Experiment # 3: Time-Resolved Spectroscopy

Summary

In this experiment you will directly monitor electronic energy transfer between molecules in solution and the rotational motion of those molecules. These experiments are performed with nanosecond time-resolved fluorescence measurements, in addition to taking steady-state fluorescence spectra. The objectives are:

1) Make fluorescence quenching measurements for a pair of donor and acceptor dye molecules in solution, and compare to Förster energy transfer theory. This process is commonly known as FRET.

2) Make time-resolved measurements to watch the reorientational motion of molecules in solution. These measurements will be fit to the Stokes-Einstein-Debye theory to determine the effective molecular volume of the dye molecule.

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

4) Develop your own data analysis routines using commercial mathematical software packages.

Note: An understanding of the methods and a considerable amount of data analysis is required before the laser experiments are performed. It is essential that you read this handout and understand the material before beginning work on the experiment. Furthermore the recommended references should be read in advance also.

I. Background

A. Introduction to Time-Resolved Methods

The study of the rates at which chemical processes, especially chemical reactions, takes place has been an active area of research for many years. Consider a simple
decomposition reaction like $A \rightarrow B$. The rate $k$ 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 which produces very clear pictures of rapid processes like bullets tearing through bananas, etc. (If not, several photographs are on display at the MIT Museum. Be sure to pay them a visit.) 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 (ps = 10$^{-12}$ s). Pulses of laser light will initially electronically excite molecules and a fast fluorescence detector will be used to measure the rate of energy transfer. 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.

**B. Electronic Spectroscopy of Dye Molecules in Solution**

For dye molecules, absorption of light in the visible and ultraviolet induces $\pi^* \rightarrow \pi$ transitions to the first excited electronic state, $S_1$. The energy of this transition is $10^5$-$10^6$ cm$^{-1}$. The process of electronic excitation (a in Fig. 1) is extremely fast (<10$^{-15}$ s or 1 femtosecond, fs), so that the nuclei do not change position during this process. Excitation is accompanied by vibrational excitation, since the new electronic configuration has a new equilibrium nuclear configuration. Vibrational relaxation and reorganization of solute molecules about the newly excited state dissipate this excess energy. These nonradiative relaxation processes rapidly equilibrate the excited state ($\sim$10$^{-13}$–10$^{-12}$ s, or 0.1 to 1 picosecond, ps), dissipating roughly $10^2$-$10^3$ cm$^{-1}$ of energy (as heat). This

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* The units cm$^{-1}$, or wavenumbers, are used in place of Joules. Wavenumbers units are reciprocal wavelengths of light, related to the transition energy by the relationship $E=hc/\lambda$. 

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Laser-2
Laser-3

relaxation is pictured as process b in Fig. 1. Once in the potential minimum of the excited state, the system can only relax back to the ground state by dissipating a large amount of energy, typically >$10^4$ cm$^{-1}$. Due to this large energy gap between the well of the excited singlet state and the ground state, it is difficult, i.e. unlikely, that this energy will be dissipated nonradiatively as heat. The most likely mechanism of relaxation is fluorescence, in which the excited molecule radiates an optical field (c). The fraction of the initially excited molecules that relax to the ground state by fluorescence (as opposed to other channels) is known as the fluorescence quantum yield, $\phi_D$. The time scale of relaxation by fluorescence – the fluorescence lifetime – is typically much longer than the initial events ($10^{-10}$-$10^{-8}$ s, 0.1-10 nanoseconds, ns). Because of the vibrational relaxation process after excitation, and the displacement of nuclei in the excited state, fluorescence is always emitted at longer wavelength than absorption. The observed absorption and fluorescence spectra in solution typically exhibit mirror symmetry, with splitting in the spectral peaks, $2\lambda$, known as the Stokes shift. This quantity reflects the amount of energy dissipated by vibrational relaxation processes on absorption.

Fluorescence is used spectroscopically as a method of probing the electronic structure and dynamics of the electronically excited state. For gas phase molecules the fluorescence spectrum (fluorescence intensity as a function of frequency) is related to the quantum mechanical electronic structure of the molecule. In solution, the spectra are featureless, but fluorescence is a valuable tool to measure the dynamics (time-dependent

Figure 1 – Electronic states of a dye molecule in solution. Absorption of light (a) causes an electronic transition to the first excited state. This is followed by rapid relaxation (b) to a new expanded nuclear configuration. The system relaxes back to the ground state by fluorescence (c). The frequency of fluorescence is lower than the frequency of absorption.
behavior) of electronic excited states. The intensity of fluorescence $I_f$ is proportional to the number of molecules in the excited state, $N$, which changes as molecules relax to the ground state

$$I_f(t) \propto N(t) \quad (1)$$

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. A first order rate equation for the relaxation of the excited state population

$$\frac{dN}{dt} = -k_f N \quad (2)$$

implies that the intensity of fluorescence will damp exponentially

$$I(t) = I_0 \exp(-k_f t) \quad (3)$$

with the fluorescence decay rate $k_f$. The fluorescence lifetime for decay to $(1/e)I_0$ is $\tau_f = 1/ k_f$. An example of a time-resolved fluorescence decay is shown in Fig. 2.

**Figure 2** – Nanosecond time-resolved fluorescence decay.
II. Experimental

A. Fluorimeter for Transient and Steady-State Measurements

The TA will help you to familiarize yourself with the optical alignment and show you how to use the oscilloscope and computer to acquire data.

The fluorimeter consists of an excitation arm that introduces the pump light for exciting the donor molecules, and a detector arm that samples the fluorescence emitted by the sample. All fluorescence measurements will be made with the laser excitation source in Room 4-470, shown in Fig. 4. The excitation laser is a small Nd:YAG (Neodymium-doped Yittrium Aluminum Garnet) laser that generates pulses of light approximately 600 ps long at $\lambda = 532$ nm. The pulses of light are emitted from the laser approximately every $200 \mu s$ (a 5 kHz repetition rate). Prior to being focused into the sample, a polarizer vertically polarizes the light. Fluorescence from the sample is collected at 90 degrees relative to excitation. A polarizer in the detection arm allows the vertically ($\parallel$) or horizontally ($\perp$) polarized fluorescence to be measured. A lens images the fluorescence onto the detector and filters allow residual excitation light or unwanted fluorescence frequencies to be blocked.

The detector for transient fluorescence measurements is a fast photodiode with approximately 500 ps time resolution. It will measure the amount of fluorescence emitted by the sample in a given time interval. This signal from the photodiode is measured by an oscilloscope with approximately 400 ps time resolution.

![Figure 4 – Schematic of the fluorimeter for time-resolved and steady-state measurements. (pol: polarizer). Scattered fluorescence is detected at 90 degrees relative to excitation by the 600 ps pulses at 532 nm.](image-url)
oscilloscope trace is triggered by the emission of the pulse from the laser head. The overall time-resolution of this instrument is dictated by the pulse-length, the detector speed, and the oscilloscope speed, and can be estimated to be $\sqrt{600^2 + 500^2 + 400^2}$ ps $\approx 870$ ps. In practice you can measure this directly by scattering a small amount of the excitation light into the detector.

For steady-state measurements of fluorescence spectra, the fluorescence is focused on the slit of a spectrometer. A grating inside of it disperses it onto an array detector that allows you to see the entire fluorescence spectrum of your sample from the visible to the near-infrared.

For each detector, position the head approximately at the focus of the fluorescence and then use the micrometers to adjust the position of the detector so that the signal is maximized on the readout. A flat top to the detector response indicates that the detector is saturated, and the excitation intensity should be reduced or neutral density filters should be inserted to reduce the fluorescence signal.

B. Laser Safety

The new lab is being performed with a compact solid-state laser source. Some of dangers associated with other lasers – chemicals and power supplies – are not a concern with this experiment. However, the light emitted from this tiny laser still has the very real potential of inflicting serious, permanent harm to anybody entering the room in which they are contained when the laser is on. This is not a reason to fear it, but it is an extraordinarily good reason to respect them. Even a partial reflection of the beam that strikes your eye can potentially cause irreversible damage to your eye. For this reason, it is imperative that you always use common sense when working around the lasers. Special laser safety goggles must be checked out of the stock room for use with this experiment. These goggles have two different filters in them because there are two different colors, or wavelengths, of light that may be present in the room. A little infrared light – that you can’t see – is emitted from the laser head, and one of the filters in your laser goggles blocks this IR light. The second filter blocks out green light, which is obviously visible to your eyes. The green light is used in the experiment and it cannot be contained, so it is important that your eyes always be protected from green light. The TAs 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 your standing). As a precaution, do not sit with your eyes level with the laser beams, and close and block your eyes when bending down to pick things up off of the floor. You must wear your goggles at all
times. If you strictly adhere to these guidelines, you can be confident that the risk of harm to you is extremely small.

Fluorescence from the sample that we detect in the experiment is not a hazard. It should be possible to perform the experiment, which involves steering the fluorescence into a detector and optimizing the signal, without removing your safety glasses. If you feel uncomfortable working with the laser, bring the specific issues that bother you up with the TAs. They will give you a more thorough discussion of laser safety.

### III. Energy Transfer

#### 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 energy transfer, is shown schematically as

\[
D^* + A \rightarrow D + A^* 
\]

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. This picture is described in more detail in Appendix 1.
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 \(1/R^6\):

\[
k_{\text{D} \rightarrow \text{A}} = \frac{1}{\tau_{\text{D}}} \left( \frac{R_0}{R} \right)^6
\]

\(\tau_{\text{D}}\) 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 \(R \approx R_0\), 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:

\[
R_0^6 = \frac{9000 \ln(10) \phi_{\text{D}} \kappa^2}{128 \pi^6 n^4 N} \int_0^\infty dv f_{\text{D}}(v) \varepsilon_{\text{A}}(v) v^4
\]

The integral – known as the overlap integral \(J_{\text{DA}}\) – is a measure of the spectral overlap of the fluorescence spectrum of the donor \(f_{\text{D}}\), and the absorption spectrum of the acceptor \(\varepsilon_{\text{A}}\). This integral indicates that for efficient energy transfer, resonance is required between the donor emission and acceptor absorption. \(v\) represents units of frequency in wavenumbers (cm\(^{-1}\)). The fluorescence spectrum must be normalized to unit area, so that \(f_{\text{D}}(v)\) will be in cm \([= 1/(\text{cm}^{-1})]\). The absorption spectrum must be expressed in molar decadic extinction coefficient units (liter/mol cm = (M cm\(^{-1}\))), using Beer’s law \(A = \varepsilon_{\text{A}}(v) l C\), where \(A\) is the absorbance or optical density, \(\varepsilon_{\text{A}}(v)\) is the molar decadic extinction coefficient for a specific frequency, \(C\) is the concentration, \(l\) is the path length. Here \(n\) is the index of refraction of the solvent and \(N\) is Avagadro’s number. \(\kappa^2\) 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. \(\phi_{\text{D}}\) is the donor fluorescence quantum yield. For \(R_0\) in units of cm, eq. 6 is often written
For dye molecules in solution typical values of $R_0$ are 10-100 Å, and the rates of energy transfer $\kappa_{D \rightarrow A}$ are often similar (0.1-1 ns$^{-1}$) to the rates of fluorescence for solutions with acceptor concentrations $\sim 10^{-3}$ 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 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 enormous
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 the first 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 and steady-state fluorescence measurements 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

$$I_D(t) = \exp \left[ -\frac{t}{\tau_D} - \frac{192\pi^3 \langle k^2 \rangle t}{18 \tau_D} n_A R_0^3 \right]$$

where $n_A$ is the number density of acceptor molecules. 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

$$\frac{I_D}{I_0} = 1 - \sqrt{\pi x \exp(x^2)}(1 - \text{erf}(x))$$

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:

$$C_0 = \frac{3000}{4\pi NR_0^3}$$

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. 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 $R_0$ will 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.
1) **Donor/Acceptor Spectral Overlap**

In advance of your energy transfer measurements, you will need to calculate the critical transfer distance for your donor/acceptor pair. The donor molecule is R6G (see Table 1), and the acceptor is NB or alternatively MG can 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 in Room 4-472. Be sure that you can express the absorption spectrum in molar decadic units (liter/mol cm). Fluorescence spectra are taken with the fluorimeter described above. 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 your 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 5.

![Normalized Intensity vs Wavelength Graph](image)

**Figure 5** – Sample overlap between donor fluorescence and acceptor absorption.

<table>
<thead>
<tr>
<th>Table 1: Dye Molecules to be used as Donors and Acceptors:</th>
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<tbody>
<tr>
<td><strong>Donor:</strong></td>
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<tr>
<td>R6G</td>
</tr>
<tr>
<td><strong>Acceptor:</strong></td>
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<tr>
<td>NB</td>
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</table>
Use your 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. The recommended approach, which will help with the later calculations also, is to write a MatLab routine (on Athena) to evaluate $R_0$. An example of a MatLab routine that describes all the important steps, from which you can work with if you are learning, is in Appendix 2. Any way that you do it, for this calculation you will have to do the following: (a) Convert your experimental donor absorption and acceptor fluorescence spectra to frequency in units of wavenumbers ($\text{cm}^{-1}$). (b) Then, using the concentration and path length of from your 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 $J_{DA} = \int_0^\infty dv f_D(v) e_A(v) v^{-4}$ 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. You can typically find values for the donor quantum yield (typically 0.5-0.9) in some of the online resources in the references (how critical is this number?).

Now from eq. 7, you can calculate $R_0$, and from $R_0$, we can calculate $C_0$ using eq. 10. We will make energy transfer measurements in a range of acceptor concentrations centered on $C_0$. Make approximately ten solutions in ethanol for which the donor concentration is approximately $10^{-4}$ M, and the acceptor concentration spans a range of concentration from roughly $0.02 C_0$ to $2 C_0$. The wider range of concentrations you have, the more precise your analysis will be. Keep the donor concentration constant for each solution and well below $10^{-3}$ M, or donor-donor interactions will become important.

2) **Transient Fluorescence Measurements**

The transient measurements are made by detecting scattered light from the sample with a fast photodetector. The output of the detector is measured with a fast digital oscilloscope, which averages the fluorescence data before being read by the computer. The TA will demonstrate the optical alignment for these experiments, as well as the use of the oscilloscope and sampling software on the computer.

a) **Measure instrument response**. 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, use a piece of paper to scatter some excitation light into the detector. Use neutral density filters to reduce the amplitude of the signal on the oscilloscope to <500 mV. The observed transient is your instrument response function. For measurements of fluorescence decays on time scales equivalent to the width of this response, deconvolution is necessary. Appendix 3 discusses deconvolution of data taken with finite time resolution if this is necessary. This may be necessary if you measure solutions with very high acceptor concentration, or perhaps in the second part of the experiment.

b) Measure fluorescence decays on dye solutions. For each of the donor/acceptor solutions prepared above, measure the transient donor fluorescence decay. After placing the sample into the holder aim the fluorescence into the detector with the focussing lens. Prior to the detector, insert a bandpass filter that only transmits wavelengths at the donor fluorescence. Also insert an orange glass filter to remove any scattered pump light. Average the fluorescence trace on the oscilloscope with a time base that allows the full decay to baseline to be resolved. Measure all solutions without changing the optics or alignment in the detection arm.

If time allows you can also measure the fluorescence rise and decay on the NB acceptor molecules for the same solutions by replacing the bandpass filter by a red-wavelength-passing filter that only samples the acceptor fluorescence. (This will not work for MG, since it relaxes almost entirely nonradiatively).

3) Steady-State Fluorescence Measurements

The steady-state fluorescence measurements are made by detecting scattered fluorescence with a small spectrometer that is sampled by the computer. Place a solution of your donor molecule (no acceptor) into the sample holder to align the fluorescence detection. Use the focussing lens to focus onto the slit of the spectrometer by maximizing the real-time fluorescence signal on the computer. Remove any bandpass or red-pass filters. Neutral density filters can be used to attenuate the light if the spectrometer is saturated. Once aligned collect the fluorescence spectra of the donor molecule and all donor/acceptor solutions without disturbing the optics and alignment of the detection arm.

4) Data Analysis

The objective of this portion of the experiment is to compare the values of $R_0$ determined by (a) eq. 6, the spectral overlap integral, (b) transient fluorescence measurements, and (c) steady state fluorescence measurements. This will involve a
comparison of how well the models for each determination of \( R_0 \) works. Be sure to include the details of all three calculations in your report. The following are some suggestions that may help in data analysis:

1. **\( R_0 \) from the spectral overlap integral.** This procedure was described in section II.C.1. See Appendix 2 for a MatLab routine with detailed annotation that will 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 \( \varepsilon_A \). You will probably need to make corrections to the input data sets – perhaps baseline correction – prior to working up \( R_0 \).

2. **\( R_0 \) from steady-state fluorescence measurements.** The quenching of the donor fluorescence should be fit to eq. 9. First clean up your spectra by dropping extraneous data and/or performing a baseline correction as necessary. Determine the integrated intensity of the donor fluorescence in the pure donor (no acceptor) solution, and normalize the integrated donor fluorescence intensity of all other solutions to this value. Integration can be done using a spreadsheet, MatLab (as with the overlap integral), or another software package. (If you are using a spreadsheet you will need to use a polynomial approximation for \( \text{erf}[x] \)). Plot \( I/I_0 \) against \( C \) and fit to determine \( R_0 \). One value of \( R_0 \) (or \( x \) or \( C_0 \)) should pass through all data points. You should do this with a least squares fitting routine.

   The best (and recommended) approach would be to use a fitting routine from a software package like MatLab. Minimally you can use a spreadsheet, plot the data points and the calculated values from eq. 9, and then vary your value of \( R_0 \) in such a way as to find the minimum value of \( \chi^2 \). (\( \chi^2 \) is the sum of the differences between the values of the data and the equation at that \( C/C_0 \) point). Calculate the uncertainty in \( R_0 \) at the 95% confidence level according to the following equation

\[
S = 1.96 \sqrt{\frac{\chi^2}{N-1}}
\]

where \( S \) is your uncertainty in \( R_0 \) at the 95% confidence level, \( N \) is the number of data points (the number of donor/acceptor fluorescence spectra used in minimizing \( \chi^2 \)). More about the error analysis is described in Appendix B. Be sure to include the final plot of \( I_D/I_0 \) vs. \( C/C_0 \) (for your data and for the theoretical curve) in your lab report.

3. **\( R_0 \) from the transient fluorescence decays.** The transient fluorescence decays for varying acceptor concentration are described by eq. 8, and should be analyzed by performing a best fit to this form. The basic idea is the same as before: a 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. Again, there are many ways to perform this fit, and you can consult your TAs. Here are instructions on how to get started:

First clean up your oscilloscope traces by chopping off the steep rise due to the time response of the system. Make sure that your baseline has returned all the way to zero on all the traces or else you may need to retake your data. Next, normalize your raw data such that each donor or donor/acceptor trace has a maximum \( y \) value of 1. You may have to “translate” your data left or right so that the maximum \( y \) value (which had better be 1!) occurs at time \( t=0 \). Now find \( \tau_D \) from your donor trace, remembering that \( \tau_D \) is defined as the time it takes for the decay to reach \( 1/e \) of the maximum value. Simultaneously fit all of the normalized traces for varying concentration to eq. 8, by varying \( R_0 \) to minimize \( \chi^2 \). Also, determine the uncertainty in \( R_0 \) at the 95% confidence level (eq. 15). Be sure to include the final plots of \( I_D(t) \) (for your data and for the theoretical curves) in your lab report.

IV. Reorientational Motion

A. Background

When a molecule is electronically excited, the transition of electrons between orbitals leads to a new charge configuration on the molecule. The change of electron density on the molecule can be pictured (and formally described) as the creation of a dipole (a vector) with a well-defined orientation. This oscillating dipole – essentially an antenna or transmitter – radiates the fluorescence that we observe. Since the dipole has a well-defined orientation, if the molecule rotates as it fluoresces, we expect that the polarization of the fluorescence will change with the rotation.

In this second part of the experiment, we will measure the rotation of a dye molecule in solution, using time-resolved fluorescence measurements in which the different polarization components of the fluorescence are observed in time. This will tell us the average rate at which molecules rotate in the liquid. Unlike the gas phase, rotation in this case does not have to be treated quantum mechanically. The solvent about the molecule exerts a force on the molecule related to the solvent viscosity, which restricts it from spinning freely. Rather, the problem is one of classical diffusion (Brownian motion). The molecular orientation changes gradually by small random steps as the solvent molecules fluctuate about the solute.
The theory of such rotational motion for a spherical object in a fluid is attributed to Debye, Stokes, and Einstein (DSE) working separately. The DSE theory predicts that if you could align a collection of molecules in a liquid and then watch the orientation randomize with their rotational motion, the net alignment would decay away exponentially with a characteristic time scale $\tau_{\text{rot}}$:

$$A(t) = A(0) \exp(-t / \tau_{\text{rot}}) \quad (11)$$

Furthermore, $\tau_{\text{rot}}$ is related to the rotational diffusion constant $D$, which can further be related to the volume of the molecule $V$ and the viscosity of the liquid $\eta$:

$$\tau_{\text{rot}} = \frac{1}{6D} = \frac{V\eta}{kT} \quad (12)$$

So, if you measure the rotational relaxation of a molecule in solutions of varying viscosity, you can determine $V$. ($V$ in fact is an effective molecular volume (or hydrodynamic volume), which usually is somewhat larger than the volume from crystal structures). Such methods can be used to measure the radius of gyration of a polymer or protein. We will measure $\tau_{\text{rot}}$ of a dye molecule in solutions made with varying ethanol/glycerol composition to vary the viscosity, and thereby determine the effective molecular volume of the dye molecule.

How do you measure the reorientational motion of molecules with fluorescence? In an equilibrium solution the orientation of molecules is isotropic – equally distributed in all directions. We need a method of breaking this isotropy (introducing an anisotropy) and watch how the system relaxes back to equilibrium. This can be done using polarized light for exciting the dye molecules and then watching the polarization of the fluorescence emitted by the sample afterward. A polarizer in the experiment sets the polarization (direction of the oscillations of the electric field) of the excitation light vertical relative to the table before it enters the sample. Since the amplitude of the electric field is oriented, it will preferentially excite those molecules aligned along the polarization. If those molecules, preferentially aligned vertically, fluoresce without rotating, then the polarization of the emitted fluorescence will be preferentially vertical also. On the other hand, if the molecules rotate before fluorescing, the polarization of the fluorescence will have a horizontal component. So if we place a polarizer before the detector and measure fluorescence relaxation such that it transmits vertically polarized fluorescence the signal should decay with $\tau_{\text{rot}}$, whereas if the polarizer transmits horizontally polarized light, fluorescence should rise in with $\tau_{\text{rot}}$. The detection geometries are known as parallel and perpendicular respectively. The decays of course
are also influenced by the fluorescence lifetime, which doesn’t change as a function of the polarizer orientation. If we measure the fluorescence decay in the parallel $I_\parallel(t)$ and perpendicular $I_\perp(t)$ configurations,

$$I_\parallel(t) = \exp(-t/\tau_r)\left(1+\frac{4}{5}\exp(-t/\tau_{\infty})\right)$$

$$I_\perp(t) = \exp(-t/\tau_r)\left(1-\frac{2}{5}\exp(-t/\tau_{\infty})\right)$$

we can remove the fluorescence lifetime component by calculating the time-dependent anisotropy:

$$r(t) = \frac{I_\parallel(t) - I_\perp(t)}{I_\parallel(t) + 2I_\perp(t)}. \quad (13)$$

The anisotropy should decay only as a function of the rotational relaxation time

$$r(t) = r(0) \exp(-t/\tau_{\text{rot}}). \quad (14)$$

These measurements are discussed in Chapter 6 of Fleming. An example of these measurements is given in Figure 3, showing the individual scans and the calculated

![Figure 3 – Parallel (dashed) and perpendicular (dotted) fluorescence decay curves, and the calculated anisotropy, which decays exponentially with the rotational relaxation time.](image-url)
anisotropy. Notice that when the rotational relaxation is somewhat faster than the fluorescence decay, the tails of each decay (when reorientation is complete) match up exactly with one another.

**B. Experimental Measurements of Reorientational Motion**

Briefly, observe the transient fluorescence of a dilute \((10^{-5}-10^{-4} \text{ M})\) solution of Rhodamine 6G. Solutions with varying weight % of glycerol in water \((100\%-20\%)\) should be made. Insert a film polarizer before the fast photodetector and orient the polarization transmission to be parallel and then horizontal to the table. Take both fluorescence decays without changing anything except the polarizer orientation. Check to see that the two decays match at long time. Repeat for several samples. Then calculate the anisotropy (eq. 13) for each solution and fit the decay of the anisotropy to an exponential to extract \(\tau_{\text{rot}}\) (eq. 14). Using the viscosity of the solutions, fit the \(\tau_{\text{rot}}\) data to eq. 12 to determine the effective molecular volume \(V\). Values of the viscosity can be extrapolated from the data in Table 2.

**Table 2. Viscosity data for water-glycerol solutions.**

<table>
<thead>
<tr>
<th>% of Glycerol by Weight</th>
<th>Grams per liter</th>
<th>Viscosity in CPu (mN·s·m(^2)) (T = 20^\circ\text{C})</th>
<th>Viscosity in CPu (mN·s·m(^2)) (T = . 25^\circ\text{C})</th>
</tr>
</thead>
<tbody>
<tr>
<td>0%</td>
<td>0</td>
<td>1.0001</td>
<td>0.89</td>
</tr>
<tr>
<td>10%</td>
<td>102.2</td>
<td>1.307</td>
<td>1.149</td>
</tr>
<tr>
<td>25%</td>
<td>265.0</td>
<td>2.089</td>
<td>1.805</td>
</tr>
<tr>
<td>50%</td>
<td>563.2</td>
<td>6.032</td>
<td>5.024</td>
</tr>
<tr>
<td>80%</td>
<td>966.8</td>
<td>61.8</td>
<td>45.72</td>
</tr>
</tbody>
</table>
III. References

The following are a variety of references (from the required to the specialized) that will help in the understanding and interpretation of the experiments.

* = Strongly recommended references for understanding the experiment.
† = References on reserve.


† 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.


* † 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.

Describes the mechanism of energy transfer in photosynthetic systems. Just for the interested.


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](http://www.lambdaphysik.com/ProductSpectrum/pdf/dyebook.pdf) or

b) [http://omlc.ogi.edu/spectra/PhotochemCAD/html/index.html](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 1 – Two Coupled Oscillators

If the solution is irradiated with light of the proper wavelength, some fraction of the dye molecules in the solution will be promoted to an electronic excited state. The two main processes by which the excited molecules can relax are fluorescence, a process by which the excited dye monomer gives off a photon and returns to the ground state, and interaction with another dye monomer in the ground state. The second of these two processes is much more rapid, and its effect will be considered first. A small number of excited donor molecules are surrounded by a large number of unexcited acceptor molecules. Although these dye molecules are electrically neutral, motion of the electrons results in Coulomb forces acting between the molecules. The result of this interaction is that the excitation can “hop” from donor to acceptor. To completely describe how and why this interaction leads to excitation hopping, it is necessary to examine quantum mechanically the dynamic behavior of interacting electrons (see the textbook by Cohen-Tannoudji et al. (1977) for some heavy-duty quantum mechanics). To get a physical feeling for the process, a simple classical model can be used to understand energy hopping in an analogous system, keeping in mind, however, that the details of the interaction are different for molecular excitation transfer.

Imagine the system of springs pictured in Figure A1:

![Two coupled oscillators](image)

**Figure A1:** Two coupled oscillators.

Mass #1 represents the donor molecule and mass #2 represents the acceptor. Spring #3 represents an interaction between the two molecules. It is important to realize that this is a drastic oversimplification of the behavior of molecules, but this model still captures the fundamental physics of energy transfer. If mass #1 were pushed and set into motion, mass #2 would not remain stationary while mass #1 oscillated. Figure 3 illustrates the time
dependence of the displacements of the two masses. The displacements of mass #1 \((x_1)\) and mass #2 \((x_2)\) are represented in Figure A2 by the solid and dashed lines respectively. It is seen that the oscillations of mass #1 gradually decrease while the oscillations of mass #2 grow larger until mass #1 is essentially at rest and mass #2 is reaching its maximum displacement. The situation then reverses itself until mass #2 is essentially at rest and mass #1 is reaching its maximum displacement. The energy of the system sloshes back and forth between the oscillations of mass #1 and mass #2. A mathematical treatment of this model is given in appendix B where it is shown that the frequency at which this energy sloshes from one mass to another is proportional to \(k'\), the spring constant of the spring which couples the two masses together.

![Figure A2: Time evolution of two coupled oscillators.](image)

Pushing mass #1 is analogous to exciting a donor with light. The donor remains excited for some period of time that inversely depends on the interaction strength between the excited donor and neighboring, ground state acceptor. Then the excitation hops to the acceptor, much like the oscillations which hop from one mass to another in the model described above. The donor that was initially excited falls to its ground state and the acceptor onto which the excitation hopped is promoted to its first excited state. This process is generally called energy transfer.
Appendix 2 –
Calculation of $R_0$ from Spectral Overlap with MatLab®

This routine was written by Joshua Vaughan, Fall 2000.

% This program will calculate the quantity $R_0$ for FRET between given
% donor and acceptor molecules. The user will need to supply a file
% containing the fluorescence spectrum of the donor (two columns, the
% first with the wavelength range in nm, the second with the intensity
% of the fluorescence) and a file containing the absorption spectrum
% of the acceptor (also two columns, the first with the wavelength in
% nanometers, and the second with the absorption of the acceptor in
% molar decadic extinction coefficients).

% It is assumed that the user will have already cleaned up these spectra by
% performing any necessary baseline corrections, and by chopping the data,
% and including zeros in place of the chopped data. The user will also need
% to supply the refractive index of the solvent used, the quantum yield of
% the donor, and the parameter $K$ (where $K=k^2$), which is usually 2/3.

% NOTE: since the absorbance spectrum is being interpolated at the set of
% fluorescence x-values, the absorbance spectrum must contain at least the
% same range of x-values that the fluorescence spectrum contains, even if
% it means adding some fake x values for which the corresponding y value
% is zero. One way to do this would be to use absorbance data that ranges
% from 500-800 nm, and fluorescence data that ranges from 532-790 nm.

q=.95;       % Enter in the quantum yield of the donor
K=2/3;       % Enter in the value K (usually 2/3)
n=1.361;

% Before Matlab can load your file, you must change the current directory to
% the directory containing your data (you can use unix commands to do this).

f=load('dcm_fl.txt');  % Load in the fluorescence spectrum
a=load('mg_abs.txt');  % Load in the absorption spectrum

% Interpolate the absorption curve at the set of fluorescence x values.
a1=[f(:,1) interp1(a(:,1),a(:,2),f(:,1))];

f1=[(1e7)*f(:,1).^(-1) f(:,2)]; % Rewrite the x axis in wavenumbers
a2=[(1e7)*a1(:,1).^(-1) a1(:,2)]; % Rewrite the x axis in wavenumbers
A=sum(diff(f1(:,1)).*f1(2:end,2)); % Calculate area of fluorescence curve
f2=[f1(:,1) f1(:,2)/abs(A)]; % Unit normalize the fluorescence. Note that
% this means the fluorescence is now in units
% of reciprocal wavenumbers, or cm.

% Calculate the overlap function $O$.
O=[f2(:,1) (a2(:,1).*f2(:,2))./(f2(:,1).^4)];

% Calculate the overlap integral $J$.
J=sum(abs(diff(f2(:,1))).*O(2:end,2));

% Calculate $R_0$ [cm].
Ro=((8.8e-25)*(K*q/(n^4))*abs(J))^(1/6)
Appendix 3 – Convolution

Determining the fluorescence decay rate from dye solutions becomes more difficult at high concentrations. We are using a photodetector with approximately 0.8 ns time resolution, and as the effective fluorescence decay time we are trying to measure approaches this value, the real fluorescence data becomes masked by the detector.

The experimental observable is determined both by the detector time-resolution and the time scale of the fluorescence relaxation. More generally, an experimental measurement is an integral over the instrumental response $R(t)$ – the detector time resolution – and the response from the sample $S(t)$ – here the fluorescence decay. The observed signal – the time-resolved fluorescence intensity – is termed a convolution integral:

$$I(t) = \int R(t - t')S(t')dt'$$

(1)

This essentially is the fluorescence relaxation summed over the profile of the detector response. When the fluorescence decay is much faster than the detector response, $S(t)$ approaches a delta function and all we see is the detector. When the detector is much faster than the fluorescence relaxation, $R(t)$ approaches a delta function and our signal looks purely like the fluorescence. When the two exist on similar time scales, we can still extract information on our decay by comparing our fluorescence data with our instrument response. Suppose we model our detector time resolution by a Gaussian function with width $\sigma$,

$$R(t) = A_1 \exp\left(-\frac{t^2}{2\sigma^2}\right)$$

(2)

and our fluorescence decay by an exponential with decay time $\tau$,

$$S(t) = A_2 \exp\left(-\frac{t}{\tau}\right)$$

(3)

Evaluating our convolution integral (and dropping constants), we obtain

$$I(t) = \exp\left(\frac{\sigma^2}{2\tau^2} - \frac{t}{\tau}\right) \left[1 - \text{erf}\left(\frac{\sigma^2 - \tau t}{\sqrt{2}\sigma\tau}\right)\right]$$

(4)

Here, $\text{erf}(x)$ is the error function, which is a standard function in most mathematical software packages. ($\text{erf}(0)=0; \text{erf}(\infty)=1$).
What does this mean for our experiment? Suppose we are trying to resolve a fluorescence decay with an effective decay constant of $\tau$ with a detector response width of $\sigma$. The linear and semi-log figures above show our calculated convolution integral for values of $\sigma/\tau$ from 0.1 to 3, compared to the exponential decay (dashed line). For small $\sigma$, we just observe the exponential relaxation, but as $\sigma$ gets larger, the observed signal approaches the detector response and we cannot extract a value.

In another comparison, we can show the exponential fluorescence decay that we are trying to recover (dashed line) from the observed signal (solid) measured with a detector response (dotted) of $\sigma=3\tau$, $\tau$, and $0.3\tau$. Clearly one cannot get a meaningful measurement for $\sigma=3\tau$, but by measuring the relaxation in the tail one can extract the decay for the other cases. (Notice that one can also measure the shift of the peak of the signal relative to the peak of the detector response to give some idea of the decay time).