Non-radioactive Section In Situ Hybridization for Embryos

Preparation and sectioning of embryos

1. For frozen section
Dissect embryos into PBS and be sure to remove all membranes. Fix the litter in 10ml of 4% paraformaldehyde in PBS at 4°C, 20 min to o/n (depending on stage) with gentle rocking (on a Nutator). Wash 2x in PBS for 5’ at RT. Transfer embryos into 15% and then 30% of sucrose in PBS at 4° C for 4-6 hr (until embryos sink to the bottom). Rinse embryos briefly with OCT and embed embryos in OCT. Section on a cryostat at 10-14 μm thickness.

2. For paraffin section
Dissect embryos into PBS and be sure to remove all membranes. Fix the litter in 10ml of 4% paraformaldehyde in PBS at 4°C, 20 min to o/n (depending on stage) with gentle rocking (on a Nutator). Wash 2x in PBS for 5’ at RT. Dehydrate in 30% EtOH 20-30 min, 50% EtOH 20-30 min then 70% EtOH 20-30 min followed by processing in paraffin processor (20 or 30 min program). Section at 5-7 μm. Dewax before implementing In Situ Hybridization: Xylene 1 min (2X), 100% EtOH 1 min (2X), 95% EtOH 1 min then PBS 1 min (2X).

Day 1: Preparation and hybridization of sections (all steps are carried out in Coplin jars – 50ml)

1. Post-fix in 4% paraformaldehyde (PFA) in PBS for 10’
2. Wash with PBS 2x for 5’
3. Drain excess PBS and incubate for 8 min in 4ug/ml proteinase K in PBS (10μl of 20 mg/ml protK to 50ml PBS) at RT. For tissue older than E17.5 use 20 mg/ml of proteinase K for 10 min at 37C.
4. Drain and wash with PBS for 2’
5. Refix in 4% PFA for 5’, then wash 5’ in PBS (3X)
6. For paraffin sections only: Acetylation: 125 ul acetic anhydride in 50 ml of 0.1 M TEA (0.1 M TEA: 18.6 g TEA+4.5ml 10N NaOH in 1 L H2O)
7. Wash with PBS 3x for 5’
8. Dehydrate in 70% (5’) and 95% (few seconds) EtOH and air dry (drain on paper towels)
Hybridization solution: (100ml, can be stored in 10ml aliquots at –20°C)

      | Final |
      |-------|
      | 50mlFormamide (deionized) | 50% |
      | 20ml 50% Dextran sulfate | 10% |
      | 1ml 100x Denhardt’s (Rnase free) | 1% |
      | 2.5ml yeast tRNA (10mg/ml) | 250μg/ml |
      | 6ml 5M NaCl | 0.3M |
      | 2ml 1M Tris-HCl, pH8 | 20mM |
      | 1ml 0.5M EDTA | 5mM |
      | 1ml 1M NaPO₄ (pH8) | 10mM |
      | 5ml 20% Sarcosyl (can’t autoclave) | 1% |
      | 11.5ml DEPC-H₂O (1ml DEPC in 1L of d H₂O; alternatively use Ultrapure bottled H₂O) |
Total: 100ml

9. Take 2μl of probe (approx. 1μg) to 1ml of hybridization solution and heat at 80°C for 2’.
   Bring the heat block to bench and keep tube warm while adding probe/hyb solution to slides in humidified chamber. This will prevent deleterious changes in probe conformation.
   (Alternatively use 3-4μl to 1 ml of hybridization solution for weak probes).
10. Place slides horizontally in a humid box with paper towels soaked in Ultrapure H₂O (or 50% formamide/2X SSC) or in cassette of hyboven. Cover sections with 200ml of hyb solution/probe and lower Rnase free coverslips over sections avoiding bubbles.
11. Seal humid box and hybridize at 55°C overnight (16-18hrs).

Joyner Lab, April 2007
Day 2: Post hybridization washes (all steps are carried out in Coplin jars – 50ml)

1. Float off coverslips by incubating slides in prewarmed 5x SSC (do not force the coverslip off or tissue may tear)
2. Place in prewarmed high stringency wash: 50% formamide, 2x SSC at 65°C for 30’ (prepare 200ml)

Prepare 50ml aliquots of Rnase buffer (5 – use 1 for Rnase A), 2x SSC (1), 0.1x SSC (1) prewarm in 37°C.

3. Wash in RNase Buffer at 37°C 3x for 10’ each

   RNase Buffer: (1L, can be stored at RT)
   100ml of 5M NaCl (0.5M)
   10ml of 1M Tris-HCl, pH7.5 (10mM)
   10ml of 0.5M EDTA, pH8 (5mM)
   880ml of dH2O

4. Using the same buffer, treat with 20µg/ml RNaseA at 37°C for 30’ (100µl 10 mg/ml RNaseA/50ml buffer)
5. Wash in RNase buffer at 37°C for 15’
6. Repeat high stringency wash (as in step 2) 2x at 65°C for 20’ each
7. Wash in 2x SSC, then in 0.1x SSC for 15’ each at 37°C
8. Wash with PBT (PBS + 0.1% Tween-20) for 15’ at RT
9. Place sections horizontally in a humid box and block for 1h at RT with 10% heat-inactivated goat serum in PBT. Use between 200-300µl per slide
10. Remove blocking solution and incubate with alkaline phosphatase-coupled anti-digoxigenin antibody (Boehringer Mannheim, BM) diluted 1:5000 in PBT with 1% goat serum at 4° overnight or for 3-4 hrs at RT. Use approximately 320µl per slide – cover with with parafilm coverslip or Rnase free coverslip.

Day 3: Visualization of reaction product

1. Remove antibody and place slides in a Coplin jar wash 4x in PBT at RT for 15’ each
2. Wash 2x 10’ in freshly prepared NTMT buffer

   NTMT: (100ml)
   2ml 5M NaCl (100mM)
   10ml 1M Tris-HCl pH9.5 (100mM)
   5ml 1M MgCl2 (50mM)
   0.1ml Tween-20 (0.1%)
   82.9ml dH2O

   before use, add 0.5mg/ml levamisole (add 50mg or 1 ml of 50mg/ml stock solution).

3. Place sections horizontally in a coplin jar containing 50ml of BM purple/25mg levamisole and incubate in the dark at RT o/n. [alternatively: place slides in humid box and add approximately 300µl of BM-purple AP substrate (BM 1442 074) containing 0.5mg/ml levamisole. Incubate in the dark at RT overnight (or as is required)].
4. Wash slides in PBS for 2-5’.
5. Postfix with 4% PFA briefly.
6. Wash with PBS and then H2O.
7. Opt.: Counterstain in 0.005% Fast Red (dilute 1/20 from 0.1% Fast Red, Poly Scientific) 1min.
8. Dehydrate and mount with Permount (Fisher SP15-100).

Additional reagents: (20x SSC can be obtained from Molecular Cloning)
5x SSC (1L): 250ml of 20x SSC (pH 4.5) diluted to 1L
2x SSC (1L): 100ml of 20x SSC (pH 4.5) diluted to 1L
0.1x SSC (500ml): 2.5ml of 20x SSC (pH 4.5) diluted to 500ml
High stringency wash (500ml): 250ml formamide:250ml of 20x SSC (pH 4.5)