**Ovary Dissection and Staining Protocol (updated as of 07.2024)**

### **Introduction:**

This protocol works well for many of the commonly used antibodies in the lab. If working with a new antibody and this protocol does not work well, see the optimization protocol for suggestions on how to improve staining. Consult with lab members to learn about any new optimizations to this IF protocol.

### **Materials:**

* 1x PBS
* 0.2% PBST: 1x PBS + Triton X-100 or Tween
* Block: 0.2% PBST + 0.5% Bovine Serum Albumin (BSA)
* 16% paraformaldehyde (PFA)
* Forceps
* Glass dissection dish
* Dissecting wires and wire holders/ or needle syringes
* Slides
* Cover slips, #1.5 (#1.5’s are 17 mm thick, which is the optimal thickness for Apotome microscopes)

### **Preparation:**

1. Female flies should be maintained on *wet* yeast in a bottle or vial with sufficient males for at least 2 days prior to dissections.—this step is very important. Wet yeasted female ovaries look bigger and are easier to see and dissect. If female flies are not fed wet yeast, ovaries will be small and hard to work with.

2. Before dissecting, prepare 4% PFA solution. Dilute 16% PFA into 1x PBS (1:4) for a final concentration of 4% PFA (100 microliters of 16% PFA to 300 microliters of 1x PBS, per sample).

**Procedure:**

***Day 1***

1. Dissect ovaries in 1x PBS— grasp a female fly from the thorax with sharp forceps and submerge ventral side up in a dissecting dish with PBS. With another pair of forceps in the other hand, puncture the cuticle near the abdomen and peel cuticle back toward the posterior. When ovaries pop out, separate away and discard the carcass.

2. Tease apart ovarioles, leaving them attached at the posterior end, if possible—using either sharp forceps or stiff wires (e.g. tungsten needles/ needle syringe), immobilize ovaries by pinning down the posterior side, which is identifiable as the larger, opaque side. With the other forceps or wire, rake through the tissue in a posterior-to-anterior direction. The ovaries are a collection of ovarioles that are bound together by a thin fibrous tissue. The goal here is to break apart this fibrous tissue at the anterior of the ovary so that fixative and antibodies can access the germaria. However, individual ovarioles are very small and hard to keep track of, so it is best if the ovarioles remain bound together at the opaque side. This allows ovary to be moved through the fixation and staining procedure as a single unit.

3. Use forceps to transfer ovaries to a microfuge tube containing 400 microliters of fix, start timer for 15 minutes - This step cross links proteins in the tissue to preserve it close to its endogenous state

4. After 15 minutes, remove fix, and rinse 1-2 times with 1x PBS. Then rinse 2 times with 0.5 ml - 1 ml block/ rinse - Block is used in preventing nonspecific binding and reducing noise levels when observing tissues under microscope

5. Remove rinse and add primary antibody (diluted in 0.5 ml - 1ml block) - Primary antibody binds directly onto proteins of interest within tissue.

**6.** Incubate overnight at 4°C on a tube rocker or nutator - Provides enough time for effective binding. Primaries can be left in for at least 16 hrs.

***Day 2***

7. Remove primary and rinse 2 times with 0.5 ml -1ml block/rinse - Prevents nonspecific binding. If you are using primary antibodies from the same host species (e.i. both mouse), do 3-4 rinses with block.

8. Wash with 0.5 ml - 1 ml of block for 1 hr at RT on tube rocker or nutator

9. Remove wash and add secondary antibody (diluted in block) - Secondary antibody is coupled with fluorescence and binds onto primary antibody. If using primary antibodies of the same host species, make sure to use isotype specific secondary antibodies for your markers.

10. Incubate 2-3 hrs at RT on tube rocker or nutator. Secondary antibodies can also be kept in 4°C overnight (if needed). - Provides enough time for effective binding

11. Remove secondary and rinse 2 times with 0.5 ml -1ml block/rinse - Prevents nonspecific binding

12. (Optional) Wash with 0.5 ml - 1 ml block for 1 hr. at RT on tube rocker or nutator

13. Remove block and rinse once with 0.5 ml - 1 ml of 1x PBS, wash once for 5 minutes 0.5 ml - 1 ml of 1x PBS to remove detergent

14. (Optional) Wash with 1x PBS + DAPI. If using phalloidin, it can be added in the DAPI wash, at 1: 200. If using vectashield + DAPI (DAPI fluoromount), do not do this step.

15. Remove 1x PBS - When removing PBS, make sure for this step to remove as much PBS as possible.

16. Add two drops of vectashield (with DAPI) into the tube with ovaries - DAPI allows us to observe the nuclei under the microscope. If not mounting right away, tubes with DAPI mount can stay in 4°C until ready to mount.

17. Add a small drop of vectashield (with DAPI) onto a single slide (to get small drops, make sure to let all excess oil drain from outside of dropper before using)

18. Transfer ovarioles to vectashield (with DAPI) with forceps or pipette tip. If using a pipette tip, cut the tip of the pipette to prevent ovaries from getting stuck inside.

19. Use a stiff needle (e.g. tungsten wire/ needle syringe) to separate apart ovarioles on slide, —it’s important to fully separate the ovarioles from each other so the germaria don’t overlap when the coverslip is placed on.

20. If vectashield (with DAPI) becomes sticky/ dries, add another drop of vectashield (with DAPI). Otherwise continue onto step 20. - Adding another drop of vectashield ,however, is not ideal, as more vectashield increases the chance that the germaria could flow out when putting on the cover slip.

21. Put on the coverslip and press slide, cover slip side down, into a folded kimwipe to remove excess vectashield - Techniques for putting on the coverslip vary, but the important part is to have the germaria positioned for excellent images under the microscope

22. Seal with nail polish

23 Label slides with genotype, channel number, primary antibody used, dissection date, and mounting date