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19.6. Electron cryomicroscopy

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19.6.1. Abbreviations used

0D	Zero-dimensional (single particles)
1D	One-dimensional (helical)
2D	Two-dimensional
3D	Three-dimensional
EM	Electron microscope/microscopy
Cryo EM	Electron cryomicroscopy
FEG	Field-emission gun
CTF	Contrast-transfer function
CCD	Charge-coupled device (slow-scan TV detector)

19.6.2. The role of electron microscopy in macromolecular structure determination

Diffraction and imaging techniques are one manifestation of the use of scattering of beams or wavefronts by objects to analyse the structure of that object (Fig. 19.6.2.1). Such methods can be used in general for the examination of structures of any size, ranging from elementary subnuclear particles all the way up to the structure of the earth's core. For macromolecular structure determination, there are two main differences between the use of electrons and X-rays to probe structure. The most important is that the scattering cross section is about 10^5 times greater for electrons than it is for X-rays, so significant scattering using electrons is obtained for crystals or other specimens that are 1 to 10 nm thick, whereas scattering or absorption of a similar fraction of an illuminating X-ray beam requires crystals that are 100 to 500 μm thick. The second main difference is that electrons are much more easily focused than X-rays since they are charged particles that can be deflected by electric or magnetic fields. As a result, electron lenses are much superior to X-ray lenses and can be used to produce a magnified image of an object as easily as a diffraction pattern. This then allows the electron microscope to be switched back and forth instantly between imaging and diffraction modes so that the image of a single molecule at any magnification can be obtained as conveniently as the electron diffraction pattern of a thin crystal. By contrast, X-ray microscopy has been much less valuable than X-ray diffraction, but may be useful for imaging at the cellular level.

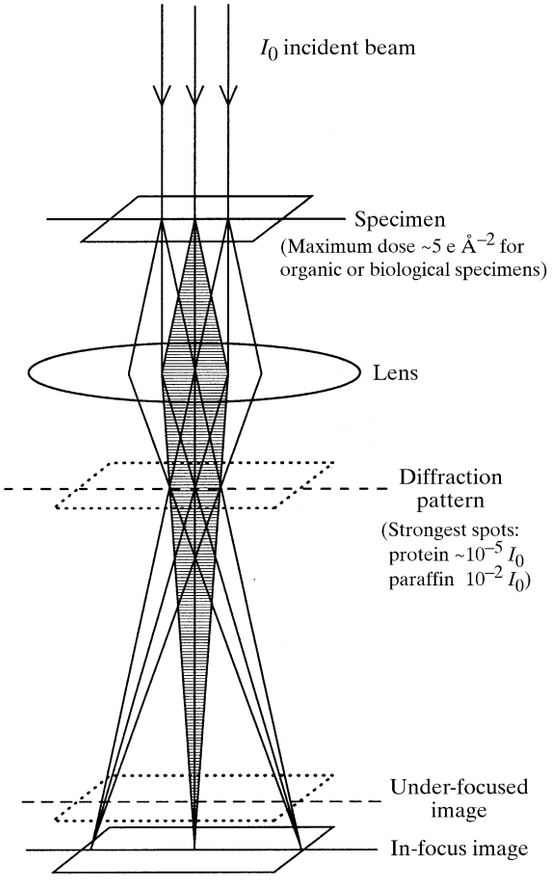

Fig. 19.6.2.1. Schematic diagram showing the principle of image formation and diffraction in the transmission electron microscope. The incident beam, I_0, illuminates the specimen. Scattered and unscattered electrons are collected by the objective lens and focused back to form first an electron-diffraction pattern and then an image. For a 2D or 3D crystal, the electron-diffraction pattern would show a lattice of spots, each of whose intensity is a small fraction of that of the incident beam. In practice, an in-focus image has virtually no contrast, so images are recorded with the objective lens slightly defocused to take advantage of the out-of-focus phase-contrast mechanism.

In the early years of electron microscopy of macromolecules, electron micrographs of molecules embedded in a thin film of heavy-atom stains (Brenner & Horne, 1959; Huxley & Zubay, 1960) were used to produce pictures which were interpreted directly. Beginning with the work of Klug (Klug & Berger, 1964), a more rigorous approach to image analysis led first to the interpretation of the two-dimensional (2D) images as the projected density summed along the direction of view and then to the ability to reconstruct the three-dimensional (3D) object from which the images arose (DeRosier & Klug, 1968; Hoppe *et al.*, 1968), with subsequent more sophisticated treatment of image contrast transfer (Erickson & Klug, 1971).

Later, macromolecules were examined by electron diffraction and imaging without the use of heavy-atom stains by embedding the specimens in either a thin film of glucose (Unwin & Henderson, 1975) or in a thin film of rapidly (Dubochet, Lepault *et al.*, 1982; Dubochet *et al.*, 1988) or slowly (Taylor & Glaeser, 1974) frozen water, which required the specimen to be cooled while it was examined in the electron microscope. This use of unstained specimens thus led to the structure determination of the molecules themselves, rather than the structure of a 'negative stain' excluding volume, and has created the burgeoning field of 3D electron microscopy of macromolecules. Many of the image-analysis techniques now used for studying unstained specimens originated from those used in the analysis of negatively stained samples.

At this point in year 2000, hundreds of medium-resolution structures of macromolecular assemblies (*e.g.* ribosomes), spherical and helical viruses, and larger protein molecules have been determined by electron cryomicroscopy in ice. Three atomic resolution structures have been obtained by electron cryomicroscopy of thin 2D crystals embedded in glucose, trehalose or tannic acid (Henderson *et al.*, 1990; Kühlbrandt *et al.*, 1994; Nogales *et al.*, 1998), where specimen cooling reduced the effect of radiation damage. The medium-resolution density distributions can often be interpreted in terms of the chemistry of the structure if a high-resolution model of one or more of the component pieces has already been obtained by X-ray, electron microscopy, or NMR methods. As a result, electron microscopy is being transformed from a niche methodology into a powerful technique for which, in some cases, no alternative approach is possible. This article outlines the key aspects of electron cryomicroscopy (cryo EM) and 3D image reconstruction. Further information can be obtained from several reviews (*e.g.* Amos *et al.*, 1982; Glaeser, 1985; Chiu, 1986;

Dubochet *et al.*, 1988; Stewart, 1990; Koster *et al.*, 1997; Walz & Grigorieff, 1998; Baker *et al.*, 1999; Yeager *et al.*, 1999) and a book (Frank, 1996). Recommended textbooks that describe general aspects of electron microscopy are those by Cowley (1975), Spence (1988) and Reimer (1989).

19.6.3. Electron scattering and radiation damage

A schematic overview of scattering and imaging in the electron microscope is depicted in Fig. 19.6.2.1. For biological electron microscopy and diffraction, the incident beam is normally parallel and monochromatic. The incident electron beam then passes through the specimen and individual electrons are either unscattered or scattered by the atoms of the specimen. This scattering occurs either elastically, with no loss of energy and therefore no energy deposition in the specimen, or inelastically, with consequent energy loss by the scattered electron and accompanying energy deposition in the specimen, resulting in radiation damage. The electrons emerging from the specimen are then collected by the imaging optics, shown here for simplicity as a single lens, but in practice consisting of a complex system of five or six lenses with intermediate images being produced at successively higher magnification at different positions down the column. Finally, in the viewing area, either the electron-diffraction pattern or the image can be seen directly by eye on the phosphor screen, or detected by a TV or CCD camera, or recorded on photographic film or an image plate.

19.6.3.1. *Elastic and inelastic scattering*

The coherent, elastically scattered electrons contain all the high-resolution information describing the structure of the specimen. The amplitudes and phases of the scattered electron beams are directly related to the amplitudes and phases of the Fourier components of the atomic distribution in the specimen. When the scattered beams are recombined with the unscattered beam in the image, they create an interference pattern (the image) which, for thin specimens, is related approximately linearly to the density variations in the specimen. The information about the structure of the specimen can then be retrieved by digitization and computer-based image processing, as described below (Sections 19.6.4.5 and 19.6.4.6). The elastic scattering cross sections for electrons are not as simply related to the atomic composition as happens with X-rays. With X-ray diffraction, the scattering factors are simply proportional to the number of electrons in each atom, normally equal to the atomic number. Since elastically scattered electrons are in effect diffracted by the electrical potential inside atoms, the scattering factor for electrons depends not only on the nuclear charge but also on the size of the surrounding electron cloud which screens the nuclear charge. As a result, electron scattering factors in the resolution range of interest in macromolecular structure determination (up to $\frac{1}{3}$ Å^{-1}) are very sensitive to the effective radius of the outer valency electrons and therefore depend sensitively on the chemistry of bonding. Although this is a fascinating field in itself with interesting work already carried out by the gas-phase electron-diffraction community (*e.g.* Hargittai & Hargittai, 1988), it is still an area where much work remains to be done. At present, it is probably adequate to think of the density obtained in macromolecular structure analysis by electron microscopy as roughly equivalent to the electron density obtained by X-ray diffraction but with the contribution from hydrogen atoms being somewhat greater relative to carbon, nitrogen and oxygen.

Those electrons which are inelastically scattered lose energy to the specimen by a number of mechanisms. The energy-loss spectrum for a typical biological specimen is dominated by the large cross section for plasmon scattering in the energy range 20–30 eV with a continuum in the distribution which decreases up to higher energies. At discrete high energies, specific inner electrons in the *K* shell of carbon, nitrogen or oxygen can be ejected with corresponding peaks in the energy-loss spectrum appearing at 200–400 eV. Any of these inelastic interactions produces an uncertainty in the position of the scattered electron (by Heisenberg's uncertainty principle) and as a result, the resolution of any information present in the energy-loss electron signal extends only to low resolutions of around 15 Å (Isaacson *et al.*, 1974). Consequently, the inelastically scattered electrons are generally considered to contribute little except noise to the images.

19.6.3.2. *Radiation damage*

The most important consequence of inelastic scattering is the deposition of energy into the specimen. This is initially transferred to secondary electrons which have an average energy (20 eV) that is five or ten times greater than the valency bond energies. These secondary electrons interact with other components of the specimen and produce numerous reactive chemical species, including free radicals. In ice-embedded samples, these would be predominantly highly reactive hydroxyl free radicals that arise from the frozen water molecules. In turn, these react with the embedded macromolecules and create a great variety of radiation products such as modified side chains, cleaved polypeptide backbones and a host of molecular fragments. From radiation-chemistry studies, it is known that thiol or disulfide groups react more quickly than aliphatic groups and that aromatic groups, including nucleic acid bases, are the most resistant. Nevertheless, the end effect of the inelastic scattering is the degradation of the specimen to produce a cascade of heterogeneous products, some of which resemble the starting structure more closely than others. Some of the secondary electrons also escape from the surface of the specimen, causing it to charge up during the exposure. As a rough rule, for 100 kV electrons the dose that can be used to produce an image in which the starting structure at high resolution is still recognizable is about 1 e Å^{-2} for organic or biological materials at room temperature, 5 e Å^{-2} for a specimen near liquid-nitrogen temperature (−170 °C) and 10 e Å^{-2} for a specimen near liquid-helium temperature (4–8 K). However, individual experimenters will often exceed these doses if they wish to enhance the low-resolution information in the images, which is less sensitive to radiation damage. The effects of radiation damage due to electron irradiation are essentially identical to those from X-ray or neutron irradiation for biological macromolecules except for the amount of energy deposition per useful coherent elastically scattered event (Henderson, 1995). For electrons scattered by biological structures at all electron energies of interest, the number of inelastic events exceeds the number of elastic events by a factor of three to four, so that 60 to 80 eV of energy is deposited for each elastically scattered electron. This limits the amount of information in an image of a single biological macromolecule. Consequently, the 3D atomic structure cannot be determined from a single molecule but requires the averaging of the information from at least 10 000 molecules in theory, and even more in practice (Henderson, 1995). Crystals used for X-ray or neutron diffraction contain many orders of magnitude more molecules.

It is possible to collect both the elastically and the inelastically scattered electrons simultaneously with an energy analyser and, if a fine electron beam is scanned over the specimen, then a scanning transmission electron micrograph displaying different properties of the specimen can be obtained. Alternatively, conventional transmission electron microscopes to which an energy filter has been added can be used to select out a certain energy band of the electrons from the image. Both these types of microscope can contribute in other ways to the knowledge of structure, but in this article, we concentrate on high-voltage phase-contrast electron microscopy

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