Clearing And Staining For Larval Fish Cartilage And Bone

(Source: the Parichy Lab from Zebrafish Book 5th Edition)

1. Fixation

Fix fish in 4% paraformaldehyde in PBS for 2 x overnight at 4°C.

2. Dehydration

For larvae less than 20 mm SL:
- a. Wash 2 x 5 minutes in PBS.
- b. Dehydrate in 50% EtOH / 50% nano water for 24 hours.
- c. Dehydrate in 100% EtOH for 24 hours.

For larvae more than 20 mm SL:
- a. Wash 2 x 5 minutes in PBS.
- b. Dehydrate in 50% EtOH / 50% nano water for 48 hours.
- c. Dehydrate in 100% EtOH for 48 hours with one intermediate solution change.

3. Staining cartilage

Cartilage staining solution:
For 100 ml final volume:
- 70 ml 100% EtOH
- 30 ml acetic acid
- 20 mg Alcian blue

- a. Transfer specimens to staining dish.
- b. Incubate in cartilage staining solution for 24 hours at room temperature with mild agitation.

4. Neutralization

Wash in saturated sodium borate solution for 9-12 hours.

5. Bleaching (optional)

Bleaching solution:
For 100 ml final volume:
- 15 ml 3% hydrogen peroxide
- 85 ml 1% potassium hydroxide

- Incubate in bleaching solution for:
  - 20 minutes for specimens less than 20 mm SL
  - 40 minutes for specimens more than 20 mm SL

6. Trypsin digestion

Digestion solution:
For 100 ml final solution:
- 35 ml saturated sodium borate
- 65 ml ddH2O
- 1 g trypsin

- Incubate in solution at room temperature with mild agitation until specimens are 60% clear.

7. Staining bone

Bone staining solution:
- 1% potassium hydroxide with
- 0.1 g/100 ml Alizarin red

- Incubate in bone staining solution for 24 hours at room temperature with mild agitation.

8. Destaining

For specimens less than 20 mm SL:
Incubate in trypsin digestion solution for 40-48 hours.

For specimens more than 20 ml SL:
Incubate in trypsin digestion solution until specimen is clear and solution remains unstained. Change to fresh solution every 2 days.

9. Preservation
a. Transfer to 30% glycerol/70% of 1% potassium hydroxide. Incubate 2-3 days at room temperature with mild agitation.
b. Transfer to 60% glycerol/40% of 1% potassium hydroxide. Incubate 2-3 days at room temperature with mild agitation.
c. Transfer to 100% glycerol with thymol.

Reference