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

Reagents and Solutions

0.36M MgCl₂.6H₂O 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 NaH₂PO₄

7.7 mM Na₂HPO₄

0.14M NaCl in DMPC-H₂O

Buffer 1 (pH 7.5)

100mM Tris

150mM NaCl

NTE (pH 8)

0.5M NaCl

10mM Tris

NTMT buffer pH 9.5

0.58 mM NaCl

0.05 mM MgCl₂

1.21 mM Tris

0.1% Triton X-100

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

**Procedure**

1. Relax anemones in MgCl₂ 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°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°C for as long as required.

NB For detailed sectioning protocol see Jodi Schwartz protocol

4. Remove Slides from -80°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°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°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°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°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°C

14. Two 15 minute rinses of 1x SSC at 37°C.

15. Slides were then placed in a separate Coplin jar containing a 37°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°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 dH₂O prior to mounting with glycerol mountant.

22. Sections viewed using an Olympus Vanox-T AH2 microscope and Sony DKL 5000 digital camera.