Video Article

Dissection and Staining of *Drosophila* Pupal Ovaries

Karen Sophia Park[1], Dorothea Godt[2], Daniel Kalderon[1]

[1]Department of Biological Sciences, Columbia University

[2]Department of Cell and Systems Biology, University of Toronto

Correspondence to: Daniel Kalderon at ddk1@columbia.edu

URL: https://www.jove.com/video/56779
DOI: doi:10.3791/56779

Keywords: Developmental Biology, Issue 133, Developmental biology, *Drosophila*, pupal ovary, dissection, immunohistochemistry, antibody

Date Published: 3/2/2018

Citation: Park, K.S., Godt, D., Kalderon, D. Dissection and Staining of *Drosophila* Pupal Ovaries. *J. Vis. Exp.* (133), e56779, doi:10.3791/56779 (2018).

Abstract

Unlike adult *Drosophila* ovaries, pupal ovaries are relatively difficult to access and examine due to their small size, translucence, and encasing within a pupal case. The challenge of dissecting pupal ovaries also lies in their physical location within the pupa: the ovaries are surrounded by fat body cells inside the pupal abdomen, and these fat cells must be removed to allow for proper antibody staining. To overcome these challenges, this protocol utilizes customized Pasteur pipets to extract fat body cells from the pupal abdomen. Moreover, a chambered coverglass is used in place of a microcentrifuge tube during the staining process to improve visibility of the pupae. However, despite these and other advantages of the tools used in this protocol, successful execution of these techniques may still involve several days of practice due to the small size of pupal ovaries. The techniques outlined in this protocol could be applied to time course experiments in which ovaries are analyzed at various stages of pupal development.

Video Link

The video component of this article can be found at https://www.jove.com/video/56779/

Introduction

Stem cell research using *Drosophila* ovaries has widely expanded since the first documentation of a stem cell niche[1,2,3,4]. Following the development of lineage tracing genetic tools, *Drosophila* ovary dissections have been commonly used to study stem cell lineages and signaling pathways that regulate stem cell maintenance, proliferation, and fate in the stem cell niche. Knowledge of these signaling pathways may yield insights into potential causes of cancers that originate from aberrant stem cell activity[5,6,7]. It has also recently been shown that somatic stem cells in the *Drosophila* ovary, known as follicle stem cells (FSCs), strongly resemble mammalian intestinal stem cells in many aspects of their organization[8]. For this reason, *Drosophila* ovaries are a highly useful model system for studying stem cell behavior.

While larval and adult ovaries offer clues to early stem cell development and final stem cell organization in the niche, respectively, the pupal ovary is an intermediate structure in which the germline and somatic cells reorganize and establish their identities[9,10]. Though several studies have examined aspects of tissue development in the pupal ovary[10,11,12,13], questions remain regarding the differentiation and spatial organization of ovarian cell types during pupal development. In particular, the specification of FSCs occurs during this period. This protocol outlines a method for dissecting and staining pupal ovaries at desired time points—a technique that can be used in time course experiments that analyze pupal ovary development in detail from the larval to the adult stage.

To account for the small size, translucence, and inaccessibility of the pupal ovary within the pupal abdomen, this protocol utilizes tools such as a custom-made thin-tipped Pasteur pipet to remove abdominal fat body tissue obstructing antibody access to the ovaries. A clear, chambered coverglass used during the antibody staining offers greater visibility of the pupae and a gentler platform for rocking the ovaries on a "Nutator." Based on a protocol for larval ovary dissections by Maimon and Gilboa[14], a relatively high concentration of Triton X-100 has been employed in the initial steps of the staining procedure to maximize cell membrane permeabilization and antibody access to the ovarian cells.

Protocol

1. EggLaying

1. Combine approximately ten male and fifteen female adult *Drosophila* flies of the desired genotype in a vial of normal rich fly food supplemented with yeast. To avoid overcrowding the vial, allow mated females to lay eggs for no longer than 2–4 h[14].
2. Transfer the adults from the vial into a new vial by tapping the vial opening against a different vial with fly food. Allow the eggs to develop into larvae at room temperature for 3–4 days.

2. Selecting Female Larvae

1. Using a moist fine brush with soft bristles, make a rolling movement with the brush along the wall of the vial to transfer wandering third-instar larvae from the vial to a glass well filled to the brim with 1x phosphate-buffered saline (PBS). Wash the food debris off the larvae by transferring them to another well filled with 1x PBS.
2. Separate the male and female larvae using forceps. Identify the male larvae by a pair of large, round, and translucent testes embedded in fat body on the lateral side of the body approximately two-thirds down from its anterior end[15]. Female larvae are on average bigger, less translucent than males, and have much smaller gonads that are more difficult to detect.
3. Select against male larvae to collect female larvae. Collect at least ten females from the well. Place the female larvae into a separate well filled with 1x PBS.
4. Use forceps to transfer the female larvae gently into a new vial of fresh fly food supplemented with yeast.
5. Place the vial with the female larvae in a dark location to facilitate pupariation[16]. Throughout the day, monitor the larvae for pupariation. As each larva immobilizes and develops into a prepupa, circle the prepupa against the vial and record the approximate time when it first forms into a prepupa. Identify prepupae by the protrusion of anterior spiracles and timing of puparium formation[17]. Allow the prepupae to develop to the desired time point (measured in hours after puparium formation, APF).
   NOTE: Any animal that undergo puparium formation within an approximately 10 h interval can be considered a prepupa. The pupal stage begins approximately 12 h after puparium formation after an internal molt has taken place.

3. Preparing Pupae for Antibody Staining

1. **Form a thin glass pipet to be used in later steps for clearing out the pupal fat body.**
   1. Melt the glass tip of a Pasteur pipet over a Bunsen burner. As the glass melts, use forceps to pull the tip horizontally away from the rest of the pipet to form a thinner tip.
   2. After cooling, break off a small portion of the pipet tip to form a neat circular opening. Attach a bulb to the other end of the pipet.
   3. Load the pipet with 1x PBS.

2. To harvest the pupae, which are glued to the wall of the vial, apply a small drop of water along the contact zone between pupa and vial. Wait 1–2 min to let the protein glue dissolve before gently lifting the pupa off the wall with a moist fine brush. Transfer the pupa to a glass well filled with 1x PBS. Dedicate a single well per pupa to avoid overcrowding the well.
3. While grasping the posterior end of the pupa with one pair of forceps, carefully tear the anterior portion of the pupal case with another pair until the head of the pupa is visible.
4. Grip the anterior-most tip of the pupal head with forceps and gently pull the pupa out of its pupal case.
   NOTE: A portion of the fat body may spill out during this process.
5. Separate and discard the anterior half of the pupa from the posterior half until only the abdominal sack remains.
6. **Extract the fat body cells from the abdominal sack.**
   1. Grasp the abdominal sack against the bottom of the well with forceps in one hand while holding the thin glass pipet filled with 1x PBS in another hand.
   2. Aim the thin pipet tip towards the opening of the sack and slowly pipet 1x PBS into the abdomen to wash away the fat body cells surrounding the pupal ovaries.
      NOTE: The ovaries are a pair of small, translucent, striated, and oblong structures that should remain inside the abdominal sack.
   3. Wash away the fat body cells until at least two-thirds of the fat body is gone or until the ovaries are visible near the opening of the abdominal sack. It is very important to execute this step slowly so as not to wash the ovaries out of the abdomen by accident.

7. Examine the well to check if the ovaries have spilled out during the fat body cell wash. If the ovaries are not visible inside the well, they should have remained in the abdomen. If the ovaries have come out, place a new pupa in the well and repeat this step.
8. Transfer the abdomen into a clear coverglass chamber filled with fixation buffer (1x PBS, 4% paraformaldehyde) and place the lid on top. To ensure a sufficient number of ovaries withstand the staining and mounting process, dissect more ovaries than needed.
   Caution: The fixation buffer contains paraformaldehyde which is toxic. Please wear appropriate protections such as gloves, safety glasses, *etc*.

4. Immunohistochemistry

1. Throughout the staining process, use forceps to gently push down any floating ovaries to the bottom of the cover glass well to ensure that the ovaries are completely immersed in antibody solution.
2. Place strips of double-sided tape onto the flat surface of a Nutator and then place the chambered coverglass on the adhesive surface.
3. Incubate the ovaries in fixation buffer from step 3.7 for 15 min at room temperature.
4. Rinse the ovaries three times in 1x PBS with 1% Triton X-100 for 5, 10, and 45 min (1 h in total), respectively, to allow thorough permeabilization.
5. Block the ovaries in 10% normal goat serum in 1x PBS with 0.5% Triton X-100 for 30 min.
6. Incubate the ovaries overnight in 600 µL of primary antibody of choice diluted to the appropriate concentration in 1x PBS with 0.5% Triton X-100 at 4 °C.
7. Rinse the ovaries three times for 5 min in 1x PBS with 0.5% Triton X-100.
8. Incubate the ovaries for 2 h in 600 µL of secondary antibody of choice diluted to the appropriate concentration in 1x PBS with 0.5% Triton X-100 at room temperature.
9. Rinse the ovaries twice for 5 min in 1x PBS with 0.5% Triton X-100 and once for 5 min in 1x PBS.

5. Dissecting and Mounting Pupal Ovaries

1. Place a small drop of 1x PBS onto a microscope slide. Use forceps to transfer the abdominal sack from the chambered coverglass to a glass well filled with 1x PBS. Dedicate a single well per pupa to avoid overcrowding the well.
2. Tear apart the abdominal sack and any remaining fat body with forceps.
   NOTE: The ovaries, which are a pair of small, translucent, striated, and oblong structures, should be visible inside the well. The ovaries are often tightly surrounded by the fat body, so it is important to make sure all fat body is thoroughly torn apart.
3. Transfer the ovaries from the well into the drop of 1x PBS on the microscope slide by grasping the center of the ovaries between the tips of the forceps. It is very important to use a firm grip without squeezing the ovaries so as not to lose or destroy them during the transfer. Add a few drops of 1x PBS to the slide when the solution dries out over time.
4. Once all ovaries have been dissected and transferred to the microscope slide, pipet 40 µL of mounting medium onto a 22 mm x 22 mm coverslip and place the coverslip gently on top of the ovaries. Let the slide dry overnight prior to imaging.

Representative Results

Successful execution of this procedure should result in clear antibody staining that reveals the structure and cellular organization of a *Drosophila* pupal ovary. Immunohistochemistry outlined in this protocol can be used to identify cell types commonly stained in larval and adult ovaries. Cells of the pupal stalk derived from swarm cells[18] (outlined by Fasciclin III in white) are shown in **Figure 3**. In addition to highlighting the cellular organization of pupal ovaries, antibody staining specific to cell proliferation (such as phospho-histone H3 staining in **Figure 3**, shown in green) can be used to study cell division patterns of stem cells and other mitotically active cell types. If fluorescent antibody signals are weak when examined under a confocal microscope, it is likely that the ovaries were not sufficiently exposed to the antibodies due to insufficient fat body extraction in the pupal abdomen. Another possibility may be that the pupal sack opening collapsed during the staining.

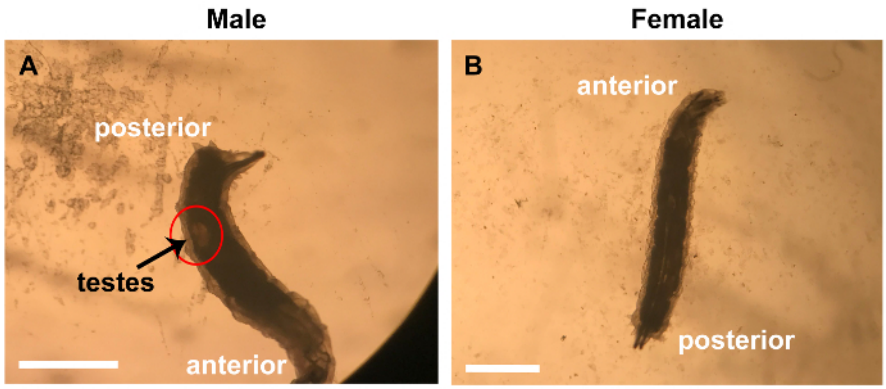

Figure 1: Side-by-side comparison of male vs. female larvae. (A) Male larva in PBS solution identified by a pair of translucent, oblong testes located approximately two-thirds down from its anterior end. **(B)** Female larva in PBS solution identified by the absence of large, translucent testes. Scale bars = 2mm. Please click here to view a larger version of this figure.

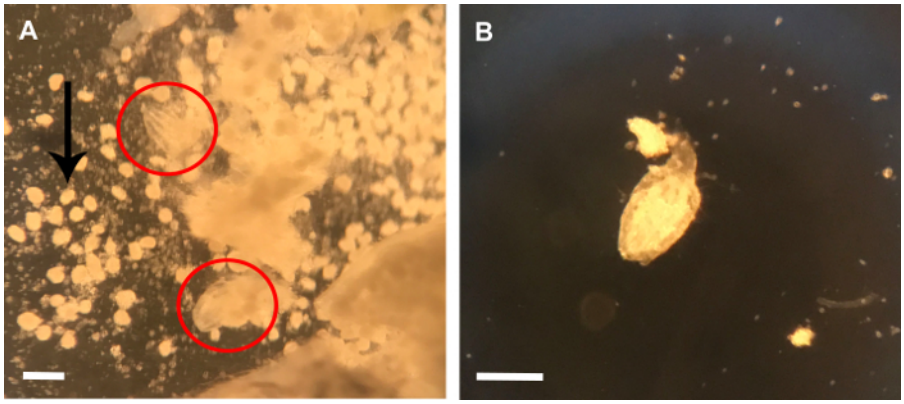

Figure 2: Dissection of pupal ovaries after immunohistochemistry. (A) Image of a pair of striated, translucent pupal ovaries (red circles) that have been removed from the abdominal sack upon immunohistochemistry staining. Pupal ovaries were dissected approximately 48 h APF. The remaining fat body cells (black arrow) that were not extracted in step 3.6 are dispersed in PBS solution. **(B)** Image of a single pupal ovary in PBS solution dissected approximately 48 h APF. Scale bars = 100 µm. Please click here to view a larger version of this figure.

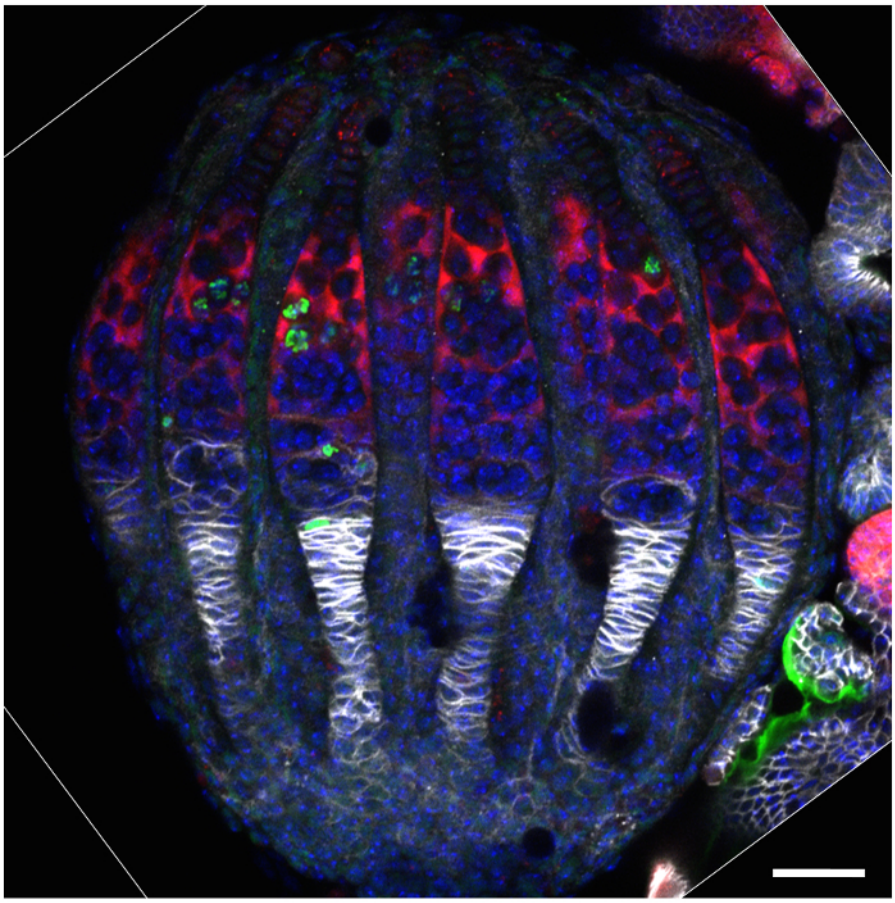

Figure 3: Stained wild-type pupal ovary dissected 48 hours APF. Image was acquired using a confocal microscope. The Wnt pathway reporter, Fz3 RFP[8] (red), is expressed in anterior somatic cells. Antibodies directed to Fasciclin III (white) outlines cells of the pupal stalk. Nuclei were counterstained with DAPI (blue). Phospho-histone H3 staining (green) highlights cells undergoing mitosis. Diagonal white lines indicate the edges of the original image. Scale bar = 20 µm. Please click here to view a larger version of this figure.

Discussion

The most critical and difficult step of this protocol involves the preparation of pupal ovaries prior to fixation. To ensure that the ovaries, small and buried by fat body cells inside the pupal abdomen, are stained sufficiently with antibodies, it is important to not only tear a large opening in the abdominal sack with forceps, but also extract the fat body cells that obstruct the ovaries from the antibodies. Successful execution of this step requires application of subtle pressure on the Pasteur pipet bulb while washing the fat body cells out of the abdominal sack (*e.g.* 1–2 fat body cells should leave the abdomen per second during this step). Failure to use gentle force will likely cause the ovaries to spill out of the abdomen and into the glass well. Because the ovaries by themselves are small, translucent, and difficult to grasp with forceps, they must remain within the abdominal sack throughout the entire staining procedure. Thus, if the ovaries come out of the abdomen during the preparation step prior to fixation, it would be extremely difficult to retrieve, stain, and mount them in their isolated form during the protocol.

Fat body cells, which primarily consist of lipid droplets, are triglyceride storage organelles commonly found in insects, including *Drosophila melanogaster*[19,20]. Since some fat body cells remain in the pupal abdomen at fixation, this protocol utilizes relatively high Triton X-100 concentrations in the first three PBS rinses to extract as many lipids as possible from both the cell membranes in the ovary and any leftover fatty tissue surrounding the ovaries. As demonstrated by the bright Fasciclin III cell membrane protein antibody staining in **Figure 3**, using 1x PBS with 1% Triton X-100 during the first three rinses works well to maximally extract lipids while still maintaining the integrity of the cell membrane.

Once the fat body has been extracted, the second most critical step is to make sure the ovaries remain within the abdominal sack throughout the fixation and antibody staining. It is helpful to place them inside a clear, chambered coverglass rather than a microcentrifuge tube for clearer visibility of the pupal abdomens during the staining. Use forceps to gently push the abdominal sacks to the bottom of the chamber so that the abdominal tissues in the sack are fully engulfed in antibody solution.

Finally, great care should be taken to transfer the ovaries from the abdominal sack to the microscope slide during the mounting process. Though the ovaries are small and difficult to grasp with forceps without pinching them, the best way to transfer them to the slide is by firmly grasping the center of the ovaries. They will remain structurally intact once placed on the slide. It is possible to dissect the ovaries from the abdomen directly on top of the microscope slide to avoid losing the ovary, but this may lead to an excessive amount of mounting medium on the slide and could complicate the mounting process.

The limitations of this protocol involve the amount of time the entire dissection process may take, the potentially low yield of well-stained ovaries, and the dexterity needed to successfully complete each step. Because the pupal ovaries require extensive fat body extraction, preparing a single pupa for fixation may take anywhere from 10 to 20 min. Moreover, to ensure a sufficient number of ovaries withstand the entire staining procedure, it is best to dissect more pupae than needed for the experiment. This means that a large amount of time may be spent simply on preparing the pupae even before the fixation step.

Pupal ovary dissections differ largely from protocols for larval[14] and adult[21] ovary dissections. The encasement of the ovary within a pupal case presents unique challenges that are met by the tools used in this protocol. These methods may be applied to lineage tracing experiments to determine when FSCs and Escort cells are specified in the pupal ovary over time. A modified version of this protocol involving insect cell culture media could also potentially be used to dissect pupal ovaries for *ex vivo* live imaging analysis.

Disclosures

The authors have no conflicts of interest to declare.

Acknowledgements

This research was supported by the National Institutes of Health (RO1 GM079351 to D.K.). We thank Dorothea Godt for her helpful advice on pupal ovary dissections based on her original protocol. We also thank Amy Reilein for her assistance and comments on the manuscript.

References

1. Losick, V., Morris, L., Fox, D., Spradling, A. *Drosophila* Stem Cell Niches: A Decade of Discovery Suggests a United View of Stem Cell Regulation. *Dev Cell.* **21** (1), 159-171 (2011).
2. Kirilly, D., Xie, T. The *Drosophila* ovary: an active stem cell community. *Cell Res.* **17** (1), 15-25 (2007).
3. Dansereau, D.A., Lasko, P. The Development of Germline Stem Cells in *Drosophila*. *Method Mol Biol.* **450**, 3-26 (2008).
4. Pearson, J., López-Onieva, L., Rojas-Ríos, P., Gonzáles-Reyes, A. Recent advances in *Drosophila* stem cell biology. *Int J Dev Biol.* **53**, 1329-1339 (2009).
5. Arwert, E.N., Hoste, E., Watt, F.M. Epithelial stem cells, wound healing and cancer. *Nat Rev Cancer.* **12** (3), 170-180 (2012).
6. Barker, N. et al. Crypt stem cells as the cells-of-origin of intestinal cancer. *Nature.* **457** (7229), 608-611 (2009).
7. Daley, G.Q. Chronic myeloid leukemia: proving ground for cancer stem cells. *Cell.* **119** (3), 314-316 (2004).
8. Reilein, A. et al. Alternative direct stem cell derivatives defined by stem cell location and graded Wnt signaling. *Nat Cell Biol.* **19** (5), 433-444 (2017).
9. Eliazer, S., Buszczack, M. Finding a niche: studies from the *Drosophila* ovary. *Stem Cell Res Ther.* **2** (6), 45 (2011).
10. Godt, D., Laski, F.A., Mechanisms of cell rearrangement and cell recruitment in *Drosophila* ovary morphogenesis and the requirement of *bric à brac*. *Development.* **121** (1), 173-187 (1995).
11. King, R.C., Aggarwal, S.K., Aggarwal, U. The development of the female *Drosophila* reproductive system. *J Morphol.* **124** (2), 143-166 (1968).
12. Vlachos, S., Jangam, S., Conder, R., Chou, M., Nystul, T., Harden, N. A Pak-regulated cell intercalation event leading to a novel radial cell polarity is involved in positioning of the follicle stem cell niche in the *Drosophila* ovary. *Development.* **142** (1), 82-91 (2015).
13. Irizarry, J., Stathopoulos, A. FGF signaling supports *Drosophila* fertility by regulating development of ovarian muscle tissues. *Dev Biol.* **404** (1), 1-13 (2015).
14. Maimon, I., Gilboa, L. Dissection and Staining of *Drosophila* Larval Ovaries. *J Vis Exp.* (51), e2537 (2011).
15. Kerkis, J. The Growth of the Gonads in *DROSOPHILA MELANOGASTER*. *Genetics.* **16** (3), 212-224 (1931).
16. Yamanaka, N. et al. Neuroendocrine Control of *Drosophila* Larval Light Preference. *Science.* **341** (6150), 1113-1116 (2013).
17. Bainbridge, S., Bownes, M. Staging the metamorphosis of *Drosophila melanogaster*. *Development.* **66**, 57-80 (1981).
18. Courdec, J. et al. The *bric à brac* locus consists of two paralagous genes encoding BTB/POZ domain proteins and acts as a homeotic and morphogenetic regulator of imaginal development in *Drosophila*. *Development.* **129**, 2419-2433 (2002).
19. Arrese, E.L., Soulages, J.L. INSECT FAT BODY: ENERGY, METABOLISM, AND REGULATION. *Annu Rev Entomol.* **55**, 207-225 (2011).
20. Zhang, Y., Xi, Y. Fat Body Development and its Function in Energy Storage and Nutrient Sensing in *Drosophila melanogaster*. *J Tissue Sci Eng.* **6** (1) (2014).
21. Wong, L.C., Schedl, P. Dissection of Drosophila Ovaries. *J Vis Exp.* (1), e52 (2006).

