Whole-mount in situ hybridization

(All the solution must be RNase free!)

1. Making digoxigenin (DIG)-labeled riboprobe

--- Design two pairs of primers:
--- Antisense pair: gene specific primer 1 (GSP1) as forward primer and gene specific primer 2 (GSP2) with T7 polymerase promoter sequence (**TAATACGACTCACTATAGGG)** as reverse primer.
--- Sense pair: gene specific primer 1 (GSP1) with T7 polymerase promoter sequence as forward primer and gene specific primer 2 (GSP2) as reverse primer.
--- PCR to amplify the region between GSP1 and GSP2 using genomic DNA or cDNA as template.
--- Purify PCR products
--- Use Ambion T7 RNA polymerase kit and DIG-UTP mix to generate DIG-labeled riboprobe.
--- Heat riboprobes at 65°C for 10 minutes in 120 mM sodium carbonate, 80 mM sodium bicarbonate, pH 10.2 to generate smaller fragments to better penetrate fixed samples.
--- Ethanol precipitated riboprobes by adding 3X volume of EtOH and 0.1X volume of sodium acetate (pH 5.2). Freeze for 20 min and full spin for 5 min. Wash once with 75% DEPC-treated EtOH and air dry for 5 min.
--- Resuspend riboprobes in hybridization buffer (50% formamide, 5XSSC, 100 µg/ml sonicated salmon sperm DNA, 50 µg/ml heparin, 0.1% Tween 20 in DEPC-treated water). The concentration of probes can be confirmed by dot blot analysis.

2. Hybridization

--- Tissues are dissected and fixed in 4% paraformadehyde-DMSO (5:1) for 30 minutes at room temperature (RT).
--- After washing in PBS containing 0.01% Tween 20 (PBST), tissues are treated with 10 µg/ml proteinase K in PBST for 10 minutes at RT, washed and refixed in 4% paraformaldehyde-DMSO (5:1) for 20 minutes.
--- Fixed tissues are prehybridized in hybridization buffer for 1 hr at 55°C
--- Then hybridized in hybridization buffer with antisense or sense riboprobes for 12 hours at 55°C.
--- After hybridization, tissues are washed in hybridization buffer over 3 hours with three changes of hybridization buffer at 55°C and one wash at RT.
--- Then hybridized tissues are blocked in 1% normal goat serum in PBST for 30 minutes
--- Then incubate tissues for 1 hr with an alkaline phosphatase-conjugated anti-digoxigenin mAb (Roche) in 1% goat serum in PBST.
--- Wash three times with PBST
--- Develop color with 5-bromo-4-chloro-3_-indolyphosphate *p*-toluidine (BCIP) and nitro-blue tetrazolium(NBT) (Roche). Stop the color reaction with EDTA.
--- Wash three times with PBST
--- Stain with DAPI (1:1000) for 5 min
--- Wash three times with PBST
--- Clean the tissue with 50% glycerol for 30 min. Mount tissues and photograph.