

## MASSACHUSETTS INSTITUTE of TECHNOLOGY

### Department of Chemistry

### 5.33 Advanced Chemical Experimentation

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## Experiment # 3: Time-Resolved Electronic 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) Learn the physical principles behind Förster energy transfer theory (FRET), a process commonly used in biochemical assays, imaging, and an important part of photosynthesis. Fluorescence quenching measurements will be made for a pair of donor and acceptor dye molecules in solution, and compare to FRET theory.
- 2) Make time-resolved measurements to watch the reorientational motion of the dye eosin bound to the protein lysozyme. These measurements will be fit to the Stokes-Einstein-Debye theory to determine the effective molecular volume of the complex.
- 3) Work with fabricated scientific instrumentation that you can assemble by yourself.
- 4) Perform data fitting and analysis 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  $A \xrightarrow{k} B$ . 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  $1000 \text{ s}^{-1}$  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 1mm. Otherwise, the image will be blurred.

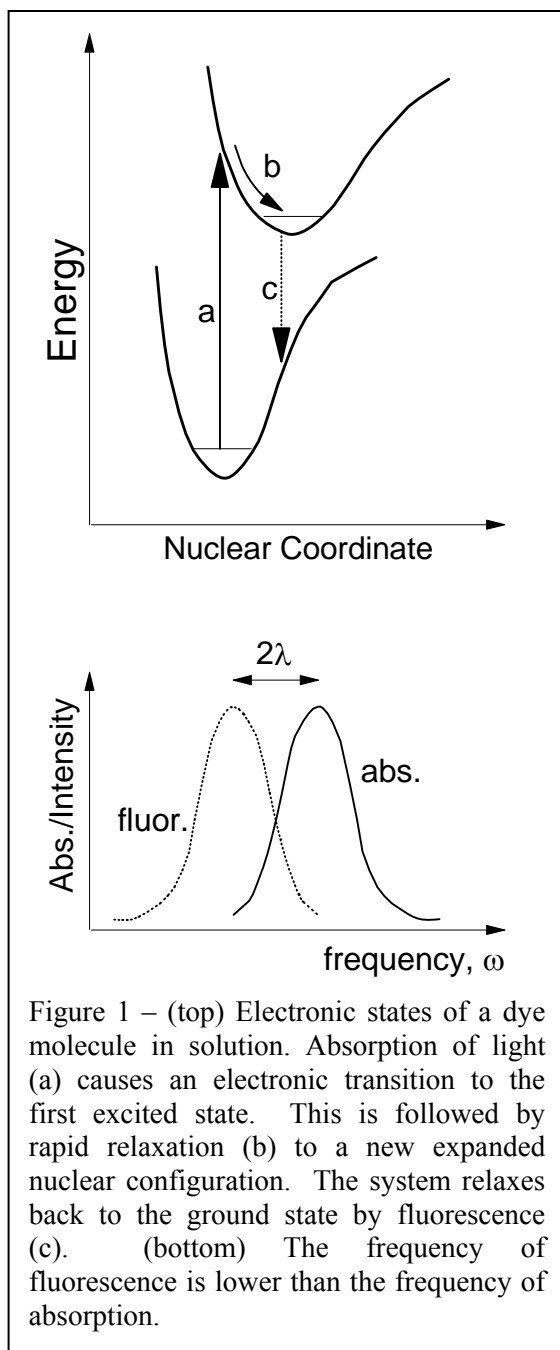
In this experiment, the rate at which energy hops from an electronically excited dye molecule (a donor) to another (acceptor) molecule is to be measured. This process can take as little as five hundred picoseconds ( $\text{ps} = 10^{-12} \text{ s}$ ). Pulses of laser light will initially electronically 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  $\pi^* \leftarrow \pi$  transitions of electrons between the occupied ground electronic state  $S_0$  and the first excited electronic state,  $S_1$ . The energy of this transition is  $10^4$ - $10^5 \text{ cm}^{-1}$ .<sup>\*</sup> The process of electronic excitation (**a** in Fig. 1) is extremely fast ( $<10^{-15} \text{ s}$  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 ( $\sim 10^{-13}$ - $10^{-12} \text{ s}$ , or 0.1 to 1 picosecond, ps),

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



dissipating roughly  $10^2$ - $10^3$   $\text{cm}^{-1}$  of energy (as heat). This 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  $>10^4$   $\text{cm}^{-1}$ . 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 light at the downward energy gap (**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

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

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

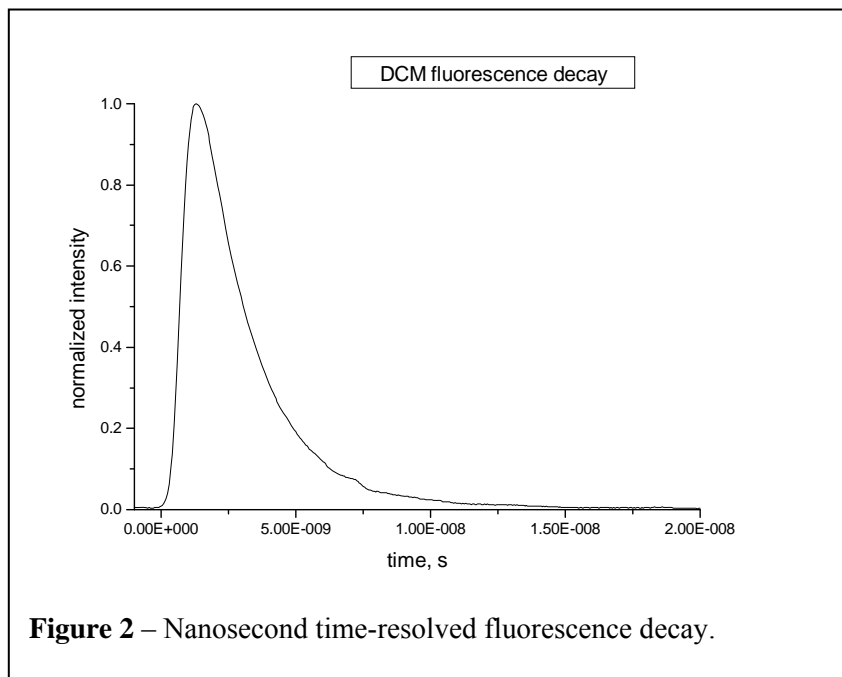
Thus watching the intensity of fluorescence emitted by the sample as a function of time (typically a few nanoseconds) allows the relaxation rate to be measured directly. A first order rate equation for the relaxation of the excited state population

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

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 is  $\tau_f = 1/k_f$ . An example of a time-resolved fluorescence decay is shown in Fig. 2.



## 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. 3. The excitation laser is a small Nd:YAG (Neodymium-doped Yttrium 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 1 ns time resolution. The oscilloscope trace is triggered

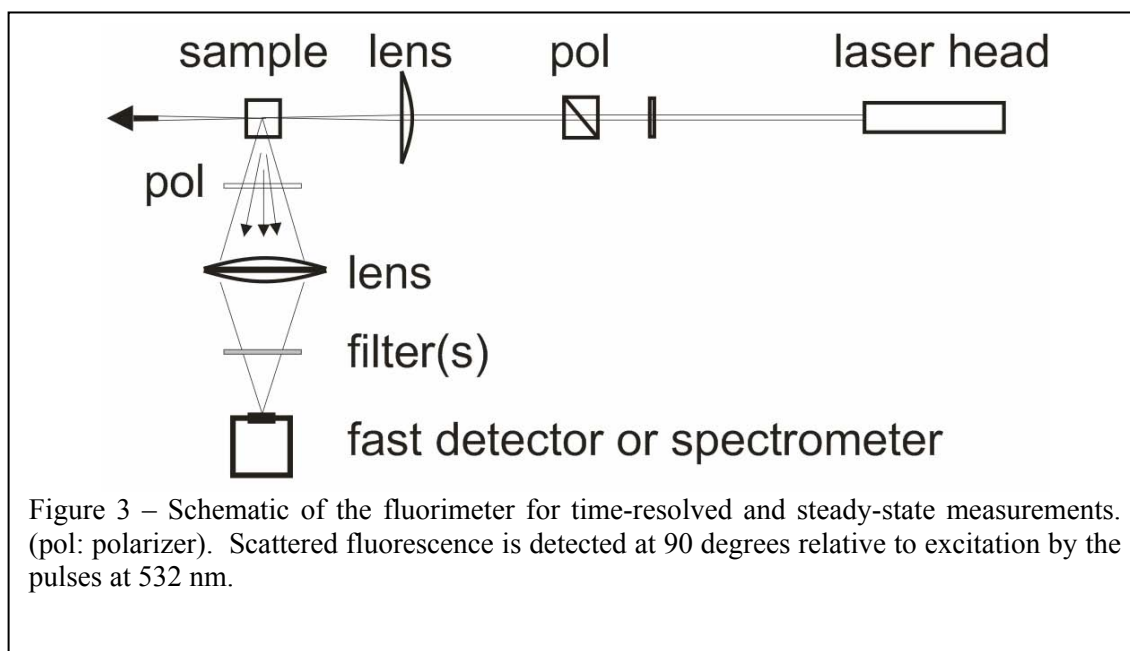


Figure 3 – Schematic of the fluorimeter for time-resolved and steady-state measurements. (pol: polarizer). Scattered fluorescence is detected at 90 degrees relative to excitation by the pulses at 532 nm.

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 is about 1 ns. 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



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  $1/R^6$ :

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

Here  $\tau_D$  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. Overall we can write the rate of excited donor relaxation as a sum of donor fluorescence and energy transfer rates:  $k_D = k_f + k_{D \rightarrow A}$ . When  $R$  is much shorter than  $R_0$  then  $k_D \approx k_{D \rightarrow A}$  and the efficiency of FRET is high. When  $R$  is much longer than  $R_0$ , the efficiency of energy transfer is low and relaxation of the donor is through fluorescence:  $k_D \approx k_f$ .

Equation 5 shows that the rates of energy transfer will change rapidly for  $R \approx R_0$ . 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:

$$R_0^6 = \frac{9000 \ln(10) \phi_D \kappa^2}{128 \pi^5 n^4 N} \int_0^\infty d\nu \frac{f_D(\nu) \epsilon_A(\nu)}{\nu^4} \quad (6)$$

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  $\epsilon_A$ . This integral indicates that for efficient energy transfer, resonance is required between the donor emission and acceptor absorption.  $\nu$  represents units of frequency in wavenumbers ( $\text{cm}^{-1}$ ). The fluorescence spectrum must be normalized to unit area, so that  $f_D(\nu)$  will be in  $\text{cm} [ = 1/(\text{cm}^{-1})]$ . The absorption spectrum must be expressed in molar decadic extinction coefficient units ( $\text{liter/mol cm} = (\text{M cm})^{-1}$ ), using Beer's law  $A = \epsilon_A(\nu) l C$ , where  $A$  is the absorbance or optical density,  $\epsilon_A(\nu)$  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.  $\phi_D$  is the donor fluorescence quantum yield. For  $R_0$  in units of cm, eq. 6 is often written



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

For dye molecules in solution typical values of  $R_0$  are 10-100 Å, and the rates of energy transfer  $k_{D \rightarrow A}$  are often similar (0.1-1 ns<sup>-1</sup>) 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 expect 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). [Note, a common misnomer is to call this “fluorescence resonant energy transfer” which incorrectly implies that the process involves absorption of donor fluorescence by the acceptor.]

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. 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 also commonly used in biochemical assays and imaging for revealing the proximity of proteins or enzyme activity.

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 possible donor-acceptor separations. This averaging process gives an expression for the time dependent decay of excited donor molecules for our measurements

$$I_D(t) = \exp\left[-\frac{t}{\tau_D} - 2\gamma\sqrt{\frac{t}{\tau_D}}\right]. \quad (8)$$

Here  $\gamma = \sqrt{2\pi^3 \langle \kappa^2 \rangle n_A R_0^3} / 3$  and  $n_A$  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 total donor fluorescence in the absence of acceptor,  $I_0$ . (*Alternatively, measure  $I_D$  relative to the total fluorescence from donor+acceptor. Why measure this? Ask your TAs*) The concentration dependence was predicted by Förster to follow the form

$$\frac{I_D}{I_0} = 1 - \sqrt{\pi} \gamma \exp(\gamma^2)(1 - \text{erf}(\gamma)) \quad (9)$$

where  $\gamma = C/C_0$ .  $C_0$  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. If  $R_0$  is expressed in cm, then

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

Note that the  $\gamma$  used in eq. 8 and eq. 9 in fact represent the same quantity. There are some slight differences, because of the different assumptions made in deriving these expressions. Eq. 9 was written for non-rotating molecules. In equation 9, the error function,  $\text{erf}(x)$ , is commonly used in statistical analyses and is included in most mathematical software packages and is also widely tabulated.

## **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

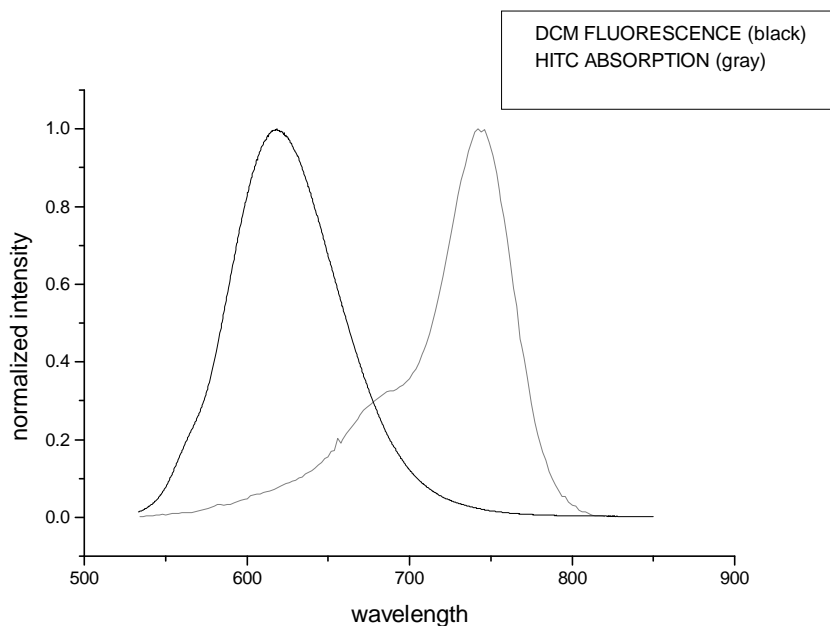


Figure 4 – Sample overlap between donor fluorescence and acceptor absorption.

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 Rhodamine 6G Perchlorate (R6G), and the acceptor is Nile Blue A Perchlorate (NB). Using dilute solutions of the individual molecules in ethanol, take absorption and fluorescence spectra of each. Absorption spectra are taken with a UV-Vis spectrometer. 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 we want the acceptor absorption near 532 nm to be minimal. *Why?*) An example of donor-fluorescence/acceptor absorption overlap is shown in Figure 4.

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 is to write a MatLab routine to evaluate  $R_0$ . An

example of a MatLab routine that calculates spectral overlap and  $R_0$  is in Appendix 2. 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  $J_{DA} = \int_0^\infty d\nu f_D(\nu)\epsilon_A(\nu)\nu^{-4}$  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 donor quantum yield for R6G is 0.985 (how critical is this number?). Now from eq. 7, you can calculate  $R_0$ , and from  $R_0$ , calculate  $C_0$  using eq. 10. Calculations of  $R_0$  and  $C_0$  from spectral overlap must be complete before further measurements can be made.

Make energy transfer measurements in a range of acceptor concentrations centered on  $C_0$ . Starting from a stock solution, make approximately ten solutions in ethanol for which the donor concentration is constant at approximately  $10^{-4}$  M, and the acceptor concentration spans a range of concentration from roughly  $0.01 C_0$  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. Note: to make the most accurate determination of  $R_0$ , it is important to over-sample the lower concentrations. Therefore only prepare two solutions greater than  $C_0$  and 8 solutions less than or equal to  $C_0$ .

## 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. The TA will demonstrate the optical alignment for these experiments, as well as the use of the oscilloscope.

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, the form of

the data is dictated by the instrument and not necessarily by FRET. [These effects can be corrected by deconvolution, which is discussed in Appendix 3. This may be necessary if you measure solutions with high acceptor concentration where FRET rates are very fast.]

**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 focusing lens. Prior to the detector, insert a band-pass 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.

### **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 focusing lens to focus onto the slit of the spectrometer by maximizing the real-time fluorescence signal on the computer. Remove any band-pass 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  $\epsilon_A$ . You will probably need to make corrections to the input data sets, such as 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.

Plot  $I_D/(I_D+I_A)$  against  $C$  and fit to equation 9 to determine  $R_0$ . One value of  $R_0$  should pass through all data points. You should do this with a least squares fitting routine. (If you are using a spreadsheet you will need to use a polynomial approximation for  $\text{erf}[x]$ ). The best (and recommended) approach would be to use a fitting routine from a software package like MatLab. Be sure to representative data and the final plot of  $I_D/(I_D+I_A)$  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. To get started:

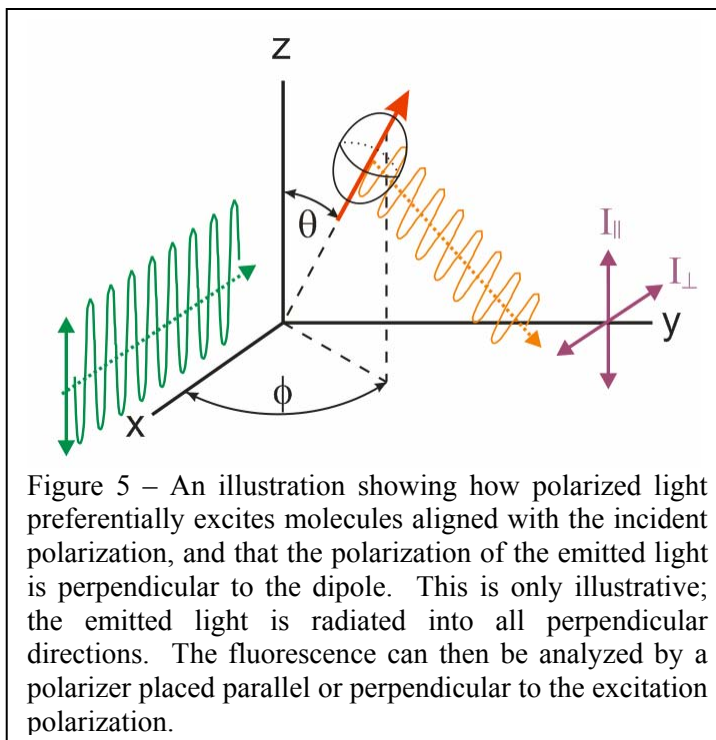
First clean up your oscilloscope traces. Set the baseline to zero using the signal prior to the arrival of the excitation light. For the purpose of fitting, chop off the steep rise due to the time response of the system. Make sure that your baseline has returned all the way to zero on all the traces 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 occurs at time  $t=0$ . Find  $\tau_D$  from an exponential fit to the pure donor decay trace.

Two options exist for fitting your data to equation 8. One approach is to simultaneously fit all of the normalized traces for varying concentration to eq. 8, varying only  $R_0$ . Alternatively, you can independently fit eq. 8 for each concentration to determine  $\gamma$ . A plot of  $\gamma$  vs.  $\sqrt{n_A}$  will give  $R_0$ . Be sure to include representative data and 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 light induces a change of charge configuration in the molecule. The change of electron density can be pictured (and formally described) as the creation of a dipole with a well-defined orientation. This oscillating dipole – essentially an antenna or transmitter – radiates the fluorescence that we observe. The direction that the molecule's dipole points dictates the direction of the fluorescence electric field.



Since the dipole has a well-defined orientation, if the molecule rotates as it fluoresces, the polarization of the fluorescence will change with the rotation. In this second part of the experiment, we will measure the rotation of molecules 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 rate at which molecules rotate in the liquid.

Unlike the gas phase, rotation of molecules in a liquid does not have to be treated quantum mechanically. The solvent about the molecule exerts rapidly varying random forces on the molecule, which restricts it from spinning freely. The reorientation is one of classical diffusion or Brownian motion. The molecular orientation changes gradually by small random steps as the solvent molecules fluctuate about the solute.

The theory of such reorientational motion for an object in a fluid is attributed to Debye, Stokes, and Einstein (DSE) working separately. The reorientation of an object through small random changes in angle is dictated by the orientational diffusion constant  $D$ . Specifically, the theory of Brownian motion describes the changes in angle  $\Delta\theta$  during a



short period of time  $\Delta t$  through  $\langle \Delta \theta^2 \rangle = 4D \Delta t$ . For the case of a sphere of volume  $V$ , the diffusion constant can be expressed as

$$D = \frac{k_B T}{6V\eta} \quad (11)$$

Here the friction of the solvent that acts to maintain the position of the sphere is expressed in terms of its viscosity  $\eta$ .

The DSE theory predicts that if you align a collection of molecules in a liquid and watch the orientation randomize through their rotational motion, the net alignment would decay away exponentially with a characteristic time scale  $\tau_{rot}$ :

$$A(t) = A(0) \exp(-t / \tau_{rot}) \quad (12)$$

where  $\tau_{rot}$  is related to  $D$  by

$$\tau_{rot} = \frac{1}{6D} = \frac{V\eta}{kT} \quad (13)$$

Thus rotation motion should slow with increased viscosity and solute size. So, if you measure the rotational relaxation of a molecule in solutions of varying viscosity, you can determine  $V$ . Such methods can be used to measure the radius of gyration of a polymer or protein. We will measure  $\tau_{rot}$  of a complex of the dye molecule eosin bound to the protein lysozyme in solutions. By varying the viscosity of the solution with sucrose we will be able to determine the effective molecular volume (the so-called hydrodynamic volume) of the eosin-lysozyme complex.

How do you measure the reorientational motion of molecules with fluorescence? In an equilibrium solution the orientation of molecules is equally distributed in all directions. We need a method of introducing an anisotropy and watching 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, using an analyzing polarizer placed in front of the signal detector. 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 whose dipoles have a projection along the excitation polarization direction. If those molecules, preferentially aligned in a direction that has a vertical projection, 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  $\tau_{\text{rot}}$  and eventually decay. 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 we get a bi-exponential decay owing to the fluorescence and rotational relaxations.

$$\begin{aligned} I_{\parallel}(t) &= \frac{1}{3} \exp(-t/\tau_f) \left( 1 + \frac{4}{5} \exp(-t/\tau_{\text{rot}}) \right) \\ I_{\perp}(t) &= \frac{1}{3} \exp(-t/\tau_f) \left( 1 - \frac{2}{5} \exp(-t/\tau_{\text{rot}}) \right) \end{aligned} \quad (14)$$

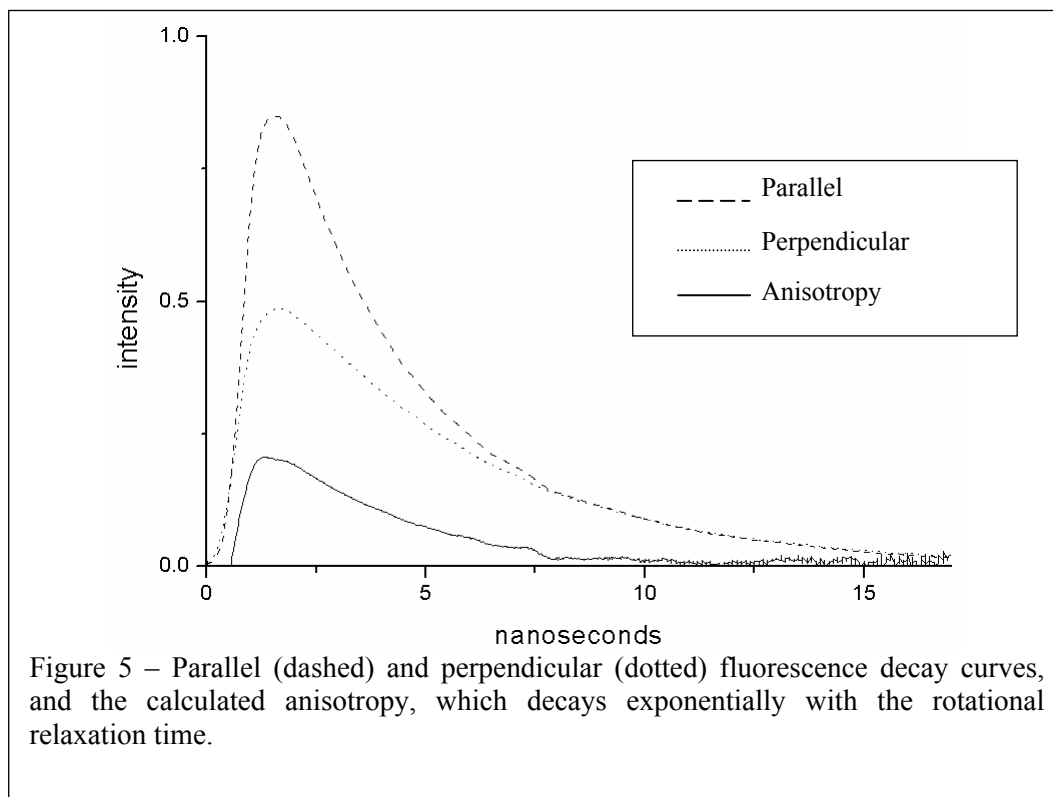
We can remove the fluorescence lifetime component by calculating the time-dependent anisotropy:

$$r(t) = \frac{I_{\parallel}(t) - I_{\perp}(t)}{I_{\parallel}(t) + 2I_{\perp}(t)}. \quad (15)$$

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

$$r(t) = \frac{2}{5} \exp(-t/\tau_{\text{rot}}). \quad (16)$$

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 anisotropy. Notice that when the rotational relaxation is somewhat faster than the fluorescence decay, the tails of the decays match up exactly with one another (when reorientation is complete). (*What would happen if you oriented the polarizer at an axis of  $\theta=54.7$  degrees [ $\tan^2(\theta)=2$ ] relative to the excitation?*)



## B. Experimental Measurements of Reorientational Motion

We will be making time-resolved fluorescence measurements on the eosin-lysozyme complex in order to calculate its hydrodynamic volume. Eosin is a strongly fluorescent dye that binds specifically to a non-active site in lysozyme, hence serving as a probe for the reorientational motion of lysozyme. The hydrodynamic volume of the lysozyme-eosin complex will be calculated from fluorescence decay times in the parallel and perpendicular polarization geometries in solutions with varying viscosities. The hydrodynamic volume of free eosin will be calculated using a high viscosity solvent and measuring its anisotropy decay.

**(1) Spectral shifts on binding.** The visible spectral band of eosin is known to shift around 8 nm to higher wavelength upon binding to lysozyme. Prepare a sample with  $10^{-6}$  M eosin in water and another sample with 5 mg of lysozyme in a  $10^{-6}$  M of eosin solution in water. Pipet these samples into fluorimeter cells and record their spectra in the UV/Vis spectrometer. *What accounts for the spectral shift?*

Images removed due to copyright reasons.

**(2) Anisotropy measurements on the eosin-lysozyme complex.** We will use measurements of  $\tau_{\text{rot}}$  as a function of  $\eta$  to determine the hydrodynamic volume of the complex. For anisotropy measurements, prepare seven solutions with different percentage by weight of sucrose in water, in the range 0% to 50%. The concentration of the stock eosin solution is  $10^{-4}$  M. Make 3 mL solutions with the above solvents, containing eosin concentration of approximately  $10^{-6}$  M and about 5 mg of lysozyme each, for a sample cuvette presenting a 1 cm path length to the laser beam. The concentration of eosin must be kept low relative to the lysozyme concentration to ensure that all the eosin molecules present in the solution bind to lysozyme. *Why?*

The polarizer for the laser excitation beam should be set vertical (perpendicular to the table). Acquire the fluorescence at 90 degrees to excitation, and insert a film polarizer before the fast photodetector. Rotate the polarizer so that transmission is either parallel or perpendicular to the excitation. Take both fluorescence decays changing nothing except the polarizer orientation. Average each data set for at least 1000 scans, so that your baseline is well-defined before and long after excitation. You will need to carefully set the common baseline for the  $I_{\parallel}$  and  $I_{\perp}$  measurements to zero; the calculated anisotropy will be very sensitive to this baseline. *Why? What is your  $r(0)$  and how does it compare to the*

*predicted value?* Begin fits to the anisotropy decay for times longer than the instrument response.

For the purpose of comparing hydrodynamic volumes of free eosin and the lysozyme-eosin complex, make an anisotropy measurement on  $10^{-6}$  M free eosin using the sucrose-water solution with highest viscosity. *On what time scale do you predict free eosin will reorient in water? From eqns. 14, we predict that the fluorescence lifetime can be measured from  $I_{\parallel}(t) + 2I_{\perp}(t)$ . What do you measure?*

**(3) Analysis.** Fit the decay of each anisotropy to an exponential to extract  $\tau_{\text{rot}}$  (eq. 12). Using the viscosity of the sucrose/water solutions, plot  $\tau_{\text{rot}}$  vs.  $\eta$  and linearly fit the data (eq. 13) to determine the effective hydrodynamic volume  $V$ . Values of the viscosity can be extracted by interpolating the data in Table 2 with the weight percent of sucrose you used. *How does your hydrodynamic volume compare with the expected volume from a lysozyme structure? Why? How linear is the DSE plot? Are there circumstances under which this model might not apply to the experiments? How do the volumes extracted for eosin and the complex compare?*

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

% Sucrose by Weight	Grams of sucrose per 5 mL	Viscosity cP ( $\text{mN}\cdot\text{s}\cdot\text{m}^{-2}$ ) at 20°C
0%	0	1.0019
5%	0.25	1.146
10%	0.5	1.336
20%	1	1.945
40%	2	6.162
50%	2.5	15.431

## V. Organization

Plan to perform this experiment over two days:

**Day 1:** Complete measurements for the spectral overlap  $R_0$  calculation and make reorientation measurements.

**Day 2:** Complete the steady state and transient FRET experiments.

Before Day 2 experiments, calculate  $R_0$  and  $C_0$  from your *Day 1* spectral overlap data.

## VI. 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.

Atkins, P. W. *Physical Chemistry, 4<sup>th</sup> 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.**

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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.**

Cross AJ and Fleming GR, "Influence of inhibitor binding on the internal motions of lysozyme." *Biophys. J.* 50, 507 (1986). **Describes fluorescence anisotropy measurements on the lysozyme-eosin complex.**

\* 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 zwischenmolekularen Uebergangs von Elektronenanregungsenergie," *Z. Naturforsch.*, **4a**, 321 (1949). **The derivation of eq. 8 (in German).**

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Jordanides XJ, Lang MJ, Song X, and Fleming GR, "Solvation dynamics in protein environments studied by photon echo spectroscopy." *J. Phys. Chem. B* 103, 7995-8005 (1999). **This describes more advanced experiments on the lysozyme-eosin complex, but has a nice discussion of the binding.**

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