1. RNA in situ hybridization in *Parhyale hawaiensis*

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1.1 Introduction

This is a slight modification of a protocol that has been established in the lab of Nipam Patel at U.C. Berkeley (we thank particularly Danielle Liubicich and Ron Parchem for communicating the protocol and some tips on embryo handling). It follows the same basic steps as most in situ protocols, but also differs in some respects – most importantly by omitting the usual pre-treatment with proteinase K and replacing it with the use of a "detergent solution" containing SDS, an unusually tough detergent to use on embryos. SDS is also included in the hybridization and post-hybridization washes.

Notes before starting

- The initial treatments and washes will be carried out in microcentrifuge tubes at room temperature, unless otherwise stated. Room temperature washes should be about 1 ml (or more) in volume.
- During the 65°C hybridization and post-hybridization washes in Hyb buffer the embryos tend to float and are hard to see, which inevitably leads to some loss of embryos. For these reasons, embryos will be transferred into small baskets just before pre-hybridization. Follow the rest steps of the protocol by transferring the baskets through the solutions/washes in the provided 12-well plates (use 1.5 ml of solution per well). Baskets can be made from small cylinders made of cut microcentrifuge tubes with a fine Nytex mesh sealed at the bottom end, or can be bought ready-made (e.g. Corning # 07-200-211).
- Be gentle with washes; apply solution gently to the side of the tube/basket; no further agitation is necessary during the course of the wash.
- Embryos can stick to pipettes, particularly in solutions lacking detergent. Prefer plastic tips of Gilson pipettes, with their tips trimmed to enlarge diameter, and "pre-coat" tips with a BSA solution (10mg/ml) before picking up embryos.

1.2 Preparation

(any time before day 1)

1.2.1 Preparation of DIG- and FITC-labeled RNA probes

This protocol is based on the MEGAscript in vitro transcription kits (Ambion), which use the T3, T7 or SP6 RNA polymerase promoters to generate RNA from any fragment cloned in the appropriate vectors (most commercial cloning vectors now have T3, T7 or SP6 promoters flanking the polylinker). The Digoxigenin-11-UTP (Roche #11 209 256 910) and Fluorescein-12-UTP (Roche #11 427 857 910) required for labeling must be bought separately.

1. Linearize 5-10µg of the plasmid template. Set up a digest at 100µl with a restriction enzyme that cleaves at the 3' end of the insert that needs to be transcribed (avoid enzymes producing 3' overhangs). Remember: you need to make an antisense probe probe to detect a transcript (it may also be useful to prepare a sense probe, which can be used as a negative control). Check digest by running a small aliquot on an agarose gel.
2. Extract once with equal volume of phenol-chloroform and once with chloroform.
3. Add 1/10th volume (10µl) sodium acetate solution and two volumes (200µl) ice-cold absolute ethanol. Incubate at -80°C for 30min and precipitate plasmid by spinning at 13,000rpm for 20min at 4°C.
4. Discard supernatant and wash pellet with RNAse-free 70% ethanol.
5. Air dry pellet for 5min, dissolve in 5-10µl nuclease-free water and quantify on a spectrophotometer.
6. Set up the following transcription reaction:

   2µl 10x buffer (Megascript)

   2µl each of ATP, CTP, GTP (Megascript)

   1.5µl UTP (Megascript)

   4µl DIG-11-UTP or FITC-12-UTP (10nmol/µl Roche stock)

   2µg linearized template

   2µl T3, T7 or SP6 polymerase (Megascript)

   DEPC-water to 20µl
7. Incubate for 6h at 37°C
8. Treat with 1µl DNase (Megascript) for 15min at 37°C
9. Stop reaction by adding 115µl nuclease-free water and 15µl ammonium acetate stop solution (Megascript).
10. Extract once with equal volume (150µl) of phenol-chloroform and once with chloroform.
11. Add 150µl of isopropanol and mix well, incubate at -20°C for 2hrs (or store for up to several months) and precipitate RNA by spinning at 13,000rpm for 20min at 4°C.
12. Carefully remove the supernatant and wash once with RNAse-free 70% EtOH.
13. Air dry pellet for 5min and resuspend in 100µl DEPC-water.
14. Check probe concentration on a spectrophotometer and probe quality by analyzing 1µl on a 1% agarose gel with EtBr.
15. If no hydrolysis is required (see below), dilute probe in Hyb buffer at a concentration of 100ng/µl. Store at -20°C for several months.
16. If the probe is larger than 1kb it may need to be hydrolyzed before use:
    - Take purified probe at about 60ng/µl (in water or TE).

- Add equal volume 80mMNaHCO3, 120mM Na2CO3.
- Incubate for 25 min at 60°C.
- Add 30vol Hyb buffer. Store at -20°C (probe concentration about 1ng/µl).

1.2.2 Embryo Preparation

1. In a clean tube, prepare 4% formaldehyde in deionized water and place on ice.
2. Collect embryos at required stages, as described in the Parhyale Microinjection section.
3. Place embryos in a clean tube, let sink and remove as much seawater as possible with a pipette. Rinse embryos once in deionized water.
4. Let embryos sink, remove water and add ice cold 4% formaldehyde (prepared earlier).
5. Let embryos fix overnight at 4°C, gently mixing on a nutator/inverting device. This cold fix in deionized water caused the egg membranes to swell and separate from the embryo.
6. Rinse embryos 2-3 times in 1xPBS, wash by gently mixing on a nutator for at least 3 hrs.
7. Transfer embryos onto a Sylgard plate (Dow Corning #634165S) in a big drop of 1xPBS. Using fine forceps or tungsten needles dissect off the membranes that surround each of the embryos, one by one. Transfer dissected embryos back into the eppendorf tube.
8. Dehydrate through a series of Methanol/PBS washes (5 min each in 50%MeOH in 1xPBS, 70%MeOH in 1xPBS, 90%MeOH in 1xPBS, 100%MeOH).
9. The embryos can now be stored in 100%MeOH at -20°C (this is the recommended step for long-term storage).

1.3 Days 1-2

1.3.1 Pre-hybridization

1. Rehydrate through a series of Methanol/PTw washes (5 min each in 70%MeOH in PTw, 50% MeOH in PTw, 30% MeOH in PTw) and 2 x 5 min in PTw.
2. Fix for 30 min in 4% formaldehyde in PTw.
3. Wash 4 x 5 min in PTw.
4. Wash 30 min in Detergent solution.
5. Wash 6 x 5 min in PTw.

   CAUTION: At this point, transfer embryos from microcentrifuge tubes into baskets in PTw. Subsequent incubations and washes will be carried out by moving the basket from one well to the next in multi-well plates.
6. Wash 10 min in 1.5ml 50%Hyb/ 50%PTw.
7. Wash 10 min in 1.5ml Hyb.

8. Transfer to fresh Hyb solution and incubate for 3hrs (or longer if preferred) in multi-well plate placed on a submerged platform in 65°C waterbath.

1.3.2 Hybridization

1. Take stock probe solutions (100ng/µl in Hyb) from -20°C. Incubate at 37-42°C for 10min to bring precipitated SDS in suspension. Mix gently and spin down shortly.
2. Dilute each probe to a final concentration 1ng/µl in 1.5ml Hyb (for a new probe might need to try a range of probe concentrations 0.5-10ng/µl).
3. Denature probes for 20 min at 80-90°C.
4. Pipette Hyb with probes into a new well in the multi-well plate kept at 65°C.
5. Transfer basket with embryos into Hyb with probes.
6. Incubate at 65°C for 30-40hrs (over day 2).

1.4 Day 3

1.4.1 Post-hybridization washes

1. Pre-heat Hyb at 65°C, mix well and aliquot 1.5 ml Hyb in 9 wells.
2. Wash 3 x 20 min in Hyb at 65°C.
3. Wash 4 x 30 min Hyb at 65°C.
4. Gradually bring to room temperature by washing 2 x 5 min in Hyb (on the bench).
5. Carry out 2-3 half washes with TBST at room temp (over 20 min) to gradually replace Hyb solution. Keep embryos in basket and continue by moving the basket from one well to the next in multi-well plate.
6. Wash 3 x 20 min in TBST, at room temp.

1.4.2 1st Antibody incubation

CAUTION: In single in situs, use DIG-labeled probes, detect with anti-DIG-AP and react with the BCIP/ NBT substrate. In double in situs, you should react one of the two probes first with the BCIP/ NBT substrate and in a second step the other probe with the Fast Red substrate! Only add the antibody for one probe at a time, e.g. you could start with the weaker anti-FITC antibody and react that probe using the stronger BCIP/ NBT reaction. It will depend on individual probe strength as tested by single label in situs.

1. Wash 1 hr in TBST+BSA, at 4°C.
2. Dilute anti-DIG-AP (1:3000) or anti-FITC-AP (1:4000) in TBST+BSA and add 1.5ml per well.
3. Incubate in antibody at 4°C overnight.

1.5 Day 4

1.5.1 1st Antibody washes and 1st AP reaction

1. Wash 4 x 30 min in TBST (room temp).
2. Wash 1 hr in TBST (room temp).
3. Wash 3 x 5 min in AP reaction buffer (freshly made!).
4. Prepare BCIP/NBT reaction solution fresh!
5. Add 1.5ml BCIP/NBT reaction solution to each well. Incubate at room temp in the dark, because the substrates are photosensitive.
6. Check the development of the colour reaction periodically under the stereoscope (first check after 15-30 min, then depending on the signal can monitor every hour); return to the dark as quickly as possible. You can refresh the reaction by replacing the reaction solution after 3-4hrs.
7. When signal has developed to the desired intensity (aim for a good signal/noise ratio) stop the AP reaction with a few TBST washes.
8. If single label, proceed with glycerol washes described at the end.
9. If double label, continue with next step

1.5.2 Removal of 1st antibody

1. Rinse quickly 1x in glycine buffer (room temp)
2. Wash 1 x 10 min in glycine buffer (room temp)
3. Wash 3 x 5 min in TBST (room temp)

1.5.3 2nd Antibody incubation

CAUTION: Put the correct antibody that detects the second probe, e.g. if you reacted the DIG-labeled probe first (using anti-DIG-AP) you should be using the anti-FITC-AP antibody in this 2nd incubation.

1. Wash 1 hr in TBST+BSA, at 4°C.
2. Dilute anti-DIG-AP (1:3000) or anti-FITC-AP (1:4000) in TBST+BSA and add 1.5ml per well.
3. Incubate in antibody at 4°C overnight.

1.6 Day 5

1.6.1 2nd Antibody washes and 2nd AP reaction

1. Wash 4 x 30 min in TBST (room temp).

2. Wash 1 hr in TBST (room temp).
3. Wash 3 x 5 min in modified AP* reaction buffer (freshly made!).
4. Prepare Fast Red reaction solution fresh!
5. Add 1.5ml Fast Red reaction solution to each well. Incubate at room temp in the dark, because the substrates are photosensitive.
6. Check the development of the colour reaction periodically under the stereoscope (first check after 30 min, then depending on the signal can monitor every hour); return to the dark as quickly as possible. You can refresh the reaction by replacing the reaction solution after 3-4hrs.
7. When signal has developed to the desired intensity (aim for a good signal/noise ratio) stop the AP reaction with a few TBST washes.
8. Optional: Wash overnight in TBST.

1.6.2 Glycerol washes and embryo storage

CAUTION: Transfer embryos from the baskets back into microcentrifuge tubes with a BSA-coated tip!

1. Replace TBST with 50% glycerol (in 1xPBS) + DAPI (0.5µg/ml), store at 4°C until embryos sink.
2. Replace with 70% glycerol (in 1xPBS).
3. Store stained embryos in 70% glycerol (in 1xPBS) at 4°C.
4. Mount embryos in 70% glycerol (in 1xPBS) for microscopy.

1.7 Solutions

10x PBS	for 1lt solution:
18.6 mM NaH2PO4	2.56g NaH2PO4•H20
84.1 mM Na2HPO4	11.94g Na2HPO4
1750.0 mM NaCl	102.2g NaCl per 1lt dH20)

Mix phosphates in about 800ml of ddH20 for a 1lt total volume. Check the pH, it should be 7.4. If it is more than 0.4 off then start over. Otherwise adjust pH to 7.4 with NaOH or HCl. Add the NaCl and the rest of the dH20. Prepare 1x PBS by diluting 1:10 with dH20 and check the pH again. Both 1x and 10xPBS can be kept indefinitely at room temp.

PTw (= 1x PBS with 0.1% Tween-20)

495ml	1x PBS
5ml	10% Tween-20

20x SSC	for 1lt solution:	
3M NaCl	175.3g NaCl	
0.3M Sodium citrate	88.2g Sodium Citrate, dihydrate	(CH6H5Na3O7•2H2O)

pH to 7.0 and sterilize by autoclaving.

pH down to 4.5 just prior to use with citric acid (HCl will do as well).

Sonicated Salmon Sperm DNA (SS DNA)

You can use Salmon or Herring Sperm DNA.

Dilute to 10mg/ml, if needed with TE or ddH20 and autoclave for 20min. Store at 4°C or in 5ml aliquots at -20°C. Can buy ready-to-use from from Sigma (#D7656-5x1ml).

Boil for 10min, chill on ice and then add in Hyb buffer.

Detergent Solution	for 500ml solution:
1.0% SDS	50ml 10% SDS (filtered)
0.5% Tween	25ml 10% Tween 20
50mM Tris-HCl (pH7.5)	25ml 1M Tris-HCl (pH 7.5)
1mM EDTA (pH8.0)	1ml 0.5M EDTA (pH 8.0)
150mM NaCl	15ml 5M NaCl
	Add ddH20 to total volume 500ml

SDS Hybridization solution (=Hyb)	for 40ml solution:
50% Formamide	20ml Formamide (Molecular Grade)
5xSSC	10ml 20xSSC (pH 4.5)
50μg/ml heparin	0.1ml 20mg/ml heparin
0.25%Tween-20	1ml 10% Tween-20
1% SDS	4ml 10% SDS
100μg/ml SS DNA	0.2ml 10mg/ml SS DNA
	add ddH20 to total volume 40ml

The pH should be between 5.0 and 6.0, if it is not, don't adjust the pH. Check your starting solutions. The lowered pH of the SDS Hyb Solution (pH 5) is essential to prevent the embryos from disintegrating at these temperatures. Be sure to keep the solution RNase free. Hyb can be

stored at -20°C, but it will have to be warmed up before use to resuspend the SDS. It can also be made fresh and kept at 65°C throughout the protocol.

TBST	for 500ml solution:
	125ml 1M Tris pH 7.5
	40g NaCl
	1g KCl

Add ddH20 to mix and bring up to 500ml volume, remove 5ml of this solution and add 5ml 10% Tween-20.

TBST +BSA	for 500ml solution:
	500ml TBST
	0.5g BSA and store at 4°C

AP Reaction Buffer	for 50ml solution:
5mM MgCl2	250µl 1M MgCl2
100mM NaCl	5ml 1M NaCl
100mM Tris pH 9.5	5ml 1M Tris pH 9.5
0.1% Tween-20	500µl 10% Tween-20
	add ddH20 to total volume 50ml

Make just prior to use – solution sitting at room temp for a few hours will not work as well for the reaction. This recipe is for AP reaction buffer pH 9.5 used for BCIP/NBT reactions.

*For Fast Red reactions, add 1.75mL 1M Tris pH 9.5 and 3.25mL 1M Tris pH 7.5 (in place of the 5mL 1M Tris pH 9.5) for a final AP reaction buffer with pH 8.2.

BCIP/NBT reaction solution

1ml AP reaction buffer (pH 9.5)

5µl NBT (4-nitro blue tetrazolium chloride; 100mg/ml in 70% DMF; Roche #11 383 213 001)

3.75µl BCIP (5-bromo-4-chloro-3-indolyl-phosphate; 50mg/ml in 100% DMF; Roche #11 383 221 001)

Mix just before use and keep in the dark. Add 1.5ml solution per well for reaction.

Fast Red reaction solution

1ml ddH20

Fast Tris tablet

Fast Red Naphthol tablet

Buy the Tris and Fast Red/Napthol tablets from SIGMA (#F4648-50SET). Dissolve the Tris tablets in ddH20 by vortexing. Once completely dissolved, dissolve the Fast Red tablets in the same buffer by vortexing. After both tablets in solution, filter through 0.2μm syringe filter and apply 1500ml solution per well for reaction.

Glycine Buffer pH 2.0 for 50ml solution:

0.375g Glycine

500μl 10% Tween-20

Dissolve glycine in 40 mL ddH2O and adjust pH to 2.0 with concentrated HCl. Add Tween-20 and adjust volume to 50mL with ddH2O