Chapter 12

Cultivation and Live Imaging of *Drosophila* Ovaries

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Abstract

Drosophila egg chamber development depends on a number of dynamic cellular processes that contribute to the final shape and function of the egg. We can gain insight into the mechanisms underlying these events by combining the power of *Drosophila* genetics and ex vivo live imaging. During developmental stages 1–8, egg chambers rotate around their anterior-posterior axes due to collective migration of the follicular epithelium. This motion is required for the proper elongation of the egg chamber. Here, we describe how to prepare stage 1–8 egg chambers for live imaging. We provide alternate protocols for the use of inverted or upright microscopes and describe ways to stabilize egg chambers to reduce drift during imaging. We discuss the advantages and limitations of these methods to assist the researcher in choosing an appropriate method based on experimental need and available resources.

Key words *Drosophila*, Egg chamber, Follicle, Live imaging, Collective cell migration, Morphogenesis

1 Introduction

The *Drosophila* egg chamber has emerged as an important model system for the study of cellular mechanisms controlling morphogenesis. The egg chamber is an ovarian structure that serves as the precursor to the fly egg. It has a core of germ cells, composed of 15 nurse cells and one oocyte, that is surrounded by a somatic epithelium of follicle cells. When an egg chamber forms it is 20 μm in diameter and spherical. As it matures, it progresses through 14 developmental stages, increases in volume almost 1000-fold, and undergoes a dramatic series of morphological changes that transform it into a highly structured, elliptical egg [1, 2].

Studies of egg chamber morphogenesis have been greatly enhanced by ex vivo live imaging [3]. Processes that occur during stages 10b–14, such as nurse cell dumping and dorsal appendage formation, have long been amenable to this approach [4–9]. There is an excellent video

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protocol currently available for working with these stages [10]. The breakthrough that allowed the live imaging of younger egg chambers came with the recent discovery that insulin needs to be added to the culture media [11]. This protocol was first used to study border cell migration at stage 9 [12, 13], but subsequently led to the discovery of two novel biological processes: oscillating contractions of the basal follicle cell surfaces, which occur during stages 9–10 [14], and egg chamber rotation, which occurs during stages 1–8 [15, 16]. The preparation of stage 1–8 egg chambers for live imaging requires particular care, as these egg chambers are small and easily damaged. This protocol will focus on these stages. Although the procedures that we present have been optimized for the study of egg chamber rotation (discussed below), they could easily be adapted for investigations of other events that occur during these stages [17, 18].

Egg chamber rotation is the result of a fascinating collective migration of the follicle cells. The follicle cell epithelium is oriented with its apical surface contacting the germ cells and its basal surface contacting the basement membrane matrix that ensheaths the egg chamber. During stages 1–8, the basal follicle cell surfaces crawl along the inside of the basement membrane, perpendicular to the egg chamber's anterior-posterior axis. This collective motion causes the entire egg chamber to rotate within its surrounding matrix, which remains largely stationary [16]. Through mechanisms that are still not well understood, rotation causes the egg chamber to elongate from a spherical to an ellipsoidal shape [19–21]. Because the important events in this system all occur near the egg chamber's outer surface, they are highly accessible for live imaging. When the basement membrane is pressed against the coverslip, the interactions between the basal follicle cell surfaces and the matrix, or between the follicle cells themselves, can be imaged at high resolution with both confocal and near-total internal reflection fluorescence (TIRF) microscopy [22, 23]. Studies of this migration are also facilitated by the powerful genetic tools of *Drosophila* and a wealth of new fluorescent markers that can be visualized in live tissue [24]. Together, these features allow for mechanistic studies of collective cell migration within the context of a living, organ-like structure [15, 16, 25, 26].

In this chapter, we describe multiple techniques that can be used to isolate and prepare stage 1–8 egg chambers for live imaging on either an inverted or upright microscope. We also describe strategies to reduce drift of the samples in the XY plane, as well as a method to correct for drift after the images have been acquired.

2 Materials

2.1 Aging Female Flies

1. Vial with fly food.
2. Yeast powder, dry active yeast ground to a fine powder in a coffee grinder.

2.2 Egg Chamber Dissection

1. Pen/Strep: penicillin G-sodium 10,000 U/ml, streptomycin sulfate 10,000 μg/ml in 0.85 % saline.
2. Acidified water: 1 μl concentrated HCl in 1 ml water.
3. Insulin: 1 mg dissolved in 100 μl acidified water.
4. Live imaging media (LI media) [11]: Schneider's S2 media, 0.6× Pen/Strep, 15% vol/vol fetal bovine serum (FBS), 0.2 mg/ml insulin (*see* **Note 1**).
5. FM4-64 dye.
6. Pyrex 9-Cavity Spot Plate.
7. Dumont forceps: #5, 0.1 × 0.06 mm tip, and #55, 0.05 × 0.02 mm tip (*see* **Note 2**).
8. Wire tool: sharpened and curved tungsten wire, original diameter 0.125 mm, inserted into a 27G½′ needle attached to a 3 ml syringe (*see* Fig. 1a, **Note 3**).
9. Eyelash tool: insert an eyelash into a slightly melted p1000 pipettor tip (*see* Fig. 1a) or attach to a toothpick with nail polish.
10. Glass Pasteur pipets, 5¾ in.
11. 5 ml pipet pump.
12. Stereomicroscope with magnification of at least 10×.

2.3 Live Imaging Setup for an Inverted Microscope

1. Aluminum slide, 76 mm × 26 mm × ~1 mm with a 12 mm diameter hole in the center surrounded by an 18 mm hole with ~0.5 mm depth on the top of the slide (*see* Fig. 1b, **Note 4**).
2. Coverslip, 50 mm × 22 mm, cleaned with ammonia-free glass cleaner and lens paper.
3. Parafilm.
4. Razor blade or needle.
5. Lumox gas permeable membrane slide, 76 mm × 26 mm, removed from a tissue culture chamber (*see* **Note 5**).

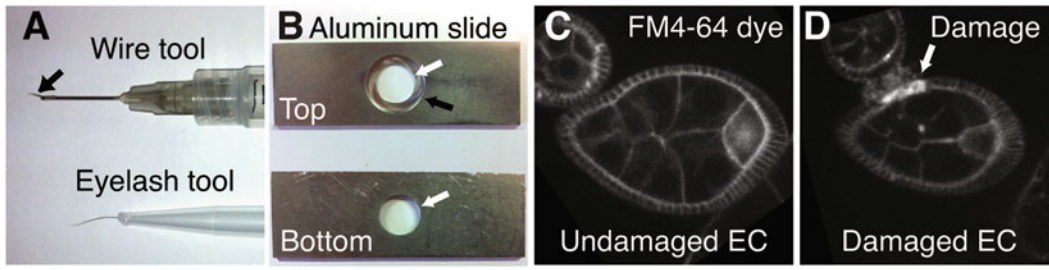

Fig. 1 Specialized tools. (**a**) The wire tool consists of a curved tungsten wire (*black arrow*) inserted into a needle attached to a syringe. The eyelash tool consists of an eyelash inserted into a pipet tip that has been partially melted. (**b**) The aluminum slide has a center hole (12 mm in diameter) that goes through the entire slide (*white arrow*). On the top of the slide, a concentric 18 mm diameter hole is cut out to a depth of ~0.5 mm (*black arrow*). (**c** and **d**) FM4-64 dye marks cell membranes and is taken up at higher levels by damaged tissue (*white arrow*)

6. Low melt agarose (LMA), 2.5% dissolved in hot water, store 1 ml aliquots at room temperature (optional).
7. Coverslip cut to approximately 4 mm × 4 mm with a diamond tip pen (optional).
8. Polystyrene beads, 20–50 μm (optional).

2.4 Live Imaging Setup for an Upright or Inverted Microscope

1. Lumox gas permeable membrane slide, 76 mm × 26 mm, removed from a tissue culture chamber (*see* **Note 5**).
2. Coverslip, 30 mm × 22 mm, cleaned with ammonia-free glass cleaner and lens paper.
3. Low melt agarose (LMA), 2.5% dissolved in hot water, store 1 ml aliquots at room temperature.
4. Vacuum grease.
5. Halocarbon oil 27.

3 Methods

3.1 Preparing Female Flies for Dissection

1. Females must be well fed with yeast for healthy egg chamber production. Incomplete nutrition will slow egg chamber production by inducing cell death in the germarium and in stage 8 egg chambers [27–29]. Sprinkle yeast powder on fly food in a vial, covering about one half of the surface. Add up to 10, 1–2 day old females and an equal number of young males to the vial.
2. Age females for 1–3 days (*see* **Note 6**). Move animals to a new vial with fresh yeast the day before dissecting.

3.2 Ovary/Ovariole Dissection

For ex vivo live imaging, it is first necessary to dissect the ovaries from the abdomen of well-fed females and then isolate the individual egg chambers from the ovary. Because this process invariably induces some tissue damage, we provide alternate techniques to avoid damaging egg chambers of particular stages (*see* **steps 5** and **6**). The entire process is documented in Video 1.

These procedures require a basic understanding of ovary structure. Here we define some key terms. Within each ovary there are 15–18 developmental arrays of egg chambers, called ovarioles. The germarium is a structure at the anterior end of each ovariole; this is the site of egg chamber production. Within the ovariole, each egg chamber is connected to its neighbors like beads on a string by thin multicellular structures called stalks. Each ovariole is then surrounded by a tubular sheath of muscle that pushes the maturing egg chambers toward the oviduct.

1. Prepare LI media and allow it to come to room temperature. Add 1 μl FM4-64 membrane dye/100 μl LI media. The dye can be used to image cell membranes, but more importantly it highlights tissue damage [12] (*see* Fig. 1c, d, **Note 7**).

2. Using a glass pipet, place 500–800 μl of LI media into a well in the spot plate. Using a black background on the stage, place the well under the stereomicroscope. At 10× magnification, focus the microscope toward the bottom of the well.
3. Anesthetize the flies using CO_2. With the #5 forceps in your nondominant hand, grab a single female from the dorsal side at the thorax. Without letting go, submerge the fly in the LI media and use the #55 forceps to grab the abdomen between the two posterior-most pigmented segments. Pull the forceps posteriorly to tear the abdomen; the ovaries should pop out of the abdomen (*see* Fig. 2a, **Note 8**). Detach the ovaries from the posterior cuticle and remove all nonovarian tissue from the well (*see* Fig. 2b). Dissect 1–3 females and collect all ovaries within the same well of the spot plate.
4. Place the tip of closed #5 forceps over the mature egg chambers at the posterior end of the ovary with your nondominant hand and gently stab through the ovary to pin it against the bottom of the well.

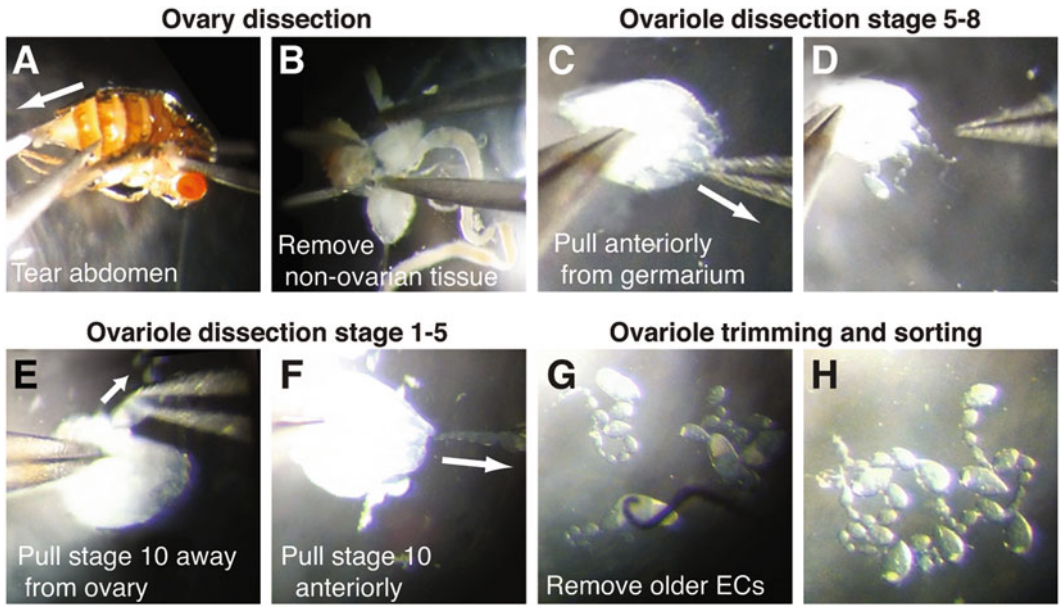

Fig. 2 Ovary/ovariole dissection. (**a**) Hold on to the dorsal side of the fly's thorax with #5 forceps (*right)*. With #55 forceps (*left*), grab between the two posterior-most pigmented segments and pull posteriorly to tear the abdomen (*white arrow*). (**b**) Separate the ovaries from the posterior cuticle and remove other tissue using forceps. (**c** and **e**) Pin the ovary against the glass well by gently stabbing with the #5 forceps in the posterior region of the ovary. (**c** and **d**) When isolating stages 5–8, grab ovarioles with #55 forceps from the anterior of the ovary near the germarium and pull anteriorly to remove them from the ovary and muscle (*white arrow*). (**e**) When imaging stages 1–5, grab near the stage 10 egg chambers with #55 forceps and pull out and away from the ovary (*white arrow*). (**f**) Then, pull the ovariole anteriorly to remove it from the muscle (*white arrow*). (**g**) Use the wire tool to remove older egg chambers by severing the stalk between two egg chambers. (**h**) Check for damage and move the ovarioles to a new well

5. To obtain stage 5–8 egg chambers, with the #55 forceps in your dominant hand, gently grab the anterior tip of the pinned ovary at or just posterior to the germaria and quickly pull anteriorly to remove single ovarioles from the ovary and their muscle sheath (*see* Fig. 2c, d). Perform this pulling motion on the same ovary until previtellogenic egg chambers are no longer visible. Continuing to pull beyond this point can induce damage, so you may only end up with a few. Repeat this process with the remaining ovaries in the well.
6. To obtain stage 1–5 egg chambers, with the #55 forceps in your dominant hand, grab single ovarioles from the region of the pinned ovary (*see* **step 4**) that contains stage 10 egg chambers. This will be approximately halfway between the anterior and posterior tips of the ovary. Pull the ovariole orthogonally away from the ovary's anterior-posterior axis, then pull anteriorly to remove the ovariole from the muscle sheath (*see* Fig. 2e, f).
7. Separate the ovarioles from the debris using an eyelash tool. Avoid ovarioles that are still in the muscle even if it does not cover the egg chamber of interest as muscle contraction will cause the ovariole to move during imaging. Remove older egg chambers with the wire tool. Place the curved wire between two egg chambers and press down to sever their connecting stalks (*see* Fig. 2g, **Note 9**). Use a sawing motion if necessary. Do not break the stalk directly adjacent to an egg chamber of interest as this process can cause damage.
8. Gather ovarioles with the eyelash tool (*see* Fig. 2h) and perform an initial check for tissue damage at 25–40× magnification. Transfer 10–15 ovarioles with a glass pipet to a new well in the spot plate with LI media.

3.3 Live Imaging Setup

In this section, we describe four options for mounting ovarioles for live imaging that are specialized for different styles of microscopy. Initially, we describe the simplest method, imaging in LI media alone using an inverted microscope. This method allows the exchange of media and the addition of pharmacological reagents. However, drift of the samples in the XY plane is common. To limit drift, a smaller coverslip can be placed on top of the ovarioles to compress them against the main coverslip. This compression is ideal for near-TIRF microscopy as it increases the surface area of the egg chamber available for imaging. Alternatively, low melt agarose (LMA) can be added to the LI media to cause it to partially solidify (LMA+). When using an upright microscope, the ovarioles can be placed between a LMA+ pad and the coverslip. The use of LMA does not allow for the exchange of media or the ability to recover the egg chambers after imaging for fixation. Although these methods do reduce XY drift, they may not eliminate it. In the final section, we describe an image-processing

method to correct for this problem. Image acquisition settings are not discussed, as they are highly specific to the microscope being used and experiment being performed.

3.3.1 Inverted Microscope Using LI Media Alone

1. Cut a piece of parafilm approximately the size of the 50 mm × 22 mm coverslip. On a flat surface, place the aluminum slide on the parafilm with the smaller hole facing down (*see* Fig. 3a). While pressing the aluminum slide down, use a razor blade or a needle to trace the hole in the aluminum slide on the parafilm (*see* Fig. 3b). Remove the parafilm circle and sandwich the parafilm between the aluminum slide and a clean coverslip (*see* Fig. 3c).

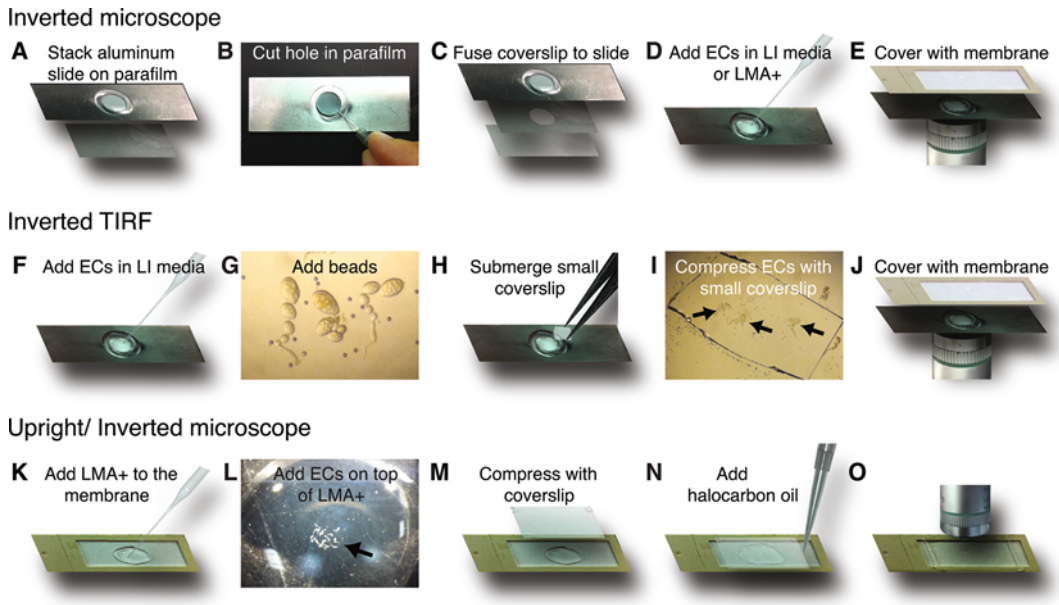

Fig. 3 Sample mounting strategies for live imaging. Preparations for an inverted microscope (**a–j**), and an upright or inverted microscope (**k–o**). (**a–c**) Preparing the aluminum slide. (**a**) Place the aluminum slide onto a piece of parafilm with the top side up. (**b**) Use a needle to trace the circumference of the inner hole on the parafilm, and remove the circular cutout. (**c**) Stack the parafilm on top of a coverslip and place the aluminum slide on top of the parafilm. Use a heat block (metal slide part down) to melt the parafilm and fuse the aluminum slide to the coverslip. (**d**) Add dissected ovarioles in LI media or LMA+ to the hole in the center of the aluminum slide. (**e**) Cover the aluminum slide with a gas permeable membrane slide to prevent evaporation. (**f**) Add ovarioles in LI media to the metal slide (prepared as described above). (**g**) Add beads to the ovarioles and LI media. (**h**) Use #5 forceps to submerge a ~4 mm × 4 mm coverslip in the LI media and gently place it on top of the beads and ovarioles. (**i**) The coverslip will compress the ovarioles (*black arrows*)**.** (**j**) Cover the aluminum slide with a gas permeable membrane slide to prevent evaporation. (**k**) Add ~100 μl LMA+ to the center of the gas permeable membrane slide and spread it out evenly before it solidifies to form the LMA+ pad. (**l**) Add dissected ovarioles to the top of the LMA+ pad; use an eyelash tool to bring the ovarioles to the center (*black arrow*). (**m**) Add vacuum grease to the corners of a coverslip, and gently lower it onto the LMA+ pad, vacuum grease side down. (**n**) Add halocarbon oil around the LMA+ pad to prevent evaporation. (**o**) Image from above (as shown) or invert the slide to image on an inverted microscope

2. Set a heat block to 70–100 °C. Place the sandwich on the heat block with the aluminum slide facing down until the coverslip is adhered to the aluminum slide (1–2 min). Gently clean coverslip with ammonia-free glass cleaner and lens paper (optional).
3. Using a glass pipet, transfer newly dissected ovarioles from Subheading 3.2 along with ~100 µl LI media into the center of the hole in the aluminum slide so that they rest on the coverslip (*see* Fig. **3**d, **Note 10**).
4. Cover the slide with the gas permeable membrane slide to prevent evaporation (*see* Fig. **3**e). The membrane should not touch the media.

3.3.2 Inverted Microscope Using Compression

1. Prepare an aluminum slide and transfer ovarioles to the slide as described in Subheading 3.3.1, **steps 1–3** (*see* Fig. **3**a–d, f).
2. Wash polystyrene beads in LI media. Add enough beads to the ovarioles and LI media on the slide so the coverslip added in the following step will lay flat against the beads, preventing the egg chambers of interest from being overcompressed (*see* Fig. **3**g).
3. Under the stereomicroscope, use forceps to submerge a ~4 mm × 4 mm coverslip in the LI media and gently place it on top of the ovarioles and beads (*see* Fig. **3**h). The egg chambers will be compressed between the two coverslips [30] (*see* Fig. **3**i, **Note 11**).
4. Cover the aluminum slide with the gas permeable membrane slide to prevent evaporation (*see* Fig. **3**j).

3.3.3 Preparing LI Media with LMA (LMA+)

1. Melt 2.5 % LMA at 65 °C.
2. Prepare LI media with FM4-64 (*see* Subheading 3.2, **step 1**) and warm to 37 °C. Add LMA to the media at a final concentration of 0.4–0.8 % to make LMA+. Place the mixture at 37 °C so it remains liquid until use.

3.3.4 Inverted Microscope Using LMA+

1. Prepare an aluminum slide as described in Subheading 3.3.1, **steps 1** and **2**.
2. Immediately following the dissection procedures in Subheading 3.2, remove as much liquid as possible from the ovarioles in the spot plate well. Using a glass pipet, add ~100 µl of liquid LMA+ to the ovarioles, and then quickly transfer the ovarioles and LMA+ to the aluminum slide (*see* Fig. **3**d).
3. Before the LMA+ solidifies, use the eyelash tool to drag the ovarioles down to the coverslip if they do not sink on their own. Allow 10 min for the LMA+ to fully solidify before imaging.
4. Cover the aluminum slide with the gas permeable membrane slide to prevent evaporation (*see* Fig. **3**e).

3.3.5 Upright or Inverted Microscope Using LMA+ Pad

1. Using a glass pipet, transfer ~100 µl of liquid LMA+ to the center of the gas permeable membrane at room temperature (*see* Fig. **3**k). Try to spread the mixture evenly before it solidifies. This will form a soft pad on which to place the ovarioles.
2. After the LMA+ solidifies, transfer the ovarioles in a minimal volume of LI media to the LMA+ pad (*see* Fig. **3**l). Remove as much liquid LI media from the pad as possible.
3. Use the eyelash tool to bring the ovarioles to the center of the LMA+ pad.
4. Place vacuum grease on the four corners of a clean 30 mm × 22 mm coverslip and gently drop it onto the LMA+ pad, vacuum grease side down (*see* Fig. **3**m). Push lightly on each corner with a pipet tip moving from corner to corner until the coverslip lays flat against the plastic frame surrounding the membrane. This will slightly compress the egg chambers (*see* **Note 12**).
5. Pipet Halocarbon oil between the coverslip and the membrane on all four sides to prevent evaporation while imaging (*see* Fig. **3**n, o).

3.4 Image Processing to Correct for Drift (See Note 13)

1. Open the image sequence as a stack in ImageJ. Duplicate the stack.
2. Apply a Gaussian blur filter to the duplicated stack so individual cells are no longer visible, Sigma (radius) of ~15–20.
3. Convert the blurred stack to a mask. Each egg chamber should be converted to a single ellipsoid shape with no holes (*see* **Note 14**).
4. Using the MultiStackReg v1.45 plugin (B.L. Busse: http://bradbusse.net/downloads.html), align the mask using translational transformation and save the transformation file.
5. On the original stack, use the same plugin but load the transformation file from the mask to align the original image sequence.

4 Notes

1. S2 media, Pen/Strep, and FBS can be combined and stored at 4 °C. We make 10 ml at a time and have used it up to 1 month later. Insulin in acidified water can be stored at 4 °C for up to a week. Insulin should be added to the S2 media/antibiotics/FBS just before use. Although the pH of the media is critical when culturing stage 9 egg chambers [11], it is less important for culturing younger egg chambers. We no longer adjust it.
2. For precise dissection with limited tissue damage, maintain the #55 forceps with great care.
3. To make the wire tool, start with a 1.5″ piece of tungsten wire. Insert the end of the wire into the needle attached to the

syringe. *Use caution while performing the following steps.* A 10 V power supply and a 1 M NaOH solution in a small beaker is required to electrolytically erode the wire. Attach a metal rod to the negative electrode of the power supply. Submerge the end of the rod in the NaOH solution. Using another alligator clip, attach the needle to the positive electrode of the power supply. Holding the syringe vertically, dip the end of the wire into the beaker for 1–2 s. Repeat this action until the wire is thinned to the desired diameter. Under a stereomicroscope, bend the thinned wire with forceps to create a curved edge or a loop. Stabilize the connection between the wire and the needle with super glue or nail polish.

4. The slide can be custom made at a machine shop. If you are unable to acquire an aluminum slide, use the setup described in Subheading 3.3.5.
5. The lumox slides have been discontinued by Grenier Bio-One but will be available from Sarstedt (94.6150.101). The slides are reusable. LI media, LMA, and halocarbon oil can be washed off. Use ethanol to remove the oil [11]. If the membrane becomes detached from the plastic slide, use nail polish to readhere it.
6. The time it takes for healthy ovaries to develop is dependent on the age and genotype of the female, and temperature. If females are too young, the ovarioles will not be fully mature, whereas females that are too old will accumulate mature egg chambers at the expense of younger egg chambers. Low temperatures will slow development and high temperatures will speed the process. If the female is of a genotype that produces round eggs, the oviduct can become blocked. Dissecting these females at earlier time points could decrease secondary defects induced in younger egg chambers from the blockage.
7. Damaged cells will take up more dye than their neighbors and will stain intensely (*see* Fig. **1**d). Briefly scan through the egg chambers before and after imaging to check for damage. Even a small amount of tissue damage can block egg chamber rotation.
8. If the ovaries do not come out of the abdomen when the posterior cuticle is removed, they can be coerced by gently squeezing the sides of the abdomen or by pulling on the ovaries directly with the forceps if they are visible. We recommend practicing dissections prior to performing live imaging experiments, as these alternate procedures can induce excessive damage.
9. When imaging, the presence of older egg chambers in the ovariole will increase the distance between the egg chambers of interest and the coverslip. Additionally, older egg chambers will deplete the media of nutrients [11], limiting the amount of time you can image.

10. When transferring the ovarioles to the aluminum slide, ensure the media either does not touch the aluminum slide or does so evenly around the circumference of the hole. If the media touches the aluminum slide unevenly, the egg chambers will drift as the media spreads along the coverslip by capillary action.
11. The size of the beads should be adjusted depending on the stage of the egg chamber you are imaging. Egg chambers that are much larger than the beads will be damaged by compression.
12. Increasing the LMA concentration of the LMA+ pad will increase egg chamber compression between the pad and the coverslip. If the concentration is too low, the ovarioles will sink into it. If it is too high, the LMA+ pad will crack when you press the coverslip against it.
13. Correcting for drift only works if the egg chambers are drifting within the XY plane. This will not correct for Z drift or egg chamber rolling.
14. If you have holes in your mask, increase the radius of the blur. If holes are present, the stack may be aligned based on the hole, not the overall shape of the egg chamber.

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Video 1 *Drosophila* ovary dissection. Video showing dissection of *Drosophila* ovaries using a stereomicroscope. Alternate dissection methods are shown for acquiring stage 6-8 or stage 1-5 egg chambers. After dissection, healthy ovarioles are sorted and older egg chambers are trimmed away. Please see Fig. 2 for stills of this video and a detailed procedural description.

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