***Tissue sectioning and* in situ *hybridization* as prepared by Simon Dunn (2/14/06)**

Reagents and Solutions

0.36M $MgCL_2$.$6H_2O$ in 50% seawater (Final concentration 0.18M)

TBS™ tissue freezing medium (Triangle Biomedical Sciences

PAP pen (Daido Sangyo Co, Ltd)

Poly-L-Lysine coated slides (LabScientific, Inc)

4% paraformaldehyde

Triton X-100

MeOH

Triethanolamine

HCl

Acetic anhydride

SSC

Formamide

RNAse A 10µg ml^{-1} (Promega)

RNAse T (Sigma)

sheep serum

Anti-Dig Digoxigenin-AP FAB conjugate (Enzo-Roche)

BCIP : NBT (Calbiochem)

1mM levamisole

PBS (pH 7.0)
2 mM NaH_2PO_4
7.7 mM Na_2HPO_4
0.14M NaCl in DMPC-H_2O

NTE (pH 8)
0.5M NaCl
10mM Tris

Hybridization buffer
50% formamide
10% 2x SSC (0.3M NaCl, 30mM sodium citrate, pH 7.0)
2.5% tRNA 10mg ml^{-1}
2% 50x Denhardt's solution
10% dextran sulfate
0.25% 10mg ml^{-1} denatured salmon sperm

Buffer 1 (pH 7.5)
100mM Tris
150mM NaCl

NTMT buffer pH 9.5
0.58 mM NaCl
0.05 mM $MgCl_2$
1.21 mM Tris
0.1% Triton X-100

NB Before Starting clean and then maintain the procedure in an RNAse free conditions until RNAse Step

Procedure

1. Relax anemones in $MgCl_2$ seawater solution for 30-60min
2. Embed Whole anemones in TBS™ tissue freezing medium (Triangle Biomedical Sciences) mix and fold within medium and repeat in fresh medium 3 times before storing at -80^{o}C.
3. Tissue sections (20-25µm) produced using a 2800 Frigocut N cryostat microtome (Reichert-Jung) and mounted within a PAP pen (Daido Sangyo Co, Ltd) restricted area of Poly-L-Lysine coated slides (LabScientific, Inc) and returned to -80^{o}C for as long as required .

NB For detailed sectioning protocol see Jodi Schwartz protocol

4. Remove Slides from -80^{o}C, allow to briefly thaw until slides first appear to liquefy and place into a Coplin jar containing 4% paraformaldehyde (important pH 7-7.5) in phosphate buffer saline (PBS) for 45 min at room temperature (RT).
5. Wash slides twice in PBST (1% Triton X-100 in PBS) for 5 minutes before a MeOH dehydration series (25%, 50%, 75%, 100%, 75%, 50%, 25% in PBST for 2 min each).
6. Rinse in PBST twice for 5 min
7. Rinse in 0.1M TEA (1.33% triethanolamine, 0.3% HCl) for 5 min
8. Incubate in acetylation buffer under the hood (0.25% acetic anhydride in 0.1M TEA) for 10 min
9. Rinse twice in PBST for 5 min.
10. For pre-hybridization, use plastic probe cover slips to incubate sections in hybridization buffer in a humid chamber at 37^{o}C for 45 min.

NB *During the pre-hybridization, antisense and sense probes in hybridization buffer (final concentration 0.5µg ml^{-1}) were heated at 80^{o}C for 10 min and then placed briefly on ice prior to application*.

11. The pre-hybridization solution was removed and replaced with the probe in hybridization buffer and incubated using probe cover slips within a humid chamber at 55^{o}C overnight.
12. The following morning, Remove probe and place slides in a Coplin jar containing 2x SSC and 50% formamide for 5 minutes at 52^{o}C (Do this under the hood).
13. Transfer slides to a new Coplin jar and do two 15 min rinses of 2x SSC at 37^{o}C
14. Two 15 minute rinses of 1x SSC at 37^{o}C.
15. Slides were then placed in a separate Coplin jar containing a 37^{o}C pre-warmed RNAse solution (2% RNAse A 10µg ml^{-1} (Promega), 0.2% RNAse T (Sigma) in NTE for 30min.
16. Rinse slides twice in 0.1x SSC at 37^{o}C for 5 min followed by two 10 min rinses in Buffer 1
17. Incubate slides in blocking solution (0.1% Triton X-100, 2% sheep serum in Buffer1) for 45 min RT.
18. Replace blocking solution with anti-DIG antibody solution (0.1% Triton X-100, 1% sheep serum, 0.001x Anti-Dig Digoxigenin-AP FAB conjugate, Enzo-Roche, in Buffer 1) and incubate for 1.25 hours.
19. Remove slides from the chamber and rinsed twice in Buffer 1 for 10 min
20. Rinse in NTMT buffer for 10 min.
21. Incubate slides in detection buffer 1:2 (BCIP : NBT, Calbiochem, 1mM levamisole in NTMT) in the dark until first visible color precipitation is observed. Stop by rinsing in dH_2O prior to mounting with glycerol mountant.
22. Sections viewed using an Olympus Vanox-T AH2 microscope and Sony DKL 5000 digital camera.