Forster resonance energy transfer (FRET): A commentary on the experimental tests for judging the applicability of a popular technique.

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Abstract

Forster Resonance Energy Transfer (FRET) is a popular spectroscopic tool applied in a vast spectrum of research activities. The technique of FRET is often found to be applied in estimation of donor (D) acceptor (A) separation in different types of systems under experiment, while remaining oblivion to the applicability of the technique at all. Only few reports are found to be concerned about establishing the operation of FRET prior to application of the technique. The aim of this commentary is to provide a critical analysis on the experimental tests for judging the applicability of this popular technique. Finally, a focus is rendered on the discussion of a number of potential and accessible experimental tools that can be exploited to decipher the authenticity of occurrence of FRET under certain given experimental conditions. Efforts are also made in making the discussion concise and general without putting emphasis on any particular experimental results. This commentary will hopefully contribute to augment a more appropriate use of the beautiful technique of FRET in various aspects of fluorescence spectroscopy.

Keywords: Forster resonance energy transfer, Fluorescence spectroscopy, Fluorescence lifetime, Experimental tests of FRET.

Accepted on 20 August, 2021

Introduction

Forster Resonance Energy Transfer (FRET) is a predominant photophysical process involving transfer of excitation energy of an electronically excited "Donor" (D) molecule to an "Acceptor" (A) molecule *via* non-radiative channels. About nine decades ago, while addressing a conundrum surrounding fluorescence quenching experiments, constructed the notion of dipole-dipole interaction underlying the operation of FRET in which the interaction between molecules separated by distances larger than their molecular diameters was possible without collision. Later on Forster designed an elegant theory in the lines of Perrin's ideas, which shaped into a quantitative interpretation of the non-radiative energy transfer:

where, the rate of energy transfer, is directly proportional to the quantum yield (ΦD) of the donor, overlap between donor emission and acceptor absorption spectra (J(λ)), and the orientation factor between the donor and acceptor molecules (dipoles) (κ2), and is inversely proportional to the sixth power of D-A separation (r) [1].

Materials and Methods

An in-depth understanding of any phenomenon on a molecular level has incessantly indulged in the fuel to all physical, chemical and biological quests. Further, with the blooming research activities focused on nano-science and nano-bio interface science, its achievement is gaining immense priority. In order to delve into a phenomenon on a molecular scale it warrants information about the spatial relationships between the molecules, and this is where the performance of the well-known technique FRET comes to commendable realization and fruition, that is, to quantitatively measure distances between molecules in the range 10 - 100 Å, thereby providing with valuable information regarding the structures and dynamics of macromolecules/nano-scale materials. Successful exploitation of FRET has fathomed into areas such as DNA sensing, proteomics, probing various processes in macromolecular assemblies like living cells, proteins etc., detection of trace metal ions and so forth.

Very recently, this wonderful technique of FRET has been enormously applied in various fields, amongst which of particular attention has been the one related to the field of interaction of small molecules with a variety of macromolecular/biological systems such as proteins. Any discernible observation of fluorescence quenching is often argued/discussed on the lexicon of operation of FRET from intrinsic protein flurophore (such as, tryptophan (Trp)) to the small molecule(s) (often referred to as the ligand in the context of protein-ligand interaction).

The motivation of the present commentary connects to discussions of the possible experimental tests that can be applied in establishing the operation of FRET in a given pair of Donor (D) and Acceptor (A). The details of the theory of FRET are not elaborated here as they can be obtained [2].

The concept of FRET in brief, common pitfalls and a general discussion on the experimental techniques to overcome them

FRET occurs between a Donor (D) molecule in the excited state and an Acceptor (A) molecule in the ground state. Resonance energy transfer is a non-radiative quantum mechanical process and requires fluorescence emission spectrum of the donor molecule (D) to overlap with the absorption spectrum of the Acceptor (A) (as illustrated

schematically in Figure 1), and the two to be within the minimal spatial range for the donor to transfer its excitation energy to the acceptor.

The Forster theory, as based on the equilibrium Fermi-golden rule approach, regards the excitation energy transfer in terms of transition between the electronic states indicate the ground and excited-states, respectively) promoted by long-range intermolecular dipole-dipole coupling between the donor and acceptor (that is, Coulombic interaction). The underlying assumptions in Forster's theory can be stated as:

- The electronic coupling between the donor and acceptor is treated on the basis of dipole-dipole interaction, typically under the assumption of point dipoles.
- The occurrence of FRET proceeds on a time-scale significantly slower in comparison to the vibrational relaxation processes of donor following electronic excitation
- The coupling between the donor and acceptor is considerably weaker in comparison to that of molecules to the surroundings, this assumption asserts the irreversibility and incoherence to the process of FRET. With a view to the assumptions stated above, the Fermi-golden rule approach is undertaken to construct the expression for the rate of energy transfer for the orientation factor (κ2) is described as follows:

Here θD and θA represent the angles made respectively by the donor dipole and acceptor dipole with the axis connecting the two, ϕ is the angle between the planes on which the two dipoles lie. The magnitude of the orientation factor will be governed by the orientation of the absorption dipole of A relative to the emission dipole of D, and its value ranges between from 0 and 4. However, precise determination of the value of κ2 is usually difficult under a given set of experimental conditions and it is often assumed to have the value of 2/3 under the assumption of random orientation of the donor emission and acceptor absorption dipoles (isotropic motion), which is certainly not fully accurate in quite a many experimental conditions, such as within the motionally constrained environments of macromolecules, in viscous medium and so forth. Thus, the use of this value (κ2 = 2/3) has always invoked controversies, however, the uncertainties can be minimized by fluorescence anisotropy studies.

In which I(λ) represents the normalized fluorescence intensity of the donor in the range λ and λ + Δλ, and ε(λ) is the absorption (extinction) coefficient of the acceptor at wavelength λ. It is imperative to state in this context that J(λ), that is, the spectral overlap term, is in principle a complicated factor particularly with a view to the fact that it incorporates such complex information as separation of the electronic coupling from the nuclear overlap factors. However, the important point to note is that such complex information can be extracted from reasonably simple experimental parameters under the framework of Forster theory.

In a typical FRET experiment the donor fluorescence intensity is gradually quenched with incremental addition of the acceptor following photoexcitation of the donor only. If the acceptor is fluorescing too, the donor fluorescence quenching is simultaneously associated with enhancement of acceptor fluorescence, though in order for FRET to be operative the acceptor need not be a fluorophore. This feature of the technique forms the actuating basis for evaluation of the efficiency of energy transfer (E) in a given FRET experiment through the following relationships:in which I and τ terms designate respectively the steady-state fluorescence intensity and fluorescence lifetime of the donor fluorophore with the subscript '0' being used to mark the parameter in the absence of the acceptor. Here, r denotes the D-A separation distance and R0 is the referred to as the critical Forster distance (or Forster distance) which signifies the condition when the energy transfer is 50% efficient [3].

This fascinating photophysical process, by virtue of its intrinsic nature, offers a number of ways to subject the experiment under study to a number of realistic tests toward deciphering the authenticity of operation of FRET within the experimental system. The extent of overlap between the donor fluorescence emission and the acceptor absorption profiles is an inevitable criterion toward not only justifying the choice of donor and acceptor molecules for FRET experiments but also assessing a legitimate estimate for the efficiency of energy transfer. However, even a good extent overlap between donor fluorescence emission spectrum and the acceptor absorption spectrum does not necessarily ascertain the operation of FRET. There are several processes which may not only potentially contaminate the FRET experiments but lead to sheer confusions as well, such as, diffusion-controlled quenching of the donor emission in the presence of progressively increasing concentration of the acceptor may produce a visually similar appearance of the spectral profile which can be easily confused for occurrence of FRET (as discussed later). Even sometimes direct excitation of the acceptor molecule may lead to similar pitfalls. Thus, it is warranted as a necessity check on the issue of direct excitation of the acceptor molecule while performing a FRET experiment. If the acceptor absorption spectral profile has a non-negligible tail on the excitation wavelength regime (illustrated schematically in Figure 1), the FRET experimental window is certainly susceptible toward contamination from simply direct excitation of the acceptor and no FRET needs occur in reality to produce a gradual fluorescence intensity decrement of the donor with increasing acceptor concentration.

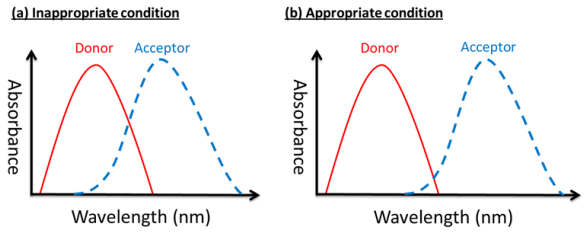

Figure 1. *Schematic presentation of the overlap between absorption profiles of the donor and the acceptor illustrating (a) an inappropriate condition for FRET when the acceptor is having a non-negligible tail within the donor absorption wavelength, and (b) an appropriate condition when there is only insignificant (or ideally no) overlap between the donor and acceptor absorption spectra and hence negating the possibility of direct excitation of acceptor during FRET.*

However, depending on relative concentrations of the donor and acceptor counterparts, optical path-length etc., other factors like diffusion-controlled quenching process, inner filter effects etc., might become functional leading to contaminate the real FRET results (or even confuse with FRET even if there is no FRET at all.

This becomes particularly more serious when the acceptor is non-fluorescent). Such factors thus obviously appear to advocate for the necessity of establishing the operation of FRET within the system under experiment before applying the FRET theories to calculate the important parameters like FRET efficiency (E), D-A separation distance (r), Forster distance (R0) and so forth.

Very often in cases involving non-fluorescent acceptors, if the donor and acceptor exhibit good spectral overlap, the reduction of the fluorescence intensity and lifetime of the donor are the only experimental parameters used to assign a dynamic quenching mechanism involving FRET.

However, when the experimental results are analyzed more carefully by raising deeper questions to fathom into the aptly operational mechanism behind the observations, which are most often disregarded, the FRET mechanism of fluorescence quenching, which appears rather obvious, actually may turn out to be inappropriate (Figure 2) [4].

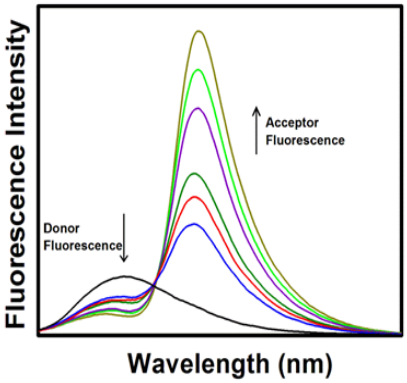

Figure 2. *An illustrative schematic describing the occurrence of FRET between a selected pair of donor (D) and acceptor (A) molecules.*

Herein, a discussion is presented highlighting the experimental tests and techniques that can be utilized to establish the genuineness of the operation of FRET in a given experiment.

Blank experiment

The imperative issue of establishing the operation of FRET should be tested against a blank experiment, that is, comparison of the total fluorescence (may be in terms of area under the fluorescence curve or fluorescence yield) of the donor in the presence of the acceptor to that from the blank experiments with the same concentration of the acceptor alone. However, this test would be applicable when the acceptor is fluorescent. In a typical FRET experiment the donor is excited at its absorption wavelength (λex = λabs (D)). Progressive addition of the acceptor results in quenching of donor fluorescence with simultaneous enhancement of acceptor fluorescence at λex = λabs (D). Simply if the acceptor fluorescence is originating from direct excitation of the acceptor molecule, its fluorescence should be comparable in the blank experiment (in the absence of the donor when excited at λex = λabs (D)) to that of the FRET experiment (that is, in the presence of the donor when excited at λex = λabs (D)). Obviously in a genuine FRET experiment (not contaminated by direct excitation of the acceptor molecule) the blank experiment should confirm negative results in terms of no significant fluorescence from the acceptor in the absence of the donor when excited at λex = λabs (D).

Excitation spectral study

Careful perusal of the excitation spectral features in the course of a FRET experiment can be exploited as a simple but strong tool for characterizing the authenticity of FRET results. However, the application of this tool also necessitates the acceptor's being fluorescent. When the excitation spectra of the donor and acceptor fluorophores are monitored individually, they should obviously reflect their own spectral characteristics, however, in a typical FRET experiment when the excitation profile is monitored at λmonitored = λem (A) (at λex = λabs (D) as usual under the FRET experimental conditions) it should display distinct excitation spectral properties of the donor molecule which can be easily characterized by a direct comparison with the individual spectra.

Stern-volmer analysis

This analysis for confirming the operation of FRET in a given D-A pair under experiment is applicable irrespective of the fluorescence properties of the acceptor counterpart (that is, this method can be applied even if the acceptor is non-fluorescent). The quenching of the donor fluorescence in the presence of incremental acceptor concentration can be meticulously analyzed on the well-known Stern-Volmer.

In which I0 and I terms designate the original fluorescence intensity and the quenched intensity of the donor, respectively, [Q] denotes the molar concentration of the quencher (here the acceptor), KSV is referred to as the Stern-Volmer quenching constant, kq is the bimolecular quenching rate constant and τ0 is the unquenched donor fluorescence lifetime.

Results and Discussion

A genuine case of FRET process should be characterized by rate constant of quenching (kq) discernibly higher than the maximum threshold for a typical diffusion-limited quenching.

Thus, if the observed magnitude of kq in a typical fluorescence quenching experiment is found to be higher than the maximum threshold for a diffusion-limited quenching process the concept of simple dynamic quenching will not lead to an unequivocal realization of the as-obtained Stern-Volmer parameters and thereby invoking one's natural inquisition to interpret the observed findings raising deeper questions. Thus, the simple

analysis based on the Stern-Volmer theory might provide fruitful acumen into assessment of the exact quenching mechanism, whether or not FRET.

Time-resolved fluorescence decay

Undoubtedly, as the most convincing experiment to confirm the operation of FRET should be utilized the technique of fluorescence lifetime measurement. If the acceptor is fluorescent, the operation of FRET in a typical experiment can be established by observing the nature of the fluorescence decay at the acceptor wavelength (that is, λmonitored = λem (A)) when the sample is excited at λex = λabs (D). If FRET is genuinely operative this experiment should manifest it through a rise (or growth) component followed by a usual decay in the time-resolved fluorescence profile monitored at λem (A) following photoexcitation at λex = λabs (D). The rise component would afford an unequivocal interpretation for an excited-state affair which can be connected to the operation of FRET in a typical experiment. Furthermore, ideally the deconvolution of this type of time-resolved fluorescence profile would be characterized by a rise time comparable to the following decay time constant. However, this experiment might prove difficult to perform in cases given issues like the availability of an appropriate instrument resolution, though smart data analysis might provide some clue to resolve such difficulties through negative amplitude in the overall time-resolved fluorescence decay profile conforming to the rise (growth) component [5].

However, in the presence of a non-fluorescent acceptor counterpart in a FRET experiment, the time-resolved fluorescence decay behavior of the donor fluorescence in the presence of increasing acceptor concentrations can be exploited to afford a sound platform in confirming the operation of FRET.

From the viewpoint of a simple fluorescence quenching phenomenon, both the intensity-weighted average lifetime and the amplitude-weighted average lifetime of the fluorophore may decrease with increasing concentration of the quencher whereby warranting the necessity of careful perusal in data analysis. A typical dynamic fluorescence quenching process should be resolved from not only the lowering of intensity-weighted average lifetime but also lowering of the individual decay time constants (τi).

Ground and excited-state complex formation

During a typical FRET experiment care should also be taken for verifying the possibilities of formation of ground and excited-state complexes between the chosen pair of molecular systems, namely, the donor and acceptor molecules. The point becomes particularly more important in the excited-state even in the absence of any substantial interaction in the ground-state. The absorption and excitation spectral studies can provide an effective way to investigate on such possibilities coupled with the tests involving variation of solvent polarity.

Conclusions

In this Commentary the use of a popular technique, namely, Forster Resonance Energy Transfer (FRET) has been discussed. The technique of FRET is being enormously applied in various research fields leading to draw many important inferences while the method of data analysis and arguments is the motivation of the present discussion. Herein, the major focus has been rendered on the propositions of some legitimate and accessible experimental tests that could be applied to establish the operation of the process of FRET in the system under investigation prior to its application. It is thus expected that careful perusal and criticism of the experimental conditions under study will lead to delve deeper into the fundamental mechanisms of interactions asking deeper questions regarding the actuating phenomenon in the experimental systems under concern.

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