Parhyale in situ hybridization

Danielle Liubicich and Francis Poulin (Patel Lab) Updated July 7, 2006

Embryo Preparation

Fix embryos in a solution containing 8 parts *in situ* fixation buffer, 1 part 10x PBS, 1 part 37% formaldehyde (thus the final concentration formaldehyde is 3.7%) for about an hour at room temp. Many alternative fixatives (such as PEM or seawater with a final formaldehyde concentration of 3.7% will also work, but the different methods have their advantages and disadvantages when it comes to being able to actually dissect the embryos free from the egg shell and surrounding membranes). Intact embryos can also be fixed overnight at 4°C and then dissected the next morning; this works well for early embryos up through the early germband stages. (For more detailed notes, see Patel Lab Parhyale dissection/fixation protocol).

After fixation, rinse the embryos in 1xPBS or PT for at least a half hour (up to a few hours), 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), then store in 100%MeOH at –20°C in eppendorf tubes.

Probe synthesis

Template Preparation

- Linearize template

- Digest 5-10µg template DNA with 10-20U restriction enzyme in a 40µl reaction overnight. ** Set up 2 reactions: one for sense probe and one for antisense probe.
- Run 1µl on an agarose gel next to uncut template DNA to confirm linearization.
- Add RNase free water to a final volume of 500µl.

- Phenol:Chloroform extraction:

- Add 500µl Phenol:Chloroform, vortex to mix
- Let stand 1 min room temp
- Spin max speed 1 min room temp
- Place aqueous phase in new eppendorf tube
- Add 500µl Chloroform, vortex to mix
- Let stand 1 min room temp
- Spin max speed 1 min room temp
- Place aqueous phase in new eppendorf tube
- Add 500µl Chloroform, vortex to mix
- Let stand 1 min room temp
- Spin max speed 1 min room temp
- Place aqueous phase in new eppendorf tube

-NaOAc/EtOH precipitation:

- Add 0.1 vol 3M sodium acetate pH5.2, mix
- Add 2 vol 100% Ethanol, mix
- Incubate on ice 30min
- Spin max speed 20min at 4°C
- Remove supernatant, add ~300µl cold 70% ethanol, flick to mix
- Spin max speed 10 min at 4°C
- Remove all ethanol and air dry 5-10 min on bench top.

-Resuspend pellet in 20µl RNase free water and store -20°C.

RNA probe synthesis

Transcription reaction

- Set up a 20µl reaction in 0.5mL eppendorf tube as follows:
  - 1µg linearized DNA template
  - 2µl DIG or Fluorescein RNA labeling mix
  - 2µl 10x transcription buffer (provided with polymerase)
  - 2µl RNA polymerase (40U) (T3,T7 or SP6)
  - RNase free water to 20µl
- Mix, pulse spin, incubate at 37°C for 3 hrs.
- After 2hrs, run 1µl on an 0.8% agarose gel for ~20min to confirm transcription occurred.
- After 3hrs, add 2µl DNaseI (10U) and incubate 15-18min 37°C.

Purification of probe: Qiagen RNAeasy columns – see protocol with kit.

this will work for both DIG and Fluorescein labeled probes

Use eluted probe at 1:500

the probe comes off the Qiagen column in a volume of 10-12 μl and will be at a concentration of roughly 250 – 500 ng/μl, adjust the dilution factor accordingly if you use a different method of purification).

A few notes before starting:

All staining should be done in eppendorf tubes. Room temp washes should be about 1 mL or more in volume. Any washes at 65°C require less volume – fill tubes so that liquid in the tube will be at the water level of the bath on the outside of the tube (probably ~ 700 µl). Be gentle with washes – apply washes to side of the tube. It is important to mix the embryos in the various solutions/washes once added, but no further agitation of the tubes is necessary (washes can be done on bench top). You may want to save all washes in medicine cups/ 3well dishes to minimize amphipod embryos loss through protocol. Embryos can be returned to tubes throughout the protocol. Glass pipettes are transparent and allow you to look at each wash as it is removed and may help prevent embryo loss. However, embryos will stick to glass in solutions lacking detergent, so use glass pipettes with caution!

Day 1

pretreatment (room temp):

- rehydration: **rehydrate a tube of flies through these washes as well
  - __ 5 min 70%MeOH in PTw
  - __ 5 min 50% MeOH in PTw
  - __ 5 min 30% MeOH in PTw
  - __ __ 2 x 5 min PTw **add flies to amphipod tubes during these washes.
- fixation:
  - __ 30 min FPTw (9 parts PTw : 1 part 37% formaldehyde)
- washes:
  - __ __ __ __ 4 x 5 min PTw
  - __ 30 min Detergent solution
  - __ __ __ __ __ __ 6 x 5 min PTw
  - __ 10 min 50%Hyb/ 50%PTw
  - __ 10 min Hyb [CAUTION: amphipods may float in Hyb!! Be careful when you place pipette into a tube with amphipods in Hyb!]

pre-hyb (65°C):

- add fresh Hyb solution (~500 µl) and incubate 65°C for 3hrs (or longer if preferred). pre-hyb at _____ to _____. Store rest of hyb at 65°C to use throughout protocol.

hyb – addition of probe: **if doing a double label, mix both probes now!

- dilute probe to a final concentration 1.0 – 0.5 ng/µl in hyb solution (see probe synthesis at beginning of protocol – this is roughly a 1:500 dilution of what comes off the Qiagen columns; depending on various parameters, you may need to increase or decrease the probe concentration, but this should get you in the right range to start with)
- heat probe 10 min 80-90°C
- equilibrate tubes containing probes 10 seconds at 65°C
- add 300-500µl probe per tube at 65°C
- hyb with probe(s) at 65°C for ~15hrs: probe in at _____ out at _____

Day 2

recover probe and store at –20°C (may be reused many times)

- washes at 65°C:
  - __ __ __ 3 x 20 min Hyb
  - __ __ __ __ 4 x 30 min Hyb
- gradually bring to room temp:
  - __ __ 2 x 5 min Hyb (washes on bench top)
- washes (room temp):
  - __ 20 min of half washes (2-3 changes) with TBST to gradually replace Hyb solution in tubes
  - __ __ __ 3 x 20 min TBST
  - __ 1 hr TBST + BSA solution

visualization of probe:

** if doing a double label, react the stronger of the two probes first! Only add the antibody for one probe at this time! Think about the strongest combinations of your two probes and reaction solutions, i.e. you could add the weaker anti-Fluorescein antibody and react that probe using the stronger BCIP/ NBT reaction or start with anti-DIG antibody and react that using BCIP/NBT, etc. It will depend on individual probe strength as tested by single label *in situs*.

- Dilute anti-DIG (1:3000) or anti-Fluorescein (1:4000) in TBST with BSA.
- Add 300-500µl per tube and incubate 4°C overnight.
- antibody in at _____ out at _____
-

Day 3

- washes (room temp):
  - __ __ __ __ 4 x 30 min TBST
  - __ 1 hr TBST
  - __ __ __ 3 x 5 min AP reaction buffer (or Fast Red reaction buffer)
- React by adding ~500µl appropriate reaction solution (BCIP/NBT or Fast Red) to each tube. React at room temp in the dark.
  **probably want to react using BCIP/NBT solutions first if you are doing a double label!
- Reaction start ____ and stop _____. (may need to react over night)
- You can refresh the reaction by replacing the reaction solution after 3-4hrs. This may make your signal stronger but you should monitor the reaction closely at first.
- Stop reaction with a few TBST washes.
- <u>If you are doing a double labeling experiment</u>, proceed to additional TBST washes and the remaining protocol on the next page (DO NOT put the embryos into glycerol solutions)
- <u>If this is a single label experiment</u> you can wash overnight in TBST (or as little as 30 min. and then stain with DAPI in 50%glycerol in PBS the next day. Store finished embryos in either 50% glycerol in PBS or 70% glycerol in PBS.

to continue with a double label experiment:

__ __ __	3 x 5 min TBST room temp
__	1 x quick rinse in glycine buffer room temp
__	1 x 10 min glycine buffer room temp
__ __ __	3 x 5 min TBST room temp
__	1 hr TBST + BSA solution

visualization of second probe:

** add the antibody for the other probe at this time!

- Dilute anti-DIG (1:3000) or anti-Fluorescein (1:4000) in TBST with BSA.
- Add 300-500µl per tube and incubate 4°C overnight.
- antibody in at _____ out at _____

Day 4 (double labeling only)

- washes (room temp):

__ __ __ __	4 x 30 min TBST
__	1 hr TBST
__ __ __	3 x 5 min Fast Red reaction buffer (or AP reaction buffer)

- React by adding ~500µl appropriate reaction solution (Fast Red or BCIP/NBT) to each tube. React at room temp in the dark.
- Reaction start ____ and stop _____. (may need to react over night)
- You can refresh the reaction by replacing the reaction solution after 3-4hrs. This may make your signal stronger but you should monitor the reaction closely at first.
- Stop reaction with a few TBST washes.
- You can wash overnight in TBST and then stain with DAPI in 50%glycerol in PBS the next day. Store finished embryos in either 50% glycerol in PBS or 70% glycerol in PBS.

Solutions:

1M Hepes

238.3g Hepes ($C_8H_{18}N_2O_4S$) per 1000mL dH_20 (Boehringer Mannheim #737-151)
pH to 6.9 with NaOH

20mM Magnesium sulfate

4.93g $MgSO_4 \bullet 7H_20$ per 1000mL of dH_20 (Sigma #M1880)

0.5M EDTA

18.612g per 100mL dH_20 (disodium salt) (Fisher #BP120-500)
pH to 8.0 with NaOH
**salt will not dissolve completely until pH is raised to around 8.

0.5M EGTA

19.02 g per 100mL dH_20 (Sigma #E3889)
pH to 8.0 with NaOH
**salt will not dissolve completely until pH around 8.

in situ fixation buffer	50mL
100mM Hepes, pH 6.9	5mL 1M Hepes, pH 6.9
2mM Magnesium sulfate	5mL 20mM Magnesium sulfate
1mM EGTA	100µl 0.5M EGTA
	dH_20 to 50mL and store 4°C
	(warm 8mLs to room temp before use)

10% Triton X-100 1L

100mL TritonX-100
900mL dH_20
Fill bottle with 100mL dH_20. Place a mark on the bottle at this 100mL level. Empty out the water and refill bottle to 100mL mark with Triton X-100 being careful not to get Triton on the bottle sides. Add 900mL of dH_20 and stir until in solution.

20% Tween-20 1L

200 mL Tween-20 (Sigma #P-1379)
800 mL dH_20
Make this in the same manner as the 10% Triton X-100.

10x PBS 1L

18.6 mM	NaH_2PO_4	(2.56g $NaH_2PO_4 \bullet H_20$ per 1000mL dH_20) (Sigma #S3522)
84.1 mM	Na_2HPO_4	(11.94g Na_2HPO_4 per 1000mL dH_20) (Sigma #S9763)
1750.0 mM	NaCl	(102.2g NaCl per 1000mL dH_20) (Sigma #S7653)

Mix phosphates in about 800mL of dH_20 for a 1L total volume. Check the pH, it should be 7.4. If it is more than .4 off then start over. Otherwise adjust pH to 7.4 with NaOH or HCl. Add the NaCl and the rest of the dH_20. Prepare 1X PBS by diluting 1:10 with dH_20 and check the pH again. Both 1X and 10X PBS can be kept indefinitely at room temp.

PTw 1L

995mL	1xPBS
5mL	20% Tween-20

(1X PBS containing 0.1% Tween-20)

PT 1L

100mL	10xPBS
10mL	10% Triton X-100
890mL	dH_20

(1X PBS containing 0.1% Triton X-100)

20%SDS 1L

200g	SDS (Gibco BRL Ultra Pure #15525-017)
800mL	dH_20

Filter sterilize after SDS is in solution. Always wear a mask and wipe down the area when you are done.

20x SSC 1L

175.3g	NaCl
88.2g	Sodium Citrate, dihydrate ($CH_6H_5Na_3O_7 \bullet 2H_2O$) (Sigma #S4641)

pH to 7.0 and sterilize by autoclaving. pH down to 4.5 just prior to use.
(0.3M Sodium citrate + 3M NaCl)

Sonicated Salmon Sperm DNA (SS DNA)
You can use Salmon of Herring Sperm DNA. Herring Sperm DNA (Fisher #PR-D1811).
Dilute to 10mg/mL, if needed with TE or ddH_20 and autoclave for 20min. Store at 4°C or in 5mL aliquots at -20°C.

Detergent Solution	500mL
1.0% SDS	25mL 20% SDS (filtered) (or 50mL of 10%SDS)
0.5% Tween	12.5mL 20% 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 dH_20 to total volume 500mL

Hybridization solution (Hyb)	40mL
50% Formamide	20mL Formamide (Fisher Mol Grade #BP227-500)
5xSSC	10mL 20xSSC (pH 4.5)
50µg/mL heparin	0.1mL 20mg/mL heparin
0.25%Tween-20	0.5mL 20% Tween-20
1% SDS	2mL 20% SDS
100µg/mL SS DNA	0.2mL 10mg/mL SS DNA
	add dH_20 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 this Hyb Solution (pH 5) is essential to prevent the embryos from disintegrating at these high temperatures. 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. The high concentration of SDS in this protocol allows you to get away without ProteinaseK treatment of the embryos.

AP (Alkaline Phosphatase) Reaction Buffer	50mL	pH 9.5
5mM $MgCl_2$	250µl 1M $MgCl_2$	
100mM NaCl	5mL 1M NaCl	
100mM Tris pH 9.5*	5mL 1M Tris pH 9.5*	
0.1% Tween-20	250µl 20% Tween-20	
	add dH_20 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.

Fast Red (and Vector Red) Reaction Buffer	50mL	pH 8.2
5mM $MgCl_2$	250µl 1M $MgCl_2$	
100mM NaCl	5mL 1M NaCl	
100mM Tris pH 8.2	1.75mL 1M Tris pH 9.5 + 3.25ml 1 M Tris pH 7.5	
0.1% Tween-20	250µl 20% Tween-20	
	add dH_20 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.

Glycine pH 2.0 50mL

0.375g Glycine
250µl 20% Tween-20

Dissolve Glycine in 40 mL dH_2O and adjust pH to 2.0 with concentrated HCl. Add Tween-20 and adjust volume to 50mL with dH_2O

BCIP/NBT solution

1mL	A.P reaction buffer (pH 9.5)
8.1µl	NBT (4-nitro blue tetrazolium chloride; 50mg/mL in 70% DMF)
6.8µl	BCIP (5-bromo-4-chloro-3-indolyl-phosphate; 50mg/mL in 100% DMF)

Mix just before use and keep in the dark. Add ~500µl solution per tube for reaction. The NBT and BCIP/NBT solutions can be purchased together from Promega (Promega # S3771), or individually (NBT is Sigma #S380C, BCIP is Sigma #S381C). A wide range of ratios of NBT to BCIP will actually work as well and in the past we have used 6.6μl NBT plus 3.3μl BCIP in 1 ml and 4.5μl NBT plus 3.5μl BCIP in 1 ml.

Fast Red reaction solution

1mL	dH_20
1	Fast Tris tablet (Sigma #T9043)
1	Fast Red Naphthol tablet (Sigma #F0775)

Dissolve the Tris tablet in 1mL dH_20. Once completely dissolved, dissolve the Fast Red tablet in the same mL. After both tablets in solution, filter through 0.2µm syringe filter (we use Gelman Sciences sterile acrodisc, product number 4192) and apply ~500µl solution per tube for reaction.

TBST 500mL

125mL	1M Tris pH 7.5
40g	NaCl
1g	KCl

add dH_20 to mix and bring up to 500mL volume,
remove 2.5mL of this solution and add 2.5mL 20% Tween-20

TBST + BSA 500mL

500mL TBST
0.5g BSA and store at 4°C

1M $MgCl_2$

20.33g $MgCl_2$, hexahydrate ($MgCl_2 \bullet 6H_20$) (Sigma #2393) per 100mL dH_20

1M and 5M NaCl

1M = 5.844g NaCl per 100mL dH_20 (Sigma #S7653)
5M = 29.22g NaCl per 100mL dH_20

1M Tris pH 7.5

12.11g Tris base (Roche #1814273) and 5.5mL concentrated HCl per 100mL dH_20
Dissolve salt in dH_20, then add HCl. Measure pH once solution is at room temp (pH will change with solution temp). Sterilize by autoclaving.

1M Tris pH 9.0

12.11g Tris base and 0.45mL concentrated HCl per 100mL dH_20
* see Tris pH 7.5 for notes on dissolving salt and reading pH. Sterilize by autoclaving.