Non-radioactive Section In Situ Hybridization for Embryos

Preparation and sectioning of embryos

Dissect embryos into PBS and be sure to remove all membranes. Fix the litter in 10ml of 4% paraformaldehyde in PBS at 4° C, o/n 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 10-14 μm thickness.

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.
4. Drain and wash with PBS for 5'
5. Refix in 4% PFA for 5', then wash 5' in PBS 3 times.
6. Acetylation: 125 μl acetic anhydride into 50 ml of 0.1M TEA-HC pH7.5 for 10 min. (optional) [0.1 MTEA (100ml: 98 ml water with 1.3 ml TEA and 260 ul HCl].
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	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 $NaPO_4$	10mM
5ml 20% Sarcosyl	1%
11.5ml DEPC-H_2O	
Total: 100ml	

9. Take 2μl of probe (approx. 1μg) to 1ml of hybridization solution and heat at 80°C for 2'.
10. Place slides horizontal in a humid box with tissues soaked in DEPC-H_2O. Cover sections with 200μl of hyb solution and lower parafilm 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 horizontally 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'
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. Use approximately 320µl per slide. No coverslip

Day 3: Visualization of reaction product

1. Remove antibody and place slides in a Coplin jar wash 4x in PBT at RT for approximately 8h in total.
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
3. Place sections horizontally in a 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. 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).