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PREPARATION OF SAMPLES FOR SCANNING ELECTRON MICROSCOPY

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INTRODUCTION

Ciliate species cannot usually be identified solely by scanning electron microscopy because only a limited number of characters are revealed. However, SEM is useful for the beginner by allowing a three-dimensional view of the object and for the specialist in documenting details which are difficult to reveal with other methods.

PROTOCOL

1. Pour ciliates into Parducz's fixative and leave for about 30 minutes. Remarks: Concentrate and clean material as thoroughly as possible (see step 2). Ratio of sample: fixative should be at least 1:1, better, 1:2. Add some drops of 5n HCl if fixative becomes milky when the material is added. Parducz's fluid fixes most ciliates very well. However, the cirri of the hypotrichous ciliates usually disintegrate into their components, i.e. cilia. Hypotrichs should thus be fixed either in concentrated sublimate (dissolve 60 g $HgCl_2$ in 1 litre hot distilled water) or in a mixture composed of 4 parts concentrated sublimate and 1 part 2% osmium tetroxide.
2. Wash ciliates at least 5 times with 0.05 M Na-cacodylate buffer. Remarks: Ciliates in the buffer may be refrigerated for years. Washing must be done in a watch-glass and a micropipette should be used to remove bacteria and detritus. This cleaning of the material is essential but rather difficult and laborious, especially with small species ($\ll$ 100 μm) and field material; thus cultures and/or pre-cleaned material (see below) should be used. The cleaning is performed as follows: Ciliates settle at the bottom of the fixation tube after 30 minutes (cp. step 1). Remove as much supernatant as possible with a pipette (do not centrifuge!). Then transfer the material to a watch-glass and allow to settle for about 5 minutes (use fume hood). Quickly remove most of the fixative with a micropipette under the dissecting microscope. Now wash the ciliates with the buffer by several passages through a large-bore (diameter about 1 mm) pipette. Bacteria and detritus adhering to the ciliates are mechanically removed. Again allow to settle, but control sedimentation with the dissecting microscope; remove supernatant containing bacteria and detritus with a micropipette as soon as ciliates settle. This procedure must be repeated until the material is clean, i.e. the detritus is removed. Use fractionated sedimentation if the sample contains several species differing in size and/or mass.
   Field material: Larger species ($\gg$ 100 μm) are picked out with a micropipette and sprinkled into the fixative. Several hundred specimens must be collected because loss of material may be considerable during the following steps. Small species can be prepared by this method only if abundant material is available. Accumulation can often be achieved by the following simple method: leave a freshly collected sample containing ample mud to stand for some hours at room temperature. Due to oxygen depletion the ciliates usually move to the surface where they can be skimmed off with a teaspoon.
3. Transfer cleaned ciliated with a small drop of buffer to the preparation chamber (see figure below). Remarks: Place the drop on the bottom plankton net of the chamber which is weighed with washer 3. The net must be dry to avoid spreading of the drop to the chamber margin and the washer. Place the top plankton net carefully on the drop, i.e. on washer 3, using forceps. Weight top net with washer 2, close chamber with lid 1 and immediately transfer into 30% ethanol. The plankton net must have a mesh-size $\ll$ 12 μm and should be used only once. It should fit exactly into the chamber, which is best achieved using an appropriate punch.

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4. Dehydrate chamber with ciliates in ethanol series (30-50-70-90-100-100%) for 5 minutes each.
5. Transfer chamber with ciliates to an ethanol:amylacetate series (2:1, 1:1, 1:2) for 5 minutes each.
6. Transfer chamber with ciliates to pure amylacetate ($C_7H_{14}O_2$) for about 12 hours.
7. Dry chamber with ciliates in a critical-point drying apparatus. Remarks: We use CO_2 and change the amylacetate at least 10 times. Sometimes good results are achieved by transferring the ciliates directly from the last ethanol step to CO_2.
8. Open chamber and place ciliates on the prepared SEM stub. Assist with a mounted eye lash if ciliates do not fall from the net. Remarks: The dried ciliates usually form a lump at the bottom plankton net. This lump is carefully transferred (by holding the net over the stub and stripping off the ciliates with the eyelash) to the SEM stub where it is dispersed under the dissecting microscope with the mounted eyelash. The ciliates spread easily if drying was sufficient. Preparation of the SEM stub: We use commercial aluminum SEM stubs. To get a black, homogenous background the stub is covered with a very thin layer of Mixtion à Dorer Clarifiée (Lefranc & Bourgeois, Le Mans) 1.5-3 hours before use. This fluid is used by artists to attach gold foil and is available in ordinary stores. It dries slowly (whereby its surface becomes smooth) and adheres the ciliates to the stub. The mixture may be diluted with turpentine. Note that small species sink into the lacquer more easily than large ones and the mixture should thus be allowed a longer drying time (2-3 hours).
9. Harden the lacquer for a least 48 hours at room temperature. Remarks: This drying is not essential but prevents the development of cracks when the preparation is placed in the vacuum of the scanning electron microscope.
10. Sputter with gold.
11. Stock Solutions

    Parducz's fixative (prepare immediately before use)

    4 ml aqueous 2% osmium tetroxide (OsO_4)

    1 ml concentrated aqueous sublimate solution ($HgCl_2$; preparation see protargol impregnation, Foissner's method)

    0.05M Na-cacodylate buffer (can be stored for several months in the refrigerator; adjust to pH 7 with HCL)

    10.7 g Dimethylarsinacid-Na salt ($C_2H_6AsNaO_2$-$3H_2O$)

    add 1000 ml distilled water

    Amylacetate ($C_7H_{14}O_2$). Use commercial product.

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SPECIMEN PROCESSING CHAMBER

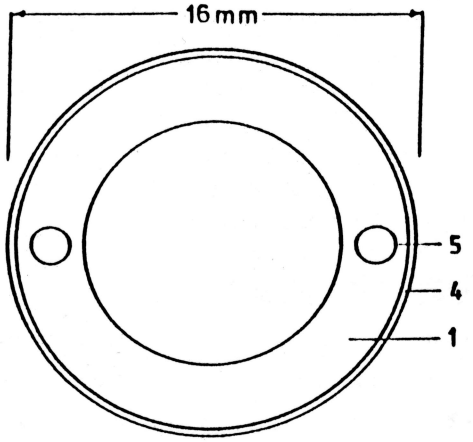

Fig. A

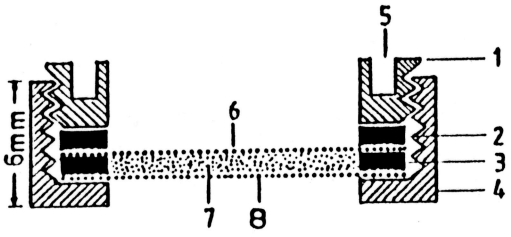

Fig. B

Legend: Figure A Top View of Chamber

Figure B Side View

Numerical Key

1	Threaded clamping ring (lid)
2-3	Washers to hold netting in place and act as spacers to create chamber
4	Base piece into which clamping ring threads
5	Holes partitially drilled into clamping ring (lid) to fasciliate tightening of the clamping ring. A stout forceps or spanner wrench can be used for tightening
6	Top net
7	Protozoa or other material to be processed
8	Lower Net

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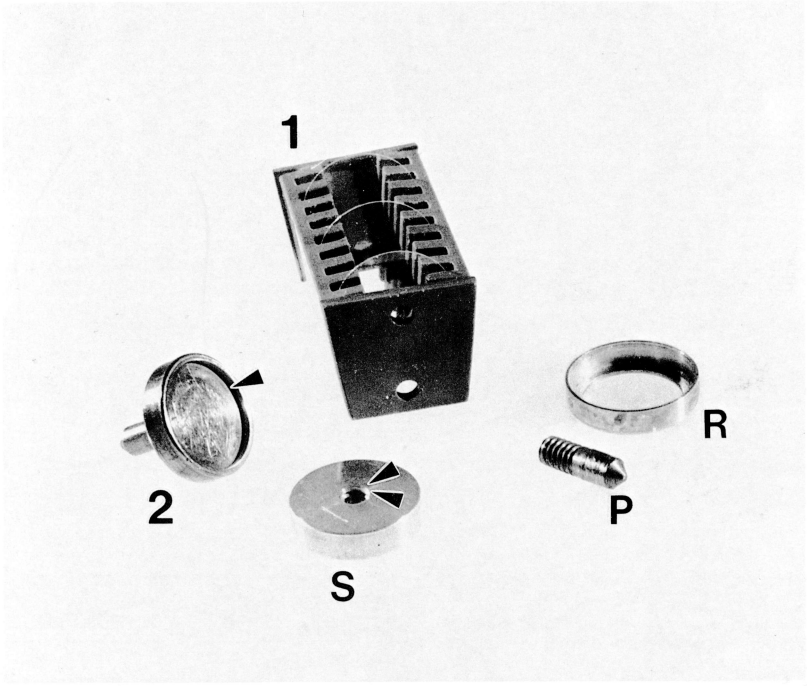

LEGENDS TO FIGURES 1 & 2

Fig. 1 Slotted carrier for processing coverslips for SEM. The carrier is 3 cm in length by 1.5 cm wide. There are 8 slots for coverslips. The end pieces are not square, but trapezoid, measuring 1.5 cm across the front (slotted side), 1.3 cm across the back and 1.5 cm deep. The end pieces being trapezoids hold the coverslips. Each slot is 1 mm thick and the carrier is made of plastic glued together with superglue. Two holes are bored on the end to facilitate handling with a forceps. The whole carrier can be loaded with coverslips and processed through the dehydration steps and finally placed in the critical point dryer. We generally use 15-25 ml beakers for our dehydrating series. Three coverslips are shown in the carrier.

Fig. 2 Specimen stub modified for SEM. The stub consists of three parts. The stub proper with a depression 1 mm deep (arrow), the peg (P) which is threaded so it can be removed during processing, and the clamping ring (R) which secures a filter to prevent specimens for floating out of the stub reservoir during processing. The clamping ring is made of aluminum and fits over the stub. We use nuclear pore filters (8 μ) or large pore size nets or screening depending on the size of the organisms we are working with. The cells are placed in the reservoir while in 100% ETOH and are never exposed to air. They are then transferred to the critical point drying apparatus. The peg (P) is screwed into the underside of the stub (S, double arrow) before or after sputter coating of specimen. The stub is standard size, 1.3 cm across the top and 1.3 cm from the base of the peg to top of stub.

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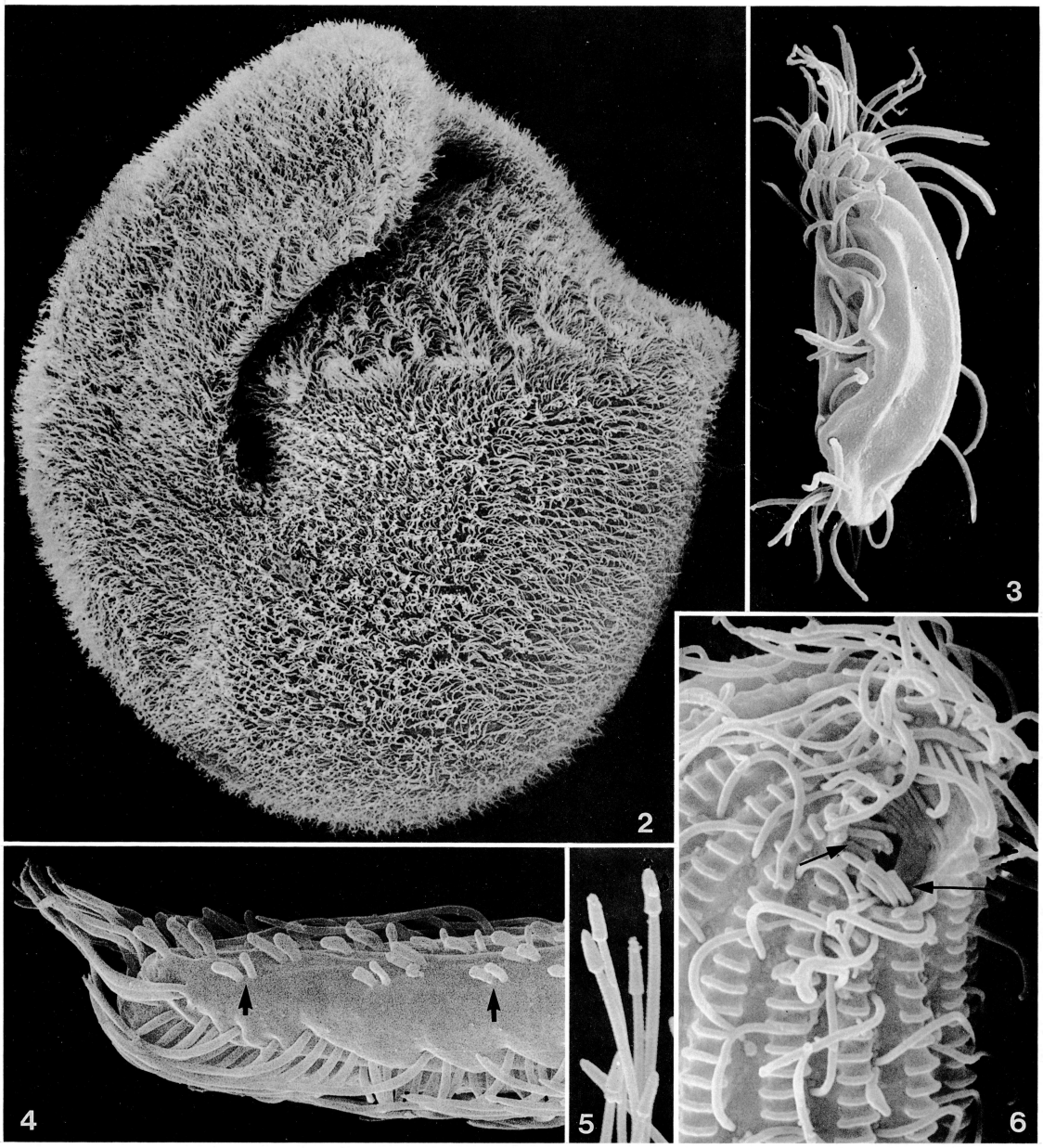

Fig. 2 - 6. Ciliates prepared with the SEM-protocol described. **2.** *Bresslauides discoides*, a huge colpodid ciliate from soil; right lateral view, length about 310 µm. **3.** *Drepanomonas exigua*, a distinctly furrowed microthoracid ciliate; ventral view, length about 30 µm. **4.** *Dileptus mucronatus*, a haptorid ciliate; left lateral view of distal portion of proboscis. Arrows mark the specialized cilia of the dorsal brush. **5.** Distal end of extruded trichocysts of *Paramecium*. **6.** *Cosmocolpoda naschbergeri*, a colpodid ciliate; ventro-lateral view showing strongly ribbed cortex and small oral organelles (arrows).