MODIFIED BISMUTH STAINING PROCEDURE FOR AXOLOTL TISSUE[1]

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ABSTRACT. A bismuth stain described by Locke and Huie (1977) was modified for the limb tissues of the axolotl, Ambystoma mexicanum. This modified procedure stains nucleoli dark brown against a light brown background in a variety of differentiated cell types as well as regenerating blastemal and developing limb bud cells. The application of this technique in cell lineage studies using the number or size of the nucleoli as a marker promises to facilitate studies of both limb development and regeneration in amphibians.

INTRODUCTION

Locke and Huie (1977) described a bismuth staining technique for light microscopy which selectively stains the nucleoli of epidermal cells in the insect Calpodes. Using this technique to study epidermal development in Calpodes, these authors demonstrated that the technique could be used to determine nucleolar number and morphology (Locke and Huie, 1980). Application of the original Locke and Huie technique to axolotl tissues did not give satisfactory results in our hands. However, various modifications of the technique are described here, which result in excellent contrast between nucleoli and other cellular constituents in axolotl tissues. The major differences between Locke and Huie's procedure and the version described here involve the length of time in bismuth solution, and the type of fixative, postfixative and rinse after postfixative. In addition, the procedure described here works for tissues which must be decalcified (as described below) and for tissues which have previously been processed as whole mounts.

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METHODS

Fixation. Mature limbs, regenerating blastemas and developing limb buds were amputated and fixed in Carnoy's fixative (Humason, 1979) for 1-2 days, then stored in 70% ethanol. Formalin and 70% ethanol, which were recommended along with Carnoy's fixative in the original procedure, gave poor results as did Bouin's fixative which is commonly used for amphibian tissues. Limbs which have been processed with Victoria Blue B as whole mount preparations (Bryant and Iten, 1974) may also be hydrated and processed in the same manner as freshly fixed material if Carnoy's is used as the fixative. However, such reprocessed limbs exhibit tissue shrinkage which makes histological analysis difficult.

Decalcification. Mature and regenerating limbs were decalcified in 10% versene (ethylene dinitrilotetraacetic acid, Matheson, Coleman and Bell, Cincinnati, Ohio) at pH 6.0 for 2 days (time variable depending on size of tissue), rinsed well in water and returned to 70% ethanol. Methods for decalcification involving acid hydrolysis, such as Decal (Omega, Cold Springs, New York) gave poor subsequent staining of sections.

Staining. Prior to staining, all tissues were dehydrated, cleared through toluene and embedded in paraffin (M.P. 56.5°C, Fischer, Fair Lawn, New Jersey). They were then sectioned at 10 and attached to albuminized slides. The staining procedure follows.

1.	Deparaffinize and hydrate sections	
2.	Postfix in 10% buffered formalin phosphate, pH 7.0 (this may be reused for a month or more)	1 hr
3.	Distilled water rinse	5 min
4.	0.1 M triethanolamine, pH 7.0	30 min
5.	Bismuth staining solution (on rotary shaker)	overnight
6.	0.1 M triethanolamine pH 7.0 (two changes, 5 min ea)	10 min
7.	Ammonium sulfide (Mallinckrodt), 1:300 in 0.1 M triethanolamine, pH 7.0 (Note: This solution may be neutralized with a few grams of ferric chloride after use)	10 min
8.	Distilled water rinse	10 min
9.	Dehydrate, clear and coverslip (Contrast may be increased by the use of a medium blue filter, BG 23)	

Staining Solution. The stock bismuth staining solution was made as follows: 20 g of sodium tartrate (Fisher) was dissolved in 500 ml of 1 N sodium hydroxide. This solution was added slowly to 10 g of bismuth subnitrate (Mallinckrodt, Paris, Kentucky) and magnetically stirred until the bismuth was in solution. This stock staining solution was then diluted 1:3 with 0.2 M triethanolamine-HCL buffer (pH 7.0) (Mallinckrodt) and the final pH adjusted to 7.0 just prior to staining (Lock and Huie, 1977). The stock bismuth staining solution is stable for a month at room temperature. Although the diluted staining solution is stable for several days, we routinely make it up fresh for more uniform results.

Two other features of this staining procedure are that poorly stained sections can be restained and that whole mount preparation may also be stained. Sections which did not stain darkly, can be restained by rehydrating and starting at step three of the staining procedure. Whole mount preparations may be fixed and stained as for sections with the exception that the triethanolamine rinses after the bismuth staining solution should be increased to 15 min. each. This tissue can then either be cleared and mounted or be embedded in paraffin and sectioned.

RESULTS. This modified procedure was found to maximize the overall staining of nucleoli in cells of developing (Figs. 1 and 2), regenerating (fig. 3) and differentiated limb tissues (Fig. 4-6) of the axolotl. The nucleoli stained dark brown against a light brown granular nucleus. The cytoplasm stained faintly or not at all in epidermal, dermal, blastemal, limb bud and cartilage cells. Myofibrils of muscle tissue stain light brown as does the extracellular connective tissue matrix of the dermis. Overall this light background staining was found to be useful for the identification of tissue types yet it still allowed the intensely stained nucleoli to stand out clearly. Muscle and cartilage occasionally show small dark staining granules.

DISCUSSION. Although other less complicated nucleolus-specific stains, such as Azure B (Dunis and Namenwirth, 1977), Toluidine Blue or Azure A plus cationic surfactant (Bennion et al., 1975) have been reported, they have not given us consistent results and the excellent contrast of this modified bismuth staining procedure on a variety of axolotl tissues. This stain is of great importance to studies which utilize nucleolar variants. In axolotls, two variants are presently obtainable, triploids (with three nucleoli) and nucleolar mutants (with abnormally sized nucleoli). Both of these are demonstrated very well by this stain. In addition, we have tested this modified stain on Xenopus laevis (which has both diploid and tetraploid lines) and have found it to give excellent results.

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FIGURE LEGENDS

Figure 1: Stage 46 (Schreckenberg and Jacobson, 1975) axolotl hind limb bud mesenchyme. Nucleoli appear as dark staining spots against a light brown granular nucleus. The cytoplasm is not stained at all. All photographs taken with bright field illumination with a medium blue filter BG 23. x 575.

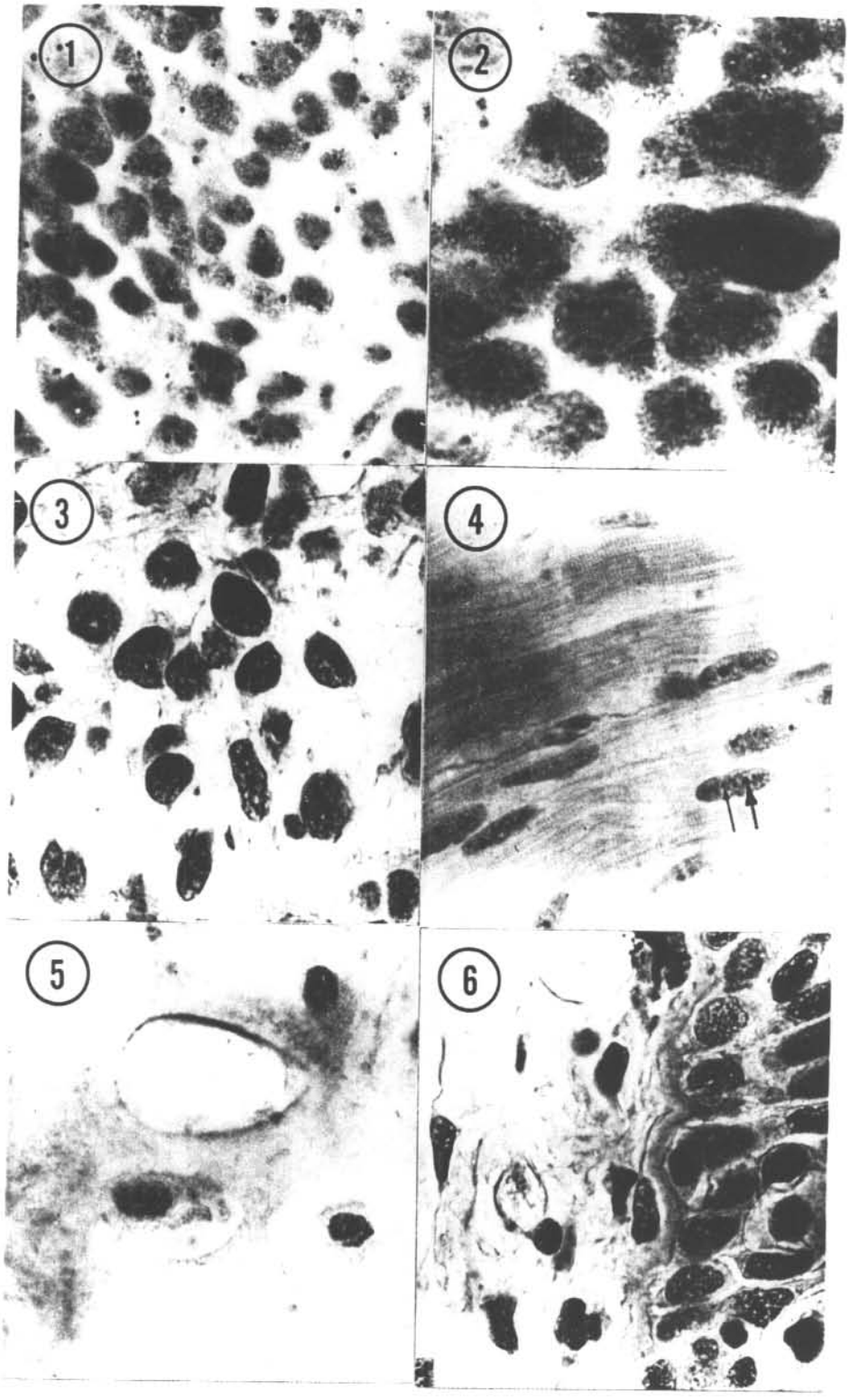
Figure 2: Higher magnification of limb bud mesenchyme shown in Figure 1. x 1150.

Figure 3: Palette stage (Tank et al., 1976) regeneration blastemal cells. x 575.

Figure 4: Muscle tissue. Myofibrils stain lightly making the striations visible. Occasionally very small dark staining granules (small arrow) are present in the nucleus along with the larger nucleoli (large arrow). Longitudinal section. x 525.

Figure 5: Cartilage cells. x 575.

Figure 6: Dermal and epidermal cells. The basal layers of the multi-layered epidermis is visible on the right side of the photograph and scattered dermal cells are present on the left side. x 525.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