Formaldehyde fixation and cytoskeletal staining by Raffi V. Aroian

Antibody staining:

1. Wash worms off plate and transfer to 1.7 ml microfuge tube. Spin down at 1500 rpm, for 30 sec (all spins are like this).

2. Wash worm pellet once with water.

3. Add 1 ml bleach solution. Bleach for 8-10 minutes

4. Spin down. Remove bleach and wash pellets 4 times with egg salts.

5. Add 1 ml Formaldehyde fix. Incubate 10 min at room temp. Invert occasionally.

6. Spin down . Remove formaldehyde and add 1 ml –20 C methanol. Invert and set in freezer for 5 min.

7. Spin down and remove methanol (save the supe and respin if it looks like there are lots of embryos here). Add PBSTx (0.5%) for 12 min at RT.

8. Remove PBST and add block + Triton-X-100 0.1% (Btw). Block for 20 min.

9. Incubate in primary antibody (1:1000) in Btx 1.5 hours or overnight.

10. WASH. Remove antibody solution. Add 1 ml PBS wash. Immediately remove and repeat. Remove second wash and let sit in 3rd wash for a minimum of 30 minutes. Remove and use block for last 10 min wash.

11. Add rabbit secondary (CY-5) at 1:500 in Btx. Incubate for at least 1 hour. WASH as in step 10.

12. Add actin Ab (mnC4 anti-actin monoclonal antibody from ICN) at 1:500 in Btx. Incubate at least 1 hour. (better if longer). WASH as in step 10.

13. Add mouse secondary (FITC) at 1:500 in Btx. Incubate for at least 1 hour.

14. Wash as in step 10 EXCEPT add DAPI to the 3rd wash at 1:1000. The fourth wash is PBS wash.

15. After the last wash leave about 100 ul on the pellet and respin in eppendorf for 1-2 min to get harder pellet. Remove as much PBS as possible and resuspend pellet in PPD. 20-50 ul depending on pellet size and amount of liquid left.

16. Flick hard to separate the embryos. Set in the dark for 15 min. These are ready to mount or can be stored in the freezer.

Solutions needed:

1) Bleach solution: Prepare fresh

3.5 ml dH2O
1.0 ml 5% bleach (be sure it hasn't expired)
0.5 ml 5 M KOH (prepare fresh)

2) Egg salts

3) Formaldehyde fixative: (1.5 ml)

0.328 ml 16% formaldehyde

0.154 ml 78% sucrose
0.375 ml 4X cytoskeletal buffer
0.643 ml d H_20
prepare fresh and keep in the dark

Note: Formaldehyde keeps best if air exposure is minimized. Transfer from ampule to small evacuated serum vial with a syringe and needle and store at 4C.

4) Methanol at -20C (easiest thing is to put a fresh aliquot back in the freezer after each fix)

5) PBST-high: PBS plus 0.5% Triton-X-100 (25 µl 20% Triton-X-100 per ml)

6) PBST wash: PBS plus 0.1% Triton-X-100 (5 µl 20% Triton-X-100 per ml)

7) Block: 1X PBS, 10% donkey serum, 1% BSA and 0.02% azide
Add detergent at 0.1% final concentration only to amount required for the day.

8) Antibodies

9) PPD (2,5-diphyenyl-1,3,4-oxadiazole; Sigma# D21,021-8): 1 mg/ml in 80% glycerol, 1X PBS

10) 4x cytoskeletal buffer: (final-1X concentration is 10 mM MES, pH 6.1, 138 mM KCl, 3 mM MgCl2, 2 mM EGTA)

800 ul	1 M MES (2-[n-mopholino]ethanesulfonic acid; Sigma #M-5287), pH 6.1
3.68 ml	3 M KCl
240 ul	1 M MgCl2
800 ul	0.2 M EGTA
14.48 ml	water

Prepare stock solutions and sterilize. MES must be filter sterilized, others can be autoclaved if volume is sufficient. Store MES in 1 ml aliquots at -20 C. 4X cytoskeletal buffer keeps 4-6 weeks at 4C.