Electron Microscopy

John J Bozzola, *Southern Illinois University, Carbondale, Illinois, USA*

Electron microscopy is a technology for examining the extremely fine detail or ultrastructure of biological specimens for use in research and medical situations.

Introductory article

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Introduction

Electron microscopy is a technology for examining the extremely fine detail or ultrastructure of biological specimens. This methodology opened a totally new vista to the human eye when biological ultrastructure was revealed as a dynamic and architecturally complex arrangement of macromolecules under the direction of genetic units termed genes. Complex subcomponents of the cell, termed organelles, were clearly visualized and biochemical activities associated with each structure. Pathogenic microorganisms, the viruses, were finally captured on film and the simple, yet beautiful, structures of these infectious subunits provided the first step to organizing them into major taxonomic categories, or virus families. The structural organization of DNA into the chromosome was elucidated using electron microscopy and the electron microscope provided insight into many disease processes. Electron microscopy is routinely used as a tool in such diverse areas as anatomy, anthropology, biochemistry, cell biology, forensic medicine, microbiology, immunology, pathology, physiology, plant biology, toxicology and zoology.

The two basic types of electron microscopy are scanning electron microscopy, for viewing surfaces of bulk specimens, and transmission electron microscopy, for scrutinizing internal as well as external features of extremely thin specimens. The scanning electron microscope is so named because a fine probe of electrons is scanned across the surface of a specimen to generate an image with a three-dimensional appearance. In the transmission electron microscope, the electrons are transmitted through the specimen to reveal a two-dimensional image of the interior of cells. Although these instruments operate in completely different ways, both use accelerated electrons and electromagnetic lenses to generate images. Both types of electron microscope require high vacuum so that electrons, having little mass, can travel down the column of the microscope without being scattered by air molecules. Images are recorded either on photographic films or captured digitally using computer interfaces.

In order to appreciate the types of information obtainable with these instruments, it is important to understand their basic design and operational principles.

The Transmission Electron Microscope

The conventional transmission electron microscope (TEM, **Figure 1**) generates a beam of electrons in the electron gun, usually by heating a tungsten filament and applying a high voltage of negative potential in the range of $-75\,000$ V. The heat and negative high voltage cause ejection of the valence electrons of tungsten near the tip of the V-shaped filament. A highly negative, cup-like device, termed a shield, forces the electrons into a cloud near the filament tip. An aperture in the shield permits some of the electrons to exit towards an anode plate. The electrons, which are travelling at about half the speed of light, then enter the magnetic fields of the first and second condenser lenses, which focus the electrons onto the specimen.

The lenses in electron microscopes are windings of copper wire, or solenoids, which generate a magnetic field when an electrical current is run through the wire. When electrons enter the magnetic fields of the condenser lenses, they come to focus at certain distances, or focal lengths, from the lens. By adjusting the current running through the condenser lens coil, the focal length is adjusted so that the electrons illuminate the specimen in the area being studied in the TEM.

After electrons strike cellular organelles in the specimen, they are deflected to various degrees, depending upon the mass of the cellular component. Areas of great mass deflect the electrons to such an extent that they are effectively removed from the optical axis of the microscope. Such areas, where electrons have been subtracted, appear dark when ultimately projected onto the viewing screen. Areas that have less mass, and so scatter the electrons to lesser degrees, appear brighter on the viewing screen. As a result of the electron scattering by the specimen, a highly detailed image forms in the objective lens. This image is then further magnified up to one million times by the remaining three to four lenses of the TEM. Even though each lens may magnify only 100 times or less, the final magnification is the product of all of the lenses and can quickly reach high magnifications.

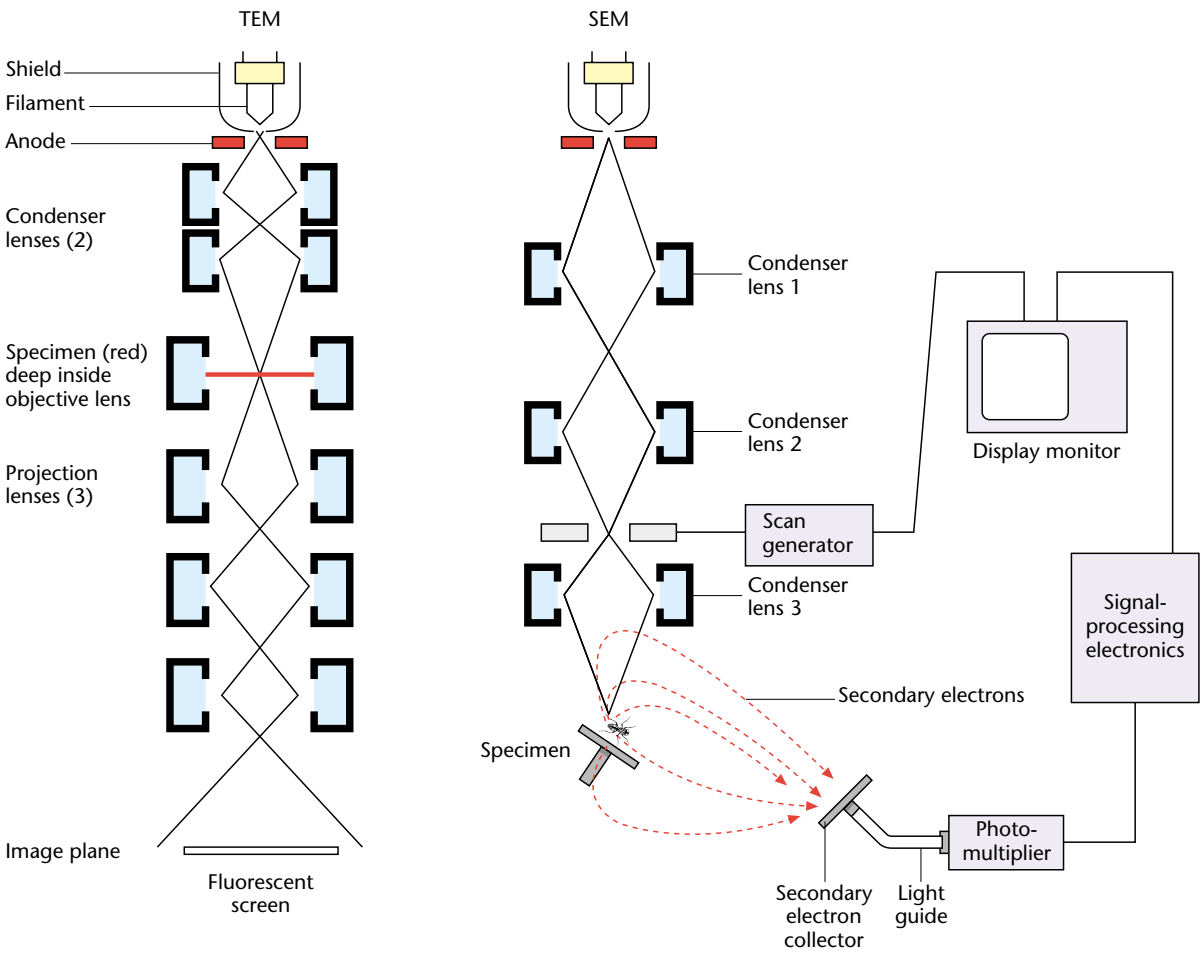

Figure 1 Diagram showing the basic components making up the transmission (TEM) scanning electron microscope (SEM).

The electromagnetic imaging lenses are constructed in the same way as the illuminating or condenser lenses. Imaging lenses are used to focus and magnify the image formed as a result of the interaction of the electrons with the specimen. Ultimately, the electrons are projected onto a viewing screen coated with a phosphorescent material that glows when struck by electrons. Greater numbers of electrons (from areas of the specimen having less density) generate brighter areas on the screen. Since photographic emulsions are sensitive to electrons as well as to photons, the image is recorded by lifting up the viewing screen and allowing the electrons to strike a photographic film placed underneath. After development, such images are termed electron micrographs.

Besides high magnification, which refers to an increase in the size of an object, TEMs produce images having high resolution. High-resolution images have tremendous detail and can withstand the high magnification that makes the details visible. A modern TEM is shown in **Figure 2**.

The Scanning Electron Microscope

The electron gun and the lenses in the scanning electron microscope (SEM, **Figure 1**) are nearly identical to the TEM. However, instead of forming an image, the SEM lenses focus the beam of electrons to a very tiny spot on the specimen. Each of the three condenser lenses in the SEM makes the spot of electrons progressively smaller. When the beam of electrons strikes the specimen, it causes the ejection of low-energy, secondary electrons from the surface of the specimen. Secondary electrons convey information about the surface of the specimen and are used to generate the image.

In contrast to the TEM, which illuminates the specimen area under examination with a single, large spot, the SEM scans a tiny spot of electrons across the specimen. This is much like the beam of electrons that is rastered across the inside of a television tube. At each point where the electron beam of the SEM strikes the specimen, various numbers of secondary electrons will be generated, depending primarily upon topography, angle of entry of the beam into the

Figure 2 This modern TEM has capabilities for scanning transmission electron microscopy (STEM) and X-ray microanalysis.

specimen and thickness of raised portions of the specimen. The secondary (image-forming) electrons are strongly attracted to a secondary electron detector that is placed at a positive high voltage of around 12 000 V. When the secondary electrons strike a phosphorescent coating on the detector, this generates a burst of light that travels down a light guide and into a photomultiplier, where photons are converted to photoelectrons and the weak signal amplified. Areas of the specimen that generate large numbers of secondary electrons appear very bright when viewed on the display screen of the SEM, whereas areas with few electrons are dark. The various shades of grey seen in an image give the impression of depth, much like a black and white photograph conveys three-dimensionality.

Two scanning events take place simultaneously, but in different locations in the SEM. The electron beam is

scanned across the specimen at the same time that a second electron beam is being scanned across a display monitor or viewing screen. For each point on the specimen, there is a corresponding point on the viewing screen, the brightness of which depends upon the number of secondary electrons generated by the specimen. The size of the area scanned on the viewing screen is fixed, based on the size of the screen (15 cm×20 cm, for example). However, the size of the area scanned on the specimen is completely variable and under the control of the operator. If one scans a line across the specimen that is 1 cm in length and then displays this line on a 20 cm monitor, the resulting line is magnified 20× . A 20 μm line scanned on a specimen when displayed to 20 cm would represent a magnification of 10 000 × .

Magnification in the SEM is expressed as:

As the length of the scan decreases on the specimen, it is necessary to make the electron spot on the specimen proportionally smaller. Otherwise, the image will appear to be out of focus. In the SEM, therefore, focusing consists of using the condenser lenses to adjust the spot size on the specimen to the appropriate size for the magnification being used. For high-magnification work involving very small scan lengths, it is necessary to decrease the size of the electron spot to extremely small spots of 1–2 nm. (For comparison, the poliovirus is about 30 nm in diameter.) A modern SEM is shown in **Figure 3**.

Outline of Methods

Specimen preparation for scanning electron microscopy

Biological specimens for SEM examination can be quite large compared with the size of specimens used for TEM. In some instances, entire organisms (insects, small plants, and animals up to several inches) may be suitable if they have been prepared properly. Hardy organisms with exoskeletons or rigid cell walls may need only air-drying. The majority of biological specimens, however, will require chemical fixation in order to stop biological processes and to stabilize the cellular architecture.

Although the details will vary considerably, depending upon the organism, a commonly followed method for specimen preparation starts with a careful cleaning of specimen surfaces using a fine brush, gentle puffs of air or mild streams of aqueous buffer. Following this, the fine

Figure 3 A scanning electron microscope (SEM) with capabilities for examining a hydrated specimen in a partial vacuum. This instrument also has X-ray analytical capabilities. A portion of the X-ray detector is shown in the top left (arrow) corner of this figure.

structure of the specimen is preserved using glutaraldehyde and osmium tetroxide. After dehydration in ethanol, and critical-point drying with liquid carbon dioxide, the specimen is mounted on a stub and coated with conductive metal.

It is necessary to apply a 40–50 nm thin coating of an inert metal, such as gold, in order to dissipate the high-voltage charge from the accelerated electrons that strike the specimen. The metal coating, normally applied by a high-voltage, sputter-coating process, serves as an excellent source of secondary electrons. **Figure 4** is a scanning electron micrograph of several *Salmonella* bacterial cells attached to a rayon fibre. The sample was prepared as described in the previous paragraphs.

Specimen preparation for transmission electron microscopy

Methods for preparing specimens for TEM examination are normally more complicated and the specimens more sensitive to processing details than are SEM samples. Since we wish to examine the interior of cells, and since the electron beam of the TEM is unable to penetrate specimens

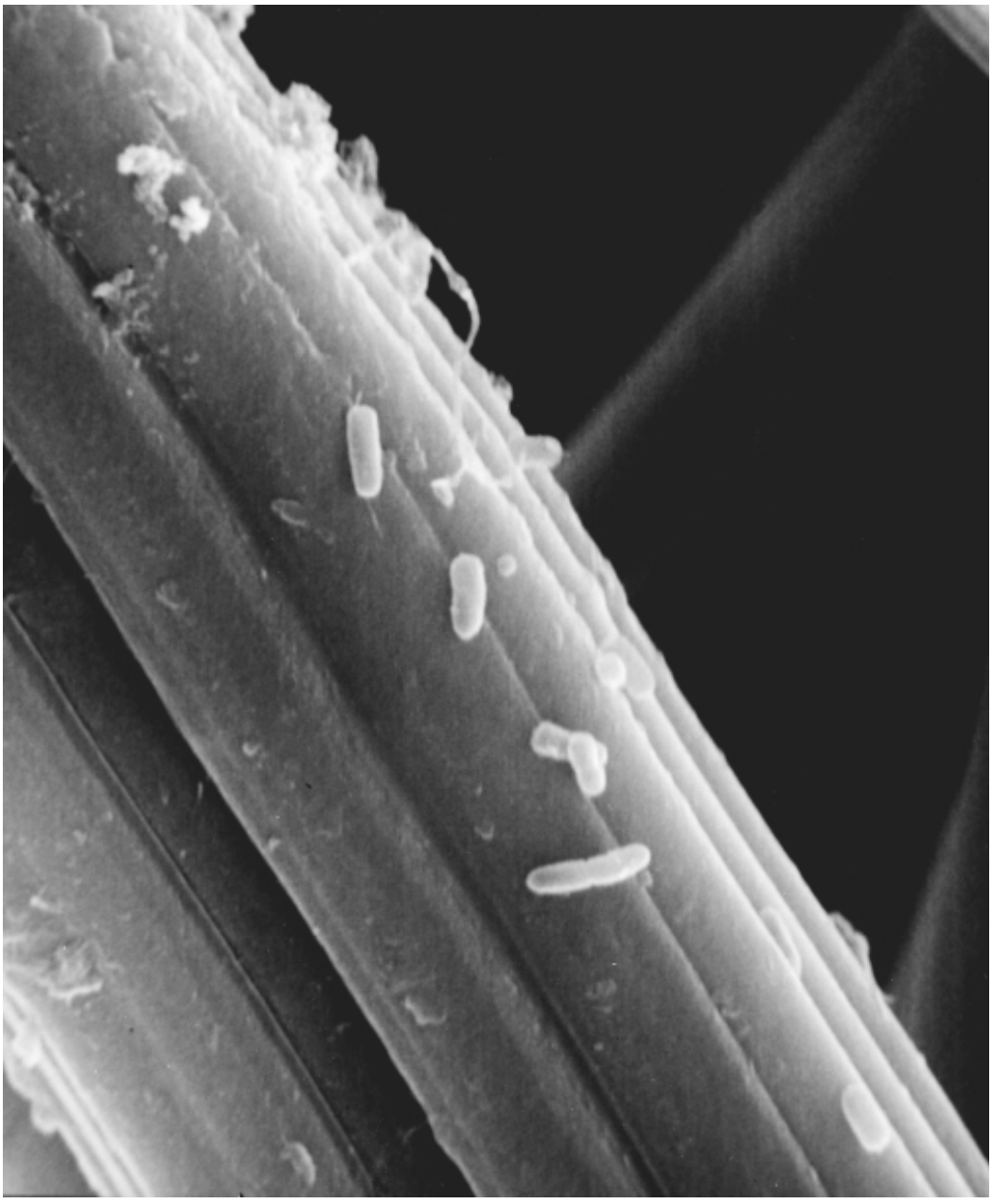

Figure 4 *Salmonella* bacteria adhering to the surface of a rayon fibre. Note the three-dimensional aspects of the image.

much thicker than 60–80 nm, it is necessary to cut extremely thin slices of most biological tissues. As is done for light microscopy, the specimen must be chemically preserved and embedded in a supporting matrix in order to prevent collapse of the tissues as the knife sections the tissue. Biological ultrastructure can rapidly deteriorate when the organism is being prepared, so it is essential to use small pieces of tissue (less than 1 mm thick) that can be penetrated quickly by the chemical fixatives.

A typical method for preparing biological tissues would employ glutaraldehyde and osmium fixatives. After the specimens have been dehydrated in ethanol, they are infiltrated with a liquid, epoxy plastic that is then hardened in an oven. At this point, the water throughout the preserved cells has been replaced completely with hard plastic and it is now possible to cut extremely thin slices. This is accomplished using an ultramicrotome that advances the specimen over a glass or diamond knife and cuts ultrathin sections from the plastic-embedded specimen. The sections are retrieved onto a specimen carrier, or grid, stained for contrast using heavy metals such as uranyl acetate and lead citrate, and placed into the TEM for viewing.

Figure 5 shows several bacterial cells that were prepared and sectioned as described. A typical plant cell is shown in **Figure 6**. Two mammalian cells, infected with a tumour virus that has been released into the space between the cells, are shown in **Figure 7**. **Figure 8** is a close-up of five of the tumour viruses.

Some specimens, such as viruses and isolated subcellular organelles (ribosomes and mitochondria, for example), are thin and do not require sectioning. In this case, the specimens are mixed with an aqueous solution of a heavy metal stain (uranyl acetate, potassium phosphotungstate) for contrast and placed directly onto the specimen grid for viewing in the TEM. This procedure is extremely rapid and permits one to examine a specimen within minutes. A single herpesvirus particle that was negatively stained with potassium phosphotungstate is shown in **Figure 9**. An explanatory diagram for this virus is presented in **Figure 10**.

As the accelerating voltage of the TEM is increased beyond the 75 000–125 000 V available in conventional instruments, it is possible to examine much thicker specimens. Intermediate and high-voltage electron microscopes are instruments that operate in the range of 200 000–500 000 and 500 000 to one million volts. High-voltage instruments have four advantages over lower accelerating voltage microscopes: increased resolution, greater penetrating ability in thicker sections, diminished specimen damage and considerably greater depth of

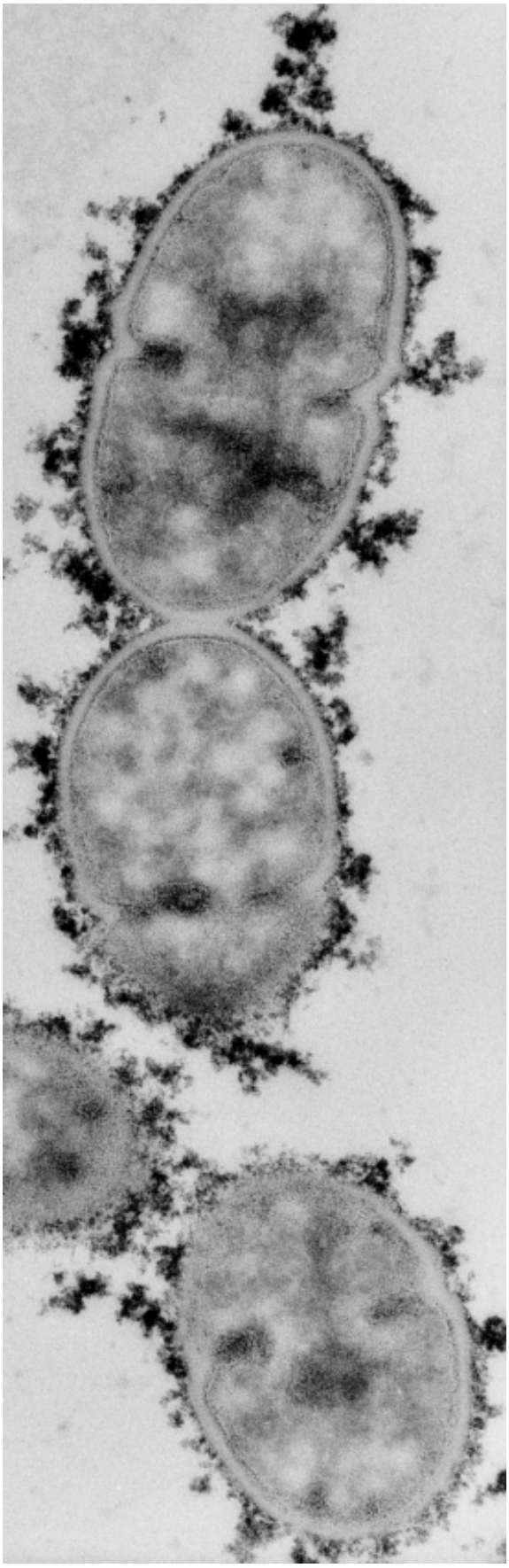

Figure 5 A chain of bacterial cells as viewed in the TEM. These bacteria, *Streptococcus mutans*, are responsible for causing tooth decay. Compare the two-dimensional appearance of this image to that shown in the previous figure. These cells have been prepared and ultrathin sections of 70 nm have been cut through the cells. These bacteria are 0.5 μm in width.

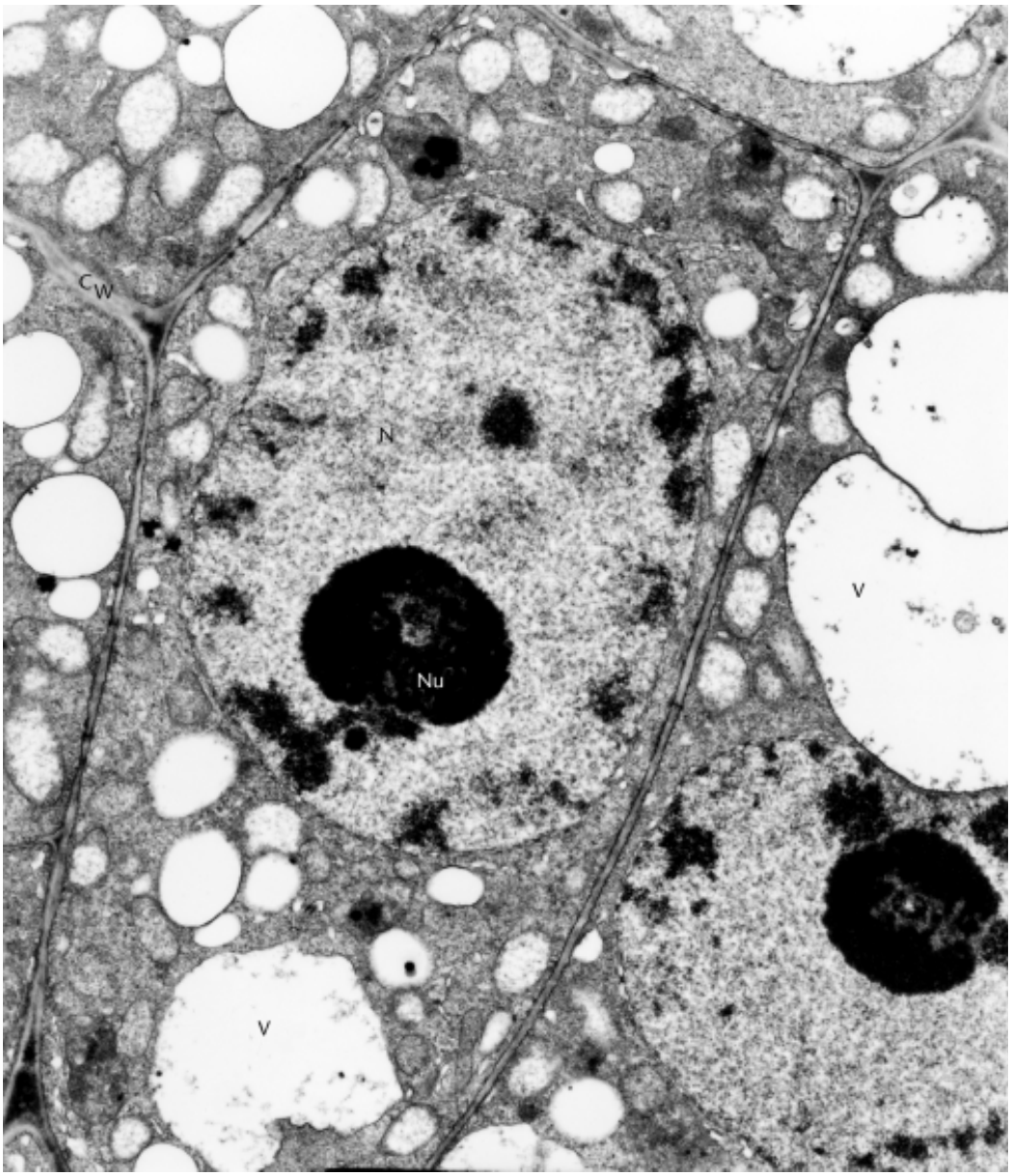

Figure 6 A typical plant cell as seen in the TEM. Ultrathin sections reveal the interior of the cell in great detail. Note the nucleus (N), nucleolus (Nu), vacuoles (V) and cell wall (CW) surrounding the cell. Courtesy of Microscopy Society of America.

information. Sections can be examined that are 10–30 times thicker than those needed for the conventional TEM.

With the right equipment, it is also possible to examine rapidly frozen specimens that have not been subjected to most of the chemicals used in preparing specimens in the conventional manner. In one method, cryoultramicrotomy, rapidly frozen specimens are sectioned at -80 to $-100^{\circ}C$ and the sections examined in the TEM. Under ideal conditions, sections can be examined in about one hour.

Another instrument, the scanning transmission electron microscope, or STEM, is similar to a TEM except that it has the capability to generate extremely small electron beams that can be rastered over a thin specimen. This technology is particular useful when one wishes to obtain X-ray analytical data from extremely small spots in the specimen.

Applications

Both types of electron microscopes have found wide use in the basic biological sciences as well as in the medical field. Strictly descriptive studies provide crucial data for newly described organisms and comparative investigations are

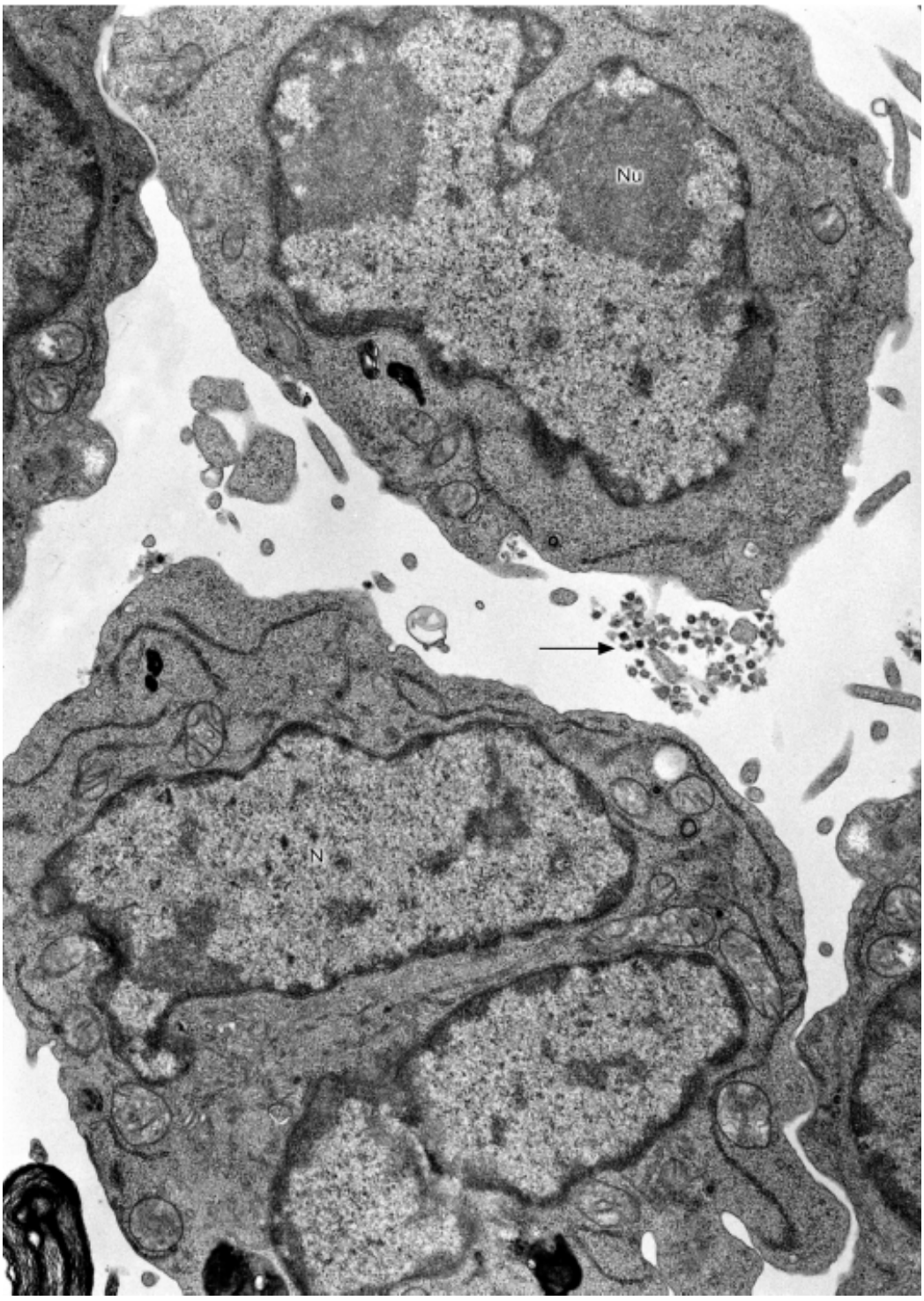

Figure 7 Sectioned mammalian cells as viewed in the TEM. These cells are infected with a tumour virus, with some virus particles visible in the space between the two cells (arrow). N, nucleus; Nu, nucleolus.

valuable to pinpoint differences between organisms (evolutionary studies, drug treatment regimes, pathological changes, cancerous transformation, physiological processes such as ageing and cell death).

The TEM has become a mainstay in the medical field as it can provide definitive evidence for a diagnosis. For example, locating certain subcellular components can readily identify certain types of tumours that are difficult to identify using conventional light microscopy. For certain types of viral infections, often the quickest diagnosis may be by electron microscopy. When newly emergent or previously undiscovered infectious agents are

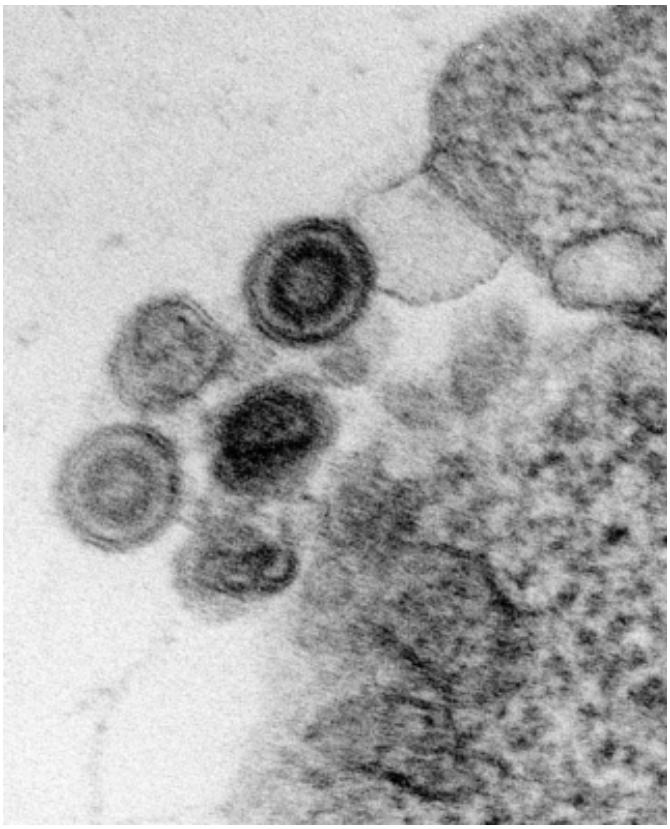

Figure 8 High-magnification view of five tumour viruses from cells shown in **Figure 7.** Note the double-layered nature of the membrane surrounding the top virus particle. This membrane is derived from the host cell and is approximately 8 nm in thickness.

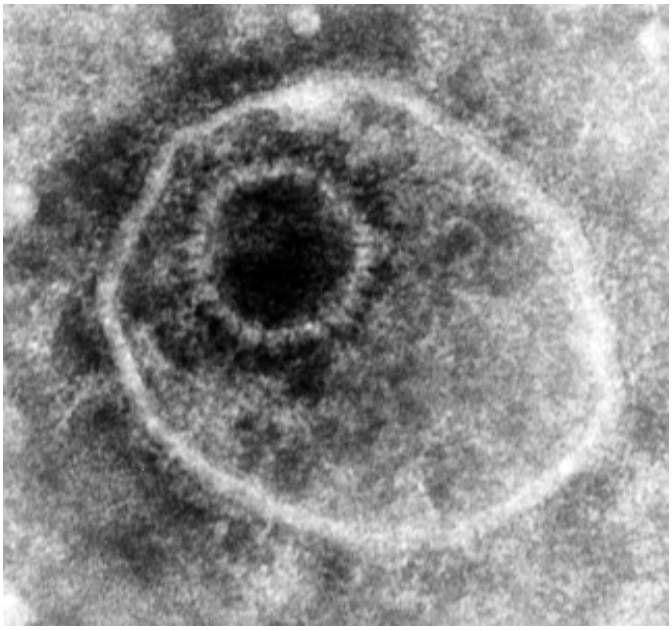

Figure 9 A herpesvirus particle from a patient suffering from shingles. This virus is responsible for causing both chickenpox and shingles. The central part of the virus particle, the capsid, is approximately 100 nm in size.

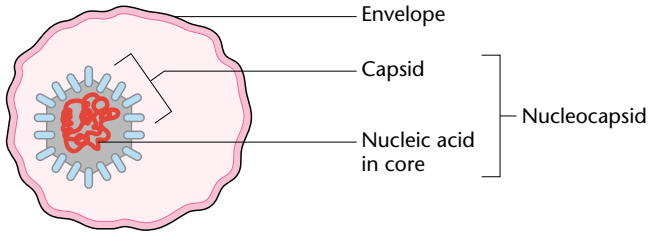

Figure 10 Diagram showing the various parts of the virus particle in **Figure 9.**

involved (hepatitis B, HIV, Ebola), electron microscopy is often the only possible way to identify the pathogen.

Besides the conventional approaches to viewing specimens in both types of electron microscopes, there exist ancillary procedures that may provide additional information. X-ray microanalysis, for example, is a technology that enables one to identify the types of elements present in a specimen. Useful in SEMs, TEMs and STEMs, the procedure is possible since the electron beam elicits the production of X-rays upon interacting with the atoms in the specimen. Since each element has a characteristic spectrum of X-rays, one can identify the elements present and sometimes the percentage composition. This may be accomplished in a very localized manner, for example over a cellular inclusion, or one may generate a distribution map of the elements present in the specimen being viewed.

A related procedure, electron energy loss spectroscopy, EELS, is useful in the TEM for localizing the lighter atomic numbered elements most likely to be present in biological tissues. EELS has the added advantage of being a more sensitive procedure for detecting trace levels of elements present in cells. EELS may also be used to increase contrast in specimens and improve resolution in thicker sections. Both EELS and X-ray analysis technologies, however, require expensive accessories to the electron microscope that are nearly half the cost of the microscope itself.

Cytochemistry, immunocytochemistry, *in situ* hybridization and autoradiography are specialized TEM techniques that allow one to localize molecules such as enzymes, viral proteins, specific sequences of DNA or RNA, cellular building blocks, hormones or practically any molecule of interest within the cell. Such studies permit one to trace the route of targeted molecules in the cell and may reveal pathways for the production of macromolecules in the dynamic interior of the cell. Once the molecules have been located in normal cells, this serves as a database for comparative studies in pathological conditions (genetic disorders, disease development, tumorigenesis), drug treatment studies (antimicrobials, anticancer agents, other pharmaceuticals) and unlimited, basic science, research investigations.

Future Developments

It has always been the goal of cell biologists to be able to examine cells in the hydrated, native state. One of the best ways of doing this involves cryomicroscopy, in which the living specimen is rapidly frozen and examined while being maintained in the frozen state. Such capabilities exist for both SEM and TEM studies. When one combines cryomicroscopy with high-voltage TEMs and modern computers, cryoelectron tomography is possible. This technology can be used to view ultrastructural details in three dimensions. For example, chromosomes, mitochondria, microtubule arrays, sites of viral replication, etc., which are unfixed, unstained and embedded in vitreous ice, can now be examined in three dimensions.

The environmental SEM, or ESEM, is a specially designed SEM that uses a newly designed, gaseous discharge detector for capturing imaging electrons. With the ESEM it is possible to examine hydrated, unfixed, nonfrozen, uncoated biological specimens with only slight vacuums present in the specimen chamber. This should permit the study of dynamic processes in hydrated, living cells.

Thanks to computers and digital imaging, electron microscopes can be directly interfaced with the Internet so that students and researchers are able to view images very shortly after they have been viewed by the microscopist. Ultimately, with faster connections, it will be possible to view the images in real time and even to operate the microscope remotely, thousands of miles away. This capability would be particularly important for medical diagnostic procedures in locations where electron microscopes are unavailable.

Summary

Electron microscopes enable investigators to obtain highly detailed images of cells for use in research and medical situations. The transmission electron microscope (TEM) has imaging and analytical capabilities over one thousand times better than light microscopes. TEMs are useful to study the interior of thinly sliced cells and subcellular entities such as viruses and may obtain images even down to the molecular level. Scanning electron microscopes (SEM), by contrast, are used to study the three-dimensional features of intact specimens (whole insects, cells, portions of organs, etc.). Modern instruments are capable of imaging frozen, hydrated specimens where one may apply various analytical procedures (elemental analysis, distribution of molecular species, for example). High-resolution, three-dimensional images of fully hydrated, frozen specimens can be obtained in high-voltage TEMs as well as in the environmental SEM. Modern computers and the Internet have increased the availability of electron microscopes to the wider teaching, research and medical communities. Further information on electron microscopy may be obtained by contacting the Microscopy Society of America website at [http://www.msa.microscopy.com].

Further Reading

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