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INVITED REVIEW

Fluorescence Spectroscopy: A Probe for Molecular Conformation and Dynamics

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Abstract

Fluorescence spectroscopic methods are growing rapidly because of their applications in diverse areas. Methods like steady-state and time-resolved fluorescence are most commonly explored to characterize emissive properties of fluorophores and their locale. Synchronous fluorescence spectroscopy (SFS) plays a significant role in analyzing multicomponent samples over conventional fluorescence spectroscopy. More importantly, among the methods, time-resolved outcomes contain more information about the kinetics of intra- and inter-molecular processes than that is not available from the steady-state. From the technique aspect, fluorescence anisotropy remains a popular tool to study biomolecular dynamics because of its sensitivity, robustness, independence of probe concentration, and simple instrumentation. Moreover, fluorescence anisotropy can be used to study many other biomolecular processes such as drug-protein and protein-protein interaction and protein aggregation. In contrast, the structural dynamics is extracted from fluorescence resonance energy transfer (FRET) data. More sophisticated techniques such as fluorescence correlation spectroscopy (FCS), very versatile and powerful in determining the dynamic behavior at a single molecular level, are based on time-averaging fluctuation analysis of fluorescence intensity generated in a tiny volume and can easily achieve single-molecule sensitivity. In addition to that, Fluorescence microscopes enable the imaging of various fluorescence probes inside the samples and help tremendously in obtaining fundamental insights into the structure and functions of biological systems.

Keywords: Time-resolved fluorescence, Synchronous, Fluorescence microscopy, Anisotropy, FCS, FRET.

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Introduction

Fluorescence typically originates from the emission of a photon from the first electronically excited, singlet state (S_1 in Figure 1) of a molecule after absorption and internal conversion. The fluorescence emission happens at lower energy (longer wavelength) than absorption, and the difference in energy (or maxima between absorption and emission peaks) is called Stokes shift after Sir G. G. Stokes, who observed the phenomenon for the first time. The loss of energy at the excited state of the fluorescent molecule has various reasons. Most importantly, fluorescence or emission is accompanied by a vertical transition, which proceeds extensively from the lowest vibrational level of the excited (S_1) state to higher vibrational levels of the ground state (S_0) as postulated by the Franck-Condon principle. In Figure 1, the violet and blue arrows for absorption are longer than the green and red arrows for emission. In addition, excited state reactions such as solvent relaxation effects, complex formation, excited-state reactions, and/or energy transfer can contribute to the Stokes shift of a fluorescent molecule. A larger stokes shift is usually considered an advantage concerning assay development because the emission light can be easily separated from the excitation light, which reduces the background from the scattered excitation light.[1]

Process	Time Scale
Absorption	10^{-15} s
Fluorescence	$10^{-10} – 10^{-7}$ s
Phosphorescence	$10^{-6} – 10$ s
Internal conversion	$10^{-11} – 10^{-9}$ s
Intersystem crossing	$10^{-10} – 10^{-8}$ s

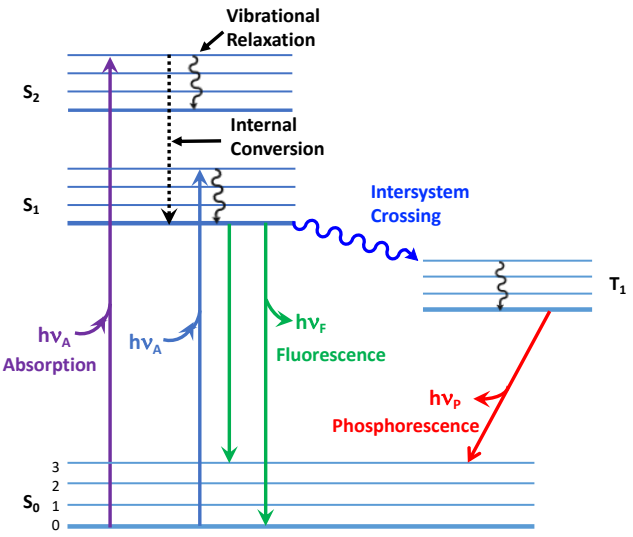

Figure 1. A typical Jablonski diagram showing the possible electronic transitions in a molecule when excited with a specific wavelength of light. The table above shows the time scale in second(s) of the various processes. Adapted from: J. R. Lakowicz, *Principles of Fluorescence Spectroscopy*. Copyright © 2013, Springer Science & Business Media.

Fluorescence can be distinguished from the other emissive deactivation pathways, including phosphorescence, by the time scale of the process. Fluorescence is a phenomenon that occurs in the range of nanosecond time scale (from the singlet excited; S_1 to ground state; S_0), while phosphorescence (happens with the excitation from lowest triplet state; T_1 to ground state; S_0) is observed at a higher time window (millisecond to second). The fluorescence is highly sensitive to molecular and environmental properties, making fluorescence spectroscopy one of the most sensitive and versatile techniques utilized for chemical and biological research. It is widely used to study the structure and dynamics of molecules in complex systems (either the molecule is self-fluorescent or tagged with a fluorescent molecule). Steady-state and time-resolved fluorescence methods are commonly explored to characterize emissive properties of fluorophores and their immediate environment at the ground and excited states, respectively. Time-resolved fluorescence measurements are generally more informative about the molecular environment of the fluorophore than steady-state fluorescence measurements. This is because time-resolved measurements capture the competing or perturbing kinetic processes such as collisional quenching, solvent relaxation, energy transfer, and rotational reorientation, which affect the fluorescence occurring in the timescale of the fluorophore's lifetime. A knowledge of these dynamics of excited states of the fluorophore is of foremost importance in deciphering various photophysical, photo-chemical, and photo-biological processes.[2-4]

Fluorescence Methods

Fluorescence measurement can be categorized into two types: steady-state and time-resolved. Steady-state measurement is the simple and most common type, while time-resolved typically requires complex and expensive instruments.

1. Steady-state fluorescence

Steady-state measurements are performed with constant illumination and observation, where the sample is illuminated with a continuous beam of light, and the emission spectra are recorded. Fluorescence intensity depends on the concentration of the fluorescent molecule, and standard concentration curves can be generated easily to determine concentrations of the molecule in the case of unknown samples. Concentration curves can also be produced to study how other molecules interact with things like proteins and can be used to systematically track protein structural changes, folding, unfolding, association, and dissociation.

Raman peaks are often visible in a fluorescence spectrum, especially when the fluorescence intensity is weak.[5] The spectrum of a blank solvent can be measured under the same conditions as that of the sample and subtracted from the fluorescent sample spectrum to remove a Raman peak. When the excitation moves more to the red or the blue, a Raman peak will move further or closer from the excitation wavelength. Thus, if

necessary, the excitation wavelength can be shifted to put the Raman peak in a slightly different location if a Raman peak interferes with a sample fluorescence spectrum. The temperature impacts fluorescence intensity, spectral wavelength, and shape as well. Along with that, the nonradiative rate constant is strongly affected and increases with increasing temperature.[6] So, in general, fluorescence will decrease with increasing temperature.

2. Time-resolved fluorescence

Time-resolved fluorescence or fluorescence lifetime or the decay time of a photoexcited molecule is defined as the time of depopulation of the singlet excited state (S_1) following a very short pulse of light (*i.e.,* a δ-pulse whose duration should be smaller than the reciprocal of the involved rate constant) which is used for excitation. The excited molecules return to S_0, either radiatively or non-radiatively, or undergo intersystem crossing.[3,4] The rate of disappearance of excited molecules, according to classical chemical kinetics, is expressed by the differential equation (Eq. 1):

$$-\frac{d[^1F^*]}{dt} = \left(k_r^S + k_{nr}^S\right)[^1F^*] \quad \ldots (1)$$

where, k_r^S and k_{nr}^S are the rate constant for radiative deactivation $S_1 \rightarrow S_0$ and overall nonradiative rate constant, respectively. The above equation upon integration yields the time evolution of the concentration of excited molecules $[^1F^*]$ of the form (Eq. 2):

$$[^1F^*] = [^1F^*]_0 \exp\left(-\frac{t}{\tau_S}\right) \quad \ldots (2)$$

where $[^1F^*]_0$ is the concentration of the excited molecule at time 0 after pulse light excitation. In fluorescence experiments, the number of excited molecules is generally not monitored; the fluorescence intensity, proportional to the fluorophore population at a given time, is used to define the rate equation. Hence, the above equation can also be expressed in terms of the time-dependent intensity *I(t)*, which upon integration yields the general expression (Eq. 3) for a single exponential decay:

$$I(t) = I_0 \exp(-t/\tau_S) \quad \ldots (3)$$

The lifetime of the S_1 excited state, τ_S, is given by (Eq. 4):

$$\tau_S = \frac{1}{k_r^S + k_{nr}^S} \quad \ldots (4)$$

where I_0 is the intensity at time 0. The fluorescence lifetime is then determined from the slope of a plot of log *I(t)* versus *t*, but generally by fitting the data to the presumed decay models. When there is more than one lifetime component, then the intensity fitted to multiexponential functions is given by (Eq. 5):

$$I(t) = \sum_i \alpha_i \exp(t/\tau_i) \quad \ldots (5)$$

where α_i is the amplitude corresponding to a lifetime component τ_i and $\Sigma\alpha_i$ is normalized to unity.

Both k_r^S and k_{nr}^S depend strongly on the fluorophore's environment, and factors like refractive index, polarity, viscosity, temperature, *etc.,* have significant effects on these; in consequence fluorescence lifetime of a molecule (fluorophore) can be utilized as a probe of its microenvironment. Since the excited state population is proportional to the fluorescence intensity, the fluorescence lifetime can be defined as the time taken for the fluorescence intensity to drop to 1/e of its initial value after the δ-pulse excitation. This is the basis of the single photon counting technique.

During the fluorescence lifetime of an excited fluorophore, various transformations are feasible, such as electron redistribution, geometrical alterations, reorganization of the surrounding molecules, *etc.* As a result of the strong influence of these factors on the nonradiative decay rates, there is an inevitable decrease in the fluorescence lifetime, and such processes are collectively known as quenching processes. The fluorescence lifetime of some fluorophores also shows solvent polarity dependence. However, unlike steady-state solvatochromism, which results from the change in the dipole moment of the excited state relative to the ground electronic state, the polarity dependence of lifetime is not properly understood.[7-9]

Fluorescence techniques

1. Synchronous

Synchronous fluorescence spectroscopy (SFS) that made a milestone in fluorescence analysis was first developed by Lloyd in constant wavelength mode.[10] Numerous synchronous fluorescence techniques have been developed depending on the scanning rates of emission and excitation monochromators, along with the type of maintained difference between them. A constant wavelength difference is maintained between the excitation and emission monochromators in the case of conventional synchronous fluorescence spectroscopy or constant wavelength synchronous fluorescence spectroscopy (CWSFS).[11,12] Inman *et al.* proposed another approach to synchronous fluorescence technique in which the excitation and the emission monochromators are scan synchronized so that a constant energy difference is maintained between the two monochromators.[13] By simultaneous scanning of the excitation and the emission monochromators, fluorescence measurement can also be carried out at different rates, which belongs to another variant of the synchronous fluorescence technique. This method is called variable-angle SFS (VASFS), which is known for its flexibility in addition to high selectivity.[14,15]

2. Fluorescence Anisotropy

Light is electromagnetic radiation with electric and magnetic vectors aligned perpendicular to each other. A molecule absorbs light while the molecular dipole is parallel to the electric vector of electromagnetic radiation (light). Therefore, the orientation of the electric vector of light is essential for molecular absorption. In depolarized light, the electric vectors are randomly oriented in all directions perpendicular to their propagation direction. On the other hand, when most electric vectors are oriented in one plane, it is known as partially polarized light.

If all the electric vectors travel in any particular plane, it is called plane-polarized light. Figure 2 depicts the schematic representations of depolarized, partially polarized, and plane-polarized light.

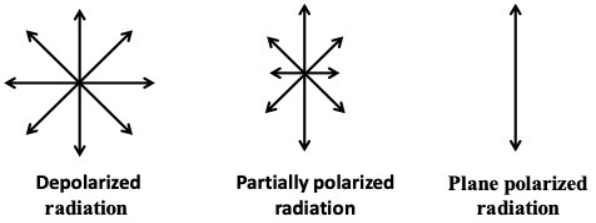

Figure 2. Electric vector representations of depolarized, partially polarized, and plane-polarized light. Adapted from *Optical Spectroscopic and Microscopic Techniques,* Ed: Harekrushna Sahoo. Copyright © 2022, Springer Singapore.

A polarizer is an optical device that converts a depolarized or partially polarized light into a beam polarized in a particular plane. The function of a plane polarizer is shown in Figure 3. The dichroic devices and double refracting calcite crystals are being used as polarizers; however, the double refracting calcite crystal polarizer is more common. While the molecules are excited with plane-polarized light, the emitted light maintains the polarization if the molecules do not rotate within the span in the excited state (fluorescence lifetime). Nevertheless, the freely rotating molecules convert the plane-polarized excitation into a depolarized emission due to a change in its orientation during fluorescence lifetime (Figure 3). The property of polarized emission against a polarized excitation provides an opportunity to monitor the probability of molecular rotation in the experimental condition. The change in orientation of the molecule, *i.e.,* the average angular displacement of the fluorophore that occurs within the span in the excited state of the fluorophore, can be measured with the help of fluorescence anisotropy. Therefore, fluorescence anisotropy is considered a measurement of the changing orientation of a molecule in space to the time between the absorption and emission events.[16] The angular displacement of the fluorophore is directly related to its rotational diffusion, and the rate of rotational diffusion is dependent on its size, shape, and viscosity of the medium. The measurement of fluorescence anisotropy for a particular fluorophore makes the anisotropy value solely dependent on the viscosity of the medium. The emission from fluorophores in a rotationally restricted environment (high viscosity) is highly polarized. In contrast, fluorophores emit depolarized light in a low viscous medium (freely rotatable), leading to nearly zero fluorescence anisotropy value (Figure 3). The following equation determines the fluorescence anisotropy of a sample:

$$r = \frac{I_{VV} - G \times I_{VH}}{I_{VV} + 2G \times I_{VH}} \quad \ldots (6)$$

where G = I_{HV}/I_{HH} (grating correction or G-factor), which corrects for any dependencies upon the plane of polarization within the instrument's optical and electrical components, I_{VV} and I_{VH} are the measured fluorescence intensities with the excitation polarizer vertically oriented, and the emission polarizer is oriented vertically and horizontally, respectively.

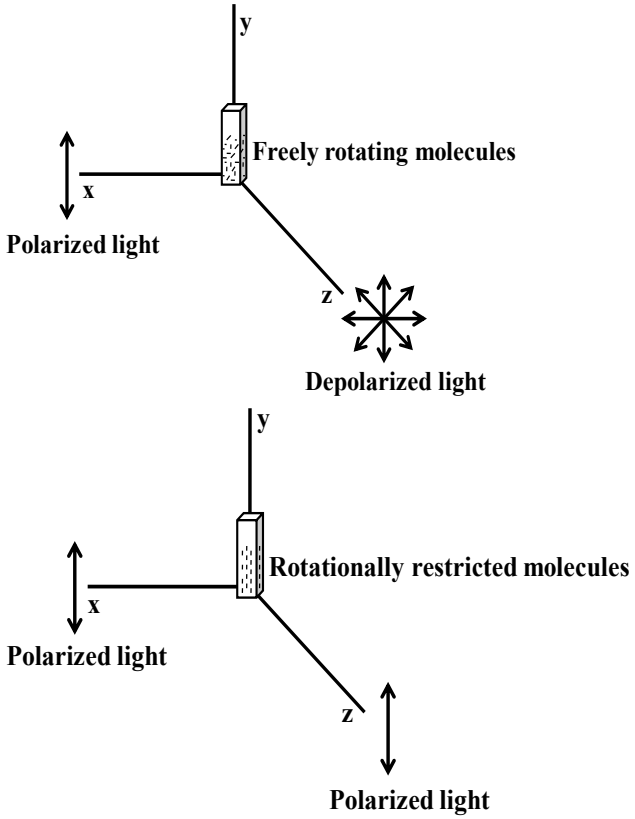

Figure 3. Functional schematic representation of a plane polarizer. Adapted from *Optical Spectroscopic and Microscopic Techniques,* Ed: Harekrushna Sahoo. Copyright © 2022, Springer Singapore.

Fluorescence anisotropy depends upon the angle between absorption and emission transition dipoles. The fundamental anisotropy is given by the Eq. 7:

$$r_0 = \frac{2}{5}\left(\frac{3\cos^2\theta - 1}{2}\right) \quad \ldots (7)$$

where r_0 is the fundamental anisotropy and θ is the angle between absorption and emission dipoles.

It has been found that 1,6-diphenyl-1,3,5-hexatriene (DPH) shows the highest value of r_0 *i.e.,* 0.39 as the θ is minimum for DPH (θ ~ 7.4º).[3] Theoretically, maximum r_0 (0.4) can be obtained when θ = 0, whereas r_0 becomes zero at θ = 54.7º. The r_0 value becomes negative and reaches -0.20 when θ = 90º. Hence, for an isotropic solution with single-photon excitation, r_0 lies between $-0.2 \leq r_0 \leq 0.4$. Since the orientation of the absorption dipole differs for each absorption band, the angle θ and r_0 vary with excitation wavelength. Since emission is mainly from the lowest singlet state, the fluorescence anisotropy value is independent of the emission wavelength. However, in the solvent relaxation process within the lifetime of a fluorophore, the lowest singlet state relaxes to lower energies. Therefore, fluorescence anisotropy depends on the emission wavelength, mainly in the restricted environment. The relaxed states of various solvents are relatively more non-degenerate in a restricted motional environment.

Rotational diffusion of the fluorophore within its fluorescence lifetime causes fluorescence depolarization. Therefore, the time required for the probe rotation (rotational correlation time) of the fluorophore is a crucial factor for fluorescence anisotropy. The rotational correlation time depends on the size of the fluorophore and the viscosity of the immediate environment of the fluorophore. The relation between rotational correlation time and fluorescence lifetime is given in the Perrin equation (Eq. 8):

$$r = \frac{r_0}{1+{}^{\tau}/_{\theta}} \qquad \ldots (8)$$

Here, r_0 is the fundamental anisotropy, which is an intrinsic property of the fluorophore, θ is the rotational correlation time, and τ is the fluorescence lifetime of the fluorophore. When θ is greater than τ, r approaches to r_0 and when θ is less than τ, the r value is close to zero. The rotational correlation time is extensively used to bind small molecules (e.g., drugs) to their macromolecular targets (e.g., proteins). As the rotation depends on the size of the molecule, free small molecules show relatively smaller θ and upon binding to their target macromolecules the θ value increases. The θ value can be monitored as a function of protein concentration to obtain the binding isotherm of any small molecule (drug). The IC_{50} value can be calculated from the binding isotherm. Therefore, the timescale of θ and τ has a significant impact on the fluorescence anisotropy value.

Fluorescence anisotropy can be measured in an L- or T-format fluorescence spectrophotometer. A single emission channel is used in the case of an L-format instrument, whereas two emission channels, one for parallel and another for perpendicular component, is used in a T-format instrument. The schematic diagram of the L- and T-format fluorescence anisotropy instrument is shown in Figure 4. Fluorescence anisotropy is calculated from the I_{VV}, I_{VH}, I_{HH}, and I_{HV} values at a particular wavelength. However, values of I_{HV} and I_{HH} are required to measure the G-factor, which is virtually constant for a particular instrument for a specific wavelength. Therefore, I_{VV} and I_{VH} are essential determinants of fluorescence anisotropy values. In an L-format instrument, these two components (I_{VV}, I_{VH}) are measured separately against two different excitations. In contrast, in a T-format instrument, one can measure two emission components simultaneously against a single excitation (Figure 5). The simultaneous measurements provide fluctuation-free signal intensity in the T-format instrument. Nevertheless, with the increased advancement in instrumentation, the importance of T-format instruments does not give much edge over L-format instruments other than minimizing experimental time. In general, an L-format instrument is widely used for fluorescence anisotropy measurement.

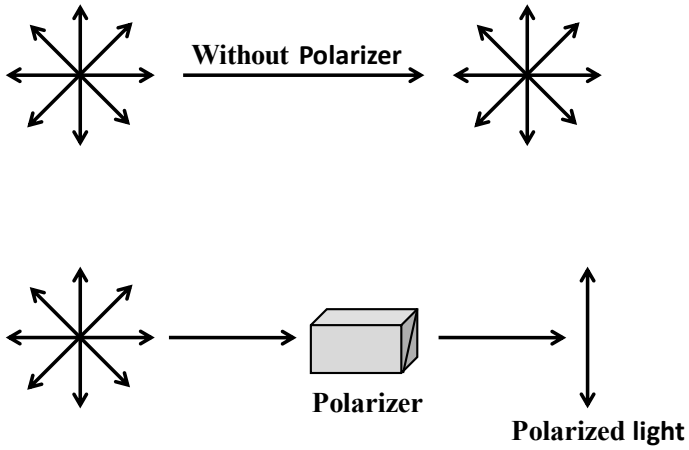

Figure 4. Schematic representation of the effect of probe rotation on emission polarization while the probe is being excited by plane-polarized light. Adapted from *Optical Spectroscopic and Microscopic Techniques,* Ed: Harekrushna Sahoo. Copyright © 2022, Springer Singapore.

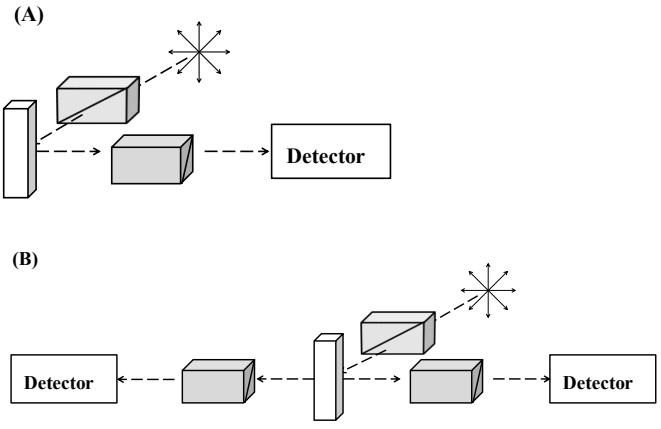

Figure 5. Schematic representations of (A) L-and (B) T-format fluorescence spectrophotometers for anisotropy measurements. Adapted from *Optical Spectroscopic and Microscopic Techniques,* Ed: Harekrushna Sahoo. Copyright © 2022, Springer Singapore.

3. Fluorescence Resonance Energy Transfer (FRET):

Fluorescence resonance energy transfer (FRET) is one of the most explored and studied phenomena in fluorescence that involves the nonradiative energy transfer due to the dipole-dipole interaction between a donor (D) fluorophore in an excited state and an acceptor (A) fluorophore in the ground state (or a quencher) when appropriate spectral overlap and proximity requirements are satisfied between the two (i.e., the donor and acceptor molecules) in close proximity (Figure 6).[17,18] As a part of the donor's energy is transferred to the acceptor; there is a fall in the donor's fluorescence emission intensity and a corresponding increase in acceptor fluorescence intensity. From the viewpoint of classical physics, FRET has been described as an electrodynamic phenomenon. The mechanism usually involves two molecules, namely, a donor in the excited state that transfers its energy to another molecule, known as an acceptor in the ground state that absorbs this energy and gets excited. However, the acceptor need not be fluorescent. If fluorescent, an enhanced acceptor emission occurs due to energy transfer from donor to acceptor. The energy transfer is due to a long-range interaction like dipole-dipole coupling. As this process usually occurs without the emission of photons, it is known as RET instead of FRET.

The FRET efficiency depends upon several factors, the most integrals of which are described below:

Spectral overlap integral, J(λ): there must be a significant overlap between the acceptor's absorption spectrum and the donor's emission spectrum. Thus, the more spectra overlap, the better the energy transfer from the donor to the acceptor (Figure 6 B). This phenomenon of energy complementarity is called resonance.[19] The overlap integral, J(λ), is given by the following equation (Eq. 9):

$$J(\lambda) = \int F_D(\lambda)\varepsilon_A(\lambda)\,\lambda^4\,d\lambda \qquad \ldots (9)$$

where F_D = normalized emission spectra of the donor, ε_A = molar absorption of the acceptor, and λ = wavelength.

Distance between D and A for FRET: the two fluorophores (donor and acceptor) must be in close vicinity of each other, having a distance range of 10 to 80 Å.[20,21] The distance between donor and acceptor corresponding to 50% transfer of energy is known as the Forster radius (R_0), which has a characteristic value for a specific donor-acceptor pair and is typically in the range of 20 to 60 Å. The value of R_0 is calculated from the spectral properties of the donors and acceptors by equations given as (Eq. 10):

$$R_0 = [8.79 \times 10^{-5}(\kappa^2\eta^{-4}Q_D J(\lambda))]^{1/6} \text{ (in Å)} \qquad \ldots (10)$$

where Q_D represents the quantum yield of the donor in the absence of acceptor, η is the refractive index of the medium, κ^2 is an orientation factor representing the transition dipoles of the donor and acceptor. It is usually assumed to be equal to 2/3, and (J(λ)) is the overlap integral representing the extent of spectral overlap between donor and acceptor. The rate of energy transfer $K_T(r)$ from donor to acceptor can be calculated if the value of R_0 is known and is given by Eq. 11:

$$K_T(r) = \frac{1}{\tau_D}\left(\frac{R_0}{r}\right)^6 \qquad \ldots (11)$$

where τ_D is the decay time of the donor in the absence of an acceptor, R_0 is the Förster distance, and r is the donor-to acceptor distance. As the overlap of the emission spectrum of the donor with the absorption spectrum of the acceptor increases, the value of R_0 increases.

$D + h\vartheta \rightarrow D^*$
$D^* + A \rightarrow D + A^*$ [D→ Donor, A→ Acceptor]
$A^* \rightarrow A + h\vartheta'$

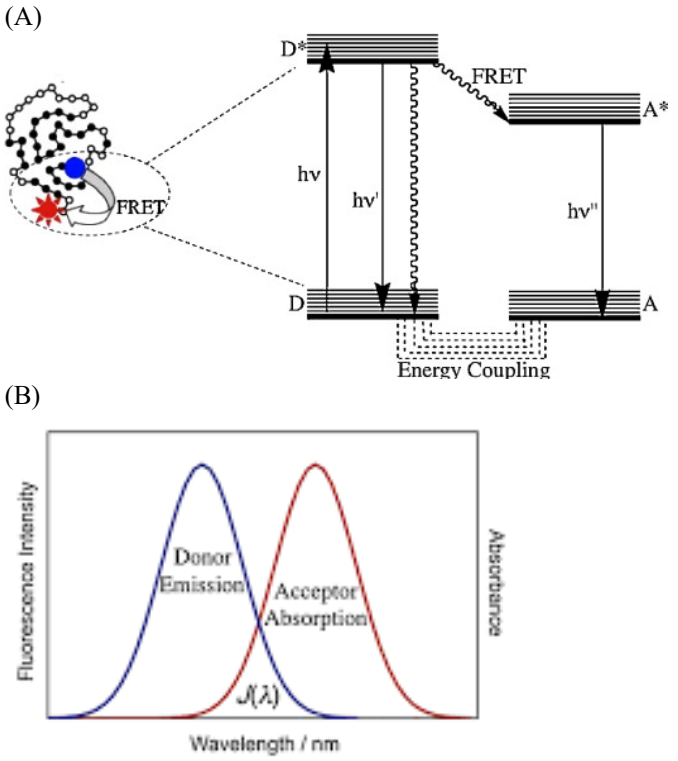

Figure 6. (A) A schematic representation of the molecular mechanism leading to FRET, and (B) the spectral overlap between donor and acceptor molecule, an essential prerequisite of FRET. Adapted from *Optical Spectroscopic and Microscopic Techniques,* Ed: Harekrushna Sahoo. Copyright © 2022, Springer Singapore.

4. Fluorescence correlation spectroscopy (FCS)

Introduced in the early 1970s, FCS is a time-averaging analysis of fluorescence fluctuation of minute molecular ensemble, thereby combining high sensitivity and statistical confidence.[22] In this method, the biophysical parameters are

extracted from the fluctuation in fluorescence intensity caused by fluorescently labeled molecules entering and leaving the probe region. Thus, labeling biomolecules with such fluorescent molecules is an essential part.[23] The detection volume is generated using a laser as an excitation source and a high-numerical-aperture objective. A highly sensitive detector called avalanche photodiodes (APD) is used to detect the fluorescence fluctuation, which collects the stream of single-photon arrival time. The parameters associated with the process responsible for fluctuations can be obtained from the analysis of the FCS data. But before that, the data need to undergo several rounds of processing.

The fluctuation in fluorescence intensity ($F(t)$) can be expressed as (Eq. 12):

$$\delta F(t) = F(t) - \langle F(t) \rangle \quad \ldots (12)$$

where $F(t)$ is the fluorescence intensity at time t, and $\langle F(t) \rangle$ denotes the average fluorescence intensity over time T. $\langle F(t) \rangle$ can be expressed as (Eq. 13):

$$\langle F(t) \rangle = \frac{1}{T} \int_1^T F(t) dt \quad \ldots (13)$$

The information on the parameters contributing to the fluorescence fluctuation is extracted from the FCS experiment by generating an autocorrelation curve that indicates the probability that the emanated signal at different times arises from the same molecule. Autocorrelation measures the Fluorescence fluctuation of the fluorophore alone with a time lag, τ. The mathematical expression for the autocorrelation function, $G(\tau)$ can be given as (Eq. 14):

$$G(\tau) = \frac{\langle \delta F(t) \rangle \langle \delta F(t+\tau) \rangle}{\langle F(t) \rangle^2} \quad \ldots (14)$$

In the above generalized autocorrelation function, G(τ) the self-similarity between $\langle \delta F(t) \rangle$ and $\langle \delta F(t+\tau) \rangle$ is more at less lag time, and hence at zero lag time, the amplitude of autocorrelation function is highest. After a long lag time, the self-similarity of the signal becomes highly reduced.

Different parameters contributing to the fluorescence intensity fluctuation can be extracted from the generalized autocorrelation function. For example, assuming the excitation profile to be a three-dimensional (3D) Gaussian illumination profile and the change in fluorescence intensity is only due to diffusion of fluorophores in and out of the observation volume, the above equation can be modified as (Eq. 15)

$$\mathrm{G}(\tau) = \frac{1}{\mathrm{N}} \left(1 + \frac{\tau}{\tau_\mathrm{D}}\right)^{-1} \left(1 + \frac{\tau}{\mathrm{K}^2 \tau_\mathrm{D}}\right)^{-1/2} \quad \ldots (15)$$

where N is the average number of fluorophores diffusing through the observation volume, and K (ω_z/ω_{xy}) is the ratio of radial to the axial radius of the three-dimensional Gaussian volume, also known as structure factor. The effective observation volume can be expressed as Eq. 16: [23,24]

$$V_{eff} = \pi^{3/2} {\omega_{xy}}^2 \omega_z \quad \ldots (16)$$

The diffusion time τ_D is related to the size of the observation volume as (Eq. 17):

$$\tau_D = \frac{{\omega_{xy}}^2}{4D} \quad \ldots (17)$$

where D is the diffusion coefficient, the hydrodynamic radius (R_h) of the sample can be calculated using the Stoke-Einstein relationship given by (Eq. 18):

$$D = \frac{k_B T}{6\pi\eta R_h} \quad \ldots (18)$$

where k_B is the Boltzmann constant, and η is the coefficient of viscosity of the solution.

The molecular size obtained from the hydrodynamic radii is very useful as it allows the characterization of the aggregated state of the molecule as well as the binding between small fluorescent ligands with large complexes. The schematic diagram of a typical confocal FCS setup is shown in Figure 7. It consists of excitation, emission, collection, and data acquisition parts. The excitation part includes first the excitation source, which is usually a laser of a particular wavelength. The beam of the laser light is expanded by using a telescope made of two lenses to overfill the back aperture of the objective. A dichroic mirror reflects the expanded beam to the back aperture of the objective lens. The objective focuses the beam to a small observation volume, where the sample in solution is placed. The fluorescence signal is collected in the epi-direction of the objective lens and passed through the dichroic mirror and subsequently through an emission filter to remove the excitation light, if any. Then the beam is fed to an achromatic lens to focus on the pinhole. The signal is then fed to a single photon counting module. The output of the detector is connected to the correlator card for the autocorrelation purpose, and the card is connected to the computer. In some set-ups, the signal is divided into two parts using a beam splitter, and the two signals are fed to two different detectors. The signals from the two detectors were cross-correlated to get the final data. This technique helps to reduce the after pulsing artefact.

The quantitative FCS measurements rely heavily on the fidelity of the calibration of the size of the detection volume, which in turn is influenced by several factors.[25] The data collected is usually in terms of the number of photon counts in a given time interval. The detected photons are then used to construct the fluctuation intensity trace, as shown in the figure. The autocorrelation curves are subsequently calculated from the fluctuation traces. The interpretation of the FCS data involves an appropriate theoretical model of the fluorescence fluctuation by fitting the autocorrelation function to a solution based on an appropriate diffusion model using nonlinear curve fitting. The diffusion coefficient D is the molecular property independent of the instrumental parameters. For this reason, FCS is calibrated with fluorophores with known diffusion coefficients. Since the

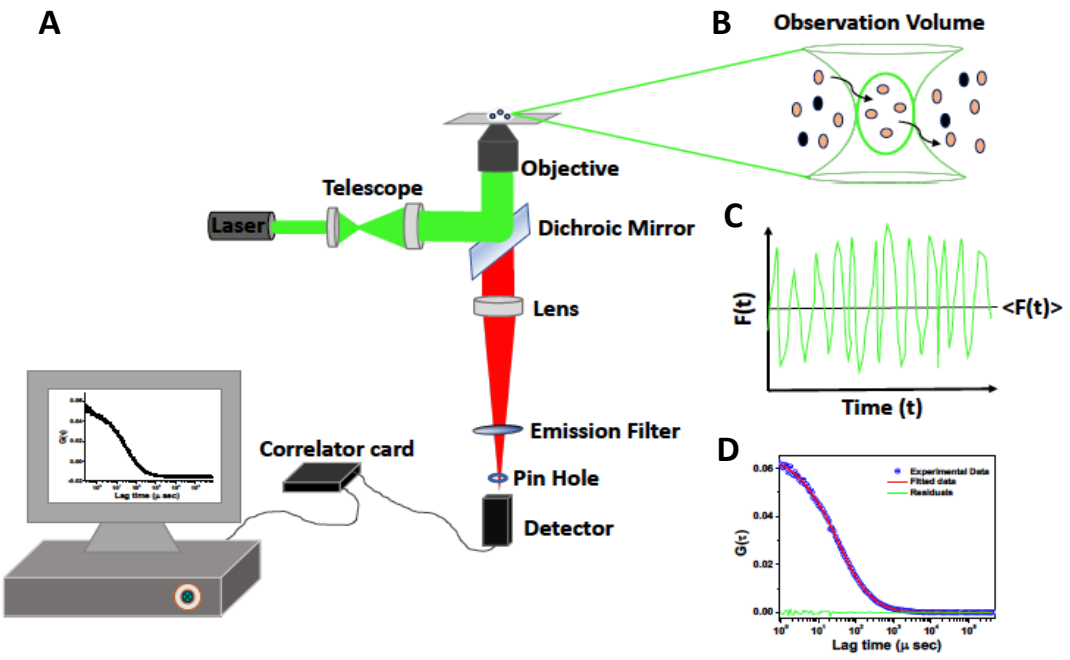

Figure 7. Schematic presentation of the FCS set-up. **(A)** The fluorescence signal emitted by the fluorescing species (fluorescently labeled molecules/ dyes) diffuses through the confocal volume element generated by a tightly focused laser spot and is transmitted to the detector. The number of fluorescent pulses sourced from the detected photons, recorded through a specific time interval, corresponds to the light intensity. Therefore, the fluctuation in fluorescence intensity over time is recorded. **(B)** The observation volume of the order of femtolitres. Fluorescent molecules diffuse in and out of this volume due to the Brownian motion. The green circle depicts the volume under observation at any given time point of the experiment. **(C)** The fluctuation in detected fluorescence intensity as a function of the time when molecules diffuse in the confocal volume is shown. **(D)** The electrical signal is collected at regular time intervals and is transferred to the signal correlation unit, and thus, the corresponding normalized autocorrelation curve G(τ) is calculated. The correlation function is plotted against the logarithm of delay time τ. The experimentally obtained autocorrelation curves are fitted with correlation function models to extract the parameters. The residual obtained from the fitting is shown in green. Adapted from *Optical Spectroscopic and Microscopic Techniques,* Ed: Harekrushna Sahoo. Copyright © 2022, Springer Singapore.

FCS measurements involve the relative diffusion coefficient of two species, the result is useful even if the absolute diffusion coefficient values are inaccurate.

5. FCS-FRET

Fluorescence correlation spectroscopy (FCS) is frequently discussed in the single-molecule fluorescence aspect. In contrast Forster resonance energy transfer (FRET) is a concept mostly implemented by both single-molecule and ensemble fluorescence approaches. FRET is a photophysical phenomenon employed in combination with FCS, i.e., FCS.FRET. Although both these concepts are treated as alternative approaches in several instances. However, in some cases, due to the involvement of two different fluorescent molecules, the application of fluorescence cross-correlation spectroscopy (FCCS), the FRET effects can cause significant complications for quantitative data analysis. For this purpose, careful calibration is needed to avoid FRET-induced artifacts.[26] This can be most elegantly done by alternative excitation schemes such as PIE (pulsed interleaved excitation) or ALEX (alternating laser excitation) and using the following mathematical expression (Eq. 19):

$$G_x(\tau) = \frac{G_{Diff}(\tau)}{V_{eff}(C)}\left[\frac{(S_2 - E_2)\left((C_g + C_{gr})\right)}{\left(S_2 - E_2(C_{gr})\right)}\right]\left[\frac{\eta_{AR} - \eta_{AG}\left((C_{gr} + C_r)\right)}{\left(\eta_{AR} - \eta_{AG}(C_{gr})\right)}\right] \quad \ldots (19)$$

Here, 'g' refers to green molecules (donor in case of FRET), 'r' represents red molecules (acceptor in case of FRET), and 'gr' stands for cross-correlating or FRET active complexes with green (donor) and red (acceptor) labeling. The above cross-correlation function capitalizes on the incomplete labeling efficiency in the data interpretation. η_{AR} and η_{AG} are the brightness of acceptor on direct (red) excitation and green (FRET) excitation, respectively. S_2 represents the FRET fraction, whereas E_2 indicates the FRET efficiency, and C stands for concentration. The effective volume is represented by V_{eff}

and G_{Diff} is the motion-related part of the correlation function. The above equation does not include the contribution from cross-talk. Graphically, the FCS-FRET system can be depicted as shown in Figure 8.

Due to the ability to resolve the processes like molecular association/dissociation on a single-molecule level, cross-correlation analysis (FCCS) may well be combined with FRET to enhance the signal specificity.

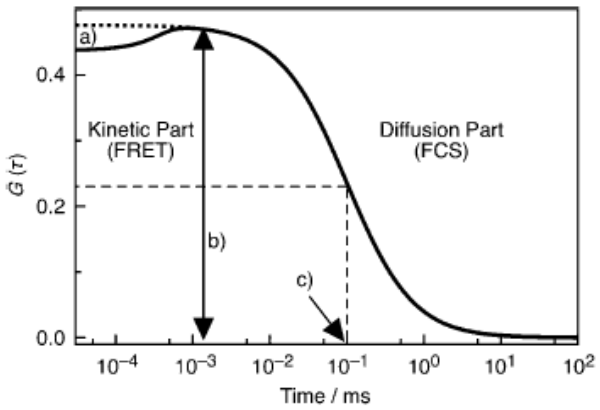

Figure 8. Cross-correlation curve showing the fluorescence intensity fluctuation due to conformational and dynamic changes: a) Intensity fluctuation as an effect of FRET, b) amplitude of the FCCS curve, and c) diffusion time, which is the correlation time at half-decay. Adapted from H. Sahoo, P. Schwille, *ChemPhysChem*, **2011**, *12*, 532-541. Copyright © 2016 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.

6. Fluorescence microscopy

Fluorescence microscopes enable the imaging of various fluorescence probes inside the samples. Depending upon the visualization requirement, a variety of fluorophores can be used to make the sample or a specific part of the sample (such as some organelles in living cells) fluorescent. Such samples can then be visualized with the help of a specially designed optical microscope. The first practical realization of a fluorescence microscope was achieved by Carl Zeiss and Carl Reichert at the beginning of the twentieth century. Since then, the technique has seen several advances that have led to the modern fluorescence microscopes used today in many research labs. Modern fluorescence microscope improved both visualizations and quantifications of fluorescent-labeled samples. The following section discusses the principle and the components of a typical fluorescence microscope.

The essential requirement of a fluorescence microscope is an excitation source, usually a mercury lamp/metal halide lamp/ Laser, an optical arrangement for selecting specific excitation and emission wavelengths, a sample containing a fluorescent molecule, and a detector for collecting the emitted fluorescence signal. The light from the source excites the sample containing the fluorescent probes. Subsequently, the fluorescent light emitted by the sample is collected using a detector (camera/eye). This process can be achieved essentially in two kinds of instrumental arrangements. One can use two different objectives for excitation and emission. However, most biological fluorescence microscopy uses a method where the excitation of fluorescent sample and collection of emission signal is achieved using the same objective. A microscope with such an arrangement is called an epifluorescence microscope.[27]

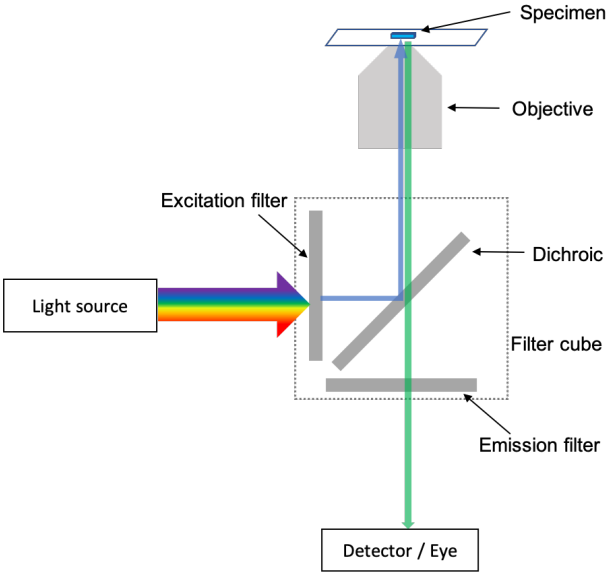

Figure 9. Excitation and emission light path in an epifluorescence microscope. The excitation of fluorophore and the collection of emitted fluorescence signal. Adapted from *Optical Spectroscopic and Microscopic Techniques,* Ed: Harekrushna Sahoo. Copyright © 2022, Springer Singapore.

A simplified diagram showing the light paths for excitation and emission in a typical epifluorescence microscope is shown in Figure 9. Briefly, a narrow band of wavelength matching the excitation spectrum of the particular dye is selected from the light source using an excitation filter and is incident upon the fluorescently labeled sample with the help of a dichroic mirror. The molecule absorbs the light, and the fluorescence emission from the sample is transmitted through the dichroic mirror and selected using an emission filter. A detector records this emitted intensity. The excitation filter, dichroic mirror, and emission filter are assembled inside a filter cube.

7. Conclusion

Fluorescence spectroscopy has wide applications in almost all research fields. It provides a wide range of detailed information concerning the molecular processes both in ensemble and at the single-molecular level. Fluorescence spectroscopy is very useful in determining the local and global changes upon environmental alternations. Its use has been extended with recent advances in technology for cellular imaging, drug delivery, and single-molecule detection. The need for complex technologies and exorbitantly priced instruments is

also significantly reduced with the technological advances in fluorescence spectroscopy. Therefore, it is rational to consider the contribution of fluorescence spectroscopy in the rapid advancement of biotechnology, biology, nanotechnology, and polymer chemistry.

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