In situ hybridization with Fluorescence TSA 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 60 minutes at RT in 1ml of Blocking Buffer.
17. Incubate for 2 hours at RT in 100ul of peroxidase-conjugated anti-Dig antibody (Roche).
18. Rinse 3X with 1ml PTw.
19. Wash 3X for 30 minutes with 1ml PTw; REMOVE ALL PTw.
20. Incubate 1.5-2 hours at RT in TSA Amplification diluents + TSA.
21. Rinse 3X with 1ml PTw.
22. Wash 3X for 30 minutes with 1ml PTw.
23. Add 70% glycerol, store at 4 °C until ready to mount.

Modifications:

a) If a second detection of Biotinylated RNA is required, heat-inactivate peroxidase-conjugated anti-Fig antibody at 70C for 15min. This step is done following step 22. After heat inactivation, start at step 17 again but substitute streptavidin-HRP (1:200) for anti-dig-POD and use a different TSA fluorophore.

b) This protocol can be adapted to immunostain following the TSA amplification of RNA probes. After the RNA detection using TSA (after step 22), block embryos for 30 min in PBT+5%NGS (1xPBS, 0.1% BSA, 0.1% Tween-20, 5% NGS). Incubate in primary antibody (time depends on antibody, but overnight at 4C usually works). Wash 3x quick and 3x 30min with PBT followed by 30min in block (PBT+5%NGS).

    a. For direct detection of fluorescent secondaries, incubate 2hrs at 4C in appropriate secondary antibody, wash, and mount.
    b. For TSA amplification of antibody; incubate 60min at RT in biotinylated secondary. Wash 3x quick in PBT, 3x 30 min in PBT, and 1x 30 min in block (PBT+5%NGS). Incubate 60 min at RT in streptavidin-HRP (Jackson labs (1mg/ml) 1:100 in PBT+NGS). Wash 3x quick in PBT, 3x 30min in PBT. Incubate 5-20min in TSA reagent diluted 1:50 in amplification diluent. The timing depends upon the antibody, but 12 min is usually good. Wash embryos in PBT thoroughly. If another immunostain using TSA is required, heat-inactivate HRP by incubating the embryos at 70C for 15 minutes, block, add primary antibody, and repeat above steps. NOTE: Heat inactivation is NOT required between TSA detection of and RNA probe and TSA detection of antibody, it is required between RNA detections and between antibody detections.

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

70% glycerol: 70%glycerol in 1XPBS

Amplification diluents + TSA. Use diluent provided with the TSA Kit. For TSA-Cy5, TSA-Cy3, and TSA-Alexa350 use 1:50 dilution. For TSA-FITC use 1:100 dilution.

TSA kits (Perkin Elmer)
NEL-705A – TSA Cy5 kit
NEL-704A – TSA Cy3 kit
NEL-701 – TSA FITC kit

TSA kit (Invitrogen)
T20937 – TSA Alexa 350

Peroxidase-conjugated anti-Digoxygenin antibody - 11 207 733 910