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Fluorescence, In Microscopy and Imaging

By P. N. Dikedi
King's College, Strand, London , United Kingdom

Abstract - This is a swift report on fluorescence and its application as a tool in imaging and microscopy; to properly comprehend this subject, with the aid of the Jablonski diagram, brief descriptions and discussions are given on fluorescence; rhodamine 6G homo-FRET reaction is considered from which both the absorption and emission spectra are drawn on a graph. Additionally, the wealth of applications of fluorescence imaging techniques such as Fluorescence Lifetime Imaging Microscopy (FLIM) and Fluorescence Polarisation and Anisotropy techniques are considered. Applications of molecular imaging techniques in cell biology and medical science are mentioned.

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Fluorescence, In Microscopy and Imaging

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I. Introduction

The aim of this report is to discuss the principle of fluorescence, and to unravel its inevitable use as a tool in imaging and microscopy. A useful feature of fluorescence is high sensitivity detection; which is its key advantage as an imaging tool. It is well reported that the fluorescence spectra from photo sensitizers can act as image guiding tool in differentiating malignant tissues from normal ones; additionally fluorescent signatures could be used to differentiate between malignant and benign diseases [1]. In section 2, the phenomenon of fluorescence will be discussed, in section 3, a brief explanation of the Jablonski diagram, will be given; thereafter in section 4, we shall probe into fluorescence imaging and microscopy, discussing its valuable applications in cell biology and medical science. Conclusions will be drawn in section 5.

II. Fluorescence

In initiating brief discussions on fluorescence, it does a lot good to start by defining what Luminescence is; it is the emission of light from any substance and it occurs from an electronically excited state. Luminescence is divided into two parts: fluorescence and phosphorescence.

One can now proceed to briefly discuss Fluorescence as a type of luminescence—which is short lived—created by electromagnetic excitation, involving absorption of light by a fluorophore—a fluorescing functional group—at a higher energy or shorter wavelength, followed by emission of light at a lower energy or longer wavelength. This process occurs at a rate called the rate of fluorescence, which is about $10^{8}s^{-1}$. Since fluorescence process involves occurrences of emission and absorption, it is important to note that time elapses between both occurrences, hence the length of time between emission and absorption or the average time between when a fluorophore is excited and when it returns to the ground state is referred to as fluorescence lifetime; this time lies between 10^{-9} and 10^{-8}s and it is a relatively short time indeed—a further discussion on this will be given in section 4.

When emission persists longer after excitation light has been extinguished, then, this phenomenon is referred to as phosphorescence—this process is not the focus for discussion in this paper and so will be left-off. Fluorescence as a tool in imaging and microscopy is well reported by Klaus *et al* (2004)[2]; fluorescence imaging techniques are optical imaging techniques which find their applications in biomedical and biological sciences because they are applicable to live cells and tissue probing in such a manner that the fluorophore environment need not be compromised with biochemical assays.

Fluorophores are molecules or functional groups which are capable of fluorescing; they are used—or selected for use—in terms of their quantum yields. Quantum yield of a fluorophore is the ratio of the number of emitted photons to the number of absorbed photons; this is expressed as:

$$\emptyset = \frac{\tau}{\tau_0} = \frac{k_r}{k_r + k_{nr}}$$

where, τ_0, is the natural or radiative lifetime, k_r and k_{nr}, are the radiative and nonradiative decay rate constants, respectively.

The highest possible quantum yield is unity or 100%, and fluorophores with high quantum yields are well sought after because of their great relevance in the biological sciences and chemistry. Fluorescein and Rhodamine 101 can each attain a quantum yield of 0.92 and 1 respectively. In discussing further on fluorescence, it is important to delve briefly into Fluorescence resonance/Förster energy transfer (FRET) phenomenon; this phenomenon is a radiationless energy transfer between two molecules, as close as < 10 nm. This is illustrated by a fluorescent emission spectra; the occurrence of FRET is illustrated by the overlaps of the emission spectrum of the donor molecule and that of the acceptor molecule.

Author : Bio, Nano and X-ray Photonics, Department of Physics, King's College, Strand, London , United Kingdom .

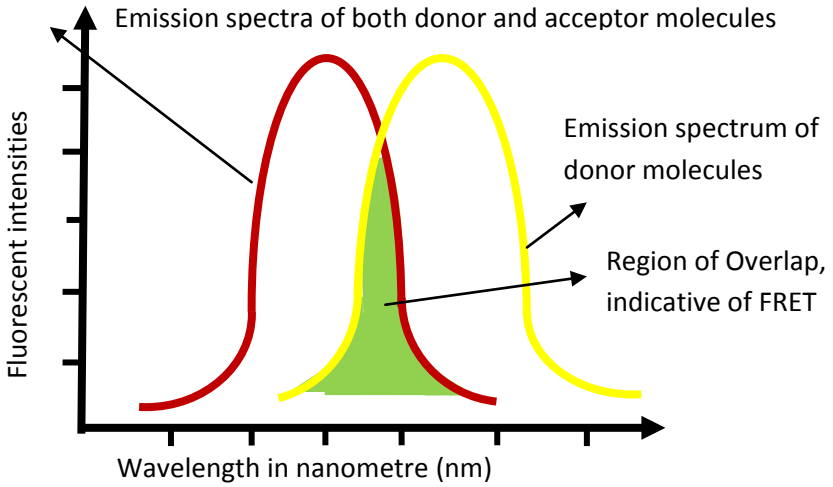

Figure 1 : A schematic of the fluorescent emission spectra with overlaps of the donor and acceptor spectra indicating FRET.

Figure 1 is a schematic of an arbitrary emission and absorption spectra; the green shaded region, an overlap of the spectra, represents spectral overlap integral.

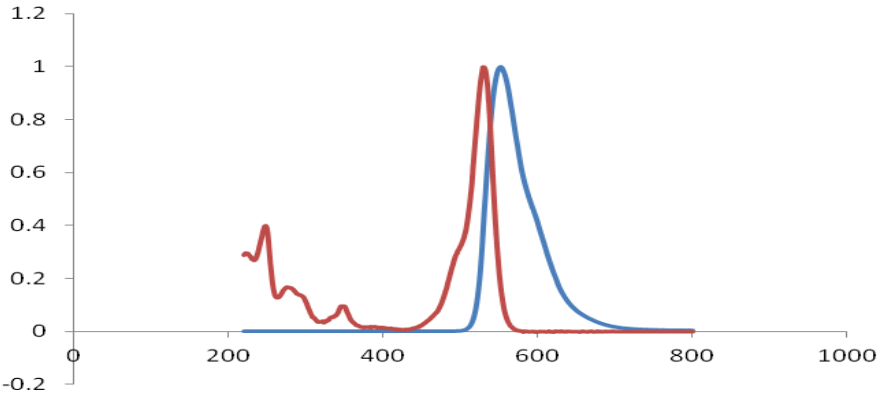

Fig. 2 : Normalized emission-absorption spectra for Rhodamine 6G Homo-FRET reaction

Fluorescent emission spectra are a plot of fluorescence intensities versus wavelength or wave numbers. They are a function of the chemical structure of a fluorophore.

Examples of these spectra are those of Rhodamine 6G homo-FRET.

Figure 2 is a schematic of the absorption and emission spectra for homo-FRET reaction of Rhodamine 6G; wavelength values ranges between $4.90x10^4$nm and $8.00x10^4$nm and corresponding molar extinction values include from $1.314874x10^4$ to $7.392346x10^4$. Below is a very simplified tabulation pertinent to the schematics in figure 2. Below is a selection from the groups of wavelengths, intensities and normalised intensities.

Table 1: shows selected values of wavelengths, intensities and normalised intensities of Rhodamine 6G for both absorption and emission spectra

ABSORPTION			EMISSION		
Wavelengths (nm)	Intensities	Normalised intensities	Wavelengths (nm)	Intensities	Normalised intensities
600	0.00006	4.07039E-05	600	8.38 X10^7	0.406225
595	0.00023	0.000156032	595	9.29 X10^6	0.450169
590	0.00047	0.000318847	590	1.01 X10^7	0.491429

585	-0.00018	-0.00012211	585	1.11 X10^7	0.538
580	0.00201	0.001363581	580	1.23 X10^7	0.594328

III. Jablonski Diagram and Photophysical Processes

The Jablonski diagram, a schematic, describes a series of photo physical occurrences, due to absorption of energy by a photon; these occurrences include: fluorescence, phosphorescence, intersystem crossing—which occurs from a singlet to triplet state—and internal conversion or vibration relaxation, in which energy is lost without emission of light [3].

A few parameters which help in describing the above mentioned processes are worth the mention; one of such is the decay rate constant, k, which describes the probability of each process taking place.

Another is the mean lifetime, τ, which is the time required for a molecule or a set of molecules to decay from one state to another. Both parameters are inversely related to each other and are described by:

$$k = \frac{1}{\tau}$$

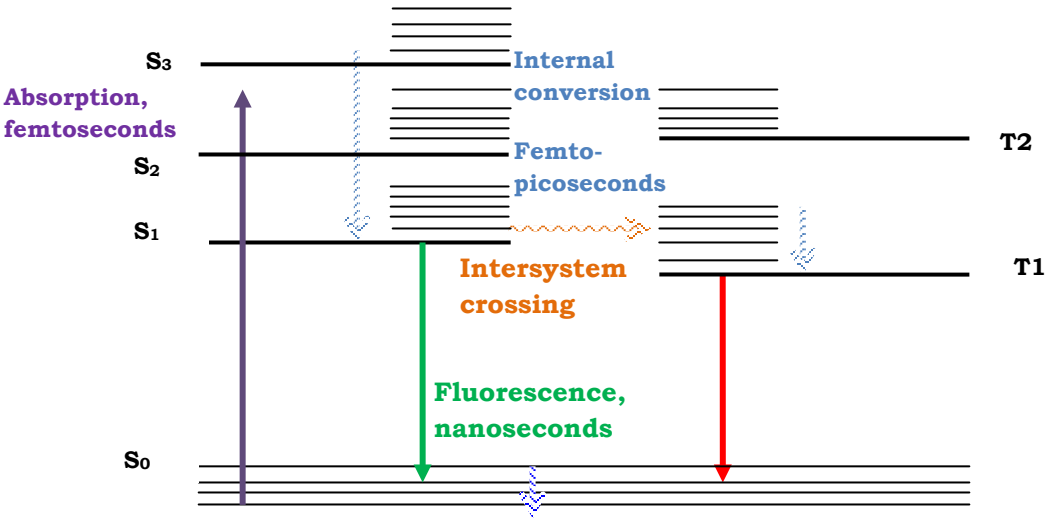

Figure 1 : An adopted Schematic of the Jablonski diagram elaborating photophysical processes [3]

It is also the time for N excited molecules to reduce by a factor, e, so that lifetime for fluorescence and other nonradiative processes also called fluorescence lifetime could be described as the time for a population of excited fluorophores to reduce exponentially to N/e.The lifetimes for fluorescence, phosphorescence and internal conversion processes are in nanoseconds (ns), microseconds (μs) and tens of femtoseconds (fs), respectively.

The expression for lifetime, in section 3, could also be expressed in an extended form—so to speak—as:

$$\tau = \frac{1}{k_r + k_{nr}},$$

Fluorescence decay time depends both on the intrinsic characteristics of the fluorophore and its local environment; local viscosity, PH, or refractive index and molecular interaction all of which affect the fluorescence decay time.

IV. Applications of Fluorescence Imaging and Microscopy

A number of fluorescence imaging techniques and their applications apposite to medical and biological sciences are either in use or underway. A few of the many applications of these techniques are presented in the following subsections:

a) Molecular imaging

This technique is employed in molecular imaging; this imaging aims to detect, localise and monitor molecular processes by the use of sensitive instruments.

Though they find their major use in clinical chemistry and *in vitro* biological analysis by measuring biological response; this technique is useful in assessing the internal environment of fluorescent probes; additionally, molecular interactions can be determined via its application as a biological imaging tool.

Fluorescence molecular probes are being designed to monitor intracellular PH—they employ ratiometric measuring method; tissues which surround tumours and diseased cells generally show abnormal pH from the rest tissues and cells and hence are detected [4]. In cell biology, abnormal PH values could impair the proper functioning of subunits of a cell such as plant vacuoles and endosomes and in medical science, they could lead to abnormal cell growth and division, a situation akin to those observed in disease types such as cancer and Alzheimer; variations in the PH value could also affect the nervous system by distrupting the synaptic transmission. The need for a qualitative measurement of PH arises; this measurement is provided by fluorescent indicators which switch off and on.

Some of the fluorescent sensors used for intra cellular PH measurements include but are not limited to, 2′,7′-Bis-(2-carboxyethyl)-5-(and-6-)-carboxyfluorescein **4** (BCECF),2′,7′-bis-(2-carboxypropyl)-5-(and-6-)-carboxy-fluorescein (BCPCF) , Fluorescein and Fluorescein sulfonic acid [5]

b) Fluorescence polarization and anisotropy

Light made to pass through polarisers, which eventually illuminate fluorophores, produces polarised fluorescence—a type of polarised emission. Polarisation, P, is related to the observed parallel and perpendicular intensities, $I\perp$ and $I\parallel$ respectively by:

$$P = \frac{I\parallel - I\perp}{I\parallel + I\perp}$$

Anisotropy, denoted by r, is another terminology under polarised emission and it is related to $I\perp$ and $I\parallel$ by:

$$r = \frac{I\parallel - I\perp}{I\parallel + 2I\perp}$$

Polarisation and anisotropy are related to each other by the following expression:

$$r = \frac{2}{3}\left(\frac{1}{P} - \frac{1}{3}\right)^{-1}$$

Fluorescence polarisation and anisotropy technique has its great relevance in clinical and biomedical fields. In the pharmaceutical industry, the discovery process of a compound of great therapeutic benefit can be a time consuming and an arduous task. Since the amount of potential molecules which could provide such benefits are overwhelming—up to millions of compounds, hence the need for a swift high-throughput screening technique arises. Fluorescence polarisation and anisotropy technique is being considered as such high-throughput screening technique which has the potential to deliver in terms of both accuracy and speed [6]

This method is strongly reputable in the study of ligand binding—binding of small molecules to proteins. Binding of molecules like acridine, naphthalene dyes, xanthenes to bovine serum albumin (BSA) have been studied.

c) Fluorescence Lifetime Imaging Microscopy (FLIM)

Fluorescence Lifetime Imaging Microscopy (FLIM), a time resolved technique for acquiring images are of two categories; the first, is the confocal scanning or multiphoton excitation technique and the second is the wide-field camera based FLIM. This technique have been employed as a tool for identifying Förster Resonance Energy Transfer (FRET), at the instance of interactions between DNA, RNA, lipids, proteins and enzymes. As cited by Klaus *et al*, steady state anisotropy-a time resolved fluorescence anisotropy technique—has been used for contrasting between Fluorescein and GFP in a cell owing to their dissimilar anisotropies in terms of their molecular sizes. Homo-FRET can be detected by employing this technique. This technique is applicable to cell imaging; it shows image contrast by utilising spatial variations in fluorescence lifetimes in single cells. Unlike time resolved measurements which are sensitive to molecular movements and interactions, molecular information can be visualised in single cells. By employing this technique, the extent of fusion of endosomes in individual cells, have been observed [7].

FLIM system has been designed and built [8]; it is specifically for intraoperative disease diagnosis. By employing a flexible imaging probe—made up of a fibre bundle and gradient index objective lens—and an intensified CCD, tissue auto fluorescence—at 33ns excitation—was imaged. Contrast in fluorescence lifetime was shown between tumour (1.77+/-0.26ns) and normal (2.50+/-0.36ns) tissue. This confirms the potential usefulness of (FLIM) technique for diagnosing intra operative diseases.

V. Conclusions

Fluorescence processes have been mentioned. Applications of FLIM and fluorescent polarisation and anisotropy have been considered as imaging and microscopic tools; additionally, molecular imagings in which fluorescent PH sensors are used for intra cellular PH measurements, in the field of cell biology and medical science have been discussed. The future of fluorescence imaging technique is far from being bleak; its applications are on the rise.

VI. Acknowledgements

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