

Whole Mount RNA In Situ Hybridization: Zebrafish Embryos

C. Moens, adapted from Thisse et al., 1993

Step 1 of 3: Probe Synthesis

A. Template preparation. You can prepare template either by linearizing a plasmid or by PCR.

Method 1: generating a plasmid template

- linearize (10 ug) plasmid DNA with the appropriate restriction enzyme for *antisense* transcription (so that the plasmid is cut at the 5' end of the insert to be transcribed).
- (optional: at the end of the digest add ProK (final concentration 100ug/ml) and SDS (0.5% final))
- phenol/chloroform, chloroform extract, ethanol precipitate, resuspend in sterile (DEPC) water
- check linearized DNA on an agarose gel to ensure that the plasmid is completely linearized.
- Proceed to step B below.

Method 2: generating a PCR template

Use primers that amplify fragment from plasmid that includes an RNA polymerase priming site at the 3' end of the insert. Many plasmids have M13 sites outside T7 and T3 polymerase sites, in these cases just use M13 forward and reverse primers. If this is not the case, you need to design primers that create a T7 or T3 site on the 3' end of the fragment.

Set up PCR:

For a 50 ul Rxn, following Hot Start ExTaq directions (TAK RR006A)

0.25 ul	Hot-Start ExTaq
5.0 ul	10X ExTaq Buffer
4.0 ul	dNTPs (2.5mM, provided with ExTaq)
2.0 ul	Fwd Primer (5 uM)
2.0 ul	Rev Prime (5uM)
small vol	DNA template
<u>38.75 ul</u>	H2O
50.0 ul	TOTAL Vol

(Note: very little plasmid DNA is necessary. 0.5 ul of a typical miniprep is plenty).

- Use annealing temp appropriate for your primers. For most generic primers (M13F, M13R, T7, T3, Sp6) use the following program:

94C 4:00
94C 0:30
53C 0:30
72C 3:00 (depending on insert length, but this is OK in generic program)
35 Cycles
72C 7:00
4C hold

- Concentrate on Microcon Montage PCR column (UFC7PCR50):
- Add entire reaction to top of column + 450 ul DEPC H₂O.
- Spin until very nearly or just dry, 15-20 min at 1000g.
- Rinse membrane with 20 ul DEPC H₂O, invert and spin into clean tube (1 min, 1000g).
- Run out a small amount of PCR (2 ul is plenty) to ensure that you have a product which is mostly a single band of expected size. Concentration by spec should be in the range of 75 ug/ml to 250 ug/ml.

B. In vitro transcription

For DNA:

If using linearized plasmid use 1-2ug.

If using PCR, I usually use 5-10 ul.

For a 20 ul reaction, assemble on ice:

Promega RNA polymerase:

to 20 total	DEPC H ₂ O
XX ul	DNA
4.0 ul	5X Trans Buffer
2.0 ul	100 mM DTT
2.0 ul	10X Dig Trans Mix (Roche)
1.0 ul	RNAasin
1.0 ul	T7 or T3 RNA Pol

Roche RNA polymerase:

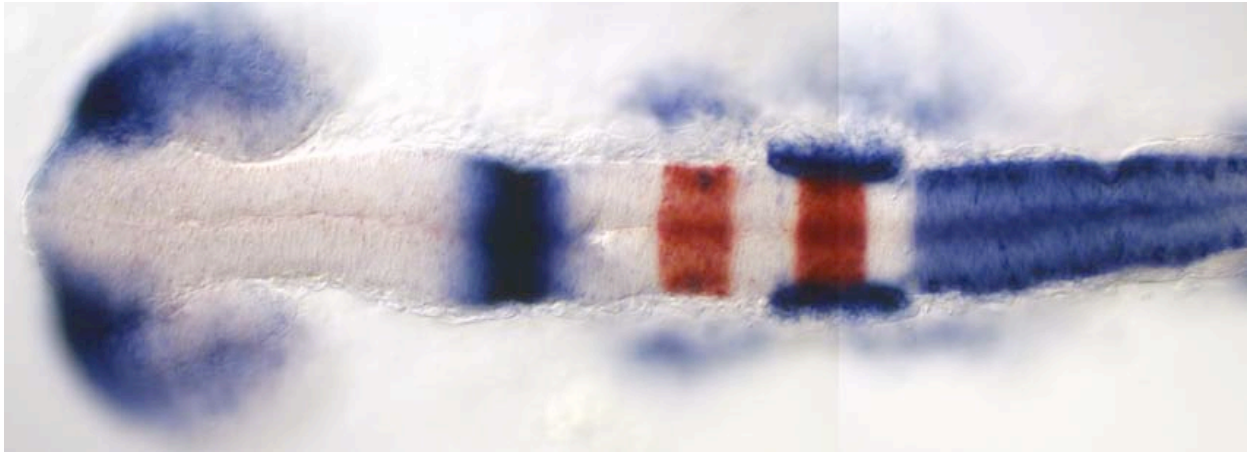
to 20 total	DEPC H ₂ O
XX ul	DNA
2.0 ul	10X Trans Buffer
	included in Roche Buffer
2.0 ul	10X Dig Trans Mix
1.0 ul	RNAse inhibitor
1.0 ul	T7 or T3 RNA Pol

- Incubate at 37C, for 2 hours. Optional: after 1 hr you can add 1ul of additional RNA polymerase.
- Digest template by adding 1ul of DNase, 15 min at 37C.
- Stop reaction by adding: 1.0 ul 0.5 M EDTA.
- Precipitate RNA by adding 2.5 ul 4M LiCl₂ and 100 ul EtOH (100%). Precipitate at least one hour at –80C. Spin down and rinse with 70% ethanol made with DEPC H₂O.
- (*Alternate: Increase volume to 50 ul with DEPC H₂O and clean up reaction on a Roche mini Quick Spin RNA column (Roche 11 814 427 001) according to Roche's directions.*)
- Resuspend in 50ul DEPC H₂O, plus RNase inhibitor, or in 50 ul Hyb mix (made without tRNA or Heparin; see recipe for Hyb mix in Appendix).
- Check probe by: running out 2-5 ul of probe on a standard 1.2-1.5% TBE agarose gel or a denaturing formaldehyde gel. RNA ladder is only informative on a denaturing gel. For samples: mix 5.0 ul probe (or less, but they can be hard to see) + 1.0 ul EtBr (1mg/ml) + 6.0 uL Ambion Sample Buffer. Heat to 65C, 5min. Cool on ice. You should see one or at most a couple of bands. Sometimes when you can barely see a band on the gel, the probe will look great in in situs. Any band is a good sign.

Notes on in vitro transcription

- a) The choice of polymerase depends on the plamid you are using: it should be the one appropriate for the priming site at the 3' end of your insert.*
- b) If you wish to do two-color in situs, you need to label one probe with digoxigenin and the other probe with fluorescein.*
- c) Add RNase inhibitor after buffer and DTT as there is a risk it may denature.*
- d) Ensure that the transcription buffer is entirely in solution (especially 10X), and add after H₂O and DNA to ensure that is does not precipitate.*
- e) If conserving reagents you can set up a 1/2 volume reaction. When adding DNase, also add 9 ul DEPC H₂O. Resuspend final pellet in only 25 ul.*
- f) Be careful not to dry out the pellet after precipitating, because it will become very difficult to re-suspend.*

g) it is possible to make probe directly from a PCR product. For this protocol go to:
<http://zfin.org/ZFIN/Methods/ThisseProtocol.html>



Step 2 of 3: In situ Hybridization

A: Embryo Fixation and Permeabilization

Embryos should be collected shortly after they are laid, fertile embryos sorted from infertile ones, and water changed to remove debris. Prior to fixation, embryos should be dechorionated manually or enzymatically according to the protocol in the online Zebrafish book (http://zfin.org/zf_info/zfbook/chapt4/4.1.html). Alternatively, they can be fixed in their chorions, but the chorions must be removed before the hybridization steps as the probe does not penetrate the fixed chorion. For embryos older than 24 hours post fertilization (hpf), the development of pigment can be blocked by raising the embryos in 0.0045% 1-phenyl-2-thiourea (P-7629 Sigma) in fish water (see the zebrafish book for recipes).

-Fix embryos in 4% Paraformaldehyde/PBS (PFA), 5 h. at room temp. or overnight at 4°C (Paraformaldehyde should be made fresh weekly as an 8% stock in sterile water and diluted 1:1 with 2x PBS before use. 8% PFA should be stored at 4°C)

optional: for long term storage, wash 1 x in 100% MeOH, 10 min., then store at -20°C in 100% MeOH. If embryos are stored in MeOH, before proceeding to next step, rehydrate embryos through 75% methanol/25% PBST, 50%methanol/50% PBST, and 25%methanol/75% PBST washes. Work with methanol and PFA should be done in the hood and waste should be put into its respective hazardous waste containers.

-Wash 5 x 5 min. at room temp in PBST (PBS/0.1% Tween-20). All PBST washes should be in approximately 0.5 mls.

-For embryos older than ~10 hpf:

-permeabilize by treating with 10 ug/ml Prot. K in PBST, 5-20 min at room temp according to age: eg: no ProK for embryos younger than 2 somites; 1 min for 2-4 somite embryos; 3min for 9-13 somite embryos; 5 min for 18-24 hour embryos, etc. *Do not over-digest or embryos will fall apart during subsequent steps.*

-refix in 4% PFA in PBS for 1 hour at room temp.

-rinse 5 x 5 min. in PBST (not necessary if embryos were not proK'd)

B: Hybridization

- Prehyb for at least 1 hour at 65°C in hyb. mix (recipe below)
- Dilute 100ng labeled RNA probe in hyb. mix (*probe dilution is arrived at empirically, however as a first approximation, dilute probe 1/200 in hyb mix.*)
- Remove prehyb and add pre-warmed hyb mix plus probe to embryos.
- Hybridize overnight at 65°C

C: Post-hybridization washes

Notes:

- a) Prewarm washing solutions before adding to embryos.*
- b) hyb mix containing probe can be removed, stored at -20°C and re-used many times without any loss of signal.*
- c) For the following washes, you can use hyb mix that does not contain tRNA or heparin if you wish to conserve these reagents.*

- Wash 5 min in 66% hyb mix, 33% 2 x SSC at 65°C.
- Wash 5 min in 33% hyb mix, 66% 2 x SSC at 65°C
- Wash 5 min in 2 x SSC at 65°C
- *Wash 1 x 20 min in 0.2 x SSC +0.1% Tween-20 at 65°C
- *Wash 2 x 20 min in 0.1 x SSC+0.1% Tween-20 at 65°C (high stringency)
- Wash 5 min in 66% 0.2 x SSC, 33% PBST at room temp.
- Wash 5 min in 33% 0.2 x SSC, 66% PBST at room temp.
- Wash 5 min in PBST at room temp

* Timing is critical

D: Anti-digoxigenin-Alkaline Phosphatase Binding

- incubate in blocking solution (PBST plus 2% sheep serum, 2 mg/ml BSA) 1 hour at room temp.
- prepare first antibody (eg, alkaline-phosphatase conjugated anti-digoxigenin) by diluting it in blocking solution; 1:5000.
- incubate in antibody for 2 hours shaking at room temp, or overnight at 4°C.

-wash 5 x 15 min in PBST (*may leave in the last PBST wash at 4°C overnight, or proceed directly to the next step*)

notes: to avoid background, leave embryos in one of the PBST washes overnight rather than in the antibody.

E: Colourization

-wash 4 x 5 min in Coloration Buffer²

-mix 45 ul nitro-blue tetrazolium (NBT) stock with 10 ml Coloration Buffer, then add 35 ul 5-bromo-4-chloro-3-indolyl phosphate (BCIP) stock.

- add 500 ul of this mix to embryos and incubate in the dark at room temperature until a blue reaction product is visible. (*colour should become visible within 3-4 hours at most. Coloration reaction can be sped up by incubating at 30°C, or slow by incubating at 4°C*).

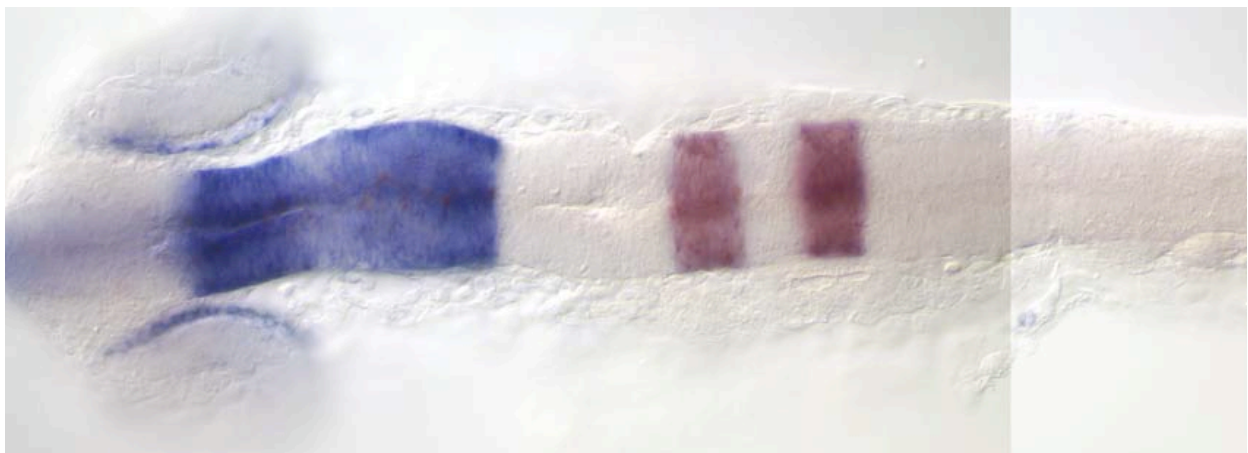
-stop reaction by washing embryos 2-4 x in sterile water.

-store at 4°C in the dark in low-pH (5.5) PBS; 1mM EDTA.

Notes:

a) the low pH PBST storage buffer prevents embryos from becoming moldy

b) if you are continuing to do a second color, wash directly from sterile H₂O into low-pH glycine (see “two-color in situs” below).



Step 3 of 3: Deyolking & Mounting

Embryos can be mounted in glycerol or dehydrated and mounted in permount, with or without the yolk. Glycerol causes less shrinkage, but is not as effective a clearing agent. If embryos are mounted on the yolk, they should be kept in the dark, and photographed immediately, because the yolk darkens rapidly (use double or triple bridged coverslips (see below), so the yolk is not crushed when mounted). Also, using BM Purple rather than NBT/BCIP in the first coloration prevents the yolk from subsequently turning purple.

Glycerol mounting

- de-yolk the embryos if required with insect pins while still in PBST. *Adherent yolk granules can be removed by brushing with a eyelash or Dalmation hair glued to a toothpick or other holder.*
- wash in 30% glycerol/ 70% PBS for about 15min. or until sunk to bottom of tube
- wash in 50% glycerol/ 50% PBS for about 15min. or until sunk to bottom of tube
- wash in 70% glycerol/ 30% PBS
- prepare a coverslip (24mm x 60mm) or slide with 4 “posts” of high vacuum grease
- transfer the embryos in a glycerol droplet to the coverslip.
- using an insect pin drag the embryo out of the drop. *Multiple embryos can be lined up in this way, each in their own micro-drop of glycerol.*
- place a coverslip (18 mm x 18 mm) on top and gently press down until the embryo is flattened but not squished (be careful not to push too hard)
- fill to the edges with 70% glycerol.
- photograph immediately or within a few days at most. *If you wish to preserve the embryos long-term, it is best to lift off the coverslip, gently dislodge the embryos and store them in an eppendorf tube in 4% PFA.*

Permount mounting:

- deyolk if required
- dehydrate the embryos through an increasing methanol/PBS series: 30%, 50%, 70%, 100% (5min each)

- clear the embryos by transferring into 66% benzyl alcohol/ 33% benzyl benzoate in the well of a glass depression dish
- once the embryos have sunk in the well (2-3 mins), pick them up with a glass hair and place in a drop of permount on a bridged coverslip (see “bridged coverslip” instructions in Appendix).
- gently lower another coverslip on top and roll until the embryo is in a desired orientation
- embryos mounted in permount can be stored indefinitely at room temperature.

Appendix

A) Two-color in situs

Two-color in situs can be done by hybridizing embryos with digoxigenin and fluorescein-labelled probes, and then detecting the digoxigenin and the fluorescein with sequential alkaline phosphatase reactions using different chromogenic substrates. In this protocol we detect the fluorescein-labelled probe with the red substrate, however either probe can be detected with either substrate. In general it is better to do the red coloration step second as the red product fades more than the blue product.

- fluorescein-labelled probes are made following the same protocol as step 1 above, but using a FITC-labeled nucleotide mix.
- hybridization is carried out as in step 2 above, except that both dig- and fluor-labelled probes are added together to the hybridization mix.
- after hybridization, the first probe (in this case the dig-labelled probe) is detected as described above in step 2.
- directly after stopping the first coloration reaction with sterile water washes, incubate embryos in 500 ul 0.1M Glycine pH2.2 for 10minutes
- wash 4x 5 min in PBST
- incubate in blocking solution (PBST plus 2% sheep serum, 2 mg/ml BSA) 1 hour at room temp.
- prepare second antibody (alkaline-phosphatase conjugated anti-fluorescein) by diluting it in blocking solution; 1:10,000.
- incubate in antibody for 2 hours shaking at room temp, or overnight at 4°C.
- wash 5x 15 min in PBST (*can leave in one of the later PBST washes at 4°C overnight*)
- wash 4 x 5 min in Coloration Buffer as in step 2 above (recipe below)

-prepare coloration reaction: add 30 ul INT/BCIP Stock Solution (33 mg/ml INT and 33 mg/ml BCIP, toluidine salt in DMSO) to 5 ml coloration buffer.

-(Optional: you can accelerate the red coloration step by diluting the coloration reagents in 10% polyvinyl alcohol. To prepare PVA, boil 0.5g (10%) Polyvinyl Alcohol into 5mls of Coloration Buffer).

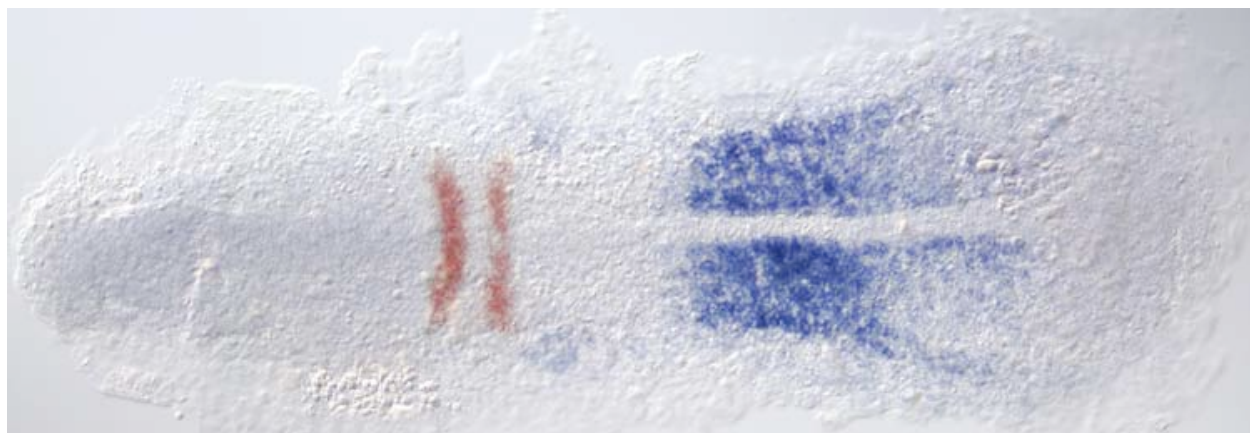
- add 500 ul of this mix to embryos and incubate in the dark at room temperature until a red reaction product is visible. *(the red colour product can take longer to become visible than the blue reaction product)*

-To stop reaction: Rinse a few times with sterile water, then store in “in situ stop buffer”: pH 5.5 PBS/ 1mM EDTA.

Appendix cont'd

B: Reagents

In vitro transcription reagents	Supplier	Catalog number
Labeled nucleotides – DIG	Roche	1277073
Labeled nucleotides – FITC	Roche	1685619
Unlabeled nucleotides	Roche	1277057
RNase Inhibitor	Promega	N2515
SP6 RNA polymerase	Promega	P1085
T7 RNA polymerase	Promega	P2075
T3 RNA polymerase	Promega	P2083
Fixative		
Paraformaldehyde	Sigma	P-6148
Hybridization reagents		
Formamide	Shelton Scientific	IB72020
Heparin	Sigma	H9399
tRNA	Sigma	R-7125
Tween 20	Sigma	P-1379
Antibody and developing reagents		
Anti-Dig AP	Roche	1093274
Anti-Fluor AP	Roche	1426338
Sheep Serum	Sigma	S-3772
BSA	Hyclone Labs	SH30070.02
NBT	Roche	11-383-213-001
BCIP	Roche	1-383-221
INT/BCIP stock solution	Roche	11-681-460-001
BM Purple	Roche	1442074
PVA	Sigma	363138



Appendix cont'd

C: solutions

1. Hybridization Solution

(store @-20 °C)

	<u>for 50 ml</u>
50% formamide	25 ml
5 x SSC	12.5 ml (20XSSC)
50 μ g/ml heparin	50 μ l of 50 mg/ml
500 μ g/ml tRNA	500 μ l of 50 mg/ml
0.1% Tween-20	250 μ l of 20%
sterile H ₂ O	to 50 ml
0.092M citric acid	460 μ l of 1M
(to adjust pH to 6.0)	

For post-hybridization washes, make and use hybridization solution without the tRNA and heparin to conserve these reagents.

2. Colouration Buffer

(make fresh each use)

100 mM Tris-Hcl, pH9.5	5 ml of 1M
50 mM MgCl ₂	2.5 ml of 1M
100 mM NaCl	1 ml of 5M
0.1% Tween-20	250 μ l of 20%
sterile water	to 50 ml

3. 0.1M Glycine, pH 2.2 (1L)

(Sigma, G-7126)

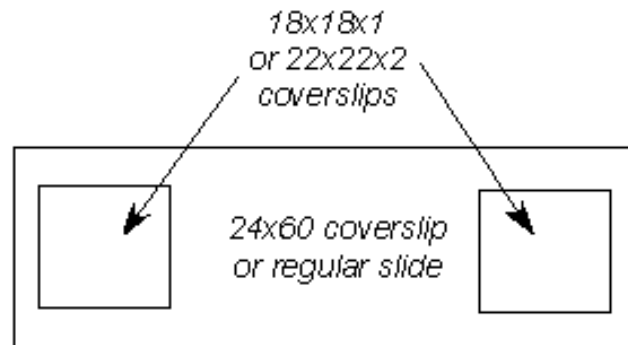
Add 7.5 grams glycine to 600 mls sterile water
Once dissolved, bring pH down to 2.2 with HCl
Bring volume up to 1L and autoclave

Appendix cont'd

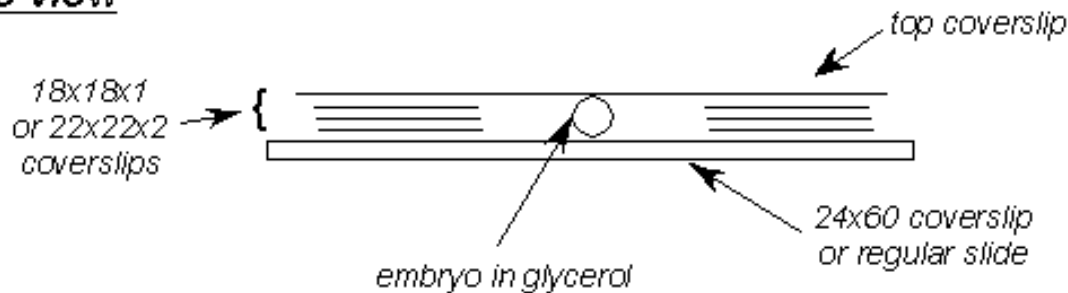
D: bridged coverslips

Bridged Coverslips

top view



side view



Instructions: use a small drop of superglue to attach 18 x 18 x 1 mm or 22 x 22 x 2 mm coverslips to either end of a microscope slide or a 24 x 60 coverslip. Add more small coverslips to the pile until the required space is accomplished - this depends on the age of the embryo and on the extent of dehydration (more dehydrated embryos are smaller). The idea is to have the top and bottom coverslips just touching the embryo but not squishing it. The exact spacing is usually arrived at empirically, but usually 2 22x22x2 coverslips is enough not to squish a bud-stage embryo.

Appendix cont'd

E: Fluorescent RNA in situ hybridization

Two protocols are available:

Julich D, Hwee Lim C, Round J, Nicolaije C, Schroeder J, Davies A, Geisler R, Lewis J, Jiang YJ, Holley SA; Tübingen 2000 Screen Consortium. (2005) *beamter/deltaC* and the role of Notch ligands in the zebrafish somite segmentation, hindbrain neurogenesis and hypochord differentiation. *Dev Biol.* 286:391-404.

Multiplex Fluorescent *In Situ* Hybridization in Zebrafish Embryos Using Tyramide Signal Amplification. Hilary Clay, Lalita Ramakrishnan *Zebrafish*. Aug 2005, Vol. 2, No. 2: 105-111

Appendix cont'd

F: In situs on sections.

It is possible to section tissues either before or after in situ hybridization. For sectioning after in situ hybridization, embed embryos in epon (plastic) and section using a glass knife according to the following protocol (from the Zebrafish Book, courtesy of Ruth BreMiller).

Resin Sections

1. Use embryos in which alkaline phosphatase has been employed as a detection enzyme. For the best results it is important to overstain heavily the whole-mount preparation.
2. Postfix embryos in 4% paraformaldehyde in PBS. The tissue can be stored in this solution for an extended period of time in the dark at 4°C.
3. Wash embryos in dH₂O and dehydrate quickly through a graded series of methanol (50%, 70%, 95% and twice 100% 2 to 3 min each).
4. Replace the methanol with propylene oxide, two changes for 5 min each.
5. Replace with a 1:1 mixture of Epon and propylene oxide for 30 min.
6. Replace with a 3:1 mixture of Epon:propylene oxide for 2 to 3 hours.
7. Transfer tissue to pure Epon for 4 hours.
8. Embed in fresh Epon. Polymerize overnight in a 60°C oven.
9. Cut sections 5 to 10 µm thick with a glass knife. Dry down on a drop of water. Cover slip in Permunt. Alternatively, cover slip in Epon and polymerize at 60°C.

For larvae and adults or adult tissues, it is not possible to do whole-mount RNA in situs because the probe will not penetrate these thick tissues. In this case it is possible to perform RNA in situs on slides after sectioning. In this case, wax sections should be made according to the following protocol: