

Fundamentals of Molecular Fluorescence and Fluorimetry

1.0 Introduction

Fluorescent light emitted by a molecule is light generated by spontaneous emission. While this might sound very simple, the emission and absorption of electro-magnetic radiation from a molecule is a fundamental topic that requires a thorough knowledge of quantum and classical physics to fully model and comprehend. Significant physical insight, however, can be gained by first understanding the molecular energy levels that are involved in the emission and absorption of photons, and then learning some simple rules about when and how these transitions can occur.

Molecules are, in essence, an assembly of atoms held together in a stable state by electromagnetic forces. Therefore, any intuition pertaining to the energy levels in an atom will help in understanding those in a molecule. Within its stable arrangement of atoms, the molecule can support a number of different energy levels. As described in this primer, these energy levels arise from both the electronic states within the molecule, as well as from the different motions within the molecule's atomic structure. These "modes" of a molecule are the rotational and vibrational movement of its atomic structure. Translational movement of the whole molecule is also a form of kinetic energy, but has less influence on direct optical transitions.

Even though quantum mechanics is required to fully explain the origin of the energy levels, a general physical insight can be gained using only classical physics. It should be emphasized that the actual energy levels of an atom or molecule are discrete in nature. Spectra, including fluorescence spectra, provide a map of the emission or absorption of radiation when transitions occur between these energy levels. When the electrons in a molecule are in the "ground state", they are in the lowest, unexcited electronic state (i.e., in a state of "rest"). When the electrons are redistributed into higher energy levels through an external stimulus, they are said to be in an "excited state". Absorption of radiation to and from these excited states is the basis for gaining insight into molecular structure or chemical reactions of the molecule. This is the foundation of optical spectroscopy and of many forms of optical sensing.

2.0 Energy Levels

In order to gain insight into the radiative transitions in molecules, it is instructive to start by understanding the origin of energy levels. We examine a diatomic molecule, because it has the simplest structure and is therefore the easiest to understand. Polyatomic molecules behave in a similar manner, but the added complication of their analysis does not generally yield better insight or understanding.

A molecule can be thought of as having several distinct reservoirs of energy including:

- the spin of its electrons
- the spin of its nuclei,
- the electronic transitions between available states,
- the vibration of the its constituent atoms,
- the rotation of the entire molecule around its center of mass, and
- translational movement of the entire molecule.

If we assume that the two atoms are held together by a spring, we can use the analogy of a vibrating string on a violin, to visualize energy levels of the basic modes of movement of the diatomic structure: vibration, rotation, and translation. In Figure 1 a – c below, the modes of a diatomic molecule are depicted. As previously mentioned, the modes of a diatomic are simple compared to a polyatomic molecule, but the basis for physical understanding is the same.

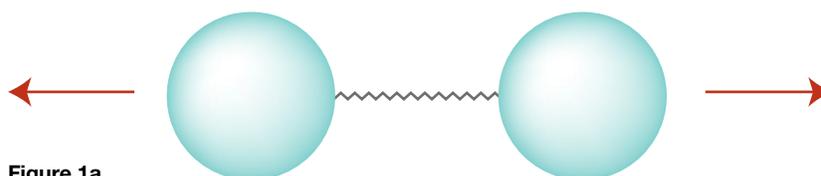


Figure 1a

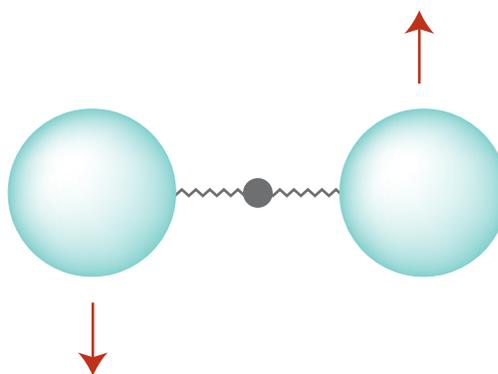


Figure 1b

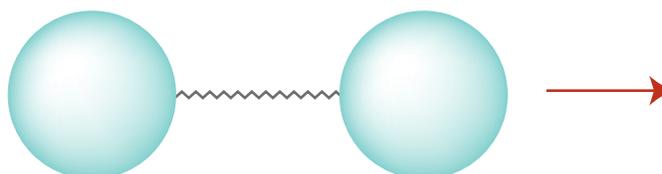


Figure 1c

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For example, figure 1a depicts a vibrational mode of oscillation called a symmetric stretch (one of several possible vibrational modes), while Figure 1b shows the rotational motion of the molecule. Figure 1c is simple translation. The molecule is represented pictorially by masses connected by springs, because the derivation of the energy levels uses this classical analogy as its starting point.

While the mathematical derivation of molecular energy levels is far beyond the scope of this application note, it can be found in full detail in many texts on quantum mechanics, statistical mechanics or physical chemistry (e.g.: Molecular Spectra and Molecular Structure: Volume I - Spectra of Diatomic Molecules. 2nd edition, G. Herzberg, D. Van Nostrand Company, Inc. (1950)). It is important to realize that while the detailed calculation of exact molecular energy levels is a complicated task, visualizing the modes of movement that give rise to the corresponding energy levels is an easy and physical exercise.

It is also helpful to note the general relative size of different types of energy levels or energy “spacings”. The relative spacing between energy levels for different modes of molecular motion is shown schematically in Figure 2. The vibrational modes are the largest, followed by the rotational, and the translational modes.

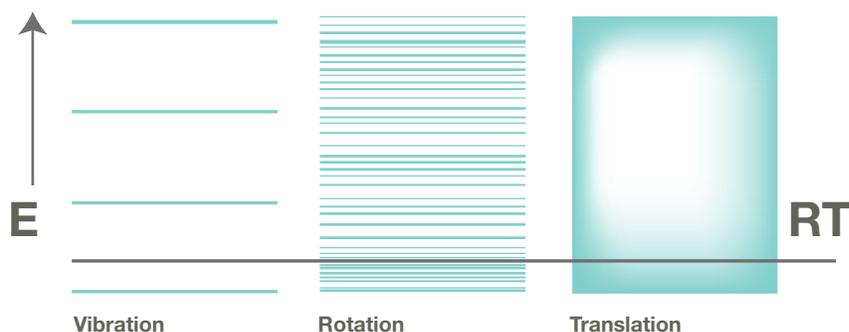


Figure 2

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It is important to note, however, that the electronic transitions have the largest energy spacing, and correspond to emission or absorption of visible photons. The electronic transitions occur from a level or state which has specific electronic, vibrational, and rotational energy to another level with a different electronic, vibrational, and rotational state. A representation of a typical electronic energy level is shown below in Figure 3.

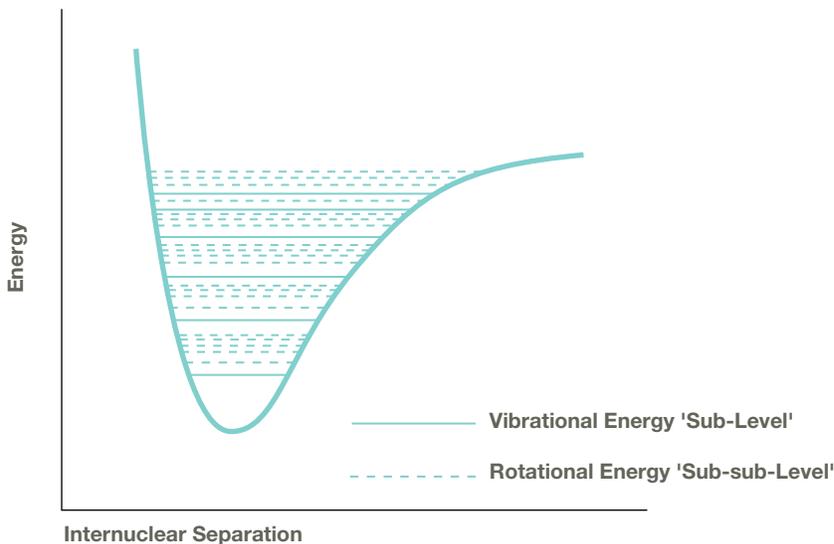


Figure 3

At equilibrium, where Boltzmann statistics hold there is a significant population in the lower energy levels, and less in the higher levels. This entails that an optical stimulus (such as a photon that is absorbed by the molecule) can excite electrons from the lower energy levels into the higher energy levels. The electrons will then “fall back” to the lower energy levels through spontaneous emission, and release a photon with a lower energy, in order to preserve the equilibrium population distribution of the molecule. The difference in photon energy becomes heat.

3.0 Wave-Functions

With this overview of how the energy levels in a molecule or molecular system arise, and their relative magnitudes, we can now examine some of the mathematical machinery used to model radiative transitions. These transitions consist of the absorption and emission of electromagnetic energy. In the optical regime, these transitions occur between electronic levels, though the rotational and vibrational states of the levels may change as well. Once again, quantum mechanics is used in order to provide the detailed models of when and why these transitions occur.

In this approach, a “wave function” that describes the position of a particle (e.g., electron) under study is calculated. The molecular (or atomic) energy levels and the wave-functions for the system are simultaneously computed using the “wave” or Schrödinger equation.” The Schrödinger equation can be expressed through so-called “operators”. The operator H is the Hamiltonian, and when acting on the wave-function, yields a constant multiplied by the same wave-function. In mathematical form, the Schrödinger equation can be expressed as:

$$H \psi(x,y,z) = E \psi(x,y,z)$$

Equation 1

H is the Hamiltonian (total energy) operator

ψ is the particle’s wave-function

E is the energy value associated with ψ

By solving the equation above for its “eigenvalues” and “eigenstates”, both E and ψ are found, and a fair amount of information about the system becomes available. The E values are the energies of the corresponding states of the particle and the wave-functions are ψ . Note that the wave-function’s precise physical meaning is the probability amplitude of finding a particle at any given position, while the absolute value of the wave-function multiplied by its complex conjugate represents the probability density of a particle’s position. Since the particle must exist somewhere in space, the integral of this probability density over all space is generally normalized to 1 to express this fact. This is expressed mathematically by the integral shown below:

$$\int \psi^* \psi dr^3 = 1$$

Equation 2

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At this point, however we have generally only solved for the “spatial” part of the wave-function. The full description of an electron’s position requires a wave-function that is the product of spatial and “spin” components, as shown in equation 3:

$$\Psi_{\text{total}} = \Psi_{\text{spatial}} \Psi_{\text{spin}}$$

Equation 3

Although spin is a fully quantum mechanical concept that has no real classical analog, some insight can be gained by knowing that it behaves in many ways like an angular momentum. The spin is important because the transitions that give rise to fluorescence occur only when certain conditions are met. These conditions or “transition rules” stipulate that for a dipole allowed transition to occur:

- 1 The spatial wave-functions of the two states must have opposite parity
- 2 The spin wave-functions must be the same

These conditions are met in many systems, and in particular many metal organic dyes. These metal organic compounds are of interest to us as they are often what are used in fluorescence quenching based optical sensors, such as dissolved oxygen or pH sensors.

With this insight into how the energy levels in a molecule or molecular system arise, and their relative magnitudes, we can now examine some more of the mathematical machinery that will help us understand radiative transitions in fluorescence. These transitions consist of the absorption and emission of electromagnetic energy. In the optical regime, these transitions occur between electronic levels, though the rotational and vibrational state of the levels may change as well. Once again, we must appeal to quantum mechanics in order to understand the details of when and why these transitions occur.

4.0 Radiative Transitions and Energy

As mentioned previously, the transitions which can release a photon in the visible spectrum occur between electronic states of a molecule. By definition, these transitions occur between a ground state and an excited state. This scenario is illustrated in Figure 4, where two wells similar to those shown in Figure 3, are now shown together. This simplified diagram still reveals the basics of how these transitions occur.

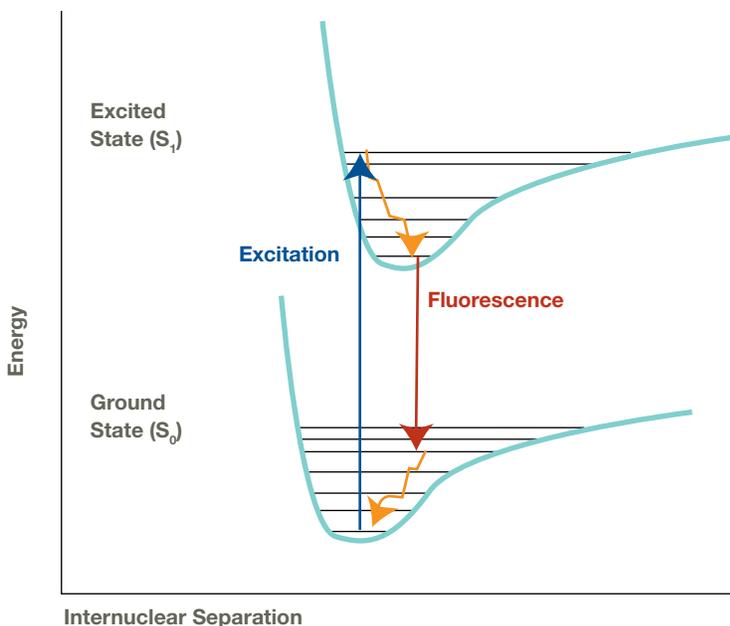


Figure 4

So how does the energy of these transitions really relate to the photon that is released? It is well known from quantum physics that a photon's energy E can be related to the photon's color or wavelength by the following relationship:

$$E = hc/\lambda$$

Equation 4

h is Planck's constant

c is the speed of light

λ is the wavelength of the light.

As can be seen in Figure 4, an electron can be promoted to an excited state using a photon with higher energy (shorter wavelength) and the subsequent fluorescent photon is emitted with a lower energy (longer wavelength). The energy difference between the two photons is taken up by the molecule and usually results in heat.

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Ideally, if the energy levels were well defined and isolated from their neighbors, the emitted photon would have a single energy value (or wavelength). Equation 4 then implies that the light emitted would be very close to monochromatic. However, at room temperature the spacing between the rotational energy levels is generally on the order of the thermal randomization energy and therefore several levels share energy or are “in communication” with each other (i.e., electrons can move about freely within these levels). This fact often causes optical transitions (e.g.: fluorescence) to be broader in spectral content compared to atomic transitions.

If the energy levels are not completely distinct because they are in thermal communication, the transition energy, E , is not sharp or distinct in wavelength and has a larger bandwidth. This fact is evidenced by the experimental spectral line shape which is usually a smeared peak with finite spectral width, rather than a delta function. This is shown in figure 5, as a comparison between a single idealized transition depicted in Figure 5a, and the actual, broadened transition (due to vibrational and rotational splitting as well as communication) shown in Figure 5b.

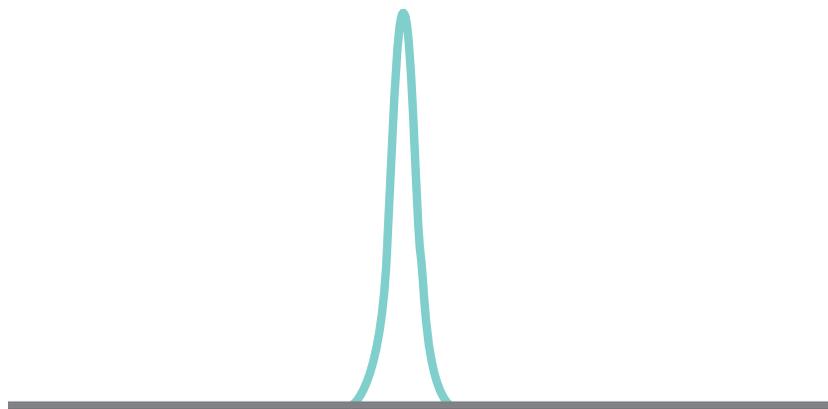


Figure 5a Wavelength

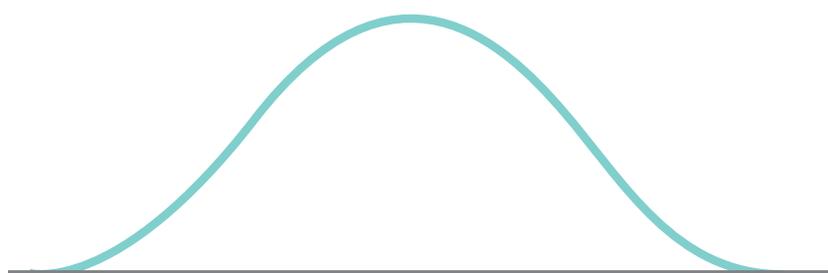


Figure 5b Wavelength

5.0 Upper-state Lifetime Measurements

While the diagrams of the full potential well shown in Figure 3 and Figure 4 above can be used to give the most physical information about the transition, these diagrams are often simplified (see Figure 6) in order to give a more direct picture of the transition.

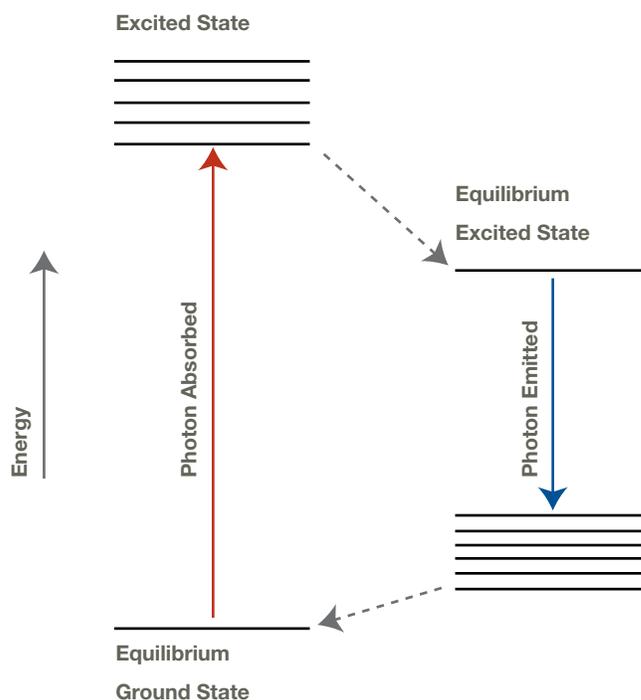


Figure 6

Figure 6 depicts the mechanics of a fluorescence transition. Specifically, as described before, a larger energy photon indicated by the blue line excites an electron from the ground state manifold to an excited state manifold. The electron may relax within the manifold to an equilibrium excited state where it can only decay further by an optical transition. This dipole allowed transition creates a lower energy photon indicated by the red line. Many organo-metallic dyes are excited in the visible region of the spectrum.

In general, the optical fluorescence depicted in Figure 6 can be monitored and a quantity known as the equilibrium excited (or upper) state lifetime can be defined. This upper state lifetime is the fundamental time it takes for the amplitude of the fluorescence to decay to $1/e$ of its initial amplitude. This measurement is often done by using a repetitively pulsed light source with very short time duration pulses (compared to any transition speed time within the system) of light, and monitoring the decay as a function of time. The decay to first order is a simple exponential decay (Figure 7).

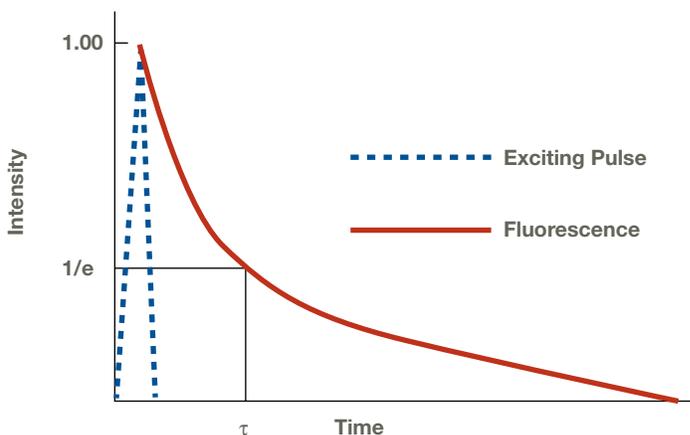


Figure 7

The exponential decay can be fitted simply as:

$$I(t) = I_0 e^{-t/\tau}$$

Equation 5

Where $I(t)$ is the time dependent intensity decay, I_0 is the initial intensity at the peak of the excitation pulse, and τ is the upper state lifetime. In order to take this measurement in the time domain (as shown in figure 7) an expensive, short-pulse laser system is required. Although this measurement is in principle a very simple measurement, it is very difficult to accurately retrieve the time constant, τ . There are many reasons for this. In general, the reasons can all be traced back to the fact that it is an amplitude based measurement. Given that the light source is repetitively pulsed, any change in the amplitude of the pulses will result in an error in calculation. Additionally, any change in the position, or size of the excitation pulse can lead to amplitude error. This is because a change in incident intensity can produce local changes in index of refraction, dye concentration, or homogeneity.

It was known that working in the frequency/phase domain would alleviate many of the aforementioned issues. Working in the frequency domain, a sinusoidally modulated continuous wave (CW) source is used and its amplitude becomes (to first order) irrelevant to the measurement. In the past, the requisite detection systems, and the electronics required to modulate the laser sources were at least as difficult to use and as expensive as the laser systems they needed to replace. In part, the telecommunications revolution that occurred in the 1990s spurred the development of inexpensive electronics, optical sources and detectors. Each of these components has enabled the development of practical phase fluorimetry systems.

6.0 Phase Fluorimetry

A phase fluorimetry system looks at the phase response of a molecular system/transition instead of the amplitude response. Specifically, instead of repeatedly using very short pulses to look at the amplitude decay of the equilibrium excited state in the time domain, the response of the system to a sinusoidally modulated excitation source is measured. This means that we take our excitation light, sinusoidally modulate it, and measure the relative phase of the fluorescently emitted light. The change in phase is meaningful because it also can be readily related to the upper state lifetime of the fluorescence system.

The system shown in Figure 6 can be viewed as a simple electrical system. Specifically, if the time response of the electron excitation and de-excitation in Figure 6 is mathematically modeled, the response looks identical to a simple, single pole, low pass filter (see figure 8, middle).

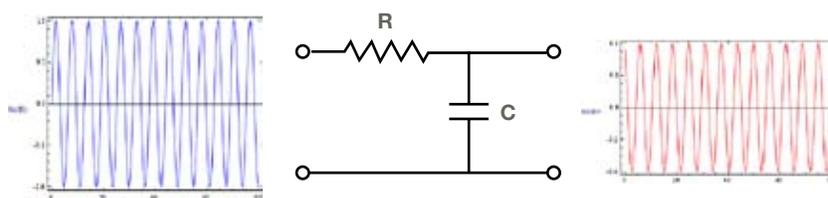


Figure 8

In Figure 8, we have conceptually represented the molecular four-level system as a simple low pass filter transfer function. A modulated blue source is incident upon the molecular system, and produces the red sinusoid output that has a lower amplitude and is phase shifted from the original signal. While this is a very simple conceptual representation of a molecular response function, it gives tremendous physical insight, and is in fact quite accurate for many calculations.

This low pass filter can be modeled using the following equations for the amplitude and phase response:

$$H(\omega) = \frac{1}{\sqrt{1+\omega^2\tau^2}}$$

Equation 7

$$\phi = \text{Tan}^{-1}(\omega\tau)$$

Equation 8

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Equation 8 tells us that if we know the modulation frequency, and we measure the phase shift between the excitation and fluorescent light, we can determine the effective upper state lifetime, τ . Experimentally this would typically be done with a phase sensitive detection system, but for large phase delays can easily be seen using an oscilloscope. A conceptual representation of what would be seen on the scope is shown below.

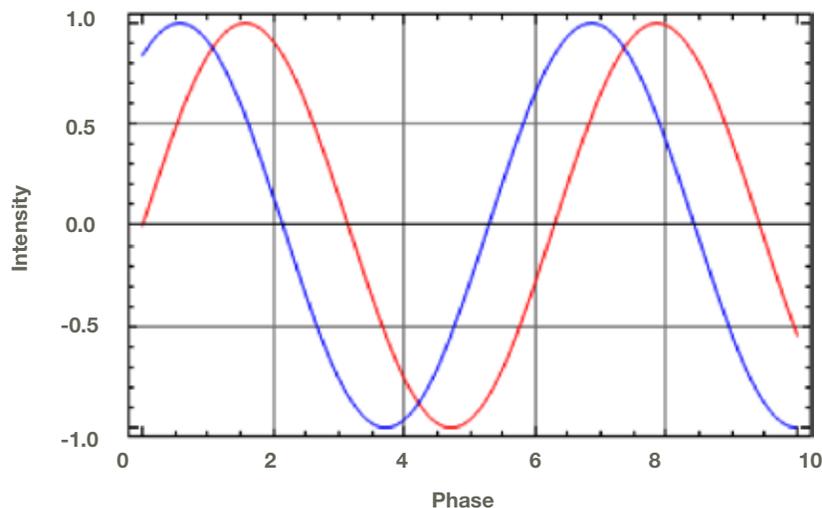


Figure 9

We can see in Figure 9 above that the red fluorescent light is phase delayed (or shifted) from the blue excitation light by $\Delta\phi$. Knowing the frequency of modulation and the phase delay the effective upper state lifetime can be calculated. This concept is the beginning of phase fluorimetry and instruments based on these concepts known as phase fluorimeters.

Please refer to the application notes entitled “Phase Fluorimetry for Dissolved Oxygen Detection” and “Phase Fluorimetry for pH Measurement” (H2 2008) for more details about using fluorescence sensors in bioprocess applications.