mRNA IN SITU HYBRIDIZATION For Sectioned Zebrafish

DAYS 1–2: Harvesting fish, tissue fixation, hybridization

1. Harvest and fix embryos in 4% paraformaldehyde overnight at 4C

**Fix should be made fresh on the day it will be used. Do not store it for long periods. Paraformaldehyde will cross link proteins.*

2. Rinse in 1x PBS for 10 minutes, then transfer to 20% sucrose in PBS
    - Incubate in sucrose until fish sink
    - Store at 4C until embedding (try to embed in same day)

3. Embed fish in OCT blocks
    - Move fish through OCT before positioning in block form to remove all sucrose
    - Slowly freeze blocks in liquid nitrogen by dipping in and out of the liquid nitrogen, store blocks in -80C

**Can embed more than one fish per block*
***Be careful not to treat blocks to roughly, because blocks can crack easily, which makes sectioning difficult*

4. Section tissue at 40-50µm, adhering sections to a superfrost-coated slide
    - Dry slides at 55C for 2-3 hours, to ensure tissue has firmly adhered to the slides

5. Wash slides in DEPC water for 3 minutes to remove OCT media from slides

6. Rinse 3x for 3 minutes each with PBST

** Do washes with slides in slide rack, dipping slides into slide boxes. Large slide boxes hold around 300ml and 30 slides. Small slide boxes hold 50ml and 10 slides (back to back).*
***Transcripts are vulnerable to RNase during these steps; work rapidly.*

7. Premeabilize tissue with proteinase-K7 in PBST

    Reasonable concentration/time combinations to try are:
    - 10 µg/ml, 5min *
    - 1 µg/ml, 20 min

**Proteinase K is expensive;make only as much solution as will be needed to allow gentle agitation of embryos. Use only proteinase K aliquoted for in situs. Left over proteinase K should not be refrozen for in situs, but can be kept and used for routine molecular biology.*

8. Replace proteinase-K with 4% paraformaldehyde, fixing for 20 min at room temperature

9. Wash embryos 3x in PBST, 3 min per wash

10. Remove PBST and replace with "fake" hybridization solution for 3 minutes

11. Outline slides with PAP pen and lay slides flat in RNAse free incubation box. Add 400µl of hybridization solution to each slide.

- Incubate slides in 55 °C for 2 hours in an RNAse free humid chamber

**Humid chamber should be moistened with same percentage of formamide as the hybridization buffer to keep the barometric pressure constant?*

12. Replace prehybridization solution of 0.5ng/µl of fragmented probe to hybridization solution. Add 130µl of hybridization solution with riboprobe.

- Preheat hybridization solution in water bath at 55 °C
- Cover slides with RNAse free cover slips
- Leave slides overnight (8–16 h) at 55 °C in an RNAse free humid chamber.

**Do not allow the slides to cool: it is best to work rapidly.*

DAY 3: Hybridization washes and antibody incubation

1. Remove hybridization solution and wash with:

- 100% fake hybridization buffer briefly, 55 °C
- 75%fake hyb/25% 2xSSC 5 min, 55 °C
- 50%fake hyb/50% 2xSSC 5 min, 55 °C
- 25%fake hyb/75% 2xSSC 5 min, 55 °C
- 2x SSC 10 min, 55 °C
- 0.2x SSC 15 min, room temperature
- 0.2x SSC 15 min, room temperature
- 75% 0.2x SSC/25% PBST 3 min, room temperature
- 50% 0.2x SSC/50% PBST 3 min, room temperature
- 25% 0.2x SSC/75% PBST 3 min, room temperature
- PBST 5 min, room temperature

**For stringency washes, do not let samples cool: work rapidly. Do washes with slides in slide rack, dipping slides into slide boxes.*

2. Outline slides with PAP pen, place slides in humid chamber, and block with 400µl of blocking solution for 2–3 hours at room temperature or overnight at 4 °C

3. Replace blocking solution with anti-DIG Fab fragments 1:5000 in blocking solution; generally 130µl.

- Cover slides with cover slips

- Place slides at room temperature for 2 hours or 4 °C, overnight (≥12 hr)

DAY 4: Antibody washes, developing, and storage

1. Return slides to slide rack and wash in PBST for 10 minutes, repeat 5 more times

2. Wash in AP buffer 2 times for 5 minutes each at room temperature in slide dishes

4. Incubate slides in staining solution (NBT/BCIP in AP buffer) at room temperature either in 50ml in a small slide dish or 400ul on individual, horizontal slides
   - Leave in staining solution covered at room temperature. Check after ~30 minutes and then at increasing intervals over several hours. If you are concerned about leaving the specimens staining unattended, place them at 4 °C
   - Change staining solution with freshly made solution when it starts to lose its yellow or straw color
   - Periodically examine the slides under the microscope to monitor staining progress

**The staining solution is light sensitive, cover slide boxes with aluminum foil*

5. Stop staining by replacing staining solution with PBS pH 5.5
   - Wash with PBS 2–3 times at room temperature, 10–30 min
   - Fix in the dark 1 hour in 4% paraformaldehyde in PBS

6. Mount in 50% glycerol in PBS
   - Allow to dry flat for 1 day, then seal edges of coverslip with clear nail polish
   - Allow nail polish to dry for a few hours, then store at 4 °C

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Solutions and Reagents:

10xPBS, phosphate buffered saline

To make 1 L of 10X stock:

NaCl	80.0 g
KCl	2.0 g
Na2HPO4	14.4 g
KH2PO4	2.4 g
nano H20	to 1.0 L

**dilute to 1X working concentration*

10xPBST, phosphate buffered saline + Tween-20

NaCl	80.0 g
KCl	2.0 g
Na2HPO4	14.4 g
KH2PO4	2.4 g
Tween-20	10.0 ml
nano H20	to 1.0 L

**dilute to 1X working concentration*

"Fake" hybridization solution

Same as hybridization solution, but without expensive components.

Formamide	250 ml
20X SSC	125 ml
10% Tween-20	10 ml
1 M citric acid	4.6 ml
nano H2O	to 500 ml total

**Store at room temperature*

Hybridization solution (T-hyb)

Formamide	250 ml
20X SSC12	125 ml
50 mg/ml yeast tRNA	5 ml
10% Tween-20	10 ml
1 M citric acid	4.60 ml ***
50 mg/ml heparin	500 µl
nano H2O	to 500 ml total

**Use citric acid to give a final solution of pH ~5.5.*

**Scale up or down as appropriate. Aliquot and store at –20 °C.*

20X SSC stock solution (3.0 M NaCl, 0.3 M trisodium citrate)

175.3 g	NaCl
88.2 g	trisodium citrate

800 ml nano H20
Adjust pH to 7.0 with a few drops of concentrated HCl
Adjust volume to 1 L with H2O
**dilute to 1X working concentration*

Antibody blocking solution
5% heat inactivated goat serum, 2 mg / ml BSA in PBST2. Sterile filter and store at 4 °C. Goat serum is heat inactivated by incubating 45 min at 50 °C, aliquot and freeze at –20 °C.

AP buffer
100 mM Tris HCl pH 9.5, 50 mM MgCl2, 100 mM NaCl, 0.1% Tween-20
Make fresh daily from stocks, to make 200 ml (scale up if necessary):

nano H20	164 ml
1 M Tris	20 ml
1 M MgCl2	10 ml
5 M NaCl	4 ml
10% Tween-20	2 ml

NBT/BCIP Staining solution

AP buffer	50 ml
50 mg/ml NBT	225 µl
50 mg/ml BCIP	175 µl

Modified From: David Parichy’s In Situ Hybridization Protocol for Larval Fish and Helena Telfer’s In Situ Hybridization Protocol for Embryos