# Journal of Visualized Experiments

## Planarian ovary dissection for ultrastructural analysis and antibody staining

--Manuscript Draft--

| | |
|---|---|
| **Article Type:** | Invited Results Article - JoVE Produced Video |
| **Manuscript Number:** | JoVE62713R1 |
| **Full Title:** | Planarian ovary dissection for ultrastructural analysis and antibody staining |
| **Corresponding Author:** | Longhua Guo<br><br>UNITED STATES |
| **Corresponding Author's Institution:** | |
| **Corresponding Author E-Mail:** | longhuaguo@mednet.ucla.edu |
| **Order of Authors:** | Fengli Guo |
| | Melainia McClain |
| | Xia Zhao |
| | Kexi Yi |
| | Tari Parmely |
| | Jay Unruh |
| | Brian Slaughter |
| | Leonid Kruglyak |
| | Longhua Guo |
| | Alejandro Sánchez Alvarado |
| **Additional Information:** | |
| **Question** | **Response** |
| Please specify the section of the submitted manuscript. | Biology |
| Please indicate whether this article will be Standard Access or Open Access. | Standard Access ($1400) |
| Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations. | Kansas City, MO, USA |
| Please confirm that you have read and agree to the terms and conditions of the author license agreement that applies below: | I agree to the Author License Agreement |
| Please provide any comments to the journal here. | |

**TITLE:**

# Planarian Ovary Dissection for Ultrastructural Analysis and Antibody Staining

**AUTHORS AND AFFILIATIONS:**

Fengli Guo[1], Melainia McClain[1], Xia Zhao[1], Kexi Yi[1], Tari Parmely[1], Jay Unruh[1], Brian Slaughter[1], Leonid Kruglyak[2,3,*], Longhua Guo[2,3,*], Alejandro Sánchez Alvarado[1,2,*]

[1] Stowers Institute for Medical Research, Kansas City, USA
[2] Howard Hughes Medical Institute, USA
[3] Department of Human Genetics, University of California Los Angeles, Los Angeles, USA

**Email addresses of co-authors:**

| | |
|---|---|
| Fengli Guo | (feg@stowers.org) |
| Melainia McClain | (mel@stowers.org) |
| Xia Zhao | (xiz@stowers.org) |
| Kexi Yi | (kyi@stowers.org) |
| Tari Parmely | (tjp@stowers.org) |
| Jay Unruh | (jru@stowers.org) |
| Brian Slaughter | (brs@stowers.org) |
| Alejandro Sánchez Alvarado | (asa@stowers.org) |

**Corresponding authors:**

| | |
|---|---|
| Leonid Kruglyak | (lkruglyak@mednet.ucla.edu) |
| Longhua Guo | (longhuaguo@mednet.ucla.edu) |
| Alejandro Sánchez Alvarado | (asa@stowers.org) |

**SUMMARY:**

This protocol presents steps taken to dissect ovaries in the freshwater planarians, *Schmidtea mediterranea.* The dissected ovaries are compatible for antibody immunostaining and ultrastructural analysis with transmission electron microscopy to study the cell biology of the oocytes and somatic cells, providing an imaging depth and quality that were previously inaccessible.

**ABSTRACT:**

Accessibility to germ cells allows the study of germ cell development, meiosis, and recombination. The sexual biotype of the freshwater planarian, *Schmidtea mediterranea,* is a powerful invertebrate model to study the epigenetic specification of germ cells. Unlike the large number of testis and male germ cells, planarian oocytes are relatively difficult to locate and examine, as there are only two ovaries, each with 5–20 oocytes. Deeper localization within the planarian body and lack of protective epithelial tissues also make it challenging to dissect planarian ovaries directly.

This protocol uses a brief fixation step to facilitate the localization and dissection of planarian ovaries for downstream analysis to overcome these difficulties. The dissected ovary is compatible

for ultrastructural examination by transmission electron microscopy (TEM) and antibody immunostaining. The dissection technique outlined in this protocol also allows for gene perturbation experiments, in which the ovaries are examined under different RNA interference (RNAi) conditions. Direct access to the intact germ cells in the ovary achieved by this protocol will greatly improve the imaging depth and quality and allow cellular and subcellular interrogation of oocyte biology.

**INTRODUCTION:**

Planarian anatomy has been examined by using TEM in many tissues[1-6]. However, little attention has been given to ovaries or oocytes. The paucity of oocyte literature is partly due to the difficulty accessing these cells, leaving the biology of planarian oocytes largely unexplored. Molecular tools have uncovered many regulatory mechanisms of ovary development in the planarians using light or fluorescence microscopy[7-20]. All these experiments were performed on whole worms or histological sections of whole worms. The antibody staining and *in situ hybridization* protocols on whole worms involve extensive bleaching, washing, and tissue clearing steps, which are time-consuming and will take several days.

The overall goal of the method described here is to provide accessibility to intact, dissected planarian ovaries and oocytes, which will remove the necessity of bleaching or histological sectioning and shorten the time for washing and tissue clearing in antibody staining and *in situ hybridization*. The dissected ovaries will also improve probe or antibody penetration and increase imaging depth and quality for light and electron microscopes. Accessibility to the dissected ovaries and oocytes allows cell biology research at cellular and subcellular resolution with whole intact oocytes. A recent study on dissected planarian ovaries characterized planarian oocyte meiosis for the first time with TEM and confocal microscopy[21]. The work provided a comprehensive description of a new phenomenon during meiosis called nuclear envelope vesiculation.

Here, we present the detailed procedures in the dissection of planarian ovaries. A fixation step was sufficient to preserve the ovary cell structure for dissection and downstream manipulation (i.e., processing for TEM and light microscope analysis). Given their similarity in body plans and tissue architecture, this protocol should also be broadly informative for studying oocytes and their nuclei in several other Platyhelminthes species (e.g., the genus of Dugesia or Polycelis). This protocol is likely irrelevant for *Macrostomum lignano* for their small sizes and almost transparent body architecture, which will allow for direct observation of the ovary and oocytes[22-24]. The body area containing the ovaries is more optically distinguishable (e.g., darker pigmented or lighter pigmented) in some species (e.g., *Dugesia ryukyuensis*[9,25]) than *S. mediterranea*. Studies in these species can rely less on the guidelines for locating the ovaries in *S. mediterranea* presented here but take advantage of the fixation and dissection conditions.

**PROTOCOL:**

**1. Preparation**

1.1. Prepare worms: feed sexual planarians twice a week with organic liver paste to achieve sexual maturity.

NOTE: Generally, such worms are bigger than 1 cm in length and have a gonopore posterior to the pharynx opening.

1.2. Prepare solutions.

1.2.1. Prepare the following reagents: 16% paraformaldehyde (PFA); 50% glutaraldehyde (GA) aqueous solution; N-acetyl-L-cysteine (NAC); 1x phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 8 mM $Na_2HPO4$, and 2 mM KH2PO4.

1.2.2. Dilute 50% GA and 16% PFA with PBS to a final mixture of 2.5% GA and 2% PFA. Dilute 16% PFA with PBS to a final concentration of 4% PFA.

1.2.3. Dissolve 5 g of NAC in 100 mL of PBS to make a 5% working solution.

## 2. Collecting ovaries

2.1. Treat sexually mature worms with 5% NAC in a dish at room temperature for 5 min.

NOTE: Large worms may curl up. Brushes or pipette tips can be used to flatten the worms. The amount of 5% NAC used is minimal, enough to cover the surface of the Petri dish.

2.2. Replace NAC with 10 mL of freshly prepared 4% paraformaldehyde and fix the worms for 1 h at room temperature with occasional shaking. Start the dissection 10 min after the addition of 4% PFA, and perform the dissection in 4% PFA as the worms are being fixed.

2.3. Observe the epithelial pigmentation to locate the ventral nerve cords, which appear as two lines with lighter pigmentation running from the anterior cephalic ganglia toward the tail.

2.4. Locate the ovaries.

NOTE: The ovary is located right next to the nerve cord on the medial side (**Figure 1A**). Rely on epithelia pigmentation to locate the position of the ovary in the anterior-posterior direction. Ideally, the pigmentation of the ventral epithelia in the ovary region is lighter than the surrounding regions. In some worms, the pigmentation of the ventral epithelia does not provide enough confidence. These worms can be discarded as some may not have well-developed ovaries.

2.5. Validate the ovaries' positions once the putative ovaries are located by pigmentation.

NOTE: First, the ovaries are posterior to the cephalic ganglia. Second, dark-colored testes are lateral to the dorsal surface above the ovary. Ovaries locate in front of the most anterior testis

(**Figure 1B**). Sometimes, testis lobes are positioned at the level of or slightly anterior to the ovaries.

2.6. Use two surgical knives to cut posterior and anterior to the two ovaries (**Figure 1C**) to remove the anterior and posterior worm fragments completely. Use one knife to anchor the worm and clean the other knife used to make the cuts.

2.7. Cut in the middle line of the ovary fragment to separate the two ovaries. Flip the fragment to have the ventral side down.

2.8. Peel off the dorsal tissue to expose the gut (**Figure 1D**) with two pairs of pointed tweezers (type 5-SA).

NOTE: The dome-shaped ovary is located beneath the gut branches.

2.9. Gently remove the gut branches sitting above the ventral tissues with the tip of the tweezers or a soft brush to expose the ovary (**Figure 1E**).

2.10. Remove the surrounding tissues to take out the ovary (**Figure 1F,G**).

2.11. Take the ovaries out and transfer them to a 1.5 mL tube to wash with 1 mL of PBS.

**3. Fixation for TEM**

3.1. Wash the ovaries in PBS for 10 min with gentle shaking. Keep the tubes upright, and let the ovaries sink to the bottom by gravity. Repeat the wash once.

NOTE: If the ovaries do not sink, apply a gentle spin for 15 s with a mini-benchtop centrifuge (maximum speed 2000 × *g*).

3.2. Replace the PBS with 2% PFA/2.5% GA/PBS.

3.3. Fix the samples at room temperature for 1 h on a shaker at 40 rpm.

3.4. Keep the samples in fixative at 4 °C overnight on a shaker at 40 rpm.

**4. Sample processing for TEM**

4.1. Wash the ovaries in PBS 3 times for 10 min each.

4.2. Wash the ovaries in double-distilled water ($ddH_2O$) 3 times for 10 min each.

4.3. Post-fix the ovaries in 2% aqueous $OsO_4$ for 1–2 h at room temperature or overnight at 4 °C.

NOTE: The sample containers need to be sealed with parafilm during this step. Prepare 2% $OsO_4$ with reverse-osmosis-treated water (see the **Table of Materials**).

4.4. Rinse the samples with dd$H_2O$ 3 times, 10 min each.

4.5. Pre-stain the ovaries in 2% aqueous uranyl acetate (UA) overnight at 4 °C.

NOTE: The UA solution needs to be filtered before use; avoid light during the *en bloc* staining. Prepare 2% $OsO_4$ with reverse-osmosis-treated water (see the **Table of Materials**).

4.6. Rinse the samples in dd$H_2O$ 4 times with gentle agitation, 10 min each.

4.7. Dehydrate the ovaries in a graded ethanol series (30%, 50%, 70%, 95%, and two times 100%, 10 min each solution) and then equilibrate them in two incubations (10 min) in propylene oxide.

4.8. For infiltration with resin, incubate the samples in 50% propylene oxide/50% liquid epoxy resin mixture overnight at 4 °C. Infiltrate the samples with 100% epoxy resin with 3 changes (1 h each change) with gentle agitation.

4.9. Embed the ovaries in 100% epoxy resin and polymerize the resin at 60 °C for 48 h.

## 5. Ultramicrotomy

5.1. Block trimming and sample check with a light microscope

5.1.1. Trim the sample blocks into a pyramid shape with a razor blade.

5.1.2. Cut sections of 1–2 μm thickness with a glass or diamond knife.

5.1.3. Transfer the sections onto a slide with a drop of water, then heat the slide on a hot plate until the sections flatten and adhere to the slide surface during water evaporation.

5.1.4. Cover the sections with a drop of 1% toluidine blue O and heat them on a hot plate for ~10 s. Rinse the slide with running water, then let it dry. Check the sections under a regular light microscope.

5.2. Ultrathin sections cutting, collection, and post-staining.

5.2.1. Cut ultrathin sections of 50–70 nm thickness with a diamond knife. Transfer the sections from the knife water boat onto mesh or single-slot copper grids.

5.2.2. Stain the sections in 2% UA for 8 min. Wash the grids in running dd$H_2O$ for 30 s.

5.2.3. Stain the sections with 1% lead solution for 6 min. Wash the sections in running dd$H_2O$ for 1 min and air-dry.

## 6. Data collection and analysis

6.1.1. Collect TEM data using appropriate software, e.g., Digital Micrograph (see the **Table of Materials** for more details).

6.1.2. Operate the scope at 80 kV. Insert the sample grid into the scope when the vacuum is ready.

6.1.3. Turn on the beam by clicking **Light** on the menu. Turn the **Intensity** knob on the left control pad until the **Auto Exposure** time is ~1 s. Click on **Start Acquire** to get a final image.

6.1.4. Construct three-dimensional EM models using the open-source image processing, modeling, and display (IMOD) package.

## 7. Antibody staining

7.1. Wash the dissected ovaries (step 2.16) in a 1.5 mL microfuge tube with 1 mL of 1x PBS supplemented with 0.5% Triton X-100 (PBST). Place the tubes on a shaker for agitation at 40 rpm for 10 min. Let the tubes stand in a rack and the ovaries sink by gravity. Replace the wash with fresh PBST, and repeat twice.

7.2 Digest the tissues with 2 µg/mL of Proteinase K and 0.1% sodium dodecylsulfate for 10 min at room temperature in PBST.

7.3. Wash the digested ovaries in PBST three times for 10 min each wash.

7.4. Incubate the ovaries with 10% horse serum in PBST for 1 h at room temperature.

7.5. Dilute primary antibodies 1 to 100 in the blocking solution (10% horse serum in PBS with 0.5% Triton X-100). Incubate the ovaries with primary antibodies overnight at 4 °C with gentle shaking.

7.6. Wash the ovaries in PBST three times for 10 min each wash.

7.7. Incubate the ovaries with secondary antibodies overnight at 4 °C with gentle shaking. Dilute all secondary antibodies 1:300 in the blocking solution.

7.8. Wash the ovaries in PBST three times, ensuring that the first and third washes last for 10 min. Stain the ovarian nuclei with Hoechst 33342 at 1:300 dilution in PBST for 30 min at room temperature in the second wash.

7.9. Mount the ovaries onto slides.

NOTE: Anti-fading mountant can be used to prolong the fluorescence signals.

**REPRESENTATIVE RESULTS:**

The method presented here has been described by Guo et al.[21]. The key to successful dissection is to identify the ovary pigmentation and position guides correctly. The strategy of the method is to move from broad positions to a specific location. First, to achieve this, rely on dorsal and ventral pigmentation patterns (**Figure 1A,B**). Ventral pigmentation, where the ovaries reside, will turn white after 5% NAC treatment and 4% PFA fixation. If the worm used does not provide a clear pigmentation distinction, we recommend using a different worm.

Next, trim away unrelated tissues in a step-by-step fashion (**Figure 1C–E**). Once the ovary is exposed (**Figure 1E**), the surrounding tissues can be trimmed away. The collected ovaries contain multiple somatic cell types and maintain ovary integrity and can be used for both ultrastructural analysis (**Figure 2A**) and immunofluorescence staining (**Figure 2B**)[21]. The method presented here provides details for TEM analysis and immunofluorescence analysis. The conditions can be adjusted as per individual antibody recommendations for antibody staining.

**FIGURE AND TABLE LEGENDS:**

**Figure 1: Locating ovaries with pigmentation patterns.** (**A**) Ventral side of a sexually mature planarian. (**B**) Dorsal side of a sexually mature planarian. (**C**) Ventral side of the fragment with ovaries, after removing the anterior and posterior tissues. (**D**) The fragment after cutting in the midline of (**C**) and removing the dorsal half of the tissues; arrowhead: gut branch on the ventral half of the worm. (**E**) Fragment from (**D**) after removing the gut and other tissues sitting above the ovary. (**F**) The isolated ovary with oviduct attached. (**G**) Image from (**F**) after contrast and brightness adjustment. Red dashed lines: ovary and oviduct (or ventral nerve cord). Red arrows: ovaries. Blue dashed lines: outline of testes. (**A–F**) Images of the field view under a dissection scope without adjustment of contrast or brightness. **A**: Scale bar = 1 mm.

**Figure 2: Representative results.** (Left) A transmission electron microscopic image of one oocyte. Magenta: nuclear envelope vesicles. Scale bar = 5 µm. (Right) A confocal image of one ovary. Magenta: nuclei stained with Hoechst 33342. Green: anti-histone H3. Scale bar = 20 µm.

**DISCUSSION:**

These fixation-based procedures for dissecting planarian ovaries will facilitate the understanding of oocyte meiosis as well as ovary development and regeneration. The sizes of the oocytes and their somatic supportive cells can range from 20 µm to 50 µm. Dissection-based methods will provide accessibility to intact single-ovary cells that sectioning or whole-mount-based methods cannot achieve. This protocol will facilitate the study of intact planarian ovary anatomy and oocyte cell biology at cellular and subcellular resolution.

These procedures require fixation, which limits its application to PFA-based experiments. Other non-PFA fixation methods (*e.g.*, methanol) may also allow for ovary dissection. If the given worm

does not develop a reasonably sized ovary In gene perturbation experiments, traditional whole methods will likely be favored.

The most critical step in the protocol is to locate the ovaries properly and perform fine dissection. A sexually mature planarian with well-developed ovaries is expected to have lighter pigmentation on the ventral epithelium right beneath the ovaries. The area can range from 0.2 to 1 mm in size. The percentage of success in locating the ovary also relies on the culturing conditions of the worms. For an actively maintained, newly regenerated or matured stock, the percentage is high (~100%). The percentage can be low (10–50%) for a stock with worms of variable sizes or health conditions. The percentage of worms with matured ovaries in an experimental population must be evaluated before planning an RNAi experiment.

In summary, the most important strength of this method is to enable a comprehensive analysis of oocyte biology at cellular and sub-cellular resolution. Combined with RNAi-based gene expression perturbations[26-28], we expect that this method will allow for oocyte functional studies, including studies into oocyte regulatory mechanisms and other diverse oocyte biological processes.

**ACKNOWLEDGMENTS:**
The work was supported by the Howard Hughes Medical Institute (LK and ASA) and the Helen Hay Whitney Foundation (LHG).

**DISCLOSURES:**
The authors have nothing to disclose.

**REFERENCES:**
1. Brubacher, J. L., Vieira, A. P., Newmark, P. A. Preparation of the planarian *Schmidtea mediterranea* for high-resolution histology and transmission electron microscopy. *Nature Protocols.* **9** (3), 661–673 (2014).
2. Carpenter, K. S., Morita, M., Best, J. B. Ultrastructure of the photoreceptor of the planarian Dugesia dorotocephala. I. Normal eye. *Cell Tissue Research.* **148** (2), 143–158 (1974).
3. Ishii, S. The ultrastructure of the protonephridial flame cell of the freshwater planarian Bdellocephala brunnea. *Cell Tissue Research.* **206** (3), 441–449 (1980).
4. Morita, M., Best, J. B., Noel, J. Electron microscopic studies of planarian regeneration: I. Fine structure of neoblasts in *Dugesia dorotocephala*. *Journal of Ultrastructure Research* **27** (1), 7–23 (1969).
5. Hyman, L. H. *The invertebrates: Platyhelminthes and Rhynchocoela, the acoelomate Bilateria*. **2**, vii + 550 (1951).
6. Higuchi, S. et al. Characterization and categorization of fluorescence activated cell sorted planarian stem cells by ultrastructural analysis. *Development, Growth & Differentiation.* **49** (7), 571–581 (2007).
7. Chong, T., Stary, J. M., Wang, Y., Newmark, P. A. Molecular markers to characterize the hermaphroditic reproductive system of the planarian *Schmidtea mediterranea*. *BMC Developmental Biology.* **11**, 69 (2011).

8. Handberg-Thorsager, M., Salo, E. The planarian nanos-like gene Smednos is expressed in germline and eye precursor cells during development and regeneration. *Development Genes and Evolution.* **217** (5), 403–411 (2007).
9. Kobayashi, K. et al. The identification of D-tryptophan as a bioactive substance for postembryonic ovarian development in the planarian *Dugesia ryukyuensis*. *Scientific Reports.* **7**, 45175 (2017).
10. Maezawa, T. et al. Planarian D-amino acid oxidase is involved in ovarian development during sexual induction. *Mechanisms of Development.* **132**, 69–78 (2014).
11. Rouhana, L., Tasaki, J., Saberi, A., Newmark, P. A. Genetic dissection of the planarian reproductive system through characterization of Schmidtea mediterranea CPEB homologs. *Developmental Biology.* **426** (1), 43–55 (2017).
12. Sato, K. et al. Identification and origin of the germline stem cells as revealed by the expression of nanos-related gene in planarians. *Development, Growth & Differentiation.* **48** (9), 615–628 (2006).
13. Tharp, M. E., Collins, J. J. 3rd., Newmark, P. A. A lophotrochozoan-specific nuclear hormone receptor is required for reproductive system development in the planarian. *Developmental Biology.* **396** (1), 150–157 (2014).
14. Wang, Y., Zayas, R. M., Guo, T., Newmark, P. A. nanos function is essential for development and regeneration of planarian germ cells. *Proceedings of the National Academy of Sciences of the United States of America.* **104** (14), 5901–5906 (2007).
15. Zhang, S. et al. A nuclear hormone receptor and lipid metabolism axis are required for the maintenance and regeneration of reproductive organs. *bioRxiv*, 279364 (2018).
16. Saberi, A. Jamal, A., Beets, I., Schoofs, L., Newmark, P. A. GPCRs direct germline development and somatic gonad function in planarians. *PLoS Biology.* **14**, e1002457 (2016).
17. Steiner, J. K., Tasaki, J., Rouhana, L. Germline defects caused by Smed-boule RNA-interference reveal that egg capsule deposition occurs independently of fertilization, ovulation, mating, or the presence of gametes in planarian flatworms. *PLoS Genetics.* **12**, e1006030 (2016).
18. Iyer, H., Issigonis, M., Sharma, P. P., Extavour, C. G., Newmark, P. A. A premeiotic function for boule in the planarian Schmidtea mediterranea. *Proceedings of the National Academy of Sciences of the United States of America.* **113** (25), E3509–E3518 (2016).
19. Rouhana, L., Vieira, A. P., Roberts-Galbraith, R. H., Newmark, P. A. PRMT5 and the role of symmetrical dimethylarginine in chromatoid bodies of planarian stem cells. *Development.* **139** (6), 1083–1094 (2012).
20. Wang, Y., Stary, J. M., Wilhelm, J. E., Newmark, P. A. A functional genomic screen in planarians identifies novel regulators of germ cell development. *Genes & Development.* **24** (18), 2081–2092 (2010).
21. Guo, L. et al. Subcellular analyses of planarian meiosis implicates a novel, double-membraned vesiculation process in nuclear envelope breakdown. *bioRxiv*. 620609 (2019).
22. Grudniewska, M. et al. Transcriptional signatures of somatic neoblasts and germline cells in *Macrostomum lignano*. *Elife*. **5**, e20607 (2016).
23. Wudarski*, J.* et al. The free-living flatworm *Macrostomum lignano*. *Evodevo.* **11**, 5 (2020).
24. Wudarski, J. et al. Efficient transgenesis and annotated genome sequence of the regenerative flatworm model *Macrostomum lignano*. *Nature Communications.* **8** (1), 2120 (2017).

25. Maezawa, T., Sekii, K., Ishikawa, M., Okamoto, H., Kobayashi, K. in *Reproductive and Developmental Strategies: The Continuity of Life.* Kobayashi, K., Kitano, T., Iwao, Y., Kondo, M. (Eds), Springer, Japan, Tokyo, 175–201 (2018).
26. Newmark, P. A., Reddien,P. W., Cebria, F., Sanchez Alvarado, A. Ingestion of bacterially expressed double-stranded RNA inhibits gene expression in planarians. *Proceedings of the National Academy of Sciences of the United States of America.* **100** (Suppl 1), 11861–11865 (2003).
27. Orii, H., Mochii, M., Watanabe, K. A simple "soaking method" for RNA interference in the planarian *Dugesia japonica*. *Development Genes and Evolution.* **213** (3), 138–141 (2003).
28. Rouhana, L. et al. RNA interference by feeding in vitro-synthesized double-stranded RNA to planarians: methodology and dynamics. *Developmental Dynamics.* **242** (6), 718–730 (2013).

Fig 1

Click here to access/download;Figure;Fig1.tif

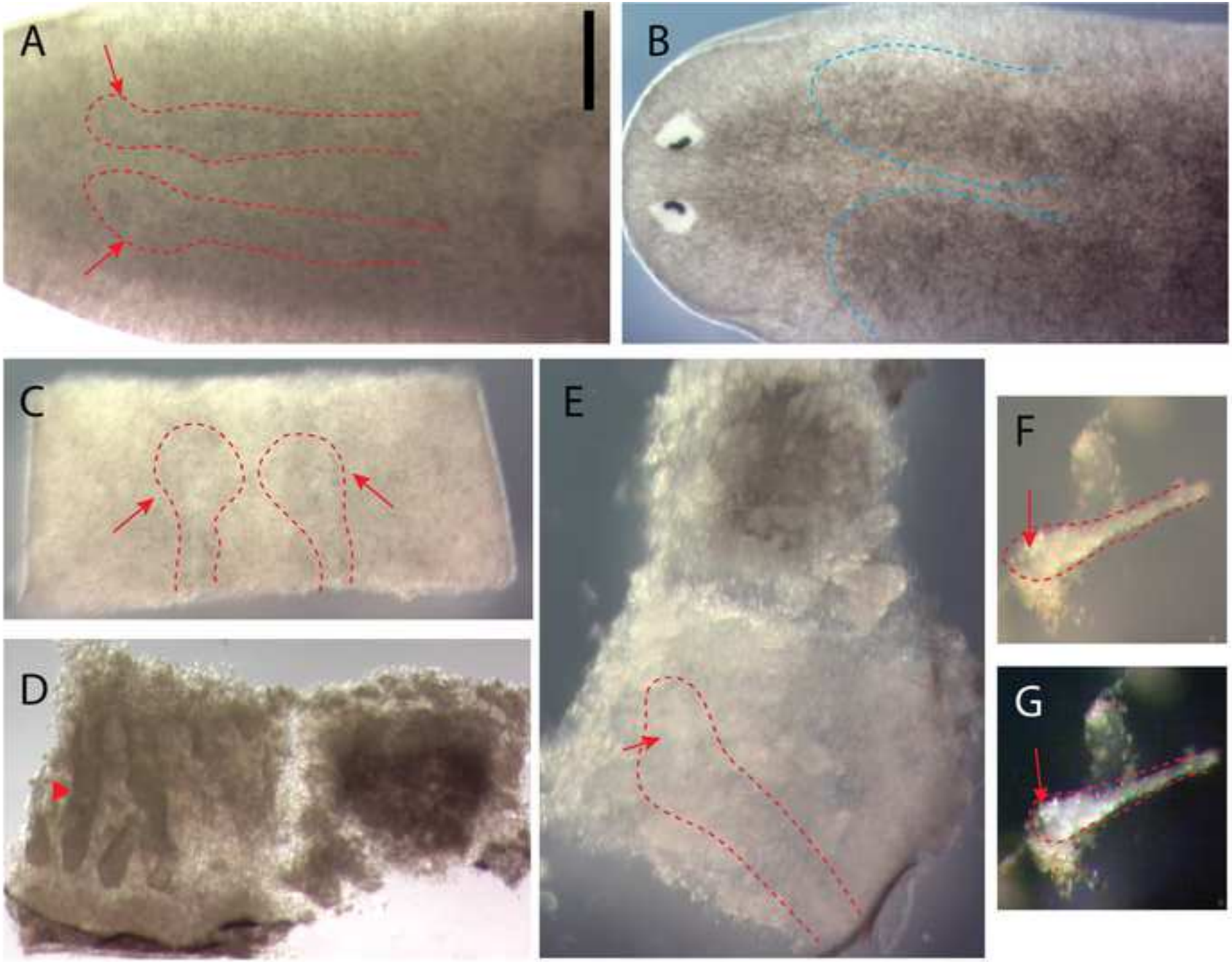

Fig 2

Click here to access/download;Figure;Fig2.tif

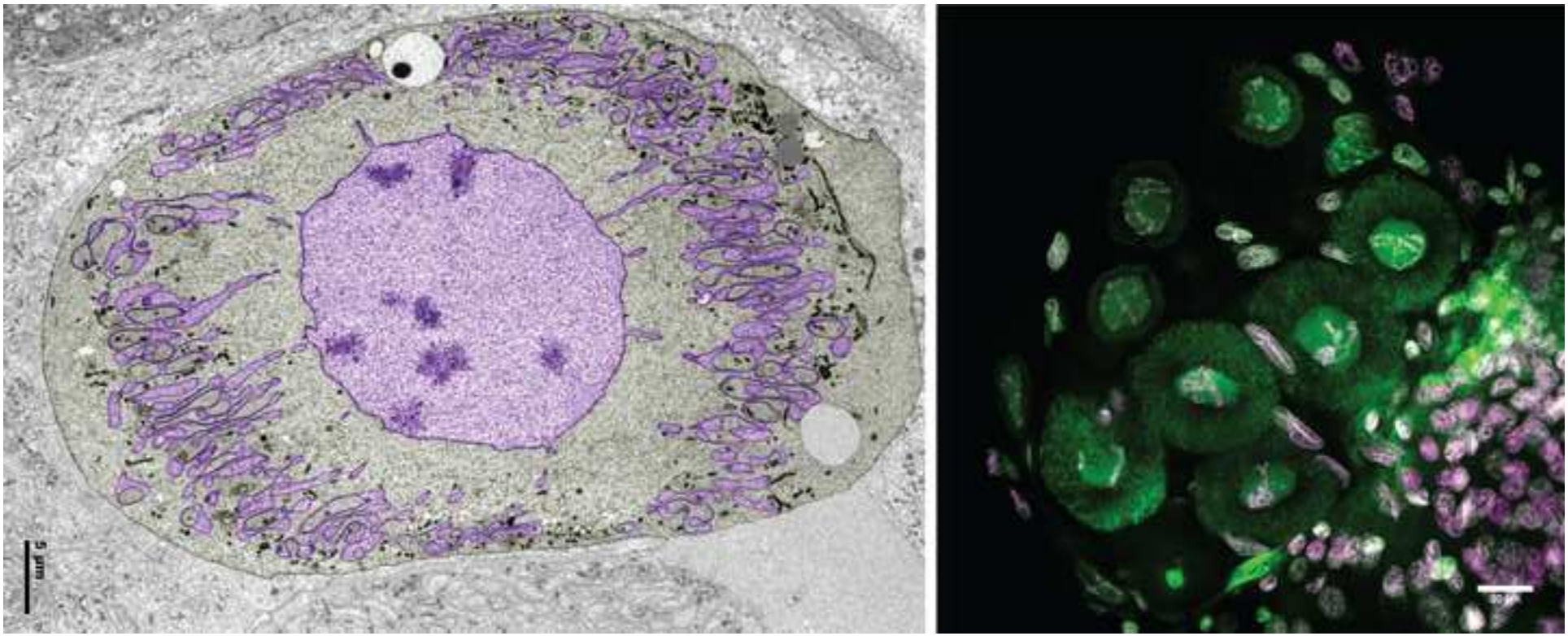

Table of Materials

Click here to access/download;Table of Materials;JoVE_Table_of_Materials.xlsx

| Name of Material/Equipment | Company | Catalog Number |
|---|---|---|
| 16% paraformaldehyde | Electron Microscopy Sciences | 15710 |
| 2% aqueous OsO4 | Electron Microscopy Sciences | 19152 |
| 50% glutaraldehyde | Electron Microscopy Sciences | 16320 |
| Digital Micrograph | Gatan Inc. | |
| Epon resin | Electron Microscopy Sciences | 14120 |
| Ethanol | Ted Pella | 19207 |
| Hoechst 33342 | Thermo Fisher Scientific | H3570 |
| Horse serum | Sigma | H1138 |
| Lead Acetate | Electron Microscopy Sciences | 6080564 |
| MilliQ water | | |
| N-Acetyl-L-cysteine | Sigma | A7250 |
| Parafilm | sigma | P7793 |
| Prolong Diamond Antifade Mountant | Thermo Fisher Scientific | P36965 |
| Propylene oxide | Electron Microscopy Sciences | 75569 |
| Proteinase K | Thermo Fisher Scientific | 25530049 |
| Toluidine blue O | Electron Microscopy Sciences | 92319 |
| Transmission Electron Microscope | FEI | |
| Uranyl acetate | Electron Microscopy Sciences | 541093 |

| Comments/Description |
| --- |
| EM grade |
| |
| EM grade |
| Version 2.33.97.1, TEM data collection |
| Embed 812 Kit, liquid, epoxy resin |
| Denatured |
| |
| |
| |
| reverse-osmosis treated water |
| |
| |
| |
| EM grade |
| |
| |
| Tecnai G2 Spirit BioTWIN |
| |

**Editorial comments:**

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
2. Please decide on one filming location between Los Angeles or Kansas City. We need to know this so we can arrange for a videographer accordingly.

Kansas City.

3. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points

This is completed.

4. Please include a Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: “This protocol presents…”

Added. Please see line 29-33.

5. Please do not include citations in the abstract. Please ensure that the long Abstract is within the 150-300-word limit and clearly states the goal of the protocol.

The references are removed from the abstract. The long Abstract is within word limit with goals stated.

6. Please expand the Introduction to include all of the following with citation:

a) A clear statement of the overall goal of this method

Please see line 48-52.

b) The rationale behind the development and/or use of this technique

Please see line 37-46.

c) The advantages over alternative techniques with applicable references to previous studies

Please see line 42-50, 54-58.

d) A description of the context of the technique in the wider body of literature

Please see line 42-72.

e) Information to help readers to determine whether the method is appropriate for their application

Please see line 64-72.

7. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: (Cat# 15710, Electron Microscopy Sciences), (Cat# 16320, Electron Microscopy Sciences), (Cat# A7250, Sigma), (EMS, Fort Washington, PA), Epon resin, (Tecnai Bio-TWIN 150 12, FEI), etc.

Commercial information was removed.

8. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”

Imperative tenses are now used throughout the protocol section.

9. The Protocol should contain all action items associated with a step in complete sentence.

The steps are now in complete sentences.

10. Please revise the protocol text to avoid the use of any personal pronouns in the protocol (e.g., "we", "you", "our" etc.).

Personal pronouns are now removed from the protocol.

11. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed?

More details are added to the steps where necessary.

12. 1.1 What do you feed the worm?

Food is added to line 76.

13. 2.4 is not an action step please convert this to a note instead.

2.4 is now converted to an action step, with supporting information in notes.

14. 2.10: How big is the cut?
"to completely remove anterior and posterior worm fragments" is added to indicate how big the cut is. "of the ovary fragment to completely separate the two ovaries" is added to 2.11.

15. 6: How do you collect data and analyze it? Please include knob turns, button clicks in the software, command lines, etc.

New information added. Please see lines 214-226.

16. There is a 10-page limit for the Protocol, but there is a 3-page limit for filmable content. Please highlight 3 pages or less of the Protocol (including headings and spacing) that identify the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Line 75-212.

17. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol

Please see lines 301-313

b) Any modifications and troubleshooting of the technique

Please see lines 301-313

c) Any limitations of the technique

Please see lines 301-304

d) The significance with respect to existing methods

Please see lines 294-299, 315-319

18. Please remove the figure legend from the figure files and place it after the representative result section of the manuscript.

Updated.

19. Please sort the materials table in alphabetical order.

Updated.

---

**Reviewers' comments:**

**Reviewer #1:**

Manuscript Summary:

Guo et al describes a protocol to dissect ovaries from S.mediterranea. the principal strength is the idea of a brief fixation in which gradually dissect the planarian body to isolate the ovaries. The paper is interesting and deserve publication in JOVE. However, I have some suggestion to ameliorate experimental reproducibility:

Minor Concerns:

1) Despite the attempt to report a detailed description, the possibility to visually detect ovaries from the ventral surface based on different pigmentation is not easy and requires experience in the field. Moreover, for other species (for example from Dugesia genus) ovaries do not correspond to a less pigmented region and, on the contrary, they can appear as darker spots. To make a more inclusive description authors should mention this aspect.

We included a paragraph in the introduction (lines 64-72) to discuss ovary identification in different species and how our protocol may help other species. We specifically mentioned the species, *Dugesia ryukyuensis*, and cited their works.

2) In paragraph 2.1, please specify what do you mean with "minimal amount"

Now “minimal amount” is deleted from 2.1. Instead, a sentence is added to the note “The amount of 5% NAC used is minimal, just to cover the surface of the petri dish” to specify the amount of NAC to use.

3) Paragraph 1.2 should include preparation of 4% PFA

A sentence is added to 1.2.2 “Dilute 16% PFA with PBS to a final concentration of 4% PFA”.

4) In paragraph 2.3: what do you mean with "10 minutes post treatment"? 10 minutes after the hour in PFA or 10 minutes after the addition of PFA (so, during the hour of fixation)? Moreover, please specify if you dissect the ovaries in PFA or in PBS.

This sentence is now replaced with two sentences to provide more clarity. “Start dissection 10min after the addition of 4% PFA. Perform dissection in 4% PFA as the worms are being fixed.” Lines 101-102.

5) I do not get what do the author mean with "position guides". Figure 1 just shows some image in which the authors putatively mark the ovary region. This is not helpful for other researcher especially if they are not expert in the field. This aspect have to be very well described in the video explanation.

This section will be filmed. The text has been re-written. See lines 104-113.

6) Paragraph 2.9: "should be" is not appropriate, are or are not the ovaries posterior to cephalic ganglions? are or are not the ovaries in front of the most anterior testis?

The ovaries are posterior to cephalic ganglions and in front of the most anterior testis for most of the cases in my experience. “should be” is removed. See lines 117-119.

7) Paragraph 3.5: the possibility of long storage in fixative is interesting under an experimental point of view, however this aspect is not properly related to the topic of the paper. So the authors should provide proof that long storage in fixative does not affect ultrastructural quality, otherwise, delete the sentence.

Now the sentence is removed. It was from experience. Indeed, we do not have comprehensive characterization and data collection to support.

8) Paragraph 5.2.3: is it correct that you stain with uranyl acetate before and after embedding?

Yes. We carried out UA staining in both steps. Staining before sectioning is not required, but helps. Staining after embedding is essential.

9) A statistic or a sentence about the percentage of success of this technique should be included (i.e. which is the chance (if there is one) that the dissection fails and the author cannot find the ovary?). This might be particularly useful to plan the number of animals to be treated, especially in RNAi experiments.

The percentage of success relies mostly on the conditions of the worms. For an actively maintained, newly regenerated or matured stock, the percentage is high (~100%). For a stock with worms of variable sizes or health conditions, the percentage can be low (10~50%). This information is added in lines 311-314.

10) Figure 2 is not cited in the text

Now Figure 2 is cited in lines 272, 273.

11) Figure 1 is very low quality, at least in the PDF for revision.

Thanks for letting us know. The filming will provide more details on the dissection steps.

**Reviewer #2:**
Manuscript Summary:
Planarian flatworms have been adopted as laboratory organisms for analysis of germline development. Ovaries in these hermaphroditic animals are relatively difficult to image compared to testes and non-reproductive organs. Ghuo et al. present a protocol for dissection of planarian ovaries and subsequent analysis via TEM, which facilitate analysis of oogenesis in these organisms.

The protocol is a useful and original addition to the tools available for analysis of oogenesis in planarians. Specifics for some steps should be added to obtain the level of detail that is expected in methods publications. I imagine that the video (which was not part of the reviewed material) will include some of the details that are not apparent in the manuscript, but there is specific information that should be updated in the text.

Major concerns:
Lines 39-41 indicates that this method of tissue dissection is compatible with downstream applications such as TEM, light microscopy, and immunofluorescence. The manuscript does include steps for TEM and provides representative results, but there is insufficient information regarding immunofluorescence. It is not clear whether ovaries isolated using this procedure are ready for analysis by immunofluorescence or whether sectioning is required. The reference provided (Guo et al., BioRxiv, 2019) only includes methods for immunofluorescence analyses on Paraffin and cryo-sectioned material. Please clarify whether ovaries dissected using this protocol are compatible with direct analysis by immunofluorescence, OR whether sectioning is required for the dissected tissue. It would be preferable if a protocol section with detailed steps for immunofluorescence was included. What is described under "Representative results" (lines 161-171; quoted below) seems insufficient for a methods publication:
"For antibody staining, collected ovaries from step 2 are washed with PBS, the same method for TEM analysis. After PBS washes, ovaries are processed through tissue penetration, blocking and antibody staining steps, as per individual antibody recommendations and conventional immunofluorescence staining protocols."

Immunofluorescence analysis presented in Guo et al. BioRxiv, 2019 also works for intact dissected ovaries. Now a more detailed step-by-step method is provided in the protocol section. Lines 228-258.

Minor concerns:
-Scalebars are missing in every panel of Figure 1 and should be included.

Scale bars are now added.

-Line 32: Please separate the references that specifically include analyses of ovaries by light microscopy or fluorescence microscopy. The following publications are worthy of including as examples for the latter: Saberi et al., PLoS Biology, 2016; Steiner et al., PLoS Genetics, 2016; Iyer et al., PNAS, 2016; Tharp et al., Dev Biol, 2014; Rouhana et al., Development, 2012; and Wang et al., Genes & Development, 2012. These references can also be used as examples for "traditional whole worm fragment (whole-mount) methods" mentioned in line 186.

Added, especially line 43.

-Line 37: It is not clear what is meant by "direct access" of oocyte nuclei. Please specify.

Now the sentence is updated as “The overall goal of the method described here is to provide accessibility to intact, dissected planarian ovaries and oocytes” in line 48. ”direct access” is replaced in other parts of the manuscript as well, into “accessibility to intact, dissected planarian ovaries”.

-Line 57: Define acronym and composition of PBS

Updated. Lines 85-86.

-Line 60: Specify which solution can be stored for 3 months. Is it the fixative solution or the NAC solution?

It is fixative. This statement is now removed as we don’t have data to support it can be stored for 3 months.

-Line 63: Define quantity or improve description of "minimal". The instruction is ambiguous as it stands and it is unclear whether there agitation during incubation. It would be good to demonstrate this step in the video or include an image.

A note is added to clarify “minimal”: The amount of 5% NAC used is minimal, enough to cover the surface of the petri dish. Lines 97-98. This will be demonstrated in the video.

-Line 67: Define volume and composition of 4% PFA solution. Is this in PBS? It was not defined in section 1.

Updated. See lines 90 and 100.

-Line 72: "ganglions" should be "ganglion" or "ganglia"

Updated.

-Lines 83-84: I don't believe that this is always accurate. Testis lobes are sometimes positioned at the level or a bit anterior to ovaries.

In my experience, it has always been. Nonetheless, this is added to line 119 for completeness. My speculation is it is probably differences in genetic backgrounds.

-Lines 84-85: I am having difficulty visualizing how to use "two surgical knives to cut posterior and anterior". Please provide an image or demonstrate in the video.

One knife is used to anchor the worm and to clean the other knife. This will be demonstrated in the video.

-Line 89: Specify size and type of forceps.

A pair of forceps is replaced with two pairs of pointed tweezers (type 5-SA). Lines 128-129.

-Line 90: revise "locate" (is located?).

Updated as “is located”. Line 130.

-Line 99: Revise "gentle" with x g value or more specific information.

Updated in lines 145-146: apply a gentle spin for 15 seconds with a mini-benchtop centrifuge (maximum speed 2000g)

-Line 101: The percentages of glutaraldehyde and paraformaldehyde in Step 3.3 are reversed from what is specified in Step 1.2.2. Please check for accuracy.

Updated to be consistent with step 1.2.2.

-Lines 102-103: Should samples be on a shaker on steps 3.4 and 3.5?

Added the information: on a shaker at 40rpm, for both steps 3.4 and 3.5.

-Lines 110 and 114: Are the OsO4 and UA solutions in MilliQ (ultrapure) water? ddH2O? or PBS?

A note is added to indicate MilliQ water was used. Lines 162-163; 169-170.

-Line 131: microns (u) should be revised to "um"

Updated.

-Line 136: "Few" is ambiguous, revise with range or approximate timing.

Revised as ~10 seconds.

**Reviewer #3:**
Although the mechanism underlying the germ cell development initiated during embryogenesis is well understood, the mechanism underlying postembryonic germ cell development is largely unknown. Planarians "post embryonically" differentiate the hermaphroditic reproductive organs from the pluripotent stem cells called neoblasts. Male reproductive organs consist of a large number of testes, while female reproductive organs consist of a pair or pairs of ovaries. Therefore, planarian is a good model animal for studying the mechanism underlying postembryonic germ cell development. However, little information is available about the ovary (the oocytes) compared with the testes. This MS by Gao et al. shows a protocol for dissection ovaries in the planarian Schmidtea mediterranea. The dissection technique outlined in this protocol allows for TEM, antibody immunostaining analysis and knockdown analysis by RNAi in planarian

ovaries. I think that this MS merits publication in Journal of Visualized Experiments. However, it is important to revise several points listed below before publication.

1. 2.8. The authors rely on pigmentation patten and position guides to locate the ovarian position. In our planaria, it is easy to find a pair of ovaries in opaque body by using a binocular microscope equipped with light source of transmitted beam. How about the ovaries in S. mediterranea? This a comment.

Thanks for letting us know. Now we added a paragraph to discuss ovary localization in multiple species in the introduction. Lines 64-72.

2. I cannot find "Fig. 2" in text.

Added. Lines 273-274.

3. 6. Data collection and analysis. The authors should show a 3D EM model as a figure or a move.

The movies and figures were presented in the cited paper (21).

4. 4.3. Parafilim is a product name. The authors should show the commercial information.

The commercial information is now added to the materials table.

5. Reference #13. "-tryptophan" is wrong. The authors miss "D-".

Corrected.

6. The authors cite two papers of bioRxiv (19, 20), namely preprint-paper. Does this journal permit to cite the preprint-papers?

The work in bioRxiv (19,20) are solid. They will be peer reviewed and published when time is appropriate.