

Methods in
Molecular Biology 2677

Springer Protocols

Michael Buszczak *Editor*

Germline Stem Cells

Methods and Protocols

Third Edition

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Germline Stem Cells

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Third Edition

Edited by

Michael Buszczak

Department of Molecular Biology, The University of Texas Southwestern Medical Center, Dallas, TX, USA

 **Humana Press**

Editor

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ISSN 1064-3745 ISSN 1940-6029 (electronic)
Methods in Molecular Biology
ISBN 978-1-0716-3258-1 ISBN 978-1-0716-3259-8 (eBook)
<https://doi.org/10.1007/978-1-0716-3259-8>

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Preface

Germ cells give rise to sperm and eggs, which represent two of the most specialized cell types within multicellular, sexually reproducing species. Germ cells have a number of unique features that have long fascinated biologists. They extensively reprogram their epigenomes back to a totipotent state. They are exceptionally good at repairing their DNA. They protect against the invasion and proliferation of transposable elements, and they are the only cells to undergo meiosis, which results in the formation of haploid gametes. Each of these areas represents an active and fundamentally important field of research. A better understanding of the mechanisms that govern germ cell formation and function will have broad impacts across biology.

Depending on sex and species, germ cells within individual organisms are often produced by populations of self-renewing germline stem cells. The study of these cells and the differentiation of their immediate progeny has served as useful models for understanding adult stem cells, the niches that support their self-renewal, competition for space within the niche, and the ability of early stem cell daughters to dedifferentiate under certain circumstances. *C. elegans*, *Drosophila*, zebrafish, naked mole rats, rats, and mice have all served as useful models for the study of germline stem cells. In addition, the derivation of primordial germ cell-like cells (PGCLCs) from embryonic stem cells and induced pluripotent stem cells has opened the door to the study of germ cell development in humans and other primates. Each of these systems has their own advantages and each has specific approaches that need to be mastered. The authors and I hope that this book provides an entrée into useful techniques that will hopefully make each of these model systems more accessible to the greater research community.

I would like to thank Prof. John M. Walker, Patrick Marton, David C. Casey, and the rest of the staff at Springer for their assistance and patience during the preparation of this book. I would also like to express my sincere appreciation and gratitude to all the contributors for sharing their knowledge and expertise. We all hope that the germ cell community benefits from the information contained within the enclosed chapters.

Dallas, TX, USA

Michael Buszczak

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Chapter 1

Analysis of the *C. elegans* Germline Stem Cell Pool

Sarah L. Crittenden, Hannah S. Seidel, and Judith Kimble

Abstract

The *Caenorhabditis elegans* germline is an excellent model for studying the genetic and molecular regulation of stem cell self-renewal and progression of cells from a stem cell state to a differentiated state. The germline tissue is organized in an assembly line with the germline stem cell (GSC) pool at one end and differentiated gametes at the other. A simple mesenchymal niche caps the GSC pool and maintains GSCs in an undifferentiated state by signaling through the conserved Notch pathway. Notch signaling activates transcription of the key GSC regulators *lst-1* and *sygl-1* proteins in a gradient through the GSC pool. LST-1 and SYGL-1 proteins work with PUF RNA regulators in a self-renewal hub to maintain the GSC pool. In this chapter, we present methods for characterizing the *C. elegans* GSC pool and early stages of germ cell differentiation. The methods include examination of germlines in living and fixed worms, cell cycle analysis, and analysis of markers. We also discuss assays to separate mutant phenotypes that affect the stem cell vs. differentiation decision from those that affect germ cell processes more generally.

Key words Stem cell pool, Progenitor zone, Notch signaling, PUF proteins, Cell cycle

1 Introduction

Identification of stem cells and the pathways that regulate them are important for both clinical research and more basic biomedical science. The *Caenorhabditis elegans* germline is a simple and well-studied model for understanding the genetic and molecular regulation of stem cells [1–3]. Several qualities distinguish the *C. elegans* gonad as a model for GSC regulation. First, in contrast to other GSC models with asymmetrically dividing stem cells, *C. elegans* GSCs are maintained as a pool (Fig. 1a) [4–7]. Second, all stages of germ cell development, from stem cell to differentiated gamete, are present in the adult gonad at one time (Fig. 1a and b). Third, establishment and maintenance of *C. elegans* germline stem cells are controlled by a simple mesenchymal niche, the distal tip cell (DTC)

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/978-1-0716-3259-8_1.

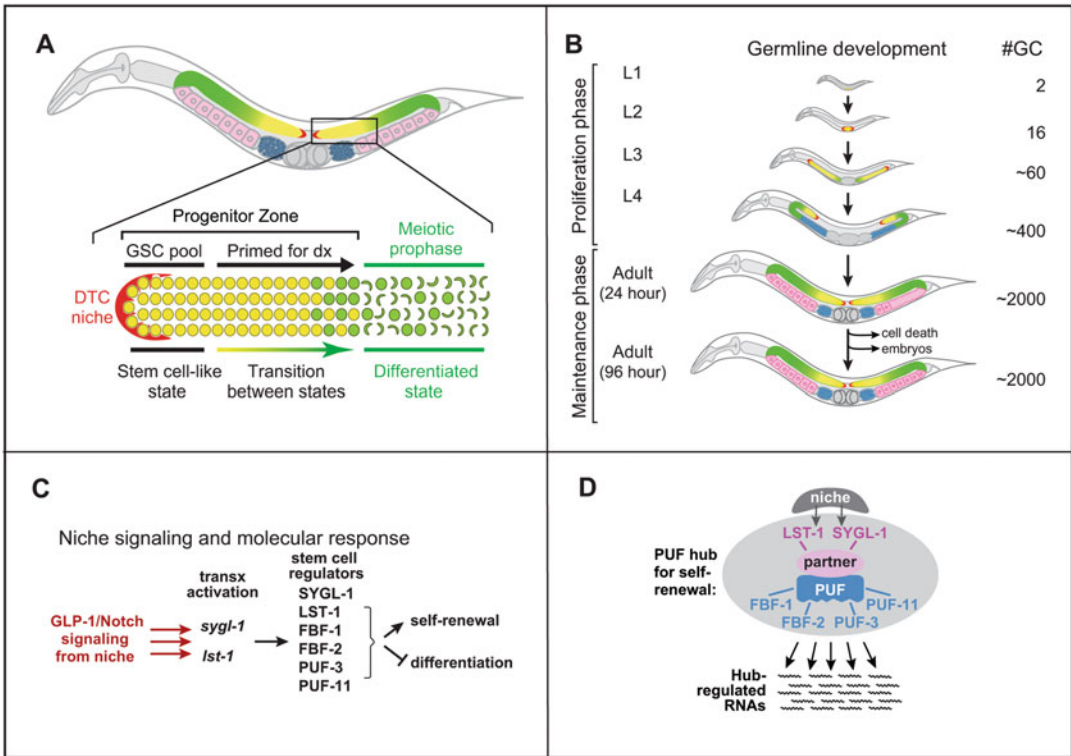


Fig. 1 Introduction to *C. elegans* germline development. (a) Diagram of adult hermaphrodite. Gonad arms are color-coded. Yellow, progenitor zone. Green, meiotic cell cycle. Pink, oocytes. Blue, sperm. Red, the somatic distal tip cell (DTC), is located at the distal end of the gonad and provides a niche for germline stem cells. Germ cells differentiate as they move proximally, away from the DTC. (b) Germline development from larval proliferative phase through adult maintenance phase. Approximate germ cell numbers for each stage are given on the right. (c) Pathway controlling GSC self-renewal/differentiation switch in the *C. elegans* germline. *sygl-1* and *lst-1* are transcriptionally activated by GLP-1/Notch signaling (transx activation) (d) Notch signaling activates *lst-1* and *sygl-1* transcription, which allows formation of the PUF regulatory hub within the niche

(Fig. 1a) [8]. Finally, regulators of stem cell self-renewal have been identified and analyzed in depth (Fig. 1c and d). The Notch receptor, GLP-1, is activated by signaling from the niche. GLP-1/Notch signaling activates transcription of two downstream targets in GSCs, *lst-1* and *sygl-1* [9–14]. LST-1 and SYGL-1 are novel proteins that partner with the PUF family of RNA regulators to form a self-renewal hub [15] that maintains GSCs in *C. elegans* [10, 16]. Both Notch signaling and PUF proteins regulate stem cells in other organisms. Notch signaling regulates numerous stem cells in vertebrates, reviewed in [2, 17] and PUF proteins regulate germline stem cell self-renewal in flies, neoblast maintenance in planaria, and maintenance of neural and hematopoietic stem cells in mice, reviewed in [18–20].

The *C. elegans* GSCs generate the germline during larval development, expanding it from 2 to 2000 cells (Fig. 1b); they maintain

the germline during adulthood, replenishing it as mature gametes are lost to cell death and fertilization (Fig. 1b); and they regenerate the germline after starvation [4, 21, 22]. These GSCs give rise to both sperm and oocytes, which produce a totipotent embryo after fertilization.

In addition to Notch signaling and its activation of the self-renewal regulatory hub, GSC behavior is influenced by many other factors, reviewed in [3] including food abundance [22–25], food quality [26, 27], age [28–30], rate of gamete production [31, 32], and sexual identity [31, 33]. In addition, screens have identified genes that modify the activity of the core pathway controlling GSCs. Modifiers include splicing factors, components of the proteasome, RNA binding proteins, proteoglycans, innexins, and genes of unknown function [1, 3, 34–40].

In this chapter, we describe methods for studying *C. elegans* germline stem and progenitor cells. Criteria to identify stem cells can vary from organism to organism and tissue to tissue. It is thus crucial to define the criteria in the system being worked on. In the *C. elegans* germline, stem and progenitor cells reside at the distal end of the germline tissue, adjacent to their niche, the distal tip cell (Fig. 1a). Older terms for this region include the “mitotic region”, “mitotic zone”, and “proliferation zone”. However, when it became clear that undifferentiated stem cells could be maintained independent of cell cycle state, the term “progenitor zone” was adopted [28, 30]. The progenitor zone includes a pool of GSCs at the distal end plus GSC progeny in various states of early differentiation. Multiple lines of evidence show that the GSC pool includes ~35–70 undifferentiated cells within the distal 5–8 rows of the germline [5]. These cells have similar cell cycle properties [4, 31, 41–43]; they can regenerate the entire germline after starvation in female germlines [22, 24]; and they express high levels of regulators that promote self-renewal, GLP-1, LAG-1, LST-1, SYGL-1, FBF-1, and FBF-2 [3, 10, 11, 13, 44, 45]. and low levels of regulators that promote differentiation, such as GLD-1 and GLD-2, reviewed in [3] (Figs. 1b and 8). The distal-most ~10 germ cells remain at the distal end [7], have extensive contact with the DTC [6, 46], the highest response to GLP-1/Notch signaling [11, 12], and the highest levels of LST-1, SYGL-1, and LAG-1 [6, 10, 13]. These characteristics are consistent with this group of cells continuing to self-renew while more proximal cells in the stem cell pool can either self-renew or differentiate. The current model is that GLP-1/Notch signaling activates transcription of *lst-1* and *sygl-1* genes in the GSC pool, and that LST-1 and SYGL-1 proteins partner with PUF RNA binding proteins to promote self-renewal and prevent differentiation. The extent of LST-1 and SYGL-1 proteins determines the size of the GSC pool. For example, increasing the extent of SYGL-1 increases GSC pool size [10] and decreasing SYGL-1 distribution shrinks GSC pool size

[14]. Male germlines similarly have a GSC pool that responds strongly to GLP-1/Notch signaling, expresses high levels of LST-1 and SYGL-1 and low levels of GLD-1 [6].

Proximal to the GSCs and thus farther from the DTC, germ cell progenitors have been triggered to enter early phases of differentiation (Fig. 1a). Two distinct decisions are made: the decision to leave the mitotic cell cycle and enter the meiotic cell cycle [5] and the decision to differentiate as sperm or oocyte [47]. These progenitors express markers of meiotic differentiation, such as GLD-1 and HIM-3 (Fig. 8) [5, 48–52], are estimated to divide 1–2 times before entering meiosis [51] and include germ cells in meiotic S phase, defined as cells that will enter meiotic prophase without passing through another mitotic division [4, 42, 49] (Figs. 1a and 8). No markers are yet available to reliably distinguish germ cells in mitotic S-phase from those in meiotic S-phase.

We will present methods to define the progenitor zone and to identify the various states of GSCs and their daughters within the progenitor zone. We will then discuss how to characterize new mutant or RNAi phenotypes. In addition to GSC regulators found in genetic screens, an expanding list of potential stem cell regulators is available from genome-wide/high-throughput studies to identify potential partners and targets of germline stem cell regulators [13, 53–57]. Inputs from other cells in the worm and the environment can affect GSC behavior, reviewed in [3, 29, 58]. Characterizing the functions of potential GSC regulators either by gene editing or RNAi can yield insights into their role in stem cell regulation. Finally, methods are now available to see where proteins interact in intact tissues, including the *C. elegans* germline, e.g., proximity ligation assay [59]. For additional information, readers are referred to excellent chapters about the germline and useful techniques available on the WormBook website (<http://www.wormbook.org>) and useful techniques on the WormBook Methods website (http://www.wormbook.org/toc_wormmethods.html).

2 Materials

2.1 Reagents

1. 4% agarose in dH₂O for microscopy of live *C. elegans*.
2. M9: 3 g/L KH₂PO₄, 6 g/L Na₂HPO₄, 5 g/L NaCl, 1 mM MgSO₄.
3. *E. coli* M9 minimal media: 3 g/L KH₂PO₄, 6 g/L Na₂HPO₄, 0.5 g/L NaCl, 1 g/L NH₄Cl, 2 mM MgSO₄, 0.1 mM CaCl₂, 0.4% glucose, 1.25 µg/ml thiamin.
4. M9 plus 0.25 mM levamisole.
5. Slides and coverslips.

6. Subbing solution:

- (a) Bring 200 ml distilled water to 60 °C.
- (b) Add 0.4 g gelatin.
- (c) Cool to 40 °C.
- (d) Add 0.04 g chrome alum.
- (e) Add 1 mM sodium azide.
- (f) Add poly-L-lysine (Sigma, catalog #P1524) to 1 mg/ml.

Store subbing solution at 4 °C. To sub slides, put subbing solution on slide for 10 min at room temperature. Wick off excess liquid. Dry in 65 °C oven for ~30 min. Slides can be stored in the oven or at room temperature.

7. Paraformaldehyde (16% stock, Electron Microscopy Sciences, catalog #I5710).
8. PBSB: PBS (for 1 liter: 8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄, 0.24 g KH₂PO₄, pH to 7.2 with NaOH) containing 0.5% BSA.
9. PBSBTw: PBSB containing 0.1% Tween 20 to prevent sticking.
10. DAPI (4',6-diamidino-2-phenylindole) (Molecular Probes, Invitrogen catalog #D1306).
11. Vectashield (Vector Labs, catalog # H-1000).
12. Hoechst 33342 (Molecular Probes, Invitrogen catalog # H3570).
13. SYTO-12 (Molecular Probes, Invitrogen catalog # S7574).
14. Rabbit anti-PH3 polyclonal antibody (Upstate Biotechnology, catalog # 06-570).
15. Mouse anti-PH3 monoclonal antibody (Cell Signaling Technology, catalog #97065).
16. Rabbit-anti-WAPL-1 (Novus Biologicals, catalog # 49300002).
17. Mouse anti-DAO-5 (DAO5 was deposited to the DSHB by Nonet, M.L. / Hadwiger, G. / Dour, S. (DSHB Hybridoma Product DAO5)).
18. M9-agar plates: 1.2% agar, 0.6% agarose in M9 salts containing 0.1 mg/ml carbenecillin (Ito, 1987 #257).
19. Click-iT® EdU Alexa Fluor® 488 Imaging Kit (ThermoFisher catalog #C10337).
20. TO-PRO-3 (Molecular Probes, Invitrogen catalog #T3605).
21. Thymidine-deficient *E. coli* MG1693 (*E. coli* Genetic Stock Center (CGSC), <http://cgsc.biology.yale.edu/top.html>, CGSC #6411).

22. P_{sygl-1}::H2B::GFP::sygl-1; P_{oma-1}::OMA-1::GFP (*Caenorhabditis* Genetics Center (CGC), <http://cbs.umn.edu/cgc>, *C. elegans* strain #JK5018).
23. P_{lag-2}::GFP (*Caenorhabditis* Genetics Center (CGC), <http://cbs.umn.edu/cgc>, *C. elegans* strain #JK2868).
24. P_{lag-2}::myr::GFP (*Caenorhabditis* Genetics Center (CGC), <http://cbs.umn.edu/cgc>, *C. elegans* strain #JK4475).
25. P_{lim-7}::GFP (*Caenorhabditis* Genetics Center (CGC), <http://cbs.umn.edu/cgc>, *C. elegans* strain #DGI575).
26. VALAP (equal parts petrolatum, lanolin, and paraffin wax, melted to combine).

2.2 Web Resources

1. in situ RNA expression database: <http://nematode.lab.nig.ac.jp/>.
2. WormBase: <http://www.wormbase.org/>.
3. modENCODE: <http://www.modencode.org/>.
4. *C. elegans* strain collection (CGC): <http://www.cbs.umn.edu/CGC/>.
5. WormBook: <http://www.wormbook.org/>.
6. WormMethods: http://www.wormbook.org/toc_wormmethods.html.
7. WormAtlas: <http://www.wormatlas.org>.

3 Methods

3.1 Identification of the Progenitor Zone in Wild-Type *C. elegans* Hermaphrodites

C. elegans hermaphrodites are self-fertile XX animals that make sperm during larval development and oocytes as adults. The adult hermaphrodite *C. elegans* germline is composed of two U-shaped tubes containing approximately 2000 germ cells in different states of differentiation which can be observed in living animals (*see* Subheading 3.2, Fig. 2a). The germline tissue is syncytial, although nuclei and their surrounding cytoplasm are largely separated from each other by plasma membranes [3 and references therein, 33]. Within the progenitor zone, these partially enclosed “units” behave independently with respect to cell cycle and marker expression and therefore are referred to as germ “cells”. The GSCs reside at the distal end of the germline tissue, adjacent to the somatic distal tip cell (Fig. 1a).

In wild-type young adult hermaphrodites (*see* Note 1), the progenitor zone is approximately 20 cell diameters in length and contains approximately 225–250 germ cells. The length of the progenitor zone is defined as the number of cell diameters between the distal tip cell and the start of meiotic prophase (Figs. 3 and 8)

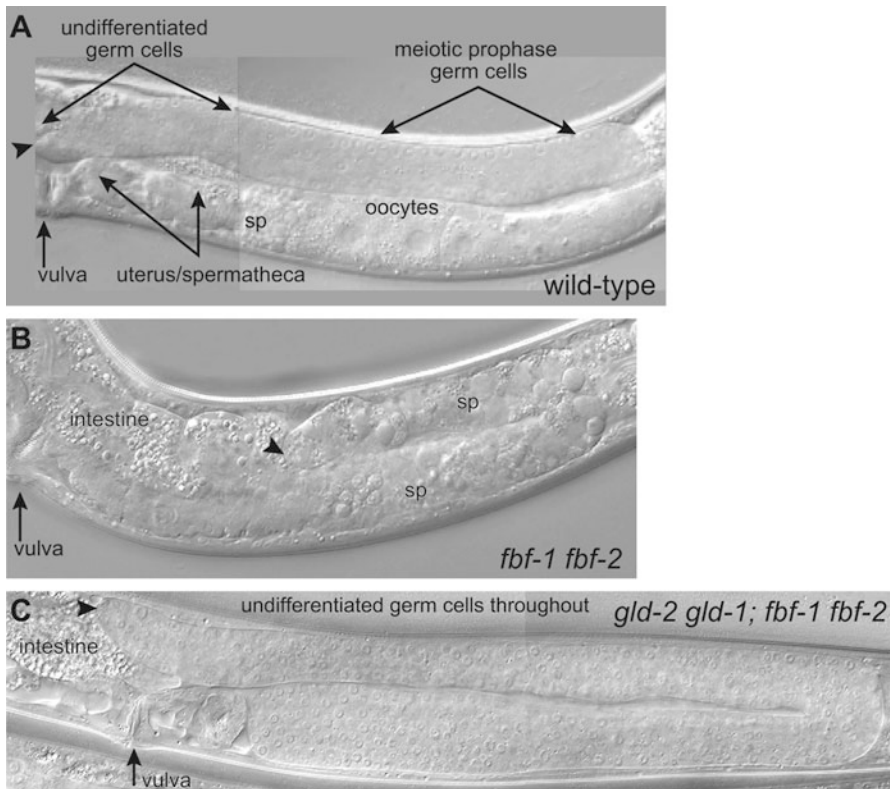


Fig. 2 Microscopy of wild-type and mutant germlines. **(a)** Differential interference contrast (DIC) micrograph of a wild-type adult hermaphrodite. **(b)** DIC micrograph of an *fbf-1 fbf-2* double mutant germline. Mature sperm are seen at the distal end. **(c)** DIC micrograph of a *gld-2 gld-1; fbf-1 fbf-2* mutant germline containing only undifferentiated germ cells. Arrowheads indicate distal end of germline

(see [3] and references therein, [4, 48]). This simple length measurement does not take into account the complex architecture of the adult hermaphrodite progenitor zone [33]. Meiotic prophase is scored by the presence of germ cells in leptotene/zygotene of early meiotic prophase, identified with meiosis-specific markers such as phospho-SUN-1 or HIM-3 (see [3] and references therein; [60]) and the distinctive crescent shape of their DNA (crescents, Fig. 3, Subheadings 3.3 and 3.5). Most cells in the progenitor zone are proliferating mitotically; however, approximately 50–100 germ cells in the most proximal rows are inferred to be undergoing meiotic S phase [4, 42]. In wild-type germlines, apoptosis occurs in some oogenic cells in the late pachytene stage of meiotic prophase (Subheading 3.4) [61, 62].

Although most work is done in the XX adult hermaphrodite germline, some work has been done on XO male germlines that only make sperm. The overall organization of progenitor and meiotic progression is similar in hermaphrodites and males, but the three-dimensional architecture of their niches and progenitor zones

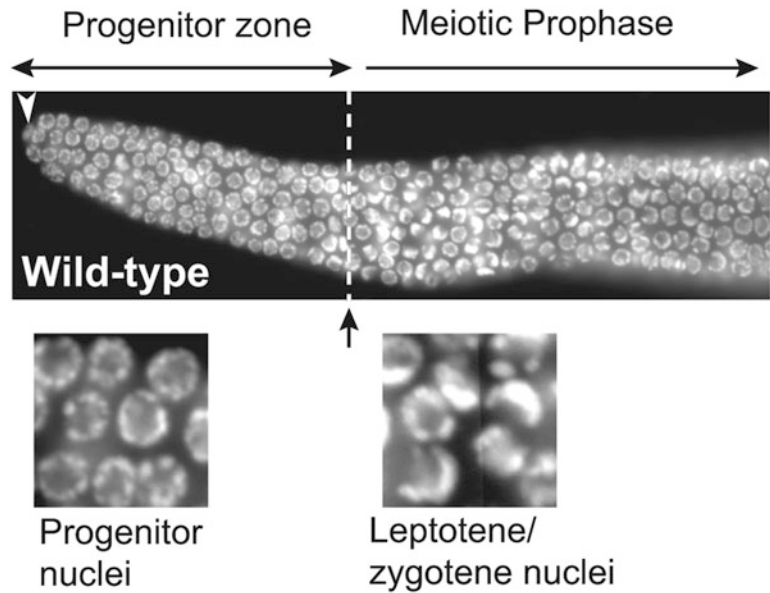


Fig. 3 Identification of progenitor zone cells and meiotic prophase germ cells. DAPI-stained distal arm of a wild-type adult hermaphrodite. In this diagram, meiotic prophase includes germ cell cells in the earliest stage of meiotic prophase, leptotene/zygotene, which is used to mark the end of the progenitor zone and is referred to as meiotic prophase. Progenitor zone and leptotene/zygotene nuclei are enlarged below. Dashed lines indicate the boundaries between the zones. Note the crescents of DNA in leptotene/zygotene nuclei. Arrowhead points to DTC nucleus. (Modified from Eckmann et al. [109])

is sex specific [31, 33, 62, 63] and apoptosis does not occur in male germlines [61]. However, both hermaphrodite and male GSCs are maintained by Notch signaling and a self-renewal hub with the same regulators [6, 15].

3.2 Mounting Live Animals for Light Microscopy (See Notes 2 and 3)

1. Make an agarose pad on slide: drop melted 4% agar on glass slide, quickly put another slide on top at a 90° angle and press to create a thin layer of agarose (about 0.4 mm thick). Slides with lab tape can be used as spacers for the top slide [64, 65].
2. Pick animals onto agarose pad next to a drop of M9 containing 0.25 mM levamisole (*see Note 4*). Levamisole will prevent animals from moving. Tricaine and tetramisole are also used [66]. Animals can be rescued from agarose pads by carefully sliding coverslip to the side and then picking the animals from the pad to a plate.
3. Cover with coverslip, avoiding bubbles. For short-term observation, it is not necessary to seal the edges of the coverslip. For longer-term imaging, coverslips can be sealed with VALAP [67].
4. Observe germline using differential interference contrast (DIC) and/or fluorescence microscopy.

3.3 Immunohistochemistry of Extruded Gonads

We use two methods for extruding and processing gonads (somatic tissues remain associated with the germline) for immunohistochemistry: extrusion on slides and extrusion in solution ([52, 68]; Schedl lab website – protocols <http://genetics.wustl.edu/tslab/>). One benefit of extruding in solution is the increased efficiency for preparing large numbers of gonads, although there are anecdotal reports of improved staining using the slide method. There are several detailed protocols for staining *C. elegans* gonads that include alternate fixation protocols [68] (*see* **Notes 5** and **6**.) If only DAPI staining is required, skip the primary antibody step and incubate the fixed germlines with DAPI. Continue on from there (*see* **Note 7**).

1. Extrude gonads. To extrude on a slide, pick 10–20 animals into 7 μL PBS containing 0.25 mM levamisole on subbed slide. Cut the animals with a syringe needle or scalpel behind the pharynx or at the tail. The germline and intestine will extrude from the cuticle (*see* **Note 8**). Make a sample area with a PAP pen to keep the liquid from spreading across the slide (*see* **Note 9**). To extrude in solution, pick or wash 10–100 animals off plate into a glass or plastic dish containing PBS, 0.25 mM levamisole plus 0.1% Tween 20 to prevent sticking. The total volume can be anywhere from 200 μL to a couple milliliters depending on your personal preference and size of the dish. Use a micropipettor to transfer samples into a microfuge tube. For the following steps, the volumes should be matched to the technique you use to extrude gonads. For extrusion on slides, generally 25–50 μL is sufficient to cover the sample area. For the solution method, volumes for fixation, permeabilization, and washes are generally 200–1000 μL , and volumes for antibody incubation are 50–100 μL ; we usually place tubes on a rotator or rocker during all steps.
2. Fix. The fixation method will depend on the antibody-antigen combination you're using (*see* **Note 5**) [68]. A good place to start is 1–3% paraformaldehyde for 10–20 min. If you extruded on a slide, keep the slides in a humidified chamber (e.g., a box with a wet paper towel taped to the lid) throughout the procedure.
3. Permeabilize. Remove paraformaldehyde and replace with 0.1% Triton X100 in PBSB for 5 min at room temperature.
4. Block in PBSBTw for 30 min at room temperature.
5. Incubate with primary antibody. Dilution, incubation time and temperature should be determined for each antibody.
6. Wash 3 times with PBSBTw for 15 min each.
7. Incubate with secondary antibody. Dilution in PBSBTw, incubation time and temperature should be determined for each

antibody (*see* **Note 10**). Include 0.1 µg/ml DAPI to stain DNA (*see* **Note 11**).

8. Wash 3 times with PBSBTw for 15 min each.
9. Add 7–10 µL Vectashield or other mounting medium; if samples are in a tube, pipet onto a slide, place coverslip over gonads and seal with nail polish. The volume of mounting medium will depend on the size of the coverslip. The volume should be large enough that gonads are not squashed, but small enough that they are not floating around. Excess liquid can be carefully wicked away using a kimwipe.
10. Collect images using either widefield or confocal microscopy.
11. We quantitate images using the plot profile function in FIJI/ImageJ [69] following the procedure described in detail in [70] (modified from [52]).
12. Fluorescence quantitation can be variable from slide to slide. Including control animals with the experimental animals throughout the staining and imaging procedure is one way to normalize this variability. Control animals can be distinguished from experimental either by a mutation that can be scored or a marker such as GFP that does not affect the level or distribution of the protein being studied [13, 52].

3.4 Visualization of DNA and Germline Apoptosis in Live Animals

1. Incubate animals in 33 µM SYTO-12 (taken up by dying cells) and/or 50 µg/ml Hoechst 33342 (DNA dye) in M9 with some *E. coli* in a microfuge tube for 4–5 h (*see* **Notes 12 and 13**).
2. Transfer to seeded plate for 30–60 min.
3. Mount on agarose pad for DIC microscopy.
4. Observe live animals on agarose pads (Subheading 3.2) using fluorescence microscopy and DIC. Hoechst can be observed using DAPI filter sets. SYTO-12 emits green fluorescence and can be observed using FITC filter sets.

3.5 Scoring Progenitor Zone Length and Cell Number

The progenitor zone extends from the distal-most germ cell to meiotic prophase. Since there are not markers for meiotic S phase, the distinctive markers of early meiotic prophase are used to determine which germ cells have begun meiosis, *see* [3, 60].

1. Using DAPI-stained germlines (Subheading 3.3), identify the distal end by locating the DTC nucleus (oval with diffuse DAPI staining, Figs. 3 and 4) (*see* **Note 14**). Immunostaining for progenitor markers is useful for scoring the extent of the PZ (*see* subheadings 3.7 and 3.8 for a detailed discussion of markers).

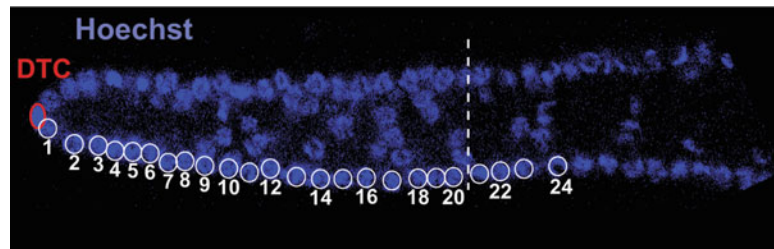


Fig. 4 Counting cell diameters from distal end of germline. Distal arm of a wild-type adult hermaphrodite gonad stained with Hoechst. DTC nucleus is circled in red, and germ cell nuclei are circled in white. Numbers begin with 1 for the nucleus closest to the DTC and increase moving proximally. Dashed line indicates the start of meiotic prophase. (Modified from Byrd et al. [46])

2. Meiotic prophase, as used to mark the proximal end of the progenitor zone, is defined in two ways. First, it can be quickly scored in DAPI-stained germlines as the position along the gonadal axis at which there are 2 or more crescents. The second method scores the position where 50% of germ cells express markers of meiotic chromosomes (*see* Subheading 3.7 for a more detailed discussion of markers) (*see* Note 15) (Fig. 3). In the proximal progenitor zone and distal region of meiotic prophase, there is a mix of germ cells expressing either progenitor zone or meiotic prophase markers. This is referred to as the meiotic entry region, for review (Fig. 8b), *see* [3].
3. Count germ cell rows and total number of cells (*see* Note 16) starting with the row immediately adjacent to the DTC nucleus and continuing to the start of meiotic prophase (Fig. 4). Counting can be done visually either through microscope eyepieces or on a computer monitor. For training yourself to count with reasonable accuracy, count the germline multiple times until your numbers are within about 5% of each other. Automated methods for counting and image analysis are also available [71–73]. It is often difficult to separate (segment) individual DAPI-stained nuclei with automated methods. Using a nucleolar marker such as DAO-5 improves the segmentation of individual nuclei, reducing the need to do this manually [33].
4. Progenitor zones in young adult wild-type hermaphrodite germlines average 19–20 cell diameters from the distal end (range 15–24) and contain 225–250 germ cells (range ~ 200–~ 300) (*see* Note 1). These numbers can vary depending on the three-dimensional architecture of individual germlines and their age. Adult hermaphrodite progenitor zones are folded which brings some germ cells into the interior of the germline tube. This means that position along the gonadal axis does not always correlate perfectly with stage of differentiation [33] and

that this position is not always the same as position along the germline tube. In addition, the number of germ cells in the progenitor zone does not always correlate with the length (e.g., male progenitor zones are longer than hermaphrodite, but contain the same number of cells) [31]. The length and number of germ cells in the PZ change with age [28, 30], so comparing staged animals is important.

3.6 Characterization of the GSC Pool and GSC Number

We present methods here to identify the GSC pool and GSC number in fixed germlines. There are also detailed protocols for live imaging in the germline, for example, using photoactivatable fluorescent proteins to track groups of germ cells over time [74], for tracking transcription sites [12], for imaging GLP-1/Notch receptor activation [75], for imaging the cell cycle [76], for imaging cellular changes in germ cells [62], and somatic cells surrounding the germline [77]. A recent methods paper compares several live and fixed imaging protocols [78].

1. *sygl-1* and *lst-1* expression. Two GLP-1/Notch targets, *sygl-1* and *lst-1*, are expressed in a subset of germ cells immediately adjacent to the DTC in the progenitor zone. The expression of these genes is dependent on GLP-1/Notch signaling, making their expression a useful marker for GLP-1/Notch responsive germ cells [9, 12, 79, 80] within the distal pool. Expression patterns for endogenous *sygl-1* and *lst-1* can be examined by in situ hybridization; detailed methods can be found in [79, 81] (see **Note 17**). *sygl-1* and *lst-1* are also expressed in maturing oocytes, so mutants that cause distal germ cells to differentiate may express *sygl-1* and *lst-1* distally due to differentiation rather than maintenance of an undifferentiated state.

sygl-1 expressing cells can also be visualized using animals carrying a single copy chromosomal insertion of a transgene containing the *sygl-1* promoter driving GFP::H2B [9]. Note that some germlines expressing this transgene are not completely normal and that expression can be partially silenced. Germline silencing is common in *C. elegans* and can be alleviated by maintaining transgenic animals in a licensing background [82–84] and/or by growing them at 25 °C (see Kershner et al. [9] Materials and methods for further details).

The extent of SYGL-1 and LST-1 protein can be determined using lines that have had epitope tags inserted at the endogenous locus using Crispr/Cas9 gene editing [10, 85].

2. *emb-30* assay. In this assay, germ cell movement is blocked and germ cells are scored for differentiation with time [5, 6, 10, 30, 80]. The *emb-30(ts)* mutant at restrictive temperature blocks the metaphase to anaphase transition, arresting cell division and cell movement [5]. After a shift to restrictive temperature, the distal-most 6–8 cell rows, containing about 50 germ cells,

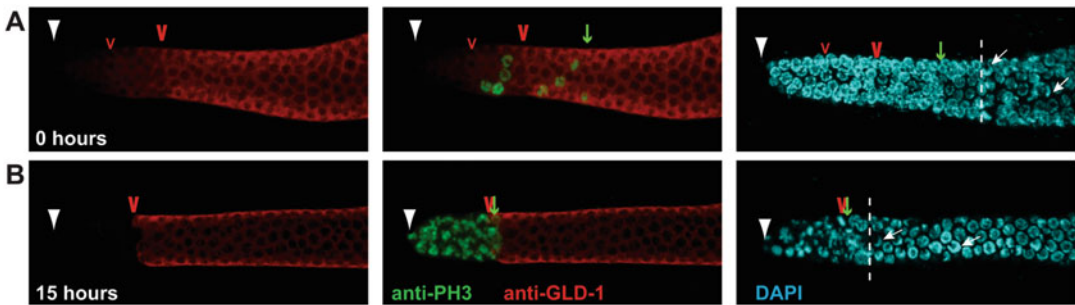


Fig. 5 *emb-30* assay. Gonads were shifted from permissive (15 °C) to restrictive (25 °C) temperatures for times shown in white lettering. Gonads were extruded and stained with anti-PH3 (green), anti-GLD-1 (red), and DAPI (cyan). Distal end is marked with white triangle, GLD-1 boundaries with red carats, most proximal PH3 with green arrow. GLD-1 often has two stepwise increases in intensity – each is marked with a caret in the 0 hour panels. The steps correspond to folds in the germline epithelium in adult hermaphrodites. These steps can be absent or look graded in some gonads, making them hard to score. Also note the variable nuclear morphology in distal end of the 15 hour DAPI-stained gonad. Dashed line indicates the start of meiotic prophase. (Modified from Cinquin et al. [5])

arrest in M-phase and remain undifferentiated, indicating that this pool of cells is maintained in an immature state. More proximal germ cells enter the meiotic cell cycle, indicating that they have been triggered to differentiate and would normally enter meiotic prophase more proximally if their movement had not been blocked (Fig. 5, compare panels A and B, *see Note 18*).

- (a) Maintain *emb-30(tn377ts)* at 15 °C. Animals are fertile; however, progenitor zones are slightly smaller than in wild-type. Maintain strains on plates that are equilibrated to 15 °C. If you are determining distal pool size in mutants, compare *mutant; emb-30* to *+/emb-30* in parallel.
- (b) Stage animals. For animals comparable to hermaphrodites grown for 24 h after L4 at 20 °C (*see Note 1*), pick L4s and let them grow at 15 °C for 36 h.
- (c) Shift staged animals to restrictive temperature (25 °C) by picking adults to plates that have been equilibrated at 25 °C.
- (d) Every hour, or at your chosen time points, fix gonads (Subheading 3.3) and stain with DAPI, anti-PH3 and anti-GLD-1 or anti-HIM-3 (Fig. 5a and b). Score the position of the boundary between metaphase-arrested nuclei (visualized with DAPI and anti-PH3) and meiotic prophase nuclei (visualized with DAPI). Also score the position of the boundary between low and high levels of meiotic proteins such as GLD-1 and/or HIM-3. Positions can be scored in germ cell diameters and/or microns (*see [5, 30] Note 18*).

3.7 Markers for the GSC Pool, Progenitor Zone and Early Stages of Differentiation

A number of markers are helpful for marking the GSC pool, the progenitor zone, differentiating progenitors, germ cells in the meiotic cell cycle, and somatic gonad cells that affect proliferation (Table 1 and Fig. 8).

Notch signaling output and GSC pool can be visualized using endogenous *sygl-1* and *lst-1* transcripts ([11], see Subheading 3.1.5.1), tagged SYGL-1 and LST-1 proteins [10, 85], or a transgene containing the *sygl-1* promoter driving H2B::GFP ([9]; see Subheading 3.1.5.1), which are abundant in the distal-most germ cells. Progenitor zone markers include the Notch receptor, GLP-1, which is abundant in membranes of progenitor zone cells [38, 39, 44, 86]; the p53 homolog, CEP-1, which is present in progenitor zone nuclei [87]; the meiotic cohesin, REC-8, which is enriched in the nuclei of progenitor zone cells under certain fixation conditions [3] and WAPL-1 [85, 88]. (Table 1 and Fig. 8). Meiotic markers include the RNA regulator, GLD-1, which increases in the cytoplasm of germ cells proximal to the GSC pool and peaks in meiotic prophase [52], the meiotic proteins HIM-3 and phospho-SUN-1, which are present in proximal progenitor cells and become localized to chromosomes in early meiotic prophase [50, 88]. Combinations of REC-8 and HIM-3 staining identify a region of overlap called the meiotic entry region where germ cells are asynchronously entering early meiotic prophase (leptotene and zygotene) [42, 88]. The meiotic entry region has substantial overlap with the meiotic S region identified by cell cycle analysis [4, 42] (Fig. 8).

3.8 Markers for the Somatic Gonad

The somatic niche, the DTC, expresses the Notch ligands, LAG-2 and APX-1 [46, 89] (see Note 19), which activate the Notch receptor, GLP-1, in GSCs. The DTC can be visualized using a transgene with the *lag-2* promoter driving GFP [4, 90]. Finer details of membrane structure can be seen using a transgene with myristoylated GFP [46]. In hermaphrodites, the DTC has a membranous cap that surrounds the distal-most germ cells (Fig. 6). The DTC produces extensive processes in early adulthood [46, 77, 91]; these processes surround and intercalate into the membranes between the germ cells in the distal pool (plexus, Fig. 6b). During adulthood, the DTC also extends long, external processes [46, 92, 93]. The DTC architecture in males differs from hermaphrodites. The single male gonadal tube has two DTCs [94] and while there is intimate contact with germ cells adjacent to the DTC cell body, the processes are not as extensive as those in the hermaphrodite [6, 31]. The *arg-1* promoter is more strongly expressed in male DTCs [6] than the *lag-2* promoter, which is quite weak [31].

The somatic sheath cells also play a role in germline patterning in hermaphrodites, especially during larval stages [3] and references therein. The sheath cells also have extensive contact with the surface of the germ cells and extensive intercalation between germ cells [93, 95], see [96, 97] for a discussion of sheath markers and the role of the sheath and innexins in patterning the progenitor zone.

Table 1
Useful markers

Marker	Molecular identity	Pattern
<i>lst-1</i> and <i>sygl-1</i> ATS	GLP-1/Notch transcriptional targets	ATS are present in the GSC pool, marker for germ cell response to active GLP-1/notch signaling [11, 12].
<i>lst-1</i> and <i>sygl-1</i> mRNA and protein	Novel GLP-1/Notch targets	Transcripts and proteins are present in the GSC pool [9–11]
P _{sygl-1} ::H2B::GFP:: <i>sygl-1</i>	<i>sygl-1</i> promoter driving histone fused to GFP	GFP is present in the GSC pool [9].
GLP-1	Notch receptor	Membrane-associated, strong in mitotic germ cells, lower levels in early meiotic region (Crittenden, 1994 [44, 86])
REC-8	Cohesin	On chromosomes in meiotic prophase (except when used as mitotic marker [3]).
CEP-1	C. elegans p53	Nuclear in progenitor zone and proximal cells [87].
PH3	Histone H3 phosphorylated on serine 10	Chromosomes in late prophase (mitotic and meiotic) and M phase [30, 99].
GLD-1	Maxi-KH/STAR domain RNA binding protein	Cytoplasmic, increasing in proximal mitotic cells, strong in meiotic germ cells [52, 155].
HIM-3	Structural component of meiotic chromosomes	Cytoplasmic in proximal progenitor zone, on chromosomes in meiotic prophase [3, 60].
PGL-1	P-granule component	Only in germ cells, not in somatic cells [150].
SP56	Sperm-specific protein	Spermatocytes and mature sperm [151].
RME-2	C. elegans yolk receptor	Oocyte membranes [156].
DAO-5	Homolog of the nucleolar phosphoprotein Nopp140	Nucleolus [33, 157].
Phospho-SUN-1	Meiotic chromosome pairing	On chromosomes in early meiotic prophase [3, 60].
WAPL-1	Cohesion chaperone	Progenitor zone nuclei [3, 88]
P _{lag-2} ::myrGFP	<i>lag-2</i> promoter driving myristoylated GFP	Somatic DTC [46]. Works best in hermaphrodites.
P _{arg-1} ::myrGFP	<i>arg-1</i> promoter driving myristoylated GFP	Somatic DTCs in males [6].
P _{lim-7} ::GFP	<i>lim-7</i> promoter driving GFP	Somatic sheath cells 1–4 [93].
P _{gld-1} ::GLD-1GFP:: <i>GLD-1</i> 3'UTR	<i>gld-1</i> promoter driving GFP tagged GLD-1	Cytoplasmic GFP, increasing in proximal mitotic cells, strong in meiotic germ cells [87].

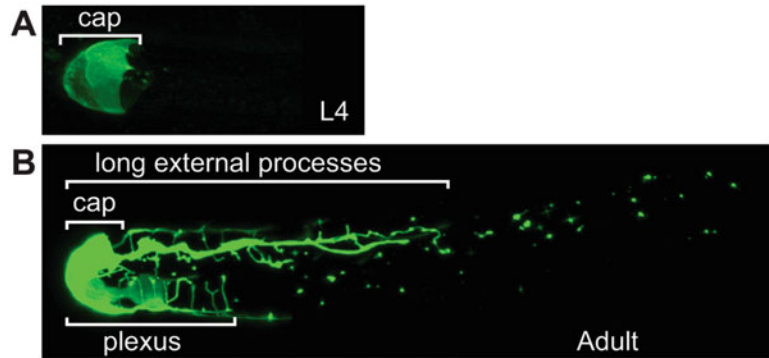


Fig. 6 DTC morphology. Gonad from hermaphrodite expressing myristoylated GFP driven by the *lag-2* promoter. Hermaphrodites have two gonadal arms, each with a single DTC at the distal end. The DTC body caps the distal-most germ cells during larval development (a) and adulthood (b). During adulthood, the DTC body extends a complex network of processes (plexus) as well as long external processes. Male gonads have one gonadal arm with two DTCs at its distal end. Male DTC bodies also cap distal germ cells, but their processes are much less complex than the hermaphrodite. (Modified from Byrd et al. [46])

3.9 Cell Cycle Analysis

Cell cycle analysis allows estimation of cell cycle lengths, identification of quiescent or slow-cycling cells, and identification of cells entering meiotic S-phase [98]. Anti-phosphohistone H3 (PH3) staining, which labels condensed chromosomes in late G2 and M phase nuclei [99], is used to determine mitotic index (% of cells in M phase) (Subheading 3.10) [30]. Alternatively, mitotic figures can be scored by DAPI staining [30, 100]. EdU labeling is used to determine the S phase labeling index (% of cells in S phase) and to estimate total cell cycle length (Subheadings 3.11–3.17). Under fixation conditions that preserve morphology well, EdU labeling combined with nuclear size can be used to identify G1 vs. G2 cells (Subheading 3.13). The proximal region of the progenitor zone, where mitotic index is low and labeling index is the same as the rest of the progenitor zone, is likely composed largely of germ cells in meiotic S phase (Subheading 3.17).

3.10 Mitotic Index with Anti-PH3

1. Prepare animals for antibody staining (Subheading 3.3).
2. Stain with anti-PH3. In our hands, mouse anti-PH3 (Cell Signaling #9706) reliably stains prophase and metaphase nuclei. Anaphase and telophase nuclei stain weakly and more variably.
3. Score position and number of PH3-positive nuclei in progenitor zone (Fig. 5a, see Note 16).
4. Count number of nuclei/row for normalizing.
5. Calculate mitotic index by dividing number of PH3-positive cells by the total number of cells in the region of interest.

3.11 EdU Labeling

EdU labeling can be performed by feeding or by soaking [30, 31, 85, 88]. Both methods are appropriate for pulse labeling (~15–30 min), but only feeding is appropriate for pulse-chase labeling or for labeling over longer time periods, because the stress of soaking can affect cell-cycle dynamics (*see Note 21*). Germ cells can also be labeled with BrdU [4] and Cy3dUTP [41]; however, BrdU is more difficult than EdU to visualize well and Cy3dUTP has to be injected.

To label with EdU by feeding:

1. Grow an overnight culture of *E. coli* MG1693 in LB or in M9 minimal media supplemented with 5 µg/ml thymine.
2. To 100 mL of *E. coli* M9 minimal media, add:
 - 3 mL of MG1693 overnight culture.
 - 100 µL 0.5 mM thymidine (0.5 µM).
 - 200 µL 10 mM EdU (Invitrogen) (20 µM) (*see Note 20*).

Grow at least 24 h at 37 °C. Spin to pellet *E. coli*. Resuspend in about 1 ml M9.
3. Seed plates with 200–400 µL labeled MG1693. Try to cover most of the plate so that animals are continuously feeding on labeled bacteria. To prevent growth of bacteria after seeding (which should be avoided because plates do not contain EdU), use nematode growth media lacking peptone and containing 60 µg/mL carbenicillin. Alternatively, plates containing *E. coli* M9 agar +60 µg/mL carbenicillin can be used, although we have found that the ammonia in *E. coli* M9 minimal media can affect the cell cycle of *C. elegans* germ cells. Plates can be stored at 4 °C for a couple of weeks.
4. Put animals on plates for the desired time period.
5. To chase, wash animals off plate with M9, wash once with M9 and place animals on plate containing unlabeled bacteria.
6. Fix with 3% PFA for 30 min at room temperature. Permeabilize with 0.1% TritonX-100 for 5 min at room temperature or with –20 °C methanol for 5–15 min. Do the Click reaction according to the instructions. We do 2 × 30 minute half-reactions (suggested by Salic, 2008). Weaker fixation conditions can also be used (e.g., 1% PFA for 10 min), but they do not preserve chromosome morphology well and therefore make it more difficult to distinguish mitotic figures and the nuclear size difference between G1 vs. G2 cells (*see Subheading 3.13*).

3.12 Label with EdU by Soaking

1. Transfer animals by picking or washing into M9 + 0.1% Tween-20 + 0.05–1 mM EdU. Incubate with rotation for 15 min.
2. Dissect gonads immediately (with no recovery period) and fix them according to the protocol for labeling with EdU by feeding, above.

3.13 Calculating Labeling Index and Identifying G1 and G2 Cells

Cell-cycle stage in mitotically dividing germ cells correlates with nuclear size: G1 and early S-phase nuclei are small; G2 and late S-phase nuclei are large; and mid S-phase nuclei are intermediate in size (Fig. 7, Movie 1). The size difference between G1 and G2 cells

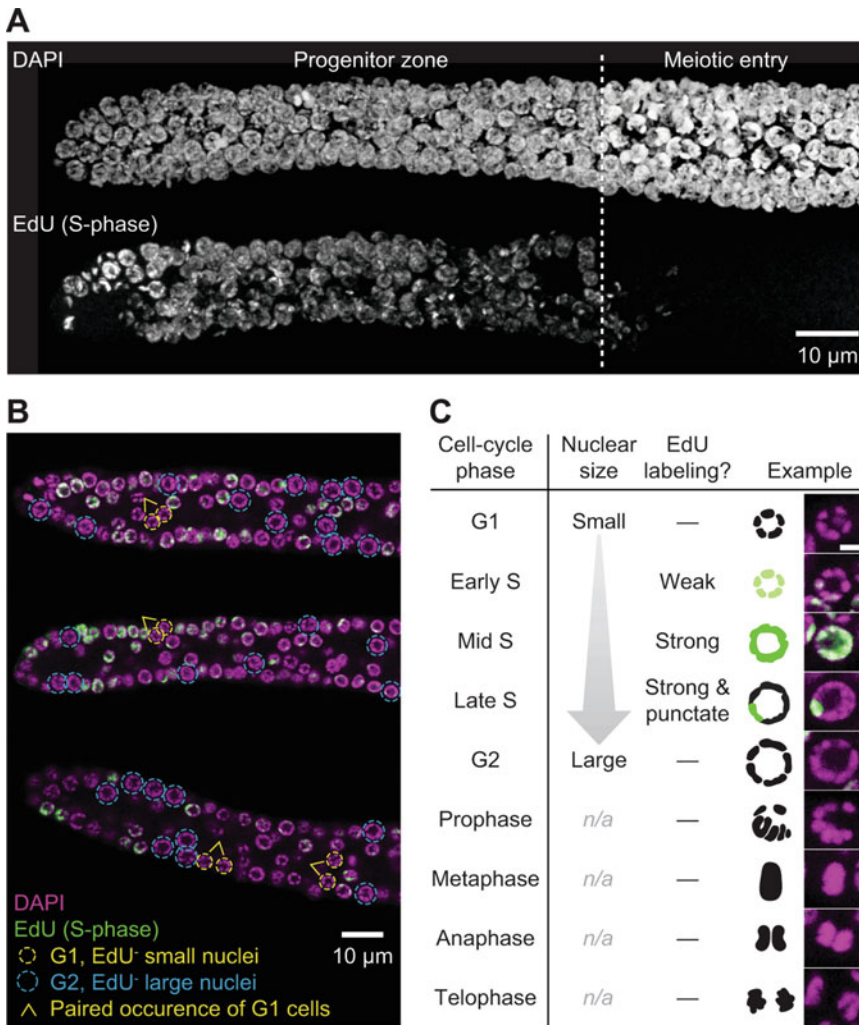


Fig. 7 EdU labeling. (a) Image of an adult hermaphrodite germline stained with DAPI to visualize DNA (top) and labeled with a 15-minute pulse of EdU to mark S-phase cells (bottom). Images are maximum-intensity z-projections. (b) Two-color images of adult hermaphrodite germlines treated as in A. Magenta, DAPI staining. Green, EdU. Yellow circles, G1 cells. Blue circles, G2 cells. Caret, occurrence of G1 cells in pairs. (c) EdU-labeling and nuclear size characteristics for cells in different stages of the cell cycle. Scale bar, 2 μm. n/a, nuclear size not applicable during M phase

is large enough that after pulse labeling with EdU to mark S-phase cells, G1 and G2 cells can be distinguished by eye. This correspondence between cell-cycle stage and nuclear size has been vetted in wild-type germlines of both sexes, but it may not hold true under conditions that alter nuclear size, such as after knockdown of certain cell-cycle regulators [42, 101]. Keep in mind that the fraction of cells detected as EdU-positive will be affected by the sensitivity of the detection system. Early S-phase nuclei label weakly with EdU and may be detected as G1 cells by image acquisition systems that are less sensitive.

1. Pulse animals with EdU for a short time, e.g., 15–30 min (*see* Subheading 3.11).
2. Fix and stain as described in Subheading 3.11. Co-stain with DAPI.
3. Examine germline at high magnification (e.g., 63X), and identify G1, S-phase, and G2 cells using the following criteria. We find it convenient to acquire z-stack images at 0.5–1 μm intervals and to identify cell-cycle stages on a computer monitor. Cells can be easily marked and categorized using the Cell Counter plug-in for ImageJ (http://fiji.sc/Cell_Counter).
 - (a) G1 cells are EdU-negative and have a nuclear size equal to or smaller than the smallest EdU-positive cells (Fig. 7c, Movie 1). Additional characteristics of G1 cells are that they are infrequent (~5–10%) and nearly always occur in pairs (Fig. 7b, Movie 1), a pattern consistent with G1 being very short [30, 42]. Chromatin in G1 cells tends to be rather condensed (Fig. 7c), such that the 12 chromosomes are often distinguishable as 12 DAPI-stained puncta (not shown).
 - (b) S-phase nuclei are EdU-positive. S-phase nuclei can be further categorized as early, mid, or late according to nuclear size and pattern of EdU labeling (Fig. 7c, Movie 1). Early S-phase nuclei are small and label weakly with EdU; mid S-phase nuclei are mid-sized and label strongly and uniformly with EdU; late S-phase nuclei are large and incorporate EdU in a punctate pattern, likely reflecting the late-replicating X-chromosome [41].
 - (c) G2 nuclei are EdU-negative and have a nuclear size equal to or larger than the largest EdU-positive nuclei (Fig. 7c, Movie 1).
4. To calculate the labeling index, count number of EdU-positive germ cells and total number of germ cells in the region of interest (e.g., entire progenitor zone, one row, or a subregion) then divide the number of EdU-positive germ cells by total germ cell number. Analogous calculations are performed to determine the G1 or G2 index.

3.14 Estimating Total Length of the Cell Cycle

There have been several estimates for the total length of the cell cycle in mitotically dividing germ cells, reviewed in [3, 30]. There is variability in cell cycle length among germ cells within a single gonad as well as at different stages of development and under different environmental conditions [30, 85, 102]. Thus, it is important to compare experimental conditions to wild-type in your own hands.

Cell-cycle length can be calculated by measuring the length of G2 or by measuring the length of G2 + M + G1. The first method involves labeling animals with EdU and determining the length of time until all M-phase cells become labeled with EdU. The second method determines the time until all cells become labeled with EdU. Measuring the length of G2 is more straightforward because there is a one-to-one correspondence between cells labeled at time zero and cells queried for their labeling status at later time points.

3.15 To Measure Cell-Cycle Length by Measuring G2

1. Label animals with EdU (on plates) by feeding for 15–30 min to ~12 h.
2. Fix and stain some animals every 30 min to 2 h.
3. Determine the fraction of M-phase cells that are EdU-positive at each time point.
4. The time it takes for 50% of M-phase cells to become EdU-positive is the median length of G2 (the time it takes for the cells in late S-phase to traverse G2 and enter M phase). The time it takes for 100% of M-phase cells to become EdU-positive is the maximum length of G2.
5. Median total length of cell cycle is estimated by dividing the median length of G2 by the fraction of cells in G2. The fraction of cells in G2 represents the proportion of cell cycle time spent in this phase.
6. Estimates of the maximum total length of the cell cycle depend on assumptions about covariance between the length of G2 and the length of M + G1 + S. If cells having a longer G2 are assumed to have a proportionally longer M + G1 + S, then the maximum length of the cell cycle is estimated by dividing the maximum length of G2 by the fraction of cells in G2. If cells having a longer G2 are not assumed to have a proportionally longer M + G1 + S, then the maximum length of the cell cycle is given by the following equation: (median total length of cell cycle * (M + G1 + S index)) + time at which all M-phase cells are EdU-positive. These two calculations can yield different estimates if the distribution of G2 lengths is long-tailed.

3.16 To Measure Cell-Cycle Length by Measuring G2 + M + G1

1. Label animals with EdU by feeding for 15–30 min to ~12 h.
2. Fix and stain some animals every 30 min to 2 h.
3. Determine labeling index (*see* Subheading 3.13) for each time point.
4. Median length of G2 + M + G1 is given by twice the time it takes for the labeling index to reach mid-way between its starting value and 100% (the time it takes for cells in early G2 to traverse through G2, M, and G1 and finally label in S phase) [103]. The time it takes for 100% of cells to become EdU-positive is the maximum length of G2 + M + G1.
5. Median total length of cell cycle is estimated by dividing the median length of G2 + M + G1 by the fraction of cells not in S-phase (i.e., 1 – the labeling index) and represents the proportion of cell cycle time spent in these phases.
6. As described for measuring cell-cycle length via G2, estimates of the maximum total length of the cell cycle depend on assumptions about covariance between the length of G2 + M + G1 and the length of S. If cells having a longer G2 + M + G1 are assumed to have a proportionally longer S, then the maximum length of the cell cycle is estimated by dividing the maximum length of G2 + M + G1 by the fraction of cells in G2 + M + G1. If cells having a longer G2 are not assumed to have a proportionally longer S, then the maximum length of the cell cycle is given by the following equation: (median total length of cell-cycle * (S index)) + time at which all cells are EdU-positive. These two calculations can yield very different estimates if the distribution of G2 + M + G1 lengths is long-tailed.

3.17 Identification of Premeiotic Cells in the Progenitor Zone

1. Calculate mitotic index at each position along the distal-proximal axis (*see* Subheading 3.10).
2. Calculate labeling index (15 minute pulse) at each position along the distal-proximal axis (*see* Subheading 3.13).
3. In wild-type, the proximal 4–6 rows of the progenitor zone have a lower mitotic index, but the labeling index remains constant. Since these cells do not progress into mitosis (no mitotic cells are seen in proximal to the start of meiotic prophase), this indicates that these cells are in meiotic S phase. There are S phase nuclei proximal to the start of meiotic prophase and it is inferred that these are in meiotic S phase (Figs. 7a and 8).

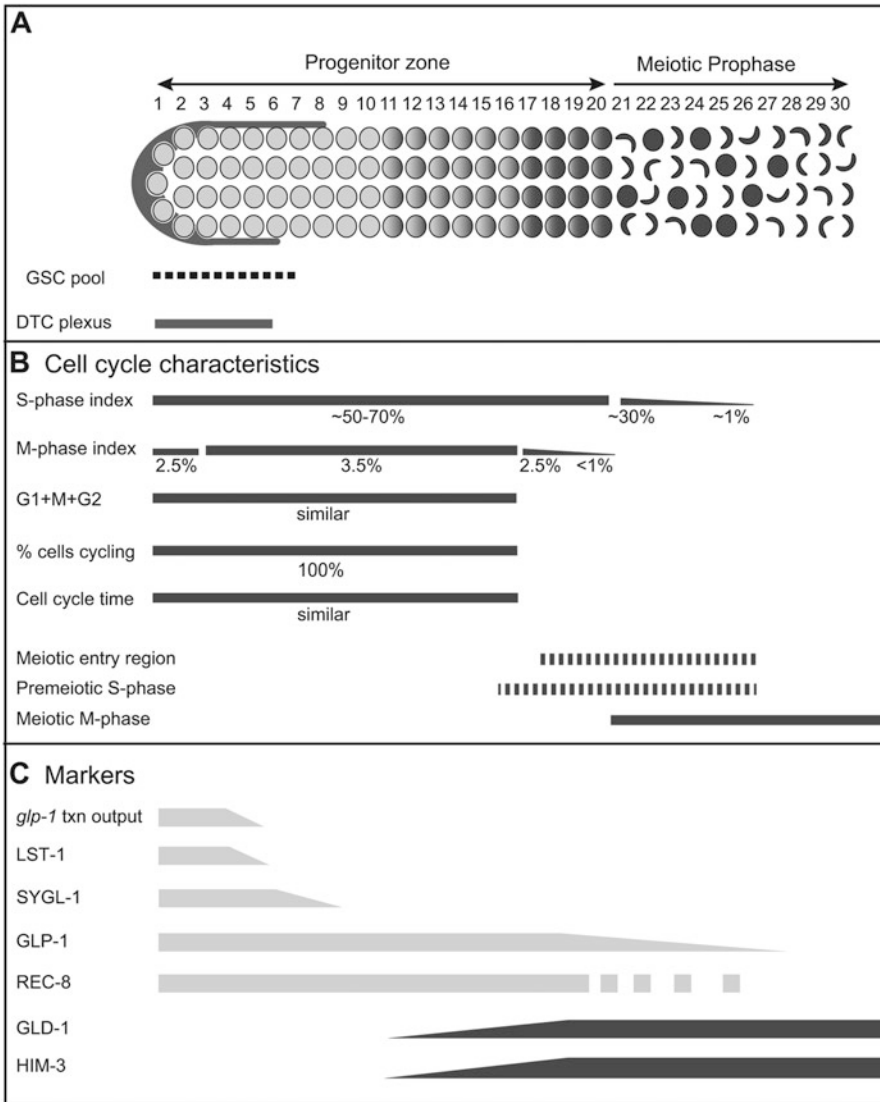


Fig. 8 Summary of cell cycle characteristics and markers for the progenitor zone in the adult hermaphrodite germline. **(a)** Diagram of the distal arm of the germline. Dark dotted line indicates region likely to contain germline stem cells (GSCs). Progenitor zone cells proximal to the GSC pool have been triggered to differentiate and are making the transition into meiosis. **(b)** Summary of cell cycle characteristics. **(c)** Summary of where markers are expressed in the distal part of the gonad (see text, Table 1 and references therein for detailed descriptions of patterns and staining methods)

3.18 Characterization of Progenitor Zone Defects in Mutants

The steps we take to characterize the phenotypes of new germline defective mutants are described briefly in this section. In particular, the goal is to distinguish mutants in the stem cell regulatory pathway, which cause a switch from self-renewal to differentiation in the GSC pool, from those affecting a more general process such as cell cycle or survival, which would cause cell cycle arrest or death of the

GSC pool. Mutants can be generated either by classical forward genetic screens [104] or by reverse genetic techniques such as RNAi [105] and CRISPR/Cas9 genome editing [106, 107]. Germline phenotypes are sensitive to age of worm, temperature, and starvation. Thus it is important to stage, grow, and score mutant animals in parallel to a wild-type control (*see* **Note 1**). Phenotypes generated by both forward and reverse genetics are annotated in WormBase.

Regulatory mutants can have either strong or subtle effects. Strong effects include complete differentiation of GSCs into mature gametes, causing the germline to be very small and contain only mature sperm or oocytes (e.g., *glp-1(lf)*) or complete loss of germline differentiation, which causes the germline to fill with undifferentiated cells (e.g., *glp-1(gf)*). Subtle effects include changes to the length of the progenitor zone or the number of cells within that region (e.g., *fbf-1*, *fbf-2*, *gld-2* and *gld-3* single mutants) [108, 109]. Some regulatory mutants only show strong phenotypes in double mutant combinations, due to redundancy (e.g., PUF proteins in GSC pool: *fbf-1* and *fbf-2* [45, 108] and *puf-3* and *puf-11* [15], and *lst-1* and *sygl-1* [10]). In addition, some regulators are pleiotropic, affecting multiple developmental processes, and can only be analyzed by isolating unusual alleles [104, 110, 111]; temperature-sensitive or weak alleles, e.g., *lag* genes [112], by using germline-specific and/or stage-specific RNAi (e.g., [10, 101] or by conditional gene expression (*see* next paragraph).

Conditional gene expression can be achieved in a number of ways and used to study gene functions in both soma and germline; however, it is especially difficult in adult GSCs. The heat shock promoter works well in the soma and proximal germline; however, it does not work well in adult GSCs [113]. FLP/FRT, Cre/lox, and the Q system work in the soma [114–116], and the Cre/lox system can also work in the larval germline [117]. Alternative methods for conditional gene expression that have been used in *C. elegans* include the use of destabilizing domains [13, 118, 119], optogenetics [120], and light-activated nuclear shuttles [121].

Expression of non-*C. elegans* genes in the germline can be challenging due to strong small RNA-mediated gene silencing. For example, GFP is often silenced, but GFP silencing can be reversed [82] and constructs can be designed to resist silencing [122]. In addition to GFP, SNAP and HALO tags can be used to fluorescently label germline proteins *in vivo* or in fixed tissues and do not have issues with silencing [44, 123, 124]. Regulatory regions that work well for germline expression, including adult GSCs, have been described, e.g., the *mex-5* promoter for general germline expression [113] and the *sygl-1* promoter for GSC

expression [9]. It is important to consider promoters, UTRs, and introns ([10, 113, 125]. Codon optimization and addition of introns are described in [122, 126] (*see Note 22*).

3.19 Initial Characterization of Mutants

1. Compare mutant to wild-type germlines at defined stages of development (Fig. 1c) (*see, for example, [15]*). Using DIC microscopy (Subheading 3.2), look first at adults to determine whether the germline has a normal size and pattern of cell fates. If the germline does not look normal, look at progressively earlier stages to determine when the mutant begins to differ from wild-type (*see Note 23*).
2. Is there a defect in the switch from GSC self-renewal to differentiation? If germ cells can no longer divide mitotically, but can still progress through meiotic prophase and gametogenesis, mature gametes will be present at the distal end of the germline where GSCs reside in wild-type animals (*see Note 24*). This defect in germline proliferation is typical of GLP-1/Notch signaling mutants, *lst-1*; *sygl-1* double mutants [9] and *puf* mutants [15, 45] (Fig. 2b). Alternatively, if germ cells can no longer enter the meiotic cell cycle, a germline tumor can result. This is typical of strong *glp-1* gain-of-function mutants, which have unregulated GLP-1/Notch signaling [127], and overexpression of either *lst-1* or *sygl-1* [10]. In addition, germline tumors can also develop from double or triple mutants, for example, *gld-2 gld-1* or *gld-2 gld-1 prom-1* loss-of-function mutants, which have decreased meiosis-promoting activity [88, 128] (Fig. 2c). Tumors resulting from an increase in GLP-1/Notch signaling are typically full of undifferentiated germ cells; little or no meiosis is seen. By contrast, tumors resulting from decreased meiotic activity often have some meiosis but revert to mitotic proliferation and can form teratomas. Teratomas lose their germ cell identity and can be identified by germ cell expression of somatic markers such as neuronal or muscle markers reviewed in [129].

Changes in timing of germ cell differentiation [130, 131], interaction with an ectopic niche [132], or failure to maintain meiosis or gametogenesis [133, 134] can result in proximal tumors. In these cases, the distal germline is relatively normal, but the proximal germline contains dividing germ cells where differentiating gametes would be found in a normal germline (*see [3]* for further description of germline tumor phenotypes).

3. Is stem cell maintenance affected early or late? Loss of the core *glp-1/Notch* signaling pathway causes GSCs to differentiate in young larvae whereas in *fbf-1fbf-2* mutants germ cells divide normally until L4 at which point GSCs differentiate. Some gain-of-function *glp-1* mutants can also generate “creeping tumors” which cause an increase in progenitor cells with age [135].

4. Is the gene required for both proliferative phase during larval development and maintenance phase in adults? RNAi can be performed at different larval and adult stages to test the temporal requirement for gene activity (*see*, for example, [9, 42, 101]).
5. Is the germline small and lacking differentiated gametes? This type of small germline can result from slowed or arrested proliferation or inappropriate cell death [136, 137]; *see* list of other genes in [3, 138].
6. If a progenitor zone is present, determine its length, cell number, and mitotic index (*see* Subheadings 3.5 and 3.10) (*see Note 1*) to identify subtle effects on proliferation.
7. Does the mutant affect germline or soma? Germline proliferation requires Notch signaling from the somatic distal tip cell (DTC) [94]. In addition, the distal somatic sheath cells and neuronal signaling can affect germline proliferation, reviewed in [3]. Several approaches can be taken to determine whether the defect is germline autonomous or due to a somatic defect.
 - (a) Is the mRNA present in the germline or soma or both? Which part of the gonad contains the mRNA? First, check the spatial transcriptomic databases [55, 56], the in situ RNA expression database, NEXTDB (<http://nematode.lab.nig.ac.jp/>), and germline-related expression databases (*see* [139] and refs therein) (*see* Web Resources Subheading 2.2). Next, perform in situ hybridizations on extruded gonads (*see Note 17*) to determine whether expression is restricted to the germline or also present in the somatic cells such as the DTC or sheath (e.g., [9, 140]).
 - (b) Transgenic rescue is much more efficient in somatic tissues. If a mutant is easily rescued with simple arrays, it is likely that the gene functions in the soma (e.g., *see* refs [130, 141]).
 - (c) RNAi in *rrf-1* mutant background. *rrf-1* mutants have decreased RNAi efficiency in the soma, but not in the germline (*see* Kumsta and Hansen, 2012 [142] for more details on *rrf-1* function in the soma).
8. Where does the mutant fit in the regulatory pathway? The genetic pathway controlling the mitosis/meiosis switch has been well characterized [1, 3] (Fig. 1b). Epistasis analysis is used to determine at what point in the pathway a gene acts [15, 88, 104].

4 Notes

1. The age of the worm affects the size of the progenitor zone, both in terms of progenitor zone length and total cell number. Traditionally, measurements are done in young adult hermaphrodites, about 24 h after mid-L4 at 20 °C. The progenitor zone shortens with age (reviewed in [29]) and animals that accumulate oocytes (stacked oocytes) due to lack of sperm have lower mitotic indices [31]. Old animals have fewer cells in the progenitor zone [28], whereas newly molted adults have more progenitor zone cells than animals aged 24 h after L4 [30]. Changes in progenitor zone length and total cell number do not always correlate (e.g., male germlines) [31] so do both counts for the most accurate description of progenitor zone size.
2. Descriptions of worm culture and genetics are available in WormBook (http://www.wormbook.org/chapters/www_introandbasics/introandbasics.html).
3. A detailed description describing the use of DIC microscopy to look at *C. elegans* can be found on the WormBook site (http://www.wormbook.org/chapters/www_intromethodscellbiology/intromethodscellbiology.html). Live imaging requires special conditions to maintain progenitor zone divisions for hours. Immobilization techniques include pads made with a higher percentage of agarose in combination with microbeads [12, 143, 144], microgrooves in agarose [76], and microfluidic devices [92, 145]. Addition of serotonin can help for short-term live imaging [12, 62, 144].
4. Placing animals directly on the agarose pad next to the drop of M9 helps them stay in place when the coverslip is put on the slide. This is especially helpful when looking at younger animals that tend to float in the M9 (see Subheading 3.2).
5. Paraformaldehyde fixation improves nuclear morphology. We generally use 1% to 3%, depending on the antigen. Most antibodies work well within this concentration range. If fixation with paraformaldehyde does not work, try fixing with -20 °C MeOH for 10 min followed by -20 °C acetone for 10 min.
6. For staining germlines in younger animals (L1-L3), it is often easier to use a whole mount protocol [68]. L3 and L4 gonads can be extruded, but they are small and take more patience and care than adult germlines.
7. Whole animals can be easily DAPI stained [146]. DAPI staining in whole animals is useful for a general look at germline size and gamete production; however, in whole mounts, it is more difficult to look at the nuclear morphology of progenitor zone cells or to identify germ cells in mutants with small gonads.

8. Gonad extrusion tips. More liquid on the slide helps prevent drying out. If animals are in levamisole too long, germlines will no longer extrude well, so pick fewer worms at a time if cutting takes a while. We try to keep dissection times to 5–10 min. Finally, an eyelash taped to a toothpick can help position or untangle germlines.
9. More gonad extrusion tips for slide method. A number of factors can affect gonad attachment to slides. First, the slides should be freshly subbed. An increase in the amount of time the subbing solution is left on the slide can improve attachment; however, too much time can result in a thick layer, which can peel off. Second, a drop of M9 on the slide should stay in a rounded drop. If it spreads, the gonads may not stick well. Third, be sure there is little or no *E. coli* picked into the M9. Fourth, a coverslip can be laid gently on the extruded gonads to increase contact with the subbed surface. The slide and coverslip are then frozen at -70°C or on dry ice, the coverslip is popped off and standard fixation protocols are followed. The need for gonad attachment to slides is eliminated using the solution method for antibody staining.
10. To reduce background in secondary antibodies, we use antibodies preabsorbed against other species for double and triple labeling. We typically incubate with the secondary antibody 1–2 h at room temperature at the supplier's recommended dilution.
11. An alternative DNA dye is TO-PRO-3, which emits a far-red signal. To use TO-PRO-3, dilute $\sim 1:50,000$, incubate with a fixed sample for about 10 min at room temperature after the secondary antibody incubation. Then wash as usual.
12. Add *E. coli* to live animals in solution. This can be done by collecting *E. coli* on a pick and swishing it off in the solution with the animals. The *E. coli* will ensure the animals have sufficient food and encourage them to eat the dye (*see* Subheading 3.4).
13. Ovulation can slow or stop in live animals kept on slides for extended periods of time. This, in turn, may influence the proliferation of the mitotic cells and the rate of movement of cells through the germline.
14. Sometimes the distal end of the gonad is lost or damaged during extrusion, so to get accurate counts, it is important to be sure the whole gonad is intact. To learn to identify the DTC nucleus look at live or fixed animals carrying a *lag-2* promoter::GFP construct (*see* Table 1).
15. The boundary between the progenitor zone and the meiotic prophase can be difficult to score using the DAPI method since crescent-shaped nuclei are mixed with nuclei in prophase.

Score a number of wild-type germlines to become comfortable with cell identification and to develop a consistent scoring method. Use markers for more accurate and precise scoring.

16. Germ cells do not line up in precise rows, so row counts are somewhat subjective and variable [33]. Counting the same germline several times gives a measure of the accuracy of the counting method. In addition, a large sample size helps increase accuracy, especially for anti-PH3 counts.
17. More RNA in situ and smFISH protocols can be found in WormBook Methods [147, 148]. Germline-specific smFISH methods can be found in [79, 81].
18. Shifted *emb-30* germ cells can be difficult to score. There is variability in response to the temperature shift, nuclear morphology degrades with time, and scoring the GLD-1 boundary can be subtle and, in some gonads, impossible. It is important to have a large number and to compare directly to controls. In addition, the interpretation is complicated by the fact that cell cycle arrest could affect cell fates by triggering a checkpoint, altering cellular metabolism, etc. (*see* refs [51, 149] for views on how to interpret this data).
19. Strains carrying transgenes that express in either the DTC or the germline often have subtle germline defects. For example, $P_{lag-2}::GFP$ germlines sometimes have a sterile phenotype similar to *glp-1* mutants. Be sure to compare the transgenic germlines to wild-type.
20. Feeding the bacteria 0.2–10 μ M EdU also works, but labeling becomes more variable as the dose of EdU decreases. We try to keep the dose low to reduce toxicity.
21. At short time points (up to a couple hours) the labeling index is similar to that for animals fed labeled bacteria; however, this method is not suitable for long-term labeling.
22. There have been anecdotal reports that some Gateway sequences can interfere with transgene expression. We use Gibson cloning rather than Gateway cloning.
23. It can be difficult to distinguish germ cells from somatic gonad cells in early development. A good germ cell-specific marker is PGL-1. Germlines can be stained for endogenous PGL-1 [150] or a PGL-1::GFP transgene can be used (*see* Table 1).
24. Markers are available to confirm the sexual identity of differentiating gametes in wild-type or mutant germlines. Good sperm-specific markers are the monoclonal antibody, SP56 [151], and anti-SPE-44 [152, 153]. Good oocyte-specific markers include LIN-41, OMA-1, and RME-2 (*see* Fig. S1 in [154]) (*see* Table 1).

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Analysis of *C. elegans* Germline Small RNA Pathways

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Abstract

Sequence-specific gene regulation by small RNA (sRNA) pathways is essential for the development and function of organisms in all domains of life. These regulatory complexes, containing an Argonaute protein (AGO) guided by a bound sRNA, have the potential to regulate thousands of individual target transcripts at both the co- and post-transcriptional level. Determining the repertoire of transcripts that an AGO is capable of regulating in a particular context is essential to understanding the function of these regulatory modules. Immunoprecipitation (IP) of AGOs and subsequent RNA sequencing of their bound sRNAs allows for the inference of their target transcripts by mapping the sequences of the co-precipitated sRNAs back to their complementary target transcripts. This approach can be complemented by sequencing sRNAs from *ago* mutants as sRNA transcripts are degraded in the absence of their AGO binding partner. Here, we describe a framework for analyzing AGO/sRNA pathways in the germline, from using CRISPR-Cas9 to tag or mutate AGOs, through protocols for the extraction, sequencing, and analysis of sRNAs from AGO IPs and *ago* mutants.

Key words sRNA, siRNA, miRNA, piRNA, Immunoprecipitation, RNA-seq, CRISPR, Argonaute, *C. elegans*

1 Introduction

Small RNA (sRNA) pathways are highly conserved gene regulatory modules that regulate a diversity of biological processes. sRNAs guide sequence-specific gene regulation by associating with effector Argonaute (AGO) proteins, which have the potential to regulate transcripts both co- and post-transcriptionally. In this complex, also known as the RISC (RNA-Induced Silencing Complex) the sRNA determines the target specificity by sequence complementarity, while the type of regulatory activity is defined by the properties of the specific AGO protein and its cofactors [1]. Across all domains of life, sRNA pathways play essential roles from the precise control of developmental timing events to protecting cells from foreign genetic elements [2].

Table 1
Relevant features of small RNA classes in *C. elegans*

Class	Biogenesis	Size (nt)	Predominant 5' Nucleotide	5' Modification	3' Modification
miRNA	Transcribed from genome by RNA polymerase II, processed by Drosha and dicer	~22	U	Mono-phosphorylated	
piRNA	Transcribed from genome by RNA polymerase II	21	U	Mono-phosphorylated, capped [44]	2'-O-methylation
26G-RNA	Transcribed from mRNA template by the RdRP, RRF-3, processed by Dicer	26	G	Mono-phosphorylated	ERGO-1 dependent 26G-RNAs possess 2'-O-methylation, other 26G-RNAs do not [45–47]
22G-RNA	Transcribed from mRNA template by RdRPs, RRF-1 and EGO-1	22	G	Tri-phosphorylated	

In many organisms, post-transcriptional gene regulation is particularly important from gametogenesis to fertilization as the genome is largely transcriptionally silent during these crucial developmental stages [3]. Therefore, it is not surprising that regulation by sRNA pathways has been tightly linked to fertility in organisms ranging from maize to humans [4, 5]. In addition to the post-transcriptional regulation of mRNAs important for germline development, sRNA pathways also play a role in silencing transposable elements, which is especially important during gametogenesis when repression of these elements by heterochromatin is in flux [6, 7].

Since the discoveries of microRNAs and the mechanism of RNA interference (RNAi) over 25 years ago in *C. elegans*, this tiny nematode has remained a premier model for studying sRNA pathways. The 19 functional AGOs in *C. elegans* partner with four endogenous classes of sRNAs: miRNAs, piRNAs, and two distinct classes of siRNAs called the 26G-RNAs and 22G-RNAs. While the miRNAs and piRNAs are genomically encoded, the siRNAs are transcribed directly from mRNA templates by RNA-dependent RNA polymerases (RdRPs). These four types of sRNAs differ in length, nucleotide composition, and chemical modifications (Table 1) [8].

An individual AGO has the ability to regulate up to thousands of distinct target transcripts. Therefore, determining the identity of these transcripts is essential to unlocking the functional roles of specific AGOs. Approaches to identify AGO target RNAs fall into

two categories: (1) directly identifying targets bound by AGO, through experiments such as RNA IP (RIP) or Cross-Linking IP (CLIP); (2) indirectly identifying sRNAs associated with AGOs and inferring targets computationally. Here we focus on identifying the sRNA partners of AGO, using two complementary approaches:

- (a) *Immunoprecipitation (IP) of an AGO and sequencing of co-precipitated sRNAs.* This approach indirectly identifies target transcripts by mapping sRNA reads that co-immunoprecipitate with an AGO back to the genome. For sRNA classes transcribed by RdRPs, which are directly complementary to their targets, the identification of targets in this way is straightforward. However, for miRNAs and piRNAs, which can regulate targets with imperfect complementarity, co-precipitated sRNA reads can be queried against databases of known miRNA and piRNA sequences and targets. This approach requires epitope tagging or generating a specific antibody for the AGO of interest.
- (b) *sRNA sequencing of an ago mutant.* This approach is based on the fact that sRNAs are generally unstable without an AGO as their chaperone. Therefore, the loss of specific sRNAs in an *ago* mutant compared to a wild-type control can be used to determine the sRNAs that are normally associated with that AGO. This approach requires a null or sRNA binding mutant for the *ago* of interest.

Using CRISPR-Cas9 genome engineering, our lab has generated a GFP::3xFLAG-tagged allele for each of the 19 functional AGOs in *C. elegans* at their endogenous loci [9]. Using these strains to examine protein expression throughout development, we determined that 16 of these AGOs are expressed in the germline, which includes members of all four endogenous sRNA pathways [9]. This set of tagged AGO strains has facilitated AGO IPs with commercially available antibodies and the sequencing of co-precipitated sRNAs. In addition, CRISPR-Cas9 genome editing has also proven useful in generating null or “designer” alleles of the *agos* to assess sRNA loss in mutants. Our analysis of *C. elegans* AGOs represents the tip of the iceberg; there remains much to be discovered about the functions of specific AGOs/sRNA pathways in various contexts (e.g., under varied growth conditions, in different developmental stages, etc.), as well as in other nematode species.

In this chapter, we discuss considerations for using CRISPR-Cas9 to epitope tag or mutate AGOs, and detail our protocols for the extraction, sequencing, and analysis of sRNAs from AGO IPs and *ago* mutants (Fig. 1). We use the well-studied nuclear AGO HRDE-1, which is expressed in the *C. elegans* germline and associates with a specific set of 22G-RNAs, as an example throughout the chapter (Fig. 2).

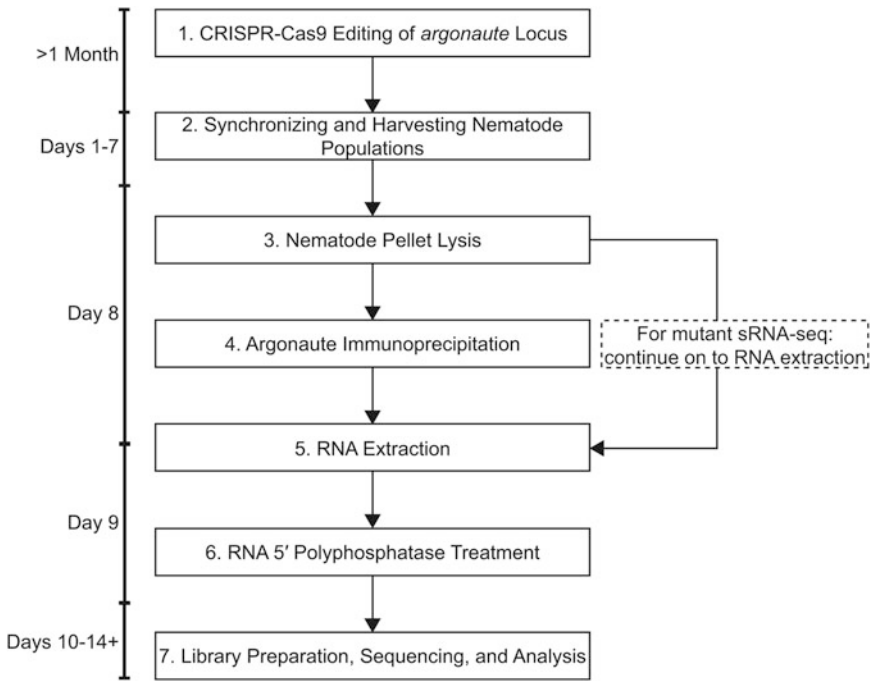


Fig. 1 Workflow and timeline for the analysis of germline small RNAs in *C. elegans*

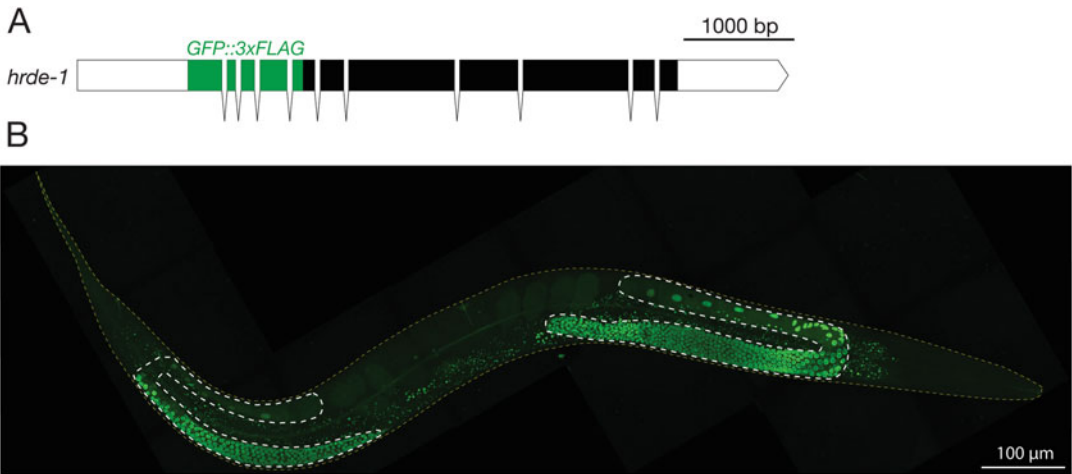


Fig. 2 Analysis of GFP::3xFLAG HRDE-1 expression in adult *C. elegans*. (a) We inserted a GFP::3xFLAG epitope tag at the N-terminus of the AGO HRDE-1 using CRISPR-Cas9 genome editing [9, 12]. Scale bar represents 1000 bp. (b) Confocal micrograph of GFP::3xFLAG::HRDE-1 expression in adult hermaphrodite *C. elegans*. The gonad arms are outlined in white and the worm body is outlined in yellow. Image was taken on a Leica DMI8 TCS SP8 confocal microscope using a 63×/1.40 HC PL APO CS2 oil objective. Scale bar represents 100 μm. HRDE-1 is found within germline nuclei

2 Materials

2.1 CRISPR-Cas9

1. PredictProtein: <https://predictprotein.org>
2. AlphaFold: <https://alphafold.ebi.ac.uk/>
3. CRISPOR: <http://crispor.tefor.net>

2.2 Synchronizing and Harvesting Nematode Populations

1. Nematode Growth Medium (NGM): 3 g NaCl, 2.5 g peptone, 17 g agar A, ddH₂O to 1 L. Autoclave NGM, MgSO₄, CaCl₂, and PPB separately. After autoclaving, cool the NGM to about 65 °C and add 1 mL of 1 M MgSO₄, 1 mL of 1 M CaCl₂, 1 mL of 5 mg/mL cholesterol and 25 mL of PPB per liter while stirring.
2. Potassium Phosphate Buffer (PPB): 108.3 g KH₂PO₄, 35.6 g K₂HPO₄, to 1 L with ddH₂O. Autoclave.
3. Terrific Broth (TB): 12 g tryptone, 24 g yeast extract, 8 mL 50% glycerol, ddH₂O to 1 L. Autoclave.
4. M9 buffer: 22 mM KH₂PO₄, 44 mM Na₂HPO₄, 85 mM NaCl, 1 mM MgSO₄. pH to ~6 with HCl.
5. 150 mm Petri dishes.
6. Serological pipettes.
7. 15 mL conical tubes.
8. Benchtop centrifuge.
9. Worm Bleach Solution: 43 mL dH₂O, 5 mL 12% sodium hypochlorite, 2 mL 5 M potassium hydroxide. Make fresh or store up to 1 week in the dark.
10. 50 mL conical tubes.
11. Dry ice.
12. 95% Ethanol.

2.3 Worm Pellet Lysis

1. EDTA base buffer: 10% glycerol, 10 mM EDTA, 30 mM HEPES, 100 mM Potassium Acetate. Filter sterilized. Store at 4 °C.
2. EDTA+ buffer: EDTA base buffer, 0.1% NP-40, 2 mM Dithiothreitol. Rock until the solution is homogeneous and chill on ice before use.
3. EDTA complete buffer: EDTA+ buffer, 1:100 Phosphatase Inhibitor Cocktail 2 (Sigma-Aldrich), 1:100 Phosphatase Inhibitor Cocktail 3 (Sigma-Aldrich), 2 tablets of cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail (Roche) per 5 mL, 1:100 SUPERase•In RNase Inhibitor (20 U/μL). Rock the solution on a rocking platform or rotary turner until tablets have dissolved and chill on ice before use.
4. Optional: Pestle (size for 15 mL conical tube).

5. Optional: Bioruptor Pico Sonicator.
6. Optional: Dounce homogenizer.
7. Optional: liquid nitrogen, mortar and pestle.
8. Nuclease-free, low-binding microcentrifuge tubes.
9. Optional: Lowry assay reagent kit.

2.4 Immunoprecipitation/Immunoprecipitation (IP)

1. Protein 6× Sample buffer (We use Invitrogen Bolt LDS sample buffer and reducing agent).
2. TRI reagent.
3. Non-conjugated magnetic beads.
4. Antibody-conjugated beads or antibody solution to be incubated with non-conjugated beads.
5. Magnetic microcentrifuge tube rack.

2.5 RNA Extraction

1. Chloroform or 1-bromo-3-chloropropane (BCP).
2. Phenol:chloroform:isoamyl alcohol (25:24:1).
3. Glycogen.
4. Isopropyl alcohol.
5. 70% ethanol.

2.6 RNA 5' Polyphosphatase Treatment

1. RNA 5' polyphosphatase (Epicentre 10 U/μL).
2. 10X reaction buffer.
3. RNasin Plus RNase inhibitor (40 U/μL).
4. Phenol:chloroform:isoamyl alcohol (25:24:1).
5. Glycogen.
6. 3 M Sodium Acetate (pH 5.2).
7. Anhydrous ethanol.

2.7 Library Preparation, Sequencing, and Sequence Analysis

1. Optional: NEBNext Small RNA Library Prep Set for Illumina.
2. FastQC: <https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>
3. Cutadapt: <https://cutadapt.readthedocs.io/en/stable/#>
4. STAR: <https://github.com/alexdobin/STAR>
5. R: <https://www.r-project.org>
6. tinyRNA: <https://github.com/MontgomeryLab/tinyRNA>
7. Wormbase: <https://wormbase.org>
8. miRbase: <https://www.mirbase.org/>
9. piRTarBase: <http://cosbi6.ee.ncku.edu.tw/piRTarBase/>
10. RepeatMasker: <https://www.repeatmasker.org/>

3 Methods

3.1 CRISPR-Cas9

The advent of genome engineering using CRISPR-Cas9 has revolutionized molecular biology. Using CRISPR-Cas9 it is now possible to engineer the genome in virtually any location with ease. In *C. elegans* there are a number of methods that have been proven for efficient CRISPR-Cas9 genome editing [10–12]. Each method has its own advantages and disadvantages so selecting an approach will depend on the goals of your experiment. For the purpose of this chapter, we will focus on considerations for using CRISPR-Cas9 to generate epitope-tagged AGO proteins and mutant *ago* genes.

3.1.1 Selection of Epitope Tag

The selection of epitope tags depends on the specific needs of the experiment. Fluorescent protein tags, such as GFP, offer an advantage where localization of AGO can be observed in live animals. In our hands, tagging with GFP yielded, in most cases, a functional GFP-AGO protein fusion that was detectable under confocal microscopy. In some cases however, a large tag such as GFP may interfere with protein function. In these cases, or in cases where a fluorescent tag is unnecessary, it is advisable to use a smaller tag such as FLAG, HA, OLLAS, or Myc. Using these tags in multiple copies (such as 3xFLAG) enables additional binding sites for anti-tag antibodies and can increase specificity and sensitivity of downstream assays. In our experience, in cases where a GFP tag yielded a non-functional AGO protein, replacing it with a smaller tag, such as 3xFLAG, alleviated the issue. Antibodies against the epitope tags mentioned above are all commercially available.

3.1.2 Selection of Tag Location

Structure-function studies of AGO proteins in several species have facilitated a better understanding of the roles for each portion of the protein [13–19]. From these studies, and the anecdotal experience of many labs, several general rules for tag placement within AGOs emerge. (1) It is generally advised to insert the tag at the N-terminus of AGO proteins. This region appears to be more flexible and less conserved among different AGOs, and our lab has had the most success with tag insertions at the N-terminus of AGOs. However, one recent observation to take under consideration is that some AGOs are subject to N-terminal peptidase processing, which could be affected by an N-terminal tag [20]. (2) It is ill advised to insert the tag at the C-terminus due to the importance of the region in PIWI domain function. C-terminal insertions may disrupt the binding of sRNAs by the AGO. In any attempt we have made to generate a C-terminal tag, we were unsuccessful in generating a functional fusion protein. (3) Internal tagging of AGOs is possible, depending on the precise location. In this case, the choice of the internal site includes the degree of conservation, the predicted structure present, and the hydrophilicity/hydrophobicity of

the selected insertion region. Broadly speaking, less conserved regions of the AGO are likely to be less critical for AGO functionally. Less structured regions and flexible loops portions of the AGO are more amenable to tagging than regions with a large proportion of alpha helices. Regions that are predicted to be more hydrophilic/less hydrophobic are likely to better position the epitope on the AGO surface. Tools such as <https://predictprotein.org/> can be used to predict protein region hydrophobicity [21]. We also recommend using Alphafold <https://alphafold.ebi.ac.uk/> to gain additional insights into tag placement [22]. Once a tag location has been selected, CRISPOR (<http://crispor.tefor.net>) can be used to design single guide RNAs.

3.1.3 Functional Validation of Tagged Protein

Introduction of an epitope tag to a protein may render the protein non-functional. Therefore, it is critical to validate that the tagged protein is functional (i.e., the tagged *ago* phenocopies wild-type animals) before any downstream experiments are undertaken to be confident about any results obtained using this reagent. Among the best assays for functionality are visible phenotypes, such as fertility, movement, and behavioral assays. In the case where no known function is attributable to the *ago* of interest, it is advisable to N-terminally tag the *ago* gene with a small tag like a 3xFLAG, as it is likely to be least disruptive. It is then possible to use this tagged AGO strain to sequence the associated sRNAs and discover the RNA targets of the AGO. Analyzing the targets may aid in inferring in which biological processes the AGO may participate (e.g., via Gene Ontology analysis) and test for relevant phenotypes in the *ago* mutant vs. wild type vs. tagged AGO strains. In addition, if specific RNA targets are discovered, one can employ molecular assays such as qRT-PCR on these targets in wild-type, *ago* mutant, and tagged AGO animals to assess changes in expression.

3.1.4 Generation of Ago Mutants

Generating *ago* null mutants is generally efficient [10, 11]. Although many approaches rely on Non-Homologous End Joining to generate a series of localized insertions and/or deletions, our strategy has generally been to use Homology Directed Repair (HDR) to introduce several stop or frameshift codons and a unique restriction site that enables PCR screening/restriction digestion to identify and distinguish the mutation of interest from the wild-type allele. We typically target the 5' coding sequence within the first exon (corresponding to the N-terminus of the AGO protein) for the introduction of stop codons, so that there is little chance for the production of a truncated protein. Like the AGO epitope tagging strategy, upon obtaining the desired homozygous mutant *ago* strain, it is critical to assess the phenotype. In this instance, we look for phenocopy of known null phenotypes [9, 23]. In addition to null *ago* mutants, it is feasible to engineer point mutations in key

residues using a similar HDR strategy. In particular, targeting residues within the 5' and 3' sRNA binding pockets are relevant here, as disruption of these binding pockets leads to disruptions in the AGO-bound sRNAs [13, 18].

3.2 Synchronizing and Harvesting Nematode Populations

3.2.1 Bleaching to Synchronize the Population

1. Chunk worms from smaller NGM plates onto 150 mm NGM plates seeded with 2–3 mL of 5X concentrated OP50 bacteria and incubate at 20 °C until most worms are gravid (full of embryos).
2. Once plates are full of gravid adults, wash the worms off the plate with M9 buffer into a 15 mL conical tube.
3. Pellet the worms by centrifugation at $700 \times g$ for 1 min and aspirate the M9 buffer from the pellet.
4. Repeat washes with M9 buffer and centrifugation as necessary until the buffer is clear of bacteria.
5. Add 10 mL of worm bleach solution to the tube of worms and rock for 10–20 min to dissolve adult worms, leaving behind only embryos (*see Note 1*).
6. Pellet the remaining embryos by centrifugation, remove bleach solution, and wash 3 times with M9 buffer, as before.
7. After the final wash, transfer the embryos in 30 mL of M9 buffer to a 50 mL conical tube by washing the 15 mL tube twice to collect all the embryos.
8. Leave the embryos rocking overnight in M9 buffer at 15 °C to hatch (*see Note 2*).

3.2.2 Plating Synchronized L1 Larvae

1. Agitate the tube of hatched L1s and transfer $5 \times 2 \mu\text{L}$ samples to a microscope slide.
2. Under a dissecting microscope, count the number of worms in each 2 μL aliquot.
3. Using the average number of worms per microliter, calculate the volume required for 100,000 worms and add that volume to a 15 mL conical tube (*see Note 3*).
4. Pellet the worms by centrifugation as before and remove the M9 buffer.
5. To each pellet, add 2–3 mL of 5X concentrated OP50 per 100,000 worms (*see Note 4*), quickly vortex to mix, spot the worm and OP50 mixture onto 150 mm NGM plates and swirl plates to spread the mixture.
6. Allow plates to air dry in a laminar flow hood or within a sterile field created by a Bunsen burner until completely dry before placing them in an incubator at the desired growth temperature.

3.2.3 Harvesting Nematode Pellets

1. Depending on the developmental stage to be harvested, the duration of growth will vary [24]. It is useful to monitor worm growth each day during the growth period to ensure that they do not starve and are developing well (*see Note 5*). At the desired time point, examine the worms under a dissecting microscope to determine that they are at the expected stage and ensure that they have not starved (*see Note 6*).
2. Wash the worms off of the plate with up to 5 mL M9 buffer per plate.
3. Transfer the worms in the buffer from one plate to wash the next to concentrate the worms from multiple plates. The number of plates to combine into one conical tube will depend on the developmental stage you are harvesting and the desired pellet size. For a ~ 1 mL pellet, combine 2–3 plates of young adult worms (200,000–300,000 worms) or 5 plates of L4-stage worms (~500,000 worms) (*see Note 7*).
4. Wash each plate a second time as in **step 2** to collect any remaining worms.
5. Once most of the worms are collected into individual 15 mL conical tubes, centrifuge at $700 \times g$ for 1 min to pellet the worms, remove the supernatant and wash with 10 mL 1X M9.
6. If harvesting pellets for lysis by Dounce homogenization or sonication (if harvesting pellets for lysis by mortar and pestle, skip to **step 24**), repeat **step 5** for a total of 3 washes with M9 buffer or until the supernatant is clear of residual OP50 bacteria.
7. Repeat 3 more washes as in **step 5**, but using autoclaved water instead of M9 buffer to wash.
8. After the last wash, aspirate as much supernatant as you can and place the tubes on ice to compact the pellet and drive any additional water to the surface.
9. After 10 min, remove any excess water and snap freeze the pellets in a dry ice and 95% ethanol bath. Store the frozen pellets at -80°C .
10. If harvesting pellets for lysis by mortar and pestle, resuspend worms in an equal volume of EDTA base buffer.
11. Pellet worms by centrifugation as above and remove supernatant.
12. Resuspend in an equal volume of EDTA complete buffer.
13. Using a Pasteur pipette, drip worms into a 100 mL beaker containing liquid nitrogen.
14. Transfer the frozen worm pellets into a 15 mL conical tube that has been chilled on liquid nitrogen.

15. Allow excess liquid nitrogen to evaporate, but do not allow the worms to thaw.
16. Close cap loosely to prevent build-up of residual liquid nitrogen vapor and store at -80°C .

3.3 Nematode Pellet Lysis

All steps in Subheadings 3.3 and 3.4 are to be performed on ice unless specified otherwise. For the remainder of this protocol, use RNA precautions when working with samples: clean surfaces, equipment, and gloves with an RNase decontamination solution (such as RNase Away), and use RNase-free barrier filter pipette tips.

1. Prepare EDTA base buffer, EDTA+ buffer, and EDTA complete buffer (*see* **Notes 8–10**).
2. The three lysis methods below differ in their requirement for specialized equipment but all disrupt the worms to produce lysate containing the protein and nucleic acids.
 - (a) *Sonication with a Biorupter*: Add 1:1 EDTA complete buffer to the pellet. Use an RNase-free pestle to break up the pellet. Sonicate in a Bioruptor Pico at 4°C for 10 cycles of 30 sec. ON/30 sec. OFF at high energy.
 - (b) *Dounce homogenization*: Clean a Wheaton Metal Dounce homogenizer thoroughly with RNase Away and water, and chill in ice. Add 1:1 EDTA complete buffer to the pellet. When the pellet releases from the walls of the tube, transfer into the Dounce homogenizer. Crush the pellet on ice and monitor the status of the lysate by removing $1\ \mu\text{L}$ every 30 crunches to observe under a dissecting microscope until worm carcasses are unrecognizable.
 - (c) *Liquid nitrogen*: Cool cryo-mortar and pestle with liquid nitrogen. Add worm pellets prepared by freezing in liquid nitrogen to the mortar and tap gently to break the pellets before grinding into a fine powder. Add additional liquid nitrogen to powder if necessary. Transfer worm powder into equal volume of EDTA complete buffer.
5. Transfer the contents of each lysed sample to pre-chilled microcentrifuge tubes and centrifuge at $13,500 \times g$ for 15 min at 4°C .
6. Transfer supernatant to new microcentrifuge tubes.
7. Determine protein concentration in the lysate (e.g., by Lowry or Bradford assay) (*see* **Note 11**).
8. When the protein concentration of the lysate is known, transfer the amount of lysate equivalent to $500\ \mu\text{g}$ of protein into a pre-chilled microcentrifuge tube on ice as the “input” RNA for total sRNA-sequencing. Add 4 volumes of TRI Reagent, flash freeze in an ethanol and dry ice bath, and store at -80°C .

9. Transfer a fraction of the lysate (*see Note 12*) into another pre-chilled microcentrifuge tube as input protein for western blot validation. Add 1X BOLT and 1X reducing agent, vortex, incubate at 70 °C for 10 min, vortex again, and store at -80 °C.
10. The remaining lysate will be used for the immunoprecipitation.

3.4 Immunoprecipitation/Immunoprecipitation (IP)

Because the total volume required to immunoprecipitate the AGO of interest (*see Note 11*) will likely exceed the capacity of a microcentrifuge tube, the lysate will be divided into multiple smaller IPs which will be combined in the last step. In parallel, one mock IP, employing non-conjugated beads, will use an equal amount of lysate to each individual IP tube. The mock IP serves as a negative control for subsequent western blot validation (Fig. 3).

Based on the protein concentration of each sample, divide the volume of lysate between multiple microcentrifuge tubes such that each tube contains a volume of lysate approximately equal to 5 mg of total protein. Bring the volume of each tube up to 500 μ L with EDTA complete buffer.

Before the IP, the lysate should be pre-cleared of proteins that non-specifically interact with the beads by incubating the lysate for each IP with non-conjugated magnetic beads (*see Note 13*).

1. Aliquot a volume of non-conjugated magnetic beads required for the number of IP and mock IP tubes and equilibrate by washing with EDTA+ buffer. After washing the beads to the

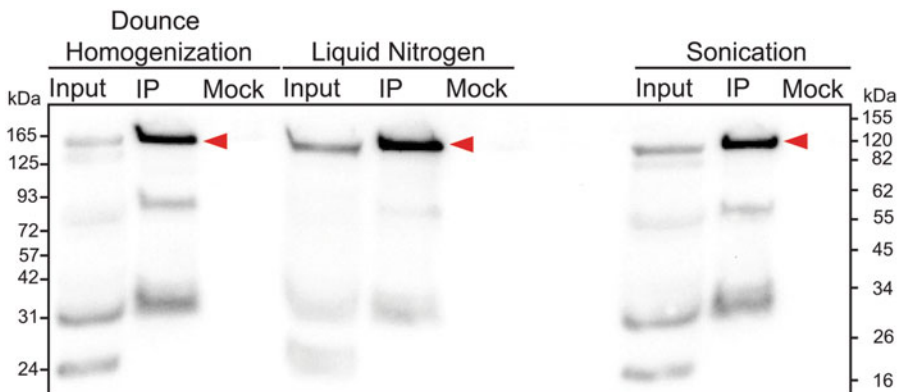


Fig. 3 Western blot analysis of HRDE-1 IPs using three different lysis methods. (a) Lysis methods are as marked. All input lanes are 100 μ g of total protein. All IPs are 5% of a 7 mg IP. GFP::3xFLAG::HRDE-1 is ~145 kDa (red arrowhead). Samplers were run on a 4–12% Bis-Tris BOLT gel with MES-SDS Running buffer (ThermoFisher Scientific). The gel was transferred to a Hybond C membrane (Amersham) using the Bio-Rad semi-dry transfer system. The membrane was probed with anti-FLAG M2 primary antibody (Sigma) and anti-mouse-HRP (Jackson ImmunoResearch) as the secondary antibody, then developed with Luminata Classico HRP substrate (Sigma) and imaged using an iBrightFL1500 (ThermoFisher)

supplier's specification, resuspend the beads in equal parts EDTA complete buffer and chill on ice before adding to lysate.

2. To each tube of lysate, add the required amount of prepared beads.
3. Pre-clear the lysate by rotating the tubes gently on a rotary turner at 4 °C for 1 h.
4. Following the procedure in **step 2**, prepare an aliquot of antibody-conjugated beads for the IP tubes as well as an aliquot of non-conjugated beads for the mock IP tubes.
5. After the pre-clearing incubation, place the pre-clearing tubes on a magnetic rack and transfer the lysate from each pre-clearing tube to its corresponding IP or mock IP tube.
6. To each tube of pre-cleared lysate, add the required amount of either antibody-conjugated (for IP tubes) or non-conjugated (for mock IP tubes) beads.
7. Gently rotate the IP tubes at 4 °C for 1 h.
8. After the IP incubation, place the tubes on a magnetic rack and discard the lysate.
9. Wash the beads with 500 µL of EDTA complete buffer, and rotate for another 10 min at 4 °C. At this point, the beads from the individual IP tubes for each sample can be concentrated into one tube.
10. Repeat **step 10** for at least 3 washes.
11. After the last wash, resuspend the beads in 200 µL of EDTA complete buffer.
12. For IP and mock IP tubes, transfer 20 µL (10%) of this volume to a new pre-chilled microcentrifuge tube for western blot validation. As was done with the input sample, add BOLT sample buffer and reducing agent to 1X, vortex, incubate at 70 °C for 10 min, vortex again, and store at –80 °C.
13. For the IP tubes, the remaining 180 µL (90%) will be used for RNA-sequencing. As was done with the input sample, add 4 volumes of TRI Reagent, flash freeze in an ethanol and dry ice bath, and store at –80 °C or immediately proceed to RNA extraction.

3.5 RNA Extraction

To obtain high-quality RNA for the generation of sequencing libraries, a series of RNA extraction and precipitation steps are required to purify the RNA in the input and IP samples from the rest of the lysate contents. In the case of sequencing sRNAs from *ago* mutants, RNA is extracted from whole worm lysates (prepared in the same way as the “input” sample for IP: *see* Subheading 3.3). The remainder of this protocol applies to both IP and whole worm samples.

1. Equilibrate phenol:chloroform:isoamyl alcohol (25:24:1, pH 4.5), which is stored at 4 °C, at room temperature for at least 30 min.
2. Retrieve samples (input and IP in 4 volumes of TRI reagent) from –80 °C and thaw by vortexing the tube at room temperature for 15 min.
3. Add 10% 1-bromo-3-chloropropane, invert to mix, and centrifuge at 12,000 × g for 15 min at 4 °C.
4. Transfer the clear aqueous phase at the top to a fresh 1.5 mL microcentrifuge tube.
5. Add 1:1 phenol:chloroform:isoamyl alcohol (25:24:1, pH 4.5) and centrifuge at 12,000 × g for 15 min at 4 °C.
6. Transfer the aqueous phase to a fresh 1.5 mL microcentrifuge tube.
7. Add 20 µg glycogen, 0.3 M NaOAc (pH 5.2) and 1 volume isopropyl alcohol.
8. Incubate at –20 °C for at least 30 min (*see Note 14*).
9. Centrifuge at 16,000 × g for 30 min at 4 °C.
10. Discard the supernatant.
11. Add 900 µL of 70% ice-cold ethanol and leave at room temperature for 10 min.
12. Centrifuge at 16,000 × g for 10 min at 4 °C.
13. Discard the supernatant.
14. Add 900 µL of 70% ice-cold ethanol and leave at room temperature for 5 min.
15. Centrifuge at 16,000 × g for 10 min at 4 °C.
16. Discard the supernatant.
17. Let the pellet air dry for approximately 10 min to evaporate any remaining ethanol.
18. Resuspend in 11 µL of RNase-free water.
19. Quantify RNA and assess quality using Nanodrop, Qubit, and/or Bioanalyzer (*see Note 15*).

3.6 RNA 5' Polyphosphatase Treatment

Different classes of *C. elegans* sRNAs possess biochemical features that render them more or less amenable to the ligation of adaptors that is generally required to generate high-throughput sequencing libraries. For example, the most common treatments of sRNAs involve removing phosphate groups from the 5' end of tri-phosphorylated 22G-RNAs. This can be achieved by several approaches [25–27], but perhaps the most straightforward is to remove pyrophosphate with 5' polyphosphatase, leaving the optimal monophosphate substrate for adaptor ligation. Importantly,

polyphosphatase treatment does not alter the pool of mono-phosphorylated sRNAs, including miRNAs, and thus enables the cloning of all sRNA classes with the least experimental manipulation (Table 1).

1. Set up the following reaction: 2 μL 10X reaction buffer, 1 μL RNA 5' polyphosphatase (10 U/ μL), 1 μL RNase inhibitor (optional), total RNA up to 4 μg . Top up to a total reaction volume of 20 μL with nuclease-free H_2O .
2. Incubate for 30 min at 37 °C.
3. Equilibrate phenol:chloroform:isoamyl alcohol (25:24:1) to room temperature during the 30 min incubation.
4. Add 130 μL H_2O to the reaction tube.
5. Add 150 μL phenol:chloroform:isoamyl alcohol (25:24:1), mix well by gentle vortexing.
6. Invert-mix until an emulsion forms (~5 times).
7. Centrifuge 16,000 \times g for 5 min at room temperature.
8. Transfer the aqueous layer to a fresh microcentrifuge tube.
9. Ethanol Precipitation: Add 20 μg glycogen, 15 μL 3 M NaOAc (pH 5.2) and 660 μL ethanol, in this order.
10. Invert to mix and incubate at -20 °C for at least 30 min (*see Note 14*).
11. Centrifuge at 16000 \times g for 30 min at 4 °C.
12. Wash the pellet with 900 μL of cold 70% ethanol, invert to mix, and incubate at room temperature for 10 min.
13. Centrifuge at 16000 \times g for 10 min at 4 °C.
14. Discard the supernatant.
15. Wash the pellet again with 900 μL of cold 70% ethanol, invert to mix, and incubate at room temperature for 5 min.
16. Centrifuge at 16000 \times g for 10 min at 4 °C.
17. Discard the supernatant.
18. Let the pellet air dry for approximately 10 min to evaporate any remaining ethanol.
19. Resuspend in 7 μL of RNase-free water (*see Note 16*). From here, samples may be immediately used to generate high-throughput sequencing libraries, or may be stored at -80 °C until further use.

3.7 Library Preparation

The next step in our workflow involves generating libraries of the isolated RNA for high-throughput Illumina sequencing. There are now many commercial options available for library construction, along with the option of generating libraries without a “kit” [25, 26, 28]. Examples of commercially available kits include

those from Illumina, Qiagen, Bio-Rad, TriLink Biotechnologies, and New England Biolabs, among others. We most often use the NEBNext Small RNA Library Prep Set for Illumina, as we found that using a kit streamlined our process and led to more consistent results across different users at different skill levels. Another benefit of using kits to clone is that they typically require total RNA as the starting material, thus eliminating the need to size fractionate small (<200 nucleotide) from larger (>200 nucleotide) RNAs prior to cloning. There are some important considerations when preparing libraries for sRNA sequencing:

- We typically follow the instructions of the manufacturer closely when using library preparation kits. For the NEBNext Small RNA Library Prep Kit, we generally use 1 µg of RNA as starting material, but have successfully used as little as 10 ng of RNA. When using lower amounts of starting material than recommended by the protocol, it is important to scale the amount of adaptor accordingly. For AGO IPs, we use the entire amount of RNA recovered up to 1 µg as starting material.
- When selecting a kit or devising a “home-made” library cloning protocol, it is important to consider which enzymatic treatments are used to generate the libraries, and ensure that the selected protocol is capable of capturing the types of sRNAs you wish to study. For example, some kits and strategies use ligation to covalently attach adaptors to sRNAs, and this is incompatible with the 5' triphosphate group present on 22G-RNAs (*see* Subheading 3.6).
- The number of PCR cycles will vary, depending on the amount of starting material. We typically perform 12–15 cycles of PCR, as recommended by the supplied protocol.
- Multiplexing samples is important, as newer sequencing modalities have much higher sequencing output than is generally necessary for a single sample. Therefore, it is important to consider how many samples can be reasonably combined in a single sequencing lane to achieve sufficient depth of coverage for particular sets of sRNAs. We typically aim to achieve ten million genome-matching reads per sRNA sample.
- Related to multiplexing, always be sure to record the index sequences that are used to distinguish each sample, and never use the same index within a single lane of sequencing.
- Particularly when sequencing sRNAs from *ago* mutant samples or samples with low input amounts, it can be useful to include unique molecular identifiers (UMIs) in the library preparation workflow. UMIs are unique sequences, like molecular barcodes, added to each sRNA prior to amplification. UMIs aid in the quantification of sRNAs by enabling the detection of PCR

duplications and stochastic variation from low-input sRNAs [29].

- In many protocols, gel extraction is required to isolate the appropriate size range of the sRNA library. In this size selection step, it is important to be attentive to the nucleotide length range that you aim to study, accounting for the added length of the adaptor oligos. For *C. elegans* sRNAs, the 18–30 nucleotide range encompasses all sRNA classes. Upon adaptor ligation and PCR amplification, the final size range is approximately 140–150 nucleotides.
- Generally speaking, 50 nucleotide, single-end sequencing runs are sufficient for sRNA sequencing. However, short sequencing runs are becoming obsolete as many approaches favor longer sequence reads. As such, we typically select the sequencing runs with the shortest read length available, depending on the sequencing technology available (MiniSeq, NovaSeq, etc.).

3.8 Sequencing Analysis

3.8.1 Choosing an Analysis Pipeline

The analysis of RNA sequencing (RNA-seq) data described here assumes that you begin with FASTQ files (.fastq) obtained from an sRNA-seq run and possess a basic familiarity with using a Unix-like command line interface. Rather than a rigid step-by-step guide, this section aims to introduce the basic principles of sRNA-seq data analysis along with the software tools that our lab uses for each task. As an alternative to creating a bespoke sRNA-seq analysis pipeline as described below, a software package currently in development called *tiny-count* (<https://github.com/MontgomeryLab/tinyRNA>) can be used to provide a more user-friendly sRNA-seq analysis workflow [30].

3.8.2 Quality Control

If there are MD5 files associated with your FASTQ files, first determine if the files are corrupted or have missing data using a *checksum* function. Next, assess the quality of the libraries using *FastQC* (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) [31]. *FastQC* can be run from the command line to generate a quality control report for each .fastq file in a directory. In your *FastQC* report, you should notice an overrepresentation of sequences corresponding to the adaptors that you ligated during library preparation which will be removed in the following step.

3.8.3 Adaptor Trimming

Use the tool *cutadapt* (<https://cutadapt.readthedocs.io/en/stable/>) to trim the adaptors from your reads [32]. You must supply the sequence of the adaptors and their location (i.e., 5' end, 3' end, or both). Of particular importance for sRNA-seq libraries is to define the length of trimmed reads that are kept for downstream analysis (i.e., 18 to 30 nucleotides to capture the repertoire of endogenous sRNA reads in *C. elegans*). Any

untrimmed reads will be discarded. After adaptor trimming, run FastQC again to reassess library quality to ensure that the appropriate adaptor sequences were successfully removed.

3.8.4 Genome Alignment

STAR (<https://github.com/alexdobin/STAR/releases>) is a tool that can be used to align RNA-seq reads to a reference genome and other provided datasets [33]. The first time performing this analysis, a reference genome will need to be indexed (e.g., Wormbase.org genome release WS283). The resulting indexed genome can then be used directly for future sequence alignment. To generate the genome index, run STAR from the command line with *runMode* set to *genomeGenerate* and provide the location to two files: [1] the reference genome assembly in FASTA format and [2] a GTF file containing the genome annotations. For nematode species, genome assemblies and corresponding annotations can be obtained from Wormbase (wormbase.org) [34]. Next, run STAR to align the trimmed sRNA-seq reads to the indexed reference genome. This will generate a BAM file containing the alignment data for each FASTQ file. Because some sRNAs are capable of targeting transcripts with imperfect complementarity, providing STAR with a mismatch allowance, usually 1 to 2 nucleotides, can ensure that sRNAs below a certain mismatch threshold are still mapped to the genome.

3.8.5 Counting Reads

Once the sRNA-seq reads have been aligned, the next step is to determine the identity of the sequences targeted by the sRNAs. We use a custom R scripts (https://github.com/ClaycombLab/Charlesworth_2020; https://github.com/ClaycombLab/Seroussi_2022) to assign reads from a BAM file as sense or anti-sense to annotated features in the genome (e.g., protein-coding genes, transposable elements, pseudogenes, etc.) [9, 27] (*see Note 17*). In addition to the BAM files generated in the previous step, this step requires annotation files. General annotations for nematode species can be obtained from Wormbase or more specialized databases such as miRbase (mirbase.org) for miRNA sequences [35], piRTarBase (cosbi6.ee.ncku.edu.tw/piRTarBase/) for piRNA targets [36], and RepeatMasker (repeatmasker.org) for repetitive elements [37] (*see Note 18*). Prior to any subsequent comparisons between libraries, read counts should be normalized to the respective library size, for example, in terms of reads per million (RPM).

3.8.6 Sequence Composition Analysis

In addition to the genome features they map to, different classes of sRNAs are also able to be distinguished based on features such as sequence length and 5' nucleotide identity (Table 1). To visualize the representation of different classes of sRNAs in your samples, it is useful to plot the frequency of read length and first nucleotide for each library. Comparing these profiles between input and IP (or wild-type and mutant) samples can be helpful in determining which class(es) of sRNA(s) are associated with the AGO of interest.

3.8.7 Differential Expression Analysis

At a minimum, we generally sequence three input (total small RNA) libraries and three AGO IP libraries in parallel to three *ago* mutant samples and three wild-type worm samples per each AGO we aim to study. We compare the AGO IP-associated sRNAs to those in the matched input sample to look for enrichment of particular sRNA classes or targets. Because 22G-RNAs and 26G-RNAs are generally completely complementary to their targets, we define them by their gene target sets. We tend to consider that a genome feature is a target of 22G-RNAs or 26G-RNAs if it is enriched at least two-fold in the IP compared to input, with a minimum of 5 RPM across independent IP replicates. Because it is difficult to determine the targets of miRNAs and piRNAs without additional experimentation, and they are encoded from their own genomic loci, we examine and define these groups only as sRNAs. In parallel to enrichment in IP samples, we look for twofold or greater depletion of sRNAs in the *ago* mutant vs. wild-type samples, because we generally expect that sRNAs bound by AGOs will be depleted in the *ago* mutant (*see Note 19*). The highest confidence sRNAs/targets of an AGO are those that are enriched in the AGO IP and depleted in the *ago* mutant.

4 Notes

1. If pellets exceed 0.5 mL, the worms should be split between multiple tubes until that volume or less is obtained. Monitor the bleaching process regularly with a dissecting microscope to ensure that embryos are not overly bleached, which can kill them.
2. Without a food source, the larvae that hatch in M9 buffer will arrest at the L1 stage, therefore synchronizing the developmental stage of the worms.
3. You can combine multiple 100,000-worm aliquots to a single tube.
4. The amount of concentrated OP50 to add will vary depending on (1) the actual number of worms per plate, (2) the preparation of concentrated OP50, and (3) the stage at which you plan to collect worms. For collecting larval worms, less OP50 is required compared to collecting adults as they will spend less time on the plates. You want to strike a balance of using enough OP50 to prevent your worms from starving without having a large excess of bacteria on the plates when it comes time to harvest. It is best to monitor the amount of OP50 on the plates under a dissecting microscope periodically. If it appears that the amount used will not last until the harvesting time point, spot an additional 1 mL or so of concentrated OP50.

5. It is well documented that many environmental factors influence the expression levels of specific sRNAs in *C. elegans*, such as temperature, food source, and starvation. Furthermore, many recent studies have shown that these changes in sRNA levels can persist for many generations after the initial stimulus [38–42]. For this reason, when it comes to growing worms for the isolation of sRNAs, it is especially important to ensure that the worms are grown at a known temperature, fed an uncontaminated food source, and not allowed to starve.
6. If a population of worms were to starve prior to harvesting, it is best to wait until at least 3 generations have passed before harvesting that population to allow for resetting of transgenerationally inherited sRNAs [41–43].
7. Aim to collect at least 3 pellets per strain/treatment in order to have 3 biological replicates for later steps (e.g., 3 input/IP samples and 3 wild type/mutant).
8. This is our preferred buffer for immunoprecipitation of AGO-sRNA complexes. Other common options include RIPA (50 mM Tris (pH 7.4), 0.1% SDS, 0.5% sodium deoxycholate, 1% IGEPAL, and 150 mM NaCl), DROSO (30 mM HEPES, 100 mM potassium acetate, 2 mM magnesium acetate, 10% glycerol, 0.1% IGEPAL, and 2 mM DTT), and Tris lysis buffer (20 mM Tris-Cl, 300 mM NaCl, 1 mM EDTA, and 1% IGEPAL).
9. While the EDTA base buffer can be prepared ahead of time and stored at 4 °C, the EDTA+ and EDTA complete buffers should be prepared fresh each time.
10. EDTA+ buffer is used for equilibrating the beads while EDTA complete buffer is used for the lysis step, resuspending the washed beads, and during the immunoprecipitation and subsequent washes.
11. On average we obtain protein concentrations of 20 +/- 5 mg/mL. At least 15 mg of protein is preferable for immunoprecipitation.
12. In our experience, 100 µg of total protein is enough to visualize any of the AGOs in *C. elegans*; however, it is advisable to hold back additional total protein, in the event that a second western blot is necessary.
13. We prefer to use magnetic beads (ChromoTek GFP-Trap® Magnetic Agarose) because of their ease in separation and recovery, but conventional beads are also appropriate here. For “non-conjugated” beads to be used for pre-clearing lysate and mock IPs, we use the same beads as for the IPs but without conjugated antibodies (ChromoTek Binding Control Magnetic Agarose Beads).

14. The sample is stable in isopropanol at this point and can be left overnight at -20 or -80 °C or for months, until ready to complete the extraction.
15. We typically obtain 25–300 ng/ μ L RNA for AGO IP samples (AGO expression levels vary greatly), and 2000–9000 ng/ μ L for input samples.
16. The volume of water to resuspend the RNA pellet depends on the volume required by downstream steps. In our typical sRNA library preparation protocol, a maximum volume of 6 μ L can be used, plus an additional 1 μ L for quantification by Nanodrop.
17. Often a read will align to more than one distinct feature. This can be handled with a weighted count approach (i.e., splitting the read count between the features based on the fraction of reads each feature contains).
18. Take care that all feature annotations used were generated using the same genome version as genomic coordinates can differ between different versions. If a set of annotations you would like to use were generated using a different genome version, you can use software tools such as LiftOver (genome.ucsc.edu) to convert between versions.
19. This protocol is useful for identifying AGO-associated sRNAs (and therefore sRNA pathway targets), and with slight modification, it can also be used to sequence the AGO-associated target mRNAs. All steps leading up to library preparation are the same, but mRNA-seq library preparation kits (those that isolate >200 nucleotide RNAs and rely on oligo-dT selection or rRNA depletion methods) would be used, and the computational analysis approaches would be focused on methods that analyze longer transcripts. This strategy provides another powerful dimension to defining and refining the targets of the AGOs, to prioritize them for further study.

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Lineage Tracing and Single-Cell RNA-seq in *C. elegans* to Analyze Transgenerational Epigenetic Phenotypes Inherited from Germ Cells

Juan D. Rodriguez and David J. Katz

Abstract

The last several years have seen an increasing number of examples of transgenerational epigenetic inheritance, in which phenotypes are inherited for three or more generations without changes to the underlying DNA sequence. One model system that has been particularly useful for studying transgenerational epigenetic inheritance is *C. elegans*. Their short generation time and hermaphroditic reproduction have allowed multiple transgenerational phenotypes to be identified, including aging, fertility, and behavior. However, it is still not clear how transgenerational epigenetic inheritance from the germline affects embryogenesis. Fortunately, the *C. elegans* embryo has a unique property that makes it ideal for addressing this question: they develop via an invariant lineage, with each cell undergoing stereotypical cell divisions to adopt the same cell fate in every individual embryo. Because of this invariant cell lineage, automated lineage tracing and single-cell RNA-seq can be employed to determine how transgenerational epigenetic inheritance from the germline affects developmental timing and cell fate. Unfortunately, difficulties with these techniques have severely limited their adoption in the community. Here, we provide a practical guide to automated lineage tracing coupled with single-cell RNA-seq to facilitate their use in studying transgenerational epigenetic inheritance in *C. elegans* embryos.

Key words *C. elegans*, Lineage tracing, Single-cell RNAseq, Epigenetic transgenerational inheritance, Germ cells

1 Introduction

The germline is a highly specialized tissue that produces gametes by the specialized cell division of meiosis. Gametes serve as the repository of all information that will be passed from one generation to the next. Predominantly, this information is encoded genetically in DNA. However, over the last few years, there have been increasing

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/978-1-0716-3259-8_3.

number of examples of transgenerational epigenetic phenotypes not encoded by changes in the DNA sequence itself. This much rarer mode of inheritance has been documented in organisms ranging from yeast to humans and has been proposed to occur via mechanisms like small non-coding RNAs, DNA methylation, and histone modifications [1, 2]. Studying epigenetic transgenerational inheritance is difficult in systems with a slow generational time, because of the time and effort it takes to monitor multiple generations. Studying epigenetic transgenerational inheritance can also be complicated by genetic variation, which can contribute to phenotypes not directly caused by transgenerational inheritance. One model organism that avoids these complications is the nematode *Caenorhabditis elegans* (*C. elegans*). *C. elegans* has a short generation time of 3 days and reproduces as self-fertilizing hermaphrodites, which limits genetic variation [3]. Partially as a result of these advantages, a large number of epigenetic transgenerational phenotypes have been observed in worms, including those that affect lifespan, fertility, and behavior [4–8].

Despite the examples of epigenetic transgenerational inheritance that have been identified, it remains unknown how heritable epigenetic transgenerational information causes phenotypes in resulting offspring. Fortunately, *C. elegans* has a unique property that makes it highly suitable for addressing this question. As originally identified by John Sulston, the *C. elegans* embryonic lineage is invariant [9], which means that the timing, cell movement, and cell fate of every cell remains the same between individual embryos, allowing the full lineage to be characterized. Because of this unique property, *C. elegans* can be used to investigate transcriptional and cell lineage defects at the single-cell level in the embryo [10]. In order to identify cell lineage defects, an automated cell tracking pipeline has been developed: StarryNite and AceTree [11]. These programs can be used to track each cell within an embryo and curate the cell lineage by utilizing live confocal imaging to follow mCherry- or GFP-labeled nuclei. By examining the cell lineage from mutant worms and comparing it with Wild Type (N2), it is possible to identify any defects in cell timing, cell migration, and inappropriate cell death, during all stages of embryogenesis. In addition, cell fate transformations can be identified by lineage conversion, as shown by the example reproduced from work by Boyle et al. [12] (Fig. 1). Along with automated lineage tracing, single-cell transcriptomics have been performed on N2 *C. elegans* embryos at all stages [13–15]. These experiments have defined the transcriptome of each cell in the *C. elegans* embryo, facilitating the identification of defects by comparison to N2.

By combining automated lineage tracing with single-cell RNA-seq (scRNA-seq) in the *C. elegans* embryo, it is now possible to determine how epigenetically inherited information affects the transcription and cell fate of embryonic cells at all embryonic stages.

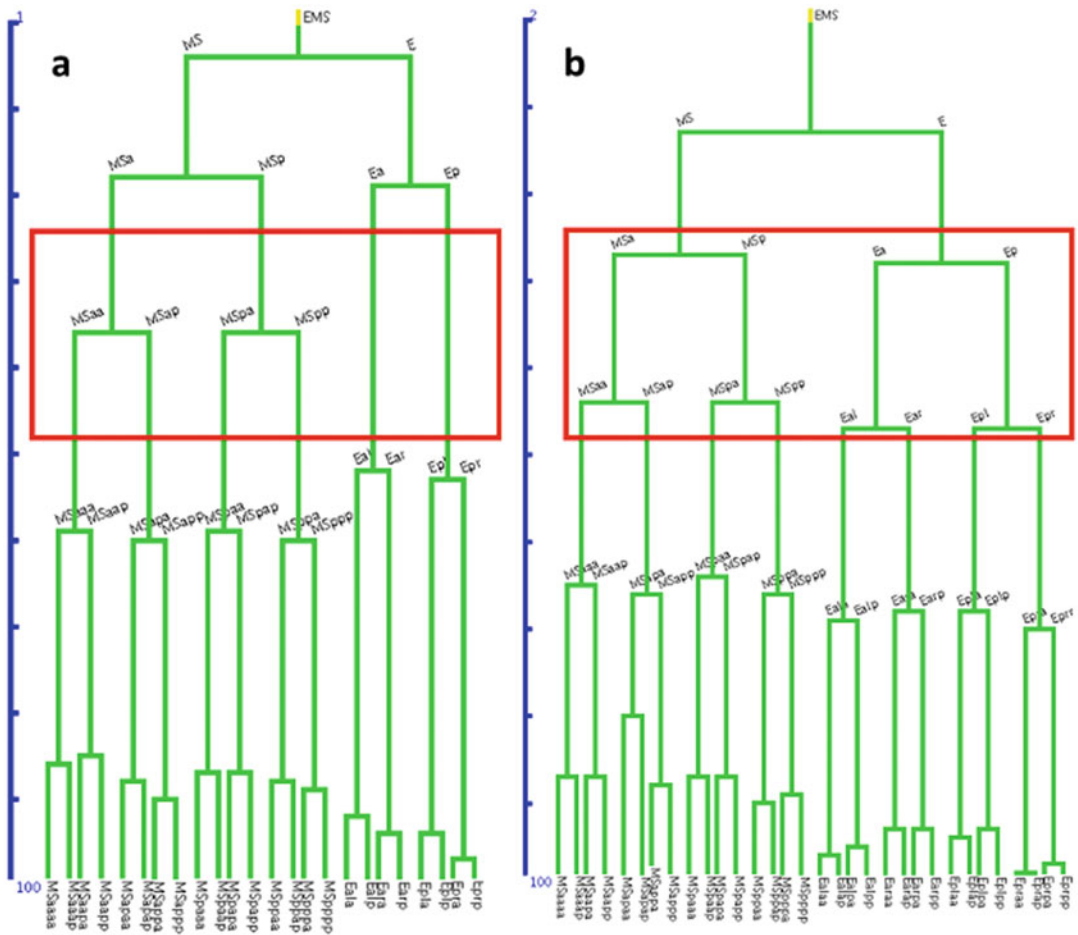


Fig. 1 Example of a lineage conversion. (a) the Wild-Type (N2) and (b) the *lit-1(RNAi)* EMS sub-lineage. Red squares highlight the difference in cell division timing between the MS (muscle) and E (intestine) lineages which are eliminated in *lit-1(RNAi)* animals. This result suggests that the E lineage adopted the cell fate of the MS. This result was previously shown by Boyle et al. [12] and colleagues, and is useful as a positive technical control

Considering the number of transgenerational phenotypes that have been identified in *C. elegans*, we believe that this will be a powerful new approach. By comparing N2 versus mutant embryos, it may be possible to determine how defects in the previous germline can give rise to phenotypes in the subsequent generation. In order to facilitate this analysis, this Methods in Molecular Biology chapter provides a practical guide for carrying out scRNA-seq and automated cell lineage tracing. The protocols that these methods are based on were originally developed and described in the following publications: scRNA-seq in Packer et al. [14], and lineage tracing in Murray et al. [11].

2 Materials

2.1 Growing Worms

1. Agar plates: 1 L Nematode Growth Media (NGM): 3 g NaCl₂, 20 g Agar, 2.5 g Bacto peptone dissolved in 975 mL with diH₂O. The remaining ~25 mL will be added after autoclaving. Be sure to add a stir bar to the solution before autoclaving. Autoclave for 1 h. Cool the flask by stirring on a heat/stir plate until the flask is ~55 °C (cool enough to briefly touch) and add the rest of the reagents: 25 mL of 1 M KPO₄ buffer pH 6.0 (108.3 g KH₂PO₄, 35.6 g K₂HPO₄ in a total of 1 L H₂O, autoclaved) (25 mM final), 1 mL 1 M MgSO₄ (1 mM final), 0.5 mL of 1 M CaCl₂ (0.5 mM final), 1 mL of 5 mg/mL Cholesterol (0.005 mg/mL final). Continue to stir and heat using a heat/stir plate while pouring into the 60 mm Petri dishes. The heating will prevent the agar from solidifying. It is recommended to use a plate pouring machine so that plates are all the same height, which eliminates the need to refocus when looking through multiple plates. Leave the plates lid-side up to dry for 2–4 days, then store lid-side down at 4 °C. Plates can be stored for several months.
2. Seeding the agar plates with bacteria for worm growth. Make Luria Broth (LB), which is used to grow *E. coli* (OP50 strain) that *C. elegans* feed upon. 1 L LB: 10 grams of Tryptone, 10 g of NaCl, and 5 g of yeast extract. Dissolve in distilled water, up to 1 L, and split into 10 glass flasks; each one should have approximately 100 mL. Autoclave. Inoculate 100 mL of LB media with a single colony of *E. coli* OP50 obtained from the Caenorhabditis Genetics Center (CGC); incubate overnight at 37 °C. From this culture, spot three OP50 drops onto each 60 mm NGM plate using a 5 mL serological pipet. The OP50 culture can be stored at 4 °C and used for several weeks.

2.2 scRNA-seq

1. M9 buffer: 22 mM KH₂PO₄, 42 mM Na₂HPO₄, 86 mM NaCl, and 1 mM MgSO₄ (store at room temperature). Prepare 1 L. Sometimes the MgSO₄ will precipitate out after autoclaving. In this case, an alternative approach is to add the MgSO₄ after autoclaving.
2. Embryo collection: Bleach solution: M9 buffer containing 20% Bleach and 10 M NaOH (store at 4 °C). Prepare 500 mL. The bleach solution can be stored for up to 1 month. After this, the bleach solution should be remade from bleach stock purchased at least every 6 months to prevent a loss of efficacy. Note: the anti-splash additive now included in many commercial bleaches is not good for bleaching worms, so do not use bleach containing anti-splash additive.

3. Egg Buffer: 118 mM NaCl, 48 mM KCl, 3 mM CaCl₂, 3 mM MgCl₂, 5 mM HEPES pH 7.2 (store at room temperature). Prepare 2 L, then add BSA to 100 mL, stored in 50 mL conical tubes (Egg Buffer +1% BSA, store at -20 °C).
4. 60% sucrose in H₂O (store at 4 °C). Prepare 100 mL and store in a sterilized container.
5. PBS (Corning 21-040-CV) (store at room temp).
6. Egg Buffer +1% BSA (-20 °C).
7. Chitinase (Sigma Aldrich C6137-5UN, ≥200 units/g solid) stored at -20 °C.
8. Pronase (Sigma Aldrich-10165921001, 1G) stored at 4 °C.
9. RNase Zap (Thermofisher AM9782).
10. Autoclaved sterile glass Pasteur pipettes.
11. Individual sterile plastic transfer pipets.
12. 3 cc syringe and 21½ G needle.
13. 10 μM filter.
14. Trypan Blue.
15. Glass hemocytometer.

2.3 Preparation of Solutions and Equipment for Microscopy

1. Boyd's buffer with methylcellulose: 60 mM NaCl, 32 mM KCl, 3 mM Na₂HPO₄, 2 mM MgCl₂, 2 mM CaCl₂; 5 mM Hepes, 0.2% Glucose, 1% methylcellulose. This buffer can be used when dissecting worms and to make the 20 μm beads dilution. Prepare 1 L (store at 4 °C).
2. M9 buffer (see Subheading 3.1).
3. Frosted microscope slides 25 × 75 × 1.0 mm (Fisherbrand: 12-550-15).
4. Coverslip 18 mm × 18 mm (sigma Aldrich: 12-548-A).
5. Two 25G × 5/8 Needles (BD:305122).
6. 10 well-cutting glass plate (Fig. 4).
7. 20 μm beads (5 mL from Polyscience, 18329).
8. Confocal microscope (Zeiss LSM 510, or other) equipped with temperature-controlled stage (Brook Industries, Lake Villa IL). Setting the temperature-controlled stage at 20 °C mimics standard laboratory conditions for *C. elegans*.
9. Petroleum jelly (1.75 ounce jar of Vaseline brand purchased from a drug store).
10. Mouth pipet with a capillary glass tube (VWR 51608) Length 75 mm ±0.05, Column 75 μL.

2.4 Website to Download Lineage Program Software

1. StarryNite launcher: Integration of StarryNite and AceTree: <https://waterston.gs.washington.edu/> [11] (see **Note 1**).
2. StarryNite: <https://wormguides.org/starry-nite/>
3. AceTree: <https://github.com/zhirongbaolab/AceTree> [11].

3 Methods

3.1 Cell Isolation for scRNA-seq

For scRNA-seq cell isolation, you will perform 3 separate synchronization steps. First, you will roughly synchronize the first generation. Second, you will collect their progeny for more precise synchronization. Finally, you will collect young embryos at the desired stage for performing scRNA-seq. For complete resuspension of the chitinase enzyme, please *see Note 2*.

1. Grow worms on 20 plastic 60 mm petri dishes, seeded with OP50 bacteria (*see Subheading 2*): place 3 L4-stage worms per plate (1 on each drop). All worms are grown in a 20 °C incubator. 20 plates of N2 will ultimately yield approximately 29,000 embryos at the ~100 cell stage (2.9×10^6 total cells). Certain mutants may be less fertile than N2 and will require starting with a larger number of worms.
2. Wait until each plate is confluent with gravid adults (worms containing two rows of embryos), but not starved (~3–4 days for N2, but the timing may be different in mutants). Using a plastic transfer pipet, rinse worms off plates with M9 buffer (by squirting onto a tilted plate ~10–20 mL for 20 plates). Collect the worms into a 50 mL conical tube, and let worms settle to the bottom by gravity (~10 min). Wash with M9 three times by removing the supernatant with a 50 mL plastic transfer pipet and adding 50 mL of M9 each time.
3. After the last wash, carefully pipet out most of the M9 with a plastic transfer pipet and add 25–30 mL of bleach solution. Place the tube onto a platform rocker and monitor degradation by looking in the tube under a dissecting light microscope (under 3–5× magnification) until most of the carcasses first disappear and embryos remain (~10 min, but this varies widely depending on the bleach, concentration of worms, etc.). Be careful not to let the embryos sit excessively in bleach as this will damage them.
4. After only embryos remain, bring the volume to 50 mL with M9 and centrifuge for 1 min at 450 rcf (Eppendorf Centrifugation 5810 R table top centrifuge). Carefully remove the supernatant using a serological pipet but leave a little bit of volume so the pellet won't be discarded by accident. Resuspend pelleted worms by bringing the volume to 50 mL with

Egg Buffer and gently mix by inversion. Centrifuge for 2 min at 450 rcf. During this time prepare a 15 mL conical tube with 5 mL of 60% sucrose (stored at 4 °C). After centrifugation is completed, discard the supernatant using a 50 mL plastic serological pipet.

5. Resuspend the embryos with 5 mL of Egg Buffer by pipetting up and down with a 5 mL plastic transfer pipet and transfer to a 15 mL conical tube with the 60% sucrose; vortex for 5 s and centrifuge for 5 min at 3220 rcf.
6. After centrifugation, two layers will form. The embryos will be in the top layer. Transfer the embryos to a 50 mL conical tube by using a glass Pasteur pipet (do not use plastic because the embryos and cells will stick to the plastic). Bring the volume to 40 mL with Egg Buffer and centrifuge for 2 min at 1260 rcf. Remove the supernatant with a 50 mL plastic serological pipet, leaving a little bit behind so the embryo pellet won't be discarded by accident. Resuspend in 1–1.5 mL of Egg Buffer (final volume, ~2 mL total). Then transfer the resuspended embryos onto 8–10 60 mm unseeded plates with a glass Pasteur pipet.
7. Allow the embryos to hatch overnight at 20 °C on unseeded plates. Without food, worms arrest at the L1 larval stage, so hatching onto unseeded plates synchronizes L1 larvae. After hatching overnight on unseeded plates move the synchronized L1 larvae to seeded plates by rinsing off the plates by squirting ~1–2 mL M9 per plate onto a tilted plate, using a glass Pasteur pipet.
8. Following the first L1 larval synchronization, proceed with a second synchronization. This second synchronization limits the number of worms you will obtain, but is necessary to make the synchronization tighter. Allow the L1 larvae to grow at 20 °C for approximately 46 h. This produces young adult worms with the first embryos in the gonad. After 46 h (this time may differ for mutant strains), repeat the bleach synchronization starting at **step 2** of Subheading [3.2](#).

3.2 Staging Worms and Collecting ~100 Cell Embryos

1. To obtain worms at approximately the 100-cell stage, allow the twice-synchronized L1 larvae to grow at 20 °C for approximately 46 h. This produces young adult worms with the first embryos in the gonad.
2. Pick individual young adults with the first embryos in the gonad onto a 60 mm plate containing a single OP50 drop from a 5 mL serological pipet. Even though the worms have been synchronized twice, picking worms is necessary to get the correct stage. It is important to pick all of the young adults within 1 h. scRNA-seq requires 10,000 cells, which generally requires starting the process with 14,000 cells. Therefore, if

embryos are at the ~100 cell stage, you will need 140 embryos. Obtaining ~140 embryos generally requires starting from 20 or more 60-mm plates of confluent worms.

3. Rinse plates with ~1–2 mL M9 (for 20 plates) by squirting onto a tilted plate with a plastic transfer pipet and collect worms into a 15 mL conical tube. Let the worms sink in the conical tube by gravity (~10 min) and wash the worm pellet with M9 three times by removing the supernatant with a 10 mL plastic serological pipet and adding 10 mL of M9 each time. The liquid should become clearer with each wash as the bacteria is removed.
4. After removing the last wash, add 7–10 mL of bleach solution, vortex for 20-s and place the 15 mL conical tube on a platform rocker for ~10 min. Monitor the worm degradation with a dissecting light microscope (3–5× magnification), vortexing occasionally, until at least 80–90% of embryos have been released and the carcasses disappear.
5. After confirming that embryos are released, bring the volume to 50 mL with M9 and centrifuge for 1 min at 450 rcf. After centrifugation is completed, carefully remove supernatant, but leave some volume behind so that the pellet won't be discarded by accident. Resuspend the pellet by bringing the volume to 50 mL with Egg Buffer and centrifuge for 2 min at 450 rcf. During this time, prepare a 15 mL tube on ice and add 5 mL of cold 60% sucrose solution. After the centrifugation is complete, discard the supernatant using a 50 mL plastic serological pipet.
6. Resuspend embryos with 5 mL of Egg Buffer and transfer to the 15 mL conical tube containing cold 60% sucrose. Vortex for 5 s to mix, then centrifuge for 5 min at 3220 rcf. The embryos should be in the top layer.
7. Transfer the embryos from the top layer to a 50 mL conical by with a glass Pasteur pipet. Bring the volume to 40 mL with Egg Buffer. At this point, you can let the embryos develop in Egg Buffer until the target cell stage is reached (*see Note 3*).
8. Once the target stage is reached, centrifuge for 2 min at 450 rcf., remove the supernatant using a 50 mL plastic serological pipet and resuspend the embryos in 1–1.5 mL Egg Buffer. Transfer the resuspended embryos to a 12-well plastic cell culture plate with a glass Pasteur pipet, then proceed with the cell membrane and cell isolation step.

3.3 Eggshell Removal and Single-cell Suspension

1. Add a ratio of 1 mL chitinase (1 U/mL) to 0.5 mL embryo suspension and incubate at room temperature for 20–30 min (if the desired embryos are greater than the 300-cell stage, *see Note 4*). Monitor eggshell removal under a dissecting light microscope (3.5–55×). It is very important to keep monitoring the cell suspension during the incubation time to observe the

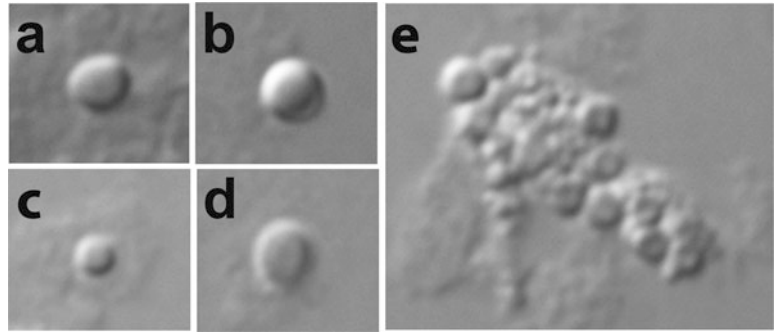


Fig. 2 Verification of single cells. DIC images of embryonic cells. (a–d), isolated single cells and (e) clump of cells. Images were taken at 100×

removal of the eggshell, so that the reaction does not proceed past the removal of the eggshell. Proceeding past the initial removal of the eggshell can result in damage to the individual cells. In the meantime, thaw the Egg Buffer with 1% BSA (stored @ -20°C) and place a new 15 mL conical tube on ice for **step 4**.

2. Optional: Another way to confirm eggshell disruption is to place 2 μL of the sample on a slide with a 2% agarose pad and examine under differential interference contrast (DIC) microscopy at 40 \times to verify the single-cell isolation (*see* Fig. 2). If there are still clumps of cells, pass the suspension through the 21 $\frac{1}{2}$ G needle again multiple times. Clumps can clog the 10 \times Chromium capture mixer (10 \times Genomics) and/or result in cell doublets within a single GEM droplet.
3. After confirmation of the disruption of the eggshell, pass the embryos repeatedly through a 21 $\frac{1}{2}$ G needle ~ 20 times to generate a single-cell suspension in one well of a 12-well cell culture plate. Incubate at room temperature for 5 min. The 12-well plate makes it easier to pass through the syringe multiple times and to monitor the single-cell isolation under a dissection light microscope (10–20 \times). Then move to **step 5** if the embryos are less than the 300-cell stage. If the desired embryonic stage is greater than 300 cells, proceed with the Subheading 3.3, **step 4**. Place 10 μL of the single-cell suspension on a 2% agarose pad and look under the DIC microscope at 40–100 \times to verify the single-cell isolation (*see* Fig. 2). If there are still clumps of cells, pass the suspension through the 21 $\frac{1}{2}$ G needle again multiple times. Clumps can clog the 10 \times Chromium capture mixer (10 \times Genomics) and/or result in cell doublets within a single Gel bead in EMulsion (GEM) droplet.
4. Stop the enzymatic reaction by adding 3–4 mL of Egg Buffer with 1% BSA (thawed in **step 1**) to the well of the 12-well plate.
5. Transfer to a chilled 15 mL tube by passing the cells through a 10 μm filter on a 3 cc syringe. Filtering through the 10 μm filter

on a 3 cc syringe removes almost all of the debris and intact embryos, while all of the single cells pass through. As a result, this filtering step should be included.

6. Centrifuge at 2500 rcf for 5 min at 4 °C. The cells will be in the pellet. In the original protocol, this centrifugation was performed at a slower speed to pellet the debris. However, after a slow speed spin, many single cells were found in the pellet with the debris and were lost during this step. The inclusion of the filtering in **step 6** makes pelleting the debris unnecessary. As a result, a slightly stronger spin (2500 rcf for 5 min at 4 °C) can be used to pellet all of the single cells in this step.
7. Using a glass Pasteur pipet, carefully remove the supernatant and wash the pellet 3× by centrifuging at 2500 rcf for 3 min. Resuspend pellet in 1 mL Egg Buffer with 1% BSA by pipetting up and down with a glass Pasteur pipet. After the first resuspension, transfer to a nonstick polypropylene Eppendorf tube for the second and third washes (*see Note 5*).
8. Following the final wash, remove most of the supernatant and add 1 mL of Egg Buffer with 1% BSA. Then centrifuge at 4 °C at 2500 rcf for 5 min.
9. Using a glass Pasteur pipet, carefully remove the supernatant and resuspend the pellet by adding 100 μL of ice-cold Egg Buffer and pipetting up and down with a glass Pasteur pipet. Then proceed with cell counting (*see Note 6*).
10. Following resuspension, count the number of cells using a hemocytometer (Fig. 3). During the counting, keep the resuspended cell on ice all the time. Clean the glass hemocytometer

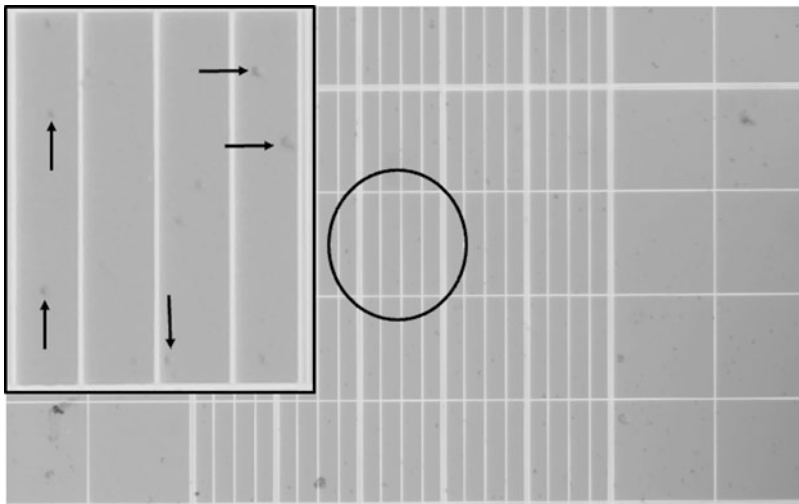


Fig. 3 Cell counting. A volume of 10 μL was loaded into the hemacytometer. The boxed inset shows a zoom in of the region of the hemacytometer that is circled. The arrows point to isolated cells. Image was taken at 20×

and coverslip with clean ethanol. Place the coverslip onto the hemocytometer, the coverslip should cover both chambers. Gently flick the Eppendorf tube that has the cells. Then take 10 μ L and apply it into the loading area of the hemocytometer, underneath the coverslip. Wait around 30–60 s for the cells to settle. Finally, count the cells at 10 \times or 20 \times magnification using a phase contrast microscope. The cells can be counted by quadrant. For example, you could count the four outside edge quadrants and the one in the center. Then the formula that would apply to the cell counting is: number of cells counted in each quadrant multiplied by the dilution factor (if you diluted the cells, i.e., with Trypan blue – *see Note 7*) equals the number of cells $10\times^4$ cells/mL, divided in the # of quadrants, in this example the # of quadrants is 5. This information will be important for the 10 \times Genomics protocol. During the cell counting process, keep cells on ice and proceed directly, as quickly as possible, to the 10 \times Genomics protocol for RNA isolation, cDNA conversion, library preparation, and sequencing guidelines (*see Note 8*).

11. After counting, immediately follow the 10 \times Genomics protocol for RNA isolation, cDNA conversion, library preparation and sequencing. Details related to the 10 \times Genomics protocol are not included here because the 10 \times Genomics protocols are constantly changing. However, it should be noted that for troubleshooting and smaller samples, currently it is recommended to use the Chromium Next GEM Single Cell 3' LT v3.1 low throughput kit (PN-1000325). For full samples, use the Chromium Next GEM Single Cell 3' LT v3.1 kit (PN-1000128). This kit is more expensive but the cell recovery is much higher. In addition, more cells can be analyzed in a single assay using this kit.

3.4 Synchronization and Worm Dissection to Obtain 2-Cell Embryos for Lineage Tracing

1. The automated lineage tracing was designed for use with the Zeiss LSM 510 microscope. It may be possible to use other confocal microscopes, but we encountered difficulty when we tried to use a Leica SP8 (*see Note 9*).
2. Embryos should start to be imaged at the 2–4-cell stage, to allow the tracking software to function correctly. The program to track the cells is StarryNite (*see Note 10*).
3. Pick around 20–30 L4 worms and place them on a seeded plate (*see Note 11*). Use the JIM113 strain: *ujIs113 [pie-1p::mCherry::H2B::pie-1 3'UTR + nhr-2p::his-24::mCherry::let-858 3'UTR + unc-119(+)]*. The goal is to compare Wild Type to certain mutants, so mutations will need to be crossed into the JIM113 genetic background.



Fig. 4 A 10-well glass plate. This plate can be used to clean the worms and perform the dissection

4. After 20–24 h, worms should have the first embryos in the uterus.
5. Prepare the imaging setup prior to placing the worms into the cutting glass plate (*see Note 12*). Place 3–4 worms into one well of a 10-well-cutting glass plate (Fig. 4) filled approximately half way with M9. Move the worms into a new well 3× to rinse and remove the bacteria.
6. Cut the worms by using two needles and slicing the worm at the boundary between the uterus and the gonad (approximately in the middle of the worm).
7. Using a mouth pipet, move ~4–6 (as many as you can find) 2–4 cell embryos into a new well of a 10-well-cutting glass plate containing M9. This helps to reduce any remaining bacteria contamination. It is best if the embryos are at the 2-cell stage or even the occasional fertilized egg at the 1-cell stage.
8. Create the bead mount: Place 3 μL of 1:20 dilution of 20 μm beads in Boyd’s buffer (final concentration of beads, 1 μM) onto a microscope slide and mouth pipet 2–4 embryos from the 10-well-cutting glass plate at the 2–4 cell stage (pick the earliest staged embryos available). Using a worm pick, gently lower the coverslip onto the embryos to avoid damaging them.
9. Seal the edges of the coverslip with enough petroleum jelly to cover the edges using a brush (e.g., a cleaned nail polish brush). Sealing the coverslip prevents evaporation. Do not use nail polish, as the acetone in the nail polish can kill the embryos (*see Note 13*).

10. Place the slide into the previously prepared temperature-controlled stage and start live-imaging with a Zeiss LSM 510 (*see Note 14*).
11. Live imaging must start with 2 or 4 cell-stage embryos and 200 min is typically sufficient to reach the 100 cell-stage. It requires ~13 h to image until the embryo hatches. At the earlier stages (2–16 cell-stage), the laser power can be high (>80). However, as cells start to divide, the signal intensity increases as nucleus size decreases, which makes it difficult for StarryNite to track the cells. As a result, the StarryNite program makes more mistakes, which requires extensive manual correction. Murray and colleagues [11] suggest setting the software to automatically adjust the laser power and other parameters in different time blocks during the imaging, but some versions of the ZEN software for running the Zeiss LSM 510 lack that option. If automatically changing the laser power and other parameters is not an option, the laser can be adjusted manually by decreasing the laser power by 20–40% after 1–2 h of live imaging. Images should be collected using a 63×, 1.4NA oil Plan-APOCHROMAT objective (*see Notes 15 and 16*).
12. When using the Zeiss LSM 510, follow the microscope settings and parameters listed in Murray et al. 2006 [11]. Images should be exported in the 8-bit TIFF format. The confocal generates individual images for every focal plane at every time point. In order to run the images on StarryNite, they must be grouped by timepoints. Murray and colleagues [11] suggest using Matlab to group the images. However, an alternative way to create folders with images grouped by timepoints is to use a Mac command. To do this use a command written in AWK to compile each image by timepoint, and group each timepoint in a separate folder (*see Note 17*). When images are grouped by timepoints, use an ImageJ macro command to compress all of individual Z-stacks into a single 8-bit TIFF file, which can then be imported to StarryNite (*see Note 17*).
13. StarryNite produces multiple outputs. Open the XML file (contained in the ZIP file) in AceTree.
14. AceTree is used to visualize the lineage and can be used to manually correct any cell division mistakes or add any cells that failed to be tracked. The most common error made by StarryNite is missing a daughter cell from the previous division. To correct this error in AceTree, click on the “edit” option and select “edit tools.” This step opens two new windows, “Edit Tracks” and “Adjust or Delete Cells.” Select the mother cell by right-clicking on the cell in the embryo image window that contains the error, then click on use active cell from the “Edit Track” window. In the “Edit Track” window, check the box

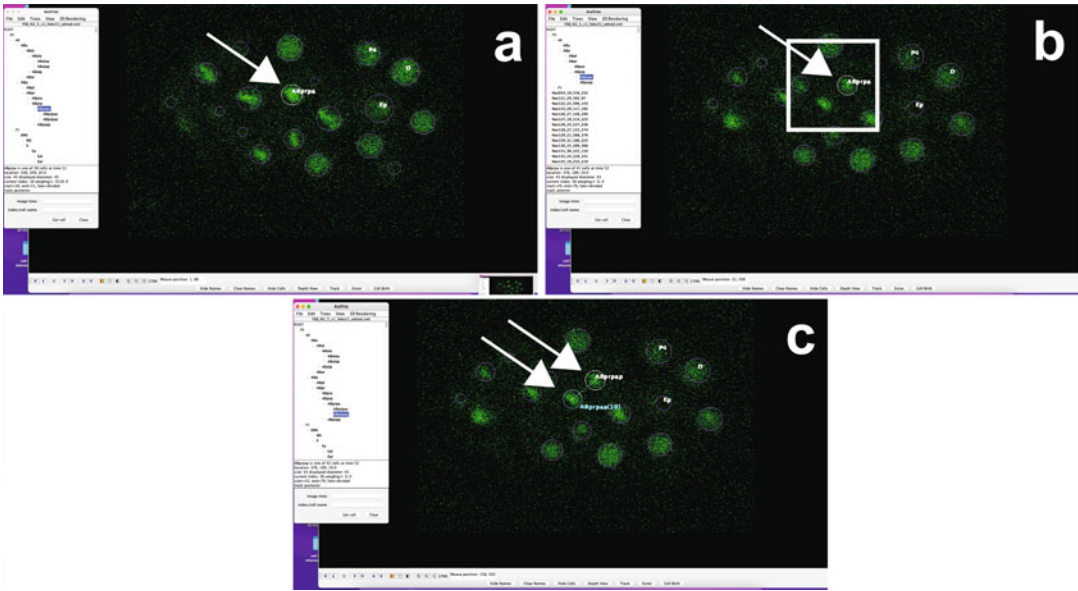


Fig. 5 Using AceTree editing tools to correct an error with StarryNite. Wild-Type (N2) embryo is shown using the AceTree program. (a) Mother cell (arrow). (b, c) The cell division (daughter cells) from the mother cell in a. In b (boxed in white), StarryNite didn't track the division. In c, the editing tool from AceTree was used to correct the cell division

that says “is early set correctly?” Then move to the next time-point by clicking on the forward arrow in the embryo image window and click on the unlabeled daughter cell that the software has failed to track. A new circle will form around the cell. Finally, click “use active cell” and “apply” from the “Edit Track” window. Now the two daughter cells will be linked to their mother cell (Fig. 5 for an example of this common mistake). Until you fix the tracking errors, AceTree will show many untracked cells as Nuc# instead of as the correct cell lineage name. Once you correct the error, AceTree will automatically assign the correct Sulston lineage name to the fixed cell.

4 Notes

1. Initially, SNlauncher software downloaded from the original website (Subheading 2.4, **item 1**) did not function. After communication with Bao lab members, an alternative version of AceTree was obtained (Subheading 2.4, **item 2**) and this version was successfully installed on a Mac OS version 12.2.1. Therefore, if SNlauncher downloaded from Subheading 2.4, **item 1** does not work, try downloading from Subheading 2.4, **item 2** as an alternative.

2. The chitinase purchased from Sigma Aldrich often does not dissolve completely, leaving very small particles that can only be observed under phase-contrast microscopy. The remaining small particles interfere with the single-cell experiment by replacing actual cells in the single GEM droplet during 10× Genomics RNA isolation. To avoid this problem, perform an ultracentrifuge step to separate the chitinase enzyme from the solid undissolved particles. Centrifuge for 30 min at 48,000 rpm at 4 °C. After centrifugation, remove the supernatant containing the particle-free chitinase enzyme and transfer to a new tube for use.
3. It typically takes ~40 min to isolate embryos via bleaching and pelleting over the sucrose cushion. To target the ~100-cell stage, let the embryos develop in Egg buffer for an additional 30–45 min. The amount of time has been empirically determined by monitoring under a dissecting light microscope (10–20× magnification) for N2 at room temperature. As a result, the amount of time will need to be separately determined for every mutant strain. This is the step before the chitinase process (Subheading 3.3).
4. If the desired embryos are greater than the 300-cell stage: The original protocol for isolating single cells from the *C. elegans* embryo incorporated both a chitinase and Pronase step. However, if targeting an embryonic cell stage of less than 300 cells, only the chitinase step is necessary, because using both chitinase and Pronase on embryos at earlier stages affects the viability of the cells. If the desired embryos are greater than the 300-cell stage, use Pronase to remove the vitelline layer of the eggshell: Add 100 µL per mL of 15 mg/mL Pronase (final concentration 1.5 mg/mL) to the sample. Using a 3 cc syringe, pass embryos repeatedly through a 21½ G needle ~20 times to generate a single-cell suspension in one well of a 12-well cell culture plate. Incubate at room temperature for 5 min. The 12-well plate makes it easier to pass through the syringe multiple times and to monitor the single-cell isolation under a dissection light microscope (10–20×).
5. Pay attention to the pellet because cells can stick to the tube. If you find that the cells are sticking to the tube, you can try centrifuging for a shorter amount of time (e.g., 30–60 s).
6. Do not use an automated cell counter instrument because the cell counting is likely to be very inaccurate. These kinds of instruments are typically designed for mammalian cells. The *C. elegans* embryonic cells are very small, often resulting in the instrument counting incorrectly. Instead, use a traditional hemocytometer to count the cells manually at 20× magnification or greater (Fig. 3).

7. Trypan blue dye is used to determine the viability of cells because dead cells take up the dye. For 10× Genomics, it is highly recommended to start with a cell population with viability of >90%, because transcripts tend to be degraded in dead cells.
8. Additional reagents not provided by the 10× Genomics kit will be needed. It's recommended that all the additional reagents be made fresh on the day of the protocol.
9. It may be possible to use a confocal other than a Zeiss LSM 510. For example, automated lineage tracing has been published using a Leica SP5 [16]. However, the StarryNite program failed to track early cell divisions from Z-stack time series generated with a Leica SP8. This may be due to differences with the metadata or with StarryNite parameters.
10. The first cell divisions in the *C. elegans* embryo occur approximately every 15 min. To slow down this process, store the buffer M9 and cutting glass at 4 °C. This will provide more time for the embryo cell stage selection, slide preparation and the microscope setup.
11. Picking L4 larvae 20–24 h prior to dissection of the adults will increase the number of worms that have 2–4 cell stage embryos 20–24 h later.
12. The temperature stage should be prepared prior to the cutting of the worms by filling the tank with distilled water and setting the temp to 20 °C.
13. Seal the slide with warm petroleum jelly. Warm petroleum jelly can be maintained permanently by storing in a glass test tube in a heat block at >65 °C.
14. Sometimes embryos will settle during the live-imaging process, which changes the focal plane and disrupts StarryNite's ability to track the lineage. If this happens, consider the following solutions:
 - (a) Setup additional Z-stacks beyond the 3 recommended by Murray and colleagues [11].
 - (b) Use a modified agar plate to prevent embryos from sinking: Using an empty 60-mm plastic petri dish, make a small opening with a heated surgical blade (sigma Aldrich:2976, No. 11) on the bottom of the plate; embryos will be placed on the exposed agar in the opening (Fig. 6). Then add 10–15 mL of hot NGM agar. Place the hole over a coverslip when pouring the NGM to prevent leakage (10–15 mL is more than needed to fill the plate, in

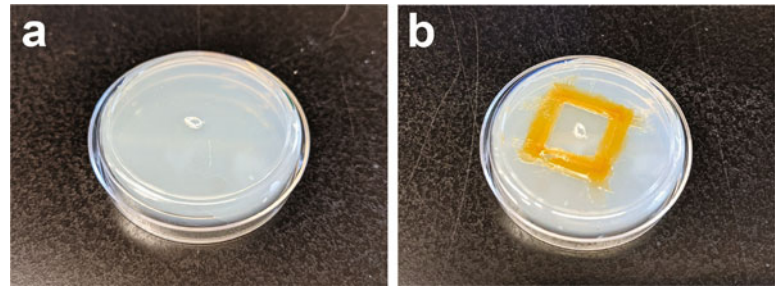


Fig. 6 Alternative method to prevent the embryo from settling during live imaging. Plate designed to prevent settling (sinking) of embryos. (a) NGM plate with hole where embryos will be placed. (b) Plate after the coverslip has been added and sealed with petroleum jelly (for details, see video link: <https://www.youtube.com/watch?v=q9GlxGfvEIQ>)

case of leakage). Let the NGM agar solidify overnight on the bench and use the next day. If you are using this technique, mouth pipet the embryos into the hole (instead of onto a slide, Subheading 3.4, step 7) and gently cover embryos with a glass coverslip; use a worm pick to gradually lower the coverslip at a diagonal. Note, when using this technique, there are no beads and no liquid on the agar. Seal the coverslip with enough petroleum jelly to prevent airflow (identical to Subheading 3.4, step 8). Place the plate on the temperature stage to start live imaging (a video link showing this process is provided in Video 1).

15. Use an upright confocal microscope rather than an inverted microscope to prevent embryos from settling out of the focal plane when inverted.
16. Any 63 \times objective (including oil, glycerol, or water) can be used for imaging. However, the standard 63 \times oil lens yielded a weaker signal. Signal intensity was improved slightly by a glycerol lens. However, a strong signal was only obtained by using the Zeiss C-Apochromat 63 \times /1.20 W Corr UV-VIS-IR water objective. Poor signal prevents StarryNite from correctly assigning cell fate, which greatly increases the amount of time that will be needed for manual correction.
17. The script used for image grouping is included in Fig. 7 (AWK code in Linux). The ImageJ macro for formatting the images prior to running StarryNite is included in Fig. 8.

```

ls -lt > qc1

ls -l | sed 's/.tif//g' | sed 's/_/ /g' | awk '{print $6}' | sed 's/_/
sort -k 1,1 | uniq | grep -v "(" | grep t | awk '{print "mkdir "$1}' >
directory.sh

sh directory.sh

ls -lt > qc2

ls -l | grep tif | sed 's/_/ /g' | sed 's/.tif/ .tif/g' | awk '{print
$6"\t"$7}' | sort -k 1,1 | uniq | awk '{print "mv *"$1"$2" "$1"/"}' > mv
sh mv.sh

```

Fig. 7 This Linux AWK command can be used to group images by each timepoint

```

/*
 * Macro template to process multiple images in a folder
 */

#@ File (label = "Input directory", style = "directory") input
#@ File (label = "Output directory", style = "directory") output
#@ String (label = "File suffix", value = ".tif") suffix

// See also Process_Folder.py for a version of this code
// in the Python scripting language.
setBatchMode(true);
processFolder(input);

// function to scan folders/subfolders/files to find files with correct suffix
function processFolder(input) {
    list = getFileList(input);
    list = Array.sort(list);
    for (i = 0; i < list.length; i++) {
        if(File.isDirectory(input + File.separator + list[i]))
            processFolder(input + File.separator + list[i]);
        if(endsWith(list[i], suffix))
            processFile(input, output, list[i]);
    }
}

function processFile(input, output, file) {
    // Do the processing here by adding your own code.
    // Leave the print statements until things work, then remove them.

    print("Processing: " + input + File.separator + file);
    open(input + File.separator + file);
    outfile = output + File.separator + file;
    ofile="[" + outfile + "];

    run("Image... ", "outputfile=&ofile display=&ofile");
    //run("Image... ", "outputfile=[&outfile] display=&file"); only does 1
    print("file:" + file);
    close();
}

setBatchMode(false)

```

Fig. 8 ImageJ macro for formatting the images prior to running StarryNite

Acknowledgments

We are grateful to the member of the Katz lab for their helpful discussion and critical reading of the chapter; J. Murray, C. Huynh, and A. Santella for advice on lineage tracing and single cell RNAseq; We thank J. Murray for the JIM113 strain. Juan D. Rodriguez was supported by NIH National Institute of Child Health and Human Development 3F31HD100145-03S1 grant and this work was supported by NSF IOS1354998.

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Methods to Analyze Nutritional and Inter-Organ Control of *Drosophila* Ovarian Germline Stem Cells

Chad Simmons, Tancia W. Bradshaw, and Alissa R. Armstrong

Abstract

Physiological status, particularly dietary input, has major impacts on the *Drosophila melanogaster* ovarian germline stem cell lineage. Moreover, several studies have shed light on the role that inter-organ communication plays in coordinating whole-organism responses to changes in physiology. For example, nutrient-sensing signaling pathways function within the fat body to regulate germline stem cells and their progeny in the ovary. Together with its incredible genetic and cell biological toolkits, *Drosophila* serves as an amenable model organism to use for uncovering molecular mechanisms that underlie physiological control of adult stem cells. In this methods chapter, we describe a general dietary manipulation paradigm, genetic manipulation of adult adipocytes, and whole-mount ovary immunofluorescence to investigate physiological control of germline stem cells.

Key words Ovary, Germline stem cells, Immunostaining, Nutrition, *Drosophila melanogaster*

1 Introduction

Drosophila melanogaster ovarian germline stem cells (GSCs) support sustained oocyte production in adult female flies and serve as an excellent model to investigate how whole-organism physiology influences adult stem cell lineages [1]. Oocyte development from GSC to mature oocyte has been well characterized [2, 3], providing a straightforward biological readout to assess the effects of physiological changes. The paired *Drosophila* ovary is composed of individual oocyte-producing units, or ovarioles (Fig. 1a, b). Developmentally progressive oocyte production occurs over 14 stages beginning at GSCs, which are housed in the anterior-most region of each ovariole, the germarium (Fig. 1b). Germaria contain on average two to three GSCs (Fig. 1c, d) that divide asymmetrically to self-renew and generate a daughter, the cystoblast. Cystoblast divisions generate germline cysts that eventually

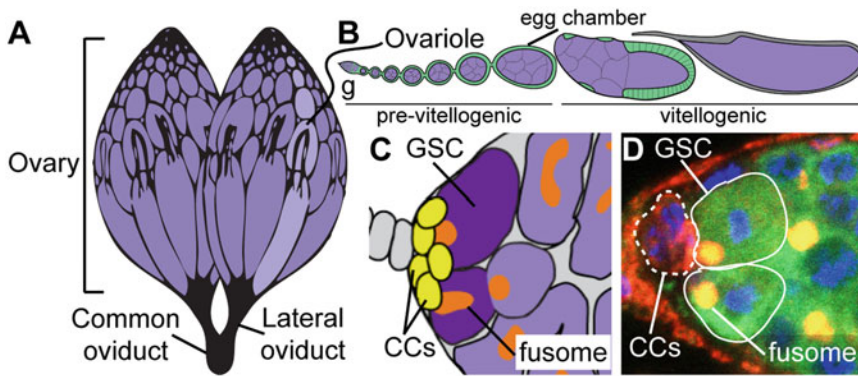


Fig. 1 Germline stem cells support the *Drosophila* ovary. (a) Each ovary in the pair is made up of 16–20 ovarioles (light purple). (b) Ovarioles contain progressively developing egg chambers composed of germ cells (purple) and somatic cells (green). The germarium (g) is located at the anterior-most region of each ovariole. (c) Germline stem cells (GSCs, dark purple) reside at the tip of each germaria adjacent to cap cells (CCs, yellow). The fusome (orange) is a specialized organelle that allows identification of GSCs. (d) Confocal image of an immunostained germarium depicting the region schematized in (c). Cap cells (dotted outline) are labeled with anti-LamC (red), GSCs (solid outline) are labeled with anti-VASA (green), and the fusome is labeled with anti-alpha spectrin (red/orange because of the overlap with VASA). Nuclei are labeled with DAPI (blue)

form a mature oocyte. Somatic cap cells are a major component of the stem cell niche that support GSC maintenance [4, 5].

A variety of physiological factors impact GSC maintenance in the ovary, including aging [6], bacterial infection [7], mating status [8], and genotoxic stress [9, 10]. Nutritional status is arguably one of the most well-understood physiological factors regulating the ovarian GSC lineage in *Drosophila* [11]. Two seminal studies demonstrated the GSCs in the *Drosophila* ovary proliferate slower [12] and are lost more rapidly from the niche [13] in females fed a protein-poor diet compared to those fed a protein-rich diet. Nutrient-responsive pathways, like insulin/insulin-like growth factor, mechanistic Target of rapamycin, ecdysone, and AMP-dependent kinase signaling, mediate the response of GSCs to dietary protein (reviewed in [1]). Interestingly, high-sugar and high-fat diets negatively impact egg production [14, 15] but it is unknown how GSCs respond to alterations in these macronutrients.

More recently, the role that inter-organ communication plays in coordinating metabolic and nutritional physiology during development and tissue homeostasis has garnered attention [16, 17]. Specifically, ovarian GSCs receive nutritional information from the *Drosophila* adipose tissue, or fat body. Amino acid sensing as well as insulin signaling function within adipocytes, the primary cell type in the fat body, to regulate GSC maintenance [18, 19]. Additionally, diet-dependent metabolic pathway activity

within adipocytes mediates activity of GSCs and their progeny [20]. Adipocyte-derived collagen transported to the germarium is also important for GSC maintenance by regulating cap cell-GSC adhesion [21]. In future studies, it will be interesting to explore how other tissues, like the gut or muscle, modulate ovarian GSCs.

Several comprehensive reviews and methods chapters describe approaches to experimentally manipulate and assess *Drosophila* physiology, such as aging [22, 23], response to infection [24], and nutrition [25, 26]. Given the prominent role that diet and the fat body play in metabolic regulation of ovarian GSCs, in this chapter we describe a general feeding paradigm for adult flies, adipocyte-specific manipulation of gene expression, and whole-mount ovary immunofluorescence to examine the GSC lineage (i.e., ovary dissection, immunolabeling, and germaria/ovariole slide mounting).

2 Materials

Unless otherwise indicated, prepare all solutions using deionized water. Most stock and working solutions should be stored at 4 °C or room temperature and will be indicated. Be sure to discard and make fresh solutions when bacterial and/or mold growth is noticed.

2.1 Fly Food Media

1. Standard medium (*see Note 1*): For 1 L, measure out 25 g of inactive dry yeast, 89.5 g of molasses solids, 57 g of yellow cornmeal, and 5.84 g of agar Type II into an appropriately sized sauce pot. Gradually add 1 L of water, stirring to moisten dry ingredients and avoid clumping. After bringing to a boil, reduce heat, cover, and simmer for 10 min, being sure to stir occasionally. Stir in 7 mL of 2 M propionic acid solution and 14 mL of 10% Tegosept. Cool to approximately 70 °C before dispensing into vials (10 mL/vial) or bottles (50 mL/vial). Cover with cheesecloth and let solidify overnight at room temperature. Store food trays in transparent office trash bags at room temperature.
2. Wet yeast paste: In a small beaker, mix inactive dry yeast and water 1:1 (g:mL). Add dry yeast or water until a creamy peanut butter consistency is reached. Cover with foil and store at 4 °C.
3. Protein-deficient (molasses-only) medium: Add 180 mL of molasses to 1112.5 mL of water (*see Note 2*) in a flask containing a stir bar. While stirring, add 44 g of agar and let stir until ingredients are combined. After autoclaving for 20–30 min, let cool while stirring. Add 9.25 mL of 10% Tegosept solution (in 95% ethanol). Dispense 5–10 mL per vial. Cover with

cheesecloth and let solidify overnight at room temperature. Store food trays in transparent office trash bags at 4 °C.

4. High-sugar (added sucrose) or high-fat (added coconut oil) diet: Prepare standard medium as described above and allow to cool to 70 °C before adding sucrose or coconut oil. For a high-sugar diet, add an appropriate weight of sucrose to achieve a 0.8 M final concentration. For a high-fat diet, mix appropriate volumes of standard medium and liquified coconut oil to achieve the desired percentage (v/v) (*see Note 3*). For both diets, stir thoroughly before and during dispensing.

2.2 Transgenic Lines

1. *Gal4* driver line for adipocyte-specific manipulation of gene expression: *tubP-Gal80^{ts}*; *3.1Lsp2-Gal4/TM6b,Tb¹* [18, 27].
2. *Gal4* driver lines for ovary-specific manipulation of gene expression: There are several routinely used *Gal4* lines used to control gene expression in various ovarian cell types and at different stages of oogenesis (described in Armstrong 2020) [26].
3. *UAS-controlled transgenes* for manipulation of nutrient sensing components: Insulin/insulin-like growth factor, mTOR-mediated, and AMPK-mediated signaling pathways can be activated or inhibited by targeting different components of each pathway. For example, RNAi-mediated knockdown of the insulin receptor blocks IIS while expression of constitutively active PI3-kinase enhances IIS. For a non-exhaustive list of transgenic lines that target these pathways, see Armstrong, 2020 [26].

2.3 Buffers and Reagents

1. 3% BSA: Make 50 mL of 3% bovine serum albumin by adding 5 mL of 30% BSA into 45 mL of sterile water in a 50 mL conical tube. Store 3% BSA at 4 °C. Be sure to discard and make fresh solutions when bacterial growth is noticed.
2. 1% PBTx stock solution (detergent): Make 250 mL of 1% Triton X-100 by adding 2.5 mL of the detergent to 247.5 mL 1× PBS (*see Note 4*). Allow the Triton X-100 to completely dissolve by mixing on a stir plate. Store at room temperature. Wash and blocking solution (#4) may contain Tween-20 instead of Triton X-100 depending on the antibody being used (*see Note 5*).
3. 0.1% PBTx wash solution: Make 50 mL of 0.1% PBTx by adding 5 mL of 1% PBTx stock solution to 45 mL of 1× PBS. Invert several times to mix. Some antibodies and/or tissues show more robust labeling when wash solutions contain a higher detergent concentration.

4. 5.3% paraformaldehyde (PFA): Prepare fresh just before or after dissection. Make the desired volume for the total number of samples necessary, 500 μ L for each 0.65 mL or 1 mL for each 1.5 mL microfuge tube, by diluting an appropriate volume of 16% PFA in 1 \times PBS.
5. Blocking solution: 5% NGS, 5% BSA, 0.1% PBT_x. Make 50 mL of blocking solution by adding 5 mL of 1% PBT_x, 2.5 mL normal goat serum 8.3 mL of 30% BSA and 34.2 mL 1 \times PBS to a centrifuge tube. Mix thoroughly and store at 4 °C. If the sample number is not likely to use the entire 50 mL volume within a week, we recommend scaling down to 15 mL.
6. Mounting medium: Vectashield containing 4',6-diamidino-2-phenylindole (DAPI).

2.4 Tools

1. BSA-coated tubes: Add an appropriate volume of 3% BSA (400 μ L for 0.65 mL or 1 mL for 1.5 mL tubes) to microfuge tubes and let rock on a nutator from 1 h to overnight. BSA-coated tubes can be made in bulk and stored at 4 °C.
2. #5 Forceps.
3. Glass Pasteur pipettes with bulbs.
4. Dissecting dish.
5. Syringes with needles.
6. Microscope slides and coverslips.
7. Nail polish.
8. 200 g weight.

2.5 Antibodies

1. The majority of primary antibodies used for GSC analyses can be purchased from Developmental Studies Hybridoma Bank (<https://dshb.biology.uiowa.edu>) and are listed in Table 1 (*see Note 6*).

Table 1
Primary antibodies routinely used in GSC analysis

Antibody	Antigen	Host species	Dilution	Recommended concentration	Cellular structure labeled
LC28.26	Lamin C	mouse	1:100	1 μ g/mL	Nuclear lamina, preferentially in cap cells
3A9	Alpha spectrin	mouse	1:50	0.5 μ g/mL	Fusomes and follicle cell membranes
1B1	Hu-li tai shao	mouse	1:10	n/a	Same as above
Anti-vasa	vasa	rat	1:100	1 μ g/mL	Germ cell cytoplasm
DCAD2	DE-cadherin	rat	1:20	5 μ g/mL	Cell-cell junctions

- We use Alexa Fluor secondary antibodies conjugated to various fluorophores (e.g., goat anti-mouse 488, 568, or 647). Upon arrival, add one volume of 100% glycerol and store in a light-protected cryobox at -20°C . All secondaries are used at 1:250.

3 Methods

Following ovary dissection and fixation, the immunostaining process consists of a series of washes and antibody incubations (Fig. 2a) that span at least 2 days. To keep track of where you are in the process, we recommend creating a streamlined checklist of the protocol described below (Fig. 2b) that can be printed and used for each experiment. Carry out all procedures with clean tools and at room temperature unless otherwise specified (*see* Notes). For all steps, use an appropriate volume; in general, 500 μL when using 0.65 mL microfuge tubes or 1 mL when using 1.5 mL microfuge tubes.

3.1 General Diet Manipulation Paradigm

- Obtain age-matched, sibling adults by collecting enough virgins to have 10 each of males and females per vial per dietary condition per timepoint, all in duplicate. Designate at least one pair of vials to be dissected prior to the dietary switch to serve as the initial timepoint (t_0).

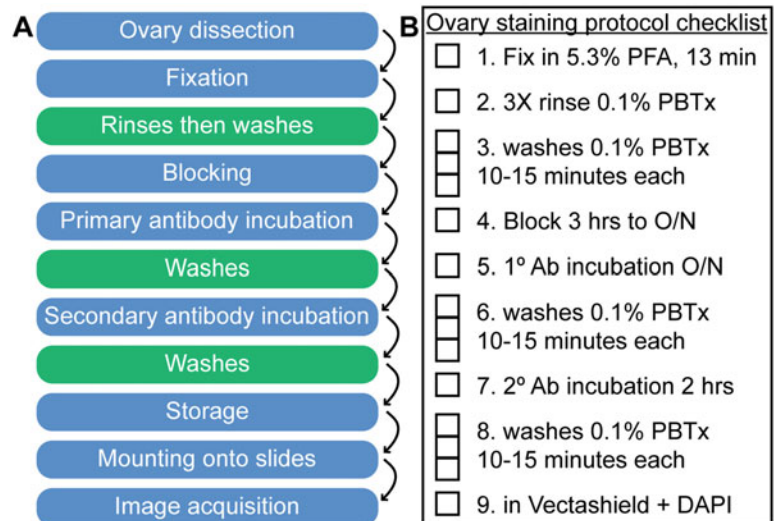


Fig. 2 Overview of the immunostaining process. (a) General order of steps involved in staining ovaries and other tissues. (b) Example of a streamlined ovary immunostaining protocol checklist that can be replicated on a single sheet to be printed and used for each experiment

2. Feed all flies a standard diet for several days (*see Note 7*) then flip into vials containing the appropriate diet—standard (controls), protein-poor, high-sugar, or high-fat (*see Note 8*). Flip into fresh vials daily until dissection.

3.2 Adult Adipocyte-specific Genetic Manipulations

1. Collect virgin females from expanded stocks of the *tubP-Gal80^{ts}*; *3.1Lsp2-Gal4/TM6b*, *Tb¹* transgenic (*see Note 9*). A good starting point is to collect enough virgins to set crosses in triplicate for each transgene, with each bottle containing 20 females (*Lsp2-Gal4*) and 20 males (*UAS-transgene*). For example, an experiment using two *UAS-transgenes* will require nine bottles (three for each cross—the control and two *UAS* lines) and total of 180 virgin *Lsp2-Gal4* females and 60 males from each *UAS* line.
2. Sprinkle the bottle food with a sparse layer of dry yeast, then add flies and label bottles appropriately.
3. Loosely cover trays containing crosses in a clear office trash bag and maintain at 18 °C flipping every 3–4 days for approximately 2 weeks.
4. Collect virgin female progeny of the appropriate genotype, add control males, and feed dry yeast for 4–5 days at 18 °C.
5. Shift vials to 29 °C to induce transgene expression and feed wet yeast paste. Flip daily until the desired dissection day. To collect samples from an initial timepoint, be sure to dissect a group of females prior to the temperature shift.

3.3 Initial Preparation for Ovary Dissection

1. Gather the tools you will need for dissection at your stereomicroscope (Fig. 3a)—BSA-coated microfuge tubes, forceps, syringe needles, dissecting dish, and moist paper towel or Kimwipe.
2. Label BSA-coated microfuge tubes.

3.4 Ovary Dissection

1. Fill wells of dissecting dish with cold 1× PBS.
2. Using forceps in your non-dominant hand grab a female fly at abdominal segment one and submerge in dissecting well (Fig. 4a). Stabilize fly by gently pressing forceps tip against dissecting well floor. With the other pair of forceps in your dominant hand, pinch the last abdominal segments and pull to remove the gut and ovaries (Fig. 4b–d) (*see Note 10*). Dispose of carcasses on the moist paper towel/Kimwipe. Transfer ovaries to new dissecting well. Repeat dissection process for the remaining flies (*see Note 11*).

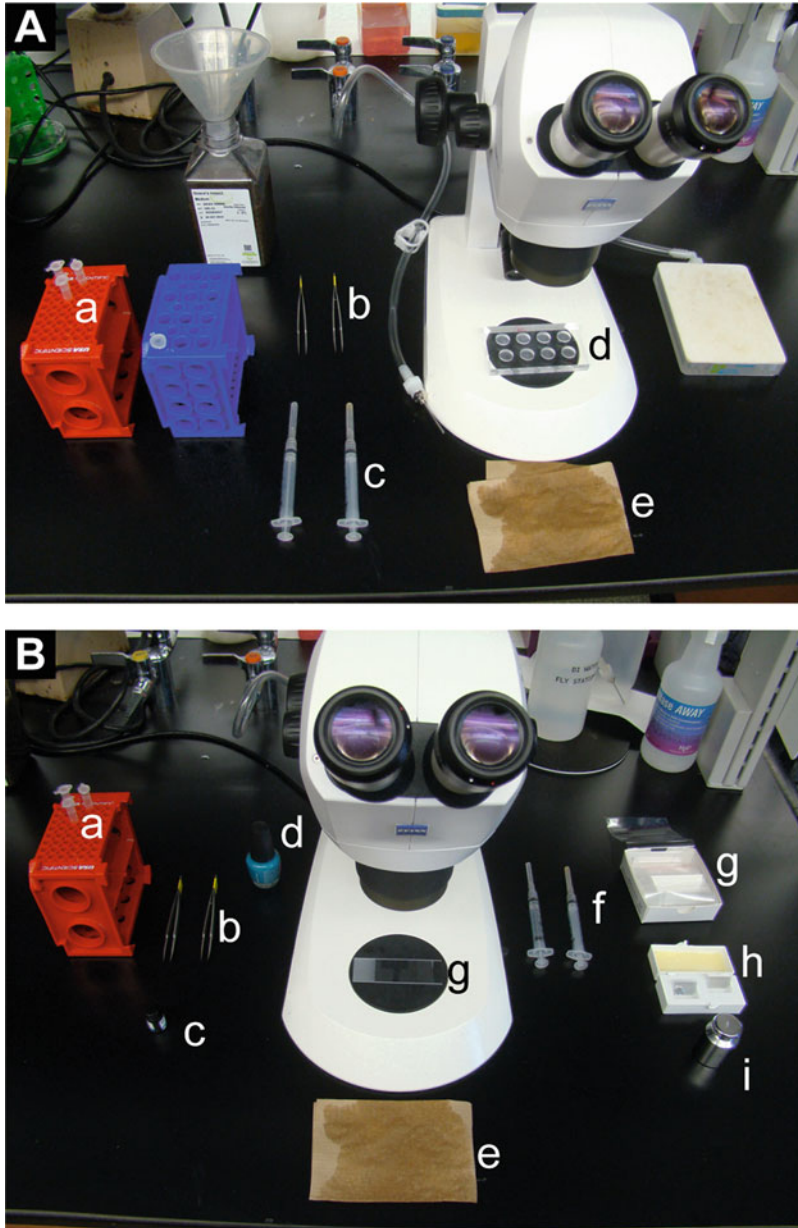


Fig. 3 Example setups for dissection and mounting. **(a)** Tools needed to dissect ovaries from adult female flies—a, 3% BSA coated microcentrifuge tubes; b, sharpened and even #5 forceps; c, syringe needles; d, dissecting dish; e, paper towel (or Kimwipe) moistened with water. **(b)** Tools needed to mount ovaries onto microscope slides—a, microfuge tubes containing stained ovaries; b, #5 forceps; c, Vectashield with DAPI (or other mounting medium); d, nail polish; e, paper towel (or Kimwipe) moistened with water; f, syringe needles; g, microscope slides; h, glass cover slips; i, weight (200 g)

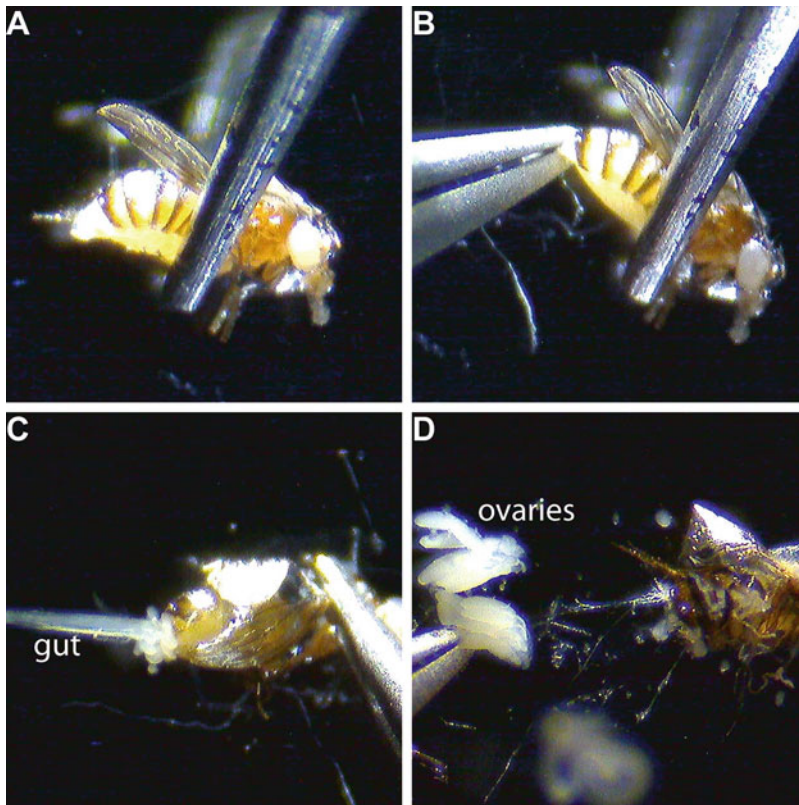


Fig. 4 Images of the ovary dissection process. (a) Pinch fly at first few abdominal segments and submerge into 1× PBS-filled dissecting well. (b, c) With a second pair of forceps pinch the last abdominal segments and pull away from the fly. As you pull, the gut and ovaries will be removed from the abdomen. (d) Fully dissected ovaries

3. Tease ovarioles apart: Stabilize ovary with a syringe needle at the lateral oviduct. With another syringe needle, gently scrape the ovary surface to loosen ovarioles from each other (*see Note 12*).
4. Transfer teased ovaries to microfuge tube: Using a glass Pasteur pipet, remove 3% BSA from microfuge tube and discard. With the same pipet, carefully aspirate ovaries, being sure to keep ovaries in the pipet tip, and transfer to the empty coated microfuge tube.

3.5 Fixation and Washing

1. Let ovaries settle and remove as much 1× PBS as possible without disturbing ovaries.
2. In the fume hood, add freshly made 5.3% PFA to samples. Let incubate on nutator at room temperature for 13 min.
3. Place microfuge tubes in a tube rack and allow ovaries to settle. Remove fix and dispose in proper waste container. Rinse

ovaries by adding 0.1% PBTx to each sample, letting ovaries settle, removing wash, and replacing with fresh wash solution. Repeat two more times. Note, for rinses, there is no incubation time outside of the time it takes for the ovaries to settle.

4. After allowing ovaries to settle, remove last rinse and replace with 0.1% PBTx. Let rock on nutator for 10–15 min at room temperature. Repeat two more times.

3.6 Blocking and Antibody Incubation

1. Blocking: Replace last wash with blocking solution at room temperature on a nutator for at least 3 h (*see Note 13*) to reduce non-specific binding of the antibodies.
2. Primary antibody incubation: Make primary antibody working solutions fresh, if not reusing from a previous immunostaining procedure (*see Note 14*), by diluting in blocking solution at the recommended concentration (see “Antibodies” sub-heading in “Materials” section). Replace blocking solution with diluted primary antibody and incubate on nutator for at least 12 h at room temperature or at 4 °C according to convention, especially if a longer incubation is planned.
3. Wash samples to remove residual primary antibody: After allowing ovaries to settle, remove primary antibody and save if planning to reuse. Add 0.1% PBTx to each sample and let rock on nutator for 10–15 min at room temperature. Repeat two more times.
4. Secondary antibody incubation: Choose secondary antibodies (*see Note 15*) with a species reactivity that matches the primary antibody host. For example, for primary antibodies generated in mice, use secondary antibodies with mouse species reactivity. Dilute secondary antibody at 1:250 in blocking solution being sure to protect from light. Care should be taken to protect samples from light for the remaining steps (i.e., cover reagents and samples with foil). Replace last wash with dilute secondary antibody and incubate on nutator for 2 h at room temperature.
5. Wash samples to remove residual secondary antibody: After allowing ovaries to settle, remove secondary antibody (do not save). Add 0.1% PBTx to each sample and let rock on nutator for 10–15 min at room temperature. Repeat two more times.
6. Store samples in mounting media: Let ovaries settle and remove as much wash solution without disturbing ovaries. Using the dropper provided with the Vectashield, add one drop, approximately 100 µL, mounting media to each sample. Gently mix ovaries and mounting media with a clean pipet tip or syringe needle to ensure that the mounting media is incorporated with the residual wash solution. Store samples overnight at 4 °C before mounting and imaging (*see Note 16*).

3.7 Mounting Ovaries on Microscope Slides

1. Gather the tools you will need to mount samples at your stereomicroscope (Fig. 3b)—samples in mounting media, forceps, Vectashield, nail polish (*see Note 17*), glass microscope slides and coverslips (*see Note 18*) moist paper towel or Kimwipe, syringe needles, and weight. Clean slides and coverslips with ethanol to remove debris and dust particles.
2. Label your microscope slide with a descriptive title before mounting.
3. Transfer ovaries in Vectashield to a microscope slide. With a syringe needle, spread the Vectashield droplet to approximately half the coverslip area. This will make it easier to manipulate individual ovarioles. Using the syringe needles, trim and remove vitellogenic stages.
4. With syringe needles, gently individualize trimmed ovarioles such that germaria do not overlap (*see Note 19*). Dispose of any debris and unwanted ovariole parts on the moist paper towel/Kimwipe. Remove large bubbles by poking with the needle tip. Remove smaller bubbles by moving to the edge and/or aspirating off with a pipet.
5. Starting from one side, slowly lower a coverslip onto the sample until the other side meets the slide, and the coverslip is completely lowered. This will minimize the formation of bubbles (*see Note 20*). Add additional mounting medium to the edge of the coverslip if there is not enough solution to cover the entire coverslip area. Wick off excess Vectashield by using a Kimwipe to gently blot the coverslip edges (moving to a dry section of Kimwipe as many times as necessary).
6. Cover slide with a Kimwipe and gently place a weight on top of the coverslip for 15–30 s. Since some larger, more developed egg chambers likely remain after trimming (**step 3**), flattening the sample on the slide provides more stabilization of germaria in aqueous mounting media such as Vectashield.
7. Remove excess mounting media that oozes from coverslip edges by gently blotting with a Kimwipe. Be sure to remove as much as possible because the nail polish will not harden over Vectashield. Seal coverslip edges with nail polish to prevent drying. Allow nail polish to dry completely before imaging. Store slides in a slide box or folder at 4 °C and protected from light (*see Note 21*).

3.8 Identifying Ovarian Germline Stem Cells

1. Before imaging, gently clean slides with a Kimwipe and glass cleaner, such as Windex.
2. To identify cap cells and germline stem cells use a 63× objective with the appropriate refractive material (air, water, or oil; indicated on the objective) whether imaging on a widefield or confocal microscope.

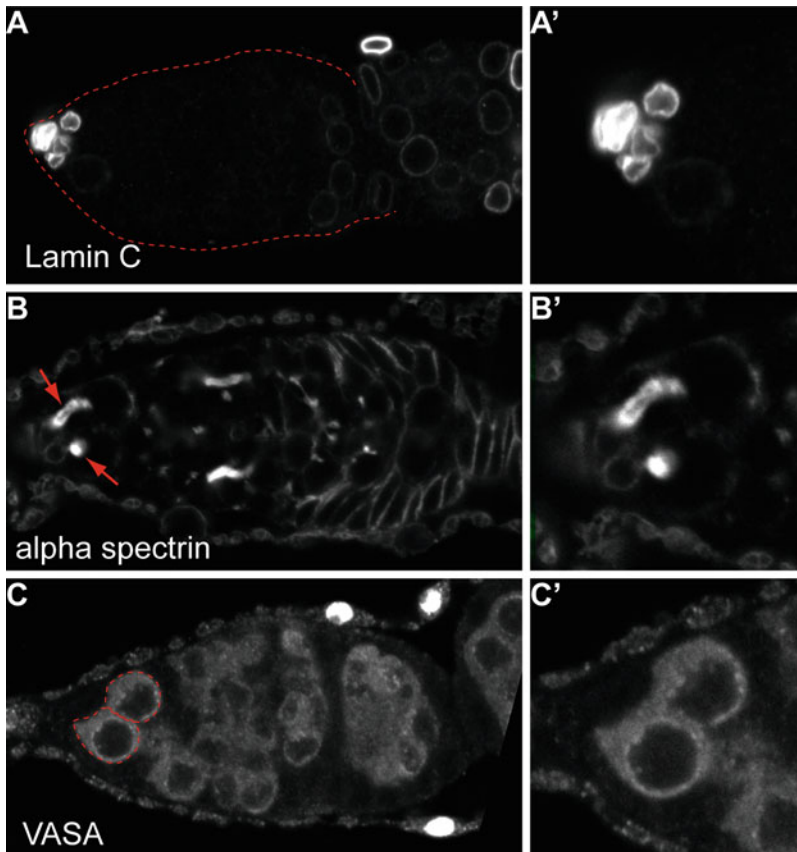


Fig. 5 Immunostaining to identify cap cells and germline stem cells in the germarium. Images with prime denotations are magnification of the apical most region of the germarium. (a, a') Lamin C immunoreactivity highlights cap cells by labeling the nuclear lamina. At this concentration (see Table 1), anti-LamC preferentially labels the nuclear lamina of cap cells and labeling of older germ cells appears as the germline cyst begins exiting the germarium to form an egg chamber. The germarium is outlined with a red dotted line. (b, b') Alpha spectrin immunoreactivity highlights the fusome of GSCs (arrows) and follicle cell membranes. (c, c') VASA immunoreactivity labels germ cells. GSCs (red dotted outline) can be identified by their location adjacent to cap cells and their size

3. Cap cell identification: Given that GSCs receive maintenance cues from the stem cell niche, cap cell number is often included in GSC analyses. On average, each germarium contains five to eight cap cells at the anterior-most region of the germarium. LamC immunoreactivity will highlight the cap cell nuclear lamina. Since cap cell nuclei occupy the majority of the cellular volume, LamC appears to outline cap cells (Fig. 5a, a').
4. Germline stem cell identification: Each germarium contains on average two to three GSCs, which can be identified by fusomes (Fig. 5b, b') adhered to cap cells. Fusome morphology will depend on GSC cell cycle phase [28]. GSCs can also be identified by cytoplasmic VASA immunoreactivity and their larger size (Fig. 5c, c').

4 Notes

1. There are a several “standard” media that vary in type and amount of macronutrients and micronutrients [25]; therefore, one might consider pilot experiments to identify a standard diet appropriate for the readout measured. For example, several meridic diets use casein as a protein source while holidic diets use defined concentrations of individual amino acids.
2. Because of its extreme viscosity, measure out the appropriate volume of molasses first. Use the same graduate cylinder to add water, being sure to dislodge and rinse as much molasses as possible from the graduated cylinder walls.
3. Standard fly food media contain little to no dietary fat making even small additions of coconut oil considered as high-fat diets. A range of 2–30% coconut oil diets have been shown to elicit effects on survival, fecundity, triglyceride and glucose levels, heart function, and gene expression [15, 29, 30].
4. Because Triton X-100 is viscous, it is important to aspirate and dispense slowly when using. Cut the very end of the pipet tip to aid in aspiration of viscous solutions. To acquire accurate volumes, be sure to keep the end of the pipet tip submerged while pausing aspiration to allow the Triton X-100 to rise by capillary action. Repeat pausing as necessary until the desired volume is reached. To ensure that all Triton X-100 is dispensed, allow the pipet to remain in the PBS while stirring for 30 min up to 1 h (i.e., allow gravity to remove as much of the Triton X-100 stuck to the inside walls of pipet).
5. Triton X-100 and Tween-20 are detergents used in *Drosophila* ovary immunostaining protocols. Different antibodies or tissues may show more consistent and robust immunolabeling when different detergents are used. For example, the anti-alpha-spectrin primary from DSHB tends to show better immunoreactivity when wash and blocking solutions contain Tween 20 instead of Triton X-100. To optimize immunostaining protocols for new antibodies and/or tissues, it is a good idea to test multiple detergents at varying concentrations.
6. The recommended concentrations of primary antibodies routinely used for ovarian GSC analysis (alpha-spectrin, LamC, DCAD2, etc.) are generally indicated as dilution ratios. However, stock concentrations can vary lot by lot. Therefore, we recommend using a specified concentration. For primary antibodies that have not been used before, we suggest a dilution/concentration series be performed to identify the ideal concentration to use for immunostaining.

7. To avoid phenotypes resulting from effects on larval fat tissue, flies should be fed from 2 to 4 days, depending on the temperature at which they are maintained, prior to any manipulations to ensure that larval fat has been cleared [31].
8. To prevent adult flies from getting stuck on the surface of high-fat and high-sugar diets, place a disc of filter paper on top of the food. Alternatively, experiments using obesogenic diets can be maintained at 18 °C.
9. Ideally, collect all the virgins needed to set crosses in 1 day. When that is not possible, the collection period can be extended to 1 week with virgins housed at 18 °C.
10. Often, the abdomen and thorax will separate when pulling off the last abdominal segments. When that happens, ovaries can be removed by using forceps to gently squeeze the abdomen starting at the posterior end to extrude ovaries from the anterior opening.
11. To avoid transferring fly debris into microfuge tubes, dissect flies in one well of the dissecting dish and transfer ovaries to a fresh well. When the well that you are dissecting in accumulates too much debris, move to a clean dissecting well.
12. So that solutions have access to and can penetrate all tissue, it is important to sufficiently tease apart ovarioles. However, it is equally important to not over-tease since this can lead to ovariole loss as you carry out the remaining incubation and wash steps. Ideally, all ovarioles will still be held together by the lateral oviduct and late-stage vitellogenic egg chambers (i.e., resembling a flower that has blossomed).
13. Samples can be left in blocking solution up to 1 week at 4 °C being sure to move to the next step before mold/bacterial growth occurs. It is possible that samples can be stored in blocking solution at 4 °C longer than 1 week but this is not recommended.
14. Primary antibodies can be reused up to three times as long as the diluted solution does not show signs of growth and has been stored at 4 °C. Be sure to keep track of how many times the antibody has been used on the label and discard after three uses.
15. When labeling with a single antibody, we recommend using secondary antibodies conjugated to fluorophores that emit in the 488/green range, since it is often the most robust. If using multiple primary antibodies, we recommend matching the least robust primary antibody with 488/FITC conjugated secondaries and the most robust primary antibodies with 568/Cy3 or 647/Cy5 conjugated secondary antibodies.

16. While it is not recommended, ovary samples can be stored in mounting medium at 4 °C for months, even years, although immunolabeling quality will deteriorate over time.
17. To avoid interference with fluorescence microscopy, use nail polish colors that are subdued (clear, white, shades of brown, or pastel colors). Avoid neon polishes and those containing glitter.
18. To help ensure that ovarioles do not overlap, use square coverslips (22 × 22 mm) for samples containing few ovaries (fewer than 5 pair) and rectangular coverslips (40 × 22 mm) for samples containing larger number of ovaries. If the sample size is too large to be mounted on one slide, split the sample in half and mount on two separate slides, being sure to label accordingly.
19. Mounting is a tedious process. We find that the following steps make the process more efficient. (A) Position ovaries close to the edge of the mounting media. (B) Drag an ovary to the middle and trim. You will need to constantly reposition individualized ovarioles so they do not move to the edge of the droplet. (C) Drag unwanted egg chambers/ovarian tissue to the outermost edge of the mounting media and remove with forceps when the pile is large enough. (D) After ovary debris has been removed, use syringe needle to sweep ovarioles toward the center of the slide. Depending on the number of ovaries being mounted, Vectashield may dry out requiring the addition of a small drop as you mount.
20. In some cases, bubbles are unavoidable. Ovarioles trapped in bubbles will not be able to be imaged. Ovarioles adjacent to bubbles can be imaged but will be somewhat hindered by the shadow of the bubble.
21. Mounted samples can be stored at 4 °C for several days. However, we recommend mounting samples just prior to imaging since immunolabeling is less stable after mounting.

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Targeting Endogenous Loci That Function in *Drosophila* Germline Stem Cells

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Abstract

CRISPR-Cas9 genome editing technology can be used to manipulate the genome of *Drosophila melanogaster*. The ability to delete genes, make specific mutations, add tags, or make other genetic manipulations is useful for studying germline stem cell biology. In this chapter, we will describe a method to use CRISPR-Cas9 genome editing technology to make knock-out and knock-in flies. We will cover everything from guideRNA (gRNA) and donor plasmid design and cloning to screening for positive edits.

Key words CRISPR-Cas9, Genome editing, *Drosophila* ovary, Germline stem cells

1 Introduction

The *Drosophila melanogaster* ovary has been used extensively to study the biology of adult stem cells [1]. Our understanding of germline stem cell (GSC) maintenance and differentiation has been expanded through studying the *Drosophila* germarium. The germarium houses the GSCs at the anterior tip of the ovariole in a well-defined niche. The progeny of GSCs differentiate in a linear and continuous fashion, allowing one to examine every stage of germ cell development in each experiment.

Previous work gained insights into the mechanisms that govern GSC maintenance and differentiation. The BMP signaling pathway maintains GSCs. Dpp produced by cap cells binds to the Tkv receptor on the surface of GSCs, resulting in phosphorylation of Mad (pMad) [1–3]. pMad and Medea translocate to the nucleus and repress *bam* transcription [4, 5]. Additionally, RNA-binding proteins Nanos and Pumilio repress the translation of differentiation transcripts [6, 7].

GSCs undergo asymmetric cell divisions to produce two daughter cells. The GSC daughter that remains in the niche continues to receive BMP signals and maintains *bam* transcriptional silencing. The daughter cystoblast cell that exits the niche no longer receives Gbb and Dpp signals from the cap cells, resulting in *bam* transcription and initiation of differentiation [8, 9]. Different mechanisms help attenuate the ability of cystoblasts to respond to niche signals. For example, Pumilio and Brat work together to repress components of the BMP pathway including Mad [6]. In addition, Smurf and Fused work together to degrade the Tkv receptor [10]. Decreasing the ability of the cell to respond to BMP signaling ensures the cell continues along the path of differentiation.

bam is the master switch of germ cell differentiation as it is both necessary and sufficient for differentiation [9]. Bam interacts with Bgcn, Mei-P26, and Sxl to repress *nanos* mRNA translation [11–13]. Reduced Nanos protein expression releases differentiation transcripts from repression, allowing for their active expression.

Sxl is necessary for the repression of *nanos* translation. The *nanos* 3' UTR has one Sxl binding site, which is necessary for the ability of the Bam, Bgcn, and Sxl complex to repress *nanos* translation [12]. Bam and Nanos have mutually exclusive expression patterns in the female germline. In contrast, both are expressed in early germ cells in the testes where there is no Sxl expression, indicating an interesting sex-specific difference. *sxl* is the master switch of sex determination in female somatic cells [14]. *sxl* also has a role in sex determination in the germline. Male-specific transcripts are expressed in *sxl* mutants indicating that *sxl* is involved in maintaining a female fate [15]. Furthermore, the expression of male-specific *phf7-RC* is a critical target of Sxl repression. *sxl* mutants express high levels of *phf7*, *upd2*, *upd3*, and *chinmo*, which are components of the Jak/Stat pathway and critical for male germ cells. A key piece of data demonstrates that knockdown of *phf7* in a *sxl* mutant background suppresses the mutant phenotype and reduces the expression of the components of the Jak/Stat pathway, indicating that *sxl* is important for maintaining a female fate and differentiation by suppressing these male-specific transcripts [16]. *sxl* expression is regulated differently in germ cells and somatic cells. In the germline, the transcription factor Ovo promotes the expression of *ovarian tumor (otu)* [17]. In addition, *otu* levels are responsive to another transcription factor, *stand still (stil)*, and as of yet unidentified signal(s) downstream of *tra2* and *dsx* in the somatic cells [18, 19]. *otu* acts upstream of *sxl*, but the nature of the regulation between these factors remains unknown.

While several mechanisms that regulate GSC maintenance and differentiation have been characterized, additional mechanisms await discovery. One key advantage to using *D. melanogaster* as a model organism is the ease of genetic manipulation. Geneticists

have been characterizing the genome of *Drosophila* for decades, but the emergence of CRISPR-Cas9 technology has greatly improved the sophistication of genetic manipulation possible in the system. CRISPR-Cas9 technology has been described extensively elsewhere [20]. Briefly, Cas9 is an endonuclease that uses an RNA called the guideRNA (gRNA) to target specific sequences in the genome for double-strand break formation. We can take advantage of Cas9's ability to form double-strand breaks to knock out genes or introduce mutations or tags. This technology can specifically be used in the ovary to knockout genes enriched in the ovary or those that have been identified as interacting partners of known factors. We can also use CRISPR-Cas9 to introduce tags like fluorescent proteins for live imaging or other tags like HA or FLAG for biochemical assays. Introducing these tags into the endogenous locus gives the advantage of the gene being expressed under the endogenous regulatory sequences. Being able to manipulate the genome in this way will greatly accelerate the discovery of additional mechanisms that function in *Drosophila* GSCs.

2 Materials

2.1 Plasmids and Other DNAs

1. pU6-BbsI-chiRNA plasmid (Fig. 1).
2. pHD-DsRed-attP plasmid (Fig. 2).
3. DNA oligos for the gRNAs (*see Note 1*).
4. DNA fragments for the homology arms (*see Note 2*).
5. Primers for PCR and sequencing.

2.2 Cloning Reagents

1. T4 DNA ligase.
2. 10× T4 DNA ligation buffer: 0.5 M Tris-HCl, 0.1 M MgCl₂, 10 mM ATP, 0.1 M DTT, pH 7.5@25 °C.
3. T4 Polynucleotide Kinase (PNK).
4. Nuclease free H₂O.
5. PCR tubes.
6. Thermocycler.
7. BbsI.
8. 10× NEBuffer r2.1: 0.5 M NaCl, 0.1 M Tris-HCl, 0.1 M MgCl₂, 1 mg/mL Recombinant Albumin, pH 7.9@25 °C.
9. Calf Intestinal Alkaline Phosphatase.
10. Agarose.
11. TAE buffer: 40 mM Tris, 20 mM acetic acid, 1 mM EDTA.
12. Ethidium bromide.
13. Gel electrophoresis equipment to run DNA gels.

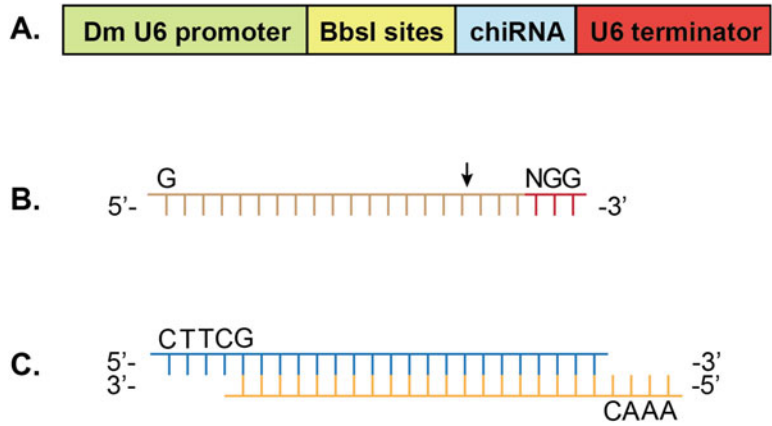


Fig. 1 (a) Diagram of pU6-BbsI-chiRNA vector. The vector has a DmU6 promoter and U6 terminator to promote transcription of small RNAs. BbsI sites are present to digest the vector with non-matching overhangs to ligate the guide sequence in frame with the rest of the chiRNA sequence. (b) Diagram of guide sequence. Guides must precede a PAM NGG sequence. Select guides that start with a G to promote U6 transcription. The scissors indicate that the Cas9 enzyme will make the double-stranded break 3 base pairs upstream of the PAM. (c) Diagram of the sense and antisense gRNA oligos. Add CTTC to the sense oligo and CAAA to the antisense oligo for ligation into the digested pU6-BbsI-chiRNA plasmid

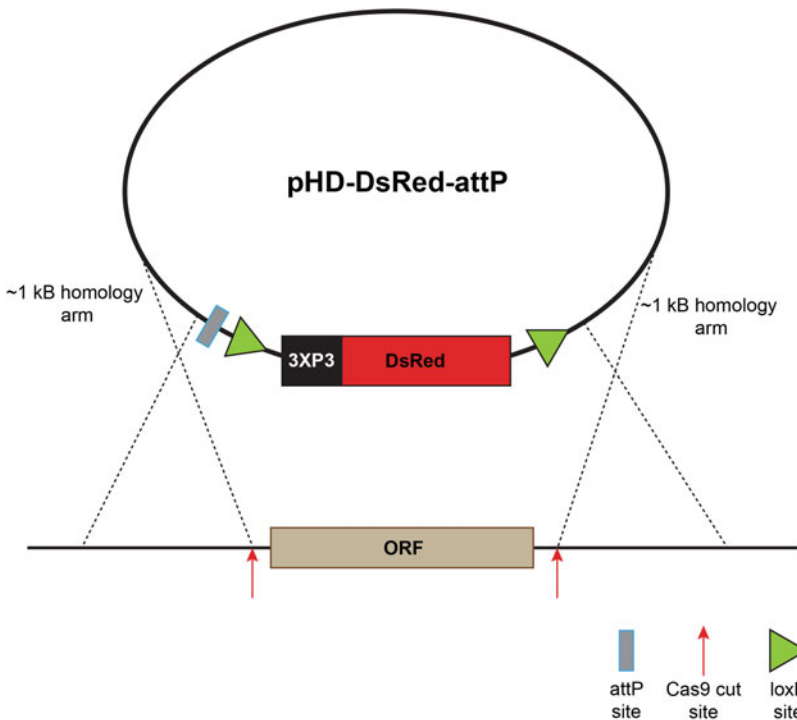


Fig. 2 Diagram of knockout donor plasmid design. gRNA sites should be selected just outside of the ORF being replaced. The pHD-DsRed-attP plasmid contains the DsRed sequence preceded by a 3XP3 promoter. This is flanked by loxP sites and preceded by an attP site. Restriction enzyme sites EcoRI and XhoI can be used to insert in homology arm sequences that match the sequences upstream and downstream of the ORF

14. DNA ladder.
15. UV transilluminator.
16. Razor blades.
17. Gel DNA recovery kit.
18. Nanodrop or spectrophotometer.
19. Chemically competent cells like Top10 or DH5 α .
20. Ampicillin.
21. LB broth.
22. SOC medium.
23. Ampicillin LB agar plates.
24. 42 °C water bath.
25. Plasmid mini prep kit.
26. EcoRI-HF.
27. XhoI.
28. 10 \times rCutsmart buffer: 0.2 M Tris-acetate, 0.1 M Magnesium acetate, 0.5 M Potassium acetate, 1 mg/mL Recombinant Albumin, pH 7.9@25 °C.
29. Gibson Assembly Kit or NEB HiFi Builder Kit.
30. DNA polymerase for colony PCRs like 2 \times Sapphire master mix (Takara).
31. Plasmid midi or maxi prep kit. Kits must provide high-quality DNA for injection. Rainbow Transgenics will only accept DNA from Qiagen or Invitrogen Plasmid Midi or Maxi Prep Kits.
32. High-fidelity polymerase for sequencing like 2 \times Primestart master mix (Takara).
33. DNAzol.

2.3 Fly Rearing Components

1. w^{1118} , w^{Berlin} , or *OreR* fly strains.
2. Balancer strains corresponding to the chromosome on which the edit is made (i.e., FM7 (first), ScO/CyO (second), and TM3/TM6b (third)).
3. Fly rearing bottles or vials with standard cornmeal agar.
4. Wet yeast paste: dry yeast mixed with water to a paste.
5. Microscope with fluorescent filters.

2.4 Ovary Dissection, Immunostaining, and Imaging Components

1. Fine tipped forceps (Dumont #5).
2. Dissecting dish with at least 2 wells. The dish should be deep enough to cover the ovaries with media.
3. Glass pipette.
4. Bulb for the pipette.

5. Grace's Insect medium.
6. PBS: 137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.4.
7. 1.7 mL Eppendorf Tubes.
8. 10× PBS: 1.37 M NaCl, 27 mM KCl, 80 mM Na₂HPO₄, 20 mM KH₂PO₄, pH 7.4.
9. 4% PFA in PBS made fresh: 250 μL 16% PFA, 100 μL 10× PBS, 650 μL H₂O.
10. Nutator.
11. PBT: 1× PBS, 0.3% Triton-X 100, 0.5% BSA.
12. Primary antibodies.
13. Fluorescently conjugated secondary antibodies.
14. Mounting solution with DAPI.
15. Microscope slides.
16. Forceps (Dumont #5).
17. Tungsten needle.
18. 22 × 22 or 22 × 40 mm coverslips.
19. Clear nail polish to seal the coverslips.
20. Confocal laser scanning microscope, spinning disk microscope, or other fluorescent microscopes.

3 Methods

3.1 Cloning guideRNA Plasmids

1. Resuspend the sense and antisense oligos to a final concentration of 100 μM in nuclease-free H₂O.
2. Phosphorylate and anneal the oligos: add 1 μL oligo 1 (sense), 1 μL oligo 2 (antisense), 1 μL 10× T4 ligation buffer, 1 μL T4 PNK, 6 μL H₂O for a total volume of 10 μL to a PCR tube.
3. Run the following thermocycler program: 37 °C for 30 min, 95 °C for 5 min, ramp to 25 °C at a rate of −0.1 °C/s. Store the annealed oligos at −20 °C until ready for use. In the case that the oligos are already phosphorylated, do not add T4 PNK to the reaction mixture and add H₂O to a final volume of 10 μL. Skip the initial 30 min incubation at 37 °C.
4. Digest 1 μg pU6-BbsI-chiRNA plasmid with BbsI restriction enzyme following the manufacturer's protocol. Halfway through the digestion add 1 μL of Calf Intestinal Alkaline Phosphatase or other comparable phosphatase. Dephosphorylating the vector will discourage re-ligation of the plasmid.

5. Make a 1% agarose gel: microwave 1% agarose solution in TAE buffer at 50% power until the agarose is melted. Allow the gel to cool and add 1 μL of ethidium bromide. Pour into a cast for the gel with a comb to form the wells.
6. Add the 6 \times loading dye to the digest reaction for a final concentration of 1 \times . Run the digest on the 1% agarose gel. Run 10 μL of DNA ladder in a separate well to compare the size of the digested fragments.
7. Image the gel on UV transilluminator.
8. Excise gel slice containing the needed DNA fragments using a razor blade.
9. Purify using gel purification kit per the manufacturer's instructions. We use the Zymo Research gel purification kit. Isolated DNA can be stored at $-20\text{ }^{\circ}\text{C}$.
10. Ligate the annealed oligos into the digested pU6-BbsI-chiRNA backbone. Set up the reaction: 50 ng of digested pU6-BbsI-chiRNA backbone, 1 μL annealed oligos, 1 μL 10 \times T4 ligation buffer, 1 μL T4 DNA ligase, X μL H_2O for a total volume of 10 μL . Incubate for 1 h at $25\text{ }^{\circ}\text{C}$. Proceed to transformation or store at $-20\text{ }^{\circ}\text{C}$.
11. Add 1.5 μL of the ligation reaction to chemically competent cells. Use chemically competent cells like Top10 or DH5 α and follow the manufacturer's protocol. Plate on ampicillin plates and incubate at $37\text{ }^{\circ}\text{C}$ overnight.
12. Mini prep the plasmid DNA from 2–4 colonies. Follow the manufacturer's protocol.
13. Sequence using T7 or T3 primers.
14. Once a correctly cloned plasmid has been identified, midi prep the plasmid following the manufacturer's instructions. We find kits from Qiagen or ThermoFisher give the highest quality DNA for injection. We have had the best results with Qiagen. Midi preps must have a concentration $>500\text{ ng}/\mu\text{L}$ for injection.

3.2 Cloning the Donor Plasmid

1. Resuspend all gene blocks to a concentration of 10 $\text{ng}/\mu\text{L}$ in nuclease-free H_2O . Follow resuspension directions from the manufacturer.
2. Digest 1 μg of vector backbone using chosen restriction enzymes following the manufacturer's instructions.
3. Run on 1% agarose gel. Run 10 μL of DNA ladder in a separate well to compare the size of the digested fragments.
4. Image the gel on UV transilluminator.
5. Excise gel slice containing the needed DNA fragments using a razor blade.

6. Purify using gel purification kit per the manufacturer's instructions.
7. PCR amplify and gel purify homology arm fragments if necessary.
8. Set up Gibson assembly reaction (we use NEB HiFi builder) following the manufacturer's protocol.
9. Transform into chemically competent cells.
10. Plate on antibiotic plates.
11. Perform a colony PCR reaction to identify correct clones.
12. Mini prep clones following the manufacturer's protocol.
13. Sequence using appropriate primers. Make sure to get full coverage of the donor sequence.
14. Once a correct plasmid has been identified midi prep the plasmid following the manufacturer's instructions. Kits from Qiagen or ThermoFisher give the highest quality DNA for injection. We have had the best results with Qiagen. Midi prep must have a concentration >500 ng/ μ L for injection.

3.3 Injection

1. We send our DNA to Rainbow transgenics to be injected into fly embryos that express Cas9. Alternatively, you can inject the plasmid DNA into embryos that express Cas9 in the pole cells and primordial germ cells. To send DNA for injection add 250 ng/ μ L of the Donor plasmid, 20 ng/ μ L of guide 1 and 20 ng/ μ L of guide 2 in a total volume of 200 μ L. If using only one guide send 40 ng/ μ L of the gRNA.
2. Request to have DNA injected into a fly where Cas9 is expressed off a different chromosome from the one being edited. We use NosCas9attp40 on the second chromosome and NosCas9attp2 on the third chromosome. The DNA will be injected into embryos, and some of the germ cells will be edited. Screen F1 progeny for desired edits.

3.4 Screening

1. Designing PCR primers: Design a PCR strategy to identify the edits. For knock-outs, have two primers outside of the homology arms and use primers from within the DsRed cassette. For knock-ins, have two primers outside the homology arms that span the edited region and primers from within the knocked-in sequence. Using primers outside of the homology arms will ensure that it hasn't been inserted in another location in the genome. Primers should have a T_m above 58 °C, ideally between 60 °C and 63 °C.
2. Fly crosses: Once the injected flies eclose, cross 1 G0 male to 3 virgin WT females (w^{1118} , w^B , etc.). Set up as many crosses as possible. If more crosses are needed, cross 2 G0 females to 3 WT males. If making a knock-out with a fluorescent marker

expressed in the eye, screen F1 flies under the microscope with the correct fluorescence filter. If using the DsRed under the 3XP3 promoter there will be red fluorescence in the eye and also in the gut of the larvae. Cross the F1 DsRed positive flies to a balancer strain for the chromosome.

3. For knock-ins that do not have a visible selection marker, cross F1 flies to balancer flies, 1 F1 male to 3 virgin females. Once larvae are clearly present (the food should have obvious churning), take the F1 fly and extract its genomic DNA. We use DNAzol (Invitrogen). Use the genomic DNA to screen by PCR for the correct knock-in. We usually have to screen 50–100 F1 flies to find a correct insertion. Less efficient knock-ins might require more screening. Once a correct knock-in has been found, PCR the whole region using the primers outside the homology arms using a high-fidelity polymerase. Sequence to make sure the knock-in is in frame and there are no SNPs, insertions, or deletions.
4. Take 10 F2 males from the crosses with the correct edit and set up crosses with the balancer chromosome again. Screen to identify the crosses that have the chromosome and from those flies establish stocks. Determine if homozygotes are viable and fertile.

3.5 Characterizing a Mutant

Once the mutant strain is isolated, many assays can be used to characterize the phenotypes of these flies. We typically stain the ovaries for different markers and image them on a confocal microscope to look at the development of the ovaries. For example, if knocking in a tag, we perform immunofluorescence to see if the tagged protein is expressed. In Fig. 3, *Rbfox1* was tagged with mCherry and the mCherry antibody and *Rbfox1* antibody signal overlapped indicating the *Rbfox1::mCherry* endogenous tag is expressed and inframe. We have included a table of common markers we use to characterize *D. melanogaster* oogenesis (Table 1).

To perform the immunofluorescence:

1. Collect 8–12 homozygous mutant females between 0 and 3 days old. Also collect control flies. Place female flies in vial with wet yeast paste with some males. Keep the vial at 25 °C for 2 days.
2. Dissect ovaries: Pour Grace's Insect Medium into two wells of a dissecting dish, enough to cover the ovaries. Place a fly in one of the wells. Using two tweezers, open up the abdomen ripping open up 2–3 segments from behind. The ovaries look like little artichokes and may be small if there is a mutant phenotype. Remove other excess tissue, but leave the ovaries attached to the oviduct as it makes it easier to see them in the tube during staining. Move the ovaries to a different well that has media. Finish dissecting the rest of the ovaries.

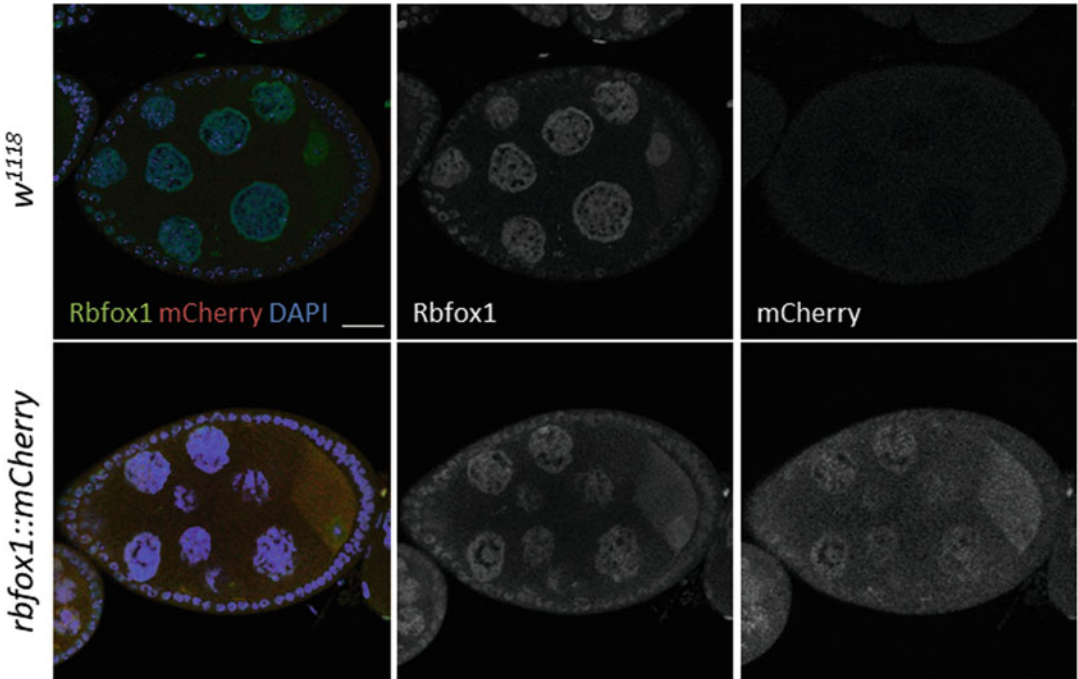


Fig. 3 *rbfox1::mCherry* and *w1118* ovaries were stained with Rbfox1 and mCherry antibodies demonstrating the colocalization of the Rbfox1 and mCherry signal in the nurse cell nuclei

Table 1
A list of common antibodies used to characterize *Drosophila* germ cells

Markers	Source	Concentration	Notes
Vasa	DSHB	1:20	Rat IgM
1B1 (Hts)	DSHB	1:20	Mouse
Sxl	DSHB M18 and M114	1:10	Mouse M18 shows one band Mouse M114 shows doublet
Bam	DSHB	1:10	Mouse. Must store at -20°C and once thawed store at 4°C and use within 2 weeks
Rbfox1	Buszczak lab	1:5000	Guinea pig

3. Transfer the ovaries to Eppendorf tube using a glass pipette and place on ice.
4. Make 4% PFA in PBS fixative.
5. Remove excess Grace’s media with glass pipette.
6. Add 500 μL of PFA fixative, and incubate on a nutator at RT for 10 min.
7. Remove PFA and rinse with 500 μL PBT. Repeat once more.

8. Wash ovaries with 500 μ L PBT for 10 min on nutator at RT. Repeat two more times.
9. Make primary antibody solutions in 200 μ L PBT per sample.
10. Remove PBT from ovaries. Add 200 μ L of primary antibody solutions. Incubate overnight at 4 °C on nutator.
11. Remove primary antibody solution.
12. Wash ovaries with 500 μ L PBT for 10 min on nutator at RT. Repeat two more times.
13. Make secondary antibody solution in 200 μ L PBT per sample. We use fluorescently conjugated secondaries at 1:300.
14. Incubate on the nutator for 6 h at RT or overnight at 4 °C. Keep protected from light to prevent photobleaching.
15. Wash ovaries with 500 μ L PBT for 10 min on nutator at RT. Repeat two more times.
16. Remove all excess PBT.
17. Add a drop of mounting solution with DAPI.
18. Store at 4 °C in the dark for at least 20 min. Samples can be stored for a couple weeks depending on the antibody until ready to mount.
19. Using a glass pipette place the ovaries and mounting media onto a microscope slide. Using a dissecting microscope use tweezers to remove any excess tissue including any remaining exoskeleton and gut tissue.
20. Using a tungsten needle and tweezers, run the needle in between the ovarioles to splay them open like a fan.
21. Add a glass coverslip on top. Go slowly to prevent bubbles from being trapped underneath. Add more mounting media from the sides using the glass pipette if necessary.
22. Seal the coverslip edges with clear nail polish.
23. Image on a confocal or other fluorescent microscope.

4 Notes

1. The gRNA sequence will have a large impact on the effectiveness of the gene edit. Many tools are available to select gRNA sequences. We have used the CRISPR track on the UCSC genome browser. Select a 20 nucleotide sequence that precedes a PAM sequence (NGG) (Fig. 1b). The first 5' nucleotide should be a G to promote U6 transcription. Choose a guide as close to the desired edit as possible to increase efficiency. Cas9 will cut in between nucleotides 17 and 18. Many of the guide design tools provide specificity and efficiency scores in

addition to the predicted number of off-target sequences. Choose a guide with the best combination of proximity, high efficiency, and no predicted off-targets. For knock-outs select two guides that are outside the genomic locus without disrupting any upstream or downstream genes.

To clone the gRNA into the pU6/BbsI/chiRNA vector, overhangs need to be added to the sense and antisense oligos to match the overhangs generated by the BbsI enzyme (Fig. 1a). Add CTTC to the 5' end of the sense oligo and AAAC to the 5' end of the antisense oligo (Fig. 1c). This design ensures the guide is cloned in the correct orientation.

2. Designing donor plasmids for knock-outs vs knock-ins requires different strategies. For knock-outs the pHD-DsRed-attP plasmid has several elements that make it a good option (Fig. 2). The plasmid has a DsRed cassette under control of a 3XP3 promoter, which drives expression in the eye making it easy to screen for knockouts. It also includes two loxP sites flanking the DsRed cassette that can be used to remove the DsRed by expressing CRE recombinase. We have utilized the restriction enzyme sites EcoRI and XhoI to insert in homology arms so the DsRed cassette can be knocked in to replace a gene locus. Homology arms should be approximately 1 kB in length. Because we use Gibson assembly to make these vectors, each of the homology arm fragments needs 20–30 bp of sequence that overlaps with the vector. We order gene block fragments from IDT for the homology arms. Alternatively, these homology arms can be PCR amplified from genomic DNA. Make sure to include the overhang sequences for the Gibson assembly in the primers to add the necessary sequences onto the PCR product. It may be necessary to PCR amplify the homology arms if the sequence is too complex or repetitive to synthesize.

Knock-in donors can be used to introduce a specific mutation in a gene or to add a tag to the protein. When designing a donor that will introduce a specific edit and it will not be possible to screen for mutants with a simple PCR, try to incorporate a restriction enzyme site into the donor design to make screening for edited flies easier. Tags are typically added at the N or C terminus. If either terminus is possible and will not interfere with protein function, we tend to opt for the one with the more optimal guide sequence. The closer a guide is to the desired insertion site for the tag, the higher the efficiency. Add in a linker sequence of your choice. Make sure that the linker is the right length for the inserted tag to be in frame with the rest of the protein. Also check if the gRNA sequence is disrupted by the insertion. If the guide sequence is still intact after the edit, we suggest mutating the PAM or guide sequence in the donor

so that it will no longer be cut once the knock-in has occurred. Choose a backbone that doesn't express other things. We typically use pUC19. Choose two restriction enzyme sites to digest the plasmid. Ideally, the homology arms will be close to 1 kB in length. We use Gibson assembly to clone the donor plasmids, so design the gene blocks and PCR sequences in such a way as to have 20–30 bp overhangs between each fragment.

Acknowledgments

We would like to thank members of the Buszczak lab for their comments on the chapter. Work in the Buszczak lab is supported by NIGMS (R35GM144043 and R01GM127569), the Center for Regenerative Science and Medicine, and the Simmons Comprehensive Cancer Center at UT Southwestern.

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Live Imaging of the *Drosophila* Testis Stem Cell Niche

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Abstract

Live imaging of adult tissue stem cell niches provides key insights into the dynamic behavior of stem cells, their differentiating progeny, and their neighboring support cells, but few niches are amenable to this approach. Here, we discuss a technique for long-term live imaging of the *Drosophila* testis stem cell niche. Culturing whole testes *ex vivo* for up to 18 h allows for tracking of cell-type-specific behaviors under normal and various chemically or genetically modified conditions. Fixing and staining tissues after live imaging allows for the molecular confirmation of cell identity and behavior. By using live imaging in intact niches, we can better uncover the cellular and molecular mechanisms that regulate stem cell function *in vivo*.

Key words *Drosophila* testis, Niche, Stem cell, Fluorescence, Time-lapse live-imaging, Confocal microscopy

1 Introduction

Adult stem cells both self-renew and differentiate in order to regulate proper tissue maintenance. While the analysis of fixed tissues provides important information regarding adult stem cell biology, live imaging of stem cells within an intact local microenvironment, or niche, reveals unique insights into stem cell behavior that cannot always be extrapolated from fixed images. For example, live imaging of the *Drosophila* testis niche has shown that although stem cells primarily undergo asymmetric divisions where one daughter cell remains a stem cell while the other daughter goes on to differentiate, germline stem cells can self-renew via symmetric renewal (in which two stem cells are made) and dedifferentiation (in which differentiated daughters migrate back to the niche and revert to stem cells) [1]. Spermatogonial fragmentation has since been shown to occur in mammalian testes using live imaging and lineage tracing [2]. In addition, the use of live imaging in combination with lineage tracing has revealed how niche cells within the

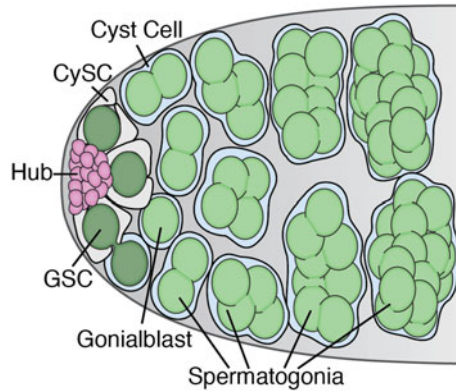


Fig. 1 The *Drosophila* testis stem cell niche consists of terminally differentiated quiescent hub cells (magenta) that signal to the surrounding germline stem cells (GSCs, dark green) and somatic cyst stem cells (CySCs, white). GSCs divide asymmetrically to produce daughter cells that transit amplify to form spermatogonial clusters (light green). These clusters undergo meiosis and differentiate into sperm. CySCs also divide asymmetrically to produce daughter cyst cells (light blue). Two cyst cells encase each spermatogonial cluster and are required for the differentiation process

testis can lose their identity and transdifferentiate into somatic stem cells upon loss of Rbf [3]. These findings thus underscore the importance of live imaging and the benefits this technique has to offer.

The well-characterized *Drosophila* testis stem cell niche provides an ideal model system to study stem cell behavior and function [4] (Fig. 1). It supports two types of stem cells, germline stem cells (GSCs) and somatic cyst stem cells (CySCs), which attach to a group of terminally differentiated hub cells that secrete ligands promoting stem cell fate. GSCs divide asymmetrically to produce a differentiating daughter (or gonialblast) that undergoes transit-amplifying divisions to form a cluster of interconnected spermatogonia. Spermatogonia subsequently enter meiosis and spermiogenesis, eventually forming sperm. CySCs also divide asymmetrically and produce daughters called cyst cells that encapsulate the germline and elongate with the growing spermatogonial clusters. The CySCs and their progeny are essential for the survival and proper differentiation of the germline [4].

Previous work in the *Drosophila* ovary provided the groundwork for live imaging in *Drosophila* gonadal tissues [5, 6] including extended live imaging (14 h) of the ovarian stem cell niche [7]. In addition, protocols for imaging embryonic testes [8, 9], adult testis tips [10], whole-mounted testes on slides [1, 11], testes covered by a membrane [12], or testes embedded in agarose [13] have been established. Here we describe a method for imaging the stem cell niche of whole *Drosophila* adult testes using glass bottom imaging

dishes coated with poly-L-lysine. This technique provides several advantages: (1) it allows for extended live imaging for up to 18 h, (2) it allows for the manipulation of imaging media so that chemicals such as inhibitors can be added and removed, and (3) it provides the capacity to fix and stain testes after long-term imaging. This protocol is updated from that published by Sheng and Matunis (2011) and Greenspan and Matunis (2017) with new adaptations developed by our lab and the DiNardo lab [3, 14, 15].

2 Materials

Prepare all solutions using ultrapure water (sensitivity 16–18 M Ω -cm dH₂O) and molecular grade reagents unless specified otherwise.

2.1 Dissection of Testes From Adult Male *Drosophila*

1. Flies expressing fluorescent reporters or proteins in cells of interest (*see Note 1*).
2. Dumont #5 forceps, blunt and fine-tipped pairs.
3. Dissecting dish: Beveled edge watch glass lined with silicone to protect forceps from damage (*see Note 2*).
4. 9" glass Pasteur pipettes.
5. Stereomicroscope (e.g., Zeiss Stemi SV 6 with a Schott-Fostec external light source).
6. CO₂ pad for anesthetizing flies.
7. Round #2 camel hair paintbrush with most of the hair removed.
8. 1 \times Becker Ringer's solution [16]: 111 mM NaCl, 1.88 mM KCl, 64 μ M NaH₂PO₄, 816 μ M CaCl₂, 2.38 mM NaHCO₃. To make a 10 \times stock solution combine 220 mL of 5 M NaCl, 18.8 mL of 1 M KCl, 3.2 mL of 0.2 M NaH₂PO₄ \cdot 2H₂O, 8.16 mL of 1 M CaCl₂ \cdot 2H₂O, and 500 mL of ultrapure water in a 1 L graduated cylinder. Add 2 g of NaHCO₃ last to prevent precipitation. Add ultrapure water to a final volume of 1 L. Cover graduated cylinder with parafilm and invert 5 times to mix. Filter-sterilize through a 0.1 μ m PES bottle-tip filter into an autoclaved 1 L glass bottle. Solution keeps many months at room temperature if the bottle is tightly sealed (wrap cap with parafilm). Dilute 1:10 with ultrapure water for 1 \times Becker Ringer's solution, store at room temperature.

2.2 Live Imaging of *Drosophila* Testes

1. Insulin stock solution: Dissolve powdered bovine pancreas insulin in acidified water (1 μ L concentrated HCl + 1 mL ultrapure water) at a final concentration of 10 mg/mL. Store 50 μ L aliquots in 1.5 mL microcentrifuge tubes at -20° C.

2. *Drosophila* tissue culture media: 15% fetal bovine serum (v/v), 0.5× penicillin/streptomycin in Schneider's media, pH 7 or Shield and Sang's M3 media. Adjust Schneider's media to pH 7, sterilize using a 0.1 µm PES syringe filter (this can be stored for many months at 4 °C). Combine 4225 µL of pH 7 Schneider's media (or Shield and Sang's M3 media), 750 µL of fetal bovine serum and 25 µL of 100× penicillin/streptomycin (10,000 U/mL of penicillin and 10,000 µg/mL streptomycin in 10 mM citrate buffer) in 50 mL sterile conical tube. Vortex briefly, then filter sterilize the solution using a 10 mL syringe with a 0.2 µm filter. Store at 4 °C and make fresh weekly.
3. Live imaging solution: 0.2 mg/mL insulin in *Drosophila* tissue culture media. Add 20 µL of the 10 mg/mL insulin stock solution to 1 mL of *Drosophila* tissue culture media. Make fresh on the day of imaging (*see Note 3*).
4. Poly-L-lysine: 1 mg/mL in 0.1 M Trizma buffer pH 8.5. Dissolve 10 mg of Poly-L-lysine in 10 mL 0.1 M filter-sterilized Trizma buffer pH 8.5. Store 200 µL aliquots in 1.5 mL microcentrifuge tubes at -20 °C.
5. Imaging dish: 35-mm glass-bottom Petri dish with 10-mm microwell.
6. Laser scanning or spinning disc confocal microscope (e.g., Zeiss LSM 780 confocal microscope with Zen software or a similar microscope).

2.3 Immunostaining Testes After Live Imaging

1. 9" and 5.75" glass Pasteur pipettes.
2. 1× Phosphate Buffered Saline (PBS): 137 mM NaCl, 2.7 mM KCl, 1.5 mM KPO₄ monobasic, 8.1 mM NaPO₄ dibasic anhydrous. To make 10× PBS combine 80 g NaCl, 2 g KCl, 2 g KPO₄ monobasic, 11.44 g NaPO₄ dibasic anhydrous, and 700 mL of ultrapure water in a 2 L beaker and stir with a magnetic stir bar. When dissolved, transfer solution to a 1 L graduated cylinder and add ultrapure water to a final volume of 1 L. Filter sterilize (PES bottle-tip filter, 0.1 µm pore size) into a 1 L autoclaved glass container and store at room temperature or at 4 °C. Dilute 1:10 with ultrapure water to make 1× PBS.
3. 1× Phosphate Buffered Saline with 0.1% Triton X-100 (v/v) (PBX): Add 1 mL of Triton X-100 to a 2 L beaker containing 1 L 1× PBS. Stir with a magnetic stir bar for 20 min. Store in 1 L glass bottle at room temperature or 4 °C.
4. Fixative solution: 4% paraformaldehyde (v/v) in PBX. Dilute 16% paraformaldehyde (open a fresh ampule each week) in PBX to a final concentration of 4%. Make fresh each time.
5. Block solution: 3% BSA (w/v), 0.02% NaN₃ (w/v) in PBX. Add 15 g bovine serum albumin (BSA) to a 1 L beaker

containing 500 mL PBX. Mix with a magnetic stir bar until dissolved. Add 500 μ L 20% sodium azide, filter sterilize (PES filter, 0.1 μ m pore size), and store at 4 °C. This solution can be used for several months.

6. Normal goat serum. Store 300 μ L aliquots in 1.5 mL microcentrifuge tubes at -20 °C. Working aliquot can be stored at 4 °C.
7. Primary antibodies.
8. Secondary antibodies with conjugated fluorophores.
9. Vectashield mounting medium.

3 Methods

All methods are performed at room temperature unless stated otherwise.

3.1 Preparing Imaging Dish

1. Thaw 200 μ L aliquot of 1 mg/mL Poly-L-Lysine and pipette onto the coverslip portion of the imaging dish.
2. Cover dish and incubate at room temperature on the bench for at least 1 h.
3. Remove Poly-L-Lysine from coverslip with a micropipettor and return to the original tube. Poly-L-Lysine can be stored at 4 °C and re-used for 1–2 weeks.
4. Wash coverslip: Add 200 μ L of sterile distilled water to coverslip, pipette up and down 3 times, and discard. Repeat 3 times.
5. Pipette 200 μ L of sterile distilled water onto coverslip, replace the cover on the dish and keep at room temperature while dissecting testes. Leave water on the coverslip until ready to directly transfer testes.

3.2 Dissecting and Mounting Whole Testes

1. Clean all forceps, dissecting dishes, CO₂ pad, and dissecting area with ethanol before dissecting.
2. Anesthetize adult flies using CO₂. Using the stereomicroscope and the paintbrush, collect 20–50 males to be dissected.
3. Pour approximately 20 mL 1 \times Becker Ringer's solution into dissecting dish.
4. Hold the blunter pair of forceps in your non-dominant hand, and use them to grasp a fly by the thorax. Anchor fly to the bottom of the dissecting dish so that it is completely submerged in Becker Ringer's solution. Without releasing the fly, use the finer pair of forceps in your dominant hand to gently puncture the cuticle near the middle of the fly's abdomen to partially release the testes into the media. Grasp the posterior

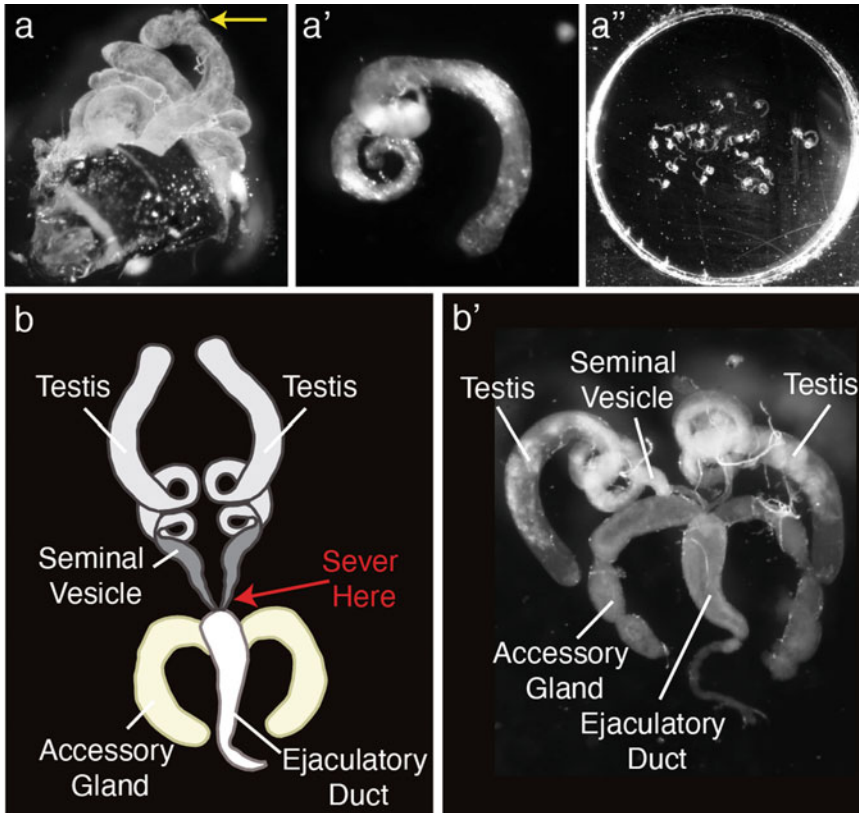


Fig. 2 Testes dissected from an adult male *Drosophila* are shown attached to the cuticle (**a**) completely detached from the cuticle, accessory glands, and ejaculatory duct (**a'**) and mounted on the circular coverslip portion of an imaging dish (**a''**). Yellow arrow (**a**) indicates a rupture in one of the testes, making it unfit for imaging. Mounted testes (**a''**) are flat on the coverslip and do not overlap. Diagram (**b**) and image (**b'**) of *Drosophila* testes, accessory glands, and ejaculatory duct. Testes with seminal vesicles attached should be separated from the accessory glands and ejaculatory duct (red arrow) prior to imaging

cuticle that contains the testes and associated structures, and gently pull until it separates from the rest of the fly (Fig. 2a). Anchor the posterior cuticle to the bottom of the dish using the blunter pair of forceps and gently use one prong of the finer pair of forceps to dissociate the testes from the cuticle. Separate the testes from the accessory glands and ejaculatory duct but not the seminal vesicle, by severing the connection between the seminal vesicle and the accessory gland (*see* **NOTE 4**) (Fig. 2a', b, b'). Testes can be left in the Becker Ringer's solution in the dissecting dish until all testes are dissected.

5. Once finished dissecting, discard any ruptured testes (Fig. 2a).
6. Coat 9" Pasteur pipette with 1× Becker Ringer's solution by pipetting up and down.

7. Remove all the water from imaging dish using a micropipettor. Immediately transfer testes from the dissecting dish to the coverslip portion of the imaging dish using the coated Pasteur pipette (*see Note 5*).
8. Using the tip of the blunter pair of forceps, carefully press testes onto the coverslip so their apical ends adhere and lie flat against the coverslip. There should be no overlap between samples (*see Note 6*) (Fig. 2a").
9. Remove all of the Becker Ringer's solution with a Pasteur pipette. Immediately add back enough fresh Becker Ringer's solution to coat testes, preventing samples from drying out. This will cause testes to strongly adhere to the coverslip allowing them to withstand several media changes if applicable.
10. When ready to image, remove Becker Ringer's solution and immediately add live imaging solution using a micropipettor (*see Note 7*).

3.3 Overnight Time-Lapse Imaging of the Testis Niche

Imaging instructions are based on using a Zeiss LSM 780 confocal microscope with Zen software.

1. Mount covered imaging dish onto movable stage of the microscope (*see Note 8*).
2. Use a 60 \times objective oil lens that will be in contact with the coverslip. For a wider field of view, a 40 \times objective oil lens can be used.
3. Locate testes using bright field or fluorescent channels (*see Note 9*). Adjust the laser power, exposure time, and gain for each wavelength used in order to obtain an optimal signal without photobleaching. This will differ depending on the microscope and fluorophore used. The more fluorophores imaged, the greater the chance for photobleaching.
4. Evaluate the fluorescent signal for each testis and select about 7 that are favorable for imaging (*see Note 10*). Save the position of those selected to image at the center plane of the Z range.
5. Once each testis position is selected, set the Z stack to range around the center of the field of interest. Typically, a 30 μm range with 1.25 μm steps will suffice.
6. Set the total time for imaging testes and the time between each scan. Typically, 25-min intervals over 18 h will be enough to follow cellular behaviors (*see Note 11*).
7. Germline divisions should be seen throughout the entire movie indicating viability of the tissue during the imaging period (Fig. 3a). Nuclear markers can be used to detect cell death since DNA will condense causing bright dense signals. While

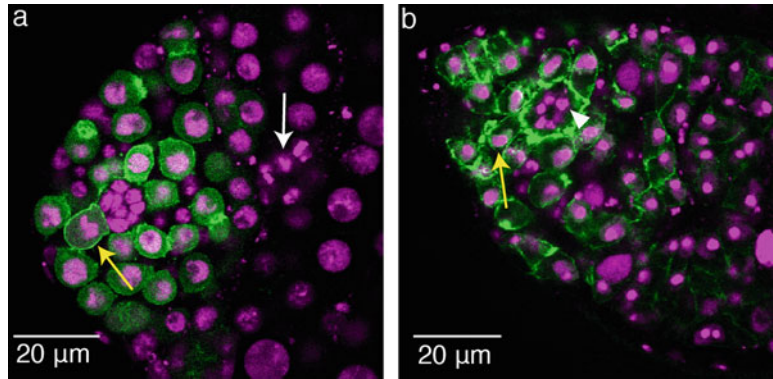


Fig. 3 Single time point images from overnight movies of a viable (a) and non-viable (b) testis expressing histone-RFP (magenta) and germline-GFP (green). (a) Viable testes show germline divisions throughout the imaging period (yellow arrow) with some cell death seen in spermatogonia (white arrow). (b) In non-viable testes, cell death is seen in most cells including germline stem cells (yellow arrow) and hub cells (white arrowhead). Dying cells can be detected through the condensation of nuclei as indicated by the dense magenta signal from the histone-RFP marker

some spermatogonial death is expected to occur in wild-type testes (Fig. 3a) [17], massive amounts of cell death, especially stem cell and hub cell death which normally does not occur in wild-type testes [18, 19], is indicative of a non-viable tissue (Fig. 3b).

3.4 *Immuno-localization After Time-lapse Imaging*

Once live imaging is complete, testes can be fixed and stained directly in the dish for additional markers.

1. Remove all imaging media from the imaging dish using a Pasteur pipette (*see Note 12*).
2. Quickly add 600 μL of fixative solution onto the coverslip portion of the imaging dish. Incubate at room temperature for 22 min.
3. Remove formaldehyde with Pasteur pipette and discard. Rinse testes twice briefly with 1 mL PBX.
4. Wash testes 3 times for 10 min each with 1 mL PBX and allow the dish to remain stationary at room temperature. Keep the dish covered to minimize evaporation.
5. Remove PBX and add 500 μL of 2% Normal goat serum in block solution to the imaging dish. Testes should remain covered and stationary for at least 1 h at room temperature or overnight at 4 $^{\circ}\text{C}$ to reduce any non-specific antibody binding (*see Note 13*).

6. Remove Normal goat serum in block solution and add 500 μ L of primary antibody diluted in block solution to an empirically determined working concentration. Incubate dish for 1–2 h at room temperature or overnight at 4 °C. Keep dish covered and stationary during incubation.
7. Remove primary antibody with a Pasteur pipette and quickly add 1 mL PBX (*see* **Note 14**).
8. Rinse twice with 1 mL PBX then wash 3 times for 10 min with 1 mL PBX as in **steps 3** and **4**.
9. Remove PBX, and add 500 μ L of secondary antibody diluted in block solution to an empirically determined working concentration. Keep imaging dish covered and stationary and incubate dish for 1–4 h at room temperature or overnight at 4 °C (*see* **Note 15**). Place dish inside a foil-covered box to protect samples from light during the secondary antibody incubation and **ALL** subsequent steps.
10. Remove secondary antibody solution and rinse twice briefly, then twice for 10 min with 1 mL PBX.
11. Remove PBX then rinse once briefly and once for 10 min with 1 mL PBS.
12. Remove PBS and immediately cover testes with a drop of Vectashield.
13. Store covered imaging dish with fixed and stained testes on a horizontal surface in a foil-covered box at –20 °C until ready to image.
14. Image fixed and stained testes with the same microscope used for live imaging, if possible, to eliminate variation between instruments.
15. Using the map of testes' locations (or tile scan), locate testes that were previously imaged live. It is helpful to compare the last frame of the movie to fixed testes to ensure they correspond (Fig. 4).

4 Notes

1. Not all fluorescent reporters or fluorescently tagged proteins are strong enough for live imaging. Those that work well in adult testes include but are not limited to: nos::Moe-GFP to visualize the germline (from R. Lehmann) [20], His2Av-RFP to visualize nuclei of all cells (Bloomington Stock Center), G-TRACE reporter for lineage tracing (Bloomington Stock Center) [21], and FUCCI reporter for cell cycle analysis (Bloomington Stock Center) [22]. Flies should be raised in incubators at constant temperature (18 °C or 25 °C) and humidity

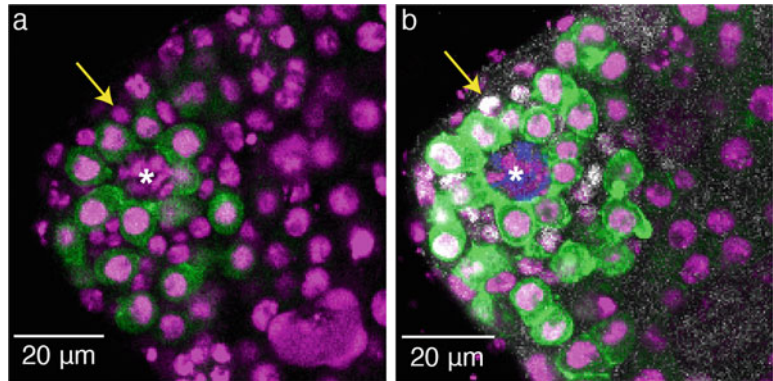


Fig. 4 Comparison of a live (a) and fixed (b) testis after 12.5 h of imaging. Staining after imaging allows for the use of cell-specific markers. Cells tracked live that are histone-RFP positive (magenta), germline-GFP negative (non-green), and close to the hub can be accurately identified as CySCs through ZFH1 staining (white) (compare a and b yellow arrow). Hub cells (white asterisk) are encircled by the germline stem cells, but can also be identified by Fasciclin 3 staining (b, blue)

(65%), and shifted to the appropriate temperatures (29 °C or 31 °C) prior to dissection if applicable to the experiment.

2. To make lined dissecting dishes, add 9 mL of elastomer base and 1 mL curing agent from a Sylgard 184 Silicone Elastomer Kit (Dow Corning) to a disposable 15 mL conical tube. Add a pinch of 100–400 mesh activated charcoal powder (Sigma Aldrich) and invert gently to mix. Continue to add charcoal until the elastomer is opaque, and then pour solution into a clean beveled edge watch glass. Remove bubbles from elastomer surface with a Pasteur pipette. Place dish in a covered container and cure overnight at room temperature.
3. Dyes such as MitoTracker Red CMXRos (1 μM) (Molecular Probes) can be added to the imaging media as well as various drugs such as the Rok inhibitor Y-27632 (380 μM) [15]. Test toxicity of new dyes or drugs to the tissue over a 18-h period prior to use in live imaging.
4. To separate the testis from the cuticle use both prongs of the forceps to pull the testis off the accessory gland or place one prong of the forceps between the spiral of the testis (careful to not puncture the testis) and pull with the anchor hand. Testes should also be separated from each other.
5. If there is excess debris in the media following dissection, testes can be gently transferred to a separate dissecting dish containing fresh Becker Ringer's solution, rinsed a few times with fresh Becker Ringer's solution, and then transferred to the imaging dish. Try to minimize handling of testes and number of transfers as this could damage the tissue.

6. Testes will loosely adhere to the coverslip portion of the imaging dish due to the Poly-L-Lysine. If repositioning of testes is necessary, be extremely gentle since too much force can damage the tissue.
7. 1 mL of media should be sufficient to cover the base of the dish completely coating the testes and ensuring they will not dry out overnight. Make sure to cover the dish with a lid before imaging to prevent any media evaporation. If desired, incubate testes in media containing dye or drug prior or during imaging.
8. Using a microscope fitted with an environmental chamber is desirable to further stabilize the dish on the stage. Utilizing the heat or humidity controls is optional.
9. Using a pen and paper, map the location of each testis on the imaging dish prior to mounting the dish on the microscope. Alternatively, create a tiled image using the 10× objective to get an overview of where each testis is located. This helps to ensure that each testis is evaluated before choosing which to image.
10. Select testes in which the fluorescent signal is strong, indicating the field of interest is closest to the coverslip, and there is little movement due to muscle contractions in order to prevent blurred images. If using multiple fluorophores, ensure all have a strong signal.
11. The interval between scans should allow for enough time for each testis to be imaged before the next cycle begins. On a LSM 780 microscope, 7–8 testes imaged with GFP and RFP with 30 μm stacks take approximately 15 min to image. This allows for 10 min between time points that the testes are not being exposed to the laser thus helping to maintain tissue viability and minimize photobleaching. If a shorter interval is desired, then the amount of testes being imaged during a single session may need to be reduced.
12. It is easiest to remove solutions from the dish using a long 9" Pasteur pipette and to add in fresh solutions when washing testes using a shorter 5.75" Pasteur pipette. Position pipette so that it isn't directly over any testes since too much suction can cause testes to dissociate from the coverslip.
13. If evaporation of the solution during overnight incubations is a concern, covered dishes can be kept in a sealed container containing a wet Kimwipe.
14. Many primary antibodies can be saved, stored at 4 °C and reused.
15. A nuclear counterstain such as 4'-6-diamidino-2-phenylindole (DAPI) can be added to the secondary antibody solution at a final concentration of 1 μg/mL.

Acknowledgments

The authors thank Steve DiNardo and Kari Lenhart for sharing adaptations to the protocol, and Margaret de Cuevas and Miriam Akeju for comments on the manuscript. This work was supported by NIH Grants GM136665 and HD052937 (EM). The Zeiss LSM 780 confocal microscope used in this study was funded by NIH Grant S10 OD016374. LJG was supported by T32 GM007445 and F31HD085748.

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Enrichment of Undifferentiated Germline and Somatic Cells from *Drosophila* Testes

Sharif M. Ridwan, Matthew Antel, and Mayu Inaba

Abstract

The *Drosophila* male germline provides a strong model system to understand numerous developmental and cell-biological processes, owing to a well-defined anatomy and cell type markers in combination with various genetic tools available for the *Drosophila* system. A major weakness of this system has been the difficulty of approaches for obtaining material for biochemical assays, proteomics, and genomic or transcriptomic profiling due to small-size and complex tissues. However, the recent development of techniques has started allowing us the usage of a low amount of material for these analyses and now we can strategize many new experiments. The method for enrichment or isolation of rare populations of cells is still challenging and should meaningfully influence the reliability of the results. Here, we provide our semi-optimized protocol of enrichment of undifferentiated germ cells and somatic cells from non-tumorous *Drosophila* testis, which we have successfully improved after multiple trials.

Key words *Drosophila* testis, Flow cytometry and cell sorting

1 Introduction

The *Drosophila* male gonad is an extremely advantaged system for whole-mount tissue imaging strategies, including IF (immunofluorescence), FISH (fluorescence in situ hybridization), and whole-mount live imaging, in which we can analyze the behavior of cells, organelles, and proteins in single-cell or sub-cellular levels in an in vivo setting. However, it has been challenging to use cells for biochemical assays, proteomics, genomic or transcriptomic profiling approaches due to the difficulty of isolation of rare populations of cells and collecting enough material. Recent advances in techniques, such as amplification techniques and multiplex labeling of DNA and proteins, enable various platforms for single-cell analysis and provide high-throughput single-cell information over thousands of cells. Moreover, recently developed enzyme-tethering strategies have dramatically improved chromatin mapping methods

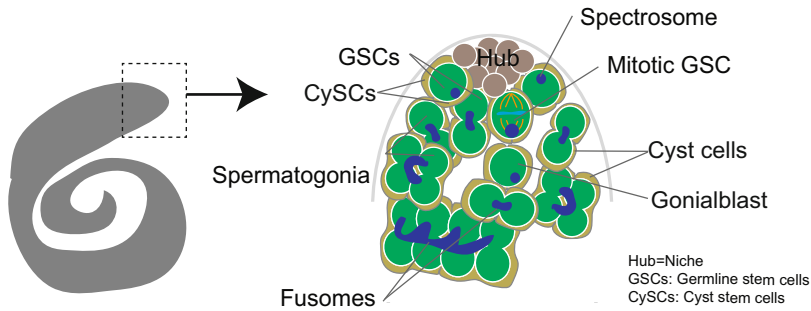


Fig. 1 Left; the *Drosophila* testis. Right panel shows schematic of the apical tip of a *Drosophila* testis (the squared portion of left diagram)

for the usage of a small number of unfixed cells [1]. Now, we are able to reconsider the methodology to apply these techniques to *Drosophila* testes. Nevertheless, challenges still remain for single-cell analyses including uncertain identification of true single cells, and technical variation of sample preparation [2]. Undoubtedly, a reliable method for cell isolation/enrichment is a top priority for successfully obtaining highly reproducible results.

In the *Drosophila* adult testis, the stem cell niche, called the hub, consists of a cluster of somatic hub cells and is located at the apical end of the testis [3, 4]. 8–11 germline stem cells (GSCs) and somatic cyst stem cells (CySCs) both attach to the hub, and GSCs divide asymmetrically to generate one daughter cell, a future GSC, that remains attached to the hub, and another daughter cell, a gonialblast (GB), which is displaced away from the hub and differentiates (Fig. 1) [4–6]. GBs then enter four rounds of transit-amplifying divisions to form spermatogonia (SGs). In these divisions, cytokinesis is incomplete and SGs remain connected by intercellular structures called ring canals and fusomes [7–9]. CySCs give rise to somatic cyst cells (CCs) and two CCs encapsulate each SG cyst. The two CCs that encapsulate SG cyst continue to grow throughout the rest of spermatogenesis. 16-cell SGs become spermatocytes and enter two meiotic divisions, then become mature sperm [7, 8]. Cells in all stages of spermatogenesis exist in the same testis and are aligned in a developmentally ordered manner from the apical tip to the distal end [3, 8].

Although dissection of testes from thousands of flies surely allows one to obtain a certain amount of protein/RNA/DNA; these are from mixed cell populations. To isolate cells or obtain material from specific cell types, several strategies have been used. Below, we describe the previous methods that have been used for *Drosophila* male and female gonad cell type isolation.

1.1 Immunoprecipitation Using Cell Type-Specific Expression of Tagged Proteins

Genetic marking of cells or proteins using the Gal4-UAS system [10] or gene-trap (GFP-trap) [11, 12] can be used for expressing GFP-tagged or epitope-tagged proteins in specific cell types. The temperature-inducible Gal4/Gal80^{ts} system [13] is useful for expressing transgenes temporally in a temperature-dependent manner so that one can avoid indirect effects of transgenes. Immunoprecipitation (IP) of whole tissue lysates allows for the isolation of interactions of protein-protein, protein-RNA, and protein-chromatin occurring within particular cell types. Samples can be used for mass spectrometry [14], RNA-IP (RIP) [14, 15], and Chromatin-IP (ChIP) [16]. If the cells expressing tagged transgenes are rare, or expression of the transgene is low, it may cause high background and unreliable results. Therefore, tumor induction for enrichment of certain cell populations in the testis is often utilized.

1.2 Labeling of Protein or RNA Using Cell-Type-Specific Expression of Enzymes

Cell-type-specific drivers can be used for proximity-based assays. The expression of proteins tagged with enzymes such as the ascorbate peroxidase APEX2 to label protein or RNA [17, 18] has been applied for *Drosophila* gonads [19]. Chemical treatment is often used for labeling steps and may create artificial effects.

1.3 Usage of Mutant Gonad for Enrichment of Specific Cell Types

Usage of mutant gonads enriched with GSC-like cells or cysts has been frequently used for RNAseq, ChIP-seq, and proteomics analyses [16, 20–23]. Male GSC-like tumors can be induced by using a loss-of-function mutant of *bag-of-marbles* (*bam*) [24], a differentiation factor, or ectopic expression of niche factors, such as Decapentaplegic (Dpp) or Unpaired (Upd) [6, 25–29]. Specific stages of cysts can be induced in the female gonad [21]. Manipulating the timing of transgene expression can be used to enrich precise stages of cyst development [30]. Mutant gonads are often used in combination with genetic marking systems or cell sorting methods. However, the conditions of tumorous gonads may be different from physiological conditions, and thus the results need to be confirmed by using more physiological conditions.

1.4 Hand Picking of Specific Cell Types

Cyst hand-picking using microneedles was used for deep-sequencing analysis to avoid the issues seen in the above methods [31]. Cells in physiological conditions can be obtained but it requires high technical skills and large efforts of dissection, producing only a small amount of material, which limits the following applications.

1.5 Flow Cytometry and Cell Sorting

Flow cytometry and cell sorting are often used for RNA analysis (RT-PCR, qRT-PCR, or RNA seq), both for testes and ovaries [32–36], and for ChIPseq [32]. Single-cell analysis platforms were also applied for sorted cells from testes [33, 34] and ovaries [32, 35]. Flow cytometry and cell sorting do not require chemical

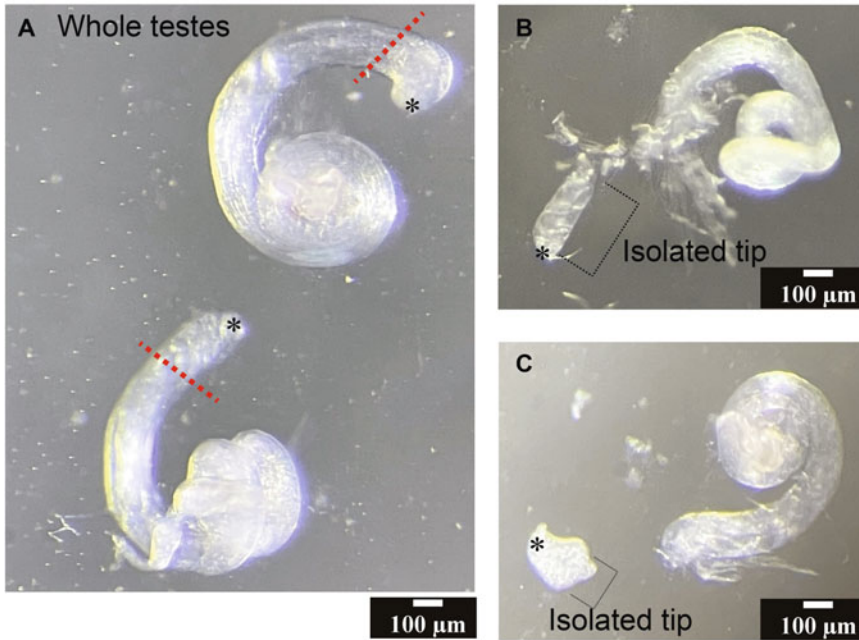


Fig. 2 Representative images of whole testes taken under the stereoscope (**a**) and testes with isolated tips (**b-c**). Red broken lines in (**a**) indicate the portion to be excised. Asterisks indicate approximate location of the hub

treatment and difficult technical skills and should be a relatively easy and reliable method that can be conducted by inexperienced researchers. However, due to the difficulty of obtaining enough numbers of cells, again, a mutant background is often utilized [35, 36].

Here, we provide a semi-optimized method for cell sorting from *Drosophila* testis and an example of the results. We have attempted to improve the method to sort early stages of germ cells (expressing histone H3-GFP [37] under the control of the nosGal4 driver [38]) or CySCs and hub cells (Chd64-GFP expressing cells [39]) from adult *Drosophila* testes without amplification of cells by genetically inducing tumors. We attempted to detect GFP-positive germline or CySCs and found that removal of the tail region of the testis is a critical step to detect the population of GFP-positive cells (Fig. 2). Moreover, an improved dissociation method also affects the percentage of GFP-positive cells that can be isolated. Cell size and GFP signal distribution were detected more broadly in the germline than in somatic CySCs, likely representing the large size of interconnected germline cysts compared to single somatic cells (Fig. 2). Therefore, we used the largest diameter of nozzle (130 μm diameter) for cell sorting and expanded the forward and side scatter (FSC/SSC) gate to include the large size of cells. We have successfully obtained cells with the efficiency of

approximately 30–50% of estimated cell number based on microscopic analysis.

This method will be useful for any high-throughput whole-genome approaches combined with new techniques optimized for low amounts of materials, such as enzyme-tethering strategies (Cut&Tag, Cut&Run), in vivo barcoding techniques, and multiple single-cell sequencing platforms.

2 Materials

2.1 Dissection

1. Schneider's insect medium.
2. Dumont #55 forceps.
3. 9 Depression Glass Spot Plates.
4. 1.7 mL microcentrifuge tubes.

2.2 Dissociation

1. Collagenase from *Clostridium histolyticum*.
2. Dispase II.
3. Phosphate buffered saline.
4. Calcium chloride (CaCl₂).
5. Magnesium chloride (MgCl₂).
6. Trypsin-EDTA (0.05%), phenol red.
7. Fetal bovine serum.
8. Metal mesh 125 µm pore size.
9. 29G insulin syringe.
10. Swinging bucket-rotor centrifuge.

2.3 Flow Cytometry and Cell Sorting

1. Collection buffer: 1X PBS, 1% Fetal bovine serum, 1 mM EDTA.
2. Dissociation buffer: 1X PBS, 2 mg/mL collagenase, 4 mg/mL dispase II, 0.5 mM MgCl₂, 0.5 mM CaCl₂ (Prepared fresh when needed).
3. Collection tube (1.7 mL microcentrifuge tubes).
4. Flow cytometry instrument.

3 Methods

3.1 Fly Culture

Flies are raised on standard Bloomington medium at 25 °C in non-overcrowded bottles and dissected 0–7 days post-eclosion. For labeling of somatic CySCs, Chd64-GFP (CB03690, FlyTrap Project [11, 40]) can be used. nosGal4 [38] and UAS-histone H3-GFP [37] flies were used for labeling the early-stage germline.

3.2 Dissection of Testes (see Note 1)

1. Dissect testes directly into ice-cold Schneider's insect medium supplemented with 10% FBS in a glass spot plate.
2. In the spot plate, carefully excise the coiled end of each testis using shape forceps; discard the coiled tail region and keep the apical tips (see Note 2, Fig. 2).
3. Collect the apical tips of testes into 1 mL of Schneider's insect medium; keep on ice (see Note 3).

3.3 Dissociation of Cells (see Notes 4–7)

1. Transfer testes tips into tubes.
2. Incubate tubes at room temperature for 5 minutes to allow tissue to settle and carefully remove supernatant.
3. Add 500 μ L of the freshly prepared dissociation buffer.
4. Incubate samples at 37 °C for 30 minutes; tap the tube every 5 minutes to aid in dissociation.
5. Centrifuge the samples at 600 x g for 5 minutes in a swinging bucket-rotor centrifuge; remove supernatant.
6. Add 200 μ L of trypsin (0.05% Trypsin/EDTA).
7. Incubate samples at 37 °C for 15 minutes; tap the tube every 5 minutes to aid in dissociation.
8. Pipet the samples up and down using a 29G insulin syringe to dissociate the cells.
9. Add 500 μ L of collection buffer.
10. Centrifuge at 600 x g for 5 minutes in a swinging bucket-rotor centrifuge.
11. Resuspend samples in 500 μ L of 1% FBS, 1 mM EDTA in PBS.
12. Pipet the samples through 125 μ m metal mesh into 5 mL FACS tube into 1% FBS, 1 mM EDTA in PBS.

3.4 Flow Cytometry and Cell Sorting

1. Set-up cell sorter with a large diameter (130 μ m) nozzle (see Note 8).
2. Sort the cells using 4-way precision purity mode. Use cells collected from wild-type tests for negative control. Expand singlet gating on FSC/SSC plot to not miss connected cells (Fig. 3a, b). GFP-positive cell populations should be gated according to negative control (see Fig. 3, Note 9).
3. Collect sorted cells in a 1.7 mL microcentrifuge tube filled with 0.4 mL of collection buffer (see Note 10).

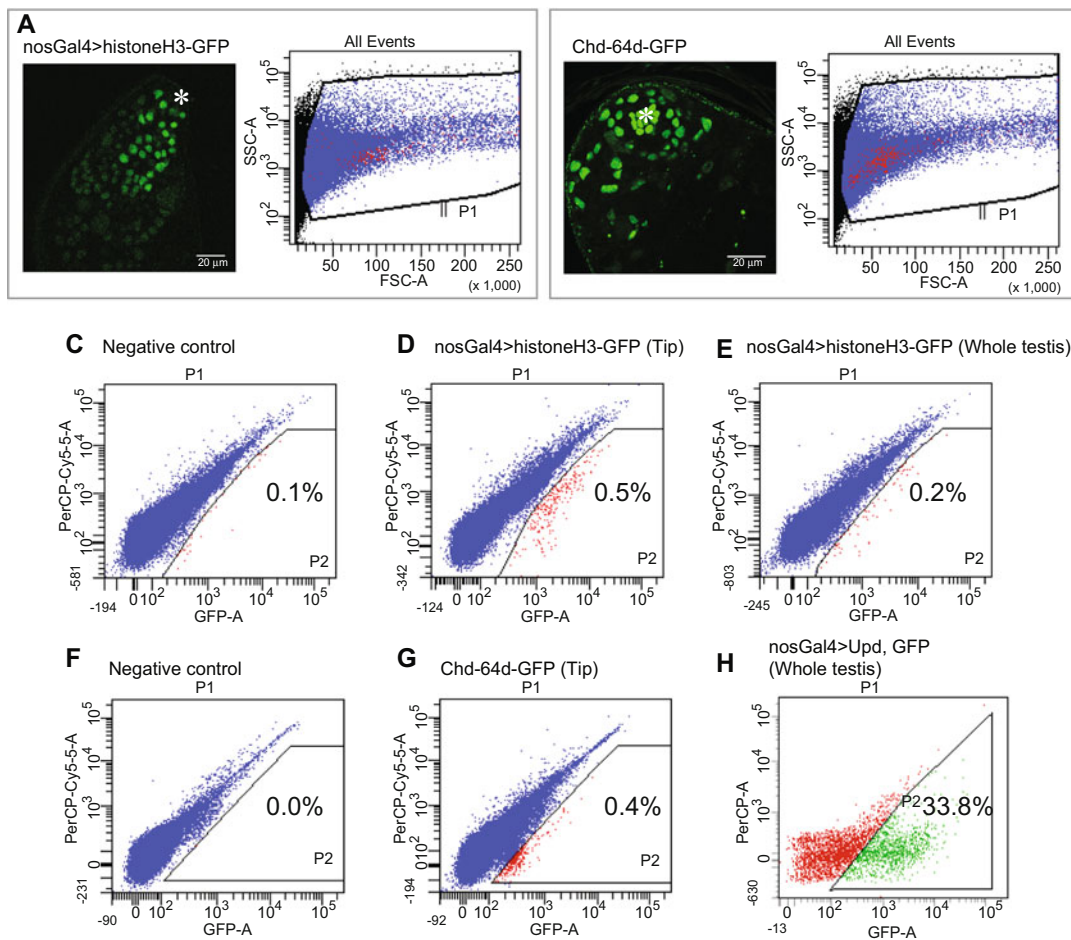


Fig. 3 (a) A representative image of the testis expressing histone H3-GFP under the *nosGal4* driver and the SSC/FSC plot using the tip dissection method. Red dots show the GFP-positive population showing broad distribution of FSC. (b) A representative image of the testis expressing Chd-64d-GFP (CySC and hub-cell marker) and the SSC/FSC plot using the tip dissection method. GFP-positive cells are mostly seen in the singlet population. GFP-positive cell number (counted under the microscope) was 81.1 ± 11.9 ($n = 7$) for (a) and 51.2 ± 12.6 ($n = 6$) for (b), respectively. (c-e) Plots of cells isolated from negative control testis tip (c), *nos* > histone H3-GFP testis tip (d) and *nos* > histone H3-GFP whole testis (e). GFP-positive population is hardly visible in (e, f-g) Plots of cells isolated from the tip of wild-type testis (f) or testes expressing Chd64d-GFP (g). (h) Plot of cells isolated from whole testes enriched with GSC by expressing *Upd* together with GFP. Asterisks indicate the hub. PerCP-Cy5-5-A or PerCP-A: empty channel. Percentages in the P2 gate (c-h) are GFP-positive cells relative to parental gate in FSC/SSC plot (P1, see a, b)

4 Notes

1. Keep samples on ice as much as you can during the entire protocol to avoid artificial gene expression or other undesired cellular processes which may occur during the procedures.
2. We use Dumont#5511255–20 forceps for excision of the testis tip under the regular dissection microscope. We could not

detect GFP-positive populations using the entire testis. Removing the tail portion is critical to increase the percentage of GFP-positive cells induced in the early stages.

3. Dissected testes can be stored overnight on ice without changing the viability of cells.
4. For the volumes of reagents given in this protocol, we dissociate up to 1000 testis tips per reaction or tube.
5. Collagenase/Trypsin mixture has been used frequently for dissociation of *Drosophila* gonads. However, collagenase requires calcium ion for its enzymatic activity but trypsin's activity can be inhibited by the presence of calcium. We found that the best dissociation results are achieved by doing the trypsin digestion step separately. This two-step method did not affect cell viability as we did not detect an increased percentage of dead cells (DAPI-positive cells).
6. Accutase solution (Sigma, A6964) works better instead of trypsin when subsequent steps require immobilization of cells on Concanavalin A (ConA) beads (such as CUT&RUN and CUT&Tag assays). Trypsin disrupts extracellular glycoproteins required for cells to bind ConA beads.
7. Usage of swing-rotor centrifugation is critical to avoid cell loss trapped on the wall of the tube.
8. We use 130 μm nozzle to avoid missing interconnected spermatogonia.
9. We obtain approximately 30,000 GFP-positive germ cells from 1000 of *nosGal4 > histoneH3-GFP* testis. This is equivalent to 30–50% of the estimated number (quantified under the microscope, *see* Fig. 3). Note, the interconnected SG cysts may be counted as a single cell in cell sorter, therefore, the sorted cell number is likely underestimated.
10. If using a plastic tube for collection, it is important to wet the walls and caps of the tube with buffer by tapping the tube or pipetting along the walls; this helps to prevent static interference with collection of the charged droplets containing cells after sorting.
11. We used isolated cells for Cut&Tag analysis [1] using the CUT&Tag-IT Assay Kit (Active motif, Cat#53160) following the manufacture's instruction. This kit is optimized for the usage of as few as 5000 cells. Library quality check result obtained from 5000 or 15,000 cells/sample is shown in Fig. 4.

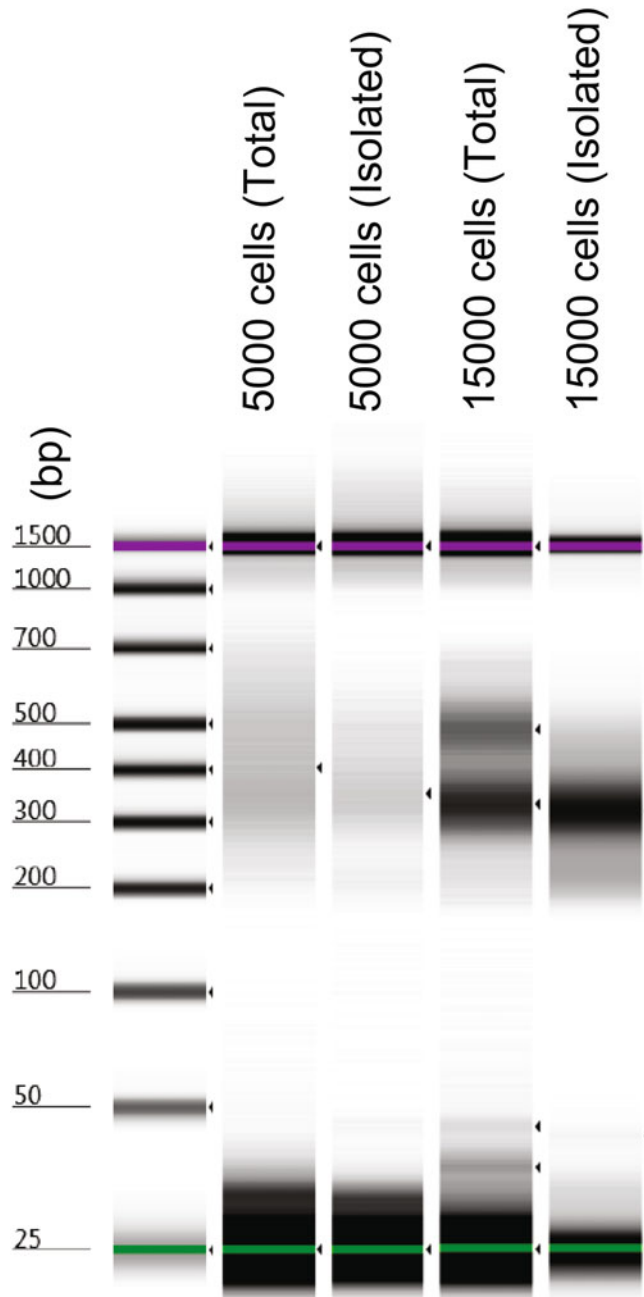


Fig. 4 Library quantification result of Agilent Bioanalyzer. Samples were prepared via CUT&Tag-IT Assay Kit (Active motif, Cat#53160) using Rabbit Histone H3K27me3 antibody (Cat#39055) (mAb) from 5000 or 15,000 total or isolated cells. Final library concentrations were quantified as 9.46 pg/ μ L, 5.65 pg/ μ L, 135.3 pg/ μ L, 495 pg/ μ L respectively (left to right) for 20 μ L final volume

Acknowledgments

We thank Evan Jellison and the Flow Cytometry Core at the UConn Health Center and Hideyuki Oguro for their assistance with cell sorting and helpful discussion and optimization of the sorting protocol, Active motif technical support team for suggestions for tissue dissociation method, Bo Reese and UConn Center for Genome Innovation for library quality check.

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Spermatogonial Dedifferentiation into Germline Stem Cells in *Drosophila* Testes

Salvador C. Herrera  and Erika A. Bach 

Abstract

Stem cell pools are dynamic and capable of reacting to insults like injury and starvation. Recent work has highlighted the key role of dedifferentiation as a conserved mechanism for replenishing stem cell pools after their loss, thereby maintaining tissue homeostasis. The testis of the fruit fly *Drosophila melanogaster* offers a simple but powerful system to study dedifferentiation, the process by which differentiating spermatogonia can revert their fate to become fully functional germline stem cells (GSCs). Dedifferentiated GSCs show interesting characteristics, such as being more proliferative than their wild-type sibling GSCs. To facilitate the study of the cellular and molecular mechanisms underlying the process of germline dedifferentiation in the *Drosophila* testis, here we describe techniques for inducing high rates of dedifferentiation and for unambiguously labeling dedifferentiated GSCs.

Key words Germline, Dedifferentiation, Stem cells, *Drosophila melanogaster*, Testis

1 Introduction

Homeostasis of highly proliferative tissues relies on the robustness of pools of resident tissue stem cells. The depletion of these stem cell pools causes tissue atrophy, and stem cell exhaustion is a hallmark of aging [1, 2]. During the life of the animal, stem cell pools are dynamic, capable of responding to insults like starvation and injury, replenishing depleted pools with new stem cells. Dedifferentiation has proven to be a key mechanism enabling this recovery, where cells on a path to terminal differentiation can revert their trajectory and regain stemness [3].

Germline dedifferentiation has been observed in *Drosophila* gonads after transiently forcing all GSCs to differentiate [4–6]. The *Drosophila* testis in particular is a suitable tissue to study this process, thanks to extensive knowledge about precise anatomical positions and molecular markers of every cell type and every stage of differentiation. In the testis, a pool of 8–14 GSCs resides

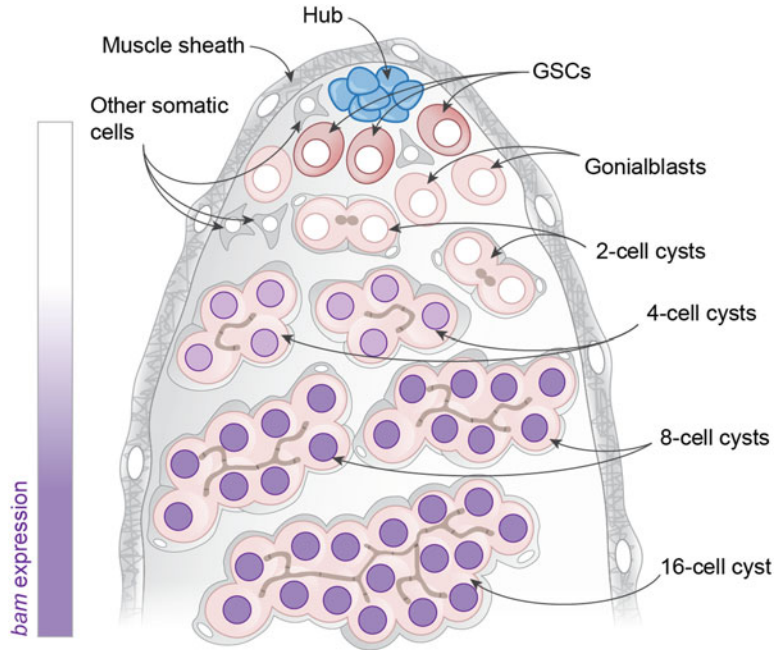


Fig. 1 Diagram of a testis, showing the different cell types and differentiation stages of the spermatogonia, up to the pre-meiotic stage. Germline cells are represented in red, the hub cells in blue, and other somatic cells in grey. The expression domain of the differentiation factor *bam* is shown as purple nuclei

attached to a niche of quiescent cells, termed the “hub” [7]. A GSC undergoes oriented mitosis that results in the production of one daughter GSC and one gonialblast. The latter begins differentiation through four rounds of mitosis with incomplete cytokinesis that results in 2-, 4-, 8-, and 16-cell spermatogonial cysts. The 16-cell cyst then undergoes meiosis and ultimately produces 64 spermatids (*see* Fig. 1).

Dedifferentiation of 4- and 8-cell spermatogonia occurs as a result of aging, mating, and after starvation [8–11]. While not essential to maintain the GSC pool during aging in standard laboratory conditions, dedifferentiation is essential for rapid recovery of the GSC pool after acute stress and for preserving robust spermatogenesis during chronic stress [10]. Furthermore, dedifferentiated GSCs display higher proliferative rates than non-dedifferentiated, “wild type”, sibling GSCs in the same testis [10].

Dedifferentiated GSCs can be monitored by indelibly labeling germline cells that have already started the differentiation process and should otherwise not be present at the stem cell niche. This can be achieved by lineage-tracing spermatogonial cells expressing *bam-Gal4*, a key factor in the germline differentiation that becomes active in 4- and 8-cell spermatogonial cysts [8, 10]. As with other lineage-tracing strategies, this *Gal4* line is used to drive the

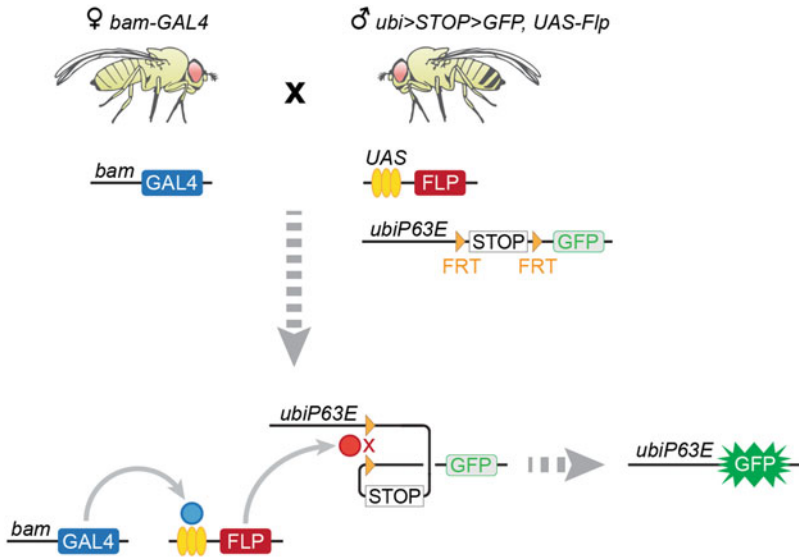


Fig. 2 Genetic methodology and crossing scheme for labeling dedifferentiated GSCs. A female expressing a *bam-Gal4* transgene is mated with a male carrying a *ubiP63E>STOP>GFP* and *UAS-Flp* transgenes. In the F1 progeny, the *bam-Gal4* driver line is used to drive the expression of a *UAS-Flp* recombinase. Flp in turn recombines the *FRT* sites flanking a stop codon on a *ubiP63E>STOP>GFP* cassette. The excision of the codon places *GFP* under the direct control of the ubiquitous *ubiP63E* promoter, thereby indelibly and permanently marking germline cells. The GFP-labeling persists even if these cells dedifferentiate into GSCs, which turn off *bam-Gal4* expression

expression of a recombinase, *UAS-Flp*, which in turn induces the excision of a stop codon flanked by *FRT* recombination sites on a cassette, in our case *ubiP63E-FRT-STOP-FRT-Stinger.GFP* (abbreviated *ubi>STOP>GFP*) [12]. Once recombined, this GFP mark remains under the genetic control of the ubiquitous promoter *ubiP63E* and persists even if the marked cell turns off the *bam* promoter (see Fig. 2). A limitation of this methodology is that it cannot detect cases of dedifferentiation among gonialblasts and 2-cell cysts, as *bam-Gal4* is not expressed at these stages. The technique, however, induces consistent and unambiguous labeling of dedifferentiation events among 4- and 8-cell spermatogonial cysts.

This method can also be modified to block dedifferentiation, and so study the effects of its absence. As the key factor that drives germline differentiation in normal conditions, transient mis-expression of *bam* is sufficient to induce the differentiation of GSCs [6, 13]. By mis-expressing additional Bam protein (with a *UAS-bam* transgene) in these *bam-Gal4*-positive cells, spermatogonia are forced to follow irreversibly the differentiation path, and as a result dedifferentiation rates drop to baseline control levels [10]. Blocking dedifferentiation can be also achieved by inhibiting

the Jun N-terminal kinase pathway, using pathway repressors such as *UAS-puc* or *UAS-bsk^{K53R}* [10].

Here, we describe a genetic methodology for labeling dedifferentiated GSCs, alternative methods for inducing high rates of dedifferentiation, and a detailed procedure for immunostaining testes and distinguishing dedifferentiated GSCs from their non-dedifferentiated siblings.

2 Materials

1. Phosphate-Buffered saline (PBS): NaCl at 137 mM, KCl at 2.7 mM, Na₂HPO₄ at 10 mM, KH₂PO₄ at 1.8 mM. Adjust the pH to 7.4 with HCl.
2. Triton X-100 detergent diluted with dH₂O to a final concentration of 10%.
3. PBS-Triton: PBS solution supplemented with both 0.2% and 0.5% Triton X-100.
4. PBTB: PBS-Triton 0.2% solution supplemented with 1% bovine serum albumin. This blocking solution can be aliquoted and frozen for better preservation (*see Note 1*).
5. Paraformaldehyde (PFA). Dilute a 16% PFA commercial solution in PBS to a final concentration of 4%. This fixative solution can be aliquoted and frozen for better preservation (*see Note 1*).
6. Dumont #5 forceps.
7. Optionally, a thin insect pin mounted on a syringe. Hold a syringe needle and very carefully place it close to/on top of a flame (but not directly inside the flame). Pull the metal needle from the plastic part using forceps when the plastic partially melts. With the plastic still molten, grab an insect pin with forceps and introduce it onto the plastic, replacing the discarded needle. Wait for the plastic to solidify, mount it on a syringe and replace the cap of the needle to protect the pin while not in use.
8. Dissecting microscope.
9. Dissecting plates: a small petri dish coated with a thin bed of Sylgard 184 Silicone Elastomer mixed with Indian ink.
10. 1.5 mL Eppendorf tubes.
11. Microscope slides and coverslips.
12. Vectashield plus DAPI mounting medium (Vector laboratories) (*see Note 9*).
13. Nail polish.

Table 1
Fly stocks used

Fly line	Source	Purpose
<i>Oregon-R</i>	BDSC #2376	Source of virgins for mating
<i>Ubi-p63E(FRT.STOP)stinger, UAS-Flp</i>	BDSC #28282	<i>Ubi>stop>GFP</i> cassette
<i>Bam-Gal4:VP16</i>	[15]	Driver for 4- and 8-cell spermatogonia
<i>UAS-bam:GFP</i>	[15]	Possible control for blocking dedifferentiation
<i>UAS-puc</i>	[16]	Possible control for blocking dedifferentiation
<i>UAS-bsk^{K53R}</i>	BDSC #9311	Possible control for blocking dedifferentiation
<i>UAS-LacZ</i>	BDSC #3955	Control for normal dedifferentiation

14. Primary antibodies: rat anti-Vasa used at a 1:20 concentration (stains the cytoplasm of germline cells) and mouse anti-Fasciclin3 used at 1:50 (stains the membrane of hub cells). Both antibodies can be obtained from the DSHB.
15. Secondary antibodies: donkey secondary antisera from Jackson ImmunoResearch. We recommend anti-rat conjugated with Alexa 555 for revealing Vasa and anti-mouse conjugated with Alexa 647 for Fasciclin3.
16. Fly food vials and plugs, filled with approximately 1 inch of standard fly food.
17. 25 °C Incubator.
18. Sucrose.
19. Agarose.
20. Cheesecloth.
21. Confocal microscope.
22. Scientific imaging software. We suggest Fiji/ImageJ (<https://imagej.net/software/fiji/>) [14].
23. Fly stocks (*see* Table 1).

3 Methods

3.1 Lineage-tracing Genetics and Husbandry

1. If the goal of the experiment is simply to observe dedifferentiated germ cells, the only stocks required will be *bam-Gal4* for virgin females and *ubi>STOP>GFP* for males. If instead the goal is studying factors affecting the dedifferentiation process, we recommend using males of the genotype *UAS-bam; ubi>STOP>GFP* (or *ubi>STOP>GFP; UAS-puc* or *ubi>STOP>GFP; UAS-bsk^{K53R}*) for the positive control

cross (where dedifferentiation will be blocked) and *ubi>STOP>GFP; UAS-LacZ* for the negative control (where a neutral *UAS* line is used to maintain the titration of the Gal4 protein). We can provide these stocks upon request, or they can be built easily through standard genetic crosses. When building these stocks, it is critical to ensure that the *hs-Flp* construct, present in many balancer stocks, does not exist on the final stock, as it would induce spurious recombination of the cassette.

2. Collect virgin females of the *bam-Gal4* stock line and cross them with the males from the stocks indicated in the previous point.
3. Ten days after setting up the crosses, collect twice daily F1 virgin males, keeping them separate from females (*see Note 6*).

3.2 Aging and Stress Assays

The different methodologies presented below will promote high rates of spermatogonial dedifferentiation. Upon normal aging, protein deprivation (commonly referred to as starvation) and mating, dedifferentiated GSCs will comprise approximately 20–25% of the GSC pool. By contrast, after chronic stress, this percentage increases to approximately 45% of the GSC pool. Bear in mind that the methodology involving chronic stress takes longer and requires more work.

If the experiment goal is to study spermatogonial cells during the dedifferentiation process, rather than observing the final result, we recommend the starvation methodology, as the spike of dedifferentiation takes place in a narrow window of time, with a sharp increase of dedifferentiated GSCs between the days 2 and 3 of refeeding [10].

3.3 Normal Aging

1. Place young (preferentially no older than 3 days) adult males into regular food vials, maintaining not more than 20–30 males per vial (*see Note 3*).
2. Flip flies into fresh food vials every two days.
3. Dedifferentiation rates increase with aging [8, 10], but age-related mortality will negatively impact the recovery of flies for dissection, especially when approaching the 60-day mark. In our experience, 45 days of adulthood is a good compromise allowing for fly survival and high levels of dedifferentiation.

3.4 Starvation

1. Prepare starvation food by melting 1% agarose with 10% sucrose in an Erlenmeyer flask. Distribute it in empty fly vials (about 1 inch of food per vial), cover them with a cheesecloth while it solidifies and then plug the vials. The vials can be stored at 4 °C for up to 2 weeks.

2. Place young (preferentially no older than 3 days) adult males on starvation vials, maintaining not more than 20–30 per vial (*see* **Note 3**). Flip them every two days into fresh vials with starvation food.
3. For refeeding, flip the males into vials with regular food. Flip them again every two days into fresh vials with regular food.
4. The maximum loss of GSCs is achieved after 6 days of starvation, while their complete recovery happens after 5 days of refeeding [9, 10]. The shortest protocol to obtain as many dedifferentiated GSCs as possible using this protocol should encompass 11 days, with 6 days of starvation and 5 of refeeding.

3.5 Stress Through Mating

1. Collect *Oregon-R* virgin females during the whole span of the experiment.
2. Place young (preferentially no older than 3 days) adult males into regular food vials together with *Oregon-R* virgin females in a ratio 1:2 (male:female). The total number of flies per vial should not exceed 20–30, *see* **Note 3**.
3. Flip flies into fresh vials every two days.
4. Every 7 days, anesthetize the flies, discard the old females and replace them with fresh virgins.
5. In our experience, good levels of dedifferentiation are achieved after 2–3 weeks.

3.6 Chronic Stress

1. Prepare starvation food as explained in Subheading 3.4, **step 1** and collect *Oregon-R* virgin females during the whole span of the experiment.
2. Place young (preferentially no older than 3 days) adult males on a starvation regime of 6 days, as explained in Subheading 3.4, **step 2**.
3. Refeed the flies by flipping them into a fresh vial of regular food for two days.
4. Transfer flies into a fresh vial of regular food together with *Oregon-R* virgin females in a ratio 1:2 (male:female). The total number of flies per vial should not exceed 20–30, *see* **Note 3**.
5. After two days, anesthetize the flies and discard the females.
6. The previous 4 steps represent a cycle of 10 days. Repeat this cycle 3 more times for a total of 40 days.
7. After the last cycle, transfer the males into a fresh vial of regular food for one additional day, so the last refeeding period is 5 days in total.

3.7 Dissection of Testes, Imaging, and Cell Characterization

1. Dissect flies in PBS on a dissection dish. Use a pair of forceps to tear apart the abdomen cuticle between segments (ideally at the anterior border of the A5, where the strong male pigmentation begins). Pull slowly and gently the posterior part of the abdomen and place it in an Eppendorf tube with PBS.
2. Remove the PBS from the tube and fix for 30 min at room temperature with gentle shaking in 0.5 mL of 4% paraformaldehyde +10 μ L of 10% Triton X-100.
3. Rinse once in PBS and then wash 2×30 min in PBS-Triton 0.5% with gentle shaking.
4. Block in PBTB for one hour with gentle shaking.
5. Incubate overnight in primary antibodies, diluted in 50 μ L of PBS-Triton 0.2%, at 4 °C with gentle shaking.
6. Rinse once in PBS and then wash 2×30 min in PBS-Triton 0.2% with gentle shaking.
7. Incubate 2 h in secondary antibodies at room temperature with gentle shaking. Dilute 1 μ L of each Alexa-labelled antibody in 200 μ L of PBS-Triton 0.2%. Protect the tubes from light from this step onward, for example, by using aluminum foil.
8. Rinse once in PBS and then wash 2×30 min in PBS-Triton 0.2% with gentle shaking.
9. Remove as much PBS-T as possible, add one drop of Vectashield plus DAPI.
10. Store at 4 °C until mounting (fluorescence can last up to a month or more) or incubate at least 30 min and proceed to mounting.
11. For mounting, take the posterior abdomens from the tube and place them on a slide, together with about 20 μ L of the Vectashield from the tube. Using forceps and the insect pin prepared in Subheading 2, **step 5** (or two forceps), separate the testes from the abdomens and move them gently toward the center of the slide. Testes can be identified as thick and coiled tubes with a fibrous content. The insect pin can be used to tear non-testis structures and to drag gently the dissected testes to the center of the slide. With the testes separated, carefully remove from the slide abdomens and other tissue debris. Put 10 μ L of Vectashield on top of the testes, and carefully place a coverslip on top and use nail polish to seal.
12. To scan the testes on a confocal microscope, locate the hub at the apical tip of the testis through Fasciclin3 staining. Use at least 63 \times magnification and acquire stacks with a z-step of 2 μ m.
13. GSCs can be identified as cells with a Vasa-positive cytoplasm directly in contact with the membranes of hub cells, labeled

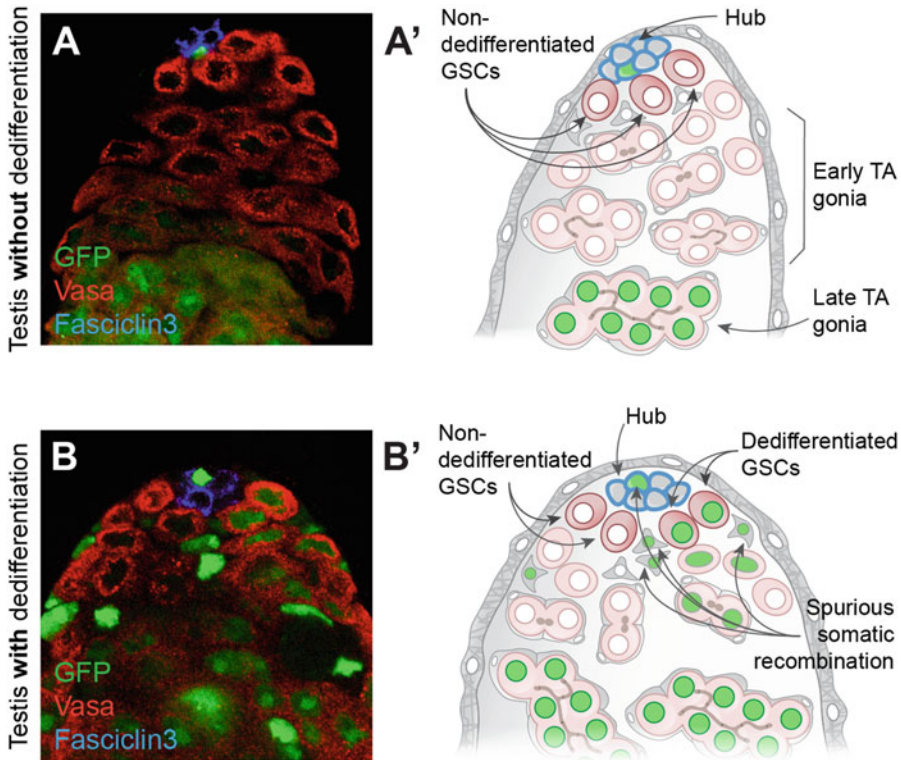


Fig. 3 Representative confocal images of a testis without (a) or with (b) dedifferentiated GSCs. A' and B' are diagrams provided as an aid for interpreting the micrographs. GSCs can be identified as cells with Vasa-positive cytoplasm (red) in direct contact with the hub (which express Fasciclin 3 (blue membranes) at apical tip of the testis). Dedifferentiated GSCs can be distinguished by the GFP-positive nuclei (green). Spurious lineage cassette recombination can happen in somatic cells, but these can be distinguished from the dedifferentiated GSCs, by the expression of cytoplasmic Vasa surrounding the GFP nuclei in the latter

with Fas3 (*see* Fig. 3a). Navigate through the image stack, as GSCs can be in a rosette around the hub or in some cases above or below the hub. Germline cells separated from the hub by a gap where another nucleus is present are gonialblasts and should not be scored as GSCs.

14. Among the GSCs, the dedifferentiated ones can be identified by GFP-positive nuclei (*see* Fig. 3b).

4 Notes

1. Once thawed, PBT-BSA and paraformaldehyde aliquots can be preserved at 4 °C. PBT-BSA must be discarded after a week and paraformaldehyde after 3 days.
2. Spurious recombination of the lineage-tracing FRT cassette is frequently observed in fly stocks in harboring both an *FRT*

cassette and a *UAS-Flp*, such as the ones used on this protocol. If this happens in the germline, the recombined cassette will be transmitted to the next generations, rendering the stock useless. Periodically check the stocks containing the *FRT* cassette for presence of GFP-positive larvae, and make new stocks again if recombination is detected.

3. Overcrowding of vials can result on suboptimal nutritional intake and affect the results – 20-30 flies is the maximum number that should be housed in a 1-inch-width vial.
4. Always flip the F1 males into fresh food every two days for optimal nutrition and better reproducibility of the results.
5. Keep at all times the F1 males on a 25 °C incubator with controlled humidity for better reproducibility. This is especially important in aging protocols.
6. Unless females are intentionally introduced to vials with adult males to induce stress, males need to be strictly separated at day 0 from females. The presence of females (and presumed mating between males and females) induces proliferative responses in GSCs [10], affecting the reproducibility of results.
7. Anticipate some lethality (in the case of chronic stress, up to 50%) after the treatments explained above. Plan to age more flies than those you strictly need for the experiment.
8. Dissection of abdomens should not take longer than 20 min before fix with paraformaldehyde. If longer times are required, PBS should be used ice-cold, while keeping the Eppendorf tube with the dissected abdomens in an ice container. Even if using ice, the total dissection time should not be longer than 30 min.
9. Staining the nuclei, while not strictly necessary, is very helpful for identifying the different cell types of the testis. If the used mounting medium does not contain DAPI, DNA can be stained alternatively by adding either DAPI or Hoechst at a 1:500 concentration during the incubation with secondary antibodies.
10. The Stinger (GFP) fluorescence from the lineage cassette shows a weaker signal in germline cells compared with somatic cells, but should be strong enough to be detected on confocal microscopes. If required, anti-GFP staining can be used to enhance the signal.
11. Occasionally the lineage cassette can spuriously recombine in some somatic cells and should not be confused with dedifferentiated GSCs. These cells can be identified by their lack of Vasa staining in their cytoplasm and denser nuclei.
12. A high variance in the proportion of dedifferentiated GSCs per testis is not unusual, especially in aged flies. It is possible to

have testes where none or all of the GSCs are positive for the dedifferentiation lineage mark.

13. If analyzing rates of dedifferentiation, always compare for each genotype the result of the chosen treatment with 0-day-old young males. A baseline of dedifferentiation of around 5% of GSCs on average is normal, possibly representing cases of dedifferentiation during development or spurious recombination of the cassette.

Acknowledgments

Work in the Bach lab is supported by grants from the NIH and NYS Department of Health/NYSTEM. Work in the Herrera lab is supported by a grant from the Fundación Bancaria “la Caixa” (ID 100010434) with the code LCF/BQ/PI20/11760005.

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Standardization of Single-Cell RNA-Sequencing Analysis Workflow to Study *Drosophila* Ovary

Deeptiman Chatterjee and Wu-Min Deng

Abstract

Developments in single-cell technology have considerably changed the way we study biology. Significant efforts have been made over the last few years to build comprehensive cell-type-specific transcriptomic atlases for a wide range of tissues in several model organisms in order to discover cell-type-specific markers and drivers of gene expression. One such tissue is the ovary of the fruit-fly *Drosophila melanogaster*, which is a popular model system with wide-ranging applications in the study of both development and disease. Three independent studies have recently produced comprehensive maps of cell-type-specific gene expression that describe both spatiotemporal regulation of the process of oogenesis and unique transcriptomic profiles of different cell types that constitute the ovary. In this chapter, we outlined the wet-lab protocol that was followed in our recent study for sample preparation and reanalyze the resultant dataset to discuss the benchmarks in data analysis, which are fundamental to comprehensive curation of the single-cell dataset representing the fly ovary.

Key words Single-cell RNA-Seq, *Drosophila*, Ovary, Transcriptomics, Single-cell, Atlas, Data analysis

1 Introduction

The adult ovary of the fruit-fly *Drosophila melanogaster* is a popular model to study a wide range of topics in the field of cell and developmental biology. The different cells in the ovary have revealed important mechanistic insights into areas such as stem-cell niche regulation [1–6], differentiation [7–9], cell cycle and size control [10–14], cell plasticity and morphogenesis [15–17], collective-cell migration [18–21], tissue repair and homeostasis [22], and apoptotic cell clearance [23, 24] besides the broader study of oogenesis and its regulation. Oogenesis is the process of egg maturation that occurs within the “factory units” of the ovary – a structure confined within the muscle sheath known as the ovariole. The structural unit of a developing egg is known as the egg chamber, which consists of the germline cells that are surrounded by a monolayer of somatic epithelial cells undergoing

morphogenetic changes over time (divided into 14 stages) to support the developing oocyte. The anterior-most structure of the ovariole, known as the germarium, contains the stem-cell niche for both the somatic and germline lineage while the posterior end of the ovariole consists of the corpus luteum, which is made up of the terminally fated somatic follicle cells that are left behind once the fully matured oocyte is released into the oviduct for oviposition.

Although the fly ovaries have served as model systems for various studies over the years, addressing cell-type-specific functions and their interactions has been impeded by a lack of cell-specific markers and drivers of gene expression. Additionally, the ovary is a highly complex tissue system consisting of several cell types with diverse constitutive proportionalities that also exhibit dynamic spatiotemporal gene-expression regulation. Which is why, spatially agnostic assays (techniques that cannot resolve cellular location) tend to exhibit low fidelity when supporting experiments done on a specific cell type within the fly ovary (e.g., somatic follicle cells) as they cannot distinguish from the properties of non-specific cell types (e.g., germline cells, hemocytes, muscle sheath, etc.). These complications make microarray or bulk RNA-Seq-based techniques to be imperfect assays for comprehensive analysis of cell- or tissue-specific genetic experiments. This impasse, however, has since been overcome in recent years as several independent as well as consorted efforts have built a comprehensive knowledge-base of the cell-type-specific gene-expression pattern in the form of a transcriptomic atlas of the fly ovary [25–29].

To uncover the anticipated (as well as unanticipated) diversity in the various cellular transcriptomes within the fly ovaries, 3 independent studies have utilized the single-cell RNA-Sequencing (scRNA-Seq) technique [26–28]. Given that scRNA-Seq analyses are generally based on the principles of clustering cells with similar features (e.g., gene expression), these studies have commonly revealed that very rarely do single marker genes identify specific cell types. Instead, annotation of distinct cell types depends on the combination of multiple markers and their relative intensities. In addition to these studies, those focused on specific groups of cell types have also revealed an unanticipated diversity in stable cell states within the transcriptome of individual cell types [30, 31]. While each of these investigations was performed with technical differences in fly lines and a different approach to both sample preparation as well as analytical methods, the obtained results were also remarkably similar. This apparent “unity in diversity” not just highlights the commonly discovered properties of the fly ovary but also sheds light on some of the technical benchmarks essential for the generation of high-quality single-cell data. In this chapter, we have discussed those benchmarks in greater detail in an attempt to standardize the pipeline with significant discussions on the more flexible aspects of data analysis.

The general application of RNA-Seq promotes interdisciplinary collaborations, as it requires the know-how of both “wet-lab” and “dry-lab” techniques. While the wet-lab protocol that deals with sample preparation for the purpose of sequencing largely depends on the target tissue, experimental design, and sequencing platform, the dry-lab protocol lacks consensus on the analysis pipeline [32]. This is because of the much greater number of computational tools available for data analysis, that are written in different programming languages for several distinct purposes, all of which are not likely to be the goal of an individual study [33]. With scRNA-Seq being the anchor to which newly emerging modalities, such as chromatin accessibility [34], DNA methylation [35], spatial resolution [36], and protein abundance [37], are linked, curation of a comprehensive scRNA-Seq dataset becomes an essential first step in compiling transcriptomic datasets that can be utilized for more than one experiment. Therefore, in this chapter, we discuss the need for a standardized protocol that would include a comprehensive set of data that can be used to link additional pipelines (e.g., RNA velocity, etc.) that build upon the information contained within the dataset. We also describe the wet-lab protocol for 10X Genomics-based library preparation as followed in the Jevitt, Chatterjee et al. paper and have also used the sequences generated in that study as a template to discuss the recreation of a single-cell dataset representing the *w¹¹¹⁸* ovary.

2 Materials

2.1 Single-Cell RNA Sequencing Sample Preparation

1. Schneider’s *Drosophila* Medium – Modified (Lonza™ Bio-Whittaker™ Insect Cell Culture Media: Catalog no. BW04-351Q).
2. S2 Complete medium (has FBS): Schneider’s *Drosophila* Medium containing 10% fetal bovine serum (FBS).
3. Earle’s Balanced Salt Solution – EBSS, no calcium, no magnesium, no phenol red (Thermo Catalog no. 14155063).
4. Papain (Worthington, Catalog no. LK003178): The papain comes as a powder. It is suggested to thoroughly suspend powder, by pipetting up and down, in EBSS to a final concentration of 50 units/mL. This comes as 50 units, so add 1 mL EBSS and aliquot into 100 ul aliquots and freeze at -20°C . Thaw the amount needed and heat activate for 10 minutes at 37°C .

3 Methods

3.1 Tissue and Cell Dissociation

The protocol described in this section was used in Jevitt, Chatterjee et al., which itself was adapted from other studies [38–40]. All procedures should be carried out in room temperature unless specified otherwise.

1. Dissect 50 flies to isolate entire ovaries in cold Schneider's *Drosophila* Medium (*see Note 1*).
2. Transfer dissected ovarian tissue into 1.5 mL microfuge tube, containing 100 μ L of S2 Complete medium on ice.
3. Spin down the tissues at room temperature by centrifuging for 2 min at 500 RCF (Relative Centrifugal Force).
4. Remove supernatant, wash tissues with 300 μ L of EBSS and re-centrifuge as described in **Step 3**.
5. Remove EBSS and add 100 μ L L-cysteine activated Papain that has been brought to room temperature after heat activation (*see Note 2*).
6. Mechanically dissociate cells from the tissue, suspended in the Papain solution, by slowly pipetting up and down 10 times for every 3 minutes with a 20 μ L pipette for the first 15 min of the reaction. Continue mixing as previously with a 10 μ L tip for the remaining time.
7. Add 500 μ L S2 Complete medium to quench the digestion.
8. Immediately, centrifuge sample for 10 min at 700 RCF. Repeat wash twice.
9. Resuspend cells from dissociated tissue in 300 μ L of S2 Complete medium.
10. Add 250 μ L of Schneider's *Drosophila* Medium and allow it to flow through a 40 μ m filter into a 1.5 mL tube (*see Note 3*).
11. Discard the flow through and repeat **Step 10**.
12. Discard collection tube and put the filter into a fresh labeled tube.
13. Transfer the cell suspension gently, without introducing bubbles, through the filter into the new 1.5 mL tube.
14. Pass 100 μ L (or more, as needed) of S2 Complete medium through the filter and dislodge any remaining cells that may stick with the filter. Repeat once.
15. Centrifuge cells down at 500 RCF for 10 min and resuspend cells in the desired volume of S2 Complete medium.
16. Count cell density with a hemocytometer and check for viability with Trypan Blue or Propidium Iodide.

17. Dilute cells to an approximate, final concentration of 2000 cells/ μ L as recommended for 10X Genomics, unless processing them for other methods.
18. Immediately process the cell suspension for fluorescence-activated cell sorting (FACS) to prevent excessive cell death.

3.2 10X Genomics Library Preparation and Sequencing

Single-cell libraries are prepared using the Single Cell 3' Library & Gel Bead Kit v2 and Chip Kit according to the recommended 10X Genomics protocol (as provided in the kit) and the single-cell suspension is loaded onto the 10X Genomics Chromium Controller. Library quantification assays and quality check analysis were performed using the 2100 Bioanalyzer instrument (Agilent Technologies). The library samples are then diluted to the final concentration of 10 nM and are loaded onto two lanes of the NovaSeq 6000 (Illumina) instrument flow-cell for a 100-cycle sequencing run. Reads are demultiplexed and the corresponding fastq files are generated for each library which are subsequently processed in the 10X Genomics' command line interface (CLI) software named Cellranger which provides files that can be used as inputs to downstream analysis pipelines such as Seurat (Fig.1 and Table 1).

3.3 Data Pre-Processing in Linux-Based Command Line Interface

Following sequencing, the user is provided with the raw fastq files that are ultimately converted to sample-specific gene-expression matrix following quality assessment of the sequences. FastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) is a popular tool for the quality assessment of both raw fastq files and processed bam files. Once the user is satisfied about the quality of data (as explained in great detail in the FastQC manual), the cellranger pipelines are employed to align the fastq reads to a reference transcriptome, which generates a number of output files compatible with several open-source software, such as the R-based package Seurat.

Using the 10X Genomics' cellranger mkfastq pipeline (which, at its core, utilizes Illumina's bcl2fastq function), the base call files (BCLs) generated by an Illumina sequencer for each flowcell directory are demultiplexed. This pipeline automatically recognizes single-indexed and dual-indexed flowcells and produces fastq files ready to be aligned to the reference transcriptome. However, before the fastq files can be aligned to the reference genome for feature counting, the 10X Genomics cellranger mkref command is run to prepare the reference genome serving as the template for sequence alignment:

```
cellranger mkref
--genome=ReferenceGenomeForCellranger
--fasta=~ /Drosophila_melanogaster.BDGP6.28.dna.toplevel.fa
--genes=~ /Drosophila_melanogaster.BDGP6.28.100.chr_filtered.
gtf
```

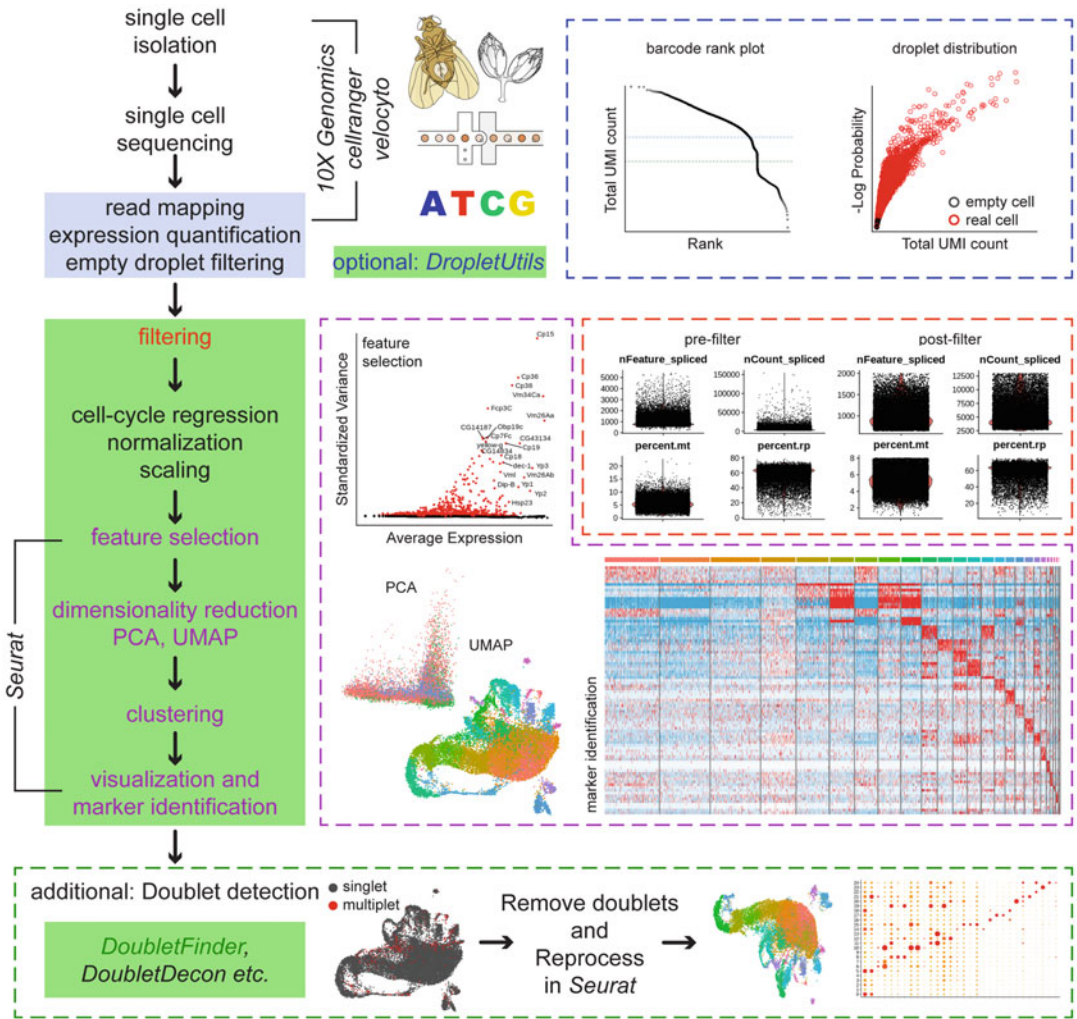


Fig. 1 Overview of the scRNA-Seq analysis workflow. Solid-color boxes represent the programming language/platform where the relevant software works: UNIX/Linux (blue) and R (green). Boxes with dashed borders are color-coded to relate to specific steps in the workflow. This workflow consists of several key stages (left column) as well as a few optional/additional steps (as mentioned) of quality control. The workflow begins with single-cell isolation from the dissected tissue and library preparation as recommended by 10X Genomics library preparation that utilizes the droplet-based technique for assigning barcodes to single cells. The sequenced reads are mapped, quantified, and filtered using the 10X Genomics’ cellranger pipelines. Unspliced reads are further quantified using velocity. Optional filtration can be performed using the R-based package DropletUtils which enables the estimation of empty droplets and visualize barcode distribution with respect to UMI counts (diagnostic plots within the box with blue-colored dashed line). The output from cellranger/velocityto can be used as input in the R-based package Seurat, which enables filtering (box with red-colored dashed lines), data processing, and iterative steps (purple colored) involving feature-selection for clustering and visualization. Additionally, the dataset can be further processed for doublet-based contamination using R-based packages such as DoubletFinder (green), which estimates and removes potential doublets, following which the final dataset is obtained after reprocessing the entire dataset in Seurat (box with green-colored dashed lines)

Table 1
List of software used in this workflow

Software	Platform	Link
Cellranger	Linux	https://support.10xgenomics.com/single-cell-gene-expression/software/pipelines/latest/what-is-cell-ranger
Velocyto [41]	Linux	http://velocyto.org/
Seurat [42, 43]	R	https://satijalab.org/seurat/
DropletUtils [48, 49]	R	https://bioconductor.org/packages/devel/bioc/html/DropletUtils.html
DoubletFinder [50]	R	https://github.com/chris-mcginnis-ucsf/DoubletFinder
GSE146040	GEO	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE146040
BDGP6.28 (release 102)	Ensembl	http://useast.ensembl.org/Drosophila_melanogaster/Info/Index

In the above command, the user provides the path to the .fasta and .gtf files for the transcriptome. Both files were downloaded from Ensembl, in the dedicated database for *Drosophila melanogaster* BDGP6.28. The reference transcriptome being built for the subsequent cellranger count pipeline was based on the dm6 build (see **Note 4**). Using this reference genome as the template, the bcl2fastq generated fastq files are subsequently aligned using the following command:

```
cellranger count
--transcriptome=~/.ReferenceGenomeForCellranger/
--id=w1118_Control          #user-defined ID
--fastqs=~/.10X/B/         #where the fastq files are stored
--sample=SI-GA-B4_1,SI-GA-B4_2,SI-GA-B4_3,SI-GA-B4_4
                           #the prefix on fastq files
--chemistry=SC3Pv2         #10XGenomics library-prep
chemistry
--indices=ACTTCATA,GAGATGAC,TGCCGTGG,CTAGACCT
                           #10XGenomics index
```

Running the above command returns gene-expression matrices for all cell barcodes (raw) as well as cell barcodes that cellranger considers to be real (filtered), i.e., the droplets containing a cell. In addition, cellranger also summarizes the associated statistics in an html file format. While the filtered output from the cellranger pipeline can directly be used for downstream analysis, we recommend the inclusion of additional information on unspliced reads contained within each cell. This enables the use of pipelines that

extract RNA-velocity-based information from the processed dataset without having to rebuild the dataset, thereby maintaining its integrity and preventing the introduction of stochastic changes common to clustering algorithms. The cellranger-derived bam file is further processed using velocityto [41] for the annotations of unspliced/spliced reads using the following command:

```
velocityto run
-b ~/outs/filtered_feature_bc_matrix/barcodes.tsv.gz
                                     #cellranger filtered barcodes
-o ~/outs/velocityto/                 #desired output folder
-m ~/dm6_rmsk.gtf                     #repeat-masker file for dm6
~/outs/possorted_genome_bam.bam       #cellranger derived bam file
~/Drosophila_melanogaster.BDGP6.28.100.chr_filtered.gtf
                                     #reference genome
```

3.4 Data Pre-Processing in R

Running cellranger and velocityto commands for every sample results in a single .loom file per dataset. This .loom file contains information on both unspliced and spliced (as well as ambiguous) reads that are detected upon alignment. After the .loom files are generated, it is used as an input into the R-based pipeline of the program Seurat [42–44] using the following command, which generates a Seurat-readable R object called scData.

```
lmdat <- ReadVelocity(file = "~/VelocityProcessed.loom")
scData <- as.Seurat(x = lmdat)
```

Once the Seurat object scData is created, we evaluate the data distribution of the raw read counts called nFeature (or nGene in previous versions of Seurat) and UMI counts called nCount (or nUMI in previous versions of Seurat). The raw read and UMI counts are stored within the object for every “assay”, i.e., the spliced, unspliced, and ambiguous assays. Given that the unspliced and ambiguous assays are utilized for RNA velocity analyses, only the spliced assay is considered for data pre-processing. Setting a minimum and maximum threshold for the raw counts, we filter out the outlying datapoints (cells) from the normally distributed data (see Fig. 1). This command attempts to filter out both empty droplets and doublets/multiplets, which are droplets that contain more than one cell’s transcriptome:

```
scData <- subset(scData, subset = nCount_spliced<13000 &
nFeature_spliced<2000 & nFeature_spliced>650 & percent.mt<8)
```

In the above command, the w^{1118} dataset is filtered by only selecting cells that contain less than 13,000 UMIs and 2000 genes but more than 650 genes. Such filtering criteria should be set after careful consideration of the data distribution in individual datasets,

which can be explored by producing diagnostic plots in Seurat. Each dataset is additionally filtered for cells expressing an excess of mitochondrial genes, which implies a state of apoptosis. In order to incorporate the information about mitochondrial-gene expression in each cell, Seurat allows the assignment of user-defined criteria (labeled `percent.mt`). Using the following command, we can assign a percentage to each cell, that describes the proportion of expressed genes whose name begins with the prefix “mt:”, which is the prefix assigned to all mitochondrial gene (for mitochondrial genes in humans, this is “MT-“).

```
scData[["percent.mt"]] <- PercentageFeatureSet(scData,
pattern = "^mt:")
```

Similarly, we can assign the percentage of ribosomal gene expression to each cell by using the prefix “Rp” (one can specifically detect percentage expression of large and small subunits of ribosomal genes by using “RpS” and “RpL” prefixes as well):

```
scData[["percent.rp"]] <- PercentageFeatureSet(scData,
pattern = "^Rp")
```

While 5% mitochondrial gene expression is suggested as a benchmark for mitochondrial gene expression per cell in the Seurat pipeline [44], several studies specific to *Drosophila* have used alternative threshold values that range between 5 and 30% [45, 46]. This lack of consensus is likely because of (a) low sequencing depth and/or (b) cell-type-specific gene-expression signatures. In our experience, the distribution of mitochondrial gene expression tends to vary for each dataset. Therefore, we suggest that a dataset-specific threshold should be considered based on the overall distribution of such criteria. A single-cell transcriptomic study of the *Drosophila* wing disc cells used a similar approach by assigning 1.5 standard deviations above the mean mitochondrial read percentage as the higher threshold [47]. Here, we applied an 8% threshold for mitochondrial expression in the *w¹¹¹⁸* dataset.

An alternative method for determining whether droplets contain cells or just ambient RNA from lysed cells is implemented in the DropletUtils package [48, 49]. Additionally, artificial libraries generated from doublets, due to errors in cell dissociation and sorting that are common to droplet-based protocol, can be detected by implementing packages such as DoubletFinder [50], DoubletDecon [51], Scrublet [52], etc. Information extracted from these pipelines can then be used to implement more stringent thresholds (*see* Fig. 1). However, applying stringent thresholds also tend to decrease the total number of cells. Hence, choosing threshold values is an iterative process which aims to build a balance between maintaining high number of cells and keeping high-quality cells, both of which are critical to downstream processing.

3.5 Dealing with Technical Noise and Confounding Factors

Data normalization is an essential step in processing counts derived from such experiments as errors in normalization can have a significant impact on downstream analyses, such as inflated false positives in differential expression analysis [53]. Standard scRNA-Seq pipelines have mostly adopted data normalization strategies from those widely used in bulk RNA-Seq analysis [54–56]. However, therein lies certain caveats that the user should be mindful of and choose a normalization strategy accordingly.

Compared to the count matrix obtained from a bulk RNA-Seq analysis, a prominent feature of scRNA-Seq is the sparsity of data, i.e., the high proportion of zeroes in the multidimensional gene-count matrix [54, 57]. This sparsity depends on several factors that are both biological and technical in nature: “zero inflation” arises in scRNA-Seq data because (1) genes not being expressed in certain cells or in transient states of cells where the cell is undergoing cell division, as well as due to (2) the presence of “dropouts”, where genes are expressed but not detected, given the stochastic nature of sequencing depth. In addition to the dropouts, the technical noise in scRNA-Seq data is also contributed by high variability in gene expression between closely related cells [58]. Lastly, because individual cells are captured from distinctly different cell types, variability in count matrix is achieved even in the absence of technical biases and noise. While this is an area of active research, most scRNA-Seq analysis pipeline uses global normalization methods that are developed mostly for bulk RNA-Seq pipelines, such as LogNormalize [44], Median of Ratios (MoR) [59], Trimmed Mean of M-values (TMM) [60], and upper quartiles (UQ) approach [61]. Seurat function `NormalizeData()` uses “Log-Normalize”, a global normalization process that divides gene counts for a cell before multiplying by a scaling factor (10,000 by default) and natural logarithmic transforming the result with $\log(x + 1)$ to account for dropouts. Other normalization methods adapted from bulk RNA-Seq include the MoR approach that is part of the popular analysis package DESeq2 [59] and the TMM approach that is part of the EdgeR [62] pipeline. UQ normalization defines scaling factor estimates as proportional to the 75th percentile of the count distribution within each cell [61]. However, while TMM have been found to overcorrect for scaling factor sizes, quantile-based normalization methods are problematic in scRNA-Seq because of zero inflation which can lead to the scaling factor estimates to be set to 0 in practice [53, 63].

Newer methods are guided by the addition of a constant volume of spike-in RNAs into each cell’s lysate, that is used to model technical noise [64]. Given that the concentration of this spike-in RNA is known, and assuming that it will experience similar technical variation as the endogenous RNA, the normalization methods model this technical noise by accounting for the difference between observed and known values of concentration. Whether the spiked-

in RNA reflects the behavior of endogenous RNA or if its detection is affected by the cellular RNA is not known [65]. Moreover, incorporation of spike-ins cannot be effectively incorporated into droplet-based platforms. Therefore, in order to standardize the analysis pipeline, suitability of all available methods must be considered for individual experimental setup and informed decision should be made to extract the best outcome for downstream analyses.

After normalization, the next step is to estimate confounding factors. Unlike experimental setups in bulk RNA-Seq which compares samples for differential gene expression under multiple conditions, a single scRNA-Seq experiment tends to capture cells from one experimental condition. This allows variability arising from systematic differences in sample preparation and batch effects. This can be solved by repeated experimentation to obtain experimental replicates [66], which can otherwise be labor-intensive and expensive. In addition, biological variables such as cell cycle stage, differences in ploidy, cell state, and apoptosis affect the overall transcriptomic profile [58]. Therefore, while apoptotic cells are removed via stringent filtering by applying an upper limit to mitochondrial gene expression for every cell, the other factors are identified and regressed from scaling using the following parameter within the `ScaleData()` function of Seurat, which removes cell-cycle differences, mitochondrial-gene expression within the permissible range, and the total number of spliced RNA reads obtained per cell respectively, from being considered as contributing factors to meaningful variability in the dataset:

```
vars.to.regress=c("CC.Difference", "percent.mt", "nCount_spliced")
```

While normalization and scaling removes unwanted technical and biological noise, the ultimate goal of scRNA-Seq analysis is to classify cells into distinct clusters based on feature similarities (between cells of the same cluster) and dissimilarities (between cells of different clusters). To facilitate this, a subset of features (such as genes) that characterize the entire dataset is selected. A popular approach is to focus on genes that exhibit high cell-to-cell variation since they emphasize the key biological signal within single-cell datasets [42–44]. These highly variable genes or HVGs (by default, a total of 2000 genes) are calculated by the `FindVariableFeatures()` function in Seurat and are used to guide dimension reduction techniques such as PCA in downstream analysis.

In newer versions of Seurat (v3.0 onwards), the `NormalizeData()`, `ScaleData()`, and `FindVariableFeatures()` functions are incorporated within the `SCTransform()` function. This was introduced in light of the realization that different groups of genes cannot be normalized by the same scaling factor and thus instead of using linear size/scaling factors, the authors recommend using a

generalized linear model (GLM) relating cellular sequencing depth to molecular gene counts [67]. This modeling framework performs normalization and variance stabilization of molecular count data and omits the need for steps that include pseudocount addition or log-transformation thereby improving common downstream analytical tasks such as variable gene selection, dimensional reduction, and differential expression. Importantly, this approach is able to remove technical variability without diminishing biological heterogeneity.

Since confounding factors, especially biological variability, would depend heavily on the heterogenous dataset, the normalization, scaling, and feature selection steps should be repeated whenever cells or clusters are isolated for subsetted downstream analyses.

3.6 *Decomplicating Single-Cell Datasets by Linear Dimension Reduction*

Major applications of scRNA-Seq analysis depend on the ability to cluster cells based on transcriptional similarity and determine co-expression patterns. However, in order to get to that step, it is essential to simplify the highly complex dataset. A typical single-cell sequencing dataset contains the “gene x count” information for every cell (multidimensional array), while also containing metadata that may apply to individual cells or to the whole dataset. As single-cell datasets continue to grow in size, demanding exponentially increasing computational requirements, data scientists have repurposed existing data formats to store large-scale omics data more efficiently. Commonly used single-cell file types, such as .loom, builds upon the Hierarchical Data Format version 5 (HDF5) file structure, which uses a hierarchical structure for data storage allowing the organization of data within the file in user-defined structures, as can be done with a directory structure on a personal computer. The HDF5 format also allows for embedding of metadata making the dataset and the information therein to be “self-describing”.

Cells are commonly projected on two- or three-dimensional space for visual inspection of the distribution of gene expression. This is first achieved by linear dimension reduction techniques such as Principal Component Analysis (PCA) or Multi-Dimensional Scaling (MDS) [68, 69]. Seurat runs PCA on the scaled data using the `RunPCA()` function, and by default uses the previously determined list of variable features as input when specified by the `features = VariableFeatures()` parameter. PCA allows the identification of major sources of heterogeneity in a dataset by determining the number of Principal Components (PCs) that order both features and cells according to their decreasing PCA scores. PC1 identifies genes that determine the biggest variance in the dataset, while PC2 identifies the group of genes that represent the second-highest variance within the dataset, and so on for PCs 3, 4, 5... to a user-defined n . Each PCs represent a robust simplification of the dataset in the form of a “meta-feature” whose corresponding

“score” is assigned to each cell as a representative of its inherent transcriptome. An appropriate number of PCs are further used as inputs to guide appropriate clustering of individual cells into local “communities”. We have chosen 60 PCs for the downstream clustering analyses of the m^{1118} ovary dataset, after carefully considering the top 100 PCs.

3.7 Clustering and Visualization

The most consequential aspect of single-cell data processing is the clustering analysis which aims to divide a set of input data (cells or whole transcriptomes of individual cells) into subset “communities” (or clusters), where each element is considered to be related by some similarity measure (gene-expression overlap). Tools such as PAGODA [70], SINCERA [71], and bigScale [72] implement hierarchical clustering, while SC3 [73] integrates hierarchical and k -means clustering to cluster cells. Seurat (as well as the popular Python-based analysis package Scanpy [74]) uses the graph-based clustering approach, where a k -nearest neighbor (KNN) graph is constructed, based on the distance metric learned from the PCA scores assigned to each cell. Here, the cells are embedded in a graphical structure with edges based on their meta-feature similarity drawn between them and the edge weights are further refined based on the shared overlap within the communities. This step is performed using the function `FindNeighbors()` that requires the number of dimensions as a required input parameter. After the cells are assigned a “position” within the graphical network, they are separated into distinct “communities” or clusters. Seurat applies modularity optimization techniques (that measure the strength of division of a network) to further classify the cells into distinct groups. The community detection algorithm is implemented by the `FindClusters()` function that also requires a resolution parameter, which affects the size and number of the recovered clusters.

As previously mentioned, single-cell datasets have a non-linear structure because of the presence of stochastic zeros in the expression matrix that arise as a result of dropouts. While linear manifold learning techniques such as PCA and MDS can reduce dimensionality, they only retain the global structure of the data and cannot resolve the relationship between closely related cells. To further explore the dataset, it is necessary to properly resolve local connectivity between the cells and visualize their distribution on two-dimensional (2D) embedding. To do so, Seurat offers both tSNE [75] (t -Distributed Stochastic Neighbor Embedding) and UMAP [76] (Uniform Manifold Approximation and Projection) non-linear dimensionality reduction algorithms, via the functions `RunTSNE()` and `RunUMAP()`. Considering that UMAP reflects the global as well as local structure of a multidimensional dataset more accurately than tSNE, we have used it to determine the 2D embedding of the ovarian cells of the m^{1118} dataset. UMAP offers several hyperparameters that can have significant impact on the

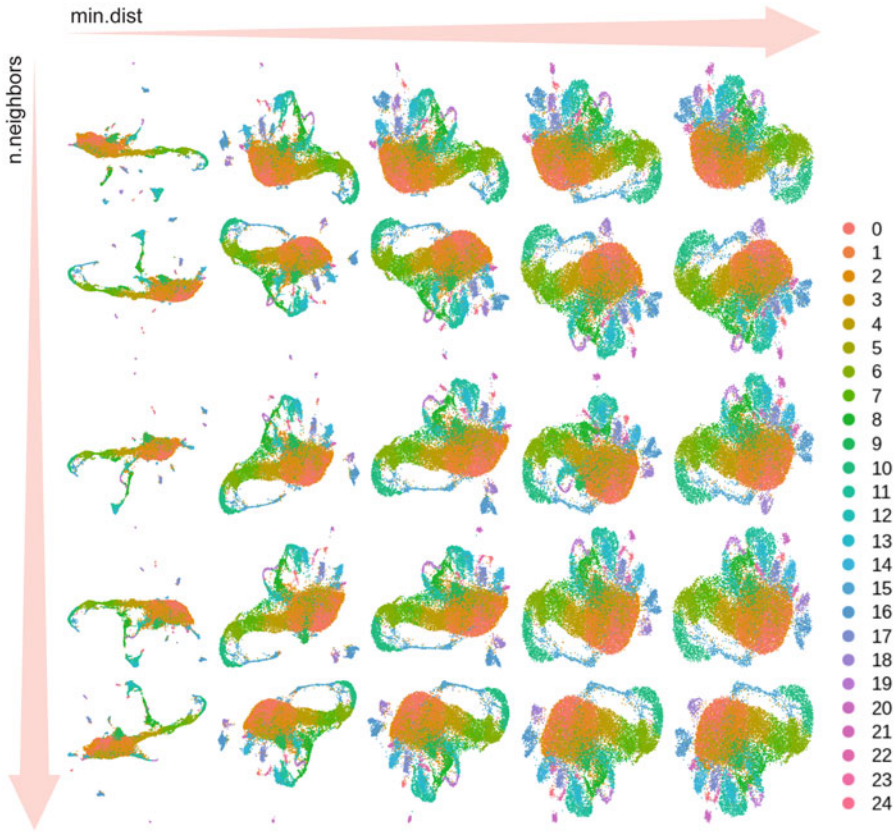


Fig. 2 Visualization of the relationship between UMAP embedding of cell clusters and changing parameters in graph-based clustering. The parameters min.dist and n.neighbors are distributed across 0, 0.25, 0.5, 0.75, 1 (columns: left to right) and 20, 40, 60, 80, 100 (rows: top to bottom), respectively. Number of cells and their cluster assignment remain unchanged

resultant embedding and understanding how they affect the visualization is critical for building the best plots. The parameters n.neighbors and min.dist have a distinct effect on the UMAP plot (*see* Fig. 2) since n.neighbors controls how the UMAP balances local vs global structures and min.dist controls how tightly the cells are allowed to pack. Assigning values to these parameters controls how the cells are embedded in the 2D space and does not affect their clustering or relationship to each other. Therefore, users must choose appropriate values to these parameters based on whichever embedding best represents how the clusters (and therefore the corresponding cell types) relate to each other (which can be obtained by running the function `BuildClusterTree()` to build a similarity-based phylogenetic tree). For the w^{1118} ovary dataset, we have chosen a min.dist = 0.5 and n.neighbors = 80.

3.8 Identifying Cell States and Assigning Cell-Type Identities

In practice, selecting the resolution parameter for clustering analysis is an iterative choice that the user makes, as lower resolution results in poor segregation of dissimilar clusters, while setting the parameter too high results in spurious categorization of communities that may lead to the false assignment of cell-type identities. While the authors of Seurat suggest that setting the resolution parameter between 0.4 and 1.2 returns good results for single-cell datasets that contain around 3000 cells, we recommend that the validity of the obtained result should be thoroughly corroborated by: (a) exploring the relationship between each cluster and hypothesizing a biological explanation for that relationship, as well as (b) biological experimentation. Our approach to setting the resolution parameter is largely informed by the subjective expectation of the minimum number of anticipated cell types in a dataset, which also depends on the tissue of origin, existing knowledge of the tissue composition, sampling efficiency, and sequencing depth (*see Note 5*). For example, an efficient sampling of the germline-cell subset of the ovarian tissue dataset should consist of the germline stem cells (GSCs), cystoblasts, germline precursor cells, oocyte, and the Nurse cells, which make up 5 cell-types in total. Hence, we may choose 5–6 sequentially increasing resolution parameters that roughly return 5 clusters (accurately reflecting the anticipated cell types). Enabled by the R package *clustree* [77], the relationship between these different resolutions is then inferred by building a “clustering tree” that shows how cells are distributed as the clustering resolution is increased. Plotting this tree helps understand how closely related cells are segregated as an effect of increasing resolution, for example, cells within cluster 1 at a lower resolution A may split to clusters 1 and 2 at higher resolution B. This method has been utilized by the Jevitt, Chatterjee et al. study [28]. To assess the biological validity of such segregation, the user must perform differential gene expression between clusters 1 and 2 (at resolution B) and identify unique markers. The presence of these unique markers in the cells must then be probed using techniques such as fluorescence in situ hybridization (FISH) and via fluorescence-tagged protein markers using Immunofluorescence (IF). Given that the resolution parameter affects the dataset globally, increasing it for one cell-type lineage may falsely increase the number of clusters for another cell-type present in the same dataset. Hence, selection of the “ideal” resolution parameter should be driven by the goal of achieving meaningful diversity in a dataset at a global level.

The manifold of single-cell datasets consistently highlights the plasticity of individual cells, as continuously differentiating cells belonging to a shared cell-type lineage appear connected in the embedding itself. The connected clusters in such a condition are closely related, thereby raising the question of whether they are distinct cell types or cell states. Our approach to assigning identities

to such clusters is first to identify the transcriptomic differences between them by applying differential gene expression (DGE) analysis. For example, to distinguish clusters 1 and 2, we perform DGE on cluster 1 vs 2 and 2 vs 1, with a focus on identifying positive markers in each cluster. Following such analysis, we aim to characterize the difference as either qualitative or quantitative. For example, if cells in cluster 1 express the same genes A and B as cluster 2 but differ only at their log-fold change, i.e., higher or lower expression of those genes, then the difference is quantitative and is possibly indicative of different cell states of the same cell type. But if cluster 1 differentially expresses gene A while cluster 2 expresses gene B, then the difference is qualitative and may be suggestive of being different cell types. This, however, depends on whether the cluster-specific genes indicate a significant transition to a distinct yet stable transcriptomic state. And while such a transition may be perpetuated by a single transcription factor or master regulator, it seldom amounts to a change in the expression of a singular gene. Thus, cell types should be further cataloged with information using their ontologies. Cellular, molecular, and biological ontology should be run on panels of cluster-specific markers and only then, logical decisions should be made regarding whether a cell cluster represents a distinct cell state or another cell type. Finally, biologists should thrive to support such decisions with biological experimentations. For example, if the expression of gene A (or group of genes represented by A) makes cells in cluster 1 transition to a stable cluster 2, then experiments can be designed to validate the necessity and sufficiency of gene A in the formation of cluster 2 cells via genetic epistasis experiments.

Applying a resolution of 1, the 19,986 cells of the w^{1118} ovary dataset are grouped into 25 clusters and are embedded on lower UMAP dimensions (*see* Fig. 2). The validity of clustering these cells as such can be inferred by the near-homogenous enrichment of specific markers in each cluster as determined by examining the heatmap (*see* Fig. 1). Identifying markers that enrich each cluster is achieved via differential gene expression (DGE) analysis. Seurat provides the FindMarkers() function to perform DGE, with specific parameters to consider. To find specific markers per cluster in a dataset, Seurat compares DGE in the cells of ident.1, i.e., the cluster of interest vs that in cells belonging to all the other clusters. However, while DGE is well established in bulk RNA-Seq applications for sample vs control comparisons, complexities of scRNA-Seq dataset introduce new aspects to consider as discussed in the subsequent section.

3.9 Inferring Enriched Markers

To specifically compare 2 clusters, one can provide a specific ident.2. Additionally, to detect only highly differential genes, one can filter out smaller differences by applying a lower limit to the DGE analysis by using the logfc.threshold parameter. Moreover,

one should consider parameters that not only speed up DE testing but also provide information regarding the sparsity of the dataset. For example, using the parameter `min.pct`, one can pre-filter genes that are detected at a user-specified frequency in the clusters being compared. One can also use the `min.diff.pct` parameter to identify differentially expressed genes that show a minimum difference in the fraction of detection between the two identities being compared. For example, a `min.diff.pct = 0.5` would only test genes that are expressed in 50% more cells in `ident.1` than `ident.2` and will likely only test highly expressed genes that are least affected by count-matrix sparsity.

While Seurat uses Wilcoxon rank sum test as the default test to analyze DGE across clusters, model-based approaches such as SCDE [54, 70], MAST [55], and scDD [78] are recommended as they account for data bimodality by using a mixture model. Candidate genes obtained from such tests can then be evaluated as binary classifiers of cell-type identity by applying the ROC or LR tests that are specifically suited for zero-inflated data. Seurat offers multiple alternatives, such as DESeq2, likelihood-ratio test assuming an underlying Poisson or negative binomial distribution, ROC, MAST, LR, etc. Given the heterogeneity, multimodality, and sparsity of single-cell datasets, there's little agreement between the different tests regarding the discovery of true, biologically relevant DE genes. In general, the available DGE tests either account for zero-inflation or multimodality but not both, hence it remains up to the users to treat the results carefully and validate them by applying biological experimentation.

4 Notes

1. The time spent on this step should be minimized to allow minimum stress to the cells.
2. The amount of Papain requires standardization depending on the amount of tissue.
3. The size of the filter depends on the knowledge of the maximum size of the cells of interest.
4. The scRNA-Seq workflow suggested here reuses sequences generated in the published *w¹¹¹⁸* ovarian cell-atlas, obtained from the SRA database (SRX7814226). In this reanalysis, the number of cells passing the filtering criteria of cellranger increased significantly from the reported number of 12,671 cells with 28,995 mean reads-per-cell in the published cell-atlas to 24,144 cells with 17,095 mean reads-per-cell, with no change in the total number of sequenced reads (429,855,892). This result is due to the remapping of sequences to the top-level dm6 reference genome, which is different from the

reference genome used in the cell-atlas study. This sequence realignment improved the genome alignment of the reads. However, the adopted analysis pipeline does not change the primary findings of the original paper as the described markers for every cell type remain the same. However, it is also interesting to note how reanalysis of available datasets using new parameters and software tends to change the output, thereby stressing on the need to compile comprehensive datasets during the time they are first prepared.

5. With the increasing accessibility of scRNA-Seq techniques and their reducing costs, efforts to characterize and catalog the cell-type composition of organisms have been significantly galvanized. However, the increased resolving power has also brought increasing complexity, challenging the very definition of what is meant by a “cell type” and how it is separate from the concept of “cell states”. Commonly, cell types are considered stable cell states that perform a certain function reflected in their gene expression signature. By this definition, a neuron cell and a germline cell could be easily categorized but separating a “firing” neuronal cell from a “non-firing” neuron may not be possible as it is not a product of transcriptional regulation. Moreover, a neuron in a “diseased” state may cluster separately from the same neuron in “normal” condition – does that make them different cell types? With increasing number of cells and high-resolution of sequencing depth, the inherent heterogeneity within an individual cell state can be clustered into multiple closely related communities at high resolutions. Given the lack of consensus regarding the interpretation of single-cell clusters as cell states or types, this creates a problem for meaningful biological inferences.

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Identification of Germline Stem Cells in Zebrafish

Bruce W. Draper

Abstract

Both male and female zebrafish have a population of germline stem cells that produce gametes throughout the life of the fish. These cells localize to specific regions in the gonads and can be identified because they uniquely express the *nanos2* gene, which encodes a conserved regulator of translation. A method is presented here for identifying germline stem cells in the ovary and testis using a combined protocol for whole-mount fluorescent RNA in situ hybridization to detect *nanos2* mRNA and immunofluorescence to detect the pan-germ cell marker Vasa.

Key words Zebrafish germline stem cells, Oogonial stem cell, Spermatogonial stem cell, Zebrafish ovary, Zebrafish testis, *Nanos2*, Vasa, Fluorescent RNA in situ hybridization, Confocal immunofluorescence

1 Introduction

The zebrafish has become an excellent model organism for studying vertebrate development. Zebrafish have a rapid generation time and are very amenable to both forward and reverse genetics. While most research has been focused on embryonic development, many labs have begun to investigate events that do not occur until later in life, such as the specification and maintenance of germline stem cells (GSC).

Unlike mammals, where GSCs are only present in the adult testis, GSCs in zebrafish are present in both the adult testis and ovary [1–3]. Members of the Nanos family of conserved translational regulators are involved in the development and maintenance of germline stem cells in most, if not all, organisms. Three Nanos ortholog-encoding genes are present in the genomes of most vertebrates (*nanos1–3*), and in several of these, including zebrafish, *nanos2* has been shown to be a specific marker of germline stem cells [1, 3–5].

The GSCs in zebrafish are only a small fraction of the total number of pre-meiotic germ cells that are present in both the testis

and ovary, but because they localize to specific regions within each tissue, have a characteristic nuclear morphology and uniquely express *nanos2*, they can be easily identified. The organizational unit of the zebrafish testis is the lobule, which is a somewhat spherical unit that has premeiotic spermatogonia and early meiotic spermatocytes localizing to the surface of the sphere while the mature sperm localize to its center [6]. In ovaries, the GSCs localize to the germinal zone, which is a discrete circumferential band of cells on the medial and lateral ovarian surface [1, 2]. Because the GSCs in both testes and ovaries are located on the surface of the tissue, it is not necessary to section the tissue prior to staining. Instead, GSCs can be identified in whole-mount tissue preparations. Below we describe a method for the identification of GSCs that is a combination of whole-mount fluorescent RNA in situ hybridization, to visualize *nanos2* expressing cells, and immunofluorescence, to visualize the pan-germ cell marker protein Vasa. The in situ protocol has been adapted from Thisse and Thisse (2014) [7] and the Vasa immunofluorescence protocol from Draper (2013) [8]. This procedure will take six days to complete.

2 Materials

2.1 Tissue Fixation and Permeabilization

1. Phosphate Buffered Saline (PBS): Prepare a 10× stock using 1.37 M NaCl; 27 mM KCl; 100 mM Na₂HPO₄; 17.6 mM KH₂PO₄. Autoclave and store at room temperature. Prepare a working solution by diluting ten-fold with sterile water.
2. Paraformaldehyde (PFA) Stock: Prepare a 4% Paraformaldehyde stock solution as follows: 40 gm paraformaldehyde (Fisher) in 400 mL water. While stirring heat to 60 °C. Add 1 N NaOH dropwise until paraformaldehyde is dissolved. Adjust to 500 mL, filter through a 45 μm vacuum filter (Fisher), aliquot into 50 mL conical tubes and store frozen at -20 °C for up to one year. Once thawed, use within one week. Alternatively, purchase pre-made 16% PFA in 10 mL ampoules (Electron Microscopy Supplies).
3. 1× Fixative: 4% PFA in 1× PBS.
4. Sylgard-coated Petri dishes: Mix Sylgard 184 Silicone Elastomer (Fisher) parts A and B in 10:1 ratio (w/w). Fill Petri dishes (10 cm and 3.5 cm) one-third full and incubate at 50 °C overnight, or until hardened.
5. Dumont No. 5 forceps (Fisher).
6. Angled scissors: e.g., Supercut 10 cm angled scissors (Fisher).

7. Proteinase K: Make a 10 mg/mL proteinase K stock solution (PCR grade; Roche) in PBT. Store 500 μ L aliquots at -20°C . Final working concentration will be 50 $\mu\text{g}/\text{mL}$.
8. 100% Methanol.

2.2 Probe Synthesis

1. Purified DNA template: *nanos2* cDNA clone pBD77 [1], linearized with SpeI enzyme (NEB) (*see Note 1*).
2. PCR purification kit (Thermo Scientific).
3. 10 \times DIG RNA labeling mix (Roche).
4. T7 RNA polymerase (Roche).
5. Dithiothreitol (DTT)(Sigma).
6. RNase inhibitor (Roche).
7. 0.5 M EDTA, pH 8.0.
8. 5 M Lithium Chloride.
9. 100% Ethanol.
10. tRNA (RNase free): Resuspend tRNA, Type V, from wheat germ (Sigma), in water and extract 3 \times with phenol:chloroform to remove proteins (or until no precipitate is visible at the interphase). Precipitate tRNA by adding 1/tenth volume 5 M LiCl, 3 \times volume 100% Ethanol, and store at -20°C for 30 min before pelleting in a centrifuge. Resuspend at 50 mg/mL in RNase-free water.
11. 5 \times RNA loading dye: 0.25% bromophenol blue (w/v) (Sigma), 0.25% xylene cyanol FF (w/v) (Sigma), 1% sodium dodecyl sulfate (SDS; v/v) (Sigma) and 30% glycerol (v/v).
12. 1% Agarose 0.5 \times TBE gel.

2.3 *nanos2* in Situ Hybridization

1. PBT: 1 \times PBS, 0.1% (v/v) Triton X-100 (Sigma).
2. Deionized formamide (IBI Scientific).
3. 20 \times SSC: Dissolve 175.3 g NaCl, 88.2 g citric acid trisodium salt in 1 L water. Autoclave.
4. 50 mg/mL Heparin: Heparin sodium salt from porcine intestinal mucosa (Sigma), dissolved in sterile water.
5. 1 M Citric Acid.
6. Pre-hybridization solution: 50% deionized formamide, 5 \times SSC, 50 $\mu\text{g}/\text{mL}$ Heparin, 500 $\mu\text{g}/\text{mL}$ RNase-free tRNA, 0.1% Tween, pH to 6.0 by adding 460 μL 1 M Citrate/50 mL pre-hyb. Store indefinitely at -20°C .
7. Hybridization Solution: In a 5 mL snap-cap tube, mix 1.8 mL prehybridization solution, 200 μL 50% dextran sulfate (5% final concentration; *see Note 2*) and 10 μL *nanos2* RNA probe (1:200 final dilution). Store indefinitely at -20°C .

8. 50% Dextran sulfate solution (Amresco).
9. Antibody Blocking solution: PBT, 2% sheep serum (v/v) (Sigma), 2 mg/mL Bovine serum albumin, fraction V, 99% pure)(Sigma).
10. AP-Anti-DIG: Sheep anti-DIG FAb conjugated to alkaline phosphatase (Roche).
11. Alkaline coloration buffer: 100 mM Tris, pH 9.5, 50 mM MgCl₂, 100 mM NaCl, 0.1% Tween 20.
12. SigmaFast™ Fast Red TR/Naphthol AS-MX tables (Sigma).
13. Labeling solution: Dissolve one Fast Red tablet in 1 mL Alkaline coloration buffer just before use.

2.4 Vasa Immunofluorescence

1. Primary antibody: rabbit anti-Vasa polyclonal sera (Knaut, 2000 #19).
2. Secondary antibody: Alexa Fluor 594 goat anti-rabbit IgG (Invitrogen).
3. Nuclear stain: 4',6-diamidino-2-phenylindole (DAPI; Fisher): Prepare a 10 mg/mL stock in water and store in dark at 4 °C.

2.5 Mounting and Confocal Microscopy

1. Glycerol: Graded glycerol (Sigma) series (30%, 50%, and 75% diluted in PBT).
2. Vacuum grease (Dow-Corning).
3. 25 × 75 × 1 mm Microscope slides (Fisher).
4. 22 × 22 mm coverslips (Fisher).

3 Methods

In adult zebrafish the ovarian germline stem cells (GSCs) localize to the germinal zone, a discreet circumferential band that runs along the lateral and medial surfaces of the ovary, oriented parallel to the anterioposterior axis [1, 2]. However, because of the large size of the adult gonad it can be difficult to reliably identify the germinal zone. By contrast, GSCs can more easily be identified in the juvenile ovary (40–50 dpf) because it is relatively planer and most of the GSCs localize to its margins, the presumed precursor to the germinal zone, and thus are easier to image using the confocal microscope [1]. In males, the germ cells are organized into spherical “lobules” and the germline stem cells as well as clones of various stage spermatocytes, localize predominantly to the surface of the lobules, whereas mature sperm are found interior [1]. In preparation for this procedure, it is important to note that the zebrafish ovary, in particular, is very fragile so it is necessary that they be fixed while still in the body cavity in order to maintain their

ultrastructure. While the testes are less delicate, they tend to curl if dissected prior to fixation, making imaging more challenging. For these reasons it is recommended that both testes and ovaries be fixed while in situ.

The method outlined below details how to identify *nanos2*-expressing GSCs using fluorescent RNA in situ hybridization, coupled with an anti-Vasa antibody staining to label all premeiotic germ cells and early-stage oocytes. In addition, all nuclei are counterstained with the DNA dye DAPI, which allows accurate germ cell staging based on nuclear morphology.

3.1 Preparation of Template DNA

1. If using the *nanos2* cDNA plasmid as a template, linearize 10 μg by digestion with SpeI restriction enzyme in a volume of 50 μL . To ensure complete digestion, incubate at 37 °C for a minimum of 2 hrs. Assess the completeness of digestion by running 1 μL of the digest on a 0.8% TBE gel with un-digested plasmid as a control. Alternatively, a DNA template can be produced by PCR (*see Note 1*).
2. Purify linearized template using a PCR purification kit. Elute in 30 μL RNase-free elution buffer and then dilute to 500 ng/ μL .

3.2 RNA Probe Synthesis

1. Assemble the components listed in Table 1 in the designated order in an RNase-free 1.7 mL snap-cap tube (*see Note 3*).
2. Incubate at 37 °C for 1 h.
3. Add an additional 1 μL of T7 RNA polymerase and incubate for an additional one hour at 37 °C.
4. Stop the transcription reaction by adding 0.8 μL 0.5 M EDTA, pH 8.0.

Table 1
In vitro transcription reaction component for in situ probe synthesis

Component	Volume
H ₂ O (RNase-free)	11.5 μL
10 \times Transcription buffer	2 μL
10 \times DIG Labeling mix	2 μL
Linearized DNA template (500 ng/ μL)	2 μL
100 mM DTT	1 μL
RNase inhibitor	0.5 μL
T7 RNA polymerase	1 μL
V _t	20 μL

5. Remove 1 μL of the reaction to run on a 1% agarose TBE gel containing ethidium bromide (or appropriate nucleic acid stain), using the RNA loading buffer, and an appropriate DNA molecular weight marker (e.g., 1 kb ladder). Because this is not a denaturing gel, the RNA will usually run as multiple bands, with one band being significantly brighter than the others. All RNA bands will run below the template DNA band, and the brightest RNA band should be at least 3 \times brighter than the DNA template band. Importantly, all bands should be discrete, and you should not see a “smear,” which would indicate degraded RNA. The *nanos2* RNA probe made with the pBD77 plasmid template has a major band at 2 kb, and a minor band at 3.5 kb.
6. While the gel is running, precipitate the RNA probe as follows: Add 1 μL 50 mg/mL RNase-free tRNA, 2 μL 5 M LiCl, and 75 μL 100% ethanol. Gently mix and incubate at -20°C for 30 min. Pellet precipitated RNA using a 4°C -cooled microcentrifuge (20 min at 14 K RPM). Rinse pellet with 75% ethanol. Let pellet slightly dry for 5 min. at room temperature. Resuspend the RNA probe in 100 μL pre-hybridization solution and store at -80°C indefinitely.

**3.3 Preparation of
Gonads for in Situ
Hybridization and
Indirect
Immunostaining**

Day 1

1. Fix gonads in situ overnight in $1\times$ PBS/4% paraformaldehyde as follows: First, euthanize fish by immersion in 0°C ice water for a minimum of 5 min (hypothermal shock). Next, decapitate fish by cutting just posterior to the gills using angled scissors. Finally, cut open the body cavity along the ventral midline, starting at the anterior and proceeding to the cloaca. To ensure that the tissue is properly and rapidly fixed, place no more than ten 40–50 day-old fish/30 mL of fixative, or no more than three adults/30 mL of fixative.

Day 2

2. Following fixation, wash fish 3×10 min in PBT.
3. Dissect gonads from the body cavity using a Sylgard-coated Petri-dish flooded with PBT and No. 5 Dumont forceps. It is useful to stabilize the fish by impaling one set of forceps into the tail just posterior to the cloaca. Using the other set of forceps, grasp the skin and body wall muscle close to the midline and peel skin and muscle off the torso to expose the gonads. The ovaries and testes are bilaterally located dorsal to the stomach and liver and ventrolateral to the swim bladder. Both ovaries and testes may have a layer of lipid covering their dorsal surface after dissection. This should be gently removed so that the tissue will sink instead of float.
4. Wash dissected ovaries in PBT 3×15 min.

5. Methanol permeabilization: Rinse ovaries 2×5 min in 100% MeOH, then add fresh 100% MeOH and incubate for 1 hr. at -20°C . Next, rehydrate ovaries in a graded MeOH series in (70%, 50%, 30% MeOH in PBT) 10 min each for 40–50 dpf gonads and 20 min each for adult gonads (*see Note 4*). Wash 3×5 min in PBT.
6. Proteinase K permeabilization: Place ovaries in a solution of $10\ \mu\text{g}$ proteinase K/mL PBT for 10 min at 37°C (*see Note 5*).
7. Inactivate the proteinase K by re-fixing ovaries in 4% PFA/1 \times PBS for 20 min.
8. Wash ovaries 3×10 min in PBT.

3.4 Whole-Mount In Situ Hybridization

1. Pre-hybridization: Place permeabilized gonads into a 1.7 mL snap-cap tube and add 1 mL of room temperature pre-hybridization solution. If large adult-size ovaries are being hybridized, place them into 5 mL snap-cap tubes (Falcon) and use 2–3 mL pre-hybridization solution (enough to cover tissue). Place tubes into a floating rack in a 65°C circulating water bath. Incubate for a minimum of 1 h.
2. Pre-heat hybridization solution to 65°C before use.
3. After removing and discarding the prehybridization solution from the tubes, add enough pre-heated hybridization solution to the tubes to cover all the tissue (typically $500\ \mu\text{L}$ –1 mL). Replace tubes into the 65°C water bath and incubate overnight (minimum 16 h). Because the hybridization solution contains dextran sulfate the tissue will initially float to the top of the solution but will eventually sink as it equilibrates.

Day 3

4. Make the solutions in Table 2 and pre-heat to 65°C :
5. Remove and save the hybridization solution: Carefully remove the hybridization mix and add back to any mix that remained in the probe stock tube (*see Note 6*). Store at -20°C .

Table 2
Post-hybridization washes

Wash duration	Solution	Temperature
1 \times 5 min	1:2 mixture of Prehybridization: 2 \times SSC	65°C
1 \times 5 min	2:1 mixture of Prehybridization: 2 \times SSC	65°C
1 \times 5 min	2 \times SSC	65°C
2 \times 20 min	0.2 \times SSC	65°C
1 \times 20 min	0.1 \times SSC	65°C
1 \times 5 min	1:1 mixture 2 \times SSC; PBSTw	RT
2 \times 5 min	PBSTw	RT

6. Wash the gonads as outlined in Table 2. Once the washes reach the room temperature steps, the tubes should be gently rocked using a rotating platform.

3.5 Antibody Staining

1. Incubate the gonads in Antibody Blocking solution for 1 h.
2. 1° antibody labeling: Incubate gonads overnight at 4 °C in Antibody Blocking solution containing goat anti-DIG FAb (1:5000 dilution) and rabbit anti-Vasa antibody (1:2000 dilution). Tubes should be gently rocked on a rotating platform.

Day 4

3. Remove 1° antibody and discard. Wash 2 × quickly, then 4 × 15 min, then 2 × 30 min in PBT at room temperature with gentle rocking.
4. Incubate the gonads in Antibody Blocking solution for 1 h.
5. 2° antibody labeling: Incubate gonads overnight at 4 °C in Antibody Blocking solution containing goat anti-Rabbit Alexa Fluor 594-labeled antibody (1:300 dilution).

Day 5

6. Remove 2° antibody and discard. Wash 2 × quickly, then 4 × 15 min, then 2 × 30 min in PBT at room temperature with gentle rocking.
7. Develop the *nanos2* in situ: Wash 3 × 10 min in Coloration buffer.
8. Dissolve one tablet of SigmaFast Fast Red in 1 mL of Coloration buffer, and then add this to gonads. While the reaction is developing, the tubes should be kept in the dark, either in a drawer or wrapped in foil.
9. Allow the *in situs* to develop in the dark for a minimum of 2 hrs and a maximum of 4 hrs. Monitor the reactions using a dissecting microscope with top lighting. At this stage it is useful to transfer the gonads to a watch glass. The signal will appear as punctate red dots. However, because germline stem cells are a very small fraction of the total cell population, it may be difficult to identify them at low magnification.
10. Stop the reaction by washing 2 × quickly in water, then 4 × 15 min, then 2 × 30 min in PBT + 2 µg/mL DAPI at room temperature with gentle rocking.
11. Place gonads in 30% glycerol in PBT + 2 µg/mL DAPI and incubate overnight at 4 °C. This step assures that the nuclei are properly stained with DAPI.

3.6 Mounting Ovaries for Confocal Analysis

Day 6

1. To mount ovaries for microscopy, the gonads are cleared by further dehydrating in a glycerol series, as follows (*see Note 7*):
 - (a) 50% glycerol in PBT + 2 $\mu\text{g}/\text{mL}$ DAPI, 1 hr. at room temp. (minimum).
 - (b) 75% glycerol in PBT, 1 hr. at room temp. (minimum).

The gonads will initially float in the 50% glycerol solution but will sink as they equilibrate. By contrast, after equilibration in the 75% glycerol solution gonads may not sink.

2. Mount gonads on microscope slides in 75% glycerol/PBT as follows: Using a 3 mL syringe filled with vacuum grease, place four spots (“post”) on a microscope slide in a square pattern that is just smaller than the coverslip. Place the gonad in the middle of the post in a small volume of 75% glycerol/PBT. Ovaries should be arranged dorsal side up for 40–50 dpf (*see Note 8*). If mounting an adult ovary, it will be necessary to carefully dissect off the lateral surface while leaving the most mature oocytes behind. This is best done with sharpened tungsten needles. Finally, place a coverslip on the vacuum grease post and, while viewing under a dissection microscope, gently depress coverslip until it is in contact with, and slightly depresses, the gonads. Care should be taken to not over-distort the tissue. Add enough 75% glycerol/PBT to fill underneath the coverslip.
3. Collect images of the stained sample using a confocal microscope equipped with 405, 488, and 543 lasers to visualize the DNA, *nanos2* mRNA, and Vasa protein, respectively. Figure 1 shows representative images of *nanos2*- expression GSCs (red) and anti-Vasa stained (green) which labels all germ cells. In addition, all nuclei are counterstained with DAPI to allow accurate staging based on nuclear morphology. For staging purposes, it is recommended to present the nuclear staining (DAPI) as a black-and-white image for maximum contrast (*see Fig. 1c and f*).

4 Notes

1. Because *nanos2* is a single exon gene, template DNA can be easily produced by PCR using genomic DNA and the following primers: nos2 Fwd: CATGGGCAAACACACCTAAAACA / T7-nos2-Rev: GCGTAATACGACTCACTATAGGGGAAG CTGCAGACCTGAAACAAAT (T7 promoter is underlined). For detailed method, see Thisse and Thisse, (2014) [7].

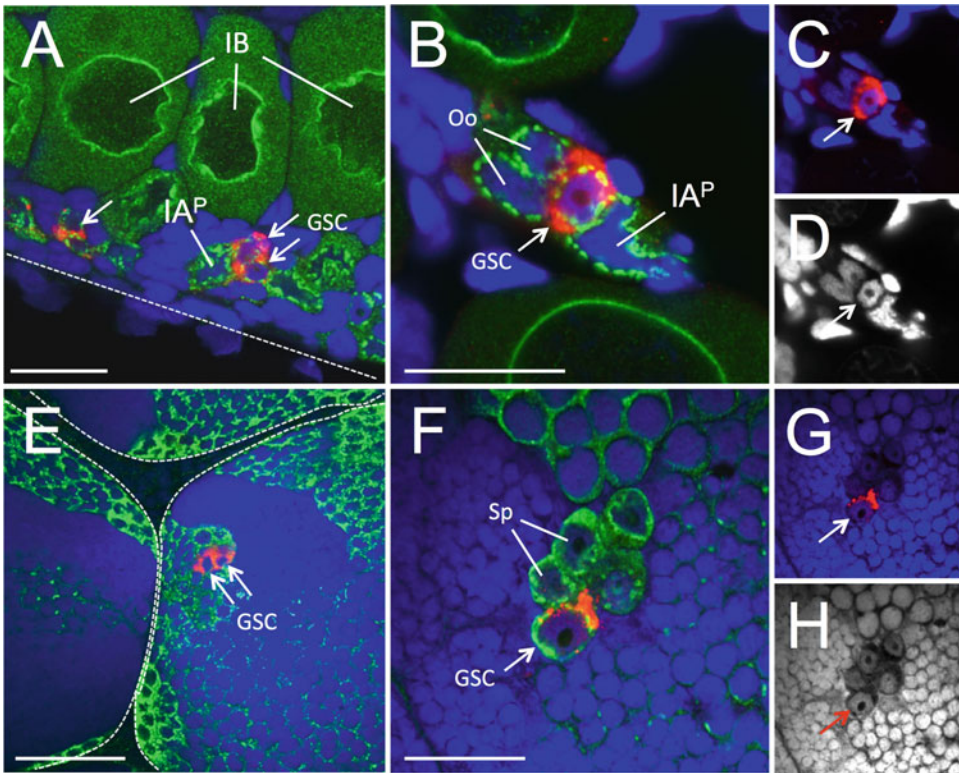


Fig. 1 Identification of germline stem cells in the zebrafish ovary and testis. Merged panels (a–d) show *nanos2* RNA (red), Vasa protein (green), and DNA (blue). Panels c and g show merged *nanos2* and DNA only. Panels D and H show DNA only. Low magnification views of *nanos2*-positive germline stem cells (GSC; arrows) that localize to the lateral edge of a 40-day-old ovary (outlined in a) or on the surface of a testicular lobule (outlined in e). Higher magnification views of a single *nanos2*-positive ovarian (b) or testicular (f) GSC within clusters of *nanos2*-negative premeiotic germ cells and early meiotic cells. Note that premeiotic germ cells can be identified as having nuclei with a single prominent nucleolus, which appears as holes in the staining. (Abbreviations: GSC germline stem cell; Oo oogonia; Sp spermatogonia; IAP^p pachytene- stage IA oocytes; IB stage IB oocytes; Scale bar: 20 μ m in a, b, and f, 50 μ m in e)

2. Dextran sulfate is a molecular crowding agent that increases the effective probe concentration in the hybridization solution. This helps to increase the signal without increasing the noise.
3. If using a template that was produced by PCR, it must be first purified using a PCR purification kit. Then use 250 ng PCR product/transcription reaction.
4. Gonads can be stored for several months in 100% MeOH at -20° C without significantly affecting this procedure.
5. Each batch of proteinase K must be tested for optimal concentration to use in this step.
6. Hybridization solution can be reused multiple times with little loss of activity. In fact the signal-to-noise ratio often improves after repeated use.

7. The precipitate that is formed by the alkaline phosphatase enzyme using the Fast Red substrate is slightly water-soluble. Because of this, it is important to image your samples soon after developing them to maintain maximum fluorescence intensity (no more than two days – post development).
8. 40–50 day-old ovaries, while mostly planer, have a slight curvature such that the convex and concave surfaces represent the dorsal and ventral surfaces, respectively. Once ovaries are on the slide, they can be flipped and positioned using fine eyelashes glued to toothpicks (hairs from short-haired dog breeds work well also).

Acknowledgments

The author would like to thank Holger Knaut for the anti-Vasa antibodies. This work was supported by a NIH grant R01-HD081551.

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Chapter 11

Characterization of the Postnatal Naked Mole-Rat Ovary: From Primordial Germ Cells to Meiotic Prophase I Oocytes

Miguel Angel Brieño-Enríquez

Abstract

The mammalian reproductive cycle, including those of humans and mice, begins very early in development. In utero, the ovaries become populated with primordial germ cells (PGCs) that will generate the oogonia. First, these cells proliferate mitotically, and then they trigger the meiotic program and initiate meiotic prophase I. Since these processes happen during gestation, their study had been very limited and challenging. Recently, we reported that, in the naked mole-rat (*Heterocephalus glaber*) ovary, there is mitotic expansion of the PGCs, and the initiation of the meiotic program occurs postnatally. In this chapter, we present a comprehensive collection of protocols that permit the analysis of naked mole-rat germ cells, from PGCs to oocytes, in meiotic prophase I, using in vivo and in vitro approaches.

Key words Naked Mole-Rat, Ovary, Primordial Germ Cells (PGCs), Meiosis, Prophase I, Culture, Ovarian Reserve, Mitotic Expansion, SOX2, OCT4, BLIMP1

1 Introduction

The adult mammalian ovary is devoid of definitive germline stem cells. As such, female reproductive senescence largely results from the depletion of a finite ovarian follicle pool that is produced during embryonic development [1]. To date, the vast majority of research on the factors that influence the establishment and maintenance of the ovarian reserve has been performed on mice, which have relatively short reproductive lifespans, measured in terms of months, while studies in humans are expensive and ethically challenging. On top of this, access to samples and the quantity/quality of the cells/tissues is very limited. In the ovary, the primordial follicles, each of which contains an oocyte surrounded by a single layer of somatic pre-granulosa cells, represent the entire ovarian reserve that a female mouse, or a woman, will ever possess. In humans and mice, these follicles are produced from a pool of primordial germ cells (PGCs), which are localized to the developing gonad early in

gestation [1, 2]. PGCs within the ovary will continue their mitotic divisions. These divisions are characterized by incomplete cytokinesis, producing an excess of interconnected oogonia, forming clusters of germ cells. Later, the PGCs enter into meiotic prophase I, where they remain arrested. The clusters of germ cells, called “cysts,” then begin to undergo “breakdown,” in which most of the oocytes are lost through apoptotic cell death, and the remaining oocytes become surrounded by a layer of somatic pre-granulosa cells, forming “primordial follicles” during mid-gestation; these comprise the ovarian reserve [3, 4]. Thus, a key determinant of the ovarian reserve is the total number and quality of the PGCs in the ovary.

The naked mole-rat is well known for being the longest-lived rodent, with a maximum lifespan of >37 years [5–7]. Female naked mole-rats are exceptional because they demonstrate no decline in fertility or fecundity during their entire lives [5, 8]. Almost none of the individuals in a naked mole-rat colony will ever be reproductively active [9]; however, those that become dominant breeding females (queens) will breed until they die [10]. This is very relevant since reproductive longevity is relatively limited in female mammals of other species, including humans, whose ovaries harbor a certain number of oocytes that progressively decreases with age. By 30 years of age, women will have already lost 90% of their oocytes, while at menopause, only 100–1000 oocytes remain [11]. Recently, we reported that the postnatal naked mole-rat ovary has a population of germ cells positive for the germ cell marker VASA (a.k.a DDX4), the pluripotency transcription factors SOX2 and OCT4, and the PGC maker BLIMP1 [12]. Importantly, in the postnatal naked mole-rats ovary, there is a concurrence of germ cells at very different stages of development, going from pre-meiotic cells, cells at meiotic prophase I, to follicles, similar to what is described in human fetal ovaries. However, and in frank opposition to humans, these different stages of germ cell development can be detected up to at least 10 years after birth. Cultures from ovaries at early postnatal ages show that germ cells positive for VASA, SOX2, and OCT are capable of mitotic expansion *in vitro*.

In this chapter, we present a compilation of protocols which have been developed to analyze the ovarian reserve, meiotic prophase I progression, and the VASA+SOX2+ germ cell mitotic expansion *in vitro* (see Fig. 1).

2 Materials

2.1 Collection of Ovaries

1. Cold (4 °C) phosphate buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, and 2 mM KH₂PO₄, pH 7.4. PBS is certified RNase-free.
2. Cold (4 °C) 10% neutral buffered formalin.

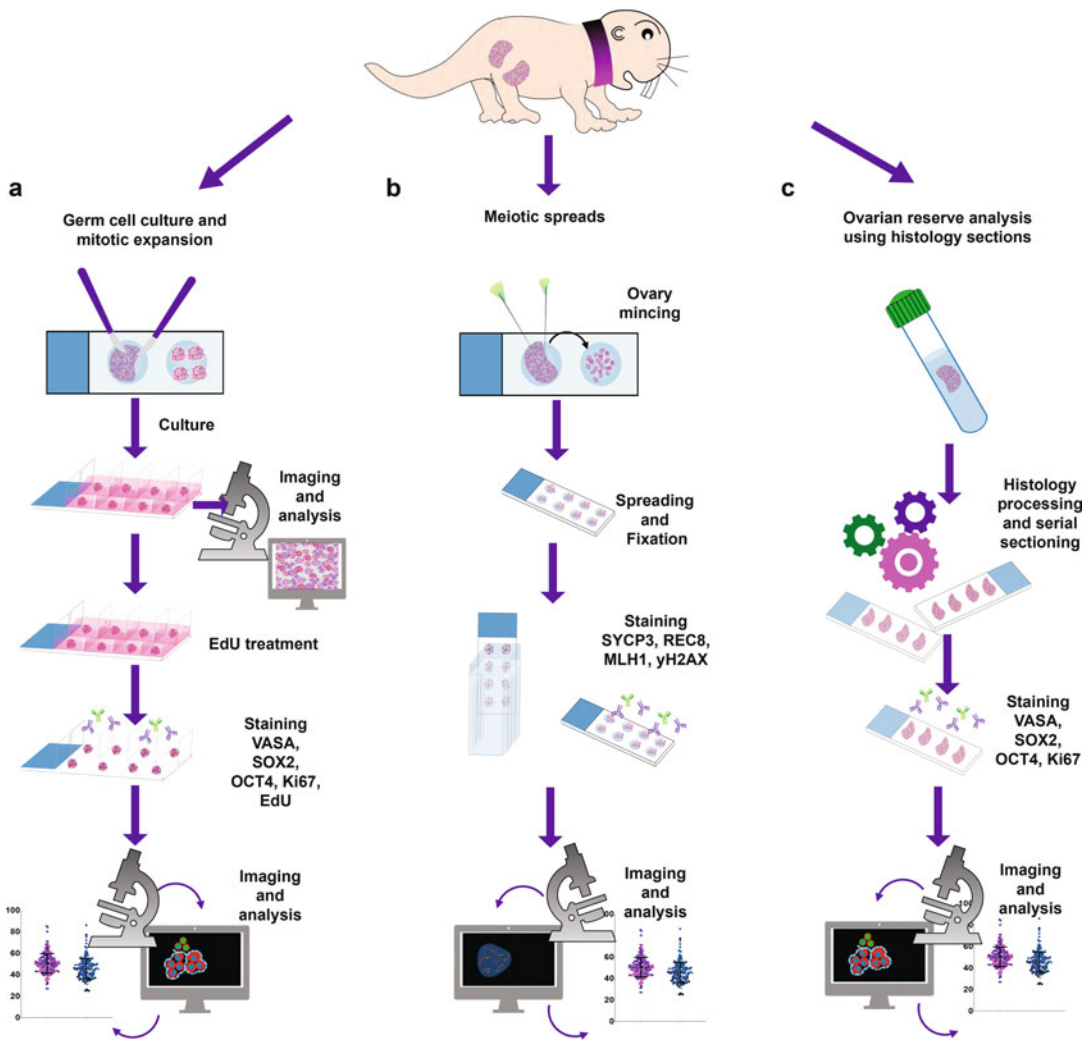


Fig. 1 Schematic diagram of the experimental design. **(a)** Germ cell culture and mitotic expansion; **(b)** meiotic spread procedure; and **(c)** ovarian reserve analysis using histology serial sections

2.2 Processing of Ovaries for Culture and Spreads

1. Cold (4 °C) PBS, sterile.
2. Stereomicroscope.
3. Dumont Tweezers #5.
4. Vannas scissors.
5. Iris forceps, curved, serrated.
6. Dissecting scissors, 10 cm, straight.
7. Scalpel and scalpel blades #11.
8. Needles, 23G×1 (0.6 mm × 25 mm), sterile.

9. Penicillin-streptomycin, with 10,000 units penicillin and 10 mg streptomycin per ml in 0.9% NaCl, 0.1 μ m, filtered.
10. Culture medium, D-MEM, high-glucose, GlutaMAX supplement, supplemented with 100 IU/mL penicillin (Gibco BLR) and 100 μ g/mL streptomycin (Gibco BLR) and 15% fetal bovine serum (FBS).
11. Falcon 35-mm Not TC-Treated, Easy-Grip Style bacteriological Petri dish, sterile.

2.3 Processing Ovaries for Ovarian Reserve Analysis and Histology Sections

1. Ethanol-water 70%.
2. 15-mL Falcon tubes.
3. Benchtop orbital shaker.

2.4 Oocyte Spreading for Immunofluorescence

1. Hypotonic extraction buffer (HEB): Tris-HCl (pH 8.2 1 M, final concentration 30 mM), Sucrose, final concentration 50 mM), Citrate trisodium, dehydrate (final concentration 17 mM), EDTA 0.5 (5 mM final concentration), DTT 0.5 M, 0.5 mM final concentration), PMSF 10 mg/mL (final concentration 0.1 mM), milliQ water. pH 8.2 (*see Note 1*).
2. Sucrose solution (100 mM).
3. Fixative solution: 1% paraformaldehyde, pH 9.2. Triton \times 0.05% (*see Note 2*).
4. Washing solution 1: 0.05% Kodak Photo-Flo 200 (Electronic Microscopy Sciences) in PBS.
5. Washing solution 2: 0.05% Kodak Photo-Flo 200 (Electronic Microscopy Sciences) in water.
6. Hydrophobic printed 8-well slides, round, 6 mm.
7. Humidity chamber.
8. Needles, 23G \times 1 (0.6 mm \times 25 mm), sterile.
9. Dumont tweezers #5.
10. Corning Pyrex 9-well glass spot plates.
11. Coplin jars.
12. Benchtop orbital shaker.

2.5 Meiotic Spreads Immunofluorescence

1. Antibody dilution buffer (ADB): Bovine serum albumin (BSA, 3 g), goat serum (10 mL), 20% Triton \times (1 mL), 10X PBS (10 mL), adjust with water to 100 mL (*see Note 3*).
2. Washing solution 1: 0.05% Kodak Photo flo 200 in PBS.
3. Washing solution 2: 0.05% Kodak Photo flo 200 in water.
4. Permeabilization solution: 0.005% Triton-X in PBS.
5. Blocking solution: 10% ADB in PBS.

6. Antibodies: dilution of antibodies, both primary and secondary, is made in ADB.
7. Vectashield mounting medium, containing 0.1 µg/mL of 4',6-diamidino-2-phenylindole (DAPI).
8. Parafilm.
9. 22- × 60-mm coverslips.
10. Coplin jars.

2.6 Culture of Ovaries

1. Cold (4 °C) IXPBS, sterile.
2. Stereomicroscope.
3. Dumont tweezers #5.
4. Vannas scissors.
5. Scalpel and scalpel blades #11.
6. Penicillin-streptomycin, with 10,000 units penicillin and 10 mg streptomycin per mL in 0.9% NaCl, 0.1 µm, filtered.
7. Culture medium, D-MEM, high-glucose, GlutaMAX Supplement, supplemented with 100 IU/mL penicillin (Gibco BLR) and 100 µg/mL streptomycin (Gibco BLR) and 15% FBS.
8. Falcon 35-mm Not TC-Treated, Easy-Grip Style bacteriological Petri dish, sterile.
9. 8-well-chambered cell culture slide.
10. 24-well glass-bottom tissue culture plate, sterile.
11. Cell culture incubator.
12. Biological safety cabinet, Class II A2.
13. Inverted microscope with Differential interference Contrast (DIC) capabilities.
14. Click-iT EdU Cell Proliferation Kit for Imaging, Alexa Fluor 647 dye.

2.7 Fixation of Ovarian Cultures

1. Fixative solution: 1% paraformaldehyde, pH 9.2. Triton × 0.05% (*see Note 2*).
2. Washing solution 1: 0.05% Kodak Photo flo 200 in PBS.
3. Washing solution 2: 0.05% Kodak Photo flo 200 in water.

2.8 Immuno-fluorescence of Ovarian Cultures

1. PBS, 2% BSA, 0.2% goat serum and 0.05% Tween 20, 0.1% Triton X.
2. Washing solution 1: 0.05% Kodak Photo flo 200 in PBS.
3. Washing solution 2: 0.05% Kodak Photo flo 200 in water.
4. Permeabilization solution: 0.05% Triton-X in PBS.

5. Blocking solution: 10% ADB in PBS.
6. Benchtop orbital shaker.
7. 0.1 µg/mL of DAPI.

2.9 Clearing of Ovarian Cultures

1. Prepare ScaleS (0) clearing solution: 40% D(-) sorbitol (w/), 10% glycerol, 4.3 M urea, and 20% dimethyl sulfoxide (DMSO), pH 8.1

2.10 Embedding of Ovary in Paraffin for Histology Sections

1. Ethanol-water (v/v) 70%, 80%, and 100%.
2. Xylene.
3. Tissue infiltration medium paraffin.
4. Embedding cassettes.

2.11 Immuno-fluorescence on Ovarian Histology Sections

1. Ethanol-water (v/v) 70%, 80%, 90%, and 100%.
2. Xylene.
3. HOC(COONa)(CH₂COONa)₂ · 2H₂O (sodium citrate, pH 6.0).
4. Permeabilization solution: 0.2% of Triton-X 100 in PBS.
5. Blocking solution: 2.52 mg/mL glycine, 10% goat serum, 3% BSA, 0.2% Tween 20 in PBS.
6. Washing solution: 0.1% Tween 20 in PBS (PBS-T).
7. Counter staining solution: 0.1 µg/mL of DAPI.
8. Coplin jars.

3 Methods

3.1 Collection of the Ovaries

All experimental procedures followed federal and institutional guidelines and were approved by the University of Pittsburgh Animal Care Committee and University of Toronto Mississauga Animal Care Committee. Naked mole-rat colonies (opposite-sex pairs) were housed in a single, medium polycarbonate cage. Naked mole-rats were kept on a 12:12 light/dark cycle at 28–30 °C and fed ad libitum with a diet consisting of sweet potato and wet 19% protein mash (Harlan Laboratories). Dissection of the ovaries can be done at room temperature by following these steps:

1. Female naked mole-rats can be euthanized by decapitation according to IACUC guidelines. Decapitate one animal at a time. Postnatal age should be determined according to what population of cells needs to be enriched. The Postnatal-Day-1 (P1) ovary has a large population of primordial germ cells (PGCs). The Postnatal-Day-5 (P5) ovary has both PGCs and cells initiating the meiotic program. P8 has a large population of meiotic cells at all stages of meiotic prophase and PGCs.

2. After decapitation, spray the animal with 70% ethanol and place it on a paper towel. Use tape on superior and inferior limbs to maintain the body in the correct position.
3. Collection of ovaries will vary, depending on the final purpose. For culture and meiotic spreads, prepare a 1.5-mL Eppendorf tube with cold PBS. For ovarian reserve and histology section analysis, prepare a 1.5-ml Eppendorf tube with fixative solution ((4 °C) 10% neutral buffered formalin).
4. With the Dumont tweezers #5 and the Vannas scissors, make an incision that allows you cross the skin and get into the abdominal cavity. The ovaries are located in the posterior wall of the abdomen. To reach them, move the intestines out of the abdominal cavity.
5. To localize the ovaries, first look for the uterine horns and, with the Dumont tweezers, follow them up. Ovaries will be at the end, semi-covered by the kidney. At this postnatal age, the amount of fat or other connective tissue surrounding the ovary is very small.
6. After localizing the ovaries, pinch the uterine horn with the tweezers and pull it gently. Using the Vannas scissors, cut the uterine horn 2 mm from the ovary and pull. The ovary should come as a block. Use the residual tissue from the uterine horn as a flap to handle the ovary without damaging it (*see Note 4*).
7. Place the ovary in either cold PBS or fixative solution ((4 °C) 10% neutral buffered formalin). Ovary fixation should be performed at 4 °C overnight and the ovary then washed 3 times for 15 min with 70% ethanol. Once the tissue has been washed, it can be processed for histological sectioning.
8. Ovaries that will be used for spreads or culture should be kept at 4 °C. Ovaries at 4 °C can be immediately used or shipped to other laboratories to perform cultures or spreads (*see Note 5*).

3.2 Oocyte Spreading for Immunofluorescence

This protocol was based on a combination of protocols developed to perform mouse spreads from ovaries [13, 14] and testis [15–17], as well as on ovarian human spreads [18–21].

1. Prepare the fixative solution (*see Note 2*).
2. Place the hydrophobic 8-well printed slide, on the humidity chamber and 50 µL of fixative solution of each printed well (*see Note 5*).
3. Prepare the HEB solution and store it on ice until use (*see Note 1*).
4. Wash the ovaries with fresh, cold PBS.
5. Under the stereomicroscope and using the 23G needles, remove any remaining uterine horn from the ovary.

6. Place the ovary on 1 mL of HEB and incubate for 10 min. If it is necessary to do culture and spreads from the same ovary (*see Note 6*).
7. Remove the ovary from HEB and place it on Corning Pyrex 9-well glass spot plates.
8. Wash the ovary with the sucrose solution (100 mM) twice (*see Note 7*).
9. Place the ovary in a new well and add 50 μ L of sucrose solution.
10. Mince the ovary; using the 23G needles, try to create a homogeneous solution. You should see a milky solution coming from the ovary. Mincing can be done with scalpels, but the use of needles facilitates the process.
11. Remove the milky solution and place it in a new well. Large pieces of ovary should be left behind.
12. Add 50 μ L of sucrose solution. If the solution is too milky, add another 50 μ L of sucrose solution. The solution needs to be slightly milky, and not completely clear (*see Note 8*).
13. Homogenize the solution and take 10 μ L of the oocyte-sucrose solution and add it to the 50 μ L of fixative solution, which was preloaded to the hydrophobic printed 8-well slide, in the humidity chamber.
14. Close the humidity chamber and let them sit overnight at room temperature.
15. After overnight incubation in the humidity chamber, open the lid and let them dry for 1 h. Do not allow the slides to get fully dry (*see Note 9*).
16. Using the benchtop orbital shaker, wash the slides with washing solution 1, twice, for 15 min each.
17. Replace the solution with washing solution 2 and wash the slides for 15 min.
18. Air dry the slides and either process for immunofluorescence or store at -80 °C. Best results have been obtained with fresh slides; however, slides can be used with good results up to 1 year after processing (*see Note 10*).

3.3 Meiotic Spread Immunofluorescence

1. Wash the slides with washing solution 1 using the benchtop orbital shaker. If frozen slides will be used, let them sit at room temperature for at least 15 min before starting the washes.
2. Using the benchtop orbital shaker wash, treat the slides with permeabilization solution for 30 min.
3. Block the slides, using the blocking solution for 1 h on the benchtop orbital shaker.

4. Dilute the antibodies in ADB, following the vendor recommendations (*see Note 11*).
5. Prepare the humidification chamber.
6. Place the slides in the humidification chamber. Do not allow them to become dry for a moment.
7. Add 25 μL of the ADB+ antibody solution to each one of the hydrophobic 8-well printed slides and incubate overnight at 4 °C. If all the wells on the slide will be tested with the same antibody combination, add 100 μL of the ADB+ antibody solution to the slide and cover the slide with parafilm (the size of the parafilm should be the same as the slide).
8. Wash the slides with washing solution 1 for 15 min using the benchtop orbital shaker.
9. Block the slides using the blocking solution for 30 min on the benchtop orbital shaker.
10. Dilute the secondary antibodies in ADB solution.
11. Add 100 μL of secondary antibody solution to each slide, cover it with parafilm (as was indicated in point 7), and incubate in the humidity chamber for 2 h at 37 °C. Protect from light.
12. While protecting the slides from light, remove the parafilm and wash the slides on the orbital shaker, using washing solution 1 three times for 15 min each.
13. Replace washing solution 1 with washing solution 2 and wash the slides for 15 min.
14. Remove the slides from the washing solution and let them dry, protecting them from light. The drying process normally takes 15–30 min.
15. Once the slides are dry, add, in the biological safety cabinet class II A2, 40 μL of Vectashield mounting medium containing 0.1 $\mu\text{g}/\text{L}$ of DAPI. Cover with 22- \times 60-mm coverslips and wait for 30 min.
16. After 30 min, remove the excess DAPI and analyze. Examples of naked mole-rat meiotic spreads are shown in Fig. 2.

3.4 Culture of Ovaries

1. Keep the P5 ovaries in PBS at 4 °C.
2. Using a stereo microscope, remove the residue of connective tissue and uterus.
3. In the Biological Safety Cabinet Class II A2, incubate the ovaries for 30 min with 150 μL of penicillin-streptomycin, with 10,000 units penicillin and 10 mg streptomycin per mL.
4. Rinse the ovaries with cold (4 °C), sterile PBS.

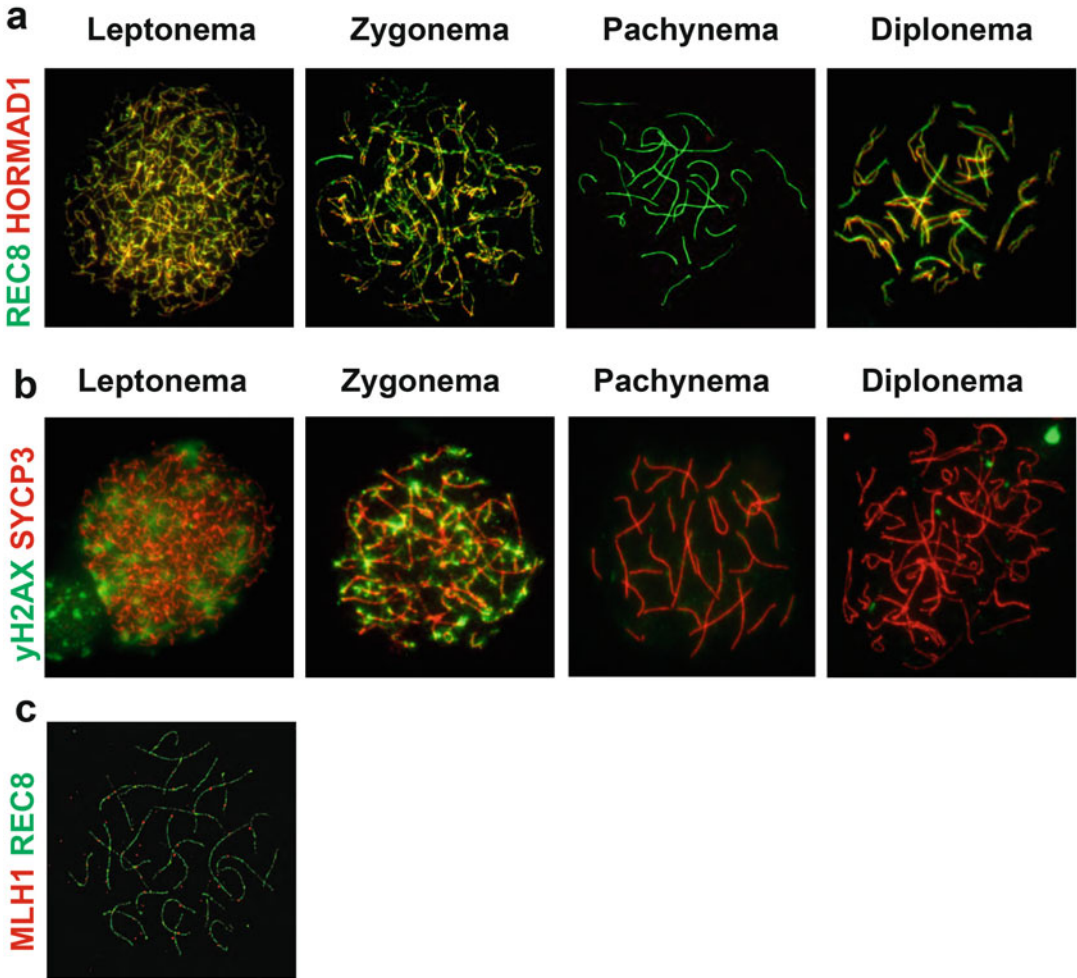


Fig. 2 Meiotic spreads of naked mole-rat ovaries. **(a)** Staining with meiotic cohesion REC8 (green) and HORMAD1 (red); **(b)** staining with the synaptonemal complex protein SYCP3 (red) and γ H2AX (green); **(c)** staining with MLH1 (red) and REC8 (green)

5. Place the ovary on a sterile Falcon 35-mm Not TC-Treated, Easy-Grip Style bacteriological Petri dish and add 100 μ L of cold sterile PBS.
6. Holding the ovary with Dumont tweezers #5, cut it, with the scalpel, into pieces of approximately 1 \times 1 \times 1 mm. Whole ovary culture is possible, following the same protocol.
7. Add 500 μ L of culture medium, D-MEM, high-glucose, GlutaMAX supplement, supplemented with 100 IU/mL penicillin (Gibco BLR) and 100 μ g/mL streptomycin (Gibco BLR) and 15% FBS.

8. Using a P1000 pipet, take the ovary pieces and seed them in an 8-well chambered cell culture slide or in plastic multi-well plates. Selection of the surface depends on the later analysis.
9. Add 300 μL of culture medium to each well and culture them at 32 °C and 5% CO_2 .
10. Every other day, remove 50% percent of culture media and replace with fresh medium.
11. Using an inverted microscope with DIC capabilities, cultures are evaluated at 5, 10, 15, 20, 30, 60, 90, and 120 days. Culture media can turn viscous; be careful (*see Note 12*).
12. To test cell proliferation, use Click-iT EdU Cell Proliferation Kit for imaging and Alexa Fluor 647 dye. Treatment with EdU should be performed 48 h before fixing the culture.

3.5 Fixation of Ovarian Cultures

1. Remove all the culture media from the wells.
2. Wash the wells with sterile PBS at room temperature, 3 times, for 15 min each, using the benchtop orbital shaker.
3. Add 400 μL of fixative solution and incubate overnight at 4 °C.
4. Wash the cultures, using 400 μL washing solution 1, twice, for 30 min each time, on the benchtop orbital shaker.
5. Wash the cultures with 400 μL washing solution 2, twice, for 10 min each time.
6. Replace the washing solution 2 with 400 μL of sterile PBS and either keep them at 4 °C or proceed to stain them.

3.6 Immuno-fluorescence of Ovarian Cultures

1. Remove the PBS and 400 μL of washing solution 1 and wash the cultures for 10 min.
2. Remove the washing solution 1 and add 400 μL of permeabilization solution and incubate overnight at 4 °C on the orbital shaker.
3. Block the cultures using blocking solution. Incubate overnight at 4 °C on the orbital shaker.
4. Prepare the primary antibodies, using ADB, and add 400 μL per well. This volume can be reduced, but the tissue should be immersed in the solution during the entire process.
5. Incubate at 4 °C on the orbital shaker for 72 h.
6. Remove the antibody solution and replace it with 400 μL of washing solution 1 and wash the cultures overnight at 4 °C on the orbital shaker. Change the washing solution at least 3 times.
7. Prepare the secondary antibodies, using ADB, and add 400 μL per well. Incubate at 4 °C on the orbital shaker for 48 h.

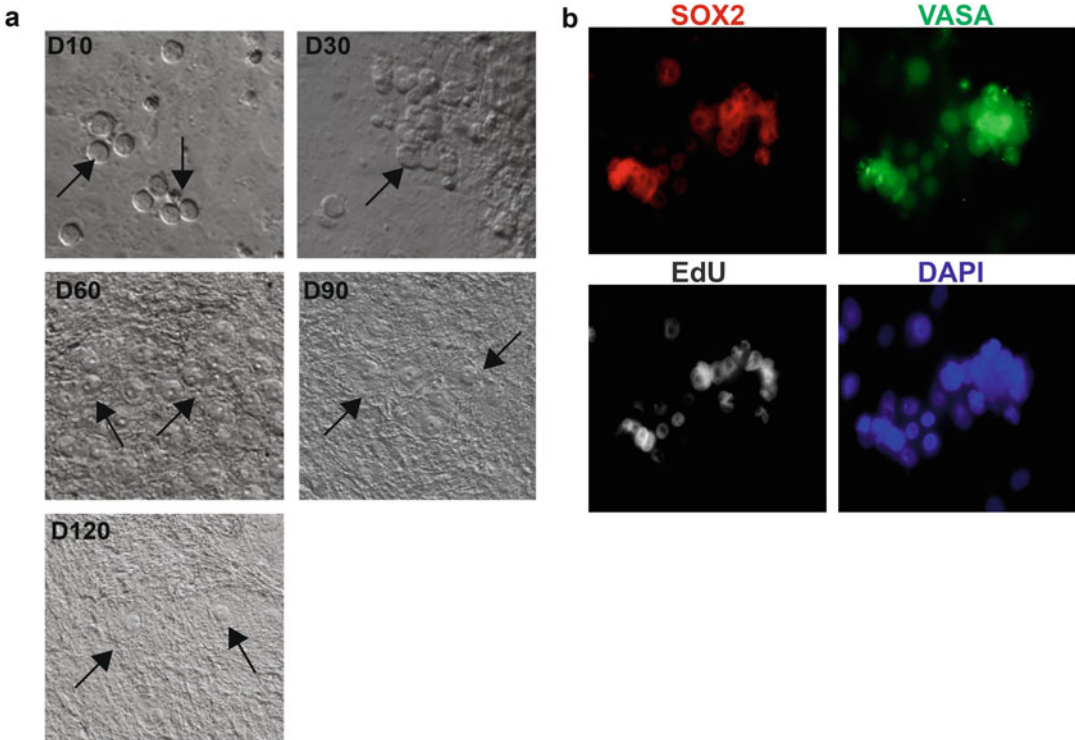


Fig. 3 Culture of postnatal ovary at Postnatal Day 5. **(a)** Culture of P5 ovary 10 days after seeding, showing clusters of PGCs at 30, 60, 90, and 120 days of culture, showing germ cell nests and follicle formation. Follicles appear 60 days after culture. **(b)** Immunofluorescence of culture of P5 ovary 10 days after seeding, treated with Edu (gray), VASA (green), SOX2 (red), and DAPI (blue)

8. Remove the secondary antibody solution and replace it with 400 μL of washing solution 1 and wash the cultures at 4 $^{\circ}\text{C}$ on the orbital shaker for 24 h. Change the washing solution at least 3 times.
9. To counterstain DNA, add 400 μL of 0.1- $\mu\text{g}/\text{mL}$ DAPI in water. Incubate overnight at 4 $^{\circ}\text{C}$ on the orbital shaker.
10. Remove the DAPI solution and add 400 μL of washing solution 2. Wash it 3 times for 15 min each.
11. Mount with VECTASHIELD and analyze. If the tissue cultures are too thick, they can be cleared and imaged once the immunofluorescence is finalized (*see* Fig. 3).

3.7 Clearing of Ovarian Cultures

The following protocol for the clearing of ovarian cultures is a modification of that previously described by Rinaldi [22], adjusted for naked mole-rat ovaries.

1. Prepare fresh ScaleS solution.
2. Add 400 μL of the clearing solution to each well and place them at room temperature on the benchtop orbital shaker.

3. Replace the clearing solution 3 times a day until the tissue becomes transparent (approximately 3 days).
4. Mount with VECTASHIELD and analyze.

3.8 Embedding of Ovary in Paraffin for Histology Sections

Using an automated Leica tissue processor with the following cycles:

1. Dehydration:
 - a. 70% ethanol 15 min.
 - b. 90% ethanol 15 min.
 - c. 100% ethanol 15 min.
 - d. 100% ethanol 15 min.
 - e. 100% ethanol 30 min.
 - f. 100% ethanol 45 min.
2. Clearing:
 - a. Xylene 20 min.
 - b. Xylene 20 min.
 - c. Xylene 45 min.
3. Wax infiltration:
 - a. Tissue infiltration medium paraffin wax (Leica) 30 min.
 - b. Tissue infiltration medium paraffin wax (Leica) 30 min.
 - c. Tissue infiltration medium paraffin wax (Leica) 45 min.
4. Place the ovary in a mold and let it cool down for at least 24 h.

3.9 Sectioning

1. Perform serial sectioning, with a thickness of 6 μ m.
2. Due to the large number of germ cells, serial sectioning was established to count every 20th section.

3.10 Immuno-fluorescence on Ovarian Histology Sections

1. Preheat steamer or water bath with staining dish/Coplin jar containing a sodium citrate buffer until the temperature reaches 95–100 °C.
2. Deparaffinize sections in 3 changes of xylene, 10 min each. Other agents can be used (*see Note 13*).
3. Hydrate in 3 changes of 100% ethanol for 5 min each.
4. Hydrate in 95%, 80%, and 70% ethanol, 2 changes each for 5 min each time.
5. Rinse two times in distilled water.
6. Immerse slides in the staining device/Coplin jar with the hot buffer.
7. Place the lid loosely on the staining device/Coplin jar and incubate for 40 min (inside the steamer or water bath).
8. Turn off steamer or water bath and return the staining dish to room temperature. Allow the slides to cool for 20 min.

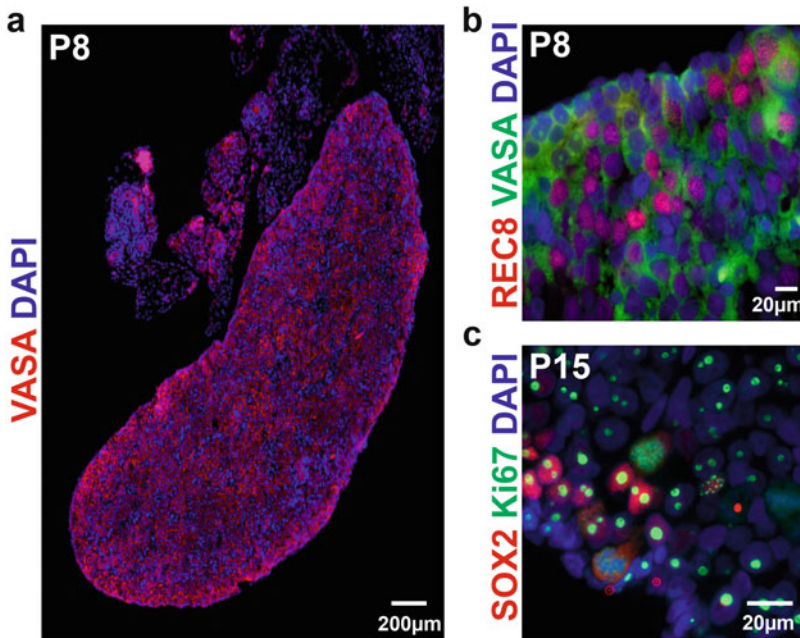


Fig. 4 Immunostaining of naked mole-rat tissue sections. **(a)** Postnatal ovary at Day 8 (P8), stained with VASA (red) and DAPI (blue); **(b)** P8 ovary stained with REC8 (red), VASA (green), and DAPI (blue); **(c)** postnatal ovary at Day 15, stained with the pluripotency marker SOX2 (red), the cell division marker Ki67 (green), and DAPI (blue)

9. Rinse slides in PBS-T two times for 5 min each.
10. Permeabilize sections with the permeabilization solution (0.2% of Triton-X 100 in PBS) for 30 min.
11. Rinse slides in PBS-T two times for 5 min each.
12. Block sections for 4 h with blocking solution.
13. Prepare the primary antibody in blocking solution. In Fig. 4, we show examples of tissue sections stained with the germ cell marker VASA, the pluripotency transcription factor SOX2, and the cell division marker Ki67. For details of antibody dilutions, see **Note 14**.
14. Incubate with primary antibody overnight at room temperature. Be sure that slides never get dry.
15. Rinse slides with PBS-T 3 times for 5 min each.
16. Incubate sections with secondary antibody at appropriate dilution for 2 h at room temperature. Secondary antibody can be diluted in blocking solution. Naked mole-rat tissue can show a lot of secondary antibody background; to reduce this artifact, we use secondary antibodies with minimal cross-reaction with human, mouse, rat, and guinea pig proteins. To see examples of secondary antibodies, check **Note 15**.

17. Rinse slides with PBS-T 3 times for 5 min each.
18. Incubate slides with 0.1 µg/mL of DAPI solution in water for 15 min at room temperature to counterstain the nucleus (*see Note 16*).
19. Rinse slides with milliQ water 2 times for 2 min each.
20. Let the slides dry for 15 to 30 min.
21. Mount with Vectashield and cover slide (*see Fig. 4*).

4 Notes

1. Hypotonic extraction buffer should be prepared fresh, no more than 2 h before it is needed. Use of old (i.e., not fresh) HEB will cause cells to not spread properly and create artifacts in the antibody signal.
2. Fixative solution should be prepared fresh, no more than 2 h before use, and kept on ice. Tritron X should be added after measuring the pH.
3. Remember to use the proper serum; if secondary antibodies were raised on donkeys or other organism, use that serum instead of goat serum.
4. The use of the uterine horn as a handle will protect the ovary and will allow you to avoid finding wrinkles or missing parts later, during staining and analysis.
5. Fixative drop should remain in the well; however, it can sometimes overflow. To avoid this, make a circle surrounding the well with a hydrophobic pen.
6. If cultures and spreads will be prepared from the same ovary, collect the ovarian leftovers and rinse them with sterile PBS, followed by 10 mins in penicillin-streptomycin, and seed the ovarian fragments as was described.
7. Sucrose wash is necessary to remove extra HEB. Wash twice and use a different slide than the one that will be used for the mincing.
8. If the solution looks very turbid or milky, the cell will not spread properly; however, if it is too clear, the number of cells will be very low.
9. Do not let the slide become fully dry; if slides are completely dry, the secondary antibody will generate a massive amount of background.
10. Abundant proteins, such as cohesin or synaptonemal complex, are properly conserved; however, proteins that form foci, such as MLH1, are not properly preserved after a year.

11. Initiate the antibody testing using a concentration of 1:100, and then optimize it according to the results obtained with this concentration.
12. Naked mole-rats produce very-high-molecular-weight hyaluronan (vHMWH) that can turn the culture media a little bit viscous. First, the culture is not contaminated, and second, the use of freshly prepared culture media helps to reduce the amount of vHMWH.
13. Xylene can be exchanged for safer products, such as Safeclear. De-waxing should be re-established using these products.
14. Initiate the standardization of the antibodies with a concentration of 1:100 and then adjust it accordingly.
15. To avoid high background, use minimal cross-reaction antibodies, Jackson ImmunoResearch gave the best results in our hands.
16. Skipping this step and trying to mount directly with Vectashield will increase the risk of a very intense DAPI signal at the borders of the tissue, making analysis more complicated.

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Chapter 12

A Roadmap for Three-Dimensional Analysis of the Intact Mouse Ovary

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Abstract

Recent advances in tissue clearing methodologies have enabled three-dimensional (3D) visualization of the ovary and, consequently, in-depth exploration of the dynamic changes occurring at the single-cell level. Here we describe methods for whole-mount immunofluorescence, clearing, imaging, and analysis of whole ovarian tissue in 3D throughout murine development and aging.

Key words Ovary, Whole-mount immunofluorescence, Tissue clearing, Imaging, 3D analysis

1 Introduction

The ovary performs two primary functions for reproduction: producing competent oocytes for ovulation with each hormonal cycle and supporting fertility and early pregnancy through hormone secretion. These dynamic and asynchronous functions require complex interactions between the oocytes, the supporting cells, and the ovarian microenvironment [1]. Two unique challenges to studying the ovary with traditional histology are the vast discrepancy in the sizes of follicles during growth and the apparent irregularity of follicle organization. As a small number of tissue sections cannot capture the global relationships between follicles, vasculature, and nerves, the ovary is an organ ripe for three-dimensional (3D) visualization. The advances in optical clearing and quantitative image analysis of the last decade have opened up new

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Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/978-1-0716-3259-8_12.

Michael Buszczak (ed.), *Germline Stem Cells: Methods and Protocols*, Methods in Molecular Biology, vol. 2677, https://doi.org/10.1007/978-1-0716-3259-8_12,

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opportunities for understanding the geography of ovarian development, disease, and aging.

The main innovations that enable whole-mount imaging of the ovary include optical tissue clearing and deeply penetrating microscopy, such as confocal and light sheet. Tissue clearing homogenizes the different refractive indices of tissue components and the surrounding medium to achieve transparency [2]. Aqueous-based optical tissue clearing approaches, such as *ScaleA2*, remove lipids using detergents while maintaining tissue hydration and, consequently, endogenous fluorescent signals [3]. In the fetal ovary, *ScaleA2*, in combination with sucrose preincubation, was used to image oocytes in situ during meiosis and fetal oocyte attrition [4], but was not sufficient for the adult ovary. Organic solvent-based clearing methods improved tissue permeabilization and transparency in large samples. Benzyl alcohol and benzyl benzoate (BABB) was first applied to visualize the gross morphology of the *Xenopus* ovary [5] and was ultimately employed to first visualize and quantify oocytes of different follicle stages in neonatal and adult mouse ovaries [6]. However, the poor preservation of fluorescent signal and limited penetration of BABB for larger tissue samples motivated the development of another organic clearing method that combined dehydration, lipid removal, and refractive index matching known as 3D imaging of solvent-cleared organs (DISCO) [7–10]. The combined use of tetrahydrofuran (THF) and dibenzyl ether (DBE) in the DISCO protocol allowed for the successful clearing and qualitative visualization of adult mouse ovaries [11].

In this chapter, we outline considerations and robust methods for clearing, immunolabeling, imaging, and performing 3D analyses on ovarian tissue throughout murine development and aging (Fig. 1). These methods may be applied toward both visualizing endogenous fluorescence in the ovaries of genetically engineered reporter mice as well as to immunofluorescence staining of ovary organoids and whole ovaries across species. We discuss different imaging platforms available at different price points, with the goal of making these techniques broadly available.

2 Materials

The following solutions should be prepared using ultrapure water with a resistivity of 18.2 M Ω .cm at 25 °C. To ensure researcher and environmental safety, follow waste disposal regulations at all times (please refer to Molbay et al. [12] for information on the toxicity of common chemicals).

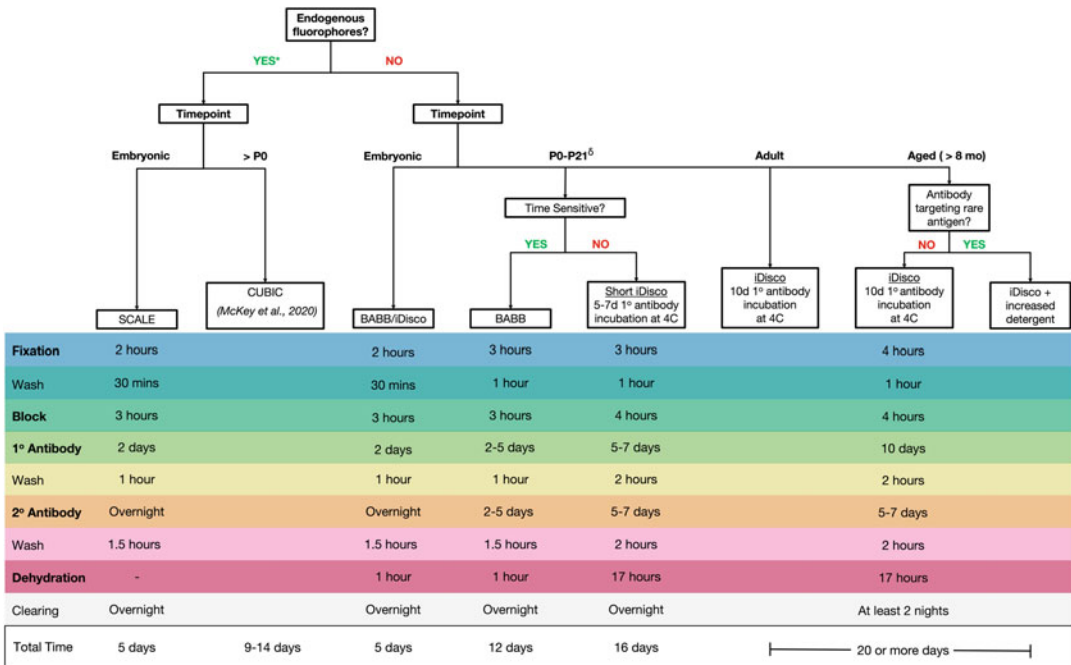


Fig. 1 Decision flow chart for choosing the optimal protocol to whole-mount stain and clear the mouse ovary. Washing steps and the final clearing step are ideal pause points if necessary. Steps in bold should be kept consistent to obtain comparable data. *: iDisco clearing is more compatible when the endogenous signal is high. δ : While both BABB and iDisco are effective at this timepoint, BABB is more time-efficient

2.1 General Supplies and Solutions

1. 1× Phosphate Buffered Saline (PBS, *CAS 7758-11-4*).
2. Dissection solution, 0.4% Bovine Serum Albumin (BSA) in PBS: Dissolve 200 mg of BSA (*CAS 90604-29-8*) in 50 mL of 1× PBS. Filter through a 0.2 μ m filter to sterilize and store at 4 °C.
3. Fixative solution, 4% Paraformaldehyde (PFA) in PBS: Combine 1.25 mL of 16% Paraformaldehyde (*CAS 30525-89-4*) and 3.75 mL of 1× PBS.
4. Methanol dehydration solutions, 25–50–75–100% Methanol in PBS: Dilute appropriate volume of 100% methanol (*CAS 67-56-1*) in 1× PBS.
5. 0.2 μ m PES (*Thermo Scientific 725-2520*).
6. Imaging cylinders: 2–3 evenly spaced 10 mm long glass cylinders (*ACE Glass 3865-10*) attached to a 24 × 50-1.5 microscope cover glass (*Fisherbrand 12544E*) with silicone glue (*GE GE280*). Prepare the imaging cylinders at least 24 h before use (*see Note 1*).
7. Transfer pipettes (*Fisherbrand 13-711-9 AM*).
8. Scale-CUBIC-1 solution: Dissolve 125 g of Urea (*CAS 57-13-6*) and 156 g of 80% Quadrol (*CAS 102-60-3*) in 144 g of water

on a stirrer. After complete dissolution, add 75 g of Triton X-100 (CAS 9036-19-5), mix on low speed and store at room temperature.

9. Leica TCS SP8 inverted scanning confocal microscope.

2.2 BABB Clearing Solutions

1. Washing solution, 0.2% BSA in PBS: Dissolve 10 mg of BSA in 5 mL 1× PBS. Filter through a 0.2 μm filter to sterilize and store at 4 °C.
2. Permeabilization solution, 0.1% Triton X-100 in PBS: Combine 50 μL of Triton X-100 (CAS 9036-19-5) and 49,950 μL of 1× PBS and mix well.
3. Blocking solution, 2% BSA, 0.1% Triton X-100 in PBS: Dissolve 200 mg of BSA in 10 mL of 0.1% Triton X-100 in PBS. Filter through a 0.2 μm filter to sterilize and store at 4 °C.
4. Antibody dilution solution, 0.2% BSA, 0.1% Triton X-100 in PBS: Dissolve 20 mg of BSA in 10 mL of 0.1% Triton X-100 in PBS. Filter through a 0.2 μm filter to sterilize and store at 4 °C.
5. Bleaching solution, 3% H₂O₂ in Methanol: Combine 1 mL of 30% H₂O₂ (CAS 7722-84-1) and 9 mL of 100% Methanol.
6. Clearing solution, Benzyl alcohol: Benzyl benzoate (1:2) (BABB) (*Refractive Index, RI: 1.559*) [13]: Combine 100 mL of Benzyl alcohol (CAS 100-51-6) and 200 mL of Benzyl benzoate (CAS 120-51-4).

2.3 iDISCO Clearing Solutions

1. Washing/Blocking solution, 0.2% Gelatin, 0.5% Triton X-100 in PBS: Dissolve 200 mg of Gelatin (CAS 9000-70-8) in 100 mL of 0.5% Triton X-100 in PBS. Filter through a 0.2 μm filter to sterilize and store at 4 °C.
2. Antibody dilution solution, 0.2% Gelatin, 0.5% Triton X-100, 0.1% Saponin in PBS: Dissolve 200 mg of Gelatin in 99.4 mL 1× PBS. Add 500 μL Triton X-100 and 100 μL 10 mg/mL Saponin in 1× PBS (CAS 8047-15-2). Filter through a 0.2 μm filter to sterilize and store at 4 °C.
3. Bleaching solution, 6% H₂O₂ in Methanol: Combine 2 mL of 30% H₂O₂ and 8 mL of 100% Methanol.
4. Tetrahydrofuran (THF) dehydration solutions, 50–80–100% THF in water: Dilute appropriate volume of 100% THF (CAS 109-99-9) in water.
5. Delipidization solution, Dichloromethane (DCM, CAS 75-09-2).
6. Clearing solution, Dibenzyl ether (DBE, CAS 103-50-4), (*RI: 1.562*) [13].

3 Methods

Dissection considerations depend on the developmental stage of the ovary (Table 1). All incubation steps should be carried out on an orbital or rocker shaker (*see Note 2*). Attach a P10 pipette tip to a disposable transfer pipette to aspirate extra solution in the tube before the subsequent step. If ovaries need to be moved (from the petri dish to the tube or from the tube to the cylinder), use a P200 wide-bore pipette tip or cut the tip of a regular P200 tip and transfer the ovaries with 100–200 μL of solution.

3.1 Whole-mount Immunofluorescence Staining for Embryonic Mouse Ovaries

1. Dissect mouse fetal ovaries in a petri dish containing 0.4% BSA/PBS then transfer them into 1.5 mL Eppendorf tubes.
2. Remove extra BSA solution from the tube and add 500 μL of 4% PFA/PBS to fix. Incubate at +4 °C for 2 h (h) (*see Note 3*).
3. Discard PFA solution and wash embryonic ovaries with 500 μL of 0.2% BSA in PBS three times for 10 min (mins) each.
4. Remove 0.2% BSA/PBS and block with 500 μL of 2% BSA, 0.1% Triton X-100 in PBS for 3 h at room temperature (*see Note 4*).
5. Dilute primary antibodies (Table 2) in 0.2% BSA, 0.1% Triton X-100 in PBS, and incubate the ovaries in 200 μL of primary antibody solution at +4 °C for two nights (*see Note 5*).
6. Wash the samples with 500 μL of 0.1% Triton X-100 in PBS four times for 15 mins each at room temperature to remove extra primary antibodies.
7. Incubate the samples with Alexa Fluor-conjugated secondary antibodies diluted in 200 μL of 0.2% BSA, 0.1% Triton X-100 in PBS at +4 °C overnight.
8. Remove the secondary antibody solution from the tube and wash the samples with 500 μL of 0.2% BSA, 0.1% Triton X-100 in PBS three times for 30 min each (proceed directly to step 9b if an organic solvent-based clearing is applied).

Table 1
Dissection considerations

Embryonic timepoints E13.5–18.5	Postnatal & adult timepoints >P0
Keep mesonephros attached for orientation when A/P information is needed	Keep oviduct/uterus attached for orientation when A/P information is needed
Dissect isolated ovaries into 0.4% BSA in PBS to prevent sticking	Remove as much of the surrounding gonadal fat pad from the ovary as possible
Between E13.5–15.5, dissect embryo submerged in 0.4% BSA in PBS for improved visualization	Remove ovarian bursa for improved antibody penetration

Table 2
Primary antibodies used to label specific structures in mouse ovaries

Approved antibodies	Catalog #	Expression	Timepoint			Notes
			E13.5- E18.5	P0- P21	Adult and aged	
AIF	4642S	Mitochondria and apoptotic cells	NT	✓	NT	
α-SMA	ab5694	Interstitial cells	✓	✓	✓	
CASPASE-3	9664	Apoptotic cells	✓	NT	NT	
c-PARP-647	BD558710	Apoptotic cells	✓	✓	NT	
COL-I	ab6308	Extracellular matrix	NT	✓	✓	
COL-IV	ab6586	Extracellular matrix	NT	NT	✓	
CYP11A1	GTX56293	Theca cells	NT	✓	NT	MeOH incompatible
DDX4/MVH	ab13840	Germ cells and oocytes	✓	✓	NT	
DMRT1	sc-377167	Transiently in germ cells	✓	NT	NT	
DPPA5	PA5-48042	Germ cells	✓	NT	NT	
E-CADHERIN	13-1900	Germ and epithelial cells	✓	NT	NT	
FMRP	ab191411	Germ cells	✓	NT	NT	
FOXL2	NB100-1277	Granulosa cells	✓	✓	✓	
GATA4	sc-1237	Somatic cells	✓	NT	NT	
GCNA1 (TRA98)	ab82527	Germ cells	✓	✓	NT	MeOH incompatible
Ki67	ab15580	Mitotically proliferative cells	✓	NT	NT	
LAMININ	L9393	Extracellular matrix	✓	NT	NT	
LIN28	ab63740	Germ cells	✓	NT	NT	
LINE-1	ab216324	Transposable elements	✓	NT	NT	
Oct-4	sc-5279	Germ cells	✓	N/A	N/A	
PECAM	557355	Vasculature	✓	✓	✓	
pHH3	9713	G2/M cell cycle	✓	✓	NT	
SSEA1	MC-480	Germ cells	✓	N/A	N/A	
StAR	8449T	Corpus luteum	N/A	✓	✓	
STELLA	ab19878	Germ cells	✓	NT	NT	
STRA8	ab49602	Germ cells	✓	N/A	N/A	

(continued)

Table 2
(continued)

Approved antibodies	Catalog #	Expression	Timepoint			Notes
			E13.5- E18.5	P0- P21	Adult and aged	
SYCP3	ab97672	Germ cells	✓	✓	N/A	
TET1	GTX125888	Germ cells	✓	NT	NT	
TH	ab112	Sympathetic nerves	✓	✓	✓	
γ -H2AX	05-636	DNA double-stranded breaks	✓	NT	NT	

AIF Apoptosis-Inducing Factor, *α -SMA* alpha-smooth muscle actin, *c-PARP* cleaved Poly(ADP-Ribose) Polymerase, *COL-1* Collagen I, *COL-IV* Collagen IV, *CYP11A1* Cytochrome P450 Family 11 Subfamily A Member 1, *DDX4/MVH* DEAD-Box Helicase 4/Mouse Vasa Homolog, *DMRT1* Doublesex And Mab-3 Related Transcription Factor 1, *DPPA5* Developmental Pluripotency Associated 5, *E-CADHERIN* Epithelial cadherin, *EMRP* The fragile X mental retardation protein, *FOXL2* Forkhead Box L2, *GATA4* GATA Binding Protein 4, *GCNA1* Germ Cell Nuclear Antigen 1, *OCT4* Octamer-Binding Transcription Factor 4, *PECAM* Platelet Endothelial Cell Adhesion Molecule, *pHH3* Phospho-histone H3, *SSEA1* Stage Specific Embryonic Antigen-1, *StAR* Steroidogenic Acute Regulatory protein, *STRA8* Stimulated By Retinoic Acid 8, *SYCP3* Synaptonemal complex protein 3, *TET1* Tet Methylcytosine Dioxygenase 1, *TH* Tyrosine Hydroxylase, γ -*H2AX* phospho-Histone H2A, *N/A* not applicable at the time point indicated, *NT* not tested at the time point indicated

9. (a) If an aqueous clearing solution is being used (i.e., Scale-CUBIC-1, *RI: 1.34*), use a transfer pipette to transfer the samples in 100 μ L of washing solution to 10 mm long glass cylinders mounted onto coverslips. Once the samples are in the cylinders, remove the extra solution then add 300 μ L of Scale-CUBIC-1 into the cylinder and incubate at +4 °C overnight. The next day, the samples are ready to be imaged (*see Note 6*).
(b) If an organic solvent-based clearing solution is being used, dehydrate the samples in an ascending methanol series: 500 μ L of 25%, 50%, 75%, 100% methanol: 1 \times PBS for 10 min each (only 100% twice) at room temperature.
10. Incubate the samples in 500 μ L of 3% H₂O₂ in methanol overnight at +4 °C (*see Note 7*).
11. To remove H₂O₂ from the samples, wash the ovaries with 500 μ L of 100% methanol two times for 30 min each (*see Note 8*).
12. Transfer the samples in 100 μ L of methanol using a P200 pipette to the 10 mm long glass cylinders mounted onto coverslips. Once the samples are in the cylinders, remove extra methanol from the cylinders (*see Note 9*).
13. Add 300 μ L of BABB clearing agent into the cylinder and incubate at +4 °C overnight. The next day, the samples are ready to be imaged (*see Note 10*).

**3.2 Whole-mount
Immunofluorescence
Staining for Postnatal
and Adult Mouse
Ovaries**

1. Dissect ovaries in a petri dish containing 0.4% BSA/PBS then transfer them into 2 mL Eppendorf tubes.
2. Remove extra BSA solution from the tube, add 1 mL of 4% PFA/PBS, and incubate at +4 °C for 3–4 h.
3. Wash the samples with 1 mL of PBS four times, 15 min each.
4. Dehydrate the samples in an ascending methanol series: in ~1 mL of 25%, 50%, 75% 100% methanol: 1× PBS for 20 min each (only 100% twice) at room temperature (*see Note 11*).
5. To decolorize hemoglobin, incubate the samples in ~1 mL of 6% H₂O₂ in methanol overnight at +4 °C.
6. Rehydrate the samples in a descending methanol series: incubate in ~1 mL of 100%, 75%, 50%, 25% methanol: 1× PBS for 20 mins each (only 100% twice) at room temperature.
7. Wash the samples with 1 mL of PBS for 20 min, at room temperature.
8. To block non-specific staining, incubate the samples with 1 mL of 0.2% Gelatin, 0.5% Triton X-100 in PBS for 3–4 h at room temperature.
9. Dilute primary antibodies (Table 2) in 400 µL of 0.2% Gelatin, 0.5% Triton X-100, 0.1% Saponin in PBS and incubate for 5–10 nights, at +37 °C (*see Note 12*).
10. Wash the samples with 1 mL of 0.2% Gelatin, 0.5% Triton X-100 in PBS four times, 30 min each at room temperature to remove extra primary antibodies.
11. Stain the samples with Alexa Fluor-conjugated secondary antibodies diluted in 400 µL of 0.2% Gelatin, 0.5% Triton X-100, 0.1% Saponin in PBS at +37 °C for 3 days (*see Note 13*).
12. Remove extra secondary antibodies by washing the sample with 1 mL of 0.2% Gelatin, 0.5% Triton X-100 in PBS four times, 30 min each at room temperature.
13. Dehydrate the sample in an ascending THF series: 1 mL of 50% THF:water overnight at room temperature, followed by 1 mL of 80% THF:water then 100% THF for 1.5 h each.
14. To remove lipids, incubate the samples with 1 mL of DCM for 30 min at room temperature.
15. Clear the samples by incubating them in 1 mL of DBE at room temperature until they become transparent (*see Note 14*).

3.3 Imaging

The following instructions are specific to the Laser Scanning Confocal microscope. Optically cleared ovaries can also be imaged using a THUNDER Imager 3D Cell Culture microscope or a Light sheet microscope (*see Note 15*).

Table 3
Whole-mount imaging set up used for mouse ovaries

Channel	Excitation laser (nm)	Detector	Spectral detection window (nm)
Hoechst	405	HyD	423–503
Alexa Fluor 488	488	PMT	506–560
Alexa Fluor 555	555	HyD	557–636
Alexa Fluor 647	647	PMT	658–777

1. To find the sample through the eyepiece, use the 10× objective and transmitted light to mitigate photobleaching. If your system is not capable of viewing samples with transmitted light, spend as little time as possible focusing using fluorescent light.
2. Consider resolvability in choosing the objective for imaging (*see Note 16*).
3. Set up your imaging channels (excitation laser, detectors, spectral detection window) (Table 3) (*see Note 17*).
4. Z-stack parameters: For setting the limits of your z-stack, base your lower and upper limits on features of your sample using the channel that represents the total germ cell population. To help image your sample evenly in z, set the starting slice closest to the coverslip/objective and the last slice furthest from the coverslip/objective. To limit movement/shifting of the sample during imaging, use the z-drive in the microscope body (z-wide) which moves the objective relative to the sample. Set the z-step size at 2 μm per slice (*see Note 18*).
5. Set laser scanning speed (pixel dwell time) to 400 (400 μs/pixel).
6. Set the pixel image size to 1024 × 1024.
7. Use bidirectional scanning if available (for 2× speed).
8. Select “between stacks” under the sequential scan tab to minimize bleaching.
9. If the image quality appears poor during setup, optimize using gain, offset, frame, or line averaging (*see Note 19*).

3.4 Image Analysis for Wholmount Stained Ovaries

Perform Image analysis using Imaris v8.3.1 (Bitplane).

1. Import the files by dragging them to Surpass mode.
2. Define the anterior and posterior tips of the ovary according to the orientation of the mesonephros. Create a surface manually on the ovary using the surface module then remove the mesonephros by masking the antibody staining channels (Fig. 4).

This step helps to reduce file size and make germ cell counts more accurate (*see Note 20*).

3. To select fluorescently labeled germ cells, use the spot detection module. To maximize accuracy when quantifying germ cells, identify the XY diameter (feature size) of labeled germ cells using the slice view to determine the XY diameter of the object of interest (Fig. 2). For a given germ cell population,

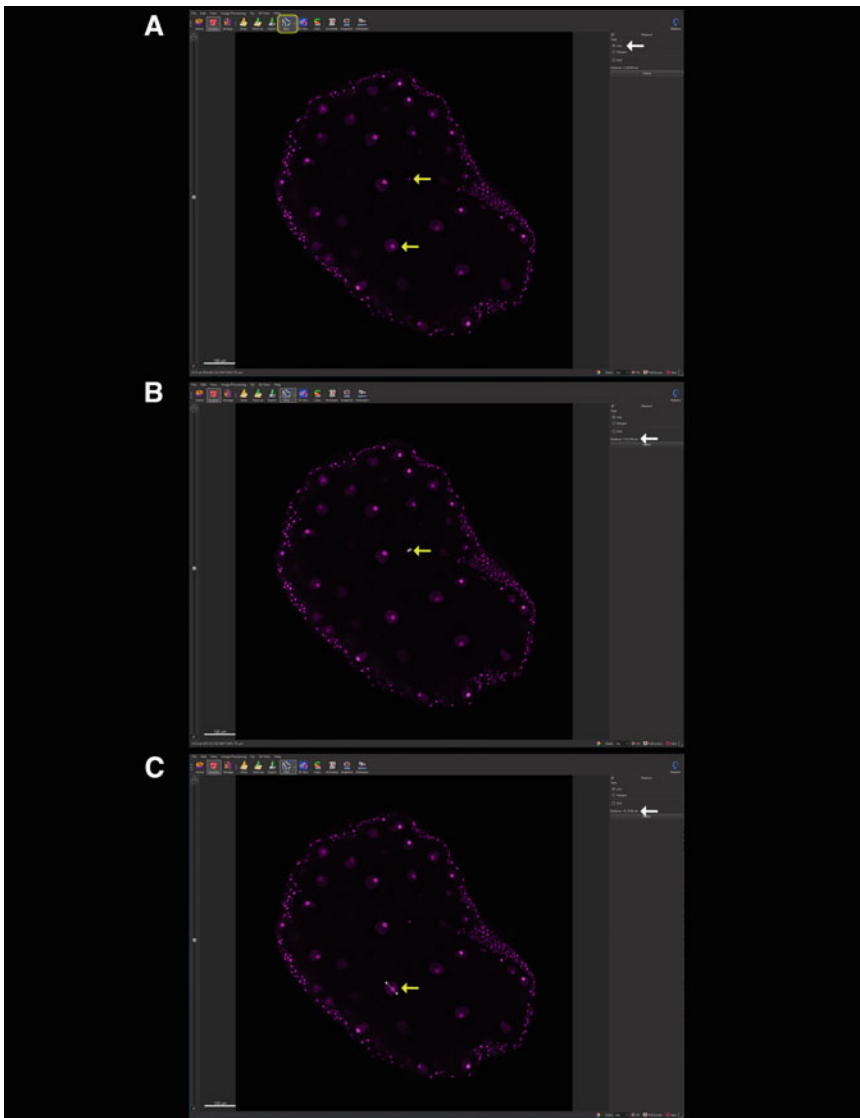


Fig. 2 Identifying the feature size (XY diameter) of NOBOX+ germ cells in postnatal day 16 (PN16) ovary. **A.** Click the section view tab (bordered by a yellow rectangle) to visualize individual z stacks. Based on the stage of the ovary, the size of objects might vary due to different stages of oocyte development (yellow arrows showing growing and non-growing follicles). Choose the line option (white arrow) under the measure tab on the right-hand side. **B** and **C.** Draw a line from one margin to another to measure XY diameter (yellow arrow), the distance value is shown under the measure tab (white arrow)

creating spots with two different object sizes (XY diameters) maximizes the number of accurately picked germ cells.

4. After creating two different spot populations (with different object sizes) for each antibody or channel used, mask the spots with larger object size from the original channel and create a new channel with a standard voxel value inside (>100) the spots (objects). To avoid over-counting, filter smaller object size spots based on the intensity median values of the previously created channel for larger object size spots. Set the threshold for filtering by moving the left slider to the far left and right slider to just before the middle of the scale. After an initial check that only smaller-sized spots are selected and larger-sized spots are eliminated, duplicate this spot population by clicking the duplicate selection to the new spots. Thus, spots are picked by using both (two different) object sizes and double-counted spots are eliminated by using the filter function of the software.
5. Create a new folder on Imaris to combine the germ cell counts; merge larger, and filter smaller object size spots under the new folder. To test the accuracy of the analysis, mask the final total spot population based on the corresponding staining channel, and set voxels inside the surface to a fixed value (>100). This creates a new masked channel based on the final spot population. Change the color of the newly created channel to white. By showing only the original staining channel and recently masked final spot population channel, the accuracy of the analysis can be verified (Movie 1). Import positive germ cell numbers for each marker to a spreadsheet application.

3.5 Spatial Analysis of Wholemount Stained Ovaries Using MATLAB

Spatial analysis of a population of interest can be performed by using a custom MATLAB script (available at <https://github.com/BIDCatUCSF/Angular-Radial-Position-Distribution>). The code assumes that the ovarian shape can be modeled as being one section of a circle. As the ovary matures, the shape changes, and to account for this, the code adapts the curvature of the evolving ovary shape by changing the radius of the circle. This curvature is calculated by a combination of user inputs defining extreme anterior and posterior points as well as with a manually created surface of the ovary from which the center of geometry is measured by the Imaris surface statistics. These three points are then used to calculate the radius of curvature that best fits the ovary. The Imaris Spots populations created for the different markers of interest further define the extent of the circle into an annulus and create bins of angular and radial sections. From these bins, the number of Spots are extracted and distribution plots are generated [14] (Fig. 4).

1. Download the code from <https://github.com/BIDCatUCSF/Angular-Radial-Position-Distribution>
2. Add the MATLAB code to your list of Imaris XTs: Open Imaris and navigate to the Custom Tools window in: File > Preferences > Custom Tools.
3. Either add the folder path where the code is stored by choosing “Add XTension Folder”, or add the code to a previously existing XT folder (except the rtmatlab folder).
4. Once your Surface and Spots objects have been created, highlight one of the object populations in the Scene and select the gear icon (list of Imaris XTs).
5. Select the code and a MATLAB Window will appear. Once prompted, select the Spots population for the general germ cell marker, and click “Ok”.
6. A window will pop up asking the user if the mouse is older than E13.5. If not, the code will calculate the radius of curvature of the surface automatically. If older than E13.5 is chosen, the user will be prompted to input the XY coordinates of the anterior and posterior positions of the surface for the curvature calculation. In this case, coordinates can be obtained by switching to Slice View within Imaris (instead of 3D View). In Slice view, Imaris will automatically display channel intensities and coordinates of your cursor in the lower left of the screen.
7. A window will pop up prompting the user to input the desired number of bins. This is the binning of the histograms produced.
8. Lastly, choose the secondary dependent Spots object (specific population of cells such as proliferating, meiotic, apoptotic germ cells, etc.) which will be compared to the primary dependent Spots object (first choice; general germ cell marker).

4 Notes

1. The thickness of the coverslip varies depending on the objective used. Although #1.5 coverslips are suitable for most objectives, check the inscription on the objective to confirm the coverslip thickness required. Some microscopes are built with custom sample holders. Thus, embedding the ovary into agarose and imaging with custom holders might be necessary [11].
2. Make sure that samples are immersed in the solution and that the solution is circulating during all incubation steps. Samples that are stuck on the wall or the cap of Eppendorf tubes can dry out and lead to suboptimal results.

3. Volumes indicated in the methods section are the minimum/ approximate volumes needed for each step. Depending on the size of the tubes used, the volumes of the solutions should be adjusted.
4. Instead of 3 h blocking at room temperature, embryonic ovaries can be blocked at +4 °C overnight. Regardless, the blocking step should be kept consistent to obtain comparable data.
5. Different ovaries that belong to the same experimental group can be pooled in one tube. In this case, the total volume of the primary antibody solution should be increased. The final volume of the solution should be at least 10 times more than the approximate volume of the ovaries.
6. Do not add Scale-CUBIC-1 solution directly into Eppendorf tubes. The Scale-CUBIC-1 solution makes samples immediately transparent and transferring cleared embryonic ovaries to cylinders is challenging. As the signal of endogenous fluorescent proteins can be lost quickly due to differences in the copy number of the transgene expressing fluorescent proteins, image the samples the next day. The Scale-CUBIC-1 solution tends to solidify when it is stored at +4 °C. To avoid this during the overnight clearing step, properly seal the cylinders by applying some vaseline on the top/edges of the cylinders and setting down a coverslip.
7. Incubation with 3% H₂O₂ in methanol is optional for smaller (E12.5–16.5) samples.
8. After the second methanol incubation step, unwanted particles and fibers attached to the sample can be removed using a dissection microscope. This prevents particles from obscuring any part of the ovary during imaging.
9. As methanol incubation turns samples opaque white, it is easier to transfer ovaries from Eppendorf tubes to the 10 mm long glass cylinders in 100–200 mL of methanol. Do not add BABB solution directly into Eppendorf tubes as this solution makes samples immediately transparent and transferring cleared embryonic ovaries to cylinders is challenging. If you prefer to image the samples in another apparatus, avoid using polystyrene products as BABB dissolves polystyrene.
10. Although samples can be stored in the dark at +4 °C for a week or even months (based on the robustness of the targeting antigen and fluorescent signal), image the samples as soon as sufficiently cleared for best results.
11. Some antibodies are not compatible with methanol incubation after fixation (Table 2). Thus, consider omitting the methanol dehydration and H₂O₂ incubation steps depending on the primary antibodies used.

Table 4
Ovary whole-mount immunofluorescence troubleshooting guide

Common problems	Recommendations
Fluorescent signal present in the periphery of the sample, but absent in the center of the sample	Increase the primary and secondary antibody incubation times Increase the concentration of detergent
Fluorescent signal is weakened or lost as the distance from the objective increases	Set z compensation during imaging
High background signal	Decrease the primary antibody concentration Increase the incubation time for the blocking step
Black spots observed on the sample during imaging	Using a dissection microscope, remove the particles and fibers from the sample Clean the inside of the cylinders and the bottom of the coverslip
No signal after clearing	As some antibodies are not compatible with methanol, omit the dehydration and H ₂ O ₂ bleaching steps after fixation

12. Incubation time with primary antibody depends on the stage of the ovary and on the targeting antigen. Please see Fig. 1 to choose the optimal incubation time and conditions.
13. Age-related increases in fibrosis and inflammation require adaptations for whole-ovary immunolabeling and clearing (Table 4). If aged ovaries are being stained, samples can be incubated for up to 7 seven days at +4 °C after incubating for 3 days at +37 °C (Fig. 1).
14. Since PN21 and older ovaries require more thorough clearing and can be located within 2 mL Eppendorf tubes (even after clearing), we recommend incubating in DBE while they are in the tubes on the shaker. We then transfer tissues to cylinders under a fluorescent dissection microscope just before imaging. As explained in **Note 8**, any particles stuck on the sample can also be removed before transferring the samples to cylinders. Samples can be stored in the dark at +4 °C for up to 6 months but consider that DBE degrades the fluorescent signal over time [8].
15. Although imaging time on the THUNDER Imager 3D Cell Culture microscope and the Lightsheet microscope is significantly shorter than on the Laser Scanning Confocal microscope, the large size of the resulting data file could be a limiting factor due to data management considerations.
16. The feature size of the object of interest varies depending on the stage and it can be identified by using the Slice View

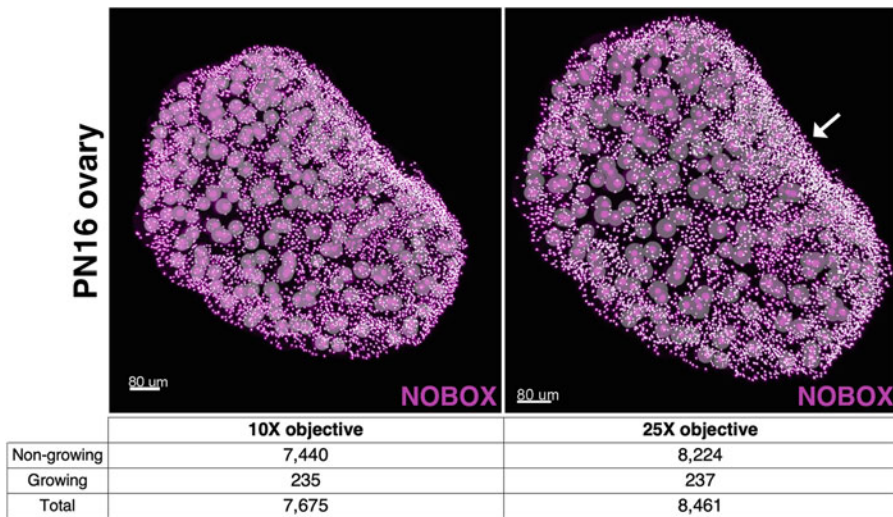


Fig. 3 Segmentation efficiency of PN16 ovary imaged with 10 \times and 25 \times objectives. The number of NOBOX+ germ cells (in magenta) of the same ovary was analyzed after imaging with the 10 \times (on the left) and the 25 \times objective (on the right). Big gray and small white circles demonstrate accurately picked growing and non-growing follicles, respectively. The table shows raw germ cell counts in the same sample after imaging with the 10 \times and 25 \times objectives and applying the same analysis pipeline. Please note that the difference in the number of non-growing follicles that are closely localized is more prominent than growing follicles. Arrow indicates the region where more non-growing germ cells were picked with 25 \times objective compared to 10 \times

function of Imaris software (Fig. 2). Based on the feature size, choosing a suitable objective is critical for the analysis. The segmentation efficiency can differ $\sim 10\%$ or more between samples imaged with 10 \times versus 25 \times objectives (Fig. 3). Using the appropriate objective is also important for reducing the mismatch between the clearing solution and the objective. Because the refractive index of water (RI: 1.33) is closer to the refractive indices of BABB (RI: 1.559) and DBE (RI: 1.562) than it is to air (RI: 1.0), using immersion objectives (such as 25 \times water objective) minimizes the effects of refractive mismatch. For choosing the image size for maximum resolution, see <https://svi.nl/NyquistCalculator>

17. Always save your microscope settings and reuse for reproducibility.
18. The thickness of the mouse ovary is $\sim 120\ \mu\text{m}$ at E13.5, $\sim 190\ \mu\text{m}$ at E15.5, $\sim 250\ \mu\text{m}$ at E18.5, $\sim 400\ \mu\text{m}$ at PN5, $\sim 700\ \mu\text{m}$ at PN21, and $\sim 1\text{--}2\ \text{mm}$ during adulthood. Avoid using the fluorescent intensity for quantifications in your analysis pipeline since it varies from the edge of tissue to the center.

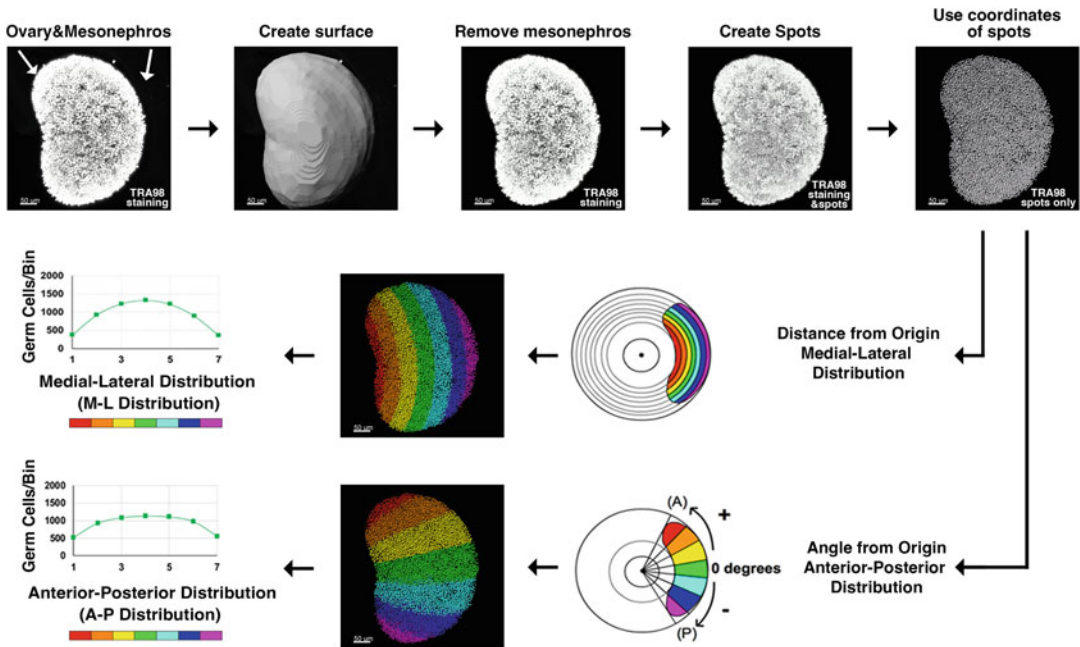


Fig. 4 Spatial analysis of germ cell distribution using the custom MATLAB script. Following whole-mount staining of the E16.5 ovary with TRA98 (in gray) and imaging with a confocal microscope, a surface is created on the ovary, and the mesonephros is removed by masking the TRA98 staining channel. Spots that represent individual TRA98+ germ cells are created and coordinates of the spots are used for downstream analysis. The ovary is divided into seven segments based on their medial (bins 3–5)—lateral (bins 1–2 and 6–7) and anterior (from bin 1)—posterior (to bin 7) distributions, respectively. Total germ cell numbers of each segment are graphed

19. Increase laser power for an increase in signal to noise, but be mindful of photobleaching your sample with high laser power. Increasing the detector gain (point scanning confocal) will produce a brighter image, but too much gain will add noise to your image. Adjust the offset to remove the unwanted autofluorescent background. Line/frame averaging during acquisition can be used to improve image quality as well as increase the pixel dwell time (reducing the scanning speed). Newer microscopes with cutting-edge technology and more sensitive detectors can be chosen to improve imaging quality.
20. Acquiring the image with optimal quality is the key to accurate data analysis. However, if the signal-to-noise ratio cannot be increased during imaging, image processing tools in the Imaris software can be implemented in the downstream analysis. Consider applying the same parameters to your samples in all groups for proper comparison.

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Chapter 13

Isolation and Culture Techniques for Fetal Mouse Germ Cells

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Abstract

The fetal gonad contains a great variety of differentiating cell populations, of which germ cells make up a relatively small percentage. In order to study germ cell-specific gene and protein expression, as well as determine direct effects of signaling molecules, it is necessary to prepare enriched populations of germ cells and maintain them in culture for several hours to multiple days. The protocols in this chapter are designed to provide a guide for the isolation or enrichment of primordial germ cells (from 9.5 days *post coitum* (*dpc*) to 18.5 *dpc*) by flow cytometry (Subheading 3.1) or magnetic sorting (Subheading 3.2), followed by feeder-free primary germ cell culture (Subheading 3.3).

Key words Germ cells, FACS, Magnetic sorting, MACS, Serum-free

1 Introduction

Mouse fetal gonads, from the time of their elaboration at about 10.5 days *post coitum* (*dpc*), are composed of both somatic and germ cells. Over the course of several days the soma becomes differentiated into either ovaries or testes and, subsequently, the germ cells undergo sex-specific differentiation into oogonia (in ovary) or pro-spermatogonia (in testis) [1]. This is a busy and critical time for the germ cell population: inappropriate prenatal germ cell development is relevant not only to potential infertility but, in humans, is also believed to underlie the formation of germ cell-derived tumors during adult life [2]. It is well established that germ cell sexual fate and development, although requiring intrinsic competency, is largely reliant on instructive signals that emanate from surrounding somatic cells. Therefore, in order to thoroughly understand both intrinsic and extrinsic responses in fetal germ cells, it is often necessary to obtain enriched or pure germ cell populations. Such cell populations can then be characterized in terms of transcriptome, epigenome, and protein expression. Isolated germ cells can also be cultured, for short periods of time, so that the effects of exposure to signaling factors can be assessed directly. Here

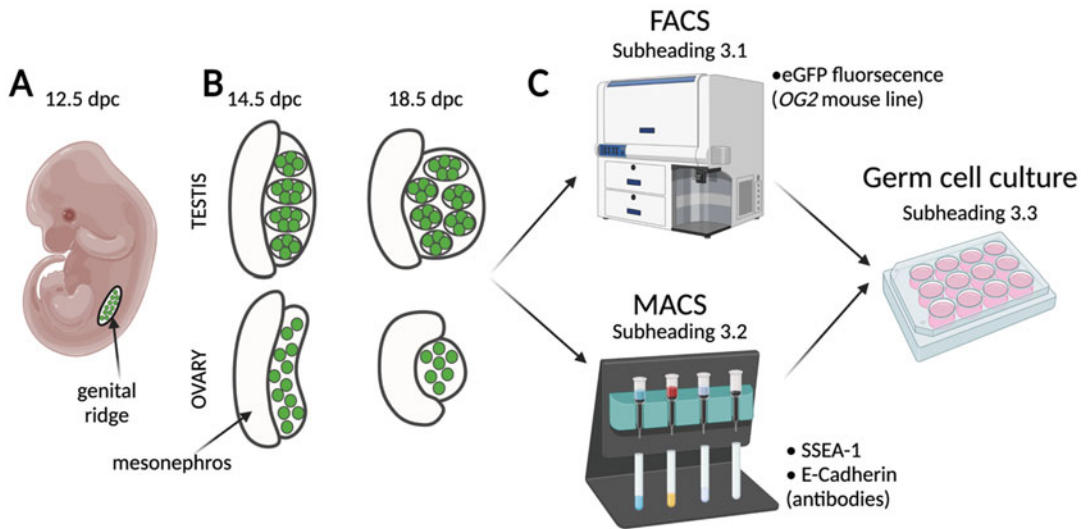


Fig. 1 Overview of gonadal location and morphology and techniques used to isolate germ cell populations. (a) Schematic of genital ridge localization within a 12.5 *dpc* embryo. (b) Schematic of testis and ovaries attached to mesonephros at 14.5 and 18.5 *dpc*. Germ cells are represented by green circles. (c) Overview of techniques that can be used to isolate germ cell populations for cell culture experiments. Fluorescent activated cell sorting (FACS) using eGFP fluorescence from the *OG2* mouse line [3]. Magnetic Activated Cell Sorting (MACS) uses either SSEA-1 or E-Cadherin antibodies. (Created with [BioRender.com](#))

we present protocols for obtaining pure (Subheading 3.1) or enriched (Subheading 3.2) populations of germ cells from mouse fetal gonads and for culturing them for short periods of time in the absence of feeder cells (Subheading 3.3). See Fig. 1 for overview.

2 Materials

2.1 Isolation of PGCs by Fluorescence Activated Cell Sorting (FACS)

In the transgenic mouse line *Oct4 Δ PE:eGFP* (*OG2*) enhanced GFP (*eGFP*) expression is controlled by the *Oct4* (*Pou5f1*) promoter and distal enhancer [3]; this line is very useful for isolating migratory germ cells (9.5–10.5 *dpc*) and gonadal germ cells (11.5–18.5 *dpc*). The eGFP expression in this line does not recapitulate *Oct4* expression faithfully in that the protein is detectable for a longer period of time, perhaps because the eGFP protein is more stable than the *Oct4* protein. Nevertheless, intensity of eGFP expression declines over time and at a faster rate in female germ cells than in male germ cells, as is the case for endogenous *Oct4*. The protocol described in Subheading 3.1 outlines isolation of gonadal germ cells (11.5–18.5 *dpc*) only. For isolation of migratory germ cells (9.5–10.5 *dpc*) please see [4] for tissue dissection. The remaining protocol(s) can be followed for isolation and culture. It is assumed the user has

sufficient understanding and experience with flow cytometry and fluorescent-activated cell sorting (or access to a facility that provides this service) for the isolation of germ cells using this protocol.

1. One or more 11.5–18.5 *dpc* pregnant CD1 females, previously timed-mated with *OG2* homozygous males (*see Note 1*).
2. 1× PBS (phosphate-buffered saline), sterile, for dissecting.
3. 10-cm culture dishes for dissection of fetuses and gonads.
4. Forceps with sharp tips (watchmaker's #5 or #55) for dissection, sterile.
5. Dissecting stereomicroscope with light source.
6. 1.5 mL microcentrifuge tubes.
7. 0.05% Trypsin/EDTA.
8. 18- and 23-gauge needles and 1 mL syringes, sterile, for tissue dissociation.
9. 1× Hank's Balanced Salt Solution (HBSS).
10. HBSS +1% BSA.
11. 5 mL FACS tubes compatible with flow cytometer.
12. Sorting flow cytometer.

2.2 Isolation of PGCs by Magnetic-Activated Cell Sorting (MACS)

Enrichment of germ cell populations can also be achieved using germ cell-specific antibodies and magnetically conjugated secondary antibodies that allow for on-column purification, first described in [5]. Due to germ cell differentiation as development progresses, different antibodies are used to identify and isolate germ cells at various stages. SSEA-1 is useful for migratory germ cells and early- to mid-gonadal germ cells (9.5–14.5 *dpc*) [6–8] while E-cadherin is useful for later gonadal germ cell isolations (13.5–15.5 *dpc*) [9]. Using this method (outlined in Subheading 3.2), germ cells can be isolated from any wildtype or transgenic line of interest.

1. One or more 11.5–15.5 *dpc* pregnant females of desired genetic background (*see Note 1*).
2. 1× PBS, sterile, for dissecting.
3. 10-cm culture dishes for dissection of fetuses and gonads.
4. Forceps with sharp tips (watchmaker's #5 or #55) for dissection, sterile.
5. Dissecting stereomicroscope with light source.
6. 1.5 mL microcentrifuge tubes.
7. 0.05% Trypsin/EDTA (Invitrogen).
8. 18- and 23-gauge needles and 1 mL syringes, sterile, for tissue dissociation.

9. MACS Buffer: 1× PBS, 2 mM EDTA and 0.5% BSA (Bovine serum albumin, Sigma Aldrich).
10. MACS MS separation columns (Miltenyi Biotech).
11. MACS pre-separation filters (Miltenyi Biotech).
12. Anti-SSEA-1 (CD115)-conjugated microbeads, mouse (Miltenyi Biotech, CAT# 130-094-530) – for 9.5–14.5 *dpc*.
13. Anti-E-Cadherin, mouse (BD Biosciences, CAT# 610182) – for 13.5–15.5 *dpc*.
14. Anti-mouse IgG microbeads (Miltenyi Biotech) – for use with anti-E-Cadherin only.
15. MACS MultiStand (Miltenyi Biotech).
16. MS Column Adapter (Miltenyi Biotech).
17. 2 mL microcentrifuge tubes.
18. 10 mL collection tubes.

2.3 Primary Fetal Germ Cell Culture

Fetal germ cells do not generally survive well in culture. A number of protocols have been developed for the culture of germ cells isolated from fetal mouse tissues, but to our knowledge these largely rely on the presence of serum, feeder cell layers or active signaling molecules (examples include [10, 11]). Although survival is certainly aided by the presence of serum, feeder layers, and “survival factors”, we have found a commercially available serum-free media (SFM) that supports germ cell survival, allowing the effect of various signaling molecules to be tested. For such studies (*see* Subheading 3.3), we find that shorter-term culture (less than or up to 48 h) is informative and that this can be supported in “serum-free” medium as detailed below. As it is well known that the combination of Kit ligand (also known as stem cell factor, SCF, or Steel factor), basic fibroblast growth factor (also known as bFGF, FGF2, FGF- β) and leukemia inhibitory factor (LIF) stimulates early gonadal germ cells to form embryonic germ cell colonies [12–14] we prefer to not include any of these compounds in our cultures. For cultures longer than 48 h, or not requiring serum-free conditions, we use “serum-containing” medium.

1. Freshly prepared 10 mL serum-free media (*see* Note 2): 9.85 mL of complete StemPro™-34 Serum-Free Media (SFM; made up as per manufacturer’s instructions, Gibco), 96 μ L of 200 mM L-Glutamine (Gibco) and 50 μ L 25 μ g/mL penicillin/streptomycin (Invitrogen) (*see* Note 3).
2. Freshly prepared 10 mL serum-containing media (*see* Note 2): 1.5 mL of 15% fetal bovine serum (FBS, AusGenx) (*see* Note 4), 8 mL of DMEM (Invitrogen), 50 μ L 25 μ g/mL penicillin/streptomycin (Invitrogen) (*see* Note 3), 100 μ L 2 mM Glutamax-1 (Invitrogen), 100 μ L 0.1 mM MEM

non-essential amino acids (Invitrogen), 100 μ L 1 \times Insulin-transferrin-sodium selenite media supplement (ITS; Sigma Aldrich), 100 μ L 1 \times N2 supplement (Invitrogen), 100 μ L 100 μ L 1 mg/mL N-acetyl-L-cysteine (NAC; Sigma Aldrich) (*see Note 5*) and 1.8 μ L of a 1/25 dilution of 0.1 mM β -mercaptoethanol (Invitrogen).

3. 12, 24, or 48-well culture plates, sterile.
4. Humidified 37 °C, 5% CO₂ incubator.

3 Methods

3.1 Isolation of PGCs by Fluorescence-Activated Cell Sorting (FACS)

1. Euthanize 11.5–18.5 *dpc* pregnant mice (*see Note 6*). Harvest embryos from the uterus and dissect embryos away from extra-embryonic membranes and place in a culture dish containing 1 \times PBS. Decapitate each embryo using forceps then cut along the ventral midline and remove the liver and gut.
2. Remove the genital ridges (with mesonephros attached) from the dorsal wall of the embryo (11.5–15.5 *dpc*), dorsal wall beneath kidneys (for ovaries, 16.5–18.5 *dpc*) or ventral posterior region (for testes; 16.5–18.5 *dpc*). Using a needle, cut away the mesonephros (Fig. 1) and collect the gonads (pooled or sexed; *see Note 7*) into a 1.5 mL microcentrifuge tube (*see Note 8*).
3. Add 1 mL of 0.05% trypsin/EDTA to each tube and incubate with gentle rocking at 37 °C for 5 min (11.5–13.5 *dpc*) or up to 10 min (14.5–18.5 *dpc*). Using an 18-gauge needle gently syringe five times to break up any larger tissue that has not dissociated, followed by use of a 23-gauge needle to achieve a single-cell suspension. Add 500 μ L of HBSS +1% BSA to dilute the trypsin (*see Note 9*). Centrifuge at 500 $\times g$ for 10 min at 4 °C. Remove supernatant and wash once with 1 mL HBSS. Resuspend the pellet in 0.5 mL HBSS and ensure a single-cell suspension by passing through a 23-gauge needle once more. Place cell suspension in 5 mL FACS tube on ice and proceed to flow cytometer.
4. Viable cells are gated based on forward scatter (FSC) vs. side scatter (SSC) profile (*see Note 10*). eGFP vs FSC will delineate a highly eGFP-positive population of cells that are the germ cells (Fig. 2). Exclude doublets (**Note 11**). Gate the eGFP-positive population for collection as germ cells and the remaining eGFP-negative cells as somatic cells. Due to the brightness of the eGFP it is not necessary to provide a GFP-negative control, although dissection of another tissue (such as kidney or limb), processed using the above method, could serve this purpose.

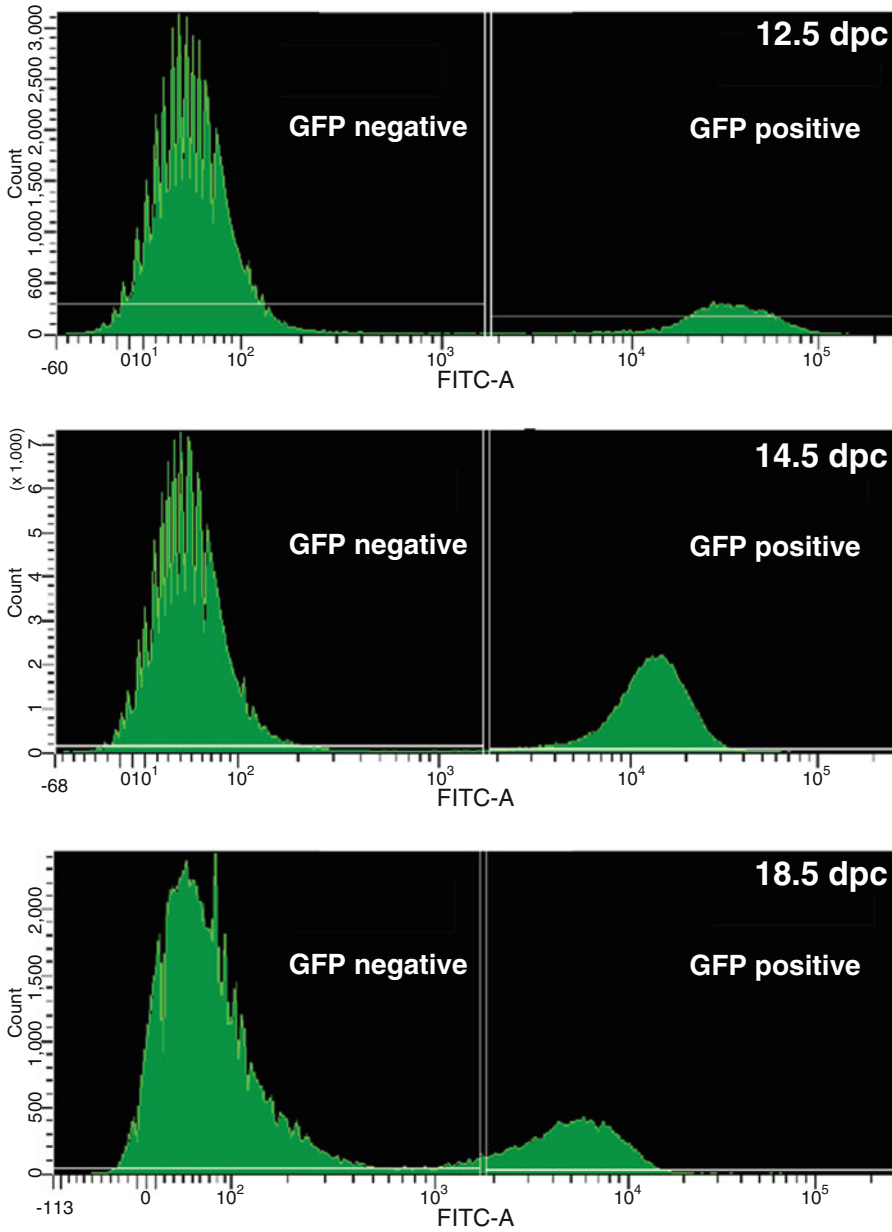


Fig. 2 Representative FACS histogram generated during purification of germ cells from *OG2* gonads. Histogram of GFP intensity relative to cell counts in gonadal tissue at 12.5, 14.5, and 18.5 *dpc*

- Sorted germ cell (eGFP+) and somatic cell (eGFP-) populations can be immediately processed for RNA extraction, DNA extraction, immunocytochemistry (following cytospinning) or primary cell culturing (see Protocol 3.3). The germ cell population isolated by this method is highly pure, although there will be some germ cell contamination of the somatic population (Fig. 3).

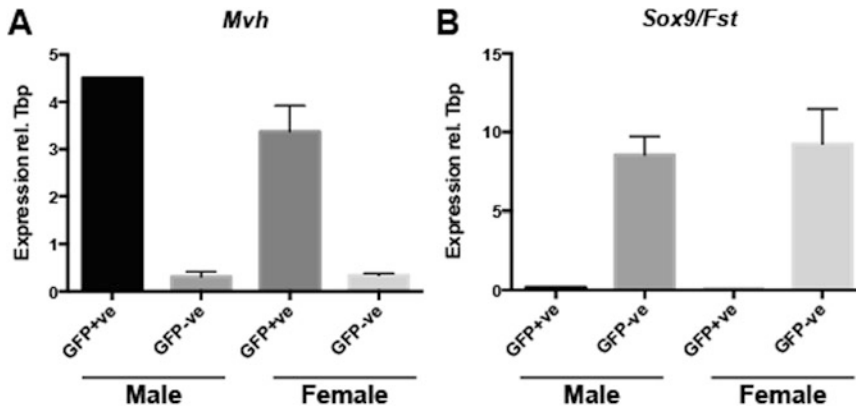


Fig. 3 Assessment of germ cell purity from *OG2* FACS isolations. **(a)** Expression of germ cell marker *Mvh* in GFP+ve (germ cell) and GFP-ve (somatic cell) populations collected via FACS from male and female gonads at 13.5 *dpc*. **(b)** Expression of somatic cell markers *Sox9* (male) and Follistatin (*Fst*; female) in GFP+ve (germ cell) and GFP-ve (somatic cell) populations collected via FACS from male and female gonads at 13.5 *dpc*

3.2 Isolation of PGCs by Magnetic-Activated Cell Sorting (MACS)

1. Euthanize 11.5–18.5 *dpc* pregnant mice (*see Note 6*). Harvest embryos from the uterus, dissect embryos away from extraembryonic membranes and place in culture dish containing PBS. Decapitate each embryo using forceps then cut along the ventral midline and remove the liver and gut.
2. Remove the genital ridges (with mesonephros attached) from the dorsal wall of the embryo (11.5–15.5 *dpc*), dorsal wall beneath kidneys (for ovaries, 16.5–18.5 *dpc*) or ventral posterior region (for testes; 16.5–18.5 *dpc*). Using a needle cut away the mesonephros (Fig. 1; *Note 12*) and collect the gonads (pooled or sexed; *see Note 7*) into a 1.5 mL microcentrifuge tube (*see Note 8*).
3. Add 1 mL of 0.05% trypsin/EDTA to each tube and incubate with gentle rocking at 37 °C for 5 min (11.5–13.5 *dpc*) or up to 10 min (14.5–18.5 *dpc*) (*see Note 9*). Using an 18-gauge needle gently syringe five times to break up any larger tissue that has not dissociated, followed by use of a 23-gauge needle to achieve a single-cell suspension. Add 1 mL of MACS buffer to dilute the trypsin. Centrifuge at $500 \times g$ for 10 min at 4 °C. Remove supernatant and wash once with 1 mL MACS buffer followed by re-centrifugation.
4. Resuspend the pellet in 80 μ L MACS buffer and 20 μ L of anti-SSEA-1 (CD115) microbeads (9.5–14.5 *dpc*) OR in 94 μ L MACS buffer and 4 μ L of anti-E-Cadherin (13.5–18.5 *dpc*) and incubate with agitation at 4 °C for 45 min. Add 1 mL of MACS buffer and centrifuge at $500 \times g$ for 10 min at 4 °C. Remove supernatant and wash once with 1 mL MACS buffer followed by re-centrifugation.

5. If using anti-SSEA-1 (CD115) microbeads (9.5–14.5 *dpc*) proceed to step 6. If using anti-E-Cadherin (13.5–18.5 *dpc*), resuspend the pellet in 80 μ L MACS buffer and 20 μ L of anti-mouse IgG microbeads and incubate with agitation at 4 °C for 15 min. Add 1 mL of MACS buffer and centrifuge at $500 \times g$ for 10 min at 4 °C. Remove supernatant and wash once with 1 mL MACS buffer followed by centrifugation.
6. Fix MiniMACS column to magnetic backing and add 1 mL MACS buffer to pre-soak the column, discarding the flow-through. Resuspend pelleted cells in 1.5 mL MACS buffer and apply to a pre-soaked MiniMACS column on magnetic backing. Somatic cells are eluted with 3 mL of MACS buffer (added in 1 mL aliquots) into a 10 mL collection tube beneath the column (*see Note 13*). Remove the column from the magnetic backing, place into a 2 mL microfuge tube and elute germ cells in 2 mL MACS buffer (*see Note 14*).
7. Sorted germ cell and somatic cell populations can be immediately processed for RNA extraction, DNA extraction, immunocytochemistry (following cytospinning) or primary cell culturing (*see Subheading 3.3*). This method provides an enriched germ cell population that will carry some somatic cell contamination (*see Note 15*). There will also be some germ cell contamination of the somatic population.

3.3 Primary Fetal Germ Cell Culture

1. Pellet germ cells by centrifugation at $500 \times g$ for 10 min at 4 °C. Remove supernatant and resuspend in an appropriate volume of serum-free or serum-containing media. The volume used for resuspension should be slightly more than half the total volume of media required for all wells. Divide suspended germ cells into culture plate cells adding half the final planned volume per well (*see Note 16*).
2. Prepare treatments (dilute active molecules/chemical inhibitors in media) in microfuge tubes at twice the final concentration required. After mixing, gently add treated media to each culture plate well to make up to the final volume required (add the same volume of media as the volume of cells in media already dispensed). We generally use 0.5 mL (48-well), 1 mL (24-well), or 2 mL (12-well plates) (*see Note 17*).
3. Incubate plates in a humidified 37 °C, 5% CO₂ incubator. Serum-free germ cell culturing can be maintained for up to 24 h. Serum-containing germ cell culturing can be maintained for up to 48 h.
4. To harvest cells pipette media up and down and transfer to a microfuge tube. Wash each well at least once with PBS and further pipetting to ensure good cell recovery (*see Note 18*). Centrifuge at $500 \times g$ for 10 min to pellet cells and proceed to RNA extraction, DNA extraction or immunocytochemistry (following cytospinning).

4 Notes

1. All protocols involving live animals must be reviewed and approved by an animal ethics committee and must conform to government regulations for the care and use of laboratory animals. The *OG2* line was originally generated on a CBA/CaJ X C57BL/6J background [3] and we maintain it on a C57BL/6 background and genotyped using the protocol outlined in [15]. In order to ensure large litter sizes and also large numbers of germ cells per gonad we mate homozygous *OG2* studs with wildtype CD1 outbred females. Usually, a minimum of two or more litters is pooled for this protocol. Noon on the day of plug is counted as 0.5 *dpc*.
2. Media prepared under sterile conditions in a Class II Biological Hazard laminar-flow hood.
3. Germ cell survival is substantially improved when penicillin and streptomycin concentrations are lower than is normal for cell culture.
4. Use FBS that has been cell culture tested.
5. NAC is an antioxidant that substantially improves germ cell survival [10].
6. Additional reagents and equipment for euthanasia of the mice are described in [16].
7. Sexing gonads at 11.5 *dpc* is only possible by PCR and therefore males and females are generally pooled at this timepoint. If sexing is required, each embryo is dissected into a 24-well plate containing warm DMEM +10%FBS, and a tail tip is taken for PCR sexing as per protocol [17]. Once results are determined (2–3 h total time) embryos are separated based on sex and gonads dissected as per this standard protocol.
8. It is possible to isolate germ cells from single gonad pairs, although germ cell recovery is very low due to loss of cells during the dissociation process. Usually two or more sexed, pooled litters are harvested at a time to ensure good germ cell yield.
9. To minimize cell damage and death, keep trypsin incubation time to a minimum and syringe gently for the least number of times to achieve a single-cell suspension.
10. The germ cells cluster with a high FSC and low SSC due to their large size and low cellular complexity and are usually identifiable by these features alone.
11. Excluding doublets, along with ensuring a single-cell suspension is achieved, will ensure highest purity of germ cells.

12. When isolating populations using E-Cadherin (13.5–18.5 *dpc*) it is imperative that all mesonephric tissue is cleanly removed due to E-Cadherin-positive cells within this tissue.
13. Flow-through containing somatic cells can be passed back through the column to maximize germ cell yield. Following this, pass another 1 mL MACS buffer through column before eluting germ cells.
14. Once the MACS buffer has flowed through the column via gravity, gently insert the pusher into the top of the column to force out the last few microliters of buffer and cells.
15. Minimize somatic cell contamination of the germ cell population by ensuring a single cell suspension after dissociation; somatic cells that are bound to germ cells will be isolated as “germ cells” using this technique. Germ cell purity, determined by alkaline phosphatase staining [18], is about 90% using this protocol.
16. Because purified germ cells are quite adherent we avoid loss by adding them straight to cell culture plates rather than preparing the various treatments in tubes.
17. Germ cells are cultured sparsely, seeded at approximately 20,000 cells per well (24-well plate) or as low as 5000 cells per well (48-well plate).
18. Germ cells become quite adherent to the culture dish during culturing. Be sure to thoroughly suspend the cells by pipetting upon harvesting.

Acknowledgments

This work was supported by the Australian Research Council [DP140104059; DP200102896], Cancer Council Queensland [APP1012325], Rebecca L Cooper Medical Research Foundation, and the Kids’ Cancer Project. CS is a University of Queensland Postdoctoral Fellow.

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***Rattus norvegicus* Spermatogenesis Colony-Forming Assays**

Karen M. Chapman, Ashutosh Pudasaini, Morgan N. Vanderbeck, and F. Kent Hamra

Abstract

Knowledge gaps persist on signaling pathways and metabolic states in germ cells sufficient to support spermatogenesis independent of a somatic environment. Consequently, methods to culture mammalian stem cells through spermatogenesis in defined systems have not been established. Lack of success at culturing mammalian stem cells through spermatogenesis in defined systems reflects an inability to experimentally recapitulate biochemical events that develop in germ cells within the testis-specific seminiferous epithelium. Complex germ and somatic cell associations that develop each seminiferous epithelial cycle support such a hypothesis, conceivably explaining why highly pure mammalian spermatogonia do not effectively develop into and through meiosis without somatic cells. Here, we outline an *in vitro* spermatogenesis colony-forming assay to study how differentiating spermatogonial syncytia develop from rat spermatogonial stem cell lines. Robust spermatogonial differentiation under defined culture conditions, once established, is anticipated to facilitate molecular biology studies on pre-meiotic steps in gametogenesis by providing soma-free bioassays to systematically identify spermatogenic factors that promote meiotic progression *in vitro*.

Key words Spermatogenesis, Gametogenesis, Spermatogonial stem cell, Germline stem cell, Self-renewal, Proliferation, Differentiation, *In vitro*, Germline gene targeting

1 Introduction

Spermatozoa are produced within the testes' seminiferous epithelium by consecutive developmental processes termed spermatogenesis and spermiogenesis [1]. Spermatogonial stem cells maintain spermatogenesis through their unique abilities to self-renew or produce spermatogonial syncytia of nascent spermatozoan progenitors that differentiate through meiosis to form haploid gametes termed round spermatids [2]. Round spermatids can then undergo the male germline-specific post-meiotic process, spermiogenesis, to differentiate into fully elongated spermatozoa [1].

In mammals, spermatogonial stem cells reside within a population of “A-single” (A_s) spermatogonia [3–5]. A_s spermatogonia divide to renew germline stem cells but can also produce syncytia of early “undifferentiated” spermatogenic progenitors termed A-paired (A_{pr}) and A-aligned (A_{al}) spermatogonia [5]. In rodents, undifferentiated spermatogonia mitotically arrest during seminiferous epithelial cycle stages VI–VIII, and then transform into “differentiating” type A1 spermatogonia under the control of the active vitamin A derivative, retinoic acid (RA), and the polypeptide growth factor, KIT Ligand (KITL) [6–8]. Type A1 spermatogonia re-enter the mitotic cell cycle and give rise to subsequent generations of differentiating spermatogonia (types $A2 > A3 > A4 > Int > B$) [9], by which time, germ cell numbers/syncytium can be amplified >100-fold prior to entering meiosis to form spermatocytes [10].

In the rat’s seminiferous epithelium, sub-populations of $Gfra1^+$ and $Gfra1^-$ undifferentiated type A_s spermatogonia are selectively enriched with nuclear *Foxa2* and are detected during all stages of a seminiferous epithelial cycle [11]. As rat germline syncytial clones develop into A_{pr} and A_{al} spermatogonia, their nuclear *Foxa2* decreases in relative abundance [11]. In rats, *Snap91* is a cytoplasmic marker enriched on endosome vesicle-like organelles in $Gfra1^+$, $Sall4^+$, $Cd9^+$, $Zbtb16^+$ undifferentiated A_s and A_{pr} spermatogonia along all stages of a spermatogenic wave [12]. Approximately 5% of total $Snap91^+$ A_s spermatogonia/wave are $ErbB3^+$, as reflected by the $ErbB3^+A_s$ spermatogonia being tightly restricted to late stage VIII and stage IX seminiferous tubule segments [12]. Epithelial cycle stage-dependent detection of *ErbB3* strictly on undifferentiated A_s spermatogonia ($Snap91^+$, $Gfra1^+$, $Foxa2^+$, $Zbtb16$, $Sall4^+$) in rat testes currently seems paradoxical to robust induction of *ErbB3* mRNA and protein by all-trans-retinoic acid (ATRA) during rat spermatogonial differentiation *in vitro* [11]. Interestingly, in testes, stage-dependent induction of $ErbB3^+$ on rat A_s spermatogonia [12] coincides with the dominant stage VIII-IX peak in RA accumulation during a mouse seminiferous epithelial cycle [13].

In rat testes, A_{al} spermatogonia robustly express *Gfra1*, and then transiently up-regulate *Foxa1* in $Gfra1^+$, $Rarg^+$, $Foxa2^-$ type A_{al} spermatogonia at the 8–32 cell steps in syncytial development [11]. Based on mouse genetics [14] and syncytia length in rat [5], the transient sharp peaks of $Foxa1^+$ spermatogonial syncytia detected along a spermatogenic wave appear to be primed for differentiation into type A1 spermatogonia ($Gfra1^-$, $Foxa1^-$, $Foxa2^-$, $Rarg^-$), prior to further development into meiosis during stage VIII of a subsequent epithelial cycle [11]. Proteins that distinctly mark pivotal steps in rat A_s (*ErbB3*, *Foxa2*), A_{pr} (*Snap91*), and A_{al} (*Foxa1*) spermatogonia maturation can be exploited to help map the molecular-anatomical topography of

germline stem cell clone development within the rat's seminiferous epithelium [11, 12].

Rodent spermatogonial stem cells can be maintained long term in culture [15], but can only be effectively cultured through meiosis in recipient testes [2, 16], or in organ culture systems with somatic testes cells [17, 18]. Still, chemically defined culture media that support soma-free spermatocyte maturation and spermatid development from mammalian spermatogonial stem cell lines remain to be established [19–21]. *Glial cell line-derived neurotrophic factor* (*Gdnf*) and *Fibroblast growth factor 2* (*Fgf2*) genes encode polypeptides that support clonal expansion of functional mouse [22, 23], rat [24, 25], shrew [26], and hamster [27] donor spermatogonial stem cell lines that maintain their sperm-forming potential for haploid genome transfer by normal mating after multiple diluting passages during subculture in vitro. Accordingly, *Gdnf* and *Fgf2* are currently essential components in serum-free media that maintain functional mouse spermatogonial stem cell proliferation long-term without somatic cells [28]. In contrast to highly defined culture systems that maintain rodent spermatogonial stem cell proliferation, prominent knowledge gaps exist on biochemical pathways in germ cells needed to effectively support full meiotic and/or post-meiotic spermatogenic differentiation steps independent of somatic cells. In the face of such a challenge to re-construct the complex nature of spermatozoan development in the “test-tube”, significant advances are continuously being made towards establishing 2D systems that, ultimately, provide more-simplified, controllable methodology to investigate molecular mechanisms in germ cells that can be used to drive spermatogenesis and spermiogenesis *in vitro* [19–21].

Recently, two polypeptides, Neuregulin-1 (NRG1) and KITL, were reported to robustly support syncytial growth of differentiating spermatogonia from authenticated rat spermatogonial stem cell lines depleted of somatic cells by signaling germ cell survival in response to the RA analog all-trans-retinoic acid (ATRA) [29]. By supplementing a serum-free rat spermatogonial culture medium (SG Medium) with ATRA and NRG1 (or KITL), an effective serum-free rat spermatogonial differentiation medium was formulated (SD Medium) [29]. Disruption of *ErbB3* in rat spermatogonial stem cell lines (CRISPR/Cas9) genetically verified that the *ErbB3* ligand, Neuregulin-1 β 1 (Nrg1 β 1), can signal differentiating spermatogonial survival in soma-free culture on laminin by activating a Lapatinib-sensitive spermatogonial *ErbB2* pathway that was distinct from an alternative Lapatinib-insensitive, *ErbB3*-independent, Kit Ligand-dependent survival pathway in rat differentiating spermatogonia [30, 31].

Spermatogonial lines sub-cultured in GDNF-containing medium commonly grow in colonies that form “clumps”, which contain stem spermatogonia based on their ability to regenerate

spermatogenesis in recipient testes [22–25, 27, 32]. Spermatogonial lines cultured in GDNF-containing medium also contain progenitor-like “undifferentiated” type A spermatogonia based on ineffectiveness at regenerating spermatogenesis in recipient testes [22, 33, 34]. Because undifferentiated spermatogonia are prone to syncytia formation when propagated in GDNF-containing medium (Fig. 1a), we find TEX14^+ syncytial clones containing 2 or more cells are commonly passaged during subculture, which hold potential to more rapidly develop into larger syncytia upon differentiation (Fig. 1b).

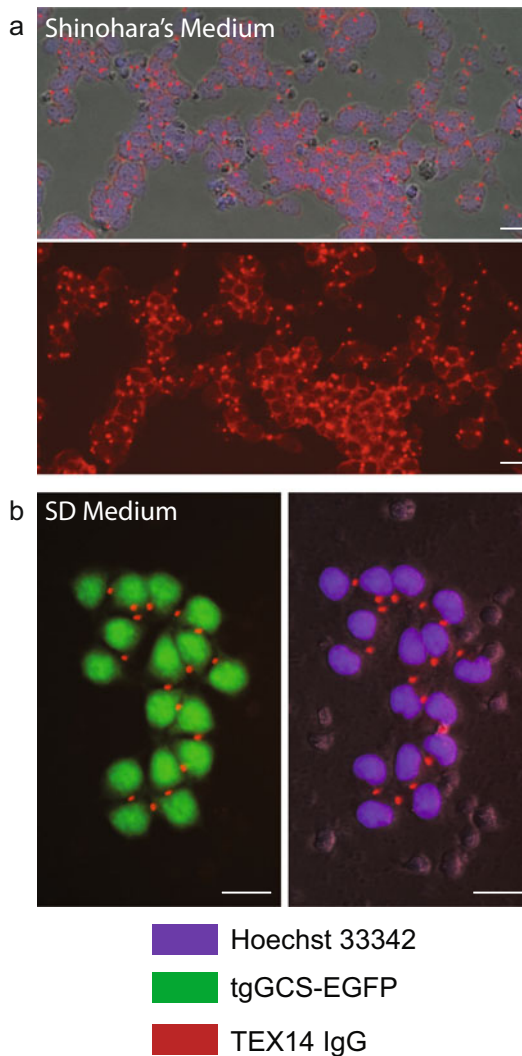


Fig. 1 TEX14 labeling in undifferentiated and differentiating spermatogonial cultures. (a) TEX14 antibody labeling in a DBA2 mouse spermatogonial line sub-cultured on laminin in Shinohara's original spermatogonial culture medium [22]. Scale, 100 μm . (b) TEX14 antibody labeling in a rat spermatogonial syncytium on laminin that developed in SD Medium from a spermatogonial line originally derived in SG Medium. Scale, 100 μm

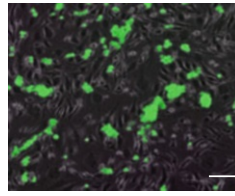
To avoid harsher dissociation treatments needed to generate single spermatogonial suspensions from spermatogonial lines, after passaging, spermatogonia are pre-incubated in a modified SG Medium termed A_s-SG Medium for 5–8 days to select against clump and syncytia formation [29]. Pre-incubation in A_s-SG Medium biologically enriches spermatogonial cultures with A_s spermatogonia prior to initiating differentiation in SD Medium. Unlike SG Medium, A_s-SG Medium does not contain GDNF (Fig. 2), which reduces spermatogonial syncytial size and heterogeneity within cultures prior to initiating differentiation in SD Medium. Shorter pre-incubation times in A_s-SG Medium prior to initiating differentiation in SD Medium increases heterogeneity in spermatogonial syncytia size and differentiation state within cultures. Conversely, longer incubation times in SD Medium increases mean nuclei number/syncytium/culture and effectively depletes spermatogonia of their ability to regenerate spermatogenesis in recipient testes [29].

Rattus norvegicus spermatogenesis colony assays provide flexible, scalable, and highly defined experimental platforms to investigate molecular mechanisms regulating spermatogonial proliferation, differentiation, and degeneration. In this chapter, we outline the methods reported by Chapman and colleagues on how rat spermatogonial stem cell lines (Foxa2⁺) derived and propagated in SG Medium are stimulated to undergo pre-meiotic spermatogenic differentiation by culturing in SD Medium with and without somatic cells [11, 30, 31]. Moreover, CRISPR/Cas9-mediated germline gene targeting in rat spermatogonial lines enables dominant and recessive traits controlling spermatogonial fate to be analyzed using the detailed *in vitro* and *in vivo* spermatogenesis colony-forming assays outlined in this edition (*see new* Subheading 3.8) [35]. Thus, herein, we tailor a spermatogenesis colony-forming assay that is highly amenable to studying molecular mechanisms that drive the clonal development of differentiating spermatogonial syncytia from highly enriched cultures of undifferentiated, rat type A_s spermatogonia (Fig. 2).

2 Materials

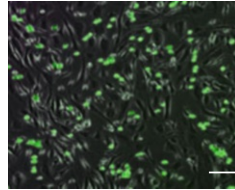
The following *in vitro* spermatogenesis colony-forming assay was established with undifferentiated, rat spermatogonial lines derived on DR4 MEFs (Fig. 3a) [25, 32, 36]. Rat spermatogonial lines are endowed with spermatogonial stem cells that regenerate and maintain spermatogenesis long-term in recipient rat testes (Fig. 3b, c) [35, 37]. Based on spermatogenic colonies formed/recipient testis/number of germ cells transplanted (~1/65), rat spermatogonial lines represent a most potent source of germline stem cells [25, 29, 32]. Functionally validated rat spermatogonial lines from wildtype

Subculture Undifferentiated Rat Spermatogonial
Line on MEFs in SG Medium



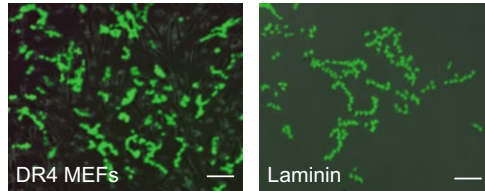
GDNF &
FGF2

Select for A-Single Spermatogonia
on MEFs or Laminin in A_s-SG Medium



FGF2 &
Lapatinib

Differentiate Spermatogonia on
MEFs or Laminin in SD Medium



DR4 MEFs

Laminin

FGF2 & ATRA**

GDNF*, FGF2,
ATRA, NRG1 or KITL**

Molecular
Biology Assays

Fig. 2 Spermatogonial culture media for *in vitro* spermatogenesis colony-forming assays: Undifferentiated rat spermatogonial lines are derived and maintained on DR4 MEFs in SG Medium (Contains GDNF & FGF2). Undifferentiated spermatogonial colonies grow in non-uniform clumps (**Top Image**) [22, 25]. Spermatogonia are passaged onto new DR4 MEF feeder layers (shown), or laminin-coated dishes and cultured in A_s-SG Medium (Contains FGF2 & Lapatinib) to enrich cultures with A_s spermatogonia (**Middle Image**). Spermatogenic differentiation is induced by culturing spermatogonia in SD Medium (Contains FGF2, GDNF, ATRA, NRG1, and/or KITL). *GDNF is not an essential component of SD Medium, but increases SD Medium effectiveness on laminin [29]. **NRG1 and KITL are interchangeable, yet essential SD Medium components required for differentiating spermatogonial syncytia to survive on laminin. DR4 MEFs produce NRG1/KITL-like factors, and thus, neither NRG1 nor KITL is required in SD Medium to support spermatogonial differentiation on MEFs [29]. Green = tgGCS-EGFP germline-specific transgene [38]. Scale, 100 μm

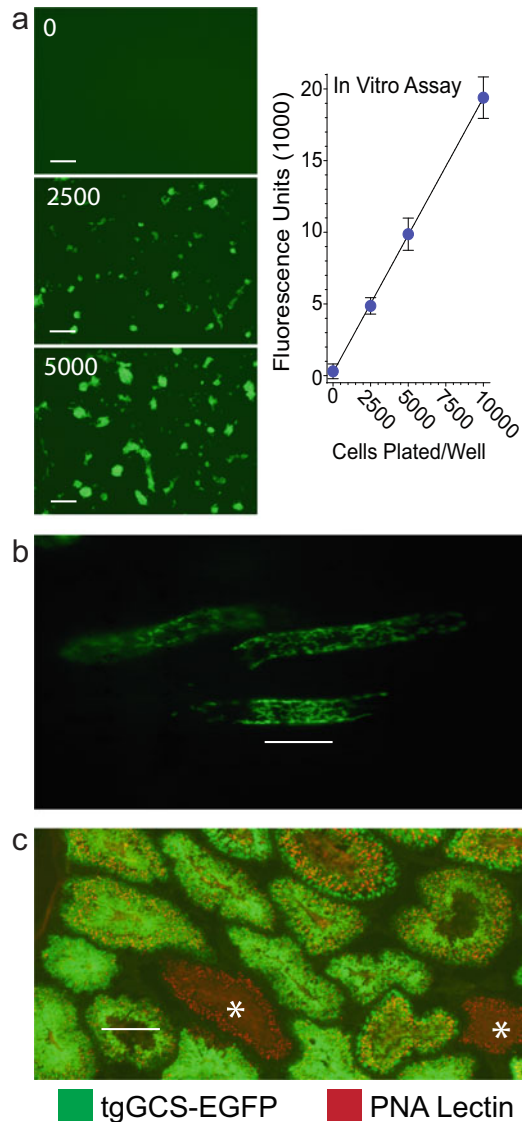


Fig. 3 Spermatogenic potential of rat spermatogonia sub-cultured in SG medium. **(a)** (Left) tgGCS-EGFP⁺ spermatogonial stem cell line (green) d7 after plating onto DR4 MEFs in 96-well plate at 0, 2500, and 5000 cells/well in SG Medium. Scale, 200 μ m. (Right) EGFP Fluorescence units/number EGFP⁺ spermatogonia plated/well from cultures in panel “a”. Cultures were lysed directly in wells and Fluorescence Units/well measured in a plate reader (lysis buffer: NaCl 100 mM, EDTA 1 mM, 10% glycerol v/v, 1% Trion X-100 v/v, 50 mM HEPES pH 8). **(b)** (Left) Image showing 3 donor-derived spermatogenic colonies (green) in a recipient rat’s seminiferous tubules d32 after transplanting tgGCS-EGFP⁺ rat spermatogonia. Scale, 200 μ m. (Right) Relationship between number tgGCS-EGFP⁺ spermatogonia transplanted and donor-derived spermatogenic colonies formed/testis scored d27 after transplantation (\pm SEM, $n = 5$ rats, right testis transplanted). Scale, 200 μ m. **(c)** Donor-derived spermatogenesis in rat seminiferous tubules d178 after transplanting tgGCS-EGFP⁺ rat spermatogonia (green) clonally expanded from an individually picked colony. Scale, 200 μ m

rats, or transgenic rats that express germline-specific EGFP [38] and tdTOMATO markers [39], can be requested from the Hamra lab. Fully functional, genetically modifiable rat spermatogonial lines can also be derived from 23 to 24-day-old Sprague Dawley rats as detailed in Chapter 12 (*Rat Spermatogonial Stem Cell-Mediated Gene Transfer*), Springer Press edition: “Advanced Protocols for Animal Transgenics” edited by Shirley Pease and Thomas L. Saunders (*see Note 1*) [36].

2.1 Spermatogenesis Colony-Forming Assays

1. Laboratory should be equipped with staff and basic equipment to conduct eukaryotic cell culture under sterile conditions: Eppendorf pipets and tips, serological pipets, 0.2 micron filters, swinging bucket table-top centrifuge, media water bath at 34–35 °C, hemocytometer, fluorescence/phase contrast microscopes, CO₂ supplied cell culture incubators set at 36–37 °C and dedicated refrigerators and freezers for storing reagents.
2. Certified biosafety cabinet in clean, low traffic environment dedicated to conducting eukaryotic cell culture under sterile conditions.
3. Rat spermatogonial stem cell line: request frozen stocks of functionally validated spermatogonial lines from F.K. Hamra. Alternatively, isolate undifferentiated rat A_s and A_{pr} spermatogonia fresh, or derive primary rat spermatogonial lines as outlined (*see Note 1*) [36].
4. Costar Clear TC-Treated Microplates, Individually Wrapped, Sterile 6-well plate, 12-well plate, 24-well plate, 48-well plate, or 96-well plate (Corning, Inc.) (*see Note 2*).
5. Dulbecco’s modified Eagle’s medium: Ham’s F12 medium 1:1 (DHF12): (Sigma, Inc.).
6. Antibiotic–antimycotic solution (100×): 10,000 U/mL penicillin G sodium (U/v), 10,000 µg/mL streptomycin sulfate (w/v), and 25 µg/mL amphotericin B (w/v) (Life Technologies Inc.).
7. DHF12 solution, Dulbecco’s modified Eagle’s medium: Ham’s F12 medium 1:1, 1% antibiotic/antimycotic solution (v/v).
8. 1 mg/mL Laminin solution (Sigma, Inc.).
9. Parafilm M (Bemis, Inc.).
10. Heat Inactivated Fetal bovine serum (FBS): (Tissue Culture Biologicals, Inc.).
11. Dulbecco’s modified Eagles Medium-high glucose (DMEM-high glucose) (Sigma, Inc.).

12. MEF medium: Dulbecco's modified Eagle's medium, 1.5 g/L sodium bicarbonate (v/v) and 15% heat-inactivated FBS (v/v).
13. DR4 Mouse Embryonic Fibroblasts (MEFs): (ATCC).
14. Irradiator source to mitotically arrest DR4 MEFs (optional: *see Note 3*).
15. Recovery Cell Culture Freezing Medium (Life Technologies, Inc.).
16. T225 Flasks Angled Neck (Corning, Inc.).
17. Gelatin from Porcine Skin-Type A (Sigma, Inc.).
18. 0.1% gelatin solution: dissolve gelatin in ultrapure laboratory grade water (1 g gelatin/L) and autoclave on liquid cycle. Store stock solution at 4 °C up to 2 months; filter-sterile before each use.
19. Dulbecco's phosphate-buffered saline (PBS) (Sigma, Inc.).
20. B-27 Supplement minus vitamin A (50x) (Life Technologies, Inc.).
21. L-glutamine (Life Technologies, Inc.).
22. Recombinant FGF2 (Sigma, Inc.).
23. Recombinant GDNF (R & D Systems, Inc.).
24. Recombinant NRG1 β 1, T176-K246 (R & D Systems, Inc.).
25. Recombinant KITL (R & D Systems, Inc.).
26. all-trans-retinoic acid (Sigma, Inc.).
27. Lapatinib (LC Laboratories, Inc.).
28. 2-Mercaptoethanol.
29. Protease-, nuclease- and fatty acid-free Bovine Serum Albumin (Calbiochem, Inc.).
30. 0.1 M Sodium Phosphate Buffer, pH 7.2.
31. 4% Paraformaldehyde Solution, pH 7.2: 10 mL 16% paraformaldehyde (Thermo Scientific, Inc.) (v/v), 30% sodium phosphate solution, pH 7.2 (v/v).
32. Methanol (Pharmco-AAPER, Inc.).
33. Germ cell marker antibodies: rabbit anti-rat DAZL IgG [40]; rabbit anti-rat TEX14 IgG [41]. (Request primary antibody stocks to DAZL and TEX14 from F.K. Hamra).
34. Spermatogonial marker antibodies: mouse anti-ZBTB16 IgG (clone 2A9, Active Motif, Inc); mouse anti-Foxa1 IgG (clone 1519, GeneTex, Inc.); rabbit anti-Foxa2 IgG (clone D56D6, Cell Signaling Technology, Inc); mouse anti-SALL4 IgG (clone 6E3, Abnova).

35. Secondary antibodies: highly cross absorbed AlexaFluor 595-conjugated, goat anti-mouse IgG (Life Technologies), AlexaFluor 488-conjugated, goat anti-rabbit IgG (Life Technologies).
36. Hoechst 33342 dye (ThermoFisher, Inc.).
37. Roche Western Blocking Reagent (Roche Applied Science; distributed by Sigma, Inc.).
38. Triton X-100 (Sigma-Aldrich, Inc.).
39. Neon Transfection System (ThermoFisher, Inc.).
40. Neon Transfection Kit—10 μ L tips (ThermoFisher, Inc.).
41. Neon Transfection Buffer R (also provided in kit from **step 40**) (ThermoFisher, Inc.).
42. Custom gene-specific Alt.-R CRISPR single guide RNA (sgRNA) (IDT, Inc.).
43. IDTE buffer (IDT, Inc.).
44. Alt-R HiFi S.p.Cas9 Nuclease V3, 62 μ M stock (IDT, Inc.).
45. Rat embryonic fibroblasts (REFs) (CellBiologics, Inc.).

3 Methods

Steps are outlined to conduct *in vitro* spermatogenesis colony-forming assays initiated by seeding rat spermatogonial stem cell lines onto laminin matrix (Subheading 3.1) or DR4 MEF feeder layers (Subheading 3.2) in A_s-SG Medium for a pre-incubation period prior to conditionally driving spermatogenic differentiation using SD Medium (Fig. 2). Spermatogenesis colony-forming assays conducted on laminin in SD Medium provide controllable conditions to study molecular mechanisms critical for spermatogonial differentiation (Fig. 4a). Optionally, MEFs produce paracrine factors that positively impact the spermatogenesis colony-forming assays outlined in Subheading 3.6. *In vitro* spermatogenesis colony-forming assays on DR4 MEFs or other feeder cell types provide a workable platform to discover/investigate factors that regulate spermatogonial biology. Spermatogenic factor activity (s) can then be probed in more detail without somatic cells on an extracellular matrix, such as a laminin matrix (Fig. 4b) [29, 41, 42]. CRISPR/Cas9-mediated gene editing technology can be combined with *in vitro* and *in vivo* spermatogenesis colony-forming assays, as detailed herein, to study how genes regulate the developmental fate of stem, progenitor, and differentiating spermatogonia (Fig. 5) [35]. Spermatogonial stem cell lines harboring targeted genomic DNA modifications can, similarly, be used as donor germline vectors to genetically engineer new rat strains by adapting the methods of Chapman, et al. (Fig. 6a) [31].

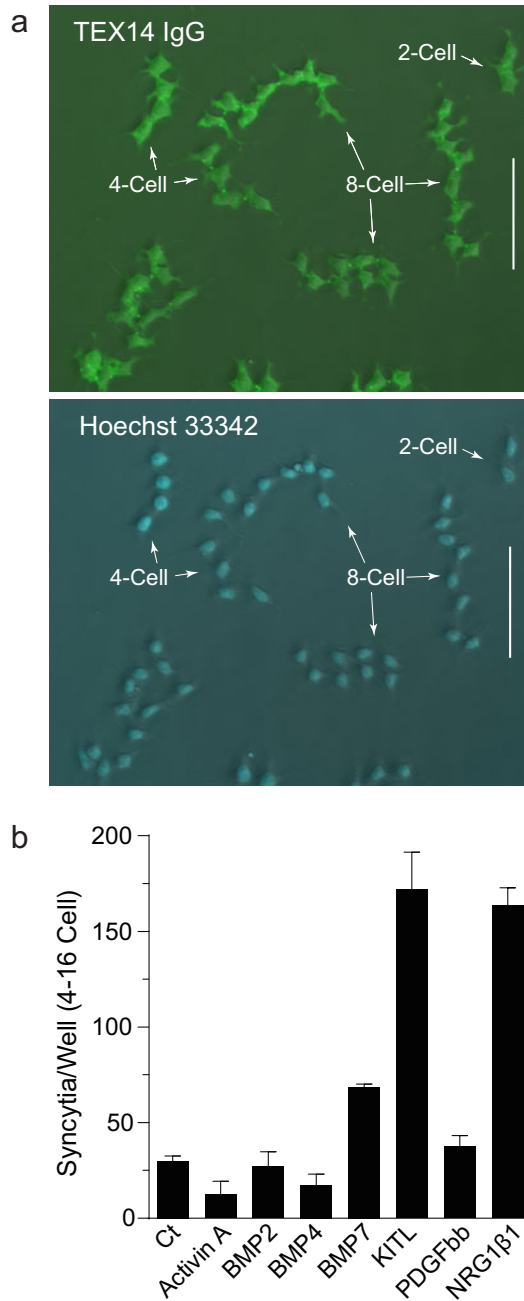


Fig. 4 Analyzing spermatogonial Syncytia development in culture. **(a)** Spermatogonial syncytia at the 2-cell, 4-cell, and 8-cell steps in development after culturing for 6 days in SD Medium containing 5 nM KITL [29]. Spermatogonia were co-labeled with an antibody to rat TEX14 (green; **Top**) and the nuclear dye, Hoechst 33342 (blue; **Bottom**). The rat TEX14 IgG selectively labels germ cell cytoplasm and forms concentrated foci at ring canals marking cytoplasmic bridges within a spermatogonial syncytium. **(b)** Spermatogonial syncytia containing 4–16 cells scored/well (0.96 cm²) by counting relative numbers of TEX14⁺ foci and Hoechst 33342⁺ nuclei/syncytium (*see Note 19*). Cultures were scored after 6 days in SD Media supplemented with respective ligands at 5 nM in place of KITL and NRG1 (±SEM, triplicate wells/condition). BMP, bone morphogenetic protein; PDGF, platelet-derived growth factor. Scale, 200 μm

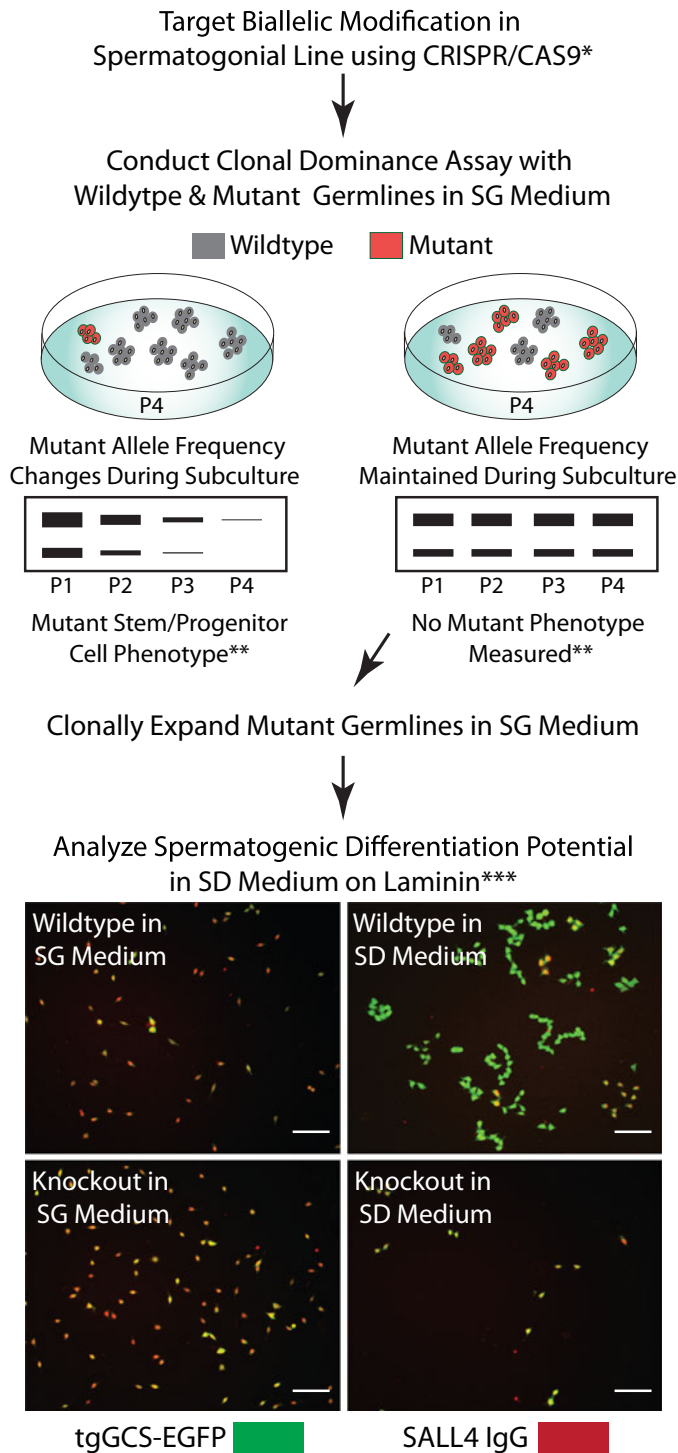


Fig. 5 Colony-forming assays to study genetic effects on spermatogonial fate. Clonal dominance assays in SG Medium and differentiation assays in SD Medium can be used to identify and measure gene mutation effects on spermatogonial fate. *Biallelic mutation frequency/cell/culture catalyzed by CRISPR/Cas9 depends on targeting construct and gene delivery efficiencies [35, 36]. **Phenotypes that disrupt spermatogonial viability or proliferation in SG Medium preclude the ability to clonally enrich for targeted germline mutations. **(Bottom Images)** Clonally expanded wildtype and *ErbB3* knockout spermatogonial lines on laminin after culturing 7 days in A₅-SG Medium and 6 days in SD Medium. ***Mutant germline analyses on DR MEFs and/or in recipient testes can also be conducted to investigate spermatogonial phenotypes identified on laminin [29]. Scale, 100 μ m

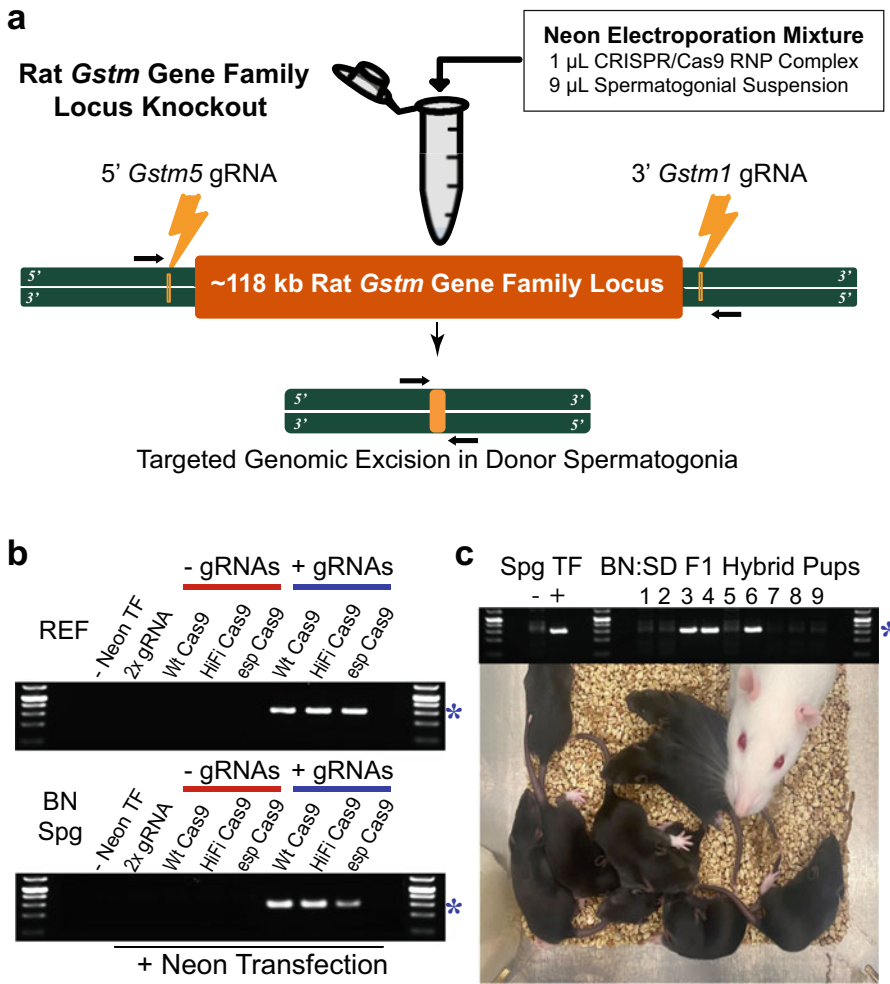


Fig. 6 Rat genetic engineering by germline gene targeting in donor spermatogonia. **(a)** Genomic excision of the Brown Norway rat (BN, Charles River) *Gstm*-gene family locus by CRISPR/HiFi S.p.Cas9 RNP-mediated gene targeting in a spermatogonial stem cell line (Spg) using gRNAs that flank *Gstm5* and *Gstm1*. The excised 118,218 bp mRatBN7.2 Chr2 sequence (nucleotides 195531627 to 195649845) harbored *Gstm5*, *Gstm6*, *Gstm7*, *Gstm6*, *Gstm31*, *Gstm2* and *Gstm1* coding genes. **(b)** Neon transfection (TF) was used to deliver recombinant CRISPR/Cas9 RNP complex variants (WtCas9, HiFiCas9, espCas9 RNPs) into REFs (top) and BN Spg (bottom) cultures. The respective RNP variant complexes were formed using CRISPR gRNA combination: 5' *Gstm5* (CCCTCCCATGTCCTACCAA GGG) + 3' *Gstm1* (ACATTGAAGTCTCAGATCGG GGG) (<http://crispor.tefor.net> gRNA design tool; NCBI *Rattus norvegicus* - genome mRatBN7.2). Predicted 309 bp amplicons (*) were generated using the respective 5'-ATCC-TTTATCACAGTGGCCC-3' and 5'-TAGCTTGAAGCTGTGTGCAC-3' *Gstm* locus primers designed to hybridize 219 bp upstream of 5'-*Gstm5* gRNA and 90 bp downstream of 3'-*Gstm1* gRNA cleavage sites in mRatBN7.2 using 20 ng REF or BN Spg DNA/reaction, minus (-) or plus (+) Neon transfection (TF) with respect CRISPR/Cas9 gRNAs and RNP complex variants. **(c)** Genomic PCR genotyping of donor germline-derived BN:SD F1 hybrid rat pups 1–9 generated by crossing a busulfan-treated BN spermatogonia recipient-male rat x wildtype female rat (Sprague Dawley, Envigo) [40]. Control lanes contain genomic DNA from BN Spg cultures transfected (TF) without (-) or with (+) HiFiCas9 RNP complex. Note: BN:SD F1 hybrid pups 3, 4 and 6 inherited the targeted 118 kb genomic excision from the genetically modified donor spermatogonia

3.1 Preparing Laminin-Coated Culture Dishes

Prepare laminin-coated dishes the day before plating spermatogonia to initiate colony-forming assays (Subheading 3.6).

1. Prepare frozen stocks of laminin to avoid multiple freeze thaws. Vials containing 1 mg/mL laminin solution are received frozen from the manufacturer. To make frozen stocks, thaw one vial of mouse laminin on ice (requires 1–2 h). Once thawed, make ~six 150 μ L laminin solution aliquots in sterile microfuge tubes on ice. Store the laminin stocks at -80°C for up to 1 year.
2. The day prior to isolating or passaging spermatogonia for colony-forming assays, coat wells in a sterile 48-well culture dish with laminin ($\sim 5.9\ \mu\text{g}/\text{cm}^2$). To prepare, thaw one 150 μ L aliquot from the 1 mg/mL laminin stocks on ice (requires ~ 30 min) and dilute the entire aliquot volume into a sterile 50 mL tube containing 8 mL DHF12 solution. *Slowly* swirl or *gently* rock the tube by hand to mix the contents.
3. Add 0.3 mL of the diluted laminin solution/well into the center 24 wells of a 48-well plastic culture dish (0.95 cm^2 wells).
4. To reduce evaporation from laminin-coated wells, add 0.4 mL PBS to the remaining 24 uncoated, “outside” wells. Additionally, wrap the dish with a Parafilm strip and store overnight (16–24 h) at 4°C .
5. The next day, equilibrate the dish to room temperature ($22\text{--}24^{\circ}\text{C}$) within a biosafety cabinet, discard laminin solution from the center 24 wells, wash each laminin-coated well once with 0.4 mL DHF12 solution and immediately proceed to steps in Subheading 3.6.

Coating 24 wells with laminin will be sufficient to conduct colony-forming assays for 6 or 8 test conditions at $n = 3$ or $n = 4$ wells/condition, respectively.

3.2 Preparing Mouse Embryonic Fibroblast Feeder Layers

DR4 MEFs are used to maintain rat spermatogonial stem cell lines [36] and can be used in colony-forming assays [29]. MEF feeder layers require ~ 2 days to prepare before plating spermatogonia to initiate colony-forming assays.

1. Primary stocks of DR4 mouse embryonic fibroblasts (MEFs) are purchased from ATCC, and expanded according to the manufacturer’s protocol after plating into MEF medium at $37^{\circ}\text{C}/5\% \text{CO}_2$. MEFs are sub-cultured up to 4 passages following their thawing and initial plating from the manufacturer’s vial (*see Note 3*).
2. Following expansion into T225 flasks, secondary stocks of MEFs are harvested according to the manufacturer’s protocol, irradiated (100 Gy) in MEF medium and then cryo-preserved in liquid nitrogen for future use by freezing in Recovery Cell

Culture Freezing Medium according to the manufacturer's protocol (*see Note 4*).

3. Pre-coat tissue culture dishes with a solution of filter-sterilized, 0.1% gelatin for 1 h at room temperature (22–24 °C). Rinse 1× with sterile PBS before plating MEFs.
4. Prior to culturing spermatogonia, thaw and plate irradiated DR4 MEFs into gelatin-coated dishes (4.5×10^5 cells/cm²) in MEF medium (0.25–0.3 mL/cm²) 16–48 h. Rinse 1× with PBS and pre-incubate in SG medium (0.25–0.3 mL/cm²) an additional 16–48 h. Discard pre-incubation medium and plate spermatogonia onto the MEFs in fresh SG medium (0.3–0.4 mL/cm²), as described in **step 1**, Subheading **3.6**.

3.3 Formulating Spermatogonial Culture Media

Spermatogonial Culture Medium (SG Medium) is prepared by supplementing Dulbecco's modified Eagle's medium: Ham's F12 medium 1:1 in the following order (*see Notes 5–7*):

1. 1× concentration antibiotic-antimycotic solution (v/v).
2. 4 mM L-glutamine (final concentration = 6 mM).
3. 100 μM 2-mercaptoethanol.
4. 1× concentration of B27 Supplement Minus Vitamin A (v/v).
5. 6 ng/ml GDNF.
6. 6 ng/ml FGF2.
7. Equilibrate to 34–35 °C and filter sterilize.

3.4 Formulating Rat A-Single Spermatogonia Culture Media (A_s-SG Medium)

1. Rat A-Single Spermatogonia Culture Medium (A_s-SG Medium) is prepared identical to that of SG Medium, with the exception that A_s-SG Medium does not contain GDNF (*see Notes 8 and 9*). Optionally, 1 μM Lapatinib (ERBB1, 2, 4 inhibitor) can be supplemented into A_s-SG Medium to help reduce syncytia formation.

3.5 Formulating Spermatogonial Differentiation Medium (SD Medium)

1. Spermatogonial Differentiation Medium (SD Medium) is prepared identical to that of SG Medium, except that the concentration of GDNF is reduced to 2 ng/mL, and SD Medium is further supplemented with 3 μM ATRA, 40 ng/ml NRG1β1 and/or 100 ng/mL KITL (*see Notes 10 and 11*).

3.6 In vitro Spermatogenesis Colony-Forming Assay

1. Day 1 (d1), plate $\sim 1 \times 10^3$ to 1.5×10^3 rat spermatogonia/well (0.95 cm²) in 0.4 ml A_s-SG Medium/well of a 48-well culture dish (0.95 cm² wells) coated with laminin (Subheading **3.1**) or containing irradiated DR4 MEFs (Subheading **3.2**) (*see Note 12*).

2. Pre-incubate spermatogonia in A_s-SG Medium at ~36.5 °C, 5% CO₂ for ~6 days (d1-d7) to enrich cultures with A_s spermatogonia. Change A_s-SG Medium (0.4 mL/well) every 2 days during the pre-incubation period using fresh A_s-SG Medium (i.e., change media on d3, d5, d7) (*see Note 13*).
3. On d7, change-out culture medium by feeding spermatogonia with SD Medium (0.4 mL/well) and incubate 5–8 days at 32.5–36.5 °C, 5% CO₂ to produce differentiating spermatogonia (*see Note 14*).
4. Feed cultures with fresh SD Medium every 2 days during the ~1 week differentiation period. Six to eight days in SD Medium promotes robust development of spermatogonial syncytia for analysis in colony-forming assays.
5. After 6–8 days in SD Medium (~d14), remove medium and fix cells directly in 0.4 mL/well ice-cold 4% paraformaldehyde, 0.1 M sodium phosphate buffer, pH 7.2, 20–30 min on ice (*see Notes 15–17*).
6. Post-fix cells in 0.4 mL/well –20 °C methanol 5 min on ice.
7. Wash fixed cultures 2× with 0.4 mL PBS/well/wash at room temperature (22–24 °C) (*see Note 18*).
8. Label spermatogonial cultures to detect desired molecular markers (*see Subheading 3.7*).
9. Score Spermatogenic Units/well ($n = 3–4$ wells/test condition/experiment, \pm S.E.M.) using a microscope to count spermatogonia/well exhibiting single, paired or longer syncytial morphologies co-labeled with reagents to detect desired molecular markers (i.e., co-labeling with anti-TEX14 IgG and Hoechst 33342 dye allow individual spermatogonial syncytia to be clearly classified) (Fig. 4) (*see Notes 19 and 20*).

3.7 Immuno-fluorescence Labeling on Spermatogonial Cultures

1. Prepare 1× stock of Roche Western Blocking Reagent in 0.1 M Phosphate Buffer pH 7.2, 0.01% Triton X-100 (v/v) (Blocking Reagent).
2. Pre-incubate fixed cultures in 0.4 mL Blocking Reagent/well 1–2 h at room temperature (22–24 °C).
3. During the pre-incubation step in Blocking Reagent (**step 2**), prepare Primary Antibody Solutions by diluting respective antibodies raised to germ cell and/or spermatogonial markers into a required volume of fresh Blocking Reagent: Mouse anti-ZBTB14 IgG at 0.2 µg/mL, Mouse anti-SALL4 IgG at 0.2 µg/mL, Mouse anti-Foxa1 IgG at 0.5 µg/mL, Rabbit anti-Foxa2 IgG at 1/200 dilution, Rabbit anti-DAZL IgG at ~5 nM (1/800 dilution) [40] and/or Rabbit anti-TEX14 IgG at ~5 nM (1/800 dilution) [42].

4. Remove Blocking Reagent from fixed cultures and then add 0.4 mL of respective Primary Antibody Solution/well and incubate overnight (14–24 h) at room temperature (22–24 °C).
5. Discard Primary Antibody Solutions and wash cultures 3 times for 10–30 min per wash with 0.4 mL PBS/well/wash (*see Note 18*).
6. During the PBS wash step (**step 5**) prepare respective AlexaFluor 488-conjugated (green fluorophore) and/or AlexaFluor 594-conjugated (red fluorophore) Secondary Antibody Solutions by diluting to ~5 µg/mL (1/400 dilution from 2 mg/mL manufacture stock) when required for indirect labeling (*see Note 21*). To directly label nuclei with a blue fluorophore, dilute Hoechst 33342 dye (1/2000) directly into the Secondary Antibody Solution.
7. Remove final PBS wash and incubate fixed cultures in 0.4 mL/Secondary Antibody Solution/well for 60–90 min at 22–24 °C.
8. Discard Primary Antibody Solutions and wash fixed cultures 3 times for 10–30 min/wash with 0.4 mL PBS/well/wash.
9. Add 0.4 mL PBS to fixed cultures for viewing and storage. Wrap culture plates with a thin strip of Parafilm to minimize evaporation during storage.
10. View labeled cultures and score Spermatogenic Units as described in **step 9** of Subheading **3.6**.

3.8 Germline Gene Targeting in Rat Spermatogonial Lines by Transfection With CRISPR/Cas9 Ribonucleoprotein (RNP) Complexes

1. On d1 prior to transfecting spermatogonia with RNP complexes, prepare respective number of 9.6 cm² culture wells coated with laminin (Subheading **3.1**) or irradiated DR4 MEFs (Subheading **3.2**)/number transfection conditions by changing to fresh SG Medium.
2. On d2 harvest and wash $1-2 \times 10^5$ spermatogonia with 4 mL PBS.
3. Suspend washed spermatogonial pellet in 9 µL Buffer R from Neon Transfection Kit.
4. Prepare Cas9 protein by adding 0.3 µL Alt-R HiFi S.p.Cas9 Nuclease V3 (62 µM IDT stock) + 0.2 µL Neon Transfection Buffer R, yielding 18 pmol/0.5 µL diluted Cas9 solution/transfection.
5. Resuspend gene target-specific Alt-R CRISPR sgRNA in IDTE buffer to 44 µM (sgRNA solution = 22 pmol/0.5 µL) (*see Notes 22 and 23*).

6. Prepare RNP complex by mixing 0.5 μL diluted Cas9 solution (**step 4**) + 0.5 μL sgRNA solution (**step 5**) = 1 μL total RNP complex mix/transfection (*see Note 24*).
7. Incubate RNP complex mix 10–20 min, 22–24 $^{\circ}\text{C}$ (=18 pmol Cas9 + 22 pmol sgRNA in Neon transfection mix) (*see Note 24*).
8. Add 1 μL RNP complex mix (**step 7**) + 9 μL spermatogonial suspension (**step 3**) and use to load a 10 μL Neon transfection tip (i.e., 18 pmol Cas9 + 22 pmol sgRNA/10 μL tip).
9. Electroporate using the Neon Transfection System set at 2 pulses, 1100 V, 20 ms per manufacturer's instructions and Chapman et al. (*see Note 25*) [31].
10. Plate electroporated spermatogonia into 9.6 cm^2 laminin-coated or DR4 MEF-coated culture well/transfection containing 3.5 mL fresh SG Medium (Subheading 3.3), A_s -SG Medium (Subheading 3.4) or SD Medium (Subheading 3.4) by directly injecting the electroporated spermatogonial solution from the Neon tip into respective culture wells (**step 1**) (*see Note 26*).
11. Utilize electroporated spermatogonial cultures for spermatogenesis colony-forming assays (Subheading 3.6) and/or to generate new genetically modified rat models following sub-culture, genetic selection, clonal expansion, and sterile-testis complementation [25, 31, 37, 39, 43].

4 Notes

1. If one chooses to derive their own rat spermatogonial lines, or utilize freshly isolated laminin-binding A_s/A_{pr} spermatogonia to initiate colony-forming assays, it should be noted that the dispase solution composition previously utilized in published protocols to digest 22–24 day old rat seminiferous tubules [32, 36, 40, 44–46] was modified by the manufacturer in 2015, and is significantly less effective. We recommend the former dispase digest be replaced by a single-step collagenase digest for 15 min, 34 $^{\circ}\text{C}$ in filter-sterilized Dulbecco's modified Eagle's medium: Ham's F12 medium 1:1 (Sigma, Inc.), 1.2 mg/mL *Clostridium histolyticum* Collogenase (Sigma; 2.1 units/mg FLGPA) [36].
2. The described *in vitro* spermatogenesis colony-forming assay (Subheading 3.6) is routinely conducted using Costar 48-well plates.

3. Irradiating MEFs prevents them from dividing and allows them to form a more stable feeder layer for spermatogonia to grow on. If an irradiator source is not available, or irradiating one's own MEFs is not desirable, several companies provide pre-irradiated DR4 MEFs, but at an elevated price compared to non-irradiated MEFs. As an alternative to irradiating MEFs, MEFs can be mitotically inactivated before use as feeder layers by a simple pre-treatment with mitomycin-C [25, 42].
4. It should also be stressed that primary DR4 MEF lots vary in quality between different companies, and lots from the same company can vary. We find ATCC's DR4 MEFs consistently reliable for culturing rat spermatogonial lines with potent sperm-forming potential [35].
5. Prepare 100× stock of 2-mercaptoethanol (10 mM) fresh by diluting 7.8 μL, 2-mercaptoethanol from the manufacture into 10 mL Dulbecco's modified Eagle's medium: Ham's F12 medium 1:1. Upon regular use, bottles containing 2-mercaptoethanol provided by the manufacture are replaced with new bottles ~every 4 months.
6. Polypeptide growth factors (GDNF, FGF2) should be reconstituted as instructed by the manufacture, but using filter sterilized 0.01% protease-, nuclease- and fatty acid-free Bovine Serum Albumin (Calbiochem, Inc.) in PBS (w/v). FGF2 is essential for undifferentiated spermatogonia and DR4 MEF viability in SG Medium. GDNF is essential for spermatogonial colony/clump formation on DR4 MEFs in SG Medium.
7. Higher GDNF and/or FGF2 concentrations can be used to increase the growth rate of some rat spermatogonial lines when cultured on some lots of DR4 MEFs in SG Medium. For example, the original SG Medium formulation consisted of 20 ng/mL GDNF and 25 ng/mL FGF2 [32].
8. A_s-SG Medium is identical to the recently reported SG^F-Medium [29]. Omitting GDNF from SG Medium selects for spermatogonia in the single (A_s) cell state, and thus, selects against syncytial growth of undifferentiated spermatogonial clones/clumps that can complicate colony counts and phenotyping.
9. It should be emphasized that after *enriching* for A_s spermatogonia by selection in A_s-SG Medium, cultures will still contain a relatively low percent A_{pr} and A_{al} spermatogonia.
10. SD Medium is a modified formulation of the serum-free, SG Medium [32]. KITL can be supplemented into SD medium at 100 ng/mL in place of NRG1β1, or in combination with up to 40 ng/mL NRG1β1 [29].

11. NRG1 β 1 and KITL stimulate respective Lapatinib-sensitive and Lapatinib-insensitive survival pathways in differentiating spermatogonia [29].
12. Plating $\sim 10^3$ undifferentiated rat spermatogonia/well in a 48-well plate format (0.95 cm²/well) from a given rat spermatogonial line typically yields 150–300 syncytial colonies containing 4–16 nuclei/syncytium/well after 6 days in SD Medium (**steps 3 and 4**, Subheading 3.6). Plating $\sim 10^3$ spermatogonia/0.95 cm² yields well-separated syncytia.
13. Pre-incubation time in A_s-SG Medium is optional, and can be shortened or omitted prior to **steps 3 and 4**, Subheading 3.6 to expedite studies on spermatogonial differentiation. Spermatogonial numbers/culture and surface area/culture in SG, A_s-SG and/or SD Medium can also be scaled to best meet scientific aims requiring other types of cellular and molecular analyses.
14. Colony-forming assays in SD Medium conducted at 32.5 °C, 34.5 °C or 36.5 °C yield similar results with respect to differentiating spermatogonial syncytia (ZBTB16⁻, SALL4⁻, DAZL⁺, TEX14⁺) development. Spermatogonial syncytia develop slower at reduced temperatures.
15. Post-fixation in MeOH promotes adherence to plates and minimizes colony loss during wash steps following primary and secondary antibody labeling.
16. Post-fixation in MeOH permeabilizes paraformaldehyde-fixed spermatogonial membranes, which facilitates antibody delivery into fixed spermatogonia to label intracellular antigens. All the antibodies listed in Subheading 2.1 are compatible with post-fixation in MeOH. Post-fixation in MeOH is optional if not compatible with antibody binding to a particular antigen under study because Blocking Reagent contains 0.01% Triton X-100 detergent. Triton X-100 also permeabilizes 4% paraformaldehyde-fixed spermatogonial membranes.
17. It is not necessary to rinse cultures with PBS before fixation with 4% paraformaldehyde, or before fixation with Methanol.
18. PBS washes should be conducted relatively gently, taking care to not add or remove each PBS wash too vigorously, which can wash colonies off the plate and increase experimental error. Minimize time between PBS washes so not to allow fixed spermatogonial cultures to air-dry. Air-drying increases background fluorescence.
19. Scoring various length syncytia is most conveniently done by co-labeling cytoplasmic bridges and nuclei with the TEX14 IgG and Hoechst 33342 dye, respectively (Fig. 1). Syncytia that have advanced to the 2, 4, 8, 16, and 32 cell steps in

development will be recognized by TEX14⁺ foci [47] localized within or adjacent to each cytoplasmic bridge separating nuclei comprising a syncytium (Fig. 1b). It should also be noted that irregular length syncytia are also observed based on the TEX14 labeling profile, primarily due to a lag in some cells within a given syncytium completing metaphase/anaphase at the time of fixation, which is revealed by nuclear labeling with Hoechst 33342 dye.

20. A_s, A_{pr}, and A_{al} spermatogonia represent “undifferentiated” stem and progenitor spermatogonia, originally classified in the rat [5]. Prior to the discovery of proteins that selectively mark distinct spermatogonial types, undifferentiated spermatogonia were distinguished by less heterochromatin, shorter syncytial length, lower abundance, and unique cell division kinetics during an epithelial cycle compared to differentiating spermatogonia (A1–A4, Int, B) [5]. Antibodies generated to protein markers can now be used to distinguish between undifferentiated and more differentiated spermatogonial types [11, 12, 48–50].

In vivo, A_s and A_{pr} spermatogonia are more refractory to differentiating into type A1 spermatogonia each epithelial cycle compared to A_{al} spermatogonia [5]. However, smaller populations of A_s and A_{pr} spermatogonial fractions are subject to differentiation more directly into A1 spermatogonia each epithelial cycle [5, 33], which has been verified by loss of molecular markers for undifferentiated spermatogonia in vivo as they progress through rodent spermatogenic stages IV–VIII [51, 52]. Similarly, retinoic acid in SD Medium effectively drives differentiation in A_s, A_{pr}, and A_{al} spermatogonia in vitro, as monitored by loss of molecular markers for undifferentiated spermatogonia (Zbtb16, Sall4) (Fig. 5b) [29], and the inability of rat spermatogonial lines to regenerate spermatogenesis in recipient rat testes following culture in SD Medium [29]. Thus, A_s, A_{pr}, and A_{al} Spermatogenic Units are experimentally defined relative to molecular markers used to distinguish between undifferentiated and differentiating spermatogonia.

21. Labeling cultures with molecular probes prior to fixation (i.e., EdU incorporation, fluorescent transgenes/tags) is compatible with procedures described in Subheadings 3.6 and 3.7.
22. Alternatively, (to using sgRNA) resuspend gene target-specific Alt-R CRISPR crRNA + tracrRNA from IDT, Inc. in IDTE buffer to 44 μM (final guide RNA solution = 22 pmol/0.5 μL).
23. For assistance resuspending guide RNAs to 44 μM, use the IDT Resuspension Calculator at www.idtdna.com/sciTools.

24. Prepare transfection suspension containing RNP mix (**steps 6 and 7**) during spermatogonia harvest/preparation period (**steps 2 and 3**) to save time.
25. Scale transfection as needed by pooling cells from multiple 10 μ l Neon tips or multiple 100 μ L Neon tips.
26. Save Neon tips by reloading the same respective Neon tip up to 3 \times with fresh spermatogonia/transfection mix after injecting each electroporation directly into its designated culture well, *taking care not to get culture medium in the tip* between each re-use.

Acknowledgments

This work was supported by National Institutes of Health grants from the Eunice Kennedy Shriver National Institute of Child Health and Human Development: R01HD053889 and R01HD061575, the Office of the Director: R24OD011108, the National Institute on Drug Abuse: U44DA044885.

Disclosure FKH is a cofounder and rat model project consultant for GenomeDesigns Laboratory, LLC.; AP is currently employed full time by The Center for Aquaculture Technologies, San Diego, California 92121; MNV is currently employed full time by the Department of Obstetrics and Gynecology, UT Southwestern Medical Center, Dallas, Texas 75390.

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Maintenance of Human Primordial Germ Cell-Like Cells in a Long-Term Culture System

Joanna J. Gell and Toshi Shioda

Abstract

Primordial germ cells (PGCs) are the earliest form of mammalian germ lineage. In humans, PGCs are present during a very early and limited window in development, limiting the ability to study fundamental developmental steps in human reproductive biology. However, recent advancements in generating in-vitro models of gametogenesis have allowed the field to generate human primordial germ cell-like cells (hPGCLCs). In this chapter, we will review the generation of hPGCLCs using the incipient mesoderm-like cell (iMeLC) protocol and the subsequent expansion of hPGCLCs in a long-term culture system.

Key words Pluripotent stem cells, In-vitro gametogenesis, Primordial germ cell, Human primordial germ cell-like cells, Long-term culture

1 Introduction

The earliest form of human germ cells, *the human primordial germ cells (hPGCs)*, have been difficult to study because the early embryonic timepoint at which they arise makes them technically and ethically challenging to interrogate. However, recent advancements in modeling human development with pluripotent stem cells (PSCs) have led to great strides in furthering our knowledge of human germ cell development through *in-vitro* gametogenesis (IVG). Numerous groups have demonstrated that either primed or naïve PSCs can be used to generate aggregates that contain human primordial germ cell-like cells (hPGCLCs) [1–5]. It has been demonstrated that regardless of the methods used to generate hPGCLCs they share similar transcriptomic and epigenomic properties [6–8].

Although multiple protocols have demonstrated the ability to generate hPGCLCs, these cells are only viable in the aggregates for a short fraction of time. Additionally, the number of hPGCLCs generated in these aggregates is limited. Therefore, means to

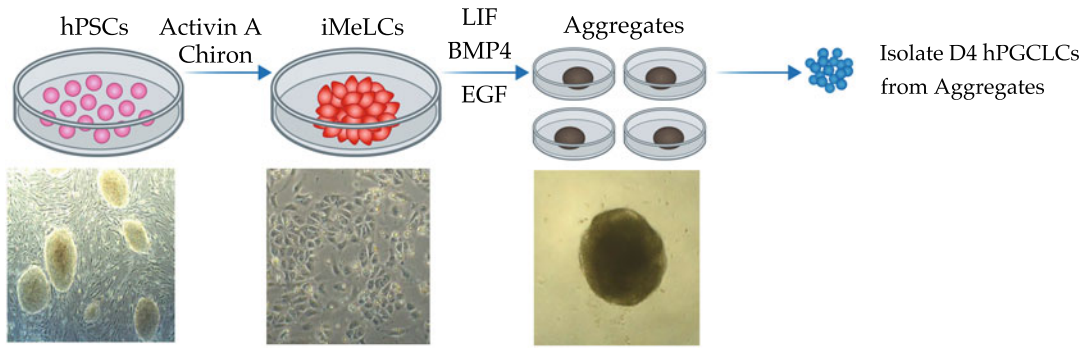


Fig. 1 Two-step differentiation method of human primordial germ cell-like cells (hPGCLCs) from human pluripotent stem cells (hPSCs). Incipient mesoderm-like cells (iMeLCs) are generated from hPSCs through signaling from Activin A and Chiron. iMeLCs are aggregated in media containing leukemia inhibitory factor (LIF), bone morphogenic protein 4 (BMP4) and epidermal growth factor (EGF). Aggregates contain hPGCLCs and are isolated from the aggregates

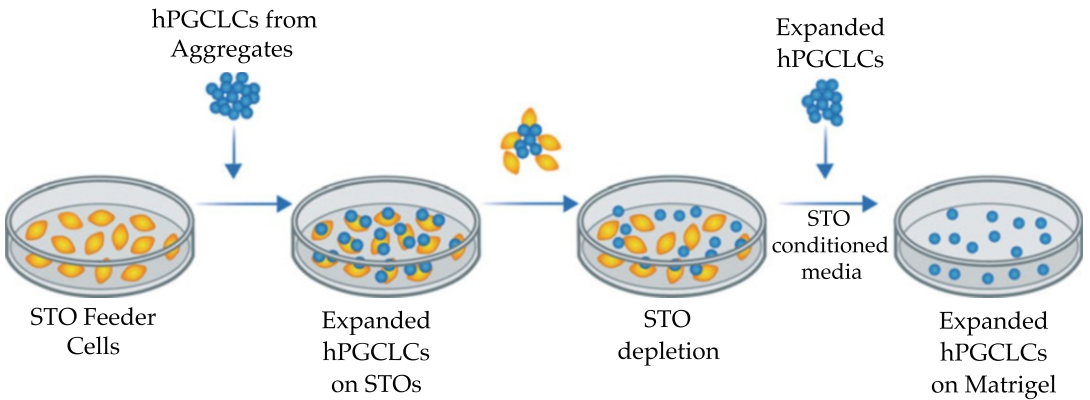


Fig. 2 Extended culture system for human primordial germ cell-like cells (hPGCLCs). hPGCLCs are seeded on a feeder layer (STO feeder cells). hPGCLCs are expanded on feeders and then transitioned to a feeder-free system, maintained on Matrigel coated plates in STO conditioned medium

efficiently maintain a homogenous population of hPGCLCs for cell number expansion and derivation of a stable cell line is of great utility. In this chapter we will review steps to generate, maintain and expand a homogenous population of hPGCLCs, deemed long-term culture hPGCLCs (LTC-hPGCLCs). Starting with primed PSCs, hPGCLCs will be generated as described by Sasaki et al. [2] (Fig. 1). The isolated hPGCLCs will then be transitioned to stable LTC-hPGCLCs with limitless expansion, as described by Kobayashi et al. [9] (Fig. 2).

2 Materials

Prior to starting, ensure all required growth factors have been stored as indicated by the manufacturer and they have not exceeded the recommended expiration date. Tables 1, 2, and 3 denote the contents of the media for incipient mesodermal-like cells (iMeLCs), hPGCLCs, and LTC-hPGCLCs, respectively.

Table 1
Incipient mesodermal-like cell (iMeLC) media components

Component	Final concentration	Stock concentration
GMEM	N.A.	N.A.
KSR	15%	N.A.
NEAA	1× (0.1 mM)	100× (10 mM)
2-mercaptoethanol	0.1 mM	55 mM
Pen/Strep/L-Glutamine	1×	100×
Sodium pyruvate	1 mM	100 mM
Activin A	50 ng/mL	0.1 mg/mL
CHIR99021	3 μM	3 mM
Y-27632 (ROCKi)	10 μM	10 mM

N.A. not applicable, KSR knockout serum replacement, NEAA non-essential amino acids

Table 2
Human primordial germ cell-like cell (hPGCLC) media components

Component	Final concentration	Stock concentration
GMEM	N.A.	N.A.
KSR	15%	N.A.
NEAA	1× (0.1 mM)	100× (10 mM)
2-mercaptoethanol	0.1 mM	55 mM
Pen/Strep/L-Glutamine	1×	100×
Sodium pyruvate	1 mM	100 mM
Human LIF	10 ng/mL	10 μg/mL
BMP4	200 ng/mL	100 μg/mL
EGF	50 ng/mL	200 μg/mL
Y-27632 (ROCKi)	10 μM	10 mM

N.A. not applicable, KSR knockout serum replacement, NEAA non-essential amino acids

Table 3
Long-term culture hPGCLC (LTC-hPGCLC) complete media components

Component	Final concentration	Stock concentration
GMEM ^a	N.A.	N.A.
KSR ^a	13%	N.A.
NEAA ^a	1× (0.1 mM)	100× (10 mM)
Pen/Strep/L-Glutamine ^a	1×	100×
Sodium pyruvate ^a	1 mM	100 mM
2-mercaptoethanol	0.1 mM	55 mM
Recombinant human SCF	100 ng/mL	100 µg/mL
L-ascorbic acid	50 µg/mL	200 mg/mL

Boldface area with ^a = components for long-term culture (LTC) hPGCLC basal media to be collected as STO-conditioned media prior to making complete LTC-hPGCLC media

N.A. not applicable, KSR knockout serum replacement, NEAA non-essential amino acids, SCF stem cell factor

2.1 Human Plasma Fibronectin (HPF)

1. In the tissue culture hood under sterile conditions, reconstitute 5 mg of lyophilized HPF in 5 mL sterile distilled water to make a 1 mg/mL stock solution.
2. Allow solution to stand for 30 minutes at room temperature to fully dissolve. Do not vortex or vigorously mix solution.
3. Aliquot 200 µL vials of 1 mg/mL stock solution and store at –20 °C (*see Note 1*).

2.2 Basal Media

1. Basal components for medium supporting iMeLCs, hPGCLCs, and LTC-PGCLCs culture can be constituted and frozen as stocks in –20 °C to –80 °C until ready to use for downstream use.
2. *Basal components for iMeLC and hPGCLC media are as follows:* Glasgow's Modified Essential Medium (GMEM) with 15% Knock-out serum (KSR), 1× (0.1 mM) non-essential amino acid (NEAA), 1 mM sodium pyruvate and 1× penicillin/streptomycin/L-glutamine (PSG).
3. *Basal components for LTC-hPGCLCs are as follows:* GMEM with 13% KSR, 1× (0.1 mM) NEAA, 1 mM sodium pyruvate and 1× PSG.

2.3 iMeLC Complete Medium (See Table 1)

1. Thaw iMeLC basal medium overnight at 4 °C.
2. Add to thawed basal medium, 0.1 mM β-mercaptoethanol (BME), 50 ng/mL Activin A, 3 µM CHIR99021, and 10 µM Y-27632.
3. Complete medium can be stored at 4 °C for 72 h.

2.4 hPGCLC**Complete Medium (See Table 2)**

1. Thaw hPGCLC basal medium overnight at 4 °C.
2. To thawed basal medium, add 0.1 mM BME, 10 ng/mL human leukemia inhibitory factor (LIF), 200 ng/mL bone morphogenic protein 4 (BMP4), 50 ng/mL epidermal growth factor (EGF), and 10 μM Y-27632.
3. Complete medium can be stored at 4 °C for 72 h.

2.5 LTC-hPGCLC**Complete Media (See Table 3)**

1. Mitotically inactivated STO feeder layers (obtained from ATCC- crl-1503) are plated at a density of 5×10^6 cell in 12 mL of basal LTC-hPGCLC medium in a T-75 flask.
2. After 24 h of culture, the STO conditioned medium (STO-CM) is collected and centrifuged to remove any cellular debris.
3. STO-CM can be stored frozen at -20 °C until ready for further use. Thaw overnight at 4 °C when ready for use.
4. Complete LTC-hPGCLC medium is derived by adding 0.1 mM BME, 50 ug/mL L-ascorbic acid, and 100 ng/mL recombinant human stem cell factor (rhSCF).
5. Complete medium can be stored at 4 °C for 1 week.

2.6 Mouse**Embryonic Fibroblast (MEF) Media**

1. Dulbecco's modification of Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 1× (0.1 mM) NEAA, and 1× PSG. Store at 4 °C.

2.7 Fluorescent-Activated Cell Sorting Buffer

1. Dissolve 5 g of bovine serum albumin in 500 mL of phosphate-buffered saline. Store at 4 °C. Filter sterilize the solution for long-term storage.

2.8 Fluorophore Conjugated Antibodies

1. EPCAM, INTEGRIN α -6, 7-aminoactinomycin D (7-AAD) (see Note 2).

2.9 hESC-Qualified Matrigel

1. Reconstitute and aliquot Matrigel as recommended in the product sheet for each lot. Aliquot volumes range from 270–350 μL.
2. Thaw aliquot in 4 °C overnight on ice.
3. Resuspend aliquot in chilled 25 mL of DMEM/F12, keep on ice.
4. Using prechilled pipette tips, add 1 mL to each well of a 6-well plate. This volume is sufficient for four 6-well plates.
5. Incubate plate at room temperature for at least 1 h before use.
6. Aspirate the remaining liquid from the well before use.
7. If not used immediately, plates can be store with the diluted Matrigel in place at 4 °C for up to 4 weeks. Wrap in paraffin to prevent evaporation.

3 Methods

3.1 Generation of iMeLCs

1. Prepare a 12-well plate with HPF by adding 200 μ L of 1 mg/mL stock solution to 12 mL of PBS.
2. Add 1 mL to each well of a 12-well plate. Incubate at 37 °C for 1 h. Coated plates can be stored at 4 °C for up to 4 weeks (*see Note 3*).
3. Grow 6-well plate of primed hPSCs (feeder or feeder-free conditions are acceptable) to 75–80% confluency.
4. Trypsinize hPSCs by adding 1 mL of 0.05% Trypsin to each well. Incubate at 37 °C for 3 min. Inactivate trypsin by adding 2 mL MEF medium to each well.
5. Transfer the cell suspension to a falcon tube, centrifuge at 1200 rpm for 5 min, and aspirate supernatant (*see Note 4* for MEF feeder depletion if hPSCs are grown on feeder layer).
6. Resuspend cell pellet with 500 μ L of iMeLC medium and pass through a 40 μ M cell strainer.
7. Plate cells into HPF-coated 12-well plate at a density of 200,000 cells per well in 2 mL of iMeLC medium.
8. Culture at 37 °C for 24 h (*see Fig. 1* for expected morphology).

3.2 Generation of hPGCLCs

1. Collect iMeLCs by adding 1 mL of 0.05% Trypsin to each well. Incubate at 37 °C for 3 min.
2. Neutralize trypsin with 1 mL/well of trypsin inhibitor. Collect cells and centrifuge at 1200 rpm for 5 min. Remove supernatant.
3. Resuspend pellet in 500 μ L of hPGCLC medium.
4. Plate iMeLCs in low attachment u-shaped bottom 96-well plates at a density of 3000 cells per well, in 200 μ L/well of hPGCLC medium. Count cells carefully, as 3000 cells per well is critical for aggregate formation.
5. Once plated swirl the 96-well plate in a circular motion, approximately 20 times clockwise and then counterclockwise, to help form aggregates.
6. Incubate at 37 °C for 4 days, don't disrupt the plate for the first 12–24 h.

3.3 Isolating hPGCLCs from Aggregate by Fluorescent-Activated Cell Sorting

1. On day 4 of culture, transfer the floating aggregates containing hPGCLCs to 1.5 mL Eppendorf tube. Allow aggregates to settle by gravity, then remove supernatant.
2. Dissociate aggregates with 500 μ L of 0.05% Trypsin for 10 min at 37 °C, agitating the tube at 5-min intervals.
3. Pipet up and down to dissociate aggregates.

4. Inactivate Trypsin by adding 1 mL of MEF medium.
5. Pellet cells by centrifuging at 1600 rpm for 5 min at 4 °C.
6. Remove supernatant, leaving ~100 μ L behind. Then resuspend in 300 μ L of FACS buffer and add INTEGRIN α -6 and EPCAM-conjugated antibodies to the cells (*see Note 5*).
7. Incubate for 15 min on ice in the dark.
8. Add 1 mL of FACS buffer to the cells and centrifuge at 1600 rpm for 5 min at 4 °C.
9. Remove supernatant, leaving ~100 μ L behind. Resuspend in 200 μ L FACS buffer.
10. Filter cells through a 40 μ m diameter cell strainer cap of a FACS tube.
11. Add 6 μ L 7-AAD (1:50 concentration), mix well and sort hPGCLCs in complete LTC-hPGCLC medium containing 10 μ M Y27632.

3.4 Generation of LTC-hPGCLCs

1. The day prior to sorting the hPGCLCs, plate STO feeder layer at a density of $8\text{--}9 \times 10^4$ cells/cm² in a well of 6-well plate.
2. Plate 2000 FACS-isolated hPGCLCs in 2 mL LTC-hPGCLCs medium containing 10 μ M Y27632.
3. After 24 h in culture, change medium to 2 mL LTC-hPGCLC medium without Y27632. Thereafter medium is changed every other day.
4. After 7–10 days of culture, cells are passaged to a fresh layer of STO feeder cells as follows: Remove medium and wash cells with 1 mL $1 \times$ PBS. Dissociate cells by incubating at 37 °C at 5 min in 1 mL of Accutase. Add 1 mL of LTC-hPGCLC medium to well and collect cells to centrifuge at 1200 rpm for 5 min. Aspirate supernatant and resuspend in 2 mL LTC-hPGCLC medium and plate on fresh STOs feeder layer.
5. When hPGCLCs reach ~20% confluency, dissociate cells with 1 mL Accutase as outlined in **step 4**.
6. Resuspend cell suspension in 10 mL MEF medium and proceed to STO feeder depletion (*see Note 4*).
7. Resuspend the STO-depleted hPGCLCs in 3 mL of LTC-hPGCLC medium and count cell number.
8. Place 2 mL of LTC-hPGCLC medium at room temperature in each well of a 6-well plated coated with hESC-qualified Matrigel plate as outlined above (Subheading 2.9).
9. Inoculate $1.0\text{--}2.0 \times 10^5$ cells into each Matrigel-coated well of the 6-well plate containing LTC-hPGCLC medium.
10. Passage with Accutase when cells are at 70–80% confluency.
11. Cells can be cryopreserved in CryoStor CS10 at a density of $1.0\text{--}2.0 \times 10^5$ cells. Store at -80 °C.

4 Notes

1. Do not freeze-thaw repeatedly. One aliquot (200 μ L vials of 1 mg/mL stock) can be used for coating one 12-well plate.
2. There are various combinations of antibodies that can be used to isolate the hPGCLCs from the aggregate by FACS. Dual staining with a combination of INTEGRIN α -6 and EPCAM or C-KIT and TNAP produce a distinct double positive population. Alternatively, CD38 also produces a pure population of hPGCLCs.
3. We recommend wrapping HPF-coated plates in paraffin for storage in 4 $^{\circ}$ C to prevent evaporation of contents and to maintain sterility.
4. *Feeder depletion*: Resuspend cell pellet containing feeder cells in 10 mL of MEF media. Deplete feeder cells (MEFs or STOs) by placing cell suspension in a tissue culture-treated plastic dish for 5 min. Collect desired cell (hPSCs or hPGCLCs) by tilting the plate and removing supernatant without rinsing the plate. Place the suspension of cells in a fresh tissue culture-treated dish for 5 min. Collect desired cell (hPSCs or hPGCLCs) by tilting the plate and removing supernatant without rinsing the plate. Centrifuge suspension at 1200 rpm for 5 min, aspirate supernatant.
5. We use antibodies at a concentration of 1:80 (i.e., 5 μ L antibody in 400 μ L FACS buffer). This may vary depending on antibodies obtained from commercial sources.

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Derivation and Primordial Germ Cell Induction of Intermediate Pluripotent Stem Cells

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Abstract

Dynamic pluripotent stem cell (PSC) states are *in vitro* adaptations of the pluripotency continuum *in vivo*. Previous studies have generated a number of PSCs with distinct properties. By modulating the FGF, TGF- β , and WNT pathways, we have derived intermediate PSCs (FTW-PSCs) that are permissive for direct primordial germ cell-like cell (PGC-LC) induction *in vitro*. Here, we describe the method for derivation and maintenance of mouse and human FTW-PSCs, as well as PGC-LC induction from FTW-PSCs.

Key words Mouse pluripotent stem cells, Human pluripotent stem cells, Intermediate state, Formative state, Primordial germ cell

1 Introduction

The first mouse embryonic stem cells (ESCs) were derived in 1981 [1, 2] but it took researchers nearly three decades to realize that these ESCs represented only one of several different pluripotent states. In 2007, a new type of pluripotent stem cell was isolated from mouse post-implantation embryos (EpiSCs), which displayed properties similar to a later stage of epiblast development [3, 4]. This led to the realization of two different pluripotency states: “naïve” for the ESCs and “primed” for the EpiSCs [5]. Naïve mouse ESCs most closely resemble the epiblast of a mature blastocyst (pre-implantation embryo, ~embryonic day 4, or E4) [6, 7], while primed mouse EpiSCs display a gene expression signature more like the anterior epiblast of a late-gastrula-stage embryo (post-implantation embryo, ~E7) [8]. Naïve and primed PSCs provide us with accessible *in vitro* models to study the molecular mechanisms underpinning different pluripotency programs. Despite these advances, however, there is a lack of stable and well-characterized PSCs to model the E5–6 epiblast; an interval between naïve and primed pluripotency.

E5–6 epiblasts putatively reside in one or more intermediate states, and these intermediate pluripotency states presumably share common functional features including permissiveness for chimera formation and primordial germ cell (PGC) specification, both of which are retained in E5–6 epiblast *in vivo* [9, 10]. In a review published in 2017, Austin Smith described this interval as a period of “formative pluripotency,” in which the transcriptional and epigenetic network of naïve pluripotency is reconfigured to render epiblast cells capable of responding to lineage induction cues [11]. Formative stem cells display a mix of naïve and primed characteristics, an intermediate transcriptomic signature, and dual competence for chimera formation and PGC induction. Initially, formative epiblast cells were modeled *in vitro* by a transient population differentiated from naïve mouse ESCs known as epiblast-like cells (EpiLCs) [12], but in recent years several groups have established culture conditions to stabilize PSCs with formative features [13–15].

Based on a culture condition that activates the FGF, TGF- β /Smad, and WNT/ β -Catenin signaling pathways, we have developed a method that enables direct derivation of intermediate formative-like PSCs (termed FTW-PSCs) from mouse blastocysts [15]. The *de novo*-derived mouse FTW-PSCs share transcriptomic similarities with the mouse E5–6 epiblast and retain high competence for direct PGC-LC induction *in vitro* and germline chimera formation *in vivo*. Using the same culture condition, we have also successfully generated transgene-free human induced pluripotent stem cells (human FTW-iPSCs). The human FTW-iPSCs also harbor intermediate pluripotency features and demonstrate PGC-LC competence *in vitro*. These intermediate FTW-PSCs can expand our knowledge of the mammalian pluripotency continuum and provide an accessible *in vitro* model to study early development as well as germ cell specification. Here, we describe the method for derivation and maintenance of mouse and human FTW-PSCs as well as the method for PGC-LC induction from these cells (Fig. 1).

2 Materials

2.1 Preparation of Mouse Embryonic Fibroblast (MEF) Feeder Cells

1. Cell culture plate, 6-well (BD Falcon, cat. no. 353046 or equivalent).
2. Tissue culture hood.
3. Cell culture incubator.
4. Micropipettes.
5. Phase contrast microscope.
6. Centrifuge.
7. 0.1% Gelatin solution (diluted in distilled water).

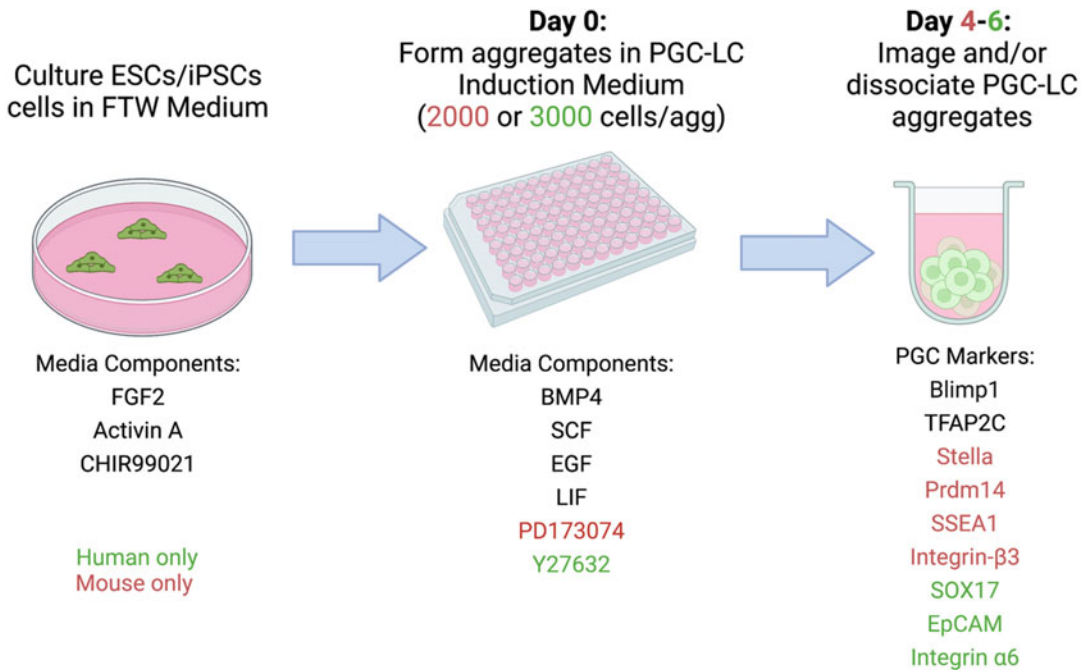


Fig. 1 Schematic of PGC-LC induction from mouse and human PSCs cultured in FTW media

8. Fetal bovine serum (FBS).
9. MEF feeder medium: 450 mL DMEM (Gibco, 10,313-021), 50 mL Fetal Bovine Serum (FBS) (HyClone, SH30071.01), 0.1 µg/mL of penicillin/streptomycin (Gibco, 15,070,063).
10. PBS (Gibco, 20,012-027).
11. 0.5% Trypsin-EDTA (Gibco, 15,400-054).

2.2 Derivation and Culture of Mouse FTW-ESCs

1. Pregnant mouse, 3.5 days post coitum (d.p.c).
2. Acidic Tyrode's solution (Millipore MR-004-D).
3. Mouth pipette.
4. Dissecting instruments: surgical scissors (FST, cat. no. 91401-12), micro forceps (Natsume, cat. no. A-7), and surgical forceps (FST, cat. no. 11252-30).
5. FBS (HyClone, SH30071.01).
6. MEF feeder cell-coated plate.
7. N2B27 medium (500 mL): 240 mL of DMEM/F12 medium (Gibco, 21,041-025), 240 mL of Neurobasal medium (Gibco, 12,348-017), 2.5 mL of N2 supplement (Gibco, 12,587-010), 5 mL of B27 supplement (Gibco, 12,587-010), 5 mL of penicillin/streptomycin (Gibco, 15,070,063), 5 mL of Glutamax (Gibco, 35,050,061),

2.5 mL of NEAA (Gibco, 11,140,050), 0.5 mL of 2-mercaptoethanol (Gibco, 21,985,023).

8. Mouse FTW derivation medium (500 mL): 500 mL N2B27 medium supplemented with 10 ng/mL of FGF2 (Peprotech, 100-18B), 10 ng/mL of Activin A (Peprotech, 120-14E), 3 μ M of CHIR99021 (Selleckchem, S2924), 0.5 μ M of PD0325901 (Selleckchem, S1036).
9. Mouse FTW culture medium (500 mL): 500 mL N2B27 medium supplemented with 10 ng/mL of FGF2 (Peprotech, 100-18B), 10 ng/mL of Activin A (Peprotech, 120-14E), 3 μ M of CHIR99021 (Selleckchem, S2924).
10. 1 \times TrypLE (Gibco, 12,605,036).
11. PBS (Gibco, 20,012-027).

2.3 Derivation and Culture of Human FTW-iPSCs

1. Human fibroblasts.
2. MEF feeder cell-coated plate.
3. Fibroblast growth medium (500 mL): 450 mL DMEM (Gibco, 10,313-021), 50 mL FBS (HyClone, SH30071.01), 0.1 μ g/mL of penicillin/streptomycin (Gibco, 15,070,063).
4. Human FTW medium (500 mL): 500 mL N2B27 medium supplemented with 20 ng/mL of FGF2 (Peprotech, 100-18B), 20 ng/mL of Activin A (Peprotech, 120-14E), 3 μ M of CHIR99021 (Selleckchem, S2924).
5. 1 \times TrypLE (Gibco, 12,605,036).
6. 0.05% Trypsin-EDTA (Gibco, 15,400-054).
7. PBS (Gibco, 20,012-027).
8. Episomal plasmids: pCXLE-hOCT3/4-shp53 (Addgene plasmid #27077), pCXLE-hSK (Addgene plasmid #27078), pCXLE-hUL (Addgene plasmid #27080), pCXLE-EGFP (Addgene plasmid #27082) (Optional).
9. Opti-MEM Reduced Serum Medium (Gibco, 31-985-070).
10. 4D-nucleofector (Lonza).
11. P3 solution (Lonza, V4XP-3032).

2.4 PGC-LC Induction from FTW-ESCs

1. 0.1% Gelatin-coated dish.
2. GK15 medium (200 mL): 163 mL of GMEM (Gibco, 11,710-035), 30 mL of KnockOut serum replacement (KSR), 2 mL of Penicillin/streptomycin (Gibco, 15,070,063), 5 mL of Glutamax (Gibco, 35,050,061), 2.5 mL of NEAA (Gibco, 11,140,050), 2 mL of Sodium pyruvate (Gibco, 11,360,070), 0.5 mL of 2-mercaptoethanol (Gibco, 21,985,023).

3. Mouse PGC-LC induction medium (20 mL): 20 mL of GK15 medium supplemented with 200 ng/mL of BMP4 (Gibco, PHC9531), 1000 U/mL of LIF (Peprotech, 300-05), 100 ng/mL of SCF (R & D, 255-SC), 50 ng/mL of EGF (Peprotech, AF-100-15), 1 μ M of PD173074 (Selleckchem, S1264).
4. Human PGC-LC induction medium (20 mL): 20 mL of GK15 medium supplemented with 200 ng/mL of BMP4 (Gibco, PHC9531), 1000 U/mL of LIF (Peprotech, 300-05), 100 ng/mL of SCF (R & D, 255-SC), 50 ng/mL of EGF (Peprotech, AF-100-15), 10 μ M ROCK Inhibitor Y27632 (APExBIO, A3008).
5. Ultra-low attachment U-bottom 96-well plate (Costar, 7007).
6. Multichannel pipette (optional).
7. Hemocytometer or automatic cell counter.

**2.5 Antibodies for
Flow Cytometry and/or
Immunostaining of
PGC-LCs**

1. SSEA-1 (480) antibody (Santa Cruz Biotechnology, sc-21,702).
2. Alexa Fluor 647 anti-mouse/rat CD61 antibody (BioLegend, 104,313).
3. Alexa Fluor 488 anti-human CD326 (EpCAM) antibody (BioLegend, 324,210).
4. Alexa Fluor 647 anti-human/mouse CD49f (Integrin alpha-6) antibody (BioLegend, 313,610).
5. Anti-Sox17 antibody (R & D Systems, MAB1924).
6. AP-2gamma (6E4/4) antibody (Santa Cruz Biotechnology, SC12762).
7. Purified anti-BLIMP-1 antibody (BioLegend, 648,202).
8. Nanos3 antibody (Abcam, ab70001).
9. Stella antibody (Abcam, ab19878).

3 Methods

3.1 Preparation of MEF Feeder Cells

1. Prepare cell culture plates coated with 0.1% gelatin, incubate at 37 degrees for at least 30 min.
2. Thaw irradiated MEF feeder cells.
3. Spin down and resuspend in MEF feeder medium.
4. Count cells and plate 400,000 cells per well of a 6-well gelatin-coated plate.
5. Allow cells to attach for 24 h before using.

3.2 Derivation and Culture of Mouse FTW-ESCs

1. Collect blastocysts from pregnant mouse (3.5 d.p.c).
2. Remove zona pellucidae from E3.5 blastocysts by brief treatment with acidic Tyrode's solution.
3. Using a mouth pipette (or alternative tools), transfer one blastocyst per well of a 6-well or 24-well MEF feeder-coated plate containing the Mouse FTW derivation medium for derivation.
4. Incubate the plate containing blastocysts at 37 °C, 5% CO₂ for 2 days (*see Note 1*).
5. On Day 3 after blastocyst plating, check whether blastocysts have successfully attached onto MEF feeder layer (*see Note 2*).
6. Gently remove ½ volume medium and add ½ volume of fresh Mouse FTW derivation medium.
7. Continue changing medium in this manner every two days.
8. From Day 7 after blastocyst plating, check the size of the outgrowth under microscope daily.
9. Once the outgrowth is large enough, gently wash the outgrowth with PBS once.
10. Aspirate the PBS, add 200 µL of 1× TrypLE to the outgrowth, and then incubate at 37 °C for 8–10 min.
11. Using 10 µL pipette, dissociate the outgrowth by gently pipetting up and down 10 times.
12. Add 1 mL Mouse FTW derivation medium +10% FBS to the well with outgrowth, and transfer everything to a new well of a MEF feeder coated plate (usually a 6-well plate).
13. Repeat step 12 one more time.
14. Incubate the new plate containing dissociated outgrowth at 37 °C, 5% CO₂ for 24 h, then change the medium to fresh Mouse FTW derivation medium without FBS.
15. Check the cells daily. Once tiny domed colonies can be observed, change the medium to Mouse FTW culture medium.
16. Replace the Mouse FTW culture medium every day and continue culture until the colonies grow large enough for passage (the cell line is now at P1) (*see Notes 3 and 4*).

3.3 Maintenance of Mouse FTW-ESCs

1. Wash the derived cells with 2 mL PBS twice.
2. Aspirate the PBS, add 500 µL of 1× TrypLE to the cells, and then incubate at 37 °C for 3 min.
3. Add 1 mL of Mouse FTW culture medium and dissociate the colonies by gently pipetting. Then transfer everything into a 15 mL falcon tube.
4. Repeat **step 3** once, and then centrifuge at 200 *g* for 3 min.

5. Aspirate the supernatant, resuspend the pellet in 1 mL of Mouse FTW culture medium.
6. Transfer 100 μ L (1:10 passage) of cell suspension into a well of a newly prepared MEF feeder plate (6-well plate) containing 2 mL of Mouse FTW culture medium.
7. Incubate at 37 °C, 5% CO₂, and change medium daily.

3.4 Derivation and Culture of Human FTW-iPSCs

1. Expand the human foreskin fibroblasts (HFFs) in fibroblast growth medium.
2. Prepare the 0.1% Gelatin solution coated dish (usually 10 cm petri dish) at least 1 h before transfection.
3. Wash the HFFs with PBS twice and add 3 mL of 0.05% Trypsin–EDTA solution (in 10 cm petri dish).
4. Incubate the dish at 37 °C, 5% CO₂ for 3–5 min.
5. Add 7 mL of fibroblast growth medium and dissociate the colonies by gently pipetting. Then transfer everything into a 15 mL falcon tube.
6. Centrifuge the tube at 200 *g* for 3 min.
7. Aspirate the supernatant, resuspend the pellet in 3 mL of Opti-MEM medium.
8. Count the cell density and transfer 2×10^6 HFFs into a new tube.
9. Centrifuge the tube at 200 *g* for 3 min.
10. Prepare P3 nucleofection solution according to the instructions and add 2.5 μ g of each episomal vector (total 10 μ g, including the vector of GFP) (*see Note 5*).
11. Aspirate the supernatant, resuspend the pellet in 100 μ L prepared P3 solution with plasmids, and transfer into a nucleofection cuvette.
12. Perform the nucleofection according to the instructions, using the NHDF program (pulse code DT130) (*see Note 6*).
13. Immediately transfer the HFFs into the gelatin-coated dish in fibroblast growth medium.
14. Wash the cuvette with 1 mL of fibroblast growth medium twice and transfer to the HFF culture dish.
15. Incubate the dish at 37 °C, 5% CO₂ for 4–5 days (*see Note 7*).
16. Prepare the MEF feeder cell-coated dish (usually 10 cm petri dish) at least 1 day before passage of HFFs.
17. Wash the HFF with PBS twice and add 3 mL of 0.5% Trypsin–EDTA solution.

18. Add 7 mL of fibroblast growth medium and dissociate the colonies by gently pipetting. Then transfer everything into a 15 mL falcon tube.
19. Centrifuge the tube at 200 *g* for 3 min.
20. Aspirate the supernatant, resuspend the pellet in 10 mL of fibroblast growth medium.
21. Transfer the cells into the prepared MEF feeder-coated dish, and incubate the dish at 37 °C, 5% CO₂.
22. Replace the medium with human FTW medium 24 h after plating.
23. Check the cells under microscope daily and change the medium every other day.
24. Manually pick and dissociate the individual colonies and transfer into separate wells of MEF feeder-coated plates in human FTW medium (*see* **Notes 8, 9, and 10**).
25. Continue culturing the picked clones at 37 °C, 5% CO₂ until colonies are large enough for passage.

3.5 Maintenance of Human FTW-iPSCs

1. Wash the cells with 2 mL PBS twice.
2. Aspirate the PBS, add 500 μL of 1× TrypLE to the cells, and then incubate at 37 °C for 3 min.
3. Add 1 mL of Human FTW culture medium and dissociate the colonies by gently pipetting. Then transfer everything into a 15 mL falcon tube.
4. Repeat step 3 once, and then centrifuge at 200 *g* for 3 min.
5. Aspirate the supernatant, resuspend the pellet in 1 mL of Human FTW culture medium.
6. Transfer 100 μL (1:10 passage) of cell suspension into a well of newly prepared MEF feeder plate (6-well plate) containing 2 mL of Human FTW culture medium.
7. Incubate at 37 °C, 5% CO₂, and change medium daily.

3.6 PGC-LC Induction from FTW-PSCs

1. PGC-LC induction is usually performed when the cells reach around 70% confluency.
2. Prepare the 0.1% gelatin-coated dish and incubate for at least 30 min before using.
3. Wash the cells twice with 2 mL of PBS to remove the dead cells.
4. Aspirate the PBS, add 500 μL of 1× TrypLE to the cells, and then incubate at 37 °C for 3 min.
5. Add 1 mL of FTW culture medium and dissociate the colonies by gently pipetting. Then transfer everything into a 15 mL falcon tube.

6. Repeat **step 3** once, and then centrifuge at 200 *g* for 3 min.
7. Aspirate the supernatant, resuspend the pellet in 1 mL of GK15 medium.
8. Transfer the cell suspension into a well of the prepared 0.1% gelatin-coated 6-well plate and incubate at 37 °C for 30 min to remove MEF feeders (*see* **Notes 11** and **12**).
9. Transfer the cell suspension into a 15 mL falcon tube without shaking or up-down pipetting. Then centrifuge at 200 *g* for 3 min. Resuspend in 1 mL of appropriate PGC-LC medium and count cells.

3.7 PGC-LC Induction from Mouse FTW-ESCs

1. Add an optimal volume of the mouse PGC-LC induction medium to adjust the concentration of mouse FTW-ESCs to 2×10^4 cells/mL (*see* **Note 13**).
2. Transfer 100 μ L of the cell suspension per well (2000 cells/well) of an ultra-low attachment 96 well plate (*see* **Note 14**).

3.8 PGC-LC Induction from Human FTW-iPSCs

1. Add an optimal volume of the human PGC-LC induction medium to adjust the concentration of human FTW-iPSCs to 3×10^4 cells/mL.
2. Transfer 100 μ L of the cell suspension into single wells (3000 cells/well) of an ultra-low attachment 96-well plate (*see* **Note 15**).

3.9 Culture of Mouse and Human PGC-LCs

1. Incubate the cells in the plate at 37 °C, 5% CO₂ and half change the PGC-LC induction medium every other day (*see* **Note 16**).
2. The cells form floating aggregates after 1 day of culture, and the PGC-LCs begin to arise after 2 days of induction.
3. Mouse PGC-LCs can be harvested at Day 4–6, and human PGC-LCs can be harvested at Day 6–8.

3.10 Dissociation and Analysis of PGC- LCs

1. At desired time point, collect aggregates in 1.5 mL tube.
2. Spin down or allow aggregates to sink to bottom. Wash once with PBS.
3. Dissociate aggregates in 500 μ L 0.05% Trypsin for 15 min at 37 °C, pipetting vigorously every 5 min.
4. Add 100 μ L FBS to quench trypsin.
5. Strain cells through 40 μ m cell strainer.
6. If cells will be analyzed at a later date, fix in 4% PFA for 15 min. Wash with PBS.
7. Percentage of PGC-LC induction can be quantified by flow cytometry for cell surface markers: SSEA1 / Integrin β 3 for mouse PGC-LCs [12], and Integrin α 6/EpCAM for human PGC-LCs [16, 17].

4 Notes

1. For the first 2 days after blastocyst plating, do not disturb the plates to allow the blastocysts to attach to the MEF feeder layer.
2. If blastocysts do not attach, use a 10 μ L pipette to mechanically push the non-attached blastocysts onto the MEF feeder layer under a dissection microscope.
3. Do not allow the outgrowth to grow too large and avoid the center of the outgrowth becoming too dark in color.
4. If flat differentiated colonies arise, manually pick the domed colonies for passage.
5. The amount of plasmid and the number of cells need to be optimized for different cell lines.
6. The transfection protocol can be different according to different equipment.
7. Do not allow the cells to become over-confluent. The transfection efficiency can be estimated by counting the ratio of the GFP-positive cells to total cells.
8. Pluripotent stem cell-like colonies arise around 2–3 weeks after passage of HFFs.
9. The colonies can be easily picked by using a 200 μ L pipette tip under the microscope and be dissociated into small pieces by gently pipetting.
10. ROCK inhibitors usually are not needed for single-cell passage of human FTW-iPSCs.
11. The MEF feeders attach to a gelatin plate earlier than PSCs, and the PSCs can be separated from MEF feeders by using this time difference. Typically, 30 min is enough for attachment of MEF feeders without the loss of too many PSCs.
12. If a large amount MEF feeders are still present in the cell suspension after 30 min incubation, transfer the suspension into another gelatin-coated well and incubate for another 30 min. The MEF feeders can be distinguished from PSCs based on cell size (MEF feeder cells are larger than PSCs).
13. Making a proper volume of cell suspension helps save the PGC-LC induction medium. Typically, 5 mL for half plate (48 wells) and 10 mL for one plate (96 wells).
14. Within the range of 1500–2000 mouse FTW-ESCs per well is acceptable.
15. Within the range of 2500–3000 human FTW-ESCs per well is acceptable.
16. During medium changing, gently remove 50 μ L old medium and add 60 μ L (taking into consideration the evaporation) fresh PGC-LC induction medium without disturbing the cell aggregates.

Acknowledgments

J.W. is a New York Stem Cell Foundation–Robertson Investigator and Virginia Murchison Linthicum Scholar in Medical Research. This project is funded by CPRIT (RR170076), NIH (GM138565-01A1, R21HD107849-01, and OD028763), and Welch (854671).

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