7
Electron Microscopy (TEM and SEM)

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7.1 Basic 'how-to-do' and 'why-do' section

7.1.1 Electron microscopy (EM)

The purpose of any microscope is to enable the observer to resolve as separate objects things that are beyond the scale of the human eye. In any microscope, the point-to-point resolution (d) is dependent on the wavelength of the illuminating source following Abbe's equation: $d = \lambda/2NA$, where NA is the Numerical Aperture, a measure of the quality of the objective – usually around 1 or slightly higher for light microscope objectives. So, in a light microscope using laser light with a wavelength (λ) of 520 nm (green) the resolution will be around $d = 200$ nm. No improvement in optical design can reduce d below this. Nowadays, however, specialized super resolution light microscopes can obtain fluorescence information below 200 nm by digital image deconvolution and reconstruction.

The electron microscope overcomes the inherent resolution limit of the light microscope by using the wavelength of electrons that are of a completely different (sub-nanometre) order. Another advantage of electron microscopy (EM) is that it will provide information on all structures present, rather than just on the location of fluorescent proteins. A disadvantage of EM, however, is that only still images can be captured, because samples have to be imaged in a vacuum. Therefore it is sometimes worthwhile to combine both LM and EM on the same sample, in one so-called Correlative Light Electron Microscopy experiment. As in the light microscope, electron microscopy can study objects inside the sample using Transmission

Essential Guide to Reading Biomedical Papers: Recognising and Interpreting Best Practice, First Edition. Edited by Phil Langton.

Electron Microscopy (TEM) or can look at the outer surface of a sample using Scanning Electron Microscopy (SEM), analogous to a stereo light microscope.

7.1.2 Transmission electron microscopy (TEM)

The greater resolving power of electron microscopes derives from the wave properties of electrons. Unlike light waves, the wavelength of an electron varies with its speed, which in turn depends on the accelerating voltage. Standard TEMs operate in the range 10,000–120,000 V. At 60 kV, the wavelength of an electron is $\approx$0.05 Å (1 nm = 10 Å), but the full theoretical resolution is not realized, owing to the aberrations of electron-focusing lenses. The practical limit of resolution in the TEM is 3–5Å or 10–15Å when observing cellular structures.

Electron microscopes consist of a vacuum column through which the electrons travel. A cathode (like the metal wire in a light bulb) will be heated and provides the source of electrons. Focusing of the beam is by electromagnetic lenses. By altering the lens currents, the magnification can be varied between about 1,000–250,000 times. Electrons are brought to focus on a phosphorescent screen (which has crystals that emit visible light). Image formation depends on differential scattering of electrons within the specimen, an effect that is proportional to the sizes of atomic nuclei in the specimen (Figure 7.1).

Biological material mainly consists of atoms of low atomic number. Therefore, to increase scattering – and thus image contrast – biological material is usually treated with solutions of heavy metals during the preparation stages (Figure 7.2). These heavy metal stains specifically bind to cellular structures like membranes or DNA.

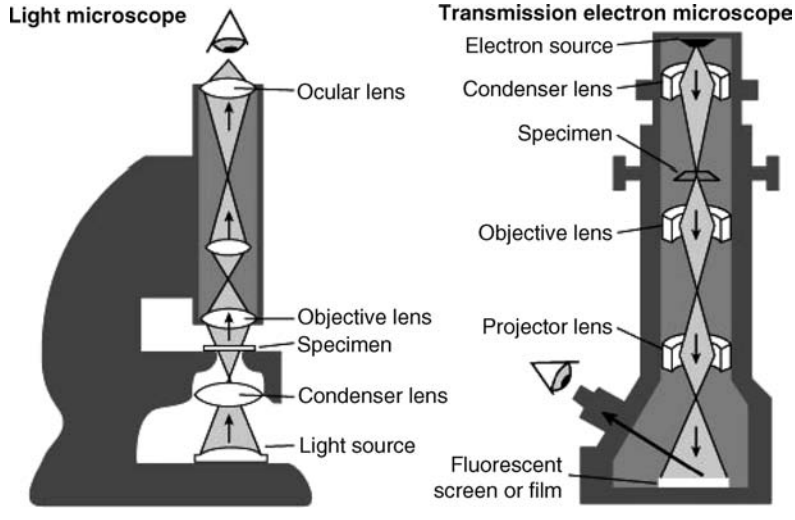

Figure 7.1 Diagrammatic representation of both the compound light (left) and Transmission Electron (right) microscopes. Blackwell Microbiology Teaching Resources (comparison of electron and light microscopes) © John Wiley & Sons Ltd.

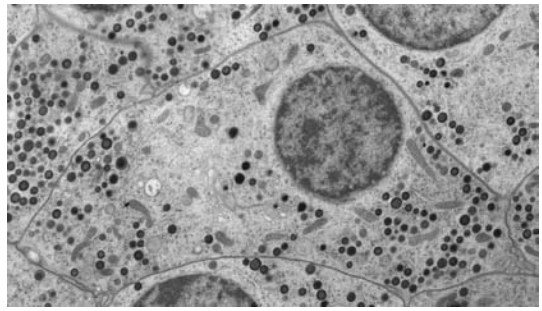

Figure 7.2 Example of a TEM image where a thin section of a piece of embedded tissue was made and imaged in the Transmission Electron Microscope. In this case, it is an insulin-producing Beta cell inside the Islet of Langerhans. It shows the nucleus as a dark grey circle and the plasma membrane is outlined by the dark lines. Inside the cytoplasm, darkly stained insulin granules can be seen. Courtesy of Dr. Paul Verkade.

The stains may, however, also limit the resolution in the EM. To record images, the phosphorescent screen is removed and a film – or more likely nowadays, a CCD – camera is brought into place.

Preparative methods for TEM

1. Most commonly, in medicine and biology, thin sections of tissue or cultured cells are cut (see Figure 7.2). The limited penetrating power of the electron beam means that ultra-thin sections ($\approx$70 nm) are necessary. Preparation of these ultra-thin sections is as follows: Fixation (often by perfusion) is the first essential step, as samples will be introduced into the vacuum. This is followed by treatment with osmium tetroxide (the heavy metal contrasting agent), then by embedding in epoxy resin (a plastic), and finally sectioned on a glass or diamond knife. Sections are collected on metal mesh 'grids' and then contrasted with uranium and lead salts before they can be analyzed in the TEM.
2. To localize proteins inside cells using electron microscopy, the thin section technique can be combined with immunocytochemical staining methods, where antibodies (see Primers 12 and 13) are used to recognize specific proteins. The main requirement is for an electron-dense marker to be attached to the reaction product. Thus, gold beads are conjugated to the secondary antibody.
3. A different technique, negative staining, is suitable for examining very small particles such as protein complexes, viruses or isolated cell organelles, etc. The mesh grid is first coated with a support film (carbon or plastic). The particles of interest are attached to the support film, after which a heavy metal stain (e.g. uranyl acetate) is applied and subsequently washed off. As it dries, it leaves an electron-dense deposit over the whole surface except where the particles lie. The specimens appear bright on the screen, with their edges and surface features defined by the (very finely granular) metal salt.

4. The above techniques all use heavy metals to stain structures in the EM so we do not actually visualize the structures themselves but rather look at a 'shadow' of them. In *cryo-TEM*, we leave out the stain and image the sample directly. One requirement for this is a different means of stabilization of the sample – cryo-fixation. This can be done via plunge freezing into liquid propane. The frozen samples can then be imaged directly in an EM, where the sample is kept well frozen while imaging. This technique is able to give us the highest resolution in EM.

5. If very high resolution imaging of thicker samples, such as cells and tissue, is required, the samples will need to be cryo-fixed via high pressure freezing. The thin section technique can be also performed on frozen specimens (without fixation and embedding). Prior protection against freezing damage is usual. This method avoids denaturation of proteins, etc. It is technically very difficult to achieve.

7.1.3 Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM), is very different from TEM in both construction and operation. A SEM utilizes electrons that have 'bounced off' the surface of the specimen. It is used primarily to examine the surfaces of objects, such as tissue microarchitecture or very small animals (see Figure 7.3).

The easiest specimens to examine are hard and dry; otherwise, the natural topography must be preserved, though devoid of fluid. To avoid the surface tension damage that accompanies air drying, a technique known as 'critical point drying' is used, involving the vaporization of liquid carbon dioxide under pressure. Once dried, specimens are coated with a thin layer of gold or gold-palladium and placed in the microscope.

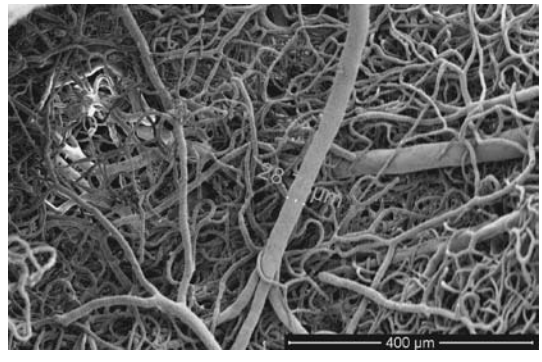

Figure 7.3 Example of a SEM image. A cast of the blood vessels in a rat brain was made using resins purchased from VasQtec, gold sputtered and imaged inside a scanning electron microscope. The image provides a 3-dimensional overview of the blood vessels (the diameter of an arteriole is shown: ≈29 microns) and shows both complexity and density of the vasculature. Courtesy of Dr. Phil Langton.

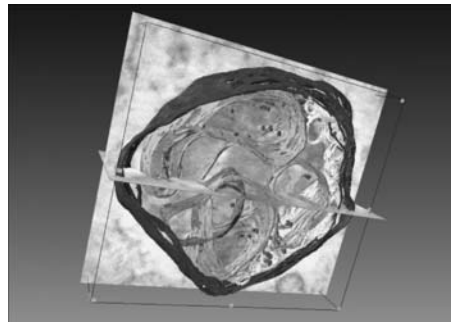

Figure 7.4 Example of a 3-D reconstruction and model of an endosome. After reconstruction of the volume, a specific slice can be analyzed. Specific features can be drawn into the reconstruction. Doing this on subsequent slices enables a 3-D model to be built. Courtesy of Dr. Paul Verkade. *A full colour version of this figure appears in the colour plate section.*

In the SEM, the electrons are accelerated as a fine beam that scans the specimen. The image is formed by back-scattered and/or secondary electrons that strike a detector near the specimen. The result is an image on the screen that corresponds to the surface features of the specimen. SEMs provide a wide range of magnification (15–150,000 $\times$) with a resolution of <5 nm and great depth of focus (see Figure 7.3).

7.1.4 Three-dimensional EM

While the SEM already provides some inherent three-dimensional information, there have been developments that allow for the acquisition of (better) 3-D information for both SEM and TEM samples. For the SEM, there are applications that cut off slices from the sample inside the microscope. This can be done using a diamond knife or a Focused Ion Beam but, rather than imaging the slices, the new surface is analyzed. By performing this procedure over and over again, one can zoom through this stack of images and build a 3-D model. These capabilities are absent for the TEM so, in order to obtain 3-D information, a thicker slice (300 instead of 70 nm) is made. Once in the microscope, the sample is tilted and a sequence of tilted images is acquired observing the sample from a large degree of angles, analogous to an MRI machine. The tilt series can be converted into a 3-D volume, where individual planes can be analyzed and models can be made (Figure 7.4).

7.2 Common problems or errors in literature

7.2.1 How to read EM images

A difficulty with EM is whether the images selected and published are truly representative. Because of the very high magnification, the samples chosen for

study may not reflect the overall complexity of the material. Statistical methods for sampling are available, but not always practicable (for details see Mayhew & Lucocq, 2008). Also, one should realize that one or two micrographs selected for inclusion in a paper may represent a large investment of time and effort.

7.2.2 Pitfalls in execution or interpretation

Any microscopical technique based on tissue sections requires the scientist to infer three-dimensional form from two-dimensional projections. Otherwise, almost every step in electron microscopy carries the risk of various artefacts. Fortunately, these are mostly well recognized and do not mislead. Although EM observations appear to present a black and white interpretation of the data, it is important to note that (3-D) models derived from electron microscopy data are based on human interpretation of the images.

The main criticism about EM is usually that because of the fixation and processing procedures, artefacts are introduced. It is very important to note that it is because the higher resolution of the EM that these artefacts are visible. Artefacts within the LM are usually much worse, but are not recognized because they are below the resolution of the LM. There are definitely artefacts introduced by the EM procedure, and electron microscopists are generally very much aware of these artefacts; however, it takes a few years of experience to be able to recognize them and fully 'read' an EM image.

7.2.3 Before starting an EM experiment

If cells can be observed by light microscopy before and after fixation, so much the better. Any EM technique involving localization of specific proteins must have appropriate controls. Techniques that capture dynamic events should have inhibited controls.

Immuno-labelling experiments especially require a number of controls – for instance, which fixative to use, or the omission of primary antibodies (for details, see Primer 13).

7.3 Complementary and/or adjunct techniques

These are dealt with in the following primers of this book:

- Primer 8: Fluorescence microscopy
- Primer 16: GFP
- Primer 13: Immunohistochemistry

Further reading and resources (biological EM only)

Bozzola, J.J. (1999). *Electron Microscopy: Principles and Techniques for Biologists*. Jones & Bartlett.

Mayhew, T.M. & Lucocq, J.M. (2008). Developments in cell biology for quantitative immunoelectron microscopy based on thin sections: a review. *Histochemistry and Cell Biology* **130** (2): 299–313.

Maunsbach, A.B. & Afzelius, B. (1999). *Biomedical Electron Microscopy*. Academic Press. (a very advanced book about interpretation of micrographs).