**FIXATION**

1. Ex-sanguinate embryos by cannulating heart and perfusing with PBS.

2. Eviscerate and behead embryos.

3. Fix for 24 h with freshly made 4% paraformaldehyde in 0.1x PBS. Fix at 4 °C and with the specimens rocking.

4. Rinse 3x with PBS and then bleach for 24 h with Dent's bleach. Bleach at 4 °C and with the specimens rocking.

5. Rinse 5x with MeOH and then fix in Dent's. At least 24 h, but may be stored indefinitely.

**ANTIBODY STAINING**

**Day 1 (single and double staining)**

1. Rinse specimens 3X with 1xPBS.

2. Wash 3x with 1xPBS for 1 hour each wash.

3. Apply 1st primary antibody in blocking solution. Incubate overnight at room temperature with the specimens gently rotating. I incubate for 18 hours.

**Day 2 (single and double staining)**

1. Rinse specimens 3X with 1xPBS.

2. Wash 5x with 1xPBS for 1 hour each wash.

3. Apply 1st secondary antibody in blocking solution. Incubate overnight at room temperature with the specimens gently rotating. I incubate for 18 hours. For the rest of the procedure, I keep the specimens in the dark by covering the rotator with a box.

**Day 3 (double staining; if single staining, proceed to Day 5)**

1. Rinse specimens 3X with 1xPBS.

2. Wash 5x with 1xPBS for 1 hour each wash.

3. Apply 2nd primary antibody in blocking solution. Incubate overnight at room temperature with the specimens gently rotating. I incubate for 18 hours.
Day 4 (double staining)

1. Rinse specimens 3X with 1xPBS.
2. Wash 5x with 1xPBS for 1 hour each wash.
3. Apply 2nd secondary antibody in blocking solution. Incubate overnight at room temperature with the specimens gently rotating. I incubate for 18 hours.

Day 5 (single and double staining)

1. Rinse specimens 3X with 1xPBS.
2. Wash 5x with 1xPBS for 1 hour each wash.
3. Withdraw 1/2 of PBS and replace with MeOH. Let stand 5 min.
4. 3x MeOH rinses for 20 min each.
5. Withdraw 1/2 of MeOH and replace with BABB. Let stand 5 min.
6. Clear in 100% BABB.

SOLUTIONS

Dent's fixative: 1 part DMSO: 4 parts MeOH
Dent's Bleach: 1 part H_2O_2: 2 parts Dent's
BABB: 1 part benzyl alcohol: 2 parts benzyl benzoate

Secondary Antibodies: I use donkey anti-mouse or donkey anti-rabbit conjugated with either DTAF or TRITC from Jackson Labs. I use at a 1:400 dilution in blocking serum.

Blocking Serum: 5% donkey serum (also from Jackson Labs), 75% 1xPBS, 20% DMSO

PBS: 20 liters=
180.0 g NaCl
20.9 g Na_2HPO_4.anhydrous
7.3 g NaH_2PO_4.H_2O
Distilled H_2O to 20 liters
pH 7.40 +/- .02