Chapter 5

Introduction to Electron Microscopy

Ellen Rosenberg
and
Michael Weis

Biology Program
University of British Columbia
Vancouver, British Columbia V6T2B1

Ellen Rosenberg has been a permanent lecturer at the University of British Columbia since 1983. She is in charge of the Cell Biology labs at the second, third and fourth year levels. Michael Weis, who is in charge of the Biological Sciences E.M. facility at U.B.C., has cooperated in this program and generated the figures in this paper.

BACKGROUND

This is a lab-tutorial designed to introduce second-year cell biology students to the principles of image formation in electron microscopy. Students have already completed a unit on light microscopy that included a theoretical consideration of resolution and the factors governing the limit of resolution.

In both the light microscopy and the electron microscopy tutorials the emphasis is on the interpretation of micrographs. The fact that the image seen is a product of the image-forming system is stressed. Many different light microscope images of the same specimen are considered **...** phase contrast, Nomarski interference, fluorescent as well as bright field. In this tutorial, we will consider scanning electron microscopy as well as transmission electron microscopy. We will also consider the effects of different specimen preparation techniques **...** negative staining, metal shadowing, etc.

The goal is for the student to look at a micrograph and recognize what technique was used and what the advantages and limitations of that technique are. This critical ability will be valuable even in reading their textbook; definitely if they go further into the literature. For the future cell biologist, this is the beginning of a basic vocabulary of tools that will be available for their research.

The tutorial begins with the electron microscope section of the Nature of Things program "Microscope: Making it Big", which gives a historical perspective as well as images of specimen preparation. An introductory lecture follows which includes a consideration of why electrons are used, the basic principles of microscope construction, specimen preparation and a comparison of SEM, TEM and STEM.

The following pages are excerpted from the Biology 200 lab manual and form the core of material for this introductory talk.

INTRODUCTION

Why the Electron Microscope?

From last Unit it was shown that the resolution of a microscope depends on 2 factors: wavelength of the illumination source (λ) and the numerical aperture of the lens (N.A.):

$$\text{limit of resolution} = \frac{0.61\,\lambda}{\text{N.A.}}$$

The maximum value of N.A. for light microscope is approx. 1.4; it is obvious, therefore, that even the short blue light ($\lambda = 436$ nm) of the visible spectrum will yield a resolution of only 190 nm. The electron microscope, however, utilizes electrons for illumination. Electrons have the characteristics of both particles and waves. The wavelength of an electron beam is about 100,000 times less than that of visible light and hence the resolution of an electron microscope is far superior to that of the light microscope.

THE TRANSMISSION ELECTRON MICROSCOPE

Construction of the Microscope:

We store an old, non-functioning Hitachi **TEM** in our lab. It is used as a comparison with a light microscope. Similarities in the construction of the two microscopes are pointed out starting with the filament as a source of electrons or light, that is focussed onto the specimen by condenser lenses. The illumination penetrates the specimen and objective lenses magnify the image. Projector or ocular lenses produce the image on the fluorescent **screen** or in the eye. Each of the components is discussed in turn.

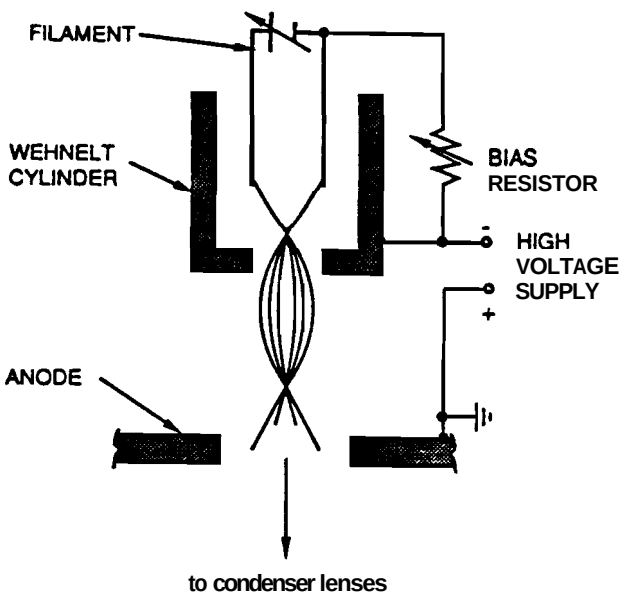
The Illumination Source

The Illumination Source or "Electron Gun": The Electron beam is generated by the electron gun located at the top portion of the microscope column. The gun consists of a V-shaped tungsten filament surrounded by a cathode shield with a circular hole in the center, During operation, a high voltage is applied between the filament (-) and the anode (+), while an electric current is regulated through the filament causing it to emit electrons. These electrons are attracted by the + anode but are forced through the hole of the cathode shield. The negative charge around the hole forces electrons from the filament into a very narrow beam.

The electron beam, as seen below, is accelerated through a potential difference of voltage between the filament and the anode. The greater the voltage (V), the higher is the speed of the electrons and the shorter the wavelength (λ), as shown in this formula:

$$\lambda = \sqrt{\frac{1.5}{V}} \text{ (nanometer)}$$

SELF-BIASED ELECTRON GUN



Wavelength of the Electron Beam

In a microscope using a routine voltage of 50 KV, the wavelength will be approximately 0.0054 nm. (1/100,000 wavelength of visible light). This value when applied to the resolution formula will yield a resolution limit about .84 nm or 8.4 Angstroms.

The Electron Lenses

The electron beam from the electron gun can be focussed and defocussed by a series of electro-magnetic lenses. Similar to the light microscope, the "Condenser Lenses" concentrate the beam onto the specimen. Electrons passing through the specimen will be focussed by the "Objective" & "Intermediate" lenses to form an intermediate image. The "Projector lens" enlarges this image into a final image on the fluorescent viewing screen at the bottom of the microscope column.

Each lens is basically a circular electro-magnet. A variable electric current through the lens will produce a magnetic field of variable strengths which will deflect or bend the electron beam passing through.

The Vacuum System

It is important to remember that the electron beam must be generated in and traverse through the microscope column under a high vacuum condition. The presence of air molecules will result in the collision and scattering of the electrons from their path. In the electron microscope the vacuum is maintained by a series of highly efficient vacuum pumps.

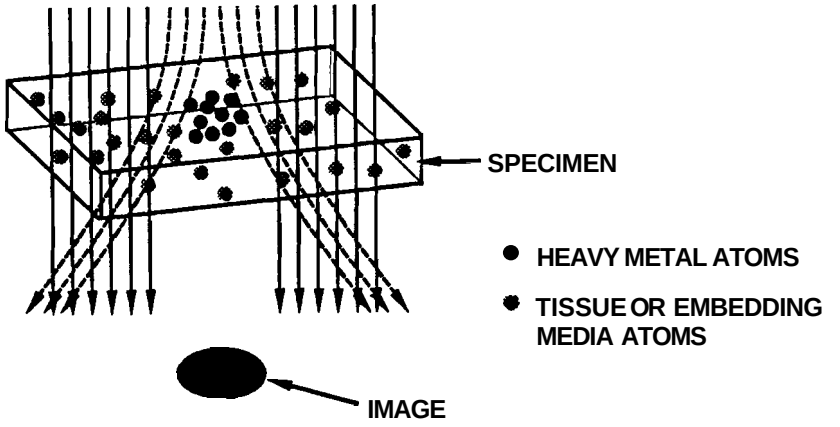
* THE VACUUM FACTOR: Biological material must be properly fixed and preserved.

Image Formation in the TEM

The basis of image formation in the TEM is the scattering of electrons caused by collisions between the beam electrons and the atoms of the specimen. The scattering results in a shadow on the viewing screen or photographic film.

Material with high atomic numbers (large number of electrons around the proton) will cause more scattering and produce a deep shadow. Such material is termed "electron dense" and has high image contrast. Biological material has low electron density and is known generally as "electron transparent". Hence, an inherent low contrast image is formed.

* BIOLOGICAL MATERIAL must, therefore, be STAINED with heavy metal salts. It must also be SLICED into very thin sections because electrons have very low penetrating power.



PREPARATION OF BIOLOGICAL MATERIAL FOR THE TEM

As mentioned before, biological tissues must be fixed and well preserved. It is necessary also to slice them into ultrathin sections which are then stained The following is a conventional method for the processing of tissues & cells for the TEM. It is known as "The Thin Sectioning Method".

Thin Sectioning Method

1. Fixation - Material is killed and preserved as life-like as possible with a chemical fixative such as glutaraldehyde and osmium tetroxide.

2. Dehydration - Water in the tissues is removed by graded alcohol or acetone solutions to allow the penetration of a supporting medium which is not miscible with tissue water.

3. Embedding - The material is embedded in a supporting medium, usually an epoxy plastic resin which when polymerized, facilitates thin sectioning.

4. Ultramicrotomy - The block of plastic containing the material is sectioned into very thin slices of 50-100 Angstroms thick.

5. Staining of the section - Sections thus obtained are first mounted onto copper grids and then stained in high electron density metal salts such as lead or uranium salts to increase the image contrast.

6. Viewing and recording of the images - The copper grids containing stained sections are viewed in the TEM. The images are recorded on photographic films and then reproduced.

THE SCANNING ELECTRON MICROSCOPE

In this section we will discuss the SEM and how it differs from the TEM.

Construction of the SEM

The upper portion of the SEM (electron gun and condenser lenses) is similar to that of the TEM. As in the TEM, the SEM condenser lenses focus the electron beam onto a small spot on the specimen surface.

Image formation in the SEM is different from the TEM in that the SEM image is a result of secondary electrons emitted from the spot on the specimen where the primary beam from the condenser strikes the specimen's surface. The event is illustrated.

After the impingement of the primary electrons on the specimens, secondary electrons as well as other forms of radiation are emitted. But only the secondary electrons will be collected by the signal detector. In the detector these electrons strike a scintillator and the light produced is converted to electric signals by a photomultiplier at the far end of the detector. The electric signal is then amplified and displayed on the cathode ray tube (CRT).

In the SEM the electron beam is rapidly scanned back and forth in an orderly pattern across the specimen surface. What you see then is a composite of many individual image spots similar to the image formed on the TV screen. The SEM has a specimen stage that allows the specimen to move freely so that the surface of the specimen can be viewed from all angles.

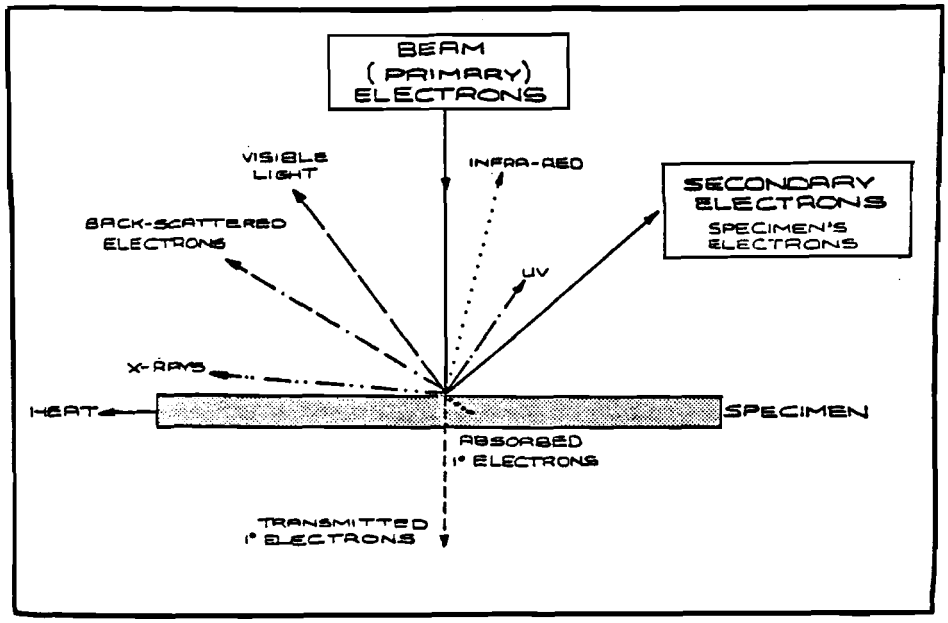
Some features of the SEM

Magnification is determined by the ratio of the viewing screen/scan area of the specimen.

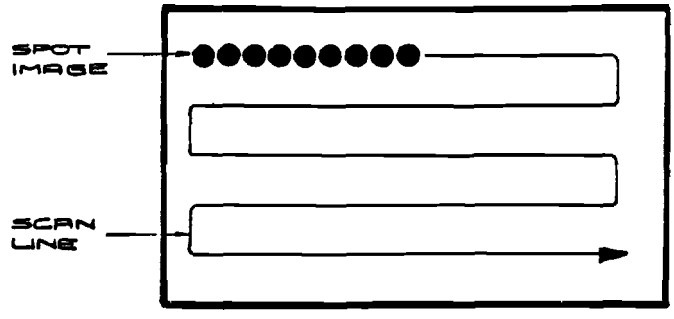
Magnification range is from 20x to 100,000x. Magnification can be changed by changing the scanning area.

Resolution of the SEM is equal approximately to the spot size of the primary electron beam; at present it lies between 4 to 20 nm.

Depth of focus is great when compared with the light microscope. The reason is that the secondary electron beam emitted is in relation to the angle of the specimen surface or the topography of the specimen.



Effects of electron beam bombardment of a specimen.



Sample scanning pattern of electron beam.

SCANNING TRANSMISSION ELECTRON MICROSCOPY (STEM)

This is a recent technological advance in the field of Electron Microscopy. The beam of electrons scans the specimen, as it does in scanning electron microscopy. However, it is the transmitted electrons that are collected and amplified and form an image on a cathode ray tube. The small spot size of the beam allows different areas of the specimen to be discriminated and analyzed. A major use of STEM is in X-ray analysis which allows the elemental composition of the specimen to be mapped.

ACTIVITIES

Following this introductory talk, tours of the EM facility begin. Students visit this facility in groups of 7-8. Other students are left with activities in the lab.

The most difficult process for second-year students is visualizing a three-dimensional object from a two-dimensional micrograph. Understanding the plane of section of a TEM micrograph requires practice. We provide posters as well as fruit and knives and stamp pads. Students are challenged to produce as many different images as possible by slicing the fruit (apple, pear, etc.) in different ways, inking the section and printing it.

In order for students to understand the importance of specimen preparation to TEM, a demonstration is set up. Each step in the process has an actual object accompanying it. Fixation and dehydration have specimens in vials. Students can handle the epon blocks with embedded specimens. Trimmed and untrimmed blocks are shown. Microtomes and glass knives are available for the sectioning demo. Grids are floated on stain. Finally, EM negatives are shown.

The heart of this demonstration tutorial is in booklets of micrographs with accompanying light microscope slides. The booklets are organized as a review of prokaryotic vs. eukaryotic organisms, as well as plant vs. animal cells. There are both SEM and TEM micrographs of each organism, as well as light microscope slides. Organisms include gram negative and gram positive bacteria, blue-green algae, euglenoids, rat liver, higher plant cells and yeast.

In summary, this is a demonstration tutorial designed for second year cell biology students. It has four components:

1. Introductory talk

   Principles of electron microscopy
   TEM, SEM, STEM
   Specimen preparation - including TEM specimen preparation demonstration

2. Light microscope exercise

   Students are given folders that contain SEM and TEM views of prokaryotic and eukaryotic cells. These accompany light microscope slides of the same organisms.

3. Tours of the EM facility

   Small groups of students are given demonstrations of SEM, TEM, STEM and image enhancement.

4. Planes of section-fruit printing

Additional reference material and posters are available.