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Preparation of Embryonic Tissue for Immunohistochemistry Using Frozen Sections

0.2M Phosphate Buffer (PB) pH 7.2
41.3 g $Na_2HPO_4•7H_2O$
6.4 g $NaH_2PO_4•H20$ or 5.57 g NaH_2PO_4
water to 1 L

4% paraformaldehyde (in 0.1 M PB) (usually made fresh, but 1 day old seems to work too)
for 100 ml:
50 ml water
4 g paraformaldehyde
Heat 50 ml water to ~60-70°C (microwave), add 1-2 drops 10 N NaOH, and then add 4 g paraformaldehyde. Stir ~10 minutes to dissolve. Add 50 ml 0.2 M PB (final conc. 0.1 M) and check pH with paper. Sterile filter and store at 4°C up to 1 day.

Other labs have been making up 4 % paraformaldehyde in larger batches and storing aliquots at -20°C indefinitely. I haven't tried this, and it remains to be tested whether this works.

Revision 4/30/07- to ensure consistency between batches of 4% paraformaldehyde, we are currently using premade aliquots of 16% PFA made by Electron Microscopy Sciences (Cat # 15710, available through Fisher Scientific). Add 10 ml of this solution to 10 ml of 0.4M Phosphate buffer and 20 ml ddH_2O (or 20 ml 0.2M Phosphate buffer and 10 ml ddH_2O). Filtration is not necessary. Chill on ice before use and store for up to 1 day.

30% sucrose in 0.1M PB solution
100 ml 0.2M PB
60 g sucrose
Dissolve and bring final volume up to 200 ml with water.
Sterile filter and store at 4°C.

Embryos should be fixed with ice cold 4% paraformaldehyde (in 0.1M PB pH 7.2). I do the fixations and subsequent wash steps in the cold room on a nutator or platform rocker.

The fixation time should be **short**: 45 minutes to 2 hours depending on the age of the embryo (45 minutes for E9.5 mouse/E2-E3 chick; 1 hour for E10.5-E11.5 mouse/E4-E5 chick; longer for older stages). The guts should be removed from E11.5 or older mouse/E4 or older chick to improve penetration into the spinal cord. It is also helpful to make small cuts in the back of the head (in the anterior hindbrain) and again somewhere in the trunk (I usually cut below the hindlimbs) at ages E10.5 and older in mouse/E4 and older in chick. Try to remove as much of the membranes surrounding the embryos as possible. For younger embryos stage, removing the membranes can be difficult, and so I will often make cuts into the membranes and/or trim them around the embryo. For much older embryo stages (E7 and older chick, E14.5 and older mouse), it may be necessary to dissect out the spinal cord or at least rip away much of the surrounding tissue.

After fixation it is important to wash the tissue extensively in cold PBS (or 0.1 M PB): 3 changes of PBS (quick changes) followed by 20 minutes to 1 hour in PBS on ice is my routine. Then transfer the tissue to cold 30% sucrose in 0.1M PB, pH 7.2 and allow it to equilibrate overnight at 4°C (or until the tissue sinks).

After sucrose treatment, embryos are equilibrated and then mounted in Tissue-Tek OCT compound using Peel-A-Way molds and crushed dry ice. Once frozen, blocks should be stored at -80°C or on dry ice before cryosectioning. Blocks should be equilibrated to the cryostat cutting temperature (~ -24°C on our machine) for at least 20-30 minutes before sectioning. We generally cut ~ 10-12 μm sections. Dry slides for at least 15 minutes and use right away, or store them indefinitely at -80°C. Though the initial fixation time is short, these sections are usually acceptable for both in situ hybridization and immunohistochemical analysis.

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Antibody Staining

For staining, hydrate slide by overlaying with blocking solution (1% serum (goat or horse) in PBS with 0.1% Triton X-100 (PBST)). Hydration doesn't have to be very long- 5 minutes is usually enough. From this point onwards, DO NOT LET THE SLIDES DRY OUT.

Remove blocking solution and replace with primary antibody diluted in blocking solution. Every primary antibody has an optimal working dilution, which is empirically determined. I generally use 300-500 µl of antibody solution per slide. Incubate in a humidified chamber overnight at 4°C.

Remove primary antibody (you can save and reuse the primary antibody several times) and wash slides several times with PBST.

Add secondary antibodies and incubate in a humidified chamber for 30 minutes or more at room temperature (in the dark or covered in foil if fluorescent secondaries are being used). Typically we use donkey anti-(species of interest) secondary antibodies from Jackson or Molecular Probes. Recommended dilutions for the different fluorescent conjugates are:
FITC: 1:500
Cy3: 1:1000
Cy5: 1:700
Alexa488: 1:1500
Hoescht 33258 (1 mg/ml solution): 1:1000

Rinse slides several times with PBST.

(Optional) Overlay slides with 4% paraformaldehyde (leftover fix used for the embryos is fine) for 3-5 minutes. This often helps to preserve the crispness of fluorescent staining, which often becomes diffuse over time- **Note that this does not appear to be necessary if you are using Prolong Gold as your mounting media.**

Rinse slides with PBST

Coverslip slides with Prolong Gold mounting media (Invitrogen/Molecular Probes).