In situ protocol for zebrafish embryos

(Elizabeth Wiellette/Jen Gutzman)

Special reagents needed

- DIG/Fluorescein RNA labeling mixes [Roche. DIG RNA labeling mix Cat.# 1 277 073; Fluor. RNA labeling mix Cat.# 1 685 619]
- Mini Quick Spin Columns [Roche. Cat.# 1 814 427]
- EM-grade Paraformaldehyde. Electron Microscopy Sciences Cat.# 15700 Comes as 16% PAF in 10mL ampules.
- Proteinase K. Roche Cat.# 1 413 783 Dilute 1 part prot.K, 1 parts H_2O. Store frozen as 1000X solution (approx. 10 mg/mL).
- Formamide redistilled Gibco Cat.# 15515-026. Store frozen
- Torula RNA. Sigma Cat.# R3629. 5 g/bottle – dissolve in 100 mL RNase free H_2O => 50 mg/mL
- BMB. (Boehringer Mannheim Blocking reagent) Roche Cat.# 1 096 176. Dissolve reagent in MAB to make 10% stock solution (w/v). Can be heated gently. Can be autoclaved. Store at –20°C.
- Lamb serum Gibco Cat.# 16070-096. To prepare, heat in 60°C water bath for 30 min. Aliquot and store at –20°C
- Antibodies: Anti-DIG-AP Fab fragments. Roche Cat.# 1 093 274. Anti-Fluor-AP Fab fragments. Roche Cat.# 1 426 338
- NBT 4-Nitroblue-tetrazolium chloride. Roche Cat.# 1 383 213. Comes as 100 mg/mL in DMF – previous solutions have been made at 75 mg/mL in 70% DMF.
- BCIP 5-Bromo-4-chloro-3-indolyl-phosphate. Roche Cat.# 1 383 221. Ready to use as 50 mg/mL in DMF
- INT Sigma Cat.# I10406. Make up at 50 mg/mL in DMSO
  (it is also possible to buy ready-to-use BCIP/INT and BCIP/NBT from Roche)
- Fast Red tablets Roche Cat.# 1 496 549
- Poly(vinyl alcohol). Aldrich Cat.# 36,313-8

Solutions needed

- 4% fix (dilute 16% paraformaldehyde in PBT)

- PBT (1:10 dilution of 10X PBS + 0.1% Tween-20)

- Hybridization buffer: 50% formamide (final – 50%)
  25% 20X SSC (final – 5X)
  10% torula RNA at 50 mg/mL (final – 5 mg/mL)
  1% Tween-20, 10% (final – 0.1%)
  0.1% Heparin 50 mg/mL (final – 50 μg/mL)

- Maleic Acid buffer (MAB):

150 mM Maleic Acid	For 20 Liters
100 mM NaCl	348.2 g Maleic Acid
0.1% Tween-20	116.9 g NaCl
7.9 g/L NaOH	20 mL Tween 20
final pH = 7.5	250 g NaOH

- Block Solution: 10% BMB (10% stock solution)

10% Lamb Serum
80% MAB

- AP buffer: for 50 mL: 1 g PVA (in H2O heat until in solution)
  1.25 mL 2M Tris pH9.5
  0.5 mL 5M NaCl
  1.25 mL 1M $MgCl_2$
  250 µL 10% Tween20
  46.75 mL H_2O

Probe preparation

- Linearize appropriate plasmid. Phenol extract and precipitate with NaOAc. (See mRNA-making protocol for preparing template)
- Set up transcription reaction: X µL linearized template (1 µg total)
  4 µL 5X transcription buffer (supplied with polymerase)
  2 µL 10X DIG or Fluor. NTP mix
  2 µL RNA polymerase (T3, T7 or SP6) (Stratagene)
  (12-X) µL H_2O
  incubate at 37°C for 2-4 hr.
- Add 1 µL (1 U) DNase, RNase free (Promega)
  incubate at 37°C for 30 min.
  heat inactivate at 65°C for 10 min.
- Add 30 µL H_2O. Optional: Remove free nucleotides by running over Roche mini Quick Spin column – follow manufacturer's directions
- precipitate RNA by adding, for every 50 µL per vol. collected: 5 µL 4M LiCl
  1 µL glycogen
  150 µL EtOH (ice cold)

keep at –20˚C overnight if possible, or on dry ice 20-30 min. Spin at max speed for 20 minutes at 4°C. Wash once with 70% EtOH, re-spin for 10 minutes. Resuspend pellet in 20-50 µL of H2O and store in aliquots at -80 C.

- Check success of transcription reaction by running 1 µL of sample on a 1% agarose gel. Look for coherence of band. Or run on a formaldehyde gel to check actual size.

Embryo preparation

- Age embryos to stage required for experiment. Transfer to eppindorf tube. Add 1.5 mL 4% fix. Incubate O/N on Nutator at 4°C (you choose if you want to dechorionate before or after fix)

---------- overnight break ----------

- Wash out fix in PBT – in eppindorf tubes.
- Dechorionate embryos: transfer to agarose plate in PBT; manually dechorionate; move back to tube.
- transfer embryos to MeOH. If for immediate use, they can be left in tubes and continue as described below, if for storage, they should be transferred to eppindorf tubes and washed at least 2X in MeOH before being left in MeOH. Store embryos for an hour (acceptable) to overnight (best) to months (OK, although embryos become more fragile in my experience)

---------- possible overnight or longer break ----------

- Rehydrate embryos. Wash for 5 min. each in: 75% MeOH: 25% PBT
  50% MeOH: 50% PBT
  25% MeOH: 75% PBT
  Wash for 20 min total in PBT, change 3-4 times. All at room temp.

- ProteinaseK treat embryos fixed at later stages. Dilute Prot.K in PBT to final concentration of 10 μg/mL. Incubate embryos at room temp. in dilute Prot.K for:
  - Times vary depending on embryos/conditions that day. Becareful not to destroy your embryos with too much ProK.
    - 24 hr: 5 min
    - 36-48 hr: 8 min
    - 72 hr: 10-15 min

  Wash in PBT for 1 min., then for 5 min. in fresh PBT at room temp. Refix embryos treated with proteinase K. Incubate embryos in 4% fix for 1 hr. at room temp. Wash out fix by incubating 4x5 min. in PBT at room temp.

Hybridization

- Remove PBT and add 0.5 mL hybe buffer to embryos in eppindorf tubes. Incubate at 60°C (although some probes might prefer a different temperature), for at least 4 hr. Enclose in sealed bag or parafilm.
- Remove pre-hybe buffer and add hybridization buffer containing probe. Usually 1-5 μL of probe/250 μL hybe buffer/tube is appropriate. Incubate overnight, enclosed in a bag or parafilm.

---------- overnight break ----------

Post-hybridization washes

- Wash embryos for 10 min each at 60°C in tubes: 75% hybe buffer: 25% 2X SSC
  50% hybe buffer: 50% 2X SSC
  25% hybe buffer: 75% 2X SSC
  100% 2X SSC
- Wash embryos for 30 min each at 70°C in 0.2X SSC (these washes can be done for longer than 10 or 30 minutes)

Antibody binding and detection

- Preblock embryos. Incubate in blocking solution (1 part 10% BMB, 1 part lamb serum, 8 parts MAB) at room temp. for at least 2 hours.
- (optional) At the same time, preblock antibodies by preparing the antibody solution: 1:10,000 dilution of α-DIG antibody or1:4000 dilution of α-Fluor. antibody in blocking solution (as above). Incubate at room temp. for 2 hours. You may need to change antibody concentrations depending on the probe/embryo stage/etc. Try up to 1:2000 dilution for each. It is also a good habit to pre-bind the antibodies to get rid of low-level non-specific staining: fix and dechorionate embryos as usual, then make a 1:100 dilution of the antibody (or some similar convenient dilution) in blocking buffer and add to the embryos. Store at 4°C and use this solution to make the final dilution of antibody used for staining.
- Incubate embryos in antibody solution. 4 hours at room temperature or overnight at 4°C

---------- possible overnight break ----------

- Wash embryos in MAB: 1X5 min., 2X10 min., 1X30 min., 1X1 hour
- Detection. Wash embryos in AP buffer. Stain embryos in AP buffer (1 mL) + BCIP (3.5 μL) + NBT (4.5 μL) [for purple stain] or INT (4.5 μL) [for orange stain]. Stain at room temp for a few hours or at 15°C overnight. AP buffer should be kept no more than a few days.
  - It is also common to add 10% PVA (poly-vinyl alcohol) to this staining solution. This helps keep noise down as signal increases. When making AP buffer, add 10% w/v PVA, and stir on hot plate at low heat until PVA dissolves.
- [alternate detection for FastRed stain. Wash embryos in 0.1M TrisCl pH8.3 + 0.1% Tween-20. Stain in 2 mL 0.1M TrisCl pH8.3 + 0.1% Tween-20 + 1 FastRed tablet]
- To stop staining, wash embryos in PBT about 30 min, changing multiple times.
- For two-color stain: Inactivate phosphatase by 1) incubate embryos in 0.1M glycine-HCl pH 2.2 + 0.1% Tween-20 for 20 min. at room temp. and/or 2) heat inactivate in MAB + 10mM EDTA 10 min. at 60°C, followed by dehydration (10 min.) and rehydration as before. Repeat with new antibody starting from the incubation in antibody.
- Storage. Refix in 4% fix overnight at 4°C. Store in PBT at 4°C.