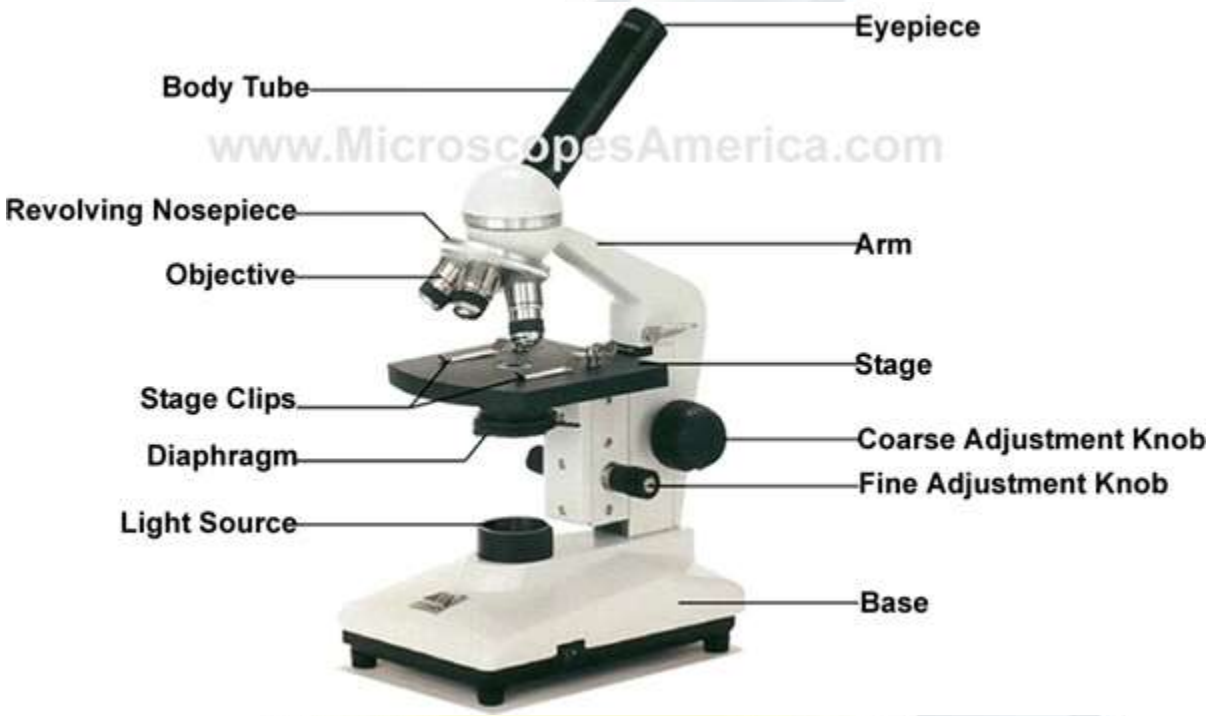
Module I
Microscopy

•**Microscopes** are instruments that produce an enlarged image of an object.

•**Microscopy** is the technical field of using microscopes

Parts of a Microscope



•A light source, which may be external to the microscope or built into its base, illuminates the specimen.

•The substage *condenser lens* gathers the diffuse rays from the light source and illuminates the specimen.

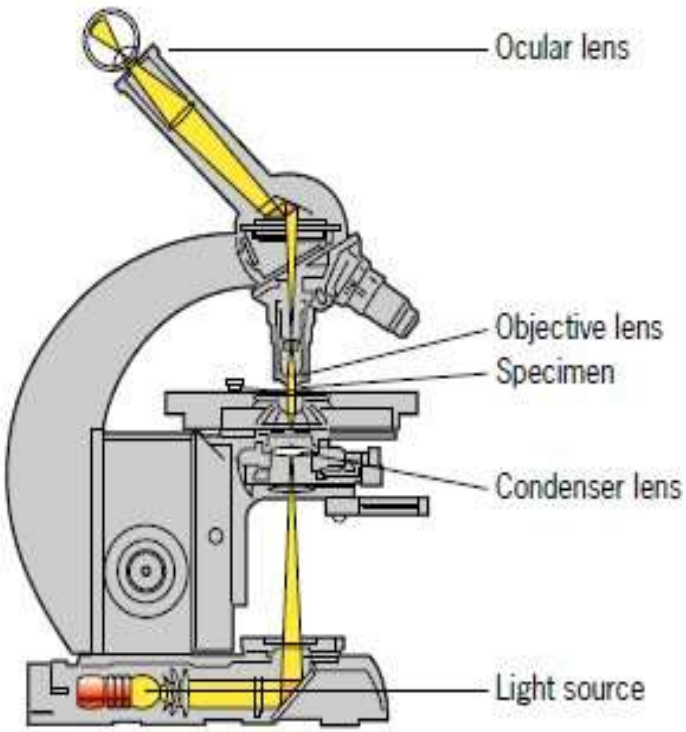


Figure 18.1 **Sectional diagram through a compound light microscope;** that is, a microscope that has both an objective and an ocular lens.

•The light rays focused on the specimen by the condenser lens are then collected by the microscope's **objective lens.**

•From this point, we need to consider two sets of light rays that enter the objective lens: those that the specimen has altered and those that it hasn't.

•The latter group consists of light from the condenser that passes directly into the objective lens, forming the background light of the visual field.

•The former group of light rays emanates from the many parts of the specimen and forms the image of the specimen.

•These light rays are brought to focus by the objective lens to form a real, enlarged image of the object.

•The image formed by the objective lens is used as an object by a second lens system, the *ocular lens*, to form an enlarged and virtual image.

•A third lens system located in the front part of the eye uses the virtual image produced by the ocular lens as an object to produce a real image on the retina.

•When the focusing knob of the light microscope is turned, the relative distance

between the specimen and the objective lens changes, allowing the final image to become focused precisely on the plane of the retina.

•The total magnification attained by the microscope is the product of the magnifications produced by the objective lens and the ocular lens.

•The optical quality of an objective lens is measured by the extent to which the fine detail present in a specimen can be discriminated, or resolved.

•The **resolving powe**r of a microscope can be defined in terms of the ability to see two neighboring points in the visual field as two distinct entities.

Types of Microscopes

1. **Differential Interference Contrast Microscope**
2. **Confocal Microscope**
3. **Electron Microscope – TEM, SEM**
4. **Scanning Tunneling Microscope**
5. **Atomic Force Microscopes**

1. Differential Interference Contrast Microscope (DIC)

•Also known as **Nomarski interference contrast** (**NIC**) or **Nomarski microscopy**, is an optical microscopy technique used to enhance the contrast in unstained, transparent samples.

•The technique was developed by Polish physicist Georges Nomarski in 1952

•The two common methods called phase-contrast microscopy and differential interference contrast (DIC) microscopy (or Nomarski interference microscopy) for imaging live cells and unstained tissues generate contrast by taking advantage of differences in the refractive index and thickness of cellular materials, produce images that differ in appearance and reveal different features of cell architecture.

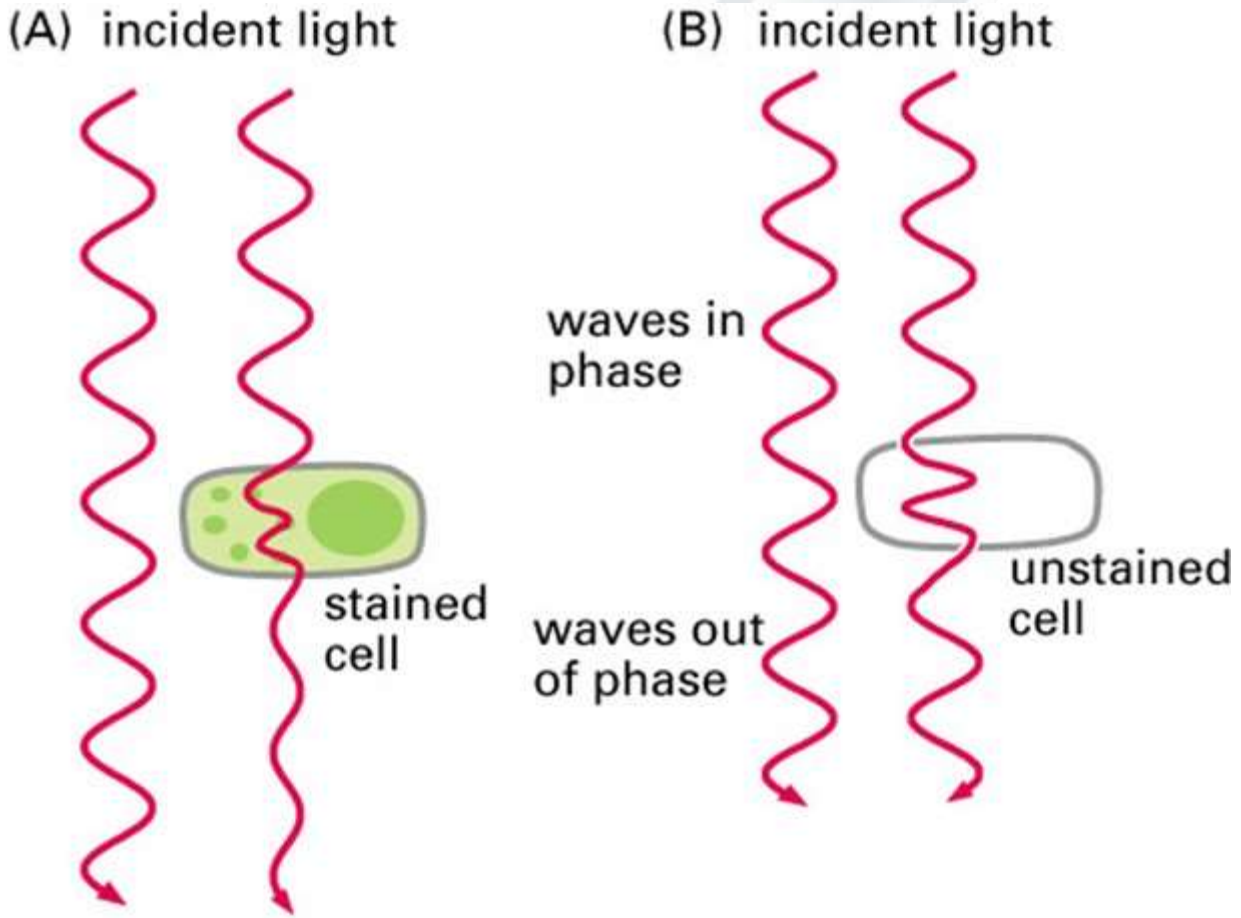
•Phase-contrast microscopy generates an image in which the degree of darkness or brightness of a region of the sample depends on the refractive index of that region.

•Light moves more slowly in a medium of higher refractive index.

•Thus, a beam of light is refracted (bent) once as it passes from air into a transparent object and again when it departs.

•Consequently, part of a light wave that passes through a specimen will be refracted and will be out of phase (out of synchrony) with the part of the wave that does not pass through the specimen.

•The refracted and unrefracted light are recombined at the image plane to form the image. Phase contrast microscopy is suitable for observing single cells or thin cell layers, but not thick tissues


(A) incident light
stained cell
(B) incident light
waves in phase
waves out of phase
unstained cell

•Differential Interference Contrast is another method of deriving contrast in an unstained specimen from differences in index of refraction of specimen components.

•As with Phase Contrast, DIC transforms the phase shift of light, induced by the specimen refractive index, into detectable amplitude differences.

•Differential interference contrast microscopes are actually microscope interferometers in that they generate contrast within the specimen by exploiting phase differences between a specimen light ray and a reference ray.

•In interferometry, a single ray of light is split into two rays, one traversing and the other missing the specimen but interacting with the background. The rays are then recombined at the image plane, where wave interference may occur.

•Image contrast can be modified by altering the phase difference between the reference and specimen rays.

•In differential interference contrast microscopy, the sample and reference rays are created *after* the ray traverses, and is phase-distorted by, the sample.

•The phased ray then is split into two equally phased, but spatially separated ("sheared") rays by the action of a Wollaston prism.

•Finally, the ray-pairs pass through a polarizing filter where they are vibrationally recombined and interact at the image plane. It is the wave interference of these two rays that creates contrast.

•In DIC images, objects appear to cast a shadow to one side. The "shadow" primarily represents a difference in the refractive index of a specimen rather than its topography. DIC microscopy easily defines the outlines of large organelles, such as the nucleus and vacuole

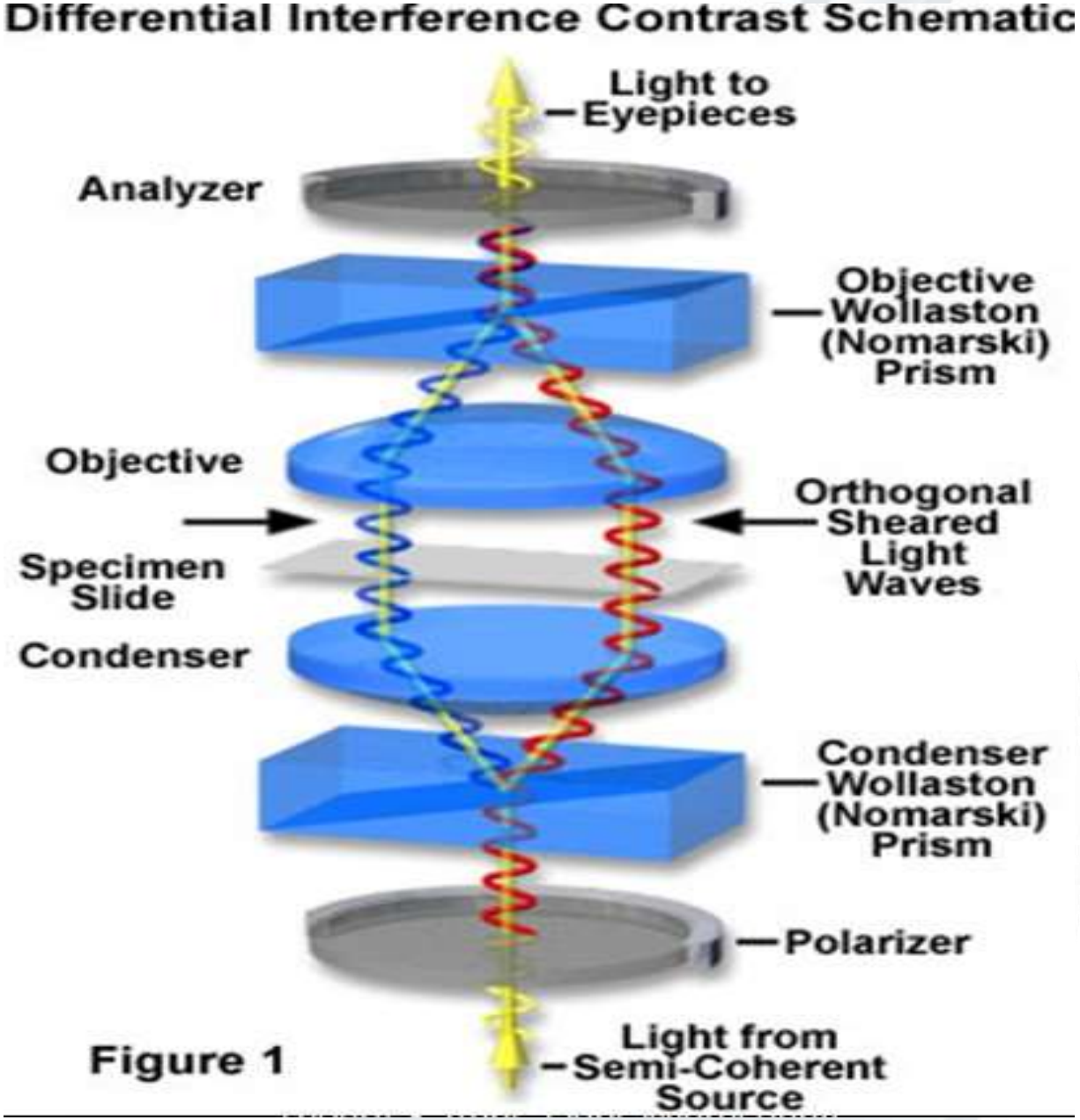

Differential Interference Contrast Schematic
Light to Eyepieces
Analyzer
Objective Wollaston (Nomarski) Prism
Objective
Orthogonal Sheared Light Waves
Specimen Slide
Condenser
Condenser Wollaston (Nomarski) Prism
Polarizer
Light from Semi-Coherent Source

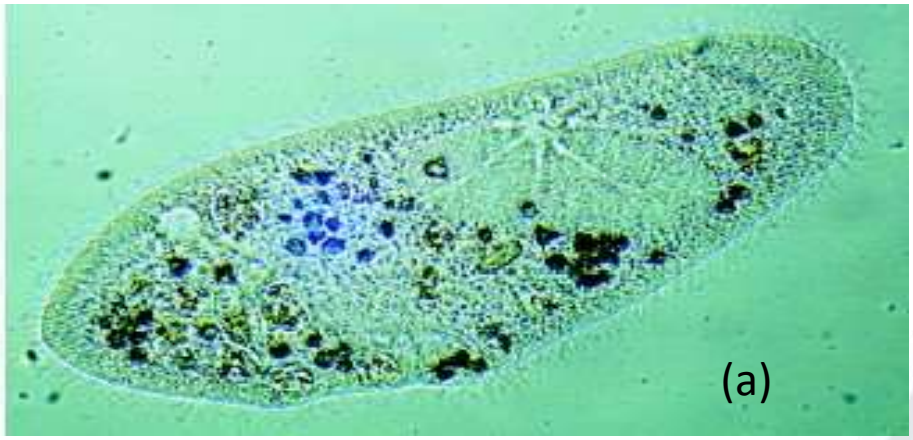
Figure 1

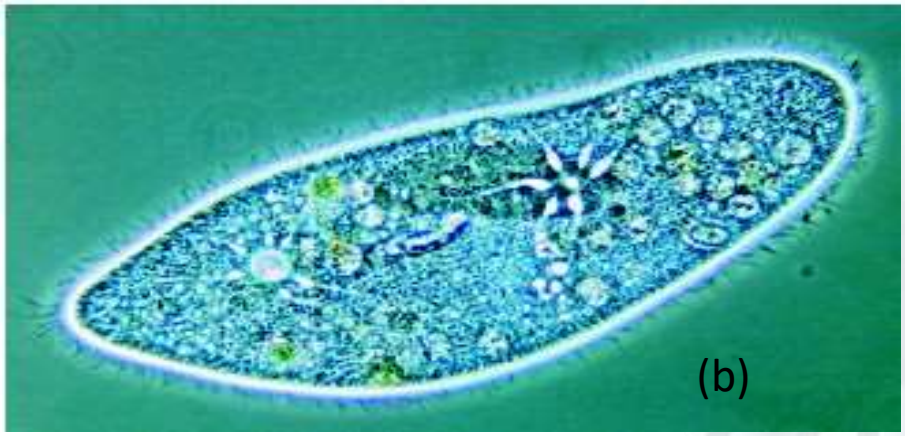
•Phase contrast microscopy produces image intensity (amplitude) values that vary as a function of specimen optical path length magnitude, with very dense regions (those having large path lengths) appearing darker than the background.

•In differential interference contrast, where optical path length **gradients** (in effect, the rate of change in the direction of wavefront **shear**) are primarily responsible for contrast.

•Steep gradients in path length generate excellent contrast, and images display the pseudo three-dimensional relief shading, which is characteristic of the DIC technique.

•Regions having very shallow optical path slopes, such as those observed in extended, flat specimens, produce insignificant contrast and often appear in the image at the same intensity level as the background.





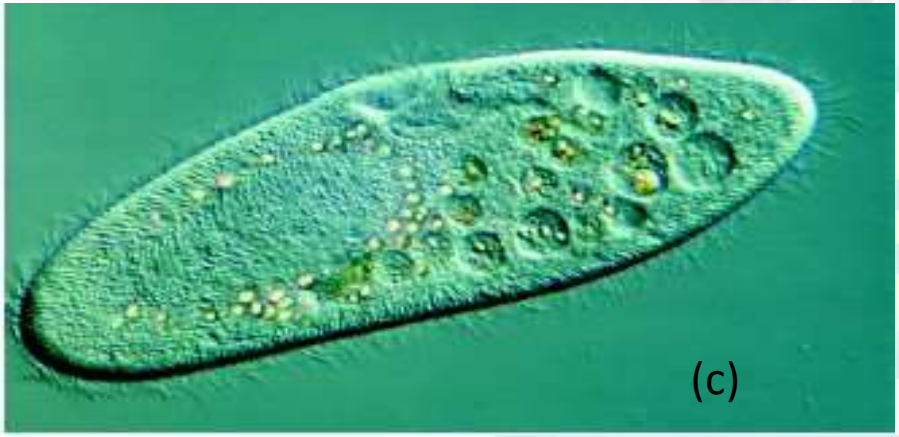


Figure 18.6 **A comparison of cells seen with different types of light microscopes.** Light micrographs of a ciliated protist as observed under bright-field (*a*), phase-contrast (*b*), and differential interference contrast (DIC) (or Nomarski) optics (*c*). The organism is barely visible under bright-field illumination but clearly seen under phase-contrast and DIC microscopy. (MICROGRAPHS BY M. I. WALKER/PHOTO RESEARCHERS, INC.)

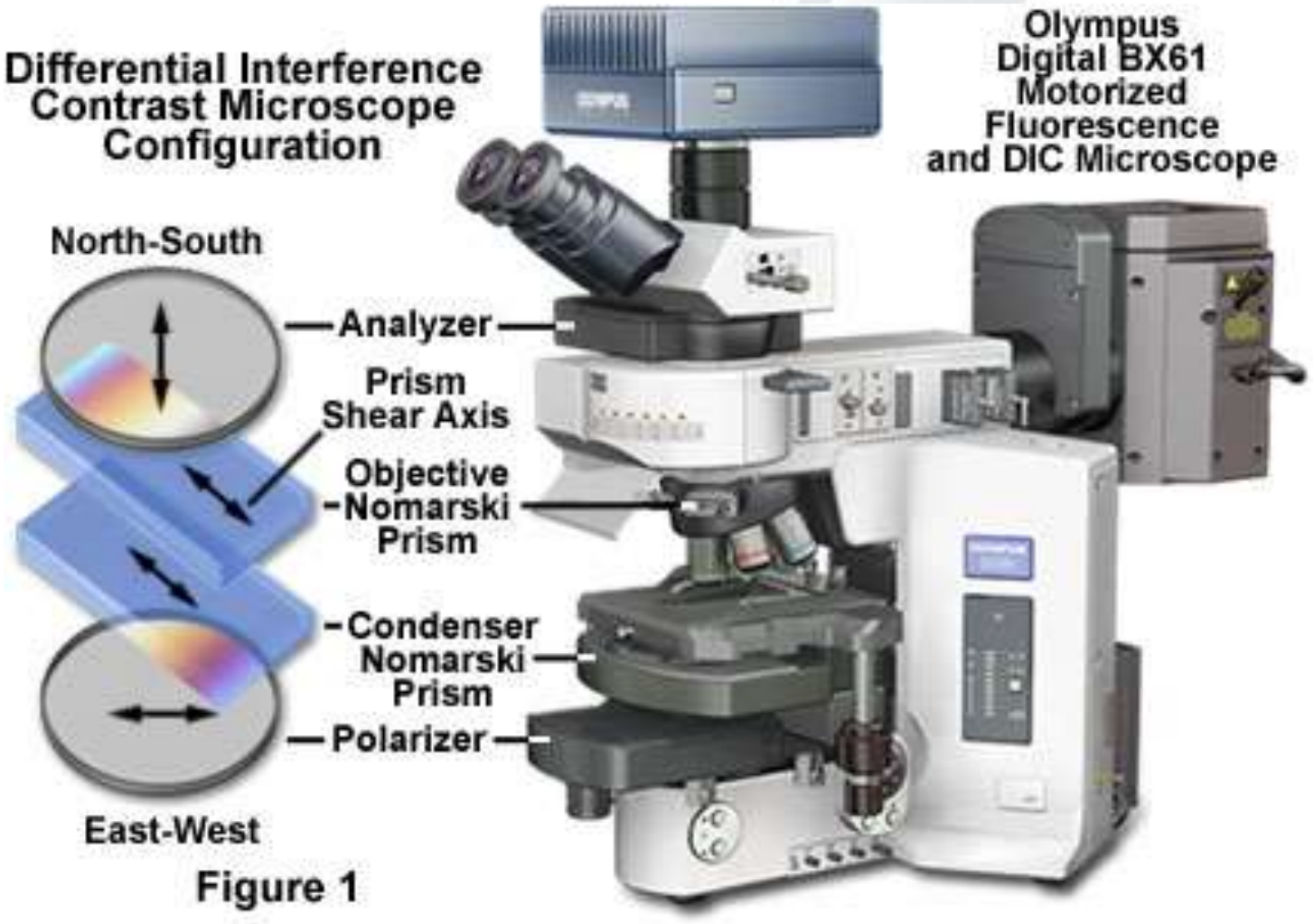

Differential Interference Contrast Microscope Configuration
Olympus Digital BX61 Motorized Fluorescence and DIC Microscope
North-South
Analyzer
Prism Shear Axis
Objective Nomarski Prism
Condenser Nomarski Prism
Polarizer
East-West

Figure 1

2. Confocal Microscope

•In this type of microscope, the specimen is illuminated by a finely focused laser beam that rapidly scans across the specimen at a single depth, thus illuminating only a thin plane (or “optical section”) within the specimen.

•Confocal microscopes are used with fluorescence optics

•Confocal systems use beams of ultraviolet laser light to excite fluorescent chemical dye molecules into emitting (returning) light.

•The exciting light beam is focused onto the specimen (usually nonliving) either through a thin optical fiber, or by passing through a small aperture shaped as a pinhole or a slit.

•Resultant fluorescent emissions are focused on a detector which also has a small aperture or slit in front of it.

- The smaller the apertures used at both sites, the greater the amounts of out-of-focus light blocked from the detector.

- A computer reconstructs an image from the emitted light with resolution that can be up to 40% better than with other types of light microscopy.

•Confocal microcopy differs from conventional fluorescence microscopy by the use of a pinhole located in front of the detector that blocks light not originating from that focal plane.

•The resulting images do not contain blurs from structures above and below the current position of the focal plane.

•short-wavelength incident light is absorbed by the fluorophores in a specimen and reemitted at longer wavelength.

•Light emitted from the specimen is brought to focus at a site within the microscope that contains a pinhole aperture.

•Thus, the aperture and the illuminated plane in the specimen are confocal.

•Light rays emitted from the illuminated plane of the specimen can pass through the aperture, whereas any light rays that might emanate from above or below this plane are prevented from participating in image formation.

• As a result, out-of-focus points in the specimen become invisible.

•The majority of confocal microscopes use a laser as the source of illumination; lasers provide a defined excitation wavelength and because of their focused energy are often well suited to penetrating thick specimen.

•Since a laser is focused on a single point on the specimen, it must be scanned across and down to build an image. The intensity of light from these in-focus areas is recorded by a photomultiplier tube, and the image is stored in a computer.

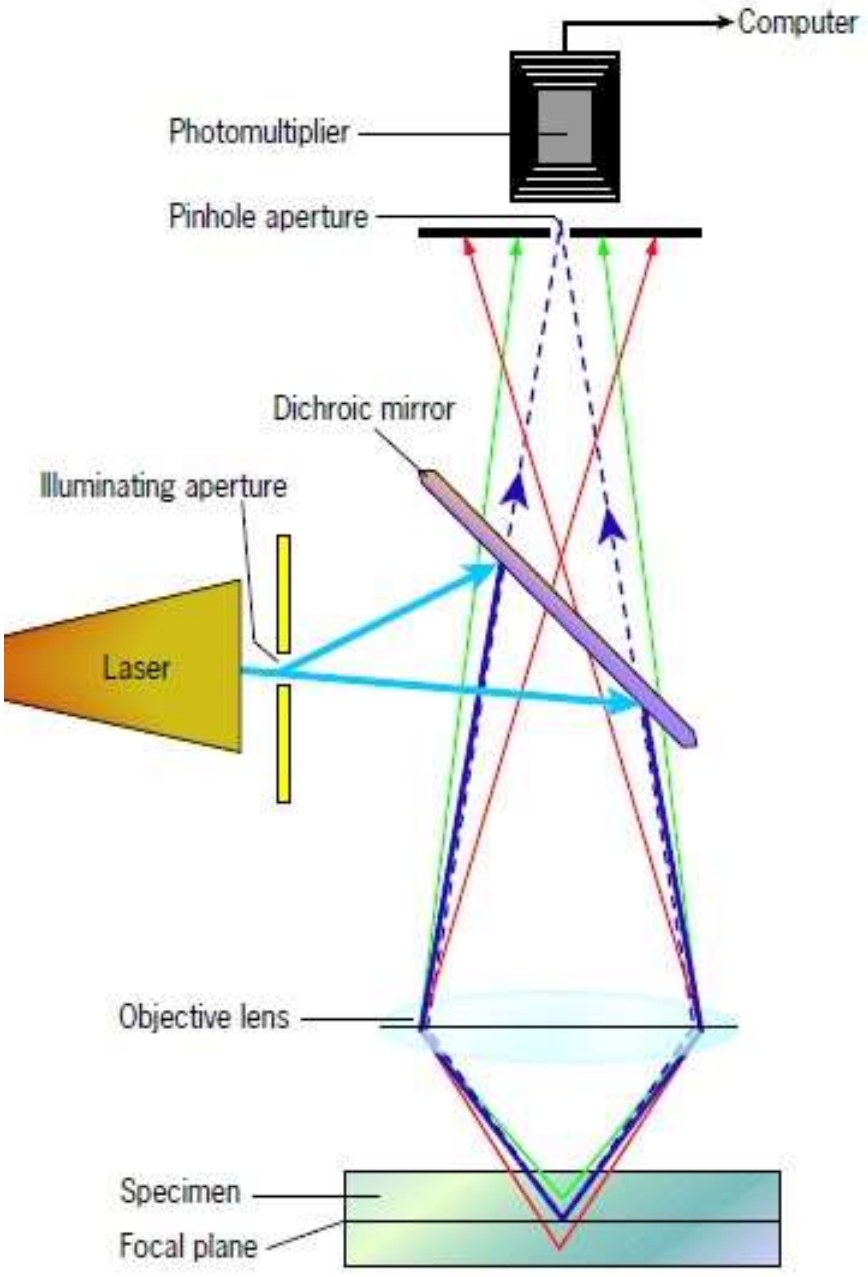


Figure 18.9 **Laser scanning confocal fluorescence microscopy.** (*a*) The light paths in a confocal fluorescence microscope. Light of short (blue) wavelength is emitted by a laser source, passes through a tiny aperture, and is reflected by a dichroic mirror (a type of mirror that reflects certain wavelengths and transmits others) into an objective lens and focused onto a spot in the plane of the specimen. Fluorophores in the specimen absorb the incident light and emit light of longer wavelength, which is able to pass through the dichroic mirror and come to focus in a plane that contains a pinhole aperture. The light then passes into a photomultiplier tube that amplifies the signal and is transmitted to a computer which forms a processed, digitized image. Any light rays that are emitted from above or below the optical plane in the specimen are prevented from passing through the pinhole aperture and thus do not contribute to formation of the image. This diagram shows the illumination of a single spot in the specimen. Different sites within this specimen plane are illuminated by means of a laser scanning process. The diameter of the pinhole aperture is adjustable. The smaller the aperture, the thinner the optical section and the greater the resolution, but the less intense the signal. (*b*) Confocal fluorescence micrographs of three separate

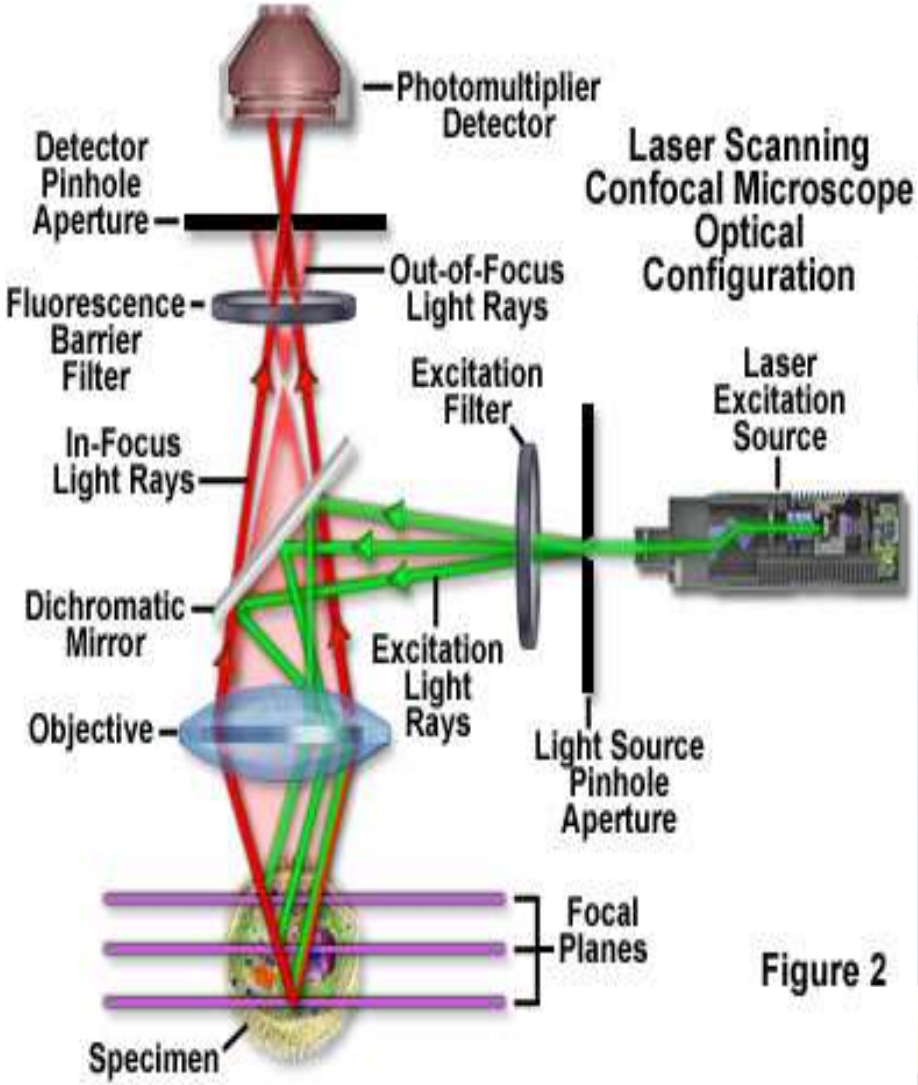

Photomultiplier Detector
Detector Pinhole Aperture
Laser Scanning Confocal Microscope Optical Configuration
Out-of-Focus Light Rays
Fluorescence Barrier Filter
Excitation Filter
Laser Excitation Source
In-Focus Light Rays
Dichromatic Mirror
Excitation Light Rays
Objective
Light Source Pinhole Aperture
Focal Planes
Specimen

Figure 2

Electron Microscope

- Uses a beam of **electrons** to create an image of the specimen.

- It is capable of much higher magnifications and has a greater resolving power than a light **microscope**, allowing it to see much smaller objects in finer detail.

- All electron microscopes use electromagnetic and/or electrostatic lenses to control the path of electrons.

•The basic design of an electromagnetic lens is a solenoid (a coil of wire around the outside of a tube) through which one can pass a current, thereby inducing an electromagnetic field.

•The electron beam passes through the centre of such solenoids on its way down the column of the electron microscope towards the sample.

- Electrons are very sensitive to magnetic fields and can therefore be controlled by changing the current through the lenses.
- The faster the electrons travel, the shorter their wavelength.
- The resolving power of a microscope is directly related to the wavelength of the irradiation used to form an image.
- Reducing wavelength increases resolution.

•Therefore, the resolution of the microscope is increased if the accelerating voltage of the electron beam is increased.

•As the wavelength of an electron can be up to 100,000 times shorter than that of visible light photons, electron microscopes have a higher resolving power than light microscopes and can reveal the structure of smaller objects.

•Modern electron microscopes can magnify objects up to about two million times.

•It is used to examine biological materials, medical biopsy samples, metals & crystalline structures, & the characteristics of various surfaces.

•Two different types of electron microscopes. Transmission electron microscopes (TEMs) form images using electrons that are transmitted through a specimen, whereas scanning electron microscopes (SEMs) utilize electrons that have bounced off the surface of the specimen.

Transmission Electron Microscope (TEM)

- The original form of electron microscopy,

- Transmission electron microscopy (TEM) involves a high voltage electron beam emitted by a cathode and formed by magnetic lenses.

- The electron beam is produced by an electron gun, commonly fitted with
a tungsten filament cathode as the electron source.

•Electrons are drawn from the hot filament and accelerated as a fine beam by the high voltage applied between the cathode and anode.

•Beam of negatively charged electrons can be focused by electromagnetic lenses, which are located in the wall of the column and the strength of the magnets is controlled by the current provided them.

•Air is pumped out of the column prior to operation, producing a vacuum through which the electrons travel.

•If the air were not removed, electrons would be prematurely scattered by collision with gas molecules.

•The electron beam is transmitted through the specimen that is in part transparent to electrons and in part scatters them out of the beam.

• When it emerges from the specimen, the electron beam carries an image of the structure of the specimen that is magnified by the objective lens system of the microscope.

•The spatial variation in this image (the "image") is then magnified by a series of magnetic lenses until it is recorded by hitting a fluorescent screen, photographic plate, or light sensitive sensor such as a CCD (charge-coupled device) camera.

•The image detected by the CCD may be displayed in real time on a monitor or computer.

•Transmission electron microscopes produce two-dimensional, black and white images.

•The major disadvantage of the transmission electron microscope is the need for extremely thin sections of the specimens, typically about 100 nanometers. (one nanometer is .001micrometere)=.1micrometer.

•Biological specimens are typically required to be chemically fixed, dehydrated and embedded in a polymer resin (epoxy resins) to stabilize them sufficiently to allow ultrathin sectioning.

•Sections of biological specimens, organic polymers and similar materials may require special treatment with heavy atom labels in order to achieve the required image contrast.

Scanning Electron Microscope (SEM)

- A **scanning electron microscope** (**SEM**) is a type of electron microscope that produces images of a sample by scanning it with a focused beam of electrons.

- The electrons interact with atoms in the sample, producing various signals that contain information about the sample's surface topography and composition.

•The most common SEM mode is detection of secondary electrons emitted by atoms excited by the electron beam.

•The number of secondary electrons that can be detected depends, among other things, on specimen topography.

•By scanning the sample and collecting the secondary electrons that are emitted using a special detector, an image displaying the topography of the surface is created.

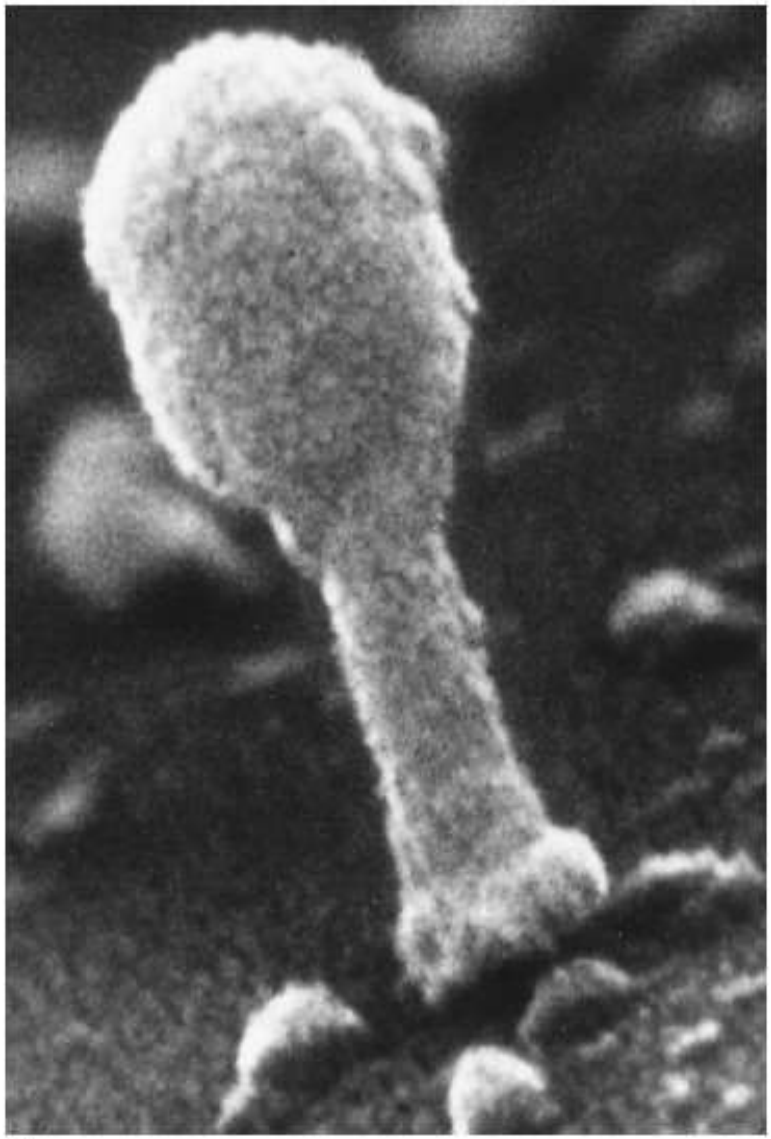
•Specimens can be observed in high vacuum, in low vacuum, in wet conditions (in environmental SEM), and at a wide range of cryogenic or elevated temperatures.

•In the SEM, we use much lower accelerating voltages to prevent beam penetration into the sample since what we require is generation of the secondary electrons from the true surface structure of a sample.

- The TEM has been exploited most widely in the examination of the internal structure of cells.

- In contrast, the scanning electron microscope (SEM) is utilized primarily to examine the surfaces of objects ranging in size from a virus to an animal head.

- SEM can achieve resolution better than 1nm.



(a)

(b)

Figure 18.19 **Scanning electron microscopy.** Scanning electron micrographs of (*a*) a T4 bacteriophage (×275,000) and (*b*) the head of an insect (×40). (A: FROM A. N. BROERS, B. J. PANESSA, AND J. F. GENNARO, SCIENCE 189:635, 1975; © 1975. REPRINTED WITH PERMISSION FROM AAAS; B: COURTESY OF H. F. HOWDEN AND L. E. C. LING.)

- In the TEM, electrons pass through the specimen to form the image.

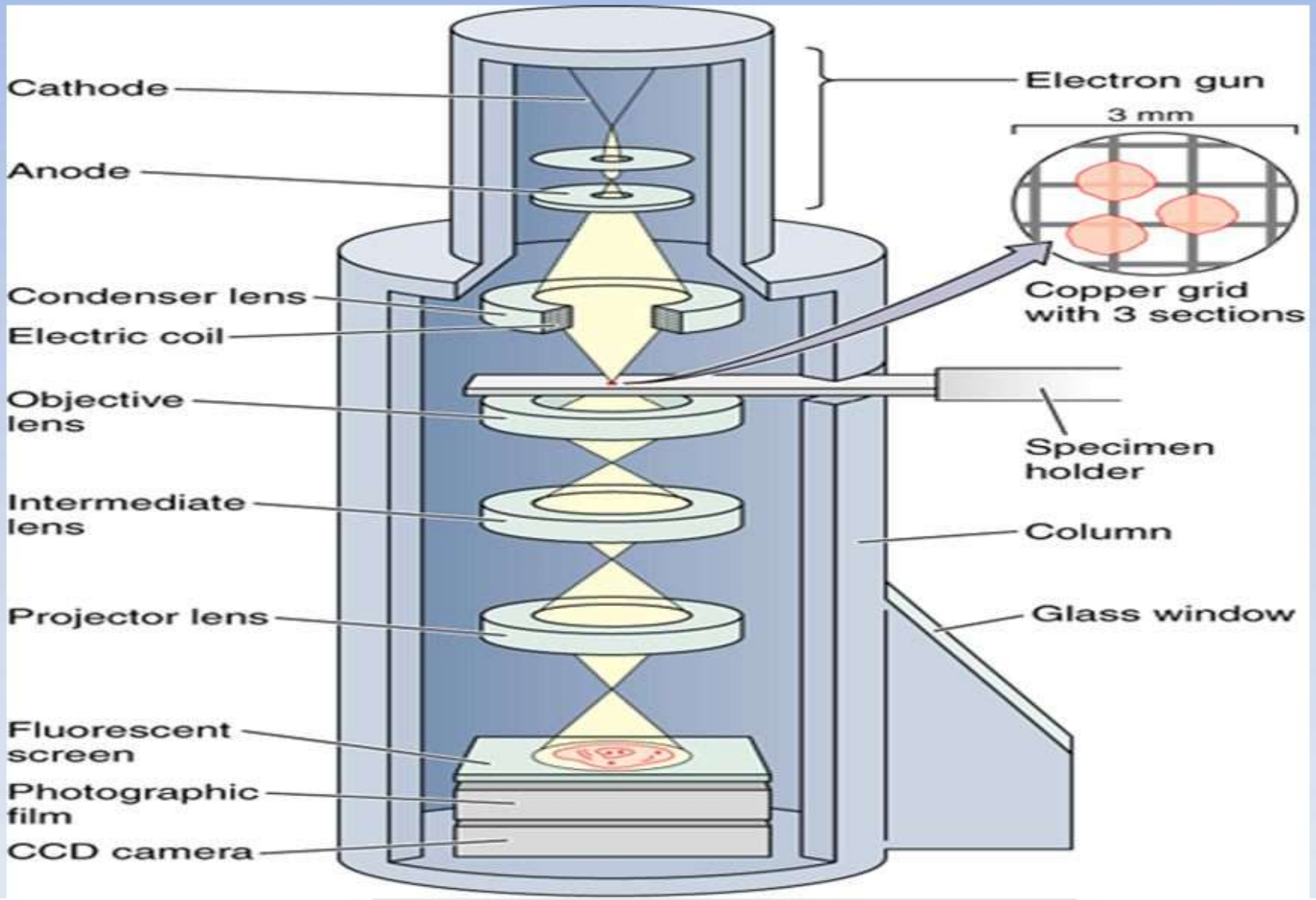
- In the SEM, the image is formed by electrons that are reflected back from the specimen (backscattered) or by secondary electrons given off by the specimen after being struck by the primary electron beam.

- These electrons strike a detector that is located near the surface of the specimen.

•The more electrons collected from the specimen at a given spot, the stronger the signal to the tube and the greater the intensity of the beam on the screen at the corresponding spot.

•The result is an image on the screen that reflects the surface topology of the specimen because it is this topology (the crevices, hills, and pits) that determines the number of electrons collected from the various parts of the surface.

Schematic view of a transmission electron microscope with its lenses and the pathway of the electrons.



A typical SEM instrument, showing the electron column, sample chamber, EDS detector, electronics console, and visual display monitors.

Scanning Tunneling Microscope (STM)

- A **scanning tunneling microscope** (**STM**) is an instrument for imaging surfaces at the atomic level.

- STM is a type of electron microscope that shows three-dimensional images of a sample.

- In the STM, the structure of a surface is studied using a stylus that scans the surface at a fixed distance from it.

- An extremely fine conducting probe is held close to the sample.

- Electrons tunnel between the surface and the stylus, producing an electrical signal.

- The stylus is extremely sharp, the tip being formed by one single atom.

- It slowly scans across the surface at a distance of only an atom's diameter.

•The stylus is raised and lowered in order to keep the signal constant and maintain the distance. This enables it to follow even the smallest details of the surface it is scanning.

•Recording the vertical movement of the stylus makes it possible to study the structure of the surface atom by atom.

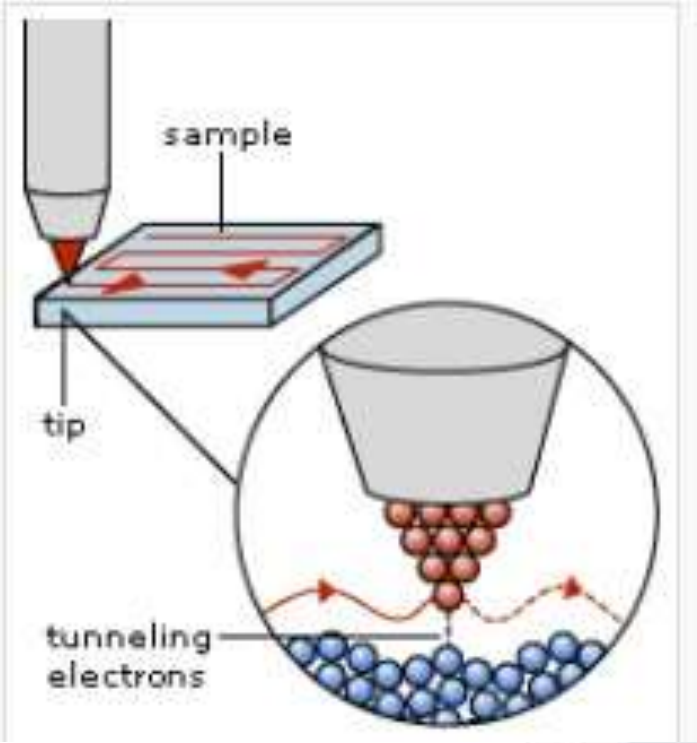
• A profile of the surface is created, and from that a computer-generated contour map of the surface is produced.

•The STM is based on the concept of quantum tunneling.

•When a conducting tip is brought very near to the surface to be examined, a bias (voltage difference) applied between the two can allow electrons to tunnel through the vacuum between them.

•The resulting *tunneling current* is a function of tip position, applied voltage, and the local density of states (LDOS) of the sample.

Information is acquired by monitoring the current as the tip's position scans across the surface, and is usually displayed in image form.



Specimen Preparation for Electron Microscopy

- As with the light microscope, tissues to be examined in the electron microscope must be fixed, embedded, and sectioned.

- Fixation of tissue for electron microscopy is much more critical than for light microscopy because the sections are subjected to much greater scrutiny.

- A fixative must stop the life of the cell without significantly altering the structure of that cell.

- At the level of resolution of the electron microscope, relatively minor damage, such as swollen mitochondria or ruptured endoplasmic reticulum, becomes very apparent.

- To obtain the most rapid fixation and the least cellular damage, very small pieces of tissue (less than 1.0 mm3) are fixed and embedded.

- Fixatives are chemicals that denature and precipitate cellular macromolecules.

- Chemicals having such action may cause the coagulation or precipitation of materials that had no structure in the living cell, leading to the formation of an artifact.

- The most common fixatives for electron microscopy are glutaraldehyde and osmium tetroxide.

- Glutaraldehyde is a 5-carbon compound with an aldehyde group at each end of the molecule.The aldehyde groups react with amino groups in proteins and cross-link the proteins into an insoluble network

- Osmium is a heavy metal that reacts primarily with fatty acids leading to the preservation of cellular membranes.

- Once the tissue has been fixed, the water is removed by dehydration in alcohol, and the tissue spaces are filled with a material that supports tissue sectioning.

- The demands of electron microscopy require the sections to be very thin. The wax sections cut for light microscopy are rarely thinner than about 5 µm, whereas sections for conventional electron microscopy are best when cut at less than 0.1 µm.

- Using ultra microtome the sections are cut by slowly bringing the plastic block down across an extremely sharp cutting edge made of cut glass or a finely polished diamond face.

- The sections coming off the knife edge float onto the surface of a trough of water that is contained just behind the knife edge.

- The sections are then picked up with the metal specimen grid and dried onto its surface.

- The tissue is stained by floating the grids on drops of heavy metal solutions, primarily uranyl acetate and lead citrate.

- These heavy metal atoms bind to macromolecules and provide the atomic density required to scatter the electron beam.

- In addition to the standard stains, tissue sections can be treated with metal-tagged antibodies or other materials that react with specific molecules in the tissue section.

Cryofixation and the Use of Frozen Specimens

- Cells and tissues do not have to be fixed with chemicals and embedded in plastic resins in order to be observed with the electron microscope.

- Alternatively, cells and tissues can be rapidly frozen. Just as a chemical fixative stops metabolic processes and preserves biological structure, so too does rapid freezing, which is called cryofixation

- Because cryofixation accomplishes these goals without altering the cell's macromolecules, it is less likely to lead to the formation of artifacts. Frozen cells do not have to be sectioned to reveal internal structure.

- To obtain the image, the computer aligns a large number of two-dimensional digital images of the cell that are captured as the specimen is tilted at defined angles relative to the axis of the electron beam. The three-dimensional, computational reconstruction is called a tomogram, and the technique is called cryoelectron tomography (cryo-ET).

Negative Staining

- The electron microscope is also well suited for examining very small particulate materials, such as viruses, ribosomes, multisubunit enzymes, cytoskeletal elements, and protein complexes.

- The shapes of individual proteins and nucleic acids can also be resolved as long as they are made to have sufficient contrast from their surroundings.

- One of the best ways to make such substances visible is to employ negative staining procedures in which heavy-metal deposits are collected everywhere on a specimen grid except where the particles are present. As a result, the specimen stands out by its relative brightness on the viewing screen

Shadow Casting

- Another technique to visualize isolated particles is to have the objects cast shadows.
- The grids containing the specimens are placed in a sealed chamber, which is then evacuated by vacuum pump.
- The chamber contains a filament composed ofa heavy metal (usually platinum) together with carbon.

- The filament is heated to high temperature, causing it to evaporate and deposit a metallic coat over accessible surfaces within the chamber.

- As a result, the metal is deposited on those surfaces facing the filament, while the opposite surfaces of the particles & the grid space in their shadow remain uncoated.

- When the grid is viewed in the electron microscope, the areas in shadow appear bright on the viewing screen, whereas the metal-coated regions appear dark.

- This relationship is reversed on the photographic plate, which is a negative of the image.

- The convention for illustrating shadowed specimens is to print a negative image in which the particle appears illuminated by a bright, white light (corresponding to the coated surface) with a dark shadow cast by the particle
- The technique provides excellent contrast and produces a three-dimensional effect.

Freeze-Fracture Replication and Freeze Etching

- As noted above, a number of electron microscopic techniques have been adapted to work with frozen tissues.
- The ultrastructure of frozen cells is often viewed using the technique of freeze fracture replication, Small pieces of tissue are placed on a small metal disk and rapidly frozen. T
- he disk is then mounted on a cooled stage within a vacuum chamber, and the frozen tissue block is struck by a knife edge

- The resulting fracture plane spreads out from the point of contact, splitting the tissue into two pieces.

- Consider what might happen as a fracture plane spreads through a cell containing a variety of organelles of different composition.

- These structures tend to cause deviations in the fracture plane, either upward or downward, giving the fracture face elevations, depressions, and ridges that reflect the contours of the protoplasm traversed. Consequently, the surfaces exposed by the fracture contain information about the contents of the cell.

- The goal is to make this information visible.
- The heavy metal is deposited onto the newly exposed surface of the frozen tissue in the same chamber where the tissue was fractured.
- The metal is deposited at an angle to provide shadows that accentuate local topography as described in the previous section on shadow casting.
- A carbon layer is then deposited on top of the metal layer to cement the patches of metal into a solid surface .

- Once this cast has been made, the tissue that provided the template can be thawed, removed, and discarded; it is the metal–carbon replica that is placed on the specimen grid and viewed in the electron microscope.

- Variations in thickness of the metal in different parts of the replica cause variations in the numbers of penetrating electrons to reach the viewing screen, producing the necessary contrast in the image.

- The fracture planes take the path of least resistance through the frozen block, which often carries them through the center of cellular membranes.

- As a result, this technique is particularly well suited for examining the distribution of integral membrane proteins as they span the lipid bilayer

- Freeze-fracture replication by itself is an extremely valuable technique, but it can be made even more informative by including a step called freeze etching.

- In this step, the frozen, fractured specimen, while still in place within the cold chamber, is exposed to a vacuum at an elevated temperature for one to a few minutes, during which a layer of ice can evaporate (sublime) from the exposed surface.

- Once some of the ice has been removed, the surface of the structure can be coated with heavy metal and carbon to create a metallic replica that reveals both the external surface and internal structure of cellular membranes.

- The deep-etching techniques, in which greater amounts of surface ice are removed, led to a fascinating look at cellular organelles.
- The technique delivers very high resolution and can be used to reveal the structure and the distribution of macromolecular complexes, such as those of the cytoskeleton, as they are presumed to exist within the living cell.

Atomic Force Microscope.

•The atomic force microscope (AFM) is a high-resolution scanning instrument.

•**AFM** also called **scanning-force microscopy** (**SFM**) is a type of scanning probe microscopy (SPM), with demonstrated resolution on the order of fractions of a nanometer, more than 1000 times better than the optical diffraction limit.

•The AFM operates by scanning a sharp, microsized tip (probe) over the surface of the specimen.

In one type of AFM, the probe is attached to a tiny oscillating beam (or cantilever),whose frequency of oscillations changes as the tip encounters variations in the topography of the specimen.

•These changes in oscillation of the beam can be converted into a three-dimensional topographic image of the surface of the specimen.

•The probe of an AFM can be used as more than a monitoring device; it can also be employed as a “nanomanipulator” to push or pull on the specimen in an attempt to measure various mechanical properties.

•In a different protocol, the AFM tip can be coated with ligands for a particular receptor, and measurements can be made of the affinity of that receptor for the ligand in question.

•Because of its many potential uses, the AFM has been referred to as “a lab on a tip.”