Title
Scanning Electron Microscope

Author
Hayes, Thomas L

Publication Date
2023-09-06

Thomas L. Hayes

Donner Laboratory

University of California

Berkeley, California 94720

Running Head: Scanning Electron Microscope

DISCLAIMER

Scanning Electron Microscope Methods for the Study of Membranes

by Thomas L. Hayes

The scanning electron microscope (SEM) forms an image of the specimen by utilizing a timed sequence of points in a fashion similar to the image forming methods used in television. The principal advantages of the SEM as compared to the conventional electron microscope do not lie in the area of resolution or greater available detail of the image, but rather in a somewhat broader area of information transfer.[1] The imaging of bulk specimens is an example. Many of the objects visualized in the SEM have the appearance of objects as we would see them in the world around us. In addition to the experiential aspect of scanning microscopy, there are several radiations which can transfer analytic information to the image. For example, cathodoluminescence, or characteristic X-rays, localize chemical information related to atomic or molecular entities within the specimen.

However, in one mode of operation, scanning transmission, the SEM can be utilized as a very high resolution microscope. The scanning transmission microscope designed by Crewe and co-workers has produced reported resolutions at the single atom level.[2] Such scanning transmission operation requires that the specimen be as thin or thinner than the conventional electron microscope specimen and the considerable advantage associated with the more general operation of the SEM in the secondary electron mode is not available for scanning transmission. For the study of membranes, however, scanning transmission may be quite acceptable if the membrane has been isolated. In general, scanning transmission would be applicable for what will

be described later as textural studies, but would find somewhat limited application to membrane studies involved with the shape of the membrane as a reflection of cellular shape.

A microscopic observation of the geometry of the membrane system might be classified into two groups: A study of the shape of the membrane as it reflects cell shape; for example an evaluation of the variety of red blood cell shapes associated with diseases such as sickle cell anemia, thalasemia, etc. In such studies it is the overall shape of the cell which is under investigation and surface texture is a secondary consideration (Fig. 1).

The second type of study would be concerned with the textural structure of the membrane surface and would be relatively independent of the overall shape of the membrane. In this category, studies of particle localization or microvilli structures on the cell membrane might be examples (Fig. 2).

In addition to geometric studies, the SEM has been utilized to study the localization of chemical elements within the specimen by utilizing characteristic X-rays as the video signal. It is also possible to localize chemical entities that will fluoresce in the visible region when bombarded by electrons, a mode of operation termed cathodoluminescence. In addition, for inorganic materials, the study of the electrical properties of the specimen has been of great value and may find future application in biological materials.

Before considering the specific methods associated with SEM, it would be appropriate to look at alternative methods which might allow an analysis of certain of these properties utilizing instruments less restrictive in their preparative requirements or more appropriate to the study of materials in a physiological state. An additional consideration would be the cost and availability of the scanning electron microscope as compared with the

alternative methods discussed below.

Alternative Methods

Studies of Shape. In investigations of the shape of the cell, many useful characteristics can be obtained through the application of the visible light microscopic method known as differential interference microscopy, or Nomarski imaging.[3] This technique allows the specimen to be viewed while in a hydrated state and permits the visualization of information that can be correlated with the three-dimensional view seen with the secondary electron mode of SEM. It is, of course, limited by the wave length considerations associated with light microscopy, but has the advantage of being able to image an optical slice of the specimen with very little interference from material above and below the plane of focus. The contrast forming mechanism utilized relies on the derivative of the optical path and thus is dependent not only on path length, but on refractive index of the material being viewed.

The Nomarski system is convenient, inexpensive, and can utilize specimens in a state more nearly approximating normal physiological conditions. Drying and fixation artifacts can be eliminated since these steps are often unnecessary for the preparation of the specimen. The Nomarski apparatus can be attached to a standard research type light microscope at a cost far below that associated with an SEM. Bessis has utilized Nomarski imaging with great effect for the study of membrane shape in the case of normal and pathological red blood cells.[4]

This same author has also compared the image obtained by Nomarski imaging to that obtained with the scanning electron microscope in the case of a study of red blood cell shapes.[4]

If the texture of the membrane rather than its shape, is to be revealed, Nomarski imaging in general does not have sufficient resolution to carry out the task. In this case an alternative method is represented by the freeze-etching technique which utilizes the very high resolution conventional electron microscope.[5]

Although the freeze-etching technique can be complicated by such possible artifacts as ice crystal formation or replication deformation, it is a well established technique offering resolutions approaching the 30 Angstrom level. When we compare this resolution limit to the accepted commercial secondary electron SEM of from 80-100 Angstroms, we can appreciate the considerable advantage of the freeze-etching technique. In addition, the freeze-etching technique utilizes conventional electron microscopy which is often available to the investigator interested in membrane structure. If such facilities are available, freeze-etching might represent a considerable saving over the rather expensive acquisition of a SEM.

The additional freeze-etching techniques of complimentary replica and stereo pairs have been utilized by Steere in the study of biological surfaces at resolutions approaching the 30 Angstrom limit.[6]

Freeze-etching techniques have also been applied to the membrane structure at the textural level as found in mitochondria.[7]

These two alternative techniques point to the necessary comparative assessment of SEM with other established available techniques of ultrastructure research. It is sometimes tempting to assign priority to the latest form of instrument design, but a newer instrument is not necessarily a better instrument, and in the case of the scanning electron microscopy of membranes, alternative techniques may well provide more information at lower cost. If, however, the SEM is selected as the instrument of choice, the next consideration

is preparative methods.

Fixation

The same careful attention to preparative methods must be applied in SEM as have been necessary in conventional electron microscopy, or for that matter, in the preparation of light microscope samples. Often the first step in this process is that of fixation. A useful procedure for fixation is to try and eliminate artifacts by simultaneously processing separate fractions of the sample through two or more separate fixatives. It has been found that in general the fixatives applied in the preparation of SEM samples are those derived from the ultrastrustructural work of conventional electron microscopy. Two fixatives will be described here which have been found useful in SEM and which might be applied in a parallel fashion with the hope that by utilizing two different methods of fixation a somewhat better estimate of artifacts can be achieved.

The first fixative is an OsO_4-$HgCl_2$ fixative developed by Parducz.[8] This fixative was first developed for so-called instantaneous fixation of ciliates and has been found to be a valuable fixative for the SEM of these small animals.[9] The Parducz fixative procedure[8] is summarized as follows:

Fixative: 6 volumes 2% aqueous OsO_4 and 1 volume saturated aqueous $HgCl_2$.

Mordant: 10 gm. pale violet iron alum crystals (ferric amonium sulfate) dissolved in 100 ml. distilled water.

Stain: Heidenhain hematoxylin: 0.5 gm. iron hematoxylin plus 10 ml. 96% ethyl alcohol plus 90 ml. distilled water. The solution to be used should be one to six weeks old and light red in color.

Procedure: To one volume of the sample add four volumes of the fixative. Fix for fifteen minutes. Wash with distilled water, add one

volume distilled water, then four volumes iron alum solution and mordant for two minutes. Wash with distilled water. Add one volume distilled water, then three to four volumes hematoxylin solution. Stain ten to twenty minutes. Wash quickly with distilled water before initiating drying steps outlined below.

The second fixative is that developed by Karnovsky.[10] This fixative is a formaldehyde-glutaraldehyde fixative of high osmolality that was developed for use in conventional electron microscopy. Karnovsky's procedure[10] can be summarized as follows: Two grams of paraformaldehyde powder are dissolved in 25 ml water by heating to 60-70° C. and stirring. One to three drops of a 1N NaOH are added with stirring until the solution clears. A slight milkiness may persist. The solution is cooled, five ml. of 50% glutaraldehyde are added, and the volume is made up to 50 ml. with 0.2M cacodylate or phophate buffer, pH 7.4 to 7.6. The final pH is 7.2. If cacodylate is used, 25 miligrams $CaCl_2$ anhydrous is added. The specimen is fixed at room temperature for 20 to 30 minutes and then if possible, diced into small blocks and then the fixation continued at room temperature for two to five hours. The blocks are washed for 3-12 hours in cold 0.1M buffer, are post-fixed in cold 1.3% osmium tetraoxide buffered with s-collidine for two hours. Drying procedures as outlined below are then commenced. The Karnovsky fixative as applied to SEM of avian lung has been reported by Nowell, et al.[11]

Drying

The SEM specimen must be dehydrated and free of liquid if it is to be inserted into the vacuum system of the instrument. These drying techniques

are most critical to the preservation of ultrastructural detail. The effects of surface tension when the specimen is allowed to dry in air are quite severe (Fig. 3) and must be avoided if faithful reproduction of the structure of the specimen is to be achieved. Two general techniques for drying have been developed, one depending on the freeze-drying process and the other on the critical point method.

Again, in general it is best to carry out the two drying methods in parallel. At this stage then, there would be four samples. Each product of the two parallel fixative steps is now divided in two for freeze drying and critical point drying.

Freeze drying is generally carried out on commercially available tissue drying apparatus where the temperature of the specimen can be controlled and monitored at all times, and where the vacuum near the specimen can also be measured. A possible difficulty may arise if there is contamination from the oil of the mechanical pump usually utilized in such apparatus, or if there are components in the specimen chamber which may lead to contamination of the cold sample by condensation. In general, it is preferable to have a well trapped system utilizing liquid nitrogen to prevent the backstreaming of oil from the pumps and to utilize standard high vacuum techniques in construction of the bell jar, gasket and other components of the specimen chamber. Freeze drying techniques have been applied to SEM preparations for some time and a review of the methods found most satisfactory can be found in the article by Boyd and Wood.[12] One of the major concerns in any freeze drying operation is production of artifact during the freezing process due to the formation of ice crystals. Such mechanical deformation can cause large changes in the specimen and in particular can account for holes punched in membranes or

for spurious particles found on the surface. Thus while freeze drying can often be suggested as the method of choice for the preparation of the samples, it is useful to run a parallel drying step utilizing a method that does not contain any freezing step. The critical point method is therefore suggested as a parallel drying procedure.

At environmental parameters above the critical point of pressure and temperature, the boundary between liquid and gas in a sample will disappear. Above the critical point there can be no liquid phase, and thus the sample can be dried by bleeding off the gas phase, avoiding surface tension effects. Critical point apparatus are available commercially which utilize either carbon dioxide or freon as the transitional fluid and this equipment is very useful for offering a non-freezing method of drying with a minimum of surface tension artifact and in a time considerably shorter than that required for freeze drying. Although the problem of ice crystals and other freezing artifacts has been eliminated, it must be pointed out that the liquids from which critical point drying can take place (carbon dioxide and freon) are quite different chemically from the normal hydrated environment and thus critical point drying by itself must be looked at with considerable care for the production of chemically induced artifact. Again, the running of parallel drying methods is recommended.

Microscopy

As mentioned above the SEM can utilize several types of radiation as its information signal. Cathodoluminescence, characteristic X-ray, transmitted electron signal and induced specimen current have all been utilized for physical and certain biological studies and may find application to the study of membranes. Manger and Bessis have suggested using paraformaldehyde stimulated cathodoluminescence to study antibodies on cell surfaces.[13] However,

the most frequently utilized signal is the secondary electron signal which conveys depth and shape information as a function of the distribution of matter on the surface of the specimen. While many of the alternative information signals would be of great significance to an understanding of the structure and function of the membrane, their application to date has been somewhat limited.

The operation of the SEM in secondary electron mode will require the optimization of several parameters including accelerating voltage, specimen current, magnification, final aperture size, and astigmatism correction. Details of a suggested procedure for optimum choice of these parameters can be found in a recent review article.[14]

In addition to these parameters, a variety of display modes are also available to the operator. The usual display mode involves modulation of the display cathode ray tube in terms of its intensity. The display point brightness is modified according to the video signal generated by the probing beam of the SEM. Instead of the signal modulating the intensity of the display point, this same signal might be utilized to deflect the display point in the vertical, or Y, direction according to the strength of the video signal, producing an image with an artificial height appearance which can enhance recognition of certain of the surface characteristics.[15] Similarly, the video signal can be utilized to modulate the color of each point at each instant on the display cathode ray tube. Such color modulation may produce an enhanced pattern recognition and is considerably used for iso-intensity mapping and for recognition of minimum grey level changes.[16]

It is also possible to process the signal electronically so that the derivative of the original information signal is displayed. Such derivative

processing can result in an image which corresponds to an obliquely lighted specimen. Such changes in lighting as a function of mathematical manipulation of the signal may help in attempts to provide maximum cognition of specimen detail by the observer.

Cognition by the Observer

The cognitive processes carried out in the mind of the observer might be divided into two general classifications. First, those processes associated with the analytic application of a system of ideas, such as physics, chemistry or biology, to the data as it is presented in the image. Secondly, subjective or experiential interaction which results from an intuitive contact between the observer and the information of the specimen.

With respect to the analytic procedures, the mathematics, physics, chemistry, etc. are appropriate to the information conveyed by a particular signal (characteristic X-ray, cathodoluminescence, etc.). Such analysis results in an awareness of certain properties of the specimen that can be represented in terms of the idea information characterized by words or numbers. For example, by applications of geometry (both metric and topologic) spatial characteristics such as length, area, angle, genus, or network characteristics can be revealed.[17]

In addition to the analytic modes of cognition, it might be useful to consider the possibilities of utilizing the scanning electron microscope as an instrument which can allow us to visit the small world of the membrane as if we ourselves were small and could experience these characteristics directly. Such experiential contact, while somewhat less clearly defined in terms of its value to our understanding of the specimen can often result in overall appreciation of its nature. The mathematician Poincare once

stated "It is by logic that we prove, but by intuition that we discover." The SEM can represent a form of instrumentation that allows us to make experiential contact with the specimen and thus allows and opens up the possibility of intuitive discoveries with respect to structure.[18]

Conclusion

Scanning electron microscopic methodology as applied to the study of membranes represents an additional tool for investigating this structure. The SEM does not in any way replace conventional electron microscope techniques but rather may be considered as complementary to the other microscopic methods. While still quite a new technique, careful application of SEM has already proven useful in several research areas[19] and it would seem that this instrument may make some useful contributions to our understanding of membrane structure.

FOOTNOTES

1. T. L. Hayes and R. F. W. Pease, "The Scanning Electron Microscope: Principles and Applications in Biology and Medicine", in Advances in Biological and Medical Physics (J. H. Lawrence and J. W. Gofman, eds.), Vol. 12, pp. 85-137. Academic Press, Inc., N. Y., 1968.

2. A. V. Crewe, J. Wall and J. Langmore, "Visibility of Single Atoms." Science, 168, 1338-1340 (1970).

3. R. D. Allen, G. B. David and G. Nomarski, "The Zeiss-Nomarski Differential Interference Equipment for Transmitted Light Microscopy." Z. Wiss. Mikr. u. Mikr. Tekn. 69, 193-221 (1969).

4. J. H. Kayden and M. Bessis, "Morphology of Normal Erythrocyte and Acanthocyte using Nomarski Optics and the Scanning Electron Microscope." Blood 35, 427-436 (1970).

5. J. K. Koehler, "The Technique and Application of Freeze-etching in Ultrastructure Research", in Advances in Biological and Medical Physics (J. H. Lawrence and J. W. Gofman, eds.), Vol. 12, pp. 1-84. Academic Press, Inc., N. Y., 1968.

6. R. L. Steere, "High Resolution Stereography of Complementary Freeze-Fracture Replicas Reveals the Presence of Depressions Opposite Membrane Associated Particles of Split Membranes", in 29th Ann. Proc. Electron. Microscopy Soc. Amer. Boston, Mass. (C. Arceneaux, ed.), pp. 442-443. Claitor's Publishing, New Orleans, 1971.

7. J. M. Wrigglesworth, L. Packer and D. Branton, "Organization of Mitochondrial Structure as Revealed by Freeze-Etching." Biochemica et Biophysica Acta 205, 125-135 (1970).

8. B. Parducz, "Ciliary Movement and Coordination in Ciliates", in Int. Rev. Cytol. (G. H. Bourne and J. F. Danielli, eds.), Vol. 21, pp. 91-128, 1967.

9. E. B. Small and D. S. Marszalek, "Scanning Electron Microscopy of Fixed, Frozen and Dried Protozoan." Science 163, 1064-1065, (1969).

10. M. J. Karnovsky, "A Formaldehyde-Glutaraldehyde Fixative of High Osmolality for Use in Electron Microscopy." J. Cell. Biol. 27, 137A-138A (1965).

11. J. A. Nowell, J. Pangborn and W. S. Tyler, "Scanning Electron Microscopy of Avian Lung", in Scanning Electron Microscopy/1970 (O. Johari, ed.), pp. 249-256. I.I.T. Research Institute, Publisher, Chicago, Ill., 1970.

12. A. Boyde and C. Wood, "Preparation of Animal Tissues for Surface-Scanning Electron Microscopy." J. Microscopy 90, 221-249 (1969).

13. W. M. Manger and M. Bessis, "Cathodoluminescence Produced in Cells and Proteins by Paraformaldehyde as Seen with the Scanning Electron Microscope", in Septieme Congres International de Microscopie Electronique, Grenoble, pp. 483-484, Societe Francaise de Microscopie Electronique, Paris, 1970.

14 T. L. Hayes, "Scanning Electron Microscope Techniques in Biology", in Electron Microscopical Techniques in Biology, (J. K. Koehler, ed.), Springer-Verlag, Berlin, in press.

15. T. E. Everhart, "Deflection Modulation CRT Display." Proc. Inst. Elect. Electron. Engrs. 54, 1480-1482 (1966).

16. J. B. Pawley, F. T. Upham, A. A. Windsor and T. L. Hayes, "Color Modulation Display and an Exposure Measuring Circuit for Scanning Electron Microscope Images." University of California Berkeley Radiation Laboratory Report UCRL 19420, 68-76 (1969).

17. T. L. Hayes, "Invariance Under Transformation: A Useful Concept in the Interpretation of SEM Images", in Scanning Electron Microscopy/1972. (O. Johari, ed.), pp. 521-528. I.I.T. Research Institute, Publisher, Chicago, Ill., 1971.

18. T. L. Hayes, "Microvision", in Scanning Electron Microscopy/1971 (O. Johari, ed.), pp. 521-528. I.I.T. Research Institute, Publisher, Chicago, Ill., 1971.

19. T. E. Everhart and T. L. Hayes, "The Scanning Electron Microscope." Scientific American, 226, No. 1, 54-69, (January, 1972).

Figure Caption

Fig. 1. (X 4,000) The overall shape of the cell and membrane can be determined by SEM. Human red blood clot after deformation by clot retraction.[20]

Footnote

[20] Originally published in L. W. McDonald and T. L. Hayes, Exp. and Mol. Path., 10, 186-198, Academic Press, N. Y. (1969).

Fig. 2. (X 19,500) Membrane texture can also sometimes be shown by SEM. Microvilli on epithelial cell membrane prepared by freeze-drying technique.

Fig. 3. (X 19,500) Preparative techniques are critical for SEM if unwanted artifacts are to be avoided. Air-dried sample of same epithelial cell membrane material shown in Figure 2. Note loss of surface detail when air-drying (Fig. 3) is used in place of freeze-drying (Fig. 2).