Chapter 8

Visualization of Actin Cytoskeletal Dynamics in Fixed and Live *Drosophila* Egg Chambers

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Abstract

Visualization of actin cytoskeletal dynamics is critical for understanding the spatial and temporal regulation of actin remodeling. *Drosophila* oogenesis provides an excellent model system for visualizing the actin cytoskeleton. Here, we present methods for imaging the actin cytoskeleton in *Drosophila* egg chambers in both fixed samples by phalloidin staining and in live egg chambers using transgenic actin labeling tools.

Key words Actin cytoskeleton, Oogenesis, Nurse cells, Microscopy, *Drosophila*

1 Introduction

Drosophila oogenesis is an ideal system to study actin cytoskeletal dynamics. Each ovary is comprised of approximately 15 ovarioles—chains of developing egg chambers or follicles. *Drosophila* oogenesis consists of 14 morphologically defined stages [1]. Mid-to-late-stage oogenesis (stage 10B–stage 13) requires the activities of numerous actin-binding proteins to mediate dynamic actin remodeling within the 15 germline-derived support or nurse cells [2]. This remodeling is necessary for the completion of oogenesis and, ultimately, female fertility. As the nurse cells are very large, these cells are an excellent system for visualizing the actin cytoskeleton in both live and fixed samples.

Fixed imaging of the actin cytoskeleton using phalloidin to label actin filaments allows for a detailed analysis of the spatial and temporal regulation of actin remodeling during mid-to-late oogenesis. Formation of cytoplasmic actin bundles begins during stage 10B in the four posterior nurse cells along the nurse cell/oocyte boundary. Actin bundles then form throughout the remaining nurse cells by the end of stage 10B [3] (Fig. 1). Additionally, the cortical actin network just beneath the nurse cell membranes is strengthened at this time. This specific pattern of spatial and

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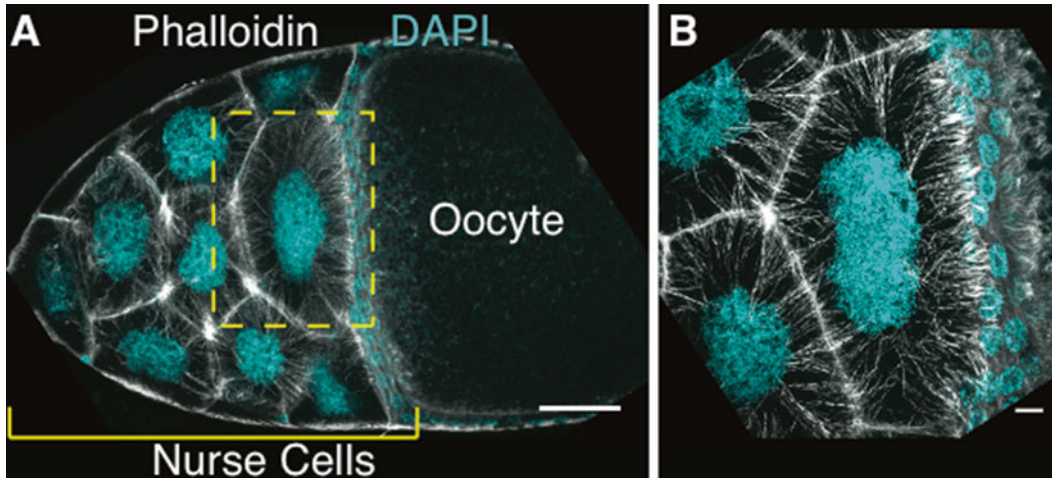

Fig. 1 Actin visualization in a stage 10B egg chamber. Maximum projections of 3–5 confocal slices of a late stage 10B egg chamber stained with phalloidin (actin, *white*) and DAPI (DNA, *cyan*). (**a**) Image taken with a 20× objective. Scale bar is 50 μm. (**b**) Image taken with a 63× objective. Scale bar is 10 μm. Nurse cells in late stage 10B egg chambers exhibit a robust network of radially aligned actin filament bundles that form at the nurse cell membranes and extend inwards towards the nucleus. The cortical actin is also strengthened during this stage of oogenesis

temporal regulation of actin remodeling provides a system for understanding how actin dynamics are regulated. For example, imaging of fixed samples can highlight defects in actin remodeling even at a relatively low magnification (20× objectives) [4].

Here, we describe two protocols for visualizing filamentous actin in fixed samples by phalloidin staining. The first, standard protocol (Subheading 3.1) works well for imaging the robust actin bundle network of stage 10B and later egg chambers. However, some less stable actin structures are formed in developing egg chambers and require a fixation protocol that stabilizes actin filaments [5, 6]. The actin fixation protocol detailed in Subheading 3.2 enhances stabilization of the less robust actin filament structures for better visualization than the standard fixed imaging protocol. The choice of method will depend upon the actin structures being analyzed.

The genetic tools available in *Drosophila* enable in vivo live imaging of actin cytoskeletal dynamics. Actin can be labeled directly via GFP or RFP tags on any of the six *Drosophila* actins (available at the Bloomington *Drosophila* Stock Center) [7]. Indirect actin labeling tools are also available for expression in the germline [8–11], including LifeAct [12], Utrophin [13], and Ftractin [14]. The actin filaments formed during mid-to-late oogenesis in live egg chambers can be visualized by expression of these UASp transgenic insertion lines with germline-specific GAL4 lines [15] (matα, oskar, nanos, matα-geneswitch). Although expression of some of these actin

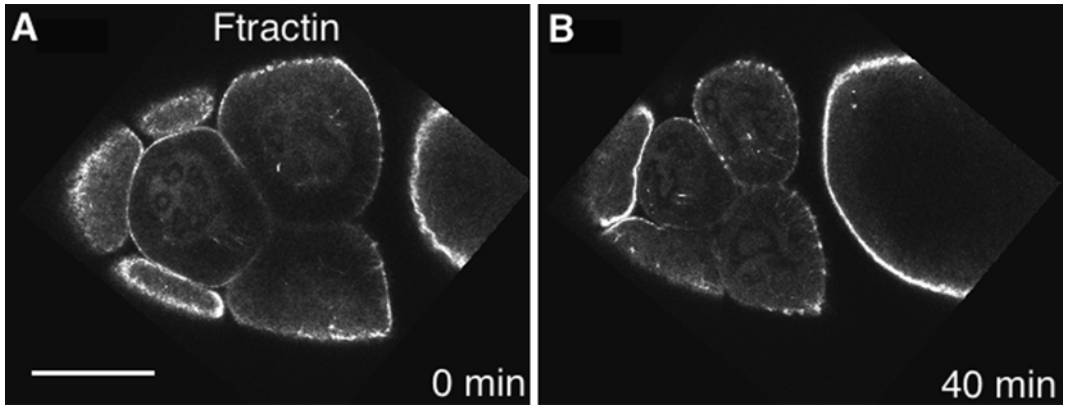

Fig. 2 Live imaging time course of a stage 10B egg chamber expressing Ftractin-tdTomato. (**a**) Image captured at $t=0$ min (stage 10B) and (**b**) 40 min (stage 11). Scale bar is 50 μm. Actin bundles labeled with Ftractin are observable in a live egg chamber as it progresses from late stage 10B to 11 and begins to undergo nurse cell dumping

labeling tools is known to cause defects, low expression of Utrophin or strong expression of Ftractin (Fig. 2) can be used to visualize the actin cytoskeleton live with minimal defects [8]. Additionally, live egg chambers can be maintained in a simple culture media for several hours while oogenesis progresses [16–19]. Therefore, the dynamics of actin remodeling can be observed in a single egg chamber as late-stage oogenesis is completed.

Here, we present methods for both short-term and long-term live imaging. Subheading 3.3, which is intended for short-term imaging, utilizes a simple liquid culture media throughout dissection and imaging. However, egg chambers may shift position in a liquid media, particularly as nurse cell dumping occurs. If a long-term time course of imaging or multipoint short-term imaging is desired, egg chambers can be embedded in an agarose media to prevent movement (Subheading 3.4).

2 Materials

2.1 Solutions

1. Wet yeast paste: 50 g active dry yeast mixed with 90 ml of ddH_2O, stored at 4 °C (*see* **Note 1**).
2. Grace's Insect Culture Medium. Store at 4 °C.
3. In vitro egg maturation (IVEM) medium: 10 % fetal bovine serum (FBS, heat inactivated) in Grace's Insect Culture Medium plus 1× penicillin/streptomycin. Prepare fresh before each use.
4. 20 % Paraformaldehyde (PFA): Prepare in fume hood. Dissolve 0.496 g of $Na_2CO_3 \cdot H_2O$ in ~800 ml of dH_2O. Add 200 g of paraformaldehyde. Heat, with stirring, to ~80 °C to get the

paraformaldehyde into solution. Bring to a final volume of 1 l with dH_2O. Filter through Whatman filter paper (#1) to remove the sludge. Store at room temperature (*see* **Notes 2** and **3**).

5. 1× Phosphate-buffered saline (1× PBS): 135 mM NaCl, 3.2 mM Na_2HPO_4, 1.3 mM KCl, 0.5 mM KH_2PO_4, pH 7.4.
6. Actin fixative [5, 6]: 4 % paraformaldehyde (diluted from 20 % solution in 1× PBS), 2 % Triton™ X-100, 1 U/ml fluorescent phalloidin (*see* **Notes 2**, **3**, and **4**).
7. Antibody wash: 0.1 % bovine serum albumin, 0.1 % Triton X-100 in 1× PBS.
8. Triton antibody wash: 0.1 % bovine serum albumin, 0.2 % Triton X-100 in 1× PBS.
9. Phenylenediamine solution: 50 mg phenylenediamine, 4.4 ml of dH_2O, 500 µl of 10× PBS. Vortex to dissolve. Bring to pH 9.0 with 1 N NaOH.
10. Phenylenediamine mounting medium [20]: 5 ml of glycerol, 4 ml of dH_2O, 1.0 ml of phenylenediamine solution. Aliquot rapidly in 200 µl volumes and store at −80 °C (*see* **Note 5**).
11. Embedding agarose: Prepare a low-melt agarose working solution (this can be kept for ~1 week in aliquots at 4 °C) with a 1 % (w/v) solution of low-melt agarose in Grace's Insect Culture Medium. Heat to 65 °C to liquefy. Mix molten agarose 1:1 with 42 °C 2× IVEM medium (20 % FBS and 2× penicillin/streptomycin in Grace's Insect Culture Medium). Keep this embedding agarose at 42 °C to keep it in its liquid state.

2.2 Dissection Materials

1. Gas-permeable fly pad.
2. CO_2 source.
3. Forceps (#5 Dumont).
4. Sharpened Tungsten dissection needles [21].
5. Pin vises and supplied needles.
6. Nine-well spot plates for dissection.
7. A dark surface (i.e., a piece of black plexiglass).
8. Pasteur pipets and pulled Pasteur pipets [16] (*see* **Note 6**).
9. Cover slip bottom dishes (MatTek Corporation, 35 mM, cover slip thickness will depend on the microscope objective used).
10. Glass slides.
11. Cover slips (cover slip thickness will depend on the microscope objective used).
12. Nail polish.
13. Dissecting microscope.
14. 0.5 ml microcentrifuge tubes.

15. Kimwipes.
16. Nutator.
17. Aluminum foil.

2.3 Antibodies/ Fluorophore Conjugates

1. Alexa488-, rhodamine-, or Alexa647-phalloidin.
2. DAPI.

3 Methods

3.1 Fixed Imaging

1. Collect newly enclosed adult flies of interest (males and females) (*see* **Note 7**).
2. Feed the flies with a dab of wet yeast paste daily for 3–4 days (*see* **Note 8**).
3. Warm Grace's Medium to room temperature before beginning the dissection (*see* **Note 9**).
4. Put the flies to sleep under CO_2 gas.
5. Fill a dissection well (9-well spot plate, etc.) with Grace's Medium, place on a dark surface (i.e., a piece of black plexiglass), and submerge a female fly in the dissection well.
6. Remove the whole ovaries using two pairs of forceps.
7. Use a sharpened dissection needle to separate the individual ovarioles for approximately the anterior third of the length of the ovary (separate between the clear egg chambers while leaving the white/opaque egg chambers at the posterior of the ovariole more closely packed; Fig. 3).
8. Transfer the ovaries to a 0.5 ml tube with Grace's Medium (*see* **Note 10**). Do not let the ovaries dry out.
9. Prepare enough 4 % PFA solution (20 % PFA diluted in Grace's Medium) to have 300 μl per sample.
10. Remove all the Grace's Medium with a pulled pipet and rapidly add 300 μl of a freshly diluted 4 % paraformaldehyde solution. Incubate for 10 min at room temperature on a nutator (*see* **Notes 11** and **12**).
11. Remove the fixative using a pulled pipet (*see* **Note 3**) and rinse with antibody wash.
12. Wash the ovaries 6 × 10 min with ~300 μl of antibody wash at room temperature on a nutator (*see* **Note 13**).
13. Dilute the desired primary antibody and phalloidin (1:250–1:500 dilution) in Antibody wash solution. Make enough for 300 μl per sample (*see* **Note 14**).

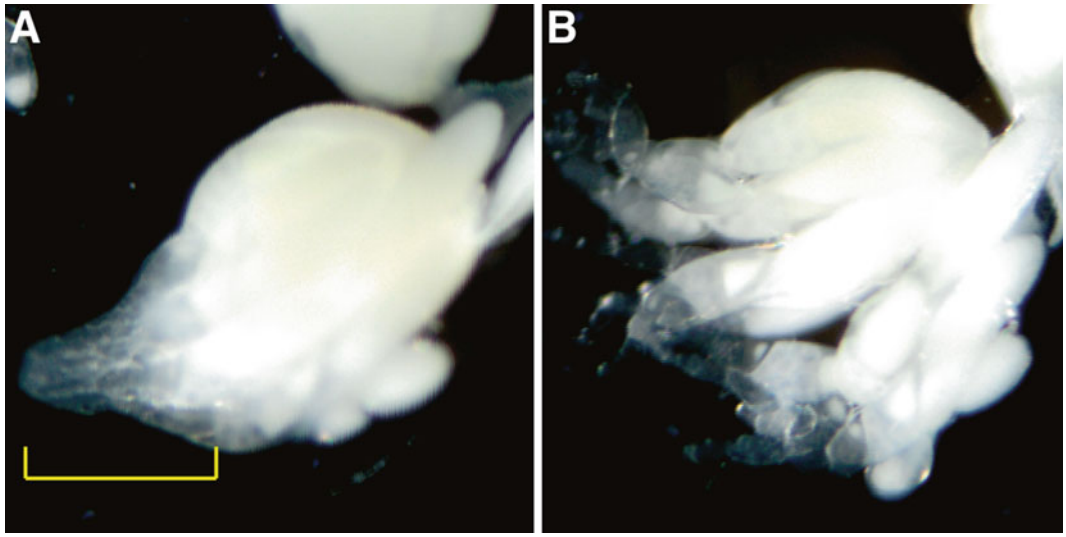

Fig. 3 Images of a dissected ovary before and after teasing the ovarioles apart. (**a**) Intact pair of ovaries. *Yellow* bracket indicates area of the ovary to be teased apart. (**b**) Pair of ovaries after sharpened dissection needles were used to separate the ovarioles over the anterior 1/3 of each ovary. Teasing the ovarioles apart prior to fixation is necessary to fully fix the sample and to allow staining reagents to evenly label the tissue

14. Completely remove the final wash (using a pulled pipet) and add 300 μl of the primary antibody/phalloidin mix to each sample. Incubate the samples at room temperature for 2–4 h or at 4 °C overnight on a nutator covered with aluminum foil (*see* **Notes 15** and **16**).
15. Wash the ovaries 6 × 10 min with ~300 μl of antibody wash at room temperature on a nutator covered with aluminum foil (*see* **Note 13**).
16. If a primary antibody was used in addition to phalloidin, incubate in the secondary antibody (make sure to select a fluorophore different than the phalloidin conjugate); otherwise proceed to **step 18**. Dilute the secondary antibody in ~300 μl of antibody wash, including phalloidin again. Completely remove the final wash (using a pulled pipet) and add ~300 μl of secondary antibody solution per sample. Incubate the samples at room temperature for 2–4 h or at 4 °C overnight on a nutator covered with aluminum foil (*see* **Note 16**).
17. Wash the ovaries 6 × 10 min with ~300 μl of antibody wash at room temperature on a nutator covered with aluminum foil (*see* **Note 13**).
18. If desired, the samples can be stained with DAPI to visualize DNA. Wash the samples at room temperature on a nutator for 10 min in 1× PBS containing DAPI at a 1:5000–1:10,000 dilution.
19. Rinse once with 1× PBS.

20. Store the ovaries in fresh 1× PBS. Samples can be stored at 4 °C or mounted immediately.
21. On a spare glass slide, place two separate drops of mounting media.
22. Pipet the mounting media onto the slide to be used for mounting.
23. Use forceps to move the stained ovaries into the first drop of mounting media on the spare slide, and then move the ovaries to the second drop. Then, place the ovaries in mounting media on a glass slide to be mounted (*see* **Note 17**).
24. Use sharpened dissection needles to separate individual ovarioles and egg chambers (*see* **Notes 5**, **18**, and **19**).
25. Apply the cover slip, invert the slide onto a Kimwipe, and apply gentle pressure to remove excess mounting media and to slightly squish the egg chambers for better imaging (*see* **Notes 20** and **21**).
26. If using a non-hardening mounting medium such as phenylenediamine, seal the cover slips using nail polish.
27. Store the slides in the dark at 4 °C (*see* **Note 22**).

3.2 Fixed Imaging for Stabilizing Actin Structure

1. Follow **steps 1–8** of Subheading 3.1.
2. Prepare enough actin fixative to have 300 μl per sample.
3. Remove the Grace's Medium completely with a pulled pipet and add 300 μl of actin fixative. Incubate for 10 min at room temperature on a nutator covered with aluminum foil (*see* **Notes 3**, **4**, and **15**).
4. Remove the fixative and rinse 2× with Triton antibody wash.
5. Wash the ovaries 3×10 min with ~300 μl of antibody wash supplemented with 1 U/ml fluorescent phalloidin at room temperature on a nutator covered with aluminum foil (*see* **Note 4**).
6. Wash the ovaries 3×10 min with ~300 μl of antibody wash at room temperature on a nutator protected covered with aluminum foil.
7. Follow **steps 13–27** of Subheading 3.1.

3.3 Short-Term Live Imaging

1. Collect and feed the flies as described in Subheading 3.1, **steps 1** and **2**.
2. Prepare a fresh aliquot of IVEM medium and warm to room temperature (*see* **Note 23**).
3. Dissect the ovaries as described in Subheading 3.1, **steps 4–6**, using IVEM medium in the dissection well instead of Grace's Medium. Move the ovaries quickly away from the debris (*see* **Note 24**).
4. Use two dissection needles to isolate individual egg chambers of the desired stage (*see* **Note 25**).

5. Pipet the egg chambers and a small drop of IVEM medium onto a cover slip bottom dish. If desired, a cover slip can be placed on top of the egg chambers to limit their movement and push the egg chambers to the bottom cover slip (*see* **Note 26**).
6. Egg chambers are now ready for imaging on an inverted confocal microscope (*see* **Note 27**).
7. Stage 10B or later egg chambers prepared in this manner will complete oocyte development. Time course imaging is feasible for several hours to capture the actin remodeling of stages 10B through 13 (Fig. 2) (*see* **Note 28**).

3.4 Long-Term Live Imaging

1. Collect and feed the flies as described in Subheading 3.1, **steps 1** and **2**.
2. Follow **step 2** of Subheading 3.3.
3. Melt the low-melt agarose working solution in a 65 °C heat block.
4. Keep the melted low-melt agarose working solution liquid (incubate in a 42 °C heat block) and warm the 2× IVEM medium in a 42 °C heat block.
5. Mix the 2× IVEM medium and the low-melt agarose working solution 1:1 to make the embedding medium and return to the 42 °C heat block.
6. Follow **steps 3** and **4** of Subheading 3.3.
7. Pipet ~200–300 μl of the embedding agarose onto the cover slip portion of a cover slip bottom dish (*see* **Note 29**).
8. Transfer isolated egg chambers to the embedding agarose using a Pasteur pipet (keep egg chambers at the end of the pipet, submerge the end into the agarose, and let the egg chambers fall into the agarose by gravity).
9. Adjust the location of the egg chambers using a dissection needle as necessary. Embedding agarose will solidify and hold the egg chambers in place.
10. Cover the solidified embedding agarose with IVEM medium.
11. Image on an inverted confocal microscope as discussed in Subheading 3.3, **steps 6** and 7.

4 Notes

1. Wet yeast paste consistency should be adjusted to be between a thick solid and a runny liquid, by either adding more ddH_2O or more active dry yeast, respectively.
2. Other fixative options: commercially available paraformaldehyde and formaldehyde (dilute to a 4 % working concentration).

3. Formaldehyde and paraformaldehyde are highly toxic. Always handle with care and the appropriate personal protective equipment. Follow all hazardous waste disposal guidelines.
4. Phalloidin is highly toxic. Always wear appropriate protective equipment when handling solutions containing phalloidin.
5. Mounting media will turn brown if left for too long at room temperature. Do not use mounting media if it has turned dark brown (light brown is fine).
6. Heat the thin portion of a 9″ Pasteur pipette over a Bunsen burner. As soon as the glass begins to soften, quickly move the pipette out of the flame and pull into a fine tube. Break off the end to produce a very thin pipet tip. Use eye protection as glass fragments may fly off.
7. The age of the females at the time of dissection can affect ovary quality and overall female fertility. Thus, it is important to be consistent with the age of dissected females. Remove all of the old adult flies from the vials 1 day before collecting flies so that all of the collected adults are approximately the same age (within ~24 h).
8. Feeding the flies with wet yeast paste daily for 3–4 days is important for the development of large ovaries and a good distribution of stages.
9. Do not use cold media or place ovaries in the cold until they are fixed. Actin filaments are disrupted by cold temperatures. Allowing the Grace's Medium to incubate at room temperature for 30 min is sufficient.
10. Some antibodies will work better if the staining is performed in 1.5 ml microcentrifuge tube. Staining conditions have to be optimized for each reagent.
11. The timing of the fixation can vary depending on the fixative and antibodies being used. For example, some antibodies work better if the fixation is 15 min with diluted 37 % formaldehyde.
12. Ovaries will not move in the tube during fixation. Ensure that all ovaries are submerged in fixative. During the washes and antibody incubation the ovaries will move freely within the microcentrifuge tube.
13. Antibody washes can be left on the ovaries for longer than 10 min if needed, with the exception of the first wash following fixation. This wash should only be 10 min so that any residual fixative is rapidly removed.
14. If the antibody is precious, smaller volumes of the primary antibody/phalloidin mix can be used. However, ovary staining will tend to be more uneven with smaller volumes.

15. Always keep solutions containing fluorophores and ovary samples stained with fluorescent reagents protected from light to avoid photobleaching the samples by covering with aluminum foil.
16. Primary and secondary antibody staining can be performed at room temperature or at 4 °C. If the antibody step will be performed at room temperature, 2–4 h is sufficient. If the step is performed at 4 °C, samples should be stained overnight (minimum of 12 h). Some antibodies work better at lower temperatures for longer periods of time. Therefore, the staining conditions must be optimized for each reagent by comparing different incubation times/temperatures for each primary antibody to determine what works best for a particular reagent.
17. It is important to completely remove all of the PBS from the ovary samples before mounting the samples on slides. This can be accomplished by moving ovaries through puddles of mounting media on an extra slide before transferring the ovaries to the mounting media on the final slide. This step is critical for obtaining the appropriate refractive index for imaging.
18. Work quickly/efficiently when mounting samples to avoid exposing the samples to light for too long. If the samples are exposed to too much light, photobleaching can occur.
19. Other options for mounting media include VectaShield and ProLong Gold.
20. The amount of pressure applied to the cover slip during mounting is important to appropriately squish the egg chambers. Too much force will result in overly flattened egg chambers that appear distorted. Too little force will result in egg chambers too thick to image across their whole depth.
21. Take care to avoid shifting the cover slip once it has been placed on the slide. Egg chambers can be stretched/distorted if the cover slip is shifted while applying pressure.
22. Fluorescent signals from slides mounted in phenylenediamine mounting medium fade after ~4–6 months of storage at 4 °C. Samples mounted in ProLong Gold and VectaShield maintain their signal for considerably longer.
23. Stage 10B or later egg chambers will develop in culture using IVEM medium. Other culture medium is required for development of egg chambers earlier than stage 10B [18].
24. It is important to move the ovaries/egg chambers away from debris from the fly dissection as quickly as possible. Exposure to the contents of a punctured digestive tract, for example, will inhibit development.
25. For stage-specific isolation of egg chambers for live imaging, the slightly larger needles supplied with the pin vises are

recommended. These needles are less likely to puncture the live egg chambers than the sharpened tungsten needles.

26. Ovaries and egg chambers dissected in Grace's Medium tend to stick to glass pipets. To avoid this, pipet IVEM medium or a 3 % bovine serum albumin (BSA) solution up and down in the pipet prior to transferring tissues. This step will prevent the tissue from sticking.
27. When performing live imaging on egg chambers, scan speed, resolution, and laser power may have to be adjusted to prevent excessive damage to the living egg chambers. If the egg chambers will be imaged repeatedly over a time course, scan speed should be increased, while resolution and laser power should be decreased, so minimal damage is done.
28. Fluorescence recovery after photobleaching (FRAP) analysis can be performed on live egg chambers expressing actin-labeling tools. 50–100 iterations of 100 % laser power are typically sufficient to bleach an area of interest. A high scan speed and lower resolution are required to capture recovery of fluorescence.
29. Work quickly once the embedding medium has been placed in the cover slip bottom dish or the agarose will solidify before the egg chambers sink to the bottom. This issue will result in an inability to get a clear image by confocal microscopy.

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