A Simple, Reliable and Inexpensive Silver Stain for Nerve Fibers in Bleached Skin

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Z. Naturforsch. **43c**, 606–608 (1988); received March 29, 1988

Lateral Line Organs, Silver Stain, Skin Nerve Fibers, Fish, Amphibians

A procedure for silver staining is described which leads to the selective and reliable impregnation of nerve fibers in bleached skin of vertebrates and invertebrates. In combination with osmium, the protocol enhances the staining of secondary sensory cells of mechanosensory and electrosensory organs so that the innervation pattern of each organ and the number of sensory cells per organ can easily be evaluated. The technique can be also used for staining nerve fibers in whole embryos.

Introduction

Silver stains, known to react specifically with neurofilaments in a variety of animals [3] have recently been revived and introduced for neuroembryological problems [1, 4] and techniques for whole mounted embryos have been worked out [2]. Most of these techniques use either the Bodian or the Liesegang technique, both of which require long staining in diluted silver protargol or silver solutions. In contrast, the Winkelmann and Schmit technique [6] is much faster by using a high concentration of silver nitrate. This technique has been shown by one of us to stain reliably nerve fibers of electrosensory organs in the whole mounted skin of weakly electric fish [7, 9]. However, some species of fish and amphibians show extremely dense melanocytes, especially after regeneration of skin and organs ([8]; Fritzsch and Zakon, in prep.). These melanocytes render the observation of the silver stained fibers virtually impossible (Fig. 1). In an attempt to overcome this problem we have modified the original protocol [6] to combine the silver stain of fibers with bleaching of the skin. In order to have a higher contrast of sensory cells we have added an osmium staining known to enhance the contrast of these cells [5].

Reagents and solutions

a. 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4);
b. 10% unbuffered formalin (= 4% Formol) with 3% H_2O_2;
c. 0.001% osmium tetroxid in 0.1 M phosphate buffer (pH 7.4);
d. 20% silver nitrate;
e. 0.2% hydroquinone in 1% sodium sulfite;
f. 0.2% gold chloride;
g. 5% sodium thiosulfate.

Protocol of the procedure

1. Remove skin from anesthetized animal, place flat on sylgard gel and fix for about 10 min with solution a.
2. Store for 1 day or up to several months in solution a.
3. Store in solution b. for at least one week until skin is bleached, several changes of solution b. may be necessary.
4. Wash in phosphate buffer for 1 h.
5. Stain with solution c. until nerve fibers are brown (several minutes). If excessively stained, bleach again in solution b.
6. Wash in phosphate buffer (several changes of 10 min each).
7. Defat in ethanol (50%, 70%, 96%), 10 min each change.
8. Three changes of isopropanol for 10 min each.
9. Clear in xylene for 1 h.
10. Three changes of isopropanol for 10 min each.
11. Three changes of ethanol (95%, 70%, 50%), 10 min each.
12. Wash in distilled water and store in 10% formalin for at least one day.
13. Wash in distilled water overnight (12 h).
14. Wash in distilled water, three times for 30 min.
15. Immerse in solution d. for 20 min.
16. Wash three times 5 min in distilled water.

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Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/88/0700–0606 $ 01.30/0

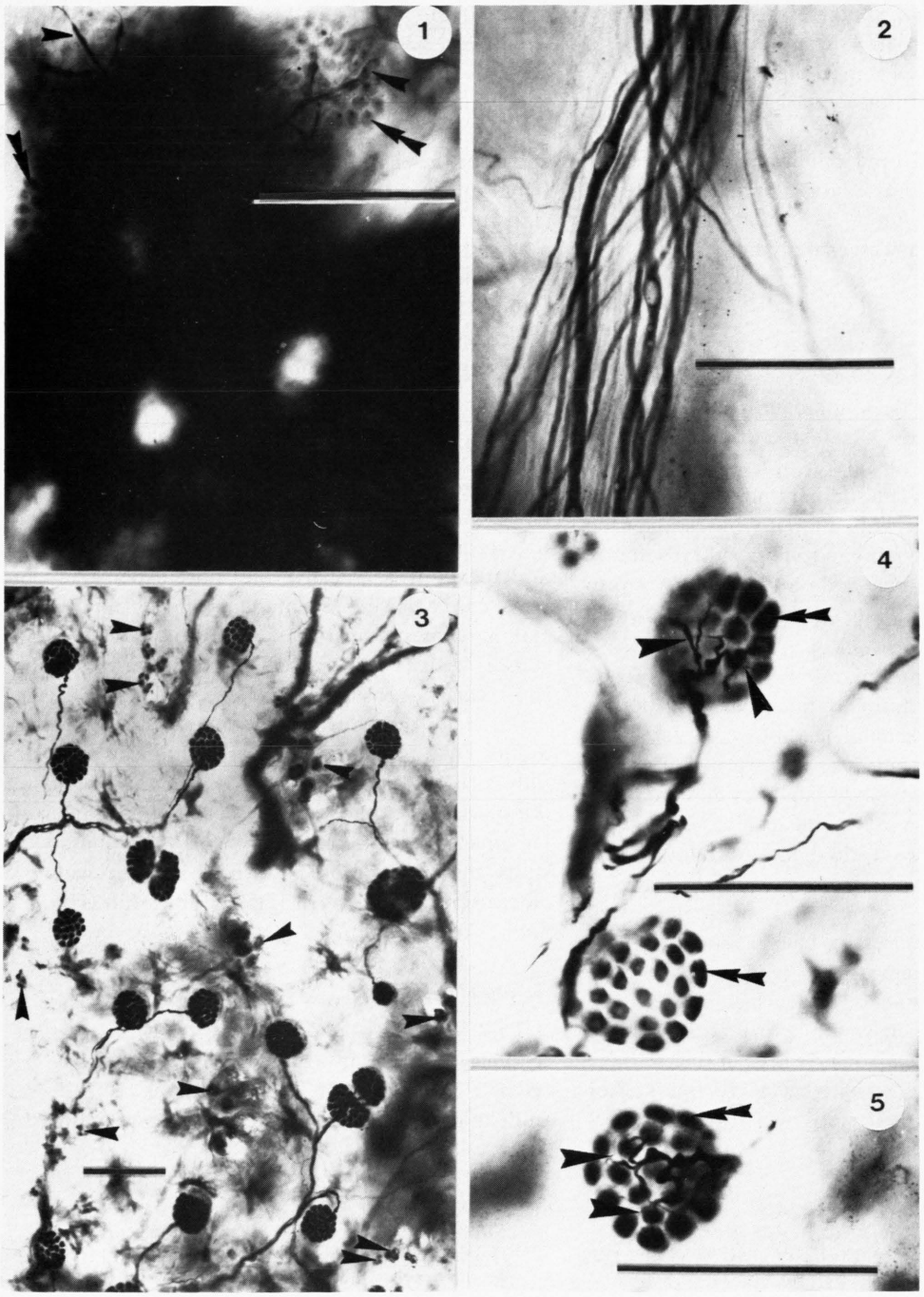

Fig. 1. This flat mounted skin of *Sternopygus macrurus* shows the appearance of the silver stained tuberous organs when adjacent to a melanocyte. Note that both nerve fibers (arrowheads) and sensory cells (double arrowheads) are visible only when remote of melanocytes. Bar indicates 100 μm.

Fig. 2. This photomicrograph shows the silver stained subcutaneous nerve fibers of a squid, *Lolliguncula spec.* Note stained thick and thin fibers and the presence of empty spaces sometimes interpreted as nuclei. Bleached and osmium treated skin. Bar indicates 100 μm.

Fig. 3. A low power micrograph shows the visibility of the silver stained nerve fibers and the electroreceptive organs in the skin of *Sternopygus* treated with the modified technique. Arrowheads indicate ampullary organs, all other organs are tuberous organs. Bar indicates 100 μm.

Fig. 4, 5. These micrographs show the delicate nerve fiber branches (arrowheads) within tuberous organs to reach every sensory cell (double arrowheads). Bleached and osmium treated skin, double impregnated. Bar indicates 100 μm.

17. Reduce in solution e. for 10 to 20 min. Nerve fibers should turn brown.
18. Wash three times 2 min in distilled water.
19. Place in solution f. for 2 min. Nerve fibers should turn purple.
20. Wash three times for 1 min in distilled water.
21. Immerse in solution g. for 5 min. Nerve fibers should be black.
22. Wash in distilled water, dehydrate in ethanol, clear and mount.

Comments

The described protocol has been used successfully for teleost (Fig. 3–5), amphibian and squid skin (Fig. 2). Both the silver nitrate and the gold chloride solution can be used repeatedly at least for one year if stored in the dark. The sodium thiosulfate solution can be stored for at least one month but can be used only once. The hydroquinone/sodium sulfite solution should be used only once and on the day it was prepared. Only distilled water is recommended for all solutions.

The fixation first in glutaraldehyde appears to be necessary for the resolution of fine branches. Bleaching alone without formalin leads to complete suppression of silver binding. Likewise, excessive bleaching affects the silver stain and should be avoided. We recommend to bleach just enough to have the melanocytes turned into a faint yellow color.

Use of osmium is optional and should be tested first in new tissue. If excessive background staining is achieved, bleach osmium stain with solution b. If white silver sulfite forms on the surface of the tissue wash longer in distilled water before and after the silver impregnation (step 15). It is desirable to have only yellow to brown staining of nerve fibers after step 17. The subsequent steps will turn the fibers black. If this is not achieved after step 21, start all over again with step 12. Multiple impregnation has been used successfully by us to achieve staining in very small fiber branches without excessive background, provided the rinsing in distilled water before and after the silver nitrate solution was long enough. If background turns to dark, start with step 12 but stop at step 17. This will result in a brown rather than a black background. We have also used the protocol for whole embryos and for the skin of embryos. Only changes in the times of rinsing in distilled water are necessary, which are shorter in the embryonic skin and longer in whole embryos.

Discussion

We describe a technique for the silver staining of nerve fibers in bleached skin of some vertebrates and invertebrates. The technique is easy, rapid, reliable and inexpensive because the most expensive solutions can be used repeatedly. It leads to detailed staining of nerve fibers and, in addition, of secondary sensory cells (Fig. 3–5), so that quantitative evaluations can be easily performed. These advantages make the proposed technique superior to other techniques [1, 2, 4], whereas the resolution of fiber details is comparable and depends presumably only on the specificity of the binding of silver ions to neurofilaments [3]. We are currently using the technique to study the innervation pattern of newly developed electrosensory organs in regenerating skin (Fritzsch and Zakon, in prep.).

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft (Fr 572, B.F.) and the NSF (BNS 8304584, H.H.Z.). We thank Dr. B. U. Budelmann for providing the cephalopod material.

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