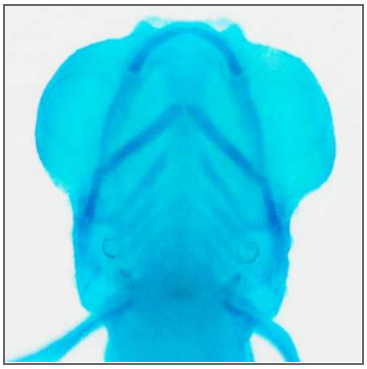

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Cartilage staining

Satheeswaran Balasubramanian[1], Ekambaram Perumal[1]

[1]Bharathiar University

Fish behavior and physiology

Satheeswaran Balasubramanian
Bharathiar University

ABSTRACT

The Alcian Blue staining technique is widely used among developmental biologists to observe the embryonic development of cartilage and bone structures in embryos and complete zebrafish larvae. Alcian blue is a positively charged dye that is thought to stain cartilage through an electrostatic interaction with negatively charged acidic mucopolysaccharides (Scott et al. 1996). The principle of this method is that different concentrations of magnesium ions can be used to compete with alcian blue for the negative charges of acidic mucopolysaccharides to differentiate different types of muco substances.

GUIDELINES

This protocol is optimized for 4 days post-fertilization of zebrafish larvae. It can be adapted for higher dpf.

MATERIALS

	A	B	C
	Abbreviation	**Chemical Name**	**Molecular Weight (g/mol)**
	Na2HPO4	Disodium hydrogen phosphate	141.96
	MgCl2	Magnesium chloride	95.21
	KCl	Potassium chloride	74.55
	KH2PO4	Potassium dihydrogen phosphate	136.09
	KOH	Potassium hydroxide	56.11
	NaCl	Sodium chloride	58.44
	dH2O	Distilled water	-
	EtOH	Ethanol	46.07

Protocol status: Working
We use this protocol and it's working

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	A	B	C
	PFA	Paraformaldehyde	30.03
	PBST	PBS plus 0.1% Tween 20	-
	PBS	Phosphate-buffered saline	-

1. 10X PBS stock; pH 7.4: To prepare 1 L stock, add 1.37 M (80 g) NaCl, 27 mM (0.2 g) KCl, 100 mM (14.4 g) Na2HPO4, and 18 mM (0.24 g) KH2PO4 in 800 mL of dH2O. Make up the volume to 1000 mL and autoclave. Store at room temperature. To prepare 1X PBS, add 100 mL of 10X PBS to 900 mL dH2O.
2. 1X PBST: Add 0.1 mL of Tween 20 to 100 mL of 1X PBS and mix.
3. 4 % PFA; pH 7.4: Dissolve 4 g of PFA in 80 mL of PBS at 60 °C, until the solution clears. Cool the solution and make it to 100 mL. To avoid repeated thawing and freezing, PFA can be aliquoted in 5 ml tubes and stored at −20 °C. Caution: PFA is a hazardous material, heat it in a fume hood.
4. Bleach solution: For 1 mL, add 100 µL of 30% solution of H2O2, 250 µL of 2% KOH and 650 µl of dH2O.
5. Staining solution: For 1 ml, add 100 µL of 10X alcian blue (0.01%), 200 µL of 1 M MgCl2 (60mM) and 700 µL of 100% EtOH (70%). a) 10X of alcian blue solution (8 GX; 05500, Sigma Aldrich): For 10 ml solution, add 10 mg alcian blue and 10 ml of methanol. b) 300 mM of Mgcl2: Add 0.29 g of MgCl2 to 10 ml of dH2O. c) 100% EtOH.
6. Storage solution: Keep 100% glycerol and 1% KOH (100 mg in 10 ml) separately.
   a. 25% solution: For 1 ml, mix 250 µL glycerol, 250 µL of KOH, and 500 µL of dH2O.
   b. 50% solution: For 1 ml, mix 500 µL glycerol, 250 µL of KOH, and 250 µL of dH2O.

SAFETY WARNINGS

Make sure to read all Safety Data Sheets for the reagents. Hydrogen peroxide and paraformaldehyde cause serious effects. Therefore, use personal protective equipment whenever manipulating it. Moreover, the preparation of hydrogen peroxide and paraformaldehyde must be performed under a fume hood at all times.

BEFORE START INSTRUCTIONS

1. Every protocol needs to be validated in the laboratory when first introduced. The present protocol describes validation steps that were taken in the Molecular Toxicology lab, at Bharathiar University.
2. Always wear personal protective equipment.
3. All the steps are optimized for 24 well plate for multiple groups such as control and treated. Each solution can be added to the well plate for convenience.

Larval fixation

3h

1 At the desired stage, the larvae for staining are taken and washed once in PBS for 5 minutes. 5m

2 The larvae were euthanized using the cold shock method (Keep at 4^{o}C for 5-10 minutes). 10m

3 Transfer the euthanized larvae into 4% PFA and keep it in the rocker for 2 hours at room temperature (2h
required, it can be kept overnight at 4^{o}C).

4 After fixation, remove the 4% PFA and wash with PBST for 5 minutes, two times. 10m

5 Dehydrate using 50% ethanol and keep it in the rocker for 10 minutes at room temperature. 10m

Staining

8h

6 After dehydration, add 1 ml of alcian blue staining solution and keep it in a rocker overnight at room temperature. 8h

Bleaching

40m

7 Remove the stain solution and rinse once using PBST. 1m

8 Transfer the larvae to a bleach solution and incubate in dark at room temperature. Assess the bleach 30m
process (using a microscope) and stop the reaction when the larvae become transparent (approximately
min for 24 hpf or 20-30 min for 96 hpf and above).

Clearing

9h

9 Remove the bleach solution and rinse once using PBST. 1m

10 Clear the beached larvae with 25% glycerol and 0.25% KOH. Keep them in a rocker at room temperatu 30m
for 30 minutes to overnight.

11 Replace the solution with 50% glycerol and 0.25% KOH. Keep them in a rocker at room temperature for 8h
hours to overnight.

Storage

12 Store larvae in a solution of 50% glycerol and 0.1% KOH at 4 ^{o}C. Avoid long-term storage as it will diminish the stain. Capture images of the cartilage using any bright field microscope

Results

13

Expected result

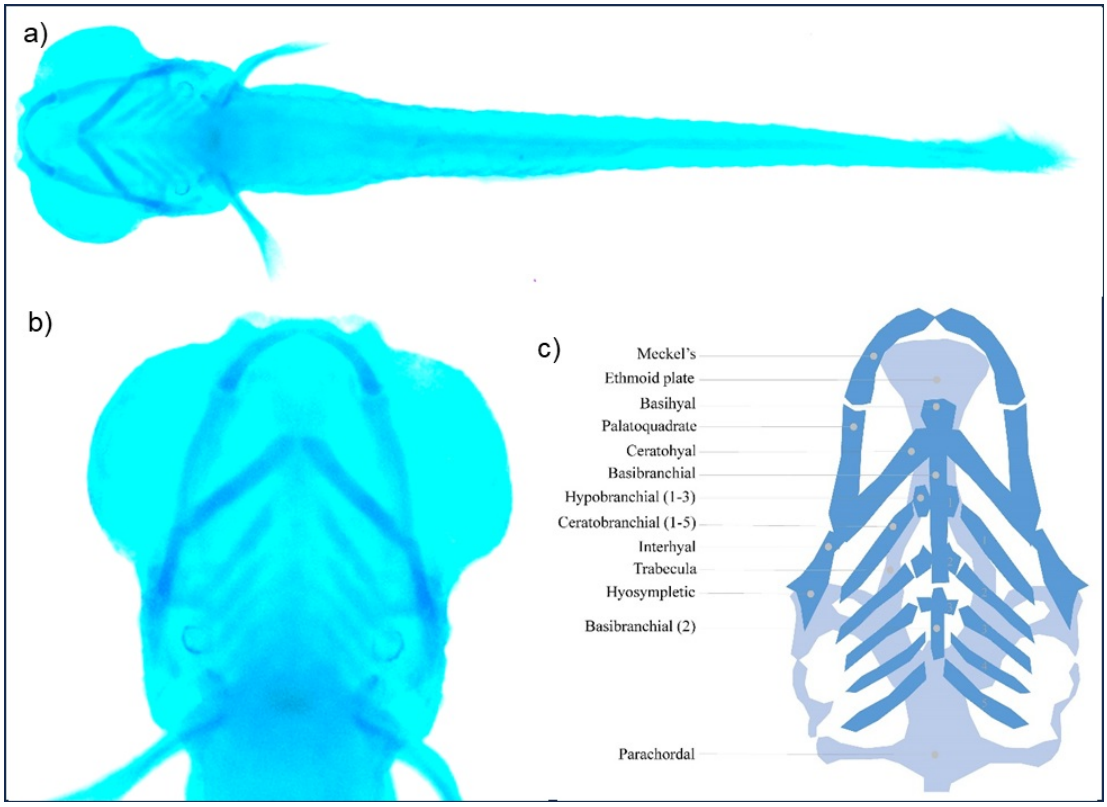

Figure 1. Representative staining images of 120 hpf larvae at 4X magnification. a) Whole body; b) head region. c) Cartilaginous structures in the head region of zebrafish.