I.E. OPERATION OF THE TRANSMISSION ELECTRON MICROSCOPE

Assuming the microscope is sufficiently aligned such that the instrument is capable of providing near-optimal performance, there are several considerations regarding the choice of conditions for recording images. Discussion of the operation of the electron microscope is divided into the following sections:

1. Choice of accelerating voltage
2. Choice of apertures
3. Specimen stage/holder
4. Choice of magnification
5. Focusing
6. Magnification calibration
7. Resolution tests
8. Image intensifiers/TV displays
9. Microscope maintenance
10. Photography

I.E.1. Choice of Accelerating Voltage

In most modern TEMs the voltages available usually range from 20,000-100,00 volts (20-100kV). Most routine biological microscopy is performed at 80 or 100kV. As the accelerating voltage **increases,** several factors are affected as follows:

a. Better penetration of the specimen enables thicker specimens to be studied.

b. Amplitude contrast decreases since the proportion of elastically and inelastically scattered electrons decreases.

c. The brightness of the electron gun increases. Gun brightness, B (current density per unit solid angle), is given by:

$$B = \frac{q_c eV}{kT} \text{ (amps/cm}^2\text{/steradian)}$$

where q_C = cathode current density
e = electronic charge
V = accelerating voltage
k = Boltzmann's constant (8.6×10^{-5} eV/°K)
T = temperature

d. The efficiency of the screen phosphor increases.

e. The efficiency of most photographic emulsions decreases.

f. Inelastic scattering decreases, thus chromatic aberration effects decrease (better resolution) and radiation damage decreases (longer specimen lifetime).

g. The electron gun is more sensitive to the vacuum, thus high voltage stability decreases.

h. Resolution limit due to diffraction effects improves (Rayleigh criteria; *i.e.* decreased □).

In general, high voltages (80-100kV) are preferred when studying biological specimens because of reduced radiation damage and improved resolution. Even though contrast is reduced at higher voltages owing to less scattering in the specimen and lower sensitivity of photographic emulsions, it is better to choose a small objective aperture and/or use staining techniques to improve specimen contrast rather than lower the voltage of the electron beam.

When the high tension on the TEM is switched on, it is best to have the filament current turned off to prevent damage to the filament or specimen by surges of voltage. The higher the kV selected, the longer the gun takes to settle down as discharges take place due to the desorption of residual column gases which are then released. It is often good practice to pre-condition the gun at a higher voltage so it will be more stable at the lower operating voltage. After the high voltage settles down,

the filament current is increased to saturate the filament. The gun should then be adjusted and aligned to give maximum beam brightness at the chosen voltage and gun bias setting. The gun bias (emission setting) can be changed to give a stronger or weaker overall illumination depending on the working conditions (specimen thickness, desired magnification, etc.).

I.E.2. Choice of Apertures

a. Condenser aperture (Figs. I.64-I.68)

The condenser aperture defines the maximum illumination aperture which can be used. It is always possible to use a smaller effective aperture by defocusing condenser lens 2 (beam spread more widely). Most TEMs use apertures in the second condenser lens in the size range 100-300 μm. As aperture size is reduced, the beam size becomes smaller at crossover and the total number of electrons are reduced. The smallest apertures used provide the optimum conditions for obtaining high resolution work since the electron beam is then more coherent and more capable of producing phase contrast interference effects. Beam coherence is "perfect" if the source of radiation is infinitely small (*i.e.* at infinite distance from the specimen) which is effectively the case when the aperture angle is significantly reduced with the smallest condenser aperture (which selects out only the narrow band of paraxial electrons in the beam). Interference (phase contrast) effects increase as the coherence of the source of radiation improves. Another way of improving coherence of the beam is to overfocus the first condenser so the second condenser aperture "sees" fewer electrons (*i.e.* from a smaller, demagnified source).

b. Objective aperture (Figs. I.85-I.90)

The objective aperture is mainly used to improve aperture or scattering contrast in the image. Objective aperture sizes generally range between 25 and 75 □m. The smaller the aperture, the greater the aperture contrast because more scattered electrons are prevented from reaching the image and regions of greater mass thickness in the specimen give rise to fewer electrons in the corresponding region of the image. Smaller apertures improve resolution limited by spherical and chromatic aberration but reduce resolution limited by diffraction effects. Thus, there is an optimum size where these effects are approximately balanced (Sec. I.B.3.b). Smaller apertures are harder to align and keep aligned and are more sensitive to contamination buildup, which introduces image astigmatism. However, the use of ultra-thin, self-cleaning objective apertures eliminates most of the problems caused by contamination because the scattered electrons heat up the edges of the aperture to a temperature high enough to prevent the formation of a contamination layer. For "pure phase objects" (*i.e.* very thin biological specimens) the objective aperture is ineffective in providing aperture contrast and thus, may be unnecessary.

I.E.3. Specimen Stage/Holder (Figs. I.84, I.91, and I.92)

The important points with regard to the specimen holder are:

- Never use bare hands to handle the tip of the holder where the specimen is held, since grease from the hands will introduce severe specimen contamination and consequent thermal drift problems.
- Make sure the specimen grid is held flat and secure in the holder to ensure proper thermal contact.

The specimen is generally inserted into the microscope with the high voltage on, the gun and condenser apertures aligned, the magnification at a low setting suitable for scanning, and the beam widely spread. Note, however, on older microscopes, such as the Philips EM300, which do not have a specimen prepumping chamber, the high voltage must be turned off before inserting a specimen. After the specimen has been introduced into the microscope column, switch to diffraction mode to obtain the diffraction pattern from a specimen area that won't be used for subsequent high resolution photography. After inserting and centering the objective aperture, return to normal mode and scan the grid at an appropriate magnification to locate areas of interest for further inspection. For radiation-sensitive specimens, the very lowest magnifications and weakest illuminating beam consistent with the ability to identify suitable areas is used (this is discussed in greater detail in Sec II.B).

I.E.4. Choice of Magnification

The choice of magnification depends on the nature of the experiments being performed. Several criteria are used to establish a suitable magnification level. These include:

a. Low magnification (<10,000X) is required to record a large field of view (>10 μm) from a specimen in a single micrograph. If a montage is made of an extensive region of the specimen by splicing together prints from several low magnification micrographs, one must be aware that if very low magnifications are used to reduce the number of prints required, pincushion and barrel distortions are likely to be significant and the prints won't match correctly where they are joined together. Using higher magnifications though, to help eliminate problems due to distortion, would necessitate an inordinately large number of micrographs to complete the montage.

b. For statistical studies such as determining relative populations or sizes of different particles, it is necessary to measure a large sample. Thus, it is best to use the lowest magnification at which the particles can still be easily and correctly identified.

c. When searching the grid for specimens that are radiation sensitive**,** it is best to use the lowest magnification and illumination level consistent with the ability to identify a suitable area for further photography.

d. High magnifications are required for obtaining the highest possible resolution**.** Note, however that it is pointless to magnify the image needlessly (empty magnification) to enable the resolution limit of the instrument to be achieved when the specimen resolution may be much lower (the usual situation for biological material). Excessive magnification will also lead to unnecessary radiation damage due to the higher doses required to obtain a suitably exposed emulsion at high magnification. The resolution of the photographic medium ultimately determines the maximum magnification needed to record details at a predetermined resolution. Most photographic emulsions used in electron microscopy can resolve image details at least as small as 20 μm, thus the resolution of object details will depend on the image magnification as shown in the table (resolution = 20 μm/magnification):

Magnification	Resolution at Object (nm)
2,000	10.0
20,000	1.0
50,000	0.4
100,000	0.2

The above table clearly demonstrates that most "typical" microscopy is performed at excessive magnifications given the limited resolution of the specimens. It is true that details resolved in the electron image will NOT be resolved by the unaided eye, so it is necessary to either magnify the micrograph photographically in an enlarger or to display on a computer a digitized version of the micrograph (digitized on a suitably small enough raster spacing). Note that photographic magnification (usually <10x) does not increase image resolution, but merely makes it possible for the eye to resolve the details contained in the original micrograph. Also, the use of unnecessarily high magnifications restricts the field of view of the specimen.

The general rule of thumb is to select the lowest magnification consistent with the required resolution, because each time the magnification is doubled, a four-fold increase in beam intensity is required at the specimen to obtain a suitably exposed micrograph.

e. At low magnification it is generally hardest to judge the correct focus position in the image using the objective lens, thus, it may be necessary to focus at a higher magnification and then return to lower magnification for photography. This procedure is generally unsatisfactory, especially for older microscopes, because the focus position "drifts" when the projector lens currents are changed to alter the magnification. It is best to use special "tricks" for focusing at low magnification, where resolution is generally not a primary concern anyway (see Sec. I.E.5 below).

I.E.5. Focusing

Focusing of the image is effected by variations in the objective lens current.

True or near focus can be defined as the condition at which no Fresnel fringe is formed at an image point. This corresponds to the point where the specimen is exactly in the conjugate plane to the image plane of the objective lens, which at any given setting of the intermediate lens is defined by the object plane of that lens. This condition is also the one in which there is, ideally, no phase contrast.

In principle, optimum results should be obtained from a focused micrograph, but a certain degree of under-focusing is generally favored. At slight under-focus, any amplitude contrast is enhanced with phase contrast.

Careful focusing is one of the most important functions required for successful operation of the TEM. Different techniques are generally used depending on the magnification chosen.

a. Low magnification focusing (<15,000X): Two methods are commonly employed to set focus at low magnification.

Focusing with a wobbler aid

The use of a wobbler focusing aid, found on most modern TEMs, makes focusing quite simple at low magnifications (Fig. I.125). The wobbler produces a cyclical deflection of the incident beam which causes a movement of the image when the objective lens is focused on a plane above or below the specimen. When the objective lens is focused, the image appears sharpest.

Minimum contrast method

Withdraw the objective aperture and set the objective lens strength to give an image with minimum contrast. Then reinsert the objective aperture before photography. This method works best with thin specimens because they will show a dramatic drop in contrast near the in-focus position (Figs. I.108 and I.124). This method has the drawback that the aperture must be removed and then reinserted after focus is set without going into diffraction mode to center the aperture. If the aperture is not properly recentered as before, the image may become astigmatic. The minimum contrast technique can be used with the objective aperture in place, but it will be somewhat more difficult to detect the "true-focus" position in the presence of strong aperture contrast.

b. High magnification focusing (usually >30,000X):

It is generally best to focus on the background support film whose "structure" is mainly a result of phase contrast. The background granularity virtually disappears at the in-focus setting where the contrast dips to a minimum (Figs. I.126-I.128). The drop in contrast is most dramatic only if the objective lens has been properly stigmated to remove residual astigmatism caused by lens asymmetry (Fig. I.129). It is generally better NOT to focus directly on the specimen, especially if it is one with which the user is unfamiliar, since it may be difficult to determine exactly what appearance it should have at different levels of focus. The support film, on the other hand, is a well-defined "specimen" with a characteristic appearance at near focus settings (Fig. I.126).

It is important to consider the phase contrast granularity when interpreting pictures of biological specimens taken at high resolution, since the granularity can be erroneously misinterpreted as genuine fine structure in the specimen. A through-focal series, in which micrographs are recorded at focus settings above and below as well as near focus, will help identify which features in the specimen are genuine and which may be attributed to phase contrast granularity (Figs. I.126-I.128).

A basic rule of focusing is that the greater the level of magnification, the more accurate the focusing must be and the closer it should be to the "in-focus" setting.

It is important for microscopists to learn **NOT** to be fooled into equating a high contrast appearance with a "sharp focus" condition. The so-called "in-focus" position is where phase contrast is at a minimum. Inexperienced TEM users invariably choose a point considerably below the in-focus setting (*i.e.* under-focus) as the "best" focus because they are mislead by the dramatic

increase of phase contrast and *apparent* increase in sharpness when the image is highly defocused.

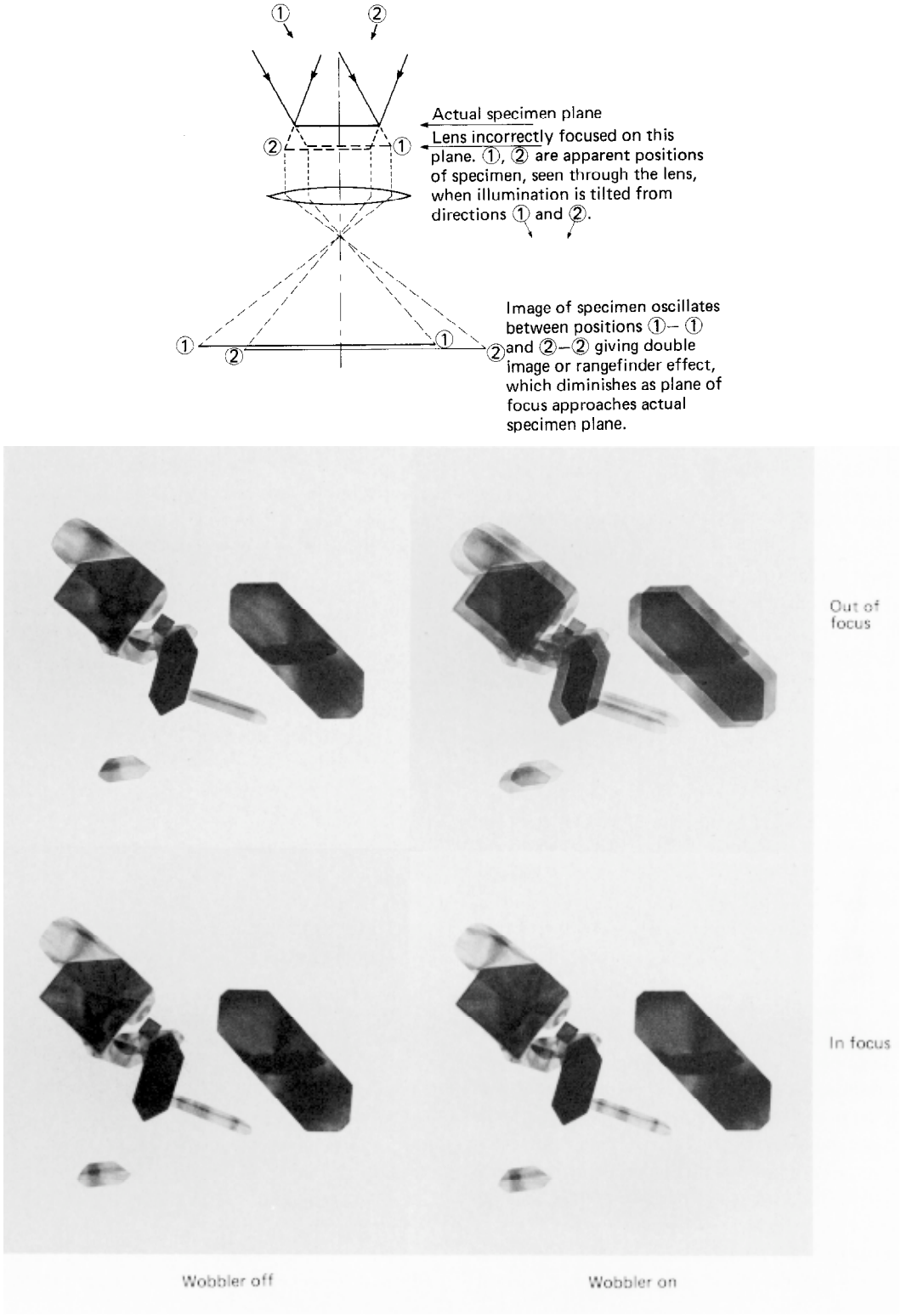

Fig. I.125. Beam wobbler focusing aid. (Top) Schematic diagram of principle of operation. (Bottom) The wobbler in practical use. (From Watt, p.30)

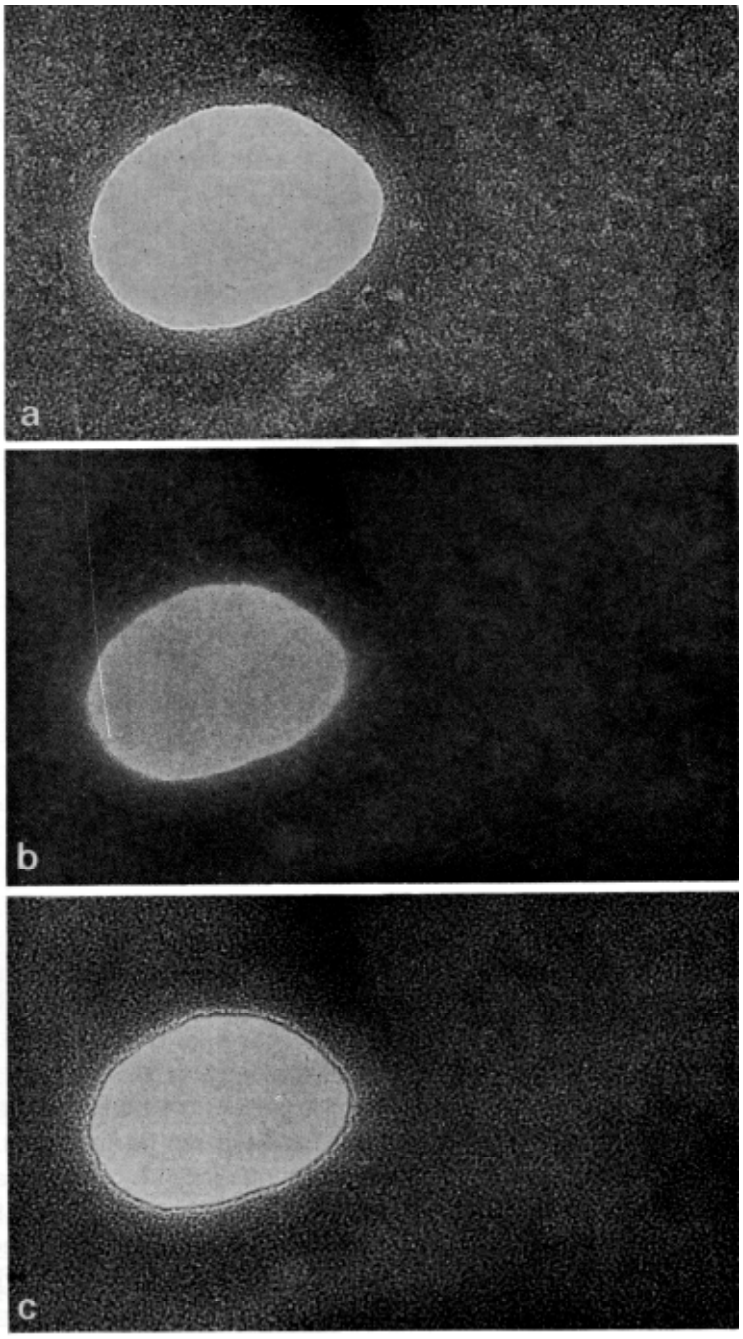

Fig. I.126. Hole in a thin carbon film: (a) under-focused objective lens (bright fringe); (b) at focus (no fringe) and (c) over-focused objective lens (dark fringe). Note also the change in appearance of the carbon fine grain. Magnification ~750,000X. (From Agar, p.137).

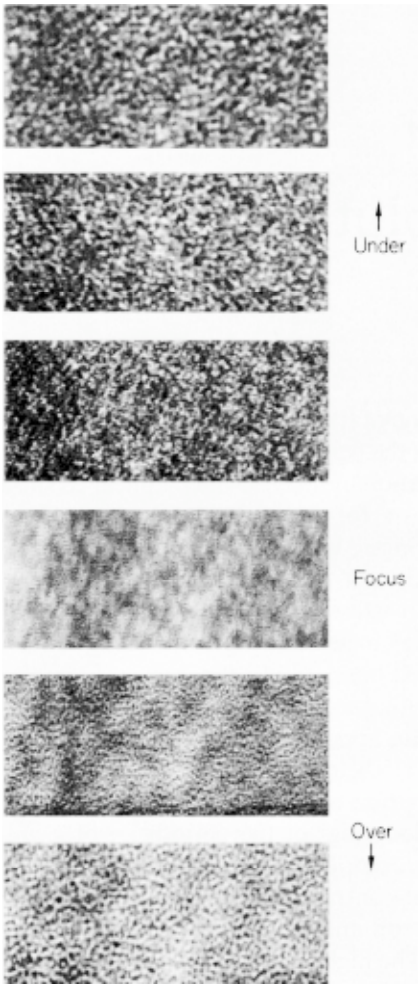

Fig. I.127. A through focus series of electron micrographs of the same area of a thin carbon film lightly shadowed with platinum, to show 'defocus granularity'. Each micrograph differs by one step or 'click' of the finest objective lens current control. From top to bottom the focus settings are: 2.1 μm under-focus, 1.4 μm under-focus, 0.7 μm under-focus, exact focus to within the resolving power of the instrument, 0.7 μm over-focus, and 1.4 μm over-focus. Magnification ~500,000X. (From Meek 1st ed., p.294)

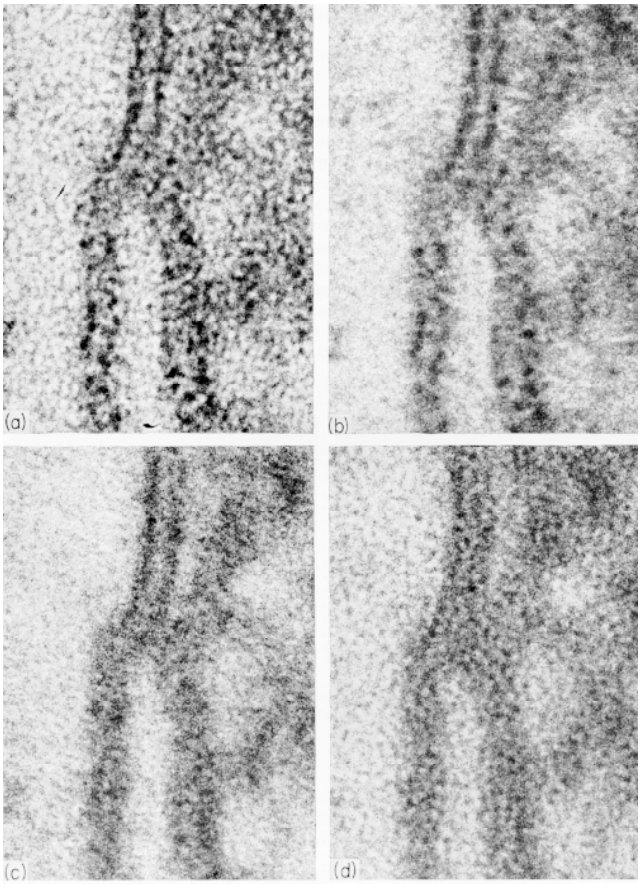

Fig. I.128. Phase contrast effects on image of structure in thin section (mitochondrial outer membrane). (a) Under-focus. (b) Close to focus. (c) Slightly over-focused. (d) Over-focused. Magnification ~1,000,000X. (From Sjostrand, p.109)

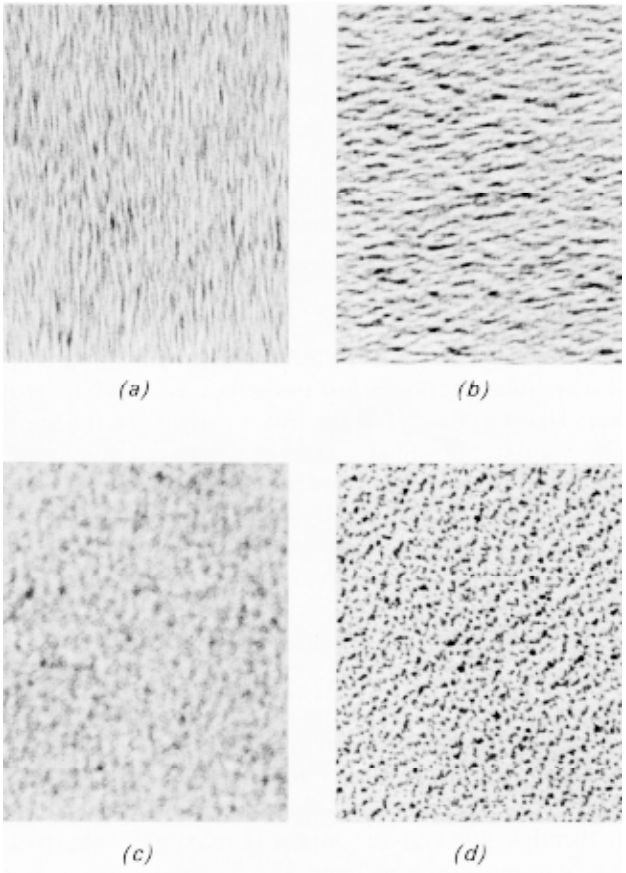

Fig. I.129. The 'line drawing' method of objective lens deastigmatism. Specimen - a highly contrasty, rather heavy coating of Pt-Ir on carbon substrate. (a) Astigmatic lens, under-focused. Astigmatic lines run N-S. (b) Same area, slight over-focused. Astigmatic lines run E-W at 90° to (a). (c) Same area at 'jump-over point' of best focus with the astigmatic lens. (d) Same area at best focus with astigmatism corrected to better than 10 Å. Magnification ~355,000X. (From Meek 1st ed., p.)

I.E.6. Magnification Calibration

The final magnification of the image is the product of the magnifications of all the imaging lenses. The strength of each lens is determined by the current flowing through it and the magnification read out on the instrument is set with reference to a known set of currents in the different lenses. Because of hysteresis in the iron circuits, the actual strength of the field depends on whether the current arrives at a given value from a higher or lower value. Thus, there is considerable uncertainty about the actual value of the magnetic field strength even though the currents are at a predetermined value. The actual magnifications may have an uncertainty of between 5-10% of the nominal value. Many modern microscopes have a facility for normalizing the magnification by cycling the lenses in a standard way, and hence improving reproducibility to about 2% or better.

In work requiring high accuracy in which knowledge of the magnification level is important, it is necessary to have an independent calibration of the magnification. For critical studies, a given calibration is only valid for the specimen holder being used for the measurements (since the specimen to objective lens distance will vary for different holders) and at a single voltage setting.

Magnification is calibrated with reference specimens of known dimensions. Specimens used for calibration purposes include:

a. **Polystyrene latex spheres** of uniform size (usually 259 nm diameter) make convenient internal calibration standards because they can be added to and photographed with a specimen

sample. However, this standard is only accurate to about 5-10%, unless a very large number are measured in the absence of contamination (which causes diameters of the particles to increase by variable amounts).

b. **Diffraction grating replicas** of known ruling spacing (usually 2160 lines/mm; Figs. I.130-I.131) are very convenient standards for low magnifications (5000-20,000X). They can be used at slightly higher magnifications (20,000-100,000X) by measuring the ratios of distances between identifiable points throughout a range of magnifications that overlap with settings where the grating spacings can be accurately measured.

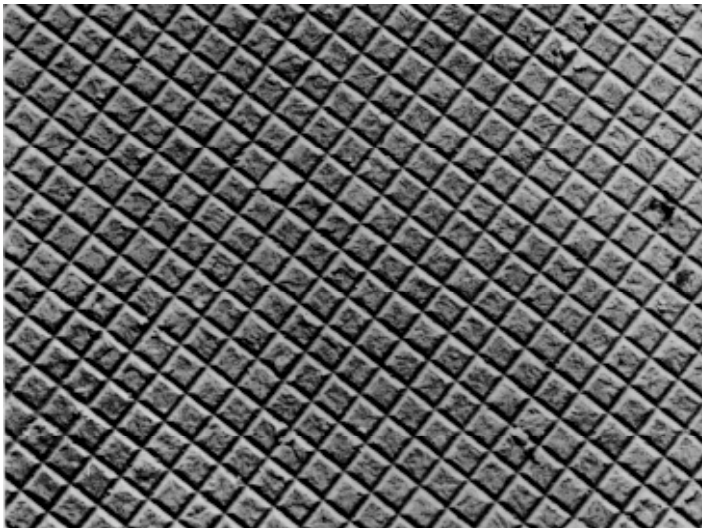

Fig. I.130. Replica of cross-ruled diffraction grating with 2160 lines/mm used as a calibration specimen. (From Agar, p.162)

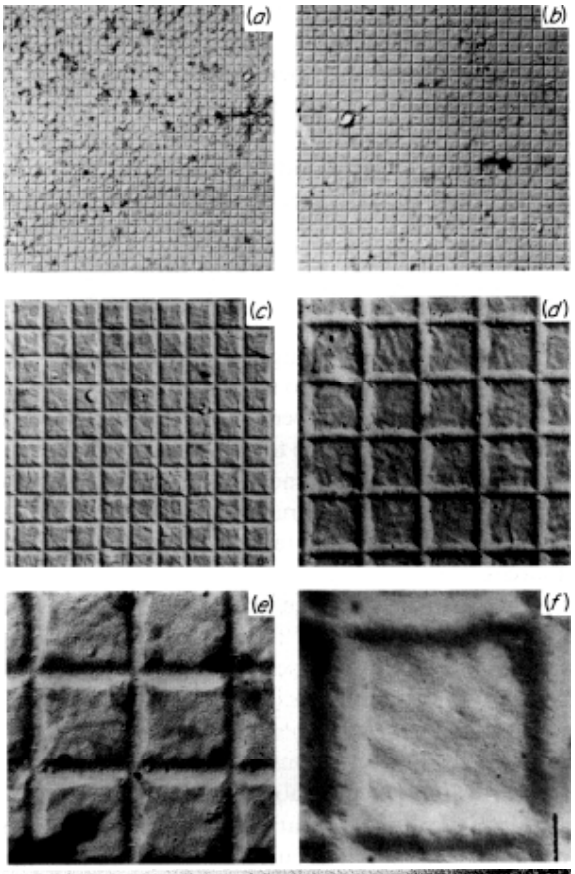

Fig. I.131. Magnification calibration with a shadowed diffraction grating replica. A square grating of 2,160 lines/mm is shown, taken at a nominal instrumental magnifications of (a) 3,000, (b) 5,000, (c) 10,000, (d) 20,000, (e) 30,000, and (f) 60,000. The optical enlargement in the figure is about 1.2 times. Sigmoid distortion can be detected in (a), but total distortion is less than 2.5%. Micrographs taken larger than 5,000X are virtually distortion-free. (f) This represents the upper useful limit of the method; the measurement of only one square is only valid to ±10% or less. (From Meek 1st ed., p.336)

c. **Crystalline specimens with known lattice spacings** such as beef liver catalase (8.75 x 6.85 nm; Fig. I.132) make excellent standards at higher magnifications as long as care is taken to assure that the sample does not shrink or expand in the electron beam or when it is dehydrated on the TEM grid. Tobacco mosaic virus (2.3 nm axial spacing) or T4 bacteriophage tails (3.9 nm axial spacing) also make excellent internal calibration standards for biological specimens examined at medium to high magnification.

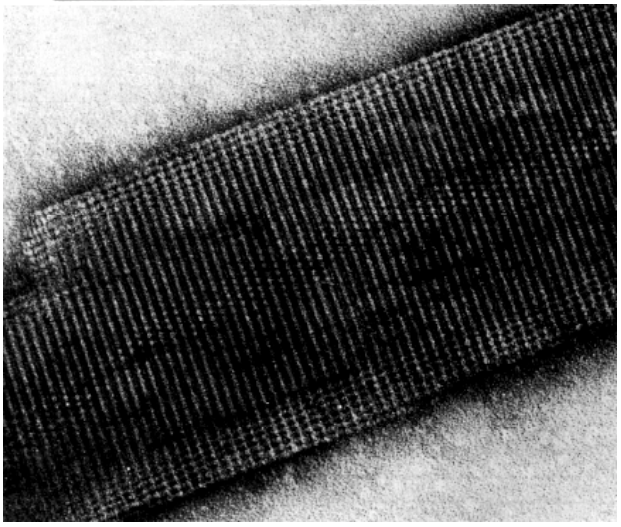

Fig. I.132. Beef live catalase crystal negatively stained with ammonium molybdate. The lattice spacing can be measured to within ±0.5% by using the electron microscope as a low-angle diffraction camera. The actual image magnification is then determined simply by counting the lines across the image. This method is useful up to about X500,000. The spacing of the lines is 8.75 nm, and the spacing of the dots on the lines is 3.43 nm. Magnification ~150,000X. (From Agar, p.163)

d. At **very high magnification** (>100,000X) **copper pthalocyanin**, with a lattice spacing of 1.24 nm, is sometimes used as a standard (Fig. I.133).

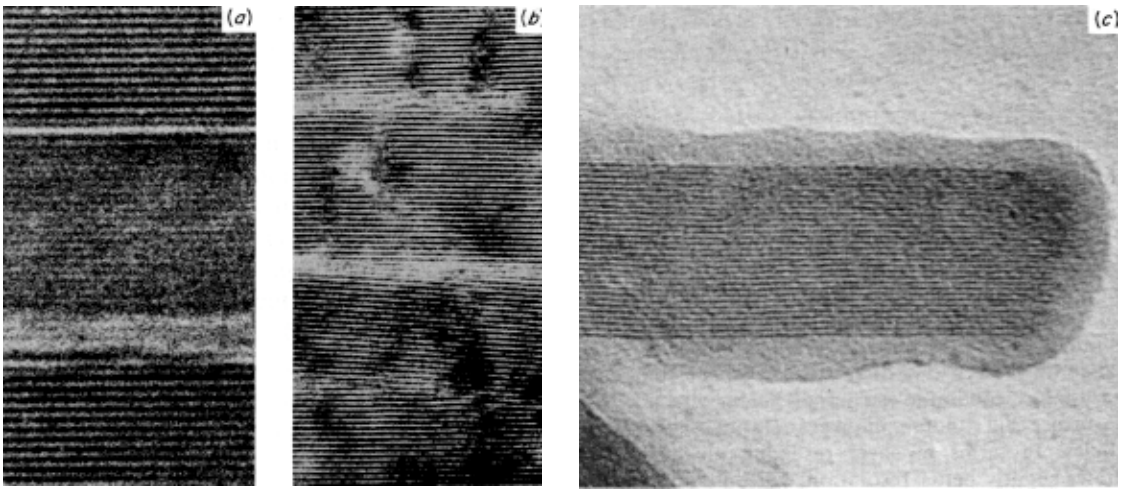

Fig. I.133. Crystal lattice plane resolution measurement. (a) copper phthalocyanine crystal showing lattice spacings of 0.98 and 1.26 nm. Because of the presence of two readily resolvable lattices of almost the same spacing, this substance is not ideal for either magnification or resolution calibration. (b) potassium chloroplatinate crystal showing 0.699 nm spacing. This substance is very susceptible to beam damage. (c) platinum phthalocyanine crystal showing 1.25 nm lattice spacing. (From Meek 1st ed., p.334)

A careful and repeated calibration of the magnification of the microscope is important for all quantitative work involving measurements of dimensions of ultra-structural and molecular components. Accurate magnification calibration of the instrument is also necessary for determining the resolving power of the instrument (see the following section).

Note that measurements of spacings in micrographs should, when possible, be made directly from the micrograph and NOT from a photographic enlargement since an enlarger may introduce distortions. In addition, photographic papers can shrink or stretch with time.

I.E.7. Resolution Tests

Normally the microscope user is not concerned with the attainment of the ultimate resolving power of the instrument because the specimen limits resolution to well below what the instrument is capable of delivering. Nonetheless, the user should be capable of checking the performance of the microscope from time to time to assure that best results can be obtained at any resolution.

The **resolving power** of the microscope is the best possible performance as limited by built-in instrumental parameters. The only practical way of checking the theoretical resolving power of a particular instrument (as quoted by the manufacturer) is to record photographs of a suitable test specimen and then measure the actual resolution achieved in the image.

There are three popular methods for testing resolution:

a. Point separation test

A photographic image of suitably small particles is searched for two small particles that can just be distinguished as separate. The distance between their centers defines the resolution achieved on the micrograph. Two successive photographs at the same focal setting must be recorded and the same pair of particles must be found on both micrographs to ensure that the effect of electron noise is negligible. Also, several pairs of particles in different orientations must be located to demonstrate that astigmatism or other image defects are not significant. If only a single micrograph is available for a resolution check, it is necessary to ensure that the magnification is sufficiently high so that specimen detail cannot possibly be confused with the electron noise in the image.

A suitable specimen for the point resolution test is a thin carbon film (5 nm thick) covered with a finely dispersed, evaporated film of platinum-iridium alloy (Fig. I.134). The pictures should be taken very close to focus to eliminate the risk that the image of the specimen will become

coarse due to out-of-focus phase contrast and diffraction effects.

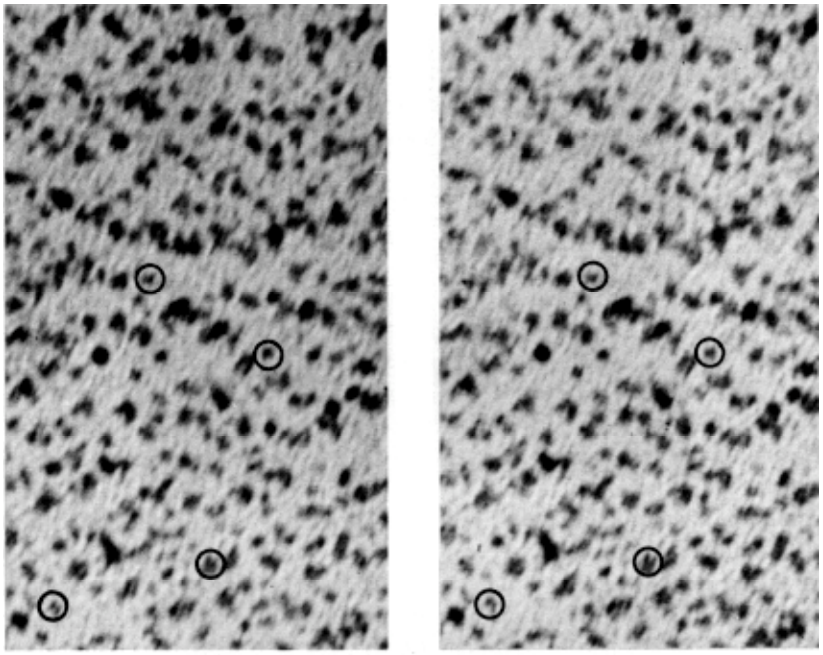

Fig. I.134. Point separation resolution measurement. The two micrographs (a) and (b) of evaporated platinum-iridium on a carbon substrate were taken in rapid succession after critical focusing. Pairs of particles (ringed) on each print show spacings of less than 1.0 mm, which at the overall magnification of approximately 2,000,000 indicates an instrumental resolving power of better than 5 Å. A trace of astigmatism in the NE-SW direction can be detected; resolution in this direction is better than resolution in the perpendicular direction. Two plates must be taken in order to ensure that random phase granularity is not measured by mistake for actual particles. (From Meek 1st ed., p.333)

b. Lattice resolution test

A suitably-oriented thin crystal with accurately known lattice spacings (*e.g.* from X-ray data) show crystal lattice planes imaged as a set of dark lines of high contrast. This type of specimen also serves as an accurate measure of the magnification (Fig. I.133). The clearly defined lines are easier to measure at a higher degree of accuracy than two image points which may be recorded at low contrast in a noisy background. It is best to use a specimen with lattice planes oriented in more than one direction. An ideal such specimen is partially graphitized carbon black since the lattice planes (0.34 nm) curve in many directions within a small region (Fig. I.135).

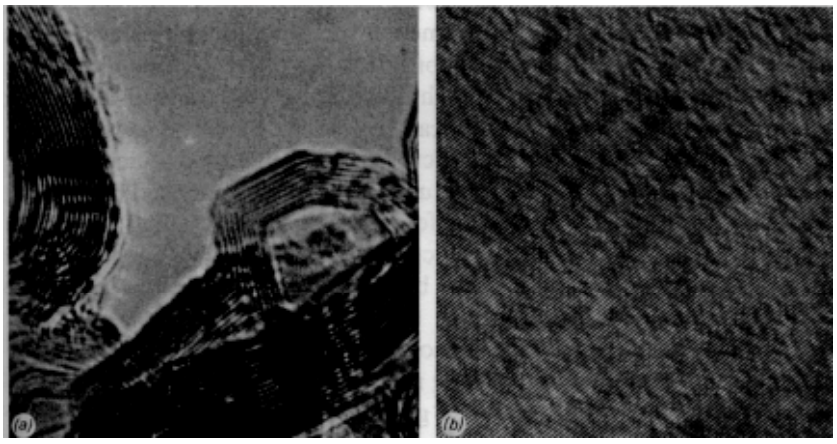

Fig. I.135. Micrographs of crystal lattice spacing in (a) graphite and (b) gold foil. In each case, the specimens have been tilted to give the optimum orientation of the lattice for maximum image contrast, using a eucentric tilt/goniometer stage. The graphite lattice has a spacing of 3.4 Å, the gold lattice has a spacing of 2.04 Å. Magnification ~2,500,000X. (From Meek 2nd ed., p.317)

The lattice line test is generally regarded as a stability test rather than as a guide to the likely performance with other specimens. It is useful to quote particle separation and lattice plane resolution figures together since they provide complementary information about microscope performance. The resolution of point objects in general is poorer than that obtained by the same instrument in the imaging of lattice planes. This is partly due to the greater ease of identifying rows of molecules in a crystal as compared to separate points in a random structure. When crystals are used as test objects, the determination of the spacing between crystal planes is made on an image, the quality of which is minimally affected by spherical aberration. Also, sharp crystal lattice planes can be obtained with a highly astigmatic optical system provided that the axis of the astigmatism coincides with that of the crystal lattices.

Contrast in images of a crystal is mainly a phase effect whereas the contrast in images of point objects is primarily an amplitude effect.

c. Fresnel fringe test

Direct visualization of the overfocused Fresnel fringe at the edges of a hole in a thin carbon film provides a convenient check on the performance of the microscope (Fig. I.136). A rough guide to the resolution is the width of the finest Fresnel fringe that can still be seen all around the circumference of a hole in the specimen film.

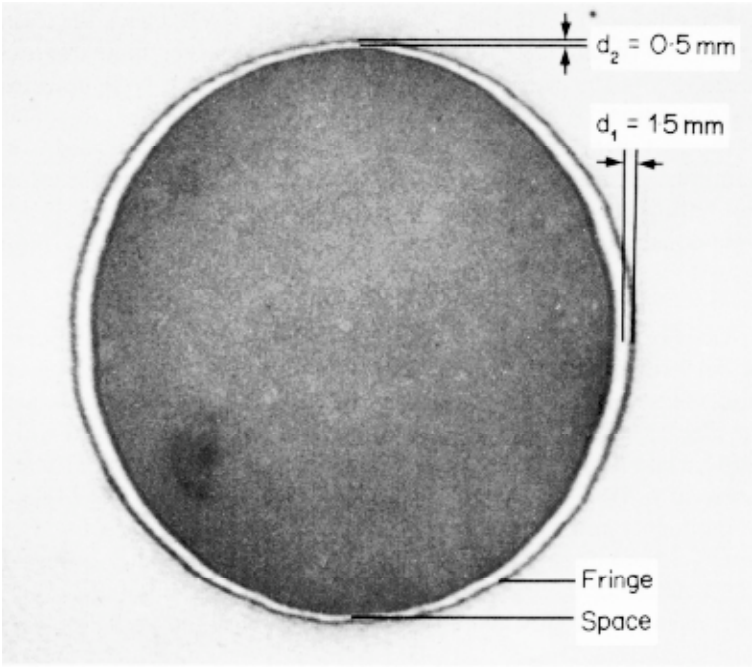

Fig. I.136. Fresnel fringe resolution measurement. This micrograph of a hole in a carbon film at X500,000 was taken with deliberately introduced astigmatism in order to demonstrate the method. The measurements are generally made direct on the processed plate exposed at top instrumental magnification without making an intermediate print; this is a reversed print corresponding to the image on the negative. The distance between the center of the black fringe and the center of the white space separating it from the edge of the hole is measured with a graticule magnifier at the points of maximum and minimum spacings, d_1 and d_2. The fringe must be almost touching the hole at its closest point. Several measurements of each spacing are made and averaged. The distances in mm are then converted to Å from knowledge of the magnification. Resolving power is simply the difference between the two measurements. In the example above, d_1 = 1.5 mm and d_2 = 0.5 mm. The difference of 1.0 mm at a magnification of X500,000 is 20 Å, which is the resolving power of the instrument under these conditions of gross astigmatism applied by the objective stigmator. (From Meek 1st ed., p.331)

I.E.8. Image Intensifiers/TV Displays

An **image intensifier** is a device that electronically scans an image and produces an amplified signal before being converted to a visual display on a second screen situated outside the TEM vacuum (Fig. I.137). The gain in contrast of the intensified image is comparable to that obtained by photographic processing. Ultimate resolution is NOT improved by use of the device.

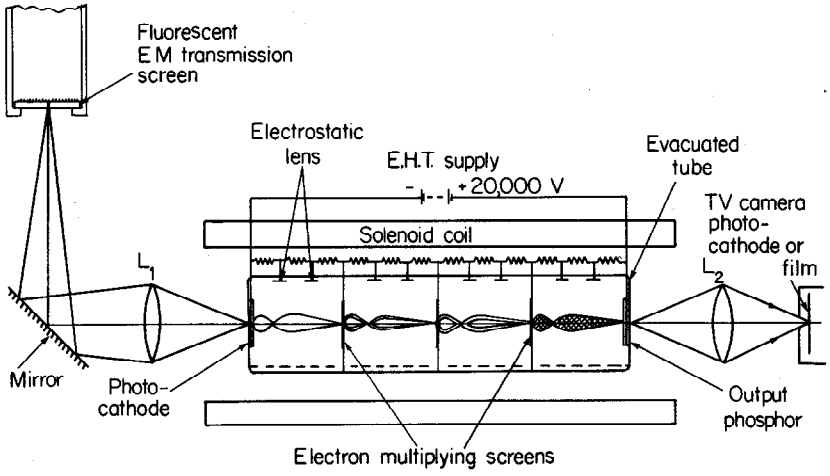

Fig. I.137. A schematic diagram of an electron microscope image intensifier and image train (developed by GEC-AEI Ltd.). The primary electron image is formed on a transparent phosphor screen below the camera in the microscope column, and is focused on to the photocathode of an electron multiplier. Four stages of electron multiplication produce an identical image of greatly increased brightness on the transparent output screen, which may be examined by eye, photographed or focused on to the photocathode of a TV camera for further amplification and display on a monitor. (From Meek 1st ed., p.401)

The basic principle of the image intensifier is to form the final electron microscope image on a transparent transmission phosphor screen mounted beneath the normal fluorescent screen and camera. The first dim image is formed on the transparent screen and all subsequent operations are performed outside of the microscope vacuum. The primary image is coupled optically to a photocathode, which emits electrons when excited by light. A lens of very large aperture or glass fiber optics are used to make the optical coupling as efficient as possible so as little light from the primary image as possible is lost in the coupling process. Electrons emitted by the first photocathode are accelerated by a high potential of several thousand volts and focused onto a second cathode that emits several secondary electrons for each primary electron striking it. The process is repeated several times, each multiplier stage increasing the total number of electrons available to excite a final transmission screen similar to the first transmission screen. The final screen may be examined and photographed directly, or it can be optically coupled to the photocathode of a TV camera scanned in the usual way. The signal from the camera is further amplified and may be displayed on the screen of a TV monitor.

Visible images can be obtained on the TV display monitor with electron image current densities as low as 10^{-14} Amp/cm^2. The human eye requires about 100 times more current density on the standard fluorescent screen for the same type of image to be visible. Electron images that are almost invisible on the screen, either because the scattered electron beams are inherently of very low intensity, or because the intensity is deliberately restricted to avoid radiation damage to the specimen, can be clearly revealed on the TV monitor screen and photographed from the screen.

The video output signal can be fed directly to a video tape recorder for immediate playback of dynamic phenomena, or to a standard closed-circuit television system for display to a large number of observers.

Among the **advantages of an image intensifier** are that they allow:

a. direct high magnification image observation at comfortable brightness levels.

b. specimen observation at lower beam intensities.

c. electronic scanning and processing of images.

d. extending the range of contrast beyond that provided by direct visualization on the screen.

e. simultaneous specimen observation by groups of viewers.

f. continuous recording of transient phenomena.

I.E.9. Microscope Maintenance

The general rule of thumb is to leave well enough alone. Generally it is best not to dismantle the TEM column to clean a suspected component until all other avenues have been explored, since it may lead to a poor vacuum for days or, at worse, may cause a leak or a misalignment due to unskillful reassembly. Most of the maintenance of a microscope is left in the hands of a qualified technician, although the user can perform some of the more common tasks.

a. Filament change

When it becomes necessary to change the filament, the condenser and objective apertures should be cleaned or replaced. The bluish coating of evaporated tungsten and contamination inside the Wehnelt shield is removed by soaking the shield in a 5% aqueous ammonia solution for a few minutes followed by polishing with a non-abrasive powdered chalk or fine alumina. The polishing medium is rinsed away with the ammonia solution and allowed to dry in a dust free place. The shield should only be handled with nylon gloves or lint-free paper since grease, fingerprints or dust specks act as nuclei for electrical discharges between the shield and gun chamber causing high tension instability. Dirt in the shield aperture will cause instability in the beam current giving rise to illumination flicker. It is usual practice to clean the anode at the same time the filament is changed.

b. Column cleaning

The parts of the column exposed to the beam slowly become coated with contamination mainly from the hydrocarbon pump oils. The greater the electron bombardment, the greater will be the

buildup of contamination. The parts that contaminate most rapidly are the lens apertures, especially in the condenser system; the gun components, especially the bias shield aperture and the anode; the condenser lens pole pieces; the gun chamber and insulator; and the anticontaminator and specimen holders. In general, the parts of the column below the objective lens are free of contamination.

Contaminated condenser apertures make the illumination beam astigmatic and reduce brightness of the focused beam, although they **do not** degrade the final image. Contaminated objective apertures and anticontaminators cause astigmatism in the final image and must be changed immediately. Contaminated gun components cause random illumination flicker.

c. Vacuum system leaks

Excessive column pressure is almost always caused by a leak at a movable vacuum seal. Fixed seals such as the O-rings between lenses usually don't spontaneously leak, except perhaps between the condenser lenses where the seals are constantly exposed to X-rays and eventually crack owing to hardening of the rubber.

Leaks are traced by manipulating suspected seals such as the aperture drives, fluorescent screen spindle, camera drive, specimen airlock, etc. while closely watching the pressure gauge.

I.E.10. Photography

The **aim of photography** is the complete and faithful reproduction of image detail. It is ***assumed*** that image intensities, in the absence of optical aberrations and specimen damage, are directly related to the structure of the object.

The basic difference between the response of photographic emulsions to electrons and light is that with electrons it is a single-hit process whereas with photons it is a multiple-hit process. The photographic characteristics that interest electron microscopists are those of speed, contrast, graininess, and resolution.

a. The photographic process

Photographic emulsions suitable for EM are normally between 10 and 30 μm thick (Fig. I.138). The **emulsion** is a suspension of tiny crystals of a silver halide (usually silver bromide) in gelatin. The mean size of the approximately spherical crystals varies in different emulsions from about 0.05 to 2 μm (Fig. I.139). The emulsion often has a supercoating of clear gelatin about 1 μm thick to protect the underlying grains against abrasion effects which render them developable without exposure. To avoid curling during processing and drying, sheet film is given a gelatin coating on the opposite side of the emulsion layer.

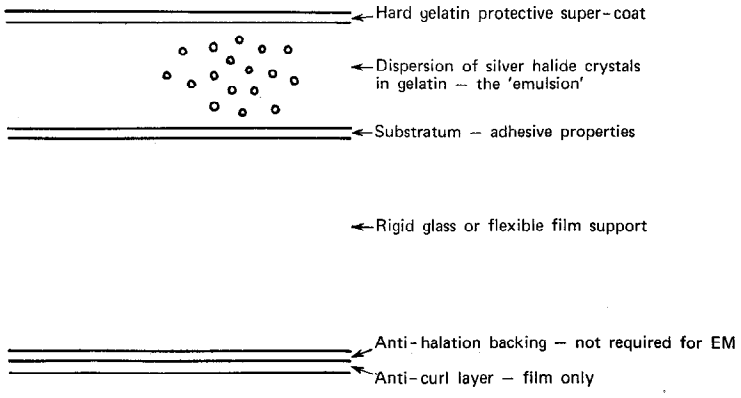

Fig. I.138. Structure of the photographic material. (From Agar, p.193)

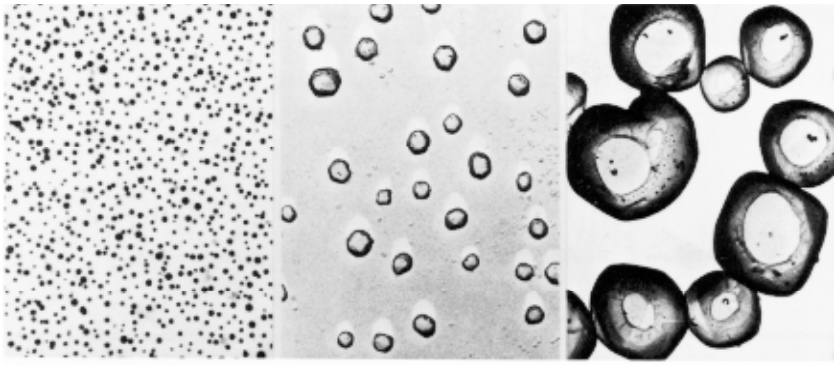

Fig. I.139. Electron micrographs of emulsions spanning the size range of commercial types of narrow spread. (Left) High resolution emulsion, (Middle) Emulsion of medium grain size of the type commonly used in electron micrography, (Right) X-ray emulsion of high sensitivity. The micrographs shown at the middle and right are of Au/Pd shadowed carbon replicas. (From Farnell and Flint, p.20)

When the emulsion is exposed to photons of energy 2.5eV or more (blue or shorter wavelengths) or to moving charged particles, tiny specks of silver atoms form at points in any crystal that has absorbed sufficient energy from the beam. For photons, the required energy is about 30eV (10 photons), but with charged particles such as electrons about 500eV is needed. Electron beams used in TEM (100kV) are sufficiently ionizing to ensure that the passage of a single electron through a crystal of silver halide transfers more than sufficient energy. As a result, exposure of a photographic emulsion to electrons is almost entirely a **single-hit process**; exposure to light requires the cooperative action of several photons on each halide crystal.

The minute specks of silver formed in the sufficiently irradiated crystals constitute the **latent image**. When **developer** (a solution containing a chemical reducing agent such as hydroquinone or p-methylaminophenol sulfate) acts on the emulsion, the crystals containing the latent image are very much more rapidly converted to metallic silver than the others (Fig. I.140). As little as three atoms of silver in its latent image is sufficient to cause development of the silver halide crystal into a slightly larger, tangled, filamentous mass of metallic silver, called **silver grains**.

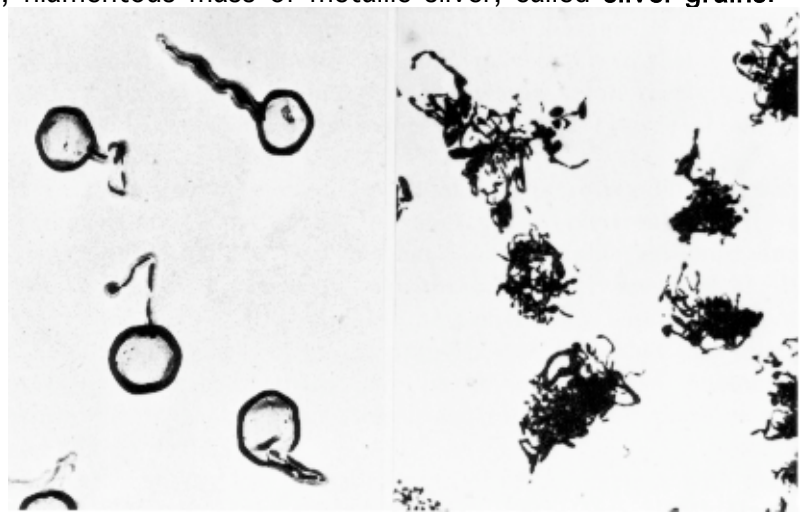

Fig. I.140. Electron micrographs of (Left) exposed grains at a very early stage of reduction by a developer, and (Right) grains of the same emulsion fully reduced to tangled masses of silver filaments. Magnification ~28,000X. (From Farnell and Flint, p.21)

Emulsions used for normal photography often have dyes added to extend their sensitivity to include red light (panchromatic emulsions). This has the disadvantage that the emulsion must be developed in total darkness. Consequently, non-panchromatic emulsions are used for TEM so developing can be done under red or yellow safelight conditions.

After development is complete, the emulsion is transferred to a solution of sodium thiosulfate (**fixer**) to dissolve the unexposed silver halide crystals and harden the gelatin matrix. The emulsion is then thoroughly washed to completely remove the fixative to avoid discoloration and eventual disappearance of the image (Fig. I.141).

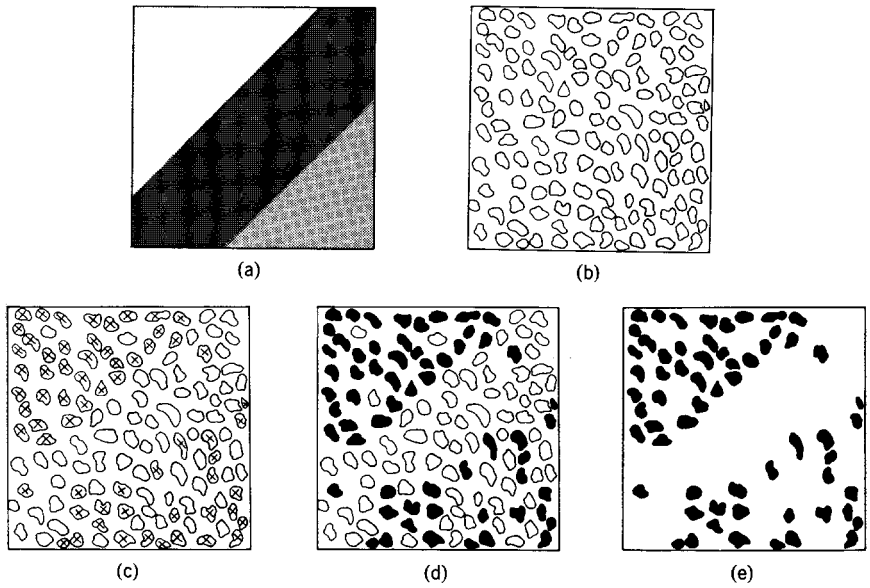

Fig. I.141. Stages in photographic processing: (a) object; (b) unexposed emulsion; (c) exposed but unprocessed emulsion; (d) developed but unfixed emulsion; (e) final image after fixation. (From Slayter, p.462)

b. Optical density of the processed emulsion

The response of the emulsion is expressed by its **optical density** which is defined as $D = -\log_{10}(1/T)$, where T (**transmission**) = I_t/I_i is the fraction of incident light transmitted by the plate or film. Thus, the optical density is a quantitative measure of the blackening of the photographic emulsion.

Thus, if T = 0.10 (only 10% of light passes through), D = 1.0. For T = 0.01 (only 1% transmitted), D = 2.0.

Most electron emulsions reach a saturation density, D_S, of about 6-8. However, the usual working range is about $D_S/4$ where the density is nearly linearly related to exposure (see below).

c. Density related to exposure

Since exposure to electrons is a single-hit process, **virtually every halide crystal hit by an electron is rendered developable.** Subsequent hits of the same crystal by other electrons are irrelevant. A single electron normally hits more than one crystal in its passage through the emulsion. D, the density of the processed emulsion, is related to E, the number of electrons that irradiate a unit area according to the following relationship:

$$D = D_s\left(1 - e^{-KE}\right)$$

K is related to the size and sensitivity characteristics of the emulsion.

$$K = na$$

where n =#grains/e-
a = area of one developed grain.

In the region where $D < D_S/4$, the curve is approximately linear, so

$$D = D_s KE$$

The result is that D is directly proportional to exposure (this does **NOT** hold for photons).

d. Density/Exposure curves

The experimental relationship between the optical density of a photographic emulsion and its exposure to light is graphically demonstrated in the so-called **H and D curve** (after Hurter and Driffield, 1890). In this curve, D is plotted against $\log_{10}E$. The form of the curve for photons is

sigmoidal with three distinct regions (Fig. I.142). In the toe region the slope increases. This is followed by a straight-line portion of nearly constant slope and then by a shoulder region where the slope decreases as the density asymptotically approaches its saturation value. The **slope**, γ (**gamma**), in the straight-line portion (working region of the emulsion) is a measure of the contrast of the emulsion.

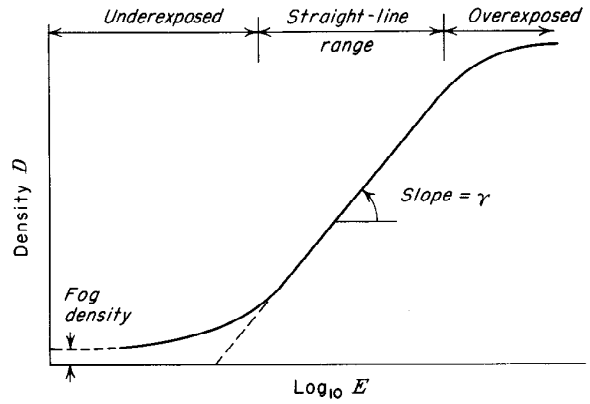

Fig. I.142. Idealized HD characteristic for photographic film. The exposure E is the product of intensity times the exposure time. (From Hall, p.172)

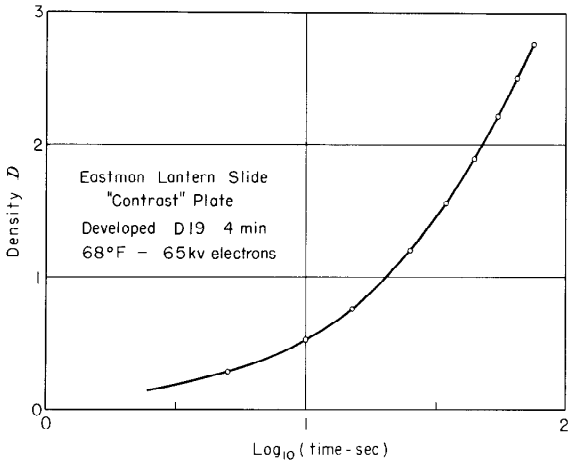

Fig. I.143. Experimental characteristic curve for a photo-graphic plate exposed to 65-kV electrons. (From Hall, p.174)

For a single-hit process the H and D curve has no special significance since the toe region is long and the straight-line portion is only reached at very high values of *D* (Fig. I.143). It is better to make a linear plot of *D* verses *E* (Fig. I.144).

e. Contrast

Contrast is defined as the difference in density for a given ratio of intensities in the image. It is equal to the slope of the *D* vs log*E* curve ($\gamma = \Delta D/\Delta \log E$). For photons, the working region of the emulsion is the straight-line portion of the H and D curve where γ is a constant. Its value, though, varies greatly with different emulsions and development conditions (time, temperature). With electrons, however, in the linear region of the *D* vs. *E* curve, contrast is linearly related to density (*i.e.* $\Delta D/\Delta \log E = 2.3D$). Thus, the contrast of the emulsion depends only on the density so, when exposed to the same density all electron emulsions will have exactly the same contrast. The contrast does not go on rising indefinitely with *D* because at high densities the linear relationship fails.

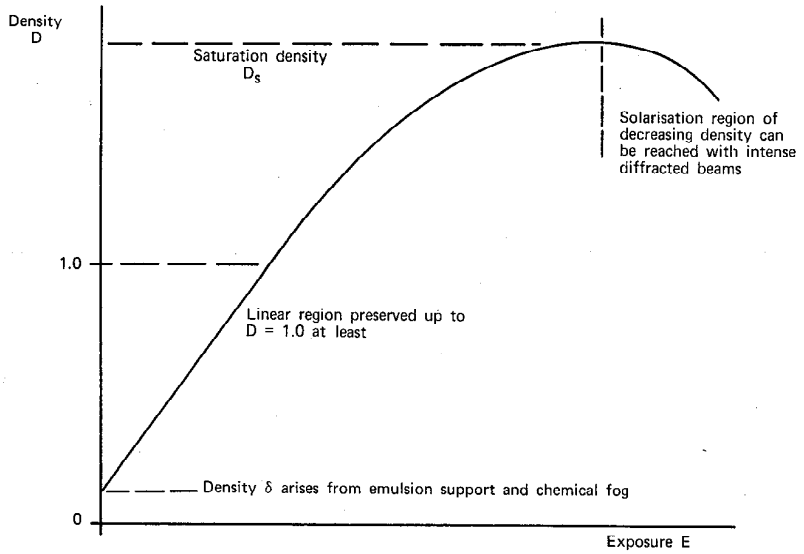

Fig. I.144. Characteristic density/exposure relationship for electron image recording. (From Agar, p.197)

f. Speed of electron emulsion

For electrons, emulsion speed is rather easy to define since the linear relation between density and exposure holds over the useful working range of the emulsion. **Speed** is expressed as the reciprocal of the number of electrons required per unit area to produce a density above the fog level equal to 1.0. For low *E*, in the linear region of the *D* vs. *E* curve, speed is equal to the slope of the straight line.

The table below demonstrates that the speed of a particular emulsion is determined by the developing conditions (See also Figs. I.145-I.146).

DEVELOPMENT AIM	FILM TYPE			DEVELOPMENT	
	SO-163	EIP	4489	D-19	Time
Maximum Speed/Minimum S/N (unstable specimens)	2.20	2.0	- - - -	Full strength	12 min
Intermediate Speed and S/N	0.80	0.7	0.4	1:2	4 min
Minimum Speed/Maximum S/N (stable specimens)	0.15	0.2	- - - -	Full strength plus anti-fog	2 min

NOTES: SO-163 and 4489 are films; EIP (Electron Image Plate) is a glass plate; D-19 developer diluted with water and used at 20°C with nitrogen burst agitation.

For example, if an emulsion with a speed = 1.0 μm^2/e- is exposed for 1 second to a beam at a normal viewing intensity (1e-/μm^2/sec), the optical density on the film will be = 1.0. For a magnification of 10,000X, the **dose-rate** at the specimen would be 100 e-/nm^2/sec. At the same viewing intensity at 50,000X the **dose-rate** at the specimen would be 2500 e-/nm^2/sec.

Emulsion speed varies only slightly with accelerating voltage in the range of 50-100kV. Above this, the speed drops significantly (Figs. I.147-I.149).

g. Electron range in emulsions

Most electron emulsions are designed to have a fully exposed (saturated) density D_S = 6. If the range of the electrons is less than the thickness of the emulsion, it will be impossible to expose it fully. For example, a 50 μm thick emulsion fully exposed to light might reach a saturation density of 6; but exposed to an electron beam which can only penetrate ~10 μm, it would only reach a density = 1.2. A rule of thumb is that the range *R* (in μm) of an electron beam in an emulsion is approximately given by:

$$R = V^2/100, \text{ where } V \text{ is the accelerating voltage in kV.}$$

Thus, a 50kV beam has a range of 25 μm, whereas a 100 kV beam can penetrate 100 μm. Recall that emulsions used for TEM are about 10-30 μm thick.

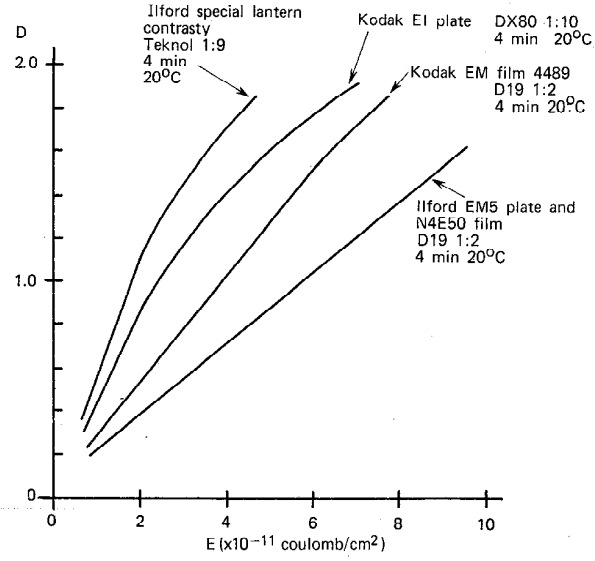

Fig. I.145. The variation of density with exposure for four emulsion/developer combinations. (From Agar, p.199)

h. Number of grains per electron

Each electron may hit more than one crystal of silver halide in passing through the emulsion. A medium-speed emulsion used for EM contains about 10% halide by volume and the diameter of the crystals is about 0.3 μm. Simple theory predicts one grain per 2 μm, or, for a 20 μm thick emulsion, about 10 grains per electron track (Fig. I.150).

Each electron may pass through as many as 100 silver halide grains, losing some energy in each. The energy loss per grain increases along the path and becomes very high at the end. Hence, the quantity of energy transmitted to each grain at the start of its track will be much less than towards the end. In consequence, the number of developed grains per unit length of track will be small at the start, but increase towards the end of the track (Fig. I.150). In almost any emulsion, a few of these grains near the end of the path are made developable. Whether or not those at the beginning of the path develop depends frequently on the development conditions.

i. Graininess

Photographic graininess limits the amount of information that can be recorded on the negative. The **word grainy**, describing the appearance of an over-enlarged emulsion, **is misleading**. It is incorrectly believed that graininess is a defect of the photographic emulsion and a result of the finite size of the grains of silver. It is quite easy to see the "photographic grain" in a ten-times enlargement of the negative, but the silver grains are so small that a high-power light microscope can barely resolve them.

Graininess is a statistical phenomenon. It is a few orders of magnitude larger than the size of the grains of silver that form the photographic image. What is seen as graininess is not the result of seeing the silver grains; it comes from the non-uniformity in density due to their random distribution.

Graininess is the product of two random processes. The first involves the random arrival of quanta or electrons in the radiation to which the emulsion is exposed. Over a large area, the radiation may be regarded to be uniform, but when subdivided into small regions, each receives comparatively few

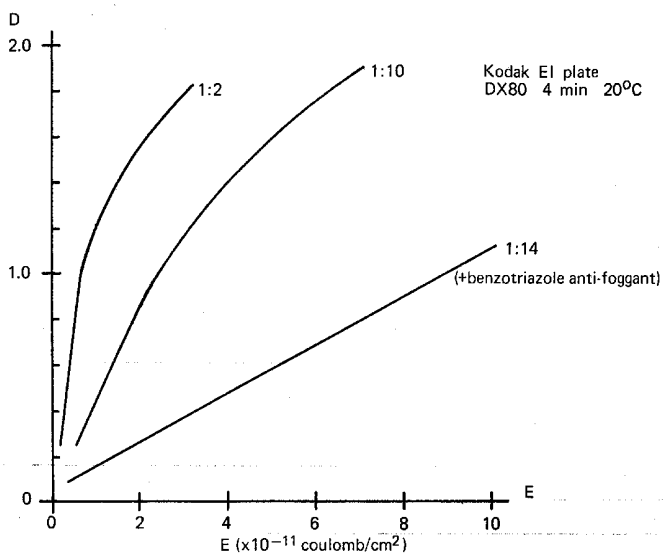

Fig. I.146. Variation of speed with developer concentration. (From Agar, p.199)

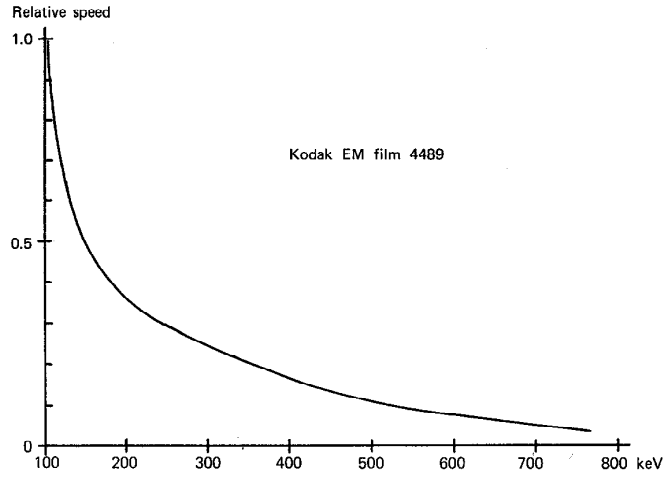

Fig. I.147. Variation of relative speed with electron energy in the range 100-800keV. (From Agar, p.206)

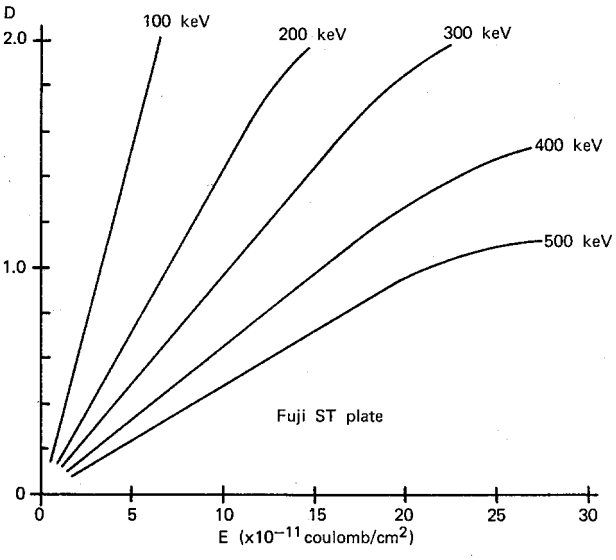

Fig. I.148. Linear relationship of density and exposure at high energies. (From Agar, p.207)

quanta and chance determines that each of these areas does not receive an exactly equal number of quanta. This random fluctuation in irradiation leads to a fluctuation in density on the negative and hence to the impression of graininess. These random fluctuations are referred to as **electron noise.** Another way to understand this is to recognize that a single electron makes an irregular track of several grains, and since the arrival of electrons is random, the pattern of tracks is irregular. This contribution to granularity far outweighs the randomness in the distribution of the silver halide grains in the emulsion.

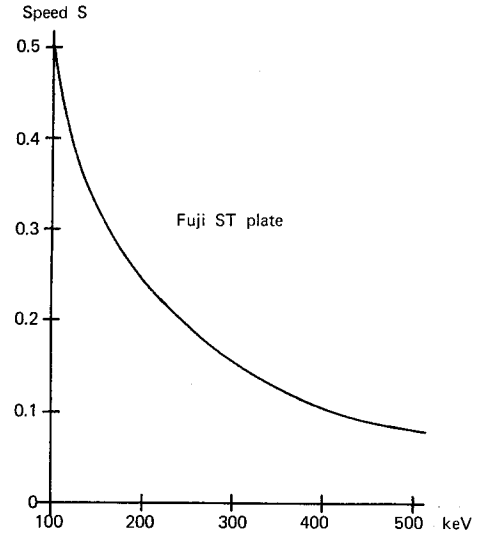

Fig. I.149. The variation of speed with energy. (From Agar, p.207)

Fig. I.150. Photomicrograph of the track of an electron having an initial energy of 75keV recorded in a nuclear track type emulsion layer. The dense aggregation of grain occurs towards the end of the track. Magnification ~4,500X. (From Farnell and Flint, p.23)

The random arrival of electron quanta follows Poisson statistics, that is, the fluctuations are proportional to $(N)^{1/2}$, where N is the number of electrons. The larger N is, the larger the fluctuation, but the less the relative fluctuation. Thus, granularity is proportional to $(N)^{1/2}/N = 1/(N)^{1/2}$. This means that the granularity is larger the smaller the area.

In converting the optical image into the photographic image, further random effects may be involved which act to amplify the quantum noise. The effect of film emulsion granularity on photographic noise amplification is proportional to $[1+(2/n)]^{1/2}$, where n is the number of grains produced per quantum event. Thus, for ordinary conditions of electron microscopy, photographic noise amplification is between 1 and 2, thus noise in the micrograph is likely to exceed noise in the electron image by a factor of less than two. In the case of photons, n is always considerably smaller than unity since many photons are required per grain. The recording of a light image by a photographic emulsion will thus always be far less efficient than an electron image, and a large amplification (up to 100) of quantum noise is expected. Thus, the photographic emulsion is an almost perfect recorder of the electron image and the graininess seen in electron micrographs is simply a faithful reflection of the information in the electron beam. Granularity can be reduced only by increasing exposure and not by any change in photographic techniques. Granularity may become significantly greater than the electron noise when emulsions are grossly underdeveloped or are exposed to very low voltage electrons. In both instances, the image is confined to a thin surface layer of the emulsion as a result of the lack of penetration of either developer or electrons.

The **detective quantum efficiency (DQE)** is a measure of how ideal the photographic emulsion is in faithfully recording the optical image. It is given by comparing the signal-to-noise ratio (S/N) in the micrograph with that in the electron image.

$$\mathrm{DQE} = \frac{\left(\frac{S}{n}\right)^2_{out}}{\left(\frac{S}{n}\right)^2_{in}}$$

where S/N = visibility of a given size detail against a grainy background
"out" = refers to the photographic image
"in" = refers to the electron image.

Since electron noise is proportional to $(N)^{1/2}$, S/N is proportional to $N/(N)^{1/2} = (N)^{1/2}$. For a perfect recorder, DQE = 1.0.

If a recorder either wastes some of the input signal by not absorbing some electrons or by having no visibly detectable response to some electrons, or if it introduces additional noise, because, for example, of excessive granular structure of its own, then DQE is less than unity. For electrons, emulsions have DQE = 0.5-1.0; for photons, DQE = 0.001.

To increase *S/N*, the exposure must be increased. This can be accomplished in several ways:

- Use more electrons to record the image.
- Develop a given emulsion longer (or with a more concentrated developer).
- Use a slower emulsion developed longer to give the same *D*.
- Use a slower developer for a longer period of time.
- Use image analysis to add images together, thereby effectively increasing the number of electrons per "image".

j. Resolution - Image spread

Emulsions have a limited resolution of their own. The track of an electron through the emulsion is not a straight line perpendicular to the surface since electrons are scattered from the incident direction by their interaction with the halide grains. Because the electron path includes sideways scatter in the emulsion, image points are spread out and contrast is reduced. The effect is described as **electron diffusion**. For example, the image of the edge of a slit would appear sharp if there were no diffusion of the electrons in the emulsion (Fig. I.151).

The magnitude of the image spread depends on the accelerating voltage of the electrons. The spread is small for low voltage electrons because their track length is limited. It is larger for medium energy electrons since more grains are hit in the non-linear path. At much higher energy (voltage) the spread becomes small again because there is less scattering of the electrons, and consequently, fewer grains are hit. Image spread will increase at all voltages as the thickness of the emulsion increases.

The **contrast transfer function (CTF)** of an emulsion defines the amount of contrast that can be recovered at given resolution limits (Fig. I.152). A perfect emulsion has a CTF = 1.0 at all resolutions. In practice, the CTF of a typical electron emulsion drops below 0.5 (*i.e.* the contrast is half what it should be) for a resolution of 40 μm. The consequence of this, is that, for example, to record 1 nm details in a specimen at 50% contrast, a magnification of **at least** 40,000X is necessary.

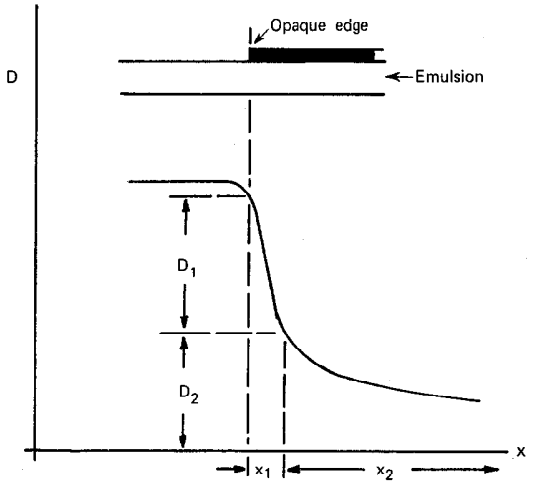

Fig. I.151. The density profile at an opaque edge. (From Agar, p.208)

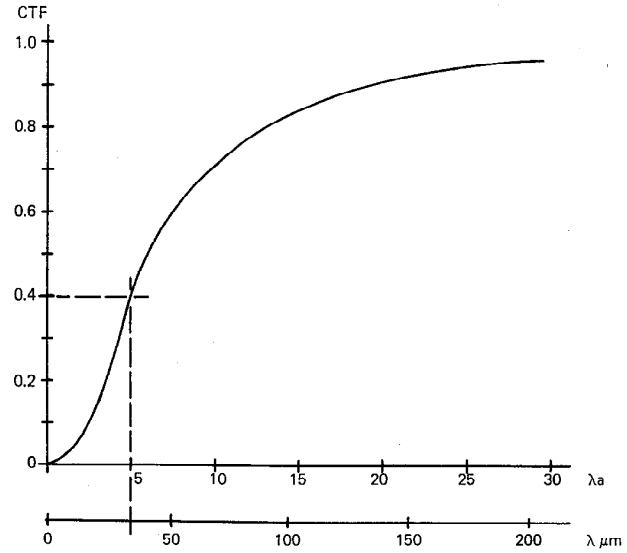

Fig. I.152. The contrast or modulation transfer function. □ is the periodicity of a sub-structure of frequency *f* lines/μm ($f = 1/$□). *a* is the diffusion constant as defined on page 209 of Agar. (From Agar, p.212)