Drosophila **Immunostaining Protocol**

Optimized for staining cytoskeletal and membrane associated proteins in *Drosophila* ovaries and larval/pupal tissues.

If you are only using stains (SYBR-Green, phalloidin, lectins) and no antibodies, you can omit the blocking step and all goat serum. Instead, all the washes and stainings are in PBT, and washes can usually be shortened.

Required:

For ovaries: Use females 2-4 days after eclosion, kept with males, and well fed (kept on fresh yeast for previous 1-2 days). This is critical to obtain stage 9-10 egg chambers.

For larvae: Scoop small larvae (2^{nd} – early 3^{rd} instar) out of a populated vial into a new vial with fresh yeast. Maintain the new vial so the larvae do not become crowded and liquefy the food (remove some larvae if necessary). The vial can be kept for several days at 18°C, or can be put at 25°C to promote GAL4-mediated expression if needed. Use wandering larvae, and wash them to remove debris before dissection.

Materials: 3- or 9-well staining dish, sharpened fine forceps and slightly heavier forceps, dissecting needle (insect pin in needle holder); all cleaned with water and ethanol. For transferring tissue, use a clean razor blade to cut the tip off a blue pipet tip. Cut at an angle, leaving a ~2-3 mm bore.

Solutions: Need PBS, formaldehyde, Triton working stock, goat serum.

Fixative, 2% formaldehyde: 125 ul of 16% EM grade formaldehyde plus 875 ul of PBS.

Make the dilution fresh each day.

PBT: PBS + 0.2% TritonX-100.

Keep on hand at least a 50 ml capped tube of PBT: 49.5 ml PBS + 500 ul 20% Triton. Store at room temp. (Triton should be pre-diluted to 20% in dH_2O before adding to any buffers)

Block: PBT + 4% goat serum

960 ul PBT + 40 ul goat serum = 1 ml of block, enough for 2 samples.

Goat serum should be kept at -20 in small aliquots. Thaw goat serum and then either re-freeze it or use it within a day.

PBTG: PBT + 1% goat serum.

You'll need ~10 ml per sample; 10 ml = 9.9 ml PBT + 100 ul goat serum. Store 4°C and use within ~3 days.

1) Place ~200 ul PBS (or Ringers) in well 1 and 1 ml PBS in well 2 of the staining dish.

2) **DISSECT**. Ovaries: Place female fly in well 1, hold thorax with heavier forceps, and remove dorsal abdomen with fine forceps.

If female is well fed and healthy, ovaries should appear as two bundles of ovarioles (similar to bunches of grapes). They attach at the oviduct; try to keep them together and remove all other tissue. They can remain attached to some dorsal cuticle.

Larvae: Grab dorsal and ventral cuticle in the posterior half of the larva, with two pair of forceps. Peel the dorsal cuticle anteriorly to expose the brain, discs, glands, etc. Pull away and remove unneeded tissue.

3) Transfer tissue to well 2. NEVER LET TISSUE DRY; if tissue is exposed to air more than a second or two, it should be discarded.

4) Take as many ovaries/larvae as you can dissect in ~10 min, and accumulate them in well 2. Dissect further as needed; ovaries should be separated at least into individual ovarioles, and ovarioles can be stroked with the needle to remove the thin outer muscle layer.

For larvae, make sure the cuticle does not cover the desired tissue (brain, discs, etc. should be visible.)

5) **FIX**. Remove PBS and add 2% formaldehyde in PBS, incubate for 15-20 min. No shaking. *(for other types of samples that are thicker, extend fixation time to an hour or more, but this can also reduce stain penetration)*

6) Wash with PBS at least 4 times. For all washes, avoid the tissue with the pipet tip and only remove the solution (tissue would become trapped inside the tip at this stage). Each wash dilutes the remaining fixative, so repeat until only trace fixative remains (at least 1:10,000 total dilution). Incubate the last wash for at least 15 minutes. No shaking.

7) Wash once with PBT in well 2.

8) Transfer the tissue along with PBT into a 1.5 ml eppendorf tube using the blue tip with the point cut off.

From here on, all washes are done in this eppendorf, by allowing the tissue to settle (takes ~1 min), and removing the supernatant. Stuff that does not settle is probably fat or trachaea and can be removed. The tube can then be tipped over slowly to let the residual solution flow down to the lip of the tube (and pipetting it away), while trapping the tissue on the side. This increases the effectiveness of each wash, but be sure to have the next solution ready so the tissue does not dry. Avoid creating bubbles.

9) **BLOCK**. Wash 1x with PBT, then incubate in PBT + 4% goat serum for 1 hr, GENTLY rocking. (For anti-phosphotyrosine staining, the block should be shortened to 45 min to preserve the antigen.)

For all rocking incubations, try to *maximize circulation* of the solution and *minimize tissue wear and tear*. Make sure the tissue is freely floating, and not stuck up on the side of the tube. Place the tube on its side on an orbital shaker, or a rotator that is nearly horizontal, and at going at very low speed. The tube should not be tumbling end-over-end.

10) Add primary antibody in PBTG (typically diluted 1:1000; dilute the antibody *before* adding to tissue.) **From this point on, the tissue stays in PBTG-based solutions; do not go back to PBS**.

11) Leave at room temp for 5 hrs, or at 4°C overnight, GENTLY rocking. If you do not have a 4°C rocker, you can rock at room temp for 2-3 hrs and then place the tube on its side at 4°C overnight. *For thicker tissues, staining steps can go 24-48 hrs at 4°C; lengthen the washes correspondingly.*

12) Wash 3-4 times with PBTG. These washes should total 45 min or more.

13) Add secondary antibody diluted 1:1000 in PBTG and leave at room temp for 5 hrs or at 4°C overnight, WRAPPED IN FOIL. Other stains may be included at this step: we often use Alexa-WGA, SYBR Green, or Alexa-phalloidin as structural markers. Phalloidin is stored in methanol, and should be air dried in the dark prior to resuspension in PBTG.

14) Wash 3-4 times with PBTG and let sample sit in last wash until mounted (at least 1 hr). Store at 4°C, in foil so the fluorochromes do not fade. Use within 5 days; after this the tissue and staining will begin to degrade.

15) Mount on a slide in a small drop of Vectashield medium (www.vectorlabs.com); dissect further as needed. Top the sample with a coverslip that has a small bit of vacuum grease on each edge to prevent the coverslip from smashing the tissue.

ABRIDGED PROTOCOL

- Dissect in PBS or Ringers as preferred
- Fix 15-20 min in 2% formaldehyde/PBS
- Wash with PBS at least 4 times
- Wash with PBT
- Transfer the tissue in PBT into a 1.5 ml eppendorf tube
- Wash 1x with PBT
- Block in PBT + 4% goat serum for 1 hr, gently rocking
- Replace with primary antibody (~1:1000 in PBTG)
- Incubate room temp for 5 hrs, or at 4°C overnight, gently rocking
- Wash 3-4 times with PBTG over 1 hour or more
- Add secondary antibody diluted 1:1000 in PBTG (and other desired stains) and rock at room temp for 5 hrs or at 4°C overnight, wrapped in foil.
- Wash 3-4 times with PBTG and let sample sit in last wash (in foil) until mounted.

K. Edwards