In Situ Hybridization Combined With Antibody Staining On Tissue Sections

Buffers

<u>0.2M Phosphate Buffer pH7.2</u>
16.6 gr $Na_2HPO_4 7H2O$
2.5 gr NaH_2PO4
water to 400 ml

<u>Hybridization Solution</u>
50% Formamide
5XSSC
5X Denharts
250ug/ml yeast RNA
500ug/ml Salmon sperm DNA

<u>TNT</u>
10ml Tris pH7.5
3 ml 5M NaCl
250ul 20% Tween 20
to 100 ml with H_20

<u>4% Paraformaldehyde (in 0.1MPB) (make fresh)</u>

50 ml water
4 gr paraformaldehyde
heat to 60-70 C, add 1 drop 10 N NaOH, and stir 10 minutes to dissolve
add 50 ml 0.2M P.B. (final conc. 0.1M)
sterile filter and store on ice up to 1 day

<u>30% Sucrose in 0.1M P.B. solution</u>

100 ml 0.2M P.B.
60 gr sucrose
dissolve and bring final volume up to 200 ml with water

Tissue Preparation (See attached for fixation protocol)

1. Dissect embryos in L15 media
2. Fix embryos/tissue for 2hrs with 4% Paraformaldehde./ 0.1M Phosphate pH7.2 at 0°C.
3. Transfer tissue to 30% Sucrose/0.1M PB until they sink or O/N.
4. Mount tissue in OTC and store at –70°C
5. Cut sections (12µ) using Fisher superfrost plus slides (No. 12-550-15)

Insitu procedure combined with Ab staining

Day1: (Slides may be freshly cut or frozen)

1. Fix slides with 4% PF/0.1M PB for 10'
2. Wash with PBS 3X for 3'
3. To block endogenous peroxidase , treat with 0.1% H202in PBS.
4. Wash with PBS 2X
5. Acetylate for 10' at RT (5.3 ml triethanolamine, 525ul glacial HCL, 750ul acetic anhydride in 400ml final volume)
6. Permeabilize 30' in PBS/0.1%TX100
7. Wash in PBS 3X for 5'
   DO NOT USE PROTEINASE K
8. Prehyb. With 500ul hyb solution for 1 hr at RT. Horizontal in a (*50%
9. Formaldehyde, 5X SSC) humidified tray.
10. Dilute probe 1/100 .(Use 100ul-200ul /slide) and heat to 80 °C for 5'.
    Then transfer to ice for 5'.
11. Replace prehyb with 100ul heated/cooled probe hybridization solution.
    Coverslip and place in a *humidified cassette (seal with tape) o/n at 72°C. Use a separate cassette for each probe.

Day2: Washing and Immunostaining

1. Remove coverslip from slides submerged in 5X SSC.
2. Wash in 5XSSC for 5' at 72°C
3. Wash in 0.2X SSC for 1hr at 72°C
4. Wash in 0.2X SSC for 5' at RT
5. Wash in B1 Buffer for 5' at RT
6. Block with TNB (0.5%Blocking Reagent /Roche cat#1096176 in TNT) for 1hr at RT. Use 1 ml /slide and keep slide horizontal in humidified tray.
7. Aspirate off blocking solution and apply 0.5 ml of anti-DIG-POD antibody in TNB and anti X (your primary antibody) o/n at 4°C in a humidified chamber.

Day3

8. Add FITC secondary antibody (to detect your protein).
9. Wash 3X with TNT (0.1Mtris pH7.5, 0.15MNaCl. 0.05%Tween20)
10. Detect DIG probe with CY3 Tyramide 1/100 in amplification diluent for 5-10 minutes at RT
11. Wash 5 times in TNT
12. Coverslip with anti quenching mounting media (ie Vectashield/Vector)

Note:
The reagents for the florescent detection (TSA-Tyramide Signal Amplification) are purchased from NEN Life Science Products/Perkin Elmer.