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TECHNIQUE FOR REVEALING THE THREE-DIMENSIONAL ARCHITECTURE OF WHOLE PRESERVED SPICULATED INVERTEBRATES

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ABSTRACT. **Procedures for revealing the three-dimensional arrangement of calcareous sclerites, spicules, or ossicles embedded within connective tissue in formalin fixed invertebrates are described. Spicules are stained with alizarin red S following maceration of preserved animals or colonies with either trypsin or KOH solutions. Connective tissue is stained with alcian blue in different samples prior to maceration. Stained animals or colonies are cleared in glycerin. This method for revealing spicular structure and arrangement and the gross morphology of connective tissues offers several advantages over either scanning electron microscopy or reconstruction from serial sections.**

Invertebrates from many phyla, including Porifera, Cnidaria, and Echinodermata, have embedded in their connective tissues small calcareous or siliceous structures known variously as spicules, sclerites, or ossicles. (For ease of discussion in this paper, we refer to all such structures as spicules.) The architecture of skeletons composed of large, regularly arranged spicules can be studied by dissection (*e.g.*, Eylers 1976). Scanning electron microscopy can resolve the arrangement of small, densely packed spicules (*e.g.*, Muzik and Wainwright 1977), but it is not appropriate for widely dispersed spicules: only spicules that lie in the fracture plane are visible, and even their shape and orientation cannot be determined fully because they are partially embedded in the surrounding tissue. Therefore, except in animals with transparent tissues, the three-dimensional arrangement of small spicules dispersed within connective tissues must be resolved by serial sectioning. This procedure, however, is laborious and time-consuming for large and complicated structures. Furthermore, sectioning of soft tissues containing hard inclusions can introduce artifacts that make the interpretation of three-dimensional spicular arrangement difficult.

To circumvent similar difficulties in analyses of skeletal tissues of vertebrates, morphologists have developed various procedures which clear whole animals and stain either calcified tissues (*e.g.*, bone and teeth), cartilage, or both (see techniques and references in Dingerkus and Uhler 1977, Hanken and Wassersug 1981, Kimmel and Trammell 1981, McLeod 1980, Simons and Van Horn 1971, Wassersug 1976). In addition to being quicker than serial reconstruction, these techniques allow immediate visualization of three-dimensional structure and surface detail. In this paper we present a modifi-

cation of procedures used for staining preserved vertebrate specimens that we have successfully applied to preserved invertebrates. This technique allows ready characterization of the distribution and anatomy of calcareous spicules vis-à-vis the surrounding matrix of connective tissue.

Two different stains are used in the technique. The first, alizarin red S, has affinity for certain insoluble mineral deposits (Humason 1979), in this case calcium carbonate ($CaCO_3$), and stains calcareous spicules red to reddish purple. The second, alcian blue, reacts with acid mucopolysaccharides (Barka and Anderson 1963), thereby staining connective tissues, such as mesoglea, blue. Successive application of alcian blue and alizarin red S in the same specimen, which provides effective differential staining of cartilage and bone in vertebrate specimens (see references above), yielded poor results when applied to invertebrates; stained spicules showed up much more vividly in unstained than in alcian blue stained connective tissue. Thus we recommend separate staining of spicules and mesoglea in different samples.

Procedure

The procedures described below are appropriate for "soft corals" such as *Alcyonium digitatum*. The fleshy colonies of these cnidarians are composed primarily of connective tissue in which calcareous spicules are embedded. The timing may be varied for different organisms as is appropriate (see Discussion).

Staining Spicules

Anesthesia. Anesthetize a colony in a solution of one part 20% magnesium sulfate ($MgSO_4 \cdot 7H_2O$) to one part sea water (Pantin 1964) until polyps do not contract when pinched with forceps.

Fixation. Fix the specimen for at least two days in 4% formalin (in sea water) buffered with calcium carbonate ($CaCO_3$). After fixation the specimen may be stored in 70% ethyl alcohol.

Washing. Wash the specimen in several changes of distilled water for at least two days.

Maceration. Transfer the specimen to an enzyme solution of 30 ml saturated aqueous sodium borate, 70 ml distilled water, and 1 g crude trypsin (*e.g.*, Matheson, Coleman and Bell TX 1590). Leave the specimen until the tissues become translucent, at which time unstained subsurface spicules may be visible. Alternatively, 0.5% potassium hydroxide (KOH) may be substituted for the enzyme solution.

Staining. Place the specimen in 0.5% KOH that has been colored deep purple with alizarin red S stock solution (Humason 1979, p. 157). Leave the tissue in this stain for 24 hours.

Clearing. Transfer the specimen through a graded series of 0.5% KOH-glycerine mixtures of increasing glycerin concentration (*e.g.*, 25%, 50%, 75%, 100%), leaving it in each solution for at least 24 hours.

Storage. Store the specimen in pure glycerin. A few crystals of thymol should be added to retard microbial growth and subsequent spoilage.

Staining Connective Tissue

Anesthesia, Fixation and *Washing* as above.

Staining. Place the tissue in a solution of 20 mg alcian blue 8GX, 70 ml absolute ethanol, and 30 ml glacial acetic acid for 12–48 hours. Staining increases progressively with time; examine the specimen periodically and remove it when staining is adequate.

Dehydration. Transfer the specimen to absolute ethanol for at least 48 hours to fix the stain, changing the alcohol bath at least once.

Hydration. Transfer the tissue through a graded series of ethanol baths of decreasing concentration (*e.g.*, 75%, 50%, 25%) and then into two changes of distilled water, leaving it at least two hours in each step. *Maceration, Clearing* and *Storage* as above.

Results and Discussion

Cleared and stained pieces of an *A. digitatum* colony are shown in Fig. 1. Alcian blue stained connective tissue in which spicules are embedded is shown in Fig. 1A; note the obvious positions of the gastrovascular cavities of the polyps which perforate the tissue in this specimen. Spicules stained with alizarin red S appear dark in Fig. 1B; preparations such as these have been used to establish differences in both size and orientation between spicules at a colony's periphery and those within (Koehl 1982).

The above staining technique is only a guideline; it should be adjusted as befits the particular situation. Adherence to the sequence of steps is necessary for proper staining, but the concentration of several solutions, as well as the length of time that a given specimen is kept in each, may be varied according to circumstances. For example, the concentration of trypsin in the enzyme solution may be increased to shorten the time necessary for complete maceration. Likewise, the concentration of both stain solutions may be increased for more rapid staining.

Specimens may be stained either *in toto* or in smaller pieces cut after fixation. We found the latter procedure preferable in our analysis of the three-

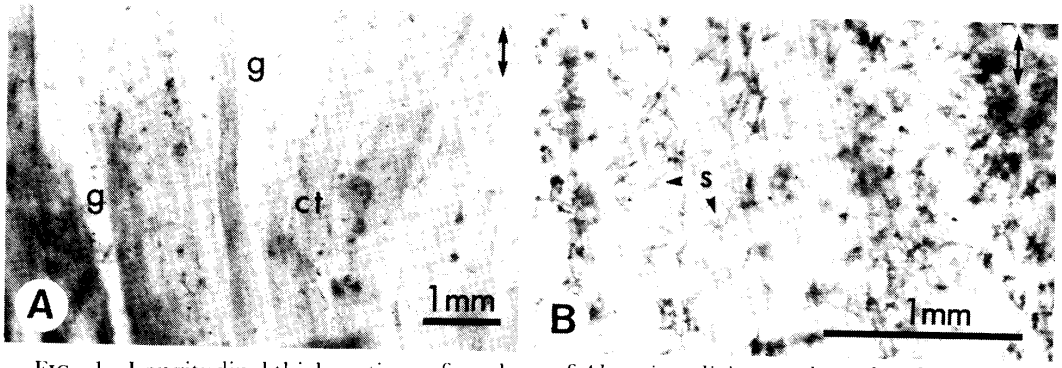

FIG. 1. Longitudinal thick sections of a colony of *Alcyonium digitatum* cleared and stained with either alcian blue (A) or alizarin red S (B). Gastrovascular cavities (g) of the polyps, which perforate the stained connective tissue (ct), are visible in A; spicules (s) are visible in B. Arrows indicate vertical axis. Scale marker equals 1 mm.

dimensional arrangement of spicules in large *A. digitatum* colonies, and for that purpose prepared multiple thick sections (5–20 mm) in radial, tangential and cross-sectional planes. Thick sections are usually preferable to whole specimens for photographing details of internal anatomy.

For maceration, we have used the enzyme solution and potassium hydroxide with equal success. However, maceration often proceeds at markedly different rates in the two treatments; specimens should be checked frequently, as they will disintegrate if left in either solution too long. We found various species of sponges, as well as the cnidarian *Alcyonium ciderium*, to require shorter maceration than did *A. digitatum*.

The technique we have described for the visualization of spicule morphology and arrangement within preserved animals or colonies is less laborious and less expensive than either serial sectioning or scanning electron microscopy, and avoids many of the artifacts that may accompany these procedures. It also permits the use of larger specimens in which the arrangement of spicules with respect to features of whole animals or colonies can be identified. Stereomicrographs of cleared and stained specimens, which vividly illustrate the three-dimensional architecture of spicules, are particularly useful for this purpose. Using this technique we have successfully prepared specimens fixed in either ethyl alcohol or formalin and stored in ethyl alcohol at room temperature for several years. Thus, this procedure also may be applicable to museum material for taxonomic investigations, as well as for analyses of functional morphology.

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References

Barka, T. and Anderson, P. J. 1963. *Histochemistry.* Harper & Row, New York.

Dingerkus, G. and Uhler, L. D. 1977. Enzyme clearing of alcian blue stained whole small vertebrates for demonstration of cartilage. Stain Technol. *52:* 229–232.

Eylers, J. P. 1976. Aspects of skeletal mechanics of the starfish *Asterias forbesii.* J. Morphol. *149:* 353–367.

Hanken, J. and Wassersug, R. J. 1981. The visible skeleton. Funct. Photog. *16*(4): 22–26, 44.

Humason, G. L. 1979. *Animal Tissue Techniques,* 4th ed. W.H. Freeman and Co., San Francisco.

Kimmel, C. A. and Trammell, C. 1981. A rapid procedure for routine double staining of cartilage and bone in fetal and adult animals. Stain Technol. *56:* 271–273.

Koehl, M. A. R. 1982. Mechanical design of spicule-reinforced connective tissue: stiffness. J. Exp. Biol. *98:* 239–267.

McLeod, M. J. 1980. Differential staining of cartilage and bone in whole mouse fetuses by alcian blue and alizarin red *S.* Teratology *22:* 299–301.

Muzik, K. and Wainwright, S. A. 1977. Morphology and habitat of five Fijian sea fans. Bull. Mar. Sci. *27:* 308–337.

Pantin, C. F. A. 1964. *Notes on Microscopical Technique for Zoologists.* Cambridge University Press, Cambridge.

Simons, E. V. and Van Horn, J. R. 1971. A new procedure for whole-mount alcian blue staining of the cartilaginous skeleton of chicken embryos, adapted to the clearing procedure in potassium hydroxide. Acta Morphol. Neerl.-Scand. *8:* 281–292.

Wassersug, R. J. 1976. A procedure for differential staining of cartilage and bone in whole formalin-fixed vertebrates. Stain Technol. *51:* 131–134.