Fast Fix Protocol for Light Sections

All steps are done on ice.

1. Cut heads off anaesthetized flies using a sharp razor blade. Cut the heads in half and fix in PLP for 20' (on ice if scoring pigment granules). The heads will float.

2. Rinse 2X5' in cold PBS.

3. Place heads in 2% glutaraldehyde in PBS. The CNS/optic lobe and overlying head cuticle (optional) should be dissected off at this stage. Fix in 2% glutaraldehyde in PBS on ice for 3 hours to overnight.

4. Post-fix in 2% glutaraldehyde, 1% OsO_4 for 2 hours on ice.

5. Post-fix in 2% OsO_4 for 30' on ice.

6. Wash 2-3 times in cold H_2O.

note: OsO_4 is an extremely volatile and potent fixative!!! Perform all steps with OsO_4 in a ventilated hood and avoid contact of the fumes with eye tissue!!! Dispose of waste via hazardous waste unit in your facility.

I find that the OsO_4 fixation step really helps in preserving morphology, particularly with short (2-3 hr.) glutaraldehyde fixation. It is possible that this step can be omitted if fixation times are long (up to overnight); however, if your goal is to score pigment granules in genetic mosaics then fixation times should be kept short to prevent diffusion of the pigment.

7. Dehydrate through EtOH series: 5-10' each in 50%, 70%, 80%, 90%, 95%, 2X100% on ice. Incubate 3X10' in 100% propylene oxide on ice. Incubate 30'-overnight in 1:1 propylene oxide:resin on ice.

8. Transfer to 100% resin and incubate 4 hours-overnight.

9. Embed in new resin in molds and heat at 65-80˚C overnight to polymerize resin.

10. Slice away!!

<u>Resin</u>

14.23 g DDSA
9.59 g Epon 812
5.05 g araldite
0.5 ml DMP-30

Mix well in disposable container. Aliquot into 10 or 20 ml syringes and seal the tips with parafilm. Store at -20˚C.

note: uncured plastic is carcinogenic!!! Bake all items used to prepare plastic overnight at 65-80˚C and bake old plastic before disposing!!! Avoid contact with uncured plastic!!!

0.1 M Lysine (500 ml)

9 g. lysine
250 ml dH_2O
add 0.1 M Na_2HPO_4 to pH=7.4 (~55 ml)
Bring volume to 500 ml

PLP

3 ml 0.1 M Lysine, pH 7.4
1 ml 8% paraformaldehyde (make fresh weekly)
10 mg sodium metaperiodate (can be left out if using immediately)

Preparation of Fly Eyes for SEM

1) Dehydrate adult flies through EtOH series: overnight each step in 30%, 50%, 70%, 80%, 90%, 95%, 2X100%.

2) HMDS (hexamethyldisilazane) series: overnight each step in 1:3, 1:1, 3:1 EtOH:HMDS.

3) Incubate 3X60' in 100% HMDS.

4) Mount using silver paste onto EM mounts. Sputtercoat and scan.

BrdU + in situ protocol

1) Dissect discs in Schneider's. Incubate 30-60' in Schneider's + 75µg/ml BrdU. BrdU stock is made fresh each time in 80% EtOH as a 7.5 mg/ml solution. **Retain larval carcasses for antibody preabsorption: fix, wash and freeze in PBT.**

2) Wash 2X5' in Schneider's, 1X5' in PBS. Transfer tissue to a basket made from fine Nitex mesh and a Sarsdedt screw-cap tube. All incubations are done in 2 ml volumes in a 24-well plate.

3) Fix 15' in 4% formaldehyde in PBS (diluted from 37%). Fix 15' in 4% formaldehyde in PBS + 0.6% Triton X-100. Wash 2X10' in PBS + 0.3% Triton X-100.

BrdU staining protocol:

4) Permeabilize 30-60 min. in PBS + 0.6% Triton X-100.

5) Transfer to 1:1 mixture of PBS + 0.6% Triton X-100:4 N HCl (2 N final conc.) for 30 min.

6) Wash 3X10 min. in PBS + 0.3% Triton X-100.

7) Incubate overnight in a 1:100 dilution of α-BrdU (Beckton-Dickinson) in PBS + 0.3% Triton X-100 + 10% goat serum.

8) Wash 3X10 min. in PBS + 0.3% Triton X-100.

9) Incubate in 2˚: Bio-Rad goat α-mouse, 1:100 in PBS + 0.3% Triton X-100.

10) Wash 1X10 min. in PBS + 0.3% Triton X-100, 2X10 min. in PBS.

11) Post-fix 10 min. in 1% glutaraldehyde in PBS; wash 3X10 min. in PBS.

12) Perform DAB reaction as usual.

in situ protocol:

13) Wash 3X10' in PBT (PBS + 0.1% Tween-20).

14) Digest with proteinase K/PBT for 5' (eye discs) -15' (optic lobe complexes) at room temp.

15) Stop with glycine/PBT for 10'. Wash 2X5' in PBT.

16) Post-fix 15' in 4% formaldehyde + 0.1-0.2% gluteraldehyde in PBS.

17) Wash 5X5' in PBT.

18) Wash in 1:1 PBT:Hyb buffer 10'.

19) Transfer the tissue to a single well in a 96-well plate which contains 50 µl of hyb buffer. Incubate at 48˚C for 1 hr.

20) Boil 50 ng of probe in 50 µl of hyb buffer 10', spin briefly and add to prehybridized tissue. Incubate at 48˚C for 16-40 hrs.

21) Transfer tissue back to baskets and wash 3X20' in hyb wash at 48˚C.

22) Wash 3:1, 1:1, 1:3 in hyb wash:PBT for 20' each at 48˚C.

23) Begin antibody preabsorption against fixed larval carcasses while washing 4X20' each in PBT at 48˚C.

24) Incubate 60' at room temp. or overnight at 4˚C in 1:2000 alkaline phophatase-conjugated anti-digoxygenin antibody preabsorbed 60' against fixed larval carcasses.

25) Wash 4X20' in PBT at room temp.

26) Incubate 2X2' in staining buffer.

27) Perform color development in 1 ml staining buffer with 4.5 µl 75 mg/ml NBT and 3.5 µl 50 mg/ml BCIP. Time required will vary according to specific activity of probe and transcript abundance and ranges from 30' to 12 hours in our hands.

28) Stop reaction by transferring tissue to PBS.

Notes

optimize proteinase K digestion for each batch of stock aliquots-general guidelines:
5-7' for eye discs
7-10' for brains
10-15' where really deep penetration is required?

use minimal glutaraldehyde to maintain morphology at post-fix step

longer hyb times seem to increase signal to noise ratio without degrading morphology significantly

overnight antibody incubations tend to give stronger, more consistent staining in thick tissue

Stock solutions

Reagents do not need to be stringently RNase-free

<u>PBT</u>
PBS + 0.1% Tween-20

<u>1000X proteinase K</u>
12.5 mg/ml in PBT
store aliquots at -80˚C
thaw aliquots just before use

10X glycine
20 mg/ml in PBT
store at -20˚C or -80˚C in one-use aliquots

hyb buffer
50 ml formamide
25 ml 20X SSC
1 ml 10 mg/ml salmon sperm DNA
1 ml 10 mg/ml tRNA
1 ml 10% Tween-20
100 µl 50 mg/ml heparin
dilute to 100 ml with dH_2O

store remaining hyb buffer at -20˚C

hyb wash
50 ml formamide
25 ml 20XSSC
1 ml 10% Tween-20
dilute to 100 ml with dH_2O

Staining buffer
1 ml 5M NaCl
2.5 ml 1 M $MgCl_2$
5 ml 1M Tris pH 9.5
0.5 ml 10% Tween-20
12 mg levamisole
dilute to 50 ml with dH_2O