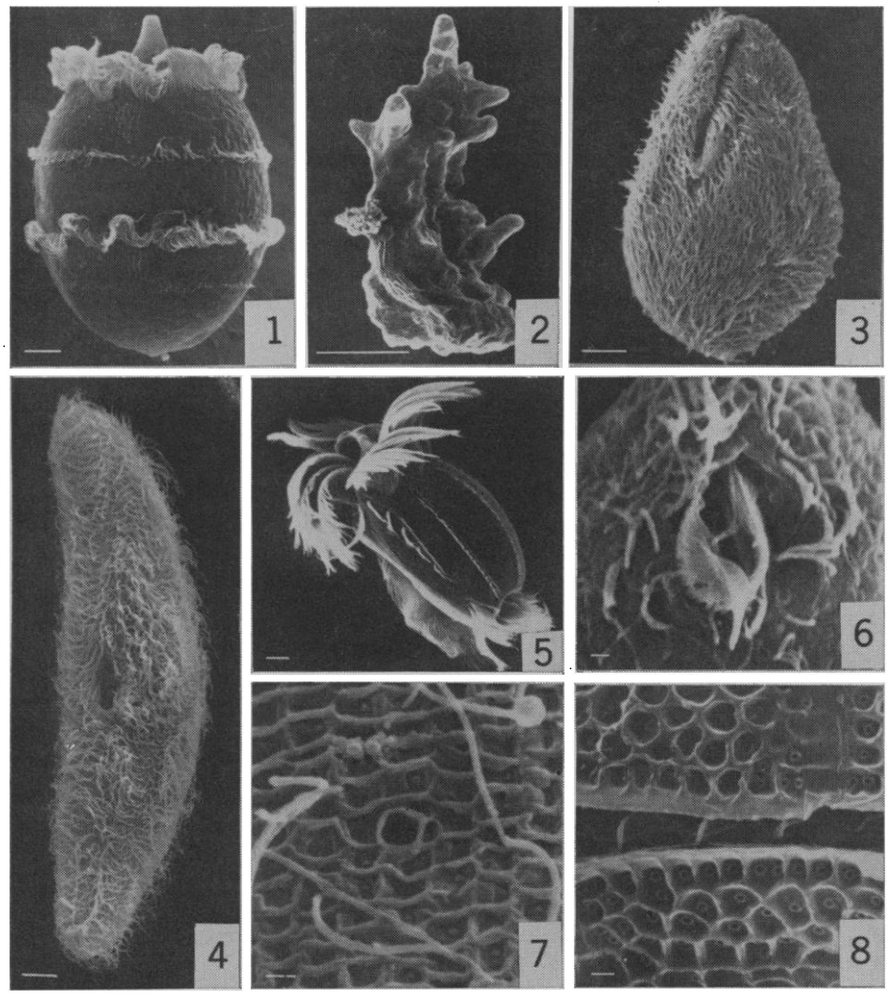
# Scanning Electron Microscopy of Fixed, Frozen, and Dried Protozoa

Abstract. *Selected protozoa were examined with a scanning electron microscope. The natural shape of the body and surface organelles were revealed. Chemical fixation and freeze-drying techniques described here permit a new approach to the study of protozoa.*

The scanning electron microscope has numerous applications in the physical sciences (*1*). In biology it has been used to study mineralized tissue (*2*), pollen grains (*3*), and the exoskeletal features of scleratized arthropods (*4*). Hayes *et al.* (*5*) discussed the biological applications of scanning electron microscopy. Most of these studies involve dehydrated tissue; in many instances, distortion occurs during the drying process so that the prepared specimen differs significantly from the original material. Pease and Hayes (*6*), and Barber and Boyde (*7*) have also reviewed the subject.




Figs. 1–8. Protozoa examined with the scanning electron microscope. Fig. 1. *Didinium nasutum*. Early stage in binary fission. Newly emergent cilia visible in anterior hemisphere between preexistent bands of cilia so fixed that metachronous wave pattern is preserved (scale, 10 $\mu$). Fig. 2. *Amoeba proteus*. Preservation of lobopods is shown (scale, 100 $\mu$). Fig. 3. *Nyctotherus ovalis*. Holotrichous ciliate from the hind gut of the roach *Blabarus discoidalis* (scale, 10 $\mu$). Fig. 4. *Paramecium multimicronucleatum*. From bacterial culture. Holotrichous ciliation (scale, 10 $\mu$). Fig. 5. *Uronychia* sp. Marine. Specialized hypotrichous ciliation, membranelles, and membranous oral cilia equally well preserved (scale, 10 $\mu$). Fig. 6. *Tetrahymena pyriformis* G1 strain. From axenic cultivation. Preservation of the oral undulating membrane and membranellar cilia that surround the oral cavity (scale, 10 $\mu$). Fig. 7. *Paramecium multimicronucleatum*. Denuded aboral surface with well-preserved pellicular surface features including prominent contractile vacuole pore (scale, 1 $\mu$). Fig. 8. *Ceratium hirudinella*. Detail of flagellum residing in the transverse girdle depression. Pores in the surface of exoskeleton are shown (scale, 1 $\mu$).

After considerable experimentation, a method was devised which produces lifelike results. Two steps are required in addition to the ordinary metal coating necessary for observation in the scanning electron microscope: (i) chemical fixation, and then (ii) freeze-dry sublimation. The technique has been applied to large and small, freshwater and marine, endocommensal and free-living species of protozoa.

The methods of preparation are as follows. Concentrate specimens by centrifugation; wash several times in filtered (pore diameter, 4 $\mu$) natural water or inorganic salt solution. With a clean, fresh micropipette, pick up specimens either singly or from a gently centrifuged concentrate, and redeposit them into a suitable volume (1000 times the volume of the concentrated organisms) of Parducz' fixative consisting of osmium tetroxide and aqueous mercuric chloride (*8*) for 1 to 5 minutes. Because this fixative preserves even the metachronous waves of cilia in forms such as *Didinium* (Fig. 1), it appears that fixation is instantaneous to surfaces exposed to it. This is particularly important in retaining a lifelike appearance of the specimen.

Next, specimens are washed in several changes of glass-distilled water, picked up with a fresh micropipette, and then released as microdrops from a height of 3 to 6 cm onto a thin aluminum container floating on liquid nitrogen (−180°C). The droplets are transferred to aluminum disks cooled with liquid nitrogen. These disks are mounted atop the specimen stubs for the microscope. The frozen specimens and disks can be temporarily stored in a disposable aluminum planchet (Sargent Co.) floated on liquid nitrogen. Thus, several samples may be prepared at one time.

The disks and specimens are transferred to the cooled (−60°C), thermoelectrically controlled cold stage of a Pearse tissue dryer (Edwards High Vacuum Ltd.). High vacuum ($5 \times 10^{-2}$ torr) is quickly restored with concomitant return of the cold stage to −60°C. Under vacuum and cold temperature, the specimens are sublimated to dryness for 5 to 12 hours. The time depends on the size of the initial microdrop and on the number of microdrop

samples. One can visually determine when the specimens have attained dryness. The stage is next warmed slowly (10 to 12 minutes) to room temperature, and the specimens are removed. The aluminum specimen holders are then attached with Duco cement to the specimen stubs of the scanning electron microscope.

The specimen stubs are placed in a vacuum evaporator, and a thin film of equal parts of gold and palladium is evaporated at a high vacuum ($5 \times 10^{-6}$ torr) onto the specimens rotated 50 to 100 times per minute so that the metal coats all parts of the specimens. Evaporation is stopped when sufficient metal has been deposited to produce a slight metallic sheen (probably about 100 Å thick). Specimens are then stored in vacuum or chemical desiccators until examination. After storage they can be used repeatedly in the scanning electron microscope.

Pseudopods of sarcodinids are well preserved in the freshwater *Amoeba proteus* (Fig. 2). The myriad patterns and arrays of cilia so diagnostic for ciliate systematics are preserved, and when coupled with the great depth of field and excellent resolution of the scanning electron microscope, they can be seen as never before. Holotrichous ciliary patterns (Fig. 3 of *Nyctotherus* and Fig. 4 of *Paramecium*) are readily observable as are the ciliary pectinelles of *Didinium* (cover photo and Fig. 1), the cirri of *Uronychia* (Fig. 5), and the adoral zone of membranelles of *Tetrahymena* (Fig. 6) and others (Figs. 3 and 5). Loss of cilia may result from the unequal distribution of chemical fixative into the microdrop precisely when the microdrop is dropped into the fixative, or from an extant physiological condition at the instant of fixation (Fig. 7). Transmission electron-microscope investigation of the feeding of *Didinium* (*8*) indicates that, at the time of ingestion of *Paramecium*, the cilia of the prey are lysed as it enters the cytopharynx of the predator (cover).

Newly emergent cilia appear in cells fixed at appropriate times during the division process. The stepwise formation of neocilia in dividing *Didinium* has been observed (Fig. 1). Between the metachronous waves of the parental-ciliary girdle in the upper hemisphere, emergent cilia of different lengths appear, none of which are as long as the cilia of similar parental structures. In the posterior hemisphere, a light band is observable—presumably the anlage of the posterior ciliary girdle.

Although the examples presented here are protozoa, the fixation-sublimation technique can be used with other biological materials of small volume. Because the scanning electron microscope provides information on spatial relations, the biologist is freed from the restriction imposed by microtome sections, and many problems can be solved by more direct, simpler means.

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### References and Notes

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## Automatic Identification and Measurement of Cells by Computer

Abstract. *A visual-input computer system was used to automatically locate and perform measurements with high accuracy on single cells from microscopic slides. The nuclear radius, irregularity, area, and density of normal and malignant cells were automatically measured. These studies agreed with hand measurements and showed differences between normal and malignant cervical cells on Papanicolaou smears.*

We have used visual-input computer systems to locate and perform quantitative measurements on cells on microscopic slides. The purpose of our study was to develop methods for the automatic machine differentiation of normal and malignant cells exfoliated from the exocervix and to serve as the basis for an automated screening procedure for Papanicolaou smears. As a result, pattern-recognition programming techniques were developed that may be of value in the automated analysis of other cytologic material.

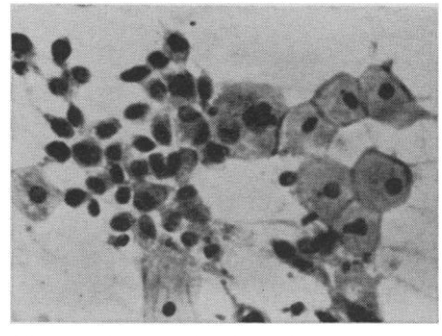

Fig. 1. Photograph of a microscopic field from a Papanicolaou smear of the cervix showing malignant cells (left) and normal squamous cells (right).

The cells were obtained by gently scraping the uterine cervix with a spatula. The material was placed on slides and fixed and stained according to the method of Papanicolaou (*1*). Both normal and malignant epithelial cells were present on the slides (Fig. 1). The smears were photographed at magnifications of ×25 to ×400 through a Zeiss microscope on Kodak Panatomic-X film. Although we have used film, the principles involved in direct examination of the slide are similar but require some equipment modification.

The photographs were scanned with a high-resolution programmable film reader (PFR-3, III) under the control