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Histological investigation of organisms with hard skeletons: a case study of siliceous sponges

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Abstract

Siliceous and calcareous sponges commonly are treated with acid to remove the spicules prior to embedding and cutting for histological investigations. Histology of spiculated sponge tissue represents a challenging problem in sponge histotechnology. Furthermore, fluorescence in situ hybridization (FISH), a key method for studying sponge-associated microbes, is not possible after acid treatment. For a broad range of siliceous sponge species, we developed and evaluated methods for embedding in paraffin, methylmethacrylate resins, LR White resin and cryomatrix. Different methods for cutting tissue blocks as well as mounting and staining sections also were tested. Our aim was to enable histological investigations and FISH without prior removal of the spicules. To obtain an overview of tissue and skeleton arrangement, we recommend embedding tissue blocks with LR White resin combined with *en bloc* staining techniques for large specimens with thick and numerous spicules, but paraffin embedding and subsequent staining for whole small specimens. For FISH on siliceous sponges, we recommend Histocryl embedding if the spicule content is high, but paraffin embedding if it is low. Classical histological techniques are used for detailed tissue examinations.

Key words: embedding, FISH, histology, LR White resin, methylmethacrylate resin, paraffin, Porifera, spicules

Sponges are ancient metazoa with simple body plans consisting of only a few specialized cell types. They have long been used as model organisms for histological studies for light and electron microscopy (Simpson 1984). Because the Porifera are one of the richest phyla for investigating the biosynthesis of bioactive natural products, they have received increasing attention from organic chemists and pharmacologists (Faulkner et al. 1994, Garson 1994, Munro et al. 1999). Whereas the intercellular matrix of higher animals is sterile, sponge tissue often contains vast numbers of microbes, which may or may not be involved in the production of the secondary metabolites (Unson et al. 1994, Debitus et al. 1998, Flowers et al. 1998, Garson et al. 1998, Turon et al. 2000, Hentschel et al. 2001). A combination of classical histology with modern molecular biological methods has become necessary to elucidate the impact of sponge-associated bacteria on the metabolism of the host sponge. Recently, fluorescence in situ hybridization (FISH) on sponge sections (e.g., Manz et al. 2000, Webster et al. 2001) became popular for identification and localization of sponge-associated bacteria within sponge tissue.

Paraffin is the classical embedding technique for light microscopic examination of any tissue (Romeis 1989), and it commonly is used for sponge histology. Cryotechniques, which involve cutting frozen tissue blocks on a special cryomicrotome, are faster and easier. For both techniques, siliceous or calcareous spicules must be removed with hydrofluoric acid (HF) or hydrochloric acid (HCC),

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respectively, to obtain sections of good quality. This procedure has several disadvantages. First, it makes observation of the skeletal arrangement in the sections impossible. Second, if rich in siliceous spicules, as most cold-water sponges are (Barthel 1995), the tissue may collapse after spicule removal. Furthermore, after HF treatment, FISH is no longer possible (personal observation). For sponge species lacking a mineralized skeleton, HF treatment can be omitted and FISH is possible on both paraffin sections (Manz et al. 2000) and cryosections (Friedrich et al. 1999, Webster and Hill 2001, Webster et al. 2001).

Embedding in resin is an alternative technique. Some methylmethacrylate (MMA) resins are used in histology, usually in kits combined with benzoyl peroxide (Romeis 1989). Trade names include Technovit and Histocryl. MMA resins have been used for histology and FISH on sponges lacking a siliceous skeleton (Wagner et al. 1998, Manz et al. 2000, Böhm et al. 2001). Magnino et al. (1999) treated the siliceous sponge, *Theonella swinhoei*, with HF prior to embedding in Technovit 8100.

Resins originally designed for transmission electron microscopy also have been used for light microscopy. Calcified biofilms were stained *en bloc* prior to embedding with either lipophilic dyes after dehydration or water soluble dyes on hydrated samples, and cut with a circular saw using a diamond knife (Reitner 1993, Arp 1999). Bhattacharyya et al. (1999) described a similar method for undecalcified rat tibia. Embedding in LR White combined with *en bloc* staining has been applied successfully on sponges with a calcareous basal skeleton (Wörheide 1997) and on siliceous sponges (Reitner 1992, 1993, Hoffmann et al. 2003).

An easy method for obtaining sections without HF treatment is simply to cut the fixed, unembedded sponges by hand with a sharp scalpel into < 1 mm sections and let them air dry (Hooper 2000, Soest et al. 2000). Sections obtained in this way are good for examination of skeletal architecture for taxonomic determination, but tissue structures are destroyed. Margot et al. (2002), however, used this method for FISH on Mediterranean axinellid demosponges.

We tested different methods for embedding, cutting and staining using a wide range of sponges from the Norwegian, Antarctic and Mediterranean Seas. The aim of the study reported here was to evaluate methods that allow preparation of tissue sections applicable for histological investigation and FISH of siliceous sponges without removing the spicules. Although some of the described embedding techniques may also be used for electron microscopy, we focus on application for light and fluorescence microscopy.

Materials and methods

Sampling and fixation

The specimens used for this report were collected during many years from different localities. Species from the Sula Ridge region, Norwegian Sea (64° 05′ N; 08° 05′ E), were sampled in May, 1999 on the Norwegian research vessel *Johan Hjort* using a triangular dredge, in July/August, 1999 on the German research vessel *Poseidon* and the manned submersible *Jago*. Sponges from the Korsfjord near the city of Bergen, Norwegian west coast (60° 09′ 12″ N; 05° 08′ 52″ E) were sampled in August and September, 2000, and in October, 2001 on the Norwegian research vessels *Hans Brattström* and *Aurelia* using a triangular dredge. Sponges from Sula Ridge were growing in association with living and decaying coral reefs of *Lophelia pertusa*, on drop stones or in soft bottom sediments near the reefs at 200–300 m depth (Freiwald et al. 2002). In Korsfjord, the sponges were growing on a hard bottom slope 100–300 m deep.

During the deep sea expedition ANDEEP II, 28.02.–1.04.2002, sponges were collected from the Antarctic Weddell Sea by Agassiz trawl and epibenthic sledge (Janussen, in press). In the Mediterranean deep sea, we recorded large populations of very small sponges, which have been collected in the course of several expeditions. During the 1993 and 1998 expeditions with the research vessel *Meteor* (Me25 and Me40), many Mediterranean deep sea stations in the Sporades Basin, Sea of Crete and throughout the Levant Basin were sampled by trawl, epibenthic sledge and box corer (Hieke et al. 1994, 1999). As part of a deep sea monitoring expedition off the coast of Israel, a few trawls tested a new sampling technique in the deep sea and retrieved very small sponges (< 1 cm).

Species used for this report are listed in Table 1. All specimen were fixed in 2% formaldehyde + 0.04% glutaraldehyde in filter-sterilized sea water, in 4% formaldehyde in sterilized sea water, or 4% formaldehyde in phosphate buffered saline (PBS) solution prepared with distilled water. Fixed samples subsequently were dehydrated in an ethanol series (15, 30, and 50% EtOH in artificial sea water or PBS), and stored in 70% ethanol.

Table 1. Sponge species used in this study

Species	Sampling site
Hexactinellida	
Malacosaccus pendunculatus TOPSENT 1910	ANT
Caulophacus antarcticus SCHULZE & KIRKPATRICK 1910	ANT
Sympagella n. sp.	SR
Demospongiae	
Homoscleromorpha	
Plakortis simplex SCHULZE 1880	SR
Tetractinomorpha	
Dragmastra normani SOLLAS 1880	SR, KO
Geodia barretti BOWERBANK 1858	SR, KO
Geodia macandrewii BOWERBANK 1858	SR, KO
Isops phlegraei SOLLAS 1880	SR, KO
Thenea muricata BOWERBANK, 1858	SR, KO
Thenea n. sp.	ME
Tentorium semisuberites SCHMIDT 1870	SR, KO
Tentorium n. sp.	ME
Suberites caminatus RIDLEY & DENDY 1886	ANT
Suberites microstomus RIDLEY & DENDY 1886	ANT
Rhizaxinella n. sp.	ME
Ceractinomorpha	
Forcepia forcipis BOWERBANK 1866	SR, KO
Mycale lingua BOWERBANK 1866	SR, KO
Axinella infundibuliformis LINNAEUS 1759	SR, KO
Phakellia ventilabrum PALLAS 1766	SR, KO
Phakellia robusta BOWERBANK 1866	KO
Oceanapia robusta BOWERBANK 1866	SR
Chondrocladia antarctica RIDLEY & DENDY 1886	ANT
Chondrocladia n. sp.	ANT
Latrunculia bocagei RIDLEY & DENDY 1886	ANT

SR, Sula Ridge; KO, Korsfjord; ANT, Weddell Sea (Antarctic); ME, Mediterranean Sea

Embedding, cutting and staining

We developed protocols for 1–2 cm^3 tissue blocks. For larger samples, times for dehydration, staining, infiltration and hardening must be longer. For smaller samples, the times should be the same, or after experimenting, may be a little shorter. Embedding techniques evaluated in our study are listed in Table 2.

Paraffin

Tissue blocks were embedded in paraffin as described earlier (Romeis 1989). To remove air, blocks soaked with paraffin were placed in a paraffin bath in a desiccator for a few minutes at 60° C. Paraffin blocks were cooled on a −5° C plate prior to cutting. Paraffin sections 3–30 µm thick were cut using a rotary microtome HM 340 E (Microm, Walldorf, Germany) with single-use blades. Thicker sections can be made by using the trimming option on the rotary microtome and cutting each section as if trimming the block. Particularly for study of the skeletal architecture, which is important for taxonomy, 30–50 µm sections are required. The slices were mounted on silanized glass slides, dewaxed by xylene treatment and dried for at least 1 h at 60° C prior to staining or FISH. Staining techniques according to Goldner and Giemsa (Romeis 1989) gave good results. Sections were mounted with Biomount or Entellan balsam (Plano W. Plannet, Wetzlar, Germany; Merck, Darmstadt, Germany) and viewed under a light microscope. For 4′,6-diamidino-2-phenylindole (DAPI) staining, sections were covered with a 1–3 µg/ml DAPI solution for 5–10 min in the dark, rinsed with distilled water, mounted with Citifluor antifading agent (Plano W. Plannet), and viewed under an epifluorescence microscope.

Cryotechniques

Tissue blocks were embedded in Cryomatrix (Shandon Inc, Pittsburgh, PA) as described by the manufacturer. Frozen blocks were stored at −20° C, sectioned with a cryomicrotome (Mikrom) at −20° C, and placed on coated glass slides (Cryo-Frost, Fisher). Sections were air dried at room temperature for 20 min prior to staining and FISH.

MMA resins: Technovit, Histocryl

Tissue blocks were embedded in Technovit 7100 (Plano W. Plannet) as described earlier (Manz et al. 2000). Sections 5–10 µm thick were cut using a rotary microtome as described above. Sections 20 µm and thicker were cut using a circular saw (Leica 1600) with diamond knife.

For embedding with Histocryl (Plano W. Plannet), dehydrated tissue blocks were infiltrated with Histocryl and benzoyl peroxide as described by the manufacturer. Samples were transferred into the polymerization solution (10 ml infiltration solution + 15–30 µl accelerator) and immediately cooled with running tab water for 5 min. Samples

Table 2. Overview of different embedding techniques evaluated in this study

	Paraffin	Cryomatrix	Histocryl	Technovit 7100	LR White
Cutting	Rotary microtome	Cryo-rotary microtome	Rotary microtome circular saw	Rotary microtome circular saw	Circular saw
Mounting	Silanized slides, heat	Not necessary	Heat	Glue, heat	Mounting medium
Removal of embedding medium	Xylol	Thawing	Not necessary	Not necessary	

remained in the water bath for a few hours until hardening was complete. Sections 5–10 µm thick were cut using a rotary microtome, and thicker sections were cut using a circular saw with diamond knife as described above.

Sections were fixed on glass slides by heat (60° C for at least 1 h) for subsequent staining and FISH.

LR White resin

If staining is desired, tissue blocks must be stained before embedding in LR White. We developed protocols for toluidine blue O (C.I. 52040), Calcein, and DAPI as follows. For staining with toluidine blue O and Calcein, tissue samples were dehydrated in an ascending EtOH series. From 99% EtOH, samples were placed in the staining solution overnight (toluidine blue: 1 g/100 ml EtOH; Calcein: saturated solution in EtOH). Samples were washed 3–4 times in EtOH before embedding. For DAPI staining, tissue blocks were placed in a 10 µg/ml DAPI in 90% EtOH staining solution overnight. Samples were washed in 99% EtOH, and dehydrated in 99% EtOH for a few hours. Tissue blocks for all staining techniques were infiltrated with 1:2, then 2:1 LR White:EtOH for at least 18 h, then embedded in LR White and hardened for 20 h at 60° C. Because all dyes described are soluble in both EtOH and LR White, infiltration solutions should contain a minor amount of the used dye. To reduce unintentional extraction of dye during the embedding steps, it is helpful to use EtOH from the washing steps to prepare infiltration solutions. Tissue sections from 20 µm to 1 mm were cut using a circular saw with a diamond knife. Sections were mounted on glass slides with Biomount balsam.

FISH and image processing

Fluorescence in situ hybridization (FISH) and wide field deconvolution epifluorescence microscopy were performed on sponge tissue sections as described earlier (Manz et al. 2000). Samples were viewed under a Zeiss axioplan microscope (Zeiss, Oberkochen, Germany). Micrographs were taken using a CCD Camera and processed with MetaMorph.

Results and discussion

A comparison of the methods described concerning embedding, section quality and staining techniques is shown in Table 3. The embedding process for the cryotechnique is quick, easy and reliable. The other methods take longer and are more complex with many pitfalls, which may cause unexpected trouble. Most of these difficulties, however, can be overcome by some training and standardization of the methods.

The best sponge tissue sections including siliceous spicules are obtained with LR White blocks cut on a circular saw; valuable information about skeleton structure and tissue-skeleton arrangement can be achieved using this method. Sections 20–1000 µm are cut, and can be ground to approximately 5 µm. Even ultramicrotome sections for electron microscopy can be obtained from spiculated tissue blocks (Uriz et al. 2000), but the knife is easily damaged if spicules are thick and numerous. Prior DAPI staining allows visualization of both different sponge cell types and associated bacteria (Fig. 1).

The calcium binding fluorochrome Calcein is commonly used in growth-since labeling experiments on calcareous skeletons (Rahn 1976a,b, Reitner and Gautret 1996, Wörheide 1997). Applied to calcified biofilms, Calcein greatly enhances the contrast of, for example, small nonphototrophic bacteria, and labels the surfaces of carbonate crystals (Reitner 1993, Reitner et al. 1995, Arp 1999). In siliceous sponges, tissue contrast is enhanced and different cell types and tissue structures are clearly visible (Fig. 2a). Staining with toluidine blue O *en bloc* cannot be recommended for sponges with dense tissue and numerous sponge-associated bacteria, because these

Table 3. Comparison of embedding methods for siliceous sponges without spicule removal

	Paraffin	Cryomatrix	Histocryl	Technovit 7100	LR White
Embedding operation	+	++	+	+	+
Section quality	+	0	++/++*	+/++*	++
Minimum section thickness (μm)	3	?	5/20*	5–10/20*	20
FISH on section	++	++	++	++	–
DAPI staining on section	++	++	++	++	–
Goldner, Giemsa staining on section	++	?	0	0	–

++ very good; + good; 0 poor; – not possible;? no data; *cut with circular saw

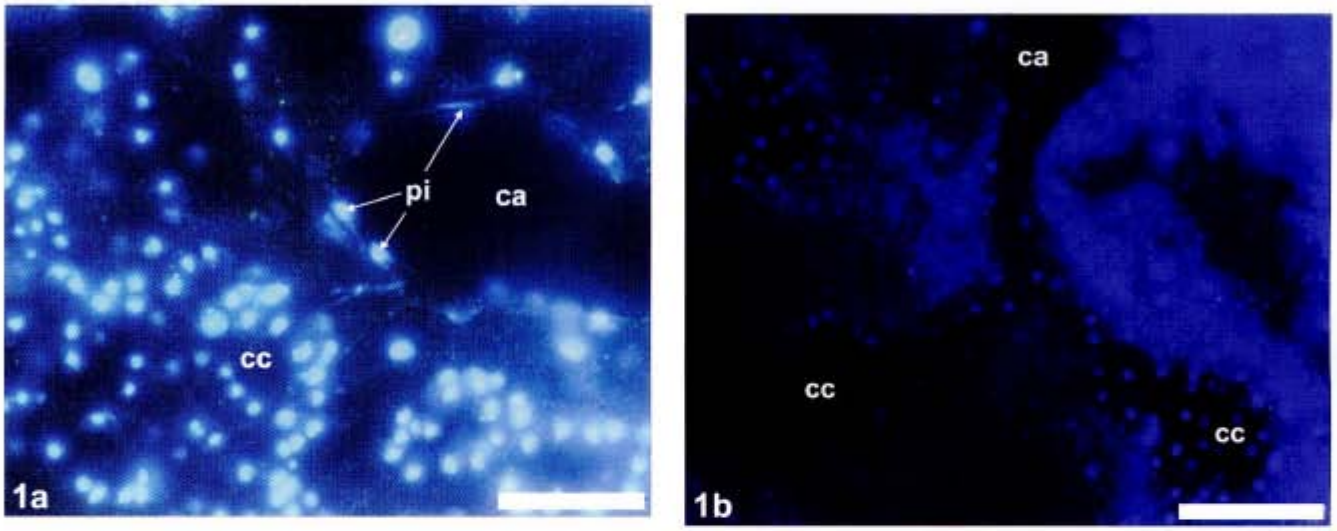

Fig. 1. DAPI stained 20 μm LR White sections of *Phakellia ventilabrum* (a) and *Plakortis simplex* (b). Choanocyte chambers (cc), canals (ca), and endopinacocytes lining canal walls (pi) are visible. This technique clearly shows differences in tissue structure and density of associated microorganisms (tiny dots in the tissue) in these two sponge species. Bars = 30 μm.

tissues are easily overstained. The protocol can be adjusted to the species of interest by inserting more and longer washing steps or by dilution of the staining solution. High contrast light microscopy images can be obtained using this method (Fig. 2b). Block staining, however, increases the amount of tissue sample needed whenever several staining techniques are to be applied on the same sponge. Material availability may be a limiting factor, especially when dealing with very small (< 10 mm) sponges such as juveniles and some deep sea species. Owing to the hydrophobic character and high density of the resin, FISH on LR White sections is impossible. Oligonucleotide probes used for FISH are water soluble and will not penetrate the resin. The same is true for DAPI and histological stains in aqueous solution.

Using a rotary microtome with sharp steel knife, the best section quality was obtained with Histocryl. This was true also for species and tissue parts containing many spicules, e.g., the cortex of *Geodia* species. Sections down to 5 μm were suitable for FISH (Fig. 3). Sections of varying thickness and quality were obtained from Technovit blocks, but parts usable for FISH could be found on most sections. Sections of better quality were obtained using a circular saw. These sections were relatively thick (> 20 μm), which makes interpretation of FISH difficult, but the thickness is advantageous for studying skeletal architecture. For observations of the skeleton, sections 30–500 μm are necessary. Because of the incompatibility of MMA resins with EtOH, only aqueous stains can be used for Histocryl and Technovit sections. Unfortunately, we observed incomplete and irregular staining with Goldner and Giemsa in both MMA resins. Gerrits (1992) described good results for different histological staining of soft tissue embedded in Technovit 7100. The failure of our attempts may be due to the rough and uneven surfaces of sections of spiculated tissue. In Histocryl sections, we sometimes observed massive stain accumulations between the spicules, whereas the surrounding tissue remained largely unstained. For paraffin sections, we

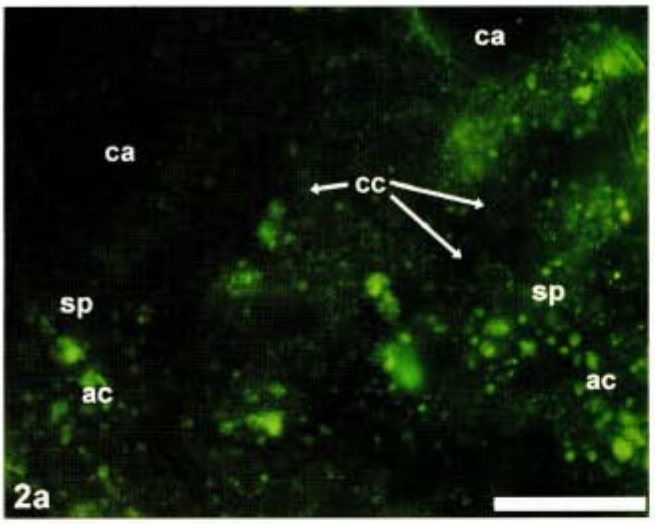

Fig. 2. (a) Fluorochrome image (Calcein) and (b) light microscopy image (toluidine blue O) of *Phakellia ventilabrum* in 100 µm LR White sections. Bundles of spicules (sp) typical for this species with accompanying cells (ac) are visible. Note choanocyte chambers (cc) and canals (ca) in the choanosome and different cell types in the ectosome (ec) at the sponge surface. Bars = 100 µm.

observed that the time needed for staining correlated negatively with section thickness. It seems that the sponge tissue in thick sections binds the stain more readily than it does in thin sections.

Paraffin sections without prior dissolution of the siliceous skeleton were of poor quality in tissue parts that contained numerous thick or long spicules, e.g., the cortex of *Geodia* or the tissue of some Hexactinellida. Results were better for tissue containing few or small spicules (Fig. 4), and although tissue sections always were partly torn, usable areas for FISH and histological staining could be found on most sections. Careful cooling of the paraffin blocks prior to cutting and the use of a new blade for each cutting event enhanced

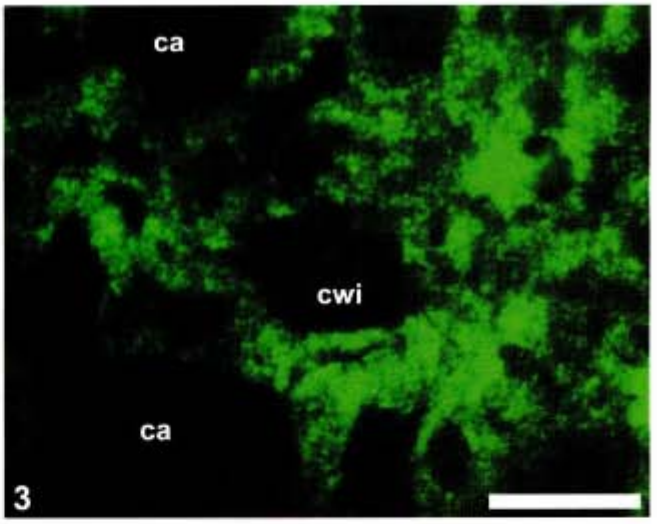

Fig. 3. Fluorescence in situ hybridization (FISH) on a 5 µm Histocryl section of *Geodia barretti*. Although this section was cut from a highly spiculated tissue part directly beneath the cortex, tissue structures as canals (ca) and a large sponge cell (cell with inclusions, cwi) are clearly visible. The oligonucleotide probe EUB338 (Amann et al. 1990), coding for all eubacteria, shows high density of associated bacteria in the sponge tissue. Bar = 30 µm.

section quality. Paraffin sections with the spicules in situ are preferable, especially for very small sponges where the sample material is limited. The extremely small spicules cause almost no disruption of the tissue, so entire sponges can be embedded, easily cut, and subsequently stained with minimal loss of material (Fig. 5).

Very poor sections are obtained from cryoblocks. The sponge tissue is entirely torn, and no tissue structures are visible. This probably is due to differences in toughness between the spicules and the embedded tissue. Histological staining of these sections was not attempted, but application of FISH probes and DAPI staining gave clear signals. Information gained from these sections, however, was comparable to that from homogenized sponge tissue. We assume that the quality of cryosections could be improved by varying cutting temperature and more practice.

For all techniques described here using rotary microtomes, section quality was never as good as that obtained with prior acid treatment. Although some histological staining could be applied successfully on sponge sections with siliceous spicules, we recommend spicule removal for histological investigations where sections < 20 µm are needed, e.g., for studying fine tissue structures. Methods for embedding and cutting siliceous sponges should be chosen according to the aim of investigation and

Fig. 4. Tentacle of *Chondrocladia* sp. from the Antarctic Weddell Sea, approximately 4970 m deep, embedded in paraffin, sectioned at 30 μm and stained according to Goldner (Romeis 1989). This section shows the central core with megascleres (styles) embedded in spongin fibers and the surrounding area packed with microscleres (isochelae). Bar = 250 μm

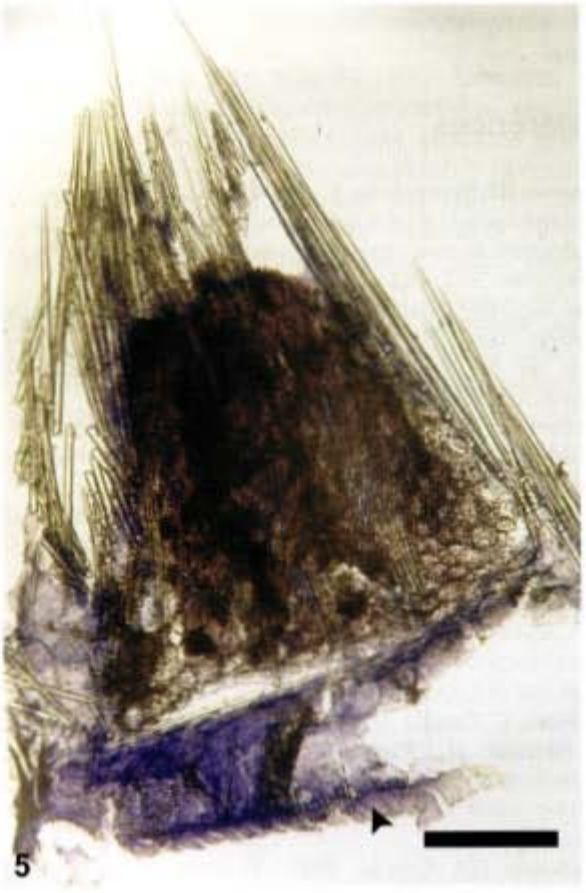

Fig. 5. Axial cut of *Tentorium* sp. from the Mediterranean Sea, Eastern Levant Basin, approximately 1200 m deep, embedded in paraffin sectioned at 30 μm, and stained according to Goldner (Romeis 1989). The section through the entire specimen shows the spicule orientation and the organic basal plate of collagen into which some of the vertical spicules protrude (arrow). Bar = 250 μm

the properties of the examined species; there is no standard method available that fits all needs.

For an overview of tissue and skeleton arrangement we recommend LR White embedding combined with *en bloc* staining and cutting with a circular saw. For very small sponges, we recommend paraffin embedding of a central longitudinal cut through the entire sponge without prior acid treatment, and cutting or trimming with rotary microtome to obtain different section thicknesses.

For FISH on siliceous sponges, we recommend Histocryl embedding for species or tissue parts with high spicule content, and paraffin embedding if the spicule content is low. Sections should be cut with a rotary microtome.

For detailed tissue examination, we recommend classical histological methods, i.e., spicule removal by acid prior to embedding, cutting and staining.

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