

## Science Note

## Basic Science

# Basic Principles of Fluorescence Microscopy

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## ABSTRACT [ENGLISH/ANGLAIS]

Fluorescence microscopy is a basic requirement in cell biology, molecular biology and biotechnology. Advancements over the years has helped scientist to trace molecules in live cells and understand the basis of cell metabolism, exchange, mutation and toxicity. In this short communication we seek to explain in simple terms the basic principles of how a fluorescence microscope works. The principles of excitation and emission focuses on the ability of fluorophores to absorb energy from photons and to emit such absorbed energy. The difference between the chemical structures of these fluorephores determines how much energy that is required to excite them and how long a fluorescence signal from a fluorophore will last. The principles of epi-illumination on the other hand describe the arrangement and function of the various components of a fluorescence microscope.

**Keywords:** Microscopy, fluorescence, light

## RÉSUMÉ [FRANÇAIS/FRENCH]

Microscopie Fluorescence est une exigence fondamentale en biologie cellulaire, moléculaire et biologie biotechnologique. Les progrès au fil des ans a permis de retracer scientifique molécules dans des cellules vivantes et de comprendre la base du métabolisme cellulaire, l'échange, la mutation et la toxicité. Dans cette communication, bref, nous cherchons à expliquer en termes simples les principes de base de la façon dont un microscope à fluorescence fonctionne. Les principes d'excitation et d'émission met l'accent sur la capacité de fluorophores pour absorber l'énergie des photons et pour émettre une énergie absorbée. La différence entre les structures chimiques de ces fluorephores détermine combien d'énergie qui est nécessaire pour les exciter et combien de temps un signal de fluorescence à partir d'un fluorophore va durer. Les principes de l'épi-illumination, d'autre part décrit le arnangement et la fonction des différentes composantes d'un microscope à fluorescence.

**Mots-clés:** Microscopie, fluorescence, la lumière

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## INTRODUCTION

For a substance to be detected by the fluorescence microscope it has to fluoresce, that is should be capable of absorbing energy quanta from photons and loose the energy by emission or fluorescence. The process of fluorescence occurs in nanoseconds (10 E-9 secs). It is the light of long wavelength seen after the one of short wavelength has passed. Fluorescence can be defined as filtering the short wavelength light in order to visualize the long wavelength light [1].

Stoke's shift describes the difference between the excitation wavelength and emission wavelength. It is a very important physical factor in fluorescence microscopy as it determines the strength of fluorescence

for fluorophores [1, 2]. It determines the wavelength of the light required to excite a fluorophore, the duration of fluorescence and the intensity of fluorescence signal that can be obtained from a fluorophore. This is to be considered in the selection of a fluorescent dye and the dichroic mirror of appropriate wavelength

## WHAT ARE FLUOROPHORES?

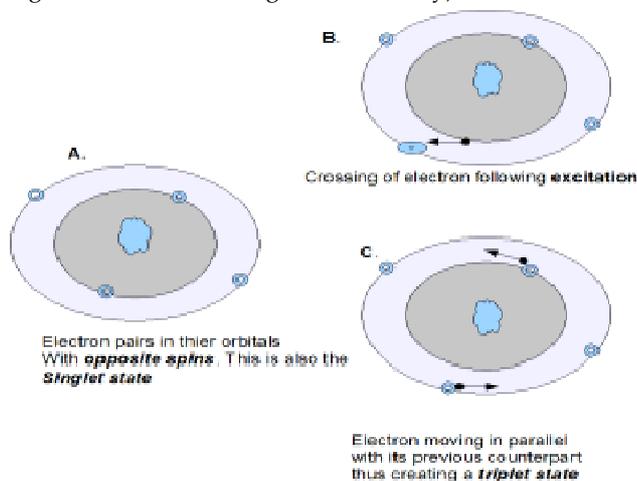
Substance used in microscopy by virtue of its ability to fluoresce are called "fluorophores". Such substances are capable of being excited. This is so because they can absorb energy from photons. The outermost orbital of this substances are important as it determines the fluorescence properties, the excitation wavelength and

emission wavelength. After excitation has passed, the molecule is capable of losing the absorbed energy by emission of fluorescence and return to the ground state by a mechanism called vibrational relaxation through which the electrons returns back to the outermost orbital. The orbitals close to the nucleus has great electrical and low vibrational energy while the outermost orbitals has high vibrational but low electrical energy. Thus, the movement of electrons from the inner orbital to the outermost orbital involves energy conversion from electrical energy to vibrational force.

### Jablinski's Diagram

To properly explain the principles of fluorescent microscopy, we must give an account of the Jablinski's diagram (principles illustrated in figure 1), introduced by Alexander Jablinski in the early 1930's. This describes the ground state ( $S_0$ ), and an excited state  $S_1$  and  $S_2$  which represents the singlet state ( $S_2$  is of higher energy than  $S_1$ ).  $T_1$  represents the triplet state in which electrons move from its orbital to another orbital farther away from the nucleus and moves in the same direction as its previous counterpart. This is called the forbidden transition and when it happens it causes phosphorescence, a phenomenon that accounts for inaccurate pixel calculation in confocal laser scanning [1, 3].

**Figure 1:** This figure shows basics of excitation and emission showing electrons in the singlet state with opposite spins (A), excitation involving movement of electro to an orbital of higher energy farther away from the nucleus (B) and movement of electrons in the same direction resulting in a triplet state (C). (Image Courtesy: Ogundele Olalekan, Bingham University)



### Autofluorescence

Certain naturally occurring fluorescent substances like green fluorescent protein (GFP) and other organic compounds are used in fluorescence microscopy [3]. These substances possess a conjugated covalent bond; usually in the form of a benzene ring (aromatic compounds). In addition to the conjugated covalent bond, they also have a pi-bond. These substances are useful because they are composed of small inter orbital distances, therefore will require small amount of energy to excite them and also it is easier for such substances to return to the ground state after emission. Previous studies have shown that the more the double bond, the longer the wavelength and the better the emission properties [4]. Other forms of fluorescent substances are those capable of being localized in specific cells or organelles and are called physiological fluorophores. The quantum yield from a fluorophore is the fluorescent signal obtainable; we can define it as the ration of fluorescent emission to non-radiative internal energy [3].

### THE SPECTRUM OF EXCITATION

When fluorophores comes in contact with fast moving photons, all the energy in the photons are transferred to the fluorophores. This can be expressed mathematically as  $E=h \times c/\lambda$  [1, 5]. Where  $h$  is the Planck's constant,  $c$  represents the speed of light in vacuum and  $\lambda$  is the wavelength of light in vacuum. When fluorophores absorb energy from photons, electrons move from orbitals close to the nucleus to orbitals far away from the nucleus (i.e. outer orbitals). If the incident photons possess more than enough energy, the fluorophores will be excited form  $S_0$  to  $S_1$  and if the energy is even higher, it will move from  $S_1$  to  $S_2$ . This implies that a required amount of energy or light of specific wavelength is required to excite a fluorophores [2, 6]. The process happens in femtoseconds. Excitation could happen if a single photon hits a fluorophores or in another instance specific number of photons summing up to the required energy to excite a fluorophore comes in contact with the fluorophore. The probability that a photon will come in contact with and excite a fluorophores in a medium is called the molar extinction coefficient ( $\epsilon$ ); expressed as  $M^{-1} cm^{-1}$ . In describing the extinction coefficient we must think of the fluorophore as the target. A wider medium increases the probability. The effective range of  $\epsilon$  for most fluorophores is between approximately 20,000-approximately 200,000. The enhanced green fluorescent

protein has a molar extinction coefficient ( $\epsilon$ ) of 60,000 [4, 5].

### THE SPECTRUM OF EMISSION

After excitation has occurred, fluorophores tend to lose the energy by emission and vibrational energy. For this to occur, the electron transferred to an orbital of higher energy (close to the nucleus) will return back to its own energy level which is always a lower energy orbital (outer orbital). This phenomenon is called "internal conversion". However, internal conversion will not involve an actual energy loss but represents a transition from an orbital of high electrical and low vibrational force to an orbital of low electrical and high vibrational force [3, 5]. At this state vibrational relaxation helps in dissipating the energy into the surrounding medium (water molecules if the containing medium is water) or other fluorophores in the form of FRET (fluorescent resonance energy transfer) [6, 7]. For most fluorophores, during the course of emission, there is a prolonged stay at S1 (first excited state) after energy loss from the highest excited state (S2). The reason being that the energy gap between S0 and S1 is wider than that between S1 and S2. The most effective fluorophores are those that can eject the photons accounting for the energy gap between S0 and S1 such that it molecules can return to S0 within a shorter duration. Since the energy required to excite a fluorophore versus the one required for it to emit is of wide difference; such a difference is called the Stoke's shift [1]. The value of the Stoke's shift (nm) accounts for the basic properties of the fluorophore (excitation time, intensity of fluorescence signal, emission time and the degree of bleaching [4, 3, 7].

### OTHER DEFINITIONS

#### Intersystem Crossing

Intersystem crossing can occur when a fluorophore stays for an extended time at S1 excitation state. If there is adequate vibrational force, a fluorophore can jump from the singlet state to a triplet [4].

#### Singlet State

It is the resting state of a fluorophore where the two electrons that forms a pair in the orbital have opposite spins. Electron pair will have  $+1/2$  and  $-1/2$ .

#### Spin

It is the angular momentum of an elementary body in its orbit. The  $+1/2$  and  $-1/2$  in the electron pair gives the energy and direction through which electrons are constantly moving in their orbit.

### Triplet State

When the electron pair in an orbital is excited, one of them can move to an orbital farther away from the nucleus, there are three possibilities, it can move parallel, anti-parallel and perpendicular thus, a triplet state. For this to occur an electron must move into another orbital of higher energy level and spin in an opposite direction in what is called the "forbidden transition". At this point the fluorophore is believed to have crossed from the singlet state of having an electron pair in a single orbit and with opposite spin ( $-$  and  $+1/2$ ) to having the electron pair in different orbits and in the same direction or possessing the same spin ( $-$  and  $-1/2$ ) or ( $+$  and  $+1/2$ ). Transition from the singlet state to the triplet state is called intersystem crossing. Casting our mind back to the Jablinski's diagram, it is almost impossible for an excited fluorophore in the triplet state (T1) to return to the normal ground state of S0, thus it returns to a triplet ground state called T0. Most fluorophore in T1 will normally return to T0 without emission but other fluorophore do give a background fluorescence effect called phosphorescence [1].

### PRINCIPLES OF EPI-ILLUMINATION

In ideal terms, epi-illumination in fluorescence microscopy involves using the objectives as the condenser. This helps in reducing light scattering and obtaining point illumination on the specimen; thus reducing the noise in the background. This arrangement will help obtain an increased signal to noise ratio (SNR) and a clearer image. To achieve this, the set up requires the help of a "dichroic mirror", that is being used as a set of prism at angle  $45^\circ$ . It has both reflective and transmission properties. The dichroic mirror reflect the excitation light to the specimen and separates the reflected light based on their wavelength. The dichroic mirror is often used with two sets of filters [1, 3]

1. Excitation filter that helps select the excitation wavelength of incident light and
2. Barrier filter that helps remove the light of short wavelength

When the illumination source (mercury or xenon lamp) is incident on the mirror, the illumination beam is reflected to the specimen and the light is being concentrated by the objectives in the form of a cone. As the light is reflected from the specimen, it is incident on the mirror which transmits the light of long wavelength in the form of fluorescence signal to the ocular region or image plane

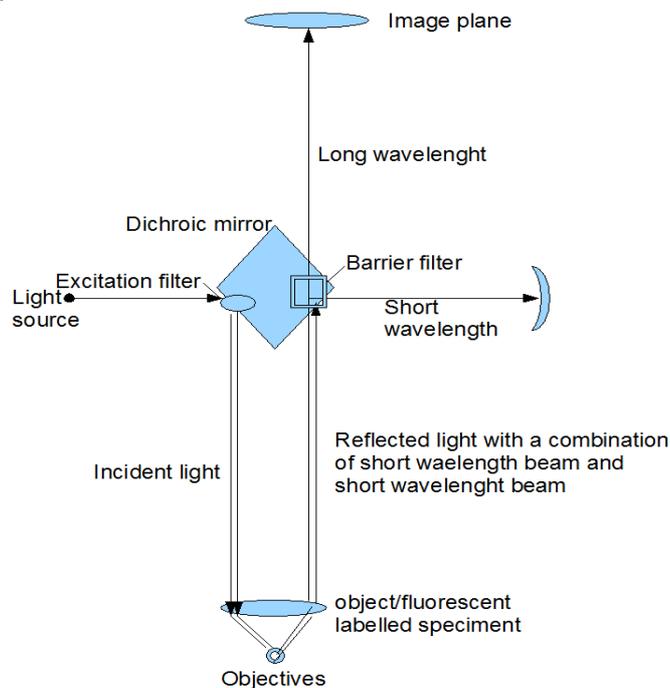
and the barrier filter will reflect the light of short wavelength to the arc lamp on the rear side of the microscope.

The microscope consists of an assembly of set of mirrors in the form of a filter cube, which will usually have 3-9 sides. Each side has its own mirror and set of filters that can be switched automatically using a motor system or manually by using the hand to switch the sides of the mirror [3]. To obtain image from a slide being stained with more than one fluorescent dye (e.g. DAPI and Cy3); the colour range for DAPI is purple and the colour range for Cy3 is red, thus we need to switch the cube to get the

excitation for Cy3 and capture the image, then the excitation wavelength for DAPI and capture the image. Softwares like Image J from the NIH (National Institute of Health) or other software from the microscope manufacturer can be used to super impose the two set of images to get a single merged image showing both DAPI and Cy3 stained regions.

In some modernized forms of the microscopes, special filter systems are used that can automatically switch the mirrors and the filters such that the two fluorophore can be captured on the same image [5, 7]

**Figure 2:** This figure shows principle of epi-illumination (schematics); the incident light is being selected by an excitation filter and sent to the object by convergence of the objective, it is being reflected and the short wavelength light is filtered out by the barrier filter while the long wavelength light is transmitted to the image plane (image Courtesy Ogundele Olalekan, Bingham University)



**CONCLUSION**

The principles of epi-illumination show the efficiency of the modern microscopes over the routine transmission fluorescence microscopes. In this set up (that is epi-illumination), the signal is improved and the noise is reduced. To obtain good images the basic principles must be taken into consideration; this will range from choice of fluorescent dye to the selection of the filters to be used in exciting and separating the emitted rays of light.

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**CONFLICT OF INTEREST**

No conflict of interests was declared by authors.

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