Unit – 2 Microscopy

Microscope is a basic tool of Microbiology. It magnifies the size of an apparent image of an object. Based on intended use, microbiologists employ specific type of microscope.

Microscope is basically grouped in main two groups based on source of illumination. These are -

1. Light Microscope: in which sunlight or artificial light is used for illumination. It is further divided as -

A. Bright field Microscope: In this an object appears dark coloured as against bright background.

B. Dark field Microscope: In this brightly illuminated object appears against dark field.

C. Phase Contrast Microscope: It is useful for visualization of an object in living condition.

D. Fluorescence Microscope: It is useful for visualization of objects that are stained with fluorescent dyes.

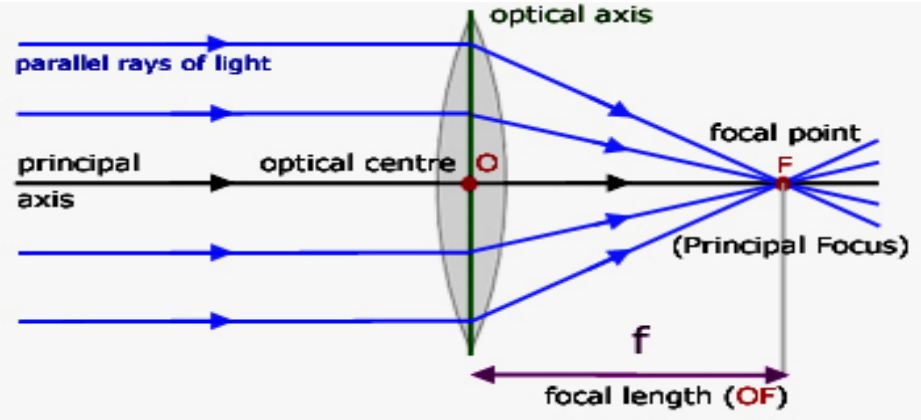
2. Electron Microscope: It is the type of microscope that is used for observation of finer details. In this microscope, instead of visible light, electrons are used for illumination. It has better resolution and high magnifying power.

To understand how does a microscope operate? Let us recall concepts of optics.

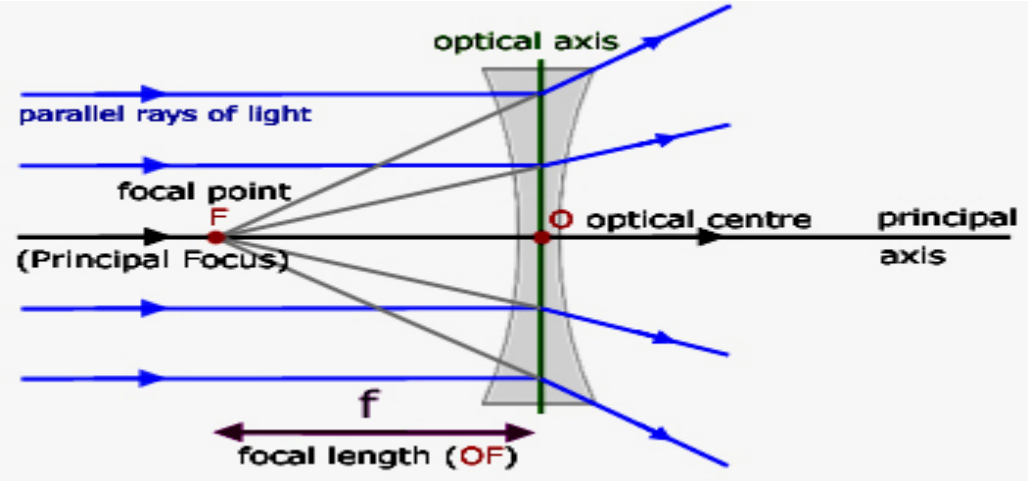
Concepts of Optics

1. **A lens** is an optical device made up of transparent material (glass) bounded by two spherical surfaces (surfaces may be convex or concave).

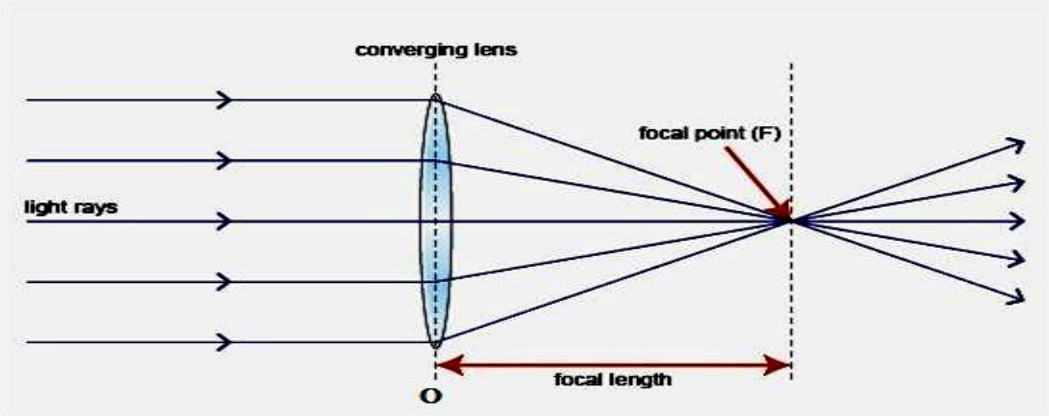
2. **Convex lens or a converging or convergent lens -** when parallel rays **pass** through a convex lens, the emerging rays converge (come together) at point F, while ray going through an optical centre emerges without bending. It forms a **real** image of the object.



3. **Concave lens or diverging or divergent lens-** Parallel rays after passing through a concave lens diverge (move away). It forms a **virtual** image of the object.



4. **Focal point (F):** It is a point at which emerging rays from a convex lens meet or in other words when parallel rays of light strike a convex **lens,** the emerging rays converges (come together) at a point called as focal point.



5. **Focal length:** The distance between an optical centre (O) of lens and focal **point (F)** is called Focal Length.

6. **Refraction:** Light rays when passing from one medium to another, **bent at** interface (border) then it is known as Refraction.

7. **Diffraction:** Bending of light rays at corners when light wave is obstructed (blocked), it is called as *Diffraction.* Thus, speed of light changes, when it passes from medium or even when it strikes on any obstacle / obstruction (blockage).

8. **Refractive index(RI) μ:** A measure of change of velocity of light is Refractive Index (RI) denoted by μ

$$\mu = \frac{\text{Speed of light in vacuum}}{\text{Speed of light in material}}$$

R-I is also a measure of direction and magnitude of changing.

When light passes from air into glass (with greater RI) the ray is slowed down and bent **towards** normal. But when it passes from glass to air, it accelerates and bent **away** from normal.

When we view an object with naked eyes, the object must be placed somewhere between infinity and nearer point. The maximum angle subtended on the eye, when the object is placed at near point is given as θ_o.

This angle can be further increased by using short focal length, converging lens, placed just before the eye. Such converging lens is called as Simple Microscope or Magnifier. It gives good magnification.

Early microscopes, used by Hooke and Leeuwenhoek were simple microscopes, of just a single lens. In the late nineteenth century major advances in microscopy were made.

Ernst Abbe developed lenses that have corrected earlier problems of chromatic aberrations. He had also developed, oil immersion lens that allows good resolution. By 1900, light microscope has reached a state of technical development that characterizes light microscopes of today.

❖ Image Formation:

In case of compound microscope, two converging lenses are used. These are arranged coaxially - one nearer to the object is an **'objective'** and another close to the eye is an **'eyepiece'**. Objective lens has smaller aperture and smaller focal length than eyepiece.

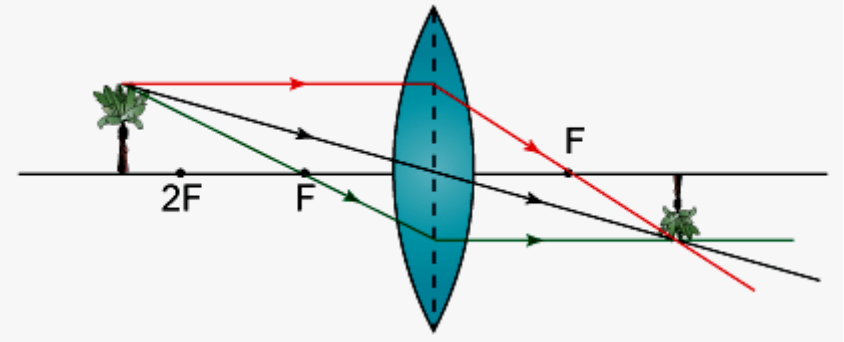
Converging lenses are often used to produce images that are **magnified**. The amount of magnification depends on:

- how curved the surface of the lens is
- how close the lenses are placed.

There are two types of image that can be seen. A **real image** is the image formed where the light rays are focussed. A **virtual image** is one from which the light rays appear to come but don't actually come from that image like in a mirror.

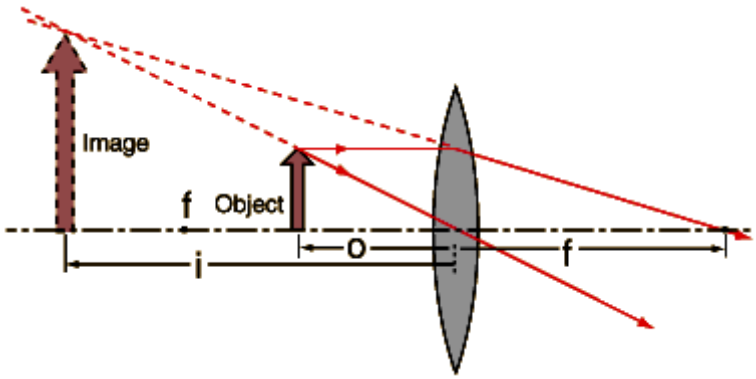
In compound microscope, separation of objective and eyepiece is achieved by means of screws. It forms two images -

1. An image formed by an objective is ***Real and Inverted***. It is thus formed because an object is placed at a distance slightly higher than the focal length of an objective. This image is called as Primary image.

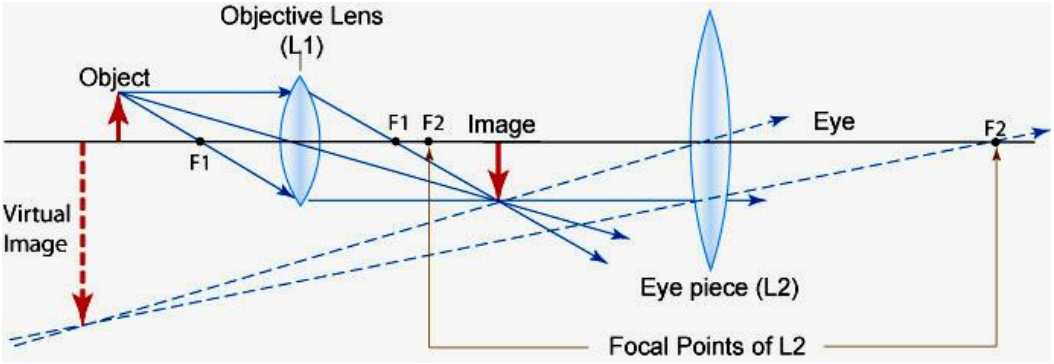


2. Second type of image is formed by an eyepiece. For eyepiece the primary image (formed by an objective) acts as an object - which falls inside focal plane of eyepiece (This is achieved by adjusting microscope.)

Image is formed at infinity. It is virtual and erect. It is the final image of the object.



In compound microscope thus, object is kept outside F of an objective and image formed by objective is placed inside F of eyepiece.



Thus, eyepiece acts as a simple microscope i.e. magnifying further image of the object.

The object is mounted on the stand below the microscope tube. The objective lens forms a real, inverted and magnified image (I_1) of the object. The image I_1 acts as an object for the eye piece. The position of the eyepiece is so adjusted that the image lies within the focus of the eyepiece (F_e). The eyepiece acts like a magnifying glass and forms a virtual erect and magnified image of the object.

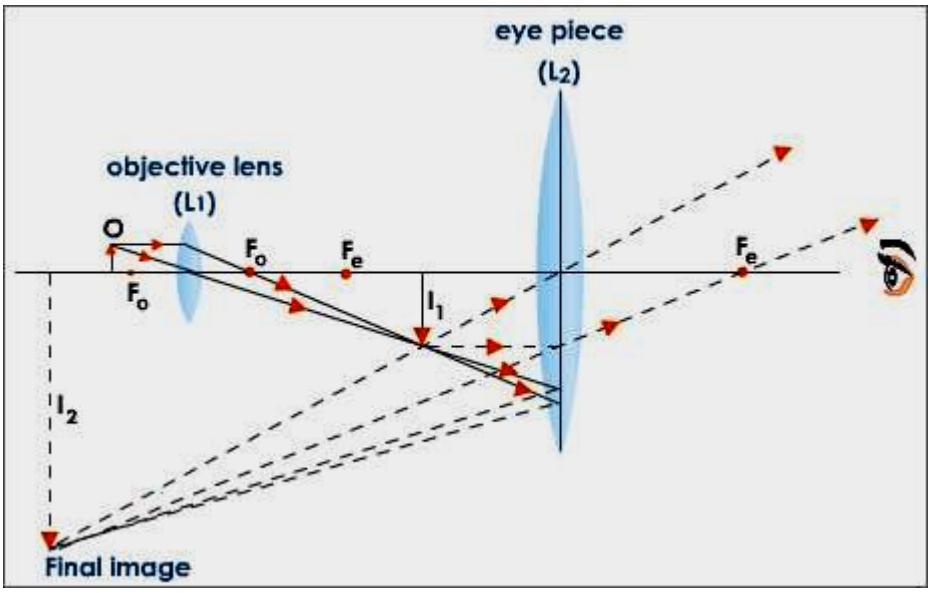


Image Formation in a Compound Microscope

- The object (O) is placed just outside F_o, the principal focus of the objective lens.
- F_e is the principal focus of the eye lens.
- A real, inverted magnified image I_1 is formed. The magnified image I_1 acts as an object for the eye lens.

- The final image I_2 is virtual and is magnified still further. It is inverted compared with the object. I_2 may appear 1000 times larger than the object.

Magnification / Magnifying Power of a Compound Microscope

The magnifying power of a compound microscope is defined as the ratio of the size of the final image (I_2) as seen through the microscope to the size of the object as seen with a naked eye.

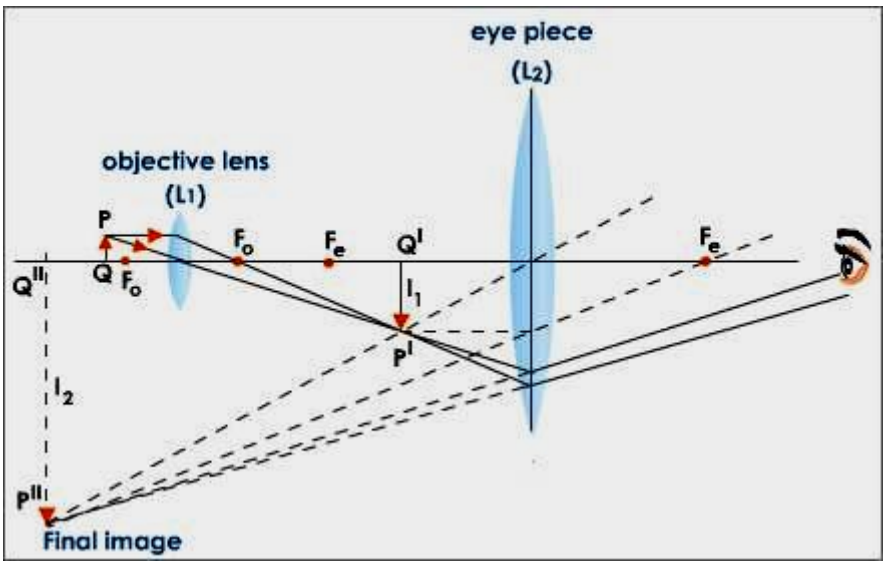


Image Formation in a Compound Microscope

$$m = \frac{\text{size of the final image as seen through the microscope}}{\text{size of the object as seen with a naked eye}}$$

$$m = \frac{h_I}{h_O} = \frac{P'Q'}{PQ}$$

$$= \frac{P'Q'}{PQ} \times \frac{P'Q''}{P'Q'}$$

$$= m_{objective} \times m_{eyepiece}$$

$$= m_o \times m_e$$

Where $m_{objective}$ (m_o) and $m_{eyepiece}$ (m_e) are the magnification produced by the objective and eyepiece respectively.

$$m = m_o \times m_e$$

Magnification is the degree of enlargement i.e. microscope should produce an enlarged image of the object. Magnification is dependent upon -
(a) Distance of object from focal point - closer the object to lens higher is the magnification.
(b) Curvature of lens - smaller the curvature larger is the magnification.

In true sense, magnification of compound microscope is the product of magnifying power of an objective and eyepiece.

When object is seen with naked eye, largest image is formed on retina provided it is near to the eye.

Practically compound microscope is provided with objectives with l0x, 40 x, 100 x magnifying power

10 x = low power
40 X = high power
100 X = oil immersion lens
and an eyepiece with 10 x magnifying power.

Total Magnification = M.P. of objective X M.P. of eyepiece

= 100 X x 10 X = 1000 X

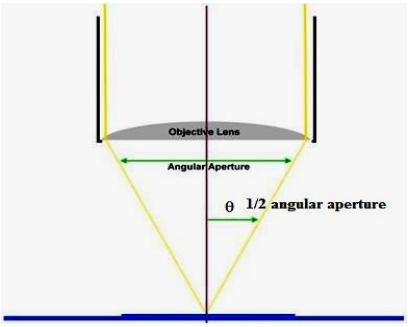
Thus, total magnification achieved is 1000 X.

Ideally, microscopes are to be parfocal *i.e.* once specimen is focused with one lens, it should remain in focus for other lenses. (Refocusing is not needed while switching over to other lens.). But such microscopes are not available.

Working distance is calculated by counting number of turns of fine adjustments required to raise the objective from surface of slide to a point where object is in sharp focus.

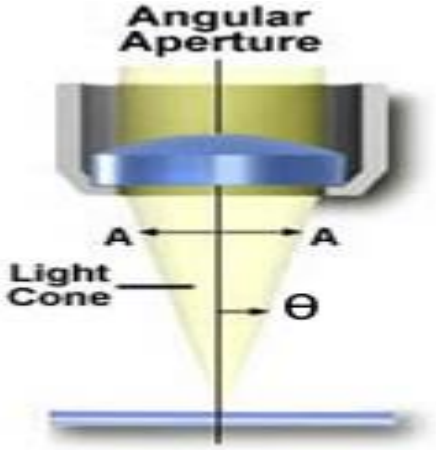
❖ Aperture angle or Angular Aperture:

It is the angle of the cone of light that can enter the lens.



❖ Numerical aperture:

The numerical aperture of a microscope objective is a measure of its ability to gather light and resolve fine specimen detail at a fixed object distance through a specific media (e.g. air, water, immersion oil). As shown in the figure, light from the specimen enter the objective in an inverted cone. Longitudinal portion of this cone of light shows the angular aperture (A), a value that is determined by the focal length of the objective. The half angle of the cone of light entering an objective is known as theta (θ).



θ= one-half of the angular aperture (A)

N**umerical Aperture (**NA) is calculated by the following equation:

$$NA = n (\sin \theta)$$

Where n is the value of the refractive index of the medium between the specimen (the cover glass) and the objective. The value of n is between 1.0 (for air) up to 1.51 (for special immersion oil).

When imaging through air then n=1 and so NA depends solely on the sin of θ which theoretically can be a maximum of 1 (sin90°=1). In practice, it is difficult to achieve NA>0.95 with "dry" microscopy (i.e. through air).Therefore, many microscope objectives (mostly for high magnifications) are designed to be used with liquid paraffin (n= 1.4), glycerin (n=1.47) or immersion oil (n=1.51).

❖ Resolving power

It is defined as an ability to distinguish between two particles situated very close.

The resolving power of an objective lens is measured by its ability to differentiate two lines or points in an object. The greater the resolving power, the smaller the minimum distance between two lines or points that can still be distinguished. The larger the N.A., the higher the resolving power.

$$d = \frac{0.5\lambda}{n\sin\theta} = \frac{\lambda}{NA(Objective) + NA(Condenser)}$$

d = Resolving Power
λ = Wavelength of light
n = Refractive index of the medium
NA= Numerical Aperture

Resolution is a subjective value in microscopy because at high magnification, an image may appear not very sharp but still it can be resolved to the maximum ability of the objective. Numerical aperture is defined as the resolving power of an objective, but the entire resolution of a

microscope system is also depends on the numerical aperture of the sub stage condenser.

Abbe equation is used to calculate the limit set on the resolution of light microscope. The oil immersion objective gives the maximum theoretical resolving power of the microscope, the numerical aperture of 1.25 and blue-green light is around 0.2µm.

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

The bright-field microscope can distinguish between two dots approximately 0.2 µm apart (the same size as a very small bacterium).

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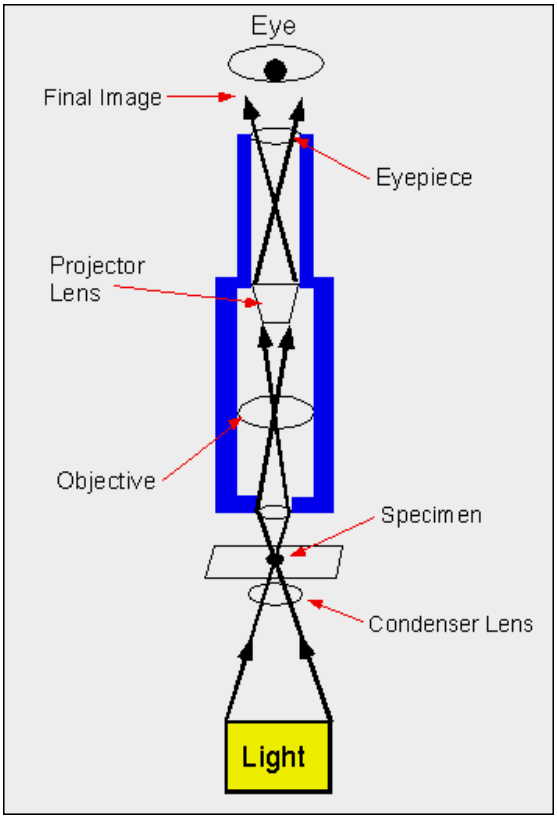
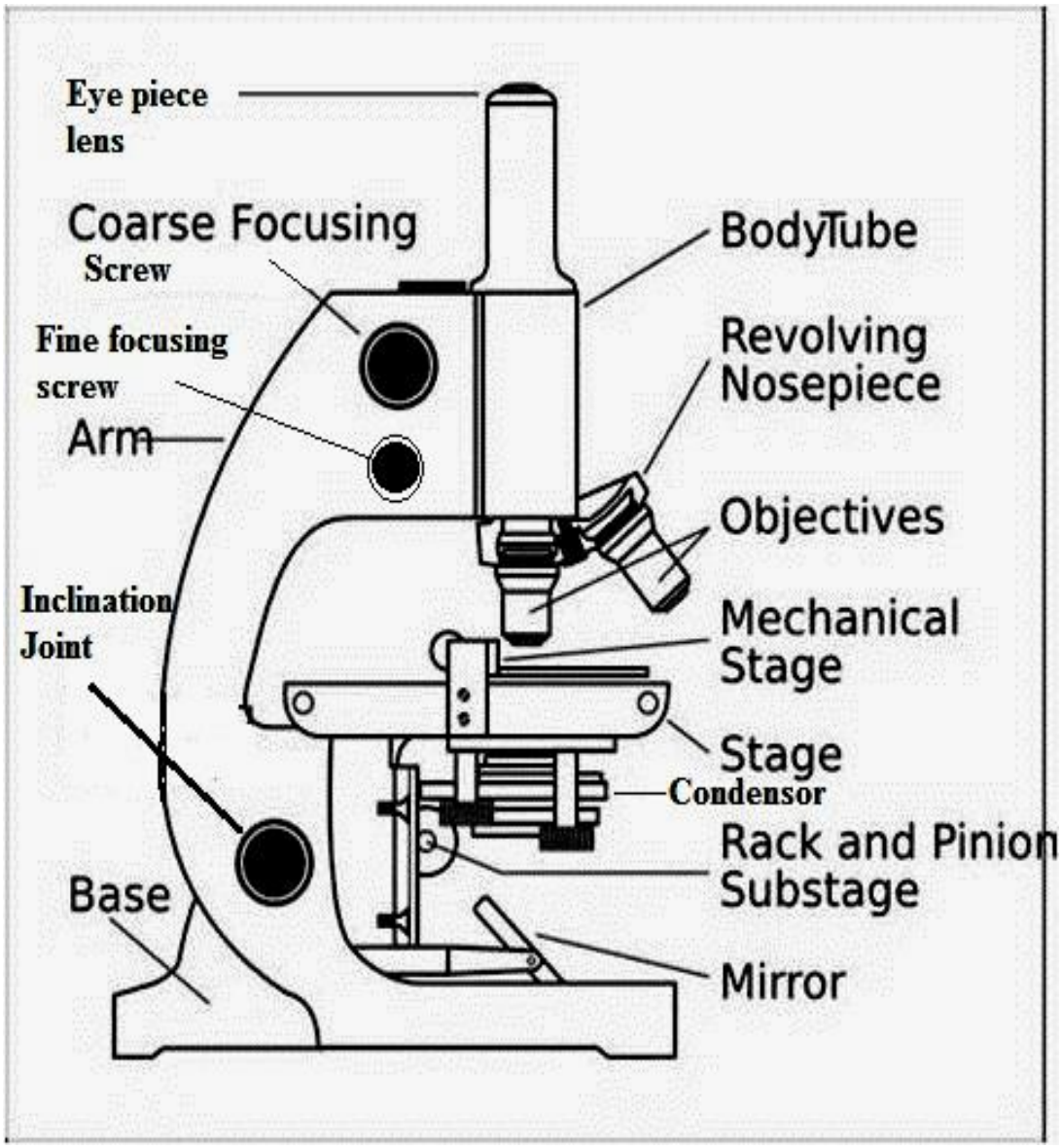
A) Bright field microscopy (Compound light microscope)

In most of our microscope activities, we normally use microscopes that utilize white light for its illumination. These microscopes have bright background and objects are stained. These are called bright field microscopes. The compound microscope consists of two optical components (thus the term compound): the objective lens system, which has a very short focal distance and is placed very close to the object; and the ocular or eyepiece system, which has a longer focal length, lower magnification; and which further magnifies and projects the image onto the retina of the eye.



Ray diagramof Compound Light Microscope



- **Compound Microscope: Parts and Functions**

1. **Base-** This is the bottom part of the microscope and supports the whole device.
2. **Light source-** Many sources of light can be used. At its simplest, daylight is directed via a mirror. Most microscopes, however, have their own adjustable and controllable light source – often a halogen lamp, although illumination using LEDs are becoming a more common provision.
3. **Mirror-** This part is found at the bottom of the stage. It reflects light from an outside source to condenser. The light passes on the whole at the center of the stage and illuminates the specimen. If the microscope is electric, the light source is generated by electricity.

4. **Condenser Lens-** The condenser lens is mounted on an adjustable bracket just below the stage. The condenser lens collects the incident beam coming from the mirror or bulb and focuses the beam into a cone that illuminates the specimen. The light is then transmitted to the objective lens as a reversed cone. The cone's angle is an important characteristic of the condenser lens.
5. **Iris diaphragm-** This part is attached just below the condenser. This is used to vary the size and shape of the light cone projected to the slide. It has a knob which you can move to control the intensity of light projected into your specimen. Iris diaphragm is an important component of microscope. The degree to which the aperture diaphragm is open determines the diameter of the beam of light reaching the condenser. Thus, the iris diaphragm affects the working numerical aperture of the condenser, and thus the resolving power of the microscope.
6. **Arm-** This metal part holds and connects the tube to the base. The whole of the optical assembly is attached to a rigid arm, which in turn is attached to a robust U-shaped foot to provide the necessary rigidity.
7. **Inclination Joint-** The arm angle may be adjustable to allow the viewing angle to be adjusted by using this joint.
8. **Stage -**The stage is a flat platform below the objective which supports the specimen being viewed. In the center of the stage is a hole through which light passes to illuminate the specimen. The stage has 2 stage clips on it which function in holding the slide in place. If the stage is mechanical, there are knobs connected to the stage clips which you can turn to move the slide to and fro and from left to right or vice versa.
9. **Objective Lenses-** These are the lenses used to magnify objects. The objective lenses differ in length; the longest having the highest magnification and the shortest having the lowest. There are 3 objective lenses in a microscope and these lenses have different magnification powers (10X, 40X, and 100X). The overall magnification of the microscope is the product of the objective lens magnification factor and the eyepiece (ocular) magnification factor. When 10 X eyepieces is used, total magnification with 10X, 40X and 100 X objectives is 100X, 400X and 1000X.

10. **Oil immersion objective (100X) -** Some microscopes make use of oil-immersion objectives for greater resolution at high magnification. These are used with index-matching material such as immersion oil between the objective lens and the sample. The refractive index of the index-matching material is higher than air allowing the objective lens to have a larger numerical aperture (greater than 1) so that the light is transmitted from the specimen to the outer face of the objective lens with minimal refraction. Numerical apertures as high as 1.6 can be achieved. The larger numerical aperture allows collection of more light making detailed observation of smaller details possible.
11. **Draw Tube-** This part of the microscope connects the objective lenses to the eyepiece. It is attached to the microscope arm for support.
12. **Revolving nosepiece (Turret) -** This is also called revolving nosepiece and directly attached to the draw tube. It is where the objective lenses are attached. As its other name implies, it can be revolved or turned by the user to select what objective lens he/she will use to magnify a particular object.
13. **Coarse Adjustment Knob-** This knob is used to adjust the distance of the objective lens to the slide and to focus the specimen. By this knob we can move the objective lens up and down until we can see the magnified image through eyepiece. Be careful not to move the high power objective into the slide too close that you can break it.
14. **Fine Adjustment Knob:** This knob is turned to focus the specimen when you are switching from one objective lens to another; for example, when you switch from LPO to HPO and vice versa.
15. **Eyepiece-** The eyepiece, or ocular lens, is a cylinder containing two or more lenses; its function is to bring the image into focus for the eye. The eyepiece is inserted into the top end of the body tube. Eyepieces are interchangeable and many different eyepieces can be inserted with different degrees of magnification. Typical magnification values for eyepieces include 10X (the most common), 15X and 20X.

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❖ Objective lenses

The objective lens is the single most important lens of the microscope. It receives a reversed cone of light from the specimen and forms the primary magnified image of the specimen. This primary image is a **magnified inverted, real image** that comes to focus at a fixed distance.

The objective lens consists of several lenses to magnify an object and project a larger image. According to the difference of the focal distance, lenses of different magnifications are available, such as 4x, 10x, 40x, and 100x mounted on a revolving nosepiece.

1. **Scanning power objective:** This is the shortest of the objective lenses and is used to scan a whole slide. The magnifying power of the scanning lens is 4X.

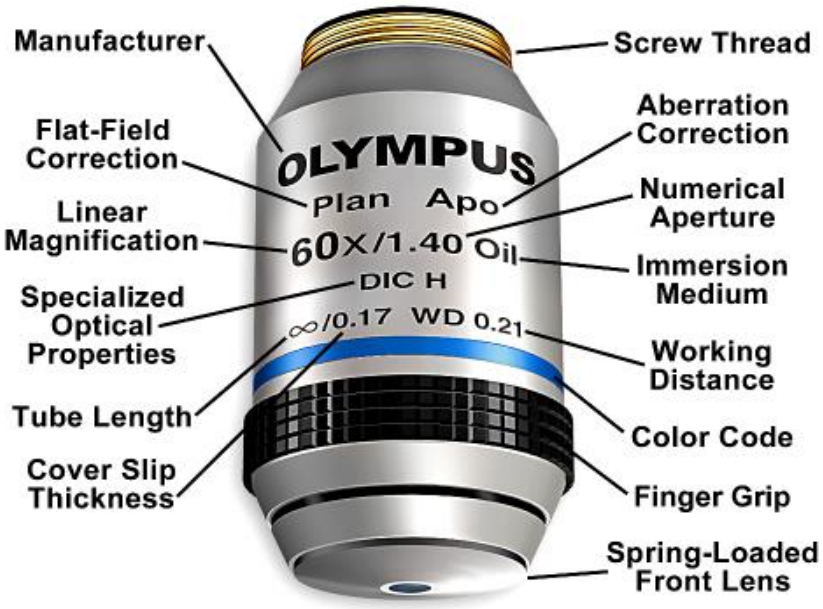
2. **Low-power objective:** Used to view objects in greater details. The magnifying power of the low-power objective lens is 10X.

3. **High-power objective:** If your microscope has three objective lenses, this lens will be the longest. It is used to view an object in even greater details. The magnifying power of the high-power objective lens is 40X.

4. **Oil immersion objective**: An immersion lens that uses oil is called an oil immersion lens. Usually it has a 100X magnification. It is used together with immersion oil to view objects with the greatest magnification. It increases the numerical aperture by filling liquid between the objective lens and the specimen to achieve a high resolution.

	Objective		
PROPERTY	**LOW POWER**	**HIGH POWER**	**OIL IMMERSION**
Magnification of objective	10x	40-45x	90-100x
Magnification of eyepiece	10x	10x	10x
Total magnification	100x	450 – 450x	900 – 1000x
Numerical aperture	0.25 – 0.30	0.55 – 0.65	1.25 – 1.4
Mirror used	Concave	Concave	Plane
Focal length (Approx)	16 mm	4 mm	1.8 – 2 mm
Working distance	4 – 8 mm	0.5 – 0.7 mm	0.1 mm
Iris diaphragm	Partially closed	Partially opened	Fully opened
Position of condenser	Lowest	Slightly raised	Fully raised
Maximum resolution(Approx)	0.9 µm	0.35µm	0.18µm

The information about manufacturer's name, magnification power, numerical aperture and the working distance are mentioned on the objective lenses.



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❖ Eye piece lens Ocular lens

The microscope eyepiece, or ocular lens, magnifies the real image projected by the objective lens to form a large virtual image. It is so named because it is closest to the eye when someone looks through the device. The objective lens or mirror collects light and brings it to focus creating an image. The eyepiece is placed near the focal point of the objective to magnify this image. The amount of magnification depends on the focal length of the eyepiece. The basic design of a microscope eyepiece consists of two lenses (or lens doublets) separated by an air space the length of half the sum of the focal lengths of the two lenses. The lens closest to the eye is called the ***eye lens*** and the other lens (closest to the objective) is called the ***field lens***. The result of having two lenses within the eyepiece is to reduce the size of the intermediate image and thus allow the observation of a larger field of view. The eyepiece focuses the real image (from the objective) outside of the eye lens at a position called the *eyepoint* or *exit pupil*, which corresponds to the position of the focal point of the eye.

The magnifying power of the eyepiece is usually 10X or 15x. Most microscopes are binocular, i.e., they have two eyepieces. Binocular compound microscopes are usually equipped with two eyepiece adjustments.

There are three major types of eyepieces that are grouped according to lens and diaphragm arrangement:

1. Negative eyepieces (***Huygenian*** eye-piece)
2. Positive eyepieces (***Ramsden*** eyepiece)
3. Hyperplane and Compensating eyepieces

1. Negative eyepieces (*Huygenian* eye-piece)

The simplest negative eyepiece design, often termed the ***Huygenian*** eye-piece, is found on most teaching and laboratory microscopes fitted with achromatic objectives. It was invented by **Christiaan Huygens** in the late 1660s and was the first compound (multi-lens) eyepiece. These have an internal diaphragm. Negative eyepieces have two lenses: the upper lens, which is closest to the observer's eye, is called the eye-lens and the lower lens (below the diaphragm) is often termed the field lens. Huygens eyepieces consist of two plano-convex lenses with the plane sides towards the eye and convex sides "facing" the specimen separated by an air gap. Between these lenses there is a fixed circular opening or internal diaphragm. The focal plane is located between the two lenses.

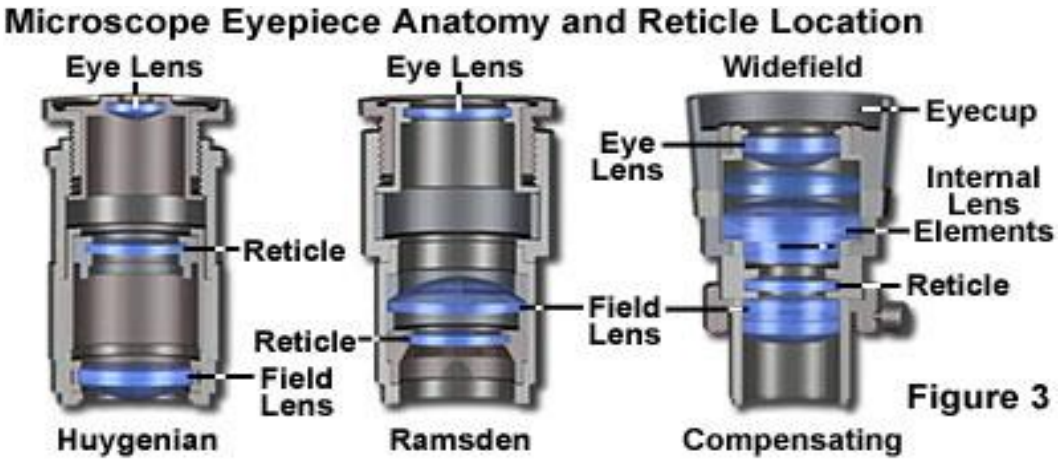


Figure 3

2. Positive eyepieces (*Ramsden* eyepiece)

These have a diaphragm below the lenses of the eyepiece commonly known as the ***Ramsden*** eyepiece. It was invented by **Jesse Ramsden** in 1782. The Ramsden eyepiece comprises two plano-convex lenses of the same glass and similar focal lengths placed less than one eye-lens focal length apart. This eyepiece has an eye lens and field lens that are also plano-convex, but the field lens is mounted with the curved surface facing towards the eye lens. The two curved surfaces face inwards. The focal plane is thus located outside of the eyepiece. The front focal plane of this eyepiece lies just below the field lens; at the level of the eyepiece diaphragm. To provide better correction, the two lenses of the Ramsden eyepiece may be cemented together.

3. Hyperplane and *compensating eyepieces*

Simple eyepieces such as the Huygenian and Ramsden and their achromatized counterparts will not correct for residual **chromatic difference of magnification** in the intermediate image, especially when used in combination with high magnification achromatic objectives as well as any fluorite or apochromatic objectives. To remove this problem, manufacturers produce **Hyperplane** and ***compensating eyepieces*** that introduce an equal, but opposite, chromatic error in the lens elements. The Hyperplane and Compensating eyepieces are similar in construction to the Huygenian eyepiece, except that the eyelens is a doublet. This construction gives more lateral color correction, as indicated in the figure. The compensating type gives the most correction for lateral color, the Hyperplane being a compromise between the Huygenian and the Compensating in this regard.

Compensating eyepieces may be either of the positive or negative type, and must be used at all magnifications with fluorite, apochromatic and all variations of plan objectives (they can also be used to advantage with achromatic objectives of 40x and higher). In recent years, modern microscope objectives have their correction for chromatic difference of magnification either built into the objectives themselves or corrected in the

tube lens. Compensating eyepieces play a crucial role in helping to eliminate residual chromatic aberrations inherent in the design of highly corrected objectives.

❖ Condenser Lens

The condenser lens is mounted on an adjustable bracket just below the stage. The condenser lens collects the incident beam coming from the mirror or bulb and focuses the beam into a cone that illuminates the specimen. The light is then transmitted to the objective lens as a reversed cone. The cone's angle is an important characteristic of the condenser lens. The greater the cone's angle, the greater the amount of light that is gathered by the lens and the better the resolving power of the microscope. The cone's angle is numerically expressed as the **angular aperture** of the lens, which is, in turn, expressed as the **numerical aperture** of the lens. Higher quality condenser lenses have higher numerical apertures. The greater the numerical aperture, the greater the cone's angle and the greater the possible resolving power of the scope.

The condenser lens and the light that passes through it are controlled by three condenser controls. The **condenser centering screws** center the condenser axis along the light path. The **condenser focusing control** focuses the tip of the light cone formed by the condenser lens onto the slide. Lastly, the **aperture diaphragm / Iris diaphragm control** controls the diameter of the **aperture diaphragm**.

The aperture / iris diaphragm is an important component of your microscope. The degree to which the aperture diaphragm is open

determines the diameter of the beam of light reaching the condenser. Thus, the iris diaphragm affects the working numerical aperture of the condenser, and thus the resolving power of the microscope.

As the aperture diaphragm is closed, the angle of the cone decreases, the working angular aperture and numerical aperature of the lens decrease, and the resolution decreases. Conversely, as the aperture diaphragm is open, the angle of the cone increases, the working angular aperture and numerical aperature of the lens increase, and the resolution increases.

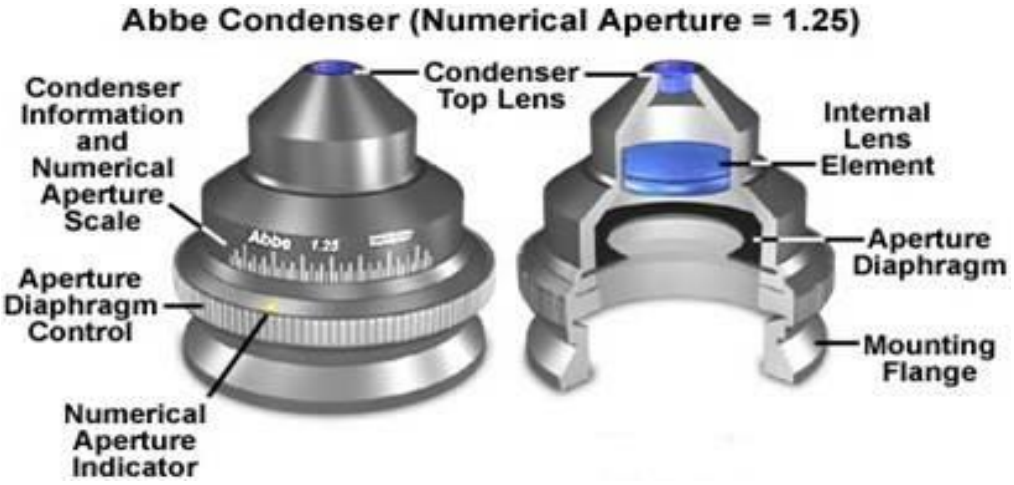
Adjusting the aperture diaphragm also affects the image contrast, and this is frequently why most users adjust the aperture diaphragm - to increase the contrast of the image. As the aperture diaphragm is closed, the image contrast increases.

There are following types of condensers used in Bright field and Dark field microscopy.

1. Abbe condenser used in Bright field microscopy
2. Abbe condenser used in Dark field microscopy
3. Cordiod Dark field condenser
4. parabolic

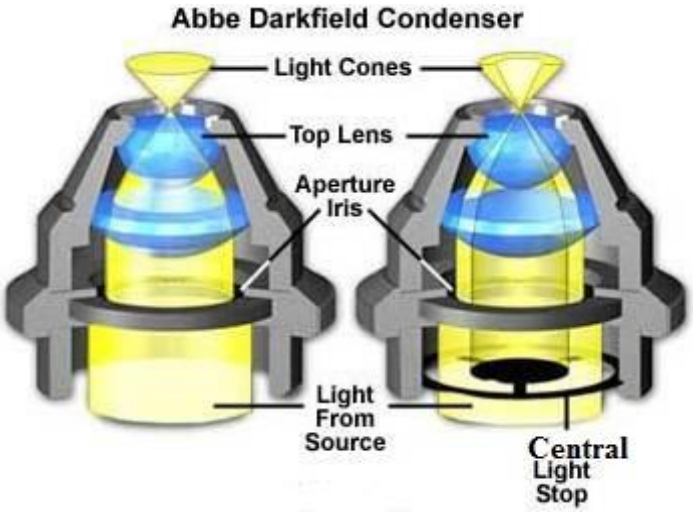
1. Abbe condenser in Bright field microscopy

The Abbe **condenser** was invented by, Ernst Karl **Abbe**, a German physicist, who developed it in 1870. The Abbe condenser is mounted below the stage of the microscope, and concentrates and controls the light that passes through the specimen and enters the objective. It has two controls, one which moves the Abbe condenser closer to or further from the stage, and another, the iris diaphragm, which controls the diameter of the beam of light. This condenser is composed of two lenses a plano-convex lens somewhat larger than a hemisphere and a large bi-convex lens serving as a collecting lens to the first. The focus of the first lens is traditionally about 2mm away from the plane face coinciding with the sample plane.

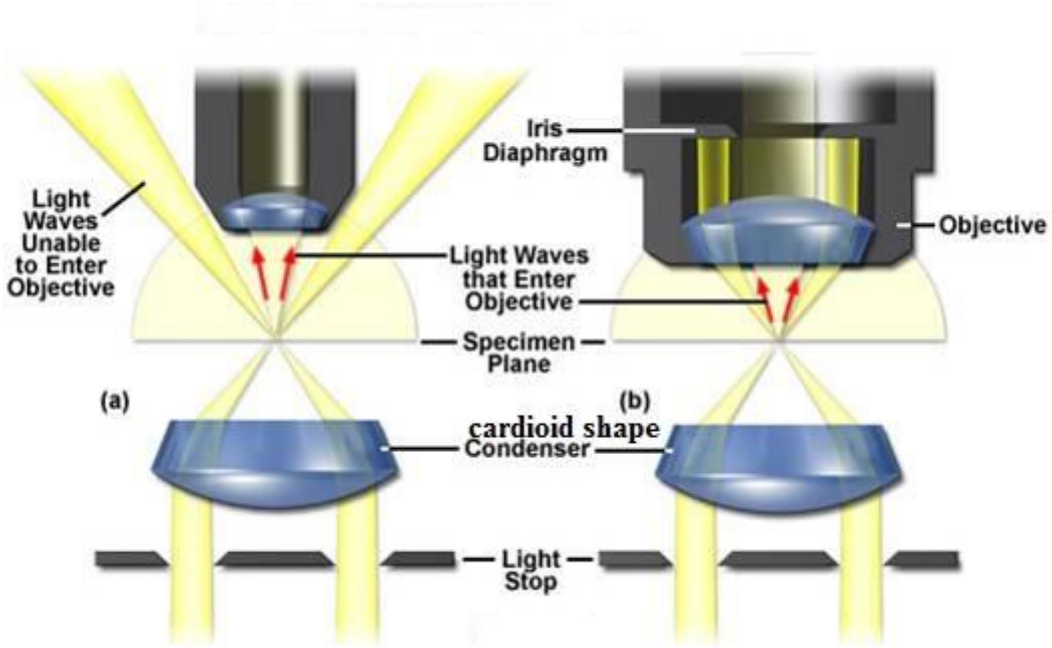


2. Abbe condenser used in Dark field microscopy

In this condenser "**Central Stop**" is placed just under the bottom lens (in the front focal plane) of the substage condenser allowing light rays emerging from the surface to form an inverted hollow cone of light with an apex centered in the specimen plane. If no specimen is present and the numerical aperture of the condenser is greater than that of the objective, the oblique rays cross and all such rays will miss entering the objective because of their obliquity. The field of view will appear dark.



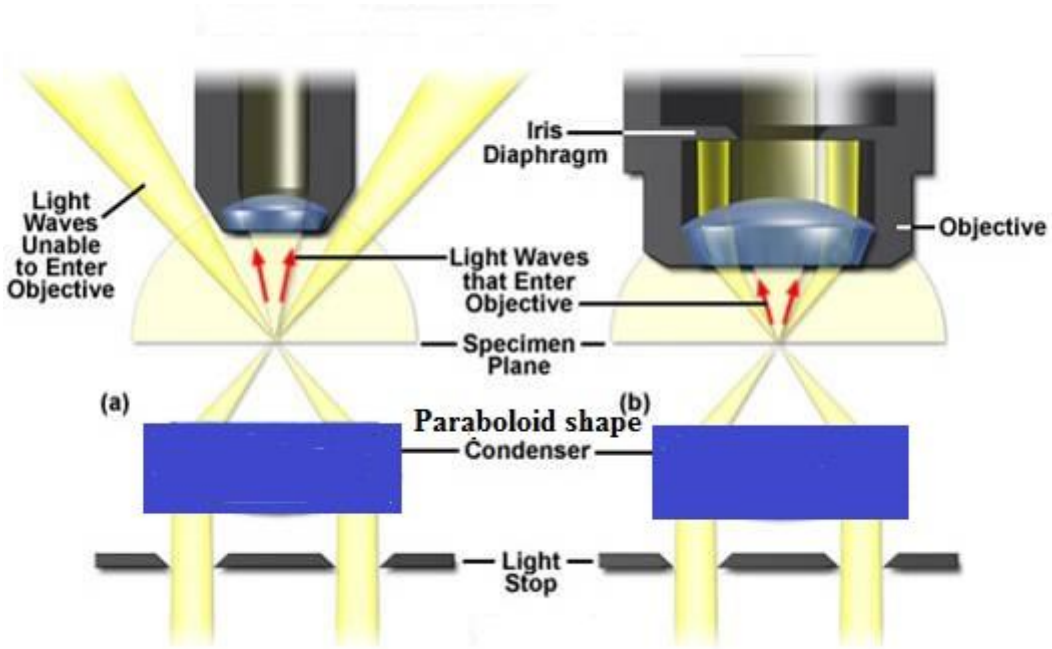
3. Cordiod Dark field condenser



This condenser design utilizes a mirrored hemisphere in the center of the condenser that serves as both a light stop and a reflector to direct light onto a second reflecting surface shaped to resemble a cardioid of revolution, from which the condenser derives its name.

4. Paraboloid Dark field condenser

The paraboloid, consisting of a solid piece of glass ground very accurately into the shape of a paraboloid. The light stop at the bottom of the glass condenser serves to block central rays from reaching the specimen.



B) DARK FIELD MICROSCOPY

A typical darkfield illumination image has a white/bright specimen with a dark background and environment filling the image. This is the exact opposite of a brightfield illumination image, and is useful for unstained specimens or images that require increased contrast. The advantage with using darkfield illumination is that unstained specimens can remain alive and vital; whereas in brightfield microscopy specimens must be treated and stained, hence do not remain alive. Also, it is possible to acquire more qualitative results with this technique through live cellular analysis.

Darkfield illumination is a technique in optical microscopy that eliminates scattered light from the sample image. This yields an image with a dark background around the specimen, and is essentially the complete opposite of the brightfield illumination technique. The primary imaging goal of the darkfield illumination technique is to enhance the contrast of an unstained sample, which is incredibly powerful, yet simple, for live cellular analysis or samples that have not gone through the staining process.

Working Principle:

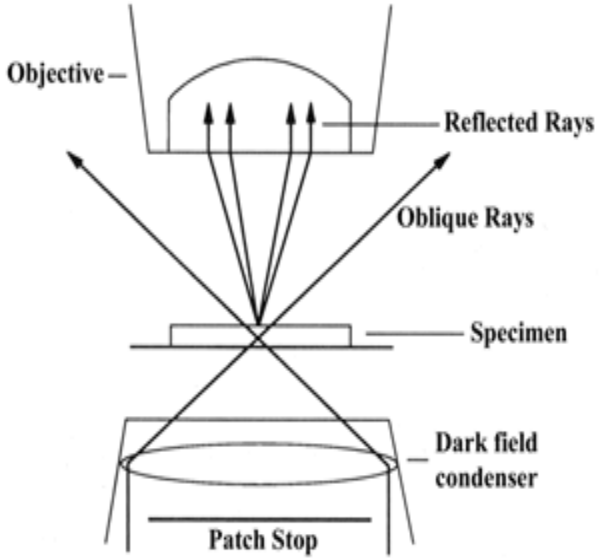
In a dark-field microscope, the object is brilliantly illuminated against a dark background. This is accomplished by equipping a light microscope with a special kind of condenser (Abbe darkfield condenser with central stop, Cardioid and Paraboloid darkfield condenser).

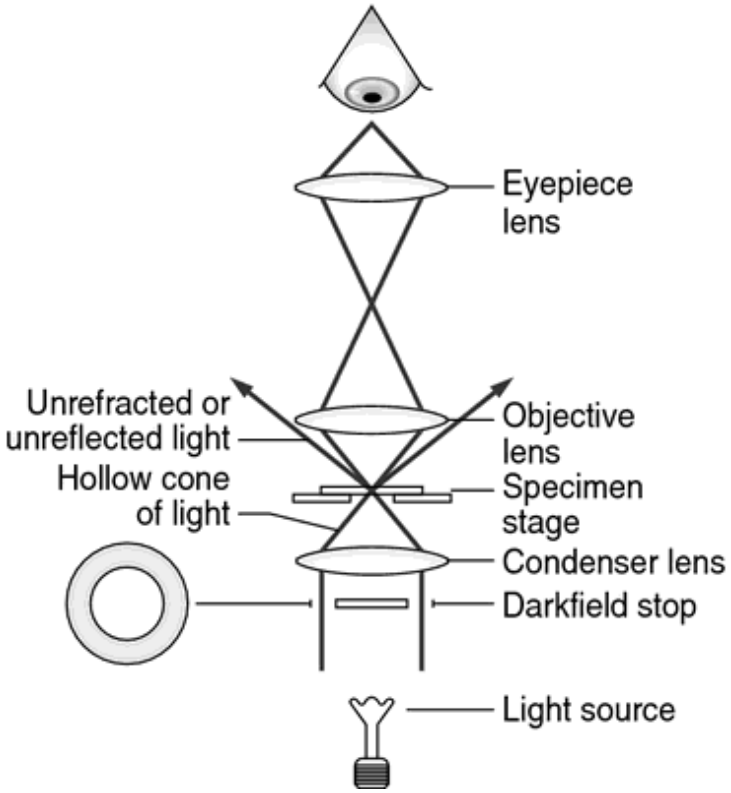
It is a condenser with a dark-field stop, which is an opaque disc obstructing the path of light from the light source centrally, but allowing a peripheral ring of light.

Thus, the condenser transmits a hollow cone of light from the light source. This cone of light converges on the object and diverges from there again as an inverted hollow cone. Thus, no light enters into the objective, as it remains in the dark cone and the field essentially appears dark in absence of any object.

However, if some objects such as microbial cells are present, some of the light rays are scattered (diffracted) by them. These diffracted rays enter into the objective and reach the eye. Thus, the object (microbial cells) appears bright in an otherwise dark microscopic field.

Dark-field microscopy is particularly valuable for examination of unstained microbes suspended in fluids (hanging drop preparation and wet mount), where live cells are to be viewed.





Dark field microscopy is also called 'dark ground microscopy'. Dark field microscopy is able to produce images of microscopic structures that are invisible or transparent in other forms of microscopy. This illumination technique can enhance the contrast in unstained microscope samples. Producing a dark to almost black background with bright specimens on it, dark field microscopy works on a principle wherein the light will not be collected by the microscope objectives and therefore do not have a direct contribution in forming the image.

In dark field microscopy, the image formation relies on the diffraction of light from the specimen which enters the microscope objective lens. The light rays in this microscopy do not directly pass through the objective lens like in bright field microscopy where light rays directly enter the microscope objectives.

By using dark field microscope fine filament structures, thin membranes, flagella etc can be observed.

The most important part of a dark field microscope is the dark field condenser. The dark field condenser blocks off the light that passes

through its central area which forming a hollow cone of light. This cone of light is directed at an angle which misses the objective lens. The image formation relies on the angle of light rays that strike and be diffracted from the cells of the specimen.

The diffracted light rays produce a specimen image that appears brilliant and clear white against a black background. The images also have a very high contrast due to which minute structures are observed clearly. These specimens do not usually need any form of staining or preparation and are usually examined in its natural living state under the microscope.

Dark field microscopy is an effective illumination technique that is ideal for biological specimens that are unstained and living.

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C) Phase Contrast Microscopy

Phase contrast microscopy, first described in 1934 by Dutch physicist Frits Zernike is a contrast-enhancing optical technique that can be utilized to produce high-contrast images of transparent specimens, such as living cells (usually in culture), microorganisms, thin tissue slices, lithographic patterns, fibers, latex dispersions, glass fragments, and subcellular particles (including nuclei and other organelles).

In effect, the phase contrast technique employs an optical mechanism to translate minute variations in phase into corresponding changes in amplitude, which can be visualized as differences in image contrast. One of the major **advantages of phase contrast microscopy** is that living cells can be examined in their natural state without previously being killed, fixed, and stained. As a result, the dynamics of ongoing biological processes can be observed and recorded in high contrast with sharp clarity of minute specimen detail.

The light rays produced by the tungsten-halogen lamp are directed through a collector lens and focused on a specialized annulus (labeled as **condenser annulus**) placed below the condenser front focal plane. The light passing through the annulus illuminate the specimen and either

passes through undeviated or is diffracted and retarded in phase by structures and phase gradients present in the specimen. Undeviated and diffracted light collected by the objective is separated at the rear focal plane by a **phase plate** and focused at the intermediate image plane to form the final phase contrast image observed in the eyepieces.

The diffracted waves being retarded in phase by a quarter wavelengths (1/4) at the specimen, while the surround waves are advanced (or retarded) in phase by a phase plate positioned in or very near the objective rear focal plane.

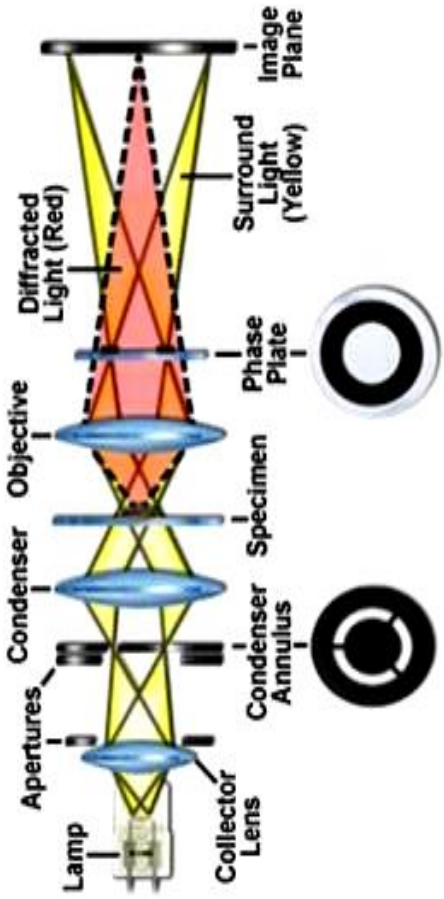
The most important concept underlying the design of a phase contrast microscope is the separation of surround and diffracted wavefronts emerging from the specimen, which are projected onto different locations in the objective rear focal plane (the **diffraction** plane at the objective rear aperture). In addition, the amplitude of the surround (undeviated) light must be reduced and the phase advanced or retarded (by a quarter wavelength) in order to maximize differences in intensity between the specimen and background in the image plane.

The mechanism for generating relative phase retardation is a two-step process, with the diffracted waves being retarded in phase by a quarter wavelength at the specimen, while the surround waves are advanced (or retarded) in phase by a phase plate positioned in or very near the objective rear focal plane.

Only two specialized accessories are required to convert a brightfield microscope for phase contrast observation.

i) **A specially designed annular diaphragm (condenser annulus)** placed in the condenser front focal plane which is matched in diameter and optically conjugates to an internal phase plate residing in the objective rear focal plane and

ii) Phase plate placed in the objective rear focal plane



The **condenser annulus** is typically constructed as an opaque flat-black (light absorbing) plate with a transparent annular ring, which is positioned in the front focal plane (aperture) of the condenser so the specimen can be illuminated by defocused, parallel light originating from the ring.

A phase plate is mounted in or near the objective rear focal plane in order to selectively alter the phase and amplitude of the surround (or undeviated) light passing through the specimen. Most of the phase plates available from modern microscope manufacturers are produced by vacuum deposition of thin **dielectric and metallic films** onto a glass plate or directly onto one of the lens surfaces within the microscope objective. The role of the dielectric thin film is to shift the phase of light, phase plates that alter the phase of surround light relative to diffracted light by 90 degrees (either positive or negative) are termed quarter wavelength plates because of their effect on the optical path difference.

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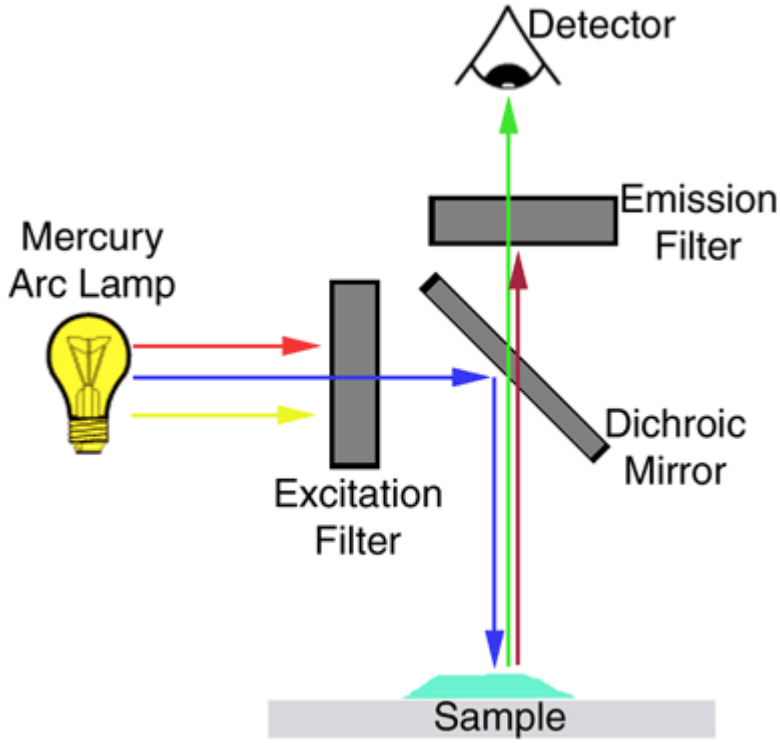
D) Fluorescence microscopy

In fluorescence microscopy, visualization of membranes and cells is done by either labeling with fluorescent molecules or by using the intrinsic fluorescent properties of molecules already present in the biological system.

Fluorescence is the optical phenomenon of illuminating a fluorescent compounds. These compounds absorb shorter wavelengths and emit longer wavelength light.

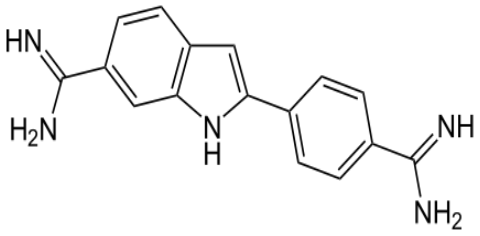
Fluorescence results from a process that occurs when certain molecules called fluorophores, fluorochromes, or fluorescent dyes absorb light. The absorption of light by a population of these molecules raises their energy level to a brief excited state. As they decay from this excited state, they emit fluorescent light.

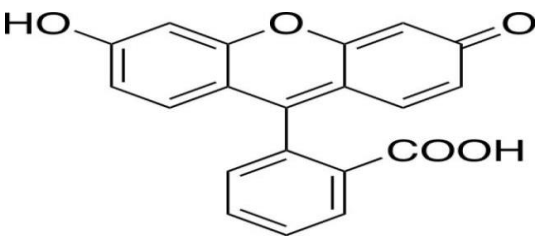
Fluorescent molecules absorb only at specific wavelengths therefore a fluorescence microscope must have a light source able to produce various wavelengths for excitation. Xenon arc lamp or Mercury-vapour lamp are used that generates white light, which is a mixture of all visible wavelengths. To view this fluorescence in the microscope, several light filtering components are needed. Specific filters are used to isolate the excitation and emission wavelengths of a fluorochrome. A dichroic beam splitter (partial mirror) reflects shorter wavelengths of light and allows longer wavelengths to pass. A dichoric beam splitter is required because the objective acts as both a condenser lens (excitation light) and objective lens (emission light); therefore the beam splitter isolates the emitted light from the excitation wavelength. This epi-illumination type of light path is required to create a dark background so that the fluorescence can be easily seen. The wavelength at which a beam splitter allows the longer wavelengths to pass must be set between the excitation and emission wavelengths of any given fluorochrome so that excitation light is reflected and emission light is allowed to pass through it.



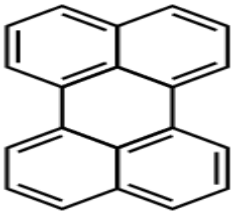
A simple illustration showing the main components of a fluorescent microscope.

Several different types of fluorescent molecules (fluorochromes) exist. Most fluorochromes tend to be organic molecules containing double, triple or aromatic systems. In order to label specific environments, these fluorophores are coupled to other molecules such as lipids, proteins, antibodies etc. These different fluorescent probes tend to accumulate in the environment for which they were designed e.g. lipid probes will accumulate in membrane structures.





DAPI diamidino-2-phenylindole fluorescein



perylene

A small selection of commonly used fluorophores used to label for instance DNA (DAPI diamidino-2-phenylindole), cells (fluorescein & green fluorescent protein, GFP) and lipid membranes (perylene & DiI). Green fluorescent protein taken from PDB file 1GFL.

Uses of fluorescence microscope -

- Imaging structural components of small specimens, such as cells
- Conducting viability studies on cell populations (are they alive or dead?)
- Imaging the genetic material within a cell (DNA and RNA)
- Viewing specific cells within a larger population

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2) Electron Microscopy

An electron microscope is a microscope that uses a beam of accelerated electrons as a source of illumination instead of visible light. As the wavelength of an electron can be up to 100,000 (1 lakh) times shorter than that of visible light photons, the electron microscope has a higher resolving power than a light microscope and can reveal the structure of smaller objects. An electron microscope can achieve better than 50 pm (picometre) / 1/100 (0.01 Angstrom Å) resolution and magnifications of up to about 10,000,000x (1 crore times) whereas most light microscopes are limited by diffraction to about 200 nm resolution and magnifications below 2000x.

Electron microscope uses electrostatic and electromagnetic lenses to control the electron beam and focus it to form an image. These electron optical lenses are analogous to the glass lenses of an optical light microscope.

Uses of electron microscope

Electron microscopes are used to study

- the ultrastructure of a wide range of biological specimens including microorganisms and cells
- inorganic specimens, large molecules, biopsy samples, metals, and crystals.
- Industrially, the electron microscope is often used for quality control and failure analysis.
- Modern electron microscopes produce electron micrographs using specialized digital cameras and frame grabbers to capture the image.

German physicist Ernst **Ruska** and the electrical engineer **Max Knoll** constructed the prototype electron microscope in 1931. There are two types of electron microscopes-

I) Transmission electron microscope (TEM)
II) Scanning electron microscope (SEM)

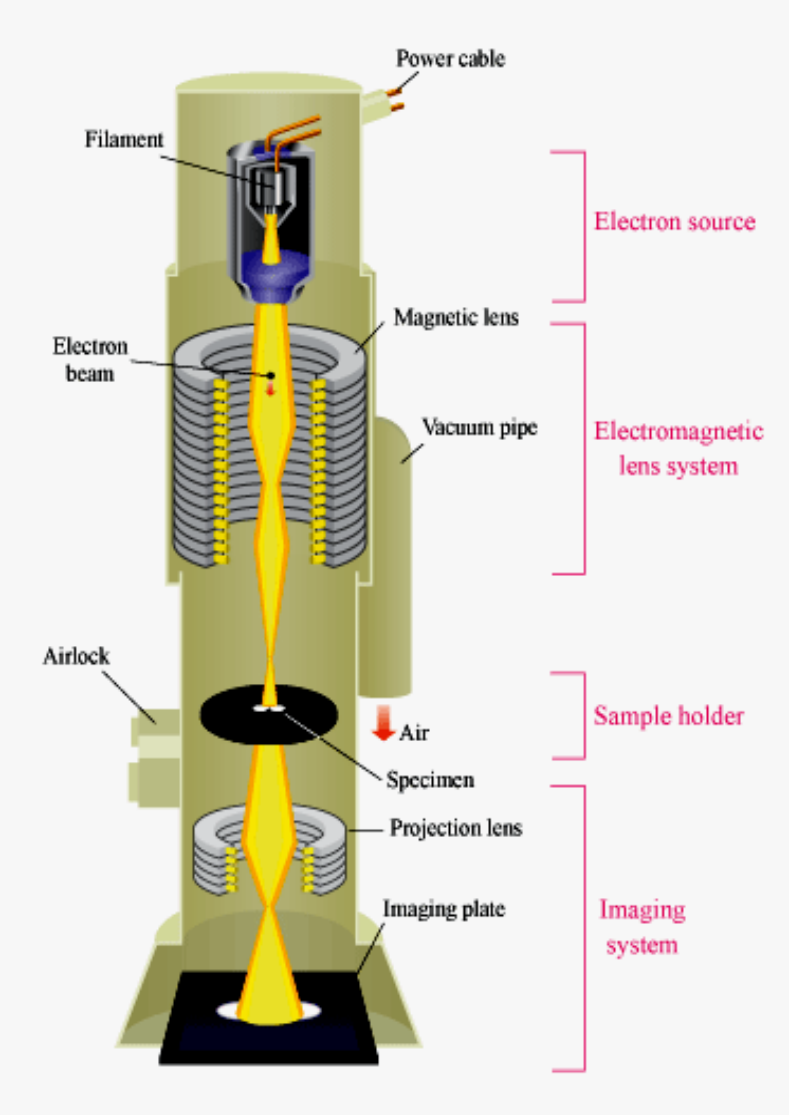
I) Transmission electron microscope (TEM)

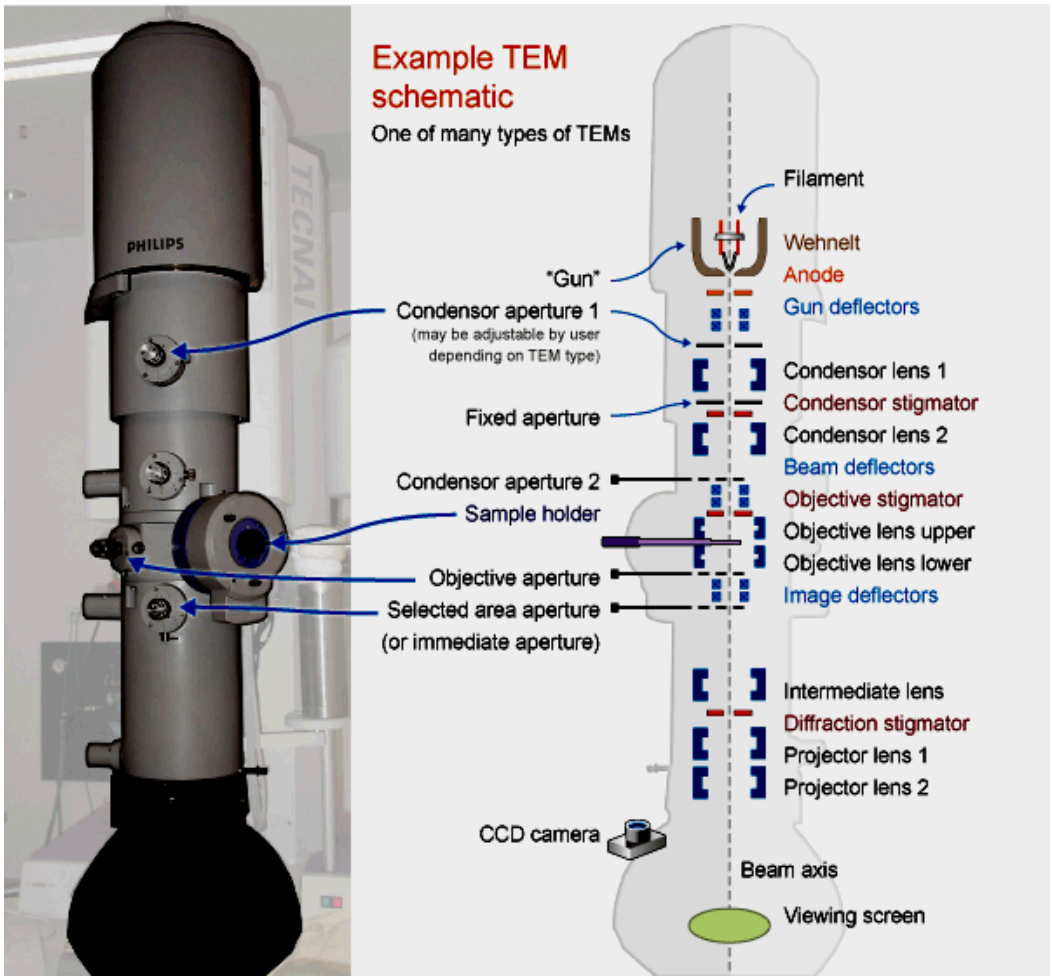
i) The transmission electron microscope (TEM) uses a high voltage electron beam to create an image. The electron beam is produced by an electron gun, commonly fitted with a tungsten filament cathode as the electron source.
ii) The electron beam is accelerated by an anode typically at +100 keV (40 to 400 keV) with respect to the cathode, focused by electrostatic and electromagnetic lenses, and transmitted through the specimen that is in part transparent to electrons and in part scatters them out of the beam.

iii) An electromagnetic coil (the first lens) concentrates the electrons into a more powerful beam.
iv) Another electromagnetic coil (the second lens) focuses the beam onto a certain part of the specimen.
v) The specimen placed on a copper grid in the middle of the main microscope tube. The beam passes through the specimen and "picks up" an image of it.
vi) The projector lens (the third lens) magnifies the image.
vii) The image may be viewed by projecting the magnified electron image onto a fluorescent viewing screen. Alternatively, the image can be photographically recorded by exposing a photographic film or plate directly to the electron beam, or a high-resolution phosphor may be coupled by means of a lens optical system or a fibre optic light-guide to the sensor of a CCD (charge-coupled device) camera. The image detected by the CCD may be displayed on a monitor or computer screen.

The high-resolution transmission electron microscopy (HRTEM) has allowed the production of images with resolution below 0.5 angstrom (50 picometres) and magnifications above 50 million times. The ability to determine the positions of atoms within materials has made the HRTEM an important tool for nano-technologies research and development.

The major disadvantage of the transmission electron microscope is the need for extremely thin sections of the specimens, typically about 100 nanometers. Biological specimens are typically required to be chemically fixed, dehydrated and embedded in a polymer resin 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.


Power cable
Filament
Electron source
Magnetic lens
Electron beam
Vacuum pipe
Electromagnetic lens system
Airlock
Sample holder
Air
Specimen
Projection lens
Imaging plate
Imaging system



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II) Scanning electron microscope (SEM)

i) A scanning electron microscope scans a beam of electrons *over* a specimen to produce a magnified image of an object. That's completely different from a TEM, where the beam of electrons goes right through the specimen.

ii) Electrons are fired into the machine.

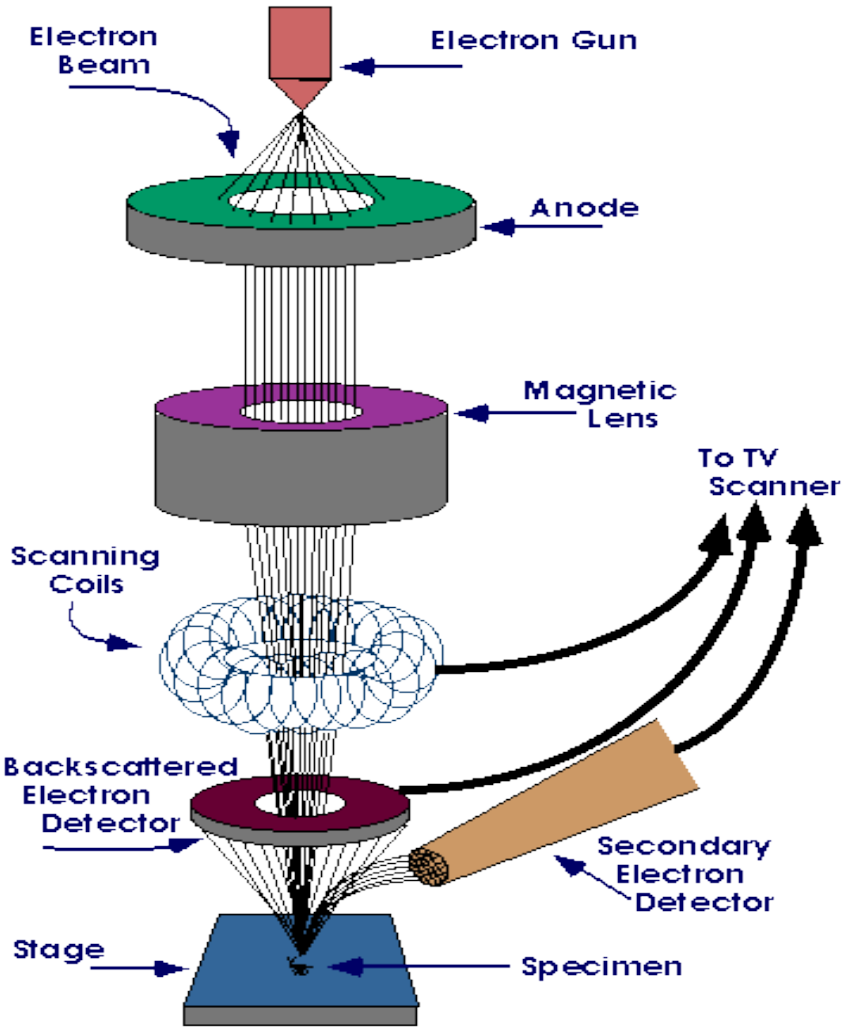
iii) The main part of the machine (where the object is scanned) is contained within a sealed vacuum chamber because accurate electron beams can't travel effectively through air.

iv) A positively charged electrode (anode) attracts the electrons and accelerates them into an energetic beam.

v) An electromagnetic coil brings the electron beam to a very particular focus, much like a lens.

vi) Another coil, lower down, guide the electron beam from side to side.

vii) The beam systematically scans across the object being viewed.
viii) Electrons from the beam hit the surface of the object and bounce off it.
ix) When the electron beam interacts with the specimen, it loses energy by a variety of mechanisms. The lost energy is converted into alternative forms such as heat, emission of low-energy secondary electrons and high-energy backscattered electrons, light emission (cathodoluminescence) or X-ray emission, all of which provide signals carrying information about the properties of the specimen surface, such as its topography and composition. A detector registers these scattered electrons and turns them into a picture.
x) A hugely magnified image of the object is displayed on a TV screen.
xi) Color: Electron microscopes produce images with a single brightness value per pixel, with the results usually rendered in grayscale. However, often these images are then colorized through the use of feature-detection software, or simply by hand-editing using a graphics editor.


Electron Beam
Electron Gun
Anode
Magnetic Lens
To TV Scanner
Scanning Coils
Backscattered Electron Detector
Secondary Electron Detector
Stage
Specimen


JEOL JSM-7001F

❖ Sample preparation

Materials to be viewed under an electron microscope may require processing to produce a suitable sample. The technique required varies depending on the specimen and the analysis required:

1. ***Chemical fixation*** – To stabilize the macromolecular structures of biological specimens, fixation of proteins with formaldehyde and glutaraldehyde and lipids with osmium tetroxide is done.
2. ***Negative stain*** **–** Suspensions containing nanoparticles or fine biological material (such as viruses and bacteria) are briefly mixed with a dilute solution of an electron-opaque solution such as ammonium molybdate, uranyl acetate (or formate), or phosphotungstic acid. This mixture is applied to a suitably coated electromagnetic (EM) grid, blotted and then allowed to dry. Negative staining is also used for observation of nanoparticles.
3. ***Staining*** **–** Uses heavy metals such as lead, uranium or tungsten to scatter imaging electrons and thus give contrast between different structures, since many (especially biological) materials are nearly "transparent" to electrons. Typically thin sections are stained for several minutes with an aqueous or alcoholic solution of uranyl acetate followed by aqueous lead citrate.

❖ Difference between Light Microscope and Electron Microscope

Light microscope	Electron microscope
Simple to use	Users require technical skills
Can view both live and dead specimens	Views only dead specimens
Poor surface view	Good surface view and internal details
Uses visible light rays of 400 to 700 nm wavelength to illuminate specimens	Uses a beam of electrons of 1nm wavelength to view specimens
Sunlight or light from simple bubs or tubes is used	High voltage electric current of 50,000 volts (50kV) is supplied to tungsten filament to produce electron beams
Lenses are made of glass	Lenses are made of electromagnets
Low resolving power, usually below 0.30µm.	High resolving power of up to 0.0001µm (0.1 Angstrom).
Low magnification of up to 1,500x	High magnification of up to 1,000,000x
Images are viewed by the eyes through the eyepiece	Images are viewed on a photographic plate or zinc sulphate fluorescent TV screen
Not used under a vacuum	Operates under a high vacuum
Specimens are stained by using dyes	Specimens are coated with heavy metals to reflect electrons
Cheap to buy and has low maintenance costs	Very expensive to buy and maintain Source:

❖ Atomic Force Microscopy

An atomic force microscope (AFM) is a type of scanning probe microscope that is used to image and manipulate surfaces at the atomic scale. Atomic force microscopy (AFM) is a form of scanning probe microscopy (SPM) with a resolution so high that it can detect molecules within a fraction of a nanometer. A mechanical probe is used to collect information by touch with the assistance of piezoelectric devices that enable very small yet precise motions via electrical control for precision scanning.

Atomic force microscopy is the most versatile and powerful microscopy technology for studying samples at nanoscale. It is versatile because an atomic force microscope can not only image in three-dimensional topography, but it also provides various types of surface measurements to the needs of scientists and engineers. It is powerful because an AFM can generate images at atomic resolution with angstrom scale resolution height information, with minimum sample preparation. It works by using a sharp probe tip to scan the surface of a sample, and measuring the forces between the tip and the surface. The AFM can produce high-resolution images of a sample's surface, as well as measure surface roughness and other physical properties. It is a powerful tool for studying the properties of materials, including metals, semiconductors, polymers, and biological samples.

Binnig, Quate, and Gerber invented the AFM in 1985.

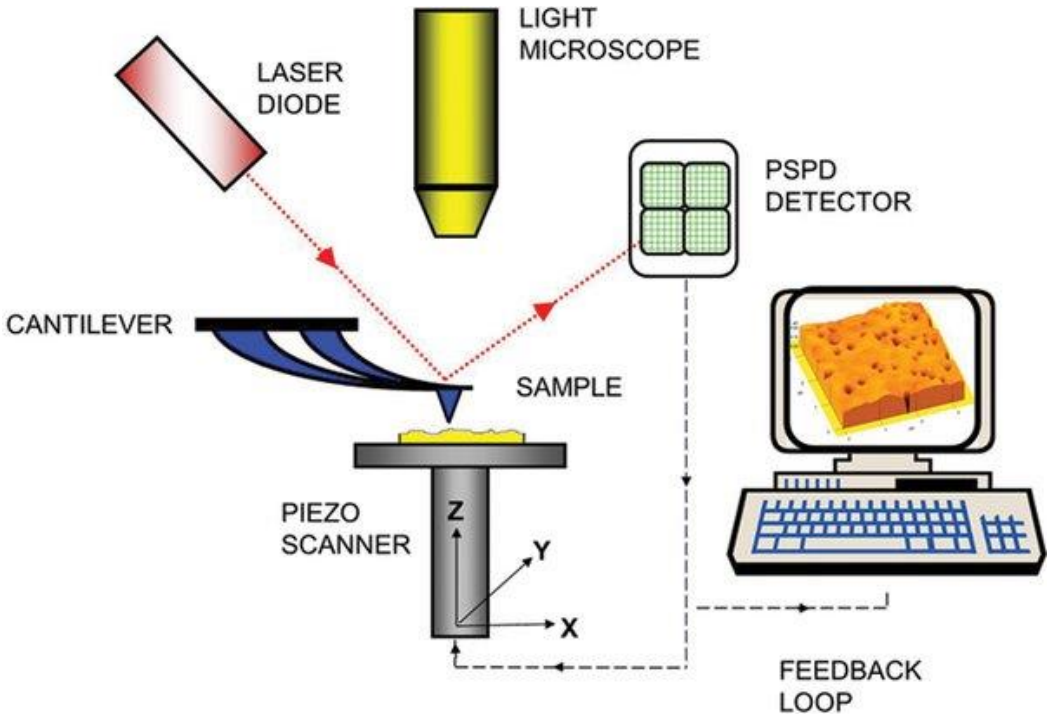
Principle of Atomic Force Microscope

Using a micromachined silicon probe with a very fine tip, AFM microscopes do surface sensing. Raster scanning the surface of a sample line by line is how this tip is used to create an image, albeit the specifics of how this is done change drastically across different modes of operation. Contact mode and dynamic mode, sometimes known as tapping mode, are the two main categories of operational modes.

AFM operates on the premise that this nanoscale tip is connected to a tiny cantilever, which acts as a spring. There is a laser diode and a split photodetector in place to detect the cantilever's bending as the tip makes contact with the surface. The force exerted by the tip on the sample can be

seen in this bending. Contact mode involves pressing the tip into the surface while an electrical feedback loop measures the force of the tip-sample interaction to maintain a constant deflection throughout raster scanning.

Tapping mode reduces the amount of time the tip is in contact with the sample surface to ensure the integrity of both the surface and the tip. When operating in this mode, the cantilever is excited to vibrate very close to its natural frequency of resonance. After then, the tip goes through a sinusoidal motion of rising and falling. As this motion approaches the sample, it is slowed by attractive or repulsive interactions. When in contact mode, a feedback loop maintains a constant quasistatic deflection; here, a feedback loop maintains a constant amplitude for the tapping motion. So doing is like drawing a line map of the sample's geography.



Parts of Atomic Force Microscope

1. Scanning probe:

This is a very fine, sharp tip that is mounted on the end of a cantilever and is used to scan the surface of a sample. The probe is typically made of a hard, durable material such as diamond or silicon, and is typically only a few nanometers in size.

2. Cantilever:

This is a small, flexible beam that supports the probe and allows it to move freely. The cantilever is typically made of a lightweight material such as silicon or silicon nitride, and is only a few micrometers long and a few hundred nanometers thick.

3. Scanning stage:

This is a platform that holds the sample and allows it to be moved relative to the probe. The stage is typically made of a lightweight, rigid material such as aluminum, and is equipped with precision motors or piezoelectric actuators that can move the sample in very small increments.

4. Detection system:

This is a system that measures the forces between the probe and the sample, as well as the position of the probe and the sample. There are several different ways to detect these quantities, including optical, capacitive, and piezoresistive methods.

5. Control and data acquisition system:

This is a computer system that controls the movement of the probe and the sample, as well as processes the data collected by the detection system. The control and data acquisition system typically includes a computer, software, and various input/output devices such as a keyboard, mouse, and display screen.

6. To detect the surface of a sample, modified tips are bent and flexed.

7. There were tweaks made in the imaging software before we utilised it on the samples.

8. Feedback loop control –

They use a laser deflector to regulate the force interactions and the tip positions, creating a feedback loop for precise manipulation. While the cantilever's tip interacts with the sample, the laser's position on the photodetector is employed in the feedback loop to track the sample's surface and make measurements.

9. Deflection –

The atomic force microscope incorporates a laser beam deflection device into its design. Back of the AFM lever is reflective surface, which reflects laser to sensitive detector. Their 10nm-sized tips are crafted from silicon compounds.

10. Force measurement –

The AFM's operation and picture quality are both reliant on the force interactions between probe and sample. With knowledge of the cantilever's rigidity, the deflection lever can be calculated, allowing one to measure the forces. Hooke's law provides the formula for this computation as follows:

$$F = -kz,$$

where F is the force, k is the stiffness of the lever, and z is the distance the lever is bent.

Operating Procedure of Atomic Force Microscope

1. **Setting up the AFM:** This involves unpacking and installing the AFM, setting up the computer and software, and ensuring that all components are properly connected and calibrated.
2. **Preparing the sample:** This involves cleaning and mounting the sample on the scanning stage, making sure that it is stable and flat, and adjusting the position and focus of the probe.

3. Scanning the sample: This involves using the computer and software to control the movement of the probe and the sample, and collecting data on the forces between the probe and the sample as the probe is scanned across the surface.

4. Analyzing the data: This involves using the software to process the data collected during the scan, and generating images and other data that can be used to study the properties of the sample.

5. Maintaining the AFM: This involves regularly cleaning and checking the probe and other components, and making any necessary repairs or adjustments to ensure optimal performance.

Types of Atomic Force Microscope

There are several different types of atomic force microscopes (AFMs), which are classified based on the type of detection system used:

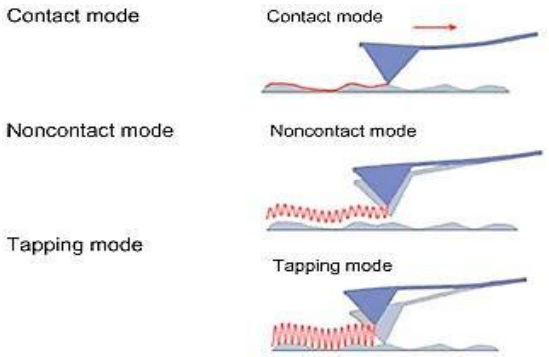
1. Contact mode AFM: This is the most basic type of AFM, in which the probe tip is held in contact with the sample surface and the forces between the tip and the surface are measured as the tip is moved across the surface. This allows the AFM to produce images of the surface topography, as well as measure surface roughness and other physical properties.

2. Non-contact mode AFM: This type of AFM uses a probe tip that is held just above the surface of the sample, and the forces between the tip and the surface are measured using the changes in the oscillation frequency of the cantilever. Non-contact mode AFM is less damaging to the sample than contact mode AFM, but it is also less sensitive and has a lower resolution.

3. Tapping mode AFM: This type of AFM is similar to non-contact mode AFM, but the probe tip is made to oscillate at or near its resonance frequency while it is scanned across the surface of the sample. Tapping mode AFM is less damaging to the sample than contact mode AFM, and it has a higher resolution than non-contact mode AFM.

4. Dynamic force microscopy (DFM): This type of AFM measures the forces between the probe and the sample as a function of the separation distance between them. DFM can be used to study the forces between atoms and molecules, and it can be used to image surfaces at the atomic scale.

5. Magnetic force microscopy (MFM): This type of AFM is used to study magnetic materials and measure the magnetic forces between the probe and the sample. MFM can be used to image the distribution of magnetic fields on the surface of a sample, as well as measure the magnetic properties of individual atoms and molecules.



Atomic Force Microscope Uses

Atomic force microscopes (AFMs) are used in a wide range of fields, including materials science, nanotechnology, and biology. Some of the key applications of AFMs include:

1. Materials science:

AFMs are used to study the properties of materials, including metals, semiconductors, polymers, and ceramics. They can be used to measure surface roughness, surface energy, surface tension, and other physical properties of materials at the nanoscale. Sample identification based on atomic number. Used to Comparing atomic force interactions. Researching the atomic structure and its dynamic physical qualities.

2. Nanotechnology:

AFMs are used to fabricate and characterize nanostructures, including nanowires, nanotubes, and nanoparticles. They can also be used to study the properties of individual atoms and molecules, and to manipulate them at the atomic scale.

3. Biology:

AFMs are used to study biological samples, such as cells, tissues, and proteins. They can be used to image the surface of biological samples at high resolution, and to measure the forces between biological molecules. Examining the physical and chemical characteristics of protein assemblies and complexes like microtubules. use to tell cancer cells apart from healthy ones. Comparing and contrasting the form and rigidity of the cell walls of nearby cells.

4. Surface science:

AFMs are used to study the properties of surfaces, including surface chemistry, surface topography, and surface roughness. They are often used to study the surface properties of materials, and to understand how these properties affect the performance of devices and systems.

5. Industrial inspection:

AFMs are used to inspect and test the quality of various industrial products, such as microelectronic devices, coatings, and MEMS devices. They can be used to detect and characterize defects in these products at the nanoscale.

Advantages of the Atomic Force Microscope

Atomic force microscopes (AFMs) have several advantages over other types of microscopes:

1. High resolution:

AFMs are capable of producing images with a resolution down to the atomic scale, which is much finer than the resolution of other types of microscopes such as optical microscopes.

2. Non-destructive imaging:

AFMs can be used to image and study samples without causing any damage, unlike other techniques such as electron microscopy, which can damage or destroy the sample.

3. Versatility:

AFMs can be used to study a wide range of samples, including metals, semiconductors, polymers, ceramics, and biological samples. They can also be used to measure a variety of physical properties, such as surface roughness, surface energy, and surface tension. You may put it to work in a variety of environments, including air, liquid, and vacuum. Useful for both studying living and nonliving things.

4. Three-dimensional imaging:

AFMs can produce three-dimensional images of the surface of a sample, which can provide valuable information about the sample's surface structure and morphology.

5. Nanoscale manipulation:

AFMs can be used to manipulate and rearrange individual atoms and molecules, which has a wide range of applications in fields such as nanotechnology and materials science.

6. Sample Preparation: Preparing samples for analysis is a breeze.
7. Reliable: Sample size calculations are reliable.

8. 3D Image: It's capable of three-dimensional imaging.