

MASSACHUSETTS INSTITUTE of TECHNOLOGY

Department of Chemistry

5.38 Module 11

SPRING SEMESTER 2009

**Applications of Time-Resolved Spectroscopy in
Biophysical Chemistry**

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. Time-resolved fluorescence lifetime quenching measurements will be used to characterize the monomer-dimer equilibrium of insulin.
- 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 protein-dye complex.
- 3) Work with fabricated scientific instrumentation that you can assemble by yourself.
- 4) Perform data fitting and analysis using commercial mathematical software packages.

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 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.

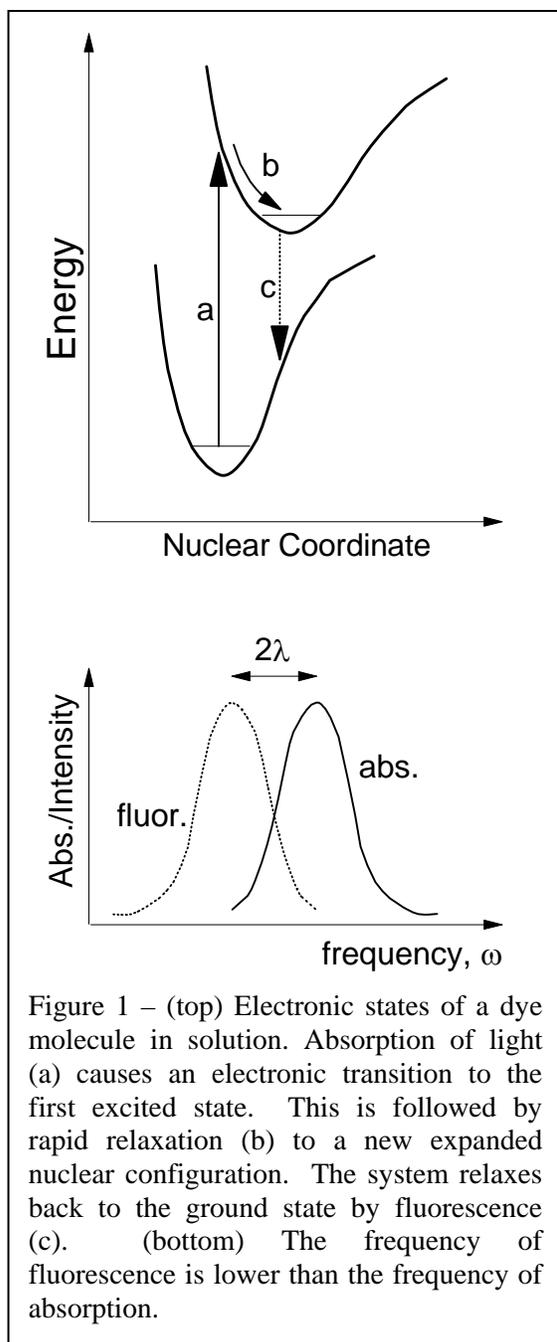
Consider a more physical example that is closely related to the experiment which will be performed. Everyone should be familiar with the photography of Prof. Edgerton which produces very clear pictures of rapid processes like bullets tearing through bananas, etc. (If not, several photographs worth a visit are on display at the MIT Museum). 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, two-types of processes that take place on the nanosecond time-scale will be investigated: the rate at which energy hops from an electronically excited dye molecule (a donor) to another (acceptor) molecule, and the reorientation of a protein in solution. Pulses of laser light will initially electronically excite molecules and a fast fluorescence detector will be used to measure the rate of energy transfer or rate of reorientation. From the preceding discussion it is clear that these pulses must be less than one nanosecond 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 cm^{-1} .* 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} s , or 0.1 to 1 picosecond, ps), dissipating roughly 10^2 - 10^3 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

* 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 $E=hc/\lambda$.



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, ϕ_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, 2λ , 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

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

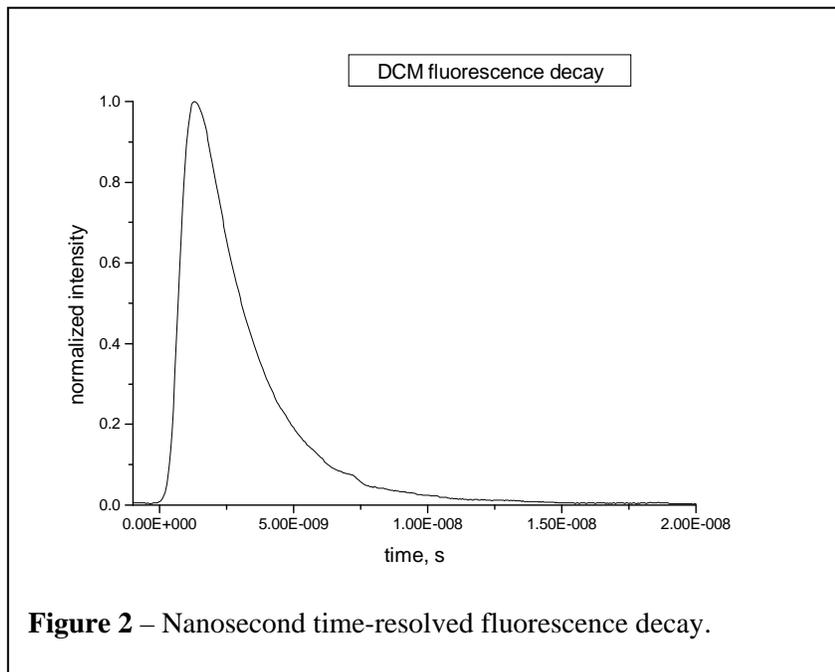
Thus watching the intensity of fluorescence emitted by the sample as a function of time (typically a few nanoseconds) allows the relaxation rate to be measured directly. A first order rate equation for the relaxation of the excited state population

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

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, where time $t=0$ refers to the point at which a short light pulse excited the sample.



II. Experimental

A. Fluorimeter for Transient 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 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 μ 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 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

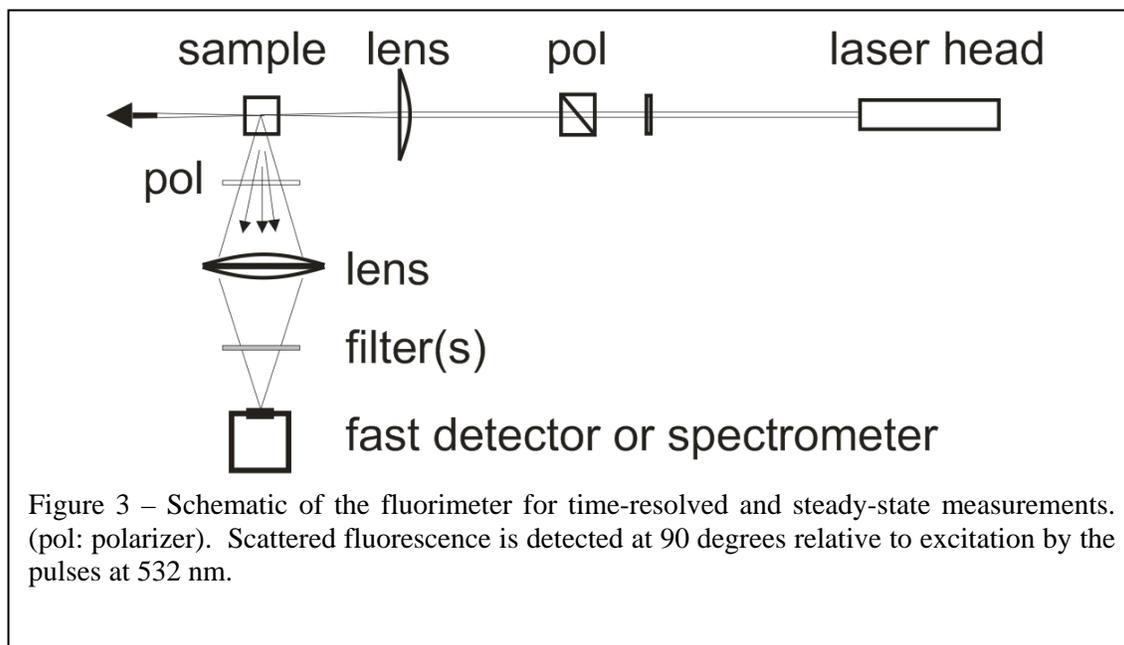


Figure 3 – Schematic of the fluorimeter for time-resolved and steady-state measurements. (pol: polarizer). Scattered fluorescence is detected at 90 degrees relative to excitation by the pulses at 532 nm.

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 the 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 you are 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. Protein Reorientation

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.

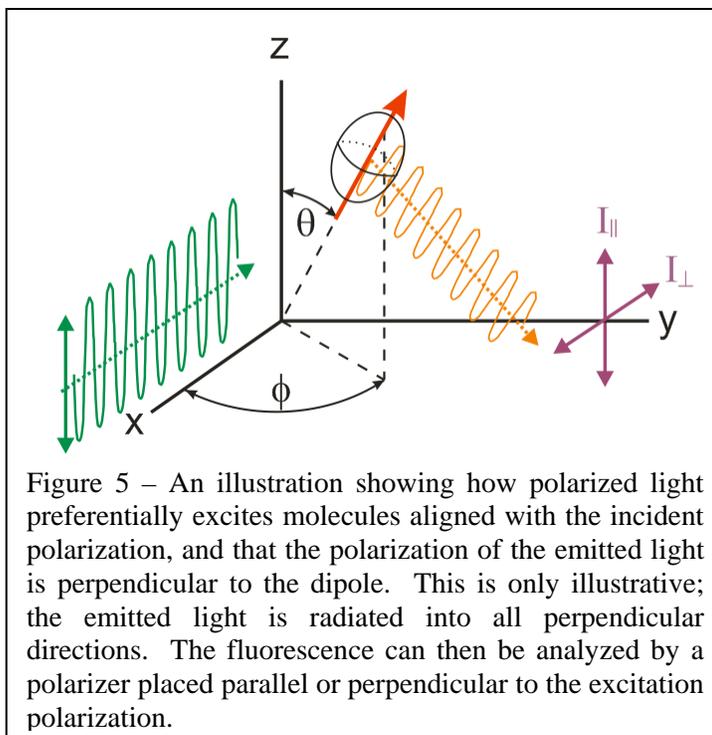


Figure 5 – An illustration showing how polarized light preferentially excites molecules aligned with the incident polarization, and that the polarization of the emitted light is perpendicular to the dipole. This is only illustrative; the emitted light is radiated into all perpendicular directions. The fluorescence can then be analyzed by a polarizer placed parallel or perpendicular to the excitation polarization.

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 Δt 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 η .

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 τ_{rot} :

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

where τ_{rot} is related to D by

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

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 τ_{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 τ_{rot} , whereas if the polarizer transmits horizontally polarized light, fluorescence should rise in with τ_{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.

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

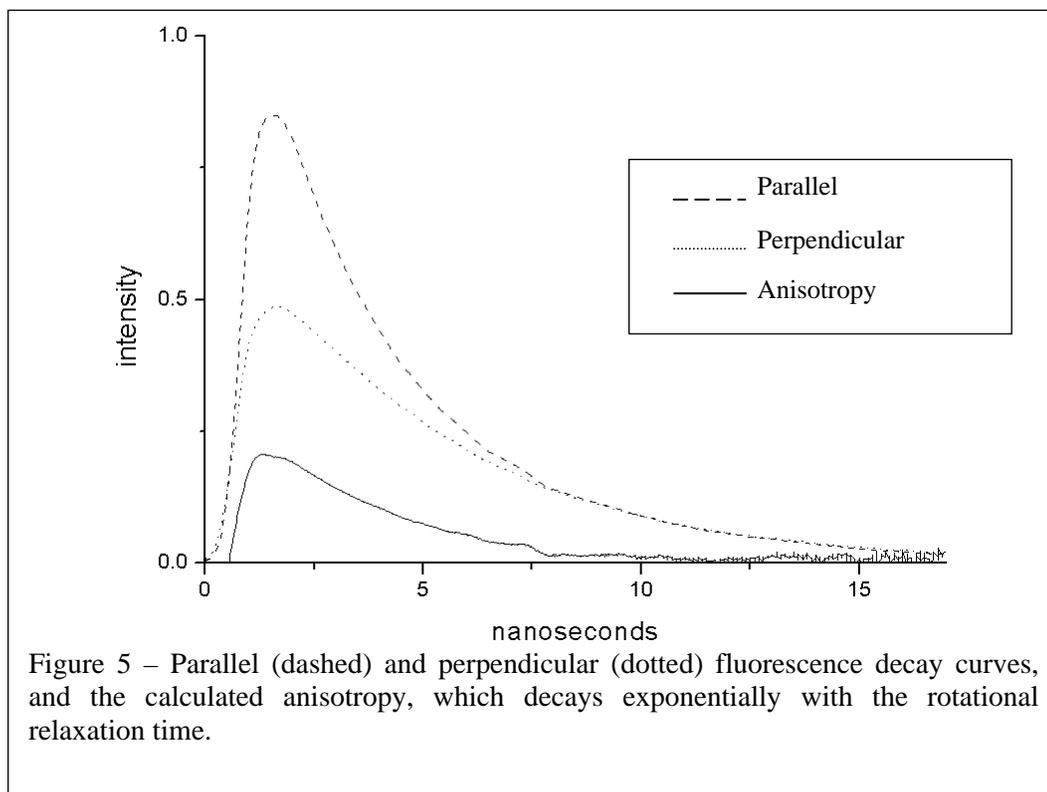
We can remove the fluorescence lifetime component by calculating the time-dependent anisotropy:

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

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?*

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Figure 6 – Figures drawn from Jordanides, et al. (A) Absorption spectrum of eosin in water shown, along with the molecular structure. The dashed line corresponds to free eosin in water, solid line is the bound eosin-lysozyme complex. (B) X-ray crystallographic structure of eosin-lysozyme complex. Eosin binds to the hydrophobic part of lysozyme.

(2) Anisotropy measurements on the eosin-lysozyme complex. We will use measurements of τ_{rot} as a function of η 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?*

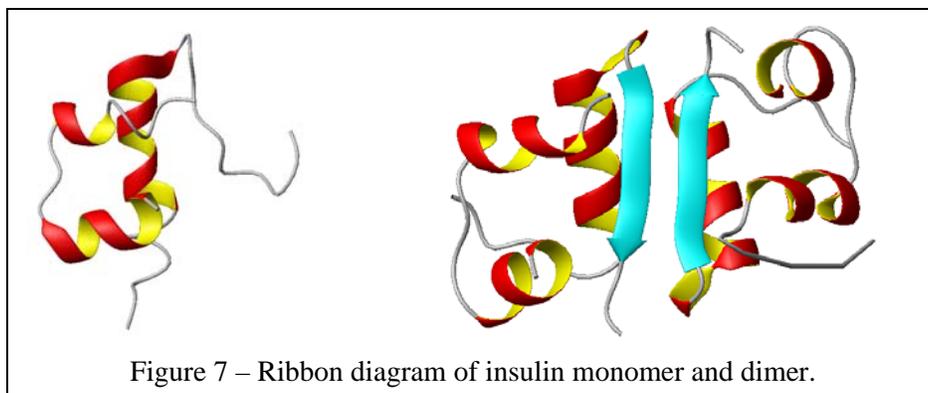
(3) Analysis. Fit the decay of each anisotropy to an exponential to extract τ_{rot} (eq. 12). Using the viscosity of the sucrose/water solutions, plot τ_{rot} vs. η and linearly fit the data (eq. 13) to determine the effective hydrodynamic volume V . Values of the viscosity can be extracted by interpolating 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

C. Organization

Plan on one full lab period to perform the sample preparation and fluorescence depolarization measurements.



IV. The Monomer-Dimer Equilibrium of Insulin

A. Background

Insulin is a small, 51 amino acid protein that is responsible for blood glucose regulation. *In vitro* and at low pH, insulin is found as both monomers and dimers (Fig. 7), making it a simple model system for mechanistic studies of protein-protein interactions. Dimer formation leads to the formation of specific contacts; the insulin monomer has a disordered chain which folds into an intermolecular β sheet in the dimer.

The monomer-dimer equilibrium is characterized by a dissociation constant K_d for the dissociation of dimers (D) into monomers (M),

$$D \xrightleftharpoons[k_a]{k_d} 2M$$

$$K_d = \frac{[M]^2}{[D]}$$

K_d has units of [concentration]⁻¹. With protein concentrations below K_d , insulin is mostly monomeric. With concentration equal to K_d , half the proteins are dimerized. From a physical perspective K_d encodes the strength of interactions between monomers: the smaller the value, the tighter the binding. We can also write it in terms of the rates of dimer dissociation, k_d , and monomer association, k_a :

$$K_d = \frac{k_d}{k_a}.$$

k_a is related to how fast monomers can diffuse to meet each other, whereas k_d depends on the barrier to dissociation.

This lab seeks to measure the dissociation constant K_d . To perform this measurement, one needs to distinguish the population of insulin in the monomer and dimer states. This is achieved here by measuring energy transfer between insulin dimers

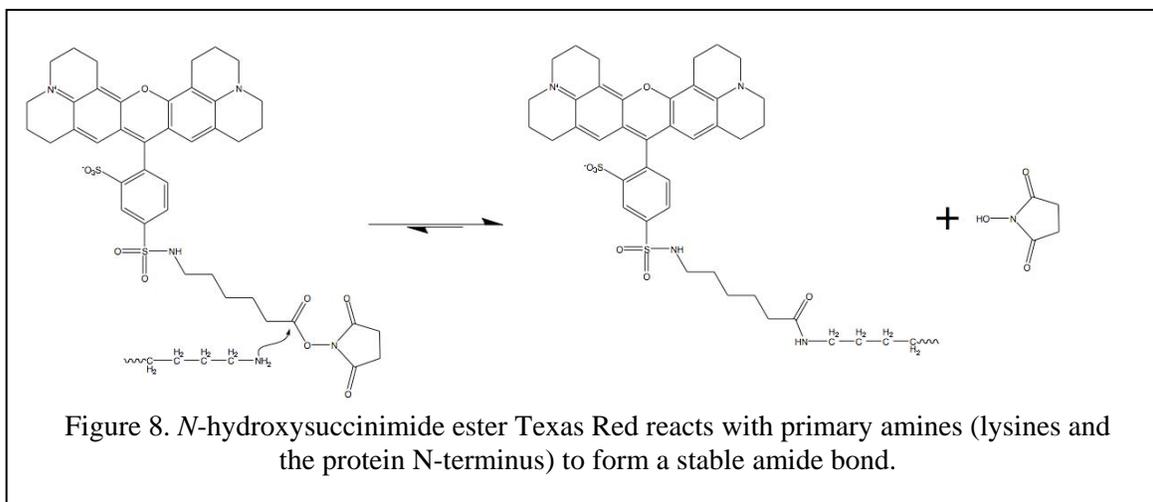
that have been labeled with a chemically attached dye molecule. When insulin is monomeric, dye molecules predominantly relax by fluorescence emission, with a rate $k_f = 1/\tau_f$. At high concentrations, where insulin is dimeric and two dye molecules are in close proximity, dye molecules can relax either by fluorescence emission or by radiationless energy transfer from one dye to another. (This is a special case of Forster resonance energy transfer, or FRET). Because dye molecules bound to insulin dimers can relax via two mechanisms, the overall rate of fluorescence relaxation (k_f+k_{ET}) will be faster than that of dyes bound to monomers.

The ensemble average fluorescence should show two rates: a slower rate indicative of the insulin monomers and a faster rate indicative of the insulin dimers. By modeling the fluorescence relaxation as this sum of contributions from monomers and dimers

$$I(t) = N_M e^{-k_f t} + N_D e^{-(k_f+k_{ET})t}$$

you will measure the ratio of monomers to dimers. Performing this experiment at several concentrations will allow extraction of K_d . *What assumptions go into this analysis?*

To label an insulin protein with a fluorescent dye, we will use the NHS-ester of Texas-Red. Primary amines react with NHS-esters to form amide bonds (Figure 8), but the esters are also hydrolysed by water. Since insulin, and most other proteins, are only stable in water, one needs to use a stoichiometric excess of NHS-ester dye when labeling. As such, the reaction mixture will contain large amounts of free dye and will require column purification. Texas Red is especially suited for looking at self-association because it is an excellent self-quencher. (Lakowicz, 2004)



B. Experimental Methods

The experimental work in the lab involves four steps: (1) Dye labeling of insulin, (2) protein purification, (3) sample characterization, and (4) time-resolved fluorescence measurements.

Labeling Reaction

1. Dissolve 9 mg of insulin in 0.9 mL of pH 8.1 phosphate buffer. *Why this pH?*
2. Using a syringe with a needle tip, add 80 μ L of anhydrous DMSO to 1 mg of NHS-Texas Red. *Why use DMSO?*
3. Using a pipettor, add the Texas Red solution to the insulin slowly while stirring. Using the pipettor instead of the syringe insures that all the Texas Red is transferred.
4. Allow the labeling reaction to proceed for at least 1 hr. The conjugated protein/dye is stable for weeks in the refrigerator.

Column Purification

1. Let 20 g of Sephadex beads swell in Millipore water at 20°C for at least 3 hours (or overnight). Do not stir the beads with anything – they are fragile. Wet them fully by shaking the beaker.
2. Using a funnel, add the beads to the column. Allow the flow-through to drain. After the beads are poured, maintain a flat layer of beads by not agitating the column.
3. Wash the beads with two column volumes of Millipore water.
4. Allow the water level to drop to a thin (< 1 mm) layer on top of the beads.

Note: The water level must always cover the beads. If the beads are allowed the dry, narrow channels will form in the column. These channels are invisible to the eye, but destroy the column's ability to separate. If this happens, the beads must be removed and the column must be repoured.

5. Add the reaction mixture dropwise to the top of the beads. Forceful pipetting will disturb the top layer of beads and cause an uneven band to form.
6. Allow the reaction mixture to settle into the column, discarding the flow-through.
7. When the reaction mixture has fully entered the column, add 2 mL of water and allow it to flow through.
8. By now, there should be a layer of free dye at the top of the column and the band of labeled protein should have passed into the column. Fill the remainder of the column with water to increase the flow rate and begin the chromatography.

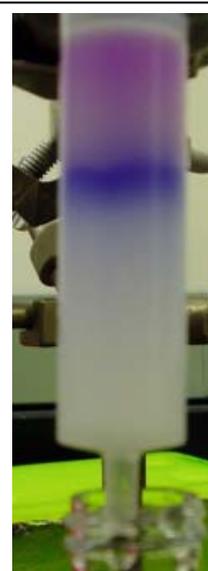


Figure 9. Free dye and protein-conjugated dye bands.

- The band of fluorescent protein should be easy to see by eye and equally easy to discriminate from the free dye- the fluorescent protein is dark purple and the free dye will be pink (see Figure 9). Collect the labeled protein fraction. It should be ~15 mL. *Why are the free dye and protein bound dye different colors?*

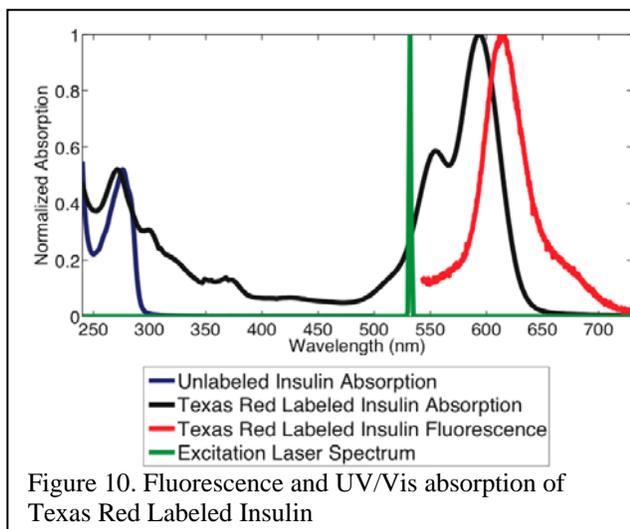
Lyophilization – (Performed with TA)

- Add the sample to a 20 mL scintillation vial.
- Cover the scintillation vial with a Kim-Wipe and seal it in place with a pipe cleaner (this is a makeshift filter).
- Freeze the scintillation vial in liquid nitrogen, making sure not to lose sample by tipping the vial or submersing it completely.
- Put the scintillation vial in a lyophilizer flask, hook the flask up the lyophilizer, and slowly turn the valve to apply high vacuum.
- Monitor the lyophilizer to insure that the pressure drops to the set point (80 mTorr)

Measuring the Sample Purity

- Dissolve the lyophilized, dye-labeled insulin in a minimal amount of pH 2 solution (20-80 μ l). This will be the stock solution that determines the maximum concentration, so it should be as concentrated as possible. *Why?*
- Prepare a sample of labeled protein for characterization by UV/Vis absorption. It should have a very faint color to the eye. This should yield an absorbance or optical density OD ~ 0.04).
- Measure the peak visible absorbance at 593 nm and use it to determine the dye concentration. Assume that the free dye and bound dye both have the same molar absorptivity, $\epsilon_{593\text{nm}} = 80 \text{ cm}^{-1} \text{ mM}^{-1}$.

- Measure the absorbance at 280 nm. This includes a contribution from protein as well as the dye. In the free dye, the ratio $\epsilon_{593\text{nm}}/\epsilon_{280\text{nm}} = 0.18$ and we assume this holds when the dye is conjugated. The protein absorbance corrected for dye absorption can be obtained from $A_{280\text{nm,protein}} = A_{280\text{nm}} - 0.18 \cdot A_{593\text{nm}}$. *What protein absorption bands occur at 280 nm?*



5. Calculate the protein concentration using the known molar absorptivity for insulin, $\epsilon_{280\text{nm}} = 5.53 \text{ cm}^{-1} \text{ mM}^{-1}$.
6. Calculate the labeling efficiency (the average number of dye molecules per insulin molecule). *What is an optimal number for this experiment?*

Time-Resolved Fluorescence

1. Prepare a set of samples varying from the most concentrated stock solution to about 1 μM . Record the fluorescence decay using the digital oscilloscope. You should expect to see a single exponential decay at low concentration when the system is largely monomers, and an additional fast component grow in with increasing concentration. Map out the approximate concentration where the amplitudes of these components are equal, and then make several measurements slightly above and below this concentration.
2. Close the laser shutter when data isn't being acquired to avoid heating the sample and changing the concentration.
3. Maintain the same time base for all acquired data so the time resolution will be the same for all samples.
4. Before taking a data set, take note of the jitter in the rising edge of the fluorescence signal. If it is not much smaller than the time resolution, check the trigger signal. The FWHM should be $< 1 \text{ ns}$ and the threshold should be set along a steep portion of the rising edge.

C. Data Analysis

1. Plot the UV/Vis spectrum you collected for the labeled protein. Report the dye concentration, the corrected protein concentration, and the labeling efficiency.
2. Load the fluorescence decay data into MATLAB or another mathematical software package and prepare a semi-log plot showing the fluorescence decay as a function of labeled protein concentration. The data may require processing such as a baseline subtraction (it should be flat around -5 ns) and intensity normalization to aid visual presentation and fitting.
3. Fit each fluorescence decay to the functional form, $I(t) = Ae^{-k_1 t} + Be^{-k_2 t}$. Your lowest concentration (well below K_d) should be the fluorescence lifetime, and can be fixed as a constraint for the fit. You will want to fit k_1 and k_2 to minimize the error for the entire data set. From the fit you will obtain amplitudes and relaxation rates. *What is the energy transfer rate you deduced in the dimer? Can you rationalize the number?*
4. Prepare a plot of the fast time coefficient ($\frac{B}{A+B}$) as a function of the labeled protein concentration. Under the assumptions in this lab, this quantity will be equal to the dimer fraction: $\theta = \frac{[D]}{[D]+[M]}$. *What are the assumptions? Are they valid? Explain.*
5. Fit the dimer fraction to the Hill Equation: $\theta = \frac{[M]^n}{K_d^n + [M]^n}$ to extract K_d and n .
6. How does your measured K_d compare to the literature value? (Nettleton, 2000; Lord, 1973.) (Often K_{12} is reported instead.)

Additional Questions and Analysis

1. In this lab, K_d is measured by attaching a fluorescent dye to the protein. What does it mean if K_d matches a value obtained without modifying the protein? What does it mean if it doesn't?
2. *N*-hydroxysuccinimide esters are reactive to primary amines. Using structure visualization software (ie: VMD from <http://www.ks.uiuc.edu/Research/vmd/>), identify all the primary amines in insulin. What is the distance between dye molecules in the dimer state? Also use this software to estimate the volume of an insulin monomer for use in question 3.
3. For chemical reactions in which diffusion of the solutes is the rate-limiting step, one can estimate the rate coefficient as $k_a = 4\pi DR_0$, where R_0 is the molecule's radius and the diffusion constant D can be approximated using the Debye-Stokes Einstein relation given in this lab manual. Using your value for K_d , what do you predict for the rate coefficients k_a and k_d ?

V. Organization

Plan to perform the in-lab portion of this experiment over three days:

In advance: Soak beads for column.

Day 1: Make lysozyme samples and complete reorientation measurements.

Day 2: Insulin labeling reaction and purification.

Overnight: Lyophilization.

Day 3: Make insulin fluorescence quenching measurements.

Reorientation measurements can be made at any point in the sequence.

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.

† = References online

General

*† 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.**

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Lord, R. S.; Gubensek, F.; Rupley, J. A. *Insulin Self-Association. Spectrum Changes and Thermodynamics*. *Biochemistry*. **12**, 4385-4392 (1973).

Nettleton, E. J.; Tito, P.; Sunde, M.; Bouchard, M.; Dobson, C. M.; Robinson, C. V. *Characterization of the Oligomeric States of Insulin in Self-Assembly and Amyloid Fibril Formation by Mass Spectroscopy*. *Biophys J*. **79**, 1053-1065 (2000).

Energy Transfer and FRET

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

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

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.**

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† Th. Förster, "Transfer Mechanisms of Electronic Excitation," *Discussions Faraday Soc.* **27**, 7 (1959). **Excellent review of the results of his theories used in this experiment.**

Appendix 1: Energy Transfer 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 electronic ground state. This picture also applies to dilute solutions of dye molecules surrounded by solvent molecules. In more concentrated solutions when the distance between dye molecules approaches distance scales of 10-100 Å, relaxation pathways that arise from intermolecular interactions between dye molecules also exist. In particular, 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 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 can 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 $1/R^6$:

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

Here τ_D is the fluorescence lifetime 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: $k_D = k_f + k_{ET}$. When R is much shorter than R_0 then $k_D \approx k_{ET}$ 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 ϵ_A . This integral indicates that for efficient energy transfer, resonance is required between the donor emission and acceptor absorption. ν represents units of frequency in wavenumbers (cm^{-1}). The fluorescence spectrum must be normalized to unit area, so that $f_D(\nu)$ will be in $\text{cm} [= 1/(\text{cm}^{-1})]$. 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. κ^2 is a constant that reflects the relative orientation of the electronic dipoles. It takes a value of $2/3$ for molecules that are rotating much faster than the energy transfer rate. ϕ_D is the donor fluorescence quantum yield. For R_0 in units of cm , eq. 6 is often written

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

For dye molecules in solution typical values of R_0 are 10-100 Å, and the rates of energy transfer k_{ET} are often similar (0.1 - 1 ns^{-1}) to the rates of fluorescence for solutions with acceptor concentrations $\sim 10^{-3} \text{ M}$. 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.

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.

Appendix 2: Relating Fluorescence and Insulin Concentration

A. Perfect Labeling

In the limit that each protein is labeled with exactly one dye molecule, the monomer dimer equilibrium constraints are

$$C = M + 2D$$
$$K_d = \frac{M^2}{D}$$

where C = total concentration, M = monomer concentration, D = dimer concentration
 K_d = dissociation constant. In this example, when dimers are formed, exactly two dye molecules are able to quench.

These equations can be rearranged to solve for the monomer and dimer concentration for a given equilibrium constant and total concentration,

$$M = \frac{1}{4} \left(\sqrt{8CK_d + K_d^2} - K_d \right)$$
$$D = \frac{1}{8} \left(4C + K_d - \sqrt{8CK_d + K_d^2} \right)$$

as well as the dimer fraction,

$$\frac{D}{D+M} = \frac{4C + K_d - \sqrt{K_d^2 + 8CK_d}}{4C - K_d + \sqrt{K_d^2 + 8CK_d}} .$$

B. Overlabeling

If each protein is labeled with more than one dye molecule, when dimerization occurs, more than two dye molecules may quench at the same time. To fit this data, we use the Hill equation (Creighton, 1993),

$$\theta = \frac{[M]^n}{K_d^n + [M]^n} ,$$

where n dictates the degree of cooperativity ($n > 1$ indicates more than one dye quenches simultaneously) and K_d is the dissociation constant.