TRANSMISSION ELECTRON MICROSCOPY IN MEDICINE

By L. G. DICKSON, MD

Contributing Authors
M. A. CLARK, PhD
C. H. DORSEY, PhD
G. W. KINDSCHI, MD

Published 1973 by
COLLEGE OF AMERICAN PATHOLOGISTS

TRANSMISSION ELECTRON MICROSCOPY IN MEDICINE

By L. G. DICKSON, MD

Contributing Authors
M. A. CLARK, PhD
C. H. DORSEY, PhD
G. W. KINDSCHI, MD

Published 1973 by
COLLEGE OF AMERICAN PATHOLOGISTS

INTRODUCTION TO THE MANUAL

Chapter I

This manual was prepared to acquaint members of the medical profession with the current status of transmission electron microscopy* and its relationship to light microscopy. Concepts, results and techniques are for hospitals now utilizing, or planning to utilize, a transmission electron microscope in patient care.

During recent years, many hospitals and medical centers have installed transmission electron microscopes. Every year more hospitals decide to take this step toward expanding their laboratory capabilities. Even the casual reader of medical literature realizes how electron microscopy aids in establishing the correct anatomic pathologic diagnosis and estimating a patient's prognosis. Some institutions desirous of doing electron microscopy do not because of fear of the microscope and its required support facilities including other equipment, personnel and unusual supplies. This fear is not realistic. When any new instrument or technique is introduced into a hospital, one anticipates problems with installation, education for use and interpretation of results. For some reason, electron microscopy is surrounded by an aura of mystery and difficulty far greater than is justified.

A reasonable approach to the understanding of the electron microscope is to consider it to be a close relative of an inverted light microscope (Figure 1). Instead of visible light, the electron microscope uses a beam of electrons. In place of glass or quartz lenses, the electron microscope uses electromagnets as lenses. Tissue stains for light microscopy interfere with passage of light; stains for electron microscopy interfere with passage of electrons. When this is appreciated, the electron microscope becomes much less mysterious. Once some of the mystery is dispelled, laboratory personnel can begin intelligent planning. First of all, they should decide if now or at a given point in

L. G. DICKSON, MD, is the author of this manual. Doctor Dickson serves as Head of the Experimental Pathology Division, Naval Medical Research Institute at the National Naval Medical Center in Bethesda, Maryland.

The opinions or assertions contained herein reflect the views of the author and are not to be construed as official or reflecting those of the Naval Department or the Naval Service at large.

*The terms transmission electron microscope and electron microscope in this manual are used as synonyms. Techniques do not apply for scanning electron microscopy.

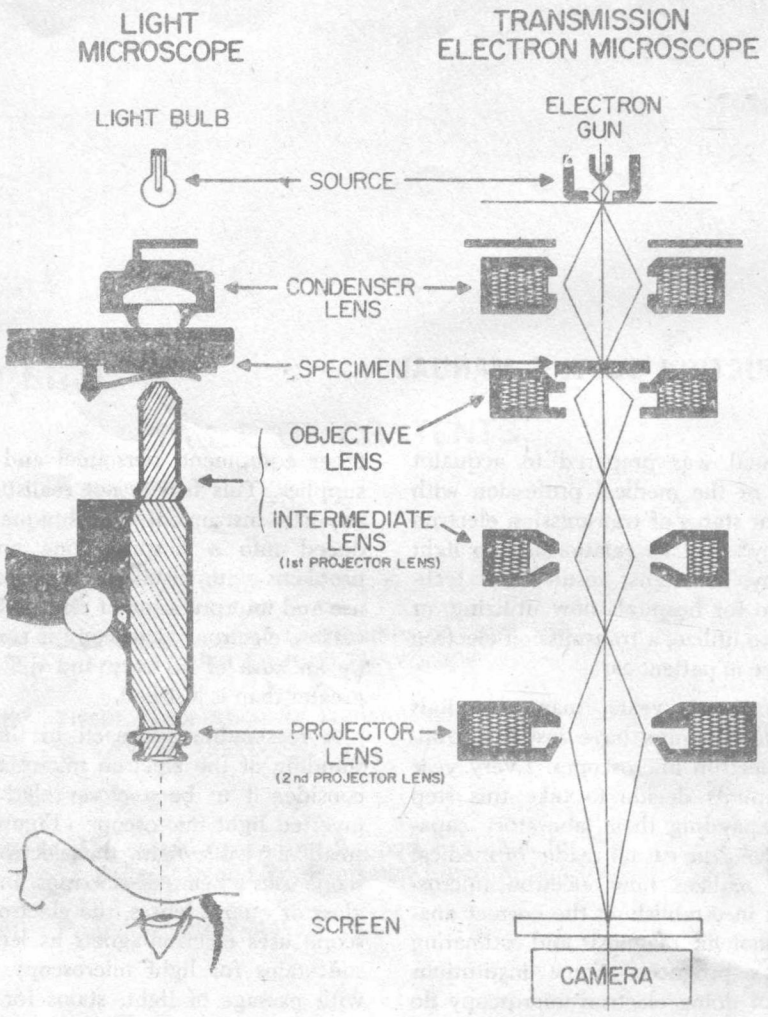

Fig. 1

the future they will want and need an electron microscope. If the answer is yes, planning for the instrument and associated space, equipment and staff should begin.

The following chapters provide a guide for either beginning hospital electron microscopy or modifying current techniques to provide broader use for an instrument already in operation. Recommended specific reagents and methods were chosen for reliability, consistency, cost and convenience. Results achieved are excellent for hospital needs. Skills and experience gained from use of these methods should allow easy introduction of more sophisticated methods as the needs arise. The net result is much more enjoyable than painful.

CHARACTERISTICS AND CAPABILITIES OF LIGHT AND TRANSMISSION ELECTRON MICROSCOPY

Chapter II

The physician using a transmission electron microscope need not understand the intricacies of design and construction to use the instrument. Proper utilization, however, does require considering some conditions occurring only in electron microscopy which markedly influence image formation.

First, information may be characterized as general and detailed. For purposes of this discussion, a situation producing *general* information is defined as examination of tissue viewing thousands of cells and their interrelationships as is best accomplished by light microscopy and less well accomplished by electron microscopy. Again, for our current purposes, *detailed* information is defined as information about the ultrastructural features of cells and their associations. In examination of a tissue section, general information is best gained by looking at more of the tissue at low magnification. Information of increased detail is obtained by examining the same tissue at higher magnification and resolution.

When large volumes of information are needed, light microscopy probably will be the best analytic method. How else can you reasonably examine relatively large volumes of tissue looking for specific arrangements of cells or individual cells of a certain kind? Once these specific features are found, usually you can readily transfer to examination of selected areas by electron microscopy. Therefore, the electron microscope usually is an adjunct tool and not a substitute for a light microscope.

The tried and true application of electron microscopy to the diagnosis of kidney disease is a specific example of good hospital application of this method. The renal glomerulus is well suited to electron microscopic examination by routine methods. Such an examination regularly provides information of diagnostic and prognostic value. There is broad agreement on interpretation of findings. In some areas electron microscopy of kidney biopsy specimens is standard practice.

Electron microscopy also is popular for the diagnosis of leukemic cell type in poorly differentiated cases. Often this is helpful. I personally feel that *in vivo* studies with supravital staining and observation of motility patterns are at least as reliable and less expensive. Our use of electron microscopy for marrow or circulating leukocyte examination is less than in some other laboratories; however, we find it useful in selected cases, especially in plasma cell associated neoplasms, and a method for processing these specimens is included in Chapter III.

Fortunately, for the pathologist, fixed human tissue is reasonably well suited for examination in the electron microscope. It survives the high vacuum of the column and heating in the focused electron beam quite well.

RESOLUTION

In microscopy, the resolving power and contrast of light or electron optical systems are major considerations. Resolving power is the smallest distance between

two structures which may be distinguished. The design of the optical system of a microscope determines its maximum resolving power. For light microscopes, the maximum resolving power usually is about 0.2 micron. Electron microscopes are capable of resolving 5 A (1 A = 10^{-4} micron) or better. This suggests that even molecules can be seen in electron photomicrographs. In practice, this is not the case because actual resolution is affected by factors such as specimen thickness and contrast. A practical rule is that maximum resolution is one-tenth the specimen thickness. Most sections of tissue are about 600 A thick, thereby limiting resolution in any electron microscopy to 60 A. Technically, it is difficult to produce 100 A sections to take advantage of full capabilities of an electron microscope with 10 A resolution. Basically, this need for ultrathin sections is the reason for going to the bother of ultramicrotome sectioning of specimens.

If sections were cut at 2 micron thickness, the resolving power of an electron microscope becomes 0.2 micron—the same as a light microscope. Ultrathin (100-900 A) sections therefore are needed to provide better resolution. It frequently is stated that ultrathin sections are necessary for penetration of the tissue by the electron beam. This is not strictly true since high voltage electron beams may penetrate even thick specimens. Use of such high voltages is now most applicable in fields other than medicine where different materials are examined and different factors influence the final result. For routine medical purposes, do not select a high voltage electron microscope.

Now, the potential purchaser of an electron microscope for medical application can begin to establish a list of performance characteristics for a microscope to fulfill his requirements. If his needs are to examine tissue cut to 400-600 A thickness, he should buy an instrument capable of 40 A. This resolution is suitable, from this single point of view, to his needs.

Even if a laboratory plans to excel in ultra-microtomy and produce 100 A thick sections as a routine, the medical electron microscopist must consider another factor limiting resolution. This is specimen contrast. To have high contrast, a specimen must have high electron scatter. High physical density is required for high electron scatter, and biologic materials just do not have it; therefore, they have low electron scatter potential and, alas, low contrast. The result is that a 100 A thick section of tissue in a high resolution electron microscope will not resolve 10 A molecules but barely be able to separate 100-200 A diameter structures. If a structure such as a small low density molecule must be demonstrated, it may be made more visible by special methods such as shadow casting or negative staining.

Fortunately, most medical electron microscopy is accomplished without having to resolve small low density molecules; but the more advanced medical electron microscopist eventually will need to develop special skills allowing this to be done. With proper equipment, these techniques are not difficult.

MAGNIFICATION RANGE

Along with resolution, an important consideration in an electron microscope is magnification range. Again, the needs for medical electron microscopy are different from those of other biological and physical applications.

The pathologist tends to use lower ranges of magnification than most other electron microscopists. Medical specimens usually are examined at 500-20,000 magnifications. Probably less than 15 per cent of hospital electron microscopy is at magnifications greater than 20,000 and less than one per cent at magnifications over 100,000. Therefore, it is important to

choose an instrument capable of "low magnification," i.e. 300-2,000 magnifications, for a routine hospital installation. Our understanding of human pathologic changes still is limited. This will probably be true for five to ten years. Highly detailed information delivered by an electron microscope at 200,000 magnifications may be useful in 5-10 years as a routine but not now.

The main point of this discussion of resolving power and magnification is to allow potential purchasers to evaluate their needs and avoid purchasing equipment which is more competent and expensive than a hospital would require.

OPERATION AND INSTALLATION

Other features and performance capabilities of transmission electron microscopes should be considered. Features bearing on operating the instrument probably are next most important. Consider two questions: How easy is it to start, operate and turn off the instrument? And, what damage may occur if the instrument is improperly operated?

The first question is an important one of convenience for the staff allowed to use the instrument. The more simple the operation of the instrument, the more service it will provide. Most recently introduced instruments have fully automated turn-on procedures. This is a major convenience item. Setting one control once is much easier than a turn-on sequence requiring setting several switches and valves to various positions at intervals. Automated control systems seem to work quite reliably. Manual control systems seem to be one major cause of fear and uncertainty among pathologists and others when first introduced to electron microscopy. This problem now may be avoided; this should not be interpreted to be a recommendation that automated control of a vacuum system is essential. Many laboratories, including our own, are satisfied with manual controls.

The question of damage to the instrument when improperly operated should be considered next. Working in a darkened room, even the most experienced electron microscopist will commit errors. Any instrument should be able to protect itself from these human errors, plus those of mechanical or electrical origin such as electrical failure, loss of cooling water flow and so on.

If installation requires more than one circuit (usually the case), the instrument should be able to protect itself if and when it experiences a partial power loss when a single circuit breaker causes part of the instrument to lose electricity. Ideally, an instrument should return to a shutdown condition if a potentially dangerous condition develops. A loud alarm is almost as satisfactory. The type of vacuum system requires consideration. Automated systems are supposedly protected from all damage. Certain manual ones will refuse to perform steps which may damage the instrument. Unfortunately, some electron microscopes can do such things as pump oil into the column if improperly operated. This can cause serious damage and require time-consuming repairs.

OTHER PERFORMANCE SPECIFICATIONS

The goal is to get as many electrons as possible from the gun (filament) at the top of the column of the microscope to the viewing screen at the bottom. The vacuum system, in addition to being safe, should develop a vacuum sufficient to do the quality of work desired. Vacuum is needed within the column. The electron beam should follow as predicable and usually linear a path as possible. Molecules of air deflect electrons. Therefore, the vacuum in the column is necessary to allow the electron beam to pass efficiently down the column, through the specimen

and produce an image with useful sharpness.

Most medical work described so far can be accomplished at a vacuum of 1×10^{-4} torr (1 torr = 1 mm Hg). Very high resolution work may require 1×10^{-6} torr. Systems producing 1×10^{-9} torr probably are unnecessary for any hospital transmission electron microscopy now and for the next ten years. In addition, such ultra-high vacuum systems are expensive and require considerable maintenance. Fortunately, electron microscope manufacturers provide vacuum systems with capacities generally suited to the other performance features of their instruments.

Voltages used for acceleration of the electron beam in examination of human tissue usually are in the 40-80 kilovolt (KV) range. Lower voltages enhance image contrast but reduce resolution. The reverse is true of higher voltages. Occasionally, use is made of this relationship. A specimen of low contrast at the usual operating voltage may sometimes be viewed satisfactorily at a lower voltage.

ALIGNMENT

Alignment procedures should also be considered before purchase of an electron microscope. The design of the instrument should allow this to be accomplished at necessary intervals without inconvenience. A procedure requiring more than one-half hour is unreasonable. The actual alignment should allow step-wise centering of the beam, through each condenser and objective lens to the final screen. In addition, all apertures should be centerable. It is also convenient if more than one objective aperture and condenser aperture are available in a carrier at one time. Different sizes of apertures, readily available, make an instrument more versatile.

Astigmation of the beam should be externally adjustable. Spherical aberration is occasionally prominent, especially at low magnifications, but should not be detectable to the unaided eye at the level of the final screen.

SPECIMEN CHAMBER

The specimen chamber should be separable from the column, and specimen changing time should be one minute or less. Movement of the specimen on the stage should be at least 1 mm in X and Y axes perpendicular to the beam. Specimen rotation and tilting relative to the beam are only rarely needed when examining human material in a hospital.

A specimen chamber anti-contamination device is desirable if high magnification examination is to be performed. The anti-contamination device is an integral part of the microscope or attachment which, by metal probe of "finger" thermo-conduction, conducts heat out of the column. This metal finger is cooled by liquid nitrogen or dry ice and acetone.

It is wise to avoid an anti-contamination device which severely limits specimen movement or has a reservoir in an inconvenient position. Such a position may interfere with safe filling of the reservoir with liquid nitrogen or the position may interfere with normal access to column controls or the viewing ports. On one microscope, it is convenient for these purposes, but I always bang my forehead on it while moving from the main viewing screen to the intermediate screen during alignment. I wish a thermo-electric anti-contamination device were available. This would eliminate the need for using potentially dangerous solidified or liquefied gases for cooling. You probably will use your anti-contamination device rarely in hospital work, so don't worry too much about selecting a given model.

VIEWING PORTS AND SCREEN

It is convenient but not necessary to have more than one viewing port or window. Two or more windows allow the

operator and observers to view the specimen image at the same time. This is an aid in education and training of all personnel concerned.

CAMERA

The camera is an important part of the electron microscope. This is the case because more reliance is placed on photographs in electron microscopy than in light microscopy. In fact, essentially no final opinions of diagnosis and prognosis are offered without study of an electron photomicrograph.

Cameras commonly use three sizes of film or photographic plates: 3¼ x 4″, 70 mm., and 35 mm. Not all microscopes offer a full range of choice. The 3¼ x 4″ glass plate or sheet film are desirable for the work of the highest quality. Roll film is less expensive from some points of view; however, results are so much poorer that the 3¼ x 4″ format is a better bargain in the long run.

Glass plates have a slight cost advantage over sheet film but require more space for storage. When you consider the magnitude of the investment necessary to buy, maintain, and operate an electron microscope, the cost differential between even 35 mm. film and glass plates is minor and more than balanced by the improvement in quality produced when 3¼ × 4″ plates or sheet film are used.

There should be an airlock between the camera and the microscope column. This allows exchange of plates or film size without letting air into the whole column. It also allows use of the microscope for specimen examination during periods when the camera chamber is open or during its evacuation after loading. While it probably is necessary to pre-dry plates or films before they enter the camera, the time required for this reloading operation should not exceed 30 minutes. Five to 10 minutes are reasonable. Automatic or semi-automatic control of exposure time is a desirable convenience.

A photocell to compute exposure times should be the minimum provision for exposure control. A feature to mark each plate or negative with the number of the exposure greatly simplifies record keeping but is not essential. If an automatic plate numbering device is present, there should be an external indicator of this number so the microscopist may maintain accurate records of exposures.

The first instrument for a hospital probably should be one of the newer small models made by several manufacturers. These are much less expensive than earlier instruments. They are simple in operation, incorporating automatic vacuum systems and very convenient controls and cameras. Basic models usually cost under $30,000. If not part of the standard basic model, the following accessories should be purchased: extra condenser lens, extra lens below the specimen whether called intermediate or projector or by some other name, automatic camera and plate or sheet film capability. The total cost for this should be under $40,000.

These small units are compact; one manufacturer's advertisement showing a microscope being delivered on a small trailer behind a small car is almost true. The modules are delivered in a small crate and assembled in two days or less. The instrument is operable immediately. Having survived one protracted installation which really wasn't complete for three years, a long time ago, I can assure you this is a real advantage.

I know of one of these compact instruments installed in an overgrown closet next to an elevator. It not only fits the space, but it has proven remarkably resistant to high magnetic fields in the area.

When buying a microscope, specify at least three full days of instruction on the

maintenance and use of your instrument for three people as a group. This time should be split into two segments, usually of two and one days. The first instruction gets you going. The second provides help polishing your technique one to two months later. One year of service should be included in the purchase price or negotiated at the time of purchase.

You may also consider buying a used electron microscope. If, for some reason, a more complicated instrument is desired but cost is a major consideration, this is a good approach. Siemens Elmiskop 1-A and Philips EM 300 microscopes would be good choices for such a purchase. Each is a very superior optical instrument and will be for 5-10 years. They lack only convenience features with which a laboratory may well dispense.

FACILITIES

Prior to installation, the room proposed for microscope installation should be surveyed for stray interfering magnetic fields, vibration, adequacy of electric power and water supplies, and ability of the floor to support the instrument's weight. The water should be examined for dissolved and suspended materials which may damage the instrument. Initial attention to these considerations will save later expense. In many parts of the country, it is wise to plan for a recirculating water system for cooling the column and oil diffusion pump (part of the vacuum system). Determine how much alteration of the available room will be required. Determine where utilities will have to be installed. Find out how much these will cost. These expenses may surprise you; it is better to get this surprise before you have used your whole equipment budget for the instrument itself.

Because an electron microscope undeniably and unavoidably is an expensive and complex addition to any hospital laboratory, it is essential to establish the required and desirable specifications and features for the hospital's use and find the best instrument to fill these needs. Advice on these matters is best obtained from other hospitals already using such equipment. If such advice is not available locally, a nearby university performing research in any of the biologic sciences can probably provide assistance.

Electron microscopists devoted purely to use of the instrument in the physical sciences can provide less specific advice because they are unfamiliar with peculiarities of biologic material including human tissue. Fortunately, many residencies in pathology in larger institutions at least partly train residents in electron microscopy. As more and more of these pathologists are trained, hospitals will, firstly, have more reason to offer electron microscopy in anatomic pathology and, secondly, be able to achieve this goal more easily.

Facilities required for tissue preparation and photography are included in Chapters III, IV and V.

CHAPTER III

TISSUE PREPARATION FOR TRANSMISSION ELECTRON MICROSCOPY

The purpose of this chapter is to describe a method of tissue processing for examination of tissue by transmission electron microscopy.

Proper demonstration of ultrastructure by electron microscopy dictates certain limitations on specimen size and fixation. Detailed methods described at the end of this chapter allow the maximum flexibility and convenience we feel are compatible with good final results. Portions of the processing may be automated if desired.

The anatomic pathologist unfamiliar with electron microscopy will notice two major requirements: 1. Fixation of tissue should be immediate. In the case of postmortem specimens, the time between death and obtaining specimens for study should be as short as possible. 2. Tissue specimens are cut to small size for fixation and later processing. At first glance, the sizes dictated seem impossible to achieve regularly. It is surprising, however, how quickly skill to do this is developed and even exceeded.

The most effective way to demonstrate the need for immediate cutting of specimens into small cubes and immersion in fixative is to process tissue handled in a way just beyond the limits set. When compared to properly prepared tissue, all personnel are quickly motivated to meet established criteria. Meeting these criteria usually means the pathologist or laboratory technician should attend the surgical procedure, receive the specimen as soon as it is removed from the patient, and begin his work.

This work involves placing the specimen (usually it is a small one) or desired portion of the specimen in a pool of fixative on wax paper, glass or nonporous surface. Portions of tissue up to 10 x 5 x 5 mm may be handled at this stage.

The tissue first is sectioned at 1-2 mm intervals to produce sheets. These are cut at 0.08-1 mm intervals to form strips and the strips are cut at 0.5 mm intervals to form cubes or rectangular tissue fragments. An ordinary razor blade is satisfactory for this. For safety, the blade should be mounted in a handle. Facility sufficient to produce 40-50 cubes within one minute of receiving the specimen is a reasonable goal. Once tissue cubes are created, the number desired are transferred to a bottle, jar, or vial containing the fixative. One or two of the narrow strips may be added to this container for insurance.

Frequently, specimens for electron microscopy are needle biopsies. These are ideal for cutting into small cubes with speed and convenience. When dealing with kidney biopsies, it may be desirable to separate cortical tissue blocks from those of medulla if the distinction can be made grossly or by using a magnifying glass or dissecting microscope.

The following methods of fixation, dehydration, clearing, embedding, sectioning and staining are selected or designed to provide consistently good results. This is not to be taken as a statement that equally

---

In Chapter III, Doctor Dickson has been joined in co-authorship by M. A. Clark, PhD; C. H. Dorsey, PhD; and G. W. Kindschi, MD.

fine results cannot be obtained by using other materials and methods. Indeed, once a laboratory achieves success with electron microscopy, it is usual to broaden the technical repertory to include special techniques and alternatives to the routine. Readily available alternatives are used because experience indicates that some tissues are best processed in ways slightly different from those proven best for other tissues. In our laboratory, we regularly handle diverse tissues such as skin, liver, kidney, lung, blood and bone marrow, so we must occasionally slightly modify fixation and embedding procedure. Advice on these matters is included in the following sections whenever appropriate.

Before launching into consideration of specific steps in tissue processing, there is one more subject of which we should not lose sight. This is the subject of artefacts. The goal of any microscopy is to be able to reproduce standard artefacts by fixation and staining and to minimize those which are variable and which distort or obscure features we want to see. Undesirable artefacts are more commonly a cause of difficulty in transmission electron microscopy than in light microscopy. Reasonable attention to detail and consideration of tissue response to physical and chemical influences, especially during fixation, greatly reduce undesirable nonstandard artefacts. It is for this reason that emphasis is placed upon types of fixatives (Table 1) and osmolarity of the fixatives. Such questions are only rarely a consideration in light microscopy. Once good fixation and staining are mastered, undesirable artefacts usually are minimized automatically, but any laboratory needs to be aware of and guard against their occurrence.

FIXATION

Fixative must reach and enter a cell before fixation occurs. Because even subtle autolytic changes may significantly alter the ultrastructural appearance of tissue, rapidity of fixation is essential. To achieve this rapid fixation, small fragments of tissue, 0.5 mm or less in thickness, are cut as rapidly as possible in a pool of fixative.

Currently, two chemicals, osmium tetroxide and glutaraldehyde, are the most popular fixatives. In our opinion, paraformaldehyde also is a satisfactory fixative deserving more use. Paraformaldehyde penetrates rapidly and more quickly than glutaraldehyde. Formulae for both glutaraldehyde and paraformaldehyde fixatives are included below. Both work well, but the paraformaldehyde option is recommended as being better. Medical electron microscopy usually requires what is called "double fixation." This involves fixing tissues in two fixatives. The most common ones in use are either glutaraldehyde or paraformaldehyde followed by osmium tetroxide. Double fixation is resorted to not only for fixation. Osmium tetroxide also stains cellular components. Uranyl acetate and lead citrate are commonly used for staining. Their staining functions are considered to predominate over their fixation effects. For this reason, they are considered under the section on stains. Fixative effects are modified by buffer systems used, as shown in Table 1.

Major cellular constituents may be listed as nucleic acids, proteins, fats, phospholipids, and carbohydrates (Table 1).

Nucleic acids, DNA and RNA, are poorly fixed by standard fixatives, but may be stained well with uranyl acetate or lead salts. *Proteins* of the cell are best fixed by aldehyde fixatives. Prolonged exposure to osmium may actually remove some protein from specimens. Therefore, exposure to osmium should be kept under two hours, unless the desire is to enhance phospholipid membranes with some loss of protein.

Buffer/ Fixative	Phosphate	Cacodylate	Collidine[1]	Chrome-osmium[2]	OsO_4[2]	$KMnO_4$[3]	Aldehyde
NUCLEUS	peripheral chromatin clumps pores not prominent		dispersed chromatin pores prominent enlarged nucleus	dense, clumped	dispersed chromatin		peripheral chromatin
MITOCHONDRIA	irregular profile prominent dense granules	dense matrix	smooth profile light matrix	irregular profile sharp outlines		swollen matrix	myelin figures
RER[4]	straight uniform profiles even RNP		RNP decreased	closely packed RNP		RNP extracted	straight uniform profiles
GLYCOGEN	dense aggregates		dispersed			aggregated	aggregated
MICROTUBULES	well preserved				not uniformly preserved		well preserved
MEMBRANES	Osmium precipitates Uranyl acetate precipitates			Excellent contrast	Good		appearance due to OsO_4 post-fixative
LIPIDS					dense, black irregular outline		smooth, grey irregular at low pH Vacuolated at high pH
EASE OF SECTIONING	Good	Excellent	Excellent	Good	Excellent	Poor	Excellent

1. Generally poorer contrast than other buffers.
2. Generally suitable only for use as post-fixatives. Suitable for use as primary fixatives only when main goal is examination only of fat and/or membranes. Cytoplasmic detail is nil.
3. Produces friable animal tissue resistant to staining. Membrane staining reverse of osmium; i.e., only the middle lamina stains. Good for plant specimens.
4. Rough Endoplasmic Reticulum

Unsaturated fats and *phospholipids* are very well fixed by osmium tetroxide and moderately well preserved by potassium permanganate. *Phospholipid* fixation and staining are important because phospholipid is the major component of most cellular membranes. Osmium tetroxide performs this task well.

Carbohydrates, as a class, are poorly fixed and stained by current techniques for light and electron microscopy. Fixation and staining methods are available only for glycogen and mucopolysaccharides. En bloc staining with uranyl acetate removes glycogen. However, when used as a stain for sections only, glycogen is well demonstrated. Glycogen is easily and routinely stained by lead salts for electron microscopy. Mucopolysaccharides are stained with metal deposition methods discussed in the section on staining techniques.

We subscribe to the philosophy that fixatives should approximate intracellular conditions with regard to pH and osmolarity. We have been especially impressed by Maumsbach's demonstration of the value of controlling such factors. It cannot be denied that excellent results can be obtained using fixatives which are not physiologic with regard to pH and osmolarity. However, we feel that fixatives with controlled pH and osmolarity produce more consistently good results than other methods. This seems to be especially true of renal tubules, liver parenchymal cells and testes. Each laboratory should probably evaluate its need for such control of tissue fixation conditions.

Selection of fixative buffers again is a personal matter with the exception that any phosphate buffer system seems to produce a fine precipitate in erythrocytes. This is probably due to formation of a random precipitate when residual buffer reacts with uranyl acetate when used as directed in this manual, that is, by block staining. This is not a problem when sections are stained with uranyl acetate. We recommend block staining with uranyl acetate to provide experience with such a method when learning to process tissue. Staining of cut sections may be substituted if desired following a procedure such as indicated for lead citrate. We prefer to avoid these problems by rising cacodylate or S-collidine buffers. Avoid S-collidine when fixing blood and bone marrow because this buffer lyses red cells and poorly preserves white cells. Neither is it essential or possible to remove all residual aldehyde before exposing tissue to osmium tetroxide. A brief wash in the buffer used in preparation of the fixative will remove sufficient aldehyde to provide satisfactory results. Washing should be for 30 to 60 minutes. Prolonged washing allows the Golgi apparatus and rough endoplasmic reticulum to collapse and loss of cytoplasmic contents.

DEHYDRATION AND CLEARING

Dehydration and clearing of tissue for electron microscopy are best conducted by passing the specimen through graded alcohol into xylene. Other combinations of solvents are in use. Some are better than the ethanol:xylene combination for some purposes. We recommend ethanol:xylene as an initial solvent system because of low cost, fire hazard, toxicity and skin sensitizing risks. The method is exactly the same as that used in routine light microscopy. In fact, we have tested diffusion rates of various recommended combinations of dehydrating and clearing agents and find ethanol and xylene to be the most rapid and efficient combination. Specific defects of other systems include the following: Methanol is sufficiently hydroscopic to absorb enough water from surrounding air and, therefore, it may not fully dehydrate tissue. Osmium tetroxide, uranyl acetate, and phosphotungstic acid either are insoluble in acetone or react with it to form a precipitate. Propylene oxide is highly volatile and diffuses slowly into other organic solvents suitable for processing tissue.

We see no reason why dehydration and clearing, along with bulk staining for electron microscopy, as described later, cannot be automated. Instructions at the conclusion of this chapter include directions on how this may be achieved. Prior to automation, each of our technicians handled tissue producing only about 15 blocks per day, including all steps from fixation to production of final sections. Automation of dehydration, clearing and a portion of embedding increased this figure to 20-30 per day. With such employees receiving $8000-$12,000 per year, this has not only produced a worthwhile saving in money but also has improved morale, since proper processing by manual meth-

ods requires exacting attention to schedules which become monotonous. We now process only suspensions of cells or organelles by manual methods.

EMBEDDING

Epoxy resins (Araldite, Epon, Vestopal) are now in general use as embedding media. The above resins are the most popular and produce excellent results. Epon 812 may be used in the processing technique described later. We prefer to use a medium of our formulation called PGE-22. This medium has a single major advantage in our opinion: The viscosity is low when compared with other standard epoxy resins. This lower viscosity shortens the time required for tissue infiltration.

PGE-22 has another advantage which generally is less important, but still worthy of mention. Hardness of the final product may not only be controlled by varying proportions of constituents, but by varying the time of exposure to heat for polymerization. Hardness of a block may be increased by subsequent reheating beyond the usual time. This advantage occasionally has proven valuable when tissues of varying consistencies are being processed at the same time.

Analysis of PGE-22 suggests that polymerization is incomplete after the usual period of heating. This was one of our goals in formulating PGE-22. The aim was to produce a low viscosity epoxy resin which could readily be dissolved from tissue sections for staining for light microscopy. It is important, especially in a laboratory beginning epoxy embedding techniques, to select one medium and master its preparation, handling, polymerization and cutting before trying alternates.

Epoxy resins, albeit almost universally used in electron microscopy, have one potentially serious disadvantage. All seem capable of causing sensitization of skin in the prepolymerized form. After polymerization, they are completely safe in this respect. Sensitization and contact dermatitis require contact between the liquid epoxy and personnel who process tissue. Such exposure by contact probably always can be prevented by proper handling techniques.

Some laboratories have a rule that requires all pre-polymerized epoxy be handled only while personnel wear rubber or plastic gloves. This seems to be a simple solution to the problem by preventing exposure and sensitization. Don't forget that epoxy will contaminate counter tops and containers. We require that counter tops and glassware be washed with acetone after an epoxy has been mixed and handled. Glass or ceramic work areas on counter tops are recommended. This clean-up also should be accomplished while wearing protective gloves.

No currently available epoxy embedding medium completely satisfies all requirements. Undoubtedly, new media will be developed and published during the future.

The low viscosity formulation currently used in our laboratory has several desirable physical properties. It has a low viscosity allowing adequate penetration of the tissue, yet in its cured state, it is soft and resilient enough to be easily cut with a diamond knife using the ultramicrotome. It has a pot life of several hours at room temperature, but will set to final hardness overnight at 70°C. It does not create major interference with the electron beam nor does it cause undue artefact formation within the tissue. Last, but not least, it is partly soluble allowing partial removal of the plastic from the final section or block, should this be needed. All of these properties can be plausibly explained by analyzing the reaction of the basic ingredients.

SECTIONING

In addition to cutting sections, many

microtomes may be used to trim the tip of the block into a pyramid suitable for final section preparation. An experienced person may perform this by hand using a razor blade. Until such experience is gained, the microtome should be used to prepare the block in this way. Separate instruments are available which perform only this step. A large laboratory may find use for such capability but a smaller one easily can produce satisfactory results without this assistance.

Instruction manuals which accompany ultramicrotomes provide instructions for block preparation and actual section cutting. These manuals generally seem to be very well written. We teach operation of the ultramicrotome by giving the manual to our new trainee and telling him that he is on his own. So far, each pupil has been successful with self-instruction in the basic operation of the ultramicrotome. This is the only area of instruction where we allow this freedom from close supervision. Basic facility with the ultramicrotome is gained quickly if the trainee has past experience with sectioning tissue for light microscopy using a rotary microtome. Such an approach has also been successful while training high school and college students who join our department for the summer.

People in training seem to appreciate this opportunity for individual work, concentrate on their task, and regularly begin producing good sections in four to twelve hours. Not every section by a long shot is good during this early period of gaining experience; however, two to four weeks of fulltime operation of the microtome should be sufficient to produce sections in good volume (more than twenty blocks per day) and of consistent quality.

Glass and diamond knives are used for cutting sections for electron microscopy. We have tried ceramic knives and can't make them work well. In our combined technique, we usually cut sections of tissue for light microscopy at the same time we make sections for electron microscopy. All instruction and much routine sectioning is done using glass knives. Our microtomists are sufficiently experienced so that they can rapidly adjust to frequent changes of knives, which is required if glass is used. A diamond knife is more resistant to dulling than glass. It, therefore, will allow a microtome operator, with modest experience, to produce significantly better sections. Diamond knives also allow for maximum production rate with technicians of any level of competence. Do not cut thick sections with a diamond knife. Thick sectioning causes excessive wear of the knive and may actually break the knife.

The convenience and efficiency of the diamond knife is offset in part by two realities of life: First is the expense. They cost from $350 to $1,000. Second is the requirement for periodic resurfacing. This process now requires three to five months with most companies. Therefore, if diamond knives are used, allowance must be made for this "down time." If possible, obtain re-sharpening service from a company which guarantees its work. When a knife is broken during sharpening and no prior replacement arrangement is made, it causes delay in work, added cost, and great frustration.

We wish we could give a definite formula to calculate how often diamond knives require such service. We calculate our knife usage in man-months. Sometimes we get six months and sometimes only three. We have noted that tissue containing calcium salts and collagen dulls a knife much more rapidly than other tissues. Some laboratories report that kidney dulls diamond knives quickly. We sometimes think we have sectioned acres of kidney and don't feel it is as hard on a knife as lung or skin. Variation in the skill

of the microtomist also must be considered. Our newer personnel seem to dull their knives more quickly. This may be due to the fact that these people must make more sections before they produce the quality they desire. If we could afford enough diamond knives to satisfy all our requirements, we would plan on owning 2.0 knives for everyone which was needed at a given time. This would allow for the current long time required for refinishing. This lengthening time results from a backlog at the service facility. Demand, therefore, exceeds supply, which is an ample testimonial of the valuable service performed by this product.

A glass knife is made logically enough in a "knife breaker." This instrument is essential and is a beautiful example of mechanical simplicity, relatively easy operation, and long life in heavy service. Only occasional replacement of certain key parts is required. This instrument allows predictable production of sharp knives of a precise configuration. The alternative is a manual glass breaking procedure. This not only wastes large amounts of relatively expensive glass, but more expensive employee time on a frustrating job.

After a strip of glass is broken into triangular knives, a small strip of tape is attached to produce a little well for water. Sections are floated on this water as they "come off" the block. Diamond knives come with, or are inserted in, similar wells.

MOUNTING SECTIONS

Cleanliness may or may not be next to godliness, but it is essential in electron microscopy. At no step is it more important than during mounting of the section. We use water passed through a small pore-size membrane filter to fill the well of the knife. As the section slides off the knife, it floats on the surface of the water. At this point, the microtomist determines the thickness of the section by evaluating the interference color. Interference colors are those commonly seen when we look at pools or lakes of water with an oil film. They are the iridescent reflections we see. The thin specimen sections form the same colors when floating. If you want 600 A thin sections, you want a section which is silver in color. Thicker sections are gold, red, and purple. Thinner sections are colorless. We usually aim for "silver sections."

Sections of the block are transferred to grids for election microscopy or usual glass slides for light microscopy. A grid is a piece of metal used to support the tissue section in the electron microscope. Staining of sections (as opposed to bulk staining) is accomplished after the section of tissue is attached to the grid for most purposes.

Grids for electron microscopy usually are thin discs of copper. The most commonly used ones have a center mesh which supports the specimen. Less commonly used are single hole grids which have a single large central opening. In use, this central hole must be covered by a thin film of electron lucent plastic. This film serves as the support for the tissue section. The single hole grid has the advantage that there is no mesh which obscures portions of the tissue section. This advantage is marked when low magnification electron microscopy is planned. The additional effort of applying the film to the grid may be amply compensated for by increased convenience.

There are two other disadvantages of such a single hole grid/film combination. For one, the film is another layer through which the electron beam must pass. This reduces the electron beam striking the final viewing screen. Any imperfections in the film appear with the specimen image. Until a laboratory learns how to produce high quality film these imperfections (usually dust and bubbles) may be more