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Dissection and Live-Imaging of the Late Embryonic Drosophila Gonad

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TITLE:

Dissection and Live-Imaging of the Late Embryonic *Drosophila* Gonad

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KEYWORDS:

Drosophila, embryo, gonad, testis, live-imaging, ex vivo, dissection, stem cell, niche development

SUMMARY:

Here, we provide a dissection protocol required to live-image the late embryonic *Drosophila* male gonad. This protocol will permit observation of dynamic cellular processes under normal conditions or after transgenic or pharmacological manipulation.

ABSTRACT:

The *Drosophila melanogaster* male embryonic gonad is an advantageous model to study various aspects of developmental biology including, but not limited to, germ cell development, piRNA biology, and niche formation. Here, we present a dissection technique to live-image the gonad ex vivo during a period when in vivo live-imaging is highly ineffective. This protocol outlines how to transfer embryos to an imaging dish, choose appropriately staged male embryos, and dissect the gonad from its surrounding tissue while still maintaining its structural integrity. Following dissection, gonads can be imaged using a confocal microscope to visualize dynamic cellular processes. The dissection procedure requires precise timing and dexterity, but we provide insight on how to prevent common mistakes and how to overcome these challenges. To our knowledge this is the first dissection protocol for the *Drosophila* embryonic gonad, and will permit live-imaging during an otherwise inaccessible window of time. This technique can be combined with pharmacological or cell-type specific transgenic manipulations to study any dynamic processes occurring within or between these cells in their natural gonadal environment.

INTRODUCTION:

The *Drosophila melanogaster* testis has served as a paradigm for our understanding of many dynamic cellular processes. Studies of this model have shed light on stem cell division regulation[1–]

[3], germ cell development[4,5], piRNA biology[6–8], and niche-stem cell signaling events[9–13]. This model is advantageous because it is genetically tractable[14,15] and is one of the few where we can live-image stem cells in their natural environment[3,16–18]. However, live-imaging of this model has been limited to adult tissue and early embryonic stages, leaving a gap in our knowledge of gonadal dynamics in the late embryo, the precise stage when the niche is first forming and beginning to function.

The late stage embryonic gonad is a sphere, consisting of somatic niche cells at the anterior, and germ cells encysted by somatic gonadal cells throughout more posterior regions[19]. This organ can be imaged live in vivo up until early embryonic Stage 17[17,20,21]. Further imaging is prevented due to initiation of large-scale muscle contractions. These contractions are so severe that they push the gonad out of the imaging frame, and such movement cannot be corrected with imaging software. Our lab is interested in unveiling the mechanisms of niche formation, which occurs during this elusive period for live-imaging. Therefore, we generated an ex vivo approach to live image the gonad starting at embryonic Stage 16, facilitating the study of the cell dynamics during this crucial period of gonad development. Previous work from our lab shows that this ex vivo imaging faithfully recapitulates in vivo gonad development[17]. This technique is the first and only of its kind for the *Drosophila* embryonic gonad.

Here, we present the dissection protocol required for ex vivo live-imaging of the gonad during late embryonic stages. This protocol can be combined with pharmacological treatments, or transgenic manipulation of specific cell lineages within the gonad. Using this technique, we have successfully imaged the steps of stem cell niche formation[17]. This imaging approach is thus instrumental for the field of stem cell biology, as it will enable visualization of the initial stages of niche formation in real time within its natural environment[15,17]. While this method is beneficial for the field of stem cell biology, it is additionally applicable for visualizing any dynamic processes occurring in the gonad during this developmental timepoint, including cellular rearrangements[22], cell adhesion[2,12,23], and cell migration[23]. This dissection protocol will thus enhance our understanding of many fundamental cell biological processes.

PROTOCOL:

1. Day-before-dissection preparation

1.1. Electrolytically sharpen a tungsten needle[24] so that the resulting diameter is approximately 0.03 mm. Adjust the voltage supplied to approximately 14 V, and use 3.3 M NaOH. Sharpening should take no more than 1 or 2 min.

CAUTION: NaOH is highly corrosive and will cause burns upon contact with skin. Wear gloves and goggles while handling, and work inside a fume hood.

NOTE: After use, store NaOH in a polypropylene tube.

1.2. Make the prepared imaging media. In a 15 mL conical tube, combine 4.25 mL of Schneider's media with 750 µL of Fetal Bovine Serum (FBS, 15% final concentration) and 27.5 µL of Penicillin-Streptomycin (0.05 U/µL of Penicillin, 0.05 µg/µL final concentration). Store this prepared imaging media at 4 °C.

1.3. Make heptane-glue solution. Add about 0.5 mL heptane to a 1 mL sealable vial stuffed with about 20 cm double-sided tape. Rock on a nutator for about an hour, or stir with a pipette tip until a consistency between that of water and glycerol is achieved. This solution of dissolved glue will last for several days before the heptane evaporates. To freshen the solution, add an additional 0.5 mL heptane, and rock.

NOTE: An effective heptane-glue solution can be prepared using various ratios of heptane to tape, as well as varying durations of rocking/stirring. The above specifications are merely suggestions for preparing or freshening one such adequate solution.

2. Embryo collection—15–17 h before dissection

2.1. Add fresh yeast paste to an apple juice agar plate[25]. Set up an embryo collection cage by adding adult flies (less than 10 days old) to an empty food bottle, perforated with small holes[26]. Cap the collection cage using the yeasted agar plate, taped to the bottle, and place the cage with the plate side down in a 25 °C dark incubator for 1 h.

NOTE: The flies must express a transgene that will fluorescently mark the gonad, for example, *six4*-GFP::Moe[20], because dissection of embryos will take place under a stereo-fluorescent microscope. See **Table of Materials** for a list of genotypes used to mark the gonad.

2.2. Remove the cage from the incubator and discard this first collection. Replace it with a freshly yeasted agar plate and place the cage back in the incubator for 2 h.

NOTE: The first embryo collection is used to clear females of fertilized, developing embryos, to achieve a tightly timed second collection of embryos.

2.3. Remove the agar plate from the cage and place it (with yeasted side facing upward) on a moist paper towel inside a sealable plastic container. Place this humid chamber in a 25 °C incubator to age the embryos for 14.5 h (final ages, 14.5–16.5 h after egg lay).

NOTE: Immediately before beginning step 2.4, complete steps 3.1 and 3.2.

2.4. Remove the agar plate from the incubator, and using a squirt bottle, add enough water to the agar plate to dissolve the yeast paste by brushing lightly with a paint brush. Rinse the embryos and dissolved yeast into a small mesh screen basket sitting inside a weigh boat. Rinse the basket with the water squirt bottle until most yeast paste has filtered through the mesh.

2.5. Remove the water from the weigh boat and place the basket back inside. Dechorionate the embryos by immersing them in a 50% bleach solution, using a squirt bottle. The bleach solution should have a depth of 3–5 mm. Keep the embryos submerged in bleach for ~2 min, with occasional swirling.

CAUTION: Bleach is corrosive and can irritate or damage eyes and the respiratory tract. Wear gloves and goggles while handling bleach.

NOTE: During this time, complete as much as possible of step 3.3. Dechorionation efficiency can be checked by placing the basket under a stereo microscope and checking for the absence of the dorsal appendages. Submerge the embryos in the bleach solution for additional time, if necessary.

2.6. Discard the bleach, and thoroughly rinse the embryos inside the basket with water from a squirt bottle (for ~3 s, blotting the mesh basket with paper towels; repeat 2–3 times).

3. Day-of-dissection preparation

3.1. Add 30 µL insulin (10 mg/mL) to 1,500 µL prepared imaging media (see section 1.2) in a 1.5 mL Eppendorf tube (0.2 mg/mL final concentration). Mix well and leave the tube on the benchtop to equilibrate to room temperature.

3.2. Prepare coverslip strips coated with glue.

3.2.1. Use a diamond-tipped knife to cut a 22 mm x 22 mm coverslip into four, equally sized strips (**Figure 1A)**.

3.2.2. Use forceps to pick up one strip and spread a total of approximately 30 µL of heptane-glue solution onto both sides of the strip (**Figure 1B**). To achieve an even layer of glue residue, tilt the strip at various angles while the heptane evaporates.

3.2.3. Store the glue-covered strip in an empty slot of a coverslip box; to maintain its stickiness, place the strip in a slanted, but upright position, leaning against the edges of the box to minimize contact with box surfaces (**Figure 1C**). Close the box so that particulates in the air do not coat the glue and lessen its stickiness.

3.3. Place the following items on the benchtop: a 6-inch glass Pasteur pipette, a microscope slide, a P200 and a P1000 pipetteman with the appropriate pipette tips, Ringer's solution[27] (pH adjusted to 7.3 with NaOH), and an uncovered, Poly-D-Lysine-coated 35 mm imaging dish.

3.4. Transfer embryos from the mesh screen basket to a small watch glass filled with 500–750 µL heptane.

3.4.1. Blot the sides and bottom of the basket dry with a tissue wipe. Moisten a paintbrush in the heptane, touch the paintbrush to the embryos (the hydrophobic vitelline membrane should adhere to the bristles), and dip the paintbrush back into the heptane in the watch glass (embryos should sink to bottom).

NOTE: The next steps must be performed as quickly as possible to prevent the embryos from drying out. Embryos should not be exposed to air for more than 20 s.

3.5. Transfer the embryos onto the microscope slide using a Pasteur pipette (**Figure 1D**). Draw embryos into the pipette slowly and limit them to the narrow portion of the pipette. Pipette embryos onto the slide slowly, such that they aggregate near the inner tip of the pipette before flowing onto the slide. Twist the corner of a tissue wipe into a fine tip, and wick away heptane from embryos on the slide. They will aggregate and cover a smaller area, making it easier to capture them on a glue-covered strip in the next step.

3.6. With forceps, pick up a glue-covered strip, and gently touch it to the embryos (**Figure 1E**). Place the strip in the imaging dish, embryo side-up, and just outside of the Poly-D-Lysine-coated center. Press the strip onto the dish using forceps, to ensure it is fixed in place. Immediately flood the dish with 2–3.5 mL Ringer's solution; submerge the embryos first to prevent them from drying out (**Figure 1F**).

4. Dissection

NOTE: These steps must be carried out under a stereo-fluorescent microscope.

4.1. Devitellinize 10–15 embryos.

NOTE: This may range from two embryos, for beginners, to fifteen embryos, for experts.

4.1.1. Select Stage 16 embryos based on gut morphology (**Figure 2**). At this stage, embryos have three gut constrictions that create four stacked gut segments (**Figure 2B,B'**). To begin devitellinization, pierce the selected embryo at one end, preferably the anterior, with the tungsten needle. The embryo may pop out of its vitelline membrane, but if not, peel the membrane off of the embryo.

NOTE: Embryos that are too young to dissect have non-regionalized guts (**Figure 2C**). Dissection of Stage 17 embryos is possible, but more challenging than dissection of Stage 16 embryos because the cuticle is beginning to develop at this stage. Early Stage 17 embryos present with four gut segments that are shifted relative to one another (**Figure 2D,D'**).

4.1.2. Hook the needle through the embryo in a region far from the gonads. Transfer the hooked embryo to the Poly-lysine-coated region of the dish (from here onwards referred to as simply "cover slip") and drag it against the bottom until it adheres. Repeat these steps, and arrange

devitellinized embryos in a row along the top of the Poly-lysine-coated cover slip (**Figure 3A**), leaving plenty of space below for further dissection.

NOTE: The gonads appear as small spherical aggregates of cells that should fluoresce brightly, depending on the specific marker used and transgene copy number. At this stage, the gonads are located laterally in segment A5, at approximately 70%–80% of embryo length. Throughout the Dissection protocol, the tissue may stick to the needle. To rid the needle of the debris, raise it just above the surface of the Ringer's solution. It is okay if devitellinization is messy, so long as the gonads are not disturbed.

4.2. Finesse the gonads out of the embryo and onto the dish (**Figure 3C,D**).

4.2.1. First, filet the embryo to expose its interior (**Figure 3C**). Slice through the embryo from its center, moving the needle posteriorly, between the gonads. Tease out some internal tissue, as this will stick to the dish much better than the external cuticle. The stickiness of the tissue will enable the next manipulations to reveal the gonad and allow it to adhere to the cover slip.

NOTE: If tissue is not sticking to the dish, try coaxing it to an uncontaminated region of the coated cover slip; the outer regions of the cover slip will be particularly sticky. As stated in step 4.1.2, it does not matter if the dissection manipulations result in a mangled embryo carcass, as long as the gonads remain unscathed.

4.2.2. Use the needle to slice around a gonad until a piece of tissue, including the gonad, is separated from the remaining carcass. With the needle, draw this tissue to a fresh region of coated cover slip, and coax it against the bottom until it adheres to the dish.

NOTE: The best imaging will be achieved for those cases where the gonad itself attaches directly to the cover slip, rather than indirect attachment via overlying tissue. Therefore, as tissue is guided away from the carcass, attempt to have the gonad touch the cover slip first, rather than extraneous tissue.

4.2.3. Remove as much surrounding tissue from the gonad as possible (**Figure 3D**) by gently dragging adherent tissue away from the gonad with the needle. Avoid touching the gonad directly, which would damage it (**Figure 4E**). To ensure the gonad is sufficiently adhered to the dish, move the needle in a gentle circular motion around the gonad—if it moves, touch the needle to the remaining adherent tissue, and direct the gonad toward a fresh region of sticky cover slip. Repeat this process until there is no detectable movement of the gonad.

4.2.4. Return to the embryo carcass and dissect out the second gonad. Repeat this process on as many embryos as possible but do NOT exceed 25 min. Tissue viability will be compromised in Ringer's solution after more than ~40–45 min.

4.3. After dissections are complete, use an indelible marker to add registration marks to the outer rim of the imaging dish to record its orientation during dissection.

4.4. Remove the glue-coated strip from the dish.

4.4.1. Gently insert the bottom prong of a pair of forceps underneath the strip, clasp and slowly tilt the strip upwards to free it from the dish with as little sloshing of the Ringer's solution as possible to minimize disturbance of the adhered gonads.

NOTE: The strip should be tilted away from the dissected-out gonads.

4.5. Gently carry the dish to the imaging microscope in a manner that avoids sloshing of the Ringer's solution.

5. Imaging

5.1. Place the imaging dish in the stage holder, using the registration marks to place the dish in the approximate orientation as during dissection. Using brightfield microscopy and a low power (~10x) objective, identify and bring into focus any piece of tissue that is adhered to the cover slip. Switch the eyepiece settings to reveal fluorescence, and using the binocular eyepieces, systematically scan the dish, marking the position of each gonad within the imaging software (see **Table of Materials**).

NOTE: Make sure gonads are centered in the field-of-view before marking positions.

5.2. Gently remove the entire stage holder assembly, with the imaging dish in its holder, and place the assembly on the work bench.

5.3. Replace the Ringer's solution with the prepared imaging media containing insulin (see sections 1.2 and 3.1).

5.3.1. Use a P1000 to remove all Ringer's solution from the inside upper ledge of the imaging dish (do not pipet Ringer's from the central cover slip region). Next, switch to a P200, and place its tip just under the surface of the remaining Ringer's in the central region. Carefully remove 50–100 µL of Ringer's; do NOT remove the entire solution.

5.3.2. Draw up ~200 µL of imaging media, and, again placing the P200 tip just under the surface of the remaining Ringer's, slowly add this imaging media. Next, add the remaining imaging media (~1,300 µL) to the dish, starting at the outermost rim of the upper ledge. While pipetting, move toward the central dome of the fluid, and eventually merge the two by brushing the pipette tip across them both. Place the lid on the dish.

NOTE: Imaging media should cover the entire inside diameter of the dish, with a depth of about 2 mm, to prevent evaporation (**Figure 4D**).

5.4. Switch the microscope to a higher power objective (63x, 1.2 NA), apply the proper immersion fluid based on the objective used (immersion fluid type and refractive index required by the objective), and then replace the stage holder assembly. Use brightfield microscopy to focus on tissue adhered to the bottom of the imaging dish.

NOTE: If the stage was not moved, the last gonad should come into view once the objective is focused.

5.5. Step through each marked gonad position and adjust that position, as necessary. Select which gonads to image based on image clarity, gonad sex, etc. Customize the imaging settings (multi-channel, exposure times, laser intensities, Z-series increments, time-lapse with appropriate intervals, etc.). Begin imaging.

NOTE: Male gonads can be identified by the presence of both a niche, and at the opposite end of the gonad, a cluster of small, highly circular somatic cells called male-specific somatic gonadal precursors (msSGPs). If the *six4*-eGFP::moesin fluorescent marker is used, the niche cells are the second-brightest cells in the gonad, the brightest being the msSGPs. At this stage, female gonads have neither a niche nor msSGPs.

REPRESENTATIVE RESULTS:

We illustrate preparation of the imaging dish in **Figure 1**, as described in "Day-Of-Dissection Preparation." These methods should ultimately result in well hydrated embryos adhered to a cover slip strip, which is temporarily fixed to the bottom of the dish and submerged in Ringer's solution (**Figure 1F**). A diamond-tipped knife allows one to cleanly slice a 22 x 22 mm cover slip into three to four smaller strips (**Figure 1A**). While handling these strips with forceps, we use a pipette to transfer enough heptane-glue to coat these strips, which gives them an adhesive, textured surface (**Figure 1B**). The coated strips are easily stored in an empty cover slip box to keep the adhesive surfaces clean for up to 2 h (**Figure 1C**). Once these coated strips are made, the goal is to adhere embryos to the strip in an aggregate, near the long edge of the strip (**Figure 1E**). It is necessary to first transfer a few embryos from the watch glass onto a clean glass slide and dry them (**Figure 1D**). Then, quickly use forceps to gently touch the coated strip to the embryos so that they adhere (**Figure 1E**). We then immediately press the strip to the bottom of the dissection dish with embryos facing up, and cover the embryos with Ringer's solution (**Figure 1F**). If this goal is met, then viewing embryos under a traditional brightfield stereomicroscope will reveal fully hydrated, healthy embryos within their vitelline membranes (**Figure 1G**). If instead, the process of transferring these embryos onto the strip and covering them with solution takes more than approximately 30 s, the embryos will dehydrate and become flaccid (**Figure 1G'**). Flaccid embryos are not healthy and are incredibly challenging to dissect, so working efficiently during this process is vital.

[Place **Figure 1** here]

Viewing an embryo under a stereo-fluorescent microscope allows clear visualization of the gut, which auto-fluoresces in the GFP channel. Gut morphology serves as a proxy for embryonic age

when choosing embryos to dissect. Because embryos adhered to the strip of cover slip will vary slightly in age, they will present a diverse array of gut morphologies (**Figure 2A**). To live-image gonad niche morphogenesis, we dissect early Stage 16 embryos. These embryos present four regionalized gut sections that are stacked in an even row (**Figure 2B', dotted lines**). Younger embryos present a sac-like, non-regionalized gut (**Figure 2C**), and do not yet have sufficient extracellular matrix (ECM) around their gonads to allow for efficient culturing of the intact organ. Older embryos that have already begun niche compaction present four gut regions that are not evenly stacked and are instead shifted relative to one another (**Figure 2D', dotted lines**). These embryos have thicker cuticle, which makes the dissection process more challenging.

[Place **Figure 2** here]

It is important to dissect embryos that express an indelible fluorescent marker in the gonad, and this marker must be visible under a stereo-fluorescent microscope. Here, we have chosen to use *six4*-eGFP::moesin[20] to mark the gonad (**Figure 2** and **Figure 3, arrows**) for the visualization during dissection.

We illustrate the gonad dissection process in **Figure 3**. The dissection of appropriately-staged embryos on the poly-lysine-coated dish allows for clean isolation of the gonads from these embryos (**Figure 3D**). Embryos that are devitellinized will adhere to the dish, and can be arranged in a convenient row prior to dissection (**Figure 3A**). Embryo devitellinization and transfer to the poly-lysine is an imprecise process such that tissue may become extruded from the confines of the embryo body (see piece of tissue between Embryo 1 and Embryo 2, and mangled Embryo 4; **Figure 3A**). This imprecision is of no importance, as long as the gonads remain unscathed. A standard stereo-fluorescent microscope enables identification of the gonad within the devitellinized embryo body (**Figure 3B, arrow**). The first few manipulations with a sharp dissection needle should separate the gonads from the embryo carcass, though some auto-fluorescent tissue will remain adhered (**Figure 3C**). Additional manipulations result in isolated gonads that adhere directly to the coated dish (**Figure 3D**).

[Place **Figure 3** here]

Once the gonads are dissected, one can replace the Ringer's solution with imaging media, and image cultured gonads directly in the same dish used for dissection. We use a spinning disk confocal microscope. Brightfield visualization of an isolated gonad on a confocal microscope reveals a dark shadow surrounding the gonad periphery (**Figure 4A–B, arrows**), which is the ECM that maintains integrity of the gonad during imaging. We present an example of a healthy, well-cultured gonad that expresses GFP-labeled F-actin in somatic gonadal cells[20] and an RFP-labeled histone marker to visualize all nuclei[28] (**Figure 4C–C'**). Because all cells in this embryo express the RFP histone marker, both gonadal cells, and cells of other adherent tissue are seen in the red channel. We discern the boundaries of the gonad using the gonad-specific GFP marker (**Figure 4C', outline**). It is clear that this cultured gonad is healthy because the gonad boundary is smooth and round (**Figure 4C', outline**), and because gonadal cells have even levels of fluorescence throughout nuclei (**Figure 4C', arrow**). If, instead, gonads are not sufficiently hydrated during

imaging, nuclei and *six4*-eGFP::moesin fluorescence can become punctate (**Figure 4D, arrow**) as the gonad tissue shrivels. Further, if the gonad ECM is damaged excessively during dissection, the gonad boundary is compromised, which is evident by the presence of gonad-specific cells (**Figure 4E, asterisks**) outside of the confines of the gonad (**Figure 4E, arrow**).

[Place **Figure 4** here]

Gonads can be cultured for about 5 h using this ex vivo imaging method, enabling image acquisition of the dynamic morphogenesis events that occur in late embryonic gonad development. In our lab, we have successfully used this protocol to image the compaction of the forming stem cell niche[17] (**Figure 5**). Prior to compaction, the niche is a loose aggregate of somatic cells at the gonad anterior (**Figure 5A, green**), surrounded by germline cells labeled with *nos*-lifeact::tdtomato[29] (see **Table of Materials**), the first tier of which will be germline stem cells (**Figure 5A, magenta**). This niche aggregate initially presents with an irregular boundary (**Figure 5A', dotted line**). Throughout the course of imaging, we observe individual niche cells rearranging their positions while the niche aggregate acquires smoother borders. These cellular rearrangements also result in a decrease in niche area (**Figure 5C**). Our observations of cell rearrangements, and neighboring germ cell divisions that occur concurrently with this phase of development informed our understanding of mechanisms underlying niche compaction[17]. This protocol, thus, affords the ability to visualize cell rearrangements, shape changes, divisions, and other cellular events with the resolution required to analyze morphogenetic events in late stage gonads.

[Place **Figure 5** here]

FIGURE AND TABLE LEGENDS:

Figure 1: Mounting and hydrating embryos prior to dissection. (**A–F**) Steps for adhering embryos to a strip of glue-coated coverslip, and securing the strip to an imaging dish. (**A**) Coverslip scored once by diamond-tipped knife (knife indicated by arrowhead) to create a strip. (**B**) Application of heptane-glue to severed coverslip strip, held with a pair of forceps. (**C**) Four glue-coated strips drying in a coverslip box. (**D–E**) Arrows point to embryos. (**D**) Dechorionated embryos that have been collected in heptane and expelled onto the edge of a microscope slide. Excess heptane was removed with a kimwipe. (**E**) Glue-coated strip with embryos attached. (**F**) The final setup of the dish immediately prior to dissection. Note the shallow layer of Ringer's solution (arrowhead) and placement of the strip (arrow) above the inner dissection circle. (**G–G'**) Embryos adhered to the strip in the dish. (**G**) Properly hydrated, turgid embryos. (**G'**) Embryos that have become dehydrated due to prolonged air exposure, evident by collapsed vitelline membranes. Asterisks indicate flaccid embryos. Scale bars are 0.5 mm.

Figure 2: Selecting appropriately aged embryos for gonad dissection. Embryos adhered to a glue-coated coverslip prior to dissection. Embryos express *six4*-eGFP::moesin (green), which marks the gonad and fat body cells. Note that the gut auto-fluoresces in green. Arrows indicate male gonads (discerned by the presence of brightly fluorescing msSGPs) that are visible in each

panel. Scale bars indicate 0.25 mm. (**A**) Embryos of various stages. (**B–D**) Embryos of distinct stages in the center of the image, oriented with anterior to the left and dorsal up. (**B**) An early Stage 16 embryo that is aged appropriately for dissection. (**B'**) The four stacked gut regions are indicated with dotted white lines. (**C**) A Stage 15 embryo that is too young to dissect (lower embryo). Note that the fluorescent gut sac just anterior to the gonad does not yet have discrete regions. (**D**) A late Stage 16 embryo that is too old to dissect. Note that the four gut regions have begun to rotate relative to one another in preparation for gut looping. (**D'**) The four gut regions are outlined.

Figure 3: The dissection process. (A–D) Embryos that express *six4*-eGFP::moesin (green) at sequential stages in the dissection protocol. (**A**) Four devitellinized embryos that have been transferred to the poly-lysine-coated dissection region of the dish. Note how the embryos are aligned in a neat row to facilitate successive downward dissections in the dish. The small piece of tissue between Embryos 1 and 2 is part of the gut from Embryo 2, extruded during hand devitellinization. (**B**) Higher magnification view of embryo from (**A**), indicated by the asterisk. (**C**) The remaining carcass of an embryo that has been filleted down the midline. The arrowhead indicates an occluded gonad, still heavily embedded in embryonic tissues. (**D**) A completely dissected gonad. Note only minimal extraneous tissue (arrowhead) remains near the gonad. Arrows point to male gonads. Scale bars are 0.25 mm.

Figure 4: Locating healthy gonads during live-imaging. (**A**) Low and (**B**) high magnification brightfield views of two dissected gonads adhered to the coverslip. (**C–C'**) One movie frame from ex vivo imaging of niche compaction in a healthy gonad. Somatic gonadal cells express *six4*-eGFP::moesin (green), and all cells express *His2Av*-mRFP (red). (**C'**) Gonad boundary marked with a white dotted line. *His2Av*-mRFP visible outside of the gonad boundary is likely fat body that is still attached to the dissected gonad. Arrow points to a germ cell nucleus with uniform *His2Av*-mRFPsignal, indicating the gonad is healthy. (**D–E**) Representative negative outcomes of the ex vivo dissection protocol. (**D**) A frame from an imaging session in which the gonad has dehydrated because of media evaporation. Note pyknotic nuclei as *His2Av*-mRFP condenses (arrow), and discontinuous spots of *six4*-eGFP::moesin along the gonad boundary. (**E**) Ex vivo imaging frame in which extracellular matrix has been compromised during dissection. Note that some germ cells (asterisks) are exiting the gonad boundary (arrow). Germ cells are labeled with *nos*-lifeact::tdtomato (magenta), and somatic gonadal cells with *six4*-eGFP::moesin (green). Scale bars show 20 µm.

Figure 5. An ex vivo cultured gonad undergoing niche compaction. Stills from an imaging series acquired promptly following dissection of gonads from early Stage 16 embryos. (**A–C**) Germ cells (magenta) are labeled by *nos*-lifeact::tdtomato and somatic gonadal cells (green) are labeled by *six4*-eGFP::moesin. The niche (dotted white line) is outlined in **A'–C'**. Scale bars show 10 µm. Anterior is to the left, and posterior is to the right. (**A**) At the first timepoint (t = 0 min) in the imaging series, niche cells have finished assembling at the gonad anterior and are at an early stage of compaction. (**B**) Midway through the imaging series and the compaction process (t = 1 h 48 min), the niche has begun to circularize, but its edge remains irregular. (**C**) Near the end of the imaging series (t = 4 h 21 min), the niche has a highly smoothened, circularized boundary.

DISCUSSION:

During gonadogenesis, the embryonic gonad, and particularly the stem cell niche within the male gonad[15], undergoes rapid morphological changes. Developmental mechanisms that underlie these dynamic changes are best understood through live-imaging techniques. However, at embryonic Stage 17, in vivo imaging of the gonad is rendered impossible by the onset of large-scale muscle contractions[17]. With this protocol, we provide a successful alternative: dissection of the gonads directly onto an imaging dish for ex vivo live-imaging. This protocol presents the only method available to accomplish live-imaging of the late stage embryonic gonad.

The critical steps of the protocol should be executed with an acute focus on dexterity and timing. Prior to dissection, rapid embryo mounting and hydration is paramount to ensure that the embryos remain healthy and turgid, which eases devitellinization and dissection. During dissection, it is vital to avoid disruption of the gonadal extracellular matrix, which is accomplished using only delicate and precise manipulations. Use of such dexterity will also ensure that the gonad is in direct contact with the imaging dish, thereby enabling clear imaging directly through the coverslip. After dissection, Ringer's solution must be switched for imaging media using only gentle suction and expulsion with a pipette to prevent gonad dislodgement. Finally, it is important to limit the overall dissection time to 25 min. This ensures that the amount of time spent in Ringer's solution during dissection, and location of the tissue at the confocal, is limited to a non-toxic exposure. With increased practice, dissection speed will improve, and personalization of the dissection sequence will occur. In our experience, sufficient dissection may be achieved after about a week of regular practice, with full mastery of the dissection attainable in about 1 month. To ease learning, we recommend practicing individual steps before executing the entire protocol.

There are a number of best practices that ameliorate the challenges associated with this protocol, starting with the genotype of the embryos used for dissection. Incorporation of multiple copies of the transgene used to mark the gonad will make the gonad brighter, and therefore easier to visualize and dissect. The dissection itself works best with a sharp needle, though it should not be overly sharpened, as it will bend against the bottom of the imaging dish and become burdened with extraneous tissue. Schneider's imaging media auto-fluoresces in the green emission spectrum, which makes it challenging to locate gonads marked with GFP once the media is added. Therefore, it is critical to use the confocal imaging software to mark the location of gonads while they are still in the Ringer's solution. If locating gonads at the confocal remains difficult, after the next dissection we suggest sketching or taking an image of the relative positioning of gonads in the dish while still at the dissection microscope. Then, mark the outside of the dish to indicate its orientation during dissection, and match this orientation when transitioning to the confocal microscope. Once located at the confocal, if a gonad appears disrupted or mangled, in the next dissection try leaving more adherent tissue surrounding the gonad. On the contrary, if a gonad is present but appears blurry throughout all planes, there is likely tissue between the coverslip and the gonad, and future dissections should strive for better isolation of the gonad from the adherent tissue. In our experience, adopting these practices has facilitated learning and execution of this protocol.

During the formation of this protocol, we identified several alterations that could be applied. For niche compaction studies, we aim to dissect embryos at Stage 16. At this stage, the embryo has not yet developed significant amounts of cuticle, and easily sticks to the poly-lysine-coated dish. However, this technique may be modified to dissect older embryos with cuticle by sticking them to the inner wall of the poly-lysine-coated region for the first incision. Once internal tissues are exposed, the embryo carcass will stick to the poly-lysine, and dissection can proceed as normal. In addition to adjustments for embryo age, we have successfully adjusted this technique to dissect multiple genotypes in the same dish. For example, homozygous mutants can be separated from sibling heterozygotes by the use of an easily discernable marker such as *deformed*-YFP on the balancer chromosome. After devitellinization, embryos should be transferred to either side of the coverslip based on the presence or absence of the marker. Dissection should proceed downwards in the dish as described in the protocol, with extra care taken to maintain segregation of different genotypes. Also, this protocol could potentially be modified to use culture medium other than Schneider's, though a superficial investigation of Shields and Sang M3 Insect Media yielded unviable gonads (data not shown). Additionally, this protocol pairs well with pharmacological manipulations by simply combining the drug with the imaging media. Over a 5 h period of imaging, re-addition of drug might be necessary to maintain an effective concentration. Finally, although this protocol was developed with the intention of visualizing niche compaction, a phenomenon specific to male gonads, in theory this protocol could also be implemented to live-image the developing ovary by simply dissecting and imaging gonads that lack a niche and msSGPs.

Limitations associated with this technique relate to the length of culturing time, and the ex vivo nature of the culture. As is the case with mid-stage *Drosophila* egg chambers[30], cultured gonads begin to die after about 5 h of ex vivo live-imaging, evidenced by loss of tissue integrity (nuclei become pyknotic and cell membranes shrivel). Thus, the window of developmental time in which this protocol is pertinent does not allow for analysis of larval stages. However, we do not rule out the possibility of ex vivo live-imaging past five hours if improved culture conditions are developed, though there may be a limit if mechanical cues from within the embryo are necessary for gonad development. Perhaps the most severe drawback of the technique is due to its inherent ex vivo nature. When cultured ex vivo, cells can have unnatural potential that is unrepresentative of in vivo biology[31]. Additionally, subtleties of culturing environments, including media content and matrix stiffness, can have drastic influence over cell behavior[32]. With this sensitivity in mind, it is vital to ensure that culturing conditions accurately reflect in vivo biology. We have taken measures to attest that in vivo niche development and signaling is recapitulated ex vivo[17]. Briefly, we ascertained that niche cell fate is maintained and niche cell count is unchanged using the markers Fasciclin-III and E-cadherin. Also, STAT signaling is present and germline stem cells divide orthogonally, suggesting that niche functionality is maintained. However, we have not verified that the relevant in vivo biology remains intact in other, non-niche tissues within the gonad. Future ex vivo investigations of these other tissues should include a comprehensive analysis to ensure that this is indeed the case.

A future direction one might take with this protocol would include incorporation of a barrier

within the imaging dish, such that one dissection session may contain both a control group as well as a drug-treatment group. This enhancement would allow for minimization of error between technical replicates, thereby improving scientific rigor.

There are no other true alternatives to this protocol, as it is the only method available to examine the live events of this organ during late embryonic development. As such, previously unanswerable questions about gonad morphogenesis are now accessible with this advancement. These questions include the intricacies of cell divisions, cytoskeletal changes, and cell intercalation events that govern stem cell niche formation and gonadogenesis. None of these events are detectable in the still images of these processes acquired using a fix-and-stain technique. Overall, this method unlocks the possibility of investigating the dynamics in the late embryonic *Drosophila* gonad, and such a breakthrough has strong implications for the advancement of a myriad of biological fields, including stem cell-niche biology, piRNA biology, and organogenesis.

ACKNOWLEDGMENTS:

We would like to thank Lindsey W. Plasschaert and Justin Sui for their substantial contributions to the early development of this protocol. The authors are grateful to the fly community for their generosity with reagents, and particularly to Ruth Lehmann and Benjamin Lin for their gift of the *nos*5'-Lifeact-tdtomato p2a tdkatushka2 Caax *nos*3' line prior to its publication. Stocks obtained from the Bloomington Drosophila Stock Center (NIH P40OD018537) were used in this study. This work was supported by NIH RO1 GM060804, R33AG04791503 and R35GM136270 (S.D) as well as training grants T32GM007229 (B.W.) and F32GM125123 (L.A.).

DISCLOSURES:

The authors have nothing to disclose.

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Figure

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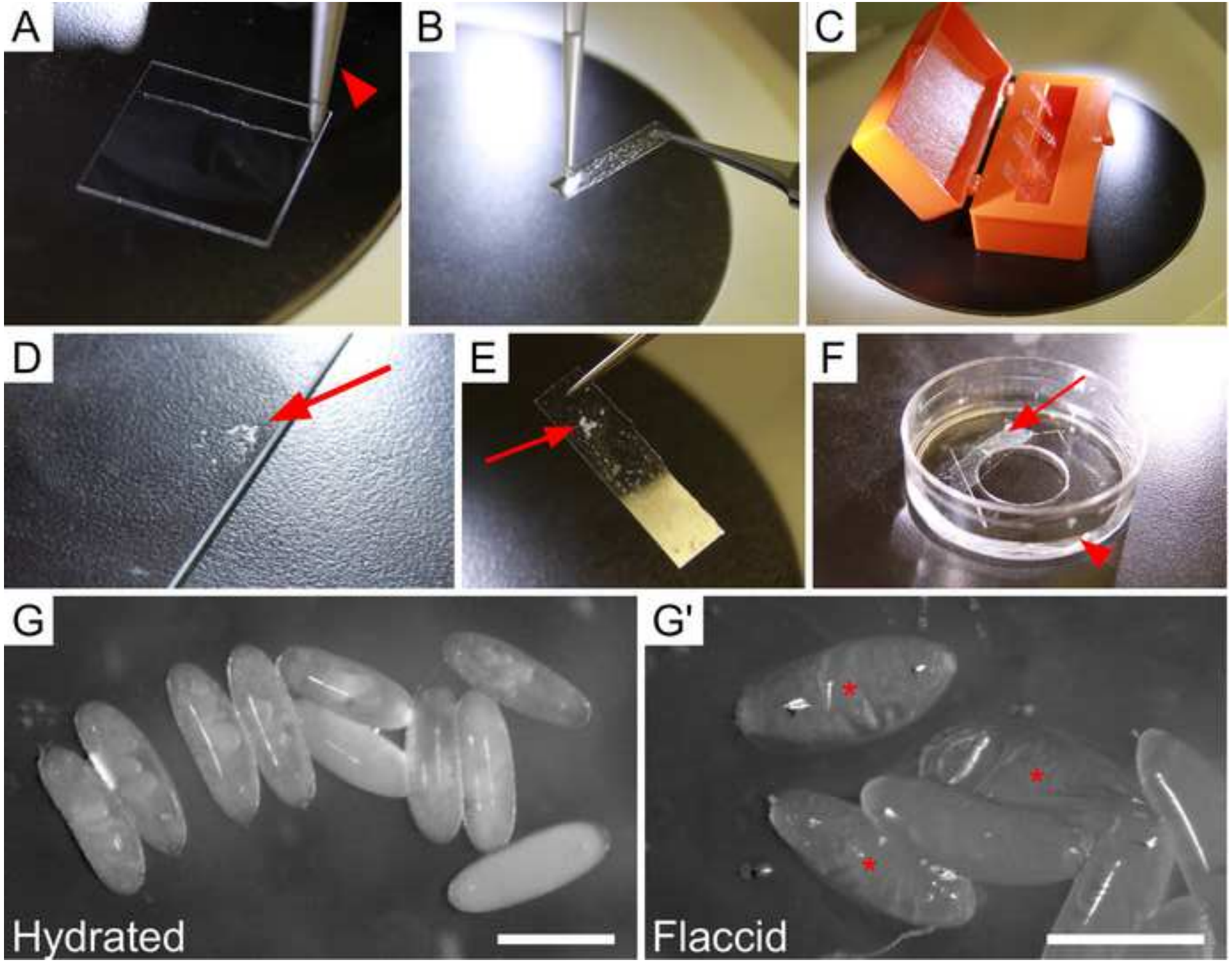
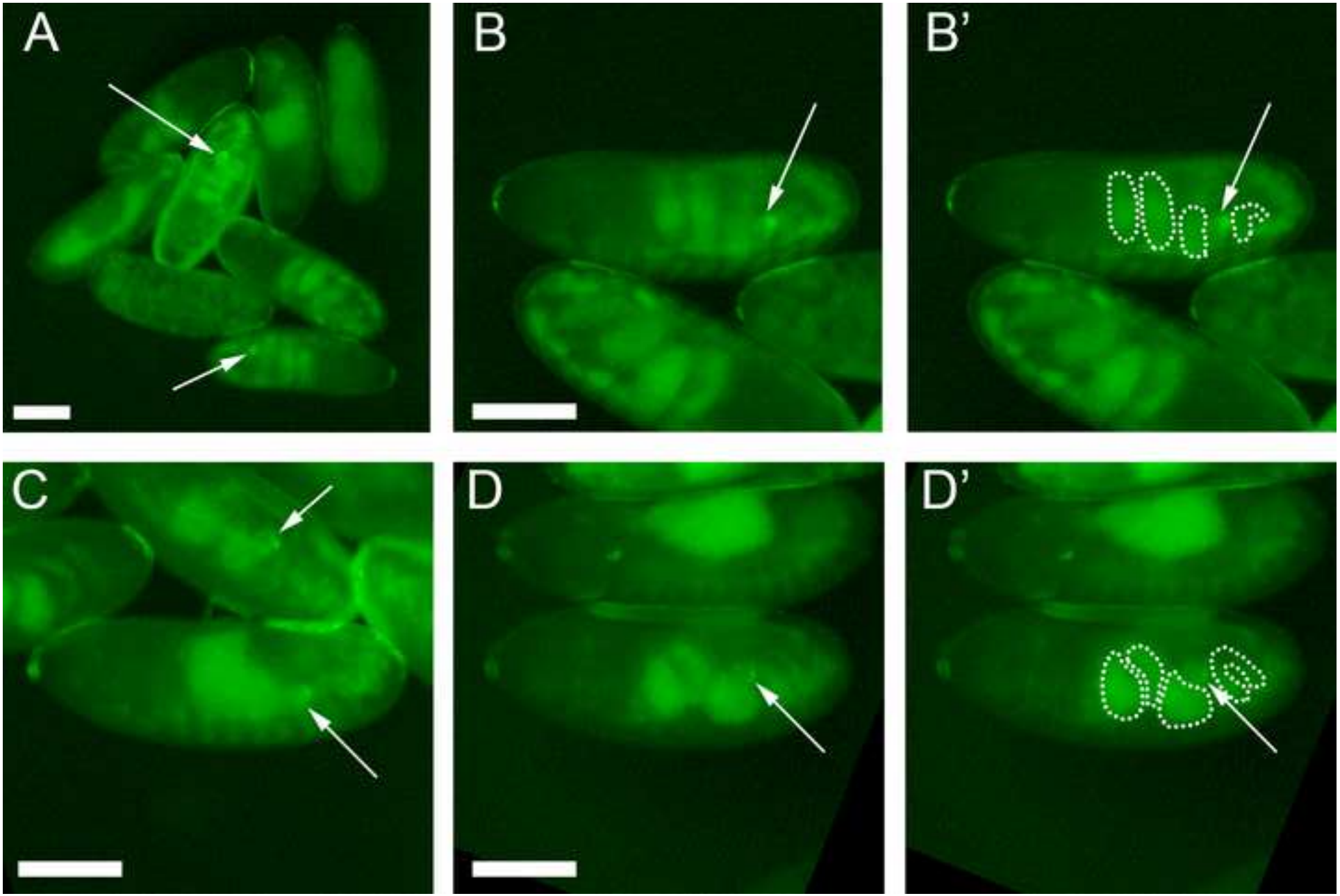


Figure



Figure

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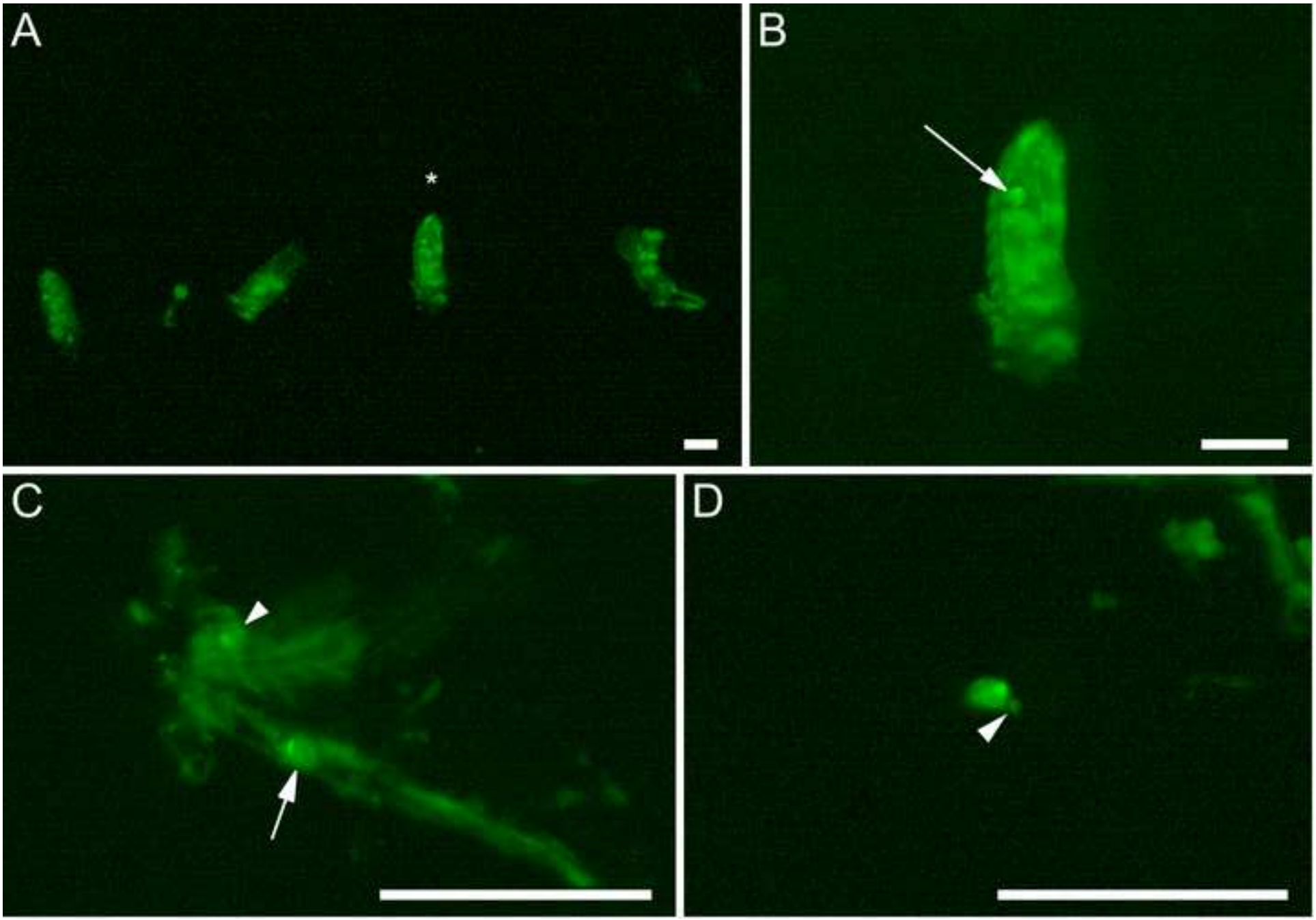
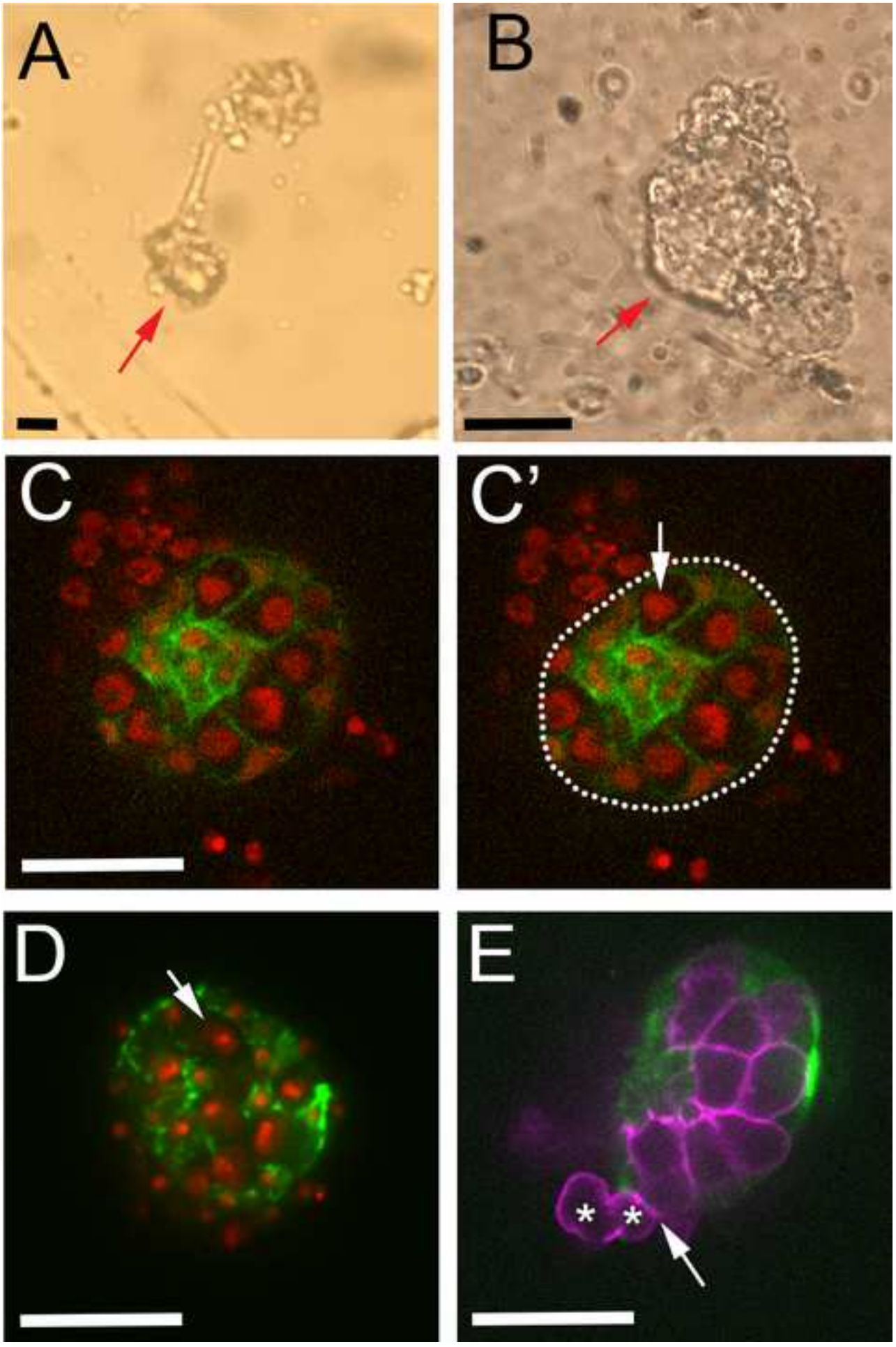


Figure



Figure

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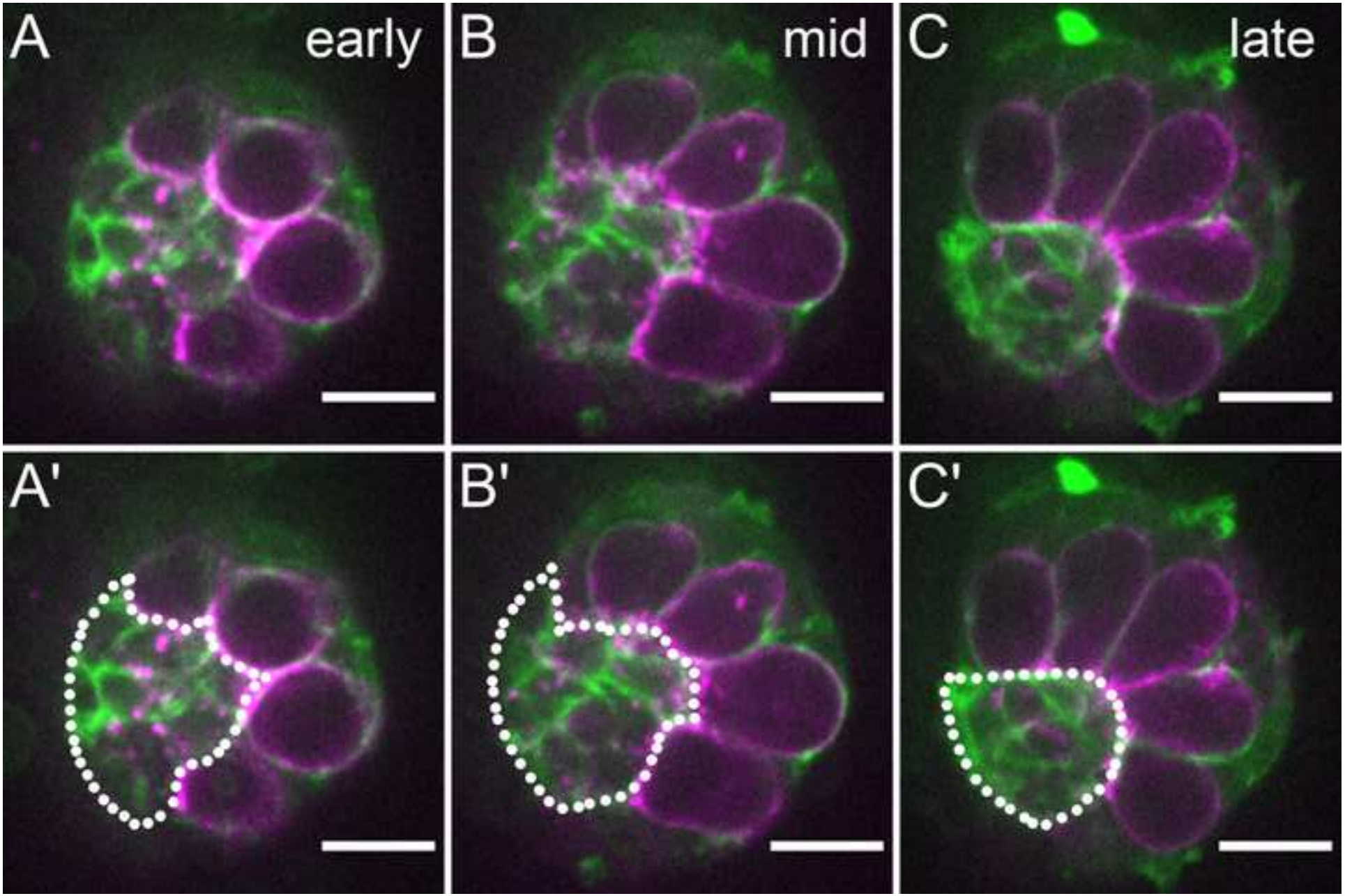

Name of Material/ Equipment	Company	Catalog Number
Alfa Aesar Tungsten wire	Fisher Scientific	AA10408G6
D. melanogaster: His2Av::mRFP1	Bloomington Drosophila Stock Center (BDSC)	FBtp0056035
D. melanogaster: nos- lifeact::tdtomato	Gift from Ruth Lehmann Lab	
D. melanogaster : P-D*six4* -eGFP::Moesin		FBtp0083398
Diamond-tipped knife		
Double-sided tape	Scotch	665
Fetal Bovine Serum	GIBCO	10082
Imaging dish	MatTek	P35GC-1.5-14-C
Imaging software	Molecular Devices	
Insulin, bovine	Sigma	I0516
Needle holder	Fisher Scientific	08-955
Nytex basket		
Penicillin/streptomycin	Corning	30-002-Cl
Ringer's solution		
Schneider's Insect Media	GIBCO	21720-024

Comments/Description
0.25mm (0.01 in.) dia., 99.95% (metals basis)
Schuh, Lehner, & Heidmann, Current Biology, 2007
Lin, Luo, & Lehmann, Nature Communications, 2020: nos5'- Lifeact-tdtomato p2a tdkatushka2 Caax nos3'
Sano et al., PLoS One, 2012
MetaMorph Microscopy Automation and Image Analysis Software v7.8.4.0
Store aliquots at 4 °C
2 mM MgCl2, 2 mM CaCl2, 130 mM NaCl, 5mM KCl, 36 mM Sucrose, 5mM Hepe's Buffer; adjusted with NaOH until pH of 7.3 is achieved

Lauren Anllo, Kara A. Nelson, Bailey N. Warder, & Stephen DiNardo
Perelman School of Medicine
The University of Pennsylvania
Department of Cell and Developmental Biology
421 Curie Boulevard
Philadelphia, PA 19104

Dr. Vineeta Bajaj
Review Editor
JOVE

September 16, 2020

Dear Dr. Bajaj:

We are happy to re-submit our manuscript "**Dissection and Live-Imaging of the Late Embryonic *Drosophila* Gonad**" to the *Journal of Visualized Experiments*.

Both Reviewers kindly acknowledged the relevance and novelty of the technique we present, and argue that our protocol is well-written, clear, and concise. The reviewers agree that we present a protocol that enables live imaging of biological processes that were previously inaccessible, and thus contributes well to the field of developmental biology. Minor suggestions were made by both reviewers. We have taken all of these on board in this re-submission.

Please find an explanation of how we incorporated all reviewer suggestions into our re-submitted manuscript in the attached point-by-point response. Our revisions include minor changes to the manuscript text, and a modification to increase clarity within Figure 3. We are pleased with the way in which all reviewer suggestions have improved the quality of the manuscript, and we are eager to share this work with the readers and viewers of *JOVE*.

Sincerely,
Lauren Anllo, Ph.D.
Kara A. Nelson
Bailey N. Warder
Stephen DiNardo, Ph.D.

Response to Reviewer #1:

Written in bold are the reviewer's comments, and beneath them are our responses. Due to our edits in the text, the line numbers have changed and we have noted the new line number for reference in parentheses after each response.

This is a well presented manuscript that provides a detailed description of a novel method to live image late male embryonic gonads from Drosophila. There has been no previous possibility of examining the cellular dynamics that occur during formation of the stem cell niche in this tissue and I believe that the described technique will be of value to developmental biologists. The adult Drosophila testis has provided general principles that govern stem cell: niche interactions and I see no reason why further such principles will not be obtained from investigation of niche formation in the embryonic gonad.

We thank the reviewer for the kind remarks, and for the helpful comments that make our manuscript stronger.

Here we address all of the concerns from reviewer 1:

1: Line 76, Please provide a little more information (or a reference) to guide readers as to the process of electrolytically sharpening a tungsten needle.

More information to guide readers through electrolytically sharpening a tungsten needle can be found in reference 24. We have moved the reference to be immediately after the statement "Electrolytically sharpen a tungsten needle" in section 1.1 of the protocol. We believe this adjustment will help clarify to the reader that this reference pertains to needle sharpening (line 76).

2: Line 86, please do not use the abbreviation "pen-strep"

We appreciate Reviewer 1 for pointing out this error, and we have changed "pen-strep" to "Penicillin-Streptomycin" in the text (lines 86-87).

3. Line 101, please provide a reference for the genotype mentioned

Reference 20 has been added to the genotype *six4*-GFP::Moe (line 108).

4. Line 136, what is the concentration of insulin?

We use an initial concentration of 10mg/mL of insulin to make a final concentration of 0.2mg/mL. This information has been added in the text (lines 145-146).

5. Line 154, pH is adjusted with NaOH (to what pH? value)

We adjust Ringers solution to a pH of 7.3. This information has been added to the text (lines 162-163) and can also be found in the table of materials.

6. Line 208, "to will" typographical error

We have made the appropriate edits by removing the word "will" (line 226).

7. Line 261, Please define "imaging media"

Here, we are referring to the Schneider's imaging media that is prepared in sections 1.2 and 3.1. To increase clarity, we refer to the Schneider's imaging media containing Fetal Bovine Serum and Penicillin-Streptomycin as "prepared imaging media" in section 1.2 (lines 85 and 88). We additionally refer to this imaging media as "prepared imaging media" in section 3.1, where we direct the reader to add insulin to the media (line 145). In section 5.3, we direct the reader to sections 1.2 and 3.1, and in the text, we also clarified that the reader should be using "prepared imaging media containing insulin" to ensure the reader uses the Schneider's imaging media prepared in these sections with Fetal Bovine Serum, Penicillin-Streptomycin, and insulin (lines 281-282).

Response to Reviewer #2:

Written in bold are the reviewer's comments, and beneath them are our responses. Due to our edits in the text, the line numbers have changed and we have noted the new line number for reference in parentheses after each response.

This manuscript describes a method for dissection and live-imaging of the gonad of late-stage Drosophila melanogaster embryos. Due to muscle movement of the embryo it is impossible to live-image the gonad in vivo at this stage (embryonic stage 17 and following). Thus, the authors present this methodology for dissecting the gonad allowing it to be imaged ex vivo. The authors outline the materials needed for the dissection technique in a clear table and add effective "Caution" and "Notes" sections, as well as bolding of some words in the text, to draw attention to important parts of the protocol. Overall, the manuscript is well-written, concise, and clearly explains the methodology and materials used. Additionally, it gives relevant background information and suggestions for applications of this technique in the discussions section. Some minor suggestions for improvement are made here, but overall, this manuscript effectively presents a relevant and novel technique to the literature.

We thank the reviewer for the kind remarks and for strengthening our manuscript through the helpful suggestions.

Here we address all of the concerns from reviewer 2:

1. In the materials table, which is very useful, please add lines delineating the different cells. This will help the reader not confuse contents - for example, what company which material is from.

We appreciate this suggestion and have made the appropriate changes to the table.

2. Bold the heading in line 74 to be consistent with the bolding of the rest of the headings.

We have made the appropriate change (line 74).

3. Specify what Voltage and Current should be used in Line 76-78 for best results. This will help readers with less experience plan their experiment more easily.

While more detail about electrolytically sharpening a needle can be found in reference 24, we have added in specifications on the Voltage we use to the text by saying "Adjust the voltage supplied to approximately 14 V…" We also specified the molarity of the NaOH solution that we use (3.3 M) in the text. Since V=IR and resistance is inherent to the circuit (remains constant), supplying the reader with either a voltage or current (either of which can be programmed on most power sources), is sufficient to achieve the relevant electrochemical properties for needle sharpening (line 77).

4. In Line 83, please specify the kind of plastic that should be used for this storage container. Polyethylene and polypropylene are the two most commonly used laboratory plastics, and readers are likely to have options to use both of these, but their chemical properties are not the same.

We have made the suggested adjustment by stating "store NaOH in a polypropylene Falcon tube." (line 83).

5. In Line 90-91, please specify the brand and catalogue number for the cellophane tape, and indicate the speed and duration of the required rocking to create the heptane glue solution such that readers can carry out this step effectively. Please also specify how much heptane should be added if needed and how to tell it is necessary to add more heptane. Like suggestion 2, this will help less experienced readers carry out this procedure.

The brand and catalogue number for the cellophane tape (we now refer to as "double-sided tape" for consistency with product description) can be found in our updated table of materials. In our experience, mixing variable amounts of double-sided tape and heptane together, with a variety of agitation methods, is all effective to create the heptane-glue. We added a note in section 1.3 to specify that making heptane-glue can be done in a variety of ways (lines 96-98). It is thus unnecessary to specify exact measurements, but as a helpful suggestion for less experience readers, we have indicated a reasonable amount of heptane required (0.5mL), as well as duration to rock on a single-speed nutator (about an hour). We also provide information to help the reader distinguish when the heptane-glue solution is ready to use by stating that it should be at a "consistency between that of water and glycerol…" Additionally, we state that the solution can be used for several days

“before the heptane evaporates.” We believe this is sufficient information for the reader to know when to add more heptane to the vial (lines 90-94).

6. In Line 120-121, specify how much of the 50% bleach solution to add to the weigh boat.
We specify the amount of bleach required by stating “the bleach solution should have a depth of 3-5mm” (lines 129-130).

7. In Line 153-154, refer to the materials table such that readers know to which pH to adjust the Ringer's solution.
We have added the required pH to the text for a more direct reference for the reader, but it can also be found in the table of materials (lines 162-163).

8. In Line 156, please specify how much heptane to add to the small watch glass. This will help readers following the procedure for the first time to add the right amount.
We have now specified to add between 500-750 uL of heptane to the watch glass in the text (lines 165-166).

9. Please place the instructions in Lines 171-173 on Line 166 immediately after the reference to Figure 1D. This will be more useful to the readers.
We thank the reviewer for this helpful suggestion, and have made the appropriate changes. We think these changes will greatly increase the clarity of the instructions for the reader (lines 176-181).

10. In Line 194, please briefly specify how to identify the gonads. This will be helpful for readers that might not have a lot of experience working with this organ.
Under section 4.1.2, we added a note to help the reader identify the gonad based on cellular fluorescence and morphology as well as position within the embryo. While gonad identification will be dependent on the transgenic fluorescent marker the reader uses, we believe this addition will be sufficient for the readers to successfully identify the gonad with an array of transgenic markers (lines 214-219).

11. In Line 208, delete the "will" in "the next manipulations to will reveal".
We appreciate Reviewer 2 pointing out this error, and have made the suggested change (line 226).

12. In Line 254, please specify the imaging software and version used, as an example of something that does work with this protocol. This will be useful even for readers who do not use that specific software, so that they know the capabilities that whatever software they do use, should have.
We have added the imaging software we use to the table of materials. We additionally refer the reader to see the table of materials when we mention “marking the position of each gonad within the imaging software” (lines 273-274).

13. Specify in Line 261 (before Line 449 where it is now) that the imaging media used is Schneider's imaging media. This will clarify for the reader which imaging media to use from the start.
In the text, we changed “imaging media” to say “prepared imaging media containing insulin” and refer the reader to sections 1.2 and 3.1 to ensure they use the Schneider’s imaging media they have prepared with Fetal Bovine Serum, Penicillin-Streptomycin, and insulin (line 281-282).

14. In Line 277, specify what the proper immersion fluid is such that inexperienced readers know which fluid to use.
The immersion fluid will depend on the objective lens that the reader decides to use. To make this clear to the reader, we have added the following clarification to the text: “apply the proper immersion fluid based on the objective used (immersion fluid type and refractive index required by the objective)” (lines 298-300).

15. In Line 334, I believe you mean that additional auto-fluorescent adherent tissue from within the embryo should be separated from the body AND from the gonads. Please rephrase this to avoid the possible interpretation that such tissue should be separated TOGETHER WITH the gonads and all moved on to the poly-L-coated region for imaging.
We actually did mean that additional auto-fluorescent tissue should be separated together with the gonad, because this is unavoidable during the dissection. We made changes to the text to increase clarity of the

sentence, by stating “The first few manipulations with a sharp dissection needle should separate the gonads from the embryo carcass, though some auto-fluorescent tissue will remain adhered” (lines 365-367). We think these changes make the sentence protocol much stronger, and thank Reviewer 2 for bringing up the previous discrepancies that could have been made.

16. In Line 355, please specify how the time limit of 5 hours was determined. Please also note if more than 5 hours might be feasible or whether you rule this out. This is also related to the comment below about Line 497.
We believe this explanation fits best in the Discussion. In our discussion, we mention that cultured gonads begin to die after about five hours of *ex vivo* live-imaging. However, we added a clause to the text stating, “we do not rule out the possibility of *ex vivo* live-imaging past five hours if improved culture conditions are developed…” We believe this addition clarifies why the 5-hour timepoint was chosen, and invites the reader to attempt optimization of culture conditions to achieve a longer imaging session. (lines 538-541)

17. In Line 389, please specify how you can tell from Figure 2 that the embryos you are examining are all male such that the gonads are all male gonads. Related, this protocol should in principle also be useful for live imaging of female gonads at this stage. Please specify for readers that may be interested in applying this technique to ovaries whether this might be feasible, If you are aware of any reason this protocol would not work for ovaries, please make that clear.
We address this comment by adding a note to section 5.5 of the protocol, stating that we discern male gonads from females due to the presence of male specific somatic gonadal precursors (msSGPs), as well as the presence of a stem cell niche at this timepoint (lines 306-310). We also added clarification into the Figure 2 legend stating that gonads were “discerned by the presence of brightly fluorescing msSGPs” (line 421). Additionally, we agree with Reviewer 2 that this protocol could in theory be used to image female gonads. We cannot think of any reasons this will not work, but have not tried such experiments ourselves. We thus added the following sentence to the discussion to invite readers who are interested in studying female gonads to try this protocol: “… in theory this protocol could also be implemented to live-image the developing ovary by simply dissecting and imaging gonads that lack a niche and msSGPs.” (lines 530-532).

18. In Line 464, please specify under what wavelengths Schneider's imaging media is autofluorescent.
We address this comment by adding “in the green emission spectrum” to the sentence stating “Schneider’s imaging media auto-fluoresces” (lines 498-499).

19. Line 491: Please indicate "data not shown" or provide the data showing the comparison of different media that is mentioned in the text.
We added “data not shown” to the text (line 526).

20. In Line 497, please specify how gonadal death was assessed. See also comment on Line 355.
To clarify how gonadal death was assessed, we added the following statement: “evidenced by loss of tissue integrity (nuclei become pyknotic and cell membranes shrivel)” (lines 536-537).

21. In Line 504, although I appreciate you have referenced your previous work (citation #17) on this point, please briefly summarize here the evidence that niche development and signaling are not disturbed by this ex vivo technique.
We made the suggested change by adding the following sentences after referencing citation 17: **“**Briefly, we ascertained that niche cell fate is maintained and niche cell count is unchanged using the markers Fasciclin-III and E-cadherin. Also, STAT signaling is present and germline stem cells divide orthogonally, suggesting that niche functionality is maintained” (lines 547-549).

22. Please refer to the explanation of the gut morphology in the instructions (not only in the representative results, where it is now) such that readers have this information while reading the procedure.
To clarify which gut morphologies indicate the appropriate embryonic stage to dissect, we added in a note under section 4.1.1. While we had previously mentioned in this section that appropriately staged embryos have three gut constrictions (lines 197-198), we believe this note further clarifies what to look for to prevent choosing improper embryonic stages (lines 203-206).

23. In Figures 2 and 3, the background levels of fluorescence are high, especially in panel A, and resolution is poor such that many images appear out of focus.
The poor resolution is a limitation of our microscope camera. However, it is an accurate representation of what the reader will see when looking through the eyepieces of a stereofluorescent dissecting microscope.
Figures 3C and 3D in particular are not helpful.
We believe figures 3C and 3D are necessary to show a typical progression through the dissection. These images are crucial for the reader to see how an embryo should look under a stereofluorescent microscope during the dissection.
In Figure 3A, what is the small green thing visible between the first and second embryos going from left to right? It is too small to be an embryo but there is no mention of it in the figure legend.
This is part of the gut from embryo 2 that has been extruded during hand devitellinization. We add in an explanation of this tissue in the Figure 3 legend so as not to distract or confuse readers (lines 434-436).
Is the rightmost thing in Figure 3A really an embryo at a comparable stage to that of the other three? Why is it curved? It looks like a larva or a piece of tissue removed from the egg.
This is an embryo that has previously been skewered onto the dissecting needle during devitellinization, and it became curved while using the adhesive poly-lysine-coated dish to remove the embryo from the needle. The process of devitellinization is an imperfect process and we address this by making the following additions in the representative results section: "Embryo devitellinization and transfer to the poly-lysine is an imprecise process such that tissue may become extruded from the confines of the embryo body (see piece of tissue between Embryo 1 and Embryo 2, and mangled Embryo 4; **Figure 3A**). This imprecision is of no importance, as long as the gonads remain unscathed" (lines 360-363). We similarly added in a note in section 4.1.2 of the protocol (where we describe how to devitellinize embryos) that "it is okay if devitellinization is messy, so long as the gonads are not disturbed" (lines 218-219). We believe these additions will make the imperfect embryos less distracting to the reader. Additionally, having such a curved embryo in our figure panel portrays an accurate representation of what one will often see during the devitellinization process, especially for beginners.
In 3C, although I am sure the authors have placed the white dotted circle over the gonad as they describe, from the reader's perspective this is not possible to see - there is nothing inside the circle that looks very different from what is outside the circle.
We appreciate Reviewer 2 pointing out this issue. We changed the dotted line to an arrowhead to make the obscured gonad more visible in Figure 3C.