Video Article

Sonication-facilitated Immunofluorescence Staining of Late-stage Embryonic and Larval *Drosophila* Tissues *In Situ*

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Abstract

Studies performed in *Drosophila melanogaster* embryos and larvae provide crucial insight into developmental processes such as cell fate specification and organogenesis. Immunostaining allows for the visualization of developing tissues and organs. However, a protective cuticle that forms at the end of embryogenesis prevents permeation of antibodies into late-stage embryos and larvae. While dissection prior to immunostaining is regularly used to analyze *Drosophila* larval tissues, it proves inefficient for some analyses because small tissues may be difficult to locate and isolate. Sonication provides an alternative to dissection in larval *Drosophila* immunostaining protocols. It allows for quick, simultaneous processing of large numbers of late-stage embryos and larvae and maintains *in situ* morphology. After fixation in formaldehyde, a sample is sonicated. Sample is then subjected to immunostaining with antigen-specific primary antibodies and fluorescently labeled secondary antibodies to visualize target cell types and specific proteins via fluorescence microscopy. During the process of sonication, proper placement of a sonicating probe above the sample, as well as the duration and intensity of sonication, is critical. Additonal minor modifications to standard immunostaining protocols may be required for high quality stains. For antibodies with low signal to noise ratio, longer incubation times are typically necessary. As a proof of concept for this sonication-facilitated protocol, we show immunostains of three tissue types (testes, ovaries, and neural tissues) at a range of developmental stages.

Video Link

The video component of this article can be found at https://www.jove.com/video/51528/

Introduction

Drosophila embryos and larvae provide an excellent model to study developmental processes in many organs and tissues. Imaging of individual cells is often necessary in these studies in order to ascertain the complex environments in which cells develop. Visualization of cells in tissues can be accomplished through immunostaining. Well-described immunostaining protocols exist for embryonic *Drosophila* tissues <17 hr after egg laying (AEL)[1-3]. However, a protective cuticle forms toward the end of embryogenesis, preventing effective antibody permeation. Thus, these immunostaining protocols are inefficient in the analysis of tissues in late-stage embryos and in subsequent stages of larval development (1st instar (L1), 2nd instar (L2), and 3rd instar (L3)). This inefficiency imposes a barrier to our understanding of dynamic processes that occur during this extended developmental period [4]. Tissue dissection is a widely employed technique to circumvent this barrier [5-7]. However, dissection can prove inefficient. Extraction may be encumbered by difficulty in locating or isolating embryonic and larval tissues. Furthermore, the physical removal of target tissues may cause damage by rupturing them or by failing to extract them in their entirety.

Sonication is a method that employs sound waves to disturb intermolecular interactions. It has been used to disrupt the integrity of the *Drosophila* larval cuticle in order to immunostain developing neural cell types [6]. This protocol has been adapted to immunostain late-stage embryonic and larval gonads, which can be as small as 50µm in diameter [8-10]. Through such studies, the process of male germline stem cell (GSC) niche formation has been characterized in late stage *Drosophila* embryos[8-10] and mechanisms regulating stem cell development and differentiation in late stage embryonic gonads and larvae have been elucidated [9-12]. Thus, sonication provides an efficient alternative to tissue dissection that may be difficult because of tissue size. Furthermore, it enables immunostaining of *Drosophila* tissues *in situ*, leaving cells within the context of the entire organism and maintaining *in situ* morphology. Here, we describe a step-by-step protocol for fluorescence immunostaining of late-stage embryonic through early/mid-L3 tissues *in situ*. Analysis of *Drosophila* gonadal and neural tissue is shown in the Representative Results to demonstrate the efficacy of this protocol. Furthermore, this immunostaining protocol may be adapted to analyze other *Drosophila* tissues as well as tissues in other organisms with an outer cuticle.

Protocol

1. Preparation of a Collection Cage

1. Anesthetize young, fertile flies with CO_2. Transfer anesthetized flies to a cage. To obtain optimal yield, use 100-120 adult flies ranging from 2-7 days of age at a 4:1 ratio of females to males. Allow flies an appropriate acclimation period, ~24 hr prior to obtaining sample for fixation. If the cage was set up with virgin females mated to males, a use a 36 - 48 hr acclimation period.
2. On the open end of the cage, place a pre-prepared apple juice agar plate with a dime-sized drop of yeast paste in the center over the opening of the cage. Then, secure with tape. Seal the junction between the apple juice agar plate and the cage with Parafilm.
3. Place the cage in a secondary container with apple juice agar plate as the base and allow the flies to reproduce at a defined temperature for an appropriate period of time as determined by the experiment.
   Note: Sample collection times will vary. For example, when collecting late stage embryos/early L1 larvae (17 - 24 hr), allow flies to lay eggs on apple juice agar plates for 7 hr at 25 °C prior to aging of sample for 17 hr at 25 °C. Similarly, for a collection of mid-late first instar larvae (36 - 48 hr) allow flies to lay eggs for 12 hr at 25 °C prior to aging of sample for 36 hr at 25 °C.
4. Once the time period is complete, tap the cage on a table so that flies drop away from the apple juice agar plate without anesthesia. Quickly replace the used plate with a fresh one containing a drop of yeast paste. Secure the fresh plate with tape and parafilm and store the cage with apple juice agar plate as base.
5. Carefully remove the yeast drop from the used plate with a metal spatula. Place lid on used apple juice plate and allow laid embryos to age if experimentally necessary.
   Note: While aging samples, plates may be stored at 25 °C unless higher temperatures are experimentally required. Examples of how to age embryos for collection are described above (see note for step 1.3). Removal of yeast paste prior to sample aging is performed to prevent larvae from crawling into the yeast and breaking up the agar underneath. The remaining apple juice agar and yeast paste residue provide nutrients for larval growth. While embryos laid directly in the yeast paste are lost through yeast paste removal, this loss is preferable to yeast and agar residue in the sample that can reduce staining efficiency. Should one choose not to remove the yeast paste, yeast may be liquefied with Phosphate Buffer Triton X-100 (PBTx), mixed with a paint brush, and then removed from the sample with a cell strainer prior to fixation.

2. Fixation

1. Once embryos laid onto the apple juice agar plate have aged to the desired time point, use a small paintbrush wetted with Phosphate Buffer Triton X-100 (PBTx) solution to carefully remove sample from the plate.
   Note: Older larvae are mobile and may crawl onto the inside of the lid. This sample may be collected similarly by removal with a paintbrush.
2. Wipe the sample-laden paintbrush against the interior-facing ridge of a cell strainer. Then, rinse the walls of the strainer and the paintbrush with a squirt bottle containing PBTx solution. Place a petri dish cover underneath the strainer to capture the PBTx solution.
3. After all the sample has been transferred to the strainer, pour enough PBTx into the strainer to raise the sample off the mesh. Then, lift the strainer and pour the contents of the petri dish into a liquid waste container.
4. Repeat Step 2.3 three times to remove yeast and fly waste that may have been transferred along with the sample.
5. Pour enough 50% bleach (NaOCl)/water (ddH_2O) solution into the strainer to raise the larvae off the mesh. Allow larvae to sit in solution for 5 min. Then, lift the strainer and pour out the contents of the petri dish in a liquid waste container.
   Note: Bleach is required only in embryonic samples aged 0 - 22 hr in order to remove the chorionic membrane. The application of bleach to older samples can be omitted, but its inclusion is not detrimental. If omission is desired, replace the bleach in this step with PBTx. Diluted bleach should be used within 24 hr of solution preparation.
6. Wash sample in the strainer with PBTx by pouring enough PBTx into the strainer to raise the sample off the mesh. Allow sample to sit in solution for 3 min. Then, lift the strainer and pour the contents of the petri dish into a liquid waste container.
7. Repeat Step 2.6 five times.
8. Dab the bottom of cell strainer dry, then transfer sample into a scintillation vial containing 1.75 ml of PEMS solution using a paintbrush.
9. Working in a ventilated fume hood, add 250 µl of 37% formaldehyde and 8 ml of reagent grade heptane to the scintillation vial.
   Note: Formaldehyde and heptane are hazardous. For safety purposes, continue to work in a ventilated fume hood and wear appropriate gloves for steps 2.9 – 3.3.
10. Allow the vial to shake at 200 rpm or a similar moderate speed for 20 min.
11. Add 10 ml of methanol to scintillation vial without removing the previous aqueous phase and allow the vial to shake vigorously at 500 rpm for 1 min.
    Note: Methanol is hazardous. Continue to work in a ventilated fume hood and wear appropriate gloves.
12. Remove vial from shaker and immediately pour contents into a cell strainer over a liquid waste container in the hood. Add extra methanol to the vial and run through the cell strainer as necessary to ensure all sample has been removed.
    Note: Cell strainers may drain slowly. To remedy this, touch a laboratory tissue to the bottom of the cell strainer in order to draw the solution through the strainer more quickly. Methanol, formaldehyde, and heptane should be stored in appropriate waste containers until proper disposal as per institutional guidelines.
13. Dry bottom of cell strainer using a laboratory tissue, then use a paintbrush to transfer sample captured in the strainer to a 1.5 ml microcentrifuge tube containing 0.5 ml of methanol.
    Note: If multiple scintillation vials are in use, complete steps 2.12 and 2.13 one vial at a time to prevent sample from drying on cell strainers.
14. Once the sample has been added to the microcentrifuge tube, remove methanol with a pipette. Ensure sample has settled to the bottom of the tube.
15. Rinse sample in the microcentrifuge tube by adding approximately 0.5 ml of methanol to tube. Wait for sample to settle then remove methanol with a pipette.
16. Repeat Step 15 three times.
17. Add 0.5 ml of methanol to the tube, and then store in a -20 °C freezer for later use.

3. Rehydration and Preparation of Sample for Immunostaining

1. Remove methanol from the microcentrifuge tube with a pipette, leaving the sample in the tube. Store methanol waste to the appropriate waste container until time of proper disposal.
   Note: To enhance sonication efficiency, use no more than 3 mm of sample settled at the bottom of the 1.5 ml microcentrifuge tube. Excess sample may be transferred to a separate tube with a pipette before beginning the rehydration step above. Cutting the pipette tip with a clean razor blade can be used to prevent clogging of the pipette tip.
2. Add 1.0 ml of a 50% methanol/Phosphate Buffer Tween (PBTw) solution to the tube and rock for 3 min.
3. Remove methanol solution to the proper waste container and rinse with 1.0 µl of PBTw twice. After each rinse, allow the sample to settle before drawing off the PBTw with a pipette.
4. Add 1.0 ml of Bovine Serum Albumin/Phosphate Buffered Tween (BBTw) mix to the vial and rock. After 3 min on a rocker, allow the sample to settle and remove the BBTw with pipette.
5. Repeat Step 3.4 two times taking care to remove as much BBTw as possible without losing sample after the last wash.
6. Add 0.5 ml of BBTw to the tube and place the tube on ice.

4. Sonication of Sample

1. Set the sonicator to 10% maximum amplitude and designate a run time of 2 sec constant sonication.
   Note: These settings are specific to the sonicator indicated in the Materials and Equipment Table and may require optimization for different sonicators.
2. Prior to sonication of sample, clean the sonicator probe by placing the probe tip into a 50 ml conical tube filled with deionized water and run sonicator to clean the probe. Dry sonicator probe with laboratory tissue after run.
3. Submerge the probe into the microcentrifuge tube containing sample so that it is positioned approximately 3 to 4 mm above the sample and start sonication.
4. Remove the microcentrifuge tube and place it on ice for at least 30 sec to dissipate heat from sonication and allow sample to settle at the bottom of the microcentrifuge tube.
5. Repeat steps 4.3 and 4.4 as needed based on age of sample being processed.
   Note: For optimum sonication sample volume, embryos younger than 17 hr do not require sonication, but those between 17 and 24 hr (stage 17/early-L1) require two sonications. Larvae between 24 - 36 (early/mid-L1), 36 - 48 (mid/late-L1), 48 - 60 (early/mid-L2), 60 - 72 (mid/late-L2), 72 - 84 (early-L3), 84 - 96 (early/mid-L3), 96 - 108 (mid/late-L3) hr require 5, 9, 11, 13, 15, 18, and 22 sonications respectively. See discussion for further elaboration on sonication times.
6. Rinse with 1.0 ml BBTw twice. After each rinse allow the sample to settle before drawing off BBTw with a pipette.
7. Add 1.0 ml of BBTw to the vial and rock. After 3 min on the rocker, allow the sample to settle and remove the BBTw with pipette.
8. Repeat Step 4.7 two times.

5. Immunostaining

1. Block sample by adding 5% normal serum diluted in BBTw to the microcentrifuge tube. Remove block after rocking for 1 hr.
   Note: Use normal serum from the species in which the secondary antibodies are generated.
2. Dilute primary antibodies to appropriate working concentration in 5% normal serum/BBTw solution.
3. Rock sample in primary antibody solution O/N at 4 °C or for at least 3 hr at RT (approximately 22 °C.
   Note: Antibody incubation times and temperatures may vary. O/N incubation at 4 °C or 3 hr at RT is typically sufficient. However, a two-day incubation period may enhance staining quality, especially with older samples or when low-affinity primary antibodies are used. Longer incubations are typically performed at 4 °C. Similar considerations must be made when using secondary antibodies (see 5.10).
4. Allow sample to settle to bottom of microfuge tube. Draw off primary antibodies with pipette. Save antibodies for reuse if desired.
5. Rinse with 1.0 ml of BBTw twice. After each rinse allow sample to settle before drawing off BBTw with a pipette.
6. Add 1.0 ml of BBTw to the vial and rock. After 3 min on the rocker, allow sample to settle and remove BBTw with pipette.
7. Repeat Step 5.6 five times.
8. Block sample by adding 5% normal serum/BBTw solution to the microcentrifuge tube. Remove block after rocking for 30 min to 1 hr.
   Note: This additional blocking step is not required, but may improve staining quality for specific antibodies.
9. Dilute secondary antibodies to appropriate working concentration in 5% normal serum/BBTw solution.
10. Wrap microcentrifuge tube in aluminum foil to reduce fading due to light exposure. Rock sample in secondary antibody solution for 12 to 48 hr at 4 °C or for at least 3 hr at RT (approximately 22 °C.
11. Allow sample to settle to bottom of microfuge tube. Draw off secondary antibodies with pipette.
12. Rinse with 1.0 ml of PBTw twice. After each rinse allow sample to settle before drawing off the PBTw with a pipette.
13. Add 1.0 ml of PBTw to the vial and rock. After 5 min on the rocker, allow the sample to settle and remove the PBTw with pipette.
14. Repeat Step 5.13 three times.
15. OPTIONAL: Add DAPI diluted 1:1,000 in PBTw. Rock sample wrapped in aluminum foil for 3 min; then remove DAPI solution with a pipette. Rinse five times with 1.0 ml of PBTw, allowing sample to settle before removing PBTw with pipette.
16. Add 80 - 100 µl of 1,4-diazabicyclo[2.2.2]octane (DABCO) solution to microcentrifuge tube and store at -20 °C.
    Note: Another glycerol-based anti-fade agent may be substituted for .

6. Analysis

1. Before mounting sample onto slides, add an extra 5 - 10 µl of DABCO/p-phenylenediamine (PPD) antifade solution to microcentrifuge tube.
   Note: Sample can be added to slides without dissection. Sample volume added to a slide varies with cover slip size. When pipetting sample onto slide, the pipette tip may be cut off using a razor blade to prevent sample shearing. It is often useful to line sample up in rows for easy

analysis. The addition of PPD as an additional antifade agent may not be required, but can prevent photobleaching if samples are stored long-term.
2. Gently place glass cover slip over sample. Then, secure cover slip in place using nail polish.
3. View mounted sample using fluorescence microscopy.

Representative Results

To demonstrate the efficacy of sonication-based immunostaining in analysis of late-stage embryonic and larval tissues *in situ*, wild-type embryos and larvae were processed for immunostaining of testes, ovaries, and neural tissue. Samples were imaged via confocal microscopy and representative results are shown (**Figure 1** and **Figure 2**). Results reveal that the described protocol is effective for visualizing morphological features as well as individual cells *in situ* during late-embryonic through early/mid-L3 stages of *Drosophila* development.

Results from Testis Immunostain:
Testis development is a particularly good system for illustrating protocol efficacy since testes maturation is dynamic throughout larval development. Adult *Drosophila* testes form a coiled tube with one blind end, where the germline stem cell (GSC) niche is situated (see [13,14] for reviews). In this niche, GSCs are arrayed around a tight cluster of non-mitotic somatic cells called the hub. GSCs undergo asymmetric division to produce one GSC that remains anchored to the hub and a daughter gonialblast that is displaced away from the stem cell niche. As the gonialblast divides incompletely, cytoplasmic extensions called fusomes form, connecting the cells within the spermatogonium. After 4 successive divisions, the spermatogonium initiates meiosis to form sperm.

Testis formation begins with the association of primordial germ cells (PGCs) and somatic gonadal precursor cells (SGPs) during embryogenesis (see [15,16] for reviews). This association results in formation of a functional GSC niche by the end of embryogenesis [8-10]. By mid-L1, asymmetric GSC divisions within the GSC niche give rise to differentiating spermatogonia with branched fusomes [9]. Asymmetric GSC division continues throughout larval development, resulting in production of additional spermatogonia and a progressive increase in gonad size. Representative images of late embryonic and early/mid-L1, L2, and L3 testes, immunostained for germ cells, hub cells, and fusomes, are shown (**Figure 1A-D**). These images illustrate the dynamic changes in gonad size and germ cell differentiation observed in testes over time.

Results from Ovary Immunostain:
Adult *Drosophila* ovaries are composed of 16-20 individual egg-producing units called ovarioles (see [17,18] for reviews). At one end of each ovariole, a structure called the germarium contains a stem cell niche. The ovariole niche is composed of undifferentiated GSCs and two populations of somatic cells: cap cells and terminal filament cells (TFs). Similar to the testis, GSCs undergo asymmetric division to produce one GSC that remains adjacent to the cap cells and a differentiating daughter cell, called a cystoblast, which is pushed away from the niche. The cystoblast subsequently undergoes 4 rounds of cell division to form a germ-line cyst, interconnected by a branched fusome. Each germline-cyst is then surrounded by follicle cells to produce an egg chamber that continues to mature as it moves down the length of the ovariole.

Like testes, ovaries are first formed during embryogenesis [16,18]. Multiple ovarioles arise from a single embryonic gonad comprised of PGCs and SGPs. By mid-L3, SGPs give rise to TF precursors at the ovary anterior, while germ cells localize to the ovary posterior and associate with SGP-derived intermingled cells (ICs)[19-22]. By late-L3, TF cells differentiate and organize into the stacks found in the adult stem cell niche, GSCs and cap cells are established, and germ-line cyst differentiation is observed [19,20,23]. Representative images of late-stage embryonic and early/mid-L3 ovaries, immunostained for germ cells, SGPs, ICs, TF precursors, and fusomes, are shown (**Figure 1E,F**). These images show normal morphology and developmental progression for their age.

Results from Neural Immunostain:
The *Drosophila* central nervous system (CNS) is derived from neural stem cells, called neuroblasts (NBs), that arise from embryonic neuroepithelium (see [24,25] for reviews). NBs in embryos and larvae divide asymmetrically to produce ganglion mother cells (GMC) that, in turn, generate neurons and glial cells found in the adult brain and ventral nerve cord. Because larval NBs give rise to the majority of adult neurons and can be distinguished based on their position within the brain, larval brains have become an important model for studying NB behavior and neural differentiation.

The L3 brain is composed of two lobes and a centrally located ventral nerve cord [24]. Representative images of mid-L3 brains immunostained for NBs, GMCs, undifferentiated neurons, immature and primary neurons and glial cells are shown (**Figure 2A-C**)[26-30]. These images show strong staining in distinct expression patterns within the brain lobes and the ventral nerve cord. Depending on mounting orientation, imaging allows for brain tissue visualization from the dorsal surface (**Figure 2A**), ventral surface (**Figure 2B**), or in sagittal cross section (**Figure 2C**).

Staining efficiency:
Our representative results demonstrate that a sonication-based immunostaining procedure is versatile. Not only can it be used to identify specific cell types but also can be used to indicate the presence of specific proteins and organelles. However, ambiguous results may stem from expected variation in staining efficiency. For example, anti-vasa stained at least one gonad in 73.2% of all larvae examined (n=781) while anti-Elav stained brains in 86% of larvae (n=115). Furthermore, we observe that staining efficiency is roughly inversely proportional to developmental stage. In late-stage embryos, anti-vasa stained 89.8% of gonads (n=206), while staining was present in only 59.8% and 46.9% of larvae at L2 and mid-L3, respectively (n=132 and 49). In addition, sonication results in sample loss. When the number of embryos and larvae present in an optimum sonication sample volume was counted before and after sonication, an average of 41.0% of sample was determined unsuitable for analysis (n=1165). To maximize staining efficiency, protocols should be optimized for specific antibodies by altering published antibody concentrations or antibody incubation times.

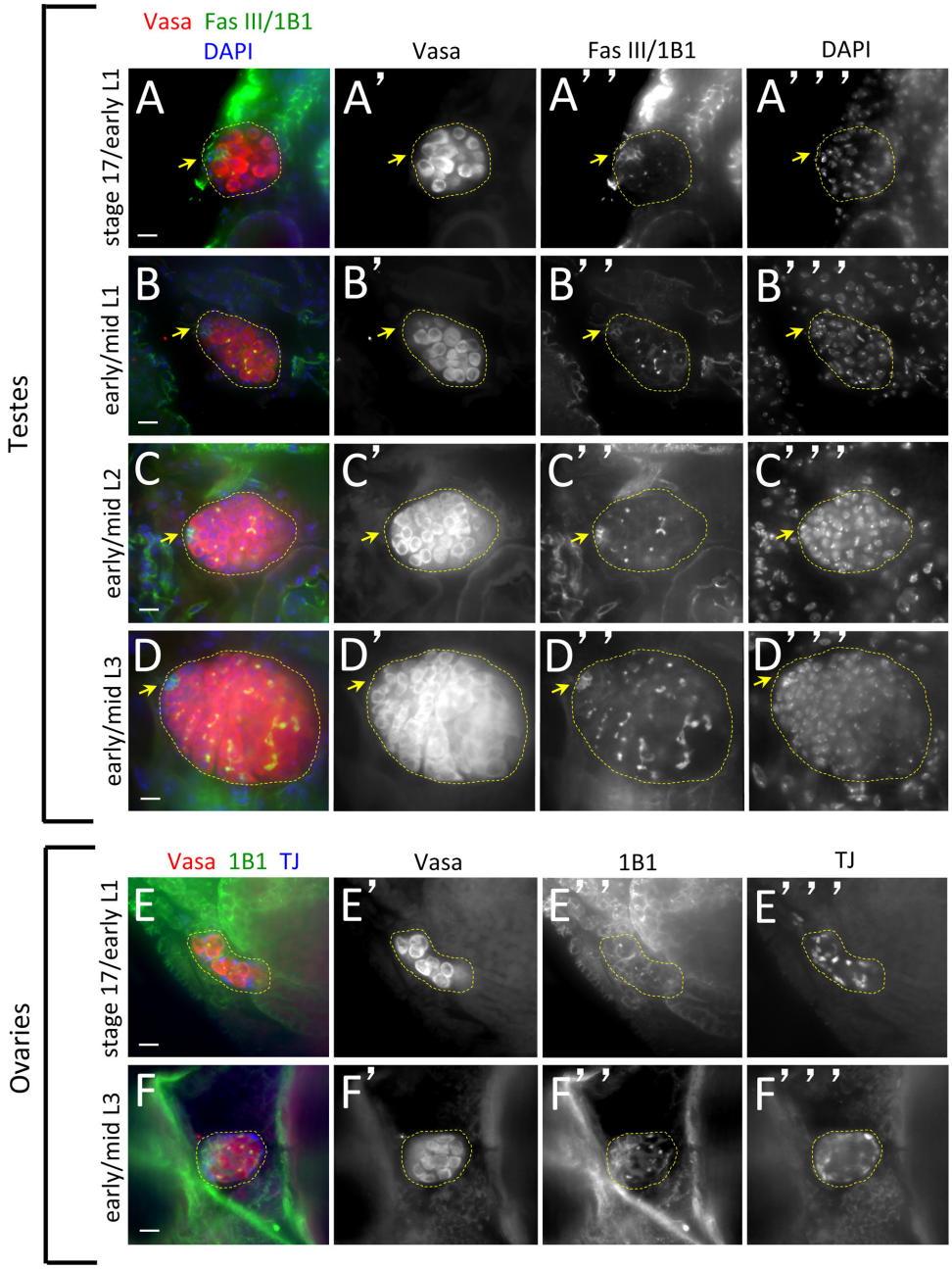

Figure 1. Immunostain of *Drosophila* gonads *in situ*. **(A-D)** Images of testes *in situ* in late-stage embryos (stage 17/early-L1) through early/mid-L3 larvae immunostained with anti-Vasa (**A-D**, red; **A'-D'** alone) to detect germ cells, anti-Fasicilin III (Fas III) and anti-1B1 (**A-D**, green; **A''-D''** alone) to detect hub cells and fusomes respectively, and DAPI (**A-D**, blue; **A'''-D'''** alone) to detect nuclei. **(A)** Stage 17/early L1 testis shows the newly coalesced hub and spherical fusomes in undifferentiated germ cells. **(B)** Early/mid-L1 testis shows spherical fusomes in GSCs localized near the hub and in some posterior germ cells elongated fusomes indicative of early spermatogonial differentiation. **(C)** Early/mid-L2 and **(D)** early/mid-L3 testes show maintenance of GSCs with spherical fusomes near the hub and fusome branching indicative of late spermatagonial differentiation in posterior germ cells. Early/mid-L3 testis has increased in size substantially and shows significant fusome branching. **(E-F)** Images of ovaries *in situ* in late-stage embryos and mid-L3 larvae immunostained with anti-Vasa (**E-F**, red; **E'-F'** alone) to detect germ cells, anti-1B1 (**E-F**, green; **E''-F''** alone) to detect fusomes and somatic cell membranes at L3, and anti-Traffic Jam (TJ)(**E-F**, blue; **E'''-F'''** alone) to detect somatic ovarian cells. **(E)** Stage 17/early L1 ovary shows that somatic cells are distributed throughout the gonad and that germ cells have spherical fusomes. **(F)** Early/mid-L3 ovary is slightly enlarged and membrane-associated 1B-1 expression indicative of TF progenitor development is detected in anterior somatic cells. Germ cells at mid-L3 are located in the testes posterior, show spherical fusomes, and associate with $TJ^{positive}/1B1^{dim}$ somatic ICs. All images are Z-projections of 4 successive confocal slices with the gonad anterior oriented to the left. Gonads are outlined and the hub is indicated with a yellow arrow in testes. Scale bar is 10 µm. Please click here to view a larger version of this figure.

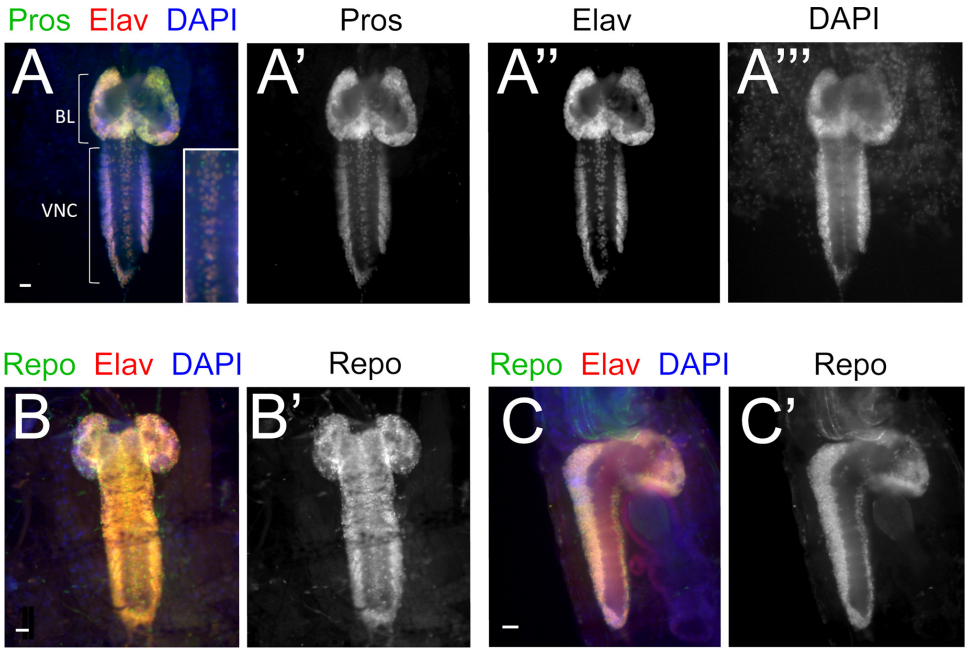

Figure 2. Immunostain of *Drosophila* neural tissues *in situ*. Early/mid-L3 larvae immunostained with anti-Elav (**A-C**, red; **A''** alone) to detect nuclei of immature and primary neurons, and either anti-Prospero (Pros) (**A**, green; **A'** alone) to detect nuclei in GMCs and undifferentiated neurons or anti-Reversed Polarity (Repo) (**B-C**, green; **B'-C'** alone) to detect glial cells. nuclei and DAPI (**A-C**, blue; **A'''** alone) was used to detect all cell nuclei. All images oriented with anterior up. Sagittal section oriented with ventral to the left. **(A)** Dorsal section shows strong staining for Pros and Elav in peripheral regions of the brain lobe (BL) and along the midline and periphery of the ventral nerve cord (VNC). Inset in panel A shows Pros and Elav staining in distinct nuclei along the VNC midline. **(B)** Ventral cross-section shows broad-based expression of Repo^{positive} glial cells with stronger staining along the VNC midline and periphery. **(C)** Sagittal section shows enrichment of Repo staining on the ventral face of the VNC. All images are single confocal sections. Scale bar is 20 µm. Please click here to view a larger version of this figure.

Discussion

This protocol provides a method to successfully immunostain target *Drosophila* embryonic and larval tissues *in situ*, thus eliminating the need for dissection. As per prior protocols for staining early embryos[1,2,3], the chorionic membrane is removed using 50% bleach (NaOCl). Samples are fixed in formaldehyde and methanol. Because the larval cuticle causes older sample to float, the entire sample is then passed through a cell-strainer to ensure larval retention. Sample is stored, if desired, in chemical-grade methanol. After rehydration, a properly implemented sonication process disrupts the integrity of the larval cuticle. This is critical after cuticle formation to allow for sufficient permeation of antibodies required for immunostaining. Before applying antibodies, rocking the sample in a 5% normal serum/BBTw solution prevents excessive nonspecific antibody binding. Primary antibodies are then added to detect specific proteins expressed in the target tissue. After washing to remove unbound primary antibodies, secondary antibodies bound to fluorophores are used to mark the location of bound primary antibodies. Analysis can then be performed with epifluorescence or confocal microscopy.

Some modifications may be required to optimize results in individual circumstances. In particular, sonication power and the number of sonications performed may require adjustment depending on the sonicator used. Adaptations to the height of the sonicator probe above the sample as well as to solution volume may also be required for different sonicators or if sufficient sample cannot be obtained. Increased solution turbidity can be used as an indicator of tissue lysis and, therefore, provides a gauge for when sonication is too severe. If solution turbidity becomes high, the experimenter should consider reducing the number of sonications performed, increasing solution volume, raising the height of the sonicator probe above the sample, and/or reducing sonicator power.

Alterations may also be required in the immunostaining protocol to enhance image quality. As with most immunostaining procedures the signal to noise ratio can be altered by modifying antibody dilution, time and temperature of antibody incubation with sample, and/or blocking reagents used. While each antibody must be considered independently, especially with respect to antibody dilution, we find that incubation at 4 °C for longer time periods can enhance staining quality after sonication, especially for antibodies with low signal to noise ratios and when older larvae are examined. In these instances, staining quality may also benefit from a slight reduction in secondary antibody dilution. In this protocol, we include both BSA and serum as blocking reagents, and the sample is re-blocked prior to addition of secondary antibodies in order to increase staining quality. Depending on the antibodies used, re-blocking and inclusion of both blocking reagents may or may not be required to enhance staining. If the re-blocking step is omitted, longer washes and more rinses may produce cleaner images. Finally, pre-absorbing polyclonal antibodies may increase the signal to noise ratio. Pre-absorption is performed by incubating the desired antibody with rehydrated embryos or larvae prior to use in an immunostain[1].

As demonstrated in our representative results, sonication allows for clear visualization of target tissues *in situ* and thus provides a reasonable alternative to tissue dissection in immunostaining protocols. Dissection can be cumbersome in *Drosophila* embryos and larvae due to difficulties in locating, isolating, and extracting undamaged target tissues. Alternately sonication allows tissues to remain in the context of the entire organism. Because sonication avoids extracting and mounting tissues between a slide and a cover slip, it more accurately preserves *in situ*

morphology. Practically, large amounts of sample can be processed more quickly for future analysis, since many organisms can be sonicated simultaneously.

Although sonication provides a great alternative to dissection, it has limitations that must be considered. Sonication beneficially disrupts the integrity of the larval cuticle but destroys some sample in the process (see Representative Results). Rinsing to remove biological debris is required following the sonication process, which can further reduce sample size. In addition, immunostaining whole embryos and larvae leaves the tissue of interest embedded in surrounding tissues. These surrounding tissues may stain positively or show non-specific antibody binding, thus reducing final image quality. Finally, staining efficiency can be variable. This variability may depend on age of sample, sonication efficacy, and antibody specificity. As a result, sonication-based immunostaining of whole-mount tissue may not always be preferable. In particular, dissection-based immunostaining may be more efficient when studying large tissues in late-L3 larvae. Furthermore, sonication can shear early- and mid-stage embryos. Despite these limitations, sonication-based immunostaining is a highly efficient technique that is well suited for analyzing development of a variety of tissues in late-stage embryos through early/mid-L3 larvae. In our lab, we regularly use this protocol to study gonad morphogenesis in *Drosophila* embryos and larvae[9,10]. Furthermore, we anticipate that implementation of this protocol will prove equally fruitful in studying development of other tissues in *Drosophila* and in other organisms with a protective cuticle.

Disclosures

The authors have nothing to disclose.

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References

1. Moore, L. A., Broihier, H. T., Van Doren, M., Lunsford, L. B., & Lehmann, R. Identification of genes controlling germ cell migration and embryonic gonad formation in *Drosophila*.. *Development.* **125**, 667-678 (1998).
2. Rothwell, W. F., & Sullivan, W. in *Drosophila Protocols.* 141-157 Cold Spring Harbor Laboratory Press (2000).
3. Jenkins, A. B., McCaffery, J. M., & Van Doren, M. *Drosophila.* E-cadherin is essential for proper germ cell-soma interaction during gonad morphogenesis. *Development.* **130**, 4417-4426 (2003).
4. Ashburner, M., Golic, K., & Hawley, R. S. in *Drosophila: A Laboratory Handbook.* Ch. 6, 122-205 Cold Spring Harbor Laboratory Press (2005).
5. Blair, S. S. in *Drosophila Protocols.* eds W. Sullivan, M. Ashburner., & R.S. Hawley) Ch. 10, 159-173 Cold Spring Harbor Laboratory Press (2000).
6. Patel, N. in *Drosophila melanogaster: Practical Uses in Cell and Molecular Biology.* Vol. 44 *Methods in Cell Biology.* eds L.S.B. Goldstei., & E.A. Fyrberg) 445-487 Academic Press (1994).
7. Maimon, I., & Gilboa, L. Dissection and staining of *Drosophila.* larval ovaries. *J Vis Exp.*, doi:10.3791/2537 (2011).
8. Le Bras, S., & Van Doren, M. Development of the male germline stem cell niche in *Drosophila*.. *Dev Biol.* **294**, 92-103 (2006).
9. Sheng, X. R. *et al.* Jak-STAT regulation of male germline stem cell establishment during *Drosophila*. embryogenesis. *Dev Biol.* **334**, 335-344, doi:10.1016/j.ydbio.2009.07.031 (2009).
10. Sinden, D. *et al.* Jak-STAT regulation of cyst stem cell development in the *Drosophila.* testis. *Dev Biol.* **372**, 5-16, doi:10.1016/j.ydbio.2012.09.009 (2012).
11. DeFalco, T., Camara, N., Le Bras, S., & Van Doren, M. Nonautonomous sex determination controls sexually dimorphic development of the *Drosophila.* gonad. *Dev Cell.* **14**, 275-286 (2008).
12. Jemc, J. C., Milutinovich, A. B., Weyers, J. J., Takeda, Y., & Van Doren, M. raw Functions through JNK signaling and cadherin-based adhesion to regulate *Drosophila.* gonad morphogenesis. *Dev Biol.* **367**, 114-125, doi:10.1016/j.ydbio.2012.04.027 (2012).
13. Fuller, M. in *The Development of Drosophila melanogaster.* Vol. I eds M. Bat., & A. Martinez Arias) 71-147 Cold Spring Harbor Press (1993).
14. Cuevas, M., Matunis, EL. The stem cell niche: Lessons from *Drosophila.* testis. *Development.* **138**, 2861-2869 (2011).
15. Williamson, A., & Lehmann, R. Germ cell development in *Drosophila*.. *Annu Rev Cell Dev Biol.* **12**, 365-391 (1996).
16. Jemc, J. C. Somatic gonadal cells: the supporting cast for the germline. *Genesis.* **49**, 753-775, doi:10.1002/dvg.20784 (2011).
17. Spradling, A. C. in *The Development of Drosophila melanogaster.* Vol. I eds M. Bat., & A. Martinez Arias) 1-70 Cold Spring Harbor Press (1993).
18. Eliazer, S., & Buszczak, M. Finding a niche: studies from the *Drosophila.* ovary. *Stem Cell Res Ther.* **2**, 45, doi:10.1186/scrt86 (2011).
19. Sahut-Barnola, I., Godt, D., Laski, F. A., & Couderc, J. L. *Drosophila.* ovary morphogenesis: Analysis of terminal filament formation and identification of a gene required for this process. *Developmental Biology.* **170**, 127-135 (1995).
20. Godt, D., & Laski, F. A. Mechanisms of cell rearrangement and cell recruitment in *Drosophila.* ovary morphogenesis and the requirement of bric a brac. *Development.* **121**, 173-187 (1995).
21. Gancz, D., Lengil, T., & Gilboa, L. Coordinated regulation of niche and stem cell precursors by hormonal signaling. *PLoS Biol.* **9**, e1001202, doi:10.1371/journal.pbio.1001202 (2011).
22. Matsuoka, S., Hiromi, Y., & Asaoka, M. Egfr signaling controls the size of the stem cell precursor pool in the *Drosophila.* ovary. *Mech Dev.* **130**, 241-253, doi:10.1016/j.mod.2013.01.002 (2013).
23. Song, X., Zhu, C. H., Doan, C., & Xie, T. Germline stem cells anchored by adherens junctions in the *Drosophila.* ovary niches. *Science.* **296**, 1855-1857 (2002).

24. Homem, C. C., & Knoblich, J. A. *Drosophila.* neuroblasts: a model for stem cell biology. *Development.* **139**, 4297-4310, doi:10.1242/dev.080515 (2012).
25. Urbach, R., & Technau, G. M. Neuroblast formation and patterning during early brain development in *Drosophila*.. *BioEssays.* **26**, 739-751, doi:10.1002/bies.20062 (2004).
26. Bello, B., Reichert, H., & Hirth, F. The brain tumor gene negatively regulates neural progenitor cell proliferation in the larval central brain of *Drosophila*.. *Development.* **133**, 2639-2648, doi:10.1242/dev.02429 (2006).
27. Lee, C. Y., Wilkinson, B. D., Siegrist, S. E., Wharton, R. P., & Doe, C. Q. Brat is a Miranda cargo protein that promotes neuronal differentiation and inhibits neuroblast self-renewal. *Dev Cell.* **10**, 441-449, doi:10.1016/j.devcel.2006.01.017 (2006).
28. Betschinger, J., Mechtler, K., & Knoblich, J. A. Asymmetric segregation of the tumor suppressor brat regulates self-renewal in *Drosophila.* neural stem cells. *Cell.* **124**, 1241-1253, doi:10.1016/j.cell.2006.01.038 (2006).
29. Pereanu, W., Shy, D., & Hartenstein, V. Morphogenesis and proliferation of the larval brain glia in *Drosophila*.. *Dev Biol.* **283**, 191-203, doi:10.1016/j.ydbio.2005.04.024 (2005).
30. Moraru, M. M., Egger, B., Bao, D. B., & Sprecher, S. G. Analysis of cell identity, morphology, apoptosis and mitotic activity in a primary neural cell culture system in *Drosophila*.. *Neural Dev.*. **7**, 14, doi:10.1186/1749-8104-7-14 (2012).

