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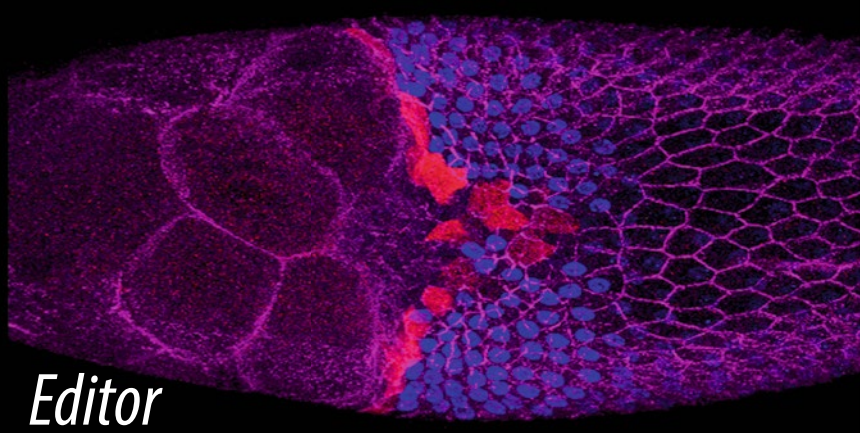

Ioannis P. Nezis *Editor*

Oogenesis

Methods and Protocols

Humana Press

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Oogenesis

Methods and Protocols

Edited by

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Preface

Oogenesis is a key biological event in the life of multicellular organisms since its final product, the oocyte, is the absolute basis for the formation of the organism. Effective oogenesis is important for evolutionary success by transferring genetic information to the next generation and it is crucial for life. Hence, comprehension of molecular and cellular mechanisms that govern oogenesis is very critical for understanding of organismal physiology. In order to achieve this, scientists have developed a large variety of methods and techniques to study oogenesis.

This book contains a collection of 20 chapters presenting methods and techniques to study oogenesis in a broad range of organisms, from plants to mammals. Specifically, the chapters include methods to study oogenesis in plants, worms, fruit flies, mosquitos, butterflies, starfish, zebrafish, frog, chicken, and mouse. The authors of the chapters are well-established and distinguished authorities in their respective areas of research.

It was a great pleasure to edit this volume and communicate with all the contributors and the Editor-in-Chief Prof. John Walker. I would like to thank all the contributors for their excellent work.

Coventry, UK *Ioannis P. Nezis*

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Chapter 1

Histological Analysis of the *Arabidopsis* Gynoecium and Ovules Using Chloral Hydrate Clearing and Differential Interference Contrast Light Microscopy

Robert G. Franks

Abstract

The use of chloral hydrate optical clearing paired with differential interference contrast microscopy allows the analysis of internal structures of developing plant organs without the need for paraffin embedding and sectioning. This approach is appropriate for the analysis of the developing gynoecium or seedpod of the flowering plant *Arabidopsis thaliana* and many other types of fixed plant material. Early stages of ovule development are observable with this approach.

Key words Ovule, Chloral hydrate, Nomarski optics, Ovary, Seed development, Seedpod

1 Introduction

The gynoecium of the flowering plant *Arabidopsis thaliana* is the female reproductive organ and the site of development of the ovules, the precursors to the seeds. Early stages of ovule development take place within the developing gynoecial tube. During development, the observation of ovules and other internal gynoecial structures is obscured by overlying tissues.

One approach to visualize internal structures of the gynoecium is histological sectioning of the material. This involves imbedding the material in paraffin or plastic resin and cutting thin sections on a microtome. Although results can be quite good with sectioning, the process is time-consuming. An alternative to sectioning is the use of a clearing agent to increase the transparency of the overlying tissue. Optical clearing is often paired with an optical sectioning technique such as the use of differential interference contrast (DIC) microscopy also known as Nomarski microscopy [1]. This allows the researcher to image optical sections of the specimen without having to physically section the material. Thus internal structures can often be visualized without the physical removal of the overlying tissue (Fig. 1). This

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Fig. 1 Differential interference contrast (DIC) microscopic image of a optically cleared gynoecium of *Camelina sativa*. Internal structures, including two rows of developing ovules, can be easily visualized without physically sectioning the gynoecium. Scale bar represents 100 μm

procedure is particularly useful for characterize morphological changes that result from experimental treatments or environmental conditions or are associated with a specific genotype. Here we present our protocol for the analysis of *Arabidopsis* gynoecial structures using chloral hydrate (*see* **Note 1**) as a component of Hoyer's media [2].

2 Materials

1. A compound microscope with differential interference contrast (DIC) optics.
2. Glass scintillation vials (20 ml capacity).
3. Forceps.
4. Dissection scope.
5. Glass slides.
6. Coverslips.
7. Eppendorf tubes.
8. Fixative solution: 90% ethanol and 10% glacial acetic acid.
9. Hoyer's solution: 70% chloral hydrate w/v, 4% glycerol, and 5% gum arabic. To prepare 20 ml of Hoyer's solution, use a

50 ml conical tube to mix 8 ml of distilled water H_2O, 2 ml of 40% glycerol, 14 g of chloral hydrate, and 1 g of gum arabic. Tightly seal the conical tube with parafilm and allow it to rock or nutate for several hours to ensure complete dissolution. Do not heat the solution. Then adjust final volume to 20 ml with distilled water. Spin in a centrifuge (100 × *g*, 5 min) to remove precipitate. Store supernatant at room temperature in a sealed tube. The solution will last at least 6 months.

10. 90% Ethanol.

3 Methods

3.1 Fixation and Initial Clearing

1. Fix inflorescences or other plant material for 2 h at room temperature in fixative solution (90% ethanol; 10% glacial acetic acid). We typically fix in glass scintillation vials. About ten *Arabidopsis* inflorescences can be fixed together in one 20 ml scintillation vial (*see* **Note 2**).
2. Remove fixative and wash the fixed tissue with 10–20 ml of 90% ethanol for 30 min. Rock or nutate to the wash tissue.
3. Remove first wash and repeat this 30 min. Wash with fresh 90% ethanol. Fixed tissue samples can be stored for long periods of time (months) in 90% ethanol in tightly capped scintillation vials if desired.
4. Transfer a single inflorescence to a clean Eppendorf tube containing approximately 500 µl of Hoyer's solution (*see* **Note 3**). When transferring the tissue, touch the tissue to a Kimwipes briefly to remove excess ethanol before transferring into Hoyer's solution. However, do not allow the tissue to dry completely, as that can alter the morphology of epidermal structures. Some ethanol will inevitably be transferred into the Hoyer's solution, but this will be diluted in subsequent steps.
5. Ensure that the Hoyer's solution is covering the tissue completely. Allow the tissue to soak in Hoyer's solution for several hours or overnight.

3.2 Dissecting and Mounting the Tissue

1. Transfer the tissue to fresh Hoyer's solution in a clean Eppendorf tube.
2. Under a dissection scope, in a drop of Hoyer's solution, dissect the sample with forceps. Often it is best to dissect the sepals, petals, and stamens away from the central gynoecium to allow best imaging of the gynoecium. As a single inflorescence has many flowers, it is good to dissect the inflorescence in stages and transfer smaller subsections to a fresh drop of Hoyer's on a clean glass slide to continue dissection (*see* **Note 4**).
3. Once plant organs are dissected from the inflorescence, proceed to mounting sample.

3.3 Mounting Gynoecia in Hoyer's Solution

1. Place a fresh drop (approximately 75 μl) of Hoyer's solution on a clean glass microscope slide. Transfer dissected gynoecium with forceps into the drop of Hoyer's on the slide. Carefully lay a coverslip over the gynoecium allowing Hoyer's to flow to the edge of the coverslip as the coverslip sinks down. For very small or delicate samples, "bridges" made from smaller coverslips can be used to support the main coverslip if needed to prevent sample from being crushed. Typically this is not necessary.
2. Allow slide to lay flat for 12–24 h to allow it to dry. Hoyer's solution will harden over this time and generate a permanent mounted specimen. There is no need to seal the edge of the coverslip as the Hoyer's will self-seal as it hardens and dries (*see* **Note 5**).

3.4 Examine the Gynoecium Under DIC Optics Using Koehler Illumination

Proper viewing of samples requires DIC or Nomarski optics to generate contrast in the otherwise clear samples. This requires proper microscopic equipment as well as proper adjusting of the microscope illumination source (i.e., to obtain Koehler illumination).

1. To set the illumination source for optimal Koehler illumination, rack up the condenser for the light source.
2. Put a sample on the microscope and focus your microscope on the sample with the 10× objective.
3. Close the lamp field diaphragm to limit the illuminated area to its smallest extent.
4. Raise or lower the condenser so that the diaphragm is in focus. The edge of the diaphragm should appear crisp through the eyepiece.
5. Use the condenser centering screws to center the condenser diaphragm if necessary.
6. Now open up the lamp field diaphragm so that the illuminated area just fills the area to be imaged (i.e., open up the diaphragm so that the edge of the diaphragm is still visible at the outer edge of the viewing area but is outside of the frame of the area that will be captured photographically).
7. Adjust the contrast of the image using the condenser diaphragm to achieve best image (*see* **Notes 6**, **7** and **8**).

4 Notes

1. Note that chloral hydrate is a controlled substance in the United States [3]. Thus state and federal regulations require registration of users to purchase, store, and use. See drug regulatory diversion site [http://www.deadiversion.usdoj.gov/] to obtain federal approval. As chloral hydrate is a sedative and a central nervous system depressant at high doses, wear gloves when handling Hoyer's or chloral hydrate and wash hands

after using. Do not eat of drink while using chloral hydrate. Visikol™ is a nonregulated alternative for chloral hydrate [4]. However, I have not yet tested Visikol™ as a replacement for chloral hydrate in my lab.

2. We usually fix many samples and then keep the tissue in 90% ethanol in parafilm-sealed glass scintillation vials for storage until ready to dissect and mount. Mesh screens can be useful for "catching" tissue when removing liquid from scintillation vials.
3. Gynoecia can be cleared directly in Hoyer's without a prefix, but these samples will eventually dissolve or deteriorate in the Hoyer's. Thus, we prefer to prefix the tissue and allow for the generation of permanent mounted specimens for long-term storage and imaging. For *Arabidopsis* inflorescences, we do not typically apply vacuum infiltration to allow penetration of fix. With other sample types, this may be more critical depending on thickness of the sample and amount of trapped air in the intracellular spaces. If using vacuum infiltration to enhance penetration of the fixative, it is best to pull the vacuum slowly and release the vacuum slowly so as to not burst sensitive cells. If needed, apply vacuum for about 10 min and then release it for 10 min. Repeat this cycle as needed.
4. There are many variant recipes for Hoyer's [2, 5, 6] and in our hands they all seem to clear well. Higher percentages of gum arabic seal faster when drying, but recipe is probably a matter of personal preference.
5. Dissecting gynoecia from the inflorescence is often done in a serial fashion (i.e., first put the inflorescence in a drop of chloral hydrate on the first slide and then pick off a few flowers of the appropriate stage and move those to a second slide with fresh drop of Hoyer's). This helps to ensure that any ethanol is well diluted in Hoyer's and generates the clearest samples for viewing. As you dissect, you may see the ethanol still leaving the tissue. Dissection of the sample in a drop of Hoyer's can be difficult under a dissection scope due to the reflection of the illumination light off the surface of the Hoyer's solution. Adjustment of the angle of the illumination or illuminating the sample from below can help with this issue. Opening the gynoecial tube by pinching with a forceps or cutting with a razor blade the apex or base (top or bottom) of the gynoecium allows better penetration of Hoyer's into center of the gynoecial tube. This is perhaps more important with older developmental stages. This is typically not needed with younger developmental stages or with many mutants since they are often not sealed up tightly into a closed tube. Allowing the Hoyer's to penetrate the gynoecial tube allows you see the ovules better through the valve without having to tear apart the gynoecium. If viewing of the internally located ovules during late developmental stages is the objective, more dissection may be required. If desired, one of the gynoecial valves (ovary walls) can be removed with forceps to

expose the ovules and image the more mature ovules. If dissected samples are small, consider using a smaller-sized Eppendorf tube during the initial Hoyer's clearing step. With small samples, it can be difficult to pick up the sample in the bottom of the Eppendorf tube with the forceps and shallower or wide-mouthed tubes may be more appropriate.

6. Gynoecia may not be completely cleared when first mounted. The final clearing and penetration of Hoyer's into the gynoecial tissues appears to happen within the first 12–24 h after mounting. After several hours, as the Hoyer's starts to dry, it may become apparent that more Hoyer's needs to be added to the sample. This will be visible as an air bubble starts to form near the edge of the cover slip. If this occurs, simply add a small amount of Hoyer's carefully under the edge of the coverslip and allow the slide to continue to dry for several more hours.
7. DIC microscopy requires the microscope to be equipped with a polarizing filter (polarizer), a condenser prism, an objective prism, and an analyzer (See Murphy and Davidson for additional details [1]). The nature and positioning of these components can vary on different microscopes; however, a few general tips are worth mentioning here to help you adjust the scope for the best-quality images. Firstly, each objective is often fitted with a different objective prism. These are paired with a particular condenser prism. Thus one should ensure that the correct condenser prism is selected for each objective lens. Selection of the condenser prism is often controlled by a "wheel" that the user turns to select different condenser prisms. Secondly, fine tuning of the contrast of the image can be achieved by adjusting the orientation/angle of the objective prism. This is typically achieved by turning a small knob or set screw that is located on the objective lens. Finally, the orientation of the sample will also dramatically alter the image due to the directionality of the polarized light source. One can adjust the image by rotating the sample using a rotating stage, if the microscope is equipped with this feature. Rotating the stage can dramatically alter the image and is a useful parameter to adjust for optimal image quality.
8. A summary of the different floral stages of *Arabidopsis* floral development can be found here in Smyth et al. [7]. A description of the stages of *Arabidopsis* ovule development can be found here [8]. A description of *Arabidopsis* gynoecial morphology can be found here [9].

Acknowledgments

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Chapter 2

Indirect Immunofluorescence of Proteins in Oogenic Germ Cells of *Caenorhabditis elegans*

John L. Brenner and Tim Schedl

Abstract

Formation of full-grown oocytes requires the control and coordination of a number of processes (e.g., oocyte growth) through multiple stages, where disruption at any one step can result in infertility. Numerous proteins are required for the regulation and execution of the various oogenic processes as well as functioning as maternal products needed for embryogenesis. Immunofluorescence microscopy combined with staining using antibodies against specific proteins, or their posttranslationally modified forms, is a standard approach to determine the temporal and spatial location of gene products that function in oocyte development. The simple linear organization of the germline in the model organism *Caenorhabditis elegans* allows easy correlation of protein localization and germ cell developmental stage, thus aiding in our understanding of protein function during gametogenesis. Here we outline co-immunofluorescence staining for two major regulators of *C. elegans* germline development, the translational repressor GLD-1 and activated form of MPK-1 (dpMPK-1) ERK MAP kinase in dissected gonads from adult *C. elegans*. Worms are first dissected and the extruded gonads are fixed and permeabilized before being bathed in primary antibodies against GLD-1 and dpMPK-1. Secondary antibodies conjugated to fluorophore dyes and that target the IgG domains of the primary antibody reagents are then used to provide a fluorescent signal that corresponds to the position of GLD-1 and dpMPK-1. The outlined procedure is amenable to many other proteins expressed in *C. elegans* germ cells.

Key words Germ cells, Indirect immunofluorescence, Gonad, Oogenesis, *C. elegans*

1 Introduction

Immunofluorescence microscopy has been an invaluable tool in cell and developmental biology since its inception in the 1940s [1, 2]. It allows localization of where proteins accumulate in specific cells of complex tissues and/or within a specific subcellular structure(s) of individual cells. The approach involves "fixing" and permeabilizing cells or tissues, incubating them with antibodies (usually affinity purified) to specific protein/peptide antigens of interest. These primary antibodies then serve as a scaffold to detect the position of the antigen either directly, where the primary antibodies are linked to fluorophores, or indirectly, where a second set

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of antibodies, termed secondary antibodies, are linked to fluorophores and these bind to the IgG domain of the primary antibodies. Indirect immunofluorescence has the added benefit of amplifying the initial signal as multiple secondary antibodies bind to the IgG domain of the primary antibody. Fluorescent microscopy is then used to identify the location of the fluorophores as a readout of protein (or other antigen) localization.

Formation of a mature gamete requires multiple developmental steps. In the nematode worm *Caenorhabditis elegans*, germline stem cells differentiate, enter meiosis, progress through meiotic prophase, and undergo gametogenesis in a linear array within a tube-shaped gonad (Fig. 1). Immunofluorescence staining of the gonad then provides information about which stage(s) of gametogenesis individual proteins accumulate and thus may function. Here we describe the general method for dissecting *C. elegans* gonads followed by

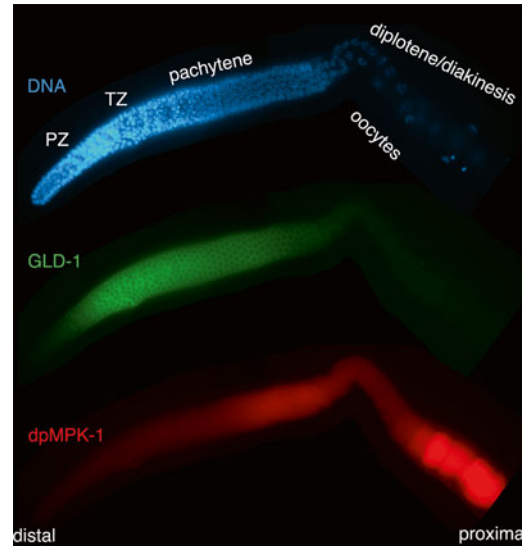

Fig. 1 Immunofluorescence of GLD-1 and dpMPK-1 in 1-day-old adult *C. elegans* hermaphrodite gonad. Germ cells develop in a distal to proximal fashion. Mitotically dividing germ cells, which include the stem cells, are located in the distal-most region, the proliferative zone (PZ). As germ cells move proximally, they overtly enter meiosis (leptotene/zygotene) in the transition zone (TZ), progress through an extended pachytene region, and then undergo oocyte growth and differentiation during diplotene and diakinesis at the proximal end. DNA in blue stained with 4′,6-diamidino-2-phenylindole (DAPI). GLD-1 in *green* stained with affinity purified antibodies [6]. Diphoshorylated MPK-1 (dpMPK-1) in *red* is stained with MAPK-YT (Sigma). Sequential images were taken using a microscope equipped with a 63× objective lens, digital CCD camera, and fluorescence optics. The images were stitched in ImageJ using the pairwise stitching plug-in [8] to generate an image of the full-length gonad, and each image corresponding to DNA, GLD-1, and dpMPK-1 were rotated and placed onto a black background, and the field of view partly removed to generate the image as shown

fixation and permeabilization, and then indirect immunofluorescence to detect proteins in germ cells, modified from procedures outlined previously [3]. For simplicity, and as a means of showing the versatility of the staining procedure, we provide directions for co-staining GLD-1 and the activated form of MPK-1 (dpMPK-1), which each show a dynamic range of expression (Fig. 1) in accordance with their important roles in germ cell development in *C. elegans*. This outlined method can be applied for detection of a wide array of known germ cell proteins, such as nucleoplasmic REC-8 [4], HIM-3 [4], CYE-1 [5], among many others.

2 Materials

Prepare all solutions with ultrapure water. All glassware should be clean, smooth, and free from scratches. Handle all solutions in accordance with institutional health and safety regulations.

2.1 Dissection Solutions

1. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, adjust to pH 7.4 with HCl. Sterilize by autoclave.
2. PBST: PBS + 0.1 % Tween-20.
3. 100 mM levamisole solution: Add 204 mg levamisole to 7 mL of PBS, mix, and raise volume to 10 mL with PBS. Store at −20 °C.

2.2 Dissection Components

1. Two 3 cc syringes affixed with a 25 gauge 5/8″ needle (*see* **Note 1**).
2. Square watch glass: Fig. 2 (*see* **Note 2**).
3. Short (5 ¾″) and (9″) long Pasteur pipettes: disposable borosilicate glass.

2.3 Fixation and Staining Solutions

1. 3% Paraformaldehyde solution (PFA): Add 1 10 mL ampule of 16% paraformaldehyde, electron microscopy grade, 3.73 mL of 1 M K_2HPO_4, 1.47 mL of 1 M KH_2PO_4 to 36.8 mL of water. Aliquot into 15 mL polypropylene centrifuge tubes and store at −20 °C.

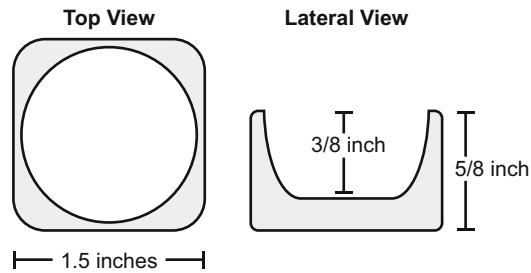

Fig. 2 Diagram of square watch glass

2. 100% Methanol (analytical grade): Store 50–100 mL aliquots at −20 °C.
3. 2.5% Agarose: 2.5 g agarose in 100 mL of water. Melt in microwave and then aliquot to small glass vials. Store at 4 °C for long-term storage or keep molten in heat block or water bath set to ~55 °C (*see* **Note 3**).
4. Blocking solution: Make 30% goat serum by diluting normal goat serum to 30% final concentration in PBS. Store 1 mL aliquots at −20 °C. Preclear the 30% goat serum prior to use by either high-speed centrifugation (10,000 × *g* at 4 °C) and/or passing through a 0.45 μm filter.
5. Primary antibody solution: Affinity purified rabbit-anti-GLD-1 antibodies [6] diluted 1:200 and MAPK-1YT (Sigma) antibodies diluted 1:400 in blocking solution.
6. Secondary antibody solution: Alexa 488-conjugated anti-rabbit IgG and Alexa 594-conjugated anti-mouse IgG each diluted 1:400 in blocking solution.
7. Anti-fade mounting solution: 0.5 g of 1,4-diazabicyclo[2.2.2]octane (DABCO), 45 mL of glycerol, 5 mL of PBS. Store at −20 °C in 50 mL conical centrifuge tube wrapped in foil.
8. DAPI solution: 100 μg/mL stock solution, store at −20 °C in a dark storage container to protect from light.
9. Nail polish (optional).

2.4 Fixation and Staining Components

1. Small glass culture tubes: 6 × 50 mm borosilicate glass disposable culture tubes.
2. Glass tubes: Pyrex centrifuge tubes, 5 mL capacity.
3. Parafilm.
4. Eyelash wand: attach a long eyelash to a wooden applicator stick with either tape or glue.
5. 75 × 25 × 1 mm glass slides.
6. Glass cover slip: 24 × 50 mm No. 1 cover slips.
7. Small glass vials. Screw top with lids, 7 mL volume, 17 mm × 60 mm × 9.5 mm.
8. Agarose pad slide. Add molten 2.5% agarose to a 75 × 25 × 1 mm glass slide. Immediately sandwich another glass slide on top length-wise such that ~3/4 of slide is covered with agarose and using two glass slides with ~3–4 strips of lab tape as a cushion (Fig. 3). Do not let agarose pad slide dry longer than 30 min before use.

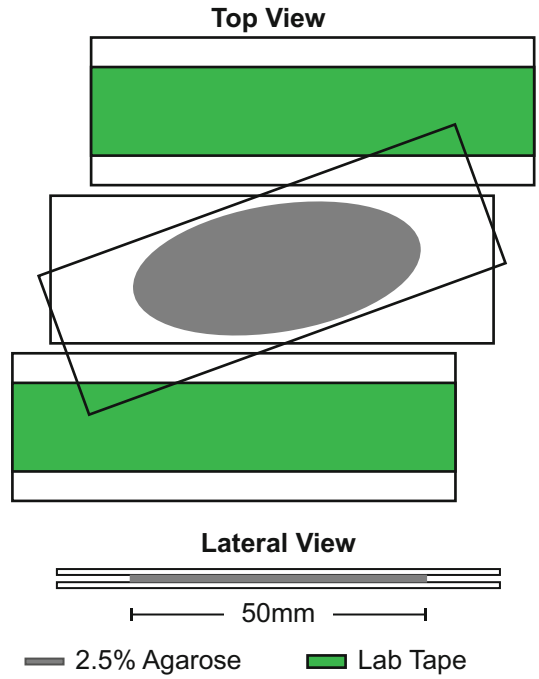

Fig. 3 Agarose pads used for mounting dissected gonads. Top view highlights the use of slides with 3–4 strips of tape (in *green*) to generate a 2.5 % agarose pad with a depth of ~1 mm (lateral view) as shown. Pad width should not exceed size of cover slip or ~50 mm

3 Methods

3.1 Dissections and Fixation

1. Pick >50 adult wild-type hermaphrodite worms, 24 h after mid-L4 stage at 20 °C, to a single 1.5 mL microcentrifuge tube containing 1 mL of PBS by direct picking (*see* **Note 4**). Fill tube with PBST to top after transfer.
2. Centrifuge tubes at ~1200 × *g* in a tabletop microcentrifuge for 1.5 min. Discard supernatant.
3. Wash worms at least once with 1–1.5 mL of PBST (*see* **Note 5**).
4. After wash, resuspend worms in 1 mL of PBS and transfer using a short glass Pasteur pipette to square watch glass.
5. Add 1 mL of PBS and 2 µL 100 mM levamisole solution.
6. After ~3–5 min (*see* **Note 6**), gently swirl the watch glass to collect worms to the center, then use two syringes affixed with needles to decapitate worms between anterior and posterior bulbs of the pharynx. This results in the gonad arms being extruded, although still attached to the carcass through the proximally located somatic gonad (the intestine is also extruded). Process worms one by one until all are dissected. Limit time to 5 min upon starting dissection.

7. Remove excess fluid from watch glass using a drawn-out long Pasteur pipette (*see* **Note 7**).
8. Add 2 mL of PFA (room temperature) directly to dissected animals in watch glass. Cover with parafilm and incubate in the dark for 10 min (*see* **Note 8**).
9. Carefully transfer the dissected worms/PFA mix to a glass conical tube using a short Pasteur pipette. Fill tube to the top with PBST (*see* **Note 9**).
10. Centrifuge tubes in a swinging bucket clinical centrifuge at ~500 × *g* for 1 min or until dissected worms collect to bottom of tube. Remove liquid. PFA-containing solutions are hazardous and should be discarded appropriately.
11. Wash dissected worms once with 5 mL of PBST, centrifuge as before, and then discard supernatant.
12. Add 2 mL of chilled (−20 °C) 100% methanol. Cover top of tube with parafilm and store at −20 °C for 30 min (*see* **Note 10**).
13. Fill tube to top with PBST (~3 mL), spin down tubes in clinical centrifuge for 1–2 min at 500 × *g*. Remove methanol-containing solution carefully (*see* **Note 11**) and discard solution.
14. Wash dissected worms three times with PBST.
15. Add 700 µL of PBST and then transfer solution containing dissected worms using a long Pasteur pipette to a small glass culture tube.
16. Spin down glass tube in clinical centrifuge at ~500 × *g*. Remove the supernatant.
17. Add 100 µL of blocking solution and incubate at room temperature for 30 min (*see* **Note 12**).

3.2 Antibody Incubations, Slide Preparation, and Imaging

1. Remove supernatant from dissected worms/gonads and discard.
2. Add 100 µL of primary antibody solution (*see* **Note 13**). Cover tube with parafilm and incubate at room temperature overnight (*see* **Note 14**).
3. Spin down tube in clinical centrifuge at 500 × *g* for 2 min. Remove the supernatant.
4. Add 500 µL of PBST, let incubate at room temperature for 5 min. Spin and remove the supernatant. Wash twice more.
5. Add 200 µL of secondary antibody solution. Cover tube with parafilm and incubate in the dark at room temperature for 4 h or overnight at 4 °C.
6. Prepare agarose pad slide but do not allow drying more than 30 min prior to use (*see* **Note 15**).
7. Spin down tube containing dissected gonads/worms in clinical centrifuge at 500 × *g* for 2 min. Remove the supernatant.

8. Add 500 µL of PBST; incubate at room temperature for 5 min in the dark. Spin and discard supernatant. Wash twice more. In final wash, add 0.5 µL of DAPI solution.
9. Spin down tube in clinical centrifuge at 500×*g* for 2 min. Remove the supernatant and add ~20 µL of anti-fade mounting solution.
10. Briefly spin tube to sediment solution to bottom.
11. Using a long Pasteur pipette, transfer whole contents of tube to previously prepared agarose pad (*see* **Note 16**).
12. While examining under dissecting microscope, use eyelash wand to gently spread out gonads along the length of the pad (*see* **Note 17**).
13. Carefully add cover slip, avoiding air bubbles (*see* **Note 18**).
14. Allow slide to dry in the dark prior to imaging (*see* **Note 19**).
15. Optional: After slide has dried, add a generous amount of nail polish around all four edges of the cover slip (*see* **Note 20**). Allow nail polish to dry ~30 min. Reapply nail polish as needed to completely seal edges of the cover slip.
16. Image gonads on a compound or confocal microscope with a 40, 63, or 100× objective equipped with appropriate filter sets and an epifluorescent lamp.
17. *See* Fig. 1 for representative image of GLD-1 and dpMPK-1 staining (*see* **Note 21**).

4 Notes

1. We typically remove the plungers from the syringes prior to use.
2. The internal part of the square watch glass holds 2–3 mL of solution; smaller versions are not compatible with our protocol and should be avoided.
3. We keep glass vials of 2.5% agarose in a heat block set to ~55 °C to keep molten. However, we avoid using agarose that has been in the heat block for longer than a week as pads from older 2.5% agarose tend to crack and are not suitable for mounting. Vials should be kept closed when not in use to prevent evaporation.
4. Worms can also be washed off adding ~1.5 mL of PBS to the worm plate and transferring solution to 1.5 mL microcentrifuge tube. However, this approach often results in higher amounts of bacteria carry-over, and additional washes should be performed prior to dissection. Also, expect to lose >10% of total worms from plate if transferring this way.
5. If solution is still cloudy, perform at least one additional wash until bacteria are no longer present.
6. Dissecting worms prior to complete paralysis seems to aid in gonads extruding from body cavity and we typically start dissecting while worms are still twitching modestly.

7. We use a Pasteur pipette attached to a vacuum manifold to remove fluid, and caution should be taken not to siphon up dissected animals.
8. We store the dish in a drawer. Alternatively the dish can be loosely covered with foil.
9. Usually some dissected animals remain after transferring solution to glass conical tube. We add 3 mL of PBST to the watch glass and then siphon up remaining dissected animals while examining under dissecting microscope. All 3 mL of PBST added to glass dish is transferred to the glass tube.
10. Dissected gonads can be stored long-term in methanol (~weeks). However, long-term storage in methanol has been observed to reduce the quality of tissue and is generally avoided when possible.
11. Dissected gonads will occasionally float in methanol solution. In such cases, do not attempt to remove all supernatant. Instead remove ~2 mL and then fill tube to top with PBST and spin again. The reduction in methanol concentration should aid dissected worms to collecting to the bottom of the tube after another round of centrifugation.
12. Blocking step can also be performed overnight at 4 °C if needed. Cover tube with parafilm to prevent contamination.
13. Using less than 100 μL of total volume for antibody incubations can lead to poor staining.
14. For longer incubations, place tubes at 4 °C. Do not incubate longer than ~2 days due to contamination concerns and elevated background staining.
15. Test the integrity of the pad by making a slide, letting dry briefly, and then remove top slide by gently sliding it off horizontally. If pad tears or cracks, then make a fresh batch of 2.5% agarose. Older agarose tends to crack or tear and are not good enough quality for use.
16. When siphoning dissected gonads from culture tube, do not allow solution to draw up too far, else tissue sticks to inside of Pasteur pipette.
17. Some lint and other "debris" are often present on the slide and should be removed using the eyelash wand. Also carefully spread the gonad away from the remaining portion of the dissected worm. The solution dries slowly, so these manipulations can be performed over the course of ~10 min if needed.
18. We add the cover slip at an angle to prevent air bubbles. Do NOT move cover slip once applied; else most gonads will be torn and damaged.
19. A short drying time is needed such that the cover slip affixes to the agarose pad without side-to-side movement; cover slip

movement may tear gonads. The amount of liquid used to place gonads onto slide typically creates a slight cushion between the pad and the cover slip and a liquid ring can be seen around the pad. In some cases, we dry slides in a container with drierite for <2 h for same-day imaging or until the liquid ring is no longer present. Alternatively, slides are allowed to air-dry overnight in the dark, e.g., in a drawer or wrapped carefully in tin foil and placed at 4 °C. Take care not to over-dry slide.

20. We use brightly colored nail polish to better observe the polish. Do not let polish seep under the cover slip and contact the pad. Uneven application of nail polish will cause distorting of the cover slip as it dries, which can cause tearing of gonads.
21. GLD-1 staining should be high in pachytene and stain throughout the rachis core. GLD-1 failing to be detected in the core of the rachis is an indicator that permeabilization failed, usually as a result of methanol becoming too hydrated. Using fresh methanol from an unopened bottle remedies the issue. To prevent this issue, we advise storing 50 mL aliquots of methanol at −20 °C in tightly closed bottles. dpMPK-1 signal in proximal oocytes is variable; however, reduced signal can also occur because of the damage to the given oocyte during dissection [7]. Co-staining against another protein present in oocytes can be used to distinguish between variable antigen levels and staining failed due to damage.

Acknowledgments

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Chapter 3

Antibody Staining in *Drosophila* Germaria

Anette Lie-Jensen and Kaisa Haglund

Abstract

Drosophila oogenesis is a powerful model for studying a wide spectrum of cellular and developmental processes in vivo. Oogenesis starts in a specialized structure called the germarium, which harbors the stem cells for both germ and somatic cells. The germarium produces egg chambers, each of which will develop into an egg. Active areas of research in *Drosophila* germaria include stem cell self-renewal, division, and maintenance, cell cycle control and differentiation, oocyte specification, intercellular communication, and signaling, among others. The solid knowledge base, the genetic tractability of the *Drosophila* model, as well as the availability and fast development of tools and imaging techniques for oogenesis research ensure that studies in this model will keep being instrumental for novel discoveries within cell and developmental biology also in the future. This chapter focuses on antibody staining in *Drosophila* germaria and provides a protocol for immunostaining as well as an overview of commonly used antibodies for visualization of different cell types and cellular structures. The protocol is well-suited for subsequent confocal microscopy analyses, and in addition we present key adaptations of the protocol that are useful when performing structured illumination microscopy (SIM) super-resolution imaging.

Key words *Drosophila* oogenesis, Germarium, Germline stem cell, Germline cyst, Fusome, Ring canal, Follicle cell, Antibody staining, Structured illumination microscopy

1 Introduction

The *Drosophila* female germline represents an excellent model to address a variety of cell and developmental biological questions. Biological processes that are actively studied in the *Drosophila* germarium include germline stem cell (GSC) self-renewal, maintenance, and niche biology [1–5], GSC division and abscission [6–10], cell cycle regulation and differentiation [11–16], germline cyst and oocyte polarization [17, 18], meiosis [19–21], GSC regeneration [22], follicle stem cell proliferation and maintenance [23–26], and many others.

A female fly has a pair of ovaries, each of which consists of 16–20 ovarioles that consist of a string of progressively more mature egg chambers from the anterior toward the posterior (Fig. 1) [27, 28]. At the anterior end of each ovariole is a specialized

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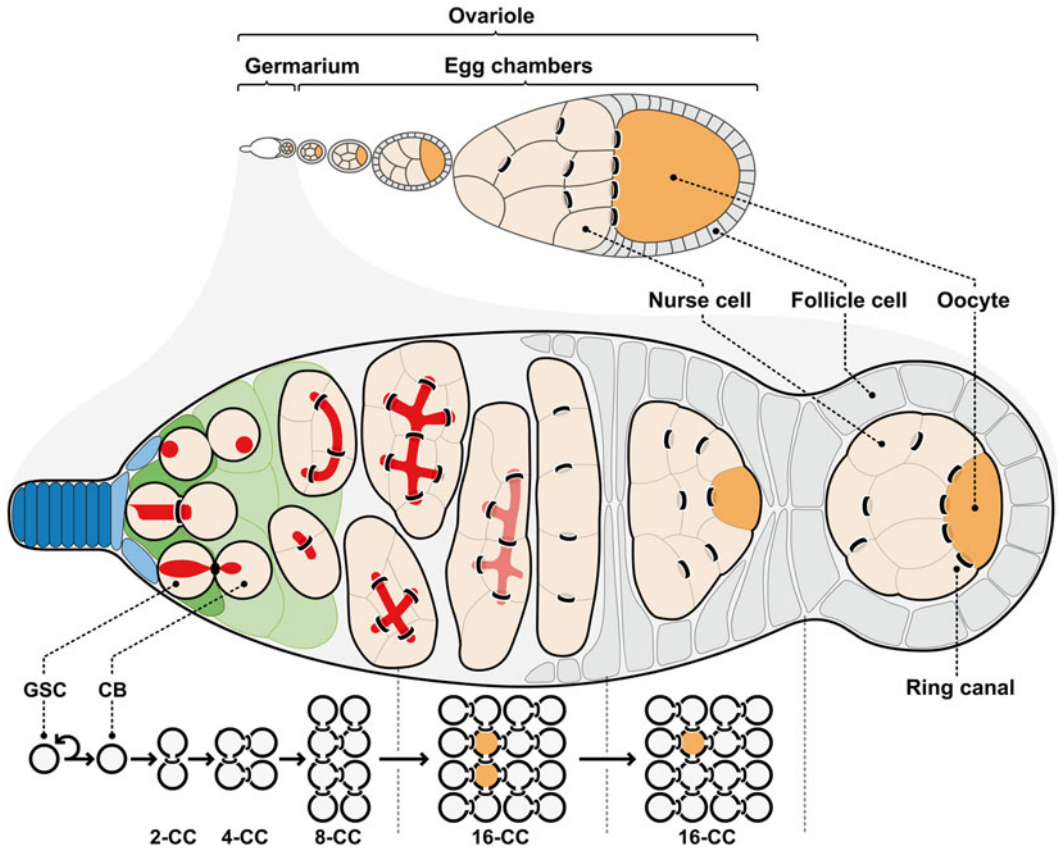

Fig. 1 Schematic illustration of a *Drosophila* ovariole and germarium. See the main text for details. Terminal filament cells are labeled in *dark blue*, cap cells in *light blue*, escort stem cells in *green* and escort cells in *light green*. The spectrosome in GSCs and fusome in GSC-CB pairs as well as the fusome in germline cysts are labeled in *red*. *GSC* germline stem cell, *CB* cystoblast, *CC* cell cyst

structure, called the germarium. The germarium contains germline and somatic stem cells and produces egg chambers that bud off at its posterior end (Fig. 1) [27, 28].

Two to three germline stem cells (GSCs) are located at the anterior tip of the germarium in contact with the stem cell niche (Fig. 1) [2]. The niche is generated by three cooperating somatic cell types: terminal filament, cap, and escort cells (Fig. 1) [2]. The attachment of GSCs to cap cells via adherens junctions ensures their sustained localization in the stem cell niche [29]. The cells of the terminal filament secrete the ligand unpaired (Upd) that activates JAK/STAT signaling in cap cells and escort cells, which in turn stimulates bone morphogenetic protein (BMP) ligand production in these cells [30, 31]. Activation of BMP receptors in GSCs initiates signal transduction that in turn results in suppression of transcription of the differentiation gene *bag of marbles* (*bam*), thereby ensuring GSC maintenance [32, 33].

Each GSC divides asymmetrically with complete cytokinesis in an oriented fashion to give rise to another GSC that remains in the stem cell niche and a daughter cell that is displaced away from the niche (Fig. 1). During GSC division, the GSC inherits the daughter centrosome and the midbody remnant [8]. Recent studies of mechanisms controlling accurate timing of cytokinetic abscission in GSCs have shown that Aurora B delays and that Cyclin B, the scaffold protein ALIX, and the ESCRT-III component Shrub promote abscission in these cells [7, 9, 10].

Following GSC division, the daughter cell that moves away from the signals of the stem cell niche initiates *bam* transcription and the cell consequently differentiates into a cystoblast (CB) [34]. The CB undergoes four mitotic divisions with incomplete cytokinesis, giving rise to a 16-cell germline cyst in which the cells are interconnected via stable intercellular bridges, called ring canals (RCs) (Fig. 1) [17, 35]. The orientation of these mitotic divisions is guided by a germline-specific organelle, the fusome, which consists of endoplasmic reticulum surrounded by cytoskeletal components and microtubules (Fig. 1) [6, 17, 36]. During each of the mitotic germ cell divisions, the fusome progressively branches in a systematic manner and anchors one pole of each mitotic spindle as the mitotic divisions proceed [6, 17, 36]. As a result a symmetric germline cyst forms, in which two of the cells, the pro-oocytes, have four RCs connected to them. One of these cells will gradually become specified as the oocyte (Fig. 1) [17]. The 16-cell cyst progressively moves toward the posterior end of the germarium where it becomes encapsulated by a single layer of epithelial follicle cells to form an egg chamber (Fig. 1) [27, 28]. At this point, the oocyte is positioned at the posterior of the germline cyst. The egg chamber then buds off from the germarium and enters the vitellarium. The oocyte polarizes and the other 15 cells, the nurse cells, begin to polyploidize [27, 28]. During the progressive egg chamber development, the RCs grow in size and mediate directed transport of cytoplasm and nutrients to the growing oocyte [27, 35]. Each egg chamber undergoes 14 developmental stages to eventually mature into an egg [37].

The genetic amenability of the *Drosophila* germarium, its well-characterized architecture, and the availability of morphological and molecular markers allow the dissection of molecular mechanisms of cell behavior and cellular processes in the context of their native environment. A wealth of GAL4 lines [37], fluorescent protein fusions [37], protein trap lines [37–39], RNAi lines [40], reagents and methods for studies of *Drosophila* oogenesis are available and are described in a recent excellent review [37]. Live imaging protocols have also been developed to visualize *Drosophila* GSC division [7, 8, 41–43] and the dynamics of different processes during *Drosophila* oogenesis [44–49].

In this chapter, we describe a protocol for antibody staining in *Drosophila* germaria. We give an overview of the main procedures for immunostaining of the *Drosophila* germarium and provide a list of commonly used antibodies to visualize different cell types and major subcellular structures in the germarium. We furthermore indicate points in the protocol at which modifications can improve staining using certain antibodies and key adaptations suitable for structured illumination microscopy (SIM) super-resolution imaging.

2 Materials

2.1 Fly Collection and Dissection

1. Vials with fresh standard fly food.
2. Yeast paste: Baker's yeast mixed with water to a thick homogenous paste.
3. 1× PBS: 8.0 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO_4 dissolved in 800 mL dH_2O. Adjust the pH to 7.4 with HCl and add dH_2O to 1 L (*see* **Note 1**).
4. Sharp, thin tip forceps (e.g., Dumont® HP) (*see* **Note 2**).
5. Pyrex® plates (22 mm well diameter, 6.4 mm well depth, Electron Microscopy Sciences).
6. Carbon dioxide (CO_2) fly pad.
7. Paint brush (e.g., size 3–6).

2.2 Fixation and Immunostaining

1. 4% formaldehyde (FA): Dilute 16% FA (methanol free, Ultra Pure, EM grade) 1:4 in PBS (*see* **Notes 3–5**).
2. 0.3% bovine serum albumin (BSA) and 0.3% Triton X-100 in PBS (PBT): Dissolve 0.15 g BSA in PBS, add 750 µL 20% Triton X-100, adjust the volume to 50 mL with PBS, and mix well.
3. Plastic dishes (with about 2 cm^2 wells, e.g., 4-well/24-well).
4. Rocking platform.
5. Primary antibody/antibodies of choice (*see* Table 1).
6. Fluorescently conjugated secondary antibodies.

2.3 Mounting and Imaging

1. Stereo microscope.
2. Object glasses (76 × 26 mm).
3. Cover slips (21 × 26 mm).
4. Antifade Mounting Medium.
5. Clear nail polish.
6. Zeiss LSM 710/780 upright confocal microscope.

Table 1
Commonly used antibodies for staining *Drosophila* germaria

Cell type/structure	Antibody	Host species	Comment	Dilution	Fixation	Source	References
Germ cells							
	pMad	Rabbit	GSCs	1:25	4% FA[a, b], RT	Cell signaling[c]	[50]
	Vasa (dC-13)	Goat	All GCs	1:200	4% FA, RT	Santa Cruz biotechnology	[9]
	Nanos	Rabbit	GSCs and GCs	1:1000	4% FA, RT	[51]	[51]
	Sxl	Mouse	GSCs, CBs and 2-CCs	1:10	4% FA, RT	DSHB	[15]
	Bam	Mouse	CBs, 2-CCs to 8-CCs	1:10	4% FA, RT	DSHB	[15]
	A2BP1	Guinea pig	8-CCs	1:5000	4% FA, RT	[15]	[15]
	C(3)G	Rabbit	Synaptonemal complex	1:3000	4% FA, RT	[52]	[52]
Somatic cells							
Stem cell niche	Lamin C (LC28.26)	Mouse	Terminal filament and cap cells	1:40	4% FA, RT	DSHB	[50]
	Engrailed (4D9)	Mouse	Cap cells, terminal filament	1:20	4% FA, RT	DSHB	[53]
	E-cadherin	Rat	Cap cells	1:20	4% FA, RT	DSHB	[29]
Follicle epithelial cells	Dlg (4 F3)	Mouse	Basolateral domains of follicle cells	1:100	4% FA, RT	DSHB	[54]
	Fasciclin III (7G10)	Mouse	Follicle cells	1:50	4% FA, RT	DSHB	[55, 56]
Fusome							
	Hts-F (1B1)	Mouse	Spectrosome in GSCs, fusome in GSC-CB pairs and fusome in germline cysts	1:50	4% FA, RT	DSHB	[6, 54]
	α-spectrin (3A9)	Mouse	Spectrosome in GSCs, fusome in GSC-CB pairs and fusome in germline cysts	1:100	4% FA, RT	DSHB	[54, 57]
	Tropomodulin	Guinea pig	Spectrosome in GSCs, fusome in GSC-CB pairs and fusome in germline cysts	1:1000	4% FA, RT	[58]	[58]

(continued)

Table 1
(continued)

Cell type/structure	Antibody	Host species	Comment	Dilution	Fixation	Source	References
Ring canals							
	Hts-RC	Mouse	RCs in germline cysts from region 2a in germarium and egg chambers	1:50	4% FA, RT	DSHB	[54, 59]
	Anillin	Guinea pig	RCs in germline cysts and until egg chamber stage 3	1:10–1:100	MeOH	[54]	[54]
	Pavarotti	Rabbit	RCs in germline cysts and egg chambers	1:500	4% FA, RT	[60]	[60]
	pTyr	Rabbit or mouse	RCs in germline cysts and egg chambers	1:500	4% FA, RT	Sigma or Santa Cruz biotechnology	[54, 59]
	Cindr	Rabbit	RCs in mitotic germline cysts	1:500	4% FA on ice	[54]	[54]
Follicle cell stable intercellular bridges							
	Anillin	Guinea pig	Contractile rings, stable intercellular bridges	1:10–1:100	MeOH	[54]	[54]
	Pavarotti	Rabbit	Spindle midzone, stable intercellular bridges, nuclei	1:500	4% FA, RT	[60]	[60]
	Cindr	Rabbit	Stable intercellular bridges	1:500	4% FA on ice	[54]	[54]
Cytoskeleton and organelles							
	Rhodamine phalloidin[d]	–	F-actin in germ and follicle cells, RCs in germline cysts and egg chambers	1:400	4% FA, RT	Molecular Probes	[54, 59]
	α-tubulin	Sheep	Microtubules, mitotic spindles	1:250	4% FA, RT or MeOH	Cytoskeleton	[9]
	Phospho-histone H3	Rabbit	Mitotic germ and somatic cells	1:500	4% FA, RT	Millipore	[7, 61]
	γ-tubulin	Mouse	Centrosomes	1:500	4% FA, RT	Sigma	[62]

GSCs germline stem cells, *CB* cystoblast, *GCs* germ cells, *CC* cell cyst, *RC* ring canal
[a]Fixation in presence of phosphatase inhibitors at RT
[b]Use 3% BSA for blocking
[c]Phospho-Smad1/5, Ser463/465 (41D10)
[d]Phalloidin is not an antibody but a toxin and should be handled with appropriate gloves and safety precautions

2.4 Specific Materials for Immunostaining for SIM Super-Resolution Imaging

1. ProLong® Diamond Antifade Mountant (Molecular Probes).
2. High Precision cover slips, 18 × 18 mm, 1.5H, 170 ± 0.5 μm (Marienfeld-Superior).
3. DeltaVision OMX microscope for SIM super-resolution imaging.

3 Methods

3.1 Fly Collection and Dissection

1. Collect newly eclosed female flies (0–24 h old) of the desired genotype and transfer them to food vials with fresh yeast paste together with some males (*see* **Notes 6** and **7**). Let the flies age for 24–48 h (*see* **Note 8**).
2. Anesthetize the flies with CO_2 and sort out the females on a CO_2 fly pad.
3. Fill the wells of a Pyrex® plate with PBS and place it on a black background under a stereo microscope.
4. Gently grasp a female over the head and thorax with a pair of forceps and submerge it in the PBS with the wings down. Use a second pair of forceps to pinch off the cuticle at the base of the abdomen and gently pull to release the pair of ovaries. If necessary gently push on the abdomen to help releasing the ovaries. Remove excess fly tissue and transfer the ovaries to a well with fresh PBS. Repeat this step for each female fly until enough ovaries have been collected (*see* **Notes 9** and **10**).
5. Grasp the oviducts between the ovaries, alternatively around the most mature egg chambers, with one forceps, and use the other to gently tease the ovaries apart to slightly separate the ovarioles (*see* **Notes 11**–**13**).

3.2 Fixation and Immunostaining

All the incubation steps are performed on a rocking platform with gentle mixing. Replacement of fixative, wash, and antibody solutions is performed under a stereo microscope on a black background. It is important that the ovaries do not dry out at any step of the protocol.

1. Add 500 μL of 4% FA to a well in a plastic dish. Transfer the ovaries to the well and fix for 30 min at room temperature (RT) with gentle shaking (*see* **Notes 14** and **15** as well as Table 1 and Fig. 2 for different fixation procedures).
2. Carefully remove the 4% FA by pipetting, wash the ovaries 3 × 15 min with 400 μL PBT, followed by incubation with 400 μL PBT for minimum 30 min at RT for efficient permeabilization and blocking (*see* **Note 16**).
3. Dilute the primary antibodies in 250 μL PBT and mix well. A list of commonly used antibodies is available in Table 1.

Remove the PBT from the ovarioles and add the antibody solution. Incubate at 4 °C over night (*see* **Note 17**).

4. Remove the antibody solution (*see* **Note 18**) and wash with 400 µL PBT 3 × 15 min at RT.
5. Dilute the secondary antibodies in 250 µL PBT and mix well (*see* **Notes 19** and **20**). Centrifuge the secondary antibody solution for 5 min at 16,000 × *g* (*see* **Note 21**). Replace the wash buffer with secondary antibody solution. Protect the samples from light and incubate for 2 h at RT (*see* **Note 22**).
6. Remove the secondary antibody solution and wash the ovaries 3 × 15 min with 400 µL PBT at RT.
7. For nuclear staining, dilute Hoechst to 2 µg/mL in PBS. Remove the wash solution and add 250 µL of the Hoechst solution to the ovaries. Incubate for 10 min at RT (*see* **Note 23**). Replace the Hoechst solution with PBS.

3.3 Mounting

1. Place a small drop of mounting medium on a clean object glass. Use the forceps and transfer the ovaries to the mounting medium.
2. At the stereo microscope, hold the posterior part of an ovary with one pair of forceps and use the other pair to gently dissociate the ovaries into individual ovarioles (*see* **Note 24**).
3. Place a cover slip over the sample, seal the edges of the coverslip with clear nail polish and let it dry. The samples are now ready for confocal imaging analyses. If imaging is not performed immediately, store the samples at 4 °C. Confocal image projections of germaria stained with antibodies from Table 1 are shown in Fig. 2.

3.4 Immunostaining for SIM Super-Resolution Imaging

Fly collection and dissection are performed as in Subheading 3.1 of the main protocol above, applying **Note 12** to remove the muscle sheath from the ovarioles.

3.4.1 Fly Collection and Dissection

3.4.2 Fixation and Immunostaining

Fixation and immunostaining are performed as in Subheading 3.2 above in Pyrex® plates with 100 µL primary or secondary antibody per sample. During incubation steps, cover the plates with Parafilm.

3.4.3 Mounting

1. Place a small drop of ProLong® Diamond Antifade Mountant on a clean object glass. In addition, place a small drop of mounting medium on a piece of Parafilm.
2. Remove the egg chamber stages older than stage 10 from an ovariole using the two pairs of forceps. Grasp the ovariole containing the germarium at the new most posterior egg chamber and gently dip it in the drop of mounting medium on the

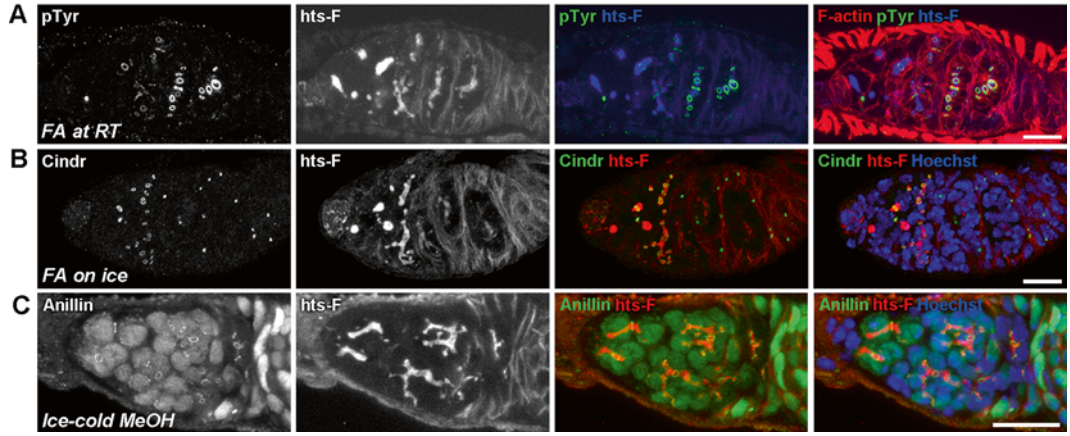

Fig. 2 Confocal image projections of *Drosophila* germaria fixed using different fixation procedures and stained with indicated antibodies. (**a**) Germarium fixed in 4% FA at RT. Fixation with 4% FA at RT is suitable for immunostaining using a wide range of antibodies. Ovaries were stained with antibodies against phosphotyrosine (pTyr, *green*) and hts-F (*blue*) and with rhodamine phalloidin (*red*) for F-actin detection. pTyr labels ring canals in germline cysts and hts-F labels fusomes. Scale bar, 10 μm. (**b**) Germarium fixed in 4% FA on ice. Some antibodies stain better when fixation with 4% FA is performed on ice. This applies, e.g., to the antibody against Cindr used here. Ovaries were stained with antibodies against Cindr (*green*) and hts-F (*red*) and with Hoechst (*blue*). Cindr marks midbody rings and midbodies in GSC-CB pairs as well as ring canals in germline cysts. Scale bar, 10 μm. (**c**) Germarium fixed in ice-cold methanol (MeOH). Fixation with ice-cold MeOH denaturates the proteins and can aid staining with certain antibodies. The antibody against Anillin used here performs better after MeOH fixation than with FA fixation. The ovaries were stained with antibodies against Anillin (*green*) and hts-F (*red*), and with Hoechst (*blue*). Anillin labels midbody rings in GSC-CB pairs and ring canals in germline cysts. Note that the antibody against hts-F works well with all three fixation methods used (**a**–**c**). Scale bar, 10 μm

Parafilm (*see* **Note 25**). Then, transfer the ovariole to the drop of mounting medium on the object glass. Transfer ~5–10 ovarioles to each object glass (*see* **Note 26**).

3. Place a High Precision cover slip over the sample (*see* **Note 27**). The sample is now ready for SIM super-resolution imaging analyses. If imaging is not performed immediately, store the sample at 4 °C. SIM super-resolution images of a germline 2-cell cyst are shown in Fig. 3.

4 Notes

1. PBS can be prepared as a 10× stock. 10× PBS: 80 g NaCl, 2 g KCl, 14.4 g Na_2HPO_4, and 2.4 g KH_2PO_4 dissolved in 800 mL dH_2O. Adjust the pH to 7.4 with HCl and add dH_2O to 1 L.
2. Tungsten or wolfram needles can be used for fine dissection of the ovarioles.

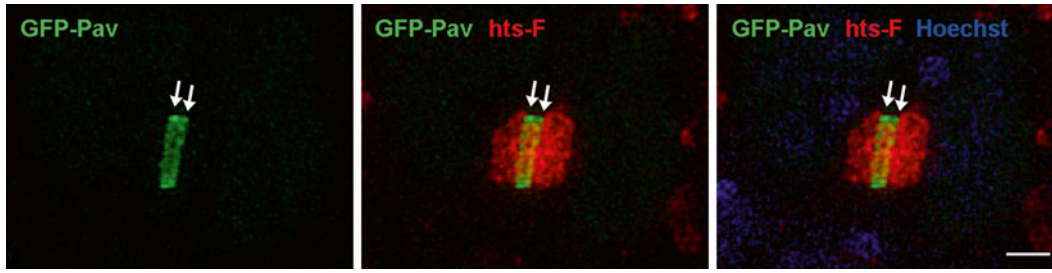

Fig. 3 A SIM super-resolution image of a *Drosophila* germline cyst. Ovaries of females ubiquitously expressing GFP-tagged Pavarotti (GFP-Pav, *green*) [60] were fixed in 4% FA at RT and stained with antibodies against hts-F (*red*) and with Hoechst (*blue*). The super-resolution image shows a 2-cell cyst. Note that the GFP-Pav signal is enriched at the rims of the RC (*arrows*) and that the hts-F signal at the fusome shows a structure that is not apparent in the confocal images in Fig. 2. Scale bar, 1 μm

3. An opened flask of 16% FA should be protected from light and air. FA is hazardous and should be handled with gloves and in a fume hood according to the material safety data sheet.
4. The 4% FA solution should be prepared fresh every time.
5. Other fixatives such as methanol can also be used, although FA is recommended for most stainings. Methanol is a precipitating fixative and causes denaturation of proteins, which might help when staining with antibodies directed toward denatured epitopes (*see* **Note 14**, Table 1 and Fig. 2).
6. Feeding the females with yeast paste and adding males promotes optimal oogenesis and ensures continued egg production.
7. The vials from which the female flies are collected should not be overcrowded during larval development to make sure that the quality of the ovaries is good.
8. The age of the flies should be selected to suit the particular experiment. We usually use 1–3-day-old flies. The ovaries from flies younger than 24 h are small and might be difficult to dissect and handle during immunostaining. To facilitate visualization and staining of such ovaries, it is helpful to retain a part of the abdomen connected to the ovaries. Moreover, to ensure that the flies have normal oogenesis, it is advisable that the females are not more than 4 days old.
9. Schematic illustrations [45, 63] and a video [64] describing the dissection of *Drosophila* ovaries in detail are available in excellent methods articles [45, 63, 64].
10. Dissect a sufficient number of ovaries that is suitable for the particular experimental setup and type of analyses to be performed.
11. This step improves the access of fixative, buffers, and antibodies to the individual ovarioles in subsequent steps of the protocol.

12. If desired, the muscle sheath surrounding the ovarioles can be removed at this step. This can be achieved by grasping a late-stage egg chamber and gently pulling the ovariole to release it from the muscle sheath. The muscle sheath can then be cut off at the border to the terminal filament of the germarium. Removal of the muscle sheath is necessary to perform SIM super-resolution imaging.
13. Some protocols for dissociation of ovaries into separate ovarioles involve rapid pipetting of the ovaries. Such protocols can however induce phenotypic artifacts and should generally be avoided [65].
14. Some antibodies stain better if fixation with 4% FA is performed 20–30 min on ice. Fixation in 100% methanol can improve staining with certain antibodies. Methanol fixation can be performed in 400 µL ice-cold methanol for 7 min at −20 °C. Note however that methanol fixation interferes with F-actin labeling with phalloidin and abolishes or reduces the signal from fluorescent proteins.
15. Addition of phosphatase inhibitors (1:200, Sigma) to the fixative and increasing the fixation time to 40 min is recommended when using antibodies against phosphorylated proteins, such as pMad (*see* Table 1).
16. Higher concentrations of BSA (e.g., 3%) can be helpful to reduce unspecific staining for certain antibodies.
17. Incubation with primary antibodies can also be performed 2–4 h at RT, but this may reduce the possibility of recycling the antibody solution.
18. The primary antibodies can be reused, so if desirable, collect the antibody solution in a tube and store it a 4 °C for later use. If bacterial growth is avoided (e.g., by adding 0.02% sodium azide), the antibody solution can be stored for months.
19. We commonly use fluorescently conjugated secondary antibodies at a dilution of 1:100–1:500.
20. If phalloidin is used for F-actin detection, it can be added to the secondary antibody solution. Phalloidin can also be added to the fixative [50]. Phalloidin is toxic and should be handled with impervious gloves and according to the material safety data sheet.
21. The centrifugation step removes aggregates of secondary antibodies. When adding the secondary antibody solution to the ovarioles, leave the last bit of it in the tube to avoid transferring any aggregates.
22. Protect the samples from light by, e.g., wrapping the plate with the samples in aluminum foil. The samples should be protected from light from this step onwards.

23. For nuclear staining, a mounting medium containing nuclear counterstain, such as Antifade Mounting Medium with DAPI, can alternatively be used.
24. Ovarioles can be separated from the muscle sheath also at this step as described by Luo et al. [50].
25. This step is performed to remove PBS from the tissue before mounting.
26. The removal of the oldest egg chamber stages and transferring a limited number of ovarioles per object glass are performed to limit the thickness of the sample.
27. The ProLong® Diamond Antifade Mountant is a curing mounting medium.

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Chapter 4

In Vitro Culturing and Live Imaging of *Drosophila* Egg Chambers: A History and Adaptable Method

Nathaniel C. Peters and Celeste A. Berg

Abstract

The development of the *Drosophila* egg chamber encompasses a myriad of diverse germline and somatic events, and as such, the egg chamber has become a widely used and influential developmental model. Advantages of this system include physical accessibility, genetic tractability, and amenability to microscopy and live culturing, the last of which is the focus of this chapter. To provide adequate context, we summarize the structure of the *Drosophila* ovary and egg chamber, the morphogenetic events of oogenesis, the history of egg-chamber live culturing, and many of the important discoveries that this culturing has afforded. Subsequently, we discuss various culturing methods that have facilitated analyses of different stages of egg-chamber development and different types of cells within the egg chamber, and we present an optimized protocol for live culturing *Drosophila* egg chambers.

We designed this protocol for culturing late-stage *Drosophila* egg chambers and live imaging epithelial tube morphogenesis, but with appropriate modifications, it can be used to culture egg chambers of any stage. The protocol employs a liquid-permeable, weighted "blanket" to gently hold egg chambers against the coverslip in a glass-bottomed culture dish so the egg chambers can be imaged on an inverted microscope. This setup provides a more buffered, stable, culturing environment than previously published methods by using a larger volume of culture media, but the setup is also compatible with small volumes. This chapter should aid researchers in their efforts to culture and live-image *Drosophila* egg chambers, further augmenting the impressive power of this model system.

Key words *Drosophila*, Ovary, Oogenesis, Egg chamber, Germline, Oocyte, Nurse cell, Somatic follicle cell, Live culturing, Live imaging, Confocal microscopy, Epithelial tube morphogenesis

1 Introduction

To understand and appreciate the live culturing methods discussed in this chapter, it is first necessary to have a basic knowledge of the *Drosophila* ovary, the *Drosophila* egg chamber, and the process of *Drosophila* oogenesis (Fig. 1). The female fruit fly possesses two artichoke-shaped ovaries, which are joined by a common oviduct and which each contain 15–20 ovarioles (i.e., parallel strings of developing egg chambers). The end of the ovariole distal to the oviduct contains a structure called the germarium, which holds the

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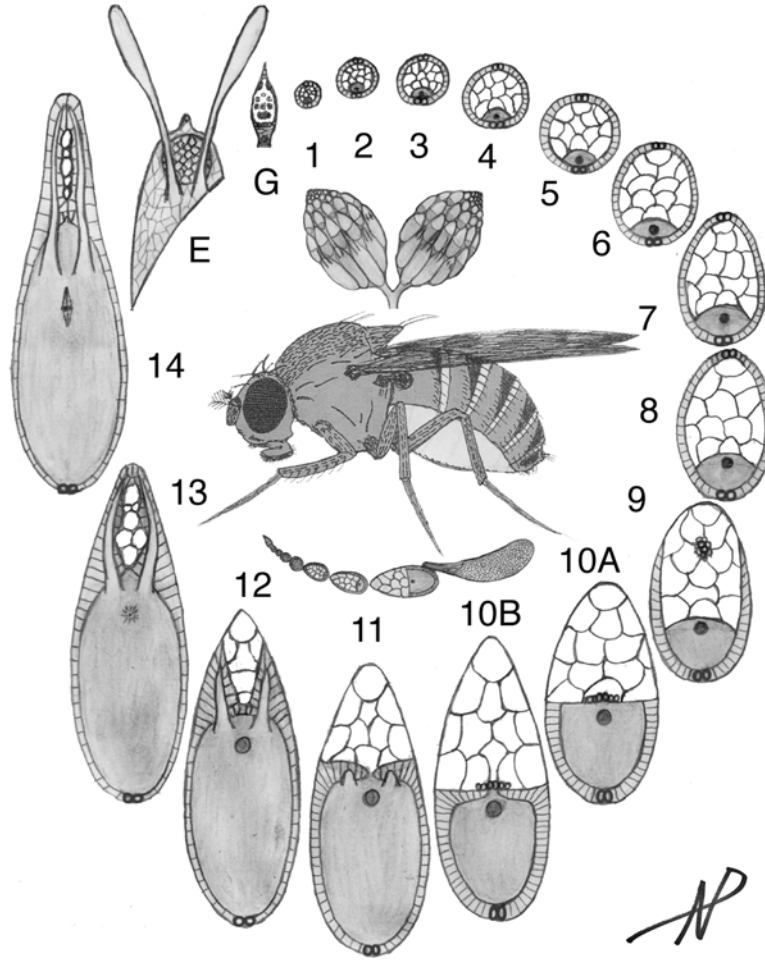

Fig. 1 Illustrations of the stages of *Drosophila melanogaster* oogenesis, arranged in a clockwise fashion around an adult female (*center*), an ovary pair (*above*), and an individual ovariole (*below*). Egg chambers are created in the germarium (G), which contains the germline and somatic stem cells. Egg-chamber development proceeds through 14 canonical stages (S1–S14) and concludes with the production of a mature egg (E), of which only the anterior portion is shown here. These illustrations are not intended to be absolutely precise in every respect, but are meant to illustrate the most obvious features that a researcher could use to distinguish egg chamber stages in a laboratory setting. These features include egg-chamber shape and relative size, the proportion of oocyte volume to total nurse-cell volume, and the morphology of the anterior end of the egg chamber when the secondary eggshell structures, such as the dorsal appendages, are created late in oogenesis

germline and somatic stem cells and their associated support cells. In the germarium, each germline stem-cell division produces a cystoblast that undergoes four incomplete mitotic divisions to produce an interconnected, 16-cell germline cyst. One cell becomes the oocyte, while the remaining cells become the oocyte's supporting nurse cells. Approximately 80 somatic follicle cells produced by the somatic stem cells of the germarium then encapsulate each germline cyst in a monolayer epithelium (apical faces inward towards the oocyte, basal faces outward); this epithelium will eventually differentiate into distinct cell types and synthesize the eggshell around the oocyte. Each somatically encapsulated germline cyst is subsequently referred to as an egg chamber until it enters the oviduct and becomes a mature egg. The overall purpose of this chapter is to provide a protocol for culturing these egg chambers, one that is adaptable for any stage of oogenesis.

During the course of oogenesis, an egg chamber proceeds through 14 stages (S1–S14) of development based on stereotyped morphological and morphogenetic changes (Fig. 1; *see* refs. 1, 2 for thorough and detailed accounts of egg-chamber development, including genetic analyses). During S1–S5 (~50 h), the growing egg chamber remains spherical in shape, the nurse cells undergo massive endoreplication (~64C), and the cuboidal follicle cells proliferate (i.e., ~80 cells become ~650 cells) and become patterned along the AP axis. During S6–S9 (~24 h), the egg chamber elongates along the AP axis, the nurse cells continue their endoreplication cycles (>500C), the oocyte expands significantly compared to its interconnected nurse-cell siblings (due in large part to uptake of yolk proteins), the follicle cells cease dividing and undergo endoreplication, the vitelline membrane begins to form, and the morphogenetic events of the follicular epithelium begin (i.e., the columnarization of posterior follicle cells contacting the oocyte, the flattening of follicle cells over the nurse cells, and the migration of the border cells). Progression through this period is variable and depends on nutrition. Early-stage S10 (S10A; ~6 h) is marked visually by the oocyte reaching a volume equivalent to that of the entire group of 15 nurse cells, and molecularly by transitions in the DV patterning of the follicular epithelium. Late S10 (S10B; ~4 h) is marked by a dramatic increase in egg-chamber volume, the almost complete separation of the oocyte from the nurse cells by the columnar follicle cells (i.e., centripetal follicle-cell migration), and the apical-basal thickening of the dorsal appendage follicle cells in preparation for their morphogenesis. As egg chambers transition into S11, the shortest of all the stages (~20 min), the nurse cells dump their cytoplasm into the oocyte, and the process of dorsal appendage (DA) tube morphogenesis begins (Fig. 2). S12–S14 (5+ hours) involves the degradation of the nurse-cell remnants following dumping, the completion of DA-tube morphogenesis, the secretion of the eggshell (Fig. 2H), and subsequent death of the

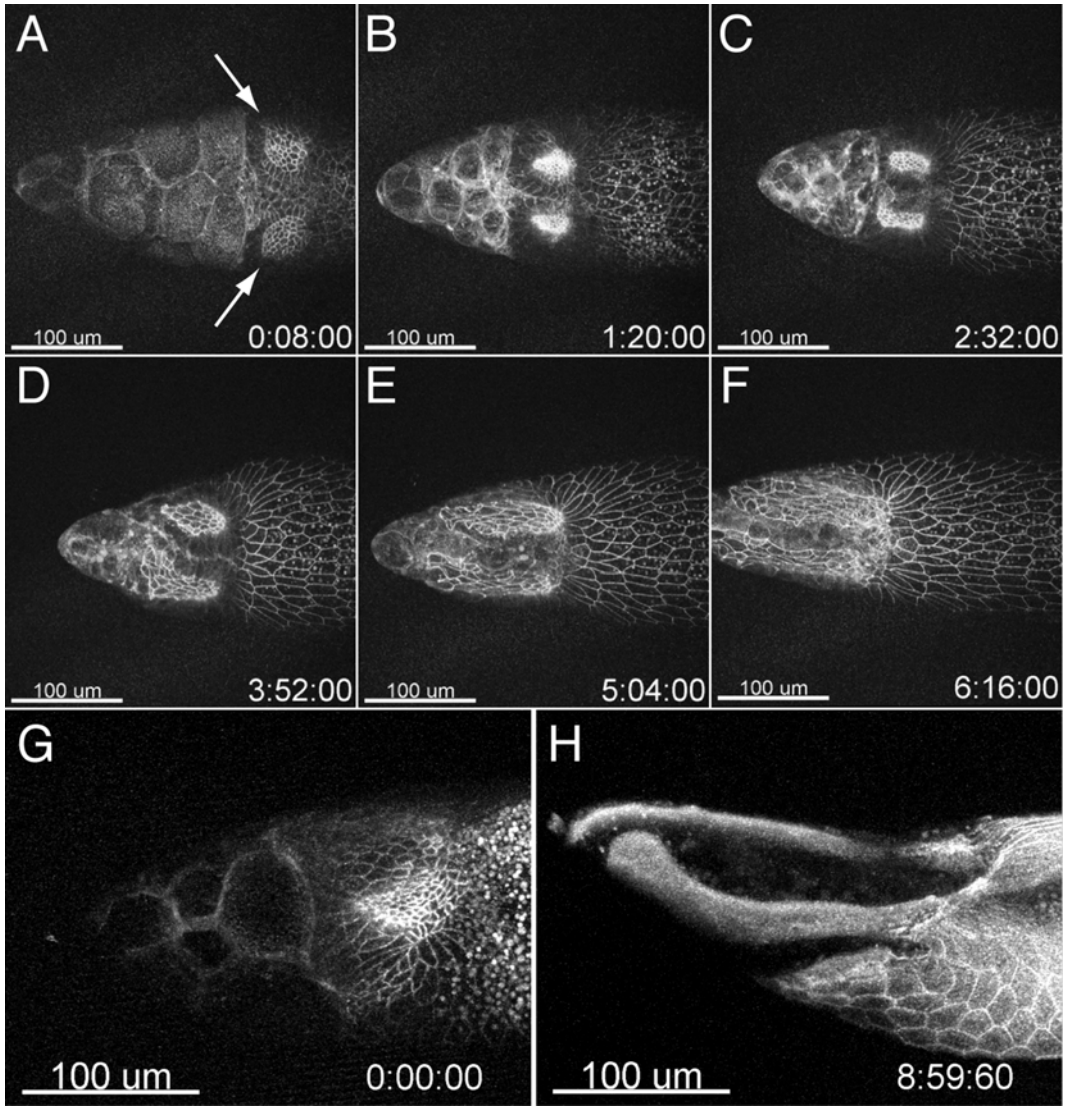

Fig. 2 Still images from two time-lapse movies of late-stage egg chambers dissected from an E-cadherin::GFP knock-in line [50], which fluorescently labels all E-cadherin with GFP. Since the highest levels of E-cadherin in the egg chamber follicle cells outline the apical surfaces of the follicle cells, this marker serves as an excellent tool for visualizing the morphology and morphogenesis of the dorsal appendage (DA) epithelial tubes between S10B–S14 [34, 47]. In the first movie (**A**–**F**), the egg chamber is homozygous for E-cadherin::GFP and is in a dorsal orientation. *Arrows* in A point to the two populations of cells that are preparing to form the DA tubes. Z-stacks were acquired every 8 min for 10 h, which encompassed both DA-tube formation (**A**, **B**) and expansion (**C**–**F**). In the second movie (**G**, **H**), the egg chamber is heterozygous for E-cadherin::GFP and is in the dorsolateral orientation. Note that the image is noisier because different imaging settings had to be used to detect the weaker signal. Z-stacks were acquired every 15 min for 10 h, which encompassed DA-tube expansion and eggshell secretion (**H**). Still images are adapted from supplementary material in [47] with permission from Elsevier

follicle cells and maturation of the oocyte, including breakdown of the nuclear envelope and movement of the meiosis-I chromosomes onto the metaphase plate. As the finished egg chamber proceeds through the oviduct, the dying follicle cells slough off and expose the eggshell, meiosis in the oocyte concludes, fertilization takes place to form an embryo, and embryogenesis begins.

Since the first detailed descriptions of *Drosophila* oogenesis in 1970 [3], nearly half a century of dedicated research and live-culturing innovation have demonstrated the awesome potential of the *Drosophila* egg chamber as a developmental model (Tables 1, 2, and 3), and these studies revealed a vast diversity of biological questions that the egg chamber can be used to address [4]. Building on the discovery that the abdomen of the fruit fly could act as an in vivo culturing chamber for transplanted tissue such as imaginal discs [5], dissected egg chambers were successfully cultured into mature eggs, even from the earliest stages, by transplanting them into new host abdomens [6]. This technique was used to facilitate development of late-stage egg chambers following ex vivo live imaging of cytoplasmic streaming [7] and mid-stage egg chambers following laser ablation of the oocyte nucleus [8]. It was also possible to culture dissected germaria in this manner, resulting in normal ovarioles and egg chambers even when germaria were transplanted into male flies, and these efforts allowed scientists to ascertain the precise timing of each stage of egg-chamber development [9]. Although this in vivo culturing approach was both elegant and innovative, particularly since it provided such a physiologically ideal environment for egg-chamber development, its primary disadvantage was its inaccessibility and visual obscurity during the culturing process. Fortunately, in vitro culturing of *Drosophila* egg chambers is also possible, enabling live-imaging studies.

Culture protocols vary depending on whether germline or somatic tissue is the focus of the analyses (Tables 1, 2, and 3) or whether the events of interest occur on a short time frame (e.g., less than 90 min) or require long-term imaging (e.g., multiple hours or days). For experiments involving microinjection of the germline (e.g., rhodamine-tubulin) and live culturing for relatively short periods of time (e.g., 100 s—[10]; 30 min—[11]; 90 min—[12]), high-grade halocarbon oil (e.g., Voltalef 10S) can act as a culturing medium. As long as the tissue of interest is not directly exposed to halocarbon oil (e.g., nurse cells, oocyte, migrating border cells), this medium can support normal development and may be preferred due to its amenable characteristics for microscopy ([13–15]). Halocarbon oil, however, is often detrimental to the proper development and morphogenesis of the external somatic follicle cells, and it limits long-term culturing experiments of egg chambers in general (personal observations; [15]). Aqueous media, such as modified Grace's medium [16], Schneider's medium [17],

Table 1
A chronological summary of 40 years of culturing and live-imaging efforts using the *D. melanogaster* ovary, concentrating on the focus/impact of research, as well as the stage(s) and tissue(s) studied

References and Date	Focus and impact of research	Stage(s)	Tissue
Srdic and Jacobs-Lorena (1978)	Transplanted egg chambers cultured in vivo can produce mature eggs (no live imaging)	< S1–S14	Both
Petri et al. (1979)	In vitro culturing media can support late-stage epithelial morphogenesis and eggshell synthesis	S10–S14	Soma
Gutzeit and Koppa (1982)	First live imaging of cytoplasmic streaming in the oocyte	S7–S14	Germline
Montell et al. (1991)	Laser ablation of the oocyte nucleus disrupts DV patterning in the oocyte	S6–S14	Germline
Lin and Spradling (1993)	Germaria transplanted and cultured in vivo can develop to the end of oogenesis	< S1–S14	Both
Wang and Hazelrigg (1994)	First live imaging of any fluorescent-labeled protein in *Drosophila*, proposed idea that microtubules could be involved in oocyte mRNA localization	S8–S11	Germline
Theurkauf (1994)	Cytoplasmic streaming in the oocyte is influenced by microtubule organization	S8–S12	Germline
Forrest and Gavis (2003)	First live imaging of fluorescent-labeled mRNA molecules, proposed a diffusion/entrapment model for mRNA localization in the oocyte	S10–S12	Germline
Cox and Spradling (2003)	Live imaging of mitochondrial inheritance in the oocyte, similarity between *Drosophila* and vertebrate mechanisms of germplasm provision	<S1–S11	Germline
Dorman et al. (2004)	First live imaging of late-stage epithelial tube morphogenesis, dorsal-appendage tubes form by wrapping	S10–S14	Soma
Gilliland et al. (2007)	First observation of female meiosis in the oocyte	S13–S14	Germline
Prasad and Montell (2007)	First live imaging of border cell migration, guidance receptors are not required for border cell protrusions	S9	Soma
Bianco et al. (2007)	Live imaging of border cell migration, there are two distinct phases of border cell migration	S9	Soma
Tekotte et al. (2007)	An alternative method for live imaging of border cell migration in halocarbon oil	S9	Soma

Tootle and Spradling (2008)	Visualization of actin dynamics during nurse-cell dumping, prostaglandins promote follicle-cell maturation	S10–S12	Germline
Fichelson et al. (2009)	Asymmetric RNP component segregation in single stem cells is required for stem cell renewal	Germarium	Germline
Wang et al. (2010)	Light-activated manipulation of border cell migration	S9	Soma
He et al. (2010)	Somatic tissue elongation is driven by a network of basal actomyosin contractions	S9–S10	Soma
Morris and Spradling (2011)	First live imaging of stem cell activity, cyst movement, and cell interactions within the germarium	Germarium	Germline
Airoldi et al. (2011)	First live imaging of somatic ring canal formation during follicle-cell mitosis	S10	Soma
Haigo and Bilder (2011)	First evidence of egg-chamber rotation; this rotation is required for egg-chamber elongation	S4–S12	Soma
Zhao et al. (2012)	During nuclear migration, the oocyte nucleus is not pulled, but is pushed via growing microtubules	S7	Germline
Osterfield et al. (2013)	A two-dimensional pattern of line tensions along apical cell-cell edges may explain the events of late-stage epithelial tube formation	S10–S12	Soma
McLean and Cooley (2013)	First evidence that cytoplasmic proteins can equilibrate between follicle cells through somatic ring canals	S10	Soma
Cetera et al. (2014)	Egg-chamber rotation promotes the global alignment of contractile machinery necessary for egg-chamber elongation	S1–S9	Soma
Cai et al. (2014)	E-cadherin-based interactions between border cells and nurse cells is required for direction-sensing during border cell migration	S9	Both
Spracklen et al. (2014)	Evaluation of actin-labeling tools in the germline reveals that each tool has both advantages and disadvantages for live imaging	S10–S11	Germline
Peters and Berg (2016)	Dynamin-mediated endocytosis is required for both late-stage epithelial tube formation and tube elongation	S10–S14	Soma

Table 2
A chronological summary of 40 years of culturing efforts using the *D. melanogaster* ovary, concentrating on the culture media and apparati used, as well as the maximum duration of culturing attempted

References	Culture medium	Culture apparatus	Max duration
[6]	Female abdomen	Female abdomen	9 days
[20]	Schneider's [17], Robb's [18], Grace's [16], D-22/ Echalier [55]	Covered glass depression slides	11 h
[7]	Imaged in Robb's, cultured in the female abdomen	Covered glass depression slides or flow-through chamber [56]	45 min
[8]	Ablations in Schneider's + 10% FBS, culturing in the female abdomen	Female abdomen	1–2 day in vivo
[9]	Short culturing in Schneider's + 10% FBS, culturing in male or female abdomens	Coverslip with dabs of vacuum grease, male or female abdomens	1 h in vitro, 1–3 day in vivo
[23]	Imaged in modified Robb's [22], treated in Schneider's + 10% FBS	Undescribed	Undescribed
[10]	Halocarbon oil (Voltalef 10S)	Oil on coverslip	100 s
[30]	Schneider's	Coverslip-bottomed culture dish with coverslip	8 h
[27]	Grace's	Greiner Lumox[a] culture dish and coverslip	3 h
[21]	Schneider's or Robb's	Machined aluminum culture slides with gas-permeable membranes [46]	14 h
[11]	Halocarbon oil (700)	Oil on coverslip	77 min
[35]	Schneider's + 200 μg/mL insulin, 10% FBS, and 0.6× Pen/Strep, pH ~6.9	Greiner Lumox[a] culture dish, coverslip supported by coverslip slivers, sealed with oil	6 h
[37]	Schneider's + 5 μg/mL insulin, 2.5% FBS, etc. [37]	Glass culture dishes	2.5 h
[13]	Halocarbon oil (Series 95)	Oil on coverslip	160 min
[28]	Modified Grace's + 10% FBS and 1× Pen/Strep	24 well tissue culture plates	10 h

[25]	Halocarbon oil (Voltalef 10S)	Oil on coverslip	40 min
[39]	Schneider's + 100 μg/mL insulin, 20% FBS, and 0.6× Pen/Strep, pH 6.95–7.0	Greiner Lumox[a] culture dish, coverslip supported by coverslip slivers, sealed with oil	1 h
[40]	Schneider's + 200 μg/mL insulin, 20% FBS, and 0.6× Pen/Strep, pH 6.95–7.0	Greiner Lumox[a] culture dish, coverslip supported by coverslip slivers, sealed with oil	20 min
[19]	Schneider's + 200 μg/mL insulin, 15% FBS, and 0.6× Pen/Strep, pH 6.95–7.0	Coverslip-bottomed culture dish, humidification KimWipe	>14 h
[32]	Schneider's + 200 μg/mL insulin	Slide and coverslip supported by vacuum grease	30 min
[43]	Schneider's + 200 μg/mL insulin and 10% FBS	Greiner Lumox[a] culture dish, coverslip supported by vacuum grease, sealed with oil	4 h
[26]	Halocarbon oil (Voltalef 10S) or Schneider's + 5 μg/mL insulin and 2.5% FBS	Oil on coverslip or poly-L-lysine coated imaging chamber	5 h
[34]	Schneider's + 10% FBS and 0.6× Pen/Strep	Coverslip-bottomed culture dish	60 min
[33]	Schneider's + 200 μg/mL insulin	Coverslip and slide separated by vacuum grease	60 min
[44]	Schneider's + 200 μg/mL insulin, 15% FBS, and 0.6× Pen/Strep, pH 6.95–7.0	Greiner Lumox[a] culture dish, coverslip supported by culture medium in low-melt agarose, sealed with oil	60 min
[42]	Schneider's + 200 μg/mL insulin, 15% FBS, and 0.6× Pen/Strep, pH 6.95–7.0	Greiner Lumox[a] culture dish, coverslip supported by coverslip slivers, sealed with oil	6 h
[29]	Modified Grace's + 10% FBS and 1× Pen/Strep	Coverslip-bottomed culture dish with low-melt agarose in modified Grace's	70 min
[47]	Schneider's	Coverslip-bottomed culture dish with weighted immobilization blanket	10 h

[a]Greiner Lumox culture dishes were previously known as petriPerm plates

Table 3
A chronological summary of 40 years of live-imaging efforts using the *D. melanogaster* ovary, concentrating on fluorescent marker(s) and imaging system(s) used, as well as the time interval(s) between data acquisition

References	Fluorescent marker(s) used	Live imaging system(s)	Interval
[6]	NA	No imaging attempted	NA
[20]	NA	Undescribed light microscope (no live imaging)	NA
[7]	NA (brightfield only)	Leitz microscope and Bolex H16 Reflex Camera	100 s
[8]	NA (brightfield only)	Zeiss 4FL fluorescence microscope	NA
[9]	NA	Laser ablations on Olympus BH2	NA
[23]	GFP::Exu	BioRad MRC600 laser confocal	Unspecified
[10]	Rhodamine-labeled tubulin	BioRad MRC600 confocal on Nikon DiaPhot	10 s
[30]	GFP-labeled *nanos* mRNA	Zeiss 510 Meta LSM confocal	1 min
[27]	mito-GFP, mitoTracker GreenFM	Leica DM IRE with Ultraview spinning disk	2–3 s
[21]	*CY2*-Gal4 > UAS-GFP::Moesin	BioRad MRC600 or Zeiss 510 Meta LSM confocals	3–20 min
[11]	rhodamine-labeled tubulin, Oli-Green DNA dye; Polo::GFP	Deltavision deconvolution microscope	54 s
[35]	*slbo*-, *Actin < flip out >* -, or *upd*-Gal4 > UAS-GFP::Moesin, UAS-actin::GFP, UAS-mCD8::GFP, UAS-dsRed::N; *slbo*-actin::GFP	Zeiss Axioplan2 with Axiocam MRm camera	2 min
[37]	*slbo*-Gal4 > UAS-mCD8::GFP; *Ubi*-NLS::GFP	Zeiss 510 Meta LSM confocal	5 min
[13]	*Ubi*-NLS::GFP, *Ubi*-EB1::GFP, *α4-tub*-Tau::GFP	Deltavision deconvolution microscope (Olympus IX70 with Coolsnap HQ CCD camera)	20 min
[28]	NA (brightfield only)	Zeiss Stereolumar (live) or Zeiss Axiophot with QImaging RETIGA 1300 camera (DIC)	10 min
[25]	*tubP*-Gal4 > UAS-Wcd::GFP or UAS-RFP::Wcd; H2B::RFP, Armadillo::GFP	Leica DM IRBE with Ultraview spinning disk (CSU10)	5 s

[39]	*slbo*-Gal4 > UAS-GFP::Moesin, UAS-mCD8::GFP, UAS-cherry-PA-RacQ61L, UAS-Rac-FRET	Zeiss 510 Meta LSM (photoactivation) and Zeiss 710 NLO-Meta (FRET) confocals	80 s
[40]	*Ay-Gal4* > UAS-Moesin::GFP, UAS-GFP::Paxillin, UAS-mCD8::GFP, UAS-NLS::GFP; *Ubi*-E-cadherin::GFP and *Sqh*-Sqh::mCherry	Zeiss 710 NLO-Meta confocal	10–60 s
[19]	*11A12*-Gal4 > UAS-Tubulin::GFP; Df31::GFP, Jupiter::GFP, His2Av::mRFP	Yokogawa CSU10 spinning disk confocal or Leica DMIRE2	≥10 min
[32]	*Act5c*- or *tubp*-Gal4 > UAS-PA::GFP, UAS-2xGFP; Vsg-GFP, Oda-GFP, Rpl30-GFP, Yps-GFP; His2Av::RFP, *Ubi*-GFP::Pav-KLP, Yps::mRFP	Zeiss Axiovert 200 with CARVII confocal imager and CoolSnap HQ2 camera or Zeiss 510 Meta LSM confocal (photoactivation)	90 s
[43]	*e22c*- or *GR1*-Gal4 > UAS-Indy::GFP, UAS-His2Av::mRFP, UAS-CollagenIV::GFP, UAS-my::mRFP; *Ubi*-NLS::mRFP	Leica TCS SL or Zeiss 510 Meta LSM confocals, Zeiss Axio M1	2–15 min
[26]	*mat-α4tub*-Gal4 > UASp-EB1::GFP; *Ubi*-EB1::GFP, *Ubi*-Cnn::GFP, *Ubi*-PACT::GFP, *Ubi*-Sas4::GFP, *Ubi*-Dlic::GFP	Olympus IX81 with Yokogawa CSU22 spinning disk and iXon DV855 camera	Variable
[34]	E-cadherin::GFP	Leica SP5 or Nikon A1 confocals	2.25 min
[33]	*c855a*- or *tubp*-Gal4 > UASt-PA::GFP, UAS-2xEGFP, 20XUAS-IVS-Syn21-mC3PAGFP, 10xUAS-IVS-myr::tdTomato; *Ubi*-RFP::LacI, *Ubi*-GFP::Pav-KLP	Zeiss 510 Meta LSM confocal	20 s–5 min
[44]	*slbo*-, *Act5c*-, *GR1*-, *tj*-, *e22c*-, or *mirr*-GAL4 > UAS-dsRed, UAS-mCD8::GFP, UAS-GFP, UAS-RFP, UAS-MoeABD::mCherry, UAS-UtrophinABD::GFP, UAS-Paxillin::GFP; Nrg-GFP, Indy-GFP, Vkg-GFP; His2Av::mRFP, *Sqh*-Sqh::mCherry	Zeiss 510 Meta LSM confocal, Nikon Ti-E with Yokogawa CSUX spinning disk confocal and HQ2 or Rolera em-c[2] cameras, or Nikon Ti-E with Andor iXon3 897 EM-CCD camera (TIRF)	30–60 s
[42]	*slbo*-, *upd*-, *triple*-, or *nos*-Gal4 > UAS-Rac-FRET, UAS-PA-RacT17N, UAS-dsRed, UAS-mCD8::GFP; *slbo*-Lifeact::GFP	Zeiss 510 Meta LSM (photoactivation, time-lapse) or Zeiss 710 LSM(FRET) confocals	2 min
[29]	*mat3*-, *c355*-, or *oskar*-Gal4 > UASp-GFP::Utrophin, UASp-Lifeact::mEGFP, UASp-F-tractin::tdTomato	Zeiss Axio Observer.Z1 with Zeiss 700 LSM confocal	10 min
[47]	E-cadherin::GFP	Zeiss 510 Meta LSM confocal	15 min

and Robb's medium [18], have facilitated the majority of egg-chamber culturing experiments, including experiments of the longest attempted duration (e.g., 14+ hour germarium culturing [19]), and all external, somatic follicle-cell culturing experiments have used aqueous media (e.g., dorsal-appendage epithelial tube morphogenesis and eggshell synthesis; 6–11 h [20, 21]).

The description of media that could support egg-chamber development and the advent of *in vitro* culturing methods resulted in a slew of influential discoveries (Tables 1, 2, and 3). In the germline, cytoplasmic streaming within the oocyte was first documented and imaged in egg chambers cultured in Robb's medium [7]. Using an optimized version of Robb's medium (composition described in [22]), the first GFP-reporter experiments ever performed in *Drosophila* allowed scientists to culture and live-image egg chambers expressing GFP::Exu in the germline [23]! This work, along with live imaging of egg chambers injected with rhodamine-tubulin and cultured in halocarbon oil [10], suggested a novel mechanism for establishing mRNA gradients via trafficking of ribonucleoprotein (RNP) complexes along microtubules (reviewed in [24]). Halocarbon-oil culturing also allowed the first live observations of female meiosis in the oocyte [11], the visualization of asymmetrical RNP component segregation within single stem cells [25], and the discovery that the oocyte nucleus is pushed by growing microtubules during DV axis specification [26]. Grace's medium facilitated analyses of organellar transport within the germ cells, including movement of mitochondria from the nurse cells to the oocyte [27]. Modified Grace's medium allowed characterization of prostaglandin signals that regulate actin dynamics during nurse-cell dumping [28], and this medium was used to show that not all actin-labeling tools available for live imaging are created equal [29]. Schneider's medium facilitated the first live imaging of fluorescently labeled mRNA molecules in the oocyte ([30]; reviewed in [31]), the discovery that somatic ring canals form through incomplete mitoses during follicle-cell proliferation [32], the observation that certain proteins can equilibrate between sibling follicle cells through somatic ring canals [33], and the first live imaging of follicle-cell morphogenesis (both Schneider's medium and Robb's medium were used successfully in [21]). Each of these culturing media has facilitated studies that provided important insights into the cell and molecular biology underlying egg-chamber development; nevertheless, most researchers now prefer aqueous culturing media for egg-chamber culturing and live imaging.

When selecting an aqueous medium for culturing egg chambers, important factors to consider are ease-of-use and availability, chemical definition, and reliability. Both modified Grace's medium and Schneider's medium are commercially available, but they are only partially defined because they contain yeastolate (i.e., yeast

medium extract). Robb's medium, which is chemically defined, must be assembled fresh and requires 56 separate ingredients, making it the most difficult of the aqueous media to use. Modified Grace's medium or Schneider's medium are used alone or with minimal supplementation, depending on the context, and are therefore preferable if complete knowledge of chemical composition is not essential. Of these aqueous culturing medium options, Schneider's medium is the most widely used and reliable and has become the preferred live-culturing medium for *Drosophila* egg chambers.

All efforts to study external, somatic follicle-cell events have utilized Schneider's medium (Tables 1 and 2). The culturing of late-stage egg chambers (i.e., S10+) is possible in unaltered, sterile Schneider's medium ([20, 21]) or in Schneider's medium supplemented with fetal bovine serum and, to suppress bacterial growth, penicillin streptomycin ([34]). For culturing younger egg chambers (i.e., prior to S10), however, supplementation of Schneider's is absolutely required. The addition of insulin and fetal bovine serum, and the adjustment of the pH to an optimal value of ~6.9, facilitated the first culturing of S9 egg chambers and the live imaging of border cell migration (S9) ([35]; method described in [36]). An alternative blend of Schneider's medium supplements, including insulin and fetal bovine serum, also enabled culturing during S9 ([37]; method described in [38] and reviewed in [36]). These culture conditions made possible the manipulation of collective cell movements via light-activated molecules in mid-stage egg chambers [39], the visualization of tissue elongation in late-stage egg chambers ([40]; method reviewed in [41]), and the use of an E-cadherin tension sensor to evaluate directional cues during collective cell migration [42]. The same conditions allowed visualization of egg-chamber rotations at mid-stages (S5–S9 [43]) and early stages (beginning at S1 [44]) and facilitated studies of the regulation of stem cells and germline–soma interactions in germaria [19]. In summary, Schneider's medium is the preferred vehicle for live culturing and analysis of the egg-chamber soma, and supplementation with insulin and fetal bovine serum as well as careful attention to pH are essential if the intent is to culture an egg chamber younger than S10 [45].

In addition to variations in culturing media, there have been numerous types of devices used for culturing *Drosophila* egg chambers (Table 2). The devices described below accommodate differences in microscope architecture, most importantly, by allowing imaging using both upright and inverted platforms. Machined aluminum culture chambers with gas-permeable Teflon membranes [46] have been used to live culture S10B–S14 egg chambers, to visualize the epithelial morphogenesis of the tubes that form the DA filaments of the mature eggshell [21]. Culturing devices constructed from gas-permeable plates, small droplets of Schneider's

medium, coverslips, risers made from coverslip fragments, and halocarbon oil have been used to culture S9 egg chambers ([35, 36, 39, 40]. Replacement of coverslip risers with vacuum grease [43] or 0.4% low-melt agarose pads and vacuum grease [44] facilitated culturing of smaller S1–S6 egg chambers.

Despite the success of previously described culturing devices, we sought to devise a culturing apparatus that would be logistically simpler to assemble than previous apparati, would hold the egg chamber securely but also gently against the coverslip with a flexible material, and would allow the egg chamber to contact a greater volume of media, to avoid complications from restricted gas exchange, temperature fluctuations, and evaporation-induced changes in ion concentration and pH. To achieve these goals, however, our method does require the use of an inverted microscope. As others have before us ([19, 30, 34]), we employ readily available and affordable glass-bottomed culture dishes. We then simply fill the culture dish with Schneider's medium; position the egg chambers in the center of the coverslip at the bottom of the dish; cover them with a small, flexible "blanket" made from a square of lab tissue (e.g., KimWipe); and weigh the lab wipe down with a brass washer. When positioned correctly, the washer applies indirect pressure to the egg chamber and holds it gently against the coverslip. Not only is assembly of this culturing device rapid and easy, but it also allows one to immobilize egg chambers during late-stage development, when the morphogenetic movements of the somatic follicle cells are most robust [47]. We see no reason why this device could not be readily used for culturing early or mid-stage egg chambers, as the immobilization "blanket" is flexible. If supplements to the culturing medium meant that using a large volume of medium is prohibitively expensive, the device could utilize a smaller volume of conditioned medium in a humidity chamber (*see* ref. 19). If the intended egg chambers were too small to be held down with the weighted lab wipe "blanket" alone, an agar pad could provide further support and immobilization (*see* ref. 44). Thus, our device, along with readily available Schneider's medium (supplemented if necessary) and minimal modifications, can provide an easy-to-assemble and cost-minimal tool for culturing and imaging living *Drosophila* egg chambers of any stage.

2 Materials

The materials described below are for the dissection, culturing, and live imaging of late-stage *Drosophila* egg chambers on an inverted confocal microscope. Notes, where appropriate, discuss details of, and reasoning for, specific steps in the protocol. Notes also indicate where the protocol can be altered to facilitate culturing of different egg-chamber stages or for use with an upright confocal microscope system.

2.1 Media and Solutions

1. Schneider's medium ([17])
   - For somatic and germline applications
   - Storage instructions (*see* **Note 1**).
   - Medium supplementation (*see* **Note 2**).
   - **-or-**
   - High-grade halocarbon oil (e.g., Voltalef 10S).
   - For internal somatic or germline applications.
2. 5× gelatin (nonstick) stock solution for coating glass transfer devices: 0.25 g (0.5%) Knox unflavored gelatin, 250 µL formalin (0.19% formaldehyde), 50 mL dH_2O.
   - Storage and coating instructions (*see* **Note 3**).
   - Alternative nonstick solutions (*see* **Note 4**).

2.2 Fly Stocks

1. Stocks for live culturing and imaging are numerous and depend on the needs of the user (Table 3; *see* **Note 5**).
2. E-cadherin::GFP (Fig. 2; *see* **Note 6**).
3. *CY2*-GAL4 > UAS-GFP::Moesin (*see* **Note 7**).

2.3 Dissection Station

1. Stereomicroscope with a dark stage (for contrast).
2. External, adjustable white-light illumination (*see* **Note 8**).
3. CO_2 source, pad, and blowgun for anesthetizing flies.
4. Fine brush or alternative implement for sorting flies.
5. Tissue wiper and tape for making a carcass disposal wipe; tissue wiper and dH_2O for cleaning forceps after dissection.
6. Three glass dissection dishes: One for dissecting ovaries from the fly, one for dissecting egg chambers from the ovaries, one for holding liberated egg chambers of the desired stage until ready for imaging (*see* **Note 9**).
7. Dissecting forceps (Dumont #5; *see* **Note 10**).
8. Pipette controller for egg-chamber transfer (e.g., Assistent micro-classic 558; *see* **Note 11**).
9. Glass transfer device (Pasteur pipette or capillary tube; *see* **Note 11**) pre-coated with nonstick gelatin solution (*see* **Note 3**).

2.4 Imaging Station

1. Inverted scanning confocal microscope with a stage adaptor that is compatible with glass-bottomed culture dishes (*see* **Note 12**).
2. Glass-bottomed 35-mm culture dish (MatTek) with lid (*see* **Note 13**).
3. Light-Duty Tissue Wipers for creating the egg-chamber immobilization "blanket" (*see* **Note 14**).

4. Brass washer for weighing down the immobilization "blanket" (*see* **Note 15**).
5. Dissecting forceps (Dumont #5; *see* **Note 10**).

3 Methods

The methods described below are for the dissection, culturing, and live imaging of late-stage *Drosophila* egg chambers on an inverted confocal microscope. Notes, where appropriate, discuss details of, and reasoning for, specific steps in the protocol. Notes also indicate where the protocol can be altered to facilitate culturing of different egg-chamber stages or for use with an upright confocal microscope system.

3.1 Care of Female Flies Prior to Dissection

1. To maximize egg-chamber production in the ovary, transfer 2–4-day-old female flies (5–15 individuals) into fresh fly food vials with several males and wet-yeast paste (*see* **Note 16**).
2. Incubate vials at 25 °C for ~24 h; these conditions should provide large numbers of S10B–S11 egg chambers (*see* **Note 17**). Female flies that are actively producing eggs will have swollen abdomens following this incubation.

3.2 Ovary Dissection

1. Equilibrate an aliquot of Schneider's medium to room temperature or desired culturing temperature (*see* **Note 18**). Fill the three dissecting dishes with Schneider's medium (*see* **Note 19**), placing the first dish on the dark surface directly under the stereomicroscope and the other two dishes off to one side.
2. Adjust the light source to illuminate the centered dissecting dish from the side (*see* **Note 8**) and focus the stereomicroscope on the bottom surface of the dish.
3. Use tape to anchor a tissue wiper near the nondominant hand for disposing of fly carcasses during dissection.
4. When the dissection station is fully assembled, turn the vial upside down and anesthetize flies with a CO_2 blowgun. Gently tip the flies onto a pad with actively flowing CO_2 and use a brush to separate males and any females with non-swollen abdomens from females with swollen abdomens (*see* **Note 20**). Position the females with swollen abdomens in a vertical column with their heads oriented towards the user's nondominant hand.
5. Take hold of a pair of forceps with the thumb and forefinger of each hand so that a pinching motion will close the forceps. During the dissection itself, the wrists should be placed on a steady surface (e.g., benchtop or stereomicroscope stage), and the forceps can be steadied against the middle finger and/or the side of the dissecting dish.

6. With the forceps in the nondominant hand, firmly grasp a female by the thorax and anterior end of the abdomen (this process will crush that part of the fly) and immerse the body in the first dissecting dish filled with Schneider's medium. Do not let go! With the forceps in the dominant hand held open, pierce the posterior cuticle of the abdomen with one tip (Fig. 3A–C), close the forceps, and remove the ovary pair (Fig. 3D) (*see* **Note 21**) while maintaining a hold on the body with the nondominant-hand forceps. Once the ovary pair is liberated with the dominant hand, use the nondominant hand to remove the body of the female from the dissecting dish, and discard the carcass by wiping the forceps across the nearby anchored tissue wiper. Return the nondominant-hand forceps to the medium and, if necessary, gently remove any non-ovarian tissue (e.g., gut, Malpighian tubules) from the ovaries (Fig. 3E) (*see* **Note 22**).
7. Use the dominant-hand forceps to gently transfer the ovary pair to the second dissecting dish with Schneider's medium (*see* **Note 23**).
8. When several females have been dissected (*see* **Note 24**), wipe both forceps clean, move the first dissecting dish to the side, and place the second dissection dish containing the clean ovaries directly under the stereomicroscope.

3.3 Ovariole Dissection and Individual Egg-Chamber Isolation

1. With the nondominant-hand forceps, grasp an individual ovary near the oviduct (potentially crushing some late-stage egg chambers). Holding the dominant-hand forceps open, use the tips to gently comb apart the ovarioles with a brushing motion (Fig. 3G); this process sometimes frees individual egg chambers (*see* **Note 25**). When an individual ovariole containing an egg chamber of the desired stage is visible, grasp it near the germarium with the dominant-hand forceps and gently tug it until it is free from the ovary (Fig. 3H) (*see* **Note 26**).
2. To isolate a late-stage egg chamber from the transparent muscle sheath of an individual ovariole (*see* **Note 27**), grasp the ovariole with the nondominant-hand forceps just past the anterior end of the desired egg chamber and hold it firmly against the base of the dissecting dish (Fig. 3J). Using the dominant-hand forceps, close the tips around the anterior end of the desired egg chamber so that they are just narrower than the maximum diameter of the egg chamber and then slowly and steadily draw those forceps away from the ovariole. If done correctly, there will be some initial resistance, and the egg chamber will deform as it passes through the narrow opening of the muscle sheath, but the egg chamber will immediately return to its normal shape as it pops out of the muscle sheath into the medium (Fig. 3J, K) (*see* **Note 28**).

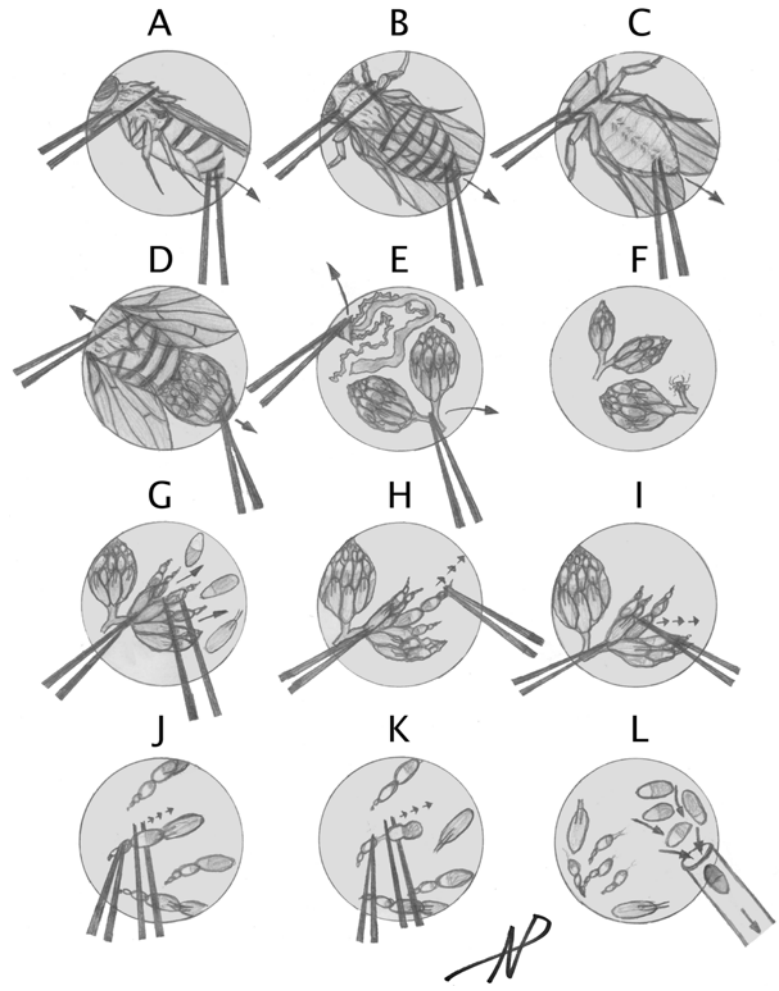

Fig. 3 Illustrated instructions for dissecting ovaries from *D. melanogaster* females, removing individual ovarioles, and isolating individual, late-stage egg chambers. In all illustrations, the dominant-hand forceps are shown on the *right*, and the nondominant-hand forceps are shown on the *left*. (**A**–**C**) illustrate three possible orientations (**A**-lateral, **B**-dorsal, **C**-ventral) for the body of the female during ovary removal and approximately where the opening in the female abdomen should be made. (**D**) illustrates the ovary pair being removed from the abdomen. When the ovaries are removed from the abdomen, the gut and Malpighian tubules are often attached, and these tissues need to be separated from the ovary pair (*top* in **E**), removed from the culturing dish, and discarded prior to any further dissection. Once isolated, the ovary pair (*bottom* in **E**) is moved to a new dissecting dish with fresh culturing medium. (**F**) illustrates how the ovaries of a very young or undernourished female might appear (*top* in **F**) or how an ovary pair might appear if one of the ovaries fails to develop (*bottom* in **F**). (**G**–**I**) illustrate the process of combing open an ovary to separate the ovarioles (**G**) and removing individual ovarioles for culturing either late-stage (**H**) or early-stage (**I**) egg chambers. (**J**, **K**) illustrate how an individual late-stage egg chamber can be removed from the muscle sheath of the ovariole by anchoring the distal end of the ovariole (nondominant forceps) and squeezing egg chambers out the proximal end with the dominant-hand forceps. This process will often deform the egg chamber as it moves out of the muscle sheath (**K**), but the egg chamber will quickly return to its normal morphology once liberated. (**L**) illustrates how egg chambers of the desired stage(s) (*right* in **L**) should be moved away from ovarioles, debris, and non-desirable egg chambers (*left* in **L**) and then moved via pipette to a glass-bottomed culture dish with fresh culturing medium

3. When a sufficient number (5–10) of late-stage egg chambers have been isolated in this manner, gently brush them into a group on one side of the dissecting dish with the dominant-hand forceps (Fig. 3L).
4. If desired, use the pipette controller and a nonstick glass pipette to transfer the group of late-stage egg chambers to the third dissecting dish with fresh Schneider's medium for observation prior to imaging (*see* **Note 29**).
5. Observe egg chambers for at least 5 min; remove and discard any egg chambers that are visibly damaged (*see* **Note 30**).

3.4 Assembly of the Culturing Chamber

1. The culturing chamber should be assembled on the stage of the inverted confocal microscope that will be used for live imaging (*see* **Note 31**).
2. Place a CLEAN, 35-mm, glass-bottomed culture dish on the stage of the confocal microscope and fill it with ~5 mL of Schneider's medium (*see* **Note 32**).
3. Using the pipette controller and a nonstick glass pipette, slowly transfer the desired egg chambers from the final dissecting dish to the center of the culture well in the bottom of the culture dish and gently group them together with the tip of the forceps (Fig. 4A) (*see* **Note 33**).
4. To make the immobilization "blanket", wet the tips of the forceps in the Schneider's medium, pinch them twice at right angles around an untouched corner of a tissue wiper to make a 1-cm X 1-cm square, and tear off the square with the forceps (*see* **Note 34**).
5. Release the immobilization "blanket" onto the surface of the Schneider's medium in the culture dish, taking care that it does not stick to the forceps; prod it until it begins to sink; carefully guide it with the forceps so that it comes to rest over the culture well and egg chambers (Fig. 4B) (*see* **Note 35**).
6. When the immobilization "blanket" is in place, grasp the clean brass washer with the forceps so that it is stable and horizontal, rinse it in the Schneider's medium in the last dissecting dish, lower it *SLOWLY* into the culture dish, and *CAREFULLY* lay it on top of the immobilization blanket so that it lies inside the culture well and against the coverslip (Fig. 4C) (*see* **Note 36**).
7. Under the microscope (widefield, 10× or 20×) confirm that the egg chambers have not been flattened (i.e., they still have a similar shape as when they were isolated from the ovarioles) and are immobile (i.e., they do not sway when the side of the culturing dish is tapped) (*see* **Note 37**). Be sure that one or preferably several egg chambers are in the desired orientation (*see* **Note 38**). If any of these criteria is not met, make any

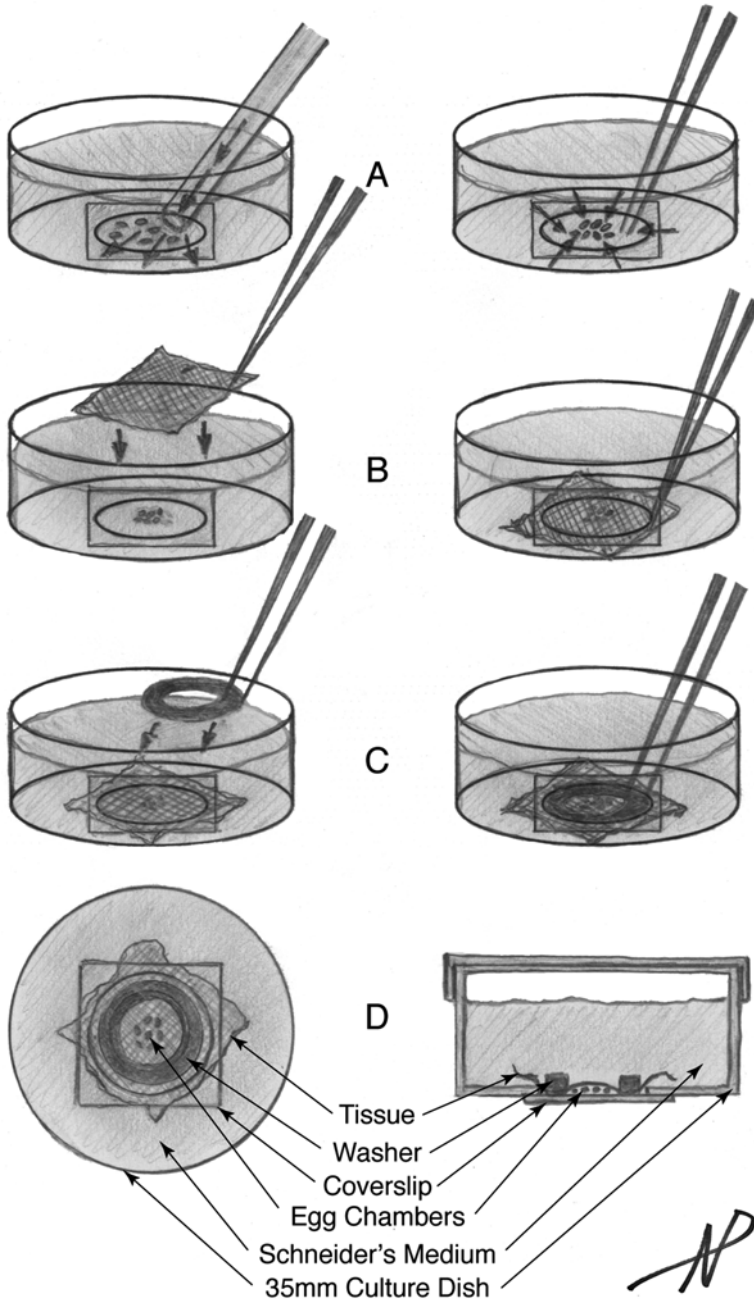

Fig. 4 Illustrated instructions for assembling an apparatus for culturing and live imaging *D. melanogaster* egg chambers. (**A**) illustrates egg chambers being dispensed from a pipette into a glass-bottomed culturing dish filled with culturing medium of the appropriate temperature and composition (*left*) and gently moved into a tight group in the center of the culturing dish (*right*). (**B**) illustrates how a small (~1 cm × 1 cm) square of tissue is first placed on the surface of the culturing medium (*left*) and then guided down to the bottom of the culturing dish so that it rests over the egg chambers (*right*). (**C**) illustrates how a brass washer, which is slightly smallerFig. 4 (Contined) than the culturing well at the bottom of the culturing dish, is first carefully lowered into the culturing medium so that it is centered over the egg chambers under the tissue (*left*) and then *VERY* gently released so that no egg chambers are crushed (*right*). (**D**) illustrates a fully-assembled culturing apparatus, viewed from the top (*left*) and from the side (*right*). Note that in the side view the culturing dish lid has been replaced, to prevent evaporation of culturing medium

necessary adjustments before proceeding to the next step (*see* **Note 39**).

8. When egg chambers are properly positioned and immobilized, place the lid on the culture dish to prevent evaporation during imaging (*see* **Note 40**). The culture chamber is now fully assembled and ready for imaging (Fig. 4D), and the forceps should be rinsed, dried, and sheathed.

3.5 Live Imaging of Egg-Chamber Development

1. Using widefield illumination (brightfield or standard fluorescence), locate a suitably oriented, late-stage egg chamber (*see* **Note 38**), and move an appropriate objective for live imaging into place (*see* **Note 41**).
2. Using the confocal microscope's time-lapse function (four dimensions: XYZT), select appropriate imaging parameters (e.g., resolution, scanning speed, *Z* parameters, laser power, gain, offset, time interval, duration) (*see* **Note 42**).
3. Upon the initiation of time-lapse imaging, monitor image acquisition during the first two time points to confirm that the microscope is imaging under the intended parameters (*see* **Note 43**).
4. If possible, monitor the sample every 30–60 min during live imaging to make sure that the egg chamber has remained in place and in focus and that development is progressing normally (Fig. 2). If necessary, pause the time-lapse imaging to make appropriate adjustments or to select a new egg chamber (*see* **Note 44**).
5. When time-lapse imaging is complete, immediately *SAVE THE EXPERIMENT* (*see* **Note 45**). The culturing chamber should then be disassembled, and the culturing dish and brass washer washed thoroughly, dried, and saved for future use.

4 Notes

1. Schneider's medium should be aliquoted under sterile conditions and can be stored at 4 °C for several months. Before use, the pH of an aliquot of Schneider's medium should be tested at room temperature to make sure that it is between 6.95 and 7.00. Aliquots should be checked for contamination by swirling prior to use, and, if the solution is cloudy, it should not be used. If salt precipitates form in the medium, the medium should be heated to 37 °C to dissolve the crystals and then brought down to the desired culturing temperature, or a new batch of medium should be used.
2. For late-stage egg-chamber culturing (>S10), we have found that no medium supplementation is necessary ([21, 47],

Fig. 2). If desired, however, 10% fetal bovine serum and 0.6× penicillin streptomycin can be added [34]. For early- or mid-stage egg-chamber culturing (<S10), a combination of 15% fetal bovine serum, 0.6× penicillin streptomycin, and 200 μg/mL insulin is optimal ([19, 36, 48].

3. The 5× gelatin (nonstick) stock solution can be stored at room temperature for several months. Prior to use, a 1× working solution should be made with dH_2O. For coating a glass Pasteur transfer pipette (or capillary tube), simply draw the 1× gelatin solution into the pipette, expel all liquid (may require several light flushings of air), allow to air dry, and rinse several times with dH_2O. Pipettes can be prepared and stored for several months or more in this manner. For more information on this method and other tips on handling tissues, see the MBL Embryology Course protocol for *Tool Making and Handling of Marine Invertebrate Embryos and Larvae* [49].
4. As alternatives for making nonstick glass transfer devices, we have successfully used 10% bovine serum albumin (BSA) or 10% normal goat serum (NGS) solutions, preparing the devices in a manner similar to that described in **Note 3**. In a rush, we have found that simply flushing a nonstick solution through a glass transfer device and rinsing with dH_2O immediately prior to use is sufficient to make the device nonstick.
5. For an additional list of strains that have been used for live imaging in egg chambers, *see* Table 3 in [48].
6. E-cadherin::GFP replaces the endogenous E-cadherin locus [50] and allows tight visualization of follicle-cell apices and DA-tube morphology (Fig. 2). Because the expression of E-cadherin::GFP is under endogenous control and is relatively lower than the expression of fluorescent reporters in many other strains that we have used for live imaging, some care is needed to avoid harming the egg chambers during imaging. Minimal laser exposure and careful tweaking of imaging parameters are required when using this strain.
7. The combination of *CY2*-GAL4 [51] and UAS-GFP::Moesin [52] can be maintained stably in a strain and allows visualization of follicle-cell outlines and actin dynamics. Since this strain illuminates all F-actin within the follicle cells, it is easier to visualize the overall movements of the follicle cells but harder to visualize the apices of the follicle cells during DA-tube morphogenesis. The expression of GFP::Moesin in this strain is very strong, requiring low-level laser power while also allowing fast image collection, making it relatively easy to image these egg chambers compared with those expressing E-cadherin::GFP.
8. We recommend using external, gooseneck, fiber-optic light sources to illuminate the glass dissection dishes from the side

and to prevent heating of the tissue. When such lighting is used in combination with a dark stage, it provides optimal illumination and contrast for dissecting ovaries, staging egg chambers, and isolating egg chambers of the desired stage.

9. We find that three dissecting dishes are optimal for egg-chamber dissection and isolation, but that at least two are required. Dissection will be easier if the dissection dishes are filled with several mL of dissecting medium so that the female can be completely submerged, but if the volume of dissecting medium is limiting, glass depression slides can be used instead. In the first dish, the transfer of dissected ovaries away from the fly carcasses, particularly the gut, is the most important step, as gut enzymes can easily disrupt egg-chamber development. In the second dissection dish, egg chambers are inevitably crushed as one separates ovarioles from each other and removes the desired egg chambers from the ovarioles. For this reason, we prefer to transfer the isolated egg chambers of the desired stage to a third dissecting dish to provide an additional rinse and for observation prior to transferring them to the culturing device.
10. We find that sharp and well-aligned dissecting forceps are essential for ovary dissection and intact egg-chamber isolation, and we prefer Dumont #5 forceps made from Dumostar alloy. Great care should be taken to protect the tips of these forceps, for even the smallest impact can blunt or bend the tips. Forceps should be rinsed with dH_2O immediately after use and stored clean and dry with a tip protector (a 20 μL pipette tip works very well for this). Prior to dissection, the forceps should be held closed and examined under the stereomicroscope from both the side and from above; the tips should align precisely from both perspectives or it will be difficult to isolate individual egg chambers without damaging them. Minimally bent or blunted tips can usually be realigned and sharpened with a wet sharpening stone.
11. We find that the Assistent-micro-classic pipette controller, when coupled with a glass, nonstick transfer device, works beautifully for transferring isolated egg chambers. The controller's thumb-controlled roller wheel enables easy, precise, one-handed operation. The controller is compatible with either Pasteur pipettes or capillary tubes, though we prefer Pasteur pipettes because they are more durable, provide a greater working volume, and are easier to coat, store, and use.
12. Our protocol is designed for imaging on an inverted scanning confocal microscope, and we have successfully used both Ziess 510 Meta and Leica SP8X confocal microscopes to this end. Spinning disk confocals allow faster image acquisition, more frequent time intervals, and lower phototoxicity, but usually

have lower resolution than scanning confocals. If only upright confocal microscopes are available, a different culturing approach using machined aluminum culture slides ([21, 46]) or gas-permeable membrane plates ([36, 48]), or a significant modification to our approach, will be required. We use adjustable stage adaptors that allow us to securely hold our 35-mm glass-bottomed culture dishes. If culturing and live imaging must be performed at a temperature other than room temperature, we use a temperature-controlled stage that is compatible with 35-mm culture dishes.

13. We use 35-mm MatTek P35G-1.5-10-C culture dishes for live culturing and imaging of late-stage egg chambers. These dishes use a No.-1.5 thickness coverslip, with a circular culturing well of 10-mm diameter. Other dish diameters, coverslip thicknesses, and culturing well diameters are also available (http://glass-bottom-dishes.com/pages/product.html). We have reused these culturing dishes several times by rinsing them thoroughly with dH_2O. Alternatively, a light detergent solution and ethanol can be used for cleaning the dishes, but all traces of detergent must be removed prior to culturing. These culture dishes require the use of an adjustable stage, or stage adaptor, and are also compatible with certain temperature-controlled stages.
14. We use VWR 82003-822 Light-Duty Tissue Wipers for creating our egg-chamber immobilization "blankets", and we find that contact with this material has no adverse effects on egg-chamber development. Alternatively, we have successfully used Kimtech Science KimWipes Delicate Task Wipers (Kimberly-Clark Professional, 34120). Since impurities in paper products might affect culture conditions, we recommend testing your brand of tissue before carrying out a critical experiment.
15. We use brass washers for weighing down our immobilization blankets because brass will not oxidize in the culture medium; oxidation could adversely affect egg-chamber viability. Because our culture dishes have a 10-mm diameter culturing well, we use a brass washer with an outer diameter of ~8 mm, so that it can lie flat against the coverslip inside the culturing well. The inner diameter of this washer is ~4 mm, a diameter that provides just enough space to cluster the egg chambers in its center without crushing them. Depending on how much compression of the tissue-immobilization "blanket" is necessary, and which glass-bottomed culture dish is selected, the outer and inner diameter of the brass washer can be altered accordingly.
16. We highly recommend the use of young, 2–4-day-old females for several reasons. First, their ovaries respond rapidly to the

presence of wet-yeast paste, which stimulates egg-chamber production. Second, a greater proportion of the ovarioles will become active at the same time, which provides greater numbers of synchronously developing egg chambers. Third, there will not be an excess of fully developed egg chambers; S14 egg chambers held within the ovary can negatively feedback on egg-chamber development, making it slower and asynchronous. Additionally, overcrowding of the vials or a lack of male flies can negatively influence the rate of egg-chamber production, so no more than 15 females should be maintained in a vial, and males should always be present. If yeast-fattened females cannot be used on a given day but can be used the following day, they should be transferred to a fresh food vial with fresh wet yeast.

17. In addition to the age of the female fly, the density of flies in a given vial, the presence of males, and the availability of good nutrition, the temperature and duration of incubation can dramatically influence the rate of egg-chamber development and the relative proportions of egg-chamber stages in a given ovary. Incubation of females for ~24 h at 25 °C usually provides large numbers of S10B–S11 egg chambers as well as some fully developed egg chambers, which provide sacrificial material for the forceps to grasp during egg-chamber isolation. Shorter incubation times at 25 °C will yield greater proportions of younger egg chambers, and longer times will yield greater proportions of older egg chambers. For experiments that utilize the GAL4-UAS system, we find that incubation at 30 °C is helpful to maximize GAL4 expression ([47, 53]). The rate of egg-chamber development is directly correlated with temperature, but incubating at temperatures higher than 30 °C will risk killing the flies. Incubating flies at temperatures lower than 25 °C will slow development proportionately, for example, 18 °C requires twice as long for egg chambers to develop. We *STRONGLY* encourage individual users to experiment with the temperature and duration of wet-yeast incubation and empirically determine the optimal incubation conditions for their experiments. This preliminary analysis will almost certainly minimize time, effort, and frustration!

18. We typically dissect flies in Schneider's medium equilibrated to 25 °C. If the flies have been raised at another temperature, such as 30 °C, and the user wishes to maintain that temperature for culturing, then the aliquot of Schneider's medium should be equilibrated to the desired temperature and poured into the dissection dishes immediately prior to dissection. Ovary dissection, egg-chamber isolation, and transfer to the culturing device should then be performed as quickly as

possible, and the culturing device should be immediately placed on a temperature-controlled microscope stage.

19. We typically fill our dissecting dishes with several mL of Schneider's medium because we find that it is easier to remove the ovaries from the female if the body of the fly is fully immersed. However, a lower volume can be used if necessary. If the user needs to use supplemented Schneider's medium for culturing and the cost of the medium is a concern, then the ovaries can be removed from the fly in non-supplemented Schneider's medium, and the ovary pair can then be transferred to a small volume of supplemented Schneider's medium in the second dissecting dish. Additionally, though we prefer to separate isolated egg chambers from ovary debris by transferring them to a third dissecting dish, a third dissecting dish is not required.
20. If the desired egg chambers for culturing are S10B–S11, as they are for our studies, the ovaries will be very large and the abdomens of the females will be swollen. In this case, females with non-swollen abdomens are not worth dissecting. If the intent is to obtain younger egg chambers and the incubation conditions have been appropriately adjusted, the ovaries may be much smaller (Fig. 2F) and the abdomens of the females may not be swollen.
21. Depending on the preference of the dissector, the female fly can be held back up, on its side, or belly up for dissection. The *Drosophila* female abdomen is divided into eight segments (A1–A8, anterior to posterior), and each of these segments is covered in a cuticular plate called a sclerite. A female's dorsal sclerites (i.e., tergites) are pigmented, relatively rigid, and have a black band at each posterior margin. A female's ventral sclerites (i.e., sternites) are nonpigmented, relatively soft, and somewhat transparent (Fig. 1). In all orientations, the goal should be to pierce the abdominal cuticle in the vicinity of A6, grab the oviduct that connects the two ovaries, and remove the ovary pair, intact, with a single motion (Fig. 3A–D). We prefer to hold the female back up and to pierce the A6 tergite with one tip of the forceps just anterior of its black band, which serves as an excellent dissection landmark. If this positioning is done correctly (Fig. 3B), the tip of the forceps will hook the "Y" of the oviduct and, when closed and pulled, will remove the posterior end of the abdomen (A6, A7, and A8), the ovary pair, and the intact gut and Malpighian tubules (Fig. 3E). If the female is held on its side, it can be harder to hook the oviduct, but the dorsal tergites can still serve as a useful dissection landmark (Fig. 3A). If the female is held belly up, the white ovaries can often be distinguished through the sternites. Although this orientation offers a better view of the ovaries

through the body of the fly, there is very little definition between the sternites to serve as a dissection landmark, and the relative softness of the sternites can resist piercing by the forceps (Fig. 3C). If, during the dissection, the oviduct separates from the posterior end of the abdomen and the ovary pair remains in the abdomen, retain the hold on the body of the fly with the nondominant-hand forceps and use the dominant-hand forceps to gently squeeze the abdomen from anterior to posterior and coax out the ovary pair. The same technique can be used if one of the ovaries separates from the oviduct and remains in the abdomen. If the abdomen separates from the thorax before the ovaries are removed, hold onto the abdomen with the dominant-hand forceps and discard the anterior of the fly on the tissue wiper. Then, either regrasp the anterior end of the abdomen with the nondominant-hand forceps and proceed as before or rotate the abdomen 180°, grasp the posterior end with the nondominant-hand forceps, and use the dominant-hand forceps to coax the ovaries out of the anterior end of the abdomen. For a useful instructional movie on removing ovaries from female flies, including what to do in these alternative situations, refer to our movie in the supplemental material of [54].

22. If the gut of a dissected fly is pierced during ovary dissection, the surrounding media will become cloudy. The user should still remove the ovaries, dip them in the cleaner medium away from the pierced gut, and transfer them to the second dissecting dish. Before dissecting any more females, the first dissecting dish should be rinsed and refilled with fresh Schneider's medium.
23. If the dissected ovary pair is still attached to the common oviduct (an ideal situation), then the dissector can grasp the oviduct with the dominant-hand forceps for transferring, as this location avoids damaging the ovaries. If an ovary has become detached from the oviduct, carefully and gently close the dominant-hand forceps around the ovary but do not close the tips all the way. The middle finger can be held against the side of the forceps to steady them and keep them from closing entirely. As the nearly closed forceps are lifted from the Schneider's medium, a small droplet of medium will remain suspended between the tips of the forceps with the ovary inside. The forceps can then be moved to the second dish and allowed to open, which will release the ovary. In this manner, even an individual egg chamber can be transferred without damaging it.
24. If females are young, well fed, and incubated for an optimal period of time, just one ovary pair can yield numerous egg chambers of a desired stage and will allow successful culturing.

To be confident that we will obtain the material we need, we usually dissect 2–3 females at a time, but this effort is not always necessary. The more ovaries that are dissected, the longer it will take to sort through the ovaries and isolate egg chambers of the desired stage. The goal of the dissector should be to dissect ovaries and isolate egg chambers as quickly and cleanly as possible, so the number of females necessary for a culturing experiment will ultimately depend on the preference and skill of the dissector and on the strain being dissected.

25. Sometimes the act of combing apart the ovarioles will liberate late-stage egg chambers on its own (Fig. 3G). While this liberation can be a fortunate situation, these egg chambers should be examined carefully to make sure the forceps have not damaged them before using them for live imaging (*see* **Note 29**).
26. If the intention is to image the germarium or very early egg chambers, do not grasp ovarioles near the germarium end. Instead, after gently combing apart the ovarioles, grasp the ovariole around a mid- or late-stage egg chamber and peel the ovariole away from the rest of the ovary (Fig. 3I).
27. Because the transparent muscle sheath of the ovariole will often continue its rhythmic contractions even after it is isolated from the ovary, and because this sheath can easily adhere to dissecting and transferring implements, we recommend the removal of individual egg chambers from the muscle sheath prior to live imaging. This removal, however, is not absolutely essential, and egg chambers can be successfully imaged while still in the transparent muscle sheath.
28. If it is unclear whether an egg chamber is still in its transparent muscle sheath, move a tip of the dominant-hand forceps in a circle around the egg chamber and see if the egg chamber catches and follows the tip. If an egg chamber ever sticks to a tip of the forceps or the dissecting dish, that is a good sign that it is still in its muscle sheath. A liberated egg chamber will not normally stick to the forceps or dissecting dish.
29. If the user opts to use only two dissecting dishes, the pipette manipulator should be used to remove all extra ovarioles, ovariole remnants, and other material from the second dissecting dish, as well as the majority, but not all, of the Schneider's medium. Never let the egg chambers dry out! Fresh Schneider's medium should then be added back to this dish, providing the desired egg chambers with an additional wash while they are held for observation.
30. Damage to egg chambers during dissection (e.g., breaking, tearing, piercing) can perturb aspects of egg-chamber development, and such damage is usually visible under widefield conditions (e.g., brightfield, fluorescence) at 10× or 20×, or even

under the stereomicroscope. The most obvious sign of egg-chamber damage is the leakage of yolk from the oocyte. While we have observed that follicle-cell morphogenesis will proceed even if all the yolk is drained from the oocyte, this situation is usually not desirable, and we typically remove the damaged egg chamber. We do not typically add dyes to our culture medium to indicate more subtle egg-chamber damage and instead rely just on visual, widefield inspection at 10× or 20×. However, incubation with a membrane marker, such as FM 4–64, or a nuclear stain, such as DAPI or Hoechst 33342, can be a useful technique for detecting subtle egg-chamber damage [48].

31. Since the culturing chamber we describe is not fixed and contains a relatively large volume of culture medium, transporting the fully assembled culturing chamber is inadvisable as it can lead to movement of the egg chambers and to egg-chamber damage.
32. We typically use ~5 mL of Schneider's medium for culturing, which fills the 35-mm culture dish ~2/3 full and leaves room to add, manipulate, and secure the egg chambers (Fig. 4). If a temperature-controlled stage is being used, the temperature of the medium should be allowed to equilibrate to the intended temperature before proceeding. If a small volume of supplemented medium is being used, the culturing dish can be made into a humidity chamber by adding a damp tissue wiper around the perimeter of the inside of the culture dish [19].
33. When transferring the egg chambers using the pipette manipulator, hold the pipette as vertically as possible to minimize contact between the walls of the pipette tip and the egg chambers. Transfer the egg chambers as quickly as possible to further minimize any contact between glass and egg chamber, but do not do so at the expense of accuracy and precision. When expelling the egg chambers into the culture dish, do so in a steady, slow motion so that the egg chambers remain in the culturing well and do not disperse throughout the culture dish (Fig. 4A). If the egg chambers need to be regrouped into the center of the culture well following transfer, make small motions in the medium with the tips of the forceps to group the egg chambers and minimize actual contact between egg chambers and forceps (Fig. 4A).
34. When making the immobilization "blanket", do not use a piece of tissue wiper that has come into contact with your hand. Wetting the forceps and pinching at the intended tear sites is not essential, but such effort greatly helps the user in making a "blanket" of the intended size. The size of the "blanket" can be altered to accommodate different sizes of culture dish and washer or to satisfy the preference of the user.

35. The immobilization "blanket" will sometimes curl and try to wrap around the tip of the forceps. If this happens, it is easier to discard that "blanket" and make a new one rather than attempt to flatten the blanket. If the user must reposition the blanket when it has sunk to the bottom of the culture dish, take care not to drag the egg chambers off to the side of the culture well, where the brass washer might crush them.
36. If the brass washer is not held horizontally, it will be very difficult to move it into place without crushing the egg chambers. It should only be released when it is just above its intended position. In order to put pressure on the immobilization "blanket" and the egg chambers, the brass washer must fit inside the culture well and lie flat against the coverslip.
37. If egg chambers are crushed and have popped, dissect new egg chambers and reassemble the culturing chamber. If the egg chambers are overly compressed and have deformed, reassemble the culturing chamber or use a different size of brass washer or culture well. To determine if the egg chambers are immobile, gently tap the side of the culture dish. Unsecured egg chambers will sway in the medium, while secured egg chambers will remain in a fixed location.
38. The optimal orientation of the egg chamber will vary depending on the goals of the user. To image DA-tube morphogenesis, the egg chambers must contact the coverslip with their dorsal or dorsolateral surfaces. These surfaces can be distinguished by the thickened placode of follicle cells that will form the DA tubes, by the initiation of apical constriction in the DA-tube cells, and by the location of the germinal vesicle (i.e., oocyte nucleus) along the dorsal midline.
39. If the egg chambers are overly compressed or unsecured, gently lift the brass washer off the immobilization "blanket", replace it, and recheck the egg chambers. Alternatively, the user can gently tug on the edges of the immobilization "blanket" with the forceps while the brass washer is in place to increase the tension on the egg chambers. If the egg chambers are in the wrong orientation, or if simply replacing the washer doesn't relieve over-compression or secure the egg chambers, remove the brass washer and immobilization "blanket", move the egg chambers around with the forceps, and reassemble the culturing chamber.
40. We find that using the culture dish lid dramatically limits evaporation of the medium, especially if the egg chambers are being cultured at a temperature higher than room temperature (*see* **Note 32**).
41. For live imaging, we have used 20× dry and 40× water or oil immersion objectives. We prefer to use at least 40× magnifica-

tion for detail, and depending on the microscope, this level of magnification does not require repositioning of the egg chamber (stage) during live imaging. Higher magnification is possible (e.g., 63×), but may require repositioning of the egg chamber during live imaging.

42. For live imaging of developing late-stage egg chambers on a scanning confocal, we typically use the following parameters: 512×512 pixel resolution, maximum or near-maximum scan speed, 3–4 line averaging, near-optimal Z-sectioning for the chosen objective, a balance of minimal laser power, minimal gain, and appropriate offset to achieve the desired level of detail, 10-min time intervals, and 10-h duration. Spinning disk confocals will usually have lower resolution than scanning confocals, but they allow for much faster Z-stack acquisition, more frequent time points and/or more recovery time between Z-stacks, and overall lower risk of phototoxicity. The imaging parameters will depend on the needs of the user, but the most important factors for minimizing phototoxicity are laser power, time interval (i.e., acquisition time vs. resting time), and duration of imaging.
43. Observing the first several time points closely is important to make sure that the egg chamber remains immobile, that the proper *Z* parameters are met, that the appropriate time interval is observed, and that the imaging software's prediction of the experimental duration is as expected.
44. Most imaging software allows the experiment to be paused, readjusted, and restarted. Alternatively, the experiment can be ended, readjustments made, a new experiment started, and the movies stitched together at a later time. Possible readjustments include changing the *Z* parameters, changing the position of the egg chamber in XY, changing exposure settings, or selecting a new egg chamber. If no development is observed after 30 min, a new egg chamber should be selected.
45. Always *SAVE THE EXPERIMENT* immediately after live imaging, especially if the imaging software does not automatically do so! Nothing is more frustrating than realizing that you have just lost an exhaustively prepared time-lapse due to user error.

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Chapter 5

Using Fluorescent Reporters to Monitor Autophagy in the Female Germline Cells in *Drosophila melanogaster*

Anne-Claire Jacomin and Ioannis P. Nezis

Abstract

Oogenesis is a fundamental biological process for the transmission of genetic information to the next generations. *Drosophila* has proven to be a valuable model for elucidating the molecular and cellular mechanisms involved in this developmental process. It has been shown that autophagy participates in the maturation of the egg chamber. Here we provide a protocol for monitoring and quantification of the autophagic process in the *Drosophila* germline cells using the fluorescent reporters mCherry-DmAtg8a and GFP-mCherry-DmAtg8a.

Key words *Drosophila*, Autophagy, Germline cells, GAL4/UAS system, mCherry-DmAtg8a, GFP-mCherry-DmAtg8a

1 Introduction

Oogenesis in *Drosophila* is a preeminent model system for studying various aspects of molecular, cellular and developmental biology. The *Drosophila* female has a pair of ovaries, which continuously produce easily dissected egg chambers highly suitable for imaging. Each ovary harbors 16 ovarioles held together by an enveloping epithelial sheath. The ovariole is the structural unit of the ovary in insects [1–3]. It consists of a tube in which egg chambers form at the anterior end and complete their development as they reach the posterior end, creating an array of developmentally ordered egg chambers [1–3]. The most anterior part of the ovariole comprises the germarium that houses the germline stem cells (GSCs) that divide to produce the egg chamber composed of 16 germline cells (15 nurse cells and 1 oocyte), surrounded by somatic follicle cells. The development of the egg chamber has been divided into a series of 14 stages. The approximate developmental stage of an egg chamber can be readily determined by morphological features [1–3].

Autophagy is a well-conserved cellular catabolic process in eukaryotes. It consists of the engulfment of cytoplasmic components

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into double-membraned autophagosomes that ultimately fuse with lysosomes, forming an acidic structure, the autolysosome, followed by content breakdown. Autophagy allows for the degradation of various cellular components such as protein aggregates, superfluous or defective organelles or intracellular pathogens [4]. LC3, the mammalian homologue of *Drosophila* Atg8a protein, is a major component of the autophagic machinery, crucial for the closure and maturation of the autophagosomes [5]. Atg8a/LC3 constitutes a widely used marker for autophagy induction and progression. Autophagy has been shown to participate in the cell death process during oogenesis [6–8].

In order to study the presence of autophagy in the germline cells during oogenesis in *Drosophila*, we created several *Drosophila* transgenic lines which express DmAtg8a protein fused to an mCherry tag [6] or the GFP-mCherry double tag [7]. While the mCherry-DmAtg8a reporter is used to detect the presence of both autophagosomes and autolysosomes, the double-tagged GFP-mCherry-DmAtg8a reporter constitutes a pH-sensitive sensor that is used to monitor both autophagy induction and flux progression through the discrimination between non-acidic (autophagosomes) and acidic (autolysosomes). Indeed, this fusion protein displays both green and red fluorescence (resulting in yellow fluorescence in the merged picture) in the autophagosomes and only red fluorescence in autolysosomes due to quenching of GFP fluorescence in these acidic structures [7, 9].

In this chapter, we describe a procedure that uses fluorescently tagged DmAtg8a reporters to investigate the induction of autophagy in the germline cells during oogenesis in *Drosophila*.

2 Materials

2.1 Drosophila Stocks and Maintenance

Flies are raised on a standard cornmeal/yeast diet and are kept at 25 °C and 70% humidity with a 12 h light-dark cycle.

The fly stocks are available from the Bloomington Drosophila Stock Center (Indiana University):

1. The germline-specific driver *nos::VP16-GAL4* (#4937) (*see* **Note 1**).
2. The transgene *UASp-mCherry-Atg8a* (#37750) [6].
3. The transgene *UASp-GFP-mCherry-Atg8a* (#37749) [7].

2.2 Dissection and Staining of the Egg Chambers

1. 4% methanol-free paraformaldehyde in PBS prepared from a 16% solution stock. The working solution (4%) can be stored at −20 °C for a few months.
2. Alexa Fluor 488-conjugated phalloidin.
3. Fly food: 1 L H_2O, 42 g inactive dry yeast, 60 g yellow cornmeal, 130 g sucrose, 5.5 g agar, 6 mL propionic acid and 15 mL Nipagin 10%.

4. Hoechst dissolved in distilled water to obtain a 1 mM (1000×) stock solution, which can be stored in a dark vial at 4 °C.
5. Phosphate-buffered saline (PBS) (1× PBS pH 7.4).
6. PBT: 1× PBS, 0.1% Triton X-100.
7. Wet yeast paste is prepared freshly by dissolving 5 g of dry active yeast in 15 mL of water.
8. Mounting medium: 70% glycerol, 2% w/v propyl gallate, 1× PBS. Can be stored for 6 months in a dark vial at 4 °C.

2.3 Equipment

1. Diffuser pad for CO_2 anesthesia.
2. Stereomicroscope.
3. Forceps and brush.
4. Vial with yeast paste-enriched fly food and vial with Whatman filter disks (Fig. 1).
5. Deep-well glass slide (Fig. 1).
6. Fine forceps (style Dumont #5) and tungsten needle and handle (Fig. 1).
7. Microscope slides and coverslips 22 × 22 mm (Fig. 1).
8. Confocal microscope, lens 40× or 63×.

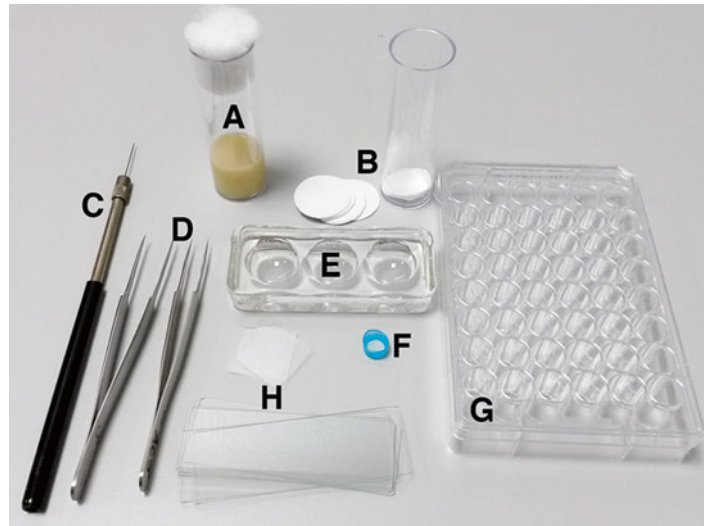

Fig. 1 Dissection tools for the isolation of the egg chambers. The figure illustrates the essential tools for the dissection, staining and mounting of the ovaries: fly tubes with fresh medium enriched with yeast paste (*A*) or with disks of Whatman paper soaked in water (*B*), tungsten needle on its handle (*C*) and fine forceps (*D*) for manipulating the flies and isolate the egg chambers in a deep-well glass slide (*E*). The isolated egg chambers are placed in a small basket (*F*) used for the various steps of the staining in a 48-well plate (*G*). Mounting is performed on microscopy glass slides and covered with cover slips 22 × 22 mm (*H*)

3 Methods

3.1 Preparation of the Flies and Induction of Autophagy by Starvation

1. Cross the UAS transgenic line of choice with the appropriate GAL4 driver (Fig. 2).
2. Parents are transferred into new vials every 2–3 days at 25 °C, and the progeny is collected 10–12 days after the transfer.
3. To obtain the ovarioles containing egg chambers at various developmental stages, 10–20 2–5-day-old male and female progenies are placed on fresh fly food supplemented with wet yeast paste and transferred every day for 2–4 days prior to dissection (*see* **Note 2**).
4. To induce autophagy, place two Whatman filter disks soaked in water at the bottom of an empty vial. Transfer the flies from the yeast-enriched tube to the vial containing the water-soaked disks. Restrict the flies' diet for 16–48 h (*see* **Notes 3** and **4**).

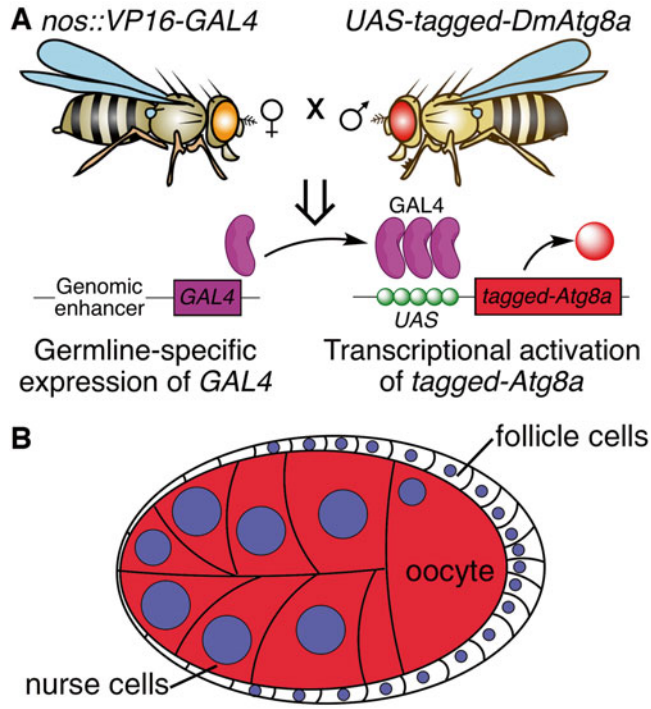

Fig. 2 Targeted expression of tagged-DmAtg8a in the germline cells of *Drosophila melanogaster*. (**A**) Virgin females *nos::VP16-GAL4* are crossed with males *UAS-tagged-DmAtg8a*, leading to F1 females constitutively expressing the *UAS-tagged-DmAtg8a* transgene in the germline cells. (**B**) Schematic representation of an egg chamber at the stage 9 of its development. The germline cells (nurse cells and oocyte) are colored in *red*. Nuclei in both germline and somatic cells are colored in *blue*

3.2 Ovary Dissection and Isolation of the Egg Chambers

1. Anesthetize the flies on a CO_2 pad.
2. Place one female in 1× PBS in a deep-well glass slide.
3. Grab the fly between the abdomen and the thorax with one set of forceps and gently squeeze the abdomen with a second pair of forceps in an anterior-posterior movement to release the ovaries (*see* **Notes 5** and **6**).
4. Isolate the ovaries from the debris and other tissues and separate the ovarioles from each other with a tungsten needle or very fine forceps.
5. Remove the muscle sheath (*see* **Note 7**).
6. Transfer the isolated egg chambers into a basket in a 48-well plate using a pipette tip that has been previously rinsed in PBT to prevent the egg chambers from sticking to the tip (*see* **Note 8**).

3.3 Fixation and Staining (See Note 9)

1. Rinse twice the egg chambers containing basket in 1× PBS to remove any trace of Triton X-100.
2. Fix the isolated egg chambers in 4% paraformaldehyde for 45 min at room temperature.
3. Rinse three times with 1× PBS (5 min each).
4. Transfer the basket into a well containing the Alexa Fluor 488 phalloidin solution and incubate for 2 h at room temperature with gentle agitation (*see* **Note 10**).
5. Rinse three times with 1× PBS (5 min each).
6. Transfer the basket into a well containing the Hoechst solution (1× dilution in PBS) and incubate for 15 min at room temperature with gentle agitation.
7. Rinse twice with 1× PBS (5 min each).
8. Place the basket into a well containing the mounting medium and gently aspirate the egg chambers and transfer them to a clean microscope slide (*see* **Note 11**).
9. Gently add a coverslip and seal with transparent nail polish.
10. Observe under a confocal microscope (*see* **Note 12**).

3.4 Image Analysis Using ImageJ/ Fiji

3.4.1 Defining the Number and Size of the mCherry-Atg8a Dots per Egg Chamber

To analyse and quantify the accumulation of autophagosomes in the egg chambers, we developed "AtgCOUNTER," a semi-automated macro based upon ImageJ/Fiji (http://rbsweb.nih.gov/ij/) programming language [10]. This macro is semi-automated because (1) the operator uses only one ImageJ tool (*Threshold*) and (2) the action of the operator-independent ImageJ/Fiji JavaScript. The macro "AtgCOUNTER" permits to measure specific mCherry-Atg8a dots while excluding background pixels and potential nuclear mCherry-Atg8a from the analysis (*see* **Note 13**):

1. Prior to running this macro, the operator must assign the correct scale (pixels/μm) of the images being analyzed using the "Set Scale" command found in the "Analyze" pulldown menu.
2. Start the macro from the "Plugins" pulldown menu. The macro performs the following commands:
   (a) The macro splits the image into individual greyscale channels (i.e., red, green, and blue); it closes the green channel and renames the red and blue channel as "Atg8a" and "Nuclei," respectively (*see* **Note 14**).
   (b) The macro selects the "Nuclei" channel and allows the user to set manually a specific range of pixel intensities using the "Threshold" function under the "Image" pulldown menu, "Adjust" submenu (*see* **Note 15**).
   (c) Then the macro selects the "Atg8a" channels and allows the user to adjust manually the pixel threshold as exposed in the previous point in order to retain only the regions corresponding to the autophagosomes.
   (d) After removing the remaining noise (via the "Despeckle" function under the "Process" pulldown menu, "Noise" submenu), the macro subtracts the "Nuclei" channel to the "Atg8a" channel in order to remove any nuclear mCherry-Atg8a staining from the analysis.
   (e) Once the two thresholded pictures have been subtracted, the macro operates the "Analyze Particles" algorithm to record mCherry-Atg8a dots number and area. The results are displayed in the "Summary" and "Results" windows. The "Summary" window shows the mean for each parameter per picture, while the "Results" window displays the detailed information for every single mCherry-Atg8a dot detected.
3. The results can easily be transferred manually by the operator to an Excel or GraphPad Prism file by using the copy and paste function in ImageJ/Fiji and used for statistical analysis (Fig. 3).

   The "AtgCOUNTER" macro is available upon request.

Fig.3 (Contined) each condition from the values extracted by the macro. The number of dots increases drastically in the abnormal egg chambers after starvation compared to the non-abnormal ones or fed condition. (**E**) Repartition of the autophagosomes according to their size for each condition. While the dots are mostly smaller than 5 px in the egg chambers from fed animals, the proportion of this class gradually decreases after starvation in favor of larger autophagosomes (classes 10–20 and >20 px)

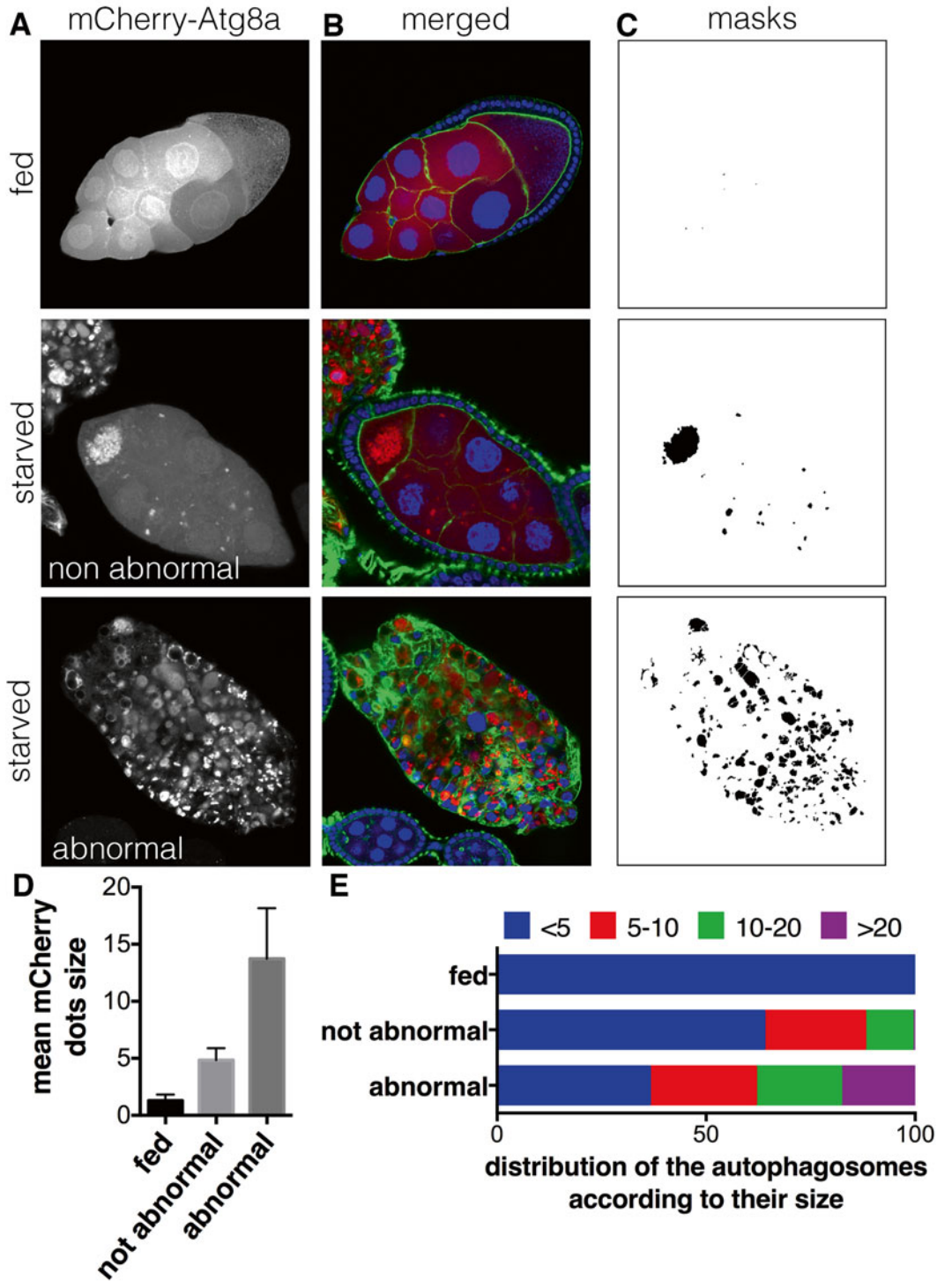

Fig. 3 Investigating the induction of autophagy using mCherry-DmAtg8a. (**A** and **B**) Greyscale images for the mCherry channel (**A**) and merged (**B**) of representative pictures of egg chambers from fed (*upper panels*) or starved (not abnormal, *middle panels*; abnormal, *lower panels*) *nos::VP16>mCherry-DmAtg8a* females. mCherry-DmAtg8a (*red*), actin (*green*), and nuclei (*blue*). (**C**) Masks obtained from the macro "AtgCOUNTER." Each *black dot* corresponds to an identified mCherry-DmAtg8a autophagic vesicle. (**D**) Quantification of the mean size of the mCherry-DmAtg8a dots for

3.4.2 Assessing the Autophagic Flux with the GFP-mCherry-DmAtg8a Construct

The induction of autophagy results in the accumulation of autophagosomes that mature to eventually fuse with the lysosomes to form the acidic autolysosomes. The expression of the double-tagged GFP-mCherry-DmAtg8a is a useful tool to make the distinction between those two autophagic vesicles. The ratio between yellow (autophagosomes) and red only (autolysosomes) can be quantitated with ImageJ/Fiji using a colocalisation plug-in. The procedure is detailed hereafter;

1. Open the pictures from the double-tagged DmAtg8a (GFP-mCherry-DmAtg8a) in the ImageJ/Fiji software.
2. Run the JACoP plug-in (http://rsb.info.nih.gov/ij/plugins/track/jacop.html) [11] from the "Plugins" pulldown menu (*see* **Note 16**).

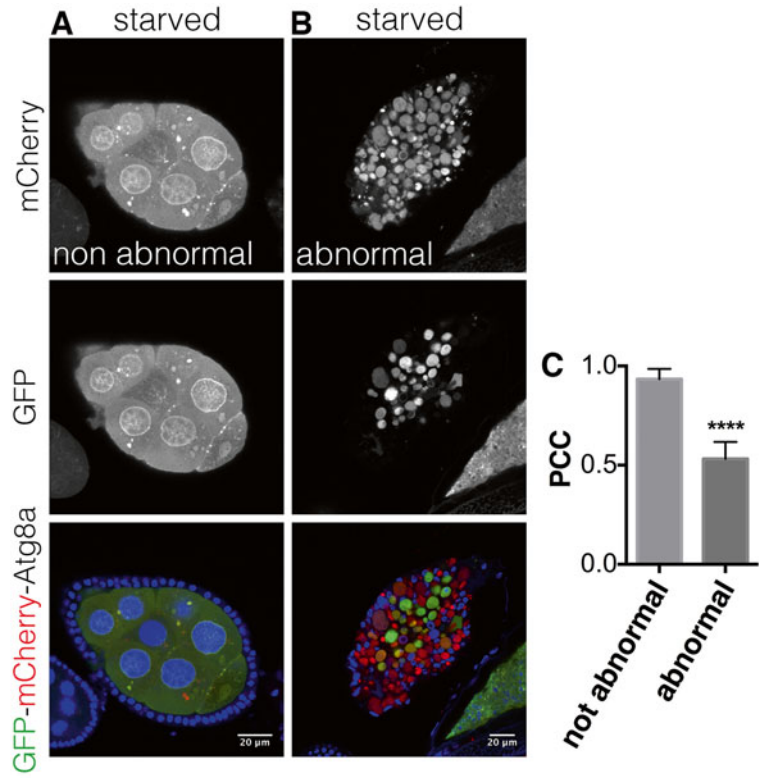

Fig. 4 Analysis of the flux in egg chambers from starved animal using the GFP-mCherry-DmAtg8a construct. Egg chambers from starved females expressing the double-tagged GFP-mCherry-Atg8a show an accumulation of autophagosomes (*yellow dots*) in non-abnormal egg chambers (**A**), while the abnormal egg chambers show an accumulation of autolysosomes (*red only dots*) (**B**). The quantitative evaluation of the colocalisation between the *green* and *red dots* is done using ImageJ/Fiji and is represented by the Pearson's correlation coefficient (PCC) (**C**)

3. Use the Pearson's correlation coefficient (original, nonthreshold) for assessing the colocalisation between the green and red staining. A ratio closes to "1" means a complete colocalisation; a decrease in the value indicates a diminution of the colocalisation between the green and red channels (Fig. 4).

4 Notes

1. Alternatively, the maternal triple driver (MTD)-GAL4 (Bloomington #31777) can be used to express GAL4-responsive UAS transgenes uniformly in the germarium and throughout oogenesis.
2. Newly hatched or very young females will have mainly previtellogenic egg chambers; therefore, it is best to dissect 3–5-days old females.
3. The duration of nutrient deprivation sufficient to induce autophagy in the germline can vary depending on the genetic background of the fly strains. As such, the time of starvation will have to be experimentally determined.
4. In order to avoid the dehydration of the flies, ensure that they are regularly supplied with water.
5. To facilitate the release of the ovaries, the posterior extremity of the abdomen (external genitalia) can be cut with a pair of micro-scissors or a set of forceps.
6. Be careful not to grab the fly abdomen directly, to avoid damaging the ovaries.
7. A good isolation of the ovarioles is a prerequisite to ensure uniform fixation and staining.
8. The baskets are prepared by carefully cutting the edge of a cell-strainer cap with a scalpel blade.
9. Due to the expression of fluorescent tagged-DmAtg8a, all incubation steps from the fixation must be performed away from light to avoid bleaching the samples.
10. If an actin staining is not required, after the fixation, move directly to the **step 5**.
11. Alternatively, if the volume of mounting medium is too important, it is possible to transfer the mounting medium and egg chambers in a 1.5 mL sample tube and centrifuge at low speed a couple of seconds before aspirate the excess of mounting medium. The egg chambers—at the bottom of the tube—can then be collected and transferred to a microscope slide.
12. If the slides are not observed immediately, they can be stored at 4 °C in a dark box. Slides conservation without fading of the fluorescent markers and staining can go up to a couple of months.

13. The macro can also be used for GFP-DmAtg8a expression.
14. This macro has been designed for the analysis of three channels pictures. The first and the last channels must be the autophagosomes and nuclei staining, respectively.
15. Consistency in the application of thresholding across the pictures is mandatory.
16. Other colocalisation analysis plug-ins are available and give the same results such as Coloc2 or intensity correlation analysis [12].

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Chapter 6

Analysis of Phagocytosis in the *Drosophila* Ovary

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Abstract

Programmed cell death (PCD) is essential for health and development. Generally, the last step of PCD is clearance, or engulfment, by phagocytes. Engulfment can be broken down into five basic steps: attraction of the phagocyte, recognition of the dying cell, internalization, phagosome maturation, and acidification of the engulfed material. The *Drosophila melanogaster* ovary serves as an excellent model to study diverse types of PCD and engulfment by epithelial cells. Here, we describe several methods to detect and analyze multiple steps of engulfment in the *Drosophila* ovary: recognition, vesicle uptake, phagosome maturation, and acidification. Annexin V detects phosphatidylserine, which is flipped to the outer leaflet of the plasma membrane of apoptotic cells, serving as an "eat me" signal. Several germline markers including *tral-GFP*, Orb, and cleaved Dcp-1 can all be used to label the germline and visualize its uptake into engulfing follicle cells. *Drosophila* strains expressing GFP and mCherry protein fusions can enable a detailed analysis of phagosome maturation. LysoTracker labels highly acidified compartments, marking phagolysosomes. Together these labels can be used to mark the progression of engulfment in *Drosophila* follicle cells.

Key words *Drosophila*, Ovary, Apoptosis, Phagocytosis, Corpse processing, Phagosome maturation, Lysosome, Acidification, Engulfment, Oogenesis

1 Introduction

Apoptosis is the most well-characterized type of programmed cell death, and it is characterized by caspase activation, DNA condensation and fragmentation, membrane blebbing, cell shrinkage, and the formation of apoptotic bodies [1]. In *Drosophila*, the genes that initiate apoptosis include *reaper*, *head involution*, and *grim* (the RHG genes or *H99* region) [2, 3]. RHG proteins inhibit anti-apoptotic death-associated IAP1 (DIAP1) via ubiquitylation to target it for degradation. In the absence of DIAP1, the initiator caspase (cysteine aspartyl protease) Dronc is relieved to kick-start the apoptotic program. Caspase activation is a key process that differentiates apoptosis from other programmed cell death modalities. Caspases are present in nearly all cells as an inactive zymogen. Initiator caspases are part of the apoptosome that activates effector

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caspases, while effector caspases cleave substrates to allow the major cellular and DNA changes that define apoptosis [4]. Interface contact between the CARD domains of Dronc and Dark are required for Dark-mediated Dronc activation [5]. Upon activation, Dronc's caspase domain dissociates from Dark to cleave effector caspase substrates Dcp-1 and Drice. Effector caspases in turn cleave substrates to enhance or reduce their activity. One substrate of effector caspases is ICAD (inhibitor of active caspase DNAse). Once ICAD is cleaved, dCAD is relieved and cleaves DNA between nucleosomes [6]. Effector caspases also cleave proteasome subunits to decrease proteasomal activity [7] and cytoskeletal actin and lamins to dismantle the cellular architecture which facilitates uptake by phagocytes [8]. To date, over 500 caspase substrates have been identified [9].

Once cells begin to undergo apoptosis, they elicit multiple signaling cues to recruit phagocytes. Three steps control apoptotic cell recognition by phagocytes: the release of "find me" signals, the presentation of "eat me" signals, and the removal of "don't eat me" signals [10]. "Find me" signals are chemoattractant signals that allow the phagocyte to migrate to the vicinity of the apoptotic cell. Some examples include ATP and UTP [10]. "Don't eat me" signals have an inhibitory role, whereby engulfment by phagocytes is prevented. Healthy cells have "don't eat me" signals so that phagocytes do not engulf them inappropriately. Some examples of "don't eat me" signals include CD31, CD46, and CD47 [11]. "Eat me" signals are ligands that are induced or trafficked to the surface of apoptotic cells. Engulfment receptors on phagocytes recognize and bind either directly to the apoptotic "eat me" signal or through bridging molecules that bind the "eat me" signal. Some examples of "eat me" signals include phosphatidylserine and calreticulin [12]. "Eat me" signals are recognized by many different receptors in mammals. In *Drosophila*, the best-characterized recognition receptors are Draper, Croquemort, and integrins [13–15].

Phosphatidylserine exposure is the best documented and evolutionarily conserved "eat me" signal reported in humans, *Drosophila*, and *C. elegans* [16]. Phosphatidylserine is a plasma membrane aminophospholipid that is maintained on the inner leaflet of healthy cells through aminophospholipid translocase (flippase) activity [17]. When a cell undergoes apoptosis, aminophospholipid translocase is inactivated, and a scramblase is activated to induce phosphatidylserine and phosphatidylethanolamine exposure to the outer leaflet (and concurrent sphingomyelin and phosphatidylcholine internalization to the inner leaflet) in an ATP-independent manner [18].

Following apoptotic cell recognition, the interaction between the "eat me" signals and engulfment receptors allows for major cellular changes within the phagocyte to facilitate uptake and degradation of the dying cell. After initial uptake, the internalized

phagosome matures and is degraded through a process called corpse processing. The GTPases Rab5 and Rab7 are required for phagosome maturation in *C. elegans*, *Drosophila*, and mammals [19–21]. Additionally, lipid composition changes have recently been shown to play an important role in corpse processing [22]. Specifically, phosphatidylinositol is phosphorylated and dephosphorylated to promote corpse processing [23]. Phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) labels the unsealed phagosomes, while newly sealed phagosomes are labeled with PI(3)P [22]. The matured phagosomes then fuse with lysosomes and mix their contents, becoming acidified and degrading the engulfed material [24–27].

Apoptotic cells can be removed by "professional" phagocytes, such as macrophages, or "nonprofessional" phagocytes, such as epithelial cells, which typically carry out other functions. The *Drosophila* ovary serves as an excellent model for investigating engulfment by nonprofessional phagocytes. Few hemocytes circulate within the ovary, and engulfment of apoptotic debris is carried out by the epithelial follicle cells. Cell death in the ovary is also inducible and reproducible, and egg chambers can be easily isolated at different steps of cell death and engulfment.

Cell death in the ovary occurs at three distinct time points: the germarium, mid-oogenesis, and late oogenesis. However, engulfment has been studied primarily during mid-oogenesis, and we focus on that time point in this chapter. During mid-oogenesis, the nurse cells die through apoptotic and autophagic mechanisms [28–30], and Dcp-1, an effector caspase, is required for nurse cell death. *Dcp-1* loss of function results in an "undead" phenotype, whereby follicle cells prematurely die and nurse cells remain intact in the presence of a death stimulus [28]. In wild-type egg chambers, the surrounding follicle cells enlarge four- to five-fold as they engulf the dying germline material, undergoing significant changes [31–34]. We have recently uncovered several molecular changes required for engulfment, including apical enrichment of the engulfment receptors Draper and integrins and activation of the JNK pathway [32, 33]. To visualize engulfment in the ovary, we and others have shown that an antibody raised against the active caspase Dcp-1 marks specifically the dying germline and the subsequent engulfed vesicles [33, 35, 36]. We have also found that Dcp-1-positive vesicles co-localize with the small GTPases Rab5 and Rab7 [36].

In this chapter, we describe methods to investigate each step of engulfment and corpse processing including the detection of "eat me" signals (Annexin V), general material uptake (cleaved Dcp-1), recognition and internalization (engulfment receptors), phagosome maturation (Rab-GFP markers and PI(3)P-mCherry lipid marker), and acidification (LysoTracker).

2 Materials

2.1 General Supplies and Reagents

1. General fly supplies: cornmeal molasses fly food, CO_2 pads, 25 °C incubator, etc.
2. Freshly made yeast paste: Mix granular yeast with dH_2O until it has a smooth consistency.
3. Apple juice agar vials for starvation: Autoclave 90 g of agar and 3 L of H_2O for 50 min. Cool to 60 °C before adding mixture of 1 L apple juice and 100 g sucrose to the autoclaved agar; mix well using a magnetic stir bar. Mix in 60 ml of 10 % *p*-hydroxy benzoic acid methyl ester (Tegosept), dissolved in ethanol. Store apple juice agar in 250 ml capped bottles at 4 °C. Melt apple juice agar solution using a microwave oven and pour into empty fly vials. Once apple juice agar has solidified, plug vials with cotton balls and store at 4 °C.
4. Fine forceps (Dumont #5), tungsten needles (Carolina Biological), and glass spot plates for dissection.
5. Glass Pasteur pipettes and bulbs.
6. Plastic fine-tipped transfer pipettes.
7. 1.5 ml microcentrifuge tubes.
8. Grace's Insect Medium, stored at 4 °C.
9. 1× Drosophila Ringer's (DR): 130 mM NaCl, 4.7 mM KCl, 1.9 mM $CaCl_2$, 10 mM HEPES, pH 6.9. Make up as a 10× solution and room temperature. Dilute to 1× for use in protocols and store at 4 °C.
10. 1× Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4. Make up as a 10× solution and store at room temperature. Dilute to 1× for use in protocols and store at 4 °C.
11. 16 % Paraformaldehyde (EM grade). Must be used within 1 week of opening.
12. Heptane.
13. 1× PBT (1× PBS + 0.1 % Triton X).
14. PBANG (1× PBT + 0.5 % Bovine Serum Albumin + 5 % Normal Goat Serum).
15. VectaShield mounting medium with DAPI, 1.5 g/ml (Vector Laboratories).
16. Rotator for use during incubation in microcentrifuge tubes.
17. Glass microscope slides and cover slips (22 × 50 mm, 0.16–0.19 mm thickness).
18. Nail polish.

19. Fluorescence or confocal microscope equipped with fluorescein (FITC), rhodamine, cyanine-5 (Cy5), ultraviolet (UV) filters, and a camera.
20. Platform shaker.

2.2 Fly Strains

Many different fly strains can be obtained from the Bloomington *Drosophila* Stock Center at Indiana University (http://flystocks.bio.indiana.edu/), including multiple GFP, RFP, or YFP-tagged Rab transgenes as well as other GFP fusions that are useful for studying cell death and engulfment. The PI(3)P-mCherry lines were a gift from the Kiger lab. *See* ref. 36 for a list of all publicly available Rab transgenes and ref. 37 for a list of lines helpful for studying oogenesis. The lines shown in this chapter are listed below:

1. Control (w^{1118}). Commonly used as a background strain for many transgenic lines.
2. Follicle cell GAL4 lines [37]:
   (a) *GR1-GAL4* [38] is expressed early in oogenesis with high levels of expression in mid-oogenesis, making it useful for driving RNAi or GFP [32].
   (b) *GR1-GAL4, G00089/TM6B* [32, 33, 36]. *G00089* is a GFP protein trap within the *trailer hitch* (*tral*) gene and specifically marks the germline cytoplasm.
3. *UAS-2XFYVE:mCherry*. FYVE is a protein domain that targets proteins to areas with phosphatidylinositol 3-phosphate (PI(3)P) composition. Kindly provided by Amy Kiger [39].
4. *w[*]; P{w[+mC] = UAS-Rab7.GFP}3*. Rab7 is a small GTPase that associates with late endosomes and is required for phagosome maturation and lysosome fusion [40].
5. *draper* (*draper*$^{\Delta 5}$, [14]). Loss of *draper* results in engulfment-defective egg chambers [32, 33].
6. *Dcp-1*$^{Prev-1}$. Imprecise excision of a *P*-element in *Dcp-1* results in an "undead" phenotype [28].

2.3 Annexin V Materials

1. 5× Annexin-binding buffer (ABB). 50 mM HEPES, 700 mM NaCl, 12.5 mM $CaCl_2$, pH 7.4. Make fresh 1× for use in protocol.
2. Annexin V Alexa Fluor 555 conjugate (ThermoFisher Scientific).

2.4 Antibody Staining

1. Recommended primary antibodies (Table 1):
   (a) Rabbit-anti-cleaved *Drosophila* Dcp-1 (Asp216) antibody (Cell Signaling Technology). Use at a dilution of 1:100.
   (b) Mouse-anti-Orb antibody (Developmental Studies Hybridoma Bank, DSHB, 6H4 and 4H8, [41]). Use in combination, both at a dilution of 1:100.

Table 1
Useful antibodies for studying cell death and phagocytosis

Recognizes	Antibody	Commercial source	Dilution	References
Germline	Cleaved Dcp-1[a]	Cell signaling	1:100	[33, 35, 36]
	Orb (rabbit)[a]		1:500	[42]
	Orb (mouse)	DSHB	1:100 each	[41, 52–55]
Membrane	Discs large[a]	DSHB (4F3)	1:100	[32, 33, 36, 43, 57–60]
	DCAD2	DSHB	1:50	[57, 60–62]
Apical surface	Draper[a]	DSHB (5D14)	1:50	[32, 33, 63]
	Integrin βPS[a]	DSHB (CF.6G11)	1:10	[33, 64–68]
	Integrin αPS3		1:500	[33, 69]
	aPKC (PKCζ)	Santa Cruz	1:1000	[33, 57, 58, 60]
	Crumbs	DSHB	1:25	[33, 58, 62]

[a]Antibodies shown in this chapter

(c) Rabbit-anti-Orb antibody [42]. Use at a dilution of 1:500.

(d) Mouse-anti-Discs large (Dlg) antibody (DSHB, 4F3 [43]). Use at a dilution of 1:100.

(e) Mouse-anti-Draper (Drpr) antibody (DSHB, 5D14 [14]). Use at a dilution of 1:50.

(f) Mouse-anti-βPS antibody (DSHB, CF.6G11 [44]). Use at a dilution of 1:10.

2. Appropriate secondary antibodies, such as:

(a) Goat-anti-mouse 647 (Jackson ImmunoResearch). Use at a dilution of 1:100.

(b) Goat-anti-mouse Cy3 or goat-anti-rabbit Cy3 (Jackson ImmunoResearch). Use at a dilution of 1:100.

(c) Goat-anti-rabbit Alexa Fluor 488 (Invitrogen). Use at a dilution of 1:200.

2.5 Indicators

1. LysoTracker Red DND-99 (Invitrogen).

3 Methods

3.1 Sample Preparation

At the time of dissection, flies should be 3–9 days old so that their ovaries are fully developed. Before dissection, adult flies should be conditioned on food vials supplemented with fresh yeast paste for 1.5–2 days, followed by starvation on apple juice agar vials for 16–20 h to induce cell death in mid-oogenesis. Optimally, each vial should contain roughly equal numbers of males and females and should not be crowded. For a video on *Drosophila* ovary dissections, *see* ref. 45.

3.1.1 Ovary Dissection

1. Anesthetize adult flies using CO_2 or ice (*see* **Note 1**).
2. Grab one female fly using a pair of forceps and submerge it completely in Grace's Insect Medium (unless otherwise noted, *see* **Note 2**) within a glass spot well.
3. Using the other pair of forceps, pin down the female by the abdomen, ventral side facing up.
4. Using the first pair of forceps, make a small hole near the base of the abdomen and remove the ovaries (*see* **Note 3**).
5. Move the newly dissected ovaries to a clean well containing Grace's Insect Medium.
6. Repeat **steps 2–5** for the remaining female flies, until seven to ten flies have been dissected. To reduce degradation of tissue, dissections should be kept to 20 min or less before moving to the next step.
7. Using a glass Pasteur pipette, move all newly dissected ovaries to a 1.5 ml microcentrifuge tube and quickly proceed to the first step of the desired protocol (*see* **Notes 4** and **5**).

3.2 Annexin V Staining

3.2.1 Step of Phagocytosis: Cell Death and Recognition

Early stages of apoptosis include cell surface membrane changes. Phosphatidylserine exposure is one of the earliest events of apoptosis and has been shown to occur before mitochondrial membrane permeabilization during apoptosis [46]. The most common phosphatidylserine marker is Annexin V [47]. Annexin V is a member of the annexin-binding protein family. Although the role of Annexin V within a cell is unknown, it binds specifically to phosphatidylserine and has been shown to label dying cells in *Drosophila* [48, 49]. Engulfment receptors can bind directly or indirectly to exposed phosphatidylserine moieties. Bridging molecules, such as milk fat globule-epidermal growth factor 8 (MFG-E8), Gas-6, and protein S bind to both the engulfment receptor and the "eat me" signal to trigger engulfment. An MFG-E8 GFP fly line that binds to exposed phosphatidylserine moieties on apoptotic cells has been generated [50], which could be used as an alternative to Annexin V:

1. Dissect ovaries as described in Subheading 3.1.1 in one drop of DR (*see* **Note 6**).
2. Transfer the egg chambers to microcentrifuge tubes and keep on ice (*see* **Note 7**).
3. Remove DR and wash egg chambers with 500 µl of fresh 1× ABB.
4. Mix 200 µl of 1× ABB with 2 µl of Annexin V conjugate. Remove wash from egg chambers and add Annexin/ABB solution. Incubate for 15 min at RT in the dark, on a platform shaker.
5. Remove Annexin/ABB solution and wash with 500 µl of 1× ABB.
6. Remove wash, add 100 µl 16% Paraformaldehyde with 400 µl ABB, and rotate for 10 min at RT (*see* **Note 8**).

7. Remove ABB/fix and wash once with excess ABB (*see* **Note 9**).
8. Remove ABB; add 50 μl of VectaShield mounting medium with DAPI and incubate at 4 °C overnight.
9. Pipette tissue onto slides and gently add coverslip. Seal with nail polish.
10. Visualize egg chambers using appropriate filters on a fluorescence or confocal microscope. *See* Fig. 1 for Annexin V staining on *Dcp-1* mutant egg chambers (*see* **Note 10**).

3.3 Markers for the Dying Germline

3.3.1 Step of Phagocytosis: General Vesicle Uptake

There are several markers for the germline cytoplasm that can be utilized for studying general vesicle uptake. Previously, we have shown that the GFP protein trap in the *trailer hitch* gene specifically marks the germline cytoplasm with GFP [32, 36]. This GFP trap is detected in the oocyte and nurse cells of both healthy and dying egg chambers (Fig. 2a–b′). Within the dying egg chambers, GFP can also be detected within the follicle cells, indicating engulfment of the germline cytoplasm. Other germline-specific fluorescent markers have been shown to be engulfed during cell death in mid-oogenesis [34, 51]. There are also markers that are specific for

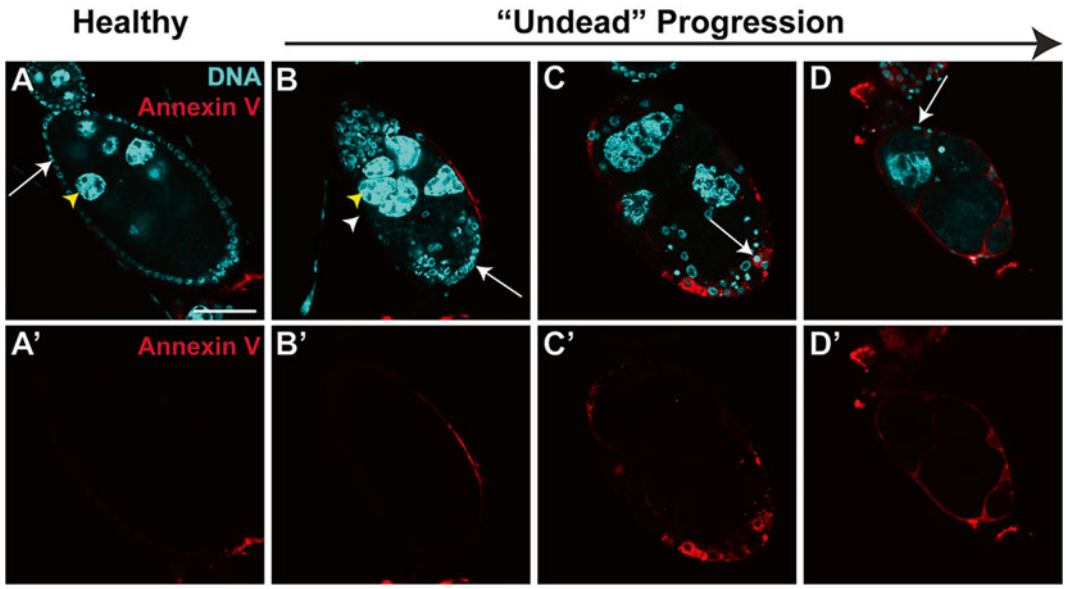

Fig. 1 Annexin V staining of *Dcp-1*^{Prev1} egg chambers. (**a**–**d**′) Nutrient-deprived *Dcp-1*$^{Prev-1}$ homozygous egg chambers stained with DAPI (*cyan*) to label DNA and Annexin V (*red*) to label exposed phosphatidylserine. (**a**) Healthy *Dcp-1* egg chamber has dispersed nurse cell chromatin (*yellow arrowhead*) and a layer of follicle cells (*white arrow*). (**b**) Early *Dcp-1* "undead" egg chamber has healthy (*white arrow*) and missing follicle cell nuclei (*white arrowhead*), and dispersed nurse cell chromatin (*yellow arrowhead*). (**c**) Mid *Dcp-1* "undead" egg chamber has nurse cells with dispersed chromatin and follicle cells that have condensed nuclei (*white arrow*) and label for Annexin V (**c**′). (**d**) Late *Dcp-1* "undead" egg chamber has very few follicle cell nuclei remaining (*white arrow*) and nurse cell membranes appear to label with Annexin V (**d**′). Scale bar is 50 μm

the oocyte cytoplasm, such as the RNA-binding protein Orb. This protein specifically marks the oocyte starting in the germarium and is maintained throughout oogenesis [41]. In early dying egg chambers, the oocyte cytoplasm marked by Orb is engulfed and can be observed within the follicle cells (Fig. 2c–d′). There are two antibodies available for Orb [41, 42, 52–55]. We show the rabbit antibody in Fig. 2; however, the mouse antibody shows the same staining pattern (not shown). Whereas *tral-GFP* and Orb mark the germline in both healthy and dying egg chambers, an antibody raised against active cleaved Dcp-1 is only detected in dying egg chambers, marking both the cytoplasm of the dying germline and the subsequent engulfed material [33, 35, 36] (Fig. 2e–f′). This antibody is discrete and specific enough that it can be used to quantify the number of engulfed vesicles and can be used to identify subtle differences between engulfment-defective mutants [33]. Here, we show *draper* egg chambers as an example (Fig. 2g–h′). Follicle cell enlargement can be visualized using membrane markers such as Discs large (Fig. 2a–b, e–h):

1. Remove all but 300 μl Grace's Insect Medium and add 200 μl heptane and 100 μl 16 % paraformaldehyde. Rotate for 20 min to fix tissue.
2. Rinse twice with PBT and wash three times with PBT for a total of an hour while rotating (*see* **Note 11**).
3. Block tissue in PBANG for an hour while rotating.
4. Incubate tissue in primary antibodies diluted in PBANG (see concentrations in Table 1) at 4 °C overnight (*see* **Note 12**).
5. Rinse twice with PBT and wash four times with PBT + 0.5 % BSA for a total of 2 h while rotating (*see* **Note 11**).
6. Incubate in secondary antibody diluted in PBANG (see concentrations in Subheading 2.4, **item 2**) for 1 h at room temperature, rotating in the dark.
7. Rinse twice with PBT and wash four times with PBT + 0.5 % BSA for a total of 2 h, while rotating.
8. Rinse with PBS and add ~2 drops VectaShield and incubate at 4 °C overnight (*see* **Note 13**).
9. Using a glass Pasteur pipette, move tissue from the microcentrifuge tube to a microscope slide, being very careful not to allow any tissue above the thin portion of the pipette (*see* **Note 4**).
10. Tease apart the tissue using tungsten needles, carefully add a cover slip, and seal the cover slip using nail polish. Visualize stained tissue using appropriate filters on a fluorescence or confocal microscope. Here, we show examples of membrane staining (Fig. 2a–b), Orb (Fig. 2c–d′) and Dcp-1 and membrane co-staining in wild-type and engulfment-defective egg chambers (Fig. 2e–h′).

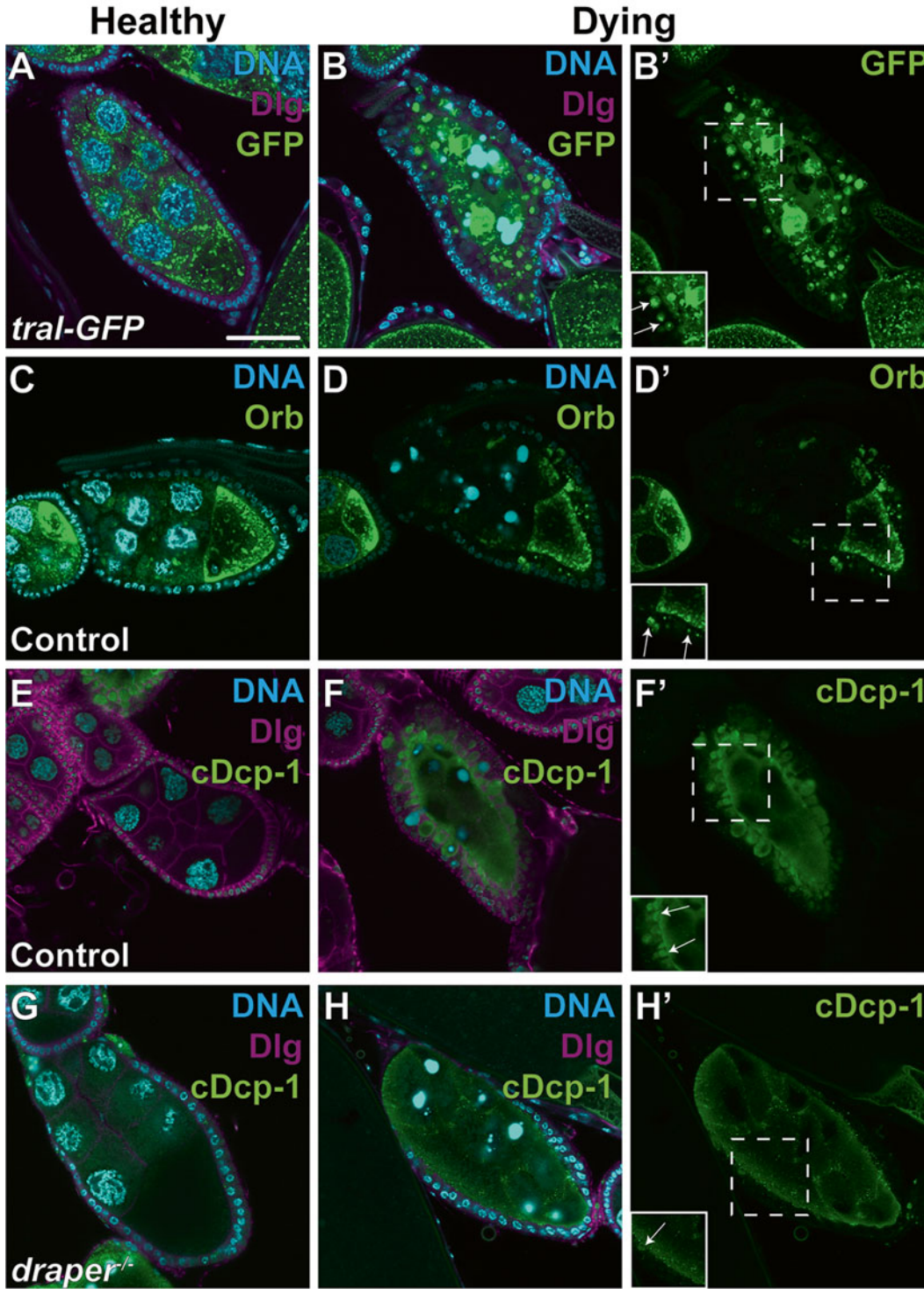

Fig. 2 Markers for germline and vesicle uptake. Egg chambers from starved flies stained with various markers to indicate germline cytoplasm and general vesicle uptake (all in *green*). *Insets* show engulfed vesicles, indicated by *arrows*. (**a**–**b**′) Egg chambers expressing a protein trap in *trailer hitch* (*tral*, *green*) stained with DAPI (*cyan*) and α-Discs large (*Dlg, magenta*). (**a**) A healthy egg chamber shows *tral-GFP* only in the germline cytoplasm. (**b** and **b**′) A dying egg chamber shows *tral-GFP* within the enlarging follicle cells. (**b**′) *tral-GFP* channel only, showing several vesicles (*arrows*) within the follicle cells. (**c**–**d**′) Egg chambers stained with DAPI (*cyan*) and α-Orb (*green*).

3.4 Markers for the Engulfing Follicle Cells

3.4.1 Step of Phagocytosis: Recognition and Phagosome Maturation

We have found that there are several molecular changes within the engulfing follicle cells, including activation of the JNK pathway (*puc-lacZ*) and enrichment of Draper, integrins (αPS3/βPS), and Croquemort [32, 33]. Several corpse processing markers are also detectable in the follicle cells during engulfment, including the GTPases Rab5 and Rab7 [36] and lipid changes (phosphatidylinositol 3-phosphate (PI(3)P)).

The engulfment receptors Draper and integrins become apically enriched early in engulfment and continue to increase as engulfment proceeds. These can be used to identify and analyze follicle cell enlargement [33] (Fig. 3a–d). We have previously shown that Rab5- and Rab7-GFP associate with Dcp-1-positive vesicles, indicating that the engulfed material is processed using the canonical corpse processing pathway [36]. Here, we show that lipid changes also occur during corpse processing in the engulfing follicle cells. Specifically, the vesicles become PI(3)P-positive near the apical surface, suggesting that lipid changes occur during phagosome maturation within the follicle cells (Fig. 3e–f, i). For this analysis, use the same protocol as in Subheading 3.3:

1. Cross a fly with an appropriate driver (*GR1-GAL4* used here) to the reporter line of interest (*UAS-Rab7-GFP* and *UAS-2XFYVE:mCherry* shown here).
2. Continue with staining as described in Subheading 3.3. GFP antibodies (Torrey Pines Biolabs) can be used to increase the signal.
3. Mount tissue and observe as described in Subheading 3.3, **step 10**. To clearly visualize vesicles, a confocal microscope is required.

3.5 LysoTracker Staining

3.5.1 Step of Phagocytosis: Acidification

LysoTracker is an acidophilic dye, marking all acidified compartments. Previously, we have shown that the nurse cells during late oogenesis cell death become acidified [36, 56]. Here, we show that the engulfed material also becomes acidified (Fig. 3g–i). LysoTracker can be used in both live imaging and on fixed tissue; we describe the protocol for use on fixed tissue here.

1. For optimal LysoTracker staining, it is best to dissect ovaries in PBS and tease apart the ovaries before transferring to a microcentrifuge tube. Adjust the volume in the tube to 600 µl PBS (*see* **Note 9**).

Fig. 2 (continued) (**c**) A healthy egg chamber shows Orb heavily enriched in the oocyte cytoplasm compared to the remainder of the germline. (**d** and **d′**) A dying egg chamber shows Orb-positive vesicles within the follicle cells. (**d′**) Orb channel only, showing several vesicles (*arrows*) within the posterior follicle cells. (**e**–**h′**) Egg chambers stained with DAPI (*cyan*), α-Discs large (*magenta*), and α-Dcp-1 (*green*). (**e**–**f′**) Control flies show no Dcp-1 staining in healthy egg chambers (**e**) and robust germline staining in dying egg chambers (**f** and **f′**). Cleaved Dcp-1 also marks the subsequent engulfed material, seen most clearly in (**f′**, *arrows*). (**g**–**h′**) *draper* flies show normal healthy egg chambers (**g**) but little to no vesicle uptake in dying egg chambers (**h** and **h′**, *arrow*). Scale bar is 50 µm

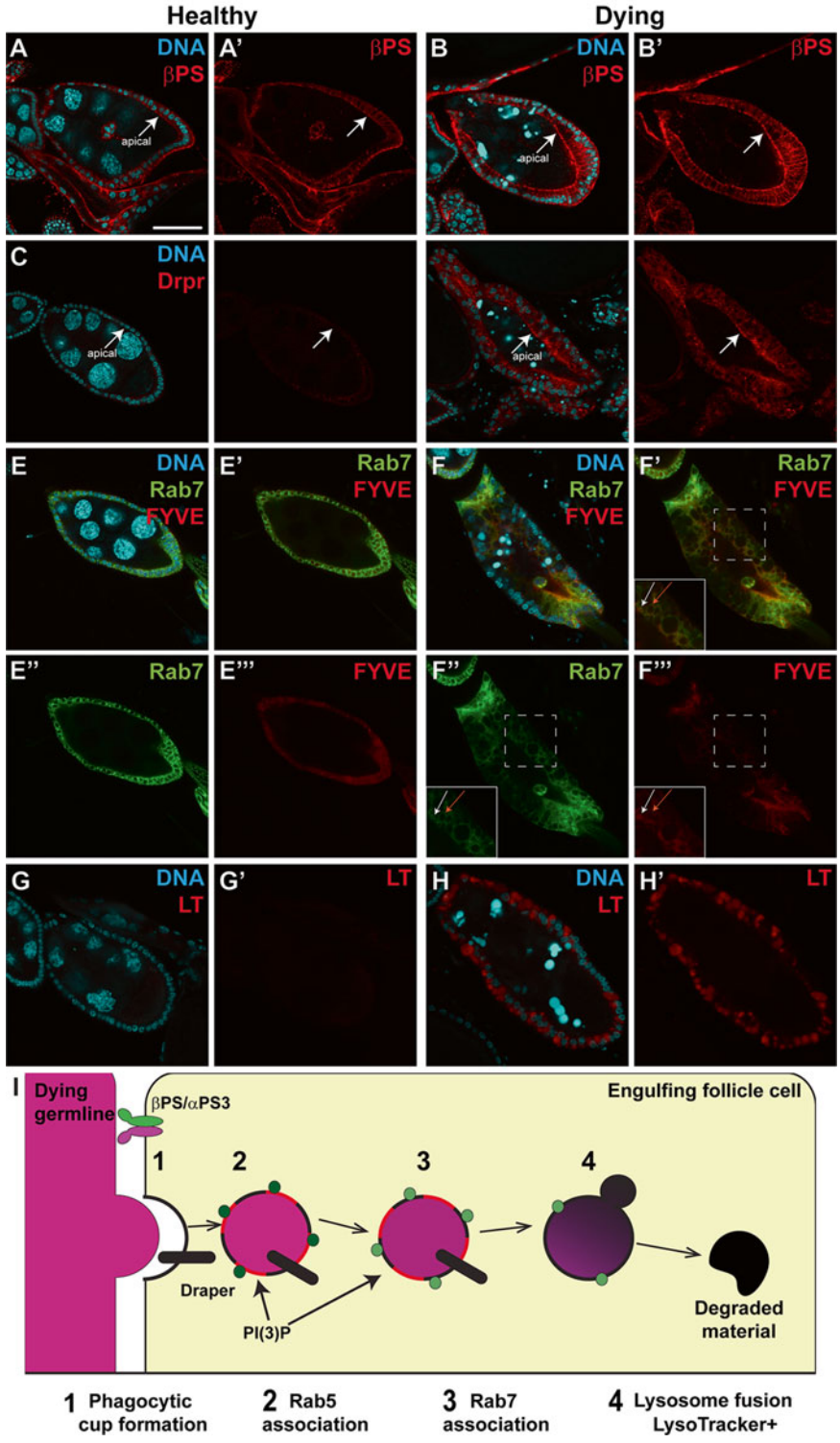

Fig. 3 Markers for follicle cell engulfment and corpse processing. Egg chambers from starved flies stained with various markers to indicate follicle cell engulfment and corpse processing (all in *red*, except Rab7 in *green* in **e**–**f**′). (**a**–**b**′) Egg chambers stained with DAPI (*cyan*) and α-integrin βPS (*red*). (**a** and **a**′) Healthy egg chambers have low levels of βPS on all surfaces of the follicle cells. (**b** and **b**′) Dying egg chambers show apically enriched βPS (*arrow*) on the enlarging follicle cells. (**c**–**d**′) Egg chambers stained with DAPI (*cyan*) and α-Draper

2. Immediately add 1 μl LysoTracker to the PBS and incubate for 5 min at room temperature on a rotator.
3. Remove LysoTracker, rinse twice with PBS, and wash tissue in PBS three times over 30 min.
4. Fix for 20 min in 300 μl PBS, 200 μl heptane, and 100 μl 16 % paraformaldehyde.
5. At this point, either rinse with PBS and add ~2 drops VectaShield or rinse with PBT and continue with the antibody staining protocol (Subheading 3.3, **step 2** onward). A high number of LysoTracker-positive vesicles can be detected within engulfing follicle cells (Fig. 3g–h′).

4 Notes

1. While adult flies can be anesthetized using ice, the effect does not last long. If CO_2 is not available, either dissect very quickly or dissect the flies while still on ice.
2. We prefer to use Grace's Insect Medium for ovary dissections. It provides sufficient nutrients such that the tissue does not degrade rapidly. Schneider's or Ringer's medium may be suitable as well. The Grace's Insect Medium should be stored at 4 °C in 50 ml aliquots prepared with sterile techniques.
3. If you remove more organs than just the ovaries, separate the ovaries before moving onto the next step.
4. The tissue can get stuck in the wider portions of the Pasteur pipettes, particularly when the tissue is in VectaShield.
5. For antibody staining or protocols with permeability problems, the ovaries should be teased apart into ovarioles before placing into a tube.

Fig. 3 (continued) (*red*). (**c** and **c**′) Healthy egg chambers have very little Draper on the follicle cells. (**d** and **d**′) Draper becomes enriched on the apical surface (*arrow*) of enlarging follicle cells. (**e**–**f**‴) Egg chambers expressing *UAS-2XFYVE:mCherry* (*red*) and *UAS-Rab7-GFP* (*green*) stained with DAPI (*cyan*). (**e**–**e**‴) Healthy egg chambers have no detectable FYVE vesicles and Rab7 endosomes are present at the apical surface. (**e**) merge. (**e**′) Rab7-GFP and FYVE-mCherry channels. (**e**″) Rab7-GFP channel only. (**e**‴) FYVE-mCherry channel only. (**f**–**f**‴) Dying egg chambers have FYVE- and Rab7-positive vesicles in engulfing follicle cells. The *inset* shows a zoom of some vesicles present within the follicle cells. *White arrow* indicates a vesicle that is FYVE- and Rab7-positive, *orange arrow* indicates a vesicle that is FYVE-negative and Rab7-positive. (**f**) merge. (**f**′) Rab7-GFP and FYVE-mCherry. (**f**″) Rab7-GFP channel only. (**f**‴) FYVE-mCherry channel only. (**g**–**h**′) Egg chambers stained with DAPI (*cyan*) and LysoTracker (*red*). (**g** and **g**′) Healthy egg chambers have no LysoTracker in the germline or follicle cells. (**h** and **h**′) Dying egg chambers have several LysoTracker-positive vesicles within the engulfing follicle cells. (**i**) A model of the canonical corpse processing pathway utilized by the follicle cells, including Draper, Rab7-GFP, FYVE-mCherry, and LysoTracker. Scale bar is 50 μm

6. It is critical that muscle sheath is removed from ovarioles because Annexin V binds to muscle sheath. To remove the muscle sheath, use forceps to pinch the tip of the germarium and slide them back and forth. The ovarioles will slide out of the muscle sheath.
7. Use nonstick microcentrifuge tubes to prevent significant tissue loss. In the event that this protocol is used on smaller tissue samples, conduct the protocol in a glass spot well.
8. Heptane can be used in the protocol, but not with the Grace's Insect Medium.
9. Avoid the use of detergents in this protocol.
10. We were unable to detect Annexin V staining on the germline membranes of *w1118* and healthy egg chambers of *Dcp-1* mutants, suggesting it may not be able to penetrate the follicle cell layer.
11. If needed, most wash steps within this chapter can be extended beyond the time specified.
12. Some primary antibodies can be reused if needed or as little as 100 μl can be used to conserve antibody. However, if under 300 μl is used, it is best not to rotate the tissue, but to nutate or shake it. When there is too little liquid, the ovarian tissue can get stuck on the lid of the tube when rotated.
13. When in a rush, tissue can be incubated in VectaShield for 2–4 h at room temperature in the dark before mounting.

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Chapter 7

Immuno-Gold Labeling of *Drosophila* Follicles for Transmission Electron Microscopy

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Abstract

Detecting the localization of cellular components using gold nanoparticles has come to offer tremendous advantages in cell biology, allowing for the high resolution imaging of the cellular organization at the subcellular level. This is further aided by the breakthroughs in the cryopreparation of samples, which focus at the retention of antigenicity in efforts to mirror the native state of the tissues and cells as closely as possible. Herein, we describe the methodology for immuno-gold labeling of *Drosophila* follicles, following preparation of the samples using the Tokuyasu method for ultracryosectioning.

Key words Cryosectioning, *Drosophila* eggs, Electron microscopy, Immuno-gold labeling, Oogenesis

1 Introduction

Spearheaded by Faulk and Taylor in 1971 [1, 2], the use of gold particles attached to colloidal sols in immuno-labeling techniques for electron microscopy (EM) has become a routinely utilized method nowadays for imaging the fine structural aspects of cell morphology with great accuracy and specificity. Considered one of the major steps in EM imaging, gold replaced ferritin as a marker of choice for high-affinity cytochemistry as it offers significant advantages for immuno-electron microscopy. Due to its higher electron density and smaller size, not only did the detection of multiple proteins on the same sample became a reality in EM imaging, but also the quality of the acquired images was vastly improved, allowing for better contrast during observation [1, 2]. Moreover, the ability to generate gold nanoparticles of various sizes has favored their usage in a wide array of EM observations [2] with larger particles regarded as well suited for scanning EM, in contrast to smaller gold molecules which are commonly used in transmission EM (TEM) [2]. In the latter case, an additional method known as "silver enhancement" has been developed, in order to further boost the emitted signal to the detection range of the electron microscope [2].

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Additionally, of the numerous methods that exist for effectively cryopreparing samples for EM, the most frequently regarded as optimal for subsequent immuno-electron imaging is the "sucrose-infusion and ultra-cryo-sectioning" developed by Taku A. Tokuyasu in 1973, simply referred to as the "Tokuyasu method" [3–5]. In TEM, this method—having undergone a series of improvements over the years—has been successfully applied to image the fine ultrastructure of various mammalian and nonmammalian samples obtained from different sources including *Drosophila* [5]. The main benefits of this technique include the primary brief fixation of the sample using aldehydes, which is the only chemical denaturation step and albeit being rather mild and slow it greatly minimizes the introduction of potential artifacts during observation [3, 4]. Furthermore, it also allows the antigens of interest to retain their antigenicity to a highest degree, which in turn enables their easier recognition and more specific binding by their respective antibodies (Abs) [2–4]. In addition, the infusion by sucrose only partially dehydrates the sample and the molecule itself acts as a cryoprotectant, enabling the more efficient vitrification of the tissue or cells during freezing, and the generation of smoother sections in the follow-up cryosectioning [3, 4]. The initial need for embedding the samples in resin during fixation was also circumvented [3, 4].

Taken together, immuno-gold labeling coupled to the latest advances in cryopreparation of biological specimens has enabled researchers to obtain samples which mirror the native state of the tissue or cells more closely, in regard to the localization, structural integrity, and interactions of the antigens under study each time [2–4]. As a result, they offer some of the best available guarantees so far, for subsequent EM investigation to depict quite reliably the actual interactions taking place at the subcellular level, in turn allowing for more solid conclusions to be drawn. In our lab, we employ the aforementioned methods for TEM, in *Drosophila* follicles (egg chambers). In *Drosophila melanogaster*, the ovary consists of two lobes that contain linear arrays of developmentally ordered follicles (egg chambers) called ovarioles [6, 7]. The follicle is the structural and functional unit of the ovary. Each follicle is composed by three cell types: the oocyte, the nurse cells, and the follicle cells. The germ line cells, nurse cells, and the oocyte are directly connected to each other by intercellular bridges, called ring canals, through which nurse cells transfer their cytoplasm to the oocyte during its maturation [7–9]. Fourteen stages of oogenesis have been described according to morphological criteria, including the appearance of the eggshell coverings, the egg chamber size, and the proportion of the follicle occupied by the oocyte [7, 9, 10]. Stage 1 represents the very first immature follicle, while stage 14 refers to the mature follicle where the nurse cells have degenerated and the eggshell is completed. In this chapter, we will describe a protocol for immune-gold labeling of thawed cryosections of *Drosophila* follicles. We also

underline any important factors for researchers to take under particular consideration, while following these step-by-step guidelines.

2 Materials

2.1 Drosophila Flies

Flies are fed on a standard cornmeal/yeast diet and are kept at 24–25 °C on a 12 h light–dark cycle and 70 % humidity.

2.2 Equipment and Reagents for Dissection of the Flies

1. Diffuser pad for CO_2 anesthesia.
2. Stereomicroscope.
3. Fine forceps (style Dumont #5) and tungsten needles.
4. Deep-well glass slide.
5. Ringer's solution: 130 mM NaCl, 4.7 mM KCl, 1.9 mM $CaCl_2$, 10 mM, HEPES, pH 7.0.

2.3 Equipment and Reagents for Fixation and Cryosectioning

1. PBS: Phosphate-Buffered Saline pH 7.4.
2. 4 % methanol free formaldehyde in PBS prepared from a 16 % solution stock.
3. 0.1 % glutaraldehyde in PBS prepared from 8 % solution stock.
4. 0.5 % toluidine blue in distilled water.
5. 2.1 M sucrose solution in distilled water.
6. 2 % methyl cellulose in distilled water.
7. Liquid nitrogen.
8. Eppendorf tubes 1.5 ml.
9. Rotator.
10. Aluminum specimen holders.
11. Aluminum plate.
12. Stainless steel loop.
13. Formvar-coated copper grids 100/200 mesh.
14. Petri dish.
15. Ultramicrocryotome.
16. Diamond trim tool and 45° ultradiamomd knife.
17. Eyelash attached to a wooden stick (to maneuver sections).
18. Static line ionizer (*see* **Note 1**).

2.4 Tools and Reagents for Immune-Gold Labeling of Thawed Cryosections

1. Parafilm.
2. Petri dish.
3. 0.2 M Glycine in PBS.
4. Blocking solution: 0.2 glycine, 0.8 % BSA (bovine serum albumin) and 1 % FCS (fetal calf serum) in PBS.

5. Primary antibodies diluted in blocking solution.
6. Secondary antibodies or Protein A gold conjugates diluted in blocking solution (*see* **Note 2**).
7. 0.45% uranyl acetate in 1.7% methyl cellulose.
8. Filter paper.
9. Transmission electron microscope.

3 Methods

3.1 Ovary Dissection

1. Anesthetize the flies under carbon dioxide.
2. Dissect the females in deep-well glass slides in 100 ml of Ringer's solution.
3. For removing the ovaries from the body, hold the fly between abdomen and thorax with forceps and pull out the terminal part of the abdomen with another pair of fine forceps. Then, gently squeeze the abdomen with the forceps to release the ovaries from the abdomen.
4. Separate ovarioles from one another with tungsten needles and/or very fine forceps and isolate the follicles (*see* **Note 3**).
5. Transfer gently the isolated follicles to the appropriate solutions using glass pipettes or plastic pipettes that have been previously rinsed in PBT (PBS containing 0.1% Triton-X 100). This helps the isolated egg chambers not to stick on the pipette (*see* **Note 4**).

3.2 Tissue Processing for Ultra-Cryosectioning

1. Fix the follicles with 4% formaldehyde in PBS for 45 min at room temperature or with 4% formaldehyde and 0.1% glutaraldehyde in PBS (*see* **Note 5**).
2. Wash three times with PBS for 5 min each time.
3. Stain the fixed follicles with 0.5% toluidine blue (*see* **Note 6**).
4. Incubate the follicles overnight in 2.1 M sucrose at 4 °C. Be sure that the samples are rotated.
5. Mount the follicles on aluminum specimen holders using one drop of 2.1 M sucrose solution. You will need to use a cold plate, in order to put the samples up on it, while you are working with them to avoid thawing (*see* **Note 7**).
6. Immerse the samples in liquid nitrogen, in order to freeze them.
7. Use an ultramicrocryotome, in order to section the frozen follicles (*see* **Note 1**).
8. Recover the ultrathin cryosections from the cryochamber of the microtome with a drop of 1:1 mixture of 2.1 M sucrose: 2%

methylcellulose using a stainless steel loop mounted on a wooden stick. Transfer the droplet toward the face of the sections, until you observe that the sections are attached to the droplet.

9. Lay the drop containing the cryosections on a carbon- or formvar-coated copper grid.
10. Until you are ready for the immuno-gold labeling, save the samples in a Petri dish at 4 °C.

3.3 Immuno-Gold Labeling

1. By using a pair of forceps, lift the grids (which contain the cryosections) and put them onto a 50 μl drop of 0.2 M glycine in PBS on a sheet of parafilm.
2. Wash three times, 5 min each on new 50 μl drops of 0.2 M glycine in PBS.
3. Incubate the grids for 10 min with blocking solution.
4. Incubate the grids for 1 h (or more depending on the antibody) with the primary antibody diluted in blocking solution (*see* **Note 8**).
5. Wash three times, 5 min each time by floating the grids on droplets of blocking solution.
6. When the primary antibody does not interact with protein A (e.g., if it is a mouse monoclonal), incubate the grids with a secondary antibody (e.g., rabbit anti-mouse) that binds protein A, diluted in blocking solution, for 30 min (*see* **Note 2**).
7. Wash grids three times, each time for 5 min with blocking solution.
8. Incubate grids for 30 min with 10 nm protein A-gold particle suspensions in blocking buffer, usually diluted 1/60 to 1/80.
9. Wash grids five times, each time for 5 min with PBS.
10. Wash grids five times, each time for 5 min with distilled water.
11. Stain the grids in 0.45 % uranyl acetate in a 1.7 % aqueous solution of methyl cellulose on ice in the dark for 10 min.
12. Retrieve the grids with a stainless steel loop of diameter slightly larger than the grid, attached to a pipette tip.
13. Drain away the excess of methyl cellulose/uranyl acetate solution by touching the loop at an angle of 45 °C to a filter paper.
14. Air-dry the grids at room temperature by placing the pipette tips upside-down in a pipette-tip rack.
15. The sample is ready for electron microscopy observation (Fig. 1).

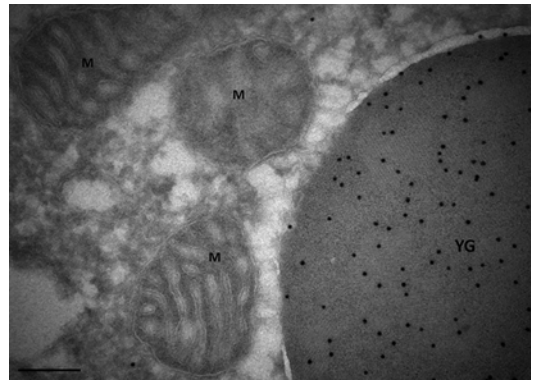

Fig. 1 Representative transmission electron micrograph of *Drosophila* oocytes. Immuno-labeling with 10 nm gold particles on cryo-sections for yolk protein. *M*: mitochondria, *YG*: yolk granule. Scale bar = 150 nm

4 Notes

1. Ultrathin cryosections can easily stick to the knife edge during sectioning. This makes it very difficult to retrieve them. The static line ionizer makes it possible to have perfect ribbons of ultrathin cryosections that will no longer stick to the knife edge or bunch up one at the top of the other. The sections can float in a nice ribbon over the surface of the knife and can be picked up easily.
2. The reactivity of protein A is very good for primary antibodies raised in rabbit, guinea pig, pig, and cow. On the contrary it is poor for primary antibodies raised in mouse, rat, goat, and sheep.
3. It is important to remove the muscle sheath from the ovarioles. To achieve this, gently squeeze the egg chambers until they exit the epithelial sheath.
4. Be gentle with pipetting because rapid pipetting of the egg chambers may cause mechanical stress which may induce phenotypic artifacts [11].
5. Duration of fixation is depended on antigenicity. If the antigen is sensitive to fixation, fixation time can be shorter or be done at 4 °C.
6. Staining with toluidine blue will give a blue color to the egg chambers. This will help to observe them easily during the following steps and will prevent tissue loss.
7. The part of the sample that you want to observe should be at the top, so make sure that you have adjusted the samples properly before you start.
8. The appropriate antibody dilution for immuno-gold labeling is usually ten times less diluted compared to the one used for immunofluorescence, or 100 times less diluted compared to the one used for Western blot analysis.

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Chapter 8

Preparation of *Drosophila* Follicles for Transmission Electron Microscopy

Anthimi Palara, Antonia P. Sagona, and Ioannis P. Nezis

Abstract

Transmission Electron Microscopy (TEM) provides high resolution and accuracy at the subcellular level for observing and investigating cellular structures. This is essential for understanding a large variety of cellular processes. In this chapter, we describe a detailed protocol for preparing *Drosophila* follicles in order to be used as a specimen for transmission electron microscopy.

Key words *Drosophila* egg chamber, Oogenesis, Subcellular morphology, Transmission electron microscopy

1 Introduction

Drosophila ovarian follicle or egg is a highly structured chamber and it is the functional unit of the fly ovary [1]. The follicle consists of three cell types: the oocyte, the nurse cells, and the follicle cells. The germ line cells, nurse cells, and the oocyte are directly connected to each other by intercellular bridges, through which nurse cells transfer their cytoplasm to the oocyte during its maturation [1]. In order to understand how this process occurs, we need to observe all the subcellular details during oocyte maturation at the ultrastructural level. Transmission Electron Microscopy (TEM) is a powerful method for observing objects at a very high resolution, with a wide range of applications in various sectors of science, such as physics, chemistry, and biology [2, 3]. In cell biology, TEM has been applied since the early 1940s. There, it has primarily been used for the imaging of cells, with the use of short wavelength electron beam, which makes the method able to achieve very high resolution, approximately 20 nm. TEM can therefore provide much more detail and information about the structures inside the cell compared to the highest resolution light microscopes and this is what makes this method so useful and unique [4]. In various instances, it has been used to analyze almost every

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cellular component of different organisms, such as cytoskeletal filaments of bacterial cells [5], mouse brain tissue compartments and organelles [6], human oocytes from cancer patients [7], and actin bundles [8], and these are only some of the examples where TEM has provided fundamental knowledge.

Sample preparation for TEM can be a complex procedure. The samples are initially fixed at room temperature using glutaraldehyde followed by osmium tetroxide. Upon fixation, samples are dehydrated with increasing concentrations of ethanol. In the biological sciences, biological specimens usually are fixed and embedded in resins. Alternatively, samples may be held at liquid nitrogen temperatures after embedding in vitreous ice [9]. The samples are then sectioned and stained before imaging. Specimens for TEM are required to be at most 100 nm thick. Preparation of TEM specimens is specific to the material under analysis and to the desired information that needs to be obtained from the specimen. As such, many generic techniques have been used for the preparation of the required thin sections. Materials that have dimensions small enough to be electron transparent can be quickly prepared by the deposition of a dilute sample containing the specimen onto support grids or films [10]. For the staining of the samples, uranyl acetate followed by lead citrate as a method to increase contrast is used. The samples in some instances can also be stained with antibodies, but this methodology will not be analyzed here. The aim of this chapter is to help researches to prepare *Drosophila* follicles for TEM observation by following a comprehensive step list.

2 Materials

2.1 Drosophila Flies

Flies are kept at 24–25 °C on a 12 h light–dark cycle and 70% humidity and are fed on a standard cornmeal/yeast diet.

2.2 Equipment and Reagents for Dissection of the Flies

1. Stereomicroscope.
2. Diffuser pad for CO_2 anesthesia.
3. Deep-well glass slide.
4. Fine forceps (style Dumont #5) and tungsten needles.
5. Ringer's solution: 130 mM NaCl, 4.7 mM KCl, 1.9 mM $CaCl_2$, 10 mM HEPES, pH 7.0.

2.3 Equipment and Reagents for Fixation and Embedding

1. PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM $KHPO_4$, pH 7.4.
2. 2% glutaraldehyde in PBS.
3. 2% osmium tetroxide, 1.5% potassium ferricyanide in distilled water (*see* **Note 1**).
4. 4% uranyl acetate in distilled water.

5. Graded series of ethanol concentrations: 30%, 50%, 70%, 85%, 100%, 100% absolute dehydrated.
6. Propylene oxide (pure).
7. Resin A mixture: 25 g of Epon 812, 20 g of Araldite, 60 g DDSA (dodecenyl succinic anhydride) (*see* **Note 2**).
8. Resin B mixture: Add eight drops of epoxy accelerator DMP-30 (2,4,6-Tris dimethylaminomethyl phenol) in 10 g of Resin A (*see* **Note 3**).
9. Rotator.
10. Flat embedding molds.
11. 60 °C oven.

2.4 Tools and Reagents for Ultra-Micro-Sectioning and Staining

1. Diamond knife.
2. Copper grids 200 mesh.
3. Petri dish.
4. Ultramicrocryotome.
5. Eyelash attached to a wooden stick (to maneuver sections).
6. 0.4% lead citrate in distilled water (*see* **Note 6**).
7. 7% uranyl acetate in distilled water.

3 Methods

3.1 Ovary Dissection

1. Anesthetize the flies with the use of carbon dioxide.
2. Dissect the females in deep-well glass slides in 100 ml of Ringer's solution.
3. In order to isolate the follicles, hold the fly between abdomen and thorax with forceps and pull out the terminal part of the abdomen with another pair of forceps. Then, gently squeeze the abdomen with the forceps to release the ovaries from the abdomen.
4. Separate ovarioles from one another with tungsten needles and/or very fine forceps to isolate the follicles.
5. Transfer the isolated follicles to the appropriate solutions gently using glass pipettes.

3.2 Fixation and Embedding

1. Fix the isolated follicles for 1.5 h at room temperature in 2% glutaraldehyde in PBS.
2. Wash in PBS three times for 5 min each, at room temperature. Be sure that the previous solution has been fully removed before you start the washes.
3. Wash with distilled water (dH_2O) three times for 5 min each, at room temperature.

4. Incubate the sample for 1 h in 2% osmium tetroxide, 1.5% potassium ferricyanide in dH_2O at 4 °C (*see* **Note 1**).
5. Repeat **step 3**, wash three times with dH_2O for 5 min at room temperature.
6. Incubate the sample, with en bloc staining that contains 4% uranyl acetate, under dark conditions, for 30 min at room temperature.
7. Wash with distilled water (dH_2O) three times for 5 min each, at room temperature.
8. Incubate the sample in graded series of dehydrated ethanol concentrations for 10 min each at 4 °C (*see* **Note 4**). The concentrations that are used for the series are 30, 50, 70, 85, 95, 100, and 100% absolute dehydrated.
9. Infiltrate with propylene oxide for two times 15 min each (*see* **Note 4**).
10. Infiltrate the sample in a mixture of propylene oxide: Resin A 3:1 for 1 h at room temperature and make sure that the sample is continuously rotated (*see* **Note 4**).
11. Repeat the same procedure as in **step 10**. The volume of Resin A: propylene oxide should now be 1:1. Infiltrate for 1 h at room temperature.
12. Repeat the procedure as before. Note that the volume now of Resin A: propylene oxide is 3:1. Infiltrate for 1 h at room temperature.
13. Make sure that the sample is rotated and let it for overnight infiltration at room temperature in pure Resin A.
14. Next day, infiltrate the sample in pure Resin B for 4 h (two times for 2 h each). Make sure that the sample is rotated. For the preparation of Resin B, 10 g of Resin A are needed, enriched with eight drops of epoxy accelerator DMP-30.
15. Fill flat embedding molds with Resin B and then place the eggs' chambers in a position that is suitable for their section (*see* **Note 5**).
16. Place the sample for 2 days incubation at 60 °C.

3.3 Sectioning and Staining

1. Section the sample by using an ultramicrotome. Place the sections on uncoated copper grids. The sections should have thickness between 60 and 80 nm.
2. The sample should be stained with 7% uranyl acetate for 10 min, following 0.4% lead citrate staining for 1 min (*see* **Note 6**).
3. Wash the grids extensively by immersing them into distilled water (into 200 ml volume beakers).
4. The sample is ready for electron microscopy (Fig. 1).

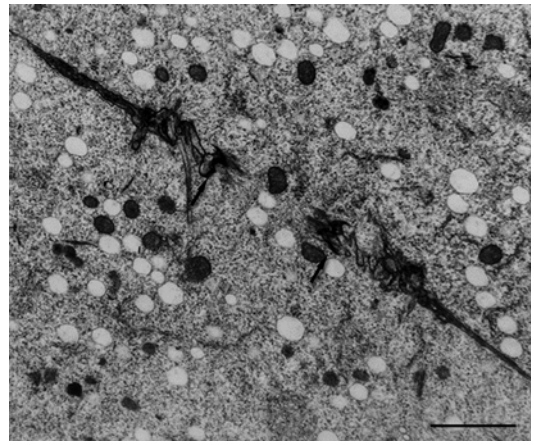

Fig. 1 Transmission electron micrograph of a *Drosophila* follicle. A ring canal is evident (*arrows*). Scale bar = 5 μm

4 Notes

1. Osmium tetroxide is very toxic and should be handled under the hood.
2. Components for making Resin A should be mixed thoroughly using an electrical mixer in 100-ml plastic disposable beakers.
3. The addition of epoxy accelerator will polymerize the resin fast and will make it very viscous. To inhibit this and gain time, Resin B can be stored at −20 °C.
4. For all the steps, 4 ml glass sample bottles that contain plastic snap caps are recommended to be used. This is necessary in order to reduce the possibility of contamination or evaporation of the samples. The volume of each solution to be used can be 1–2 ml. The glass vials need to be closed carefully and to be thoroughly sealed before the infiltration of the sample in propylene oxide, so as to avoid evaporation.
5. The tip of wooden stick can be used to orient the egg chambers.
6. To prepare 0.4% lead citrate, dissolve 0.4 g lead citrate in 100 ml freshly distilled water.

   Add 1 ml drop by drop of 10 N NaOH (0.4 g NaOH per 1 ml) after the lead is dissolved in water until the solution is clear. Store in airtight containers at room temperature. It is good to prepare fresh solution every time. When staining with lead citrate it is important to add a few pellets of NaOH inside the petri dish and keep the lid closed. This will create carbon dioxide free conditions. This step is necessary to avoid possible lead citrate interaction with CO_2 and lead carbonate precipitation on the sections.

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Chapter 9

Quantitative Real-Time PCR Analysis of Gene Transcripts of Mosquito Follicles

Aparna Telang

Abstract

Real-time (quantitative) PCR, or QPCR, has become an indispensible tool for characterizing gene expression. Depending on the experimental design, researchers can use either the relative or absolute (standard curve) method to quantify transcript abundance. Characterizing the expression of genes in mosquito ovaries will require use of the standard curve method of quantification. Here, I describe reagents and equipment necessary to run standard curve QPCR. I also provide details on the construction of the standard linear curve and calculations required to determine transcript abundance.

Key words Real-time PCR, Quantitative PCR, Standard curve PCR, Transcript abundance, Mosquito ovary, Mosquito follicles, RNA, Gene expression

1 Introduction

Mosquitoes fall into two broad categories based on their reproductive physiology. First, most mosquito species are obligatory blood feeders, term anautogenous, and require a blood meal as a source of protein for egg production. Anautogenous mosquitoes are medically important because they can feed on humans and potentially transmit disease-causing pathogens. Second, fewer species of mosquitoes are able to produce their first batch of eggs without a protein meal. These females are called autogenous and rely on larval stores of protein in place of adult protein feeding [1]. Thus, mosquito ovaries need to coordinate oogenesis with availability of protein, requiring changes in gene expression to drive changes in underlying ovarian tissue.

Gene expression profiles give researchers a snapshot of cellular activity at a molecular level because changes to an expression profile indicate changes to that cellular activity. Techniques for measuring gene expression fall into either broad coverage or single gene types. Broad coverage is accomplished with hybridization-based methods, such as microarrays [2, 3], or sequence-based

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ones, such as cDNA or EST libraries [4]. Both approaches have advantages and disadvantages, and such considerations have been reviewed elsewhere [5]. High-throughput sequencing approaches, like RNA-Seq (RNA sequencing), have been quickly replacing gene expression microarray in labs due to its many comparative advantages [5]. Regardless of the technique that is used to provide broad coverage of a transcriptome, examining the expression of individual genes is still an important step in validating high-throughput data. Traditionally, Northern blotting allowed one to detect the abundance of specific RNA molecules within an RNA population from a particular cell type or tissue [6–8]. The two disadvantages of the Northern blot are that the intensity of bands indicates only relative abundance of transcripts, and a relatively large amount of RNA starting material is required.

Real-time PCR has become an indispensible method to quantify transcripts in real time. However, one point of confusion is that we have two methods named RT-PCR. First, there is the use of reverse transcriptase (RT) enzyme to produce cDNA from mRNA. Second, there is the use of real-time PCR (RT-PCR) to quantify transcripts in real time (measuring the buildup of fluorescence with each PCR cycle). As the second is considered a quantitative PCR method, the use of the term QPCR is preferred over RT-PCR to avoid further confusion. The strength of QPCR is that it generates quantitative (fluorescence) data at the early, truly linear, phases of PCR. There are two methods of quantification, each suitable for different applications: relative and absolute (standard curve) quantification. Many research questions regarding gene expression can be suitably answered using the relative quantification method. In this method, the abundance of the GOI in an experimental sample is compared to a calibrator (control sample). The calibrator sample serves as a baseline for the expression of target GOI. Differences in C_T value between experimental and calibrator samples are presented as fold changes relative to the calibrator sample. In addition, this method requires the user to normalize values with a reference gene whose expression is constant in both the calibrator (control) and experimental samples [9]. A more precise method of quantification is to use a linear standard curve prepared from a dilution series of a template of known concentration. The absolute quantity of initial template in the experimental samples is then interpolated from the standard samples' C_T values. The standard curve method should be utilized when the objective of the project requires exact measurement of template in samples (e.g., determining viral load disseminated in mosquito tissues) [10]. The standard method is also required when the tissue under study is actively developing such that transcripts are expected to continually change levels [4, 11], thereby precluding the use of reference genes and the relative quantification method. In this chapter, I will present details of the setup and the use of the

absolute (standard curve) method of quantification that is required to examine expression of GOIs in mosquito ovaries.

2 Materials

Prior to beginning work outlined there, a number of preparatory steps are required (*see* **Notes 1** and **2**).

2.1 Ovary Dissection in Saline Solution (Christopher's Staging Used) and then RNA Preservative

1. Saline solution: 128 mM NaCl, 4.7 mM KCl, and 1.9 mM $CaCl_2$. Add about 80 mL water to a glass beaker. Add small magnetic stir bar to beaker and place beaker on stir plate with gentle stirring. Weigh 0.748 g NaCl and transfer to beaker. Weigh 0.035 g KCl and transfer to beaker. Weigh 0.028 g $CaCl_2$ and transfer to beaker. Allow to stir/mix until all salts are dissolved. Transfer solution to a 100 mL graduated cylinder and bring up volume to 100 mL with water. Mix again and store batches at −20 °C (*see* **Note 3**).
2. Use a preservative solution that maintains integrity of RNA molecules (*see* **Note 4**).
3. Use 1.5 mL microcentrifuge tubes (certified DNase/RNase free).
4. Use fine tip (#5) microdissection forceps made of nonmagnetic alloy.

2.2 RNA and cDNA Prep

1. Total RNA should be prepared using quality kits such as RNeasy (Qiagen) or SV total RNA isolation (Promega). Follow instructions according to manufacture purifying total RNA from animal tissues. We briefly summarize RNeasy material preparation below.
2. Prior to using most kits for the first time, some reagents require preparation before use. For example, a volume of 95 % ethanol needs to be added to buffer RPE concentrate as listed on bottle.
3. Also, for example, buffer RLT must have β-mercaptoethanol (β-ME) added for use as lysis solution. After adding β-ME, the RLT solution remains fresh for only 1 month at room temperature. Therefore, it is advisable to calculate a volume of prepared RLT buffer needed for samples that will be processed. Remove required volume of buffer RLT and place into an RNase-free tube and then add 10 μL of β-ME for every 1 mL of buffer RLT. Place date on the tube so users know how to use the prepared RLT buffer within 1 month.
4. It is advisable to include a DNase digestion treatment in order to remove any genomic DNA that may remain during the preparation of total RNA. Total RNA kits typically offer RNase-free DNase sets that digest DNA during the RNA purification process.

Prepare the DNase stock solution using RNase-free water according to manufacturer instructions (*see* **Note 5**).

5. When total RNA preparation is complete, it is advisable to remove a small aliquot for use in gel electrophoresis (**items 6** and **7** below) and quantification (**item 8** below). With this precaution, the main tube of total RNA can be kept sterile on ice while quantification proceeds. Since frequent freeze-thaw steps degrade RNA at a faster rate, it is best to synthesize first-strand cDNA soon after total RNA preparation and quantification.
6. RNA integrity is checked using agarose/TAE (Tris-acetate-EDTA) gel electrophoresis. 1× TAE buffer: 40 mM Tris acetate, 20 mM acetic acid, and 1 mM EDTA, pH 8.0. It is best to make a 50× TAE stock (500 mL). First make 0.5 M EDTA, pH 8.0, by combining 80 mL water, 18.61 g EDTA, and 2 g NaOH pellets. Mix and check pH is 8.0 before bringing the volume to 100 mL. Then, to make the 50× TAE buffer, combine 300 mL water, 50 mL 0.5 M EDTA, pH 8.0, 121 g Tris base, and 28.5 mL glacial acetic acid. Add water to a final volume of 500 mL. Sterilize by autoclaving. To make 1× TAE buffer, combine 20 mL 50× TAE stock with 980 mL water. This 1× buffer will be used to make 1% agarose gel and serve as electrophoresis running buffer.
7. To make a 40 mL 1% agarose/TAE gel, add 0.4 g agarose (analytical grade for electrophoretic separation of DNA/RNA molecules) to 40.0 mL 1× TAE buffer in a 250 mL glass flask. Heat this mix to boiling in order to melt agarose. Microwaving the solution is fastest as it only takes about a minute of heating on high setting to completely melt agarose. If microwave is not available, flask can be heated using a hotplate. For this, flask should be placed within a larger glass container with water that is placed on a hot plate. Bring water in secondary container to boiling to indirectly heat the agarose solution until agarose is completely melted. Allow agarose solution to cool until comfortable to touch glass flask and then add 3.0 μL of 10 mg/mL solution of ethidium bromide and mix solution. Pour right away in gel casting tray that has been set up in gel rig. Gels can be prepared in advanced, but it is best to do so on the day needed.
8. Quantify total RNA on a microvolume UV-Vis spectrophotometer. Follow manufacturer instructions on the use of the equipment.
9. Synthesis of cDNA from quantified RNA will most likely rely on the use of a PCR thermal cycler. Use 1.0 μg of total RNA to synthesize cDNA using a cDNA synthesis kit to convert total RNA to cDNA. Follow manufacturer's instructions on the use of kit and cycler parameters to program in order to complete synthesis reaction.

2.3 Primer Design

1. There are numerous software programs available to help design both reverse-transcription PCR (RT-PCR) and quantitative, real-time PCR primers (QPCR). Most sequence analysis software packages automatically come with primer design modules, and free web resources exist. Therefore, only general considerations for the design of gene-specific primers will be given here.
2. The following are important characteristics to use as settings to design RT-PCR primers from an input gene sequence: primer length = 18–25 bp, primer Tm = 55–65 °C, and primers with a GC content of 50–60 % are best. From primer list results, select primers that have a 3′-end that hybridizes well to template. The 5′-end can be initially less complementary. Software programs should also offer detailed analyses of selected primers in regard to undesirable structures, such as hairpin loops, self-dimer, and heterodimer. Lastly, it is customary to conduct BLAST analyses using selected primers as input sequences to check product specificity.
3. The following are important characteristics to use as settings to design QPCR primers from an input gene sequence: product size range = 80–150 bp, primer length = 18–22 (optimum is 20) bp, primer Tm = 58–63 °C (optimum is 60 °C), and primers with a GC content of 50–60 % are best. From primer list results, select primers that end with G/C at their 3′-ends and avoid primers that end with three or more A/T at their 3′-ends. Software programs should also offer detailed analyses of selected primers in regard to undesirable structures, such as hairpin loops, self-dimer, and heterodimer. Lastly, it is customary to conduct BLAST analyses using selected primers as input sequences to check product specificity.
4. For each gene of interest (GOI), it is advisable to purchase two to three primer sets that encompass the above primer characteristics.

2.4 Preparation of Standards (for Standard Curve Method of Quantitation) (See Note 6)

1. The most straightforward quantitation method is to build a standard line using the purified PCR product of the GOI (*see* **Note** 7). This method consists of amplifying a section of the GOI using reverse transcription PCR (RT-PCR), examining PCR products on agarose gel, excising the slice of gel containing PCR product band, and eluting the purified PCR product for use as a standard.
2. The PCR product is first examined using agarose gel electrophoresis. Next, the gel band containing the DNA of interest should be excised (sliced) and purified (*see* **Note** 8).
3. Quantify purified PCR product on a microvolume UV-Vis spectrophotometer. Using this quantity, make up a 1.0 ng/μL

standard using fresh PCR grade water. From this 1.0 ng/μL standard, make the following 1:10 serial dilutions of standards: 100 pg/μL, 10 pg/μL, 1 pg/μL, 100 fg/μL, and 10 fg/μL.

2.5 Master Mix for QPCR and Number of Replicates to Include in QPCR Experiment (See Note 6)

1. Use low-profile 0.2 mL thin-walled polypropylene tubes as these are ideal for small reaction volumes and light capture in fluorescence assays. Most vendors sell tubes in natural and opaque white walls (some vendors report that white tubes may offer increased signal in these optical assays).
2. This QPCR protocol, and subsequent calculations, will incorporate use of a SYBR Green supermix that is typically supplied at a 2× concentration. The use of mixes at different concentrations will require minor changes to calculations. Most kits contain a concentrated qPCR mix with dNTPs, Taq DNA polymerase, $MgCl_2$, nucleic acid stain (such as SYBR Green in the case of this kit), and reference dye fluorescein (which normalizes the fluorescent signal between reactions, for instruments that can support this option, and adjusts for non-PCR-related fluctuations in fluorescence between reactions).
3. Calculate volume of master mix (on per 10 μL reaction basis):

Reagent	1× Reaction
2× Supermix	5.0 μL
Forward primer	0.25 μL (*see* **Note 9**).
Reverse primer	0.25 μL (*see* **Note 9**)
PCR grade H_2O	3.50 μL
Template	1.0 μL

2.6 Setup of Fluorescent Signal Detector and Software (PCR Program + Melt Curve Analysis)

1. The instructions given here are generalized as much as possible to accommodate most well-known QPCR machines and software.
2. First, the master file is a new or blank "plate" that consists of a 96-well format. This "plate" needs to be set up to reflect a number of parameters. You will indicate the plate type (type and color—white or clear—of the vessels used) and the dyes used (such as SYBR Green). Most QPCR machines can allow for the detection of multiple dyes in any given well. Plate setup will vary accordingly. Then, you will need to specify the placement of standards and samples in the wells (the well contents). Most software will use this information for calculations.
3. Once placement of standards has been indicated, typically the software will ask you to specify the quantitation standards. This is where the user can enter the standard quantities and unit (ng, pg, mol, etc.).

4. Software will also require you to set up a "protocol" file. This is the window in which the thermal-cycling parameters for an experiment can be set: temperature of sample holder and heating the lid, reaction volume, and the temperature steps (incubation temperature and duration). Once this is done for the first time, the user only need edit an existing file.

2.7 Agarose Gel Electrophoresis

1. As in all PCR experiments, it is advisable to run reaction products on agarose gel to confirm product size (*see* Subheading 2.2, **items 6** and **7**).

3 Methods

3.1 Ovary Dissection in Saline Solution (Christopher's Staging Used) and then RNA Preservative

1. Prior to preparing the workstation, mosquitoes should be cold anesthetized on ice.
2. Prepare the workstation and dissection tools by wiping down these surfaces using a decontaminating solution to remove RNase enzymes that may degrade RNA in a sample tissue.
3. Cut a cap from a 1.5 mL microfuge tube and keep both. This size cap can hold about 100 μL of RNA preservative. Place the volume of RNA preservative into a cap and place the cap on ice to maintain ovaries at cold temperatures.
4. Dissect ovaries that are at a desired Christopher's stage [12]. Ovaries dissected in saline solution are transferred immediately to a microfuge tube cap containing RNA preservative.
5. When dissections are complete, invert the tube portion and snap down onto a cap with dissected ovaries. Centrifuge the tube at low RPM to spin down contents from the cap to the bottom of the tube. Incubate ovaries in that tube with preservative overnight at 4 °C and then transfer to −80 °C until ready to process. These incubation steps will ensure that tissues have adequate opportunity to absorb preservative but doing so at colder temperatures to maintain integrity of tissues.

3.2 RNA and cDNA Prep

1. Prepare total RNA using a kit. Follow instructions according to manufacture in order to purify a total RNA from the animal tissue. We outline steps using RNeasy kit as a sample.
2. Prior to this procedure, some buffers require advance preparation as outlined in methods, Subheading 2.2, **items 2** and **3**.
3. Buffer RLT, prepared with β-mercaptoethanol (β-ME), is used as a lysis solution. To each sample of dissected ovaries stabilized in RNA preservative, add 600 μL of prepared buffer RLT directly into the 1.5 mL microfuge tubes holding preserved samples. Disrupt and thoroughly homogenize ovary samples in buffer RLT using a sterile, polypropylene pestle for 1.5 mL microfuge tube.

4. Centrifuge the lysate for 3 min at full speed. Pipette supernatant to a new microfuge tube (*see* **Note 10**).
5. Add 600 μL of 70 % ethanol to the supernatant (cleared lysate) and mix immediately by pipetting volume up and down. Do not centrifuge. Transfer 700 μL of this mix to an RNeasy spin column already provided in a 2.0 mL collection tube (from a kit). Close lid gently and centrifuge for 30 s at 10,000 rpm (8000 × *g*). Discard the flow-through and place the same spin column back onto the same collection tube. Transfer the remaining volume of cleared lysate mix into the same spin column and centrifuge and discard the flow-through.
6. For each sample, perform an on-column DNase digestion treatment to remove any genomic DNA that may remain during the preparation of total RNA. An RNase-free DNase set, which digests DNA during the RNA purification process, typically requires an advance preparation. Thaw the required number of single-use aliquots of DNase stock and apply to column according to manufacturer instructions. This will typically entail adding DNase stock directly to the spin column membrane, centrifugation, and wash.
7. After DNase digestion treatment, add 500 μL buffer RPE to each spin column. Close lid and centrifuge for 30 s at 10,000 rpm (8000 × *g*). This serves as the first RPE wash. Discard the flow-through. Again, add 500 μL buffer RPE to each spin column. Close lid and centrifuge for 2 min at 10,000 rpm (8000 × *g*). This serves as the second RPE wash.
8. After centrifugation, remove each RNeasy spin column from its collection tube and discard the collection tube with the flow-through. Place spin column in a new 2 mL collection tube (from kit), close lid, and centrifuge at full speed for 1 min. Place the spin column in a new 1.5 mL collection tube (from a kit) and add 30 μL RNase-free water directly to each spin column membrane. Close lid and centrifuge at full speed for 1 min. This step elutes the RNA.
9. When total RNA preparation is complete, remove a small aliquot for use in gel electrophoresis and quantification on a microvolume UV-Vis spectrophotometer. With this precaution, the main tube of total RNA can be kept sterile on ice while quantification proceeds. Since frequent freeze-thaw steps degrade RNA at a faster rate, it is best to synthesize first-strand cDNA soon after total RNA preparation and quantification.
10. Run all RNA samples on a 1 % agarose/TAE (Tris-acetate-EDTA) gel to check integrity of RNA (Fig. 1). A gel used at a concentration of 1 % agarose can be run at 80 V for 30 min.
11. Both the quantity and quality of RNA can be analyzed from spectrophotometer 280/260 ratios (using microvolume

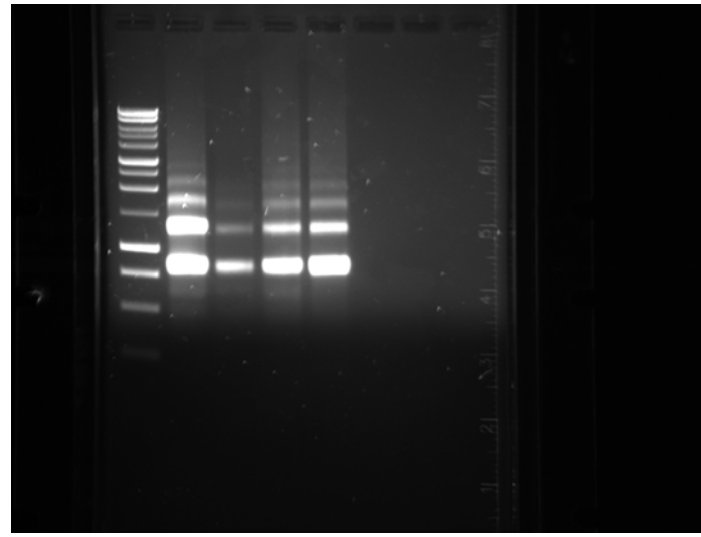

Fig. 1 A successful RNA preparation should exhibit faint smearing that ranges 200 bp –4 kb or greater with well-defined bands for the 18S RNA subunit and the 28S large subunit. Heavily degraded RNA will appear as a very low molecular weight smear. In this figure, RNA samples were run on a native (non-denaturing) gel. As such, the secondary structure of RNA alters its migration pattern in such gels so that the RNA will not migrate according to its true size. However, a non-denaturing gel is sufficient for purposes of RNA quality check

UV-Vis spectrophotometer). Follow manufacturer instructions on the use of this equipment. Good quality RNA gives 280/260 ratio readings of 2.0–2.2.

12. Use 1.0 μg of total RNA to synthesize cDNA. Kits for cDNA synthesis are used to convert total RNA to cDNA. First, determine concentration of total RNA from a microvolume UV-Vis spectrophotometer. Next, calculate the volume of total RNA that gives 1.0 μg. For example, if the sample of total RNA is at a concentration of 0.276 μg/μL, then 3.6 μL of this sample will be equivalent of 1.0 μg RNA. Following manufacturer's instructions, for a total reaction volume of 20 μL, the reaction mix would consist of 4.0 μL 5× synthesis reaction mix, 1.0 μL reverse transcriptase enzyme, 3.6 μL of total RNA template, and 11.4 μL nuclease-free water. The synthesis reaction can be completed in a PCR machine with a program setup with a series of incubation steps (see manufacturer instructions for required program).
13. For a long-term storage, all total RNA samples should be stored at −70 °C or colder.

3.3 Primer Check

1. Follow guidelines given in Subheading 2.3 to design primers. As stated, it is advisable to purchase two to three sets of primers for RT-PCR and QPCR (*see* **Note 11**).

2. It is also advisable to conduct a gradient check for primers. Most QPCR machines offer this feature in which a sample with test primers are incubated at several different anneal temperatures simultaneously so as to optimize annealing conditions.
3. Lastly, it is customary to implement a dissociation (melting curve) analysis to check that a given primer set will generate a homogeneous product. This is a standard feature in QPCR software. During a melt curve analysis, all products generated during a QPCR reaction are melted at 95 °C, then annealed at a specified start temperature (usually 55 °C), and subjected to gradual increases (user can specify temperature increment and hold time for which temperature increment should be maintained before fluorescence is read) until a specified end temperature. I have found a temperature increment of 0.2 °C and hold time of 1–2 s to work in most cases. Fluorescence data are collected during the incremental temperature increases until reaction reaches the specified end temperature.

3.4 GOI PCR and Preparation of Standards (for Standard Curve Method of Quantitation)

1. Prior to QPCR assay, researcher will need to conduct RT-PCR assay for GOI to obtain PCR product. Annealing temperatures for RT-PCR primers are determined from primer design and are then incorporated into thermal cycler program to obtain amplicon. The PCR product (amplicon) will require purification (*see* **Note 8**) and quantitation prior to use as standards for QPCR assay (Subheading 3.5 below).
2. Begin by quantifying GOI PCR product on a microvolume UV-Vis spectrophotometer. Using this quantity, make up a 1.0 ng/μL standard using fresh PCR grade water. From this 1.0 ng/μL standard, make the following 1:10 serial dilutions of standards: 100 pg/μL, 10 pg/μL, 1 pg/μL, 100 fg/μL, and 10 fg/μL.

3.5 Master Mix Recipe for QPCR and Number of Replicates to Include in QPCR Experiment

1. Calculate how much master mix you will need using the formula shown in Subheading 2.5, **item 2** for a single reaction. The exact formula will depend on the master mix purchased. The total volume will be based on the number of standards [6], experimental samples, as well as the optimized concentration of primers. The total volume should also accommodate replicates of standards and samples, as well as control reaction (the absence of template). We recommend running quadruplicates of standards, samples, and no-template controls (NTC).
2. It would be easiest to prepare one tube to house a total volume of master mix (main mix). Then separate template-specific master mix tubes should be prepared.
3. When preparing all QPCR tubes, label only the sides of tube, not the lids, as that will interfere with fluorescence reading.

4. Once main master mix is prepared, aliquot appropriate volume of this main mix to separate template-specific tubes. It would be best to aliquot main master mix to tubes dedicated to NTC last, in case volume is less than needed due to pipetting error.
5. Prepare NTC master mix by adding the appropriate volume of PCR grade water. Stir with the pipette tip to ensure mixing. With a new pipette tip, dispense 10 μL of the NTC master mix into each NTC-labeled QPCR tubes using the same pipette tip for all quadruplicates. Cap NTC QPCR tubes and set aside away from all tubes to be dedicated to samples and standards.
6. Prepare specific master mixes for each concentration of standards (a total of six tubes). Pipette 10 μL of each standard-specific master mix into each standard-labeled QPCR tubes using the same pipette tip for all quadruplicates. Cap standard QPCR tubes and set aside.
7. Repeat procedure for all samples of cDNA prepared from ovary total RNA.

3.6 Set Up QPCR Machine and Software (PCR Program + Melt Curve Analysis)

1. The QPCR instructions given here have been generalized as much as possible to accommodate most QPCR machines and software. Vendors will provide specific instructions for their machines and software for new users.
2. Start by preparing a new plate by following these steps. Click "new"—A new screen should open.
3. Under "dyes" on the left hand side, indicate SBG1 (SYBR Green or other fluorescence dye utilized).
4. Highlight the wells you want to use and indicate whether they are standards or samples by clicking the Std (standard) button in the "contents" section or the Sa (sample) button. A total of four replicates should be indicated for each standard or sample. Once you have indicated where your samples will be, click "specify quant standards" in the bottom right. A new box should open allowing one to change the units to ng and then to specify the amount, in nanograms, of standard present (per μL) in each standard well (1 ng, 0.1 ng, 0.01 ng, 0.001 ng, etc.). When done, click ok.
5. Click sets in the upper right—now click new and highlight each set of four replicates to indicate which four replicates is part of a set. Return to the "wells" view and enter descriptions for each well. When finished, click ok in the upper left.
6. Under the protocol setup, click "edit". Click on the step you need to edit (usually the annealing temperature). Click edit step and change the temperature. Click ok in the upper right.
7. Each QPCR run should include a dissociation (melting curve) analysis to assess homogeneity of products. Typically, one can

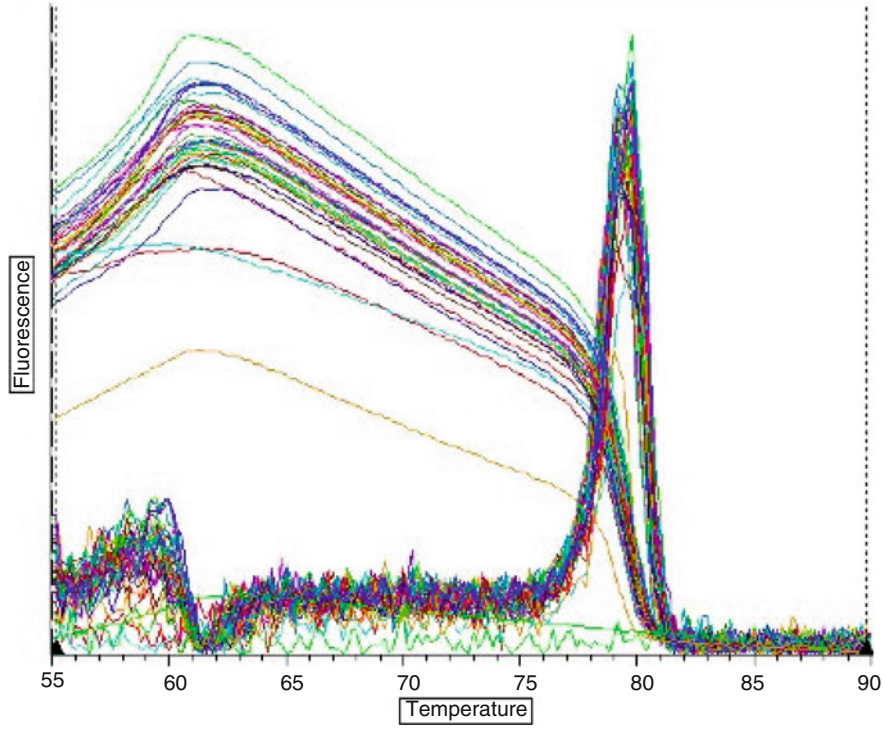

Fig. 2 The dye SYBR Green binds to all dsDNA. As such, it is important to check the specificity of each QPCR assay by analyzing the reaction products. This includes a melting curve analysis at the end of each QPCR amplification and running products on an agarose gel. In a melt curve analysis, sample fluorescence is measured repeatedly as the temperature is gradually increased. A decrease in fluorescence is observed as melting progresses with a sharp decrease at the melting temperature. This sharp decrease appears as a peak, and this represents complete dissociation of the dsDNA. This technique helps to differentiate products by their melting temperatures, confirming the existence of a single product

add a melting curve step when setting up a program. During a melt curve analysis, all products generated during a QPCR reaction are melted at 95 °C and then annealed at a specified start temperature (usually 55 °C) and subjected to gradual increases until a specified end temperature. Our program typically included temperature increments of 0.2 °C and hold time of 1–2 s. Fluorescence data are collected during the incremental temperature increases until reaction reaches the specified end temperature (Fig. 2).

3.7 Agarose Gel Electrophoresis

1. Electrophorese all QPCR reaction products on a 1 % agarose/TAE gel to confirm product size. A gel used at a concentration of 1 % agarose can be run at 80 V for 30 min.

3.8 QPCR Calculations (Described in Ref. 13)

1. At the end of real-time fluorescence detection, software generates data in the form of threshold cycle or C_T value (number of cycles required to reach threshold). As fluorescence intensity

reflects template amount, samples with more template will require fewer PCR cycles to reach threshold (a user defined level of fluorescence). Therefore, the number of cycles required to reach threshold (C_T value) is inversely proportional to the log of starting template quantity so that C_T values are used to calculate starting template quantities.

2. The threshold line (or C_T line) usually appears as a horizontal line (dashed, solid, or other, depending on machine and software) in the data window of software. This line will require positioning by the user so that it intersects fluorescence curves at a point where signal surpasses background level and is increasing exponentially (Fig. 3).

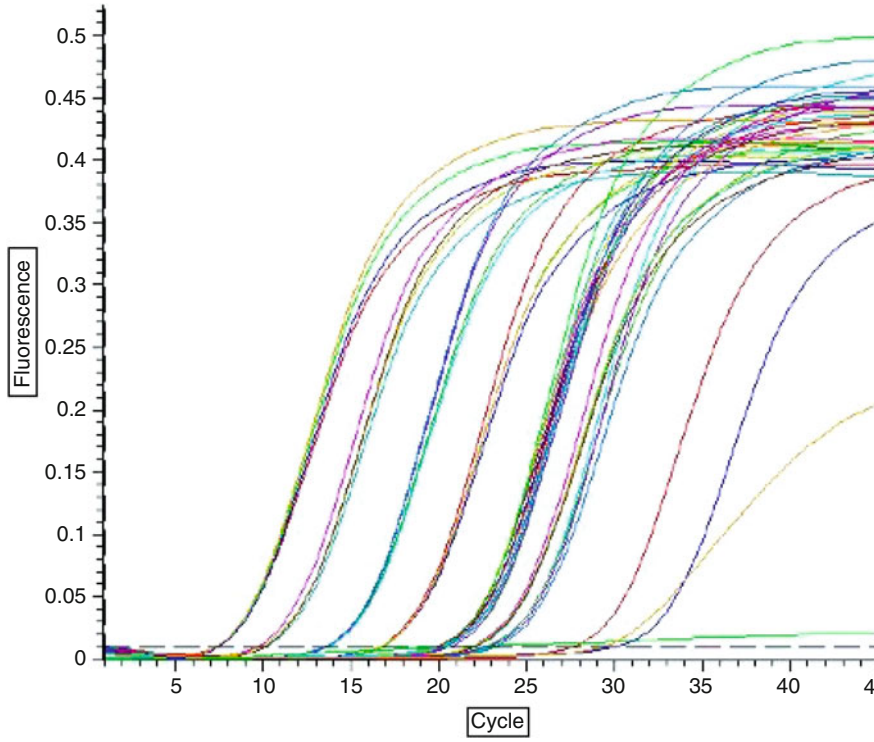

Fig. 3 Sigmoidal amplification profile of samples, this one shown is specific for oxysterol-binding protein [4]. The PCR cycle number is shown on the ***x***-axis, and the fluorescence from the amplification reaction is shown on the ***y***-axis. The fluorescence signal is proportional to the amount of amplified product in each tube. During the exponential phase, the amount of PCR product approximately doubles in each cycle. As the reaction proceeds, reagent components are consumed, and the reaction slows and enters the plateau phase. The cycle number at which enough amplified product accumulates to give a detectable fluorescence signal is called the threshold cycle. If a large amount of template is present at the start of the amplification reaction, fewer amplification cycles will be needed to accumulate enough product to give a fluorescence signal, and that reaction will have a low or early $\boldsymbol{C_T}$ value. In contrast, if a small amount of template is present at the start, more amplification cycles will be needed, and the reaction will have a high $\boldsymbol{C_T}$ value

3. Export C_T values for standards and samples to a spreadsheet program for data analysis. The following calculations will be required to build the standard line.

   An initial step entails calculating the MW of purified PCR product used as standards.

   MW (g) = PCR product size (bp) $\times 1.096 \times 10^{-21}$. The 1.096×10^{-21} constant represents the grams/bp for the four bases of DNA taking into account their molecular weights.

   Convert MW (g) to MW (Da): MW (g) $\times 6.023 \times 10^{23}$.

   Next, convert all serial dilutions of PCR product (10 fg–1 ng) to grams and then divide each standard value by MW (Da) value to obtain the total mole PCR product.

   Calculate copy numbers for each dilution: Total the mole PCR product $\times 6.023 \times 10^{23}$.

   Convert all copy number values to log copy #.

   Finally, C_T values for each standard point can be plotted against log copy #. Regression analysis will provide best-fit line (Fig. 4).

4. Regression analysis provides the line equation in the form of $y = mx + b$, where m = slope of the line and b = the y-intercept. For all samples, in which transcript copy numbers are not known and require calculation, each C_T value is entered into line equation as x-value in order to calculate its log copy #. Each log copy # can then be converted to copy number. Copy numbers can then be statistically analyzed [4].

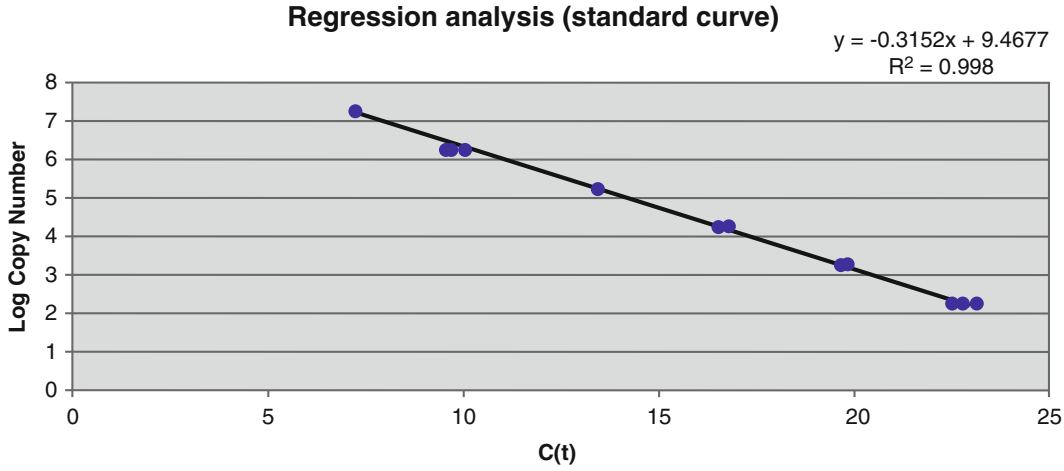

Fig. 4 The absolute quantification method relies on the accurate production of a standard curve to study transcript expression. In the standard curve method, the log of the known copy numbers of the serially diluted PCR product standards is plotted against the cycle at which the fluorescence from the standard crosses an indicated critical threshold (C_T). As is the case for any linear regression, we want to see an R^2 value that is 0.98 or higher, and the value of the slope helps us calculate the amplification efficiency of reactions

4 Notes

1. Preparation of the intact total RNA is critical to gene expression analysis. Prior to sampling and collecting, the workstation and tools must be rendered free of RNase. Use an RNase surface decontamination solution to clean work surfaces and microdissection forceps prior to dissection. Use only microcentrifuge (microfuge) tubes that are certified DNase/RNase free. Use clean tubes straight from vendor bag without further processing like autoclaving, as that additional processing may introduce contaminants. Prepare necessary PCR mixes and reagents using PCR grade water that can be purchased from a variety of vendors. Purchased and prepared reagents are stored at −20 °C (unless indicated otherwise). Prepare all other solutions using ultrapure water (prepared by purifying deionized water to attain a sensitivity of 18 MΩ cm at 25 °C).
2. Precise quantitation of standards is needed for this QPCR method. Given the small volumes of PCR reagents, use of a microvolume UV-Vis spectrophotometer is suggested to quantify RNA and DNA samples.
3. It is best to store saline solution in various working volumes. For example, one can dispense 25 mL as 1.0 mL aliquots that are stored at −20 °C. These serve as single-use aliquots for dissections and are thawed when needed and discarded after use. The remaining saline solution can be dispensed as 25 mL batches in clean plastic tubes and stored at −20 °C. When more 1.0 mL aliquots are needed, thaw 1 × 25 mL tube of saline solution and dispense as 1.0 mL aliquots that are once again stored at −20 °C for single use.
4. We strongly recommend the use of a product that is a preservative for RNA molecules to stabilize harvested mosquito follicle tissue for isolating total RNA. If a preservative is not available, kits will advise that animal tissue can be flash-frozen in liquid nitrogen and stored at −70 °C or that tissue can be immediately homogenized in a solution that contains reagents that inhibit or denature RNase enzymes. However, we do not recommend these last two options for mosquito follicles.
5. Divide DNase stock solution into single-use aliquots of various volumes. The following example is if Qiagen kit is used: 20 μL aliquots for two RNA samples, 40 μL aliquots for four RNA samples, etc. Volumes will differ according to manufacturer used. Store aliquots at −20 °C.
6. Prior to preparation of PCR reagents and mix, it is critical to prepare your workstation to rid it of possible contaminants by wiping all work surfaces with a 10% bleach solution. This is especially crucial if using plasmids containing cloned target sequences as QPCR standards (*see* **Note** 7).

7. Plasmids containing cloned target sequences are also commonly used as standards in QPCR. However, because plasmid sequences are common and abundant, they can easily be sources of PCR contamination. A great deal of caution is required to prevent exposing work surfaces, PCR reagents, and solvents to plasmid DNA.
8. The simplest method is to purify and recover PCR product from ethidium bromide-stained agarose gel. A number of high-quality kits exist. The purified PCR product is then ready for use as standard.
9. The volume of forward and reverse primer will depend on optimum concentration of primers for individual experiments. The volume listed (0.25 μL) is based on a primer concentration of 250 nM. A final concentration of 200–250 nM per primer is effective for most reactions. However, it may be necessary to optimize primer concentration, which will then require a titration of primer concentration between 100 and 500 nM.
10. For ovary samples, only a very small amount of insoluble material will be present after centrifugation. In this regard, the pellet will be invisible. To ensure collection of only supernatant, place sample microfuge tube with cap hinge facing away from rotor center at centrifugation. This will ensure that pellet, if present, will deposit at the hinge side of the bottom of the microfuge tube. Collect supernatant by directing pipette tip to the side opposite of expected pellet and slowly pipette supernatant (cleared lysate) into a new microfuge tube.
11. Vendors supply oligos in lyophilized form. Briefly centrifuge each tube of lyophilized oligo prior to opening to ensure contents are at bottom of the tube. Vendors also provide the exact amount of nmoles of each oligo purchased on the tube label and on the oligo paperwork. A concentration of 100 pmols/μL (100 μM, micromolar) is standard for an oligo stock solution. To obtain this stock concentration, multiply the "nmole" amount by 10 to calculate the volume of solvent (sterile 1× TE buffer recommended: 10 mM Tris–HCL, pH 7.5, or 8.0, 0.1 mM EDTA) to be added and add that volume of 1× TE directly to the tube of lyophilized oligo. Mix well to fully solubilize oligos. Once stock solutions are made, a working solution of oligos can be obtained by diluting to a concentration desired.

Acknowledgment

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Chapter 10

Studying Oogenesis in a Non-model Organism Using Transcriptomics: Assembling, Annotating, and Analyzing Your Data

Jean-Michel Carter, Melanie Gibbs, and Casper J. Breuker

Abstract

This chapter provides a guide to processing and analyzing RNA-Seq data in a non-model organism. This approach was implemented for studying oogenesis in the Speckled Wood Butterfly *Pararge aegeria*. We focus in particular on how to perform a more informative primary annotation of your non-model organism by implementing our multi-BLAST annotation strategy. We also provide a general guide to other essential steps in the next-generation sequencing analysis workflow. Before undertaking these methods, we recommend you familiarize yourself with command line usage and fundamental concepts of database handling. Most of the operations in the primary annotation pipeline can be performed in Galaxy (or equivalent standalone versions of the tools) and through the use of common database operations (e.g. to remove duplicates) but other equivalent programs and/or custom scripts can be implemented for further automation.

Key words Transcriptome, De-novo assembly, Annotation, Non-model organism, Oogenesis

1 Introduction

Identification of key genes involved in any biological process has always been a rather laborious process and more often than not the approach was biased toward candidate genes and the genes they interact with. Furthermore, such studies largely focused on model systems only, such as *Drosophila melanogaster* [1, 2]. Thus progress was to an extent limited in terms of elucidating the complete spectrum of genes involved in a particular biological process, their divergences in expression under different conditions and across species. In the last decade, the availability of next-generation sequencing (NGS) has addressed this to a large extent and advanced a great many research fields, including the study of insect oogenesis. Now that a significant number of non-model organisms have been sequenced [3], it is possible to both identify the gene expression patterns underpinning a particular biological process in any

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given tissue across a relevant time-scale within a species, and also to perform powerful across species comparisons.

One of the major advances in sequencing was the introduction of massively parallel sequencing platforms such as Illumina [4]. Despite the phenomenal increase in sequencing output (by a factor of 108), the construction of contigs and the assembly of genomic and transcriptomic datasets are far from trivial (e.g. issues with short read length, repetitive elements, and annotation). As de novo assembly algorithms have improved [5–7], transcriptomic data obtained through RNA sequencing (RNA-Seq) can now be reliably analyzed in terms of expression levels [8, 9]. The high throughput of RNA-Seq also means that multiple experimental samples can be run simultaneously (e.g. multiplexing) allowing for an accurate insight into the expression changes across different combinations of tissues, time points, and/or conditions [10, 11]. Using a main model system to compare against, RNA-Seq in non-model species provides the opportunity to rapidly identify the candidate genes involved in the diversification of particular developmental pathways such as oogenesis [3, 12–15]. Sequence submissions are in fact accumulating exponentially, in the order of hundreds of millions of sequences every year, but with varying levels of (accurate) annotation [16]. For any study wishing to characterize the mechanisms and the genes involved underpinning a biological process a key step in processing the RNA-seq data will be the annotation, and it is this step in particular that may prove challenging. It is inefficient to perform individual BLAST searches and decide on the suitability of the hit for each contig generated from a de novo transcriptome for a non-model system without prior sequencing data.

In order to determine the genes involved in regulating oogenesis, as well as maternal effect gene expression and genes expressed in embryogenesis, in a non-model organism, the Speckled Wood butterfly *Pararge aegeria* [15], we have developed a protocol for an accurate annotation of conserved genes in such a non-model system to facilitate subsequent identification and designing experiments targeting these genes.

2 Materials

2.1 Hardware Requirements

Computer with Multi-Core Processor >2 GHz (number of cores and processor speed will increase computing speed depending on the program), RAM >8 GB (memory demands increase with transcriptome/genome size), HDD or SSD with ample space for your data and a Unix-based OS (Linux/MacOS).

2.2 Recommended Software

Detailed instructions on the use of the various software packages can be found on the provided websites.

2.2.1 Raw Data Quality Control

1. FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/).

2.2.2 Raw Data Editing

2. CLC Gx Workbench (http://www.clcbio.com/products/clc-genomics-workbench/).

De Novo Assembler

3. CLC Gx Workbench (http://www.clcbio.com/files/whitepapers/whitepaper-denovo-assembly-4.pdf) or.
4. Trinity (https://github.com/trinityrnaseq/trinityrnaseq/wiki).
5. CAP3 (http://seq.cs.iastate.edu/cap3.html).

2.2.3 Read Mapping and Counting

6. Tophat (http://ccb.jhu.edu/software/tophat/index.shtml).
7. HTSeq (http://www-huber.embl.de/users/anders/HTSeq/doc/install.html).
8. Cufflinks (http://cole-trapnell-lab.github.io/cufflinks/) including Cuffmerge (http://cole-trapnell-lab.github.io/cufflinks/cuffmerge/).

2.2.4 Annotation Pipeline

9. EMBOSS (http://emboss.sourceforge.net).
10. BLAST+ (https://www.ncbi.nlm.nih.gov/books/NBK279671/).

2.2.5 Further Annotations

11. BLAST2GO (https://www.blast2go.com/blast2go-pro/b2g-register-basic).

3 Methods

3.1 Sample Preparation

Sample preparation will vary for every tissue and/or species but in order to obtain the best results for your sequencing experiment the following key steps must be optimized/determined, and sequencing facilities require full disclosure of such relevant information:

1. Perform tissue isolation, RNA extraction, DNA removal (*see* **Note 1**).
2. Verify the integrity of your RNA on a BioAnalyzer or equivalent (*see* **Note 2**).
3. Perform the cDNA library preparation according to the chosen sequencing method (*see* **Note 3**).

3.2 Sequencing

There are a number of options available for sequencing depending on the biological question and budget. Key questions to be answered include:

1. What length of reads and therefore what method do you require/can you afford?
2. What depth of sequencing is required for each sample?

3. How many samples can share each lane (multiplexing)?
4. How many technical replicates can you get or do you need (*see* **Note 4**)?

3.3 Quality Control

In some cases the reads produced may contain contaminants, resulting from read-throughs into the adaptors, adaptor dimerization as well as general sequencing artifacts. A number of tools can perform analyses and trimming/cleaning of the raw data.

1. Get **input**: FASTQ file containing the raw reads to be checked.
2. Run **program**: *FastQC* (Galaxy or standalone).
3. Resulting **output**: HTML Report with 12 sections informing you of the quality of the raw data (*see* **Note 5**).

   If reads require editing:
4. Get **input**: FASTQ file containing the raw reads to be edited and Adaptor sequences (*see* **Note 6**).
5. Run **program**: *CLC Gx* (standalone only) or equivalent.

   Example (GUI): Mismatch cost: 4; Gap cost: 5; Internal match threshold: 19; End match threshold: 5 (*see* **Note 7**)
6. Resulting **output**: Clipped FASTQ file [File 3.3.6].

3.4 Assembly

Multiple algorithms exist for de novo assembly, with most of them employing *de Bruijn* graph resolution algorithms with alterations. Ultimately, it is ideal to experiment with different tools and parameters. It is also possible to merge multiple assemblies into one with a tool such as CAP3 [17].

1. Select assembly methods suitable for your data (*see* **Note 8**) such as CLC Gx Workbench or Trinity.
2. Set insert size and/or assembler parameters (*see* **Note 9**).
3. Quality Control of assemblies and merging (optional).
4. Resulting **output**: FASTA containing de novo assembled contigs [File 3.4.4].

3.5 Mapping

At this point there are two possibilities, either your organism has a reliable reference genome or not. In the latter case the reads are mapped against the de novo assembly of the transcriptome generated. Numerous mapping tools exist and should be chosen on the basis of the type of sequencing performed [18]. If you have different experimental groups for which you would like to test for differential expression you must map the reads from each condition separately until Subheading 3.8 (Merging).

1. Get **input**: FASTQ files containing the clean reads to be mapped (*see* **Note 10**) [File 3.3.6] and FASTA file containing de novo assembled contigs (or reference genome) [File 3.4.4].

2. Run **program**: *Tophat* (Galaxy or standalone).
   `Example (CLI): $ tophat options* [reference].fasta [reads].fastq`
3. Resulting **output**: "accepted_hits.bam" SAM file containing alignment of reads to the reference [File 3.5.3].
4. Repeat the above steps or run in parallel for the other conditions.

3.6 Read Counts

1. Get **input**: SAM file [File 3.5.3].
2. Run **program**: *HTSeq-count* (Galaxy or standalone).
   `Example (CLI): $ htseq-count options* [alignment_file].bam [features].gff`
3. Resulting **output**: Table with reads for each feature [File 3.6.3].
4. Repeat the above steps or run in parallel for the other conditions.

3.7 FPKM Counts (via Cufflinks)

1. Get **input**: SAM file with aligned reads [File 3.5.3].
2. Run **program**: *Cufflinks* (Galaxy or standalone).
   `Example (CLI): $ cufflinks options* [alignment_file].bam`
3. Resulting **output**: "transcripts.gtf" a GTF file containing assembled isoforms [File 3.7.3].
4. Repeat the above steps or run in parallel for the other conditions.

3.8 Merging

1. Get **input**: Assembled isoforms from Condition A, B, C… [File 3.7.3].
2. Generate a simple text file with list of paths for all files generated (for each condition).
3. Resulting **output**: List of paths to all GTF assembly files [File 3.8.2].
4. Get **input**: Manifest file [File 3.8.2].
5. Run **program**: *Cuffmerge* (Galaxy or standalone).
   `Example (CLI): $ cuffmerge options* [assembly_GTF_list].txt`
6. Resulting **output**: GTF file containing merged assembly of all isoforms in all conditions [File 3.8.6].

3.9 Differential Expression

With the reads mapped, there is now information available on how many reads have been mapped to each isoform, gene, or contig and this can be used to estimate the abundance of transcripts sequenced. Multiple statistical methods exist with varying false-positive or false-negative discovery rates [9], such as CuffDiff2 (following on from the Cufflinks workflow) [19], edgeR [20], and DESeq2 [21]. Please refer to references for details on how to apply these methods to your data.

3.10 Primary Annotation: Determine Lengths and Open Reading Frames from Assembled Contigs

At this stage many transcriptomic data sets are simply annotated based on the best BLAST hit against the current NCBI repository of sequences with a simple *e*-value threshold. The following Primary Annotation sections detail the workflow (Fig. 1) designed to improve on this, offering more informative annotations and levels of confidence (*see* ref. 15 for an example of such analyses).

1. Get **input**: FASTA file containing the assembled contigs to be identified and annotated [File 3.4.4].
2. Run **program**: *Compute Sequence Length* (Galaxy tool) or equivalent script (*see* **Note 11**).
3. Resulting **output**: Tab delimited two-column table: FASTA title and length in nucleotides [File 3.10.3].
4. Get **input**: FASTA file containing the contigs to be identified and annotated [File 3.4.4].
5. Run **program**: *getORF* (EMBOSS tool; Galaxy or standalone; *see* **Note 12**).
   Example A (CLI): $ getorf -minsize 200 –find `1 options* –sequence [contigs].fasta –outseq [output].fasta`
   `Example B (CLI): $ getorf –minsize 200 –find 3 options* –sequence [contigs].fasta –outseq [output].fasta`
6. Resulting **output**: FASTA file containing translated ORF sequences (from example A; *see* **Note 13**) [File 3.10.6A] and FASTA file with ORF as nucleotide sequences (from example B) [File 3.10.6B].

3.11 Primary Annotation: Prepare BLAST Databases

1. Download the latest “nr” and “nt” databases from NCBI.
2. Depending on your chosen model system you will want to identify groups of reliable sequences and compile GI number lists (*see* **Note 14**) by robustness of annotation that will in turn allow better inferred annotations for your sequences (*see* **Note 15** and Table 1).
3. Create a GI List for curated protein database such as UniProtKB/Swiss-Prot (for sources A and D).
4. Create a GI List with a selection of the most related and well annotated protein sequences (for sources B and E).
5. Create a GI List for Mitochondrial proteins (for source C).
6. Create a GI List for an extended scope of protein sequences (e.g. Protosomia; for source F).
7. Create a GI List for an extended scope of nucleotide coding sequences (for sources G and H).
8. No GI list needed for the full “nr” nucleotide database (for source I).

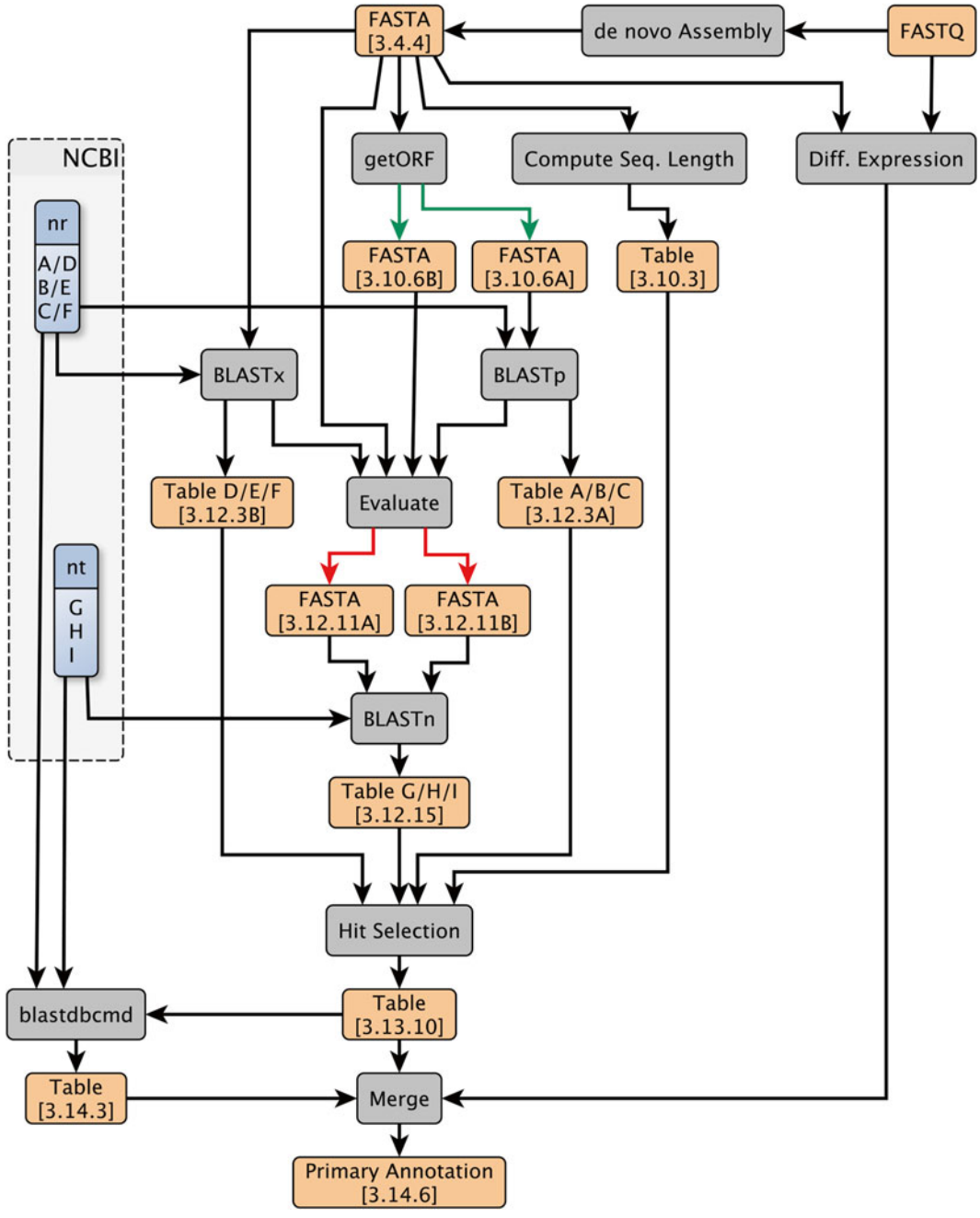

Fig. 1 Overview of the primary annotation workflow. Overview of the steps performed to achieve a primary annotation of de novo assembled contigs. Annotations are inferred on the basis of similarity with existing sequences using a multi-BLAST strategy that prioritizes sources on the basis of their defined reliability

Table 1
BLAST strategies for hit selection

DB	BLAST+	Group	Query	Filter	Source
nr	BLASTp	Alpha	ORF (aa)	*UniProtKB/Swiss-prot*	A
				Selected	B
			Mt ORF (aa)	*Mitochondrion*	C
	BLASTx	Beta	Contig (nt)	*UniProtKB/Swiss-prot*	D
				Selected	E
		Charlie	Contig (nt)	*Extended*	F
nt	BLASTn		ORF (nt)	*CDS*	G
		Delta			
			Contig (nt)	–	I

Summary of the different combinations of queries and databases that result in different sources of hits. Hits that pass the similarity threshold (user defined) are given priority relative to the next group/source down (e.g. Alpha has priority over Beta; A has priority over B). Filters applied to each database restrict the scope according to the general quality of annotations attached to the subject sequences. In three cases this is user defined (in *red*) as selection of the closest and most reliable annotated depends on the organism studied

3.12 Primary Annotation: Perform BLAST Searches for Each Group

1. Get **input**: Predicted ORF protein sequences [File 3.10.6A] and the relevant GI List to limit the search to databases A/B/C/D/E/F.
2. Run **program**: *BLASTP* (BLAST+ tool; standalone only).
   ```
   Example (CLI): $ blastp -query [input].fasta -db [dbname] -out [output].tsv -gilist [gilist].txt -evalue 1 -outfmt "6 qseqid sgi length positive evalue bitscore qstart qend sstart send" -max_target_seqs 1 -num_threads 4
   ```
3. Resulting **output**: Tabular hit list for Alpha (Source A/B/C) [File 3.12.3A] and Beta/Charlie (Source D/E/F) [File 3.12.3B].
4. **Evaluate which contigs failed to find any hits in protein databases:**
5. Get **input**: Tables from Alpha [File 3.12.3A] and Beta BLASTs [File 3.12.3B].
6. Run **program**: Database manager or custom script (Excel, Python, Perl) for **steps 7–11**:
7. Merge tables, duplicate the file and remove hits below threshold.
8. Sort and remove duplicate entries.
9. Find entries that passed in a copy of the original FASTA with Contig sequences [File 3.4.4]. Remove these entries.

10. Find entries that passed in a copy of the FASTA with ORF nucleotide sequences [File 3.10.6B]. Remove the found entries.
11. Resulting **output**: FASTA file containing Contig sequences that failed to find a match in protein databases [File 3.12.11A] and FASTA file containing ORF nucleotide sequences that failed to find a match in protein databases [File 3.12.11B].
12. **Perform BLAST searches against the Delta and Zeta nucleotide databases (G/H/I):**
13. Get **input**: Failed ORF sequences [File 3.12.11B] and Contig sequences [File 3.12.11A].
14. Run **program**: *BLASTN* (BLAST+ tool; standalone only).
    ```
    Example (CLI): $ blastn -query [input].fasta -db [dbname] -out [output].tsv -gilist [gilist].txt -evalue 1 -outfmt "6 qseqid sgi length positive evalue bitscore qstart qend sstart send" -max_target_seqs 1 -num_threads 4
    ```
15. Resulting **output**: Tabular hit list (Source G/H/I) [File 3.12.15].

3.13 Primary Annotation: Hit Selection

1. Get **input**: Tabulated hits from sources A to F [File 3.12.3B], Tabulated hits from sources G to I [File 3.12.15], and Table of Contig lengths [3.10.3].
2. Use a database manager or custom script (Excel, Python, Perl) for **steps 3–10**:
3. Merge into a table.
4. Calculate Similarity Score (*SS*) for each hit:

$$SS = \text{bitscore} \times \frac{\text{positives}}{\text{Contiglength}}$$

5. Set threshold (pass if $SS \geq 18$; 45% positive matches for a bitscore of 40) and perform Similarity Score Test (*see* **Note 16**).
6. Sort by Similarity Score Test (pass or fail).
7. Pass hits are then sorted by Group (Alpha-Zeta), Positive matches (Descending), Source (A–I), and Similarity Score (Descending).
8. Failed hits are sorted directly by Similarity Score (Descending, *see* **Note 17**).
9. Duplicate the table, remove all duplicate Contig ID entries (leaving only the top filtered hit).
10. Resulting **output**: Table with top hits for each Contig (e.g. ContigID, ORF information, Hit metrics, Similarity Score; *see* **Note 18**) [File 3.13.10].

3.14 Primary Annotation: Retrieve Hit Information and Merge

1. Get **input**: List of GI numbers for all top hits [File 3.13.10].
2. Run **program**: blastdbcmd (BLAST+ tool; standalone only). Specify the output format to provide the GI (%g), taxid (%T), scientific name (%S), and title (%t) separated by a delimiter of your choice (;).
   ```
   Example (CLI): $ blastdbcmd -db nr -entry_batch [gilist].txt -out [output].csv -outfmt "%g;%T;%S;%t"
   ```
3. Resulting **output**: CSV file with Hit description [File 3.14.3].
4. Get **input**: Table with Contig ID, ORF details and Hit metrics [File 3.13.10], Hit Descriptions [File 3.14.3], and Differential Expression Results.
5. Use a database manager or custom script (Excel, Python, Perl) to combine datasets on the basis of ContigIDs and GI numbers.
6. Resulting **output**: Table with putative annotations based on BLAST similarity and differential expression data [File 3.14.6].

With basic putative transcript features and annotations based on similarity to existing sequences, you may want to focus further analyses on a specific biological process, developmental stage, protein type, organelle, or sequence property. There are a number of methods to approach this, the most suitable of which will be dependent on the model system you are working with.

3.15 Further Annotations: Comparison to an Existing Well-Established Model System

In this case, you are investigating a species that is not phylogenetically isolated and may share both sequence similarity with a well-established model system, such as *Drosophila*, or morphological similarity (e.g. butterflies and *Drosophila* both have meroistic ovaries). Depending on the resources available for the model system you are comparing to, it may allow you to compile a list of the genes that are known to be of interest for your study in this well-established system (as done in [15]). Once you have obtained the sequences for these genes you can then attempt to identify possible orthologs in your transcriptome by using BLAST. The original annotation and the best hit from this approach may not match for a number of reasons but the primary annotation should be the most informative. Further verification can be performed manually or through an automated semantic analysis.

1. Get **input**: Gene Identifier List of genes of interest and Database (example: nr)
2. Run **program**: *BLASTX* (BLAST+ tool; standalone only).
   ```
   Example (CLI): $ blastx -query [input].fasta -db [dbname] -out [output].tsv -gilist [gilist].txt -evalue 1 -outfmt "6 qseqid sgi length positive evalue bitscore qstart qend sstart send" -max_target_seqs 1 -num_threads 4
   ```

3. Resulting **output**: Table with putative orthologous hits [File 3.15.3].
4. Get **input**: Orthologous Hit Table and Table/Database [File 3.15.3].
5. Merge information and optionally filter on basis of the bitscore.

3.16 Further Annotations: Gene Ontology (GO) Analysis

This may be performed in addition or as an alternative to the previous section. The GO initiative provides a hierarchical classification of terms that help describe genes through their biological processes, cellular components, and molecular function. GO terms can be annotated on the basis of BLAST results through the use of BLAST2GO a tool that cross-references sequences with existing GO terms with your hits.

1. Get **input**: Sequences or Subset of sequences to be annotated with GO terms, Database (example: nr), and Gene Identifier List of sequences to base GO term annotation on (gilist.txt; *see* **Note 19**).
2. Run **program**: *BLASTX* (BLAST+ tool; standalone only).
   ```
   Example (CLI): $ blastx -query [input].fasta
   -db [dbname] -out [output].tsv -gilist [gilist].
   txt -evalue 1 -outfmt 5 -num_threads 4
   ```
3. Resulting **output**: BLAST Hits in XML format [File 3.16.3].
4. Get **input**: XML Hits [File 3.16.3].
5. Run **program**: *BLAST2GO* (standalone only): Import XML, run mapping and annotation tools within the software.
6. Resulting **output**: Various export options exist.

3.17 Investigating Novel Sequences

If you are interested in identifying sequences unique to your species or unknown sequences that you have found to be differentially expressed in your conditions of interest, the following approaches form a good starting point.

1. **Conserved Domain identification**: The primary solution to generally begin identification is to scan these sequences for known domains as defined in the NCBI Conserved Domain Database (CDD) or in InterPro databases. This can be accomplished by running RPS-BLAST (included in the BLAST+ package) and/or the InterProScan tool on your sequences. Alternatively, tools integrating these approaches exist such as afterParty [22] and offer a solution for visualization of your data and annotations.
2. **De novo domain identification and/or structure prediction**: You may want to further predict much more specialized features such as binding sites, signal peptides sequences, or transmembrane helices. There is no single tool that can accomplish all of these predictions but many specialized tools that should be consulted in each case.

3. **Investigating specific sequence features**: In this case, you are interested in identifying very specific sequence features that belong to a certain type of RNA (ncRNA, UTR, etc.) or protein family. The approach is similar as for the two previous cases, you may simply restrict the initial search or subsequently filter the results for your features of interest (*see* **Note 20**).

3.18 Sequence Submission (NCBI)

When you are ready to publish and share the results of your analyses on the transcriptome you may upload the sequences as well as all the annotation information. There may be however some formatting differences between the way your annotations are currently organized and the requirements of the public host (for example NCBI):

1. Adjust all coordinates to match requirements (The stop codon is included for NCBI CDS features).
2. Convert your features (such as 5′UTR, CDS, 3′UTR) into the Feature Table format. Partial features should have the translation frame indicated.
3. Format sequences in FASTA with a unique SeqID followed by source information in square brackets.
   `Example: >Contig42 [organism=Drosophila melanogaster]`
4. Perform format validation in Sequin and export to submission file.
5. Create Bioproject, Biosample, SRA, and TSA entries.

4 Notes

1. Initial trials can be optimized on the basis of nanodrop measurements with 260/280 and 230/260 ratios providing an indication of purity from contaminants.
2. The BioAnalyzer will provide an indication of the quality of the RNA sample analyzed on the basis of the ribosomal RNA detected expressed as the [28S/18S] ratio. Location of the 28S and 18S peaks as well as the optimum ratio will differ across species so make sure you know what the correct values are for your species of interest. Insects in particular differ significantly in this respect from vertebrates.
3. There are different methods for building a cDNA library including simply outsourcing this step to a sequencing company. If you decide to perform the library preparation yourself you must ensure it is compatible with the downstream sequencing method.

4. Running aliquots of the same cDNA library on different lanes will act as technical replicates to control for variation between lanes.
5. The FastQC "Per Base Sequence Quality" and "Overrepresented Sequences" sections are especially useful for informing whether trimming or adaptor clipping should be performed on your dataset. Extensive documentation for FastQC is available on the website (*see* Subheading 2).
6. Adaptor sequences depend on the library preparation and sequencing method used.
7. CLC Gx Trimming tool only trims from the 5′ end therefore you have to bear this in mind when specifying the strand for the target sequence. In other words, a minus strand target search will remove the 3′ end complementary to the 5′ minus strand target.
8. There are many alternative de novo assembly programs and new implementations are released continuously.
9. These vary depending on the program/method used. CLC Gx utilizes for example word and bubble size parameters to guide the resolution of *de Bruijn* graphs. CLC Bio provides a white paper with further information (*see* Subheading 2).
10. Beware of the FASTQ file format, as multiple versions exist which encode the quality differently and the read mapper (such as Bowtie) may not be able to automatically detect what encoding has been used. Please read carefully the input specifications for your chosen mapping tool.
11. Specify number of letters to keep from the title of each FASTA entry. 0 = All. Only capitals are counted.
12. You will need to limit ORF to 200 nucleotides if uploading to NCBI as they do not accept sequences below 200 base pairs. A contig may yield more than 1 open reading frame.
13. FASTA titles will be amended with an underscore followed by a unique incremental digit and coordinates of the ORF relative to the input sequence in square brackets.
14. From the NCBI Browser you can filter what sequences to display and download the corresponding GI Number lists.
15. The logic underlying the grouping is that during the filtering and sorting steps, hits from different sources in each group will be competing to be the top hit but hits from different groups cannot. Group Alpha integrates reliable sources and reliable coding sequence coordinates. Group Beta integrates reliable sources but feature coordinates are more uncertain (likely yielding "partial" features). Group Charlie is an optional user-defined extension of the search scope. Delta and Zeta are last

resort groups for identifying sequences that failed to find any similarity with protein databases. Annotations inferred from Charlie, Delta, and Zeta may be less informative but will indicate if homologs are found in other species (thus excluding them from potential novel/species-specific sequences). Essentially, this approach adds a dimension of semantic reliability to standard inference of features by sequence similarity.

16. You may also use your own method or threshold for the Similarity Score Test.
17. This sorting will effectively allow hits to be prioritized by reliability of databases consulted. If acceptable hits exist in two or more groups, the most reliable group is prioritized. In the case that an alignment is identical (exact number of positive matches) priority will be given to the most reliable source. If no hits exist with a suitable similarity score, the hit with the highest similarity score should be selected by default.
18. A possible layout for such a table would include the following columns: Contig ID, Contig Length, ORF coordinates, Group, Source, Hit GI, Positives, evalue, bitscore, Similarity score.
19. The gene ontologies are regularly updated and you will want to select species that have been well annotated with GO terms.
20. You may find it helpful to make use of the basic annotation features to limit such de novo analyses to specific segments of the sequences from the transcriptome such as the predicted UTRs or coding sequences.

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Chapter 11

Live Imaging of Centriole Dynamics by Fluorescently Tagged Proteins in Starfish Oocyte Meiosis

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Abstract

High throughput DNA sequencing, the decreasing costs of DNA synthesis, and universal techniques for genetic manipulation have made it much easier and quicker to establish molecular tools for any organism than it has been 5 years ago. This opens a great opportunity for reviving "nonconventional" model organisms, which are particularly suited to study a specific biological process and many of which have already been established before the era of molecular biology. By taking advantage of transcriptomics, in particular, these systems can now be easily turned into full fetched models for molecular cell biology.

As an example, here we describe how we established molecular tools in the starfish *Patiria miniata*, which has been a popular model for cell and developmental biology due to the synchronous and rapid development, transparency, and easy handling of oocytes, eggs, and embryos. Here, we detail how we used a de novo assembled transcriptome to produce molecular markers and established conditions for live imaging to investigate the molecular mechanisms underlying centriole elimination—a poorly understood process essential for sexual reproduction of animal species.

Key words Starfish, Meiosis, Transcriptome, Fluorescent markers, Centrosome, Centriole elimination, Microinjection, Live imaging

1 Introduction

What we call now "nonconventional" model organisms have in fact been very popular model systems starting from the end of nineteenth century that contributed to key discoveries, setting the ground for modern cell biology (such as Boveri's chromosome theory or his discovery of the centrosome, just to name two examples [1–3]). Many of these were marine species, eggs and embryos of which are easily accessible, available in large amounts, and transparent to allow direct observations under the microscope. Indeed, in sea urchin embryos, one of Boveri's favorite model systems, the centrosomal microtubule asters are visible under a dissecting microscope simply by transmitted light.

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These model systems then "went out of fashion," because they were not amenable for genetics mostly due to their long life cycle. This led to the establishment of the currently known set of genetically tractable model organisms such as yeast, *C. elegans*, *D. melanogaster*, and more recently zebrafish and mouse. All these organisms have a well-characterized life cycle, relatively short generation times that allowed the establishment of inbred strains, which in turn enabled genome sequencing and setting up large collections of mutant lines through genetic screens.

Advances in molecular biology, such as DNA sequencing and genome editing, now provide a unique opportunity to broaden the range and revive some of the nontraditional organisms as systems for modern molecular cell biology. This will allow selection of the most suitable system from a much wider choice of organisms to study a specific physiological process. Additionally, the characterization of biological processes in multiple species will allow sampling of nature's diversity providing an understanding of how these processes might have evolved.

Here, we use centriole elimination to exemplify the advantages of characterizing a conserved process in a nonconventional model organism. Centrioles compose the major microtubule-organizing center of the cell, the centrosome. Centriole number is tightly controlled over cell generations, which is achieved by coupling the centriole duplication cycle with the cell cycle [4–6]. However, this cycle needs to be modified during fertilization: to avoid a surplus of centrioles in the zygote upon gamete fusion, centrioles are eliminated during female meiosis and only the sperm contributes with the active centrioles to the zygote [7, 8]. Centriole elimination occurs in all metazoan species, but the mechanism is far from being understood.

A main reason for this lack of understanding is that in most conventional model organisms including *C. elegans*, *D. melanogaster*, *X. laevis*, and mouse, centriole elimination occurs during the long meiotic prophase that is difficult to access experimentally [9–13]. Therefore, despite the availability of molecular tools in these species, a mechanism for centriole elimination has not been described. To the contrary, in some other "nonconventional" organisms including annelids, echinoderms, nemerteans, and mollusks centriole elimination occurs later, during the actual meiotic divisions [14–18]. This is a major advantage, because in these species centriole elimination can be followed live in the transparent oocytes undergoing synchronous and rapid meiotic divisions. However, the lack of specific molecular tools has complicated the study of this process in this group of animals.

Specifically, starfish (*Patiria miniata*) oocytes are available in large quantities and meiosis can be easily induced by addition of the maturation hormone. Moreover, starfish oocytes are transparent, and appear quite resistant to photodamage that are major advantages for live cell imaging. These advantages render starfish oocytes an

excellent model to study centriole elimination. Indeed, centriole elimination has been extensively studied in starfish oocytes in the pre-molecular era, for example by performing very elegant experiments of centriole transplantation, retention, and oocyte fusion [19–22].

Here, we describe how we established the required fluorescent molecular markers by first characterizing centriole composition in starfish by homology searches in a de-novo assembled transcriptome dataset. We then cloned several of these centriolar proteins as fluorescent protein fusions to test them as centriolar markers. We then established conditions for live imaging in the oocyte during meiosis, which allowed us to follow the entire centriole cycle and directly visualize when and where centrioles are eliminated.

2 Materials

2.1 Identification of Starfish Centriole Homologs

2.1.1 BlastP

1. Human protein database: http://www.ncbi.nlm.nih.gov/protein/
2. Sea urchin protein database: http://sugp.caltech.edu/SpBase/wwwblast/blast.php
3. Starfish database: http://www.lenartlab.embl.de:4567/
4. Tool to identify conserved protein domains: SMART (http://smart.embl-heidelberg.de/) or PFAM (http://pfam.xfam.org)
5. Multiple alignment tools: CloneManager (Sci-Ed Software), Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) or ESPript [23] (http://espript.ibcp.fr/ESPript/ESPript/index.php).

2.1.2 cDNA Library Preparation

6. Microcentrifuge tubes.
7. Centrifuge and rotor capable of reaching up to 12,000 × *g*.
8. TRIzol LS reagent (Thermo Fisher Scientific).
9. 18G syringe.
10. RNase-free water.
11. RNase-free DNase and corresponding buffer.
12. Phenol–chloroform solution (phenol, non-stabilized–chloroform–isoamyl alcohol 25:24:1).
13. Chloroform.
14. Isopropanol.
15. 80% cold ethanol (−20 °C).
16. Dynabeads Oligo (dT) kit (Invitrogen).
17. GeneRacer kit (Invitrogen).

2.1.3 Cloning

1. HotStar HiFidelity DNA polymerase and PCR kit (Qiagen).
2. Kapa Hifi DNA polymerase and PCR kit (Peqlab).

3. PCR Purification Kit.
4. pGEM®-T easy plasmid (Promega).
5. pJET1.2 plasmid (Thermo Fisher Scientific).
6. Restriction enzymes and respective buffers.
7. pmEGFP/pmCherry plasmids (with different coding frames).
8. Custom pGEM-HE plasmid (available at Addgene).
9. CloneManager (Sci-Ed Software).

2.1.4 In Vitro Transcription

1. DNA from midiprep.
2. RNase-free microcentrifuge tubes.
3. Restriction enzymes (single cutter) and respective buffers.
4. Phenol–chloroform solution (phenol non stabilized–chloroform–isoamyl alcohol 25:24:1).
5. Chloroform.
6. 100% ethanol.
7. 3 M Na-acetate.
8. 70% ethanol.
9. RNase-free water.
10. Amplicap Max™ T7 High Yield Message Maker kit (Cellscript).
11. A-Plus™ Poly(A) Polymerase Tailing Kit (Cellscript).
12. 100% isopropanol.
13. NanoDrop spectrophotometer.
14. Gel electrophoresis set.
15. RNA gel loading dye 2×.
16. Quantitative RNA ladder.

2.2 Oocyte and Sperm Collection

1. Biopsy puncher (3 mm).
2. Tweezers (No. 5).
3. Calcium-free seawater (437 mM NaCl, 9 mM KCl, 22.9 mM $MgCl_2$, 25.5 mM $MgSO_4$, 2.1 mM $NaHCO_3$, 50 mM phenylalanine, pH = 8).
4. Filtered seawater (use natural sea water or add 9.3 mM of $CaCl_2$ to the recipe above and leave out phenylalanine).
5. Small beakers (25 ml).
6. Disposable sterile petri dish (100 × 20 mm).
7. Pasteur pipette.
8. 100 μM acetylcholine.
9. 10 μM 1-methyladenine (1-MA).
10. Microcentrifuge tubes.

2.3 Chamber Preparation and Microinjection

1. U-shaped plastic microinjection chambers (EMBL mechanical workshop, technical drawings available upon request).
2. Double stick tape.
3. No. 1 22×22 mm coverslips.
4. Diamond pen.
5. Silicone grease, high viscosity.
6. CellTram oil microinjector (Eppendorf).
7. Mineral oil (to load the syringe, Sigma).
8. MM-92 motor-drive manipulator (Narishige).
9. Microscope adaptor (EMBL mechanical workshop).
10. Glass capillaries (50 μl microcaps, Drummond).
11. Loading capillaries (outer diameter 0.8 mm, inner diameter 0.6 mm, length 100 mm, Drummond).
12. Narishige PN-3 glass microelectrode horizontal needle puller.
13. 10 μl Hamilton syringe (Hamilton).
14. Mercury.
15. Dimethylpolysiloxane, viscosity: 20 cts (for the loading capillary, Sigma).
16. mRNA fluorescently tagged.
17. Inverted transmitted light microscope with movable stage (e.g., Nikon Ti-U).
18. Long working distance 10× objective (e.g., Nikon Plan fluor 10× N.A. 0.30 W.D. 16.0 mm).
19. Eyepiece micrometer.
20. Disposable sterile petri dish (100×20 mm).

2.4 Live Imaging

1. Confocal microscope (e.g., Leica SP5II equipped with a motorized XY stage, SuperZ Galvo stage and a 40× HCX PL APO 1.10 NA objective lens).

3 Methods

3.1 Identification of Starfish Centriole Homologs

Recently, it became fairly easy to obtain a transcriptome by following standard RNA isolation and purification protocols, followed by sequencing and assembly. We assembled de novo a *P. miniata* transcriptome from starfish eggs. We followed the manufacturer's instructions to extract total RNA using the TRIzol LS reagent, isolated polyA+ RNAs, and chemically fragmented mRNAs. The sequencing was performed on an Illumina HiSeq2000 platform using 50 bp paired-end reads at EMBL's Genomics Core Facility. For the assembly of the resulting reads, the Trinity software

package was used [24]. The technology still evolves rapidly, and therefore, it is difficult to give more precise recommendations. Note that companies such as GATC also provide transcriptome sequencing as a service.

3.1.1 BlastP

The first step is to select the candidate proteins that are likely to work as a fluorescent marker. Our general strategy starts with an extensive literature search that might also provide examples of functional fluorescent fusions (e.g., linker sequence, N- or C-terminal tag). Here we use Poc1 as an example to demonstrate the individual steps we followed from selection to cloning.

1. Finding the human protein: Poc1, as many other centriole protein, is known to be well conserved across species [25, 26] (Fig. 1a). Full sequence of human Poc1 can be found at http://www.ncbi.nlm.nih.gov/protein/NP_758440.1. Frequently, human proteins have several isoforms. Literature can help to choose the one most generally used in experiments. Otherwise, selecting the longest isoform might be the safest way to find homologs in other species. In case of Poc1 we selected the 478 aa "isoform A" (NP_758440.1) (*see* **Note 1**).
2. Perform an intermediate BlastP search (Fig. 1a): if the model organism does not have a reliable annotated genome database, searching homologs in a database of a more closely related species might be a good strategy. In our case, sea urchin is the closest related species with a sequenced and partially annotated genome (see http://sugp.caltech.edu/SpBase/wwwblast/blast.php).

   Our BlastP search found a single 353 aa isoform of a Poc1 homolog in the sea urchin proteome (database identification number: SPU_023342.3a) with a low *E*-value of 1×10^{-164}. The human and sea urchin proteins share 63% of sequence identity (*see* **Note 2**).
3. Finding starfish homologs (Fig. 1a): the starfish BlastP search was performed in our recently assembled transcriptome database that has been translated to proteins using the online tool available at http://kirschner.med.harvard.edu/tools/mz_ref_db.html [27]. The starfish protein database is publicly available at http://www.lenartlab.embl.de:4567/. Here, the best hit with an *E*-value of 0 is a 480-aa protein with the ID CL1998Contig1. This and sea urchin sequences share an identity of 89%.
4. Identification of conserved domains (Fig. 1c): many proteins carry conserved domains, which are shared between homologs. These domains can be identified using domain prediction tools (e.g., SMART (http://smart.embl-heidelberg.de/), or PFAM (http://pfam.xfam.org)). The presence of the same domain(s) provides an important verification of the presumed homology. In case of our starfish Poc1 candidate, similar to the

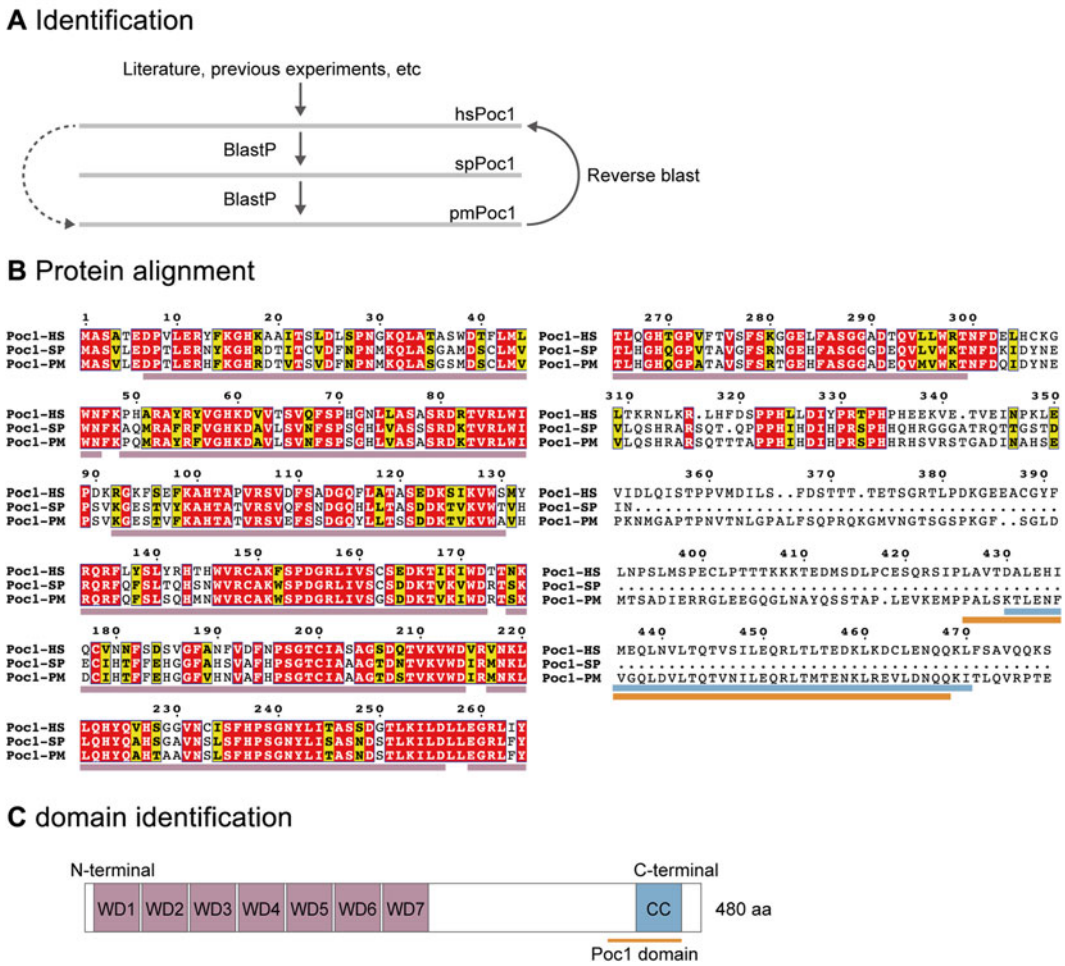

Fig. 1 Identification of starfish centriole homologs by BlastP. (**a**) BlastP steps are shown in a schematic. "hs," "sp," "pm" indicate human (*Homo sapiens*), sea urchin (*Strongylocentrotus purpuratus*), and starfish (*Patiria miniata*) homologs, respectively. The *dashed arrow* indicates that this intermediary step is not necessary in case the proteins are highly conserved. (**b**) Protein alignment between the different protein sequences identified. Amino acid identity is shown in *red. Yellow* indicates amino acids with similar chemical properties. (**c**) pmPoc1 is represented with domains color-coded. "cc" indicates a coiled coil domain, which partially overlaps with the conserved Poc1 domain (*orange*)

human and sea urchin sequences, seven WD40, one coiled coil and a characteristic Poc1 domains were identified.

5. Perform a reciprocal BlastP search (Fig. 1a): if your assumption regarding homology between the selected human and starfish sequences is correct, BlastP search in the human protein database using the starfish sequence should return the human sequence that you started with, as (one of) the highest ranking hit(s).
6. Align protein sequences: aligning the homologous sequences provides a good overview of the length, similarity and domain

structure of the selected proteins. It can also highlight larger insertions or deletions, which can be caused by sequencing/annotation errors, and should be carefully considered (for example, the Poc1 domain seems to be missing in the sea urchin Poc1. This might be caused by an assembly error—*see* Fig. 1b). Several online tools are available as Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/), ESPript [23] (http://espript.ibcp.fr/ESPript/ESPript/index.php).

3.1.2 cDNA Library Preparation

1. Pellet oocytes or embryos by centrifugation at high speed, and remove the supernatant before lysis.
2. Lyse pelleted oocytes by addition of TRIzol LS reagent (10 ml for each 0.75–1 ml of cells). We followed the manufacturer's instructions for homogenizing samples, phase separation, RNA precipitation, and RNA wash with a single modification: in the lysis step we used an 18G syringe.
3. Dissolve RNA in 100 μl RNase-free water—in this step all cellular RNAs are isolated (*see* **Note 3**).
4. Digest DNA by treatment with RNase-free DNase. Below, an example of this reaction for a total volume of 100 μl is given, which can be scaled up if necessary:
   (a) 87.5 μl RNA solution.
   (b) 10 μl enzyme buffer.
   (c) 2.5 μl RNase-free water.
5. Perform a phenol–chloroform purification:
   (a) Treat your RNA with a phenol–chloroform solution (1:1 volume).
   (b) Vortex for 30 s.
   (c) Spin for 5 min at 4 °C at maximum speed.
   (d) Recover upper phase to a fresh tube and repeat same procedure by adding chloroform (1:1 volume).
   (e) Recover upper phase to a fresh tube and repeat same procedure by adding isopropanol (1:1 volume).
   (f) Incubate for at least 20 min at −20 °C to precipitate RNA.
   (g) Centrifuge for 30 min at 4 °C at maximum speed.
   (h) Remove supernatant and wash pellet with 80% cold ethanol (−20 °C).
6. Centrifuge for 5 min at 4 °C and air dry pellet.
7. Dilute the pellet in RNase-free water (30–50 μl) and store at −80 °C.
8. Isolate all poly(A)-tailed mRNAs—we used Dynabeads Oligo (dT) kit.

9. Reverse-transcribe mRNAs—we used the GeneRacer kit following the manufacturer's instructions.

3.1.3 Cloning (See Fig. 2a)

1. Primer design: using the cDNA sequence encoding for the protein, design forward and reverse primers to amplify the full length DNA fragment carrying your gene of interest (*see* **Note 4**).
2. PCR amplification: using the cDNA library as template and the designed primers amplify your sequence by PCR—we used the HotStar HiFidelity DNA polymerase, which generates A-overhangs. Alternatively, we also used KapaHifi polymerase: this enzyme generates blunt end fragments. To avoid the introduction of unwanted mutations the PCR reactions are done at least in two parallel reactions and products are sequenced and compared with each other and with the transcriptome.
3. Transfer your purified PCR product to an intermediary vector. The DNA fragments generated by the previous PCR step can be ligated into plasmid vectors designed for this purpose: for fragments with A-overhangs, we used the pGEMT-Easy system, for blunt end fragments the pJET1.2 system.
4. Prepare a miniprep, providing a template for the next PCR step, in which you can extend your coding sequence with restriction sites for subsequent cloning steps. This step also removes the endogenous stop codon for C-terminal tagging. These modifications could have been added in **step 2**, but in our experience the success rate is higher by using this intermediary step.
5. Transfer your purified PCR product to an intermediary vector (repeat **step 3**).
6. *IMPORTANT NOTE*: as an alternative to the above described PCR amplification, the selected ORF can be synthetized directly, a service now offered by several companies (e.g., Genewiz, GeneScript, GeneArt). The costs are significant (currently about EUR 1 per amino acid), but unlike PCR the success is independent of the DNA sequence (e.g., GC content), abundance, etc. Additionally, any modification of the sequence such as introduction of additional restriction sites, codon optimization, if required, mutations, come for free.
7. Building the final construct:
   (a) pGEM-HE vector: this is a modified version of the plasmid pGEM optimized for in vitro transcription featuring a T7 promoter and a polyadenylation sequence. The multiple cloning site was adapted in order to be compatible with the mammalian pmEGFP/pmCherry plasmids. Restriction sites for rare enzymes such as SgrAI and AscI were introduced outside the multiple cloning site for linearization of the plasmid.

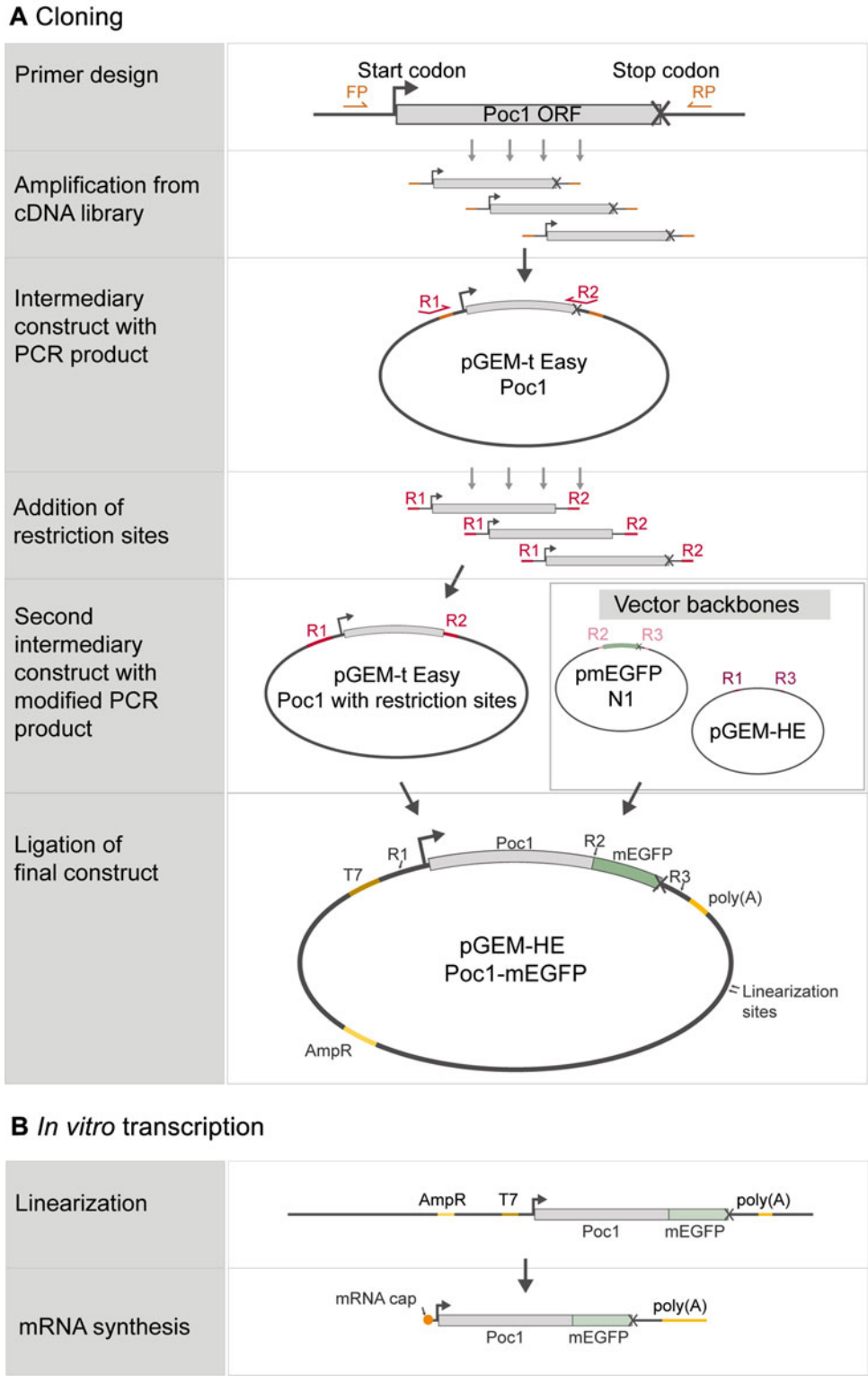

Fig. 2 Preparation of markers by cloning and in vitro transcription. (**a**) Schematics of the cloning steps. "FP" and "RP" indicate forward and reverse primers, respectively. (**b**) The process of in vitro transcription. The first step requires linearization of the plasmid, followed by in vitro mRNA synthesis

(b) Fluorescent tag: the family of the pmEGFP/pmCherry plasmids provides a variety of mEGFP and mCherry sequences with different coding frames, allowing tagging an ORF at the 3′ or 5′ end.

(c) ORF of your gene of interest (here Poc1): tagged with the required restriction sites.

8. We tagged Poc1 C- and N-terminally with mEGFP and mCherry (*see* **Note 5**).

3.1.4 In Vitro Transcription (Fig. 2b)

1. The first step to perform in vitro transcription is to linearize the pGEM-HE construct. We typically set up a digestion for a total volume of 200 µl, and digest about 15–30 µg of DNA (*see* **Note 6**).
2. Purify the DNA obtained by a phenol–chloroform extraction.
   (a) Add 200 µl of phenol–chloroform solution.
   (b) Vortex for 30 s.
   (c) Spin for 1 min at maximum speed and recover upper phase.
   (d) Repeat by adding 200 µl of chloroform, vortexing for 30 s and spinning for 1 min at maximum speed.
   (e) Transfer the upper phase to a clean microcentrifuge tube, and add 500 µl of 100% ethanol and 20 µl of 3 M of Na-acetate. Mix by pipetting up and down.
   (f) Centrifuge for 15 min at 4 °C at maximum speed.
   (g) Remove supernatant and wash pellet with 70% ethanol.
   (h) Centrifuge for 2 min at 4 °C at maximum speed.
   (i) Dry pellet and dissolve in 5 µl of RNase-free water. Out of this, dilute 1 µl in 9 µl of RNase-free water to test quality and quantity of your RNA by NanoDrop spectrophotometer. Run the rest on a 1% agarose gel, to assess successful linearization (*see* **Note 7**).
3. Transcribe DNA and cap its 5′ end. We used the AmpliCap-Max™ T7 High Yield Message Maker kit, and at room temperature (RT) conditions, assembled the following reaction, in the following order (all reagents are provided with the kit):

   x µl RNase-free water.

   y µl linearized DNA (at least 1 µg), where $x+y=5.5$ µl.

   2 µl 10× AmpliCap-Max T7 Transcription Buffer.

   8 µl AmpliCap-Max Cap/NTP PreMix.

   2 µl 100 mM DTT.

   0.5 µl ScriptGuard RNase Inhibitor.

   2 µl AmpliCap-Max T7 Enzyme Solution.

4. Incubate for 2 h at 37 °C.
5. Add 1 µl RNase-Free DNase I (provided with the kit).
6. Add a 3′ end poly(A) tail to the capped mRNA. We used the A-Plus™ Poly(A) Polymerase Tailing Kit, and assembled a reaction in the following order at RT conditions to a total volume of 132 µl (all reagents are provided in the kit):

   80 µl RNase-free water.

   0.6 µl ScriptGuard RNase Inhibitor.

   13.2 µl 10× A-Plus Poly(A) Tailing Buffer.

   13.2 µl 10 mM ATP.

   5 µl A-Plus Poly(A) Polymerase (20 U).
7. Incubate for 1 h at 37 °C.
8. To stop the reaction, add 15 µl ammonium-acetate stop solution (provided in the kit).
9. Purify the in vitro transcribed RNA by performing a phenol–chloroform purification: add sequentially phenol–chloroform solution (150 µl), chloroform (150 µl). After addition of each reagent:

   (a) Vortex for 30 s.

   (b) Spin for 1 min at maximum speed.

   (c) Transfer supernatant to a fresh tube.

   Finally precipitate mRNA in 100% isopropanol (150 µl).
10. After isopropanol addition, incubate for at least 15 min at 4 °C (*see* **Note 8**).
11. Remove supernatant and dry pellet.
12. Dilute the purified mRNA in 11 µl of RNase-free water (*see* **Note 9**).
13. Check RNA concentration:

    (a) Add 9 µl of RNase-free water to 1 µl of purified RNA.

    (b) Use 1 µl to check concentration and quality in a NanoDrop spectrophotometer.

    (c) To the rest add 9 µl of RNA gel loading dye 2×.

    (d) Denature at 65 °C for 10 min.

    (e) Prepare 18 µl RNA ladder (premixed with RNA gel loading dye) (see Note 10)—we used RiboRuler high range RNA ladder (Thermo Fisher Scientific).

    (f) Run a standard agarose gel to check the RNA produced.
14. Inject your mRNA the day before imaging, to allow protein expression overnight (*see* **Note 11**).

3.2 Oocyte and Sperm Collection

The protocol below is based on the methods described in detail by Jaffe and Terasaki [28] with minor modifications.

3.2.1 Oocyte Collection

1. Puncture a small hole in one arm of a female starfish with a disposable biopsy punch (*see* **Note 12**).
2. Use the forceps to pull a piece of ovary out and transfer them to the beakers containing approximately 20 ml of calcium-free seawater supplemented with phenylalanine (phenylalanine helps to prevent spontaneous maturation). Calcium-free seawater removes the surrounding follicle cells.
3. Keep ovaries for 20 min in calcium-free seawater supplemented with phenylalanine. Wash once (*see* **Note 13**).
4. Transfer ovaries to a petri dish with filtered seawater supplemented with 100 μM acetylcholine, which cause the ovary to contract releasing the oocytes.
5. Collect oocytes with a Pasteur pipette and transfer them to a fresh petri dish containing fresh filtered seawater. Oocytes can be kept for 2 days at 14 °C.

3.2.2 Sperm Collection

1. Puncture a small hole in the arm of a male starfish.
2. Pull a piece of testis out and transfer it to a microcentrifuge tube.
3. Keep the testis "dry" at 4 °C for up to 3–4 days.
4. Prepare a dilution of a small piece of the testis of around 1:8000 in sea water just before fertilization (*see* **Note 14**).

3.3 Chamber Preparation and Injection

For mRNA injection and imaging, we mount oocytes in a U-shaped plastic microinjection chamber originally described by Kiehart [29] and described in detail at http://mterasaki.us/panda/injection/ and by Jaffe and Terasaki [28] (Fig. 3). In these chambers, oocytes are lined up, in a row on a shelf formed between two coverslip using double stick tape as spacer. Typically, 1–2 μl of dense suspension oocytes (settled by gravity) are loaded (Fig. 3). The assembled chamber allows to keep oocytes in place and in seawater during injection, for overnight incubation and imaging. Additionally, seawater can be exchanged by seawater containing either the maturation inducing hormone (1-MA) or inhibitors, while oocytes remain in the chamber without moving or being lost. Fertilization can also be performed in this chamber.

For microinjection, we also use the setup described by Jaffe and Terasaki [28] with a few adaptations: the micromanipulator is mounted on an inverted microscope and consequently, the objective lens is moved for focusing while the stage remains at a stable Z position—unlike on the original setup by Terasaki that uses an upright microscope. Therefore, during injection the injection chamber is only moved in XY, and if everything is properly aligned, a simple U-shape movement is sufficient to bring the needle from the loading

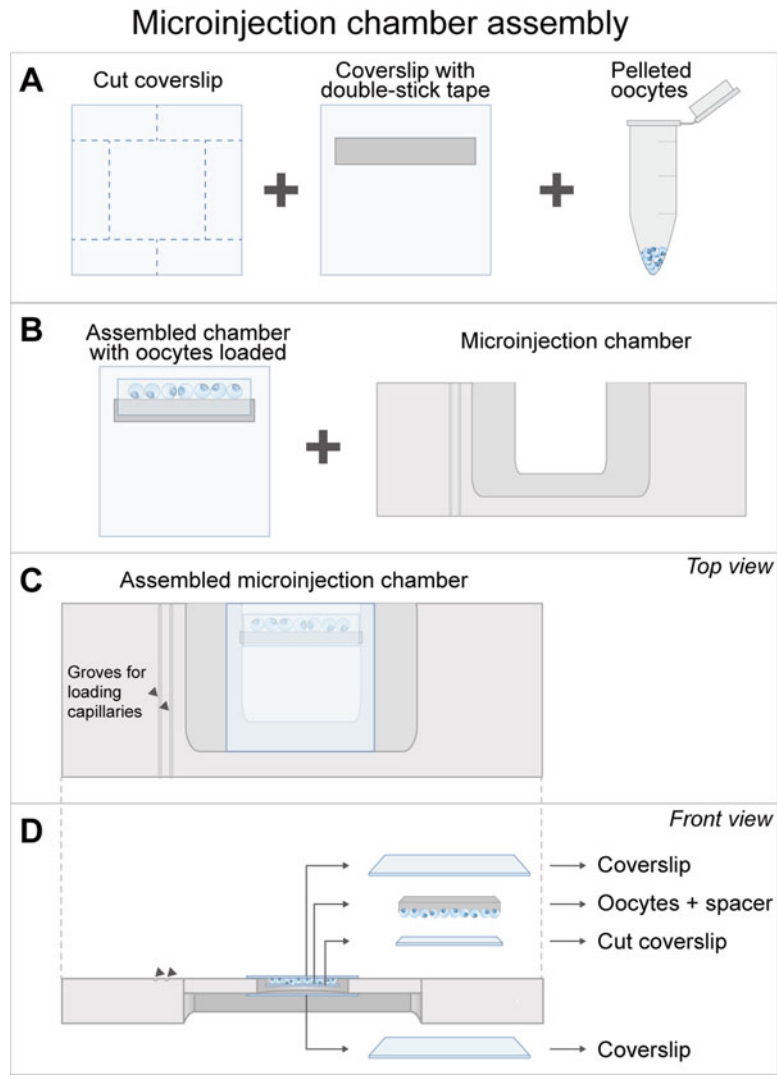

Fig. 3 Assembly of the microinjection chamber. (**a**) Cut coverslips with a diamond pen. Stick a double layer of double sided tapes and onto a coverslip. Let oocytes settle in a microcentrifuge tube. (**b**) Stick the cut coverslip piece onto the double-stick tape, leaving a small "shelf" between the two. Load 1–2 μl of oocytes into the shelf. (**c** and **d**) Top and front view of the assembled microinjection chamber. The coverslip containing the oocytes is mounted on the top, and an additional coverslip without oocytes seals the chamber on the bottom side. Silicone grease is used to stick the pieces of the assembly together. The chamber is then filled with seawater (not shown in the figure). The loading capillaries are placed in the groves (*arrowheads*)

capillary to the oocytes and back. One just has to refocus when the needle enters in the chamber due to a difference in the refraction index of air and water. Additionally, we use a different injection syringe (CellTRam Oil) and a motor-drive micromanipulator (MM-92) to control the movements of the needle in *XYZ*.

1. Pull needles from Drummond Microcaps 50 with a Narishige PN-3 glass needle puller.
2. Fill the needles with approximately 1 μl of mercury, using a Hamilton syringe. The mercury helps balancing the pressure in the system required for smooth and precise control of injection.
3. Load the loading capillary that will contain your mRNA and non-reactive silicone oil (see http://mterasaki.us/panda/injection and [28]) (Fig. 4a) (*see* **Note 15**).
4. Insert the loading capillary into the groves on the U-shaped chamber (Fig. 4a).
5. Insert the injection needle (filled with mercury) into the needle holder.
6. Find the loading capillary in the eyepiece and focus on the middle of the capillary. Then, move the needle into the field of view and in focus. Align the needle with the scale in the ocular, so that the needle tip is aligned with the "0" of the scale. Once the needle is positioned this way, no additional movements of the needle should be required. The repeated cycles of needle loading and injection are performed by moving the stage in XY and turning the focus wheel to refocus as required (Fig. 4b) (*see* **Note 16**).
7. Break the tip of the needle by bumping it against the wall of the loading capillary to result in an opening of a couple of micrometers (Fig. 4c).
8. Push the mercury to the front of the needle (Fig. 4c).
9. Front load the needle with a large amount of oil (Fig. 4d) (approx. 100 units on the eyepiece micrometer). If the oil does not flow in, try breaking the tip of needle a bit more.
10. Load mRNA (normally around 30–50 units of injected mRNA) (Fig. 4e). The ocular micrometer allows precise control over the amounts of mRNA injected.
11. Load an oil cap (around 10–20 units) (Fig. 4f). The presence of a small oil cap is important to avoid mRNA getting in contact with seawater.
12. In a U-movement with the stage, bring the needle inside the chamber (Fig. 4f, g) (*see* **Note 17**).
13. Once inside the chamber, the focus of your needle will change and it is important that you refocus before going deeper into the chamber. Seawater might flow inside your needle, which you will also have to control (Fig. 4g).
14. Bring the needle close to the oocyte. Find the "equator" of the oocyte (the plane where is diameter is largest) by turning the focus wheel and move the needle to the same plane (Fig. 4g).
15. Inject the oocyte with mRNA and oil cap. The oil forms a drop inside the oocyte and labels the oocytes that were injected (Fig. 4h, i) (*see* **Note 18**).

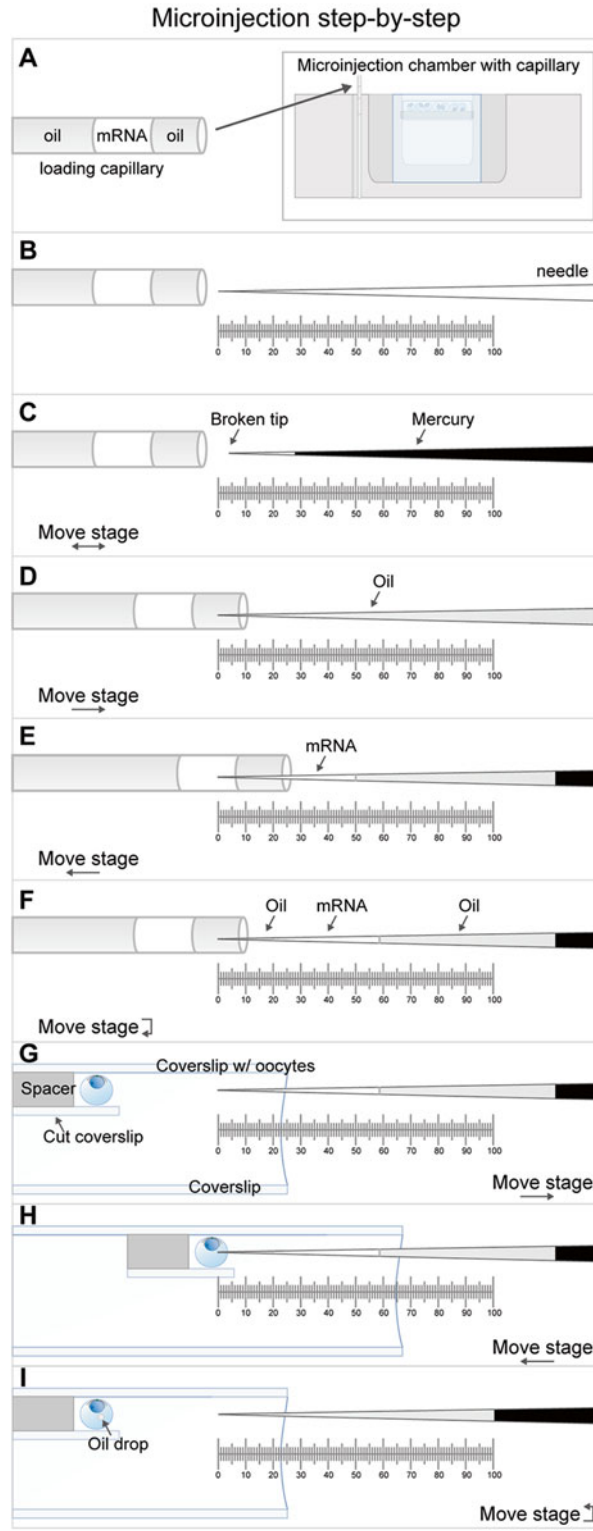

Fig. 4 Steps of microinjection (see text for descriptions)

16. Take the needle out of the chamber—some oil might flow out once you are out of the chamber, which you should control.
17. By moving the stage, return the needle to its position in front of the loading capillary, refocus needle with the center of the capillary. Reload the needle with oil, mRNA, oil.
18. Repeat the procedure to inject as many oocytes as desired.
19. Leave the microinjection chamber in a wet chamber (e.g., a petri dish with a wet piece of paper tissue in it) at 14 °C overnight to allow protein expression.

3.4 Live Imaging

In the following section, we summarize some considerations for live imaging of centriolar markers in starfish oocytes that we think might be generally helpful when planning similar experiments. Here we give specific instructions for using a Leica SP5II confocal microscope, but very similar settings are available on any commercial laser scanning confocal system.

Our SP5II confocal is equipped with a motorized XY stage, a fast Z-focusing device (SuperZ Galvo stage) and a 40× HCX PL APO 1.10 NA water immersion lens. The imaging was performed in a room air conditioned to 20 °C. For the example used here the oocyte is expressing Poc1-mEGFP (Fig. 5).

1. Turn on the computer and microscope.
2. Find the oocyte to be imaged by transmitted light.
3. Turn on the laser(s), 488 nm in the example used here for imaging mEGFP. Choose settings and then 12-bits in the properties to record data at a wider dynamic range.
4. Select mode of acquisition. As we are imaging in 3D and over time, we used XYZT mode.
5. Select the properties of laser scan: scan speed and unidirectional or bidirectional scan. We typically use bidirectional scan, which allows faster imaging in combination with relatively high scan speeds (700 or 1000 Hz).
6. Select the number of lines to average. Normally, we select a three to five-times line average, depending on the required frame rate (more averaging will proportionally increase scan time). As most of the processes we image are quite dynamic, we chose line instead of frame averaging to avoid blurring effects caused by the movement of the sample.
7. Pick image size. Ideally, the physical pixel size should match the resolution limit of the objective (for the objective used here the resolution is ~250 nm). This can be achieved either by adjusting the zoom or changing the number of pixels. In the example shown, we used a zoom of 5.5 and an image size of 400 × 400 pixels resulting in a pixel size of approx. 164 nm (in this case the image is oversampled), conveniently covering the meiotic spindle area of approx. 65 × 65 μm.

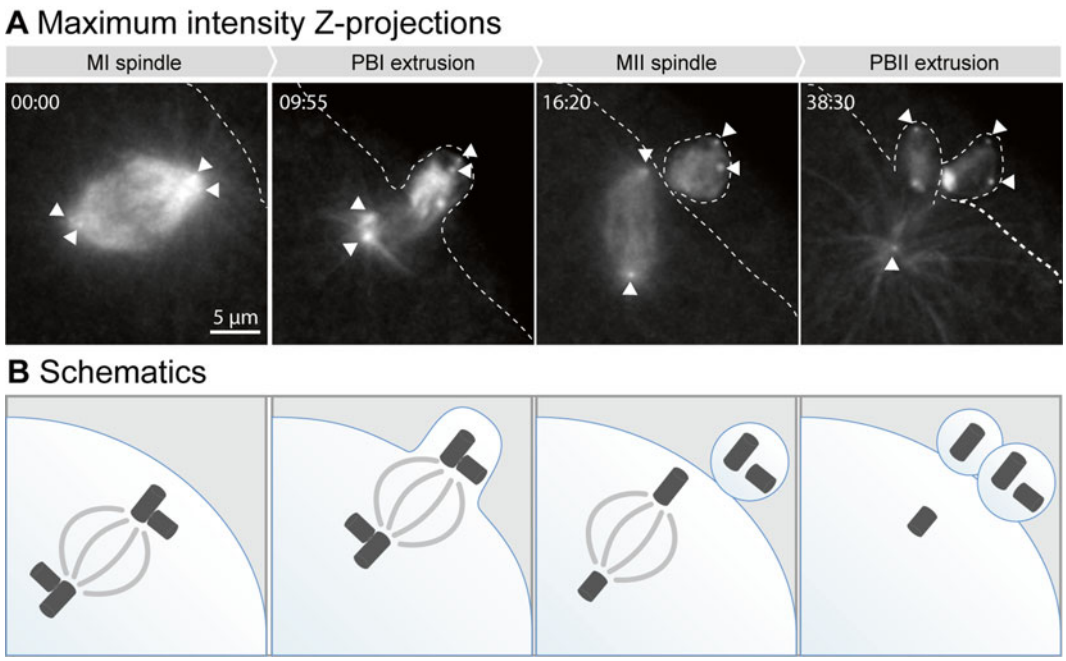

Fig. 5 Poc1 labels centrioles and the microtubule spindle during starfish meiosis. (**a**) Key steps of meiosis. During "MI," metaphase I, two centrioles localize at each pole of the spindle (partially hidden behind the dense microtubule arrays). During "PBI," the first polar body extrusion, one pair of centrioles is extruded into the first polar body, and one pair remains. During "MII," metaphase II, single centrioles localize at the poles of the spindle. A single centriole is extruded during "PBII," the second polar body extrusion, and one single centriole remains in the cytoplasm of the mature egg. Panels correspond to maximum intensity projections of selected Z-stacks. Raw data was filtered in Fiji with a Gaussian blur 3D (sigma: 0.6), cropped and resized by cubic interpolation. Z-stacks were acquired every 35 s. Time is indicated in mm:ss. Scale bar: 5 µm. Centrioles are indicated by *arrowheads*. (**b**) A schematic representation of each stage is shown

8. Pick the Z-step size and the number of Z-stacks you want to acquire. We typically select around 15–25 stacks with a Z-step size of 1–1.5 µm to cover an about 20 µm thick volume occupied by the spindle with overlapping optical sections. These imaging settings allowed us to image a single oocyte in approx. 10–35 s (depending of the imaging settings). In this specific case we imaged a single oocyte with a scan speed of 700 Hz, five-times line average, with 22 Z-stacks and Z-step size of 1.5 µm, in 35 s.
9. Select the pinhole size. We selected a pinhole size of approx. 1.7 Airy units—we found this pinhole size to be a good balance between the signal collected and Z resolution (*see* **Note 19**).
10. Choose the appropriate lasers to the fluorescent proteins the oocytes are expressing, and select their intensity. For our example, we used the 488 nm laser. We are using powers between 5 and 15 %. The laser intensity that is still tolerated

by the cells will need to be empirically determined and optimized for not only every cell type, but every assay: different cellular processes will have different light sensitivity and changing imaging settings (e.g., zoom) also changes the light dose.

11. Select the appropriate dichroic mirrors: here we used a 488/561 nm double dichroic mirror.
12. Select detectors and the range of wavelengths to be detected based on the emission spectra of the fluorophores imaged (available at for example https://www.chroma.com/spectra-viewer). In our example 490–550 nm for mEGFP. Semiconductor based HyD detectors are a great advantage for live imaging applications as these are about twice as sensitive as photomultiplier tubes (PMT).
13. Adjust the gain to spread the signal across the dynamic range while avoiding saturation.
14. If the microscope is equipped with a motorized XY stage, mark the positions of the oocytes. This allows simultaneous imaging of multiple oocytes in the chamber. In our example, we could image four to five oocytes per chamber every 50–60 s.
15. Start recording. Poc1-mEGFP labels centrioles and microtubules simultaneously.
16. *ADDITONAL CONSIDERATIONS:* for all live imaging applications, one needs to consider the "photon budget," the number of photons that the sample is able to emit in a given amount of time without damaging the sample. It is always a compromise to use this budget that should be divided in the best possible way between resolution in XY, in Z, in time as well as for signal-to-noise.

    For example, if the goal is fast imaging and good image quality:

    (a) Reduce the number of Z-stacks acquired.

    (b) Select bidirectional scan.

    (c) Zoom in the area of interest.

    (d) Reduce the number of oocytes imaged in parallel.

    If bleaching and light sensitivity are issues:

    (a) Decrease the time resolution.

    (b) Decrease laser power.

    (c) Increase gain.

    (d) Optimize imaging setting for fast frame times: reduce the number of pixels, increase scan speed, reduce averaging.

    (e) Consider opening the pinhole.

4 Notes

1. It is important to keep a table with the records of the name of the proteins, GenBank ID or NCBI reference sequence, coding and protein sequence and respective number of nucleotides and amino acids (aa). Other factors as % identity and *E*-value might be important to note as well.
2. Although this intermediate blast search might be very useful, in many cases it may not be necessary, as highly conserved proteins can possess enough similarity to allow identification of homologs between distantly related species (Fig. 1a, dashed line).
3. As lipids are often hard to remove, you might see some indissoluble lipid droplets at this stage. Avoid pipetting those into the next tube.
4. Depending on the redundancy and the length of the selected cDNA sequence, PCR amplification of longer fragments from a cDNA library might be problematic. We tend to have complications above the size of 2 kb. In such cases, amplification in two smaller pieces could be an effective strategy. In that case, you can amplify overlapping fragments carrying a unique restriction site in the shared region, which can then be ligated. Our primers were produced by the company Sigma-Aldrich.
5. Virtual DNA cloning software, as for example CloneManager, is very helpful in designing PCR primers and cloning strategies.
6. We have noticed that the in vitro transcription works better if we start with DNA purified from a midiprep. Use single cuter enzymes that do not cut in your sequence of interest but can linearize the plasmid. We normally use SgrAI and AscI simultaneously to increase the yield of plasmid linearization.
7. Linearized DNA can be stored at −20 °C.
8. A longer incubation at −20 °C seems to facilitate RNA precipitation and consequently increase its concentration. RNA can be left overnight at this step.
9. We get typical mRNA concentrations in the range of 2–6 μg/μl.
10. This RNA ladder will help you to quantitatively assess the amount of RNA on the gel. Load respectively 2 μl, 5 μl, and 10 μl of the ladder on your gel. For each 2 μl, 120 ng of RNA is present.
11. Initially we inject oocytes with different amounts of mRNA to assess the correct amount to be injected.
12. Starfish have two rows of ovaries per arm, thus ten ovaries in total. As the wound heals typically after 2 days one starfish can be used for over a month of experiments.
13. Additionally, keeping oocytes in large amounts (~10 ml) of seawater helps to prevent spontaneous maturation.

14. As starfish fertilization is external, seawater activates the sperm, which are motile just for a few minutes after dilution.
15. Amounts of mRNA as little as 0.4 μl can be used for loading and the same capillary can be reused several times, and stored at 4 °C for about a week.
16. This only works if the needle and holder are aligned horizontally to the stage—make sure this is the case.
17. To prevent the needle to bump against the coverslip upon chamber entry (in case the system is misaligned), you might move the needle slightly down, just until the needle becomes slightly unfocused. This also means that you will have to readjust the level of the needle by going up inside the microinjection chamber.
18. We prefer to inject only the oocytes with the nucleus in the upper half of the oocyte, i.e., facing the top of the chamber (*see* Fig. 4). During imaging, this side of the chamber faces to the objective, thus the distance between the objective and the area to be imaged will be significantly less.
19. A larger pinhole size will increase the signal but degrade Z resolution. An important consideration is to compare the Z-step with the optical slice thickness defined by the pinhole size. We recommend acquiring slightly overlapping optical slices to avoid loss of information between Z slices (that will be visible as empty stripes between the slices in a Z-projection).

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Chapter 12

High-Pressure Freezing Electron Microscopy of Zebrafish Oocytes

Palsamy Kanagaraj, Dietmar Riedel, and Roland Dosch

Abstract

Oogenesis is an essential cellular and developmental process to prepare the oocyte for propagation of a species after fertilization. Oocytes of oviparous animals are enormous cells endowed with many, big cellular compartments, which are interconnected through active intracellular transport. The dynamic transport pathways and the big organelles of the oocyte provide the opportunity to study cellular trafficking with outstanding resolution. Hence, oocytes were classically used to investigate cellular compartments. Though many novel regulators of vesicle trafficking have been discovered in yeast, tissue culture cells and invertebrates, recent forward genetic screens in invertebrate and vertebrate oocytes isolated novel control proteins specific to multicellular organisms. Zebrafish is a widely used vertebrate model to study cellular and developmental processes in an entire animal. The transparency of zebrafish embryos allows following cellular events during early development with in vivo imaging. Unfortunately, the active endocytosis of the oocyte also represents a drawback for imaging. The massive amounts of yolk globules prevent the penetration of light-beams and currently make in vivo microscopy a challenge. As a consequence, electron microscopy (EM) still provides the highest resolution to analyze the ultra-structural details of compartments and organelles and the mechanisms controlling many cellular pathways of the oocyte. Among different fixation approaches for EM, High Pressure Freezing (HPF) in combination with freeze substitution significantly improves the samples preservation closest to their natural status. Here, we describe the HPF with freeze substitution embedding method for analyzing cellular processes in zebrafish oocytes using electron microscopy.

Key words Zebrafish, Oocyte, High pressure freezing, Electron microscopy

1 Introduction

Zebrafish emerged in recent years as an excellent model system to study cellular processes in an entire vertebrate. The embryo rapidly grows outside the mother and finishes developing major organs within 36 h post fertilization (hpf) [1]. Furthermore, the transparent embryo makes zebrafish an easily accessible vertebrate model for microscopic observations and live imaging without the need for dissection. This feature is used extensively in combination with forward genetic screens to isolate the endogenous key factors regulating cellular processes (reviewed in [2]). Moreover, the

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panoply of reverse genetic tools, including the high efficiency of CRISPR-mediated mutagenesis, makes zebrafish amenable to examine the endogenous role of control genes quickly [3]. A critical asset for efficient molecular genetics is its sequenced genome [4], of which 75% is conserved to humans [5], and 84% of human disease genes have a zebrafish homolog [6]. This genomic homology to humans positions zebrafish as an important model to discover the cellular mechanisms of health and disease.

Oocytes are highly specialized cells with multiple cellular events preparing the egg for fertilization and early embryogenesis. For instance, in oviparous animals the mother needs to provide the entire nutrition through yolk proteins until the embryo develops the ability to take up food. Hence, the oocyte is very active in vesicle transport, as the cell needs to import vast amounts of Vitellogenin protein through receptor-mediated endocytosis [7]. As a consequence, its cellular organelles and compartments are larger in size compared to somatic cell types. The large compartments help to observe cellular events easily as compared to other cells and hence, the clathrin coat was first discovered in mosquito oocytes [8]. Subsequently, the oocyte system was very popular to study endocytosis and vesicle trafficking of yolk and led to the isolation of interesting proteins such as the Vitellogenin receptor [9]. However, the power of biochemistry in tissue culture cells and forward genetic screens in yeast *S. cerevisiae* led to a decline of the oocyte as a vesicle trafficking model. Interestingly, in recent years vesicle trafficking research in eggs enjoyed a renaissance mainly in *C. elegans* (a nematode), where genetic screens isolated numerous novel regulators using the oocyte with its active transport system as a sensitive readout [10, 11]. Therefore, the highly active endocytosis in oocytes combined with genetic screens has been quite successful in discovering novel regulators of vesicle trafficking.

Zebrafish eggs are large cells with a diameter of about 700 μm [12, 13] and their cellular compartments and organelles are also relatively big, e.g., lysosomes in zebrafish eggs have on average a diameter of around 5 μm, which is close to the size of a yeast cell. This enormous growth of a single cell occurs during oogenesis, whereby the zebrafish oocyte increases its volume by 3000-fold within 10 days, mostly by importing Vitellogenin [7]. In addition to the high import activity, the zebrafish oocyte also prepares for a massive exocytosis event at fertilization. As soon as the egg is released into the water the cortical reaction is activated. During this process, a vast amount of secretory granules release their contents by exocytosis comparable to an active neuron. The cortical reaction leads to the elevation of the acellular chorion membrane [14, 15]. This reaction is a critical process to prevent polyspermy and thereby guarantees the survival of the embryo. As the contents of the secretory granules are synthesized by the oocyte itself, it needs to tightly control endocytic and secretory pathways

simultaneously. Interestingly, a recent study revealed that import and export routes in the oocyte are connected [16].

The combination of zebrafish genetics and the oocyte with its high vesicle trafficking activity provides an excellent system to characterize novel regulators in vertebrates. Furthermore, they are amenable to in vitro culture, which for example, permits chemical treatments or trafficking assays [16, 17]. However, the large size of the oocytes and their high lipid and yolk protein content currently renders them unsuitable for high-resolution imaging like STED microscopy or live imaging.

Electron microscopy (EM) achieves the highest resolution at the cellular level to study the subcellular defect in mutants, which is crucial to determine the specific function of a gene [18]. Hence, a critical step during sample preparation for EM is fixation and dehydration to maintain the architecture of the tissue in its natural status. However, conventional methods of sample processing at room temperature sometimes create artifacts like shrinkage and distortion of samples such as oocytes. Many different sample preparation methods have been developed to overcome these problems. Among them, High Pressure Freezing (HPF) combines numerous advantages, as it keeps the tissue close to its native status [19]. Moreover, other groups have shown in *C. elegans* that HPF maintains fluorescent signals after freeze substitution and HPF, which allows using the same section for fluorescent imaging, immunogold labeling and electron microscopy [20]. HPF is a physical method of fixing by ultra-quick freezing a biological sample in the vitreous state [21, 22]. HPF together with freeze substitution is applicable to thicker samples like zebrafish embryos [20]. However, it is still limited to sample thickness of 200 μm, because the reduction in cooling rate by thicker tissues causes less vitrification of the sample. Fortunately, early stage oocytes in zebrafish are still small enough to use in HPF. As HPF achieved excellent resolution in our hands, we wanted to make this method accessible to researchers interested in oogenesis and/or zebrafish embryogenesis by providing a step-by-step methodology.

2 Materials

2.1 Ovary Dissection and Purifying Staged Oocytes

1. Castroviejo Corneal scissors (HSB 517-10, Micro scissors) (*see* **Note 1**).
2. Scalpel blade.
3. Two pairs of forceps (FST Dumont #5).
4. Tricaine (A-5040, Sigma): 0.015 %—Stock solution: 0.4 % wt/vol., 400 mg tricaine in 97.9 ml water and 2.1 ml Tris–HCl (pH 9.0). Dilute 4.2 ml of stock solution up to 100 ml in fishwater and store at 4 °C.

5. Ice (to prepare ice water).
6. Dissection plate (90 mm petri dish).
7. Plastic Pasteur pipette.
8. Spin column—DNA binding filter is replaced by nylon mesh (*see* **Notes 2** and **3**).
9. Cell strainer (BD Falcon, 40 μm, Blue, Ref no. 352340).
10. Nylon mesh with pore sizes 125, 300, and 700 μm.
11. 6-well plate.
12. Sterile OR2 buffer: 82.5 mM NaCl, 2.5 mM KCl, 1.0 mM $MgCl_2$, 5.0 mM HEPES pH 7.8, sterile filter using 0.2 μm filters.
13. 60% Leibovitz L-15 medium: 0.3 mg/ml glutamine, 50 U/ml penicillin, 0.05 mg/ml streptomycin, 0.8 mM $CaCl_2$, and 3% fetal calf serum, use after sterile filtration.
14. Oocyte culture media: 60% L-15, 15 mM HEPES pH 7.5, 1 μM insulin, 1 mM glutamine, 100 μg/ml gentamycin, 50 U/ml penicillin/streptomycin, 1 mM nystatin, and 5 μl of DHP (17α-20β-dihydroxy-4 pregnen-3-one) 0.5 mg/ml for inducing oocyte maturation.
15. Liberase (Roche TM research grade #05401119001) (*see* **Note 4**).
16. Dissection microscope.

2.2 High Pressure Freezing and Imaging

1. Electron Microscope: Philips CM120 electron microscope, TemCam 224A slow scan CCD camera (TVIPS, Gauting, Germany).
2. Fixative: anhydrous acetone, 0.1% tannic acid low molecular weight (CAS #1401-55-4), 0.5% anhydrous glutaraldehyde, 1/10 g OsO_4 crystal (EMS Electron Microscopy Science, Ft. Washington, USA).
3. High pressure freezer: Leica EM HPM100 high pressure freezer (Leica Mikrosystem Vertrieb GmbH, Wetzlar, Germany).
4. Freeze substitution: Automatic Freeze Substitution Unit Leica EM AFS2.
5. Aluminum platelets 150 μm cavity (Engineering Office M. Wohlwend GmbH CH-Sennwald), Agar 100 (Epon 812 equivalent, Agar Scientific LtD Essex, England) and 1-hexadecane.

3 Methods

3.1 Ovary Dissection

1. Anesthetize the female fish in 0.015% tricaine and euthanize it in ice water for 5 min.
2. Decapitate the fish with a scalpel blade (*see* **Note 5**).

3. Cut the skin along the ventral midline from the decapitated end to the anal fin. Be careful not to penetrate into the ovary with the scissor blade (*see* **Note 6**).
4. Remove the gastrointestinal tract, swim bladder and liver. With the two forceps reorient the fish with the incision upwards and pull the skin apart to expose the yellowish ovary in the body cavity.
5. Pull or scratch the bilobed ovary from the body cavity using the forceps without damaging the oocytes.
6. Transfer the ovary to a small petri dish on ice with precooled OR2 buffer for short time periods until all dissections are finished (*see* **Note 7**).

3.2 Purifying Staged Oocytes

1. Prepare a 2 ml collection tube: Insert a spin column with a 600 μm pore size nylon mesh at the bottom into a 2 ml collection tube and fill the tube with OR2 buffer (Fig. 1a).
2. Incubate the ovary with 12 μl Liberase enzyme (14 Wünsch/ml) in OR2 buffer for 30–60 min and keep checking the ovary until the tissue is loosened (*see* **Note 8**).
3. To accelerate the dissociation, carefully pipette the ovary up and down every 15 min with a plastic pipette. Simultaneously, collect the separated oocytes from the enzyme solution to avoid over-digestion of oocytes (*see* **Note 9**).
4. Once the ovary is loosened, remove the enzyme solution and wash twice with OR2 buffer.
5. Transfer the loosened ovary into the column with a nylon mesh at the bottom in OR2 buffer.
6. Slowly move the column up and down manually inside the collection tube to separate single oocytes from the ovary.
7. Carefully transfer the single oocytes using a Pasteur pipette into 6-well plate containing 2 ml OR2 for further staging (*see* **Note 10**).
8. Prepare cell strainers with different nylon mesh size (40, 125, 300, and 700 μm pore size) to collect the corresponding oocyte stage. Stage I oocytes are collected using the filter with the pore size of 40 μm, stage II with 125 μm, stage III with 300 μm, and stage IV with 700 μm (e.g., the 300 μm sieve retains stage III and IV oocytes, whereas stage II and I will pass through the filter which can be separated using the filter with the small pore size) (Fig. 1b).
9. Stage IA oocytes can be concentrated by spinning at 25 rpm for 5 min to pellet the oocytes after separation (*see* **Note 11**).
10. Wash the oocytes with OR2 buffer or 60% L-15 media, if you want to culture the oocytes further.

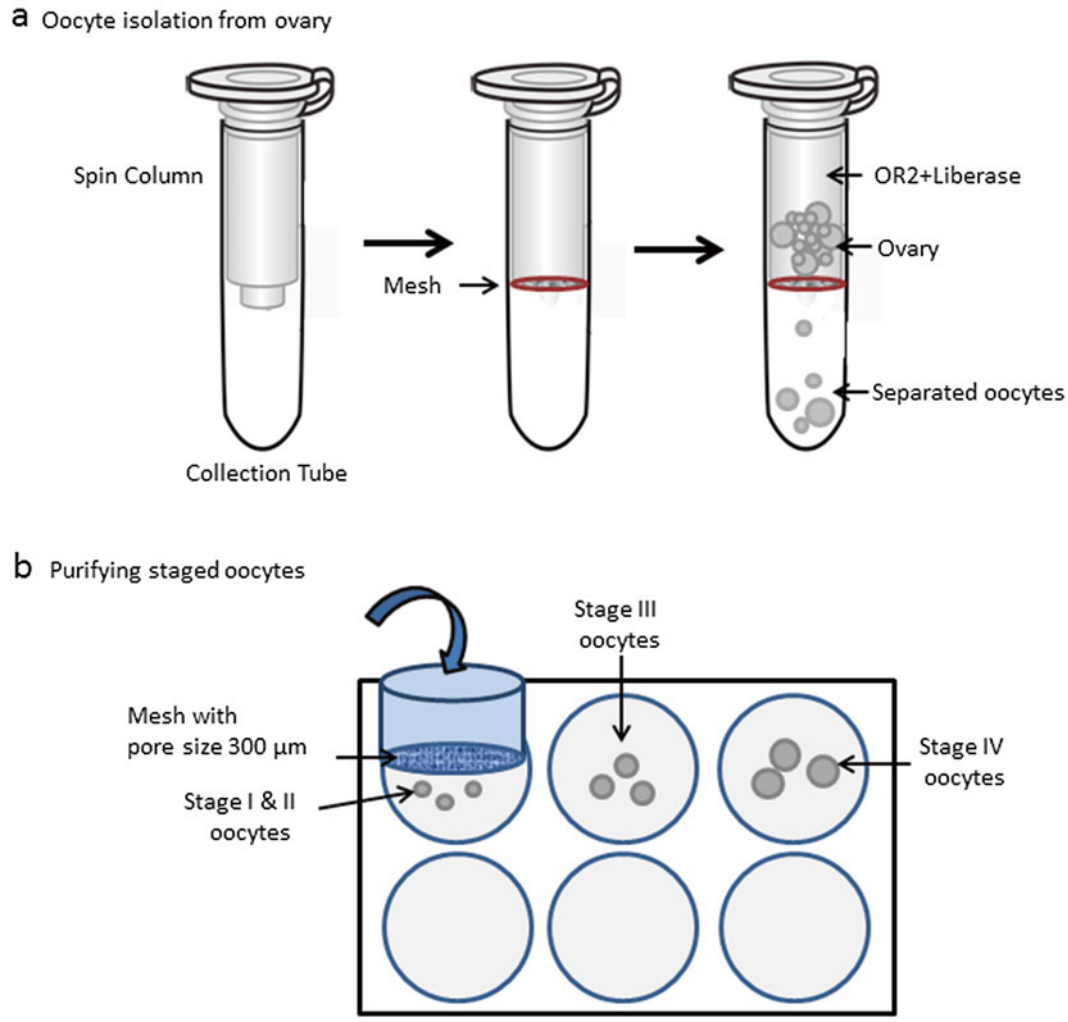

Fig. 1 Oocyte isolation and staging. (**a**) Oocyte isolation. The cartoon illustrates the modification of a spin column for the purification of staged oocytes by sticking a 700 µm nylon mesh at the bottom. Then the oocytes are placed inside the column with the buffer containing Liberase enzyme. Oocytes will drop into the collection tube, while the connective tissues of the ovary will be retained by the nylon mesh. (**b**) Oocyte staging. Different sized nylon meshes are used to purify oocytes at selected stages

11. Keep the oocyte for 30–45 min in buffer or medium and then remove damaged oocytes based on the structure and yolk granulation (*see* **Note 12**).
12. Oocytes are now ready for injection or drug treatments followed by culture or for imaging (*see* **Note 13**).

3.3 Oocyte Culture

1. Normal oocytes or injected/treated oocytes are cultured at 26 °C in oocyte culture media (*see* **Note 14**).

2. Oocytes have to be cultured under aseptic conditions and dead cells should be removed as soon as possible to avoid contamination (*see* **Note 15**).

3.4 High Pressure Freezing

1. Place living oocytes in aluminum platelets of 150 μm depth containing 1-hexadecene [21].
2. Freeze the platelets using a high pressure freezer and store them under liquid nitrogen until further treatments.

3.4.1 Freezing and Substitution (Fixing–Dehydration–Embedding) (*See* **Note 13**)

3. Transfer the vitrified oocytes to an automatic Freeze Substitution Unit, where the samples are substituted at −90 °C in a solution containing anhydrous acetone, 0.1% tannic acid for 72 h.
4. Remove the solution and wash the samples three times with acetone at −90 °C.
5. Continue the incubation for 7 h at −90 °C in 1% OsO_4/0.5% glutaraldehyde in acetone.
6. Increase the temperature with +5 °C per hour, up to −20 °C.
7. Leave the samples for 18 h at −20 °C.
8. Increase the temperature with +10 °C per hour, up to +4 °C.
9. Keep the sample for 1 h at 4 °C.
10. Remove OsO_4 from the sample.
11. Wash the sample three times with acetone for 30 min each.
12. Increase the temperature with +10 °C per hour, up to +20 °C.
13. Remove aluminum platelets.
14. Rock the sample in Epon–acetone 1:1 for 1 h in rocker table at +24 °C, with a closed cover to avoid evaporation of acetone.
15. Leave it in Epon for 1 h at +24 °C, with open cover in rocker table to remove acetone.
16. Incubate in fresh Epon overnight on rocker table.
17. Embed samples in flat embedding molds.
18. Leave sample for polymerization at +60 °C for 48 h.

3.4.2 Staining and Sectioning

If needed, the ultrathin section of the embedded tissue with 70 nm can be counterstained using different poststaining methods using 1% uranyl acetate aqua., Reynolds lead citrate [23], or 1% uranyl acetate in methanol.

3.4.3 Imaging

Images were taken in a Philips CM120 electron microscope using a TemCam 224A slow scan CCD camera (Figs. 2 and 3).

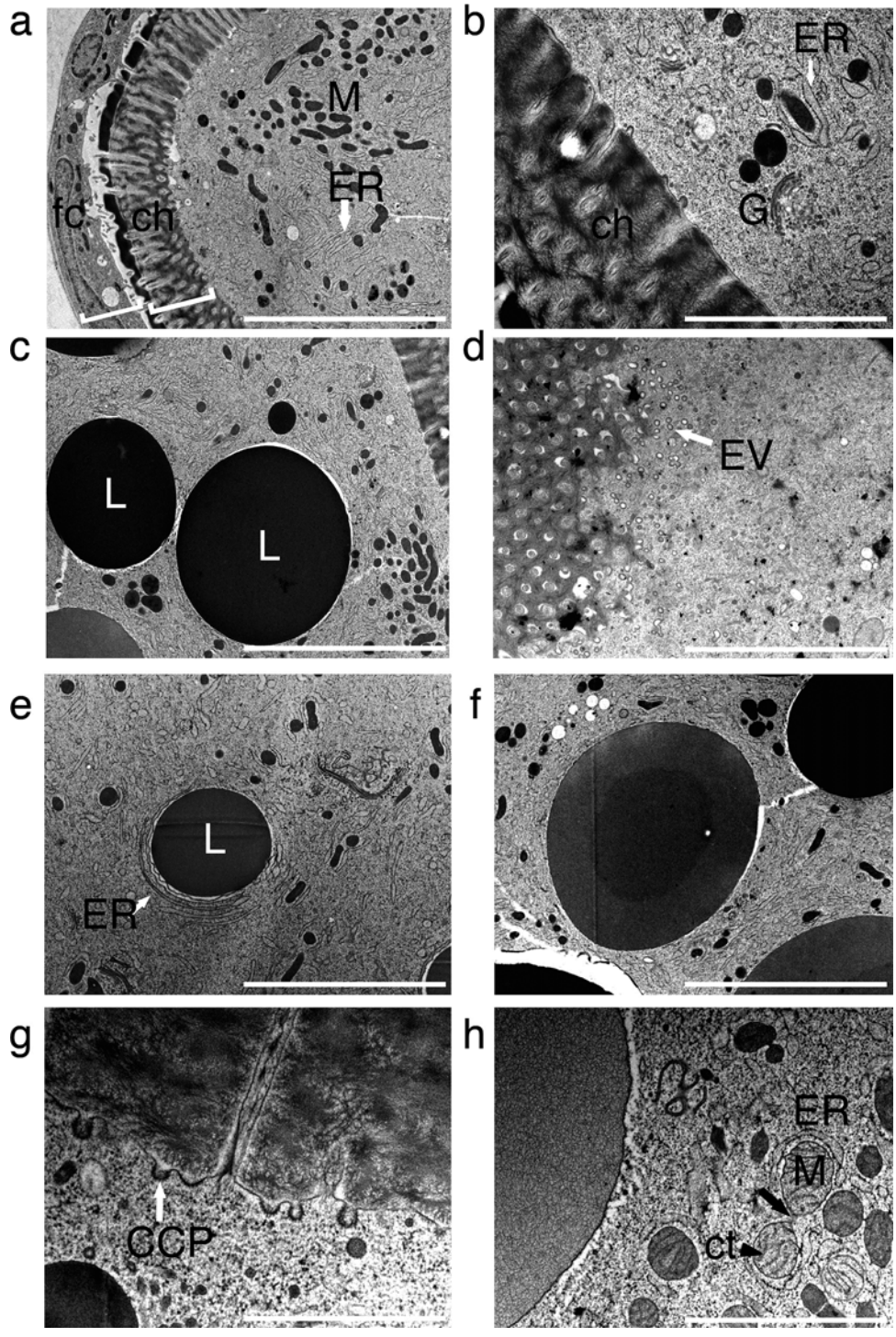

Fig. 2 Oocyte compartments. (**a**) Micrograph showing the oocyte cortex covered with follicle cells (fc) (Flat cells on the *left*) and acellular membrane (Chorion—ch). The cytoplasm contains mitochondria (M), endoplasmic reticulum (ER). (**b**) Micrograph showing a zoomed-in version of (**a**). Chorion (ch), ER and Golgi stacks (G). (**c**) yolk-filled lysosomes/yolk globules (L). (**d**) Endocytic vesicle (EV) (*arrow*). (**e**) A lysosome (L) surrounded by endoplasmic reticulum (ER). (**f**) A large dense core secretory vesicle (LDCV). (**g**) Clathrin coated pits (CCP) at the inner oocyte membrane. (**h**) Mitochondria during fission or fusion (*Black arrow*) surrounded by endoplasmic reticulum. Also notice the mitochondria cristae (*arrow head*—ct). Scale bar 1 μm for panel **a**, **c**, **e**, and **f**; 0.5 μm for panel **b**, **d**, and **h**; 0.25 μm for panel **g**

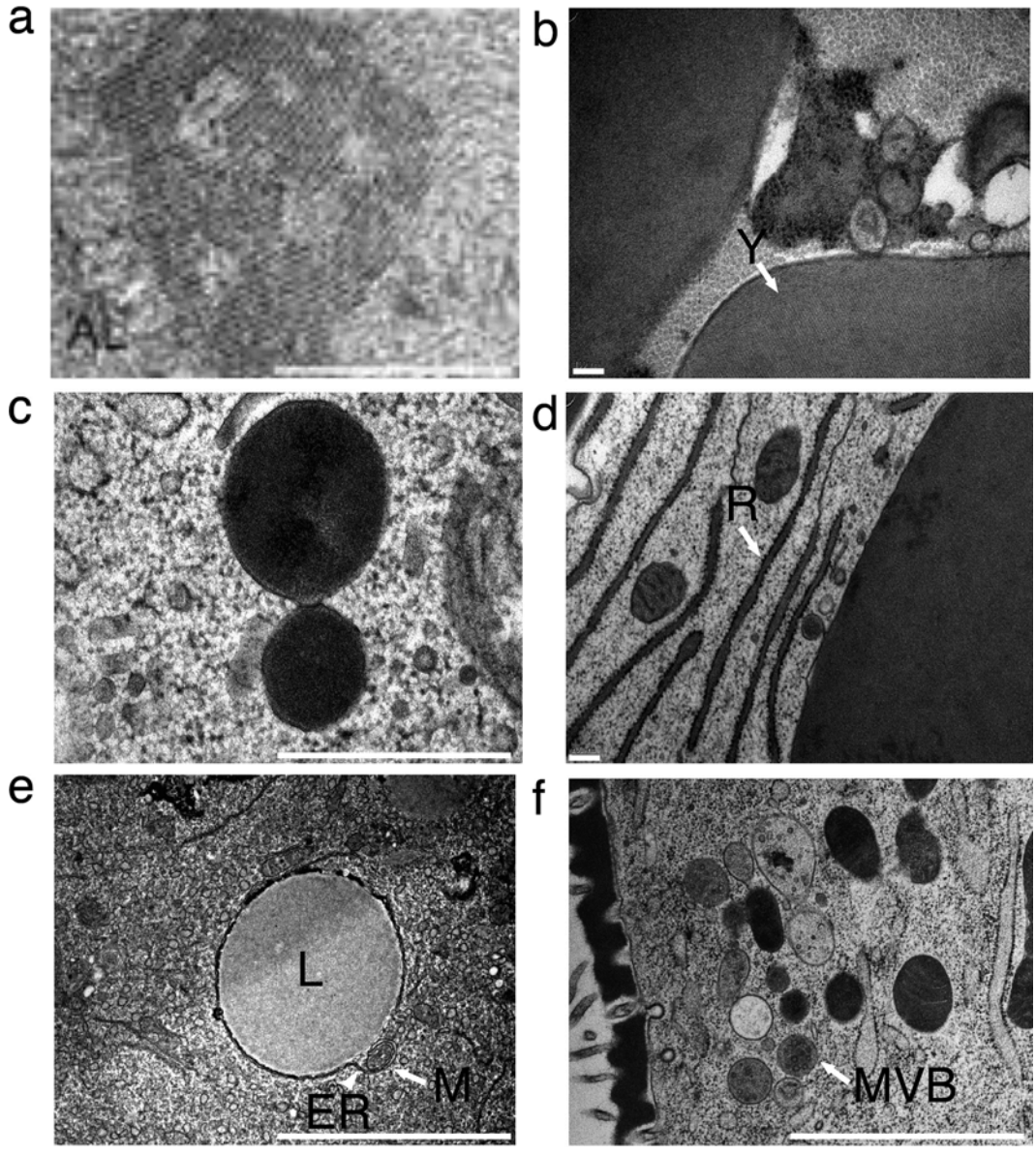

Fig. 3 Subcompartmental structures. (**a**) Annulate lamellae (AL) [13, 24, 25]. (**b**) Linear arrays of the crystalline yolk (Y) in lysosomes. (**c**) Vesicle budding. (**d**) Rough endoplasmic reticulum with ribosomes (R) arranged on its surface. (**e**) Lysosome (L), mitochondria (M—*arrow*), and endoplasmic reticulum (ER—*Arrowhead*) connected to each other. (**f**) Multivesicular bodies (*arrow*—MVB) with internal vesicles at the oocyte cortex. Scale bar 0.1 μm for panel (**a**, **c**, **d**), and (**e**); 0.5 μm for panel (**f**) and 0.1 μm for panel (**b**)

4 Notes

1. Use sharp scissors to avoid crushing or destroying the oocytes close to the skin. This is especially important for smaller oocytes, which are attached to the ventral side of the fish.
2. The nylon meshes are used to separate the single oocytes from the ovary tissue and to separate different stages. You can also use the whole ovary for high pressure freezing but it might disturb the structure. A set of nylon meshes with different pore size is used to purify selected stages from ovaries. Small, purified oocytes are better for high pressure freezing.
3. Once you obtained single oocytes, then pass them through different sized meshes into a 6-well plate to collect the desired stage. Handle the oocytes very gently to prevent physical damage.
4. We use Liberase enzyme (Purified form of collgenase) to digest the ovary tissue and to release the individual oocytes. When the tissue is incubated with Liberase, control the digestion frequently to avoid damaging the oocytes. As soon as the ovary tissue is loosened, remove the enzyme and wash it with OR2 buffer.
5. While dissecting the ovary, do not apply any physical pressure on the belly to circumvent crushing smaller oocytes.
6. We prefer Castroviejo Corneal Scissors with their bent tip to avoid cutting into the ovary. Normal straight scissors require more care during dissection.
7. Immediately transfer the ovary into buffer or medium to prevent desiccation of the oocytes.
8. Be careful with handling the oocyte at this step during incubation. Over-digestion will rupture the oocyte, and therefore, the precise concentration of the enzyme is critical.
9. Move the column up and down slowly to separate oocytes from the ovary after enzymatic treatment, otherwise the pressure will burst the cells.
10. During the purification process of staged oocytes, pour them slowly with the buffer on the filter. To remove the oocytes from the filter, dip the filter into buffer instead of pouring the buffer on the filter. Wash the filters several times during oocyte collection after separating different stages from the 6 well plates.
11. Performing most steps slowly and gently achieves good quality oocytes with minimal damage.
12. After the purification process, remove damaged oocytes to reduce contamination.

13. If the oocytes are not required for further treatment or culture, then purify the oocytes as quickly as possible to keep the nature of the sample before freezing.
14. The L-15 medium has to be aseptic and should contain all antibiotics and fungicides.
15. When you culture the oocytes, fungicides and antibiotics should be present in the medium.

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Chapter 13

Cytoskeleton and Cytoskeleton-Bound RNA Visualization in Frog and Insect Oocytes

Malgorzata Kloc, Szczepan Bilinski, and Jacek Z. Kubiak

Abstract

The majority of oocyte functions involves and depends on the cytoskeletal elements, which include microtubules and actin and cytokeratin filaments. Various structures and molecules are temporarily or permanently bound to the cytoskeletal elements and their functions rely on cytoskeleton integrity and its timely assembly. Thus the accurate visualization of cytoskeleton is often crucial for studies and analyses of oocyte structure and functions. Here we describe several reliable methods for microtubule and/or microfilaments preservation and visualization in *Xenopus* oocyte extracts, and in situ in live and fixed insect and frog (*Xenopus*) oocytes. In addition, we describe visualization of cytoskeleton-bound RNAs using molecular beacons in live *Xenopus* oocytes.

Key words Cytoskeleton, Cytokeratin, Actin, Tubulin, Mitotic spindle, Cell-free extract, Xenopus, Oocyte, Molecular beacon, Localized RNA

1 Introduction

The invertebrate and vertebrate oocytes have an intricate cytoskeleton built of actin and cytokeratin filaments and microtubules. The cytokeratin and actin filaments extend through the oocyte cytoplasm and underneath the outer membrane to form the oocyte cortex. Oocyte cortex not only gives physical sturdiness but also plays an indispensable role in anchoring and proper segregation (after fertilization and during embryogenesis) of localized RNAs, organelles, and germ cell determinants (germ plasm islands) [1–5]. The oocyte microtubules span in orderly and purposely fashion through the oocyte cytoplasm where they participate in the transport of miscellaneous proteins, RNAs, organelles, and vesicles to a variety of destinations. In addition, during mitotic divisions (during oogenesis) or meiosis (in fully grown and activated oocytes), the oocyte cytoplasm assembles (polymerizes) the mitotic or meiotic spindles and asters from the stockpile of unpolymerized cytoplasmic tubulin, which assures accurate segregation of genetic material [6]. Thus, the ability to adequately fix and visualize

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various cytoskeletal elements is often an important endeavor in the studies of oocyte and its organelles' structure and functions [7]. Here we present several reliable methods to preserve and visualize oocyte cytoskeletal elements in insect and frog oocytes. In addition we describe the procedure for labeling the cytoskeleton-bound localized RNAs in live frog oocytes.

2 Materials

2.1 Cytoskeleton Visualization in Insect Oocytes

It is quite challenging to analyze cytoskeleton and its 3D organization in relatively large cells such as insect oocytes. This difficulty is due not only to their large size but also to the presence of numerous "reserve materials," i.e., yolk spheres, lipid droplets and glycogen particles accumulated in oocyte cytoplasm (ooplasm). Yet another cause of this difficulty is follicular epithelium that tightly encapsulates growing oocytes and may block or at least delay penetration of fixatives. One way to overcome this problem is to remove the unwanted cell constituents (cytoplasm, reserve materials) and make follicular cells permeable with detergent (Triton X-100) extraction. This method is particularly suitable for visualization and analysis of microtubule distribution (Fig. 1a, b).

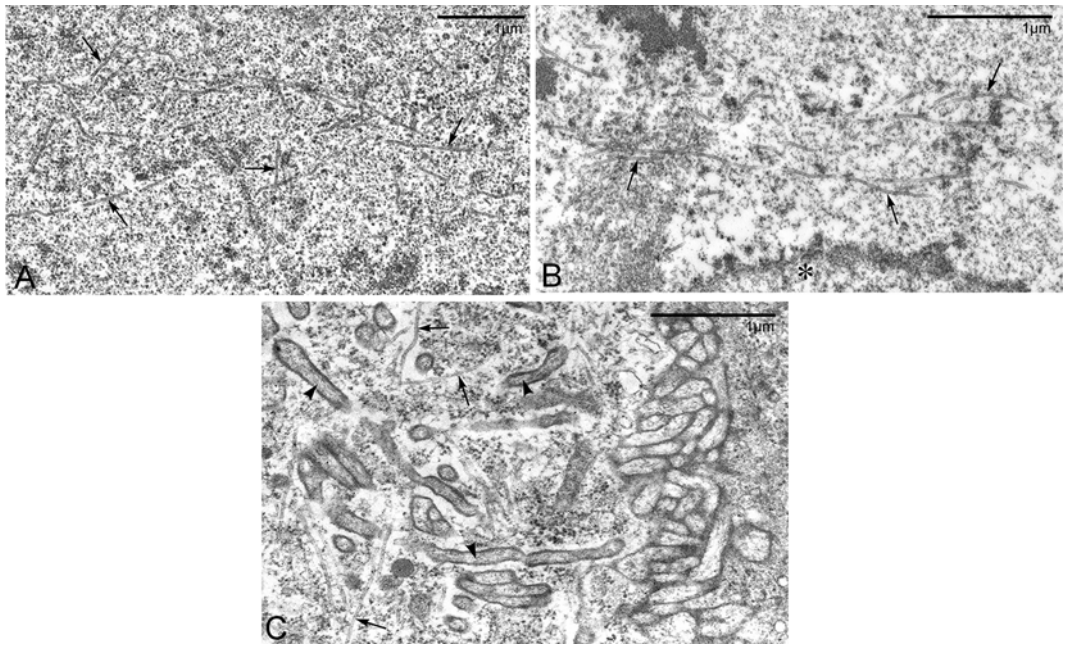

Fig. 1 Cytoskeleton in insect ovaries. (**a**) *Cosmoconus meridionator* (parasitic wasp). Microtubules (*arrows*) separating the periplasm from the ooplasm. Tangential section through the oocyte periphery; Triton X-100 extraction. (**b**) *C. meridionator.* Microtubules (*arrows*) surrounding the nurse cell nucleus (*asterisk*). Triton X-100 extraction. (**c**) *Cicindela campestris* (tiger beetle). The oocyte periphery penetrated by microvilli of the somatic follicular cells. Microtubules (*arrows*), microfilaments inside microvilli (*arrowheads*). Contrast enhancement. Scale bar: 1 μm

2.2 Insect Oocytes Fixatives for Extraction

1. Extraction buffer for insect oocytes: 137 mM NaCl, 5 mM KCl, 1.1 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 4 mM $NaHCO_3$, 12 mM $MgCl_2$, 2 mM EGTA, 50 mM 2-(*N*-morpholino) ethanesulfonic acid (MES), 5.5 mM glucose, 1 % Triton X-100.
2. Fixative 1: 2.5 % glutaraldehyde in the extraction buffer.
3. Fixative 2: Mixture of 2 % osmium tetroxide and 0.8 potassium ferrocyanide in 0.1 M phosphate buffer, pH 7.4. The mixture should be prepared just before use and kept at 4 °C.
4. Fixative 3: 0.15 % tannic acid in 0.1 phosphate buffer, pH 7.4.

2.3 Insect Oocytes Fixatives for Contrast Enhancement

1. Fixative 1: a mixture of 2 % formaldehyde and 2.5 % glutaraldehyde in 0.1 M phosphate buffer, pH 7.3.
2. Fixative 2: a mixture of 2 % osmium tetroxide and 0.8 % potassium ferrocyanide in 0.1 M phosphate buffer. The mixture should be prepared just before use and kept at 4 °C.

2.4 Cytoskeleton and Cytoskeleton-Bound RNAs Visualization in Xenopus Oocytes

1. OR2 buffer 10× (modified from Wallace et al. [8]): 0.35 M NaCl (24.11 g), 25 mM KCl (0.932 g) 10 mM Na_2HPO_4 (0.710 g), 38 mM NaOH 0.76 g, 50 mM Hepes 5.96 g. Add water to 400 ml, adjust pH to 7.8. Add water to 500 ml.
2. 10× Barth's solution (from Gurdon [9]): 0.88 M NaCl (23.65 g), 0.01 M KCl (0.375 g).
3. 0.1 M HEPES (11.9 g), 0.008 M $MgSO_4 \times 7H_2O$ (1.0 g), 0.003 M $Ca(NO_3)_2 \times 4H_2O$ (0.39 g).
4. 0.004 M $CaCl_2 \times 2H_2O$ (0.30 g). Add water to 400 ml, adjust pH to 7.6. Add water to 500 ml.

2.5 Fixative for Xenopus Spindle Visualization

1. BRB80 buffer (80 mM K-1,4-piperazinediethane-sulfonic acid, pH 6.8, 1 mM ethyleneglycol-bis(aminoethylether)-tetraacetic acid, 1 mM $MgCl_2$), 30 % glycerol, 1 % paraformaldehyde, 0.5 % Triton X-100.

3 Methods

3.1 Extraction Procedure of Insect Oocytes

1. Immerse small pieces of insect ovaries or isolated ovarioles in the extraction buffer (Subheading 2.2 and **Note 1**).
2. Fix in fixative 1 (Subheading 2.2) for 15–30 min at room temperature.
3. Wash in 0.1 M phosphate buffer, pH 7.3–7.4, 3 × 15 min at room temperature.
4. Fix in fixative 2 (Subheading 2.2) for 1–2 h at 4 °C.
5. Wash in 0.1 M phosphate buffer, pH 7.3–7.4, 3 × 20 min at room temperature.

6. Fix in fixative 3 (Subheading 2.2) for 1–1.5 h at room temperature.
7. Rinse in tap water, 3 × 30 min at room temperature.
8. Stain (en block) in 2 % aqueous uranyl acetate for 2 h at room temperature.
9. Rinse in tap water, 3 × 10 min at room temperature.
10. Dehydrate in increasing concentrations of ethanol (30 %—2 × 10 min, 50 %—15 min, 70 %—15 min, 90 %—15 min, 96 %—15 min, 100 %—2 × 15 min) and in acetone (3 × 10 min); all steps in room temperature.
11. Embed in Epon 812 or its equivalents according to manufacturer's protocol.
12. Cut blocks into ultrathin sections. According to our experience the best results are obtained with relatively thick (dark gold) sections.
13. Contrast sections with lead citrate according to standard protocol.

3.2 Contrast Enhancement of Insect Oocytes

Another method to improve visualization of the cytoskeleton at the EM level is through the enhancement of oocyte/tissue contrast [10]. This can be achieved by using improved/modified fixatives. The procedure is less complicated than Triton X-100 extraction and usually ensures nice preservation of cytoskeletal elements (Fig. 1c). The steps of the procedure developed in our laboratory are as follows:

1. Fix in fixative 1 (Subheading 2.3) for 1–1.5 h at 4 °C.
2. Wash in 0.1 M phosphate buffer, pH 7.3–7.4, 3 × 15 min at 4 °C.
3. Fix in fixative 2 (Subheading 2.3) for 30–60 min at 4 °C.
4. Rinse in tap water, 3 × 30 min at room temperature.
5. Dehydrate in increasing concentrations of ethanol (30 %—2 × 10 min, 50 %—15 min, 70 %—15 min, 90 %—15 min, 96 %—15 min, 100 %—2 × 15 min) and in acetone (3 × 10 min); all steps in room temperature.
6. Embed in Epon 812 or its equivalents.
7. Cut blocks into ultrathin (grey or silver) sections.
8. Contrast sections with uranyl acetate (saturated aqueous solution) and lead citrate according to standard protocol.

In addition to "laborious and time-consuming" EM methods the oocyte cytoskeleton (distribution of microfilaments or microtubules) can be also analyzed by fluorescence/confocal microscopy using whole mount preparations of ovarioles or individual oocytes. Here we present two procedures developed in our laboratory [11].

3.3 Visualization of Microtubules in Insect Oocytes

1. Dissect and isolate ovarioles or ovarian follicles in phosphate buffer saline (PBS).
2. Fix in 4% formaldehyde (freshly prepared from paraformaldehyde) in PBS for 15–30 min at room temperature.
3. Place samples in 0.1% Triton X-100 for 30 min at room temperature.
4. Wash in PBS, 3 × 30 min at room temperature.
5. Incubate in a primary antibody, e.g., anti-β-tubulin. Dilution of the antibody and time of incubation should be determined experimentally.
6. Wash in PBS, 5 × 15 min at room temperature.
7. Incubate in the appropriate secondary antibody, e.g., fluorescein isothiocyanate (FITC) conjugated secondary antibody (dilution 1:32) for 2 h at room temperature or overnight at 4 °C.
8. Wash in PBS, 3 × 15 min at room temperature.
9. Mount on microscopic slides.

3.4 Visualization of Microfilaments in Insect Oocytes

Steps 1–3: as described above in Subheading 3.3

4. Incubate in rhodamine-conjugated phalloidin (1 μg/ml) for 30 min at room temperature in darkness.
5. Wash in PBS, 3 × 15 min at room temperature.
6. Mount on microscopic slides.

3.5 Xenopus Oocyte Defolliculation

1. Prepare collagenase in 1× OR2 (Subheading 2.4).
2. Dissolve 0.002 g of collagenase per 1 ml 1× OR2 (Do not waste—Collagenase is expensive).
   - Use ~0.05 g/25 ml for small frogs.
   - Use ~0.1 g/50 ml for medium frogs.
   - Use ~0.2 g/100 ml for large frogs.
   - Use ~0.5 g/250 ml for four large frogs.
3. Break the ovarian sacks with forceps.
4. Transfer ovarian fragments into a dish with collagenase and OR2.
5. Shake gently at 30 °C for 2 h.
6. Discard the buffer and wash oocytes once with 1× OR2 to remove dead cells and floating tissue debris.
7. After collagenasing rinse oocytes three times in large volume (~100 ml) of Barth's solution (Subheading 2.4 and **Note 2**).

3.6 Actin and Cytokeratin Staining in Fixed Xenopus Oocytes

3.6.1 Actin Staining (Modified from Mazurkiewicz and Kubrakiewicz and Kloc et al. [12, 13])

1. Fix defolliculated oocytes in 4% formaldehyde (EM grade) in PBS with 0.1% Triton X-100 for 30 min at room temperature.
2. Wash twice, 15 min each wash in PBS-0.05% Tween 20.
3. Block oocytes for 1–6 h at room temperature or overnight at 4 °C in casein blocking buffer in PBS supplemented with 0.05% Tween 20.
4. Stain overnight at 4 °C in rhodamine-phalloidin (5 μl of methanolic stock solution of 200 U/ml per 200 μl of casein blocking solution).
5. After staining wash oocytes three times (15 min each wash) in PBS-0.05% Tween 20 in the dark.
6. Mount oocytes (squash them under coverslip) in an antifade reagent containing Hoechst (nuclear staining) and photograph using a Nikon fluorescence microscope ([13]; Fig. 2b).

3.6.2 Cytokeratin Staining (Modified from Alarcon and Elinson [16] Was described before in Bilinski et al. [7])

1. Fix defolliculated oocytes in 2% formaldehyde in 100% methanol overnight at –20 °C.
2. Rehydrate and wash fixed oocytes with slow shaking on a rotating platform as follows: 100% ethanol 2 × 10 min, 70% ethanol 15 min, 50% ethanol 15 min, 2 × 5 min washes in phosphate buffered saline (PBS) containing 0.05% Tween 20 (cat #166-2404, Bio-Rad Laboratories, Hercules, CA), 10 min in PBS-0.25% Triton X-100 (cat # X100, Sigma-Aldrich, St. Louis, MO, USA), and 2 × 5 min with PBS-0.05% Tween 20.
3. Block nonspecific binding by 1–6 h incubation at room temperature in casein blocking buffer in PBS supplemented with 0.05% Tween 20 at room temperature.
4. Incubate with FITC-conjugated anti-pan cytokeratin clone C-11 mouse monoclonal antibody at 1:50 dilution in casein blocking buffer containing 0.05% Tween 20, overnight at 4 °C. If the FITC-conjugated cytokeratin antibody is not available one can stain oocytes with 1:50 dilution of any anti-pancytokeratin unconjugated antibody in casein blocking buffer, followed by three 1 h washes in PBS-0.05% Tween 20 (or last overnight wash at 4 °C) and subsequent incubation with 1:200 dilution of appropriate secondary antibody in casein blocking solution either 1–3 h at room temperature or overnight at 4 °C.
5. Wash the oocytes at room temperature overnight at 4 °C with PBS-0.05% Tween 20.
6. Mount washed oocytes in antifade solution (squash them under coverslip on the microscope slide) containing Hoechst and observe under fluorescence microscope (Fig. 2a).

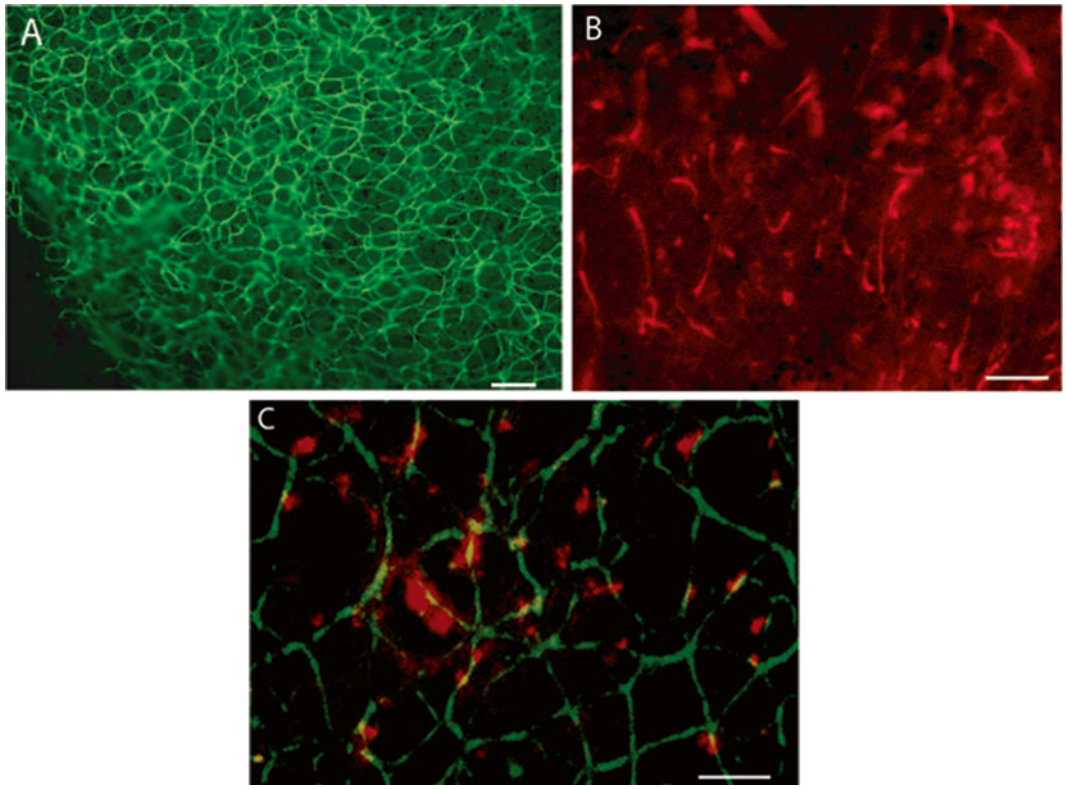

Fig. 2 Cytoskeleton and RNA in *Xenopus* oocytes. (**a**) Network of cytokeratin filaments labeled green with FITC-conjugated pancytokeratin antibody in vegetal cortex of stage VI *Xenopus* oocyte. (**b**) Actin labeled with rhodamine-phalloidin in the cortex of stage VI *Xenopus* oocyte injected with AS fatvg. Image from Kloc [14] with permission from John Wiley and Sons. (**c**) VegT mRNA labeled red with Texas Red-conjugated molecular beacon on cytokeratin filaments labeled green with FITC-conjugated pan-cytokeratin antibody in the vegetal cortex of *Xenopus* stage VI oocyte. (from Kloc[15]. Scale bars: (**a**) 12 μm, (**b** and **c**): 5 μm

3.7 Cytokeratin Staining in Live Xenopus Oocytes

1. Inject defolliculated oocytes with 1 nl of an anti-pancytokeratin-FITC antibody (C11 antibody, Sigma).
2. Immediately after the injection, cut off the tip (or larger fragment) of the oocyte and mount in PBS on a microscope slide under a coverslip. Because this is unfixed oocyte, which will lyse fast, observe under fluorescence microscope within 15–30 min of C11-FITC injection.

3.8 Labeling of RNAs with Molecular Beacons in Live Xenopus Oocytes

Molecular beacons are hairpin shaped short oligonucleotides with 2′-*O*-methylribonucleotide backbone, and fluorophore (for example Texas Red) at the 5′ end and BHQ2 (Black hole quencher 2) at the 3′ end. When they are in hairpin conformation their fluorophore is quenched, but after they hybridize to nucleic acid target in the cell, the hairpin configuration is lost and fluorescence is restored [17]. We successfully used such molecular beacons to detect various vegetally localized RNAs in *Xenopus* oocytes [3, 14, 15].

1. Inject 10 ng of molecular beacon dissolved in 10 nl of Barth's into the cytoplasm of defolliculated oocyte. Incubate oocyte for several (4–6) hours at 18 °C in Barth's solution.
2. Cut off the tip or larger fragment of the oocyte, mount in PBS on a microscope slide under a coverslip.
3. Observe under fluorescence microscope within 15–30 min from mounting.

3.9 Co-labeling of Cytokeratin with Antibody and RNAs with Molecular Beacons in Live Xenopus Oocytes

1. Inject 10 ng of molecular beacon (conjugated with red fluorophore such as Texas Red) dissolved in 10 nl of Barth's (Subheading 2.4) into the cytoplasm of defolliculated oocyte.
2. Incubate oocyte for several (4–6) hours at 18 °C in Barth's solution.
3. Subsequently inject oocytes with 1 nl of an anti-pancytokeratin-FITC antibody (C11 antibody, Sigma).
4. Immediately after the injection, cut off the tip (or larger fragment) of the oocyte and mount in PBS on a microscope slide under a coverslip.
5. Observe under fluorescence microscope within 15–30 min of C11-FITC injection (Fig. 2c; [3, 14, 15]) (*see* **Note 3**).

3.10 Visualization of Spindles Formed in Xenopus Oocyte/Egg CSF Cell-Free Extract

Meiotic spindles are very tiny structures in comparison to the huge size of *Xenopus laevis* egg. In addition, there is only a single spindle per egg. Therefore, it is very difficult to visualize spindles *in ovo*. To avoid this problem and facilitate spindle studies the isolated sperm heads may be added to the cell-free extracts obtained from ovulated oocytes arrested in the second meiotic M-phase by a cytoplasmic factor called CSF (CytoStatic Factor) [18]. In such M-phase-arrested extracts the sperm head chromatin undergoes initial decondensation followed by condensation and formation of chromosomes. The presence of chromatin induces microtubules assembly and the bipolar spindles are formed around the chromosomes originating from the sperm heads. This process can be followed by the fluoresecence of rhodamine-labeled bovine brain tubulin added to the extract. Such sperm-induced spindles are much easier to observe than the native egg spindle.

3.10.1 Preparation of Spindles in Cell-Free Extracts

1. Prepare CSF cell-free extract by crushing MII-arrested oocytes (called also eggs) at 10,000 × *g* in Beckman ultracentrifuge [19].
2. Prepare sperm heads from *Xenopus laevis* male testis [20].
3. Place 50 μl of the extract in Eppendorf tube and add 0.5 μl (0.2 mg/ml) of rhodamine-labeled bovine brain tubulin (Cytoskeleton, via TEBU-Bio, Le Perray en Yvelines, France).
4. Add 2 μl of sperm heads to 50 μl of the extract, which makes the final concentration about 1000 nuclei/μl.
5. Incubate the extract for 60–90 min at 21 °C to allow chromatin condensation into chromosomes and spindle assembly.

3.10.2 Visualization and Imaging of "Crude" Spindles Embedded in the Cytoplasm

1. Place aliquots of extracts containing spindles (2–3 μl) on microscope slide.
2. Cover with a coverslip.
3. Press with a finger.
4. Immediately fix in liquid nitrogen for 20 s.
5. Remove the cover glass with fine forceps.
6. Wash in PBS (room temperature).
7. Proceed with classical immunofluorescence staining of the protein of interest (in our case TCTP) using FITC-conjugated secondary antibody (microtubules are stained red with rhodamine) (Fig. 3 and **Note 4**).

3.10.3 Visualization and Imaging of Purified Spindles Free of Cytoplasm

1. Prepare spindle fixative in BRB80 buffer (Subheading 2.5) to prefix spindles.
2. Mix 15 μl of spindle-containing CSF extract with 1 ml of the above solution.
3. Spin down the sample at 2300 × *g* for 30 min at room temperature through a 40 % glycerol cushion in BRB80 onto glass coverslips in 12-well plate.

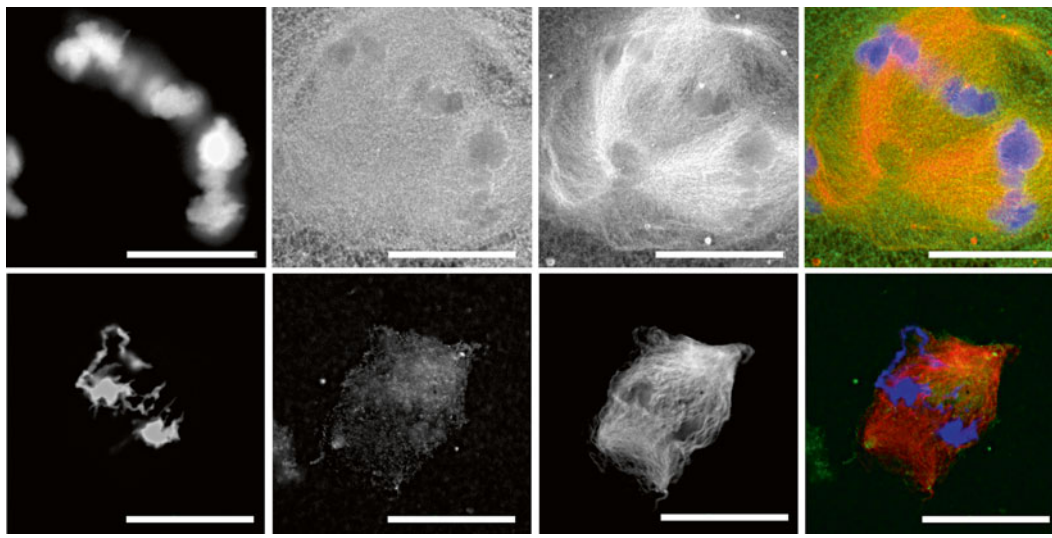

Fig. 3 Spindle in *Xenopus* cell-free extract. TCTP is abundant in the mitotic spindle, but it is not associated with spindle microtubules. *Upper row*: Crude spindles. *Bottom row*: Purified spindles. In each row: *left panel*: DNA; *middle-left*: TCTP immunofluorescence; *middle-right*: rhodamine microtubule staining; *right panel*: merge of TCTP and microtubule staining. Crude spindles (*upper row*) show bright (non-filamentous) TCTP staining and a granular network of TCTP in surrounding cytoplasm that does not co-localize with microtubules. In contrast, in purified spindles (*bottom row*) the majority of cytoplasmic TCTP staining disappeared and discrete fluorescent dots of TCTP remained only on the spindle poles. The merged image of DNA, TCTP, and rhodamine tubulin shows clear red staining of spindle microtubules with *yellow dots* on its poles indicating co-localization of TCTP and tubulin in these areas. Scale bars: 20 μm

4. Fix spindles attached to the cover glass by adding 1 ml of cold methanol (−20 °C) for 10 min.
5. Wash with PBS at room temperature.
6. Proceed with classical immunofluorescence to stain the protein of interest (in our case TCTP) using FITC-conjugated secondary antibody (microtubules are stained red with rhodamine) (Fig. 3) (*see* **Note 5**).

4 Notes

1. Extraction procedure of insect oocytes is relatively time consuming and complicated [11, 21, 22]; it involves 13 subsequent steps. According to our experience, the first step is the most critical. The extraction time depends strongly on the species analyzed and the size of the oocyte, i.e., its developmental stage, and usually must be determined experimentally.
2. *Xenopus* stage VI oocyte is covered by several layers of follicular cells. Before the injection or fixation the oocytes have to be defolliculated. Defolliculation can be performed either manually or enzymatically. Although manual defolliculation under the stereoscope using very fine forceps is a preferred method it requires skills and great patience. Thus, many researchers prefer enzymatic defolliculation, when oocytes can be defolliculated in bulk.
   Enzymatic defolliculation is performed by gently shaking oocytes for about 2 h at 30 °C in Petri dish in 1× OR2 (which is calcium and magnesium free buffer) containing 0.2% collagenase (Sigma-Aldrich, St Lous, MO, USA cat # C6885). The effectiveness of collagenasing should be checked under the microscope after 30 min and every 15 min afterwards—the shorter collagenasing the better integrity of oocytes.
3. It is important to remember that because the live oocytes fragments are mounted in pure PBS without a glycerol or any antifade compound, the illumination with UV light during sample viewing and taking images in fluorescent microscope will cause fast bleaching and has to be performed expeditiously.
4. We successfully used visualization of *Xenopus* spindles in cell-free extracts to verify the association of Translationally Controlled Tumor-Associated Protein (TCTP) with spindle microtubules [23]. Initially, TCTP was described as MAP (Microtubule-Associated Protein) and was used in immunofluorescence studies as a marker for spindle in mouse oocytes [6]. However, our studies suggested that TCTP associates rather with actin microfilaments than with microtubules. To verify if and why the TCTP staining is present on the spindles we have

used the method described above and showed that although TCTP accumulated within the spindles it did not directly associate with spindle microtubules [23]. The complex relationship between TCTP, microtubules, and actin microfilaments regulates cell shape in normal and cancer cells. We compared the fluorescence images of microtubules and TCTP in "crude" spindles embedded in the cytoplasm and in purified spindles after removal of the cytoplasm. This type of analysis of the localization of proteins of interest within the spindle allows answering the question whether the protein of interest is associated with spindle microtubules or not.

5. The comparison of images of "crude" extracts and isolated spindles allows distinguishing whether the protein of interest is indeed associated with microtubules or just present within the spindle.

Competing Interests

The authors declare that they have no competing interests.

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Chapter 14

RNA FISH, DNA FISH and Chromosome Painting of Chicken Oocytes

Silvana Guioli and Robin Lovell-Badge

Abstract

Fluorescence in situ hybridization (FISH) is a molecular cytogenetic technique. It identifies the location of DNA loci and RNAs, including nascent RNAs in the process of being transcribed, within individual cells. Great advances in fluorescent dye technology and technique sensitivity, combined with developments in light microscopy and imaging software have made it widely accessible and have expanded the range of applications in basic research as well as in diagnostics. Being able to perform RNA hybridization, DNA hybridization, and protein immunofluorescence consecutively on the same sample is an invaluable tool to study RNA expression in relation to their gene loci and to map RNA and DNA in relation to nuclear or cellular structures. This has contributed to enormous progress in understanding basal mechanisms of male and female meiosis in different animal model systems. In this chapter we describe in detail the protocols for FISH based techniques applied to study gene expression dynamics and nuclear architecture of chicken oocytes during meiotic prophase I. These techniques can be easily performed in any molecular and cell biology laboratory and be adapted to different systems and to different phases of gametogenesis.

Key words Chicken oocyte, Meiosis, Fluorescence in situ hybridization, Chromosome painting

1 Introduction

Originally developed in 1969 by Gall and Pardue [1] to detect the position of DNA sequences within the cell, the in situ hybridization procedure brought cytogenetics to the molecular era. Today most in situ hybridization protocols make use of fluorescence for target detection and therefore it is referred to as Fluorescence In Situ Hybridization or FISH [2]. The basic technique implies the labeling of a DNA probe and its hybridization to cells fixed on a slide. The target sequence is visualized as a fluorescent signal within the cell. Since its development, FISH has greatly improved its sensitivity, specificity, and resolution power. This, combined with technological advancements bringing a wide range of detection methods, multiplex-detection, sensitive quantitative measurements and the development of microfluidic arrays, has transformed

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molecular cytology. FISH has now evolved into a plethora of versatile methods that allow the examination of DNA loci (DNA FISH) as well as RNAs (RNA FISH) [3]. The range of applications in research and diagnostics continues to expand and now includes karyotyping, genotyping, cancer diagnostics, genome evolution, and gene expression studies [4]. Chromosome painting, one of the first applications of DNA FISH, identifies whole chromosomes using chromosome-specific DNA libraries as probes [5]. Now each chromosome can be labeled with a different fluorophore to generate a spectral karyotyping [6].

The possibility to combine RNA FISH with DNA FISH and protein immunofluorescence means that we can now visualize RNA and DNA in situ in direct relation with endogenous proteins, in particular proteins that define specific cytoplasmic or nuclear cell compartments. This has made FISH an invaluable tool in the field of reproductive science and in particular to study oogenesis and spermatogenesis. For example, it has been instrumental in understanding fundamental processes in meiosis, such as the mechanism of meiotic sex chromosome inactivation (MSCI) and its evolution [7–10].

Many labs are using RNA/DNA FISH to investigate the topographic relations between DNA, nascent RNA and chromatin-associated proteins in late diplotene oocyte from species with chromosomes in the lampbrush form. The high resolution power of this system has made it into an ideal model to study the epigenetic mechanisms of transcriptional regulation [11]. In human, multicolor FISH and more recently comparative genomic hybridization (CGH) and array CGH are the basis for prenatal diagnosis. The routine use of these techniques to screen embryos generated by in vitro fertilization are starting to shed light on the types of maternal and paternal meiotic errors that lead to aneuploidy [12].

In this chapter we describe the FISH protocols we use to detect nascent RNA transcripts and DNA loci, or whole chromosomes, on spreads from chicken ovaries containing oocytes in early stages of meiotic prophase I. We also discuss how to combine these labeling techniques with protein immunofluorescence. These protocols have been adapted from methods developed for the study of mouse meiosis [13] and can be further adapted to be used in other animal models.

2 Materials

2.1 Preparation of BAC Probes

1. LB agar.
2. LB broth.
3. 25 mg/ml chloramphenicol.
4. P1 solution: 15 mM Tris–HCl pH 8, 10 mM EDTA pH 8, 100 μg/ml RNase A (ice-cold).
5. P2 solution: 0.2 M NaOH, 1% SDS.

6. P3 solution: 3 M potassium acetate (KCH_3COO) pH 5.5 (ice-cold).
7. Ice-cold isopropanol.
8. 1× TE pH 7.5: 10 mM Tris–HCl pH 7.5, 1 mM EDTA pH 8.
9. 10 mg/ml RNase A.
10. Phenol.
11. (Phenol–chloroform)–isoamyl alcohol 24:1.
12. Chloroform–isoamyl alcohol 24:1.
13. 100% Ethanol (EtOH).
14. 70% EtOH.
15. 5 M NaCl.
16. Commercial kits for alternative DNA extraction: Norgen BAC DNA MiniPrep kit (#18000), or Sigma PhasePrep BAC DNA kit (#NA0100).
17. DIG-Nick translation mix (Roche, 11745816910).
18. BioNick™ DNA labeling System (Invitrogen, 18247-015).
19. 10 mg/ml salmon sperm DNA.
20. Deionized formamide.
21. 37 °C incubator.
22. 37 °C shaking incubator.
23. Centrifuge.
24. Refrigerated microcentrifuge.
25. 15 °C water bath.
26. Agarose.
27. Gel electrophoresis system.

2.2 Labeled Chicken Chromosome Paint Probes

Specific chicken chromosome paints labeled with Biotin-16-dUTP or DIG-11-dUTP can be obtained from FARMACHROM (*see* **Note 1**).

2.3 Preparation of Oocyte Spreads for RNA FISH

1. Ice-cold PBS buffer (Phosphate-buffered saline).
2. Ice-cold CSK buffer: 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 10 mM PIPES, adjusted to pH 6.8 and filtered; it can be aliquoted and stored at 4 °C for short term storage or –20 °C for long term storage—Supplements to be added fresh: 0.5% Triton X-100, 1 mM EGTA, 2 mM vanadyl ribonucleoside.
3. Ice-cold 4% paraformaldehyde (PFA) in PBS pH 7 (made fresh and filtered).
4. Ice-cold EtOH series: 70%, 80%, 95%, 100%.
5. 6 cm petri dish.
6. Scalpel blades.

7. Superfrost® slides.
8. Ice-cold platform (glass plate on top of small ice-bucket).
9. Coplin jars.

2.4 Alternative Preparation of Oocyte Spreads

1. PBS buffer.
2. Cell suspension buffer: 9% sucrose, 0.05% Triton X-100 in PBS, adjusted to pH 7; it can be aliquoted and stored at 4 °C for short term storage or −20 °C for long term storage.
3. 2% formaldehyde (FA), 0.02% SDS in PBS, adjusted to pH 7 (made fresh and filtered).
4. 6 cm petri dish.
5. Scalpel blades.
6. Superfrost® slides.
7. Ice-cold platform (glass plate on top of small ice-bucket).
8. Coplin jars.

2.5 RNA FISH

1. 2× hybridization buffer: 4× SSC, 50% dextran sulfate, 2 mg/ml BSA, 2 mM Vanadyl ribonucleoside.
2. Wash solution 1: 50% formamide, 1× SSC.
3. Wash solution 2: 2× SSC.
4. Equilibration buffer: 4× SSC, 0.1% Tween 20.
5. Blocking buffer: 4× SSC, 4 mg/ml BSA, 0.1% Tween 20.
6. Detection buffer: 4× SSC, 1 mg/ml BSA, 0.1% Tween 20.
7. FITC-conjugated anti-digoxigenin (affinity purified sheep polyclonal antibody, part 90426 of Apoptag® kit (S7111) from Merck-Millipore).
8. Vectashield mounting medium with DAPI (Vector).
9. Coplin jars, coverslips.
10. 85 °C dry block.
11. Shaker.
12. 37 °C incubator.
13. 42 °C incubator.
14. Tip Top vulcanizing solution (Rema) (optional).
15. Humidified chambers: box containing paper towels soaked in 50% formamide, 2× SSC; box containing paper towels soaked in 4× SSC.
16. Refrigerated microfuge.

2.6 DNA FISH/ Chromosome Painting

1. PBS buffer.
2. Pepsin solution: 500 µg/ml pepsin in 10 mM HCl.
3. 4% PFA in PBS pH 7.

4. 2× SSC.
5. 100 µg/ml RNase A in 2× SSC (optional).
6. Denaturation solution: 70% deionized formamide, 30% 2× SSC.
7. Ice-cold EtOH series: 70%, 80%, 95%, 100%.
8. 2× hybridization buffer: 4× SSC, 50% dextran sulfate, 2 mg/ml BSA.
9. Wash solution A: 2× SSC.
10. Wash solution B: 0.1× SSC prewarmed at 60 °C.
11. Equilibration buffer: 4× SSC, 0.1% Tween 20.
12. Blocking buffer: 4× SSC, 5% dry milk.
13. Streptavidin, Alexa Fluor® 555 conjugate (Invitrogen S21381).
14. Biotinylated anti-streptavidin antibody (Goat antibody, Vector BA05000).
15. Vectashield mounting medium with DAPI (Vector).
16. 37 °C water bath (optional).
17. Coplin jars, coverslips.
18. 80 °C and 60 °C incubators.
19. 85 °C dry block.
20. Shaker.
21. 37 °C incubator.
22. 42 °C incubator.
23. Tip Top vulcanizing solution (Rema) (optional).
24. Humidified chambers: box containing paper towels soaked in 50% formamide, 2× SSC; box containing paper towels soaked in 4× SSC.
25. Refrigerated microfuge.

3 Methods

Before starting a FISH experiment on chicken oocytes there are several things to consider that are discussed below.

If analyzing early prophase I, bear in mind that the oocytes enter meiosis in an asynchronous fashion. Before hatching the ovary mostly contains oocytes in pre-leptotene, leptotene, and zygotene phases. Pachytene cells start to be common at hatching and become the predominant population between day 1 and 3. Diplotene cells commonly appear at around day 3 and become the major type by day 6. The synaptonemal complex protein SYCP3 is a good marker to stage the cells up to this point [8], but later diplotene cells need to be identified in other ways (e.g., with markers for the centromere or for cohesins).

In multi-label experiments it is advisable to start with RNA FISH, followed by protein immunofluorescence and finally DNA FISH. This is because RNA can be easily degraded. The only instance immunostaining should be done first is when the protein epitope could be harmed by the RNA FISH protocol. In this case try to preserve the RNA by using RNase free solutions also for immunofluorescence and add an RNase inhibitor, such as RNasin® or RNaseOUT™ to the antibody.

Efficient and reliable hybridization to nascent RNAs and corresponding DNA targets is routinely achieved with the use of double strand DNA probes at least 10 kb long. We generally use BAC DNA probes because longer target sequences yield stronger signal (*see* Subheading 3.1 for DNA preparation), but it is possible to generate the probes by PCR. To this end you can choose to do a long range PCR or multiple PCRs generating fragments of the same size covering around 10 KB of the target sequence. After PCR DNA purification, the long probe or the pool of probes can be labeled following the procedure described for BAC DNA probes in Subheadings 3.2, 3.3 and 3.4. When generating the probe by synthesis, if the target sequence is a nascent RNA transcript, make sure to design a probe that contains both intronic and exonic sequences.

We generally use the same DNA sequence to make probes to detect the RNA and the corresponding DNA locus. One probe is labeled with digoxigenin and the other with biotin. These probes are then indirectly detected using proteins/antibodies conjugated to different fluorochromes.

Probe labeling is achieved by nick-translation, which replaces some of the nucleotides with their labeled analogs. During this process the probe is fragmented to generate a pool of tagged fragments whose size is a function of the reaction time. The optimal range of fragments for both RNA and DNA FISH hybridization is around 200 bp. Long probes do not penetrate the cell nucleus very easily and tend to give a high background.

We routinely analyze the results using a compound fluorescence microscope and digital imaging. We generally avoid confocal microscopy to limit fluorescence bleaching. We score the cells using a high magnification lens, generally a 100× oil immersion objective with high numerical aperture. Nascent RNA targets appear as bright small spots. These are generally close to the corresponding DNA sequence. Nonspecific signals in RNA FISH are generally large and irregular in shape. Remember that RNA FISH targeted to nascent transcripts reflects the real transcription status of the individual cell. It is therefore normal that only a percentage of the oocytes display the signal. It is also advisable to score positive and negative cells under the microscope and only then to proceed to imaging the results.

DNA FISH can also be used as a control of the reliability of the RNA FISH to study gene expression, as probes that work for DNA FISH should also work for RNA FISH.

3.1 BAC DNA Preparation

Inoculate a single colony from a freshly streaked plate into 25 ml of LB medium with the appropriate antibiotic and grow overnight in a 37 °C shaking incubator (*see* **Note 2**).

Proceed to isolate the BAC DNA using a basic alkaline lysis miniprep method as described below.

1. Transfer the culture into two 15 ml tubes and centrifuge for 10 min at 3500 × *g* (best in a swinging bucket centrifuge) at room temperature (RT).
2. Resuspend the pellet in 1 ml of P1 solution by vortexing.
3. Add 1 ml of P2 solution and mix by gently inverting the tubes. Incubate at RT for 5 min (the suspension becomes translucent).
4. Add 1 ml of ice-cold P3 solution and mix by inverting the tubes. Incubate on ice for 5 min (a thick precipitate will form).
5. Aliquot the suspension into two 1.5 ml tubes (about 1.4 ml each) (leaving behind most of the precipitate) and centrifuge in a microfuge at 13,000 rpm (16,200 RCF) for 10 min at 4 °C.
6. Transfer 1.2 ml of supernatant into two 2 ml tubes (avoiding the white precipitate) (*see* **Note 3**).
7. Add 0.6 Volumes (V) of ice-cold isopropanol, mix by inverting the tubes, and centrifuge in a microfuge at max speed for 15 min at 4 °C.
8. Discard the supernatant, add 1 ml of 70% EtOH, mix by inverting the tubes and centrifuge 5 min at max speed.
9. Air-dry the pellet at RT until it turns translucent (avoid over-drying as it makes it difficult to resuspend the DNA), then resuspend it in 120 μl of 1× TE per tube, by tapping the tubes and by incubating them at 37 °C for at least 1 h.
10. Add 1 μl of RNase A to each tube and incubate at 37 °C for at least 1 h (you can leave it overnight).
11. Combine the two tubes (240 μl in total), add 1× TE to have 400 μl total volume and remove RNase A by phenol–chloroform extraction: add 400 μl of phenol, invert the tube several times, centrifuge in microfuge at max speed and transfer upper phase to a new tube. Repeat this step using 400 μl of (phenol–chloroform)–isoamyl alcohol 24:1 and repeat again using 400 μl of chloroform–isoamyl alcohol 24:1. Avoid carryover of solvents.
12. Precipitate the DNA by adding 200 mM NaCl and 2 V of 100% ice-cold EtOH.
13. Wash pellet with 500 μl of 70% EtOH, centrifuge in microfuge at max speed for 5 min.
14. Air-dry the pellet and resuspend it in 32 μl RNase free H_2O (*see* **Note 4**).
15. Measure the DNA concentration using a spectrophotometer (*see* **Note 5**).

3.2 Generating a BAC DNA Probe Using the BioNick™ Labeling System

1. Use 1–2 μg of BAC DNA in 40 μl H_2O (*see* **Note 6**), mix by pipetting.
2. Add 5 μl of dNTP mix from the kit.
3. Add 5 μl of 10× enzyme mix from the kit.
4. Mix well and spin down briefly.
5. Incubate at 15 °C until the probe length is in the range of 200 bp. Check probe length by gel electrophoresis, running an aliquot of the reaction (5 μl) on a 2% agarose gel; if necessary extend the incubation time and run another aliquot on the gel (*see* **Note 7**).
6. Stop the reaction by adding 5 μl of stop buffer from the kit.
7. Store the probe at −20 °C until ready to use.

3.3 Generating a BAC DNA Probe Using the DIG-Nick Translation Mix

1. Use 1–2 μg of BAC DNA in 16 μl H_2O (*see* **Note 6**), mix by pipetting.
2. Add 4 μl of DIG-Mix (5×) from the kit.
3. Mix well and spin down briefly.
4. Incubate at 15 °C until the probe length is in the range of 200 bp. Check probe length by gel electrophoresis, running an aliquot of the reaction (2 μl) on a 2% agarose gel; if necessary extend the incubation time and run another aliquot on the gel (*see* **Note 7**) (Fig. 1).
5. Store the probe at −20 °C until ready to use.

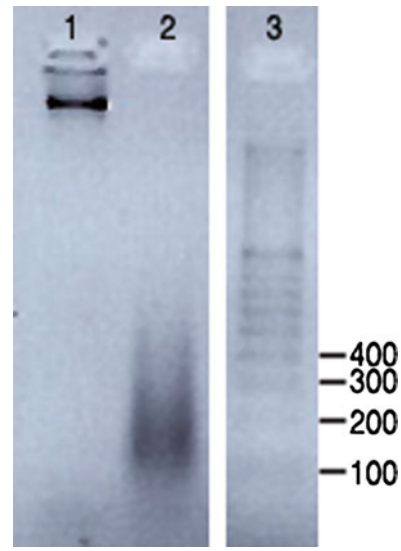

Fig. 1 DIG-labeling of a BAC DNA probe. *Lane 1*, an aliquot (2 μl/32 μl) of a BAC DNA from the alkaline miniprep; *lane 2*, an aliquot (2 μl/20 μl) of the BAC DNA after DIG-labeling showing a smear in the range of 100–300 bp; This smear range is optimal for RNA or DNA hybridization. *Lane 3*, 100 bp ladder

3.4 Preparation of the Probe for Hybridization

Use 1/10 V of the BAC DNA labeled probe generated as described in Subheading 3.2 or 3.3 per slide to be hybridized. Scale up based on number of slides.

1. Transfer 5 μl of biotinylated probe or 2 μl of DIG probe to a 0.5 ml tube and add 1 μl of salmon sperm DNA; adjust the total volume to 9 μl with H_2O (*see* **Note 8**).
2. Precipitate the DNA by adding 3 V of 100% EtOH; store at −80 °C for 10 min then centrifuge at max speed in a microfuge for 15 min at 4 °C.
3. Wash the pellet with 20 μl of 70% EtOH and centrifuge again at max speed for 5 min at 4 °C.
4. Air-dry and resuspend the pellet in 15 μl of formamide by vortexing.
5. To prepare chromosome paint probes *see* **Note 9**.
6. Store at −20 °C, or proceed to use in hybridization: denature the probe at 85 °C for 10 min, add 15 μl of 2× hybridization buffer preheated at 37 °C and incubate at 37 °C for 20 min before placing it onto the slide (*see* **Note 10**).

3.5 RNA FISH

1. Before starting to process the ovary, boil Superfrost® slides in distilled H_2O for 10 min (*see* **Note 11**), air-dry the slides and then place on an ice-cold, horizontal platform.
2. Dissect the left ovary in cold PBS ensuring the connected mesonephros is removed without compromising the surface epithelium of the ovary, as the germ cells are just beneath. From one embryonic or early post-hatching ovary you can generate six to eight spreads (*see* **Note 12**).
3. Transfer the ovary to a 150 μl drop of ice-cold PBS on a small Petri dish (6 cm) and mince it with scalpel blades to obtain a milky suspension.
4. Tilt the Petri dish, add another 300 μl of cold PBS and pipette up and down a few times to maximize the release of germ cells.
5. Wait for 5 min to allow for the debris to sink.
6. Place 70 μl of the suspension on the center of each slide using a micropipette and avoid aspirating the debris. Drop the suspension from a good height (few cm) to allow spreading and leave for 5 min to settle down.
7. Permeabilize the cells by flooding the slide with ice-cold CSK + supplements; leave for 10 min.
8. Drain the liquid, first by gentle aspiration with a micropipette at one end of each slide, then by placing paper towels at one end of the slides and tilting the platform.
9. Fix the cells by flooding the slide with 4% ice-cold PFA and leave for 10 min.

10. Remove the slides from the cold platform and pour off the fixative.
11. Rinse the slides in a Coplin jar filled with ice-cold RNase free H_2O.
12. Transfer to a Coplin jar with ice-cold 70% EtOH for 3 min and proceed to dehydrate via an ethanol series starting with a second wash in 70% EtOH, then 80, 95, and 100% for 3 min each.
13. Air-dry thoroughly at RT (*see* **Note 13**).
14. In the meantime process the DNA probe as described in Subheading 3.4. Be ready to start the probe denaturation step (described in **step 6** of Subheading 3.4) while performing the dehydration of the slides in EtOH (**step 12**).
15. Place the probe from **step 6** in Subheading 3.4 on the center of the slide and add a coverslip avoiding bubble formation (*see* **Note 14**).
16. Put the slides in a humidified chamber and incubate overnight at 37 °C (*see* **Note 15**).
17. On the next day preheat a Coplin jar and stringency wash solutions 1 and 2 at 42 °C.
18. Perform three 5 min washes in the Coplin jar filled with prewarmed wash solution 1 on a shaker at RT. If the coverslip is too adherent to the slide remove it only after the first wash.
19. Perform another three 5 min washes in wash solution 2 at 37 °C with shaking.
20. Transfer slides in equilibration buffer at RT and leave until ready to proceed to the next step.
21. Transfer the slides to a humidified chamber (containing paper towels soaked in 4× SSC) and drop 100 μl of blocking buffer on top; place the coverslip and incubate at 37 °C for 30 min.
22. If the hybridization was performed using a DIG-labeled DNA probe, dilute the FITC-conjugated anti-digoxigenin antibody 1:10 in detection buffer and spin 10 min at 4 °C in a microfuge to remove potential precipitates (*see* **Note 16**).
23. Pour off the blocking buffer from the slides and add 100 μl of the diluted antibody per slide; place the coverslip and incubate in the humidified chamber at 37 °C for 60 min.
24. Remove the coverslip and perform three 5 min washes in equilibration buffer at RT with shaking.
25. Drain the slides; at this point you can proceed immediately to immunostaining and/or, if needed, DNA FISH/chromosome painting, or you can add mounting medium plus DAPI and image. Slides in mounting medium can be stored at −20 °C and processed for antibody staining and/or DNA FISH in the next few days (*see* **Note 17**) (Fig. 2).

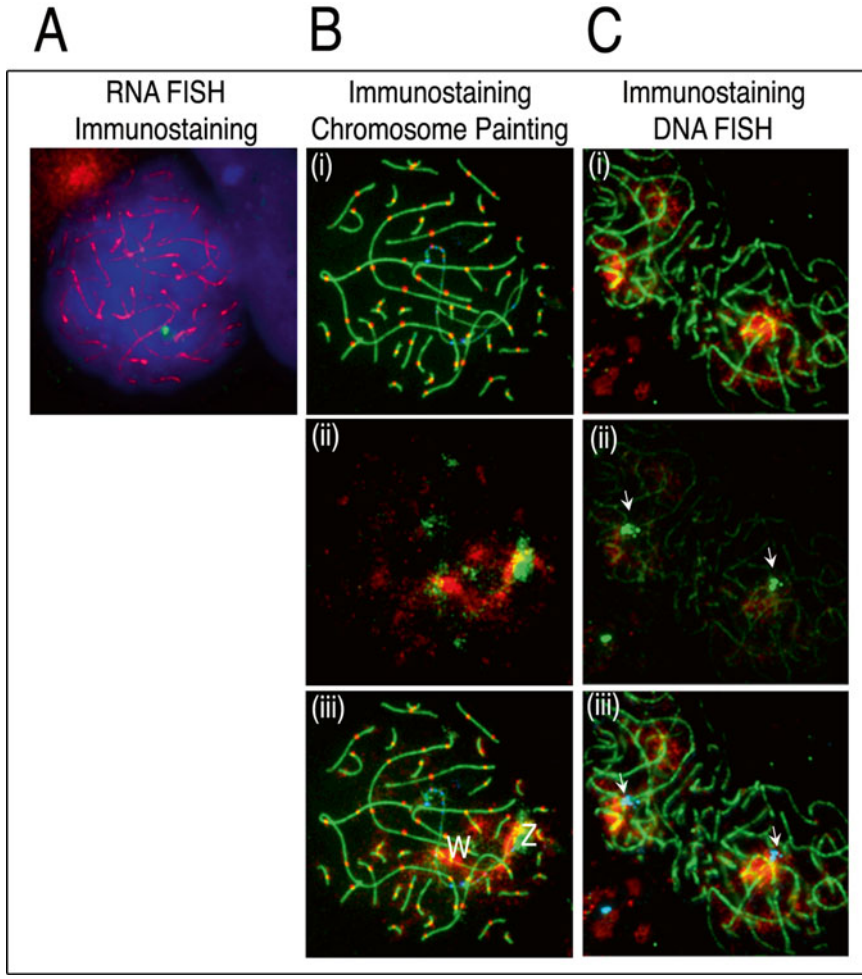

Fig. 2 Oocyte nuclei from 1 day post hatching ovarian spreads. (**A**) Late zygotene/early pachytene cell processed for RNA FISH followed by immunostaining; the RNA FISH signal in *green* identifies nascent transcripts detected with a BAC DNA probe mapping to the Z chromosome. *Red* is the immunofluorescence signal for the SYCP3 protein, which labels the lateral elements of the synaptonemal complex (SC). (**B**) (*i*) Late pachytene cell immunostained for SYCP3 (*green*), MLH1 (*red*) and RPA-32 (*blue*) which label SCs, crossovers and sites of unrepaired double strand breaks, respectively. (*ii*) Same cell from (*i*) reimaged after processing the slide for Z (*green*) and W (*red*) chromosome painting. (*iii*) Composite image from (*i*) plus (*ii*). This late pachytene cell carries over unrepaired double strand breaks on the Z chromosome. (**C**) (*i*) Two pachytene cells Immunostained for SYCP3 (*green*) and H3K9me3 (*red*) which label SCs and heterochromatin blocks, respectively; (*ii*) same cells from (*i*) reimaged after DNA FISH using the BAC DNA probe used in panel **A** (the signal, in *green*, is indicated by *arrows*); (*iii*) Composite image from (*i*) plus (*ii*) where the DNA FISH signal is pseudocolored in *light blue*. The locus detected by the probe is outside the heterochromatin block. Note that the RNA FISH signal in panel **A** is small and tight, while the DNA FISH signal (using the same probe) in panel **C** is made by multiple spots

3.6 Oocyte Spreads for Immuno-fluorescence and DNA FISH/Chromosome Painting

To generate slides with well spread chicken oocytes from embryonic ovaries and 1–2 weeks post-hatch ovaries we use a variation of the protocol used for RNA FISH (*see* **Note 18**). Essentially follow **steps 1–6** in Subheading 3.5 to release the germ cells from the ovary then:

1. Permeabilize the cells by flooding the slide with 800 μl of cell suspension buffer; leave for 10 min.
2. Drain the liquid, first by gentle aspiration at one end of each slide using a micropipette, then by placing paper towels and tilting the slide.
3. Fix the cells by flooding the slides with PBS containing 2% formaldehyde, 0.02% SDS (pH 7) and leave for 1 h (cover the platform with a lid to generate a humidified chamber).
4. Drain the liquid on paper towels by tilting the slides or the all platform; rinse the slides in a Coplin jar filled with H_2O and then let them air-dry upright (*see* **Note 13**).

3.7 DNA FISH and Chromosome Painting

The protocols for DNA FISH and chromosome painting are essentially the same with a minor modification concerning the preparation of the probe, which is addressed in Subheading 3.4. Moreover many of the steps are the same as for RNA FISH.

DNA FISH or chromosome painting can be performed on newly prepared spreads (fresh or after storage). In this case it is important to promote access to chromatin by aging the slides. This can be done by incubating the slides at 60 °C for 2–3 h, or at RT in pepsin solution for 10 min just before starting the protocol.

If DNA FISH or chromosome painting is performed after immunohistochemistry, RNA FISH, or RNA FISH plus immunohistochemistry, the aging step is not necessary; instead, post-fix the slides in 4% PFA for 10 min at RT before starting.

Then:

1. Three 5 min washes in PBS, at RT.
2. One 5 min wash in 2× SSC, at RT (*see* **Note 19**).
3. Incubate 5 min in preheated 2× SSC at 80 °C.
4. Denature the chromatin by incubating the slides in preheated denaturation solution for 3 min at 80 °C.
5. Transfer to a Coplin jar with ice-cold 70% EtOH for 3 min and proceed to dehydrate via an ethanol series starting with a second wash in 70% EtOH, then 80, 95, and 100% for 3 min each.
6. Air-dry thoroughly at RT.
7. In the meantime process the DNA probe as described in Subheading 3.4. Be ready to start the probe denaturation step (described in **step 6** of Subheading 3.4) while performing the dehydration of the slides in EtOH (**step 5**).

8. Place the probe from **step 6** in Subheading 3.4 on the center of the slide and add a coverslip avoiding bubble formation (*see* **Note 14**).
9. Put the slides in a humidified chamber and incubate overnight at 37 °C (*see* **Note 15**).
10. On the next day transfer the slides to a Coplin jar.
11. Perform four 3 min washes in wash solution A at 42 °C (the coverslip should fall off after the first wash, if not, carefully ease it off the slide).
12. Perform four 3 min stringency washes in preheated wash solution B at 60 °C.
13. Wash briefly in equilibration buffer preheated at 37 °C.
14. Drain the slides, transfer them to a humidified chamber (containing paper towels soaked in 4× SSC) and drop 100 μl of blocking buffer on top; place a coverslip and incubate at 37 °C for 30 min.
15. In the meantime prepare substrates and antibodies for the detection steps. To detect the biotinylated probe, dilute the streptavidin–Alexa 555 1:100 in blocking buffer and spin the solution in a microfuge at 10,000 rpm (9,600 RCF) for 10 min at 4 °C to remove any precipitate (*see* **Note 20**).
16. Slide off the coverslip and drain the buffer.
17. Add 50 μl of diluted streptavidin–Alexa 555 on top of each slide, add the coverslip and place the slides back in the humidified chamber at 37 °C for 30 min.
18. Remove the coverslip, transfer the slides to a Coplin jar and do four 3 min washes in equilibration buffer at 37 °C with shaking.
19. Drain the slides, replace the coverslip and observe under a fluorescence microscope if the signal is present. If so, proceed to **step 24**. If the signal is not present, or it is too weak, proceed with amplification **steps 20–23**. If the slides were simultaneously hybridized to a DIG probe, proceed to step 25 now or after the biotin signal amplification (**steps 20–23**).
20. Dilute the biotin conjugated anti-streptavidin antibody 1:100 in blocking buffer and spin the solution in a microfuge at 10,000 rpm for 10 min at 4 °C to remove any precipitate.
21. Add 50 μl of diluted biotin conjugated anti-streptavidin antibody on top of each slide, add the coverslip and place the slides back in the humidified chamber at 37 °C for 30 min.
22. Repeat **step 18** washes.
23. Repeat **steps 16** and **18** to reapply the streptavidin–Alexa 555.
24. Drain the slides and mount it with a drop of mounting medium plus DAPI and place a coverslip. The slides are ready for imaging or can be stored at –20 °C.

25. To reveal the DIG probe dilute the FITC conjugated anti-DIG antibody 1:10 in blocking buffer and spin the solution in a microfuge at 10,000 rpm for 10 min at 4 °C to remove any precipitate.
26. Add 50 μl of diluted FITC-conjugated anti-DIG antibody to each slide, place the coverslip and place the slides back in the humidified chamber at 37 °C for 60 min.
27. Repeat **step 18** washes and proceed to finalize as in **step 24** (Fig. 2).

4 Notes

1. FARMACHROM is a resource center based at the University of Kent, Canterbury, CT2 7NJ, UK, and it is a part of the laboratory of Prof. D. Griffin. Chicken chromosome paints and other avian resources can be requested at the site www.farmachrom.net by filling in a resource request form. The paints are generated and labeled by degenerate oligonucleotide primed polymerase chain reaction (DOP-PCR) on flow sorted or microdissected chromosomes [14]. It is possible to ask for unlabeled or labeled paints.
2. A 25 ml culture should yield enough BAC DNA for at least two labeling reactions.
3. Alternatively distribute ~900 μl of the supernatant into three 1.5 ml tubes.
4. Alternatively to this basic protocol you can use commercially available kits for small scale preparation of BAC DNA. We tested the BAC DNA miniPrep kit from Norgen and the PhasePrep BAC DNA kit from Sigma and they are both suitable. The Norgen kit is based on spin column chromatography. Follow the manufacturer's instructions, with some modifications as follows. After spinning the bacterial culture, resuspend pellet in 800 μl of resuspension buffer; scale up next steps of lysate preparation and column binding accordingly. You will use two columns per culture. Wash the DNA bound to the columns with 600 μl of wash solution twice; elute the DNA from each column in 70 μl of elution buffer. Combine the elution from the two columns and extend the manufacturer protocol to include an EtOH precipitation step (add 200 mM NaCl, 2 V of 100% EtOH and spin at max speed in a microfuge for 20 min at 4 °C; also perform a 70% EtOH wash) to get better purification. Resuspend in 32 μl of H_2O.

   The Sigma PhasePrep BAC DNA kit is based on temperature mediated extraction of impurities and phase separation. It consistently produces good quality DNA that is suitable for

labeling without further steps; it is also easily scalable. Follow the manufacturer's instructions for micro scale preparation, scaling up the prep accordingly. After spinning the 25 ml bacterial culture, resuspend pellet in 800 μl of resuspension solution (at this point it is best to transfer the sample into two 2 ml tubes for easy processing) and scale up the next steps accordingly until the nucleic acid precipitation step (**step 5** in their protocol). Resuspend the pellet in 800 μl of elution solution and scale up again the next steps accordingly. Dissolve the purified DNA in 32 μl of H_2O.

5. We use a NanoDrop spectrophotometer for microvolumes. Alternatively you can estimate the concentration by gel electrophoresis.
6. Generally around half of the DNA prepared from the miniprep.
7. The labeling step is very important and therefore it has to be monitored. Electrophoresis is used to visualize the smear produced by nick-translation. Under-labeled probes will appear as high molecular weight smears. These long probes poorly penetrate cells and therefore seldom generate good specific signals while promote nonspecific background in the form of aggregates outside the nuclei. It is safe to check the labeling after 30 min of incubation and decide whether to continue the reaction and for how long. It is possible to speed up the reaction by a short burst at 37 °C. The incubation time is mainly dependent on DNA concentration and purity, so it has to be determined empirically every time a new preparation is made.
8. Many FISH protocols add Cot-1 DNA (the repetitive DNA fraction) to the probe as unlabeled competitor, to neutralize the repetitive DNA present in the probe. We routinely do RNA FISH and DNA FISH using BAC probes without Cot-1 and do not generally have much background problems. At the moment chicken Cot-1 DNA is not commonly available on the market and you have to find your own source, or generate it in your laboratory. If adding Cot-1 DNA, do so at this step before adding the EtOH for the precipitation: typically 3 μg.
9. Labeled chicken chromosome paint probes provided by FARMACHROM are ready to be used for hybridization, so there is no need for the precipitation procedure. Normally we use 1 μl of labeled paint per slide (check with the provider for differences between batches). Dilute 1 μl of paint in 15 μl of formamide per slide to be hybridized, and proceed to **step 6**.
10. We use the same hybridization buffer for RNA FISH, DNA FISH and chromosome painting. For RNA FISH we add an RNase inhibitor (Vanadyl-ribonucleoside).
11. Put the slides in a staining trough, add enough H_2O to completely cover the slides, place the cover on top and put in a microwave at full power for 10 min.

12. At this point it is possible to store the ovary at −80 °C; place the ovary in a 1.5 ml tube, snap-freeze in dry ice and transfer to −80 °C. When ready you can proceed to **step 2** (ovaries can be safely stored for long periods). The RNA FISH signal will not be compromised by freezing. Use the all ovary to generate a batch of spreads, as meiotic progression is asynchronous and oocytes localized in different parts of the ovarian cortex are in slightly different phases of the process.
13. At this point the slides can be used immediately or stored in a box at −80 °C for later use (spreads can be safely stored at −80 °C for long periods).
14. An alternative way to add the probe is to place it on the coverslip and then invert the slide on top of the coverslip.
15. We put individual slides in a slide tray plate and the plate in a box containing paper towels soaked with 50% formamide in 2× SSC. Alternatively seal the slide with tip-top solution. In this case you have the choice of using a box in a 37 °C dry incubator, or of using a floating tray, covered with foil and placed in a 37 °C incubator bath. This last option is feasible with only a few slides.
16. In our experience DIG probes result in cleaner signal, therefore we prefer to use them for RNA FISH, or for single labeling experiments. The detection of biotin labeled probes is described in **steps 15–24** of Subheading 3.7.
17. Immunostaining is generally complementary to the RNA FISH protocol as it allows, amongst other aims, at least for identification and staging of the oocytes on the slide. We prefer performing it after the RNA FISH protocol to limit the possibility of RNA degradation. Check if the RNA FISH has worked by inspecting the slide under a fluorescence microscope. If the signal is fine transfer the slide to PBS and proceed with the immunostaining protocol specific to the antibodies to be used. The RNA FISH signal should survive the immunostaining treatment and the results from the two experiments can be imaged at the end. If RNA FISH has to be followed by DNA FISH or chromosome painting, do the immunostaining after the RNA FISH as indicated earlier. Record the images and then start the DNA FISH/chromosome paint protocol. Then record the new images. RNA and immunostaining signals may survive DNA FISH, but they become much weaker and a bit brittle because of the harsh treatment. It is therefore best to record the images before and after the DNA FISH. For this purpose it is necessary to use a fluorescence microscope equipped with a stage ensuring accuracy and precision of slide position and with motorized XY positioning. The XY coordinates of the cells imaged for RNA and antibody signals stored in a file are used after DNA FISH to retrieve the selected cells for reimaging.

18. This protocol generates better chicken oocyte spreads than the one described for the RNA FISH protocol and therefore it is preferred for immunohistochemistry with or without DNA FISH or chromosome painting, or, anyway, when the priority is a clear nuclear architecture.
19. We do not generally perform an RNase step before starting the chromosome painting/DNA FISH protocol on newly prepared slides, but if you have background problems, you can try troubleshooting by adding the RNase step at this point. After the rinse in 2× SSC, transfer the slides to a humidified chamber and add 100 µl of RNase solution per slide (100 µg/ml RNase A in 2× SSC). Incubate at 37 °C for 1 h and continue the protocol from **step 2**.
20. As mentioned in **Note 16**, in our experience DIG probes give cleaner signal in RNA FISH and DNA FISH, so we prefer to use DIG probes for single labeling experiments. This is not true for chromosome painting where both DIG or biotin probes produce equally good signals. When sequentially performing RNA and DNA FISH on the same slide we advise to use DIG probes for RNA FISH and biotinylated probes for DNA FISH because the detection of native RNA is trickier. When performing a simultaneous hybridization with two different probes, one labeled with biotin and the other with digoxigenin, always proceed by detecting first the biotinylated probe and then the DIG probe (this is valid for RNA FISH, DNA FISH, and chromosome painting).

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Chapter 15

Preparation of Cell Lysate from Mouse Oocytes for Western Blotting Analysis

Petros Marangos

Abstract

Western Blotting has been used extensively for the identification of the protein factors that regulate mammalian oocyte meiosis. However, the limitations in collecting sufficient numbers of oocytes can hinder the efficiency of the technique. Here we provide a detailed protocol for the accurate preparation of mouse oocyte samples for Western Blotting analysis.

Key words Western blotting, SDS-PAGE, Mouse oocyte, Primary antibody, HRP-conjugated antibody, Chemiluminescence, Securin, Morpholino

1 Introduction

One of the most important techniques for mammalian oocyte experimentation is Western Blotting. Western Blotting is an experimental method used to determine the presence and the total amount of a specific protein in a cell or tissue sample. The method involves the separation of all the sample proteins through SDS-PAGE depending on their molecular weight. Electrophoresis is followed by the transfer (blotting) of the proteins on a membrane and the detection of the target protein by specific antibodies.

Western blotting has been imperative for determining the protein factors necessary for mammalian oocyte regulation. Western blotting has been used for determining the presence and abundance of protein factors and for verifying the effectiveness of RNA interference (RNAi) approaches [1–3]. Nevertheless, in mammalian oocytes, it has always been challenging to collect sufficient amounts of protein for efficient detection. Unlike proliferating somatic cells, fully grown mammalian oocytes are nondividing cells, arrested at G2/Prophase of meiosis and can only be collected from sacrificed laboratory animals [4]. For somatic cell protein detection, a sample for Western Blotting usually contains 10–20 μg

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of total protein. Unlike a fully grown (stage VI) *Xenopus laevis* oocyte which contains more than 500 μg of protein [5], the mouse oocyte total protein content is approximately 25 ng [6]. If the detection of a specific protein factor requires 1/10 of one oocyte in *X. laevis*, it would require 200 mouse oocytes and the sacrifice of approximately five animals. Therefore, due to the limitations in material availability and recovery, it is imperative that sample preparation and Western Blotting is performed with increased efficiency when experimenting on mammalian oocytes.

Here we describe all the necessary steps for optimized sample preparation for the efficient detection of specific proteins by using the method of Western Blotting in mouse fully grown oocytes.

2 Materials

2.1 Collection and Culture of Oocytes

1. 20–25 old female MF1 or CD1 mice.
2. Pregnant mare's serum gonadotropin (PMSG).
3. M2 medium, with BSA.
4. M16 medium with BSA.
5. 200 μM 3-isobutyl-1-methylxanthine (IBMX). Make up 200 mM stock solution by adding 22 mg IBMX to 0.5 ml DMSO. Aliquot and store at −20 °C.
6. Plastic Petri dishes, 35 mm diameter.
7. 0.22 μm filters.
8. Hot-block set to 37 °C.
9. Incubator set at 37 °C with an ambient atmosphere of 5% CO_2.
10. 27 gauge needle.
11. Mouth pipette.
12. Mineral oil.

2.2 Sample Preparation for SDS-PAGE

1. 1% Polyvinylalcohol (PVA).
2. Phosphate-buffered saline (PBS) tablets.
3. Sample lysis buffer (4×): 424 mM Tris–HCl, 564 mM Tris, 8% lithium dodecyl sulfate (LDS), 40% glycerol, 2.04 mM ethylenediamine tetraacetic acid (EDTA), 0.88 mM Coomassie Blue, 0.7 mM Phenol Red.
4. Nuclease-free water.

2.3 SDS-PAGE and Western Blotting

1. Running Buffer (20×): 1 M MOPS, 1 M Tris, 2% SDS, 20 mM EDTA.
2. Precast 4–12% Bis–Tris mini-gels.
3. Electrophoresis system compatible with mini-gels.

4. Prestained molecular weight markers.
5. Western blotting buffer: 25 mM Tris, 0.192 M Glycine, 20% methanol.
6. Blotting paper (3 MM).
7. Polyvinylidene fluoride (PVDF) membrane.
8. Electrophoretic Transfer Cell.
9. PBS containing 0.05% Tween-20 (PBS-T).
10. Blocking buffer: 3% bovine serum albumin (BSA) in PBS-T.
11. Primary antibody.
12. Secondary antibody conjugated to horseradish peroxidase (HRP).
13. Enhanced chemiluminescent (ECL) reagents.

3 Methods

3.1 Mouse Oocyte Collection and Culture

1. Inject, intraperitoneally, 7 IU of PMSG into 21–25 days old female mice.
2. For collection of fully grown germinal vesicle stage (GV) oocytes, sacrifice the mice 40–50 h post-injection and collect the ovaries.
3. Filter approximately 10 ml M2 medium and add IBMX to a final concentration of 200 mM (M2-IBMX). IBMX should be kept as a stock solution of 200 mM at –20 °C (*see* **Note 1**).
4. Prewarm medium at 37 °C on a hot-block 30 min prior to collection of tissues.
5. Filter M16 medium and equilibrate in a CO_2 incubator at 37 °C at least 2 h prior to collection of tissues.
6. Prepare Petri dishes containing drops of M2 and/or M16 medium covered by mineral oil and place on hot-block and/or incubator, respectively.
7. Pull glass Pasteur pipettes over a flame to use for mouth-operated pipetting.
8. Place the collected ovaries in a Petri dish containing 1–2 ml M2-IBMX.
9. Release cumulus-enclosed GV oocytes by repeated puncturing of the surface of the ovary with a 27-guage needle.
10. Transfer GV oocytes into fresh drops of M2-IBMX.
11. After all the oocytes are transferred, mechanically remove the surrounding cumulus cells by repeated pipetting with an appropriately sized glass pipette and transfer the cumulus free oocytes to fresh drops of M2-IBMX (*see* **Note 2**).
12. Maintain oocytes on the hot-block if the immunoblotting sample is to be prepared within 1–2 h. Otherwise, place oocytes in M16 in a CO_2 incubator until the time of sample preparation.

3.2 Sample Preparation for SDS-PAGE

1. Dissolve PVA into PBS in order to prepare a solution of 1% PVA (PBS–PVA) (*see* **Note 3**).
2. Filter PBS–PVA and prewarm at 37 °C at least 30 min prior to use (*see* **Note 4**).
3. Prepare Eppendorf tubes containing 5 μl of sample lysis buffer (4×) (*see* **Note 5**) mixed with 10 μl of sterile-filtered water (*see* **Note 6**).
4. Calibrate a pulled glass pipette for 5 μl by aspirating 5 μl of PBS or water and mark the level of the meniscus with an indelible marker.
5. Prepare a Petri dish with three drops of PBS–PVA covered by mineral oil.
6. Wash oocytes in PBS–PVA by transferring the cells between drops (*see* **Note 7**).
7. At the last washing drop, place all the oocytes in close proximity to each other (*see* **Note 8**).
8. Aspirate all the oocytes in 5 μl of PBS–PVA by using the calibrated glass pipette, transfer to the sample Eppendorf tube and release the content of the pipette within the sample lysis buffer-water solution (*see* **step 3**) while mixing (*see* **Note 9**).
9. Snap-freeze sample in dry ice for 5 min.
10. Repeat thawing–freezing twice and store at −80 °C.

3.3 SDS-PAGE and Western Blotting

1. Prepare 800 ml of running buffer from 20× stock solution and ultra-pure water (*see* **Note 10**).
2. Assemble the electrophoresis unit by placing the gel and filling the tank with the running buffer (*see* **step 1**).
3. Thaw, mix, and centrifuge samples at (6800×*g*) for 10 s.
4. Heat samples at 90 °C for 5 min (*see* **Note 11**).
5. Centrifuge samples at (6800×*g*) for 10 s and load each sample, including a prestained marker, into a separate well of the gel (*see* **Note 12**).
6. Run the gel at 150 V until the dye front reaches the edge of the gel.
7. While electrophoresis is under way, prepare 2 l of blotting buffer, six sheets of blotting paper (9 × 7 cm), and a segment of PVDF membrane (*see* **Notes 13** and **14**).
8. Shake the blot gently in methanol for 1 min, followed by rinsing with ultra-pure water for 1 min and shaking in blotting buffer until the time of blotting.
9. Assemble the holder cassette by placing in the following order: black side of cassette, three sheets of paper, the gel, the membrane, three sheets of paper, white side of cassette (*see* **Note 15**).
10. Assemble the blotting apparatus by placing in the tank precooled blotting buffer, the cassette (*see* **step 9**), a magnetic stir bar and a cooling unit (*see* **Note 16**).

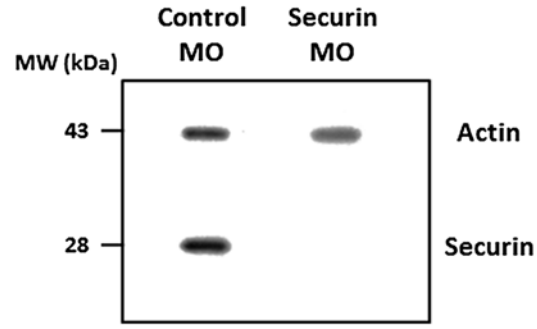

Fig. 1 Western blot showing Securin and Actin expression and Morpholino (MO)-induced depletion in GV-stage oocytes. Oocytes were micro-injected with either a Securin MO or control MO [9] and maintained in IBMX for 24 h before collecting/freezing for Western Blotting. Fifty oocytes per sample were immunoblotted with antibodies against Securin and Actin. In this blot, the membrane was first probed for Securin and then for Actin

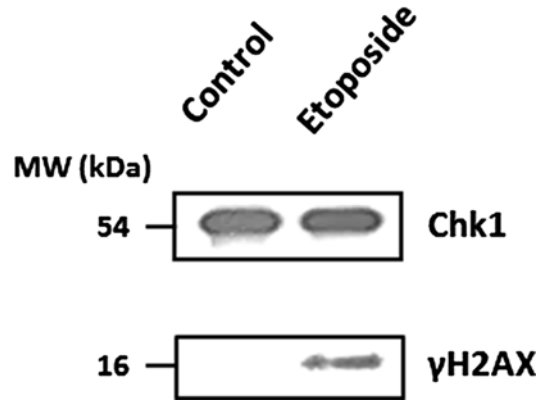

Fig. 2 Western blot showing phosphorylated histone H2AX (γH2AX) and Checkpoint kinase 1 (Chk1; total protein) expression following Etoposide-induced DNA damage in GV-stage oocytes. Oocytes were incubated with 100 μg/ml of the Topoisomerase II inhibitor Etoposide and maintained in IBMX for 1 h before collecting/freezing for Western Blotting. One hundred oocytes per sample were immunoblotted with antibodies against γH2AX and Chk1. The blot was cut into different fragments before probing with antibodies. The blot fragment incorporating the 16 kDa MW was probed with the γH2AX antibody and the fragment incorporating the 54 kDa MW was blotted with the Chk1 antibody. We see that DNA damage induces γH2AX activation, while the total protein levels of Chk1 remain unaltered

11. Perform the transfer on a magnetic stirrer in a cold room (*see* **Note 17**) at 500 mA for 2 h.
12. Following transfer, place the membrane in blocking buffer and shake gently for 1 h.
13. Incubate the membrane with the primary antibody at 4 °C overnight while shaking gently (*see* **Note 18**).

14. Wash three times with PBS-T (5 min each time).
15. Incubate the membrane with the HRP-conjugated secondary antibody at room temperature for 1 h while shaking gently (*see* **Note 18**).
16. Repeat **step 14**.
17. Detect specific protein bands according to the chemiluminescence method by using ECL Plus substrates and a digital Western Blot imaging system (*see* **Note 19**) (*see* Figs. 1 and 2).

4 Notes

1. IBMX (3-isobutyl-1-methylxanthine) is a phosphodiesterase inhibitor that allows the accumulation of cyclic AMP (cAMP) in GV oocytes. cAMP activates a cascade reaction that leads to inhibition of CDK1-Cyclin B and to subsequent maintenance of G2/Prophase arrest of GV oocytes [7].
2. All cumulus cells should be removed from the oocytes. Otherwise, their protein content would be included in the oocyte protein immunoblotting samples and therefore interfere with the immunoblotting results.
3. Polyvinyl alcohol (PVA) is a water-soluble synthetic polymer and is used for preventing oocytes from attaching to the Petri dish when oocytes are removed from the culture media.
4. The PBS–PVA washing buffer must be warm. Otherwise, oocytes could be destroyed before transferring to the sample buffer.
5. The sample buffer is used for the lysis of the cell membranes.
6. Water provides a hypotonic environment that accelerates the lysis of the oocyte plasma membrane.
7. Washing removes the culture medium. This is imperative because the proteins in the medium and especially BSA (bovine serum albumin), which is present in large amounts, may interfere with the mobility of the sample proteins during electrophoresis.
8. If oocytes are dispersed within the drop, it will be difficult to collect them all in 5 μl.
9. The total volume of the sample is 20 μl because this is the volume of the well of the electrophoresis gel used in this protocol. It is important that the volume of the sample does not exceed that of the well of the gel. The sample volume can be reduced to 16 μl if the experimenter is not confident that 20 μl of sample can be placed in the gel well.
10. An XCell *SureLock* Mini-Cell gel unit and Novex precast 4–12% mini-gels have been used for SDS-PAGE in this protocol. Precast gels are more reliable compared to self-made SDS-PAGE gels; it

is important to avoid mistakes due to the limited availability of mammalian oocytes. The 4–12% polyacrylamide concentration allows greater separation of proteins during electrophoresis.

11. Heating at high temperatures causes the denaturing of the proteins which is necessary for the proteins to run on the gel only according to their molecular weight and not their secondary or other structures.
12. Keep the number of air bubbles introduced to the well to a minimum and load the samples at the bottom of the wells to avoid spillage.
13. A mini trans-blot cell has been used for blotting in this protocol.
14. The dimensions of the PVDF membrane depend on the number of wells/samples used. It should not exceed the dimensions of the gel.
15. It is vital that no air bubbles are trapped within the cassette. Bubbles can be transferred during blotting and distort the protein signal.
16. It is crucial to obtain the correct orientation in order to prevent the proteins being transferred into the buffer instead of the membrane.
17. If a cold room is not available, the blotting apparatus can be placed on ice during transfer. It is also advised to replace the blotting buffer with fresh cool buffer half-way during transfer.
18. All antibody dilutions should be prepared in blocking buffer.
19. For a more detailed description of the immunoblotting of specific proteins in mouse oocytes *see* ref. [8].

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Chapter 16

Quantitative Microinjection of Morpholino Antisense Oligonucleotides into Mouse Oocytes to Examine Gene Function in Meiosis-I

Shoma Nakagawa and Greg FitzHarris

Abstract

Specific protein depletion is a powerful approach for assessing individual gene function in cellular processes, and has been extensively employed in recent years in mammalian oocyte meiosis-I. Conditional knockout mice and RNA interference (RNAi) methods such as siRNA or dsRNA microinjection are among several approaches to have been applied in this system over the past decade. RNAi by microinjection of Morpholino antisense Oligonucleotides (MO), in particular, has proven highly popular and tractable in many studies, since MOs have high specificity of interaction, low cell toxicity, and are more stable than other microinjected RNAi molecules. Here, we describe a method of MO microinjection into the mouse germinal vesicle-stage (GV) oocyte followed by a simple immunofluorescence approach for examination of gene function in meiosis-I.

Key words Morpholino antisense oligonucleotides, Microinjection, Mouse oocyte, Meiosis-I, Immunofluorescence

1 Introduction

Mammalian oocyte meiosis-I, also known as oocyte maturation, is the essential final stage of oogenesis. Understanding the molecular mechanisms of this cell division is paramount, as defects can lead to chromosomal imbalances [1–4] or cytoplasmic deficiencies [5, 6] that can endanger reproductive potential. Experimental means for determining the function of potentially important genes during meiosis-I are essential in unraveling the complex cellular events that cause these defects.

The past 10 years have seen the emergence and application of several modes of gene interrogation in oocyte meiosis-I, each with their own *pros* and *cons* depending upon the experimental plan. The oocyte-specific conditional knockout mouse approach was first reported in 2006 [7], allowing investigators to delete a target gene of interest in a specific tissue and at specific

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developmental stage using the Cre-LoxP system, bypassing embryonic lethality which is common for genes regulating cell division (for review *see* ref. 8). Generation of conditional knockouts can be expensive and time consuming, however, and deletion efficiency of a target gene can be variable [8]. While the recent advent of CRISPR/Cas9 approaches will doubtless expedite the generation of tissue-specific knockouts [9–11], mutant mouse approaches nonetheless require extensive animal breeding and husbandry, which can be impractical in some research programs. Recent leaps forward in small-molecule biology have yielded well-characterized target specific inhibitors that have allowed acute examination of protein function in oocytes from normal (wild-type) laboratory mice [12–14]. However, this approach is critically dependent on the discovery and extensive characterization of such agents. Specific gene interrogation in wild-type mammalian oocytes has also been achieved by overexpression of mutant dominant negative forms of a protein [15–17], or by overexpression of a subunit to disturb protein complex stoichiometry [18, 19], each of which can be introduced by microinjection. However, the precise functioning of such constructs in the highly unusual cytoplasmic milieu of the mammalian oocyte requires careful validation.

Bypassing many of the pitfalls of these approaches, microinjection of RNA antisense constructs into the oocyte (RNA interference, RNAi) to deplete a protein of interest has become increasingly popular over the past 10 years [20, 21]. The major advantage of RNAi microinjection is that constructs can often be obtained from commercial sources inexpensively and, provided constructs are effective, results can be arrived at relatively quickly. RNAi has been achieved in oocytes using a variety of approaches, including small interfering RNA (siRNA) [22–25] and double stranded RNAs (dsRNA) [15]. Probably the most frequently used approach, however, is to inject commercially purchased Morpholino antisense Oligonucleotides (MOs). MOs are short chains of approximately 25 nucleotides assembled on a synthetic backbone of methylenemorpholine rings and phosphorodiamidate links, and are attractive tools as they have very high target-sequence specificity and are, in contrast to RNA-based microinjections, extremely stable [20, 26–28]. To our knowledge MOs were first used to assess gene function in mammalian oocyte meiosis by the Verlhac Lab in 2002 [29] and have since been used by a host of laboratories including ours to examine the effect of depleting numerous targets including cell cycle proteins [26, 27, 30, 31], signaling molecules [32, 33], and motor proteins [34]. Here we describe our standard routine protocol for MO microinjection into the mouse GV oocyte, followed by a simple immunofluorescence approach to confirm protein depletion and examine the resulting phenotype.

2 Materials

2.1 Materials for Mouse Ovarian Stimulation

1. 6–12-week-old female CD1 mice (*see* **Note 1**).
2. Pregnant mare serum gonadotropin (PMSG) (*see* **Note 2**).
3. Phosphate buffered saline (PBS).
4. 1 ml syringes.
5. Needles.

2.2 Materials and Reagents for Mouse Gv-Stage Oocyte Collection and Culture

1. M2 medium (*see* **Note 3**).
2. M16 medium.
3. 3-Isobutyl-1-methylxanthine (IBMX) for germinal vesicle break down (GVBD) inhibition is dissolved in DMSO at a stock concentration of 100–200 mM. Final concentration is 0.1–0.2 mM (1:1000). Store at −20 °C.
4. M2 medium supplemented with IBMX (M2 + IBMX) for collection and handling of GV oocytes outside the incubator (10 ml, keep at 37 °C).
5. M2 + IBMX for MO microinjection (1 ml, keep at room temperature).
6. M16 medium supplemented with IBMX (M16 + IBMX) for GV oocyte culture in the incubator before and after MO microinjection (~5 ml, keep in the incubator). We recommend making this the night and leaving in incubator overnight to ensure it is properly equilibrated at 5 % CO_2).
7. Mineral oil (*see* **Note 4**).
8. Transfer pipette for pouring mineral oil to the tissue culture.
9. 0.22 μm syringe filter.
10. 10 ml syringe to filter the media.
11. 14 ml polystyrene round-bottom tube.
12. Dissection microscope.
13. Digital dry bath.
14. High precision tweezers.
15. 27G × ½″ needle.
16. Petri dishes.
17. Pasteur pipette (*see* **Note 5**).
18. Mouth-controlled aspiration tube for the Pasteur pipette.
19. Cell culture incubator.

2.3 Materials and Reagents for Microinjection of Morpholino Antisense Oligos (MO)

1. MOs designed and purchased from Gene Tools LLC (www.gene.tools.com) (*see* **Note 6**).
2. Dry bath or PCR machine for preheating MO before microinjection.
3. Centrifuge.
4. Oocyte microinjection station, comprising the following (components and setup illustrated in Fig. 1): Leica DMIL-LED inverted scope, Hydraulic micromanipulator with universal joint, Coarse manipulators, Injectors, Iron plates as magnetic bases for injectors, PV820 pneumatic picopump (World precision instruments), Electrometer intracellular amplifier IE-251A (Harvard apparatus), Silver wire for intracellular amplifier IE-251A (Advent research materials), Magnetic base for intracellular amplifier. The apparatus is assembled and placed on an antivibration table (*see* **Note 7**, Fig. 1).
5. Holding pipettes (commercially available readymade from Hunter scientific http://www.hunterscientific.com or homemade pipettes *see* **Note 8**).
6. Vertical micropipette puller for the injection pipette.
7. Glass capillaries with filament for MO injection (Harvard apparatus).

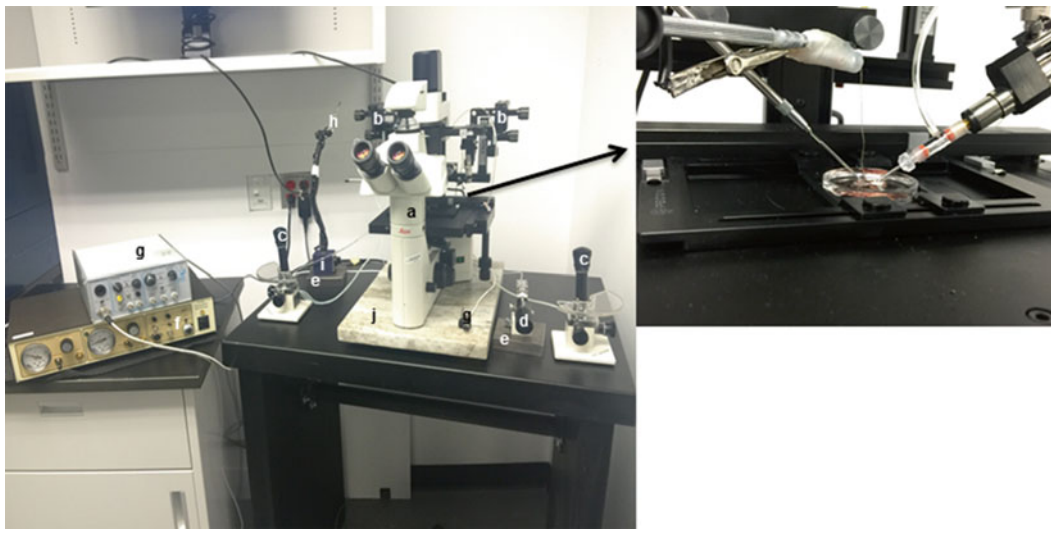

Fig. 1 Oocyte microinjection station. (**a**) Leica DMIL-LED inverted scope, (**b**) Hydraulic micromanipulators with universal joint, (**c**) Coarse manipulators, (**d**) Injector, (**e**) Iron plates for as magnetic bases for injectors, (**f**) PV820 pneumatic pico-pump, (**g**) Electrometer intracellular amplifier IE-251A, (**h**) Silver wire for electro intracellular amplifier IE-251A, (**i**) Magnetic base for electro intracellular amplifier, (**j**) An antivibration table

2.4 Materials and Methods for Preparing Oocytes for Immuno-Fluorescence Microscopy

1. Paraformaldehyde (PFA).
2. Triton X-100.
3. Bovine serum albumin (BSA).
4. PBS tablets.
5. 96-well plates with round bottom.
6. 35 mm Glass bottom culture dishes (*see* **Note 9**).

Using the above reagents the following working solutions should be prepared. The solutions are filtered and stable for several weeks at 4 °C.

1. 4 % PFA + PBS for fixing.
2. 1 % BSA + PBS for washing.
3. 3 % BSA + PBS for blocking.
4. 0.25 % Triton X + PBS for permeabilization.

3 Methods

3.1 Collection and Preparation of GV Mouse Oocytes

1. Administer 5 IU PMSG intraperitoneally to mice 44–48 h before GV stage oocytes are to be collected (*see* **Note 10**).
2. Prepare 35 mm tissue culture dish with 50 µl drops (~5 drops/dish) of M16 + IBMX and M16 medium. Put 3 ml of mineral oil to cover the drops and place in the incubator (37 °C, 5 % CO_2 in air) (*see* Fig. 2). M16 medium has to be equilibrated for at least 2 h before oocyte collection. Prepare similar dishes of M2 + IBMX drops and place on the dry bath (37 °C).
3. Collect the ovaries from the PMSG-injected females. Place in 2 ml of prewarmed M2 + IBMX (37 °C) in a 35 mm or 60 mm dish under the dissection microscope.
4. Breach antral follicles on the ovary surface by immobilizing the ovary using tweezers while repeatedly puncturing the ovary surface with a 27G needle mounted on a 1 ml syringe.
5. After dissection of ovaries, collect only fully grown oocytes with two or three layers of cumulus cells (*see* Fig. 2) using a large diameter (~150 µm) Pasteur pipette connected to a mouth pipette. Transfer the collected cumulus–oocyte complexes (COCs) into a drop of prewarmed M2 + IBMX (*see* **Note 11**).
6. Remove all cumulus cells around the oocyte by pipetting COCs back and forth using a Pasteur pipette with diameter approximately similar to the diameter of an oocyte (~80 µm, *see* **Note 11**).
7. Transfer cumulus-cell-free ("denuded") GV oocytes into a clean drop of prewarmed M2 + IBMX. Repeat 3–5 times in successive clean drops to wash the oocytes until all somatic

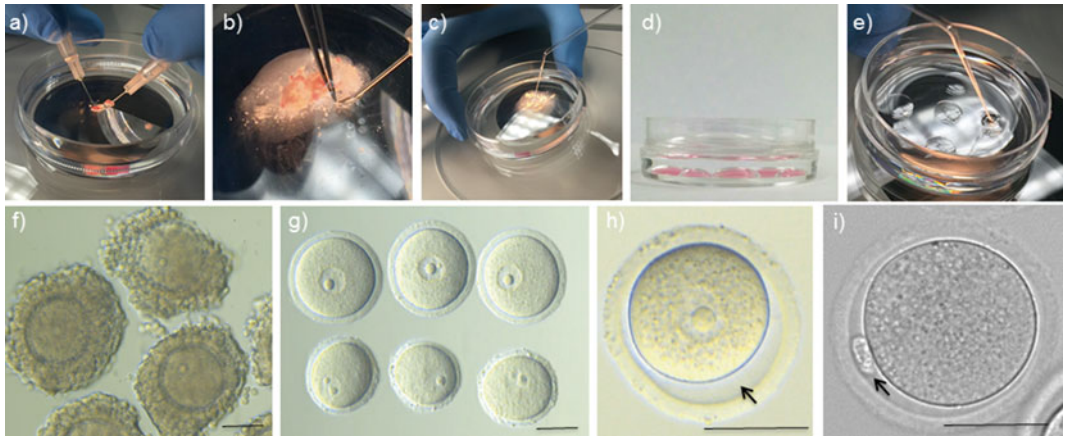

Fig. 2 Dissection of mouse ovaries and collection of GV oocytes. (**a**) Ovaries are placed in the 60 mm dish with 7 ml of prewarmed (37 °C) M2 + IBMX. (**b**) Ovaries are dissected using 27G needle and tweezers under the dissection microscope. (**c**) Fully grown oocytes are collected using a large diameter Pasteur pipette and mouth-controlled aspiration tube. (**d**) 50 μl drops of culture medium are covered with mineral oil and placed in the incubator at least for 2 h for equilibration before performing the experiment. (**e**) Collected oocytes are washed using a large diameter Pasteur pipette. Oocytes are washed through 3–5 drops before the start of in vitro culture. (**f**) Fully grown mouse GV oocytes with two or three layers of cumulus cells. (**g**) Denuded fully grown oocytes (*top* three oocytes), and growing oocytes (*bottom* three oocytes) which we would not use for this type of experiment. (**h**) A fully grown oocyte with a perivitelline space (*black arrow*) 1 h after incubation in M16 + IBMX. (**i**) Metaphase-II egg with a first polar body (*black arrow*). Scale bars are all 50 μm

cells are removed (*see* Fig. 2). Take care to only collect fully grown GV stage oocytes. Transfer oocytes to a dish of M16 + IBMX drops, washing the oocytes through for 3–5 drops to remove M2 and then place them in the incubator for 30–60 min prior to MO microinjection (*see* **Note 12**).

3.2 Microinjection of MO Oligonucleotides

1. Prewarm MO oligonucleotides with dry bath or PCR machine for 5–10 min at 65 °C followed by centrifugation at full speed for 1 min (*see* **Note 13**).
2. Prepare the microinjection chamber: place 600–800 μl of M2 + IBMX (room temperature) on the upturned lid of 35 mm tissue culture dish and cover with mineral oil. Place on the microinjection stage.
3. Prepare the microinjection pipette using a vertical pipette puller (*see* **Note 14**).
4. Insert holding pipette into the holding shaft. Adjust holding pipette using micromanipulators such that holding pipette is at center of the field of view, ~200 μm above the floor of the injection chamber (*see* **Note 15**).
5. Wash GV oocytes through three drops of M2 + IBMX, before mouth pipetting into the M2 + IMBX component of the microinjection chamber (*see* **Note 16**).

6. Clean the silver wire that is attached to the injection probe by gentle wiping with 70% EtOH and tissues.
7. Load 1 μl of the MO solution into the injection pipette using a microloader tip (Eppendorf) fitted on a "P10" Gilson pipette, with the silver wire close to the MO solution, and fit into injection probe.
8. Place the silver wire connected with "grounds chassis" jack back side of the intracellular electrometer into M2 + IBMX using the swan-neck attachment (*see* **Note 17**).
9. Focus on the GV oocytes then lower the holding pipette slowly until it touches the GV oocyte. Adjust pressure on the holding pipette to adhere the oocyte to holding pipette. Lift oocyte to a position ~200 μm above the injection chamber surface.
10. Lower the injection pipette into M2 + IBMX in line with oocyte. Confirm that neither the "amplifier" nor "current injection" LEDs are illuminated on the intracellular electrometer.
11. Using micromanipulators, smoothly insert the injection pipette into the cytoplasm through the zona pellucida.
12. Apply a brief pulse of negative capacitance to the Oolema by pressing the "buzz" button on the intracellular electrometer.
13. Microinject MO into the GV oocyte by pressing "start" button on the pico-pump and remove the injection pipette gently (*see* **Note 18**).
14. Injection size can vary day to day depending upon injection pipette diameter. Therefore, perform **steps 12** and **13** on a small number of "test" oocytes to adjust and see the size of the injection before microinjecting the main cohort of oocytes. Adjust injection size by controlling injection pressure and duration on pico-pump. The size of the injection can be judged by cytoplasmic displacement (*see* **Note 19**).
15. Once the appropriate injection size has been established, inject the remaining cohort of oocytes without changing injection settings, to ensure consistent injection size for each oocyte.
16. Following MO microinjection, transfer oocytes to M16 + IBMX in the incubator using the mouth-controlled Pasteur pipette.
17. Repeat the above steps with other microinjection solutions according to experimental design (*see* **Note 20**).
18. Leave oocytes in M16 + IBMX for between 1 and 24 h prior to in vitro maturation to permit protein turnover (*see* **Note 21**).
19. Wash MO-microinjected GV oocytes through 3–5 clean drops of pre-equilibrated IBMX-free M16 medium and return to the incubator to initiate meiosis-I completion, which takes ~9 to 12 h in most laboratory mouse strains (*see* **Note 22**, Fig. 2).

3.3 Preparation of Oocytes for Immuno-Fluorescence Analysis

Immunofluorescence provides a simple but tractable approach for determining the effectiveness of a MO in depleting its target protein, and for analyzing resulting phenotypes. The effectiveness of antibodies for oocyte immunofluorescence varies depending upon the precise makeup of immunofluorescence processing reagents. Here we describe a simple protocol that is effective with many commercial antibodies.

1. Prepare a round-bottom 96-well plate for immunofluorescence staining, arranging solutions in vertical columns, and oocyte treatment groups in horizontal rows. Place 50 μl of solution in each well, covering each 50 μl filled well with a single drop of mineral oil.
2. Using a bent Pasteur pipette (*see* **Note 23**), transfer oocytes into column 1.
3. Fix the oocyte in 4% PFA + BSA. Allow to sit for 40 min at room temperature (RT). Subsequent steps described later entail moving oocytes to successive wells in the 96-well plate.
4. Wash the oocytes with 1% BSA + PBS 3 times for 5 min each at RT.
5. Permeabilization with 0.25% Triton-X + PBS for 10 min each at RT.
6. Wash with 1% BSA + PBS three times for 5 min each at RT.
7. Block with 3% BSA + PBS for 60 min at 37 °C or 4 C overnight.
8. Incubate with primary antibody for 60 min at 37 °C.
9. Wash with 1% BSA + PBS three times for 5 min each at RT.
10. Incubate with secondary antibody for 60 min at 37 °C. Protect from the light (*see* **Note 24**).
11. Wash with 1% BSA + PBS three times for 5 min each at RT.
12. Incubate with 10 μg/ml Hoechst 33343 for 5 min at RT.
13. Prepare 5 μl of 1% BSA + PBS drops on the glass bottom dish and cover with mineral oil.
14. Pipette the fixed oocytes into the drops.
15. Analyze with epifluorescence or confocal microscopy (*see* **Note 25**).

4 Notes

1. Animals should be acquired and stored in accordance with local animal welfare regulations.
2. To make injection syringes, dissolve PMSG (1000 IU/vial) into pre-chilled (4 °C) filtered 40 ml PBS (stock concentration 25 IU/ml). Aliquot into 1 ml syringes attached with 27G needle, working on ice. Freeze immediately and store at −20 °C. Inject 200 μl per mouse to administer 5 IU.

3. We normally use commercially provided M2 and M16 medium, but these solutions can be made in-house [35].
4. Mineral oils can contain contaminants. We therefore recommend a lot-test even this product is supplied as "embryo culture grade." Autoclave to sterilize if necessary.
5. Pasteur pipette need to be pulled to appropriate thickness using an alcohol lamp or Bunsen burner. Large diameter pipettes (150 μm opening) are needed for initial oocyte collection and narrow diameter pipette (approximately the same size of the oocyte diameter, 80 μm) are needed for denudation of the cumulus cells. Pasteur pipettes are attached to a mouth-controlled aspiration tube to allow oocytes to be transferred between drops. Moving oocytes with a mouth-controlled Pasteur pipette takes several weeks' practice, but once mastered allows very precise and controlled movement.
6. MOs are typically purchased as 300 nanomoles in powder form. We typically make a 1 mM stock by dissolving in molecular biology grade water warming at 65 °C and vortexing 3–4 times. The 1 mM stock should be kept at room temperature in a good quality tube. 1 mM injection (final concentration ~50 nM, *see* ref. 34) represents a good starting concentration for experiments. At higher concentrations MOs can occasionally dimerize and become inactive. If a previously functioning MO stock appears to stop working, we recommend trying to separate the dimers by diluting the stock further (0.5 mM), autoclaving, and allowing the solution to return to room temperature. For further tips on MO use *see* ref. [21].
7. Precise micromanipulation setup may vary. The amplifier and negative capacitance pulse is essential when performing injections into metaphase-stage eggs and embryos, which are extremely sensitive to injection. The apparatus is also beneficial when injecting GV-stage oocytes also, but is dispensed with by some labs. An antivibration assembly is essential. Commercial isolation tables are effective, but a homemade apparatus comprising a marble slab and sponge balls is cost effective and works well (*see* Fig. 1).
8. We recommend investigators with minimal micromanipulation experience to buy pre-made holding pipettes in the first instance. However, holding pipettes can also be manufactured in house using a pipette puller and microforge, which allows for the holding pipette to be replaced regularly at low cost, and for pipettes to be tailored to individual users tastes. For further information on holding pipettes manufacture *see*: (http://narishige-group.com/movie/MF-900_cutting/index.html, http://narishige-group.com/movie/MF-900_bending/index.html, http://narishige-group.com/movie/MF-900_fire-polishing/index.html).

9. We normally use Petri dishes fitted with No. 0 coverglass (0.085–0.13 mm) for immunofluorescence microscopy.
10. The number of fully grown oocytes retrieved per mouse is highly dependent upon mouse strain, age, and PMSG administration [36, 37]. We most frequently use ~12-week-old female CD1 mice, and administer 5 IU of PMSG, retrieving 20–25 oocytes per mouse.
11. An overly narrow Pasteur pipette can damage or kill the oocyte. Care must be taken in selecting a pipette with the correct diameter.
12. Selecting only fully grown GV stage oocytes from antral follicles is critical, as oocytes less than full size may not be competent to resume meiosis [38–41]. Non-fully grown oocytes typically feature a thinner zona pellucida that is less smooth in appearance and should be discarded (at least for the purpose of the experiment described herein). 30–60 min incubation allows for the formation of the perivitelline space [42] in fully grown oocytes but not mid-grown phase oocytes and is thus a further hallmark of a fully grown oocyte (*see* Fig. 2).
13. MO solution can be gloopy. To prevent injection pipette blockage, before use incubate the MO for 5–10 min at 65 °C and centrifuge for 1 min before use.
14. Establishing pipette-puller settings to manufacture appropriate pipettes is achieved empirically and can be time consuming. Once settings are established, pipette size can remain reasonably consistent over the course of several weeks. We recommend that pipette pullers be placed away from areas of air flow (e.g., air conditioning).
15. Setting up the microinjection rig and aligning pipettes can be fiddly, time consuming, and frustrating, and takes time and practice for new experimenters. We typically find that a new investigator within our lab can take upward of 10–20 experimental days of training in this technique before being competent to perform robust experiments.
16. GV oocytes should not be kept on the microinjection stage for more than 20 min. We therefore recommend that beginners microinject in small groups, transferring injected oocytes back to warmed M2 + IBMX on the dry bath/warm plate.
17. The silver wire can be placed anywhere within the M2 + IBMX of the injection chamber to establish an electrical circuit. The negative capacitance enables smooth delivery of the pipette tip into the ooplasm. The red lamp tuens off when an electrical circuit is successfully made, Care must be taken as the injection pipette tip is very fragile and easily broken.
18. Before MO microinjection into the oocyte, change “duration” setting to “gated” and keep press “start” button briefly to

expel a small amount of injection solution. This is useful to counteract capillary action. Change "duration" setting to "timed" prior to oocyte injection.

19. The injection size should be no more than 5 % of the oocyte volume. As a useful estimate, an injection with cytoplasmic displacement diameter 1/6 of the diameter of the diameter of the oocyte provides less than 1 % by volume (*see* Fig. 3). Larger injection size can be detrimental to oocyte health. To increase or reduce the injection size, change either "eject pressure" or "period" settings of pico-pump. In general, once the correct injection size is established, the injection should be nondamaging as evidenced by >95 % oocyte survival. No cytoplasmic displacement upon injection indicates a failed injection. The most likely explanation for this is a blocked pipette. Change the injection pipette, using freshly preheated MO solution. Make sure that the injection pipette does not touch the germinal vesicle (the oocyte nucleus), as this can damage the oocyte and block the injection pipette. Microinjection of fluorescent tracer (Rhodamine B isothiocyanate-Dextran, Sigma Cat # R8881-100MG or Fluorescein isothiocyanate-Dextran, Sigma Cat# FD10S-100MG) can be used when first learning microinjection into the oocyte to confirm successful delivery.
20. Experimental groups and controls depend upon the experimental design. However, since off-target effects have been occasionally reported with oligonucleotides [21], we strongly recommend that controls include specific MO-injected, water-injected, and random-sequence MO control-injected oocytes. MO-induced phenotypes should in most cases be reversible by expression of exogenous protein, and we consider corroboration of results by an alternative intervention (e.g., expression of a dominant-negative mutant version of the target) highly desirable to confirm specificity. For an example of such experimental design from our lab, *see* ref. [34]. For further notes on control experiments, *see* ref. [21].

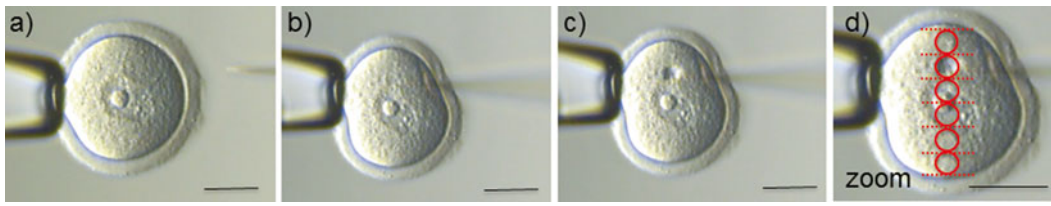

Fig. 3 MO microinjection into the mouse GV oocyte. (**a**) Oocyte is immobilized using a holding pipette (*left side*) and set at the center of the field of view. Injection pipette is to the *right*. (**b**) The injection pipette is gently inserted into the cytoplasm through the zona pellucida, avoiding the nucleus. (**c**) Injection of MO into the cytoplasm. (**d**) A zoom of the image in (**c**) to illustrate estimation of injection size. Overlay illustrates that cytoplasmic displacement diameter is approximately 1/6 of the diameter of the oocyte. Scale bars 50 μm

21. Required incubation time is highly dependent upon the rate of protein turnover. Cell cycle-related proteins often have quick turnover therefore making them good candidates for RNAi approaches. Some proteins are inappropriate targets due to slow turnover.
22. The time required to complete in vitro oocyte maturation (IVM) depends upon the mouse strain but is 9–12 h in most cases. Completion of IVM is confirmed by the formation of a first polar body (*see* Fig. 2). In most laboratory mouse strains the conditions described herein should permit meiosis-I completion in 90% or more of wild-type oocytes.
23. We use a 96-well plate for immunofluorescence for examination of gene function in MO-injected oocytes. A bent Pasteur pipette with ~45–90 ° angle is required to use a 96-well plate. Add 50 μl of fixing/washing solution and 2–5 μl of antibody into the each well.
24. Alexa-labeled secondary antibodies are light sensitive. Antibodies should be stored in nontranslucent tubes (or cover tubes in silver foil). Once oocytes have been exposed to fluorescent antibodies dishes, 96-well plates should be covered in silver foil.
25. Immunolabeled oocytes can be stored in PBS + 1% BSA in 96-well plates for many months and the fluorescence remain stable (depending upon the antibodies). Hoechst fluorescence is less stable, but can be reapplied on the day of imaging as required. Immunofluorescence of the target protein itself can provide a simple test of the effectiveness of depletion, and allows quantitative analysis of reduced abundance at a specific location (e.g., at the spindle). Western blotting provides an excellent quantitative alternative especially if the protein is predominantly cytoplasmic (*see* Chapter 15). For many cell division targets, analysis of spindle morphology (microtubule antibody, Sigma) can provide an informative first analysis of knockdown effect.

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Chapter 17

Monitoring Calcium Oscillations in Fertilized Mouse Eggs

Guillaume Halet

Abstract

In mammalian species, including human, fertilization is characterized by the triggering of long-lasting calcium (Ca^{2+}) oscillations in the egg cytoplasm. The monitoring of these Ca^{2+} oscillations is a valuable technique to demonstrate that fertilization has occurred, to study egg activation events elicited downstream of the Ca^{2+} signal, as well as to evaluate sperm quality. This chapter describes our protocol to monitor sperm-induced Ca^{2+} oscillations in mouse eggs, using fluorescence microscopy techniques and the Fura-2-AM ratiometric Ca^{2+} indicator.

Key words Oocyte, Egg, Sperm, Fertilization, Calcium, Ca^{2+}, Fluorescence, Imaging

1 Introduction

In the great majority of mammalian species, the ovulated egg is blocked at metaphase of the second meiotic division (Metaphase II, or MII). Only fertilization will allow for meiosis to resume, leading to the extrusion of one set of the segregated maternal chromatids, into the second polar body. This is immediately followed by meiotic exit, formation of male and female pronuclei, and progression to the first embryonic mitotic division (cleavage). In conjunction with these cell cycle events, fertilization also elicits the release of the egg's cortical granules in order to prevent polyspermy and the recruitment of maternal mRNAs for translation of new proteins important for the early stages of embryo development. These events are collectively referred to as "egg activation" [1–4].

In all mammalian species studied to date, the earliest activation event that is detected in the fertilized egg is the triggering of cytoplasmic Ca^{2+} oscillations (Fig. 1a) that last for several hours [1, 4]. Detailed description of the spatiotemporal dynamics of these Ca^{2+} oscillations, and the mechanism of their generation, have been provided using the mouse egg as a model [5–7]. The importance of these Ca^{2+} oscillations is demonstrated by the fact that their inhibition, using Ca^{2+} chelators, results in a complete failure of meiotic

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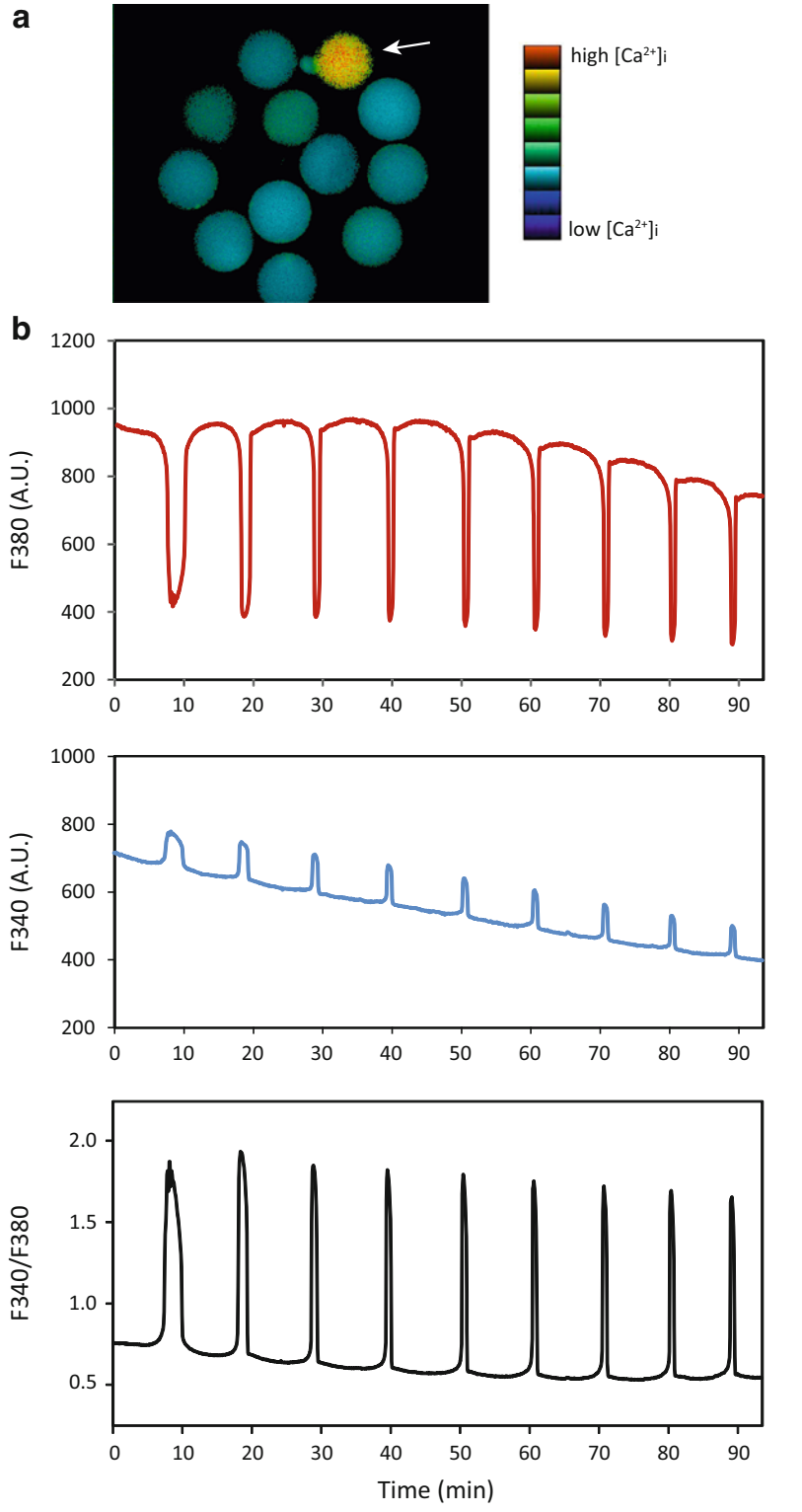

Fig. 1 Monitoring of Ca^{2+} oscillations in mouse eggs using Fura-2. (**a**) Pseudocolor ratio image of a field of mouse eggs loaded with Fura-2-AM and undergoing fertilization. The *arrow* points to an egg in which cytosolic [Ca^{2+}] is rising (Ca^{2+} oscillation). (**b**) *Line graphs* showing the fluctuations in F340 and F380 (one acquisition every 5 s), and the corresponding ratio trace, during fertilization-induced Ca^{2+} oscillations in a mouse egg

resumption and egg activation [8]. Interestingly, the initiation and completion of the various egg activation events are differentially sensitive to the number, amplitude, and duration of the Ca^{2+} spikes, suggesting that the timing and temporal sequence of these events are encoded in the pattern of the fertilization-induced Ca^{2+} oscillations [9–12]. Intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) also increases in fertilized eggs from nonmammalian species, such as amphibians, fish, and marine invertebrates, where the sperm-induced Ca^{2+} signal takes the form of a single transient or a series of oscillations [13].

The monitoring of Ca^{2+} oscillations in fertilized eggs can serve both research and clinical purposes. On the egg side, Ca^{2+} oscillations are recorded to investigate the dynamics and molecular aspects of egg activation events, such as the regulation of cell cycle resumption by Ca^{2+}-calmodulin-dependent protein kinase II, a downstream effector of the $[Ca^{2+}]i$ rise [14]. There has been also considerable effort to uncover the molecular identity of the sperm factor responsible for initiating these Ca^{2+} oscillations, with compelling evidence pointing to the sperm-specific phospholipase C zeta [4, 7, 15]. Accordingly, the recording of $[Ca^{2+}]$ fluctuations in the egg cytoplasm represents a valuable assay to evaluate sperm "quality," i.e., its ability to induce long-lasting Ca^{2+} oscillations that are required for full egg activation. In line with this, a number of studies have reported the use of mouse eggs as "reporter" of the fertilizing capacity of human sperm via the monitoring of cytoplasmic Ca^{2+} oscillations elicited after ICSI, or injection of human sperm extract or of human recombinant PLCzeta [16–19].

A number of fluorescent Ca^{2+} indicators have been used to investigate fluctuations in $[Ca^{2+}]i$ in mammalian eggs [20]. In this chapter, I describe our protocol for the monitoring of Ca^{2+} oscillations in fertilized mouse eggs, using the popular ratiometric fluorescent Ca^{2+} indicator Fura-2. Fura-2 is a high affinity Ca^{2+} indicator (Kd = 145 nM, in vitro), which shows a shift in its excitation spectrum when bound to Ca^{2+}, while the fluorescence emission peak (510 nm) remains unchanged. It is therefore classified as a dual-excitation indicator. Free Fura-2 shows an excitation peak around 380 nm, while Ca^{2+}-bound Fura-2 shows an excitation peak around 340 nm. Fluctuations in $[Ca^{2+}]i$ are therefore translated into reciprocal fluctuations in Fura-2 emitted fluorescence (F) when excited at 340 or 380 nm (Fig. 1): upon $[Ca^{2+}]i$ increase, the emitted fluorescence at 340-nm excitation (F340) will increase; conversely, the emitted fluorescence at 380-nm excitation (F380) will decrease. The corresponding fluctuations in $[Ca^{2+}]i$ are displayed as the F340/F380 ratio (Fig. 1b). The advantage of a ratiometric measurement is that the ratio signal is less affected by fluorescence bleaching or by variations in Fura-2 loading among the egg population. Note that the F340/F380 signal is not a quantitative measurement of the actual Ca^{2+} concentration, which would require calibration of the signal [20]. It provides however useful information regarding the rate, duration, frequency, and relative amplitude of the Ca^{2+} oscillations.

2 Materials

All solutions and culture media are prepared with ultrapure water with a resistivity of 18.2 MΩ-cm.

2.1 PMSG and hCG Hormones for Superovulation

1. PMSG: Lyophilized pellet of Pregnant Mare Serum Gonadotrophin/PMSG is resuspended in filtered (0.20 μm) ice-cold PBS to a final concentration of 25 IU/ml. The solution is then distributed into 1-ml syringes, which are next fitted with 27-gauge needles and immediately frozen at –20 °C.
2. hCG: Lyophilized pellet of human Chorionic Gonadotrophin/hCG is resuspended in filtered (0.20 μm) ice-cold PBS to a final concentration of 25 IU/ml, and aliquoted as readymade syringes as described for PMSG.

2.2 T6 Medium for Sperm Capacitation

100 mM NaCl, 1.42 mM KCl, 0.47 mM $MgCl_2$, 0.36 mM Na_2HPO_4, 1.78 mM $CaCl_2$, 25 mM $NaHCO_3$, 25 mM Na lactate, 0.47 mM Na pyruvate, 5.56 mM glucose, 10 mg/l phenol red. Store in fridge for 2 weeks. The day before the experiment, add 16 mg/ml BSA, filter (0.20 μm) and incubate overnight in a 5% CO_2 incubator.

2.3 Egg Handling Media

1. M2 medium: for egg collection. We use commercial M2 medium.
2. M2-hyaluronidase: A 10× stock solution is prepared by dissolving hyaluronidase (from bovine testes) into M2 medium to a final concentration of 3 mg/ml, and immediately frozen into 250-μl aliquots.
3. H-KSOM medium for $[Ca^{2+}]i$ recordings: 95 mM NaCl, 2.5 mM KCl, 0.35 mM KH_2PO_4, 0.2 mM Na pyruvate, 1 mM l-glutamine, 0.01 mM EDTA, 0.2 mM $MgSO_4$, 10 mM Na lactate, 4 mM $NaHCO_3$, 1.71 mM $CaCl_2$, 0.2 mM glucose, 20 mM HEPES, 10 mg/l phenol red (*see* **Note 1**), pH 7.4. This medium does not contain BSA, so as to promote egg adhesion to the glass coverslip.

2.4 Acidic Tyrode for Zona Removal

We use commercial acidic Tyrode's solution (pH 2.5) that is stored at –20 °C as 500-μl aliquots.

2.5 Keeping the Eggs Warm

Eggs must be kept warm for the whole duration of the isolation and dye-loading steps. To do so, culture media and dishes are stored on a hot block set to 37 °C (*see* **Note 2**).

2.6 Preventing Evaporation

When drops of medium need to be kept at 37 °C on the hot block for extended duration, or on the stage of the fluorescence microscope during image acquisition, the drop is covered with a layer of mineral oil in order to prevent evaporation.

2.7 Egg Manipulation Using a Mouth Pipette

The manipulation and transfer of eggs from one dish to another is facilitated using a "mouth pipette," consisting of a rubber aspiration tube connected to a glass Pasteur pipette. The Pasteur pipette needs to be narrowed at the tip to an inside diameter slightly bigger than the eggs diameter. This is achieved by heating the glass using a Bunsen burner followed by sharp pulling. Cut the pulled glass pipette to the desired length and allow for the pipette to cool down to room temperature before manipulating the eggs.

2.8 Fura-2

Prepare a stock solution by resuspending Fura-2-AM in DMSO to a final concentration of 1 mM. Aliquote and store at −20 °C. Avoid exposure of the aliquots to light.

2.9 Fluorescence Microscopy

In brief, ratiometric Ca^{2+} imaging using Fura-2 is performed with an inverted epifluorescence microscope equipped with a 20× objective, a UV light source, and a filter wheel with the relevant filters for Fura-2 dual excitation (peak transmission at 340 and 380-nm), a 510-nm dichroic mirror, and a 520-nm long-pass emission filter. Fluorescence images (F340 and F380) are captured using a cooled CCD camera and stored on a computer running a fluorescence imaging software that controls the camera shutter and the excitation filter wheel. To prevent phototoxicity, a neutral density filter is placed in the path of the excitation light. Importantly, the microscope must also be fitted with a stage incubator, in order to provide a warm (37 °C) environment to the cells during imaging (*see* **Note 3**).

2.10 Fluorescence Analysis

Fluorescence measurements (F340 and F380) are realized by drawing a circular region of interest on the fluorescence images, such as to collect fluorescence intensity from the bulk of the egg cytoplasm. The numerical values reporting the fluctuations in F340 and F380 intensities are exported toward Microsoft Excel to generate line graphs and for calculation of the F340/F380 ratio.

3 Methods

3.1 Superovulation of Mice

We use mice from either the CD-1 or MF-1 strains. In order to obtain large amounts of ovulated eggs (*see* **Note 4**), inject female mice intraperitoneally with 5–7.5 units pregnant mare's serum gonadotrophin (PMSG), followed 44–48 h later with 5–7.5 units human chorionic gonadotrophin (hCG) to induce ovulation. Ovulation occurs around 12 h following hCG injection.

3.2 Preparation of Mouse Sperm

1. On the day preceding the experiment, prepare two 35-mm dishes each containing 0.5 ml of T6 medium with BSA, under a layer of mineral oil to prevent evaporation. Leave overnight in a 5 % CO_2 incubator at 37 °C for equilibration.

2. On the day of the experiment, sacrifice a male mouse of proven fertility, dissect out the epididymides and place them in the drop of T6-BSA under oil. With the help of the dissection binocular and a sterile needle, puncture the epididymides in order to release sperm into the medium. Discard the epididymal tissue fragments and return the dish to the incubator. Allow 20 min for the sperm to disperse.
3. Add 100 µl of this sperm suspension into the second dish containing 0.5 ml T6-BSA. Make sure the drop remains covered with oil. Return the dish to the incubator and incubate for 3 h to achieve sperm capacitation.

3.3 Egg Collection

1. Ovulated MII eggs are collected 13–15 h after hCG injection. Sacrifice the mice by cervical dislocation and collect the two oviducts in a small tube containing 1 ml warm M2 medium.
2. Thaw one aliquot (250 µl) of M2-hyaluronidase and dissolve into a final volume of 2.5 ml M2, to reach a working concentration of 0.3 mg/ml.
3. Using a dissection binocular, slice open the oviducts using a sterile needle, in order to release the cumulus masses (i.e., ovulated oocytes surrounded by a cloud of granulosa cells, *see* **Note 5**) into a 35-mm dish containing prewarmed M2-hyaluronidase.
4. Following a 3–5 min incubation at 37 °C on a hot block, the cumulus cells are dispersed under the action of hyaluronidase and cumulus-free oocytes can be collected by mouth-pipetting (*see* **Note 6**). This is followed by two extensive washes in fresh M2 in order to rinse out hyaluronidase.

3.4 Preparation of the Eggs for Ca^{2+} Imaging

In the following procedures, it is advisable to protect the eggs from light, as Fura-2 is light sensitive. During egg incubation on the hot block, an opaque plastic box is placed on top of the dish to reduce light exposure to a minimum.

1. Thaw a Fura-2-AM aliquot and incubate the MII eggs in M2 medium containing 2 µM Fura-2-AM (*see* **Note 7**), for 10 min at 37 °C on a hot block. Next, transfer the eggs in a new dish filled with fresh M2 in order to wash off the residual Fura-2-AM. Leave the eggs on the hot block for an additional 15 min, to allow for cytoplasmic esterases to cleave off the AM groups.
2. Remove the zona pellucida, in order to accelerate the fusion of the fertilizing sperm with the egg. Place a drop (500 µl) of acidic Tyrode medium in the center of a culture dish, on the hot block at 37 °C for warming. Using a pipette filled with acidic Tyrode, transfer the eggs into the drop of acidic Tyrode, then return the dish on the hot block. The acidic medium will induce a progressive dissolving of the zona pellucida (*see* **Note 8**). Once the zona has fully dissolved, return the eggs to M2 medium on the hot block.

3. Prepare the imaging chamber. We use 35-mm glass-bottom dishes with a 20-mm glass diameter. A 500-μl drop of H-KSOM without BSA is deposited on the glass coverslip and covered with mineral oil to prevent evaporation, avoiding flattening of the drop. Place the imaging dish on the prewarmed stage of the inverted microscope inside the incubation system (*see* **Note 3**).
4. Using a mouth pipette filled with H-KSOM, transfer the zona-free eggs to the center of the imaging dish. This is accomplished by careful mouth pipetting, while looking down the microscope eyepieces to adjust the positioning of the pipette close to the surface of the glass coverslip. Slowly release the eggs so that they remain grouped in the field of view.

3.5 Setting Up Fluorescence Recordings

1. Start acquiring F340 and F380 fluorescence in order to adjust the exposure time for each wavelength (in the order of 100–300 ms) to obtain a good fluorescence signal, and to set the wavelength-switching time and the frequency of acquisition (such as one image every 5 s, *see* Fig. 1).
2. Using the ROI (Region Of Interest) function of the imaging software, draw a large circle in the cytoplasmic area of each egg to collect F340 and F380 fluorescence values to be exported in Excel. Background fluorescence should be subtracted from each excitation wavelength before creating the ratio image.

3.6 Fertilization

1. Add 5–10 μl of the capacitated sperm suspension to the imaging dish containing the eggs loaded with Fura-2 (*see* **Note 9**). Add the sperm slowly and away from the egg area, to avoid creating a drift that could lead to the eggs detaching from the coverslip.
2. Look down the eyepieces to check for sperm binding to the eggs surface, before starting the actual experimental recording.

4 Notes

1. The use of phenol red as an indicator of pH changes in the culture medium is not an absolute requirement. Actually, it can be omitted in the medium used for performing the actual Ca^{2+} imaging experiment (H-KSOM), as the dye generates background fluorescence at the wavelengths used to excite Fura-2.
2. It is recommended to adjust the hot block temperature slightly above 37 °C, such that a temperature of 37 °C is effectively delivered in the culture dish where the eggs are seated, and which is exposed to ambient air. This calibration is accomplished by using a wire temperature probe that is positioned against the bottom of a test dish filled with M2.
3. To ensure eggs and sperm will be kept warm for the duration of the Ca^{2+} recording, we use a multiple temperature control

scheme: in addition to the large size incubator covering the entire microscope, an additional mini-incubator (Tokai Hit) is fitted on the stage of the microscope, while the 20× objective is fitted with a lens heater (Tokai Hit). Temperature controls are set to generate 37 °C inside the drop of medium in the imaging dish, as verified with a wire temperature probe.

4. Superovulation is not an absolute requirement to obtain mouse eggs, i.e., injection of hCG alone can be envisaged. However, superovulation (PMSG + hCG) will substantially increase the egg yield, allowing performing the experiment using a minimal number of animals (one or two mice). The efficiency of superovulation may vary with the mouse strain, the age of the females, and the dose of hormone administered. Using the common CD-1 strain, young females (4–5 weeks old) will be efficiently stimulated with 5 units of PMSG and hCG. Older females may require up to 7.5 units of each hormone. In addition to the increased overall body weight, the increased amount of fat in the peritoneal cavity of older females may act as a sink for the injected hormones. Using the CD-1 strain, we routinely obtain 30–40 healthy MII eggs from one mouse.
5. The cumulus mass is easy to spot as it induces a distinct dilation of the oviduct. The size of the cumulus mass is generally proportional to the number of MII eggs that have been ovulated and which are buried into the mass.
6. Healthy MII eggs should fill most of the space within the zona pellucida, except in the region where the first polar body was emitted. In contrast, eggs that appear shrunk and that make no contact with the ZP must be discarded. Occasionally, some eggs will be found already separated from the rest of the mass, before hyaluronidase had started to disperse the cumulus cells. These eggs generally display a shrunk morphology and must be discarded.
7. The "AM" form of Fura-2 means it is modified by addition of acetoxymethyl groups via an ester bond, which renders the dye membrane permeant (passive diffusion), thus avoiding invasive loading techniques. Once the indicator has penetrated the egg, cytosolic esterases cleave the ester bond to release anionic Fura-2, which remains trapped inside the cell. However, a fraction of Fura-2-AM may also penetrate and remain sequestered into cytoplasmic organelles (compartmentalization), and generate a contaminating fluorescent signal that is unaffected by changes in $[Ca^{2+}]i$. To avoid this, Fura-2 salt (such as pentapotassium salt) can be used instead of Fura-2-AM, however it has to be microinjected into the egg cytoplasm.
8. It is important to manipulate the eggs with a pipette filled with warm acidic Tyrode and not M2 medium, as the latter would contaminate the drop of acidic Tyrode and slow down the zona dissolving process. In addition, occasional gentle pipetting of

the eggs using a pipette filled with acidic Tyrode will accelerate zona removal, by promoting the rupture of the thinned zona pellucida. Note that the eggs are more fragile and prone to damage and lysis when denuded of their protective zona pellucida, and must therefore be manipulated with extra care.

9. Zona-free eggs have lost their barrier against polyspermy, which is provided primarily by the zona pellucida. It is therefore frequent to observe several sperm fusing with the same egg. This can be an issue when the aim of the experiment is to investigate Ca^{2+} oscillations parameters, because polyspermy notoriously increases Ca^{2+} oscillation frequency [21]. For this reason, it is recommended to start by adding little amount of the sperm suspension at first, to increase the probability to obtain monospermic fertilization. This also means that the time required to obtain sperm-egg binding may be longer, only a fraction of the eggs layered on the imaging dish may be fertilized, and fertilization events in the egg pool may happen asynchronously. If no fertilization event happens during a prolonged period, add a second sample of the sperm suspension.

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Chapter 18

Live Imaging of Intracellular Dynamics During Meiotic Maturation in Mouse Oocytes

Shuhei Yoshida, Yogo Sakakibara, and Tomoya S. Kitajima

Abstract

Fluorescence live imaging is a powerful approach to study intracellular dynamics during cellular events such as cell division. By applying automated confocal live imaging to mouse oocytes, in which meiotic maturation can be induced in vitro after the introduction of fluorescent proteins through microinjection, the meiotic dynamics of intracellular structures, such as chromosomes, can be monitored at high resolution. A combination of this method with approaches for the perturbation of specific proteins opens up opportunities for understanding the molecular and intracellular basis of mammalian meiosis.

Key words Meiosis, Oocyte, Live imaging, Chromosome

1 Introduction

Live imaging is a powerful method to study dynamic cellular events such as cell divisions, including mitosis and meiosis. Much of our knowledge about the mechanism of cell division has come from studies of mitosis, which can be analyzed by live imaging approaches using various types of cells that proliferate in in vitro cultures. However, relatively less is known about meiosis, a specialized cell division that takes place specifically in spermatocytes and oocytes to produce gametes. Mouse oocytes, which can be cultured in vitro from prophase of meiosis I to the end of meiosis II, provide a unique opportunity to analyze the intracellular dynamics that occur during meiosis using fluorescence live imaging. Fully-grown oocytes arrested at the germinal vesicle (GV) stage, which are optically transparent and arrested at the prophase of meiosis I, can be collected from the ovaries of female mice and maintained in an in vitro culture. Exogenous proteins, such as fluorescently tagged proteins, can be expressed by the introduction of in vitro-synthesized RNAs or proteins through microinjection. Meiotic resumption can be induced by a simple medium change, which allows for the monitoring of meiotic maturation, the process from the end of prophase I

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to metaphase II, by live microscopy. Mouse oocytes are much thicker (~80 μm in diameter) than flat-shaped mammalian cultured cells, but this disadvantage can be largely overcome by collecting optical sections in confocal microscopy. By automating a confocal microscope, the intracellular structures of interest can be tracked throughout meiotic maturation, which enables high-resolution and high-throughput live imaging. In this chapter, we describe a particular technique for the visualization of chromosome and kinetochore dynamics by automated confocal microscopy, which can be applied to many intracellular structures such as microtubules and F-actin in mouse oocytes (*see* **Note 1**). We have used this method to quantitatively and systematically analyze individual chromosome dynamics prior to chromosome segregation by completely tracking all of the kinetochores and chromosomes in 3D throughout meiosis I [1, 2]. This approach can be applied to the functional analysis of proteins of interest by introducing short-interference RNAs, morpholinos, inhibitory antibodies and dominant negatives through microinjection or by adding specific inhibitors to the medium, thus opening opportunities for studying molecular and cellular biology of mammalian meiosis.

2 Materials

2.1 RNA Preparation

1. Plasmids for in vitro RNA synthesis. We use the plasmid pGEMHE [3] as a standard backbone. In pGEMHE, a T7 promoter is located upstream of an open reading frame (ORF) for a fluorescently tagged protein. A poly (A) sequence is located downstream of the ORF. A list of the published plasmid constructs is shown in Table 1 (*see* **Note 1**).
2. Phenol/chloroform.
3. 70% Ethanol (v/v).
4. RNase-free water.
5. mMESSAGE mMACHINE® T7 Transcription Kit (Thermo Fisher Scientific, AM1344).
6. Chloroform.
7. Isopropanol.

2.2 Oocyte Isolation

1. The female mice are 8–12 weeks old. We use BDF1 as a standard strain (*see* **Note 2**).
2. Pregnant mare's serum gonadotropin (PMSG, e.g., Serotoropin, ASKA Pharmaceutical).
3. Culture dish (35 × 10 mm).
4. M2 medium.
5. 3-Isobutyl-1-methyl-xanthine (IBMX, Sigma, 15879).

Table 1
A list of fluorescent markers used in mouse oocytes

Protein	Fluorescent tag	Localization	Reference
Histone 2B (H2B)	mRFP, mCherry	Chromosome	[1, 7, 8]
CENP-C	EGFP, 2mEGFP, 3mCherry	Kinetochore	[1, 2]
MAP4	EGFP	Microtubule	[8]
EB3	mEGFP	Microtubule plus-end	[8]
UtrCH	EGFP	F-actin	[9, 10]
Plk1	Venus, mEGFP	MTOC and kinetochore	[11, 12]
Cep192	EGFP, tdEos	MTOC	[13]

6. Stereomicroscope with an eyepiece micrometer.
7. Heating plate.
8. 23-G needles.
9. Glass pipettes for oocyte collection with inner diameters of ~120 and ~100 μm. To prepare the glass pipettes, we pull a glass capillary (Blaubrand Disposable micropipettes, intraMARK 100 μl) over the flame of a burner and then cut it at a position that produces a desired inner diameter under a stereomicroscope.
10. Aspirator tube assemblies (e.g., Sigma-Aldrich, A5177-5EA).
11. Mineral oil (e.g., Wako, Liquid Paraffin, 128-04375, lot tested for oocyte culture).

2.3 Microinjection

1. Glass capillary (e.g., Narishige, G-100) for the microinjection pipette.
2. Glass capillary (e.g., Drummond, Microcaps 10 μl, 1-000-0100) for the RNA loading capillary.
3. Puller (e.g., Sutter, P-97/IVF).
4. Mercury.
5. 23-G needle.
6. Syringe.
7. Coverslips.
8. Microinjection chamber. The assembly of the microinjection chamber was previously described [4]. One layer of double-sided tape is used to form a space to place the oocytes.
9. Silicone oil (Sigma, Dimethylpolysiloxane, DMPS2X).
10. Inverted microscope with an eyepiece micrometer. The microscope is placed on a vibration-free table.

11. Microinjector (e.g., Narishige IM-9B).
12. Incubator.

2.4 Induction of Meiotic Resumption

1. Chambered coverslip (e.g., Nunc, Lab-Tek Chambered #1.0 Borosilicate Coverglass System 4 chamber 155383 or 8 chamber 155411).

2.5 Live Imaging

1. Confocal microscope. We use an LSM 710 or 780 (Zeiss) equipped with a 40× C-Apochromat 1.2NA water-immersion objective lens, a piezoelectric drive acting on a stage, and a large incubator (PeCon, XLmulti S1).
2. Autofocus software. We use AutofocusScreen for ZEN (EMBLEM, download available at http://www.embl-em.de) to achieve automated multi-position and multi-time imaging with a 3D autofocus [5].
3. Mineral oil for imaging (e.g., Nacalai tesque, Paraffin Liquid, 26133-54, lot checked for oocyte culture).

3 Methods

Briefly, mouse oocytes at the GV stage are collected. The oocytes are microinjected with in vitro-synthesized RNAs encoding fluorescently tagged proteins that localize to an intracellular structure of interest. After the fluorescently tagged proteins are expressed, meiotic resumption is induced. The dynamics of the intracellular structures probed by the fluorescently tagged proteins during meiotic maturation is monitored by live imaging with an automated confocal microscope. In this method, the dynamics of various intracellular structures such as chromosomes, kinetochores, microtubules, and F-actin can be observed (*see* **Note 1**). A list of published fluorescent proteins that mark various structures is shown in Table 1. In particular, this chapter provides a protocol to achieve dual-color fluorescence imaging of kinetochores and chromosomes, which are labeled by 2mEGFP-CENP-C and H2B-mCherry, respectively [2].

3.1 RNA Preparation

1. Linearize 10 μg of a plasmid DNA in a final volume of 200 μl. We use *Asc*I for the linearization of pGEMHE-2mEGFP-CENP-C and pGEMHE-H2B-mCherry.
2. Purify the DNA using a standard method of phenol/chloroform extraction followed by ethanol precipitation.
3. Wash the DNA with 70% ethanol, and allow it to dry.
4. Dissolve the DNA in 5 μl of RNase-free water.
5. Measure the concentration of the purified DNA, and adjust it to 1 μg/μl.

6. Use 0.5–2 μg of the DNA for the in vitro RNA synthesis reaction with the mMessage mMachine® T7 Transcription Kit in a final volume of 10 μl. Incubate the reaction mixture at 37 °C for 2 h.
7. Add 1 μl of DNase I (provided as a part of the transcription kit) and incubate at 37 °C for 15 min.
8. Add 57.5 μl of RNase-free water and 7.5 μl of the Ammonium Acetate Stop Solution (provided as a part of the transcription kit), and then mix thoroughly.
9. Extract the RNA solution with 75 μl of phenol/chloroform.
10. Extract the RNA solution with 75 μl of chloroform.
11. Add 75 μl of isopropanol and mix thoroughly.
12. Incubate the mixture for at least 15 min at −20 °C.
13. Centrifuge at 4 °C for 15 min at 16.9 × *g*.
14. Carefully remove the supernatant.
15. Centrifuge again at 4 °C for 30 s at 16.9 × *g*.
16. Completely remove the remaining supernatant. Do not allow the RNA pellet to dry. Dissolve the pellet in a total volume of 5 μl of RNase-free water.
17. Measure the concentration of the RNA and check the quality by agarose gel electrophoresis. We usually obtain a single band of RNA at a concentration of 1–2 μg/μl. Adjust the RNA concentration to 1 μg/μl.
18. Mix 4 μl of the 2mEGFP-CENP-C RNA and 1 μl of the H2B-mCherry RNA. Dilute the RNA mixture with 2.5 μl of RNase-free water.
19. Dispense and store the RNA mixture at −80 °C (*see* **Note 3**).

3.2 Oocyte Isolation

1. Inject a female mouse with PMSG (5 unit) 2 days before the experiment.
2. On the day of the experiment, pre-warm 5 ml of M2 medium containing IBMX (M2 + IBMX) at a final concentration of 200 nM at 37 °C.
3. Pre-warm 15 ml of mineral oil at 37 °C.
4. Forty-eight hours after the PMSG injection, dissect the mouse and collect both of the ovaries in 2 ml of M2 + IBMX on a culture dish.
5. Place the culture dish on a stereomicroscope equipped with a heating plate set at 37 °C (*see* **Note 4**). All of the procedures for oocyte isolation, collection, and transfer should be performed on the heating plate unless otherwise stated.
6. While looking through the stereomicroscope, punctuate the follicles with the 23-G needles to liberate the oocytes.

7. Collect the oocytes surrounded by the cumulus cells with a glass pipette (inner diameter of ~120 μm) with a tube assembly, and transfer them into a 50 μl drop of M2 + IMBX overlaid with mineral oil on a culture dish.
8. Partially remove the cumulus cells from the oocytes by vigorously pipetting with a glass pipette (inner diameter of ~120 μm). Transfer the oocytes into another 50 μl drop of M2 + IMBX overlaid with mineral oil.
9. Completely remove cumulus cells from oocytes by vigorously pipetting with a glass pipette (inner diameter of ~100 μm). Transfer the oocytes into another 50 μl drop of M2 + IMBX overlaid with mineral oil.
10. Select fully-grown GV-stage oocytes that have a diameter of ~80 μm and a nucleus at the center (*see* **Note 5**) [6]. We usually obtain ~30 such oocytes from one mouse.

3.3 Microinjection

We use a microinjection method previously described for mouse follicular oocytes [4] with some modifications. For details of this method, *see* ref. [4]. The microinjection is carried out at room temperature.

1. Pull a glass capillary with a puller (*see* **Note 6**).
2. Insert an ~1 mm column of mercury into the pulled capillary from its back using a syringe with a 23-G needle. This capillary is used as a microinjection pipette.
3. Prepare an RNA loading capillary. Insert an ~1 mm column of the silicone oil, and then an ~1 mm column of the RNA mix solution, and then another ~1 mm column of the silicone oil into a glass capillary. The capillary will contain a stack of three columns at a tip: silicone oil, RNA solution, and silicone oil.
4. Assemble a microinjection chamber as described in the ref. [4] with one layer of double-sided tape. Fill the chamber with M2 + IBMX.
5. While looking through a stereomicroscope, insert the oocytes into a space between the coverslips in the chamber.
6. Place the chamber on an inverted microscope.
7. Set a microinjection pipette with the microinjector.
8. Slightly break the tip of the microinjection pipette by gently tapping the tip against the face of the loading capillary.
9. Using the microinjector, push the mercury to the tip of the microinjection pipette.
10. Move the tip of the microinjection pipette into the loading capillary and aspirate the silicone oil into the microinjection pipette. Measure the length of the aspirated silicone oil inside of the microinjection pipette.

11. Move the tip of the microinjection pipette into the medium in the chamber.
12. Push the silicone oil into the medium. The silicone oil will form a sphere. Measure the diameter of the sphere to calculate the volume of the silicone oil. Repeat **steps 10–12** until you determine the length in the microinjection pipette that gives the desired volume (2 pl).
13. Move the tip of the microinjection pipette into the loading capillary, and aspirate the silicone oil into the microinjection pipette. This is to prevent mercury from mixing with the RNA solution.
14. Aspirate the RNA solution. The length of the aspirated RNA solution is same as the length determined in **step 12**.
15. Aspirate the silicone oil. This is to prevent RNA from mixing with the medium. The microinjection pipette will carry a stack of silicone oil, RNA solution, silicone oil, and mercury at the tip.
16. Move the tip of the microinjection pipette near to an oocyte. Push the oocyte to the wall of the double-sided tape.
17. Introduce the tip of the microinjection pipette inside the oocyte. Move the tip as close as possible to the center of the oocyte, but avoid the nucleus.
18. Inject the first column of silicone oil and the RNA solution into the oocyte. The silicone oil serves as a marker for the microinjected oocytes.
19. Move the tip of the microinjection pipette out of the medium. In some cases the microinjected oocyte will stick to the microinjection pipette in the medium, but when the tip is removed from the medium, these oocytes will be separated from the tip and stay inside the medium.
20. Repeat the **steps 13–19**. We usually microinject ~30 oocytes within 30 min.
21. Collect the microinjected oocytes in the chamber and transfer them to a 50 μl drop of M2 + IBMX overlaid with mineral oil.
22. Incubate at 37 °C for 2–3 h.
23. Pre-warm the confocal microscope incubator to 37 °C.

3.4 Induction of Meiotic Resumption

1. Pre-warm 2 ml of M2 medium without IBMX to 37 °C.
2. Place six 50 μl drops of M2 medium in a culture dish, and overlay the drops with mineral oil.
3. With a glass pipette (inner diameter of ~100 μm), aspirate the microinjected oocytes in as little volume of medium as possible. Transfer the oocytes to one of the M2 drops.
4. Pipette the oocytes several times, aspirate them in as little volume of medium as possible, and transfer them to another drop.

5. Wash the oocytes by repeating **step 4** six times.
6. Prepare a chambered coverslip. We use four-well Lab-Tek (Nunc) chamber wells. Place one small drop (~3 μl) of M2 around the center of a well and overlay it with mineral oil for imaging (*see* **Note 7**).
7. Aspirate the washed oocytes in as low a volume of medium as possible, and transfer them into the small drop of M2 in a chambered coverslip. Position the oocytes at the center of the drop on the surface of the coverslip. The oocytes are stable when they are placed next to each other in the drop.

3.5 Live Imaging

1. Check that the microscope is warmed (*see* **Note 8**).
2. Place the chambered coverslip on the stage of the confocal microscope.
3. Verify that the fluorescent proteins are expressed in the oocytes (*see* **Note 9**). Compare oocytes with those that did not receive

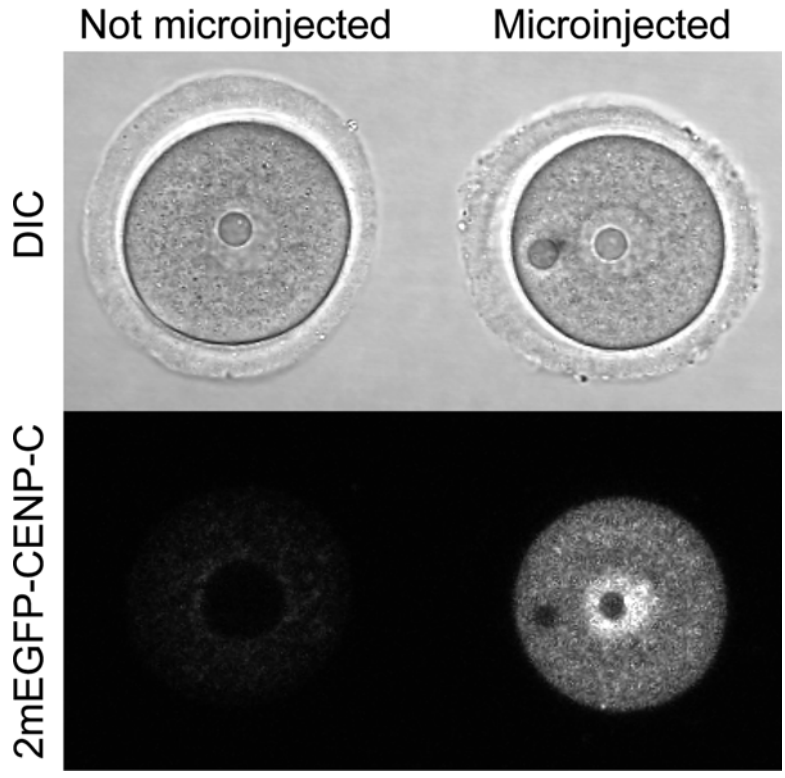

Fig. 1 Oocytes expressing a fluorescent protein. The image shows an oocyte that did not receive microinjection (*left*) and an oocyte 2.5 h after 2mEGFP-CENP-C RNA microinjection (*right*). Note that the oocyte without the microinjection exhibits autofluorescence. In the microinjected oocyte, a significant amount of the fluorescence signal from 2mEGFP-CENP-C is detected. 2mEGFP-CENP-C is enriched at the kinetochores, although the individual kinetochores are not visible under this particular imaging condition at low resolution

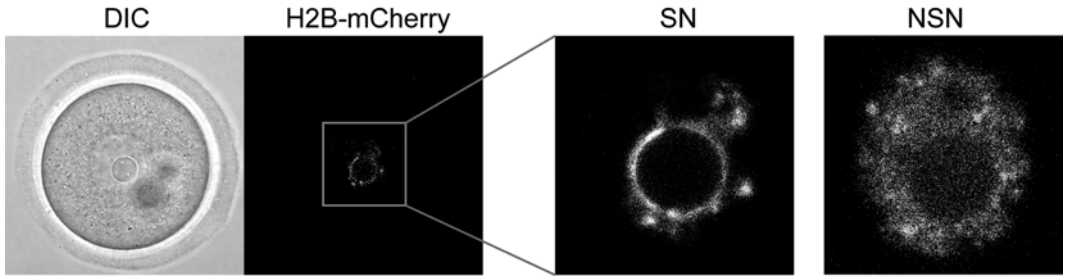

Fig. 2 SN oocytes. The image shows oocytes 2.5 h after H2B-mCherry RNA microinjection. The nucleus is magnified. In the SN oocyte (*left*), the H2B-mCherry signal is enriched and around the rim of the nucleolus and compacted in the nucleus. In contrast, in the non-SN (NSN) oocytes (*right*), the enrichment and compaction are relatively poor

microinjection, in which a low level of autofluorescence will be detected in the GFP-detection channel (Fig. 1).

4. Take a snapshot of the H2B-mCherry signal. Select the surrounded nucleolus (SN) oocytes that exhibit an H2B-mCherry signal forming a ring around the nucleolus, which serves as a marker for meiotic competence [6] (Fig. 2).
5. Set the imaging conditions. We achieve 3D tracking of the kinetochores by acquiring 17 confocal z-sections (every 1.5 μm) of 256×256 pixel xy images (a total volume of 30.4×30.4×25.5 μm) at 3-min intervals (*see* **Note 10**). Under this imaging condition, we obtain 4D images of 33 oocytes [2]. See the instructions at www.embl-em.de for setting up the automated multi-position and multi-time imaging with 3D autofocus by Autofocusscreen for ZEN.
6. Start imaging (*see* **Note 11**). The oocytes from the BDF1 mice exhibit GV breakdown (GVBD) 45–60 min after an IBMX washout, and complete meiotic maturation is observed 6.5–7.5 h after GVBD (*see* **Note 12**).

4 Notes

1. This method relies on the capacity of the expressed proteins to localize to the intracellular structure of interest. Therefore, proteins that shuttle between the intracellular structures of interest and the cytoplasm in the GV stage or during meiotic maturation should be selected as marker proteins.
2. The time required for the completion of meiotic maturation varies among mouse strains. In BDF1, the time from GVBD to the onset of anaphase I is 6.5–7.5 h. The time also depends on the culturing conditions in individual laboratories.
3. Spin before use.

4. **Steps 6** and 7 are carried out as quickly as possible to avoid evaporation of the medium.
5. Fully-grown GV-stage oocytes exhibit a small gap (perivitelline space) between the oocyte cortex and zona pellucida in an in vitro culture.
6. With a Sutter P-97/IVF puller, we use the values of 752 for HEAT, 50 for PULL, 120 for VEL, and 200 for TIME. Individual laboratories and investigators should optimize these values. Pulling with a smaller HEAT value produces a thicker tip.
7. The use of mineral oil should be avoided when experiments require the addition of drugs (such as protein inhibitors) in the medium because the drugs can be absorbed by the mineral oil. In such cases, we use 8-well Lab-Tek chamber wells filled with 200 μl of M2 with no mineral oil, place the oocytes in the bottom corner of the well to avoid oocyte movement, and seal the well by placing silicon grease on the top of the well before placing the lid on it to avoid evaporation.
8. To achieve high experimental reproducibility, temperature control is critical. Even if a microscope is equipped with a large incubator, the temperature of the samples can be affected by room temperature. Therefore, the room temperature should be kept constant by an air conditioner during imaging. In a pilot experiment, we monitored temperature stability at the sample position overnight by inserting a temperature probe into a medium drop on a chambered coverslip that was placed on the microscope stage and touched by an immersion liquid on an objective lens.
9. It should be noted that overexpression could cause adverse effects. For example, excessive 2mEGFP-CENP-C expression accelerates chromosome stretching during prometaphase I. When there is excessive H2B-mCherry expression, chromosome bridges are often found at anaphase I. Therefore, observations obtained from live imaging should be confirmed by other experimental approaches such as immunostaining of fixed oocytes in a time-course.
10. It is important to find a proper balance between image quality (achieved by higher light exposure) and cell viability (achieved by lower light exposure). With laser-scanning confocal microscopes, a lower scanning speed, a larger number of averaging, a larger number of pixels, and a higher laser power provide a higher image quality but greater damage to the oocytes and fluorophores. Limiting the intracellular region to be scanned by 3D autofocus helps to achieve both a higher image quality and less oocyte damage.
11. Start imaging within 45 min after the IBMX washout to image GVBD.
12. Under optimized conditions, we detected no chromosome segregation errors in 169 oocytes from 8-week-old BDF1 mice [2].

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Chapter 19

Isolation of Mammalian Oogonial Stem Cells by Antibody-Based Fluorescence-Activated Cell Sorting

Deanna M. Navaroli, Jonathan L. Tilly, and Dori C. Woods

Abstract

The ability to isolate and subsequently culture mitotically active female germ cells from adult ovaries, referred to as either oogonial stem cells (OSCs) or adult female germline stem cells (aFGSCs), has provided a robust system to study female germ cell development under multiple experimental conditions, and in many species. Flow cytometry or fluorescence-activated cell sorting (FACS) is an integral part of many isolation and characterization protocols. Here, we provide methodological details for antibody-based flow cytometric isolation of OSCs using antibodies specific for external epitopes of the proteins Ddx4 or Ifitm3, alone or in combination with the use of fluorescent reporter mice. Beginning with sample preparation, we provide point-by-point instructions to guide researchers on how to isolate OSCs using flow cytometry.

Key words Female germline stem cell, Oogonial stem cell, Ovary, Flow cytometry

1 Introduction

The recent identification of a relatively rare population of mitotically active germline cells that have the ability to generate oocytes and viable offspring in adult mammalian females has led to multiple published reports from different laboratories detailing isolation strategies [1–10]. Of the described methods, two approaches using antibodies targeting the germline-expressed proteins, DEAD-box polypeptide 4 (Ddx4, also referred to as Vasa or mouse Vasa homolog) and interferon-induced transmembrane protein 3, Ifitm3—also referred to as Fragilis), have been demonstrated to successfully yield a live cell population that can be expanded ex vivo, spontaneously differentiates in vitro into immature oocytes, and generates fertilization competent eggs that give rise to live offspring following intraovarian transplantation [1, 4, 11–13]. Although both FACS and magnetic-assisted cell sorting (MACS) have been used for the isolation of OSCs [1, 4, 5, 10, 12–15], only flow cytometry offers the ability to perform

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characterizations based on cell size, viability [4, 5] and, if desired, multiple fluorescent parameters [2, 6].

OSCs represent a relatively rare population of ovarian cells, with estimates approximating $0.014 \pm 0.002\%$ of the total ovarian cell population in mice [4]. Although OSCs can be expanded to large numbers in vitro, a small starting number of cells obtained after isolation is to be expected. The relative rarity and small size (between 5 and 10 μm, depending on species; [4, 5, 16]) can make OSCs difficult to sort, and so additional considerations such as setting a size gate, inclusion of a viability marker, and proper secondary antibody and IgG controls are essential for successful isolation. Additionally, tissue dissociation, sample preparation, and primary antibody selection are important factors that, when not performed properly, can lead to atypical results.

Regarding antibody selection, two proteins have been reported to have cell-surface epitopes suitable for antibody-based live cell isolation: Ddx4 and Ifitm3 [1, 3–5, 15–17]. While the initial report describing Ddx4 in ovarian tissues designated expression as cytosolic [18], experimental evidence has since verified specific localization of the C-terminus on the surface of OSCs using multiple approaches [1, 4]. Alternatively, Ifitm3 is expressed in OSCs [3], contains a well-characterized extracellular N-terminus [19], and has been used to isolate functional OSCs in mice and rats [3, 11–13]. However, two caveats to the potential use of Ifitm3 as a cell-surface target for purification of OSCs lead us to recommend anti-Ddx4 as the antibody of choice for OSC isolation. First, Ifitm3 protein expression is not restricted to germline cells (http://www.proteinatlas.org/), and thus its utility for isolation of only OSCs is questionable. Second, while the C-terminus of Ddx4 is highly conserved among species (Fig. 1), and therefore a single antibody will work for multiple animal models, the Ifitm3 amino acid sequence that encodes for the extracellular N-terminus is poorly conserved (Fig. 1). This then requires sequence analysis and species-specific antibodies, increasing time and cost. Nonetheless, when applied to dispersed ovarian tissue, antibodies targeting Ifitm3 will successfully yield OSCs following FACS (although we caution that purity may be compromised), and so we have elected to include instruction and examples using both antibody-based FACS strategies here.

Another important factor to consider when attempting isolation of OSCs is the proper preparation of ovarian tissue into a single-cell suspension prior to antibody labeling. Collagenase type IV, which contains no tryptic activity, is utilized to effectively release cells from tissue [5]. Use of trypsin is not recommended, as multiple trypsin digest sites exist within the extracellular C-terminus of Ddx4 and N-terminus of Ifitm3 (Fig. 2). Accordingly, inclusion of trypsin leads to a high probability of cleaving the antigen required

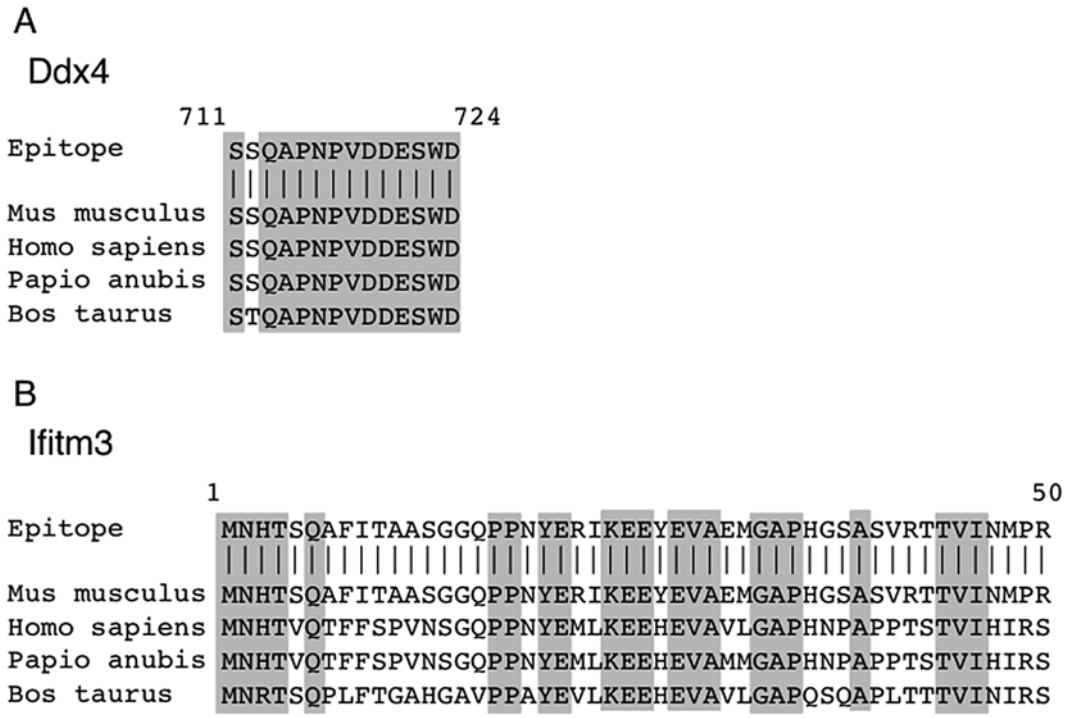

Fig. 1 Protein alignments demonstrate conserved peptides in Ddx4 or Ifitm3 extracellular domains recognized by anti-Ddx4 (Abcam #ab13840) or Ifitm3 (Abcam #ab15592) antibodies. (**a**) Protein alignment of the C-terminus of Ddx4 between mouse (*mus musculus*), human (*homo sapiens*), baboon (*papio anubis*), and bovine (*bos taurus*). (**b**) Protein alignment of the N-terminus of Ifitm3 between mouse (*mus musculus*), human (*homo sapiens*), baboon (*papio anubis*), and bovine (*bos taurus*). *Gray boxes*, conserved residues across species

for antibody-based FACS isolation. Additionally, overdigestion or incomplete neutralization of collagenase type IV results in loss of viability, and as a result can lead to promiscuous (nonspecific) antibody binding.

Finally, FACS-based OSC isolation protocols allow for detection and analysis of multiple fluorescent parameters in a single sample, as we have previously demonstrated using Ddx4-Cre;Rosa26$^{tdTm/+}$ mouse ovaries. These mice express restricted *tomato red* (*tdTm*) gene expression in cells in which the *Ddx4* gene promoter has been activated. We have previously demonstrated by flow cytometry that dispersed ovaries from these animals yield a large population of non-germline tdTm-positive cells [6], indicative of promoter "leakiness" in the Ddx4-Cre mouse line. Nonetheless, a small subset of these tdTm-positive cells exhibit externalized expression of Ddx4 protein, consistent with their identification as OSCs [6]. Accordingly, genetic reporter mouse lines coupled with antibody-based FACS strategies can be useful tools for downstream studies and applications focussed on OSC biology and function.

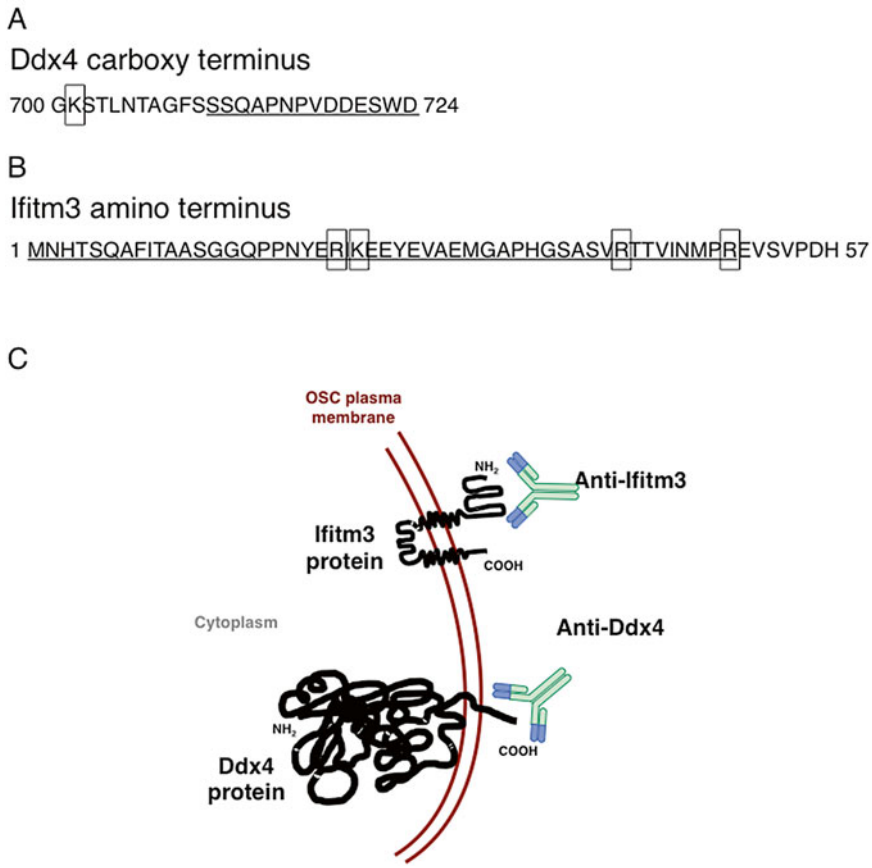

Fig. 2 Trypsin digest sites within the extracellular mouse Ddx4 ((**a**), *boxes*) and Ifitm3 ((**b**), *boxes*) antibody recognition sites (*underlined*). (**c**) Schematic of anti-Ddx4 and anti-Ifitm3 recognition sites localized on the membranes of OSCs

The following protocol describes in detail a procedure to successfully identify and isolate OSCs by antibody-based FACS, using adult mouse ovarian tissue as an example. All steps are included for sample preparation and enzymatic tissue dissociation, antibody-labeling, fluorescent controls, and gating strategies for flow cytometry.

2 Equipment

1. Laminar flow hood.
2. Analytical balance.
3. Heated orbital shaker.
4. CO_2 asphyxiation chamber.
5. Dissecting microscope.

6. Swinging bucket centrifuge.
7. Fixed angle bench-top centrifuge.
8. 37°C bead or water bath.
9. Assorted calibrated pipettes.
10. Pipette aid.
11. Laboratory timer.
12. Fluorescence-activated cell sorter with 100 μm or 70 μm nozzle and FACS software (FACS Diva 8.0.1).
13. GentleMACS tissue dissociator (Miltenyi Biotech #130-093-235).

3 Materials

1. 5 C57Bl/6 mice, 6 weeks of age (Charles River Laboratories/Jackson Laboratory).
2. Surgical scissors.
3. Surgical forceps.
4. #9 single edge utility 2″ razor blades.
5. No. 10 sterile scalpel.
6. 60 mm sterile tissue culture-treated dishes.
7. BD Luer-Lok 10 ml syringe.
8. 0.2 μm PES syringe filter.
9. Pyrex 7740 glass petri dish (Corning #3160101).
10. 50 ml polypropylene conical centrifuge tubes.
11. 5 ml borosilicate glass sterile disposable serological pipette.
12. Cell strainer, 70 μm nylon mesh (BD Falcon #352350).
13. Kimwipes.
14. 15 ml polypropylene conical centrifuge tubes.
15. 12 × 75 mm 5 ml round bottom polypropylene tubes with 35 μm cell strainer cap (BD Falcon #352235).
16. Steriflip 50 ml sterile disposable vacuum 0.22 μm filter unit.
17. Nalgene 500 ml sterile disposable filter unit with 0.2 μm PES membrane.
18. 24-well sterile tissue culture-treated plate.
19. Laboratory tape.
20. GentleMACS C-tube (Miltenyi Biotech #130-093-237).

3.1 Chemicals/Reagents

1. Flow Cytometry Size Calibration Kit (Life Technologies #F13838).

2. 0.15 M sodium chloride.
3. 70% ethanol.
4. Hanks Balanced Salt Solution (HBSS) with $MgCl_2$ and $CaCl_2$.
5. HBSS without $MgCl_2$ and $CaCl_2$.
6. Collagenase type IV (Worthington #CLS-4).
7. DNase I (Sigma #D4263).
8. IgG and protease-free bovine serum albumin (BSA).
9. Normal goat serum (NGS).
10. Viability dye (SYTOX blue, Life Technologies #S34857; 7-Amino-actinomycin D (7AAD), BD #555816; 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI), Sigma D9542; Propidium Iodide (PI), Life Technologies #P3566).
11. Rabbit IgG (Life Technologies #10500C).
12. Rabbit anti-DDX4 Antibody (Abcam #ab13840) or rabbit anti-Ifitm3 Antibody (Abcam #ab15592).
13. Fluorophore microscale protein labeling kit of choice; recommend Alexa Fluor 488 microscale protein labeling kit (Life Technologies #A30006).
14. 1 M HEPES buffer for cell culture.
15. Phosphate buffered saline, no calcium, no magnesium (PBS).
16. 10 mM Tris pH 7.6.
17. MEMαGlutaMax (Life Technologies #32561-102).
18. Qualified fetal bovine serum (FBS), one shot (Life Technologies).
19. Sodium pyruvate.
20. MEM Non-Essential Amino Acids Solution (NEAA).
21. 2-Mercaptoethanol (Sigma #M3148).
22. Leukemia inhibitory factor (LIF, ESGRO©, Milipore #ESG1106).
23. N-2 MAX Media Supplement (R&D Systems #AR009).
24. Recombinant human epidermal growth factor (EGF, Life Technologies #PHG0311).
25. Recombinant human glial cell line-derived neurotrophic factor (GDNF, R&D Systems #212GD050).
26. Recombinant human basic fibroblast growth factor (bFGF, Life Technologies #13256-029).
27. Penicillin–streptomycin–glutamine (PSG).

3.2 Reagent Preparation

1. Using the microscale protein labeling kit, conjugate primary antibody to selected fluorophore according to the manufacturer's protocol.

2. DNase I: 6 U/μl (*see* **Note 1**). Store at −20°C for up to 3 months.
3. Mouse dissociation buffer: 800 U/ml collagenase type IV, 6 U/ml DNase I in HBSS with $MgCl_2$ and $CaCl_2$ (*see* **Note 2**).
4. Blocking buffer: 2% BSA, 2% NGS HBSS without $MgCl_2$ and $CaCl_2$ (*see* **Note 3**).
5. FACS buffer: 25 mM HEPES, 2% BSA in HBSS without $MgCl_2$ and $CaCl_2$ (*see* **Note 4**).
6. Recombinant human EGF: 0.1 mg/ml (*see* **Note 5**). Store at −20°C.
7. Recombinant human GDNF: 0.1 mg/ml (*see* **Note 6**). Store at −20°C.
8. Recombinant human bFGF: 0.1 mg/ml (*see* **Note 7**). Store at −20°C.
9. OSC culture medium: 10% FBS, 1× PSG, 1 mM sodium pyruvate, 0.1 mM NEAA, 1× N-2 Max, 0.1 mM 2-mercaptoethanol, 10^3 Units LIF, 0.01 μg/ml EGF, 0.04 μg/ml GDNF, 0.001 μg/ml bFGF in MEMαGlutaMax (*see* **Note 8**).
10. Dissociation buffer: 400 U/ml collagenase type IV, 6 U/ml DNase I in HBSS with $MgCl_2$ and $CaCl_2$ (*see* **Note 9**).

4 Methods

4.1 Mouse Dissection Procedure

1. Carbon dioxide asphyxiate and cervically dislocate five mice.
2. Spray mice with 70% ethanol to minimize hair sticking to surgical instruments.
3. With the mouse supine, hold the lower abdominal skin with surgical forceps and make a small incision using surgical scissors into the lower abdominal region, through the peritoneal cavity.
4. Enlarge the incision medial and lateral to expose the abdominal region. To expose the uterus flip the intestines proximal and the fat pad distal so that each is out of the field of view.
5. Moving proximal up the uterus, the oviduct attached to the ovary and ovarian fat pad will be visible.
6. Remove the ovary and place in a 60 mm tissue culture dish containing HBSS with $MgCl_2$ and $CaCl_2$.
7. Using forceps and a scalpel remove any fat and oviduct from the ovary. Combine and place all cleaned ovaries (10 total) in a new 60 mm tissue culture dish containing HBSS with $MgCl_2$ and $CaCl_2$.

4.2 Mouse Ovary Dissociation Procedure

1. Transfer ovaries to a glass petri dish with 50 μl of prewarmed dissociation buffer (800 U/ml collagenase type IV with 6 U/ml DNase I in HBSS with $MgCl_2$ and $CaCl_2$).
2. Mince ovaries in a scissoring motion between two razor blades for 2–7 min until a slurry has formed.
3. Transfer ovarian slurry to a 50 ml conical tube containing dissociation buffer, rinsing petri dish as needed with dissociation buffer to collect maximum amount of ovary sample.
4. Affix tube horizontally to the heated orbital shaker surface with laboratory tape and shake tube at 225 rpm at 37°C for 15 min.
5. Pipette solution ten times with 5 ml glass serological pipette (Note: do not use plastic serological pipettes, as these can damage cells).
6. Shake tube at 225 rpm at 37°C for 15 min.
7. Pipette solution ten times with 5 ml glass serological pipette.
8. Shake tube at 225 rpm at 37°C for 15 min.
9. Pipette solution ten times with 5 ml glass serological pipette.
10. Filter solution through a 70 μm cell strainer into a fresh 50 ml conical tube.
11. Rinse the original 50 ml tube with 5 ml HBSS without $MgCl_2$ and $CaCl_2$ and filter through same 70 μm nylon mesh cell strainer into same new 50 ml tube as sample.
12. Centrifuge the cell suspension at 600 × *g* for 5 min, maximum acceleration, minimum brake in a swinging bucket centrifuge.
13. Decant and discard the supernatant, blot the top of the tube with a Kimwipe.

4.3 Antibody Labeling

1. Resuspend the cell pellet (*see* **section 4.2, step 13**) with 0.5 ml of blocking buffer (2% BSA, 2% NGS in HBSS without $MgCl_2$ and $CaCl_2$).
2. Incubate cells in blocking buffer at room temperature for 20 min.
3. Set up the following controls and samples in separate 15 ml conical tubes:

Control/sample	Cells (*see* **step 2**)	IgG or primary antibody
Unstained	50 μl	
Viability dye	50 μl	
Rabbit IgG control	100 μl	3.3 μg fluorophore-conjugated rabbit IgG
Stained sample	300 μl	10 μg fluorophore-conjugated rabbit anti-Ddx4 or rabbit anti-Ifitm3

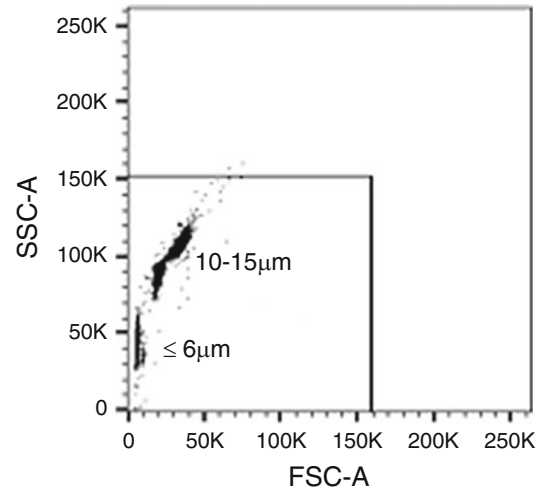

Fig. 3 Size gating strategy using calibrated beads 2–15 μm in diameter. Size gate using forward scatter (FSC-A) and side scatter (SSC-A) parameters is set to include all events in the size range

4. Incubate controls and samples (*see* **step 3**) in 15 ml conical tubes for 20 min at room temperature.
5. Add 5 ml of HBSS without $MgCl_2$ and $CaCl_2$ to each 15 ml conical tube to wash.
6. Centrifuge at 600 × *g* for 5 min at room temperature with maximum acceleration and brake in a swinging bucket centrifuge.
7. Decant supernatants and resuspend samples/controls in 0.3–0.5 ml FACS buffer (25 mM HEPES pH 7.0 and 2% BSA in HBSS without $MgCl_2$ and $CaCl_2$) and strain into 5 ml round bottom tubes with 35 μm cell strainer caps).
8. Set up flow cytometer, perform any necessary calibration, and determine the drop delay for sorting collection.
9. Set the forward- (FSC) and side- (SSC) scatter parameters in linear scale.
   (a) FSC: Size; larger cells have greater FSC.
   (b) SSC: complexity/granularity; "smoother" or less granular cells will have a lower SSC.
10. Prepare the flow cytometry size calibration kit (*see* **Note 10**). Run the microspheres and set the gate on the FSC/SSC dot plot to include all objects in the 2–15 μm range of the agranular objects (Fig. 3). OSC diameter varies across species, murine OSCs are ≤10 μm.
11. Run unstained control and adjust voltages as needed.
12. Depending on the fluorophores selected, multicolor experiments require spillover correction between fluorochromes. Set up compensation as needed.

13. Use a viability dye to distinguish live/dead events. Depending on the fluorophore of your secondary antibody utilize SYTOX Blue at a final concentration of 1 μM, 7AAD at 10 μl per sample, propidium iodide at a final concentration of 1 μg/ml, or DAPI as described in [5]. Dead cells can bind antibodies nonspecifically leading to atypical results. It is important to minimize the dead cells in the analysis. Add viability dye to the viability dye-only tube, IgG control tube, and stained sample tube. Run the viability dye control and confirm the voltages from **step 31**.
14. Run IgG control and prepare a gating strategy to visualize a minimum of three plots: FSC/SSC (Fig. 4a, e), SSC/viability (Fig. 4b, f), and viable SSC/antigen (Fig. 4c, g or d, h).
15. Run the samples and include agranular objects 2–15 μm in size within the gating strategy.
16. Downstream of the nozzle, droplets detach from the flow cytometer stream. The drop delay is the time between a particle being interrogated by the laser and reaching the droplet break-off point. To ensure the drop collected contains the particle of interest, the drop delay must be set on the instrument.
17. With the cytometer sample chiller off, run stained sample using the gating strategy in **step 34** (Fig. 4). Collect events of interest in microcentrifuge tube(s) containing 0.5 ml of prewarmed OSC culture medium.
18. Following event collection, centrifuge the microcentrifuge tubes at 800 × *g* for 5 min in a fixed angle centrifuge at room temperature. Carefully remove the supernatant and proceed to downstream application. If subculture and expansion of cell numbers is desired, resuspend the pellet, which may not be visible to the eye due to the small diameter and small number of cells collected, in 0.5 ml fresh prewarmed OSC culture medium and seed into one well of a 24-well tissue culture-treated plate. Culture cells in a humidified incubator at 37°C with 5% CO_2 as described [5].

4.4 Dissociation Procedure: Other Mammalian Species

For isolation of OSCs from species with larger ovaries (e.g., human, cow, non-human primate), we recommend using a dissociation instrument, such as the GentleMACS tissue dissociator. Harvest only the cortex of the ovary and proceed stepwise through the following protocol:

Fig. 4 (continued) by dot plot showing FSC vs. SSC signals, which are related to the size and complexity/granularity of the cell, respectively. Based on size calibration shown in Fig. 3, agranular, small events ranging from 1 to 15 μm are included in gate. (**b**)/(**f**). Events from gated region in (**a**)/(**e**) are further analyzed for SYTOX Blue to exclude nonviable events. Events from gated region in (**b**)/(**f**) are subsequently distinguished based on IgG binding (**c**)/(**d**), Ddx4 expression (**g**) or Ifitm3 expression (**h**). Samples were run using a 100 μm nozzle at 20 psi

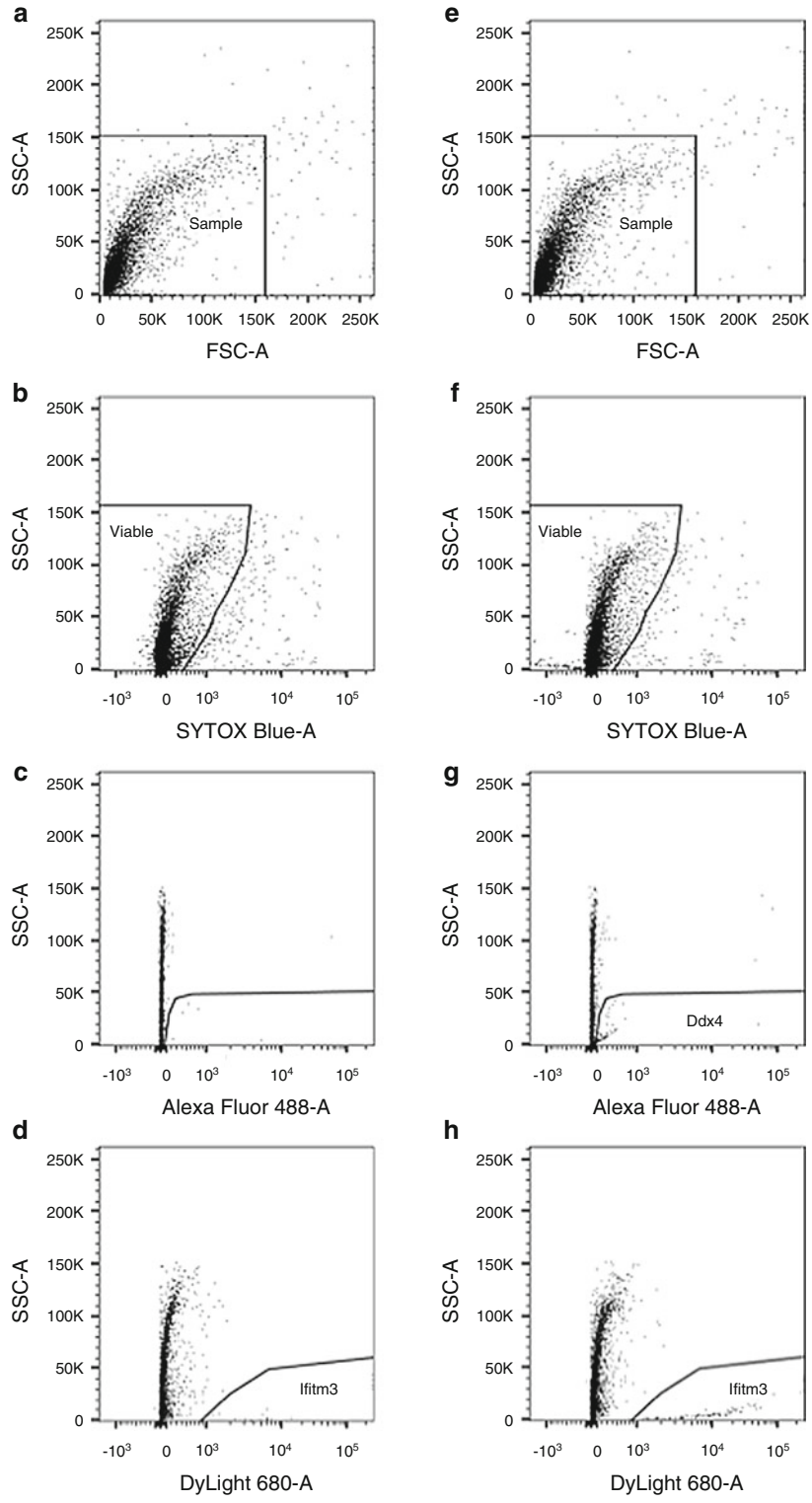

Fig. 4 Gating strategy for sorting murine OSCs by FACS. Ovarian dispersates were prepared from 6-week-old mouse ovaries and labeled with Ddx4-AlexaFluor-488 and Ifitm3-DyLight-680. Rabbit IgG controls (fluorescent-labeled IgG; (**a**–**d**)) and antibody-labeled samples (**e**–**h**) are shown. (**a**)/(**e**). Dispersed mouse ovary is visualized

1. Prepare dissociation buffer (400 U/ml collagenase type IV with 6 U/ml DNase I in HBSS with $MgCl_2$ and $CaCl_2$; *see* **Note 9**) and prewarm the solution to 37 °C.
2. Using a scalpel, cut the ovarian cortex into ~2 × 2 mm pieces. Do not cut the pieces smaller than 2 × 2 mm as they will be too small for the GentleMACS. Do not cut the pieces larger than 2 × 2 mm as they will be too large and can jam the GentleMACS.
3. Transfer pieces of ovarian cortex to a GentleMACS C-tube with 5 ml of prewarmed dissociation buffer.
4. Load the GentleMACS C-tube into the GentleMACS tissue dissociator.
5. Run GentleMACS pre-programmed protocol for Human Tumor 1. If any pieces of tissue become trapped in the C-tube mechanism use forceps to return tissue to the dissociation buffer.
6. Affix tube horizontally to the heated orbital shaker surface with laboratory tape and shake tube at 225 rpm at 37°C for 15 min.
7. Pipette solution ten times with 5 ml glass serological pipette.
8. Run GentleMACS pre-programmed protocol for Human Tumor 2. If any pieces of tissue have become trapped in the C-tube mechanism use forceps to return tissue to the dissociation buffer.
9. Shake tube at 225 rpm at 37 °C for 15 min.
10. Pipette solution ten times with 5 ml glass serological pipette.
11. Run GentleMACS pre-programmed protocol for Human Tumor 3. If any pieces of tissue have become trapped in the C-tube mechanism use forceps to return tissue to the dissociation buffer.
12. Shake tube at 225 rpm at 37°C for 15 min.
13. Pipette solution ten times with 5 ml glass serological pipette.
14. Continue at **step 10** from mouse ovary protocol (*see* **section 4.2**).

4.5 Antibody Labeling with Ddx4-Cre;Rosa26$^{tdTm/+}$ Mice

The reporter mouse model Ddx4-Cre;Rosa26$^{tdTm/+}$, which can exhibit leaky expression of the promoter in nongermline cells [6], can be utilized in combination with antibody-based FACS to successfully isolate OSCs.

1. Harvest, prepare, and stain mouse ovarian dispersates as described in mouse protocol above (mouse dissection **steps 1–7**, mouse ovary dissociation **steps 8–20**, antibody labeling **steps 21–29**). We recommend allophycocyanin (APC) for the Ddx4 secondary fluorophore (antibody labeling **step 23**).

2. Prepare the flow cytometry size calibration kit (*see* **Note 10**). Run the microspheres and set the gate on the FSC/SSC dot plot to include all objects in the 2–15 μm range of the agranular objects (as demonstrated in Fig. 3). OSC diameter varies across species, murine OSCs are ≤10 μm.
3. Run unstained control and adjust voltages as needed.
4. Dead cells bind antibodies nonspecifically, and each antibody can bind differently. Therefore, every attempt must be made to minimize unintended detection of dead cells in the analysis. Use a viability dye to distinguish live/dead cell events. For this experiment, tomato red expression will be visualized with a phycoerythrin (PE) filter and Ddx4 with an APC filter. Therefore, the recommended viability dyes are SYTOX Blue at a final concentration of 1 μM or DAPI as described in [5]. Add viability dye to viability dye-only sample, control sample, and stained sample. Run viability dye control and confirm voltages from **step 3**.
5. Run negative control and prepare a gating strategy to visualize a minimum of four plots: FSC/SSC (Fig. 5a/f), SSC/viability (Fig. 5b/g), viable SSC/Tomato (PE, Fig. 5c/h), and viable Tomato + (PE+) SSC/Ddx4 (APC) (Fig. 5d/i or e/j).
6. Run the samples and include agranular objects 2–15 μm in size within the gating strategy.
7. Downstream of the nozzle, droplets detach from the flow cytometer stream. The drop delay is the time between a particle being interrogated by the laser and reaching the droplet breakoff point. To ensure the drop collected contains the particle of interest, the drop delay on the instrument must be set.
8. With the cytometer sample chiller off, run Ddx4 stained sample using the gating strategy set up in **step 5** (Fig. 5) and collect events of interest in microfuge tubes containing 0.5 ml of prewarmed OSC culture medium.

Following event collection, centrifuge the microfuge tubes at 800 × *g* for 5 min in a fixed angle centrifuge at room temperature. Carefully remove the supernatant. Resuspend the pellet, which may not be visible due to the small number and diameter of the cells collected, in 0.5 ml fresh prewarmed OSC culture medium and seed into one well of a 24-well tissue culture treated plate. Culture cells in a humidified incubator at 37 °C with 5% CO_2 as described [5].

5 Notes

1. Reconstitute one vial of 2000 U DNase I with 333.3 μl of sterile 0.15 M sodium chloride to achieve a 6 U/μl solution of DNase I. Aliquot and store at −20 °C for up to 3 months.

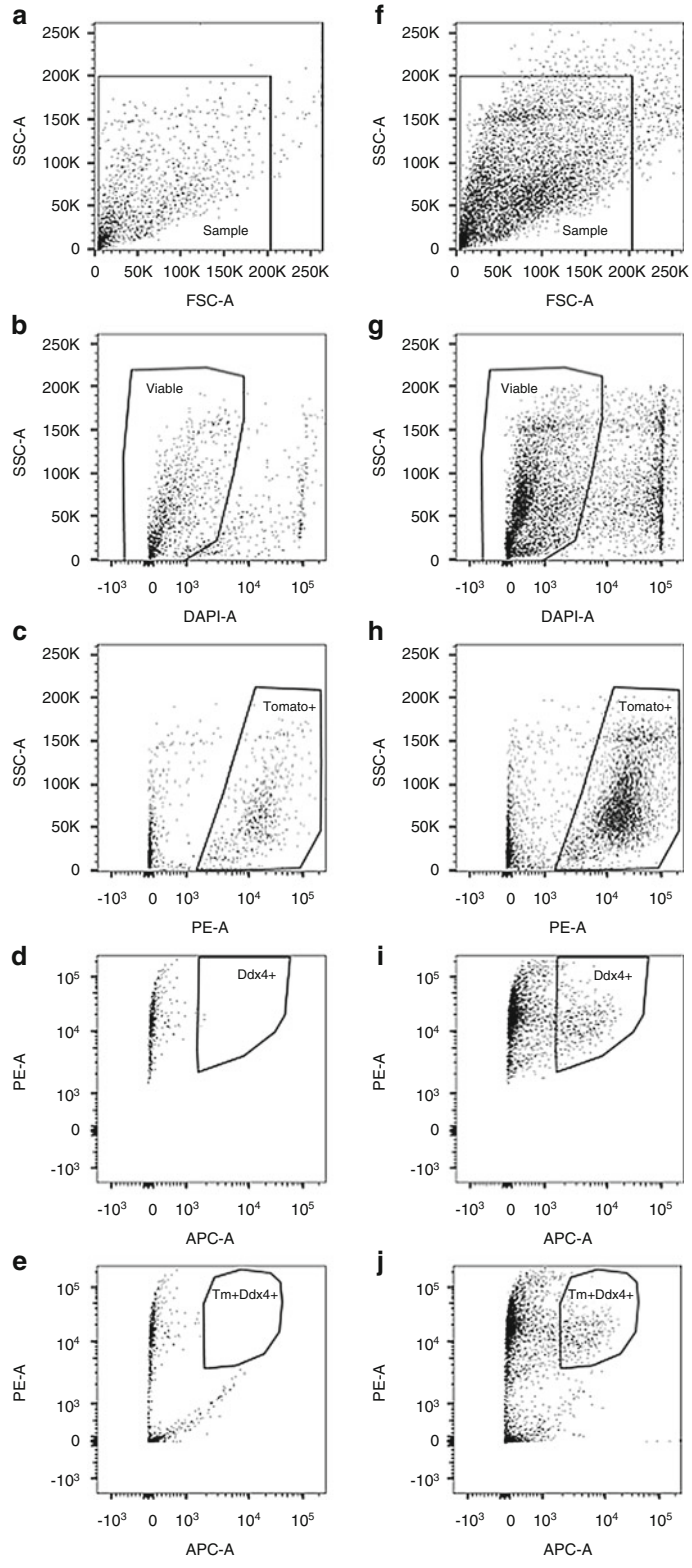

Fig. 5 Gating strategy for sorting murine OSCs by FACS from Ddx4-Cre;Rosa26$^{tdTm/+}$ mice. OSCs were isolated from 8-week-old Ddx4-Cre;Rosa26$^{tdTm/+}$ mouse ovaries

2. Dissolve collagenase type IV in 10 ml of HBSS with $MgCl_2$ and $CaCl_2$ to a concentration of 800 U/ml. Syringe filter into a sterile 50 ml polypropylene conical tube. Add 10 μl of 6 U/μl DNase I to bring the solution to 6 U/ml DNase I. Place in 37 °C water or bead bath to warm prior to use on tissue.
3. Dissolve 0.5 g IgG and protease-free BSA in 24.5 ml HBSS without $MgCl_2$ and $CaCl_2$, add 0.5 ml NGS and filter-sterilize using a 0.2 μm filter.
4. Dissolve 0.5 g IgG and protease-free BSA in 24.375 ml HBSS without $MgCl_2$ and $CaCl_2$, add 625 μl of 1 M HEPES and filter-sterilize using a 0.2 μm filter.
5. Dissolve 100 μg EGF in 1 ml sterile PBS for a concentration of 0.1 mg/ml. Aliquot and store at −20 °C.
6. Dissolve 50 μg GDNF in 0.5 ml sterile PBS with 0.1 % IgG and protease-free BSA for a final concentration of 0.1 mg/ml. Aliquot and store at −20 °C.
7. Dissolve 10 μg bFGF in 0.1 ml sterile-filtered 10 mM Tris pH 7.6 for a final concentration of 0.1 mg/ml. Aliquot and store at −20 °C.
8. Prepare OSC culture medium using the following recipe: 430 ml MEMαGlutaMax, 50 ml FBS, 5 ml of 100× PSG, 5 ml of 100 mM sodium pyruvate, 5 ml of 10 mM NEAA, 5 ml of 100× N-2 Max, 3.5 μl of 14.3 M2-mercaptoethanol, 0.5 ml of 10^6 Units LIF, 50 μl of 0.1 mg/ml EGF, 200 μl of 0.1 mg/ml GDNF, 5 μl of 0.1 mg/ml bFGF. Filter-sterilize through 0.22 μm PES filter. Store at 4 °C for 2 weeks or −80 °C for 3 months.
9. Dissolve collagenase type IV in 10 ml of HBSS with $MgCl_2$ and $CaCl_2$ to a concentration of 400 U/ml. Syringe filter into a sterile 50 ml polypropylene conical tube. Add 10 μl of 6 U/μl DNase I to bring the solution to 6 U/ml DNase I. Place in 37 °C water or bead bath to warm prior to use on tissue.
10. Mix the flow cytometry size calibration kit microsphere suspensions and add 20 μl of each size suspension to 0.5 ml flow cytometer sheath fluid and gently vortex.

Fig. 5 (continued) and stained with Ddx4-APC. Non-antibody (IgG) control (**a**–**e**) and labeled sample (**f**–**j**) are shown. (**a**)/(**f**). Dispersed mouse ovary is visualized by dot plot showing FSC vs. SSC signals, which are related to the size and complexity/granularity of the cells, respectively. Agranular, small events ranging from 2 to 15 μm are included in gate. (**b**)/(**g**). Events from gated region in (**a**)/(**f**) are further analyzed for DAPI to exclude nonviable cells. Events from gated region in (**b**)/(**g**) is subsequently distinguished based on tomato red (Tm) expression into Tomato− and Tomato+ events (**c**)/(**h**). Tomato + events are further analyzed for non-specific secondary antibody binding (**d**) and Ddx4 expression (**i**). Alternatively, events from the gated region in (**b**)/(**g**) can be distinguished based on Tomato+/nonspecific antibody binding (**e**) and Tomato+/Ddx4+ expression (**j**). Samples were run using a 70 μm nozzle at 70 psi

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Chapter 20

Immunohistochemistry of Paraffin Sections from Mouse Ovaries

Gokhan Akkoyunlu and Filiz Tepekoy

Abstract

Immunohistochemistry (IHC) is an efficient technique to detect cellular localizations of the proteins in paraffin-embedded tissues. It allows specific proteins to be visualized by the interaction of antibodies with an enzyme–substrate–chromogen system. Here, we describe indirect immunohistochemistry method for paraffin-embedded mouse ovaries fixed with Bouin's Fixative.

Key words Immunohistochemistry, Mouse ovary, Paraffin sections, Bouin's Fixative, Biotinylated antibodies

1 Introduction

Immunohistochemistry (IHC) is an outstanding method for detecting the localization of specific proteins in paraffin-embedded tissues fixed with appropriate fixatives. Coons and co-workers first demonstrated that we can detect antigens using fluorescence-labeled antibodies [1]. Later IHC for formalin-fixed, paraffin-embedded tissues was developed by Taylor and Burns in 1974 [2]. The technique is widely used in ovaries for both research [3, 4] and diagnostic purposes [5, 6]. The advantage of the technique is its allowance for determination of cell type-specific localization of specific proteins within a tissue. The disadvantage of the technique might be nonspecific binding of the antibodies in spite of application of the blocking methods.

The technique is based on antibodies that bind to their specific antigens. Antigen detection can be made through direct and indirect immunohistochemistry. The direct technique includes only one labeled antibody that is directly linked to the antigen. The indirect immunohistochemistry includes a primary antibody that binds to the antigen and a labeled secondary antibody that binds to the primary antibody. The indirect technique is more sensitive due to the involvement of signal amplification [7].

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The signal amplification can be provided through amplifiers such as biotin that is conjugated to the secondary antibodies. An enzyme such as peroxidase is linked to this antibody system. Peroxidase is usually conjugated with streptavidin protein that has high affinity to the biotin [8]. Consequently, there is a substrate–chromogen system which reacts with peroxidase. This reaction is a color-producing reaction that finally allows to visualize the localization of the antigen in the color specific to the applied chromogen [9].

The most notable results are obtained through application of the most appropriate fixation protocol in adequate time for the tissue as well as for the antibodies [10]. In our studies, we fix mouse ovaries with Bouin's Fixative to ideally protect the tissue morphology as well as to have reliable results in immunohistochemistry method. We conduct indirect immunohistochemistry technique in order to detect localization of specific proteins in mouse ovary for research purposes [11].

2 Materials

1. Fixative.

   Bouin's Fixative: Add 75 mL of saturated aqueous solution of picric acid (*see* **Note 1**), 25 mL of formalin and 5 mL of glacial acetic acid. Store at room temperature.

2. Tissue Dehydration Reagent.

   A graded series of ethanol (70, 80, 90, and 100%) is used for dehydration of the tissue.

3. Tissue Clearing Reagent.

   Three changes of xylene is used for clearing the tissue.

4. Tissue Embedding.

   Parrafin wax is used for embedding the tissue.

5. Antigen Retrieval Buffer.

   10 mM Citrate Buffer, **pH**: **6.0**: Add about 100 mL water to a 1-L graduated cylinder or volumetric flask. Weigh 2.1 g Citric Acid and transfer to the cylinder. Add water to a volume of 800 mL. Mix and adjust pH with HCl (12 N). Store at 4 °C.

6. Blocking Reagents

   3% H2O2: Add 63 mL methanol and 7 mL 30% of H_2O_2 to a glass jar covered with aluminum foil.
   Ultra V Block: Polyvalent blocking serum to reduce nonspecific background staining.

7. Washing Buffer
   Phosphate Buffered Saline (**PBS**), **pH**: **7.2–7.4**: Add about 200 mL water to a 2-L volumetric flask. Weigh 0.8 g KH_2PO_4

(2.9 mM), 2.85 g Na_2HPO_4 (10 mM) and 16 g NaCl (137 mM) and transfer to the flask. Add water to a final volume of 2 L. Mix and adjust pH with NaOH (2 M). Store at 4 °C.

8. **Primary Antibody**.
   The antibody that binds to the specific antigen (studied protein).
9. **Biotinylated Secondary Antibody**.
   The antibody that binds to the Fc domain of the primary antibody with its Fab domain. The Fc domain of the secondary antibody is conjugated with biotin that will bind to streptavidin to amplify the reaction.
10. **Streptavidin Enzyme Conjugate**.
    Ready to use streptavidin conjugated with Horseradish Peroxidase (HRP) (Thermo Scientific, TS-XXX-HR).
11. **Chromogenic Substrate Solution**.
    The standard working dilution of 3,3′-diaminobenzidine (DAB) is 20 µL of DAB plus chromogen per 1 mL of DAB substrate according to the directions of the manufacturer (Thermo Scientific, TA-002-HCX, TA-060-HSX).

3 Methods

Carry out all procedures at room temperature unless otherwise specified.

1. Fix freshly dissected total ovary with Bouin's Fixative for 4 h at room temperature. (Caution: Bouin's Fixative includes suspect carcinogens. It can cause eye, skin, and respiratory tract irritation. It should be handled in a hood) (*see* **Note 2**).
2. Treat the tissue with 70% ethanol for 24 h (*see* **Note 3**).
3. Dehydrate the tissue through 80 and 90% ethanol, 24 h each, followed by dehydration through 100% ethanol for 3 h.
4. Clear the tissue through three changes of xylene, 2 min each.
5. Immerse the tissue in three changes of paraffin, 1 h each.
6. Embed the tissue in a paraffin block. The paraffin tissue block can be stored at room temperature for years.
7. Take 5 µm sections from the paraffin-embedded ovarian tissue block on a microtome.
8. Float the sections in a 40 °C water bath containing distilled water.
9. Transfer the sections onto superfrost ultra plus adhesion slides.
10. Allow the slides to dry and incubate at 37 °C overnight and store the slides at room temperature.
11. Deparaffinize slides in two changes of xylene, 10 min each.

12. Hydrate the tissue sections with decreasing concentration series of ethanol (100, 90, 80, 70%, 5 min each).
13. Transfer slides to the distilled water.
14. To unmask the antigenic epitope, perform antigen retrieval. Incubate the slides in citrate buffer at 95–100 °C for 5 min and allow the slides to cool for 20 min at room temperature in the citrate buffer.
15. Wash the slides in PBS three times for 5 min.
16. Use a hydrophobic pen (PAP Pen) to circle each section on the slide in order to concentrate the reagents on each section and avoid leaking from the slide.
17. To block endogenous peroxidase activity, incubate the slides with 3% H_2O_2 solution at room temperature for 20 min.
18. Wash the slides in PBS three times for 5 min.
19. Add 100 μL Ultra V Block on the sections of the slides and incubate in a humidified chamber at room temperature for 7 min (*see* **Note 4**).
20. Drain off the excess serum from the slides.
21. Apply 100 μL appropriately diluted (*see* **Note 5**) primary antibody (in PBS) to the sections on the slides and incubate in the humidified chamber at 4 °C overnight (*see* **Note 6**).
22. Wash the slides in PBS three times for 5 min.
23. Apply 100 μL appropriately diluted (*see* **Note 5**) biotinylated secondary antibody (in PBS) to the sections on the slides and incubate in the humidified chamber at room temperature for 30 min.
24. Wash the slides in PBS three times for 5 min.
25. Apply 100 μL Streptavidin-HRP conjugates to the sections on the slides and incubate in the humidified chamber at room temperature for 20 min.
26. Wash the slides in PBS three times for 5 min.
27. Apply 100 μL DAB substrate solution to the sections on the slides to reveal the color of antibody staining (*see* **Note 7**). Caution: DAB is a suspect carcinogen. Handle with care. Wear gloves, lab coat, and eye protection.
28. Wash the slides in distilled water.
29. Counterstain slides by immersing sides in Hematoxylin for 1–2 min (*see* **Note 8**).
30. Wash the slides in running tap water for 1–2 min.
31. Dehydrate the sections through a graded series of ethanol (70, 80, 90, and 100% each for 5 min).

32. Clear the tissue slides in two changes of xylene (5 min for each) and coverslip using mounting solution. The mounted slides can be stored at room temperature temporarily (*see* **Note 9**).
33. Observe the color of the antibody staining in the tissue sections under microscopy.

4 Notes

1. Filter the picric acid with a filter paper before adding to the fixative to avoid any eventual remaining undissolved particles.
2. The adipose tissue around the ovary must be carefully removed under a stereomicroscope for better fixation of the ovarian tissue.
3. The tissue might be rinsed with tap water to remove the excess amount of Bouin's Fixative. However, since the water cannot remove the picric acid invaded in the tissue, washing must be done via 70% ethanol on a shaker. The ethanol must be changed several times until the yellow color of picric acid disappears.
4. All incubations should be carried out in the humidified chamber to avoid drying of the tissue. Drying at any stage will lead to nonspecific binding and ultimately high background staining.
5. The antibody must be diluted as recommended by the manufacturer. The most common dilution is 1:200 for primary and 1:500 for secondary antibodies. If the desired reaction cannot be observed, firstly different dilutions (starting from 1:50 up to 1:500) of primary antibody must be applied. The dilution of secondary antibody can also be modified if background staining appears. Make sure the primary antibody is raised in a species different from the tissue being stained.
6. Perform negative controls on each slide by replacing the primary antibody with PBS.
7. Allow the color development for <5 min until the desired color intensity is reached.
8. Hematoxylin staining must be controlled under the microscope to obtain the desired intensity.
9. The slides can be stored at 4 °C to delay fading of the DAB reaction.

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