Double *in situ* Hybridization

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[Abstract] Double *in situ* hybridization is very useful to examine the relationship between the expression of two genes. However, it can be a tricky experiment because of the cross reaction of two different antibodies. This protocol provides a solution to overcome this problem and perform double color *in situ* hybridization in zebrafish embryos.

Materials and Reagents

1. Methanol
2. 5-Bromo-4-Chloro-3-Indolyl Phosphate (BCIP)
3. Nitrotetrazolium blue chloride (NBT)
4. EDTA
5. INT
6. Tris
7. Tween 20
8. Glycine
9. Na_2HPO_4
10. HCl
11. NaCl
12. Sodium Citrate
13. $MgCl_2$
14. KCl
15. PFA
16. Sheep serum
17. BSA
18. Citric acid
19. Formamide
20. Dechorionate
21. Hybe+ buffer (5ml/tube)
22. Torula Yeast RNA (Sigma-Aldrich, catalog number: R6225)
23. Heparin (Sigma-Aldrich, catalog number: H0777)

24. Lamb Serum (GibcoBRL, catalog number: 16070096)
25. Fast Red staining buffer (FRSB) [1 M Tris (pH 8.2), 0.1% Tween]
26. Proteinase K (Roche Diagnostics, catalog number: 10165921001)
27. Fast Red talets (Roche Diagnostics, catalog number: 11496549001) or INT/BCIP (Roche Diagnostics, catalog number: 11681460001)
28. NBT/BCIP (Promega Corporation, catalog number: S3771)
29. Anti-DIG -AP (Roche Diagnostics, catalog number: 11093274910)
30. Anti-Fluorescine AP (Roche Diagnostics, catalog number: 11426338910)
31. NBT/BCIP (see Recipes)
32. 1x PBT (see Recipes)
33. 20x SSC (see Recipes)
34. 10x PBS (see Recipes)
35. 4% Paraformaldehyde (see Recipes)
36. Hybe+ buffer (5ml/tube) (see Recipes)
37. Heat Inactivated Lamb Serum (see Recipes)
38. Blocking solution (see Recipes)
39. Staining buffer (see Recipes)
40. Stop solution (see Recipes)

Equipment

1. Rotator
2. Nalgene filter
3. Hybridization Incubator

Procedure

A. Preparation of Embryos

1. Fix in p-formaldehyde (4%) o/n at 4 °C.
2. Wash twice in PBT (fresh made stock) and dechorionate.
3. Wash and equilibrate with methanol (3x, for 5 min each).
4. Store at -20 °C.

Day 1

B. Rehydration of Embryos

1. Wash for 5 min each in Methanol: PBT sequentially (3:1, 1:1, 1:3).
2. Wash 4x, 5 min each in 100% PBT.

3. Incubate in Proteinase K (dilute 1 mg ml^{-1} stock 100 fold. 100 µl in 10 ml PBT).
    a. Younger than “bud”: 30 sec.
    b. Early Somitogenesis: 1-2 min.
    c. Late Somitogenesis (14-22 s): 2-4 min.
    d. 24 hpf: 10 min.
    e. 36/48 hpf: 20 min.
4. Wash once (quick) in PBT to get rid of the proteinase K. (optional).
5. Refix for 20 min in 4% p-formaldehyde at room temperature (RT).
6. Rinse 5x, 5 min in PBT.

C. Hybridization

1. Prehybridize embryos in hybe+ buffer (5 ml/tube) at 70 °C for 2-5 h.
2. Replace prehybe with hybe+ buffer containing the two probes of choice (~150-200 ng of each probe/200 µl hybe+ buffer).
3. Incubate o/n at 70 °C.

Day 2

4. Remove hybe/probe mixture and store at -20 °C (can be used up to 3x).
5. Washes:
    a. 100% prewarmed hybe- buffer, 10 min, 70 °C.
    b. 75% hybe-/25% 2x SSC, 15 min, 70 °C.
    c. 50% hybe-/50% 2x SSC, 15 min, 70 °C.
    d. 25% hybe-/75% 2x SSC, 15 min, 70 °C.
    e. 100% 2x SSC, 15 min, 70 °C.
    f. Wash 2 times in 0.2x SSC, 30 min, 70 °C.
    g. 75% 0.2x SSC/ 25% PBT, 10 min, RT.
    h. 50% 0.2x SSC/ 50% PBT, 10 min, RT.
    i. 25% 0.2x SSC/ 75% PBT, 10 min, RT.
    j. PBT, 10 min, RT.
6. Block embryos in PBT/2% sheep serum/2 mg/ml BSA at RT for 2 h.

D. First Ab Incubation (anti-fluorescein-AP)

1. Incubate embryos with 500 µl of antibody solution (1:2,000 dilution) for 2 h at RT or o/n at 4 °C, rocking on a rotator.

E. Staining the embryos (Fast red method)

1. Wash excess ab off embryos 6x, 15 min in PBT, shaking at RT.

2. Wash 2-3x in FRSB (1 M Tris, pH 8.2, 0.1% Tween).
3. Stain in Fash Red Solution (1 tablet in 2 ml FRSB).
4. After staining is complete wash 3x, 5 min each at RT in 0.1 M glycine (pH 2.2), 0.1% tween to remove the antibody.
5. Wash 3-4x in PBT to remove all the glycine.

Or staining the embryos (INT method)

1. Wash embryos 2x for 5 min each in staining buffer.
2. Stain embryos in the following solution: 31.5 µl INT, 35 µl BCIP to 10 ml with staining buffer.
3. To stop reaction fix for 20 min at RT in 4% PFA.
4. To get rid of primary ab, wash 3x for 5 min each at RT in 0.1 M glycine (pH 2.2), 0.1% tween 20.
5. Wash 3x for 15 min each at RT in PBT to remove all the glycine.

F. Second Ab Incubation (anti-DIG-AP)

1. Incubate embryos with 500 µl of antibody solution (1:5,000 dilution) o/n, rocking on anutator, at 4 °C or for 2 h at RT.

Day 3

G. DIG Staining

1. Wash quickly in PBT.
2. Wash 6x, 15 min in PBT, shaking at RT.
3. Wash 2-3x, 5 min in staining buffer.
4. Add 90 µl of 50 mg/ml NBT and 70 µl of 50 mg/ml BCIP to 20 ml staining buffer.
5. Add about 500 ml of staining buffer to embryos and wrap rack in aluminum foil and shake at RT. Check new probes every 30 min to 1 h.
6. Stop reaction by washing in Stop Solution 3x [PBS (pH 5.5), EDTA 1 mM)] or 4% PFA.
7. Store embryos in 4% PFA at 4 °C in a closed box.

Recipes

1. 20x SSC (pH 7.0)
   NaCl 175.3 g
   NaCitrate 88.2 g
   for 1 L
2. 10x PBS
   To 800 ml ddH_2O dissolve

NaCl 80 g

KCl 2 g

Na_2HPO_4 14.4 g

KH_2PO_4 2.4 g

pH to 7.4 with HCl and add ddH_2O to 1 L.

* Filter 1x PBS through a .2 µm Nalgene filter. Store at RT.

3. 1x PBT

   10x PBS (pH 7.4) to 1x PBS

   Make a 20% Tween stock. The final concentration of Tween for PBT should be 0.1%.

4. 4% Paraformaldehyde/PBS

   4 g in 100 ml of PBS, dissolve at 650 C, (or microwave while carefully watching).

5. Hybe+ buffer

   50% Formamide 25 ml of 100% stock

   5x SSC 12.5 ml of 20x stock

   0.5 mg/ml Torula Yeast RNA 1.25 ml of 20 mg/ml stock

   50 mg/ml heparin 50 µl of 50 mg/ml stock

   0.1% Tween 250 µl of 20% Tween

   1 M citric acid 460 µl

   50 ml total volume

6. For Hybe-, leave out the torula yeast RNA and the heparin.

7. Heat Inactivated Lamb Serum

   Thaw Lamb Serum and heat inactivate at 55 °C for 30 min. Store in aliquots at -20 °C.

8. Blocking solution

   100 mg BSA (Sigma-Aldrich)

   1 ml 100% Lamb/Sheep Serum

   50 ml PBT

9. Staining buffer

   10 ml 1 M Tris (pH 9.5)

   5 ml 1 M $MgCl_2$

   2 ml 5 M NaCl

   500 µl Tween 20

   to 100 ml with water

10. NBT/BCIP

    225 µl 50 mg/ml NBT

    175 µl 50 mg/ml BCIP

    to 50 ml staining buffer

11. 3x stop solution

PBS (pH 5.5)

EDTA 1 mM

12. Pre-Staining Buffer

    10 ml 1 M Tris (pH 9.5)

    5 ml 1 M $MgCl_2$

    2 ml 5 M NaCl

    500 µl Tween 20

    To 100 ml with water

13. Satining buffer
14. NBT/BCIP

    225 µl 50 mg/ml NBT, 175 µl 50 mg/ml BCIP, to 50 ml w/ staining buffer

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