I.C. CONTRAST AND IMAGE FORMATION

Resolution in electron images is normally limited by contrast, NOT by lack of resolving power. Whereas the resolving power of the microscope is of the order 0.2-0.3 nm, for most biological specimens, resolution is limited to about 1-5 nm. Contrast of images is determined by the nature and extent of interactions between the electron beam and the specimen. Properties of both the specimen (**inherent contrast**) and of the microscope system (**instrumental contrast**) are of importance.

Contrast is defined as the relative difference in intensity between an image point and its surroundings.

$$Percent\ contrast = 100 \frac{|I_o - I_b|}{I_b}$$

where I_o = intensity of the object point

I_b = intensity of the background adjacent to the object point

I.C.1. Electron Scattering

In light microscopy, **differential absorption** of light, which depends mainly on staining the specimen, results in the visible differences in various parts of the image. In the TEM, for specimens of "normal" thickness (<100-200 nm), the portion of the beam absorbed is minimal. To be absorbed, an electron must lose **all** its energy to the specimen. Those electrons that give up part of their energy are defined as **inelastically** scattered, whereas those that give up none of their energy when scattered are **elastically** scattered.

The amount of scattering that occurs at any particular specimen point is dependent on its density and overall thickness and is relatively independent of the atomic number, chemical composition, or other specimen properties. The scattering power of a particular area of specimen is directly proportional to its mass density (mass per unit area = density x thickness). With increasing mass thickness the probability of scattering increases. Since the level of contrast is determined by the average atomic number of the specimen and biological specimens consist mainly of carbon, nitrogen, oxygen, and hydrogen atoms, weak contrast is a limiting problem in the imaging of biological specimens. Inherent contrast may be increased by the preferential addition of materials of high atomic number during specimen preparation. A number of specimen preparation techniques for increasing the mass density of biological specimens to increase contrast will be discussed in Section II of the course and Lecture Notes.

The path of the beam of electrons is changed when passing through the specimen either through collisions or electrostatic interactions with the atomic nuclei or the electrons in the electron shells surrounding the nuclei (Fig. I.103). Beam electrons that pass outside the range of the electrostatic field of atomic nuclei and atomic electrons are not scattered. Electrons that are scattered (deflected) may be associated with no loss of energy (**elastic collision**) or a loss of energy (**inelastic collision**).

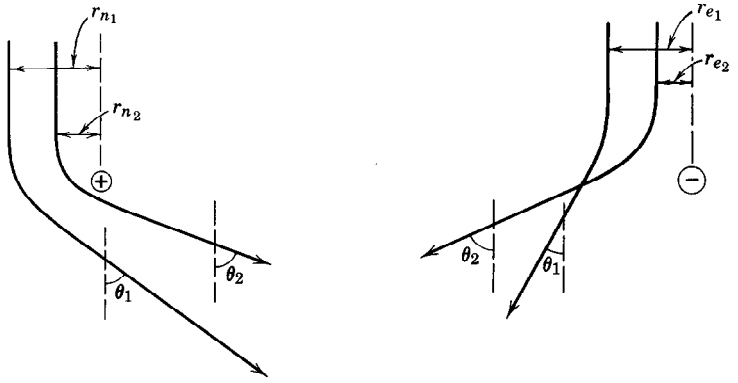

Fig. I.103. Left: Electron trajectories in the vicinity of a nucleus (elastic scattering). Right: Electron trajectories in the vicinity of a stationary electron (inelastic scattering). (From Slayter, p.423).

Deflection of the beam by direct collisions as well as electrostatic interactions between electrons in the beam and the massive nuclei result in negligible energy losses of the beam electrons (Fig. I.104). Interactions that produce no change in the energy (*i.e.* wavelength or velocity) of the incident electron are termed elastic collisions. Inelastic collisions occur when energy is transferred from the beam to the electrons (negligible mass) of the specimen atoms. The proportions of inelastic and elastic collisions depend on the accelerating voltage and the nature of the specimen. For example, for a 50 nm thick carbon film illuminated with 50kV electrons, 34% of the beam is undeflected, while 11% is elastically scattered and 55% is inelastically scattered.

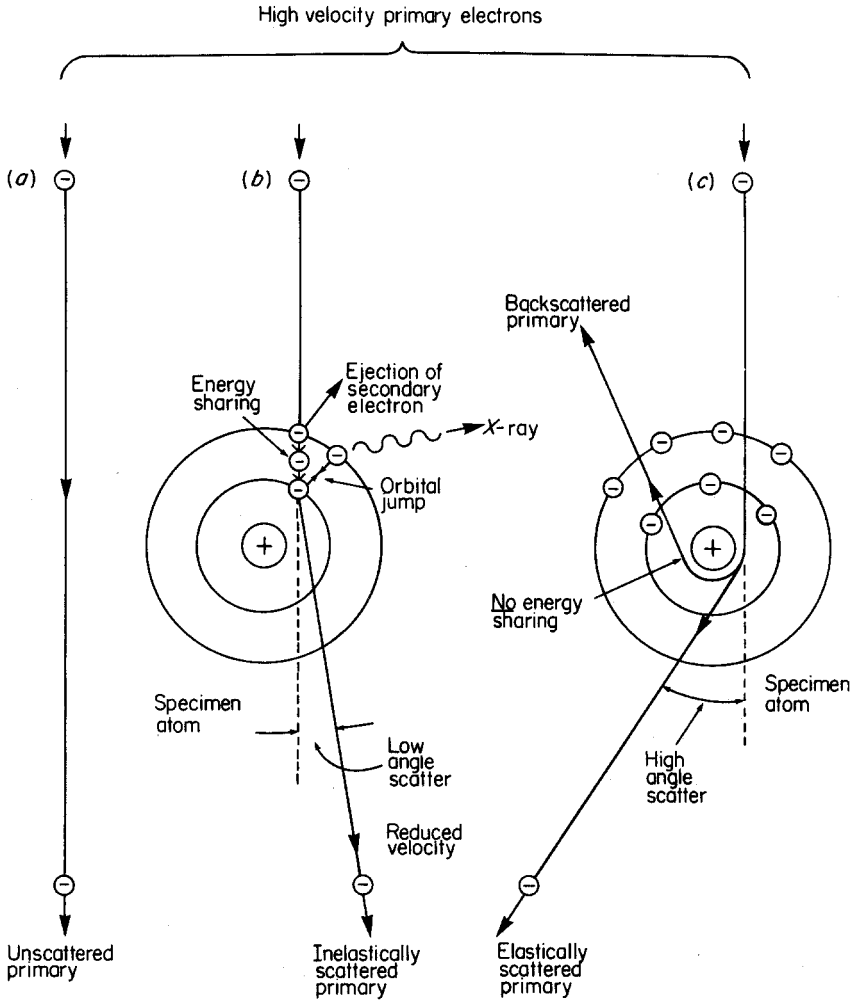

Fig. I.104. The principal modes of interaction of high-energy electrons with the atoms of matter constituting the specimen. (From Meek 2nd ed., p.95)

Since matter is mainly empty space, the trajectory of an electron must pass very close to an atomic nucleus or electron before it is deflected. Scattering of the beam electrons from direct collisions with the atomic nuclei and electrons represents a negligible factor contributing to image contrast compared with scattering due to electrostatic deflection since the probability of a direct collision is very low because nuclei and electrons have such small cross-sections.

a. Elastic scattering (Figs. I.103 and I.104)

An electron passing close to a nucleus is attracted toward the positive charge. The electron travels a **hyperbolic path** near the nucleus and then travels a straight line, but at some angle θ with respect to the original trajectory. An electron passing closer to the nucleus is more strongly attracted by the positive charge and is therefore deflected through a larger angle. The momentum of the incident electrons is sufficient that they escape capture by the nucleus. Elastic scattering results in deflection of the incident electrons through angles up to but not much greater than 10^{-2}

radians.

The angular deflection, θ_n, of the beam electron passing a distance r_n from the atomic nucleus is given by the following relationship (based on a very simplistic model for scattering):

$$\theta_n = \frac{Ze}{Vr_n}$$

where Z = the atomic number of the specimen atom
e = the charge of an electron
V = the accelerating voltage of the illumination beam (in electrostatic units)
r_n = distance of the beam electron from the stationary atomic nucleus

Thus, the deflection of the electron is directly proportional to the atomic number, Z, and decreases with distance from the nucleus, r_n, as well as with increasing beam potential, V. This expression, however, represents an oversimplification neglecting the electrostatic shielding of the nucleus by atomic electrons.

b. Inelastic scattering (Figs. I.103 and I.104)

Forces of repulsion determine electron trajectories in the vicinity of the specimen electrons. The closer the approach, the larger is the deviation of the electron trajectory. In the case of electrostatic interactions and collisions between the beam electrons and the electrons surrounding the atomic nucleus, the deflected electrons are likely to undergo a loss of energy (*i.e.* shift to longer wavelength and lower velocity). Scattering, in which the incident electrons lose some energy, is called **inelastic scattering**. The energy loss is generally on the order of 10-20 eV for thin specimens (<100 nm) where there is generally only one scattering event as the electron passes through the specimen. The beam electrons suffering such an energy loss are deflected through very small angles (~10^{-4} radians), thus nearly all of them pass through the objective aperture.

The proportional energy loss due to inelastic collisions corresponds to fluctuations in the accelerating voltage of the order one part in 10^4-10^5. Thus, the change in wavelength produced by a single electron scattering event is relatively insignificant. However, **multiple scattering**, which can occur in relatively thick specimens, can be a serious source of chromatic aberration in the image.

Inelastic scattering of the beam electrons by electrostatic repulsion from the atomic electrons leads to an angular deflection, θ_e, of the electrons according to the following expression:

$$\theta_e = \frac{e}{Vr_e}$$

where r_e = distance of the beam electron from the atomic electron.

Thus, the nucleus has a higher scattering power than the atomic electron by a factor of Z, due to the greater concentration of charge in the nucleus.

Since electrons approach the "stationary" atom from various directions (defined by the angular aperture of the beam), each atom behaves as a point source of scattered electrons. Thus, nuclei and individual electrons are comparable to "self luminous" points considered in light optics.

I.C.2. Amplitude/Phase Contrast

Contrast in the electron image can arise from both "amplitude" and "phase" effects. **Amplitude contrast** is produced by the loss of amplitude (i.e. electrons) from the beam; **Phase contrast** originates from shifts in the relative phases of the portions of the beam that contribute to the image. Unfortunately, the terms "amplitude" and "phase" contrast can be somewhat misleading, especially to those who are familiar with similar terms used in crystallography. More appropriate terms are **scattering contrast** and **interference contrast** instead of amplitude and phase contrast. However, to be consistent with most texts and articles dealing with electron microscopy, the terms "amplitude" and "phase" contrast will continued to be used in these notes. Please remember the distinction though, because this becomes important in discussing the fundamentals of image analysis (taught in BIO595W: Lecture Notes Sec. III).

The relative importance of various contrast mechanisms is quite different in light and electron microscopy. For the majority of specimens, **differential electron scattering,** an amplitude effect, is the primary source of electron contrast. In **light microscopy** the important mechanism is one of differential absorption (also an amplitude effect). Absorption by usefully thin electron microscope specimens is negligible, whereas scattering contrast is only occasionally of importance in light microscopy. As the limit of resolution in the TEM is approached, the importance of phase contrast effects increases. Electron phase contrast originates from two factors: 1) defocusing the image and 2) from interference between rays which are misfocused by spherically aberrant lenses. The second mechanism is of consequence only very close to the limit of resolution.

In general, amplitude contrast is dominant for structures with large mass thickness, while phase contrast increases in importance for small and thin structures and becomes the dominant source of contrast for very small objects of low atomic number.

a. Amplitude contrast

The angular distribution of scattered intensities varies as a function of the atomic composition and density of the object (Fig. I.105). **Electron opaque** object points produce appreciable scattering through relatively large angles. Thus, many of the electrons incident on such points are excluded from the lens aperture and the intensity of the images of these points is correspondingly low. Conversely, **electron transparent** regions in the object, which are of lower average atomic number and/or thickness (mass density) produces little scattering beyond the lens aperture. The intensity of images of these points is correspondingly high.

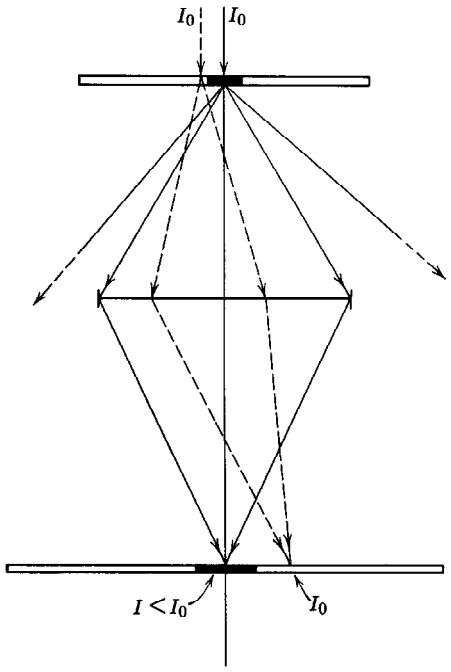

Fig. I.105. Scattering contrast in electron imaging (From Slayter, p.427)

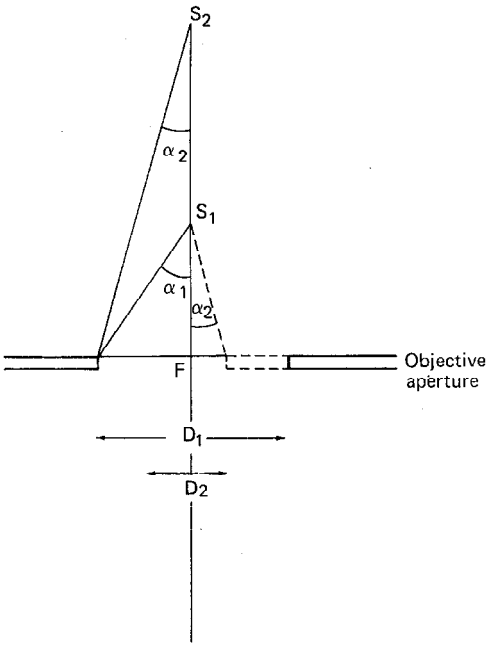

Fig. I.106. Diagram showing how the amplitude contrast is increased by reduction of the effective angular aperture of the objective lens as the specimen is moved away. The dotted rays on the right-hand side of the axis show how a similar objective angular aperture may be obtained by fitting a smaller objective aperture of diameter D_2. (From Agar, p.90)

Amplitude contrast can be controlled to some extent by i) **choice of accelerating voltage** and ii) **size of objective aperture** (Figs. I.87 and I.106). Contrast is improved at lower voltages and with smaller apertures. However, unless the specimen is very thin, the higher chromatic aberration at lower accelerating voltages may lead to unacceptable loss of resolution. Gun brightness also decreases as the accelerating voltage is decreased.

Limiting the angular aperture of the objective lens can diminish the background intensity produced by spherical aberration. This is done with the **objective aperture** placed at the back focal plane of the objective lens (Fig. I.88). The aperture works to reduce the background intensity due to spherical aberration and thus increases the contrast of image points. As the size of the objective aperture is reduced, more scattered electrons are stopped and contrast improves. If the aperture size is made too small (<20 μm) there will be a loss of resolution due to diffraction effects (this may be useful though at low magnifications where resolution is not as important a factor). A practical problem with small apertures is that they are more difficult to align in the back focal plane of the objective lens and are more susceptible to the effects of contamination, which produces lens asymmetry and thereby reduces resolution. These problems can be partially overcome with the use of a high contrast specimen holder that allows the objective lens to be operated at a longer focal length, thus reducing the semi-angular aperture (Fig. I.106). However, these changes may lead to some loss of resolution due to increased aberration effects.

b. Phase contrast

Electron scattering can be described as the diffraction of electron waves. If the interference of diffracted waves produces differences in intensity at the image, phase contrast is generated. Electrons scattered through large angles may fall outside the lens aperture, giving rise to amplitude contrast. Ideally, the lens focuses all other scattered electrons at the corresponding image points, where they arrive **in phase**. At planes above or below the ideal image plane, interference produces differences in intensity. These differences consist of the focus (Fresnel) fringes. Because of lens aberrations, a slight phase contrast **PERSISTS AT EXACT FOCUS**. At other focal settings, phase contrast is produced by a combination of defocusing and lens aberration.

Phase contrast in images arises from differences in phase between scattered and unscattered rays in different parts of the image and interference between these rays. In a fully transparent (*i.e.* no variation in refractive index) object, there are no phase differences and, hence no phase contrast in the image. **Defocusing**, in which path lengths for scattered rays are changed more than for the unscattered rays, can be used to enhance phase contrast. Contrast due to phase differences is more important for thin objects and when working near the resolution limit than contrast due to amplitude differences.

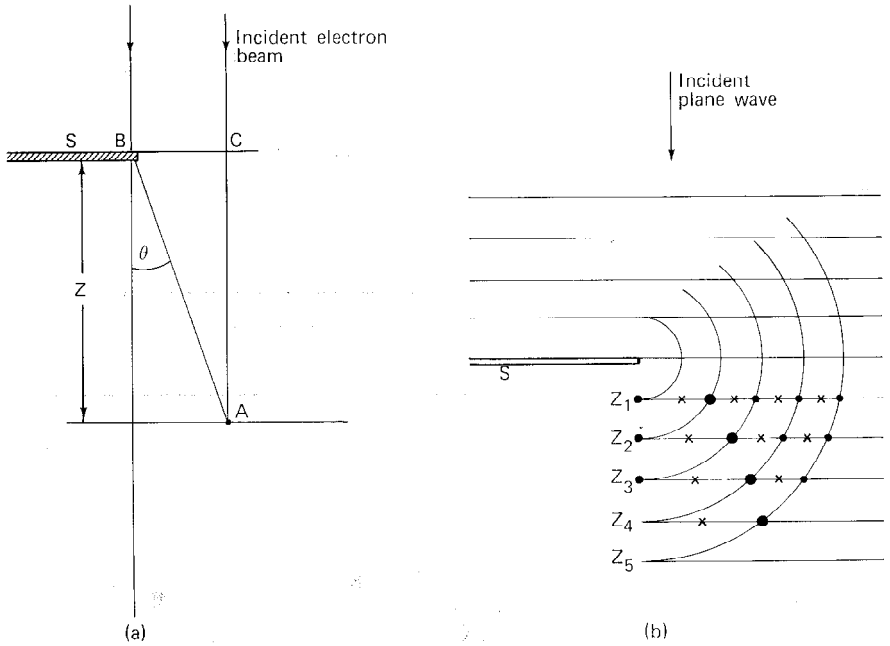

Fig. I.107. (a) A specimen S scatters the incident electron beam which interacts with the unscattered beam at a point A, distance Z behind the specimen. If this plane is viewed with the objective lens (*i.e.* the lens is overfocused) a Fresnel diffraction fringe will be observed. (b) A plane wave meets a specimen S and scattered spherical waves interfere with the plane waves. Maxima of intensity are denoted by dots, and minima by crosses. (From Agar, p.97)

Defocus or **phase contrast** results in the formation of strong Fresnel fringes about any part of the specimen where there is a rapid change in mass thickness (Fig. I.107). The fringes serve to enhance such points or edges by delineating them with a bright line in the underfocused position (objective lens too weak) or with a dark line in the overfocused position (objective lens too strong). The Fresnel fringes virtually disappear at the point of "exact" focus and image contrast due to phase effects is at a minimum. It is common practice with thin specimens studied at high resolution to defocus (underfocus) slightly to improve image contrast (Fig. I.108). At slight overfocus the image is generally confusing. Initially, at slight defocus, resolution is not significantly reduced, but further defocusing reduces resolution considerably. Critical focusing is a skill learned by experience. Excessive defocusing may lead to spurious details in the image that may be erroneously interpreted as real structural details in the specimen. The defocus effect described here is an interference phenomenon similar to the effect used in phase contrast light microscopy.

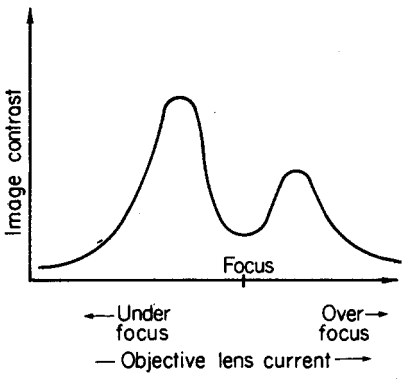

Fig. I.108. A curve showing the variation of image contrast with objective lens focus. Fresnel fringes produce a so-called 'defocus granularity' or spurious out-of-focus contrast which is maximal at a point just below focus, where detail is enhanced by the bright underfocus line. (From Meek 2nd ed., p.100)

I.C.3. Phase Contrast Transfer Function

The phase contrast phenomenon is responsible for the general granular background observed in pictures of supporting films. The coarseness of the granularity varies with the degree of defocusing and virtually disappears at the "true" (theoretically perfect) focus setting. This granularity must be considered when interpreting high resolution images of biological specimens since it can lead to erroneous conclusions regarding the fine structure in particles. The **contrast transfer function** (CTF) of the electron microscope (Fig. I.109) specifies the relative contrast of features in the image for all spatial frequency ranges and depends both on the spherical aberration and the level of focus of the objective lens. Note that, for typical biological specimens, "optimum focus" is actually at a point where the objective lens is slightly under-focused (*i.e.* longer focal length than required for an "ideal" system).

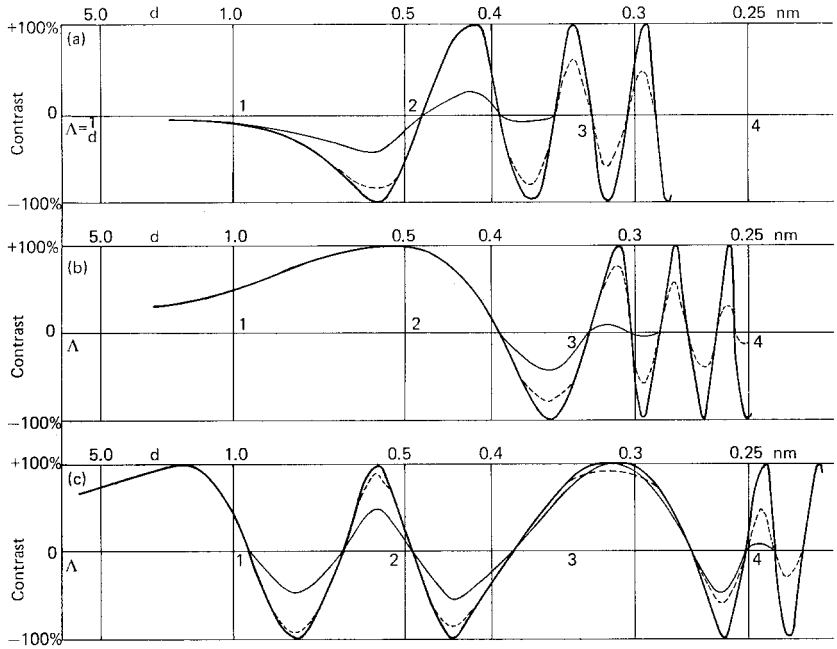

Fig. I.109. Plot of phase contrast as a function of structure size. (a) Objective lens in focus. (b) Objective lens 78 nm underfocus. (c) Objective lens 234 nm underfocus. (From Agar, p.282)

I.C.4. Multiple Scattering in Thick Specimens

In thick specimens (>>100 nm), each beam electron on average suffers a number of encounters with atoms of the specimen and, hence, multiple energy losses. The energy spread in the beam emerging from the specimen may be as high as 100 eV or more and there may be relatively few unscattered electrons. Under these conditions there is no possibility of phase contrast from the specimen since there are no longer one or two well-defined electron wavelengths in the beam suitable for coherent interference. Thick specimens, therefore, mainly exhibit amplitude contrast.

Because of the energy losses, the **chromatic aberration** of the objective lens will produce a limit to the resolution of the image of:

$$d_{cv} = C_c \cdot \alpha_o \cdot \Delta V / V \quad \text{(see also section I.B.3.d)}$$

where d_{cv} = separation of two object points which are just resolved, considering voltage
C_c = chromatic aberration coefficient of the objective lens (usually 1-3 mm)
α_o = objective semi-angular aperture angle
V = accelerating potential
ΔV = maximum departure from V of electrons contributing to the image

Thus, for C_c = 2 mm, α = 5 x 10^{-3} radians, ΔV = 100 volts and V = 100,000 volts:

$$d_{cv} = (2 \times 10^6 \text{ nm})(5 \times 10^{-3})(10^2 \text{ volts})/(10^5 \text{ volts}) = 10 \text{ nm !}$$

Since instabilities in the electrical supply are only on the order of 1-2 eV, the specimen itself becomes the main limitation to the resolution obtainable in the image.

I.C.5. Other Methods for Enhancing Contrast

Inherent contrast in biological specimens is very weak because such specimens contain atoms of low average atomic number, therefore contrast is generally enhanced by i) directly increasing the specimen contrast using various preparation procedures (*i.e.* staining, shadowing, etc.) or ii) using longer exposure times in recording the photographic image. These methods will be discussed in greater detail later (Section II of the Lecture Notes).