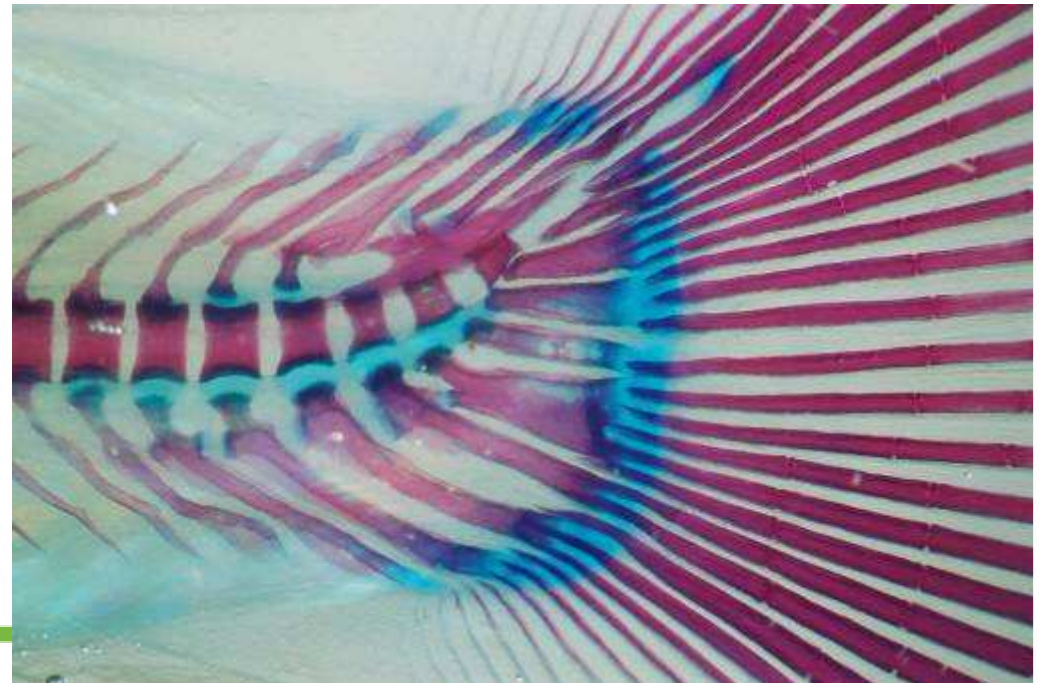




FineFish training course cod - Cartilage and bone staining protocol

Synnøve Helland





Staining of bone and cartilage

1. Some examples of use
2. When use cartilage and bone staining?
3. Staining protocol
4. Imaging
5. Diagnostic

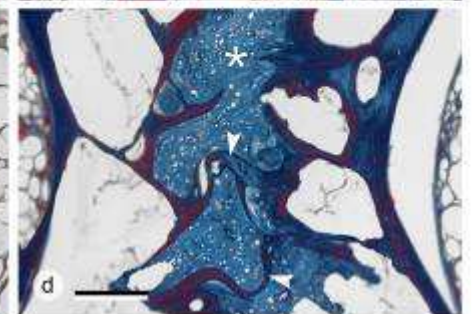
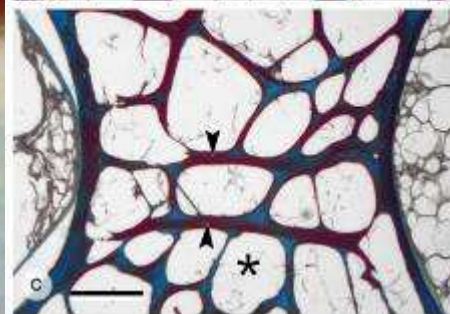
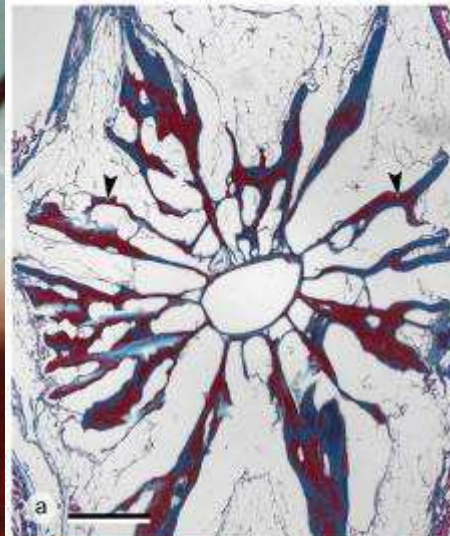
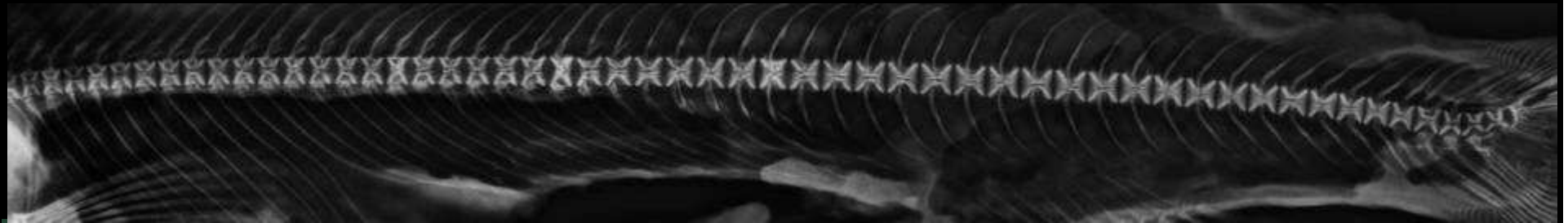


Some examples of use

Effect of high and low phosphorus content in the feed for A. salmon



Hyper dense vertebrae





When use cartilage and/or bone staining?



Two main areas of use

1. Diagnostics

- small fish, inkl salmonids (<1.0 g)
- marine fish (<0.5 g)

2. Illustration

- any size



Staining protocol



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

There are a lot of methods available, but they all follow the same basic principle:

- killing
- fixation
- staining
- clearing
- preservation



Single and double staining

(Cartilage or bone or both cartilage and bone)

It is possible to do double staining, i.e. both cartilage and bone staining.

I prefer to stain fish either for cartilage or bone, sometimes also with a double staining on surplus fish.

I will show the method that I most often use, mainly based on Potthof 1984, and with some explanation and adaptations made based on experience in using this and other staining and clearing methods.



Procedure step 1 to 9

1. Killing

Lethal dose of MSS222

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)



Procedure step 1 to 9

3. Staining of cartilage

Dehydration

- Rinse thoroughly with fresh water
- 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
- Transfer to absolute ethanol (95% ethanol may 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



Procedure step 1 to 9

Staining with Alcian blue

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.

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. 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)

- Monitor the staining process
- Staining can not be removed from the cartilage by any chemicals used later in the clearing and staining process



Procedure step 1 to 9

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, like inside the vertebrae. Change to 1% KOH immediately if bubbles are formed and repeat changes until no bubbles are formed



Procedure step 1 to 9

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.

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



Procedure step 1 to 9

7. Staining of bone

Fish that comes directly from formalin (if step 5 and 6 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

Continues on next slide..



Procedure step 1 to 9

Staining solution

100 ml 1% KOH solution

1 mg Alizarin Red stain

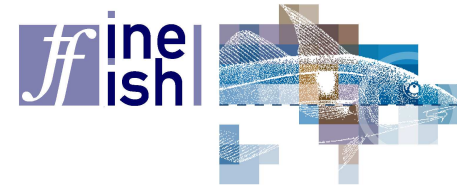
Mix the KOH and the Alizarin Red stain. The solution will now turn purple due to alkali pH. Place on stirrer until dissolved. Filter the solution.

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 (ex. covered with aluminium foil).



Procedure step 1 to 9

8. Destaining

Destaining solution 1.

35 ml saturated sodium borate solution

65 ml distilled H₂O,

Trypsin powder

Destaining solution 2.

1% KOH

Fish standard length 10 to 20 mm: 2 days

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.



Procedure step 1 to 9

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 final preservative for long time storing)

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.



Imaging



Imaging

1. Direct photo (film or digital)
2. Photo using stereoscope (film or digital)
3. Photo using microscope (film or digital)
4. Scanning

Which method to use depend on size of fish, number of fish, necessary image quality, and use of image

Direct photo (film or digital)

- Place the fish on a Petri dish or another similar tray and fill half way up with glycerol (100%)
- A tip here is to place the fish on a light board
- Use a tripod or similar for the camera



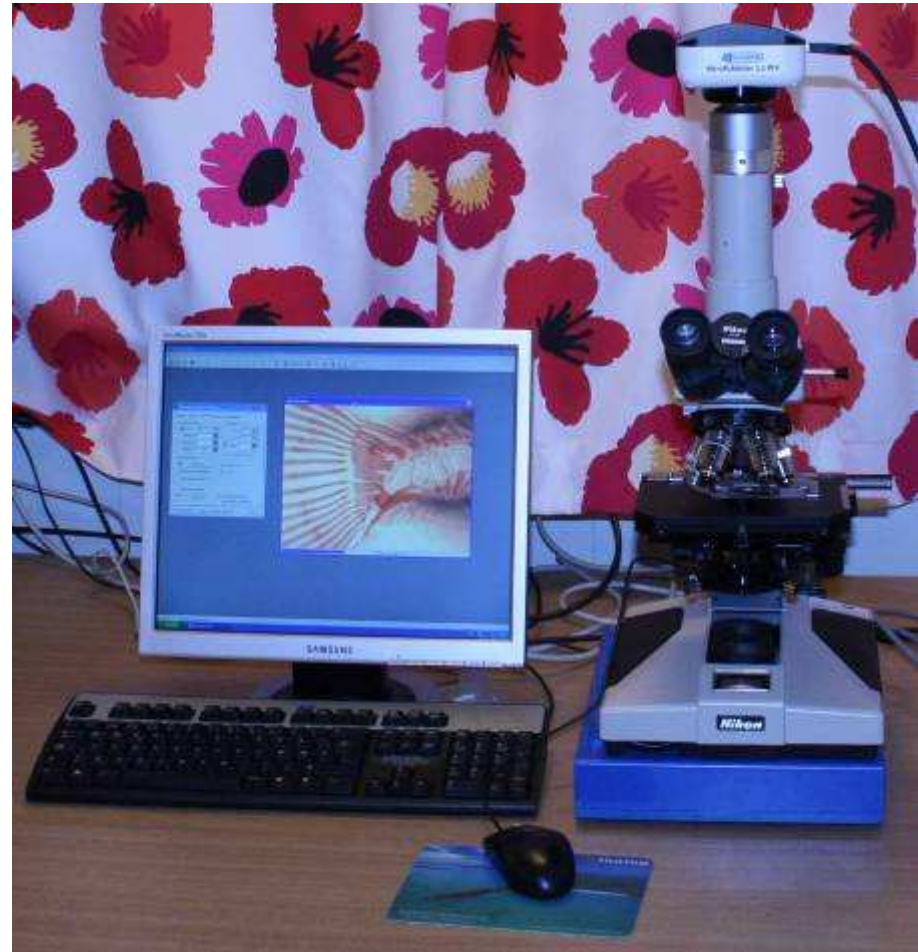
Photo using stereoscope (film or digital)

- Place the fish on a petridish or another similar tray and fill half way up with glycerol (100%)
- Make sure that external light sources don't create unnecessary reflections
- Work with the reflector to get the right lighting
- Take pictures of larger areas, and crop later



Photo using microscope (film or digital)

- This is only for a few vertebrae of small fish
- Short field of depth
- A tip to enhance the field of depth is to take several photos with sharpness at different depths, for then later to merge these images with a software tool like Helicon Focus



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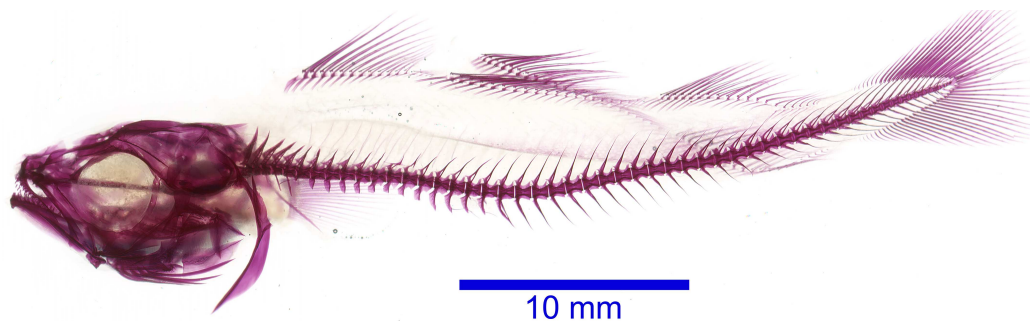
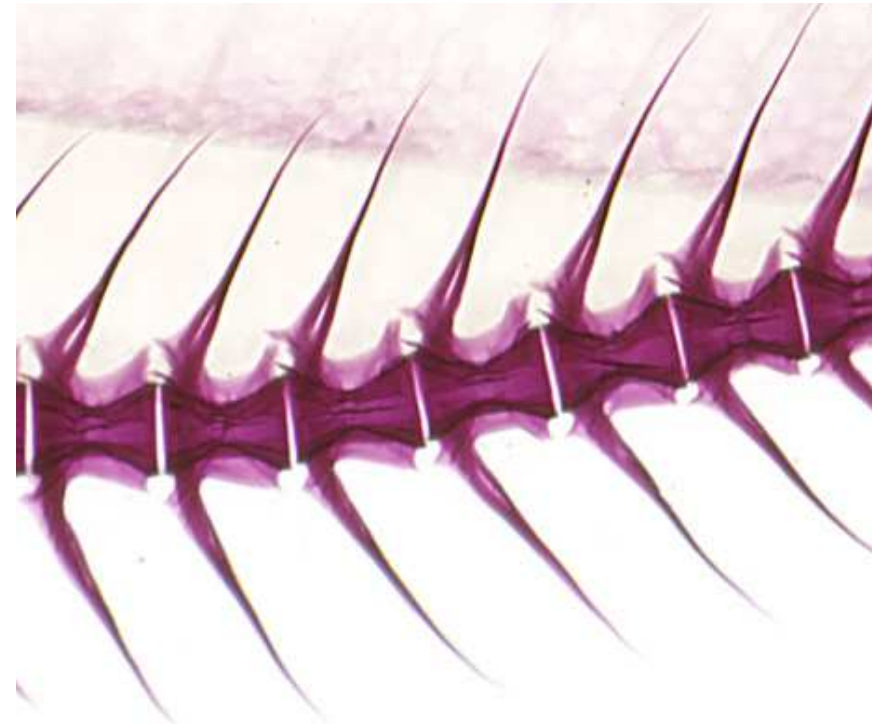
<http://HeliconFocus.com>

focus@helicon.com.ua

Authors: Danylo Kozub, Vitaly Khmelik, Jury Shapoval, Vitaly Chentsov, Stanislav Yatsenko

Scanning

- This is OK for larger fish
- It is also usable for comparing several smaller fish
- Use an image scanner



10 mm

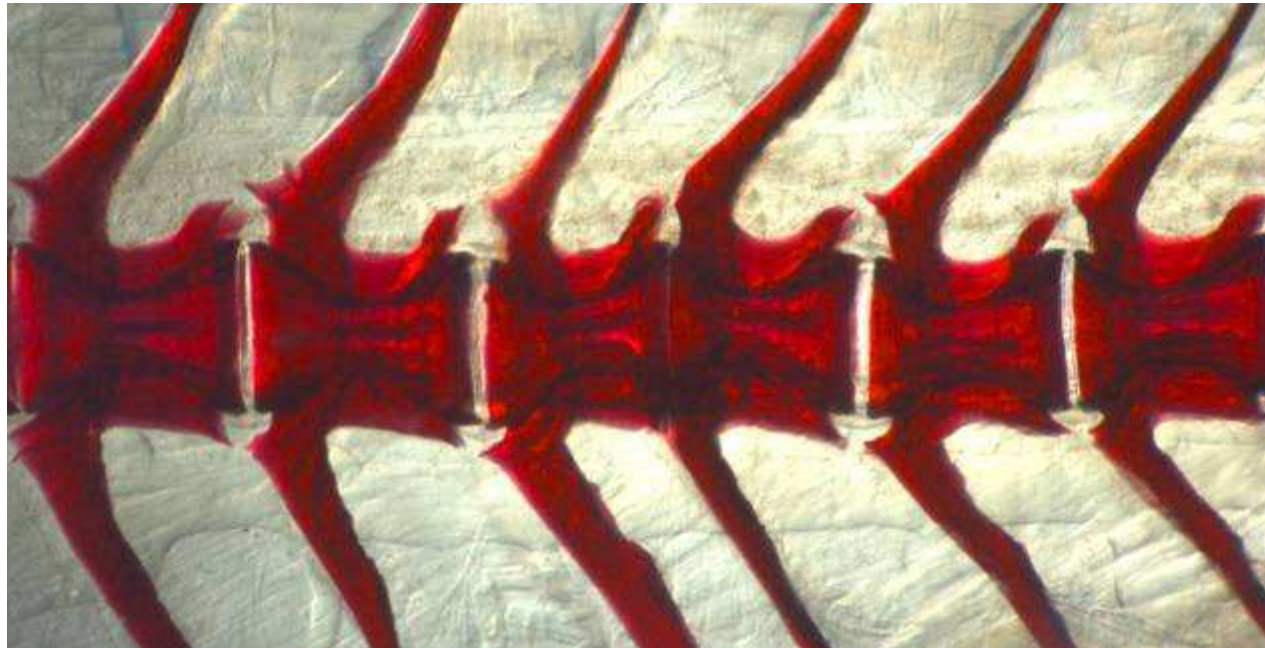


Identify the diagnostic

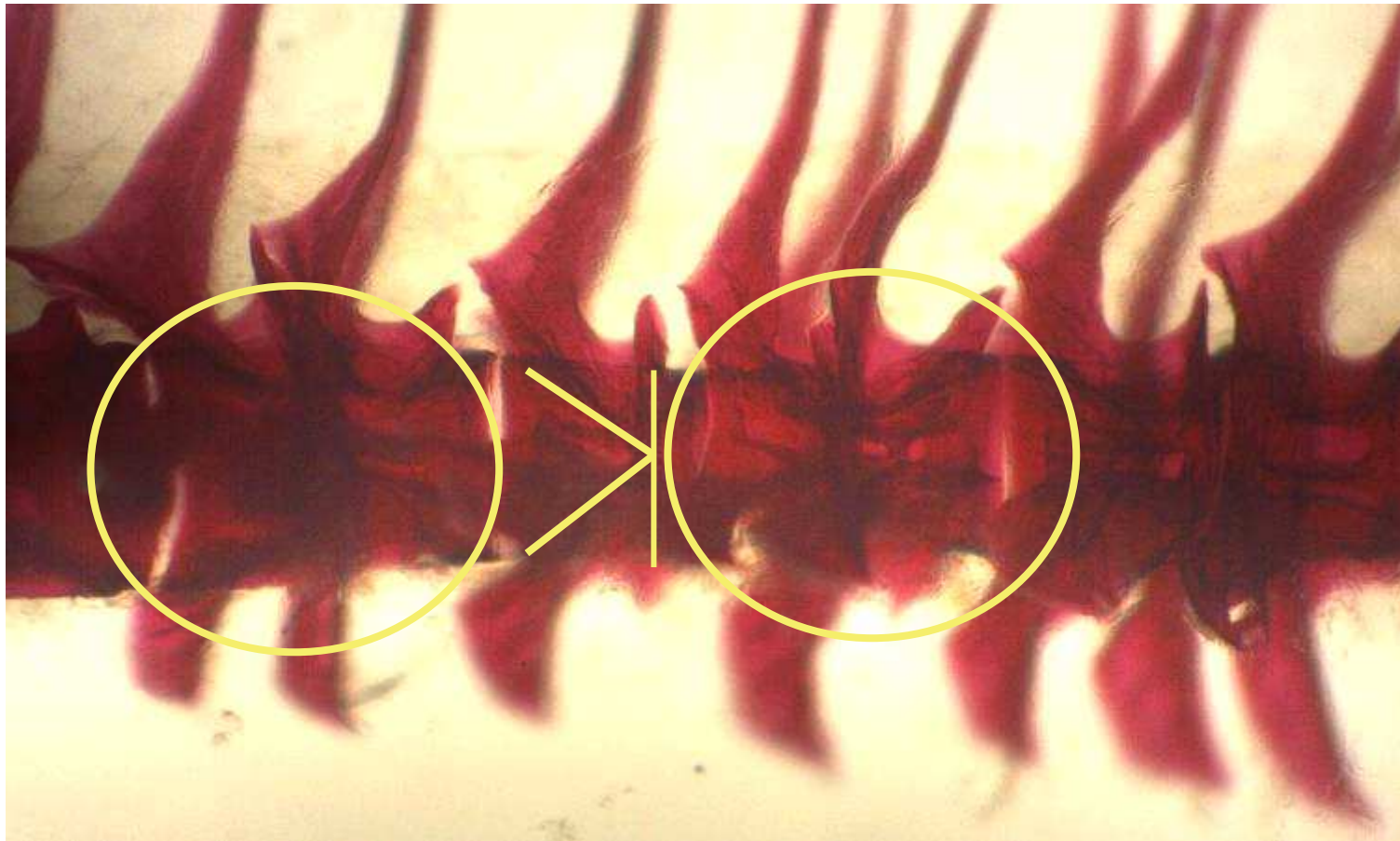
Lordosis



Early fusion



Several fusions and K vertebrae



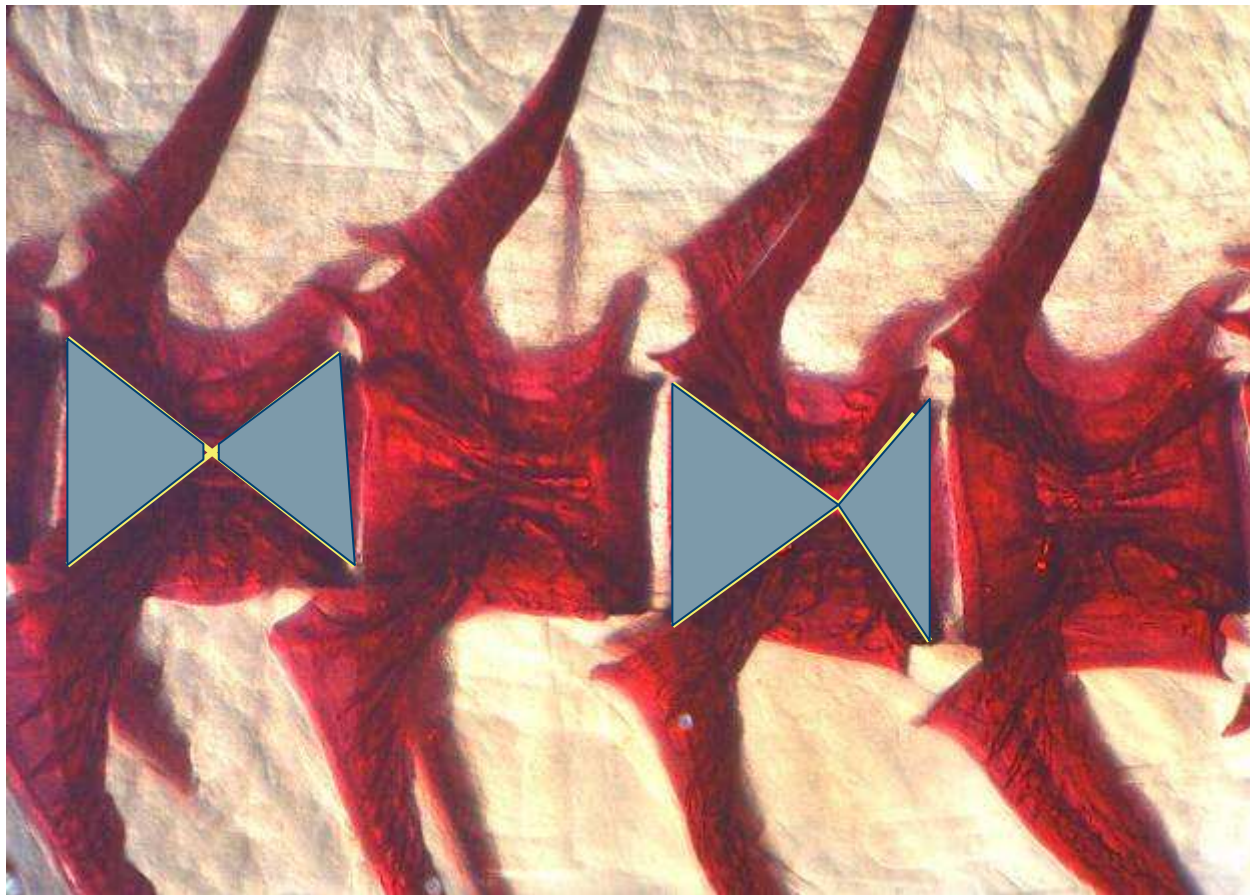
Several fusions



K vertebrae



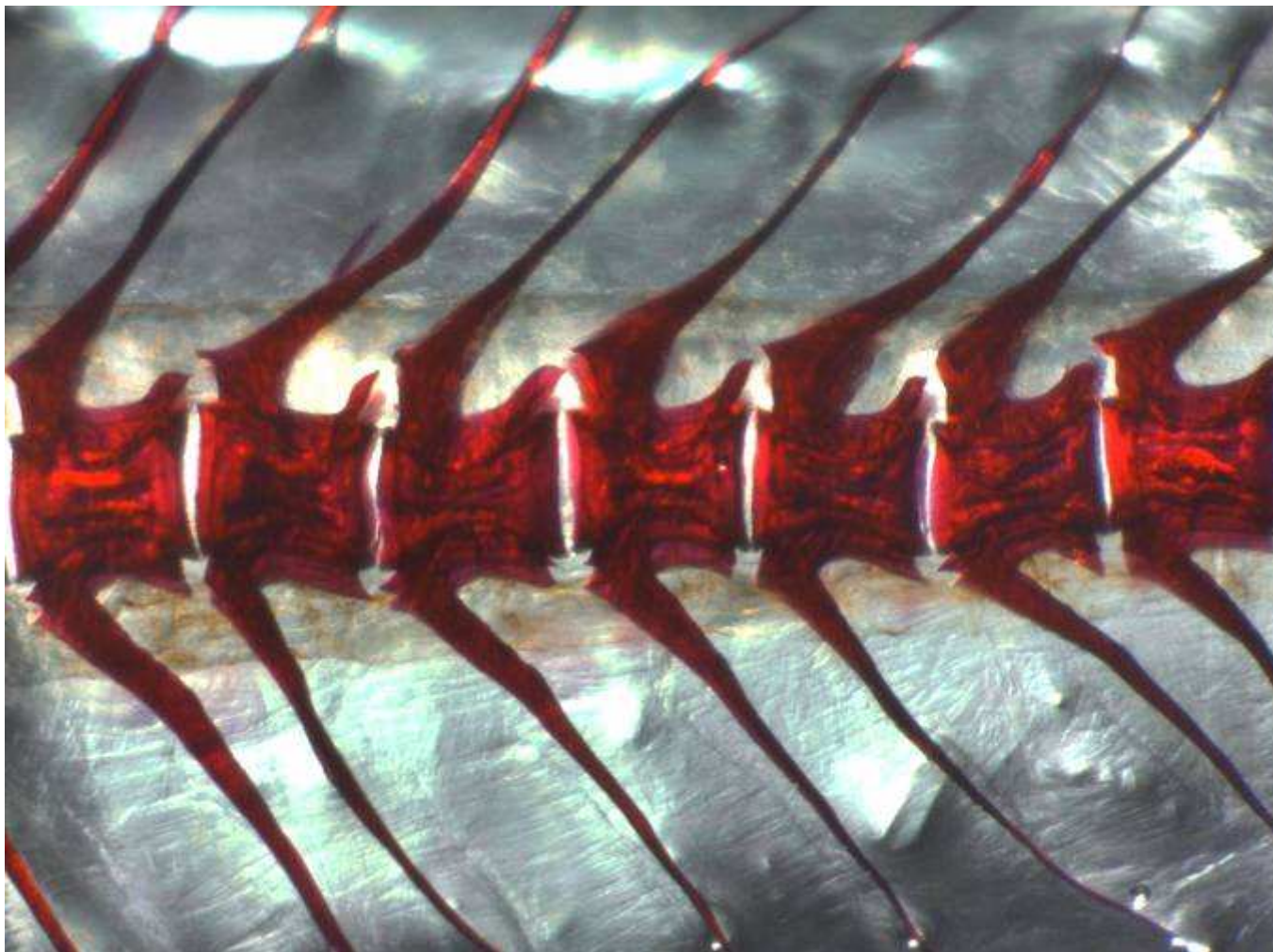
K vertebrae



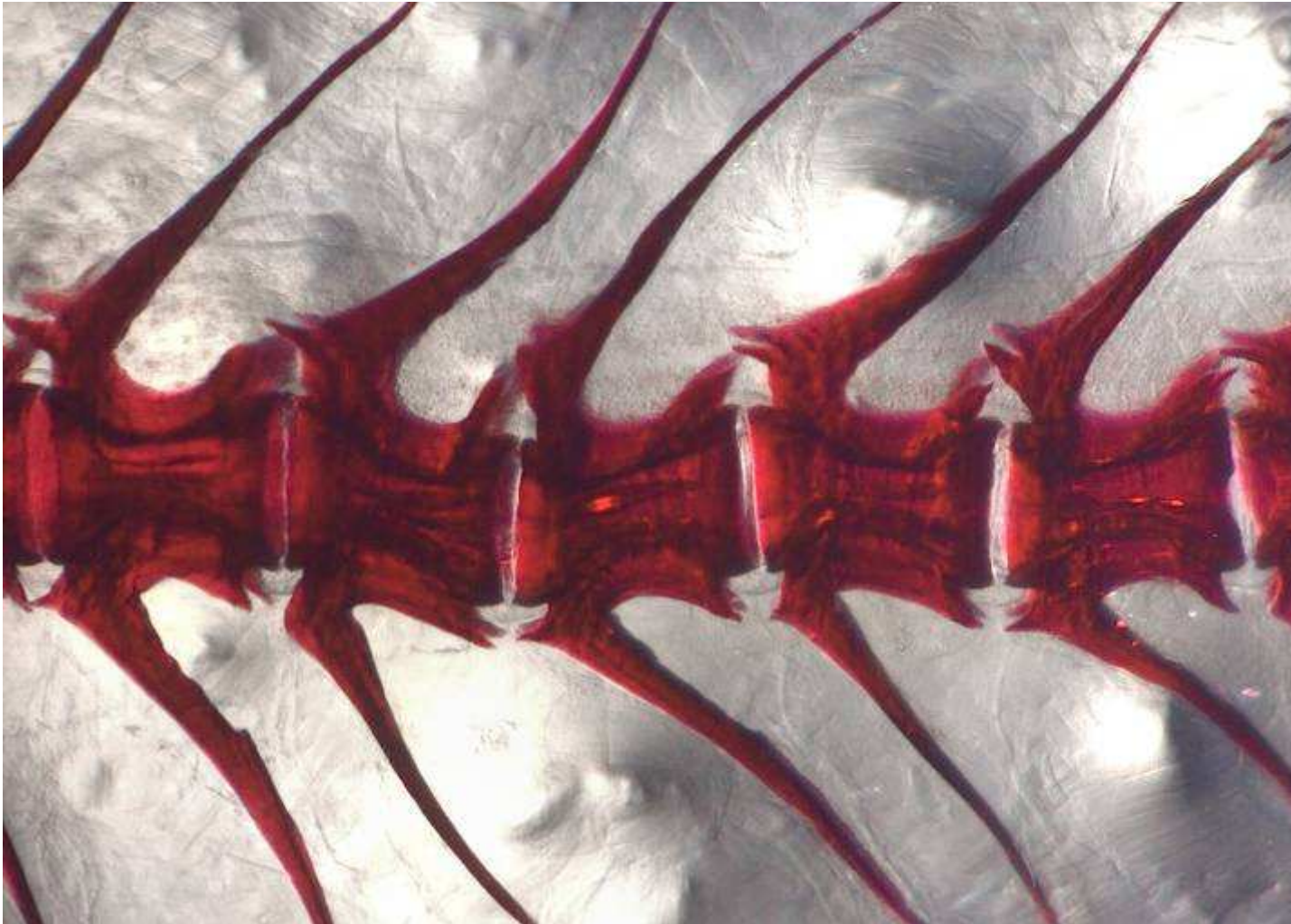
Platypsondyli



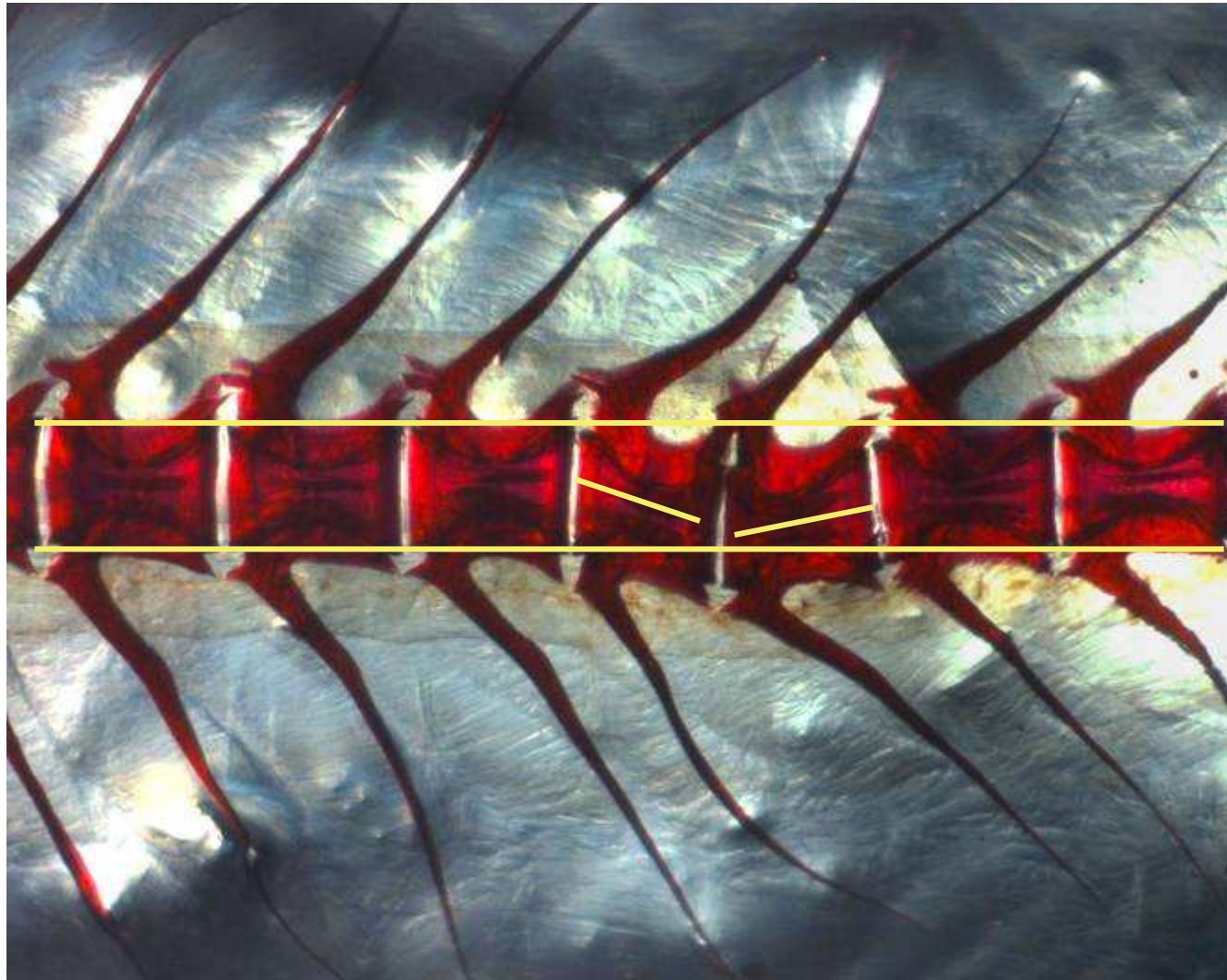
Other

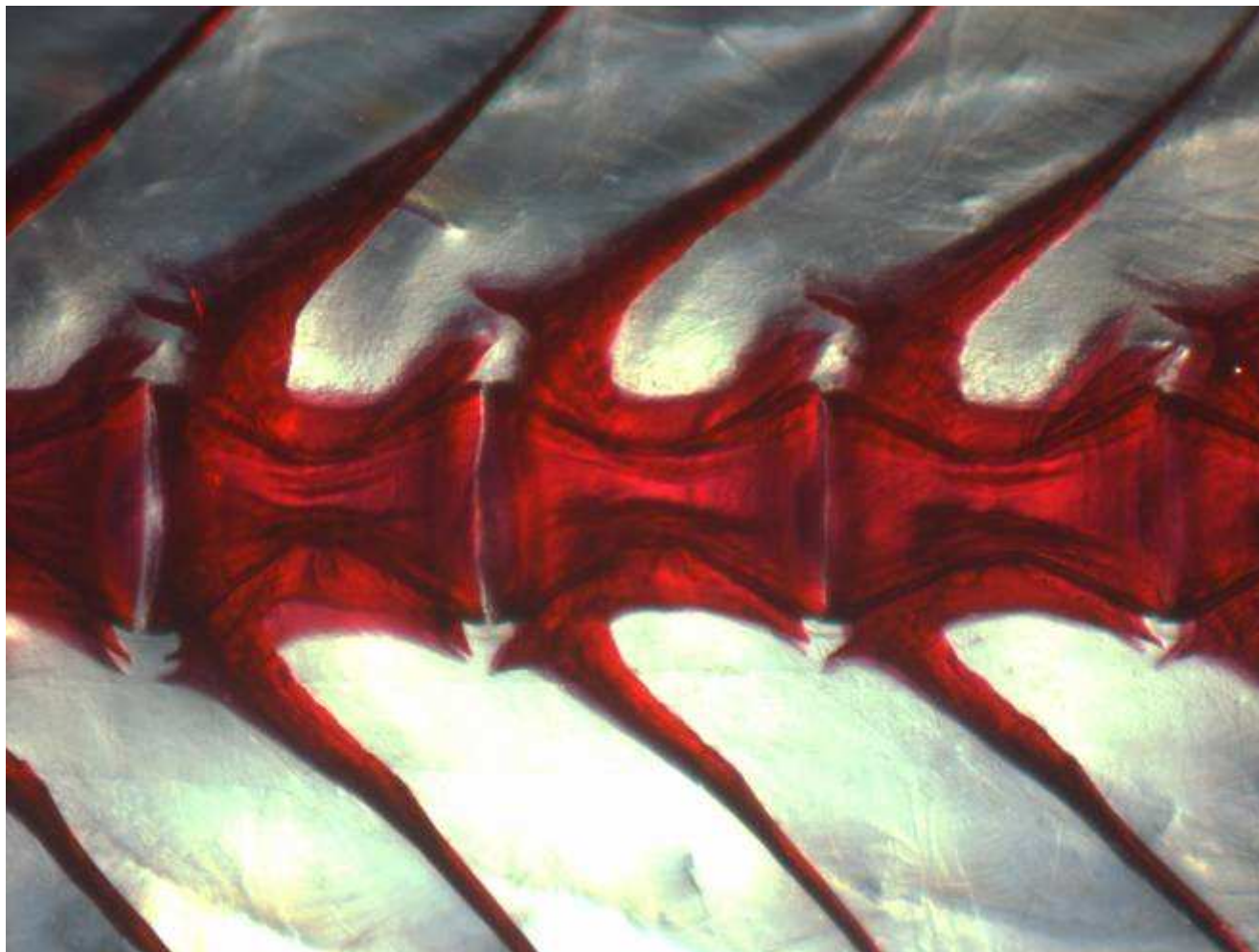


Other



Other





Thank you for the attention!

