In situ Hybridization in Zebrafish Embryos

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[Abstract] *In situ* hybridization is routinely used to examine the gene expression level and location of embryos. This protocol is modified from Thisse *et al.* (2008), and is a detailed description of the *in situ* hybridization procedure in zebrafish embryos.

Materials and Reagents

1. Bovine serum albumin (BSA) (Sigma-Aldrich, catalog number: A9418)
2. PFA (powder) (Thermo Fisher Scientific)
3. DEPC (Sigma-Aldrich, catalog number: D5758)
4. Glutaradehyde (Sigma-Aldrich, catalog number: G5882)
5. Roche anti-DIG AP (Roche Diagnostics, catalog number: 11093274910)
6. Torula yeast RNA (Sigma-Aldrich, catalog number: R6225)
7. Heparin (Sigma-Aldrich, catalog number: H0777)
8. Lamb/Sheep Serum (Thermo Fisher Scientific, catalog number: 16070-096)
9. Formamide (High purity grade)
10. Methanol
11. Sodium Citrate
12. EDTA
13. NaCl
14. KCl
15. $MgCl_2$
16. Na_2HPO_4
17. KH_2PO_4
18. HCl
19. Tween 20
20. Citric acid
21. 1x PBT(made from DEPC H_2O) (see Recipes)
22. Pronase (Roche Diagnostics, catalog number: 10165921001) (see Recipes)
23. NBT/BCIP color substrate (Promega Corporation, catalog number: S3771) (see Recipes)
24. Hybe+ buffer (see Recipes)

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25. Hybe- buffer (see Recipes)
26. 20x SSC (see Recipes)
27. 10x PBS (see Recipes)
28. 4% paraformaldehyde/PBS (see Recipes)
29. Blocking solution (see Recipes)
30. Stop solution (see Recipes)
31. Heat inactivated lamb serum (see Recipes)
32. Pre-staining buffer (see Recipes)

Equipment

1. Hybridization Incubator
2. 1.5 ml tube
3. 24-well plate
4. Aluminum foil
5. Nalgene filter

Procedure

A. Preparation of embryos
   1. Dechorinate the embryos using pronase. (Add 1 drop of pronase solution to 1 ml of E3). Keep the embryos at room temperature (RT) for 3-5 min (do not let the embryos in pronase solution for too long!).
   2. Pipet the embryos to break the chorion.
   3. Collect the embryos in 1.5 ml tubes (usually 20 embryos/per tube).
   4. Remove E3 buffer and rinse embryos with 1 ml of new E3 buffer to get rid of pronase.
   5. Remove all the E3 and add 500 μl of 4% PFA to fix the embryos O/N at 4 °C.

B. Dehydrate embryos
   1. Remove PFA (collect PFA in waste collecting tube) and wash with PBT three times (use PBT made from DEPC H_2O. 400 μl, 10 minutes each wash, total 3 times).
   2. Equilibrate with methanol to dehydrate the embryos (400 μl, 5 min each wash, three times).
   3. Remove methanol (collect methanol in waste collecting tube).
   4. Add 500 μl to each tube. Store the embryos in at -20 °C (at least 2 h).

Day 1

A. Rehydrate embryos

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Wash for 5 min each sequentially in Methanol: PBT (3:1, 1:1, 1:3). (400 µl each wash, use DEPC-PBT.)

Wash 4x, 5' each in 100% PBT. (400 µl each wash)

See note 1 in the end.

Fix embryos in 4% PFA (+0.2% glutaradehyde) at RT for 20 min.

Wash 4x, 5' each in 100% PBT (400 µl each wash).

B. Hybridization

Prehybridize embryos in hybe+ buffer (300 µl/tube) at 70 °C for 30 min-3 h.

Replace prehybe with hybe+ buffer containing the probe(s) of choice. (0.1 ng-1 ng probe/µl hybe+ buffer)

Incubate o/n at 70 °C.

Day 2

Note: After the RNA probe is hybridized to its template, RNA becomes double stranded and is more stable than single stranded. DEPC-PBT is not necessary.

1. Remove hybe/probe mixture and store at -20 °C. (can be used up to 3x)
2. Washes

   75% hybe-/25% 2x SSC 15min, 70 °C
   50% hybe-/50% 2x SSC 15min, 70 °C
   25% hybe-/75% 2x SSC 15min, 70 °C
   100% 2x SSC 15min, 70 °C
   Wash 2 times in 0.2x SSC 30min, 70 °C
   75% 0.2x SSC/ 25% PBT 10min, RT
   50% 0.2x SSC/ 50% PBT 10min, RT
   25% 0.2x SSC/ 75% PBT 10min, RT
   PBT 10min, RT
3. Add blocking solution to block embryos in at RT for several hours (30 min minimum).

C. Antibody incubation

1. After incubation, change buffer for antibody solution (1:5,000 dilution of Roche anti-DIG AP in blocking solution, 500 µl/tube), rock on a platform rotator, at 4 °C O/N.

Day 3

DIG staining

1. Wash quickly in PBT. Use 500 µl/tube.
2. Wash 6x, 15 min in PBT, shaking at RT. 500 µl/tube.

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3. Wash 1x, 5 min in pre-staining buffer (fresh made). 500 μl/tube. Transfer embryos to 24-well plate use plastic pipets.
4. Change the buffer to staining buffer+ (1 ml/well) to the embryos and wrap the plate with aluminum foil and shake at RT.
   a. Staining buffer+: Add 4.5 μl of NBT and 3.5 μl BCIP to 1 ml pre-staining buffer.
   b. Check new probes every 30 min to 1 h.
   c. When the staining is done, collect the staining buffer waste. Wash the stained embryos with 1 ml 2x PBT.
   d. Stop reaction by washing in stop solution.
   e. Leave the embryos in stop solution (1 ml/well) or fix in 4% PFA.
5. Store embryos in stop solution or 4% PFA at 4°C in a closed box.
   Note: For embryos older than 20 somite stage, permeabilization with proteinase K is required to allow the probe to enter the cells.
6. Incubate in Proteinase K (dilute 1 mg/ml stock 100 fold. 100 μl in 10 ml PBT).
   a. Late somitogenesis (14-22 sec): 2-4 min.
   b. 24 hpf: 10 min.
   c. 36/48 hpf: 20 min.
7. Wash once (quick) in PBT to get rid of the proteinase K (optional) and continue the fixation.

Recipes

1. Pronase
   30 mg/ml in E3
2. 20x SSC (pH7.0)
   NaCl 175.3 g
   NaCitrate 88.2 g
   for 1 L
3. 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 0.2 μm Nalgene filter. Store at RT.
4. 1x PBT

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10x PBS (pH 7.4) to 1x PBS

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

5. 4% Paraformaldehyde/PBS

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

6. Hybe+ buffer

50% Formamide	25 ml of 100% stock
5x SSC	12.5 ml of 20x stock
0.5 mg ml^{-1} Torula yeast RNA	1.25 ml of 20 mg/ml stock
50 mg ml^{-1} heparin	50 µl of 50 mg/ml stock
0.1% Tween	250 µl of 20% Tween
ddH_2O	up to 50 ml
	50 ml total volume

   pH to 6-6.5 with 1 M citric acid ~460 µl.

   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 3 min. Store in aliquots at -20 °C.

8. Blocking solution

   100 mg BSA

   1 ml 100% Lamb/Sheep Serum

   50 ml PBT

9. 2x stop solution

   PBS (pH 5.5)

   EDTA 1 mM

10. Pre-staining buffer

    10 ml 1 M Tris (pH9.5)

    5 ml 1 M $MgCl_2$

    2 ml 5 M NaCl

    500 µl Tween 20

    to 100 ml with water.

11. Staining buffer +

12. NBT/BCIP

    225 µl 50 mg/ml NBT

    175 µl 50 mg/ml BCIP

    to 50 ml w/ staining buffer

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