Luminescence Quenching Background

Excited State Processes

The fate of excited states can be divided into the processes that take place within the initially prepared excited state and transitions from that state. Within the initial excited state, a molecule can relax to a new equilibrium position of the nuclei. That position is different from the nuclear equilibrium position both in terms of the bonds and the salvation shell around the molecule. Vibrational relaxation (shown in Figure 1) leads to a new equilibrium distribution of the vibrational energy in the excited state. Likewise, following fluorescence or other processes that take the system back to the ground state, there will be vibrational relaxation in the ground state manifold. We refer to a manifold as collection of vibrational levels in a potential energy surface.

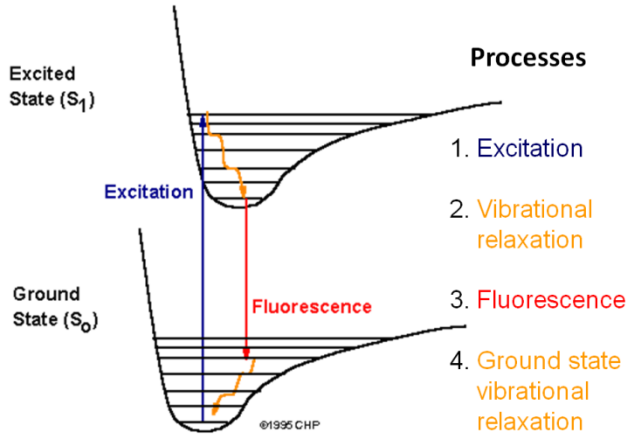

Figure 1. Relaxation processes within an excited state or the ground state.

Relaxation between states is usually represented using a Jablonski diagram, such as that shown in Figure 24. The ground state S_0 refers to a singlet. Not all ground states are singlets, but this is a common situation. The first singlet excited state is S_1. The triplet state is T_1. The non-radiative processes shown include internal conversion (IC) and intersystem crossing (ISC), which represent conversion of the S_1 state to the singlet ground state and triplet states, respectively. Both of these processes compete with fluorescence and reduce the fluorescence quantum yield. Internal conversation involves coupling of the excited state to the ground state by means of the Born-Oppenheimer breakdown operator,

$$H = \frac{\partial}{\partial Q}$$

This operator represents a part of the Hamiltonian neglected when the Born-Oppenheimer approximation is made. This term can couple states perturbatively. Coupling of the S_1 state to T_1 involves the spin-orbit coupling operator. The relaxation of the triplet state to the ground state can be non-radiative, i.e. yet another ISC process, or radiative. A radiative $T_1 \rightarrow S_0$ process is called phosphorescence. Since triplet-singlet processes are formally forbidden the lifetime of the triplet state is typically quite long compared to the singlet state.

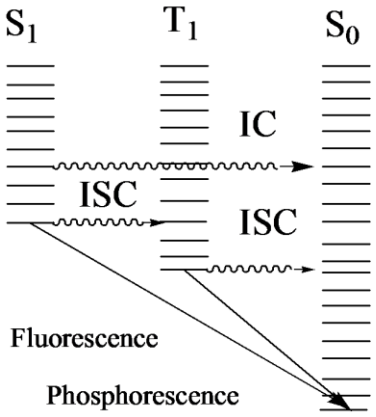

Figure 2. Example Jablonski diagram shown the processes of internal conversion (IC), intersystem crossing (ISC), $S_1 \rightarrow S_0$ fluorescence and $T_1 \rightarrow S_0$ phosphorescence.

Stimulated emission is only practical from a singlet state. The ideal laser material has a large absorption, and therefore stimulated emission, coefficient. However, spontaneous emission can compete with the stimulated emission. In practice organic laser dyes are fluorophores as well.

Emission – the mirror image relationship

Once a photon has been absorbed, the excited state may live for many nanoseconds. During that time vibrational relaxation can occur so that the population of vibrational states in the excited state potential surface is equilibrated. Fluorescence, shown in Figure 3, occurs following vibrational relaxation so that the initial state for fluorescence will be mostly 0' with some contribution from thermally populated vibrational states. The overlaps of 0'-0, 0'-1, 0'-2 etc. are the same as 0-0', 0-1', 0-2' etc. Thus, FC factor for emission is the same as that for absorption. The difference is that in absorption we add quanta of energy and in emission we subtract them. This can be seen in Figure 4. It is clear that the emission energies will all be lower than 0-0', while the absorption energies will all be higher. The relationship shown in Figure 4 leads to the "mirror image" relationship between absorption and emission lines shown in Figure 5.

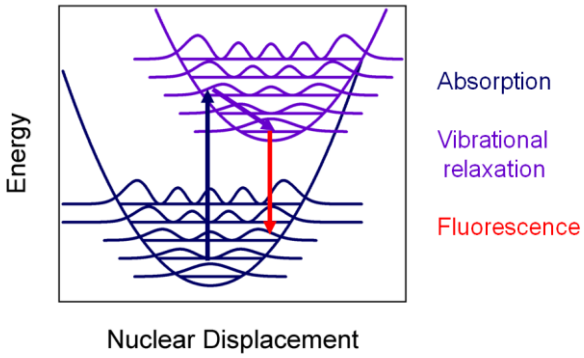

Figure 3. Illustration of the events leading to fluorescence. Absorption is followed by vibrational relaxation. Fluorescence occurs from a relaxed nuclear geometry in the excited state.

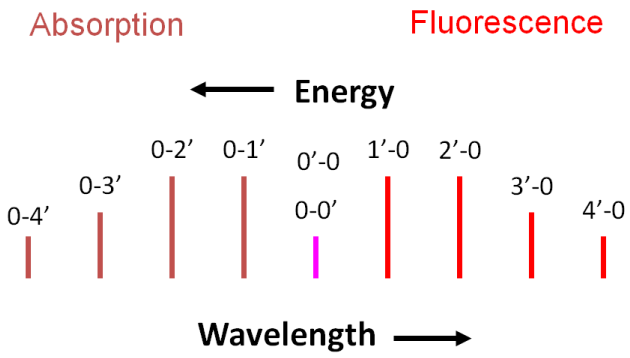

Figure 4. Illustration of the mirror image relationship for absorption and fluorescence spectra. The commonly used dye Rhodamine shows the mirror image relationship.

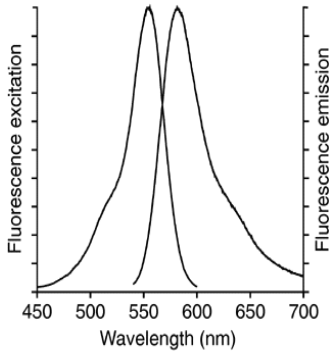

Figure 5. Mirror image relationship of excitation and emission.

Figure 5 shows the excitation spectrum and the emission spectrum. The excitation spectrum is has the same theoretical line shape as the absorption spectrum.

The excited state lifetime

When a molecule is excited, an electron is promoted from the ground state to an excited state. The electron will return to the ground state with lifetime that is determined by both the fluorescence rate constant, k_f and the non-radiative rate constant, k_{nr}. The measured the excited state lifetime, τ_{obs}, depends on both of these processes. First, we note that the rate constant is the inverse of the lifetime,

$$k_{obs} = \frac{1}{\tau_{obs}} \tag{1}$$

The observed rate constant is the sum of both intrinsic rate constants,

$$k_{obs} = k_f + k_{nr} \tag{2}$$

The measured population of the excited state decreases exponentially according to

$$N(t) = N(0)e^{-k_{obs}t} \tag{3}$$

The quantum yield of the fluorescence is given by,

$$\Phi_f = \frac{k_f}{k_f + k_{nr}} \tag{4}$$

A single measurement of the kinetics will give only the observed rate constant, k_{obs}. In order to measure the intrinsic rate constant, k_f, we need to measure both the observed lifetime by a kinetics

measurement and the fluorescence quantum yield. The lifetime can be measured using time-correlated single photon counting, which is a widely used technique for determining the time course for excited state emission. The fluorescence quantum yield, Φ_f, can be measured in a fluorometer by comparing the emission of an unknown to that of a known standard.

Practical fluorescence spectroscopy

Tryptophan fluorescence

The fluorescence of the amino acid tryptophan is widely used as a probe of protein structural changes. Tryptophan is an aromatic amino acid, whose structure is shown in Figure 13.4. Tryptophan absorbs light by excitation of $\pi \rightarrow \pi^*$ transitions near 290 nm. We can model the absorption of tryptophan using the particle-on-circle model. Although tryptophan is not truly circular, it is aromatic with 10 electrons in the □-system. Note that we count the nitrogen heteroatom contribution of 2 electrons in addition to the 8 electrons from p-orbitals of the carbon atoms. The model predicts that there will be a strongly absorbing band (allowed) with $\Delta m = \pm 1$, and a weak absorption band with $\Delta m = \pm 5$.

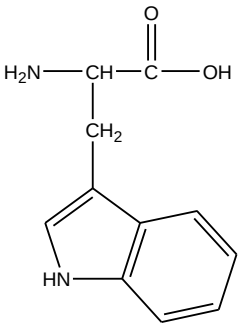

Figure 13.4 Structure of tryptophan

The observed band at 290 nm is the weak band, which is formally forbidden. As is the case for benzene, porphyrins and other aromatic molecules, the low energy "forbidden band" has structure, which is known as vibronic structure. The structure arises from the fact that vibrational distortions of the molecule lead to coupling of the weak L band to the stronger B band. This is shown in Figure 13.5, which shows the extinction coefficient of tryptophan.

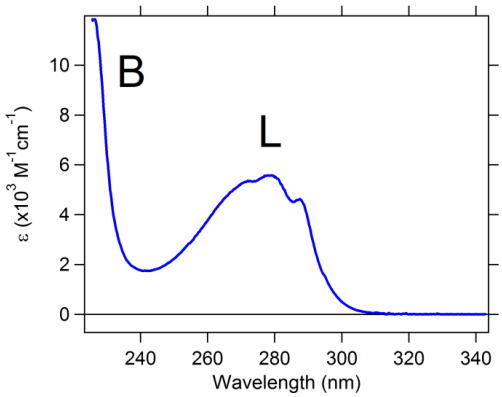

Figure 6. Absorption spectrum of tryptophan.

The emission spectrum of tryptophan is shown along with the absorption spectrum in Figure 7.

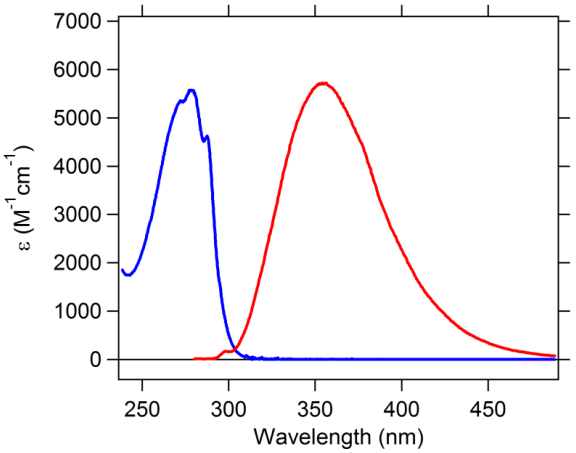

Figure 7. Tryptophan absorption (blue) and fluorescence (red).

At first, it may appear that the mirror image relationship is not obeyed. The red-colored emission spectrum has significantly different shape, i.e. it is much broader than the absorption spectrum. However, this different is an artifact due to the fact that we have plotted both absorption and emission as a function of wavelength, λ, rather than wave number, $\tilde{\nu}$. If we convert to units of cm^{-1}, the appearance of these data is shown in Figure 7.

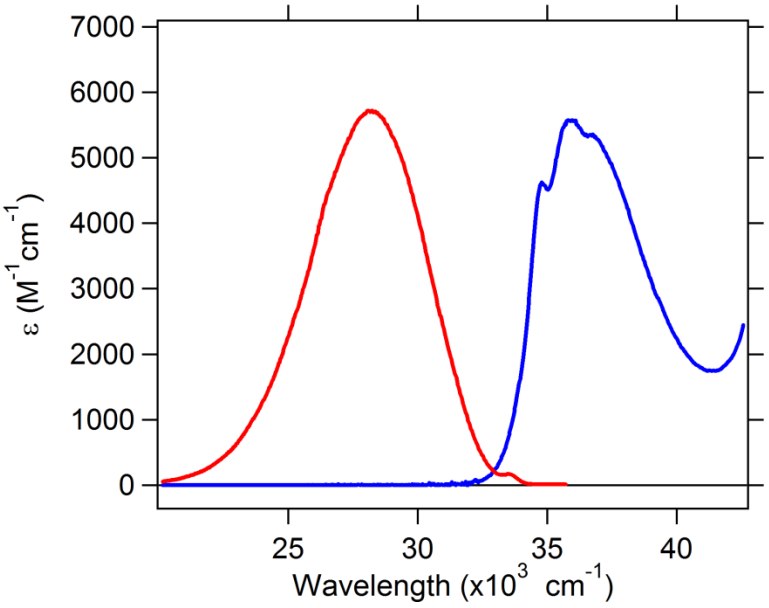

Figure 8. Absorption and emission spectra of tryptophan plotted as a function of wave number.

There is still some difference owing to the apparent broadening of the emission line shape relative to the absorption line shape. This occurs due to rapid dephasing processes in the excited state that

give rise to broadened lines in the emission spectrum, relative to the absorption spectrum, which is initiated from the ground state. The absorption and fluorescence data for tryptophan were obtained from the resource known as PhotochemCAD. For more information on this resource students should consult the publication, H. Du, R. A. Fuh, J. Li, A. Corkan, J. S. Lindsey, "PhotochemCAD: A computer-aided design and research tool in photochemistry," *Photochemistry and Photobiology*, 68, 141-142, 19913.

Green fluorescent protein

The green fluorescent protein (GFP) is found in a jellyfish that lives in the cold waters of the north Pacific. The jellyfish contains a bioluminescent protein-- aequorin--that emits blue light.

Green fluorescent protein converts this light to green light, which is what we actually see when the jellyfish lights up. Solutions of purified GFP look yellow under typical room lights, but when taken outdoors in sunlight, they glow with a bright green color. The protein absorbs ultraviolet light from the sunlight, and then emits it as lower-energy green light.

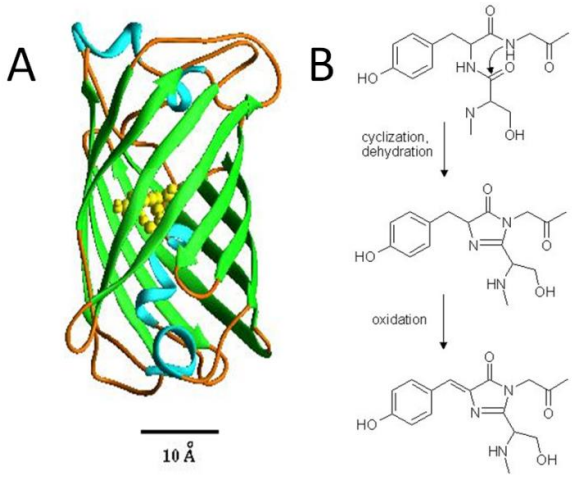

Figure 9. A. Overall structure of green fluorescent protein.

B. Sequence of reactions leading to the formation of the fluorophore.

GFP is useful in scientific research, because it allows us to look directly into the inner workings of cells. It is easy to see where GFP is at any given time: you just have to shine UV light, and any GFP will glow bright green. So the trick is to attach GFP to any object that you are interested in watching. The structure of GFP (Figure 9) is a β-barrel, with the GFP chromophore in the center of the cylindrical fold. The remarkable property of GFP is that the chromophore forms spontaneously in any cell where GFP is expressed and can fold. Following protein folding, several chemical transformations occur: As shown in Figure 9B, the glycine forms a chemical bond with the serine, forming a new closed ring, which then spontaneously dehydrates. Then, over the course of an hour or so, oxygen from the surrounding environment attacks a bond in the tyrosine, forming a new double bond and creating the fluorescent chromophore. Since GFP makes its own chromophore, it is perfect for genetic engineering. You don't have to worry about

manipulating any strange chromophores; you simply engineer the cell with the genetic instructions for building the GFP protein, and GFP folds up by itself and starts to glow.

The reporter gene technology uses GFP as an indicator of gene expression. As shown in Figure 10, the GFP gene is placed next to a gene of interest. Then when the gene of interest is expressed, the cells will also express GFP. An example shown in Figure 10 comes from research into the development of the nematode, *C. elegans*. As genes are expressed during *C. elegans* development in genetically modified organisms, different regions of the nematode show fluorescence from GFP.

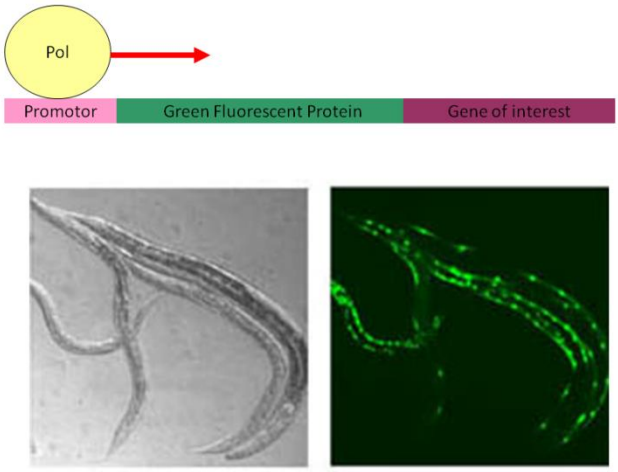

Figure 10 Illustration of the reporter gene concept.

Fluorescence quenching and energy transfer

The quantum yield gives the intrinsic fraction of the molecules that decay by a emitting light. In addition, the fluorescence emission can be further quenched by:

1. Collisional quenching – molecular collisions in solution
2. Intersystem crossing – conversion from singlet to triplet
3. Electron transfer – $^1DA \rightarrow D^+A^-$
4. Energy transfer – emission is transferred to an acceptor

Fluorescence quenching can be both beneficial and a source of error in experiments. Since fluorescence is subject to quenching, one must be careful to account for any possible fluorescence quenching that may occur during an experiment. However, the intentional addition of quenchers can be used to monitor the accessibility of fluorophores, and is thus a useful method for determining the location of fluorophores in a cell. Collisional quenching is a function of concentration, and is found in all solutions. This type of quenching can be minimized by keeping the concentration of fluorophores low. Typically, it is best to work in the micromolar range or lower. Intersystem crossing occurs due to the "heavy atom effect". Halogens like bromine and iodine, metals, and other elements, including heavy noble gases such as Xenon can give rise to

spin flips that change the singlet excited state to a triplet excited state. Since fluorescence is emission from the singlet excited state, the singlet→triplet conversion, known as intersystem crossing, causes a decrease the fluorescence quantum yield. Electron transfer occurs when electron acceptors are in solution. The loss of an electron from a molecular excited state leads to a reduction in the fluorescence quantum yield. Energy transfer is similar, but the energy transfer process can lead to fluorescence from another molecule. Energy transfer is very useful in biomolecular studies since it is a strong function of the distance between the donor and acceptor. Thus, energy transfer can be used to determine whether there are changes in the distance of the donor and acceptor on the nanometer length scale. It is a molecular ruler.

Fluorescence resonance energy transfer (FRET) is also considered a molecular-optical ruler. The distance dependence can be plotted and the true distance between the fluorophore and quencher obtained from a distribution. Quenching and FRET obey the same distance dependence. They are analogous phenomena. Quenching is energy transfer followed by rapid deactivation. But the energy transfer part has the same spatial and temporal properties as FRET.

Stern-Volmer quenching

The Stern-Volmer equation is:

$$\frac{\Phi_0}{\Phi} = 1 + k_q \tau_{obs}[Q] \qquad (5)$$

k_q is the quenching rate constant, τ_{obs} is the observed lifetime in the absence of quencher, [Q] is the quencher concentration. An example of quenching is shown in Figure 7.17. The molecular EDANS (5-((2-aminoethyl)amino)naphthalene-1-sulfonic acid) is a fluorophore. When the energy is transferred to DABCYL (e-(4-dimethylaminophenylazobenzoyl) -L-lysine), there is no emission. DABCYL loses its energy exclusively by a non-radiative decay.

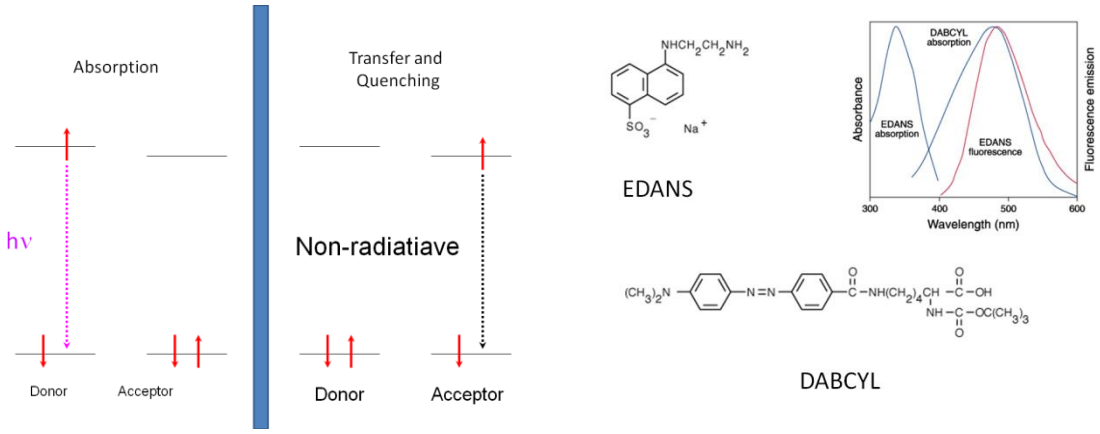

Figure 11. Quenching of radiation by energy transfer to a molecule with no emission.

A discussion of electron transfer quenching of the $Ru(bipy)_3^{2+}$ excited state.

The electron transfer rate constant, k_{ET}, depends:
(i) distance between the donor and acceptor,
(ii) the degree of quantum mechanical coupling between the molecular orbitals of donor and acceptor, and
(iii) the free energy change, $\Delta G°$
(iv) the reorganization energy, λ.
The latter parameter refers to the energy cost incurred by molecular rearrangements that must result from the transfer of charge along a finite distance. Generally, it is expected that rates of electron transfer will increase with decreasing donor-acceptor distances, and that the maximum rate will be observed when the reaction is activationless, i.e., when $\Delta G°$ is **negative**, preferably when $\mathbf{\Delta G° = -\lambda}$. In other words, the rate is optimized when the standard free energy change for the reaction is matched exactly by the energy required for reorganization of the donor, acceptor, and solvent molecules. In the table below you see the redox potentials for the species involved in this experiment. By analysing it you should conclude what quenching mechanisms are plausible for different quenchers. **NB**. The exact expression for

Species	Redox potential
$Ru(bpy)_3^{3+} + e^- \rightarrow Ru(bpy)_3^{2+}$	+ 1.26 V
$Ru(bpy)_3^{2+} + e^- \rightarrow Ru(bpy)_3^{+}$	+1.28 V
$*Ru(bpy)_3^{2+} \rightarrow Ru(bpy)_3^{3+} + e^-$	+ 0.84 V
$Ru(bpy)_3^{2+} \rightarrow *Ru(bpy)_3^{2+}$	(+ 2.1 V)
$Fe(CN)_6^{3-} + e^- \rightarrow Fe(CN)_6^{4-}$	+ 0.36 V
$Fe(H_2O)_6^{3+} + e^- \rightarrow Fe(H_2O)_6^{2+}$	+ 0.77 V
$O_2 + e^- \rightarrow O_2^-$	– 0.365 V

In principle, there is sufficient potential energy in a photo-generated redox pair that one devise a scheme to split water into H_2 and O_2. The goal of light-driven water splitting has been approached by hundreds of different research teams. The processes relevant to the process using $Ru(bipy)_3^{2+}$ are summarized below, where L represents the diimine ligand and Q represents the oxidant:

$$RuL_3^{2+} + h\nu \rightarrow *RuL_3^{2+}$$
$$*RuL_3^{2+} + Q \rightarrow RuL_3^{3+} + Q^-$$
$$2\ RuL_3^{3+} + H_2O \rightarrow 2\ RuL_3^{2+} + 1/2\ O_2 + 2H^+$$
$$Q^- + H^+ \rightarrow Q + 1/2\ H_2$$

In other words, solar energy can be is used to make fuels from water. Although the chemistry of such a solar cell is straightforward, there are some technological barriers to be overcome before ruthenium (II) complexes can form the basis of a commercial photovoltaic device.

Other applications for photooxidation of ruthenium (II) complexes involves development of due-sensitized photovoltaic devices, where $*RuL_3^{2+}$ is oxydized by a network of TiO_2 nanoparticles and consequently RuL_3^{3+} is reduced with a help of I_3^-/I^- redox pair. Resulting charges recombine

at the electrodes, thus, producing a phovoltage equal (without a load) to the overall redox potential through all the steps.

Fluorescence resonant energy transfer (FRET)

Fluorescence resonance energy transfer (FRET) is a distance-dependent interaction between the electronic excited states of two dye molecules in which excitation is transferred from a donor molecule to an acceptor molecule without emission of a photon. FRET is dependent on the inverse sixth power of the intermolecular separation, making it useful over distances comparable with the dimensions of biological macromolecules. Thus, FRET is an important technique for investigating a variety of biological phenomena that produce changes in molecular proximity.

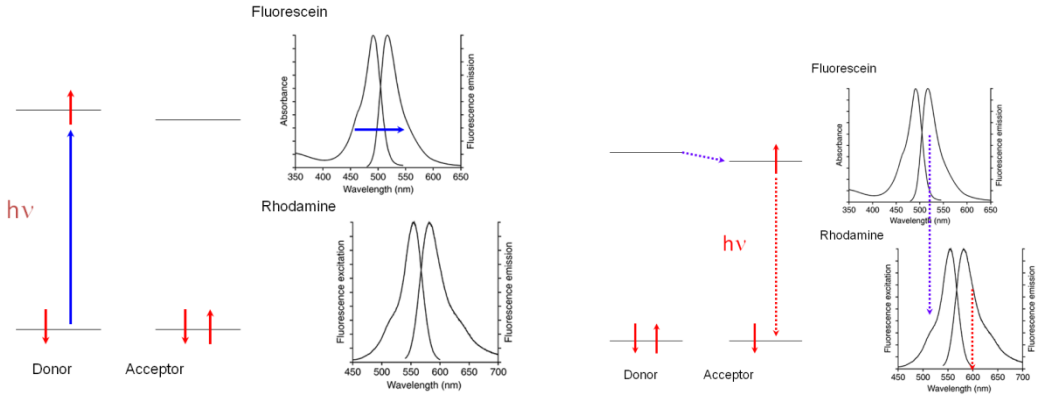

Figure 12. Energy transfer from one fluorophore (fluorescein) to another (rhodamine).

There are several requirements for energy transfer to occur. Because of the steep distance dependence, the donor and acceptor molecules must be in close proximity to one another (typically 10–100 Å). The absorption spectrum of the acceptor must overlap fluorescence emission spectrum of the donor. The donor and acceptor transition dipole orientations must be approximately parallel for maximum effect. The orientation dependence is $\sin^2\theta$, where θ is the angle between the transition dipole moments of the donor and acceptor.

The rate constant for energy transfer can be written in a simple form that emphasizes the distance dependence,

$$k_{DA} = (R_0/R)^6 \qquad (6)$$

The $1/R^6$ distance dependence arises from the fact that energy transfer is a dipole-dipole interaction. Speaking more precisely, it is the interaction of the transition dipole moment on the donor and the

acceptor that gives rise to the distance dependence. In Eqn. 6, the constant R_0 represents the distance at which the energy transfer efficiency is 50%. Solving for that constant we obtain:

$$R_0 = \sqrt[1/6]{\frac{13.8x\ 10^{-28}\kappa^2 \Phi_f J(\lambda)}{\tau_{obs} n^4}} \tag{7}$$

In Eqn. 7 the following quantities are defined as:

κ^2 - orientation factor (2/3 for an isotropic sample)

n - index of refraction

Φ_f - quantum yield of the donor

The spectral overlap integral is,

$$J(\lambda) = \int \varepsilon(\lambda) F_D(\lambda) \lambda^4 d\lambda \tag{8}$$

Two examples of so-called FRET pairs are given in Table 1

Table 1 Example of FRET pairs, including the distance of 50% FRET efficiency, R_o.

Donor	Acceptor	R_o (Å)
Fluorescein	Tetramethylrhodamine	55
IAEDANS	Fluorescein	46

Using these definitions, we can predict the distance dependence of the FRET yield, Φ_{FRET}. A FRET yield of zero means that all of the fluorescence originates from the donor and a FRET yield of one signifies that all of the energy has been transferred to the acceptor, and all of the fluorescence is observed at the wavelength of the acceptor. An intermediate yield implies that there will be two fluorescence bands, one from the donor and one from the acceptor, with varying yield. The FRET yield is

$$\Phi_{FRET} = \frac{1}{1 + \left(\frac{R}{R_0}\right)^6}$$

We can see that when $R = R_0$ the FRET yield is 0.5, which corresponds to the definition of R_0 given above.

Relaxed and resonance fluorescence: the time-dependent Stokes shift

Franck-Condon transitions are vertical transitions that occur from the ground to the excited state (or in emission from the excited state to the ground state). The pattern of vibronic bands is

determined by the displacement of the excited state relative to the ground state potential energy surface as shown below. The overlap of the vibrational wavefunctions in the two states gives rise to the progression in terms such as <0|0'>, <0|1'> , <0|2'> , <0|n'> etc. We can represent this within the harmonic approximation

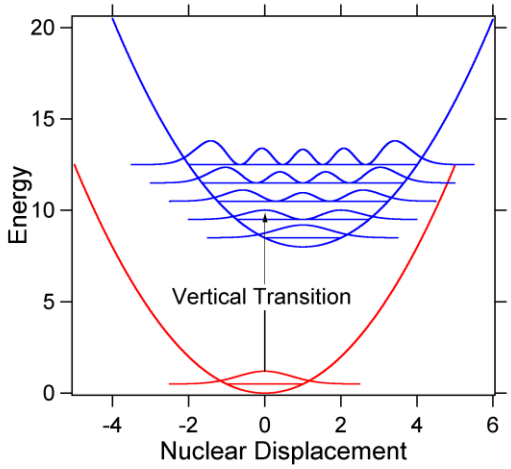

Figure 13. Vertical transition according to Franck-Condon principle that nuclei are frozen during an electron promotion to an excited state.

The shape of the absorption band is determined by the displacement of the potential energy surface in both the harmonic and realistic picture. In addition, we can identify two types of emission that can occur. The emission that occurs instantaneously following photoexcitation is called resonance fluorescence. It is shown below.

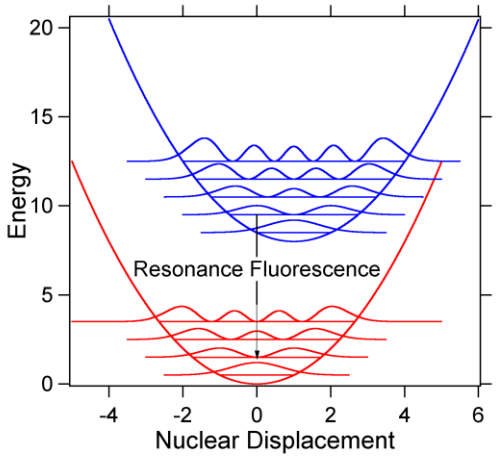

Figure 14. Resonance fluorescence or unrelaxed fluorescence that occurs prior to vibrational relaxation.

However, typical excited states live long enough that the vibrational energy of the excited state can be dissipated to the surroundings. This energy dissipation is known as vibrational relaxation. Following vibrational relaxation the populated vibrational modes in the excited state are in thermal equilibrium. If emission occurs from these modes the Franck-Condon vertical transition occurs from the equilibrium nuclear position of the excited state. This is called relaxed fluorescence and is shown below.

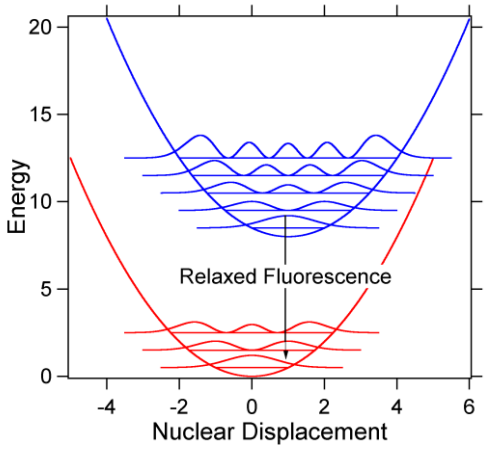

Figure 15. Relaxed fluorescence, which is normal fluorescence that occurs after vibrational relaxation.

Thus, resonance fluorescence occurs at the λ_{max} of the absorption spectrum but the λ_{max} of relaxed fluorescence is shifted. The shift between resonance and relaxed fluorescence is known as the Stokes shift. We usually quote the values of these shifts in cm^{-1} (wavenumbers) where wavenumbers $= 10^7/\lambda$ (nm). The energy difference between resonance and relaxed fluorescence is called the reorganization energy. If we consider the coordinates of the molecule the reorganization energy is $\Delta^2/2$ for each Franck-Condon active mode. This is illustrated for a single vibrational mode below:

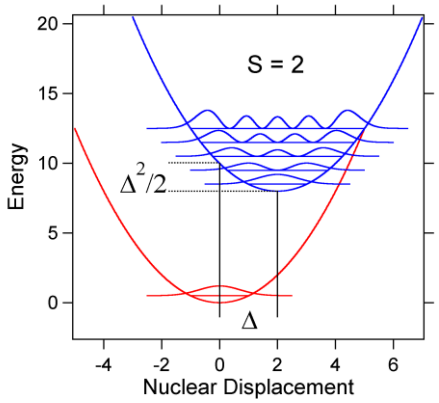

Figure 16. Depiction of an excited state displacement of S = 2.

For N coupled Franck-Condon active modes we have

$$E_{reorganization} = \sum_{i=1}^{N} \frac{\Delta_i^2}{2} \hbar\omega_i$$

for a classical model. The reorganization energy is the energy required to distort the molecule to the equilibrium nuclear position of the ground state along the excited state potential energy surface. This is equivalent to the energy required to distort the molecule to the equilibrium position of excited state along the ground state potential energy surface. Note that quantum-mechanically for each mode the energy required is S (the electron-phonon coupling constant) time the mode energy. Thus, for a quantum-mechanical model we have:

$$E_{reorganization} = \sum_{i=1}^{N} S_i \hbar\omega_i$$

The discussion thus far assumes that there is no excited state photochemistry. We treat photodissociation and predissociation phenomena below.

The Franck-Condon active modes will be totally symmetric modes of the molecule. This is because the excited state must have the same symmetry as the ground state in a Franck-Condon transition. Recall that since the transition is vertical the nuclei do not have a chance to change their positions during the time of the electronic transition. This alone requires that the symmetry remain the same. Thus, the relevant vibrational modes depicted in the Figures above must be totally symmetric modes (belonging the symmetries A, A_1, A_{1g} etc. if the molecule has high enough symmetry to belong to one of the point groups in the character tables).