Electron microscopy

Introduction

In the first lesson on light microscopy, we have learned that the resolution is limited by the wavelength. This is because due to the wave-like nature of light, it is diffracted by obstacles and objects that are too close together can no longer be distinguished as two separate objects. Now, the logical question is: If the resolution is limited by the wavelength, why do we not use light of a smaller wavelength, e.g., X-rays, in order to resolve smaller objects? The answer is that there are no lenses to bend light of lower wavelengths[1].

Consequently, in order to extend the range of resolution, we are looking for a physical entity that has a smaller wavelength than visible light, but whose beam path can be manipulated by lenses. This is where electrons come into play: Although we are used to thinking of electrons as particles they also have properties of a wave. For example, just as for light, their propagation through space is best described to behave like a wave[2]. In fact, it is possible to produce electrons that are liberated from atomic nuclei with a wavelength below 1 nm, allowing visualizing structures at atomic resolution. This is the basic principle of electron microscopy, where a beam of electrons is used as source of radiation instead of light. This, however, comes at the cost of extensive and demanding sample preparation – especially for samples in aqueous solution, which is true for virtually all biological samples. Another major difference between light microscopy and electron microscopy is that the electron beam is not focused using glass lenses, but by electromagnets (remember, electrons carry charge and can be manipulated in an electromagnetic field).

In light microscopy, we can obtain information about the specimen from the transmission and diffraction pattern of the light. Similarly, the information in electron microscopy is contained in the transmission and scattering pattern of the electron beam. The electrons can, e.g., interact with the nuclei of the atoms in the sample and be scattered by their electromagnetic field; or they can interact with orbiting electrons of sample atoms in a variety of ways, giving up some of their energy in the process. Each type of interaction can serve as a signal that carries information about the sample.

Different types of electron microscopy

Electron microscopy comes in mainly two flavors[3] transmission electron microscopy and scanning electron microscopy. They produce vastly different kinds of images and have consequently distinct applications. Both techniques will be introduced in this lesson.

Transmission electron microscopy (TEM) uses high-energy electrons to produce 2D projections of thinly sliced samples. It can achieve extremely high resolutions of up to 0.05 nm. As a typical C-C bond is 1.4 Å (0.14 nm) long, TEM can be used to look at samples on atomic level. However, radiation damage arising in biological samples from exposure to the electron beam limit the resolution to $\sim$ 1 nm – which is still excellent when compared to light microscopy. Figure 2-1 below shows a TEM image of a macrophage.

[1] This is because the refractive index of materials is dependent on the wavelength, and for X-rays, the refractive index of most materials is close to $n = 1$ and very little refraction occurs when X-rays pass from one material into the next. If we want to use light with lower wavelengths than visible light, e.g., X-rays, we have to use other methods to read the information contained in the diffraction pattern (as done in X-ray crystallography).

[2] Note that, since, unlike photons, electrons have a mass, there is a different correlation between speed, energy and wavelength: Electrons do not and cannot travel at the speed of light; as the velocity of an electron increases, its wavelength decreases and its energy increases.

[3] There are a few other types of electron microscopy, such as scanning transmission electron microscopy or reflection electron microscopy, but here, we will focus on TEM and SEM.

Scanning electron microscopy (SEM) uses a focused beam of low-energy electrons to scan the surface of a bulky object. It provides an extremely high depth of field, but is limited to show the specimen's surface topography and has typically a lower resolution as TEM – often around 10 nm. Thus, SEM is typically used to image entire cells or small organisms rather than subcellular structures. An example of an SE micrograph of a macrophage is shown in figure 2-2.

Figure 2-1 Transmission electron micrograph of a macrophage, a type of large white blood cell that recognizes foreign particles and engulfs and digests them. (From sciencesource.com)

Introduction to transmission electron microscopy (TEM)

TEM uses a high-energy electron beam to measure the density of a sample. This is done in a process called amplitude contrast[4] and works similar to X-ray imaging of doctors: TEM measures the share of the electron beam that moves through the sample unscattered and thus produces a negative image of the density of the sample.

To achieve this, TEM microscopes are designed similarly to light microscopes – but flipped by 180°. On top, there is an electron-emitting cathode (electron gun); below, a series of electromagnetic coils are used as lenses that focus the beam on the specimen. Unlike in light microscopy, the specimen has to be placed in a vacuum to avoid that any electrons from the air scatter the electron beam of the microscope. The basic layout is depicted in figure 2-3.

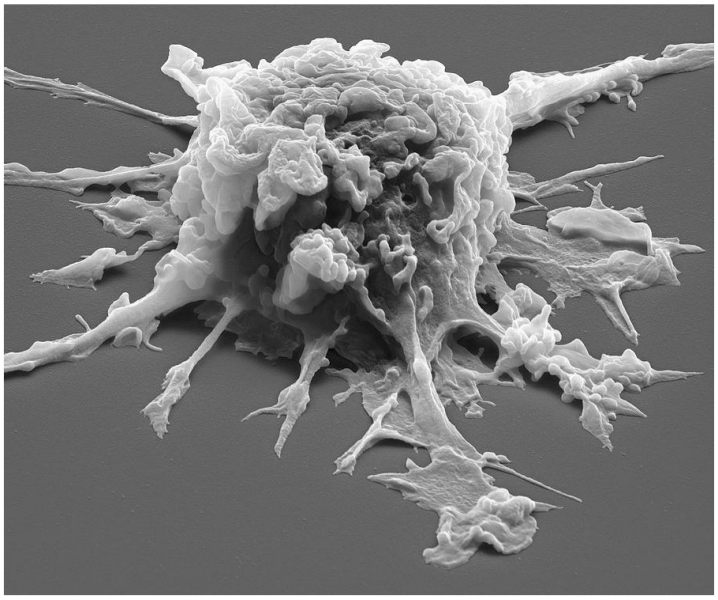

Figure 2-2 Scanning electron micrograph of a macrophage.

[4] To increase resolution, TEM uses also phase contrast which interferes with information of scattered electrons. However, the basis for phase contrast - the relativistic view on electrons - goes beyond the scope of this introduction.

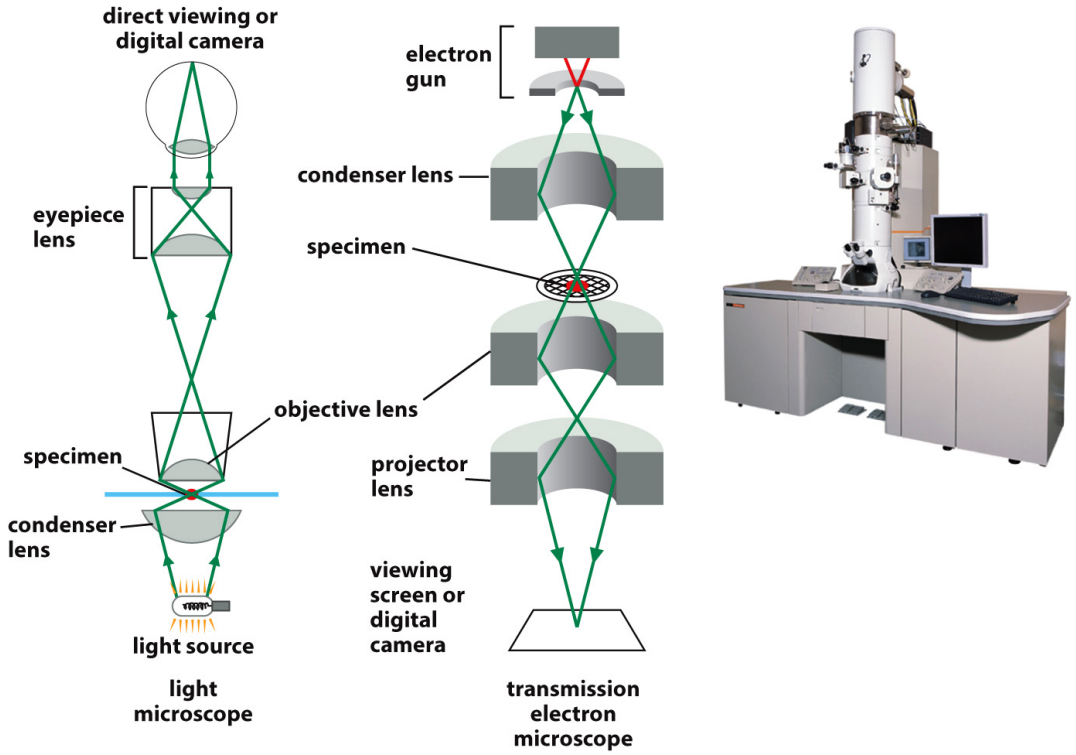

Figure 2-3 The principal features of a light microscope and a transmission electron microscope. These drawings emphasize the similarities of overall design. Whereas the lenses in the light microscope are made of glass, those in the electron microscope are magnetic coils. The electron microscope requires that the specimen be placed in a vacuum. The inset shows a transmission electron microscope in use. (From Molecular Biology of the Cell, Alberts, 6th edition, Garland Science)

Introduction to scanning electron microscopy (SEM)

SEM uses a focused low-energy electron beam to raster around a bulky object. Whereas TEM uses the electrons that pass through the specimen to render an image, SEM uses the electrons that scatter from the specimen's surface to create a picture. As the scattering depends on the angle between the beam and the surface, SEM allows a detailed reconstruction of the surface of an object. Figure 2-4 shows an illustration of the scanning electron microscope. The basic layout resembles the one of a transmission electron microscope, but the scanning electron microscopes is generally a bit smaller and cheaper. Note that in SEM, the detector is arranged to measure scattering electrons, whereas in TEM the detector is arranged to catch the electrons that pass through the specimen. SEM results in pictures with a great depth of field, but is limited to the analysis of surfaces and has a lower resolution than TEM.

SEM to study subcellular structures

The comparatively low resolution of SEM typically means that SEM is used to study larger structures such as cells or even entire organisms. Recent developments in the electron source allow SEM with high resolution and thus the analysis of small subcellular structures. Figure 2-5 shows high resolution SEM images of both sides of a rapidly frozen nuclear pore complex. The resolution is good enough that molecular details such as the basket structure can be visualized.

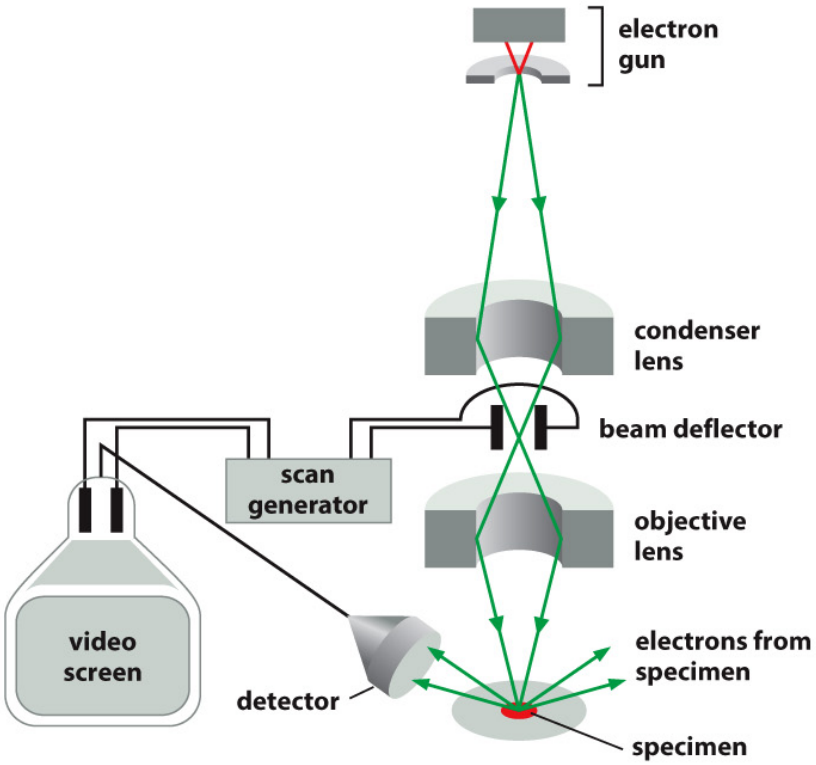

Figure 2-4 The scanning electron microscope. In an SEM, the specimen is scanned by a beam of electrons brought to a focus on the specimen by the electromagnetic coils that act as lenses. The detector measures the quantity of electrons scattered or emitted as the beam bombards each successive point on the surface of the specimen and controls the intensity of successice points in an image built up on a screen. The SEM creates striking images of three-dimensional objects with great depth of focus and a resolution between 3nm and 20nm depending on the instrument. (From Molecular Biology of the Cell, Alberts, 6th edition, Garland Science)

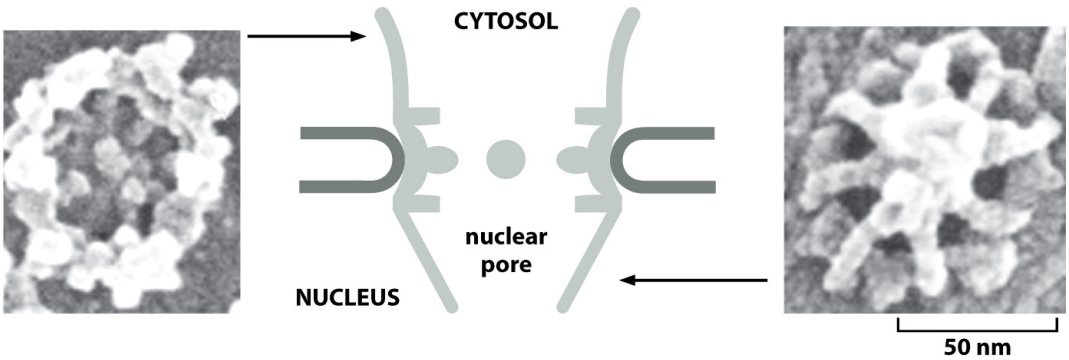

Figure 2-5 The nuclear pore. Rapidly frozen nuclear envelopes were imaged in a high-resolution SEM, equipped with a field emission gun as the electron source. These views of each side of a nuclear pore represent the limit of resolution of the SEM. (From Molecular Biology of the Cell, Alberts, 6th edition, Garland Science)

Sample preparation in electron microscopy

The combination of electron microscopy and biological samples confronts researches with three key challenges:

- Biological samples are typically in aqueous solution – which prevents direct exposure to the vacuum
- Biological samples have low intrinsic contrast
- Biological samples are sensitive to the radiation of the electron beam

There are two basic approaches to deal with these challenges: Negative staining and cryo-electron microscopy (cryo-EM).

In *negative staining*, the sample is embedded in low concentrations of salts of heavy atoms, such as uranium. This solution is then dried out to "cast" a negative of the sample. This negative can then be analyzed in the electron microscope. As the sample is dried, there is no problem with water in the vacuum. The salt provides high contrast and is relatively stable to radiation. The downside of this method is, that the resolution is limited to the grains of the salt and that there are frequent artefacts due to collapsing structures as the sample dries.

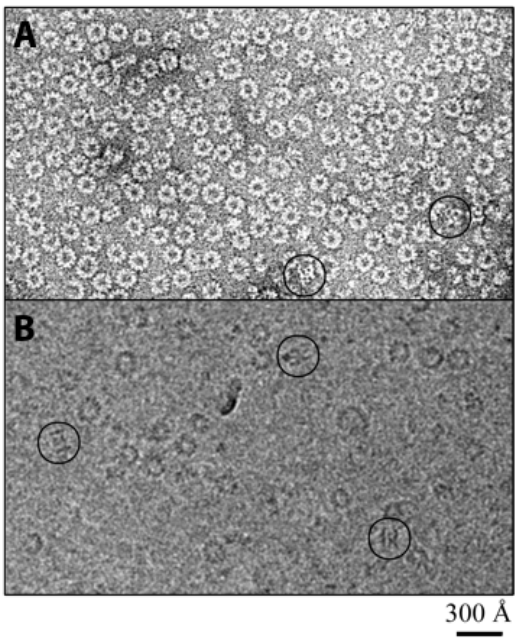

Figure 2-6 Comparison of negative staining (A) and cryo-EM (B) images of recombinant rabies virus RNA rings. Negative staining increases the contrast of the sample. (From Schoen *et al.*, *J Virol*, 2001)

In *cryo-EM*, the sample is quickly frozen to very low temperatures (cryogenic temperatures). This results in supercooled water, which is rigid but not organized as crystals. Basically, the sample is hydrated in a solid state. These samples can be analyzed without vaporization of the water. Typically, there are no artefacts and analysis at very high resolutions is possible. However, Cryo-EM results in lower contrast than negative staining and is more sensitive to radiation. As a consequence, the samples have to be treated with less electrons which results in a worse signal-to-noise ratio. Therefore, in order to obtain enough information about an object to define an image at atomic resolution, tens of thousands of images from different molecules (of the same type, of course) have to be combined.

Figure 2-6 shows a negative-staining (top) and a cryo-EM image (bottom) of a nucleoprotein-RNA complex from a rabies virus (*dt.* Tollwutvirus). Notice the striking differences in contrast.