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Fluorescence Resonance Energy Transfer FRET

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Abstract:

FRET is analogous to near-field communication, in that the radius of interaction is much smaller than the wavelength of light emitted. In the near-field region, the excited chromophore emits a virtual photon that is instantly absorbed by a receiving chromophore. These virtual photons are undetectable, since their existence violates the conservation of energy and momentum, and hence FRET is known as a radiationless mechanism. Quantum electrodynamical calculations have been used to determine that radiationless (FRET) and radiative energy transfer are the short- and long-range asymptotes of a single unified mechanism.

1. Introduction

1.1 Introduction to fluorescence spectroscopy

Fluorescence is the emission of light by a substance that has absorbed light or otherelectromagnetic radiation. It is a form of luminescence. In most cases, the emitted light has a longer wavelength, and therefore a lower photon energy, than the absorbed radiation. A perceptible example of fluorescence occurs when the absorbed radiation is in the ultraviolet region of the electromagnetic spectrum (invisible to the human eye), while the emitted light is in the visible region; this gives the fluorescent substance a distinct color that can only be seen when the substance has been exposed to UV light. Fluorescent materials cease to glownearly immediately when the radiation source stops, unlike phosphorescent materials, which continue to emit light for some time after.[1]

Fluorescence has many practical applications, including mineralogy, gemology, medicine, chemical sensors (fluorescence spectroscopy), fluorescent labelling, dyes, biological detectors, cosmic-ray detection, vacuum fluorescent displays, and cathode-ray tubes. Its most common everyday application is in (gas-discharge) fluorescent lamps and LED lamps, in which fluorescent coatings convert UV or blue light into longer-wavelengths resulting in white light which can even appear in distinguishable from that of the traditional but energy in efficient incandescent lamp.

Fluorescence also occurs frequently in nature in some minerals and in many biological forms across all kingdoms of life. The latter may be referred to as biofluorescence, indicating that the fluorophore is part of or is extracted from a living organism (rather than an inorganic dye or stain). But since fluorescence is due to a specific chemical, which can also be synthesized artificially in most cases, it is sufficient to describe the substance itself as fluorescent fluorescence spectroscopy (also known as fluorimetry or spectrofluorometry) is a type of electromagnetic spectroscopy that analyzes fluorescence from a sample. It involvesusing a beam of light, usually ultraviolet light, that excites the electrons in molecules of certain compounds and causes them to emit light; typically, but not necessarily, visible light, a complementary technique is absorption spectroscopy. In the special case of single molecule fluorescence spectroscopy, intensity fluctuations from the emitted light are measured from either single fluorophores, or pairs of fluorophores. Devices that measure fluorescence are called fluorometers. [2]

1.2 Theory

Molecules have various states referred to as energy levels. Fluorescence spectroscopy is primarily concerned with electronic and vibrational states. Generally, the species being examined has a ground electronic state (a low energy state) of interest, and an excited electronic state of higher energy. Within each of these electronic states there are various vibrational states. In fluorescence, the species is first excited, by absorbing a photon, from its ground electronic state to one of the various vibrational states in the excited electronic state. Collisions with other molecules cause the excited molecule to lose vibrational energy until it reaches the lowest vibrational state from the excited electronic state. This process is often visualized with a Jablonski diagram, figure 1. The molecule then drops down to one of the various vibrational levels of the ground electronic state again, emitting a photon in the process.[3] As molecules may drop down into any of several vibrational levels in the ground state, the emitted photons will have different energies, and thus frequencies. Therefore, by analysing the different frequencies of light emitted in fluorescent spectroscopy, along with their relative intensities, the structure of the different vibrational levels can be determined.

For atomic species, the process is similar; however, since atomic species do not have vibrational energy levels, the emitted photons are often at the same wavelength as the incident radiation. This process of re-emitting the absorbed photon is "resonance fluorescence" and while it is characteristic of atomic fluorescence, is seen in molecular fluorescence as well.

In a typical fluorescence (emission) measurement, the excitation wavelength is fixed and the detection wavelength varies, while in a fluorescence excitation measurement the detection wavelength is fixed and the excitation wavelength is varied across a region of interest. An emission map is measured by recording the emission spectra resulting from a range of excitation wavelengths and combining them all together. This is a three dimensional surface data set: emission intensity asa function of excitation and emission wavelengths, and is typically depicted as a contour map.

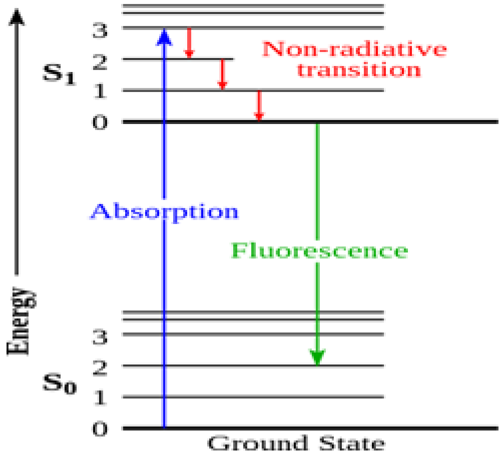
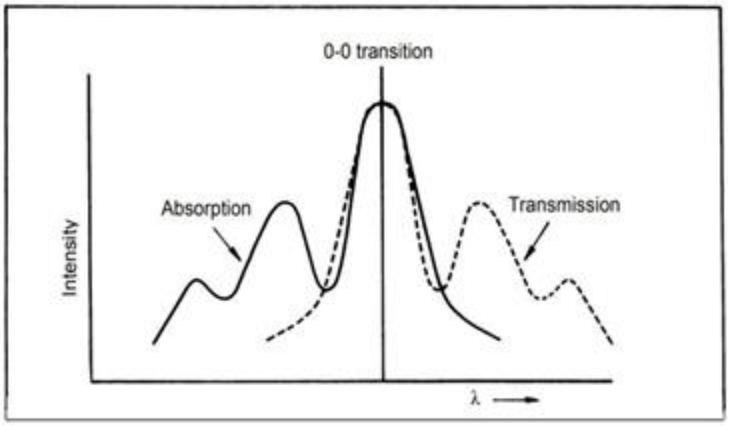


Figure (1) Jablonski diagram including vibrational levels for absorbance, non-radiative decay, and fluorescence.

At room temperature most molecules occupy the lowest vibrational level of the ground electronic state, and on absorption of light they are elevated to produce excited states. Excitation can result in the molecule reaching any of the vibrational sub-levels associated with each electronic state. Since the energy is absorbed as discrete quanta, this should result in a series of distinct absorption bands. Therefore, most compounds have broad absorption spectra except for those where rotational levels are restricted (for example, planar, aromatic compounds).

Having absorbed energy and reached one of the higher vibrational levels of an excited state, the molecule rapidly loses its excess of vibrational energy by collision and falls to the lowest vibrational level of the excited state. In addition, almost all molecules occupying an electronic state higher than the second undergo internal conversion and pass from the lowest vibrational level of the upper state toa higher vibrational level of a lower excited state which has the same energy. From there the molecules again lose energy until the lowest vibrational level of the first excited state is reached.[4]



From this level, the moleculecan return to any of the vibrational levels of the ground state, emitting its energy in the form of fluorescence. If this process takes place for all the molecules that absorbed light, then the quantum efficiency of the solution will be a maximum, unity. If, however, any other route is followed, the quantum efficiency will be less than one and may even be almost zero. One transition, that from the lowest vibrational level in the ground electronic state to the lowest vibrational level in the first excited state, the 0-0 transition, is common to both the absorption and emission phenomena, whereas all other absorption transitions require more energy than any transition in the fluorescence emission. We can therefore expect the emission spectrum to overlap the absorption spectrum at the wavelength corresponding tothe 0-0 transition and the rest of the emission spectrum to be of lower energy, or longer wavelength.

In practice, the 0-0 transitions in the absorption and emission spectra rarely coincide exactly, the difference representing a small loss of energy by interaction of the absorbing molecule with surrounding solvent molecules.

The absorption of energy to produce the first excited state does not perturb the shape of the molecule greatly and this means that the distribution of vibrational levels is very similar in both the ground and first excited states. The energydifferences between the bands in the emission spectrum will be similar to those in the absorption spectrum and frequently the emission spectrum will be approximate to a mirror image of the absorption spectrum. Since the emission of fluorescence always takes place from the lowest vibrational level of the first excited state, the shape of the emission spectrum is always the same, despite changing the wavelength of exciting light. A plot of emission against wavelength for any given excitation wavelength is known as the emission spectrum. If the wavelength of the exciting light is changed and the emission from the sample plotted against the wavelength of exciting light, the result is known as the excitation spectrum. Furthermore, if the intensity of exciting light is kept constant as its wavelength is changed, the plot of emission against exciting wavelength is known as the corrected excitation spectrum. [5]

1.3. Förster resonance energy transfer (FRET(

Förster resonance energy transfer (FRET), fluorescence resonance energy transfer, resonance energy transfer (RET) or electronic energy transfer (EET) is a mechanism describing energy transfer between two light-sensitive molecules (chromophores). [6] A donor chromophore, initially in its electronic excited state, may transfer energy to an acceptor chromophore through nonradiative dipole– dipole coupling. [7] The efficiency of this energy transfer is inversely proportional to the sixth power of the distance between donor and acceptor, making FRET extremely sensitive to small changes in distance.[8][9] Measurements of FRET efficiency can be used to determine if two fluorophores are within a certain distance of each other.[10] Such measurements are used as a research tool in fields including biology and chemistry. FRET is analogous to near-field communication, in that the radius of interaction is much smaller than the wavelength of light emitted. In the near-field region, the excited chromophore emits a virtual photon that is instantly absorbed by a receiving chromophore. These virtual photons are undetectable, since their existence violates the conservation of energy and momentum, and hence FRET is known as a radiationless mechanism. Quantum electrodynamical calculations have been used to determine that radiationless (FRET) and radiative energy transfer are the short- and long-range asymptotes of a single unified mechanism.

Förster resonance energy transfer is named after the German scientist Theodor Förster.[11] When both chromophores are fluorescent, the term "fluorescence resonance energy transfer" is often used instead, although the energy is not actuallytransferred by fluorescence.[12][13] In order to avoid an erroneous interpretationof the phenomenon that is always a nonradiative transfer of energy (even when occurring between two fluorescent chromophores), the name "Förster resonance energy transfer" is preferred to "fluorescence resonance energy transfer"; however, the latter enjoys common usage in scientificliterature.[14]

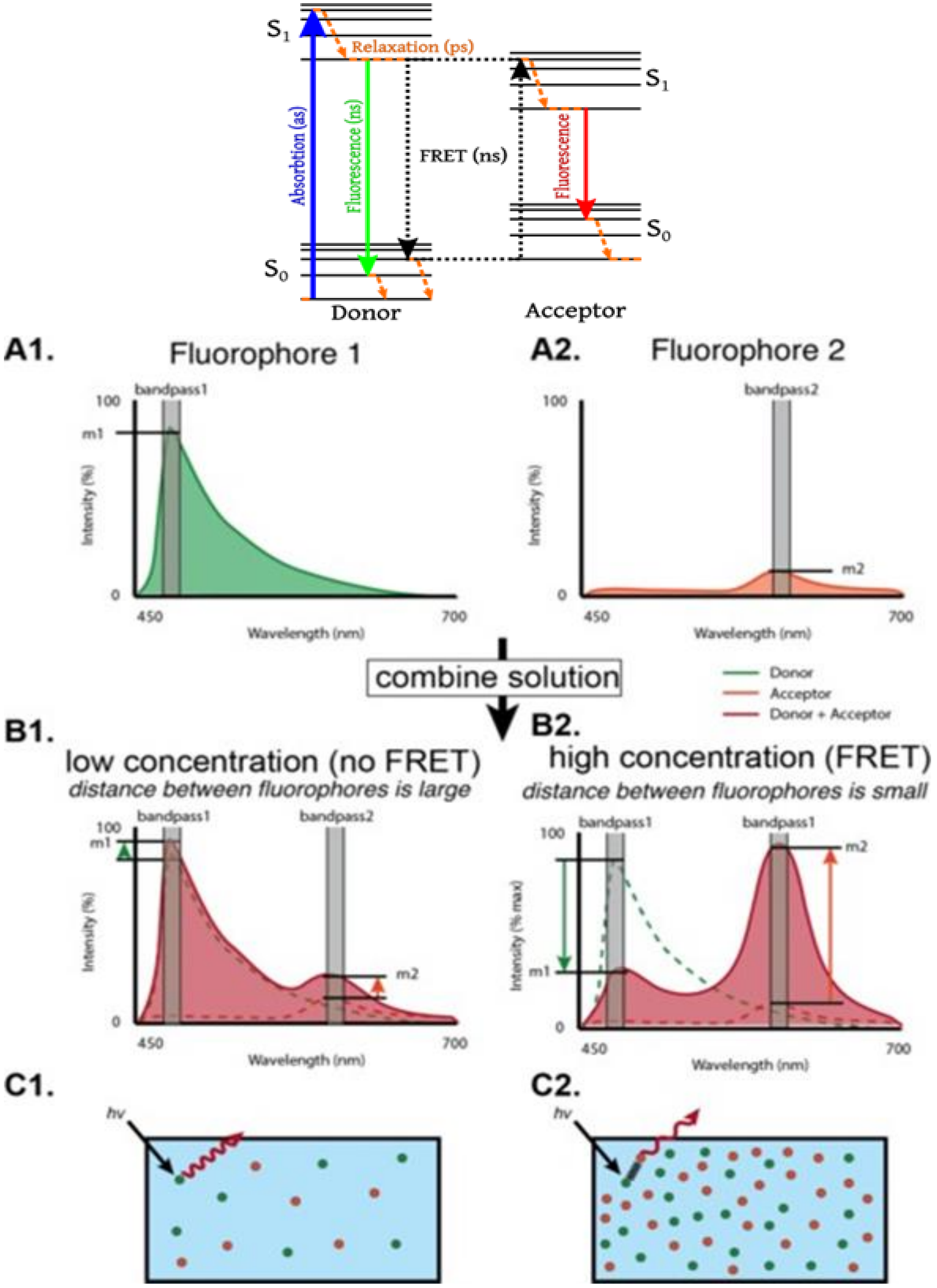


Figure (2) Jablonski diagram of FRET with typical timescales indicated. Note that the black dashed line indicates a virtual photon.

2. Experimental part

The peculiar parameters that characterize fluorescence are measured using "spectrofluorometers"; sometimes, instruments for the measurement of excitation and emission spectra are termed "spectrofluorimeters", while the ones for the measurements of the decay times termed "spectrofluorometers". Yet, the distinction is not anymore as clearly demarked as several instruments allow, in the same unit, to measure both the steady-state (excitation and emission spectra) and the dynamic (decay times and rotational correlation times) properties of the fluorescence. Usually, in all of the instruments, the fluorescence is collected at an angle of 90 degrees with respect to the optical axis set by the excitation light beam. This geometry maximizes the efficiency of the emission collection and reduces the background due to the excitation light.[15]

It is worthy to mention that absorption spectra can be measured using a spectrophotometer. In this type of instrument the light detector is placed on the same optical axis of the excitation light beam and the instrument detects the amount of light that is being transmitted (that is, not absorbed) through the sample. A spectrophotometer measures the difference in the intensity of two signals (typically, sample transmittance is compared to 100% transmittance); instead, a spectrofluorometer measures a signal (the fluorescence) over a zero background.

The key elements of a spectrofluorometer are the light source, the monochromator and the light detector.[16]

2.1. Light Source

The typical light source utilized in a spectrofluorometer is a high-pressure xenonarc lamp. The bulb of this lamp includes xenon at high pressure that is excited tohigher level by the electrical arc established by the current running through the electrodes. The emitted light is a continuous spectrum from (depending upon the models and geometries) about 250 nm up to 1100 nm. Figure 3 displays the spectrum of the lamp utilized by ISS. Although the spectrum is relatively flat up to about 800 nm, several sharp resonances are present above that wavelength**.**

It is worth noting that a variation of this lamp is the Hg-Xe lamp, which contains traces of mercury; this element displays resonances at around 295 nm and this feature allowed for its use as an excitation source for the proteins containing tryptophan. Light emitting diodes (LEDs) are also utilized as light sources especially in the region from 240 nm to 350 nm, where lasers are not available (with exceptions at 266 nm, 315 nm, 325 nm). They are compact, relatively inexpensive and the source of choice when building an instrument dedicated to a specific application.[17]

2.2. Monochromator

Monochromators are utilized to select the wavelength used for irradiating the sample when using a xenon arc lamp; in the collection channel of a spectrofluorometer they are utilized to record the range of wavelengths emitted by a fluorophore (emission spectrum, see below). The simplest monochromator includes a diffraction grating and slits at the entrance and at the output. Light impinging at an angle on the grating is diffracted at a series of angles; usually, thefirst angle (or first order) is selected for the measurement.

It is important to realize that the transmission of the light traversing a monochromator is affected by two parameters:

the wavelength; the grating has a specific transmission curve and some wavelengths are transmitted with a higher efficiency than other wavelengths, a feature to remember when collecting excitation and emission spectra**.**

1. the polarization status of the radiation; the grating of the monochromator transmits differently radiation with different planes of polarization .[15][17]

A characterization of every monochromator is the amount of stray light, that is radiation present at any wavelength other than the specific wavelength the monochromator is set at. The stray light is usually measured as the amount oflight that is transmitted outside the band pass of the 632.8 nm HeNe laser line. Gratings can be arranged in different designs to build a monochromator, the two more popular being the Czerny-Turner and the Seya-Namioka.[16]

2.3. Light Detectors

In all the instruments the fluorescence signal is converted into current by a photomultiplier tube (PMT), or photodiode (instruments for lifetime measurements may utilize other types of detectors too, such as hybrid PMTs, microchannel plate detectors or streak cameras).

Photomultiplier tubes are sensitive within a set wavelength range that is determined by the material used in the photocathode. Figure 3 displays the region of sensitivity for the PMT Model R928 by Hamamatsu. The PMT can be utilized in the region from about 230 nm to about 830 nm. It is apparent that even within the operational wavelength region, the sensitivity is not the same; the non- linearity of the sensitivity introduces an artifact in the data such that a correction to the data has to be introduced.[18]

A spectrofluorometer includes other optical elements such as lenses and mirrors; moreover polarizers are utilized for anisotropy measurements. The operational region of the instrument is given by the superposition of the wavelength transmission of the various elements of the instruments. Even within this region, the variation in transmission has to be taken into account when measuring the fluorescence parameters. The standard light source is a 300 W xenon arc lamp. Continuous wave (cw) lasers, pulsed lasers (including the multi-photon laser) and light emitting diodes (LEDs) can be coupled to the K2 as well; typically these sources are utilized for the measurement of the decay times of fluorescence[19]

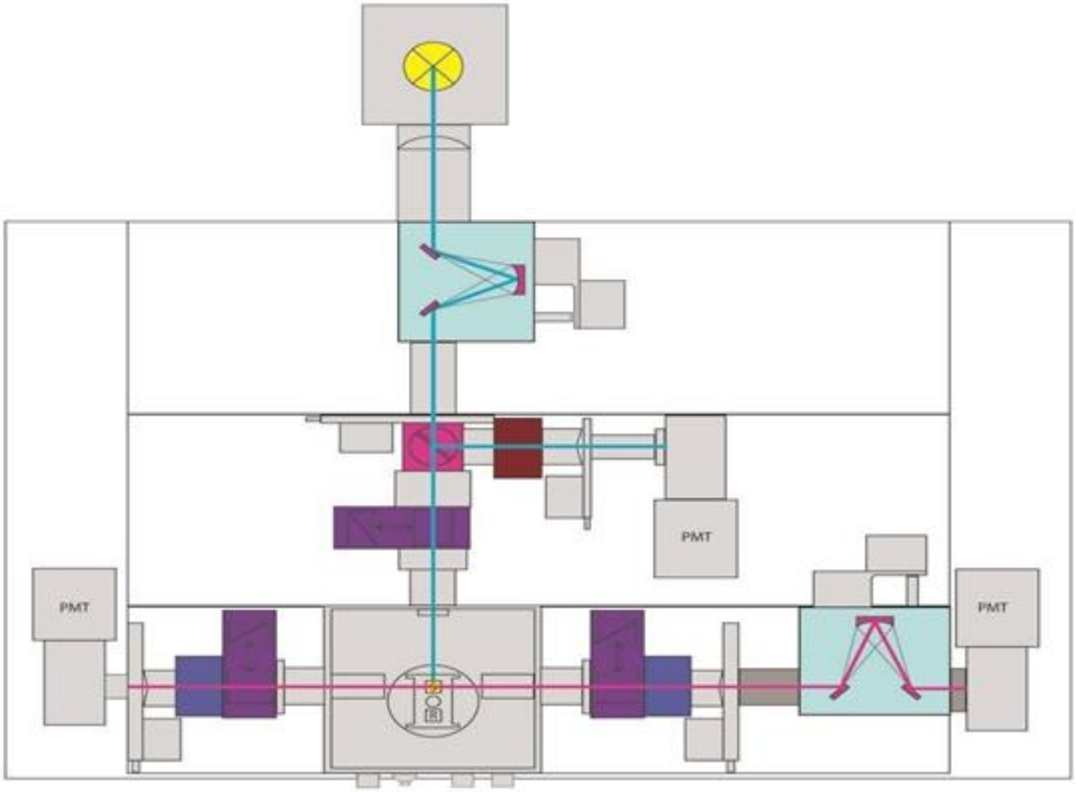


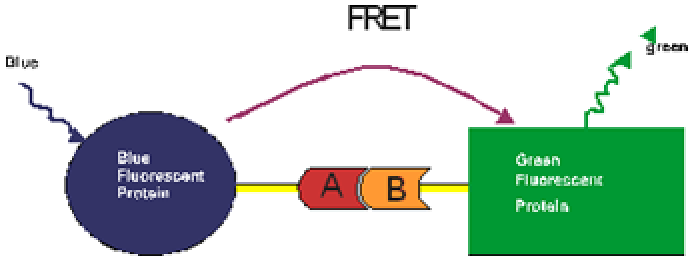
Figure (3) PC1 Photon Counting Spectrofluorometer (courtesy of ISS)

The light emitted by the source travels through the excitation channel that comprises the monochromator, a filter holder and the polarizer holder; the monochromator selects the wavelength of the light that excites the sample. The fluorescence emitted by the sample is collected through the left or the right channels; the right channel includes the emission monochromator.

The instrument includes polarizers' holders, filters holders, shutters for blocking the light from reaching the sample and the detectors. All of these components are required for automated measurement acquisition.[20][15][16]

3. Results and discussion

3.1. Acceptor-Photobleaching FRET in Cells



There are several ways to measure FRET in cells including sensitized emission, FLIM, fluorescence anisotropy and Acceptor-photobleaching FRET has several advantages that make it suitable for studying protein interactions in cells. First, FLIM and anisotropy measurements require access to instruments that often must be custom designed and built by the user. In contrast, suitable laser scanning confocal microscopes that can be used for acceptor-photobleaching experiments are both commercially available and accessible at most institutions. In addition, even a standard fluorescence microscope with a mercury lamp can be used to perform acceptor photobleaching (Kenworthy and Edidin, 1998). Second, sensitized emission depends on acceptor emission and often suffers from signal bleed-through from the donor, requires multiple correction factors[21][22], and provides no information about the relative populations of associated proteins. In contrast, acceptor photobleaching can be quantitated with a simple arithmetic equation, is unaffected by bleed-through, and can be used to gain insights into associated and unassociated populations of proteins. A noteworthy disadvantage of acceptor-photobleaching FRET is that it requires destruction of the probe, which prevents more than one measurement in a region of a cell. In addition, photobleaching to backgroundlevels of fluorescence intensity is often slow and either requires cells to be fixed or the proteins of interest to be relatively immobile, An important consideration for experimental design is whether the goal of the study is to distinguish between two distinct readout states or a continuum of states. It is often not feasible to synchronize multiple protein complexes in their dynamic changes. In contrast, measuring the change between a treated and untreated sample is more experimentally tractable for samples in cells. The measurement of discrete states permits cells to be fixed. While the aesthetic and intellectual appeal of live celldata is undeniable, the actual requirement for using live cells is worthconsidering[23]. It is still possible to perform live-cell acceptor-photobleaching FRET studies, though the investigator must now contend with the issue of diffusion. Many proteins are not immobile. [25]Because complete photobleaching of a fluorophore can take several seconds to minutes, acceptor photobleaching benefits substantially from immobilizing proteins with fixation. Before investing the time and resources into performing FRET experiments, the investigator will benefit from critical analyses of the questions to be addressed, the available reagents, and anticipated outcomes under idealized conditions. For example, a failure to consider expression levels when choosing which of two proteins to label as the donor, or attempting to measure absolute distances between proteins with antibody-based FRET will result in low probabilities of success. To enhance the probability of detecting the highest possible FRET signal for a pair of proteins, several conditions need to be empirically optimized.[26]

3.2. Determine Which Protein Will Be the Donor and the Acceptor

In acceptor-photobleaching experiments, this choice can significantly impact the observed FRET value. First, unpaired donors (i.e., those not in complex with the acceptor) dilute the detectable FRET signal. Second, unpaired acceptors have little effect on acceptor-photobleaching FRET values, and thus, are effectively invisible in this assay format. Thus, the donor in most cases should be the protein of lower stoichiometry to minimize the percent of unpaired molecules. Note that, because of this asymmetry, a discrepancy in FRET values upon exchanging donor and acceptor status can provide insights into the stoichiometry of complex components.[27]

3.3. Select an Appropriate Labeling Scheme

The method of labeling the proteins of interest will directly affect the ability of FRET to detect changes in protein proximities. For this unit, it is assumed that proteins of interest will be proteins expressed in the cell. These proteins can be labeled either with fluorescent dye-labeled antibodies, variant fluorescent proteins (i.e., GFP) or newer fluorescent tags including FlAsh and ReAsh [28]. After designating which protein will be the donor and which will be the acceptor, the investigator must choose a suitable labeling scheme. If a donor protein is present at low levels and overexpression changes a cellular phenotype, then antibody labelingis likely to be better than using a fluorescent fusion protein. Not only will the antibody boost the fluorescent signal due to multiple dyes on the antibody, but the multiple dyes can also enhance the probability of detecting FRET. If the donor and acceptor are abundant proteins and the donor remains functional when fused to a fluorescent protein, such as GFP, then addition of a fluorescent protein tag or epitope tag may provide flexibility in experimental design. It is not recommended to have an antibody donor and a fluorescent protein acceptor. In this case, there would be multiple dyes on the antibody that might fail to exhibit FRET with a single fluorophore acceptor. Again, the key principle is to avoid situations with excess donor molecules or limiting acceptor molecules.[29]

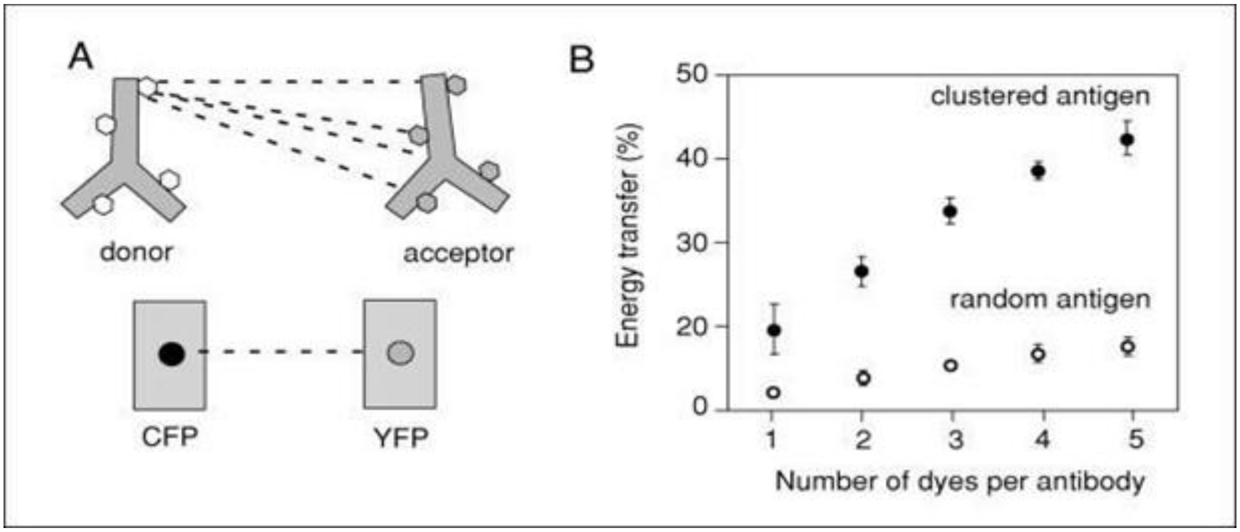


Figure (4) Shape above ,llustration of the advantage of using antibody probes labeled with multiple dyes. (**A**) Cartoon illustrates how each donor dye on an antibody has the potential to transfer energy to four different acceptor dyes on an acceptor antibody. In contrast, a cyan fluorescent protein (CFP) molecule can only potentially transfer energy to one yellow fluorescent protein (YFP) acceptor. The consequence is that multiple dye-labeled acceptor antibodies enhance the probability of detecting FRET. (**B**) The relationship of dye number on each donor and acceptor antibody as plotted with simulated data for antibodies bound to a multiprotein complex. Note that the value calculated for a single dye will not be equivalent to the CFP-YFP pair, because the simulated dye placement on the antibodies was random and could include distances up to three times greater than for the fluorescent fusion proteins.

If using antibodies to label the protein(s) of interest, either monoclonal or mono- specific polyclonal antibodies (raised against short peptide sequences of ~8 to 20 amino acids) are preferable to broad specificity antibodies[30]. A single protein epitope is likely to ensure that only a single antibody will bind a single protein.This is an especially useful quality when an investigator is trying to determine whether a complex contains more than one copy of a protein[31]. A single epitope will provide more specific information concerning protein organization and can aid in experimental design. For example, an epitope against the cytoplasmic domain ofa membrane protein has a higher probability of undergoing FRET with the cytoplasmic epitope of a partner protein[32].

3.4. Create a Model for the FRET Experiment

sufficient information about the proteins of interest is available, it is worthwhile to model and simulate FRET experiments to assist with experimental design and expectations for results. Simulated results serve to illustrate the capabilities, sensitivity, and specificity of an approach and provide boundaries for the type of questions that can be addressed. In particular, two issues are of direct relevance to most studies of protein complex assembly: the discrimination of assembled from disassembled multiprotein (or oligomeric) complexes and the discrimination of small changes in the structure of a complex that remains assembled in the same general configuration.

Monte Carlo simulations, rather than theoretical calculations using simplifying assumptions, can provide insight into not only the expected FRET efficiency, but also the degree of variability that can be anticipated from measurement to measurement due to the stochastic nature of many of the variables involved. [33] For example, FRET between subunits of a membrane protein complex containing three subunits was modeled (figue5-a). Donor and acceptor antibodies labeled with four dyes at random positions were simulated in binding to the proteins with varying ratios. A visual representation of the relative proximities of subunit proteins and fluorescent dyes in the disassembled and assembled states for a small section of membrane is shown in (figure 5-b) as a two-dimensional illustration.

Then the expected FRET efficiency for each ensemble of fluorophores was calculated according to previously established equations [34]

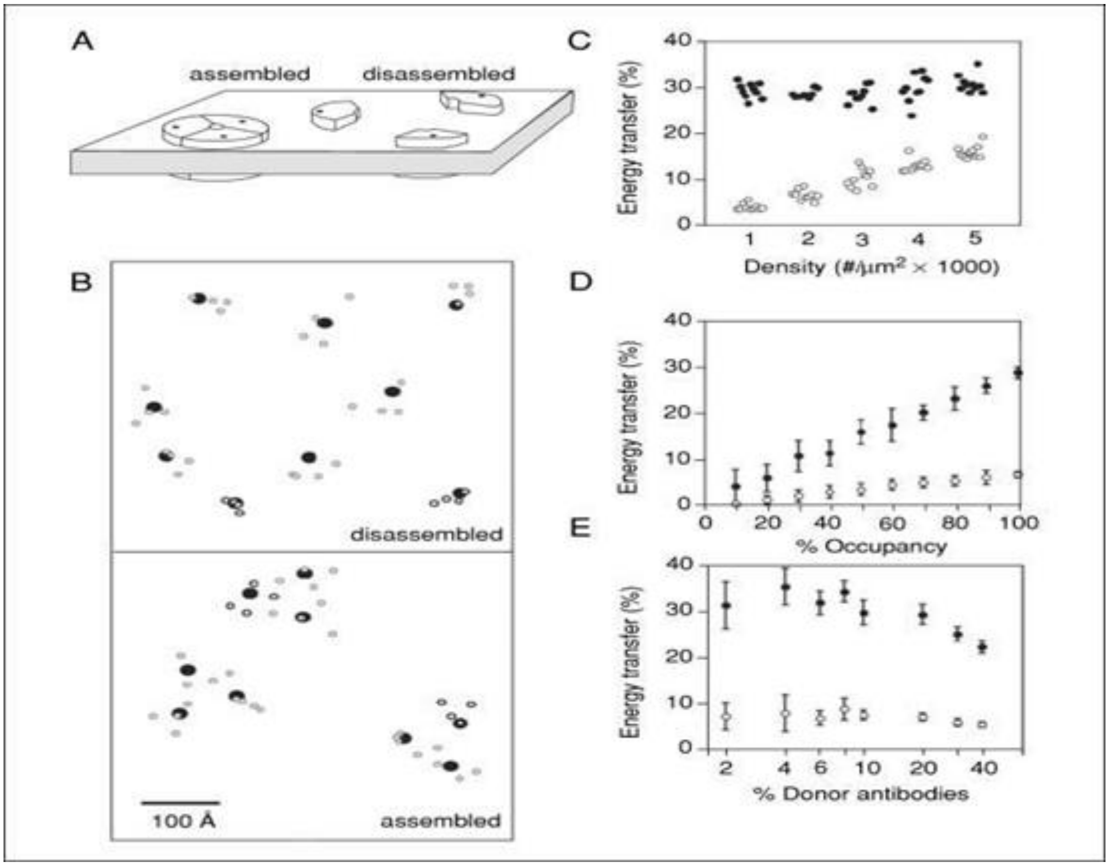


Figure (5) above shows Simulations of FRET for multiprotein complexes undergoing a conformational change. (**A**) Scale diagram of a complex of 90- versus 100-Å diameter. The modeled complex is of three antigens around a circle of the indicated diameter. (**B**) Clusters of three antigens in circles of diameters ranging from 9 to 11-nm were randomly distributed in 0.25 μm^2 areas, and the FRET between the bound antibodies calculated by Monte Carlo simulations.

The details of the simulation model system benefit from data on the size, structure, and abundance of the complex to be studied. Parameters to test include: simulated FRET between fluorophores with varying parameters such density of antigens (figure 5-c), percent occupancy of antigens by antibodies (figure 5-d), relative ratios of donor to acceptor antibodies (figure 5-e), proximities of protein subunits, and configuration of antigens. It is beyond the scope of this unit to provide a detailed guide for model design and simulation. For more background on designing molecular simulations, [35]

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