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MICROMANIPULATION OF BIOLOGICAL TISSUE
IN THE SCANNING ELECTRON MICROSCOPE

James Binfield Pawley
(Ph. D. Thesis)
DONNER LABORATORY

October 1972

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Dedication

In memory of my father, Kenneth Francis Binfield Pawley, for years of patience and encouragement
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PREFACE

The work described herein was conducted between the fall of 1969 and the summer of 1972, while the author was a graduate student at the Donner Laboratory of the University of California, Berkeley, under the supervision of Drs. Thomas L. Hayes and Robert M. Glaeser of Donner and Dr. William Spencer (M.D.) of UCSF. The author would like to thank the Donner Mechanical Shop under Mr. Edward Dowling and the Donner Electronics Support Group under Mr. Frank Upham, as well as Mr. Parker Smiley of Physics International Co. and Mr. Duane Norgren of the Mechanical Engineering Group, Lawrence Berkeley Laboratory, for their great help in assisting the author in the design and construction of his apparatus.

Thanks are also due to Drs. Edwin Lewis, Thomas Budinger and Walter Tyler (Davis campus) for providing some of the biological specimens used to ascertain the efficacy of the technique and to Dr. Thomas Everhart for many helpful discussions, especially on the subject of charging artifacts. Jan Nowell provided the CEM micrographs of the dissected equine lung for which the author is deeply grateful. Finally, I would like to thank the Graphic Arts Departments of the Lawrence Berkeley Laboratory and Donner Laboratory for their essential contribution in preparing the figures, and Mrs. Rowena Prelock, Mrs. Beverly Heinrichs and Mrs. Dorothy Sprague for their help in typing the manuscript.
Micromanipulation of Biological Tissue in the Scanning Electron Microscope

by

James Binfield Pawley

Co-Chairman of Thesis Committee

In this thesis, the technique of microdissection is used to obtain anatomical information on an unprecedentedly small scale. Previous work was limited by the system used to view the process of microdissection: the light microscope. The light microscope image is severely limited in terms of resolution, depth of field and the fact that it "sees" through samples while in everyday life we are accustomed to looking at outside surfaces. Furthermore it is these surfaces which are most often anatomically important.

The recent introduction of the Scanning Electron Microscope (SEM) used at T.V. scan rate, while it very effectively overcomes the optical limitations of the light microscope, presents its own problems to microdissection. This is because the sample must be in a vacuum and must therefore be dry. Special techniques of specimen preparation and a unique micromanipulator design are required.

A micromanipulator capable of performing simple microdissection in the SEM has been designed and built. It consists of a piezoelectrically driven head mounted on a 33 cm long, 9.5 mm dia. stainless steel tube which can slide through an O-ring in a modified airlock cap of a JSM-1 SEM. This allows the head to be placed near the sample when in use or retracted into the airlock for removal.
The position of the head can be controlled from outside of the vacuum by coarse and fine mechanical adjustments. These either cause the tube to pivot around the O-ring seal (X and Z directions) or move a spring-loaded rod which passes down the center of the tube and is connected to the head (Y direction). The coarse adjustments are made using dovetail slides and hand-driven 40-thread/in leadscrews (X and Z) or a 2-thread/in helicoid gear (Y). The fine mechanical adjustments are made using servomotor-driven differential screws in all 3 directions. Their range is 1.25 mm with an accuracy \( \pm 1 \mu \) and a maximum speed of 30 \( \mu /\text{sec} \).

The piezoelectric head is capable of controlling each of two electro-etched tungsten needles in three directions over a range of 100 microns and with an accuracy of .1 microns. Response is instantaneous. The head also has provisions for aligning the two probe tips, prior to their insertion into the microscope, so that they can interact as a pair of pincers.

The controls for all motions in the XY plane are coordinated by means of a joy stick.

Microdissections on four separate animal tissues, prepared by critical point or freeze drying, are described to demonstrate the micromanipulator's ability to perform three distinct, though often interrelated tasks: (1) to reveal details below the surface created during sample preparation, (2) to remove parts of an SEM sample for additional study in the conventional (transmission) electron microscope, (3) to make qualitative determinations of the mechanical properties of the sample on a micro scale.
Also included is a discussion of present inadequacies and possible future improvements in SEM micromanipulation techniques and apparatus. Finally, there are three appendices and a bibliography. The appendices cover: (1) the process of making color-modulated SEM micrographs, (2) a synopsis of critical point and freeze-drying techniques, (3) the production of charging artifacts in the SEM.
ACKNOWLEDGMENTS

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INTRODUCTION

The morphological characteristics of biological systems have been widely and successfully investigated at the cellular and subcellular level using the conventional electron microscope (CEM)* and at the tissue level using the light microscope (Cosslett 1966, Sjostrand 1967, Martin 1966). Between these two levels, especially in neuronal systems, there is a range of size where the morphological and topological relationships between small groups of cells are important but hard to decipher using either of these tools (Hayes and Pease, 1968). Microscopical investigation of neuronal systems generally attempts to discover two types of information: structural features which may give clues to the operation of various sensory systems and axon-synapse-dendrite topology, the "wiring diagram" of the nervous system.

Before the advent of the CEM, the light microscope was almost the sole source of information on structural relationships in neuronal tissue aside from that available indirectly from electrophysiology. Though the light microscope is effectively limited to stainable structure, a vast amount of information on neuronal systems has been acquired using this instrument. Its major limitations, insufficient resolution and narrow depth of field, are set by optical considerations.

* Until recently, it was common to refer to non-scanning electron microscopes as transmission electron microscopes (TEM). The rise of the scanning transmission electron microscope (STEM), however, has made the name TEM ambiguous and it has been replaced in this paper by the term conventional electron microscope (CEM).
The CEM, while having more than adequate resolution, is limited to either thin sections of material or to replicas of natural or fractured surfaces. Though the internal structural features of many sensory systems have been discovered, the important topological relationships of their active membranes are not so easily investigated. Thin sections are not well suited to investigating the topological characteristics of even a single large neuron. The few studies which have been done, have required hundreds or even thousands of serial sections, to discover the configuration of a single complete cell (Karlsson et al. 1965) and have indicated by their complexity that other methods will be necessary to probe the far greater complexity of small networks of neurons. Of course, CEM serial sections offer higher resolution than the SEM and they do provide internal structural information in addition to the shape of the outer surface. However, the internal information is often of secondary importance and much of the increased resolution is lost in the process of converting the thousands of SEM micrographs into an interpretable three-dimensional model.

Because of the small size and chance nature of the area replicated, the replica technique has also not been very useful in studies of topological relationships between small groups of cells.

Part of the problem lies in the fact that these CEM techniques, like architect's drawings, display information about only two dimensions while the object itself has three dimensions. If
neurons were regularly shaped, like rooms in a hotel, architect's drawings might be sufficient. Unfortunately they are seldom this highly ordered and in general, they have more in common with a plate of spaghetti. What we need are not architect's drawings but realistic pictures of the surfaces of the spaghetti and possibly a "fork" to remove the outer strands and reveal the interior structure.

The scanning electron microscope (SEM) affords the investigator such an image and this instrument has been used to study neuronal systems with some success (Lewis, 1969a, b, Hillman and Lewis, 1971). The most important limitation was that too often the details of interest lay not only on, but also below, the outer surface which is produced during specimen preparation and which is the only surface that the SEM can "see." Though some authors (Stewart and Boyde, 1962) have used ion etching to remove the surface layers from SEM samples, the results are often very difficult to interpret and the method is not sufficiently specific to remove only the unwanted part of the sample. Instead a "fork" was indeed needed and so we decided to build a micromanipulator to enable the SEM investigator to look below the original surface when necessary. (Pawley & Hayes 1971, Pawley 1972b)

The central goal of the research reported in this thesis was to prove that such a micromanipulator could be built and that it was useful. First, micromanipulation is discussed generally and then the features of the SEM which make it a particularly suitable means of observing the process of microdissection are explained.
This is followed by criteria affecting the construction of such a device and a description of the final design of the manipulator as constructed.

The performance of the device is considered and its ability to perform three types of tasks (removal of objects to reveal subsurface detail, removal of small pieces of sample for further investigations in the CEM and qualitative determination of the mechanical properties of the sample) is discussed in some detail. The main body of the thesis ends with a short appraisal of the future of SEM micromanipulation.

Finally, two subjects which do not bear directly on micromanipulation, but which are mentioned in the main text and which are not well covered in the general literature, are included in appendices on color modulation, and the production of charging artifacts in the SEM. Another appendix is included in an attempt to summarize the relevant parts of the extensive literature relating to the drying of SEM samples.
MICROMANIPULATION

Though vision undoubtedly provides most of man's everyday knowledge of physical objects, the ability to touch them, to perturb them, to take them apart adds greatly to his understanding of how they function. Leonardo da Vinci made great discoveries in anatomy not because he looked at the human body but because he dissected it. When simple magnifying lenses made it possible for man to see objects too small to be touched conveniently with his fingers (Leeuwenhoeck, 1674), he turned to the hand-held dissecting needle. However, as improved optics provided higher magnification he discovered the necessity of interposing a mechanical device between his hand and the needle in order to reduce the magnitude of his hand motions.

The first recorded device for performing this function was built by Schmidt and reported in 1859 (Schmidt, 1859). It was very crude by today's standards but it had many useful attributes (Figure 1). It provided 3 needles which could each be moved, independently, in 3 spherical coordinates. Not only were the possible motions more precise than they would be by hand, but unlike any probe held in the hand, each needle, once positioned, required no further effort in order to keep it in the same position.

The development of micromanipulation for the light microscope is long and detailed (El Badry 1963, Kopac 1964). Only the briefest description can be given here. The applications of the micromanipulator were extremely diverse but instrumental improvements
Figure 1. Schmidt's Micromanipulator, (Schmidt, 1859).
were directed against only a few specific, though often interrelated, problems which were encountered with early instruments: (1) vibration, (2) backlash and overshoot, (3) coordinated control.

VIBRATION

Early instruments were direct mechanical linkages usually involving screws driven by the fingers. Because the linkages were usually very fragile, the tremor normally present in bodily movements was passed on to the manipulator tip. This problem was attacked in two ways which we might call the non-massive and the massive.

The first was to interpose a transducer between the actual manipulator mechanism and the fingers. The intermediate transducer was variously pneumatic (de Fonbrune 1934, 37, 39), hydraulic (Cailloux 1934), piezoelectric (Ellis 1962) or thermoelectric (depending on the expansion of heated wires) (Bush et al. 1953). The pneumatic system was perhaps the most widely used because of its simplicity and lack of overshoot (when compared to the hydraulic system with its higher moving mass). However, the piezoelectric did have the advantage of greater frequency response and the ability to be driven by electrical signals other than those derivable from simple hand movements.

The other more recent approach was to make the manipulator so massive and robust that hand tremors did not affect it (Taylor 1925, Chambers and Kopca 1950). This approach utilized modern spring-
loaded ball-bearing slides positioned by micrometers which could be driven by sensitive feedback-controlled motors (Kopac and Harris 1962). Such devices are only practical because of the technologically sophisticated component parts which have been developed in other disciplines. They are more akin to the robots used to handle radioactive materials than to the micromanipulators of the past. Their complexity is somewhat offset by the high accuracy ( < 1.0 micron) they can maintain over long working ranges (1 m.) (Alston 1971).

BACKLASH AND OVERSHEET

Backlash refers to the lag which exists between the time or position when a driving element reverses direction and the time a driven element does likewise. It is often caused by the threads of a screw bearing first against one side of the groove and then against the other. Overshoot is similar and it occurs when the driven element continues to move after the driving element has stopped. These problems are customarily controlled by spring loading the driven elements so that, for instance, the screw is always riding on only one side of the groove. In the control of backlash, the size of the spring depends on the friction associated with the motion of the driven element. More friction requires a bigger spring and creates a greater load for the driving element. As large driving elements often produce greater vibration, especially if they are motors, low friction in the motion of the driven element is very important.
In the non-massive manipulators mentioned above, it was common to mount the driven element directly on springs so that the friction involved was the small internal resistance of the springs themselves. While this solves the problem well enough, the springs involved are quite weak making the driven end susceptible to vibration other than that associated with the driver (environmental vibration). The massive type manipulators, however, require low friction ball (or sometimes air) bearings combined with springs.

Overshoot, on the other hand, is proportional to the momentum of the moving mass. It can be reduced by increasing the friction associated with the motion of the driven element but, because this increases backlash (see above), it is common to limit either the mass (non-massive systems) or the velocity (massive systems) of the driven element.

COORDINATED CONTROL

In all the manipulators mentioned above it has been necessary to resolve the motion of the manipulator into at least three orthogonal or semi-orthogonal spatial coordinates. Each coordinate has separate control and driving elements. In order to produce motions involving movements along more than one axis at a time, the controls for 2 or 3 axes are combined. Usually this combined control is a so-called joy stick, the handle of which can move anywhere within the circle. It is connected to the control pistons (hydraulic or pneumatic) or potentiometers (providing either
thermoelectric or piezoelectric driving or servo reference voltages) in such a way that each position within the circle corresponds to a certain position of the driven end and so that motions of the control stick produce motions of the driven end which are similar in shape though smaller in magnitude. Control of the third axis can be achieved by turning or extending the handle of the joy stick.

The responsiveness and precision of these controls greatly affect the ease of use of the manipulator and much work has been done on this aspect of design.
THE SUITABILITY OF THE SEM FOR VIEWING MICRODISSECTION

The SEM is virtually the first instrument to challenge the light microscope as a means of viewing biological structure on the micron scale. This section examines some of the reasons for this, while explaining why it is also an ideal method for viewing micromanipulation. The general design and principles of operation of an SEM are by now widely understood and the reader is referred to several excellent review articles for further details (Oatley et al. 1965, Hayes and Pease 1968, Carr 1971, Everhart and Hayes 1972). We shall deal here only with those aspects of the SEM which make it the most effective means of observing the process of dissection on a truly micro scale.

SECONDARY ELECTRON MODE IMAGES

Coding

The most commonly used mode of SEM operation and the one used predominantly in this investigation makes use of the low energy (< 50 eV) secondary electrons ejected from the sample by the impinging primary beam. The number of electrons from any particular data point is a function of many variables (Clarke 1971) but the most important of these is the angle, \( \theta \), between the local specimen normal and the beam. In general this will be a monotonically increasing function between \( \theta = 0^\circ \) and \( \theta = 90^\circ \). This relationship is important because it closely parallels that governing the apparent brightness of a diffrusely illuminated, flat, white macroscopic surface as it is tilted at different angles to the line of vision. For this reason, the image produced
when the secondary electron signal is allowed to intensity-modulate the display cathode ray tube (CRT) appears as though it is a picture of a macroscopic object viewed by diffuse illumination.

The importance of this particular coding mechanism can be seen by comparing the two parts of Figure 2. The upper half shows a normal intensity-modulated image of the corner of the compound eye of a Tribolium Confusum. It has the realness we normally associate with scanning electron micrographs. In the lower images, however, the same secondary electron signal information is displayed using color modulation. This process (Pawley et al. 1969) was developed by the author and his colleagues and in it the video signal is electronically discriminated so that only signals lying between two specific voltages are displayed at any one time. (See Appendix I.) By placing this electronic window over different regions of the video signal on four successive sweeps and by coupling these changes in window with arbitrary changes in the type of filter used to record each sweep, the lower micrograph was recorded using Polacolor film. This micrograph may appeal to the viewer for other reasons but not because it resembles the images associated with normal experienced vision. The comfortable feeling of familiarity is lost simply because, though the information in both pictures is the same, the coding method used to display the information has been changed. The magnitude of the loss in interpretability is a measure of the importance of this particular perceptual code.
Figure 2a. Intensity modulated image of the eye of *Tribolium confusum*. 2b. Color modulated images of the same sample.
This type of image of micron-sized objects cannot be produced by any other type of microscope.

Other Characteristics of Scanning Electron Micrographs

There are other reasons, aside from the way the information about the specimen surface is coded, which enhance the feeling of realism of an SEM micrograph and they include the following:

(1) Outside surface seen: overlap code

Because the radiation must be spatially focused after it interacts with the sample in both the CEM and the high resolution light microscope, specimens for these instruments are usually transparent to the radiation used. As a result the observer finds himself looking through the specimen, not at its outside surface as is the case with normal, experiential vision.

In the SEM, however, the information is obtained from the outside surface and just those parts of the outside surface that would be seen by an observer looking at the sample from the objective lens aperture. This allows us to take advantage of the fact that, in experiential vision, we normally observe these same parts of the outside surfaces and we have developed mental image-processing procedures such as the overlap code to extract useful information from images of such surfaces (Rubin and Walls, 1969).

(2) Collection shadowing

The collection of secondary electrons usually involves their being drawn to a positively charged electrode situated to the side of the sample. If we imagine a sample consisting of a small sphere resting on a flat plane it is reasonable to assume that electrons
emerging from a part of the plane such that their trajectory to the collector is intersected by the sphere will be less likely to be collected. Therefore this area will appear darker in the micrograph. In practice, it is just as though the sample was a real macroscopic object being illuminated from the position of the collector with the sphere casting a shadow onto the plane. The resulting micrograph is more understandable because our brain is accustomed to decoding useful information from such shadows (Rubin and Walls 1965).

(3) Depth of field

Up to now, we have considered that the high degree to which the SEM image mimics the light image of experienced vision, was an index of its excellence. We have neglected to mention why some sort of light microscope might not provide equally interpretable images with far less fuss. The diffraction limit on resolution of about 0.2 microns in the light microscope compared with about 0.02 microns for an optimum secondary electron image in our SEM, is, of course, a consideration. It is made somewhat less important by the fact that this resolution drops to .08 -.12 microns when using T.V. scan rate and the long working distance required for micromanipulation. Also, as we have already indicated, on a micro scale, light does not interact with matter as it appears to do on a macro or experiential scale. (We "see" through many thin things rather than just observing their outside surfaces.) A third important consideration is that the optics of our eyes have made us accustomed to a depth of field far greater than that afforded by
a diffraction limited light microscope. In all these respects, the SEM parallels our macro experienced vision far more closely than is possible with high magnification light optics. The CEM is similar to the SEM in depth of field but it is limited to thin samples and therefore this virtue can only be utilized on replicas (see (4) below) while low power "dissecting" microscopes have greater depths of field but limited resolution.

(4) Bulk sample

It is almost trivial to point out that all the coding mechanisms above would be almost useless if we were confined to observing the thin samples used in the transmission electron microscope. Carbon replicas, shadowed with metal, also produce very realistic images, but one will learn little by dissecting a replica. There are no theoretical limitations to the size of sample which can be viewed in the SEM because it need not be transparent to the electron beam and therefore large pieces of biological tissue can be viewed.

(5) True stereoscopic images available

Partially as a consequence of (3) and (4) above, truly stereoscopic images of SEM samples are possible. The techniques for producing them are widely known (Boyde, 1970) and they basically involve taking pictures of the same area of the sample from two different angles and then viewing these pictures with a mirror stereoscope or similar apparatus. The required change of viewing angle (7 - 10 degrees) is usually accomplished by tilting the specimen between micrographs. Another alternative (Dinnis 1971) is to electronically tilt the SEM beam so that it approaches the
sample from different angles. This second approach not only holds the promise of 'live' stereo at T.V. frequencies but also would allow the position of the manipulator probe above the sample to be ascertained. This is not possible in stereoscopic images made by tilting the specimen since the probe does not tilt with the specimen. (Boyde 1972)
Prior to the advent of the micro-electronics industry, few man-made systems required the fine motions provided by a micro-manipulator. Though a certain amount of work was done on micro-chemistry and the investigation of fine particulate inorganic material, virtually all manipulators were made to operate on biological specimens. For optical and biological reasons these samples were usually wet.

However, to preserve the high vacuum in the SEM, all specimens must be dry, at least near the surface where any dissection must take place. Methods for freeze or critical point drying of biological tissue with little shrinkage or distortion are well known (Boyde and Wood 1969, Cohen et al. 1968, Anderson 1951), and are briefly discussed in Appendix II. However, as many of the structural features of biological systems depend on the presence of water for their integrity, it was reasonable to assume that dried tissue might cleave in a different and hopefully more interesting way than wet tissue. Preliminary investigations of freeze-dried tissue under both the dissecting microscope and the high resolution light microscope were inconclusive as it was difficult to see where the breaks were actually occurring. It was evident, however, that pieces of carefully dried bulk tissue are often very tough and elastic. This implied that small blocks of tissue were preferable to large ones and that at least two
probes would probably be required: one to hold the bulk and one to pull away pieces of it.

Previous manipulators had made great use of glass micro tools which would be easily formed into diverse useful shapes on an apparatus known as a micro-forge (de Fonbrune 1934). Unfortunately the glass tips were found to lack the rigidity required for use on dried tissue and tungsten tips were chosen instead. Such tips can be easily formed by immersing the tip of a piece of tungsten wire in a solution of 1.0 N NaOH and applying 4-6 volts a.c. between it and some other large electrode in the same solution (Figure 3a) (Hubel 1957). The tip will slowly etch away as seen in Figure 3b. The process can be stopped at any time to obtain tips of varying geometries (Figure 4). Such tips are not only stronger and more rigid than glass, they also conduct well and so do not require coating with metal to avoid charging up in the SEM primary beam. They are still very delicate objects, however, and the manipulator must be built so that they can be replaced and realigned easily.

CONSTRAINTS IMPOSED BY THE SEM

Five different types of constraints on micromanipulator design are imposed by the SEM itself.

(1) Vacuum

A vacuum of about $5 \times 10^{-5}$ torr must exist in the vicinity of the specimen. Therefore, no part of the manipulator inside the column may affect or be affected by this vacuum.
ETCHING TUNGSTEN TIPS

A.

Power supply 0-6.3 V ac

Electrode

Clip

1.0 N NaOH

10x magnifier

0.02" tungsten wire

B.

Figure 3a. Apparatus for electro-etching tungsten tips.

3b. Tip profiles showing how differing geometries can be obtained by varying etching time.
Figure 4. Micrographs of two etched tungsten tips. A slender tip at 2500X (a), and 8000X (b), and a shorter tip at 2500X (c), and 8000X (d).
(2) **Stray fields**

Stray electric (> $10 \frac{V}{cm}$) or magnetic (> 20 g) fields near the specimen may deflect emerging secondary electrons and prevent them from being collected. This must be avoided.

(3) **Limited space**

There are certain advantages to mounting the most precise and responsive drive elements of the manipulator near the sample. Basically the smaller the moving part of the device, the easier it can be controlled. By mounting the drive near the sample, the size of the moving parts can be substantially reduced. Unfortunately, though the specimen chamber of an SEM is much larger than that of a CEM, it still is not very spacious. For electron optical considerations it is desirable to have the specimen surface not more than 2.5 cm below the lower pole piece of the objective lens. If the probe tip of the manipulator is to approach a level sample at some convenient angle, say 30°, then there is very little space for a drive mechanism to be installed. The drive mechanism could, of course, be put to one side or below the sample and curved needles used, but such needles are harder to fabricate and not as strong as straight needles. Alternatively the sample could be tilted slightly toward the manipulator, however, this usually entails tilting it away from the collector and reduces the efficiency with which the secondary electron signal can be collected from the sample. For these reasons, any drive mechanism mounted near the sample will have to be small ($\approx 1.5 \times 2 \times 2$ cm).
(4) **Limited access**

This problem could be sidestepped by somehow mounting the entire manipulator on the specimen stage. Access to it (necessary to change probe tips) would then be obtained in the same way that the specimen was exchanged. However, we felt it important to have the movements of the specimen (X and Y translation, rotation, and tilt) independent of the motion of the manipulator (see next section, Degrees of Freedom). Therefore, it must be anchored to the side of the specimen chamber in some way that permits access to the probe tips.

(5) **Resolution**

In the T.V. mode, which is necessary for continuous viewing of manipulator motions, the resolution of the author's SEM is limited to about 0.1 microns. Accuracy on this order of size was a requirement for the SEM micromanipulator.

**OTHER CONSIDERATIONS**

Very briefly, given the constraints mentioned above, we wished to retain as much as possible the ease of use and the precision of earlier manipulators made for use with the light microscope.

**Controls**

Vibration, backlash and overshoot should be held to a minimum and controls should be coordinated wherever possible. Ideally, one would like to be able to maintain maximum accuracy of control over the greatest possible range. Leaving aside the problem of developing transducers having high accuracy and long range, the
controls for such transducers would also pose problems. For instance, a transducer operating over a range of 1 mm with an accuracy of 0.1 micron requires a control which can be set within one in $10^4$. This is far beyond the capabilities of a simple joystick which is capable of one part in 400 at best. The simplest way out of this dilemma is to have two separate joysticks operating in series; one covering the full range and the other covering only a small part of it. Given this constraint on the controls, it could be appropriate for the transducer also to be divided into coarse and fine components.

Degrees of Freedom

A final consideration is the number of degrees of freedom required in the movement of the probe with respect to the sample. Specimen stages in SEM's commonly have between three and six (three translations and three rotations) degrees of freedom (Houghton et al. 1971) and in fact quite useful studies can be made by simply using the stage controls to move the sample past a fixed probe (Smith 1956, Gane and Bowder 1968, Hepworth et al. 1969). Such simple approaches are limited by the difficulty of making complex coordinated movements with the stage controls and by the difficulty of aligning the probe directly on the lens axis so it can be "viewed" with the electron beam. For this reason it is desirable for the probe itself to move and for it to have as many degrees of freedom as possible. The effect of each control on the position of the probe tip should be as independent as possible of the other controls. A consequence of this requirement is that any rotational degrees of freedom must have their axes pass through the probe tip. This is
very difficult to achieve and as a consequence previous micro-
manipulators as well as the model described here have confined
themselves to the three translational motions. It is possible,
however, to combine any rotational movements available on the stage
with the three translational movements of the manipulator as long
as the manipulator mechanism doesn't interfere with the stage
motion. This is especially useful since some SEM stages are
designed so that all axes of rotation intersect at the point on
the sample which is being observed.

In this way, up to six degrees of freedom between the sample
and one probe can be obtained although the range on the rotational
motions is often somewhat restricted by the requirements of signal
collection and by mechanical interference with the manipulator.
The stage translation controls are of course still needed to place
the area of the sample to be dissected within the field of view.
SEM MICROMANIPULATOR DESIGN

OVERALL DESIGN

The most important single influence on the final design (Figures 5, 6, and 7) revolved around the problem of replacing the probe tips easily. We planned to have at least two of these mounted on the manipulator. They would require precise alignment with each other and this could not be accomplished while they were inside the vacuum. Unlike many modern SEMs, the one available to the author* provided only a small airlock for specimen exchange rather than allowing for one entire side of the specimen chamber to open so that the specimen could be exchanged manually. In other words, whereas in many modern microscopes access to any apparatus mounted near the specimen could be had merely by opening the side of the specimen chamber, as if to change specimens, in our microscope such access could only be gained by a rather complicated disassembly of the microscope.

For this reason it was decided that the manipulator must enter the specimen chamber through the airlock. Once this had been decided, the other major features of the design followed. To reach the specimen area from the airlock would require a long shaft (Figure 5). To reduce the effects of vibration it would have to be rigid and therefore heavy, and as a result, it would be necessary to control it with the massive drive system: motor-driven

* Japan Electron Optics Co. JSM-1 (modified) with simple goniometer stage (X, Y translation, tilt, rotation.
Figure 5. A sketch showing the spatial relationship between the main stainless steel shaft, the airlock, the specimen and the mechanical drives.
Figure 6. The manipulator mechanical drive assembly viewed from below.
2.54 cm dia., 24 tooth pinion wire gear attached to differential screw barrel

Motor mounting barrel

Differential screw

Bellows coupling to restrict motor rotation

Motor and transmission

Pin in slot

5.08 cm dia., 48 tooth potentiometer drive gear

5k Control potentiometer

20 turn 5k potentiometer

5.1 V dc

Figure 7. Differential screw drive with feed-back network.
micrometers. If the shaft were allowed to pivot and slide in its vacuum seal, the drive system could be mounted outside the microscope where size and vacuum considerations are not so restricting. We needed controls for two needles, however, and as two such bulky systems could not be mounted on one airlock and because mechanical systems involving shafts of this length and mass are somewhat limited in their accuracy and frequency response, a dual piezoelectric drive system would be mounted on the end of the shaft near the specimen.

MECHANICAL DESIGN

Let us start with a modified airlock cap having in it a hole with an O-ring gasket through which passes the main shaft. (Figure 6, right inset.) The shaft of stainless steel tubing (O.D. = .954 cm, I.D. = .47 cm) is capable of pivoting on the O-ring and of sliding back and forth through it, thereby allowing the piezoelectric head to be positioned near the sample or retracted into the airlock for removal. Figure 5.

When the modified cap is mounted on the airlock the distance from the O-ring to the sample is about 20 cm. If the drive mechanisms for the angular position of the shaft (X and Z) were mounted near the O-ring but outside the vacuum, their purposely small displacements would unfortunately be increased by the mechanical disadvantage of the lever system composed of the shaft and the O-ring fulcrum. For this reason the X and Z drive mechanisms are attached to an aluminum plate which is mounted 18.5 cm behind the O-ring and are held there by a 7.2 cm diameter brass tube attached to the modified airlock cap, Figure 6.
The Mechanical Drive System

The purpose of the three Mechanical Drive Systems (X, Y, Z) is to position the piezoelectric head near the sample by acting on the outside end of the main shaft either by pivoting it about its O-ring seal (X, Z) or by pushing on a spring-loaded rod passing down the center of the shaft (Y). A two-stage system was developed. A coarse stage for positioning the head in the area of interest and a fine stage capable of carrying out coordinated motions on a scale about ten times larger than possible with the piezoelectric drive.

1. Coarse Mechanical Drive

To allow for changing specimen geometries and for variation in the size and mounting of the replaceable probe tips (needles), a coarse adjustment of about 1 cm was required along each axis. This adjustment could be manually driven and needed no more than 50-micron accuracy. It must be rigid, however, and be connected mechanically in series with the fine motion so that the final setting is the sum of the coarse plus the fine setting.

Dovetail slides (2.54 cm nominal width, 1.27 cm travel and 7.62 cm guide length*) were chosen for the coarse controls in the X and Z directions because of their rigidity and economy. Though high in friction (and therefore in backlash), these slides have sufficient accuracy when driven by hand using a 40-thread/in lead screw. To reduce the transfer of vibration from the hand to the drive system, the knobs controlling the lead screw were mounted on .317 cm diameter flexible shafts.

* 3" x 1" Unislide, Velmex Inc., East Bloomfield, Holcomb, N.Y., 14469.
As the controls in the Y direction must act down the axis of the shaft, it was found most convenient to mount the Y mechanical drive on the end of the shaft itself. Mounting a dovetail slide here, however, would be clumsy and a more convenient method is to use the focusing mount of a 16-mm movie lens. The one used had a usable travel of about 1 cm in 1.8 turns.

(2) Fine Mechanical Drive

The requirements of each of these systems are that it position the outside end of the main shaft or the Y control rod passing down the center of this shaft with an accuracy of about 3 microns over a range of about 1 mm and that it do this in such a way that controls for motions in the two directions lying in the plane of the SEM image can be coordinated. Figure 7.

The simplest type of drive mechanism capable of providing this accuracy over this range is a differential screw. A differential screw uses a shaft which is free to rotate and which has two precision threads of slightly varying pitches, each one ground onto one half of its length. The nuts which fit on these threads are fixed so that they cannot rotate but can slide axially with respect to each other. If one of the two nuts is also fixed axially and the threaded shaft is rotated, the second nut will move axially the distance that the shaft has moved through the first nut less the distance the second nut has moved up the shaft. Mechanisms of this type are available having total ranges of .25 mm and 1.25 mm in 40 turns. We used the second type.*

* #22.505 Lansing Research Corp., 705 Willow Ave., Ithaca, N.Y. 14850.
The main disadvantage of differential screws is that they have five to twenty times the friction of simple micrometer screws and special lubricants* along with a general loosening of the adjustments on the female threads were needed in order to keep the required motors small.

These motors** and their accompanying metal transmissions (141:1***) were capable of driving the screws at approximately 60 RPM or 30 microns/sec. The resulting motor speed $141 \times 60 \text{ RPM} = 8460 \text{ RPM}$ is near the limit of the motor's range. The motors are mounted in stainless steel shells having two $2.5 \text{ cm} \times 0.316 \text{ cm}$ slots on opposite sides of the shell and to the rear of the motor, Figure 8. A $0.310 \text{ cm}$ diameter pin in these slots supports the rear end of the shell and restricts rotation of the motor-transmission while allowing the shell to slide in and out as the screw handle unwinds. Torque is transferred from the transmission output shaft to the rear of the screw through a flexible copper bellows to prevent binding and to support the front end of the motor mounting shell.

As the motors are not free of overshoot and other mass effects, a feedback loop surrounds them. The details will be discussed in the Controls section and all that need concern us now is the sensing element: a gear-driven potentiometer. The differential screw handle turns 40 times and moves $2.54 \text{ cm}$ axially in covering its range; however, as 40-turn potentiometers are bulky and expensive,

* STP and Convoil 20 (vacuum pump oil); 1:4.
** Motor #250/007, 6 volts, 0.3 amps with 141:1 all-metal transmission, Micro-Mo-Electronics, 3691 Lee Rd., Cleveland, Ohio.
a 20-turn potentiometer* is gear driven at a 2:1 reduction to cover this range. To allow for the axial motion of the screw handle, the 2.54 cm dia. driving gear attached to the handle is made of pinion wire and is 3 cm long.

The entire fine mechanical drive for either the X or the Z direction is mounted on a carriage which is attached to the moving part of one of the dovetail slides. These are in turn mounted on the aluminum plate mentioned previously. On the Y direction the screw is mounted directly on the rear of the focusing mount while the potentiometer and the pin restricting motor rotation are attached to a tube (3.7 cm dia. 17.8 cm long) which is also attached to the rear of the focusing mount.

(3) Mechanical Linkage

To transmit to the shaft the orthogonal displacements created by the X and Z mechanical drives, the small end of a teardrop-shaped connecting link is connected to the output piston of each differential screw with a loose pin so that it is free to rotate slightly in the X, Z plane. The shaft passes through a hole in the center of the link’s large end and a guide prevents the links from binding when the shaft is advanced or retracted. This large hole has a cutout on one side into which a key (attached to the shaft) fits when the shaft is advanced. This restricts shaft rotation which would otherwise occur when the Y coarse control (camera focusing mount) is turned. As the manipulator is designed so that the needle tips are almost on the axis of the shaft, small rotations caused by imperfections of this system are not an important problem.

* International Rectifier Corp., #1220-1454B, 5 K ohms.
Backlash is controlled in the Z and X directions by mass loading of the Z linkage and by a spring between the X carriage and a metal ring encircling the main shaft. The axial rod, which the Y drive system bears upon, is also spring loaded. (Figure 6, left inset.)

PIEZOELECTRIC DRIVE

History

The reverse piezoelectric effect was first described by the Curies (Curie and Curie 1880). On theoretical grounds, Lippmann then proposed the piezoelectric effect (Lippmann 1881) the existence of which was soon verified (Curie and Curie 1881). Good reviews by several authors are available. (Nye 1957, Lion 1959, Jaffe 1966)

Basically, it concerns the distortion of an ionic crystal lattice produced by an applied electric field and resulting in a change in the shape of the crystal. The effect is very small; at fields approaching dielectric breakdown (in practice about $2.5 \times 10^4$ V/cm) changes in length are usually on the order of 0.1 per cent (Kittel 1967). Although effective single crystal piezoelectric materials such as Rochelle salt can be grown quite large, they are fragile, unable to be formed into complex shapes and thermally unstable near room temperature (Kittel 1967). As a result many piezoelectric devices are made of ceramics of lead or barium zirconate and/or titanate. These ceramics can be formed, molded or ground to shape first and then artificially polarized by imposing a high electric field in the same way that a ceramic magnet is polarized in a magnetic field. In fact, such piezoelectric ceramics are sometimes referred to as electrets.
Figure 8. Dual-needle piezoelectrically driven head viewed from below and showing the three crystals controlling the lower needle. Insets show how the $r$, $\theta$ and $\phi$ adjustments operate to allow the probe tips to be aligned. The electrical connections and electrostatic shield have been omitted for clarity.
Figure 9. A single piezoelectric subunit. The crystal is held between two lucite end caps, one of which can be adjusted to hold it firmly. Expansion of the crystal produces motion through an 8:1 lever arm.
Most problems associated with the design of electrical to mechanical piezoelectric transducers revolve around the small size of the effect. Ellis (Ellis 1962) solved this problem by using devices called bimorphs (Sawyer 1931). These are composed of two thin crystals which have different directions of expansion with a given applied voltage and which have been glued together so that the resulting sandwich either twists or bends when a voltage is applied. Ellis used twister bimorphs attached to a very delicate system of levers in his apparatus and was able to obtain a working range of 0.6 mm. Unfortunately his system was far too bulky either to install in our specimen chamber or to pass through the microscope airlock.

In view of these disadvantages, we should explain the reasons for choosing piezoelectric transducers. It had been decided that the drivers for the finest movement should be near the sample and this cannot be done with mechanical, pneumatic or thermal-electric systems because of vacuum considerations and a lack of space. This left piezoelectric and magneto-strictive drivers. The former was considerably smaller, easier to shield, and dissipated less power. It was chosen on this basis.

Subunits

The system we finally adopted is shown in Figure 8. The principle of operation is very simple and is based on 6 subunits (3 for each drive unit) such as the one shown schematically in Figure 9. A tube of high strain ceramic crystal* (1.27 cm long, 0.63 cm O.D., 0.49 mm I.D.) is held between a fixed L-shaped

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* Part 8-4020-5H Vernitron Piezoelectric Division, 232 Forbes Rd., Bedford, Ohio, 44146.
member and a moving 7.5:1 lever arm which is attached to the fixed member by a leaf spring. Lucite caps serve to hold the crystal in place and to insulate it from the stainless steel frame. One of the caps is threaded into the frame to provide an adjustment to assure intimate contact between the crystal, the caps and the lever arm.

The tubular shape was chosen for several reasons, the first being strength. Ceramics are very brittle; however, in the configuration described above all the loading is compressive and axial and the chance of damage is small.

An equally important consideration is electrical. If one wishes to produce a useful motion of approximately 10 microns one will need a crystal at least \( \frac{10^{-3} \text{ cm}}{1 \text{ cm}} = 1 \text{ cm} \) long. To produce the maximum required field of \( 2.5 \times 10^4 \) volt/cm one could apply \( 2.5 \times 10^4 \) volts between electrodes on the ends of a 1 cm crystal or alternately one could apply \( 2.5 \times 10^3 \) volts to a stack of ten 1 mm thick crystals with electrodes in between and with the correct polarization to allow them to be excited in parallel. The first of these alternatives would require high voltages and the second would require rather complex fabrication. A third alternative is to take advantage of the fact that the piezoelectric effect is almost isovolumetric; that is to say increases in length in the direction parallel to the field are offset by decreases in the directions perpendicular to it and vice versa. For this reason a thin radically polarized tube produces a total motion proportional to its length but requires a voltage proportional only to its wall
thickness. The tubes used in our subunits had an 0.5 mm wall thickness and a maximum applied voltage was +1350 V dc.

A third advantage of the tubular design is that, if the outer electrode is grounded, stray fields resulting from the ungrounded (inner) electrode are much smaller than would be the case in the other configurations described.

To avoid depolarizing the ceramic, reverse bias is limited to only -350 V producing a field \( D = \frac{350 \text{ V}}{0.5 \text{ mm}} = 7 \times 10^2 \frac{\text{V}}{\text{cm}} \). Between these extremes (+1350 to -350 V dc) the response of the crystals is fairly linear and the total displacement is approximately \( 1.5 \times 10^{-3} \text{ cm} \) resulting in a motion at the needle tip of approximately 0.1 mm as desired.

Because the field present between the inside and outside electrodes at the ends of the tubes is well above the breakdown field both in air and in the vacuum which we use, the end tubes must be covered with an insulator. At first we used a hard wax* applied by touching the freshly sanded end of the tube to a small amount of wax which had been melted on a heated glass slide. Unfortunately, though effective in suppressing electrical breakdown, the wax had a tendency to creep and allow the crystals to loosen. To remedy this, the wax was replaced with a standard electrical grade epoxy** with satisfactory results.

* Stickywax®, Corning Rubber Co., 577 Gates Ave., Brooklyn, N.Y.

** Epocast 202, Furane Plastics Inc., 16 Spielman Road, Fairfield, New Jersey, 07006.
Mechanical Connections

Each set of three subunits is arranged as shown in Figure 10 with the result that the probe tip moves the vector sum of the motions of the independent subunits. The leaf springs not only provide support but also eliminate backlash. These motions are unfortunately more curvilinear than rectilinear at the probe tip. To reduce this effect, the axes of motion have been moved as far from the probe tip as possible considering the limited space available.

Electrical Connections

Connections to the electrodes on the crystal surfaces are made using 7 x 45 stranded wire* having a polyethylene insulation sufficient to withstand 1500 V dc. The actual connections make use of conductive epoxy on the inside electrodes and clips made of gold-plated stainless steel wire on the outside. A small multiconductor "strip" connector mounted on each side of the head,** Figure 11, facilitates rapid connections when the head is removed from the shaft to replace or adjust the probe tips.

* Alpha Wire Corp., #1101 Stereo.

** Strip connector - Amphenol, Model #221 with Fire-Form contacts.
Figure 10. A single piezoelectric drive unit composed of three subunits interconnected in such a way that the probe tip moves the vector sum of their individual displacements. The axis of rotation and resultant motion produced by each crystal is indicated.
Figure 11. Dual-needle piezoelectric head with electrostatic shield removed.
As each of the piezoelectric drives has a range of only .1 mm, it is necessary to mount the tips of the probes quite close together so that they can interact as a pair of pincers. The following is a description of the mechanism which makes this possible.

First, it was necessary to find a convenient method of attaching the probe tips to the drive mechanism. As we used .512 mm tungsten wire for the tips it was found convenient to slide them into sections of a #22 hypodermic needle. The way these hypodermic needles are used can be seen in Figure 8.

To bring the tips together three orthogonal degrees of freedom are required. As it is difficult to apply all these adjustments to one needle holder in the limited space available, the radial (r) adjustment is applied to the upper needle and the $\theta$ and $\phi$ adjustments to the lower needle.

**Radial Adjustment**

The upper hypodermic needle is soldered to a small ring of metal which acts as a piston and rides in a cylinder, like the moving part of a bicycle pump. The metal ring is held against the end of a #0-80 hex-head screw with a small spring (Figure 8, lower detail). Turning the screw moves the needle in or out with an accuracy of 0.01 mm over a range of 5 mm.

**Angular Adjustment**

The lower hypodermic needle is soldered to a short piece of .51 mm dia. stainless steel spring wire. It is bent in two
separate directions by a $\Theta$ adjustment screw acting on a small metal block soldered to the needle and a $\phi$ adjustment screw acting through an 0.15 mm thick, flat, brass spring. The needle passes through a slot in this spring which is parallel to the $\Theta$ direction and perpendicular to the $\phi$ direction so as to allow the two adjustments to work independently (Figure 8, upper detail). Referenced to the probe tip, these controls provide an accuracy of 0.02 mm over a range of 3 mm.

Figure 12 shows a view of the entire assembly mounted on the microscope including the control console, T.V. monitor, and video tape recorder. Figure 13 shows a more detailed view of mechanical drive system attached to the specimen chamber airlock.
Figure 12. Equipment layout. (1) Micromanipulator control console in front of SEM controls. (2) Video tape recorder with remote control (3). (4) Signal monitoring oscilloscope for setting CRT brightness and contrast. (5) Micromanipulator mechanical drive assembly mounted in airlock at base of SEM column.
Figure 13. Close-up of the mechanical drive system mounted on the microscope.
CONTROLS

MOTOR CONTROLS

The position-referenced feedback control systems used for the three motors are nearly identical electrically. The theory of such systems is widely known and has been reviewed. (Wiener 1961, Kuo 1962) Mechanically, the control potentiometers for the X and Y directions are coordinated by use of an Army-surplus joy stick and other associated controls explained later, while the Z control is a single 10-turn potentiometer (pot.).

A schematic diagram of one complete control is shown in Figure 1, while block diagrams are shown in Figures 6 and 7. The integrated circuit (IC) high-gain amplifier (#IM-201) amplifies the difference between a sensed voltage and a reference or control voltage. If this difference is not zero, a transistorized power amplifier composed of $T_1$, $T_2$, $T_3$, and $T_4$ drives the motor in the correct direction to reduce it. The gain of the amplifier is adjusted with resistor $R_1$, a 25-turn trim-pot., so that critical damping without overshoot can be obtained.

The sensed voltage is obtained from the sliding contact of the 20-turn, 5 kilohm pot. mentioned in the section on Fine Mechanical Drive. This pot. is connected across a +5.1 V reference power supply as is the circuit which produces the control voltage. For the Z direction, this control voltage is set with a 10-turn, 10 kilohm pot. but in the X and Y directions a more complicated system is used.
The gearing of the joystick is such that standard one turn 10-kilohm pots are turned through their full 270° range as the stick moves across its full 90° range. By careful manipulation, the stick can be positioned with an accuracy of 1.0% of the total range. Referred to the probe tip this corresponds to an accuracy of 1% × 1.250 mm = 12.5 microns which is insufficient. For this reason the X and Y control voltages are each derived from a set of 3 pots. One, referred to as Fine, is attached to the joystick, a second, also 10 kilohms is referred to as Coarse and a third 100 kilohms is referred to as Magnitude. The purpose of this last is to vary the fraction of the total range (1250 microns) over which the Fine control operates from a maximum of 90% (1125 microns) with an accuracy of 1.0% (1125) = 11.25 microns, to a minimum of 10% (125 microns) with an accuracy of 1.0% (125) = 1.25 microns. The Coarse pot controls motion over that part of the total range not controlled by the Fine control. The arrangement is not ideal since changing the Magnitude setting also results in a small change in the control voltage. This means that the manipulator translates slightly as you change the range over which the joystick controls operate. However, the design is simple and works sufficiently well in practice.

PIEZOELECTRIC CONTROLS

The six piezoelectric controls are very simple and each consists of a 5-megohm pot connected between power supplies of +1350 V dc and -350 V dc with the sliding contact connected to the
MICROMANIPULATOR MOTOR CONTROL CIRCUIT

Figure 14.
inner electrode of the appropriate crystal. Two joy sticks coordinate the X and Y controls for the two separate drive assemblies while the Z controls are separate 1-turn 5-megohm pots. As the total voltage across these pots is $350 + 1350 = 1700$ V dc, which is well above the rating of most single turn pots, some care was necessary in choosing the actual units to be used.*

**POWER SUPPLIES**

The motor controls are powered by the regulated power supply shown in Figure 13. The ICs require ±15 V dc while the amplifiers use 6 V dc supplies so that the voltage ratings of the motors will not be exceeded.

The +1350 V dc supply is a simple voltage doubler circuit with zener diode regulation (ripple > 0.1%). The particular capacitors we used are oil-filled and rated very conservatively; as a result their maximum voltage can be slightly exceeded without damage. Because of their greater availability, large numbers of low-voltage diodes, both zener and normal, are used in series in place of single units with higher voltage ratings. The -350 V dc supply uses a half-wave rectifier followed by filter capacitors and zener diode regulation (ripple < 0.1%). None of the components of the power supplies are rigidly specified and others having similar specifications could be substituted without affecting the performance.

* Allen-Bradley, Type J, 5 megohm.
Figure 15.  HIGH VOLTAGE POWER SUPPLY FOR PIEZOELECTRIC HEAD

[Diagram of high voltage power supply with labels and components: 115 V ac, 470 V ac, 43 k, 51 k, 22 k, 10 mA, 8 N989, 5 M, 1N2225, Crystal 1, Crystal 2, Piezoelectric controls, ALL DIODES 1N2225, +1350 V, -350 V]
USES OF AN SEM MICROMANIPULATOR TO INVESTIGATE BIOLOGICAL TISSUE

LIMITATIONS IMPOSED BY RADIATION DAMAGE

Ideally, one would like to be able to use an SEM micromanipulator to perform, with increased spatial resolution and ease of observation, all the functions of a conventional manipulator. In practice this turns out to be impossible because many uses of conventional manipulators, especially those involving electrophysiological observations, require that the specimen be wet and alive. Although it may be possible to construct a "wet" specimen stage for the SEM capable of preventing a drop of water from evaporating in the $10^{-5}$ torr. vacuum to which it is exposed (Lane 1970*), it has not yet been demonstrated that biological samples can be kept wet without the water obscuring surface detail. In any case, the limiting variable of the specimen environment for live samples is not the availability of water but presence of large amounts of radiation. It has been noted that even at the comparatively low primary beam currents ($10^{-11}$ A) required at normal scanning rates (1 sec/frame - 40 sec/frame), the radiation dose to the upper few microns of the sample is on the order of $10^9$ rads/sec. (Pease et al. 1966). At T.V. scan rate, which is likely to be used to view micromanipulation, the dose rate will be more like $10^{11}$ rads/sec. because of the higher beam current required. As even extremely hardy cells such as Micrococcus Radiodurans lyse after only $10^7$ rads, they would live only $10^{-4}$ sec, and their membranes would probably polymerize in 0.1 sec. (Dean et al. 1966).

* The water drop shown in Lane's study may in fact be ice.
It should be pointed out that this limitation is in no way contradictory to the finding that some insects can be viewed, alive in the SEM, and that they lead reasonably normal lives after their removal from the microscope. These insects have thick (50 microns) chitinous outer coverings which not only protect the animal against desiccation but also absorb most of the beam-produced radiation. As this covering is practically inert biologically, and as the main portion of the radiation is not very penetrating (kilovolt β particles, range 4-5 microns), the living part of the insect is well protected from radiation damage. In general, however, living cells of interest cannot be viewed if they are covered by some sort of a thick coating and cannot live if they are not. Therefore, the SEM micromanipulator will have to restrict itself to studying micro-anatomy by microdissecting samples which are dead though not necessarily dry.

MICRODISSECTION

Three general categories of application were envisioned for the SEM manipulator. (1) Removing objects, which are obscuring details of interest, from the sample surface. (2) Removing interesting parts of the sample for additional study, using the CEM or other technique. (3) Distorting or deforming the sample in order to gain a subjective idea of its mechanical properties.

*Please et al., 1966
Sample Preparation

(1) Dehydration

Unless some sort of wet stage is used, biological samples must be dehydrated before being observed in the SEM. This can be accomplished in a variety of ways including simple air drying from aqueous solution, air drying from a volatile solvent, critical point drying and freeze drying. Air drying invariably results in large distortion of the sample caused by the surface tension forces present at the meniscus formed when the outside surface of the evaporating liquid approaches the specimen surface. Critical point and freeze drying avoid this problem by removing the liquid from the sample without creating a meniscus. These are therefore the methods of choice and are described briefly in Appendix II.

(2) Coating

Carefully dried biological specimens are very good insulators. If they are viewed in the SEM without further treatment, the primary beam will inject a charge slightly below the sample surface because, at commonly used primary beam voltages (5 - 50 KV), the secondary electron coefficient, $\delta$, is less than unity. This means that the number of primary electrons impinging on the sample's surface is greater than the number of electrons emitted from it. The result is, of course, that a negative charge accumulates in the sample. This in turn causes a number of untoward effects some of which are described in Appendix III. The normal solution is to vacuum evaporate a thin (100-200Å nominal) layer of conducting material onto the surface of the sample thereby providing a low
resistance path to ground.

However, when one plans to manipulate such material while it is being observed in the SEM, it is inevitable that this coating must be disrupted and it is necessary to consider how the resulting charging artifacts will limit the use of the manipulator. As a matter of fact, charging becomes an important factor in only the first of the preliminary missions established earlier for the manipulator; removing objects which obscure details of interest from the sample surface. In this case, what is originally not on the outer surface, and hence not coated, is what is of interest. The problem has been attacked in two ways: (a) by varying the beam energy, (b) by perfusing the sample with antistatic agent prior to dehydration.

(a) **Lower beam energy.** Generally speaking, at normal incidence on flat specimens $\delta$ increases to unity as the energy of the incident electrons is reduced to 1-3 keV. Because $\delta$ is roughly proportional to the secant of the angle between the local surface normal and the beam, however, it may reach unity at considerably higher primary beam energies on rough samples where much of the surface is not normal to the beam. When $\delta = 1$, of course there is no charge accumulation and no artifact. In practice charging artifacts caused by disruption of the surface conducting coat can be reduced though not quite eliminated by using primary beam voltages in the range 5-10 kV (Figure 18, 10 kV). Unfortunately the resolution of the microscope is also reduced at these voltages.

There are three principal reasons for this. The first is the linear relationship between the energy and the current density of a focused spot of electrons which was derived by Langmuir (Langmuir 1937):
\[ J = J_o \left( \frac{eV}{kT} + 1 \right) \sin^2 \alpha \]

\( J \) = current density in the spot.
\( J_o \) = current density at the surface of the cathode
\( e \) = electron charge
\( V \) = accelerating voltage
\( k \) = Boltzman constant
\( T \) = absolute temperature of the cathode
\( \alpha \) = the half-angle of the beam of electrons converging on the spot.

As the total current in the beam is fixed by noise considerations (Everhart et al. 1959), a decrease in current density implies an increase in spot size and hence a lower resolution.

The second reason is that it has been shown (Pfeiffer 1972) that all SEMs which have a cross-over just outside the Wehnelt (i.e., all except those with field emission guns) are limited by chromatic aberration. The disk of confusion attributable to chromatic aberration is proportional to \( \frac{\Delta E}{E} \) where \( \Delta E \) is the uncertainty of the energy, \( E \), of the electrons leaving the gun. The energy uncertainty is basically fixed by the gun geometry and the filament temperature. It follows that as \( E \) is reduced \( \frac{\Delta E}{E} \) will increase and so will the disk of confusion due to chromatic aberration.

The final reason is that low energy electrons are far more easily deflected by stray fields than high energy electrons. Such fields, which produce marked astigmatism, arise from inadequate shielding or cleaning of the column and are often difficult to eliminate completely in commercial instruments.
(b) **Antistatic treatment.** For the reasons stated above it was felt desirable to attempt to solve the charging problem by means other than reducing the beam voltage. The use of the antistatic sprays, similar to those used in the textile industry, as a means of reducing charging artifacts in the SEM was first suggested by Sikorski (Sikorski et al. 1968). In theory such a treatment should increase the bulk conductivity of the sample and this would not be affected by disruption of the surface. Antistatic compounds are fairly closely related to detergents and therefore we might expect some damage to biological tissue if they were applied in a polar solution such as water. When applied in nonpolar solvents prior to freeze or critical point drying as described in Appendix II, however, they do not seem to cause any damage to the specimen. Unfortunately, we also found that such a treatment was only slightly effective in preventing charging. In fact even when applied to wool fibers by spraying from an aerosol can to the extent that large drops can be seen on the surface (Figure 16a) such compounds do not seem to provide increased conductivity to the cortex of the fiber. This can be seen in Figure 16b where the fiber has been broken using the manipulator and increased brightness, which is often characteristic of charging samples, can be seen on the broken end.

(3) **Conclusion**

Although at first the failure to solve the charging problem effectively would appear to be a fatal blow to micromanipulation in the SEM, in fact this is not the case. There are at least two reasons for this. The first is that, as explained in Appendix III, if the sample is sufficiently rough, relatively low
Figure 16. Where the gold coated wool fiber passes around the hooked probe tip, a meniscus formed by excess antistatic agent can be seen (a). In (b) anomalous brightness indicates charging on the broken surface of a similarly treated fiber in spite of the antistatic treatment.
surface potentials are capable of increasing the number of electrons actually leaving the sample per incident electron, until $\delta_{\text{eff}} = 1$. This is because, on a rough sample many of the secondaries originally ejected by the beam collide with adjacent parts of the sample and are thereby prevented from leaving it. Such electrons are said to be "specimen collected." A small negative charge inhomogeneously distributed over such a rough surface, is capable of repelling emerging secondaries in such a way that less of them will be specimen collected and $\delta_{\text{eff}}$ will be increased.

This sort of effect can be seen in Fig. 17 which shows two pieces of critical point dried rabbit retina. Their surface has been coated with carbon and gold and then disturbed with the probes of the manipulator. The upper micrograph is of the outside surface of the retina which is covered with rod or cone outer segments and is therefore rough while the lower micrograph is of the inside surface (inner limiting membrane partially obscured by condensed vitreous) and is relatively smooth. Charging artifacts on the smooth sample can be easily seen to be more severe than those on the rough one.

The second reason, which is actually related to the first, is called the "window effect." It was first described by Thornton (Thornton 1968) and more recently by Pfefferkorn (Pfefferkorn 1972) and it refers to the fact that charging artifacts are reduced by keeping the nonconducting area small. This can be done either by covering the bulk of the sample with a sheet of metal foil which has a millimeter-sized hole to reveal the area of interest, or by using conductive paint to
Figure 17. Surface disruption produced during microdissection produces less severe charging artifacts when the sample is rough (a) than when it is smooth (b). The sample is critical point dried rabbit retina, outer (a) and inner (b) surfaces.
cover all but a small area of the sample.

There are two mechanisms whereby such a procedure might reduce charging. The first is that by reducing the distance between the charging area and a grounded surface, resistance is reduced allowing current to flow through the sample more easily. The second and probably more important mechanism can be explained by considering the electric field immediately above the sample. Imagine a large insulating sample having a small area on it which is charged to some surface potential $V_c$. The field above the sample which is due to this voltage is, roughly speaking, inversely proportional to the distance from the charged area to the nearest grounded surface. The effect of the metal foil or the silver paint is to bring this grounded surface nearer to the charged area. As a result, the field above the surface is increased, specimen collection is reduced, and $\varepsilon_{\text{eff}}$ increased to unity. Either or both of these mechanisms could explain why small uncoated areas on generally well-coated samples do not produce particularly objectionable charging artifacts, especially if the samples are rough.

Therefore, our procedure is simply to mount the dried sample on a standard $3/8" \times 3/8$" long Dural stub, using conductive paint or preferably conductive epoxy, and coat with 100Å (nominal) of carbon followed by 100Å of gold. Precautions are taken to avoid over-heating and the sample is rotated during evaporation. To increase further the number of directions from which evaporant atoms approach the sample, the evaporations are carried out in a relatively poor ($5 \times 10^{-4}$ Torr) vacuum allowing the evaporant
atoms to scatter off molecules of the residual gas.

If necessary, the sample can be recoated after having been dissected so that optimal micrographs of newly revealed structures can be obtained.

**Use of the Micromanipulator**

The prepared sample stub is placed in a specimen holder which has been slightly modified to prevent interference with the motion of the probe head electrostatic shield. The specimen and holder are then introduced into the specimen chamber through the airlock and an SEM-T.V. image of the sample is obtained in the conventional way. With the stage in the lower position, the sample is moved to the top right corner of the viewing area at the lowest magnification (60 x) so that the probes, which will enter from the bottom left, will not inadvertently strike the specimen holder as they are inserted. The sample is titled from the horizontal at -20° away from the collector.

Next, the outer airlock cap and the specimen insertion rod are removed from the airlock and the micromanipulator, with the probe head retracted, is mounted in their place. The airlock is pumped out and the interlock switch, which normally disables the PMT power supply when the inner airlock door opens, is bypassed with a separate switch. The coarse mechanical adjustments on the manipulator are set to standard values, the inner airlock door is opened and the probe head is introduced. As it approaches the sample, it can be seen on the T.V. screen and moved so that the probe tips are on the axis of the electron optical system. The part of the sample to
be dissected is then returned to the center of the screen, taking care that the probe tips are above it (i.e., that they do not hit the side of the specimen holder).

When the probe tips are within ~0.5 mm of the area to be dissected, the manipulator power supplies are turned on and the fine mechanical controls are used. Finally, the two sets of piezoelectric controls are employed for more precise work over short distances or to pick up or release pieces of the sample held between the probe tips.

**Recording**

The most convenient way to record the process of micromanipulation in the SEM is to use a video tape recorder (VTR). Though at one time a VTR was a luxury instrument, recent improvements have greatly reduced both initial and operating costs and increased the fidelity and ease of use. In micromanipulation, it is not always easy to know what images need to be recorded until it is too late to do so because the object in question no longer exists. The VTR avoids this problem by recording everything without any effort on the part of the operator, and it also permits "instant-replay" when the course of some motion is unclear.

For these reasons most of the micrographs in the present work which show manipulation are made from VTR recordings. Some of them show why such recordings, though convenient, are not ideal, because of low micrograph quality.
At T.V. rate, the time required to scan the entire raster (1/30 sec) is about $10^{-3}$ of the scan rate (40 sec) usually used to record SEM micrographs. Unless the collected signal is correspondingly increased, the signal-to-noise ratio,

$$\frac{S}{N} = (iK \gamma T)^{\frac{1}{2}}$$

$i$ = the beam current

$K$ = a constant related to $S_{\text{eff}}$ and the dimensions of the raster

$\gamma$ = the collection efficiency

$T$ = the time for one complete scan of the raster

will be reduced and the micrograph will have poor quality (Everhart 1958).

In practice, $i$ can be increased by only a factor of 50 while the collection efficiency, $\gamma$, is reduced to about 15% of its normal value by the presence of the probe head, which distorts the collection field, and by the fact that the sample is tilted slightly away from the collector. As a result of all these factors

$$\frac{S}{N} \ (40 \text{ sec}, 2 \times 10^{-11}A) = 20 \frac{S}{N} \ (1/30 \text{ sec}, 10^{-9}A)$$

no manipulator

This can be remedied to some extent by photographing a large number (10-100) of consecutive T.V. images on a single negative, thereby increasing $T$. This can only be done if there is no movement of the image, however, and it is therefore also limited by the poor
stability of the T.V. scan generator.

To obtain better quality micrographs it is necessary to stop the dissection periodically and take a normal slow scan picture. This has been done in one of the following series of micrographs (dissection of equine lung).
RESULTS

Four different tissues were chosen for the microdissections described in this section to give an indication of the micro-manipulator's ability to perform the three tasks for which it was designed.

Removing Objects from the Specimen Surface to Reveal Hidden Structures

(1) Duodenum

One of the first samples investigated in this way was a piece of freeze-dried mouse duodenum. The villi in this sample are covered with, and embedded in, a thick coat of dried mucous and it was of interest to know where the fracture planes would occur. Accordingly a probe was used to pry a row of villi away from the neighboring mucous blanket (Figures 18a and b). The resulting fracture can be seen in the low magnification micrograph, Figure 18c. A closer examination of the villi and mucous surfaces at higher magnification yielded Figures 18d and e (resp.). In the former we can see the microvilli on the surface of the villi and in the latter the impression left in the mucous by similar microvilli. This indicates that the split did occur along the villi surface as expected.

(2) Frog sacculus

Hillman and Lewis (1971) have proposed a morphological basis for a mechanical linkage in otolithic receptor transduction in the frog. Their explanation involved the mechanical properties of the tufts of stereocilia, the cuticle to which these tufts were attached and their relationship to the nearby kinocilium.
Figure 18. Using the micromanipulator to create new surfaces. One can see the manipulator needle pull apart this sample of freeze dried mouse duodenum in (a) and (b). The result can be seen in (c). Closer examination reveals that the microvilli on the surface of the villi (d) have produced an imprint on nearby deposits of mucous (e).
It was felt that additional information might be forthcoming from microdissection and one of the samples used in their study was obtained for this purpose.

When first received, part of the otolithic membrane obscured much of the receptor surface (Figure 19a). This was easily removed using the micromanipulator (Figure 19b. Note: The damage to the receptor surface was caused by later investigations below this surface not by removal of the otolithic membrane.)

In Figure 20a, b the manipulator needle was used to test the strength of the connection thought to exist between the bulbous head of the kinocilium and the tuft of stereocilia. The absence of any significant attachment may be attributable to weakening during specimen preparation.

Figure 20, c and d, e and f are before and after micrographs showing the removal of two different ciliary tufts, including the cuticles.

In order to evaluate the structures revealed by the removal of the tufts, high resolution, slow scan, stereo-micrographs are very useful and they are shown in Figures 21 and 22. The top pair (Stereo-pairs should be viewed through a mirror stereoscope or other appropriate device) indicates the general area of the investigation and some holes made accidentally because of the difficulty experienced in judging probe motion in the direction parallel to the SEM beam (see 'Stereo T.V.' under 'Future Improvements'). The higher magnification micrographs below, show several examples of a structure which may anchor the cuticle to the cell. Investigation of
Figure 19. In (a), part of the otolithic membrane can be seen still obscuring much of the surface in this preparation of frog saccus (Hillman and Lewis, 1971). In (b), it has been removed by the manipulator needles seen coming from the left.
Figure 20. T.V. images of micromanipulation of frog sacculus (Hillman and Lewis, 1971). In (a) and (b) the kinocilia can be seen to be pulled away from the stereo cilia. The removal of one ciliary tuft can be seen in (c) and (d) and a second in (e) and (f).
Figure 21. SEM microdissection of frog sacculus reveals structures, not yet fully identified, present beneath the excised ciliary tufts. These micrographs form the left half of stereo pairs with Figure 22 (a) and (b).
Figure 21. SEM microdissection of frog sacculus reveals structures, not yet fully identified, present beneath the excised ciliary tufts. These micrographs form the left half of stereo pairs with Figure 22 (c) and (d).
Figure 22. SEM microdissection of frog sacculus reveals structures, not yet fully identified, present beneath the excised ciliary tufts. These micrographs form the right half of stereo pairs with Figure 21 (a) and (b).
Figure 22. SEM microdissection of frog sacculus reveals structures, not yet fully identified, present beneath the excised ciliary tufts. These micrographs form the right half of stereo pairs with Figure 21 (c) and (d).
this structure and its effect on the mechanical properties of the receptor is continuing.

Removing Parts of Sample for Subsequent Study

Investigators using the SEM to study critical point dried emphysematous equine lung have described objects which seem to be associated with this disease and which they call plaques (Nowell et al. 1971). These plaques are found attached to the trabeculae formed as the alveolar walls are destroyed by this disease. One such plaque attached to at least four trabeculae can be seen just beyond the end of the probes in Figure 23a. Because plaques compose only a small percentage of the total volume and because their main distinguishing characteristic is their topological relationship to the trabeculae, it had proven impossible to identify them in CEM micrographs. For this reason it was not even known if they were cellular or composed of mucous or some preparative artifactual material. It was felt that the SEM micromanipulator might permit sampling of a single plaque so it could be investigated and characterized using the CEM.

As can be seen in Figures 23 and 24 the micromanipulator was first used to snip the two trabeculae on the right side of the plaque (Figure 23b). A trabeculum attached to the plaque on the back side was next broken leaving (Figure 23c) the plaque dangling from the remaining two trabeculae (Figure 23d). Figure 24a is a higher magnification micrograph of one of these remaining trabeculae showing a cracked coat of mucous and squamous epithelial cells covering a bundle of collagen fibrils. Figure 24b shows these final trabeculae being cut. The plaque is then lifted away from
Figure 23. Removal of "plaque" from a specimen of equine lung (Nowell et al. 1971) for further study. In (b), two trabeculae attached to plaque have been snipped and two more (one beneath) cut in (c) leaving the plaque dangling by the last two in (d).
Figure 24. (a) A high magnification view of a cracked trabeculum showing the coat of squamous epithelial cells surrounding a collagen bundle. The manipulator needles grasp the plaque (b) hold it over a coated EM embedding capsule (c) and release it (d).
Figure 25. The dissected plaque can be seen in the bottom of the capsule (a). C.E.M. micrographs of the plaque after embedding and sectioning show a cell near the edge of the plaque (b) and a trabeculum passing diagonally between two segments of the plaque (c).
the sample and held pinched between the two probes while a standard EM embedding capsule, coated to make it conductive, is moved beneath it (Figure 24c). The probes are then separated so that they release the plaque (Figure 24d). The magnification was then increased so that the entire raster struck the plaque, causing it to charge up and "pith ball" into the bottom of the capsule (Figure 25a).

The plaque was then fume fixed in OsO₄ and embedded in epoxy in the conventional way.* Three hundred serial sections of the plaque area were made from this sample and part of two of these is shown in Figures 25b and c. The upper one shows a trabeculum passing diagonally between two segments of the plaque. The plaque can be seen to be cellular and remnants of the gold conductive coating can be seen. The lower micrograph shows a higher magnification of a cell near the tip of the plaque in another section.

Although these are only preliminary results, at least some questions are now answered. The plaque is cellular and we have learned a little about the morphology of its cells, in spite of the rather horrendous treatment they have gone through.**

Qualitative Determination of the Mechanical Properties of the Surface

It is difficult to define concisely what is meant by this task and for this reason it may appear to be the least "scientific."

* The author is very grateful to Miss Janice Nowell who prepared the samples and obtained the CEM micrographs.

** The sample we used had been dry for at least 18 months. During this time the internal cell components had little if any mechanical support and received very high doses of radiation from repeated SEM examinations. They were then subjected to surface tension effects as the dry sample was placed into propylene-oxide prior to embedding.
Figure 26. Segments of critical point dried rabbit retina were found to be very brittle (a and b). Part c shows a portion of retina (viewed edge on) from which some of the outer and inner segments have been removed with the manipulator. Together with Figure 27e it forms a stereo-pair of which it is the right hand member.
Figure 27. T.V. rate micrographs showing removal of outer and inner segments from a sample of critical point dried rabbit retina (a, b, c and d). Part (e) forms the left hand half of a stereo-pair with Figure 26c.
Figure 28. A view of undisturbed retina (a). Receptor cells are to the right, inner limiting membrane to the left. After dissection a clump of receptors can be seen dangling to the right of the retinal surface (b). These micrographs form the left hand member of a stereo pair with Figure 29 a and b.
Figure 28. A series of increasing magnification, c and d, e and f (next page) shows that breaks have occurred between the inner and outer segments and between the inner segments and the cell bodies (i.e., along the outer limiting membrane which can be seen clearly in e). These micrographs form the left-hand members of stereo pairs with Figure 29.
Figure 28. See description on preceding page.
Figure 28. A magnification series of a slightly different area of dissected rabbit retina is shown in (g and h). These micrographs form the left hand member of a stereo pair with Figure 29 g and h.
Figure 29. A view of undisturbed retina (a). Receptor cells are to the left, inner limiting membrane to the right. After dissection a clump of receptors can be seen dangling to the left of the retinal surface (b). These micrographs form the right hand member of a stereo pair with Figure 28 a and b.
Figure 29. A series of increasing magnification, c and d, e and f (next page) shows that breaks have occurred between the inner and outer segments and between the inner segments and the cell bodies (i.e., along the outer limiting membrane which can be seen clearly in e). These micrographs form the right-hand members of stereo pairs with Figure 28.
Figure 29. See description on preceding page.
Figure 29. A magnification series of a slightly different area of dissected rabbit retina is shown in (g and h). These micrographs form the right hand member of a stereo pair with Figure 28 g and h.
However, in the end it may be the most useful of all. A suitable piece of low density pumice can appear very similar to a cellulose sponge and although both can be used for scrubbing one's back, qualitative information on their mechanical properties is needed before they can be applied to this task with efficiency and safety.

Of course, there is a definite risk in applying to living tissue, mechanical information gained from samples which are both fixed and dry. In this section, we will investigate the mechanical properties of various dried tissues and attempt to relate these properties to known anatomical facts.

In the preceding section, a small piece of tissue was removed from a sample of equine lung (Figures 23, 24). The elasticity of an in vivo lung is well known and as can be seen in the micrographs (and as was even more evident to one watching the dissection) this elasticity is well preserved in the dried tissue.

Likewise, in the in vivo frog succulus, the individual sensory cilia are known to be capable of considerable flexibility. As can be seen in Figure 20b, they retain much of this flexibility in the dried state.

The third case we would like to present involves a piece of critical point dried rabbit retina. Although the retinas of other vertebrates have been investigated in the SEM previously (Lewis et al. 1969a), it is believed that this is one of the first critical point dried SEM preparations of a mammalian retina.* McAlear has briefly described the dissection of frozen (but not freeze dried)

* The author would like to express his appreciation to Dr. Thomas Budinger for providing the sample for this work.
mouse eyes in the SEM (McAlear et al. 1971). This work involves the use of a fixed probe past which the sample is moved using the stage motion controls. It is therefore not considered micro-manipulation by the criteria adopted previously in this paper. This research is further flawed by the presence of what appear to be excessive ice crystal artifacts present in the sample.

In contrast with the lung, critical point dried retina as a whole is very brittle (though individual rods are quite flexible). This can be seen in Figure 26a and b where a slight pressure has caused a piece of retina to fracture like a piece of glass. This behavior is not surprising when one considers the comparatively low incidence of collagen fibrils and other connective structures in the retina. In Figure 28a-29a such a break can be seen to be very clean compared to Figure 24a (broken trabeculum). The fracture surface also is seen to pass between adjacent rod inner and outer segments and not down the center of the membrane, as one would find in a frozen fracture (Koehler 1968).

Finally, we will consider two other planes in the retina along which we might expect to find mechanical weakness. The first is the plane which separates the rod inner and outer segments. Each outer segment is connected to its inner segment by a very narrow neck of tissue composed primarily of a single cilia (Sjöstrand 1961, Cohen 1963, Prince 1964). Any stress on the outer segment might be expected to rupture this neck. The second plane, the so-called outer limiting membrane, is not really a membrane as such but is a sheet of marginal cell junctions which connect all adjacent receptor cells on a plane passing between the inner segments and the cell bodies
(Cohen 1963).

Figure 27a, b, and c show successive steps in a dissection attempting to cause fractures along these two planes. The outer segments can be identified by the fact that they appear much lighter than the adjacent inner segments. Figure 27d shows the result of a second similar motion and it is possible to see that in this case most of the fractures have occurred between the inner and outer segments. Figures 27e and 26c form a stereo-pair showing the results of the first motion. A crack can be seen where the inner segments have pulled away along the outer limiting membrane.

Figure 28 and 29 are composed of eight stereo-pairs. All except (a) were taken immediately after dissection and without recoating. A clump of receptors removed during the dissection can be seen dangling to the right of the receptor surface in (b). Figures 28-29 (c, d, e and f) show progressively higher magnifications of the dissected sample. In (e) particularly, it is possible to see that breaks have occurred along the two expected planes of weakness. The outer limiting membrane can be seen (arrow, dark surface) and more to the left, a surface composed of the broken ciliary connections is visible. On the far left, one can observe the undisturbed receptor surface. These last two surfaces are perhaps seen more easily in (f). A shorter magnification series is shown in (g) and (h). Here again the three surfaces are visible (g) and the attachment between the inner segments and the outer limiting membrane can be seen to be quite tenuous (h).

What does all this show? It shows that fracture planes produced during the microdissection of critical point dried tissue in the SEM
occur where one would expect them to occur. Microdissection does not reduce the sample to useless rubble but tends to break it along anatomically important surfaces and to preserve the structural integrity of the sample’s subunits.
FUTURE IMPROVEMENTS

It is the firm belief of the author that the SEM micro-manipulator will soon become an essential tool. It will aid investigators from many disciplines to explore the exquisitely comprehensible world revealed by the SEM. Although this world has been slowly appearing for at least fifteen years scientists are only now beginning to realize that the micro world which many subconsciously believed to be as flat as an architect's drawing really consists of the same three-dimensional shapes and surfaces as our everyday macroscopic world. Analytically, of course, the third dimension was always recognized as important but at the same time it was so terribly difficult to get at, because of technical reasons, that it became very easy to accept the flat world as being real. Once the SEM removed many of these difficulties, investigators rapidly found that they needed what the third dimension could tell them.

A person attempting to discover the workings of a carburetor or an old clock would probably start by taking it apart. We are accustomed to examining mechanical objects this way. When the structural features can be visualized, they often suggest functional relationships. However, because our optical devices have made us feel that small objects are somehow different in kind, we have not often resorted to manipulative exploration as a means of studying them. Just as it has taken time for investigators to become aware that the micro world can appear as real as the one we walk around
it will take a while for them to feel comfortable with the idea that it can be disassembled.

On the other hand, the discrepancy between the capabilities of the instrument described above and those which are necessary to properly disassemble a retina or a small ganglion is far from trivial. It will be the object of this last section to discuss some of the limitations of the present instrument and possible ways of overcoming them. We will start with present limitations in the system of observation, continue with considerations of the manipulator itself and end with a brief discussion of sample preparation.

**IMPROVEMENTS IN OBSERVATION**

**Stereo T.V.**

Because of the SEM's great depth of field, ascertaining the distance of the probe tip above the sample and also the angle between the sample surface and the probe tip was found to be very difficult. Binocular stereopsis will definitely be necessary for routine work on a precise level, although it was possible to do the relatively crude procedures described in the preceding section without it. Fortunately, as mentioned on page 12, after-lens-deflection has already been used to produce stereo pairs at slow scan rates (Dinnis 1971). Work is currently under way to extend this to T.V. scan rates where the angle at which the beam approaches the sample is switched electrically at either the frame speed (1/30 sec) or the interlace speed (1/60 sec).
The requirements of such a system include a long working distance between the sample and the objective lens pole piece and a noise-free T.V. image. The former requirement is necessary to allow the deflection coils to be placed below the lens and is not so important in some proposed systems which attempt to use the field of the objective lens (with its aperture removed) as the final deflector (Boyde, 1972, personal communication). The second requirement stems from the fact that much binocular information is decoded by matching small details which are visible to both eyes. If the noise level is too high, such small details cannot be easily discerned. This effect can be seen in Figures 30 and 31 (stereo-pair) where the upper set of T.V. rate micrographs were taken at $10^{-10}$ A and the lower set at $3 \times 10^{-9}$ A. Beam currents near this latter figure can be produced in most SEMs, though resolution is then limited to about 0.2 - 0.3 microns.

**Charging**

This problem will become harder to ignore as the beam current increases. There are two possible approaches. The first is to use very low beam voltages (around 1-3 kV). We have previously mentioned the problems involved with using low accelerating voltages in conventional SEMs such as the one used by the author. In this section, however, where future improvements are being considered, it would be improper not to point out that an SEM of rather unusual design has recently been introduced (Welter and Coates 1971). This instrument uses a field emission source which is far more intense than the conventional heated tungsten hairpin filaments. As a
Figure 30. T.V. rate micrographs of a screw thread at $10^{-10}$ and $3 \times 10^{-9}$ A beam current, left of stereo-pairs.
Figure 31. Same as Figure 30, right of stereo-pairs.
result it can produce noise-free T.V. images at much higher resolution than is possible with conventional SEM. It also has a working distance of up to 6 cm compared with 1-2 cm on most machines. Finally, because of its unique electron optical design and its extremely clean vacuum, it is capable of producing images of 500Å resolution using beam energies of 500-1000 eV where charging is no longer a problem because $5 \leq 1$ for most samples.

The purpose of this paper is not to explain the theoretical basis for these capabilities but to point out that they do exist and that they might solve some of the problems presently complicating the observation of microdissection. A word of caution is also in order. The increased beam current in the Welter & Coates instrument implies increases in both radiation damage and thermal loading. The effect of such increases on sensitive biological samples is not presently known.

A second approach to the charging problem is to improve the way that the secondary electron signal is collected. As explained in Appendix III, many charging artifacts result from the fact that secondary electrons emitted from some areas of a charging sample are deflected away from the collector causing these areas of the sample to appear dark. So far, most efforts at redesigning the secondary electron collector have attempted to increase this effect in order to improve the detection of small surface potentials (Banbury and Nixon 1970). However, it is possible to take the opposite approach and try to decrease the effect of the surface potentials produced by surface charges. The simplest way of doing this is to increase the
collection field at the sample in order to make this field more
difficult to distort. This could be accomplished simply by
increasing the bias on the collector grid or by removing the grid
altogether allowing the collection field to be established by the
+10 kV collection electrode on the scintillator. We used this
latter approach with moderate success.

Another way to avoid the effects of charging on secondary
electron trajectory is to measure the current which remains in the
sample (specimen current). The information should be the inverse
of that obtained from the current of secondaries and backscattered
electrons leaving the specimen (assuming the beam current is constant).

Recent developments in solid state technology make it possible to
develop a specimen current amplifier nearly as noise-free as is the
scintillator-photomultiplier secondary electron amplifier. The
specimen current is not affected by the electric fields above the
sample, however, and it is possible, though not yet confirmed, that
the specimen current signal would be less subject to charging
artifacts.

IMPROVEMENTS IN THE MANIPULATOR

The present instrument is disquietingly complex. Much of the
complexity stems from the necessity of having to pass the probe head
through the specimen chamber airlock; a difficulty future manipulators
may be able to avoid.

The accuracy and response speed of the present piezoelectric
drive units are more than sufficient; however, the range and linearity
are still less than desirable. This problem also will be more easily overcome once some of the size constraints imposed by the airlock are removed. It is possible that a system using bimorphs might be more successful in this case (Ellis, 1962). Alternatively, where the specimen chamber is sufficiently small, it may be possible to use needles attached to relatively short shafts passing through the chamber wall and controlled solely from outside the chamber. Increases in the moving mass and the high viscous friction in the feed-through would be offset by ease of construction and the fact that any commercially available light microscope micromanipulator could be used as a drive unit. Two feed-throughs of this type are now commercially available.*

Another limitation of the present instrument is the small number of probes available. Experience has indicated that many tissues are so elastic that several probes are needed to hold the tissue locally before careful dissection can take place. The more probes involved the more reasonable it seems that the drive units for at least some of them should be mounted entirely outside the column. A satisfactory second generation SEM manipulator might have two such feed-throughs on each side of the specimen chamber. Tungsten needles bent at a point well away from the tip could be used to allow needles from both sides to meet the sample at a reasonable angle. The problem of lining up these tips would not be difficult in a microscope permitting easy visual access to the specimen chamber.

* Ernest F. Fullam, Inc., Schenectady, N. Y.
ETEC Corp., Hayward, Calif.
Another area of improvement involves the number of degrees of freedom. Anyone attempting to eat a plate of spaghetti without bending his wrist has some idea of the problem. The only reasonable solution at present is a precise eucentric stage with two orthogonal tilt directions which are controlled by a rapid and sensitive servo system.

**Probe tips.** The tungsten probe tips used in this work are also far from ideal. Though it is common to think of tungsten as a hard, brittle metal, this is not a good description of tungsten points in the micron size range. The most common problem is bending of the tip upwards. This can be seen in Figure 32a which shows a probe tip cutting a flat surface and Figure 32b which shows the resulting groove. Since the groove is shorter than the tip, the tip must be pointed upwards. As this happens quite often, a loop of 0.005" dia. wire is attached to the edge of the specimen holder to allow crude repairs to be made. Figure 32c shows the offending tip (lower) pressing up against the loop. As the probe is pulled to the left, the elastic limit of the tip is reached, allowing the probe to flip up as its tip is bent down (Figure 32d). The reverse tactic is now employed in Figure 32e with the result that the tip is still bent up somewhat though less than originally (Figure 32f). Such operations would, of course, be greatly facilitated by stereo T.V.

Tantalum carbide was recommended to the author as a superior alternative material, however, we failed in our attempts either to purchase or to synthesize this compound in wire form. Further research on tip fabrication seems desirable.
Figure 32. By comparing the position of the needle in (a) and the resulting groove in (b), it is obvious that the probe tip is bent upwards.
Figure 32. Attempts to straighten it using a fixed loop of 0.005" wire are shown. In (c) it is pushing up under the wire. As the needle is withdrawn it flips up, bending the tip down (d).
Figure 32. The procedure is reversed (e) and the result (f) is still pointed down somewhat.
Controls. Finally, a word about the controls. The two-dimensional joy stick will be inadequate when stereo T.V. becomes a reality. Furthermore, the mechanical precision of the present controls was at best marginally acceptable and should be improved.

The ultimate control system would employ some sort of simple computer (Alston et al. 1971). This would simplify the problem of varying the reduction ratio without affecting the position of the probe. It could also be used to correct non-linearities in the control and drive transducers. Another use might be analyzing feedback data on the position of the drive units. We have employed this sort of feedback only around the fine mechanical drives. However, as the charge required to maintain a certain voltage between the electrodes of a piezoelectric crystal is a measure of the stress on the crystal, it might be possible to place feedback loops around these also. (Alternately, small sensing crystals could be placed in series with the driving crystals.)

One thing which will probably not be possible in this regard is kinesthetic feedback. This would allow the operator to "feel" the pressure exerted on the probe tip by the sample, in the form of mechanical resistance to the motion of the control transducer. Though catastrophic contacts (such as forcing the needle holder into the lens pole piece) could be sensed, the forces which a tiny tungsten probe could exert would probably be so small in comparison to those exerted by antibacklash and other springs that they probably could not be detected. Therefore, they could not be used to modulate the mechanical resistance of the transducer. The most effective way...
of assessing the pressure on the tip will remain the practice of watching it bend.

IMPROVEMENTS IN SPECIMEN PREPARATION

Heretofore fixatives have usually been chosen on their ability to preserve some particular cellular or subcellular detail. Except when part of the tissue is to be excised for future examination in the CEM, this is probably not the most important criterion for tissue which is to be microdissected in the SEM. More important are: (1) hardness and toughness of the tissue after drying and (2) type of fracture planes favored (whether inter- or intracellular). Considerable experimentation with various fixatives, possibly used after treatment with digestive enzymes, will be necessary to find which are most suitable.
Color modulation display system

The SEM, like many other instruments, usually displays the data which it has obtained in the form of a picture on the face of a cathode ray tube (CRT). This picture is made up as the electron beam of the CRT sequentially illuminates all the points on a rectangular raster having the same proportions but larger in size than the raster scanned by the probing beam in the microscope column. Information gained at each raster point is usually displayed by intensity modulation. That is to say the more signal detected from the sample at a given position on the raster the greater the current in the CRT beam and the brighter the corresponding point on the display raster.

This is a simple and effective system and is similar to that used in television. However, because sample interaction with the primary beam is so rich in information, it is often desirable to be able to display and record more of it than is possible using only the ten tones of grey which can be distinguished easily on a photograph of the CRT screen. One answer to this problem is deflection modulation (Everhart 1966), which uses the fact that small spatial displacements are more easily seen than small changes in brightness. A limitation of this method is that the strict one-to-one spatial relationship between points on the sample and points on the image is broken down and as a result only samples with simple geometries can be usefully displayed using this method of display.
A second alternative is to use color. We will distinguish here between color coding and color modulation. The first refers to the practice of recording information obtained using several different signal modalities (secondary electrons, cathodeluminescence, X-ray, etc.) on one piece of color film with each type of signal being recorded in a different color. The second refers to the display of just one type of signal by using changes in hue rather than changes in intensity (Pawley et al. 1969).

Figure I-1 is a conceptual sketch of an apparatus which can be used to produce color modulated images and Figure I-2 shows a schematic diagram of the required circuitry.

The continuous signal which is present on the CRT grid, is applied to two Schmitt trigger (Schmitt 1938, General Electric 1964) sensing circuits composed of the micro-logic integrated circuit, the buffer amplifier T₁ and T₂ and two 20-kilohm threshold potentiometers (labeled UPPER LIMIT and LOWER LIMIT). The output of this circuit triggers the CRT blanking circuit when the signal is greater than that set by the lower limit or less than that set by the upper limit. The blanking circuit places a -20 volt signal on the CRT cathode. The effect of this circuitry can be seen in the middle of Figure I-1.

If the CRT phosphor produced white light this circuitry would have been sufficient; however, as it was predominantly green, the following additions were necessary: (1) the capability to shift the D.C. level of the signal after it had been applied to the sensing circuit (i.e., without changing which part of the signal
Figure I-1. Block diagram of the elements in the color modulation circuit. The three graphs above show the effect on the signal of the various processing steps.
COLOR MODULATION
VIDEO DISCRIMINATOR CIRCUIT

FROM PMT

PMT AMP

2N3638

2N3638

(A - B) DTµL 946

BLANK TRIGGER

Figure I-2

KM47C

+15 V

COM

-15 V

OUT

BAL

+5 V

0.47

0.47

0.47

1N3826

100 k

16 k

10 k

10 k

100 pF

100 k

1 k

100 pF

100 k

1 k

100 pF

100 k

1 k

100 pF

100 k

1 k

100 pF

100 k

1 k

100 pF

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100 pF

100 k

1 k

100 pF

100 k

1 k

100 pF

100 k

1 k

100 pF

100 k

1 k

100 pF

100 k
would be blanked). This was necessary to remove the d.c. voltage corresponding to the lower Schmitt trigger threshold to allow the a.c. part of the signal to be amplified without saturating the amplifier. (2) Amplification of the a.c. signal. This was necessary because, for instance, with the red filter a signal of 7 volts peak to peak was required to produce the various red colors from black to light pink while only 2 volts peak to peak were needed to produce all the recordable green tones.

The operational amplifier KM47C, and its associated circuitry including the d.c. LEVEL and GAIN controls accomplishes these ends and the resulting signal was applied to the CRT grid.

A monitor oscilloscope was connected to measure either the CRT grid or the CRT grid-cathode voltage so that signal manipulations could be observed. The blanking signal from the microscope was used to partially blank the monitor CRT so that successive "windows" could be set properly. This monitor also allowed the proper photographic exposure to be set repeatedly.

To make a color-modulated micrograph, using this circuitry requires three or four successive exposures. Each exposure couples a particular colored filter on the camera lens and a particular setting of the electronic window. Adjacent windows usually overlap each other slightly.
Critical point drying

The critical point of a substance is characterized by a pressure ($P_c$) and temperature ($T_c$) at which the liquid phase of the substance is indistinguishable from its gaseous phase. Under these conditions (and when $T > T_c$ or $P > P_c$) there can be no meniscus between the two phases as only one phase exists. Ideally, when trying to take advantage of this fact to dehydrate biological tissue, one would heat and pressurize the sample to the critical point of water and then slowly remove liquid-gaseous water and replace it with something else (dry nitrogen, for example). Unfortunately the critical point of water occurs at prohibitively high pressures and temperatures: $P_c(H_2O) = 218$ atm, $T_c(H_2O) = 374^\circ C$ and, for this reason, the water in biological tissues must be replaced with a liquid having a more easily attainable critical point. The two liquids most often used are liquid CO$_2$ and Freon 13*. As these liquids are immiscible with water, mutually miscible intermediate liquids are required. In the case of CO$_2$ these are usually ethanol followed by amyl-acetate while Freon 13 requires ethanol and then Freon TF**. Differences between the two systems center on the effects produced by immersing the specimen in liquids of varying chemistry (most are potent lipid solvents) and on the fact that $P_c$ is 72.9 atm for CO$_2$ and 32.2 atm for Freon 13. This second difference makes Freon easier to use, both because the apparatus is less expensive and because it is possible to view the

* CCl$_3$F$_3$

** CCl$_2$F - CCl$_2$F
drying process through a glass pressure window so that the appearance of a liquid phase caused by inadvertently allowing $T < T_c$ can be readily observed and corrected.

Because of equipment availability we have used only the Freon system and have found it very satisfactory.

The protocol is as follows: Dissect out tissue of interest in some isotonic solution using dissecting light microscope if necessary. Cut the tissue into small pieces ($1 \text{ mm}^2$), wash in isotonic solution, and fix in Karnovski's fixative for 24 hours (Karnovski 1965) (or other fixative, if found to be appropriate from other studies), then rinse. Take sample through a graded series of ethanol and water (25%, 50%, 75%, 90%, 100% ethanol) and then through a graded series of ethanol and Freon TF (25%, 50%, 100%). Place the sample, in Freon TF (mixed with antistatic agent, if desired), into a precooled bomb (specimen chamber of critical point drying apparatus*) (Cohen et al. 1968), being careful not to allow the surface to air dry. This allows Freon 13 to enter under pressure. Bleed off mixture of 13 and TF until most of the TF is removed. Heat bomb to increase temperature and pressure while observing the attainment of critical conditions (no meniscus) through the window. Continue heating and bleed the now gaseous Freon 13 from the chamber until ambient pressure is attained. This must be done slowly to stop the gas from cooling below $T_c$ as it expands. Remove sample.

* Bowmar SPC-900.
Freeze drying

The process of freeze drying might more properly be called sublimation drying. In freeze drying from aqueous solution, the sample is first frozen as rapidly as possible. While still frozen it is kept in an environment having a low partial pressure of water vapor and the ice is allowed to sublime away. This method has an advantage over critical point drying in that no solvent chemicals are required but is limited by the formation of ice crystals. These crystals can puncture and distort the structure of the sample and, especially in homogeneous samples such as agar or plasma which have a high water content, they can produce interesting but totally artificial structure where none previously existed. An example of this is Figure II-1A which shows ice crystal artifacts in a piece of freeze dried plasma clot. A properly dried sample is shown in Figure II-1B.

When the freeze fracture technique is used to prepare replicas for the CEM, the formation of ice crystals is limited by cryoprotective agents such as DMSO or glycerols. It is not possible to do this in freeze drying, however, because in the process of trying to sublime these substances from the sample a eutectic liquid is formed. Removal of this liquid results in the usual surface tension artifacts. A related but more successful treatment makes use of the fact that clathrate forming compounds such as chloroform seem to act as artificial ice crystal nucleators and produce a smaller average crystal size. For this reason the last wash following fixation is carried out in a saturated solution (2%) of chloroform
Figure II-1. Freeze-dried samples showing ice crystal artifacts in a plasma clot (a) compared to a properly dried clot (b) and salt deposits on nerve tissue culture preparation which was inadequately washed prior to freezing.
in water. Washing in distilled water is important to remove electrolytes which will otherwise be deposited on the surface of the sample when it freezes, Fig. II-1C.

Ice crystals form most readily in the range -40°C - 0°C (MacKensie 1972). In order to avoid this range, care must be taken to cool the specimen to lower temperatures as rapidly as possible. This can be done most effectively by plunging (quenching) small (< 1 mm³) pieces of fixed and washed tissue into a liquid which is held at liquid nitrogen (LN) temperature (-190°C). This is preferable to quenching in LN directly because bubbles of nitrogen gas produced as the LN boils at the specimen surface effectively insulate the sample from the bulk of the LN and reduce the cooling rate.

The ideal intermediate liquid should not boil at room (specimen) temperature or freeze at LN temperature and should have high heat conductivity. The common possibilities are:

<table>
<thead>
<tr>
<th></th>
<th>MP (°C)</th>
<th>BP (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Propane</td>
<td>-189</td>
<td>-44.5</td>
</tr>
<tr>
<td>2. Freon 22 (monochlorodifluoromethane)</td>
<td>-146</td>
<td>-39</td>
</tr>
<tr>
<td>3. Freon 12 (dichlorodifluoromethane)</td>
<td>-155</td>
<td>-29</td>
</tr>
</tbody>
</table>

We normally use Freon 12 or 22 because they are not flammable. The apparatus used to quench in this fashion is shown in Figure II-2. The quenching liquid is held in a small Dural cup having a serrated rim and called a castle. This is cooled by resting on the polished upper end of a Dural rod, the lower end of which is immersed in LN. Gaseous Freon passes through the copper cooling tube where it condenses before being deposited in the castle.
SPECIMEN QUENCHING APPARATUS

- Alternate castle
- Quenching liquid (Freon-22)
- Standoffs
- Copper cooling tube
- Castle
- Cap
- Support screen
- Aluminum centerpost
- Liquid nitrogen
- 2-liter Dewar
- Stand

Figure II-2
After the samples have been frozen, the quenching liquid is allowed to freeze around them and the entire castle is moved to the pre-cooled stage of the freeze drying apparatus.* A pre-cooled Dural lid is placed on the castle and the specimen chamber is evacuated. As the castle and lid will remain at essentially the same temperature as the thermo-electrically cooled stage, the specimen inside the castle is effectively shielded from radiation heating. The castle also forms a cold trap to prevent backstreaming vacuum pump oil from condensing on the sample. The serrations on the castle's rim allow the water vapor which sublimes from the sample to escape from the castle so that it can be absorbed by a P₂O₅ desiccant also present in the chamber.

Once the specimen is frozen it must be kept as cool as possible to prevent the formation of ice crystals, which occurs to some extent at temperatures down to -100°C. Unfortunately, the sublimation rate is too slow for practical freeze drying at this temperature so it is common to use -70°C where ice crystal formation is very slow and sublimation rates are acceptable.

When all the ice has sublimed, the pressure in the specimen chamber drops and this is a signal that the sample can be slowly heated to room temperature for removal. The amount of time required for total sublimation at -70°C varies somewhat with the type of tissue and to a much greater extent with its thickness. In general, it is on the order of days for samples 1-2 mm thick. Occasionally when ice crystal formation in the center of a rather large sample (> 10 mm³)

* Speedivac-Pearse tissue dryer, Edwards High Vacuum.
is not deemed to be too damaging, the temperature (and therefore the sublimation rate) may be increased to about -30°C after the outer layers of the sample are believed to be free of ice. In general, however, freeze drying of SEM specimens from aqueous solution, has proved to be most useful when applied to small samples (single cells, cultured cells) (Small and Marszalek 1969) or to samples sufficiently tough to withstand ice crystal formation (bones and teeth) (Boyde 1967).

Freeze drying from non-polar solvents

This approach combines elements of both critical point drying and conventional freeze drying. To get around the problem of ice crystal artifacts, the water is substituted with a non-polar liquid (chloroform, amylacetate, etc.) after an intermediate ethanol step. Non-polar liquids show little inclination to form crystals even at temperatures near their melting point. Therefore they can be held at temperatures near their melting points where sublimation occurs much more rapidly than in normal freeze drying.

Freeze drying from non-polar solvents, therefore, offers the advantage of larger samples, shorter drying times and the ability to distribute an antistatic agent throughout the bulk of the sample by dissolving it in the solvent. These are all characteristics of critical point drying and so is the disadvantage of having to use liquids which may damage some biological samples. A problem which should be noted, is associated with the fact that such solvents have rather large melting ranges and it is usually necessary to keep the sample cooled to at least -100°C to prevent melting. This requirement is met with difficulty in commercially available freeze drying apparatus.
APPENDIX III

CHARGING ARTIFACTS IN THE SCANNING ELECTRON MICROSCOPE

The artifact known as "charging" is familiar to investigators using the scanning electron microscope (SEM) to study insulating materials. Because the average secondary electron coefficient, $\delta$, is less than unity at commonly used beam voltages, a negative charge accumulates just below the surface unless there is some low resistance path to ground. Such a path is normally provided by the thin surface coat of vacuum evaporated metal. However, occasionally there are discontinuities in this coat, and as a result, the area not connected to ground will appear to be much brighter, in a secondary electron mode micrograph, than surrounding areas which are so connected (Figure III-1a).

Common Brightness Artifacts

This increased brightness is the most frequently encountered charging effect. It cannot be explained by the familiar theories attributing secondary electron contrast to variations in surface angle (Everhart et al. 1958, 1959), atomic composition (Wells 1957), or crystallographic orientations (Coates 1967), and, as described below, it is not wholly similar to voltage contrast (Everhart 1968). Nor is it the same as the edging or beam penetration artifacts which occur when low density samples are viewed using high accelerating voltages. This last effect produces bright edges because large numbers of collectible secondary electrons are excited from sample surfaces spatially distinct from the point of primary beam impact.
Figure III-1. Micrograph of a plasma clot on a glass cover slip exhibiting common brightness charging artifact (a). Distorted image of a 3/8" uncoated Lucite stub (b) produced by reducing the beam voltage after the surface has acquired a high potential from bombardment with a beam of higher voltage. Nominal mag. 50 X.
The exact form of the charging effect may vary with beam current and voltage, specimen orientation, collector voltage, sweep speed, and magnification. However, it usually involves only very small geometrical distortion of the image and areas of increased brightness will be seen to contain the usual topographic detail, although the contrast setting may need to be reduced before it can be seen. The signal intensity from a charging area may be two or three times that from an adjacent non-charging area if both are measured with respect to zero signal intensity, as measured with the beam turned off.

**Previous Explanations**

There have been several explanations of the relationship between the accumulation of negative charge (and therefore lowered surface potential) and the increased brightness of the observed signal. Because the presence of a surface charge implies a surface potential, the first and most important explanation was the discovery, by Everhart (Everhart 1968), of voltage contrast between the two sides of a reverse biased semiconductor diode. After a computer analysis of the trajectories followed by secondary electrons leaving a sample of this kind, he determined that, depending on their initial energy, they would enter spatially separate parts of the collector and that by masking the detector appropriately he could selectively collect electrons leaving only one side of the diode. Thus secondary electron trajectory, as altered by the "charging" field, affected the collection efficiency, \( \gamma \), and was responsible for changes in brightness.
A second explanation was suggested by Van Veld and Shaffner (Van Veld and Shaffner 1971) who observed that on large sheets or fibers of insulating material, surface potentials large enough to deflect the primary beam directly into the collector could be generated. Such direct collection of the primary beam results in a greatly increased brightness, and these authors suggest that most charging artifacts are due to this type of mechanism. Finally, Thornton (Thornton 1968) suggested that the effect is the same as that described by users of electron diffraction devices. If surface charge is allowed to accumulate on the sample, the surface potential will become more negative, thereby reducing the incident energy of the incoming electrons. As the energy of the incident electrons is reduced (to a few kilovolts), $\beta$ increases toward unity. The surface potential continues to become more negative until the beam electrons arrive at the energy at which $\beta = 1$; at this point charge accumulation ceases. The increased $\beta$ is considered responsible for the increased brightness associated with a charging sample.

Though these explanations may be valid for certain special situations, they do not adequately explain the effects commonly seen by investigators of biological and other insulating materials (Figure III-1a), in the following respects. In the first case (electron trajectory), Everhart's collector was specially designed to collect only those electrons leaving the sample with a certain energy, whereas most collectors are designed to collect all the electrons possible. There are different types of collectors, of course, and a collecting field created by a grid biased at only +200 volts may be more easily distorted than one created by a collection
electrode, at ±10 kV which is not masked by such a grid. More important, is the fact that objects which might be expected to charge, such as dust particles or fragments broken off the sample after it has been coated, always appear brighter and never darker than their surroundings (although the area immediately adjacent to them may appear darker). If changes in $\gamma$, caused by variations in trajectory between the sample and the collector were responsible for this effect, however, we would expect that some, if not most, of the time it would result in a lower $\gamma$ (and therefore reduced brightness) because the electrons leaving the negatively charged area would be more energetic and therefore less susceptible to direction by the collection fields.

As charging artifacts are readily observable at accelerating voltages of 25 kV and higher, the other two hypotheses (deflection of primary beam into collector, reduction of incident beam electron energy) would require development of surface potentials of many kilovolts. However, charging parts of a sample are often separated from areas at ground potential by only a few microns. It would not be possible to sustain such voltages across this short a distance. In addition, when such voltages can be produced on parts of a sample, the associated fields also bend the beam when it scans adjacent areas producing wildly distorted pictures of these areas (Figure III-1b). (Note image of collector grid in top left.) Finally, as the signal from the charging area usually contains the same sort of topographic information as that from a noncharging area, the primary beam must strike the specimen.
The Present Theory

We must explain an increase in brightness produced by the accumulation of charge near the specimen surface and associated with surface potentials of at most a few tens or hundreds of volts. Logically, we must attribute it to an increase in either the signal collection efficiency ($\gamma$) or the amount of signal leaving the sample ($6_{\text{eff}}$).

While we have acknowledged that variations in surface potential can, and do produce important changes in $\gamma$ by what we might call trajectory contrast, we do not feel that such changes adequately explain why negatively charged objects always appear bright and their immediate surroundings appear dark and not vice versa.

A second mechanism must be at work: a change in $6_{\text{eff}}$. This does not necessarily imply an increase in $5_{\text{-intrinsic}}$: the number of secondaries per incident electron, originally ejected from the surface by the primary beam or by emerging backscattered electrons. It could be explained instead by a reduction in the "specimen collection" of these secondaries.

The term "specimen collection" was first used by Everhart (Everhart 1958) to explain the observation that the side of a hole produces less secondary electron signal near its bottom than it does near its rim. This can be understood in the following way. The number of secondary electrons leaving the surface in a particular direction, $\phi$ (where $\phi$ is the angle between the local surface normal, and the path of the exiting electron) is proportional to cosine $\phi$.

If the sample is flat, all electrons can reach the collector; however, if the sample is rough, a large fraction ($\lambda$) will collide with adjacent areas of the sample again (specimen collection) and not reach
Figure III-2. Specimen collection evident in (a) where some secondaries collide with the sample again can be reduced by the presence of a negative charge below the surface (b).
the collector (Figure III-2a). Briefly,

\[ \delta_{\text{eff}} = \delta_{\text{int}} (1 - \lambda) \]

and we can see that a brighter signal (greater \( \delta_{\text{eff}} \)) can be obtained by reducing \( \lambda \). Figure III-2b shows schematically how this could result from a negative charge being present near the surface. Any such charge will always repel free electrons, reducing the chance that they will hit the sample again, and increasing the chance that they will be collected. This mechanism would explain why charged samples always appear bright.

To test this simple hypothesis, a sample consisting of an insulated brass electrode 0.25 mm in diameter and centered in a 0.5 mm hole in a larger electrode was fabricated, and the end of the center electrode was made coplanar with the outer electrode. In Figure III-3a, this sample has been lapped smooth, and the center electrode is biased at -90, -45, and 0 volts (top to bottom) and viewed at normal incidence. The outer, grounded electrode becomes darker with increasing bias because the field created by the negative electrode repels secondary electrons from this area back into the specimen or at least away from the collector. The center electrode, however, changes brightness very little except where \( \lambda \) was originally high: in scratches and on its steep sides. The fact that only the side of the electrode facing the right or collector side of the picture is brightened is an indication that trajectory contrast also plays a role.

In Figure III-3b the same sample has been scratched with 600 grit emery paper. The contrast setting is somewhat different than
that in Figure III-3a, but one can easily see that the major brightening effect is evident in the scratched area. Actual measurements showed the average increase in signal from the center electrode produced by the -90 volt bias to be about 5% in Figure III-3a and 50% in Figure III-3b. However, when all the currents entering and leaving the center electrode were measured, it was found that the fraction of the electrons leaving the center electrode which reached the collector was almost the same in each case. This shows that trajectory contrast is not the cause.

Let us now consider some of the numbers involved. The collecting field at the surface of the sample is normally on the order of 10^2 V/cm, with the result that secondary electrons having average energies of 4 eV can be expected to travel in essentially straight lines in the direction in which they leave the surface for hundreds of microns before being bent toward the collector. Any roughness on this order of size or smaller can therefore cause specimen collection. Furthermore, any local field appreciably larger than the collecting field can be expected to reduce \( \lambda \). Even on a sample as gross as that shown in Figure III-3a and b, fields of \( 10^3 - 10^4 \) volts/cm can be expected with only 90 V bias, while a 10 micron diameter charged sphere could do the same with only 3 or 4 volts. What is important is not so much the surface potential but localized fields created by the inhomogeneous distribution of charge which we would expect on a rough insulating specimen. The amount of charge accumulation required to reach a steady state (\( \frac{\delta_{eff}}{\delta} = 1 \) averaged over the charging area) will depend on the amount of specimen collection.
Figure III-3. The center electrode in this sample, viewed at normal incidence simulates a smooth charging object which has little specimen collection ($\lambda$) because it has been polished flat (a). Therefore the indicated imposed negative biases cannot increase its brightness by reducing $\lambda$. When this sample is scratched, however, $\lambda$ is increased in the scratches. Therefore greater brightness (more signal) can be realized by reducing $\lambda$ in these scratches with a negative bias (b).
originally present, and will be set by a negative feedback mechanism because the presence of increased charge decreases the rate of charge buildup. The size of this charge depends not only on the roughness of the sample but also on the primary beam voltage and what we might call the porosity of the sample. Though, as we have noted, $\delta_{\text{int}}$ generally increases at lower voltages, this is for smooth, flat surfaces at near normal incidence. If the sample is spongy or fibrous, however, large numbers of secondary electrons (per incident primary electron) can be produced if the beam is energetic enough to emerge from a second surface. Usually, most of these second-surface electrons are collected by the specimen but because they are so numerous, only a small reduction in $\lambda$ is sufficient to reach a steady state. Therefore we have a situation where higher primary beam potential, with its greater penetration, is associated with lower equilibrium surface potential. Coupled with changes in beam induced conductivity this explains why, in some cases, there seems to be more charging artifact at 5 kV than at 50 kV (Figures III-4a and b, respectively), a situation contrary to the conventional wisdom.

**Discharge Artifacts**

In Figure III-5 are three T.V. micrographs of a live, coated beetle taken under nearly identical conditions ($10^{-10}$ A, 25 kV, stub normal to beam). The coating is not continuous with the stub, however, and the beetle only makes contact with ground through the micromanipulator needles seen coming in from the right side (Figure III-5a). When the needles are moved away slightly, the beetle
Figure III-4. Charging artifacts on 18 μ latex spheres which are visible at 5 kV (a) are less obvious at 50 kV (b). In (c) the bright frame on the uncoated 1/2 of this Millipore R filter specimen is produced by stray electrons which have diffused away from the area of the higher magnification raster. They were trapped there because beam-induced conductivity was limited to the raster area and they produce the bright frame by reducing λ on the surface above them. The image of a charged scorpion tail shows the effects of beam bending in the distortion of a piece of EM grid which lies below the tail's tip (d).
charges up and a shifting pattern of light and dark areas is caused by distortion of the collection fields (Figures III-5b). After a reproducible interval of about 3 seconds, however, a second phenomenon is noted: the appearance of bright spots in the image (Figure III-5c). These spots are random in size and in position with respect to the image, and they carry no topographic information. The beam is still striking the sample, however, because the relatively undistorted image of the beetle can still be seen between the flashes. Reconnecting the beetle to ground immediately causes Figure III-5a to reappear.

Tracing down the origin of such transient signals is difficult at this time although, based on the limited amount of information available, we might speculate as to the cause. As the detector is sensitive to light, as well as to electrons, it is possible that in the breakdown of a dielectric somewhere in the sample, light is produced. However, in our experimental set-up, we feel that it is unlikely that such light would be detected. Therefore, we are left with the fact that somehow bursts of electrons are being emitted in a random manner. Due to temperature considerations (which bar thermionic emission), and the fact that the beetle is covered with very sharp hairs, the most likely candidate is either corona discharge or field emission from the tips of the hairs. Rough calculations show that the $10^5 - 10^6$ V/cm fields necessary could exist, and the bad vacuum (compared to that required for continuous field emission) would go far to explain why the bursts are so sporadic.
Figure III-5. Discharge Artifacts. The coated beetle is only grounded through the manipulator needles seen coming in from the right side (a). If contact with the needles is lost, charge accumulates on the beetle and strong trajectory contrast is seen (b). Finally, after a repeatable interval of about 3 seconds, sporadic discharge occurs (c).
High Voltage Charging Artifacts

So far we have mainly discussed charging phenomena which often afflict investigators of rough insulating specimens in spite of their best efforts at coating or other strategies for charge removal. In doing so we have tried to take what might be called the low voltage approach and have attempted to show that many effects can be explained without invoking kilovolt surface potentials. However, we should point out that such large potentials (> V beam/2) can exist on appropriate samples, and that they do produce effects in addition to those mentioned previously.

One of these effects is "pith-balling" or motion of part of the specimen caused by electrostatic repulsion. It is normally possible to distinguish such motion from that caused by heating, drying or radiation damage because of its abruptness. Motion of this type can introduce important errors into the evaluation of stereo-pairs and can also mislead investigators who are attempting to determine the original position of a small particle. It can also be useful. Small pieces dislodged from the sample during micromanipulation often charge up and "pith-ball" away making it unnecessary to go to the trouble of removing them mechanically.

A second effect is image distortion. Weitzenkamp (Weitzenkamp 1969) and others have shown that negatively charged fibers can deflect the primary beam and so produce large, anisotropic deviations in observed magnification. An extreme case of such distortion is Figures III-1b and 4d where the beam has been deflected completely
off the sample in some places, and the grid of the collector can be observed toward the top left corner. This picture was made at 9 kV after the flat, uncoated, Lucite sample, 1 cm in diameter, had first been observed at 25 kV. During the earlier observation the surface potential had reached a steady state value of approximately 7 kV. This produced only slight distortion until the beam voltage is changed from a high to a lower potential. In fact it is quite simple to view the objective aperture in this way by making the cathode potential less than the steady state surface potential previously established by a high voltage beam.

Some have felt it necessary to propose rather complicated mechanisms to explain similar phenomena (Clarke and Stuart 1970) because it is known that $5 = 1$ and charge accumulation ceases before the surface potential equals that of the cathode. Therefore, at a fixed beam voltage, mirroring should not occur. It would seem in this case, however, that one need only assume that the charge injected at the higher voltage does not subsequently leak away very rapidly.

We should point out that when the Lucite sample mentioned above was viewed at 50 kV, electrical breakdown occurred before the steady state surface potential could be reached and the charge alternately built up and then abruptly discharged in a cyclical fashion.

**Resistivity and Time Effects**

Up to now we have considered that the resistance from charging surface to ground was infinite. Obviously, this is an approximation especially when we remember that even a specimen resistance as high
as $10^{11}$ ohms will produce a surface potential of only 2 or 3 volts at commonly used beam currents. The approximation is made even less valid by beam-induced conductivity changes.

The electrical conductivity of a substance depends on the concentration and mobility of charged carriers within it. In solids at room temperature, these carriers are usually unbound electrons and their concentration varies widely between metals ($>10^{21} \text{ cm}^{-3}$), semiconductors ($10^{13} \text{ cm}^{-3}$), and insulators ($<10^8 \text{ cm}^{-3}$). In the process of thermalizing a single low energy (25 kV) electron in a solid, a large number ($>10^3$) of ionizations occur. The free electrons produced in this way are unbound and, until they recombine ($10^{-6}$ to $10^{-10}$ sec), they are capable of acting as carriers and of increasing the conductivity of the area in which they are present.

If the $10^3$ ionizations produced by each of the primary electrons in a $10^{-11}$ A beam occur uniformly spaced within a sphere 10 microns in diameter (corresponding roughly to the volume of beam penetration) and last for $10^{-8}$ sec each, the resulting average carrier density is $1.2 \times 10^{12} \text{ cm}^{-3}$. Therefore, the irradiated region has a conductivity near that of silicon (assuming similar mobilities).

As the irradiated volume increases in low density samples, the free carrier density must necessarily decrease, however, if there are many voids in the sample as there are in freeze dried biological material, it seems reasonable that the mobility may correspondingly increase. Therefore the effect of specimen density may be slight.

As the charge lifetime is short compared to the amount of time that the beam is on any single point on the sample at all but T.V. scan rates, the carriers would act the same way whether the beam is
moving or not. Thus the free charges exist only in the area
directly under the beam. As they can move laterally as well as
vertically, however, the increased conductivity effectively exists
over the entire raster area to the depth of beam penetration (1-50μ
depending on beam voltage and specimen density). This is illustrated
well in Figure III-4c. This is a low magnification micrograph, at
25 kV, of a piece of millipore filter, which has been coated on
only the lower right hand side. It was produced by taking a picture
of the T.V. display immediately after the magnification had been
reduced by a factor of three. At the higher magnification, induced
conductivity was sufficient to prevent charge buildup in the uncoated
part of the raster area but insufficient outside it. Here a charge
of stray electrons has diffused away from the raster area and become
trapped. This trapped charge is now producing the bright frame
around the first raster by reducing \( \lambda \) in the area immediately above
it.

Charging artifacts often appear to be less severe at T.V.
scan rate although they definitely do occur (Figures 4a and 5b and
c). Part of this effect is due to the fact that most T.V. video
amplifiers are a.c. coupled. This removes any d.c. component of
the signal and forces the average brightness of the T.V. screen to
remain constant. Other effects are more speculative but the follow-
ing mechanism is reasonable. Consider two adjacent areas of a
rough insulating sample the first one, \( A \), having \( \delta_{\text{eff}} < 1 \) and the
second, \( B \), having \( \delta_{\text{eff}} > 1 \). Let us assume that as the beam sweeps
from \( A \) to \( B \), negative charge which has accumulated in \( A \) can flow to
\( B \), using beam-induced conductivity. The amount of charge which
accumulates in A will be proportional to the length of time that the beam remains on A before moving to B and will therefore be reduced at higher scan rates.

Most time dependent aspects of charging artifacts can be explained by changes in conductivity, produced by the beam, and by effects resulting from the finite capacitance and resistance between the charged surface and ground.
Summary of Charging Artifacts

The distinguishing characteristics of the various artifacts discussed above are summarized below. More than one may be present at any given time.

1. Reduced Specimen Collection ($\lambda$)
   - produces greater signal from rough charged samples at all surface voltages.

2. Trajectory contrast (voltage contrast)
   - produces dark as well as light areas on the charging part of the sample
   - sensitive to specimen orientation.

3. Sporadic discharge
   - bright flashes of variable length and unrelated to sample topography.
   - a higher voltage phenomenon particularly common on samples with sharp protrusions.

4. High surface potentials ( $> 1/2$ cathode potential)
   - can cause large distortions in the image especially if beam voltage is reduced.
   - may produce pith-balling.
   - can only exist where permitted by the resistive, geometrical, and dielectric properties of the sample.
Complications

Charging is a very complicated topic. It takes a computer to calculate all the secondary electron trajectories from the surface of a uniformly charged sphere just above a ground plane, and most SEM specimens are far more complex than that. It is also a rather important topic because the secondary electron SEM signal, even from a conducting sample, is a function of so many variables. The added complication of charging artifacts has already been the cause of much misinterpretation, at least in part because there is widespread misunderstanding of how they are produced. We have attempted to provide possible and reasonable mechanisms both new and old which, individually or in combination, can be used to explain a large number of effects. In particular, we believe that reduced specimen collection, together with changes in collection efficiency caused by distortion of the collection fields (voltage or trajectory contrast), are responsible for most brightness artifacts. However, we expect to see other mechanisms elucidated for certain special cases.
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Note: IITRI stands for Illinois Institute of Technology Research Institute.


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