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 (2X), 70% 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. <u>For paraffin sections only</u>: 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
	50ml	Formamide (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 $NaPO_4$ (pH8)	10mM
	5ml	20% Sarcosyl (can't autoclave)	1%
	11.5ml	DEPC-H_2O (1ml DEPC in 1L of d H_2O; alternatively use Ultrapure bottled H_2O)	
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 H_2O (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).

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 dH_2O
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 (Boerhringer 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 $MgCl_2$ (50mM)
   0.1ml Tween-20 (0.1%)
   82.9ml dH_2O
   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 containg 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 H_2O.
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)