THE USE OF THE SCANNING ELECTRON MICROSCOPE
FOR BIOLOGICAL MATERIAL
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Introduction

The use of the scanning electron microscope (SEM) in biology is becoming widespread. It is an excellent tool for studying the morphology of cells and tissues. Its magnification, 20 to 30 thousand times, overlaps both the optical and conventional electron microscope. Its depth of field is at least a hundred times greater than that of the optical microscope. Many objects may be studied with minimal preparation. For example, some material may be fixed in glutaraldehyde, dehydrated in alcohol, air dried and then coated with a thin (200 Angstroms) film of gold. This preparation is much simpler than that used in conventional electron microscopy and avoids the necessity of thin sectioning the specimen.

The use of the scanning electron microscope in biology is still in its infancy. Sophisticated techniques must still be developed so the use of the microscope will be enhanced. Since the biologist depends on morphology a great deal, this microscope has become a very useful instrument.

The Basic Instrument

A stream of electrons is scanned across the surface of the biological material in a vacuum. This primary beam generates secondary electrons on the specimen surface which are gathered into an electron collector, point by point, as the material is scanned. The magnitude of this secondary collection is converted to intensity of light as displayed on a cathode ray screen.

A more detailed account of the instrument is as follows. Electrons are emitted from an electron gun at the top of the instrument and are accelerated towards the anode which is at earth potential relative to the 2 to 30 kilovolts supplied to the electron gun (see Figure 1). The electron beam passes through one or more magnetic lens so that the specimen is bombarded with a fine stream of electrons. The electron beam is deflected by a scanning generator which allows the beam to successively scan the complete specimen surface. The primary electrons produce lower energy secondary electrons at the surface of the specimen which are then electrically attracted to a collector. The magnitude of the collector signal is amplified and fed to a cathode ray tube. The brightness of its beam is related to the number of secondary electrons collected. The electron beam scanning of the specimen is coupled to a similar scanning across the display tube such that an image of the specimen is displayed. The inherent noise in the system can be decreased by increasing the time of scanning. Thus, for better pictures, the photographic exposure time is much greater than that used for the monitor display tube.

Specimens several centimeters in diameter may be placed into the instrument. A manipulator allows the surface to be translated and rotated respective to the impinging electron beam. Since the specimen is in vacuum, most biological specimens must be dried before being used. A thin (200 A)
coating of gold is evaporated over the surface of the specimen to increase secondary electron emission and also to leak off the electrical charge generated by the scanning electron beam.

Radiation other than secondary electrons are also given off by the specimen. These can also be put to good use. For example, the amount of cathodoluminescence released by the specimen is related to the amount of fluorescent materials within a given region of the specimen. A part of the electron beam that penetrates the specimen may also be collected beneath it so as to simulate a conventional transmission microscope with poor resolution. However, there is some advantage in obtaining simultaneously a transmitted image and a surface image from the same piece of biological material.

It is often convenient to obtain images from two different angles to obtain a three dimensional picture of the specimen. This is very useful when looking into holes or crevices of biological structures. Stereoscopic viewers have been utilized to make quantitative measurements with a good deal of accuracy. It should be noted, however, that even without the use of special stereoscopic techniques, all scanning electron microscope pictures have a three dimensional appearance due to the great depth of focus.

Tissue Preparation

As in conventional histology, the preparation techniques depend upon the particular type of biological material used. The surface of soft tissue is usually washed with a jet of isotonic solution although the removal of mucus may require special treatment. Many fixation and drying methods have been developed in our laboratory (Beidler, 1969). However, most small tissue specimens are treated as follows.

The tissue (1 cm diameter or less) is placed for 24 hours in 6.25% gluteraldehyde made in buffered sucrose. Water is replaced by placing the tissue in a series of ethanol-water solutions (50, 70, 80, 90, 95 and 100%) for intervals of 2 hours each. The alcohol is then replaced by amyl acetate by placing the tissue in 25, 50, 75, and 100% amyl acetate-ethanol solutions for 10 minutes each. To avoid artifacts caused by phase boundaries or crystal formations the amyl acetate is replaced by liquid CO₂ and then heated to above 31°C, the critical point of CO₂, so that the liquid CO₂ becomes a gas and is released from the tissue without tissue distortion. A special apparatus (see Figure 2, 3) for CO₂ critical point drying has been designed by Mr. Webbers of our laboratory using the principles outlined previously by Anderson (1951) and exploited for certain scanning electron microscopic applications by Horridge and Tamm (1969).

The tissue is placed in a small stainless steel container (5) with a bottom of stainless steel wire cloth. It is then closed with a cover made of stainless steel wire cloth. This container is placed in a high pressure chamber made of stainless steel about 2" on a side, sealed with an "O" ring (3) and the top (2) affixed with four socket screws. The pressure chamber is connected on one side (6) with a CO₂ cylinder via a valve. The other side (4) is connected to atmospheric pressure by means of a regulating valve and an on-off valve. A pressure gauge is also connected (8) to the chamber. By opening inlet (6) and regulating the flow from outlet (4), the amyl acetate in the tissue is replaced by liquid CO₂ at room temperature.
When completely filled with liquid CO₂, the outlet (4) and inlet (6) are closed and the temperature of the chamber is raised by a built-in cartridge heater (1) to a temperature of 50°C. At this temperature the pressure in the chamber is 1400 PSI. The liquid CO₂ is changed to a gas. The outlet (4) is slowly and continuously opened over a period of 15 minutes for the CO₂ gas to escape. The slow escape is designed to avoid a destructive pressure gradient within the tissue.

The dried tissue is placed with Duco cement onto a conventional aluminum stub and then coated with Palladium-gold (40:60) in a Denton high vacuum evaporator. The stub is then placed into the scanning electron microscope and positioned properly for scanning.

Sample Photographs

Raw potatoes (Red Bliss), untreated stew beef, "Protem"-treated stew beef and fresh parsley leaves were selected for their possible interest to members of the American Meat Institute Conference. Special methods of tissue preparation were not researched for these materials, but the above general procedure was followed. It is hoped that the results shown will illustrate some of the possibilities that exist in the study of foods using the scanning electron microscope.

A low magnification SEM photograph of the center of the raw potato is shown in Figure 4. A better cellular representation is given in Figure 5 where the individual starch granules may now be seen with clarity. A photograph of similar type of tissue and similar magnification (see Figure 6) was taken from a publication by Reeve (1967) where he used conventional histological procedures and polarizing optical microscope. There is a great difference in the type of information one may obtain with the SEM versus the optical microscope. Note the three dimensional aspect of the SEM photograph. By merely turning a switch, a higher magnification of one group of starch granules is obtained as shown in Figure 7. A single granule is shown in Figure 8. This is a magnification of only 4,000 whereas the SEM can still give good resolution at above 30,000.

Untreated stew beef is shown in Figure 9. Note the large amount of connective tissue and collagen. Individual bundles of fibers are usually enclosed by a sheath as shown in Figure 10. Sometimes part of the sheath has been mechanically disturbed when we open the muscle although usually this sheath is intact. Figure 11 and 12 illustrate where a small portion of such a bundle has been damaged and the individual skeletal fibers beneath are exposed.

"Protem"-treated stew beef is much cleaner in appearance as shown in Figure 13. Much of the connective tissue is not apparent nor is much of the collagen. If a single fiber bundle is magnified to over 2,000 times, one can easily see the Z-bands of the muscle fibers (Figure 14). The A and I-bands are shown at magnifications of 5,000 in Figure 15. It is interesting to note that when these fibers are damaged they appear to break at the Z-bands.

Parsley leaves have been selected for illustration since the tissue is quite different from that of beef or potato. Figure 16 illustrates a small portion of the leaf at low magnification. At slightly higher magnification
the stoma become apparent, as shown in Figures 17 and 18. At a magnification of 5,000, the structures of the stoma are easily observed (Figure 19).

I hope the above illustrations have indicated some of the possibilities that exist in the study of foods with the SEM. My own laboratory is primarily interested in the chemical receptors in a variety of different animals, including man. Special techniques may be developed for specific tissues. For example, one may need to study the surface of the human tongue over a period of several months. This can easily be accomplished by coating the surface of the tongue with silicone rubber and making a mold of the natural tongue surface. A positive of the mold is made, then coated with gold and examined with the scanning electron microscope. This can be done day after day without any discomfort to the subject.

I cannot emphasize too strongly that research for methods to study biological material is still in its infancy. The scanning electron microscope is in many ways easier to use than the conventional electron microscope. It has been greatly used in the textile and electronic industries in a rather routine manner and there is no reason why it cannot be adapted to the food industries.

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References


Figure 1. The basic instrument.
Figure 2. Photograph of CO$_2$ critical-point drying bomb together with valves controlling inlets and outlets.
Figure 3. Drawing of bomb showing method of construction. See text for details.

Figure 4. SEM photograph of slice of raw potato. Magnification of 52X.
Figure 5. Potato slice magnified 210X. Notice starch granules in each compartment.

Figure 6. Parenchyma of water care of potato shown after histological preparation and viewed with polarizing microscope at 150X.
Figure 7. Enlargement of group of starch granules at 1050X.

Figure 8. A single starch granule at 4000X.
Figure 9. Commercial stewing beef showing connective tissue and collagen 500X.

Figure 10. Single muscle bundle of beef showing ruptured sheath. 1100X.
Figure 11. A bundle of muscle fibers mechanically damaged. 1100X.

Figure 12. Enlargement of Figure 11 showing striated muscle fibers beneath sheath. 5500X.
Figure 13. Cut end of stewing beef "Protem" treated. Note how clean is the muscle. 100X.

Figure 14. Disrupted bundle of treated beef muscle showing striations of fibers. 2100X.
Figure 15. Muscle fiber striations showing prominent Z bands and faint A and I bands. 5000X.

Figure 16. Top view of parsley leaf. 56X.
Figure 17. Underside of parsley leaf. 180X.

Figure 18. Enlargement of leaf to show numerous stoma. 500X.
Figure 19. One of the parsley leaf stomata magnified 5000X.
J. D. SINK: We will open the session for discussion from the floor again reminding you to use one of the aisle mikes. State your name, institution and then the question. Any questions?

V. K. JOHNSON: What is the cost of the apparatus?

L. M. BEIDLER: Now I'm not here to sell this instrument you understand.

V. K. JOHNSON: Well I know that, but I'm interested.

L. M. BEIDLER: Well, when we got our instrument it was around 60 thousand dollars, which is the cost of a very good transmission microscope. I think the Gerocol, (?) is also cheaper than the Cambridge.

J. D. SINK: Other comments. Questions?

UNIDENTIFIED: What kind of treatment did you subject the material to when the collagen disappeared from the fibers?

L. M. BEIDLER: Let me say I didn't take fresh muscle. If I had I doubt that I'd have gotten the same pictures. I was talking to this group so I went out to a store and I bought stewing beef and I took stewing beef not treated and Proten-treated. It was the Proten-beef, that did not have much connective tissue around it. I used this material as an illustration for this group. I didn't do any research on this meat and I don't want to act as if I'm an expert meats researcher or anything like that. So this was right off the shelf, this meat.

UNIDENTIFIED: What is the difference between the electron microscope and the stereoscan especially in the preparations and in the magnification?

L. M. BEIDLER: Well, the usable magnification of the stereoscan is about 30,000 with the transmission maybe a million although people go up normally to about 200,000, so it conventionally is higher. The big difference is in resolution. In ease of preparation for this instrument -- you can take a butterfly and put it in the instrument without any preparation, or you can gold coat it. You can go from that extreme to very sophisticated techniques. You could take formaldehyde-treated soft tissue, dehydrate it with alcohol, air dry it and put it in the instrument, no microtoming, no sectioning or anything like that. A conventional morphologist who doesn't know anything about modern techniques could use the instrument very very well. Now if you go to the transmission electron microscope, as you probably know, it takes a long time to learn to use the instrument. It takes a long time to learn to microtome properly and all the other techniques. So this is a simple instrument compared to the transmission microscope.

R. L. HENRICKSON (Oklahoma): How effective is the instrument for measuring thicknesses of tissues such as the sarcolemma or some other smaller item?

L. M. BEIDLER: I don't think it would be too good for thickness, unless you could get it on an edge because the electrons generate their secondary electrons in very thin sheets on the surface. In other words, it