Clearing and Staining Protocol - Draft 5

Harold Hodgins

June 15, 2016

The following procedure is a combination of the protocols of Dr. Armbruster [1] and Dr. Helland[2]. It has been modified specifically for use with zebra fish in small petri dishes. The volumes for the stock chemicals and required solutions are based on 4 fish and 10mL / required solution / fish. Rounded to 50mL total volume of each required solution.

1 Stock Chemicals

- Glacial Acetic Acid - 15mL
- Alcian Blue - cheapest source is fine - 10mg
- Alizarin red - a little goes a long way - 0.5mg
- 95% Ethanol - 165mL
- Potassium Hydroxide Crystals - 0.5g[1]
- Sodium Borate - borax from grocery store is fine - 80mL saturated solution
- Trypsin Powder - cheapest source is fine - $\approx 0.5g$ total

If preserving

- 10% Formalin - 50mL

If Bleaching

- Hydrogen Peroxide - pharmacy grade (4%) is sufficient, replace every 6 months - 7.5mL

If Storing in Glycerin

- Glycerin - cheapest source is fine
- Thymol Crystals - a little goes a long way

[1] If not bleaching. If also bleaching then 1g total. Glycerin storage amounts not included since we aren't storing our specimens in Glycerin

If Storing in Resin

- Clear polyester casting resin
- Catalyst

2 Required Solutions

- Preservation/Dehydration
  - 50mL 10% Formalin
  - 50mL 30% Ethanol
    - 16mL 95% Ethanol
    - 34mL DI water
  - 50mL 50% Ethanol
    - 26mL 95% Ethanol
    - 24mL DI water
  - 50mL 70% Ethanol
    - 37mL 95% Ethanol
    - 13mL DI water
  - 50mL 95% Ethanol
- 50mL Cartilage Stain
  - 15mL acetic acid
  - 35mL 95% Ethanol
  - 10mg Alcian Blue stain
- 50mL Bone Stain
  - 50ml 1% pottassium hydroxide
  - 0.5mg Alizarin Red stain
- Neutralization
  - 50mL saturated sodium borate
- Bleaching
  - 7.5mL 4% Hydrogen Peroxide
  - 42.5mL 1% Potassium Hydroxide
- Clearing Stage 1,2
  - 100mL 30% sodium borate
  - 0.5g trypsin

3 Procedure

1. Obtain the fish post-mortem.
2. Preserve the fish.
   (a) place in **10% formalin** for 5 days
   (b) rinse with water
   (c) store in water for 2 days
   (d) rinse with water
   (e) store in **30% ethanol** for 2-5 days (<150 mm 2 days)
   (f) store in **70% ethanol** for 2-5 days (<150 mm 2 days)
3. **Remove the guts (optional)**
4. Dehydration
   (a) rinse with fresh DI water
   (b) transfer to **50% ethanol**
      - 10-20mm : 1 day
      - 20-80mm : 2 days
      - 80-200mm : 3 days
      - >200mm : 5 days
   (c) place in **95% ethanol**
      - 10-20mm : 1 day
      - 20-80mm : 2 days
      - 80-200mm : 3 days
      - >200mm : 7 days
5. Cartilage Staining
   (a) place specimens in **30/70 acetic acid/absolute ethanol+ 20mg alcian blue stain (per 100ml)**
      - (<80mm) 1 day
      - (80-500mm) 1.5 days
      - (>500mm) 2 days
6. Neutralization
   (a) place specimens in **saturated sodium borate** directly post staining
      - (<100mm) $\frac{1}{2}$ day
      - (>100mm) 2 days (may need to change solution half way through)
7. **Bleaching (optional)**

(a) place specimens in **15/85 4% hydrogen peroxide/1% potassium hydroxide**, most will depigment within an hour, darker will take longer, ***DO NOT LEAVE IN TOO LONG***

(b) If bubbles form in skeleton immediately move to 1% potassium hydroxide solution

8. Clearing (Stage 1)

   (a) place specimens in **30% sodium borate + trypson powder** for 7-10 days till 60% clear(you can see the vertebral column but no fully clear yet)

   (b) change solution every 10 days

   (c) change solution when it turns blue

   (d) keeping specimens in light may help speed things up

9. Bone Staining

   (a) place specimens in **1% potassium hydroxide + 1mg Alizarin Red**

   (b) leave till bones are desired darkness level

   - 10-80mm : 1 day
   - 80-200mm : 2 days (replace solution after 1 day)
   - > 200mm : 4 days

   (c) ***DO NOT LEAVE IN TOO LONG***

   (d) Helland recommends staining with a stirer at room temp in the dark

10. Clearing (Stage 2)

    (a) place specimens back into **30% sodium borate + trypson powder** for 7-10 days till fully clear

    (b) change solution when it turns blue

    (c) keeping specimens in light may help speed things up

11. Preservation

    - If in Glycerin
      (a) put specimens in **30/70 of glycerin/1% potassium hydroxide** for 2-7 days (depending on size)
      (b) put specimens in solution **60/40 of glycerin/1% potassium hydroxide** for 2-7 days (depending on size)
      (c) put specimens in **glycerin with thymol** for final storage
      (d) move to next solution after the specimen(s) sink to the bottom
    - If in Resin (per specimen)
      (a) Mix correct volume of resin and catalyst
      (b) Pour in tube around specimen
      (c) top off resin and let fully cure

4 Time Line

Stage	Time (days)
Aquisition	1
Preservation	11-17
Gutting	1
Deyhydration	2-7
Cartilage Staining	1-2
Neutralization	1-2
Bleaching	1
Clearing 1	7-10
Bone Staining	1-3
Clearing 2	7-10
Preservation	4-14
Total (days)	**37-74**
Total (weeks)	**5-10**

References

[1] Jonathan W. Armbruster. Clearing and staining methods. `http://www.cypriniformes.org/files/Clearing_and_staining.pdf`.

[2] Synnove Helland. Diagnostics - staining protocol of cartilage & bone. `"www.feap.info/shortcut.asp?FILE=1082`.