Immunocytochemistry on dissected *Drosophila* embryos & larvae

1. Dissect wandering third instar larvae* (in sylgard-bottom Petri dish). Note: Ringer with 2mM glutamate (which is approximate in vivo [glutamate]) optimally preserves morphology of NMJ-associated peripheral glia and GluR clusters. This is less important with fast fixatives (e.g. methanol).

2. Fix tissues (incubate preps 45 minutes (30'-2h) with 4% paraformaldehyde in PBS, or Bouin's, or whatever…).

3. Unpin larva, transfer to 1.5 ml microcentrifuge tube.

4. Rinse 3x in PBTX (Change solution three times).

5. Incubate 1 hour (30'-2h) in PBTX (rotating/shaking).

6. Incubate in primary antibody (diluted in PBTX) rotating/shaking at 4 degrees C overnight**.

7. Rinse 3x in PBTX.

8. Wash 30 minutes (rotating/shaking) in PBTX. Repeat.

9. Replace wash with secondary antibody (diluted in PBTX ~1:300-1:500)

10. Incubate 2 hours (1h-3h) (rotating/shaking, 4 C).

11. Rinse 3x in PBTX.

12. Wash 30 minutes (rotating/shaking) in PBTX. Repeat.

Note: You can't wash too much. Washing for several (2-3) days at 4C seems to make no noticeable difference, and sometimes even helps.

If using biotinylated Ab:

13. Add appropriate conjugate (~1:500) and incubate 30 minutes (rotating/shaking)

14. Repeat steps 11 and 12.

*Dissected embryos are same, except fix 10-30 min, and all procedures are on embryos glued to sylgard-coated coverslips in multi-well plates

** Fluorescent conjugated anti-HRP antibodies can be treated as if staining with secondary Abs alone (dissect, fix, rinse, stain 2h, rinse, and view). Jackson Labs' anti-HRP abs work well at 1:200 (5 ul/ml).

Antibody staining solutions

PBS = Solution A: 0.2M NaH_2PO_4 (*H_2O, monobasic) = 27.8g/L (10.84g/390ml H_2O)[1]
Solution B: 0.2M Na_2HPO_4 (anhydrous, dibasic) = 28.38g/L (17.3g/610ml H_2O)[2]

To make 1L of 10X PBS: Combine 390ml A + 610 ml B + 90g NaCl
(pH to 7.0, keep frozen in aliquots)

[1] If using NaH_2PO_4 (*$2H_2O$), then use 12.16g/390ml)
[2] If using Na_2HPO_4 (*$7H_2O$), then use 32.7g/610ml)

PTX = PBS + 0.1% Triton X-100 (1 ml Triton / L)

PBTX = PTX + 0.1% (by weight) Bovine serum albumin (1g BSA / L)

[To make PBTX: 1) Thaw 100 ml frozen 10X PBS, 2) Add 1 ml Triton X-100, 3) Add 1 g BSA, 4) Add 900 ml water.]

4% paraformaldehyde fix (in PBS)
4 g paraformaldehyde/100 ml PBS
Stir on moderate heat (<70C), covered in fume hood, until dissolved (sol'n clear)

Bouin's fix:
7.5 ml saturated picric acid
2.5 ml formalin (34% formaldehyde)
0.5 ml glacial acetic acid
(or purchase pre-made from Fisher Scientific)

Glutaraldehyde-based fix (make fresh, keep at 4C):
1% glutaraldehyde
1% sodium metabisulfite in: 0.1M phosphate buffer (pH 7.2)
0.1M phosphate buffer = 1.008g KH_2PO4 + 2.535g Na_2HPO_4 (anhydrous) + 250 ml H2O

Methanol fix: (fine for staining NMJ GluRs or other membrane proteins in dissected preps, but might not fix deep tissues or cell interior)
Pure methanol, use ice cold (from freezer)
Fix 5 min.

For neurotransmitter staining:
1) Dissect in ice-cold 0 Ca ringer with 10 mM EGTA
2) Fix in 4% paraformaldehyde fix (or 3-4h in glutaraldehyde)