Time Resolved Fluorescence Spectroscopy

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This experiment was adapted by Prof P. Callis for Chem 326 (chmy374) from the following: Department of Chemistry MASSACHUSETTS INSTITUTE of TECHNOLOGY 5.33 Advanced Chemical Experimentation FALL SEMESTER 2003 Experiment # 3: Time-Resolved Spectroscopy http://ocw.mit.edu/NR/rdonlyres/Chemistry/5-33Advanced-Chemical-Experimentation-and-InstrumentationFall2002/AF5354F3-7E09-45BE-BDC6-2A5E14881E0C/0/Exp3.pdf

Revisions: April 10, 2007; March 24, 2011(Appendix B has errors); April 1 2013 (new Appendix B response curve measurement); March 23, 2014: general reorganizing; March 25, 2015: parts I and II only.

To the Instructor: Read this first. ***The laser used in this experiment, although not powerful, can in principle cause eye damage. Also, unlike most experiments in this course, there are several ways to destroy components costing thousands of dollars. It is imperative that you read the Appendix A on the critical procedures.***

Overview

Earlier in the semester, we used a conventional fluorometer to observe typical fluorescence and fluorescence excitation spectra of a dye to demonstrate some basic principles. We measured the rate of fluorescence quenching by iodide, which required knowledge of the fluorescence "lifetime". In this experiment, we will directly measure the fluorescence lifetime of a common dye molecule, and how quenching by electronic energy transfer to another dye molecule changes that lifetime. This experiment is performed with nanosecond time-resolved fluorescence measurements and uses ~$10,000 worth of components assembled from University Equipment Fee funds specifically for this course. These include a fast digitizing oscilloscope, a small nanosecond pulsed laser, and a fast photomultiplier tube, all assembled on a table (no black boxes).

The objectives are:

1) Make fluorescence quenching measurements—using fluorescence "lifetime" instead of quantum yield—for a pair of donor and acceptor dye molecules in solution, and compare to **F**örster **R**esonance **E**nergy **T**ransfer theory. This process is commonly known as **FRET**.

2) Work with fabricated scientific instrumentation that you can assemble by yourself.

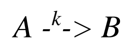
3) Learn and apply the mathematical procedures called ***convolution (and "deconvolution")***.

I. Background

A. Introduction to Time-Resolved Methods

Please review the introductory comments from your earlier experiment on the fluorescence of dyes in solution.

The study of the rates at which chemical processes, especially chemical reactions, take place has been an active area of research for many years. Consider a simple decomposition reaction (effectively irreversible),



$A \xrightarrow{k} B$

The rate constant k can be determined by simply measuring the rate of disappearance of A (or appearance of B). The maximum rate that can be measured is established by the amount of time that it takes to measure the amount of A (or B) present in the system. For example, one could not measure a decay rate of 1000 s^{-1} with a technique that takes 15 seconds to measure the amount of A present. The experiment would only determine that after 15 seconds all of A is gone.

Consider a more physical example that is closely related to the experiment which will be performed. Everyone should be familiar with the photography of Prof. Edgerton that produces very clear pictures of rapid processes like bullets tearing through bananas, etc. These pictures are taken by exposing film to a very short burst of light produced by a strobe light while the event of interest is taking place. Take, for example, the bullet impacting the banana. If 1 mm resolution is desired in the photograph, the strobe light must be on for much less time than it takes for the bullet to travel 1 mm. Otherwise, the image will be blurred.

In this experiment, the rate at which energy hops from an electronically excited dye molecule (a donor) to another (acceptor) molecule is to be measured. This process can take as little as five hundred picoseconds (1 ps = 10^{-12} s). Pulses of laser light will electronically excite molecules, and a fast fluorescence detector will be used to measure the fluorescence signal as a function of time following the pulse. Without the energy transfer, that decay will be exponential (because it is a ***first order*** process, i.e., depends only on the concentration of *excited* molecules. The decay will be faster and a different function of time when the energy also being transferred to the quencher dye. From the preceding discussion it is clear that these pulses must be less than several hundred picoseconds in duration, and the detector must be equally fast. In this experiment we will be limited to decay times on the order of 1 ns (1000 ps).

Fluorescence is closely related to UV-Vis absorption, and is used spectroscopically as a method

of probing the electronic structure of the electronically excited state. In addition, fluorescence is a valuable tool to measure the *dynamics* (time-dependent behavior) of electronic excited states. The fluorescence decay rate and lifetime not only provide physical information, but may serve as an additional signature of a particular molecule and/or its environment.

As just noted, the intensity of fluorescence ($\mathbf{I_f}$) is proportional ***only*** to the number of molecules in the excited state, N, which changes as molecules relax to the ground state

$I_f(t) \propto N(t)$ or more precisely: $dN/dt = - k_f N$ (1)

Note that for a 1st order process units do not matter, but of course they must be the same on both sides of the equation.

Thus, the process is ***first order*** in N. It is also a first order overall process, because only N is involved. Thus watching the intensity of fluorescence emitted by the sample as a function of time (typically a few nanoseconds) allows the relaxation rate to be measured directly. All chemists

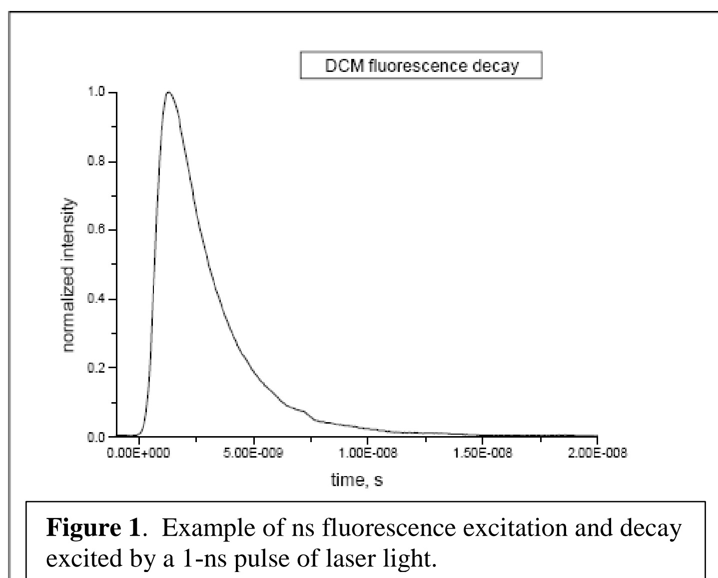


Figure 1. Example of ns fluorescence excitation and decay excited by a 1-ns pulse of laser light.

and physicists will memorize that the solution of this simplest and most common of differential equations gives ***exponential*** decay: $I(t) = I_0 \exp(- k_f t)$, or equivalently, $I_0 \exp(-t/ \tau_f)$. (3)

The fluorescence lifetime for decay to $(1/e)I_0$ is obviously, $\boldsymbol{\tau_f = 1/ k_f}$. An example of a time-resolved fluorescence decay is shown in Figure 1.

II. Experimental

A. Fluorimeter for Transient Measurements

We will help you to familiarize yourself with the optical alignment, shown in Figure 2, and show you how to use the oscilloscope and computer to acquire data. The fluorimeter consists of a laser that produces the "pump" light for exciting the donor molecules, and a detector arm that samples the fluorescence emitted by the sample. The excitation laser is a small JDS-Uniphase Neodymium-doped Yttrium Aluminum Garnet (Nd:YAG) laser that generates pulses of light

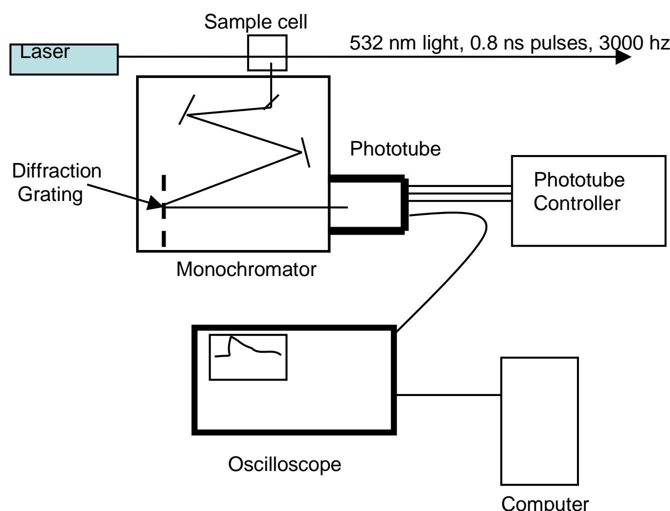


Figure 2. Schematic of lifetime apparatus.

approximately 0.8 ns long at λ = 532 nm. The pulses of light are emitted from the laser approximately every 330 µs (a 3 kHz repetition rate). Fluorescence from the sample is collected at 90 degrees relative to excitation. A lens is not needed to image the fluorescence onto the entrance slit of the monochromator in this experiment because the fluorescence is almost too bright to look at, and the cell can be placed near the slit. Filters allow residual excitation light or unwanted fluorescence frequencies to be blocked if necessary. However, the monochromator is set to allow only the desired wavelength range. The detector for transient fluorescence measurements is a fast phototube with approximately 0.8 ns time resolution. It will measure the amount of fluorescence emitted by the sample in a given time interval. This signal from the phototube is measured by a *digital storage oscilloscope* with approximately 1 ns time resolution. The oscilloscope trace is triggered by the periodic sudden rise of signal. The overall time-resolution of this instrument is dictated by the pulse-length, the detector speed (response time), and the oscilloscope speed, and can be estimated to be about 1 ns. In practice you can measure this directly by *(very carefully with instructor supervision)* scattering a small amount of the *excitation light only* into the detector. This produces what is called the ***instrument response***

function, or **IRF.**

B. Laser Safety

Some of the general dangers associated with other lasers – chemicals and power supplies – are not a concern with this small solid state laser. However, it is ***not a laser pointer*** of the type used in presentations. The energy is concentrated into 1 ns pulses, meaning the power is very high during the pulse. Therefore, the light emitted from this tiny laser (JDS Uniphase NG-10320-100, 3 micro Joule/pulse @3000 hz) still has a small potential of inflicting serious, permanent retinal damage if even a stray partial reflection should become focused on your retina. This is because it is *coherent and remains a beam even after reflections*. If the lens of your eye should happen to be such that a pulse of 1 micro Joule was focused on your retina, damage might occur. The probability of this is very low, but the policy in this lab that you always use common sense when working around the lasers. *****Special laser safety goggles must be worn when the laser power is on, even if the beam is blocked.***** (You don't know when someone may unblock the beam.) The danger comes only when the beam is being changed (e.g., placing a cell in the cell holder). These goggles have a high absorbance (optical density) of ~5 (meaning the transmittance is 10^{-5} for 532 nm light (a shade of green) that blocks the YAG laser light. Within the laser is an even more powerful IR beam having wavelength = 1064 nm (*invisible)*, safely contained within the laser, where it is passed through a crystal which creates the green beam at twice the frequency by a non-linear optical process. The green light is used in the experiment, so it is important that your eyes be protected from green light while the laser is on. The Instructors take special precautions to ensure that all of the beams stay in the plane of the optical table (i.e., no beams will be aimed at your eyes while you are standing). As a precaution, do not sit with your eyes level with the laser beam.

In contrast to the laser, the *fluorescence* emitted by the sample, even though extremely bright, poses no unusual danger of retinal damage (it is like the sun). The fluorescence is emitted in all directions and it is not possible for it to be concentrated once it has traveled several inches.

C. Procedure

1) Oscilloscope orientation and "observing" photons

Prior to making the transient fluorescence measurements, the Instructor will introduce the concepts of dark noise and shot noise. Dark noise is from individual electrons being thermally emitted from the photocathode (alkali metal), which is, naturally, proportional to the Boltzmann factor. It can be totally eliminated, therefore, by simple cooling the phototube to ~0 degrees F. *Shot noise* is noticeable for very weak light from the room when the slit is opened and will be seen as an increase of the signal pulses due to individual photons that happen to knock an electron out of the metal.

2) Measurements
We will make energy transfer measurements in a range of quencher (acceptor) dye concentrations based on a certain concentration, $\mathbf{C_0}$, which the Instructors *believe* to be about 10^{-3} M. (C_0 is that quencher concentration that will reduce the quantum yield by 50 %. It is related to R_0, which is the intermolecular distance at which there is a 50 % probability of energy transfer)

1. Make 6 solutions in ethanol for which the donor concentration is approximately 10^{-4} M, and the acceptor concentrations are more precisely: **0. , 2 x 10^{-3}, 5 x 10^{-4}, 1 x 10^{-4}, and 3 x 10^{-5}**. (The wider range of concentrations you have, the more precise your analysis will be. Keep the donor concentration constant for each solution and close to **10^{-4} M**, to avoid interfering donor-donor interactions.)

2. Position the monochromator so that a stable, maximum signal is obtained. It is best to have the light travel in a straight line perpendicular to the laser beam from the front of the cell into the monochromator. The signal strength should not be sensitive to small movements of the monochromator. The idea is to never have to move the monochromator during the experiment.

4. Obtain fluorescence decay curves for each of the 5 solutions.

5. Measure the instrument response function measuring the light scattered from a cell of clean, clear water with the monochromator set at **532 nm**. ***It is necessary to take great care not to damage the phototube when doing this by keeping the peak signal very similar to the low values used for the fluorescence!*** (the water scatters weakly, so the slit should be opened gradually to give a signal similar to that seen for the fluorescence.)

3) Notes on the Transient Fluorescence Measurements

(a)Alignment (if necessary).

Important: ALWAYS close the shutter on the laser while placing or removing cells from the holder. The cell surface could direct the laser beam into someone's eye. (This is only because someone might forget to have their goggles on.)

First, be sure the *slit is closed and high voltage is off.* Too much light on the phototube with the high voltage on can damage the phototube.

With the shutter closed on the laser, place a dye sample into the holder. Open the shutter and make sure the beam is passing through the middle of the cell. Fluorescence from the incident surface of the cell is lined up with the entrance slit of the monochromator by *translating the*

monochromator (not the cell).

The wavelength setting on the monochromator should be set to near the maximum of the emission spectrum of the R6G dye. There is no wavelength dial. ***With the high voltage off,*** remove the top. ***With the exit slit blocked to protect the phototube,*** visually verify that the fluorescence (or laser light when obtaining the IRF) is focused on the exit slit.

b) Measuring fluorescence decays of dye solutions.

The monochromator slit must be closed prior to turning on high voltage.

Ensure that the scope is set in a way to guarantee detection of the pulses.

Turn on the high voltage. The phototube voltage settings must not be too high or too low. The instructor will make these settings.

When the high voltage is on, the entrance slit should be opened very slowly to a very small width so that the ***signal is <500 mV or < 10 mA.*** The high time resolution of a few ns requires the input impedance be **50 Ω.**

NOTE: Unfortunately, the slit is sticky and does not shut immediately when it is set to zero width. Putting gentle sideways stress on micrometer can coax the slit to shut

For each of the donor/acceptor solutions prepared above, measure the transient donor fluorescence decay. Average the fluorescence trace over enough pulses that the trace on the oscilloscope is free of noise. Use a time base that starts ~5 ns prior to the pulse and ends after the **full decay to baseline** is captured on the screen. **Measure all solutions without changing the optics or alignment in the detection arm once it has been set.**

c) Measure instrument response ***after*** *measurement of dye solutions,* ***without changing settings if possible***. The monochromator setting should be changed to match the laser wavelength. As outlined above, the time resolution of the transient measurement is dictated by several elements in the experiment. To measure the instrumental response, which dictates the time scales of the fastest dynamics that can be observed, measure the laser light scattered from pure water, as directed in the Measurement section above. For measurements of fluorescence decays on time scales equivalent to the width of this response, deconvolution is necessary. (We will do this following directions in **Appendix B**). This is increasingly necessary as the acceptor concentration increases.

4) Data Analysis

1. Compare the signal from the laser light to that of the dark pulses observed with the slit closed and the laser off. What does this tell you?

The main objective of the experiment is to compare the values of R_0 determined by (a) Eq. 6, the spectral overlap integral **(may be <u>optional</u> at Instructor's judgment**), and (b) transient fluorescence measurements. This could involve comparison of how well the models for each determination of R_0 works. Be sure to include the details of calculations in your report. The following are some suggestions that may help in data analysis:

2. <u>R_0 from the spectral overlap integral (**OPTIONAL**).</u> This procedure is described in section III A. to calculate the spectral overlap integral and critical transfer distance starting with a fluorescence data file and an absorption spectrum in which the amplitude is expressed as the molar decadic extinction coefficient ε . You would probably need to make corrections to the input data sets – perhaps baseline correction -- prior to working up R_0 if they were not provided. In the calculations use 76,800 for the maximum extinction coefficient of NB, 0.95 for the donor quantum yield, $\langle\kappa^2\rangle = 2/3$, and n = 1.361 (the refractive index).
Note: the data files are given with constant increments between wavelengths. (not necessarily the same increment in the two spectra). However, the integral in Equation 6 uses cm^{-1} as the variable. It is most convenient to change the variable to wavelength in the integral ($d\nu/\nu^4 = -\lambda^2 d\lambda$). The units of λ must be cm.

3. <u>R_0 from the transient fluorescence decays.</u> The theoretical transient fluorescence decays for varying acceptor concentration are described by **eq. (8)**, and should be analyzed by performing a best fit to this form. Ideally a non-linear least squares fitting routine should be used to numerically vary the value of R_0 to find the best fit between your data and the set of curves predicted by equation 8. There are many ways to perform this fit, and you can consult your TA. It is acceptable here to do this by trial and error until a satisfying visual fit is found.

See Appendix B below for details for extracting the dye fluorescence decay curves and R_0.

In your report, be sure to include the final plots of the intensity decay curves, comparing the experimental and theoretical curves. Do this with one graph for each acceptor concentration (including 0), that plot both the experimental and theoretical result on the same graph for easy comparison.

III. Energy Transfer Theory

A. Background

For an isolated molecule in an electronically excited state, the mechanism of relaxation is primarily radiative. The molecule fluoresces in the process of returning to the ground state. This picture also applies to dilute solutions of dye molecules surrounded by solvent molecules. In more concentrated solutions, other relaxation pathways exist, which arise from intermolecular interactions between dye molecules. On distance scales of 10-100 Å, the motion of electrons in the excited electronic state on one molecule can exert a Coulomb force on another, allowing the excitation to “hop” from one molecule to another. The initially excited molecule is referred to as the donor (D), and the energy hops to the acceptor molecule (A). This process, referred to as resonance energy transfer, is shown schematically as

$$D^* + A \rightarrow D + A^* \qquad (4)$$

where the asterisk implies electronic excitation. Thus, relaxation of the donor to the ground electronic state is accompanied by excitation of the acceptor to its excited electronic state. The acceptor then finally relaxes to its ground state, typically by fluorescence. A complete description of this process is quantum mechanical (for instance, see Förster or Cohen-Tannoudji), but classical models provide most of the information that we need to build a physical picture of this process. The simplest picture is that of two oscillators (masses on springs that represent the donor and acceptor), which are coupled to one another by a third spring. Displacing one mass from equilibrium will lead a displacement of the second mass at a rate proportional to the force constant of the spring coupling the masses together. A demonstration will be provided.

The Coulomb interaction that leads to energy transfer is a dipole-dipole interaction. This is similar to a transmitter antenna communicating with a receiver antenna. If the distance between these two dipoles (or antenna) is R, then the strength of interaction (the potential) is proportional to $1/R^3$. You should review the dipole-dipole interaction in a P. Chem. text, like Atkins (see references), or in a general physics text.

Förster (see references) developed the theory for energy transfer between a donor and acceptor molecular dipole, and showed that the rate of energy transfer from donor to acceptor is proportional to $1/R^6$:

$$k_{D\to A} = \frac{1}{\tau_D}\left(\frac{R_0}{R}\right)^6 \qquad (5)$$

τ_D is the fluorescence decay time of the isolated donor molecule. The fundamental quantity in Förster's theory is R_0, the critical transfer distance. If a donor and acceptor are separated by R_0, there is equal probability that the electronically excited donor molecule will relax by fluorescence or by transfer to the acceptor. Equation 5 shows that the rates of energy transfer will change rapidly for R . R_0, which is typically between 10 Å and 100 Å. So if R_0 is known, measurement of energy transfer rates can be used for distance measurements on molecular scales (a "molecular ruler"). Förster showed that R_0 could be calculated from a few experimental observables:

$$R_0^6 = \frac{9000\ln(10)\phi_D\kappa^2}{128\pi^6 n^4 N}\int_0^\infty d\nu \frac{f_D(\nu)\varepsilon_A(\nu)}{\nu^4} \qquad (6)$$

*** The above equation has a famous, long-propagated error: π^6 *__should be π^5.__***

The integral – known as the overlap integral J_{DA} – is a measure of the spectral overlap of the fluorescence spectrum of the donor f_D, and the absorption spectrum of the acceptor ε_A. This integral indicates that for efficient energy transfer, resonance is required between the donor emission and acceptor absorption. ν represents units of frequency in wavenumbers (cm^{-1}). The fluorescence spectrum must be normalized to unit area, so that $f_D(\nu)$ will be in cm [= 1/(cm^{-1})]. The absorption spectrum must be expressed in molar decadic extinction coefficient units (liter/mol cm = $(M\ cm)^{-1}$), using Beer's law $A = \varepsilon_A(\nu) l C$, where A is the absorbance or optical density, $\varepsilon_A(\nu)$ is the molar decadic extinction coefficient for a specific frequency, C is the molar concentration, and l is the path length in cm. Here n is the index of refraction of the solvent and N is Avagadro's number. κ^2 is a constant that reflects the relative orientation of the electronic dipoles. It takes a value of 2/3 for molecules that are rotating much faster than the energy transfer rate. Φ_D is the donor fluorescence quantum yield. For R_0 in units of cm, eq. 6 is often written as:

$$R_0^6 = 8.8\times 10^{-25}\ \kappa^2\phi_D J_{DA}/n^4. \qquad (7)$$

For dye molecules in solution typical values of R_0 are 10-100 Å, and the rates of energy transfer $k_{D\text{-->}A}$ are often similar (0.1-1 ns^{-1}) to the rates of fluorescence for solutions with acceptor concentrations ~10^{-3} M. In this limit, two efficient decay channels exist for relaxation of an electronically excited donor: fluorescence and energy transfer to the acceptor. For a particular separation between donor and acceptor molecules, we would observe the fluorescence to decay exponentially with a rate given by the sum of rates for fluorescence and energy transfer. In solution, a distribution of donor-acceptor distances can exist, and the problem becomes more complicated (see below). The most important

requirement for energy transfer, shown in eq. 6, is that of resonance. The fluorescence spectrum of the donor represents the oscillation frequency of the excited donor dipole. This must match the frequency of electronic transitions of the acceptor from the ground to the excited state, i.e. the absorption spectrum. The amplitude of the overlap integral J_{DA} reflects the extent of this frequency matching. If this resonance between the excited donor and ground state acceptor is not present, energy transfer is not possible. Thus, this energy transfer mechanism is often referred to as Förster resonance energy transfer (or FRET). Resonance, in combination with vibrational relaxation, dictates that FRET is an energetically downhill process. The fluorescence of the donor is at a lower frequency than the donor absorption. Likewise, once energy is transferred from donor to acceptor, vibrational relaxation on the excited state of the acceptor dissipates more of the initially excited energy, and ensures that energy cannot hop back to the donor. In concentrated donor solutions, the partial overlap of donor fluorescence with donor absorption allows energy to hop from one donor molecule to another, before relaxing through other pathways.

> [More generally there are a number of energy transfer pathways that can exist for interactions between two electronic states: FRET, the exchange interaction, and radiative coupling. Radiative coupling, sometimes known as the trivial mechanism or two-step mechanism, represents emission of fluorescence by the donor followed by absorption of the fluorescence by the acceptor. This mechanism depends on sample length and is present on long distance scales or dilute solutions. The exchange interaction requires orbital overlap between the electronic states of the donor and acceptor and thus is operative on very short distance scales. For more discussion see Birks or Fleming.]

Resonance energy transfer is a particularly important process in photosynthesis and light harvesting (see references). Plants and photosynthetic bacteria use large arrays of chlorophyll and carotenoid molecules arranged in ring-like structures to absorb light and funnel the excitation through energy transfer processes to the reaction center, where the primary photosynthetic charge separation event occurs. In these light harvesting arrays, carotenoids and outermost chlorophylls absorb the highest energy light, and transfer these in a cascading process to other chlorophylls with lower energy near the reaction center. A variation of the protein environment about different chlorophyll molecules acts to tune the spectral overlap between the donor and acceptor chlorophylls in this array. The reaction center is itself built of chlorophyll molecules with even lower absorption energy.

Energy transfer measurements are now increasingly used as a molecular ruler in solution in which distances – for instance between amino acid residues and prosthetic groups in a protein – can be determined.

In this part of the experiment, measurements of energy transfer will be made on solutions of donor and acceptor dye molecules. We will use solutions of dilute donor concentration and variable acceptor concentration to vary the mean distance between donor and acceptor molecules. The critical transfer distance R_0 will be determined using

time-resolved fluorescence on these solutions. For such solutions, acceptor molecules are statistically distributed with varying number density (concentration) about donor molecules. While the population of donors decays due to energy transfer for any particular donor/acceptor pair by a rate given in eq. 5, a distribution of donor/acceptor separations exist, as do energy transfer rates. To obtain the time-dependent population of donors in such a solution, the energy transfer rate must be averaged over all R. This ensemble-averaging gives an expression for the time dependent decay of excited donor molecules that describes time-dependent fluorescence measurements

$$I_D(t) = \exp\left[-t/\tau_D - \sqrt{\frac{192\pi^3\langle\kappa^2\rangle t}{18\tau_D}}\, n_A R_0^3\right] \qquad (8)$$

*** Note that $n_A R_0^3$ is *not* under the radical**

where n_A is the number density of acceptor molecules (same units as $R^3{}_0$). Notice that the donor decay has two contributions, fluorescence from the donor and energy transfer to the acceptor. The donor fluorescence decays away exponentially, while the energy transfer terms decays as an exponential in the square-root of time. Eq. 8 allows R_0 to be accurately determined from a study of several solutions with varying acceptor concentration. The form of the transient acceptor fluorescence decay can also be seen from eq. 8. The acceptor fluorescence will decay with the fluorescence lifetime of the donor, but rise with the rate of energy transfer to the acceptor given by the second term in eq. 8. A description of these expressions are given in the original work by Förster and in Fleming.

Steady-state measurements of donor fluorescence in solutions of varying acceptor concentration can also be used to determine R_0. As the acceptor concentration is raised, the rates of energy transfer to the acceptors increase, the rate of donor fluorescence decay increases, and thus the total intensity of fluorescence from the donor decreases. With the solutions of varying acceptor concentration C, the integrated intensity of donor fluorescence I_D can be measured and compared to the intensity observed with no acceptor molecules I_0. The concentration dependence was shown by Förster to follow the form

$$\frac{I_D}{I_0} = 1 - \sqrt{\pi}\, x \exp(x^2)(1 - \mathrm{erf}\,(x)) \qquad (9)$$

where $x = C/C_0$. C_0 is the critical transfer concentration, which represents a concentration of acceptors such that, on average, one acceptor molecule exists within a sphere of radius R_0 from each donor molecule:

$$C_0 = \frac{3000}{4\pi N R_0^3} \qquad (10)$$

The error function, erf(x), is commonly used in statistical analyses and is included in most mathematical software packages and is also widely tabulated. While the steady state method requires no time-domain measurements it has some limitations, such as contributions from reabsorption that are difficult to correct for. (see Birks)

B. More Notes on Experimental Measurements of Energy Transfer

Overview and Objective: FRET measurements will be made on solutions of donor and acceptor dye molecules. The critical transfer distance R_0 will be determined using time resolved fluorescence and steady-state fluorescence measurements on these solutions.
The determined values of R_0 may be compared with the value calculated from eq. 6. To study the FRET mechanism, we will study acceptor molecules statistically distributed with varying number density (concentration) about donor molecules. By making solutions of dilute donor concentration and variable acceptor concentration we can vary the mean distance between donor and acceptor molecules

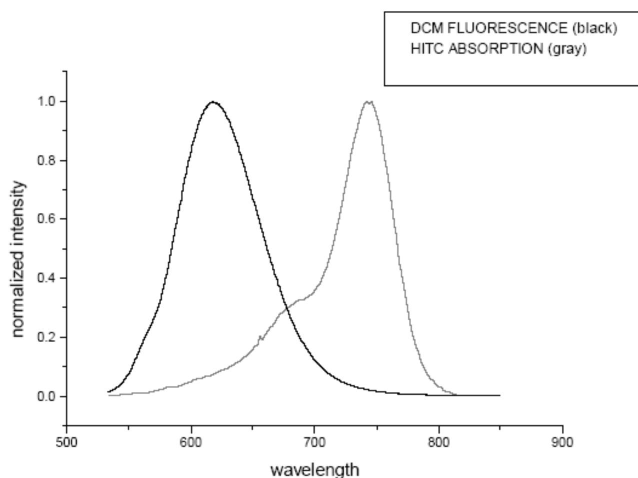


Figure 3. Example of overlap of fluorescence of donor (left) and absorption of acceptor (right) required for resonance energy transfer.

1) Donor/Acceptor Spectral Overlap

Generally, you would need to calculate the critical transfer distance for the donor/acceptor pair in advance of the energy transfer measurements. This is because you would not know the proper concentration range to use for the acceptor. In this lab, we will tell you a useful range of concentrations to use. You will calculate R_0 as part of your analysis to see if you were provided with concentrations that should result in observable energy transfer.

The donor molecule is Rhodamine-6G (R6G), and the acceptor is Nile Blue (NB). Any dye with a similar absorption spectrum could be used. Using dilute solutions of the individual molecules in ethanol, take absorption and fluorescence spectra of each. Absorption spectra are taken with the UV-Vis spectrometer. Be sure that you can express the absorption spectrum in molar decadic units (liter/mol cm). Fluorescence spectra may be taken with a fluorimeter.

Alternatively, you may be supplied the spectra in the form of data files in order to save time. R6G has been selected as a donor that absorbs the excitation light at 532 nm, and the acceptor should have a favorable absorption overlap with the donor fluorescence. (Also the acceptor absorption near 532 nm should be minimal. Why?) An example of donor fluorescence/acceptor absorption overlap is shown in Figure 3.

You may use the donor fluorescence and acceptor absorption spectra to calculate R_0 from eq. 6. The primary step is to calculate the spectral overlap integral. This can be done in a number of ways (devise your own method), but one of the simplest approaches is a numerical integration using the trapezoid approximation or Simpson's Rule in a spreadsheet. Any way that you do it, for this calculation you will have to do the following: (a) Convert the experimental donor absorption and acceptor fluorescence spectra to frequency in units of wavenumbers (cm^{-1}). (b) Then, using the concentration and path length from the acceptor absorption measurement, convert the absorbance to molar decadic extinction units (from Beer's law). (c) Normalize the amplitude of the fluorescence spectrum so that the numerically integrated area is unity. (d) These two spectra now allow you to calculate the overlap integral $J_{DA} =$

$$\int_0^\infty d\nu\, f_D(\nu)\varepsilon_A(\nu)\nu^{-4}$$

from their product divided by the fourth power of frequency, and integrated over the range of overlap. Since the experimental data sets will have different frequency axes, some interpolation will be needed to match them. The quantum yield is 0.95 for R6G in ethanol. Now from eq. 7, you can calculate R_0, and from R_0, we can calculate C_0 using eq. 10.

IV. References

The following are a variety of references (from the introductory to the specialized) that will help in the understanding and interpretation of the experiments.
* = Strongly recommended references for understanding the experiment.
† = Most can be found in the MSU library

† Atkins, P. W. *Physical Chemistry, 4th Edition*. W. H. Freeman & Co., NY (1990), pp. 655-661. **These pages review the dipole-dipole interaction.**

J. B. Birks, *Photophysics of Aromatic Molecules*, Wiley, 1970. **Chapter 11: Detailed discussion of energy transfer and other intermolecular interaction mechanisms for electronic states.**

† I. B. Berlman, *Energy Transfer Parameters of Aromatic Compounds*, Academic Press, New York, 1973. (Chapter 4) **Basic outline of the results of Förster theory, and lots of experimental data on absorption and fluorescence.**
Cohen-Tannoudji, C., B. Diu, et al., *Quantum Mechanics, Volume Two*, New York. John Wiley and Sons, 1977. **Quantum mechanical discussion of excitation hopping. Just for the extremely interested. See pages 1130-1139.**

*† G. R. Fleming, *Chemical Applications of Ultrafast Spectroscopy*, Oxford University Press, New York, 1986. **Discussion of time-resolved measurements in chemistry. In particular: Chapter 6.6 – energy transfer, Chapter 6.2 – rotational relaxation.**

Th. Förster, "Experimentelle und theoretische Untersuchung des zwischenmolecularen Uebergangs von Electronenanregungsenergie," *Z. Naturforsch*, **4a**, 321 (1949). **The derivation of eq. 8 (in German).**

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* † Th. Förster, "Transfer Mechanisms of Electronic Excitation," *Discussions Faraday Soc.* **27**, 7 (1959). **Excellent review of the results of his theories used in this experiment. Also see Fleming, Berlman, and Birks for this material.**

† Grondelle, R. v. (1985). "Excitation Transfer, trapping and annihilation in photosynthetic systems." *Biochimica et Biophysica Acta* **811**: 147-195. **Describes the mechanism of energy transfer in photosynthetic systems. Just for the interested.**

† Hunter, C. N., R. v. Grondelle, et al. (1989). Photosynthetic antenna proteins: 100 ps before photochemistry starts. *Trends in Biochemical Sciences***:** 72-76. **Describes the structure of the photosynthetic proteins and the time scale for energy transfer to the photosynthetic reaction center. Just for the interested.**
Svelto, O., *Principles of Lasers*, NY., Plenum, 1989. **Text on laser physics. Not required, but recommended for those who want to learn something about lasers.**

Two online references that are useful for getting data on the dye molecules used:
a) the Lambda-Physik Laser Dye Catalog at

http://www.lambdaphysik.com/ProductSpectrum/pdf/dyebook.pdf or
b) http://omlc.ogi.edu/spectra/PhotochemCAD/html/index.html. This is a great reference for spectral data on dyes, and for calculating overlap integrals. Look for their software.

Appendix A: Critical Procedures and Settings

1. The laser MUST be tightly (but not *too* tightly) constrained for two reasons. (a) It could cause eye damage if it were to be accidentally bumped
(b) It can overheat and be destroyed if it is not in tight contact with a heat dissipating object. Check the temperature with your hand during the first 30 minutes of operation after a new setup. It should not feel hot.

2. The phototube can be damaged, again in two ways:
(a) If the voltage controller wires are not connected correctly.

(b) If the phototube is exposed to room light, direct laser light, or overly intense fluorescence *while the high voltage is on* the tube may destroyed. Also, it is not good to expose the tube to bright light even if the voltage is off. It is okay to have it exposed to subdued room light for a few minutes at a time—*with the voltage off*.

Appendix B: Advanced Data Analysis: Convolution

(will be available "soon")