Fluorescence Spectroscopy

1. 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 3.1, 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 3.1. 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 1 leads to the "mirror image" relationship between absorption and emission lines.
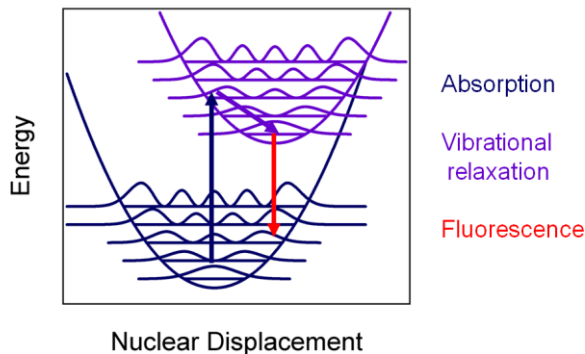

Figure 1. Illustration of the events leading to fluorescence. Absorption is followed by vibrational relaxation. Fluorescence occurs from a relaxed nuclear geometry in the excited state.
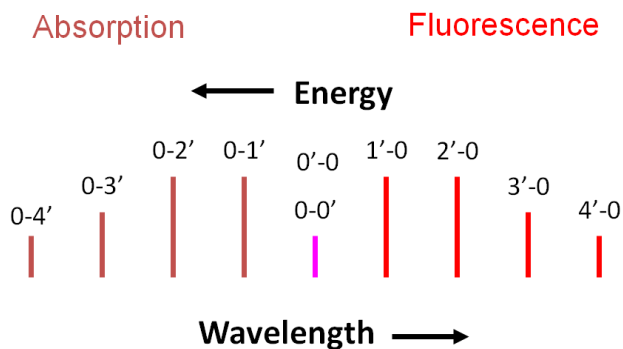
Figure 2. Illustration of the mirror image relationship for absorption and fluorescence spectra.

The commonly used dye Rhodamine shows the mirror image relationship.
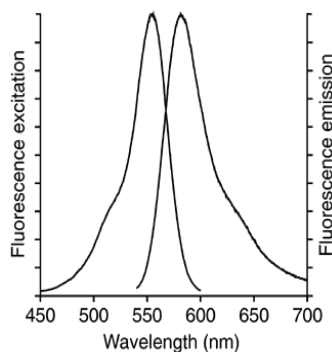

Figure 3. Mirror image relationship of excitation and emission.

Figure 3 shows the excitation spectrum and the emission spectrum. The excitation spectrum is has the same theoretical line shape as the absorption spectrum.

2. 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,

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

(2.1)

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

$$k_{obs} = k_f + k_{nr}$$

(2.2)

The measured population of the excited state decreases exponentially according to

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

(2.3)

The quantum yield of the fluorescence is given by,

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

(2.4)

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, $\Box_f$, can be measured in a fluorometer by comparing the emission of an unknown to that of a known standard.

3. 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 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 π-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 $\Delta m = \pm 5$.
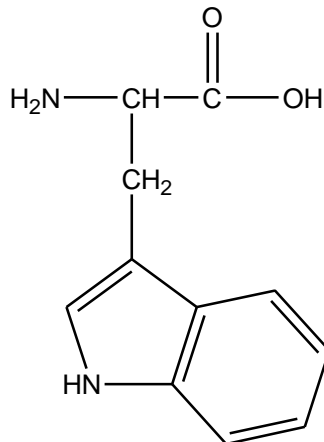
Figure 4 Structure of tryptophan

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 5, which shows the extinction coefficient of tryptophan.
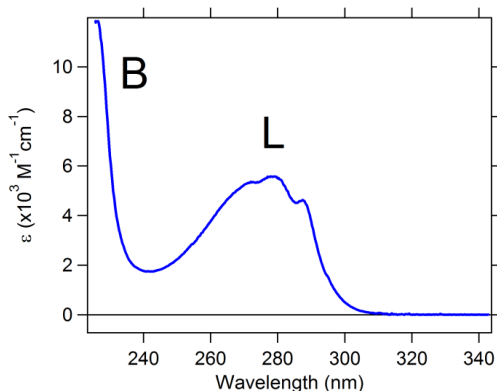

Figure 5 Absorption spectrum of tryptophan.

The emission spectrum of tryptophan is shown along with the absorption spectrum in Figure 6.
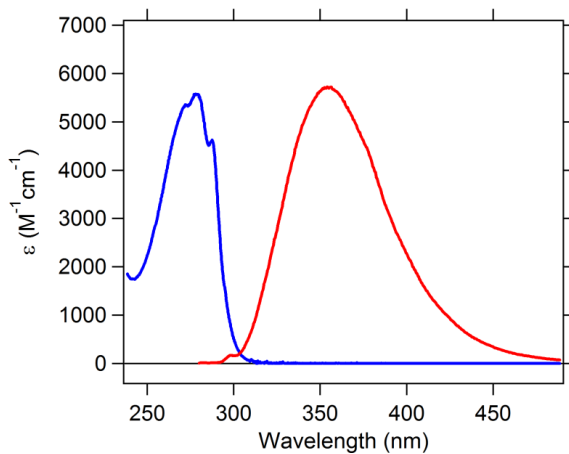

Figure 6 Tryptophan absorption (blue) and fluorescence (red).

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, $\widetilde{\nu}$. If we convert to units of cm^{-1}, the appearance of these data is shown in Figure 7.
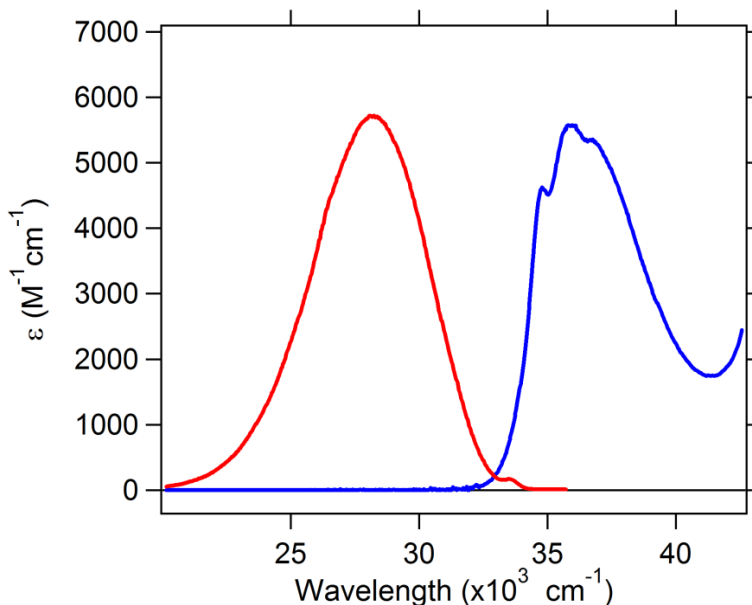

Figure 7. Absorption and emission spectra of tryptophan plotted as a function of wave number.

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, 199

The wavelength and intensity of tryptophan emission is sensitive to hydrogen bonding and hydrophobicity. If a protein unfolds, for example, there will be a large change in the fluorescence yield and fluorescence wavelength. Figure 8 shows the example of a folding study of the enzyme, caspase 3. Caspases are cysteine proteases that are activated during apoptosis. As with other proteases they have an inactive form, the zymogen form, and an active form. Splicing of caspases to remove a short peptide, called a pro-peptide, is required to initiate their function in controlled cell death. Shown in the figure is the structure of caspase-1. Note that the structure indicates that two subunits have been cleaved and are intermingled. This type of fold following removal of the pro-peptide produces the active form of the enzyme.

One can study the folding of the protein by monitoring its unfolding as the concentration of urea is increased. This is shown in Figure The tryptophan fluorescence wavelength changes as a function of the temperature. Of course, the interpretation of such wavelength changes depends on experience, but the significant point is that the changes in wavelength are sensitive to

the environment so that protein unfolding can be successfully followed in an experiment. In this particular case, the data were interpreted to indicate that there are two folding intermediates. One of them consists of monomer caspase and one of them of dimer caspase protein.
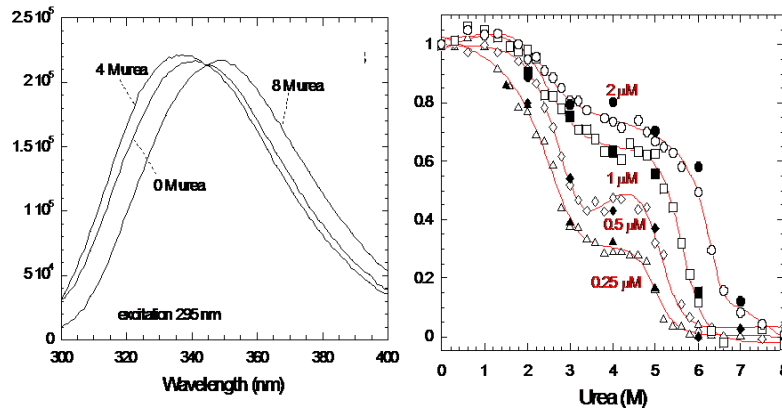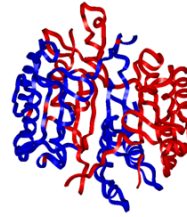

Figure 8 Study of the unfolding of caspase using

tryptophan fluorescence to monitor protein structure.

Data obtained courtesy of the laboratory of Prof. Clay Clark, NCSU, Biochemistry.

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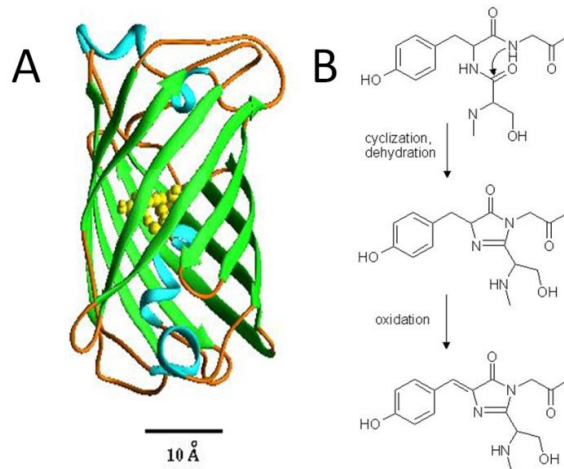
Figure 9. A. Overall structure of green fluorescent protein.

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 9A) 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.
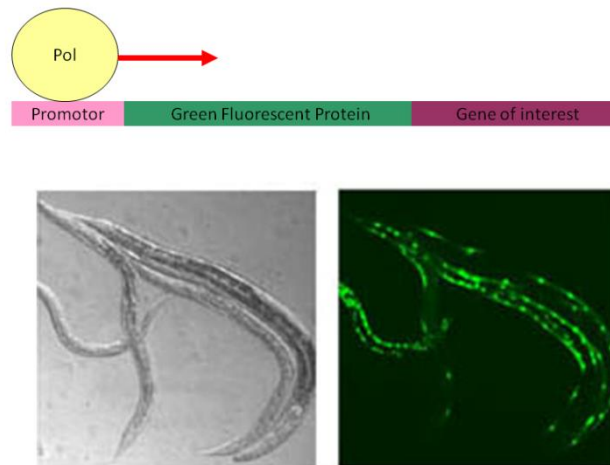
Figure 10 Illustration of the reporter gene concept.

2.1.4 Fluorescence quenching and energy transfer

The topic of resonance energy transfer include fluorescent resonant energy transfer, but has a number of other applications in quantum chemistry.[1] When applied to fluorophores we need to understand both absorption and emission spectra, including their line shapes. Since the fluorescence energy transfer rate is proportional to spectral overlap of the donor and acceptor, there is a further relationship between static and time-resolved spectroscopy. One can measure energy using both. The quantum yield gives the intrinsic fraction of the molecules that decay by emitting light.

As we consider the energy transfer pathway, we must always be aware that it competes with a number of other deactivation pathways:

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 can occur when electron acceptors are in solution. Studies of solutions reveal much general information useful for more localized and defined FRET measurements.[2] 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.

Stern-Volmer quenching

The Stern-Volmer equation is:

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

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 11. 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.
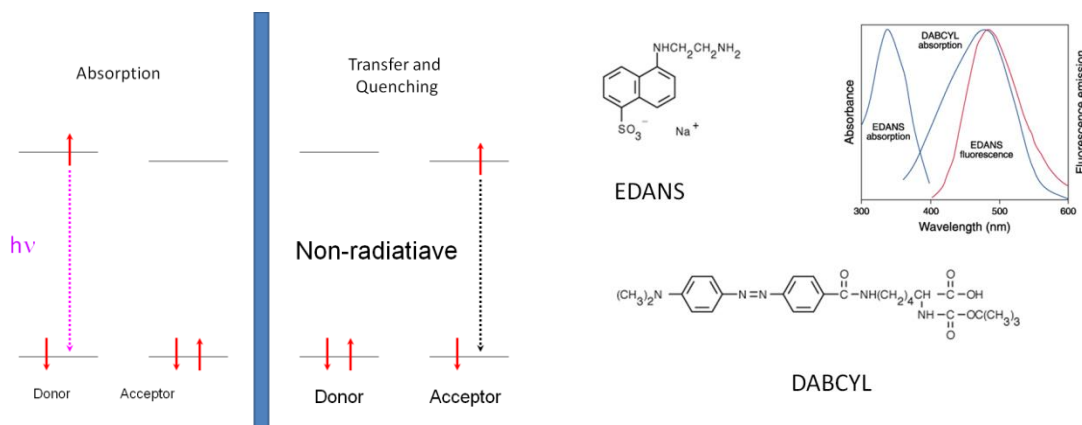

Figure 11. Quenching of radiation by energy transfer to a molecule with no emission.

2.1.6 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
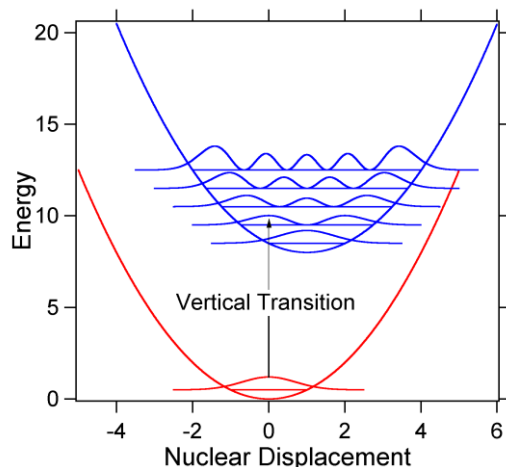

Figure 13. Vertical transition according to Franck-Condon principle that nuclei are frozen during an electron promotion to an excited state.

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.
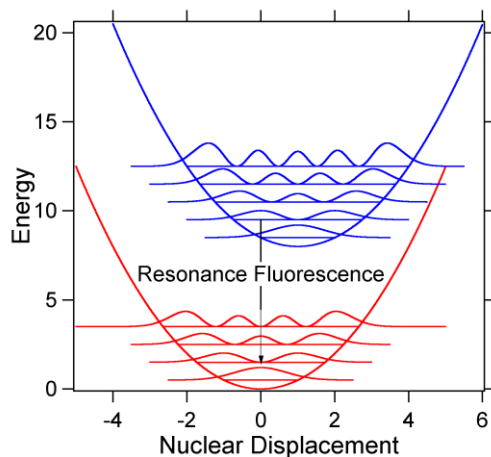

Figure 14. Resonance fluorescence or unrelaxed fluorescence that occurs prior to vibrational relaxation.

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.
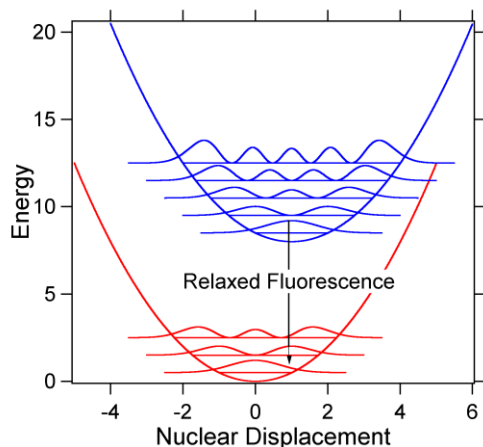

Figure 15. Relaxed fluorescence, which is normal fluorescence that occurs after vibrational relaxation.

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:
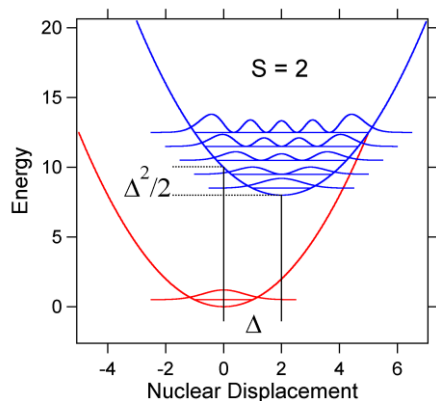

Figure 16 Depiction of an excited state displacement of S = 2.

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).

2.1.7 Summary of important aspects of fluorescence

The mirror image relationship in fluorescence spectra

The Franck-Condon factors are identical for absorption and emission. This means that the line shapes are related. However, for emission the energies are lower than the 0 → 0' transition, while those for absorption are greater than 0 →0' transition resulting a mirror image relationship for fluorescence spectra.

Measurement of the intrinsic fluorescence rate constant

The fluorescence emission rate constant, k_f, is a fundamental quantity that can be determined by measurement of two experimental quantities; the observed lifetime, k_{obs}, and the

fluorescence quantum yield, Φ_f. A single measurement will not suffice because neither has enough information to uniquely determine k_f.

$$\Phi_f = \frac{k_f}{k_f + k_{nr}} \;\; ; \; k_{obs} = k_f + k_{nr}$$

Applications of fluorescence

Fluorescence is probably the most used kind of spectroscopy. Since fluorophores can be used to stain cells and as indicators in complex materials, one can measure local effects that include the dielectric function and the proximity of the fluorophores to quenchers. Quenchers include dyes that can undergo energy transfer. Fluorescence energy transfer can be applied as a molecular ruler to determine the distance between two fluorescence molecules in a sample. This has applications in polymer science, cell biology, DNA hybridization, protein folding, and single molecule detection.