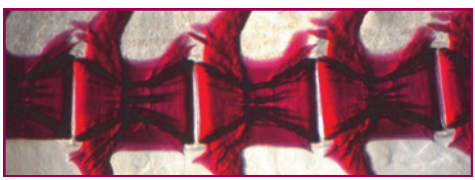
Synnøve Helland

General information about the staining of cartilage and bone, and the clearing technique

There are a lot of methods available, but these all follow the same basic principle of killing, fixation, staining, clearing, and preservation. It is possible to do double staining, i.e. both cartilage and bone staining. The following is a method for staining for bone and cartilage mainly based on Potthof, 1984, and adapted by S. Helland, Nofima Marin, based on experience in using this and other staining and clearing methods.



- For double staining follow all steps, 1 to 9.
- For cartilage staining follow steps 1 to 6, and then step 8 and 9.
- For bone staining follow steps 1 and 2, and then step 5 to 9.

List of Chemicals

MS222	Hydrogen peroxide (H_2O_2)
4% phosphate buffered formalin	Potassium hydroxide (KOH)
96% Ethanol	Distilled water
Absolute ethanol	Trypsin powder
Acetic acid	Alizarin Red S
Alcian Blue 8GX	Glycerine
Sodium borate (Borax, Di sodium tetraborate decahydrate)	Thymol crystals (optional)

List of Materials

Glass containers	Forceps	Sieve

Method for cartilage and bone staining, and clearing – Steps 1 to 9

1. Killing

Use a lethal dose of MS222

2. Fixation

Fixation in 4% phosphate buffered Formalin (10:1 ratio of formalin:fish).

- Fish standard length 10 to 80 mm: 2 days
- Fish standard length 80 to 100 mm: 3 days
- Fish standard length >100 mm: 5 days or more (remove some flesh on left side)

3. Staining of cartilage

Dehydration

A.) Rinse thoroughly with fresh water

B.) Transfer to 50% ethanol.

- Fish standard length 10 to 20 mm: 1 day
- Fish standard length 20 to 80 mm: 2 days
- Fish standard length 80 to 200 mm: 3 days
- Fish standard length >200 mm: 5 days

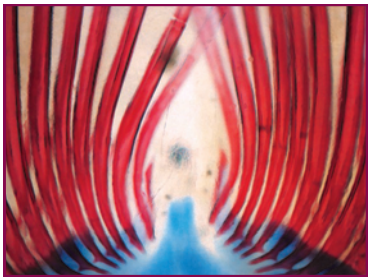
C.) Transfer to absolute ethanol *(95% ethanol may also be used)*

- Fish standard length 10 to 20 mm: 1 day
- Fish standard length 20 to 80 mm: 2 days
- Fish standard length 80 to 200 mm: 3 days
- Fish standard length >200 mm: 7 days

Staining with Alcian blue

⇒ Fish standard length 10 to 80 mm: use *Solution A*

⇒ Fish standard length 80 to >500 mm: use *Solution B*



Solution A

70 ml absolute ethanol, 30 ml acetic acid, 20 mg Alcian blue.

Solution B

60 ml absolute ethanol, 40 ml acetic acid, 30 mg Alcian blue.

A.) Mix the ethanol and the acetic acid and then add the Alcian blue. Place on stirrer until dissolved. Filter the solution. The staining solution can be used twice.

B.) Soak the fish in the staining solution

- Fish standard length 10 to 80 mm: max 1 day (Solution A)
- Fish standard length 80 to 500 mm: 1.5 days (Solution B)
- Fish standard length >500 mm: 2 days (Solution B)

C.) Monitor the staining process by taking the specimen and examine through a stereoscope, do not overstain the specimen. Staining can not be removed from the cartilage by any of the chemicals that are used later in the clearing and staining process.

4. Neutralisation (to prevent calcium loss during bleaching)

Move the fish directly from the staining solution to a saturated sodium borate solution

- Fish standard length 10 to 80 mm: 0.5 day
- Fish standard length 80 to 500 mm: 2 days (change solution after 1 day)

5. Bleaching (optional)

Bleaching solution

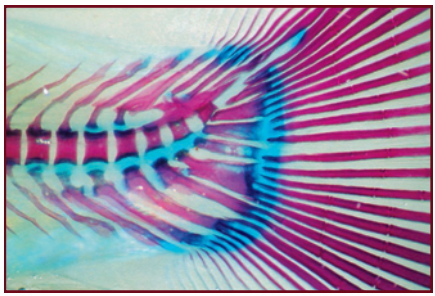
15 ml of 3% hydrogen peroxide (H_2O_2) with 85 ml of 1% potassium hydroxide (KOH)

Be very careful and do not bleach for too long, observe continuously! If done for too long, gas bubbles will form within the skeleton, e.g. inside the vertebrae. Change to 1% KOH immediately if bubbles are formed and repeat changes until no bubbles are formed.

6. Clearing (trypsin digestion)

Clearing solution

35 ml saturated sodium borate, 65 ml distilled water, trypsin powder



Keep the fish in the clearing solution until about 60% clear, change solution at least every 10th day. Illumination speeds up the clearing process.

The amount of trypsin powder must be evaluated - based on the amount of tissue surrounding the fish; for small fish, no trypsin is needed.

7. Staining of bone

Fish that comes directly from formalin (if step 3 and 4 are not used) should first be washed with running tap water and then pre-soaked in 1% KOH.

- Fish standard length 10 to 80 mm: 1 day
- Fish standard length 80 to 200 mm: 2 days (change solution after 1 day)
- Fish standard length >200 mm: 4 days

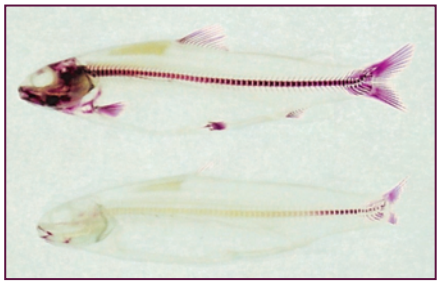
Staining solution

100 ml 1% KOH solution, 1 mg Alizarin Red stain

Mix the KOH and the Alizarin Red stain. The solution will now turn purple due to the alkaline pH. Place on stirrer until fully dissolved. Filter the solution.

- Fish standard length 10 to 80 mm: 1 day
- Fish standard length 80 to 200 mm: 2 days (change solution after 1 day)
- Fish standard length >200 mm: 4 days

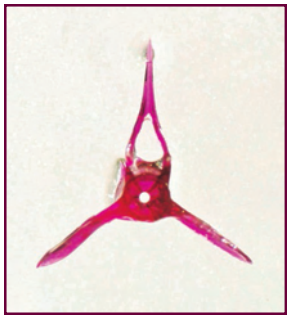
If possible the staining should take place on a rotor at room temperature. If not, stir regularly. The containers should be kept in darkness (e.g. covered with aluminium foil).



8. Destaining

Destaining solution 1.

35 ml saturated sodium borate solution, 65 ml distilled H2O, trypsin powder



Destaining solution 2.

1% KOH

- For fish standard length 10 to 20 mm: 2 days
- For fish standard length >20 mm: Change to fresh solution every 10 days until solution remains unstained and specimen is clear. Alternate between destaining solution 1 and 2.

9. Preservation

Preservation solution 1.

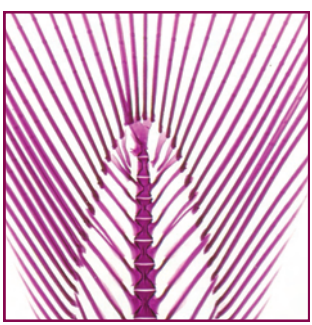
30% glycerine and 70% KOH

Preservation solution 2.

60% glycerine and 40% KOH

Preservation solution 3.

100% glycerine (with thymol as a final preservative for long time storage)

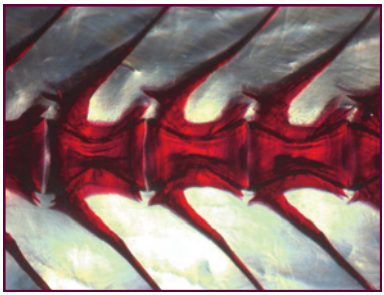


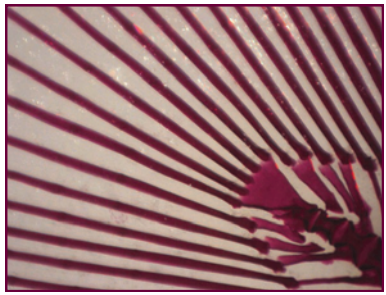
Place the fish in the preservation solutions 1, 2, and 3, and change of solution first after the fish has sunk to the bottom of the container. They are then ready for the next solution in the series. Direct sunlight and glycerine helps to clear and destain difficult specimens.

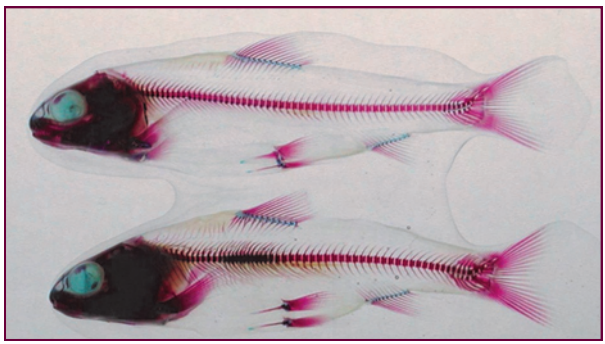
- Fish standard length 10 to 20 mm: 1 week
- Fish standard length 20 to 100 mm: 2 weeks
- Fish standard length >100 mm: 4 weeks

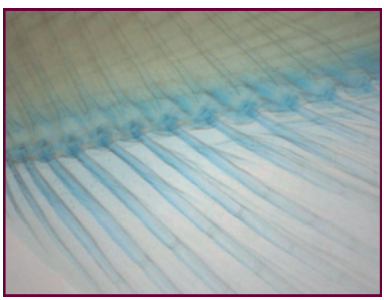
Most often the preservation step takes less time than foreseen

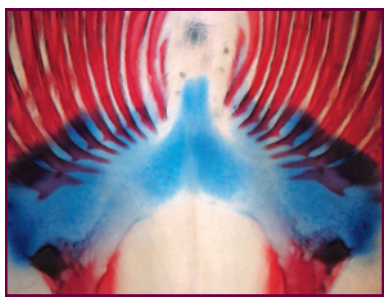
Some examples of staining using this protocol











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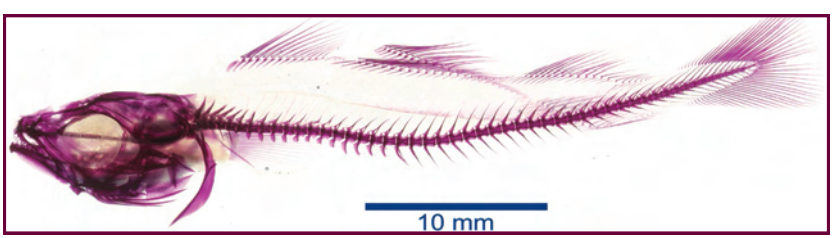

All photographs taken by Nofima Marin AS