In situ hybridization with Alkaline-Phosphatase detection

Embryos for in situ:
Embryos should be fixed in 4% formaldehyde/PBS (see fixation protocol) and washed 3 times in 100% methanol (instead of ethanol) prior to storage at –20C. It is recommended that freshly fixed embryos (less than 1 month old) be used for best results. Approximately 25ul of embryos is a good amount to use per in situ probe, although more or less can be used.

Antibody staining following in situ:
It is common to stain with antibodies after fluorescence in situ hybridization. No special steps are required between the in situ protocol and antibody staining protocol. After step 22 of this protocol, begin the antibody staining protocol at step 3 (the initial blocking step).

In situ hybridization using DIG-labeled RNA probes:
Day 1:

1. Remove methanol from formaldehyde-fixed embryos.
2. Rehydrate for 2 minutes in 3:1 Methanol: (4%Formaldehye/1xPBS).
3. Rehydrate for 5 minutes in 1:3 Methanol: (4% Formaldehyde/1xPBS).
4. Fix for 10 minutes in 4% Formaldehyde/ 1xPBS.
5. Wash 6X in 1X PTw. Remove final wash.
6. Add 1ml Hybridization buffer without dextran sulfate.
7. Rock for 1 hour at room temperature.
8. Dilute probe 1:100 in Hybridization buffer with 5% dextran sulfate (I use 1ul of probe in 100ul of Hybridization buffer).
9. Heat probe dilution to 85°C for 5 minutes, place on ice 2 minutes, then place at 55°C for 2 minutes.
10. Remove hybridization buffer without dextran sulfate from embryos. Add warmed probe. Incubate O/N at 55°C.

Day 2:

11. Add 150ul of wash buffer to embryos. Place at 55°C until embryos settle. Remove liquid and wash 2X in 150ul of wash buffer at 55°C.
12. Wash 8X for 30 minutes in 150ul of wash buffer at 55°C.
13. Wash O/N in wash buffer at 55°C. (I have left embryos washing for 3 days at 55°C, in general, the longer you wash the more background you remove).

Day 3:

14. Rinse in 1ml of PTw.
15. Rock for 30 minutes at RT in 1ml of PTw; remove PTw.
16. Rock for 2 hours at RT in 100ul of PTw containing 5% goat serum and 1:2000 dilution of anti-DIG-AP Fab fragments (Roche).
17. Rinse 2X with 1ml PTw.
18. Wash 9X for 10 minutes with 1ml PTw.
19. Rinse 2X with 1ml AP buffer (make up fresh).
20. Wash for 5 minutes at RT in 1ml AP buffer; remove AP buffer.
21. Add 1ml BCIP/NBT color development solution.
22. Rock at RT until desired color is achieved. (Can be anywhere from 20 minutes to 5 hours at RT. If the color has not developed by 2 hours, you can place at 4°C O/N)

23. Rinse 6X in PTw to stop color reaction.
24. Add 70% glycerol, store at 4 °C until ready to mount.

Solutions:

4% formaldehyde/1xPBS: 1x PBS containing 4% formaldehyde. Example: for 10ml use 1.05ml 37% formaldehyde, 1ml 10xPBS, 7.95 ml water.

PTw: 1X PBS containing 0.1% Tween 20

Hybridization buffer: 4X SSC, 50% deionized formamide, 0.01% Tween 20

Hybridization buffer with dextran sulfate: Add 5% dextran sulfate to hybridization buffer

Wash buffer: 2X SSC, 50% deionized formamide, 0.01% Tween 20

AP buffer: 50mM MgCl2, 100mM NaCl, 100mM Tris pH 9.5, 0.01% Tween 20

BCIP/NBT color development solution: 3.5ul of 50 mg/ml BCIP and 4.5ul of 50mg/ml NBT in 1 ml of AP buffer.

70% glycerol: 70%glycerol in 1XPBS