MICROSCOPY

Microscopy is defined as the technique of using microscopes to view objects and areas of objects that cannot be seen with the naked eye. There are two well-known branches of microscopy: optical and electron microscopy, along with the emerging field of X-ray microscopy.

Optical microscopy and electron microscopy involve the diffraction, reflection, or refraction of electromagnetic radiation/ electron beams interacting with the specimen, and the collection of the scattered radiation or another signal in order to create an image. This process may be carried out by wide-field irradiation of the sample (for example standard light microscopy and transmission electron microscopy) or by scanning a fine beam over the sample (for example confocal laser scanning microscopy and scanning electron microscopy). The development of microscopy revolutionized biology, gave rise to the field of histology and so remains an essential technique in the life and physical sciences. X-ray microscopy is three-dimensional and non-destructive, allowing for repeated imaging of the same sample for in situ or 4D studies, and providing the ability to "see inside" the sample being studied before sacrificing it to higher resolution techniques. A 3D X-ray microscope uses the technique of computed tomography, rotating the sample 360 degrees and reconstructing the images.

OPTICAL OR LIGHT MICROSCOPY

Optical or light microscopy involves passing visible light transmitted through or reflected from the sample through a single lens or multiple lenses to allow a magnified view of the sample. The resulting image can be detected directly by the eye, imaged on a photographic plate, or captured digitally. The single lens with its attachments, or the system of lenses and imaging equipment, along with the appropriate lighting equipment, sample stage, and support, makes up the basic light microscope. The most recent development is the digital microscope, which uses a CCD camera to focus on the exhibit of interest. The image is shown on a computer screen, so eye-pieces are unnecessary.

There are different types of light microscopes, e.g. brightfield or compound light microscope, darkfield microscope, phase-contrast microscope, interference microscope, polarizing microscope, ultraviolet microscope, fluorescence microscope, etc.

Brightfield Microscope

Brightfield Microscope is also known as the **Compound Light Microscope**. It is an optical microscope that uses light rays to produce a dark image against a bright background. It is the standard microscope that is used in Biology, Cellular Biology, and Microbiological Laboratory studies.

This microscope is used to view fixed and live specimens that have been stained with basic stains which give a contrast between the image and the image background. It is specially designed with magnifying glasses known as lenses that modify the specimen to produce an image seen through the eyepiece.

Principle:- The ray diagram given below gives the principle of a compound microscope. The object is mounted on the stand below the microscope tube. The objective lens forms a real, inverted and magnified image ($\mathbf{I_1}$) of the object. The image I1 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 ($\mathbf{F_e}$). The eyepiece acts like a magnifying glass and forms a virtual erect and magnified image of the object.

Image formation in a compound microscope takes place as follows:
The object (**O**) is placed just outside F_o, the principal focus of the objective lens. $\mathbf{F_e}$ is the principal focus of the eye lens. A real, inverted magnified image $\mathbf{I_1}$ is formed. The magnified image $\mathbf{I_1}$ acts as an object for the eye lens. The final image $\mathbf{I_2}$ is virtual and is magnified still further. It is inverted compared with the object. $\mathbf{I_2}$ may appear 1000 times larger than the object.

For a specimen to be the focus and produce an image under the Brightfield Microscope, the specimen must pass through a uniform beam of the illuminating light. Through differential absorption and differential refraction, the microscope will produce a contrasting image.

The specimens used are prepared initially by staining to introduce color for easy contrasting characterization. The colored specimens will have a refractive index that will differentiate it from the surrounding, presenting a combination of absorption and refractive contrast.

The functioning of the microscope is based on its ability to produce a high-resolution image from an adequately provided light source, focused on the image, producing a high-quality image. The specimen which is placed on a microscopic slide is viewed under oil immersion or/and covered with a coverslip.

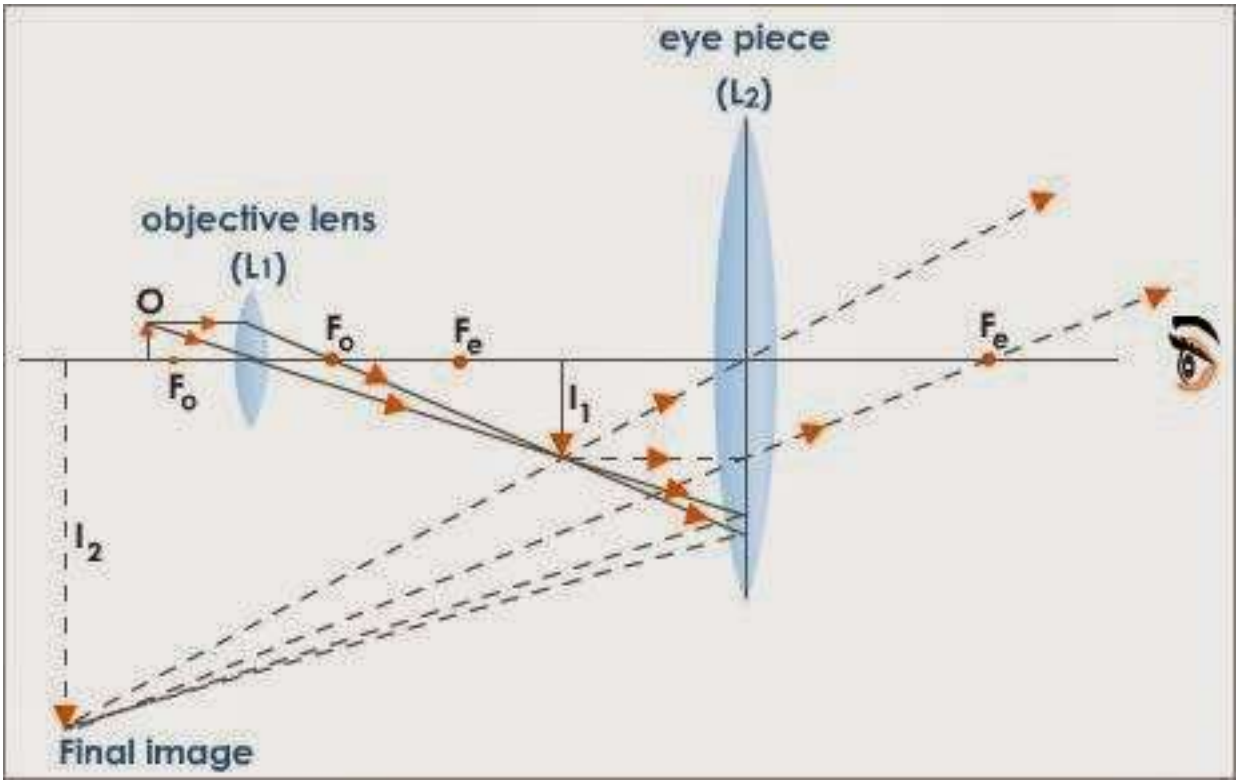


Fig.1 Ray diagram of a compound microscope

Parts Of Compound Microscope:- The parts of the compound microscope can be categorized into (**Fig.2**):

- Mechanical parts
- Optical parts

(A) Mechanical Parts of a Compound Microscope

1. Base or Metal Stand:- The whole microscope rests on this base. Mirror, if present, is fitted to it.

2. Pillars:- It is a pair of elevations on the base, by which the body of the microscope is held to the base

3. Inclination joint:- It is a movable joint, through which the body of the microscope is held to the base by the pillars. The body can be bent at this joint into any inclined position, as desired by the observer, for easier observation. In new models, the body is permanently fixed to the base in an inclined position, thus needing no pillar or joint.

4. Curved Arm:- It is a curved structure held by the pillars. It holds the stage, body tube, fine adjustment and coarse adjustment.

5. Body Tube:- It is usually a vertical tube holding the eyepiece at the top and the revolving nosepiece with the objectives at the bottom. The length of the draw tube is called 'mechanical tube length' and is usually 140-180 mm (mostly 160 mm).

6. Draw Tube:- It is the upper part of the body tube, slightly narrower, into which the eyepiece is slipped during observation.

7. Coarse Adjustment:- It is a knob with rack and pinion mechanism to move the body tube up and down for focusing the object in the visible field. As rotation of the knob through a small angle moves the body tube through a long distance relative to the object, it can perform coarse adjustment. In modern microscopes, it moves the stage up and down and the body tube is fixed to the arm.

8. Fine Adjustment:- It is a relatively smaller knob. Its rotation through a large angle can move the body tube only through a small vertical distance. It is used for fine adjustment to get the final clear image. In modern microscopes, fine adjustment is done by moving the stage up and down by the fine adjustment.

9. Stage:- It is a horizontal platform projecting from the curved arm. It has a hole at the center, upon which the object to be viewed is placed on a slide. Light from the light source below the stage passes through the object into the objective.

10. Mechanical Stage (Slide Mover):- Mechanical stage consists of two knobs with rack and pinion mechanism. The slide containing the object is clipped to it and moved on the stage in two dimensions by rotating the knobs, so as to focus the required portion of the object.

11. Revolving Nosepiece:- It is a rotatable disc at the bottom of the body tube with three or four objectives screwed to it. The objectives have different magnifying powers. Based on the required magnification, the nosepiece is rotated, so that only the objective specified for the required magnification remains in line with the light path.

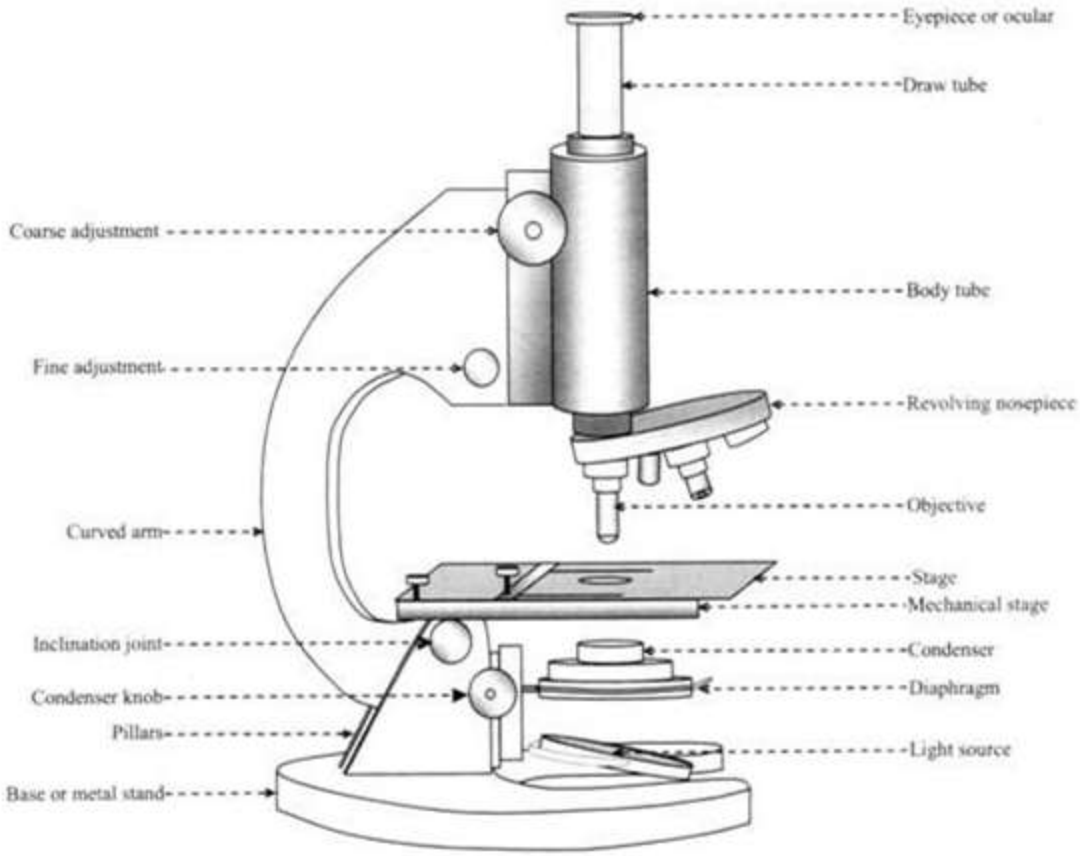


Fig.2 Components of a compound microscope

(B) Optical Parts of Compound Microscope;- These parts are involved in passing the light through the object and magnifying its size.

1. Light Source:- Modern microscopes have in-built electric light source in the base. The source is connected to the mains through a regulator, which controls the brightness of the field. But in old models, a mirror is used as the light source. It is fixed to the base by a binnacle, through which it can be rotated, so as to converge light on the object. The mirror is plane on one side and concave on the other.

It should be used in the following manner:

(a) Condenser Present:- Only plane side of the mirror should be used, as the condenser converges the light rays.

(b) Condenser Absent:- (i) Daylight: Plane or concave (plane is easier) (ii) Small artificial light: High power objective: Plane side, Low power objective: Concave side

2. Diaphragm:- If light coming from the light source is brilliant and all the light is allowed to pass to the object through the condenser, the object gets brilliantly illuminated and cannot be visualized properly. Therefore, an iris diaphragm is fixed below the condenser to control the amount of light entering into the condenser.

3. Condenser:- The condenser or sub-stage condenser is located between the light source and the stage. It has a series of lenses to converge on the object, light rays coming from the light source. After passing through the object, the light rays enter into the objective. The 'light condensing', 'light converging' or 'light gathering' capacity of a condenser is called 'numerical aperture of the condenser'. Similarly, the 'light gathering' capacity of an objective is called 'numerical aperture of the objective'. If the condenser converges light in a wide angle, its numerical aperture is greater and vice versa.

If the condenser has such numerical aperture that it sends light through the object with an angle sufficiently large to fill the aperture back lens of the objective, the objective shows its highest numerical aperture (**Fig. 3**). Most common condensers have numerical aperture 1.25.

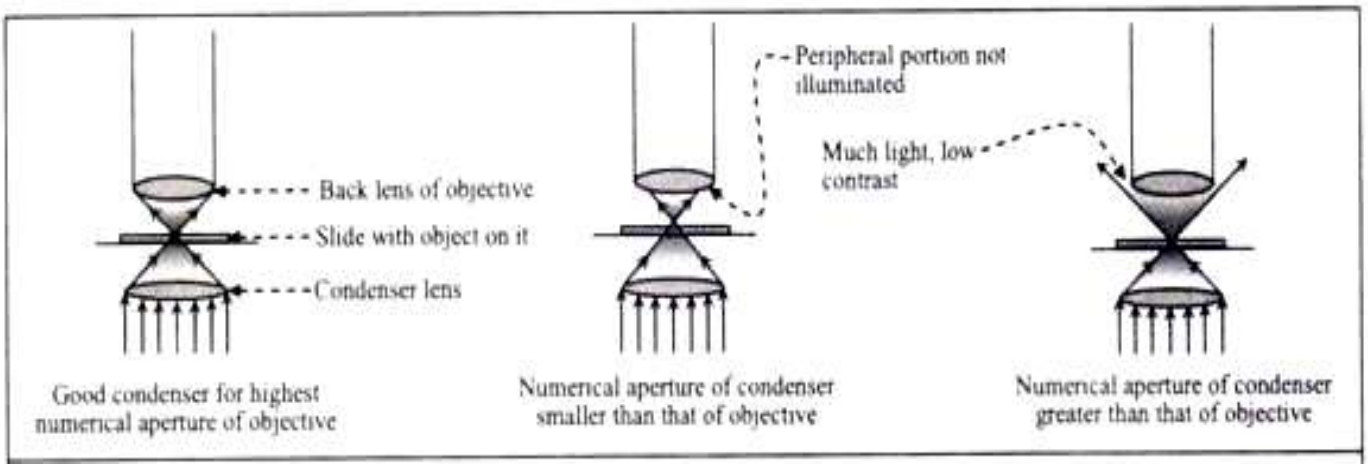


Fig.3 Numerical aperture of condensers

If the numerical aperture of the condenser is smaller than that of the objective, the peripheral portion of the back lens of the objective is not illuminated and the image has poor visibility. On the other hand, if the numerical aperture of condenser is greater than that of the objective, the back lens may receive too much light resulting in a decrease in contrast.

There are three types of condensers as follows:

(a) Abbe condenser (Numerical aperture=1.25): It is extensively used.

(b) Variable focus condenser (Numerical aperture =1.25)

(c) Achromatic condenser (Numerical aperture =1.40): It has been corrected for both spherical and chromatic aberration and is used in research microscopes and photomicrographs.

4. Objective:- It is the most important lens in a microscope. Usually three objectives with different magnifying powers are screwed to the revolving nosepiece.

The objectives are:

(a) Low power objective (X 10):- It produces ten times magnification of the object.

(b) High dry objective (X 40):- It gives a magnification of forty times.

(c) Oil-immersion objective (X100):- It gives a magnification of hundred times, when immersion oil fills the space between the object and the objective

The primary magnification (X4, X10, X40 or X100) provided by each objective is engraved on its barrel. The oil-immersion objective has a ring engraved on it towards the tip of the barrel.

Resolving Power of Objective:

It is the ability of the objective to resolve each point on the minute object into widely spaced points, so that the points in the image can be seen as distinct and separate from one another, so as to get a clear un-blurred image.

It may appear that very high magnification can be obtained by using more number of high power lenses. Though possible, the highly magnified image obtained in this way is a blurred, one. That means, each point in the object cannot be found as widely spaced distinct and separate point on the image. Mere increase in size (greater magnification) without the ability to distinguish structural details (greater resolution) is of little value. Therefore, the basic limitation in light microscopes is one not of magnification, but of resolving power, the ability to distinguish two adjacent points as distinct and separate, i.e. to resolve small components in the object into finer details on the image.

Resolving power is a function of two factors: (a) Numerical aperture (n.a.) & (b) Wavelength of the light (λ)

(a) Numerical aperture:- Numerical aperture is a numerical value concerned with the diameter of the objective lens in relation to its focal length. Thus, it is related to the size of the lower aperture of the objective, through which light enters into it. In a microscope, light is focused on the object as a narrow pencil of light, from where it enters into the objective as a diverging pencil (**Fig.4**).

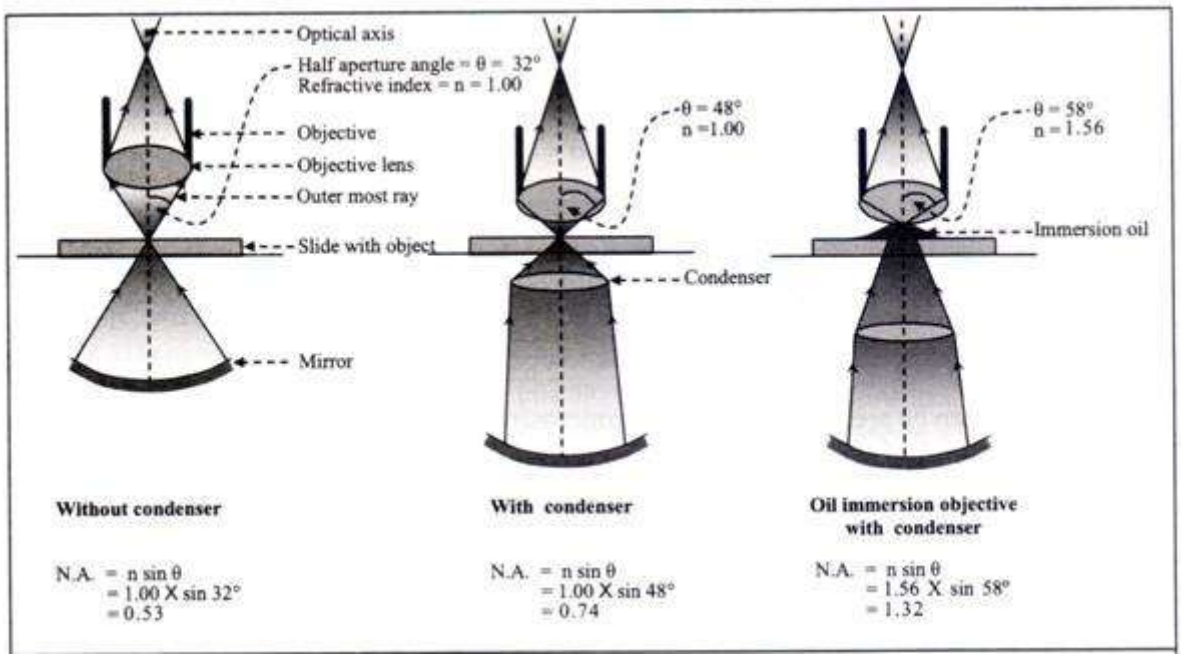


Fig.4 Numerical aperture of objectives

The angle θ subtended by the optical axis (the line joining the centers of all the lenses) and the outermost ray still covered by the objective is a measure of the aperture called 'half aperture angle'. A wide pencil of light passing through the object 'resolves' the points in the object into widely spaced points on the lens, so that the lens can produce these points as distinct and separate on the image. Here, the lens gathers more light.

On the other hand, a narrow pencil of light cannot 'resolve' the points in the object into widely spaced points on the lens, so that the lens produces a blurred image. Here, the lens gathers less light. Thus, the greater is the width of the pencil of light entering into the objective (2θ), the higher is its 'resolving power'.

The numerical aperture of an objective is its light gathering capacity, which depends on the site of the angle θ and the refractive index of the medium existing between the object and the objective. Thus,

Numerical aperture (n.a.) = n sin θ

Where,

n = Refractive index of the medium between the object and the objective and

θ = Half aperture angle

For air, the value of 'n' is 1.00. When the space between the lower tip of the objective and the slide carrying the object is air, the rays emerging through the glass slide into this air are bent or refracted, so that some portion of it do not pass into the objective. Thus, loss of some light rays reduces numerical aperture and decreases the resolving power.

However, when this space is filled with an immersion oil, which has greater refractive index (n=1.56) than that of air (n=1.00), light rays are refracted or bent more towards the objective. Thus, more light rays enter into the objective and greater resolution is obtained. In oil immersion objective, which provides the highest magnification, the size of the aperture is very small.

Therefore, it needs bending of more rays into the aperture, so that the object can be distinctly resolved. That is why, immersion oils, such as cedar wood oil and liquid paraffin are used to fill the gap between the object and the objective, while using oil-immersion objective.

(b) Wavelength of light (λ):- The smaller is the wavelength of light (λ), the greater is its ability to resolve the points on the object into distinctly visible finer details in the image. Thus, the smaller is the wavelength of light, the greater is its resolving power.

Limit of resolution of objective (d):

The limit of resolution of an objective (d) is the distance between any two closest points on the microscopic object, which can be resolved into two separate and distinct points on the enlarged image. Points with their in-between distance less than'd' or objects smaller than 'd' cannot be resolved into

separate points on the image. If the resolving power is high, points very close to each other can be seen as clear and distinct.

Thus, the limit of resolution (the distance between the two resolvable points) is smaller. Therefore, smaller objects or finer details can be seen, when'd' is smaller. Smaller'd' is obtained by increasing the resolving power, which in turn is obtained by using shorter wavelength of light (λ) and greater numerical aperture. Thus,

Limit of resolution = d = λ/2 n.a.

Where,

λ = Wave length of light and

n.a. = Numerical aperture of the objective.

If λ green = 0.55 p and n.a. = 1.30, then d = λ/2 n.a. = 0.55/2 X 1.30 = 0.21 µ. Therefore, the smallest details that can be seen by a typical light microscope is having the dimension of approximately 0.2 µ. Smaller objects or finer details than this cannot be resolved in a compound microscope.

5. Eyepiece:- The eyepiece is a drum, which fits loosely into the draw tube. It magnifies the magnified real image formed by the objective to a still greatly magnified virtual image to be seen by the eye (**Fig.5**).

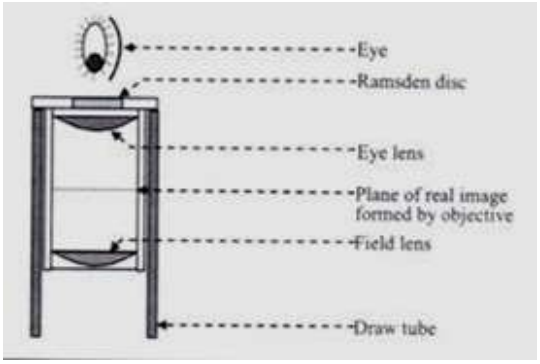


Fig.5 Eyepiece or ocular

Magnification Power of Compound Microscope

The total magnification of image formed by the compound microscopes is calculated b this following formula:

$m = D/ f_o \times L/f_e$

Where, D = Least distance of distinct vision (25 cm)

L = Length of the microscope tube

f_o = Focal length of the objective lens

f_e = Focal length of the eye-piece lens

Practically the total magnification is calculated as follows:

$M_t = M_{ob} \times M_{oc}$

Where,

M_t = Total magnification,

M_{ob} = Objective magnification and

M_{oc} = Ocular magnification

If the magnification obtained by the objective (M_{ob}) is 100 and that by the ocular (M_{oc}) is 10, then total magnification (M_t) = 100 X 10 =1000. Thus, an object of l µ will appear as 1000 µ.

Phase contrast microscope

The Phase Contrast Microscope was designed by Frits Zernike, a Dutch physicist who was awarded the Nobel Prize in 1953. It is the microscope that permits living cells to be observed. To achieve contrast, this microscopy employs unique optical components that take advantage of minute changes in the refractive indices of water and cytoplasmic components of living cells.

Principle:- The phase contrast microscopy is based on the principle that small phase changes in the light rays, induced by differences in the thickness and refractive index of the different parts of an object, can be transformed into differences in brightness or light intensity. When light passes through a transparent

object in a medium having different refractive index, the amplitude of the light waves remain unchanged while their velocity changes leading to phase change. Human eye is sensitive to amplitude change but not phase change. Thus, transparent objects causing phase change of light waves remain invisible to human eye. Different protoplasmic constituents of the cell produce phase changes as they vary in thickness and refractive index. Diffraction of light generates a set of new waves which differ in phase from the direct light not passing through the object. The diffracted light waves are retarded in phase by about a quarter of a wavelength, i.e. λ/4 for a cell. The microscope image is formed due to interference between the diffracted and direct light waves differing in amplitude. If the phase difference between these two sets of waves is further increased to λ/2, then maximal destructive interference will result leading to amplitude difference as well as intensity difference. This can be perceived by the eye and the transparent object is seen as an absorbing object which appears dark to a greater or lesser extent depending on the degree of phase change.

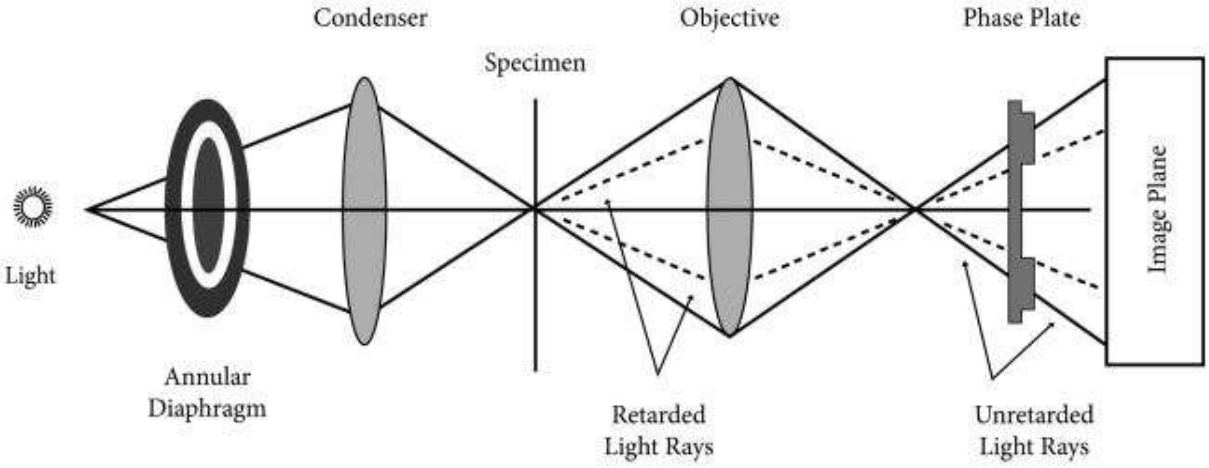


Fig.1 Ray diagram of a phase-contrast microscope

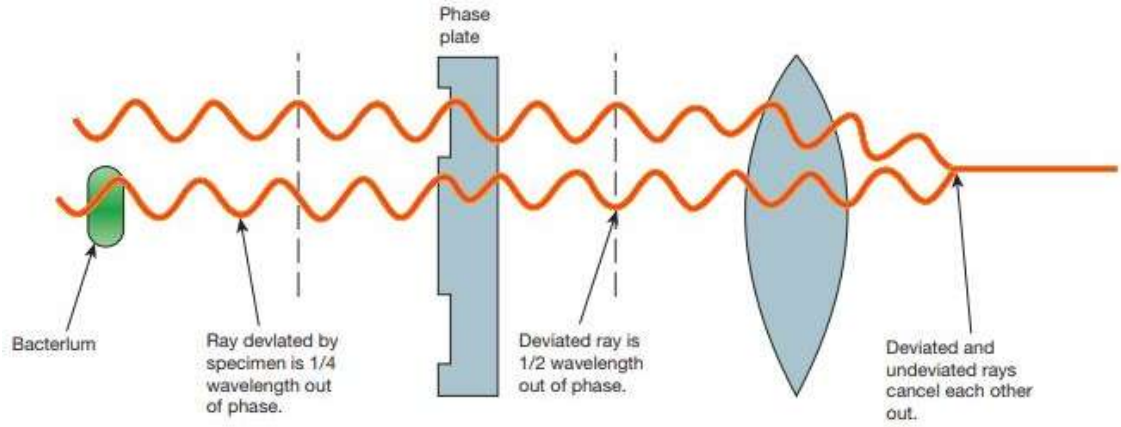


Fig.2 Ray diagram depicting phase changes

The phase contrast microscope has special devices such as annular diaphragm and phase plate, which convert these minute phase changes into brightness (amplitude) changes, so that a contrast difference can be created in the final image. This contrast difference can be easily detected by human eyes. In phase contrast microscope, to get contrast, the diffracted waves have to be separated from the direct waves. This separation is achieved by the sub-stage annular diaphragm. The annular diaphragm illuminates the specimen with a hollow cone of light. Some rays (direct rays) pass through the thinner region of the specimen and do not undergo any deviation and they directly enter into the objective lens. The light rays passing through the denser region of the specimen get regarded and they run with a delayed phase than the non-deviated rays. Both the deviated and non- deviated light has to pass through the phase plate kept on the back focal plane of the objective to form the final image. The difference in phase (Wavelength) gives the contrast for clear visibility of the object.

Optical Components of PCM:- The phase contrast microscope is similar to an ordinary compound microscope in its optical components. It possesses a light source, condenser system, objective lens system

and ocular lens system. A phase contrast microscope differs from bright field microscope in having a phase condenser and phase plate.

Phase condenser:- An annular diaphragm in the sub-stage (phase condenser) In the focal plane of the sub-stage, an annular aperture in the diaphragm regulates the illumination of the object. This is found behind the microscope's condenser. This annular diaphragm serves to highlight the object by forming a narrow, hollow cone of light.

Phase Plate:- The back focal plane of the objective lenses houses this plate. The phase plate is divided into two sections, one of which is covered with a light-retarding material (Magnesium fluoride) and the other of which is light-absorbing but not light-retarding. This plate aids in the reduction of incident light phase.

Applications of PCM:

1. Phase contrast microscope enables the visualization of unstained living cells.
2. It makes highly transparent objects more visible.
3. It is used to examine various intracellular components of living cells at relatively high resolution.
4. It helps in studying cellular events such as cell division.
5. It is used to visualize all types of cellular movements such as chromosomal and flagellar movements.

Fluorescence Microscopy

It refers to the microscopic technique that uses chemical substances that stain the specimen by absorbing white light of a specific wavelength and emitting light of a longer wavelength to form an image. Fluorescent dyes are the chemical substances used in this advanced microscopic method. Chemical particles within the fluorescent dye come into action under visible light. Some portions of the specimen may take up the stain, while a few portions may not after staining the specimen with fluorescent dyes. Therefore, the portion stained with fluorescent dye will appear fluorescent green against the dark black background, while the unstained portions will remain invisible.

It is available in different designs. The epifluorescence microscopes have the most common and simple setup, while confocal microscopes have a sophisticated setup. Epifluorescence microscope has prominent applicability in different laboratories, which allow excitation of reactive dyes and detection of the fluorescent light emitted through the same objective. Therefore, the working of a fluorescent microscope depends upon the reactive species within the fluorescent dye, which absorb photons of the exciting light (low wavelength) and later transmit fluorescent light (high wavelength). There is a short delay between the excitation and emission of fluorescent light that is generally negligible (takes nanoseconds). This post mainly explains the definition, working and phenomena of fluorescence.

Principle of Fluorescence Microscopy:- The working of a fluorescent microscope depends upon the principle of fluorescence rather than scattering and reflection of light. Some chemical substances or reactive dyes possess the property of light absorption and emission, which can undergo the fluorescence phenomenon. George G. Stokes was the first to study the mechanism of fluorescence and also coined the term in 1852.

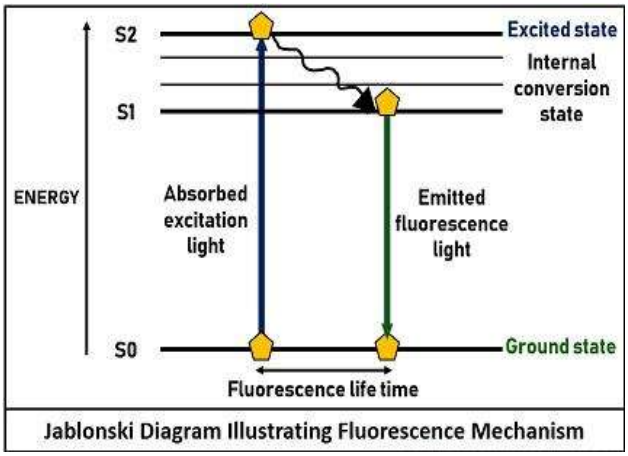


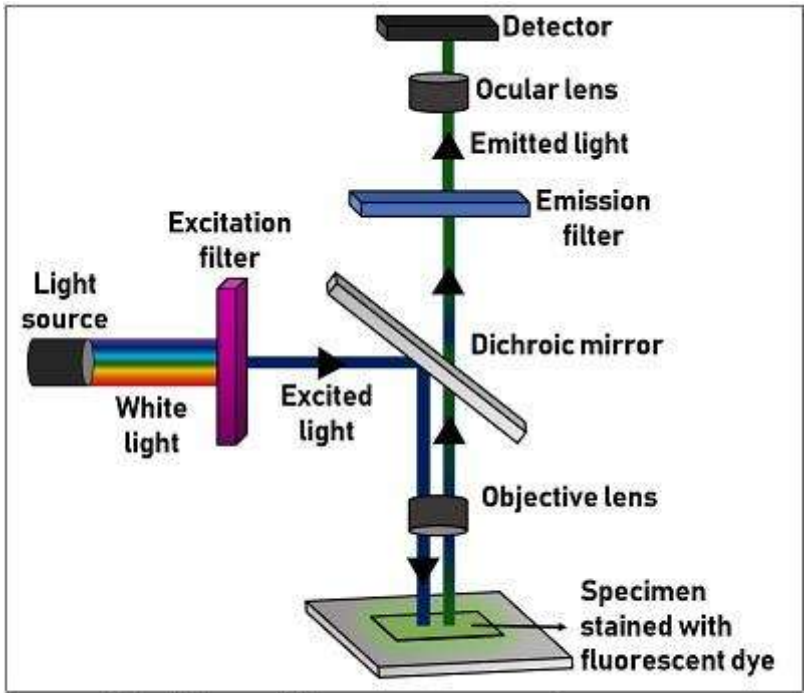
Fig.1

Fluorescent molecules absorb the light of a short wavelength and excite due to high energy photons through the excitation mechanism. Here, the light triggering the fluorescent particles refers to the X-rays and UV-rays.

On the other hand, fluorescent molecules emit high wavelength light via an emission mechanism. Here, the light reflected by these chemical substances after absorption of photons merely refers to the emitting light generally in the form of visible light. The fluorescence is achieved when the high energy photon molecules in the excited state will go back to their normal ground state by releasing some energy in the form of fluorescence. The difference between the phase of excitation and emission is known as "Strokes shift", which can be relatively short and sometimes far apart.

Working principle of Fluorescence Microscopy:-

1. Firstly, a light source falls onto the excitation filter.
2. The function of the excitation filter is only to pass the light of a particular wavelength that can excite the fluorescent molecules tagged in the specimen.
3. Then, the excitation light falls onto the dichroic mirror.
4. A diachronic mirror reflects the emitted light towards the objective lens and then onto the specimen.
5. As the specimen is stained with a fluorescent dye, the fluorescent molecules will excite and emit a high wavelength light in a reverse manner.
6. The emitted light will first go through the dichroic mirror that permits most of the green light to pass along with some blue light towards the emission filter.
7. The emission filter only permits the green light of a longer wavelength and totally rejects the blue light.
8. Finally, the green light goes to the ocular lens and finally to the detector.
9. The detector detects and allows the green light to fall back onto the specimen.
10. Thus, the majority of specimens appear fluorescent green against a jet black background.



Working of fluorescence microscopy

Parts of Fluorescent Microscope:-

Typical components of a fluorescence microscope are as follows:

Fluorescent dyes: These are the chemical compounds called **fluorophore** or **reactive dyes**. Fluorescent dyes possess a property of fluorescence, by which they can form a fluorescent image by emitting highly contrast visible green light after getting excited by the highly illuminating UV light. A fluorophore highlights a wide range of biomolecules like antibodies, nucleic acids, proteins, etc. For instance, Hoechst and phalloidin dyes stain actin fibres in mammalian cells.

Light Source: It includes xenon arc lamps, mercury-vapour lamps, lasers, and high-power LEDs. The sophisticated confocal fluorescent microscope mainly uses laser light. On the contrary, a simple epifluorescent microscope uses a light source from xenon lamps, mercury lamps, and LEDs. Epifluorescence microscopy has wide applicability in different laboratories.
Excitation Filter: The excitation filters are designed with high-resolution capacity and interference optics. It passes the light of a shorter wavelength, which the fluorescent dye could absorb. Also, it blocks the other sources of exciting light.
Dichroic Mirror: It is a dichromatic beam splitter that works as an interference colour filter. Thus, a dichroic mirror selectively reflects or transmits light of determined wavelengths. It generally sets at an angle of 45 degrees along the path of light coming from the exciter filter in the fluorescent microscope.
Emission Filter: It has a coloured glass or interference coating or a combination of both. It works as a barrier filter that only permits the fluorophore's passage and blocks excitation light.

Advantages

- Fluorescence microscopy helps in the study of cell behaviour.
- It is the specific microscopic method that highlights the biomolecule of interest.
- The fluorescent microscope can also highlight the image of particular structural components within the microscopic organisms.
- It is a highly sensitive technique that can detect around 50 molecules/µm3.
- Fluorescent dyes tag biomolecules differently, by which we can analyze or track the physiochemical properties of multiple biomolecules simultaneously.

Disadvantages

- It only allows the observation of specific structures inside a cell tagged with the fluorescent dye.
- The photobleaching due to the electron excitation during the process of fluorescence may affect reactive molecules of the fluorescent dyes. As a result, the reactive dyes might lose their chemical property of fluorescence emission intensity.
- The cells are susceptible to the phototoxic effect after staining with fluorescent dyes, as the fluorophore molecules absorb the high energy photons from the short-wavelength light.

Confocal microscopy

Confocal microscopy, most frequently confocal laser scanning microscopy (CLSM) or laser confocal scanning microscopy (LCSM), is an optical imaging technique for increasing optical resolution and contrast of a micrograph by means of using a spatial pinhole to block out-of-focus light in image formation. Capturing multiple two-dimensional images at different depths in a sample enables the reconstruction of three-dimensional structures (a process known as optical sectioning) within an object. This technique is used extensively in the scientific and industrial communities and typical applications are in life sciences, semiconductor inspection and materials science.

Light travels through the sample under a conventional microscope as far into the specimen as it can penetrate, while a confocal microscope only focuses a smaller beam of light at one narrow depth level at a time. The CLSM achieves a controlled and highly limited depth of field.

Principle:

The principle of confocal imaging was patented in 1957 by Marvin Minsky and aims to overcome some limitations of traditional wide-field fluorescence microscopes. In a conventional (i.e., wide-field) fluorescence microscope, the entire specimen is flooded evenly in light from a light source. All parts of the sample can be excited at the same time and the resulting fluorescence is detected by the microscope's photodetector or camera including a large unfocused background part. In contrast, a confocal microscope uses point illumination and a pinhole in an optically conjugate plane in front of the detector to eliminate out-of-focus signal – the name "confocal" stems from this configuration. As only light produced by fluorescence very close to the focal plane can be detected, the image's optical resolution, particularly in the sample depth direction, is much better than that of wide-field microscopes. However, as much of the

light from sample fluorescence is blocked at the pinhole, this increased resolution is at the cost of decreased signal intensity – so long exposures are often required. To offset this drop in signal after the pinhole, the light intensity is detected by a sensitive detector, usually a photomultiplier tube (PMT) or avalanche photodiode, transforming the light signal into an electrical one.

As only one point in the sample is illuminated at a time, 2D or 3D imaging requires scanning over a regular raster (i.e. a rectangular pattern of parallel scanning lines) in the specimen. The beam is scanned across the sample in the horizontal plane by using one or more (servo controlled) oscillating mirrors. This scanning method usually has a low reaction latency and the scan speed can be varied. Slower scans provide a better signal-to-noise ratio, resulting in better contrast.

The achievable thickness of the focal plane is defined mostly by the wavelength of the used light divided by the numerical aperture of the objective lens, but also by the optical properties of the specimen. The thin optical sectioning possible makes these types of microscopes particularly good at 3D imaging and surface profiling of samples.

Successive slices make up a 'z-stack', which can either be processed to create a 3D image, or it is merged into a 2D stack (predominately the maximum pixel intensity is taken; other common methods include using the standard deviation or summing the pixels).

Confocal microscopy provides the capacity for direct, noninvasive, serial optical sectioning of intact, thick, living specimens with a minimum of sample preparation as well as a marginal improvement in lateral resolution compared to wide-field microscopy. Biological samples are often treated with fluorescent dyes to make selected objects visible. However, the actual dye concentration can be low to minimize the disturbance of biological systems: some instruments can track single fluorescent molecules. Also, transgenic techniques can create organisms that produce their own fluorescent chimeric molecules (such as a fusion of GFP, green fluorescent protein with the protein of interest). Confocal microscopes work on the principle of point excitation in the specimen (diffraction limited spot) and point detection of the resulting fluorescent signal. A pinhole at the detector provides a physical barrier that blocks out-of-focus fluorescence. Only the in-focus, or central spot of the Airy disk, is recorded.

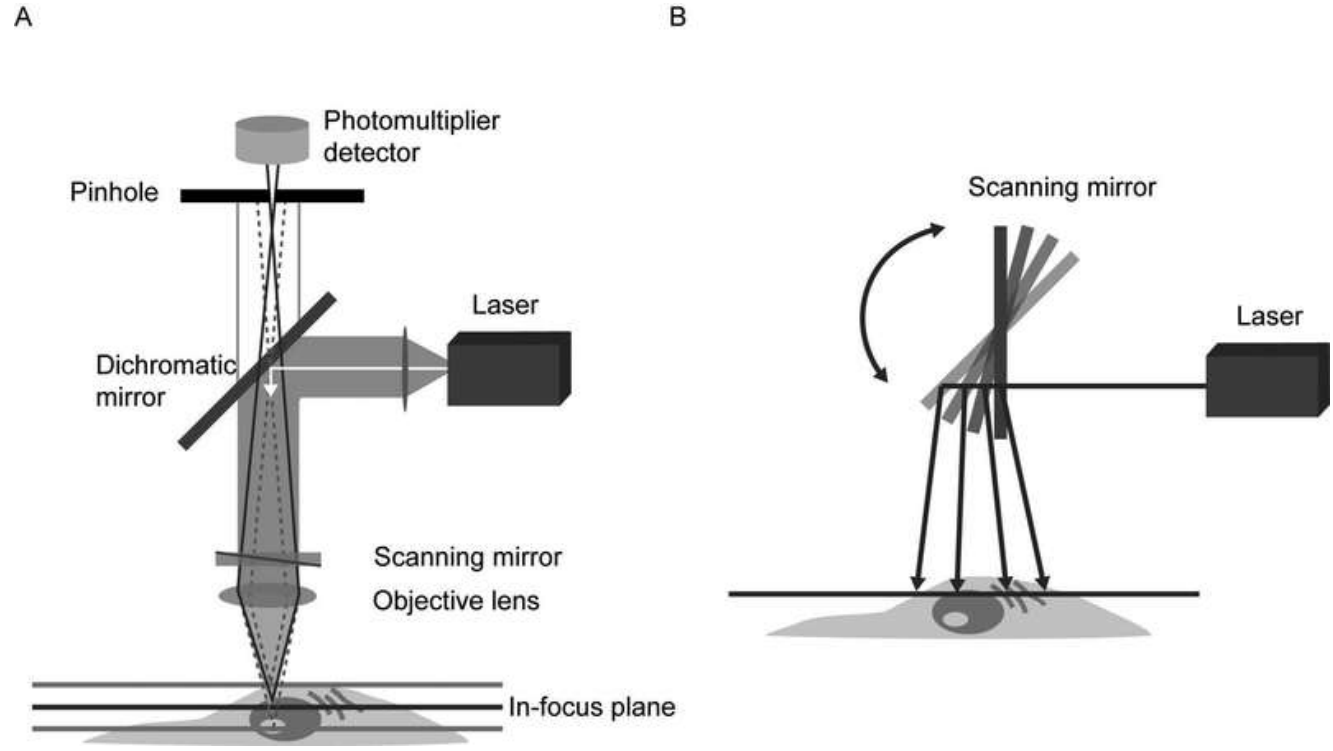


Fig. Working principle of a confocal microscope. A. Light from a laser source is passed through collimating optics to a variable dichromatic mirror or AOBS and reflected to the objective lens which focuses the beam on a point in the sample. Scanning mirrors sweep the excitation beam over the sample point by point to

build the image. Emitted fluorescence passes back through the objective lens, the dichromatic mirror or AOBS, and is detected by the PMT(s). A pinhole placed in the conjugate image plane to the focal point in the sample serves to reject out-of-focus light, which does is not picked up by the detector. In this epifluorescence configuration, the illumination and emission light both pass through the same lens, thus requiring only the detector-side pinhole. Varying the size of the pinhole changes the amount of light collected and the optical section thickness. Spectral imaging can be achieved with an array of PMTs and a diffraction grating, or prism, placed in the emission light path. B. A schematic of the scanning mirrors employed by confocal microscopes to sweep the excitation light across the sample.

Components of confocal microscope:

The basic components of a modern confocal microscope are the pinholes, the objective lenses, and low-noise detectors in common with the original design but also typically include fast scanning mirrors, filters for wavelength selection, and laser illumination. While gas lasers (argon and helium-neon) are still in use, diode lasers, fiber lasers, and solid-state lasers are increasingly common. These light sources are more stable, more uniform, produce less heat, and emit a broad range of visible wavelengths. Detectors are still primarily highly sensitive photomultipliers (PMTs) due to the light-rejecting nature of a confocal microscope. These are essentially one spot cameras that maximize the light budget by amplifying the signal over a photoelectric device.

In light microscopy, the resolution is determined by the numerical aperture (NA) of the objective lens, the properties of the sample (index of refraction), and the wavelength of light. The lateral resolution of a confocal microscope is improved over a conventional widefield fluorescence microscope when the pinholes are closed to the minimum size providing a diffraction-limited imaging system. The best resolution that can be obtained is ~ 0.2 µm laterally and ~ 0.6 µm axially, though in practice that is not always achieved. Despite the pinholes, the axial resolution in a confocal microscope is still worse than the lateral resolution, as in widefield fluorescence microscopy. The equations used to determine lateral and axial resolution are as follows:

$R_{lateral}=0.4\lambda/NA$----------Equation 1

$R_{axial}=1.4\lambda\eta/(NA)2$----------Equation 2

Where R is the resolution, λ is the emission light wavelength, η is the refractive index of the mounting medium (speed at which light propagates through the material), and NA is the objective's numerical aperture. There is a tradeoff in confocal microscopy between the light collection efficiency and resolution. For dimly fluorescing samples, the pinhole may be opened to collect more light toward improving the contrast at the cost of resolution. Similarly, the resolution can be improved by closing the detection-side pinhole to a size smaller than one Airy unit at the cost of signal-to-noise. An Airy unit is defined as the zeroth order portion of the airy disc (central diffraction spot) at the image plane. At one Airy unit, the system is diffraction-limited.

Applications:

CLSM is widely used in various biological science disciplines, from cell biology and genetics to microbiology and developmental biology. It is also used in quantum optics and nano-crystal imaging and spectroscopy.

Biology and medicine: Clinically, CLSM is used in the evaluation of various eye diseases, and is particularly useful for imaging, qualitative analysis, and quantification of endothelial cells of the cornea. It is used for localizing and identifying the presence of filamentary fungal elements in the corneal stroma in cases of keratomycosis, enabling rapid diagnosis and thereby early institution of definitive therapy. Research into CLSM techniques for endoscopic procedures (endomicroscopy) is also showing promise. In the pharmaceutical industry, it was recommended to follow the manufacturing process of thin film pharmaceutical forms, to control the quality and uniformity of the drug distribution. Confocal microscopy is also used to study biofilms — complex porous structures that are the preferred habitat of microorganisms. Some of temporal and spatial function of biofilms can be understood only by studying

their structure on micro- and meso-scales. The study of microscale is needed to detect the activity and organization of single microorganisms.

Optics and crystallography: CLSM is used as the data retrieval mechanism in some 3D optical data storage systems and has helped determine the age of the Magdalen papyrus.

Audio preservation: The IRENE system makes use of confocal microscopy for optical scanning and recovery of damaged historical audio.