Clearing and Staining Methods
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Small specimens, up to about 50cm, can have skeletons prepared via clearing and staining, a process that renders the flesh invisible, the bone red, and the cartilage blue. The general methods to follow were described by Potthoff (1984) and Taylor and van Dyke (1985). Although these methods provide exact measurements for the various solutions, in practice approximation is good enough. For a more detailed protocol see (http://www.aquamedia.org/FileLibrary/27/Diagnostics%20-%20Staining.pdf)

What you need

You will need certain chemicals and should make some stock solutions. Some of these can be purchased cheaply, some are more expensive.

1. Hydrogen peroxide – buy at a pharmacy, replace about every six months. Do not mix stock solutions of hydrogen peroxide.
2. Potassium hydroxide – mix a stock solution of approximately 1% potassium hydroxide by weight with deionized or distilled water. An easy approximation is to put a single layer of potassium hydroxide chips in a one-gallon jar lid and mix with one gallon (4l) of water.
3. 95% ethanol.
4. Glacial acetic acid.
5. Alcian blue stain – used to stain cartilage. Cheapest form available works fine
6. Alizarin red stain – used to stain bone. A little alizarin goes a long way..
7. Trypsin – a digestive enzyme. Buy in powdered form in the cheapest form available.
8. Glycerin – used to house the specimens upon completion of the process. Again, use the cheapest form available. Beauty supply stores sometimes carry it as it is used in soaps. It is also a food additive. Glycerin does come in reagent grade, which is very expensive and unnecessary.
9. Sodium borate – used with the trypsin in the clearing liquid and to buffer the specimens after immersion in the alcian blue stain. Use borax, available in the laundry soap aisle at the grocery store – much cheaper than reagent grade and just as effective.
10. Thymol – used to prevent fungal growth in specimens stored in glycerin. A little goes a very long way.

Chemicals	
Acetic Acid	Hydrogen Peroxide
Alcian Blue	Potassium Hydroxide Crystals
Alizarin Red	Sodium Borate
Ethanol (95-100%)	Thymol Crystals
Glycerin	Trypsin Powder

Solutions needed

1. 15% hydrogen peroxide/85% Potassium hydroxide – mix as needed (I often do this in the jar).
2. 30% acetic acid/70% ethanol/alcian blue – mix liquids and then add enough alcian blue to make a deep blue liquid. You may reuse this solution many times. When the alcian blue no longer stays in solution, replace.
3. Staurated sodium borate – add a lot to a jar of deionized water and shake vigorously. Let sit for awhile, shake again. Make sure that there is still sodium borate at the bottom of the jar and you have saturated sodium borate.
4. 30% Sodium Borate – take your stock saturated sodium borate solution and mix it with water – 30% sodium borate solution/70% water.
5. 30% sodium borate + trypsin – mix when you place specimens into trypsin. I usually add approximately ¼ teaspoon/specimen. A little less if they are small, a little more if they are large.
6. 70% potassium hydroxide/30% glycerin – mix by volume. Solution can be reused unless it is too stained
7. 40% potassium hydroxide/60% glycerin – as above.
8. Glycerin + thymol – add a few crystals of the thymol to the glycerin solution. Alternatively, you can dissolve the thymol in alcohol so that it is saturated and add a couple of drops to the glycerin. You can store specimens in a glycerin/ethanol solution (no greater than 25% ethanol), but the specimens will not appear as clear.

Chemicals	
10% Formalin	30% Sodium Borate
Ethanol (30%, 70%. 95-100%)	30% Sodium Borate + Trypsin (mix as needed)
15% Hydrogen Peroxide/85% Potassium Hydroxide (mix as needed)	70% Potassium Hydroxide/30% Glycerin
30% Acetic Acid/70% Ethanol/Alcian Blue	40% Potassium Hydroxide/60% Glycerin
Saturated Sodium Borate	Glycerin + Thymol (mix as needed)

Step 1, Preservation: If your fish is not preserved, preserve in 10% formalin solution for 5 days, rinse with water, store in water for a couple of days, rinse again, and step up alcohol (30%, 70%) with 2-5 days in each level depending on size (<150 mm, 2 days). If specimen in preserved already, go to step 1a.

Step 1a, Dissection: If the specimen is large, you may wish to filet one side of the fish and remove the skin. If scales are large, you may wish to remove skin.

Step 2, Remove guts: Remove the gastrointestinal tract and gonads of the specimen and place in a vial that can be returned to the original specimen jar.

Step 3, Dehydration: Place specimen in 95% or 100% ethanol (95% is much cheaper). For small specimens (<150mm) leave in for 2 days, larger specimens up to a week.

Step 4, Alcian Blue Staining for cartilage: Place specimens in 30% acetic acid/70% ethanol/alcian blue stain for 1 day (<80mm), 1.5 days (80-500 mm), or 2 days (>500 mm).

Step 5, Neutralization: Return alcian blue stain solution to stock bottle, and place specimens in saturated sodium borate for half a day (<100 mm) to 2 days (>100 mm; you may wish to change solution halfway through).

Step 6, Bleaching: Place specimens in 15% hydrogen peroxide/85% potassium hydroxide solution. Most specimens will be depigmented within an hour (darker specimens may take a little longer). If the specimen has little pigment, you may skip this step. DO NOT LEAVE SPECIMENS TOO LONG IN THIS SOLUTION!

Step 7, Clearing 1: Place specimens in 30% sodium borate solution and add approximately ¼ teaspoon of trypsin per specimen (if you are clearing a lot in a jar at the same time, you can add less). You may need to change this solution in a couple of days if it turns blue. Replace solution every 7-10 days until the specimen is about 60% clear (you can see the bones of the vertebral column, but it isn't perfectly clear yet). Keeping the specimens in light may speed the clearing process.

Step 8, Alizarin Staining for Bone: Add enough alizarin red dye to 1% potassium hydroxide solution to make it a deep reddish purple. This will take only a tiny amount of alizarin (about what you can grab with a small forceps). Shake vigorously to mix. Leave specimens in until the bones reach the level of darkness that you prefer (some like lightly stained specimens and some like them almost purple). Usually 1-3 days is sufficient. DO NOT LEAVE SPECIMENS TOO LONG IN THIS SOLUTION!

Step 9, Clearing 2: Place specimens back into trypsin solution until suitably clear. What is this? It is when the flesh seems almost invisible. It will not be perfectly clear because the clarity in glycerin is part optical illusion (the refractive index of the glycerin and the cleared flesh is about the same). Usually a week will suffice.

Step 10, Stepping to Glycerin: This step is not necessary, but it helps to fix the dyes in the fish (I think) as well as prepare the tissues for full immersion in glycerin. Place specimens in stock 70% potassium hydroxide/30% glycerin for 2-7 days (depending on size) and then 40% potassium hydroxide/60% glycerin for 2-7 days. Then place in glycerin with thymol for final storage.

NOTE 1: Specimens that are poorly preserved may begin to fall apart at any stage during this process. If this begins to happen, rush the specimens through the procedure and avoid potassium hydroxide! Specimens will take the dye in a solution of 30% sodium borate if necessary.

NOTE 2: For extremely delicate specimens, you can mix the alizarin with either sodium borate or glycerin for staining. Avoid potassium hydroxide. This will not work as well, but it will work.

NOTE 3: Old specimens rarely stain well for cartilage. If you wish to stain only bone, skip steps 3-5.

Literature Cited

Potthoff, T., 1984. Clearing and staining techniques. In: Moser, H.G. (Ed.), Ontog eny and Systematics of Fishes. Special Publication-American Society of Ichthyolo gists and Herpetologists, vol. 1. Allen Press, Lawrence, KS, USA, pp. 35–37.

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