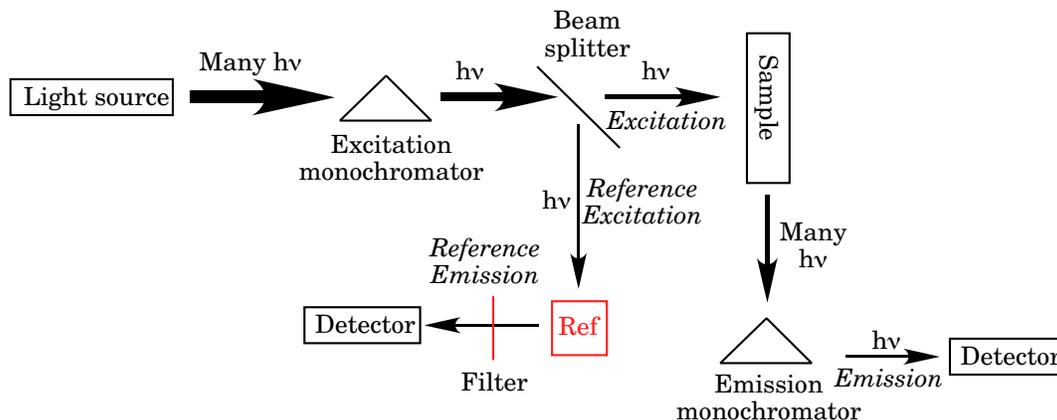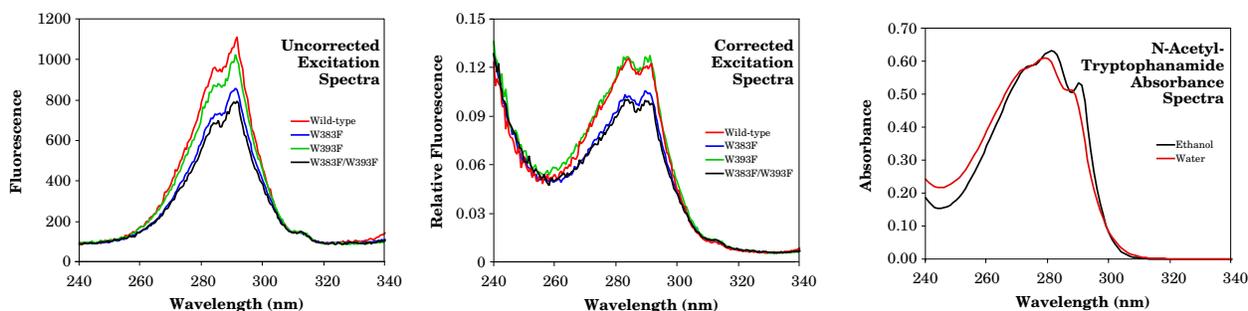## Spectrofluorometers and Fluorescence Phenomena

In practice, the fluorescence spectroscopic measurements are more complex than the relatively simple emission intensity equations imply. To see why, it is necessary to look at a simple diagram for a spectrofluorometer.

Light source → Many hν → Excitation monochromator → hν → Beam splitter → hν Excitation → Sample → Many hν → Emission monochromator → hν Emission → Detector

Beam splitter → hν Reference Excitation → Ref → Reference Emission → Filter → Detector

The spectrofluorometer consists of a light source, an excitation monochromator, a sample holder, an emission monochromator, and a detector.

Most spectrofluorometers also have a reference sample. The reference is generally a solution of a strongly fluorescent molecule with a broad absorbance spectrum such as rhodamine. The reference is necessary to correct for lamp output, especially when varying the excitation wavelength, and to correct for differences in detector sensitivity. The graphs below show excitation spectra for proteins containing different amounts of tryptophan. The spectra uncorrected for lamp output are distorted, while the spectra corrected for lamp output are much more similar to the absorption spectrum of N-acetyl-tryptophanamide.

Uncorrected Excitation Spectra (Fluorescence vs Wavelength (nm)): Wild-type, W383F, W393F, W383F/W393F

Corrected Excitation Spectra (Relative Fluorescence vs Wavelength (nm)): Wild-type, W383F, W393F, W383F/W393F

N-Acetyl-Tryptophanamide Absorbance Spectra (Absorbance vs Wavelength (nm)): Ethanol, Water

Inner filter effect

At low concentrations of fluorophore, the fluorescence emission intensity of a sample is essentially linearly proportional to concentration. However, as the concentration increases, a point is reached at which the intensity increase is progressively less linear, and the intensity eventually decreases as concentration increases further. The most common reason for this is that, as the absorbance of the sample increases, the light intensity experienced by some of the fluorescent molecules is lower than that experienced by others. When excitation intensity

decreases, so does fluorescence emission intensity. It is generally necessary to use concentrations that result in absorbance values of 0.1 or lower to observe concentration dependent emission.
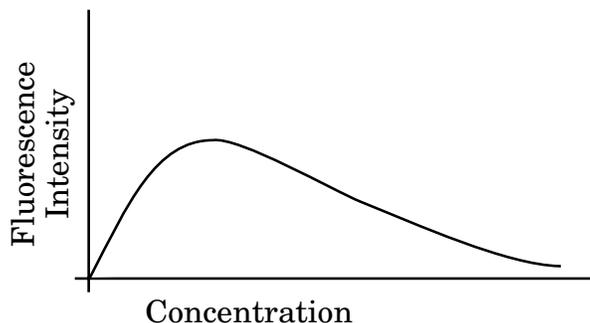
Emission spectra for single fluorophores
In most cases, the emission occurs at longer wavelengths than are required for excitation. This difference, the **Stokes shift**, is due to a variety of factors; some of these factors are intrinsic to the fluorophore, and some are due to interactions of the fluorophore with its environment.

Photon absorption occurs on a very short time scale ($\sim 10^{-15}$ seconds). Several processes then may occur. If the molecule is excited beyond the first excited electronic state (*e.g.*, the S_2 state), it will usually rapidly lose energy via internal conversion, to reach the lowest energy singlet excited state, S_1, in a process that takes $\sim 10^{-12}$ seconds. In most cases, the excited state will vibrationally relax to reach the lowest energy state within the excited state.
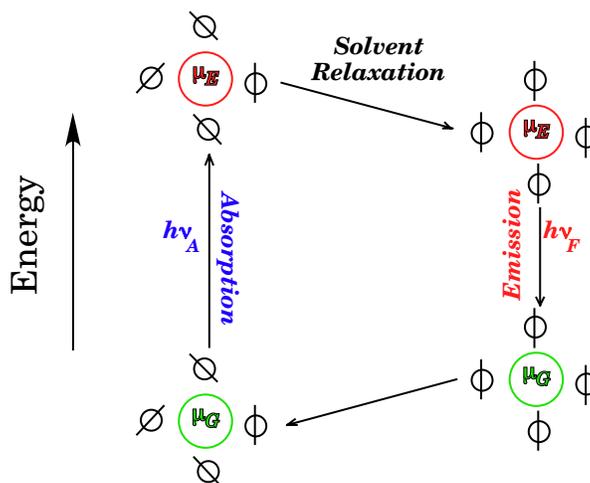
If the fluorophore has a larger dipole moment in the excited state (which is usually the case, especially for polar fluorophores), the solvent will rearrange to stabilize the greater charge separation. In the diagram above, the fluorophore in the ground state (μ_G) absorbs a photon to transition to an excited state (μ_E). The surrounding solvent molecules do not move during the absorption process. The solvent molecules can then rearrange to adapt to the altered properties of the excited fluorophore in a process that takes 10^{-11} to 10^{-10} seconds. The solvent rearrangement effect is too

slow to occur during absorption processes, and therefore absorption tends to be much less sensitive to solvent effects than is fluorescence. However, the excited state lifetime is easily long enough (usually ~10^{-9} seconds) to allow solvent rearrangement. The rearranged solvent stabilizes the excited fluorophore, and therefore lowers the overall energy of the system. However, when the fluorophore emits a photon to transition back to the ground state, once again the solvent molecules do not move during the process. As a result, the immediately reached ground state is higher in energy until the solvent molecules rearrange again.

All of these factors mean that the wavelength of the fluorescence emission is usually longer than that of the absorbed radiation. Because the excited state that decays to the ground state is the same, the fluorescent spectrum is usually independent of the excitation wavelength. This is useful for molecules that contain more than one fluorophore; choosing an excitation wavelength that excites one fluorophore without affecting the other will give an excitation spectrum similar to any other wavelength that would excite the fluorophore if that fluorophore were alone. For example, proteins contain tryptophan and tyrosine. Tyrosine has little absorbance at 295 nm; although this is not the absorbance peak of tryptophan, excitation at this wavelength will result in tryptophan fluorescence unaffected by tyrosine contributions.

Theoretical considerations regarding solvent rearrangement

The solvent rearrangement effect depends on the solvent, with more polar solvents typically exhibiting greater effects. The Lippert equation describes the energy change that results from solvent effects.

$$\bar{v}_A - \bar{v}_F = \frac{2}{hc}\left(\frac{d-1}{2d+1} - \frac{n^2-1}{2n^2+1}\right)\frac{(\mu_E - \mu_G)^2}{a^3} + constant$$

where $\bar{v}_A$ and $\bar{v}_F$ are the frequencies of absorption and emission in wavenumbers (cm^{-1}); the difference for these terms is the Stokes shift; d is the solvent dielectric constant, n is the index of refraction, μ_E and μ_G are the dipole moments of the excited and ground states, and a^3 is the volume of the cavity surrounding the fluorophore. The "constant" reflects the Stokes shift that results from the vibrational relaxation and internal conversion effects mentioned earlier. The term within the brackets is the orientation polarizability of the solvent, Δf:

$$\Delta f = \frac{d-1}{2d+1} - \frac{n^2-1}{2n^2+1}$$

The Lippert equation derivation makes several assumptions. It assumes a spherical fluorophore. It assumes that the dipole vectors for the excited and ground states are similar (this is a reasonable assumption for most fluorophores). Finally, it ignores specific solvent interactions, such as hydrogen bonds that stabilize the excited state.

The effects of the index of refraction differ from those of the dielectric constant. The index of refraction is a property of the electrons of the solvent molecules, while the

dielectric effects require both electronic and molecular orientation effects. The difference means that the index of refraction affects both the excitation and emission properties, while the solvent dielectric affects only the state that results in emission.

The Lippert equation works fairly well for non-protic solvents; protic solvents (such as water and ethanol) tend to form hydrogen bonds, and tend to result in greater than predicted Stokes shifts. In some cases, the specific solvent effect can be corrected for by using solvent mixtures. For example, both ethanol and water have the ability to form hydrogen bonds, but these solvents have ~3-fold differences in dielectric constant.

Observations of Stokes shift can be used to assess environment polarity. The solvent also affects the quantum yield by differential effects on the rate constants for fluorescent emission relative to other relaxation mechanisms. This property can also be used to assess changes in environment. However, the change in quantum yield does not change systematically with polarity. An example of this is shown in the graph below, which considers the fluorescence of N-acetyl tryptophanamide in different solvents. Note that while the position of the peak emission varies with solvent polarity, the relative quantum yield varies in a non-regular fashion.
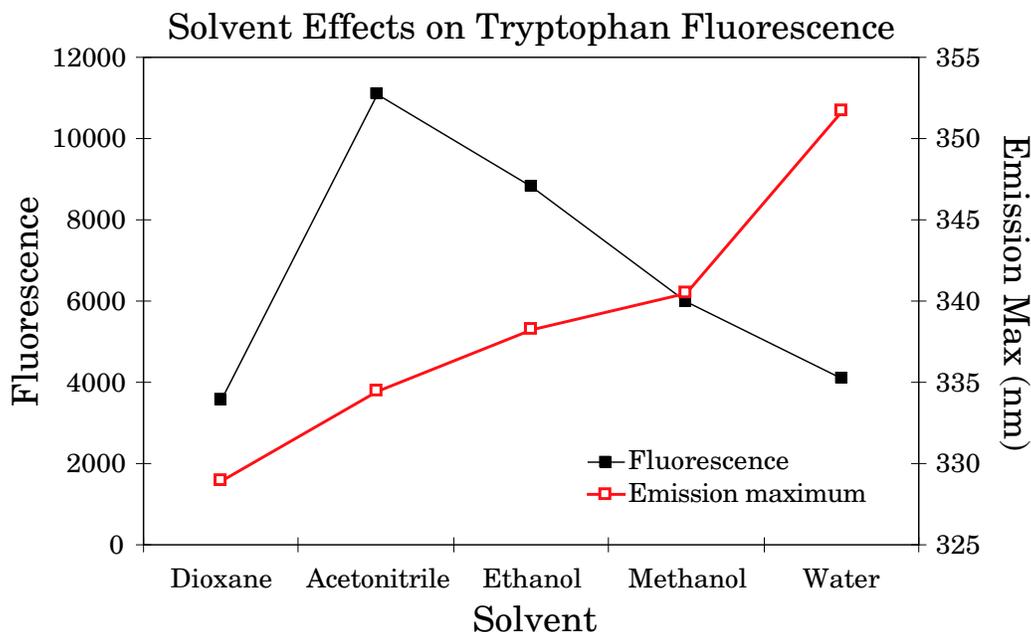
The Stokes shift phenomenon is a large part of the reason that fluorescence spectroscopy is more sensitive to the environment around the chromophore. Because no rearrangement of the environment occurs during the absorption process, spectral changes in absorbance spectroscopy only reflect differences in the ground state environment. In contrast, fluorescence allows measurements of changes in the environment around both the ground state and the excited state of the molecule.