Lenses and the Bending of Light

Light microscopes were the first microscopes invented and they continue to be the most commonly used type. Therefore to understand light microscopy, we must consider the way lenses bendand focus light to form images. When a ray of light passes from one medium to another, refraction occurs; that is, the ray is bent at the interface. The refractive index is a measure of how greatly a substance slows the velocity of light; the direction and magnitude of bending are determined by the refractive indices of the two media forming the interface. For example, when light passes from air into glass, a medium with a greater refractive index, it is slowed and bent toward the normal, a line perpendicular to the surface.As light leaves glass and returns to air, a medium with a lower refractive index, it accelerates and is bent away from the normal. Thus a prism bends light because glass has a different refractive index from air and the light strikes its surface at an angle.

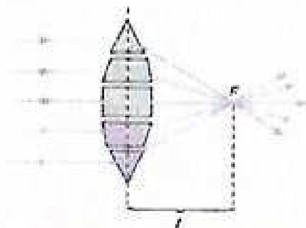


Figure 2.2 Lens Function. A lens functions somewhat like a collection of prisms. Light rays from a distant source are focused at the focal point F. The focal point lies a distance f, the focal length, from the lens center

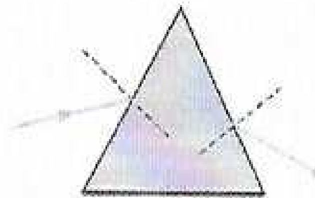


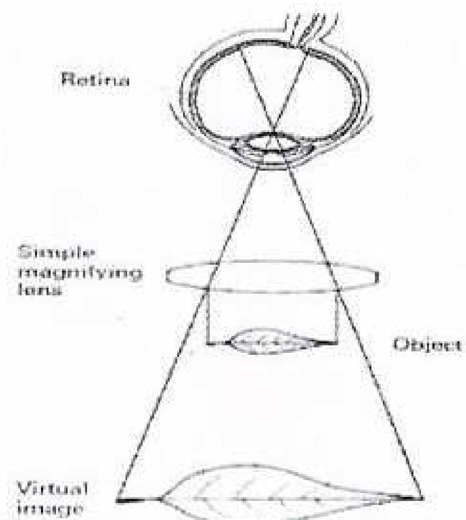
Figure 2.1 The Bending of Light by a Prism. Normals (lines perpendicular to the surface of the prism) are indicated by dashed lines. As light enters the glass, it is bent toward the first normal. When light leaves the glass and returns to air, it is bent away from the second normal. As a result, the prism bends light passing through it.

MAGNIFICATION

• The magnification or linear magnification of a microscope is defined as the ratio of the image size to the object (specimen) size.

• If the image and object are in the same medium, then it is just the image distance divided by the object distance.

• There is a difference in the meaning of the two terms, magnification and **magnifying power.**
Magnifying power or **angular magnification** is the ratio of the angle subtended by object and image.



The magnification of the image is determined by the magnification of the objective against the magnification of the eyepiece lens. The objectives have a magnification power of 40x-1000x depending on the type of brightfield microscope while the eyepiece lens has a standard magnification power of 10x.
Therefore to calculate:

Total Magnification power = Magnification of the objective lens x Magnification of the eyepiece

For example: if the magnification of the objective is 45x and that of the eyepiece is 10x, the total magnification of the specimen will be 450x.

The magnification is standard, i.e not too high nor too low, and therefore depending on the magnification power of the lenses, it will range between 40X and 100oX.

FACTORS AFFECTING MAGNIFICATION RESOLUTION AND NUMERICAL APERTURE

• The most important part of the microscope is the objective, which must produce a clear and magnified image
• Resolution is the ability of a lens to separate or distinguish between small objects that are close together.
• Much of the optical theory underlying microscope design was developed by the German physicist **Ernst Abbé**, in the 1870s.
• Resolution is described mathematically by an equation developed by Ernst Abbé, called **Abbé equation**

• The Abbé equation states that the minimal distance (d) between two objects that allows them to be seen as two separate entities, depends on the wavelength of light (λ) used to illuminate the specimen and on the numerical aperture of the lens ($n \sin \theta$), which is the ability of the lens to gather light. $d = 0.5\ \lambda / n \text{ Sin } \theta$
• As d becomes smaller, the resolution increases, and fine details can be observed in a specimen
• As d becomes smaller as the wavelength of light used decreases and as the numerical aperture (NA) increases.
• Thus the greatest resolution is obtained using a lens with the largest possible NA and light of the shortest wavelength

NUMERICAL APERTURE

The numerical aperture is the light gathering ability of a lens and is defined by two components: n is the refractive index of the medium in which the lens works (e.g., air) and θ is 1/2 the angle of the cone of light entering an objective.
• When this cone has a narrow angle **bcz working distance high** and tapers to a sharp point, it does not spread out much after leaving the slide and therefore does not properly separate images of closely packed objects.
• If the cone of light has a very wide angle and spreads out rapidly after passing through a specimen, closely packed objects appear widely separated and are resolved.
• The angle of the cone of light that can enter a lens depends on the refractive index (n) of the medium in which the lens works, as well as upon the objective itself.

• The refractive index for air is 1.00 and $\sin\theta$ cannot be greater than 1 (the maximum θ is 90° and sin 90° is 1.00).
Therefore, no lens working in air can have a numerical aperture greater than 1.00.

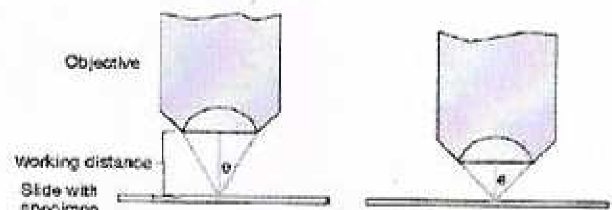
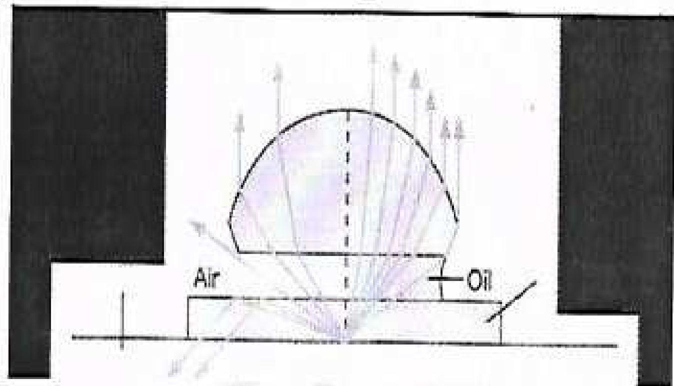


Figure 2.4 Numerical Aperture in Microscopy. The angular aperture θ is 1/2 the angle of the cone of light that enters a lens from a specimen, and the numerical aperture is $n \sin \theta$. In the right hand illustration, the lens has larger angular and numerical apertures; its resolution is greater and its working distance smaller.

IMMERSION OIL

In light microscopy, oil immersion is a technique used to increase the resolving power of a microscope. This is achieved by immersing both the objective lens and the specimen in a transparent oil of high refractive index, thereby increasing the numerical aperture of the objective lens.



The only practical way to raise the numerical aperture above 1.00, and therefore achieve higher resolution, is to increase the refractive index with immersion oil, a colorless liquid with the same refractive index as glass . If air is replaced with immersion oil, many light rays that did not enter the objective due to reflection and refraction at the surfaces of the objective lens and slide will now do so . This results in an increase in numerical aperture and resolution.

VARIOUS TYPES OF IMMERSION OILS

Before the development of synthetic immersion oils in the 1940s, cedar tree oil was widely used. Cedar oil has an index of refraction of approximately 1.516. The numerical aperture of cedar tree oil objectives is generally around 1.3. Cedar oil has a number of disadvantages however: it absorbs blue and ultraviolet light, yellows with age, has sufficient acidity to potentially damage objectives with repeated use (by attacking the cement used to join lenses), and diluting it with solvent changes its viscosity (and refraction index and dispersion). Cedar oil must be removed from the objective immediately after use before it can harden, since removing hardened cedar oil can damage the lens. In modern microscopy synthetic immersion oils are more commonly used, as they eliminate most of these problems. NA values of 1.6 can be achieved with different oils. Unlike natural oils synthetic ones do not harden on the lens and can typically be left on the objective for months at a time, although to best maintain a microscope it is best to remove the oil daily. Over time oil can enter for the front lens of the objective or into the barrel of the objective and damage the objective.

There are different types of immersion oils with different properties based on the type of microscopy you will be performing. Type A and Type B are both general purpose immersion oils with different viscosities. Type F immersion oil is best used for fluorescent imaging at room temperature (23 °C), while type N oil is made to be used at body temperature (37 °C) for live cell imaging applications.

Limits of Resolution:

The maximum theoretical resolving power of a microscope when viewing a specimen using an oil immersion objective (numerical aperture of 1.25) and blue-green light is approximately 0.2 µm

$$d = \frac{(0.5)(530\ \text{nm})}{1.25} = 212\ \text{nm or } 0.2\ \mu\text{m}$$

WORKING DISTANCE:

Numerical aperture is related to another characteristic of an objective lens, the working distance. The working distance of an objective is the distance between the surface of the lens and the surface of the cover glass (if one is used) or the specimen when it is in sharp focus. Objectives with large numerical apertures and great resolving power have short working distances.

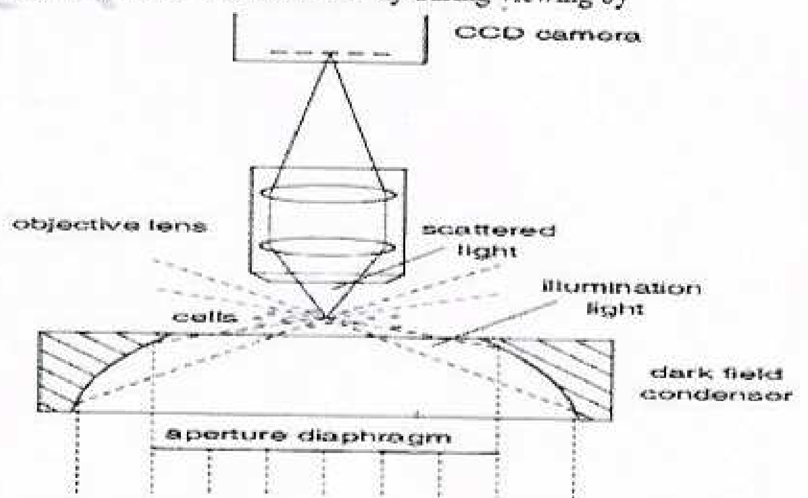
BRIGHT FIELD MICROSCOPY

PRINCIPLE: Brightfield microscopy is the most elementary form of microscope illumination techniques and is generally used with compound microscopes.

The name "brightfield" is derived from the fact that the specimen is dark and contrasted by the surrounding bright viewing field. Simple light microscopes are sometimes referred to as brightfield microscopes.

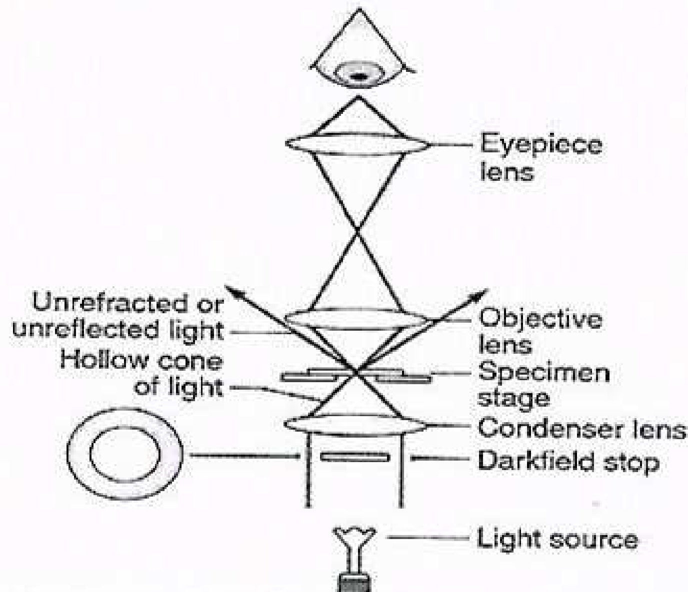
- The microscope consists of a sturdy metal body or stand composed of a base and an arm to which the remaining parts are attached.
- A **light source**, either a mirror or an electric illuminator, is located at the base.
- Two focusing knobs, the **fine and coarse adjustment knobs**, are located on the arm and can move either the stage or the nosepiece to focus the image.
- The stage is positioned about halfway up the arm and holds microscope slides by either simple slide clips or a mechanical stage clip.

- A mechanical stage allows the operator to move a slide around smoothly during viewing by use of stage control knobs.
- The substage condenser is mounted within or beneath the stage and focuses a cone of light on the slide.
- Its position often is fixed in simpler microscopes but can be adjusted vertically in more advanced models.
- The curved upper part of the arm holds the ordinary body assembly, to which a nosepiece and one or more eyepieces or oculars are attached.
- More advanced microscopes have eyepieces for both eyes and are called **binocular microscopes.**
- The body assembly itself contains a series of mirrors and prisms so that the barrel holding the eyepiece may be tilted for ease in viewing.
- It is opposite to Dark field microscopy in which a brighter image is formed against a darker Background



DARK FIELD MICROSCOPY

- The dark-field microscope allows a viewer to observe living, **unstained cells** and organisms
- It produces images of **brightly illuminated** objects on a black background.
- Only light that has been reflected or refracted by the specimen forms an image.
- A hollow cone of light is focused on the specimen in such a way that unreflected and unrefracted rays do not enter the objective.
- This technique has traditionally been used for viewing the outlines of objects in liquid media such as living spermatozoa, microorganisms, cells



- For lower magnifications, a simple darkfield setting on the condenser will be sufficient.
- For more critical darkfield imaging at a higher magnification, a **darkfield condenser** with a darkfield **objective lens** will be required.
- If the object surface is of uneven thickness, hence different portions in an object absorb light to different extent then Phase contrast Microscopy will be used.

Principle:

It creates a contrast between the object and the surrounding field so that the back ground is dark and the object is bright. The objective and the ocular lenses are used in this microscope are same like ordinary microscope but a special condenser is used that prevent the transmitted light from directly illuminating the specimen. Only oblique scattered light reaches the specimen and passes on to the lens and causing the bright objects against dark Background.

Applications:

1. It also is used to identify certain bacteria like the thin and distinctively shaped Treponema pallidum, the causative agent of syphilis.
2. Viewing bacteria, algae and blood cells.
3. Viewing hair line metal fracture.

Advantages:

1. It is ideal for viewing unstained, transparent and little absorbed objects.
2. It is ideal to study marine organisms such as diatoms, algae, plankton etc.
3. It is used for research on live bacterium, mounted cells and tissues.
4. It is used to examine external details like out lines, edges, grain boundaries etc.

Disadvantages:

1. The images are prone to degradation and distortion.
2. It needs an intense amount of light to work.
3. It is not reliable tool to obtain accurate measurement of specimens.
4. If oil or water is used on the condenser then it is impossible to avoid air bubbles on slides.

Definition of Phase Contrast Microscopy

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A phase contrast microscopy converts slight differences in refractive index and cell density into easily detected variation in light intensity to observe living cells.

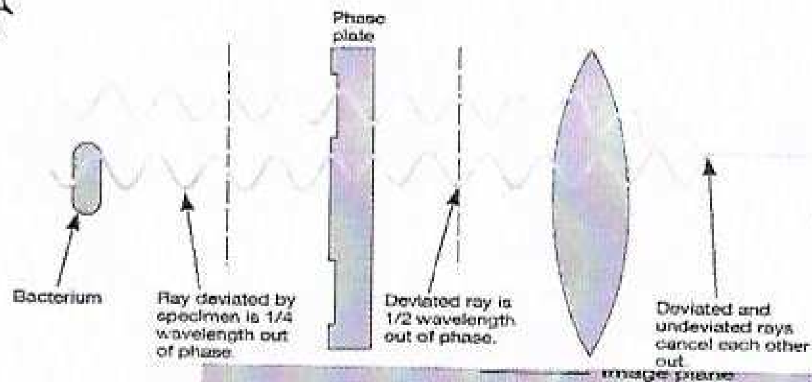
This microscope is used for visualization of cell culture and live cells. Living cells can be observed without any staining.

Unstained specimens have absorbed no light, as a result it creates extremely small differences in the intensity distribution in the image. Therefore in a bright field microscope, the specimen is not clearly visualized. Because a small phase shifts occurred, when light passes through specimens, which we can't see with our eyes.

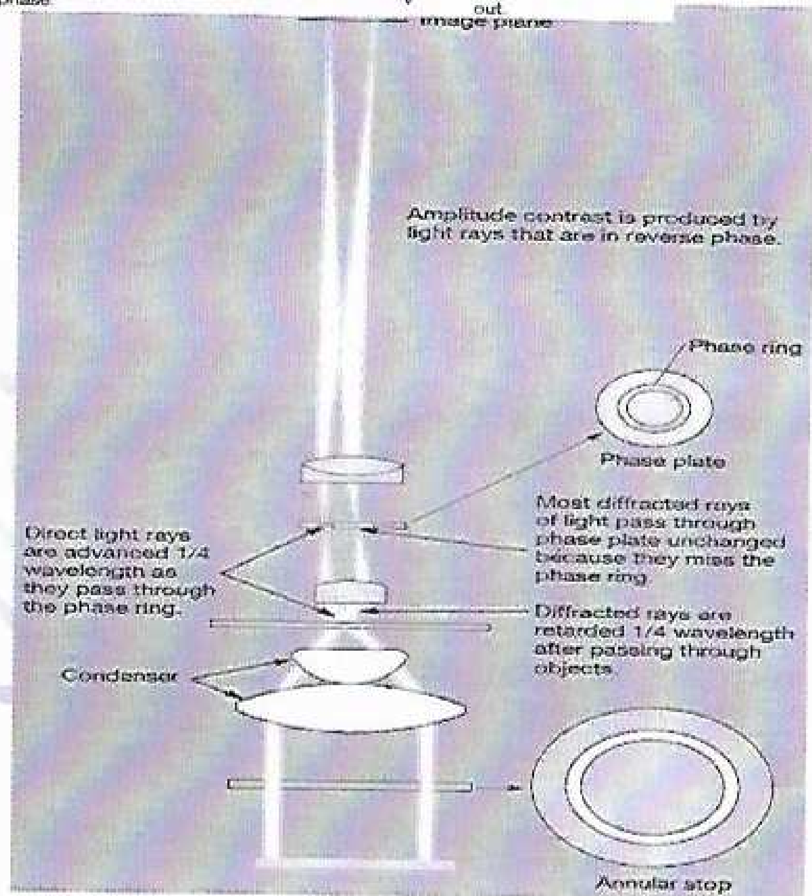
In a phase contrast microscopy, these phase shifts are transformed into changes in amplitude, which can be observed as differences in image contrast.

Principle of Phase Contrast Microscope

The condenser of a phase-contrast microscope has an annular stop an opaque disk with a thin transparent ring that produces a hollow cone of light.
As this cone passes through a cell some light rays are bent due to variation in density and refractive index within the specimen and are retarded by 1/4 wavelength. The deviated light is focused to form an image of the object.
The undeviated light rays strike a phase ring in the phase plate a special optical disks located in the objective, while the deviated rays miss the ring and passed through the rest of the plate.
The undeviated light which strikes the phase ring gets advance by 1/4 wavelength when passing through this ring.
The deviated and undeviated waves become 1/2 wavelength to each other and will cancel each other to come together to form an image. Therefore deviated and undeviated lights from different image.
The background formed by undeviated light is bright while the unstained object appears dark and well-defined.



Light Path of Phase Contrast Microscope



The light rays enter the annular diaphragm from its source.

Then it passes through the condenser lens, which focused the rays on the specimen.

The light transmitted through the specimen and then enter the objective lens where an image of the specimen is created.

As the light transmitted through the specimen it creates a deviated and undeviated light rays.

The deviated light rays miss the phase ring over the objective lens. Whereas the undeviated light rays strike a phase ring. As a result, deviated and undeviated rays formed different images.

The undeviated light rays formed the background of specimen's image.

Parts of Phase Contrast Microscope

A phase contrast microscope is basically a modified form of a simple microscope. It contains all the components of a normal microscope, except for two important parts that are missing in a simple light microscope such as, Annular diaphragm and Phase plate. This two component helps to form the phase contrast, by separating the direct rays from the diffracted rays.

Annular diaphragm:

Annular diaphragm is consists of a circular disc that had a circular annular groove. This annular groove passes the light rays to the object.

- It is located under the condenser lens.

Phase plate

- It is a transparent disc.
- There are present two types of phase plates such as the positive phase plate and the negative phase plate.
- The positive phase plate contain a thick circular area where the negative phase plate contain a thin circular groove.
- These thin and thin areas in phase plate called conjugate area.
- The direct light rays and diffracted light are basically passed through the annular groove and through the region outside the groove.

What is the negative and positive phase contrast?

- In the positive phase contrast, the object appears as dark gray on a brighter grey background.
- In the negative phase contrast, the object appears as brighter on a dark background.

Applications of Phase Contrast Microscope

1. Phase contrast microscopy is specially useful for the detection of bacterial components such as endospore and inclusion bodies.
2. Phase contrast microscopy also are widely used in studying Eukaryotic cells.
3. It also used to visualize a thin tissue slice.

Advantages

- Living cells are observed in the natural state. It will provide more information about the specimen than specimens that need to be killed, fixed or stain to view under a microscope.
- It produces a resolution and high contrast images of the specimen.
- It is useful for studying on thin specimen.
- The modern form of this microscope can capture photos or can record videos.
- Phase Contrast Microscopy can create a visible image of a highly transparent objects.

- No need for staining and fixation of the specimen.
- Intercellular components of living cells can be observed with high resolution.

Disadvantages

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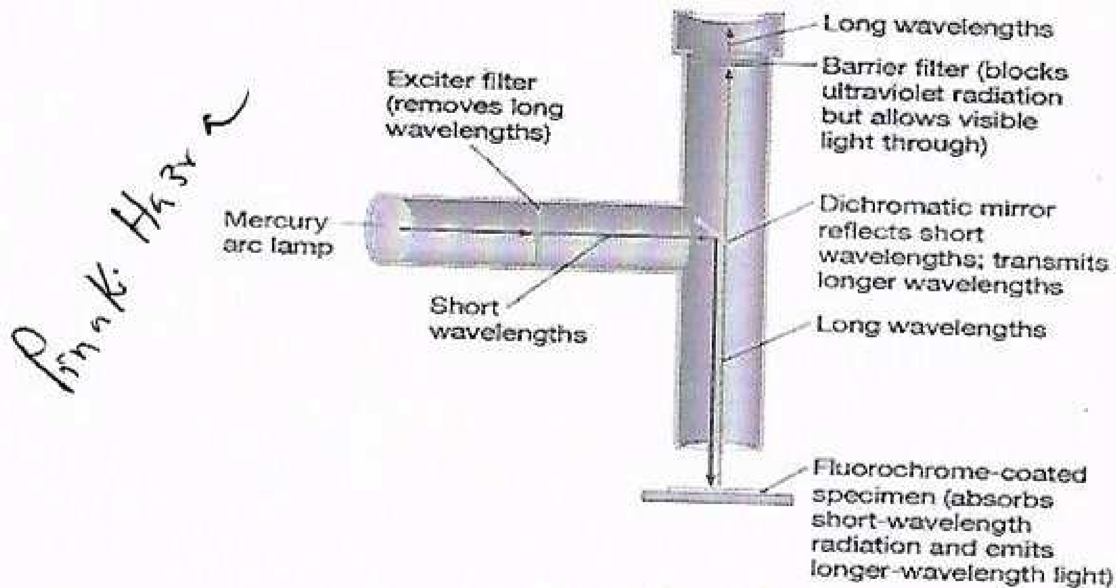
- The Annuli or rings of phase contrast microscope limit the aperture to some extent, which decreases the resolution of image.
- Thick organisms or specimens can not be observed by this microscope, a distorted image can appear.
- With the uses of white or green lights Images appear as grey or green, which results a poor photomicrography.
- Shade-off and halo effects can occur in this microscope.

FLUORESCENT MICROSCOPY

• Fluorescence microscopy is currently the most widely used contrast technique.
• The most commonly used fluorescence technique is called **epifluorescence light microscopy,** where 'epi' simply means 'from above'.
• Here, the light source comes from above the sample, and the objective lens acts as both condenser and objective lens.
• Fluorescence is popular because of the ability to achieve highly **specific labelling** of cellular compartments.
• The images usually consist of distinct regions of fluorescence (white) over large regions of no fluorescence (black), which gives excellent signal-to-noise ratios.
• The light source is usually a high pressure **mercury or xenon vapour lamp**, which emits from the ultraviolet into the red wavelengths.
• A specific wavelength of light is used to excite a fluorescent molecule or **fluorophore** in the specimen
• Light of longer wavelength from the excitation of the fluorophore is then imaged.
• This is achieved in the fluorescence microscope using combinations of **filters** that are specific for the excitation and emission characteristics of the fluorophore of interest.
• There are usually three main filters: an **exciter filter**, a **dichromatic mirror** (often called a **dichroic)** and a **barrier filter**, mounted in a single housing above the objective lens.
• The **exciter filter** transmits only the desired wavelength of excitation light.
• The **dichromatic mirror** reflects light of shorter wavelengths (i.e., the excitation light) but allows light of longer wavelengths to pass through.
• The **barrier filter** blocks ultraviolet radiation but allows visible light to pass through.
• For very tiny specimen (eg. viruses), the detailed internal structure can be observed only with electron microscopes.



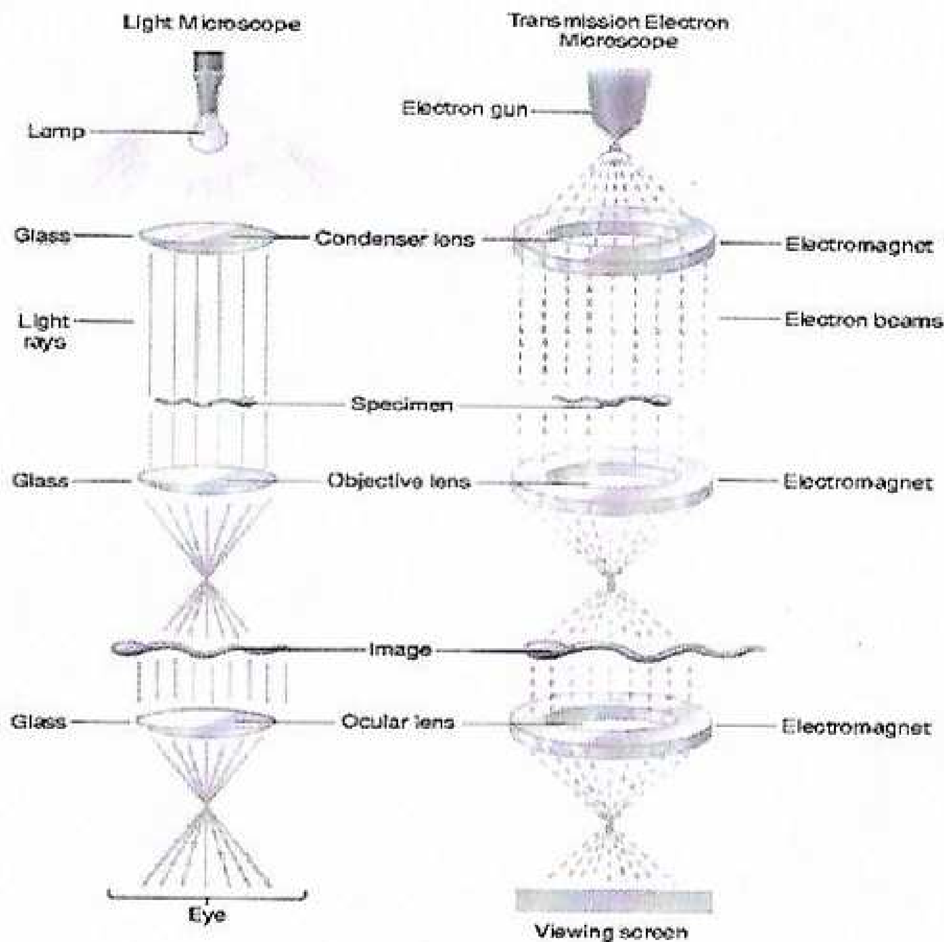
ELECTRON MICROSCOPY

- The light microscopes are most important instrument for studying microorganisms.
- But even the best light microscopes have a resolution of only 0.2 µm
- Hence, they cannot be used for detailed studies of many microorganisms.
- Viruses, for example, are too small to be seen with light microscopes.
- Prokaryotes can be observed, but only their general shape and major morphological features are visible.
- The detailed internal structure of larger microorganisms also cannot be effectively studied by light microscopy.
- These overcome these limitations, Electron microscopes have been used that have much greater resolution.
- Electron Microscopes are scientific instruments that use a beam of highly energetic electrons to examine objects on a very fine scale.
- The electron microscope is able to achieve greater magnification and resolution because it uses a high voltage beam of electrons, whose wavelength is very much shorter than that of visible light.
- As a result, it is able to resolve points that are much closer together than is possible even with the very best light microscope.
- The resolving power of an electron microscope may be as low as 1–2 nm, enabling visualization of viruses and the internal structure of several cells.
- The greatly improved resolution means that specimens can be successfully magnified over 100000 times.

TRANSMISSION ELECTRON MICROSCOPY

- The transmission electron microscope (TEM) operates on the same basic principles as the light microscope but uses **electrons** instead of light.

- It consists largely of a tall, hollow cylindrical column through which the electron beam passes and a console containing a panel of dials that electronically control the operation in the column.
- The top of the column contains the **cathode, a tungsten wire filament** that is heated to provide a source of electrons.
- Electrons are drawn from the hot filament and **accelerated** as a fine beam by the high voltage applied between the cathode and anode.
- Air is pumped out of the column prior to operation, producing a vacuum through which the electrons travel.
- A beam of negatively charged electrons is focused by **electromagnetic lenses**, which are located in the wall of the column.
- The strength of the magnets is controlled by the current provided them, which is determined by the positions of the various dials of the console.
- The **condenser lenses** are placed between the electron source and the specimen, and they focus the electron beam on the specimen.
- The specimen is supported on a small, thin metal grid (3 mm diameter) that is inserted with **tweezers** into a grid holder, which is inserted into the column of the microscope.
- For examination of surface of microorganisms in detail, scanning electron microscope would be employed, that produce an image from electrons released from atoms on an object's surface.



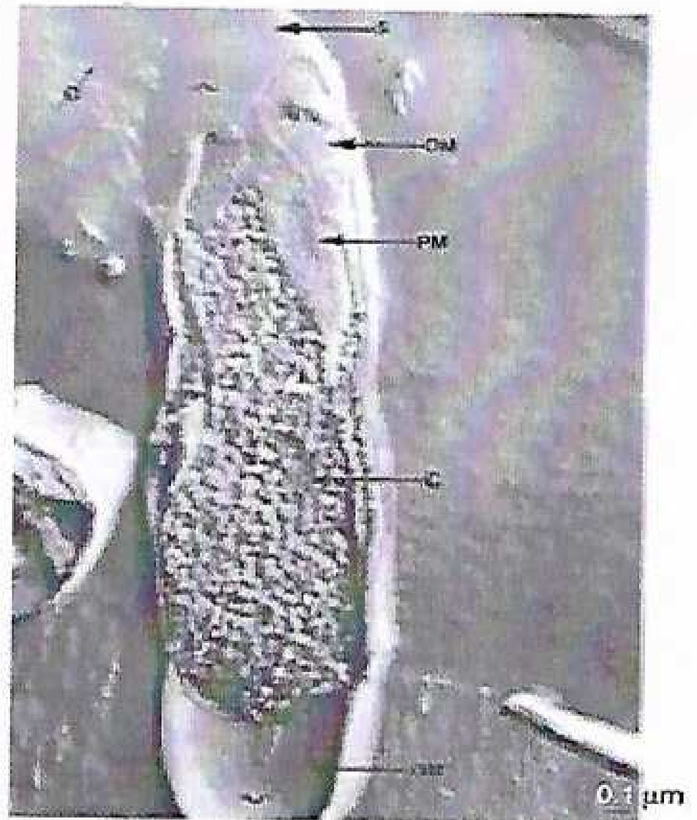
Sample Preparation:

As with bright-field light microscopy, cells usually must be stained before they can be seen clearly with a TEM. The probability of electron scattering is determined by the density (atomic number) of atoms in the specimen. Biological molecules are composed primarily of atoms with low atomic numbers (H, C, N, and 0), and electron scattering is fairly constant throughout an unstained cell or virus. Therefore specimens are prepared for observation by soaking thin sections with solutions of heavy metal salts such as lead citrate and uranyl acetate. The lead and uranium ions bind to structures in the specimen and make them more electron opaque, thus increasing contrast in the material. Heavy osmium atoms from the osmium tetroxide fixative also stain specimens and increase their contrast. The stained thin sections are then mounted on tiny copper grids and viewed.

Two other important techniques for preparing specimens are **negative staining and shadowing. In negative staining,** the specimen is spread out in a thin film with either phosphotungstic acid or uranyl acetate. Just as in negative staining for light microscopy, heavy metals do not penetrate the specimen but render the background dark, whereas the specimen ap pears bright in photographs. Negative staining is an excellent way to study the structure of virus particles, bacterial gas vacuoles, and other similar objects **. In shadowing**, a specimen is coated with a thin film of platinum or other heavy metal by evaporation at an angle of about 45° from horizontal so that the metal strikes the microorganism on only one side. In one commonly used imaging method, the area coated with metal appears dark in photographs, whereas the uncoated side and the shadow region created by the object are light. **This technique is particularly useful in studying virus particle morphology, bacterial and archaeal flagella, and DNA.**

Freeze-etching procedure:

The shapes of organelles within cells can be observed by TEM if specimens are prepared by the freeze-etching procedure. When cells are rapidly frozen in liquid nitrogen, they become very brittle and can be broken along lines of greatest weakness, usually down the middle of internal membranes. The exposed surfaces are then shadowed and coated with layers of platinum and carbon to form a replica of the surface. After the specimen has been removed chemically this replica is studied in the TEM, providing a detailed view of intracellular structure. **An advantage of freeze-etching** is that it
minimizes the danger of artifacts because the cells are frozen quickly, rather than being subjected to chemical fixation, dehydration, and embedding in plastic.



The scanning electron microscope (SEM)

• The scanning electron microscope (SEM) is utilized primarily to examine the surfaces of objects ranging in size from a virus to an animal head.
• The construction and operation of the SEM are very different from that of the TEM.
• Specimens to be examined in the SEM are fixed, passed through a series of alcohols, and then dried by a process of **critical-point drying.**
• At this point, there is no surface tension between the gas and the liquid.
• The solvent of the cells is replaced with a liquid transitional fluid (generally carbon dioxide), which is vaporized under pressure so that the cells are not exposed to any surface tension that might distort their three-dimensional configuration.
• Once the specimen is dried, it is coated with a thin layer of metal, which makes it suitable as a target for an electron beam.
• **Electrons** are **accelerated** as a fine beam that scans the specimen.
• 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.
• Image formation in the SEM is **indirect.**
• In addition to the beam that scans the surface of the specimen, another electron beam synchronously scans the face of a **cathode-ray tube**, producing an image similar to that seen on a television screen. The electrons that bounce off the specimen and reach the detector control the strength of the beam in the cathode-ray tube.
• 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 that determines the number of electrons collected from the various parts of the surface.

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