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An embedding, polishing and etching procedure for examining the 3-D structure of diatoms with SEM

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Abstract – A technique for obtaining cross-sections of diatom frustules which retains the integrity of the frustule without recourse to an ultra-microtome and allows their examination under SEM is described. The technique employs the polishing and etching of resin-embedded material and can be applied to cleaned diatoms from field samples or cultures. Examples are shown of the types of frustule ultrastructure information that can be obtained using this technique.

Diatoms / Embedding / Etching / Polishing / Scanning electron microscopy

Résumé – Il est décrit une technique pour obtenir des sections transversales de frustules diatomiques qui conserve l'intégrité de la frustule sans l'emploi d'une microtome et qui permet ensuite de les examiner au microscope électronique à balayage. Cette technique se base sur le polissage et « etching » de matériel en résine. Elle est appropriée pour les diatomées prélevées sur le terrain ou élevées en culture après nettoyage. Quelques exemples sont donnés pour illustrer quel genre de renseignements sur l'ultrastructure des frustules on peut obtenir grâce à cette technique.

Diatomées / Etching / Inclusion / Microscopie électronique à balayage / Polissage

INTRODUCTION

The study of diatom wall structure was revolutionised by the introduction of scanning electron microscopy (SEM) (e.g. Ross & Sims, 1971, 1974; Hasle, 1972; Cox, 1975; Crawford, 1975; Mann, 1981; Round *et al.*, 1990; Cox, 2003) and its use is now virtually obligatory for taxonomic and systematic studies of the group. While the siliceous nature of diatom valves lends them to preparation and study with SEM, the necessary cleaning and mounting procedures often result in

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dissociation of the frustule components. Thus it is sometimes difficult to reconstruct the relationships between cingulum elements and between them and the valves, while understanding cross-sectional structure relies on fortuitous breaks in frustule elements.

Transmission electron microscopy has provided information on the valve – cingulum junction, raphe slit and wall cross-sections, but its techniques require expertise, are time-consuming, and siliceous elements are prone to fracturing during ultramicrotomy. It is therefore rarely used to study 3D relationships of the wall components. In addition, most TEM diatom work relies on cultured material because precise identification from fixed sections would be difficult (if not impossible) and field material often includes particulate matter which would potentially damage diamond knives.

Massé *et al.* (2001) described a technique for obtaining cross-sections of raphe and intact frustule structures, combining fixation, sectioning, removal of organic matter and SEM. They embedded glutaraldehyde fixed samples in Spurr's resin and cut 250-500nm sections with a diamond knife, which were placed on a glass coverslip and heated in a muffle furnace to remove all the resin. This procedure produced some elegant images of valve and cingulum sections, but the technique does not seem to have been used elsewhere. Although they describe it as straightforward, the sectioning procedures require expertise and access to an ultramicrotome, both of which are not invariably available. Earlier, Pocock & Cox (1982) had developed an embedding and etching technique that could be applied to field and cultured material, showing its value for examining complex cingula. They embedded samples in resin, used an ultramicrotome to cut the face of the resin block, and then etched away the resin to expose sections of diatom before attaching the resin block to an aluminium stub, coating it with gold-palladium and examining with SEM.

The method that we describe in this paper is a development of the Pocock & Cox (1982) approach, but by incorporating a polishing stage, eliminates the need for an ultramicrotome and sectioning expertise, and also permits the same sample to be repeatedly studied. Our alternative method quickly achieves cross-sections of embedded diatoms, and with appropriate etching, results in three-dimensional views of the specimens, revealing internal wall detail and cingulum construction, as well as detail of internal and external surfaces.

METHODS

Diatom preparation – Field or cultured diatoms were cleaned in the usual way by gently heating in a beaker with 50% nitric acid, 50% distilled water solution until 3/4 of the solution had evaporated. The remaining solution was then diluted with distilled water and allowed to stand overnight so that the diatoms settle at the bottom of the beaker. Next day, most of the solution was decanted off, replaced with distilled water and left overnight. This step was repeated several times until the solution was neutral. (Subsamples of cleaned material are made into permanent mounts in the normal manner.)

Embedding – Cleaned diatoms were pipetted into a 1.5ml Eppendorf tube, if possible avoiding contaminants such as sand to make the polishing stage easier, and the Eppendorf tube was spun at 14,300 revs/min for 10 minutes. Water was

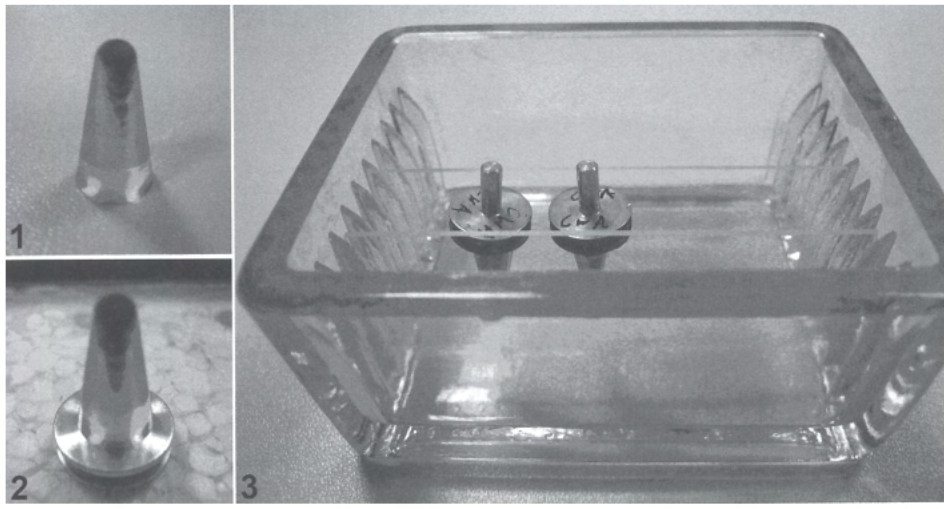
pipetted off and replaced with 20% acetone/water solution, shaken/flicked to resuspend the diatoms, and spun again for another 10 minutes. This procedure was repeated through a graded acetone/water series (30%, 40%, 50%, 70%, 80%, 90% and 95%) up to 100% acetone. The 100% acetone wash was repeated three times.

After the final spin, half the acetone was removed and replaced with TAAB 810 resin. (Spurr's resin was not used because the samples were spun in a centrifuge in an open laboratory.) Shaking the mixture ensured diatoms mixing in the Eppendorf tube, and the tube was then rotated for 2 hours to enhance mixing. The samples were spun for 15 minutes and as much of the resin/acetone solution as possible was removed and replaced with 100% resin. The mixture was shaken and then rotated for 2 hours, before spinning for 15 minutes. Samples were heated in a water bath at 75°C overnight (16 hours) to cure.

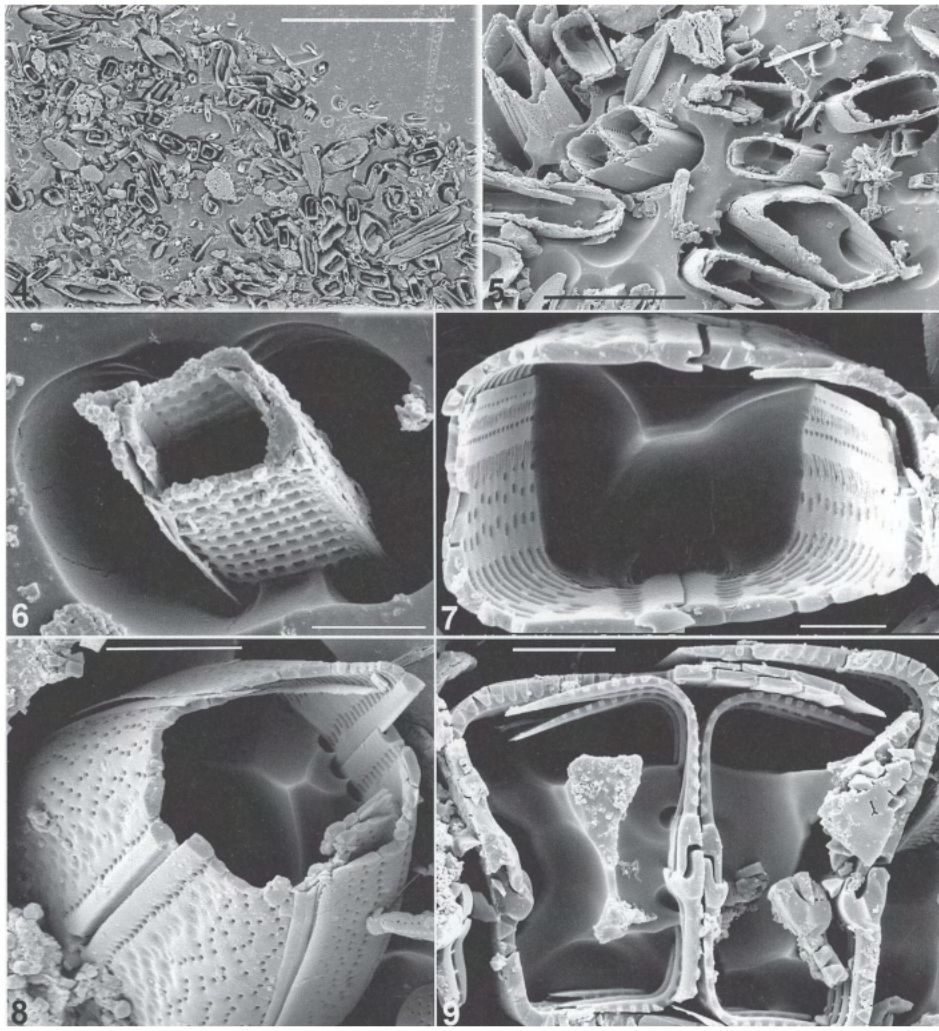
The cured samples were removed from the Eppendorf tubes (Fig. 1) and inscribed with their identity. Excess resin (above the sample in the Eppendorf) was removed with a diamond saw to ensure the samples would fit easily into the SEM. Final samples were 10-15mm in height (Fig. 2) and were attached to aluminium stubs using Araldite™ Precision.

Polishing – The tips of the samples were roughly polished by hand using a 2500 grit polishing wheel lubricated with distilled water. Only a very small layer was removed, average contact time being around 2 seconds per sample. After washing in water, the samples were polished by hand using 0.3 micron alpha alumina on a felt wheel lubricated with distilled water, for an average contact time of around 3 minutes. Samples were then placed upside down in a rectangular glass slide holder, supported by pairs of glass slides (Fig. 3). The slide holder was filled with methanol and placed in an ultrasonic bath for 10 minutes to wash off any resin fragments and alumina from the surface of the samples. After cleaning, the samples were removed and air dried before being replaced in the glass slide holder as before.

Etching – A saturated solution of sodium methoxide and dry methanol was used for the etching solution, stored in a glass bottle with a glass lid. Sodium methoxide

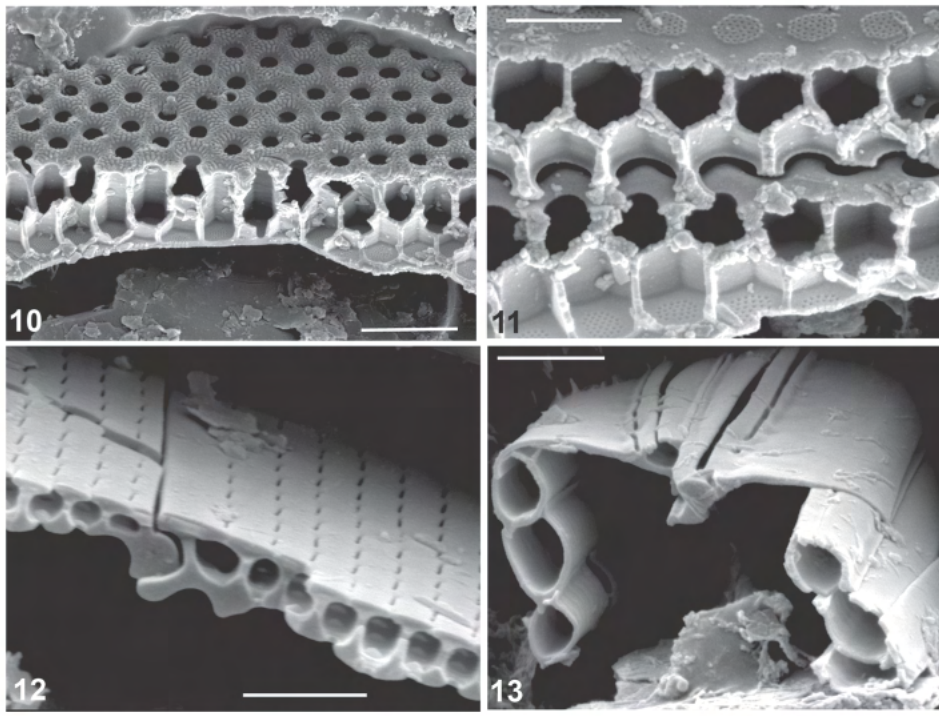


Figs 1-3. Mounting and etching embedded material. **1.** Cured resin with diatoms removed from Eppendorf tube. **2.** Resin attached to SEM stub. **3.** Slide holder with inverted resin blocks suspended across pairs of microscope slides.



Figs 4-9. SEM micrographs of polished and etched resin blocks containing diatoms. **4.** Low magnification view of polished and etched block face, showing junction between side of block and polished face with diatoms projecting after etching. **5.** General view showing diatoms projecting at different angles from the resin. **6.** Cross section through a *Nitzschia* frustule, showing preferential etching around and within the diatom. **7.** Cross section through a frustule of *Anomoeoneis sphaerophora* showing relationship between the epi- and hypothecae and the lock and key nature of the raphe slit. **8.** Oblique section towards a cell apex of *A. sphaerophora* showing both external and internal valve detail. **9.** Cross section of dividing cell of *A. sphaerophora* showing forming sibling valves within the parent frustule. Scale bars: 4 = 100 µm, 5 = 20 µm, 6, 7 = 2 µm and 8, 9 = 1 µm.

is very caustic and hence was only handled in a fume cupboard with gloves (preferably thick washing-up gloves). 25 ml of the saturated solution was diluted with 25 ml of dry methanol and poured into the glass slide holder so that the polished diatom surfaces were completely covered with etching solution to a depth



Figs 10-13. SEM micrographs showing valve structure. **10.** Oblique section through valve of *Thalassiosira* showing loculate structure with internal vela. **11.** Oblique section through sibling valves of *Thalassiosira* showing appressed external faces and both faces of internal vela. **12.** Cross section through *Gyrosigma* valve, showing loculate pores and oblique internal opening of raphe slit. **13.** Cross section through valve and cingulum of *Proschkinia complanatula*, showing conopeum adjacent to raphe slit and folded girdle bands. Scale bars: 10 = 2 µm, 11-13 = 1 µm.

of around 3 mm. The samples remained in the etching solution in a fume cupboard for 30 minutes. They were then placed in a beaker containing methanol and washed. This step was repeated with fresh methanol after which the samples were washed with a large amount of water.

The samples were again placed upside-down in the glass slide holder as before, placed in methanol and ultrasonicated for 3 minutes to remove any debris from the surfaces. Three washes of 1 minute each in fresh alcohol ensured there was no loose silica on the samples. Samples were air dried and coated with 20nm of gold/palladium and examined in a Philips XL30 field emission SEM at magnifications of 40 × to 35,000 ×.

RESULTS AND DISCUSSION

The use of polishing allows samples with large amounts of detritus to be handled and viewed without difficulty (Fig. 4) and diatoms are seen in a variety of orientations within the resin (Fig. 5). The presence of a large number of

diatoms in the sample increases the likelihood of obtaining the required orientation without elaborate manipulation, however, if the required section/ orientation is not present, it is a relatively simple task to repeat the finest polishing stage for 2 minutes, etch, clean and coat the sample again and re-view. Nevertheless, it should be remembered that, once re-polished, all information from the previous section is lost. Thus, if permanent sections are required, it is advisable to embed several subsamples in different resin plugs for the initial processing.

During etching, the amount of resin removed varied across the sample face. After half an hour, only a few microns of resin were removed from the sample as a whole, however, the etching was far greater adjacent to silica surfaces (Figs 5, 6). Thus, up to 10-20 microns of resin were removed around the diatoms, so it was often possible to see external and internal detail simultaneously making identification relatively straightforward. If a small amount of the cleaned material has been made up as a permanent mount or used for conventional SEM, this can be used to check the identity of diatoms in the etched sample.

Figs 5-13 illustrate some of the structural information that can be obtained using this method. Cross-sections of intact frustules reveal details of raphe structure (Figs 7-9), pore chambering (Figs 10-12) and the relationship between valves and cingulum (Figs 7-9, 13). In several cases, diatoms were seen with daughter valves inside the parent frustule (Figs 5, 9, 11), while oblique sections revealed the close match between sibling valves (Fig. 11). It was particularly useful for interpreting complex girdle structures and conopea, as in *Proschkinia* (Fig. 13).

CONCLUSIONS

We consider that there are several advantages to this method:

1. Samples with large amounts of detritus, such as sand, can be polished and viewed without difficulty or additional processing steps. Thus field and cultured material can be studied in the same way, whereas techniques involving ultramicrotomes require the removal (or absence) of detritus to minimise damage to glass or diamond knives.
2. Both outer and inner diatom surfaces can often be viewed simultaneously along with raphe features.
3. Delicate girdle bands and developing valves are retained intact, allowing their relative position and orientation to be determined. (This assumes that the preliminary cleaning process has been relatively gentle and has not separated the frustule components.)
4. Samples can be easily re-polished, etched and coated many times to obtain additional views.
5. Etching is preferential around silica surfaces allowing several surfaces to be viewed simultaneously, aiding identification in mixed samples.

One potential disadvantage is the need for larger amounts of diatoms than for usual embedding and sectioning techniques. This would not usually be a problem for field samples, however greater care would be needed with smaller amounts of cultured samples to ensure that material was not lost in the first polish.

The technique would also probably benefit from including a final polishing step using 0.05 micron gamma alumina powder. This should result in cleaner polished surfaces with reduced fracturing. The polished samples should also be sonicated several times in methanol to ensure all small particles are removed from the polished surfaces.

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