Frontiers in Developmental Biology: Concepts, Techniques and Model Organisms

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PROTOCOLS for ZEBRAFISH PRACTICAL SESSIONS

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Microinjection Techniques
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Staining and Observation of Cartilaginous Structures in Zebrafish Complete Larvae

In situ Hybridization

1. **Fix** embryos in 4% Paraformaldehyde-PBS for 3 hours at RT or 4ºC ON. Manually dechorionate.

Day 1

2. **Dehydrate**: Methanol 10 min at RT
Methanol at least 60 min at -20ºC, store like this for up to 6 months.

(Prepare 4% Paraformaldehyde in PBS before starting: 1g in 25ml PBS, 65ºC for 1 hour)

3. **Rehydrate**: 5 min 75% methanol/25% PBS
5 min 50% methanol/50% PBS
5 min 25% methanol/75% PBST
5 min PBST x4

Optional Bleaching: For removing unsightly pigment in day two or older fish.
Prepare bleaching solution (for 10 ml):

ddH_2O	5.95 ml
20X SSC	0.25 ml
formamide	0.5 ml
30%H_2O_2	3.3 ml

Expose on light box or sunlight windowsill for 10-30 min. Wash 3 more times 5 min in PBST.

4. **Digestion**: 15 min at RT with 1ul 10mg/ml Prot K for embryos <24 hr

5. **Refix**: 30 min at RT in 4%PFA-PBS

6. **Rinse**: 5X 5 min in PBST.

7. **Prehybridization**: Add 0.5 ml of hybridization buffer (hyb); and incubate at 65ºC for 10 min. Replace hyb and incubate for 4 hr to O/N at 65ºC.

8. **Hybridize**: O/N at 65ºC with 0.5 to 1µg of probe per 1 ml of hyb solution (5 min at 70ºC before use, to denature).

Day 2

9. **Wash**: 10 min 75% formamide, 2X SSC at 65ºC
10 min 50% formamide, 2X SSC at 65ºC
10 min 25% formamide, 2X SSC at 65ºC
10 min 2X SSC at 65ºC
2X 30 min 0.2X SSC at 65ºC

10. **Block**: incubate 4hr to O/N in MAB+BMB+LS***
***At this time add antibody (1:2000) to MAB+BMB+LS and rock to preabsorb while embryos are blocking.

11. **Antibody:** 8 hr to O/N with MAB+BMB+LS+anti dg

12. **Washes**: 5X 20 min
3X 5 min with alkaline phosphatase buffer

13. **Stain**: RT from 30 min to 2 hours with 1 ml staining buffer.

14. **Stop reaction**: rinse twice in PBST and refix in 4% paraformaldehyde. This will fix stain and make it hard to remove: don't fix if you want to get rid of background.

15. **Clear:** Glycerol: 50% for 1-2 hours with mixing and then to 100% for 1-2 hours
Methyl Salicilate: dehydrate in an EtOH series (50-70-80-90-95-100) 5 min each and transfer to MethylSal (note: stain will fade in this solvent, after a few hours or days).

Solutions:

Hybridization buffer:
50% Formamide
5X SSC
50µg/ml Heparin
500µg/ml tRNA
0.1% Tween-20
Water to 10 ml
92 µl of 1M Citric Acid (pH 6.0)

Alkaline phosphatase buffer:
100mM TrisHCl pH 9.5
50mM $MgCl_2$
100mM NaCl
0.2% Tween-20
0.2% Triton-X100
60 mg/50 ml Levamisole

MAB:
100mM Maleic Acid
150mM NaCl
0.1% Tween
pH 7.5 (adj with conc. NaOH)

MAB+BMB:
MAB+2% Boehringer Blocking Reagent (1096176)

MAB+BMB+LS:
MAB+BMB+20% Inactivated Lamb Serum

Staining buffer:
3.5µl X-Phosphate (75mg/ml)
3.5µl NBT (100mg/ml)
Per 1 ml of Alk. phosphatase buffer

Wholemount in situ hybridizations on zebrafish embryos using digoxigenin probes

no DEPC treated water used, all buffers except paraformaldehyde contain 0.1% Tween 20

- **Probe synthesis**

1. Cut 10 μg of plasmid DN with appropriate enzyme in 100 μL. Use Geneclean II kit to isolate linear plasmid. Resuspend linear plasmid in H_2O as directed by kit.
2. Mix together: 2 μL linearized plasmid (1 ug of insert DNA)
   2 μL 10x transcription buffer (400 mM Tris-HCl pH 8.0; 60 mM $MgCl_2$; 100 mM dithiothreitol; 20 mM spermidine; 100 mM NaCl; RNAse inhibitor 1 unit/μL)
   2μL 10x nucleotide mix (10 mM each ATP, GTP, CTP; 6.5 mM UTP, 3.5 mM digoxigenin-UTP)
   1 μL (20 units) of RNAse inhibitor
   11 μL RNAse free H_2O for a final reaction volume of 20 μL
   2 μL (40 units) of T7, SP6, or T3 RNA polymerase
   incubate for 2 hr at 37°C.
3. Add 2 μL (40 units) DNAse I and incubate for 30 min at 37°C to remove plasmid DNA
4. Stop reaction by adding 2 μL EDTA (optional). Precipitate RNA with 2.5 μL 4M LiCl and 75 μL of prechilled ethanol for 30 min on dry ice. Can be stored for days at -80°C this way.
5. Spin down pellet and redissolve in 100 μL of RNAse-free water. Add 900 μL of hyb and store at -20°C. Run a small sample (5 μL) on a gel to check probe.

- **Preparation of Embryos**

1. Dechorionate embryos with pronase (1-2 mg/mL [final])[If embryos are less than 20 somites, wash off pronase well and let "recover" for 20-10 min. in egg water]. Dehydrate in MeOH (2x washes) for at least 20 minutes and store embryos at -20°C in MeOH.
2. Rehydrate fixed embryos, wash: 1x 1 min 50% MeOH/50% PTw
   1x 1 min 100% PTw
   Up in 500 μl PTw
3. Add 5μl 1mg/ml Prot K stock (10μg/mL final) Incubate at RT for times dependan. For 24 hr embryos, digest for 5 min.
4. Refix embryos in 4% paraformaldehyde in 1x PTw for 20 min at RT.
5. Wash 5 changes with PTw

- **Hybridization**

1. Prehybridize embryos in 500 μl hybridization buffer for at least 1 hr (4-5 hr is better) at hybridization temperature (65-70°C). Temperature is probe specific, but 65-70°C works well for almost all probes.
2. Replace prehybridization buffer with hybridization buffer, 15 μL probe/200 μL hyb (or already diluted probe).
3. Incubate 4h - overnight at hybridization temperature in a water bath.
4. Remove and save probe, this solution can be reused up to 15 times!
5. The following washes are performed at hybridization temperature with preheated solutions, 2 min washes, mix after 1 min: 75% hyb/25% 2x SSC
   50% hyb/50% 2x SSC
   25% hyb/75% 2x SSC
   100% 2x SSC

6. Wash 2x 30 min with 0.2x SSC at 70°C. (down to 0.05x SSC for high stringency)
7. The following washes are performed at RT:

1x 1 min 75% 0.2x SSC/25% PTw
1x 1 min 50% 0.2x SSC/50% PTw
1x 1 min 25% 0.2x SSC/75% PTw
1x 5 min 100% PTw

- **Preabsorption of the anti-digoxigenin antibodies**

1. The anti-digoxigenin antibodies can be preabsorbed against 20-40 whole embryos prior to use to improve clarity and specificity of staining. Any non-specific interactions will occur in these fish, and the supernatent can be taken and used against embryos containing the digoxigenin antigen. (1:100 in Block Solution)

- **Antibody incubation and staining**

1. Block with 2 mg/mL BSA and 5% sheep serum in PTw for at least 1 hr. Bring to 500 µl.
2. Add 10 µl of the 1:100 pre-abs antibody solution that has anti-digoxigenin-phosphatase antibody*** (1:5000 final)
   [*For 2 color in situ: add 2 µl anti-fluorescein-AP antibody (1:250 final)]**
3. Incubate 2 hr at RT to overnight at 4°C
4. Wash 2x 1 min with PTw, then 6x 15 min with PTw.
5. Equilibrate 3x 5 min in freshly made NTMT. Stain with 3.5 µL 50 mg/mL x-phosphate and 4.5 µL 75 mg/mL NBT per 1 mL of NTMT. [*** For 2 color in situ: use INT substrate for red reaction product first: 7.5 µl INT/BCIP stock solution in 1 mL NTMT]. Transfer embryos to staining tray. Take care that embryos do not stick. Incubate at 37°C in humid chamber to accelerate the staining process.
6. Stop reaction with 4% paraformaldehyde for 30 min to overnight.
7. Clear in 75% glycerol.

***For Two Color In Situ:

1. Wash back out of glycerol into PBT. (4x5 min)
2. Deactivate AP using 30 min 1) at 65° or 2) in100mM Glycine/.1%Tween-20 ph 2.2
3. Wash back to PBT (4x5min) if necc.
4. Block with 2 mg/mL BSA and 5% sheep serum in PTw 30 min to 1 hr (or o/n 4°), bring to 500µl
5. Add 10 µl of a 1:100 pre-abs antibody solution that has anti-digoxigenin-phosphatase antibody =(1:500 final)
6. Incubate 2 hr at RT to overnight at 4°C
7. Wash 2x 1 min with PTw, then 6x 15 min with PTw (or O/N 4°)
8. Equilibrate 3x 5 min in freshly made NTMT. Stain with 3.5 µL 50 mg/mL x-phosphate and 4.5 µL 75 mg/mL NBT per 1 mL of NTMT.
9. Transfer embryos to staining tray. Take care that embryos do not stick. Incubate at 37°C in humid chamber to accelerate the staining process.
10. Stop reaction with 4% paraformaldehyde for 30 min to overnight.
11. Clear in 75% glycerol.

- **Reagents**

<u>Dig RNA labeling mixture (10x)</u>
For 20 RNA samples Roche 1 277 073

Anti-digoxigenin Fab fragment- alkyline phosphatase
150 units Roche 1 093 274

T3 RNA polymerase
1000 units + 10x buffer Roche 1 031 163

T7 RNA polymerase
2000 untis Roche 881 767

RNAse inhibitor
2000 units Roche 799 017

DNAse I
RNAse free 10000 units Roche 776 785

- **Solutions**

1x PBS
for 10 L: 80 g NaCl; 2 g KCl; 14.4 g Na_2HPO_4; 2.4 g KH_2PO_4, pH 7.4

1x PTw
1x PBS, 0.1% Tween 20; 1 mL Tween/1 L PBS

20x SSC
for 40 mL: 7.012 g NaCl; 3.528 g sodiumcitrate, pH 7.0

hyb
50% formamide, 5x SSC (pH 7.0), 500 µg/mL torula RNA, 50 µg/mL heparin, 0.1% Tween 20, 9 mM citric acid, pH 6.0-6.5

NTMT
0.1 M Tris-HCl, pH 9.5; 50 mM $MgCl_2$; 0.1 M NaCl; 0.1% Tween 20.
For 50 mL add: 5 mL Tris-HCl, pH 9.5…….. 1 M stock
5 mL $MgCl_2$ ……0.5 M stock
5 mL NaCl……..1 M stock
0.5 mL Tween…….10% stock

NBT
75 mg/mL NBT in 70 dimethyformamide

X-phosphate
50 mg/mL x-phosphate in dimethylformamide

Digoxygenin Probe synthesis for in situs

Digest DNA with a suitable restriction enzyme (5' over-hangs are best), clean up and resuspend in H_2O.

Transcription reaction:
1-2µg DNA (linearized template)
10µl 2.5mM Dig mix NTPs 5X
10µl 5X transcription buffer
2µl RNasin (40U/ul)
5ul polimerase (T7/T3/Sp6)
H_2O to 50µl

2 hr at 37ºC

Add: 5µl DNase, incubate 15' 37ºC

Precipitation (or clean probe with column)

2µl 0.5M EDTA
2µl 10M LiCl
200µl EtOH
Store at -20 for 2 hr or at -70 for 30 min.
Centrifuge 14K 20 min.Wash 80% EtOH.
Resuspend in 11 ul water and then dilute in hybridation buffer.

DNA Extraction from adult or embryonic zebrafish

A batch of embryos or a caudal fin clip can be used for genotyping by PCR.

- Anesthetize fish in Tricaine (4ml Tricaine /100ml fish water)
- Place tail clipping in an eppendorf tube containing 100µl lysis buffer (10µl for a single embryo.

Lysis Buffer: 100 mM Tris pH 8
100 mM NaCl
10 mM EDTA
0.2 % SDS
200 µg/ml Proteinase K

- Incubate at 55°C for 2 hours at least, mixing or vortexing 2-3 times. At this point, samples may be stored ON at –20°C.
- Centrifuge 10 min at 14.000g
- Extract 50 µl of supernatant and add 150 µl of 100% ethanol mixing slowly to genreate a white precipitate. Centrifuge full speed for 10 min and then wash pellet with 70 % ethanol. Resuspend pellet after drying in 50 µl TE buffer.
- Heat DNA samples to 65 °C for at least an hour. Mix until proper resuspension is achieved.

Immunostaining

Day 1

• Wash 2 x in PBST	15 min
• Add protK 10ug/ml in PBST	15min
• Fix with PFA 4%	30min
• Wash 4 x in PBS	5min
• Wash 1 x in distilled water	1hr
• Incubate in precooled acetone (-20°C)	7 min
• Wash 4 x in PBST	5 min
• Block in NCS-PBST	1hr
• Add primery antibody	O/N

Day 2

• Wash 4 x with PBST	20 min
• Add secondary antibody	O/N

Day3

• Wash 4 x with PBST	20 min
• Preincubate with DAB	30 min
• Stain in peroxidase sol. With H_2O_2	(usually 15 min)

Notes :
NCS-PBST: 20 ml lamb serum,
2 ml DMSO
bring to 200ml with PBST

DAB solution: Add 1µl of 1/10 dil H_2O_2 to 200µl DAB solution (0.03%w/v in PBS)