Section in situ hybridization (SISH)

<Preparation of the embryos for frozen section>, <Cutting frozen sections> : as for the immunofluorescence. Better to put 2 sections per slide for this purpose.

<In situ hybridization>
For all the steps up to hybridization, be extra-clean to prevent RNase contamination.

Defrost slides at room temp. Dry for 20 min.
Wash in PBS (for all SISH, PBS with Ca, Mg) (use glass jar, 10 slides/200 ml capacity) 5 min x3 to remove OCT.
Fix in 4% paraformaldehyde in PBS at room temp. for 10 min.
Rinse in PBS 3 min x3.
Proteinase K (cat# 3 115 836, unit: 25 mg, Roche; prepare 10 mg/ml stock, aliquot 100 ul, store at –20 C) treatment : 1 ug/ml, 15-20 min at room temp.
Rinse quickly in PBS x1
Fix in 4% paraformaldehyde in PBS at room temp. for 5 min.
Rinse in PBS 3 min x3
Acetylation : In the glass jar,

DDW	200 ml
Triethanolamine	2.66 ml (cat# 90279, unit 100 ml, Fluka)
37% HCl	0.35 ml

start stirring with magnetic bar, then add

Acetic anhydride	0.75 ml (cat# A-6404, unit 200 ml, sigma)

Wait for a few seconds for big drops to disappear.
Immediately place slides, incubate for 10 min with rocking.
Rinse in PBS 5 min x3.
Prehybridization – prepare a chamber (slide box) humidified with 50% formamide/5x SSC. Add 200 ul of hybridization buffer (warmed at 65 C to dissolve SSD precipitation) per section, and incubate at room temp for 2-4 hrs.

Hybridization buffer:
- 50% Formamide (cat# FX0420-6, unit 1 kg, EMD)
- 5x SSC, pH 4.5 (Sigma, premixed powder for 20x SSC, use citric acid to pH, filter 0.2 um; citric acid: monohydrate, granular, cat# 0627-12, 500 g, Mallinckrodt chemicals)
- 50 ug/ml yeast tRNA (cat# 15401-029, unit 50 mg, Invitrogen)
- 1% SDS (AB01920, 1 kg, American Bioanalytical)
- 50 ug/ml Heparin (sigma H-8514)

Hybridization – Aspirate prehyb sol., add DIG probe 500 ng/ml (prewarm to 70 C) (or 1 mg/ml for weak probe) 120 ul per section, coverslip, wrap the slide box tightly with plastic wrap, and incubate at 72 C (or 60 C, depending on signal intensity and background) o/n.
Prewarm 5x SSC and 0.2x SSC sol. to 72 (or 60) C.

Remove the coverslip by immersing the slides in 5x SSC, 74 C (or 63 C) for 5 min. If the parafilm doesn't come off, shake gently to let it fall. Do not peel it off the slide manually.
Incubate in 0.2x SSC, 74 C or (63 C), 30 min x2, then 0.2x SSC RT 5 min.
Transfer slides to NTT for 5 min RT.

NTT: 0.15 M NaCl
0.1 M Tris pH 8.0 (UltraPure Tris, cat# 15504-020, 1 kg, Invitrogen)
0.1% Tween-20 (Sigma, P-1379, 100 ml)

Blocking – 5% HISS (see Immunofluorescence protocol) + 2% Blocking reagent (cat# 1 096 176, unit 50 g, Roche; heat at 65 C to dissolve, cool to RT before adding serum) in NTT. Add 200 ul per section and incubate in humidified chamber (slide box with DDW) or slide mailer at room temp. 1-2 hrs.
Antibody binding – 2 ul of anti-Digoxigenin-AP (Fab fragments, cat# 1 093 274, 150 U/200 ul, Roche) to 10 ml of 1% HISS/2% Blocking reagent in NTT. Add 200 ul per section, cover with coverslip, and incubate in humidified chamber (slide box with DDW), wrapped with plastic wrap, at 4 C o/n.

Remove parafilm by immersing slides in NTT (from this step, use 20 slide-capacity plastic green bucket, 10 slides/bucket). Wash in NTT 30 min x3, room temp., with agitation.
Wash in NTTML 5 min x3, room temp., with agitation.

NTTML: 0.15 M NaCl
0.1 M Tris pH 9.5 (from 2 M Tris pH 9.5, filtered)
0.1% Tween-20
50 mM $MgCl_2$ (from 1 M $MgCl_2$, filtered)
2 mM Levamisole (Sigma, L-9756, unit: 10 g; prepare 2 M stock, aliquot 500 ul and store at –20 C)

Incubate in BM purple (AP substrate, precipitating, cat# 1 442 074, unit 100 ml, Roche) in slide mailer, upright, in dark. Room temp. 1-4 days.

Rinse in PBS 5 min x3.
Fix in 0.2% glutaraldehyde (cat# G-5882, unit 10 ml, Sigma)/4% paraformaldehyde in PBS, at room temp. for 1-2 hrs.
Rinse in water briefly x3.
Air dry o/n.
Xylene 2 min x2
Coverslip with Permount