Two Color Whole-Mount RNA In Situ Hybridization

(Source: G. Hauptmann from Zebrafish Book 5th Edition)

I. Preparation of RNA Probe

Reagents:

- **NTP mix:**
  - ATP, CTP, GTP 10 mM each
  - UTP 6.5 mM (all Boehringer)
- **Digoxigenin 11 UTP 10 mM (Boehringer)***
- **Fluorescein 12 UTP 10 mM (Boehringer)***
- **RNAsin 20 40 u/µl (Promega)***
- **T7 RNA Polymerase 15 u/µl (Promega)***
- **T3 RNA Polymerase 15 u/µl (Promega) or 40 u/µl (Boehringer)***
- **5x transcription buffer (Promega):**
  - 200 mM Tris HCl pH 7.9
  - 30 mM MgCl2
  - 10 mM spermidine
  - 50 mM NaCl
  - DNAse 1 u/µl (Promega)
- **TNE:**
  - 20 mM Tris HCl pH 7.5
  - 100 mM NaCl
  - 10 mM EDTA
- **NucTrapTM push columns (Stratagene)***
- **HB4 H2O:**
  - 10 ml formamide
  - 2.5 ml 2xSSC
  - 10 µl heparin (50 mg/ml)
  - 10 µl Tween 20
  - 50 mg torula RNA (Sigma) final volume is 7.6 ml

1. Add in the following order:
   - 1.0 µg linearized, phenol extracted and precipitated template DNA in 9.5 µl H20DEPC
   - 2 µl 100 mM DTT
   - 1.3 µl NTP mix
   - 0.7 µl Digoxigenin 11 UTP or Fluorescein 12 UTP
   - 0.5 µl RNAsin

2. Mix and spin down.

3. Add:
   - 4 µl 5x transcription buffer
   - 2 µl T7 RNA Polymerase or T3 RNA Polymerase
   - final volume is 20 µl

4. Incubate 2 3 hours at 37°C.

5. Add 4 µl DNAse and incubate 30 minutes at 37°C.

6. Add 40 µl H20DEPC

7. Prepare push column device:
   - Push 70 µl TNE through column.
   - Push probe through column.
   - Push 70 µl TNE through column.
   - Volume of purified probe will be about 110 µl.

8. Precipitate with 0.5 volumes 7.8 M NH4Ac and 3 volumes 100% EtOH at 20°C for 30 minutes.

9. Wash with 80% EtOH.

10. Redissolve in 25 µl H20DEPC

11. Remove 1 µl and analyze on a 1% Agarose gel. To check for incorporation you can blot and stain the gel or just do a dot blot.

12. Add 76 µl HB4 H20 and store at 20°C.

13. First try 1 µl probe in 100 µl HB4 for in situ hybridization

Comments

If you get high background with this probe concentration after a short staining time, dilute your probe. If you get no staining at all, this usually means the probe is not very good. Make a new one.
T7 RNA Polymerase from Promega usually works and will produce about 10 µg of RNA. In my hands, T3 RNA Polymerase from Promega for some strange reason often gives a very low amount of RNA. But with good templates, you still get a very good probe. For difficult templates try T3 RNA Polymerase from Boehringer which appears to produce more RNA. Overall, it is better to use T7 RNA Polymerase to produce antisense RNA.

Wizard Miniprep DNA can be used to make the templates. Dissolve in H2O/DEPC. It is best to use 1 µg of template. More than 2 µg does not increase the amount of RNA significantly and less than 0.5 µg is not very effective.

You can also precipitate with NaAc instead of NH4Ac. If you omit the push column and just precipitate with NH4Ac, to save time you will also get a good separation of nucleotides. However, precipitation with LiCl only is not very effective in removing free nucleotides. Hydrolysis of the probe is not advisable because this reduces the sensitivity of the probes. Probes from 0.5-2.5 KB can be used without hydrolysis. Probes longer than 1 kb work best. If you want to combine different fragments of the same gene to get a stronger probe, mix them before the push column step.

II. Fixation and Storage of Embryos
1. Transfer embryos of the desired stage to a small Petri dish.
2. Remove embryos from their chorions using watchmaker forceps.
3. Fix embryos with 4% paraformaldehyde in PBS overnight at 4°C.
4. Wash 4 x 5 minutes in PBSTw at room temperature.
5. Transfer embryos to 100% methanol. Replace with clean methanol after 5 minutes. Store embryos at 20°C in a 24 well tissue culture plate (Falcon 3047) sealed with Parafilm. Embryos should be cooled to 20°C for at least 30 minutes for permeabilization even if you do not want to store them.

III. Proteinase Digestion and Postfixation
All steps are performed at room temperature on a shaker.
1. Transfer embryos to a small Petri dish.
2. Immerse 5 minutes in 75% MeOH in PBSTw at room temperature.
3. Immerse 5 minutes in 50% MeOH in PBSTw.
4. Immerse 5 minutes in 25% MeOH in PBSTw.
5. Rinse 2 x 5 minutes in PBSTw.
6. Digest zebrafish embryos with Proteinase K (10 µg/ml in PBSTw) at room temperature for 1 to 15 minutes depending on the stage: 1 cell to high for 1 minute; 30% epiboly to 10 somites for 2-3 minutes; 10-20 somites for 3-4 minutes 24-32 hpf for 5-6 minutes; 40-50 hpf for 10-15 minutes.
7. Rinse 2 x briefly in 2 mg/ml glycine in PBSTw.
8. Fix in 4% paraformaldehyde in PBSTw for 20 minutes.
9. Wash 5 x 5 minutes in PBSTw.

IV. Hybridization
Following steps are performed at 55°C for zebrafish.
1. Transfer embryos into 2.0 ml Eppendorf tubes.
2. Prehybridize in 350 µl Hb4 for 1-8 hpf.
3. Heat the probe in 100 µl Hb4 at 80°C for 5-10 minutes, quickly cool on ice/ethanol, spin down and keep on ice/ethanol. Replace prehybridization solution with probe solution. Incubate overnight.

V. Washes
1. Wash embryos 2 x 30 minutes in 50% formamide in 2xSSCTw at 55°C or at 65°C.
2. Wash 15 minutes in 2xSSCTw at 55°C or at 65°C.
3. Wash 2 x 30 minutes in 0.2xSSCTw at 55°C or at 65°C.

VI. Detection
All detection steps are performed at room temperature and on a shaker except the staining reactions.
1. Block for 1 h hpf with PBSTw plus 5% sheep serum.
2. Incubate embryos in 100 µl preabsorbed sheep anti Fluorescein AP Fab fragments at a 1:2000 dilution in PBSTw. You can reuse antibody twice.
3. Shake for 2 hours at room temperature or overnight at 4°C. Save used antibody in a new tube designated 1x used or 2x used.
4. Wash 6 x 20 minutes with PBSTw (you can perform one of the washes overnight).
5. Wash 2 x 5 minutes in 0.1 M Tris HCl pH 8.2, 0.1% Tween.
6. Dissolve Fast Red tablets in 0.1 M Tris HCl pH 8.2, 0.1% Tween (2 ml/tablet) and sterile filter.

7. Stain in Fast Red solution for up to 48 hpf.

8. Wash 3 x 5 minutes with PBSTw. (Storage at 4°C possible.)

9. Incubate 10 minutes in 0.1 M glycine HCl pH 2.2 plus 0.1% Tween at room temperature to remove first antibody.

10. Wash 4 x 5 minutes in PBSTw.

11. Incubate embryos in 100 µl preabsorbed sheep anti Digoxigenin AP Fab fragments at a 1:2000 dilution in PBSTw. You can reuse the antibody twice.

12. Shake for 2 hours at room temperature or overnight at 4°C.

13. Wash 6 x 20 minutes with PBSTw (one of the washes overnight possible).

14. Wash 2 x 5 minutes in SB.

15. Stain in SS for up to 48 hours.

16. Wash 3 times with PBSTw.

17. Embryos may be fixed for at least 30 minutes and stored at 4°C in PBSTw (add NaNO₃).

18. Mount in glycerol.

Do not clear embryos in alcohol because Fast Red precipitate is supposed to be unstable in ethanol.

**Comments**

To detect two different RNA transcripts in whole embryos make one RNA probe using Fluorescein UTP and the other using Digoxigenin UTP. Both probes are hybridized at the same time and detected one after another in two rounds of detection. The crucial step is the inactivation and removal of the first applied Fab AP conjugate using the low pH step after the first staining round. Inactivation of the first applied Fab AP conjugate by heating is not recommended, since incubation at 65°C for 20 minutes or at 80°C for 10 minutes causes fading of the red precipitate resulting in a weaker and more diffuse signal. In contrast, the blue precipitate darkens resulting in an increased background in the embryo and a nearly black yolk.

Because Fast Red is less sensitive than BCIP/NBT (5 10x), the stronger probe is always visualized in red. Because the second round of detection is less sensitive than the first, stain the red first. Nevertheless, if necessary, it is also possible to do the purple staining first.

Fluorescein labeled probes are slightly less sensitive than digoxigenin probes. Therefore, the stronger probe is usually detected in red using a fluorescein probe.

To detect transcripts of a single gene use just one probe and one round of detection with BCIP/NBT as substrate.

**Antibodies, Stock Solutions and Reagents**

**Preabsorption of antibody with zebrafish embryos**

Zebrafish embryos need not to be removed from their chorions. The number of embryos you use for preabsorption depends on the amount of antibody you want to preabsorb. The stages used for preabsorption should include the same or older stages you will use in your in situ hybridizations. 2-3 d embryos work well.

1. Fix embryos in 4% paraformaldehyde at 4°C overnight.

2. Wash 4 x 5 minutes in PBSTw.

3. Store embryos in 100% methanol at 20°C.

4. Rehydrate 1 ml of embryos by rinsing 3 x 5 minutes in PBSTw.

5. Transfer embryos to a 2.0 ml Eppendorf tube.

6. Homogenize embryos with a pestle and adjust to about 1.0 ml with PBSTw.

7. Add 10 µl antibody (final 1:100).

8. Preabsorb antibody by shaking at least overnight or store for a few days at 4°C. Spin down embryonic debris and sterile filter (Millex GV 0.22 µm, Millipore) supernatant.

9. Resuspend embryonic debris in PBSTw and sterile filter again.

10. Fill up with PBSTw to a final 1:2000 dilution of antibody.

11. Store preabsorbed antibody (20 ml) at 4°C.

12. The antibody preabsorbed with zebrafish embryos works well also for staining of Drosophila embryos.

**Stock solutions and other reagents**
• 0.5 M PO4 buffer pH 7.3: 
80 ml 0.5 M Na2HPO4 + 20 ml 0.5 M Na2HPO4

• 10xPBS: 
8% NaCl 
0.2% KCl 
0.2 M PO4 buffer pH 7.3

• 1xPBSTw: 
1x PBS + 0.1% Tween 20

• 100 mg/ml glycine in ddH2O: 
store at 20°C

• 20 mg/ml proteinase K in ddH2O: 
store at 20°C in aliquots

• 4% paraformaldehyde in 1x PBS is prepared under a fume hood: 
1. Mix in a beaker: 
2 g paraformaldehyde 
5 ml 10xPBS 
45 ml ddH2O 
2. Heat to 70°C. 
3. Stir for about 2 hours until all paraformaldehyde is dissolved. 
4. Cool to 4°C. 
5. Add 4 µl 1N NaOH. 
6. Mix and store at 20°C in 5 ml aliquots.

• HB4: 
50% formamide (Merck) 
5xSSC 
50 µg/ml heparin 
0.1% Tween 20 
5 mg/ml torula RNA (Sigma)

• 50 mg/ml heparin: 
store at 20°C

• 2xSSCTw: 2xSSC + 0.1% Tween 20

• 0.2xSSCTw: 0.2xSSC + 0.1% Tween 20

• Sheep serum: 
heat inactivate at 56°C for 30 minutes and store at 20°C

• Fast Red tablets (Boehringer): 
0.5 mg napthol substrate 
2.0 mg Fast Red chromogen 
0.4 mg levamisole per tablet

• SB: 
100 mM NaCl 
50 mM MgCl2 
100 mM Tris HCl pH 9.5 
1 mM levamisol 
0.1% Tween 20

• NBT (Boehringer): 
75 mg/ml in 70% DMF/H2O

• BCIP (Boehringer): 
50 mg/ml in 100% DMF

• SS: 
4.5 µl NBT + 3.5 µl BCIP in 1 ml SB Final: 337.5 µg/ml NBT and 175 µg/ml BCIP

Reference: 