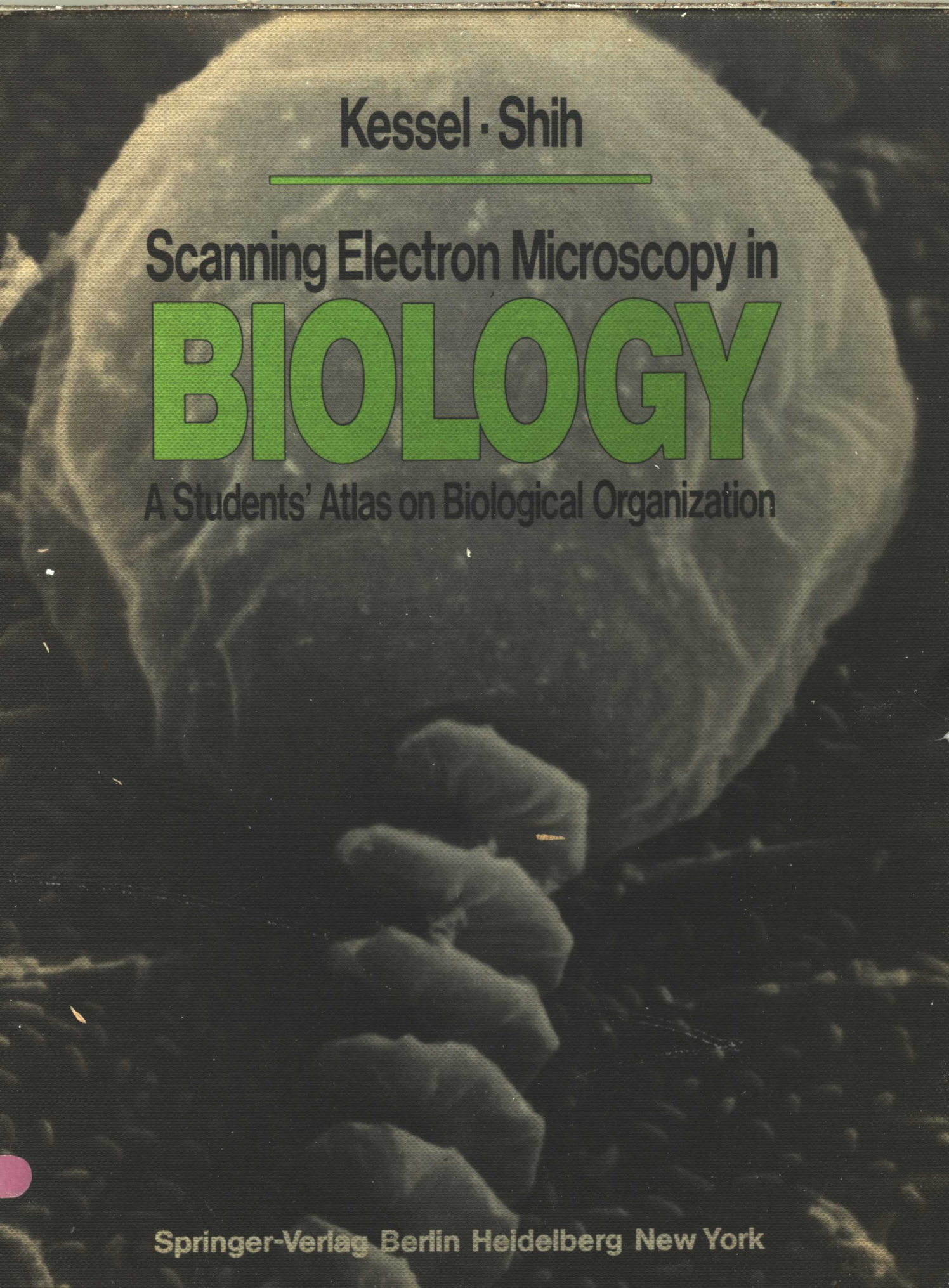

Kessel · Shih

Scanning Electron Microscopy in BIOLOGY

A Students' Atlas on Biological Organization

Springer-Verlag Berlin Heidelberg New York

R. G. Kessel C. Y. Shih

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Corrected Reprint of the First Edition

With 132 Plates and 22 Figures

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Preface

In the continuing quest to explore structure and to relate structural organization to functional significance, the scientist has developed a vast array of microscopes. The scanning electron microscope (SEM) represents a recent and important advance in the development of useful tools for investigating the structural organization of matter. Recent progress in both technology and methodology has resulted in numerous biological publications in which the SEM has been utilized exclusively or in connection with other types of microscopes to reveal surface as well as intracellular details in plant and animal tissues and organs. Because of the resolution and depth of focus presented in the SEM photograph when compared, for example, with that in the light microscope photographs, images recorded with the SEM have widely circulated in newspapers, periodicals and scientific journals in recent times.

Considering the utility and present status of scanning electron microscopy, it seemed to us to be a particularly appropriate time to assemble a text-atlas dealing with biological applications of scanning electron microscopy so that such information might be presented to the student and to others not yet familiar with its capabilities in teaching and research. The major goal of this book, therefore, has been to assemble material that would be useful to those students beginning their study of botany or zoology, as well as to beginning medical students and students in advanced biology courses.

It should be emphasized that the SEM is only one tool capable of providing a particular kind of useful information, just as, for example, the light microscope or the transmission electron microscope. While a limited number of light photomicrographs and transmission electron micrographs have been included for clarity in certain cases, the emphasis is on the view obtainable with the SEM, since this represents new information in some cases or has provided additional clarity in other cases. The student, therefore, should consult freely the large number of books in which there is information on the finer intracellular and extra-

cellular details such as can be obtained with the transmission electron microscope.

A phylogenetic and systematic approach is utilized in the organization of this book and is intended to serve students as an adjunct to their textbooks and laboratory manuals. The format of this book was designed for maximum utility on the part of the student. In most sections we have attempted to cover one subject in each plate, placing the corresponding descriptive information close at hand so that it can be quickly and easily related to the illustrations. Present-day textbooks in biology tend to extensively utilize drawings to illustrate structural details and interrelationships as well as processes or concepts. The student of science can well appreciate the significance of the old adages "one look is worth a thousand words" and "seeing is believing." In this connection, the three-dimensional quality of the SEM image as well as the excellent resolution and large surface area in the SEM picture serve a particularly valuable and realistic teaching function.

In our desire to provide rather broad coverage of both plants and animals, many of the preparations were made specifically for this book. In a few cases where information was already available we have used illustrations kindly provided by other investigators. In addition to our desire to select and present information scientifically useful to the student, we have attempted to present images that were esthetically pleasing and interesting as well.

The bibliographic citations in this book have been held to a minimum since a complete and excellent bibliography dealing with research papers involving scanning electron microscopy is published each year as part of the Proceedings of the Annual Scanning Electron Microscope Symposium sponsored by the Illinois Institute of Technology Research Institute and organized by Dr. OM JOHARI.

It is not difficult to predict that the SEM will be increasingly used in the biological, medical, and physical sciences. The number of instruments in use and the number of users continue to increase yearly. Furthermore, scientists continue their efforts to improve the operation of the instrument, the methods of specimen preparation, and the instrumental accessories that can enhance the information output. We are hopeful that this book will stimulate additional interest and progress in this field.

Iowa, Summer 1974

R. G. KESSEL
C. Y. SHIH

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Contents

Chapter 1 Introduction

A number of scanning electron microscopes are now commercially available, and one such instrument is illustrated in Fig. 1. The control console of a scanning electron microscope consists of two major assemblies. The electron-optical column with the specimen chamber is located at the left of Fig. 1 and is mounted on a plinth containing the vacuum pumping unit. The assembly illustrated in the right portion of Fig. 1 includes display racks containing most of the operator controls, display cathode ray tube (CRT) screens, processing units, meters, etc.

Comparison of the Scanning Electron Microscope with other Microscopes

Magnification

The maximum effective magnification of the light microscope (LM) is about 1200 diameters, whereas the effective magnification of the best transmission electron microscope (TEM) approaches 1000000 diameters. The scanning electron microscope (SEM) can provide a range of magnification varying from about 15 diameters to about 50000 diameters, depending on the nature and form of the material examined. The value of expanding the low magnification range of the SEM from about 2× to 50× has been emphasized (MEAKIN and FALLON, 1973). Useful pictures in this range can be obtained by increasing the working distances to reduce distortion.

Resolution

The maximum resolution (the minimum distance two objects may be separated and still observed as distinct) of the LM is about 2000 to 3000 Å (200 to 300 nm). The best TEM's have a resolving power of 2 to 5 Å (0.2 to 0.5 nm). Most SEM's have a resolution of 100 to 200 Å (10 to 20 nm), depending upon the nature and conditions of the sample as well as on the operating voltage of the instrument and final aperture size. A resolution of 100 Å at 30 KV is common.

Depth of Field

The outstanding feature of SEM micrographs is the remarkable three-dimensional quality. In contrast, the typical micrograph obtained with the LM or the TEM is a two-dimensional image. The LM can be focused in only one plane; therefore, its depth of field is severely limited. The LM is useful for revealing the shape of specimens only at low magnification since the depth of field of a LM decreases with increasing magnification. For internal detail the LM is best used with thin or flat samples, and even thinner sections are required for the TEM. Useful sections for study in the LM commonly range from 8 to 15 μm, but sections to be studied in the TEM generally range from 300 to 700 Å in thickness and must be cut on a special microtome. Because the specimens examined in the TEM must be so thin, the depth of field is limited and an essentially two-dimensional image is obtained. No such limits are found with the SEM. In contrast to the LM or the TEM, in which the illuminating source (light or electrons) must pass through the specimen, in the SEM the electrons that are recorded do not pass through the specimen, but rather secondary electrons are collected from the surface of the specimen and are used to form an image. These secondary electrons need not be focused, only collected. Therefore, in the SEM there is virtually no constraint on the size of the specimen to be studied, the size being limited only by the capacity of the specimen stage.

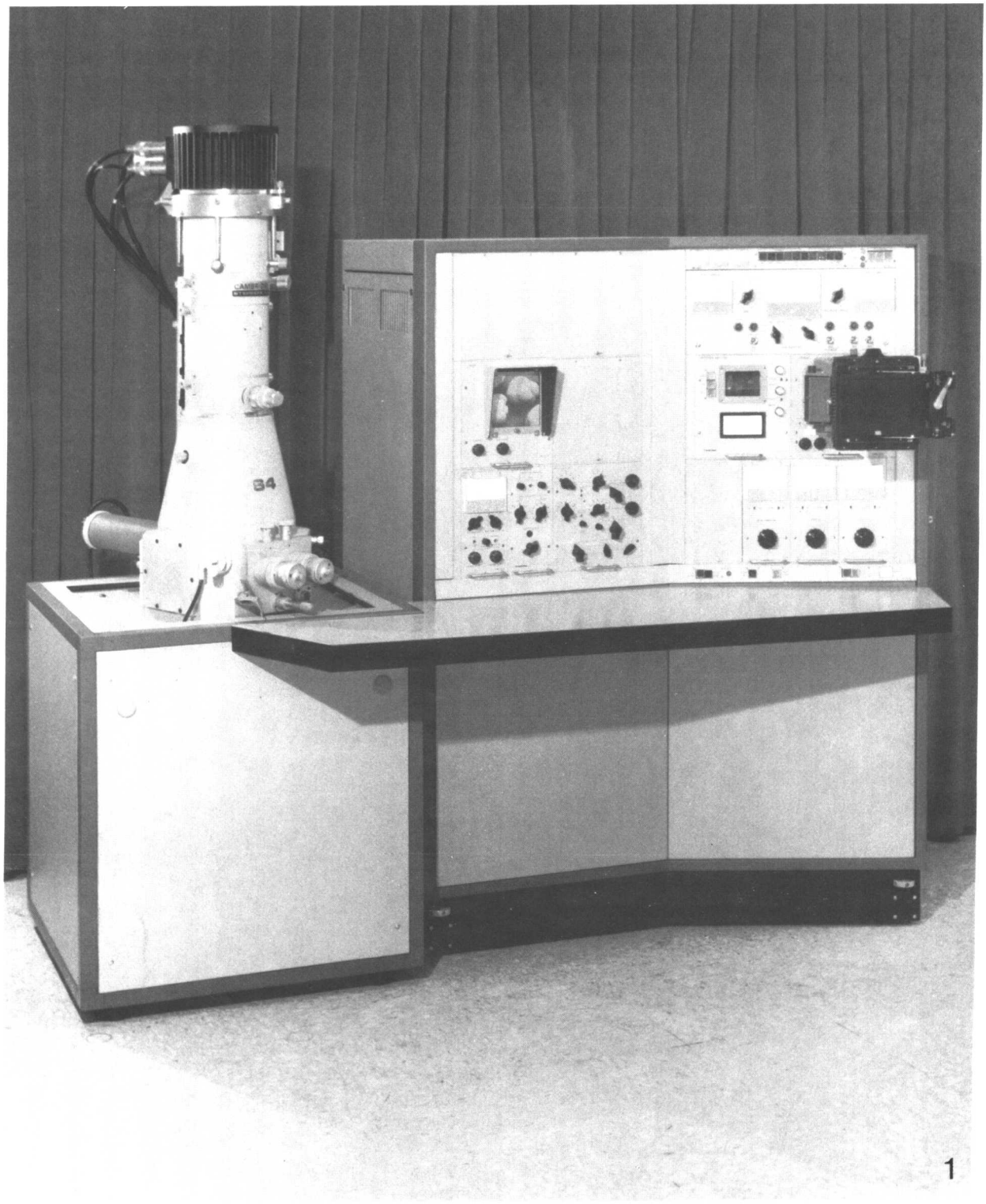


Fig. 1. A Cambridge Stereoscan S4 Scanning Electron Microscope (photograph courtesy of Kent Cambridge Scientific Co.)

In terms of both magnification and resolution the SEM has a range generally intermediate between that of the LM and the SEM. Truly unique features of the SEM include the large specimen size that can be observed and the tremendous depth of field. The depth of field in the SEM, compared with the LM, is better at comparable magnifications by a factor of at least 300.

Operating Modes

The SEM can also obtain other kinds of information not found with the LM or the TEM. The major modes of imaging in the SEM include (1) emissive, (2) reflective, (3) absorptive, (4) transmission, (5) cathodoluminescence, (6) x-ray, and (7) beam-induced conductivity.

In summary, the overall advantages of the SEM include (1) a tremendous depth of field and the resulting three-dimensional image, (2) the capability of viewing a large size specimen, (3) a broad magnification range, (4) resolution, (5) ease of varying magnification without changing the focal length so that the depth of field remains constant, and (6) various kinds of information that may be obtained from the specimen. Extensive work is presently underway to improve various technological features of the SEM. Among the important efforts being made are those concerned with providing a better electron beam source and a better vacuum system. This effort is reflected in the development of a field emission electron gun, an ion pump-lanthanum hexaboride gun, and a scanning-transmission electron microscope (cf. CREWE and WALL, 1970; AHMED and NIXON, 1973; KUYPERS *et al.*, 1973; SWANN and KYNASTON, 1973).

Basic Theory and Operation of the Scanning Electron Microscope

A schematic comparison of the light optical and a transmission electron optical system with the scanning electron microscope system is illustrated in Fig. 2. The SEM can be simply characterized as a closed-circuit television system in which the object observed is illuminated by a constantly moving spot of electrons. The electrons in a SEM are emitted by the heating of a tungsten filament (cathode) located within the gun in the upper portion of the microscope column. The cathode is generally held at 20000 V (but is variable over 1 to 30 KV range) below ground potential of the anode. The electron gun thus produces the electron source (a crossover of high current density). Below the gun are three prealigned electromagnetic condenser lenses which serve to accelerate the electrons and focus them to a small point. The condenser lenses also serve to progressively demagnify the electron beam into a probe which is focused on the surface of the specimen. The electron probe thus formed may range from 10 to 50 nm in diameter. The probe of electrons is deflected by scanning coils which drive the beam over the specimen surface in a square raster comparable to the situation in a television screen. This electron beam or probe is synchronized with the electron beam of a cathode ray picture tube. The scanning coil assembly is located in the bore of the final or lowest condenser lens, which is sometimes referred to as an objective lens. The final lens is fitted with three interchangeable apertures. The specimen chamber is situated at the base of the microscope column in line with the electron beam. The final aperture limits the divergence of the electron beam at the specimen surface. Usually three different sizes of apertures can be manually selected by a control external to the specimen chamber (Fig. 1). This control (isolation valve) is also used to seal off the specimen chamber from the column when samples are changed. It is important that the final aperture, which is located just above the specimen, be clean and in alignment during high-resolution work so as to avoid astigmatism and charging artifacts. Otherwise the image will shift during focusing or other lens adjustments. Larger aperture sizes are used at higher magnifications, but they limit the depth of field obtainable in a focused picture.

Separate diffusion pumps are provided for the column and the specimen chamber and are backed by a single rotary pump. Column vacuum can be maintained during specimen changes and during replacement of the filament (electron source). Both the column and specimen chamber must be under adequate vacuum (less than 10^{-4} torr) for operation of the instrument. The quality of the vacuum in the specimen chamber, very important in high-resolution operation, minimizes contamination. With an inadequate vacuum, contamination of the system occurs rapidly as the hydrocarbons undergo cracking in the electron beam. This, in turn, decreases the signal amplitude and results in poorer resolution. Some SEM's utilize vacuum systems with liquid nitrogen cold traps to reduce the possibility of oil from back-streaming into the chamber from diffusion pumps.

Movements of the specimen in the x-, y-, and z-directions, as well as tilting and rotation, are possible

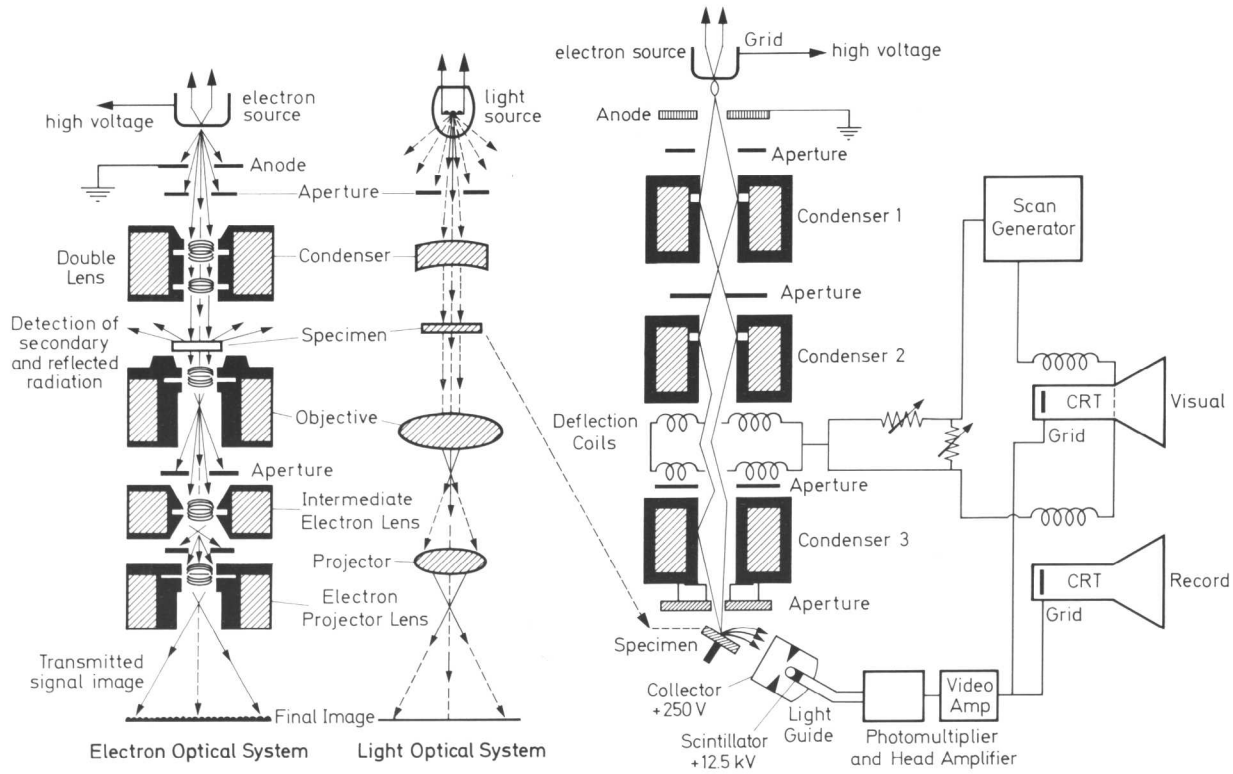


Fig. 2. Diagram illustrating details of a transmission electron optical system, light optical system and SEM (From P.E. MEE: "Microscopy and its contributions to computer technology." *Microstructures*, Oct./Nov. 1972)

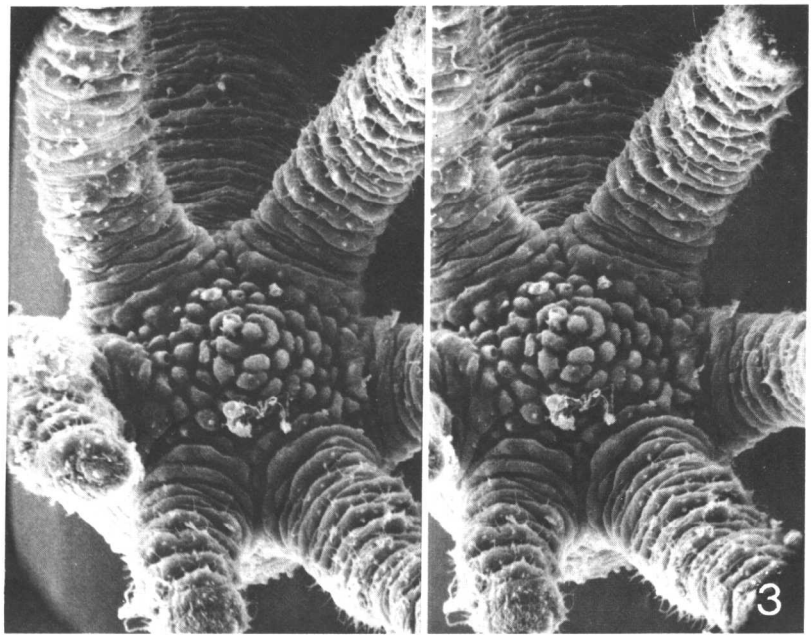


Fig. 3. Stereopair, oral view of *Hydra*. ×46

by means of controls outside the specimen chamber. If a specimen is tilted 8 to 10° between successive exposures, stereo pairs can be obtained and viewed with a stereoviewer. The stereo pairs illustrated in Fig. 3 are oral views of the coelenterate *Hydra oligactis*, showing the mouth and tentacles. A simple stereoviewer placed in proper position over this micrograph should illustrate a three-dimensional depth.

Modes of Operation of the Scanning Electron Microscope

As the electron beam is passed over or scanned across the specimen surface, the interaction of electrons with matter (the specimen) results in the production of a variety of electron types as well as x-rays and cathodoluminescense. This results in the possibility of several major modes of imaging in the SEM (Fig. 4).

Emissive Mode

The most important and widely used operating mode is the emissive. When the high-energy electrons in the probe strike the specimen, secondary electrons are excited so that a signal is formed from these low-energy secondary electrons (energies of about 4 eV) emitted from the top 50 to 100 Å of the specimen. Secondary electrons can provide three kinds of specimen information: topographical detail, superimposed voltage contrast, and superimposed crystallographic orientation patterns. The number of secondary electrons produced depends on the surface topography as well as the composition of the specimen. It is generally necessary to evaporate a metal of high atomic number (usually gold-palladium alloy) over the specimen, particularly those that do not conduct well or those that are poor emitters of secondary electrons. The secondary electrons emitted from the specimen pass to the collector (+charge), which accelerates the electrons to strike the scintillator (+charge, but higher voltage). Here photons are produced which enter and pass through a photomultiplier, which serves to produce large numbers of additional electrons (100000 to 50 million). The electrons leaving the photomultiplier pass to display and record cathode ray tubes. Each of the cathode ray tube (CRT) displays in an instrument such as illustrated in Fig. 1 is 100 mm square. One of these magnetically focused displays is for visual observations and has a long-persistence phosphor coating (about 600 lines resolution), whereas the other CRT has a short-persistence phosphor coating for high-resolution photographic recording (about 1000 lines center resolution). The magnification in a SEM is the ratio of the scan length on the visual display cathode ray tube (constant) to the scan length on the specimen (variable). The magnification can thus be increased by decreasing the length of the specimen scan.

In summary, in the emissive mode, low-energy secondary electrons emitted from the specimen by primary electron-probe excitation are drawn usually in a curved trajectory to a positively biased scintillator—light-guide—photomultiplier collector system (Fig. 2). After amplification, this is the signal that modulates the CRT beam.

Applications of the emissive mode in the study of surface topography are virtually unlimited. In addition, large single-crystal specimens examined at low magnifications illustrate patterns of crystallographic orientation similar in appearance to Kikuchi patterns. Finally, it also is possible to display "voltage contrast" with this mode. Generally, negative potentials appear bright and positive potentials appear dark. This provides a useful way of studying semiconductor devices and integrated circuits.

Reflective Mode

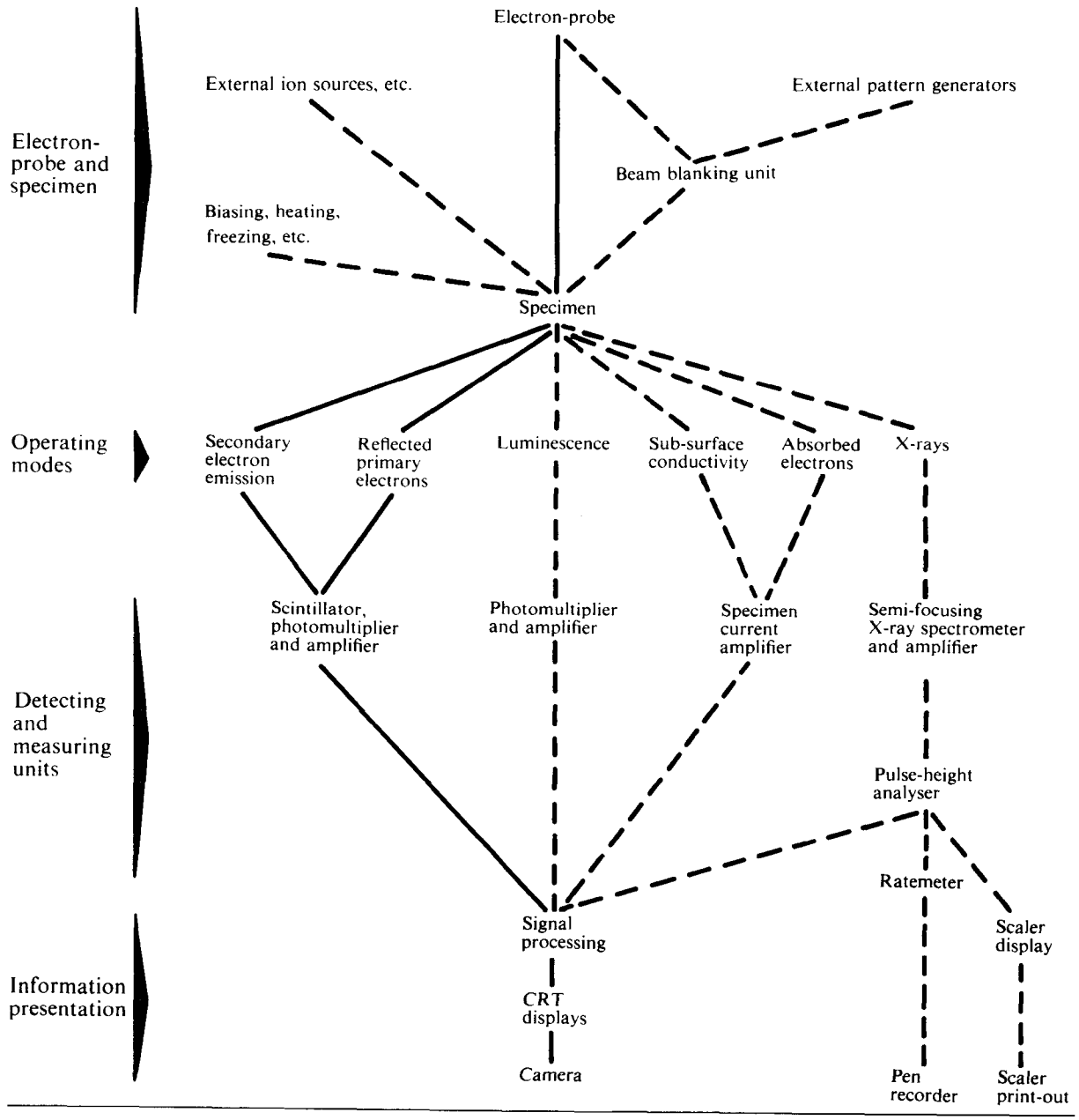
In this mode of operation backscattered electrons with a higher energy than secondary electrons are collected. Since they have their origin from depths of up to several microns in the specimen surface, these backscattered images have lower resolution than the secondary images. Collected backscattered electrons can be used to obtain information regarding the kinds of atoms in a specimen. The resolution limit is about 500 to 2000 Å. The resolution limit for the other modes indicated below is typically 0.1 to 1 μm.

Absorptive Mode

In this condition the signal is collected by monitoring the current flowing to earth from the specimen.

Transmission Mode

In this mode electrons pass through a specimen (as with a section of biological material), and the electrons which have penetrated the specimen are collected. Here



Information provided

Emissive mode
*1. Topography
*2. Surface voltage
*3. Crystallographic orientation

Conductive mode
*1. Sub-surface conductivity

Reflective mode
1. Topography
2. Element discrimination
3. Crystallographic orientation

Absorptive mode
1. Topography
2. Crystallographic orientation
3. Surface voltage
4. Element discrimination

Luminescent mode
*1. Luminescence

X-ray mode
*1. Quantitative microanalysis
*2. Qualitative microanalysis

*Preferred method

◀ Fig. 4. Major modes of imaging in the SEM (courtesy of Cambridge Scientific Instruments Limited)

it is possible to examine thicker specimens than can be viewed with a conventional transmission electron microscope.

X-Ray Mode

In the x-ray mode the x-rays generated when the electron beam reacts with the specimen are collected, analyzed, and used to form a signal. The ability to perform x-ray microanalysis of elements in the SEM is one of its most valuable assets. It is accomplished by adding to the SEM crystal diffraction spectrometers, energy dispersion spectrometers, or a combination of both. Crystal spectrometers can operate at high resolution and have good linearity at high counts. Generally, they have been preferred for *quantitative* analysis with high-probe currents. Energy-dispersive spectrometers are useful for rapid *qualitative* analysis (sometimes also with quantitative analysis), and they have a high collection efficiency but poorer energy resolution (LIFSHIN and CICCARELLI, 1973).

Cathodoluminescence Mode

When a high-energy electron beam interacts with a solid specimen, one measurable phenomenon that can be displayed is cathodoluminescence. When a substance that is bombarded with electrons emits light, this phenomenon can provide useful information, such as structural details, that would otherwise not be visible. It is also possible to examine the behavior of cathodoluminescence material. The effect produced is somewhat similar to optical fluorescence microscopy. The cathodoluminescence mode of the SEM has not as yet been extensively used (YOFFE *et al.*, 1973). An initial application of this mode to biological material was made by PEASE and HAYES (1966), who used the microscope in the luminescent mode to observe sections of spinach leaves treated with Thioflavin T. This dye appears to selectively bind with the cell walls to make these structures clearly visible. This mode is also useful in semiconductor work.

Beam-Induced Conductivity Mode

In this condition electrical connections are made to the specimen and an external voltage is applied. The current flow that results is monitored to provide a signal. When coupled with the changes possible with the incident beam itself, the technique is of great value in the study of junction field regions in semiconductor devices.

Thus, we have seen that a scanning electron microscope system can detect, display, and in some cases measure information derived from the action of a very fine beam of electrons scanning a specimen surface in a square raster. In general terms the information derived includes surface detail (microscopy), x-ray microanalysis, surface voltage contrast, sub-surface current, crystallographic orientation, and luminescence. The specimen can be manipulated and its state changed during investigation. Further, the specimen can be moved linearly, rotated, and tilted. With the addition of suitable modules, the specimen can also be heated, cooled, bombarded with ions, etc. With the addition of suitable external pattern generators, the SEM can also be used for electron-beam processing on an experimental basis.

Further, there are many factors contributing to the acquisition of high-quality pictures of biological material in the SEM. These include factors of (1) specimen fixation and drying, which are dealt with subsequently, (2) suitable coating of the specimen to reduce charging artifacts, (3) probe size and signal-to-noise ratio (the diameter of the probe is dependent to a large extent on the accelerating voltage used as well as lens aberration and settings), (4) properly aligned gun, (5) accelerating voltage of electrons, (6) final aperture size and its cleanliness, and (7) quality of the vacuum.

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Methods of Specimen Preparation in Scanning Electron Microscopy

It is only within the past several years that the SEM has been used appreciably in the study of cells and soft tissues. This is somewhat surprising, especially in view of the fact that this technique can provide a picture of whole cell surfaces that markedly improves on the images of the light microscope (even Nomarski optics) yet avoids the limitations of replication techniques. The depth of field and the increased resolution afforded by the scanning electron microscope when compared with the light microscope permit the visualization of a completely new realm of biological structure.

The reasons for the limited use of scanning electron microscopy in biology in the past are doubtless several. Not only has the instrument been of limited availability, but perhaps more important, techniques available for the preparation of soft, hydrated tissues have only recently been developed at a level providing useful images. Hard objects such as insect mouthparts, pollen grains, and teeth lend themselves well to examination by scanning electron microscopy because they do not require specialized methods to preserve their natural morphology. Until recently, soft hydrated tissues have not been prepared with sufficient care so as to avoid drying artifacts. As a consequence published images in many cases have been only slightly representative of the original cells. However, it is now quite possible with the variety of preparative procedures available and now in use to observe structures associated with cell surfaces and with cellular interrelationships in a manner impossible to obtain by any other method.

Fresh Material

Initial studies of biological material in the scanning electron microscope (BOYDE and STEWART, 1962) involved hard tissues such as teeth, hair, bone, and nails after treatment to remove debris such as dust or mucus. Such specimens retain their original shape in the dried state and can be examined without a metal coating in the SEM by using low accelerating voltages and low magnification. Hard objects such as rocks, shells, and small insects, which have a hard exoskeleton and plant structures including pollen grains can still be usefully studied in the SEM without fixation. However, before viewing, the specimens are sometimes coated with a thin layer of carbon and gold-palladium to reduce charging effects in the electron beam.

Specimens in an electron microscope are exposed to a vacuum pressure generally in the range of 10^{-4} to 10^{-6} torr. If a wet specimen is introduced to such vacuums, its water content will evaporate and the specimen will freeze. The surface tension forces generated during evaporation and the resulting ice crystal formation and growth is sufficently harmful so as to render the resultant images almost useless.

Air-Drying

The problem of drying artifacts became critical when cells and soft tissues were to be studied in the SEM. In the past, specimens were often fixed, rinsed with distilled water, dried overnight in a vacuum dessicator, and examined directly in the SEM. Initial preparations were air-dried from such solvents as water, alcohol, or acetone. Air-drying of cells and tissues is now known to produce a number of drying artifacts in which surface structures may be obliterated because of their collapse during the drying process. There is, however, less distortion and artifact production if soft objects are air-dried from a solvent that has a low surface tension (e.g., acetone or propylene oxide).

The ileum of the rat or mouse is commonly infected with a long bacterium, *Streptobacillus moniliformis.*

This condition is useful to illustrate the effects of various drying methods after fixation. A low magnification view of the villi in the rat ileum is illustrated in Fig. 5 and a higher magnification in Fig. 6. In both cases the material was air-dried from water. At low magnification the bacteria are not visible, but at higher magnification it can be seen that they have collapsed onto the surface of the villi during the air-drying process. The surface of epithelial and goblet cells are not distinct under these conditions. In Figs. 7 and 8 portions of intestinal villi and bacteria from the mouse are illustrated as dried from absolute ethanol and propylene oxide, respectively. Under these conditions, the bacteria are more apparent and not as collapsed onto the surface of the villi. Further, the cell boundaries of epithelial cells and goblet cells are more apparent. A low magnification view of the rat ileum dried by the critical point method from carbon dioxide is illustrated in Fig. 9. At this magnification the bacteria are quite apparent and many of them stand erect and project into or through the intervillous spaces. This condition is also illustrated at higher magnification in Fig. 10, which includes a portion of two villi and the intervening space. The bacteria are erect and well preserved. The effect of various drying methods on cultured cells has been studied by PORTER *et al.* (1972).

Chemical Fixation of Cells, Tissues, and Organs

A wide variety of chemical fixatives have been employed in scanning electron microscopy of biological tissues. In many cases the fixatives are similar to those used in preparating tissues for examination by transmission electron microscopy. Fixatives commonly employed include

(1) 1 or 2% osmium tetroxide in 0.5 or 1.0 M sodium phosphate or sodium cacodylate buffer (pH 7.2 to 7.4).

(2) 1.5 to 3% glutaraldehyde prepared in 0.5 or 1.0 M sodium phosphate or sodium cacodylate buffer (pH 7.2 to 7.4).

(3) KARNOVSKY'S (1965) paraformaldehyde-glutaraldehyde fixative prepared using one of the buffers already indicated. This fixative has also been used at dilutions of one-half to one-fourth of the full strength mixture depending upon the osmolality of the particular tissue to be studied.

(4) A "cocktail" fixative containing both glutaraldehyde and osmium tetroxide has been employed to good advantage to preserve structural details in biological specimens. The mixture frequently used consists of cold 1.25% glutaraldehyde and 2% osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.2 to 7.4). In this case the glutaraldehyde and osmium tetroxide are combined from stock solution just prior to use. Fixation is carried out for 1 to 3 hours at 4° C.

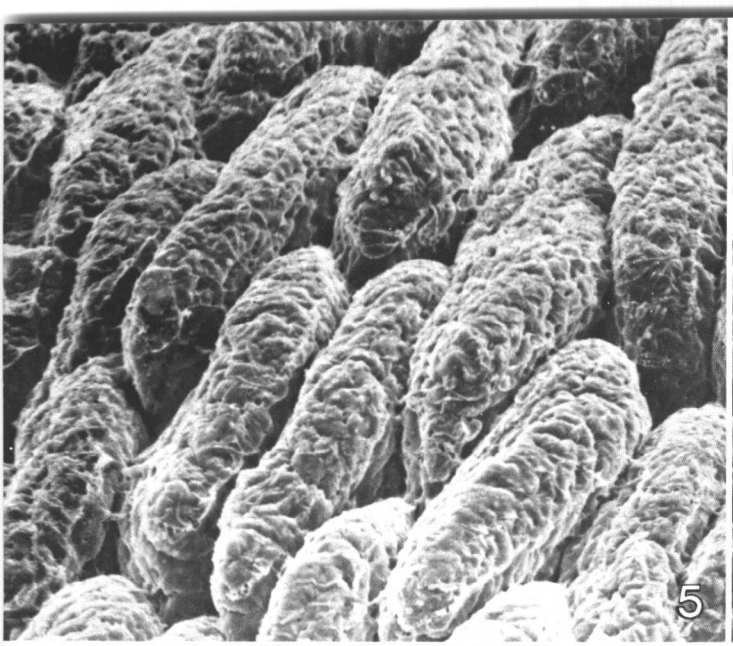
Commonly, the tissues are initially fixed in glutaraldehyde (primary fixative) followed by a buffer rinse and secondary fixation in osmium tetroxide. Thus, a dual fixation consisting of an aldehyde (reducing fixative) and osmium tetroxide (oxidizing fixative) is frequently employed. Both glutaraldehyde and osmium tetroxide are noncoagulating fixatives (*cf.* HAYAT, 1970). In some cases the tissues are fixed only in osmium tetroxide.

Both glutaraldehyde and osmium tetroxide penetrate tissue blocks slowly. Therefore, care should be exercised so that relatively small tissue blocks are exposed to the fixative to insure good fixation. Further, fixation in glutaraldehyde is often for 24 to 48 hours or more during which time the fixative is changed twice.

Many individual cells such as protozoa, blood cells, and sperm are extremely sensitive to the osmolality of the fixative (BESSIS and WEED, 1972). For example, if mammalian erythrocytes are placed in a hypertonic fixative, they become covered with short spine-like processes (crenated) due to shrinkage of cells (Fig. 11). In hypotonic fixatives the erythrocytes tend to swell and become spherical rather than biconcave. In these cases especially, it is important that the concentration of the fixative and buffer be adjusted to approximate that of the tissues being fixed. In some cases it is useful to add sucrose to the fixative to increase its osmolality.

Fixation in òsmium tetroxide is especially useful for those specimens covered by a thin mucus film since osmium tetroxide tends to remove this potentially obscuring layer. In those structures where mucus is more abundant (e.g., stomach, intestine, trachea, etc.) the tissues must be carefully rinsed with buffer or Ringer's solution prior to fixation. In some cases it is helpful to remove mucus by rinsing such tissue blocks prior to fixation with a 1% solution of sodium carbonate (prepared with the appropriate Ringer's solution and adjusted to pH 8 with hydrochloric acid) for about one minute.

In general, primary fixation of biological material for SEM observation involves an aldehyde and occurs over a period of several hours to several days. The tissue is then rinsed several times with buffer, saline, or distilled water (for about 30 to 60 minutes) and then 1 or 2% osmium tetroxide is used as a secondary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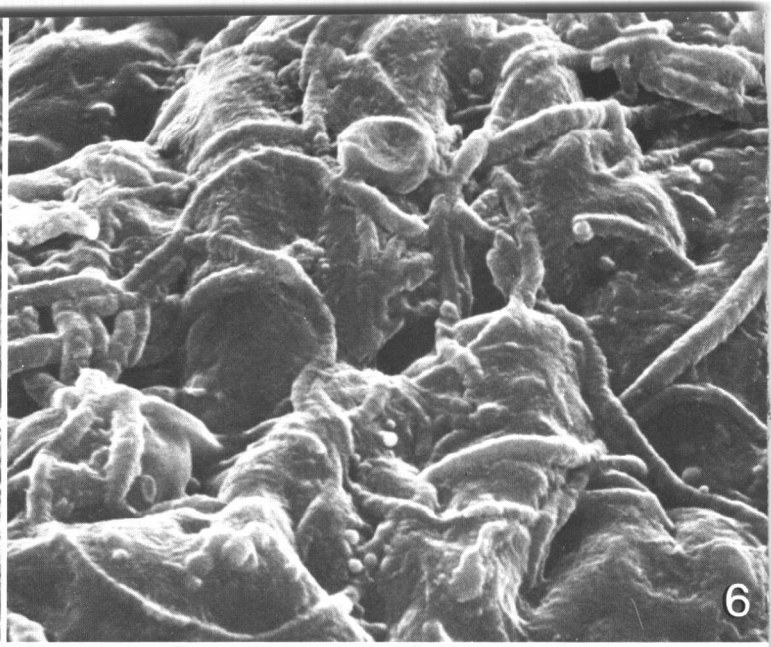
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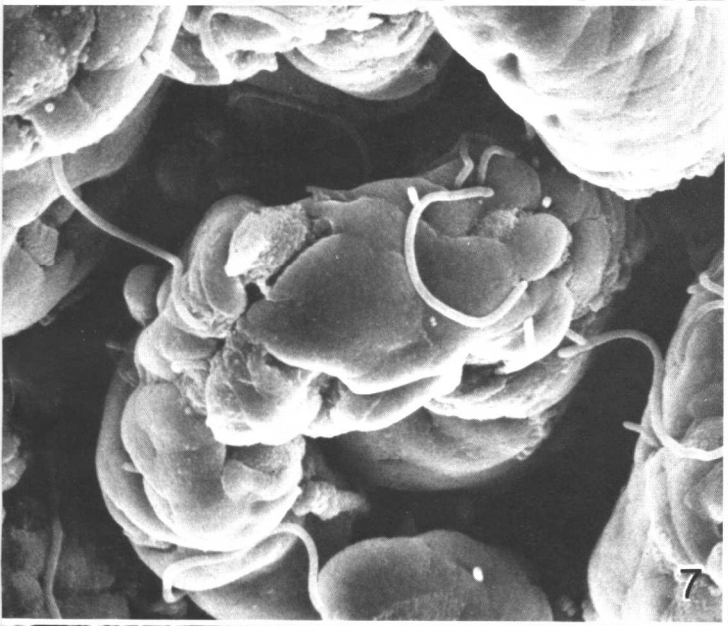
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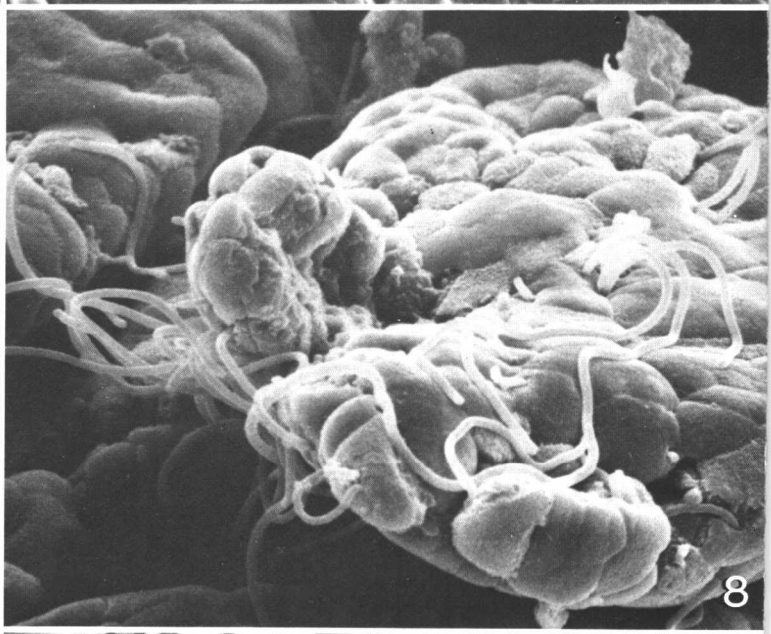
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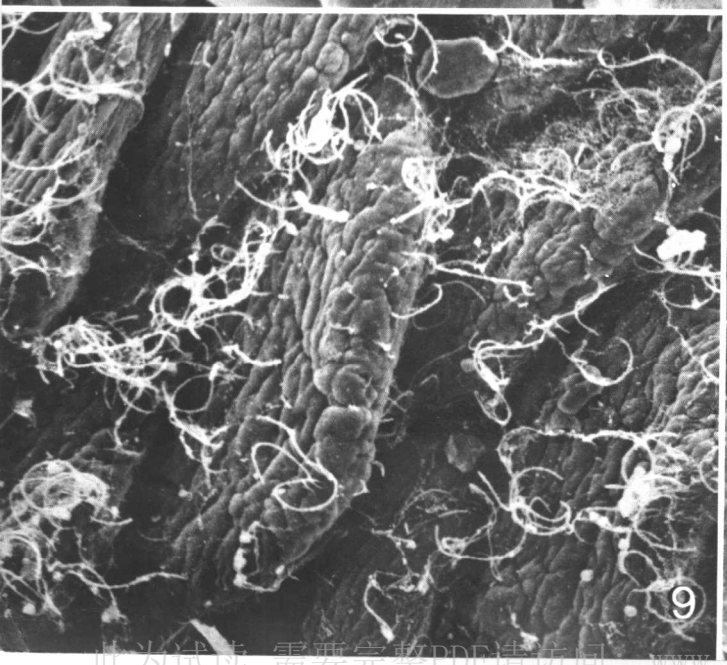
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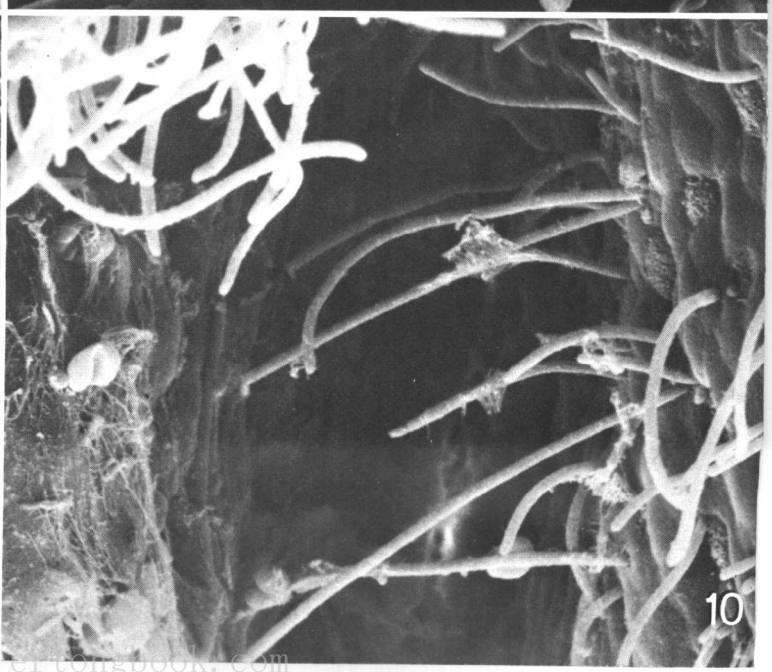
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