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Fluorescence microscopy—A literature review

Zaid T. ALdabbagh

Laser and Photonics Center ,Department of Laser and Spectroscopy , Hamdaniya University, Nineveh, Iraq
Email: zaidaldabbagh@uohamdaniya.edu.iq

Abstract: One important technique for keeping an eye on cell physiology is fluorescence microscopy. While the principles of fluorescence and its optical separation through filters are still the same, the design of microscopes varies in order to improve spatial resolution and image contrast. The choices, benefits, and proper use of laser scanning confocal microscopy, two-photon microscopy, scanning disk confocal microscopy, total internal reflection, and super resolution microscopy are highlighted in this overview of the fundamentals of wide-field microscopy. Additionally, the fundamentals of how these microscopes create images are examined in order to understand their potential, limits, and limitations. Fluorescence microscopy's versatility, specificity, and high sensitivity make it an effective and distinctive method for studying both living and fixed cells. Labeled molecules in biological samples can emit fluorescence, which fluorescence microscopes can detect as pictures or photometric data from which emission spectra and intensities can be inferred. Numerous methods have been created to visualize and analyze complicated dynamic events in cells, organelles, and sub-organelle components inside the biological material by taking advantage of fluorescence's properties.

Keywords—fluorescence microscopy;Multiphoton microscope;Fluorescence Resonance Energy (FRET)

1. INTRODUCTION

Several fluorescence techniques, including fluorochrome-based enzymes, are essential to experimentalimmunology.assays,fluorescencemicroscopy,and flowcytometry.Theprimarybeft of fluorescence instruments is their intrinsically higher sensitivity and range as compared to techniques that use chemiluminescent emission or variations in optical density; the latter approach releases one photon per molecule, whereas a single fluorochrome generates hundreds to thousands of photons. Over the last two decades, flow cytometer has made significant contributions to the advancement of immunology in a variety of fields, including hybridism selection and cell population characterization (show figure1). While fluorescence microscopy, a complementary technique, provides us with a wealth of information about a relatively small number of cells, flow cytometry only provides us with a small amount of information on a large number of cells. However, flow cytometer cannot be used in in vivo research and provides little information about the spatial positions or dynamics of constituent molecules. Fluorescence microscopy has become increasingly significant in immunology for these and other reasons. Significant advancements in the last two decades, including confocal and two-photon fluorescence microscopy, have made it possible to analyze the structure of immune cells and tissues. Fluorescence microscopy's influence is about to explode. Among the recent or ongoing achievements made possible by fluorescence microscopy are:

- Single molecule investigations of individual proteins,
- fluctuation correlation spectroscopy and fluorescence resonance energy transfer to study molecular connections in living cells,
- Stimulated emission depletion microscopy, 4Pi imaging, and structured illumination provide significantly higher resolution;
- In vivo trafficking of immune cells or targets using fluorescent labels;
- Total internal reflection microscopy to investigate single channel opening events; and 6-Quantum dots for single molecule studies in living cells.

Figure 1: Fluorescence microscopy

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2 FLUORESCENCE MICROSCOPES

GIVEN THE WIDE VARIETY OF FLUORESCENCE MICROSCOPES AVAILABLE, IT IS HELPFUL TO LIST SOME OF THEIR FUNDAMENTAL CHARACTERISTICS. I'LL START BY THINKING ABOUTHOWIN THE TYPE OF FLUORESCENCE MICROSCOPE IS GENERALLY DETERMINED BY THE EXCITATION LIGHT THAT IS APPLIED TO THE MATERIAL (FIG. 1).[1]

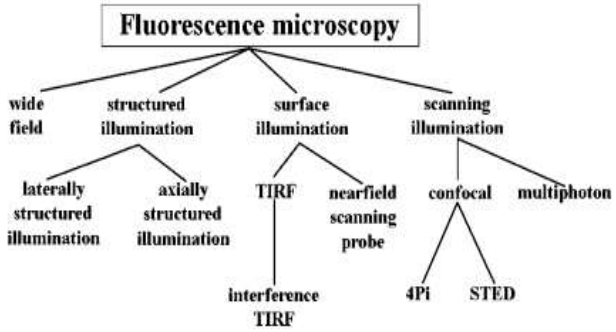

FIG1:FOUR PRIMARY GROUPS OF FLUORESCENCE MICROSCOPY: SCANNING ILLUMINATION, SURFACE ILLUMINATION, WIDE FIELD, AND STRUCTURED ILLUMINATION[1].

Structured and Wide-Field Lighting. Readers should be familiar with conventional wide-field fluorescence microscopes. A compound microscope with an objective lens and eyepieces is used for conventional fluorescence microscopy. Wide-field imaging allows the entire sample to be seen through the eyes after being lit by excitation light. The fluorescence micrograph resolution produced by traditional fluorescence microscopy.

3 Principle of Operation

When one thinks about the equipment needed to detect fluorescence in a microscope, the comparison between a firefly and a fluorescent object that was helpful in explaining fundamental concepts comes to an end. A firefly uses ATP energy to produce light on its own through a biochemical process known as bioluminescence, which does not require light to commence. In contrast, a microscopic object's fluorescence must be triggered by incident light with a shorter wavelength. Therefore, it is necessary to build a fluorescence microscope in a way that enables fluorescence to be excited, the comparatively weak emission to be separated from the intense exciting light, and then the fluorescence to be detected. To achieve great image contrast, the exciting and fluorescence light must be effectively separated before they can reach the observer's sight or the electronic detector. Figure 3.4 shows a schematic of a typical widefield fluorescence microscope.

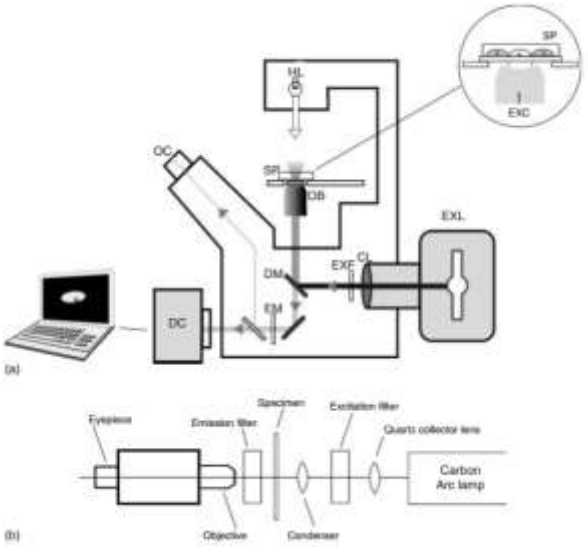

Figure 3.4 Fluorescence microscope – principle of operation. (a) A schematic diagram of an inverted fluorescence microscope (b) A schematic diagram of an early fluorescence microscope, with dia-illumination.[2]

Under a microscope, the majority of cellular components are colorless and indistinguishable. Fluorescence dyes, sometimes referred to as fluorophores or fluorochromes, are chemicals that absorb excitation light at a specific wavelength (usually UV) and then release light at a longer wavelength after a brief delay. This is the fundamental idea behind fluorescence microscopy.

4 *Applications of fluorescence microscopy*

The specimen is exposed to the exciting light through a fluorescence microscope, which also produces a high contrast image and separates the fluorescence light from the exciting light. It makes it simple for users to view minuscule cells and their contents in incredibly detailed microscopic specimens. Features in a specimen that are invisible to ordinary bright-light microscopy are frequently revealed by fluorescence microscopy, particularly interior structures. Fluorochromes can be used to safely analyze living tissue and microorganisms since secondary fluorescence requires incredibly low fluorochrome concentrations (1:10,000 or greater). Table 2.1 lists the applications of fluorescence microscopy.[3]

Table 2.1 Fluorescence microscopy applications[3]

Infectious disease diagnosis	***Industrial process control***
Agent detection and identification	**Monitoring of clean rooms**
Antibody titration	**Detection of fluid contamination**
Toxin detection	**Sampling of food and beverages**
Autoimmune disease diagnosis	*Disease diagnosis*
Antibody detection and identification	Etiological agent detection and identification Antibody titration
Antibody titration	
Environment monitoring	*Environment microorganism monitoring*
Microbial aerosol monitoring Water analysis	Air testing (aerosols) Water analysis (bacteriological safety)
Limnology	Sewage treatment control
Emergency support	*Biological attack defence*
Casualty diagnosis	Rapid diagnosis of casualties
Microorganism aerosol detection and identification	Detection of biological aerosols Sampling of suspicious munitions
Biological research	*Industrial process control*
Location and identification of cells Identification of tissues	Monitoring of clean rooms (electronics) Detection of fluid contamination (electronics, pharmaceuticals)
Detection of nucleic acids	Detection of product contamination (food, beverages)

5 Equipment for fluorescence microscopy

Fluorescence microscopy requires the following tools: a compound microscope (basic platform and lenses); a high-power illuminator (excitation light);

- Exciter light filter, which chooses light hues
- dichroic mirror (which only emits epifluorescence and reflects or transmits specific light colors)
- barrier filter, which prevents light with low wavelengths.

The platform that unifies all the other components and offers specimen picture magnification and resolution is a compound microscope. Bright light with the proper wavelength (color) is supplied by a light source to induce fluorescence. Only the light wavelength (color) required to trigger the fluorescence of the employed fluorochrome is transmitted by an excitation (primary) filter. It filters out unnecessary wavelengths from the light source.
While a dichroic mirror does not reflect the higher wavelength fluorescence light, it does reflect the exciting light color. The higher wavelength fluorescence light is passed via a barrier (secondary) filter, which also prevents any interesting light that might get past the other filters. Figure 3.1 shows the above-described schema.[3]

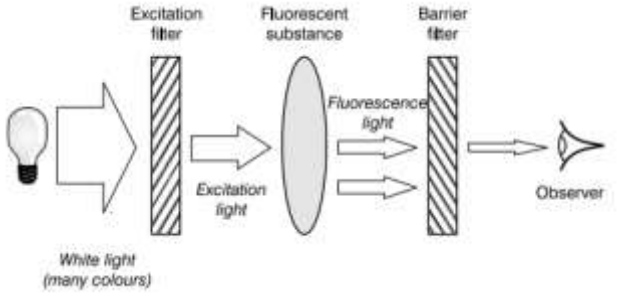

Figure 3.1 **Schematic diagram of a fluorescence microscopy system**[3]

6 Types of Fluorescence microscope:

One of the most popular imaging techniques for living things and molecular biology is fluorescence microscopy. In order to improve spatial resolution and picture contrast, various forms of fluorescence microscopy have been created. Nonetheless, fluorescence microscopy comes in four primary varieties:
a) The most popular kind of fluorescence microscopy is the epifluorescence microscope. This microscope uses the same light path—that is, the objective—for both fluorophore excitation and fluorescence detection.
b) Confocal microscope: This kind of fluorescence microscope uses fluorescent-labeled dye to evaluate high-resolution imaging of thick materials without physical sectioning.
c) Multiphoton microscope: This kind of microscope takes high-resolution, three dimensional pictures of objects that have been tagged with extremely specific fluorophores using multiphotonfluorescenceexcitation.
d) The total internal reflection fluorescence (TIRF) microscope: TIRFM takes advantage of the special characteristics of an induced evanescent wave or field in a small specimen area that is right next to the interface between two media with different refractive indices. [4]

7 Fluorescence Resonance Energy

FRET, or nonradiative fluorescence resonance energy transfer, is a significant illustration of a nonradiative decay mechanism. One can measure distances of the order of 1–5 nm using the "spectroscopic rule" that FRET gives (Fo¨rster, 1948; Stryer and Haugland, 1967). Figs. 6 show the FRET concept in action. The donor-acceptor pair, which consists of two fluorophores, is used in FRET-based systems. The fluorescence excitation and emission spectra of a well-matched donor-acceptor pair are displayed in Figure 6.[5]

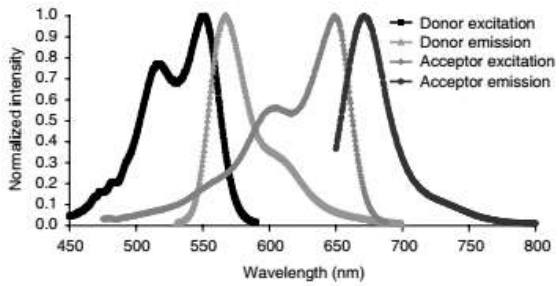

Fig. 6 Spectral overlap between donor emission and acceptor excitation spectra in fluorescence resonance energy transfer.[5]

8 Conclusion

The fact that the majority of life sciences discoveries over the last 50 years or more have been based on imaging biological and pathological processes highlights the importance of imaging-based approaches. Not to mention, the sophisticated fluorescence microscopic methods discussed in this review all significantly influenced (bio)medicine and cell biology research. A wide variety of fluorescence-based research tools have been made possible by complementary evolutionary growth in fluorescence microscopy and fluorochrome development, from newly synthesized dyes to tailored FPs and nanoparticles, as well as ongoing technological exchange and traffic between these. Specifically, the FP-color palette's notable extension beyond the traditional green fluore

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