Classic Clear and Double Stain Protocol

Ryan Kerney December 2013*

1) *Fixation*. Fix specimens for overnight in 10% Neutral Buffered Formalin (NBF)[1]. For at least two nights.
    1. If the specimen is still in its jelly coat then remove the coat after one day of fixation and keep exposed embryo in fix overnight.
    2. Refix EtOH stored specimens overnight in 10% NBF before beginning the protocol,

2) *Skinning*. Skin heavily pigmented specimens as much as possible remove them from the yolk. This may also be done following the alcian blue step.

3) *Washes*. Wash the specimens 3X30 minutes in diH2O. Rock if possible, if not then change at least 5 times and shake occasionally by hand (gently).

4) *Dehydration*. Dehydrate the specimens through 1 hour changes in 50%, and 70% EtOH. Make sure the specimens sink by the end of each change.
    1. Specimens can be left in 70% overnight.

5) *Cartilage staining*. Transfer specimens to alician blue working solution[2] for 12-24 hours. Watch closely for excessive staining in the muscles. These should never turn deep blue.
    1. The skin should be brittle by this point and easy to remove.

6) *Rehydration*. Transfer to 2 changes of 2 hours in 100% EtOH. Then walk through 2 hour changes of 95%, 70%, 40%, and 15% EtOH or until the specimens sink. Transfer to distilled H_2O for 2-3 hours or until the specimens sink.
    1. If you leave them in 70% EtOH for several days the muscles will remain light blue through the staining.

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* Based on:
1. G. Dingerkus, L. D. Uhler, *Stain Technology* **52**, 229-232 (1977).
2. R. J. Wassersug, *Stain Technology* **51**, 131-134 (1976).
3. M. W. Klymkowsky, J. Hanken, in *Methods in Cell Biology*. B. K. Kay, H. B. Peng, Eds. (Academic Press, San Diego, 1991), vol. 36, pp. 419-441.

7) *Trypsin clearing*. Place in 1% trypsin solution[3] and incubate at 37C. Change the solution every 12 hours. Continue until the cartilage is clearly visible and the muscles have lost all bluish tint. Specimens should be limp. May take several days.

8) *Bone staining and maceration*. Transfer to Alizarin red working solution[4] , stain overnight or until the bone is clearly bright red.

9) *Second clearing and glycerol walk*. Walk specimens through a 0.5% KOH : Glycerol series of 3:1, 1:1, and 1:3 keeping the specimens in each stage until they sink.
    1. Leave in 3:1 for several days for additional clearing. This step may also decrease the bone staining. Specimens can be returned to alizarin to intensify staining.
    2. A single drop of 3% H_2O_2 per 50mls may be added to the 3:1 for bleaching. Watch bleaching step closely for the formation of bubbles. These will destroy your tissues.
    3. The yolk and hypobranchials can be removed easily while in 3:1 solution. It is better to keep the yolk in tact during the bone staining, since leaking yolk will be messy.

10) *Storage*. Store specimens in 50% glycerol 50% Ethanol

Solutions

Filter each before use with Watman Paper

1. 10% NBF (can order liquid from sigma HT501-128)
    - 4.0g Sodium acid phosphate $NaH_2PO_4*H_2O$ (Fisher S369-500)
    - 6.5g Anhydrous disodium phosphate NaH_2PO_4 (Fisher S374-500)
    - 100ml of 37% Formaldehyde
    - 900ml of Filtered or distilled water

    pH 7.4

2. 8GX Alcian Blue Working Solution
    - 0.01 g alican blue 8GX (Sigma A5268)
    - 80 ml of 95% EtOH
    - 20 ml of glacial acetic acid (Fisher A38-500)

Mix thoroughly. Make take a few hours to go into solution, Filter and store at 4C.

3. 1% Trypsin solution (make fresh)
    - 30 mls of saturated sodium borate
    - 1 g of powdered trypsin (Fisher T360-500)
    - 70 mls of filtered or distilled water

Mix thoroughly and filter before use on embryos. Don't filter for adult frogs or larger samples.

4. Alizarin red working solution
    - Add 1-2 drops of 0.1% Alizarin red working solution to 0.5% KOH
    - Working solution: 0.5 g Alizarin red (Sigma A5533) in 50 mls filtered or distilled water
    - 0.25 g KOH (Sigma P1767) in 50 mls filtered or distilled water