Staining and observation of cartilaginous structures in zebrafish complete larvae

- **Introduction:**

Alcian Blue (AB) is a cationic dye derivative from the group of phthalocyanines. Phthalocyanines are synthetic analogues of porphyrins that contain nitrogen-aromatic rings surrounding a central atom. These substances are basic molecules that bind to the acidic groups of their targets leading to the formation of a saline compound, especially when the pH is acidic (pH $\leq$ 2.2). The blue colour of AB is due to the presence of a copper atom in the molecule. The copper phthalocyanine (CuPC) has got universal applications and is dominant in color blue and green.

Alcian Blue staining technique is widely used among developmental biologists to observe the embryonic development of cartilage and bone structures in embryos and complete larvae. In the fish, preparations of Alcian Blue can be used to observe individual cartilage from two days post-fertilization (dpf) onwards.

Cartilage and bone are connective tissues in which the cells are surrounded by a solid and relatively rigid extracellular matrix. The hydrated and viscose matrix of the extracellular matrix, in which are embedded fibers of collagen and elastin, consists mostly of proteoglycans and glycoproteins. Extracellular glycoproteins usually have bound a large number of glycosaminoglycans (GAGs). GAGs are molecules with hydrophilic carboxyl groups and contain negatively charged sulphate groups that attract both water and cations, thus, forming a hydrated and gelatinous matrix. The four most important GAGs types are: Hyaluronic Acid, Heparin Sulphate, Chondroitin sulphate (present in bone and cartilage tissue) and Keratin Sulphate.

Fish cartilage is stained because of the selectively of the AB binding to acidic GAGs in acid solution (pH $\leq$ 2.2). AB displays specific affinity for GAGs containing acid sulphate groups, staining them of blue color. Instead, neutral GAGs are not stained in acidic conditions.

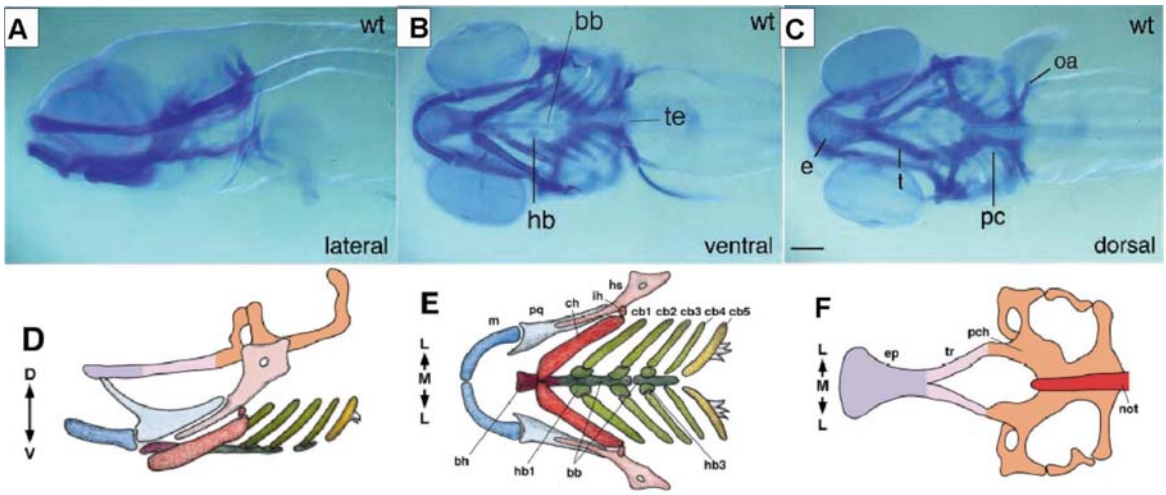
- **General Objectives:**

The principal goal of this experimental activity is that the students learn to recognize the craniofacial cartilaginous structures of zebrafish larvae.

- **Specific Objectives:**

The specific objectives are that students learn to:

1) Manipulate zebrafish 5 dpf larvae and recognize their main body structures.
2) Stain cartilage of zebrafish 5 dpf larvae.
3) Recognize craniofacial structures normally or abnormally develop



Photomicrographs and schematic drawings of Alcian blue-stained (A-C) wild-type larvae. Color-coded digrams (D-F) of the stained larvae shown in D-F depict distinct cartilaginous elements of the head. (A) Lateral view; (B) Lateral view (C) Dorsal view. (D) The neurocranium (diagrammatically elevated dorsalward for the sake of clarity) and the pharyngeal skeleton in side view. (E) The pharyngeal skeleton in ventral view. (F) The neurocranial (or basicranial) cartilages and notochord from the dorsal aspect. The eyes fit into the shallow grooves along the sides of the ethmoid plate and trabeculae. The otic vesicles fit into the prominent cavities to either side of the notochord and arachordal cartilages. The brain's posterior pituitary fits into the prominent midline cavity ahead of the notochord, the hypophysial fenestra. *Abbreviations used*: A, anterior; bb, basibranchial; bh, basihyal; cb, ceratobranchial; ch, ceratohyal; D, dorsal; ep, ethmoid plate; hb, hypobranchial; hs, hyosymplectic; ih, interhyal; L, lateral; M, medial; m, Meckel's; not, notochord; P, posterior; pch, parachordal; pq, palatoquadrate; tr, trabecula; V, ventral. Scale bars: A, B, C, 100 μm.

Alcian Blue staining

Five days post fertilization (dpf) or older zebrafish embryos are fixed overnight in 4% (p/v) phosphate-buffered paraformaldehyde and maintained in methanol 100% at -20ºC until use.

1- Wash the embryos several times in phosphate-buffered saline with 0.1% Tween-20 (PBT)

2- Bleach specimens in 30% hydrogen peroxide for 2 hours or until the eyes became sufficiently translucent.

3- Rinse embryos twice with 1 ml of PBT, transfer into an Alcian blue solution (1% concentrated hydrochloric acid, 70% ethanol, 0.1% Alcian blue), and stain specimens overnight.

4- Rinse 3 or 4-times with 1-1.5 ml acidic ethanol (5% concentrated hydrochloric acid, 70% ethanol, HCl-EtOH)

5- Rinse embryos during 20 min in 1-1.5 ml solución HCl-EtOH.

6- Re-hydrate embryos during 5-10 min in 1,5 ml HCl-EtOH/H_2O_d series, as follows:

- 1º 75% / 25% HCl-EtOH/H_2O_d;

- 2º 50/50 HCl-EtOH/H_2O_d;
- 3º 25/75 HCl-EtOH/H_2O_d;
- 4º 100% H_2O_d.

7- Remove the H_2O_d and store specimens in 1 ml of glycerol-KOH

NOTE: The students will receive wild-type (WT) or treated (*cnbp*-MO) 5 dpf larvae in Alcian Blue and have to continue with the protocol from step number 4 (four).

RECOMENDATIONS:

- Students will receive a fixed pool of larvae of 5 dpf in Eppendorf tubes with Alcian Blue.
- All washes have to be made with plastic Pasteur **labeled** for each solution. Note that the volumes indicated from the different solutions are approximated (between 1 and 1.5 mL)
- After incubation in glycerol-KOH, larvae are taken with plastic pipette, placed on small Petri dishes, and observed under a microscope binocular.
- Larvae have to be handled with care to avoid the loss of all or part of the sample. Moreover, they tend to stick to the tube walls as well as the Pasteur pipettes. Hence, a slow release of the solutions on the larvae is recommended.