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Author	Family Name	**Tastan**
	Particle	
	Given Name	**Ömür Y.**
	Suffix	
	Division	MRC Functional Genomics Unit, Department of Physiology, Anatomy and Genetics
	Organization/University	University of Oxford
	City	Oxford
	Postcode	OX1 3PT
	Country	UK
Corresponding Author	Family Name	**Liu**
	Particle	
	Given Name	**Ji-Long**
	Suffix	
	Division	MRC Functional Genomics Unit, Department of Physiology, Anatomy and Genetics
	Organization/University	University of Oxford
	City	Oxford
	Postcode	OX1 3PT
	Country	UK
	Email	jilong.liu@dpag.ox.ac.uk
Abstract	We describe a user-friendly immunohistochemical approach for the detection of protein localization in *Drosophila* ovaries, here focusing on CTP synthase. This approach mainly uses fluorescently labeled antibodies to detect single, double, or multiple antigens. We provide a step-by-step protocol with detailed notes and tips, a simplified method that can also be adapted to detect protein localization beyond *Drosophila* ovaries.	
Keywords (separated by " - ")	*Drosophila* - Ovary - Antibody - Antigen - Immunohistochemistry - Follicle cell - Cytoophidium	

Chapter 13

Visualizing Cytoophidia Expression in *Drosophila* Follicle Cells via Immunohistochemistry

Ömür Y. Tastan and Ji-Long Liu

Abstract

We describe a user-friendly immunohistochemical approach for the detection of protein localization in *Drosophila* ovaries, here focusing on CTP synthase. This approach mainly uses fluorescently labeled antibodies to detect single, double, or multiple antigens. We provide a step-by-step protocol with detailed notes and tips, a simplified method that can also be adapted to detect protein localization beyond *Drosophila* ovaries.

Key words *Drosophila*, Ovary, Antibody, Antigen, Immunohistochemistry, Follicle cell, Cytoophidium

1 Introduction

A pair of ovaries is the most prominent and easily found tissue in the abdomen of *Drosophila melanogaster* [1]. A female has a pair of ovaries that contain approximately 16 ovarioles, each of which continuously produces functional eggs. Egg chambers bud off and mature as they move along the ovarioles, reaching the posterior as mature eggs ready for fertilization. Oogenesis takes only around 7 or 8 days, depending on the temperature and availability of nutrition [2, 3]. *Drosophila* ovaries house a number of different cell populations, including germ cell lineage and follicular epithelium, which serve as excellent models for the study of different aspects of development [4].

Drosophila ovaries are particularly amenable to immunohistochemistry (IHC). IHC is a technique used to visualize the presence and location of proteins within tissues. In IHC, proteins are detected with antibodies that specifically bind to "epitopes" within the protein of interest. The advance of a fluorescent toolbox empowers IHC and many new approaches to detect protein localization and function [5]. In order to preserve these epitopes, tissues must be fixed prior to staining. Furthermore, in order for antibodies to penetrate the membranes, cells must be permeabilized with detergents. Depending on the nature of antibodies,

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IHC can be carried out with either a "two-step method" or "one-step method." In two-step IHC, the primary antibodies are not labeled with any fluorophores. Once the cells are fixed and permeabilized, a variety of primary antibodies can be mixed and put on the cells for the detection of different proteins (i.e., "the first step"). After primary antibody staining, adding secondary antibodies labeled with different fluorophores (i.e., "the second step") will make targeted proteins visible under either a fluorescent or a laser-scanning confocal microscope. In one-step IHC, primary antibodies are directly labeled with fluorophores that can be visualized either under a fluorescent or a laser-scanning confocal microscope. Combining with fluorescent proteins (such as green fluorescent protein, GFP [6], or mCherry), IHC can be used for detecting the spatial and temporal distributions of multiple proteins [7–11]. IHC can also be used in combination with fluorescence in situ hybridization (FISH) for detection of the relative localization of protein, RNA, and/or DNA [12, 13].

It was recently discovered that the enzyme CTP synthase forms filamentous subcellular structures (called cytoophidia) that are highly conserved from prokaryotes to eukaryotes [7, 14–16]. CTP synthase is expressed at moderate levels in *Drosophila* ovaries [17, 18]. It is cytoplasmic in the germarium and cytoophidium formation starts around stage 2 and the size of the cytoophidia seems to correlate with the cell size [7]. Two types of cytoophidia containing CTP synthase exist in nurse cells: macro-cytoophidia (those that are long and thick) and micro-cytoophidia (short and thin) [7]. Macro-cytoophidia and micro-cytoophidia coexist in *Drosophila* nurse cells and oocytes. The abundance of cytoophidia varies in different cells and tissues [7].

Here we describe an IHC protocol that we used routinely in our laboratory. Our aim was to make IHC as simple as possible. To this end, we have simplified the protocol by skipping some steps used in many other IHC protocols. For example, we do not wash the samples after staining with secondary antibodies and we do not add special mounting medium onto our slides. These changes seem to be against our intuition. However, our IHC results suggest that these changes work well. Furthermore, we have adapted this protocol to study other *Drosophila* tissues such as testes, gut, brain, various glands and imaginal discs in adult animals or larvae [7, 18], as well as to stain culture cells in mammals [19–21].

2 Materials

1. Tweezers (Dumont HP Tweezers 5 stainless steel. 0.10×0.06 mm^2 tip) (*see* **Note 1**).
2. Micro needles (angled stainless steel needles 0.25 mm diameter, 36 mm long) (*see* **Note 2**).

3. Disposable petri dish (35 mm) (*see* **Note 3**).
4. Grace's Insect Medium [22] (*see* **Note 4**).
5. Either paraformaldehyde (PFA) or 16 % formaldehyde (*see* **Note 5**).
6. Phosphate-buffered saline (1× PBS): 135 mM NaCl, 2.5 mM KCl, 4.3 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, pH 7.2 (*see* **Note 6**).
7. Triton™ X-100 (*see* **Note 7**).
8. Horse serum (*see* **Note 8**).
9. PST (1×): 1× PBS, 0.3 % Triton™ X-100, 0.5 % horse serum (*see* **Note 9**).
10. Primary antibodies (*see* **Notes 10–14**).
11. Fluorescently labeled secondary antibodies (*see* **Notes 15** and **16**).
12. Vaseline (*see* **Note 17**).
13. Nail polish (*see* **Note 18**).
14. Glass slides.
15. Coverslips (*see* **Note 19**).

3 Methods

3.1 Preparing the Flies for Dissection

Two to three days before dissection, transfer the stocks into new vials with standard fly food. Tap the flies to the bottom and quickly add wet yeast paste on the inner wall of the new vials (*see* **Note 20**).

3.2 Dissection

1. Anesthetize the flies with CO_2.
2. Separate the flies by sex, discarding the males.
3. Separate the female flies into different groups by genotype.
4. Dissect the ovaries from each group in 3 ml of Grace's Insect medium (*see* **Note 4**) in a 35 mm petri dish at room temperature (Fig. 1a, b) (*see* **Notes 21** and **22**). Do this as quickly as possible without worrying about cleaning the accessory tissues (*see* **Note 23**).
5. Transfer the tissues to a 1.5 ml eppendorf tube using a 20 µl pipette (cut the pipette tip so that the opening is big enough to transfer the fly ovaries) (Fig. 1c, d) (*see* **Note 24**).
6. Remove as much liquid from the tube as possible.
7. In a fume hood, make a 4 % PFA solution by adding 50 µl of 16 % PFA into 150 µl of 1× PBS in a new 1.5 ml tube (*see* **Note 25**).
8. Add newly prepared 4 % PFA into the tube with the ovaries and incubate at room temperature for 10 min.

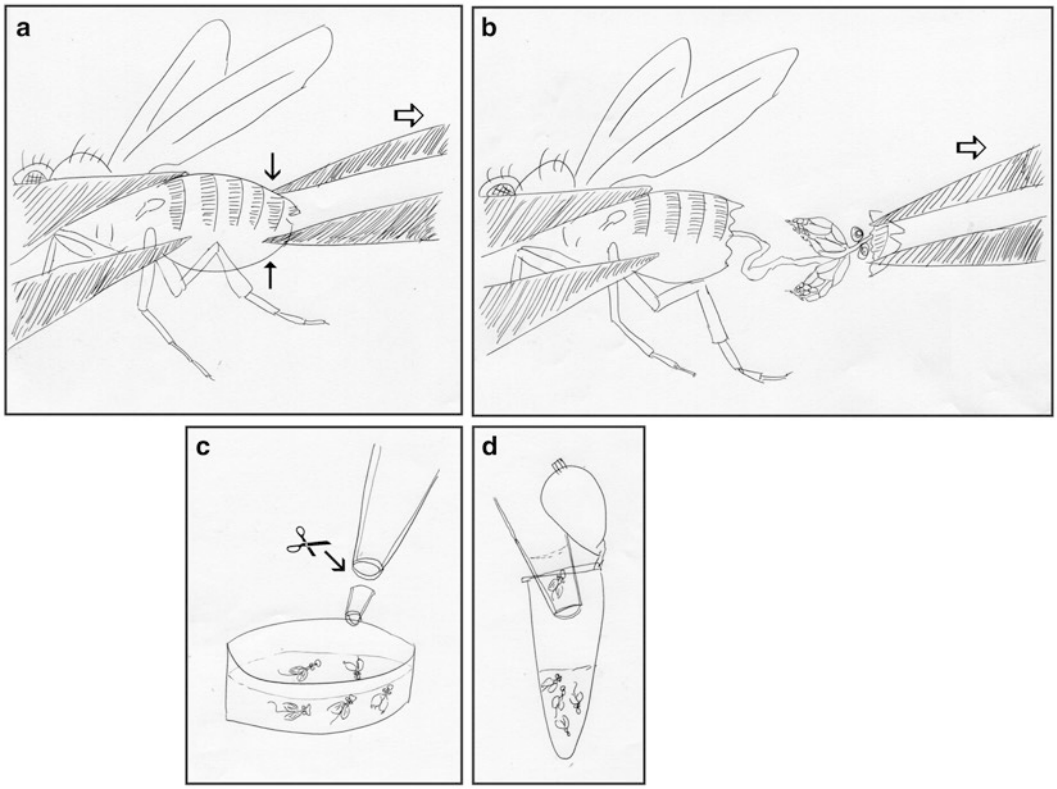

Fig. 1 Dissection of *Drosophila* ovaries. (**a**) Hold a female fly at her thorax and the anterior region of the abdomen with a pair of tweezers. Use the other pair of tweezers to pinch the segment (*solid arrows*) that is second to the most posterior segment, and then pull away from the flies main body (*open arrow*). (**b**) A pair of ovaries and accessory tissues can be easily removed from the flies' abdomen. (**c**) Cut a pipette tip with a blade or a pair of scissors and (**d**) transfer the ovaries and the attached accessory tissues from the dissecting petri dish into a 1.5 ml eppendorf tube

9. Remove as much of the 4 % PFA from the tube as possible (*see* **Note 26**).
10. Add 1 ml of PST to the tube. Remove the PST from the tube.
11. Add 1 ml of PST to the tube. Transfer the ovaries into a new petri dish.
12. Under the dissecting microscope, remove the accessory tissues from the ovaries (*see* **Note 27**).
13. Transfer the clean ovaries into a 500 µl microcentrifuge tube (cut the pipette tip to pipette up the ovaries).
14. Remove as much liquid as possible and then add 500 µl of PST.
15. Use the samples for immunostaining immediately or store them at 4 °C (*see* **Note 28**).

3.3 Immunostaining: Primary Antibody Incubation

1. Make the primary antibody mix for multiple tissue preparations. Target a final aliquot volume of 15 µl (*see* **Notes 29** and **30**).
2. Use 600 µl centrifuge tubes for immunostaining and transfer 2–5 pairs of ovaries into each tube.
3. Remove as much liquid as possible.
4. Add 15 µl of primary antibody mix to each tube.
5. Keep at room temperature overnight (*see* **Note 31**).

3.4 Immunostaining: Secondary Antibody Incubation

1. Make secondary antibody mix for multiple tissue preparations. Add a DNA dye (e.g., Hoechst or DAPI) to the secondary mix. Target a final aliquot volume of 15 µl (*see* **Notes 15** and **16).**
2. Remove as much of the primary antibody mix as possible.
3. Wash in 200 µl of 1× PST at room temperature (*see* **Note 32**).
4. Remove as much 1× PST as possible.
5. Add 15 µl of the secondary antibody mix per tube.
6. Keep at room temperature overnight (*see* **Note 31**).

3.5 Slide Preparation (~30 min Before Imaging)

1. Place a coverslip over paper towel (Fig. 2).
2. Put Vaseline on the four corners of the coverslip (*see* **Note 17**).
3. Transfer the ovaries in 12–15 µl of the secondary antibody mix onto the coverslip (*see* **Notes 33–36**).
4. Put a glass slide onto the coverslip and gently push the slide against the paper towel.
5. Flip the glass slide so the coverslip sits on the top of the glass slide.
6. Use a pipette tip to press the corners of the coverslip so that the liquid spreads evenly between the coverslip and the glass slide.
7. If there is extra liquid outside the coverslip region, gently wipe the liquid off with paper towel.
8. Seal the coverslip with nail polish (*see* **Note 37**).
9. Once the nail polish is dried (5–10 min), the slide is ready for microscopy (*see* **Notes 38** and **39**).

3.6 Confocal Microscopy

Images were taken with an objective (10×, 40× or 63×) on a fluorescence microscope or a laser-scanning confocal microscope (Fig. 3) (*see* **Notes 40** and **41**).

4 Notes

1. The tweezers used for dissection are very sharp and this helps to pull the bottom of the fly with precision preventing damage to the ovaries.

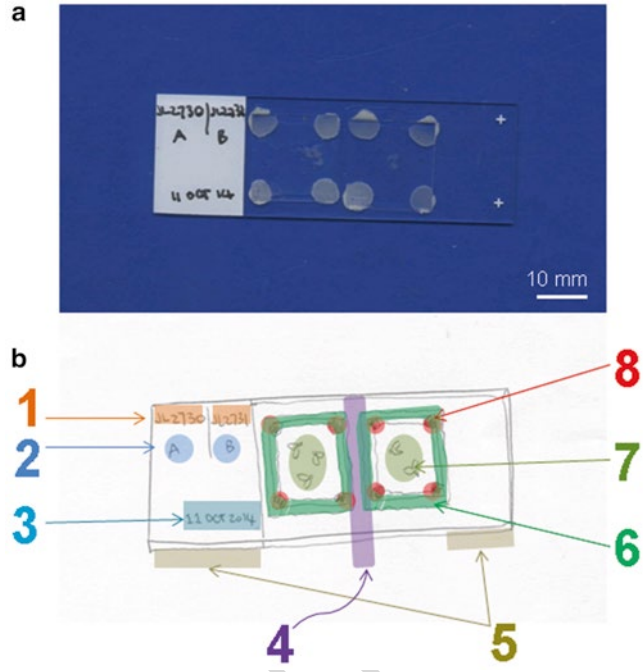

Fig. 2 Preparation of slides. (**a**) A slide with two coverslips. (**b**) The layout of the slide showing in (**a**). (*1*) Labels of the two coverslips showing on this slide. It is convenient to label the coverslips/samples continuously so each sample has a unique identification number in notebooks and digital files. (*2*) Labels of the two samples groups. Detail information should be written in the notebook. (*3*) Date of slide preparation. (*4*) A gap between the two coverslips to avoid contamination from one another. (*5*) Sufficient space from the coverslips to the edges of the slide so samples are accessible for detection under microscopy. (*6*) Nail polish to seal coverslips. (*7*) Samples positioning at the center of the coverslips. (*8*) Vaseline at the corners of coverslips to protect samples from being squashed during scanning

2. A micro needle is useful for fine dissection, such as removing accessory tissues or combing the ovarioles after fixation.
3. We prefer to use a disposable petri dish for the dissection rather than a reusable glass dish.
4. It is important to dissect the tissues in a medium that mimics physiological conditions. By this standard, we chose Grace's insect medium. We strongly recommend not using PBS for the dissection step.
5. As regards whether to use paraformaldehyde or formaldehyde, in our experience this did not make much difference. Paraformaldehyde seems to be more durable at room temperature, and for this reason it was preferred. (a) Preparation of PFA: Open a vial of 16 % PFA, decant the contents into a

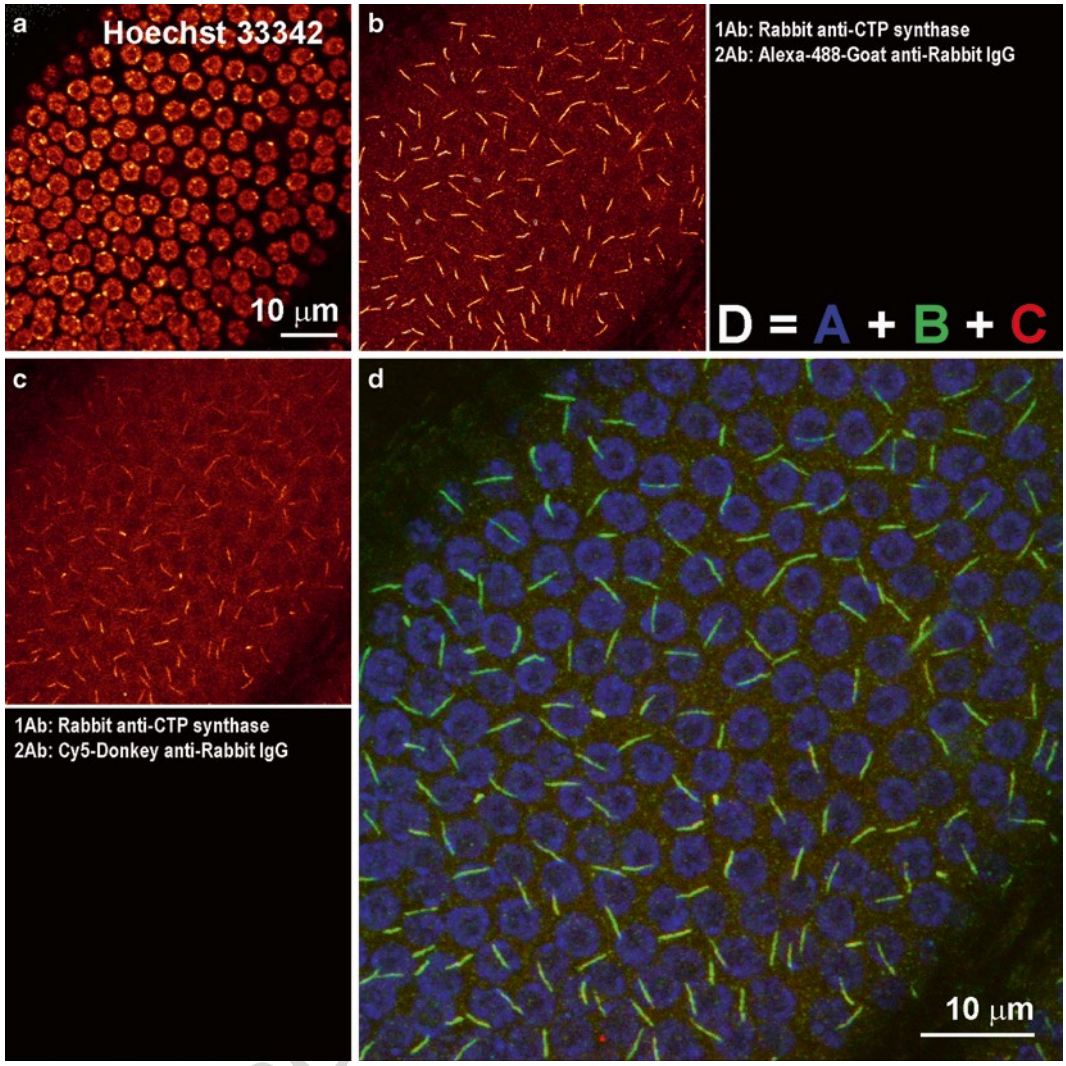

Fig. 3 *Drosophila* cytoophidia expression in the follicle cells monolayer. Images were taken under a Leica TCS SP5 II laser-scanning confocal microscope. (**a**) Follicle cell nuclei labeled with a DNA dye Hoechst 33342. (**b**, **c**) Cytoophidia, filamentous structures that contain CTP synthase [7]. The primary antibody used here is rabbit anti-CTP synthase and the secondary antibodies are Alexa 488 labeled goat anti-Rabbit IgG (**b**) and Cy5-labeled donkey anti-rabbit IgG (**c**). Note that although (**b**) and (**c**) show identical pattern, the signal showing in (**b**) is more intense and clearer than that in (**c**). Images in (**a**, **b,** and **c**) are shown in "glow-over display." (**d**) A merge image of (**a**, **b,** and **c**). Scale bar is 10 μm

15 ml falcon tube and store at room temperature. (b) Preparation of formaldehyde: Open a vial of 16 % formaldehyde, make 200 μl aliquots and store at −20 °C.

6. Use PBS Tablets for consistency. From a pack of 100 PBS tablets, each tablet makes 100 ml of 1× PBS. After dissolving the tablets in water, autoclave the 1× PBS solutions and store at room temperature. Alternatively, PBS can be prepared as a 20× stock.

7. Triton™ X-100 vs. Tween 20. Triton™-X 100 is the default detergent used in IHC. However, Tween 20 is a milder detergent, and in rare cases some antibodies tend to work only with Tween 20 in PST, and not with Triton™ X-100.
8. Powdered bovine serum albumin (BSA) vs horse serum. Both work similarly in blocking nonspecific protein binding sites.
9. For 100 ml of PST, add 6 ml of 5 % Triton™ X-100 and 5 ml of 10 % horse serum into 89 ml of 1× PBS. PST may be stored at room temperature for several months.
10. The amount of the primary antibody needed is 10–15 μl, just enough to cover 2–5 pairs of ovaries. To accomplish this, we generate dilutions of primary antibodies and use these dilutions for the final primary antibody solutions (e.g., use 1 μl of 1:100 dilution in a 9.0 μl of PBT to achieve a 1:1000 final concentration.). If the original concentration of the antibody is known, the final concentration of the antibody used was normally 1 μg/ml.
11. The concentration of antibodies used for immunofluorescence is generally 10× higher than that used in Western blotting. For example, if the dilution for an anti-myc antibody is 1:10,000 for Western blotting, 1:1000 should be used as a starting point for immunofluorescence. Although this is a guideline, it may, however, not apply to all primary antibodies.
12. We generally make 10× stocks for primary and secondary antibodies. For example, if the optimal final concentration for an antibody is 1:1000, we will make 500 μl or 1 ml of a 1:100 dilution, that is, ten times the final concentration. We store 10× stocks at 4 °C.
13. Primary antibody concentration. Sometimes a lower amount may be more effective, and optimizing the primary antibody concentration for the first time can sometimes prove difficult. For a new antibody, the starting trial concentration for a polyclonal antibody may be higher (e.g., 1:250 or 1:500) than the concentration used for most antibodies (1:1000). After looking at our samples, we tend to decide whether to use less or more of the antibody. Generally, when there is no signal or a lot of background, one's instinctive reaction is to add more of the antibody. In contrast to this, however, what we found was that using a smaller quantity of a polyclonal antibody can increase signal quality. Sometimes, therefore, using a smaller quantity of primary antibody (increasing dilution from 1:1000 to 1:10,000) may give much better images with low background.
14. For staining multiple targeted proteins with multiple antibodies simultaneously, it is important to use primary antibodies raised from different species.
15. Antibodies arriving in powder form should be diluted in glycerol in accordance with the manufacturer's instructions.

16. Secondary antibodies may be labeled with fluorophores such as Alexa 488, Alexa 546, Cy3, Alexa 633, or Cy5 (Fig. 1). For the staining of multiple antibodies with different fluorophores, it is important to choose those with well separated spectra. For double labeling, we prefer the combination of Alexa 488 (or EGFP) and Cy5, along with staining the DNA with Hoechst 33342 or DAPI at the same time.
17. Vaseline is used to support the coverslip so the sample is not damaged during microscopy. We normally squeeze Vaseline into a 35-mm petri dish and let it dry for at least 3 days. The traditional method is to heat paraffin wax and Vaseline at a 1:1 ratio to make soft wax. However, for us, dried Vaseline has worked better than traditional soft wax, as well as being easier, cheaper, and simpler.
18. Any brand of nail polish will suffice, though our preference is for a cheap, transparent one.
19. We prefer 18×18 mm^2 coverslips.
20. Put 5–10 female flies together with 2–3 males in a vial with wet yeast paste on standard fly food and keep at 25 °C for 1–3 days. This ensures that the ovaries are fat and contain all developmental stages.
21. It is important to dissect flies at room temperature to maintain natural conditions for as long as possible prior to fixation. Once Grace's insect medium is removed from a fridge, pour it into a petri dish and wait for at least 10 min for the medium to warm up to room temperature. We have found that many proteins change their distribution pattern when the tissues are heat-shocked or cold-shocked before fixation.
22. The flies should be dissected one at a time with a pair of forceps/tweezers [4]. Pull the bottom of a single fly with one tweezer while holding the thorax of the fly with the other tweezer. Ideally, the ovaries will come out very easily. If not, push the ovaries out using the flat side of the tweezer (closed) while holding the fly at the thorax with the other tweezer.
23. Try to minimize the time between dissection and fixation.
24. It is easier to control pressure during tissue transfer with a 20 µl pipette than with a 200 µl pipette. This helps to avoid the tissues becoming stuck onto the walls of the tips during transfer. In addition, use pipette tips for the transfer of tissues only if the medium contains a detergent or serum.
25. When handling PFA, use gloves and a fume hood, as it is toxic. If no fume hood is available, ensure that the eppendorf tubes are closed once the PFA is added.
26. Used PFA should be kept in a capped bottle or a tightly closed container and disposed of appropriately.

27. After fixation and washing, the accessory tissues may be removed in the dissecting dish using tweezers. The ovaries can be opened up more using a pair of micro needles to allow penetration of the fixing solution. It is even possible to carefully comb between the ovarioles, making sure that both ovaries stay attached to the oviduct for easy transport.
28. We prefer to use freshly fixed tissues for immunostaining. However, in our experience, good immunostaining results can be obtained from fixed tissues stored for months at 4 °C.
29. It is good practice to centrifuge the 10× antibody stocks before making the primary or secondary antibody mix (e.g., full speed for 2 min on a benchtop centrifuge). This will dramatically reduce background staining, especially when the antibody solution is not very clear.
30. In the case of a primary antibody directly labeled with a fluorophore, a DNA dye (Hoechst 33342 or DAPI) may be added directly to the mix.
31. We prefer to keep the samples in primary antibody at room temperature (not at 4 °C) for at least one night. This may be extended to several days if there is sufficient time. The same is true for the incubations in secondary antibody.
32. The duration of the washing stage is flexible.
33. We mount samples in secondary antibody mix.
34. We often skip the washing stage. We do not wash off secondary antibodies and do not recommend using a special mounting medium.
35. In our experience, background issues were of low importance, as we used low concentrations of secondary antibodies and confocal microscopy.
36. In our experience, a special mounting medium can dampen the staining signal.
37. Make sure the coverslip is sealed well with nail polish. If there are air bubbles under the coverslip, it helps by adding another layer of nail polish.
38. Prepare the samples fresh before confocal microscopy. Once a slide is made, try to observe it under confocal microscopy within 2 h.
39. If there are too many samples to observe in 1 day, it is better to keep them in a secondary antibody mix in eppendorf tubes rather than mounting them on slides.
40. We prefer to prepare fewer than four slides for a 2 h confocal session, so that we can spend about 30 min of confocal time on each slide. For those slides that require a more extended observation, our aim is to revisit them within 3 days.

41. If using a laser-scanning confocal microscope, make sure the settings of the laser beams and photomultiplier tubes correspond to the excitation and emission spectra of the antibody-labeling fluorophores.

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