

Introduction to Fluorescence Spectroscopies I. Theory

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All life appears to be nurtured by the excitation of electrons by light in photosynthesis. The vision enjoyed by higher life forms begins with the electronic excitation of a conjugated polyene.

1. Introduction

Motivation

- **To understand our very existence we must understand the absorption and emission of light.**
- **From a practical standpoint, there is an explosion in the use of fluorescent dyes to visualize processes in living cells and other materials at unprecedented detail, and in breathtaking color.**
- **These images are a new art form. Fluorescence-based assays are also playing a rapidly increasing role in areas traditionally served by radioactive assays. Major reasons for this are the high sensitivity of fluorescence detection and its environmental friendliness.**
- **In addition, light emitted within 100 fs following pulses of light as short as 10 fs represent the current frontier for the observation of fast chemical events.**

During this experiment we will learn about a conventional (steady state) fluorometer and use it to observe behavior of a common fluorescent dye, *fluorescein* that illustrates general principles of fluorescence and fluorescence excitation spectra, including fluorescence quenching. We will also learn about and observe *light scattering*. Both fluorescence and light scattering involve the irradiation of a sample with light and the detection of the light emitted **spontaneously** by the sample (generally at right angles to the incident beam). Because the experimental set-up for the observation of both phenomena is the same, fluorescence and light scattering are often both observed in a single experiment.

In order to avoid misinterpretation of the data obtained, it is, therefore, important to recognize the origin of any particular signal. The aim of this experiment is to develop tactics for distinguishing between signals originating from fluorescence and those originating from *two*

types of light scattering. Before this can be done, however, we must first have an understanding of the physical basis of both phenomena.

2. PRINCIPLES AND THEORY

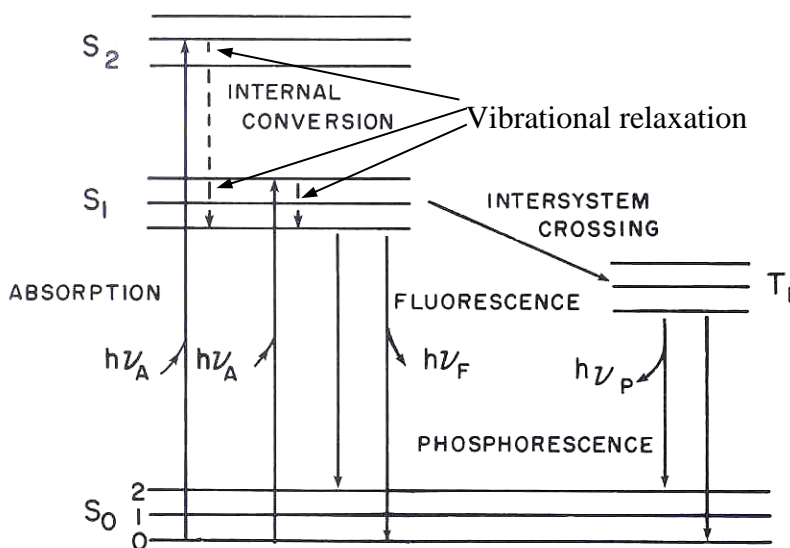
2.1 Fluorescence

If a molecule is irradiated with light whose energy corresponds to the energy difference between its ground electronic state and one of its excited electronic states, absorption of a photon can occur and a rearrangement of the molecule's electronic distribution occurs as it reaches the excited state. For example, an electron may be excited from a π molecular orbital to a π^* molecular orbital. Because the initial light absorption only involves the movement of electrons (no nuclear rearrangement) and electrons are very light (approx. $1/2000^{\text{th}}$ of the mass of a proton), light absorption is a very fast process. The “quantum event” is over a very short time interval, about 1 femto-second (10^{-15} s). Once excitation of the molecule has occurred by the absorption of the energy of a photon, the molecule can subsequently return to the ground state by losing its energy in two possible ways:

- 1) by transferring heat to the surroundings (*radiationless transition*),
- 2) by the emission of light (*fluorescence* or *phosphorescence*).

Either path may dominate, depending on the nature of the molecule. The fluorescence *quantum yield* (i.e., the ratio: photons emitted/photons absorbed) can range from $< 10^{-5}$ to near 1.

Figure 1: Jablonski diagram: photophysical processes (*Note: Internal conversion happens from S_1 also.*)



The absorption and emission of light is nicely illustrated by the energy level diagram suggested by A. Jablonski (1935) (see Fig. 1). The ground, first, and second electronic states are depicted by S_0 , S_1 , S_2 , ..., respectively. The letter S (in this context) indicates a *singlet* excited state, in which the electron spin *vectors* remain “paired” (spin *vectors* cancel, $S = 0$) during the excitation. In a *triplet* state (T), the electron spin *vectors* are locked in a more parallel direction, giving a total spin, $S = 1$). At each of these electronic energy levels the fluorophores can exist in a number of vibrational energy levels, depicted by 0, 1, 2, etc. (*That is a cartoon. They extend to S_1 and beyond, and the number of states get enormously crowded!*) It is important to note that the energy levels can be modified by interaction of the fluorophore with surrounding solvent molecules, but such solvent effects are not included in the diagram. Following light absorption, several processes usually occur.

A fluorophore is usually excited to some higher vibrational level of either S_1 , S_2 , or higher. (You are familiar with this from the UV absorption experiment earlier in the course.) The overlap of ground state vibrational wavefunction with higher vibrational states of the upper electronic state (which gives the Franck-Condon factors) allow vibrational energy of up to $\sim 4000 \text{ cm}^{-1}$ to be reached along with the electronic excitation. **This is very hot!** (Recall that $k_B T = 200 \text{ cm}^{-1}$ near room temperature.) So, immediately ($\sim 1 \text{ ps}$) the molecule cools off by spreading the energy to the cool solvent. That is called *vibrational relaxation* on the diagram. Molecules in condensed phases (liquid or solid) rapidly relax to the lowest vibrational energy level of S_1 by a combination of vibrational relaxation and changing electronic state if initially above S_1 . Changing electronic states without changing spin is called *internal conversion* and generally occurs on the time-scale of 1 ps (10^{-12} s) also. Again the excess energy is lost as heat to the surroundings.

This process *could* continue all the way to becoming a cold ground state, S_0 , but except in rare instances, the process of internal conversion from S_1 to S_0 is “slow”, because of the larger energy gap between S_1 and S_0 . “Slow” means about the same rate as that for emitting a photon of fluorescence, which is about 10^8 s^{-1} (but varies from 10^6 - 10^9 , depending on the transition dipole, which is the Hamiltonian matrix element connecting the excited and ground *electronic* states), and therefore when in S_1 , fluorescence usually competes well with internal conversion to S_0 .

Hence, **fluorescence emission generally results from the *thermally equilibrated S_1 excited state.***

As for absorption, electronic transitions down to a lower electronic level most often results in an excited vibrational state (see Fig. 1). This state will also “cool off” in about 10^{-12} s .

2.2 Phosphorescence

Molecules in the S_1 state can also “unpair their spins”. “Paired” means the LUMO electron and HOMO electron not only have different m_s values (one is $+1/2$ and the other is $-1/2$) but are correlated *in such a way* that the two spin *vectors* point in opposite directions and cancel, making the total spin $S = 0$, and the degeneracy, $2S+1 = 1$ (i.e., singlet). “Unpaired” means both m_s values may be $+1/2$ or both $-1/2$ or one is $+1/2$ and the other is $-1/2$, but *in such a way* that they

don't cancel. This is 3 states (triplet), corresponding to total spin angular momentum quantum number, S , of $S=1$. The degeneracy, $2S+1 = 3$. All of that was a long way to say the molecule may undergo **intersystem crossing** to a triplet state and cascade down in energy to the first excited triplet state, T_1 , just as it does in the singlet state. **T_1 is always lower than S_1** because the two electron spin *vectors* point roughly the same direction. If they get too close, there is a kind of *violation of the Pauli exclusion principle*, so they avoid each other more than they do in the singlet state. Less repulsion means lower energy. (This kind of avoidance is neither because of some repulsion of the magnetic dipoles nor the very strong Coulombic repulsion. It is because the triplet state Slater determinant wavefunction actually **vanishes** if any two electrons are at the same position if in the triplet state. That is not true for the singlet state) This is true for **all fermions** (particles with half-integer spin and obey the exclusion principle)

The Pauli exclusion effect is stronger than the Coulomb repulsion.

Emission from T_1 is termed phosphorescence, and for reasons just mentioned, is *always* at longer wavelengths (lower energy) relative to the fluorescence. Because spin unpairing is almost quantum mechanically "forbidden" for light atoms, it is slow compared to what internal conversion would be between states of the small energy difference between S_1 and T_1 . Therefore the S_1 lifetime turns out to be typically $\sim 10^{-8}$ s (10 ns). Getting from T_1 to S_0 , on the other hand, is really slow because it is *both* spin forbidden *and* the energy change is much greater than for the $S_1 \rightarrow T_1$ intersystem crossing. As a result, phosphorescence lifetimes are typically in the 10^{-3} s range or longer, i.e. five orders of magnitude longer than those of fluorescence. ***In solids devoid of oxygen, the phosphorescence can glow for many seconds after turning off the excitation light.***

2.3 A important note about 1st order rate constants and "lifetime".

Like radioactive decay, emission of light is a 1st order process. Recall that this means an *elementary* reaction like $A \rightarrow B$ so that the rate of decay depends only on the amount of A present at any time, i.e., $d[A]/dt = -k[A]$. Such processes in nature are ubiquitous.

The solution of this simplest of differential equations is the familiar **exponential** behavior: $[A]_t = [A]_0 \exp(-kt) = [A]_0 \exp(-t/\tau)$, **a result all chemists should memorize.**

τ (tau), is called the **"lifetime"** (because it is the average time that passes before A "instantly" (but randomly) becomes B . Obviously $\tau = 1/k$, where k is called the **rate constant**.

2.4 Summary

Now let us summarize the various photophysical processes, and their lifetimes.

(get in the habit of noting that the corresponding rate constants are just the reciprocal of the lifetimes)

1. **Absorption and emission** events: happen "instantly", $\sim 10^{-15}$ s (~ 1 femto second, 1 fs)

The waiting time for such an event, however, is typically thousands or millions of times longer.

2. **Internal conversion** from S_2 or higher: $\tau \sim 10^{-12}$ s (1 picosecond, 1 ps)
Internal conversion from S_1 : $\sim 10^{-8}$ s to 10^{-13} s. (This determines the quantum yield)

3. **Fluorescence** : lifetime: $\tau \sim 10^{-8} - 10^{-9}$ s (1-10 ns) or $k = 10^8 - 10^9$ s⁻¹ typically, but can be $\sim 10^{-12}$ s for “non-fluorescent” molecules such as DNA bases. (again, note that the time for the actual emission of a photon by a single molecule is $\sim 10^{-15}$ s, i.e., same as for absorption, but the probability to emit is typically about once every 10 ns.)

4. **Intersystem crossing**: $\sim 10^{-8}$ s from S_1 , but $\sim 10^{-6}$ s to ~ 60 s from T_1 , depending on temperature, viscosity, and impurities.

Phosphorescence: lifetime $\sim 10^{-6}$ s to ~ 60 s from T_1 , depending on temperature, viscosity, and impurities

Recall that internal conversion and intersystem crossing are known as *radiationless transitions*.

2.5 Kasha's Rule

By looking at the rates just summarized, one can note what Michael Kasha did in 1950. Because vibrational relaxation and internal conversion from higher states is over in a very short time (picoseconds), fluorescence will generally occur from the lowest vibrational level (within $k_B T$) of S_1 . The important consequence of this is: ***The wavelength (or energy) of fluorescence emission is generally independent of the wavelength (or frequency) of the exciting light. This is known as Kasha's Rule.***

In addition, there is a near symmetry of the Franck-Condon factors for absorption and emission. This means the ***fluorescence band spectral shape will appear nearly as the mirror image of the lowest energy absorption band—independent of the excitation light wavelength.***

2.6 Fluorescence Quenching

Quenching is a generic term usually referring to a process that leads to a decrease in fluorescence intensity emitted by the fluorophore (fluorescing molecule), either because of electron transfer to or from another molecule (called the quencher), or, transfer of the fluorophore's excitation energy to another molecule. (***quenching is therefore technically a form of internal conversion if one considers the fluorophore and quencher as a single system***)

One important class of quenching that decreases quantum yields is *collisional* quenching. This refers to quenching that requires close contact of the quencher and fluorophore during the excited-state lifetime of the fluorophore and leads to a loss in fluorescence. This effectively means such a process is ***diffusion controlled***. A common example of this is the interaction between a fluorophore and a heavy atom such as bromide or iodide. Collision of the fluorophore and heavy atom quencher brings the particles close enough that spin-orbit coupling or electron transfer occurs and the excited electron in the fluorophore cannot fluoresce well.

It is straightforward to calculate the change in fluorescence intensity as a function of quencher concentration:

Absorption:	$M + h\nu \text{ (photon)} \rightarrow M^*$	rate: $= \epsilon(\lambda) [M] \times \text{(Intensity of light)}$
Fluorescence:	$M^* \rightarrow M + h\nu'$	rate $= k_{rad} [M^*]$
Internal conversion:	$M^* \rightarrow M + \text{heat}$	rate $= k_{ic} [M^*]$
Intersystem crossing	$M^* \rightarrow M(\text{triplet}) + \text{heat}$	rate $= k_{isc} [M^*]$
Quenching:	$M^* + Q \rightarrow M + Q + \text{heat}$	rate $= k_q[Q][M^*]$

Fluorescence Quantum yield $= \Phi_f =$ the rate of fluorescing divided by total rate of leaving the excited state:

$$\Phi_f = \frac{k_{rad}[M^*]}{(k_{rad} + k_{ic} + k_{isc})[M^*] + k_q[Q][M^*]} = \frac{k_{rad}}{k_{rad} + k_{ic} + k_{isc} + k_q[Q]}$$

For the well-known dye fluorescein, $\Phi_f = 0.97$, $\tau_{rad} = 4.74 \text{ ns}$, $k_{ic} + k_{isc} = 7 \times 10^6 \text{ s}^{-1}$, and for quenching by iodide ion, $k_q = 2 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$ (a typical value for pairs of smallish molecules)

We will also make use of the maximum molar decadic extinction coefficient, $\epsilon_{max} = 92,300 \text{ M}^{-1}\text{cm}^{-1}$, a very high value typical of dye molecules.

Another type of quenching, often called “*static*” quenching, or *binding-related* quenching comes about if the quencher binds significantly to the fluorophore in the ground state such that there exists equilibrium between highly quenched fluorophore and unquenched fluorophore. Quenching in this case begins immediately upon excitation (*there is no waiting for the quencher to diffuse to the fluorophore*), and the fluorophore may be quenched so fast that one does not detect any fluoresce from the complexed form). Another form of quenching would be the binding of Ca^{2+} ions that lead to structural changes in the fluorophore which causes it to become non-fluorescent. (such a molecule is termed a *calcium sensor*)

3. Light Scattering

Light *scattering* is closely related to absorption and emission just described. The difference is that somewhere in the 1 fs of photon-molecule interaction, the photon “decides” not to become trapped in the molecule. Instead it continues on its way after about 1 fs, usually in a completely different direction, i.e., *the photon “bounces” off the molecule.*

This happens for *all wavelengths*, but it happens more strongly if in resonance or near resonance, i.e., for wavelengths near an absorption band.

When a beam of light shines on an assembly of molecules the photons of energy $h\nu$ collide with the molecules, and one of two things can happen. If the collision is *elastic* (i.e. no net change in

energy of the photons or of the molecules), the deflected photons, that is, the scattered radiation, will have the same energy (and therefore same wavelength) as the incident photons. On the other hand, if the collision is *inelastic*, the deflected photons will have either a higher or lower energy than the incident photons. In this case energy is either given up or taken away from the molecule as a result of the interaction.

The law of conservation of energy requires that: $h\nu + E = h\nu' + E'$ (1)

where E represents the rotational, vibrational and electronic energy of the molecule before collision and E' represents the same values after the collision. The scattered radiation is classified as

$E = E'$ ($\nu = \nu'$)	Rayleigh scattering
$E < E'$ ($\nu > \nu'$)	Raman scattering (Stokes lines)
$E > E'$ ($\nu < \nu'$)	" " (Anti-Stokes lines)

Thus, in the Raman scattering case, energy is either given to or taken away from the molecule as a result of the interaction, whereas in the case of Rayleigh scattering there is no net change in energy. Nevertheless, the vibrational energy exchange comes from the same mechanism from which Franck-Condon factors comes: excitation makes bond lengths change. The actual interaction between the incident light and the molecules occurs because the electric field of the electromagnetic radiation interacts with the electron cloud of the molecule causing a transient electrical polarization., which is like being excited, but only for ~1 fs in the case of scattering.

Figure 2 shows energy level diagrams for Rayleigh and Raman scattering as well as, for comparison, the process of infra-red absorption.

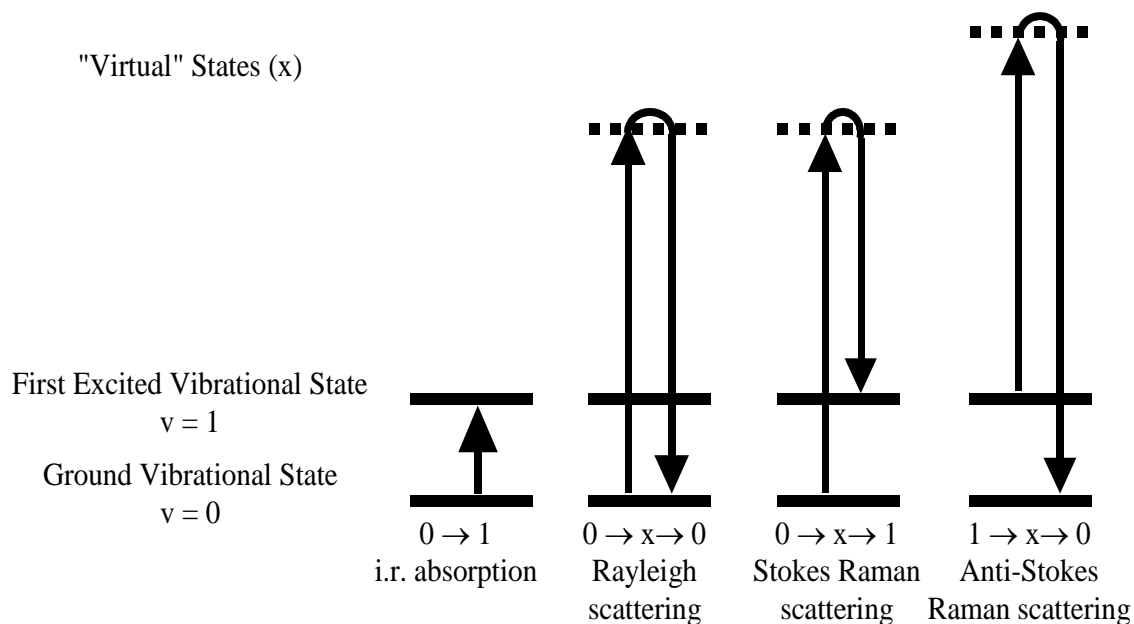


Figure 2: Illustration of Infrared absorption, Rayleigh and Raman scattering processes

In the case of *infrared absorption*, the low energy infrared radiation is absorbed by the molecule causing a transition normally from its ground vibrational state to a vibrationally-excited state. In the case of scattering measurements, much higher energy radiation is used, usually in the visible or ultraviolet region. (Light scattering intensity is proportional to the **frequency raised to the 4th power!** This is why the sky is blue.) If a molecule is initially in its ground vibrational state (the normal case), interaction with a photon of visible or ultraviolet radiation *that is not absorbed* can be thought of as inducing a transition of the molecule to a so-called "virtual" state (x), a higher state which may be considered to be a mixture (superposition) of all possible excited states of the molecule that absorb light, and therefore has an extremely short lifetime ($<10^{-15}$ s). Because the virtual state is so highly unstable, the excited molecule immediately emits (scatters) a photon before any excited state radiationless relaxation processes can occur, and the molecule drops back down to either its ground state (Rayleigh scattering) or to an excited vibrational state (*Stokes* Raman scattering). If a molecule is initially in the first excited vibrational state (note: this is unlikely at room temperature unless the vibrational energy is near kT), the molecule can be promoted to a virtual state and drop back down to the ground state. In this case the energy of the scattered light is greater than the incident light and this phenomenon gives rise to the so-called *anti-Stokes* lines of a Raman scattering spectrum.

It is important to note that the virtual states can be considered to be virtually energy-continuous, i.e. there is a negligible energy gap between adjacent virtual states. As a consequence of this light of **any** wavelength or frequency in the visible or ultraviolet range can cause a transition from the ground or first excited vibrational state to a virtual state. This is a major difference to fluorescence spectroscopy, where only light of **particular** wavelengths or frequencies can induce a transition from the ground electronic state to the discrete excited electronic state.

3.1 Raman Selection Rules

In order for Raman scattering to occur, light must exchange energy with the molecule via one of its stretching or bending vibrations. However, not all stretching and bending vibrations of any particular molecule are Raman active. Raman scattering can only occur when the vibration results in a *change in polarizability* of the molecule, since only then can there be an interchange of energy between the oscillating electric field of the incident light and the oscillating polarizability of the molecule. This is termed a *selection rule*. In both Rayleigh and Raman scattering the oscillating induced dipole moment of the molecule caused by its interaction with light allows it to act as a "transmitter" and re-emit or scatter a photon. Scattering is thus termed a *two-photon process*.

In the case of infrared absorption a selection rule also applies. IR absorption will only occur when the vibration motions results in an oscillating *change in dipole moment* of the molecule. The change in dipole moment due to the vibration of the molecule causes an oscillating electric field, which is able to interact with the oscillating electric field vector of the

incident radiation and cause the absorption of energy. Since only one photon is involved in the effect, IR absorption is termed a *one-photon process*.

For simple molecules, one can often deduce which vibrations are infrared active and which are Raman active simply by inspecting the change in symmetry of the molecule due to the vibrations. As stated above, any vibration leading to a change in dipole moment will be infrared active. This is relatively easy to predict. The assignment of Raman activity is somewhat more difficult. However, a change in polarizability (necessary for Raman activity) implies that the molecule's electrons are being held more or less strongly as a consequence of the vibration. Hence any vibration leading to a change in size of the molecule will be particularly Raman active.

4. Some simple Pre-Lab exercises

1. Using the data given for fluorescein in Sec. 2.6,

(a) Calculate the fluorescence quantum yield in the absence of quencher to see how close it comes to the stated value of 0.97.

(b) Calculate the lifetime of the excited state. Hint: this comes from the sum of the rates leaving the excited state.

(c) Now calculate the quantum yield and the lifetime of the excited state in the presence of 0.1 M iodide.

(d) What concentration of fluorescein is required to make the transmittance of the solution be 0.90. (90% is transmitted)? (We will need to have the concentration this low for the experiments we will do.)