Unit 6

OPTICAL MICROSCOPY

Structure

6.1 INTRODUCTION

In the previous unit 5, you have learned about the principles and applications of advanced spectroscopy methods which are used to study the biochemical characterization and three-dimensional structure of biomolecules. Furthermore, there is another crucial scientific instrument known as a microscope, which is utilized for examining living things, cellular structures and biomolecules. This unit will cover the fundamental principles, setup, and practical use of optical microscope. The microscope is a widely recognized symbol of scientific inquiry. It allows scientists to make discovery about invisible world and enhances our understanding about life. We use microscope to view smaller entities like cells (organelles), microorganisms, viruses, biomolecules etc. Without microscope, we cannot see little things. Microscope allows us to examine living organisms at cellular and molecular level in order to understand their structure and function. We believe that you would have used light microscope to view specimen slides in your undergraduate biology

Fig. 6.1: Foldscope

The Foldscope is a novel and economical optical paper microscope for scientific research and education. Manu Prakash and his Stanford University team created it to make microscopy accessible to everyone, especially in resource-limited environments.

lab. Theoretically, a light microscope is an array of two optical lenses which produce an image using the light source. However, all microscopes are magnifying devices which produce enlarge image of small objects. In a nutshell, microscope makes science accessible. Microscopy is generally characterized by magnification and resolution about which you will study in this unit.

We begin this unit by learning the history of microscopy and the principle of microscopy. Thereafter you will learn about basic parts, working principle and application of different microscopes like bright field and dark field microscopy, Interference and Phase contrast Microscopy, fluorescence microscopy and confocal microscopy. The importance of cell fixation in microscopy is also discussed. The advanced super imaging microscopes such as fluorescence resonance energy transfer (FRET), and stimulated emission depletion (STED) are also discussed. The biolumenisnce and biolumenisnce microscope are also discussed in the end of unit. In next unit 7 you will study about electron microscopy.

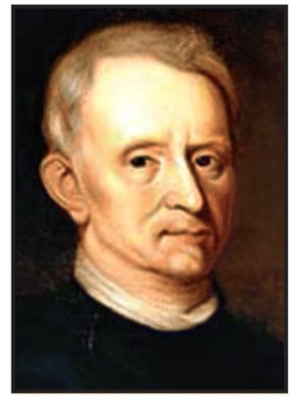
Expected Learning Outcomes

After studying this unit, you should be able to:

- explain the basic principle of Light microscopy;
- differentiate between magnification and resolving power;
- indicate the methods and purpose of cell fixation;
- describe the principles, basic parts and applications of :
- bright field and Dark field Microscopy
- interference and phase contrast microscopy
- fluorescence Microscopy
- confocal Microscopy
- fluorescence resonance energy transfer (FRET) and Super resolution microscopy; and
- Explain bioluminescence and its application.

6.2 HISTORY OF MICROSCOPY

The history of the microscope is very interesting because it takes us through hundreds of years of science progress and technological progress. The microscope has changed the way we think about and study the natural world and has made significant contributions to biology, biochemistry, microbiology, virology, medical science, materials science, and nanotechnology, among others. Its journey began with the use of glass to see smaller things in ancient times. The main credit goes to two Dutch sight makers Zacharias Janssen and his father Hans who invented the compound microscope in the late 1600s. This microscope contains more than one lens in tube like shape that work together to magnify things. Their first invention led it possible for more advanced microscope and telescopes to be made that could see details that had not been seen before. However, the early microscopes were of two kinds: simple microscopes and compound microscopes. The image viewed by the eye is a result of the magnification of lenses. The history of microscope is summarized in Table 6.1.



Robert Hook

Antonie van Leeuwenhoek

Table 6.1: Development journey of microscopy

Scientists	Contribution	Year
Zacharias and Hans Janssen (Dutch spectacle makers)	Invented the first compound microscope by combining multiple lenses in a tube	1590s
Galileo Galilei	Developed a compound microscope with a convex and a concave lens	1609
Robert Hooke	Developed basic compound microscope to see cells of dead cork cell wall and coined the term "cells"	1665
Antonie van Leeuwenhoek	Improved the magnification (200 to 300X) of simple microscope to see first living cells (bacteria)	1674
Ernst Ruska and Max Knoll	Designed and developed first transmission electron microscopes,	1931
Frits Zernike	Invented the first Phase contrast microscopy (PCM) and awarded by Nobel prize in1953	1934
Ernst Ruska	Made the first scanning electron microscope (SEM)	1942
Marvin Minsky	Built confocal microscope imaging.	1957
Gerd Binnig and Heinrich Rohrer	invented the scanning tunnelling microscope to view atom surface in 3D image	1982
Stefan Hell	Pioneer to develop Super-resolution microscopy	1993-1996
Eric Betzig, Stefan Hell and William Moerne	*Nobel Prize winner in 2014 for development of super-resolved fluorescence microscopy to see things less than 0.2µm*	2014

It is crucial to highlight that several significant microscopic breakthroughs were achieved using basic microscopes in the past. Over the past few decades, there have been significant improvements in microscopy technology, which have greatly enhanced our ability to capture and examine images. Advanced imaging techniques, such as confocal microscopy, fluorescence microscopy, and super-resolution microscopy, have provided researchers with the ability to investigate biological processes at the molecular and cellular levels. These microcopy is also discussed in this unit. Therefore, the history of the microscope demonstrates human curiosity, creativity, and unwavering dedication to acquiring information in the fields of life sciences and biochemistry. In next section you will study principle of optical microscope.

SAQ 1

Match the column I with that column II

Column I	**Column II**
a. Marvin Minsky	i. See first living things
b. Zacharias and Hans Janssen	ii. scanning tunnelling microscope
c. Antoine van Leeuwenhoek	iii. Confocal microscope
d. Frits Zernike	iv. Inventor of the first microscope
e. Gerd Binnig and Heinrich Rohrer	v. Phase contrast microscope

6.3 PRINCIPLE OF LIGHT MICROSCOPY

A microscope is an optical device that uses lenses to enlarge and produce visual or photographic images of very small objects. However, the underlying idea of microscopy is based on the interaction between light, lenses, and specimens, resulting in the formation of magnified and clear images. The limitations of human vision in seeing tiny details highlight the significance of the microscopy. Our eye has a minimum distance of 25cm, called the least distance of distinct vision (LDDV), beyond which it is unable to focus an objects. Therefore, things that are smaller than about 0.1mm cannot be seen without the use of microscope. We believe that you have studied about basic elements of a compound microscope in your undergraduate Biology/ Biochemistry course.

Here, we first discuss the working mechanism of light microscopy. Fig. 6.2 depicts the working mechanism by which the two lenses of a compound microscope collaborate to produce a magnified image of an object. Microscopy involves the interaction of light with the specimen through the process of absorption, refraction and transmission. Light interacts with the specimen through the processes of absorption, refraction, and transmission in microscopy. When light interacts with a specimen, some of the light may be absorbed by it, while the remaining may be transmitted through the specimen or refracted out of it. Some of the refraction may occur because of a variation in the medium. In microscopy, refraction—the bending of light—is more important than absorption and transmission. Therefore, light refraction is used to provide a clear and detailed image of the specimen in microscopy. The image that is formed is the inverted virtual image under the microscope. The inverted image is formed because the path of light changes (refraction) when it possess through the specimen due to the difference in refractive index of medium.

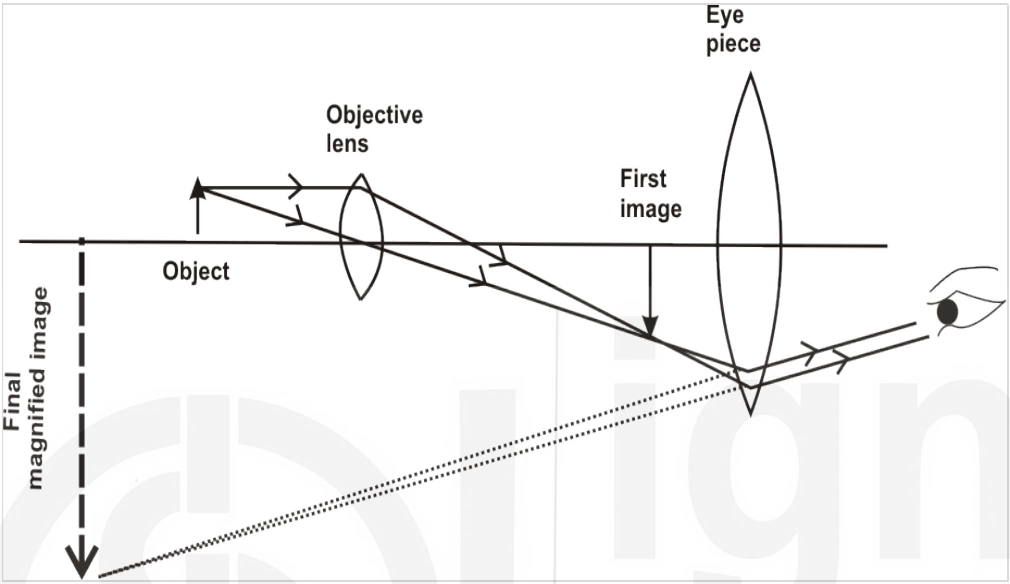


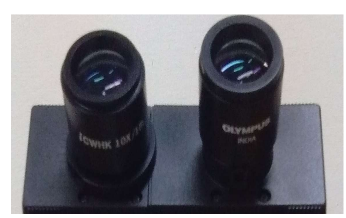
Fig. 6.2: Image magnification in a compound microscope.

Now we will learn the important terms-magnification, resolution and contrast used in microscopy. There are three major parameters that must be considered in order to produce a magnified image of great quality:

6.3.1 Magnification

Magnification is a characteristic that shows the ability of the microscope to magnify and enlarge an object, which provides the viewer with a more detailed visual depiction of the object. Basically, it is the ratio of the apparent size of object as seen in its image under the microscope and the actual size of object. You can calculate magnification power (M) by the given formula

$$\text{Magnification (M)} = \frac{25}{f} + 1$$



Eyepiece (10X)

<u>Where,</u>

25 = the least distance (D, in cm) of vision for a normal eye

F = focal length of the lens

Magnification (X) of a microscope can be calculated by multiplying the objective lens and eyepiece lens magnification. For instance, when you combine a 10X objective lens with a 10X ocular lens, the resulting magnification is calculated as (10X10) X = 100X. But higher magnification doesn't mean the higher resolution.

Now we will discuss the resolving power and numerical aperture which are important in the light microscopy.

6.3.2 Resolution and Resolving Power

The resolving power of a microscope determines the degree of clarity in differentiating between two closely spaced spots at a specific level of magnification. However, there is little difference between the resolution and resolving power. **Resolution** is about the clarity of two closely object seen in a microscope while **resolving power** is the ability to visualize such images separately by the microscope. It means, two closely points within an object seen as separate entities are the resolution. Therefore, the quality or clarity of a picture is determined by optimising the separation between the any two points that are close in proximity within the image (Fig. 6.3).

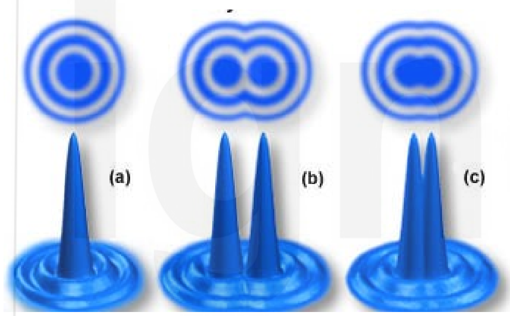


Fig. 6.3 : Resolution is to see clearly and separate two closely point within the specimen.

Adapted from http://microscopy.fsu.edu/primer/anatomy/numaperture.html

A microscope's **resolving power** is inversely proportional to its numerical aperture and directly relates to the wavelength of light it uses. These values are related by the **Abbe's equation** (including Rayleigh's correction):

$$\text{Resolving power (d)} = \frac{0.612\,\lambda}{n\,.\,\sin\theta}$$

Where, **λ** = wavelength of light used (0.612 constant λ)

n = refractive index of medium between the specimen and objective lens

α = half aperture angle in radians.

n sin θ = numerical aperture (NA) of objective,

The equation provided clearly indicates that enhancing the resolution can be achieved by modifying either the wavelength or the numerical aperture. The resolving power formula is employed to calculate the resolution of an image in the field of light microscopy.

The resolution of the compound microscope is constrained by the wavelength of light employed to illuminate the specimen, which normally ranges from 400 to 700 nm. The objective provides more specific information as the wavelength of light drops. The ideal compound microscope has a restricted capacity to differentiate elements of a specimen that are less than 200nm apart.

Contrast: Some biological samples require staining technique to examine clearly them under microscope. So, it is necessary to have a contrast image that is sufficiently different from the background or the medium that is around it. Microscopes use a variety of techniques such as staining to increase contrast and the clarity of the image as well as the visibility of the details against their surroundings.

Let us learn the concept of numerical aperture.

6.3.3 Numerical Aperture (NA)

Numerical Aperture is a measure of the resolving power of a microscope objective. It is equal to the product of the refractive index of the medium (n) in front of the objective and the sine of the angle (sin θ) between the outermost ray entering the objective and the optical axis (Fig.6.4). Therefore, NA is an estimate of how much light from the sample is collected by the objective lens.

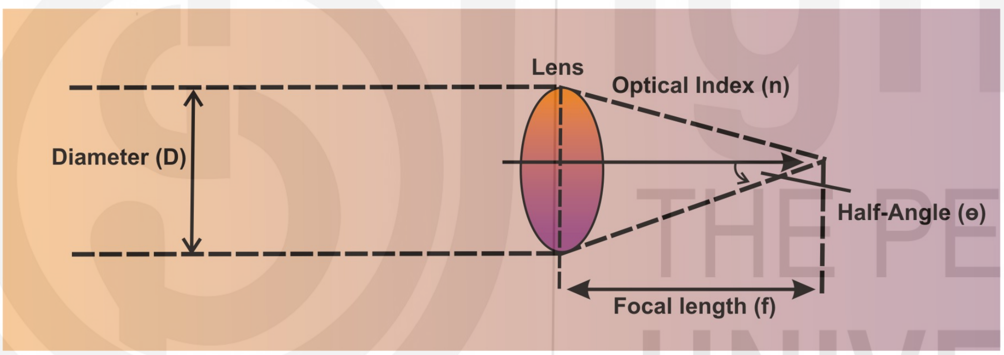


Fig. 6.4: Numerical aperture (Focal length (f) is the distance from an object and optical lens and D is the diameter of lens.

The numerical aperture (NA) can be determined by the following formula

$$NA = n \cdot \sin\theta$$

Where

n = Refraction index of medium between specimen and objective lenses (Air; n = 1; Oil; n = 1.51 and 1.33 for pure water);

θ= is the half angle of maximum cone of light formed by the optical axis and the refraction of light from the center of the lens.

Resolving power improves as the objective lens's numerical aperture increases, but only to a certain extent. Since oil has a greater refractive index than air, it can be used to fill the space between the specimen and the objective to increase the numerical aperture value even further. The numerical aperture can be raised to as high as 1.4 using oil immersion lenses.

Now you know how to obtain optimal resolution and magnification. If the contrast is inadequate, the image quality will remain inappropriate for analysis even at extremely high magnification and resolution.

In order to achieve this, we must lastly create contrast so that less light passes through the sample than through the medium. Because of the cells' absorption of light and the medium's refractive index difference, less light passes through the microscope's optical path, resulting in a reduction in light transmission. By staining the specimen, we can enhance the degree of contrast of an image or by optimizing the optical settings in the microscopes.

SAQ 2

Do as directed:

a) Differentiate between magnification and resolution.

b) **What will be magnification if** 40X objective lens and a 10X ocular?

   i) 40X

   ii) 10X

   iii) 400X

   iv) 30X

c) The resolving power will be if NA = 1.4 and **λ** =400nm or 500nm.

d) Calculate magnification power if focal length 12.5.

6.4 CELL FIXATION

In the previous section, you learned about the fundamental principle of microscopy and the functioning of a compound microscope. Prior to commencing the observation of specimens, it is important to immobilise cells or tissues in order to maintain their preservation in a state closely resembling life. Cell fixation serves to inhibit autolysis and putrefaction of the specimen, as well as safeguard the tissue/cells throughout further processing procedures.

Cell fixation can be achieved by either physical or chemical means. Physical fixation is achieved by manipulating temperature, either by increasing it through heating or decreasing it through cryopreservation. On the other hand, chemical fixation involves the use of organic solvents or cross-linking agents. Typically, chemical fixation procedures are employed to preserve the majority of specimens. The organic solvents, such as alcohol and acetone, induce lipid loss, tissue drying, protein denaturation and precipitation, as well as alterations in protein structure and solubility. They are commonly known as coagulants because of the effects they cause. The dehydration effect of ethanol can be mitigated by the swelling properties of acetic acid, and these two substances are combined in certain cytological fixatives.

The crosslinking methods employ aldehydes (such as paraformaldehyde and glutaraldehyde) or osmium tetroxide, which react with proteins and other biomolecules by creating intra- and intermolecular crosslinks. Covalent bonding provide superior preservation of cellular structure compared to organic solvents. The process of cross-linking provides cells with increased stiffness, which is advantageous when sectioning delicate tissues.

Fixation rapidly terminates cellular activity while enabling us to observe its form and internal components as they existed before to fixation. This is advantageous because once the cells have stabilised, the investigator can easily see them. Fixing also increases cell permeability. This enables the visualisation of cellular proteins or other molecules by facilitating the entry of antibodies or stains, such as DAPI for DNA, into the cells and their subsequent binding to specific targets. Stains provide varying colours and colour intensity, enhancing the visual distinction between different parts of a specimen.

There is no perfect fixative, hence it is recommended to select the most suitable fixative as per your sample. Several variables that can be standardised include the concentration of the fixative, the duration of exposure, the temperature, and the rate of penetration for fixation of biological sample. Non-toxic fixative may be used for tissue fixation if possible,

It is important to remember that fixed cells are no longer alive (dead cells), are not appropriate for studying the dynamic or time-dependent changes in cellular processes. Live cell imaging techniques allow us to examine living cells in native state.

SAQ 3

a) What is the advantage and disadvantage of tissue fixation?

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b) Match the term in column I with that in column II

Column I	Column II
1. Physical method	a. binds to proteins
2. Cross linking methods	b. dehydration solvent
3. Alcohol	c. Chemical fixation
4. Staining dye	d. increasing and decreasing temperature
5. Paraformaldehyde	e. DAPI

6.5 BRIGHT FIELD AND DARK FIELD MICROSCOPY

When you prepare biological specimen, the primary step to consider is the selection of an appropriate light source for illuminating the sample. The compound microscope discussed in section 2.2 is classified as a bright field microscope because to its ability to produce a dark image against a brighter background. In this process, the condenser lens focuses and transmits the light directly through the specimen. In transmitted light microscopy, an image is created through the interference between undiffracted light and diffracted light emitted by the specimen. Less transparent regions of the sample will look dark against the bright background, while more transparent regions will appear less dark. Completely transparent regions will have the same brightness as the background and may thus be indistinguishable. It is important to note that the majority of biological samples are highly transparent. Thus, in order to enhance visibility, we can utilise different stains that decrease the transparency of the sample and provide a noticeable difference in brightness between the background light and the sample. Bright field microscopy is typically appropriate for biological samples that have been stained. Table 6.2 provides a list of the stains often utilised.

Table 6.2: Stains commonly used for animal and plant tissues

Stain	Suitable for	Color
Hematoxylin	Nucleus	Purple
Eosin	Cytoplasm and plasma membrane	Pink to red
Methylene blue	Cell nucleus and bacteria	Blue
Toluidine blue	nucleus of plant cells (in mitosis)	Blue
Iodine	Xylem and Starch content in plant cells	Blue-black
Leishman /Giemsa/ Wright's.	Blood cell analysis	Violet
Safranin	Plant cell nucleus	Red yellow

Another method for examining transparent objects is the **dark field microscopy**, when the object is illuminated with bright light against a black backdrop. Fig. 6.5 depicts a schematic representation of bright field and dark field microscopes. Dark field microscopy employs a technique that blocks the direct entry of light into the front of the objective, allowing the microscope to specifically detect light that is scattered (diffracted) from the object. The light that has undergone diffraction will pass through the objective and ultimately reach the eye. The condenser is fitted with a dark field stop to obstruct the passage of light. Typically, obtaining a high-quality image necessitates the use of specialised intense light sources. The black background offers a significant contrast, allowing for clear visibility of scattered objects. This is particularly beneficial when examining low magnification outlines of individual cells, such as sperms, which have a strong light scattering effect.

Compound or stereomicroscopes can be easily switched from bright field to dark field microscopy by adjusting the knob. It is utilised for the examination of unstained samples, particularly those organisms that are resistant to staining or are altered by the staining process. Furthermore, staining is lethal to living cells, whereas dark field microscopy is employed to acquire real-time images of transparent materials that emit brilliant light.

The resolution in dark field microscopy is constrained by the need that the numerical aperture (NA) of the condenser must be higher than the NA of the objective. This ensures that the cone angle of the illuminating light is not recorded by the objective. Nevertheless, the inclusion of dust particles in the optical path would adversely affect the quality of the dark field microscopic images due to their ability to scatter light.

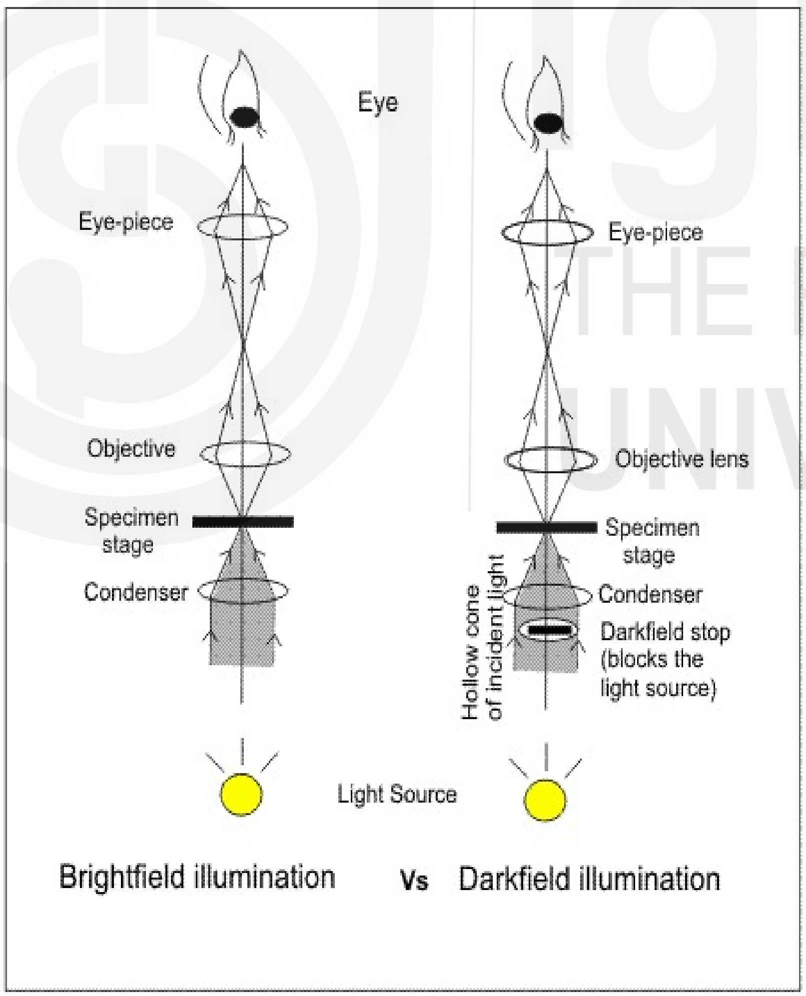


Fig 6.5: A schematic view of Bright field and Dark field microscopes.

SAQ 4

a) What is the difference in the sample illumination technique used in bright field and darkfield microscopy?

b) **Which of the following statements are true or false?** Write (T) for true and (F) for false in the given boxes.

i) Bright field microscope forms a bright image against a dark background.

ii) Scattered light is used to view objects in bright field microscopy.

iii) Dark field microscope is used to observe transparent samples

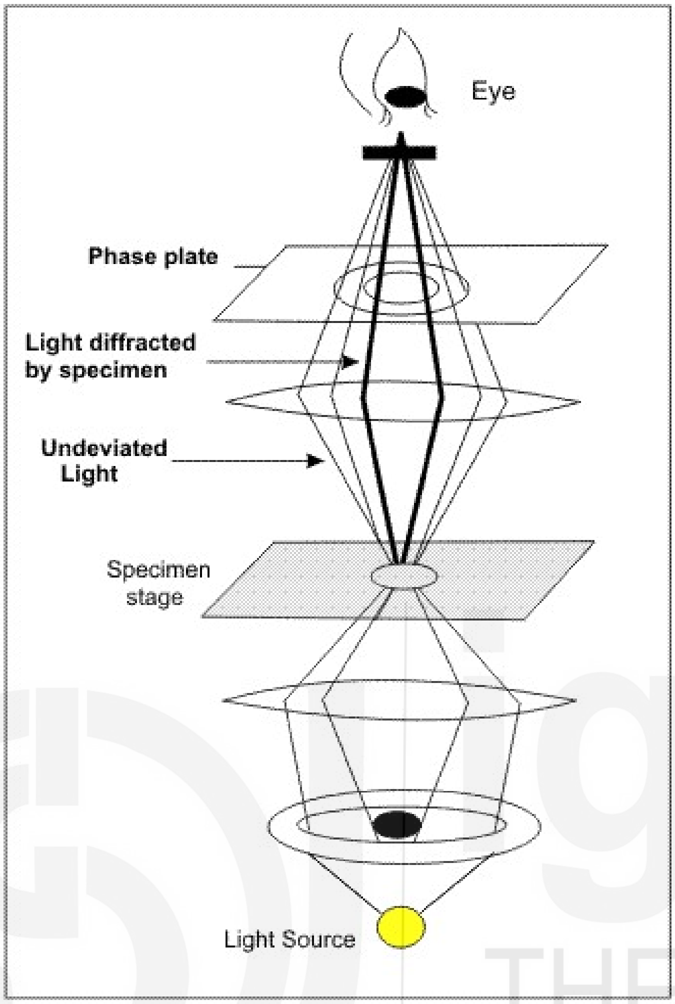
iv) Staining improves contrast between background light and the sample.

v) Bright field microscope is suitable for observing stained samples.

6.6 PHASE CONTRAST AND INTERFERENCE MICROSCOPY

This section discusses the phase contrast microscopy (PCM) and Nomarski differential interference contrast microscopy (NDIC). These microscopes are designed to produce the contrast image of unstained biological sample. Hence, PCM and NDIC are called the contrast-enhancing microscopy. Unlike compound microscope, these microscopes enable us to view live cells growing in cell culture. This microscopy is helpful to view transparent specimens (unstained sample) with high-contrast images including living cells, thin tissue slices, and subcellular organelles. Let us first study the PCM.

Frits Zernike, a Dutch physicist, developed the phase contrast microscopy (PCM) in 1934 and was awarded the Nobel Prize in physics in 1953 for his invention of this microscope. In phase contrast microscopy, a tungsten-halogen lamp emits light which is then directed through a collector lens and focussed on a ring-shaped annulus positioned in the sub stage phase contrast turret condenser. The annulus only allows light to pass through, resulting in a hollow cone of light reaching the specimen. The light waves that traverse the annulus illuminate the specimen and either flow through without any delay (surround waves) or undergo diffraction and delay when they interact with various parts of the material (Fig.6.6).



Frits Zernike

Fig. 6.6: Phase contrast microscope.

Optical path in phase contrast microscopy are summarised below:

Light source → Condenser with annulus → Specimen → Objective → Phase plate with annulus → Image plane

The phase contrast microscope finds changes in the refractive index of different cellular structures to produce contrast images. When light waves go through an object that has a higher refractive index than the medium, the waves are slowed down or delayed. We call this phase change. Different parts of the cellular structure have different refractive indices and thicknesses, which cause them to change phases. These changes in phase are tiny, and a compound microscope can't detect them. Small changes in phase can be seen with the naked eye or picked up by a photoelectric plate. PCM amplifies these changes.

In PCM, a specific phase condenser and phase objective lenses are provided to detect the refractive index of sample medium. The phase setting of the condenser lens is aligned with the phase setting of the objective lens. The objective collects both the undeviated and diffracted light, which is then separated by a phase plate. The objective phase includes a dark annulus that delays the diffracted waves by ¼ wavelengths, causing them to be more out of sync with each other and with the undeviated waves. The phenomenon we observe is the result of the simultaneous occurrence of constructive and destructive interference when these light waves intersect. Coherent light waves create a luminous image, but incoherent waves interfere with each other to different extents, resulting in varied degrees of darkness in the produced images (ranging from grey to black).

Let us learn about Differential interference contrast (DIC) microscopy. The DIC was developed by Polish physicist G. Nomarski as a technique for observing specimens that have low optical contrast. It is a procedure that does not require any incisions or penetration of the body. Fig. 6.7 illustrates the optical pathway of Differential Interference Contrast (DIC) microscopy, which consists of a polarising filter, two double prisms, and an analyzer.

Georges Nomarski

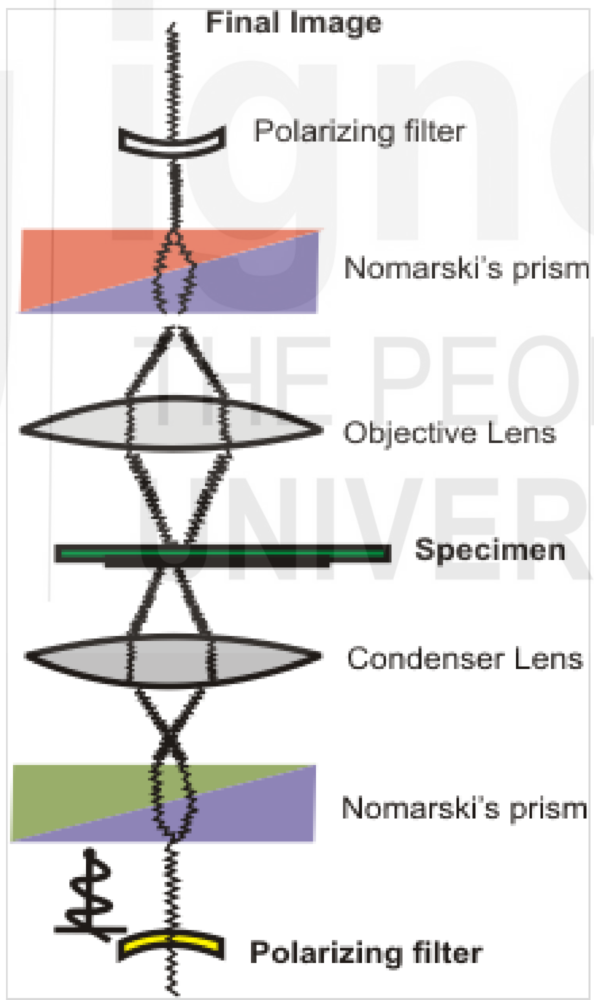


Fig. 6.7: Optical path of DIC microscope.

DIC utilises a pair of light beams. The unpolarized light emitted by the lamp is initially passed through a polarising filter, which polarises it at an angle of 450 degrees. It is thereafter directed towards a Nomarski-modified Wollaston prism positioned beneath the condenser. The prism divides the incoming beam into two separate beams of polarised light that travel in slightly divergent directions. The recombination of these beams does not result in interference. The distance separating the two beams is smaller than the objective's resolving capability and is commonly known as the 'shear distance'. Subsequently, the two beams traverse the condenser lens, aligning them in a parallel manner, enabling them to flow alongside each other through the specimen. As the beams traverse the specimen, their trajectories are altered by the variations in thickness and refractive index that they meet. Contrast in DIC is primarily determined by gradients in the optical path length. Upon entering the objective, the parallel beams converge above the back focal plane. Subsequently, the beams pass through the second prism, where they are combined into a single polarised beam. Recombination results in interference. The second polarizer, also known as the analyzer, is necessary to align the vibrations of the beams along the same plane and axis, so enabling interference to take place. The ultimate visual representation is observed via the eyepiece, displaying variations in both brightness and hue. The DIC image provides high-resolution surface information and is capable of generating three-dimensional-like images. The cells exhibit a protruding structure on the surface of the slide, and even the nuclei are elevated above the cytoplasm's surface.

SAQ 5

Distinguish between Phase contrast microscopy and DIC microscopy.

6.7 FLUORESCENCE MICROSCOPY

Fluorescence microscopy is also contrast optical microscopy technique based on the capturing the images of fluorescent sample. It involves illuminating a fluorescent sample with light of a shorter wavelength, often blue or ultraviolet (UV) light, and then measuring the released photons of longer wavelength. This phenomenon is based on the emission of visible light energy by particular compounds.

The first recorded instance of fluorescent properties was observed in quinine (a blue fluorescence molecule) which was extracted from the cinchona’s bark by Sir John F.W. Herschel. Fluorochromes were first utilised for biological investigations in the 1930s. By that time, numerous distinct stains had been identified, which prompted the advancement of fluorescence microscopes. Today, it has become an essential tool in biological and medicinal research.

Next, we shall know the basic parts of a fluorescent microscope (Fig. 6.8). A fluorescence microscope is designed to illuminate the specimen with intense light of a certain wavelength and then separate the faint emitted light (fluorescence) from the excitation light. This allows for the creation of a high-contrast image of the specimen against a dark backdrop.

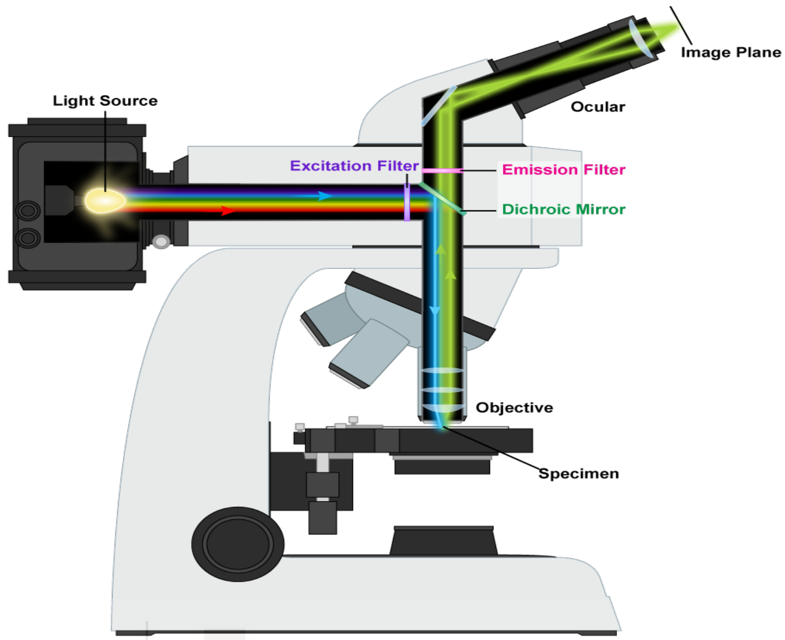


Fig. 6.8: Fluorescence microscope.

Image source: By Masur (Own work) [GFDL, CC-BY-SA-3.0 or CC BY-SA 2.5-2.0-1.0], via Wikimedia Commons

The basic parts of a fluorescence microscope are:

❖ **Light source**: A variety of light sources can be used. Mercury or xenon arc lights and lasers are used. Only the desired wavelength of excitation light passes through the excitation filter.

❖ **Filters:** Three categories of filters are essentially required for fluorescence microscopy. They are excitation, barrier (emission) and dichromatic beam splitters (dichroic mirrors) which provide high resolution with interference optics. These filters are combined in filter cubes and there are multiple such cubes that can be rotated easily to change filters. The excitation filter transmits only the excitation light of the desired wavelength. Dichroic mirrors are placed in the light path at an angle and they reflect the excitation light from the objective to the sample. A barrier filter removes any excitation light that could otherwise reduce image's contrast. Incidentally the objective is designed both to direct excitation light and to function as an image forming light gather. An objective of higher numerical aperture is better for getting a bright image.

❖ **Detection:** The fluorescent image can be viewed or recorded using a camera with sensors in a dark background.

Let us discuss why fluorescence molecules emit fluorescence radiations

6.6.1 Stock Shift

Fluorescent molecules have ability to emit photons of higher wavelength after absorbing certain photons. This phenomenon was observed by the scientist George Gabriel Stokes (1852) in fluorescent molecules when these molecules are exposed to a specific wavelength of light; they undergo excitation and subsequently release fluorescence radiation. He called this phenomenon as the stock shift. This emission occurs when the excited electrons within molecule return back to its initial energy state (ground state). During this process, electrons that have left the system transition to a higher energy state, where their vibrational energy level is unstable. Then they return back to the ground state within a short interval of less than 10^{-8} seconds by producing photons with a higher wavelength in order to stabilize. The distinction between emission spectra and absorption spectra is commonly known as the stock shift as shown in the Fig. 6.9. In nut shell, stock shift is the shift of wavelength between the absorption spectra and emission spectra.

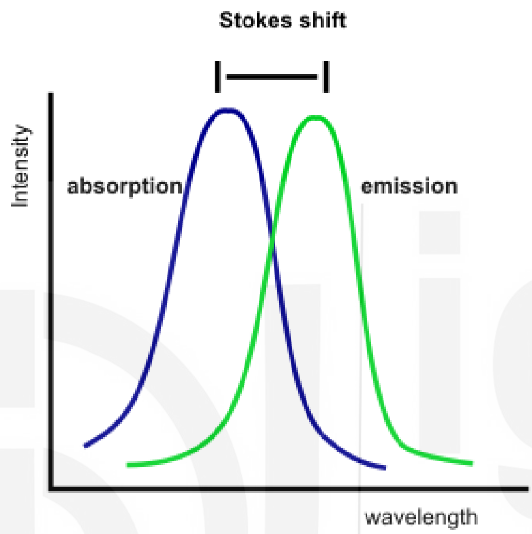


Fig. 6.9: Stokes shift.

Additionally, as the Stokes shift values increase, it becomes more convenient to distinguish excitation light from emission light by employing filters. Fig.6.10 illustrates Jablonski's energy diagrams, which are valuable for elucidating the molecular transitions between the absorption and emission of light energy.

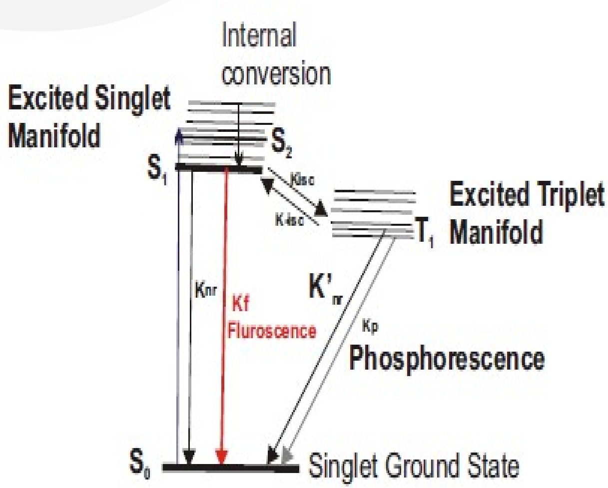


Fig 6.10: Jablonski energy diagram.

Some samples are auto fluorescing and other specimens are stained with fluorochromes that fluorescence brightly upon exposure to light of a specific wavelength. A fluorochrome is a fluorescent chemical that can reemit light upon excitation. A fluorochrome s generally excited at wavelength at or near the peak of the excitation curve. Table 6.3 gives the excitation and emission wavelength of some fluorochromes. It is also possible to use multiple fluorochromes to stain different components with different colors.

An important consideration in the choice of the dye is its **quantum efficiency (QE).** It is defined as the fraction of absorbed photons resulting in emission of fluorescence. Compounds with a higher QE are preferred.

Table 6.3: Excitation and Emission wavelengths of common fluorochromes

Fluorescent Dye	Excitation wavelength (nm)	Emission wavelength (nm)
Hoechst 33342	343	483
DAPI	345	455
Ethidium Bromide	493	620
Acridine Orange	503	530/640
SYTOX Green	504	523
Propidium Iodide (PI)	536	617
Fluorescein	495	519
TRITC	547	572
EGFP	488	507
mCherry	587	610

Photo bleaching is the main limitation in fluorescence imaging. It results from the excited state fluorescent molecule's irreversible breakdown from its contact with oxygen before emission. Using fade-resistant chemicals (Quantum dots), adding anti-fade compounds, or opening the shutter only when necessary are some solutions to this issue. Because fluorescence microscopy offers great sensitivity, specificity, contrast, and quantification, it has become an essential tool for biologists. It can detect the existence of a single molecule.

Here, we now discuss the application of fluorescence microscopy.

❖ **Live cell imaging**: It is possible to obtain information about cell dynamics. These may include movements and interactions of different biomolecules. Thus, one can measure the rates of physiological reactions, binding constants of bio- molecular interactions, dynamic changes in cellular structures etc. Such studies require time lapse fluorescence imaging where the same area is imaged continuously at regular time intervals and the changes are analyzed.

- **Cell viability:** Viability of cells can be ascertained using vital stains during fluorescence microscopy. Vital stains are dyes that can enter only dead cells and therefore make the dead cells appear fluorescent.

- **Immunofluorescence:** It requires fluorescent labeling of proteins such as antibodies. It may be either direct or indirect labeling. In direct immunofluorescence the labeled antibody (primary) is against the target antigen whereas in case of indirect immunofluorescence the labeled antibody (secondary antibody) is against the primary antibody.

  Most often indirect immunofluorescence is used as it does not require specific labeled antibody against the target antigen and labeled secondary antibodies can be purchased. In addition, the sensitivity of staining is enhanced as multiple fluorochromes labeled antibodies can bind to each primary antibody. It is most often used to localise antigens in tissue sections or sub cellular compartments.

[**You can also listen to a video lecture by N. Stuurman on Fluorescence microscopy that is freely accessible at ibioseminars.org**]

SAQ 6

Define Stokes shift. When EGFP fluoresces, what color of light will be seen?

6.8 CONFOCAL MICROSCOPY

Confocal microscopy or more appropriately confocal laser scanning microscopy (CLSM) includes a family of techniques that essentially have two parts – optical sectioning and confocal. It is advancement over conventional **wide field microscopy** that is known to generate both in focus and out of focus images (non confocal), especially in case of thicker specimens and this blurring effect eludes the desired details. Confocal microscopy on the other hand produces high resolution in focus (confocal) images.

The basic principle of confocal microscopy was developed by **Marvin Minsky** in 1953 but it took almost three decades for it to become a standard technique. The development of lasers has made this technique to take off and reach the level of refinements we see today. An important issue to address in the successful development of this kind of microscopy was to get rid of the out of focus images. The innovation was simply to place a **pinhole** in the same focal plane as the sample (and hence the term ‘confocal’).

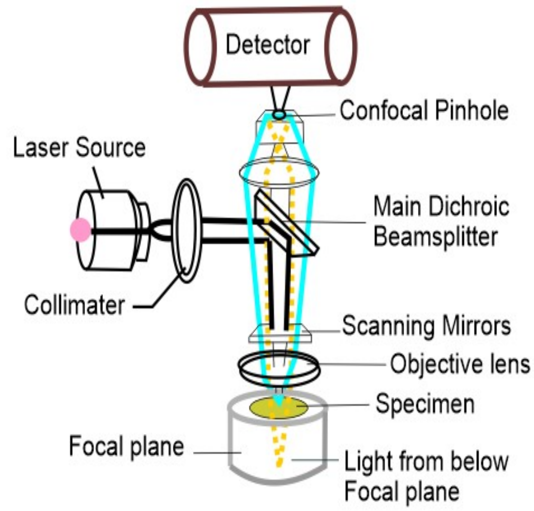


Fig. 6.11: Basic setup of Confocal Microscope.

A confocal microscope is made up of the following components (Fig. 6.11):

- ❖ The optical system
- ❖ The laser light source
- ❖ Scanning device
- ❖ The detection system

We will now proceed to describe the scanning device and the detection system. The image is created by **scanning** laser point by point over the fluorescent sample. The intensity is recorded at each point. This method is called ***z-scanning*** as the optical sectioning is along the z-axis of the microscope. As we move the illumination point, the emission point also moves and so it is necessary to check that the pinhole is coincident with it. The stack of two dimensional images generated can be viewed either individually or is reconstructed by the computer imaging software. The reconstructed image provides a 3D view.

The increased resolution in confocal microscopy is at the expense of decreased signal intensity as only the fluorescent light that passes through the pinhole at the focal point is picked up by the detector. This implies the **detectors** must be sensitive to detect these weak signals and in many cases longer exposures are needed. Since we are scanning spot by spot the detectors must also be fast as the confocal beam spends only a few microseconds at each pixel. Some of the detectors use photomultiplier tubes (PMT).

Applications of confocal microscopy

- ➢ It is useful for **3D imaging** of cells in tissues and surface profiling of samples.
- ➢ It is a **non invasive** technique involving optical sectioning of thick fluorescent labeled live biological specimens.

- It is possible to observe dynamic changes in a cell by continuous scanning in the same plane.
- It can be used for visual analysis of expression patterns of genes during development.

Drawbacks and possible solutions

- It is relatively **slow t**o generate an image as it scans excitation spot point by point. It is not suitable to visualise fast processes.
- It is **not very sensitive** because PMT detectors record only a small percentage of the light that hit them.
- These limitations have been addressed by incorporating multiple pinholes and CCD camera as detector. In another variation- the multi photon excitation approach for optical sectioning eliminates the need for even a pinhole.

[You can listen to a video lecture on confocal microscopy by Kurt Thorn at ibioseminars.org]

SAQ 7

Which structure in a confocal microscope is used to obtain images from a single focal plane of the specimen sample?

6.9 ADVANCED MICROSCOPY

Microscopy is a fast developing field since its inception. The advance form of microscopy employs fluorescence imaging techniques to overcome the resolution limit of light microscopy. However, traditional microscopes cannot resolve the smaller structure below 240nm due the diffraction limit of light. SRM enables biologist to study cellular architecture and dynamics in living cells. It helps to reveal ground breaking discovery in the field of molecular cell biology, medicine and drug research. Biological scientist can get three dimentational super resolution images. The main aim of super resolution microscopy is to enhance of an image from low resolution to the higher resolution. It is useful to **examine molecular interactions, single molecule and subcellular structures** at nanoscale level. It gives high resolution image with high density pixel. Some SRM is combined with intrinsic optical sectioning with fast data acquisition and dual color super resolution to get good quality image in timely fashion. The fast advancement of fluorescence microscopy, coupled with digital imaging and, most importantly, the emergence of novel fluorescent probes helps in development of super imaging microscopy such as Fluorescence resonance energy transfer microscopy and Stimulated Emission Depletion (STED) microscopy. Hence, this technique is also called is imaging enhancing microscopy. Therefore, in this section, we will study principle and application of FRET and STED.

6.9.1 Fluorescence Resonance Energy Transfer (FRET) Imaging Microscopy

The word FRET stands for **Fluorescence resonance energy transfer**. FRET microscopy involves the energy transfer between two fluorophores that are in close proximity to each other. However, the traditional optical microscope has a limitation to visualize protein-protein interactions using a fluorescent probe due to its limited optical resolution power (about 0.2µm). To overcome this problem, FRET imaging microscopy was developed to facilitate to study of molecular interactions in an intact living cells. FRET imaging microscopy helps to investigate protein-protein interactions, protein-nucleic acid interactions, molecular conformational changes, enzyme-substrate interactions, and signal transduction pathways in living cells. It is frequently utilized in the research area of cell biology, neuroscience, immunology, and medicine.

Principle:

The basic principle of FRET is based on the shifting of energy transfer from electrically excited molecule (donor) to a neabouring molecule (acceptor). This energy transfer happens only when the two fluorescent molecules (donor-acceptor pair) are enough proximity less than10nm and the excited donor transfers energy (not electron) to an acceptor group through non-radiative process (dipole-dipole interactions). This phenomena result in overlapping fluorescence emission. In this way, the energy transfers decreases donor fluorescence intensity and increases acceptor fluorescence intensity, indicating the tagged molecules' closeness and interaction.

<u>FRET experiment</u> <u>example</u>

FRET involves the utilization of spectral variations of green fluorescent protein (GFP). In this experiment, the activation of a cyan fluorescent protein (CFP)-tagged protein is utilized to observe the emission of a yellow fluorescent protein (YFP)-tagged protein. YFP fluorescence is only detectable when the proteins are in close proximity and excited by CFP as shown in Fig. 6.12.

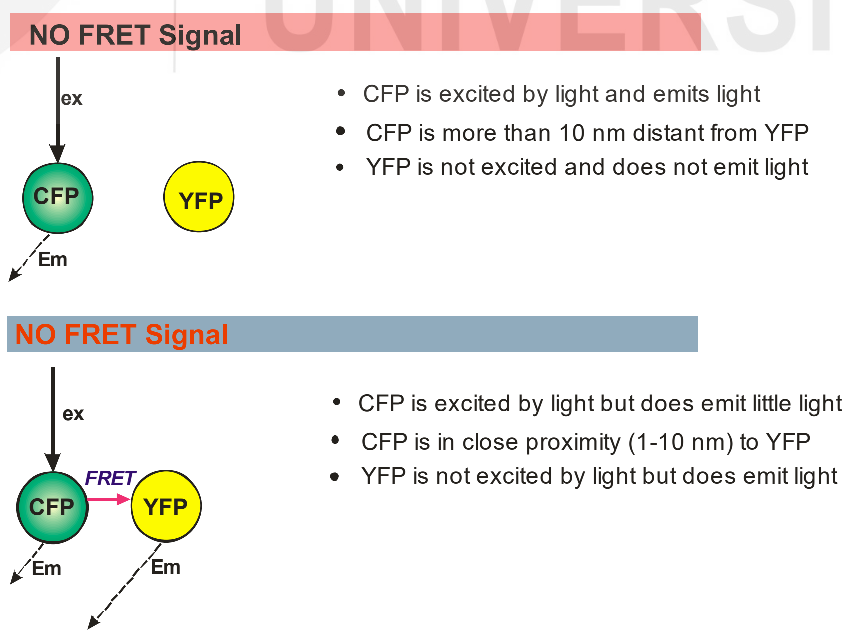


Fig. 6.12: A schematic description of the principle of FRET microscopy.

The fluorescence emitted by the donor fluorophore is detected by appropriate filters of FRET microscopy. The captured image showed the overlapping spectra of CYP/YFP pair and ideal for FRET. Given its ability to be observed over a period of time, FRET can be utilized to quantify the direct interaction between proteins or protein complexes (Fig. 6.13).

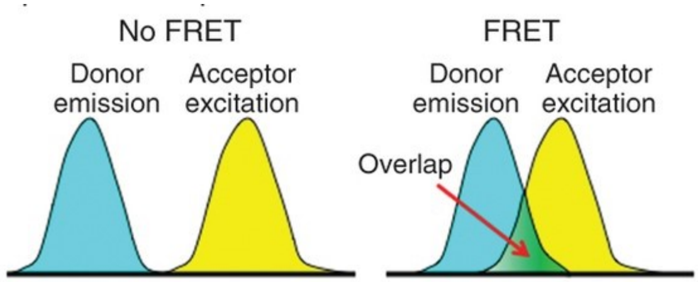


Fig. 6.13: Spectral overlap in FRET.

6.9.2 Stimulated Emission Depletion Microscopy (STED)

The stimulated emission depletion microscope is abbreviated as STED. STED microscopy is based on the principle of diminishing fluorescence emission from the adjacent areas of the focus point, leading to a decrease in the effective excitation volume. This is a fluorescence based super imaging microscopy technique. It employs the fluorescent molecules and the laser beam to visualize ultra cellular structure. The laser beam scans over the sample. Fig. shows the setup of STED. This microscopy procedure utilizes two laser beams: **a donut-shaped excitation beam and a depletion beam.** The excitation beam is responsible for inducing fluorescence within the sample, while the depletion beam reduces the excitation of fluorophores in the surrounding area of the excitation spot. As a result, the extent of the fluorescence-permitting region is diminished. This is the first method to resolve the ultrastructure below the limit of optical resolution.

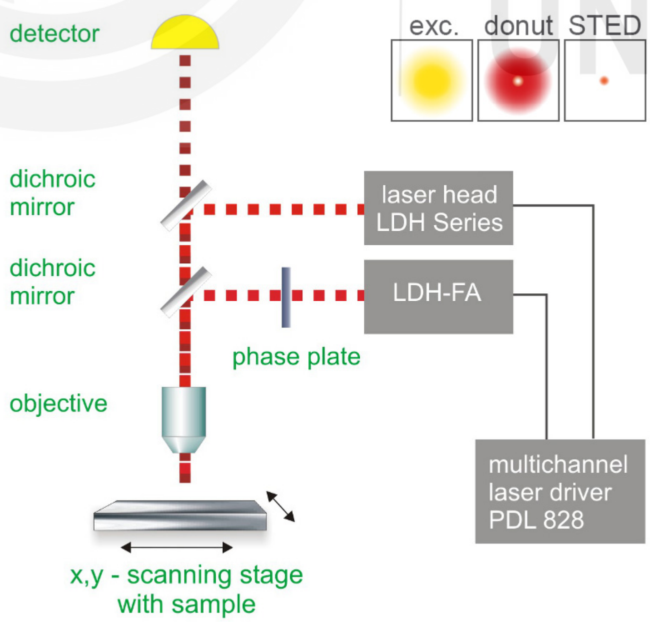


Fig. 6.14: STED setup.

6.10 CONCEPT OF BIOLUMINESCENCE AND ITS USE IN MICROSCOPY

Bioluminescence is an intriguing natural occurrence in which organisms produce light as a result of a chemical reaction occurring within their bodies. Bioluminescence is the term used to describe the emission of light by living organisms. Fireflies are a prime example of this phenomenon, since they generate a glow through a biochemical process that involves the enzyme luciferase, a luciferin substrate, ATP, and calcium ions. Therefore, it is an energy dependent process.

Bioluminescence imaging is a technique that utilises the distinctive characteristic of bioluminescence. It involves the detection of light emitted by cells that have been genetically engineered to express the luciferase gene or other luminous genes. This imaging technique utilises ultra-sensitive cameras or detectors to capture images of live cells without the requirement of excitation light, as seen in fluorescence microscopy. Bioluminescence microscopy overcomes the problems of phototoxicity and photobleaching that can occur with fluorescence microscopy as a result of extended exposure to excitation light.

However, bioluminescence microscopy enables non-invasive imaging of live cells, which complements the fluorescence microscopy. This allows to study of molecular cell biology and the observation of live-cell dynamics. Conventional fluorescence microscopes exhibit limited efficacy in transmitting light from the specimen to the sensor, necessitating prolonged exposure durations and have an issue of phototoxicity and photobleaching. In contrast to fluorescence microscopy, bioluminescence microscopy does not require excitation light, and it lacks also phototoxicity. However, a drawback of bioluminescence microscopy is the week bioluminescent signals, which can impact the sensitivity of imaging.

SAQ 8

Do as directed:

a) Differentiate between FRET and STED.

b) define the bioluminescence.

6.11 SUMMARY

In this unit you have learnt that:

- The cells of most organisms are smaller than what the human eye is capable of seeing. A microscope is therefore an indispensable tool because of the inherent limitations of the human eye.

- Most present day microscopes are compound microscopes that have two type of magnifying lenses. In order to generate good quality magnified image, three most important parameters are magnification, resolution and contrast.
- Cells and tissues are generally fixed by physical or chemical methods to preserve them.
- A bright field microscope forms a dark image against a brighter background. In this the condenser concentrates and transmits the light directly through the specimen. In dark field microscopy the object is brightly illuminated against a dark background. It prevents direct light from entering the front of the objective and so the microscope detects light scattered from the object. Most bright field microscopes can be used for dark field microscopy by simply shifting the knob from bright field to dark field.
- Phase contrast microscopy (PCM) and Nomarski differential interference contrast microscopy (NDIC) are contrast enhancing techniques. They were developed by Frits Zernike and G. Nomarski, respectively.
- Fluorescence microscopy is a special type of optical microscopy where a fluorescent sample is illuminated with light of shorter wavelength, usually blue or UV light and the emitted photons (longer wavelength) are measured. It finds application in live cell imaging, immunofluorescence, etc.
- Confocal microscopy includes a family of techniques that essentially have two parts – optical sectioning and confocal. Confocal microscopy produces high resolution in focus (confocal) images. The image is created by scanning laser point by point over the fluorescent sample.
- FRET and STED microscopy are both advanced imaging methods utilized in biological and materials science research. It allows resolution below the diffraction limit of optical microscopy. FRET is the process of transferring energy from an excited fluorophore (donor) to a nearby non-excited fluorescent molecule (acceptor) without light emission.
- STED microscopy operates on the principle of selectively diminishing the fluorescence emission in the vicinity of the focal point. By reducing the excited state of fluorophores, a STED laser improves resolution and narrows the effective point spread function.
- Bioluminescence microscopy detects light from luciferase and luciferin chemical reactions.

6.12 TERMINAL QUESTIONS

1. Explain the working principle of light microscopy.
2. Elaborate the important parameters for a good quality magnified image?
3. Write a brief note on Numerical Aperture.
4. What is fixation? What are the two general types of fixation?

5. Name the three types of filters used in fluorescence microscopy? Indicate their role.
6. Explain stock shift with the help of jabonski diagram.
7. How does a confocal microscope eliminate out of focus light?
8. Explain working principle and application FRET and STED
9. Differentiate between bioluminescence microscopy and fluorescence microscopy?

6.13 ANSWERS

Self-Assessment Questions

1. a) iii)
   b) iv)
   c) i)
   d) v)
   e) ii)

2. a) Magnification is the enlargement of image by the optical lens while resolution is to see separate two closely space point within specimen.
   b) iii)
   c) We may use this formula to calculate the resolving power,

$$\text{Resolving power (d)} = \frac{0.612\,\lambda}{n\,.\,\sin\theta}$$

(i) d = 0.61 ×400 / 1.4 = 174 nm,

(ii) d = 0.61 × 500/1.4 = 217 nm

d) As you know focal length = 12.5cm and least distance of distinct vision D = 25cm

$$\text{Magnification (M)} = \frac{25}{f} + 1$$

M= 25/12.5 =2+1= 3

Magnification power is 3

3. a) The advantage of fixing cells is that the samples can be preserved for a long time. Cell fixation prevents autolysis and decay of the

specimen. Further, fixing also permeabilises the sample and makes it easier for dyes or antibodies against specific structures or proteins to gain entry into the cells.

b) 1- d; 2 - c; 3 – b; 4 - e; 5 – a

4. a) A bright field microscope forms a dark image against a brighter background. In this the condenser concentrates and transmits the light directly through the specimen. A less transparent part of the sample would therefore appear dark against the bright background while more transparent parts will appear less dark and completely transparent part will have the same brightness as the background. In **dark field microscopy** the object is brightly illuminated against a dark background. It prevents direct light from entering the front of the objective and so the microscope detects light scattered from the object. The diffracted light will enter the objective and reach the eye. The condenser is equipped with a dark field stop to block the light.

b) i) False; ii)False; (iii) True; iv) True; v) True

5.

Phase contrast microscopy (PCM)	**DIC microscopy**
It is not sensitive to sample orientation.	The contrast is directional and so sample orientation can change the regions of max and min contrast.
In PCM light traveling through the transparent sample is made to interfere with the illuminating light to produce contrast.	It is a variation of polarization microscopy. The condenser DIC prism splits illumination into two divergent polarised beams.
Thick samples can't be imaged properly.	Even thick samples can be imaged.

6. The shift in the wavelength of emitted light by an excited fluorochrome is called Stokes shift. The fluorescence always occurs at a longer wavelength than the excitation light. The emission maxima of EGFP are 507nm which falls in the "Green" region. Please refer to the subsection 6.6.1.

7. It is the pinhole screen placed in front of the eye-piece.

8. a)

FRET	STED
It is the fluorescence resonance energy transfer microscopy. It is based on the transfer of energy between two fluorescent molecules which acts as a donor and an acceptor fluorophore, when they are in close proximity.	It is the stimulated emission depletion microscope. It works by selectively depleting the fluorescence emission from regions surrounding the focal spot.
Fluorescent probes or tags are linked to sample proteins.	Fluorescent labeling is required, but it does not related to energy transfer between fluorophores. It uses standard laser beam to scan the sample
It useful to study about molecular interactions between proteins and confirmation changes.	Provides super-resolution imaging of nano size cellular structure.

b) Bioluminescence is an inherently phenomena of light production by chemical reaction occur within the living things. This light emission is usually caused by luciferase enzymes and luciferin substrates in the presence of ATP and calcium ion.

<u>Terminal Questions</u>

1. Please refer to section 6.3.

2. The resolving power, magnification and contrast are most important parameters for getting a good quality image in light microscopy. Please refer to section 6.3 for more details

3. Please refer to section 6.3

4. Fixation is the process of preserving of cells / tissues in a near life like condition. It also protects the sample during further processing steps. Fixation can be done by either physical (temperature variations) or chemical methods (organic solvents or cross linking agents). Please refer to section 6.4

5. Three categories of filters are essentially required. They are excitation, barrier (emission) and dichromatic beam splitters (dichroic mirrors). The filters are high resolution with interference optics.

   (a) The excitation filter transmits only the excitation light of the desired wavelength. (b) Dichroic mirrors are placed in the light path at an angle and they reflect the excitation light from the objective to the sample.

(c)The barrier filter removes any excitation light that could otherwise reduce image's contrast. Filters enable the florescence light to form higher resolution final image.

6. Please refer to the section 6.6.1.

7. By using a pinhole screen that allows only light from the focal plane to pass through and reach the detector.

8. Please refer to subsection 6.8.1 and 6.8.1.

9. Please refer to section 6.9.

6.14 FURTHER READINGS

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