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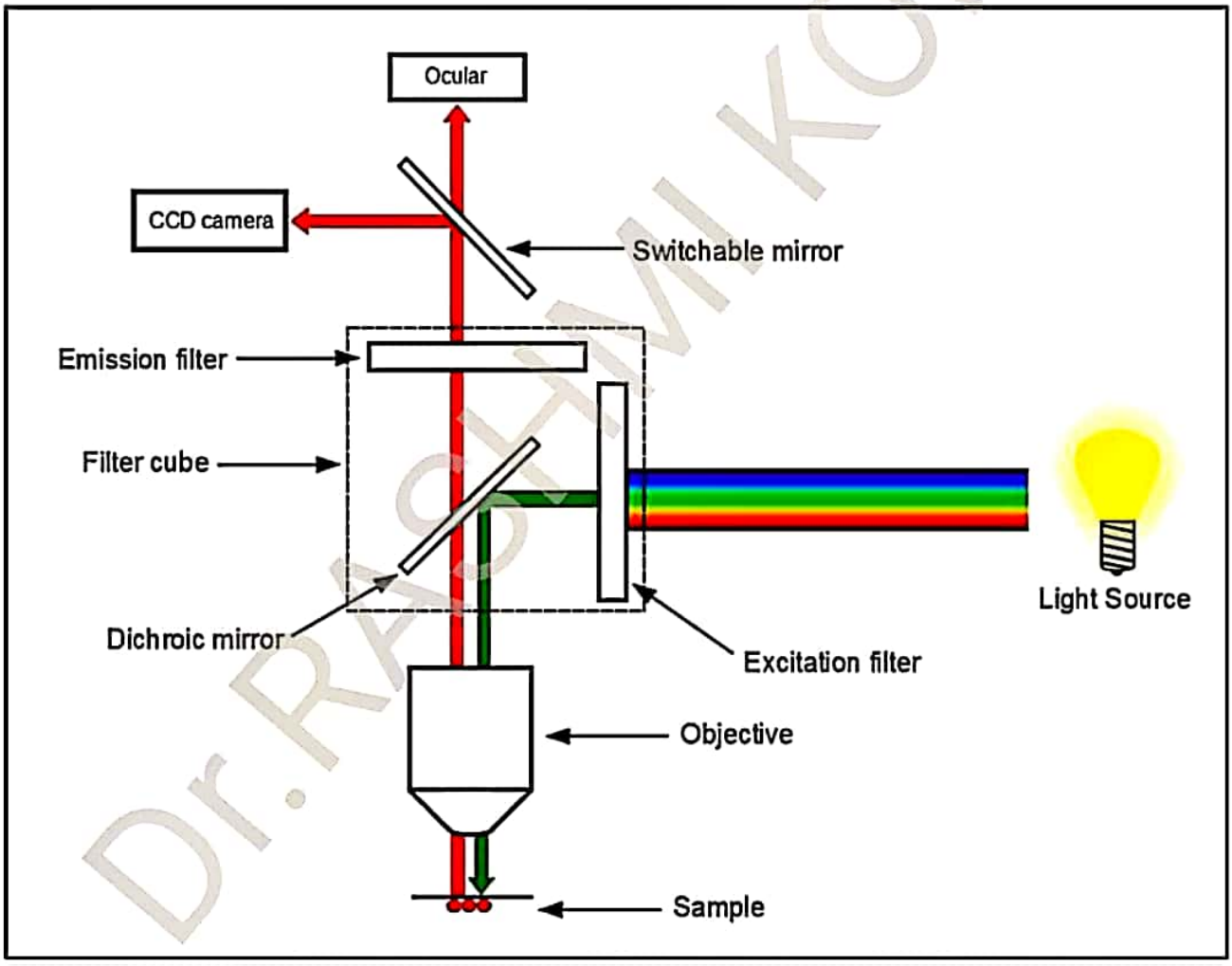
Fluorescence Microscope

A fluorescence microscope is an optical microscope that uses fluorescence instead of, or in addition to, scattering, reflection, and attenuation or absorption, to study the properties of organic or inorganic substances



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Principle :

1. 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 dyes.
2. Fluorescent dyes, also known as fluorophores or fluorochromes, are molecules that absorb excitation light at a given wavelength (generally UV), and after a short delay emit light at a longer wavelength. The delay between absorption and emission is negligible, generally on the order of nanoseconds.
3. The emission light can then be filtered from the excitation light to reveal the location of the fluorophores.

- Fluorescence microscopy uses a much higher intensity light to illuminate the sample. This light excites fluorescence species in the sample, which then emit light of a longer wavelength.
- The image produced is based on the second light source or the emission wavelength of the fluorescent species rather than from the light originally used to illuminate, and excite, the sample.

Working :

In most cases the sample of interest is labeled with a fluorescent substance known as a fluorophore and then illuminated through the lens with the higher energy source. The illumination light is absorbed by the fluorophores (now attached to the sample) and causes them to emit a longer lower energy wavelength light. This fluorescent light can be separated from the surrounding radiation with filters designed for that specific wavelength allowing the viewer to see only that which is fluorescing.

The basic task of the fluorescence microscope is to let excitation of light radiate the specimen and then sort out the much weaker emitted light from the image. First, the microscope has a filter that only lets through radiation with the specific wavelength that matches your fluorescing material. The radiation collides with the atoms in your specimen and electrons are excited to a higher energy level. When they relax to a lower level, they emit light. To become detectable (visible to the human eye)

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the fluorescence emitted from the sample is separated from the much brighter excitation light in a second filter. This works because the emitted light is of lower energy and has a longer wavelength than the light that is used for illumination.

In short, light of the excitation wavelength is focused on the specimen through the objective lens. The fluorescence emitted by the specimen is focused on the detector by the objective. Since most of the excitation light is transmitted through the specimen, only reflected excitatory light reaches the objective together with the emitted light.

Most of the fluorescence microscopes used in biology today are epi-fluorescence microscopes, meaning that both the excitation and the observation of the fluorescence occur above the sample. Most use a Xenon or Mercury arc-discharge lamp for the more intense light source.

Parts :

Typical components of a fluorescence microscope are:

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 of these are small molecules that are intrinsically fluorescent and bind a biological molecule of interest. Major examples of these are nucleic acid stains like DAPI and Hoechst, phalloidin which is used to stain actin fibers in mammalian cells.

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.

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- Lasers are mostly used for complex fluorescence microscopy techniques, while xenon lamps, and mercury lamps, and LEDs with a dichroic excitation filter are commonly used for wide-field epifluorescence microscopes.

3.The excitation filter

- The exciter is typically a bandpass filter that passes only the wavelengths absorbed by the fluorophore, thus minimizing the excitation of other sources of fluorescence 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.

Applications :

The refinement of epi-fluorescent microscopes and advent of more powerful focused light sources, such as lasers, has led to more technically advanced scopes such as the confocal laser scanning microscopes and total internal reflection fluorescence microscopes (TIRF).

These are invaluable tools for producing high resolution 3-D images of subsurfaces in specimens such as microbes.

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These microscopes are often used for -

- Imaging structural components of small specimens, such as cells
- Conducting viability studies on cell populations
- Imaging the genetic material within a cell (DNA and RNA)
- Viewing specific cells within a larger population with techniques such as FISH
- To identify structures in fixed and live biological samples. 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.

Strengths :

- They are able to produce sharp images of thick samples at various depths by taking images point by point and reconstructing them with a computer rather than viewing whole images through an eyepiece.
- Fluorescence microscopy is the most popular method for studying the dynamic behaviour exhibited in live-cell imaging.
- This stems from its ability to isolate individual proteins with a high degree of specificity amidst non-fluorescing material.
- The sensitivity is high enough to detect as few as 50 molecules per cubic micrometre.
- Different molecules can now be stained with different colors, allowing multiple types of the molecule to be tracked simultaneously.
- These factors combine to give fluorescence microscopy a clear advantage over other optical imaging techniques, for both in vitro and in vivo imaging.

Limitations :

- Fluorophores lose their ability to fluoresce as they are illuminated in a process called photobleaching. Photobleaching occurs as the fluorescent molecules accumulate chemical damage from the electrons excited during fluorescence.

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- Cells are susceptible to phototoxicity, particularly with short-wavelength light. Furthermore, fluorescent molecules have a tendency to generate reactive chemical species when under illumination which enhances the phototoxic effect.
- Unlike transmitted and reflected light microscopy techniques fluorescence microscopy only allows observation of the specific structures which have been labeled for fluorescence.

FIG : Fluorescence Microscope

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Reference :

- microscopemaster.com
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- wlkipedia.com

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