RPL49* gene levels by PCR helped in making these precise calculations. In conditions where the ubiquitous knockdown appears lethal or harmful, we explored the option of tissue-specific knockdown. Additionally, when a gene's function confines to a particular tissue, the power of tissue-specific promoter mediated knockdown can be utilized. Any potential off-target gene of utilized RNAi must also be analyzed to build a correlation between gene and phenotype. Mehta *et al.*, 2020 reported that two off-targets of *dElys* RNAi were not perturbed significantly. Simultaneously, a gentle lowering of RNA transcript for a dosage-sensitive protein can also bring about the observable phenotypes. *dElys* levels reduced strongly under *dElys* knockdown conditions (*Actin5C*>*dElys*). The ubiquitous knockdown of

dElys and subsequent analysis of the salivary gland nuclei indicated that nuclear pore assembly is affected by *dElys* depletion. Under similar conditions, *dElys* levels are unperturbed in control knockdown (*Actin5C>Control*) (Figure 5). mAb414 antibody recognizes four distinct FG-repeat rich nucleoporins. A noticeable reduction in mAb414 staining of nuclear pores is also evident upon *dElys* RNAi. Based on these observations, we suggest an essential role for *dElys* in nuclear pore assembly in *Drosophila*.

We further utilized the UAS-GAL4 system's power for RNAi mediated knockdown of *Nup160* in *Drosophila* eye and wings. Eye-specific *Ey*-GAL4 and wing-specific *wg*-GAL4 promoters depleted *Nup160* to produce significant perturbation in the development of these two tissues. In *Nup160* knockdown animals, the entire compound eye is shrunken and seems absent. The SEM imaging of knockdown eyes shows drastic changes compared with eye-specific control knockdown (Figure 6, panel A). Similarly, the wing-specific knockdown of *Nup160* caused a reduction in the wing blade size, and the veins are completely missing (Figure 6, panel B).

The observations made with RNAi mediated knockdown of nucleoporin genes *dElys* and *Nup160* established the robustness of this gene knockdown paradigm. Further, the tissue-specific knockdown and analysis from salivary glands, eyes, and wings highlighted the functional significance of nucleoporins in *Drosophila* development.

Recipes

1. Phosphate Buffer Saline (1×)

Sodium Chloride 137 mM
Potassium Chloride 2.7 mM
di-Sodium hydrogen phosphate heptahydrate 10 mM
Potassium dihydrogen phosphate 1.8 Mm

2. PBS-T

Lukewarm the falcon tube containing 1× PBS and add Triton X-100 (100%) to a final concentration of 0.2% with a cut pipette tip.

3. Fly food ingredients

Ingredient	Amount/liter
Corn flour	80 g
Sugar	40 g
Dextrose	20 g
Yeast Extract	15 g
Agar	10 g
Methyl-4-hydroxybenzoate	1 g
Propionic acid	4 mL
Orthophosphoric acid	0.6 mL
Ethanol absolute	10 mL

a. Weigh corn flour, sugar, dextrose, yeast extract, Agar separately, and add to the flask.

Note: Follow the ratio of different components mentioned in the table above.

- b. Autoclave at 121°C for 20 min and let it cool for some time till the temperature reaches ~50-55°C.
- c. Meanwhile, weigh 1 g of methyl-4-hydroxybenzoate and dissolve in 10 mL ethanol.
- d. Once the medium has cooled down to ~50-55°C (check it with a thermometer), add methyl-4-hydroxybenzoate solution, propionic acid, and orthophosphoric acid to the media.
- e. Mix it thoroughly and pour 8-10 mL into each glass vial.

Note: Avoid the incorporation of bubbles. Do not let it cool below ~50°C; otherwise, Agar will start to solidify.

- f. Once the media has solidified inside the vial, put a tight cotton plug on each vial, and use it as required.

Note: Keep vials inside a cage for cooling. The cage helps avoid contamination of vials by stray flies in the room.

4. RNA loading dye

- 50 µL Formamide
- 35 µL 6× DNA loading dye
- 15 µL Ultrapure water (to make up the final volume)

5. DEPC treated water

- a. Add 1 mL of DEPC to 1,000 mL of ultrapure water (0.1%).
- b. Mix well, cover the bottle and leave it overnight at room temperature.
- c. Autoclave two times, let it cool down to room temperature.
- d. Make aliquots and store them at -20°C for use.

6. TAE Buffer (50×)

- a. Add 242 g of Tris base to 700 mL of double-distilled water.
- b. Add 57.1 mL of Glacial acetic acid carefully.
- c. Mix 100 mL of 0.5 M EDTA having pH 8.0 to it.
- d. Adjust volume to 1,000 mL.

Note: The pH should be around 8.5 and need not be adjusted.

- e. Use 20 mL of 50× TAE stock to make a 1 liter of 1× TAE buffer.
- f. 1× TAE buffer will have the following final concentrations: 40 mM Tris base, 20 mM Acetic acid, and 1 mM EDTA

7. 4% Paraformaldehyde

- a. Take 50 mL of 1× PBS in a clean glass bottle.
- b. Add 4 g of paraformaldehyde while keeping the bottle on the heating plate at ~60°C

Note: Make sure that to work inside a ventilated hood as paraformaldehyde is hazardous.

- c. Add 1 N NaOH slowly and dropwise to dissolve all the paraformaldehyde.
- d. When all paraformaldehyde dissolves in PBS, adjust the pH to 7.2-7.4 using 1 N HCl.
- e. Adjust the final volume to 100 mL using 1× PBS.
- f. Filter and store paraformaldehyde as 10 mL aliquots at 0-4°C.

Note: Do not store the paraformaldehyde solution for more than a month.

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Competing interests

No competing interest to declare.

Ethics

1. No human subjects or mouse model was used in the study.
2. For rearing, maintaining, and experimenting with *Drosophila*, work followed the applicable parts of animal Ethics guidelines of the host Institute.

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Live Intravital Intestine with Blood Flow Visualization in Neonatal Mice Using Two-photon Laser Scanning Microscopy

Yuhki Koike^{1,2}, Bo Li¹, Yong Chen¹, Niloofar Ganji¹, Mashriq Alganabi¹, Hiromu Miyake¹, Carol Lee¹, Alison Hock¹, Richard Wu³, Keiichi Uchida², Mikihiro Inoue², Paul Delgado-Olguin^{4,5,6}, Agostino Pierro^{1,*}

¹Division of General and Thoracic Surgery, Physiology and Experimental Medicine Program, The Hospital for Sick Children, Toronto, ON, Canada

²Department of Gastrointestinal and Pediatric Surgery, Mie University Graduate School of Medicine, Tsu, Mie, Japan

³Division of Gastroenterology, Hepatology and Nutrition, The Hospital for Sick Children, Toronto, ON, Canada

⁴Translational Medicine, The Hospital for Sick Children, Toronto, Ontario, Canada; ⁵Department of Medical Biophysics, University of Toronto, Toronto, Ontario, Canada; ⁶Heart & Stroke Richard Lewar Centre of Excellence, Toronto, Ontario, Canada

*For correspondence: agostino.pierro@sickkids.ca

Abstract

This protocol describes a novel technique to investigate the microcirculation dynamics underlying the pathology in the small intestine of neonatal mice using two-photon laser-scanning microscopy (TPLSM). Recent technological advances in multi-photon microscopy allow intravital analysis of different organs such as the liver, brain and intestine. Despite these advances, live visualization and analysis of the small intestine in neonatal rodents remain technically challenging. We herein provide a detailed description of a novel method to capture high resolution and stable images of the small intestine in neonatal mice as early as postnatal day 0. This imaging technique allows a comprehensive understanding of the development and blood flow dynamics in small intestine microcirculation.

Keywords: Intravital imaging, *In vivo* imaging, Two-photon laser scanning microscopy (TPLSM), Necrotizing enterocolitis (NEC), Neonatal mouse imaging

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Background

Development of *in vivo* real-time imaging

Recent technological advances are overcoming the limitations of conventional histological analysis by enabling live imaging on experimental animals. In contrast to conventional histological microscopy techniques, the intravital approach provides insight into previously unknown morphogenetic and functional processes in live tissues. Furthermore, this information can be acquired in real time lapses, whereas alternative techniques are limited to a snapshot in time. The alternative techniques have the added requirement of determining the best timing to acquire images during a specific process in an experimental protocol.

Two-photon laser-scanning microscopy (TPLSM), compared to confocal microscopy, offers *in vivo* imaging that is superior for deep optical sectioning of living tissue (Pittet and Weissleder, 2011). The higher resolution and reduced phototoxicity of this method allow longer time periods of continuous real-time imaging on intact organs. We have standardized *in vivo* real-time imaging of intra-abdominal organs through TPLSM and used it to study the contribution of abnormal intestinal microcirculation in pathophysiological processes. For example, we have used this technology to study bacterial translocation in dextran sodium sulfate (DSS)-induced colitis (Toiyama *et al.*, 2010), neutrophil extracellular traps (Tanaka *et al.*, 2014a), thrombus formation in laser-induced endothelium injury (Koike *et al.*, 2011), visualization of chemotherapy responses of colorectal liver metastases to the tumor microenvironment (Tanaka *et al.*, 2014b), and the dynamics of circulating free DNA in a model of DSS-induced colitis (Koike *et al.*, 2014). Additionally, previous reports have shown the application of intravital multiphoton microscopy to study pathophysiological processes in other abdominal organs including the liver (Honda *et al.*, 2013; Lu *et al.*, 2014; Liang *et al.*, 2015), pancreas (Coppieters *et al.*, 2010; Martinic and von Herrath, 2008), spleen (Ferrer *et al.*, 2012), and kidneys (Peti-Peterdi *et al.*, 2012; Hackl *et al.*, 2013; Devi *et al.*, 2013; Schiessl and Castrop, 2013).

In recent years TPLSM has become increasingly popular in *in vivo* research. However, intravital imaging of the small intestine in neonatal mice has been challenging due to their small body size and fragility of the intestinal wall. We have developed a novel application of TPLSM to visualize and study the small intestine of neonatal mice *in vivo*.

Advantages of the method

Two of the key advances of TPLSM are deeper tissue penetration and reduced photobleaching. These advances have facilitated the study of dynamic processes *in vivo* while minimizing injury to the organ or cell under investigation. Obtaining intravital stable images of the gastrointestinal tract has been difficult due to the movements caused by heartbeat, respiration, and intestinal peristalsis. To reduce the effects of these movements, we and others have purposefully designed devices and equipment that improve live image stability of the gastrointestinal tract (Watson *et al.*, 2005; McDole *et al.*, 2012; Ritsma *et al.*, 2013). However, setting up such devices is time consuming, costly, and present technical challenges that require extensive trial and error. Current systems of choice for stabilizing intravital imaging are: a viewing window with a vacuum chamber (Looney *et al.*, 2011), a microstaging device (Cao *et al.*, 2012), and our previously designed organ stabilizer (Japanese Patent No. 5268282) (Toiyama *et al.*, 2010). These methods have been used for imaging the small intestine in adult mice, however, none of them have been applied to the imaging of the neonatal small intestine. One reason for this is that these devices are too large and limit the working space for the neonatal small intestine. To limit the movements induced by heartbeat and breathing during imaging, it is important that the organ stabilizer is detached from the neonatal abdominal wall. In addition, fixation maneuvers required when using previously developed organ stabilizing devices can cause injury or affect the neonatal intestinal microcirculation.

Our method allows for direct microvascular blood flow analysis. Stappenbeck TS *et al.* (2002) reported a method to analyze intestinal microcirculation indirectly from intestinal tissue samples harvested immediately after injecting fluorescein isothiocyanate-labeled dextran into the heart (Yu *et al.*, 2009; Watkins and Besner, 2013; Yazji *et al.*, 2013). However, this and other similar methods for intravital imaging of the adult mouse intestine are not functional in blood flow dynamics. Our method allows for blood flow dynamics analysis, and facilitates investigation of intestinal villi development and establishment of the capillary network complexity (Stappenbeck *et al.*, 2002). This

protocol will allow the investigation of pathological processes associated with intestinal blood flow dynamics *in vivo*, thus promoting translational research.

The method we describe in this protocol is simple, overcomes the limitations of previous systems, and allows for stable live imaging of the neonatal mouse small intestine. This method interferes minimally with the microcirculation and enables high resolution intravital imaging of the small intestine for long periods of time. This protocol allows for unprecedented stability of intravital imaging of the neonatal small intestine.

Applications of the method

The TPLSM imaging method described here can be easily applied to investigate different physiological processes in the neonatal intestine in multiple mouse models. For example, we are using this method to study blood flow dynamics and inflammatory responses in necrotizing enterocolitis (NEC), intestinal epithelium and microvasculature development in short bowel syndrome (SBS), inflammatory and immunological status in inflammatory bowel disease (IBD), and ischemia-reperfusion injury in midgut volvulus. Previous studies investigated neonatal intestinal microcirculation in experimental NEC *ex vivo*, however, these studies did not consider the effects of blood flow dynamics in capillary-vessels in the villi. Our new method allowed us to measure neonatal intestinal microcirculation from movies of live blood flow and to derive blood flow velocity, vessel diameter and inflammation, and assess irrigation of the serosal and mucosal layers. Moreover, this technique is being used to visualize and quantify live blood flow dynamics during reperfusion and ischemia in experimental midgut volvulus, which will be useful to identify the primary intestinal tissues affected.

Here, we provide a step-by-step methodology to set up the neonatal mouse small intestine using a simple stabilizing device to evaluate the intestinal microcirculation by TPLSM.

Limitation of the approach

The proximal jejunum close to the ligament of Treitz is difficult to study due to its proximity and attachment to the abdominal wall. The described technique allows for analysis of the small intestine between the anterior superior iliac spine and the xiphoid process transversely, and between the sternum and the posterior abdominal wall longitudinally. Analyzing the intestine outside these marking points (for instance, in portions of the colon) may lead to potential bleeding from the mesentery due to excessive stretching applied on the intestine for appropriate positioning. Additionally, the device is limited to areas of the intestine that are naturally close to the abdominal wall without the need for heavy manipulation to avoid potential intestinal damage. The organ stabilizing device should not be in direct contact with the mouse abdominal wall to avoid image instability caused by movements due to breathing and heartbeat.

Considerations for intestinal preparation

The neonatal mouse should be fasted for at least 4 hours before microscopic observation since food residue within the intestinal lumen could affect the imaging results by affecting intestinal blood flow and/or potential for development of ischemia. Some reports have shown that intestinal blood flow varies by gestational age and feeding time (Pezzati *et al.*, 2004; Watkins and Besner, 2013; Thompson *et al.*, 2014; Morgan *et al.*, 2014), suggesting that feeding tolerance should also be considered in the study protocol. Therefore, for analyzing the small intestine microcirculation using this method, a fixed fasting duration and gestational age between all mice being used should be considered before starting the experiment to allow for appropriate comparison. The ideal fasting time to use will vary depending on the specific parameters to be measured in the neonatal small intestine. For example, 6-hour fasting allows proper imaging of the neonatal ileum in 5-day old pups subjected to experimental NEC, but this may vary if examining a different disease or mice of a different age. The varying effects on blood circulation secondary to feeding should be minimized by maintaining a homogenous feeding schedule and fasting duration across all mice being examined.

Procedure for NEC induction

NEC is induced by gavage feeding a hyperosmolar formula gavage, exposure to temporal hypoxia and oral administration of lipopolysaccharide (LPS) (Zani *et al.*, 2008). Gavage feeding is given 3 times a day, using a 1.9-Fr silicon catheter (VYGON UK Ltd, Gloucestershire, UK). The hyperosmolar formula is prepared with 15 g of SMA Gold (SMA Nutrition, Berkshire, UK) in 75 mL of Esbilac canine supplement (Pet-Ag Inc., Hampshire, IL) (Barlow *et al.*, 1974). Pups are exposed to hypoxia before each feed by placing them in a hypoxic chamber containing a gas mixture of 5% O₂ and 95% N₂ for 10 min, monitored with an O₂ gas detector (BW O₂ Gas Alert Clip Extreme, Rockall Confined & Safety, Cardiff, UK). LPS is administered on the 1st and 2nd day after NEC induction; mice are gavaged with 4 mg/kg/day LPS (lipopolysaccharide from Escherichia coli 0111:B4, Sigma-Aldrich Company Ltd., Dorset, UK) mixed within the formula feed. During the whole experiment, mice are kept in a neonatal incubator to maintain temperature (30 °C) and humidity (40%).

Materials and Reagents

Note: All reagents may be substituted with appropriate alternatives from other manufacturers.

Consumables

1. Sterile 1.0 mL syringe with 26 Gauge needle or smaller (VWR, catalog number: 309597)
2. Sterile gauze (VWR, catalog number: CA95041-740)
3. Soft absorbable pad (VWR, catalog number: 95057-862)
4. Rubber glove
5. Kimwipes (VWR, catalog number: 102097-615)
6. Microscope slides (VWR, catalog number: 48311-703, 1.0 mm thickness)
7. Microscope cover glass (VWR, catalog number: 48393-172, 0.13-0.17 mm thickness)
8. Falcon tube (15 mL) (VWR, catalog number: CA60819-761)
9. Adhesive tape (VWR, catalog number: 89097-912)

Animals

Neonatal *ROSA*^{mT/mG}, *Tie2-Cre* mice of both sexes were used to visualize the hemodynamics of small intestinal microcirculation and leukocyte movement. Table 1 shows a comprehensive list of transgenic mouse lines carrying fluorescent reporters that could be used in this protocol. Alternative transgenic reporter lines expressing fluorescent proteins can also be used in this method.

Table 1. Fluorescent positive mouse lines

Name	Strain	Type	Promoter	Specificity
GFP mouse	C57BL/6	Tg(CAG-EGFP)	Chicken β-Actin and cytomegalovirus enhancer	All tissues except for erythrocytes and hair appear green under excitation light.
mTmG mouse	C57BL/6	Rosa ^{mTmG}	Chicken Actin/pCA	These mice possess loxP sites on either side of a membrane-targeted tdTomato (mT) cassette and express strong red fluorescence in all tissues and cell types examined. When bred to Cre recombinase expressing mice, the resulting offspring have the mT cassette deleted in the Cre expressing tissue(s), allowing expression of the membrane-targeted EGFP (mG) cassette located just downstream.

Tie2 Cre	(C57BL/6 x SJL)F1	Tek-cre	Tek, endothelial- specific receptor tyrosine kinase	These transgenic mice express Cre recombinase under the control of a mouse Tek promoter and enhancer. This promoter is active in endothelial cells.
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!Caution: Please note that all animal experiments should be performed following ethical guidelines and regulations of both the animal and imaging facilities. Research should not begin until use live animals is approved by the facility's Animal Care Committee.

Reagents

1. Sterile Phosphate-buffered saline (PBS, 1×, pH 7.2)
2. Ultra-purified water
3. Disinfectant: 70% ethanol solution (70 mL of 100% ethanol added to 30 mL of water)

Equipment

Note: All Equipment may be substituted with appropriate alternatives from other manufacturers.

General Equipment

1. Appropriate microscope stage (Zeiss LSM710 motorized X, Y stage with Z focus) with heating pad (FHC Inc., catalog number: 40-90-2-07, Bowdoin, ME, USA)
2. Gas anesthesia vaporizer (IsoTec4; Datex-Ohmeda GE Healthcare, Waukesha, WI, USA)
3. Oxygen gas
4. Hair removal cream (Nair® hair remover cream for face)
5. Curved blunt forceps (VWR, catalog number 76319-850)
6. Fine forceps (VWR, catalog number 82027-408)
7. Scissors

!Caution: Only use the tip of sharp scissors when conducting fine neonatal mouse surgery. This will allow for more precise work with less tissue damage.

8. Solder lug terminal; 0.3 mm; M4 (Manufacturer OSTERRATH, manufacturer part number 60-2814-51/0030, Figure2A)
9. Holding devices for both the cover glass and solder lug terminal (TEKTON 7521 Helping Hand with Magnifier, Figure 2C)

Microscope

1. Inverted two-photon laser scanning microscope (TPLSM, e.g., Zeiss LSM710)
2. Laser: Ti:Sapphire Chameleon Vision (Coherent)
3. Objectives: 20× (Water immersion lens, e.g., Zeiss W Plan APOCHROMAT, 1.0 DIC (UV) VIS-IR∞/0.17)
4. Software application: ZEN (Zeiss, imaging Software)

Note: Magnifiers are not needed in this study and can be removed from the device.

Anesthesia

1. Portable anesthesia machine
2. Isoflurane vaporizer
3. Anesthesia breathing circuit
4. O₂ gas flow regulator for E-cylinders (Praxair)
5. O₂ tank (E-cylinder)
6. Anesthesia breathing circuit and nose cone

Procedure

Stage setup

1. Make sure the heating pad of the stage is plugged in and that the stage is at 37 °C before starting the animal procedure.

Microscope setup

2. Turn on the laser source, microscope and start the imaging applications, and all imaging components (data analysis workstation/computer, monitor, laser sources, camera, raster scanning unit, and detectors).

!CAUTION: Prepare all microscopic settings before subjecting the animals to anesthesia.

Anesthetic cone setup

3. Uncouple the normal sized anesthetic cone from the anesthetic breathing circuit and cover the end with a cut-out finger from a rubber glove. Cut a small hole at the tip of the rubber finger to fit the size of the neonatal mouse head (Figure 1A).

!CAUTION: The hole size of the rubber glove finger should fit tightly around the head of the neonate mouse head, otherwise the isoflurane gas might leak.

Mice and surgery for image preparation

4. A neonatal *ROSA^{mT/mG}; Tie2-Cre* mouse of either sex is used in this protocol to visualize the hemodynamics of the small intestinal microcirculation.
5. Place the mouse in the chamber for anesthesia with 2% isoflurane in oxygen. Confirm appropriate anesthesia by the absence of the withdrawal reflex after a toe pinch, as well as physiological responses including reduced respiratory and heartbeat rate. Once the mouse is anesthetized, reduce the isoflurane gas flow to 1-1.5%.
6. Put the mouse on the preheated heating pad on a diaper pad, and the mouse head into the end of the anesthetic breathing circuit, with the mouse's upper extremities fixed to the rubber finger with tape (Figure 1B).

!CAUTION: To ensure that the animal is unconscious during the procedure, isoflurane is delivered continuously via the end of the anesthetic breathing circuit with the rubber finger covering the nose.

7. Tape the lower extremities of the mouse to fix the body on the stage of the microscope and position it with a downward slope towards the tail (Figure 1C).
8. The level of the downward slope should be such that it makes a triangular space on the neonatal abdomen from a lateral view. The angle θ from 30 to 45 degree is the best range for this experiment (Figure 1D, see also Table 2).

▲CRITICAL STEP: Making this triangular space is critical to stabilize the image or movie. The top side of

this triangle should be aligned at the same height as the sternum, and the bottom of the angle should be positioned above the Anterior Superior Iliac Spine (Figure 1D).

Table 2. Troubleshooting table

Step	Problem	Possible reason	Solution
8	Can not make the triangle space	The level of downward slope is not enough	Put a small back pillow made by Kimwipe or a small folded cloth between the soft absorbable pad and mouse's back. See also Figure 1D.
12	Bleeding from the abdominal wall	Incision line may have transversed the inferior epigastric artery and vein	Make a muscle incision vertically at the middle of abdominal wall. You can distinguish the vertical abdominal midline from other muscle lesion because only the midline can see through to the inside of the abdominal wall clearly.
13	Bleeding from the intestine or mesenteric lesion	Too much force was used to pulled the intestine outside of the abdominal cavity	During picking up of the intestine and putting it back into the abdominal cavity, be careful not to pull the intestine lower than the superior anterior iliac spine level to avoid the intestinal and mesenteric injury induced by tensile force.
17	Having a difficulty making the gentle curve of the solder lug	Fine manual work could be difficult by hand	Use two fine pliers and gently make the curve.
23	The fixed intestinal color is getting dark after putting the cover glass on it	The pressure on the fixed intestine is too high	Remove the cover glass quickly and release the compressed intestine. Re-check the diameter of both the intestine and the space created by the curved solder lug, and regulate the shape of the solder lug to fit the intestinal diameter.
24	Can not keep the optimal intestinal observation position without intestinal physical injury.	Pulling up the intestine over the heart level or pulling down the intestine over the superior anterior iliac spine level.	Re-check the anesthetic mouse body condition. Make sure that there is a enough triangular square under the horizontal line of sternum. See also step 5 troubleshooting.
27	Noisy Background signal	Laser power is too high	Too high of a laser power can cause not only photobleaching or photo-damage to the observed intestine but also non-specific autofluorescence. Decreasing the laser power (regulation of both laser intensity and fluorescent gain) is necessary, and then keep the imaging lesion clear as long as possible when long observation time is needed.

Difficulty focusing the images	Focusing area is outside of the contact area	Control the x and y axes and find the appropriate contact region. Possible intestinal scan area is limited in the attached lesion with coverglass. See also step 20.
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9. Disinfect the whole abdominal skin area with 70% ethanol. Note that normally, mice have no hair in the neonatal period. If the mice have the hair on the operational skin area, you should use the hair remover gel to remove the hair softly with a small cotton swab.
10. Put a sterile gauze on top of the disinfected abdominal area. The sterile gauze should have a small hole in the middle large enough to allow making a small incision.
11. Make a vertical skin incision at the lower to middle abdominal area. This incision will expose the intestine and urinary bladder through the abdominal muscle layer.
12. Cut the abdominal muscle and peritoneal layer vertically in the middle (Figure 1E, see also Table 2).

!CAUTION: *The length of the abdominal muscle incision should be shorter than the length of the skin incision to prevent intestinal protrusion and allow the mouse's body to maintain its position throughout the imaging.*

13. Push gently on the lateral sides of the abdominal wall to allow a portion of the intestine to be completely externalized from the abdominal cavity. Using the cecum as a landmark, locate the segment of the small intestine that is of interest to your study, and place the rest of the intestinal length back into the abdominal cavity (Figure 1F, see also Table 2).

▲CRITICAL STEP: *It is not recommended to use forceps to locate the cecum because even with gentle maneuvers, picking with forceps could easily injure the neonatal intestine. Additionally, it is possible that with an intestinal disease model, the microcirculation of the target area is already compromised, making the intestine and blood vessels even more delicate. With confirmation of the location of the cecum, which allows to distinguish the small intestine from the colon, the intestine should be carefully handled from a region away from the target area. The rest of the intestine should be put back into the abdominal cavity using blunt forceps.*

!CAUTION: *Gentle maneuvers are necessary while moving the intestine around. Using blunt forceps, try to move the intestine by grasping tissue away from target region. Avoid unnecessary picking of the intestine with the forceps to reduce the number of contact points and to prevent puncturing/injuring the intestine. Avoid grasping the mesentery when moving the intestine.*

14. For the small intestine section that is selected for observation, use blunt forceps to gently pull out a small portion of the intestine that you will be observing. It is important to do this gently and allow the tissue to be under no tension to prevent potential ischemia or damage. Place one drop of PBS on the intestine to avoid drying.

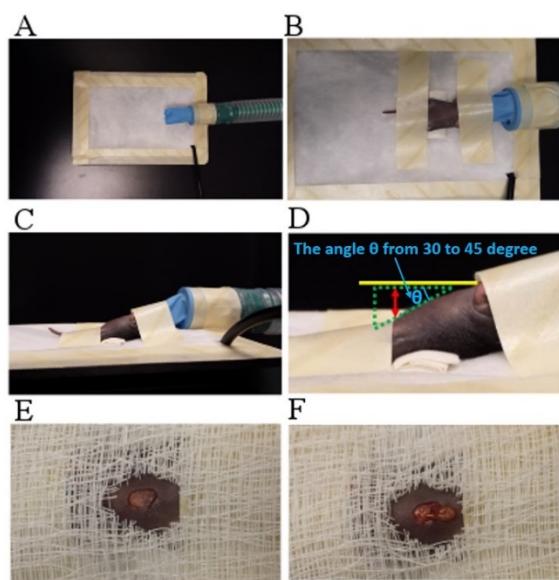


Figure 1. Neonatal mouse preparation for the TPLSM analysis.

(A-D) Mouse anesthesia and appropriate positioning. (E, F) Intestine exposure.

Preparation of custom fixing device

15. Bend the solder lug to a gentle curve, creating a space to sandwich the intestine between the ring of the solder lug and the cover glass (Figures 2A, 2B).
16. Attach the other end of the bent solder lug to the holding device with tape (Figure 2C).
17. Put the cover glass on the end of the solder lug that is attached to the holding device and clip it in place using the holding device. Ensure that there is space between the ring of the solder lug and the cover glass. The thickness of the space between the cover glass and the ring of the solder lug should be exactly the same as the maximum diameter of the intestine (Figure 2D, see also Table 2).

!CAUTION: Maintaining a fixed space between the solder lug and cover glass according to the actual diameter of the intestine is crucial to keep the intestine in place while allowing proper intestinal blood circulation.

18. Bend the ring of the solder lug to a complete horizontal plane parallel to the cover glass (Figure 2E, 2F).

▲CRITICAL STEP: Precise bending of the solder lug to sandwich the intestine between the ring of the solder lug and the cover glass is crucial to keep the small intestine in position during microscopic observation.

19. After creating the appropriate space between the bent solder lug and cover glass and orienting the solder lug ring parallel to the cover glass on a horizontal plane, remove the cover glass.

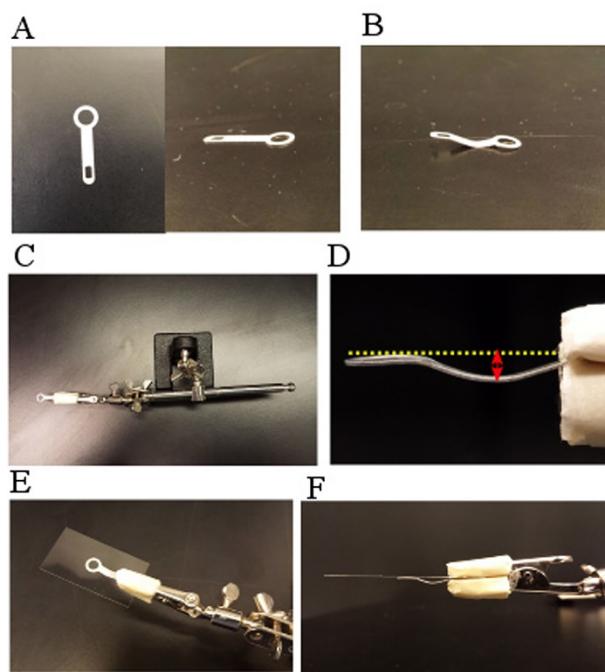


Figure 2. Fixing device setup for intestinal microscopic observation.

(A, B) Solder lug. (C, D). Solder lug attached to holding device with appropriate spacing, the spacing should be the same as the diameter of the intestine being used (generally between 1 and 3 mm). (E, F) Cover glass attached on top of the solder lug and clipped with the holding device.

Preparation of the microscopic stage

20. Transfer the customized fixing device to the microscopic stage where the mouse is mounted.
21. Put the 'U' shaped intestine on the ring of the solder lug. Wet the intestine with a drop of PBS and place the cover glass directly on top of the area, fixing the cover glass with the clip of the holding device. The bottom area of the 'U' shaped intestine should be slightly over the solder lug (Figure 3A).

▲CRITICAL STEP: If the bottom area of 'U' shaped intestine is not over the solder lug, the position of the intestine should be moved, as miss positioning may prevent stable intravital imaging.

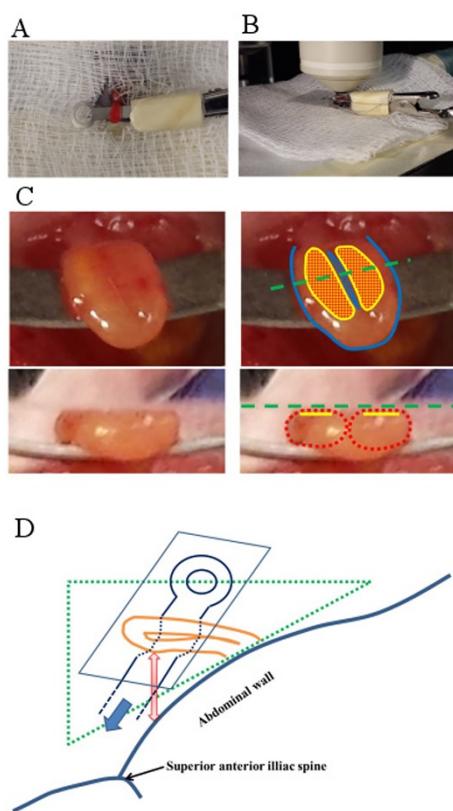


Figure 3. Neonatal mouse intestine exposed for TPLSM observation.

(A-C) Mouse intestine preparation and areas of observation. The yellow marking or alternatively in the red dotted area shows the contact lesion area between small intestinal wall and the cover glass. The green dashed line (under image) shows the cover glass level (the top picture in C: view from above the cover glass, the bottom picture in C: view from the frontal view. The observable area is between the green dotted line and the yellow line from the frontal view. The red dashed circle represents the outer intestinal wall line from the frontal view. (D) Illustration of mouse intestine for microscopic observation.

22. Put one drop of distilled water on the cover glass where the intestine is touching the cover glass. Bring down the objective lens immersion into the waterdrop dome and begin the microscopic observation (Figure 3B).
23. Figure 3C shows the ideal set up of the customized holding device, the microscope, and the mouse. After putting the cover glass on top of the intestine, only a small portion of the intestine will be in contact with the cover glass (shown in Figure 3C inside the yellow marking or alternatively in the red dotted area). This area will be the best location to observe the whole intestinal wall from the serosa level to the villi. When viewing the set up from the side, the segment of intestine sandwiched between the solder lug and the cover glass should look slightly oval shaped (Figure 3C red dotted circles). This positioning of the intestine is essential to prevent ischemia (see also Table 2).

▲ CRITICAL STEP: Make sure that the intestine is not damaged after fixation on the device and putting on the coverglass. Damaged tissue can be identified by increased redness. If the color of the intestine turns dark soon after fixation, remove the cover glass immediately and release the compression to restore intestinal blood flow circulation.

!CAUTION: Placing of the cover glass and securing it with the clip must be done very carefully. Applying too much pressure with the clip can break the cover glass.

24. Once the intestine is fixed, ensure that any parts of the device are not in direct contact with the mouse body (Figure 3D). Additionally, confirm that the fixed intestine that is being observed lies between the sternum and

the Anterior Superior Iliac Spine in position (see also Table 2).

!CAUTION: Do not pull the intestine above the level of the heart (towards the head) to avoid both the potential positional effects on blood pressure and the potential effects on blood flow from stretching the tissue. As well, do not pull the intestine below the level of the Anterior Superior Iliac Spine, this would impose too much tension and cause ischemia.

Intravital imaging

25. Intravital observation can now be performed using a Zeiss LPM710 inverted microscope (Zeiss) with $\times 20$ water immersion objective lens (W Plan-Apochromat 20 \times /1.0 DIC, VIS-IR M27 75mm). TPLSM images are acquired at 512 \times 512 pixels of spatial resolution from a 386.45 μm field of view. The excitation wavelength to detect GFP is 910 nm. The scan speed should be set at 1.27 $\mu\text{s}/\text{pixel}$. Two-photon fluorescence signals are collected by an internal detector (non-descanned detection method) at an excitation wavelength.

!CAUTION: The laser power is adjusted according to the imaging depth and intestinal diameter. When imaging at greater depths, the laser power level should be increased (up to 100%) manually using the laser power level controller. The laser power should be adjusted slowly from a low to high level to avoid photo-bleaching of the target area, which may occur at higher laser power. If the observation area is photo-bleached the stage must be moved to focus on another area.

26. Each area of interest is scanned at a high magnification (water-immersion objective 20 \times or higher if desired) by manually setting the X/Y plane and adjusting the Z axis to obtain high-resolution, clear TPLSM images.
27. In our experimental setting, the imaging depth ranged from 100 μm to 400 μm . Optimal high-resolution images were obtained from the tissue surface up to 200 μm in depth. For optimal simultaneous imaging of EGFP and tdTomato, detection gain should be adjusted for EGFP (to 500-570) or tdTomato (to 580-650)

!CAUTION: The range of gain value should be changed according to each intestinal region. A higher gain produces a stronger signal; however, it also produces more Background.

▲CRITICAL STEP: The combination of laser power and gain value determines the intensity of the laser signal. The settings of these two values depend on the quality of image focus and on the length of time of continuous imaging (see also Table 2).

28. Start the observation and recording by TPLSM and save the acquired data. Saving the data as a czi file is recommended because czi files can be used to analyze the blood flow dynamics or make Z-stack movies/3-dimensional images using ZEN 2 lite Software after imaging.

!CAUTION: For the analysis of blood flow dynamics, including blood flow velocity, blood flow volume, and leukocyte rolling speed, we recommend capturing 80-100 frames within 30 s.

29. After microscopic intravital observations, euthanize the mouse under general anesthesia according to approved protocols.

Data analysis

Processing of image data

1. Launch the ZEN 2.0 lite Software (freely available from the ZEISS company website after registration).
2. Open the saved czi files and place a properly sized scale bar. The czi file contains information about each imaging setting, as well as scanned area size and time. To create image data from sequential time course images,

choose one image and select the ‘export/import’ button from the file tag. Then, select ‘export’ and choose the appropriate file type (e.g., JPEG, TIFF, PNG *etc.*). To create a movie from the data, select the ‘movie export’ function and choose the appropriate file type (e.g., AVI, WMF, MOV *etc.*). The czi files can be used for the analysis.

Blood flow velocity

The blood flow velocity (V) is calculated as described in several studies that quantified the blood flow in vessels of different caliber, from arteries to capillaries (Tang *et al.*, 2015). In one movie (20-30 s), select a sequence of the four to six most clear images of a blood vessel. Measure the tangent length that is parallel to the blood flow direction (ΔX) and the cosine length of the strand that is vertical to the blood flow direction (ΔT). The blood flow velocity is calculated using the following formula (Figures 4A, 4B and Figure 5):

$$V [\mu\text{m}/\text{ms}] = \Delta X / \Delta T$$

Velocity can be defined as a mean value calculated from four or six strands selected from the movie.

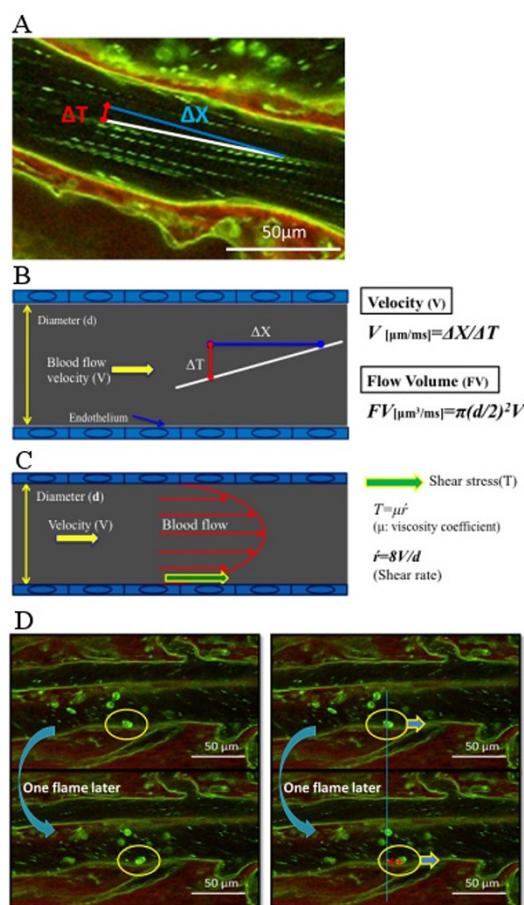


Figure 4. Dynamic intestinal microcirculation in neonatal mice.

A. Intestinal microcirculation image obtained by TPLSM. B. Blood flow volume calculation. C. Blood vessel wall shear rate calculation. D. Leukocyte rolling speed calculation.

Blood flow volume

Blood flow volume (FV) is calculated using the following formula (Figure 4B and Figure 5):

$$FV [\mu\text{m}^3/\text{ms}] = \pi(d/2)^2 \Delta X / \Delta T$$

[d] is the maximum diameter of the blood vessel

Shear rate of blood vessel wall

Blood vessel wall shear rate (W) is calculated in a 100-μm segment of the vessel using the formula based on the Newtonian definition (Russell *et al.*, 2003) (Figure 4C and Figure 5):

$$W [1/\text{ms}] = 8V/d$$

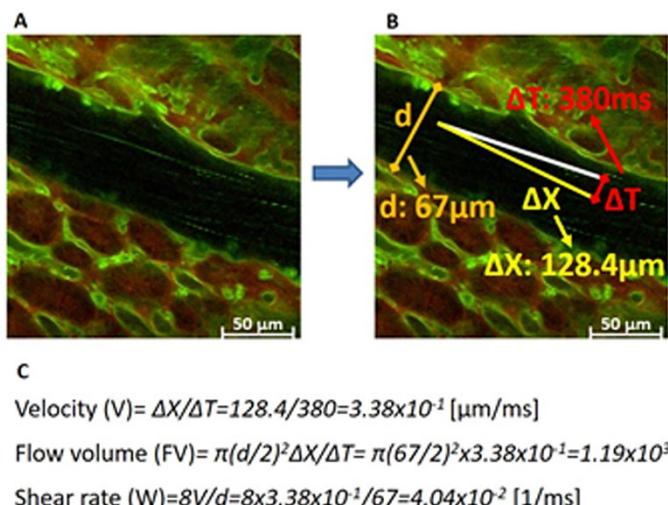


Figure 5. Example of experimental result and data analysis.

A. One still/flame image cutting from the recorded video (one flame scan speed: 380 ms). From this find a straight line of the platelet. B. Measure the tangent length of the straight line that is parallel to the blood flow direction (ΔX), scan time (ΔT), and blood vessel maximum diameter (d). C. Calculate the velocity, flow volume, and shear rate using the specific formula.

Number of adherent leukocytes

Adherent leukocytes can be defined in each vessel segment as cells that do not move or detach from the endothelial lining within a specified observation period of 5-10 s. Quantification is done by counting the number of adherent leukocytes sticking to the endothelial surface within a 100-μm length of a single vessel (Kubes *et al.*, 2003; Nakagawa *et al.*, 2006).

Leukocytes rolling speed

Leukocytes' rolling phenomenon is observed mainly in the post capillary venule. The post capillary venule is a V1 or V2 level vein that is located in the submucosal level, made from the combined branches of the capillary vessels (Yu *et al.*, 2009). The maximum diameter of this venule in the neonatal mouse intestine is approximately 20-60 μm. Choose a frame in which there is a consistent maximum diameter of post-capillary venules and select a single rolling leukocyte to follow as it rolls on the inner venule wall. Measure the distance travelled by the leukocyte and record

the length of travel time, as determined by the time from the first frame observed to the last frame observed. Leukocyte rolling speed [$\mu\text{m}/\text{ms}$] is calculated in $[\mu\text{m}/\text{ms}]$ (Figure 4D):

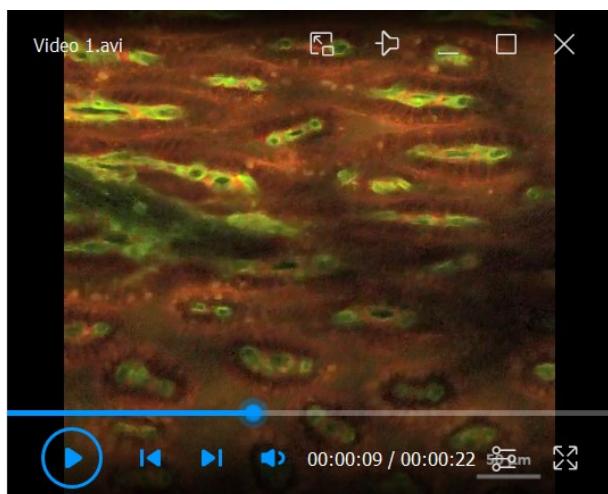
$$\text{Leukocyte rolling speed} = [\mu\text{m}/\text{ms}]$$

Where: leukocyte traveled distance length [μm]/ the scan time span between the two selected time frames [ms].

Anticipated results

By using this method of TPLSM imaging of the neonatal intestine, we are able to observe and analyze the microcirculation and blood flow dynamics of the neonatal small intestine in detail (Video 1). Video 1 starts at tips of the villi and travels downwards towards the based of the villi and then through the underlying vascular supply and smooth musculature at the end. Moreover, acquisition of z-stack and x-y data allows creating three-dimensional images of the entire structure of the small intestine, from the top of the villi to the bottom, including the submucosal area. Administration of additional fluorescent dyes such as SYTOX green or red prior to imaging enables the localization of affected cells in a disease model (Video 2). Using this approach, we revealed that the top of the villi is the main area affected by necrosis in the experimental NEC mouse model (Video 2). This approach is also useful for long-term analysis of the effects of drugs and other procedures on the neonatal small intestine. Furthermore, this approach allows the user to visualize changes in intestinal microcirculation in a target area.

In summary, the combination of the organ stabilizing method for TPLSM described in this protocol yields reliable results for studying neonatal intestine development and intestinal pathophysiology.



Video 1. Microcirculation of the neonatal small intestine



Video 2. 3-D image of the whole villi in a control neonatal mouse and the necrotizing enterocolitis mouse model

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Competing interests

The authors have no conflict of interest to declare.

Ethics

All procedures described in this protocol were approved by the Institutional Animal Care Committee at the Toronto Medical Discovery Tower (No.4886.0), and by the Advanced Optical Microscopy Facility.

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A Workflow for High-pressure Freezing and Freeze Substitution of the *Caenorhabditis elegans* Embryo for Ultrastructural Analysis by Conventional and Volume Electron Microscopy

Mohammad M. Rahman^{1, #, *}, Irene Y. Chang^{2, 3, #}, Orna Cohen-Fix¹ and Kedar Narayan^{2, 3, *}

¹The Laboratory of Biochemistry and Genetics, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD, USA

²Cancer Research Technology Program, Frederick National Laboratory for Cancer Research, Frederick, MD, USA

³Center for Molecular Microscopy, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Frederick, MD, USA

#Contributed equally to this work

*For correspondence: mohammad.rahman@nih.gov; kedar.narayan@nih.gov

Abstract

The free-living nematode *Caenorhabditis elegans* is a popular model system for studying developmental biology. Here we describe a detailed protocol to high-pressure freeze the *C. elegans* embryo (either *ex vivo* after dissection, or within the intact worm) followed by quick freeze substitution. Processed samples are suitable for ultrastructural analysis by conventional electron microscopy (EM) or newer volume EM (vEM) approaches such as Focused Ion Beam Scanning Electron Microscopy (FIB-SEM). The ultrastructure of cellular features such as the nuclear envelope, chromosomes, endoplasmic reticulum and mitochondria are well preserved after these experimental procedures and yield accurate 3D models for visualization and analysis (Chang *et al.*, 2020). This protocol was used in the 3D reconstruction of membranes and chromosomes after pronuclear meeting in the *C. elegans* zygote (Rahman *et al.*, 2020).

Keywords: High-pressure freezing, Freeze substitution, *C. elegans*, FIB-SEM, Volume electron microscopy, vEM

This protocol was validated in: J Cell Biol (2021), DOI: 10.1083/jcb.201909137

Background

C. elegans is a free-living nematode with many properties that make it amenable to scientific study: (1) the worms are ~1 mm long; (2) they are easy to grow, handle and maintain; (3) they proliferate rapidly and (4) they are amenable to genetic manipulations. The reader is encouraged to consult Corsi *et al.* (2015) for an excellent primer to *C. elegans* and its use as a model organism in biology. Embryonic cell division events in *C. elegans* are largely invariant both spatially and temporally, making the organism a robust eukaryotic model system (Oegema and Hyman, 2006). Transient changes in dynamic cellular components like the nuclear envelope and chromosomes during embryonic development can be well described at resolutions afforded by fluorescence microscopy (Cohen-Fix and Askjaer, 2017), but capturing the corresponding ultrastructural changes at higher resolutions, whether in two or three dimensions, is challenging (Altun *et al.*, 2002). The chitinous shell of the embryos poses a diffusion barrier to chemicals, precluding conventional aldehyde-based fixation protocols; thus, rapid freezing of samples followed by freeze-substitution and imaging by EM is the preferred way to capture ultrastructural intermediates at nanometer resolutions. As these samples are too large for simple plunge freezing, they have to be high-pressure frozen, ideally in a manner conducive to screening for the correct developmental stage (Muller-Reichert *et al.*, 2003; McDonald *et al.*, 2010). Recently, we published a report describing architectural intermediates in nuclear envelope breakdown during embryonic development (Rahman *et al.*, 2020). We visually followed *C. elegans* embryos trapped in capillaries until just before high-pressure freezing to ensure that the correct stages were frozen. In an accompanying methods paper, we also reported cryo-fluorescence microscopy of high-pressure frozen whole *C. elegans* worms followed by correlative FIB-SEM (Chang *et al.*, 2020) to image such structures in the intact worm. In both these advances, central experimental steps included the appropriate high-pressure freezing, freeze-substitution and resin embedding of trapped worms and/or embryos. Here we provide a step-by-step protocol to correctly execute these procedures for downstream vEM analysis, either to replicate our findings, or to answer other questions of interest in *C. elegans*.

Materials and Reagents

A. *C. elegans* maintenance

1. Tissue culture dish, 35 × 10 mm (Corning, Falcon, catalog number: 353001)
2. Worm pick (Genesee Scientific, catalog number: 59-30P16)
3. Micropipette tips with barrier (any brand, 1 µL, 20 µL and 1,000 µL)
4. Serological pipets, 10 mL for OP50 seeding (Corning, Falcon, catalog number: 357551)
5. *C. elegans* Bristol N2 [Caenorhabditis Genetics Center (CGC), <https://cgc.umn.edu> (Stiernagle, 2006)]
6. *E. coli* OP50 strain DA735 [Caenorhabditis Genetics Center (CGC), <https://cgc.umn.edu>]
7. Water, molecular biology grade (GE Healthcare, HyClone, catalog number: SH30538.02)
8. Agar (RPI, catalog number: A20020-5000)
9. Yeast extract (ThermoFisher Scientific, catalog number: BP9727-2)
10. Bacto Peptone (BD, Bacto, catalog number: 211677)
11. Sodium chloride (Avantor Performance Materials, J.T. Baker, catalog number: 3624-05)
12. Cholesterol (Sigma, catalog number: 1580-01)
13. Ethanol 200 proof (Decon Labs, catalog number: 2716)
14. Trizma-Cl (Roche, catalog number: 10812846001)
15. Trizma-OH (Roche, catalog number: 10708976001)
16. Luria-Bertani broth, bacterial culture medium (KD medical, catalog number: BLC-5020)
17. M9 buffer (IPM Scientific USA, catalog number: 11006-517)
18. Levamisole hydrochloride (Sigma, catalog number: 196142)

B. High-pressure freezing and freeze substitution

1. Glass microscopy slides (ThermoFisher Scientific, catalog number: 10144633B)
2. Tubes, 1.5 mL for BSA solution aliquots (ThermoFisher Scientific, catalog number: 05-408-129)
3. Micropipette tips with barrier (any brand, 1 µL, 20 µL and 1,000 µL)
4. Micropipette tips without barrier (Eppendorf, 1 µL, catalog number: 30072.014)
5. Cellulose capillary tube (Leica, catalog number: 16706869)
6. Needles, 21-gauge (G) × 1½ inch (Covidien Monoject, catalog number: 305167)
7. Syringe, disposable (VWR, EMS, catalog number: 72508 for 2.5 mL or 72509 for 5 mL)
8. Alcohol swabs (BD medical, catalog number: 326895)
9. Type A gold-coated copper planchette (Leica, catalog number: 16770152)
10. Type B gold-coated copper planchette (Leica, catalog number: 16770153)
11. Cryomarker, Black (ThermoFisher Scientific, Nalgene, catalog number: 22-026-700)
12. Sample holder Cartridge system, D3 mm half cylinder (Leica, catalog number: 771849)
13. Sample holder Cartridge system, D3 mm middle plate (Leica, catalog number: 771813)
14. Nalgene cryovials (ThermoFisher Scientific, catalog number: 5000-1012)
15. Polypropylene tubes, 50 mL (BD Falcon, catalog number: 352098)
16. String or twine (VWR, Twine, catalog number: 30-33113)
17. Disposable cellulose acetate filter, 0.22 µm mesh size, diameter 25 mm (Millipore, Cameo syringe filter, catalog number: 1213657)
18. Erlenmeyer glass flasks, 250 mL (VWR, Pyrex, catalog number: 4444-250)
19. Pasteur pipettes, Borosilicate glass, 5 mL (ThermoFisher Scientific, catalog number: 13-678-20A)
20. Pasteur pipettes, plastic, 2 mL and 7 mL (Globe Scientific, catalog numbers: 137040 and 134090)
21. Serological pipette, Borosilicate glass, 1 mL (VWR, catalog number: 93000-682)
22. Plastic cups (for weighing resin) (VWR, Therapak, catalog number: 74850)
23. Beem capsules (Ted Pella, catalog number: 69910-01)
24. Beem capsule holder (Ted Pella, catalog number: 132-B)
25. Double Edge Carbon steel blade (Ted Pella, Feather, catalog number: 121-9)
26. Kimwipes 05517 (ThermoFisher Scientific, Kimberly-Clark Kimtech Science Precision wipes, catalog number: 06-677-72)
27. Styrofoam container or tray (e.g., a shipping container lid, typically 15 × 20 cm and 2 cm deep)
28. Disposable polypropylene spatula for osmium handling (VWR, catalog number: 80081-194)
29. Bovine Serum Albumin (BSA), heat shock fraction (Sigma, catalog number: A3294)
30. Nail polish (any color)
31. Osmium tetroxide (OsO₄) granules (EMS, catalog number: 19134)
32. Acetone (ThermoFisher Scientific, catalog number: 9011)
33. Uranyl acetate (EMS, catalog number: 22400)
34. Methanol (Mallinckrodt, catalog number: 3016)
35. Poly/Bed 812 embedding kit with DMP-30 (Polysciences, catalog numbers: 08792 and 08791)
36. Dry ice (in-house supply)
37. Double deionized water (in-house supply)
38. Dry liquid nitrogen (LN₂) (in-house supply)
39. Dodecetyl succinic anhydride (DDSA) (Polysciences, catalog number: 08792, kit same as 35)
40. Nadic Methyl anhydride (NMA) (Polysciences, catalog number: 08792, kit same as 35)
41. Modified Youngren's, Only Bacto-peptone (MYOB) plates for worm maintenance (see Recipes)
42. Cholesterol stock solution (see Recipes)
43. *E. coli* OP50 stock (see Recipes)
44. Cellulose capillary attachment (see Recipes)
45. 20% BSA solution (see Recipes)
46. 25 mM Levamisole solution (see Recipes)
47. Quick Freeze-substitution (QFS) cocktail (see Recipes)
48. Poly/Bed 812 resin mix (see Recipes)

Equipment

1. Micropipettes (any brand, for 1 µL, 20 µL and 1,000 µL volumes)
2. Sharp-point tweezers (EMS, Dumont, catalog numbers: 78320-51T, 72919-0A, and 78340-51S) (Figure 1a-1c)
3. Tweezers insulated with PVC (EMS, Dumont, catalog number: 3C-inox-E) (Figure 1d)
4. Forceps insulated with PVC, long (Leica, VOMM, catalog number: 22SAESD) (Figure 1e)
5. Crimping tool, scalpel #20 (Bard-Parker, catalog number: 371620) (Figure 1f)



Figure 1. Tools for sample handling.

a-c. Sharp-point tweezers for sample handling (Section A Steps 4-5). d-e. Insulated tweezers for frozen sample recovery and transfer (Section B Steps 4-9, and Section C Steps 6-7). f. Crimping tool: #20 scalpel manually blunted by rubbing the blade gently on a metal surface (McDonald *et al.*, 2010).

6. Table-top centrifuge (Eppendorf, model: 5424 or similar)
7. Bottom illuminated stereomicroscope with frosted glass stage (Leica, model: SM2745 or similar)
8. Leica high-pressure freezer, ultra-low temperature equipped with stereomicroscope and funnel to fill LN₂ (Leica, model: ICE)
9. Frozen sample recovery cryobox (stainless steel tray, deep) with frozen sample release station and 3 mm punch, rod, and plug (Leica, Austria; EM ICE high-pressure freezer package)
10. Mini benchtop orbital shaker (VWR, catalog number: 97109-890)
11. Large surface slide warmer (Premiere, catalog number: XH-2002)
12. Infrared thermometer (General Tools & Instruments, catalog number: IRT207)
13. Analytical balance (Sartorius Corp, model: BCE64-15)
14. Stirring plate (Corning, catalog number: PC420D or similar)
15. Standard desiccator connected to a mechanical pump (Ted Pella, model: VRD4)
16. Metal blocks with 12 mm holes (ThermoFisher Scientific, catalog number: 88880152)
17. Dry ice buckets, multiple (VWR, Scienceware Magic Touch 2 with lid, catalog number: M16807-2001)
18. Bunsen burner
19. Chemical safety hood
20. Laboratory timer
21. Chemical scale (Mettler, model: AE240)

22. Temperature-controlled oven (Quincy Lab, model: 20GC)
23. Standard autoclave
24. Hairdryer (any brand with hot air fan)
25. Sample storage, large LN₂ dewar (Taylor-Wharton, catalog number: HC34)
26. Portable LN₂ dewar on a rolling base, 25 L (Worthington, catalog number: LD25)
27. Portable LN₂ dewar, 4 L (Worthington, catalog number: LD4)
28. PPE (lab coat, face shield and cold-resistant gloves) for handling LN₂ (any brand)
29. Facemask, surgical (Halyard health, catalog number: 28806; or any brand)

Procedure

A. High-pressure freezing (HPF) of *C. elegans* individual embryos

1. Start a worm culture by placing 20-30 starved L1 larva on a new MYOB agar plate seeded with *E. coli* OP50 bacteria (see Recipes). Maintain worms by transferring 2-3 adults to a new MYOB plate every fourth morning (Stiernagle, 2006).
2. 72 h prior to the HPF experiment, transfer 5-6 gravid adults to each of several new MYOB plates (at least 3 separate plates to ensure enough young adults).
3. In the morning of the HPF experiment, take out two aliquots (500 µL each) of 20% w/v BSA solution from 4 °C and spin at 94 × g (~1,000 rpm) for 5 min in a table-top centrifuge to remove bubbles. Keep at room temperature.
4. Place 3-4 pairs of clean sharp-point tweezers (Figure 1a and 1b) next to the dissecting scope, and 1-2 pairs (Figure 1c) next to Leica ICE high-pressure freezer. Tweezers 1a and 1b are suitable for cellulose tube transfer while tweezer 1c is suitable for planchette transfer. It is critical to have alcohol swabs next to the tweezers (Figure 2a): unless you clean them frequently, the tweezers become sticky with 20% BSA solution, resulting in sample loss during transfer.
5. Place one micropipette (1 µL) set at 0.8 µL and another micropipette (20 µL) set at 12 µL next to alcohol swab, tweezers, crimping tool (white arrow), and worm pick (yellow arrow) as in Figure 2a. Set up worm dissecting tool (syringe with 21G needles, Figure 2b). Place multiple cellulose tubes attached to pipette tips (Figure 2c, purple arrows and inset). Keep one micropipette (1 µL) dedicated for capillary tube handling (Figure 2c).
6. Take out 9-10 planchettes (type A and type B separately) in 35 mm Petri dishes (Figure 2c), and mark type A cavities with a black marker to distinguish it from type B later.

Note: Type A planchettes have a single 300 µm deep cavity on one face while the other face is flat; the samples will be placed later in the shallow cavity of a type B planchette (Step A14).

7. Check liquid nitrogen (LN₂) transport dewars to make sure they are empty and dry. Fill one large dewar (25 L) and two medium dewars (4 L) with LN₂.
8. Turn on the Leica ICE high-pressure freezer (Figure 3a) and wait to hear the compressor turn on (identified by a hum and mild vibration), which should also be reflected in the monitor status screen. The loading station (Figure 3b) should be clean and dry. Fill the freezer chamber (Figure 3c) with LN₂ from the 25 L dewar slowly with multiple brief pauses to avoid triggering the alarm and a false indication that the tank is full. A significant amount of LN₂ evaporates in the process of chilling the tank.

Note: You may need help from a second person to pour ~18 liters of LN₂ safely.

9. Assemble HPF sample storage dewar (Figures 3d-3f). Note the trisection pod in the dewar can hold up to three cartridge systems in each of three cups, equating to a maximum of nine HPF “shots” before the sample storage dewar is full. The high-pressure freezer requires ~20 min to equilibrate to LN₂ temperature;

once equilibrated, fill the HPF sample storage dewar (Figure 3f) with LN₂ and insert into Leica ICE freezer chamber (Figure 3g, underneath the loading station in Figure 3a). You must wait until the freezer is ready, otherwise the container will collect and freeze condensation from the air forming ice crystals. Crystalline ice or frost must be avoided in this procedure, as with other cryogenic experiments. So, maintain dry conditions, and work at a quick pace to minimize exposure of the sample and tools to humidity.

10. Assemble plastic adapters on the two halves of the Leica ICE high-pressure freezer loading station (Figures 3h-3j, and arrows in Figure 3b). Place the planchette holder with a hole in it (Figure 3i) on top of a plastic adapter placed on the bottom steel surface (Figure 3b, yellow arrow). Run a blank HPF cycle by manually closing the red lid (Figure 3b) to ensure the freezer is working properly (Figure 5).

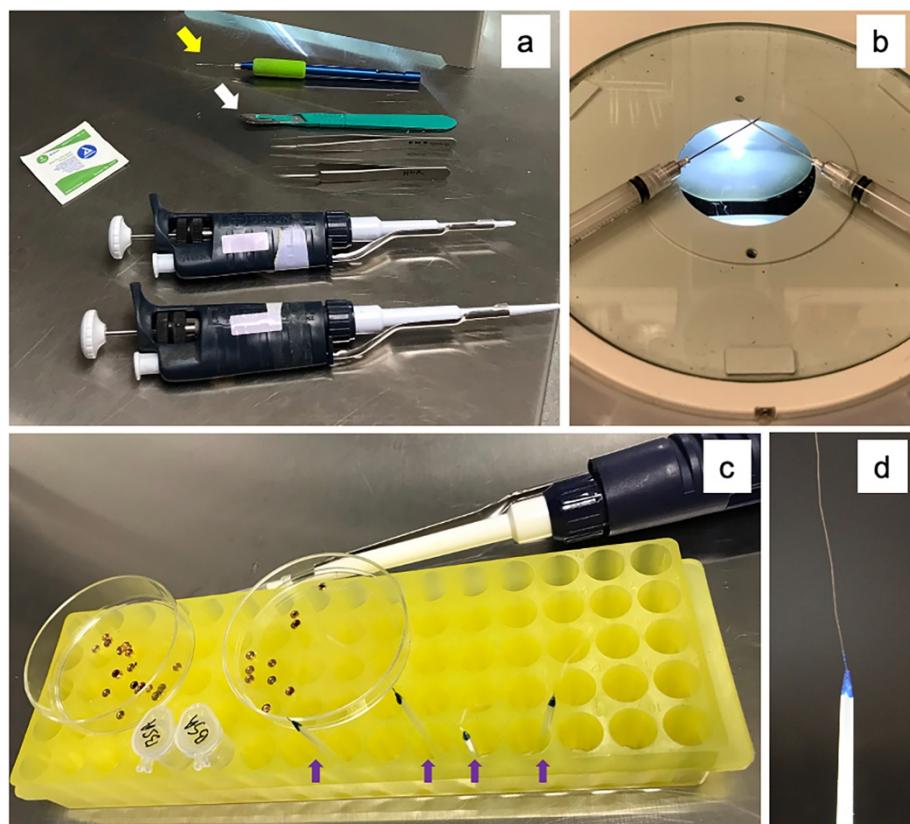


Figure 2. Setup for worm dissection prior to HPF.

- Micropipettes (1 μ L and 20 μ L), sharp-point tweezers, alcohol swab, crimping tool (white arrow) and worm pick (yellow arrow).
- b. Worm dissecting tool – a pair of 21G needles on 2.5 mL syringe to be used as scissors to cut worms.
- c. A dedicated micropipette (1 μ L) for handling cellulose capillaries attached to pipette tips (purple arrows and d).
- d. Planchettes type A and type B are placed in separate plastic Petri dishes.
- A cellulose capillary attachment (see Recipe 4) for embryo and/or worm collection.

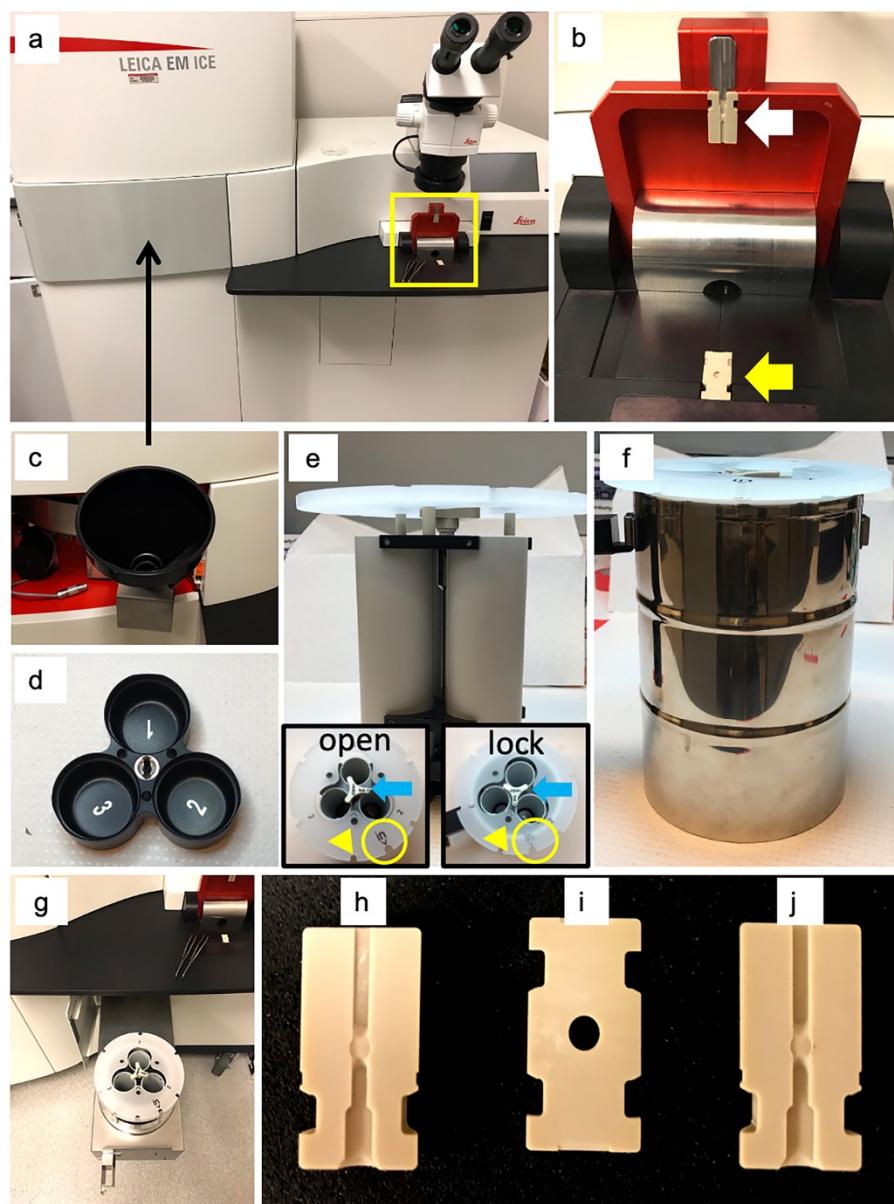


Figure 3. Setting up the Leica EM ICE for HPF.

a. Leica EM ICE high-pressure freezer. b. Loading station, enlarged from boxed area in 3a. Arrows show where planchette holders are placed for each HPF run, with a half cylinder on top (white arrow and 3h), and planchette holder with a hole in it (3i) atop the other half cylinder placed on the bottom black steel half (yellow arrow and 3j). c. LN₂ filling port behind grey door (long black arrow). d-f. Assembly of HPF sample storage dewar comprising the trisection pod (3d) and segmenting insert (grey cylinders in 3e). Insets in 3e show top view after assembly; blue arrow, “bayonet” or release button and yellow arrowhead, lock/open position. The bayonet is pressed and rotated counter-clockwise or clockwise to open or lock respectively (left inset – bayonet at open position, and right inset – bayonet at close position). g. Collection holder unit inserted into receptacle before automatic withdrawal into the freezer unit, seen as a black outlined panel below the loading station in 3a. The notch with the “in >” label (3e inset, yellow ring) should face the HPF instrument holding unit. h-j. Leica cartridge system contains two identical half cylinders and a planchette holder with a hole in it.

11. Collect 3-4 young adult worms with a worm pick and place in a drop (12 μ L) of 20% BSA solution on a clean glass slide. Use two needles as shears (Figure 4a) to cut the worms open (at the middle of the body) to release the embryos (McDonald, 1999; Muller-Reichert *et al.*, 2007; McDonald *et al.*, 2010).

Note: If whole worms are collected, use 20% BSA solution with 25 mM levamisole and incubate 2-3 min to anesthetize the worms. The worms can be taken up into capillaries individually, as below.

12. Visually scan the embryos to find one at the desired stage of embryonic development, keeping in mind that the steps leading to HPF will take approximately 2 min. Avoid prolonged observation under the dissecting scope as the small volume of 20% BSA solution will quickly dry out due to evaporation.
13. To collect an embryo of desired stage, place the open end of a cellulose capillary tube, attached to pipette tip (Figure 4b, cartoon) close to the embryo. Due to capillary action, the embryo will get into the capillary tube and there is no need to pipette it in (Figure 4c, cartoon).
14. Use a crimping tool to press gently on the capillary tube (otherwise it will cut open and embryo will float away) on both sides of the trapped embryo to seal the capillary tube (refer to Figure 4c, cartoon). Use the same tool to separate the section (Figure 4c, inset) with trapped embryo away from dissected worms. Remember to keep the length of this section around or below 2 mm (Figure 4c, inset) so that it will fit in the cavity of the type B planchette (Muller-Reichert *et al.*, 2008). Follow embryonic development of the trapped embryo under a dissecting scope.
15. Approximately 1 min prior to the desired embryonic developmental stage, add 0.8 μ L of 20% BSA solution into the 100 μ m cavity of the type B planchette (Figure 4d). The planchette must be filled to the top to avoid air bubbles, but not overfilled to avoid excessive liquid wicking into the loading area. A gentle positive meniscus typically suffices. Transfer the capillary tube section with the embryo (Figure 4c, inset) with sharp-point tweezers into the type B planchette filled with 20% BSA solution (for ease of visualization, capillary tube sections with intact worms are shown in Figure 4d). Place a type A planchette (flat side) on top of the type B planchette (Figure 4g).
16. Transfer the planchette sandwich with the embryo secured inside (Figures 4e-g) to the Leica EM ICE loading station (Figure 3d, pre-assembled cartridge system; also see Figures 4h-i). Manually closing the red flap (Figure 4j) will initiate the freezing process, where the embryo secured inside the cartridge system is cooled to LN₂ temperatures within tens of milliseconds and under high pressures of approximately 2,000 bar (refer to Figure 5).

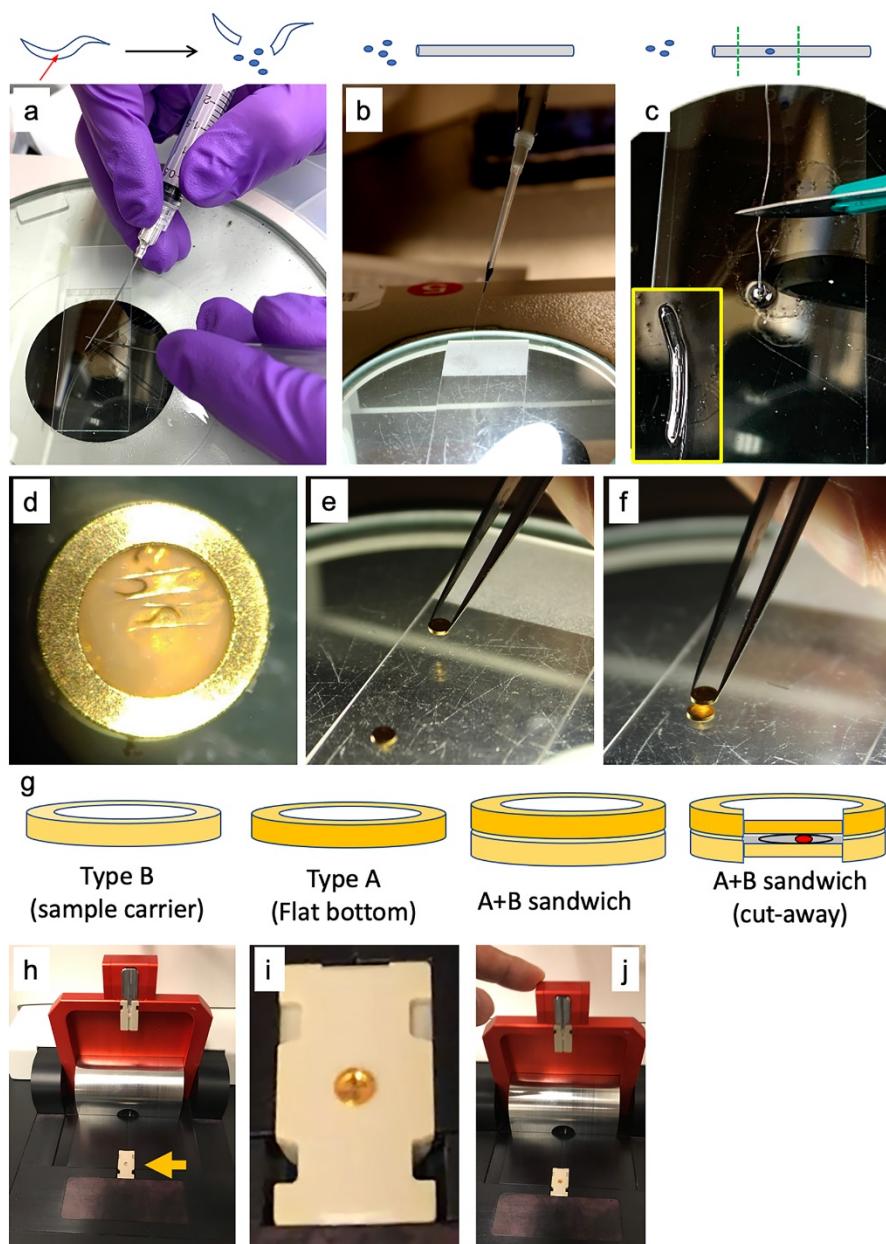


Figure 4. Sample preparation for HPE.

a-c. Dissection of young adult worms in a drop of 20% BSA solution. Steps are depicted in a cartoon above each image. An embryo of choice is collected into a cellulose capillary and trapped with the crimping tool (c, inset). d. Planchette with whole worms inside cellulose capillaries in 20% BSA solution filled to the top. e-g. A sharp-point tweezers which is used to transfer the capillary piece (c, inset) to the type B planchette cavity, is used to place the type A planchette (flat side) on top of type B planchette. h-j. The planchette sandwich (g, right) is placed securely in the holder (h, orange arrow). Manually closing the red lid (j) on the loading station will plunge the sample into LN₂ under high pressure.

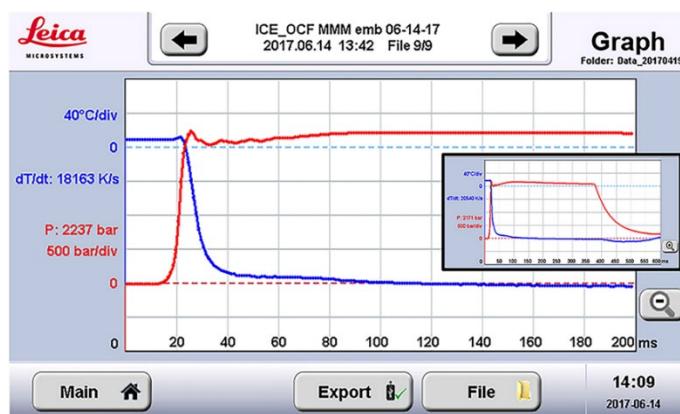


Figure 5. Temperature and pressure plot of a high-pressure freezing cycle.

The x axis is time in milliseconds (ms), and the y axis is either pressure in bar, red, or temperature in Kelvin (K), blue. For only illustration purpose, here is a screenshot of an acceptable execution of a single run from the high-pressure freezer, with pressure reading of 2237 bar at time sample cooling started at a rate (dT/dt) of 18163 K/s. Using the zoom out button (right bottom corner) pressure, the temperature status can be obtained for up to 600 ms (see inset). For details refer to Muller-Reichert *et al.* (2007 and 2008).

17. Repeat the Steps A10 to A15 until you have frozen the necessary number of samples. The maximum capacity of a single sample storage dewar in the Leica ICE high-pressure freezer is nine samples, however a blank HPF run is typically executed at the start of the experiment, leaving space for up to eight samples. Note that it is possible to switch the sample collection to a new and dry holder (Figure 3d) to collect another set of up to nine samples. Other high-pressure freezers do not have a limit of nine samples.
18. High-pressure frozen samples can be stored under LN₂ for years. For long-term storage of samples go to protocol B. For freeze substitution, go to protocol C directly, skipping protocol B.

B. Recovery of frozen samples from HPF machine

1. To store frozen samples, prepare a 50 mL polypropylene tube by perforating its side wall with a hot end of a sharp mini screwdriver (place the tip on a Bunsen burner briefly to heat). The hole prevents pressure build up and a potential explosion hazard in case of accidental warming. Attach a long (~3 feet) string securely in the cap (Figure 6) and a tape label so the tube holding frozen samples can be pulled out of a large LN₂ tank.

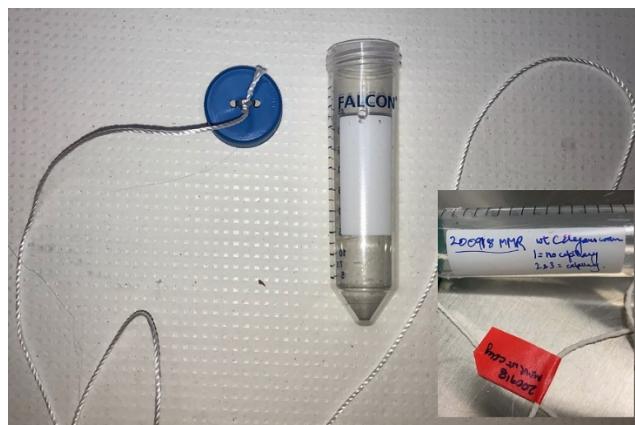


Figure 6. Preparation of storage tube.

A 50 mL blue cap tube is prepared as described in Section B Step 1. Inset: An example of label on the tube

- and tape label to the string.
2. Prepare multiple 1 mL cryovials by perforating its side wall (similar to Step B1). The hole prevents pressure buildup in the tube. Label each cryovial with date, experiment number and sample number using a pencil. Pencil marks are stable in acetone and help prevent mix-up later.
 3. Use a hairdryer to remove moisture completely from the sample unloading chamber and accessory units (Figure 7a). Fill the chamber slowly with LN₂, and wait until there are no bubbles, that is, the LN₂ surface is calm. Keep the plexiglass cover closed to minimize ice or frost contamination.
 4. Place all tweezers and other accessories on a slide warmer set at 45°C next to the chamber and keep the hairdryer plugged in as you will need it again. Be quick and alternate the tweezers kept on a slide warmer to prevent depositing ice crystals into sample unloading chamber.

Note: Wearing a surgical facemask reduces condensation and fog in chamber filled with LN₂.

5. Take the sample storage dewar out from Leica ICE high-pressure freezer. Transfer the segmenting insert and attached trisection pod (Figure 7c) to the designated slot (Figure 7a, left side) in the cryobox and release the trisection pod containing the planchette assemblies (Figures 7c-7d) by pressing and twisting the “bayonet” or release button from the locked to open position on the segmenting insert (Figure 7c, blue arrow).
6. Dip a dry insulated tweezer (Figure 1d) in LN₂ away from the samples for about 20 s to chill it to LN₂ temperature. Watch for the bubbles and wait until the hissing sound ceases.
7. Remove plastic adapters one at a time out of the sample holder (Figure 7e) leaving only the holder with frozen planchettes. Quickly transfer the holder with frozen planchettes (keeping submerged in LN₂ all the time) to the sample release station (Figures 7e-7f, right side).
8. With the release handle (Figure 7a, white arrow), punch out the frozen planchettes from the holder into the reservoir (Figure 7g, yellow arrow). Keep the plexiglass cover closed to minimize ice contamination when not transferring/releasing samples.
9. Place prelabeled cryovials into the cryobox and allow to chill to LN₂ temperature for a few minutes. Use dry tweezers (Figure 1d) to transfer frozen planchettes into respective cryovials (keeping submerged in LN₂ all the time). Warm up each cryovial cap with your palm, and then quickly close. If the cap is not warm and moisture free, then it will freeze to the tube and it is nearly impossible to reopen the tube without warming up the whole tube, causing damage to the frozen sample.
10. Final Check: Inspect for any split planchettes, and if found, collect the ones without the black dot. The type-A plachette (flat bottom) used as a cover is marked with a black dot, and can be easily identified under LN₂ and discarded, as the frozen embryo is inside the cavity of an unmarked, type B plachette.
11. Collect all cryovials into a prelabeled 50 mL polypropylene tube (prechilled in LN₂ in a portable dewar), close it with a cap with the string with tape label visible outside (see Figure 6), and deposit into a LN₂ storage tank. Samples can be stored for years with appropriate care.

C. Freeze substitution of HPF embryos for electron microscopy

Note: This process takes 3-4 days in total and likely more than 8 h on the first day due to long incubation times. Confirm that you have enough dry ice and LN₂ supply. Reagents must be freshly prepared, so plan your day accordingly. Use a slide warmer and hairdryer to keep all instruments moisture free!

1. Prepare the QFS cocktail as per recipe 7 (Rahman *et al.*, 2020), and aliquot 1 mL each in prelabeled cryovials (label as per sample number, one frozen sample per vial).
2. Place a metal block with 12 mm holes facing up in dry ice bucket. Fill with LN₂ and allow to chill to LN₂ temperature. Add LN₂ frequently to keep the block submerged. Place a lid on the bucket to prevent condensation. The bucket must be placed in a chemical fume hood, and subsequent steps performed in a fume hood as osmium is toxic and volatile.
3. Freeze the QFS cocktail by placing the tubes into the metal block submerged in LN₂. Do not add too much LN₂, otherwise the tubes will float out of the block.

4. Collect the sample cryovials (HPF embryos or worms) from the LN₂ storage tank into a transfer dewar filled with LN₂ (Figure 8a, blue arrow).

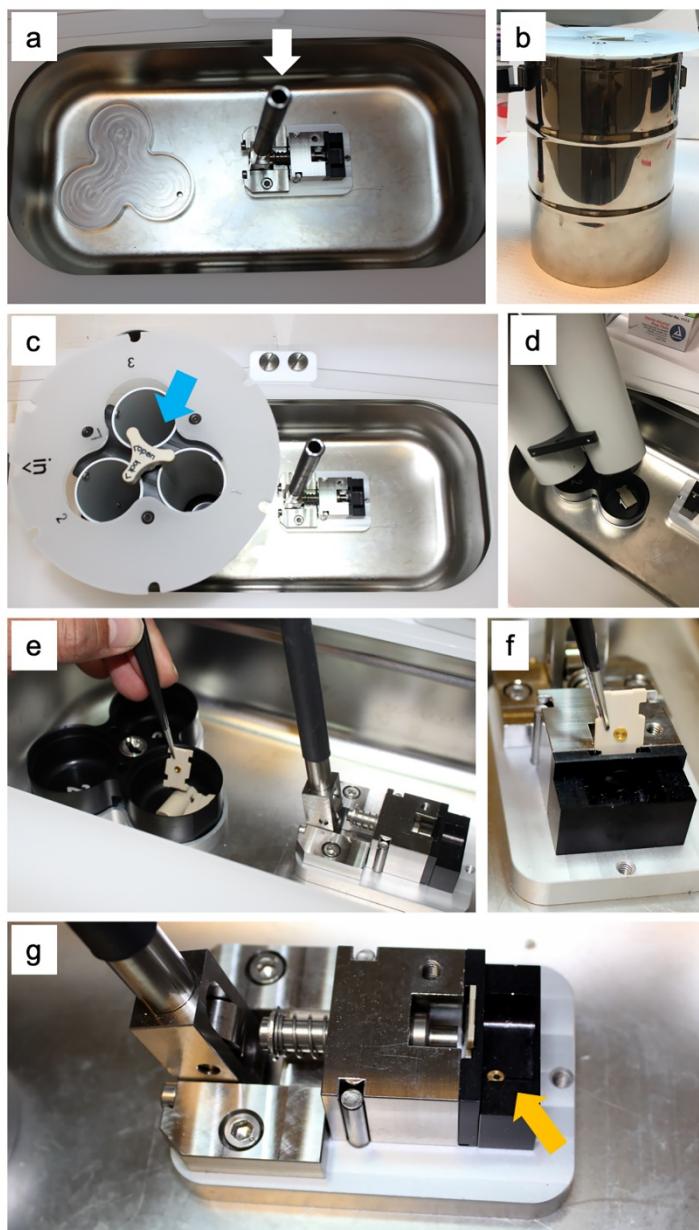


Figure 7. Sample recovery after high-pressure freezing.

All photographs were taken without LN₂ for clarity. a. Sample recovery cryobox showing a tray for sample holder (left) and sample release station with release handle (white arrow) and collection reservoir on the right. b-d. Sample holder from ICE high-pressure freezer transferred to sample recovery cryobox. Twisting the bayonet (c, blue arrow) counter clockwise releases the sample collection container unit from the cylinders. e-g. One sample holder at a time is transferred to the release station. Frozen planchettes are collected into the reservoir (g, orange arrow).

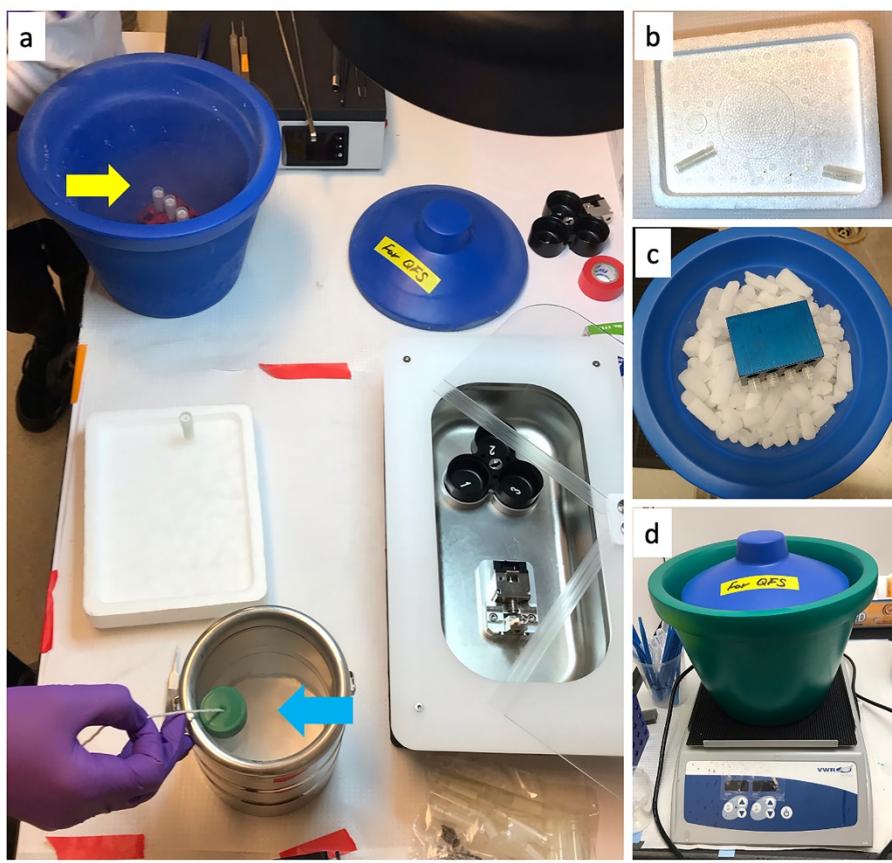


Figure 8. Sample transfer for QFS.

a. Frozen samples are transferred in LN₂ dewar (bottom, blue arrow). QFS cocktail submerged in LN₂ in a dry ice bucket (top left, yellow arrow). b. A shallow Styrofoam container with frozen QFS cocktail cryovial (top left) and HPF sample cryovial (top right) laid flat submerged in LN₂. One tube at a time, cryovials are opened (top right), frozen planchettes are spilled into LN₂ on a shallow Styrofoam container, and then transferred into frozen QFS cocktail in cryovials laid flat on the same surface (top left) as in Steps C5 to C7 with tweezers. c. QFS tubes are placed on dry ice sideways (in a prechilled metal block). d. The bucket is filled with more dry ice, covered with a lid, and then placed on an orbital shaker (McDonald and Webb, 2011).

5. Take a clean and dry shallow Styrofoam container or tray. A lid of a shipping container, typically 15 × 20 cm and 2 cm deep, will suffice. Add LN₂ to the tray and allow it to cool just prior to the subsequent steps.
6. Take out one cryovial at a time. Uncap the cryovial, and quickly lay down the vial flat on a shallow Styrofoam container filled with LN₂ and spill out the planchette under LN₂. Next to it also uncap and lay down a frozen QFS-containing vial flat in the LN₂. Keep the cap of this vial warm in your palm or on a slide warmer to avoid condensation otherwise it will freeze and get stuck.
7. Transfer one planchette into each vial with frozen QFS cocktail with a sharp-point tweezer. Quickly stand up the cryovial (keeping most of the vial submerged in LN₂) and screw the warm cap onto the tube. Immediately transfer it to the metal block submerged in LN₂ (Step C2). It is possible to place multiple planchettes in a single QFS cocktail tube if samples are easily identifiable, however we warn that the planchettes can jostle against each other and dislodge large samples during QFS.
8. Frequently check and add LN₂ to keep the metal block submerged the whole time. Place the lid on top to restrict condensation (as much as possible) from the moisture in the air.
9. Repeat Steps C4 to C7 until all sample-containing planchettes are transferred to individually labeled QFS-containing cryovials. We advise not to attempt more than eight tubes at a time.

10. Carefully decant LN₂ and fill the ice bucket halfway with dry ice. Rotate the metal block so cryovials are now lying flat on their side. Add more dry ice to cover the metal block completely and cover the bucket with a lid. Place it on an orbital shaker set at 60 cycles/min for 3 h inside a chemical safety hood (McDonald and Webb, 2011).
11. Discard dry ice after 3 h. Place the lid back and continue to rotate at 60 cycles/min for another hour.
12. Remove the lid and continue to shake at 60 cycles/min for another hour (or as needed) until the metal block reaches a temperature of about 4°C. Frequently check the temperature of the block with an infrared thermometer. This is a critical step! Long exposure at 4 °C or higher may significantly darken the entire volume, making it hard to identify a single embryo inside the capillary.
13. Replace the QFS cocktail with 100% acetone (stored at 4°C). First, carefully remove the QFS cocktail with a glass pipette (take care to leave the planchette undisturbed). Use a fresh pipette, and gently refill by releasing the solution (100% acetone) against the side wall of the cryovial. Replace the solution with the following mixture after 1 h incubation in each (use an orbital shaker set at 60 cycles/min).
 - a. 1:2 resin:acetone
 - b. 1:1 resin:acetone
 - c. 2:1 resin:acetone

Discard all pipettes, used and unused reagents, paper towels and kimwipes used in the process in accordance with institutional biohazard disposal procedure.
14. Transfer the planchettes into 100% resin and leave overnight (14 h to 16 h) at room temperature on an orbital shaker set at 60 cycles/min.
15. Next morning, replace overnight resin with freshly prepared 100% resin and immediately proceed to the next step.
16. Use a razor blade to cut off the bottom of a Beem capsule; uncap the capsule and flex the hinge so that the lid lies flat on a surface. Place the planchette sample side up along with a few drops of resin on the inside of the Beem capsule lid. Carefully close the capsule onto the lid, keeping the planchette sample side up. The bottom of the capsule with the cut-out hole should be facing up. Fill the capsule through this opening with 100% resin (see Figures 9a-9i), and bake it to cure in an oven at 75°C for 60 h to 65 h.
17. Once cured, use a razor to cut off the plastic beam capsule (see Figures 9j-9m). Now carefully remove all the resin around the metal planchette under a dissecting scope. Important: be sure to completely remove all resin on the sides – the shiny metal of the planchette side should be totally exposed (see Figures 9o-9p). Now quickly immerse the exposed metal base of the planchette into LN₂ until it reaches cryogenic temperature, then heat with a hairdryer until all condensation has disappeared and the planchette is warm. Repeat the cold/hot cycle multiple times as required, until the planchette or the metal carrier pops out and falls away due to differential thermal expansion between the metal and resin. This will leave the resin-embedded sample intact (Figure 9r). Do not use force to pry the planchette loose, as this risks fracturing the resin leaving the resin-embedded sample still in the cavity. You can store resin-embedded samples either with or without planchette attached for years in a dustproof storage container at room temperature before sectioning for imaging (TEM or vEM).

Note: The QFS protocol described here is adequate for FIB-SEM imaging and array tomography in our hands. For conventional TEM imaging, the samples may be sectioned and are typically post-stained to enhance contrast (Hayat, 2000). For other volume EM approaches such as serial block face SEM, users may wish to further enhance metallization of the sample for high-resolution work by adapting room temperature en-bloc staining protocols (Hayat and Giaquinta, 1970; Hua et al., 2015).

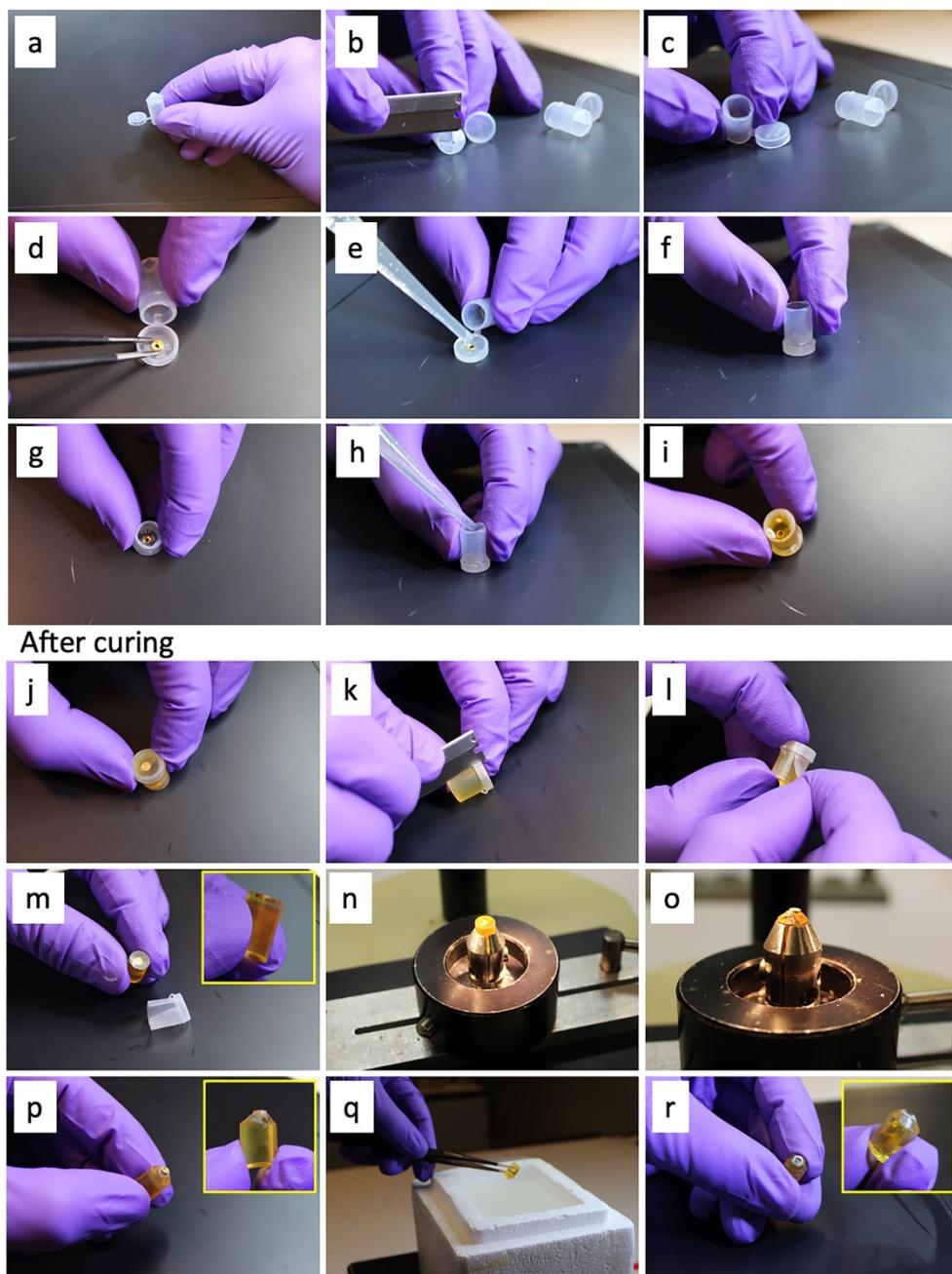


Figure 9. Resin embedding and sample processing.

a-c. A razor blade is used to cut off the bottom of a Beem capsule. d-e. Flex the hinge so that the lid lies flat on surface. Place the planchette (from QFS Step C15) sample side up and add a few drops of resin with a Pasteur pipette. f-g. Carefully close the capsule on the lid keeping sample side of the planchette up. h-i. Fill the Beem capsule with 100% resin and transfer to an oven (see Step C16) to cure. After curing, the stained sample is at the bottom of the capsule (9j, back view). k-m. Use a razor blade to remove the Beem capsule. n-p. Under a dissecting scope carefully remove the resin using a razor blade to expose the planchette. q. Once the planchette is exposed multiple times, immerse it in LN₂ followed by heating with a hairdryer until the metal carrier pops out. r. Exposed sample embedded in 100% resin after removal of planchette or metal carrier.

Notes

Working with liquid nitrogen is potentially dangerous. Appropriate Personal Protective Equipment (PPE) must be used to protect your eyes, face and exposed skin. Also see your institute's safety procedure.
Osmium, a heavy metal, poses health risk if inhaled. You must wear a surgical mask and prepare the freeze substitution cocktail mix in a chemical hood connected to an exhaust system.

Recipes

1. Modified Youngren's, Only Bacto-peptone (MYOB) plates for worm maintenance

- Bacto Agar 20 g
Sodium chloride (NaCl) 2 g
Trizma-HCl 0.55 g
Trizma-OH 0.24 g
Bacto Peptone 3.1 g
Deionized water to 1 liter
- Autoclave for 20 min (liquid cycle), allow to cool down
 - Add 1.6 mL cholesterol from stock solution (see below)
 - Mix thoroughly with a magnetic stirrer prior to pouring into 35 mm tissue culture dishes, about 5 mL per plate (~200 plates per liter)
 - Once the agar plates have solidified and are dry, apply ~200 μ L *E. coli* OP50 culture (see below) and let stand for two days at room temperature until the bacterial solution has dried and a bacterial lawn is formed (Church *et al.*, 1995)

2. Cholesterol stock solution

- Cholesterol 5 mg
Ethanol (200 proof) 100 mL
Use a small magnetic stir bar to dissolve cholesterol (slow speed for about 2 h)

3. *E. coli* OP50 stock

From a frozen stock, streak bacteria on an agar plate (without any antibiotics) and incubate overnight (~14-16 h) at 37°C. Inoculate a few isolated colonies into 100 mL LB media (without any antibiotics) and incubate for 6-7 h at 37°C without shaking. Seed MYOB plates with 200 μ L culture (Stiernagle, 2006). Dispose of unused bacterial culture appropriately.

4. Cellulose capillary attachment

See Figure 10

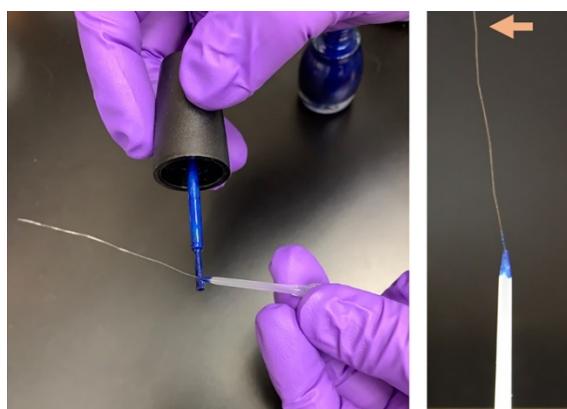


Figure 10. Cellulose capillary attachment for sample collection.

Cut cellulose capillary into ~4 cm pieces using sharp scissors. Under a dissecting scope, push or pull the capillary through a 1 μ L pipette tip (without a barrier) until the edge of the capillary tube is close to the edge of the tip. Apply nail polish to glue the capillary tube to the tip of the pipette tip and allow it to dry at room temperature for an hour (Muller-Reichert *et al.*, 2003). Ensure the other end of the capillary tube is open; if not, cut to open near the edge with sharp scissors (see arrowhead).

5. 20% BSA solution

BSA 10 g

M9 buffer to 50 mL

- Prepare the solution in multiple steps, that is, each time add a small amount of BSA into 35 mL of M9 buffer (in a 50 mL blue cap tube)
- Mix gently on a table-top orbital shaker at low speed to avoid generating bubbles
- Once BSA is completely dissolved, bring volume to 50 mL. Mix gently to homogeneity and store at 4°C, or -20°C in small aliquots (long-term preservation) for up to one year

6. 25 mM Levamisole solution

- Add 0.051 g levamisole into 10 mL of 20% BSA solution

- Mix gently on a table-top orbital shaker at low speed to avoid generating bubbles

Note: The solution can be used for up to two to three months.

7. Quick Freeze-substitution (QFS) cocktail

Note: Must prepare inside a chemical hood. Use a borosilicate glass pipette to measure acetone and methanol. Please follow institutional chemical safety regulations for handling Osmium and Uranium compounds. These are extremely toxic; safety is paramount.

OsO₄ granule 0.1 g

Acetone 13.5 mL

At room temperature stir to mix completely for about 10-15 min. Once no residue is seen, add from a freshly opened vial of:

2% Uranyl acetate (UA) in methanol 0.75 mL

Distilled deionized (DD2) water 0.75 mL

At room temperature mix completely and pass through a 0.22 μ m cellulose acetate filter to remove any residual undissolved elements. Aliquot 1 mL into separate cryovials, flash freeze in LN₂ and store at -80°C (Rahman *et al.*, 2020)

8. Poly/Bed 812 resin mix

Poly/Bed 812 14.6 g

Dodeceny succinic anhydride (DDSA) 8.4 g

Nadic Methyl anhydride (NMA) 7.0 g

DMP-30 0.42 mL

Mix gently with magnetic stirrer until homogeneity is achieved. Prepare fresh from the kit which contains all the chemicals mentioned above (Rahman *et al.*, 2020)

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Competing interests

The authors declare no competing financial interests.

Ethics

Hazardous chemicals were disposed according to institutional guidelines.

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Production of Phenotypically Uniform Human Cerebral Organoids from Pluripotent Stem Cells

Adam Sivitilli^{1, #}, Parisa Ghiasi^{1, #} and Liliana Attisano^{1, *}

¹Department of Biochemistry, Donnelly Centre, University of Toronto, Toronto, ON, Canada

*For correspondence: liliana.attisano@utoronto.ca.

#Contributed equally to this work

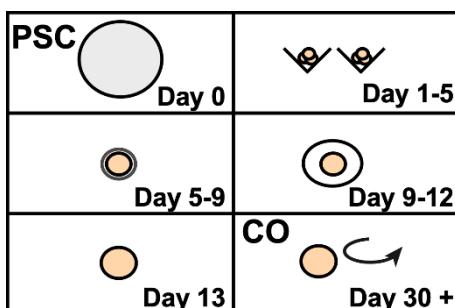
Abstract

Recent advances in stem cell technology have allowed researchers to generate 3D cerebral organoids (COs) from human pluripotent stem cells (hPSCs). Indeed, COs have provided an unprecedented opportunity to model the developing human brain in a 3D context, and in turn, are suitable for addressing complex neurological questions by leveraging advancements in genetic engineering, high resolution microscopy, and tissue transcriptomics. However, the use of this model is limited by substantial variations in the overall morphology and cellular composition of organoids derived from the same pluripotent cell line. To address these limitations, we established a robust, high-efficiency protocol for the production of consistent COs by optimizing the initial phase of embryoid body (EB) formation and neural induction. Using this protocol, COs can be reproducibly generated with a uniform size, shape, and cellular composition across multiple batches. Furthermore, organoids that developed over extended periods of time (3–6 months) showed the establishment of relatively mature features, including electrophysiologically active neurons, and the emergence of oligodendrocyte progenitors. Thus, this platform provides a robust experimental model that can be used to study human brain development and associated disorders.

Keywords: Stem cells, Organoids, Cerebral organoids, Brain organoids, 3D culture, Neural development, Neuroscience

This protocol was validated in: Life Sci Alliance (2020), DOI: 10.26508/lsa.202000707

Graphical Abstract:



Overview of cerebral organoid development from pluripotent stem cells

Background

Recent advancements in the *in vitro* development of 3D cerebral organoids (COs) derived from human pluripotent stem cells (hPSCs) have provided an unprecedented opportunity to model the developing human brain and relevant complex diseases in an experimentally tractable system. Indeed, this approach has allowed researchers to study early brain development and the consequences of alterations associated with various human neurological disorders, such as Alzheimer's, blindness, Autism Spectrum Disorder (ASD), and Zika virus infection (Lancaster and Knoblich, 2014b; Quadrato *et al.*, 2016; Di and Kriegstein, 2017; Huch *et al.*, 2017; Amin and Paşa, 2018; Rossi *et al.*, 2018; Chen *et al.*, 2019). In addition, several groups have applied COs to study and establish preclinical models of human brain cancers such as glioblastoma multiforme (Drost and Clevers, 2018; Linkous *et al.*, 2018). In recent years, numerous protocols have emerged to facilitate the development of region specific-COs by controlling the underlying cell signaling pathways with exogenous growth factors and small molecule inhibitors to guide cell fate changes as the organoid matures (Lancaster *et al.*, 2013; Mariani *et al.*, 2015; Jo *et al.*, 2016; Qian *et al.*, 2016; Birey *et al.*, 2017; Quadrato *et al.*, 2017; Watanabe *et al.*, 2017; Pollen *et al.*, 2019; Velasco *et al.*, 2019; Yoon *et al.*, 2019). However, due to the fact that human whole-brain organoids are largely produced by intrinsic self-patterning and do not rely on controllable exogenous factors, stochastic differentiation often leads to cellular diversity, which is amplified with extended culture. Unfortunately, the considerable variability between individual organoids obtained using whole-brain differentiation platforms can therefore limit the utility of these COs for studying disease mechanisms or the development of potential therapeutics. Here, we describe our robust protocol for efficiently and reproducibly generating mature, uniform human COs (Figure 1). By optimizing an established protocol for the creation of self-patterned whole-brain organoids (Lancaster and Knoblich, 2014a; Lancaster *et al.*, 2013), we successfully generated phenotypically uniform forebrain organoids with reproducible cell-type compositions.

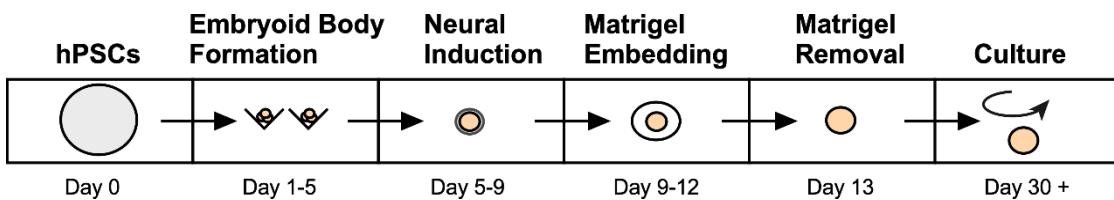


Figure 1. Overview of the developed method to generate human COs from pluripotent stem cells

Materials and Reagents

1. 96-well V-bottomed non-binding plates (Greiner Bio-One, catalog number: 651970)
2. 24-well clear flat-bottomed ultra-low attachment plates (Corning, catalog number: 3473)
3. 4-well cell culture plates (ASI, catalog number: TP9004)

4. 50 mL and 15 mL conical centrifuge tubes (Corning, Falcon, catalog numbers: 352096, 352070)
5. Low-retention microcentrifuge tubes (1.5 mL and 0.6 mL, Thermo Fisher Scientific, catalog numbers: 3451 and 3446)
6. 50 mL sterile disposable reagent reservoirs (Corning, catalog number: 4870)
7. DMEM/F-12, HEPES (Thermo Fisher Scientific, Gibco, catalog number: 11330032, store at 4 °C)
8. KnockOut™ Serum Replacement – Multi-Species (Thermo Fisher Scientific, Gibco, catalog number: A3181502, store at -20°C)
9. MEM Non-Essential Amino Acid Solution (100×) (Thermo Fisher Scientific, Gibco, catalog number: 11140050, store at 4°C)
10. 2-Mercaptoethanol (1,000×) (Thermo Fisher Scientific, Gibco, catalog number: 21985023, store at 4°C)
11. Animal-Free Recombinant Human FGF-basic (Peprotech, catalog number: AF-100-18B, store at -80°C)
12. TrypLE™ Express Enzyme (1×) (Thermo Fisher Scientific, Gibco, catalog number: 12604013, stored at room temperature [22°C] in the dark)
13. Neural Basal Medium (Thermo Fisher Scientific, Gibco, catalog number: 21103049, store at 4°C)
14. Y27632 ROCK Inhibitor (Cedarlane, catalog number: S1049-10MG, store at -80°C)
15. GlutaMAX™ Supplement (Thermo Fisher Scientific, Gibco, catalog number: 35050-061, store at 4°C)
16. Heparin sodium salt (Sigma-Aldrich, catalog number: H4784, store at -20°C)
17. Insulin solution human (Sigma-Aldrich, catalog number: I9278, store at 4°C)
18. N-2 Supplement (100×) (Thermo Fisher Scientific, Gibco, catalog number: 17502001, aliquots of 500 µL, store at -20°C)
19. B-27™ Supplement (50×), minus vitamin A (Thermo Fisher Scientific, Gibco, catalog number: 12587010, aliquots of 500 µL, store at -20°C)
20. B-27™ Supplement (50×), serum-free (Thermo Fisher Scientific, Gibco, catalog number: 17504044, aliquots of 500 µL, store at -20°C)
21. Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix (Corning, catalog number: 356231, aliquots of 500 µL, store at -20°C)
22. D-PBS^{-/-}, 1×, without calcium and magnesium (Wisent Bioproducts, catalog number: 311-425-CL, store at 4°C)
23. Embryoid Body Media (EB Media) (see Recipes)
24. Neural Induction Media (see Recipes)
25. Cerebral Organoid Differentiation Media without Vitamin A (CDM-A) (see Recipes)
26. Cerebral Organoid Differentiation Media with Vitamin A (CDM+A) (see Recipes)

Equipment

1. Nalgene™ Square PETG media bottles (250 ml) (Gibco, Thermo Fisher Scientific, catalog number: 2019-0250)
2. Pipettes (5 mL, 10 mL, 25 mL, 50 mL), micro-pipettes (10 µL, 20 µL, 200 µL, 1,000 µL)
3. Multi-channel pipette (Eppendorf, model: Research plus 12 channel pipette, catalog number: ES-12-300)
4. Water bath
5. Centrifuge
6. Phase contrast microscope
7. Hemocytometer
8. 37°C, 5% CO₂ cell culture incubator
9. Orbital shaker that can be installed inside the incubator (such as Thermo Fisher, catalog number: 88881101)
10. Blade and scalpel

Procedure

A. Embryoid Body Production: Singularizing and Plating Human Embryonic Stem Cells (hESCs)

1. Prepare EB Media without growth factors and prewarm to 37°C using a water bath. EB Media (without growth factors) can be stored for up to 1 week at 4°C, and once warmed, the stock should be used or discarded.
2. Wash hESCs, which have reached 70–80% confluence, twice with 3 volumes of D-PBS^{-/-}.

Note: This protocol was established using hESCs cultured in mouse embryoinic fibroblast (MEF) conditioned media supplemented with bFGF to a final concentration of 4 ng/mL (Sivitilli et al., 2020).

3. Add 1 mL TrypLE and incubate for 5 min at room temperature (22°C).
4. Gently pipette up and down to dissociate colonies to a single-cell suspension.
5. Neutralize TrypLE with 4 volumes (*i.e.*, 4 mL) EB media and add the resulting suspension to a 15-mL Falcon tube.
6. Use a hemocytometer to count the cells. Avoid using an automated cell counter, which can result in inaccurate cell numbers because hESCs are prone to clumping.
7. Centrifuge cells at 150 × g for 5 min at room temperature (22°C).
8. During centrifugation, prepare EB Media by supplementing with 4 ng/mL bFGF and 50 μM Y-27632 (ROCK inhibitor). For this, use 15 mL EB media with 1.5 μL bFGF (from a 40 ng/μL stock) and 7.5 μL Y-27635 (from a 100 mM stock).
9. Resuspend single hESCs to a final concentration of 80,000 cells/mL in the freshly prepared EB Media supplemented with bFGF and Y-27632.
10. Transfer the resuspended cells to a sterile disposable reagent reservoir and add 150 μL cell suspension (12,000 cells) to each well of a 96-well V-bottomed non-binding plate using a multi-channel pipette.
11. Place the plate at 37°C in a CO₂ incubator for 2 days.

B. Embryoid Body Production: Feeding

1. Prepare EB Media fresh daily and prewarm to 37°C using a water bath.
2. On day 2, feed the EBs with EB media supplemented with 2 ng/mL bFGF. To reach the required concentration, mix 15 mL EB media with 0.75 μL bFGF (40 ng/μL).
3. Remove 135 μL media from each well of the incubated 96-well plate using the multi-channel pipette.
4. Transfer the EB media (supplemented with 2 ng/mL bFGF) into a sterile disposable reagent reservoir and add 150 μL EB media using the multi-channel pipette.
5. Return the plate to the CO₂ incubator for a further 2 days.

Note: Due to evaporation, wells toward the peripheral edge of the 96-well plate may have a slightly lower volume. This is normal and EBs should still form correctly.

C. Embryoid Body Production: Assessment Checkpoint

On day 5, measure the EB size using a brightfield microscope to determine whether the EBs are ready for neural induction (Figure 2). If the size of the EBs is 350–600 μm, proceed to Procedure D (Neural Induction); if the size is below 350 μm, then repeat Procedure B; if the size is above 600 μm, we do not recommend continuing with the protocol. Restart the protocol and double check cell counting in Procedure A to ensure accuracy when seeding EBs and start another batch.

D. Neural Induction: Transfer to Neural Induction

1. Prepare Neural Induction Media fresh for each differentiation and prewarm an aliquot to 37°C using a water bath. Neural Induction Media can be stored for 1 week at 4°C. Once warmed, Neural Induction Media should not be reused.
2. Choose EBs that meet the size and visual criteria (Figure 2).
3. Cut 2–3 mm off the extremity of a 200-μL filtered pipette tip using a razor blade to make a wider opening.
4. Prepare a 24-well ultra-low attachment plate by adding 500 μL prewarmed Neural Induction Media to each well.
5. Using a dissecting scope and a cut 200 μL pipette tip, pick up the EB and transfer to a 24-well ultra-low attachment plate. Set the pipette to 20 μL, place the end at the bottom of the well and pipette up the EB. A maximum of 2 EBs per well can be added; additional EBs increase the likelihood of further aggregation.
6. Place the plate in a CO₂ incubator for 2 days.

E. Neural Induction: Feeding

1. Prewarm Neural Induction Media to 37°C using a water bath.
2. Feed the EBs by adding an additional 500 μL Neural Induction Media to each well.
3. Return the plate to a CO₂ incubator for a further 2 days.

F. Neural Induction: Assessment Checkpoint

1. Use a brightfield microscope to examine the neuralized EBs for optical clearing in the outer 100 μm of the EB (Figure 3). The outer ring structure should be prominent and marked by a stark change in opacity as compared with the central region of the EB. This is a critical checkpoint; EBs that do not have the correct organization of the neuroepithelial ring will not form organoids.

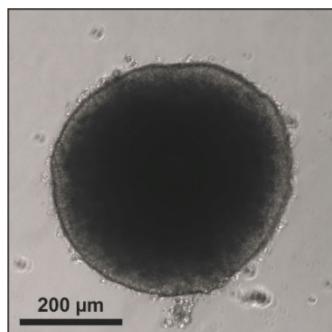


Figure 2. Representative EB derived from H9 hESCs on Day 5

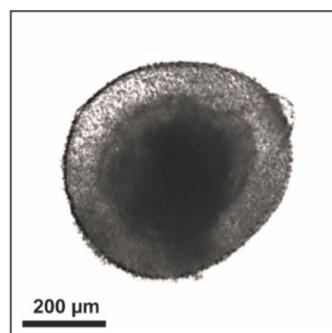


Figure 3. Neuroectoderm formation in an EB derived from H9 hESCs at Day 9

G. Neuroepithelial Expansion: Transfer to Solid Phase Matrigel Scaffold

1. Thaw Growth Factor Reduced Matrigel on ice for 2 h. Once thawed, Matrigel can be used for up to 6 h but must be kept on ice. Matrigel cannot be refrozen once thawed.
2. Prepare CO Differentiation Media without Vitamin A (CDM-A) fresh for each differentiation and prewarm an aliquot to 37°C using a water bath. CDM-A can be stored for 2 weeks at 4°C. Once warmed, CDM-A should not be reused.
3. Prepare a box of wide-bore tips by cutting 3–4 mm off the extremity of a 200 µL filtered pipette tip using a razor blade to make a wider opening. Pick up the EBs one by one using a 200 µL cut tip and transfer to the center of each well of the 4-well plate (1 EB per well).
4. Using an uncut 200 µL tip, draw 25 µL to aspirate the remaining Neural Induction Media from each well. It is critical to remove all media from the EB in the 4-well plate; failure to do so will prevent the Matrigel from attaching to the plate surface and prevent removal of the Matrigel in Procedure J.
5. Cover the neuralized EB with a drop of cold Matrigel (30 µL) using an uncut 200 µL tip.
6. Position the EB in the center of the Matrigel droplet using a 10 µL tip. Ensure that the EB is not resting on the bottom of the plate.
7. Once a plate has been filled (4 EBs), transfer the full plate to a CO₂ incubator for 10 min at 37°C to polymerize the Matrigel.
8. After 10 min, carefully add 500 µL prewarmed CDM-A and return to a CO₂ incubator for a further 2 days.

Note: Be consistent with the amount of media collected when transferring the EB. We recommend collecting EBs with 25 µL media to create a droplet in the 4-well plates.

H. Neuroepithelial Expansion: Feeding

1. Prewarm an aliquot of CDM-A to 37°C using a water bath.
2. Carefully aspirate all media and add 500 µL CDM-A to each well of the 4-well plate.
3. Return the plate to the incubator for a further 2 days.

I. Neuroepithelial Expansion: Assessment of Checkpoint

1. Use a brightfield microscope to examine the COs for ring structures in the peripheral regions of the organoid (Figure 4). COs should be approximately 500–700 µm in diameter and have multiple ring structures throughout; this is a critical checkpoint, those without ring structures will not form organoids.

J. Transfer to Spinning Culture: Extraction from Solid Phase Matrigel

1. Prepare CO Differentiation Media (CDM) fresh and prewarm an aliquot to 37°C using a water bath. CDM can be stored for 2 weeks at 4°C. Once warmed, CDM should not be reused.
2. Prepare wide-bore pipette tips by cutting 4–5 mm off the extremity of 200 µL pipette tips to make a wider opening.
3. Prepare a 6-well plate with 3 ml prewarmed CDM per well.
4. Using a dissection microscope, carefully cut around the COs implanted in the Matrigel droplet; the goal here is to remove as much Matrigel as possible without damaging the CO. Excess Matrigel remaining on the CO at this stage will result in formation of cyst-like structures in the spinning culture.
5. Use a cut 200 µL tip to transfer the COs to the 6-well plate containing CDM; a maximum of 4 hCOs per well is acceptable.
6. Once all the COs have been transferred, place the 6-well plate on an orbital shaker (ThermoFisher Cat: 88881101) in a 37°C incubator at a speed of 80–90 rpm (rpm may vary if a different orbital shaker model is used).

K. Transfer to Spinning Culture: Feeding in Spinning Culture

1. Prewarm CDM using a 37°C water bath.
2. Pause the orbital shaker and collect the plate containing the COs.
3. Carefully aspirate 2/3 of the total media volume from each well; be mindful not to aspirate the COs with the media since they are in suspension.
4. Add 3 ml prewarmed CDM to each well.
5. Return the plate to the orbital shaker in the incubator and initialize (Figure 5).

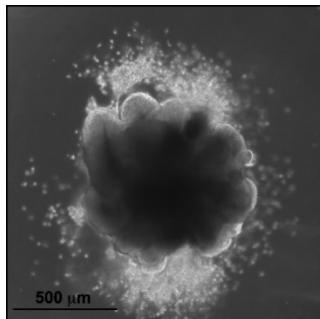


Figure 4. Representative hCOs derived from H9 hESCs on day 13 prior to Matrigel extraction

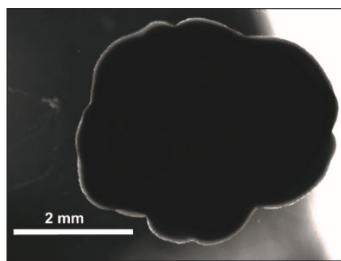


Figure 5. Representative hCO in a spinning culture derived from H9 hESCs at 12 weeks

Recipes

1. Embryoid Body Media (EB Media) (Table 1)

Table 1. EB Media Composition.

A list of components, stock and final concentrations (Con.), and volumes for working stocks are indicated.

Components	Stock Con.	Final Con.	/50 mL medium
DMEM/F12			39.5 mL
KOSR			10 mL
MEM:NEAA	100×	1×	500 μL
2-mercaptoethanol	100×	1×	50 μL

2. Neural Induction Media (prepared fresh every week) (Table 2)

Table 2. Composition of Neural Induction Media (prepared fresh every week).

Once warmed, Neural Induction Media should not be reused. A list of components, stock and final concentrations (Con.), and volumes for working stocks are indicated.

Components	Stock Con.	Final Con.	/50 mL medium
DMEM/F12			48.5 mL
GlutaMax	100×	1×	500 µL
MEM:NEAA	100×	1×	500 µL
N2	100×	1×	500 µL
Heparin	10 mg/ml	0.001 mg/mL	5 µL

3. Cerebral Organoid Differentiation Media without Vitamin A (CDM-A) (Table 3)

Table 3. Composition of Cerebral Organoid Differentiation Media without Vitamin A.

A list of components, stock and final concentrations (Con.), and volumes for working stocks are indicated.

Components	Stock Con.	Final Con.	/50 mL medium
DMEM/F12			24 mL
Neural Basal Media			24 mL
MEM:NEAA	100×	0.5×	250 µL
GlutaMax	100×	1×	500 µL
B27-Vit A	50×	0.5×	500 µL
N2	100×	0.5×	250 µL
2-mercaptoethanol	1,000×	1×	50 µL
Insulin	9.5–11.5 mg/mL	2–3 µg/mL	12.5 µL

4. Cerebral Organoid Differentiation Media with Vitamin A (CDM+A) (Table 4)

Table 4. Composition of Cerebral Organoid Differentiation Media with Vitamin A.

A list of components, stock and final concentrations (Con.), and volumes for working stocks are indicated.

Components	Stock Con.	Final Con.	/100 mL medium
DMEM/F12			48 mL
Neural Basal Media			48 mL
MEM:NEAA	100×	0.5×	500 µL
GlutaMax	100×	1×	1 mL
B27 (with Vit A)	50×	0.5×	1 mL
N2	100×	0.5×	500 µL
2-mercaptoethanol	1,000×	1×	100 µL
Insulin	9.5–11.5 mg/mL	2–3 µg/mL	25 µL

Acknowledgments

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Competing interests

The authors declare that they have no conflicts of interest.

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Development of a Chemical Reproductive Aging Model in Female Rats

Nayara Pestana-Oliveira^{1, 2, *}, Ruither O. G. Carolino⁵, Bruna Kalil-Cutti³, Cristiane M. Leite⁴, Litamara C. Dalpogetto⁵, Bruna Balbino De Paula⁶, Jonh P. Collister¹ and Janete Anselmo-Franci⁵

¹Department of Veterinary and Biomedical Sciences, College of Veterinary Medicine, University of Minnesota, Saint Paul, MN, USA

²Department of Physiology, School of Medicine of Ribeirão Preto, University of São Paulo, Ribeirão Preto, São Paulo, Brazil

³Department of Physiology, Institute of Biomedical Science, Federal University of Alfenas, Alfenas, MG, Brazil

⁴University of Northern Paraná (UNOPAR), Londrina, PR, Brazil

⁵Department of Basic and Oral Science, School of Dentistry of Ribeirão Preto, University of São Paulo, Ribeirão Preto, São Paulo, Brazil

⁶Department of Psychology, School of Philosophy, Science and Letter of Ribeirão Preto, University of São Paulo, Ribeirão Preto, SP, Brazil

*For correspondence: pesta016@umn.edu

Abstract

Women are born with an abundant but finite pool of ovarian follicles, which naturally and progressively decreased during their reproductive years until menstrual periods stop permanently (menopause). Perimenopause represents the transition from reproductive to non-reproductive life. It is usually characterized by neuroendocrine, metabolic and behavioral changes, which result from a follicular depletion and reduced number of ovarian follicles. During this period, around 45-50 years old, women are more likely to express mood disorders, anxiety, irritability and vasomotor symptoms. The current animal models of reproductive aging do not successfully replicate human perimenopause and the gradual changes that occur in this phase. While the traditional rat model of menopause involves ovariectomy or surgical menopause consisting of the rapid and definitive removal of the ovaries resulting in a complete loss of all ovarian hormones, natural or transitional menopause is achieved by the selective loss of ovarian follicles (perimenopause period). However, the natural aging rodent (around 18-24 months) model fails to reach very low estrogen concentrations and overlaps the processes of somatic and reproductive aging. The chronic exposure of young rodents to 4-vinylcyclohexene diepoxide (VCD) is a well-established experimental model for perimenopause and menopause studies. VCD induces loss of ovarian small follicles (primary and primordial) in mice and rats by accelerating the natural process of atresia (apoptosis). The VCD, ovary-intact or accelerated ovarian failure (AOF) model is the experimental model that most closely matches natural human progression to menopause mimicking both hormonal and behavioral changes typically manifested by women in perimenopause.

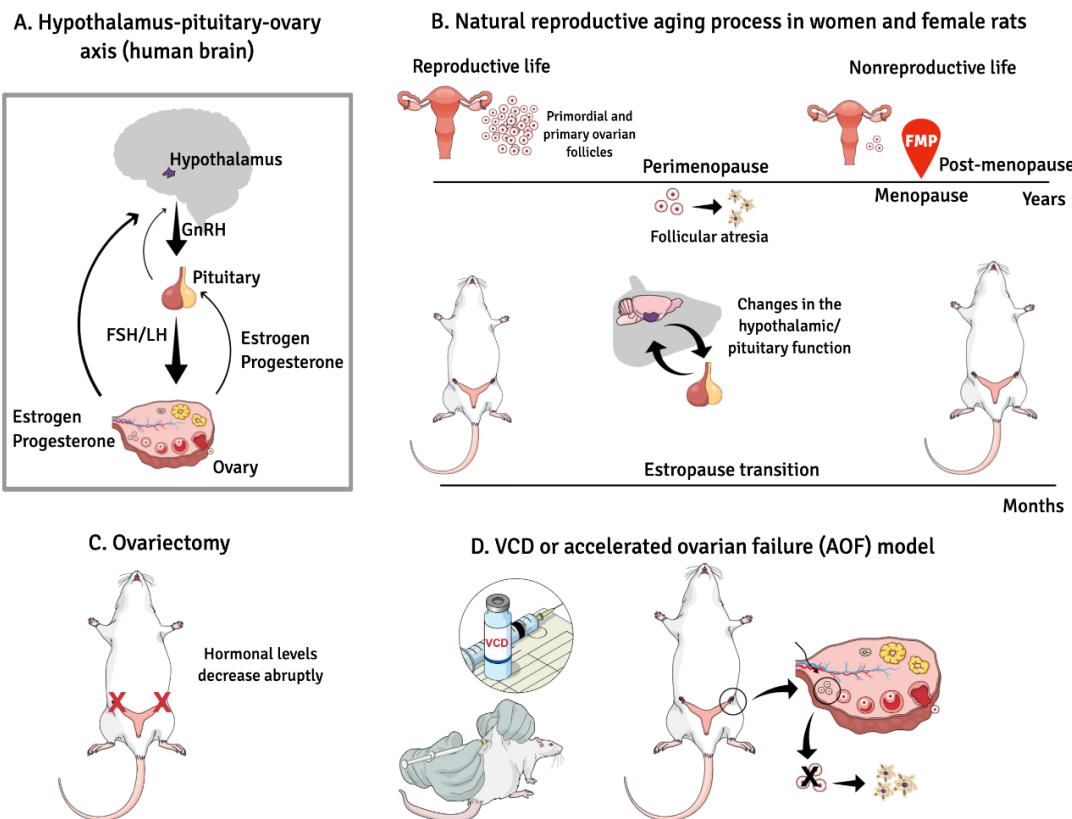
Keywords: Perimenopause, Chemical reproductive aging, 4-vinylcyclohexene diepoxide (VCD), Accelerated ovarian failure (AOF), Follicular depletion

This protocol was validated in: eNeuro (2018), DOI: 10.1523/ENEURO.0247-17.2017

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Graphical Abstract:



The female reproductive system is regulated by a series of neuroendocrine events controlled by central and peripheral components.

(A). The mechanisms involved in this control are extremely complex and have not yet been fully clarified. In female mammals whose ovulation (the most important event in a reproductive cycle) occurs spontaneously, reproductive success is achieved through the precise functional and temporal integration of the hypothalamus-pituitary-ovary (HPO) axis. (B). In women, loss of fertility appears to be primarily associated with exhaustion of ovarian follicles, and this process occurs progressively until complete follicular exhaustion marked by the final menstrual period (FMP). (C). While in female rodents, reproductive aging seems to begin as a neuroendocrine process, in which changes in hypothalamic/pituitary function appear independently of follicular atresia. The traditional rat model of menopause, ovariectomy or surgical menopause consists of the rapid and definitive removal of the ovaries resulting in a complete loss of all ovarian hormones. (D). The chronic exposure (15-30 days) to the chemical compound 4-vinylcyclohexene diepoxide (VCD) in young rodents accelerates gradual failure of ovarian function by progressive depletion of primordial and primary follicles, but retains residual ovarian tissue before brain alterations that occurs in women in perimenopause. Low doses of VCD cause the selective destruction of the small preantral follicles of the ovary without affecting other peripheral tissues.

Background

Perimenopause, the transition period from reproductive to non-reproductive life, is defined as the period immediately before menopause. This period is marked by the onset of endocrine and biological changes, as well as clinical symptoms suggestive of the approach of menopause and can extend up to twelve months after the final menstruation, with an average duration of five years (WHO, 1996; Soules *et al.*, 2001; Bacon, 2017; Wang *et al.*, 2019). In addition

to menopause or definitive cessation of menstrual cycling, perimenopause is a uniquely human process, but it can be mimicked by experimental models, especially in rodents. According to Prior and Hitchcock, perimenopause, previously seen as a period of hypoestrogenism, can be characterized by three main hormonal changes in women whose menstrual cycle remains regular: 1) normal or erratically high concentrations of estradiol; 2) decline in plasma progesterone concentrations and 3) changes at all levels of the reproductive axis (Prior and Hitchcock, 2011). During perimenopause a high percentage of women manifest typical symptoms of this period, which include: vasomotor changes, variations in menstrual cycle duration, sleep disorders, worsening cognitive functions, behavioral and mood changes (irritability, nervousness, anxiety and depression), in addition to metabolic and physiological changes (Mitchell and Woods, 1996; Brinton *et al.*, 2015; Chalouhi, 2017). Considering the brain changes during this period, Brinton *et al.* (2015) defined perimenopause as a “state of neurological transition”.

The process of reproductive senescence in mammal species is complex and poorly understood, especially in humans (Brinton *et al.*, 2009; Brinton, 2010). Consequently, animal models of menopause and perimenopause function as windows into the complex mechanisms involved in reproductive biology senescence at different levels (systemic, cellular, molecular, and genomic), which are not possible to perform in humans (Brinton, 2012). Nonetheless, current animal models of menopause do not successfully replicate human perimenopause and the gradual changes that occur in this phase. While the traditional rat model of menopause, ovariectomy or surgical menopause that consist of the rapid and definitive removal of the ovaries resulting in a complete loss of all ovarian hormones, natural or transitional menopause is achieved by the selective loss of ovarian follicle (perimenopause period). However, natural aging models fail to reach very low estrogen concentrations and overlapping changes related to somatic aging and those of reproductive aging (Kermath and Gore, 2012; Frye *et al.*, 2012; Kirshner *et al.*, 2020). Additionally, these models do not reproduce what occurs in women, since the primary causes of reproductive aging between species diverge significantly. In women, loss of fertility appears to be primarily associated with exhaustion of ovarian follicles (Faddy *et al.*, 1992; Rubin, 2000), while in female rodents, reproductive aging seems to begin as a neuroendocrine process, with the changes in hypothalamic/pituitary function appearing independently of the follicular atresia (Gore *et al.*, 2000).

A well-established experimental model in the literature for studying perimenopause and menopause is exposure of rodents to the chemical 4-vinylcyclohexene diepoxide (VCD), which leads a gradual failure of ovarian function by progressive depletion of primordial and primary follicles, but retains residual ovarian tissue similar to women in perimenopause (Springer *et al.*, 1996; Kao *et al.*, 1999; Hoyer *et al.*, 2001). Importantly, the VCD model or model of Accelerated Ovarian Failure (AOF, Brooks *et al.*, 2016) mimic both hormonal (Reis *et al.*, 2014; Pestana-Oliveira *et al.*, 2018; Carolino *et al.*, 2019) and behavioral changes such as anxiety (Reis *et al.*, 2014), impaired memory (Koebele *et al.*, 2016), depression (Kalil *et al.*, 2020) and aggressiveness (Dalpogeto *et al.*, 2016; Scafuto *et al.*, 2017) typically manifested by women in perimenopause. Low doses of VCD specifically cause selective destruction of ovarian small pre-antral follicles without affecting other peripheral tissues. Furthermore this occupational chemical doesn't cross the blood-brain barrier (Lukefahr *et al.*, 2012).

Therefore, the VCD-induced follicular depletion model, followed by ovarian failure has been widely used in experimental research on perimenopause and menopause (Reis *et al.*, 2014; Liu *et al.*, 2015; Brooks *et al.*, 2016; Koebele *et al.*, 2016; Pestana-Oliveira *et al.*, 2018; Wang *et al.*, 2019; Carolino *et al.*, 2019; Kirshner *et al.*, 2020) and is the experimental model that most closely matches the natural human progression to menopause, since the majority of women enter menopause through a gradual and irreversible process of reduction in ovarian function, while retaining the residual tissue of the ovary (Brooks *et al.*, 2016). Thus, considering that it is a critical number of ovarian follicles and not the woman's age that determines the onset of menopause (Faddy *et al.*, 1992), VCD-induced perimenopause is a translational model that presents analogy, predictability and homology, and allows plausible inferences to be made about the dynamics of follicular loss and its effects on the neurochemistry of women in perimenopause and menopause, periods in which affective disorders, vasomotor alterations and several other symptoms compromise the quality of life of middle-aged women.

Recently (2015-2017) the AOF model was applied to the streets and subways of large North American cities such as Chicago, New York, San Francisco, and Los Angeles with the aim of reducing the population of rats that has infested those cities (<https://www.chicagomag.com/Chicago-Magazine/March-2015/birth-control-for-rats/>).

Materials and Reagents

1. Polyethylene tubing (Thermo Scientific™ Immuno Tubes and Stoppers, catalog number: 12-565-150), stored at room temperature (RT)
2. Plastic funnel
3. Pipet tips (Eppendorf®, catalog numbers: 1300 RN [1-100 µL, yellow]; 1400 [101-1,000 µL, blue]), stored at room temperature
4. Glass slides for immunofluorescence (dimensions: size 25.4 × 76.2 mm; thickness: 1.0 × 1.2 mm), twelve transparent circles (Perfecta, catalog number: 214-6), stored at RT
5. Flexible, translucent silicone elastomer tubing (Silastic, Dow Corning™, 7.8 mm × 12.7 mm × 2.38, catalog number: 11-189-13B), stored at RT
6. Syringe (1 ml Tuberculin Syringe Regular Tip) (Monoject™, catalog number: 8881501400), stored at RT
7. Sterile Gloves (Synthetical Surgical Gloves Powder-Free, Confiderm® SPT), stored at RT
8. Gauze sponges (Non-woven Gauze Sponges, 2 in. × 2 in. [5.08 cm × 5.08 cm]) (AVANT GAUZE®, catalog number: 25223), stored at RT
9. Needles (Standard Hypodermic Needles, Monoject™, catalog numbers: 8881250255 [23G], 8881250149 [21G]), stored at RT
10. Females Wistar rats (age 28 days) from the animal facilities of the University of São Paulo, campus Ribeirão Preto, Brazil
11. 4-vinylcyclohexene diepoxide (VCD [C₈H₁₂O₂] Sigma-Aldrich, catalog number: 94956-250ML), stored at RT
12. 17-β-estradiol (Sigma-Aldrich, catalog number: E8875-1G), stored at RT
13. Corn oil (Liza-900ML), stored at RT
14. Ketamine HCl Injection, USP (Ketaset®, 100 mg/kg; NDC: 0856-2013-1), RT
15. Xylazine (Schering-Plough, Coopers of Brazil, Cotia, São Paulo, 14 mg/kg), RT
16. Small Veterinary Pentabiotic® (Zoetis, 1.7 g/3 mL, catalog number: 232092), RT
17. Banamine Solution Injectable (Schering-Plough Animal Health, 2.5 mg/kg; catalog number: 12080097), stored at room temperature Sodium Chloride (NaCl) 0.9% (Samtec Biotechnology, 10 mL), RT
18. 5% Povidone-iodine antiseptic microbicide for animal use (Betadine® Solution, catalog number: 12265), stored at RT
19. 70% Isopropyl Alcohol (473 mL) (Medline Industries, catalog number: 53329-800-06), stored at RT
20. ELISA estradiol kit (DRG® Instruments GmbH DRG, EIA 2693), storage: 2°C-8°C
21. Progesterone double antibody RIA kit (P4) (MP Biomedicals, catalog number: SKU 07-170105 CF), storage: 2°C-8°C
22. Standard Rat Chow (23.2% Protein Rodent Diet, LabDiet®, catalog number: 5012), stored at RT
23. Heparin 50 units/ml (Heparin Sodium Injection USP, 50 units USP per ml) (B. Braun Medical Inc., catalog number: 0264-9577-10), stored at RT
24. 4-vinylcyclohexene diepoxide dilution (VCD) (see Recipes)

Equipment

1. Standard cages (Bonther, 40 × 33 × 17 cm)
2. Guillotine (Bonther, model: Inox 420)
3. Surgical scissors
4. Forceps
5. Needle holder
6. Microscope (Zeiss Axioskop 2 Plus Ergonomic Trinocular, catalog number: 452342)
7. Centrifuge (Eppendorf, model: 5425, catalog number: 5405000042; 24 × 1.5/2.0 mL Capacity, up to 21,330 × g (15,060 rpm), includes FA-24x2 rotor with aerosol-tight QuickLock lid, keypad control, 120 V)
8. Magnetic Stirrer Magnetic Stirrer with Heating (Thermo Scientific, catalog number: N2400-3010)
9. Shaker/Vortexer with racks (BenchMixer XLQ, catalog number: BV 1010-TST)

10. Detector Gamma Counter (Wizard2™, PerkinElmer®, catalog number: 2470-0020)
11. Absorbance Reader 800 TS (BioTek)
12. Pipette single channel (Eppendorf™ Research [0.5-10 µL, EP: 3123000020 yellow; 20-200 µL, EP: 312300055 yellow; 100-1,000 µL, EP: 312300063 blue]
13. Electronic Pipette Multichannel (Eppendorf™ Research, 100 µL, SKU #184287407894)

Software

1. GraphPad Prism 7 Software (GraphPad Software, La Jolla, CA)
2. Adobe Photoshop (Adobe Photoshop Lightroom, version 5.3; Adobe Systems, Inc.)
3. Mind the graph (www.mindthegraph.com)
4. Microsoft Excel

Procedure

Although many women in perimenopause have normal or erratically high estradiol plasma concentrations (Santoro *et al.*, 1996), estrogenic therapy is very common in clinical practice. Interestingly, improvement was observed regarding the typical symptoms of this transition period (Schmidt *et al.*, 2000). The study that inspired this protocol (Pestana-Oliveira *et al.*, 2018) had as one of its objectives to understand the possible effects of estradiol in the model of accelerated ovarian failure (Figure 1).

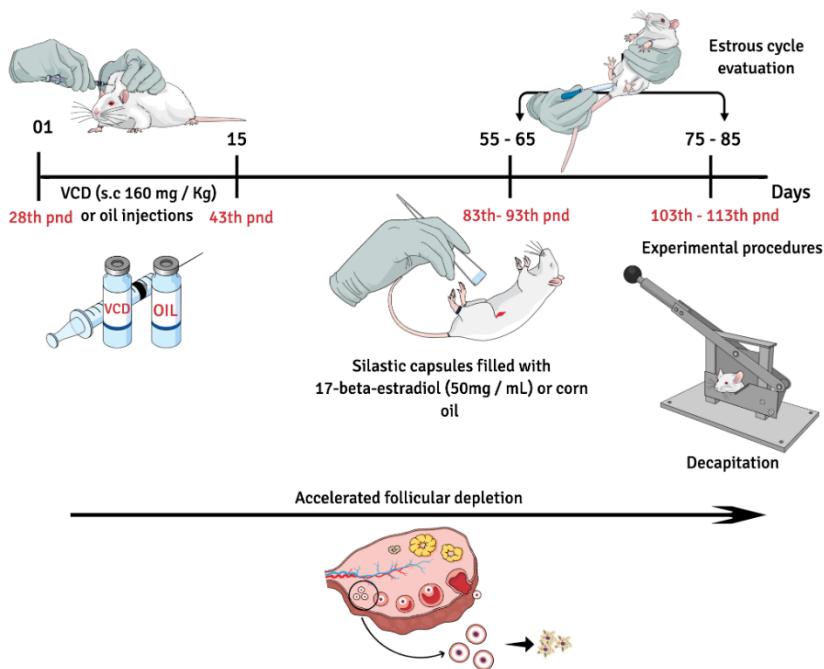


Figure 1. Schematic diagram showing the timeline of the experimental protocol.

Female Wistar rats (28 post-natal days) must be daily injected with VCD (160 mg/Kg) or corn oil (O; 1.25 mL/kg of body weight) for 15 days. Approximately 55 days after the first VCD or O injection, insert implant pellets of 17 β -estradiol or O s.c. in the dorso-lateral region (Groups O + O; VCD + O; VCD + E). 21 days after oil or estradiol pellets implantation rats must be decapitated in the morning of diestrus.

A. VCD or corn oil injections (Figure 2)

1. Weigh and identify the female rats (28th post-natal day) individually and calculate the volume of VCD or corn oil that each will receive. It is important that this procedure is performed every two days. Then prepare and arrange the environment to give the injections. Cover the table or bench with paper and fill the syringes with the volume of VCD or oil previously calculated.
2. Take one animal and apply the subcutaneous injection on the back near to the neck region. Repeat the same procedure for all animals. Perform the applications slowly as the oil is viscous and the syringe may burst from the needle with pressure. In the case of animals that will receive injections containing VCD, avoid wasting liquid after the injection.
3. After finishing all the injections, return the animals to the specific animal facility. Repeat the entire procedure for 15 consecutive days. To avoid cutaneous and subcutaneous injuries we strongly suggest that injections be applied to different parts of the animal's body.

Note: The contact of the skin and mucous membranes with the VCD should be avoided as much as possible, especially if it is handled by female individuals. Therefore, the use of individual safety Equipment such as gloves, masks and glasses is strongly recommended.

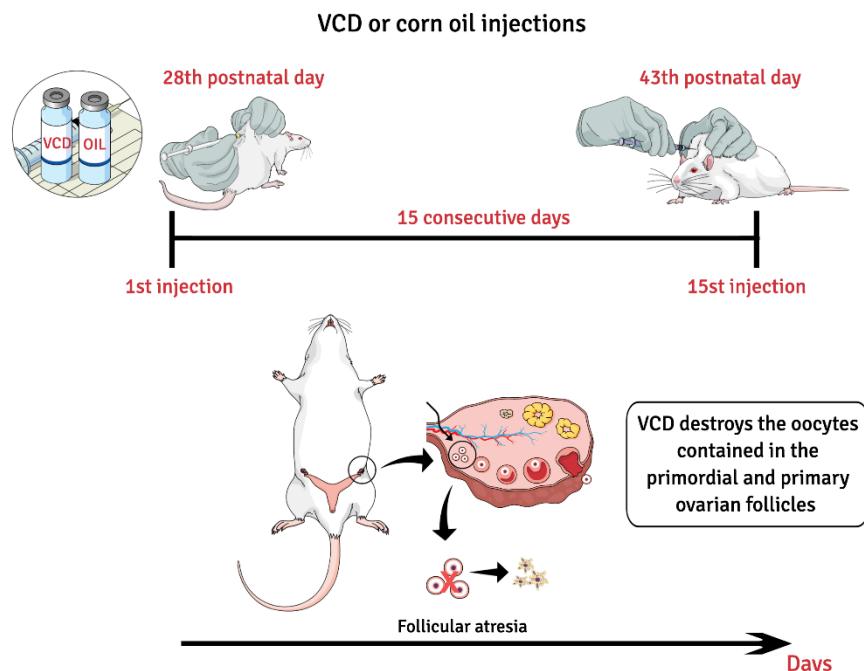


Figure 2. Schematic diagram showing the preparation of the accelerated ovarian failure model and control groups

B. Estrogen therapy

Silastic capsules preparation (Figure 3)

1. Extend the silastic tubing on a table or bench, with the aid of a ruler, divide it into 15 mm long pieces. Then cut the pieces. Insert the tip of one end of the silastic into a drop of silicone glue, press the opposite end so that the glue enters into tube. With the aid of hemostatic scissors, clamp the end filled with glue. Repeat this procedure with the remaining pieces of silastic. Place the tubes on a flat surface for 24 h so that the glue can dry and thus seal the tube end (Figure 3-1).

2. Use a pipette and a plastic tip (0.5-10 μ L) to fill the silastic capsules with 8 μ L of 17- β -estradiol (50 mg/mL, previously diluted in corn oil). The estradiol suspension must be placed on a magnetic stirrer to ensure homogeneity throughout the process of filling the pellets. In addition, on the previous day it is important to subject the solution to ultrasound for 3 h followed by overnight stirring. When ready, it should appear as a homogeneous milky solution. At rest, the suspension settles, and it is possible to observe two phases: the upper one (vehicle) represented by the oil is transparent while the lower one is whitish. The oil pellets must be filled with 8 μ L of corn oil. The sealing process of the open end of the capsules is the same as described above except that it requires greater care considering that the capsules are filled with liquid. After complete sealing, it is important to trim the ends using small scissors (Figure 3-2).

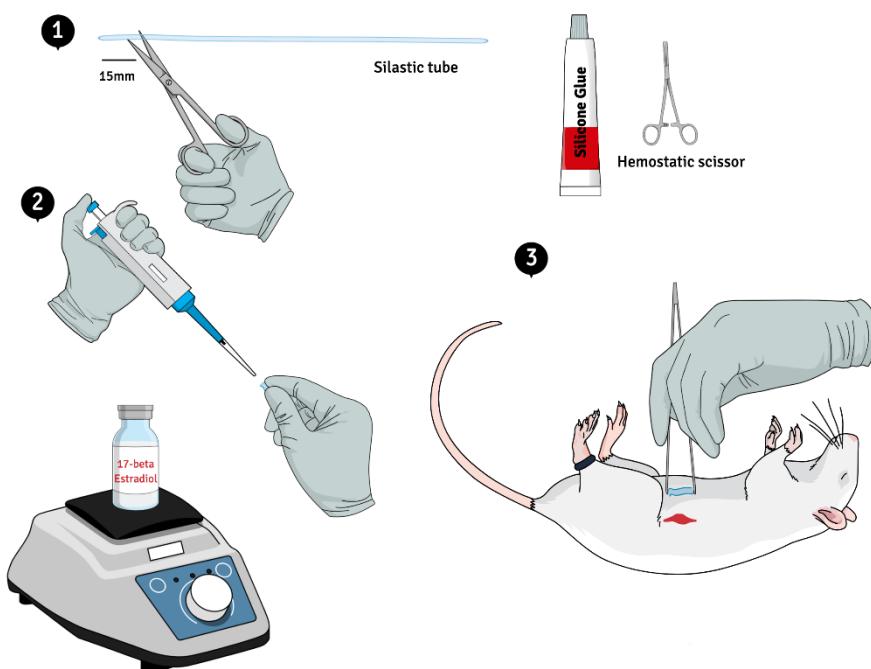


Figure 3. Schematic diagram showing the silastic capsules preparation and surgery implantation

Pellets implantation surgery

All Instruments and materials used for surgery must be previously sterilized. We recommend steam sterilization (autoclaving). Furthermore, female rats should be weighed for the doses of anesthetics and medications be given according to individual body weight.

3. The animals must be anesthetized with ketamine (55 mg/kg, IP) and xylazine (10 mg/kg, IP) and perform aseptic procedures as follows: shave the rats, scrub the surgical site with antiseptic solution (Betadine).

Note: In this protocol we use injectable anesthesia. However, is possible to use other approach such as inhaled anesthetics.

4. With the aid of a small scissors, make a discreet incision (0.5 cm) and insert the tip of the scissors to separate the tissue, a subcutaneous pocket is formed to store the pellet. This surgery is minimally invasive and quick. After surgery, the animals must receive prophylactic antibiotics (Pentabiotico 0.2 mL/rat, i.m.) and anti-inflammatory treatment (Banamine, 2.5 mg/kg, s.c).

C. Vaginal smears collection and estrous cycle evaluation (Figure 4)

The estrous cycle of female rats lasts an average of 4 to 5 days and has 4 distinct phases: proestrus, estrus, metestrus (or diestrus I) and diestrus (or diestrus II), which can be easily identified by the cell types observed in the vaginal smear (Marcondes *et al.*, 2002).

Vaginal smear collection and estrous cycle evaluation

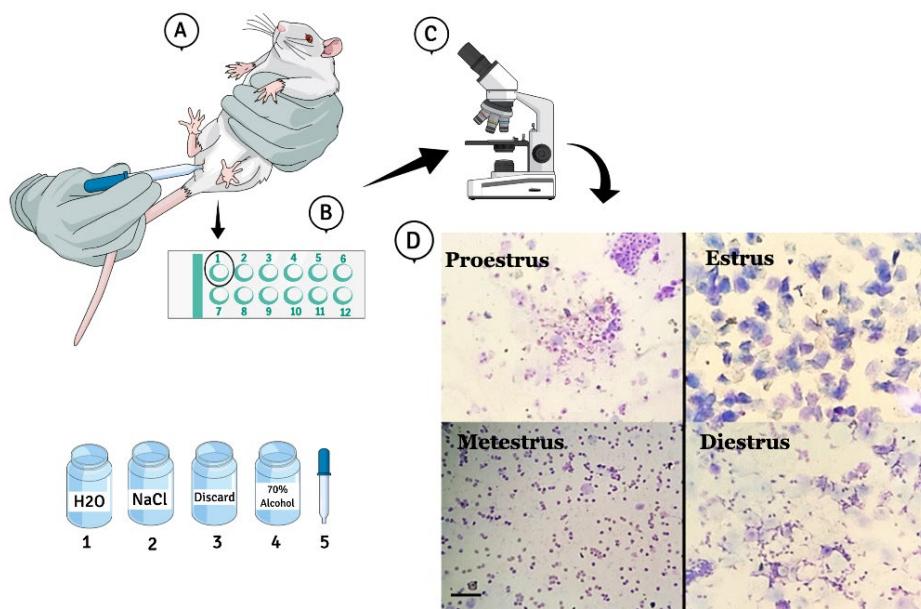


Figure 4. Schematic diagram showing the vaginal smear collection and estrous cycle evaluation.

Photomicrography (10×) kindly provided by Thalita de Oliveira Gonçalves and Guilherme de Souza Gagliano.

1. With one hand, hold the animal firmly so that the vagina is visible. With the other hand, gently insert the tip of a plastic pipette previously filled with 0.9% NaCl (approx. 10 µL) into the vagina of the rat, it is not necessary to deepen the pipette. Press the rubber on the surface of the pipette to push the NaCl, repeat this process twice as if washing (Figure 4A-1).
2. Remove the vaginal fluid and deposit a drop on a glass slide (Figure 4B). Discard the excess of the collected smear in the specific container (Figure 4-3). The pipette must be sanitized between one animal and another, filling it with water (Figure 4-1) and discarding it in container 3. Repeat the wash 3 times, then fill the pipette with 70% alcohol (Figure 4-4) and discard, the pipette will dry immediately and be ready to be reused in another animal. One drop from each rat must to be placed sequentially on the slide (Figure 4B).
3. After collecting the vaginal smear from all rats, the glass slide is ready to be observed under an optical or light microscope without the need for a lens condenser. 10× and 40× objective lenses are suitable for observing fresh material (Figure 4C).
4. When observing the slide under the microscope, it will be possible to recognize 3 distinct cell types: 1) the round and nucleated ones that resemble bunches of grape or fried eggs are non-cornified squamous epithelial cells typically observed during the proestrus; 2) irregular cells, in the shape of a dry leaf or corn flakes, are the cornified epithelial cells, characteristic of estrus; 3) small round cells are the leukocytes present in the diestrus (Figure 4D). Animals in metestrus will present a variety of these cells. It is important to emphasize that the proportion between them should be used to determine the estrous cycle phases (Marcondes *et al.*, 2002).

Note: The collection of the vaginal smear and evaluation of the estrous cycle must be carried out in the early morning, between 7:30 and 9:30 am to prevent the cells present in the vaginal fluid from moving from one

phase to the next. Since until close to 90 days after the onset of VCD treatment the proportion of rats that cycle regularly (about 20%) is the same in control rats and those treated with VCD (Carolina et al., 2019). Only control and VCD treated rats cycling regularly be used in the experiments performed before 90 days after starting VCD injections.

D. Euthanasia and blood samples collection

Between 75 and 85 days after the start of the VCD/oil injections, the animals should be euthanized in the morning, by decapitation or anesthetic overdose (ketamine [110 mg/kg, IP] and xylazine [20 mg/kg, IP]).

1. Before starting the decapitation, make sure the guillotine is sharp enough. Quickly and firmly hold the animal and position the head at the height of the trunk between the guillotine blades, once positioned, lower the lever.
2. With the aid of a plastic funnel previously heparinized and identified attached to the polyethylene tube, position the animal's body downwards, facilitating the exit of the blood. Shake the tube gently, facilitating the mixing of blood and heparin to prevent clotting. Store the tube containing blood in a refrigerated environment (2°C-8°C).
3. Remove the brain quickly and gently from the skull and freeze it immediately either on dry ice or liquid nitrogen. Store the brain at -70°C.
4. Centrifuge the blood samples in a refrigerated centrifuge at 1,200 × g for 20 min at 4°C. Separate the plasma and store at -70°C until the assay.

Note: The decapitation must be done the most humane way possible, guaranteeing the animal's well-being as well as the safety of the experimenter. Avoid noise, odors and crowding in the experimental room. The environment should be well lit with circulating air. We recommend using exhaust fans to help dispel specific odors such as blood. We recommend that the guillotine, the sink and the counter be cleaned with water and 70% alcohol to avoid the odor of blood between becapitations.

Data analysis

All comparisons were performed using one-way ANOVA followed by Newman-Keuls *post hoc* test. Data are presented as the mean ± SEM. Significance was accepted at $P > 0.05$. All statistical analyses and graphs were performed using GraphPad Prism 7 Software.

Notes

An important question regarding the accelerated ovarian failure model is: how to assess whether the VCD injections were effective in promoting the depletion of primordial and primary follicles?

We suggest performing at least 1 of the three positive control tests:

1. **Ovarian histology.** This procedure is highly effective, since significant qualitative and quantitative changes are expected in ovarian follicles (Reis et al., 2014). The disadvantage of this method is the time to obtain the results. Depending on the demand of each laboratory, between the collection of samples and obtaining the slides ready for microscopy, it will require at least 1 week.

After euthanasia (decapitation or perfusion), remove the ovaries, separate them from the connective tissue and fat and fix them in 10% formaldehyde for 24 h. Perform the histological processing that includes the dehydration processes (subject the material successively in increasing concentrations of alcohol to 70%, 90%, 100%), clearing with xylol, assembling the paraffin blocks and obtaining sections with a microtome of 8 µm thickness. To assemble the slides, we recommend semi-serial sections and stained with hematoxylin and eosin (HE). With the aid of an optical microscope, count and classify the ovarian follicles. The Leica Biosystems website provides the detailed guidance on procedures (Overview of the steps in tissue processing for paraffin

sections; <https://www.leicabiosystems.com/knowledge-pathway/an-introduction-to-specimen-processing/>).

2. **Measurement of estradiol and progesterone plasma concentrations.** Most perimenopause women have a typical hormonal profile, with reduction in progesterone plasma concentrations while estradiol concentrations are normal or erratically high (Santoro *et al.*, 1996; Prior and Hitchcock, 2011). The same pattern was observed in rats submitted to chronic VCD injections (Reis *et al.*, 2014; Pestana-Oliveira *et al.*, 2018; Carolino *et al.*, 2019). Therefore, we consider this an effective way to assess whether perimenopause was induced in female rats.

Immediately after the decapitation collect the blood samples and centrifuge at $1,200 \times g$ for 20 min at 4°C . The plasma must then be separated and be stored at -70°C until the assay. All samples must be measured in the same assay to avoid intraassay variation.

Progesterone assay: To measure progesterone plasma concentrations we recommend specific radioimmunoassay (RIA) kits for humans provided by MP Biomedicals. Polyethylene tubes must be numbered with a permanent pen in ascending order. For a more accurate assay we recommend that samples be measured in duplicate or triplicate. However, before the test it is necessary to perform a dilution test in order to identify the volume of plasma needed so that the value obtained from the samples plasma concentrations is in accordance with the kit standard curve. The MP Biomedicals website provides the step-by-step instructions on how to perform the assay.

Estradiol assay: To measure estradiol plasma concentrations we recommend specific kit for an enzyme-linked immunosorbent assay provided by DRG.

3. **Measurement of anti-Mullerian plasma concentrations.** It is well established in the literature that the anti-Mullerian hormone concentrations (AMH) have a direct correlation with the number of ovarian follicles, being thus considered a marker of ovarian follicular reserve whose levels decrease with increasing age, and therefore practically undetectable after menopause (Cui *et al.*, 2015). Also, the AOF model in both monkeys and mice has demonstrated a linear correlation between reduced levels of AMH and reduced number of developing follicles (Sahambi *et al.*, 2008). In a recently published study using the AOF model in female rats, Dr. Anselmo-Franci's laboratory (Carolino *et al.*, 2019) demonstrated that in fact plasma AMH concentrations can be considered a good index of ovarian follicular reserve as well as effectiveness of VCD injections in follicular depletion. To measure AMH plasma concentrations, use a specific enzyme-linked immunosorbent assay kit for mice and rat provided by Ansh Labs (Webster, TX, USA).

Recipes

1. 4-vinylcyclohexene diepoxide dilution (VCD, Figure 5)

The chemical compound VCD is obtained through epoxidation catalyzed by cytochrome P450 from 4-vinylcyclohexene (HCV), an occupational ovarian toxin produced from the dimerization of 1,3-butadiene during the manufacture of synthetic rubber, flame retardants, insecticides and plasticizers (Mayer *et al.*, 2002). The occupational chemical 4-vinylcyclohexene diepoxide (VCD) has been shown to cause selective destruction of ovarian small pre-antral (primordial and primary) follicles in rats and mice by accelerating the natural, apoptotic process of atresia (Kappeler and Hoyer, 2012).

To obtain the dose of 160 mg/Kg (1.25 $\mu\text{L/g}$ body weight) dilute the VCD reagent in corn oil at a concentration of 12%, in other words, if you want to prepare 100 mL of stock solution, measure 12 mL of VCD in a graduated cylinder and make up to 100 mL with corn oil. With the aid of a magnetic stirrer, homogenize the solution. The ultrasound is not necessary to improve the dilution.

Notes:

- a. *The amount of solution can be prepared according to the demand of each laboratory. On average every 6 months it is possible to prepare 1 L of stock solution that should be stored at RT and kept in an amber bottle or wrapped in aluminum foil to avoid exposure to light. We recommend distributing the stock solution in smaller bottles that will be used routinely.*

- b. In all processes involving the handling of the chemical compound VCD, the use of personal protective Equipment is essential. In addition, dilution of the VCD must be performed under ventilation and properly cleaned with 70% alcohol. Avoid skin contact and inhalation.

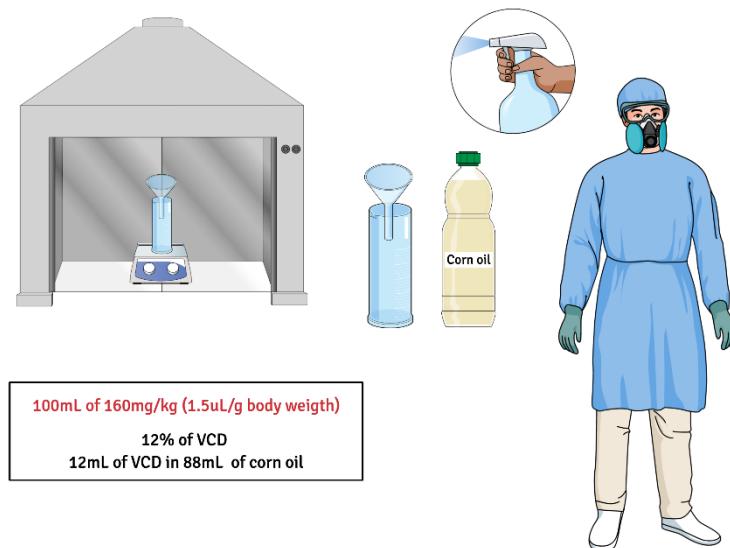


Figure 5. Schematic diagram showing the 4-vinylcyclohexene diepoxide (VCD) dilution

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Competing interests

The authors declare that they have no conflict of interest.

Ethics

All procedures were approved by the Committee for Animal Care and Use (2013.1.1412.58.7), University of São Paulo.

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Identification of R-loop-forming Sequences in *Drosophila melanogaster* Embryos and Tissue Culture Cells Using DRIP-seq

Célia Alecki^{1,2} and Nicole J. Francis^{1,2,3,*}

¹Institut de recherches cliniques de Montréal, 110 Avenue des Pins Ouest, Montréal, QC, H2W 1R7, Canada;

²Département de biochimie et médecine moléculaire Université de Montréal, 2900 Boulevard Edouard-Montpetit, Montréal, QC, H3T 1J4, Canada; ³Division of Experimental Medicine, McGill University, 1001 Decarie Boulevard, Montreal, QC, H4A 3J1, Canada

For correspondence: nicole.francis@ircm.qc.ca

Abstract

R-loops are non-canonical nucleic structures composed of an RNA-DNA hybrid and a displaced ssDNA. Originally identified as a source of genomic instability, R-loops have been shown over the last decade to be involved in the targeting of proteins and to be associated with different histone modifications, suggesting a regulatory function. In addition, R-loops have been demonstrated to form differentially during the development of different tissues in plants and to be associated with diseases in mammals. Here, we provide a single-strand DRIP-seq protocol to identify R-loop-forming sequences in *Drosophila melanogaster* embryos and tissue culture cells. This protocol differs from earlier DRIP protocols in the fragmentation step. Sonication, unlike restriction enzymes, generates a homogeneous and highly reproducible nucleic acid fragment pool. In addition, it allows the use of this protocol in any organism with minimal optimization. This protocol integrates several steps from published protocols to identify R-loop-forming sequences with high stringency, suitable for *de novo* characterization.

Keywords: DRIP, R-loop, S9.6 antibody, *Drosophila melanogaster*, RNA-DNA hybrid, Tissue culture cells

This protocol was validated in: Nat Commun (2020), DOI: 10.1038/s41467-020-15609-x

Graphical Abstract:

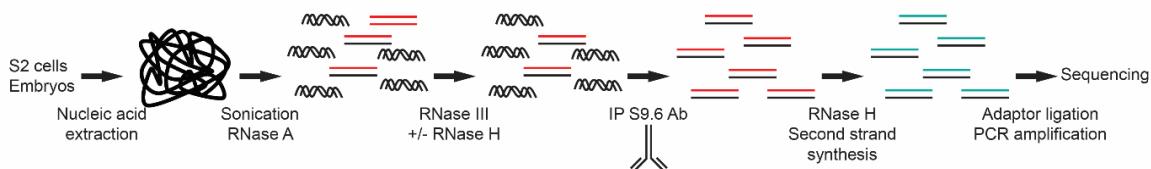


Figure 1. Overview of the strand-specific DRIP-seq protocol

Background

Overview

R-loops are triple-stranded nucleic acid structures that form when an RNA hybridizes with a complementary ssDNA, leading to displacement of the second DNA strand. R-loops were first described as a by-product of transcription and a source of genomic instability that needed to be resolved. However, research over the last decade has shown that R-loops can be associated with specific histone modifications and transcriptional status, induce the targeting of proteins, or act as promoters (Chedin and Benham, 2020; Niehrs and Luke, 2020). To study R-loop function, protocols have been developed to map these structures in multiple organisms. Differences have been observed between species: R-loop-forming sequences in mammals have a GC skew; those in *Saccharomyces cerevisiae* have an AT skew; and in plants, both GC and AT skews have been reported (Sanz *et al.*, 2016; Wahba *et al.*, 2016; Xu *et al.*, 2017; Hartono *et al.*, 2018). Differences in the properties and localization of R-loop-forming sequences between species and the association of R-loops with different diseases and gene misexpression highlight the importance of developing standard R-loop mapping protocols to perform rigorous comparisons.

R-loop maps in plants have shown that some R-loops can form differentially during development (Fang *et al.*, 2019; Xu *et al.*, 2020) and may potentially be involved in changes in transcriptional status. In mammals, active enhancers have been associated with R-loop formation, and these R-loops can act as promoters (Tan-Wong *et al.*, 2019). The study of R-loops in the context of development in intact organisms with available genetic tools to evaluate their formation, resolution, and function, will allow a better understanding of R-loop biology.

Here, we provide a detailed strand-specific DRIP-seq protocol to identify R-loop-forming sequences in *Drosophila melanogaster* embryos and tissue culture cells (Figure 1). Similar to other DRIP protocols, our protocol relies on the S9.6 antibody to identify R-loop-forming sequences but also considers that the weak affinity of the S9.6 antibody for dsRNA can generate false positive signals (Phillips *et al.*, 2013; Hartono *et al.*, 2018; König *et al.*, 2017). This single-strand DRIP-seq protocol in association with the genetic tools available for *Drosophila melanogaster* should be a powerful system to study R-loop function in the whole organism, during development, and in different tissues (Figure 2, Table 1). DRIP-seq can be combined with genetic manipulation to overexpress or knockdown genes with a view to identifying their effect on R-loop formation, or with cell synchronization to follow R-loop formation during the cell cycle. Finally, the use of sonication rather than restriction enzymes to fragment the genomic DNA means that this protocol could potentially be adapted to other organisms with minimal optimization.

Limitations

The protocol relies on the S9.6 antibody to immunoprecipitate RNA-DNA hybrid-containing fragments. This antibody is not a perfect tool: it shows differential binding affinity based on the sequence of the R-loop with no correlation with the GC content and a preference for longer RNA-DNA hybrids. It can bind dsRNA, albeit with an affinity 5-times lower than that for RNA-DNA hybrids (Phillips *et al.*, 2013; König *et al.*, 2017). The latter two limitations can be overcome during the preparation of nucleic acids. After nucleic acid extraction, ssRNA and dsRNA are removed by incubating the nucleic acids with RNase A in the presence of 0.5 M NaCl to avoid the

degradation of RNA in the RNA-DNA hybrids. This first digestion step is followed by an incubation with RNase III to degrade any dsRNA that were not degraded by RNase A. By using two different RNases before immunoprecipitation, we removed all ssRNA and dsRNA but also potentially some R-loops, which can be sensitive to these RNases. Thus, these steps make the experiment more stringent and limit false positives but could be modified to balance stringency with sensitivity, since some R-loops may be sensitive to these treatments. Removal of RNA that can interact with S9.6 is most relevant when the RNA component of the R-loop is to be sequenced; although, we have observed that the RNA-DNA hybrid pulldown efficiency is increased when stringent RNase treatment is used. It is also essential to have an RNase H-treated negative control to evaluate the specificity of the pulldown (Figure 3). We found that commercially available *E. coli* RNase H does not consistently completely digest RNA-DNA hybrids in nucleic acid preparations. This can be solved by producing and using highly active human RNase H1 and RNase H2. This negative control is especially important during optimization of the DRIP protocol; it ensures that the technique is specific. It is also used to call peaks when DRIP-seq is performed (Figures 2, 4, and 5).

To avoid bias toward longer fragments during the immunoprecipitation step, we fragment the nucleic acids by sonication (Figure 4A). Sonication allows us to obtain a homogenous population of fragments with an average size of 300 bp. Sonication is performed after RNase A treatment to avoid the possibility of generating new RNA-DNA hybrids during the DRIP procedure. Sonication is reported to reduce the recovery of some RNA-DNA hybrids as compared with restriction digestion (Crossley *et al.*, 2020).

Advantages

Advantages compared with other DRIP protocols

This protocol uses a gentle lysis step to extract the nucleic acids. Cell lysis can be performed on tissue culture cells, whole embryos, or dissected tissues from larval, pupal, or adult *Drosophila melanogaster*. Although the ~200 mg *Drosophila* embryos needed to perform one DRIP-seq experiment is relatively high, the possibility to freeze tissues and pool them for a single nucleic acid extraction makes it feasible. By dissecting *Drosophila melanogaster* and performing DRIP on discs, organs, or sorted cell populations, it should be possible to identify cell- or tissue-specific R-loops.

This DRIP protocol, contrary to several protocols developed for mammalian cells (*e.g.*, Sanz *et al.*, 2016), does not use a restriction enzyme cocktail to fragment the nucleic acids. Instead, sonication is used, which leads to the fragmentation of nucleic acids at an average size of 300 bp (Figure 4A). These fragments have a homogenous size and the sonication is highly reproducible. An increase in resolution by using sonication, as compared with restriction digestion, in mammalian cells has recently been demonstrated (Crossley *et al.*, 2020). By using sonication, the protocol can easily be adapted to other organisms with little optimization; the only step that may require optimization is lysis. Sonication has another advantage: it leads to disruption of the displaced single-strand DNA of the R-loop, which makes it possible to prepare strand-specific sequencing libraries using either the DNA or the RNA moiety of the RNA-DNA hybrid (Wahba *et al.*, 2016).

Advantages compared with other methods

Another method to identify R-loop-forming sequences relies on a catalytically inactive form of the RNase H1 enzyme (dRNase H1) (Ginno *et al.*, 2012; Chen *et al.*, 2017). The use of dRNase H1 in cells presents several potential problems, which may explain the differences in the identification of R-loop-forming sequences with this method versus the S9.6 antibody. Firstly, RNase H1 is not the only regulator of R-loops in cells; it has been suggested that topoisomerases are the main enzymes responsible for the resolution of R-loops that form co-transcriptionally in human cells (Manzo *et al.*, 2018; Zhang *et al.*, 2019), while RNase H1 and H2 target R-loops once they are formed. Secondly, a recent article by Lockhart *et al.*, 2019 demonstrated that RNase H1 is activated upon stress, while RNase H2 displays the main activity under physiological conditions in *S. cerevisiae*. Thirdly, with dRNase H1, R-loop identification may be limited to R-loops that are accessible, protein-free, and normally degraded by RNase H enzymes. Fourthly, expression of dRNase H1 may stabilize R-loops. While this may allow detection of transient R-loops, it could also skew interpretation of where stable R-loops exist. Finally, RNase H1 has two RNA-DNA hybrid binding domains, both of which need to bind to induce degradation of the RNA moiety of the hybrid (Nowotny *et al.*, 2008). This may prevent or limit the detection of smaller RNA-DNA hybrids. Thus, while dRNase

H1 may be a useful tool in some contexts, the interpretation of DRIP results may be more straightforward. Comparison of results from both methods could also yield complementary information.

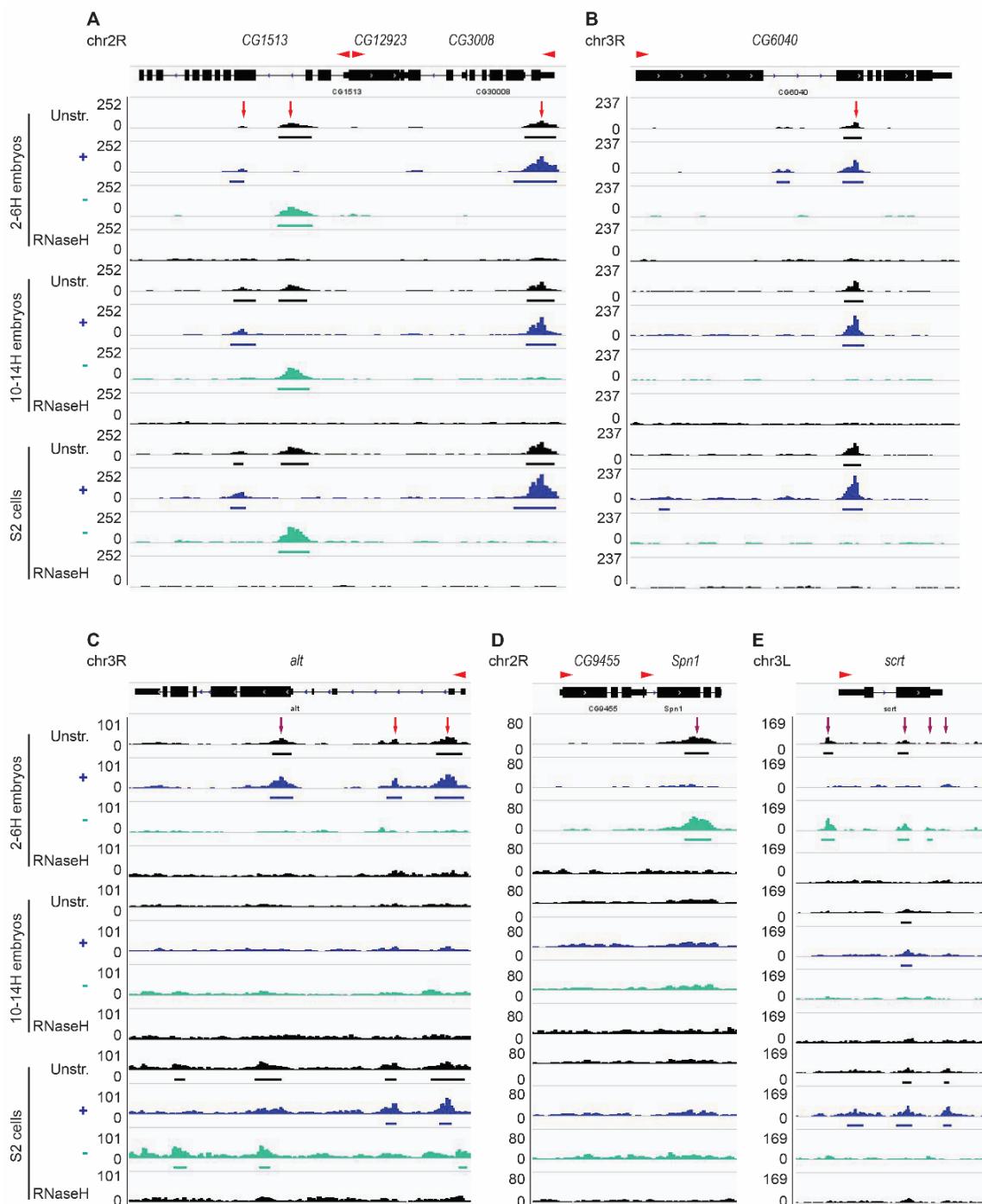


Figure 2. Examples of shared and distinct R-loops among S2 cells and early and later stage embryos.

A-B. R-loops form over the *CG1513*, *CG3008*, and *CG6040* genes in S2 cells and 2-6 H and 10-14 H embryos (red arrow). No R-loop is formed over the *CG12923* gene. C. At the *alt* gene, three R-loops are detected in 2-6 H but not in 10-14 H embryos. Two of these R-loops (red arrows) are also present in S2 cells, while one of them is replaced by an R-loop on the opposite strand (purple arrow). D. R-loop formed over *Spn1* is only present in 2-6 H embryos (purple arrows). E. R-loops form over *scrt* on the + strand in 2-6 H embryos and the - strand in 10-14 H embryos

and in S2 cells. “Unstr” corresponds to unstranded DRIP-seq data, “+” and “-” indicate the strand-specific track, and RNase H-treated sample acts as a negative control. The red arrowhead indicates the orientation of the transcript. Note that the “+” and “-” strands refer to the DNA strand such that “+” strand R-loops should arise from transcription in the leftward direction. Thus, in A, the R-loop in the *CG3008* gene is in the expected orientation to have arisen from gene transcription, while that in the gene *CG1513* would be derived from antisense transcription.

Similar to DRIP, dRNase H1 has been used to isolate RNA-DNA hybrids after extraction of nucleic acids from tissue culture cells; however, bias of the enzyme toward longer hybrids and its weaker affinity as compared with the S9.6 antibody make it less efficient (Ginno *et al.*, 2012).

Finally, native bisulfite sequencing is an alternative high-resolution method for identifying R-loops. Bisulfite converts cytosine to uracil in single-stranded DNA (Yu *et al.*, 2003); thus, this method does not detect RNA-DNA hybrids but the ssDNA strand that is displaced when they form. However, the presence of methyl-cytosine in the genome blocks modification of the ssDNA, and ssDNA can be displaced by the formation of other non-canonical DNA structures such as G-quadruplexes and I-motifs, which could lead to false negative or false positive results, respectively.

The single-strand DRIP-seq protocol presented here has been optimized to identify R-loop-forming sequences in *Drosophila melanogaster* embryos and tissue culture cells, and can potentially be used in other organisms with minimal optimization. This protocol could be a standardized means to evaluate R-loops across developmental stages or in different tissues or organisms.

Table 1. Data availability

Deposited data (from Alecki <i>et al.</i> , 2020)	
DRIP-seq 2-6H Oregon R embryos	GSE127329
DRIP-seq 10-14H Oregon R embryos	GSE127329
DRIP-seq S2 cells	GSE127329

Materials and Reagents

A. RNase H1 and H2 expression and purification

1. Amicon® ultra centrifugal filter, 0.5 mL 10K (EMD Millipore, catalog number: UFC5010BK)
2. Amicon® ultra centrifugal filter, 0.5 mL 3K (EMD Millipore, catalog number: UFC5003BK)
3. Econo-column (2.5*20 cm) (Bio-Rad, catalog number: 7374252)
4. *E. coli* Rosetta™ 2(DE3)pLysS Singles™ Competent Cells – Novagen (Sigma, catalog number: 71401)
5. Ni-NTA agarose (Qiagen, catalog number: 1018244)
6. Glutathione-superflow resin (Takara, catalog number: 635607)
7. PreScission protease plus (Homemade; commercial enzyme could also be used)
8. Ampicillin (Bioshop, catalog number: AMP201.100)
9. LB Broth Miller (Bioshop, catalog number: LBL407.500)
10. Albumin, bovine serum (Bioshop, catalog number: ALB001.100)
11. Lysozyme (Bioshop, catalog number: LYS702.5)
12. Imidazole (OmniPur) (Millipore, catalog number: 5710-OP)
13. Glycerol (Bioshop, catalog number: GLY001.4)
14. IPTG (Bioshop, catalog number: IPT002.5)
15. RNase H2 extraction buffer (see Recipes)
16. RNase H1 lysis buffer (see Recipes)
17. RNase H1 wash buffer (see Recipes)
18. RNase H1 elution buffer (see Recipes)
19. RNase H size column buffer (see Recipes)
20. RNase H storage buffer (see Recipes)

21. Protease inhibitors (see Recipes)

B. Protease inhibitors and additives

1. TLCK (Sigma, catalog number: T7254)
2. Benzamidine (Bioshop, catalog number: BEN601.25)
3. Pepstatin A (Bioshop, catalog number: PEP605.25)
4. 1,10-Phenanthroline (Sigma, catalog number: 131377-5G)
5. PMSF (Fisher Scientific, catalog number: 19538125)
6. Aprotinin (Bioshop, catalog number: APR200)
7. Leupeptin (Bioshop, catalog number: LEU011.50)
8. NP40 (Nonidet P40 substitute, Fluka catalog number: 74385) (can substitute Sigma, catalog number: 74385)
9. DTT (Bioshop, catalog number: DTT002.100)

C. Activity testing of RNase H1 and H2

1. rNTP (NEB, catalog number: N0450S)
2. UTP α -P32 (Perkin Elmer, catalog number: BLU007H250UC)
3. T7 RNA polymerase (NEB, catalog number: M0251L)
4. SSC 20 \times (see Recipes)
5. Tri-sodium citrate (Bioshop, catalog number: CIT001.205)

D. *Drosophila melanogaster* embryo collection

1. Fly bottles
2. Oregon R flies (Dr Éric Lécuyer lab; available through Bloomington Drosophila stock center)
3. Agar A (Bioshop, catalog number: FB0010)
4. Sugar (RedPath)
5. Apple juice
6. TEGOSEPT, 1 KG (Nipagin) (Diamed.ca, catalog number: GEN20-258)
7. Homemade sieve made with NITEX (can be obtained from <https://flystuff.com/>)
8. Funnel
9. Fly cages (fly cages, food, and bottles can be obtained from <https://flystuff.com/>)
10. Fly food (prepared in-house)
11. Methanol (Bioshop, catalog number: MET302.4)
12. Embryo lysis buffer (see Recipes)
13. Apple juice plate (see Recipes)
14. 1 \times PBS (see Recipes)
15. 1 \times PBT (see Recipes)

E. Cell culture

1. S2 cells (Invitrogen, catalog number: R69007)
2. GibcoTM Schneider's Drosophila Sterile Medium (Thermo Fisher Scientific, catalog number: 21720-024)
3. FBS (Thermo Fisher Scientific, catalog number: 16140-089)

F. Nucleic acid extraction and preparation

1. Proteinase K (Biobasic, catalog number: PB0451)

2. Phase lock gel, heavy (VWR, catalog number: 10847-802)
3. DNase I (RNase-free) (NEB, catalog number: M0303L)
4. Ambion™ RNase III (Thermo Fisher Scientific, catalog number: AM2290)
5. RNase A (Qiagen, catalog number: 19101)
6. UltraPure™ DNase/RNase-free distilled water (Thermo Fisher Scientific, catalog number: 10977015)
7. Covaris microTUBE AFA fiber pre-split snap-cap 6*16 mm (Covaris, catalog number: 520045)
8. Phenol-chloroform isoamylalcohol (Bioshop, catalog number: PHE512.400)
9. Chloroform (Bioshop, catalog number: CCL402.1)
10. Reagent alcohol (Sigma, catalog number: 277649-1)
11. Tris (Bioshop, catalog number: TRS001.10)
12. Acetic acid, glacial (Thermo Fisher Scientific, catalog number: 351271-212)
13. EDTA (Bioshop, catalog number: EDT002.500)
14. Sodium acetate (Bioshop, catalog number: SAA304.5)
15. Sodium chloride (Bioshop, catalog number: SOD002.10)
16. 1× RNase H buffer (see Recipes)
17. TE (see Recipes)

G. DRIP

1. Anti-DNA-RNA Hybrid [S9.6] antibody (Kerafast, catalog number: ENH002, hybridomas are available through ATCC, HB-8730)
2. Dynabeads™ Protein G for Immunoprecipitation (Thermo Fisher Scientific, catalog number: 10004D)
3. NucleoSpin® Gel and PCR (Macherey-Nagel, catalog number: 740609.250)
4. DNA Clean & Concentrator™ (Zymo Research, catalog number: D4014)
5. BSA, molecular biology grade (NEB, catalog number: B9000S)
6. 10× DRIP binding buffer (see Recipes)
7. 1× DRIP binding buffer (see Recipes)
8. DRIP elution buffer (see Recipes)

H. Library preparation and qPCR

1. NEBNext® Multiplex Oligos for Illumina® (Index Primers Set 1) (NEB, catalog number: E7735S)
2. NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina® (NEB, catalog number: E7760S)
3. RNase H (New England Biolabs (NEB), catalog number: M0297L)
4. PowerUp SYBR Green PCR master mix (Thermo Fisher Scientific, catalog number: A25741)

I. Agarose and SDS-PAGE gel preparation and staining

1. SYBR™ Gold Nucleic Acid Gel Stain (Thermo Fisher Scientific, catalog number: S11494)
2. SYPRO Ruby Solution (Lonza, catalog number: 50562)
3. Glycine (Bioshop, catalog number: GLN001.10)
4. 0.5× TBE (see Recipes)
5. 10× DNA loading buffer (see Recipes)
6. SDS-PAGE running buffer (see Recipes)
7. Protein loading buffer (see Recipes)

J. Plasmids

1. pHis-MBP-hRNaseH1 (Alecki *et al.*, 2020)
2. pGEX6P1-hsRNASEH2BCA (Addgene, catalog number: 108693)
3. vg-pETBlue (Alecki *et al.*, 2020)

Equipment

1. Typhoon Imager (GE Healthcare) for in-gel fluorescence imaging and phosphorimaging
2. ViiA7 PCR system (Thermo Fisher Scientific) for real-time PCR
3. Covaris E220 (Covaris) for nucleic acid sonication
4. Centrifuge Avanti® J-E (Beckman Coulter)
5. AKTA FPLC (or equivalent chromatography system)
6. HiLoad 20/60 Superdex 200 column (GE Healthcare, catalog number: GE28-9893-36) (available from Sigma)
7. Superdex 200 10/300 GL size exclusion column (GE Healthcare, catalog number: 28990944)
8. Sonicator (Sonics, Vibra cells™) (probe sonicator for cell lysis)
9. Nutator rotating platform to gently mix tubes
10. Bacterial shaker
11. 10 cm Petri dish
12. Liquid nitrogen for flash freezing
13. -80°C Freezer
14. Nanodrop spectrophotometer
15. Water bath
16. 24°C Incubator
17. 27°C Shaking incubator for S2 cells; S2 cells can also be grown on plates at room temperature

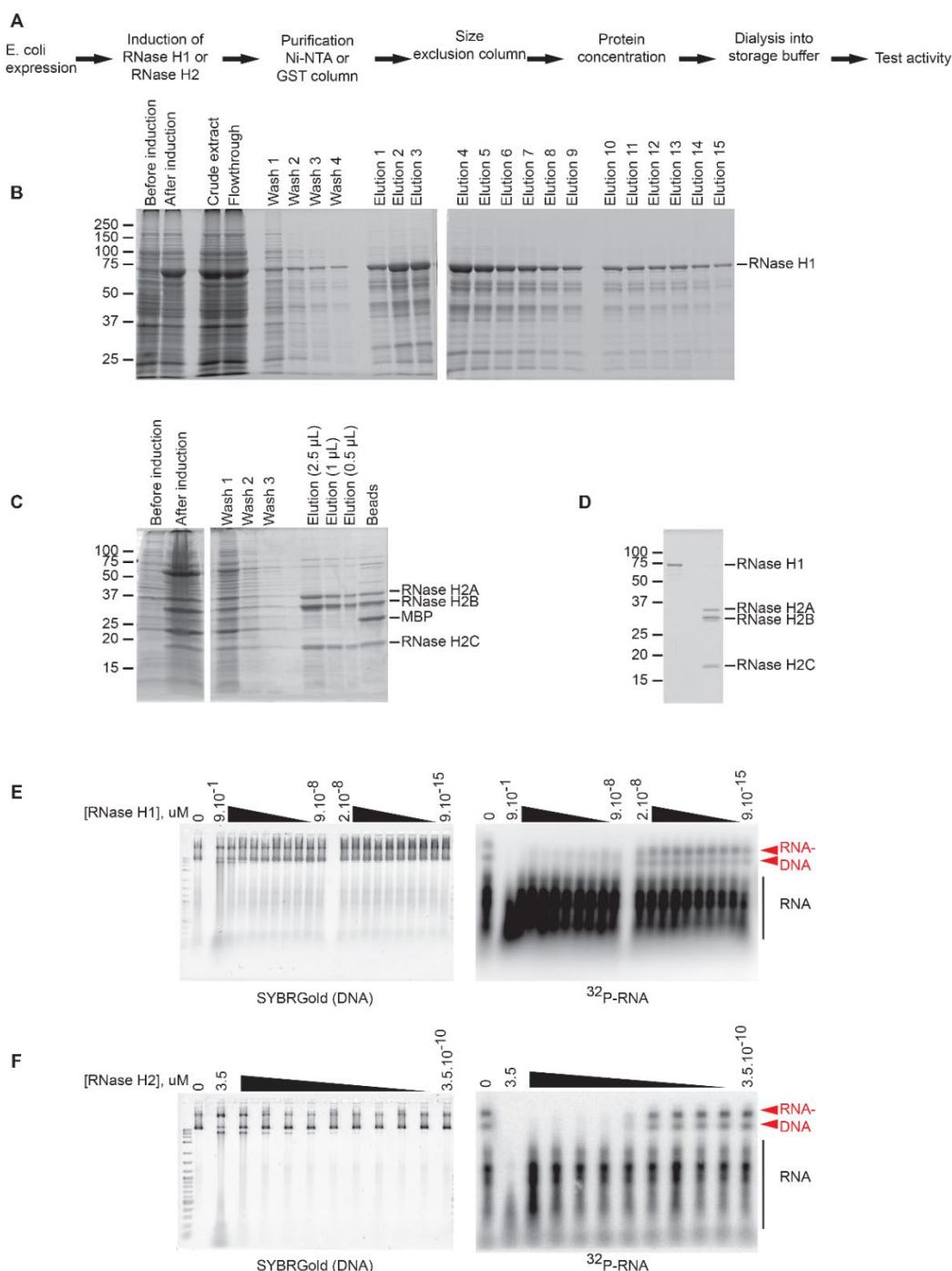
Procedure

A. RNase H1 and H2 expression and purification

1. RNase H1 expression and purification (Figures 3A-3D)
 - a. Transform *E. coli* Rosetta cells with pHis-MBP-hRNaseH1. The next day, inoculate 2 mL LB-ampicillin (100 ng/μL) with a single colony and allow it to grow O.N. at 37°C in a shaking incubator.
 - b. Add 1 mL culture from (a) to 200 mL LB-ampicillin and allow it to grow O.N. at 37°C in a shaking incubator.
 - c. Add 100 mL culture from (b) to 1 L LB-ampicillin and induce RNase H1 expression with 0.1 mM IPTG for 4 h at 37°C.
 - d. Harvest the cells by centrifuging for 20 min at 4,000 × g, 4°C.
 - e. Discard the supernatant, freeze the cell pellet in liquid nitrogen, and store for at least a few hours at -80°C.
 - f. Thaw the cell pellet on ice and add 20 mL RNase H1 lysis buffer containing 0.1 mg/mL lysozyme, 100 μg RNase A, 100 μg DNase I, and protease inhibitors.
 - g. Incubate for 30 min on ice.
 - h. Keep the cells on ice and sonicate 12 times for 15 s ON, 15 s OFF at an amplitude of 60%.
 - i. Centrifuge for 20 min at 20,000 × g, 4°C.
 - j. Keep the supernatant as the crude extract. The supernatant can be frozen in liquid nitrogen and stored at -80°C for future purification.
 - k. Wash 3 mL Ni-NTA beads 3 times with 15 ml RNase H1 lysis buffer in a 50-mL tube by centrifuging for 2 min at 207 × g.
 - l. Add the crude extract to the beads and incubate O.N. at 4°C on a nutator. Starting from this step, all the washes and elutions must be performed at 4°C to ensure minimal protein degradation.
 - m. Wash an Econo column with RNase H1 lysis buffer and transfer the beads and crude extract into the column.
 - n. Save the flowthrough (if all the RNase H1 protein did not bind to the beads, the flowthrough can be incubated with the Ni-NTA resin a second time).

- o. Wash 4 times with 15 mL RNase H1 wash buffer supplemented with protease inhibitors and collect each wash.
 - p. Elute the protein 5 times with 3 mL RNase H1 elution buffer containing protease inhibitors; collect 1-mL fractions.
 - q. Load 2.5 μ L each fraction on a 10% SDS-PAGE gel and stain with SYPRO Ruby following the manufacturer's instructions (Figure 3B).
 - r. Pool the elutions with similar concentrations and dialyze them against 1 L chilled RNase H storage buffer.
 - s. Equilibrate a HiLoad 20/60 Superdex 200 size exclusion column on an AKTA FPLC with 2-3 CV 20 mM Tris-HCl pH 7.0 containing 150 mM NaCl and 0.2 mM PMSF at 4°C (equilibration can be performed overnight).
 - t. Concentrate the sample to \leq 5 mL if necessary. Centrifuge at \geq 10,000 \times g for 10 min at 4°C to remove any precipitate and load on the Superdex 200 column.
 - u. Run the Superdex 200 column at 4°C. Collect 1.5-mL fractions.
 - v. Run 5 μ L each fraction on a 10% SDS-PAGE gel and stain with SYPRO Ruby.
 - w. Pool the fractions containing RNase H1 protein without degradation products and dialyze them twice O.N. at 4°C against 2 L chilled RNase H storage buffer.
 - x. Concentrate the protein 2-3-fold with an Amicon ultra column 10,000 MCWO following the manufacturer's instructions to obtain a protein concentration of 0.5-1 μ g/ μ L.
 - y. Aliquot, freeze in liquid nitrogen, and store at -80°C.
 - z. Run 2.5 μ L on a 10% SDS-PAGE gel and stain with SYPRO Ruby to confirm the quality of the preparation (Figure 3D).
 - aa. Determine the protein concentration on a NanoDrop using an extinction coefficient = 81,050 M⁻¹cm⁻¹ and a MW = 32 kDa. For 1 L of culture, ~9 mg purified RNase H1 is expected.
2. RNase H2 expression and purification (Figures 3A-3D)
 - a. Transform *E. coli* Rosetta cells with pGEX6P1-hsRNASEH2BCA. Inoculate two 5-mL starter cultures of LB-ampicillin (100 ng/ μ L) with a single colony and grow O.N. at 37°C with shaking.
 - b. The next day, inoculate 1 L LB-ampicillin with the 10-mL culture and allow to grow for 4 h at 37°C on a nutator.
 - c. Allow the culture to sit for 1 h at RT to cool. Save 500 μ L to run on an SDS-PAGE gel as the uninduced fraction.
 - d. Induce with 0.1 mM IPTG O.N. at 20°C with shaking.
 - e. Harvest the cells by centrifuging for 20 min at 15,000 \times g, 4°C.
 - f. Resuspend the pellet in 15 mL 1 \times PBS containing 0.2% Tween-20, 10 mM MgCl₂, and protease inhibitors.
 - g. Freeze in liquid nitrogen and thaw at 37°C (3 times) to facilitate cell lysis.
 - h. Sonicate on ice 30 times for 30 s ON, 30 s OFF at an amplitude of 60%.
 - i. Centrifuge for 20 min at 20,000 \times g, 4°C to pellet the cell debris.
 - j. Save the supernatant as the crude extract and run 10 μ L on a 12% SDS-PAGE gel to verify the induction. The crude extract can be frozen in liquid nitrogen and stored at -80°C for future purification.
 - k. Wash 1.5 mL glutathione superflow beads 3 times with 1 \times RNase H2 extraction buffer.
 - l. Add the crude extract to the beads and incubate O.N. at 4°C on a nutator. All the remaining steps should be carried out at 4°C.
 - m. Wash the beads twice with 10 mL 1 \times RNase H2 extraction buffer containing protease inhibitors and once with 4 mL 1 \times RNase H2 extraction buffer without protease inhibitors.
 - n. Resuspend the beads in 2 mL 1 \times extraction buffer supplemented with 25 μ g PreScission protease plus (we prepare our own PreScission; commercial enzyme could also be used).
 - o. Incubate O.N. at 4°C on a nutator.
 - p. Collect the flowthrough containing the RNase H2 released by PreScission cleavage.
 - q. Repeat the incubation with 1 \times RNase H2 extraction buffer and PreScission protease.
 - r. Analyze 10 μ L input and washes, 1 μ L each elution, and 10 μ L beads on a 12% SDS-PAGE gel.
 - s. Stain the gel with SYPRO Ruby following the manufacturer's instructions (Figure 3C).

- t. Pool the elutions containing protein and concentrate them 5-fold with an Amicon ultra column 3,000 MCWO following the manufacturer's instructions. The final concentration should be ~30 mg/mL in a volume of ~750 µL.
- u. Equilibrate a 24-mL Superdex 200 10/300 GL size exclusion column with 2 CV 20 mM Tris-HCl pH 7.0 containing 150 mM NaCl and 0.2 mM PMSF.
- v. Load the RNase H2 on the column and collect 500-µL fractions.
- w. Run 5 µL each fraction on a 12% SDS-PAGE gel and stain with SYPRO Ruby.
- x. Pool all the fractions containing the RNase H2 complex and dialyze them O.N. at 4°C against 1 L chilled RNase H storage buffer.
- y. Aliquot, freeze in liquid nitrogen, and store at -80°C.
- z. Run 0.1, 0.5, and 2.5 µL on a 12% SDS-PAGE gel next to a standard dilution of BSA and stain with SYPRO Ruby to determine the protein concentration (Figure 3D).
 - aa. For 2 L of culture, ~5 mg purified RNase H2 trimer is expected.

**Figure 3. Preparation of human RNase H1 and H2 proteins.**

A. Overview of RNase H1 and H2 expression and purification. B-C. SDS-PAGE gels of RNase H1 (B) and RNase H2, and (C) induction and purification on an Ni-NTA or GST column. D. SDS-PAGE gel of RNase H1 and H2 protein after purification and dialysis, stained with SYPRO Ruby. E-F. Agarose gel of transcribed DNA after incubation with RNase H1 (E) or RNase H2 (F), showing that both enzymes are active and degrade RNA-DNA hybrids.

3. Activity testing of RNase H1 and H2 (Figures 3E-3F)

A plasmid containing a sequence prone to forming R-loops is transcribed and used as a template to measure the ability of RNase H1 or RNase H2 to degrade R-loops. We use a plasmid with the vg PRE cloned into pETBlue (Alecki *et al.*, 2020).

- a. Assemble on ice (Table 2).

Table 2. *In vitro* transcription assay

	Final concentration	Volume (μL)
Tris-HCl 1 M pH 8.0	40 mM	2
MgCl ₂ 100 mM	8 mM	4
NaCl 1 M	25 mM	1.26
Spermidine 100 mM	2 mM	1
DTT 1M	30 mM	1.5
ATP 1 mM	40 nM	2
CTP 1 mM	40 nM	2
GTP 1 mM	40 nM	2
UTP 1 mM	8 nM	0.4
UTP α -P32	2.6 nM	0.2
DNA (vgcorePRE-pET) 100 ng/ μL		5
T7 RNA polymerase (NEB)		0.125
H ₂ O DNase/RNase-free		30.3
Final volume		50

- b. Incubate for 30 min at 30°C.
- c. Stop the reaction by heating the sample for 10 min at 65°C.
Titrate RNase H1 or RNase H2.
- d. Assemble on ice (Table 3).

Table 3. Activity testing of purified RNase H1 or RNase H2

	Per reaction (μL)
H ₂ O DNase/RNase-free	5
10× RNase H buffer	1
Transcribed vgcorePRE-pET	3
RNase H1 or RNase H2	1
Final volume	10

Prepare serial dilutions of RNase H1 (10-fold steps) or RNase H2 (5-fold steps) in storage buffer.

- e. Incubate for 1 h at 37°C.
- f. Add 1 μL 10× DNA loading buffer.
- g. Load on a 1% agarose 0.5× TBE gel.
- h. Stain with SYBRGold and image.
- i. Incubate the gel for 15 min in H₂O.
- j. Incubate the gel for 20 min in 75 mM NaOH.
- k. Incubate the gel for 15 min in 0.5 M Tris-HCl 1.5 M NaCl.
- l. Incubate the gel for 30 min in 6× SSC.
- m. Transfer the gel O.N. to a HYBOND membrane (downward transfer) with the wick in 20× SSC.
- n. Expose the membrane to a phosphorimager screen.
- o. Scan on a Typhoon or equivalent phosphorimager (Figures 3E-3F).

B. Embryo collection

Note: This site (https://openspim.org/Drosophila_embryo_sample_preparation) provides a more detailed explanation of how to collect embryos.

1. Transfer 5 bottles of flies into a cage containing a 10-cm apple juice plate smeared with yeast paste to allow the flies to lay eggs. To collect enough embryos, 2-4 cages are used.
2. Allow the flies to lay eggs on the apple juice plates for 1 h before starting a timed collection.
3. Change the apple juice plates and allow the flies to lay eggs for 4 h.
4. Change the apple juice plates and allow the embryos to age in the incubator on the plate for 2 h or 10 h in order to collect 2-6 h or 10-14 h *Drosophila* embryos.
5. Remove the yeast, add household bleach diluted 1:2 with H₂O to the plate, and incubate the embryos for 2 min to dechorionate them.
6. Transfer the embryos to a homemade sieve (we cut a large hole in the cap of a 50-mL tube and glue nylon mesh over it (a cell strainer (40-100 µm mesh size) can also be used) and wash with water.
7. Dry excess liquid on paper towel and transfer the embryos to a pre-weighed 1.7-mL tube.
8. Record the weight of the embryos, freeze in liquid nitrogen, and store at -80°C.

C. Nucleic acid extraction from *Drosophila melanogaster* embryos

Notes:

- a. To have enough material for one DRIP-seq experiment, start with 500 µL or 200 mg embryos
- b. Before they are washed with methanol, embryos stick to plastic. To minimize the loss of material, only glass vials and glass pipettes are used until the methanol wash; 8-mL glass scintillation vials work well for this step. For all steps involved in nucleic acid preparation, low-binding tubes and tips are used.

1. Transfer 500 µL or 200 mg embryos to a glass vial.
2. Wash embryos with 4 mL 1× PBS.
3. Remove the PBS and wash the embryos with 4 mL 1× PBT.
4. Transfer the embryos to a bottle containing 3 mL 1× PBS and 3 mL N-heptane.
5. Shake for a few seconds to mix the 2 phases.
6. Remove the lower phase (PBS) and leave the interphase intact.
7. Add 3 mL methanol and shake vigorously for 1 min.
8. Remove the top and interphase.
9. Wash the embryos with 3 mL methanol.
10. Transfer the embryos to a 15-mL tube and wash with 4 mL 1× PBS.
11. Resuspend the embryos in 4 ml embryo lysis buffer and incubate for 2 h at 50°C. Every 15 min, invert the tube to mix.
12. Centrifuge at 4,000 × g for 15 min. Transfer the supernatant to a 50-mL tube.
13. Add 4 mL phenol/chloroform/isoamyl alcohol. Incubate for 1 h on a nutator at 4°C.
14. Centrifuge at 4,000 × g for 15 min. Transfer the upper phase to a 50-mL tube.
15. Add 4 mL phenol/chloroform/isoamyl alcohol. Incubate for 1 h on a nutator at 4°C.
16. Centrifuge at 4,000 × g for 10 min. Transfer the upper phase to a clean 50-mL tube.
17. Add 4 mL chloroform/isoamyl alcohol. Incubate for 1 h on a nutator at 4°C.
18. Centrifuge at 4,000 × g for 10 min. Transfer the upper phase to a clean 50-mL tube.
19. To precipitate the nucleic acids, add 200 µL 3 M KOAc pH 5.2 and 2.8 mL isopropanol. Incubate for 30 min on a nutator at 4°C.
20. Gently transfer the white filaments to a 1.7-mL microfuge tube containing 1 mL 70% ethanol. Use a 1-mL pipette tip or cut the end off a 200-µL pipet tip to transfer the filaments without breaking.
21. Wash 3 times with 1 mL 70% ethanol without centrifugation.
22. Remove as much ethanol as possible.

23. Centrifuge for 1 min at $1,000 \times g$ and remove the ethanol. This step can be repeated to remove the residual ethanol.
24. Air-dry the nucleic acid pellet for 1-4 h depending on the size of the pellet (until it becomes transparent).
25. Resuspend in 1 ml TE O.N. at 4°C on a nutator. At this step, the nucleic acid is viscous. Nucleic acid can be stored for a few weeks at -20°C before nuclease digestion and purification for DRIP.

D. Nucleic acid extraction from *Drosophila* S2 cells

1. *Drosophila* S2 cells are grown at room temperature in Schneider's media containing 10% FBS.
2. Transfer 2×10^7 cells to a 50-mL tube.
3. Pellet the cells at $500 \times g$ for 5 min.
4. Remove the supernatant and wash the cell pellet with 10 mL 1 \times PBS.
5. Pellet the cells at $500 \times g$ for 5 min.
6. Resuspend the cells in 3 mL TE and transfer to three 1.7-mL tubes.
7. Add 26 μL 20% SDS and 60 μg proteinase K to each tube. Mix gently by inverting the tube several times.
8. Incubate O.N. at 37°C .
9. Transfer to 2-mL phase lock tubes.
10. Add 1 volume phenol/chloroform/isoamyl alcohol. Shake and centrifuge at $14,000 \times g$ for 5 min.
11. Transfer the supernatant to a 50-ml tube containing 2.4 volumes of 100% ethanol and a 1/10 volume of 3 M NaOAc pH 5.2.

Note: It should be possible to substitute the KOAc used for Drosophila embryos for NaOAc.

12. Invert the tube gently to precipitate the nucleic acids.
13. Transfer the nucleic acids to a 1.7-mL tube containing 1 mL 70% ethanol.
14. Wash 5 times with 1 mL 70% ethanol by removing as much ethanol as possible without centrifugation.
15. Remove all the ethanol.
16. Centrifuge for 1 min at $1,000 \times g$ and remove all the ethanol. This step can be repeated to remove the residual ethanol.
17. Air-dry the nucleic acid pellet for a few hours until the pellet becomes transparent.
18. Resuspend in 1 ml TE O.N. at 4°C on a nutator. At this step, the nucleic acid solution is viscous. Nucleic acid can be stored for a few weeks at -20°C before nuclease digestion and purification for DRIP.

E. Nuclease digestion and sonication of nucleic acids

Note: All the measurements on the NanoDrop are carried out using dsDNA parameters. The samples collected to measure the concentration on the NanoDrop and run on a gel are: 1) gDNA prior and 2) after RNase A treatment; 3) after sonication; 4, 5) after RNase III \pm RNase H treatment.

1. Quantitate nucleic acids on a NanoDrop (concentration should be around 2 $\mu\text{g}/\text{μl}$ and $A_{260}/A_{280} \geq 2.0$).
2. Run 1 μL nucleic acids on a 1% agarose 1 \times TAE gel to verify that the gDNA is intact. Stain the gel with SYBR Gold. gDNA should be above 10 kb and a smear of RNA should be visible between 100 bp and 1.5 kb.
3. RNAase A digestion: Incubate 250 mg nucleic acids in the presence of 0.5 M NaCl and 0.1 mg/mL RNase A in a final volume of 1.5 mL for 3 h at 37°C .
4. Transfer to 2-mL phase lock tubes and add 1 volume phenol/chloroform/isoamyl alcohol.
5. Mix vigorously and centrifuge at $14,000 \times g$ for 5 min.
6. Transfer 500 μL upper phase to a 1.7-mL tube containing 1 mL 100% ethanol and 50 μL 3 M NaOAc pH 5.2.
7. Invert gently to precipitate the nucleic acids.
8. Transfer the nucleic acids to a 1.7-mL tube containing 400 μL 70% ethanol.

9. Remove all the ethanol.
10. Dry the nucleic acids for 10-30 min depending on the size of the pellet.
11. Resuspend in 500 μ L TE by pipetting gently up and down and incubate on ice for at least 30 min.
12. Measure the concentration of nucleic acids on a NanoDrop and adjust the volume to a concentration below 40 ng/ μ L (A_{260}/A_{280} should be 1.8-2.0). Save 1 μ L to run on an agarose gel to verify complete degradation of free RNA.
13. Sonication using a Covaris E220: Split the sample into 130- μ L aliquots (~5 μ g) for sonication in a 130- μ L Covaris microtube. Sonicate using the following parameters (Table 4):

Table 4. Parameters for the sonication using a Covaris E220

Peak incident power (W)	140
Duty factor	10%
Cycles per burst	200
Treatment (s)	80

14. Pool the sonicated nucleic acids.
15. Save 1 μ L to load on the gel to verify sonication. From a band above 10 kb before sonication, a smear should be observed with an average size of 300-400 bp after sonication.
16. RNase H and RNase III digestion: Split the nucleic acids from step 14 into two aliquots. To both aliquots, add 100 μ L 10 \times RNase H buffer and 2 units RNase III. Bring the volume to 1 mL. To one of the aliquots (RNase H-treated), add 10 μ g RNase H1 and 10 μ g RNase H2.
17. Incubate both digests O.N. at 37°C.
18. Transfer to 2-mL phase lock tubes and add 1 volume phenol/chloroform/isoamyl alcohol.
19. Mix vigorously and centrifuge for 5 min at 14,000 \times g.
20. Transfer 500 μ L upper phase to a 1.7-mL tube containing 1 mL 100% ethanol and 50 μ L 3 M NaOAc pH 5.2.
21. Invert gently to precipitate the nucleic acids.
22. Centrifuge for 15 min at 16,000 \times g.
23. Remove the supernatant and wash the pellet with 400 μ L 70% ethanol.
24. Centrifuge for 5 min at 16,000 \times g and remove all the ethanol.
25. Allow the nucleic acid pellet to dry for approximately 10 min.
26. Resuspend in 400 μ L TE by gently pipetting up and down and incubate on ice for at least 30 min.
27. Measure the concentration on a NanoDrop and save 1 μ L to run on a gel. This measurement is used to calculate the volume needed for the 4.4 μ g used for DRIP (DRIP Step F2a).
28. Analyze the test aliquots from each step of the procedure (Steps E1-E5) on a 1% agarose 1 \times TAE gel and stain with SYBR Gold (Figure 4A). Nucleic acids can be stored for a few days at -20°C before DRIP.

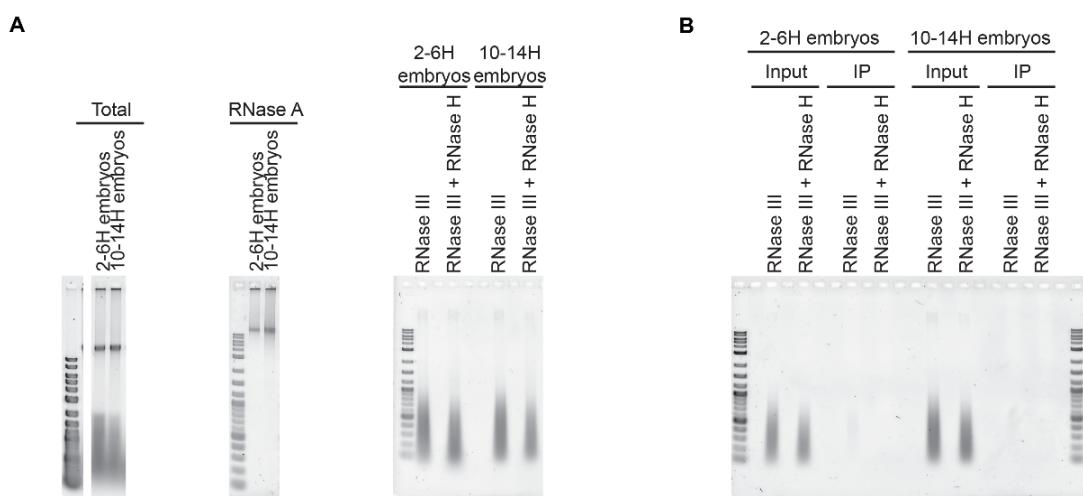


Figure 4. DRIP and nucleic acid preparation.

A. Nucleic acid preparation from *Drosophila melanogaster* embryos. Total nucleic acids after extraction from embryos (left). Nucleic acids after RNase A digestion and before sonication (middle). Nucleic acids after sonication and RNase III +/- RNase H digestion (right). B. Input and elution from a DRIP experiment performed on 2-6 h and 10-14 h *Drosophila melanogaster* embryos. A faint smear is observed in the elution but not in the elution of the RNase H-treated sample.

F. DRIP

Notes:

- a. For DRIP, all the steps are carried out using low-retention tubes and pipette tips.
- b. For sequencing, we perform 3 DRIP experiments as described above in parallel and pool the elutions after the final purification.

1. Bead preparation
 - a. Wash 40 µL Protein G Dynabeads twice with 1× PBS containing 5 mg/mL BSA.
 - b. Resuspend the beads in 2 volumes 1× PBS containing 5 mg/mL BSA and add 10 µg S9.6 antibody.
 - c. Incubate O.N. at 4°C on a nutator.
 - d. Wash the beads once with 1× PBS containing 5 mg/mL BSA.
 - e. Keep the antibody-bound beads on ice or at 4°C.
2. DRIP
 - a. For each DRIP, dilute 4.4 µg previously purified DNA (Step E27) based on the NanoDrop measurement in 440 µL TE and add 50 µL 10× DRIP binding buffer.
 - b. Save 50 µL for input and incubate the remaining sample with the S9.6-Dynabeads O.N. at 4°C with rotation.
 - c. Capture the beads for 30 s using a magnetic rack, remove the supernatant, resuspend the beads in 700 µL 1× DRIP binding buffer, and incubate for 10 min at RT with rotation.
 - d. Repeat the washes twice, for a total of 3 washes.
 - e. Resuspend the beads in 250 µL DRIP elution buffer and 140 µg proteinase K.
 - f. Incubate for 45 min at 50°C; invert the tube every 5 min.
 - g. Collect the supernatant (IP/elution).
3. Input and IP purification

Note: The nucleic acids are purified on two successive columns, the first of which is used to eliminate SDS from the samples.

- The input and IP are purified on Macherey-Nagel (MN) PCR cleanup columns using NTB buffer following the kit instructions. The elution is performed with 50 µL MN elution buffer.
- The input and elution are purified on Zymoresearch DNA purification columns following the kit instructions. Elute the input and IP with 50 µL 10 mM Tris-HCl pH 8.0. For sequencing, elute the IP with 8.5 µL 10 mM Tris-HCl pH 8.0 and pool the 3 IPs together.
- Run 2 µL each sample on a 1.5% agarose 1× TAE gel and stain for at least 1 h with SYBR Gold. A smear should be visible in the IP but not in the RNase H-treated IP (Figure 4B). We store the samples overnight at -20°C and prepare the libraries the next day. It should also be possible to store the samples at -80°C for a few weeks until library preparation.

G. Library preparation and qPCR

- Library preparation using an NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina®.

Notes:

- The DNA moiety of the RNA-DNA hybrid is sequenced by starting with the second strand synthesis; the RNA moiety is removed in the second strand synthesis by RNase H digestion.*
 - The number of PCR cycles is determined following the manufacturer's instructions based on the amount of nucleic acid (Step G1a).*
- Estimate the amount of material in the IP and input on a bioanalyzer. The amount of nucleic acid should be above 1 ng to make the library (Figure 5A).

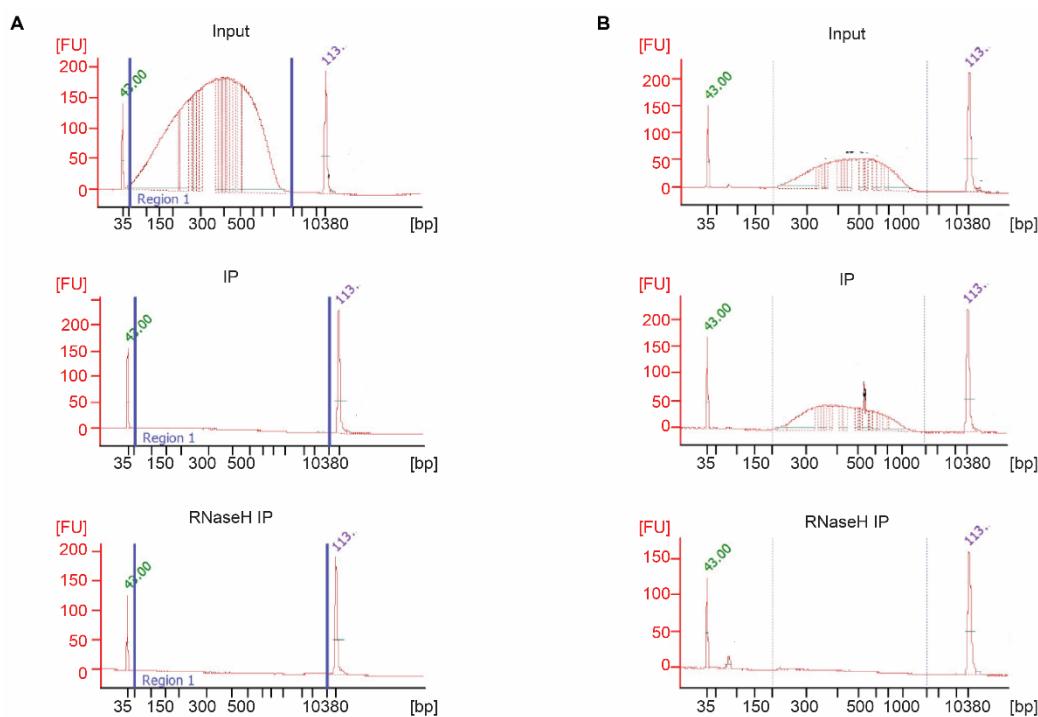


Figure 5. DRIP library preparation.

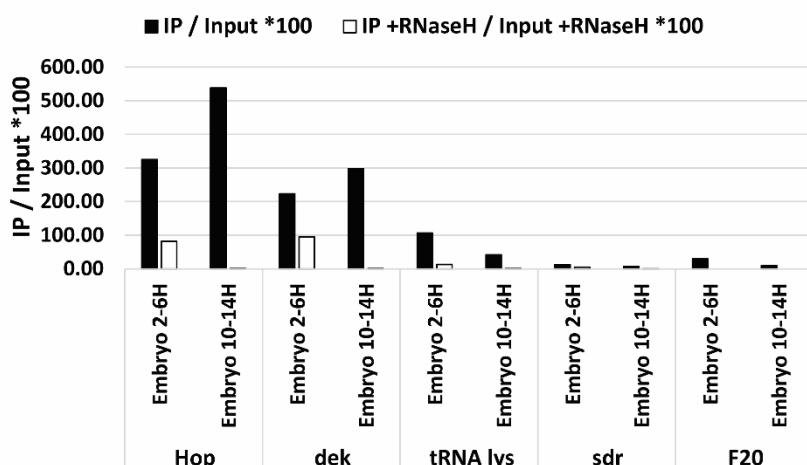
Input and elution from 10-14 h embryos before (A) and after (B) the library preparation. The elution is quantitated by a bioanalyzer. The same amount of input and RNase H elution is used to prepare the libraries. The entire immunoprecipitated sample of the RNase H-treated nucleic acid is used to prepare the library since we are unable to determine the amount of nucleic acid present.

- b. Adjust the amount of input to the IP. For the RNase H-treated IP, the amount of material recovered cannot be quantitated. We use the whole sample and are able to generate a high complexity library, although the number of reads is typically 2-5× lower.
- c. Prepare the library using NEB RNA Ultra II for Illumina, start at the second strand cDNA synthesis step (Table 5).

Table 5. Modified second strand synthesis reaction

Second strand synthesis reaction	Volume
Input and IP	20 µL
NEBNext second strand synthesis reaction buffer with dUTP mix (10×)	8 µL
NEBNext second strand synthesis enzyme mix	4 µL
Random primer	1 µL
RNase H (NEB), 1.6 U	0.3 µL
Nuclease-free water	46.7 µL

- d. Assemble on ice and incubate for 1 h at 16°C.
 - e. For the next steps, follow the kit instructions.
2. Quality control of the library
- a. Using qPCR, verify enrichment of R-loops at 3 positive sites and their absence at 2 negative sites (Figure 6; primer sequences in Table 6).
 - i. Dilute 1 µL each library in 10 µL 0.1× TE.
 - ii. Set up the qPCR plate with a standard curve using gDNA from S2 cells. This gDNA can be prepared as described in section D, digested with RNase A, and stored at -80°C. We prepare a dilution series in 10-fold steps from 25 to 0.025 ng/µL and use 2 µL each standard for qPCR with each primer set (standard curve is 50-5-0.5-0.05 ng total gDNA).
 - iii. qPCR reactions (5 µL total volume, run in a 384-well plate) consist of 2.5 µL Power Up SYBR Green master mix, 2 µL diluted library or standard genomic DNA from S2 cells, and 0.25 µL each primer diluted to 1 µM in water.
 - iv. qPCR is run on a ViiA7 instrument with 40 cycles, Tm = 60°C, and extension time = 1 min.

**Figure 6. qPCR after library preparation.**

3 positive and 2 negative sites confirmed the DRIP and library preparation of the input, IP, and RNase H-treated IP by qPCR.

Table 6. qPCR primers

Primer name	Sequence	
Hop F	CTACAAGCAGGCGAAGGTTT	R-loop (positive)
Hop R	CTTGATCTCAGGGGTGCGAT	
dek F	GCGATGAGCCAGAACAGATGAG	R-loop (positive)
dek R	CTTGGACTCATCAGTGGCAT	
tRNA lys F	GCCAAGCTCATTCTACGATCT	R-loop (highly transcribed gene, positive)
tRNA lys R	GTCCGACAAACGCCGATGATA	
Sdr F	ACAGCTGATGTCGCTCACAT	No R-loop (negative)
Sdr R	CGCTGAATGATCACCAGGTGA	
F20 F (<i>CG12754</i>)	TCAAGCCGAACCCTCTAAAAT	No R-loop (negative)
F20 R	AACGCCAACAAACAGAAAATG	

- b. Using the bioanalyzer
 - i. Run 1 µL each library on a bioanalyzer (Figure 5B).
 - ii. The average size of the fragments should be 400 bp.
- 3. Sequence the library on a HiSeq™ sequencing system with a depth of 50 million reads per sample. Paired end sequencing was used here, with a read length of 50 bases.

Data analysis

The work-flow for DRIP-seq analysis as described in Alecki *et al.* (2020) is similar to standard ChIP-seq analysis. The basic work-flow is trimming of the adaptors, quality control of the FASTQ files, alignment to the genome, removal of PCR duplicates, and calling of the peaks. The protocol diverges from ChIP-seq in the steps of splitting data by strand before peak calling and calling peaks against both the input and the RNase H-treated samples. All the analysis steps can be carried out on a Galaxy (usegalaxy.org) or using simple bash scripts. This workflow identifies a stringent set of reproducible peaks that meet strict criteria for being R-loops (*i.e.*, they are sensitive to RNase H).

Notes:

- a. *Others have described the use of a two-fold reduction in read counts in RNase H-treated samples to filter DRIP-seq peaks (Crossley *et al.*, 2020) rather than comparing peaks called in DRIP versus RNase H-treated DRIP. This approach may be more flexible to accommodate sequencing experiments in which the RNase H digestion is suboptimal and should be considered, particularly if RNase H filtering removes a large number of peaks (>10%).*
- b. *We call peaks from both strands together or after separating the bam files into forward (F) and reverse (R) strands. The strand-specific analysis is more informative since it makes it possible to infer the orientation of transcription that produced the RNA. A fraction of peaks have an R-loop signal on both strands (~10% of peaks); we do not know whether these are technical artifacts (Alecki *et al.*, 2020). Some, particularly in the*

case of embryo analysis, may be the result of mixed cell populations. It may be desirable to remove them for downstream analysis (Crossley *et al.*, 2020), which can be done using bedtools to intersect the F and R strands.

- c. Crossley *et al.* (2020) recently described a synthetic spike-in strategy that can be used to normalize DRIP-seq data, allowing quantitative comparisons across conditions. This could be especially valuable when comparing *Drosophila* of different genotypes.
1. Trim the adaptors and remove low-quality reads using Trimmomatic (Bolger *et al.*, 2014) or fastp (v0.20.0) (Chen *et al.*, 2018).
2. Align the reads to the *Drosophila* genome using Bowtie2 (v 2.3.1) (Langmead and Salzberg, 2012) (--fr --no-mixed--no-unal).
3. Use Samtools (v. 1.4.1) (Li *et al.*, 2009) to convert the sam files generated by Bowtie2 to bam files, sort the bam files, and create a bam index for each file.
4. Use Picard (<http://broadinstitute.github.io/picard>). Mark duplicates to filter PCR duplicates. Sambamba (v0.7.1) (Tarasov *et al.*, 2015) can also be used for this step.
5. Generate strand-specific bam files (for all samples: DRIP, RNase H DRIP, and Input) based on samflags using the samtools view as follows:
Forward strand: samtools view -f 99; samtools view -f 147, followed by samtools merge.
Reverse strand: samtools view -f 83; samtools view -f 163, followed by samtools merge.
To carry out this procedure in Galaxy, use the samtools view and select “A filtered/subsampled selection of reads”; then set the correct combination of “Require that these flags are set.”
For example, for the forward strand, first output the -f99 reads with these flags set:
–read is paired –read is mapped in a proper pair =mate strand =read is the first in a pair;
then output -f147 reads with these flags set;
–read is paired –read is mapped in a proper pair –read strand =read is the second in a pair;
samtools merge is used as above to merge f99 and f147 (F strand) and f83 and f163 (R strand).
6. Use MACS2 (v2.1.1) (Zhang *et al.*, 2008) to call peaks of DRIP versus Input using broad peak settings, with DRIP as the treatment and Input as the control. For example, for peak calling with paired-end bam files aligned to the dm6 version of the *Drosophila* genome:
(macs2 callpeak -t DRIP.bam -c DRIP_input.bam -f BAMPE -g 1.4e+08 -n DRIP -outdir DRIP_peaks –broad).
7. Use MACS2 to call peaks of DRIP versus RNase H-treated DRIP with broad peak settings, with DRIP as the treatment and RNase H-treated as the control.
8. Use the BEDTools (Quinlan and Hall, 2010) intersect to retain peaks present in IP versus input and IP versus RNase H IP (bedtools intersect -a IP_versus_IN_peaks.bed -b IP_versus_RNaseHIP_peaks.bed -u >filtered_peaks.bed).
9. Use the BEDtools intersect to retain only peaks present in both replicates.

Notes

1. This DRIP protocol consists of 4 steps, with the most critical being the preparation of the nucleic acids for immunoprecipitation. We encourage the users to perform DRIP-qPCR several times to ensure reproducibility before performing sequencing.
2. Time-line (Table 7).

Table 7. Time-line of DRIP-seq on *Drosophila melanogaster* embryos and tissue culture cells

Day 1	Embryo collection	Tissue culture cell lysis
Day 2	Embryo lysis and nucleic acid extraction	Nucleic acid extraction
Day 3	Nucleic acid preparation I + bead preparation	
Day 4	Nucleic acid preparation II + immunoprecipitation	

Day 5	Bead washing + elution + purification
Day 6	Library preparation and qPCR

3. Embryo collection

Note that embryos can be collected and stored at -80°C for a few months. This protocol can be performed in one week but it is also possible to stop at several steps. The nucleic acids can be stored at -20°C for a few days after extraction from tissue culture cells or embryos, after the preparation of the nucleic acids (before immunoprecipitation) and before the qPCR or library preparation.

4. Nucleic acid extraction and preparation

For DRIP protocols performed on protein-free nucleic acids after cell lysis, it is essential that the cell lysis and purification be performed gently to avoid breaking R-loops or creating new RNA-DNA hybrid by spurious annealing of RNA and complementary DNA. Thus, from extraction until sonication, do not centrifuge the nucleic acids at high speed. To resuspend the nucleic acids, do not vortex; instead pipette gently up and down.

5. DRIP

Nucleic acids from other species can be included before immunoprecipitation as a spike-in control to normalize the results between different samples or experimental conditions (Chen *et al.*, 2016).

Recipes

1. Embryo lysis buffer

50 mM Tris-HCl pH 8.0
100 mM EDTA
100 mM NaCl
0.5% SDS
5 mg/mL proteinase K

2. 1× RNase H buffer

50 mM Tris-HCl
75 mM KCl
3 mM MgCl₂
10 mM DTT
pH 8.3

3. 10× DRIP binding buffer

100 mM NaPO₄ pH 7.0
1.4 mM NaCl
0.5% Triton X-100

4. 1× DRIP binding buffer

10× DRIP binding buffer diluted in TE

5. DRIP elution buffer

50 mM Tris-HCl pH 8.0
10 mM EDTA
0.5% SDS

6. TE

10 mM Tris pH 8.0
1 mM EDTA

7. Apple juice plate

3.5% agar
4% sugar
40% apple juice
0.30% nipagin diluted in ethanol (2.5% of the final volume)

8. RNase H2 extraction buffer

1× PBS
0.2% Tween-20
10 mM MgCl₂

9. RNase H1 lysis buffer

25 mM Tris-HCl
300 mM NaCl
5 mM imidazole
pH 8.0 adjusted with NaOH

10. RNase H1 wash buffer

50 mM Tris-HCl
300 mM NaCl
20 mM imidazole
pH 8.0 adjusted with NaOH

11. RNase H1 elution buffer

50 mM Tris-HCl
300 mM NaCl
250 mM imidazole
pH 8.0 adjusted with NaOH

12. RNase H size column buffer

20 mM Tris-HCl pH 7.0
150 mM NaCl
Add 0.1 mM PMSF immediately before use

13. RNase H storage buffer

20 mM Tris-HCl pH 7.5
50 mM NaCl
1 mM DTT
0.1 mM EDTA
20% glycerol
Add 0.1 mM PMSF immediately before use

14. Protease inhibitors and additives (Table 8)

Note: The buffers used to prepare RNase H1 and H2 proteins contain freshly added protease inhibitors, NP40, and DTT, except for the size column buffers, which have only PMSF and DTT added.

Table 8. List of protease inhibitors and additives

	Final concentration
TLCK	13.5 μ M
Benzamidine	100 μ M
Pepstatin	3 μ M
Phenanthroline	55 μ M
PMSF	100 μ M
Aprotinin	1.5 μ M
Leupeptin	23 μ M
NP40	0.05%
DTT	1 mM

15. 0.5× TBE

44.5 mM Tris
44.5 mM Boric acid
1 mM EDTA pH 8.0

16. 10× DNA loading buffer

20% glycerol
0.1 M EDTA pH 8.0
1% SDS
0.25% Bromophenol Blue
0.25% xylene cyanol

17. SSC 20×

3 M NaCl
0.3 sodium citrate
pH 7.0 adjusted with HCl

18. 1× TAE

40 mM Tris-HCl
20 mM Acetate
1 mM EDTA

19. 1× PBS

137 mM NaCl
2.7 mM KCl
10 mM Na₂HPO₄
1.8 mM KH₂HPO₄

20. 1× PBT

1× PBS
0.1% Triton X-100

21. 3 M KOAc pH 5.2

3 M potassium acetate
pH 5.2 adjusted with glacial acetic acid

22. 3 M NaOAc pH 5.2

3 M sodium acetate

pH 5.2 adjusted with glacial acetic acid

23. SDS-PAGE running buffer

25 mM Tris-HCl
192 mM Glycine
0.1% SDS

24. Protein loading buffer

6× SDS-sample buffer, added to samples at a final concentration of 1×
0.35 M Tris, pH 6.8
30% glycerol
1% SDS
0.0001% Bromophenol Blue

Acknowledgments

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Competing interests

The authors declare no competing interests.

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Generation of Mouse Pluripotent Stem Cell-derived Trunk-like Structures: An *in vitro* Model of Post-implantation Embryogenesis

Adriano Bolondi^{1, 2}, Leah Haut¹, Seher Ipek Gassaloglu³, Polly Burton³, Helene Kretzmer¹, René Buschow⁴, Alexander Meissner^{1, 2, 5, 6, *}, Bernhard G. Herrmann^{3, 7, *} and Jesse V. Veenvliet^{3, 8, *}

¹Dept. of Genome Regulation, Max Planck Institute for Molecular Genetics, 14195 Berlin, Germany

²Institute of Chemistry and Biochemistry, Freie Universität Berlin, 14195 Berlin, Germany

³Dept. of Developmental Genetics, Max Planck Institute for Molecular Genetics, 14195 Berlin, Germany

⁴Microscopy and Cryo-Electron Microscopy, Max Planck Institute for Molecular Genetics, 14195 Berlin, Germany

⁵Department of Stem Cell and Regenerative Biology, Harvard University, Cambridge, MA 02138, USA

⁶Broad Institute of MIT and Harvard, Cambridge, MA 02142, USA

⁷Institute for Medical Genetics, Charité - University Medicine Berlin, Campus Benjamin Franklin, 12203 Berlin, Germany

⁸Max Planck Institute of Molecular Cell Biology and Genetics, 01307 Dresden, Germany

*For correspondence: meissner@molgen.mpg.de; herrmann@molgen.mpg.de; veenvliet@mpi-cbg.de

Abstract

Post-implantation mammalian embryogenesis involves profound molecular, cellular, and morphogenetic changes. The study of these highly dynamic processes is complicated by the limited accessibility of *in utero* development. In recent years, several complementary *in vitro* systems comprising self-organized assemblies of mouse embryonic stem cells, such as gastruloids, have been reported. We recently demonstrated that the morphogenetic potential of gastruloids can be further unlocked by the addition of a low percentage of Matrigel as an extracellular matrix surrogate. This resulted in the formation of highly organized trunk-like structures (TLSs) with a neural tube that is frequently flanked by bilateral somites. Notably, development at the molecular and morphogenetic levels is highly reminiscent of the natural embryo. To facilitate access to this powerful model, here we provide a detailed step-by-step protocol that should allow any lab with access to standard cell culture techniques to implement the culture system. This will provide the user with a means to investigate early mid-gestational mouse embryogenesis at an unprecedented spatiotemporal resolution.

Keywords: Trunk-like structures, Gastrulation, Somites, Self-organization, Morphogenesis, Gastruloids, Embryoids, Organoids, *In vitro* models, Stem cells

This protocol was validated in: SCIENCE (2020) DOI: 10.1126/science.aba4937.

Background

Gastrulation and early organogenesis represent developmental events that are crucial for the successful generation of a functional body plan. In mammals, these processes start just after the embryo implants in utero and within few days, a variety of morphologically and functionally diverse tissues emerge. It is currently difficult to study these highly dynamic changes *in vivo*, and *ex vivo* culture of post-implantation mouse embryos is laborious, costly, and requires rigorous training, which often renders it impractical for most laboratories. These impediments have led to extensive efforts to model post-implantation and early mid-gestational development *in vitro* using embryonic stem cells (reviewed in Shahbazi and Zernicka-Goetz 2018; Shahbazi *et al.*, 2019; Baillie-Benson *et al.*, 2020; Veenvliet and Herrmann, 2021). In particular, post-implantation development can be modeled with gastruloids, mouse or human embryonic stem cell (mESC/hESC) aggregates that self-organize (van den Brink *et al.*, 2014 and 2020; Moris *et al.*, 2020). The original mouse gastruloid culture protocol resulted in elongated structures with embryo-like expression domains similar to the post-occipital mouse embryo and with correct positioning of the three body axes, but limited morphogenesis (van den Brink *et al.*, 2014; Baillie-Johnson *et al.*, 2015; Beccari *et al.*, 2018a and 2018b; Turner *et al.*, 2017). More recent efforts have managed to introduce embryo-like morphological features by changing the cellular environment, such as the formation of somite-like structures or a heart tube (van den Brink *et al.*, 2020; Rossi *et al.*, 2021). Further advances have demonstrated that the addition of an extracellular matrix (ECM) surrogate to gastruloids can trigger a more embryo-like architecture with a gut tube as well as somites flanking a neural tube (Veenvliet *et al.*, 2020). We dubbed these embryonic organoids trunk-like structures (TLSs), since they resemble the core part of the trunk of an early mid-gestational embryo (~embryonic stage (E) 8.5-9). Importantly, during the timeframe of TLS induction (96-120 h post-aggregation), the gene regulatory programs are highly similar to the developing embryo. Moreover, the segmentation clock, an oscillator driving the rhythmic deposition of somites *in vivo*, is active at an embryo-like pace in the TLS (Pourquié, 2003; Veenvliet *et al.*, 2020).

The TLS model is easy to access, track, manipulate, and scale, which makes it a powerful tool to study post-implantation and early mid-gestational mammalian development in a dish. Here, we provide a comprehensive step-by-step procedure to facilitate the generation of trunk-like structures. We also describe how to process TLSs for downstream analysis, including whole-mount immunofluorescent staining and (single cell) RNA sequencing.

Materials and Reagents

1. Pipet tips, variable volumes (Biozym, SafeSeal SurPhob VT)
2. 1.5 mL tubes (Sarstedt, catalog number: 72.706)
3. 15 mL Falcon tubes (Sarstedt, catalog number: 62.554.502)
4. 50 mL Falcon tubes (Sarstedt, catalog number: 62.547.254)
5. 6 cm cell culture plates (Sarstedt, catalog number: 83.3901.300)
6. Ultra-low attachment 96-well plates (Corning, Costar, catalog number: CLS7007)
7. 6-well cell culture plates (Corning, catalog number: 3516)
8. 10 cm cell culture plates (Corning, catalog number: 430167)
9. Luna cell counting slides (Logos Biosystems, catalog number: L12001)
10. μ -Slide 8-well glass bottom (Ibidi, catalog number: 80827)
11. Flowmi cell strainers 40 μ m (Merck, catalog number: BAH136800040)
12. Bottle top vacuum filter unit (Corning, catalog number: CLS431096)
13. KnockOut DMEM (Gibco, catalog number: 10829018)
14. 100 \times Penicillin (5000 U/mL)-Streptomycin (5,000 μ g/mL) (Lonza, catalog number: DE17-603E)
15. 100 \times Glutamine, 200 mM (Lonza, catalog number: BE17-605E)
16. 100 \times Nucleosides (Sigma, catalog number: ES-008D)
17. Gibco 2-Mercaptoethanol, 55 mM solution in DPBS (Gibco, catalog number: 21985023)
18. Fetal Calf Serum (FCS), both regular (Pan Biotech, catalog number: P30-3306) and qualified and embryonic stem cell culture tested (Pan Biotech, catalog number: P30-2602)

19. TrypLE (Gibco, catalog number: 12604013) OR 0.05% Trypsin-EDTA (1×) (Gibco, catalog number: 25300-054)
20. NDiff 227 medium (Takara, catalog number: Y40002)
21. CHIR99021 InSolution (Sigma, catalog number: 361571) OR 10 mM in dimethyl sulfoxide (DMSO) (Tocris Biosciences, catalog number: 4423)
22. LDN193189 (Reprocell, catalog number: 04-0074-10)
23. DMSO (Sigma, catalog number: D2650)
24. Matrigel Growth Factor Reduced (GFR), Phenol Red-free (Corning, catalog number: 356231) – multiple lots/batches have been tested yielding similar results in terms of trunk-like-structure generation efficiency
25. Gelatin 2% solution (Sigma, catalog number: G1393)
26. DPBS, w/o MgCl₂/CaCl₂ (Gibco, catalog number: 14190144)
27. PBS with MgCl₂/CaCl₂ (Sigma, catalog number: D8662)
28. Murine Leukemia Inhibitory Factor (LIF) ESGRO™ (10⁷U/mL) (Millipore, catalog number: ESG1107)
29. Trypan Blue (Bio-Rad, catalog number: 1450021)
30. UltraPure Dnase/Rnase-Free Distilled Water (Invitrogen, catalog number: 10977049)
31. Reagent Reservoirs 60 mL (Merck, catalog number: BR703411)
32. Bovine Serum Albumin powder (BSA) (Sigma, catalog number: A2153)
33. Dulbecco's Modified Eagle's Medium (DMEM) 4,500 mg/mL glucose, without sodium pyruvate (Lonza, catalog number: BE12-733F)
34. Cell culture grade water (Lonza, catalog number: BE17-724Q)
35. 0.1% Gelatin solution (see Recipes)
36. Mouse embryonic fibroblast (MEF) medium (see Recipes)
37. Mouse embryonic stem cell (mESC) medium (see Recipes)
38. PBS/0.5% BSA solution (see Recipes)

Equipment

1. Biological safety cabinet (Thermo Fisher Scientific, model: Herasafe KS12)
2. Clean horizontal laminar flow hood (Thermo Fisher Scientific, model: HeraGuard ECO)
3. Cell culture incubator (Thermo Fisher Scientific, model: Heracell Vios 160i)
4. Cell culture centrifuge (Eppendorf, model: Centrifuge 5804R)
5. Variable volume pipets and multichannel pipets (Eppendorf, model: Research® plus pipette)
6. Horizontal light source, Light ring (Nikon, P-DF LED Darkfield Unit) or other stereomicroscope stand
7. Automated cell counter (Logos biosystems, Luna automated cell counter, L10001)
8. Cell culture water bath (LAUDA Aqualine, catalog number: AL18)
9. Tissue culture vacuum pump (Vacuuubrand, catalog number: 20727200)
10. Microcentrifuge (Eppendorf, model: 5424R)

Equipment set up:

1. Cell culture incubators are set to 37°C, 5% CO₂.

Note: We have also successfully generated TLSs at 7.5% CO₂, but routinely use 5%.

2. Cell culture water bath (set to 37°C).
3. All centrifugation steps are performed at room temperature, unless otherwise indicated.

Procedure

A. Seeding mouse embryonic fibroblasts (MEFs)

Notes:

- a. Seed MEFs at least one day prior to seeding the mESCs.
- b. Pre-warm MEF medium in the water bath for at least 20 min before starting.
- c. MEF plates should be used within one week of seeding.

1. Coat a 6 cm cell culture plate with 3 mL 0.1% gelatin solution.

Note: Gelatin-coated culture plates have to be prepared fresh on the day of seeding MEFs and cannot be stored.

2. Leave the plate at room temperature for 15 min.

Next, thaw a vial of mitotically inactive MEFs at 37°C in the water bath.

Notes:

- a. Inactive MEFs are mitotically inactivated in-house using mitomycin C treatment (3 h at 37°C).
- b. You need 1.0×10^6 MEFs to coat a 6 cm cell culture plate. Thaw the appropriate number depending on the number of 6 cm cell culture plates needed.

3. Add the MEFs to a 15 mL Falcon tube containing 5 mL pre-warmed MEF medium.

4. Centrifuge the cells at $200 \times g$ for 5 min.

5. While centrifuging, aspirate gelatin from each 6 cm plate and add 2 mL MEF medium.

6. Aspirate the supernatant from the 15 mL tube containing MEFs and resuspend the cell pellet at a concentration of 1.0×10^6 cells per mL.

Note: Viable MEFs are counted at the time of freezing and there is no need to count them again after thawing.

7. Add 1 mL cell suspension to each prepared 6 cm plate.

8. Place the plate in the incubator and swirl the plate to ensure even distribution of cells.

B. Seeding mouse embryonic stem cells (mESCs)

Notes:

- a. Pre-warm mESC medium in the water bath for at least 20 min before starting.
- b. We routinely use mESCs with an F1G4 genetic Background for TLS protocol generation (George et al., 2007).

1. Thaw a vial of mESCs at 37°C in the water bath immediately before plating.

Note: You need 3.5×10^5 mESCs for a 6 cm MEF-coated plate. Thaw the appropriate number depending on the number of 6 cm cell culture plates needed.

2. Add the mESCs to a 15 mL Falcon tube containing 5 mL pre-warmed mESC medium.

3. Centrifuge the cells at $200 \times g$ for 5 min.

4. While centrifuging, aspirate MEF medium from each 6 cm plate containing MEFs and add 2 mL mESC medium.

5. Aspirate the supernatant from the 15 mL tube containing mESCs and resuspend the cell pellet at a concentration of 3.5×10^5 cells per mL.

Note: Viable mESCs are counted at the time of freezing and there is no need to count them again after thawing.

6. Add 1 ml cell suspension to each MEF-coated 6 cm plate.
7. Place the plate in the incubator and swirl the plate to ensure even distribution of cells.
8. Replace the medium daily with 3 ml fresh mESC medium.

C. Passaging mESCs

Notes:

- a. Passage mESCs every 48 h at a splitting ratio of 1:8-1:10. Colony density and morphology should look similar to that shown in Figure 1A. Do not let your culture overgrow (Figure 1B).
- b. The splitting time and ratios detailed here are optimized for the mESC lines used in Veenvliet et al. (2020). Based on the proliferation rate of the mESC line used, splitting times and ratios may need to be adjusted. This may be especially true if transgenic lines and/or mESCs with different genetic backgrounds are used.
- c. Pre-warm mESC medium and TrypLE in the water bath for at least 20 min before starting.
- d. Prepare MEF-coated plates one day prior to passaging mESCs.
- e. Instead of TrypLE, 0.05% Trypsin-EDTA can be used.

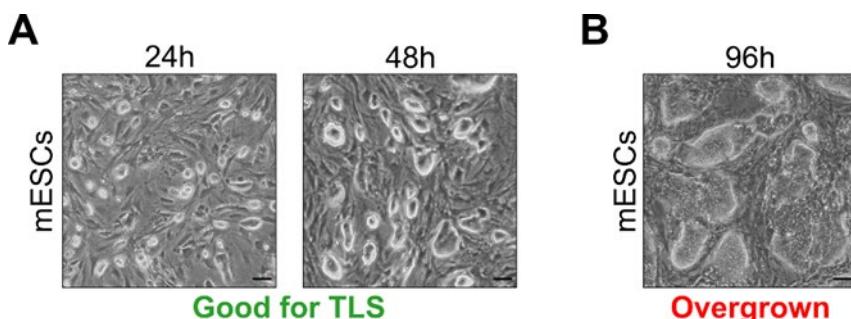


Figure 1. Optimal embryonic stem cell culture densities for successful TLS generation.

A. mESC culture densities suitable for TLS generation (24 h and 48 h after seeding). B. mESC culture density unsuitable for TLS generation (96 h after seeding). Scale bars for all panels, 50 μ m.

1. Aspirate the medium from the mESC plate and wash with 3 mL DPBS.
2. Aspirate the DPBS and add 1 mL TrypLE.
3. Ensure that the plate surface is evenly covered with TrypLE and place it in the incubator at 37°C for 5 min.
4. After 5 min, dislodge the colonies with a P1000 pipet set to 800 μ L by pipetting up and down in the plate 20 times.
5. Inactivate the TrypLE by adding 1 mL mESC medium and pipet further to obtain a single cell suspension.
6. Transfer the cell suspension to a 15 mL Falcon tube and wash the plate with an additional 3 mL mESC medium to recover all cells. Transfer these cells to the same 15 mL tube.
7. Centrifuge the cells at 200 \times g for 5 min.
8. While centrifuging, aspirate the MEF medium from the previously prepared 6 cm plate containing MEFs and add 2 mL mESC medium.
9. Aspirate the supernatant from the 15 mL tube containing mESCs and resuspend the cell pellet in 2 mL mESCs medium.
10. Add the appropriate amount of cell suspension to each MEF-coated 6 cm plate (ratio 1:8-1:10 \rightarrow 200-250 μ L). Adjust the final volume to 3 mL.
11. Place the plate in the incubator and swirl the plate to ensure even distribution of cells.
12. Replace the medium daily with 3 mL fresh pre-warmed mESC medium.

D. Generation of trunk-like structures (TLSs)

Notes:

- The input cell number for each well detailed here is optimized for the mESC lines used in Veenvliet et al. (2020). Based on the proliferation rate of the mESC line (especially for transgenic lines and/or mESCs with a different genetic Background), the cell amount may need to be adjusted to reach the same efficiency reported in Veenvliet et al. (2020).
- We recommend first optimizing the standard gastruloid protocol for new cell lines, using gastruloid elongation efficiency as a fast experimental readout (Cermola et al., 2021). In our experience, good gastruloid elongation efficiency (>95%) is essential to achieve a similar TLS efficiency to that reported in Veenvliet et al. (2020). A routine optimization procedure involves the seeding of 100-600 mESCs per well, with a stepwise increase of 50 cells.
- mESCs must be in culture for at least one passage before starting.
- Pre-warm mESC medium and TrypLE in the water bath for at least 20 min before starting.
- Here, we use commercially available, quality controlled NDiff 227 medium (N2B27). We and others have successfully generated gastruloids with home-made N2B27 (Beccari et al., 2018b); however, in our hands, more robust results of the gastruloid and TLS protocols are obtained with the NDiff 227 medium.
- Instead of TrypLE, 0.05% Trypsin-EDTA can be used.
- A schematic overview of the TLS generation protocol indicating critical timepoints is provided in Figure 2.

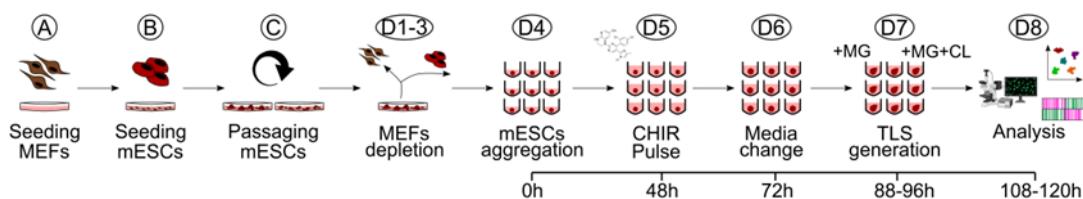


Figure 2. Schematic overview of the TLS generation protocol.

Workflow for the generation of trunk-like-structures (TLS) from seeding of MEFs to downstream analysis. MG, Matrigel; CL, CHIR+LDN; MEFs, mouse embryonic fibroblasts; mESCs, mouse embryonic stem cells.

D1. Prepare 6-well plate for MEF depletion

- Coat three wells of a 6-well plate with 2 mL 0.1% gelatin solution for each 6 cm plate that will be used for TLS generation.
- Incubate the 6-well plate at room temperature for 15 min.
- Aspirate gelatin solution and add 1 mL mESC medium to each well.
- Store plate in the incubator until use.

D2. Prepare a single cell suspension

- Aspirate the medium from the mESC plate and wash with 3 mL DPBS.
- Aspirate the DPBS and add 1 mL TrypLE.
- Ensure that the plate surface is evenly covered with TrypLE and place it in the incubator at 37°C for 5 min.
- After 5 min, dislodge the colonies with a P1000 pipet set to 800 µL by pipetting up and down in the plate 20 times.
- Inactivate the TrypLE by adding 1 mL mESC medium and pipet-mix.
- Transfer the cell suspension to a 15 mL Falcon tube and wash the plate with an additional 3 mL mESC medium to recover all cells. Transfer these cells to the same 15 mL tube.

7. Centrifuge the cells at $200 \times g$ for 5 min.
8. Resuspend the cell pellet in 1 mL mESC medium and pipet up and down 50 times.

Note: Here, it is critical to achieve a proper single cell suspension to avoid losing mESCs (or retaining MEFs) during MEF depletion and to ensure the best protocol performance. We recommend checking for a proper single cell suspension under a microscope.

D3. MEF depletion

Note: An example how the wells with cells attached to the bottom should look like after each step of MEF depletion is provided in Figure 3.

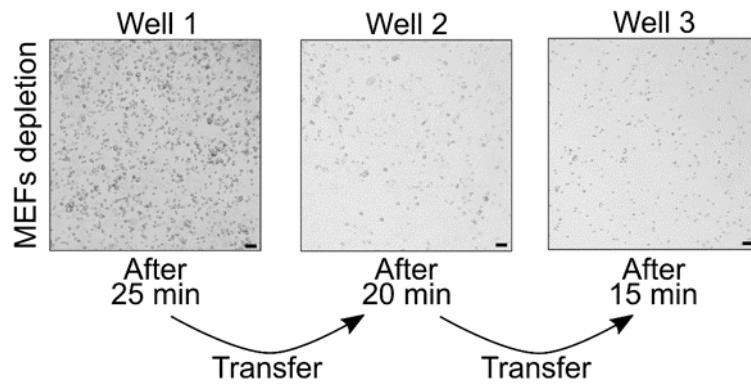


Figure 3. MEF depletion prior to mESC aggregation.

MEFs attach to the 0.1% gelatin-coated wells. Scale bars, 50 μm .

Note: With consecutive transfers, the amount of attached cells decreases. After the third incubation, MEF depletion is completed and mESCs are ready to be used for aggregation.

1. Transfer the obtained cell suspension to a well of the prepared 6-well plate (see D1).

Note: Transfer the amount of mESCs present in one 6 cm plate into one well of the prepared 6-well plate. The presence of too many cells could result in decreased depletion efficiency.

2. Pipet-mix 10 times.
3. Place the plate in the incubator and swirl the plate to ensure even distribution of cells. Leave untouched for 25 min.
4. Next use a P1000 pipet set to 1 mL to carefully transfer all the cells in suspension to another well.

Note: It is critical not to dislodge the MEFs, which are attached to the bottom of the wells.

5. Pipet up and down 10 times in the new well to ensure a single cell suspension.

Note: Cells may clump during the incubation; therefore, it is critical to pipet once the cells are transferred to the new well. We recommend confirming under the microscope that you have obtained a proper single cell suspension.

6. Place the plate in the incubator and swirl the plate to ensure even distribution of cells. Leave untouched for 20 min.
7. Next, use a P1000 pipet set to 1 mL to carefully transfer all the cells in suspension to another well.

Note: It is critical not to dislodge the MEFs, which are attached to the bottom of the wells.

8. Pipet up and down 10 times in the new well to ensure a single cell suspension.

Note: Cells may clump during the incubation; therefore, it is critical to pipet once the cells are transferred in the new well. We recommend confirming under the microscope that you have obtained a proper single cell suspension.

9. Place the plate in the incubator and swirl the plate to ensure even distribution of cells. Leave untouched for 15 min.
10. During this last 15-min step, equilibrate the required amount of NDiff 227 in a 10 cm dish in the incubator for at least 20 min. Longer incubation is also possible (e.g., NDiff 227 can be placed in the incubator after step 6. See Table 1 for the volume needed as a function of the number of 96-well plates to seed).

Note: NDiff 227 is light-sensitive and should be protected from (direct) light as much as possible.

11. Carefully transfer all MEF-depleted mESCs to a 15 ml Falcon tube with a P1000 pipet.

Note: It is critical not to dislodge the MEFs, which are attached to the bottom of the wells.

Table 1. Cell numbers and volumes required for mESC aggregation.

The amounts are calculated for an input of 200 cells/well in 35 µL. Volumes and cell numbers in column 4 are calculated for 100 samples (instead of 96) per plate *plus* a 10% excess dead volume. In column 2, the volume of NDiff 227 to equilibrate is calculated based on the amount needed for washing and counting the cells (5.5 mL per experiment, independent of the number of plates, see step D4.4 and D4.6), *plus* the amount indicated in column 4, *plus* an extra volume to account for dead space in the dish and evaporation during medium equilibration.

Number of well plates	Volume of NDiff 227 to equilibrate (mL)	Total number of cells needed	Volume of NDiff 227 required to reach 5.7×10^3 cells/mL (mL)
1	12	2.2×10^4	3.85 <i>minus</i> cell volume
2	16	4.4×10^4	7.70 <i>minus</i> cell volume
3	20	6.6×10^4	11.55 <i>minus</i> cell volume
4	24	8.8×10^4	15.40 <i>minus</i> cell volume
5	28	11×10^4	19.25 <i>minus</i> cell volume

D4. mESC aggregation (0 h)

Note: The first 96 h of the TLS protocol are similar to the gastruloids protocol (Baillie-Johnson et al., 2015; van den Brink et al., 2014; Beccari et al., 2018b; Anlas et al., 2021). Detailed protocols for gastruloid formation, including troubleshooting, are provided elsewhere (Baillie-Johnson et al., 2015; Beccari et al., 2018b; Anlas et al., 2021).

1. Centrifuge the cells at $200 \times g$ for 5 min.
2. Resuspend the cell pellet in 5 mL PBS with MgCl₂/CaCl₂ and pipet up and down 20 times (Wash 1).
3. Centrifuge the cells at $200 \times g$ for 5 min.

Note: In case of low starting cell numbers, steps 2 and 3 can be omitted. This may however slightly compromise protocol efficiency.

4. Resuspend the cell pellet in 5 mL pre-equilibrated NDiff 227 and pipet up and down 20 times (Wash 2).
5. Centrifuge the cells at $200 \times g$ for 5 min.
6. Resuspend the cell pellet in 500 μL pre-equilibrated NDiff 227 and pipet up and down 30 times.
Note: It is critical to obtain a single cell suspension prior to counting and plating.
7. For counting, prepare a 1:2 dilution of the cell suspension by adding 10 μL cell suspension to 10 μL Trypan Blue.
8. Count using the Luna automated cell counter with the following set up: Dilution factor → 2; Noise reduction → 5; Live detection sensitivity → 5; Roundness → 85%; Min cell size → 10 μm ; Max cell size → 20 μm ; Declustering level → High.
9. Transfer the amount of cells needed for the experiment to a new Falcon tube (see Table 1 for the number of cells needed as a function of the number of 96-well plates to seed).
10. Add the pre-incubated NDiff 227 volume required to bring the cell suspension to a concentration of 5.7×10^3 cells/mL (see Table 1 for the volume to add as a function of the number of 96-well plates to seed).

Note: This cell concentration is optimized for an input of 200 cells/well, which has been shown to give high TLS generation efficiency for all cell lines tested (Veenvliet et al., 2020). Similar results were obtained for inputs ranging from 200 to 250 cells/well.

11. Mix the new cell suspension vigorously and transfer it to a reservoir.
12. Use a multichannel pipet to transfer 35 μL to each well of an ultra-low attachment 96-well plate. Pipet gently up and down in the reservoir between each transfer.
13. Gently tap the plate 5 times on a clean bench, transfer to the incubator, and allow undisturbed aggregation for 48 h.

Note: Keeping NDiff 227 outside the incubator for longer periods of time (more than 5 min) will lead to disequilibration of the medium. Therefore, try to avoid keeping NDiff 227 or plates with freshly seeded cells in NDiff 227 out the incubator for too long. In the case of handling multiple plates, we recommend putting each plate into the incubator directly after pipetting.

D5. CHIR pulse (48 h)

Notes:

- a. Start this procedure at least one hour before the end of the 48 h.
- b. Cells at 48 h should have formed one single round aggregate with a diameter measuring $214 \pm 13 \mu\text{m}$ (see Figure 4A).
- c. CHIR99021 is light-sensitive and should be protected from (direct) light as much as possible.
- d. CHIR99021 should be aliquoted in single-use aliquots in brown (light-protected) sterile tubes upon arrival and not subjected to repeated freeze-thaw cycles.

1. Equilibrate the needed amount of NDiff 227 in a 10-cm dish in the incubator for at least 20 min (see Table 2 for the volume needed as a function of the number of 96-well plates used in the experiment).
2. Transfer the required amount of NDiff 227 to a 50-ml Falcon tube (see Table 2 for the volume needed as a function of the number of 96-well plates used in the experiment).

Table 2. Volumes of NDiff 227, CHIR99021, and Matrigel required during the last three steps (protocol steps D5, D6, D7) of the TLS generation protocol.

In column 2, the volume of NDiff 227 to equilibrate is calculated including an excess volume (2 mL for each dish that is used for medium equilibration) to account for dead space in the dish(es) and medium evaporation during equilibration.

Number of 96-well plates	Volume of NDiff 227 to equilibrate (D5, D6, D7) (mL)	Volume of NDiff 227 to transfer in the 50-ml tube/s (D5, D6) (mL)**	Volume of NDiff 227 to transfer in the 50-ml tube/s (D7) (mL)**	Volume of 10 mM CHIR99021 to add for 3 µM (D5 only) (µL)	Volume of Matrigel to add for 5% final (D7 only) (mL)***
1	18	16	15.01	4.8	0.99
2	38*	32	30.03	9.6	1.97
3	52*	48	45.04	14.4	2.96
4	70*	64	60.05	19.2	3.95
5	86*	80	75.06	24	4.94

*For volumes higher than 30 ml, use more than one 10-cm plate to equilibrate NDiff 227 in the incubator.

**Use two 50-mL tubes if the volume exceeds 50 mL.

***Note that we calculate 5% v/v Matrigel as a function of the final volume in each well ($35 \mu\text{L} + 150 \mu\text{L} = 185 \mu\text{L}$) (as opposed to CHIR99021). This means that in the NDiff 227, the concentration of Matrigel is slightly higher than 5% (6.17%). For instance, for 1 plate of TLSs, the volume of Matrigel added is calculated as $(0.05*16)*(185/150) = 0.987 \text{ mL}$.

3. Add CHIR99021 to the NDiff 227 medium to obtain a final concentration of 3 µM.
4. Mix the medium vigorously and transfer it to a reservoir.
5. Use a multichannel pipet to add 150 µL CHIR99021-supplemented medium to each well of the plates containing the aggregates.
6. Gently tap the plate 10 times on a clean bench, transfer to the incubator, and allow further undisturbed development for 24 h.

Notes:

- a. *The tapping is critical to prevent cell aggregates from attaching to the culture plates. Ensure that the aggregates are freely moving immediately after tapping (this can be checked under the microscope).*
- b. *Take caution to avoid splashing medium on the lid while tapping the plates.*

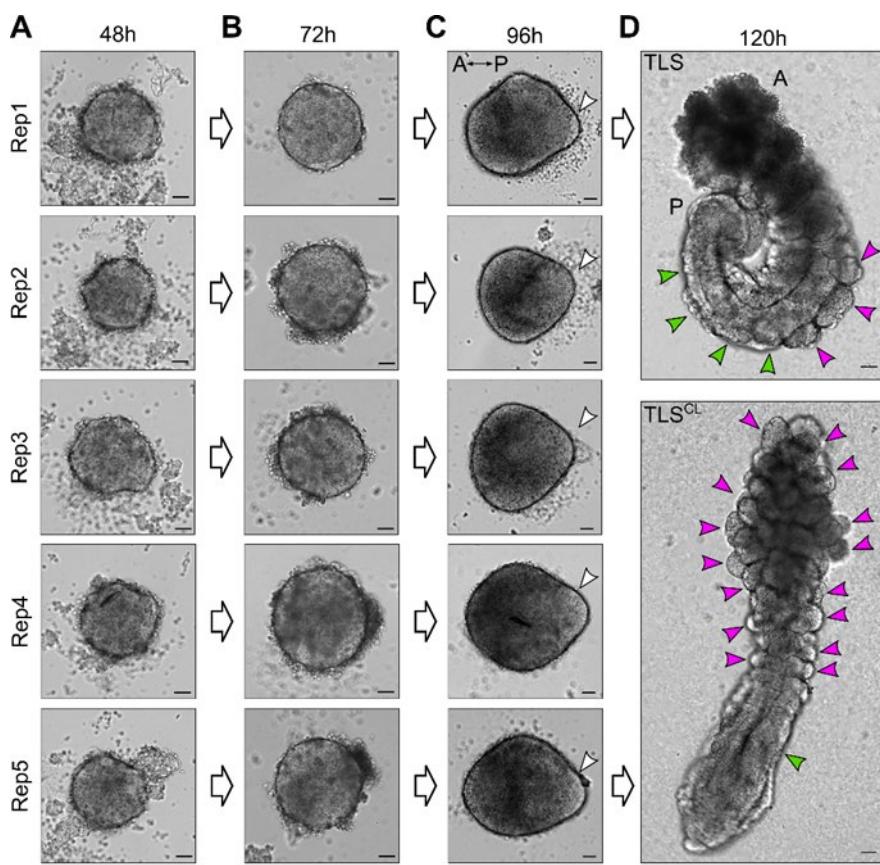


Figure 4. Examples of expected morphology of mESC-derived aggregates at several timepoints during TLS generation.

A, B. mESC-derived aggregates at 48 h and 72 h after aggregation are round without clear signs of symmetry breaking. C. At 96 h after aggregation, the structures have clearly broken symmetry and are teardrop shaped. The white arrowheads indicate the posterior pole, where localized expression of Brachyury is expected. Note that, depending on the cell line used, aggregates may establish the teardrop-like morphology prior to 96 h. In that case, structures should be embedded in Matrigel earlier (as soon as the teardrop-like morphology is observed) to achieve optimal TLS efficiency (see main text for details). D. Upon addition of 5% Matrigel, the aggregates will establish an architecture reminiscent of the embryonic trunk, with somites (magenta arrowheads) flanking a neural tube (green arrowheads). Chemical modulation with a WNT agonist (5 μ M CHIR99021) and BMP inhibitor (600 nM LDN193189) results in compromised neural tube development and formation of excess somites arranged like a “bunch-of-grapes” (TLSCL). Scale bars for all panels, 50 μ m. A, Anterior; P, Posterior.

D6. Media change (72 h)

Notes:

- a. Start this procedure at least one hour before the end of the 72 h.
- b. Aggregates at 72 h should look like the example given in Figure 4B and measure $244 \pm 15 \mu\text{m}$ in diameter.
- c. If available, perform this step on a clean bench containing a stereoscope or a light ring to help visualize the structures and avoid loss of structures while pipetting off the old medium.

1. Equilibrate the required amount of NDiff 227 in a 10-cm dish in the incubator for at least 20 min (see Table 2 for the volume needed as a function of the number of 96-well plates used in the experiment).
2. Transfer the required amount of NDiff 227 to a 50-mL tube (see Table 2 for the volume needed as a function of the number of 96-well plates used in the experiment).

3. Use a multichannel pipet to remove 150 μ L from each well without disturbing the structure.

Note: CRITICAL STEP. Avoid losing structures while pipetting off the medium. This is best achieved by keeping the plate under a 30-40° angle and putting the pipet tips against the side opposite to that where the aggregate should be located. As a visual aid, a stereoscope or light ring could be used as stated above (see Figure 5 for a schematic of how to position the plate and tips).

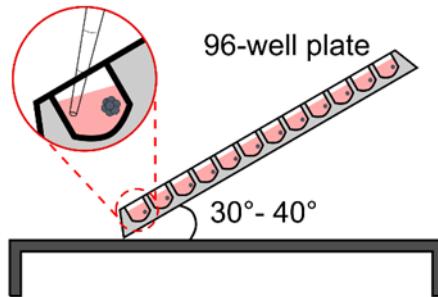


Figure 5. Schematic representation of plate positioning for media changes during the TLS generation procedure.

The plate is tilted at a 30-40° angle on a clean bench and the media is carefully aspirated with a multichannel pipet, avoiding disturbance of the aggregates.

4. Pour the pre-equilibrated medium in a reservoir.
5. Use a multichannel pipet to add 150 μ L medium to each well of the plates containing the aggregates.
6. Gently tap the plate 10 times on a clean bench, transfer to the incubator, and allow further undisturbed development for 16-24 h.

Notes:

- The tapping is critical to prevent cell aggregates from attaching to the culture plates. Ensure that the aggregates are freely moving immediately after tapping (this can be checked under the microscope).*
- Take caution to avoid splashing medium on the lid while tapping the plates.*
- Thaw overnight at 4 °C on ice the amount of Matrigel needed the following day (see Table 2 for the volume needed as a function of the number of 96-well plates used in the experiment).*
- We have used multiple Matrigel batches with comparable results.*

D7. TLS generation (88-96 h)

Notes:

- Start monitoring the TLSs around 88 h after aggregation for the appearance of a “teardrop-like” shape (see Figure 4C). Structures should present a longer axis ($421 \pm 33 \mu\text{m}$) and a shorter axis ($337 \pm 30 \mu\text{m}$), with an axis ratio of 0.8 ± 0.07 .*
 - Start this procedure immediately when a “teardrop-like” shape is observed in the majority of the TLSs (or latest 96 h after aggregation) to achieve optimal TLS formation efficiency.*
 - If available, perform this step on a clean bench containing a stereoscope or a light ring to help visualize the structures and avoid losing structures while pipetting off the old medium.*
 - If performing chemical modulation at this step, follow the “Variant protocol: chemical modulation during TLS generation.”*
1. Equilibrate the required amount of NDiff 227 in a 10-cm dish in the incubator for at least 20 min (see Table 2 for the volume needed as a function of the number of 96-well plates used in the experiment).
 2. Transfer the required amount of NDiff 227 to a 50-mL Falcon tube and place it on ice (see Table 2 for the

volume needed as a function of the number of 96-well plates used in the experiment).

3. Once the medium has cooled down, supplement it with the correct volume of Matrigel on ice and mix vigorously.

Note: It is critical, while handling Matrigel, that every step is performed on ice to avoid clumping. It is also recommended to pre-cool the pipet tips used for the handling of 100% Matrigel by placing the box in the fridge until use; see Table 2 for the volume needed as a function of the number of 96-well plates used in the experiment.

4. Move the Falcon tube with Matrigel-supplemented medium to room temperature.
5. Use a multichannel pipet to remove 150 μ L from each well without disturbing the structure.

Notes:

- a. *CRITICAL STEP. Avoid losing structures while pipetting out the medium. This is best achieved by keeping the plate under a 30-40° angle and putting the pipet tips against the side opposite to that where the aggregate should be located. As a visual aid, a stereoscope or light ring could be used as stated above (see Figure 5 for a schematic of how to position the plate and tips).*
- b. *This step should not take more than 5 min after Matrigel-supplemented medium has been placed at room temperature. If there are multiple plates, it is advisable to remove the medium from all plates before equilibrating the Matrigel-supplemented medium at room temperature and keep the structures in the incubator.*

6. Pour the Matrigel-supplemented medium into a reservoir.
7. Use a multichannel pipet to add 150 μ l Matrigel-supplemented medium in each well of the plates containing the aggregates.
8. Gently tap the plate 10 times on a clean bench and transfer to the incubator.

Notes:

- a. *The tapping is critical to prevent cell aggregates from attaching to the culture plates. Ensure that aggregates are freely moving immediately after tapping (this can be checked under the microscope).*
- b. *Take caution to avoid splashing medium on the lid while tapping the plates.*
- c. *If performing Live Cell Imaging after embedding, allow the TLSs to settle for 1 h in the incubator before starting imaging.*

Variant protocol: chemical modulation during TLS embedding

Follow “D7. TLS generation (88-96 h)” for all passages except for point 3.

- 3'. Once the medium has cooled down, supplement it with the correct volume of Matrigel and appropriate chemical compounds for your modulations on ice and mix vigorously.

Notes:

- a. *It is critical, while handling Matrigel, that every step is performed on ice to avoid clumping; see Table 2 for the volume needed as a function of the number of 96-well plates used in the experiment.*
- b. *Add an equal volume of diluent to the control sample TLSs when performing chemical modulations.*
- c. *In Veenvliet et al. (2020), we induced excess somite production and compromised neural tube development by supplementing the medium with 5 μ M CHIR99021, alone (TLS^C) or in combination with 600 nM LDN193189 (TLS^{CL}).*

EXPECTED OUTCOME:

TLSs at 120 h should look elongated with a clear anterior and posterior domain (Figure 4D upper panel; Figure 6A). Moreover, they should show clear segmentation (somites) on one or both sides of a tubular structure (neural tube). TLSs subjected to chemical modulation (TLS^{CL}) should display compromised neural tube

development and formation of excess somites arranged like a “bunch-of-grapes” (Figure 4D lower panel; Figure 6B).

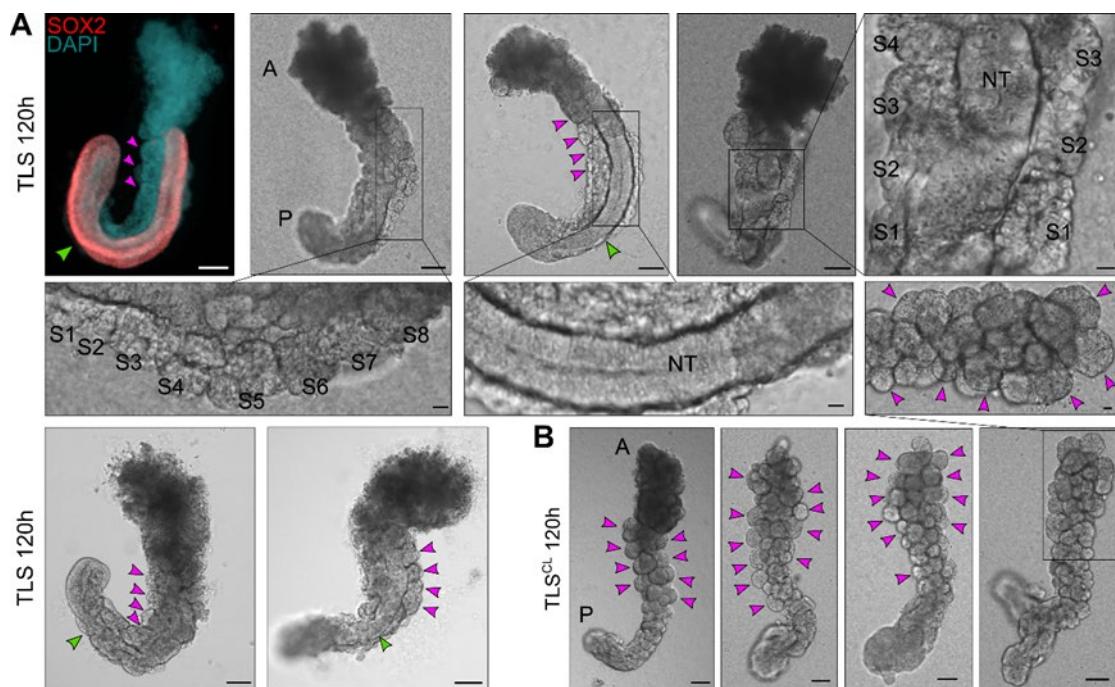


Figure 6. Expected outcome of the TLS protocol.

A. Examples of trunk-like structures (TLSs) 24 h after addition of 5% Matrigel (total culture time 120 h). The left upper structure is immunostained with a SOX2 antibody and counterstained with DAPI, labeling the neural tube and nuclei, respectively. Somites are indicated with magenta arrowheads or with S1, S2, etc. (in magnifications); S1-S8, Somite 1-Somite 8. Neural tube (NT) is indicated with a green arrowhead. A, Anterior; P, Posterior. B. Expected outcome of TLSs subjected to chemical modulation (TLS^{CL}) is compromised neural tube development and formation of excess somites arranged like a “bunch-of-grapes.” Somites are indicated with magenta arrowheads. Scale bars, 100 μ m (whole structure) or 20 μ m (magnifications). A, Anterior; P, Posterior.

D8. TLS analysis (108-120 h)

Notes:

- Depending on the specific biological question, the exact time of analysis may vary (see Veenvliet *et al.*, 2020 for time-resolved expression dynamics).
- If available, perform this step on a clean bench containing a stereoscope or a light ring to help visualize the structures and avoid losing structures while processing them.
- The following protocol variants are performed (D8', D8'', D8''') depending on the downstream applications.
- Prepare a P200 tip box with the tip cut-off at the 50- μ L mark for TLS picking (if using unmarked tips, cut approximately 9 mm off the tip).

D8'. Staining and imaging

- Use a P200 pipet set to 50 μ L and the cut-off tips to manually pick each individual TLS that needs to be analyzed in a well of an Ibidi 8-chamber plate.
- Add 200 μ L cold PBS/0.5% BSA solution to each well containing a TLS.
- Remove 200 μ L with a pipet and perform the same 200 μ L cold PBS/0.5% BSA solution wash three times.

4. Use the fixative of interest to fix TLSs in the Ibidi plate for the downstream protocol.
5. Perform the rest of the staining protocol in the Ibidi plate and image the structure with the desired microscope and settings.

*Note: We have so far used 4% PFA fixation for whole-mount immunofluorescence (WIFC) as well as whole-mount *in situ* hybridization (WISH). A detailed description of the protocols used for WIFC and WISH, including method-specific fixation times and downstream processing, is provided in the Supplemental Information of Veenvliet et al. (2020).*

D8''. RNA extraction

1. Use a P200 pipet set to 50 µL and the cut-off tips to manually pick and transfer each individual TLS that needs to be analyzed to a 1.5-ml tube containing 1 mL cold PBS/0.5% BSA solution.

Note: The number of TLSs that are pooled in one tube depends on the downstream application and experiment.

2. Centrifuge the TLSs at 200 × g for 1 min at 4°C.
3. Remove the supernatant with a P1000 pipet while being careful not to disturb the TLS pellet.
4. Wash the structures with 1 mL cold PBS/0.5% BSA solution.

Note: Ensure loosening of the pellet without aspirating into the pipet tip.

5. Centrifuge the TLSs at 200 × g for 1 min at 4°C.
6. Remove the supernatant with a P1000 pipette while being careful not to disturb the TLS pellet.
7. Add the indicated amount of RNA lysis buffer or TRIzol depending on the desired RNA extraction strategy.

D8'''. 10× Genomics single-cell RNA sequencing

Notes:

- a. This section explains how to process TLSs to generate a single cell suspension suitable for efficient Gel Bead-in-Emulsion (GEMs) generation. Follow the manufacturer's instructions for every step after the single cell suspension has been generated and counted.
 - b. Pre-warm TrypLE in the water bath for at least 20 min before starting.
1. Use a P200 pipette set to 20 µL and the cut-off tips to manually pick each individual TLS that needs to be analyzed in a well of an ultra-low attachment 96-well plate containing 200 µL cold PBS.
 2. Transfer each TLS serially five times to new wells containing 200 µL cold PBS.

Notes:

- a. It is CRITICAL to carry over as little volume as possible from the culture to minimize the amount of Matrigel transferred. Carrying over excess amounts of Matrigel can lead to microfluidics clogging during GEM generation.
 - b. Since washing of TLSs is performed in PBS without BSA, the structures may become sticky and get stuck to the tip wall. Avoid this by pipetting a very low volume to maintain the structure at the liquid/air interphase in the tip.
3. After the five washes, transfer all the structures into a single drop of 200 µL pre-warmed TrypLE in the center of a 6-cm plate.
 4. Transfer the plate to the incubator and allow cell dissociation for 25 min with pipetting every 5 min to ensure that a single cell suspension is achieved.

Note: Perform the pipetting steps under a stereoscope to monitor the degree of cell dissociation and ensure no loss of material.

5. At the end of the 25 min, and after verifying correct achievement of a single cell suspension, transfer the cell suspension to a 1.5-mL tube on ice.
6. To ensure maximum cell recovery and to quench the trypsinization reaction, wash the part of the plate where the drop was located four times with 200 µL PBS/0.5% BSA solution. Add every wash to the same tube (from step 5) containing the cell suspension.
7. Filter the cell suspension using a P1000 set to 1 mL through a 40-µm Flowmi Cell Strainer in a new 1.5-mL tube on ice.
8. Centrifuge the cell suspension at 300 × g for 5 min at 4°C.
9. Remove 800 µL supernatant with a P1000 while being careful not to disturb the cell pellet.

Note: The cell pellet may be very small and barely visible, so be extremely careful during these steps.

10. Wash with 1 mL PBS/0.5% BSA solution.
11. Centrifuge the cell suspension at 300 × g for 5 min at 4°C.
12. Remove 800 µL supernatant with a P1000 while being careful not to disturb the cell pellet.

Note: The cell pellet may be very small and barely visible, so be extremely careful during these steps.

13. Resuspend the pellet in the remaining 200 µL left in the tube.
14. Centrifuge the cell suspension at 300 × g for 5 min at 4°C.
15. Remove the supernatant with a P200 pipet, leaving ~42 µL in the tube.

Note: Use another tube containing exactly 42 µL PBS/0.5% BSA solution as a guide to evaluate the approximate volume to leave in the tube.

16. Resuspend the cell pellet in the ~42 µL left and determine the cell suspension concentration using a manual hemocytometer (analyze a 1:5 cell suspension dilution by adding 2 µL cell suspension to 8 µL Trypan Blue).
17. Proceed with the desired amount of cells for GEM generation following the manufacturer's instructions.

Data analysis

All data and analysis needed for the development and characterization of this protocol are available in the main text or Supplemental Information of Veenvliet *et al.* (2020).

Recipes

1. 0.1% Gelatin solution

Dilute sterile 2% Gelatin to 0.1% in cell culture grade water. Store at 4°C.

2. Mouse embryonic fibroblast (MEF) medium

Note: Heat inactivate the FCS for 30 min at 56°C before use.

500 mL Dulbecco's Modified Eagle's Medium (DMEM)

55 mL regular FCS (Pan Biotech, catalog number: P30-3306)

5.5 mL 100× Glutamine

5.5 mL 100× Penicillin/Streptomycin

Sterile filter
Store at 4°C

3. Mouse embryonic stem cell (mESC) medium

Note: Heat inactivate the FCS for 30 min at 56°C before use.

400 mL Knockout Dulbecco's Modified Eagle's Medium (KO-DMEM)

75 mL **mESC tested** FCS (Pan Biotech, catalog number: P30-2602)

5 mL 100× Glutamine

5 mL 100× Penicillin/Streptomycin

5 mL 100× Nucleosides

1 mL Gibco 2-Mercaptoethanol

Sterile filter

Aliquot mESC medium without LIF in 40-mL portions and freeze at -20°C

Thaw before use, then store at 4°C

Add 1:10,000 LIF immediately before use

Store at 4°C

Note: Homemade LIF has also been successfully used; however, the right concentration has to be tested based on the purification protocol and batch concentrations.

4. PBS/0.5% BSA solution

PBS with MgCl₂/CaCl₂

0.5% BSA powder

Prepare fresh, sterile filter, keep on ice for the procedure

Store at 4°C

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Competing interests

The authors declare no competing interests.

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GeneWeld: Efficient Targeted Integration Directed by Short Homology in Zebrafish

Jordan M. Welker¹, Wesley A. Wierson¹, Maira P. Almeida¹, Carla M. Mann¹, Melanie E. Torrie¹, Zhitao Ming¹, Stephen C. Ekker², Karl J. Clark², Drena L. Dobbs¹, Jeffrey J. Essner^{1,*} and Maura McGrail^{1,*}

¹Department of Genetics, Development and Cell Biology, Iowa State University, IA, USA

²Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN, USA

*For correspondence: mmcgrail@iastate.edu; jessner@iastate.edu

Abstract

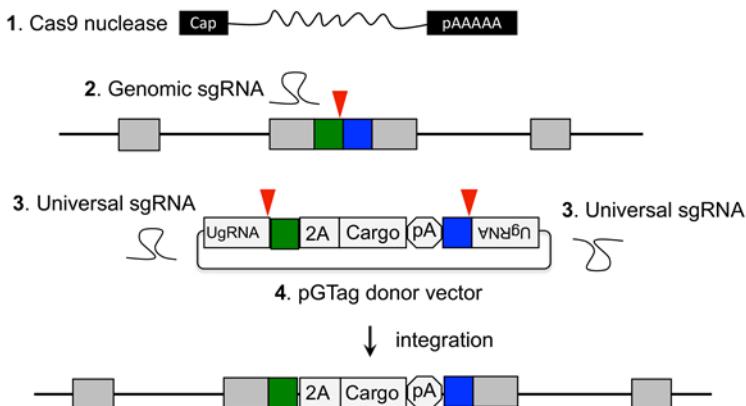
Efficient precision genome engineering requires high frequency and specificity of integration at the genomic target site. Multiple design strategies for zebrafish gene targeting have previously been reported with widely varying frequencies for germline recovery of integration alleles. The GeneWeld protocol and pGTag (plasmids for Gene Tagging) vector series provide a set of resources to streamline precision gene targeting in zebrafish. Our approach uses short homology of 24-48 bp to drive targeted integration of DNA reporter cassettes by homology-mediated end joining (HMEJ) at a CRISPR/Cas induced DNA double-strand break. The pGTag vectors contain reporters flanked by a universal CRISPR sgRNA sequence to liberate the targeting cassette *in vivo* and expose homology arms for homology-driven integration. Germline transmission rates for precision-targeted integration alleles range 22-100%. Our system provides a streamlined, straightforward, and cost-effective approach for high-efficiency gene targeting applications in zebrafish.

Keywords: CRISPR/Cas9, Knock-in, Homology mediated-end joining, Targeted integration, Zebrafish

This protocol was validated in: eLife (2020). DOI: 10.7554/eLife.53968

Graphical Abstract:

GeneWeld



GeneWeld method for CRISPR/Cas9 targeted integration.

Background

Designer nucleases have rapidly expanded the way in which researchers can utilize endogenous DNA repair mechanisms for creating gene knock-outs, reporter gene knock-ins, gene deletions, single nucleotide polymorphisms, and epitope-tagged alleles in diverse species (Bedell *et al.*, 2012; Beumer *et al.*, 2008; Carlson *et al.*, 2012; Geurts *et al.*, 2009; Yang *et al.*, 2013). A single dsDNA break in the genome results in increased frequencies of recombination and promotes integration of homologous recombination (HR)-based vectors (Hasty *et al.*, 1991; Hoshijima *et al.*, 2016; Orr-Weaver *et al.*, 1981; Rong and Golic, 2000; Shin *et al.*, 2014; Zu *et al.*, 2013). Additionally, *in vitro* or *in vivo* linearization of targeting vectors stimulates homology-directed repair (HDR) (Hasty *et al.*, 1991; Hoshijima *et al.*, 2016; Jasin and Berg, 1988; Orr-Weaver *et al.*, 1981; Rong and Golic, 2000; Shin *et al.*, 2014; Zu *et al.*, 2013). Utilizing HDR or HR at a targeted double-strand break (DSB) allows directional knock-in of exogenous DNA with base-pair precision; however, reported frequencies vary widely, and engineering targeting vectors with long homology arms is not straightforward.

Previous work has shown that Xenopus oocytes have the ability to join or recombine linear DNA molecules that contain short regions of homology at their ends, and this activity is likely mediated by exonuclease activity, allowing base pairing of the resected homology (Grzesiuk and Carroll, 1987). More recently, it was shown in *Xenopus*, silkworm, zebrafish, and mouse cells that a plasmid donor containing short (≤ 40 bp) regions of homology to a genomic target site can promote precise integration at the genomic cut site when the donor plasmid is cut adjacent to the homology (Aida *et al.*, 2016; Hisano *et al.*, 2015; Nakade *et al.*, 2014). Gene targeting is likely mediated by the alternative-end joining/microhomology-mediated end joining (MMEJ) pathway or by a single-strand annealing (SSA) mechanism (Ceccaldi *et al.*, 2016), collectively referred to as a homology mediated end joining (HMEJ). In contrast, in human cell culture, linear donors using a similar strategy with homologous ends have been reported to show inefficient integration until homology domains reach ~ 600 bp (Zhang *et al.*, 2017), suggesting different repair pathways may predominate depending on cell type. In the initial reports using short regions of homology for *in vivo* gene targeting in zebrafish, the level of mosaicism in F0 injected animals was high, resulting in inefficient recovery of targeted alleles through the germline (Aida *et al.*, 2016; Hisano *et al.*, 2015; Luo *et al.*, 2018; Nakade *et al.*, 2014). Most recently, studies in Drosophila show efficient integration of exogenous DNA in flies and S2 cells using 100 bp homology arms flanked by a CRISPR target site for *in vivo* homology liberation (Kanca *et al.*, 2019). Together, these studies suggest that a strategy that combines short homology flanked donors with *in vivo* homology arm liberation should lead to efficient precision targeting in zebrafish and mammalian cells.

Here, we present GeneWeld, a HMEJ strategy for targeted integration directed by short homology that we demonstrated leads to efficient germline transmission rates for recovery of targeted alleles in zebrafish (Wierson *et*

al., 2020). We provide a suite of donor vectors, called pGTag, that can be easily engineered with homologous sequences (homology arms) to a gene of interest and a web interface for designing homology arms (Mann *et al.*, 2019). Homology of 24 or 48 base pairs directly flanking the cargo DNA promotes efficient gene targeting in zebrafish, with germline transmission rates averaging approximately 50%. The tools and methodology described here provide a tractable solution to creating precise targeted integrations and open the door for more advanced genome editing strategies using short homology. The following detailed protocol outlines steps for gRNA selection, homology arm design, vector construction, CRISPR/Cas targeting, and recovery of targeted integration alleles.

Materials and Reagents

1. Polystyrene Petri dishes (Thermo Fisher, catalog number: FB0875713)
2. Borosilicate Glass Capillaries (World Precision Instruments, catalog number: 1B100-04)
3. Microloader tips (Eppendorf, catalog number: 920001007)
4. Commercially available molds for injection plates available from https://www.agnthos.se/index.php?id_product=204&controller=product
5. Kwik-Fill borosilicate glass capillaries (World Precision Instruments, catalog number: 1B100-4)
6. EasyStrip Plus Tube Strip with Attached Ultra Clear Caps (Thermo Fisher Scientific catalog number: AB2005)
7. pGTag vectors are available through Addgene (<https://www.addgene.org/kits/essner-geneWeld/>)
8. NEB Stable Competent *E. coli* (New England Biolabs, catalog number: C3040I)
9. One Shot TOP10 Chemically Competent *E. coli* (Thermo Fisher, Invitrogen, catalog number: C404010)
10. pT3TS-nCas9n expression vector (Addgene, catalog number: 46757)
11. PureYield Plasmid Miniprep System (Promega, catalog number: A1223)
12. Ambion mMessage Machine T3 Transcription Kit (Thermo Fisher, catalog number: AM1348)
13. miRNeasy Mini Kit (Qiagen, catalog number: 217004)
14. NEBNext Ultra II DNA Library Prep Kit for Illumina (New England Biolabs, catalog number: E7645L)
15. Zebrafish Tg (*miniTol2<14XUAS:mRFP,γcry:GFP>*)^{T2} (Balciuniene *et al.*, 2013)
16. Zebrafish wild-type WIK strain (Zebrafish International Resource Center, catalog number: ZL84, <https://zebrafish.org/home/guide.php>)
17. Agarose (Thermo Fisher, catalog number: BP160-500)
18. Ethidium Bromide (Fisher Scientific, catalog number: BP1302-10)
19. Ethyl 3-aminobenzoate methanesulfonate, Tricaine MS-22 C₉H₁₁NO₂·CH₄SO₃ (Millipore Sigma catalog number 886-86-2)
20. 1-phenyl-2-thiourea C₇H₈N₂S (Thermo Fisher, catalog number: AC207250050)
21. NorthernMax-Gly Sample Loading Dye (Thermo Fisher, catalog number: AM8551)
22. Decon ELIMINase (Fisher Scientific, catalog number: 04-355-31)
23. Molecular Grade RNase/DNase-Free water (e.g., Invitrogen, catalog number: 10977023)
24. XbaI restriction endonuclease (New England Biolabs, catalog number: R0145S)
25. BfuAI restriction endonuclease (New England Biolabs, catalog number: R0701S)
26. BspQI restriction endonuclease (New England Biolabs, catalog number: R0712S)
27. GoTag Green 2× MasterMix (Promega, catalog number: M7123)
28. X-Gal solution, ready-to-use, 20mg/ml (Thermo Fisher Scientific, catalog number: R0941)
29. LB broth (Fisher Scientific, catalog number: BP9723-500)
30. LB agar (Fisher Scientific, catalog number: BP9724-2)
31. SOC medium (Thermo Fisher Scientific, catalog number: 15544034)
32. Kanamycin (Fisher Scientific, catalog number: BP9065)
33. T4 Quick Ligase, Rapid DNA Ligation Kit (Thermo Fisher Scientific, catalog number: K1422)
34. Sodium Hydroxide NaOH (Millipore, Sigma-Aldrich, catalog number: 30620)
35. Tris Base (Fisher Scientific, catalog number: BP152-500)
36. UgRNA
5'-
GGGAGGCGUUCGGGCCACAGGUUUUAGAGCUAGAAAUGCAAGUUAAAAUAAGGCUAGUCC

- GUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCGGAUC-3' and gene-specific sgRNAs ordered from Synthego (https://orders.synthego.com/products/crisprevolution-sgrna-ez-kit-13/#/tubes?mod_code=1) or IDT (<https://www.idtdna.com/site/order/oligoentry/index/crispr> (see Section B.)
37. Primers, can be ordered from IDT:
F3'-check: 5'-GGCGTTGTCTAGCAAGGAAG-3'
R3' pgtag seq: 5'-ATGGCTCATAACACCCCTTG-3'
R-Gal4-5'juncM: 5'-GCCTGATTCCACTTCTGTCA-3'
R-RFP-5'junc: 5'-CCTTAATCAGTTCCCTGCCCTTAGA-3'
R-eGFP-5'junc: 5'-GCTGAACTTGTGGCCGTT-3'
F-Gal4-3'juncM: 5'-GCAAACGGCCTTAACCTTC-3'
F-Gal4-3'junc: 5'-CTACGGCGCTCTGGATATGT-3'
F-RFP-3'junc: 5'-CGACCTCCCTAGCAAACGGGG-3'
F-eGFP-3'junc: 5'-ACATGGTCCTGCTGGAGTTC-3'
 38. Zebrafish embryo E2 Medium (see Recipes)

Equipment

1. Microcap Microliter Pipets (Drummond Scientific, catalog number: 1-000-0010)
2. Flaming/Brown Micropipette Puller (Sutter Instrument, model: P-97)
3. X-Cite 120W Metal Halide lamp (Excilitas Technologies, model: X-Cite 120Q)
4. Pico-Injector (Harvard Apparatus, model: PLI-100)
5. MM-3 Micromanipulator (Narishige, model: MM-3)
6. Nitrogen gas tank, or JUN-AIR Oil-lubricated Piston Air Compressor (Cole-Parmer, catalog number: 1152000)
7. Nanodrop (Thermo Fisher Scientific, model: NanoDrop 2000)
8. iBright FL1500 Imaging System (Thermo Fisher Scientific, model: A44241)
9. Shaking Incubator (Thermo Scientific, model: MaxQ8000)
10. Isotemp Standard Laboratory Incubator (*e.g.*, Thermo Scientific, model: 51-028-065HPM)
11. Precision General Purpose Baths (*e.g.*, Thermo Scientific, model: TSGP02)
12. Thermal Cycler (Eppendorf, 6335000020)
13. Zeiss SteREO Discovery.V8 Stereomicroscope or similar and epi-illumination X-Cite 120W metal halide light source with fiber optic cable (Excilitas Technologies)
14. Owl EasyCast B2 Mini Gel Electrophoresis Systems (Thermo Fisher Scientific, model: B2-12)

Software

1. GTagHd, Gene Sculpt Suite www.genesculpt.org/gtaghd/ (Mann *et al.*, 2019)
2. CRISPRScan <http://www.crisprscan.org/> (Moreno-Mateos *et al.*, 2015)
3. Primer 3 http://biotools.umassmed.edu/bioapps/primer3_www.cgi
4. Synthego ICE Analysis <https://ice.synthego.com/#/>
5. Cas-Analyzer at CRISPR RGEN Tools <http://www.rgenome.net/cas-analyzer/#>
6. GraphPad Prism <https://www.graphpad.com/scientific-Software/prism/>

Procedure

The GeneWeld protocol is associated with the publication Wierson *et al.* (2020).

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A. Introduction

The GeneWeld strategy (Figure 1) and pGTag vector series are designed for straightforward assembly of vectors containing short homology arms for efficient CRISPR/Cas9-directed recovery of germline precision targeted integration alleles.

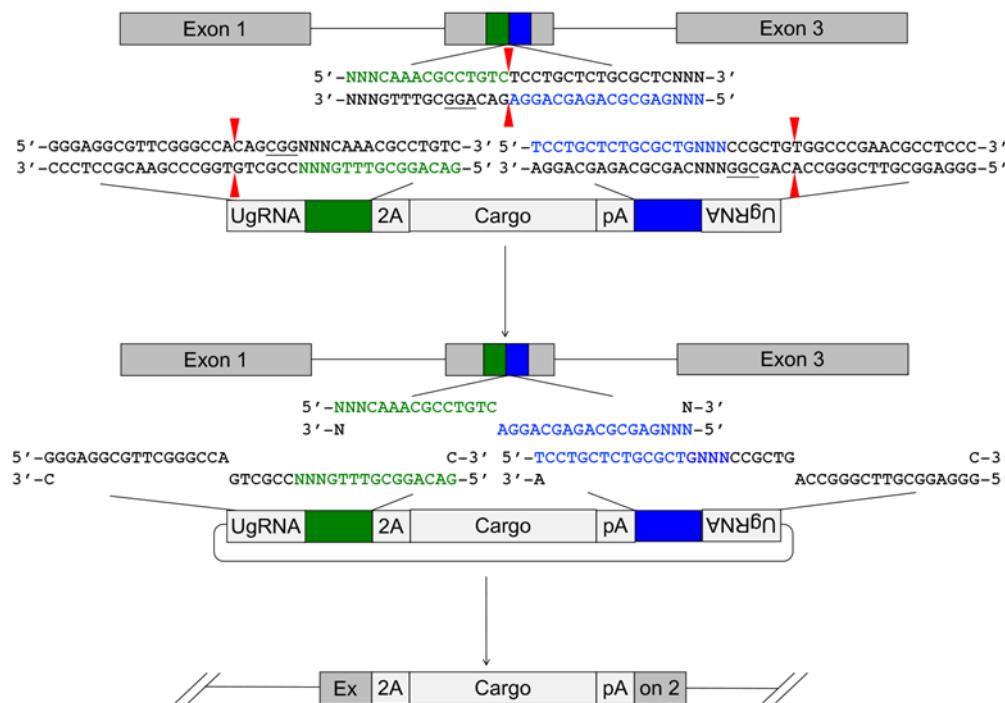


Figure 1. Targeted integration of pGTag vector cargo DNA into a 5' coding exon.

Short homology arms complementary to the 5' (green) and 3' (blue) sequences of the genomic target site are cloned on the 5' and 3' sides of the vector cargo DNA. The short homology arm cargo cassette is flanked by two universal guide RNA UgRNA sites. CRISPR/Cas9 simultaneously targets double-strand breaks at the sgRNA genomic target site and at the UgRNA sites flanking the cargo on the plasmid donor. Exonuclease end resection liberates single-stranded DNA in the vector homology arms that is complementary to the resected strands on the 5' and 3' sides of the genomic double-strand break. The complementary sequences direct homology mediated end joining integration of the cargo DNA at the exon target site. PAM sequences are underlined, and small red arrows indicate Cas9 cut sites in the genome and vector.

B. Selection of a CRISPR/spCas9 target site in the gene of interest

- Zebrafish wild-type strains in common use are polymorphic.

Note: It is highly recommended to first sequence the target exon in the genomic DNA from your fish strain and use this sequence to design gRNAs.

- To identify an sgRNA site for targeting, first, view the gene model on a genome browser and download the gene sequences.
 - At <ensembl.org> Search for the gene name of interest for the species of interest and open the Transcript page (Figure 2).

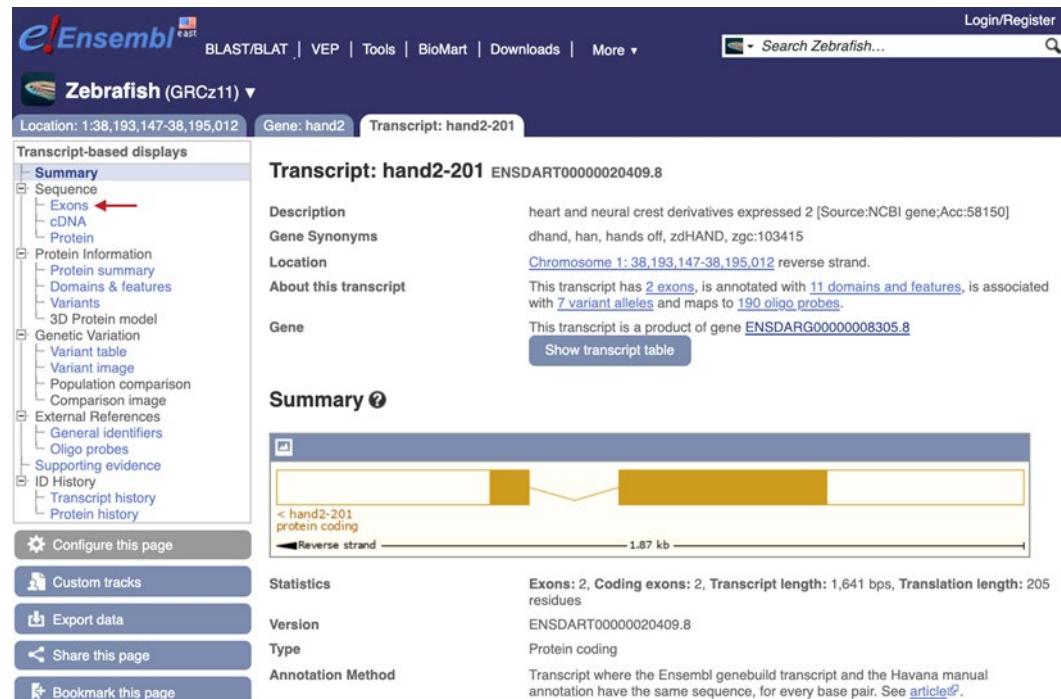


Figure 2. Screenshot of zebrafish hand2 Transcript page on the ensembl.org genome browser (https://useast.ensembl.org/Danio_reario/Transcript/Summary?db=core;g=ENSDARG0000008305;r=1;38193147-38195012;t=ENSDART00000020409)

- In the left-hand side bar click on “Exons” to find the first coding exon and initiation ATG (Figure 3).

Note: If there are alternative transcripts for the gene, make sure there are not alternative initiation ATGs. If there are alternative start codons, target the first exon that is conserved in all transcripts to generate a strong loss-of-function allele.

- Click on the Download sequence button (Figure 3, red arrow). A list of choices for genomic, coding, untranslated regions, and intronic sequences is shown. Select the cDNA and target exon and download as separate sequence files.
- Using ApE <<http://biologylabs.utah.edu/jorgensen/wayned/ape/>> or SnapGene, annotate the coding sequence with the exons.
- Design primers to amplify the target exon from fin clip genomic DNA and sequence the amplicon. Use this sequence to identify gRNA sites either manually, by searching for PAM sequences, or using CRISPRScan. Remove additional “Gs” CRISPRScan added to the 5’ end. Select a sgRNA location that does not have an in-frame ATG downstream of the sgRNA target site. Annotate the

selected target sequence and NGG PAM in the cDNA sequence files.

3. Synthetic sgRNAs can be ordered from Synthego or IDT. On the Synthego ordering page, select “2’-O-Methyl at 3 first and last bases, 3’ phosphorothioate bonds between first 3 and last 2 bases” the Modifications tab.
4. Design ~20 bp DNA primers for a PCR amplicon of ~130 bp of DNA surrounding the sgRNA target site. These primers will be used to amplify genomic DNA from embryos after injection of CRISPR reagents to test for mutagenesis at the target site. The presence of indels at the target site can be detected in the PCR products in multiple ways, including gel electrophoresis to visualize heteroduplex formation, resistance to restriction enzyme digestion at a site overlapping the sgRNA target and direct sequencing followed by ICE Analysis.
 - a. Users can design primers with Primer 3 http://biotools.umassmed.edu/bioapps/primer3_www.cgi.
 - b. Paste DNA sequence surrounding the target site into the web interface. It is recommended to use 160-300 bp of exon sequence centered on the cut site for primer design. Intron sequence can be used, but this often contains polymorphisms that can lead to amplification failure.
 - c. Locate the target sequence, including the PAM sequence NGG (underlined, Bold in the example below), and predict the cut site (3 bp upstream of the PAM represented here by the ‘x’). Mark the targeted exon sequence approximately 65-150 bp on both sides of the cut site by putting [square brackets, highlighted in yellow] around it. Primer3 will design primers outside this sequence. This design allows the primers to be used for both checking of mutagenesis and for junction fragment analysis when checking for integration.

Example:

CGGCCTCGGGATCCACCGGCC[AGAATCGATACTACGATGAACAGAGCAAATTGTG
TGTAATACGGTCGCCACCATGGCCTxCCTCGGTTGCTACGATGCATTGCACCACTCT
CTCATGTCCGGTTCTGGG]AGGACGTCATCAAGGAGTTCATGCGCTCAAGGTGCGCA
TGGAGGGCTCCGTGAAC

- d. Set the “Primer Size” variables to Min = 130, Opt = 170, and Max = 300. Everything else can be left at the defaults.
- e. Click on “Pick Primers.”
- f. Select primers from the output. Note the “product size” expected and the “tm” or melting temperature of each primer/pair. Mutagenesis is easier to visualize in smaller product sizes.

Figure 3. Exon sequences and Download page for zebrafish *hand2* gene on the ensembl.org genome browser

5. Preparation of SpCas9 mRNA
 - a. Digest ~5-10 µg pT3TS-nCas9n plasmid (plasmid Addgene #46757) (Jao *et al.*, 2013) with XbaI to linearize the vector.
 - b. Purify linearized DNA with a Qiagen PCR cleanup kit or Promega PureYield Plasmid Miniprep System. Elute in RNase-free water.
 - c. Run 100-500 ng on a 1.2% agarose gel in 1× TAE to confirm the plasmid is linearized.
 - d. Use 100 ng to 1 µg DNA as template for *in vitro* transcription reaction with the mMESSAGE mMACHINE T3 kit Life Technologies (AM1348). Follow the manufacturer’s instructions provided with the kit. Save a 1 µL aliquot of the *in vitro* synthesis reaction.
 - e. Use the miRNeasy Qiagen kit to purify the nCas9n mRNA according to the manufacturer’s instructions.
 - f. Verify mRNA integrity by running a sample of the *in vitro* synthesized mRNA on a gel, before and after the miRNeasy Qiagen kit cleanup. Mix 1 µL of Cas9 mRNA, 4 µL of Molecular Grade RNase/DNase-free water, and 5 µL glyoxal dye (NorthernMax-Gly Sample Loading Dye, Thermo Fisher, AM8551).
 - g. Heat mixture at 50°C for 30 min in a water bath or thermal cycler, then place on ice.
 - h. Clean the gel box, comb, and tray with Decon ELIMINase and rinse with DI water.
 - i. Run all 10 µL of RNA mixture on a 1.2% agarose gel in 1× TAE at 100 V for 1 h as described above. Image gel in an iBright FL1500 Imaging System or another gel-documentation imaging system. One band should be visible at ~4.5 kb.
 - j. Determine the concentration of the RNA sample using a Nanodrop. Concentrations between 0.45 and 1 µg/µL are expected.
 - k. Aliquot and store RNA at -80°C.

C. Injection of sgRNA and spCas9 mRNA

Deliver 25 pg sgRNA and 300 pg Cas9 mRNA in a 2 nl volume to embryos at the one-cell stage. Below is a step-by-step protocol for zebrafish embryo injection.

1. A detailed video of zebrafish embryo injection can be found in Rosen *et al.* (2009). Cast zebrafish embryo injection trays with custom molds that create 45° troughs for lining up and holding embryos (Figure 4A). Molds are also available commercially (see Materials and Reagents). Melt 1.2% agarose in 1× E2 Medium and pour into a polystyrene Petri dish. The mold is set on top (Figure 4B), and, once the plates have set, gently remove the mold (Figure 4C). Injection trays can be used multiple times and are stored inverted at 4°C for up to three weeks between use.

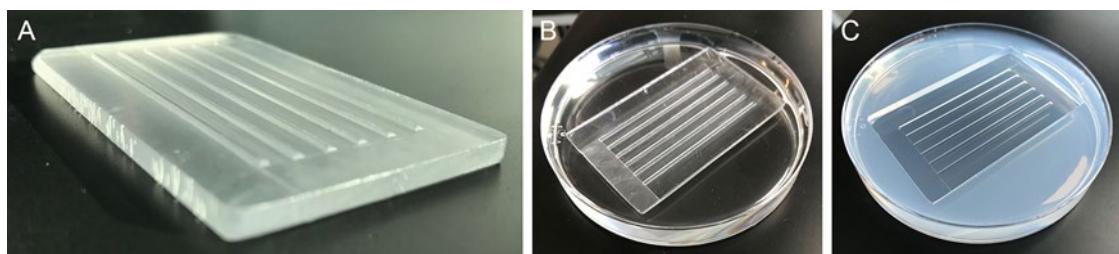


Figure 4 . Injection tray mold.

The injection tray mold (A) is set on top of melted 1.2% agarose (B). Solidified injection plate with troughs to hold embryos (C).

2. Pre-warm injection trays to 28.5°C prior to injection by placing them in a 28.5°C incubator for 20 min.
3. Pull microcapillary glass needles using Kwik-Fill borosilicate glass capillaries on a Sutter Instrument Flaming/Brown Micropipette Puller.
4. Prepare injection samples containing the following diluted in Molecular Grade RNase/DNase-free water:
12.5 ng/μL of genomic sgRNA
150 ng/μL of mRNA for Cas9
Keep injection solution on ice
5. Backload needles with injection solution using microloader pipet tips and attach to a micromanipulator. Connect the needle holder tubing to a Harvard Apparatus PLI-90 Pico-injector. Turn on nitrogen gas or the air compressor to pressurize the system and set injection pressure to 40 PSI with an injection time of 100-200 ms.
6. Calibrate injection needle by first breaking the end of the tip off with sterile tweezers (Figure 5A, B). Use the pedal to expel 10 droplets and capture each droplet with a 30 mm long capillary tube that represents a volume of 1 μL (Figure 5C). Measure the distance from the end of the capillary to the meniscus of the liquid and convert to a volume of 1 mm = 30 nL; therefore, 2/3 of a mm = 20 nL. The volume of each droplet is adjusted by changing the injection time to achieve 2 nL droplets. There is a linear relationship between volume and time at a set pressure. Avoid injection times lower than 100 ms and higher than 400 ms.
7. Transfer one-cell embryos from collection Petri dishes to the troughs on the pre-warmed agarose injection tray (Figure 5D). Each embryo is encased in a chorion. As the embryos near the first cell division at 45 minutes after fertilization, the single cell is clearly visible atop the yolk (Figure 5D).
8. Use the micromanipulator to pierce the needle through the chorion and into the embryo. Inject 2 nL of sample at the center of the yolk interface/boundary between the single cell and the yolk (Figure 5E, white arrow points to interface where needle tip is placed). Inject embryos before the first cell division begins.

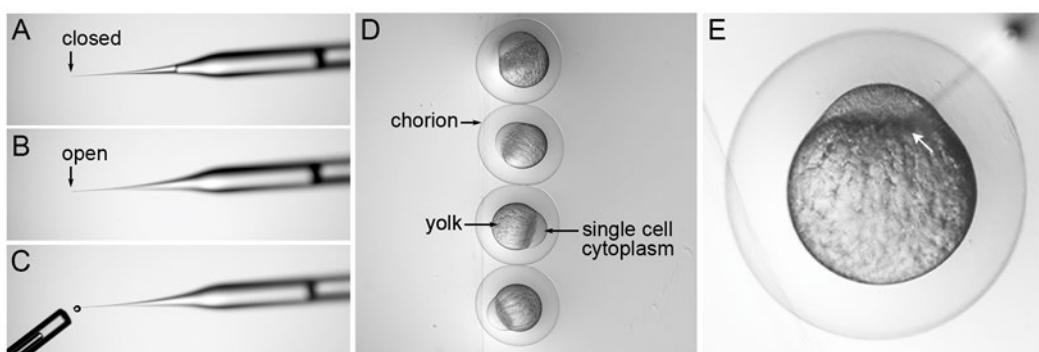


Figure 5. Microinjection needle calibration and zebrafish single-cell embryo microinjection.

(A) Backloaded injection needle with closed tip. (B) A small portion at the tip of the needle is removed using forceps to create an open end. (C) A single droplet of injection solution is expelled by pressing on the injection apparatus pedal. The tip of a 1 μ L Drummond capillary tube that was used to capture 10 drops is shown. (D) Embryos lined up in an injection tray trough with labels indicating the chorion, yolk, and single-cell embryo. (E) Needle inserted through the chorion and into the embryo. The tip of the injection needle is positioned at the yolk interface (white arrow) between the single cell on top and the yolk below. The image in (E) was published in Almeida *et al.* (2021).

9. After injection, wash the embryos from the injection tray into a clean Petri dish with embryo media.
10. Keep 20-40 embryos separate to use as uninjected controls.
11. At 3-5 h post injection, remove any unfertilized or dead embryos from the dishes.

D. Test for sgRNA mutagenesis efficiency and indel production

1. Biallelic inactivation can lead to loss of function of essential genes that may be lethal. After injection, count and remove dead embryos from the dish. If all embryos are defective and unlikely to survive, reduce the amount of guide sgRNA that is injected to 12.5 pg. If embryos still fail to survive, reduce the amount of sgRNA further to 6.25 pg. As we reported previously, for a ubiquitously expressed, essential gene such as the tumor suppressor *rb1*, the amount of injected sgRNA needs to be reduced to 6.25 pg to recover viable juvenile fish that survive to adulthood and transmit germline gene-edited alleles (Solin *et al.*, 2015).
2. Digestion of embryos for isolation of genomic DNA for mutagenesis analysis.
Extract genomic DNA from either individual or pools of five embryos from the same injection, aged between 1 and 5 dpf, using the following protocol previously published (Wierson *et al.*, 2020).
 - a. Dechorionate embryos if they have not emerged from the chorion.
 - b. Place embryo into a PCR tube and remove as much of the fish water as possible. Collect at least three injected embryos and one uninjected control embryo.
 - c. Add 20 μ L of 50 mM NaOH per embryo.
 - d. Heat the embryos at 95°C in a thermocycler for 30 min.
 - e. Vortex samples and spin the tubes down. The embryos should be completely dissolved.
 - f. Neutralize the samples by adding 1 μ L of 1 M Tris pH 8.0 per 10 μ L NaOH. Mix by vortexing, then spin down.
 - g. Genomic DNA is stored at -20°C.
3. Analysis of CRISPR/Cas9 mutagenesis efficiency at targeted gene locus.
 - a. Remove genomic DNA samples from -20°C and place on ice to thaw. Keep thawed genomic DNA on ice at all times.
 - b. Set up the following PCR reactions for each tube of embryo digested genomic DNA using the gene-specific Forward and Reverse primers that were designed to create an amplicon around the CRISPR/Cas9 target site.

- 12.5 μ L of 2 \times GoTaq Mastermix
 1 μ L of Forward Primer (10 μ M)
 1 μ L of Reverse Primer (10 μ M)
 1 μ L of gDNA template (digested embryos)
 9.5 μ L of nuclease-free water
 For a 25 μ L total
- c. Flick the tubes to mix and briefly spin down the PCR reactions.
- d. Run the following PCR program to amplify the targeted locus.
- 95°C 2 min
 95°C 30 s }
 55°C 30 s } 35 cycles
 72°C 30 s }
 72°C 5 min
 4°C hold
- e. Run up to 7 μ L of PCR product on a 2.5 to 3% agarose gel in 1 \times TAE, for 1 h at 80-100V. Image gel in the iBright FL1500 Imaging System or another gel-documentation imaging system.
- An example of sgRNA injection and validation targeted exon 1 of the *hand2* gene is shown in Figure 6A. The control uninjected embryo PCR amplicon runs as a single, tight band on the gel (Figure 6B, U). The amplicons from eight injected embryos show multiple bands or are diffuse in appearance relative to the control (Figure 6B, 1-8). This indicates heteroduplex formation in the PCR product caused by the presence of indel mutations at the CRISPR target site in the gene of interest.

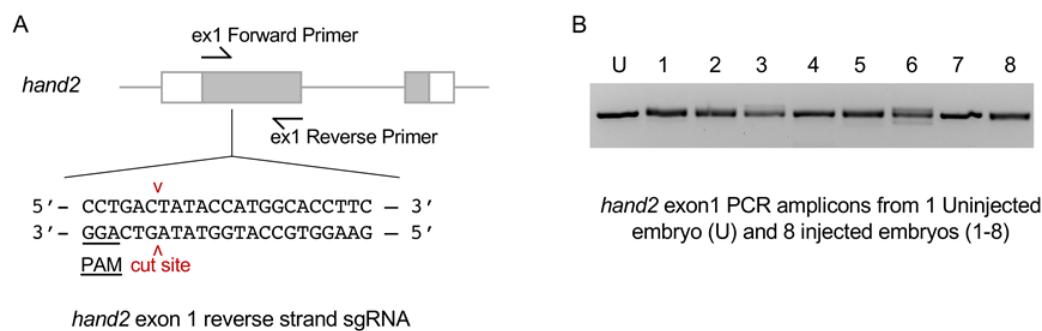


Figure 6. *hand2* exon 1 sgRNA validation.

A. Sequence of the *hand2* reverse strand sgRNA site located in exon 1. B. PCR amplicons with primers flanking the sgRNA target site. Diffuse bands in injected embryos represent heteroduplex DNA caused by indel mutations at the target site.

- f. For quantitative analysis of mutagenesis efficiency, Sanger sequence PCR products to verify the presence of indels. The percentage of indel formation can be analyzed using Synthego's ICE Analysis. Alternatively, Illumina MiSeq multiplex next-generation sequencing can be used to test the efficiency of multiple gRNAs in parallel.

An example of ICE analysis of amplicons from the *hand2* exon 1 targeted embryos #3 and #6 from Figure 6 is shown below in Figure 7. Embryo #3 and #6 show 84% and 80% indel alleles after targeting, respectively, indicating high mutagenesis efficiency of the sgRNA.

A. ICE analysis of *hand2* exon 1 sgRNA targeted embryo #3B. ICE analysis of *hand2* exon 1 sgRNA targeted embryo #6**Figure 7. Validation of sgRNA mutagenesis efficiency by ICE analysis.**

PCR amplicons from *hand2* exon 1 targeted embryos #3 (A) and #6 (B) were Sanger sequenced and the results analyzed with Synthego ICE Analysis, revealing 84% and 80% of sequences contained indel mutations. Plots on the right show the range of indel mutations recovered.

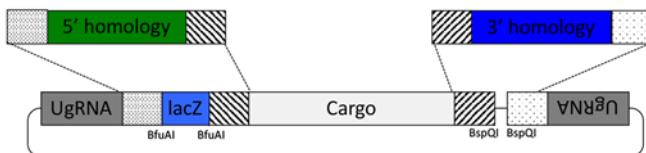
E. Design short homology arms for pGTag targeting vector assembly

Homology-directed gene targeting allows the seamless integration of exogenous DNA into the genome with precise repair events at the target site. However, designing and cloning individual targeting vectors and homology arms for each gene of interest can be time consuming. The pGTag vector series and web design tools provide versatility and ease to generate knock-out alleles (Figure 8). The vectors contain BfuAI and BspQI type II restriction enzymes for cloning of short homology arms (24 or 48 bp) using Golden Gate cloning. The pGTag vectors require in-frame integration for proper reporter gene function. The reporter gene consists of several parts. A 2A peptide sequence causes translational skipping, allowing the following protein to dissociate from the locus peptide. The eGFP, TagRFP, or Gal4VP16 reporter coding sequences have several options for localization signals, including cytosolic (no signal), a nuclear localization signal (NLS), or a membrane localization CAAX sequence. Finally, translation is terminated by one of two different transcription termination polyadenylation (pA) sequences, the 3'UTR region of the zebrafish β -actin gene or the SV40 viral transcription termination sequence.

For many genes, the level of endogenous gene expression is not high enough to produce a detectable fluorescence signal from the integrated reporter gene. The Gal4VP16 pGTag vector in combination with the transgenic Tol2<14XUAS/RFP> reporter line (Balciuniene *et al.*, 2013) allows for amplification of the signal. The Tol2<14XUAS/RFP> reporter line is available upon request from the lab of Dr. Darius Balciunas, Temple University.

Plasmid sequence maps can be downloaded at www.genesculpt.org/gtaghd/.

A pGTag vector:



B Cargo Suite:

Localization Signal	Reporter	Polyadenylation Signal
NLS	eGFP	SV40 pA
CAAX	tagRFP	β-actin pA
		Gal4VP16

Figure 8. The pGTag vectors allow one step cloning of homology arms.

All vectors (Figure 8) can be obtained through Addgene (www.addgene.org). Because the pGTag plasmids contain repeated sequences, vector recombination can occur in bacteria. We recommend using NEB Stable Competent *E. coli* (New England Biolabs, C3040I). Bacteria should be grown at 30°C to further reduce vector recombination.

Homology Arm Design at GTagHD

The web tool GTagHD allows for quick design of oligos to generate short 24 or 48 bp homology arms complementary to the target site in a gene of interest.

Two complementary oligos with overhangs are annealed to generate the double stranded homology arm for cloning into the pGTag vector.

To use the tool, choose the "Submit Single Job" tab. Follow the instructions in the tab.

The sequences of two pairs of complementary oligos will be returned, one pair for the 5' homology arm and the other for the 3' homology arm. If there are problems with the sequences and values that were entered, the web page will display the errors and advice on how to fix them. Double-check your output as below.

Manual Homology Arm Design

The following protocol describes how to design homology arm oligos manually:

Note: In the following section, orientation of target sites and homology is in the context of the reading frame of the genetic locus of interest. Example: A forward strand CRISPR gRNA means that the gRNA and PAM are encoded on the sense strand of the gene. Upstream homology domains are 5' to the CRISPR/Cas9 cut site, and downstream homology domains are 3' to the cut site with respect to the reading frame of the gene being targeted.

Also note: Upper case and lower case bases are not specially modified; this is simply a visual marker of the different parts of the homology arms.

Upstream Homology Arm Design

1. Open the sequence file for the gene of interest and identify the CRISPR site (in this example, it is a Reverse CRISPR target in Yellow, the PAM is in Orange, and the coding sequence is in purple). Copy the 48 bp 5' of the CRISPR cut (the highlighted section below) into a new sequence file; this is the upstream homology (Figure 9).



Figure 9. Screenshot of a targeted gene displayed in ApE, highlighting the target sequence (yellow), PAM (orange), coding sequence (purple), and the gene sequence of the upstream homology arm (highlighted white).

- Observe the next three bases immediately upstream of the 48 bp of homology, and pick a base not present to be the 3 bp spacer between the homology and the Universal PAM in the vector. Here, the three bases are “GGA,” so “ccc” was chosen for the spacer. Add the spacer to the new file 5’ (in front) of the homology (see below). The spacer acts as a non-homologous buffer between the homology and the eventual 6 bp flap from the universal guide sequence that will occur when the cassette is liberated and may improve intended integration rates over MMEJ events (Figure 10).

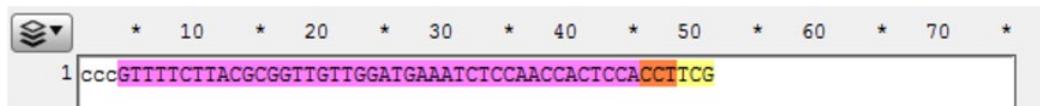


Figure 10. Screenshot of the gene sequence of the upstream homology arm (purple), the PAM (orange), and the remaining target sequence to the cut site (yellow).

ccc was added as a spacer with a non-homologous sequence.

- Determine where the last codon is in the homology. Here, the 3’ G in the homology domain is the first base in the codon cut by this CRISPR target. Complete the codon by adding the remaining bases (called padding on GTagHD) for that codon from your sequence to ensure your integration event will be in frame (Figure 11).

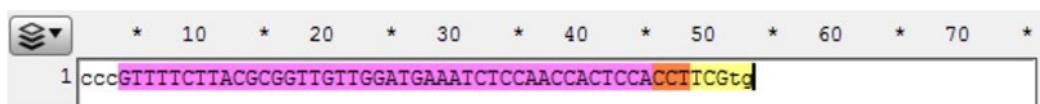


Figure 11. Screenshot of the gene sequence of the upstream homology arm (purple), the PAM (orange), and the remaining target sequence to the cut site with the padding nucleotides (tg) to keep the integration in frame (yellow).

- Add the BfuAI enzyme overhang sequences for cloning to the ends of the homology domain. Here, both overhangs, 5’-GCGG and 3’-GGAT, are added to prevent errors in copying sequence for the oligos in the next two steps (Figure 12).

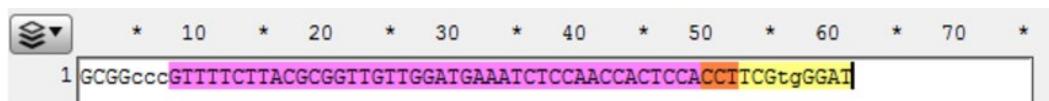


Figure 12. Screenshot of the gene sequence of the upstream homology arm (purple), the PAM (orange), and the remaining target sequence to the cut site with the padding nucleotides (tg) to keep the integration in frame (yellow) and the BfuAI sites added to each end.

5. The Upstream Homology Oligo A will be this sequence from the beginning to the end of the last codon (see highlighted below). Copy and paste this sequence into a new file and save it. In this example, this oligo sequence is 5'-GCGGcccGTTTCTTACCGGGTTGGATGAAATCTCCAACCACTCCACCTTCGtg-3' (Figure 13).



Figure 13. Screenshot of the gene sequence of the upstream homology arm (purple), the PAM (orange), and the remaining target sequence to the cut site with the padding nucleotides (tg) to keep the integration in frame (yellow) and the BfuAI sites added to each end.

The sequence of Oligo A is in white.

6. The Upstream Homology Oligo B will be the reverse complement of this sequence from the beginning of the spacer to the end of the sequence (see highlighted below in Figure 14). Copy the reverse complement, paste it into a new file, and save it. In this example, this oligo sequence is 5'-ATCCcaCGAAGGTGGAGTGGTTGGAGATTTCATCCAACAAACCGCGTAAGAAAACggg-3'.



Figure 14. Screenshot of the gene sequence of the upstream homology arm (purple), the PAM (orange), and the remaining target sequence to the cut site with the padding nucleotides (tg) to keep the integration in frame (yellow) and the BfuAI sites added to each end.

The sequence of Oligo B is highlighted. Use the reverse complement of the highlighted sequence.

Downstream Homology Arm Design

7. Open sequence file for the gene of interest and identify the CRISPR site. The reverse CRISPR target is in Yellow, the PAM in Orange, and coding sequence in purple. Copy the 48 bp 3' of the CRISPR cut into a new sequence file; this is the downstream homology (Figure 15).

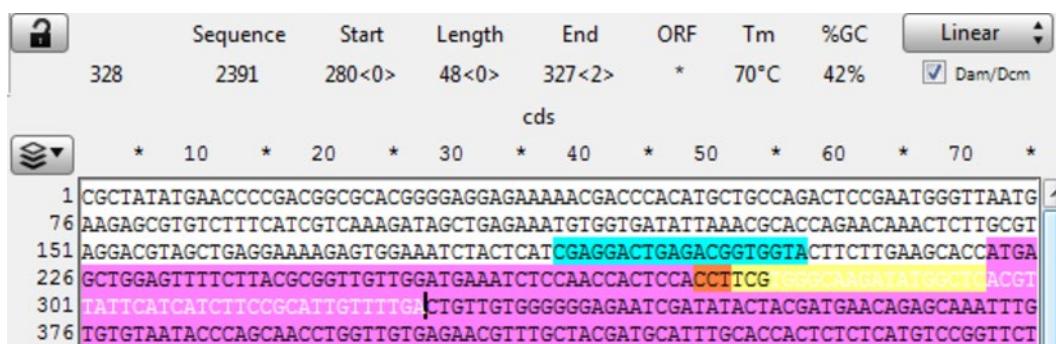


Figure 15. Screenshot of a targeted gene, highlighting the target sequence (yellow), PAM (orange), coding sequence (purple), and the gene sequence of the downstream homology arm (in white).

- Observe the next three bases downstream of the 48 bp of homology and pick a base not present to be the 3 bp spacer between the homology and the Universal PAM in the vector. Here, the bases are “CTG;” therefore, “aaa” was chosen for the spacer. Add the spacer to the new file 3’ of (after) the homology (Figure 16).

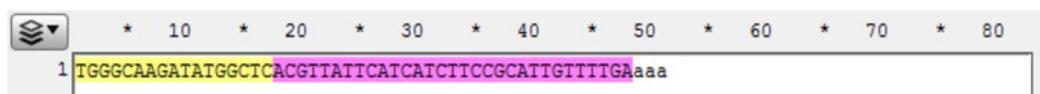


Figure 16. Screenshot of the gene sequence in the downstream homology arm from the targeted gene.

This comprises part of the target sequence (yellow) and additional 3’ coding sequence (purple). aaa was added as padding nucleotides.

- Add the BspQI enzyme overhang sequences for cloning to the ends of the homology domain. Here, both overhangs, 5’-AAG and 3’-CCG, are added to prevent errors in copying sequence for the oligos in the next two steps (Figure 17).

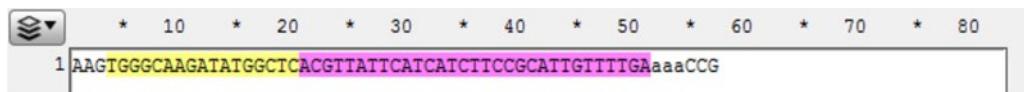


Figure 17. Screenshot of the gene sequence in the downstream homology arm from the targeted gene with part of the target sequence (yellow) and additional 3’ coding sequence (purple). BspQI enzyme overhang sequences are added to each end.

- The Downstream Homology Oligo A will be this sequence from the beginning of the sequence to the end of the spacer (see highlighted below). In this example, this oligo sequence is 5’-AAGTGGGCAAGATAATGGCTCACGTTATTACATCATCTTCCGCATTGTTTGAaaa-3’ (Figure 18).

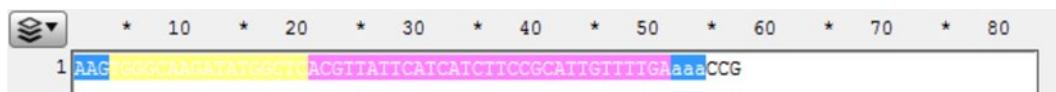


Figure 18. Screenshot of the gene sequence in the downstream homology arm from the targeted gene with part of the target sequence (yellow) and additional 3’ coding sequence (purple). The sequence for Oligo A is in white.

11. The Downstream Homology Oligo B (will be the reverse complement of this sequence from the beginning of the homology to the end of the sequence (see highlighted below). In this example this oligo sequence is 5'-CGGttTCAAAACAATGCGGAAGATGATGAATAACGTGAGCCATATCTGCCCA-3' (Figure 19).



Figure 19. Screenshot of the gene sequence in the downstream homology arm from the targeted gene with part of the target sequence (yellow) and additional 3' coding sequence (purple). The sequence for Oligo B is highlighted in white. The reverse complement should be ordered.

12. An example of correct homology arm design, showing complementary overhangs for cloning into the pGTag and pPRISM BfuAI and BspQ1 sites for a hand2 gRNA site (Figure 20).

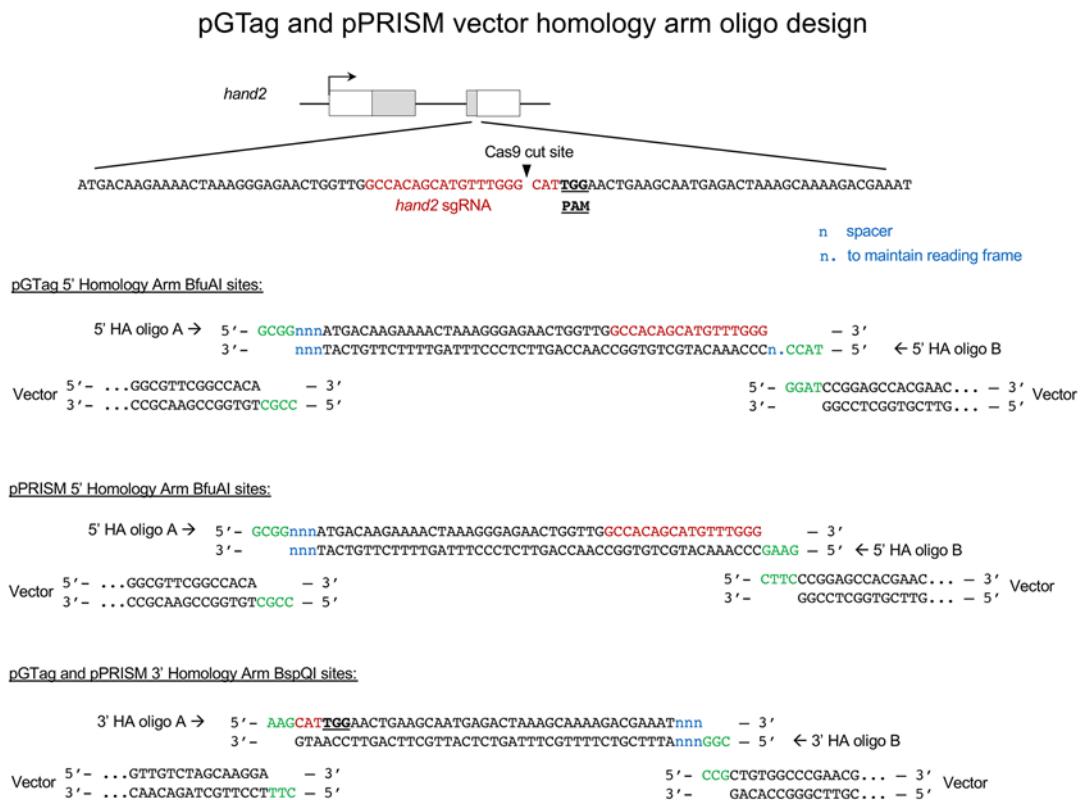


Figure 20. Example of pGTag and pPRISM vector homology arm design showing complementary 5' overhangs for cloning into the BfuAI and BspQ1 type II restriction enzyme sites.

Diagram of CRISPR/Cas9 target site in the *hand2* gene. gRNA sequence in red and PAM sequence underlined and in bold. Annealed homology arm oligos A and B are shown with overhangs (green) complementary to the vector overhangs after enzyme digestion. n, spacer nucleotides; n., nucleotides included to maintain the reading frame of pGTag integration alleles.

F. One Pot Cloning of Homology Arms into pGTag Vectors

Notes:

- a. If the homology arm oligos contain the 5'-ACCTGC-3' or 5'-GAAGAGC-3' sequences (or their complements), the cloning reaction will be less efficient.
 - b. If One Pot cloning is unsuccessful, the 5' and 3' homology arms can be cloned sequentially into the vector using gel-purified linear plasmids digested with the appropriate enzyme.
1. Homology Arm Annealing
Anneal upstream and downstream homology oligo pairs separately:
4.5 μL oligo A at 10 μM
4.5 μL oligo B at 10 μM
4 μL 10× Buffer 3.1 from NEB
27 μL dH₂O
For a 40 μL total
To anneal the oligos, run the following program in a thermal cycler: Step 1, incubate at 98°C for 5 minutes; Step 2, incubate at 97°C for 45 seconds; repeat Step 2 for 90 cycles during which the temperature is decreased by 1°C/cycle; hold at 4°C for the final step. Alternatively, boil water in a glass beaker on a hot plate and incubate the tube in the boiling water for 5 minutes. Remove the beaker from the heat source and allow it to cool to room temperature. Store the annealed homology arms on ice or in a -20°C freezer.
 2. 1-Pot Digest
Mix the following solutions:
4.0 μL dH₂O
2 μL Plasmid at 50 ng/μL
1 μL 10× Buffer 3.1 from NEB
1 μL 5' annealed homology arm
1 μL 3' annealed homology arm
0.5 μL BfuAI enzyme from NEB
0.5 μL BspQI enzyme from NEB
For a 10 μL total
Incubate at 50°C for 1 h, place on ice.
 3. Ligation
Add the following:
3 μL 5× T4 quick ligase buffer
1.5 μL dH₂O
0.5 μL T4 quick ligase
For a 15 μL total
Incubate 8-10 min at room temperature (to overnight). Store at -20°C.
 4. Transformation: To prevent recombination at repetitive elements in the plasmid, grow transformations and overnight cultures at 30°C. Our standard protocol uses NEB Stable Competent *E. coli* (C3040H) cells for cloning and propagation of the GeneWeld pGTag and pPRISM plasmid series to limit recombination.
 - a. On ice, thaw 1 (one) vial competent cells (50 μL) for every 2 ligation reactions (approx. 5 min).
 - b. While cells are thawing, label the microcentrifuge tubes for each ligation and put on ice.
 - c. Once the cells have thawed, use a pipette to transfer 25 μL of the competent cells into each labeled tube.
 - d. Add 1.5 μL of a ligation reaction into competent cells to transform.
The amount of ligation reaction added should be less than 5% of the volume of competent cells.
 - e. Mix by tapping the tube several times or gently mixing with the pipet tip.

Note: Do NOT mix by pipetting; this will lyse the cells.

- f. Incubate on ice for 5 to 20 min.
- g. Heat-shock the cells by submerging the portion of the tube containing the cells in a 42°C water bath for 40-50 s.

- h. Incubate cells on ice for 2 min.
 - i. Add 125 µL of room temperature LB or SOC to each transformation.
 - j. Incubate cells at 30°C for 1-1.5 h in a shaking incubator.
 - k. While the transformed cells are recovering, spread 40 µL of X-Gal solution and 40 µL IPTG 0.8 M on LB Kanamycin selection plates.
 - l. X-Gal is lethal to cells while wet; it is recommended to first label the plates and then place them in a 30°C incubator to dry.
 - m. After recovery and the X-Gal is dry, plate 150 µL of each transformation on the corresponding correctly labeled plate.
 - n. Incubate plates overnight at 30°C.
5. Growing colonies
Pick three white colonies from each plate and grow in separate glass culture tubes with 3 mL LB/Kanamycin, overnight at 30°C, or to pre-screen colonies by colony PCR:
 - a. Pick up to 8 colonies with a pipet tip and resuspend them in separate aliquots of 5 µL dH₂O. Place the tip in 3 mL of LB/Kan, label, and store at 4°C.
 - b. Make a master mix for your PCR reactions containing the following amounts times the number of colonies you picked.

7.5 µL 2× GoTaq mastermix
5.5 µL dH₂O
0.5 µL primer at 10 µM “F3'-check” 5'-GGCGTTGTCTAGCAAGGAAG-3'
0.5 µL primer at 10 µM “3' _pgtag_seq”5'-ATGGCTCATACACCCCTTG-3'
For a 14 µL total
 - c. Aliquot 14 µL of mixed master mix into separate labeled PCR tubes.
 - d. Add 1 µL of colony to each reaction as template, or 20 ng purified plasmid as control.
 - e. Cycle in a thermocycler

95°C 2 min
95°C 30 s }
57°C 30 s } 35 cycles
72°C 30 s
72°C 5 min
4°C hold
 - f. Run 5 µL of PCR product on a 1% agarose gel. Image gel on iBright FL1500 Imaging System or another gel-documentation imaging system. There should be bands that are a different size than the control.
 6. Mini Prep Cultures
Follow Qiagen Protocol
 7. Sequencing of Plasmids
The 5' homology arm can be sequenced by the 5'_pgtag_seq primer:
5'-GCATGGATGTTTCCAGTC-3'.
The 3' homology arm can be sequenced with the “3'_pgtag_seq” primer:
5'-ATGGCTCATACACCCCTTG-3'.

G. Injection of GeneWeld Reagents (spCas9 mRNA, Universal sgRNA (UgRNA), genomic sgRNA, and pGTag homology vector) into 1-cell zebrafish embryos

Prepare and collect the following reagents for injection:

1. Prepare nCas9n mRNA from pT3TS-nCas9n (Addgene #46757) (Jao *et al.*, 2013) (See Section B5. Preparation of SpCas9 mRNA).
2. The UgRNA and genomic sgRNA can be directly ordered from IDT or Synthego and resuspended in Molecular Grade RNase/DNase-free water.
3. The pGTag homology vectors should be purified a second time prior to microinjection under RNase free conditions with the Promega PureYield Plasmid Miniprep System beginning at the endotoxin removal

- wash. Plasmid DNA is eluted in Molecular Grade RNase/DNase-Free water.
4. Embryo Injections for Integration of pGTag vectors.
Injections are performed into single cell embryos at a volume of 2 nl per embryo containing the following concentration of RNAs and vector:
- | | |
|-----------------------------|------------------------|
| In injection solution | In embryo |
| 75 pg/nl of nCas9n mRNA | 150 pg of nCas9n mRNA |
| 12.5 pg/nl of genomic sgRNA | 25 pg of genomic sgRNA |
| 12.5 pg/nl of UgRNA | 25 pg of UgRNA |
| 5 pg/nl of pGTag DNA | 10 pg of pGTag DNA |

H. Test injected embryos for evidence of precision on-target integration

1. Examine injected embryos for fluorescence under a Zeiss Discovery dissecting microscope with a 1× objective at 70-100× magnification. If weak signals are observed, manually dechorionate the embryos, and view on a glass depression well slide on a standard upright compound microscope with epi-illumination. High resolution confocal live imaging can also be carried out, as shown in Wierson *et al.* (2020) Figure 3 (<https://elifesciences.org/articles/53968/figures#fig3>).
The type of light source used for fluorescent protein activation significantly affects the ability to visualize fluorescence signals. The X-Cite 120W metal halide light source with fiber optic cable (Excilitas Technologies) works well to visualize fluorescence after somatic targeting. The TagRFP protein also is shifted in its excitation, and filters optimized for this protein are recommended. Filters optimized for GFP and BFP are also recommended. If no or weak signal is observed, integration of pGTag-Gal4VP16 can be used to amplify reporter expression in the 14XUAS-RFP transgenic line (Balciuniene *et al.*, 2013).
2. Perform junction fragment PCR analysis on positive embryos that display widespread fluorescence in expression domains consistent with the targeted gene. Isolate genomic DNA from individual embryos and a control embryo (See Section D 2. Digestion of embryos for isolation of genomic DNA for mutagenesis analysis). PCR amplifies the genomic DNA-integrated cassette 5' and 3' junctions fragments. An example of F0 embryo junction analysis is shown in Figure 3s1 in Wierson *et al.*, 2020 (<https://elifesciences.org/articles/53968/figures#fig3s1>).

The following primers are used for junction fragment analysis and must be paired with gene-specific primers (5' to 3'):

5' pGTag junctions:

R-Gal4-5'juncM GCCTTGATTCCACTTCTGTCA and a gene-specific forward primer
R-RFP-5'junc CCTTAATCAGTTCCCTGCCCTAGA
R-eGFP-5'-junc GCTGAACTTGTGGCCGTTT

3' pGTag junctions:

F-Gal4-3'juncM GCAAACGGCCTTAACTTCC and a gene specific reverse primer
F-Gal4-3'junc CTACGGCGCTCTGGATATGT
F-RFP-3'junc CGACCTCCCTAGCAAATGGGG
F-eGFP-3'junc ACATGGTCCTGCTGGAGTTC

To control for PCR amplification artifacts as described in (Won and Dawid, 2017), perform PCR junction analysis on embryos injected with all targeting reagents minus the genomic sgRNA.

The alternate primers F-Gal4-3'juncM and F-Gal4-3'juncJ may increase primer specificity, depending on the target gene.

7.5 μL 2× GoTaq mastermix

5.5 μL dH₂O

0.5 μL primer at 10 μM genomic primer

0.5 μL primer at 10 μM pGTag primer

For a 14 μL total

a. Aliquot 14 μL of mixed master mix into separate labeled PCR tubes.

b. Add 1 μL of genomic DNA to each reaction as template.

- c. Cycle in a thermocycler with the following steps:
95°C 2 min
95°C 30 s }
55°C 30 s } 35 cycles
72°C 30 s }
72°C 5 min
4°C hold
- d. Run 5 µL of PCR product on a 1.2% agarose gel in 1× TAE. Image gel on the iBright FL1500 Imaging System or another gel-documentation imaging system. Putative junction fragments should give bands that are of the predicted size.

I. Establish a new transgenic line of a precision targeted integration allele

1. Raise to adulthood fluorescence reporter-expressing F0 siblings of injected embryos that showed positive bands on the 5' and 3' junction analysis, indicating precision targeted integration. Outcross F0 adults to wild type and examine the progeny for reporter gene fluorescence as described above to identify F1 embryos that have inherited a stable germline integration allele. For Gal4Vp16 integration alleles, cross the F0 adults to the 14XUAS:RFP reporter line. Silencing of the 14XUAS:RFP reporter may result in mosaic expression patterns in Gal4Vp16 targeted F1 embryos.
2. Test F1 fluorescence positive embryos for precise transgene integration by junction fragment PCR analysis as described above. Raise F1 siblings to adulthood and fin-clip to identify individuals with precise targeted transgene integration as shown in Figure 4s 2-4 of Wierson *et al.* (2020) (<https://elifesciences.org/articles/53968/figures#fig4s2>).
3. Outcross a single positive F1 adult to establish F2 families. F1s can also be sacrificed 3 weeks post-fertilization to the confirm location and precision of targeted integrations by genomic Southern Blot RFLP analysis as in Figure 4s1 of Wierson *et al.* (2020) (<https://elifesciences.org/articles/53968/figures#fig4s1>). Continue to maintain lines by outcrossing to wild type in subsequent generations. Tables 1 and 2 of Wierson *et al.* (2020) (<https://elifesciences.org/articles/53968/figures>) show the range of germline transmission frequencies of precision targeted integration alleles at eight zebrafish loci.
4. To perform an initial assessment of whether the targeted integration allele causes a loss of function phenotype, F0 and F1 identified fish can be incrossed or crossed to a known indel allele of the targeted gene.

Data analysis

Links to numerical data in the original article (Wierson *et al.*, 2020) are included in the protocol.

Recipes

Zebrafish embryo E2 Medium (Westerfield, 1995)
(<https://wahoo.cns.umass.edu/book/export/html/867>)

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Competing interests

JJE, MM, and KJC have a financial conflict of interest with Recombinetics, Inc.; JJE and SCE with Immusoft, Inc.; JJE, MM, WAW, KJC, and SCE with LifEngine and LifEngine Animal Technologies.

Ethics

All zebrafish experiments described in this protocol were carried out under approved protocols from Iowa State University Animal Care and Use Committee Log#11-06-6252, in compliance with American Veterinary Medical Association and NIH guidelines for the humane use of animals in research.

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Direct-TRI: High-throughput RNA-extracting Method for All Stages of Zebrafish Development

Kota Ujibe, Kanako Nishimura, Makoto Kashima and Hiromi Hirata*

Department of Chemistry and Biological Science, College of Science and Engineering, Aoyama Gakuin University, Sagamihara, Japan

*For correspondence: hhirata@chem.aoyama.ac.jp

Abstract

Recent popularization of next-generation sequencing enables conducting easy transcriptome analysis. Nevertheless, substantial RNA isolation work prior to RNA sequencing, as well as the high cost involved, still makes the routine use of large-scale transcriptome analysis difficult. For example, conventional phenol-chloroform RNA extraction cannot be easily applied to hundreds of samples. Therefore, we developed Direct-TRI, a new cost-effective and high throughput RNA-extraction method that uses a commercial guanidine-phenol-based RNA extraction reagent and a 96-well silica column plate. We applied Direct-TRI to zebrafish whole larvae and juvenile samples and obtained comparable RNA qualities by several different homogenization methods such as vortexing, manual homogenizing, and freezing/crushing. Direct-TRI enabled the extraction of 192 RNA samples in an hour with a cost of less than a dollar per sample. Direct-TRI is useful for large-scale transcriptome studies, manipulating hundreds of zebrafish individuals, and may be used with other animal samples.

Keywords: Zebrafish, RNA isolation, RNA-Seq, Gene expression, High throughput

This protocol was validated in: Sci Rep (2020), DOI: 10.1038/s41598-020-70110-1.

Background

RNA-sequencing (RNA-Seq) is one of the standard methods in various fields of biology (Cheng *et al.*, 2021; Sun *et al.*, 2021) because it provides comprehensive information about gene expression. Recently, several researchers have developed cost-effective and high-throughput RNA-Seq library preparation protocols such as Lasy-Seq (Kamitani *et al.*, 2019), BRB-Seq (Alpern *et al.*, 2019), and Decode-Seq (Li *et al.*, 2020). Although these techniques and the lower cost of massive parallel sequencing have enabled large-scale RNA-Seq analyses (Hoang *et al.*, 2020; Miller *et al.*, 2013), the RNA extraction process prior to RNA-Seq remains laborious and time-consuming. For example, a conventional RNA extraction method using phenol-chloroform (Peterson *et al.*, 2009) requires careful liquid handling and a number of steps. Another method using silica columns, the RNeasy of Qiagen, provides easy and time-saving operation but is costly. In addition, the lysis solutions included in the kit do not contain phenol and, thus, are less effective in disrupting cells and tissues than phenol-containing lysis solutions. Recently, a new easy and time-saving method, Direct-zol (Sosanya *et al.*, 2013), has been developed by combining these two methods. In the Direct-zol protocol, samples are lysed in phenol-containing solution and subsequently subjected to silica column-based RNA purification. However, the kit is costly (approximately 7 dollars per sample) for a large number of samples. From the viewpoint of high throughput, low cost, and robustness, the aforementioned methods are not feasible for large-scale sampling. Here, we introduce Direct-TRI, a high-throughput, cost-effective, and reliable RNA-extraction method using TRI Reagent-LS and 96-well silica column plate. This protocol consists of two steps: homogenization of samples in phenol-containing TRI Reagent-LS followed by the direct isolation of RNA from the phenol lysate with handmade washing solutions. Direct-TRI makes it possible to simultaneously process hundreds of RNA samples individually with a simple procedure and at an affordable cost. We show an example of a Direct-TRI application using whole zebrafish samples. Zebrafish is a model organism to study development, behavior, and disease in vertebrates (Goldsmith *et al.*, 2012; Gore *et al.*, 2018; Maeta *et al.*, 2020). The large-scale gene expression analysis of zebrafish is a powerful tool for investigating the molecular basis underlying developmental events and diseases (Lee *et al.*, 2020; Scholz, 2013). The combination of Direct-TRI and the cost-effective and high throughput RNA-Seq library preparation methods will make large-scale transcriptome analysis more useful not only for zebrafish samples but also for other organisms.

Materials and Reagents

1. 1.5 mL microcentrifuge tube (Rikaken, catalog number: RSV-MTT1.5)
2. Transfer pipet, 3 mL (Falcon, catalog number: 357575)
3. 8-strip tube, dome type (Rikaken, catalog number: RS-PCR-8D)
4. Cell culture dish, 100 mm (Nippon Genetics, catalog number: FG-2090)
5. Cell culture dish, 35 mm (Nippon Genetics, catalog number: TR4000)
6. Gemma Micro ZF 75 (Skretting)
7. Otohime B2 (Marubeni Nisshin Feed)
8. Kimwipe (Kimberly Clark)
9. AcroPrep Advance 96-well Long Tip Filter Plate for Nucleic Acid Binding (Pall, catalog number: 8133)
10. A reservoir for AcroPrep Advance (Merck, catalog number: BR701340-50EA)
11. Eppendorf twin.tec 96-well LoBind PCR plates (Eppendorf, catalog number: 0030129512)
12. DuraCross zebrafish breeding tank with divider (Laboratory Product Sales, catalog number: T233795)
13. Zebrafish (*Danio rerio*)
14. TRI Reagent-LS (Molecular Research Center, catalog number: TS120)

Notes:

- a. *TRIzol-LS Reagent* (Thermo Fisher Scientific, catalog number: 10296028) or *ISOGEN-LS* (Nippon Gene, catalog number: 311-02621) can also be used.

- b. *Sepasol-RNA II Super* (Nacalai Tesque, catalog number: 30487-46), an equivalent product, could not be used for RNA extraction from adult zebrafish because a precipitate was produced during the freezing and crushing processes.
15. Ethanol, 99.5% (Nacalai Tesque, catalog number: 08948-25)
 16. Nuclease-free water (Merck, catalog number: H20MB1006)
 17. BioMasher II (Nippi, catalog number: 320102; Figure 1)



Figure 1. Image of BioMasher II

18. Liquid nitrogen
19. Pronase (Merck, catalog number: 53702)
20. QuantiFluor RNA System (Promega, catalog number: E3310)
21. Agilent RNA 6000 Pico kit (Agilent Technologies, catalog number: 5067-1513)
22. 10× Loading Buffer (Takara Bio, catalog number: 9157)
23. ExcelBand 1 KB (0.25-10 kb) DNA Ladder (Smobio, catalog number: DM3100)
24. Tricaine (Wako, catalog number: 051-06571)
25. Tris(hydroxymethyl)aminomethane (Rikaken, catalog number: RSP-THA500G)
26. Boric acid (Wako, catalog number: 021-02195)
27. EDTA-2Na (Wako, catalog number: 343-01861)
28. Agarose Powder (Rikaken, catalog number: RSV-AGRP-500G)
29. 10 mg/mL Ethidium bromide (Nippon Gene, catalog number: 315-90051)
30. Ethanol, 80% (see Recipes)
31. 1% agarose gel (see Recipes)
32. Tricaine (see Recipes)
33. E3 medium (see Recipes)
34. 20 mg/mL Pronase (see Recipes)
35. 5× TBE (see Recipes)

Equipment

1. Mortar and pestle, 120 mm (AsOne, catalog number: 6-549-03)
2. Vortex-Genie 2 (Scientific Industries)
3. PlateSpin II (Kubota)
4. Quantus Fluorometer (Promega, catalog number: E6150)
5. Agilent 2100 Bioanalyzer System (Agilent, catalog number: G2939B)

Procedure

A. Zebrafish maintenance and larvae collection

1. Maintain adult zebrafish in a recirculation system at 28.5°C under 14:10 h light:dark cycle according to the standard protocol (Westerfield, 2000).
2. Place female and male adult zebrafish in a breeding tank with a divider that separates fish to avoid immediate mating.
3. Obtain fertilized eggs [embryos, 0 days post fertilization (dpf)] by removing the divider the next morning.
4. Raise zebrafish embryos in E3 medium in a 100 mm cell culture dish at 28.5°C.
5. At 1 dpf, transfer embryos with 2.5 mL of E3 medium into a 35 mm cell culture dish and add 150 µL of 20 mg/ml pronase.
6. After 15 min incubation at room temperature (RT), dechorionate embryos by stirring the dish.
7. Transfer embryos to a 100 mm cell culture dish with 20 mL of E3 medium and raise them up to 5 dpf at 28.5°C.
8. Place zebrafish larvae (5 dpf larvae) in the recirculation system (30 larvae/tank).
9. Feed zebrafish larvae in the recirculation system both paramecia and Gemma Micro ZF 75 twice a day from 5 to 30 dpf.
10. Feed juveniles (30-90 dpf) and adults (~90 dpf) both brine shrimp and Otohime B2 twice a day after 30 dpf (Wakamatsu *et al.*, 2019).

B. RNA extraction from zebrafish larvae and juveniles

Note: Use nuclease-free tips in all RNA extraction steps.

1. Larvae (approximately 4-12 dpf, size smaller than 5 mm)
 - a. Anesthetize a zebrafish larva in 20 mL of 0.2 mg/ml Tricaine at RT for 3 min.
 - b. Prepare 100 µL of TRI Reagent-LS in 8-strip tube.
 - c. Place a single zebrafish larva inside of a cap of the 8-strip tube and remove water using a micropipette (Figure 2).

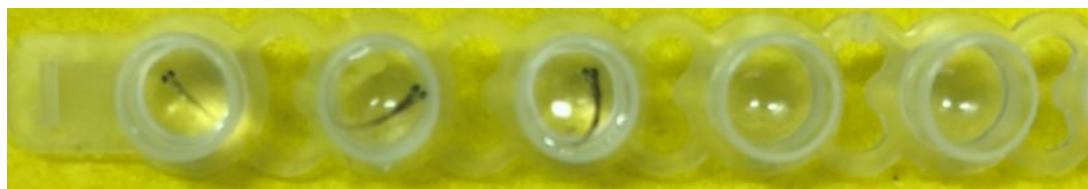


Figure 2. Zebrafish larvae placed in the tip of the 8-strip tube dome cap

- d. Assemble the cap to the 8-strip tube with each well containing the TRI Reagent, and lyse larvae completely by vortexing for 1 min at RT.
- e. Move to the RNA purification steps immediately or store the 100 µL lysates at -80°C for 1 month.
2. Larva (approximately 14-38 dpf, size smaller than 10 mm)
 - a. Anesthetize a zebrafish larva in 20 mL of 0.2 mg/mL Tricaine at RT for 3 min.
 - b. Transfer a zebrafish larva in a 1.5 mL microcentrifuge tube with a Biomasher II and remove excess water using a micropipette.
 - c. Add 50 µL of TRI Reagent-LS to the larva and immediately mash it at RT using a pestle until it is completely crushed.
 - d. Add 50 µL of TRI Reagent-LS and vortex the sample for 1 min at RT.
 - e. Move to the RNA purification steps immediately or store the 100 µL lysates at -80°C for 1 month.
3. Juvenile (approximately 38 dpf, size larger than 10 mm)

- a. Anesthetize a zebrafish juvenile in 20 mL of 0.2 mg/mL Tricaine at RT for 3 min.
- b. Place a juvenile zebrafish in a 120 mm mortar and remove water by wiping the juvenile using Kimwipes.
- c. Pour liquid nitrogen to quickly freeze the juvenile.
- d. Grind the frozen juvenile into powder using a pestle (Figure 3).



Figure 3. Image of a juvenile zebrafish on a mortar for grinding

- e. Add 1 mL of TRI Reagent-LS to the frozen powder and mix using a pestle until the mixture becomes liquid.

Note: TRI Reagent-LS is frozen before the completion of tissue lysis. To lyse tissues completely, defrosting the regents is essential. Placing the mortar on a water bath (40-50°C) is sufficient to defrost the regents in a short time. The integrity of the RNA is not affected by this warming.

- f. Transfer 100 µL of the lysate to a new 1.5 mL microcentrifuge tube.
- g. Move to the RNA purification steps immediately or store the 100 µL lysates at -80°C for 1 month.

Note: For reuse, the mortar should be washed with distilled water until the lysate is completely removed.

4. RNA Purification

- a. Add 100 µL of 99.5% ethanol to the 100 µL lysate and mix by vortexing at RT.

Note: For reuse, the mortar should be washed with distilled water until the lysate is completely removed.

- b. Prepare the AcroPrep Advance 96-Well Filter Plate on a reservoir (Figure 4).

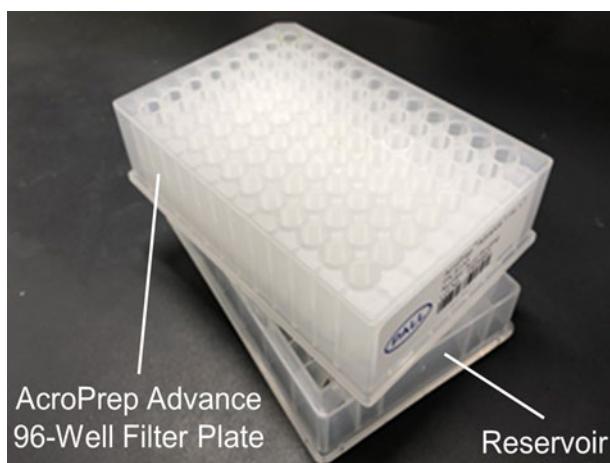


Figure 4. AcroPrep Advance 96-Well Filter Plate on a reservoir

- c. Transfer the 200 μL mixture into each well of the AcroPrep Advance plate.
- d. Centrifuge at 1,300 $\times g$ for 4 min at RT. Discard the flowthrough.
- e. Add 400 μL of 99.5% ethanol and centrifuge the plate at 1,300 $\times g$ for 4 min at RT. Discard the flowthrough.
- f. Repeat adding 400 μL of 99.5% ethanol and centrifuge the plate at 1,300 $\times g$ for 4 min at RT. Discard the flowthrough.
- g. Add 700 μL of 80% ethanol to the plate and centrifuge at 1,300 $\times g$ for 10 min at RT. Discard the flowthrough.
- h. Elute the RNA by adding 30 μL of nuclease-free water to the center of each well followed by incubation for 1 min at RT. Then, centrifuge at 1,300 $\times g$ for 4 min at RT. RNA solution can be stored at -80°C.

C. Determination of the RNA concentration

Determine RNA concentration with the QuantiFluor RNA System kit and Quantus Fluorometer according to the manufacturer's manual. Typical results are shown in Note 1.

Note: The expected RNA concentration from an individual is too low to be measured with a spectrophotometer (e.g., NanoDrop).

D. RNA quality check

We recommend checking the quality of RNA through agarose gel electrophoresis or with the Bioanalyzer for some but not all samples because the amounts of RNA are low in some cases.

1. Agarose gel electrophoresis
 - a. Add 15 μL (300 ng/lane) of the RNA sample into a well of 1% agarose gel.
 - b. Run electrophoresis at 135 V for 25 min in 0.5 \times TBE. Typical results are shown in Note 2.
2. Bioanalyzer

This procedure requires the use of the Bioanalyzer.

Check the RNA quality with the Agilent 2100 Bioanalyzer and the Agilent RNA 6000 Pico kit according to the manufacturer's manual. Typical results are shown in Note 3.

Notes

1. Typical yields for RNA isolation with the Direct-TRI and phenol-chloroform are shown in Table 1. Owing to the limitation in the capacity of RNA to bind to the AcroPrep Advance 96 Filter Plate (approximately 3 µg), the yield of Direct-TRI tends to be smaller than that of the phenol-chloroform method (Peterson *et al.*, 2009) when adult zebrafish are used.

Table 1. Total RNA amounts obtained with the Direct-TRI and phenol-chloroform methods

	6 dpf (Vortex)	17 dpf (Biomasher)	84 dpf (Freezing homogenize)
Direct-TRI	330 ng	3480 ng	2.8 µg
Phenol-chloroform	170 ng	1500 ng	132 µg

Note: RNA concentration was determined with the QuantusTM Fluorometer.

2. A typical result of the RNA quality check through electrophoresis is shown in Figure 2. Zebrafish (6 dpf) were used to extract RNA using the TRI Reagent-LS to compare two different RNA-extracting methods, and then the RNA quality was checked. Two major bands (28S and 18S rRNA) indicate that RNA was not degraded (Figure 5). The small-size RNA was successfully obtained with the Direct-TRI method but not with the conventional phenol-chloroform method.

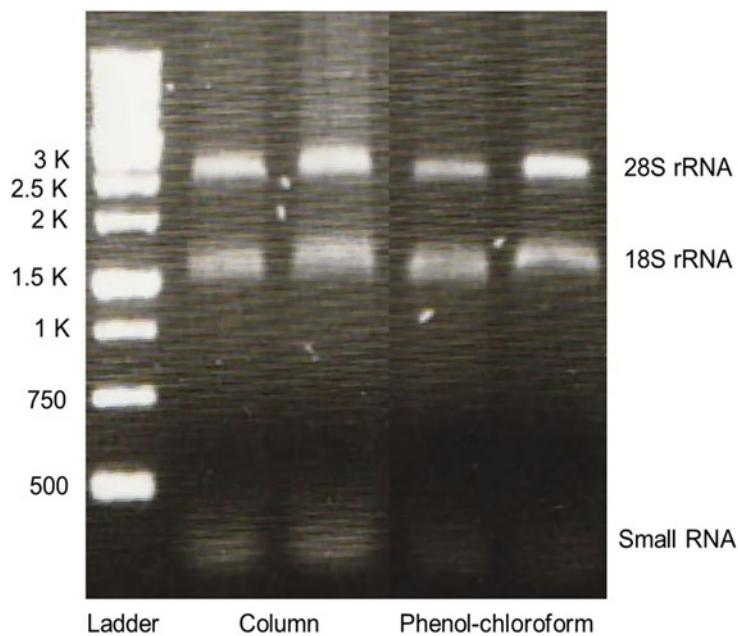


Figure 5. Typical results of the RNA quality check through agarose electrophoresis

3. A typical result of the RNA quality check using the Bioanalyzer is shown in Figure 6. RNA was isolated from a 15 dpf larva with Direct-TRI, and the RNA quality was checked with the Bioanalyzer. Two RNA peaks (18S and 28S rRNA) were clearly observed, indicating that the RNA was not degraded (RIN 9.10). In the Direct-TRI method, the RIN value was approximately 7-9.5.

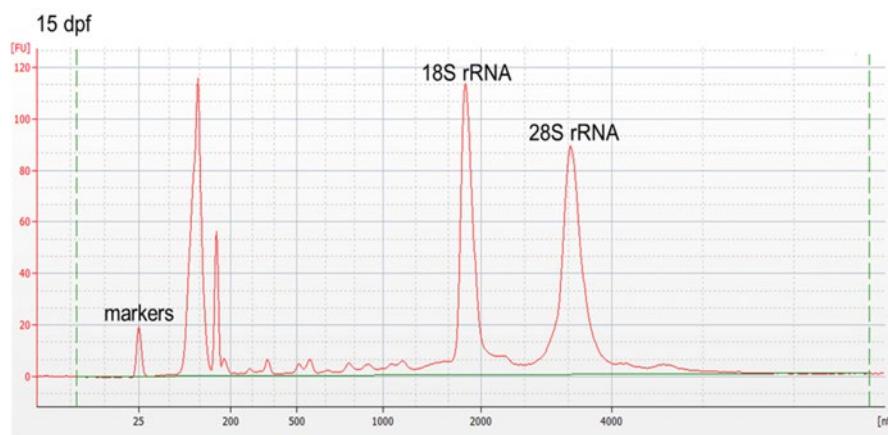


Figure 6. Typical results of the RNA quality check with the Bioanalyzer

Recipes

1. E3 medium

50× E3 stock solution

NaCl 14.65 g (500 mM)

KCl 0.63 g (17 mM)

CaCl₂·2H₂O 2.43 g (33 mM)

MgSO₄·7H₂O 4.07 g (33 mM)

Distilled water mess up to 1 L

Adjust pH to 7.2 with ~100 µL of 0.1 M NaOH

Total 1 L

Autoclave at 121°C for 20 min

Store at RT

E3 medium (1× E3 working solution)

50× E3 stock solution 20 ml

Distilled water mess up to 1 L

Total 1 L

Store at RT

2. 20 mg/mL Pronase

Pronase 1 g

Distilled water mess up to 50 mL

Total 50 mL

Divide into 1 mL aliquots

Store at -20°C

3. Tricaine (Ethyl 3-aminobenzoate methanesulfonate)

10 mg/mL Tricaine (Ethyl 3-aminobenzoate methanesulfonate)

Tricaine 1 g

Distilled water mess up to 100 mL

Total 100 mL

Divide into 1 mL aliquots

Store at -20°C

0.2 mg/mL Tricaine (Ethyl 3-aminobenzoate methanesulfonate)

10 mg/ml Tricaine 1 mL
Distilled water mess up to 50 mL
Total 50 Ml

4. 80% Ethanol

Ethanol, 99.5% 40 mL
Nuclease-free water 10 mL
Total 50 mL
Store at RT

5. 5× TBE

Tris(hydroxymethyl)aminomethane 54.0 g
Boric acid 27.5 g
EDTA-2Na 1.85 g
Distilled water mess up to 1 L
Total 1 L
Store at RT

6. 1% agarose gel

5× TBE 35 mL
Agarose Powder 3.5 g
Distilled water 315 mL
Total 350 mL
Autoclave at 121°C for 1 min and cool to 50°C
Add 17.5 µL of 10 mg/mL Ethidium bromide and pour into a gel plate

Acknowledgments

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Competing interests

No competing interests declared.

Ethics

This study was approved by the Animal Care and Use Committee of the Aoyama Gakuin University (A9/2020) and conducted according to the Aoyama Gakuin University Animal Care and Use Guidelines and the Animal Research of *In VIVO* Experiments (ARRIVE) guidelines.

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Isolation of Myofibres and Culture of Muscle Stem Cells from Adult Zebrafish

Massimo Ganassi*, Peter S. Zammit and Simon M. Hughes*

Randall Centre for Cell and Molecular Biophysics, King's College London, SE1 1UL, UK

*For correspondence: massimo.ganassi@kcl.ac.uk; simon.hughes@kcl.ac.uk

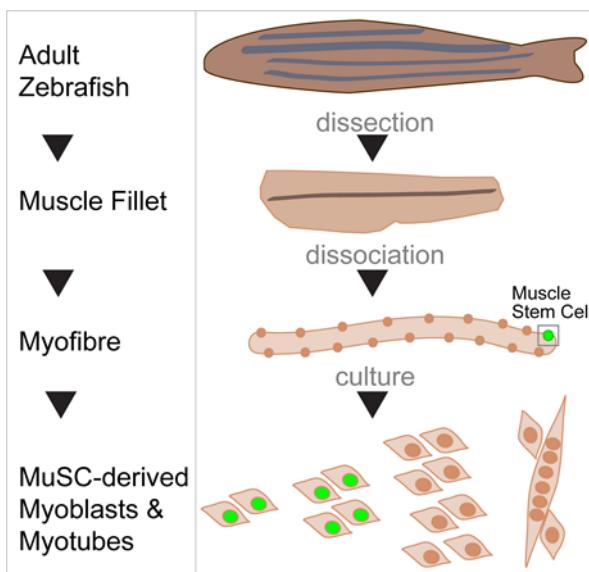
Abstract

Skeletal muscles generate force throughout life and require maintenance and repair to ensure efficiency. The population of resident muscle stem cells (MuSCs), termed satellite cells, dwells beneath the basal lamina of adult myofibres and contributes to both muscle growth and regeneration. Upon exposure to activating signals, MuSCs proliferate to generate myoblasts that differentiate and fuse to grow or regenerate myofibres. This myogenic progression resembles aspects of muscle formation and development during embryogenesis. Therefore, the study of MuSCs and their associated myofibres permits the exploration of muscle stem cell biology, including the cellular and molecular mechanisms underlying muscle formation, maintenance and repair. As most aspects of MuSC biology have been described in rodents, their relevance to other species, including humans, is unclear and would benefit from comparison to an alternative vertebrate system. Here, we describe a procedure for the isolation and immunolabelling or culture of adult zebrafish myofibres that allows examination of both myofibre characteristics and MuSC biology *ex vivo*. Isolated myofibres can be analysed for morphometric characteristics such as the myofibre volume and myonuclear domain to assess the dynamics of muscle growth. Immunolabelling for canonical stemness markers or reporter transgenes identifies MuSCs on isolated myofibres for cellular/molecular studies. Furthermore, viable myofibres can be plated, allowing MuSC myogenesis and analysis of proliferative and differentiative dynamics in primary progenitor cells. In conclusion, we provide a comparative system to amniote models for the study of vertebrate myogenesis, which will reveal fundamental genetic and cellular mechanisms of MuSC biology and inform aquaculture.

Keywords: Zebrafish, Muscle fibre, Myofibre, Stem cell, Skeletal muscle, Myonucleus, Pax7, MuSC, Adult, Satellite cell

This protocol was validated in: eLife (2020). DOI: 10.7554/eLife.60445

Graphic Abstract:



Schematic of Myofibre Isolation and Culture of Muscle Stem Cells from Adult Zebrafish.

Background

Skeletal musculature provides lifelong body support and movement through coordinated contraction of myofibres, highly specialised syncytial cells containing hundreds of post-mitotic myonuclei. Myofibres constantly adapt to both exogenous and endogenous stimuli in part thanks to resident muscle stem cells (MuSCs), also known as satellite cells, located underneath the basal lamina of most myofibres (Katz, 1961; Mauro, 1961; Relaix and Zammit, 2012; Purohit and Dhawan, 2019). In response to exercise or damage, quiescent MuSCs quickly activate and become muscle progenitor cells (MPCs) called myoblasts, which proliferate, differentiate and fuse either to pre-existing multinucleated myofibres or to one another to form new myofibres (Fukada *et al.*, 2020). Isolation of rodent myofibres and culture of associated MuSCs is a well-established tool to explore muscle stem cell biology, providing not only an understanding of MuSC behaviour and regulation of quiescence, activation, proliferation, self-renewal and differentiation (Zammit *et al.*, 2004), but also yielding insights into both embryonic muscle development and adult myofibre growth and maintenance (Buckingham and Relaix, 2015). However, mechanisms underlying murine MuSC biology may not fully resemble those found in human myogenesis. Therefore, alternative vertebrate models to study adult/MuSC myogenesis are desirable to consolidate findings of ancestral mechanisms in vertebrate muscle. Notably, despite substantial overlap of molecular pathways shared in myogenesis between zebrafish and amniotes (Hammond *et al.*, 2007; Hinitz *et al.*, 2009 and 2011; Ganassi *et al.*, 2018; Osborn *et al.*, 2020), the complementary study of adult muscle homeostasis in zebrafish is limited to mechanical trituration of bulk muscle, hindering the purity of myogenic yield (Alexander *et al.*, 2011; Froehlich *et al.*, 2014). Adult myofibre isolation in fish has permitted the study of their physical or contractile properties (Johnston and Altringham 1988; Davies 1995; Johnston *et al.*, 2004), and some studies have more recently exploited it to investigate zebrafish MuSC biology (Anderson *et al.*, 2012; Zhang and Anderson, 2014; Ganassi *et al.*, 2018 and 2020).

Here, we describe how to isolate single viable myofibres and associated MuSCs through enzymatic digestion and fine trituration of the trunk musculature of adult zebrafish. This method is adapted from the standard mouse protocol (Bischoff, 1975; Cardasis and Cooper, 1975a and 1975b; Bekoff and Betz, 1977a and 1977b; Rosenblatt *et al.*, 1995; Moyle and Zammit, 2014). We explain how to plate myofibres to study MuSC activation and progression through myogenesis *in vitro*, ensuring a virtually pure myogenic population (Ganassi *et al.*, 2018 and 2020). Our protocol provides an appropriate toolbox for comparative analysis of adult myogenesis across vertebrates and has been

recently developed and used to explore the function of the transcription factor Myogenin in adult MuSC activation, proliferation and differentiation (Ganassi *et al.*, 2018 and 2020). Myofibre isolation, culture, and analysis from adult fish exploits the advantages of the zebrafish model, such as the spatial segregation of slow and fast myofibres that facilitates fibre-type specific studies (Blagden *et al.*, 1997; Pipalia *et al.*, 2016; Hromowyk *et al.*, 2020), and provides insight useful to aquaculture. As an alternative to the classical rodent procedure, the analysis of fish MuSC also offers an independent benchmark to verify genetic and cellular mechanisms identified using rodent models. Therefore, application of our techniques to adult zebrafish muscle has the potential to contribute to understanding genetic, molecular, and cellular mechanisms maintaining and adapting human musculature.

The method is simple, efficient, and cost-effective and permits the study of 1) myofibre characteristics *ex vivo*, 2) MuSC-derived myoblasts/myotubes *ex vivo* and 3) mechanisms of adult muscle formation, development, and maintenance.

Materials and Reagents

Materials required for dissection and dissociation of adult muscle

1. Deep Petri dishes (150 mm and 100 mm) sterile, cell culture grade (Corning, catalog numbers: 430599 and 430167)
2. Glass Pasteur pipettes (22 cm), sterile (Volac, catalog number: D812)
3. 0.45 µm and 0.2 µm sterile syringe filters (ThermoFisher, catalog numbers: 15216869 and 15206869)
4. Sterile syringe, 50 mL (Terumo, catalog number: SS+50ES1)
5. Aluminium foil
6. Bijou tubes, 7 mL
7. Tricaine methanesulfonate (MS-222) solution (Sigma-Aldrich, catalog number: E10521)
8. 70% Ethanol solution (in deionised water) (70% EtOH) (Ethanol absolute; Sigma-Aldrich, catalog number: 1024282500)
9. 5% Bovine serum albumin (BSA) (powder, Sigma-Aldrich, catalog number: A7906)
10. Collagenase from Clostridium histolyticum (Sigma-Aldrich, catalog number: C0130)
11. Dulbecco's modified Eagle's medium (DMEM), high glucose, GlutaMAX, Pyruvate (ThermoFisher, catalog number: 31966)
12. Phosphate-buffered saline Ca²⁺ and Mg²⁺ free (PBS), sterile (Oxoid, catalog number: BR0014G)
13. Penicillin and Streptomycin solution (Sigma-Aldrich, catalog number: P0781)
14. 1% Virkon solution (in deionised water) (powder, 3S Healthcare) (see Recipes)
15. P/S-PBS (see Recipes)
16. BSA-PBS (see Recipes)
17. cDMEM (complete DMEM) (see Recipes)
18. Collagenase-cDMEM (see Recipes)

Materials required for myofibre and MuSC-derived cell immunolabelling

1. Cover glasses 50 mm × 22 mm (Academy, catalog number: 400-04-17)
2. Glass Slides (Fisher, catalog number: 1157-2203)
3. Crystal-clear plastic microcentrifuge tubes, 2 mL (Starlab, catalog number: S-1620-2700)
4. Transparent nail varnish
5. Paraformaldehyde (PFA) solution, 4% in PBS (PFA-PBS) (Alfa Aesar, catalog number: J61899)
6. Liquid blocker super pap pen (Pyramid Innovation, catalog number: R62002-E)
7. Triton X-100 detergent solution (Sigma-Aldrich, catalog number: X100)
8. Chicken anti-GFP (RRID:AB_300798; Abcam, catalog number: 13970; use 1:400)
9. Goat anti-chicken IgY (H+L), Alexa Fluor® 488 (RRID:AB_2534091; Thermo Fisher Scientific, catalog number: A11032, use 1:1,000)

10. Hoechst 33342 solution (ThermoFisher, catalog number: H3570, use 1:1000)
11. Normal goat serum (NGS) (Agilent, catalog number: x0907)
12. Glycerol-based mounting medium (Agilent, catalog number: 50001)
13. PBSTx (see Recipes)

Materials required for myofibre and MuSC-derived cell culture

1. 24-well plates cell culture grade (ThermoFisher, catalog number: 142475)
2. Fetal bovine serum (FBS), heat inactivated (ThermoFisher, catalog number: 10500-064)
3. Horse serum (HS) (ThermoFisher, catalog number: 26050088)
4. Matrigel (Corning, catalog number: 354263)
5. 5-ethynyl-2'-deoxyuridine (EdU) solution (From Click-iT EdU kit; ThermoFisher, catalog number: C10646)
6. Gentamicin (Gibco, catalog number: 15750-060)
7. Matrigel solution (see Recipes)
8. Proliferation Medium (PM) (see Recipes)
9. Differentiation Medium (DM) (see Recipes)

Equipment

Dissection and dissociation of adult muscle

1. Tissue culture hood or lamina flow cabinet
2. Tissue culture incubator (humidified, 28.5°C, 5% CO₂)
3. Cork dissection board (IKEA, catalog number: 870.777.00)
4. Dissection metal pins
5. Fine forceps, one pair (Idealtek, No. 5A.s)
6. Sterile disposable scalpels No. 10 (Swann-Morton, catalog number: 0501)
7. Bunsen burner
8. Diamond-tipped pen (VWR, catalog number: 201-0392)
9. Dissection microscope with transmission illumination (Zeiss Stemi SV6 and Leica M50)

Software

1. Image Analysis: Fiji; NIH (www.Fiji.sc)
2. Data presentation: GraphPad Prism 8 (<https://www.graphpad.com/scientific-Software/prism/>)

Procedure

A. Muscle Dissection

Where possible, perform steps under sterile conditions in a tissue culture hood or laminar flow cabinet.

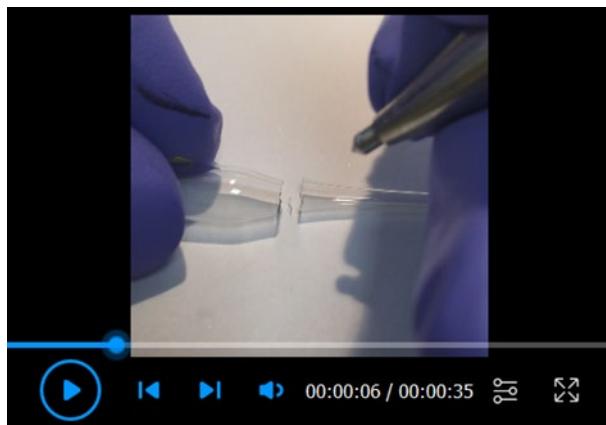
1. Euthanise the fish by immersion in ice-cold 0.3 mg/mL tricaine solution. Immerse fish in chilled tricaine solution aliquoted into a 50 mL tube for the required amount of time. To minimise animal distress, keep the tube on ice during incubation. Please note that the duration of tricaine incubation must be determined empirically, depending on fish size and age as described before (Westerfield, 2000; Harper and Lawrence, 2011) (see Note 1).
2. Remove fish carcass from tricaine solution and immerse it in 25 mL of 1% Virkon (see Recipe 1) solution

- in a 100 mm dish. Incubate for 5 min to kill bacteria and fungi.
3. Use clean forceps to transfer the fish carcass to a new 100 mm dish containing 25 mL P/S-PBS (see Recipe 2) and incubate for 5 min.
 4. Transfer fish carcass into a new empty 100 mm Petri dish. Use a disposable scalpel to remove scales. To increase scaling efficiency, position the blade perpendicular to the antero-posterior axis of the fish body and gently scrub the skin surface from tail to head (see Note 2).
 5. Wash the descaled carcass into a new 100 mm dish containing 25 mL of fresh P/S-PBS for 5 min. Meanwhile, carefully wipe dissection metal pins, corkboard and fine forceps with 70% EtOH to reduce chances of contamination.
 6. Move fish carcass to a new 100 mm dish, gently dry residual P/S-PBS with cloth and spray with 70% EtOH on both sides.
 7. Move fish to the dissecting corkboard and place one pin passing through tissue just behind the gill operculum and a second pin penetrating the tissue just anterior to the base of the caudal fin (Figure 1A).
 8. Use the scalpel to cut fins as close as possible to the fish body. Removed fins can be processed to extract genomic DNA for fish genotyping (Figures 1A and 1B) (see Note 3).
 9. Make a curved incision along the ventral side of the carcass to facilitate evisceration using blade and fine forceps (Figure 1A).
 10. At this point, different portions of the carcass can be collected for required analyses. As indicated in Figure 1B: i) fins are useful for retrospective genotyping, ii) the muscle region near the tail tip is usually damaged by the dissecting pin but can be used for whole muscle RNA/Protein analysis, iii) the adjacent 5 mm section of muscle can be cryopreserved for histological analysis and iv) most of the trunk musculature is processed for myofibre isolation.
 11. Use the scalpel to make a light incision on the skin just behind the gill operculum and perpendicular to the antero-posterior axis, carefully avoiding incision of the muscle beneath. Use the fine forceps to gently pinch and lift the skin along the incision edge. Carefully grab and pull the skin toward the fish tail to expose the underlying muscle (Figure 1C).
 12. Continue to pull gently until reaching the pin positioned close to the tail (Figure 1C). Slow muscle is strongly attached to the overlying skin, so pull very gently to avoid damaging the slow myofibres. Most of the trunk musculature should now be exposed.
 13. Use the same procedure to remove skin from the contralateral side.
 14. When skinning is completed, unpin the fish and rotate it 90° onto its back so that the ventral side (belly) points upward towards the operator (Figures 1D-1F).
 15. Re-pin the fish to the corkboard in the new position, using one pin passing through the lower jaw and head and the second at the base of the tail. The vertebral column should be visible and accessible through the opening in the belly (Figures 1E and 1F).
 16. Use the blade to cut on the right of the vertebral column along the entire antero-posterior axis to create two muscle fillets, one bearing the associated vertebral column and spinal cord and the other without. It is important to angle the scalpel so that its tip points toward the dorsal midline, penetrating the anterior-most part of the muscle tissue close to the vertebral column (Figure 1E). Draw the blade posteriorly until the tail pin is reached, leaving the ribs in the fillet (Figure 1E'). Stopping or hesitating whilst cutting along the column can lead to varying fillet thickness and damage the medial-most muscle.
 17. Use scalpel to remove the fish head and fully release the two muscle fillets. The fish fillets display slow and fast muscle compartments (Figure 1G). The spinal cord should be visible in the right fillet and can be removed with the scalpel, but this is not essential. We usually do not remove it to reduce possible damage to the surrounding muscle tissue.
 18. Muscle dissection should require 30 min and can be performed on multiple fish in parallel.

B. Myofibre Dissociation and Isolation

1. Rinse one 150 mm and two 100 mm new sterile Petri dishes per fish with BSA-PBS solution (see Recipe 3) to prevent myofibre adhesion to the dish. Remove excess BSA-PBS solution and add 25 mL and 10 mL of complete DMEM (cDMEM, see Recipe 4) to the 150 mm and 100 mm dishes, respectively. Place

- dishes in a 28.5°C 5% CO₂ incubator for at least 30 min to allow the cDMEM to warm.
2. Place the freshly dissected fillets in the bijou tube with Collagenase-cDMEM solution (see Recipe 5 and Note 4), apply cap loosely and incubate at 28.5°C in 5% CO₂ incubator for 120 min with occasional (every 30 min) very gentle swirling of the tube (Figure 1H).
 3. Meanwhile, use a diamond pen to score two glass Pasteur pipettes per fish and create openings with diameters of approximately 1 and 3-4 mm, respectively (Figure 1I). Use a Bunsen burner to melt the glass around the opening to smoothly polish any sharp edges (Figure 1I and Video 1). Test the polishing by circling the pipette edge on aluminium foil. No cut/tear should be produced. Quickly flame the prepared glass pipettes to sterilise, wrap in aluminium foil and store in the tissue culture hood until use.



Video 1. Glass Pasteur pipette cut and heat-polish process

4. When incubation is complete, place the bijou tube in the tissue culture hood. A well-digested muscle looks slightly swollen and, under the microscope, hair-like myofibres appear dislodged around the edge of the muscle mass (Figures 1J and J' and see Note 5). Also collect the 150 mm dish with warm cDMEM from the incubator and place it in the culture hood.
5. Gently decant and discard most of Collagenase-cDMEM solution from the bijou tube. Rapidly invert the bijou tube to pour the muscle fillets into the 150 mm Petri containing cDMEM. Return the Petri dish with fillets to the incubator for 20-30 min. This allows the muscle to rest and dilute the Collagenase, promoting inactivation of the enzyme.
6. Place the dissecting microscope in the culture hood, if possible; otherwise, use a clean area away from doors, windows and draughts or other contamination sources. Collect the 150 mm dish with fillets and place under the lens of the microscope.
7. Rinse the heat-polished glass pipettes with BSA-PBS solution to prevent myofibre adhesion.
8. Using the pipette with the larger diameter (~3-4 mm), direct cDMEM onto the fillets repeatedly for at least 10 min, pipetting up and down to expel a continuous stream of liquid (Figure 1K). Tissue dissociation can be enhanced by carefully passing the fillets once or twice in and out of the glass pipette, but not continuously as this will damage the myofibres and reduce the final yield. Myofibres are visible hair-like structures that will be released from the muscle bulk (Figures 1K' and 1L).
9. Continue the trituration process until most myofibres have been released. The procedure will also result in the release of debris, including fat droplets and hypercontracted myofibres (Figure 1L), which will increase the turbidity of the medium. If trituration is prolonged, allow a further 5-10 min incubation at 28.5°C, 5% CO₂ to re-equilibrate the temperature and pH of the medium. If medium reaches below the range of physiological temperatures (22-29°C) for an extended time, myofibers will hypercontract and die.
10. Place the 150 mm plate back in the incubator for 10-15 min to allow released myofibres to rest and sink to the bottom.
11. Using the glass pipette with the smaller diameter (~1 mm), carefully collect intact myofibres and transfer

them onto a 100 mm dish with fresh cDMEM (Figure 1K'). If needed, the remaining muscle bulk can be further processed to enhance the release of residual myofibres, but not longer than 30-60 min as this will reduce the viability of residual myofibres.

12. Place the 100 mm dish containing cleaned myofibres back in the incubator for another 10-15 min to allow them to rest and sink to the bottom.
13. Viable myofibres appear translucent and with a smooth surface (Figure 1M). If needed, myofibres can be transferred into a new cDMEM-containing 100 mm dish to clean further. If significant debris is still present in the dish, repeat Steps B11 and B12.
14. The entire muscle dissection procedure should require 180 min.

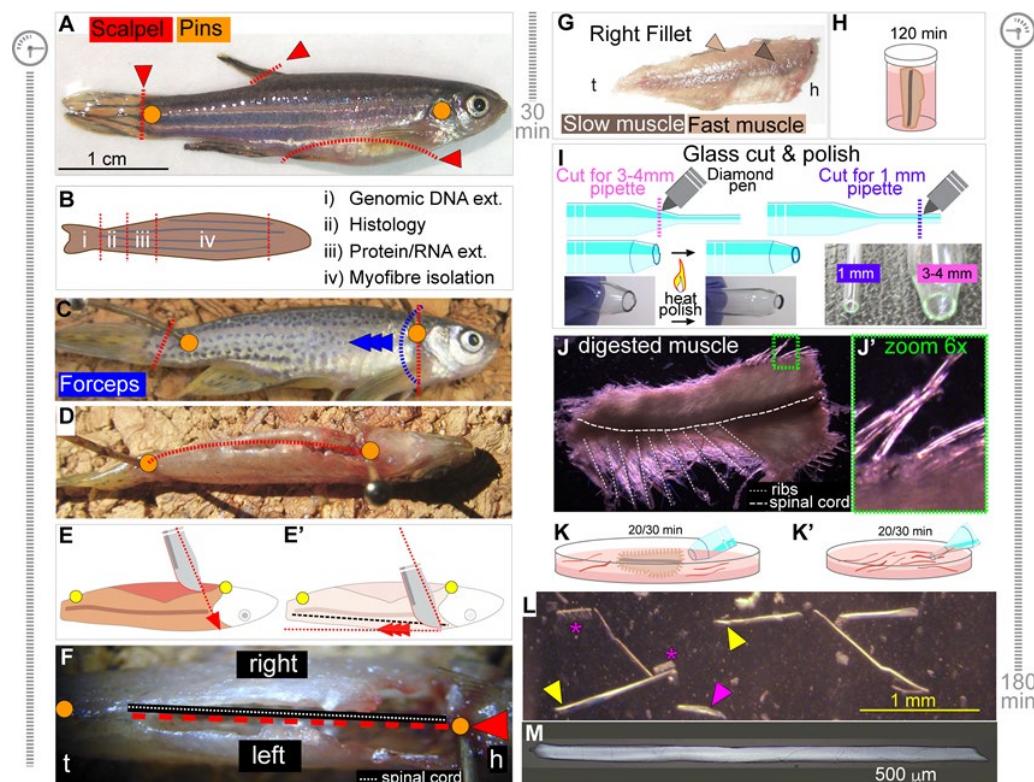


Figure 1. Dissection and Isolation of Myofibres from Adult Zebrafish.

A. Representative picture of an 8-month-old adult zebrafish depicts pin positioning (orange dots) to anchor fish carcass to the dissecting board. Red dashed lines indicate cuts to remove fins and to perform ventral incision for evisceration. B. Summary of analysis performable from different portions of the fish (ext; extraction). C. Blue dashed line and arrowheads indicate position of the skin incision, pinch and pulling direction for skinning with forceps. D. After skinning, 90° rotation of the carcass on its dorsal side exposes the ventral incision upward. Red dashed line and orange dots indicate cut direction and pin positioning, respectively. E, E'. Diagram of scalpel angle and cut direction during fish filleting. Red dashed arrows indicate inclination toward the dorsal midline (E) and cut direction towards tail pin (E'). Dashed black line shows position of spinal cord (E'). F. View of the ventral incision upward. Dashed red line and arrow indicate cut direction for filleting, with the spinal cord used as a guide (dashed white line). Antero-posterior orientation is indicated (h; head, t; tail). G. Slow (dark arrowhead) and fast (light arrowhead) muscle portions are visible in the dissected fillets (h; head, t; tail). H. Fillet is incubated with Collagenase-cDMEM solution for 120 min in the bijou tube at 28.5°C. I. Diagram of Pasteur glass pipettes cut and heat-polished to obtain two pipettes with wide (3-4 mm, pink) and small diameter (1 mm, purple) apertures, for muscle trituration and single muscle fibre handling, respectively. Purple (cut for 1 mm opening) or pink (cut for 3-4 mm opening) dashed lines indicate cut position on each pipette with diamond pen and heat polishing with a flame. Desired result for cut and edge heat-polish is shown

in bottom pictures (see Video 1). J, J'. Representative images of muscle fillet after 120 min incubation in Collagenase-cDMEM solution (J). Note the hair-like myofibres dislodged around the edge of the muscle mass (J', zoomed area in green). Position of ribs and spinal cord is also indicated. K, K'. Schematic of fillet trituration (K) and single myofibre isolation and wash (K') with estimated duration in minutes. L. Representative images of single myofibres during washes. Yellow arrowheads denote intact viable myofibres, while the magenta arrowhead and asterisks indicate damaged or hypercontracted myofibres, respectively. Estimated time for fish preparation/muscle dissection (C-F) and myofibre dissociation/isolation (G-J) are reported beside panels. M. Representative single viable myofibre following muscle dissection and isolation.

C. Analysis of Isolated Myofibres

1. Isolated myofibres are now ready for analysis, such as morphometrical measurements. Myofibres can be photographed prior to fixation using a brightfield microscope, which allows exclusion of those that are hypercontracted or damaged. Depending on the microscope used, damaged myofibres that are not yet fully hypercontracted appear shorter and more opaque, with a rough and irregular surface (Figure 1L).
2. Nuclear counting and subsequent analyses may require myofibre fixation. Under the microscope, use the BSA-PBS pre-rinsed glass pipette with smaller diameter (Figures 1I and 1K') to collect isolated myofibres and place them in a 2 mL clear round-bottomed microcentrifuge tube that has been rinsed with BSA-PBS to prevent myofibre adhesion. Gently swirl the dish to gather all myofibres at the centre of the plate to reduce the volume of cDMEM medium collected with myofibres. We suggest limiting the number of myofibres to 40 per tube to avoid clumping and possible damage.
3. Leave the collection microcentrifuge tube standing upright for 5 min at room temperature to allow the myofibres to sink to the bottom of the tube.
4. Carefully remove the medium above the myofibres with a pipette and replenish the tube with 1 mL of 4% PFA in PBS solution (PFA-PBS) by gentle trickling down the side of the tilted tube. Incubate for 10-15 min at room temperature.
5. Remove PFA-PBS solution and gently replenish with 1.5 mL of PBS to wash the myofibres. Incubate for 5 min and repeat the wash with fresh PBS. Lysine or BSA may be added to more efficiently inhibit the PFA. Fixed myofibres can be stored at 4°C for at least 2 weeks.
6. Remove PBS, wash and replace with freshly prepared Hoechst 33342 dye solution diluted in PBS to stain myofibre nuclei. Incubate for 15 min and replace with fresh PBS (as in Step C5). Myofibres are now ready to be mounted on glass slides for detailed analysis.
7. Use a water-repellent pen to outline a rectangular area (size depending on the size of the coverslip, e.g., 50 mm × 22 mm) on several glass slides. Under the microscope, use a clean smaller diameter pipette, pre-rinsed with BSA-PBS to collect myofibres from the 2 mL tube and transfer them onto the prepared glass slide. We suggest limiting to 10/15 myofibres per slide to facilitate handling.
8. Remove as much PBS as possible from the glass slide with a pipette to ease myofibre adhesion and reduce the risk of damage and/or loss. A 200 µL micropipette tip wrapped in aluminium foil and pre-immersed in BSA-PBS can be used to carefully reposition myofibres after PBS removal. We suggest being quick as residual liquid, along with immersed myofibres, will dry out rapidly.
9. Place two/three drops of glycerol-based mounting medium on the glass slide, position a 50 mm × 22 mm coverslip with an edge on the slide touching glycerol and gently lower the coverslip to avoid trapping air bubbles that can mis-position or sweep away myofibres. Wait 5 min to allow the mounting medium to spread beneath the coverslip.
10. Seal the coverslip and secure to the glass slide by brushing on a small amount of nail varnish, first at the corners and then seal the edges.
11. Mounted myofibres can be photographed using an epifluorescence or confocal microscope.
12. Using imaging Software, measure myofibre width (diameter) at a minimum of two different positions along each myofibre. Concomitantly, measure myofibre length and count the number of nuclei using Hoechst (Figure 2A).
13. Myofibre volume can be calculated with the formula reported in the Data analysis section and Figure 2A.

An example of myofibre volume in *Myog*^{+/−} adults from Ganassi *et al.* (2020) is shown (Figure 2B). Alternatively, confocal scanning may give full myofibre morphology profile (see Ganassi *et al.*, 2020).

D. Myofibre Immunolabelling

1. Fixed myofibres (Step C5) can be processed for immunolabelling. Here, we deploy the transgenic fish *TgBAC(pax7a:GFP)*^{β32239Tg} (Nüsslein-Volhard C.; MPI Tübingen) (Mahalwar *et al.*, 2014) and provide a template immunolabelling protocol using anti-GFP antibody to detect MuSCs. Although GFP fluorescence encoded by the *pax7a:GFP* transgene does resist PFA fixation, we suggest enhancement using the anti-GFP antibody, especially if co-labelling with multiple antibodies and fluorophores (Ganassi *et al.*, 2020).
2. Remove the PBS from the fixed myofibres with the BSA-PBS pre-rinsed smaller-diameter glass pipette and replace with 0.5% Triton-X100 detergent in PBS (PBSTx; see Recipe 6). Incubate for 15 min to permeabilise the cell membranes of both myofibres and associated MuSCs.
3. Remove PBSTx and gently add a blocking solution of 10% normal goat serum (NGS) in PBS to block non-specific antibody binding. Incubate for at least 30 min, occasionally tilting the tube (see Note 6). Alternatively, 5% NGS in PBS solution can be used to incubate for 1 h.
4. Prepare antibody solution by diluting the anti-GFP primary antibody in 0.1% Triton-X100 detergent PBS solution (PBSTx0.1) containing 2% NGS. Remove blocking solution from tube and gently add the primary antibody solution. Incubate overnight (16 h) at 4°C.
5. Remove the primary antibody solution and replace with fresh PBSTx0.1 to wash myofibres for 5 min (see Note 7). Primary antibody solution can be stored at 4°C and re-used reliably within one week (perhaps longer if 0.002% sodium azide in PBS is added).
6. Wash myofibres three times for 5 min each using PBSTx0.1 with occasional gentle tilting of the tube.
7. Dilute fluorochrome-conjugated (*e.g.*, Alexa Fluor 488) secondary antibodies and Hoechst 33342 dye solution (10 µg/mL final) in PBSTx0.1 and incubate for at least 60 min at room temperature, protected from light, with occasional tube swirling. Secondary antibody solution can be stored at 4°C in the dark and re-used reliably within one week or longer if 0.002% sodium azide in PBS is added.
8. Transfer myofibres onto a prepared glass slide and mount under a coverslip as described in Steps C7-C11. An example of a *pax7a:GFP* MuSC on a myofibre immunolabelled for GFP is shown in Figure 2C and can be found in Ganassi *et al.* (2020). Store slides at 4°C in the dark; GFP fluorescence lasts for up to 14 days.

E. Myofibre-derived MuSC Culture and Immunolabelling

1. Coat the desired number of wells of a 24-well plate by rinsing with Matrigel solution (see Recipe 7). Be sure to completely cover the surface of each well. Immediately remove excess solution using a sterile pipette and return the Matrigel solution to 4°C to avoid precocious gelling. Place the prepared plate in a 28.5°C 5% CO₂ incubator for 30-45 min to allow Matrigel gelling.
2. Prepare the proliferation medium (PM; see Recipe 8) and pre-warm at 28.5°C in the incubator prior to aliquoting 200 µL per Matrigel-coated well.
3. Transfer myofibres with a pipette into a new 100 mm dish containing 5 mL of 40% FBS-cDMEM solution. Each transfer of myofibres leads to concomitant carry-over of nearly 150-200 µL of cDMEM from the original dish that dilutes the serum concentration. At the end of the transfer, the final 100 mm dish now contains about 10 mL of DMEM with approximately 20% FSB, thus mimicking PM.
4. Gently swirl the 100 mm dish to gather myofibres at its centre. Use the small diameter glass pipette BSA-PBS pre-rinsed to transfer approximately 90-100 freshly isolated myofibres into each Matrigel-coated well. Ensure that the myofibres are evenly spaced across the well by placing the plate on a flat surface and moving laterally in cross-like orthogonal directions several times.
5. Place the 24-well plate(s) in the incubator and culture the myofibres undisturbed for at least 48 h. During the initial 24 h, myofibres can easily be dislodged, impacting MuSC activation, proliferation, migration

and adhesion to the culture plate. Even opening/closing the incubator door can cause vibrations that dislodge myofibres.

6. While a fraction of MuSCs activates within 24 h after plating, migrate off the myofibre and adhere to the Matrigel coating (see Note 8), we suggest waiting 48 h before analysis on MuSC. MPCs/myoblasts can be followed in culture and assayed for proliferation or differentiation capacity over time (Figure 2D).
7. At 48 h, cells can be immunolabelled with anti-Desmin antibody to confirm the purity of the myogenic population. Myofibre plating usually yields almost pure myogenic progenitors in culture (Figure 2E). The original data is presented in Figure 4-figure supplement 1 (Ganassi *et al.*, 2020), and a similar calculation can be found in Supplementary Figure 6g (Ganassi *et al.*, 2018). Cell fixation is described below (Step E12).
8. Cells can be collected at desired time point(s) for RNA extraction and gene expression analysis by RT-qPCR (see Note 9 and Figure 4 in Ganassi *et al.*, 2020).
9. Analysis of MuSC proliferation using 5-ethynyl-2'-deoxyuridine (EdU) is best performed no earlier than 2 days (48 h) from myofibre plating. Dilute EdU to a final concentration of 10 µM in fresh pre-warmed PM (EdU-PM).
10. Remove PM from culture well and rinse vigorously twice with freshly prepared PM to remove plated myofibres. At this time point, most myofibres should be either floating or loosely adhering to Matrigel and thus easily removed.
11. Remove PM and quickly rinse twice with PBS. Replace PBS with EdU-PM solution and place for 2-8 h in 28.5°C 5% CO₂ incubator. Duration of incubation can be changed according to the experimental design; the example shown here refers to 8 h treatment.
12. At the end of incubation, remove EdU-PM solution, wash vigorously twice with PBS and fix with PFA-PBS (4% PFA in PBS) for 15 min. If next step is immunolabelling, continue as described above for myofibres (Steps D2-D6).
13. After the final wash in PBSTx0.1, perform click chemistry to reveal EdU incorporation following the manufacturer's instructions.
14. Remove PBS, wash and replace with freshly prepared Hoechst 33342 dye solution diluted in PBS to stain nuclei. Incubate for 15 min.
15. Wash twice with fresh PBS, then replenish each well with 300 µL of PBS. The cells are now ready to be visualised using an inverted epifluorescence microscope (Figure 2F). Cells can be stored at 4°C (2-4 weeks) by replacing PBS with PBS containing 0.002% sodium azide to prevent microbial growth.
16. Dynamics of MuSC/MPC proliferation can be followed over time, as reported in Figure 2F (adapted from Figures 4E and 4F [Ganassi *et al.*, 2020]).
17. Zebrafish myoblasts can also be induced to differentiate to evaluate the myogenic program. After 96 h from initial plating, remove PM and wash twice with sterile PBS to eliminate serum residues. Remove PBS and add 500 µL of differentiation medium (DM; see Recipe 9) to each well. Replace DM every 48 h. We previously assessed myogenic differentiation by culturing zebrafish primary myoblasts in low serum medium for 5 days prior to immunolabelling for structural components such as Myosins (Figure 2D). Figure 2G shows myotubes differentiated for 5 days and co-immunolabelled for myosin heavy chain (MyHC) using MF20 and A4.1025 concomitantly (Blagden *et al.*, 1997) and counterstained with Hoechst 33342 to assess differentiation and cell fusion (original data is in Figure 8 in Ganassi *et al.*, 2018) and Figure 4 - Supplement 1E (Ganassi *et al.*, 2020).

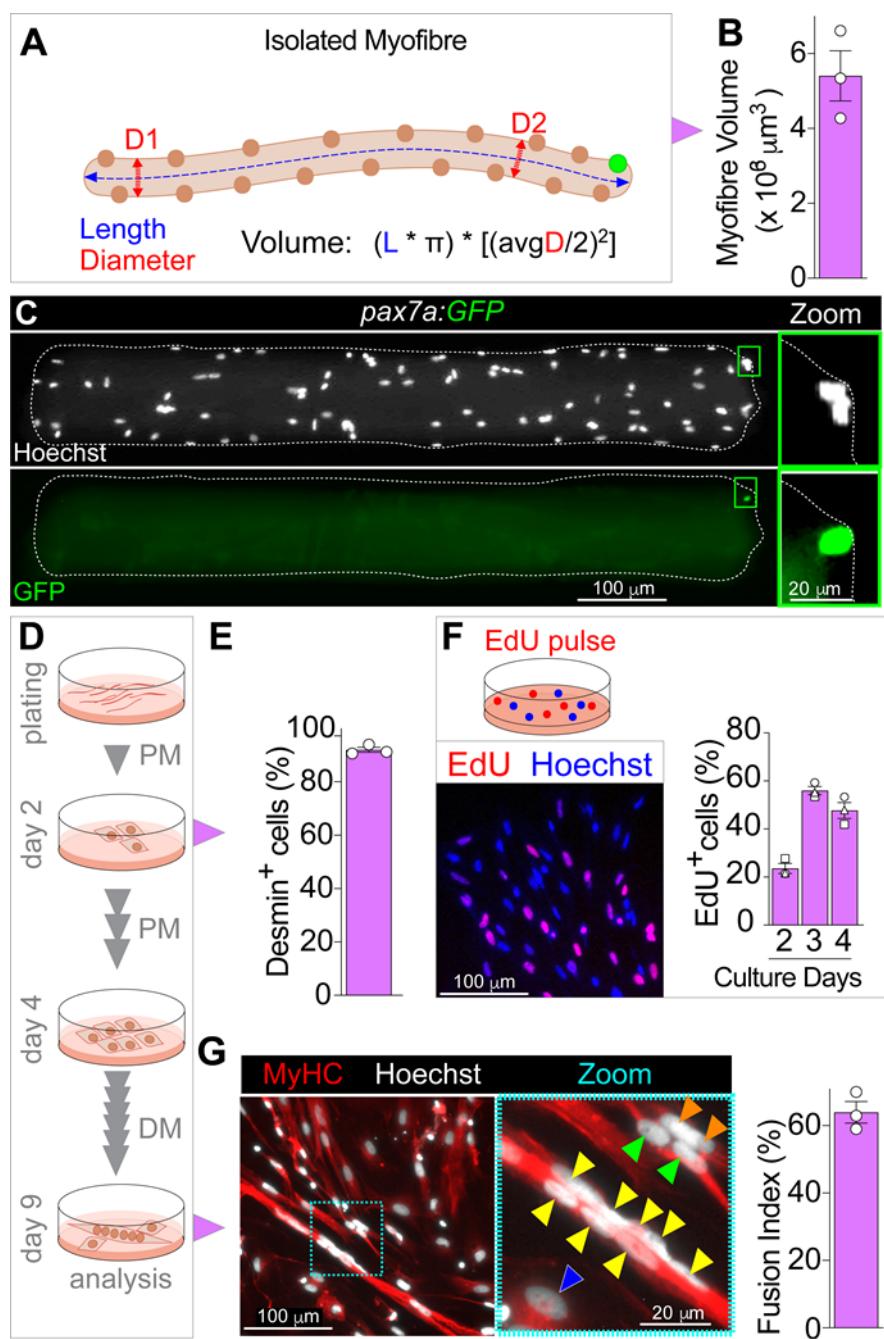


Figure 2. Analysis of Isolated Myofibres and Cultured MuSCs.

A. Diagram of myofibre average diameter (myofibre width, red arrowed dashed line) and unit length (blue arrowed dashed line) measurements to calculate myofibre volume. Avg, indicates arithmetic mean of diameter measure across myofibre length (D1 and D2). B. Example results of 8-month-old adult myofibre volume (adapted from Ganassi *et al.*, 2020). Symbols represent average values from 20–30 myofibres from each of three different fish. C. *TgBAC(pax7a:GFP)^{l32239Tg}* (*pax7a:GFP*) myofibre immunolabelled for GFP (green) reveals the position of a MuSC (green rectangle, magnified at right) near the myofibre-end and myofibre nuclei counterstained with Hoechst 33342 (white). D. Schematic of myofibre plating, MuSCs/myoblasts proliferation (PM; proliferation medium), expansion and myotube differentiation protocol (DM; differentiation medium) with indicated timing, medium change and analysis. E. Example calculation of fraction of cells that were Desmin⁺ (myoblasts) two days after

myofibre plating. F. MuSC-derived myoblasts can be EdU pulsed (red) two days after myofibre plating in proliferation medium. Nuclei were counterstained with Hoechst 33342 (blue). G. Representative image of differentiated multinucleated myotubes or mononucleated cells containing MyHC (red) after 5 days of culture in differentiation medium, coloured arrowheads indicate nuclei within the same cell (from Ganassi *et al.*, 2020). Cyan dashed rectangle indicates the magnified area in the right panel. Nuclei were counterstained with Hoechst 33342 (white). Example results of fusion index (adapted from Ganassi *et al.*, 2018). All graphs report mean \pm SEM, and symbols represent biological replicates.

Data analysis

1. Use the measured average myofibre width (avg diameter) and length to calculate the volume of the myofibre following the formula:
$$[(\text{Length} \times \pi) \times ((\text{average Diameter}/2)^2)]$$
 (Figure 2A),
where ‘average’ is the arithmetic mean of diameter measurements at a minimum of two different positions along the myofibre length. Values can be compared with chosen statistical analysis (*e.g.*, unpaired two-tailed t-test). Graphs were produced in GraphPad Prism 8 (see Software).
2. Myofibre volume and number of nuclei can be combined to calculate the myonuclear domain using the formula:
$$[(\text{Length} \times \pi) \times ((\text{average Diameter}/2)^2)/\text{Number of myofibre nuclei}]$$
. Alternatively, average myofibre width and length can be used to calculate the surface area of the myofibre following the formula: Length \times $\pi \times$ avg Diameter. The Surface Area Domain Size (SADS), the notional SA occupied by each myofibre nucleus, is calculated using the formula: SA/number of myofibre nuclei. Examples of the calculation are available in Ganassi *et al.* (2020) or in Brack *et al.* (2005) for mouse myofibres.

Notes

1. For zebrafish weighing 0.3-0.6 g, euthanasia is usually reached within 5-10 min after incubation in ice-cold 0.3 mg/mL tricaine solution. However, this is only indicative timing and must be determined empirically and according to local guidelines.
2. It is essential to wash and thoroughly sterilise Equipment and dissecting tools to avoid microbial contamination.
3. Removing fish fins is not essential but facilitates handling and reduces the risk of microbial contamination.
4. It is important to batch-test replacement reagents, such as Collagenase, against existing, optimised components. There are variable amounts of proteases in batches of Collagenase, but Collagenase with neutral protease around 53 U and clostripain at approximately 0.6 U is ideal, as described before (Rosenblatt *et al.*, 1995).
5. Adult (8-15 months old) zebrafish trunk muscle is usually digested after 2 h. Although longer incubations (\geq 3 h) have a marginal effect on myofibre viability, shorter incubation may reduce digestion efficiency, hindering the isolation of viable myofibers. The precise time depends upon both the age and size of the fish and the activity of the batch of Collagenase used and should be determined empirically.
6. The normal serum used for blocking should derive from the species in which the secondary antibody was raised.
7. Myofibres can be stained with fluorochrome-conjugated toxins to detect the subcellular structure, such as filamentous actin (F-actin) using Phalloidin (ThermoFisher, A12379) or neuromuscular junction (acetylcholine receptor) using α -Bungarotoxin (ThermoFisher, B35451).
8. If myofibres/MuSCs are to be cultured for longer periods, replace half the volume of the medium with fresh medium every 48 h.
9. For gene expression analysis, collect cells by removing medium and washing twice with PBS. Incubate with the appropriate volume (*e.g.*, 200 μ L for a 24-well plate well) of Accutase® reagent to detach the cells from Matrigel for 10 min (or until complete detachment of all cells; check under a microscope, but this should not take longer than 15 min) at 28.5°C, 5% CO₂ (see Ganassi *et al.*, 2020). Collect cells in a 1.5 mL clear tube, pellet by centrifugation at 200 \times g at 4°C and wash once in PBS. Pelleted cells are now ready for RNA extraction and analysis or can be stored at -80°C.

Recipes

1. 1% Virkon

Prepare 1% Virkon by dissolving 5 g of powder in 500 mL of deionised sterile water.

1% Virkon solution can be aliquoted and stored at -20°C for at least 2 months. Virkon powder can be stored at room temperature.

2. P/S-PBS

Prepare 10% vol/vol Penicillin/Streptomycin solution in PBS (P/S-PBS) and aliquot 25 mL into a 100 mm plastic Petri plate.

3. BSA-PBS

Prepare 5% BSA solution in sterile PBS (BSA-PBS) and heat-inactivate at 60°C for 60 min before filtering through a 0.45 µm syringe filter.

4. cDMEM (complete DMEM)

Prepare cDMEM by adding Penicillin/Streptomycin solution at 1% vol/vol and gentamicin to 50 µg/mL to DMEM. Prepared cDMEM can be stored at 4°C for 2-3 weeks.

5. Collagenase-cDMEM

Immediately before dissection, prepare a 0.2% collagenase solution in cDMEM. In the tissue culture hood, filter-sterilise the Collagenase-cDMEM solution using a sterile syringe with a 0.2 µm filter. Consider approximately 2 mL Collagenase-cDMEM for each fish (two fillets) and aliquot into separate 7 mL bijou tubes.

6. PBSTx

Prepare PBSTx (0.5%) by diluting 100% Triton X100 in sterile PBS. Solutions containing Triton X-100 can be prepared in advance, but for long term storage, use PBS containing 0.002% sodium azide instead of PBS and wrap the tube/bottle in aluminium foil to protect from light.

7. Matrigel solution

Defrost Matrigel stock overnight at 4°C. Dilute it to 1 mg/mL in DMEM and aliquot into 2 mL micro-centrifuge tubes. It is essential to complete this step on ice or the Matrigel will gel and form lumps. Aliquots of diluted Matrigel can be stored at 4°C for up to 2 weeks or frozen at -20°C for longer term storage.

8. Proliferation Medium (PM)

Prepare PM by supplementing DMEM with 1% Penicillin/Streptomycin, 10 µg/mL gentamicin and 20% FBS. Pre-warm PM to 28.5°C in the incubator. We recommend preparing PM fresh prior to starting muscle dissection/dissociation and to use within 2 to 3 days.

9. Differentiation Medium (DM)

Prepare DM by supplementing DMEM with 1% Penicillin/Streptomycin, 10 µg/mL gentamicin and 2% horse serum. Pre-warm DM to 28.5°C in the incubator. We recommend preparing fresh DM as needed and using it within 2 to 3 days.

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Ganassi *et al.* (2018) and Ganassi *et al.* (2020).

Competing interests

The authors declare that they have no competing interests.

Ethics

All procedures were performed on adult zebrafish in accordance with the PPL license held under the UK Animals (Scientific Procedures) Act 1986, and later modifications conformed to all relevant guidelines and regulations. All lines used were reared at King's College London on a 14/10 h light/ dark cycle at 28.5°C, with staging and husbandry as described before (Westerfield, 2000).

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En masse DNA Electroporation for *in vivo* Transcriptional Assay in Ascidian Embryos

Sébastien Darras*

Sorbonne Université, CNRS, Biologie Intégrative des Organismes Marins (BIOM), Banyuls-sur-Mer, France

*For correspondence: sebastien.darras@obs-banyuls.fr

Abstract

Ascidian embryos are powerful models for functional genomics, in particular, due to the ease of generating a large number of transgenic embryos by electroporation. In addition, the small size of their genome makes them an attractive model for studying *cis*-regulatory elements that control gene expression during embryonic development. Here, I describe the adaptation of the seminal method developed 25 years ago in *Ciona robusta* for *en masse* DNA electroporation for *in vivo* transcription to additional species belonging to three genera. It is likely that similar optimizations would make electroporation successful in other ascidian species, where *in vitro* fertilization can be performed on a large number of eggs.

Keywords: Ascidians, Tunicates, Electroporation, Transgenesis, *Cis*-regulatory DNA, *Phallusia*, *Ascidia*, *Molgula*

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Background

Ascidians are marine invertebrates that are the closest vertebrate relatives. Their fast, stereotyped, and external embryonic development gives rise to tadpole-like larvae closely resembling other chordates (vertebrates and amphioxus). Their compact genome, together with the availability of simple and efficient methods to manipulate gene expression and function, have made ascidians interesting model organisms for functional genomics (Lemaire, 2011; Satoh, 2014). In particular, the introduction of plasmid DNA into a fertilized egg by electric shock (electroporation) allows the generation of hundreds to thousands of transient transgenic embryos, which promoted *Ciona robusta* as the reference ascidian species almost 25 years ago (Corbo *et al.*, 1997). DNA electroporation is a widely used method for *in vivo* transcriptional assays (for characterization of the activity of candidate *cis*-regulatory elements using reporter genes), over-expression/knockdown, and live imaging.

Ascidians are a diverse group of animals containing around 3,000 species that have been proven as interesting systems to study the evolution of developmental mechanisms since their genomes have been extensively rearranged, but their embryonic development is strongly conserved (Dardaillon *et al.*, 2020). In particular, DNA electroporation has been applied to various species (Roure *et al.*, 2014; Stolfi *et al.*, 2014; Colgan *et al.*, 2019; Coulcher *et al.*, 2020). Here, I provide a detailed protocol to perform electroporation in four species: *Phallusia mammillata*, *Phallusia fumigata*, *Ascidia mentula*, and *Molgula appendiculata* (Figure 1), which cover almost 400 million years of evolution.

Materials and Reagents

1. Disposable scalpels
2. 300- and 120- μm homemade sieves

Note: Cut a 50-ml plastic tube at ~4-5 cm from the opening. Cover a heating plate with aluminum foil. Fuse a piece of nylon mesh (Sefar Nitex, catalog numbers: 03-300/51 [300- μm opening], 03-120/49 [120- μm opening]) to the cut tube using the heated plate.

3. 15-mL glass centrifuge tubes (Dutscher, catalog number: 092305)
4. 15-mL plastic conical tubes (*e.g.*, Sarstedt, catalog number: 62.554.502)
5. 60-mm plastic Petri dishes (*e.g.*, Sarstedt, catalog number: 82.1194.500)
6. 92-mm plastic Petri dishes (*e.g.*, Sarstedt, catalog number: 82.1473.001)
7. 60-mm and 92-mm agarose-coated Petri dishes. Alternatively, gelatin-coated dishes (GF) can be used as in Sardet *et al.* (2011)

Note: Melt the agarose in sea water (1 g per 100 ml sea water). Fill a 92-mm dish with the hot solution. Make a thin agarose layer by pouring the agarose solution into the next dish; repeat until you have enough dishes (you will need two dishes per electroporation, plus a dozen for dechorionation, fertilization, and washing).

8. 6-well plates

Note: Eggs tend to stick to new plastic material. To avoid this, reuse the same plates; simply wash with tap water and air dry.

9. Gloves (*Phallusia* blood is a powerful stain!)
10. Glass Pasteur pipets

Note: Soak them in tap water to avoid sticking of the embryos. Smooth the opening using a lighter. Prepare some with a larger opening (2-3 mm) using a diamond for egg collection in *Phallusia*.

11. 100 mL glass beaker
12. Horizontal rotating shaker (*e.g.*, VWR, catalog number: 444-2900)
13. 1.5 mL microtubes
14. Low-binding 1.5 mL (*e.g.*, VWR, catalog number: 525-0230) and 2 mL (*e.g.*, VWR, catalog number: 525-0232) microtubes
15. 4 mm electroporation cuvettes (*e.g.*, Dutscher, catalog number: 38191)
16. Depression slides: 4 mm (Dutscher, catalog number: 065230) and 1.5 mm (Dutscher, catalog number: 65227) of thickness
17. Tungsten needle (Roboz Surgical Instrument Co., catalog number: RS-6065)
18. Adult ascidians are collected from the ocean by diving, using a trawl or a dredge and are provided by the French node of the European research infrastructure EMBRC (Station biologique de Roscoff: *Phallusia mammillata* and *Ascidia mentula*; Station marine de Banyuls-sur-mer: *Phallusia mammillata*, *Phallusia fumigata*, *Ascidia mentula*, and *Molgula appendiculata*)
19. Supercoiled circular plasmid DNA (20-100 µg) of the reporter construct
20. NaOH, 2.5 M
21. NaOH, 1 M
22. 1 M Tris, pH 9.5
23. 0.96 M D-mannitol solution (VWR, catalog number: 25311.297)
24. TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0)
25. 25% glutaraldehyde solution (Sigma-Aldrich, catalog number: G6257)
26. Sodium thioglycolate (Sigma-Aldrich, catalog number: T0632)
27. Pronase (Sigma-Aldrich, catalog number: P5147)
28. 0.2 µm filtered natural sea water or artificial sea water (BASWH: see Recipes)
29. *Phallusia* and *Ascidia* dechorionation solution (see Recipes)
30. *Molgula* dechorionation solution (see Recipes)
31. PBTw (see Recipes)
32. 10× PBS (see Recipes)
33. X-gal staining solution (see Recipes)
34. X-gal stock solution (see Recipes)

Equipment

1. Scissors and tweezers
2. Temperature-controlled room set at 18°C
3. Dissecting scope for live embryo manipulation (*e.g.*, Discovery V8 from Zeiss)
4. Square wave electroporator (Harvard Apparatus, BTX ECM830, catalog number: 45-0052)
5. Incubators
6. Dissecting scope equipped with a digital camera for staining analysis (*e.g.*, Discovery V20+Axiocam Erc 5s from Zeiss)

Procedure

The *in vivo* transcriptional assay proceeds according to these successive steps: 1) gamete collection, 2) egg dechorionation, 3) fertilization, 4) DNA electroporation, 5) embryo fixation and staining, and 6) data collection and analysis. The first 4 steps differ between species. Here, I present two versions: one for *P. mammillata*, *P. fumigata*, and *A. mentula* (with minor changes among species); and one for *M. appendiculata*.

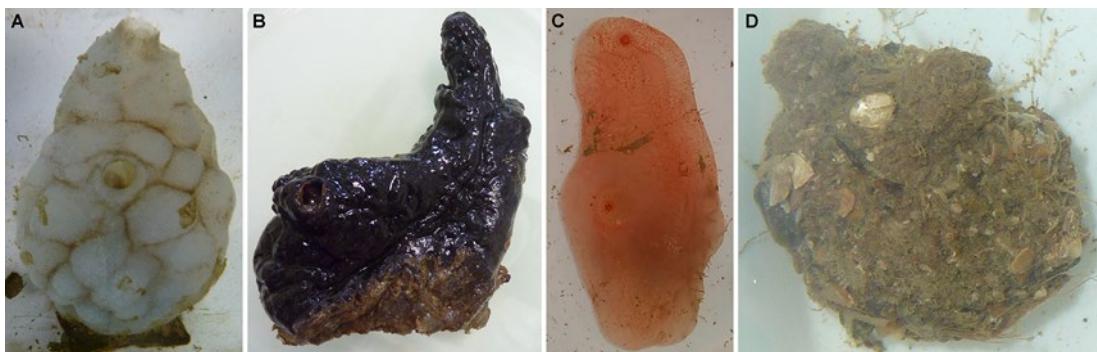


Figure 1. Adult ascidian species.

(A) *Phallusia mammillata* (5-20 cm long). (B) *Phallusia fumigata* (5-20 cm long). (C) *Ascidia mentula* (4-10 cm long). (D) *Molgula appendiculata* (3-6 cm long).

A. Gamete collection

Ascidians are hermaphrodites. The aim is to collect both eggs and gametes, separately, from each individual.

1. *Phallusia* and *Ascidia*

P. mammillata, *P. fumigata*, and *A. mentula* belong to the same family, the Phlebobranchia. Gametes are collected from the gonoducts by dissection. To avoid unwanted self-fertilization, eggs are collected first. All species have transparent eggs.

- a. Open the animals with a scalpel by cutting between the two siphons (Figure 2A). *Phallusia* tunic is thick and hard.
- b. Incise the tunic toward the base of the animal on both sides. Do not cut too deep to avoid damaging the animal.
- c. Open the tunic by pulling apart with your fingers.
- d. Transfer the animal to a dish on its left side (you should see the heart beating at the base of the animal, opposite to the siphons). If the animal is on the right side, you should see a large, yellowish (*Phallusia*, Figure 2C) or reddish (*Ascidia*, Figure 2B) oviduct full of eggs.
- e. Make a small cut in the oviduct and collect the eggs with a Pasteur pipet (Figure 2D).

*Note: The eggs in the oviduct are very compact and embedded in a gelatinous substance. Use a wide-bore Pasteur pipet for *Phallusia*; otherwise, their aspiration through the small opening of a pipet may cause extreme deformation and egg death. Dead eggs are easily spotted since they turn opaque.*

- f. Transfer the eggs to a 6-well plate filled with sea water. *Phallusia* individuals normally have a high number of eggs (0.5-2 ml eggs). *Ascidia* usually have fewer eggs, but the amount can reach 0.5-1 mL.
- g. Check the egg quality (transparent and uniform egg cytoplasm, Figures 2E and 2F) and transfer them to a glass tube for several washes with sea water. Eggs can be kept overnight at 14°C, spread on a Petri dish, with no major decrease in quality.
- h. You should see the spermiduct (sharp white duct) underneath the oviduct once you have removed the eggs. Make a small cut and collect the sperm into a 1.5-ml tube using a Pasteur pipet for *Phallusia* or a micropipet with a yellow tip. Avoid collecting sea water, debris, and blood. Dry sperm remains active for several days at 4°C.

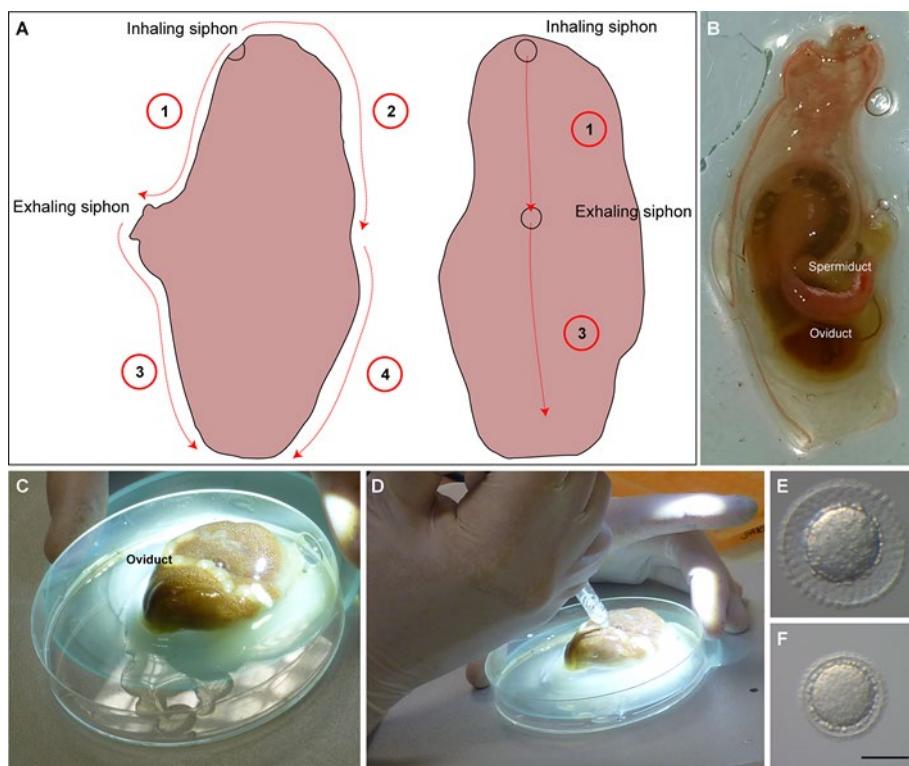


Figure 2. Gamete collection in *Phallusia* and *Ascidia*.

(A) Schematic diagram illustrating the successive incisions (numbered red dotted lines) to open the tunic (left: lateral view with the two siphons pointing to the left; right: side view with the two siphons pointing toward the experimenter). (B) Dissected *A. mentula* individual where the gonoducts are exposed. (C) Dissected *P. mammillata* individual where the oviduct is clearly visible. (D) Egg collection in *P. mammillata* using a wide-bore Pasteur pipet. (E) *P. mammillata* egg. (F) *A. mentula* egg. Scale bar: 100 µm in D and E.

2. *Molgula*

Contrary to *Phallusia* and *Ascidia*, very few eggs are present in the tiny oviduct (Figure 3D). Gametes are therefore collected by gonadal dissection. The oocytes collected from the gonad represent all stages of oogenesis. The procedure aims at collecting mainly fully grown oocytes that are not yet mature, as evidenced by the presence of a large germinal vesicle (GV). Fortunately, these oocytes mature spontaneously in sea water, and germinal vesicle breakdown (GVBD) occurs within 30-60 min.

- Open the animals with scissors, starting from one siphon around the animal all the way to the other siphon (Figure 3A) (*M. appendiculata* are covered with debris, shells, and stones; use large scissors and make your way around these obstacles).
- Pull open the tunic and drag the animal still attached by the siphons (Figure 3B).
- Cut the body wall from siphon to siphon with fine scissors and pull open the animal (Figure 3C).
- Under the dissecting scope, locate the gonads (one on each side of the animal, Figure 3C) under the pharyngeal basket with tweezers. You should see the short gonoducts pointing toward the siphons and the large ovary covered on one side by the testis (Figure 3D).
- Cut out each gonad with scissors and place them in the same well of a 6-well plate filled with sea water (Figure 3E).

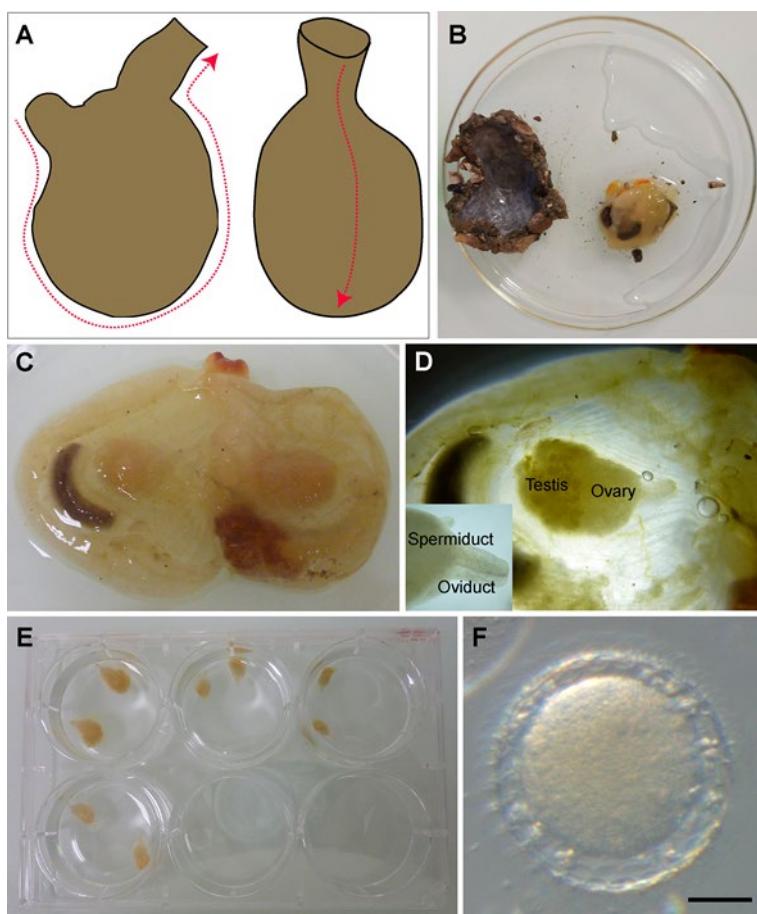


Figure 3. Gamete collection in *Molgula appendiculata*.

(A) Schematic diagram illustrating the incision (red dotted line) to perform to open the tunic (left: lateral view with the two siphons pointing to the top; right: side view). (B) An individual (right side) after tunic (left side) removal. (C) Cut open the animal with a gonad visible at the center on each side. (D) Each gonad is composed of an ovary surrounded by a testis. The inset shows a close-up view of the tiny spermiduct (top) and the oviduct (bottom), both openings pointing to the right. (E) A 6-well plate where pairs of gonads from 4 individuals have been collected. (F) A fertilizable egg is obtained after spontaneous maturation of a fully grown oocyte (scale bar: 50 μ m).

- f. Under the dissecting scope, release the oocytes from the ovary using tweezers, and transfer the testis to a Petri dish filled with sea water (60-mm diameter). You should see a full range of oocytes (from tiny transparent oocytes to large, opaque, fully grown oocytes with GV).
- g. Transfer all the oocytes from one individual (2 ovaries) through a 300 μ m sieve placed in a 100 ml glass beaker (gonad debris will not go through).
- h. Transfer the contents of the beaker to a 120 μ m sieve and wash extensively with sea water to remove sperm and small oocytes.
- i. Transfer the oocytes to a 60 mm agarose-coated dish.
- j. Wait 30-60 min until GVBD occurs and produces fertilizable oocytes.
- k. Release the sperm from the testis of all individuals that have been collected in the same Petri dish by dissociating each testis with tweezers (discard the testes afterwards). Collect the concentrated sperm solution into a 15-ml plastic tube and keep at 4°C until use.

B. Egg dechorionation

The chorion that protects the egg is made of an inner vitelline membrane consisting of extracellular material and an outer cellular layer of follicle cells. To perform electroporation, it is necessary to remove it; this is achieved using a mixture of sodium thioglycolate and pronase. The concentration of each compound and the duration of the dechorionation varies between species.

Note: Dechorionation is usually performed before fertilization, and the naked eggs can still be fertilized. For Molgula, since the procedure is rather quick, this can be performed after fertilization.

1. Collect the eggs in a glass tube and allow them to settle to the bottom.
2. Discard the excess sea water.
3. Activate the dechorionation solution by raising the pH (add 3 drops (around 100 µL) (*Phallusia/Ascidia*) or 6 drops (around 200 µL) (*Molgula*) 2.5 M NaOH to 7 ml dechorionation solution using a Pasteur pipet, and mix).
4. Proceed to dechorionation
 - a. In an agarose-coated dish for *Phallusia/Ascidia*, by adding the eggs to the dechorionation solution (~7 mL for a 60-mm dish and ~14 ml for a 92-mm dish). Mix well and place on a horizontal shaker at ~70 rpm.
 - b. In a glass tube for *Molgula*, by adding 3-4 mL dechorionation solution. Mix well by pipetting up and down.
5. Regularly check the dechorionation status under the scope. Dechorionation takes 30-45 min (*Phallusia/Ascidia*) and 7-15 min (*Molgula*) depending on the egg density.
6. Stop dechorionation when most eggs are clearly devoid of the chorion. *Phallusia/Ascidia*: gather the eggs at the center by swirling the dish and transfer them to a 15 ml glass tube. Add sea water to the top and gently mix by pipetting up and down. *Molgula*: discard as much dechorionation solution as possible. Add sea water to the top and gently mix by pipetting up and down.

Note: Dechorionation is more difficult to follow in Molgula: while follicle cells are quickly lost, the vitelline membrane is more difficult to see (look closely, change the scope mirror orientation; the presence of test cells on the egg's surface is a good indication that the eggs are not fully dechorionated).

7. Wash extensively 2-4 times by allowing the eggs to settle down, replacing the sea water, and gently mixing with a Pasteur pipet. Debris (chorion and dead eggs) will float, while naked eggs rapidly sink to the bottom of the tube. Naked eggs are fragile and explode easily; be gentle and avoid air bubbles.
8. You can proceed to the next step using the same tube or collect the eggs in an agarose-coated dish for later use. Dechorionated, unfertilized eggs can be stored overnight at 4-14°C with no major decrease in quality.

C. Fertilization

1. (*Phallusia/Ascidia* only) Add 5 µL dry sperm to a 1.5-mL microtube containing 1 mL sea water.
2. (*Optional*) Fertilization is usually efficient with straight sperm solution; however, to achieve a complete fertilization rate and high synchrony, it is better to activate the sperm solution by raising the pH (in the ocean, sperm are activated by the slightly basic pH of the sea water). Add either 50 µL 1 M Tris pH 9.5 (*Phallusia*), 4 µL 1 M NaOH (*Ascidia*), or 8 µL 1 M NaOH (*Molgula*) to the sperm solution.

Note: Phallusia sperm can also be activated by incubating the 1 mL mixture for 15 min with 20 µL chorionated eggs as in Sardet et al. (2011).

3. Check the sperm motility by diluting an aliquot 50-100× in sea water and looking under the dissecting scope at high magnification. Sperm should be swimming frantically.
4. Add ~200 µL (60-mm dish) or 400-500 µL (92-mm dish or 15-mL glass tube) sperm solution.
5. Mix well, making the eggs float in the medium. You should see the sperm swimming intensively.
6. Check for egg deformation that occurs following fertilization and that should be visible within the first few minutes.
7. At 10-15 min post-fertilization, proceed to the washes to remove the sperm, since this protocol uses enormous quantities of sperm. (Fertilization in a Petri dish) Gather the eggs at the center by swirling the dish and transfer them to a 15-ml glass tube using a Pasteur pipet. Allow the eggs to settle to the bottom of the tube, discard most of the sea water using a Pasteur pipet, add clean sea water up to the top by gently pouring down the side of the tilted tube; resuspend the eggs thoroughly by flushing sea water with the pipet. Repeat this wash 1-3 ×.

Note: Embryos without their protective chorion are fragile and explode as soon that they come into contact with the water/air interface. Be cautious; gently manipulate them, avoiding air bubbles.

D. DNA electroporation

1. Prepare the DNA (20-100 µg) in 50 µL TE.
2. Add 200 µL 0.96 M D-mannitol and mix well.
3. Collect the fertilized eggs at the bottom of a glass tube.
4. Mark with a pen, the 100 µL level on the low-binding 1.5 mL tubes.
5. Transfer the fertilized eggs into the 1.5-mL low-binding tubes. Try to add the same number of eggs to each tube (one per construct to be tested).
6. Adjust the volume to the 100 µL mark with sea water.
7. To each tube, add 250 µL DNA/mannitol solution.

Note: Mannitol is used to reduce the salt concentration (and thus avoid electric arcing during electroporation) while keeping the osmolarity at a sufficient level.

8. Transfer the total mixture to an electroporation cuvette (use a different Pasteur pipet for each tube to avoid mixing DNA).
9. Place the cuvette into the electroporator cuvette holder.
10. Apply a single electrical pulse of 37 V (*Phallusia/Ascidia*) or 20 V (*Molgula*) for 32 ms. The pulse should be done toward the end of the first cell cycle (but before cleavage): at 50-60 min post-fertilization.

Note: Electroporation can be performed any time after fertilization, and the timing does not affect the electroporation efficiency; however, the fertilized eggs are more robust to electroporation (but also to microinjection) during the last third of the first cell cycle.

11. Proceed to the electroporation of the next construct.
12. Once all electroporations are done, add some sea water to the cuvette and transfer the eggs to 92 mm agarose-coated Petri dishes (usually at least two dishes for one electroporation).

Note: A total of 4-6 electroporations are routinely done per fertilization round. With experience, 10-12 electroporations can be performed.

13. Check the dish under the dissecting scope. You should see intact embryos (since they are toward the end of the first cell cycle, you should see the myoplasm and/or deformations corresponding to the preparation of the first cleavage) and debris of exploded eggs.

*Note: For species with transparent eggs (*Phallusia/Ascidia*), dead eggs are easily spotted since they become opaque. In most cases, there is a small proportion of eggs that do not survive the electroporation; these correspond to fragile or unfertilized eggs. In cases where you find no surviving embryos, there are two main explanations: the fertilization failed (unfertilized eggs systematically explode after the electrical pulse) or the voltage was too high. Try to make two experimental controls: non-dechorionated and non-electroporated embryos, and dechorionated and non-electroporated embryos.*

14. Transfer the dishes to an incubator set at the desired temperature.

*Note: 14-19°C is the common range of temperature for all species. They can also develop well at lower temperatures with a significant decrease in speed. *Phallusia/Ascidia* also develop well up to 22°C.*

15. Spread the embryos by flushing sea water with a Pasteur pipet (they have a very high tendency to stick to each other if they are too close). Avoid moving the dishes before the 8-cell stage since blastomeres are very loosely attached to each other.

E. Embryo fixation and staining

Depending on the reporter gene, the course of action may differ. Here, I describe the use of *LacZ* as a reporter gene (detecting β-galactosidase activity using the chromogenic substrate X-gal).

1. Allow the embryos to develop until the desired stage.
2. Swirl the dish to gather the embryos at the center.
3. Collect the embryos using a glass Pasteur pipet and transfer them to a 2-mL low-binding microtube.
4. Allow the embryos to settle and discard the excess sea water.
5. Fill the tube with fixative (0.2% glutaraldehyde in sea water) and rotate the tube in your fingers for 30 s.
6. Fix for exactly 30 min at room temperature (over-fixing will abolish β-galactosidase enzymatic activity, too light a fixation may lead to embryo disintegration).
7. Remove half of the fixative and add 1 mL PBTw. Mix by inverting 2-3 ×.
8. Once the embryos have settled, replace the solution with 2 mL PBTw, mix by inversion, and wait 10 min. Repeat this wash 1 ×.
9. Rinse for 5 min in X-gal staining solution.
10. Replace with 250 μL staining solution containing 0.4 mg/mL X-gal (dilute the X-gal stock solution 100×).
11. Incubate at 37°C (an incubator is preferable to a water bath since no evaporation takes place in the tube).
12. Check the blue staining from time to time (it starts within a couple of hours depending on the strength of the tested regulatory region).
13. Once the staining is adequate (this may take several days, but this is a staining procedure that never produces any Background), wash 2 × 5 min in PBTw. Typical examples of staining with a version of β-galactosidase targeted to the nucleus may be found in Roure *et al.* (2014) and Coulcher *et al.* (2020).
14. Post-fix for 1 h at room temperature or O/N at 4°C in PBTw containing 3.7% formaldehyde.
15. Samples can be stored long-term.

Data analysis

A. Data collection

1. Discard the fixative and wash 2 × 10 min in PBTw.
2. Transfer all the embryos to a thick depression slide.
3. Transfer part of the embryos to a thin depression slide.

4. Using a dissecting scope and a tungsten needle, score the number of stained embryos in the target tissue until you reach 100-200 scored embryos or until all the embryos in the tube have been scored (score only those embryos that have developed normally) (Roure *et al.*, 2014; Coulcher *et al.*, 2020).

Notes:

- a. *Embryos can be manipulated easily using a tungsten needle, a cat whisker, or an eyelash.*
- b. *Depending on the aim, the approach can be refined by scoring not only the number of stained embryos, but also the number of stained cells per embryo; however, this may be time consuming.*
- c. *Do not forget that this transgenesis method yields mosaic transgene expression (each cell has randomly inherited a variable amount of plasmid DNA) and that each embryo is an independent event; hence, the activity of a given region is determined by compiling the expression in all the embryos that are analyzed.*

5. Take images of representative embryos.

B. Evaluation of *cis*-regulatory activity and comparisons

1. The assay provides qualitative data (*i.e.*, the spatial domain of activity of a given piece of genomic DNA), but the percentage of stained embryos also reflects the strength of the regulatory region.
2. The score obtained may vary from experiment to experiment according to the ascidian batch, plasmid DNA preparation, and embryo concentration during electroporation; thus, we usually perform at least 3 biological replicates (different ascidian, different day of experimentation) and calculate an average score and standard deviation.

Note: Long (4-10 kb) genomic regions are usually strongly active (staining is visible within 1 h of incubation at 37°C, with over 90% of the embryos stained) and do not display much variation between experiments.

3. Often, the goal of this assay is to locate essential *cis*-regulatory elements; thus, one needs to compare the activity of various constructs. Obviously, the plasmid backbone should be the same. We also favor comparing the results of constructs that have been electroporated during the same experiment.

Recipes

1. Banyuls-sur-mer Artificial Sea Water HEPES (BASWH)

BASWH is a ‘Mediterranean type’ sea water with the following features: salinity of 37.9 g/L, osmolality of 1219 mOsmole, Na⁺/K⁺ ratio of 47, and a pH of ~8.0.

For 1 L, mix:

30.34 g NaCl

0.83 g KCl

1.47 g CaCl₂·2H₂O

4.98 g MgCl₂·6H₂O

6.29 g MgSO₄·7H₂O in MilliQ water

Adjust to 1 L

At this step, the solution can be stored at room temperature for months.

Before use, add 0.18 g NaHCO₃ and 5 mL HEPES 1 M pH 8.0, and filtrate (0.2 µm). Can be stored for 1-2 months at 4°C.

2. *Phallusia* and *Ascidia* dechorionation solution

1% sodium thioglycolate

0.05% pronase in sea water

Mix thioglycolate in sea water by shaking, add pronase on top – no mixing – and keep at 4°C for a few hours before use.

Can be used for 1-2 weeks when kept at 4°C.

Bring aliquot to room temperature before use.

3. *Molgula* dechorionation solution

0.5% sodium thioglycolate

0.1% pronase in sea water

Mix thioglycolate in sea water by shaking, add pronase on top – no mixing – and keep at 4°C for a few hours before use.

Can be used for 1-2 weeks when kept at 4°C.

Bring aliquot to room temperature before use.

4. PBTw

PBS 1× containing 0.1% Tween-20

5. PBS 10×

For 1 L, add:

80.0 g NaCl

26.8 g Na₂HPO₄·7H₂O

2.0 g KCl

2.4 g KH₂PO₄

Adjust pH to 7.4 with NaOH

Bring to 1 L, and autoclave

6. X-gal staining solution

1 mM MgCl₂

3 mM K₄Fe(CN)₆

3 mM K₃Fe(CN)₆ in PBTw

7. X-gal stock solution

40 mg/mL X-gal in dimethylformamide

Acknowledgments

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Competing interests

There are no conflicts of interest or competing interests.

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Inter-species Transplantation of Blastocysts between Medaka and Zebrafish

Jana F. Fuhrmann^{1,§}, Jasmin Onitschenko^{1,2} and Lázaro Centanin^{1,*}

¹Laboratory of Clonal Analysis of Post-Embryonic Stem Cells, Centre for Organismal Studies (COS) Heidelberg, Heidelberg Universität, Heidelberg, Germany

²The Heidelberg Bioscience International Graduate School (HBIGS), University of Heidelberg, Heidelberg, Germany

§Current address: Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany

*For correspondence: lazaro.centanin@cos.uni-heidelberg.de

Abstract

Transplantation of blastocysts from a donor to a host blastula constitutes a powerful experimental tool to tackle major developmental biology questions. The technique is widely implemented in diverse biological models including teleost fish, where it is typically used for intra-species blastula transplantsations – *i.e.*, labeled blastocysts into a non-labeled host to follow lineages, or mutant blastocysts into a wild-type host to address autonomous vs. non-autonomous roles of a gene of interest. We have recently implemented a protocol to transplant blastocysts between zebrafish (*D. rerio*) and medaka (*O. latipes*), two species in which blastocysts show different developmental dynamics and sizes (Fuhrmann *et al.*, 2020). We present here a detailed protocol on how to overcome the early differences in chorion structure, blastula size, and speed of development to achieve trans-species blastocyst transplantation.

Keywords: Blastomere transplantation, Zebrafish, Medaka, Chimeras.

This protocol was validated in: Development (2020) DOI: 10.1242/dev.192500

Background

The *Chimera* is a mythological creature containing parts of different animals – the body of one species, the head of another species, the tail of yet another species, and so on. In genetics, the term is used to define an organism where cells have diverse genetic information – usually a different genetic origin. In the field of developmental biology, chimeras and genetic mosaics have long been used as powerful tools to differentiate cell intrinsic from cell extrinsic processes, as well as to establish lineages (Le Douarin, 1993; Le Douarin and Dupin, 1993; Buckingham and Meilhac, 2011; Kretzschmar and Watt, 2012). The generation of chimeras and mosaics can be done genetically or mechanically, depending on the animal model of choice. For teleost fish, their external development allows the early, mechanical mixing of blastomeres among siblings and/or between a wild type and a mutant embryo. The use of a tracer in donor cells is typically used to identify the *foreign* cells in the developing host. There are well-established protocols for blastomere transplantation in different species of teleost fish – examples of blastula transplantation in the context of a specific biological question and explanatory videos on the process can be found (Haas and Gilmour, 2006; Rembold *et al.*, 2006; Kemp *et al.*, 2009; Centanin *et al.*, 2011). Each of them takes into account the species-specific morphology of the early embryos and their developmental dynamics. Here, we modify existing protocols and establish a pipeline to generate zebrafish/medaka chimeras (Fuhrmann *et al.*, 2020).

Materials and Reagents

1. Petri dish, 100 mm diameter (Greiner Bio-One, catalog number: 627102)
2. 12/24 well plate (Corning, catalog numbers: 3512 [12-well]; 3527 [24-well])
3. Agarose coated dishes (homemade): 10 cm diameter, 6 cm diameter (Greiner Bio-One, catalog numbers: 627102 [10 cm diameter]; 628102 [6 cm diameter]) and 6-well plates (Roth, catalog number: ATCO.01). For the coating, use agarose 1% in ERM (medaka) or E3 (zebrafish). The base of the plastic plate should be covered by agarose; 1-2 mm thick is enough.
4. Cell saver tips (200 µl) (Biozyme, catalog number: 729051)
5. Borosilicate glass capillaries, 1.2 mm outer diameter × 0.94 mm internal diameter (Mind that these are different from the injection needles. They should NOT contain an internal filament. HARVARD Apparatus, model: 30-0016 GC120T-10 or GC120T-15)
6. Razor blade (to cut open the borosilicate needle) (Schreiber Instrumente, catalog number: 11-0240)
7. For Homemade (Air) transplantation device
 - a. Tube connector, Syringe Mouthpiece (Narishige, model: CI-1)
 - b. Teflon Tubing (Narishige, model: CT-1)
 - c. Handle Probe (WPI, catalog number: 2505)
 - d. Needle Holder (2 MM Press Port 1.2 MM, WPI catalog number: MPH412)
 - e. Epoxy Potting (Gray, WPI, catalog number: 4886)
 - f. 1 mL Syringe (BD Plastipak, catalog number: REF300013)
8. Glass Pasteur pipette (WU Mainz, catalog number: 200760)
9. Glass Pasteur pipette, opened tip
10. Glass plates (40 mm diameter; Roth, catalog number: T937.1)
11. Forceps (Dumont No. 5 or No. 55)
12. Long tips – to handle medaka embryos after dechorionation (Eppendorf, model: 5242956003, Microloader 20 µL in Racks)
13. Sandpaper (240/180)
14. Plastic Pasteur pipette (Biosigma, catalog number: 390512)
15. Sieve to wash zebrafish embryos
16. Net to collect medaka females (homemade)
17. Hook to collect medaka embryos (homemade, see details in Figure 1)
18. Glass flasks (100 mL, 500 mL)
19. 100 mL Beakers (Schott, catalog number: 211062402)

20. Low melting point agarose (Roth, catalog number: 6351.5)
21. Pronase (Roche, catalog number: 10165921001)
22. Hatching enzyme (HE) medaka (home-made, can be ordered at NBRP Medaka: <https://shigen.nig.ac.jp/medaka/strain/hatchingEnzyme.jsp;jsessionid=8F08DDE7593CDD7D1BFA32E6382E7E0F>)
23. Transplantation mold (we use homemade molds; see Figure 2. Similar commercial molds can be found at: <https://www.wpi-europe.com/products/pumps-and-microinjection/oocyte-injection/z-molds.aspx>)
24. Embryo rearing medium for medaka (ERM) (see Recipes)
25. Zebrafish medium (see Recipes)
26. 20× Tricaine (Sigma-Aldrich, catalog number: A5040-100G) (See Recipes)

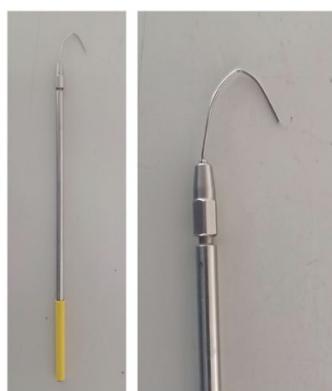


Figure 1. Hook to collect medaka embryos.

Inoculation loop holder (Roth, KL97.1), length 21 cm; wire 1.4301: length 2 cm, width 2.5 cm.

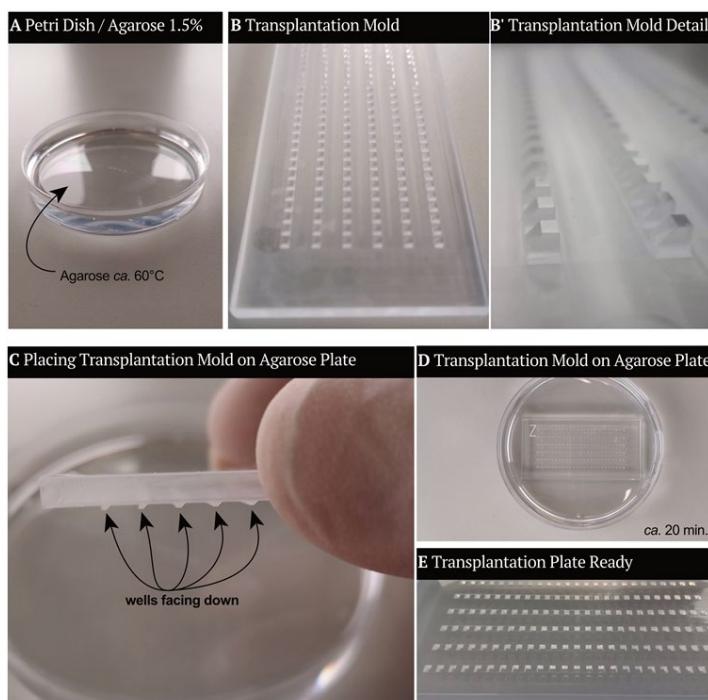


Figure 2. Transplantation molds.

To create the transplantation plate, molds are inserted top-down in 1.5% Agarose (see section B1). (A) Petri dish filled with 1.5% agarose in water, ca. 60°C. (B-B') Transplantation molds with a detailed view of the

structures that will form the wells. (C) Transplantation molds are placed in the Petri dish containing agarose, with the wells facing the agarose. (D) Transplantation mold stays in the Petri dish until the agarose has formed a stable gel. (E) When the transplantation mold is removed, the agarose gel contains wells in which the embryos will be placed. The transplantation plate is ready then.

Equipment

1. Zebrafish housing system (Tecniplast ZEBTEC equipped with 3.5 L tanks)
2. Zebrafish mating boxes (homemade 5 L boxes; see Figure 3. Alternatively, we have used breeding tanks from Tecniplast – 0.8 or 1.7 L, sloped tank)

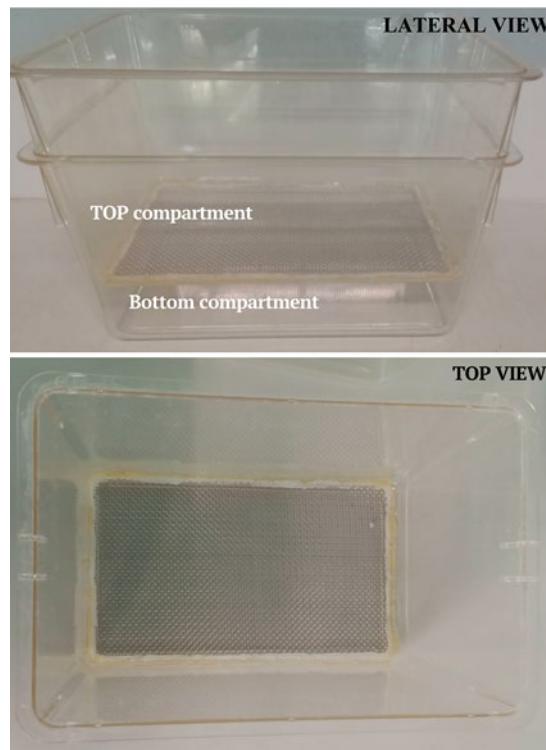


Figure 3. Zebrafish mating box.

Lateral view (top) and top view (bottom) of the zebrafish mating box. This setup accommodates up to five couples.

3. Medaka housing system (Tecniplast ZEBTEC equipped with 3.5 L tanks and Müller & Pflege, 4 L tanks)
4. Incubator, 23°C (no need of CO₂ control) (Binder, model: 9020-0339)
5. Incubator, 28°C (no need of CO₂ control) (Rumed, model: 200330160)
6. Incubator, 32°C (no need of CO₂ control) (Binder, model: 9010-0081)
7. Micropipette puller (we use a horizontal puller, Sutter Instrument CO, model: P-97)
To pull Harvard Apparatus GC120T – 10 capillaries, we use the following program: Heat 505; Pull 25; Vel 250; Time 10)
8. Transplantation device (a homemade air system; see Figure 4. We have also successfully used the Eppendorf CellTram^R Air/Oil system)
9. Stereoscope with light source (Olympus, model: SZX7)
10. Micromanipulator (Eppendorf, model: InjectMan NI 2)
11. Pipette (Gilson 20-200 µL, 200-1,000 µL)

12. Fluorescence stereomicroscope (Nikon, model: SMZ18, solar light engine from Lumencor)
13. Microwave

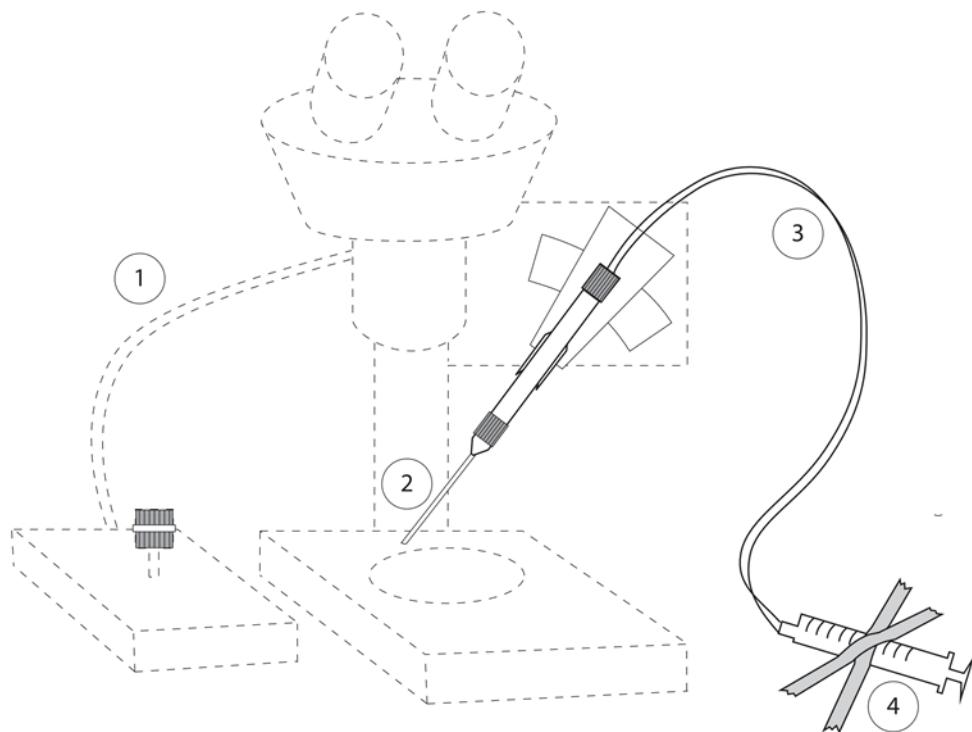


Figure 4. Set up for Transplantations.

Main components: (1) Micromanipulator and Stereoscope with light source; (2) transplantation needle inserted into needle holder; (3) tube attached to the needle and a syringe (airtight); (4) syringe (volume 1 mL) attached to desk.

Procedure

Please consider Figure 5 for the order of steps taken to complete the protocol and the respective temperatures settings used.

A. Set up fish to produce embryos

Medaka

1. Medaka crosses

Medaka are kept in their respective breeding group to allow for early breeding on the next day. There is no need to split females and males the day before. Collection of embryos can be done every day for each female, which lays approximately 20 eggs each. Embryos are attached to the mother for some hours after fertilization. Beware that you need to use more medaka females than zebrafish females.

2. 9:00-9:30 am – Embryo collection

a. On the morning of the experiment (~9:00 am), identify a female harboring embryos and gently catch it with a fishnet (Figure 6).

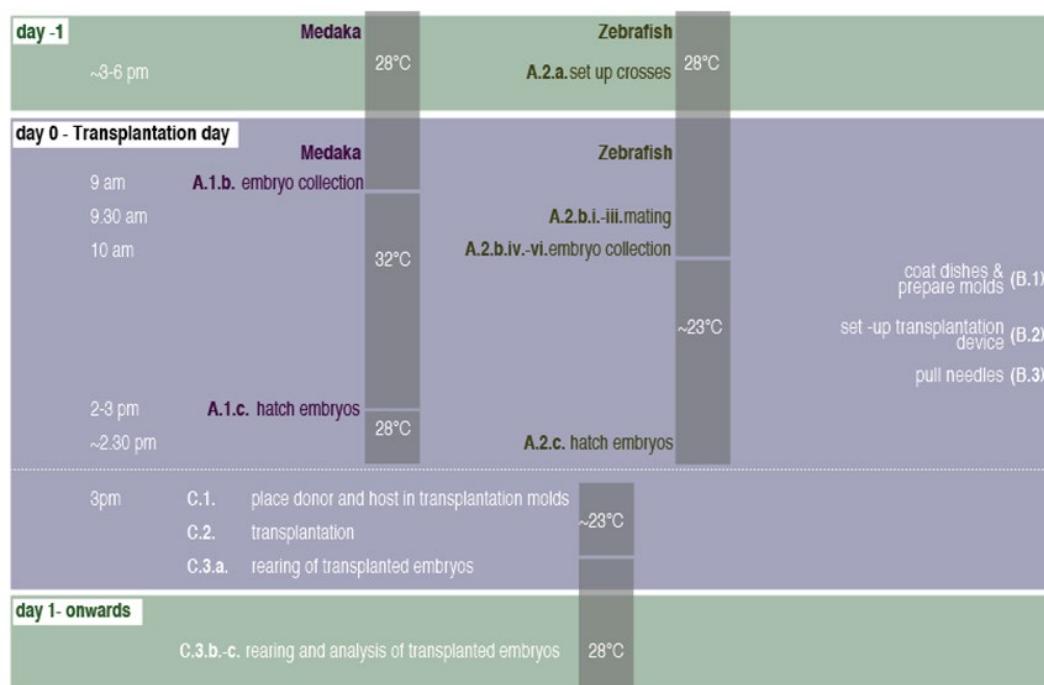


Figure 5. Timeline for inter-species transplantsations

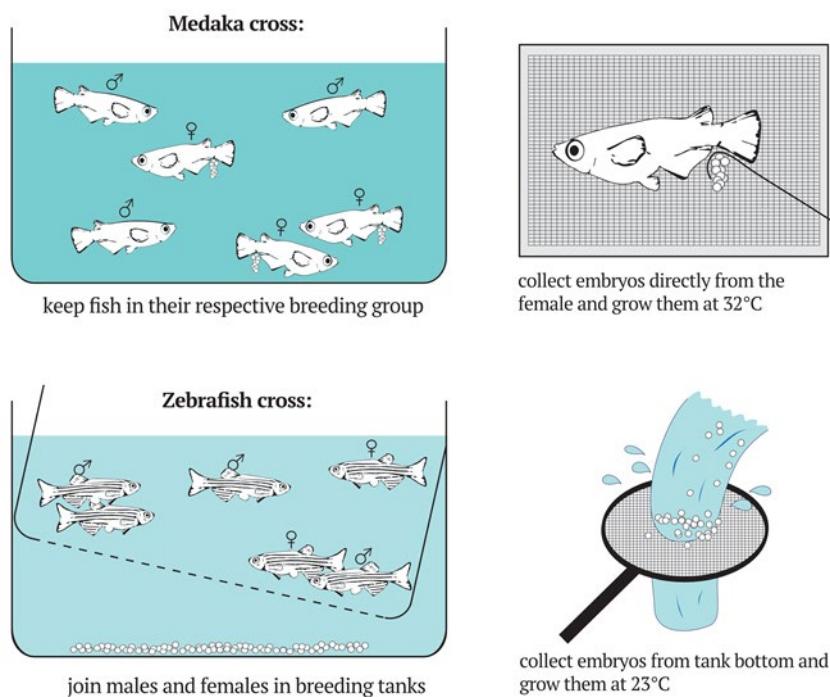


Figure 6. Setup for medaka and zebrafish crosses and embryos collection

- Collect the embryos using a thin metal hook (Figure 1), brushing them gently from the female's belly. Place the female back in the tank.
- Transfer the embryos from the net or from the hook into a 10 cm Petri dish filled with ERM.
- Place medaka embryos in ERM at 32°C. This helps embryos to develop faster to catch up with

- zebrafish blastulae (Furutani-Seiki and Wittbrodt, 2004).
3. 2:00-3:00 pm – Hatch medaka embryos
 - a. Hatching of medaka embryos is best performed at the 256-512 cell stage (~2 pm). Younger embryos are more fragile; the mortality rate increases if hatched earlier.
 - b. Take medaka embryos out of the fish medium and use your fingers to roll them gently on sandpaper (grain 240) to remove hairs from the chorion.

Note: The goal is to create holes in the external part of the chorion to favor the hatching enzyme degrading the chorion from the inside. We use the removal of hairs as a proxy for efficient creation of holes.

 - i. Check embryos under the stereomicroscope to monitor hair removal. Repeat the rolling step until all hairs are gone.
 - ii. Discard dead embryos before moving forward.
 - c. Rinse the embryos in ERM medium
 - d. Transfer the embryos to a glass tube using a Pasteur pipette and remove all liquid.
 - i. Use a plastic Pasteur pipette from which the tip has been cut out
 - ii. Do not put more than 20 embryos per tube.
 - e. Cover embryos with hatching enzyme (HE) and incubate at 28°C for 30 to 60 min maximum.
 - i. Use the minimal possible amount of HE, just covering the embryos.
 - ii. Always cover the tube with parafilm to avoid evaporation of HE.
 - iii. Incubation time depends on: a) the quality of the HE, especially when using homemade HE, and b) the number of embryos in the tube.

Notes:

- a. *Increasing the HE-volume to embryo ratio leads to faster hatching.*
- b. *if hair removal was inefficient, hatching takes more time or can even fail entirely.*
- iv. Check the progress of the HE reaction under the stereomicroscope every 10-15 min.
- f. When embryos have holes in the chorion, wash them carefully four times with ERM.
 - i. Rinse embryos by letting the ERM flow down slowly on one side of the tube.
 - ii. Be aware that the embryos are extremely fragile when dechorionated.
 - iii. It is important to use ERM at 28°C to avoid a heat shock.
- g. Transfer the embryos to an agarose-coated Petri dish – follow steps in Figure 7.
 - i. Fill the glass tube entirely with ERM.
 - ii. Place a finger on top, sealing the tube completely - avoid air bubbles.
 - iii. Invert slowly and place the top of the tube underneath the surface of the medium in the agarose-coated dish.
 - iv. Release finger and let the embryos sink.

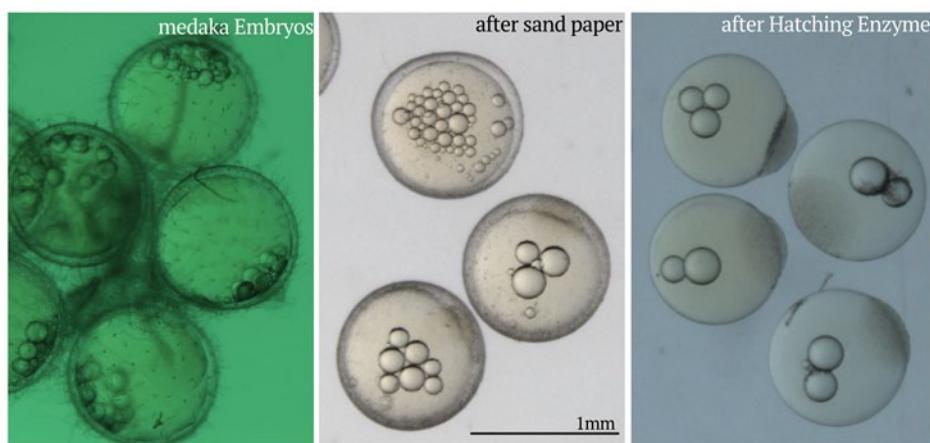
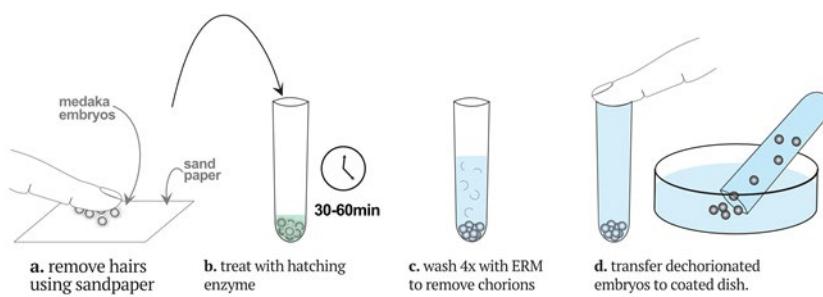


Figure 7. Steps to dechorionate medaka embryos

Zebrafish

1. 3:00-6:00 pm (day -1) Set up zebrafish crosses (Westerfield, 2007)

On the afternoon before the transplantation experiment, split females and males using a large breeding tank.

- a. Fill a breeding tank with 2:1 fishwater:deionized water.
b. The breeding tank consists of 2 compartments separated by a mesh. Place the males in the bottom compartment and the females in the upper compartment.
c. One cross consists of 5 males and females; depending on your experiment, you might need to set up more than one cross.
2. 9:30-10:30 (day 0, transplantation day): Embryo collection
a. On the morning of the experiment (9.30 am – 10:00 am), transfer the males into the upper compartment – they are now together with the females.
b. Tilt the mesh separator to create a slope with a shallower and a deeper region (Figure 6).

Note: Zebrafish usually breed in shallow waters; you should observe couples mating in the upper extremity.

- c. Leave crosses undisturbed for 20-30 min.

Note: You should observe embryos accumulated at the bottom of the tank afterwards.

- d. Transfer fish into their tank and remove the mesh bottom separator.
e. Collect eggs by running the breeding tank water through a fine sieve.
f. Transfer embryos in a 10 cm diameter Petri dish and rinse with zebrafish medium
g. Keep embryos at room temperature (~23°C).

3. 2:30-3:30 pm – Hatching of zebrafish at early blastulae stage, 258 to 512 cell stage (Westerfield, 2007)
a. Transfer zebrafish blastulae to 100 mL glass beakers.

- b. Remove the zebrafish medium and add 30 mL of fresh zebrafish medium.
- c. Add 1 mL Pronase (30 mg/mL).
Note: Thaw Pronase and keep it on ice before using it.
- d. Move the beaker in circles every 30 s, checking Pronase activity under the stereoscope (Figure 8).
 - i. Embryos without the chorion are the first to accumulate at the center.
 - ii. Time ranges from 3 to 7 min and depends on the quality of the Pronase and the number of embryos per beaker. Pre-aliquot Pronase to avoid multiple thawing and freezing cycles, which impair its quality and extend the hatching time.
 - iii. When you see a few embryos without the chorion, it is time to stop the reaction. The chorion of the other embryos will be gone during the washes.

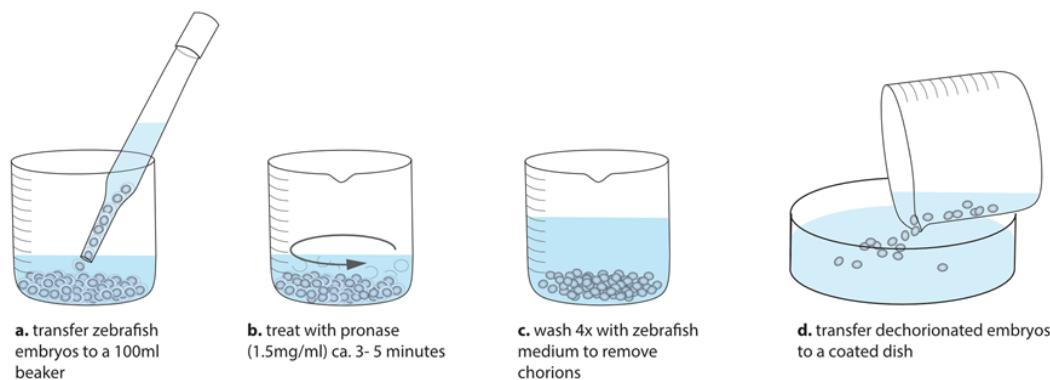


Figure 8. Steps to dechorionated zebrafish embryos

- e. Discard the Pronase solution and wash zebrafish medium at least four times.
Note: Each wash will remove a considerable number of chorions
- f. Transfer freshly hatched embryos into a coated Petri dish and keep them in zebrafish medium until transplantation.

B. 2:30-4:00 pm Preparing the transplantation setup

1. Agarose coated dishes and transplantation molds
Coated dishes
 - a. Prepare a 1.5% solution of agarose in ddH₂O and boil it in a microwave until dissolved. Agarose solution can be prepared fresh or stored pre-made:
 - i. Use 1 L Bottles and prepare 800 mL of agarose solution.
 - ii. Keep agarose solution at 65°C until used.
 - b. If using plastic dishes, coat them with 1.5% agarose.
 - i. Glass dishes do not need to be coated.
 - ii. To coat: pour the Agarose solution into a 10 cm Petri Dish until the bottom is just covered (*ca.* 20 mL).
 - iii. Prepare at least one 10 cm Petri Dish for each genotype to keep dechorionated embryos before transplantation.
 - iv. Prepare a 10 cm Petri Dish or 6-/12-/24-well plates for the transplanted blastulae.
 - v. Which of the above is used depends on the experimental design. If donors need to be kept for later screening (*i.e.*, to check genetic Background), smaller well plates are useful to keep donors and hosts individually, allowing for the identification of the most relevant chimeras.
 - vi. When using medaka, few embryos per shared volume is preferential as death of neighboring embryos affects survival rates. Keep one blastula per well in a 24-well or 12-well plate, or approximately three blastulae per well in a 6-well plate.
 - vii. When using zebrafish (and if donor/host combinations are not tracked), 10 cm Petri dishes

give a good survival rate.

Transplantation plates

a. Pour the Agarose solution into a 10 cm diameter Petri dish until the bottom is covered.

b. Place the transplantation mold (Figure 2) on top of the agarose.

i. Wells should face down.

ii. Avoid bubbles.

iii. The transplantation mold should not be covered by agarose.

c. Once the agarose is firm, the mold can be removed using forceps.

Prepared plates can be stored in the fridge for one day but need to be equilibrated at room temperature before usage.

2. Prepare transplantation needles

a. Pull glass capillary with a horizontal needle puller.

Transplantation needles should be long and thin (see Figure 9).

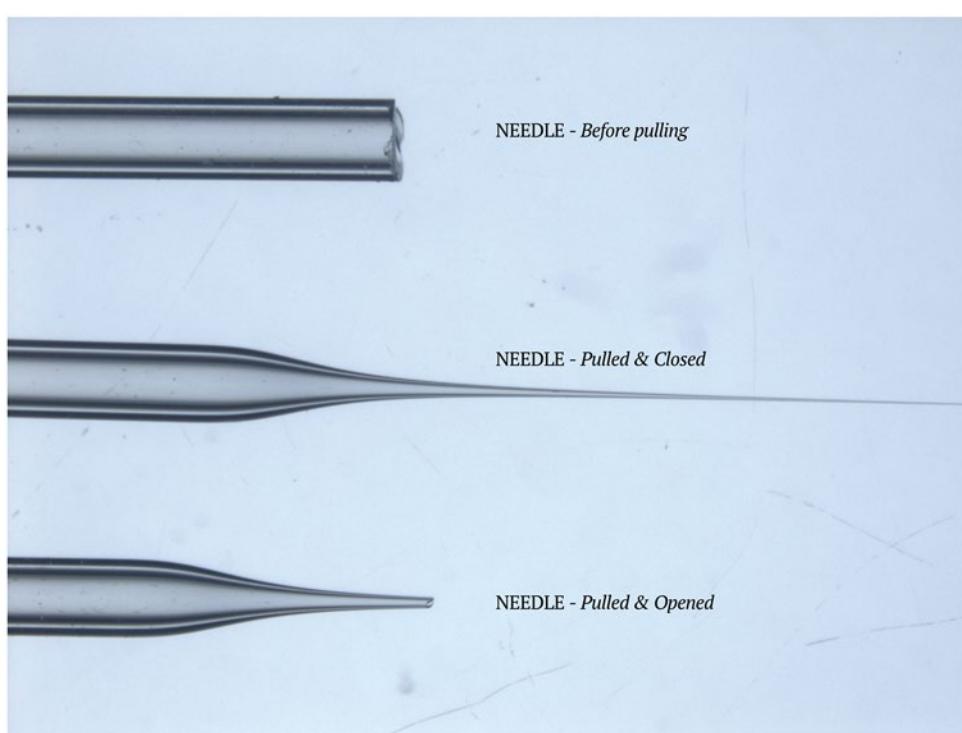


Figure 9. Transplantation needle.

Images showing the needle before pulling as from the manufacturer (Materials #5), after pulling with the needle puller (settings described in Equipment #7), and after being opened with a razor blade (B2a).

b. Open needle with a razor blade

i. The diameter of the needle should be larger than a cell diameter. Since blastula cells have different diameters according to their developmental stage, you will need to adjust the opening of the needle to the stage you are using and the number of cells to be transplanted.

ii. A needle with small diameter ($1\text{-}1.5 \times$ cell width) takes up cells individually and leads to higher dispersal of cells in the needle, which gives a better control of the number of transplanted cells.

iii. A needle with larger diameter allows for the transplantation of more cells with less liquid, preserving the integrity of the donor cells.

iv. The edge of the needle should not be too jagged. However, a slight tilt helps to push through the donor and host blastulae.

- v. A good needle can be reused for multiple transplants. To store it, be sure to clean it properly by taking ERM/zebrafish medium many times after the last transplantation.
3. Setting up the micromanipulation device
 - a. Use a stereomicroscope with a ~0.7-7:1 magnification and an integrated light source connected to a 3-axis motorized manipulator with a micromanipulation controller and a needle holder (see set up in Figure 4).
 - b. Attach a tube to the needle holder and a syringe (volume 1 mL) and seal airtight.
 - c. Attach the syringe to the desk using tape.

Note: This will allow you to one-handedly control the syringe and thereby the cells entering and exiting the transplantation needle.

- d. Place transplantation needle in the needle holder.
- e. Place a plate with fish medium in the field of view and submerge the tip of the needle in the water using the micromanipulator.
- f. Move the syringe plunger and check if there is medium entering and exiting the needle.
Note: If the liquid is responding directly and predictably to the movement of the plunger; then you are ready to go.

C. 3:30 pm onwards – Transplantation

Check that the embryos of both species are at blastula stage (~3 pm) (Kimmel *et al.*, 1995; Iwamatsu *et al.*, 2004). Move plates to the microinjection setup to avoid subsequent water movement.

1. Place donor and host blastulae in alternating columns (Figure 10).
 - a. Fill in the transplantation plate with medium for the HOST species.
 - b. Transfer zebrafish embryos to individual wells using a glass Pasteur pipette.
 - i. Dechorionated zebrafish blastulae have a diameter slightly smaller than that of the glass Pasteur pipette.
 - ii. Take up a few blastulae from the plate with the pipette and release them individually in the transplantation plate, placing them along a column, and filling one embryo per well.
 - iii. Donors should be placed in the left-most column; hosts should be placed in the second to fifth column. Since cells from one donor can be transplanted to many hosts, it is convenient to arrange multiple putative hosts per donor.
 - c. Accommodate the embryos with the cells facing upwards. Embryos usually fall into the molds with the yolk facing down, and no further re-positioning of the blastulae is necessary. However, if necessary, take the blastula up with the pipette and retry, or use Microloader™ tips as described below.
 - d. Transfer medaka blastulae individually and place each next to a mold using cell saver tips. Clip the end of a plastic Microloader™ tip to 2-3 cm. Use the tip to gently push the blastulae into the molds with the animal pole (blastula cells) facing upwards. Only touch the cells at the animal pole and not the yolk cell with the plastic tip.

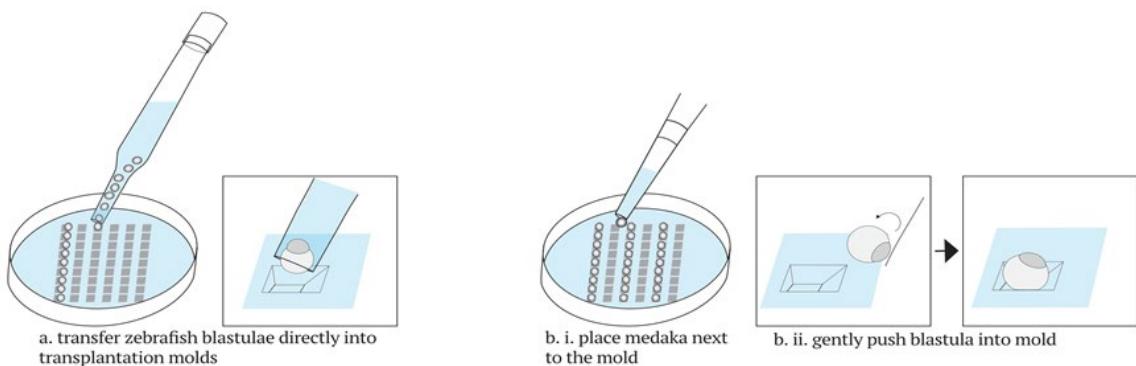


Figure 10. Disposition and orientation of embryos in the transplantation plate.

Zebrafish embryos (a) can be handled with a glass Pasteur pipet and accommodated directly into the agarose well. Medaka embryos (b) are bigger and need to be transferred with a plastic Pasteur pipet into the dish and then handled into the wells with a Microloader™ tip.

2. Transplantation of blastocysts (Figure 11)

a. Calibrate the transplantation needle.

- i. Take up a small amount of medium in the transplantation needle by applying a gentle negative pressure through the syringe. Use this to adjust yourself to the response of the water flow inside the needle to the applied pressure.
- ii. The surface of the medium inside the thin part of the needle should be visible through the stereomicroscope before attempting to take up cells from the donor, thereby giving direct feedback of the applied flows.

b. Place the tip of the needle touching the host cells.

- i. Blastula cells can be taken from any position, but caution must be taken for cells close to the yolk cell to avoid sucking up yolk.
- ii. In our setup, we use cells closest to the animal pole of the embryo; however, chimeras are also observed when taking up cells in the peripheral regions.

c. Apply negative pressure to take cells up

- i. Depending on the setup of the transplantation experiment, you can take one or up to a hundred cells from a donor.
- ii. When the desired number of cells is reached, apply a gentle opposing force to the syringe to stop the flow.

d. Remove the needle from the donor blastula and move it to the host blastula.

e. Place the needle in the desired location of the host and release cells by carefully applying positive pressure.

The position of the cells in the host affects which organs the donor cells will contribute to. In general terms, cells in central positions will be incorporated in anterior structures like the forebrain and retina.

f. Move the needle to the following host and repeat the procedure until finishing the donor cells.

One blastula can serve as donor for multiple hosts, and the number of cells transplanted can be adjusted to the experimental conditions. Transplanted embryos with up to ~150 donor cells can survive; yet, we usually transplant up to 50 cells.

g. Clean your needle before going to the next donor blastula.

- i. Allow medium to enter and exit the needle to remove the remaining cells.
- ii. Avoid air bubbles in the transplantation dish; this can damage the blastulae.

h. Repeat the procedure for as many donor blastulae as needed.

After transplants are completed, remove all dead and non-transplanted blastulae from the transplantation plate.

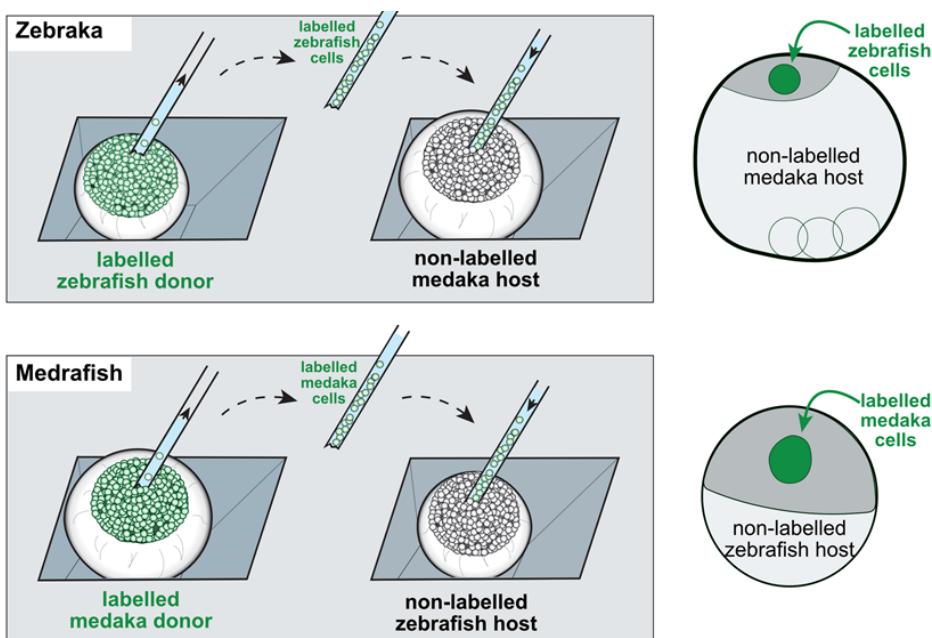


Figure 11. Transplantation of blastomeres between medaka and zebrafish

3. Rearing of transplanted embryos
 - a. Keep transplanted embryos in the transplantation plate at 28°C overnight.
 - b. For Medrafish (zebrafish hosts)
 - i. Transfer surviving embryos to a fresh Petri dish on the next day and exchange the zebrafish medium again in the afternoon of the same day.
 - ii. Replace medium daily and remove dead embryos. We keep embryos up to day 5 of development.
 - c. For Zebraka (medaka hosts)
 - i. Place surviving embryos individually using cell saver tips in 1.5 mL ERM in a coated 24 well plate (Agarose 1.5%). Alternatively, embryos can be kept in glass dishes (2 cm diameter) or differently sized multi-well dishes.
 - ii. Exchange $\frac{1}{2}$ the medium daily, avoiding mechanical perturbation during the first 3 days. We keep embryos up to day 9 of development.

Notes

A. Fish maintenance and mating

1. Both medaka and zebrafish fish are maintained on a 14 h day – 10 h night cycle at 28°C.
2. Medaka breeding group:
 - a. Each breeding group consists of a single male and two to three females.
 - b. Fish are maintained in the same tank; courting behavior starts with the first light (8:00 am in our fish room).
 - c. Embryos are collected from each female daily.
3. Zebrafish breeding group:
 - a. Each breeding group consists of five males and five females.
 - b. Fish are not fed in the morning of the cross.
 - c. Use breeding groups maximum once per week.

B. Temperature

1. Medaka embryos tolerate a high temperature range; in our hands, a temperature of 32°C before transplantation (from 2 to 512 cell stage) worked best to speed up development without long-term consequences. Higher temperatures cause mold and increase evaporation but should still be possible in a respective setup.
2. Zebrafish tolerate only a narrow temperature range, but embryos develop normally at room temperature (~23°C), which delays development sufficiently to achieve synchronization with medaka at the blastula stage.

C. Handling of dechorionated embryos

1. Hatched embryos are more fragile than intact embryos. Special care must be taken before the completion of gastrulation as the yolk does not sustain contact with plastic.
2. Medaka embryos are more fragile than zebrafish embryos due to their larger yolk cell. Mechanical perturbations, such as those induced by water movement, are sufficient to cause ruptures in the yolk and result in the death of the respective embryos. Especially in the first two days after transplantation, the embryos should be perturbed as little as possible.
3. Dechorionated embryos appear to be more sensitive to the chemical nature of their surroundings. This means that extra care must be taken when handling the fish medium, and dead embryos should be removed from the dish.
4. If only a few zebrafish embryos are available, dechorionate them with fine forceps (Dupont 55) under a stereomicroscope (Skip step A2c). If done correctly, manual dechoronation is gentler than chemical treatments and improves the survival rate of embryos, but is more time consuming. You cannot hatch medaka embryos with forceps as the chorion is very stiff and the attempt will damage your embryo. However, it is possible to remove a partially opened chorion after an incomplete hatch with HE using forceps.

D. Imaging

1. Every transfer to a microscope causes mechanical strain and thus reduces the survival rate and should be done carefully.
2. Mount embryos for live imaging as follows:
 - a. For general observation and imaging in a stereomicroscope, transfer to 3% Methylcellulose (in fish medium).
 - b. For confocal live imaging, mount in a 0.6% solution of low melting agarose (should be <40°C before mounting). This is preferential over Methylcellulose as Methylcellulose creates fluorescent Background signals.
3. Once embryos start moving, anesthetize dechorionated live embryos in 1× (1 mg/mL) tricaine in the respective medium (Neiffer *et al.*, 2009; Lischik *et al.*, 2019).
4. In all stages of development, we check embryos using a fluorescent stereomicroscope if a fluorescent marker is available in the donor. This allows to:
 - a. Select for successfully transplanted embryos.
 - b. Report the location of most donor cells.
 - c. Follow the outgrowth dynamics of larger structures, such as neurons.
5. Use confocal (live) imaging, for example, to:
 - a. Spot smaller clones of donor cells.
 - b. Identifying cellular and sub-cellular behaviors.
 - c. Visualize donor-host interactions.
6. Dechorionated zebrafish embryos survive live imaging early on; medaka embryos are more sensitive and survive better at older stages.

Recipes

1. Embryo rearing medium for medaka (10× ERM)

170 mM NaCl
4 mM KCl
2.7 mM CaCl₂·H₂O
6.6 mM MgSO₄·7H₂O
170 mM HEPES
Fill up to 10 L with deionized water.

2. Zebrafish medium

3 g red sea salt in 100 mL deionized water

3. 20× Tricaine

2 g Ethyl 3-aminobenzoate methanesulfonate
5 g Na₂HPO₄·2H₂O in 500 ml deionized water
Adjust pH 7.0-7.5

Acknowledgments

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Competing interests

The authors declare no conflict of interest.

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Fixation and Immunostaining of Endogenous Proteins or Post-translational Modifications in *Caenorhabditis elegans*

Robert O'Hagan^{1, #, *} and Irini Topalidou^{2, #}

¹Biology Department, Montclair State University , Montclair, USA

²Department of Biochemistry, University of Washington, Seattle, Washington, USA

*For correspondence: ohaganr@montclair.edu

#Contributed equally to this work

Abstract

Although the advent of genetically-encoded fluorescent markers, such as the green fluorescent protein (GFP; Chalfie *et al.*, 1994), has enabled convenient visualization of gene expression *in vivo*, this method is generally not effective for detecting post-translational modifications because they are not translated from DNA sequences. Genetically-encoded, fluorescently-tagged transgene products can also be misleading for observing expression patterns because transgenes may lack endogenous regulatory DNA elements needed for precise regulation of expression that could result in over or under expression. Fluorescently-tagged proteins created by CRISPR genome editing are less prone to defective expression patterns because the loci retain endogenous DNA elements that regulate their transcription (Nance and Frøkjær-Jensen, 2019). However, even CRISPR alleles encoding heritable fluorescently-tagged protein markers can result in defects in function or localization of the gene product if the fluorescent tag obstructs or otherwise interferes with important protein interaction domains or affects the protein structure.

Indirect immunofluorescence is a method for detecting endogenous gene expression or post-translational modifications without the need for transgenesis or genome editing. Here, we present a reliable protocol in which *C. elegans* nematodes are fixed, preserved, and permeabilized for staining with a primary antibody to bind proteins or post-translational modifications, which are then labeled with a secondary antibody conjugated to a fluorescent dye. Use of this method may be limited by the availability of (or ability to generate) a primary antibody that binds the epitope of interest in fixed animals. Thousands of animals are simultaneously subjected to a series of chemical treatments and washes in a single centrifuge tube, allowing large numbers of identically-treated stained animals to be examined. We have successfully used this protocol (O'Hagan *et al.*, 2011 and 2017; Power *et al.*, 2020) to preserve and detect post-translational modifications of tubulin in *C. elegans* ciliated sensory neurons and to detect non-modified endogenous protein (Topalidou and Chalfie, 2011).

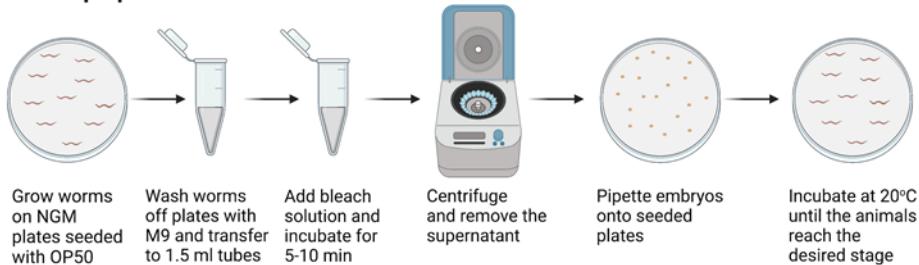
Keywords: Fixation, Immunofluorescence, Antibody, Staining, Tubulin post-translational modifications

This protocol was validated in: PLoS Genet (2020), DOI: 10.1371/journal.pgen.1009052

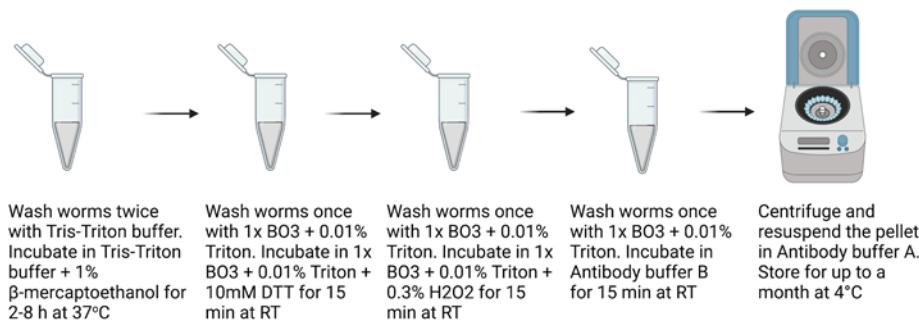
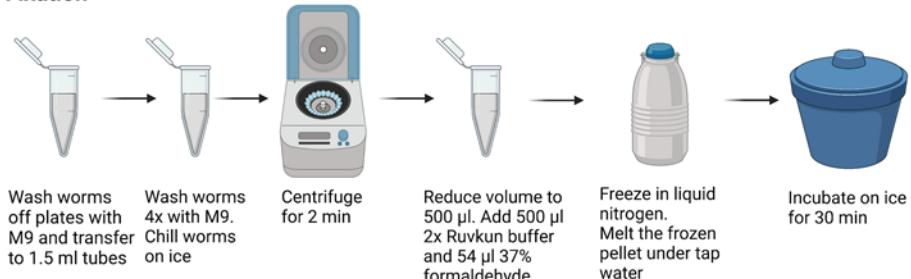
Background

The ability to detect the presence and localization of endogenous biological molecules or epitopes in organisms is essential to understand their function. Previous methods of fixation and permeabilization of *C. elegans* for immunofluorescent detection of molecules include both freeze cracking and tube fixations (Finney and Ruvkun, 1990; Miller and Shakes, 1995; Duerr *et al.*, 2006). Here, we present a robust method (see overview in Figure 1), modified from the one described by Finney and Ruvkun (1990).

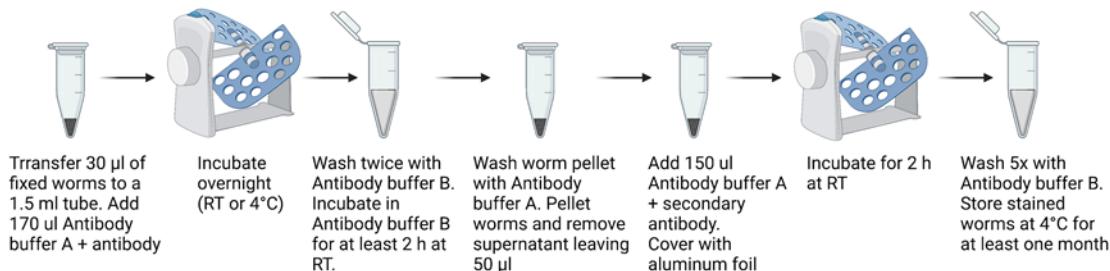
Animal preparation



Fixation



Primary Antibody Binding



Secondary Antibody Binding

Figure 1. Overview of procedures used for animal preparation, fixation, and primary and secondary antibody binding. Figure 1 was created with Biorender.com.

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Materials and Reagents

1. *C. elegans* strains of interest (<https://cgc.umn.edu/>)
2. OP50 *E. coli* strain (<https://cgc.umn.edu/>)
3. NGM plates seeded with OP50
4. PBS/phosphate Buffered Saline, 1× Powder, pH 7.4 (Fisher Scientific, catalog number: BP661-10)
5. Tris Base (Sigma-Aldrich, catalog number: 648311)
6. EDTA (Sigma-Aldrich, catalog number: E9884)
7. EGTA (Sigma-Aldrich, Calbiochem, catalog number: 324626)
8. Spermidine trihydrochloride (Sigma-Aldrich, catalog number: S2501)
9. PIPES (Sigma-Aldrich, catalog number: P1851)
10. BME/β-mercaptoethanol (Sigma-Aldrich, catalog number: M6250)
11. DTT/dithiothreitol (Fisher Scientific, catalog number: FERR0861). Store at 4°C, or in aliquots at -20°C
12. H₂O₂/Hydrogen Peroxide 30% with stabilizer (Sigma-Aldrich, catalog number: H1009)
13. H₃BO₃/Boric Acid Powder 99.5% (Fisher Scientific, catalog number: 18-609-176)
14. Formaldehyde Solution, 37% (Sigma-Aldrich, catalog number: 252549). Store at 4°C
15. Triton X-100 non-ionic detergent (Sigma-Aldrich, catalog number: X100)
16. BSA (Sigma-Aldrich, catalog number: A2153). Store at 4°C, or in aliquots at -20°C
17. NaN₃/Sodium Azide (Sigma-Aldrich, catalog number: S2002)
18. GT335 (AdipoGen Life Sciences, catalog number: AG-20B-0020-C100). Store at -20°C
19. Alexa-fluor 568-conjugated donkey anti-mouse secondary antibody (Fisher Scientific, Invitrogen, catalog number: A10037). Store in the dark at 4°C
20. Sodium hypochlorite 5.65-6% solution (Fisher Scientific, catalog number: SS290-4) or Clorox bleach (~7.5% sodium hypochlorite)
21. Potassium hydroxide (KOH) (Fisher Scientific, catalog number: P251-500)
22. Agarose (Fisher Scientific, catalog number: 16-520-050)
23. 2× Ruvkun Buffer (see Recipes)
24. Tris-Triton buffer (see Recipes)
25. 20× BO₃ buffer (see Recipes)
26. 1× BO₃ + 0.01% Triton buffer (see Recipes)
27. Antibody buffer A (see Recipes)
28. Antibody buffer B (see Recipes)
29. M9 solution (see Recipes)
30. 5 M KOH (see Recipes)
31. Tris-Triton buffer +1% β-mercaptoethanol (see Recipes)
32. 1 M DTT solution (see Recipes)
33. 1× BO₃ + 0.01% Triton buffer + 10 mM DTT (see Recipes)
34. 1× BO₃ + 0.01% Triton buffer + 0.3% H₂O₂ (see Recipes)
35. Liquid 2% agarose (see Recipes)

Equipment

Note: Equipment similar to the items described below is required.

1. Platform Rocker (Fisher Scientific, Thermo Scientific Vari-Mix, catalog number: 09-047-113Q)
2. Microcentrifuge (Fisher Scientific, Thermo Scientific Sorvall Legend 21, catalog number: 75-77-2488)
3. Compound epifluorescence or confocal microscope with 63× (NA 1.4) or 100× (NA 1.4) oil-immersion objectives
4. Digital microscope camera (Examples of microscope cameras we have used successfully for this protocol include:

- Retiga-SRV Fast 1394 digital camera
- Photometrics Cascade 512B CCD camera
- Hamamatsu C11440-42U ORCA-Flash4.0 LT Digital CMOS camera
- Photometrics CoolSNAP HQ2-FW camera
- 5. Microscope slides (Fisher Scientific, catalog number: 22-265446)
- 6. Carl Zeiss High Performance Coverslips (Fisher Scientific, catalog number: 10474379)
- 7. Glass Pasteur pipettes (Fisher Scientific, catalog number: 13-678-20A)
- 8. Eyelash glued to a toothpick

Software

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

Procedure

A. Optional: Synchronization of animals for fixation

To compare results across control and mutant strains, it is important that animals are age-matched. However, this step may be omitted if the purpose of the experiment is to detect an epitope across all stages of the development of *C. elegans* nematodes.

1. Grow *C. elegans* using standard methods on a 6 cm NGM plate seeded with OP50 *E. coli* (see wormbook.org for standard growth conditions and instructions for M9 solution, NGM plates, and culture of OP50).
2. When many gravid hermaphrodites are present, wash worms off the plate(s) by pipetting 0.5 mL M9 solution onto the plate, tilting the plate to wash hermaphrodites off, and pipetting worm suspension into a 1.5 mL centrifuge tube. Add 20 µL of 5 M KOH and 100 µL of 5.65-7.5% sodium hypochlorite (bleach; we use Clorox. Optionally, a 5.65-6% sodium hypochlorite solution works just as well). Incubate for about 5-10 min, occasionally inverting tube to keep worms suspended. This step dissolves the adults but spares the embryos, which are surrounded by a tough eggshell.
3. Every few minutes, check for disappearance of adults, either by viewing the tube under a dissecting microscope or holding it up to the light and viewing by eye. When few adults are visible in the suspension, spin tube(s) at ~900 × g (~3,000 rpm) in a benchtop centrifuge for ~20 s to pellet embryos (eggs) released by hypochlorite digestion of adults.
4. Optional: Remove most of the supernatant by pipetting, but leave ~100 µL so that the pellet is undisturbed. Then add 900 µL M9 solution and resuspend to wash, eliminating most of the sodium hypochlorite. Sodium hypochlorite could induce oxidative stress that could alter expression of some genes. Spin tube(s) at ~900 × g (~3,000 rpm) in a benchtop centrifuge for ~20 s to pellet embryos (eggs).
5. Remove most of the supernatant by pipetting but leave ~100 µL.
6. Pipette up and down gently to resuspend the pellet of embryos in the 100 µL. (If few worms survive the sodium hypochlorite treatment, consider including one or two washes (Step A4) with M9 solution to remove the sodium hypochlorite).
7. Pipette drops of the embryo suspension onto fresh NGM plates with lawns of OP50. Place the drops outside the lawn of OP50, and tilt the plates to spread the drop out so that the liquid (which still includes sodium hypochlorite) will more rapidly soak into the NGM plate and/or evaporate. (If embryos continue to soak in the hypochlorite mix too long, they will become non-viable). Try adding approximately 100-200 embryos per plate to each of five plates. If you don't have enough embryos, scale up the number of starting plates that you synchronize by bleaching.
8. Incubate at 20°C for approximately three days to reach the first day of adulthood. Temperature or time can be adjusted depending on differences in aging rate and reproduction of your worm strain to provide a

loosely synchronized population of young adults or your desired larval stage.

Note: This method isolates only embryos that are contained in the uterus of adult hermaphrodites. Embryonic development is occurring inside the adult hermaphrodite uterus, such that embryos could be several hours apart in terms of development. Therefore, embryos will be only loosely developmentally synchronized using this method. For tighter synchronization, other methods must be used.

B. Fixation

1. Collect worms from 3-5 non-starved, non-contaminated plates by washing them off with M9 and transfer them to 1.5 mL centrifuge tubes. Many worms from several plates are needed because some worms are lost at each subsequent step. Because worms stick to the inside of plastic pipette tips, using sterile glass Pasteur pipettes to transfer worms in this step can help prevent the loss of worms.
2. Wash four times over ~60 min by resuspending in 1 mL M9 so that the bacteria in the gut are excreted. Between washes, pellet worms by centrifuging at $\sim 900 \times g$ (~3,000 rpm) for 2 min before carefully withdrawing most of the supernatant using a pipette. Be sure to leave the pellet of worms untouched.
3. Chill worms on ice and centrifuge at $\sim 900 \times g$ (~3,000 rpm) for 2 min. Remove supernatant to reduce volume to 500 μL , then add 500 μL ice-cold 2 \times Ruvkun buffer to a final concentration of 1 \times . Add 54 μL 37% formaldehyde (final concentration ~2%). Fixation by formaldehyde may negatively affect some epitopes, and its concentration (within a range of ~1-4%) may need to be adjusted by trial and error. Mix by inverting the tubes or pipetting.
4. Freeze rapidly in liquid nitrogen or dry ice/ethanol. This step may help crack or permeabilize the tough worm cuticle. Frozen samples can be stored at -80°C.
5. Melt the frozen worm pellet under tap water.
6. Incubate on ice, inverting the tube occasionally, for 30 min.
7. Wash worms twice in Tris-Triton buffer by centrifuging at $\sim 900 \times g$ (~3,000 rpm) for 2 min, carefully withdrawing supernatant, and resuspending in 1,000 μL Tris-Triton buffer. Triton detergent helps prevent worms from sticking to the pipettes, tubes, and one another.
8. Pellet worms by centrifuging at $\sim 400 \times g$ (~2,000 rpm) for 2 min. After this step, the animals should be considered fragile. From this point on, when pelleting worms, centrifuge at no more than $\sim 400 \times g$ (~2,000 rpm). Remove supernatant, carefully avoiding pellet, and resuspend the worms in 500 μL Tris-Triton buffer +1% β -mercaptoethanol. Incubate for 2-8 h with gentle agitation on a rocker platform in a 37°C incubator. β -mercaptoethanol can reduce disulfide bonds in proteins, which may improve permeability of the cuticle. The incubation with β -mercaptoethanol at high temperature may also help to denature enzymes like DNases, proteases, and peroxidases that could damage epitopes.
9. Wash the worms once by pelleting in the centrifuge, carefully removing supernatant, and resuspending with 1,000 μL 1 \times BO₃ + 0.01% Triton buffer. The BO₃ buffer has a basic pH that benefits the redox reactions in the subsequent steps.
10. Pellet worms, remove supernatant leaving pellet undisturbed, and resuspend the worms in 500 μL of 1 \times BO₃ + 0.01% Triton buffer + 10 mM DTT. Incubate for 15 min with gentle agitation on the rocker platform at room temperature. DTT also may improve cuticle permeability by further reducing disulfide bonds.
11. Wash the worms once by pelleting in the centrifuge, carefully removing supernatant, and resuspending with 1,000 μL of 1 \times BO₃ + 0.01% Triton buffer.
12. Pellet worms, remove supernatant leaving the pellet undisturbed, and resuspend the worms in 500 μL of 1 \times BO₃ + 0.01% Triton buffer + 0.3% H₂O₂. Incubate for 15 min with gentle agitation at room temperature. H₂O₂ may oxidize sulfhydryl groups to prevent disulfide bonds from reforming.
13. Wash the worms once by pelleting in the centrifuge, carefully removing supernatant, and resuspending with 1,000 μL of 1 \times BO₃ + 0.01% Triton buffer.
14. Pellet by centrifugation, remove supernatant, and resuspend with 1,000 μL Antibody buffer B. Allow to wash for at least 15 min with gentle agitation at room temperature to remove any residual BO₃ buffer or H₂O₂. Pellet by spinning at $\sim 400 \times g$ (~2,000 rpm) for 2 min and resuspend in ~100-300 μL of Antibody buffer A, depending on how many worms are present. Worms are now fixed and can be safely stored for

up to a month at 4°C in Antibody buffer A.

C. Primary Antibody Binding

1. Resuspend fixed worms by gently inverting the tube, then transfer 30 µL of fixed worms to a fresh 1.5 mL tube using a pipette tip that has been cut to enlarge the opening (this may prevent damage to the fragile fixed worms). Visually confirm that many worms are present and, if necessary, adjust volume. There should be hundreds of worms.
2. Add 170 µL Antibody buffer A with the appropriate amount of the desired primary antibody. For example, we often use GT335 (a monoclonal antibody that binds the branch point glutamate of post-translationally polyglutamylated proteins, such as tubulins) at a dilution of 1:450. If we had three *C. elegans* strains to compare, we would dilute 1.33 µL GT335 into 510 µL Antibody buffer A, and then add 170 µL of the dilution to the 30 µL worm suspension for the final dilution of 1:450 in a final volume of 200 µL. Incubate overnight, either at room temperature or at 4 °C in a cold room, depending on the antibody documentation. (Incubation time can be reduced, but a minimum of 2 h is recommended to allow diffusion through cuticle and tissues.) Dilution and incubation temperature may need to be determined empirically. Especially when trying a new antibody, it is also worthwhile to include a control in which no primary antibody is added. Use a rocker to provide gentle agitation.
3. Wash the worms twice with Antibody buffer B by pelleting in the centrifuge, carefully removing all but the last ~50-100 µL of supernatant, and resuspending in 500 µL Antibody buffer B. Then pellet worms, remove supernatant, and resuspend worms for a third time in at least 500 µL Antibody buffer B and incubate for at least 2 h to overnight with gentle rocking at room temperature to eliminate all unbound primary antibody.
4. Wash the worms once by pelleting in the centrifuge, carefully removing all but the last ~50-100 µL of supernatant, and resuspending in 500 µL Antibody buffer A. Pellet worms and remove supernatant leaving approximately the last 50 µL.

D. Secondary Antibody Binding

1. Add 150 µL of the secondary antibody diluted in Antibody buffer A for a final volume of 200 µL. We typically use Alexa-fluor 568-conjugated donkey anti-mouse secondary antibody at a dilution of 1:2,000 or 1:2,500 in the final volume of 200 µL of worm suspension. Enclose the tubes in a cardboard freezer box or cover with aluminum foil to protect from light, and incubate at room temperature for 2 h on the rocker.
2. Wash worms five times over several hours by pelleting in the centrifuge, carefully removing all but the last ~50-100 µL of supernatant, resuspending in 500 µL Antibody buffer B, and gently rocking to remove unbound secondary antibody. If excessive non-specific secondary antibody staining is observed, continue washing worms with Antibody buffer B for several more hours and/or leave on rocker in Antibody buffer B overnight. Fixed stained worms can be stored for a month or more at 4°C.
3. Make an agarose pad by adding a drop of liquid 2% agarose to a slide and immediately placing another slide on top to create a flat thin layer. Remove the top slide and mount worms for imaging by using a pipette tip that has been cut to slightly enlarge the opening to transfer a drop of 3-5 µL onto the agarose pad. Position gently using an eyelash glued to a toothpick and apply a cover slip before imaging. Use the eyelash to manually clear away any debris or precipitates that might remain from the fixation or staining process. If few stained worms are transferred to the slide, allow worms to settle to the bottom of the tube and either reduce the volume in the tube or draw worms up directly from the pellet rather than resuspending, before attempting to mount worms again.

Data analysis

Because Alexa-fluor 568 fluoresces red, we typically acquire two-color images to capture red immunofluorescence

in transgenic animals expressing green fluorescent protein markers. GFP fluorescence appears dimmer after the fixation and staining treatment than in live animals but can often be used to provide visual landmarks to interpret the tissues, cells, or subcellular structures in which red immunofluorescence is detected. We acquire epifluorescence or confocal data by optical sectioning using a monochrome CCD or CMOS camera and observe color channels individually or in combination in z-projections created using ImageJ Software.

Results are generally consistent across trials, but some variation is to be expected in the intensity of staining of worms (Figure 2). To confidently report on the tissues, cells, or subcellular structures in which immunofluorescence is detected, it is important to see consistent staining in multiple animals. We typically first examine many animals microscopically and capture images of at least 10 animals for quantitative analysis (presence or absence of staining, area/region of structures stained, or fluorescence intensity of staining). We have used image analysis of animals subjected to this antibody staining method to detect differences in glutamylation across genotypes (O'Hagan *et al.*, 2011), and others have detected differences based on environmental regulation (Kimura *et al.*, 2018).

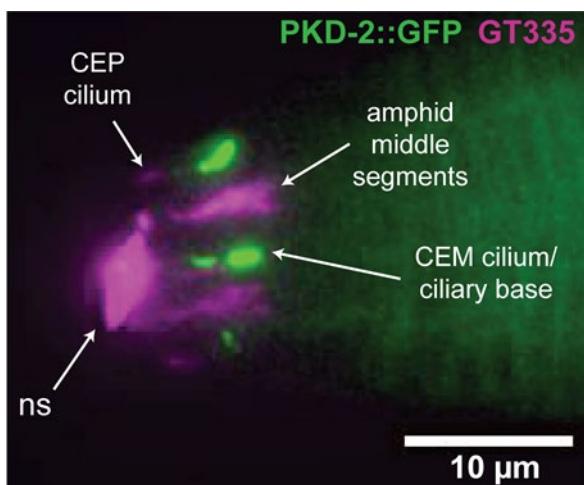


Figure 2. Representative image of staining with GT335 primary antibody and Alexa-fluor 568-conjugated secondary antibody.

GT335 detects post-translational glutamylation by labeling the branch point glutamate (Wolf *et al.*, 1992). In this picture of a *C. elegans* adult male nose, indirect immunofluorescence (magenta) of the GT335 monoclonal primary antibody, labeled with Alexa-fluor 568-conjugated secondary antibody, labels the ciliary middle segments of the amphid channel neurons, as well as CEP (cephalic) cilia. PKD-2::GFP (green) labels cilia of the male-specific CEM (cephalic male) sensory neurons in the nose. This epifluorescence image was captured using a Photometrics Cascade 512B CCD and Zeiss AxioPlan2 microscope with 100× (NA 1.4) oil-immersion objective. ns indicates non-specific staining – a clump of debris and fluorescent secondary antibody was stuck to the buccal opening. Inconsistent or variable staining of a structure sometimes indicates that it may be non-specific. The buccal cavity is a common location to observe non-specific staining. Depending on the secondary antibody, non-specific staining is sometimes observed on annuli or other cuticular structures, and may be seen in controls in which the primary antibody is omitted. If available, use genetic mutants that lack the epitope or protein of interest to validate the antibodies used in the experiment.

Recipes

1. 2× Ruvkun Buffer

- 160 mM KCl
- 40 mM NaCl
- 20 mM EGTA
- 10 mM spermidine-HCl

30 mM PIPES buffer, pH 7.4

50% methanol

Store at 4°C

PIPES buffer pH 7.4 is typically made as a 1 M stock by adding 302.37 g of PIPES powder to 600 mL ddH₂O and bringing to pH 7.4 by adding approximately 153 mL of 10 N NaOH before bringing to volume by adding ddH₂O. PIPES will not readily dissolve until pH is above 6.5.

2. Tris-Triton buffer

100 mM Tris-HCl, pH 7.4

1% Triton X-100

1 mM EDTA

Tris-HCl pH 7.4 solution is typically made as a 1 M stock by diluting Tris base in ddH₂O and bringing the pH to 7.4 by slowly adding HCl.

3. 20× BO₃ buffer

1 M H₃BO₃

0.5 M NaOH

Dilute to 1× with ddH₂O

4. 1× BO₃ + 0.01% Triton buffer

Dilute Tris-Triton buffer 1:100 in 1× BO₃ buffer

5. Antibody buffer A

1× PBS

1% BSA

0.5% Triton X-100

0.05% sodium azide

1 mM EDTA

Store at 4°C

6. Antibody Buffer B

1× PBS

0.1% BSA

0.5% Triton X-100

0.05% sodium azide

1 mM EDTA (The same as Antibody buffer A, but with only 0.1% BSA)

Store at 4°C

Note: Antibody dilutions should be made freshly from refrigerated or frozen aliquots to minimize freeze-thaw cycles.

7. M9 Buffer

3 g KH₂PO₄

6 g Na₂HPO₄

5 g NaCl

1 mL 1 M MgSO₄

ddH₂O to 1 L

Sterilize by autoclaving

8. 5 M KOH

Add 28.05 g KOH pellets to approximately 75 mL ddH₂O.

Allow to dissolve, with occasional shaking. Dissolving KOH generates heat, so do not cap tightly. Then bring to 100 mL with ddH₂O.

9. Tris-Triton buffer +1% β-mercaptoethanol

Working in a fume hood, add 100 μL β-mercaptoethanol to a 15 mL conical tube.

Add Tris-Triton buffer to a final volume of 10 mL.

10. 1 M DTT solution

Working in a fume hood, dissolve 1.55 g of DTT powder in 10 mL ddH₂O.

Distribute into 1 mL aliquots and store at -2°C.

11. 1× BO₃ + 0.01% Triton buffer + 10 mM DTT

Working in a fume hood, add 100 μL 1 M DTT solution to a 15 mL conical tube.

Add 1× BO₃ + 0.01% Triton buffer to a final volume of 10 mL.

12. 1× BO₃ + 0.01% Triton buffer + 0.3% H₂O₂

Working in a fume hood, add 100 μL Hydrogen Peroxide 30% solution to a 15 mL conical tube.

Add 1× BO₃ + 0.01% Triton buffer to a final volume of 10 mL.

13. Liquid 2% agarose

Add 2 g agarose to 100 mL ddH₂O.

Heat in microwave until agarose is fully dissolved.

Use while still liquid.

Acknowledgments

This work was funded by the New Jersey Commission for Spinal Cord Research (NJCSCR) CSCR15IRG014. This method was used in our recent research publication, Power *et al.* (2020), as well as in previous publications (Topalidou and Chalfie, 2011; O'Hagan *et al.*, 2011 and 2017). This method is minimally modified from the Finney and Ruvkun method (Finney and Ruvkun, 1990)

Competing interests

The authors declare no competing interests.

Ethics

Use of *C. elegans* does not require institutional approval.

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Structural and Functional Mapping of Mesenchymal Bodies

Sébastien Sart^{1, 2}, Raphaël F.-X.Tomasi^{1, 2}, Antoine Barizien^{1, 2}, Gabriel Amselem¹, Ana Cumano^{3, 4} and Charles N. Baroud^{1, 2, *}

¹LadHyX and Department of Mechanics, Ecole Polytechnique CNRS - UMR 7646, Palaiseau, France

²Physical Microfluidics and Bio-Engineering, Department of Genomes and Genetics, Institut Pasteur, Paris, France

³Unit for Lymphopoiesis, Department of Immunology – INSERM U1223, Institut Pasteur, Paris, France

⁴Université Paris Diderot, Sorbonne Paris Cité, Cellule Pasteur, 75018, Paris, France

*For correspondence: charles.baroud@pasteur.fr

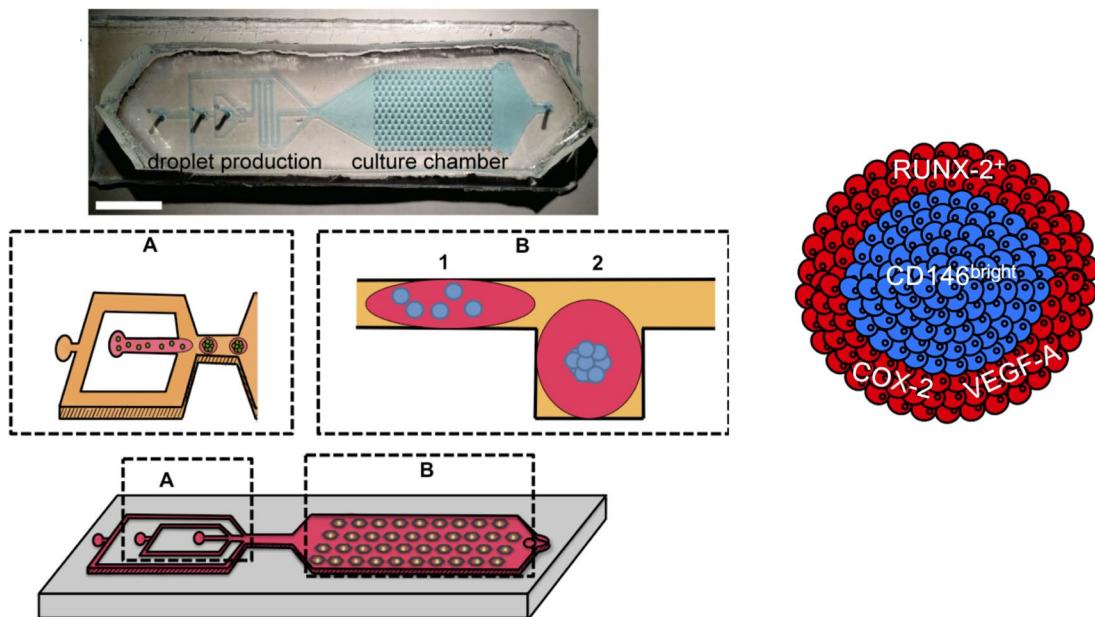
Abstract

The formation of spheroids with mesenchymal stem/stromal cells (MSCs), mesenchymal bodies (MBs), is usually performed using bioreactors or conventional well plates. While these methods promote the formation of a large number of spheroids, they provide limited control over their structure or over the regulation of their environment. It has therefore been hard to elucidate the mechanisms orchestrating the structural organization and the induction of the trophic functions of MBs until now. We have recently demonstrated an integrated droplet-based microfluidic platform for the high-density formation and culture of MBs, as well as for the quantitative characterization of the structural and functional organization of cells within them. The protocol starts with a suspension of a few hundred MSCs encapsulated within microfluidic droplets held in capillary traps. After droplet immobilization, MSCs start clustering and form densely packed spherical aggregates that display a tight size distribution. Quantitative imaging is used to provide a robust demonstration that human MSCs self-organize in a hierarchical manner, by taking advantage of the good fit between the microfluidic chip and conventional microscopy techniques. Moreover, the structural organization within the MBs is found to correlate with the induction of osteo-endocrine functions (*i.e.*, COX-2 and VEGF-A expression). Therefore, the present platform provides a unique method to link the structural organization in MBs to their functional properties.

Keywords: Mesenchymal stromal cells, Mesenchymal bodies, Microfluidics, Droplets, Spheroids, Quantitative imaging

This protocol was validated in: Sci Adv (2020), DOI: 10.1126/sciadv.aaw7853

Graphical Abstract:



Droplet microfluidic platform for integrated formation, culture, and characterization of mesenchymal bodies (MBs).

The device is equipped with a droplet production area (flow focusing) and a culture chamber that enables the culture of 270 MBs in parallel. A layer-by-layer analysis revealed a hierarchical developmental organization within MBs.

Background

Mesenchymal stem/stromal cells (MSCs) comprise a heterogeneous population of mesenchymal progenitors that are capable of differentiation into osteoblastic, chondrogenic, and adipogenic lineages (Dominici *et al.*, 2006). MSCs also bear important trophic functions that regulate immune cell activities, promote angiogenesis, reduce tissue inflammation, and activate tissue-resident progenitors, making this cell type particularly suited for many tissue engineering/regeneration applications (Caplan and Correa, 2011). The formation of spheroids with MSCs (*i.e.*, mesenchymal bodies, MBs) was recently found to enhance their differentiation potential and their secretory activities (Sart *et al.*, 2014). However, it remains poorly understood how heterogeneous hMSCs self-organize in 3D, as well as the mechanisms linking their structural organization to their functional activities (Cesarz and Tamama, 2016).

Several methods have been used for the formation of MBs, including bioreactors and conventional well plates (Sart *et al.*, 2014 and 2016; Sart and Agathos, 2018). However, while these methods enable the formation of a large number of MBs, they provide limited control over single aggregate stimulation or characterization (Sart *et al.*, 2016; Sart and Agathos, 2018). Because MSCs constitute a heterogeneous population, the analysis of single aggregates at single-cell resolution is required to understand the mechanisms by which 3D cultivation induces functional changes on the behavior of a minority of very responsive cells or a global population shift. The macro-scale culture vessels and global population analysis therefore do not allow the MB structure and the cellular functions to be related (Sart *et al.*, 2017; Sart and Agathos, 2018).

Here, we use a microfluidic platform that allows high density and controlled-sized MB formation within nanoliter drops. The microfluidic format enables a robust quantitative demonstration, using quantitative imaging at a single-cell level, that human MSCs self-organize in a hierarchical manner: The most undifferentiated MSCs are located in the core, while partially committed cells are located at the boundaries of the MBs. Moreover, we found that such

structural organization correlated with the induction of osteo-endocrine functions. The microfluidic method and protocols developed here can find applications to characterize other types of organoids, to link cell sorting processes to phenotypic commitment, in view of understanding the mechanisms leading to tissue patterning in 3D stem cell cultures. The current method can be applied to any kind of stem cells cultivated in 3D, although the volume of droplets should be adapted to a specific type of stem cells. The data-driven approach developed in this protocol allows us to obtain a robust quantitative characterization of the structural and functional organization within organoids, which would be difficult to obtain using conventional population-based approaches that require the alteration of the cellular microenvironment.

Materials and Reagents

A. Cell culture reagents

1. T-175 cm² flasks (Greiner, Cellstar, catalog number: 660175)
2. Human mesenchymal stromal cells derived from the Wharton's jelly of the umbilical cord (hMSCs), purchased from American Type Culture Collection (ATCC) (ref #PCS-500-010)
3. α -modified Eagle's medium (α -MEM) (Life Technologies, catalog number: 32561-029)
4. TrypLE™ Express (Life Technologies, catalog number: 12604013)
5. Phosphate buffer saline (PBS) (Sigma-Aldrich, catalog number: D8662)
6. Fetal bovine serum (FBS) (Life Technologies, catalog number: 10500-064)
7. Penicillin-streptomycin (pen-strep) (Life Technologies, catalog number: 10378-016)
8. Triton X-100 (Sigma-Aldrich, catalog number: X100)
9. Ultra-low-melting agarose (Sigma-Aldrich, catalog number: A5030)
10. Paraformaldehyde (PFA), 16% (Alpha Aesar, catalog number: 43368)
11. Mouse anti-human CD146-Alexa Fluor 647 (clone P1-H12) (BD Biosciences, catalog number: 563619)
12. Rabbit anti-COX-2 polyclonal antibody (Abcam, catalog number: ab15191)
13. Rabbit anti-human VEGF-A monoclonal antibody (Abcam, catalog number: ab52917)
14. Mouse anti-human RUNX-2 monoclonal antibody (Abcam, catalog number: ab76956)
15. Alexa Fluor 488-conjugated goat anti-mouse IgG2a secondary antibody (Life Technologies, catalog number: A-21131)
16. Alexa Fluor 594-conjugated goat polyclonal anti-rabbit IgG secondary antibody (Life Technologies, catalog number: A-11012)
17. DAPI (Sigma-Aldrich, catalog number: 10236276001)
18. Vybrant™ multicolor cell labeling kit (Life Technologies, catalog number: V22889)
19. hMSC culture medium (see Recipes)
20. Staining buffer (see Recipes)
21. CD146 staining solution (see Recipes)
22. Fixative solution (see Recipes)
23. Agarose solution (see Recipes)
24. Permeabilization buffer (see Recipes)
25. Blocking buffer (see Recipes)
26. Primary antibodies solution (see Recipes)
27. Secondary antibodies solution (see Recipes)

B. Materials for microfabrication and microfluidics

1. Brass plates (5 × 5 cm)
2. Dry-film photoresists: Eternal Laminar E8020, Eternal Laminar E8013 (Eternal Materials), and Alpho NIT215 (Nichigo-Morton)
3. K₂CO₃ (Sigma-Aldrich, catalog number: P5833)

4. Poly(dimethylsiloxane) (PDMS) (Dow Corning, catalog number: SYLGARD 184)
5. 3MTM NovecTM 1720 Electronic Grade Coating (3M)
6. 3MTM FluorinertTM Electronic Liquid FC-40 (3M)
7. PEG-di-Krytox (RAN Biotechnologies)

Equipment

A. Equipment for cell culture

1. CO₂ incubator (Binder, CB 170)
2. Cell sorter (flow cytometer) (BD Biosciences, FACS Aria III)

B. Equipment for microfabrication and microfluidics

1. Office laminator (PEAK, pro PS320)
2. Ultraviolet lamp (Hamamatsu, Lightningcure)
3. Micromilling machine (Minitech Machinery, CNC Mini-Mill/GX)
4. Plasma cleaner (Harric, PDC-32G)
5. 100 µL and 1 mL glass syringes (SGE, Analytical Science, Gas tight luer lock syringes)
6. 1 mL plastic syringe (Terumo, SS+01T1)
7. Syringe pumps (neMESYS Low-Pressure Syringe Pump, Cetoni GmbH)

C. Microscopy

1. Motorized wide-field microscope (Ti, Eclipse, Nikon), equipped with a CMOS (complementary metal-oxide semiconductor) camera (ORCA-Flash4.0, Hamamatsu), a fluorescence light-emitting diode source (Spectra X, Lumencor), and a 10× objective with a 4-mm working distance (extra-long working distance) and a 0.45 numerical aperture (NA) (Plan Apo λ, Nikon)
2. Motorized (Ti2, Nikon) confocal spinning disc microscope equipped with lasers (W1, Yokogawa) and the same camera and objective as above

Software

1. MATLAB (r2016a, MathWorks, Natick, MA)

Procedure

A. Microfabrication of the microfluidic chips

1. A detailed protocol on the fabrication of the chips can be found in Amselem *et al.* (2018). The chips consist of two parts: 1) The top part comprises a flow focusing junction, a serpentine, diverging rails, and a culture chamber. The molds are fabricated using dry resins that are etched to produce the shape of the droplet generators and guiding channels by standard soft lithography. 2) The bottom of the chips consists of an array of 270 hexagonal capillary traps. The molds of the bottoms part of the chips are fabricated by micromilling the brass plates. The geometry and dimensions of the features can be found in Sart *et al.* (2020) (**Figure 1**).

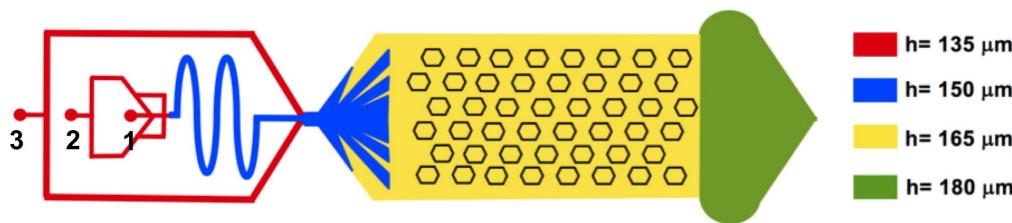


Figure 1. Chip design and depth of the different regions.

1. Inlet for the injection of the aqueous phase containing cells and culture medium; 2. Inlet for oil injection at the junction; 3. Inlet for oil injection to push the drops within the trapping chamber.

2. Fabricate the tops and the floors of the chips by casting poly(dimethylsiloxane) (PDMS) (a mix of 90% of base and 10% of curing agents) into the molds.
3. Place the two molds in an oven set up at 80°C for 2 h to promote the polymerization of the PDMS.
4. Extract the two parts of the chips from the molds and cut them with a scalpel.
5. Assemble the two parts of the chips by bonding the two surfaces for 40 s using a plasma cleaner.
6. Flush the chips with Novec and heat them at 110°C for three cycles to render the inner walls of the chip fluorophilic.

B. Cell culture, sorting, and loading in microfluidic droplets

1. Cultivate hMSCs using hMSC culture medium, from passage 2 to 7 in regular T-175 cm² flasks, and place them into a culture incubator, set up at 37°C and 5% CO₂.
2. Seed the cells into the flask at a density of 5 × 10³ cells/cm², subcultivate them every week using TrypLE, and change the medium every 2 days.
Optionally, sort hMSCs based on their level of expression of CD146 by flow cytometry. CD146 is a marker of undifferentiated status, whose level of expression decreases upon differentiation (Sacchetti *et al.*, 2007). To separate the CD146^{dim} and CD146^{bright} hMSCs, isolate the cells (passage 5) from the flasks, and incubate them with CD146 staining solution for 30 min. Wash the cells with straining buffer. Identify and select the hMSC population by plotting FSC-A and SSC-A signals using the flow cytometer. Eliminate cell doublets by gating the main population obtained by plotting FSC-H versus FSC-A signals. From this selected population, plot the FITC-A (or any fluorochrome for which the cells are not labeled) versus Alexa-647-A signal distribution; analyze the spread of expression of CD146 with the cytometer and sort 25% of the brightest and 25% of the dimmest cells.
3. Connect two 1 mL syringes containing a 1% RAN in FC-40 solution to inlets #3 and #2, block inlet #1, then flush the oil of each syringe at 50 μL/min to remove the air from the chip (Figure 2, Step 1).
4. Load a suspension of 6 × 10⁶ bulk- or a mix of 50:50 CD146^{dim} and CD146^{bright} sorted- hMSCs/mL in culture medium supplemented with the agarose solution into a 100 μL glass syringe.
5. Connect this syringe into inlet #1 (Figure 2, Step 2).
6. Apply a flow rate of 8-8.5 μL/min to syringe 1 and a flow rate of 11 μL/min to syringe 2. The solution containing the cells is pinched at the junction. This yields to the formation of monodispersed droplets containing a suspension of about 380 cells each (Figure 2, Step 2).
7. Apply a flow rate of 50 μL/min on syringe 3: the droplets are pushed on the rails that guide them evenly within the culture chamber. The drops are then captured within the capillary traps (Figure 2, Step 2).
8. Stop the flow of syringe #1 and apply a flow rate of 100 μL/min to syringes #2 and #3 to remove the excess of non-anchored droplets in the culture chamber (Figure 2, step 3).
9. Stop the oil flows; the cells settle down at the bottom of the drops and start clustering.

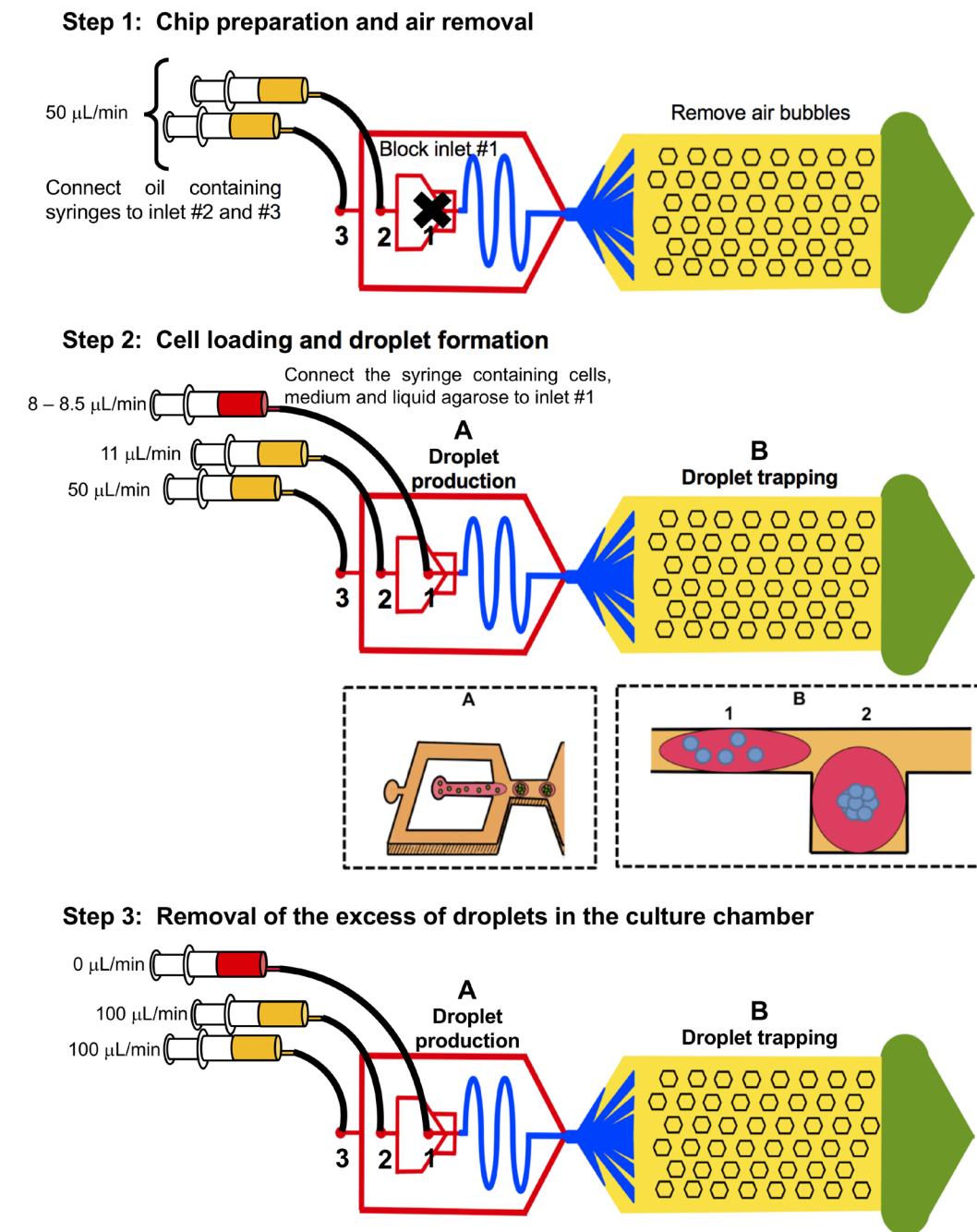


Figure 2. Protocol for cell loading and droplet formation

C. Spheroid formation and culture in microfluidic droplets

1. Place the chips into the CO₂ incubator overnight to let the cells form spheroids (Figure 3 and Video 1).

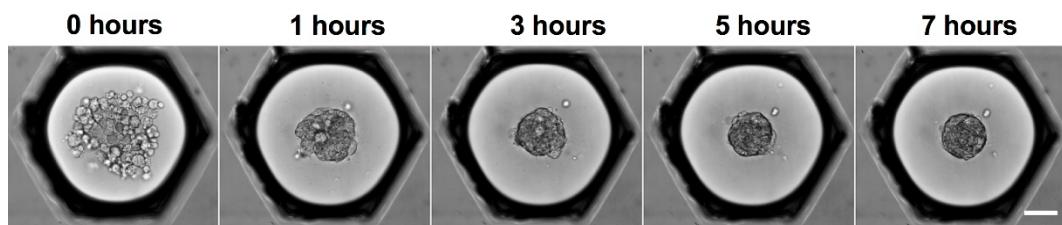
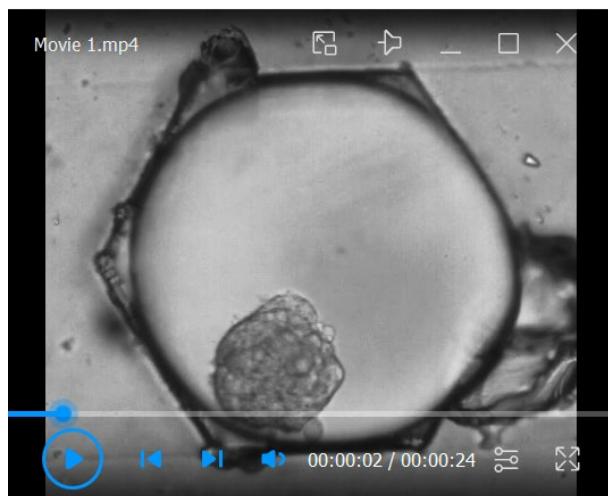


Figure 3. Kinetics of MB formation in microfluidic droplets.

After stopping the oil flow, the cells are allowed to settle down to the bottom of the drops, where they start clustering. Cells are monitored continuously by live imaging, while forming MBs. Scale bar = 100 μm .



Video 1. Morphology of MB in 3D.

The MBs in liquid drops are subjected to recirculation by applying an oil flow around them. The protocol allows the MBs to rotate on their axis. The movie demonstrates the 3D structural organization of MBs.

2. On the second day, place the chips at 4°C for 30 min to gel the agarose.
3. Flush the chips with 1 mL of pure FC-40 oil, at 80 $\mu\text{L}/\text{min}$, to dilute the surfactant.
4. Replace the oil phase with culture medium by slowly flowing the aqueous solution into the device. At this stage, the 3D aggregates are mechanically retained into the traps by the hydrogel. They can be regularly perfused with culture medium; thus, they are now ready for long-term culture while remaining fully viable (Sart *et al.*, 2020).

D. Spheroid labeling and imaging within microfluidic droplets

To interrogate the structural organization within MBs:

1. Label CD146^{dim}- and CD146^{bright}- hMSCs with Vybrant DiO (green) or Vybrant DiD (red) dyes (5 μL for 1 mL culture medium) for 30 min, prior to loading them into the microfluidic drops.

Note: CD146 protein and the Vybrant dyes are soluble in Triton-X 100 solution; thus, this protocol does not allow to combine immunostaining with the detection of the different CD146 subpopulations.

2. After spheroid formation and culture, image MBs using a fluorescent microscope (*e.g.*, wide-field or confocal microscope equipped with large working distance objectives) (Figure 4).

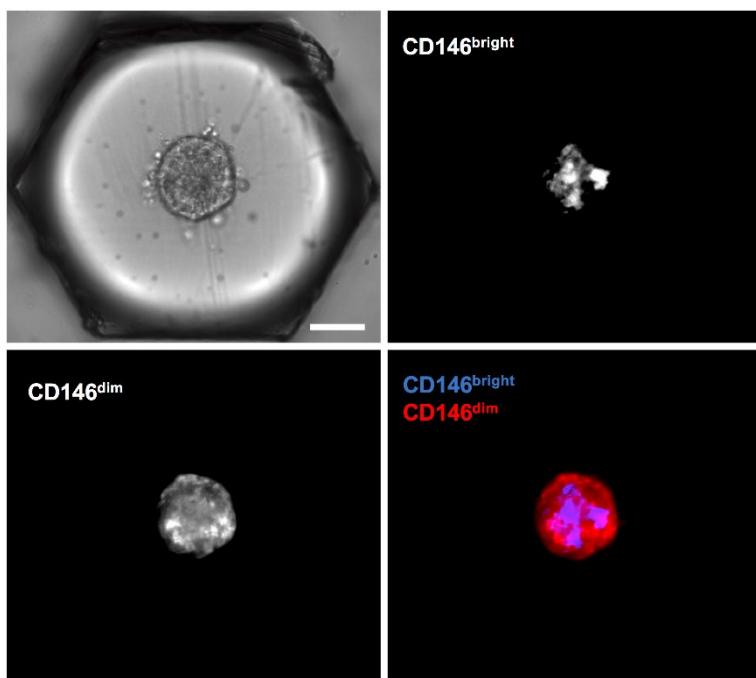


Figure 4. Spatial organization of CD146^{dim} and CD146^{bright} hMSCs within MBs.

CD146^{dim} and CD146^{bright} are isolated by cell sorting, then labeled with Vybrant DiO (green) or Vybrant DiD (red) dyes prior to their loading into drops. Scale bar = 100 μ m.

To interrogate the functional organization within MBs (*e.g.*, the regional expression of VEGF-A, COX-2, or RUNX-2):

1. Fix the MBs by perfusing a solution of 200 μ L of fixative solution, by first filling a 1 mL plastic syringe with PFA, then flowing at a flow rate of 80 μ L/min.
2. Incubate the aggregates of hMSCs with the PFA solution for 30 min at room temperature.
3. Wash the culture chamber with PBS at the same flow rate (200 μ L at 80 μ L/min).
4. Permeabilize the MBs by perfusing at 80 μ L/min with a 200 μ L of permeabilization buffer, and incubate for 5 min.
5. Wash the culture chamber with PBS (200 μ L at 80 μ L/min).
6. Block MBs by perfusion of a blocking buffer (200 μ L at 80 μ L/min) and incubate for 30 min.
7. After blocking, perfuse the chamber with a solution of primary antibody (*e.g.*, anti-COX-2 or anti-VEGF-A antibody or anti-RUNX-2; 200 μ L at 80 μ L/min), and incubate for 4 h.
8. Wash the primary antibody solution by perfusing PBS (200 μ L at 80 μ L/min).
9. Perfuse the solution of secondary antibodies (200 μ L at 80 μ L/min) and incubate for 1 h 30 min.
10. Wash with PBS (200 μ L at 80 μ L/min).
11. Image the MBs in the traps using a fluorescent microscope (Figure 5).
12. To validate the specificity of the primary antibodies, incubate the sample with the secondary antibody only, then wash with PBS. Absence of fluorescent signal validates that the primary antibody is specific to its target (*i.e.*, VEGF-A) and that excess of antibodies is effectively washed with PBS rinsing (Sart *et al.*, 2020).
13. To validate the absence of diffusion limitation, omit the blocking step and incubate with the secondary antibody only. Absence of diffusion limitation is demonstrated by homogeneous fluorescent signal distribution within the aggregate (Sart *et al.*, 2020).

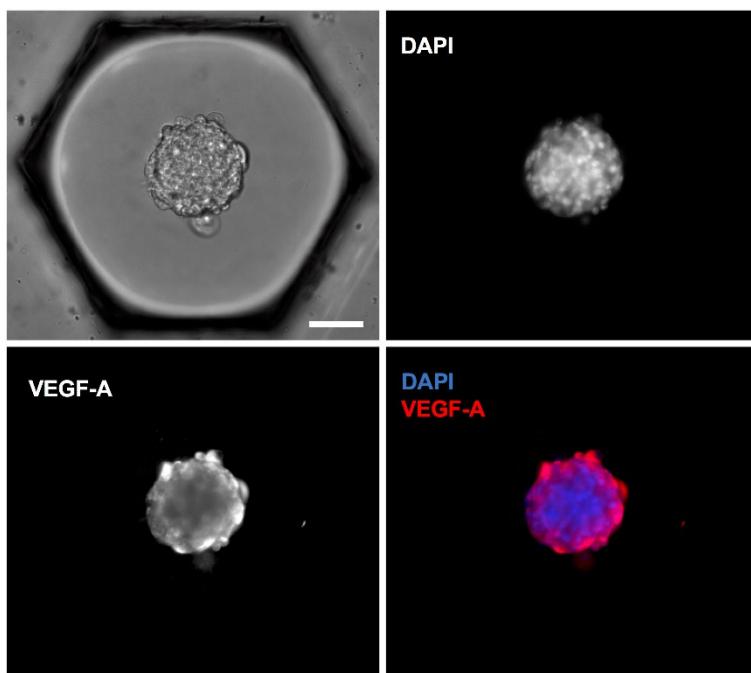


Figure 5. Functional organization hMSCs within MBs: VEGF-A expression detected by immunostaining.
Similar results were obtained for RUNX-2 and COX-2 immunostaining (Sart *et al.*, 2020). Scale bar = 100 μm .

Data analysis

The objective of the data analysis is to provide a quantitative characterization of the structural and functional organization within mesenchymal bodies using image analysis. In the following experiments, the data are generated from about 270 replicates that have been reproduced in at least three different chips.

A. Quantification of the structural organization within MBs using Matlab[®]

Detailed information on data analysis can be found in Sart *et al.* (2017).

1. Identify the centroid of each spheroid.
2. Measure the area (A) of each spheroid.
3. Calculate R, the radius of each spheroid, defined as the square root of A/π .
4. Measure the fluorescent intensity of each pixel of Vybrant DiO (green, corresponding to CD146^{dim} hMSCs) or Vybrant DiD (red, corresponding to CD146^{bright} hMSCs) stained cells, as well as their normalized distance (r/R) to the centroid (**Figures 6A and 6B**).
5. Bin the values of fluorescence at specific (r/R).
6. Normalize the two fluorescent signals against the DAPI signal to take into account the spherical shape of the aggregates.
7. The relative intensity of the two different fluorescence signals indicates the relative abundance of the CD146^{dim} vs. CD146^{bright} hMSCs at different radii within the MBs (**Figure 6C**) (Sart *et al.*, 2020).

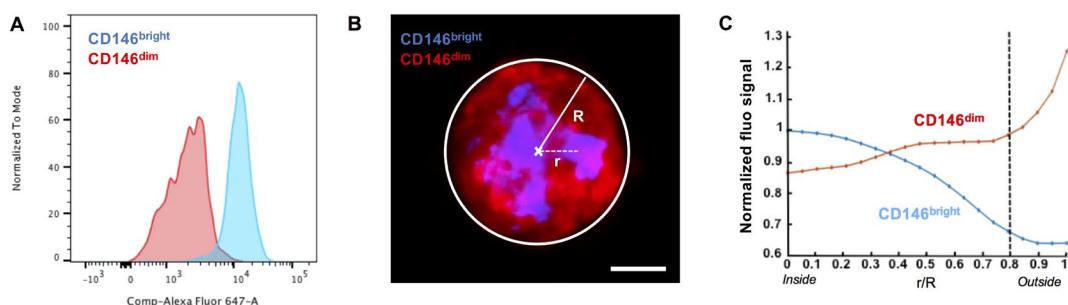


Figure 6. Data analysis methodology to quantify the structural organization in MBs.

(A) Cell sorting yielded to the separation of $CD146^{\text{dim}}$ and $CD146^{\text{bright}}$ populations. (B) Position of the fluorescent signal of $CD146^{\text{dim}}$ and $CD146^{\text{bright}}$ labeled with Vybrant DiO (green) or Vybrant DiD (red) identified by their radial coordinates (r/R). Scale bar = 100 μm . (C) Quantification of the distribution of the different $CD146$ populations within MBs, calculated based on radial coordinates. Part C is extracted from Sart *et al.* (2020).

B. Quantification of the functional organization within MB using Matlab[®]

Detailed information on data analysis can be found in Sart *et al.* (2020):

1. Identify each local maximum of DAPI-stained nuclei (Figure 7A).
2. Construct Voronoi diagrams (Chang *et al.*, 2007) by drawing the perpendicular bisectors of the segments between each neighboring local DAPI maxima, which approximate the cell shapes inside the MBs (Figure 7A).
3. Identify the position of each layer of cells within the aggregate (Figure 7B).
4. Quantify the cellular cytoplasmic fluorescent signal (COX-2, VEGF-A, and RUNX-2) for each cell of the MBs (Figure 7C).
5. Correlate the fluorescent signal of each cell to their cell layer position within the MBs (Figure 7D). To account for the variability of the cytoplasmic signal across the entire cell (nucleus included), the fluorescence signal of a single cell was defined as the mean signal of the 10% highest pixels of the corresponding Voronoi cell (Sart *et al.*, 2020).
6. Bin the values of fluorescence at specific cell layers.

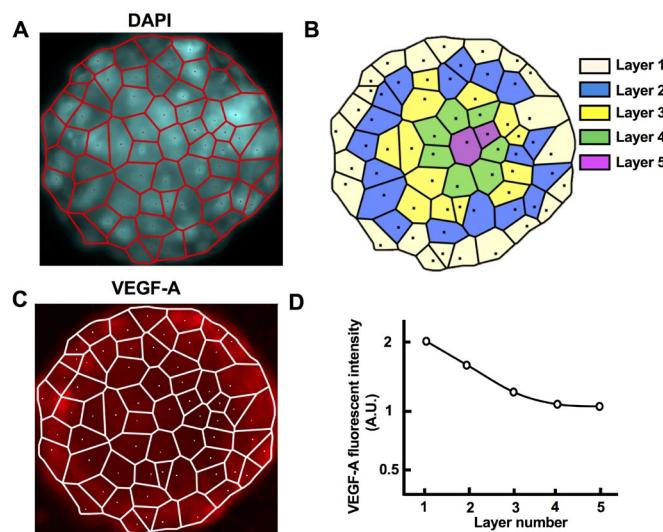


Figure 7. Data analysis methodology to quantify the functional organization in MBs: VEGF-A spatial

distribution.

(A) Detecting the DAPI signal enables drawing a Voronoi diagram, which allows estimating the area occupied by each cell in the aggregate. (B) The position of each cell is assigned to a layer number. (C) The fluorescent intensity in each cell defined from the Voronoi diagram is measured. (D) The fluorescent intensity of the protein of interest in each cell is correlated to the cell position (layer number) within the MBs, which allows mapping the cell function within the MBs. Similar results were obtained for RUNX-2 and COX-2 immunostaining (Sart *et al.*, 2020).

The results show that hMSCs self-organize in a hierarchical manner: most undifferentiated MSCs are located in the core, while partially committed cells are located at the boundaries of the MBs (**Figure 6**). Moreover, we found that such structural organization correlated with the induction of osteo-endocrine functions (*i.e.*, COX-2 and VEGF-A expression) (**Figure 7** and Sart *et al.*, 2020 for RUNX-2 and COX-2).

Recipes

1. hMSC culture medium

Mix 50 mL of FBS and 5 mL of the pen-strep (100×) with 500 mL of α-MEM basal medium, which results in a medium containing 10% (v/v) FBS and 1× pen-strep.

2. Staining buffer

Mix 200 μL of FBS with 9.8 mL PBS, which results in a 2% FBS (v/v) solution in PBS.

3. CD146 staining solution

Mix 10 μL of Alexa Fluor 647-conjugated anti-CD146 antibody stock solution with 990 μL of staining buffer, which results 1:100 diluted CD146 antibody solution.

4. Fixative solution

Mix 1 mL of PFA stock solution (16%, v/v) with 3 mL of PBS, which results in a 4% PFA (v/v) solution.

5. Agarose solution

Weight 30 mg of agarose powder and mix it with 1 mL PBS. Place this solution in a oven set at 80°C for at least one hour, in order to melt the agarose. It results in a liquid agarose solution at concentration of 3% (w/v). Prior to cell loading into drops, mix 30 μL of the the 3% (w/v) agarose solution with 70 μL of medium containing 6×10^6 cells. This results in a solution containing cells at a concentration of 6×10^6 cells/mL and agarose at 0.9% (w/v).

6. Permeabilization buffer

Mix 500 μL of Triton-X100 with 10 mL PBS, which results in a permeabilization buffer containing 0.5% Triton-X100 (v/v).

7. Blocking buffer

Mix 500 μL of FBS with 9.5 mL PBS, which results in a bolocking buffer buffer containing a 5% FBS (v/v) solution.

8. Primary antibodies solution

Mix 10 μL of primary antibody stock solution (anti-COX2, anti-VEGF-A, anti-RUNX-2) with 990 μL of staining buffer, which results 1:100 diluted primary antibody solution.

9. Secondary antibodies solution

Mix 10 µL of Alexa Fluor 594-conjugated secondary antibody stock solution and 20 µL DAPI solution at 14 µM (prepared from 5 mg DAPI resuspended in 10 mL PBS, which stock solution (1.4 mM) is then diluted at 1:100, *i.e.*, 10 µL into 990 µL PBS) with 970 µL of staining buffer, which results in a 1:100 diluted solution of secondary antibodies containing 0.3 µM DAPI.

Acknowledgments

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Competing interests

The authors declare that they have no competing interests.

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Proximity Dependent Biotin Labelling in Zebrafish for Proteome and Interactome Profiling

Zherui Xiong¹, Harriet P. Lo¹, Kerrie-Ann McMahon¹, Robert G. Parton^{1, 2, *} and Thomas E. Hall^{1, *}

¹Institute for Molecular Bioscience, the University of Queensland, Queensland 4072, Australia

²Centre for Microscopy and Microanalysis, The University of Queensland, Brisbane, Queensland 4072, Australia

*For correspondence: parton@imb.uq.edu.au; thomas.hall@imb.uq.edu.au

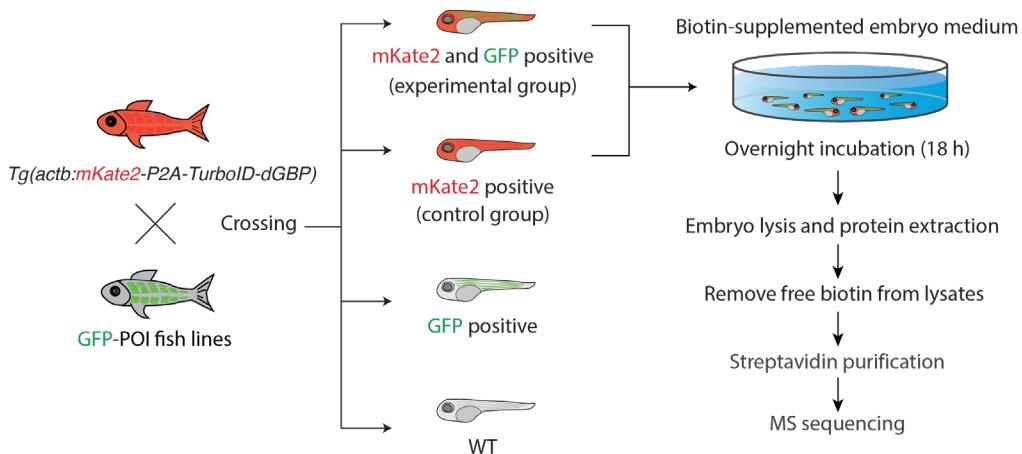
Abstract

Identification of protein interaction networks is key for understanding intricate biological processes, but mapping such networks is challenging with conventional biochemical methods, especially for weak or transient interactions. Proximity-dependent biotin labelling (BioID) using promiscuous biotin ligases and mass spectrometry (MS)-based proteomics has emerged in the past decade as a powerful method for probing local proteomes and protein interactors. Here, we describe the application of an engineered biotin ligase, TurboID, for proteomic mapping and interactor screening *in vivo* in zebrafish. We generated novel transgenic zebrafish lines that express TurboID fused to a conditionally stabilised GFP-binding nanobody, dGBP, which targets TurboID to the GFP-tagged proteins of interest. The TurboID-dGBP zebrafish lines enable proximity-dependent biotin labelling in live zebrafish simply through outcrossing with existing GFP-tagged lines. Here, we outline a detailed protocol of the BLITZ method (Biotin Labelling In Tagged Zebrafish) for utilising TurboID-dGBP fish lines to map local proteomes and screen novel interactors.

Keywords: Proximity-dependent biotin labelling, GFP Nanobody, Zebrafish, TurboID, Proteomics, BioID

This protocol was validated in: eLife (2021), DOI: 10.7554/eLife.64631

Graphical Abstract:



Schematic overview of the BLITZ method.

TurboID-dGBP fish are crossed with GFP-tagged lines to obtain embryos co-expressing *TurboID-dGBP* (indicated by mKate2) and the GFP-POI (protein of interest). Embryos expressing only *TurboID* are used as a negative control. Embryos (2 to 7 dpf) are incubated overnight with a 500 μ M biotin-supplemented embryo medium. This biotin incubation step allows *TurboID* to catalyse proximity-dependent biotinylation in live zebrafish embryos. After biotin incubation, embryos are solubilised in lysis buffer, and free biotin is removed using a PD-10 desalting column. The biotinylated proteins are captured by streptavidin affinity purification, and captured proteins are analysed by MS sequencing.

Background

In the past decade, proximity-dependent biotin labelling (BioID) has emerged as a powerful method for studying local proteome and protein interaction networks in living cells. This method utilises a genetically modified biotin ligase to covalently attach biotin molecules to proteins in the immediate vicinity. *TurboID* is one such biotin ligase that converts ATP and biotin into a highly reactive biotin-AMP that covalently labels proximal proteins (Branon *et al.*, 2018). Compared with other existing biotin ligases, *TurboID* has the most robust enzymatic kinetics, which enables its application in living organisms such as *Drosophila* and *C. elegans* (Branon *et al.*, 2018; May *et al.*, 2020). However, the applicability of *TurboID* has been limited by the necessity of genetically tagging the biotin ligase to each protein of interest (POI) and generating transgenic organisms.

Recently, we developed a platform for proteomic mapping through proximity-dependent biotin labelling in live zebrafish (Xiong *et al.*, 2021). Compared with conventional proximity-dependent biotin labelling methods, our platform circumvents the necessity of fusing a POI with the biotin ligase and creating transgenic organisms. Instead, the platform uses a modular system for GFP-directed proteomic mapping by combining *TurboID* with a GFP-binding nanobody (dGBP). By crossing the *TurboID-dGBP* fish line, *Tg(actb2:mKate2-P2A-TurboID-dGBP)^{uq23rp}*, with existing GFP-tagged fish lines, the dGBP targets *TurboID* to the GFP-labelled protein/region of interest through the binding of dGBP to GFP, which allows *TurboID* to capture the interactors of the POI or local proteome (Branon *et al.*, 2018). This system couples the power of the *TurboID* with the ability to use existing GFP-tagged transgenic zebrafish lines for proteomic mapping *in vivo*. The rapid degradation of unbound *TurboID-dGBP*, due to the conditionally stabilised nanobody (Tang *et al.*, 2016), improves the signal to noise ratio by preventing unspecific labelling from unbound *TurboID-dGBP* and achieves tissue specificity by restricting the *TurboID*-catalysed biotinylation only to tissues expressing GFP-tagged proteins. Our platform allows *in vivo* proteomic studies in specialised cells/tissues such as skeletal muscle, endothelia, and neurons. Furthermore, the method can be extended to study proteome/interactome changes under different physiological conditions and in disease models.

Materials and Reagents

1. Petri dish (Sarstedt, catalog number: 82.1473.001)
2. 24-well plate (Sigma-Aldrich, catalog number: CLS3526-50EA)
3. Eppendorf tube (Eppendorf, catalog number: EP0030108132)
4. PD-10 desalting column (GE Healthcare, catalog number: 17-0851-01)
5. *Tg(actb2:mKate2-P2A-TurboID-dGBP)^{uq23rp}* fish line or similar [this line was generated using the Tol2kit system according to established methods (Kawakami, 2004; Kwan *et al.*, 2007)]. The TurboID construct was codon-optimised for zebrafish expression based on the protein sequence from Branen *et al.* (2018). A red fluorescent reporter, mKate2, was indirectly linked to the N-terminus of TurboID-dGBP through a ribosome-skipping sequence, P2A (Shcherbo *et al.*, 2009; Kim *et al.*, 2011). The expression of the construct was driven by the ubiquitous beta actin2 promoter *actb2* (Higashijima *et al.*, 1997; Casadei *et al.*, 2011). The plasmid constructs were co-injected with tol2 mRNA into one-cell-stage WT zebrafish embryos for genomic integration. Injected F₀s were raised and screened for founders producing positive F₁s with Mendelian frequency, indicative of a single genomic integration. The fish line was maintained in a heterozygous state.
6. Streptavidin-HRP (Abcam, catalog number: Ab7403)
7. Biotin (Sigma-Aldrich, catalog number: B4639-1G)
8. Tricaine (Sigma-Aldrich, catalog number: E10521)
9. Pronase (if necessary) (Roche, catalog number: 10165921001)
10. Trypsin/Lys-C Mix, Mass Spec Grade (Promega, catalog number: V5073)
11. InstantBlue™ Coomassie Protein Stain (Expedeon, catalog number: ISB1L)
12. Bolt™ 10% Bis-Tris Plus Gels (Invitrogen, catalog number: NW00100BOX)
13. NeutrAvidin-DyLight 405 (Invitrogen, catalog number: 22831)
14. Sodium deoxycholate (Sigma-Aldrich, catalog number: D6750-10G)
15. NP-40 (Sigma-Aldrich, catalog number: 18896-50ML)
16. EDTA (Astral Scientific, catalog number: BIOEB0185-500G)
17. NaCl (Sigma-Aldrich, catalog number: S9888)
18. Paraformaldehyde (Sigma-Aldrich, catalog number: 158127)
19. Proteinase K (Invitrogen, catalog number: 25530015)
20. Tween 20 (Sigma-Aldrich, catalog number: P1379-500ML)
21. KCl (Sigma-Aldrich, catalog number: P9541-500G)
22. CaCl₂·2H₂O (Sigma-Aldrich, catalog number: C5080-500G)
23. MgCl₂·6H₂O (Sigma-Aldrich, catalog number: M2670-500G)
24. Complete™ Protease Inhibitor Cocktail (Sigma-Aldrich, catalog number: 11836145001)
25. Pierce™ BCA protein assay kit (Thermo Scientific, catalog number: 23225)
26. PBS tablets (Medicago, catalog number: 09-8912-100)
27. Triton-X100 (Sigma-Aldrich, catalog number: T9284-500ML)
28. Dynabeads™ MyOne™ Streptavidin C1 (Invitrogen, catalog number: 65001)
29. Blot LDS sample buffer (Invitrogen, catalog number: B0008)
30. DAPI (Sigma-Aldrich, catalog number: D9542-5MG)
31. Acetonitrile, LC-MS grade (Thermo Scientific, catalog number: 51101)
32. Formic acid (Thermo Scientific, catalog number: 28905)
33. Trifluoroacetic acid (Thermo Scientific, catalog number: 28904)
34. Embryo medium (see Recipes)
35. Tricaine stock (25×) (see Recipes)
36. Biotin stock solution (see Recipes)
37. Deyolkning solution (see Recipes)
38. Embryo blocking solution (see Recipes)
39. DAPI stock and intermediate solutions (see Recipes)
40. Trypsin/Lys C working solution (see Recipes)
41. Formic acid/acetonitrile solution (see Recipes)

42. Trifluoroacetic acid solution (see Recipes)
43. 4% paraformaldehyde (see Recipes)
44. Fish embryo lysis buffer (see Recipes)
45. SDS Washing buffer (see Recipes)
46. Urea Washing buffer (see Recipes)
47. Destaining solution (see Recipes)

Equipment

1. TripleTOF 6600 quadrupole time-of-flight mass analyser or similar (SCIEX)
2. Eksigent ekspert™ nanoLC 400 system or similar (SCIEX)
3. Dynabeads magnetic particle concentrator or similar (Applied Biosystems, catalog number: A13346)
4. Nikon SMZ18 stereo microscope with Nikon Intensilight Mercury White Light source or similar (Nikon)
5. Virtis Virsonic 100 ultrasonic cell disruptor or similar (Virtis Virsonic)
6. Zeiss LSM 710 meta upright confocal microscopes or similar (Zeiss)
7. Vortex
8. Thermo Fisher Pico™ 17 Microcentrifuge or similar (Thermo Fisher Scientific)
9. VWR® Tube Rotator or similar (VWR)
10. PowerPac™ HC High-Current Power Supply or similar (Bio-Rad)
11. Mini-PROTEAN Tetra Vertical Electrophoresis Cell or similar (Bio-Rad)
12. Vacufuge plus centrifuge concentrator or similar (Eppendorf)
13. Elmasonic S150 ultrasonic sonicator bath or similar (Elma)

Software

1. Analyst® TF 1.7 (SCIEX, <https://sciex.com/support/Software-support/Software-downloads>; require purchasing a licence or request a free trial)
2. ProteinPilot™ 5.0.1 (SCIEX, <https://sciex.com/support/Software-support/Software-downloads>; require purchasing a licence or request a free trial)
3. Microsoft Excel (Microsoft, <https://www.microsoft.com/en-au/microsoft-365/excel>)

Procedure

A. Outcross TurboID-dGBP lines with GFP-tagged lines of interest (e.g., zebrafish lines expressing GFP-labelled proteins or regions of interest)

1. Set up zebrafish pairs in mating tanks. It is preferable to use males from the TurboID-dGBP line and females from the GFP-tagged lines for protein extraction from embryos younger than 3 dpf (see Note 1).
2. Collect embryos the next morning and incubate them in the embryo medium at 28.5°C.
3. Sort embryos at 3 dpf according to fluorescent marker expression under a fluorescence microscope. The transgenic fish lines are normally maintained as heterozygote carrying a single copy of the transgene. Therefore, an outcross will produce offspring with all four genotypes: WT embryos with no fluorescent marker, POI-GFP embryos with GFP fluorescent marker, TurboID-dGBP embryos with mKate2 fluorescent marker, POI-GFP and TurboID-dGBP co-expressing embryos with both GFP, and mKate2 fluorescent markers (Figure 1). For protein extraction from 3 dpf embryos or younger, sort the embryos after biotin incubation.

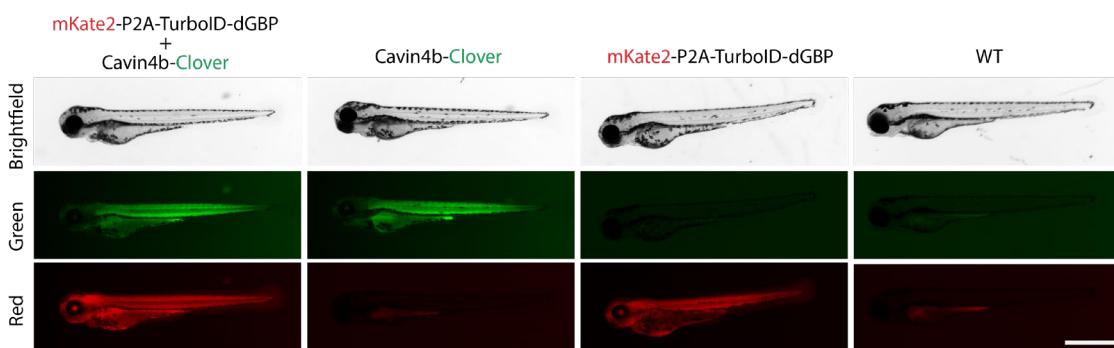


Figure 1. Representative embryos (3 dpf) from outcrossing the mKate2-P2A-TurboID-dGBP with Cavin4a-Clover line.

The transgenic fish lines are maintained as heterozygotes carrying a single copy of the transgene. Therefore, the crossing produces four combinations of genotypes indicated by corresponding fluorescent markers. Scale bar = 1 mm.

B. In vivo biotinylation

1. Prepare fresh biotin-supplemented embryo medium by diluting biotin stock solution into embryo medium to reach a concentration of 500 μ M biotin.
2. Incubate sorted embryos (~350 each group) in ~25 mL biotin-supplemented embryo medium in a 10 cm Petri dish at 28.5°C for 18 h.
3. Remove unincorporated biotin by three changes of fresh embryo medium (20 min each change) after the biotin incubation. Add ~1 mL tricaine to the medium to anaesthetise embryos before changing the medium.
4. Check the biotin labelling in the embryos by immunostaining with NeutrAvidin-DyLight 405 and immunofluorescence imaging (see next section).

C. Examining *in vivo* biotinylation by NeutrAvidin immunostaining

Note: This should be performed prior to streptavidin affinity pulldown and mass spectrometry (MS) analysis. A trial experiment of NeutrAvidin immunostaining with a small number of embryos is recommended before conducting a large-scale pulldown.

1. Sample three embryos for each group after *in vivo* biotinylation step and anaesthetise them in 500 mL ice-cold tricaine solution in a 24 well plate for 20 min.
2. Replace the tricaine solution with 500 μ L of 4% paraformaldehyde (PFA) by carefully pipetting, and fix the embryos overnight at 4°C.
3. After fixation, wash embryos 3 times with PBS and permeabilise the embryos with 300 μ L of 10 μ g/mL proteinase K (10 min for embryos at 2 dpf; 15 min for embryos at 3 dpf; or 20 min for embryos at 4 dpf). Fix embryos again with 4% PFA for 15 min.
4. Wash embryos twice in 1 mL of 0.1% Tween 20 in PBS by gently swirling on a shaker for 5 min.
5. Transfer embryos into embryo blocking solution (see Recipes) for 3 h at room temperature.

Note: Embryos can be kept in blocking solution overnight at 4°C.

6. Stain the embryos with NeutrAvidin-DyLight 405 (1:500 dilution in blocking solution) overnight at 4°C followed by four washes with 0.3% Triton X-100 in PBS, 30 min each wash on a shaker. For nuclear staining (if required), stain embryos with DAPI (see Recipes) for 10 min followed by 3 washes with PBS 0.3% Triton X-100, 20 min each wash on a shaker.

7. Mount embryos on a slide with coverslip; alternatively, the embryos can be imaged directly with a water dipping lens. Fluorophore DyLight 405 can be visualised using a blue filter on a fluorescent microscope or use laser 405 nm for excitation and detect emission between 415-460 nm on a confocal microscope.

Note: Embryos co-expressing mKate2-P2A-TurboID-dGBP and GFP-POI should show a clear colocalization of NeutrAvidin and GFP signals, whereas embryos carrying only one of the transgenes should give no or minimum NeutrAvidin staining. An example is provided in Figure 2.

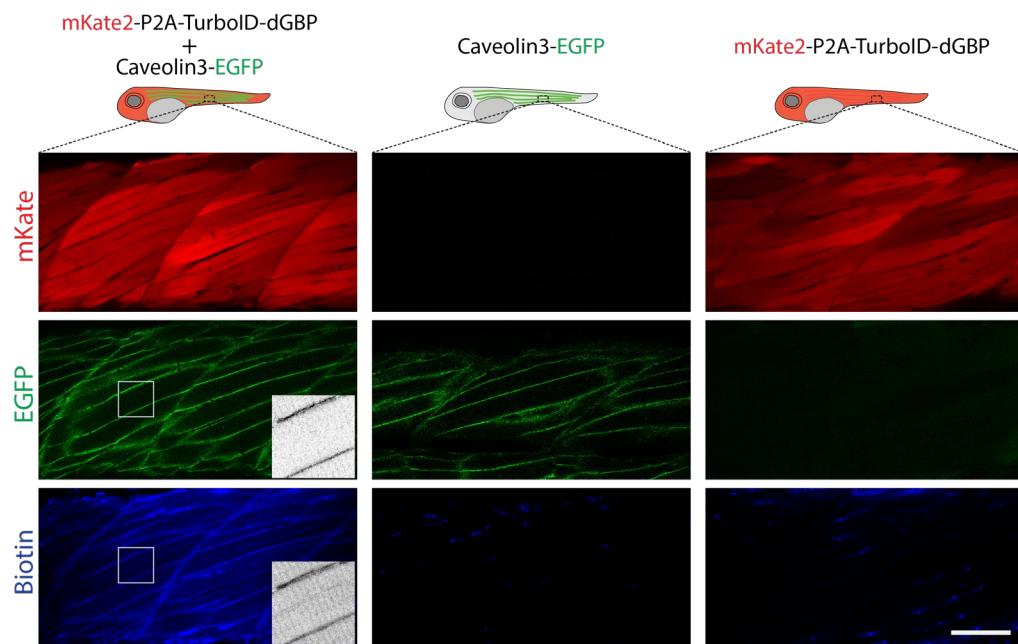


Figure 2. Representative confocal images showing TurboID-catalysed biotinylation in zebrafish embryos. Embryos were obtained from outcrossing the TurboID-dGBP line with a Caveolin3-EGFP line. They were subjected to overnight biotin incubation at day 4 and fixed at day 5. TurboID-catalysed biotin labelling was visualised by NeutrAvidin-DyLight 405 staining. Strong biotin labelling was observed at the plasma membrane of muscle cells in the embryos co-expressing TurboID-dGBP and Caveolin3-EGFP. This staining pattern corresponds to the subcellular localisation of membrane protein Caveolin3, and it is absent in the siblings expressing either TurboID-dGBP or Caveolin3-EGFP. The inserts show boxed area in inverted grayscale. Scale bar = 40 μ m.

D. Yolk removal (strongly recommended for embryos younger than 5 dpf; otherwise, skip to section E)

1. Anesthetise embryos by adding tricaine solution and incubate on ice. Transfer the embryos into 2 mL Eppendorf tubes.
2. Replace the medium with ice-cold deyolking solution and disrupt yolk sac by pipetting embryos through a 200 μ L pipette tip (Figure 3).
3. Pellet the embryos by brief centrifugation and remove supernatant, which contains yolk proteins. Rinse embryos twice with deyolking solution on ice.

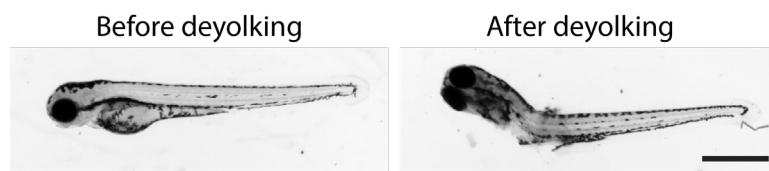


Figure 3. Representative images showing the 3 dpf embryos before and after deyolking.
The disrupted yolk sac was resulted from pipetting through a 200 μ L pipette tip.

E. Protein extraction and biotin affinity purification

1. Homogenise deyolked zebrafish embryos in 1 mL of embryo lysis buffer (with freshly added protease inhibitors) by brief pulse sonication (1s sonication at intensity 5 with 1s interval for 1 min). Add another 1 mL of embryo lysis buffer and vortex for 30 min at 4°C. Centrifuge the lysates at 14,000 \times g for 10 min at 4°C to remove insoluble tissue debris.

Note: Zebrafish embryo lysates are prone to protein degradation. Add protease inhibitors and keep lysates on ice to minimise protein breakdown.

2. Transfer the supernatant to a new tube and measure the protein concentration using the Pierce™ BCA protein assay kit. For each sample, we used 4 mg of crude fish proteins for biotin affinity purification. However, the amount of crude protein required depends on the GFP-POI expression level and tissue types. For low expressing GFP lines or expression in low abundance cell types, a larger amount of crude protein lysates may be required.
3. Pass the fish lysates through a PD-10 desalting column to remove free biotin. This step is important for subsequent streptavidin pulldown because it increases the efficiency of capturing biotinylated proteins by streptavidin-conjugated beads.
4. Prewash Streptavidin-conjugated Dynabeads™ in embryo lysis buffer three times. Add 200 μ L of washed Dynabeads into each lysate sample and constantly mix the lysate/beads on a rotor wheel at 4°C overnight.
5. After the incubation, separate the beads from the fish lysates using a magnetic particle concentrator and transfer to a new Eppendorf tube. Wash the beads twice with fish embryo lysis buffer, once with SDS washing buffer, once with urea washing buffer, and twice in embryo lysis buffer again. Note that the magnetic beads tend to clump together after capturing biotinylated proteins. Vortex the beads in the washing buffer to break down the aggregate and ensure a thorough wash.

F. Protein electrophoresis and protein gel staining (skip this step if on-bead digestion is used)

1. To elute captured proteins, incubate beads in 60 μ L of 2 \times Blot LDS sample buffer with 2 mM biotin and 20 mM DTT at 95°C for 10 min. Briefly vortex the beads after the first 5 min of incubation. Note that the combination of free biotin and excess heat is able to efficiently elute the biotinylated proteins from streptavidin-conjugated beads (Cheah and Yamada, 2017).
2. Load the eluted protein sample to a Bolt™ 4-12% Bis-Tris Plus Gel (or similar) and separate the proteins at 100 V for 30 min.
3. Stain the protein gel with InstantBlue™ Coomassie Protein Stain (or Colloidal Coomassie Blue) (Figure 4). Note that the incubation time varies depending on the amount of protein. For maximal detection sensitivity, incubate the gel overnight in the staining solution.
4. Cut the protein gel into slices of 1-2 mm thickness. Avoid any blank gel in an excised gel slice since blank gel will reduce in-gel digestion efficiency. The excised gel slice can be stored in the fridge for one week.

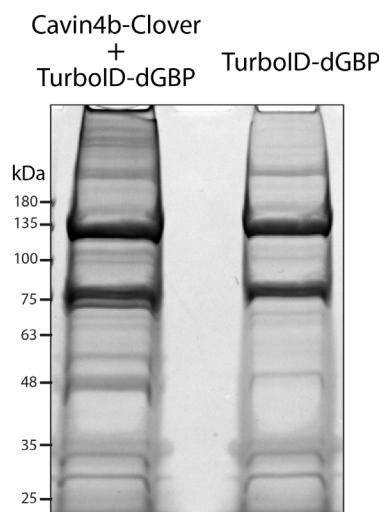


Figure 4. Protein gel stained by InstantBlue™ Coomassie Protein Stain.

The left lane contains a streptavidin pulldown from embryos co-expressing TurboID-dGBP and Cavin4b-Clover, while the right lane contains a streptavidin pulldown from control embryos expressing only TurboID-dGBP.

G. Sample preparation for mass spectrometry

1. For in-gel digestion, destain the gel slices with 500 µL of destaining solution and incubate for 30 min with occasional vortexing.
2. Replace the destaining solution with 200 µL of 100% acetonitrile and leave for 15 min. Replace the acetonitrile again with another 200 µL of 100% acetonitrile for 15-30 min to ensure dehydration of the protein gel. Acetonitrile is removed in preparation for trypsin digestion.
3. Add 200 µL of sequencing grade Trypsin/Lys-C (20 ng/µL in 50 mM ammonium bicarbonate pH 8 buffer). Note that the Trypsin/Lys-C solution should cover the gel pieces, and if required, a further 100 µL of Trypsin/Lys-C solution can be added. Incubate the samples at 37°C overnight.
4. Add 200 µL of 5% formic acid/acetonitrile (3:1, vol/vol) to each sample and incubate for 15 min at room temperature in a shaker. Transfer the supernatant into clean Eppendorf tubes and dry down in a vacuum centrifuge.
5. Add 12 µL of 1.0% (vol/vol) trifluoroacetic acid in H₂O to the tube and vortex. Incubate in a sonication bath for 2 min and then centrifuge for 1 min at 6,700 × g. Transfer the solution to an autosampler vial for MS analysis.

Data analysis

MS data were acquired and processed using Analyst TF 1.7 Software (SCIEX). Protein identification was carried out using ProteinPilot™ Software v5.0 (SCIEX) with Paragon™ database search algorithm (Figure 5). MS/MS spectra were searched against the zebrafish proteome in the UniProt database. The search parameter was set to thorough with False Discovery Rate (FDR) analysis. The cut-off for identified proteins was set to 1% global FDR. Endogenous biotinylated proteins, common contaminants and Background proteins present in control embryos were subtracted. The MS2Count of each protein was used to compare the relative abundance of identified proteins.

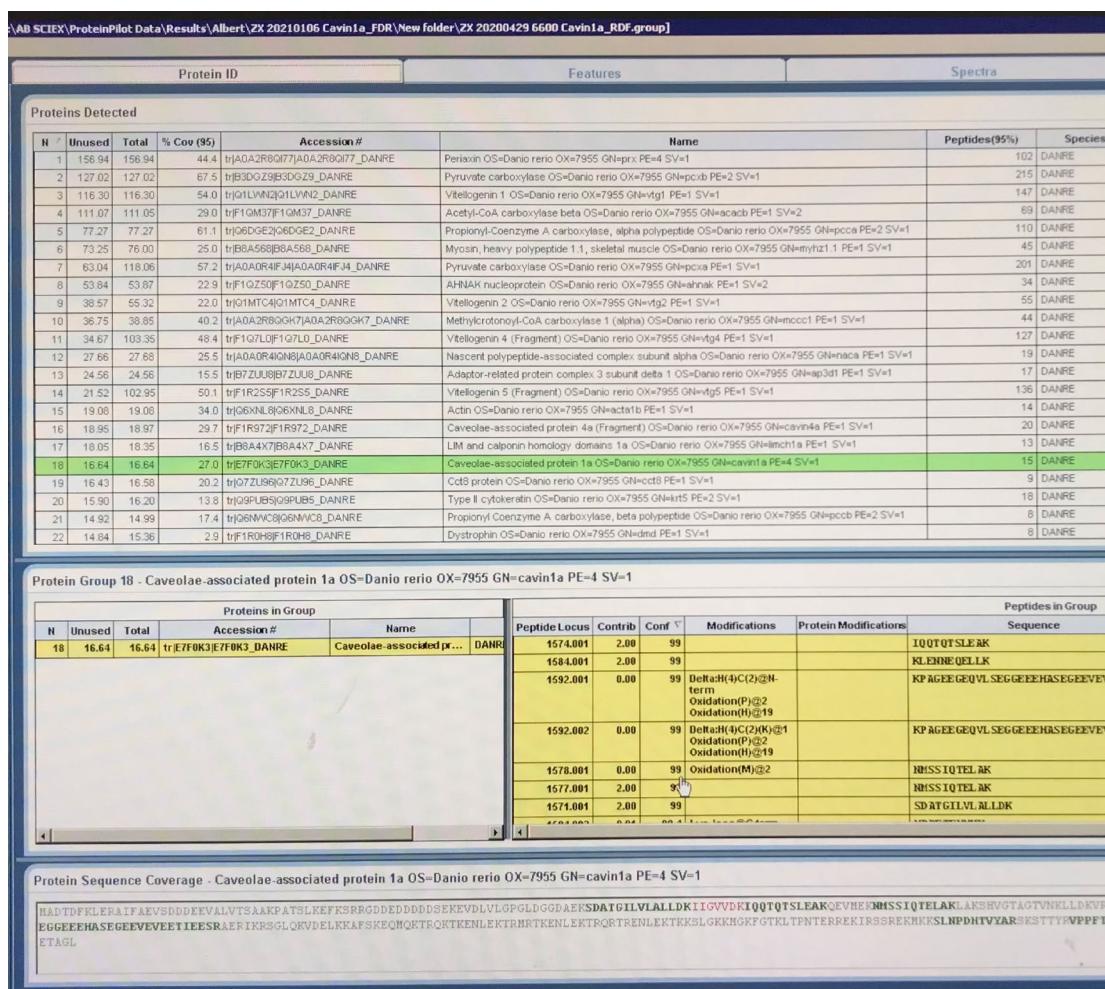


Figure 5. A screenshot of ProteinPilot™ Software showing the proteins identified from the embryos co-expressing TurboID-dGBP and Cavin1a-Clover

Notes

- Select males from the TurboID-dGBP line to cross with females from the GFP line if the proteomic analysis is conducted on embryos that are 3 dpf or younger. This prevents the presence of TurboID-dGBP in the yolk due to maternal inheritance (Pelegrí, 2003) and reduces potential interference caused by yolk proteins.
- The delivery of biotin to target cells/tissues is a prerequisite for TurboID to catalyse proximity-dependent biotinylation in zebrafish. The overnight incubation of 3 dpf zebrafish embryos in 500 µM biotin-supplemented embryo medium is sufficient for biotin to reach the musculature, endovascular system, and motor neurons in which TurboID-catalysed biotinylation was observed (Xiong *et al.*, 2021). The overnight incubation method was also tested with embryos from 2 to 8 dpf, and TurboID-catalysed biotinylation was observed in the musculature of all developmental stages tested (unpublished data).
- The amount of protein biotinylated by TurboID is highly dependent on the expression level of the GFP-POI. For low GFP-POI expressing lines, older embryos or juvenile zebrafish may be required for producing detectable biotinylated proteins by TurboID. For GFP-POI expressed in low abundance cell types, specific tissue isolation after *in vivo* biotinylation may be required to improve the detection of TurboID-biotinylated proteins over Background proteins.
- For MS analysis, the in-gel digestion method is described in this protocol. Alternatively, on-bead digestion may

also be possible for downstream MS analysis. Note that zebrafish embryos have a considerable amount of endogenous biotinylated proteins (carboxylases). The presence of these endogenous biotinylated proteins might compromise the detection of low-abundance proteins in the sample. The SDS-PAGE step in the in-gel digestion method separates protein samples based on their size, which allows MS analysis on each individual protein band at different molecular weights. Therefore, in-gel digestion allows separate detection of endogenous biotinylated protein (around 70 and 135 kDa) from the proteins biotinylated by TurboID. This might improve MS detection for low-abundance proteins present in samples.

Recipes

1. Embryo medium

5 mM NaCl
0.17 mM KCl
0.33 mM CaCl₂
0.33 mM MgSO₄

2. Tricaine stock (25×)

Dissolve 400 mg of Tricaine powder in 98 mL of H₂O and add 2 mL of 1 M Tris base (pH 9).
Adjust pH to 7.
Store at -20°C.

3. Biotin stock solution (100 mM biotin in DMSO)

Dissolve 244.31 mg biotin powder in 10 mL of DMSO with vortexing.
Keep stock solution in an airtight amber bottle (or a Falcon tube wrapped with aluminium foil).

4. Deyolking solution

55 mM NaCl
1.8 mM KCl
1.25 mM NaHCO₃
Add cComplete™ Protease Inhibitor Cocktail (2 tablets per 50 mL) before use.

5. Embryo blocking solution

0.3% (vol/vol) Triton X-100 and 4% (wt/vol) bovine serum albumin in 1× PBS solution.
Store solution at 4°C up to 2 weeks.

6. DAPI stock and intermediate solutions

To make 14.3 mM DAPI stock solution, dissolve 5 mg of DAPI in 1 ml of deionised water (sonication might be required to facilitate dissolution). The stock solution can be aliquoted and stored at -20°C.
Add 2.1 μL of the 14.3 mM DAPI stock solution to 100 μL PBS to make a 300 nM DAPI intermediate dilution.
Dilute the intermediate solution 1:1,000 in PBS to make a 300 nM DAPI stain solution.

7. Trypsin/Lys C working solution

Rehydrate lyophilised Trypsin/Lys-C Mix in Reconstitution Buffer to 1 μg/μL.
Aliquot and store the rehydrated Trypsin/Lys-C Mix in -20°C.
Dilute an aliquot of the 1 μg/μL Trypsin/Lys-C Mix in 50 mM ammonium bicarbonate pH 8 to a final concentration of 20 ng/μL.

8. Formic acid/acetonitrile solution

Combine 5% (vol/vol) formic acid in ultrapure H₂O with acetonitrile at a ratio of 3:1 (vol/vol).

9. Trifluoroacetic acid solution

1.0% (vol/vol) Trifluoroacetic acid in ultrapure H₂O

10. 4% paraformaldehyde

Dissolve 40 g of paraformaldehyde powder in 800 mL of heated 1× PBS solution (~ 60°C) in a glass beaker on a stir plate (in a ventilated hood).

Slowly add 1 N NaOH dropwise until the solution clears.

Adjust the volume of the solution to 1 L with 1× PBS.

Adjust the pH to approximately 6.9.

Store the solution at 4°C.

11. Fish embryo lysis buffer

50 mM Tris-HCl, pH 7.5

150 mM NaCl

1% NP-40

0.1% SDS

5 mM EDTA

0.5% Na-deoxycholate

12. SDS Washing buffer

2% SDS in 50 mM Tris-HCl pH 7

13. Urea Washing buffer

2 M urea in 10 mM Tris-HCl pH 8

14. Destaining solution

100 mM ammonium bicarbonate/acetonitrile (1:1, vol/vol).

Dissolve 1.58 g ammonium bicarbonate in 100 ml acetonitrile and add 100 mL ultrapure water.

Store this solution at 4°C for up to 2 months.

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The TurboID is based on the engineered biotin ligase by Branon *et al.* (2018), and the destabilised nanobody (dGBP) is based on the studies of Tang *et al.* (2016). This protocol describes the methodology used in the research paper (doi: 10.7554/eLife.64631) by Xiong *et al.* (2021)

Competing interests

The authors declare that they have no conflicts of interest and no competing interests.

Ethics

All experiments were approved by the University of Queensland Animal Ethics Committee.
Approval ID IMB/271/19/BREED. Approval valid 01/01/2019 – 01/01/2022.

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Phalloidin Staining of Actin Filaments for Visualization of Muscle Fibers in *Caenorhabditis elegans*

Mario Romani and Johan Auwerx*

Laboratory of Integrative Systems Physiology, École Polytechnique Fédérale de Lausanne, Lausanne, Switzerland

*For correspondence: admin.auwerx@epfl.ch

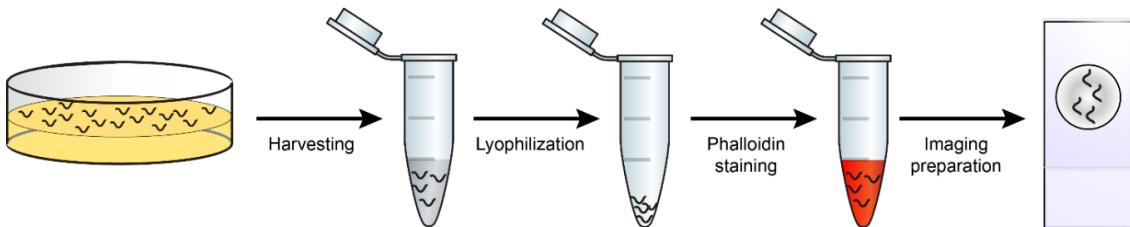
Abstract

Advances in *C. elegans* research have allowed scientists to recapitulate different human disorders, from neurodegenerative diseases to muscle dysfunction, in these nematodes. Concomitantly, the interest in visualizing organs affected by these conditions has grown, leading to the establishment of different antibody- and dye-based staining protocols to verify tissue morphology. In particular, the quality of muscle tissue has been largely used in nematodes as a readout for fitness and healthspan. Phalloidin derivatives, which are commonly used to stain actin filaments in cells and tissues, have been implemented in the context of *C. elegans* research for visualization of muscle fibers. However, the majority of the phalloidin-based protocols depend on fixation steps using harmful compounds, preparation of specific buffers, and large amounts of worms. Herein, we implemented a safer and more flexible experimental procedure to stain actin filaments in *C. elegans* using phalloidin-based dyes. Lyophilization of the worms followed by their acetone permeabilization allows bypassing the fixation process while also providing the opportunity to suspend the experiment at different steps. Moreover, by using conventional buffers throughout our protocol, we avoid the additional preparation of solutions. Finally, our protocol requires a limited number of worms, making it suitable for slow-growing *C. elegans* strains. Overall, this protocol provides an efficient, fast, and safer method to stain actin filaments and

Keywords: Phalloidin, *C. elegans*, Actin, Muscle fibers, Cytoskeleton, Nucleus, Muscle disease, Aging

This protocol was validated in: Cell Rep (2021), DOI: 10.1016/j.celrep.2020.10866

Graphic Abstract:



Schematic overview of phalloidin staining in *C. elegans* for assessing muscle fiber morphology.

Background

In the last decades, the nematode *Caenorhabditis elegans* has emerged as a powerful model organism and has provided an unprecedented opportunity to expand our knowledge in different fields of research, from developmental biology (Mello *et al.*, 1992; Bowerman *et al.*, 1993; Hubbard and Greenstein, 2000), to neuromuscular degeneration (Braungart *et al.*, 2004; McColl *et al.*, 2012; Sorrentino *et al.*, 2017; Romani *et al.*, 2021) and aging (Kimura *et al.*, 1997; Herndon *et al.*, 2002). One of the main reasons that promoted the usage of *C. elegans* as a laboratory model is that despite its simplicity, it has defined tissues. In particular, it consists of five main tissues: epidermis, reproductive tissues, digestive system, nervous system, and muscle tissue (Spencer *et al.*, 2011). Among these, muscle tissue morphology and function have been widely used to typify *C. elegans* fitness and healthspan (Fire *et al.*, 1991; Ryu *et al.*, 2016; Fang *et al.*, 2017; D'Amico *et al.*, 2019; Romani *et al.*, 2021). In recent years, numerous protocols have emerged to facilitate the visualization of *C. elegans* muscles, taking advantage of the transparent nature of this nematode. Phalloidin is a mushroom-derived molecule that specifically binds filamentous actin (F-actin); hence, phalloidin derivatives tagged with fluorescent molecules are commonly used in microscopy to visualize F-actin in different fields of research (Chazotte, 2010). Body wall muscles of the nematode *C. elegans* possess obliquely striated myofibrils composed of highly organized filaments of actin, whose disposition is altered in pathological conditions (Gieseler *et al.*, 2018). For this reason, phalloidin-based staining of actin filaments has been successfully implemented in nematodes for the detection of cytoskeleton and muscle structure, with important applications in muscle disease and degeneration (Gieseler *et al.*, 2018). However, phalloidin-based protocols typically rely on tedious fixation steps using harmful compounds, such as PFA, and require the preparation of specific, complicated buffers (Costa *et al.*, 1997; Ono, 2001; Bansal *et al.*, 2015; Geisler *et al.*, 2020). We present here a method for *C. elegans* research that allows phalloidin staining of actin filaments, bypassing fixation, and using conventional buffers. Moreover, by integrating 4',6-diamidino-2-phenylindole (DAPI) staining in our protocol, we allow the visualization of muscle cell nuclei, whose morphology can be used to interpret cellular homeostasis. Finally, our protocol requires a limited amount of worms and can be suspended at different steps, ensuring a versatile experimental procedure. Overall, our methodology provides a robust, simple, and safer pipeline for the visualization of muscle fibers and nuclei in *C. elegans* with applications in multiple fields of biomedical research. We predict that this protocol could be coupled with additional dyes specific for other tissues or cellular components, such as endoplasmic reticulum and mitochondria, allowing an even broader overview of worm physiology and homeostasis.

Materials and Reagents

1. 1.5 mL Eppendorf tube (Eppendorf, catalog number: 11.3817.01)
2. Glass pipette
3. Microscope slides (Thermo Scientific, catalog number: 11950657)

4. Cover slips (VWR, catalog number: 43211.KG)
5. Plastic dropper (VWR, catalog number: AMP-30)
6. Aluminum foil
7. Liquid nitrogen
8. Acetone (Millipore, catalog number: 1.00014.1011)
9. Rhodamine Phalloidin (Invitrogen, catalog number: 219920-04-4)
10. Methanol (Sigma-Aldrich, catalog number: 646377-1L)
11. BSA (Sigma-Aldrich, catalog number: A7906-100G)
12. Tween-20 (Sigma-Aldrich, catalog number: P1379)
13. DAPI (Invitrogen, catalog number: D1306)
14. Agarose (Promega, catalog number: V3125)
15. M9 buffer (see Recipes)
16. Washing buffer (see Recipes)
17. 2 µg/mL DAPI in M9 (see Recipes)
18. 2% (w/v) agarose (see Recipes)

Equipment

1. Standard Equipment for worm culture (see Brenner, 1974)
2. Centrifuge (Eppendorf, Centrifuge 5424)
3. SpeedVac Vacuum Concentrator (Labogene, Speed Scan 40)
4. Laboratory fume hood
5. Leica SP8 confocal microscope (Stand: Upright Leica DM6 CS; Illumination: Lumencor Sola SM II LED, Laser; Software: LAS-X; Camera: DFC 7000 GT (B/W); Objective: HC PL APO, 63×/1.40 oil, DIC POL0.14)

Software

1. Fiji (Schindelin *et al.*, 2012)

Procedure

A. Culture 50 worms on a NGM agar plate. Each plate of 50 worms serves as a replicate

Note: The protocol will work with any number of worms between 50 and 100. We suggest using two replicates for each experimental condition.

B. Worm retrieval from agar plates

1. Wash worms off the plate using 1 mL of M9 buffer and place the solution in a 1.5 mL Eppendorf tube.

Note: We suggest retrieving the M9 solution from the plates using a glass pipette, which reduces the adhesion of worms to the pipette's walls.

2. Spin briefly in a centrifuge to pellet the worms but not the bacteria (do not exceed 400 × g); 10 s is sufficient to pellet adult worms.

3. Remove as much supernatant as possible using a micro-pipette (1,000 µL and 200 µL).

Note: C. elegans tend to swim, so the pellet is not stable; avoid bringing the tip of the pipette too close to the pellet to prevent sucking in any worms.

4. Repeat steps from 1 to 3 one more time to ensure that all the worms are retrieved from the plate.
5. Snap freeze the worms by immersing the Eppendorf tube in liquid nitrogen.

Note: At this stage, worms can be stored at -80°C for months.

C. Worm permeabilization

1. Open the Eppendorf tube and place it in a SpeedVac Vacuum Concentrator to dry the worm pellet at 20°C. This step typically takes ~10 min but can vary according to the amount of supernatant that is left from Step B3.
2. Add three drops of ice-cold acetone to the dry worms. Gently tap the Eppendorf tube five times.

Note: It is important to cover all the worms; additional drops of acetone could be used if necessary. If worms are not correctly permeabilized, the protocol will fail.

3. Incubate 5 min at room temperature.
4. Remove as much of the acetone as possible using a micro-pipette (1,000 µL and 200 µL) and air dry the remaining acetone in a laboratory fume hood.
5. After complete evaporation of the acetone, worms can be stored at -20°C for months.

D. Phalloidin staining

Note: We suggest working in a dark environment to prevent phalloidin degradation and consequent failure of the protocol.

1. Place 2.5 U (for each replicate) of Rhodamine Phalloidin in an Eppendorf tube.
2. Use the SpeedVac Vacuum Concentrator to remove the methanol in which the Rhodamine Phalloidin is dissolved.

Note: It takes ~5-10 min to dry the Rhodamine Phalloidin, but this varies according to the volume that is used.

3. Resuspend the dried Rhodamine Phalloidin in 20 µL (for each replicate) of M9.
4. Add 20 µL of the M9-resuspended Rhodamine Phalloidin to the dried worms. Gently tap the Eppendorf tube five times.
5. Incubate in the dark at room temperature for 30 min.
6. Wash the worms twice in 1 mL of washing buffer.
7. If nuclei staining is not required, proceed to Procedure F.

E. DAPI staining

Note: We suggest working in a dark environment to prevent DAPI degradation.

1. Resuspend the worms in 20 µL of 2 µg/mL DAPI in M9. Gently tap the Eppendorf tube five times.
2. Incubate in the dark at room temperature for 5 min.
3. Wash the worms twice in 1 mL of washing buffer.

F. Preparation of the worms for imaging

Note: We suggest working in a dark environment to prevent degradation of the fluorescent dyes and consequent failure of the protocol.

1. Resuspend the worms in 10 μ L of M9.
2. Prepare a 2% (w/v) agarose pad on a microscope slide for each replicate. To prepare the agarose pad, apply lab tape on two microscope slides and place a third one in between them (Figure 1a). Apply 2-3 drops of agarose in the center of the microscope slide not wrapped in tape using a micro-pipette (1,000 μ L). Place another microscope slide on the top of the agarose (Figure 1b). Wait 1-2 min for the agarose to solidify and remove the microscope slide on the top. Store the agarose pads in a humid place before usage to prevent dryness.

Note: One layer of tape is sufficient to create the space needed for the formation of an agarose pad. Cut the micro-pipette tip to facilitate the dispensation of agarose. Gently put the last microscope slide on the liquid agarose to avoid the formation of bubbles in the agarose pad.

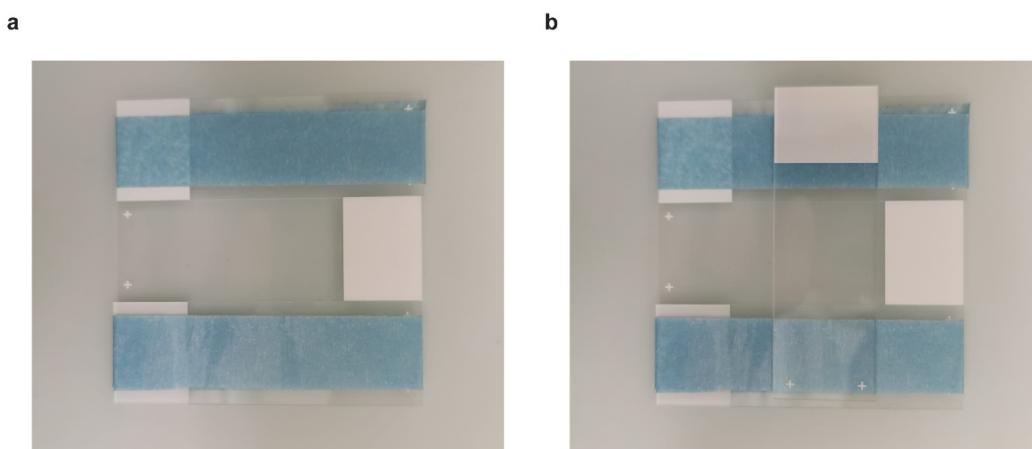


Figure 1. Agarose pads preparation.

First (a) and second (b) steps for the preparation of agarose pads described in Step F2.

3. Transfer all the M9 containing the worms to the agarose pad using a glass pipette.
4. Apply a cover slip, avoiding the formation of bubbles. Surface tension of M9 will keep the cover slip steady during the imaging process but avoid touching it.

G. Imaging using a confocal microscope

Note: We suggest not using inverted microscopes to keep the cover slip steady during the imaging process.

H. Images analysis

1. Use Fiji to open the images recorded with the confocal microscope.
2. Add scale bar: “Analyse” -> “Tools” -> “Scale bar” (Figure 2).

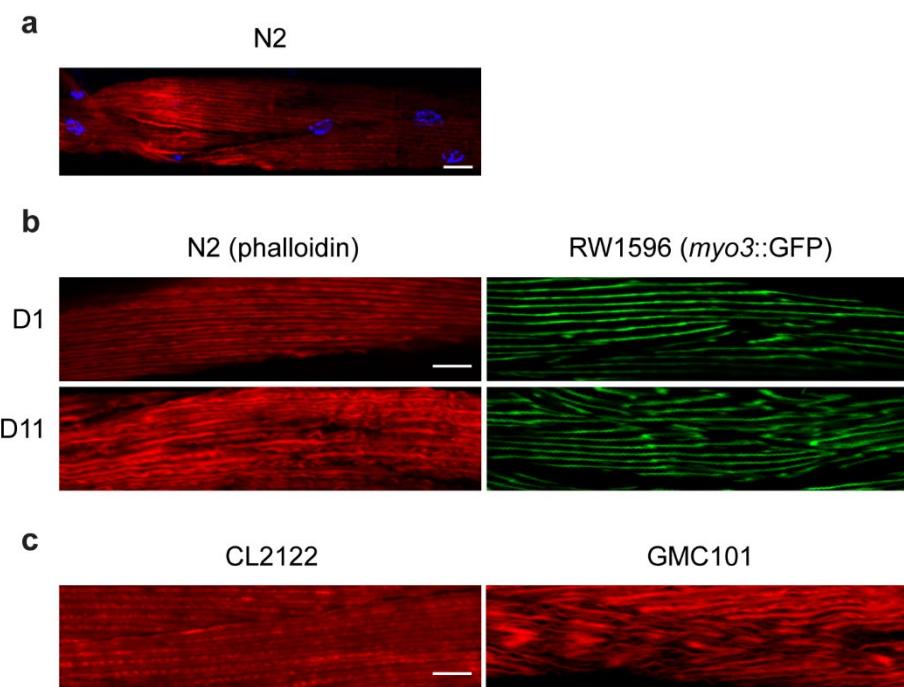


Figure 2. Phalloidin staining applications and comparison with GFP-tagged muscle fibers.

(a) Representative image of day 1 adult N2 worms; in red, actin filaments stained with Rhodamine-Phalloidin; in blue, nuclei stained with DAPI. (b) Day 11 adult N2 worms present disorganized muscle fibers, visualized here through Rhodamine-Phalloidin staining (in red), when compared to young (day 1) worms as described before (Romani *et al.*, 2021). These results are comparable to those obtained using a transgenic worm strain (RW1596) in which the muscle fibers are labeled with GFP (in green). (c) Rhodamine-Phalloidin staining of the inducible muscle proteotoxicity model, GMC101, reveals altered muscle fiber morphology compared to the control strain CL2122, consistent with the data obtained in young and old worms and as described before (McColl *et al.*, 2012). Scale bar, 10 μ m; magnification, 63 \times .

Recipes

1. NGM agar plates (see Brenner, 1974)

2. M9 buffer (Table 1)

Table 1. M9 buffer composition.

List of components and volumes for 1 L of working stock.

Components	Quantity
Na ₂ HPO ₄	6 g
KH ₂ PO ₄	3 g
NaCl	5 g
MgSO ₄ ·7H ₂ O	250 mg
Water	1 L

Note: Filter before using.

3. Washing buffer

M9 1 mL
BSA 50 mg
Tween-20 50 μL

4. 2 μg/mL DAPI in M9

To make a 5 mg/mL DAPI stock solution, dissolve the content of one vial (10 mg) in 2 mL of water. For long-term storage the stock solution can be aliquoted and stored at $\leq -20^{\circ}\text{C}$. For short-term storage the solution can be kept at $2\text{--}6^{\circ}\text{C}$, protected from light.

5. 2% (w/v) agarose

Dissolve 500 mg of agarose in 25 mL of water.

Note: Warm up the solution to near-boiling temperature to dissolve the agarose. We suggest using a microwave for this step.

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Competing interests

The authors have no competing interests to declare.

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Collection of *in vivo* Capacitated Sperm from Different Locations Along the Reproductive Tract of Time-Mated Female Mice by Microdissection

Lukas Ded^{1, 2, *} and Jean-Ju Chung^{1, 3, *}

¹Department of Cellular & Molecular Physiology, Yale School of Medicine, New Haven, USA

²Laboratory of Reproductive Biology, Institute of Biotechnology, Czech Academy of Sciences, BIOCEV, Vestec, Czech Republic

³Department of Obstetrics, Gynecology, and Reproductive Sciences, Yale School of Medicine, New Haven, USA

*For correspondence: Lukas.Ded@ibt.cas.cz; jean-ju.chung@yale.edu

Abstract

Mammalian sperm cells are not capable of fertilizing an egg immediately after ejaculation; instead, they must gradually acquire the capacity to fertilize while they travel inside the female reproductive tract. Sperm cells are transported by the muscular activity of the myometrium to the utero-tubal junction (UTJ) before entering the oviduct where they undergo this physiological process, termed capacitation. Since the successful emulation of mammalian sperm capacitation *in vitro*, which led to the development of *in vitro* fertilization techniques, sperm capacitation and gamete interaction studies have been mostly carried out under *in vitro* conditions. Sperm cells are typically incubated *in vitro* for up to several hours at a concentration of more than 1 million cells per milliliter in the capacitation media inside a 37°C incubator with 5% CO₂, mimicking the tubal fluid composed of serum albumin, bicarbonate, and Ca²⁺. The resultant sperm are functionally and molecularly heterogeneous with respect to acrosome reaction, motility, and phosphorylation. By contrast, *in vivo* sperm capacitation occurs in a time- and space-dependent manner, with limits on the number of capacitating sperm in the oviduct. The small number of sperm at the fertilization site *in vivo* are highly homogeneous and uniformly capable of fertilization. This discrepancy makes the degree of correlation between the changes observed from *in vitro* capacitation as a population average and the fertilizing capacity of sperm less clear. To overcome this issue, we used CLARITY tissue clearing to visualize sperm directly inside the female tract *in situ* and isolated sperm capacitated *in vivo* from the oviducts of the female mice after timed mating (Ded *et al.*, 2020). Here, we present a step-by-step protocol to collect *in vivo* capacitated sperm by detailing a microdissection technique and subsequent preparation steps for fluorescent imaging. The advantage of the microdissection technique over *in vitro* capacitation is the ability to collect physiologically segregated, homogeneous sperm populations at different stages of capacitation. Compared to CLARITY, this technique is more straightforward and compatible with a broader spectrum of antibodies for downstream imaging studies, as it allows the researcher to avoid a potentially high background from non-sperm cells in the tissue. The disadvantage of this technique is the potential contamination of the isolated sperm from different regions of the oviduct and disruption of the fine molecular structures (e.g., CatSper nanodomains) during sperm isolation, especially when the preparation is not performed swiftly. Hence, we suggest that the combination of both *in situ* and *ex vivo* isolated sperm imaging is the best way how to address the

Keywords: Mice, Oviduct, Sperm, Microdissection, Timed mating, Microscopy

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Background

The discovery of sperm capacitation occurred in the 1950s when researchers failed to fertilize eggs *in vitro* by directly adding sperm to the oocytes without any prior incubation in media of similar composition close to the oviductal fluid (Austin, 1951; Chang, 1951). Since then, a vast majority of the existing information about the physiological and molecular processes surrounding capacitation was obtained using *in vitro* systems, which were first used in the 1970s for the conception of the first child through *in vitro* fertilization (IVF) (Steptoe and Edwards, 1976). Nowadays, up to 10% of children in developed countries are conceived by assisted reproductive technologies such as IVF and intracytoplasmic sperm injection (ICSI). With this development, there is an even greater need to obtain detailed knowledge about the natural capacitation process and to understand its differences from *in vitro* capacitation. This protocol enables researchers to study the molecular features of sperm at the different stages of the capacitation process by isolating sperm from different locations along the reproductive tract (UTJ, isthmus, and ampulla) of time-mated female mice. This protocol can also be applied to recover freshly ejaculated sperm from mice (Li *et al.*, 2015; Chung *et al.*, 2017) as epididymal sperm are normally used for mouse experiments, as opposed to ejaculate sperm for large domestic animals or humans.

Materials and Reagents

1. Mini Petri dishes, 30 mm (Sigma-Aldrich, catalog number: SLW1480/12D)
2. Microsurgical scalpel (Stab Knife, 22.5 Degree, Straight; Sharpoint, catalog number: 72-2201)
3. Glass microcapillaries (50- μ l calibrated pipets; Drummond, catalog number: 2-000-050)
4. Aspirator tube assemblies for calibrated microcapillary mouth pipette (Sigma-Aldrich, catalog number: A5177-5EA)
5. Syringe filter (0.2 μ m; Pall Life Sciences, catalog number: PN 4612)
6. EprediaTM PTFE Diagnostic Slides, 3-well (Thermo Fisher Scientific, catalog number: 10632391)
7. EprediaTM PTFE Diagnostic Slides, 8-well (Thermo Fisher Scientific, catalog number: 10727951)
8. Animals: C57BL/6J female mice (3-5 weeks old)
9. Gonadotropin from pregnant mare serum; PMSG (Sigma-Aldrich, catalog number: G4877)
10. Chorionic gonadotropin human; hCG (Sigma-Aldrich, catalog number: CG10)
11. Fibronectin [Sigma-Aldrich, catalog number: F1141-2MG (solution; from bovine plasma) or 11051407001 (powder; from human plasma)]
12. M2 medium (Sigma-Aldrich, catalog number: M7167)
13. Mineral oil (Sigma-Aldrich, catalog number: M8410)
14. PBS (Sigma-Aldrich, catalog number: P4417)

Equipment

1. An animal room with a 12-hour light/dark cycle, red-light option, and a light timer that allows the researcher to shift the light schedule to be more compatible with normal working hours
2. Stereo microscope (Nikon, model: SMZ1270)

Procedure

A. Timed mating of female mice in the estrous stage

1. For timed mating, hyper-stimulated (super-ovulated) or non-stimulated female mice can be used. The hyper-stimulation procedure increases the chance of successful timed mating.

- a. Hyper-stimulation: Hyper stimulate female mouse (22-24 days old optimally but up to 35 days old) with an intraperitoneal (i.p.) 5-IU injection of PMSG, followed by a 5-IU i.p. injection of hCG 48 h after PMSG. Calculate the time of both injections to start timed mating 12-14 h after the hCG injection. The protocol can be modified according to the animal stock provider's suggestions (e.g., The Jackson Laboratory; <https://www.jax.org/>). Alternatively, the estrous cycle can be determined by examining washes of cell smears of the vagina to increase the success rate of the timed mating.
 - b. Determining the stage of the estrous cycle in female mice is described in detail in Champlin *et al.* (1973) and Caligioni (2009).
2. Introduce a male to a female mouse housed in the single cage during the last dark hour of the 12 h long dark cycle (30 min to 1 h). Work under red light when handling mice for mating to not disrupt the light/dark cycle.
 3. Check the presence of vaginal plugs as soon as the light cycle begins (set this time as 1 h post-coitus). Females with plugs can be used for the experiment at a desired time point (*i.e.*, 3 h or 7 h post-coitus). Save females without plugs as they can be used for another run of timed mating.
 4. Hormone injection and mating time should be customized according to the availability of the animal room with flexible light/dark cycle adjustment and the experimenter's schedule. The following example is based on the lack of such facility and flexible schedule of an experimenter: In a facility with a 12-h light/dark cycle with the light on at 7 am and off at 7 pm, PMSG can be injected on Day 1 at 6 pm, followed by 6 pm hCG injection on Day 3. At 6 am (12 h after hCG) on Day 4, start timed mating under red light without disrupting the light/dark cycle by introducing a male to females. When the light is on at 7 am, the females are allowed to mate for 2 h. In the next 30 min or so, check the plug and sacrifice the plugged females at 9:30-10:30 am for collecting *in vivo* capacitated sperm from 3-4 h post-coitus or 4:30-5:30 pm for collection *in vivo* capacitated sperm at 7-8 h post-coitus.

B. Microdissection and isolation of *in vivo* capacitated sperm

1. After the sperm remain inside the female reproductive tract for a sufficient duration to allow *in vivo* capacitation, sacrifice the female mice with the confirmed vaginal plugs either by CO₂ asphyxiation or cervical dislocation.
2. Under the well-lit stereo microscope, grip the fat pad around the ovary and stretch gently to clearly identify the membranous boundary between the ovary and the oviduct (*line 1*) and the oviduct and the uterus (*line 2*) (Figure 1).
3. Carefully cut through the membrane around the proximal oviduct to slightly de-coil the oviduct first (*line 2*), followed by the membrane around the bursa and just outside infundibulum (*line 1*), and lastly, cut through the uterus just outside UTJ (*line 3*) (Figure 1).

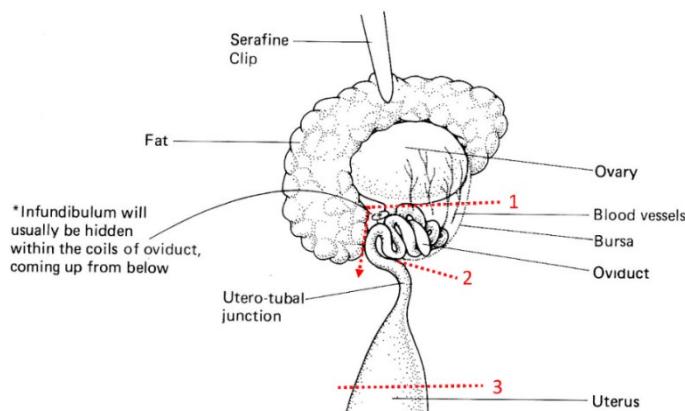


Figure 1. Scheme of mouse female reproductive tract dissection.

Drawing slightly modified from Nagy (2003) in *Manipulating the Mouse Embryo*.

4. The trimmed female tract (*right*) and the number and distribution of *in vivo* capacitated sperm (*left*) will look like Figure 2A.
5. (Optional) If Acr-EGFP or Su9-DsRed/Acr-EGF mice are used, the distribution and number of sperm can be validated under the fluorescent microscope (Figure 2B).

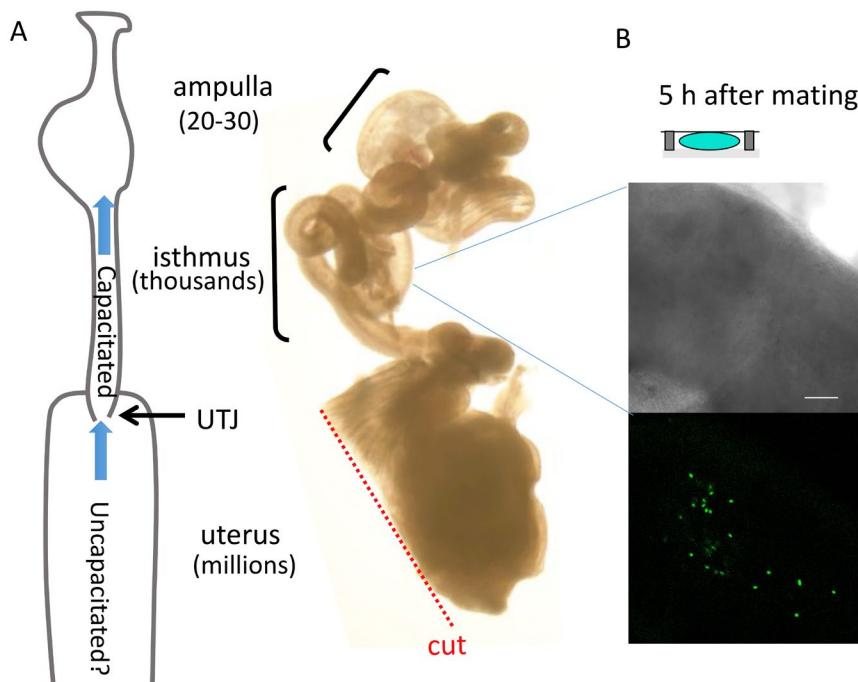


Figure 2. Distribution of sperm along the female reproductive tract.

(A-B) Number of sperm recovered from each region of the female tract, aligned with an actual example image of a dissected female tract as described. Scale bar, 50 μ m. (C) Validating location and number of Acr-EGF sperm in the isthmus with fluorescence microscopy.

6. Place individual trimmed tract into a 0.3 mL-droplet of M2 media under mineral oil (Figure 3A) and further dissect them carefully with a microsurgical scalpel into ampullar, middle isthmus (2-3 mm), and UTJ parts (Figure 3B).
7. Immediately place each part into an individual a 100- μ L droplet of M2 media in a mini-Petri dish under mineral oil and gently press to release sperm (Figure 3B).

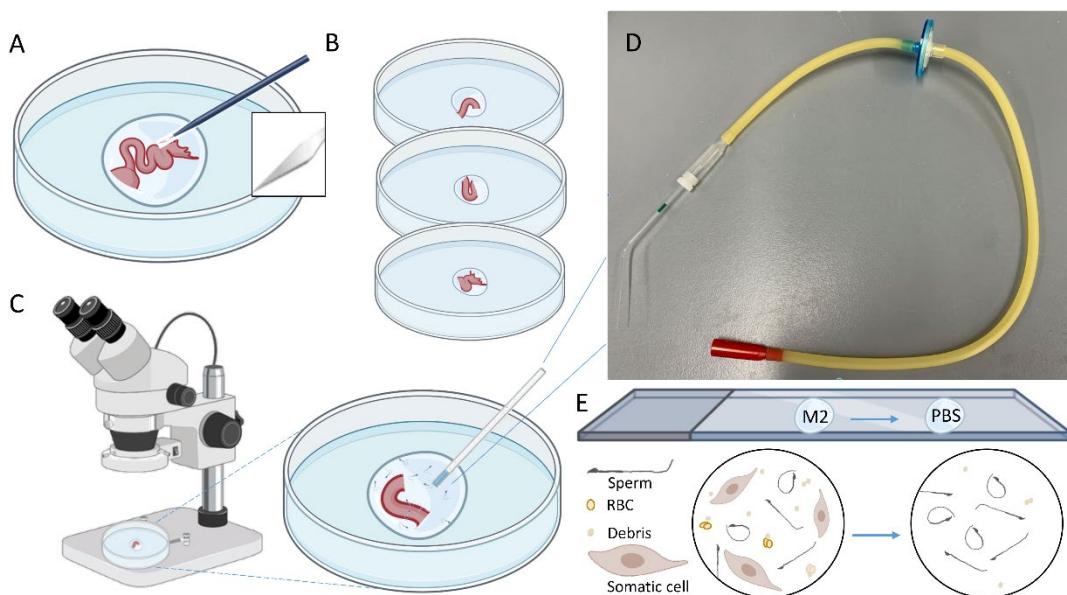


Figure 3. Microdissection and aspiration of sperm from different regions of the female reproductive tract.
 (A) Oviduct dissected by microsurgical scalpel placed into a 0.3-mL M2 droplet. (B) Placing individual oviductal parts into a 0.1-mL M2 droplet. (C) Observation under stereo microscope and picking sperm by glass microcapillary. (D) A setup of mouth aspiration pipette with a glass capillary tube and a filter. (E) A schematic cartoon representation of visible components of the droplet under the microscope before and after additional transfer/washing.

8. Observe the droplets (optimally by three persons) under (stereo) microscopes (e.g., for 10 min) and pick individual sperm with an aspirator connected to a filter in the middle and 50-ul glass microcapillary by mouth pipetting (Figure 3C and 3D).
9. Depending on the speed of dissection and handling of the tissue, there will be other cell types, such as discharged epithelial cells and red blood cells (RBC), and debris together with sperm cells (Figure 3E). As sperm cells are smaller than these somatic cells (except fine debris), transferring the liquid to the 2nd droplet by avoiding these contaminants can help to obtain a preparation more enriched with sperm cells.

C. Slide preparation

1. Coat the surface of the slides with fibronectin. (A) Method A uses the fibronectin solution from bovine plasma: take 50-100 μ L directly from the vial and spot onto the surface multiples times, making a domed solution with the desirable size. After 2-5 min when the edge of the dome is about to dry, rinse the coating solution with water. The slide/coverslip is ready to use for the next step. Method A does not require additional incubation time. (B) Method B prepares fresh fibronectin solution from fibronectin powder from human plasma. Reconstitute in sterile water by incubating for 30 min at 37°C to yield 1 mg/mL stock solution. Make small aliquots and keep frozen at -20°C until use. Prepare a fresh working solution (50 μ g/ml in water) from an aliquot and apply 50 μ L per well (3-well slide) or 10 μ L per well (8-well slide). Incubate the slide/coverslip in a humid chamber for ~1 h. Rinse the coating solution with water and keep the slides in the chamber prior to sperm deposition. The time between the coating and sperm deposition should be as short as possible. Ideally, the whole procedure should be tested to optimize the cell attachment and reduce the Background for the subsequent immunofluorescent microscopy due to the lot-to-lot variation of fibronectin.
2. Place the picked-up sperm to 10 μ L PBS droplet on the fibronectin-coated 8-well Teflon slides (Figure 4A: At a minimum, an average of 10 sperm per sample should be obtained). If too much tissue material is

transferred by micropipette, a PBS washing step can be added (Figure 3E).

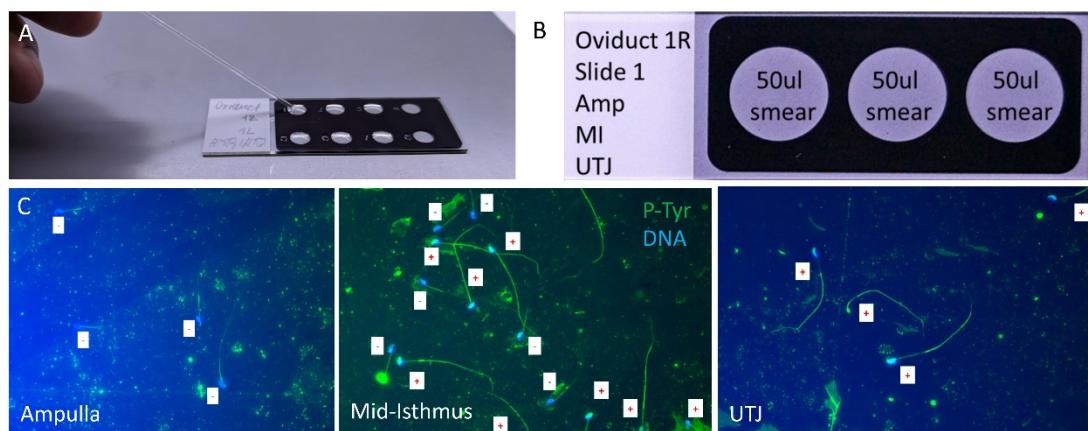


Figure 4. Slide preparation for downstream experiments and example images.

(A) Sperm transfer by glass micropipette into 8-well Teflon microdroplet slides. (B) An example of setting up a 3-well Teflon smeared slide.

3. Subject the rest of the material to finer dissection, remove macroscopic pieces manually, mix the rest of the material with PBS (material/PBS 1:10), and smear it on the fibronectin-coated 3-well Teflon slides (Figure 4B: ~30-50 μ L per well).

Data analysis

Obtained microscopic slides can be subjected to classical procedures for immunofluorescent staining, followed by confocal or super-resolution imaging. Shown here is an example of raw data (Figure 4C) used for P-Tyr quantification shown in Ded *et al.* (2020) to illustrate a downstream experimental outcome.

Notes

Mice hyperstimulation and vaginal smear analysis procedures are complex general protocols, and their detailed description can be found in provided references/links.

Acknowledgments

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Competing interests

Authors declare no competing interests.

Ethics

This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All the mice were treated in accordance with guidelines approved by Yale (protocol number: 20079, valid 2015-current) Animal Care and Use Committees (IACUC).

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Targeting the Expression of Long Noncoding RNAs in Murine Satellite Cells from Single Myofibers

Martina Macino^{1, 2, \$a}, Beatrice Biferali^{1, 2, \$b}, Andrea Cipriano^{2, \$c}, Monica Ballarino² and Chiara Mozzetta¹
*

¹Institute of Molecular Biology and Pathology (IBPM), National Research Council (CNR) at Sapienza University of Rome, Rome, Italy

²Department of Biology and Biotechnologies “Charles Darwin”, Sapienza University of Rome, Rome, Italy

\$aCurrent address: Berlin Institute for Medical Systems Biology, Max Delbrück Center for Molecular Medicine in the Helmholtz Association, Berlin, Germany

\$bCurrent address: Gene Expression and Muscular Dystrophy Unit, Division of Genetics and Cell Biology, IRCCS San Raffaele Scientific Institute, Milan 20132, Italy

\$cCurrent address: Department of Obstetrics & Gynecology, Institute for Stem Cell Biology & Regenerative Medicine, Stanford University, 265 Campus Drive, Stanford, 94305, United States

*For correspondence: chiara.mozzetta@uniroma1.it

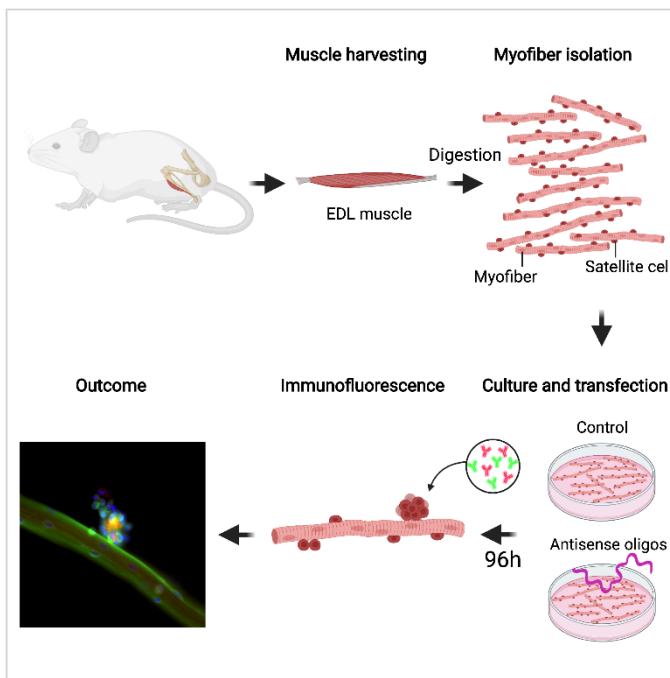
Abstract

LncRNAs have been recently implicated in the epigenetic control of muscle differentiation and their functional characterization has traditionally relied upon *in vitro* models of myogenic differentiation. However, the use of experimental paradigms to specifically target lncRNAs expression in muscle stem cells (MuSCs), also known as satellite cells, represents an important requisite to interrogate their function in more physiological contexts. Since isolation and culture of single myofibers preserves satellite cells within their physiological niche underneath the surrounding basal lamina, this procedure represents the optimal approach to follow satellite cell dynamics *ex-vivo*, such as activation from quiescence, expansion of committed progenitors, differentiation, and self-renewal. Here, we detail an optimized protocol to isolate viable single myofibers from the extensor digitorum longus (EDL) skeletal muscle of adult mice and to manipulate the expression of lncRNAs by antisense LNA GapmeRs-mediated knockdown (KD). Furthermore, we describe a method of EdU incorporation that, coupled to lncRNA KD and subsequent immunofluorescence analysis of proliferating, differentiating, and satellite cell-specific markers, permits the inference of lncRNAs function on muscle stem cells dynamics.

Keywords: Single myofiber, Mouse, Skeletal muscle, Satellite cells, Knockdown, LncRNA, EdU, Immunofluorescence

This protocol was validated in: eLife (2021), DOI: 10.7554/eLife.54782

Graphical Abstract:



Graphical representation of the single myofiber isolation method.

Experimental workflow showing the main steps of the protocol procedure: EDL muscle harvesting from the mouse hindlimb; EDL digestion into single myofibers; transfection with antisense oligos and culture for 96h; immunofluorescence protocol and image outcome.

Background

Long noncoding RNAs (lncRNAs) represent a class of transcripts longer than 200 nucleotides, which are mostly devoid of protein-coding capacity (Rinn and Chang 2012; Fatica and Bozzoni, 2014). They are found both in the cytoplasm and in the nucleus, and modulate gene expression by influencing genome organization *via* epigenetic, transcriptional, and post-transcriptional mechanisms (Quan *et al.*, 2015; Cai *et al.*, 2016). As such, they are emerging as critical modulators of several biological processes, including stem-cell mediated regeneration and differentiation.

LncRNAs have been recently implicated in the epigenetic control of different steps of muscle differentiation (Lu *et al.*, 2013; Ballarino *et al.*, 2015 and 2018; Zhu *et al.*, 2017; Desideri *et al.*, 2020). However, examination of lncRNA function during myogenesis has traditionally relied on ablation studies performed on established myoblast cell lines (*i.e.*, C2C12) or primary cells cultured in proliferating (high-serum) and differentiating (low-serum) conditions. These experimental settings allow discrimination of the role of lncRNAs only in the proliferation and differentiation steps, preventing their functional characterization on other crucial phases of *in vivo* myogenesis. Indeed, formation of new skeletal myofibers *in vivo*, or repair of damaged ones, relies on the activity of specialized adult muscle stem cells (MuSCs), located at the periphery of myofibers, between the sarcolemma and the basal lamina, that have been usually referred to as satellite cells (Mauro, 1961). In resting adult muscles, satellite cells are present in a post-mitotic quiescent state. However, upon injury, satellite cells activate and proliferate to expand a pool of committed myogenic progenitors that, upon expression of muscle-regulatory factors, enter the muscle differentiation program to form new muscle fibers (Figure 1). A small fraction of the expanded pool avoids terminal differentiation to self-renew and replenish the pool of quiescent stem cells (Biferali and Mozzetta, 2019).

Therefore, to infer the function of lncRNAs along the different stages of satellite cell-mediated myogenesis, it is important to rely on an experimental model that allows for monitoring of satellite cell activation from the quiescent

state to their expansion and commitment to differentiation, and even to self-renewal (Mozzetta, 2016). To this end, we optimized previous protocols to isolate intact and viable single myofibers (Pasut *et al.*, 2013; Mozzetta, 2016; Pegoli *et al.*, 2020) from the extensor digitorum longus (EDL) muscle of wild-type mice for the purposes of subsequent manipulation of lncRNAs expression and phenotypical characterization by EdU incorporation and immunostaining (Cipriano *et al.*, 2021).

This isolation procedure preserves satellite cells within their physiological niche underneath the surrounding basal lamina. Thus, once cultured *ex-vivo* in non-adherent conditions and in the presence of serum and growth factors, satellite cells associated with the myofibers start to proliferate, giving rise to a progeny visible as a round group of cells attached to the fiber (Figure 1). This cluster represents the progeny of a single satellite cell that, upon 72–96 h of culture, comprises either differentiating myoblasts or self-renewing cells. The protocol described here details how to interfere with lncRNAs expression by LNA GapmeRs-mediated knock-down and optimization of subsequent immunostaining assays for assessment of MuSCs-associated myofibers.

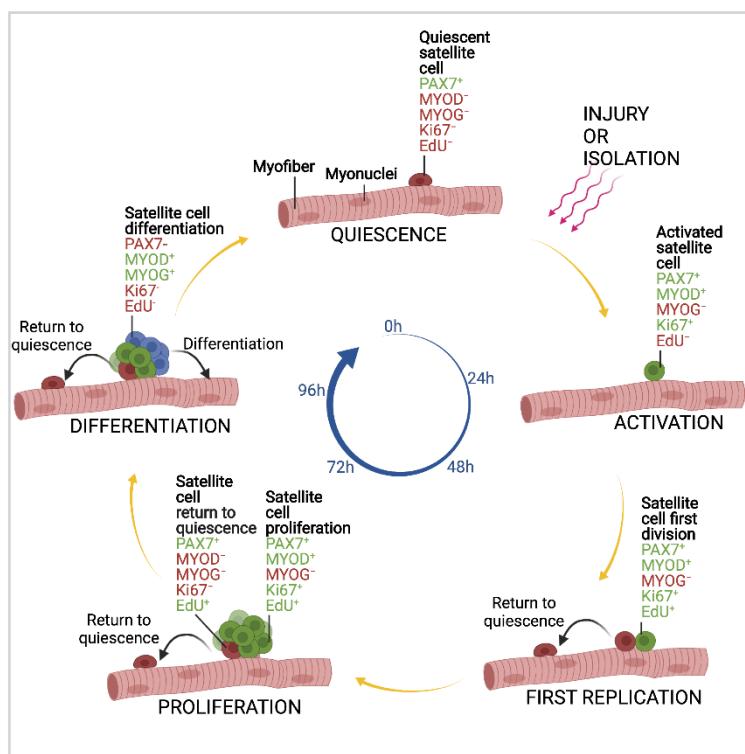


Figure 1. Satellite cells are present in a post-mitotic quiescent state ($PAX7^+$; $MYOD^-$; $MYOG^-$; $Ki67^-$; EdU^-) on single myofibers.

Upon injury or isolation, satellite cells become activated and start to express early muscle-regulatory factors (such as MYOD), undergoing the first replication within 48 h, in which one cell is a committed myogenic progenitor, while the other one can return to quiescence. The activated cell starts to proliferate, creating a cluster of round cells that then enter the muscle differentiation program by expressing Myogenin (MYOG) to form new muscle fibers.

Materials and Reagents

1. 2 mL tube (Eppendorf, catalog number: 0030.120.094)
2. 1.5 mL tube (Eppendorf, catalog number: 0030.120.086)
3. 1,000 μ L Tips (Axygen, catalog number: TF-1000-LRS)
4. 200 μ L Tips (Axygen, catalog number: TF-200-LRS)
5. 50 mL tube (Corning, catalog number: 430829)

6. Syringe filter 0.22 µm (Millipore, catalog number: SLGS033SS)
7. 50 mL syringe (Fisher BD, catalog number: 309653)
8. Petri dish, tissue culture treated (Falcon, catalog number: 353003)
9. 12 well plate, tissue culture treated (Falcon, catalog number: 353043)
10. 60 mm plate, tissue culture treated (Falcon, catalog number: 353002)
11. Cover glasses (Thermo Scientific, Menzel-Glaser 12mm diameter #1)
12. Glass slides (Thermo Scientific, Menzel-Glaser AA00000102E01FST20)
13. C57BL/10 (WT) (JAX, catalog number: 000665)
14. Ice
15. Collagenase I (Sigma, catalog number: C0130)
16. Fibroblast growth factor (FGF) (Gibco, catalog number: PHG0026)
17. Chick embryo extract (CEE) (Life Science Production, catalog number: MD-004D-UK)
18. Anti-Pax7 mouse monoclonal (DSHB, catalog number: Pax7-s [1:10])
19. Anti-Ki67 rabbit polyclonal (Abcam, catalog number: ab15580 [1:100])
20. Alexa Fluor 488, rabbit (Invitrogen, catalog number: A21206 [1:250])
21. Alexa Fluor 594, mouse (Invitrogen, catalog number: A21203 [1:250])
22. Antisense oligos: LNA GapmeRs: non-targeting control (Qiagen, catalog number: 300610) or lncRNA sequence-specific custom GapmeRs
23. Lipofectamine 2000 (Invitrogen, catalog number: 11668019)
24. Optimem (Gibco, catalog number: 31985047)
25. Click-iT EdU Alexa Fluor 594 HCS Assay (Invitrogen, catalog number: C10354)
26. Ethanol (Sigma, catalog number: 32221)
27. Phosphate buffered saline (PBS) (Sigma, catalog number: D8537)
28. Triton-X 100 (Sigma, catalog number: T8787)
29. Glycerol (Applichem, catalog number: A2926)
30. Paraformaldehyde (PFA) (Sigma, catalog number: P6148)
31. Penicillin-Streptomycin (Sigma, catalog number: P0781)
32. 4',6-diamidino-2-phenylindole (DAPI) (Sigma, catalog number: 28718-90-3 [1:10,000])
33. Dulbecco's Modified Eagle Medium (DMEM), high glucose, pyruvate (Gibco, catalog number: 41966-052)
34. Horse serum (Gibco, catalog number: 26050088)
35. Fetal Bovine Serum (FBS) (Corning, catalog number: 35-015-CV)
36. Digestion solution (see Recipes)
37. Wash solution (see Recipes)
38. Growth medium for fibers (see Recipes)
39. IF solutions (see Recipes)

Equipment

1. Micropipette (P200, P1000)
2. Surgical scissors (F.S.T, catalog number: 14060-10)
3. Jewelers' forceps, Dumont No. 5, L 4 1/4 (Sigma, catalog number: F6521)
4. Microscope (Zeiss, model: Stemi DV4)
5. Axio Observer 3 inverted fluorescence microscope (ZEISS)
6. Shaker (Labnet, model: Rocker 25)
7. Vortex (Heidolph, model: Reax 2000)
8. Water Bath (Thermo Scientific, Precision Water Bath)
9. CO₂ Incubator (Euroclone, model: S@fegrow 188)
10. Ice bucket

Software

1. Fiji image processing package (Open-source Software (OSS) projects, <https://imagej.net/Fiji>)
2. ZEN 3.0 (Blue edition, ZEISS)

Procedure

Attention: Keep everything sterile if planning to culture the fibers.

A. Preparation

1. Fill an ice bucket with ice.
2. Prepare “digestion solution,” “wash solution,” and “growth medium for fibers” (see Recipes).
3. Pre-heat the “wash solution” and “growth medium for fibers” solution at 37°C.
4. Coat 2 ml tubes (one tube per sample) with FBS. Add 1 ml of FBS to the tube. Move it up and down until the sides of the tube are completely covered, and then discard the FBS.

Note: The tube needs to be coated to keep the myofibers in suspension; otherwise, they will stick to the tube’s wall. We use FBS for coating as this will also be in the growth medium.

5. Add 2 ml of the “digestion solution” to each coated tube. Keep it on ice.
6. Pre-heat the water bath at 37°C.

B. EDL isolation

Attention: This step must be performed as fast as possible before rigor mortis. For this reason, we suggest performing EDL isolation from one mouse at a time.

Note: Always handle the muscle from tendon to tendon and avoid touching the bulk of the muscle to preserve myofiber integrity.

1. Sacrifice the mouse using a method approved for your research, such as CO₂ or cervical dislocation.
2. Wet the skin and the fur of the mouse with 70% ethanol.
3. Position the mouse under the hood. Pull up and cut the skin from the ankle to remove the skin and to expose the tibialis anterior (TA) and the extensor digitorum longus (EDL) (Video 1).



Video 1. Procedure for EDL isolation.

This video was made at the Department of Biology and Biotechnologies of University Sapienza, according to guidelines approved by the Institutional Animal Care and Use Committee of the Department of Biology and Biotechnology of University Sapienza, the Italian Ministry of Health, and local authorities according to Italian law; Protocol N° 7FF2C.4—Authorization N° 746/2016-PR.

4. Carefully cut the tendon of the TA muscle from the ankle, grab the loose tendon with the tweezers, and gently pull up the muscle. Then cut the TA muscle under the knee to expose and have a good visualization of the EDL muscle (Video 1).
5. Cut the tendon of the EDL at the base, just over the ankle, and gently pull the muscle until it remains attached only by the tendon on the knee. By keeping the EDL from the ankle tendon, isolate the muscle cutting the tendon over the knee (Video 1). Place the muscle inside a coated tube with the “digestion solution” and leave the tube on ice.
6. Repeat Steps B4 and B5 with the other leg. Add the second muscle to the coated tube with the “digestion solution” and place it on ice.

C. Digestion

1. Incubate the tube from 45 min to 1 h in a pre-warmed bath at 37°C. Gently shake every 10 min.

Note: Invert the tubes a couple of times; do not shake vigorously to avoid breaking the myofibers.

Tip: It is possible to see the state of the digested muscle looking through the tube against a source of light. The muscle should be in a loose state.

2. At the end of the incubation, give a strong shake to release the myofibers from the digested muscle.

Tip: It is possible to see the released myofibers looking through the tube against light before and after the final shake.

D. Single myofiber isolation

Attention:

1. *This step needs to be performed as fast as possible to avoid fiber contraction. When the myofibers are kept for too long on the “digestion plate,” they will start to shrink and contract, leading to myofiber damage.*
2. *To avoid contraction, pre-heat all the solutions to 37°C. The myofibers are sensitive to temperature and should be kept constantly at 37°C.*

Tip: If it is possible, perform myofiber isolation from one mouse at a time. In any case, keep all the plates containing the fibers in the incubator while they are not being used.

1. Prepare two 60 mm plates per mouse with 4 mL of pre-heated “wash solution” for the cleaning step.
2. Transfer the digestion solution containing the fibers to one of the 60 mm plates with the “wash solution.”

Note: Myofibers should appear long, smooth, unbroken, and slightly translucent (Figure 2, red arrow).

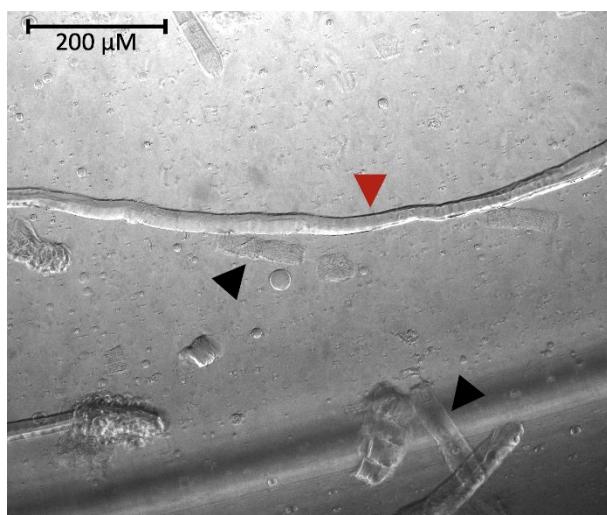


Figure 2. Representative image of a newly isolated single myofiber (red arrow) in the washing plate. Black arrows indicate broken pieces that should be avoided when collecting the single myofiber.

3. Coat the tip of a P200 micropipette by pipetting one time with the “wash solution.” Under the dissecting microscope (Zeiss; Stemi DV4), with the coated tip, take one by one every single myofiber in the direction of the fiber to avoid damage, collect multiple fibers in the tip, and gently release them into the second clean 60 mm plate containing 4 mL of pre-heated “wash solution” (cleaning step).
Tip: Slightly slope the plate (and consequently move the media) to better visualize the translucent myofibers.

Attention:

- a. Avoid the broken or contracted pieces (Figure 2, black arrow) and take only the intact single myofibers.
- b. Critical step! Be as gentle and as fast as possible. All the cleaning procedures over the hood should not take more than 10 min.

4. After picking all the visible intact myofibers, gently pipette the digested muscle to release the myofibers still attached to it. Under the microscope, it is possible to see the myofibers being released.
Tip: It is possible to perform this step using the tip of a P1000 micropipette cut with scissors or with a sterile glass Pasteur pipette previously broken at the neck.

Note: Be sure to coat the tip with the media before using it.

5. When more myofibers are released, pick out single myofibers with the coated tip of a P200 micropipette (as done in Step D3) and place them in the 60 mm plate used before in the cleaning step. In this case, also avoid the broken pieces, taking only the single myofibers.
6. When all the myofibers are in the cleaning plate, pick single myofibers in the direction of the fiber and plate them in the final dish (e.g., 24 well plate ~10-15 fiber/well or 12 well plate ~20-25 fiber/well) for culture with the pre-heated “Growth medium for fiber.”
Return the plates to the incubator at 37°C.

Attention: The myofibers are cultured in suspension, so the volume should not be too small to allow fibers to float in the medium (e.g., in a 12 well plate, it should not be less than 1 mL).

The fibers can be fixed and analyzed immediately or cultured for satellite cell amplification.

E. Culture and transfection

Attention: The myofibers are sensitive to changes in temperature.

1. Keep the myofiber in the incubator for at least 4 h before transfection. The first hour after plating is critical, so keep the myofibers in the incubator to stabilize them.
2. To perform the transfection, mix 1 μ L/mL of lipofectamine with 49 μ L of Optimem in one 1.5 mL tube per sample. In another 1.5 mL tube, add 1 μ L/mL of antisense oligos (GapmeRs) from a stock at a concentration of 50 μ M per sample and mix 49 μ L of Optimem. After 5 min, mix the content of the lipofectamine tube with the content of the GapmeRs tube and wait for 20 min. Pipet 100 μ L of the mix directly to the culture media in each well, drop by drop. Incubate overnight (13-14 h).

Notes:

- a. The final volume should be 1 mL per well (for a 12 well plate).
- b. We strongly recommended not performing more than one overnight (13-14 h) transfection to avoid myofiber disruption.

3. In the morning, change the media, paying attention to not remove fibers. To this end, gently remove as much medium as possible by tilting the plate on one edge and aspirating the medium from the opposite side. Always leave some media in the plate and add new pre-heated “growth media for fibers” on top of the plate, drop by drop. Let the myofibers sink for 10 min (preferably in the incubator), and then repeat the procedure once or twice more.

Note: Aspire the media from the surface very gently to avoid loss of the suspended myofibers.

4. Leave in culture until the desired time point. It is safe to keep them in culture for at least 96 h after plating (Figure 3).

Note: If left only for 96 h in culture, there is no need for media change to prevent fiber loss. For longer culture, we suggest changing the media every 2-3 days.



Figure 3. Experimental timeline used for the culture and transfection of single myofibers and EdU incorporation pulse.

F. EdU incorporation

1. Add 1 μ L/mL of EdU directly to the media, as indicated in the manufacturer's instructions.
2. The recommended pulse is 24 h (Figure 3).

Note: The pulse should start 24 h before the selected time point.

G. Myofiber fixation

Attention: The myofibers are in suspension, so, after each step, let the fibers settle to the bottom for 10 min and then gently remove the supernatant close to the surface.

Note: Myofiber fixation is performed in the well.

1. Gently remove 500 μ L of media from each well, paying attention not to remove fibers.

Attention: The fibers should be left in ~500 μ L.

2. Add 1 mL of PFA 4% directly in the media to obtain a final concentration of 3% PFA. Leave for 30 min at room temperature.
3. Remove as much PFA as possible and add 1 mL of PBS to wash the myofibers for 10 min. Repeat this washing step three times. Then leave the myofibers in at least 1 mL of PBS.

Note: It is possible to keep them at 4°C for a few weeks. To avoid PBS evaporation, carefully close the lid of the plate with parafilm and, if EdU incorporation was performed, keep the plate in the dark.

H. Edu detection and immunofluorescence

Note: For immunofluorescence analysis, transfer approximately 50 myofibers in 2 mL tubes per condition.

1. Coat a 2 mL tube/each sample with FBS (as described in Step A3).
2. Under the microscope, remove as much supernatant as possible, and then pick the single myofibers (in the direction of the myofiber) with a coated P200 micropipette tip and transfer them to the bottom of the 2 mL coated tube.

Attention: When pipetting the myofibers, always coat the micropipette tip with FBS to prevent myofiber loss or disruption (they tend to attach to the sides of the uncoated tube or tip).

3. Let the fibers settle down to the bottom of the tube for 10 min and then remove the supernatant. Leave the myofibers in no less than 150 μ L of PBS.
Tip: It is a good trick to compare the volume with another 2 mL tube containing 150 μ L of water.
4. Add 500 μ L of Triton 0.5% to each tube and incubate for 10 min at room temperature.
5. Remove the supernatant and wash with 1 mL of PBS for 10 min.
6. For EdU detection, myofibers are stained using Click-iT EdU Alexa Fluor 594 HCS Assay (Invitrogen):
 - a. Dilute the 10 \times solutions (Click-iT EdU buffer additive Component E 10 \times and Click-iT reaction buffer Component C 10 \times) provided in the kit ten times to 1 \times by adding the proper volume of water (e.g., 50 μ L of E 10 \times + 450 μ L of water).
 - b. Then prepare the Click-iT reaction cocktail mix, mixing 425 μ L of Component C solution 1 \times , 20 μ L of CuSO₄, 1.25 μ L of Alexa Fluor azide, and 50 μ L of Component E solution 1 \times to obtain ~500 μ L of the mix.

Note: Reagents should be added to the mix in this specified order.

- c. Then add 200 μ L of cocktail mix per sample of fibers to be stained. Incubate 30 min in the dark at room temperature, and then wash with 200 μ L of reaction rinse buffer provided in the kit.

Notes:

- i. Perform the EdU detection assay before standard immunofluorescence.
- ii. If you perform EdU detection, all the steps of the immunofluorescence should be performed in the dark.

7. Remove the supernatant and wash with 1 mL of PBS for 10 min.

8. Remove the supernatant and add 1 mL of block solution (10% FBS in PBS). Leave for 1 hour at room temperature, preferably on a shaker in slow agitation.
9. Let the tube settle for 10 min in a vertical position and then remove the supernatant, leaving the myofibers in ~150 µL. Add the primary antibodies diluted in 10% FBS in PBS (Ki67 at 1:100 and Pax7 at 1:10). The final volume should not be less than 300 µL. Incubate the tube overnight (13-14 h) at 4°C.
Tip: When preparing the mix with the antibodies, we recommend calculating the needed concentration of the antibody for the final volume but adding 150 µL less of FBS 10% in the mix (this volume is already present in the tube with the myofibers).
10. Remove the supernatant and wash twice with 1 mL of 0.1% FBS in PBS for 10 min.
11. Remove the supernatant, leaving the myofibers in ~150 µL. Add the secondary antibodies diluted in 10% FBS in PBS (Alexa Fluor 488 and 594, both at 1:250). The final volume should not be less than 300 µL. Incubate for 1 hour at room temperature in the dark.
12. Remove the supernatant and wash once with 1 mL of 0.1% FBS in PBS for 10 min.
13. Remove the supernatant and wash once with 1 ml of PBS for 10 min.
14. Add 500-800 µL of DAPI (1:10,000 in PBS) and incubate for 10 min at room temperature.
15. Remove the supernatant and perform two washes with 1 ml of 0.1% FBS in PBS.
16. Mount the fibers on the cover glass by removing all the supernatant and leaving the myofibers in as little volume as possible.
17. Add 5-10 µL of Glycerol:PBS. Cut a small piece of the tip of a P200 micropipette with scissors and coat it with FBS. Use it to gently take the myofibers from the bottom of the 2 ml tube and place them on the glass cover, drop by drop, spanning the center of the glass slide.

Note: This is a critical step, so be careful not to discard myofibers that might remain attached to the tip. Check the tip content before discarding it, by looking inside the tip against a source of light. If necessary, pipette more Glycerol:PBS.

Tips:

- a. It is possible to see the myofibers on the glass slide at this stage.
- b. Check the bottom of the 2 mL tube, looking through it against a light source to ensure that there are no myofibers left.
18. Delicately close the cover glass with a rectangular coverslip.
19. Let the mounted fibers dry in the dark and then seal the cover glass on the sides with nail polish.
20. Image with a fluorescence microscope (Figure 4).

Note: It is suggested to use a 40× or 63× objective to visualize the satellite cell clusters and 10× or 20× objective for the entire myofiber.

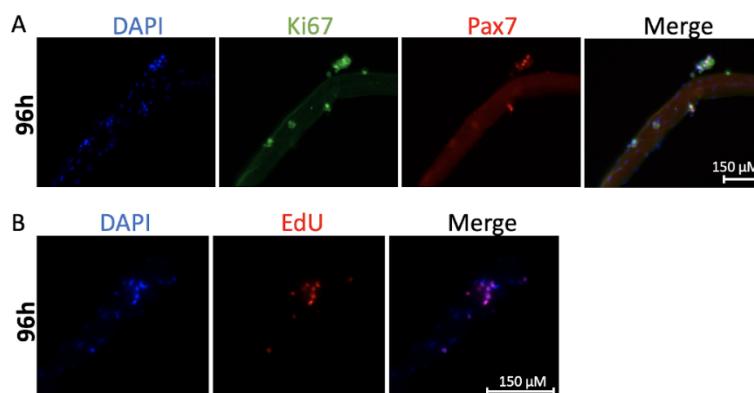


Figure 4. Example of immunofluorescence images obtained with our protocol.

A. Representative images of immunofluorescence performed on a single myofiber kept in culture for 96 h.

Ki67, proliferation marker (green) and Pax7, satellite cells specific marker (red). B. Representative images of EdU detection (red) in a single myofiber kept in culture for 96 h and with a 24 h EdU pulse. DAPI (blue) was used to visualize the nuclei.

Data analysis

The data obtained with the single myofiber immunofluorescence and EdU detection can be used to perform different analyses, depending on the biological question that is being addressed.

For example, we wanted to understand the role of LncRewind in the context of the activation and proliferation of satellite cells (Cipriano *et al.*, 2021), so we performed co-staining with Pax7, a specific marker for satellite cells, and Ki67, a marker for proliferation, on the single myofibers isolated from five mice. To obtain statistically significant results, we suggest quantifying at least 50 fibers per condition, isolated from a minimum of three mice. Exclude short and broken myofibers from the analysis.

For the statistical analysis, apply unpaired Student's *t*-tests when comparing two conditions and one-way Anova with Tukey's multiple comparison test when comparing more than two conditions.

We used the data to execute different analyses:

1. We performed a manual count under the microscope of the number of Pax7⁺ cells and Pax7⁺Ki67⁺ double positive cells per cluster, comparing the results among the different conditions (Control vs. KD). For the statistical analysis, we compared 40 clusters per condition.
2. We also counted the mean number of clusters [composed by a number of nuclei (n) > 2], pairs (n = 2), or single Pax7⁺ (n = 1) cells per fiber. For the statistical analysis, we compared 80 fibers per condition.
3. We also compared the total number of Pax7⁺ cells per fiber between control and KD. In this case, we used the data from 50 fibers per condition for the statistical analysis.

Notes

1. There is great variability among different mice, even with the same sex and age.
2. The mean number of myofibers that we collect per experiment is approximately 100 per mouse, from two EDL muscles, but approximately 20% do not survive during culture.
3. We also noticed that performing this procedure with more than three mice at a time can be detrimental, as the cleaning step after isolation should be performed quickly to avoid fiber contraction.
4. We also performed myofiber isolation from gastrocnemius muscle, dividing it into four longitudinal pieces. However, in our hands, the results are cleaner and the myofibers are stronger just with the EDL muscle. We found that the EDL muscle was ideal to use in this procedure because its small size allows the digestion of the entire muscle without the need for manual trituration that could cause myofiber damage.

Recipes

1. Digestion solution (2 mL per mouse)

Collagenase I 10 mg
DMEM high-glucose + Pyruvate (Gibco) 2 mL
Pen/Strep 100× 20 µL

Attention:

- i. The quantity and the state of the Collagenase I is critical for the success of this procedure
- ii. Always keep the collagenase I and the solution on ice. When using mice with fluorescent reporter genes, we recommend keeping this solution in the dark.

- a. Measure the quantity of Collagenase I very carefully.
- b. Place it in a 50 mL tube. In a sterile environment, add the media and the antibiotic and vortex carefully until the Collagenase I is completely dissolved.
- c. Filter the solution through a 0.22 μm filter with a 50 mL syringe into a new 50 mL tube.
Tip: It is possible to lose some of the volume in the filtering step, so it is better to start with a larger volume.

2. Wash solution (50 mL)

DMEM high-glucose + Pyruvate (Gibco) 45 mL

Horse serum 10% 5 mL

Mix the ingredients

Before use, pre-heat the solution at 37°C

Note: Ensure that the solutions are sterile.

Tip: If the fibers are to be transfected, we recommend not adding any antibiotics in this solution, as they will interfere with the transfection efficiency.

3. Growth medium for fibers

DMEM high-glucose + Pyruvate (Gibco), 39.5 mL

FBS 20%, 10 mL

FGF 2.5 ng/mL

Chick embryo extract (CEE) 1%, 500 μL

Mix the ingredients and filter them through a 0.22 μm filter with a 50 mL syringe into a new 50 mL tube.

Before use, pre-heat the solution at 37°C.

Note: Ensure that the solutions are sterile.

Tip: If the fibers are to be transfected, we recommend not adding any antibiotics in this solution, as they will interfere with the transfection efficiency.

4. IF solutions

PFA 4% in PBS

a. Dissolve 4 g paraformaldehyde powder in 100 mL PBS.

b. Heat the solution to 65°C until the paraformaldehyde is completely dissolved.

c. Store 10 mL aliquots at -20°C.

Triton X-100 0.5% in PBS

Mix 50 μL of Triton with 9.95 mL of PBS

FBS 10% in PBS

Mix 5 mL of FBS with 45 mL of PBS

FBS 0.1% in PBS

Mix 50 μL of FBS with 50 mL of PBS

Glycerol:PBS 3:1

Mix 3 mL of Glycerol with 1 mL of PBS

Acknowledgments

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This protocol is adapted from Cipriano *et al.* (2021; DOI: 10.7554/eLife.54782).

Competing interests

The authors declare that no competing interests exist.

Ethics

Animal experimentation: For the experiments described in this study, C57BL/10 wild-type mice were used, and differences that were observed in both male and female mice were included in experiments. With respect to housing, nutrition, and care, animals were treated according to the guidelines of good laboratory practice (GLP). All experimental protocols were approved and conformed to the regulatory standards (Protocol N° 7FF2C.4—Authorization N° 746/2016-PR, Cipriano *et al.* eLife 2021; 10: e54782. DOI: <https://doi.org/10.7554/eLife.54782> 20 of 25 Research article Cell Biology). All animals were kept in animal cages with at least five animals, at a temperature of $22^{\circ}\text{C} \pm 3^{\circ}\text{C}$, and with humidity between 50% and 60%.

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APEX-mediated Proximity Labeling of Proteins in Cells Targeted by Extracellular Vesicles

Lu Song^{1,§}, Jun Chen², Angela Sun¹ and Randy Schekman^{1,*}

¹Department of Molecular and Cell Biology, Howard Hughes Medical Institute, University of California, Berkeley, Berkeley, CA 94720, USA

²California Institute for Quantitative Biosciences at UC Berkeley (QB3-Berkeley), University of California, Berkeley, Berkeley, CA 94720, USA

[§]Present address: Janssen Biotherapeutics, The Janssen Pharmaceutical Companies of Johnson & Johnson, 169 Harbor Way, South San Francisco, CA 94080, USA

*For correspondence: schekman@berkeley.edu

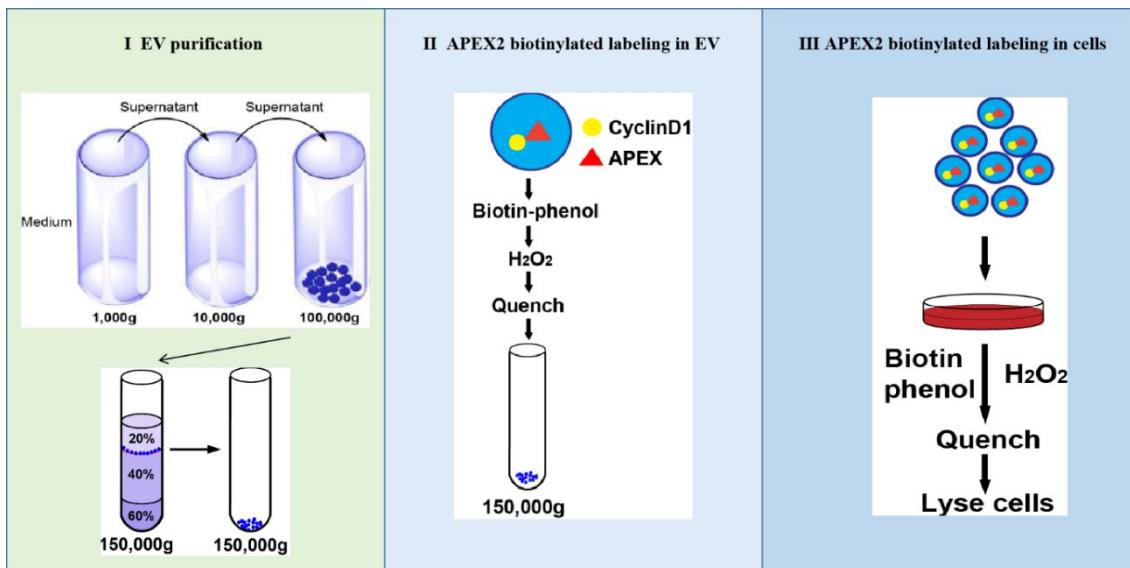
Abstract

Extracellular vesicles (EVs) are thought to mediate intercellular communication through the delivery of cargo proteins and RNA to target cells. The uptake of EVs is often followed visually using lipophilic-dyes or fluorescently-tagged proteins to label membrane constituents that are then internalized into recipient cells (Christianson *et al.*, 2013; De Jong *et al.*, 2019). However, these methods do not probe the exposure of EV cargo to intracellular compartments, such as the cytoplasm and nucleus, where protein or RNA molecules could elicit functional changes in recipient cells. In this protocol, we employ an EV cargo protein-APEX fusion to detect proximity interactions with recipient cell cytoplasmic/nuclear targets. This approach results in the biotinylation of proteins in close contact with the reporter fusion and thus permits profiling of biotinylated proteins affinity purified on immobilized streptavidin beads.

Keywords: Extracellular vesicles (EVs), APEX Proximity Labeling, mESC differentiation, EV uptake, Mass Spectrometry (MS)

This protocol was validated in: *J Cell Biol* (2021), DOI: 10.1083/jcb.202101075

Graphical Abstract:



Schematic showing three steps of APEX-mediated proximity labeling of proteins in cells targeted by EVs.

Background

Extracellular vesicles (EVs) are secreted by cells, circulate in body fluids, and ultimately generate functional changes through interaction with or uptake into recipient cells (Raposo and Stoorvogel, 2013). Protein, RNA, and possibly DNA are packaged by EVs and may be delivered into target cells to elicit changes in gene expression and cell behavior (Budnik *et al.*, 2016; Van Niel *et al.*, 2018; Shurtleff *et al.*, 2016; Temoche-Diaz *et al.*, 2019; Song *et al.*, 2021). Most of the current methodologies used to visualize EV uptake, however, do not identify the molecular targets of molecules released into recipient cells. An approach to define such targets should be of broad utility.

APEX biotinylation labeling is a newly-developed technique that reveals the subcellular proteomes of many landmarks in the nucleus and cytoplasm (Chen and Perrimon, 2017). Using hydrogen peroxide (H₂O₂) as an electron donor, the enzyme APEX catalyzes the oxidation of the substrate biotin-phenol (BP) (Hung *et al.*, 2016). The biotin-phenoxyl radical is a highly reactive, short-lived (<1 ms) species that conjugates to other proteins that are proximal to the APEX active site. Biotinylated protein products may then be isolated by streptavidin affinity purification and identified using conventional mass spectrometry (MS) techniques (Lobingier *et al.*, 2017).

We used this approach to investigate the contacts made by the protein cyclinD1 when it is delivered to mouse embryonic cells (mESCs) from EVs produced by differentiating neural progenitor cells (Song *et al.*, 2021). The use of a fusion of APEX to other EV cargo proteins should prove useful to identify molecular contacts within target cells.

Materials and Reagents

1. Syringe, 10 mL (BD, catalog number: 302995)
2. Syringe filter (0.45 µm; Thermo Scientific, catalog number: 44525-PP)
3. Falcon tube (Fisher Scientific, catalog number: 08-771-23)
4. 35 mm dishes (tissue culture dish) (Corning, catalog number: CLS430165)
5. 10 cm dishes (tissue culture dish) (Corning, catalog number: CLS430167)

6. 15 cm dishes (tissue culture dish) (Corning, catalog number: CLS430597)
7. Open-Top Thinwall Ultra-Clear Tube (38.5 mL), 25 × 89 mm (Beckman Coulter, catalog number: 344058)
Note: On text "38.5 mL ultra-clear tube".
8. Open-Top Thinwall Ultra-Clear Tube (13.2 mL), 14 × 89 mm (Beckman Coulter, catalog number: 344059)
Note: On text "13.2 mL ultra-clear tube".
9. Open-Top Thinwall Ultra-Clear Tube (5 mL), 13 × 51 mm (Beckman Coulter, catalog number: 344057)
Note: On text "5 mL ultra-clear tube".
10. Transfer pipettes (Fisherbrand, catalog number: 13-711-7M)
11. 293T cells (ATCC, catalog number: CRL-3216)
12. N2A cells (ATCC, catalog number: CCL-131)
13. mESCs (R1, ATCC, catalog number: SCRC-1011)
14. Retinoic acid (RA) (Sigma, catalog number: R2625)
15. Fetal bovine serum (FBS) (VWR, catalog number: 89510-194)
16. Exosome-depleted FBS (System Biosciences (SBI), catalog number: EXO-FBS-250A-1)
17. Puromycin (Sigma-Aldrich, catalog number: P8833-100MG)
18. pcDNA3 APEX-nes (Addgene, catalog number: 49386)
19. XPack CMV-XP-MCS-EF1 α -Puro Cloning Lentivector (System Biosciences)
20. psPAX (Addgene, catalog number: 12260)
21. pMD2.G (Addgene, catalog number: 12259)
22. Lipofectamine 2000 (Life Technologies, catalog number: 11668019)
23. OPTI-MEM (Thermo Scientific, catalog number: 31985062)
24. Sucrose (Fisher Chemical, catalog number: S5-3)
25. Biotin-phenol (Sigma-Aldrich, catalog number: SML2135)
26. Protease inhibitor cocktail (100 \times) (Sigma-Aldrich, catalog number: P8340)
27. Streptavidin-HRP (Thermo Fisher Scientific; catalog number: 21130)
28. H₂O₂ (Thermo Fisher, catalog number: 34062)
29. Sodium ascorbate (Sigma-Aldrich, catalog number: A7631)
30. Sodium azide (Sigma-Aldrich, catalog number: 26628-22-8)
31. Trolox (Sigma-Aldrich, catalog number: 238813-5G)
32. Biotin (Thermo Fisher Scientific, catalog number: 29129)
33. DTT (Gold Biotechnology, catalog number: DTT25)
34. DPBS (Dulbecco's phosphate-buffered saline; Thermo Fisher, catalog number: 14190144)
35. Streptavidin-agarose beads (Sigma, catalog number: 16-126)
36. NovexTM WedgeWellTM 10% Tris-glycine mini gels, 10-well (Thermo Fisher, catalog number: XP10200BOX)
37. Ponceau S (Thermo Fisher Scientific, catalog number: XP00100PK2)
38. Coomassie G250 (Sigma, catalog number: 1.15444)
39. DMEM/F12 culture medium (Thermo Fisher Scientific, catalog number: 11320033)
40. DMEM culture medium (Life Technologies, catalog number: 10566-024)
41. Neurobasal culture medium (Thermo Fisher Scientific, catalog number: 21103049)
42. B-27TM Supplement (50 \times) (Thermo Fisher Scientific, catalog number: 17504044)
43. N-2 Supplement (100 \times) (Thermo Fisher Scientific, catalog number: 17502048)
44. L-glutamine (Thermo Fisher Scientific, catalog number: 25030081)
45. Non-essential amino acids (100 \times) (Thermo Fisher Scientific, catalog number: 11140050)
46. β -mercaptoethanol (0.1 M) (Sigma, catalog number: M3148)
47. DMEM + 10% FBS (see Recipes)
48. RA (10 μ M) + 1% exosome-depleted FBS in DMEM (see Recipes)
49. Buffer C (see Recipes)
50. Quencher solution (see Recipes)
51. 4 \times Loading buffer (20 mL) (see Recipes)
52. N2B27 medium (1 L) (see Recipes)
53. RIPA (see Recipes)
54. Buffer D (see Recipes)

55. TBS-T (see Recipes)

Equipment

1. Sorvall RC 6+ centrifuge (Thermo Scientific, model: 46910)
2. Fixed angle rotor F14S-6X250y FiberLite (Thermo Scientific, catalog number: 78500)
3. Ultracentrifuge (Beckman Coulter, model: Optima XE-90, catalog number: A94471)
4. Swinging-bucket rotor SW 32 Ti and bucket set (Beckman Coulter, catalog number: 369694)
5. Swinging-bucket rotor SW 28 Ti and bucket set (Beckman Coulter, catalog number: 342204)
6. Swinging-bucket rotor SW 55 Ti and bucket set (Beckman Coulter, catalog number: 342194)
7. Swinging-bucket rotor SW 41 Ti and bucket set (Beckman Coulter, catalog number: 331362)
8. ChemiDoc MP Imaging System (Bio-Rad Laboratories, model: ChemiDoc MP 10)
9. NanoSight NS300 instrument equipped with a 405-nm laser (Malvern Instruments, Malvern, United Kingdom)
10. Refractometer (Fisher Scientific)
11. Bath sonicator (Covaris, model: S220)

Software

1. Nanosight NTA 3.1 Software (Malvern Instruments)
2. Excel (Microsoft, 2016)
3. GraphPad Prism 7
4. ImageLab Software v4.0
5. PEAKS Studio X+
6. Vsn R package

Procedure

Part I: EV purification

A. Plasmid construction

1. Clone PCR fragments of cyclin D1 and APEX from pcDNA3 APEX-nes into the XPack CMV-XP-MCS-EF1 α -Puro Cloning Lentivector.
 - a. PCR cyclinD1 from the cDNA of mESCs by using the following primers:
CyclinD1-F: acgGGATCCCATGGAACACCAGCTCCTG
CyclinD1-R: acgGAATTGATGTCCACATCTCGCACG
 - b. Insert cyclinD1 PCR fragments into pcDNA3 APEX-nes through BamHI and EcoRI to get pcDNA3 cyclinD1-APEX-nes plasmid.
 - c. PCR cyclinD1-Flag-APEX from the above vector by using the following primers:
acgCTCGAGTATGGAACACCAGCTCCTG
acgGGATCCtGATGTCCACATCTCGCACG
 - d. Insert cyclinD1-Flag-APEX PCR fragments into XPack CMV-XP-MCS-EF1 α -Puro through Xhol and BamHI to get XPack CMV-XP-cyclinD1-Flag-APEX -EF1 α -Puro plasmid.
2. Confirm vectors by using Sanger DNA sequencing (UC Berkeley DNA sequencing facility).

B. Lentivirus package and transfection

1. Vector transfection
 - a. Seed 293T cells at early passage (P1 in our experiments) on 10 cm culture dish and culture to get 60–70% confluency.
 - b. Mix 6 µg of transfer vector (XPack CMV-XP-cyclinD1-APEX-EF1α-Puro), 3.9 µg of psPAX, and 2.1 µg of pMD2.G in 0.5 mL of OPTI-MEM (total DNA 12 µg).
 - c. In another tube, mix 36 µL of lipofectamine in 0.5 mL of OPTI-MEM.
 - d. Mix the DNA and the lipofectamine, and incubate at room temperature for 20 min.
 - e. Change the culture medium into 5 mL OPTI-MEM.
 - f. Add the DNA and liposome mixture (1 mL) to the culture dish.
 - g. After 8 h, remove OPTI-MEM media and discard into 100% bleach.
 - h. Incubate cells in fresh growth medium (see Recipe 1) for 2 days.
2. Harvest virus
 - a. Centrifuge the medium at 1,000 × g for 5 min at 4°C.
 - b. Filter the medium, which has the cyclinD1-APEX lentivirus, slowly through a 0.45 µm filter into a 15 mL Falcon tube.
3. Transduce 50% confluence N2A cells with lentivirus
 - a. Add 1.5 mL cyclinD1-APEX lentivirus to N2A cells in 35 mm dish and culture for 24 h.
 - b. Replace medium with growth medium (see Recipe 1) containing 5 µg/mL puromycin.
 - c. Culture cells in 5 µg/mL puromycin for 4 days to select N2A cells stably expressing cyclinD1-APEX.

C. Cell culture and differentiation

1. Cell culture

Culture cyclinD1-APEX stably expressing N2A cells in four 15 cm dishes in 30 mL cell culture growth medium (see Recipe 1) until cells reach 80% confluency (the cell number is ~3 × 10⁷ cells per dish).

Note: The surface available for cell growth in these dishes is 151.9 sq. cm. The total volume of cell culture growth medium used for these dishes is 30 mL per plate.

2. Cell differentiation

- a. Split cells in the four 15 cm dishes to a total of fourteen 15 cm dishes, each containing approximately 8 × 10⁶ cells, and incubate in 30 mL of RA (10 µM) containing medium with 1% exosome-depleted FBS (see Recipe 2).

Note: The maximum volume of medium that can be processed in the steps below is 420 mL at each time point.

- b. Incubate cells for 6 days. Feed the cells with RA-containing medium (see Recipe 2) every 3 days. Collect medium afterward at day 6.

D. Collect conditioned medium and sediment EVs

1. Collect the conditioned medium from the fourteen 15 cm plates into separate containers.

Note: Approximately 420 mL of total condition medium should be collected.

2. Centrifuge conditioned medium at 1,000 × g for 15 min at 4°C using Sorvall RC 6+ centrifuge with a fixed rotor of F14S-6X250y FiberLite (or equivalent centrifuge) in 250 mL tube to remove floating cells (Low Spin Speed).

3. Decant the supernatant fraction into a new container immediately after the centrifuge finishes.

Note: It is important to remember not to disturb the pellet.

4. Use a Sorvall RC 6+ Centrifuge (with F14S-6X250y FiberLite fixed angle rotor) to centrifuge the supernatant at $10,000 \times g$ for 15 min at 4°C and sediment large EVs and cellular debris (medium speed spin).
5. After the centrifuge stops, immediately but carefully decant the supernatant fraction into a new container.

Note: It is important to remember not to disturb the pellet.

6. Transfer 32 mL of the collected supernatant into one 38.5 mL ultra-clear tube. Keep transferring until twelve 38.5 mL ultra-clear tubes are filled.
7. Use SW-28 and SW32 Ti rotors at $\sim 100,000 \times g$ (28,000 RPM) for 1.5 h at 4°C at maximum acceleration/brake to centrifuge.
8. Gently remove supernatant and resuspend the pellet fraction by pipetting two to three times in 200 μL of phosphate buffered saline (PBS, pH 7.4).
9. Pipette the resuspended pellet fraction into a new 5 mL ultra-clear tube.

Note: After combining the pellet fractions from 12 tubes, ~ 3 mL of resuspend pellet may be collected.

10. Dilute resuspension from the above step with PBS to a total volume of ~ 4.8 mL.
11. Use SW-55 rotor to centrifuge sample at approximately $150,000 \times g$ (38,500 RPM) for 1 h at 4°C and with maximum acceleration and brake.
12. Gently remove the supernatant. Add 100 μL of PBS into the tube and incubate at room temperature for 30 min.
13. Resuspend pellet by gently pipetting.
14. Add 900 μL of 60% sucrose. Mix thoroughly until a homogenous suspension is made.

Note: Sucrose is dissolved in buffer C (see Recipe 3). Use the refractometer to measure the sucrose concentration. The read of the refractometer should be 60.

15. First, carefully add 2 mL of 40% sucrose and then carefully place 1 mL of 20% sucrose on top. Add the sucrose medium very slowly and smoothly to avoid disturbing the layer between the cushion. Three separate layers of the different densities of sucrose should be apparent.

Note: This step requires extreme caution. Dispense sucrose slowly to the side of the clear tube, and let it trickle down the wall of the tube.

16. Use the SW-55 rotor to centrifuge sample at approximately $150,000 \times g$ (38,500 RPM) for 16 h, at 4°C and with minimum acceleration with no brake.

Note: It is important to have no brake during deceleration. Having the brake on can cause disruption of the gradient.

17. Once the centrifugation stops, aspirate ~ 0.8 mL of supernatant from top to bottom. Collect the 20%/40% sucrose interface (Figure 1) (the total volume at this step is ~ 1 mL) into a new 5 mL ultra-clear tube.

Note: Be careful not to disrupt the second gradient of the 40%/60% sucrose interface.

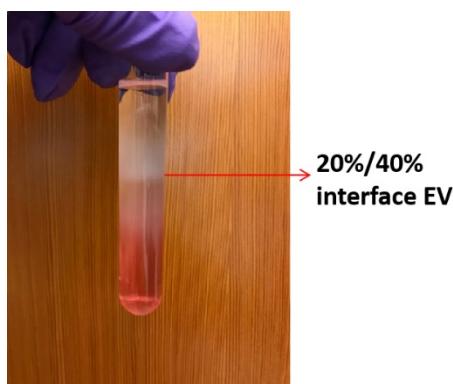


Figure 1. EVs at 20%/40% interface after overnight centrifugation

18. Dilute up to ~4.8 mL of PBS in the 5 mL ultra-clear tube.
19. Centrifuge at $\sim 150,000 \times g$ (38,500 RPM) for 1 h in an SW-55 rotator at 4°C and with maximum acceleration and deceleration.
20. Gently decant the supernatant fraction and add 100 μ L of PBS into the tube to incubate at room temperature for 30 min.
21. Resuspend the EV pellet by gently pipetting.

E. EV measurement by NTA

1. Estimate EV sizes and quantities using the NanoSight NS300 instrument equipped with a 405-nm laser; analyze the data in the scatter mode.
2. Dilute collected vesicles as described above (D21) at 1,000 \times with filtered PBS.
3. Introduce samples into the chamber automatically, at a constant flow rate during five repeats of 60-s captures, and at camera level 13 in scatter mode with Nanosight NTA 3.1 Software.
4. Estimate the EV size at the detection threshold using the Nanosight NTA 3.1 Software, after which export “experiment summary” and “particle data” (Figure 2).

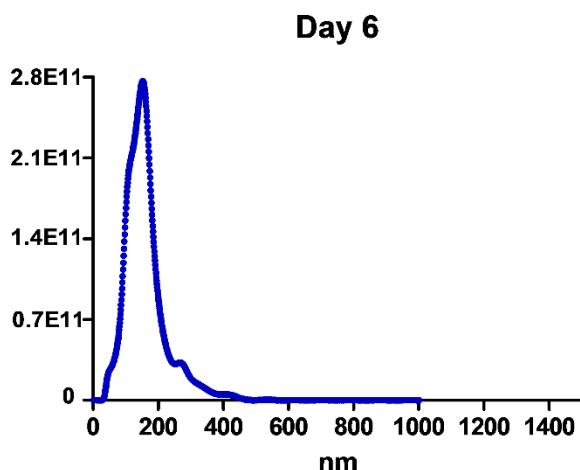


Figure 2. Nanoparticle tracking analysis of the size distribution and the number of purified EVs from the 420-mL medium of N2A cells treated with RA for 6 days.

5. Calculate the particle numbers in each size category from the particle data, and pool, bin, and count “true” particles with track length >3 with Excel.

Note: $\sim 5 \times 10^8$ to $\sim 2.5 \times 10^9$ per 1 μL particle number is normally counted in step D21 EV sample.

Part II: APEX biotinylated labeling in EV

1. Incubate biotin-phenol (500 μM) with $\sim 1 \times 10^{10}$ purified EVs (see Step D21 in Part I) for 30 min at 37°C in a total mixture volume <50 μL in an Eppendorf tube. Prepare two samples at this step.

Note: Keep in a small volume to ensure there is less of the biotin-phenol residual after the quencher solution wash.

2. Transfer the mixture into two 13.2 mL ultra-clear tubes.
3. Initiate APEX labeling by adding 1 mM (0.003%) H_2O_2 in one tube.

Note: For the other tube, perform the same treatment as follows, but do not add H_2O_2 . This serves as a negative control.

4. After 1 min, add 12 mL quencher solution (see Recipe 4) immediately.
5. Centrifuge at $\sim 110,000 \times g$ (31,500 RPM) for 1 h in an SW-41 rotor and 4°C with maximum acceleration and brake.
6. Use quencher solution (see Recipe 4) to sediment and wash EVs once. Once stopped, aspirate the supernatant and add 12 mL quencher solution (see Recipe 4).
7. Centrifuge at $\sim 110,000 \times g$ (31,500 RPM) for 1 h in an SW-41 rotor at 4°C and with maximum acceleration and deceleration.
8. Gently decant the supernatant fraction. Add 40 μL of PBS into the tube and incubate at room temperature for 30 min.
9. Resuspend the EV pellet fraction in 40 μL PBS by gently pipetting.
10. Transfer the two EV samples into new Eppendorf tubes.
11. Add 4 \times SDS-loading buffer (see Recipe 5) to prepare samples for SDS PAGE and streptavidin-HRP blotting.
 - a. Incubate samples at 95°C for 10 min.
 - b. Load 15 μL of samples per lane in a 10% Tris-glycine mini gels.
 - c. Run gels at a constant voltage of 150 V for 1 h.
 - d. Transfer proteins from gels onto a PVDF membrane with constant amps of 0.6 A for 1.5 h.
 - e. Block membrane(s) with 5% BSA in TBST overnight in the cold room (4°C).
 - f. Incubate membrane(s) with the Streptavidin-HRP (1:10,000) for 1 h at room temperature.
 - g. Wash three times with TBST for 8 min of each wash.
 - h. Add the HRP substrate following the manufacturer's specifications. Develop signal using Chemidoc Imaging System.

Note: The remaining EVs can be used for the following steps after proving that the APEX biotinylated labeling works well in EVs.

Part III: APEX biotinylated labeling in cells

A. APEX reaction in receipt mES cells

1. Prepare two 35 mm dishes of ES cells at a density of 4×10^4 - $5 \times 10^4/\text{cm}^2$.
2. Incubate cylinD1-APEX EVs with mESCs in N2B27 medium (see Recipe 6) for 2 days at a concentration of $\sim 5 \times 10^9$ EV/mL medium.
3. Pre-warm N2B27 medium (see Recipe 6) at 37°C for 30 min and then add 2 μL of the biotin-phenol stock solution (500 mM) into 2 mL of N2B27 medium to make a 500 μM biotin-phenol solution.
4. Incubate the above solution with cells for 30 min at 37°C.

5. Immediately prior to use, dilute 1 mM (0.003%) H₂O₂ into the medium for each dish for a 1-min labeling reaction at room temperature. Use the other dish without H₂O₂ as a negative control.
6. Quench the reaction by immediately washing cells thoroughly with room temperature quencher solution three times (see Recipe 4).

Note: Ensure that the washes are performed using the quencher solution instead of purely DPB.

7. Lyse cells in 1 mL RIPA (see Recipe 7) supplemented with 10 mM sodium ascorbate, 1 mM sodium azide, and 1× protease inhibitors.
8. Sonicate cell lysate in a bath sonicator for 10 min on ice and then centrifuge at 10,000 × g for 10 min at 4°C.
9. Add 40 µL of concentrated streptavidin-agarose beads to the supernatant fraction (800 µL), and then rotate the mixture overnight at 4°C.

Note: Wash 40 µL of streptavidin-agarose beads once with 1 mL of RIPA.

10. Centrifuge above sample at 3,000 × g for 10 min at 4°C. Discard the supernatant fraction.

Note: Ensure all the following buffers and samples are kept on ice.

11. Add 1 mL of RIPA buffer (see Recipe 7) to streptavidin-agarose beads and rotate the mixture at 4°C for 10 min.
12. Centrifuge above sample at 3,000 × g for 10 min at 4°C. Discard the supernatant fraction.

Note: The process from 10 to 11 is named the beads wash in the following steps.

13. Wash beads once again by RIPA buffer (see Recipe 7).
14. Wash beads once with buffer D (see Recipe 8).
15. Elute biotinylated proteins from the beads by heating the sample in a 4× SDS-loading buffer (see Recipe 5) supplemented with 2 mM biotin and 20 mM DTT for 10 min at 95°C.
16. Load 15 µL of sample per lane in 10% Tris-glycine mini gels. Run at a constant voltage of 150 V for 1 h.
17. Ponceau S stain gels at room temperature for 3 min (Figure 3).
18. Wash gel three times with ddH₂O.
19. Evaluate biotinylated proteins by blotting with streptavidin-HRP (Figure 3).

Note: The same procedure in Part II, Step 11 from d to h.

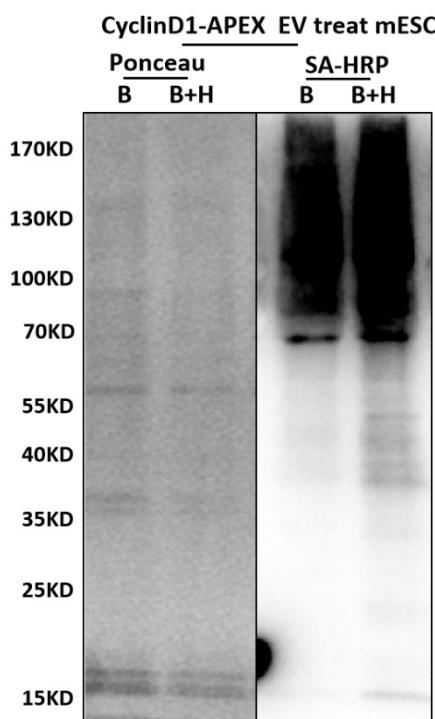


Figure 3. Streptavidin-HRP blotting after cyclinD1-APEX EV incubation with mESC

Note: Treat EV pre-incubation mESC with biotin-phenol with H₂O₂ (B+H) or not (B). Detect biotinylated protein by blotting with streptavidin-horseradish peroxidase (SA-HRP). Ponceau S staining (left of panel) of the same membrane serves as a loading control.

B. Mass Spectrometry (MS) analysis

1. Electrophoresis heated samples from Part III, Step A16 in a 10% Tris-glycine mini gel for ~3 min.
2. Stain the proteins with Coomassie and excise stained bands from the gel with a fresh razor blade.
3. Submit samples to a Spectrometry Facility (we used the Taplin Mass at Harvard Medical School) for in-gel tryptic digestion of proteins followed by liquid chromatography and mass spectrometry analysis according to their standards.
4. MS Data analysis
 - a. After raw MS data is acquired, perform peptide identification, quantification, and filtering on the Peaks Studio X+ platform with the default settings via the Proteome Discoverer database.
 - b. Implement Variance stabilization normalization (Vsn) with the justvsn function from the R/Bioconductor-package Vsn to normalize the quantified protein data.
 - c. Perform T-test analyses to examine significant changes in protein abundances between two different groups.

Data analysis

1. Export immunoblot images using ImageLab Software v4.0 as .tif format.
2. Use ImageJ to open and process immunoblot images, including rotating, cropping, adjusting brightness, and contrast when necessary.
3. Prepare figures with GraphPad Prism or preferred program (Figure 2).

Recipes

1. DMEM + 10% FBS (500 mL)

50 mL FBS
450 mL DMEM

2. RA (10 µM) + 1% exosome-depleted FBS in DMEM (500 mL)

5 mL exosome-depleted FBS
RA (10 µM)
Add DMEM to 500 mL

3. Buffer C

20 mM Tris-HCl pH 7.4
137 mM NaCl

4. Quencher solution

10 mM sodium ascorbate
5 mM Trolox
10 mM sodium azide
In DPBS (Dulbecco's phosphate-buffered saline)

5. 4× Loading buffer (20 mL)

Dissolve 1.6 g of SDS in 6 mL of ddH₂O
Add 5 mL of 1 M Tris-HCl pH 6.8
Add 1.23 g of DTT. Dissolve
Add 8 mg of bromophenol blue
Add 8 mL of glycerol
Add ddH₂O to 20 mL
Make 1 mL aliquots. Store at -20°C

6. N2B27 medium (1 L)

475 mL DMEM/F12
475 mL Neurobasal
20 mL B27
10 mL N2
10 mL L-glutamine (200mM)
10 mL non-essential amino acids (100×)
1 mL 0.1 M β-mercaptoethanol

7. RIPA

50 mM Tris, pH 7.4
150 mM NaCl
1% Triton X-100
0.5% deoxycholate
0.1% SDS
1 mM Trolox
1 mM DTT

8. Buffer D

1 M KCl

0.1 M Na₂CO₃
2 M urea
10 mM Tris-HCl, (pH 7.4)

9. TBS-T

0.1% Tween 20 (v/v) in 1× TBS

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Competing interests

The authors declare no competing interests.

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