Swayam Course - Analytical Techniques

Week: 6, Module15 - Light Microscopy and Confocal Microscopy

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Introduction

We all human beings see nature, its valuable creations and wonderful events by our precious eyes. In general, eyes can see major objects present on earth, however our eyes have a limit to see upto millimetre range and after that we require an aid to visualize the small objects (Figure 1). From this need, different visualizing aids has been developed which are collectively comes under a common term known as "**microscopy**".

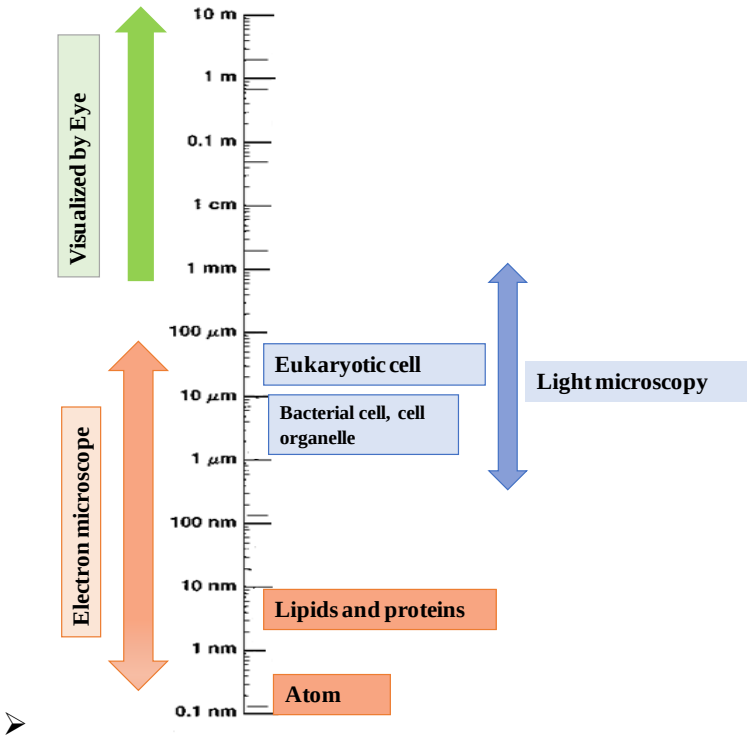


Figure 1- A scale presenting size of various molecules which are visible to human eye along with the need of microscope to visualize tiny molecules which are invisible to naked eye.

Microscopy is the term used to describe the use of lenses to reveal details of an object that are not visible to the eye unaided. A microscope (from Greek **mikrós** "**small**" and **skopeîn**, "**to look**") is an instrument used to visualize objects that are too small to be seen by the naked eye. Prime focus of the module is mainly on the basic components and methods of Optical

microscopy. Majority of the concepts discussed in later sections can also be applicable to some extent to the methods of various other microscopic procedures. In view of this, following learning objectives are prepared to achieve the above said information.

- To provide Introduction, history of advancement and importance of microscopy.
- To explain the basic principles and structural components of a typical microscope.
- To elucidate the procedure of sample preparation of microscopy experiment.
- To provide basic principles and structural components of a confocal microscope.

Historical perspectives

The microscope was first built in 1595 by Hans and Zacharias Janssen in Holland. It was perfected in the 17^{th} century in several countries, including by Robert Hooke. In 1660s, Antony van Leeuwenhoek made a simple microscope using powerful magnifying glass. Leeuwenhoek's simple microscope could magnify an object up to 200 times and he recorded minute details of various natural samples of animal and plant tissues, blood, fossils etc. However, there were some limitations of poor image resolutions and colour distortion associated with earlier microscopes. These limitations were removed in the subsequent years. Later in 19^{th} century, Ernst Abbe discovered that oil-immersion lenses could prevent light distortion at highest magnification power that is still used with 100X objective microscopes.

Basics of microscopy

A compound light microscope is an optical instrument that uses visible light to produce a magnified image of an object (or specimen) that is projected onto the retina of the eye or onto an imaging device (Figure 2).

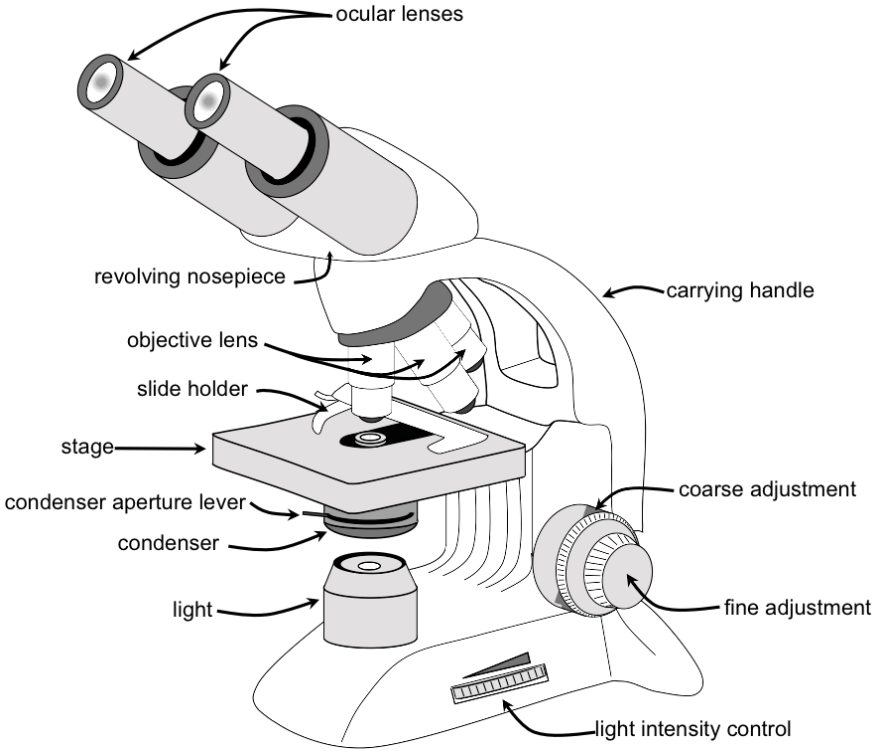


Figure 2a- Schematic representation of a compound light microscope. (Source- https://commons.wikimedia.org/wiki/File:Parts_of_a_Microscope_(english).png)

The most important concepts of microscope include- magnification and resolution. The understating of these two concepts is important to comprehend the applications and limitations of various microscopic variants.

Magnification- The unaided human eye can visualize the small objects up to 1mm size. Therefore, the use of convex lens is required to see small things like microorganisms, which are less than 1mm as mentioned in figure 1. Therefore, magnification is the process of enlarging the apparent size, not physical size, of something e.g. a simple hand held magnifying lens is the simplest form of microscopy which is generally used for magnifying the image of a specimen (Figure 2b).

Figure 2b- Magnification.

The word compound refers to the fact that two lenses, the objective lens and the eyepiece (or ocular lens), work together to produce the final magnification M of the image such that

$M_{final} = M_{obj} \times M_{oc}$

Where, M_{obj} and M_{oc} is magnification of objective and ocular lens, respectively.

The other important aspect of a compound microscope is **focal length (f)** and **numerical aperture (NA)** of its lenses. The focal point (F) is the specific place where light rays focus and the distance between center of lens and focal point is known as focal length (f) (Figure 2c). The magnification of each lens depends upon its focal length, and shorter the focal length, higher is the magnification power e.g., a lens of 10X magnification have f=16mm whereas same of 100X have f=1.8.

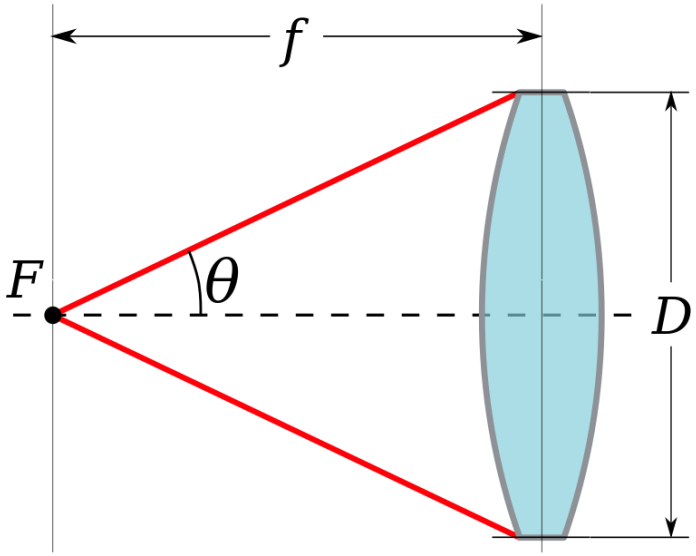
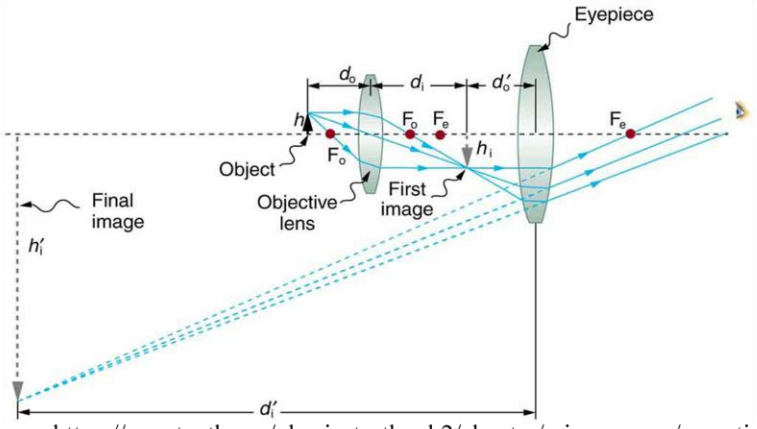


Figure 2c: Schematic representation of the focal point (F) and focal length (f).

Numerical aperture (NA) of a lens is the ratio of the diameter of the lens to its focal length and calculated as nSinθ, where 'n' is the refractive index of a medium. It is an index of the resolving power and can be decreased by decreasing the amount of light that passes through a lens.

Two components of microscope are of utmost importance in forming an image: the **objective lens**, which collects light, diffracted by the specimen and forms a magnified real image at intermediate image plane near the eyepieces, and **the condenser lens**, which focuses light from the illuminator onto a small area of the specimen. The arrangement of these and other components is shown in Figure 2.

In a compound microscope, an image is formed by the objective lens that serves as an object for eyepiece lens. The image formed by eyepiece is visualized by eye and this is a product of two lenses shown in figure 3. The parallel light beams passed through convex lens get converged at a point called as focal length (f) of the lens.



Source: https://opentextbc.ca/physicstestbook2/chapter/microscopes/, creative commons

Where, f_0 and Fe are the focal length of the objective and eye piece lens, d_0, d_i and d'_i are the object distance, image distances and location of the final image respectively, for the objective lens.

Figure 3-A magnified virtual image of a specimen in the microscope. The objective lens forms a magnified image of the object (called the real intermediate image) in or near the eyepiece; the intermediate image is examined by the eyepiece and eye, which together form a real image on the retina. Source- https://courses.lumenlearning.com/boundless-physics/chapter/other-optical-instruments/

Resolution- The ability to see two close objects as two distinct objects called resolution. The limit of resolution (d) is the smallest distance between two points, where the points can be distinguished as separate thing. The combination of good resolution with higher magnification is important to visualize small organisms; otherwise magnification alone will produce blurred images. The resolution defines the distance at which two points can be visualized as two distinct objects. This limit of resolution (d) can be calculated as,

$d = 0.61\lambda/NA = 0.61\lambda/n\sin\theta$

Where, NA is the numerical aperture, n=refractive index, λ is the wavelength (Visible light is 400-700nm). The cone of light collected by the lens ($n\sin\theta$) determines the resolution.

The resolution will increase with decreased d, hence smaller is the wavelength of light, and higher is the resolution. In a compound microscope generally 10X, 40X and 100X (oil immersion) object lenses are used with the combination of 10X or 15X ocular lenses. The magnification of a 100X (oil immersion) object lens and 10X eye lens can produce an image with 1000X magnification.

In the above, a term known as refractive index got utilized which is usually considered for increasing the resolution. This term is derived from refraction (light rays get deviation/bent, when pass from one medium to another).The refractive index is a measure of how greatly a substance slows the velocity of light. The direction and magnitude of bending is determined by the refractive indexes of the two media forming the interface.

Need of oil immersion lens- The high magnification (100X) objectives have small lens diameter which is not sufficient to gather all refracted rays. The refracted rays when enter the air

and high power lens in incapable to capture them, a fuzzy image with low resolution will result. Hence, the use of immersion oil maintain the direction of light rays at 100X, as it has similar refractive index to that of glass and it does not allow the emerging rays from specimen to get away as shown in figure 4.

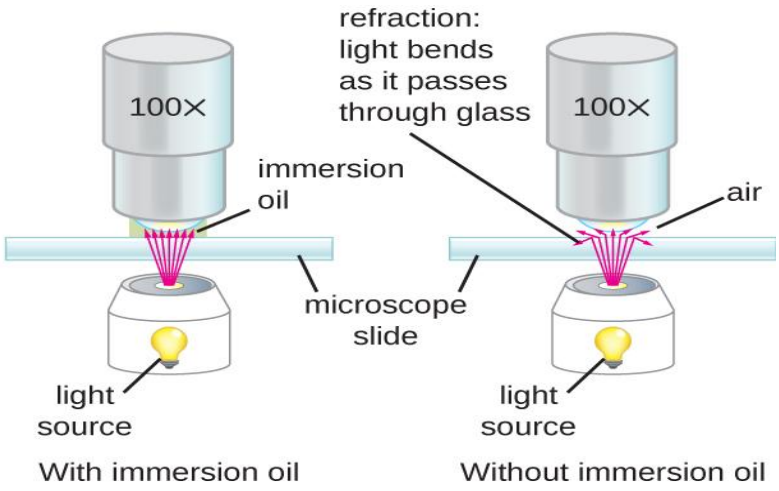


Figure 4- A diagram showing the effect of immersion oil on the numerical aperture. **Source-** http://www.alyvea.com/micro/instruments.php

The use of oil immersion lens can also be justified by the following mathematical calculations.

The limit of resolution (d) can be calculated as **$d = 0.61\lambda/NA = 0.61\lambda/n\sin\theta$**
The resolution will increase with decreased d, and higher NA

- Refractive index of air= 1; and maximum $\sin\theta = 90$ i.e. 1 therefore, no lens working in air can have a numerical aperture value greater than 1.

- Refractive index of oil is higher than air and maximum NA is 1.4. Therefore, it increase NA and decrease the value of d, hence increase resolution.

Effect of staining specimen- The refractive index of specimen also gets changed by staining. For example, in an unstained sample, light rays move in straight line through a single medium but after staining, the incident light has to pass through two different media, *i.e.*, air and stained specimen. The difference in refractive indices results in increased contrast between the medium and specimen.

Types of light microscope: Based upon transmitted-light observation, compound light microscope has been classified into 4 major categories.

- Bright-field microscope
- Dark-field microscope
- Phase-contrast microscope
- Confocal microscope

Bright field microscope- The name bright-field has derived from the fact that the dark specimen is surrounded by bright viewing field. Bright-field microscopy makes the use of light from the lamp source beneath the microscope stage to illuminate the specimen. This light is collected in the condenser, and then shaped into a cone where the apex is focused on the plane of the specimen. The light rays that pass through the specimen must be changed enough in order to interfere with each other that build an image of specimen under a bright viewing field. Sometimes, when a specimen will have a refractive index very similar to the surrounding medium between the microscope stage and the objective lens, the image cannot be seen. In order to visualize these biological materials well, they must have a contrast caused by the proper refractive indices, or be artificially stained. Since staining can kill specimens, there are times when dark-field microscopy is used instead.

Dark-field microscope- In dark-field microscopy no direct light from the condenser enters the objective lens, as opposed to bright-field microscopy that illuminates the sample as shown in figure 5a. Only the light that is reflected, refracted or diffracted by the specimen enters the objective lens. The dark field condenser produces a circle of light (hollow cone of light) at an extremely oblique angle to the surface of the slide. This oblique light used to a focus specimen and it diverges so strongly that no direct light enters the objective. The entire viewing field appears dark when there is no sample on the microscope stage. However, when a specimen is placed, it appears bright against a dark background. In dark-field microscopy, it is important that the NA of condenser must be larger than that of objective lens in order to prevent the entry of direct light into objective lens.

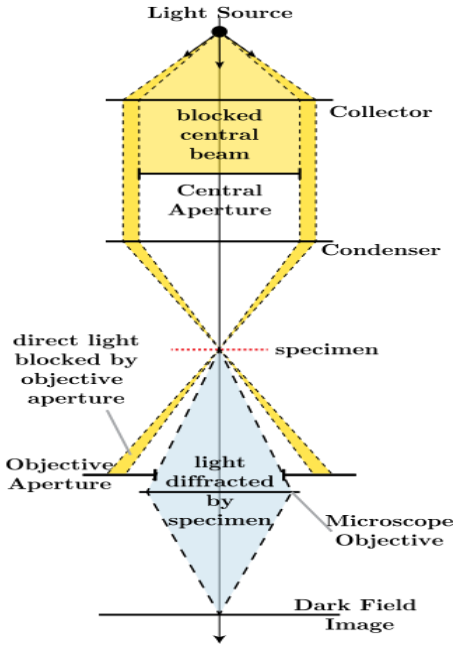


Figure 5a- Illustration of the light path through a dark-field microscope. **Source-** https://en.wikipedia.org/wiki/Dark-field_microscopy

In order to achieve higher magnification, the dark field condensers must always be oiled to the specimen slide. The oiling is important because the angle of the light leaving the top of the condenser is much larger than the critical angle for glass-air interface; thus, no light appear from the condenser until immersion oil applied to its surface. If this angle is greater than the critical angle at any interface, the illumination will be totally internally reflected; indicating the importance of specimen's immersion medium. The critical angle for glass to air is 41 degrees and for glass to water it is 61 degrees. Low power dark field condensers work fine for specimens in water, whereas, high power dark field condenser may not be useful with a water immersed specimen. The immersion of the dark field condenser to the slide with oil even for low power dark field condensers is always recommended. Figure 5b shows the comparison of two microscopic images i.e. bright field and dark field.

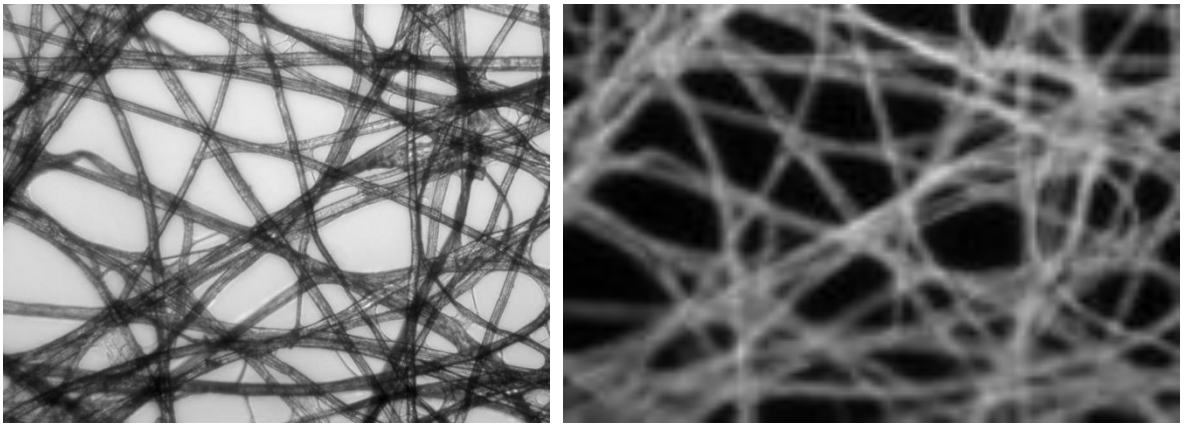


Figure 5b: Left panel: Bright-field microscopic image and **right panel:** dark-field microscopic image. (**Source-**https://en.wikipedia.org/wiki/Bright-field_microscopy#/media/File:Paper_Micrograph_Bright.png).

Phase contrast microscopy- Phase contrast microscopy was described in 1934 by Dutch physicist Frits Zernike. This is a contrast enhancing optical technique that allows us to produce high-contrast images of transparent specimens, including living cells, thin tissue slices, sub-cellular particles (including nuclei and other organelles), lithographic patterns, fibers, latex dispersions, and glass fragments.

Presented in figure 6 is a schematic illustration of a modern upright phase contrast microscope, including the phase contrast optical train. A phase contrast microscope possessed a light source, condenser, objective and ocular lens, similar to normal microscope. A phase contrast microscope differs from normal compound microscope as they contain addition of sub-stage annular diaphragm and phase plate. **Sub stage annular diaphragm** is located below the sub-stage condenser and it helps to create a narrow hollow cone of light that illuminate the object. The tungsten-halogen lamp produces partially coherent illumination that is directed through a collector lens and focused on a sub-stage annular diaphragm. **The phase plate** is transparent glass disc with few channels. The channels are coated with material that can absorb

light and cannot retard it, and simultaneously the portion other than channel is coated with materials like magnesium fluoride that retard the light.

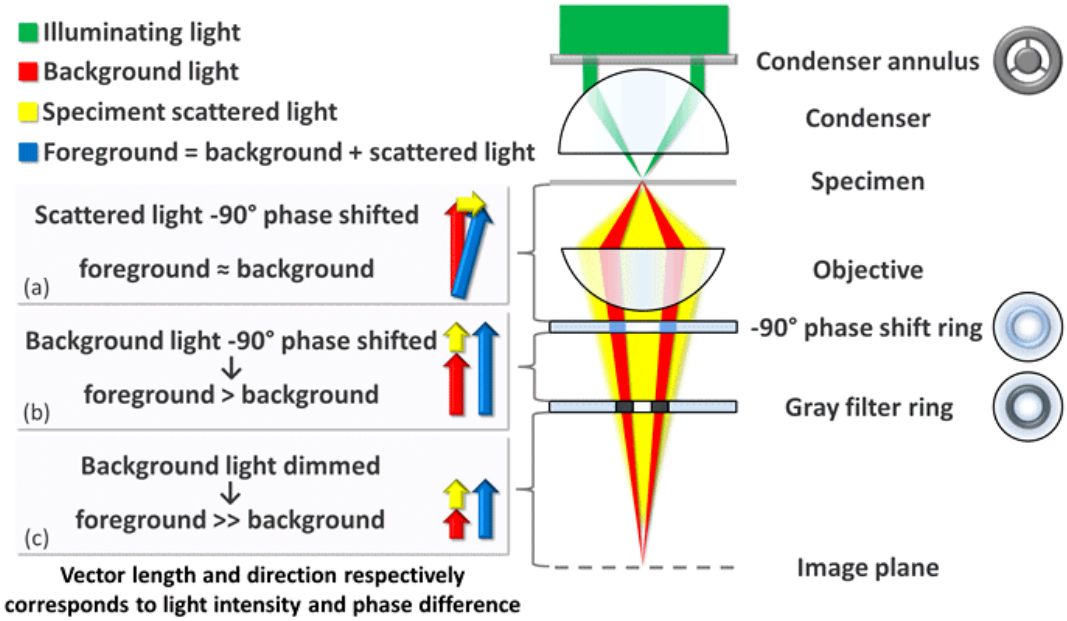


Figure 6- An illustration of a modern upright phase contrast microscope with its working principle.**Source:**https://en.wikipedia.org/wiki/Phasecontrast_microscopy#/media/File:Working_principle_of_phase_contrast_microscopy.gif

Creation of high contrast images- As the light encounters the regions of variable thickness and refractive indices, it get deviated from the normal path and light experiences phase change. Similarly, when light pass through an area of less refractive index, it remains un-deviated.

The phase difference between retarded and un-retarded light beams is about one fourth of the original wavelength. An unaided human eye in incapable to detect these small changes in the phase of light and hence, no contrast images will appears. The addition of sub-stage annular diaphragm and phase plate convert minute changes corresponding in amplitude, which results in visual differences in image contrast and brightness. Because the light rays tend to cancel each other out, the image of the specimen will be dark against a brighter background.

Applications and advantages of Phase contrast microscopy: Phase contrast microscope enhances the contrast between intracellular structures having slight differences in refractive index and it is the excellent way to observe living cells. One of the key advantages of phase contrast microscopy allows the examination of living cells in their natural state without being killed, fixed, and stained. Consequently, clear dynamics of ongoing biological processes can be observed with sharp contrast of minute specimen detail. Phase contrast microscopy can also be utilized to study the cellular events like cell division, cellular movements and phagocytosis. It also helps to study membrane permeability of cells and membrane bound organelles. It also extensively utilized to observe living cells in tissue culture to monitor their growth. However, phase contrast microscopy is only useful for observing individual cells or thin layer of cells.

Confocal Microscope:

The term confocal derives from the coincidence of two focal planes (objective lens focus point and the focus point), for removal of out-of-focus light that result in increased optical resolution and contrast of a micrograph. A confocal microscope creates images with sharp contrast of a specimen that would otherwise appear blurred if viewed with a conventional microscope. The creation of sharp images is possible through the exclusion of most of the light from the specimen that is not coming from the microscope's focal plane. Apart from better observation of fine details, it is also possible to construct three-dimensional (3D) images of the specimen by assembling a series of thin slices taken along the vertical axis.

Background- Confocal microscopy was invented by Marvin Minsky in 1955. This invention would execute to construct images by sequentially focusing a point of light across the specimen and then collecting some of the returning rays. Illumination of a single point at a time avoided the majority of the unwanted scattered light that abstruse an image when the entire specimen is illuminated. The additional use of second pinhole aperture allows the light returning from the specimen to pass through and reject rays that were not directly coming from the focal point. The desirable light rays would then be collected by a photomultiplier and the image steadily constructed on a long-persistence screen. The movement of the stage rather than the light rays to scan the specimen was the key feature.

Modern confocal microscopy- Modern confocal microscopes are based on the key elements of Minsky's design: a pinhole aperture and point-by-point sequential illumination of the specimen. The incorporation of advanced optics and electronics into existing designs, provide better speed, image quality, and storage of the generated images. The majority of images captured by confocal microscopes are either through reflecting light of the specimen or by stimulating fluorescence from dyes that applied to the specimen. The fluorescence confocal microscopy is most commonly used in biological applications.

Working principle of fluorescence confocal microscope- Confocal imaging is accomplished by using a two-step process. First, the excitation light that is focused on the specimen by the objective is passed through a small aperture. A pinhole is placed in front of the illumination source allow transmission only through small area and only a point of the specimen is illuminated at one time (figure 7). Fluorescence excited in this manner at focal plane is imagined onto a confocal pinhole placed right in front of detector. By exciting the light this way, the amount of fluorescence not in focus can be controlled or minimized. Only fluorescence excited within the focal plane of specimen will go through the detector pinhole.

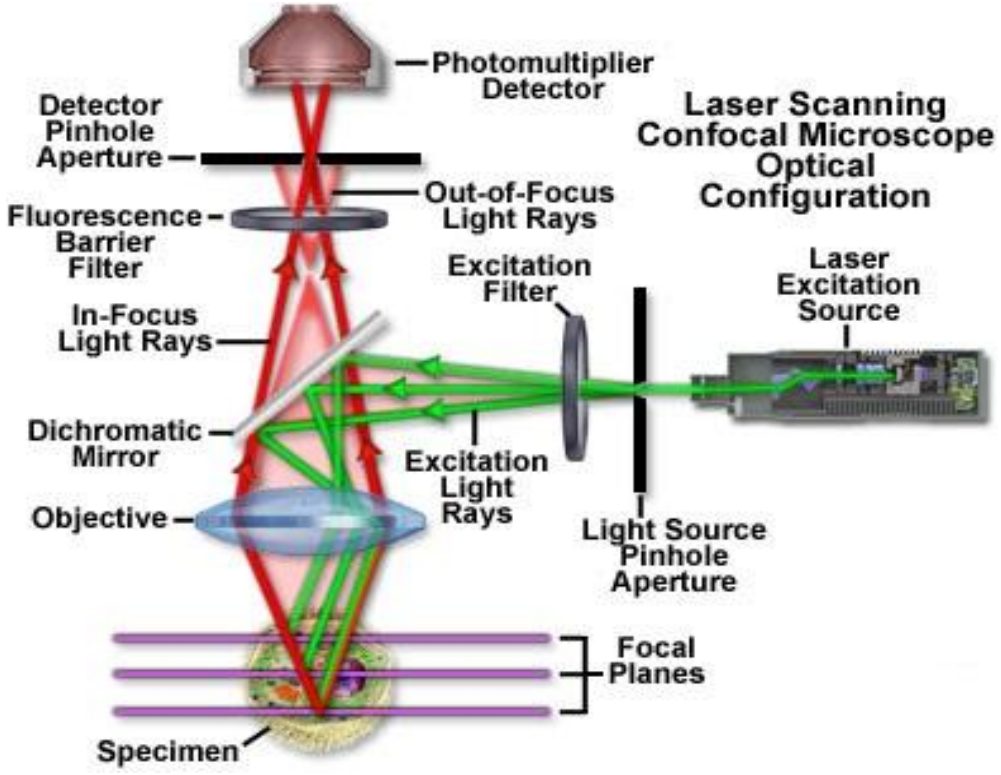


Figure 7- Confocal microscope setup for capturing fluorescent images. Source- Basil and Wassef. 2013. IntechOpen.

Second, fluorescence emissions that originate from above or below the plane of focus are blocked by a second aperture or slit in front of the detector. The smaller this second opening, the higher the rejection rates of out-of-focus light and the thinner the optical section. These thin optical sections have greatly improved contrast and axial resolution, but they are obtained at the expense of overall specimen brightness. The capture of multiple two-dimensional images at different depths in a sample enables the reconstruction of a three-dimensional structure (a process known as optical sectioning) within an object.

There are following different parts of a confocal microscope which are in general considered in advancement.

(i) Laser- Different Light Amplification by Stimulated Emission of Radiation (Laser) are used because they are an intense coherent monochromatic source of light and capable of being expanded to fill an aperture or focused to a spot. A list of most commonly used lasers has been listed in figure 8. In general, fluorescent specimens get efficiently excited by the laser which emits wavelength in the same general region as a mercury lamp.The argon ion laser is proved as a good candidate for this and utilized in many confocal microscopes. Argon lasers are capable of emitting the wavelengths 364, 458, 488, and 514 nm. The laser light is focused on an area of interest and back scattered light is then refocused onto the detection system by the lens.

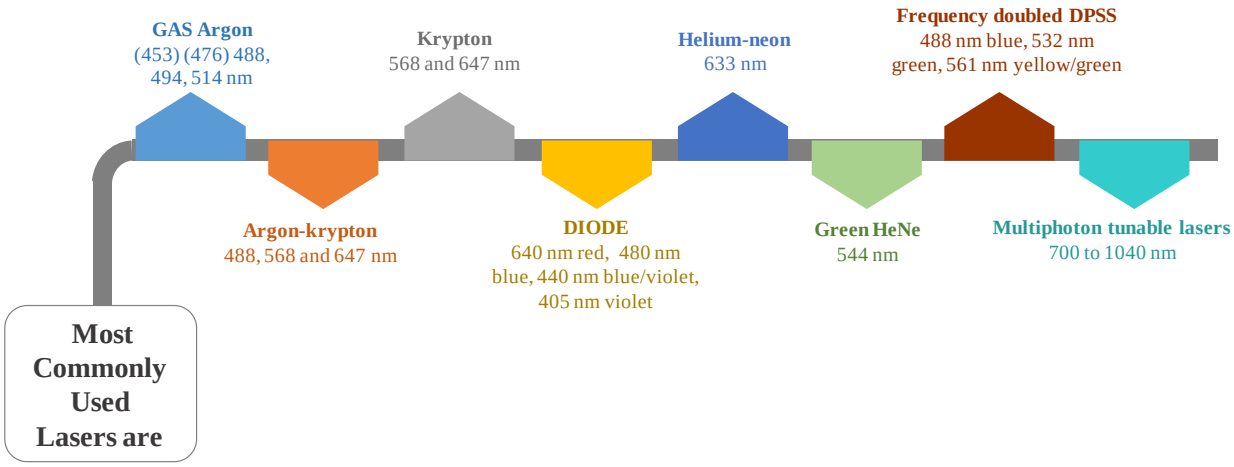


Figure 8- A list of lasers commonly used in confocal microscopy.

The main drawback in using lasers is that to cover a large excitation range you will need several lasers.

(ii) Types of filters in a confocal microscope:

The excitation and barrier filter can be either a **short, long, beam splitter or bandpass** (Figure 9). Its purpose is to only allow the required excitation light to pass to the specimen.

The **dichroic** is positioned at an angle of 45 degrees and will reflect down the excitation light to the specimen and then allow the longer fluorescent light to pass through it to the detector.

The final filter is known as a **barrier filter** can once again be any of the short, long or bandpass. Its purpose is to block any of the excitation light and only pass the required emitted fluorescent light.

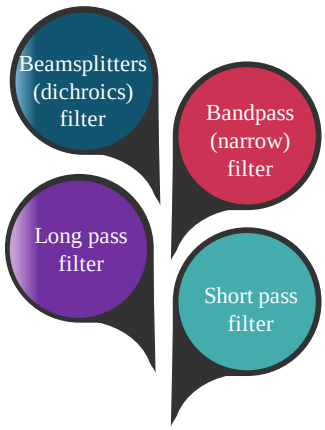


Figure 9- Examples of filters used in confocal microscopy.

(iii) Detectors of confocal microscopy:

Photomultiplier tubes (PMTs) are the most common detectors in point scanning confocal microscopes. An incoming photon liberates an electron from the photocathode which starts a chain reaction so that a cascade of electrons finally reaches the anode. The main reason that PMT's have been used in confocal microscopy is they are very sensitive.

Two important factors are considered for successful confocal microscopy experiment.

Photo-damage- Photo-damage of biological molecules must be avoided at all costs. This will often require lowering the illuminating laser power or using a laser wavelength that minimizes any photo-damage.

Photo-bleaching- Photo-bleaching is the irreversible destruction of a fluorescent molecule so that it no longer fluoresces. Photo-bleaching results when a fluorescent molecule is in its excited semi-stable state & is then hit by a second photon causing permanent damage to the molecule.

Generally occurs when using higher laser powers for longer times. Apart from decreasing the strength of any fluorescent signal, biologically reactive molecules are often produced as a consequence of photo-bleaching, thereby potentially altering the very events one is trying to measure. Reducing the laser power (and pixel dwell time) can minimize photo bleaching.

Advantages and applications of confocal microscopy

- Confocal laser scanning microscopy allows live 3-dimensional cell imaging with limited of cell damage as only a point of specimen is being illuminated at a time. It has faster acquisition time and allows visualization of rapid processes.
- Confocal laser scanning microscopy creates sharp images of specimen that would appear otherwise blurred with conventional microscope.
- It is ideal visualization technique for deep tissue penetration or thick samples. Further, a series of thin slices of specimen can be assembled to create 3D structures and analysis of x-z and y-z planes can be readily generated by confocal software programs.

PREPARATION FOR MICROSCOPY EXPERIMENT

There are two factors important in microscopy, a) resolution and b) contrast of specimen from near area. Resolution has been discussed in above sections. In this section, several basic methods will be briefly looked for specimen preparation for microscopy and for creating contrast. The need for contrast techniques is obvious to everyone who has worked with biological samples in microscopy. The biological samples are generally hydrated in which transparent cellular structures are embedded with a very thin and highly transparent surrounded cell membrane. Such a biological specimen does not give a proper image during microscopy, regardless of the resolution capabilities of the microscope. In general, there are two major methods for creating contrast in a microscopic specimen. In one category the specimen gets labeled with a chemical substance that changes the light refraction/reflection properties or gets fluorescent when illuminated by a suitable wavelength.

The other basic category is to visualize the specimen by minor change in refractive index. The oldest and general contrast technique is done with the help of different dyes. In general, the specimen is treated for short duration with dyes (often brightly colored), which preferentially binds to specific parts of the specimen, e.g. nucleus, membrane etc. This process is generally known as sample staining. After staining, a specimen shows a better contrast under microscope

through absorption of light in the labeled parts. These dyes have two common features a) chromophore groups: chemical groups with conjugated double bonds which give dye its color and b) ability to bind to the cells and their subcellular structures. In general, basic dyes e.g., crystal violet, methylene blue etc. are frequently used. Basic dyes bind to negatively charged molecules like nucleic acids and many proteins. Because the surfaces of bacterial cells also are negatively charged, basic dyes are most often used in bacteriology. One of the basic applications of these dyes comes in differentiating the bacteria as gram positive and gram negative type. The procedure of differentiating the bacteria is based on dye retaining capacity of a bacterium. Briefly, the bacterial smear is stained with the basic dye crystal violet (primary stain), followed by treatment with an iodine solution which functions as a mordant i.e, the iodine increases the interaction between the cell and the dye so that the cell is stained more strongly. The smear is next decolorized by washing with ethanol or acetone. Gram-positive bacteria retain the crystal violet, whereas gram-negative bacteria lose their crystal violet and become colorless. Finally, the smear is counterstained with a simple, basic dye different in color from crystal violet. Safranin, the most common counterstain, colors gram-negative bacteria pink to red and leaves gram-positive bacteria dark purple.

Summary

Above, basic information related to microscopy is summarized in following points. However, newer, updated techniques have been evolved in past decades with more resolved structures through microscopy.

- Microscopy is the term used to describe the use of lenses to reveal details of an object that are not visible to the eye unaided.
- Magnification is the ratio of the size of an object seen by microscope to the actual size observed by naked eye.
- The minimum distance two points can be separated is limit of resolution.
- Light microscope may be of Bright field, dark field or Phase-Contrast. Bright field microscopy produces a dark image against a brighter background.
- Light Amplification by Stimulated Emission of Radiation (Laser) are used in **Confocal microscopy** because they are an intense coherent monochromatic source of light, capable of being expanded to fill an aperture or focused to a spot.
- There are **four types** of filters in a Confocal microscope. short pass filter , long pass, bandpass (narrow) and beamsplitters (dichroics).
- **Photo-damage** of biological molecules must be avoided at all costs in **Confocal microscopy**. This will often require lowering the illuminating laser power or using a laser wavelength that minimizes any photo-damage.

- Ratio of signal to noise (often shown as S/N ratio) is critical in **Confocal microscopy.** Sometimes it is easier to reduce the noise in a detector system (perhaps by cooling the detector) than to increase the absolute signal emanating from sample. Such a system would give a higher S/N ratio and, therefore, more robust data and a better image.

- The oldest and general contrast technique is done with the help of different dyes. In general, the specimen is treated for short duration with dyes (often brightly colored), which preferentially binds to specific parts of the specimen, e.g. nucleus, membrane etc. This process is generally known as sample staining. After staining, a specimen shows a better contrast under microscope through absorption of light in the labeled parts.