Coating Techniques for SEM and Microanalysis

10.1. Introduction

Nearly all nonconductive specimens examined in the scanning electron microscope or analyzed in an electron probe microanalyzer need to be coated with a thin film of conducting material. This coating is necessary to eliminate or reduce the electric charge which builds up rapidly in a nonconducting specimen when scanned by a beam of high-energy electrons. Figures 10.1a and 10.1b show examples of pronounced and minor charging as observed in the SEM. In the absence of a coating layer, nonconductive specimens examined at optimal instrumental parameters invariably exhibit charging phenomena which result in image distortion and thermal and radiation damage which can lead to a significant loss of material from the specimen. In extreme situations the specimen may acquire a sufficiently high charge to decelerate the primary beam and the specimen may act as an electron mirror. Numerous alternatives to coating have been proposed and some of these will be discussed in this chapter. Much of what will be discussed is directed towards biological material and organic samples simply because these types of specimens are invariably poor conductors and more readily damaged by the electron beam than most inorganic materials. However, it is safe to assume that the methods which will be described for organic samples will be equally effective for nonconducting inorganic specimens.

This chapter will concentrate on the practical aspects of some of the more commonly used vacuum evaporation and sputter coating techniques which are now standard procedures in most electron microscope and analytical laboratories. It is not proposed to enter into a detailed discussion of the theoretical aspects of thin film technology, but those readers inter-
10.1.1. Specimen Characteristics

10.1.1.1. Conductivity

The single most important reason for coating is to increase the electrical conductivity of the sample. Materials of high resistivity, i.e., exceeding $10^{10} \Omega \cdot \text{cm}$, will rapidly charge under the incident beam and may develop a potential sufficient to cause a dielectric breakdown in certain regions of the specimen. This leads to variations in the surface potential, giving rise to the complex image artifacts commonly referred to as “charging.” These artifacts are manifest as deflection of low-energy secondary electrons, increased emission of secondaries within the crevices of a rough specimen, periodic bursts of secondary electron emission, and deflection of the electron beam, all of which degrade the resolving power and analytical capabilities of the system by introducing astigmatism instabilities, undue brightness, and spurious X-ray signals (Figure 10.2). This undesirable situation is frequently compounded because many of the adhesives used to attach the specimen to the substrate are themselves nonconductors and may prevent any electrical charge leaking away, even from conductive samples. A suitable conducting path may be established with silver or carbon paint. If one is concerned only about electrical conductivity, then a thin layer of gold, silver, or copper will suffice to eliminate the problems associated with charging. Even though metallic samples are usually conductive, there are situations where one may wish to examine nonconducting areas, e.g., inclusions, and in these cases it is necessary to apply a thin coating layer. The conductivity of the thin film should be sufficient to ensure that the specimen current is drained to ground without the development of a significant surface potential.

10.1.1.2. Thermal Damage

Specimen heating is not usually a problem in most samples examined in the SEM, because the probe current is usually in the picoampere range. Although higher currents are frequently used for TV scanning, these are unlikely to seriously degrade the specimen. Thermal effects are potentially more serious for cathodoluminescence and X-ray microanalysis, for which the probe currents are in the nanoampere and even the microampere range.
10.1.1.3. Secondary and Backscattered Electron Emission

The thin layer of metal which is usually applied to an insulator to make it electrically and thermally conductive is also the source of the bulk of the secondary electrons. A 10-nm layer of metal such as gold would certainly improve the coefficient of secondary-electron emission, $\delta$, for an organic specimen examined at low kilovoltage, but might well diminish $\delta$ for a ceramic containing significant amounts of alkaline earth oxides.

Backscattered electrons have also been used in conjunction with standard cytological techniques to localize regions of physiological interest in biological tissue. Thus if one has gone to considerable effort to obtain deposits of lead or silver at specific sites in a piece of tissue it seems inappropriate to mask the atomic-number contrast these locations will give by covering them with a layer of heavy metal. The appropriate technique would be to apply a thin layer of a low-atomic-number conductor such as carbon which would not significantly scatter the incident beam, allowing it to reach the specimen.

For high-resolution, low-loss scanning electron microscopy where the image is dependent on the scattering of high-energy electrons from the specimen surface, it is necessary to coat the samples with a thin layer of a heavy metal which shows no structure at the 1-nm resolution level. Experimental evidence suggests that the refractory metals such as tantalum or tungsten yield such a coating.

10.1.1.4. Mechanical Stability

Particulate matter and fragile organic material are more firmly held in position on the specimen stub after coating with a thin layer of carbon. In many cases it is possible to place such material directly onto the specimen support and stabilize it with a very thin layer of carbon applied from two directions. This simple technique avoids the use of adhesives, most of which are highly nonconductive. Metal coatings, particularly those deposited by a sputtering process, are quite strong and contribute to the increased mechanical strength of otherwise fragile material.

10.1.1.5. Uncoated Specimens

Several methods can be used to examine uncoated specimens in the SEM, including operation at low beam energy, incorporating a second beam of electrons or ions to discharge the specimen, and examining the specimen in the presence of water.

Reduction of charging at low accelerating voltages is due to the characteristics of electron emission from solids, as indicated in the follow-
established with the effective $E$ equal to $E_{II}$. Oatley (1972) points out that this equilibrium is unsatisfactory, since $E_{II}$ can vary significantly about the surface, giving rise to large variations in the final surface potential from place to place. Slight leakage through surface conduction can greatly disturb such a situation, producing complicated image behavior as a function of time. It is thus desirable to operate with $E_1 < E < E_{II}$, which is often achieved with $E$ set to approximately 1 keV. Under such conditions, insulators can be examined uncoated. However, SEM performance is usually significantly poorer at such low accelerating potentials, since source brightness is greatly decreased under such conditions. With an SEM equipped with a field emission gun, the high brightness can be utilized to obtain good image resolution even at low beam energies (Welter and Coates, 1974).

Low beam energy prohibits x-ray microanalysis, since the overvoltage is inadequate except for x-rays of extremely low energies. To avoid this limitation several authors have reported methods which utilize a second beam of low-energy electrons or ions to discharge the sample during bombardment by the high-energy electron beam. Spivak et al. (1972) employed a pulsed beam of low-energy electrons to bombard the specimen during the scan line flybacks. The principle of the sample discharge is the same as that shown in Figure 10.3. Crawford (1979), described a charge neutralization method based on a low-energy beam of positive alkali ions which are attracted to the sites of negative surface charges.

If the specimen contains water, it will have sufficient conductivity to discharge. Considerable success has been obtained with examining and analyzing uncoated biological material at low temperatures (Echlin and Saubermann, 1977). In conditions where water is retained in the frozen state, so too are ions and electrolytes (Echlin, 1978) which provide conductivity. If specimens can be examined in an environmental cell, water can also be retained in the liquid state to discharge the primary beam (Robinson and Robinson, 1978).

### 10.1.2. Alternatives to Coating

One of the more useful techniques which has been devised is to increase the bulk conductivity of the sample as distinct from the surface conductivity. An increase in bulk conductivity may be achieved by metal impregnation from fixative solutions of osmium and manganese, with or without the use of organic metal ligands or mordants such as thiocarbohydrazide, galloglucose, paraphenylenediamine, by exposing specimens to OsO$_4$ vapor or by bulk staining the specimens after fixation with metallic salts. Kubota and Veda (1980) have devised a useful modification to the osmium vapor technique by also exposing the sample to hydrazine hydrate.
vapors which result in the deposition of metallic osmium. The relative merits of these methods are discussed in the recent review papers by Munger (1977) and Murphey (1978, 1980).

Alternatively, specimen charging may be reduced by spraying or impregnating with organic antistatic agents derived from polyamines, i.e., Duron, Denkil, or sodium alkyl-benzene sulfonate, soaking in conducting colloids of noble metals or graphite, or covering the sample with a thin (~1.0–20.0-nm) polymer film such as Formvar or styrene-vinylpyridine (Pease and Bauley, 1975).

With the possible exception of the techniques which increase the bulk conductivity of the sample, none of these methods gives anything like the resolution and information content which may be obtained from properly coated samples. Indeed, these alternative methods have diminished in usefulness now that it has been shown (Panay et al., 1977) that one can adequately coat even the most delicate and thermally sensitive specimens in the sputter coater. Further, it can be argued that if a sample cannot survive the moderate vacuum found in a sputter coater it is unlikely to survive the high vacuum of an electron beam instrument.

The techniques devised to increase bulk sample conductivity are useful especially when they are used in conjunction with coating techniques. Bulk sample conductivity methods are frequently used in connection with the examination of fractured surfaces of three-dimensional specimens whose internal morphology is being investigated. For example, the substructure of fractured surfaces may be revealed by atomic number contrast from osmium incorporation, rather than topographic contrast derived from surface irregularities.

### 10.1.3. Thin-Film Technology

Thin films can be produced in a variety of ways (Maissel and Glang, 1970), but of these methods only thermal evaporation and sputtering are useful for coating specimens for SEM and x-ray microanalysis. In discussing these methods below, it is important to consider the properties of the ideal film. Such a film should exhibit any structural features above a scale of 3–4 nm resolution to avoid introducing unnecessary image artifacts. The ideal film should be of uniform thickness regardless of the specimen topography and should not contribute to the apparent chemical composition of the specimen or significantly modify the x-ray intensity emitted from the sample.

### 10.2. Thermal Evaporation

Many metals and some inorganic insulators when heated by one means or another in a vacuum begin to evaporate rapidly into a monoatomic state when their temperature has been raised sufficiently for the vapor pressure to reach a value in excess of 1.3 Pa (10⁻¹⁻ Torr). The high temperatures which are necessary to permit the evaporation of the materials can be achieved by three different methods.

In the resistive heating technique an electric current is used to heat a container made of a refractory material such as one of the metal oxides or a wire support made of a high-melting-point material such as tungsten, molybdenum, or tantalum. The material to be evaporated is placed in or on the container, which is gradually heated until the substance melts and evaporates. In the electric are method an arc is struck between two conductors separated by a few millimeters. Rapid evaporation of the conductor surface occurs. This is the usual way by which some of the high-melting-point metals are evaporated. For most high-melting-point materials such as tungsten, tantalum, and molybdenum, the most effective way of heating the substance is to use an electron beam. In this method the metal evaporant is the anode target and is heated by radiation from a cathode maintained at 2–3 keV. The electron gun can also be used for evaporating some of the lower-melting-point metals such as chromium and platinum which have a very small particle size.

We can conveniently consider evaporation methods under two headings, high- and low-vacuum techniques.

#### 10.2.1. High-Vacuum Evaporation

In this context high-vacuum is considered to be the range between 10⁻⁶ Pa and 10⁻¹⁰ Pa (~10⁻¹⁻ Torr). High-vacuum evaporation techniques are commonly used in electron microscope laboratories.

The formation of a thin film is a complex process and proceeds through a series of well characterized steps: nucleation and coalescence to form a continuous film. The first atoms arriving at the surface of the specimen will only stay there if they can diffuse, collide, and adhere to each other on the surface to form nucleus sites of a critical size. The stronger the binding between the adsorbed atoms and the substrate, the higher the nucleation frequency and the smaller are critical nuclei. Most biological and organic samples are likely to have variable binding energy, which would result in variation of critical nuclei across the surface, producing uneven film deposition. For this reason precoating with carbon at low vacuum to cover the specimen with a homogeneous layer results in smaller, even-sized critical nuclei when subsequently coated with metal. For example the nucleation density of gold can be substantially increased by 5–10 nm carbon precoating. As deposition continues, the nucleation centers
grow in three dimensions to form islands, which gradually coalesce to give a continuous film (Figures 10.4a-10.4e). The rate of continuous film formation and the average thickness at which a given film becomes continuous are influenced by a large number of variables. These include the nature of the evaporant and the substrate, the relative temperatures of the evaporant and substrates, the rate of deposition and final film thickness, and the surface topography of the specimen.

10.2.1.1. The Apparatus

There are four basic requirements for a high-vacuum evaporator. (1) It should have high pumping speeds at low gas pressure to ensure that there is a rapid removal of gases liberated from the evaporant source and specimen during coating. (2) There should be minimum backstreaming into the coating chamber of vapors from the pumps. (3) The system should be readily taken apart for cleaning and maintenance. (4) Adequate provision should be made for electrical connections for multiple evaporation schemes and sample manipulation.

Most units are pumped out using a diffusion pump backed by a rotary pump. The larger the throat size of the diffusion pump, i.e., 150 mm versus 75 mm, the faster the pump-down time of the evaporation chamber, although there will only be a marginal improvement in the ultimate pressure which is obtained. Some units are equipped with turbomolecular pumps backed by rotary pumps. Such units give faster pump-down times and a cleaner vacuum, although the ultimate pressure is no better than that obtained using a diffusion pump. The rotary pump should be a two-stage pump capable of being ballasted. Gas ballasting is one of the most effective means of preventing vapor condensation in a rotary pump. The method

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Figure 10.4a. Formation stages of a thin film (from Leaver and Chapman, 1971).

Figure 10.4b-e. Electron micrographs of ultrathin gold films evaporated at 0.05 nm/s. Thicknesses are (b) 1 nm, (c) 4 nm, (d) 6 nm, and (e) 15 nm. (From Kazmerski and Racine, 1975.)
consists of leaking a small quantity of air into the rotary pump during the compression cycle so that the exhausted vapor is mixed with a noncondensable gas. This decreases the compression necessary to raise the exhaust valve and prevents vapor condensation. Rotary pump vapor condensation can also be decreased by raising the pump temperature and by placing a desiccant such as P_{2}O_{5} in the exhaust line. To avoid backstreaming, the system should not be pumped below 10 Pa (10^{-1} Torr) on the rotary pump alone. The rotary pump should not be exhausted into the laboratory but vented into a fume cupboard or to the outside. There seems little necessity to resort to ion pumps, sublimation pumps, or exotic cryopumping systems as ultrahigh vacuum (130 nPa to 130 pPa) is not required for the commonly used evaporation methods.

Whatever system is used it is necessary to ensure that the backing and roughing lines from the pumping unit to the chamber are fitted with baffles and/or an activated alumina foreline trap to minimize backstreaming of pump oil. Water-cooled baffles are quite effective, although liquid-nitrogen baffles are better. A liquid-nitrogen trap in the roughing line will minimize diffusion of forepump oil into the evaporation chamber during roughing operations and maintain the forepressure of a diffusion pump at approximately 10 mPa. It is also useful to have a liquid-nitrogen trap above the diffusion pump between it and the coating chamber. Care should be taken to note where the vacuum pressure is read in the system. If the pressure is read near the pumps it is likely to be ten times better than that found in the evaporation chamber. The evaporation chamber should be made of glass and as small as is convenient for the work to be carried out. A safety guard should be fitted over the evaporation chamber to minimize danger from implosion fragments. The chamber should contain at least four sets of electrical connections to allow evaporation of two different materials, specimen rotation and thin-film measurement. The electric power for the evaporation sources should be variable, and it is useful to have a pushbutton control to allow maximum power to be applied in short bursts. The unit should be brought up to atmospheric pressure by means of a controllable needle valve which can be connected to a dry inert gas.

### 10.2.1.2. Choice of Evaporant

The choice of material to be evaporated and the manner by which it is to be applied is very dependent on the particular application in hand. A tabulation of selected elements and their properties, which are useful for coatings, is listed in Table 14.11 of the data base, Chapter 14. For most SEM work, gold, gold–palladium, or platinum–carbon is used. Silver has a high secondary-electron coefficient and is one of the best substances for faithfully following the surface contours. Unfortunately silver suffers from the disadvantages that it easily tarnishes and has a larger grain size than other metals. Gold has a high secondary emission, is easily evaporated from tungsten wire, but has a tendency to graininess at agglomerates during coating, requiring a thicker coating layer to ensure a continuous film. A 60:40 gold–palladium alloy or palladium alone shows less granularity than gold and yields one of the thinnest continuous films. Unfortunately both metals easily alloy with the tungsten holder. Platinum–carbon when evaporated simultaneously produces a finer grain size but of rather low conductivity. Maeki and Benoki (1977) have found that low-angle (~30°) shadowing of specimens with evaporated chromium before carbon and gold–palladium coating improves the image of samples examined in the SEM. The finest granularity is obtained from high-melting-point metals, but they can only be evaporated with electron beam heaters. Figures 10.5 and 10.6 show the results.
of interest. Particular problems can occur if the element of interest is present in small or trace amounts. The usual thicknesses used range between 5 and 50 nm (50 and 500 Å). For 5–10-nm (50–100-Å) films of carbon, aluminum, gold, and gold–palladium, the energy loss of the primary beam appears to be of small consequence even at low nominal voltages. However, the beam and backscattered electrons obtained from the specimen could excite x-ray radiation from the film. This process may be particularly serious for gold and gold–palladium coatings on specimens with average atomic numbers greater than 10.

Examination of mass attenuation coefficients (\(\mu/\rho\)) shows that for x-ray lines from 8 to 40 Å, aluminum is lowest of all four, followed in order by carbon, gold, and gold–palladium. In the region below 8 Å, the carbon \(\mu/\rho\) value is lower than those for aluminum, gold, and gold–palladium. However, gold is best when electrical and thermal conductivities are considered, with aluminum about one-third as good, and carbon poor. It would seem that, for general purposes, aluminum is favored by its physical properties for use with x-ray lines of 0.8–4 nm (8–40 Å), while carbon is favored outside this region.

Most of the commonly used evaporants are available in the form of wire. It is recommended that thick wire, i.e., 0.5–1.0 mm is used, as short pieces can be easily looped over the appropriate refractory wire. Substances not available in wire form are available as powders or chips and can be conveniently evaporated from refractory crucibles or from boats made of high-melting-point metals. Care should be taken to ensure that the evaporant does not alloy or form compounds with the refractory. Most metals with a melting point below 2000 K can be evaporated from a support wire or boat made from a refractory metal such as tungsten, molybdenum, or tantalum. Such supports should be good electrical conductors, have a very low vapor pressure, and be mechanically stable.

10.2.1.3. Evaporation Techniques

When a pressure of about 10 mPa has been reached, the refractory support wire and, where appropriate, the carbon rods, can be heated to a dull red color. Gentle heating will cause a sharp rise in the vacuum pressure brought about by outgassing and removal of residual contamination. Once the outgassing is completed, the current is turned down and pumping continued. If a carbon coating is to be applied to the specimen before metal coating, this is best done at a pressure of about 10 mPa.

During coating the specimens are rotated and tilted to give an even coating on all surfaces. Following the deposition of the carbon layer the vacuum pressure is decreased to a pressure between 1.0 mPa and 100 μPa. An electric current may now be passed through the tungsten wire holding the metal to be evaporated. This should be done carefully and it is best to
gradually increase the current until the tungsten wire just begins to glow and then back off a small amount. This allows the metal evaporant to heat up slowly and melt as the tungsten wire current is slowly increased.

The metals commonly used in evaporation form a molten sphere in the V of the tungsten wire. This stage should be allowed to remain for a few moments to remove any residual contamination. The current should then be further increased until the sphere of molten metal appears to shimmer and "rotate." When this point is reached evaporation has started and the shutter may be opened to expose the rotating specimen to the evaporation source. In order to achieve a uniform coating on complexly sculptured specimens it is essential that they be rotated rapidly (6–8 rps) during coating. The ideal arrangement is to rotate the specimen in a planar motion while continuously tilting it through 180°. This slow heating, melting, and evaporation of the metal evaporant is most important, and more particularly so with aluminum which alloys with tungsten. If the source is heated too rapidly the metal evaporant melts at its point of contact with the tungsten wire and falls off. However, the speed of coating is important in obtaining films of good quality, and the faster the speed of evaporation the finer the structure of the layer. This optimal high speed of coating must be balanced against the higher thermal output from the source, and the increased chance of the wire support alloying with the substrate and melting. The thickness of the evaporated film may be measured by a number of different techniques which will be described later, the most convenient of which is a quartz crystal thin-film monitor mounted inside the vacuum chamber. The thickness deposited also depends on the particular specimen being studied and the type of information required from the sample. It has usually been considered necessary to apply sufficient metal to give a continuous film since it has been assumed that only a continuous film would form a surface conductive layer on a nonconductive sample. Although the physical nature of charge transport in particulate films is not clearly understood, recent work suggests that particulate layers may be a useful method for SEM specimens because discontinuous metal films exhibit a significant dc conductivity. Paulson and Pierce (1972) have shown that discontinuous films can conduct a limited current by electron tunneling between evaporated island structures and suggest that such discontinuous films may be useful for the examination of nonconductive specimens viewed at very low current. Specimens have been successfully examined at 20 keV which have only been coated with 2.5 nm of gold (Figure 10.7).

The color of the layer on a white card or glass slide can give a quick practical estimate of thickness. For most specimens a carbon layer visible as a chocolate color and a gold layer which is a reddish-bronze color by reflected light and blue-green to transmitted light will be sufficient. For aluminum coating sufficient metal is deposited when the layer is a deep

Figure 10.7. Cleared diatom frustules coated by evaporation, with increasing thickness of gold. (a) 2.5 nm; (b) 5.0 nm; (c) 10 nm; (d) 20 nm; (e) 50 nm; (f) 100 nm and examined in the secondary mode of operation at 30 kV. Note that the very thin layers give optimal information, whereas the thicker layers obscure the specimen surface.
blue to transmitted light. Once these parameters have been worked out for a particular evaporation unit with a fixed geometry of specimen and source, it is only necessary to cut off a standard length of evaporant metal wire for each coating.

10.2.1.4. Coating Artifacts

If the coating procedure has been carried out properly, artifacts are rarely seen. Some of the possible causes of artifacts are discussed below.

(a) Heat of Radiation. The intensity of thermal radiation reaching the specimen depends on the source temperature and the source-to-specimen distance. The heat of radiation may be diminished by using a small source and/or moving the specimen further away. The best practical solution is to use a small source at high temperature and have at least 150 mm between the source and the sample. Provided the specimen is adequately shielded from the target, and the shutter only opened at the working temperature, little damage is likely to occur.

Thermal artifacts appear as smooth micromelted areas on inclined fracture faces of biological materials which are bombarded vertically, minute holes, and surface distortions. If specimens are being damaged by heat radiation, the damage can be diminished by placing a cold plate with an aperture over the specimen.

(b) Contamination. Contamination is due primarily to dirt and volatile substances in the vacuum system being deposited on the specimen, and it is for this reason that care must be taken to clean the system properly before use. The most effective way to reduce this problem is to surround the specimen with a cold surface. However, this is probably unnecessary except for very high-resolution studies. Contamination may be recognized as uneven coating and hence charging, as small randomly arranged particulate matter, and in the most extreme situations as irregular dark areas on the specimen.

(c) Decoration Effects. Decoration or agglomeration of the evaporated material occurs to some extent with most metal coatings and is a result of uneven deposition of the evaporated metal. Agglomeration occurs if the cohesive forces of the film material are greater than the forces between the film molecules and the substrate. Because of geometrical effects, rough surfaces are particularly difficult to coat evenly, and it is inevitable that those parts which protrude will receive more coating than crevices and holes.

(d) Film Adhesion. Poor film adhesion is associated with hydrocarbon and water contamination, and, in the case of plastics, with the presence of a thin liquid layer of exuded plasticizer. Discontinuous and poorly cohesive films are recognized by a "crazed" appearance and have a tendency to flake easily. In the microscope there are variations in the image brightness, and charging occurs on isolated "islands" of material which are not in contact with the rest of the film.

10.2.2. Low-Vacuum Evaporation

In low-vacuum evaporation, carbon is evaporated in an atmosphere of argon at a pressure of about 1 Pa. The carbon atoms undergo multiple collisions and scatter in all directions. This technique is useful for preparing tough carbon films and for coating highly sculptured samples prior to analysis by x-rays, cathodoluminescence, and backscattered electrons. However, its general usefulness for SEM specimens is questionable, particularly as the yield of secondaries from carbon is very low. There is little doubt that the multiple scattering and surface diffusion of the carbon allows it to more effectively cover rough specimens, and for this reason would be a useful method to use if a sputter coater was not available.

10.3. Sputter Coating

Although sputter coating has been known for a long time it is only recently that it has become more widely used for producing thin films. In the process of sputtering an energetic ion or neutral atom strikes the surface of a target and imparts momentum to the atoms over a range of a few nanometers. Some of the atoms receive enough energy in the collision to break the bonds with their neighbors and be dislodged. If the velocity imparted to them is sufficient, they are carried away from the solid (Wehner and Anderson, 1970).

The glow discharge normally associated with sputter coating is a result of electrons being ejected from the negatively charged target. Under the influence of an applied voltage the electron accelerates towards the positive anode and may collide with a gas molecule, leaving behind an ion and an extra free electron. The glow discharge is located some distance from the target. The positive ions are then accelerated towards the negatively biased target where they cause sputtering. At high accelerating voltages many electrons are ejected per impinging ion, and these electrons have sufficient energy to damage delicate targets.

A number of factors affect the deposition rate. The sputtering yield increases slowly with the energy of the bombarding gas ion (Figure 10.8). The current density has a greater effect than the voltage on the number of ions striking the target and thus is the more important parameter determining the deposition rate (Figure 10.9). Variations in the power input can have a dramatic effect on the properties and composition of sputtered
films; for example, with increasing power levels, aluminum films became smoother with fewer oxide particles. As the pressure of the sputtering system is increased, the ion density also increases. There is a linear increase in current, and hence sputtering rate, between pressures of 3 and 20 Pa. But because there is an increased tendency at higher pressures for the eroded material to return to the target, a compromise pressure of between 3 and 10 Pa is commonly used.

Impurities in the bombarding gas can appreciably reduce the deposition rate. Gases such as CO₂ and H₂O are decomposed in the glow discharge to form O₂, and the presence of this gas can halve the deposition rate. Deposition rate decreases with an increase in specimen temperature, although this phenomenon may not be peculiar to sputter coating. Finally, the deposition rate is higher the closer the target is to the specimen, but this also increases the heat load on the specimen. The ejected particles arrive at the substrate surface with high kinetic energy either as atoms or clusters of atoms, but not as vapor. There is some evidence that the sputtered atoms have sufficient energy to penetrate one or two atomic layers into the surface on which they land.

There are several different ways to produce the sputtering process, including ion beam sputtering, plasma sputtering, radiofrequency sputtering, triode sputtering, diode (DC) sputtering, and cool diode sputtering. As the technique has evolved for SEM and electron probe specimen coating, only ion beam, diode, and cool diode sputtering are now commonly employed.

10.3.1. Ion Beam Sputtering

The technique of ion beam sputtering is illustrated in Figure 10.10a. An inert gas such as argon is ionized in a cold cathode discharge, and the resulting ions from the ion gun are accelerated to an energy of 1–30 keV.

Figure 10.9. Variations in film thickness in relation to the sputtering voltage and current.

Figure 10.10. Sputter coating techniques: (a) ion beam sputtering; (b) diode sputtering; (c) cool diode sputtering. Key to symbols: A, specimen; B, target or evaporative source; C, target atoms; D, low-pressure argon discharge; D', high vacuum; E, electrode; F, glass vacuum vessel; G, anode; H, permanent magnet; I, line to vacuum pumps; J, ion gun. K, inert gas supply; L, high-voltage power supply; M, insulator; O, direct current power supply (1–3 keV); P, aluminum shield; Q, iron pole piece; R, cooling module; S, lines of magnetic flux.
The ion beam may be formed by collimation or focused with a simple lens system to strike the target. The energetic ions strike the target atoms and impart momentum in plastic collisions; atoms lying near the surface of the target are ejected with energies in the range 0-100 eV. These sputtered atoms then deposit on the sample and all surfaces which have a line of sight to the target. An advantage of this arrangement is that a field-free region exists between the target and substrate so that negative ions and electrons are not accelerated toward the substrate. Multiple coatings can be applied from different targets, providing care is taken to prevent cross-contamination of the targets during sputtering. If a nonconducting target is used, the buildup of positive charge can be suppressed by an electron flood gun. Ion beam sputtering has been used by Adachi et al. (1976) and Higson et al. (1977) for high-resolution shadowing. By using sputtered films of tungsten–tantalum, tungsten, and carbon, they were able to see details smaller than 1.0 nm.

10.3.2. Diode or Direct Current Sputtering

This is the simplest, most reliable, and most economical type of sputtering and is the basis of a number of commercially available dedicated instruments as well as sputtering attachments for thermal evaporators. These instruments, which operate between 1 and 3 keV, are sometimes referred to as diode sputtering units as well as DC sputtering units. DC sputter coating units consist of a small bell jar containing the cathode target and water-cooled specimen holder anode, which sits on top of a control module containing the vacuum gauge, high-voltage power supplies, leak valve, and a small timer (Figure 10.10b). The detailed mode of operation and application of this type of instrument has been previously described (Ehlin, 1975a). One of the potential problems of this type of coater is that delicate specimens can be thermally damaged.

10.3.3. Cool Diode Sputtering

The heat problem of diode sputtering has been overcome by redesigning the diode coater to incorporate devices to keep the specimen cool throughout the coating procedure (Panayi et al., 1977) as shown in Figure 10.10c. The electron bombardment of the specimens is significantly reduced by replacing the disk-shaped target of the diode coater with an annular target. Thermal damage to the specimen is further reduced by fitting a permanent magnet at the center of the target and an annular pole piece around the target. This arrangement deflects the electrons outside the periphery of the specimen holder. As a further precaution, the specimen holder is cooled by a small Peltier effect cooling module. Using this improved equipment it has been possible to coat crystalline hydrocarbon waxes with melting points as low as 305 K, and thermolabile sensitive plastic films, neither of which had been previously coated successfully by any of the methods available.

10.3.4. Sputtering Techniques

It is important that the coater is fitted to an adequate supply of clean, dry argon, or, where appropriate, another noble gas. A small trace of nitrogen in the argon probably does not matter, but it is important that the gas is free of water, carbon dioxide, and oxygen. Traces of water vapor, which are recognizable by the blue tint in the glow discharge, can easily be removed by passing the gas through a column of desiccant. The final traces of volatile material may be removed from the target by bombarding it with a current of 20 mA at 2.5 kV at a pressure of between 2 and 8 pA. The target can be considered clean when there is no degradation of vacuum when the high-voltage discharge is switched on.

Specimens are placed on the specimen table and the unit pumped down to about 10-15 Pa (10^-1 Torr) using a two-stage rotary pump fitted with an activated alumina foreline trap to prevent oil backstreaming. It is most important not to let the unit pump for a long time at the ultimate pressure which may be obtained by the rotary pump, because this will cause backstreaming, resulting in contamination. Because of this it is advisable to have the argon leak valve slightly open, to ensure a continual flow of inert gas through the system giving a pressure of about 6-7 Pa. If the unit is fitted with a water-cooled or, better still, a Peltier module cold stage, this should be turned on and the specimens cooled to the working temperature.

One of the commonest sources of poor pumping performance in both sputter and evaporative coaters is the continual outgassing of the specimen and the adhesive used to attach it to the support. It is recommended that biological and organic material, after it has been dried and attached to the specimen support, be placed in a 310 K vacuum oven overnight to ensure that all volatile substances are removed before coating.

Once the specimen has been cooled and an adequate pressure has been reached, the sputter coating may proceed. The system is pumped down to about 2 pA and the high voltage turned to 2.5 kV. The argon leak valve is opened until a plasma discharge current of 12-15 mA is recorded at a pressure of 6-7 Pa. The timer is set and coating continued until the desired thickness has been deposited on the specimen.

It is possible to use nitrogen as an alternative to argon. However, extended discharge times and/or higher plasma currents are necessary to give the same thickness of coating. Sputter coating in air should be avoided.
because the presence of water vapor, carbon dioxide, and oxygen gives rise to highly reactive ions which can quickly degrade the specimen.

10.3.5. Choice of Target

Platinum or the gold–palladium targets have been found to be satisfactory for the routine specimen preparation for the SEM. Targets are available in most of the other noble metals and their alloys as well as elements such as nickel, chromium, and copper. There are differences in the sputtering yield from different elements and these must be borne in mind when calculating the coating thickness. There are difficulties with a carbon target, for although it is possible to very slowly erode the target with argon, the sputtering rate falls off rather rapidly. This decrease is due either to the presence of forms of carbon which have a binding energy higher than the energy of the argon ions, or the poorer conductivity of the carbon gives rise to charging and a decreased erosion rate. The claim that carbon can be sputtered at low kilovoltage in a diode sputtering coater are probably erroneous. The deposits of “carbon” which are achieved are more likely to be hydrocarbon contaminants degraded in the plasma than material eroded from the target. There seems to be little likelihood that a simple technique will be devised for sputter coating aluminum. The oxide layer which rapidly forms on the surface of aluminum is resistant to erosion at low keV, and the rather poor vacuum makes it difficult to deposit the metal. For details of other targets, particularly those made by nonconductive materials and bombarding gases, the reader is referred to the book by Maissel and Glang (1970).

10.3.6. Coating Thickness

For a given instrumental setting there is a linear relationship between the deposition rate and the power input. In practical terms this means the thickness of the coating layer is dependent on gas pressure, the plasma current, voltage, and duration of discharge. It is difficult to accurately deposit thin films, i.e., below 10 nm, and it is necessary to rely on evaporative techniques for such films. However, most biological material requires about 15–25 nm of metal coating, and once one has established the ideal film thickness for a given specimen it is easy to accurately reproduce this thickness on other samples.

10.3.7. Advantages of Sputter Coating

One of the main advantages of the technique is that it provides a continuous coating layer even on those parts of the specimen which are not in line of sight of the target. Figure 10.11 compares the major coating processes. The continuous layer is achieved since sputtering is carried out at relatively high pressures and the target atoms suffer many collisions and are traveling in all directions as they arrive at the surface of the specimen. Highly sculptured structures or complexly reticulate surfaces are adequately coated. This ability of the target atoms to “go round corners” is particularly important in coating nonconductive biological materials, porous ceramics, and fibers. Complete coating is achieved without rotuting or tilting the specimen, and using only a single source of coating material. Provided the accelerating voltage is sufficiently high it should be possible to sputter coat a number of nonconducting substances such as the alkaline halides and alkaline earth oxides, both of which have a high secondary-electron emission. Similarly, it should be possible to sputter substances which dissociate during evaporation. Film thickness control is relatively simple, and sputtering can be accomplished from large-area targets which
contain sufficient material for many deposition runs. There are no difficulties with large agglomerations of material landing on the specimen, and the specimens may more conveniently be coated from above. The surface of specimens can be easily cleaned before coating either by ion bombardment or by reversing the polarity of the electrodes. The plasma may be manipulated with magnetic fields, which gives greater film uniformity and reduces specimen heating.

10.3.8. Artifacts Associated with Sputter Coating

Sputter coating of SEM samples has attracted some unfavorable comments, because in the hands of certain users it has resulted in thermal damage to delicate specimens and to surface decoration artifacts. There is little doubt that the earlier diode sputter coaters could cause damage to heat-sensitive specimens, particularly if coating was carried out for a long time at high plasma currents on uncooled specimens. The thermal damage problem has been greatly reduced with the advent of the new series of cool sputter coaters. A careful examination of the examples of decoration artifacts, which have been reported in the literature reveals that in most cases some regard has been paid to cleanliness during coating, and that the artifacts were due to contamination from backstreaming oil vapors and/or the use of impure or inappropriate bombarding gases. Nevertheless it would be improper to suggest that artifacts never occur with sputter coated materials and some of the more common problems are discussed below.

10.3.8.1. Thermal Damage

A significant rise in the temperature of the specimen can be obtained during sputter coating. The sources of heat are radiation from the target and electron bombardment of the specimen. There is an initial rapid rise in temperature which then begins to level out and, depending on the nature of the material being coated, may cause thermal damage. Depending on the accelerating voltage and the plasma current, the temperature rise can be as much as 40 K above ambient. However, as mentioned earlier heating effects can be entirely avoided by using the modified diode cool coater where the heat input due to electron bombardment is only 200 mW or somewhat reduced by operating a conventional diode coater intermittently at low power input.

When thermal damage occurs it is manifest as melting, pitting, and, in extreme cases, complete destruction of the specimen. While accepting that thermal damage can be a problem in sputter coating, in nearly all cases where this has been reported it is due to the specimen being subjected to inordinately high power fluxes.

10.3.8.2. Surface Etching

This is a potential hazard in sputter coating and may be caused either by stray bombarding gas ions or, more likely, by metal particles hitting the surface with sufficient force to erode it away. It is possible to find very small holes in the surface of sputter coated specimens examined at high resolution. It is not clear whether these are the result of surface etching or simply thermal damage.

10.3.8.3. Film Adhesion

Film adhesion is much less of a problem with sputter coated films than with evaporated films and is probably due to the fact that the metal particle penetrates the surface of the specimen, forming a strong bond. However, sputter coated samples should not be subject to wide excursions of temperature or humidity, both of which can give rise to expansion and contraction with a consequent rupture of the surface film.

10.3.8.4. Contamination

Because of the low vacuum used in most sputter coaters, the problem of backstreaming from the rotary pumps, and the difficulty of placing effective cold traps in the backing lines, contamination can be a potentially serious problem, particularly if no foreline traps have been fitted. Many of the artifacts which have been described are probably due to contamination, and care should be taken in setting up and using the sputter coater.

10.4. Specialized Coating Methods

Although a description has been given of the general principles of evaporative and sputter coating, there are a number of specialized coating methods which should be discussed because they are applicable to both scanning electron microscopy and x-ray microanalysis.

10.4.1. High-Resolution Coating

As the resolution of scanning electron microscopes increases, greater attention must be given to the resolution limits of the coating layer. For many instruments which can resolve about 10 nm, the granularity of the film is of little consequence, with the possible exception of agglomeration of gold particles. A number of microscopes are routinely operating in the 5-10 nm range and the STEM instruments can give between 2- and 5-nm
resolution in the secondary mode. Transmission electron microscopists have long been concerned about high-resolution films, as they are necessary for specimen support, for shadowing, and for making replicas of frozen and ambient temperature fractured material. It is interesting to note that one of the favorite coating materials for scanning electron microscopists—gold—has a coarse granularity when examined at high resolution in the TEM. Chromium, gold–palladium, platinum, and zirconium have much finer grain size, and carbon, platinum–carbon, tungsten, and tantalum all have virtually no grain size when examined in the TEM. The graininess of the deposits generally decreases with an increase in the melting temperature. The studies of Adachi et al. (1976) and Hjojou et al. (1977) show that sputter coated films of tantalum and tungsten have very small grain size and would be most suitable for high-resolution SEM studies.

Braten (1978) found that thermally evaporated gold–platinum and carbon–gold–palladium gave the best result as far as specimen resolution and surface smoothness was concerned. Thermally evaporated and sputter coated gold gave a far more grainy appearance, and interweaving cracks could be seen on the surface of the specimen. The ultimate particle size is also dependent on the nature of the substrate. Echlin and Kaye (1979) and Echlin et al. (1980) found that for high-resolution (2–3 nm) SEM, electron beam evaporation of refractory metals (W, Ta) or carbon–platinum gave the best results. The most convenient means of preparing thin films for medium resolution (5–8 nm) SEM is to sputter coat films of platinum or gold–palladium onto specimens maintained below ambient temperature. Slower sputtering rates also result in smaller particle size. Advantage can also be made of the conductivity of discontinuous metal films which can provide an effective coating layer when only a few nanometers thick. Franks (1980) has used another sputtering technique based on a cold cathode saddle-field source to produce an ion beam. The high-energy beam was used to bombard a target producing coatings at a pressure of 10 mPa. The beam is directed onto the target with the substrate at a fixed or variable angle, and provides very fine grain deposits. Although the coating times are rather long (10–15 min) the films which are produced are ideal for high-resolution SEM studies. Figure 10.12 compares the results obtained with ion beam sputtering with those obtained with cold DC sputtering and evaporative coating. Whereas the sputter coated material and the evaporative coated material show artifacts at high resolution, only random electron noise is evident in the ion beam sputtered material. In a recent study, Clay and Peace (1981) have used ion beam sputtering to coat biological samples. They showed a significant reduction in surface artifacts compared to material which has been diode sputter coated (Figure 10.13). Peters (1980a) has used penning sputtering, which generates high-energy target atoms resulting in very small crystallites. This technique allows very thin, i.e., 1–2-nm-thick films, to be produced with small particle size. In an earlier study, Peters (1979), has showed that provided great care is taken to minimize contamination, high-resolution films can also be obtained by thermal evaporation of high-melting-point metals.
10.4.2. Low-Temperature Coating

The advantages of examining and analyzing frozen specimens in the scanning electron microscope and the x-ray microprobe have been discussed (Echlin and Saubermann, 1977; Echlin, 1978; Saubermann et al., 1981). The main problems associated with low-temperature coating are contamination and maintaining the specimen below 143 K during the coating procedures. The main source of contamination is residual water vapor, which can readily interact with the metal during the coating process. Saubermann and Echlin (1975) found that unless great care was taken during the coating process, evaporated aluminum was deposited as a grey granular layer onto thin sections of frozen biological material held at below 123 K. A similar phenomenon was occasionally observed during gold coating of fractured, bulk frozen material. These effects are entirely eliminated if the coating is carried in a coating unit interfaced with the microscope via an airlock (Fuchs et al., 1978; Pawley and Norton, 1978; Echlin et al., 1979, 1980).

Low-temperature coating has only been successfully achieved using evaporative techniques, and care has to be taken not to melt the surface of the specimen during the coating procedure. Clark et al. (1976), using thin-film thermocouples, recorded an insignificant temperature rise on a thin-film substrate coated from a collimated and shuttered evaporative source. Although attempts have been made to sputter coat frozen samples (Robards and Crosby, 1979), the results bear no comparison with those which can be obtained using evaporative methods.

10.5. Determination of Coating Thickness

There are a number of techniques which can be used to determine coating thickness, and a recent paper by Flood (1980) reviews the methods which are available. However, all the methods give an average value on a flat surface and it is necessary to briefly consider this type of measurement in relation to the coating thickness one might expect on rough surfaces. If the coating thickness is too thin it will be ineffective in eliminating the charge; if it is too thick it will obscure surface detail. A coating layer of a certain minimum thickness is necessary to form a continuous layer, and this thickness varies for different elements and for the roughness of the surface. On an absolutely flat surface a layer of carbon 0.5 nm thick will form a continuous coat. A layer ten times as thick is required to form a coat on an irregular surface. As a general rule, thicker coatings are required for irregular surfaces to ensure a continuous film over all edges, cavities, and protrusions.

It is clear that the film thickness we can measure on a flat surface bears little relation to the thickness we obtain on an irregular surface which may have been rotated and tilted during coating. For practical purposes, a given set of rough specimens should be coated with different thicknesses under standardized conditions, each film layer being measured and re-
corded from a reference flat surface such as a glass slide. The specimen should be examined in the microscope and the one which gives the optimum image in terms of resolution and information content can be considered to have the most suitable coating thickness.

10.5.1. Estimation of Coating Thickness

In high-vacuum thermal evaporation, one can assume that all the vapor molecules leave each part of the evaporant surface without preferred direction and pass to the substrate surface without colliding with residual gas molecules. By allowing for the vapor incident angle at the specimen, and assuming that all the incident vapor molecules have the same condensation coefficient, the coating thickness distribution may be calculated. The formula below may be applied to a flat, nonrotating surface, oriented at an angle \( \theta \) to the source direction and can be used to calculate the thickness from the quantity of material to be evaporated:

\[
T = \frac{MR^2}{4\pi R^2 \rho} \cos \theta
\]  

(10.1)

where \( T \) is the thickness in centimeters; \( M \) is the mass of the evaporant in grams; \( \rho \) is the density in g cm\(^{-3}\); \( R \) is the distance from source to specimen in centimeters; and \( \theta \) is the angle between the normal to the specimen and the evaporation direction. With Equation (10.1), the thickness can be calculated to within \( \pm 50\% \) if the geometry of the evaporation rig and substrate is known and if the material in question evaporates slowly and does not alloy with the heater. It is assumed that evaporation is from a point source and occurs uniformly in all directions. The uncertainty in \( T \) arises in part because of uncertainty in the sticking coefficient of the evaporant or the substrate. The efficiency is material dependent and is rarely better than \( 75\% \). The thickness calculated by Equation (10.1) must therefore be reduced by multiplying by the sticking efficiency (approximately \( 0.75 \)).

The thickness of coating \( T \) in nanometers which may be obtained using a diode sputter coater is given by

\[
T = \frac{CV_{1k}}{10}
\]  

(10.2)

where \( C \) is the plasma discharge in milliamperes, \( V \) the accelerating voltage in kilovolts, \( t \) the time in minutes, and \( k \) a constant depending on the bombarding gas and the target material. The constant \( k \) must be calculated for each sputter coater, target material, and bombarding gas from experimental measurements.

10.5.2. Measurement during Coating

A number of different methods are available which vary in their sensitivity and accuracy. The devices are placed in the coating chamber close to the specimen and measure the thickness during the coating process. It is possible to measure the density of the evaporant vapor stream either by measuring the ionization which occurs when vapor molecules collide with electrons, or by measuring the force which impinging particles exert on a surface. Mass-sensing devices may be used for all evaporant materials; these operate by determining the weight of a deposit on a microbalance or by detecting the change in oscillating frequency of a small quartz crystal on which the evaporant is deposited. The quartz crystal thin-film monitor resonates at a frequency dependent on the mass of material deposited on its surface. The frequency of a "loaded" crystal is compared to the frequency of a clean crystal and the decrease in frequency gives a measure of the film thickness. A typical sensitivity value for a crystal monitor is a 1-Hz frequency change for an equivalent film of 0.1 nm C, 0.13 nm Al, and 0.9 nm Au. The uncertainty of these devices is of the order \( \pm 0.1 \mu\text{g cm}^{-2} \). By multiplying this value of uncertainty by the evaporant density, the uncertainty in the film thickness can be determined.

It is also possible to monitor specific film properties such as light absorption, transmittance, reflection, and interference effects by various optical monitors. Film thickness of conductive materials can be measured by in situ resistance measurements and the thickness of dielectric materials measured by capacitance monitors. A further sophistication of most of these in situ techniques is that they can be used to control the coating process.

10.5.3. Measurement after Coating

The most accurate measurements of film thickness are made on the films themselves. These methods are based on optical techniques, graviometric measurements, and x-ray absorption and emission. Multiple-beam interferometry is the most precise and depending on the method used can be as accurate to within one or two nanometers. The Fizeau method can be used to check film thickness and involves placing a reflective coating on top of the deposited film step and measuring a series of interference fringes. The film thickness can also be measured by sectioning flat pieces of resin on which a coating has been deposited and measuring the thickness of the metal deposit in the transmission electron microscope. The accuracy of this method is dependent on being able to section the resin and photograph the section at right angles to the metal deposit. A simple method for accurately determining film thickness and grain size has been recently described by
Roli and Flood (1978). They found that linear aggregates of latex spheres only accumulate coating material along their free surface. Transverse to the linear aggregate, the sphere diameter will increase in thickness by twice the film thickness, while parallel to the aggregate the thickness increase will correspond to the film thickness. Using this technique they have measured the film thickness derived from different coating procedures to within $\pm 2$ nm. Film thickness may be estimated by interference colors or in the case of carbon by the density of the deposit on a white tile.

### 10.5.4. Removing Coating Layers

Having taken all the trouble to deposit a coating layer on a sample it is sometimes necessary to remove it. Provided care is taken, hard specimens can be restored to their pristine uncoated state.

If the sample is a polished section, the coating can be easily removed by returning to one of the final polishing operations (6 $\mu$m diamond or 1 $\mu$m Al$_2$O$_3$). If the sample is rough or a flat sample cannot be repolished, chemical means must be used to remove the coating.

If it is known that the coating layer is to be removed after examination of a rough sample, it is preferable to use aluminum as the coating material because of its ease of removal. Sylvester-Bradley (1969) removed aluminum from geological samples by immersing them in a freshly prepared solution of dilute alkali for a few minutes. Sela and Boyde (1977) describe a technique for removing gold films from mineralized samples by immersing the specimen in a cyanide solution. Gold-palladium may be removed using a 10\% FeCl$_3$ in ethanol for 1–8 h (Crissman and McCann 1979).

Carbon layers can be removed by reversing the polarity in the sputter coater and allowing the argon ions to strike the specimen. Care must be taken only to remove the carbon layer and not to damage the underlying specimen. Carbon layers on inorganic samples such as rocks, powders, and particulates can be efficiently removed in an oxygen-rich radiofrequency plasma.

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**Preparation of Biological Samples for Scanning Electron Microscopy**

### 11.1. Introduction

In this and the next chapter we will consider the practical aspects of specimen preparation for both the scanning electron microscope and the x-ray microanalyzer. Although the two types of instrument are very similar and in many respects can be used interchangeably, it is useful, from the biologist's point of view, to consider the preparatory techniques separately. The scanning electron microscope gives morphological information, whereas the x-ray microanalyzer gives analytical information about the specimen. It is important for the user to appreciate fully these differences as they have a significant bearing on the rationale behind the specimen preparation techniques. The methods and techniques which are given in these two chapters will provide the optimal specimen preparation conditions for scanning microscopy or x-ray microanalysis. It must be realized that anything less than the optimal conditions will result in a diminished information transfer from the specimen. It will also become apparent that it will be frequently necessary to make some sort of compromise between the two approaches, which must result in less information from the specimen.

The practical, do-it-yourself aspects of specimen preparation will be emphasized, and while it is appreciated that a certain understanding of the theoretical aspects might be useful, such considerations will be kept to a minimum and readers will be directed to key review papers on the subject. It is not our intention to provide the biologists with a series of recipes. These already abound in the literature and very few of them can result in optimal specimen preparation for anything other than the specimens for