MICROSCOPY

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INTRODUCTION:

The science of cytology emerged after the discovery of microscope and its application by Robert Hooke (1665) in studying a slice of cork. Later, with advancement of technology and science the power, resolution and clarity of microscopic images enhanced to the present stage of excellence. Thus from a simple microscope with feeble magnification, electron microscope has emerged that can resolve sub-cellular organelle with the precision of an experienced surgeon.

HISTORICAL REVIEW:

- First optical microscope was built by Janseen and Janseen (1590).
- First compound microscope was built by Kelper (1611).
- First laboratory microscope was built by Robert Hooke (1665); its magnification was 14 - 42x.
- TEM was developed by Knoll and Ruska (1932).
- SEM was developed by Knoll in 1935 but was first used in 1965.
- Phase contrast microscope was developed by Zernicke (1935, Nobel prize in 1953).
- Ultraviolet microscope was developed by Casperson (1938).

LIGHT MICROSCOPE / COMPOUND MICROSCOPE:

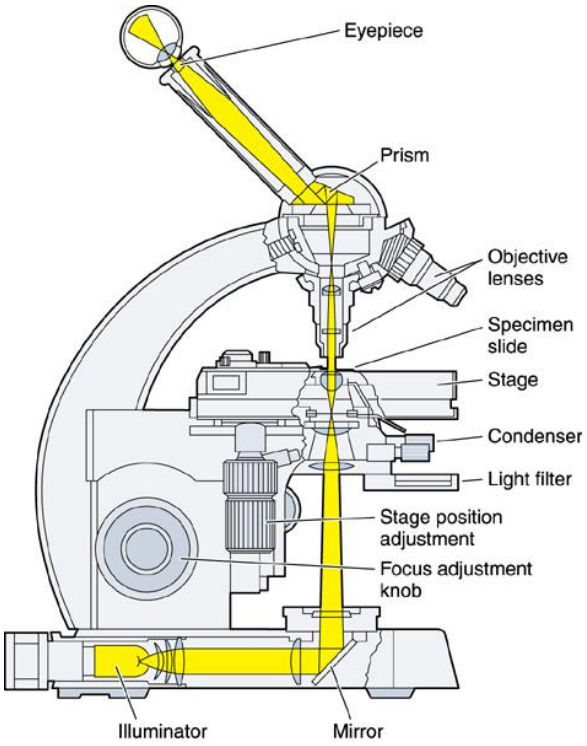


Figure : Schematic drawing of a light microscope showing its main components and the pathway of light from the substage lamp to the eye of the observer.

PROPERTIES OF LIGHT MICROSCOPE:

A brief description of the characteristics features of light microscope is as discussed below:

§ Source of light:

Within the light microscope, stained preparations are usually examined by means of light that passes through the specimen. The source of light may be sunlight reflected on the specimen via condenser lens by a reflector (prism, plano-concvave mirror etc.) or an electric bulb connected to a 220V power supply. The average wave length of the visible light spectrum is around 390-760nm.

§ Medium of light transmission:

The light reaches the eye of the observer via air and series of glass lenses.

§ Nature of the specimen:

The specimen for examination might be dehydrated as well as hydrated as both are penetrable by light.

§ Lens:

- Condenser lens: It is a convex glass lens that helps in focusing the beam of reflected light from the reflector on the specimen.
- Objective lens: The emerging light from the specimen passes through a convex, objective lens and get magnified while traveling towards the eye- piece. It may be of 4x, 10x, 40x, 60x, 100x, etc. variable magnification power.
- Eye-piece lens: It is the convex lens on which an observer sets his eye(s). It magnifies the image formed by the objective lens by 5x or 10x or 15x times.

§ Nature of image:

The image formed by the set of lenses of the microscope is perceptible to human eye. The colour and texture of the image is an exact replica of the specimen under observation. The image can be photomicrographed by suitable camera in desired tone.

§ Magnification:

The magnifying power of image by a light microscope is appreciable for ordinary viewing and it ranges for a maximum 1500x under immersion oil (15x eye-piece x 100x objective =1500x). Compared to an EM, it has a very low magnification.

§Contrast:

The contrast of an image is developed upon differentiation of the varied parts of the object under observation. The varied wave length of the components of the beam of light emerging from the specimen under observation is responsible for generation of contrast in the image of a microscope.

§ Resolution:

The resolving power of a microscope can be defined as the ability of the device to view distinct images of point objects separated from each other by finest distance. The resolution of light microscope has a maximum range of 0.25µm i.e. the light microscope will be incapable of perceiving separately two different objects separated by a distance less than 0.25µm and in that case the two different objects will be viewed as a single object.

§ Tissue preparation:

- **Fixation:** Fixation of sample tissue is done primarily to preserve the conformity and stability of the cellular architecture. Alterations in conformity of the cellular components are generally not taken into consideration. Besides fixation also prevent unwanted decomposition and infection of the tissue. The common fixatives are alcohols, formaldehyde, acetic acid , mercuric chloride etc. or mixture of the above in definite proportions.
- **Embeddening:** The fixed tissue samples are embedded in molten paraffin (melting point: 55^0-60^0 C) which hardens on cooling at the room temperature.
- **Sectioning:** Thin tissue section of 5-7µ thickness is ideal for light microscopy. Sectioning of the paraffin block is done on a tissue-microtome with steel blade knife. The sectioned tissue appears as ribbons which are then mounted on slides (in Mayer's albumin) on hot plate for stretching and adherence.
- **Staining:** Staining is done to generate differential colour pattern of the tissue samples based on their chemical nature. Acidic and/or basic dyes are used to generate such colour differentiation. Haematoxylin and eosin are popularly used for counter staining- the former stains the nucleus dark blue while the later stains the cytoplasm faint red. Giemsa's stain is also a very popular stain with proven nuclear affinity.

Remark: Before staining, the stain impermeable paraffin layer on the tissue sample of the slide has to be removed by dipping the specimen in xylene- an organic solvent.

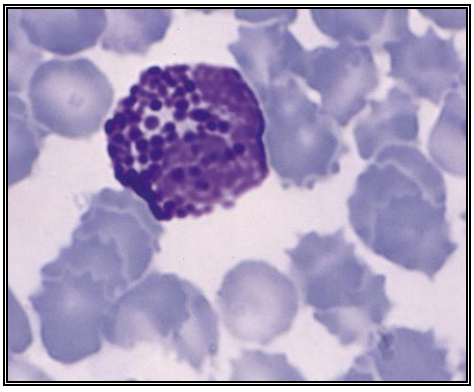
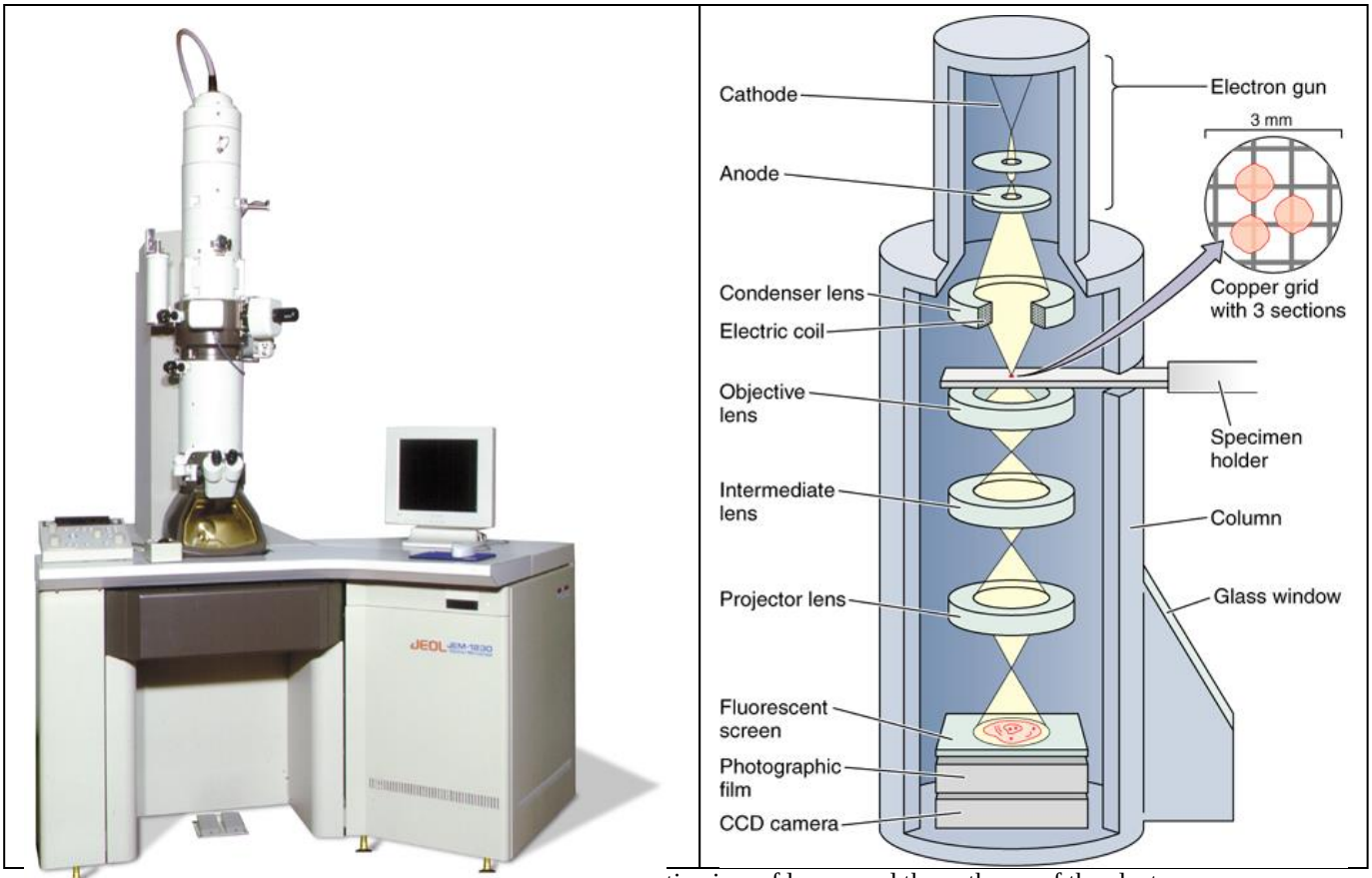


Figure: A basophil with many granules covering the cell nucleus. This makes it difficult to see the nucleus clearly. Giemsa stain. High magnification.

ELECTRON MICROSCOPES:



tic view of lenses and the pathway of the electrons.

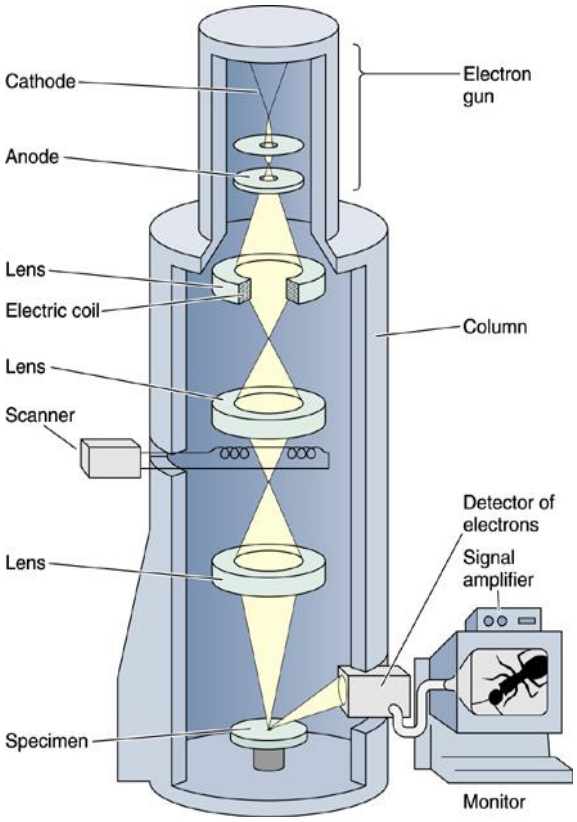
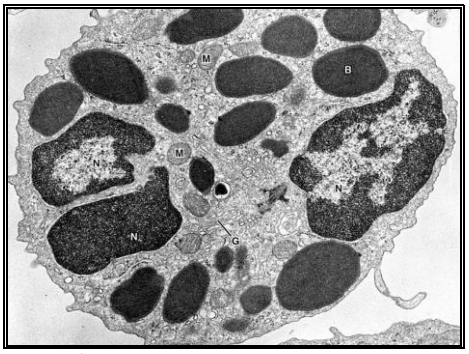
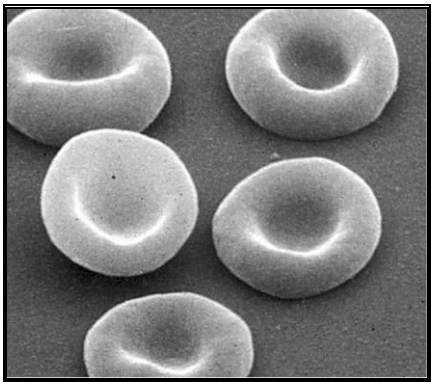


Figure: Schematic view of a scanning electron microscope



(Fig: a)



(Fig:b)

Figure a: Electron micrograph of a rabbit basophil. The lobulated nucleus (N) appears as 3 separated portions. Note the basophilic granule (B), mitochondria (M), and Golgi complex (G). x 16,000.

Figure b: Scanning electron micrograph of normal human erythrocytes. Note their biconcave shape. x3300.

PROPERTIES OF TRANSMISSION ELECTRON MICROSCOPE:

A brief description of the characteristics features of light microscope is as discussed below:

§ Source of light:

Any light source is not in use. Instead a beam of flowing electron passes through the specimen under observation. The beam of electron is generated from a heated tungsten wire at 3000^0C based on the principle of "thermionic emission" (when a metal is heated, the electrons of the outermost orbital get over-energized and get expelled to the next higher orbital). The electron gun consists of:

- a V-shaped tungsten filament that acts as cathode
- a metal plate with a central hole that acts as cathode shield
- a metal plate with a central hole that acts as anode
- a series of condenser lens.

The entire set up is connected to a 40-120KV supply. The cathode tungsten filament on heating at 3000^0C emits stream of electrons that passes through the central hole of the negatively charged cathode shield and falls on the anode to form a condensed beam of flowing electron. The entire phenomenon occurs within a vacuum tube at reduced pressure of less than 0.0001mm of mercury.

§ Medium of electron transmission:

The transmission of electron is made to happen inside a vacuum tube since the electron might get scattered or deflected and their speed will get reduced in contact with air.

§ Nature of the specimen:

The specimen for examination should be made absolutely dehydrated as the electrons prior to their incidence on the sample tissue, might get scattered on collision with the water molecules. Hydrated sample will form obscure image.

§ Lens:

All the lenses used within a TEM are electromagnetic lenses that have specialized property of concentrating and condensing a flowing electron stream. The lenses are be of following types and numbers:

- Condenser lens: Two in number. They help to produce condensed and narrower beam of electrons.
- Objective lens: A series of objective lenses helps to gather the electrons deflected from the specimen and magnify the image manifold.
- Projector lens: The image formed by the objective lenses is further magnified by the projector lens.

Remark: The power of the electromagnetic lenses can be altered by regulating the amount of current flowing through the electromagnets.

Nature of image:

The image formed by the flowing electrons is not visible by naked eye. It becomes perceptible to our eye only when it is casted on a fluorescent screen of zinc sulphide. The image formed is always in black and white tone and con be photomicrographed accordingly in desired magnification.

§ Magnification:

The magnification power of EM is of the range of 1, 20,000x for tissue samples and 4 00,000x for isolated molecules. A series of objective lenses help to generate such huge magnification.

§ Resolution: The resolving power of TEM is extraordinarily high: 0.5Å-5.0 Å.

§ Tissue preparation:

- **Fixation:** The fixation of sample tissue for electron microscopy is done not only to preserve the conformity and stability of the cellular architecture but also to keep intact the volume and dimensional properties cellular and sub-cellular organization. Ordinary fixatives are of no use for EM sample fixation because:
  - ¥ alcohols cause shrinkage of cells and dislocation of lipid based cellular components
  - ¥ acids cause swelling of cells
  - ¥ mercury salts cause distortion in protein components of cells
  - ¥ aldehydes cause stiffening of the tissue fraction

  As such fixation of tissue for TEM is done either with glutaraldehyde or with osmium tetroxide.
- **Embeddening:** Embeddening of the tissue sample is inappropriate with paraffin. The reasons are:
  - ¥ paraffin undergoes considerable volume change on solidification from molten stage.
  - ¥ it is too soft for making section less than 0.1µm thick.
  - ¥ it is not permeable to stains.
  - ¥ it is too volatile to tolerate the heating caused by electron bombardment.

  To avoid this difficulty, polymerized resin is used for embeddening which is harder and durable than paraffin. Removal of the embeddening medium is not necessary prior to staining as the embeddening medium is pervious to the staining salts.
- **Sectioning:** Ultrathin section of the magnitude of less than 0.1µm is of absolute necessity. Sectioning is done with high quality crystal or diamond knife. Tissue sections are placed on gold or copper meshed grids. Sectioning is done in ultra-microtome.
- **Staining:** Staining of the SEM tissue sample is done to increase the scattering effect of the electrons. As such high density heavy metal salts are used to serve the purpose. The common salts used are:
  - * lead hydroxide or lead citrate to bind specifically with nucleic acid and proteins
  - * phosphotungstenic acid, palladium, gold etc for binding collagen.

* uranyl acetate to bind nucleic acid and protein etc.

The black and white image generated in the process shows dark electron dense regions and faded electron lucent regions.

	Light Microscope	**Electron Microscope**
1	Illuminating source is visible light	Illuminating source is beam of electrons
2	Microscope is compact and handy	Microscope is large and complicated
3	Both living and fixed specimen can be studied	Only fixed specimen can be studied
4	Specimen may be hydrated or dehydrated	Specimens are strictly dehydrated
5	No vacuum tube is required for transmission of light	A vacuum tube is necessary for transmission of electrons in low pressure condition
6	No need for high voltage electricity supply; a mere 220V supply is enough	A high voltage supply of the magnitude 40-120 KV is necessary
7	Anode or cathode is not necessary	An electron emitting tungsten filament is used as cathode.
8	Cooling system is not necessary	A cooling system to reduce the heat generated by high voltage power supply present
9	Risk of radiation is absent	Risk of radiation through leakage is present
10	Glass lens is used	Electromagnetic lens is used
11	Image is formed due to absorption of light waves	Image is formed due to scattering of electrons
12	Image can be seen directly	Image is seen only on fluorescent screen
13	Specimen is stained with colour dyes	Specimen is impregnated with heavy metal salts
14	Image may be coloured or black and white as per specimen	Image is black and white
15	Resolving power is 0.25μm or 250nm or 2500 Å	Resolving power is 0.5-5.0 Å
16	Maximum magnification is 1500x	Magnification may range up to 4,00,000x
17	Embeddening of tissue is done in molten paraffin; steel knife is used in sectioning	Embeddening of tissue is done in polymeric resin; diamond knife is used in sectioning
18	It is used for studying gross internal structure of tissue.	It is used to study external surface (SEM) and ultrastructure of cell and small organisms (TEM)

Phase Contrast Microscopy

Frits Zernike, a Dutch physicist and mathematician, built the first phase contrast microscope in 1938. It took some time before the scientific community recognized the potential of Zernike's discovery; he won the Nobel Prize in 1953 and the German-based company Zeiss began manufacturing his phase contrast microscope during World War II. Most of the detail of living cells is undetectable in bright field microscopy because there is too little contrast between structures with similar transparency and there is insufficient natural pigmentation. However the various organelles show wide variation in refractive index, that is, the tendency of the materials to bend light, providing an opportunity to distinguish them.

Principle

Highly refractive structures bend light to a much greater angle than do structures of low refractive index. The same properties that cause the light to bend also delay the passage of light by a quarter of a wavelength or so. In a light microscope in bright field mode, light from highly refractive structures bends farther away from the center of the lens than light from less refractive structures and arrives about a quarter of a wavelength out of phase. Light from most objects passes through the center of the lens as well as to the periphery. Now if the light from an object to the edges of the objective lens is retarded a half wavelength and the light to the center is not retarded at all, then the light rays are out of phase by a half wavelength. They cancel each other when the objective lens brings the image into focus. A reduction in brightness of the object is observed. The degree of reduction in brightness depends on the refractive index of the object.

Applications for phase contrast microscopy

Phase contrast is preferable to bright field microscopy when high magnifications (400x, 1000x) are needed and the specimen is colorless or the details so fine that color does not show up well. Cilia and flagella, for example, are nearly invisible in bright field but show up in sharp contrast in phase contrast. Amoebae look like vague outlines in bright field, but show a great deal of detail in phase. Most living microscopic organisms are much more obvious in phase contrast.

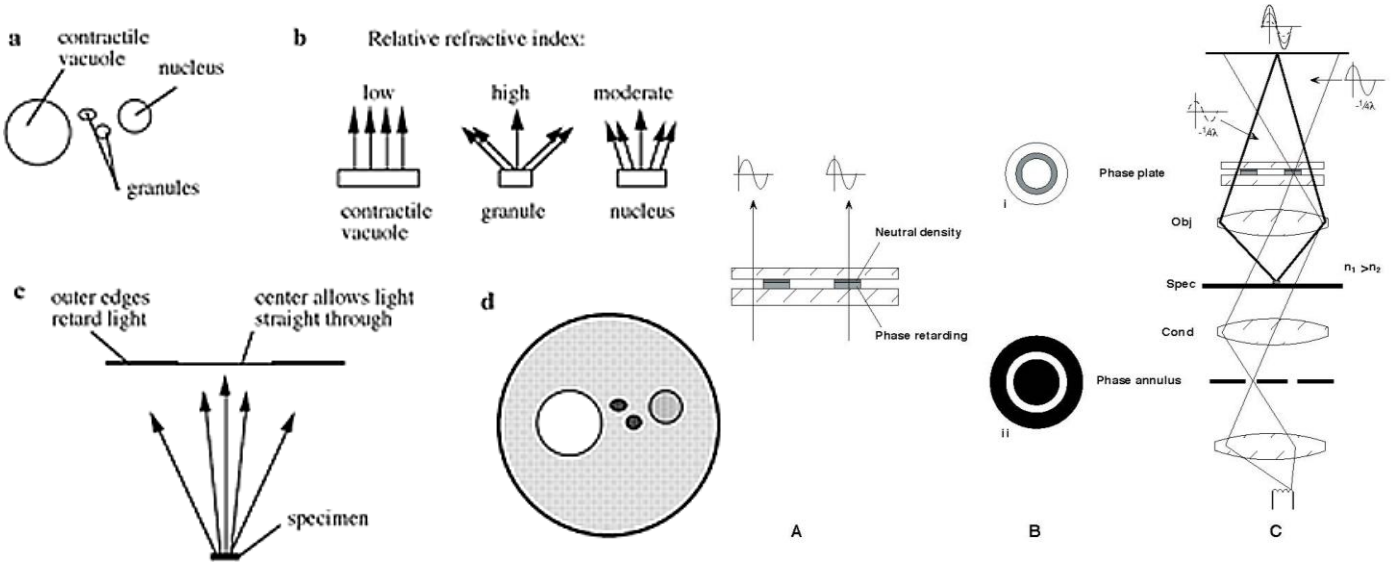


Figure. (a) organelles are nearly invisible in bright field although they have different refractive indexes; (b) light is bent and retarded more by objects with a high refractive index; (c) in phase contrast a phase plate is placed in the light path. Barely refracted light passes through the center of the plate and is not retarded. Highly refracted light passes through the plate farther from center and is held back another one quarter wavelength.; (d) The microscope field shows a darker background (in this case the cell cytoplasm has a higher refractive index than the contractile vacuole), with the organelles in sharp contrast.

Using phase contrast

Phase contrast condensers and objective lenses add considerable cost to a microscope, and so phase contrast is often not used in teaching labs except perhaps in classes in the health professions and in some university undergraduate programs. This is unfortunate since the images obtainable in phase contrast mode can be very dramatic. To use phase contrast the light path must be aligned. An element in the condenser is aligned with an element in a specialized phase contrast lens. This usually involves sliding a component into the light path or rotating a condenser turret. The elements are either lined up in a fixed position or are adjusted by the observer until the phase effect is optimized. Generally, more light is needed for phase contrast than for corresponding bright field viewing, since the technique is based on a diminishment of brightness of most objects.

Advantages

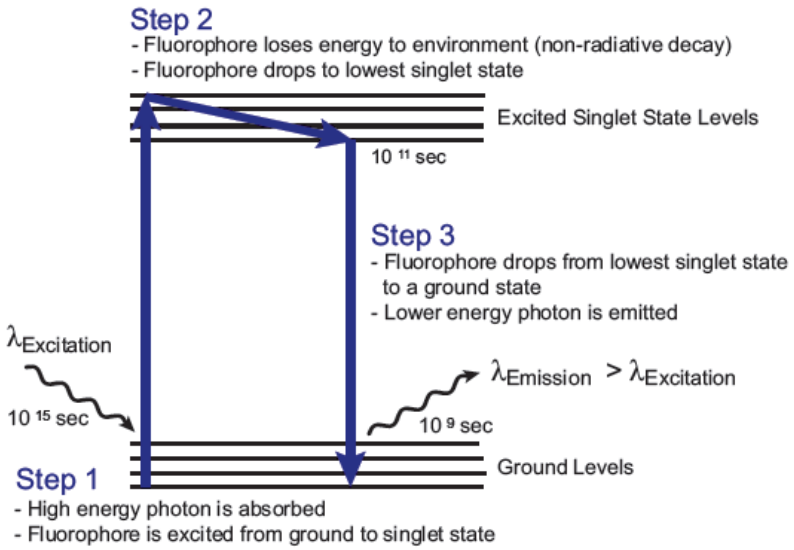
- The capacity to observe living cells and, as such, the ability to examine cells in a natural state
- Observing a living organism in its natural state and/or environment can provide far more information than specimens that need to be killed, fixed or stain to view under a microscope
- High-contrast, high-resolution images; ideal for studying and interpreting thin specimens
- Ability to combine with other means of observation, such as fluorescence
- Modern phase contrast microscopes, with CCD or CMOS computer devices, can capture photo and/or video images

Disadvantages

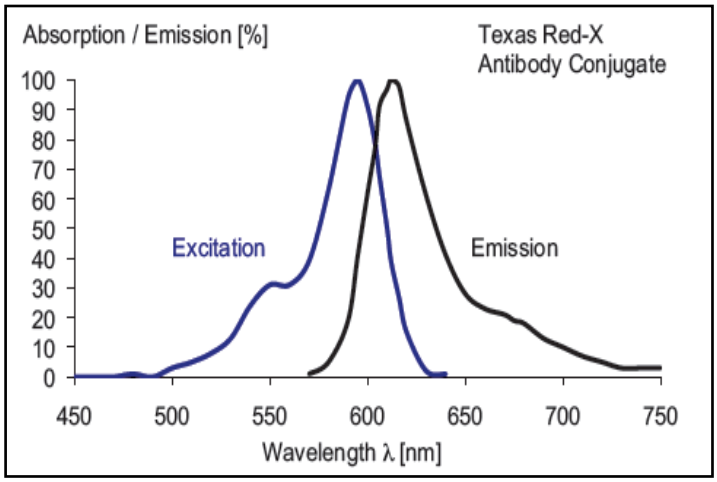
- Annuli or rings limit the aperture to some extent, which decreases resolution
- This method of observation is not ideal for thick organisms or particles
- Thick specimens can appear distorted
- Images may appear grey or green, if white or green lights are used, respectively, resulting in poor photomicrography
- Shade-off and halo effect, referred to a phase artifacts
- Shade-off occurs with larger particles, results in a steady reduction of contrast moving from the center of the object toward its edges
- Halo effect, where images are often surrounded by bright areas, which obscure details along the perimeter of the specimen

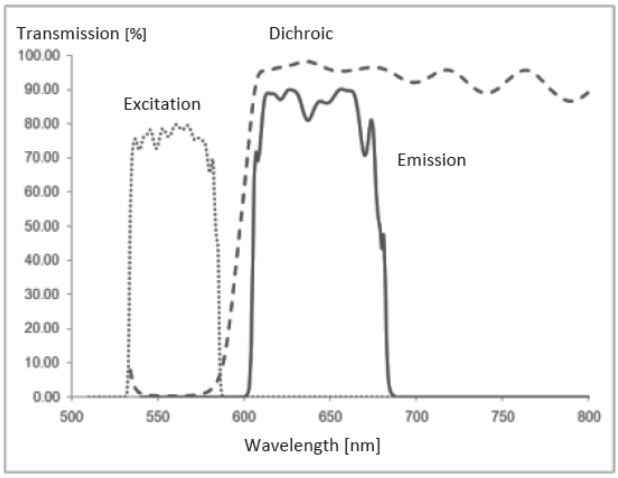
Some basic information on fluorescence

Specimens can absorb and re-emit light through **fluorescence**. The specific wavelength of light absorbed or emitted depends on the energy level structure of each molecule. When a molecule absorbs the energy of light, it briefly enters an excited state before releasing part of the energy as fluorescence. Since the emitted energy must be lower than the absorbed energy, fluorescent light is always at longer wavelengths than the excitation light. Absorption and emission take place between multiple sub-levels within the ground and excited states, resulting in absorption and emission spectra covering a range of wavelengths. The loss of energy during the fluorescence process causes the **Stokes shift** (a shift of wavelength peak from that of excitation to that of emission). Larger Stokes shifts make it easier to separate excitation and fluorescent light in the microscope. Note that energy quanta and wavelength are related by $E = hc/\lambda$



- ✓ A fluorescence microscope includes a set of three filters: an excitation filter, emission filter, and a dichroic mirror (also called a dichroic beam splitter).
- ✓ These filters separate weak emission signals from strong excitation illumination.
- ✓ The most common fluorescence microscopes are configured in epi-illumination mode.
- ✓ The dichroic mirror reflects incoming light from the lamp (at short wavelengths) onto the specimen.
- ✓ Fluorescent light (at longer wavelengths) collected by the objective lens is transmitted through the dichroic mirror to the eyepieces or camera.
- ✓ The transmission and reflection properties of the dichroic mirror must be matched to the excitation and emission spectra of the fluorophore being used.





Confocal Microscope:

It is a scanning technique that employs pinholes at the illumination and detection planes so that only in-focus light reaches the detector.

- This means that the intensity for each sample point must be obtained in sequence through scanning in the x and y directions.
- An illumination pinhole is used in conjunction with the imaged sample plane and only illuminates the point of interest.
- The detection pinhole rejects the majority of out-of-focus light.

Basic working principle

A laser scanning confocal microscope (LSCM) incorporates two principal ideas: point by point illumination of the sample and rejection of out of focus light..

- Laser light (blue line) is directed by a dichroic mirror towards a pair of mirrors that scan the light in x and y.
- The light then passes through the microscope objective and excites the fluorescent sample.
- The fluoresced (light green) light from the sample passes back through the objective and is descanned by the same mirrors used to scan the sample.
- The light then passes through the dichroic mirror through a pinhole placed in the *conjugate focal* (hence the term confocal) plane of the sample; the pinhole thus rejects all out-of-focus light arriving from the sample.
- The light that emerges from the pinhole is finally measured by a detector such as a photomultiplier tube.

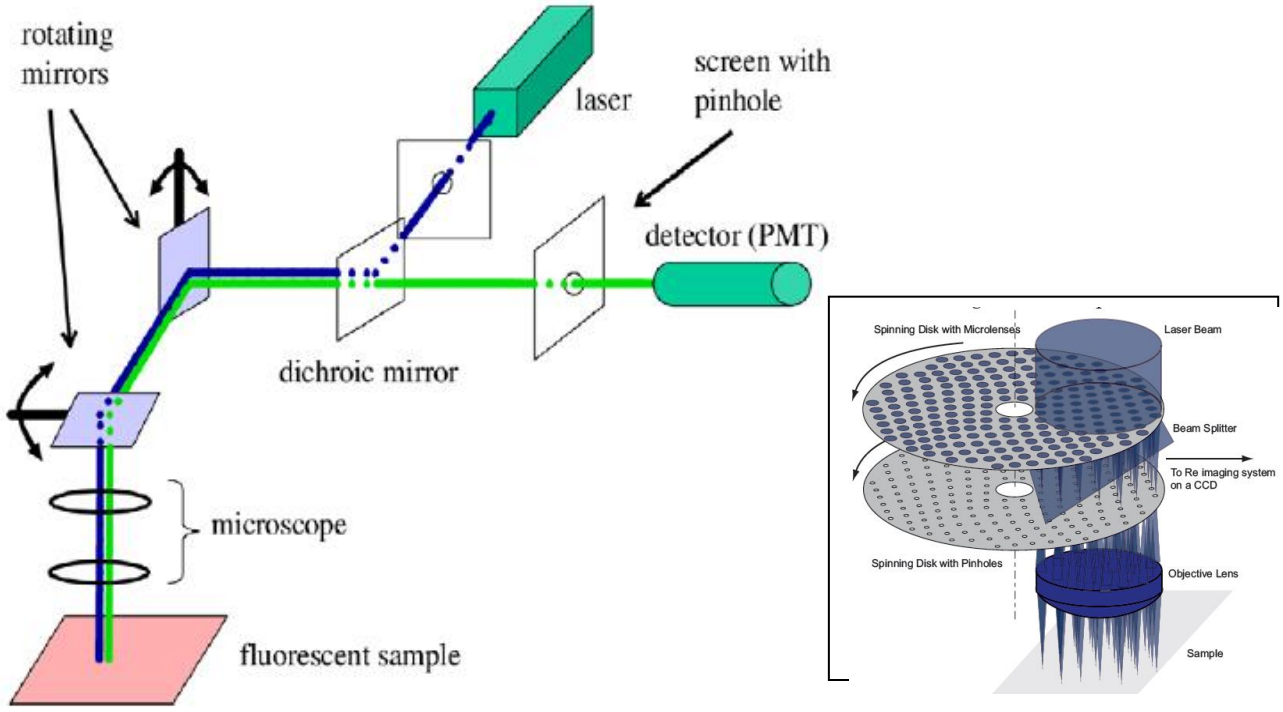


Figure: Schematic diagram of a conventional confocal microscope. The screen with the pinhole lies in the back focal plane of the sample with respect to the objective, thus rejecting most out-of focus light. The rotating mirrors scan the sample pixel by pixel, and are the rate-limiting step for obtaining an image.

Characteristics:

- At any particular instant only one point of the sample is observed;
- A computer reconstructs the 2D image plane one pixel at a time.
- A 3D reconstruction of the sample can be performed by combining a series of such slices at different depths.
- The pinhole filters out background fluorescence that would normally prevent clear imaging of a high volume fraction sample

Resolution of a confocal microscope

- ✓ Like a conventional optical microscope, the resolution of a confocal microscope is limited by diffraction of light.
- ✓ The image of an ideal point viewed through a circular aperture is blurred, and the diffracted image is known as an Airy disc.
- ✓ The size of the Airy disc depends on the wavelength of the laser source and the numerical aperture of the objective lens.
- ✓ This Airy disc limits the maximum resolution of the microscope in the sample plane due to the Rayleigh criterion, which states that two Airy discs must be separated by at least their radius in order to be resolved.
- ✓ For the optical setup of most commercially available confocal microscopes this limit is about 200 nm.
- ✓ More generally, the Airy disc is the image of a perfectly focused point; an out-of-focus image tends to be even more blurred due to diffraction.
- ✓ The 3D generalization of the Airy disc function is termed the 'point-spread function'. Just as the intensity of light smoothly decreases away from the centre of the Airy disc in x and y, the intensity also decreases in z for the point spread function.
- ✓ Limitations in the optics make this decrease slower in z than in x or y and thus the z resolution is poorer, typically at best 500 nm .

Note:

*I*n practice the size of the confocal pinhole is set to be the size of the Airy disc after it is magnified by the microscope optics. A larger pinhole allows too much out-of-focus light to pass through; a smaller pinhole degrades the signal to noise ratio.

Confocal microscopy has the advantage of lowering the background light from out-of-focus layers, increasing spatial resolution, and providing the capability of imaging thick 3D samples, if combined with z scanning. Due to detection of only the in-focus light, confocal microscopy can provide images of thin sample sections.

- The system usually employs a photo-multiplier tube (PMT), avalanche photodiodes (APD), or a charge-coupled device (CCD) camera as a detector.
- For point detectors, recorded data is processed to assemble x-y images. This makes it capable of quantitative studies of an imaged sample's properties.
- Systems can be built for both reflectance and fluorescence imaging.

The number of points in the image, scanning technique, and the frame rate are related to the signal-to-noise ratio SNR (through time dedicated to detection of a single point). To balance these parameters, three major approaches were developed: point scanning, line scanning, and disk scanning.

- A **point-scanning confocal microscope** is based on pointby- point scanning using, for example, two mirror galvanometers or resonant scanners. Scanning mirrors should be located in pupil conjugates (or close to them) to avoid light fluctuations. Maximum spatial resolution and maximum background rejection are achieved with this technique.
- A **line-scanning confocal microscope** uses a slit aperture that scans in a direction perpendicular to the slit. It uses a cylindrical lens to focus light onto the slit to maximize throughput. Scanning in one direction makes this technique significantly faster than a point approach. However, the drawbacks are a loss of resolution and sectioning performance for the direction parallel to the slit.
- **Spinning-disk confocal imaging** is a parallel-imaging method that maximizes the scanning rate and can achieve a 100–1000 speed gain over point scanning. It uses an array of pinholes/slits (e.g., Nipkow disk, Yokogawa, Olympus DSU approach). To minimize light loss, it can be combined with an array of lenses, so each pinhole has a dedicated focusing component. Pinhole disks contain several thousand pinholes, but only a portion is illuminated at one time.

Feature	Point Scanning	Slit Slit Scanning	Disk Spinning
z resolution	High	Depends on slit spacing	Depends on pinhole distribution
x,y resolution	High	Lower for one direction	Depends on pinhole spacing
Speed	Low to moderate	High	High
Light sources	Lasers	Lasers	Laser and other
Photobleaching	High	High	Low
QE of detectors	Low (PMT) Good (APD)	Good (CCD)	Good (CCD)
Cost	High	High	Moderate