Section 3.1

Electron Microscopy

Fundamentals

In 1872, Ernest Abbe found out why a light microscope, even with the most refined optics, is unable to go above 0.2-um resolution. The reason was found in the wavelength of light (lambda), because resolution is defined as follows:

$$d = \frac{\text{lambda} \times 0.61}{(n \text{ sen} \propto)}$$

where n sen ∝ is the number aperture of the objective lens (AN).

Accordingly, resolution could only be increased by reducing lambda.

In 1934, 65 years later, Ernest Ruska designed the first electron microscope with an electron beam instead of light, and electromagnets instead of lenses. So, at 100,000 volts, lambda is approximately 0.05 angstroms (A). Thus Abbe's limit was overcome.

The first commercial electron microscope (EM) was designed by Ruska and marketed by Siemens in 1939, making Ruska the founder of electron microscopy.

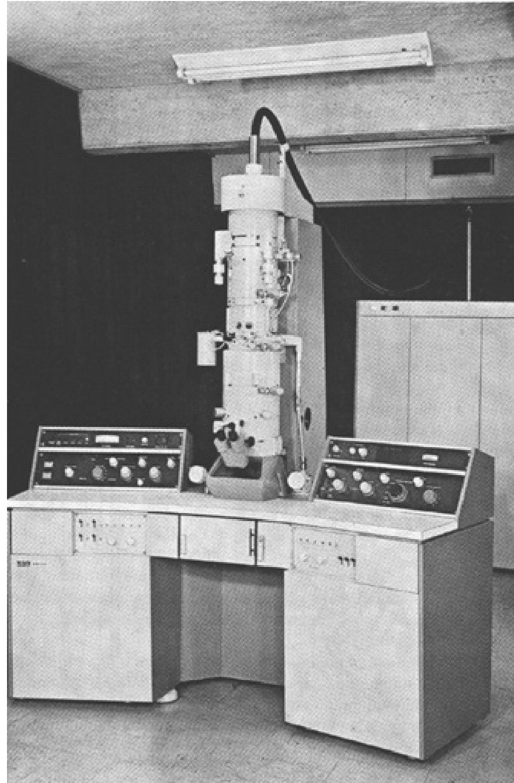

Figure 1. High-resolution electron microscope (JEM-100U).

There are two basic types of electron microscopes: the transmission electron microscope (TEM), and the scanning electron microscope (SEM). The former can be compared with a light microscope (LM) and the latter with a stereoscopic or a dissection microscope. TEM uses transmitted electrons that can penetrate the sample. Obviously this sample must be very thin, since conventional methods render a flat image. SEM uses scattered electrons from the sample surface, either secondary or backscattered, thus rendering a tridimensional image.

1. Electron Beam and Sample Interaction

When electrons of a given energy impinge on a substance, these electrons are changed into transmitted electrons, backscattered electrons (reflected electrons), and absorbed electrons (absorption) by an interaction of atoms constituting the said substance. The transmitted electrons may be classified into two types:

a) Elastically scattered electrons (elastically scattered waves), which change direction due to atomic collisions but retain their energy, and

b) Inelastically elastic scattered electrons (non-elastic scattered waves), which suffer a change in direction and a partial energy loss. (These electrons are the most important in the image formation on the TEM.)

The elastically scattered electrons can be classified into two types:

a) Coherent electrons (Bragg reflection electrons), which have passed through a substance with a regular atomic structure order, and

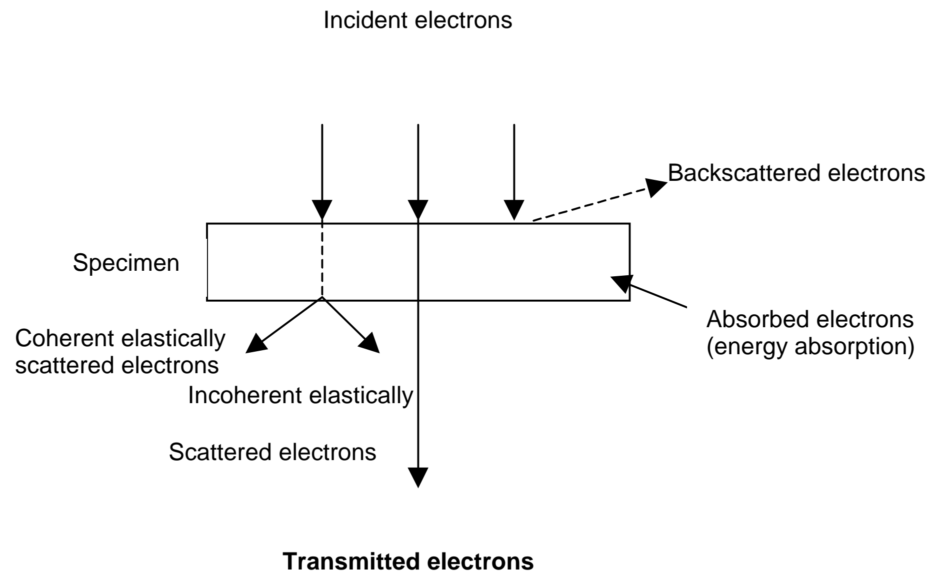

Figure 2. Electron beam and sample interaction. The electrons hitting the sample are turned into transmitted electrons, inelastic scattered electrons, and elastic scattered electrons. According to sample structure, the latter are coherent or incoherent.

b) Incoherent electrons, which have passed through a substance having irregular order.

Image contrast in the transmission electron microscope is related to the transmitted electron rate among elastic scattered electrons.

2. Image Formation and Contrast

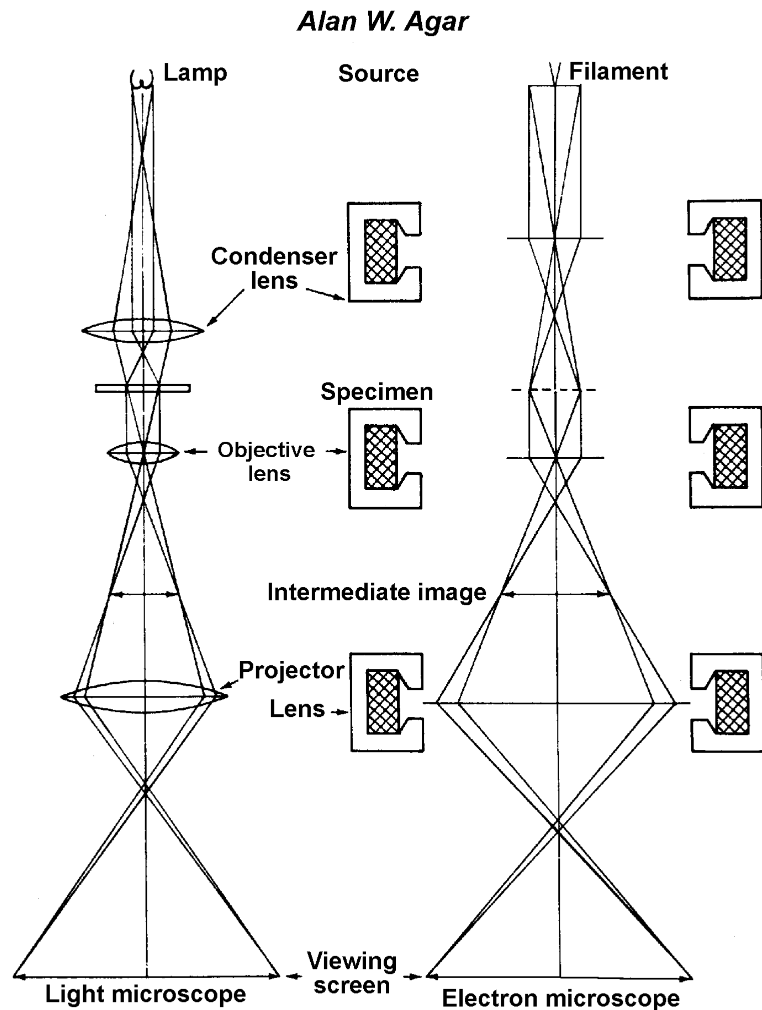

Figure 3. Comparison of light microscope and electron microscope. In both types, the source (lamp, filament in the electron gun) is focused by the condenser lens onto the specimen. A first magnified image is formed by the objective lens. This image is further magnified by the projector lens onto a ground glass screen (light) or fluorescent screen (electrons).

In optical microscopy, image contrast is determined by the difference in the absorption coefficient between different portions of the specimen and (partially) by the difference in reflectivity.

However, in electron microscopy, image contrast is determined by the scattering absorption contrast, diffraction contrast, and phase contrast.

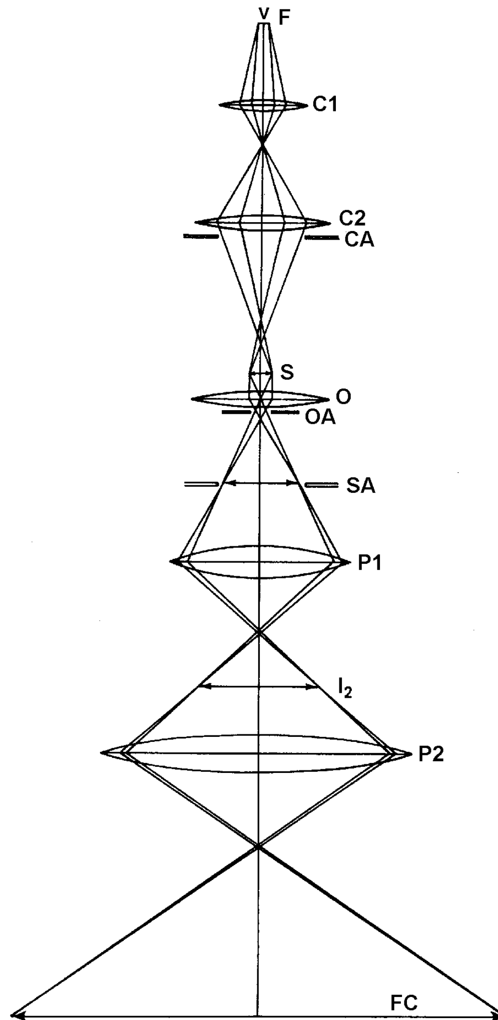

Figure 4. TEM ray diagram: filament F; condenser 1 lens C1; condenser 2 lens C2; condenser aperture CA; specimen S; objective lens O; objective aperture OA; first intermediate image and selector aperture SA; intermediate lens P1; second intermediate image I_2; projector lens P2; final image on the fluorescent screen FC.

If the specimen is an amorphous substance, the electrons scattered by the specimen are captured by the aperture, which is located near the back focal plane of the objective lens, thus generating the scattering absorption contrast. The lens aperture is very small (several tens of micrometers) in order to minimize the spherical aberration. Therefore, if the scattering (especially incoherent elastic scattering) is large, most of the scattered electrons will be obstructed by the aperture, thus forming the dark portion of the image. The extent to which the electrons are scattered is proportional to the mass thickness of the specimen. Thus, scattering absorption contrast provides information concerning the existence and configuration of the object.

3. TEM Structure and Function

Electron microscopes are principally used to observe high-resolution and magnification images. Images of very high quality can be easily obtained within a wide magnification range, from very low magnifications to ultra high magnifications, which can reach the atomic observation level.

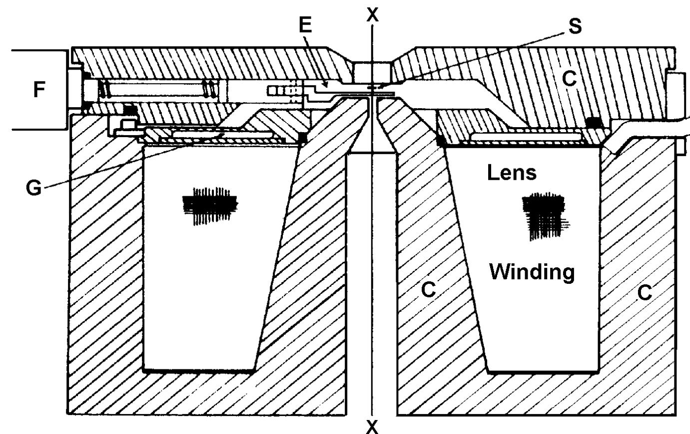

Figure 5. Cross-section of an objective lens. The specimen is at S. The objective apertures are carried on the rod E and are adjusted by control knob F. The water-cooling channels G are above the lens coik. The heavy iron circuit C and XX is the lens axis.

With the use of an image formation system of six electromagnetic lenses, high-quality images, wide visual field, minimal image distortion, and high contrast can be obtained. At 50x magnification, a 2mm-wide visual field can be observed without image distortion. Furthermore, it is possible to obtain a 0.14-nm resolution between lines by using a high-resolution polar piece objective lens.

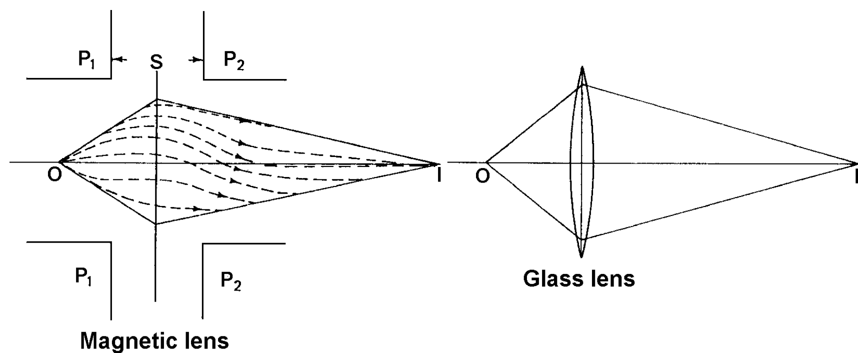

Figure 6. Comparison of light and electron lenses. Each lens forms an image I from the object point O, so that the geometrical image formation is identical, but the electrons rotate in a spiral trajectory about the lens axis as they pass through the magnetic field formed between the pole pieces P1 and P2.

In principle, electrostatic or magnetic lenses can be used to focus an electron beam, but in practice, magnetic lenses with less defects than electrostatic lenses are used. The magnetic field required to develop an electronic lens is a strong axial field in the direction of the electron beam.

The envelope of the electron beam figure is exactly analogous to that of a light beam passing through a converging lens, except that the electrons move along a helical path through the lens.

This additional rotation movement is characteristic of magnetic lenses.

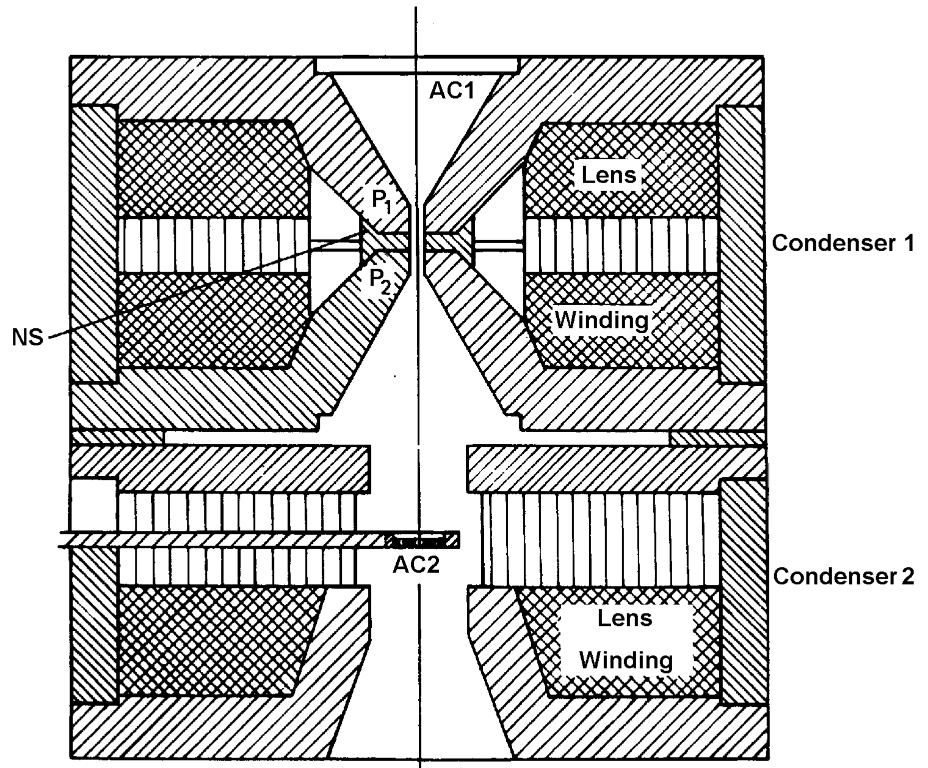

Figure 7. Cross section through a double condenser lens assembly. The upper lens is condenser 1, with fixed aperture; the lower lens is condenser 2 with an adjustable aperture. Poles pieces are indicated by P1 and P2, and the non-magnetic spacer is indicated by NS.

The electron microscope consists of a column, which basically has a system of electron lenses; an energy supply system used for electron voltage acceleration and lens charge up; a control circuit system to control the flow of energy; and an evacuation system, which provides electrons with a vacuum route.

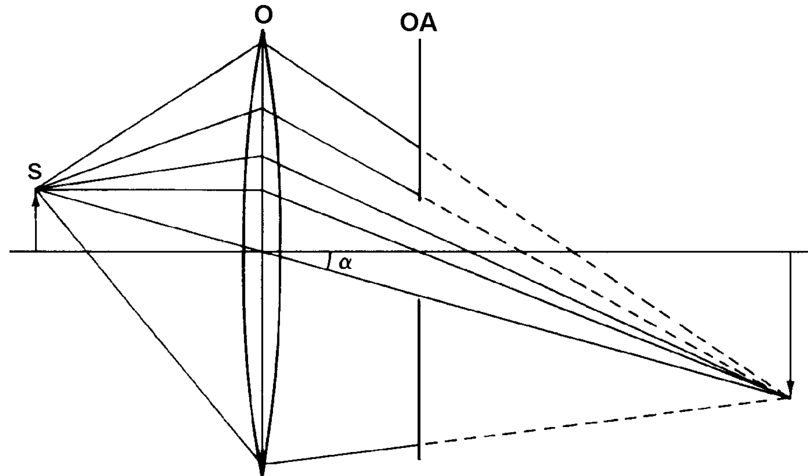

Figure 8. Function of the objective aperture OA in stopping scattered electrons from the sample S in front of the objective lens O; α is the semi-angular aperture of the lens.

The illumination system comprises part of the column, which goes from the electron gun to the condensing lenses. These condensing lenses gather the accelerating electrons in very fine beams of parallel rays and guide them to the sample surface, which remains illuminated. The part spreading from the objective lens to the second projecting lens is called the "image lens system," where the object is focused by the objective lens. The image produced is increasingly magnified by the intermediate lenses, and even more finely by the first and the second projecting lens. The condensing and objective lenses each have movable apertures with diameters in the range of 0.1–1 mm and 0.01–0.1 mm, respectively.

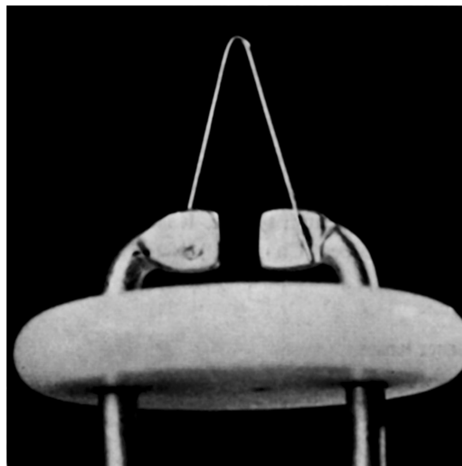

Figure 9. Standard type of hairpin filament; uniform tungsten wire in V shape.

The electronic gun operates under the same principles as a direct hot-type triode. The cathode consists of a V-shaped tungsten filament, which is heated up to 2600°K to generate thermo ions. Between this and the

anode, voltage acceleration is applied to accomplish electron jump and acceleration.

A hood, called a "Wehnelt cylinder," is located between the filament and the anode, and is useful not only to prevent electrons from scattering but also to control their intensity. Most electron microscopes operate in the voltage range of 25–200 Kv. A few operate at 1000 Kv or more.

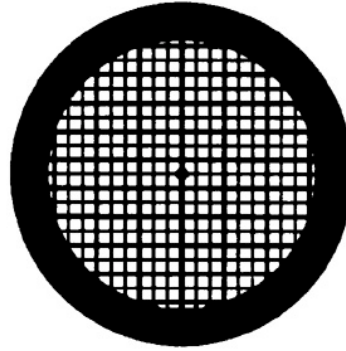

Figure 10. Electron microscope specimen grid.

The sample is generally placed on a 3 mm-wide electro-forming grid (usually copper). This is known as a sample holder grid. This grid may have a variety of designs. For better resolution, place the grid between the space of the pole pieces of the objective lens.

The observation chamber is located under the projecting system, which incorporates the fluorescent screen on which the image is barely visible to the human eye. To obtain a permanent image record, photographic material has been incorporated into the microscope for direct expositions to the electron beam.

One surprising characteristic is that photographic material (plates) are generally located on a plane several centimeters away from the fluorescent screen, where the image is focused. This is behind the wide field depth of the electron microscope, which is increased as smaller diameter apertures are used in the objective lens.

For an image resolution of d, and an objective aperture α, the depth of field in the object plane is Do.

$$Do = \frac{d}{\alpha}$$

When depth of field refers to the image plane, it is known as depth of focus.

For a total instrumental magnification M, depth of focus Di becomes very large indeed:

$$Di = M^2 x d$$

α

For M = 10,000x; d = 1.5 nm; α = 0.01 rad; then Di = 50 m. Therefore, it is immaterial that the fluorescent screen and the camera are in different planes, as their separation is negligible compared with the depth of focus. Since, in general, the photographic plate is below the viewing screen, the viewing screen magnification may be as much as 20% lower than the indicated magnification.

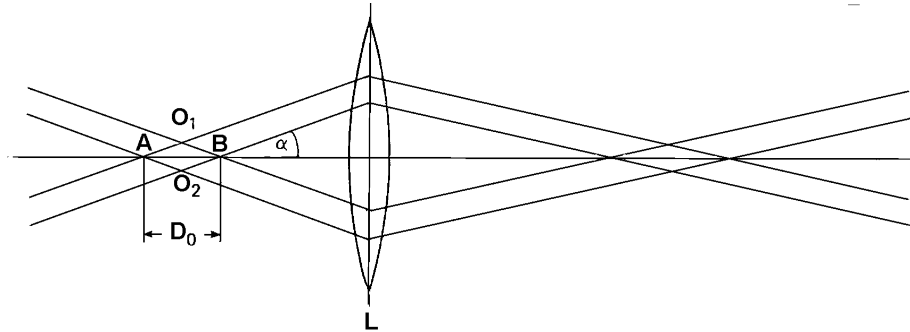

Figure 11. Depth of field. Object points O1 **and** O2 **objects are separated by the resolution limit** d **of the lens. Rays from these points cross the axis at** A **and** B **equally. Hence, points between** A **and** B **will look equally sharp, and** AB **is the depth of field** Do **of the lens for a semi-angular aperture α.**

The final magnification of the image Mt is the product of the magnifications of the individual lens. Thus

$$Mt = Mo \times M1 \times M2$$

where Mo is the magnification of the objective lens, M1 is the magnification of the first projector lens (sometimes called the intermediate lens), and M2 is the magnification of the second projecting lens. Some typical values of these magnifications would be:

Mo = 50x
M1 = 16x
M2 = 250x
Therefore, Mt = 200,000x

Moreover, standard specimens are used to measure magnification. These samples can be replicas of optical diffraction grids, which have spaces between lines of 1 um or 0.5 um. These replicas are widely used for magnifications above 50,000x.

For high magnifications, known crystal lattice spacing are used, such as from 126 nm (copper phtalocyanine, etc.). When these specimens are photographed and the **d** mm distance between lines is measured from the developed negative (and the grid space known is **do** nm), the magnification reached by the instrument would be

$$\frac{d}{do}$$

For example, if a "Line Grating Replica" is used (2160 lines/mm), and a photograph is taken with a nominal magnification of 20,000x, then the calibrated magnification would be:

d = 9.3 mm (= $9.3x10^6$nm)
do = 462.9 nm

Calibrated magnification = 20,090x

To find the resolution, the observed image should be photographed to a slightly lower magnification and then amplified. Since photos obtained from an electronic microscope have a d2 resolution of about 20 nm in good conditions, the increased M1 magnification required by the resolution is 5x and is estimated by the following equation:

$$M1 = d1/d2$$

where **d1** is the eye's power resolution (approximately 0.1 mm).

But, to determine the resolving power **d** of the image in the observation chamber, a suitable magnification must be achieved to the measure permitted by electron microscope. For example, in a JEOL JEM100S, which has a resolution power of 0.34nm, the minimal effective magnification **M** is determined by the resolving power of the viewer eye **d1** (approximately 0.1–0.2 mm).

if M = d1/d
d1 = 0.1mm (=$0.1x10^6$nm)
d = 0.34 nm ("lattice resolution")

then, M = 294,118x, which means it is impossible to observe smaller distances (0.34 nm) because the JEM 100S model have a magnification power of only 200,000x. Nevertheless, this limitation can be overcome by using binoculars that can amplify the image 10 more times, to a maximal magnification of 2,000,000x with the JEM100S.

Ultramicrotomy

Ultramicrotomy is a special technique used to produce ultrathin sections (25–90 nm) from fixed, dehydrated, and embedded biological materials, using special microtomes (thermal or mechanical advance microtomes), and glass or diamond knives with very sharp and hard cutting edges. The first model was designed by Sorvall in 1953, and it is known as Sorvall-MT1. A Swedish company manufactures commercial thermal ultramicrotomes under the "Ultratome" brand name.

Glass knives are made by cutting a special glass to obtain a sharp cutting edge. The Swedish firm has developed a machine to make knives. The knife's cutting edges must not be touched or rubbed against each other and should be discarded when blunt.

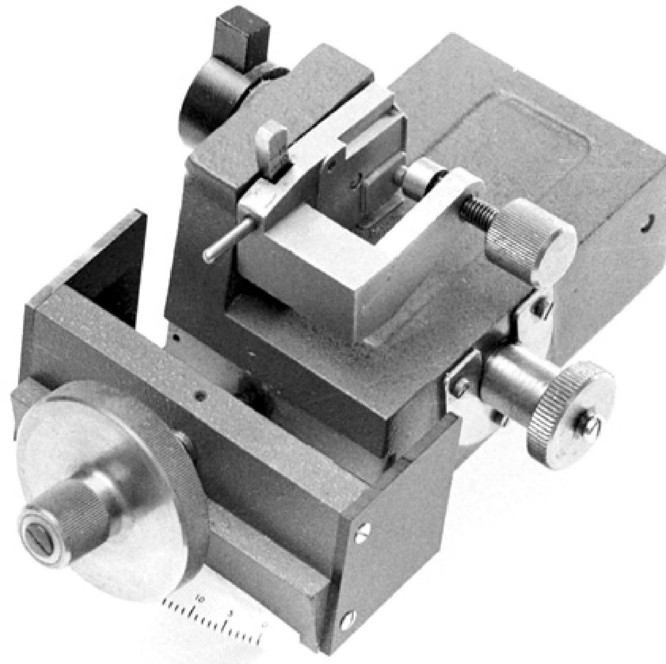

Figure 12. Ultramicrotome “Cambridge Huxley Mark 2”

Obtaining ultrathin sections by microtomy is the most important technique in electron microscopy. The optimal characteristics of an ultrathin section are:

a) Thickness of 30 to 60 nm.
b) Enough consistency to support the electron beam.
c) Susceptible to good contrast through its affinity with the tinctures.

Fine cuttings should stay afloat on water behind the sharp edge of the knife as they are produced.

Thus, their thickness can be estimated by observing the interference color (which is the reflection of a white fluorescent light illuminating the cutting areas). The cuttings can then be easily picked up.

Gray and silver-plating cuttings (25–60 nm) are more useful for ultrastructural studies. These cuttings are developed on copper grids of 200-mesh without a support membrane, but plastic membranes should be used when grids are under 200-mesh.

Staining

In electron microscopy, specimens are stained to increase contrast as well as to facilitate cytochemical studies. Increased contrast is achieved by depositing atoms of high atomic number in organic components.

Staining methods in electron microscopy include:

a) Wohlfarth and Botterman phosphotungstic acid (1956),

b) Watson uranyl acetate (1968), and

c) Reynolds' lead citrate or high pH (1963).

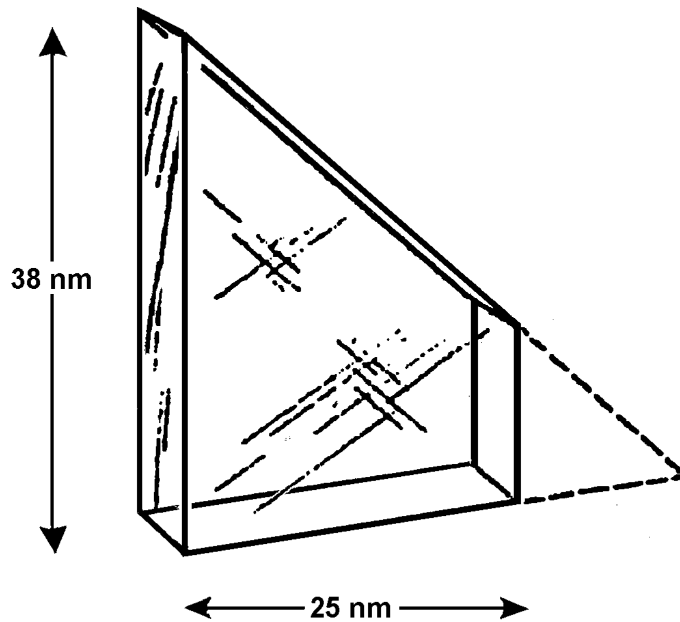

Figure 13. Glass knife for the ultramicrotome

1. Preparation and use of the lead citrate stain for tissue sections

Solution 1: Weigh 3 grams of lead citrate and transfer to a 30–50 ml flask.

Add 30 ml NaOH 1N. Shake well, and allow to settle for 1 hour.

Solution 2: Prepare 30 ml NaOH 0.05N.

Stain Solution: Centrifuge solution 1 for 5 minutes at 2000 g. Dissolve the supernatant 1:5 or 1:10 with solution 2. Use immediately for staining the grids.

Staining of tissue sections should be made in a carbon dioxide free environment to prevent reactions with lead. The procedure is as follows:

a) Prepare a Petri-wax plate with a suitable hole to put NaOH "pellets." Add 5 NaOH pellets in the center hole (this works as a trap to capture the carbon dioxide).

b) For each grid (maximum 10 at a time), put 1 drop of distilled water and 1 drop of the staining solution onto the waxed surface.

c) Put the grids on the water drops for 30 seconds and then in the tincture for 5 minutes.

d) Remove the grids, wash immediately with 10 or 20 drops of water, dry, and examine them under the electron microscope.

2. Stains used in virus electronic microscopy

Negative staining was first applied by Hall in 1955 to stain virus. These stains are used for negative stainings; that is, stain metal components are deposited around viral particles in such a way that they delineate the surrounding virus and make it electrodense. By contrast, the entire virus particle area remains translucid to the electrons.

When the tincture enters the viral capsicle and reaches the virus core, a positive staining is achieved, in which the whole particle is electrodense and its surrounding is translucid to the electrons.

For the biological material to achieve suitable negative staining, its constituents should be free in the suspension, with diameters under 5 nm. A minimum material concentration should be used, and the material should not interact with tinctures; nor should it have an affinity with the support membrane. No detergents should be used in the preparation.

It is assumed that a metallic-salt solution is capable of occupying the hydrated regions inside or around the object.

a) Phosphotungstic Acid (PTA)

Generally, 1 or 2% of PTA in distilled water (p/v) is used. The solution can be adjusted to the required pH by adding (drop by drop) NaOH 1N. The pH may be 5, 6, 6.5, 7, and 8.

b) Ammonium Molybdate

Commonly, 0.5–2% in distilled water is used.

Use NaOH 1N to adjust pH to between 6 and 8.

c) Uranyl Formate (UrF)

Prepare 1–2% in distilled water to use (freshly made).

The solution is unstable to heat and tungsten light. This is an acid stain with pH 3 whose high resolution and contrast make it particularly appropriate.

d) Uranyl Acetate (UAc)

Generally, 1–2% is used in distilled water, or 4% in ethyl alcohol at 50%. The solution is unstable to heat and tungsten light. This is an acid stain with pH 3. It gets high resolution and has more contrast than UrF.

3. Methods for Staining Virus Particles

Before staining a virus sample, it is necessary to prepare grids for electron microscopy covered with adequate membranes. Copper grids of 200-, 300-, or 400-mesh are used, depending on the size of the object studied and the kind of substrate used as a support membrane.

Charcoal and plastic membranes are the two types commonly used ("formvar," "collodion," etc.). Charcoal membranes are better than plastic ones because they are very thin (1.5–2 nm), they are pure, and they have a low image contribution. Unfortunately, they are often hydrophobic and costly.

On the other hand, plastic membranes are more practical, easy to prepare, and cheaper.

"Formvar" membranes are prepared as follows:

Dip the glass-slide plates in a formvar solution at 0.8–1% (p/v) in pure chloroform. Let dry and scrape the slide edges with a razor blade or a scalpel. Detach the membrane by dipping the plate (at an angle of 45°) in distilled water.

Carefully place the grids upside down the membrane and lift them with a paper. Let dry.

Methods to stain virus particles depend on whether the preparation is semipurified or purified. Charcoal grids are normally used:

a) For purified preparations with sucrose or contaminating buffer solutions:

Put a drop of bacitracin at 0.01% on the side of the grid with the coal membrane for 15 seconds. Drain and replace the bacitracin drop with a drop of the sample for 30 seconds. Drain and dry. Then, let the grids float on 6 or 7 drops of ammonium molybdate at 2%, with pH 6 or 7, on paraffin paper. Allow to rest for 3 to 5 minutes, remove, dry, and examine.

b) For high resolution from purified preparations free of contaminants (final purifications of virus free of cellular components):

Place a bacitracin drop at 0.01% on the side of the grid with the charcoal membrane for 15 seconds.

Wash with 10 to 15 drops of ammonium acetate 0.05 M, leaving a drop on the grid. Dissolve the virus in the drop with capillarity of 10 ul. Without draining, wash with 6 to 10 drops of uranyl acetate or with uranyl formate at 2%. Drain, dry, and examine.

4. Methods used for examining fresh leaf material.

a) "Epidermal Strip" Method

Take a grid using clamps and put a drop of distilled water, buffer solution, or PTA on the grid. Use tweezers to detach pieces of leaf epidermis. Put the pieces on the drop of solution and shake softly, being careful not to break the charcoal membrane. After 30 seconds, remove the epidermal pieces, drain the drop with a filter paper, and wash with 3 or 4 drops of same if tincture was not used. Drain, dry, and examine under the electron microscope.

b) "Leaf Dip" Method

Take a grid using tweezers and add a drop of distilled water, buffer solution, or PTA. Cut a small piece (1 x 3 mm) of a leaf, and lift it from one end with another tweezers. Touch the drop 10 times with the cut edge of the leaf. Remove the leaf, drain with filter paper, and dye (if necessary).

c) "Leaf Squash" Method

Place a piece of leaf of 1 cm^2 on a slide plate. Use a second plate, at 45°, to macerate the sample. Add some drops of distilled water, buffer solution, or PTA to the sample and, using the second plate, macerate the material completely.

Press the macerate between the two plates until the liquid reaches one of the edges and drain 1 or 2 drops on the test tube. Dissolve with the original liquid until it turns pale-green. With a Pasteur pipette, take a sample and put a drop on the grid. Dye as usual.

Fixing

In some cases, it is better to fix the material before staining. A degradation effect would take place during the negative staining process, tenuivirus, rhabdovirus, and some other virus groups.

At present, we have two excellent types of fixatives for electronic microscopy: glutaraldehyde and osmium tetraoxide.

Glutaraldehyde is used as primary fixative, mainly due to the facts that it reacts very rapidly with proteins and that, being dialdehyde, it stabilizes structures by cross-linking before there is any opportunity for extraction by the buffer to occur. The osmium tetraoxide is very useful in post-fixing due to its reactivity with a great variety of cellular components, its total solubility (in either the aqueous or lipidic phases), and its powerful inhibitor effect on enzymatic reactions.

Buffer solutions are necessary to prepare fixatives, to maintain a pH closer to the physiological pH of the tissue to be fixed. The most commonly used are veronal acetate, sodium phosphate, and sodium cacodylate.

1. Preparation of buffer solutions

a) Sodium cacodylate 0.1M pH 7.4
Add (drop by drop) HCl 0.2N to solution A to reach a pH of 7.4. Then increase volume to 100 ml with bi-distilled water.

Dissolve the calcium dichloride (2 hydrate) in the solution.

Solution A*	50 ml
HCl 0.2N	2.7 ml
$CaCl_2.2H_2O$	100 mg

*Sodium cacodylate 0.2M

b) Veronal acetate 0.28M

Stock solution:

Sodium veronal	2.89 g
$CH_3COO.Na$	1.15 g
distilled water	100 ml

Buffer solution:	
stock solution	5 ml
distilled water	15 ml
*Add (drop by drop) to reach a pH of 5.1.	

2. Preparation of fixative solutions

a) Karnovsky's fixative solution

paraformaldehyde at 8%*	12 ml
sodium cacodylate 0.1M	33 ml
glutaraldehyde at 70%	2 ml

*In a 100-ml Erlenmeyer, dissolve 4 grams of paraformaldehyde in 50 ml of bi-distilled water. Raise temperature to 60°C. Add 1 drop of NaOH 1N until the turbid solution clarifies. Cool and filter.

b) Uranyl acetate fixative solution

uranyl acetate	100 mg
veronal acetate	10 ml

c) Osmium tetraoxide fixative solution

Prepare an osmium tetraoxide solution at 1% in cacodylate buffer solution 0.1M, pH 7.4.

3. Fixation method

Fresh material should be used, and the sample (the leaves, for example) may be collected in plastic bags. Biological samples should be immediately fixed in vials with the fixative solution.

a) Place Karnovsky's dampened leaf in the plastic bag, and cut into small pieces of 1 mm^2.

Put the leaf pieces in vials using the fixative and leave them for 1 hour at room temperature.

b) Place the vials at 50 vacuum Torr for 30 minutes.

c) Wash with Sodium cacodylate 0.1M pH 7.4 buffer solution. Change 6 times at 10-minute intervals.

d) Fix a second time with osmium tetraoxide at 1% in Sodium cacodylate buffer solution for 1 hour. Use a fumehood bell to avoid inhaling highly toxic osmium gases.

e) Wash with Sodium cacodylate buffer solution 0.1M. Change two times (5 minutes each) and wash with veronal acetate buffer solution for 5 minutes.

f) Fix a third time with uranyl acetate (at 1%) in veronal acetate buffer solution for 1 hour at ambient temperature.

g) Wash with buffer solution of veronal acetate. Change 2 times (5 minute each) and wash with bi-distilled water for 5 minutes.

Dehydration

The samples are dehydrated in an acetone or ethanol schedule at 30, 50, 70, 80, and 90% for 5 minutes each time, and then in acetone or ethanol at 96% three times at 5 minutes each. Finally, place the samples in acetone or ethanol at 100%, three times, for 10 minutes each.

If ethanol is used first, replace it with another dehydrating agent such as propylene oxide:

3:1* for 20 minutes.
2:2* for 20 minutes.
1:3* for 20 minutes.
Pure propylene oxide for 20 minutes.
Pure propylene oxide for 20 minutes.

*Proportion of absolute ethanol to propylene oxide.

Infiltration

For infiltration, place the tissues in a mix of acetone and the embedding medium (or propylene oxide and the embedding medium).

It is advisable to use epoxy resins like the Spurr mixture (1969), which is a low viscosity resin with excellent penetration in all kinds of tissues:

Acetona: Spurr (3:1) 30 minutes
Acetona: Spurr (1:1) 30 minutes
Acetona: Spurr (1:2) 30 minutes
Pure resin overnight
Pure resin for one hour

When using the propylene oxide as a dehydrating agent:

Propylene oxide: Spurr (3:1) all night.
Propylene oxide: Spurr (2:2) for 2 hours.
Propylene oxide: Spurr (1:3) for 2 hours.
Pure resin for 2 hours.
Pure resin for 2 hours.

Put the samples in dry gelatin capsules or in fresh-resin plastic molds.

For polymerization, place in an oven at 60°C for 24–48 hours.

Preparation of the Spurr Mixture

"Epoxy"-type resins cause little damage during polymerization. They form transversal bridges, which, once polymerized, are not soluble in any solvent. Epoxy resins do not sublimate when irradiated by electrons, thus protecting structures from distortion.

Embedding of cells and tissues in plastic is a fundamental advance in electron microscopy. It allows us to manipulate the tissue in a solid medium, helping to obtain ultrathin sections.

The Spurr absorption medium is composed of the following substances:

Cyclohexane vinyl dioxide (ERL-4206)
Diglycidic ether of propylene-glycol (DER-736)
Nonenylsuccinic anhydryde
Amino ethanol dimethyl (S-1)

The mix shelf life is 3 to 4 days. If the components are mixed in a single test tube and are correctly absorbed, the mix can be prepared easily. It is desirable to add the catalyst (S-1) at the end. The air in the mixture can be eliminated with a vacuum bell.

In a graded test tube, mix the following amounts in the prescribed order:

ERL-4206	10 ml
DER-736	6 ml
NSA	26 ml
S-1	0.4ml

Recommended Literature

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