mRNA IN SITU HYBRIDIZATION FOR LARVAL FISH[1]

PROBE PREPARATION

Probes may be prepared either from existing plasmids (1A) or from PCR products (1B).

1A. cDNA template preparation

a. Linearize plasmid containing cDNA of interest by digesting with appropriate restriction enzyme for 2 hr, 37 °C
Typically digest enough DNA so it can be used for multiple riboprobe syntheses, e.g.:

- 20 ug plasmid DNA + nano H_20 174 µl
- 10x restriction enzyme buffer 20 µl
- BSA 2 µl
- restriction enzyme (10U/ul) 4 µl

Avoid using restriction enzymes (e.g., Sac I, Kpn I, Pst I) that leave a 3' overhang, as this can result in inappropriate transcription of sense RNA that may contribute to background. Alternatively, overhangs can be filled using DNA polymerase I (Klenow) after restriction digest.

b. Add equal volume phenol-chloroform-isoamyl alcohol (pH 8.0); vortex 10 sec; centrifuge at maximum speed 5 min.
All steps after this point must be performed RNase-free.

c. Remove aqueous (upper) phase to new RNase-free 1.5 ml tube (do not disturb interface between phases).

d. Add 0.1x volume 3 M sodium acetate pH 5.2 and 2.5x volume 100% ethanol; vortex and leave on ice 15 min.

e. Centrifuge at maximum speed 15 min at room temperature.

f. Remove supernatant and add 1 ml 70% ethanol; centrifuge at maximum speed 5 minutes at room temperature; draw off supernatant, air dry briefly.

g. Resuspend in nano H_20 at 1–2x original volume taken from plasmid prep.

h. Check cutting efficiency and determine template concentration on a 1% agarose gel.
Run 0.1 µl of sample on gel (dilute 1 µl DNA into 9 µl TE, then dilute 1 µl of this into 9 µl TE). Run alongside 250, 500 ng lambda–Hind III to estimate concentration. Make sure that templates are completely linearized, as circular DNA will result in probes including the vector sequence

i. Store cut DNA at –20 °C.

1B. PCR template preparation

a. Amplify in a 50 µl PCR from plasmid or cDNA using PCR primers in which one primer includes an RNA polymerase binding site at its 5' end (for T7: taatacgactcactatagggc); for antisense probes the RNA polymerase binding site should be on the reverse primer, for sense probes it should be on the forward primer.

b. Check 5–10 µl of PCR on an agarose gel and if the amplification has generated a single strong fragment, proceed to step c.
All steps after this point must be performed RNase-free.

c. Purify the remainder of the PCR by spin column and elute in 10 mM Tris pH 8.0 or (Qiagen EB buffer).

d. Check concentration using the Nanodrop spectrophotometer and proceed as indicated below or store purified PCR product at –20 °C.

2. Riboprobe synthesis

a. Set-up in vitro transcription reaction at room temperature.
Low temperature and excessive DNA concentrations can result in precipitation. Mix reaction at room temperature but keep enzymes and RNase inhibitor on ice. Reaction (and precipitation) volumes can be scaled up as needed.

• 1 μg (maximum) linear template DNA	x μl
• nano H_20	9 – x μl
• 5x transcription buffer	4 μl
• 100 mM DTT	2 μl
• RNase inhibitor	1 μl
• DIG-labeling mix	2 μl
• RNA polymerase (T3, T7, or Sp6)	2 μl
	20 μl total

b. Vortex gently to mix, spin to bottom of tube.

c. Place in a 37 °C air incubator for 2–4 hr.

d. Digest the template cDNA by adding 2 μl RNase-free DNase, 15 min at 37 °C.

e. Stop the reaction and precipitate the probe by adding:

• 0.5 M EDTA, pH 8.0	2.0 μl
• 4 M LiCl	2.5 μl
• 100% EtOH	75.0 μl

f. Place at –80 °C, 10 min to overnight ; spin at maximum speed 30 min.

g. Remove supernatant and wash with 70% EtOH; spin 5 min; remove supernatant and air dry briefly.

h. Resuspend by vortexing in 100 μl nano H_20; add 1 μl Superasin RNase inhibitor.

i. Probe fractionation: alkaline hydrolysis is used to fragment probes greater than 300 nt.
Do <u>not</u> hydrolyze all of the probe, as the intact probe will be analyzed for quality, below.

• intact probe	50 μl
• nano H_20	30 μl
• 0.4 M sodium bicarbonate *(1.68 g in 50 ml nano H_20)*	10 μl
• 0.6 M sodium carbonate *(3.18 g in 50 ml nano H_20)* for a final volume of 100 μl	10 μl

heat in a water bath at 60 °C for time indicated in Appendix, or by calculating:

time (min) = (starting kb – desired kb) / (0.11 x starting kb x desired kb)
so, for a ~3 kb probe fractionated to 0.6 kb, time = 12.1 min

For larval in situs it is important to fractionate the riboprobe to allow efficient penetration into the tissues; we use 300–600 nt as average final sizes.

j. Hydrolyzed probe precipitation:

• fractionated probe	100 µl
• nano H_20	100 µl
• 3 M sodium acetate pH 4.5	20 µl
• glacial acetic acid	2.6 µl
• 100% ethanol	600 µl

chill at –80 °C for 10 min to overnight, spin at maximum speed 30 min;
pour off supernatant and wash with 70% EtOH; spin 5 min; reomve supernatant and air dry briefly; resuspend by vortexing in 50 µl nano H_2O; add 1 µl Superasin RNase inhibitor

k. Check riboprobe quality on an agarose/MOPS/formaldehyde gel:

prepare gel:

- 33 ml nano H_20
- 4 ml 10X MOPS buffer[2]
- 0.6 g agarose
  melt in microwave and when cool enough to touch add 2.2 ml 37% formaldehyde, swirl, and pour immediately; cover gel box while gel sets

prepare samples:
to PCR tubes, add:

- 3 µl RNA loading buffer[3]
- 2 µl nano H_20
- 1 µl riboprobe

prepare an extra tube as above for RNA ladder (stored at –80 °C)
heat all tubes at 65 °C for 15 min; quick chill on ice
It is essential to denature the single stranded RNA molecules, which otherwise will migrate as a smear or as irregular bands that cannot be evaluated.

run gel:
use 1X MOPS as running buffer, load all of sample and run at medium voltage (~100V); do not add ethidium bromide (it is already in the RNA loading buffer)

inspect probe:
Successful probe synthesis should yield a single fragment at the expected size for intact probes and a smear around the predicted final average size for fragmented probes. If this is not observed, DO NOT PROCEED with in situ hybridization!

l. Check riboprobe concentration by Nanodrop spectrophotometer or dot blot.
Accurate quantification of probe concentration is critical for successful in situs as excess probe will result in surface background and the failure of normal staining!

Nanodrop: Set the Nanodrop to read for single stranded RNA and calculate the concentration for 2 µl of each probe. Note that the Nanodrop cannot provide information on the efficiency of probe labeling.

Dot blots: Blots provide information on probe concentration and labeling efficiency, and also serve to verify the activity as well as antibody and developing reagent efficacy.

prepare dilutions

To 6 rows of a microtiter plate add 9 ml of nano H_2O in as many columns as you have probes to check; add 2 additional columns of wells for controls. To the uppermost row, add 1 ml of each sample and mix into the 9 µl of H_2O. With the same pipette tip, take 1

ml of the diluted probe and add to the next row; repeat to make a series of dilutions ranging from 10^{-1} to 10^{-6}. To control wells, add 1 µl of 100 ng/µl control DIG-labeled RNA (Roche) and serially dilute as for probes.

prepare dot blot

To a small piece of Amersham Hybond N+nylon membrane in a 60 mm or 100 mm petri dish, add 1 µl of each dilution in rows and columns with a micropipette. Avoid touching the membrane with the pipette tip and place rows and columns just far enough apart so that spots do not merge. Label tops of columns with pencil or extra fine point sharpie. Array control RNA dilutions on either side of probe dilutions. Once spotted, place membrane in 60 °C air incubator to dry.

incubate dot blot

Wash blot briefly with PBST[5] two times, then block with blocking reagent[13] for 10 min. Add anti-DIG alkaline phosphatase conjugated antibody to a final concentration of ~1:5,000–1:10,000. Incubate for 20 min. Pour off antibody and wash four times 5 min each with PBST.

develop dot blot

Replace with PBST with AP buffer[16]; rinse 3x with AP buffer. Replace with NBT/BCIP staining solution[17] and allow to develop without agitation until a range of control dots become visible (typically 5–10 min). Rinse twice with nano H_2O and dry in air incubator.

estimate probe concentration

Compare staining of control DIG-labeled RNA dots (10^{-1}=10 ng, 10^{-2}=1 ng, etc.) to probe dilutions to estimate probe concentration. Successful probe synthesis, hydrolysis and recovery should yield probe at 50–100 ng/µl.

m. Adding a small amount (~1 µl) of Ambion SUPERaseIn RNase inhibitor to the probe stock(s).

n. Log probe(s) into the probe storage inventory; be sure to fill-in all inventory fields: https://spreadsheets0.google.com/a/uw.edu/spreadsheet/ccc?key=tPLlqNVw0FQaB1CpwPjvM9g#gid=0

o. Store well-labeled probe (with ID number) in a designated location within the probe storage boxes at –80 °C. Store diagnostic images, dot blots, etc. for probe(s) with their corresponding numbers in the probe diagnostics loose lead binder.

IN SITU HYBRIDIZATION

DAYS 1–3: Pretreating fish, harvesting, and tissue fixation

1. Methylene blue treatment of fish to be fixed.

Methylene blue treatment helps to remove surface fungal or bacterial contamination that may increase background during staining.

a. At the end of the day before harvesting larvae for in situ hybridization, collect larvae to be used in a fine screen net (be gentle, larvae are fragile) and place them in a new clean tank containing system water and enough methylene blue to give a light to moderate blue tint to the water.

b. Mark the tank with stock information and a tag stating "Do not feed" with your initials and date. Place the tank in the quarantine room or in the fish room with gentle aeration from a bubbler. Do NOT put the tank on flowing water as methylene blue will harm the essential denitrifying bacteria in the fish system.

c. Continue processing larvae early the next morning, beginning with step 2, below.
Larvae may not be left for an extended period of time without food. Additionally, there is some indication that owing to circadian effects, some transcripts may be more abundant very early in the morning than in the middle of the afternoon.

2. Prepare fix: 4% paraformaldehyde, 1% DMSO in PBS

Fix should be made fresh on the day it will be used. Do not store it for long periods. Paraformaldehyde will cross link proteins; DMSO will assist penetration into tissues. Scale-up recipe as appropriate for amount of tissue to be fixed.

a. Add to an Erlenmeyer flask 200 ml PBS[4]; heat in microwave 45 sec

b. Add 8 g paraformaldehyde (toxic: wear gloves, do not breathe powder)

c. Add stir bar, cover, and place on hot plate at medium heat; do not boil

d. Powder should dissolve within a few minutes

e. Place on ice to cool

f. Add 2 ml DMSO

3. Harvest and fix larvae

It is important to avoid contaminating with fix fish room dishes, nets, and tanks. The transfers described are intended to prevent such contamination from occurring.

a. Collect larvae in a fine screen net; be gentle, larvae are fragile

b. Optional: add several drops to milliliters of 10 mg/ml epinephrine (or a dash of epinephrine powder); be sure to wear gloves when handling the epinephrine
After several minutes this will cause melanosomes to move to melanophores centers. Useful for examining expression in melanophores themselves, or to get melanin out of the way for visualizing deeper tissues.

c. Transfer larvae to a large disposable Petri dish with a small amount of fish water and euthanize by MS222 overdose

d. Fill a second large disposable Petri dish half way with fix

e. Pour larvae into fine net (for fix use only), and then transfer them into the dish with fix

f. Leave at room temperature for ~10 min

g. Use a scalpel or razor blade to remove heads, tails, or both, to improve reagent penetration

h. Trim a plastic transfer pipette so it has a wide bore opening; use this to transfer the trunks to a 50 ml blue cap plastic centrifuge tube

i. Top off with fix and place on a rocker at 4 °C, overnight at least
We typically perform in situs on larvae fixed over one or two nights at 4 °C.

4. Dehydration
Methanol precipitates proteins and removes cell membranes, permeabilizing the tissue. Although embryos can be stored for long times in methanol at –20 °C, we prefer to store larvae no more than 1–2 days as signal can be lost if not fixed thoroughly. Storing in "fake hybridization" solution at –20 °C is likely preferable.

a. Use an RNase-free pipette to remove fix

b. Replace fix with 100% methanol

c. Rinse 2x ~5 min each with 100% methanol (pouring usually works unless the larvae float)

d. Place at –20 °C overnight, or proceed immediately

DAY 4: Prehybridization

1. Rehydration
Transcripts are vulnerable to RNase during these steps; work rapidly.

a. Remove larvae from freezer and allow them to warm ~10-15 min.

b. While larvae are warming, prepare fresh 4% paraformaldehyde (as above but without DMSO); cool to room temperature.

c. Remove methanol from larvae and serially replace 3–5 min each with:
- 75% methanol / 25% PBST[5]
- 50% methanol / 50% PBST
- 25% methanol / 75% PBST
- 100% PBST
- 100% PBST
We use a plastic squirt bottle for more rapidly dispensing PBST in these and all subsequent steps. Trim the tip of the bottle to allow a larger volume to be dispensed with less force on the tissue. Always start each round of in situs with freshly made PBST.

d. Replace one more time with 100% PBST and leave on rocker with gentle agitation.

2. Proteinase-K[7] treatment
Proteinase-K (PK) treatment is critical for permeabilizing tissue and degrading RNA binding proteins that may hinder riboprobe access to target. Different times and concentrations are appropriate for different stages, tissues of interest and probes. When first trying a probe it is best to try a couple of different PK concentrations and incubation times to determine which will be appropriate (e.g., a more

extensive treatment may be ideal for deeply situated bones, but a more gentle treatment for pigment cells immediately under the skin). Too little or too much PK treatment can reduce signal. Also beware the lots of PK can vary in activity, so whenever possible make up large quantities of concentrated stocks and use the same lot number for a given set of experiments. Treatments below include DMSO to facilitate PK penetration into tissues. Additional enzymatic digestions to reduce incubation times are being tested as well.

a. Prepare a solution of 10–20 µg/ml proteinase-K[7] in PBST containing 1% DMSO. Reasonable concentration/time combinations to try are:
   • 20 µg/ml, 20–25 min (early metamorphic larvae)
   • 20 µg/ml, 25–30 min (mid metamorphic larvae)
   • 20 µg/ml, 30–40 min (late metamorphic larvae)
   • 20 µg/ml, 40–60 min (juveniles with scales)
   • 10 µg/ml, 12 min (very superficial tissues)
   Proteinase K concentrations and incubation times should be determined for each probe and stage. Deep tissues or longer fixation times may require longer PK treatments. The smaller or more accessible the specimen, the less treatment required (e.g., less time for larvae with heads removed compared to heads intact). If possible, use >1 treatment per probe to ensure staining as well as positive control probes known to stain tissue layer at the depths of interest.
   Proteinase K is expensive; make only as much solution as will be needed to allow gentle agitation of larvae. 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.

b. Replace PBST with proteinase-K solution.

c. Gently rock on shaker for 12–60 min depending on probe, tissue, stage.

d. Optional: To rapidly stop PK activity, rinse 2x with PBST then incubate 10 min in freshly prepared 2 mg/ml glycine in PBST, followed by rinsing 2x in PBST.
   We do not normally do this.

e. Replace with 4% paraformaldehyde (which should already be at room temperature).

f. Fix for 20 min at room temperature with gentle rocking.

3. Prehybridization

a. Wash larvae 3x in PBST, 5 min per wash.

b. Remove PBST and replace with "fake" hybridization[8] solution.

c. Allow larvae to sink and replace with fresh "fake" hybridization solution; repeat once more for three total washes in fake hybridization solution.
   Specimens may be safely stored at –20 °C at this point.

d. Use an RNase-free wide mouth transfer pipette to divide larvae (and fake hybridization solution) amongst glass 5 ml vials that will be used for hybridization.
   For labeling vials, it is best to number them 1,…,n and keep a log of the respective treatments; this will simplify labeling during later steps. A wide black permanent Sharpie can be used to label the caps, but this should not be relied upon as the label can sometimes come off. A better method is to use a diamond scribe to etch the number onto the side of the glass.

e. Replace solution in vials with hybridization solution[9] (fill vials at least two-thirds full).

f. Place vials in shaking water bath set to 58 °C – 68 °C[10] with ~30 gentle oscillations per minute

Normally incubation should be done at 68 °C unless a probe is known or discovered to only work at lower temperatures. Vials are placed on their sides in a covered tray lined with paper towels and containing water a few mm deep.[11] The tray is allowed to float within a larger, water filled tray that is stationary on the moving water bath platform. Larvae should be moving gently.

g. Leave 3 hours to overnight.

DAY 4: Hybridization

1. Prepare hybridization solution[9]

a. For each probe to be used, fill sealable tubes with enough hybridization solution for each sample vial (one tube per sample vial, or make a master mix with about 10% more solution than you expect to need).
Typically ~1500 µl of hybridization solution is used per vial. Use enough to cover the larvae and allow them to move back and forth; the vial should not need to be filled. To keep about the same ratio of tissue to solution, scale up or down as needed.

b. Add appropriate volume of fragmented probe to hybridization solution. Dilute probe to 0.55 ng/µl. Excessive probe concentrations will lead to extensive background and failed in situs, so be sure to quantitate your probe concentrations carefully.

c. Preheat hybridization solution in water bath at 58 °C – 68 °C.[10,11]
We prefer to use high temperatures, and try to ensure probe penetration, rather than using lower temperatures which may allow for binding of small quantities of probe that has reached the interior of specimens, but non-specific binding of larger quantities of probe near the surface. A temperature of 66 °C often works well.

2. Hybridization

a. Replace prehybridization solution with hybridization solution.
Do not allow the larvae to cool: it is best to work rapidly and remove only one vial at a time from the water bath.

b. Leave vials overnight (8–16 h) at 58 °C – 68 °C with gentle agitation.[10,11]

c. Periodically check water bath and refill as needed due to evaporation; if a large volume must be added, preheat water in microwave.

DAY 5: Hybridization washes and antibody block

1. Washes

For stringency washes, use the same temperature as for hybridization (58 °C – 68 °C); do not let samples cool: work rapidly and remove only one vial at a time from the water bath. These do not need to be performed RNase free.

a. Preheat all wash solutions in 50 ml blue cap centrifuge tubes or flasks as appropriate. Assume 5 ml solution per sample, for washes done in vials (2x SSCT, steb 1b). Use 2 L beakers covered with saran wrap or sealable tupperware containers for washes done in plastic carriers (0.2x SSCT, 2x SSCT step 1c).

b. Remove hybridization solution and wash with:

- 2x SSCT — 5 min, 58 °C – 68 °C
- 2x SSCT — 5 min, 58 °C – 68 °C
- 2x SSCT — 5 min, 58 °C – 68 °C
-

c. Transfer larvae from glass vials to plastic carriers, and immerse in:
   - 2x SSCT — 1 h, 58–68 °C
   - 2x SSCT — 1 h, 58–68 °C
   - 0.2x SSCT — 45 min, 58–68 °C
   - 0.2x SSCT — 45 min, 58–68 °C

d. Using a wide mouth pipette, transfer larvae from carriers to new vials at room temperature and perform additional washes to transfer larvae to PBST (or MAB[13]). Larvae may be sticky at this stage so do not rock vials; instead swirl gently a few times at each step.
   - 66% 0.2x SSCT / 33% PBST (or MAB) — 3 min, RT
   - 33% 0.2x SSCT / 66% PBST (or MAB) — 3 min, RT
   - PBST (or MAB) — 3 min, RT

Note: Sensitivity might be enhanced by avoiding PBS, as the phosphate may under some circumstances inhibit alkaline phosphatase activity. Maleic acid buffer (MAB) is a standard substitute but is expensive. Tris buffered saline (TBS) may be a reasonable compromise. We have seen little difference among buffers, however, and routinely use PBS.

2. Antibody block and antibody preparation

a. Remove PBST from vials and fill vials ~half full with standard blocking solution[14] for PBST-based incubations (or Roche block[15] for MAB-based incubations)

b. Allow larvae to sit several minutes in blocking solution with only occasional swirling

c. Place vials on a rocker with gentle agitation overnight at 4 °C.
Longer, overnight incubations may allow more time for additional unbound probe to diffuse from the tissue.

DAY 6: Antibody incubation

1. Preadsorb anti-DIG Fab fragments for 1 hr at room temperature or overnight at 4 °C by diluting antibody 1:500 in blocking solution containing a small amount of fish powder[16]
2. Filter through a 0.22 μm syringe filter and dilute tenfold with fresh blocking solution to 1:5000 final working strength; generally we use 1.5 ml per vial (extra filtered preadsorbed antibody can be kept at 4 °C at 1:500 dilution for later use)
3. Replace blocking solution with antibody
4. Place vials on rocker with gentle agitation for 24 h at 4 °C

DAY 7: Antibody washes

1. Rinses

a. Remove Ab from vials and replace with PBST (or MAB)
Diluted antibody can be re-used: pass through a 0.2 μm syringe filter to sterilize and store appropriately labeled at 4 °C. Generally we use only fresh antibody for larval in situs, but used antibody is appropriate for embryos or for blotting.

b. Immediately replace with fresh PBST (or MAB) and agitate gently at room temperature 5–10 min

2. Dialysis washes

a. Use pre-numbered plastic carrier vials with mesh-covered holes on top and bottom[17]
Inspect the carriers to see if relabeling is needed, if so use a black Sharpie on the mesh, not on the plastic (it will wash off). Verify that the mesh is firmly attached to the plastic.

b. Fill a 2 L glass beaker with 2 L of PBST2[6] (or MAB[13]); add a medium size stir bar

c. Place open plastic carrier in a Petri dish and pour larvae into it; if necessary use a wide mouth transfer pipette to assist with this; screw cap on tightly (otherwise it will come off during the washes and the larvae will float out)

d. Place the plastic carrier into the 2 L PBST2 (MAB) beaker ; use a transfer pipette placed against one end to suck out any air bubbles

e. Once all plastic carriers are in, place the beaker on a stir plate at 4 °C
Stir just fast enough that carriers are drawn down to the bottom, but not so fast that the carriers will be damaged.

f. Stir gently for 2x overnight (36–48 h) at 4 °C with ~5 changes of PBST2 (or MAB)
Since washing depends on the diffusion of excess antibody out of the specimens, wash for at least as long as the initial antibody incubation. Be careful that vials are spinning gently; excessive speed and agitation damages the specimens.

DAY 8–9: Developing and storage

1. Rinse into freshly made alkaline phosphatase (AP) buffer[18]

a. Pour larvae from plastic carriers into Falcon 50 x 9 mm (#1006) sealable plastic Petri dishes
Label the edges of both top and bottom with black Sharpie.

b. Remove PBST and replace with enough AP buffer to cover larvae; rinse with gentle agitation ~10 min at room temperature.
We dispense AP buffer and staining solution (below) from a squirt bottle, as for PBST above.

c. Change AP buffer 2 more times ~10 min each.

2. Developing

a. Replace AP buffer with staining solution[17] (NBT/BCIP in AP buffer) at room temperature
Typically ~8–10 ml staining solution per dish is appropriate. The staining solution is light sensitive.

b. Leave in staining solution covered at room temperature with minimal agitation on a slow rotator. Check after ~1 hour and then at increasing intervals over several hours to days. If you are concerned about leaving the specimens staining unattended through the night, place them in fresh staining solution (or AP buffer) and leave at 4 °C; if left at room temperature, be sure to change the staining solution before the end of the day.
The staining solution is light sensitive.

c. Change staining solution with freshly made solution when it starts to lose its yellow or straw color.
We use frequent changes, especially early in the reaction, assuming this is when the alkaline phosphatase is most active.

d. Periodically examine the larvae under the microscope to monitor staining progress.
Reasonably stained larvae may appear nearly uniformly black to the naked eye. It is crucial that the specimens be viewed with appropriate magnification. Illumination from the side, as opposed to directly from above or below is usually helpful, as are different microscope stage backgrounds (black, white, etc.) depending on specimen thickness, contrast, etc. Good staining may take anywhere from 2–72 hr, so do not discard samples if they do not develop color quickly. It may be advisable to stop sets of larvae at different times to avoid potential over staining as well.

3. Stopping

a. Replace staining solution with alkaline phosphatase stop solution: PBS pH 5.5.

b. Wash with PBS 2–3 times at room temperature, 10–30 min with gentle rocking.

c. Fix in the dark overnight in 4% paraformaldehyde in PBS.

4. Storage

a. Store in fix or transfer to glycerol:

- Wash 3 times with PBST ~10 min each at room temperature
- Replace with 50% PBS / 50% glycerol / 0.1% sodium azide; allow larvae to sink.
- Replace with 100% glycerol.
Use only high quality glycerol as bad batches can leach purple precipitate from specimens.

b. Store in the dark or filet and mount on slides for immediate viewing

NOTES AND REAGENTS

[1] Adapted from:
C. Thisse and B. Thisse, *High resolution whole-mount in situ hybridization*. Unpublished.
T. Jowett and Y.-L. Yan. 1996. Two-color whole-mount in situ hybridization. In: P. Krieg (ed.), *A Laboratory Guide to RNA: Isolation, Analysis, and Synthesis*. Wiley.
If you publish results obtained with this protocol, please cite one of the Parichy lab's recent publications or as a personal communication from D.M. Parichy.

[2] 10X MOPS buffer:

0.4 M MOPS, pH 7.0
0.1 M sodium acetate
10 mM EDTA

Autoclave for 1 h and cool before using. Store at 4 °C.

[3] RNA loading buffer:

1.5X dyes (0.06% bromophenol blue, 0.06% xylene cyanol)
1.5X MOPS
9% formaldehyde
60% formamide
0.1 mg / ml ethidium bromide
Many other possible versions.

[4] PBS, phosphate buffered saline:

To make 1 L of 10X stock:

NaCl	80.0 g
KCl	2.0 g
Na_2HPO_4	14.4 g
KH_2PO_4	2.4 g
nano H_20	to 1.0 L

dilute to 1X working concentration

[5] PBST, phosphate buffered saline + Tween-20:

To make 1 L of 10X stock:

NaCl	80.0 g
KCl	2.0 g
Na_2HPO_4	14.4 g
KH_2PO_4	2.4 g
Tween-20	10.0 ml
nano H_20	to 1.0 L

dilute to 1X working concentration

[6] PBST2, phosphate buffered saline + Tween-20:

To make 1 L of 10X stock:	
NaCl	80.0 g
KCl	2.0 g
Na_2HPO_4	14.4 g
KH_2PO_4	2.4 g
Tween-20	20.0 ml
nano H_20	to 1.0 L

dilute to 1X working concentration

[7] Proteinase-K (Roche) stock for in situs:
Aliquots are 10 mg/ml proteinase-K in proteinase-K storage buffer, stored at –80 °C. Once thawed, aliquots may be stored at —20 °C, but should not be refrozen at –80 °C. To make aliquots use an unopened 250 mg vial of lyophilized proteinase-K. Add to the vial xxxx ml nano H_20 and gently dissolve the powder. Add 12.5 ml glycerol, 250 µl 1 M Tris pH 7.5, and 1 ml 500 mM $CaCl_2$. Mix gently but thoroughly. Aliquot and store at –80 °C. Storage buffer final composition is: 50% glycerol, 10 mM Tris pH 7.5, 20 mM $CaCl_2$ in nano H_2O.

[8] "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 H_2O	to 500 ml total

Store at room temperature.

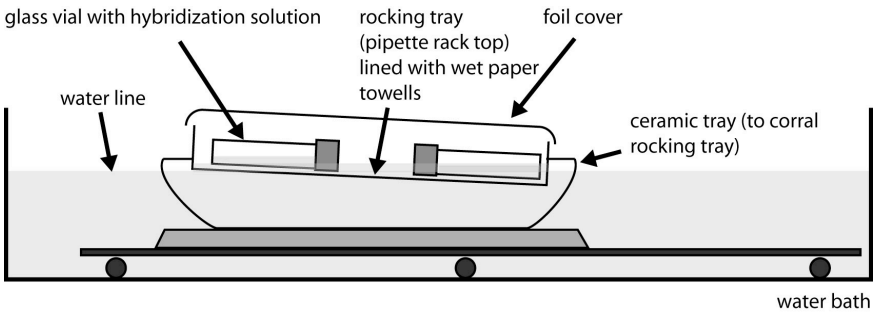
[9] Hybridization solution (T-hyb):

formamide	250 ml
20X SSC[12]	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 H_2O	to 500 ml total

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

[10] Hybridization and pre-hybridization temperature:
Appropriate prehybridization and hybridization temperatures need to be determined empirically. 65 °C is a good starting point.

[11] Hybridization set-up:



[12] SSC and SSCT:
Make 20X SSC stock for dilution to working concentrations of 2X, 0.2X, 0.05x and for use in hybridization solution.

20X SSC stock solution (3.0 M NaCl, 0.3 M sodium acetate): Dissolve 175.3 g NaCl, 88.2 g sodium citrate (dihydrate) in 800 ml nano H_20. Adjust pH to 7.0 with a few drops of concentrated HCl. Adjust volume to 1 L with H_2O. Sterilize by autoclaving.

To make 2X, 0.2X or 0.05X SSCT, dilute 20X SSC with nano H_2O and add 2 ml Tween-20 per L.

[13] 10x MAB stock:
For 1 L of concentrated stock solution, begin with ~750 ml nano H_2O then add:

1 M maleic acid	116.07 g
1.5 M NaCl	87.66 g

Adjust to pH 7.5 with NaOH pellets initially (~72 g per liter) then 10 M NaOH solution. Buffer will clear around pH 6.0. Desired pH is easy to overshoot!

fill with nano H_2O to 1000 ml

Use at 1x for washes, etc.

[14] 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.

[15] Roche blocking reagent:
To make a 10x stock (10% w/v), dissolve 10 g Roche blocking reagent in 100 ml 1x MAB over moderate heat with stirring. Aliquot and store at -20 °C. For working strength, dilute to 1x with MAB.

[16] Fish powder:
Fix larval and adult fish as well as some larval tank debris in formalin for 1–2 hr at room temperature. Wash in tap water 1–2 hr and place in blender. Puree. Add acetone or methanol and centrifuge in SS34 or larger rotor. Pour off supernatant and repeat several times. Transfer pellet to open dish, spread, and allow to dry in 60–70 °C air incubator. Once dried, store in sealed vial at 4 °C.

[17] Plastic vials:
Nalgene 4 ml polypropylene with holes melted through top and bottom, then covered with 250 μm Nytex mesh attached with heat.

[18] AP buffer:
100 mM Tris HCl pH 9.5, 50 mM $MgCl_2$, 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 $MgCl_2$	10 ml
5 M NaCl	4 ml
10% Tween-20	2 ml

[17] NBT/BCIP Staining solution

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

Make up NBT stock from powder in 70% dimethylformamide, 30% H_2O in a glass bottle (DMF will dissolve some plastics). Protect both NBT and BCIP soutions from light at store at –20 °C. Verify that NBT is bright yellow before each use. If it has started to turn brown, discard it.

APPENDIX: Hydrolysis times (min at 60 °C)

Starting nt \ Final nt	200	250	300	350	400	450	500	550	600
200									
250	9.1								
300	15.2	6.1							
350	19.5	10.4	4.3						
400	22.7	13.6	7.6	3.2					
450	25.3	16.2	10.1	5.8	2.5				
500	27.3	18.2	12.1	7.8	4.5	2.0			
550	28.9	19.8	13.8	9.4	6.2	3.7	1.7		
600	30.3	21.2	15.2	10.8	7.6	5.1	3.0	1.4	
650	31.5	22.4	16.3	12.0	8.7	6.2	4.2	2.5	1.2
700	32.5	23.4	17.3	13.0	9.7	7.2	5.2	3.5	2.2
750	33.3	24.2	18.2	13.9	10.6	8.1	6.1	4.4	3.0
800	34.1	25.0	18.9	14.6	11.4	8.8	6.8	5.2	3.8
850	34.8	25.7	19.6	15.3	12.0	9.5	7.5	5.8	4.5
900	35.4	26.3	20.2	15.9	12.6	10.1	8.1	6.4	5.1
950	35.9	26.8	20.7	16.4	13.2	10.6	8.6	7.0	5.6
1000	36.4	27.3	21.2	16.9	13.6	11.1	9.1	7.4	6.1
1050	36.8	27.7	21.6	17.3	14.1	11.5	9.5	7.9	6.5
1100	37.2	28.1	22.0	17.7	14.5	11.9	9.9	8.3	6.9
1150	37.5	28.5	22.4	18.1	14.8	12.3	10.3	8.6	7.2
1200	37.9	28.8	22.7	18.4	15.2	12.6	10.6	9.0	7.6
1250	38.2	29.1	23.0	18.7	15.5	12.9	10.9	9.3	7.9
1300	38.5	29.4	23.3	19.0	15.7	13.2	11.2	9.5	8.2
1350	38.7	29.6	23.6	19.2	16.0	13.5	11.4	9.8	8.4
1400	39.0	29.9	23.8	19.5	16.2	13.7	11.7	10.0	8.7
1450	39.2	30.1	24.0	19.7	16.5	13.9	11.9	10.3	8.9
1500	39.4	30.3	24.2	19.9	16.7	14.1	12.1	10.5	9.1
1550	39.6	30.5	24.4	20.1	16.9	14.3	12.3	10.7	9.3
1600	39.8	30.7	24.6	20.3	17.0	14.5	12.5	10.8	9.5
1650	39.9	30.9	24.8	20.5	17.2	14.7	12.7	11.0	9.6
1700	40.1	31.0	25.0	20.6	17.4	14.9	12.8	11.2	9.8
1750	40.3	31.2	25.1	20.8	17.5	15.0	13.0	11.3	10.0
1800	40.4	31.3	25.3	20.9	17.7	15.2	13.1	11.5	10.1
1850	40.5	31.4	25.4	21.1	17.8	15.3	13.3	11.6	10.2
1900	40.7	31.6	25.5	21.2	17.9	15.4	13.4	11.7	10.4
1950	40.8	31.7	25.6	21.3	18.1	15.5	13.5	11.9	10.5
2000	40.9	31.8	25.8	21.4	18.2	15.7	13.6	12.0	10.6
2050	41.0	31.9	25.9	21.5	18.3	15.8	13.7	12.1	10.7
2100	41.1	32.0	26.0	21.6	18.4	15.9	13.9	12.2	10.8
2150	41.2	32.1	26.1	21.7	18.5	16.0	14.0	12.3	10.9
2200	41.3	32.2	26.2	21.8	18.6	16.1	14.0	12.4	11.0
2250	41.4	32.3	26.3	21.9	18.7	16.2	14.1	12.5	11.1
2300	41.5	32.4	26.4	22.0	18.8	16.2	14.2	12.6	11.2
2350	41.6	32.5	26.4	22.1	18.9	16.3	14.3	12.7	11.3
2400	41.7	32.6	26.5	22.2	18.9	16.4	14.4	12.7	11.4

Starting nt \ Final nt	200	250	300	350	400	450	500	550	600
2450	41.7	32.7	26.6	22.3	19.0	16.5	14.5	12.8	11.4
2500	41.8	32.7	26.7	22.3	19.1	16.6	14.5	12.9	11.5
2550	41.9	32.8	26.7	22.4	19.2	16.6	14.6	13.0	11.6
2600	42.0	32.9	26.8	22.5	19.2	16.7	14.7	13.0	11.7
2650	42.0	32.9	26.9	22.5	19.3	16.8	14.8	13.1	11.7
2700	42.1	33.0	26.9	22.6	19.4	16.8	14.8	13.2	11.8
2750	42.1	33.1	27.0	22.7	19.4	16.9	14.9	13.2	11.8
2800	42.2	33.1	27.1	22.7	19.5	17.0	14.9	13.3	11.9
2850	42.3	33.2	27.1	22.8	19.5	17.0	15.0	13.3	12.0
2900	42.3	33.2	27.2	22.8	19.6	17.1	15.0	13.4	12.0
2950	42.4	33.3	27.2	22.9	19.6	17.1	15.1	13.4	12.1
3000	42.4	33.3	27.3	22.9	19.7	17.2	15.2	13.5	12.1
3050	42.5	33.4	27.3	23.0	19.7	17.2	15.2	13.5	12.2
3100	42.5	33.4	27.4	23.0	19.8	17.3	15.2	13.6	12.2
3150	42.6	33.5	27.4	23.1	19.8	17.3	15.3	13.6	12.3
3200	42.6	33.5	27.5	23.1	19.9	17.4	15.3	13.7	12.3
3250	42.7	33.6	27.5	23.2	19.9	17.4	15.4	13.7	12.4
3300	42.7	33.6	27.5	23.2	20.0	17.4	15.4	13.8	12.4
3350	42.7	33.6	27.6	23.3	20.0	17.5	15.5	13.8	12.4
3400	42.8	33.7	27.6	23.3	20.1	17.5	15.5	13.9	12.5
3450	42.8	33.7	27.7	23.3	20.1	17.6	15.5	13.9	12.5
3500	42.9	33.8	27.7	23.4	20.1	17.6	15.6	13.9	12.6
3550	42.9	33.8	27.7	23.4	20.2	17.6	15.6	14.0	12.6
3600	42.9	33.8	27.8	23.4	20.2	17.7	15.7	14.0	12.6
3650	43.0	33.9	27.8	23.5	20.2	17.7	15.7	14.0	12.7
3700	43.0	33.9	27.8	23.5	20.3	17.7	15.7	14.1	12.7
3750	43.0	33.9	27.9	23.5	20.3	17.8	15.8	14.1	12.7
3800	43.1	34.0	27.9	23.6	20.3	17.8	15.8	14.1	12.8
3850	43.1	34.0	27.9	23.6	20.4	17.8	15.8	14.2	12.8
3900	43.1	34.0	28.0	23.6	20.4	17.9	15.9	14.2	12.8
3950	43.2	34.1	28.0	23.7	20.4	17.9	15.9	14.2	12.9
4000	43.2	34.1	28.0	23.7	20.5	17.9	15.9	14.3	12.9
4050	43.2	34.1	28.1	23.7	20.5	18.0	15.9	14.3	12.9
4100	43.2	34.1	28.1	23.8	20.5	18.0	16.0	14.3	12.9
4150	43.3	34.2	28.1	23.8	20.5	18.0	16.0	14.3	13.0
4200	43.3	34.2	28.1	23.8	20.6	18.0	16.0	14.4	13.0
4250	43.3	34.2	28.2	23.8	20.6	18.1	16.0	14.4	13.0
4300	43.3	34.2	28.2	23.9	20.6	18.1	16.1	14.4	13.0
4350	43.4	34.3	28.2	23.9	20.6	18.1	16.1	14.4	13.1
4400	43.4	34.3	28.2	23.9	20.7	18.1	16.1	14.5	13.1
4450	43.4	34.3	28.3	23.9	20.7	18.2	16.1	14.5	13.1
4500	43.4	34.3	28.3	24.0	20.7	18.2	16.2	14.5	13.1
4550	43.5	34.4	28.3	24.0	20.7	18.2	16.2	14.5	13.2
4600	43.5	34.4	28.3	24.0	20.8	18.2	16.2	14.6	13.2
4650	43.5	34.4	28.3	24.0	20.8	18.2	16.2	14.6	13.2
4700	43.5	34.4	28.4	24.0	20.8	18.3	16.2	14.6	13.2
4750	43.5	34.4	28.4	24.1	20.8	18.3	16.3	14.6	13.2
4800	43.6	34.5	28.4	24.1	20.8	18.3	16.3	14.6	13.3
4850	43.6	34.5	28.4	24.1	20.9	18.3	16.3	14.7	13.3
4900	43.6	34.5	28.4	24.1	20.9	18.3	16.3	14.7	13.3
4950	43.6	34.5	28.5	24.1	20.9	18.4	16.3	14.7	13.3
5000	43.6	34.5	28.5	24.2	20.9	18.4	16.4	14.7	13.3

Notes for updating: