E-Study material
For 5th semester Botany Honours (CBCS)
Course Code: BD501T
Core Course VII: Genetics
DSE Course – I: Analytical Techniques in Plant Sciences
Unit 1: Imaging and related techniques

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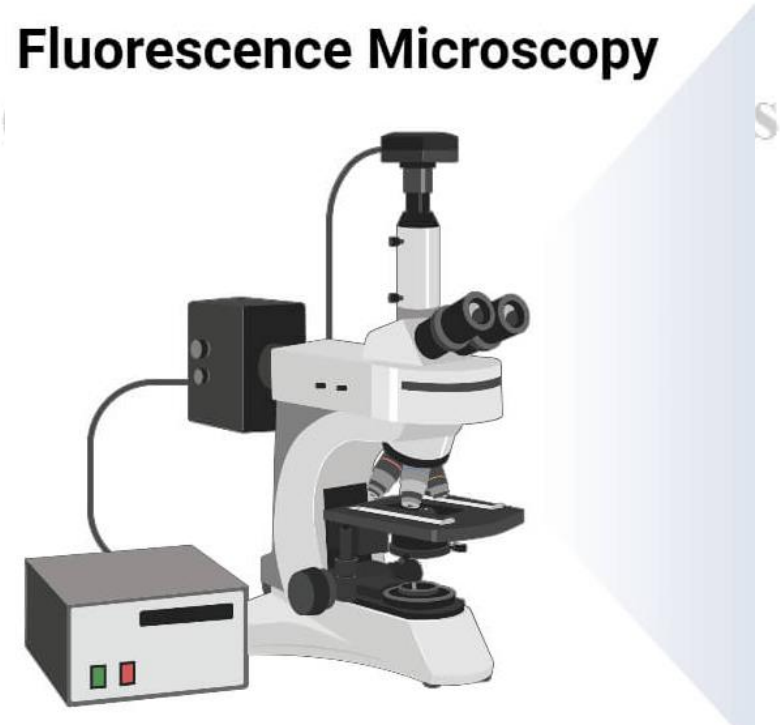
Fluorescence microscopy:

Question: What do you mean by fluorescence microscope? Who discovered it? Describe briefly its parts, types and working principle with diagram. State briefly the application of fluorescence microscope.



A fluorescence microscope is similar to a regular light microscope, but it has several extra qualities that enhance its usefulness. The typical microscope

magnifies a sample using visible light (400-700 nanometers).On the other hand, Fluorescence microscopes use high-intensity light to stimulate fluorescent organisms in a sample. This fluorescent species emits a longer-wavelength, lower-energy light that magnifies the image. Therefore, a fluorescence microscope is an optical microscope that studies the properties of organic or inorganic substances by using fluorescence and phosphorescence instead of or in addition to reflection and absorption.

Fluorescence was first discovered in 1845 by Fredrick W. Herschel. However, the first working fluorescent microscope was developed by Oskar Heimstaedt in 1911.

Parts of fluorescence microscope:

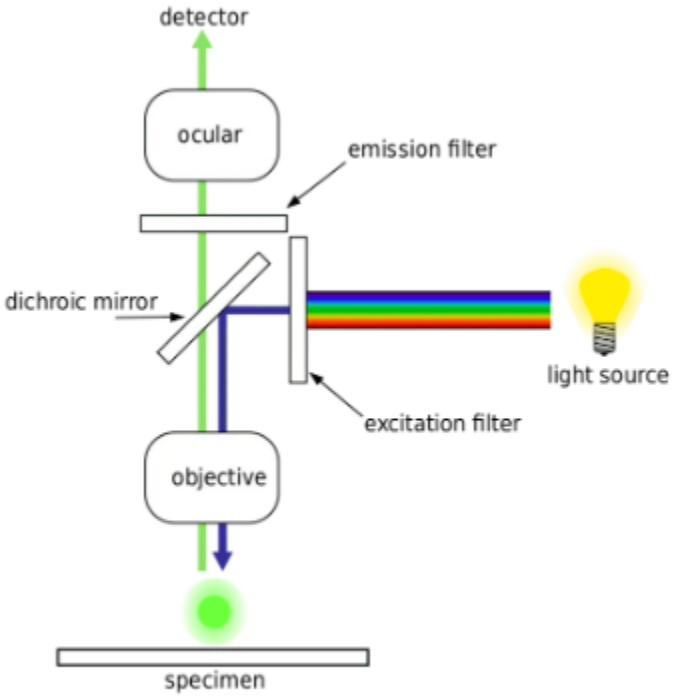
Fluorescence microscope components includes

1. **Fluorescent dyes (Fluorophore):** A fluorophore is a fluorescent chemical compound that can re-emit light upon light excitation. Fluorophores typically contain several combined aromatic groups, or plane or cyclic molecules with several π bonds. Many fluorescent stains have been designed for a range of biological molecules. Some are fluorescent tiny compounds that bind a biological molecule. Nucleic acid stains like DAPI and Hoechst, and phalloidin are examples.
2. **A light source:** Four main types of light sources are used, including xenon arc lamps or mercury-vapor lamps with an excitation filter, lasers, and high- power LEDs. Complex fluorescence microscopy techniques require lasers, while wide-field epifluorescence microscopes use xenon lamps, mercury lamps, and LEDs with a dichroic excitation filter.
3. **The excitation filter:** Typically, the exciter is a bandpass filter that transmits only the wavelengths absorbed by the fluorophore, reducing the excitation of other fluorescence sources and blocking excitation light in the fluorescence emission band.
4. **The dichroic mirror:** A dichroic filter or thin-film filter is a very accurate color filter used to selectively pass light of a small range of colors while reflecting other colors.

5. **The emission filter:** The emitter is typically a bandpass filter that passes only the wavelengths emitted by the fluorophore and blocks all undesired light outside this band – especially the excitation light. By blocking unwanted excitation energy (including UV and IR) or sample and system autofluorescence, optical filters ensure the darkest background.

Working Principle of Fluorescence microscope:

Most cellular components are colorless and cannot be clearly distinguished under a microscope. The basic premise of fluorescence microscopy is to stain the components with fluorescent dyes, also known as fluorophores or fluorochromes, which are molecules that absorb excitation light at a given wavelength (generally UV), and after a short delay emit light at a longer wavelength.



Working mechanism of Fluorescence Microscope

To observe the sample through a fluorescence microscope, it should first be labeled with fluorescent dyes/substances known as fluorophores. Higher

energy shorter wavelength lights (UV rays or blue light) generated from xenon arc lamp or mercury vapor arc lamp passes through the excitation filter. The excitation filter allows only the short wavelength of light to pass through and removes all other non-specific wavelengths of light. The filtered light is reflected by the dichroic filter and falls on the fluorophore-labeled sample. The fluorochrome absorbs shorter wavelength rays and emits rays of longer wavelength (lower energy) that pass through the emission filter. The emission filter blocks (suppresses) any residual excitation light and passes the desired longer emission wavelengths to the detector. Thus the microscope forms glowing images of the fluorochrome-labeled microorganisms against a dark background.

To the observer, the background is dark, as there is no visible light and only the labeled specimen (cells, microorganisms, etc.) appear bright (fluoresce).

Types of Fluorescence microscope:

Fluorescence microscopy is one of the most used imaging modalities in molecular biology and living specimens. To increase image contrast and spatial resolution, different type of fluorescence microscopy has been developed. However there are 4 main types of fluorescence microscopy:

a) **Epifluorescence microscopes:** It is the most common type of fluorescence microscope. In this microscope, excitation of the fluorophore and detection of the fluorescence are done through the same light path (i.e., through the objective).
b) **Confocal microscope:** In this type of fluorescence microscope, high-resolution imaging of thick specimens (without physical sectioning) can be analyzed using fluorescent-labeled dye.
c) **Multiphoton microscope:** In this type of microscope, multiphoton fluorescence excitation captures high-resolution three-dimensional images of specimens tagged with highly specific fluorophores.
d) **Total internal reflection fluorescence (TIRF) microscope :**Total internal reflection fluorescence microscopy (TIRFM) exploits the unique properties of an induced evanescent wave or field in a limited specimen

region immediately adjacent to the interface between two media having different refractive indices.

Applications of Fluorescence Microscope:

1. Fluorescence microscopy is widely used in diagnostic microbiology and microbial ecology for enumerating bacteria in natural environments. Some organisms, such as Pseudomonas, fluoresce naturally when irradiated with ultraviolet light. Other organisms, such as *Mycobacterium tuberculosis* and *Treponema pallidum*, are treated with fluorochrome. Acid-fast bacilli (AFB) in sputum or CSF are detected when stained with auramine fluorescent dye. Detection of Trichomonas vaginalis, intracellular gonococci, and other parasites when stained by acridine orange. In immunodiagnosis of infectious diseases, using both direct and indirect antibody techniques. Immunofluorescence is especially useful in diagnosing syphilis and rabies.
2. It is used to identify structures in fixed and live biological samples.
3. Fluorescence microscopy is a common tool for today's life science research because it allows the use of multicolor staining, labeling of structures within cells, and the measurement of the physiological state of a cell.
4. It is used to imaging structural components of small specimens, such as cells
5. It is used to conducting viability studies on cell populations (are they alive or dead?)
6. It is used to imaging the genetic material within a cell (DNA and RNA)
7. It is used to viewing specific cells within a larger population with techniques such as FISH

Advantages of Fluorescence Microscope:

1. Fluorescence microscopy is the most popular method for studying the dynamic behavior exhibited in live-cell imaging.
2. This stems from its ability to isolate individual proteins with a high degree of specificity amidst non-fluorescing material.

3. The sensitivity is high enough to detect as few as 50 molecules per cubic micrometer.
4. Different molecules can now be stained with different colors, allowing multiple types of the molecule to be tracked simultaneously.
5. These factors combine to give fluorescence microscopy a clear advantage over other optical imaging techniques, for both in vitro and in vivo imaging.

Limitations of Fluorescence Microscope:

Fluorophores gradually lose their ability to fluoresce as they are illuminated in photobleaching. Photobleaching can severely limit the time a sample can be observed by fluorescence microscopy. However, several techniques exist to reduce photobleaching, such as using more robust fluorophores, minimizing illumination, or using photoreactive scavenger chemicals.

Fluorescence microscopy has enabled the analysis of live cells, but fluorescent molecules generate reactive chemical species under illumination that enhances the phototoxic effect, to which live cells are susceptible.

Fluorescence microscopy only allows observation of the specific structures labeled for fluorescence. For example, observing a tissue sample prepared with a fluorescent DNA stain by fluorescence microscopy only reveals the organization of the DNA within the cells and reveals nothing else about the cell morphologies.