Protocol for Whole Mount In Situ Hybridization

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0. Prepare 100% EtOH
   100% MetOH
   50/50 Xylene/EtOH (1 ml/sample)
1. Transfer fixed embryos stored in 100% EtOH at -20 degrees to a 1.5 ml tube with 100% EtOH
2. Rinse 100% EtOH, x5 (All rinses and washes are done in 1 ml, unless otherwise mentioned.)
3. Rinse 50/50 Xylene/EtOH, x1
4. Rinse 100% EtOH, x5
5. Rinse 100% MetOH, x2
6. Prepare H_2O_2/MetOH (60 μl H_2O_2 + 840 μl MetOH; at least 450 μl/sample)
7. Incubate with rocking for 20 min in H_2O_2/MetOH.
   During this incubation period, prepare
   PBStw (1x PBS + 0.1% Tween 20)
   Fixative (1.4 ml formalin + 8.6 ml PBStw)
   50/50 MetOH/Fixative (1 ml/sample)
8. Rince 100% MetOH, x3
9. Incubate with rocking for 5 min in 50/50 MetOH/Fixative
   Turn ON the Heat Block (55 degrees)
   Prepare Ice Bucket
   Take out Salmon Sperm DNA (ssDNA), Dextran, and Heparin from the freezer, and put them on ice after melting
10. Rinse PBStw, x5
11. Prepare ProK solution (1 ml PBStw + 4 μl Proteinase K (1 mg/ml); at least 300 μl/sample)
12. Incubate for 3-4 min in ProK solution with occasional shaking
13. Rinse PBStw, x5
14. Post-fix in Fixative for 20-25 min with rocking
    Boil ssDNA in a 1.5 ml tube for 5 min (12 μl/sample)
    Prepare Hybridization solution (see next page) and
    50/50 Hybri(w)/PBStw (1 ml/sample)

15. Rinse PBStw, x5
16. Incubate with rocking for more than 10 min in 50/50 Hybri(w)/PBStw
17. Rinse Hybri (w), x1
18. Pre-hybridize for at least 1.5 hr in 1 ml Hybri (h) at 55 degrees
19. Turn ON another Heat Block (80 degrees)
20. Prepare probe solution (just before use); add 0.5-1 μl RNA probe to 100 μl Hybri (h), heat for 5 min at 80 degrees, and put immediately on ice.
21. Remove Pre-Hybridization solution
22. Add Probe solution, mix well, and let hybridize for more than 18 (12?) hr at 55 degrees.

Preparation of Hybridization Solutions, Hybri(w) and Hybri(h):

a. Mix followings in a 50 ml tube:

Formamide	25 ml
20x SSC	12.5 ml
50 mg/ml heparin	50 μl
20% Tween20	250 μl
H_2O	2.2 ml
	40 ml

b. Hybri (h): This solution is used for Pre-hybridization and Hybridization.
Transfer 4 ml of the solution (made at step a) into another tube.
Separate 880 μl of this solution into a 1.5 ml tube/sample.
Add 220 μl of 250 mg/ml Dextran/tube, mix well, and centrifuge.
Add 11 μl boiled ssDNA/tube, mix well, and centrifuge.

c. Hybri(w): This solution is used in washing steps.
Add 9 ml H_2O into the remaining 36 ml solution (step a).
(Volume of water is 1/4 of the volume of the solution in step a.)
(To prepare 50 ml Hybri(w), add 12.2 ml H_2O instead of 2.2 ml at step a.)

23. Rinse Hybri (w), x1 at room temperature (RT)
24. Wash Hybri (w), for 10-15 min, x5 at 55 degrees
    At each step, pre-warm Hybri (w) for the next step.
25. Wash 50/50 Hybri(w)/PBStw with rocking for 10 min, x1 (at RT hereafter)
26. Wash 25/75 Hybri(w)/PBStw with rocking for 10 min, x1
27. Wash PBStw with rocking for 10 min, x5
    <<For Tyramide reaction, go to page 5>>
28. For AP staining, prepare an antibody solution by diluting the antibody stock with PBStw.
        Anti-DIG-AP (Roche), 1:1000
        Anti-FITC-AP (DAKO), 1:10
        Anti-DNP-AP (Vector), 1:50
29. Incubate in the antibody solution with rocking for more than 1.5 hr
30. Rinse PBStw, x1
31. Wash PBStw, for 5-10 min, x3
        Prepare AP buffer (see next page)
32. Incubate in AP buffer, 5 min, x2
33. Transfer embryos into 24- or 4-dish.
34. Prepare Staining solution (see next page)
35. Incubate in Staining solution, protect from light.
    Examine the coloring reaction at some intervals, without turning on light.
36. When good staining is observed, stop the reaction by rinse with PBStw several times.
37. Post-fix for more than 20 min at RT or over night at 4 degrees.
38. Rinse PBStw, x3-5
39. Incubate in AP inactivation solution with rocking for more than one hour.
40. Rinse PBStw, x3-5
41. For samples stained with NBT/BCIP, EtOH treatment: Incubate in 50/50 PBStw/EtOH for 5 min; 100% EtOH 5-10 min, x1 or 2; 50/50 PBStw/EtOH for 5 min; Rinse PBStw, x3-5
42. Detection of another probe (go to step 28), Detection of Biotin-dextran,

Counter-stain with DAPI, or Observation.

Preparation of AP buffer and Staining solution

<Staining with NBT/BCIP>

AP buffer

5M NaCl	400 μl
1M $MgCl_2$	1 ml
2M Tris-Cl (pH9.5)	1 ml
20% Tween20	100 μl
H_2O	17.5 ml
	20 ml

Staining solution

Mix followings in 1ml AP buffer and centrifuge At least 500 μl/sample.

50 mg/ml NBT 6.6 μl

50 mg/ml BCIP 3.3 μl

Tyramide Reaction

Samples from Step 27 (page 3)

1. Rinse with TNT (0.1 M Tris (pH7.5), 0.15 M NaCl, 0.05% Tween20).
   1 M Tris, 5 ml; 5 M NaCl, 1.5 ml; 20% Tween20, 125 μl/50 ml
2. Blocking: Incubate with rocking in Blocking solution (PE) for more than 30 min at RT.
3. Dilute an Antibody stock solution with Blocking solution
   Anti-DNP-HRP (PE), 1:100
4. Incubate with rocking in Antibody solution for more than 1.5 hr at RT
5. Rinse TNT, x1
6. Wash TNT, for 4 min x3
   Prepare 4-Iodophenol: 30 mg 4-Iodophenol dissolve in 1 ml of 100% EtOH
   Prepare H_2O_2: add 1 μl H_2O_2 in 100 μl H_2O
   Take out 250 mg/ml Dextran from freezer
7. Rinse Borate Buffer (pH8.5), 4min x2
   Borate, 0.309 g; 5M NaOH, approx. 100 μl; 20% Tween20, 250 μl/50 ml
8. Prepare Tyramide Reaction solution:

H_2O_2 (1/100)	1 μl
4-Iodophenol (30 mg/ml)	1 μl
Dextran (250 mg/ml)	8 μl
Borate Buffer 100 μl ß	

   Mix well, centrifuge, and add appropriate Tyramide.
   1 μl for DNP (DNP amplification reagent)
   Mix well and centrifuge
9. Incubate in Tyramide Reaction solution for 15 min.
10. Rinse with PBStw x2
11. Wash PBStw for 4 min x3

<Staining with Vector Red>

1. Dilute an Antibody stock solution with Blocking solution
   Anti-DNP-AP (Vector), 1:100
2. Incubate with rocking in Antibody solution for 1.0 hr at RT
3. Rinse PBStw x1
4. Wash PBStw for 5min x 3
6. Wash APtw (pH8.5) buffer 5min x2
7. Rinse AP (pH8.5) buffer x1 (not entirely)
8. Incubate in Staining solution for (protect from light).

Examine the coloring reaction at some intervals. If the signal is not strong at 45 min, the staining solution may be replaced with a new one.

9. Stop the reaction by rinsing with PBStw several times.
10. Post-fix for more than 20 min at RT or over night at 4 degrees.
11. Rinse with PBStw, x3-5
12. Incubate in AP inactivation solution with rocking for more than one hour.
13. Rinse PBStw, x3-5

AP buffer

5M NaCl	400 μl
1M $MgCl_2$	1 ml
2M Tris-Cl (pH8.5)	1 ml
H_2O	17.5 ml
	20 ml

Separate an appropriate volume (2.5-10 ml) of this buffer.
Add 20% Tween20 in the remaining AP buffer for final 0.1%.

Staining solution

Add 1 drop of sol. 1 (supplied in a Vector Red kit), 1drop of sol. 2, and 1 drop of sol. 3 into 2.5 ml AP buffer without Tween20.
Change to fresh Vector Red Staining solution every 45 min.
*NBT/BCIP should be used first, and Vector Red is used second.
**Signal can be amplified by combining a-DNP-HRP + DNP-Tyramide and a-DNP-AP.