*Copeia*, 1995(1), pp. 114–118

# Clearing and Staining Whole Fish Specimens for Simultaneous Demonstration of Bone, Cartilage, and Nerves

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**Comparative anatomical studies of nerves alone, or combined with those of bone and cartilage, are rare in ichthyology because of lack of a reliable, repeatable protocol for preparing specimens that demonstrate all three character systems. We present a method for enzyme clearing and staining whole fish specimens for simultaneous demonstration of bone, cartilage, and nerves. Nerves are stained dark blue with Sudan Black B following alcian blue-alizarin red S staining. Our protocol for Sudan Black B staining differs from others in concentration of Sudan Black B solution, length of time in that solution, and method of differentiation.**

CLEARING and staining of whole small vertebrates for demonstration of bone (Taylor, 1967a, 1967b) or bone and cartilage (Dingerkus and Uhler, 1977; Kawamura and Hosoya, 1991) are methods used widely in comparative vertebrate osteology. Such cleared-and-stained preparations of whole fish specimens provide osteological data, source of a large portion of the characters used in studies of fish phylogeny (see contributions in Johnson and Anderson, 1993). In contrast, few systematic ichthyological studies incorporate comparative anatomical data from nerves. The exceptional work of Freihofer and collaborators (Freihofer, 1966, 1978; Freihofer et al., 1977) introduced the systematic ichthyological community to the Sihler technique in which hematoxylin is used to stain nerves. For reasons discussed in Filipski and Wilson (1984, 1985) and Nishikawa (1987), the Sihler technique proved too difficult to apply regularly and never became popular.

Sudan Black B was used by Rasmussen (1961) to stain acoustic nerve branches in the whole, but not cleared, vertebrate ear for gross dissection. Enzyme clearing and Sudan Black B nerve staining was combined by Filipski and Wilson (1984, 1986) in a so-called regressive staining method and by Nishikawa (1987) in a progressive staining method. Filipski and Wilson (1985) combined enzyme clearing with alizarin red S bone and Sudan Black B nerve staining. Filipski and Wilson (1984:208) concluded that "The possibility also exists of adding Alcian Blue as a cartilage stain," but they did not describe a protocol. A method that reliably provides a cleared specimen with stained bone, cartilage, and peripheral nerves for study of nerve-skeletal relationships has not been described. Furthermore, and perhaps more important, to be used widely in systematic ichthyology, such a method must use formalin-fixed, alcohol-stored specimens; be straightforward enough to allow for preparation of specimens in a reasonable amount of time; and result in specimens that remain stained during long-term storage. Here, we report such a method that we have used to clear muscle and stain bone, cartilage, and nerves in whole fish specimens.

By design, our method begins with a counterstaining technique modified slightly from Dingerkus and Uhler (1977) and with our annotations, followed by nerve staining. One advantage of such a protocol is that specimens may be cleared and stained for bone and cartilage, examined, and then stained for nerves.

Our contribution to Sudan Black B nerve staining methods involves differentiation (Step 11, below), modified from Rasmussen (1961), a step developed to obtain satisfactory results with small as well as large specimens. As Filipski and Wilson (1984:208) note, small, often young, specimens that they prepared retained little or no Sudan Black B. In Filipski and Wilson's (1984) regressive method, after immersion in Sudan Black B, specimens were destained in 70% ethanol before being placed in 0.5% KOH overnight to remove ethanol. The specimen is transferred next to a fresh trypsin solution which removes excess stain from surrounding tissues more slowly than the ethanol bath. In Nishikawa's (1987) progressive method, after immersion in Sudan Black B, specimens were rinsed in distilled water prior to clearing in a glycerin series. Whether destaining takes place in 70% ethanol or distilled water, excess Sudan Black B is removed not only from skin, muscles, and other tissues but from peripheral nerve branches as well. It is difficult to obtain satisfactory results with small specimens using the above so-called regressive and progressive protocols. Our differentiation step is designed to solve this problem by processing specimens individually, varying length of destaining time, and manually pushing or squeezing excess Sudan Black B from the specimen while it is immersed in 50% ethanol. For reasons discussed below, we are not

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optimistic that this method can be applied regularly to existing large collections of cleared-and-counterstained specimens.

## Materials and Methods

We developed this method to prepare specimens for study of homology of superficial neuromast innervation patterns in gobioid fishes that could be used also for study of nerve-skeletal relationships. We prepared 20 specimens representing 19 species in 16 genera of acanthomorph fishes from the National Museum of Natural History (USNM) collection. All specimens chosen for triple-staining had been relatively well fixed in approximately 10% formalin and stored in 70–75% ethanol. Specimen size ranges from 16.1–232 mm TL.

*Procedure.*—Step 1. Fixation: Fix fresh material in 10% formalin for three days or more depending on size of specimen, or use a formalin-fixed specimen (stored either in formalin or ethanol).

Step 2. Wash: Wash specimen in several changes of distilled water two to three days or more depending on specimen size. We found that washing specimens in cold, running tap water overnight also proved satisfactory. The purpose of this step is to wash fixative thoroughly from the specimen. Skin and eviscerate specimens or not depending on the purpose of study. We recommend no dissection until after step 13 (permanent storage) except for medium to large specimens that may be hemisectioned in this step. Skinned specimens are useless for studying innervation of superficial neuromasts. Also, scales and superficial dermal bones may be dislodged during skinning or evisceration.

Step 3. Cartilage staining: Place specimen in the alcian blue solution for one to two days. The solution should be made within the previous week (alcian blue solution: 10mg alcian blue 8GN, 80 ml 95% ethanol, and 20ml glacial acetic acid).

Step 4. Rehydration: Transfer specimen through two changes of 95% ethanol, two to three hours in each change, then through successive solutions of 75%, 50%, and 30% ethanol for two to three hours each, finally to distilled water (two to three changes) for two to three hours or until specimen sinks.

Step 5. Muscle digestion: Place specimen in trypsin solution for several days to weeks depending on specimen size and the amount of fat it contains. In large fish with thick skin or a layer of fat under the dermal tissue, muscle digestion may take several weeks. Placing the specimen on a light-box or warming-tray (temperature higher than room temperature) may accelerate clearing. Change enzyme solution completely, and clean the specimen container every two to three days to prevent contamination until bone and cartilage are visible [enzyme solution: 1 gm or about 1/4 teaspoon of trypsin in 30% sodium borate buffer (30ml saturated aqueous sodium borate, 70ml distilled water)].

Step 6. Build KOH environment: Transfer specimen to 0.5% aqueous potassium hydroxide (KOH) for about one hour (length of time is flexible). The purpose of this step is to wash away the enzyme solution and build a KOH environment to help the alizarin red S penetrate bone.

Step 7. Bone staining: Transfer specimen to alizarin red S solution for about 24 h or until bones are distinctly red or reddish purple. Do not overstain; otherwise undigested muscles may become reddish (alizarin red S solution: add alizarin red S powder slowly to 0.5% KOH while stirring until the solution turns deep purple).

Step 8. Destaining and bleaching: Transfer specimen to 0.5% KOH for 15–30 min. Specimen may be bleached at this stage to remove natural, darker colored pigments by leaving the specimen in the bleaching solution for one-half to one hour (bleaching solution: 3 or 4 drops of 3% $H_2O_2$ in 100ml 0.5% KOH solution).

Step 9. Dehydration: Transfer specimen through solutions of 30%, 50%, and 70% ethanol, leaving specimen in each solution for about 30 min.

Step 10. Nerve staining: Place specimen in 30–50% Sudan Black B solution for about five to eight hours for small specimens (total length of 16–125 mm), overnight for medium-sized specimens (total length of 232 mm), or several days for large specimens (total length 450–560 mm; Song and Brood, 1993). Concentration of, and length of time a specimen is placed in, the Sudan Black B solution is flexible. In general, larger specimens need to be placed in a more concentrated solution for a longer period of time than do smaller specimens. A specimen that is found to be understained when differentiation (step 11) is started may be placed back in the Sudan Black B solution. Likewise, specimens overstained may be destained. (Sudan Black B solution: Make saturated Sudan Black B solution with 70% ethanol, filter, then dilute the solution with seven or five parts 70% ethanol to make a 30% or 50% Sudan Black B working solution. The Sudan Black B solution ideally should be freshly made and not more than two weeks old.)

Step 11. Differentiation: This is the critical

step that determines quality of nerve staining. Destain the specimen by dipping it quickly in 70% ethanol for 30 sec to 1 or 2 min to wash off excess Sudan Black B solution. Dip the specimen quickly in 60% ethanol for 30 sec to 5 min depending on size of the specimen, thickness of the skin, and amount of extra stain it has taken up. The purpose of these two ethanol washes is to remove excess Sudan Black B from the surface of the specimen. Avoid destaining small, peripheral nerve branches.

Transfer the specimen to 50% ethanol to remove Sudan Black B from muscle and other tissues. We recommend strongly that as much of this process as possible be observed using a dissecting microscope. Use forceps to gently "push" or "squeeze" the specimen occasionally to accelerate destaining. If the solution becomes medium to dark blue, change it. This step should be stopped at the point remaining muscle fibers become clear and nerve branches may still be differentiated or until the solution is light blue to clear. Time of this destaining step can be from about one hour (for the gobioid *Xenisthmus polyzonatus*, USNM 245270, 16.1 mm TL) to one or two days depending on size of the specimen and time spent gently pushing or squeezing it. Specimens may be left overnight in 50% ethanol to destain gradually.

Step 12. Transfer the specimen to 0.5% KOH overnight.

Step 13. Storage: Transfer the specimen to 30% glycerin and 70% 0.5% KOH until it sinks, then to 70% glycerin and 30% 0.5% KOH, finally storing in 100% glycerin without thymol. Thymol has been added for long-term storage of cleared-and-stained specimens, but, for reasons unknown to us, thymol may destain nerves (see Discussion).

## Results and Discussion

Our triple-stained specimens have been stored in 70% or 30% glycerin in 0.5% KOH for more that one year (since Sept. and Oct. 1992), and all three stains are well maintained (Fig. 1). Our method of nerve staining may be considered intermediate between the so-called regressive and progressive staining methods, which differ in concentration of the Sudan Black B solution, length of time in the solution, and method of destaining (Nishikawa, 1987). To stain nerves, Nishikawa (1987) immersed specimens for 7–10 days in 5% saturated solution of Sudan Black B in 70% ethanol; whereas Filipski and Wilson (1984) immersed specimens for one minute in a 100% saturated solution of Sudan Black B in 70% ethanol. Longer staining time means better penetration of the specimen with Sudan Black B; however, too much time in Sudan Black B means that bones and other fat-containing structures will stain (Nishikawa, 1987). Here, Step 10 above, we immersed specimens in 30–50% saturated solution of Sudan Black B in 70% ethanol for five to eight hours. We were able to stain peripheral nerves of relatively small (e.g., *Xenisthmus polyzonatus*, USNM 245270, 16.1 mm TL) as well as larger specimens.

Our differentiation step involves processing of specimens individually, varying length of destaining time, and manually pushing or squeezing excess Sudan Black B from muscle, fat, and other tissues while the specimen is immersed in 50% ethanol. For each specimen, the preparer can decide when to stop differentiation. Moreover, differentiation (Step 11) and the previous step, nerve staining (Step 10), are reversible. It is possible to move a specimen back and forth between nerve staining and differentiation until satisfactory results are obtained; backtracking is usually not necessary, in our experience.

Filipski and Wilson (1984) stored their nerve preparations in 100% glycerin with thymol crystals added to inhibit mold growth. They noted (p. 208) that ". . .the few specimens that did not destain sufficiently in the head region did in fact destain after prolonged storage for 30–60 days in glycerin." We observed rapid destaining of a triple-stained specimen when it was placed in glycerin with thymol. The specimen was restained and stored in glycerin without thymol; it is still well stained. Also, we place cleared and counterstained ricefishes (Adrianichthyidae) that had been stored in glycerin and thymol for over 10 years and were in good condition in Sudan Black B solution. Results were unsatisfactory for observation of nerves of the larger specimen, *Adrianichthys* sp., 70 mm TL, (USNM 326628); undigested muscles were stained dark blue, and nerves could not be differentiated. The smaller specimen, *Oryzias melastigma*, 29 mm TL, AMNH 20560SW, retained less of the Sudan Black B in undigested muscles. The trunk portion of the lateral-line nerve may be differentiated clearly, but the anterior portion of the lateral-line nerve may not. Specimens stained for bone or counterstained for bone and cartilage and stored in glycerin with thymol, a common practice advocated by Dingerkus and Uhler (1977) and others, may not be optimal for Sudan Black B nerve staining. Nishikawa (1987) recommended that hydrogen peroxide and KOH be omitted from nerve staining procedures because both damage nerves. In our experience, $H_2O_2$ and KOH can be used with care in the triple-staining procedure.

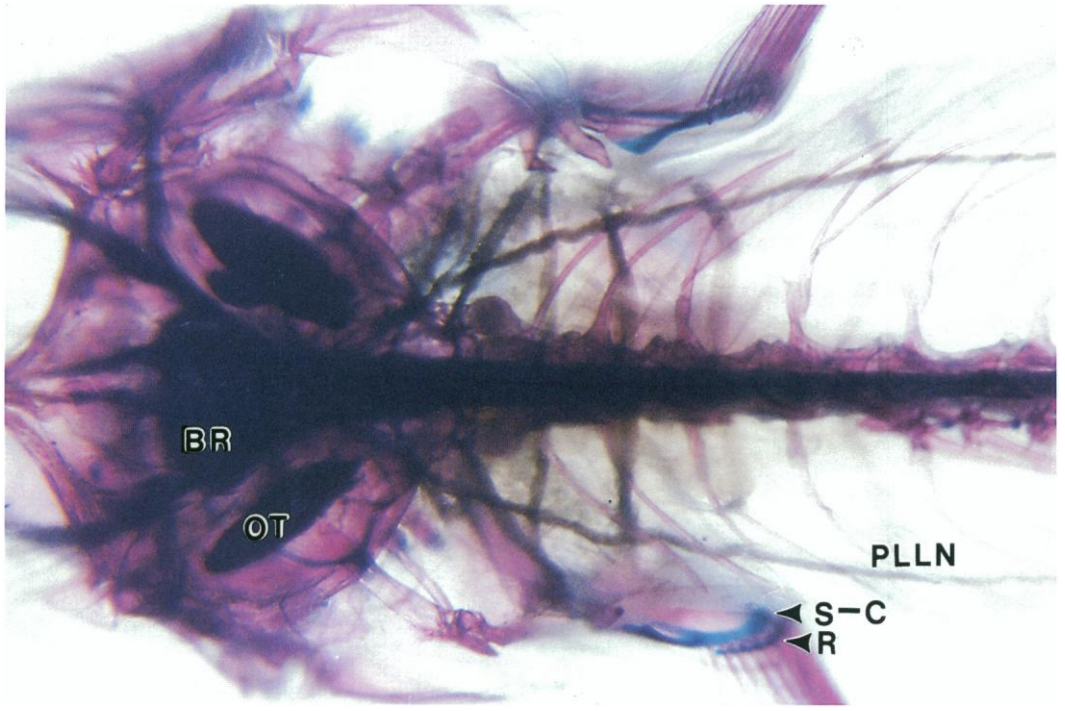


Fig. 1. *Callogobius depressus*, USNM 214613, 46 mm TL. Dorsal view of posterior part of head, anterior vertebrae, and dorsal portion of pectoral-fin skeleton. BR = brain, OT = otolith, PLLN = posterior lateral line nerve, R = pectoral-fin radial cartilages, S-C = scapulo-coracoid cartilage.

Specimens cleared with trypsin and stained solely with Sudan Black B by one of us (JS) as early as 1985 (Song, 1986) and stored in glycerin without thymol retain well-stained, well-differentiated nerves; and the specimens are in good condition (Song and Northcutt, 1991). We placed one specimen, *Fundulus heteroclitus*, USNM 326631, prepared in 1988, in a solution of alizarin and 70% glycerin in 0.5% KOH. The bones were stained a deep red after several days. Backtracking to alcian blue is not possible after trypsin digestion (Dingerkus and Uhler, 1977).

## Acknowledgments

JS thanks G. T. Filipski and M. V. H. Wilson for correspondence and references. LRP has benefited from numerous discussions with G. Dingerkus on methods of clearing and staining. M. N. Feinberg, G. Nelson, and M. L. J. Stiassny (American Museum of Natural History, AMNH) provided a loan of specimens in their care. This research was supported in part by a Smithsonian Institution postdoctoral fellowship to JS. Comments of R. P. Vari and reviewers improved the text.

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*Copeia*, 1995(1), pp. 118–124

# New *Uroplatus* Duméril (Reptilia: Squamata: Gekkonidae) of the *ebenaui*-Group from the Anosy Mountains of Southern Madagascar

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**A new species of *Uroplatus* from the southern Anosy Mountains of southern Madagascar is described. It is a small, short-headed, and short-snouted species with a laterally compressed body. It lacks membranous fringes on the lower jaw, neck, body, and limbs. It is most similar to *Uroplatus ebenaui*, from which it differs in scalation, coloration, and structure of the hemipenis. The new species is known from a single specimen from unprotected montane rainforest in a region where rainforest is rapidly disappearing.**

THE gekkonine genus *Uroplatus*, endemic to Madagascar, was reviewed most recently by Bauer and Russell (1989). These authors recognized six species: *alluaudi*, *ebenaui*, *fimbriatus*, *guentheri*, *lineatus*, and *sikorae*. Böhme and Ibisch (1990) subsequently described a seventh species, which they named *Uroplatus henkeli*.

The species of *Uroplatus* can be assigned tentatively to three species groups: the *fimbriatus*-Group, including *fimbriatus*, *lineatus*, *sikorae*, and *henkeli*; the *ebenaui*-Group, including only *ebenaui*; and the *alluaudi*-Group, including *alluaudi* and *guentheri*. Bauer and Russell (1989) placed another species, *U. phantasticus*, in the synonymy of *U. ebenaui*. Nussbaum and Raxworthy (1994) described another new *Uroplatus*, which is the third member of the *alluaudi*-Group.

Species of the *fimbriatus*-group are relatively large, long-tailed, long-headed, and long-snouted forms with depressed heads and lateral membranous fringes placed variously on parts of the head, neck, body, and limbs. The *ebenaui*- and *alluaudi*-groups consist of smaller, short-tailed, short-headed, and short-snouted forms with heads that are not depressed, at least posteriorly; and they lack membranous fringes except on the tail. The *ebenaui*-group is further characterized by having a laterally compressed body; scattered, posteriorly directed, spines on the dorsolateral surfaces of the head, neck, body,

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