Lifetime of fluorescence

Key Words

Laser, Fluorescent molecules, Lifetime measurement, Fluorescence lifetime, pH-dependent fluorescence, Fluorescence quantum yield, Fluorescence quenching, Isomerization, Photo-induced electron transfer

Object

It aims to learn basic principles of chemical luminescence by measuring the fluorescence lifetime of various fluorescent dye molecules at different pH values. After this experiment, students may be familiar with the various relaxation modes of electronically excited molecules as well as the concept of spectroscopy using laser.

Introduction

Luminescence is the emission of light from any substance, and occurs from electronically excited states. Luminescence is formally divided into two categories—fluorescence and phosphorescence—depending on the nature of the excited state. In excited singlet states, the electron in the excited orbital is paired (by opposite spin) to the second electron in the ground-state orbital. Consequently, return to the ground state is spin allowed and occurs rapidly by emission of a photon due to the spin selection rule ($\Delta S=0$). The emission rates of fluorescence are typically 10^8 s^{-1}, so that a typical fluorescence lifetime (that is the inverse of the emission rate) is near 10 ns (10×10^{-9} s).

Fluorescence spectroscopy can be applied to a wide range of problems in the chemical and biological sciences. The measurements can provide information on a wide range of molecular processes, including the interactions of solvent molecules with fluorophores, rotational diffusion of biomolecules, distances between sites on biomolecules, conformational changes, and binding interactions. The usefulness of fluorescence is being expanded by advances in technology for cellular imaging and single-molecule detection. These advances in fluorescence technology are decreasing the cost and complexity of previously complex instruments. Fluorescence spectroscopy will continue to contribute to rapid advances in biology, biotechnology and nanotechnology.

In particular, there has been a remarkable growth in the use of fluorescence in the biological sciences. Fluorescence spectroscopy and time-resolved fluorescence are considered to be primarily research tools in biochemistry and biophysics. This emphasis has changed, and the use of fluorescence has expanded. Fluorescence is now a dominant methodology used extensively in biotechnology, flow cytometry, medical diagnostics, DNA sequencing, forensics, and genetic analysis, to name a few. Fluorescence detection is highly sensitive, and

there is no longer the need for the expense and difficulties of handling radioactive tracers for most biochemical measurements. There has been dramatic growth in the use of fluorescence for cellular and molecular imaging. Fluorescence imaging can reveal the localization and measurements of intracellular molecules, sometimes at the level of single-molecule detection.

Background Information

Theory

Principles of fluorescence spectroscopy[1]

It is highly recommended to read chapters 1, 2, and 4 (8 and 9, if your time is available) of reference 1 (pdf file available from KAIST library website) if you want to fully learn about the fluorescence spectroscopy. What follows is a summary of a part of chapter 1.

Fluorescence typically occurs from aromatic molecules. Some typical fluorescent substances (fluorophores) are shown in Figure 1. One widely encountered fluorophore is quinine, which is present in tonic water. If one observes a glass of tonic water that is exposed to sunlight, a faint blue glow is frequently visible at the surface.

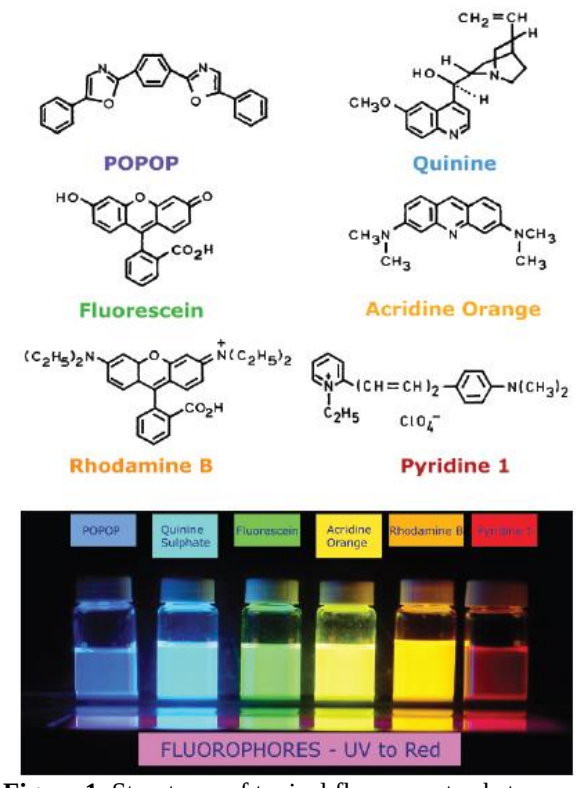


Figure 1. Structures of typical fluorescent substances.

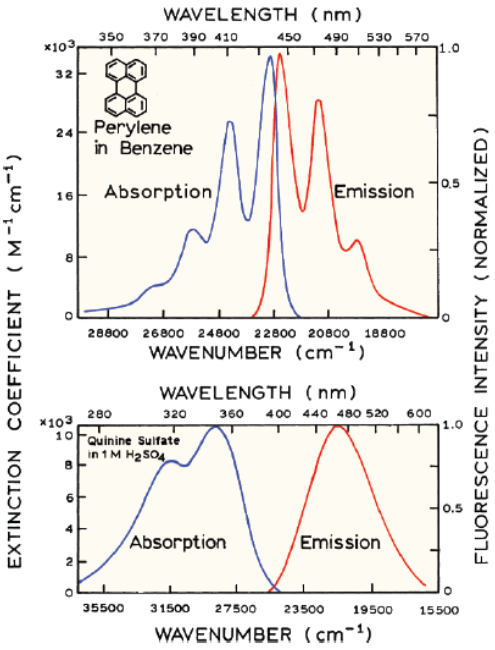


Figure 2. Absorption and fluorescence emission spectra of perylene and quinine. Emission spectra cannot be correctly presented on both the wavelength and wavenumber scales. The wavenumber presentation is correct in this instance. Wavelengths are shown for convenience.

Fluorescence spectral data are generally presented as emission spectra. A fluorescence emission spectrum is a plot of the fluorescence intensity versus wavelength (nanometers) or wavenumber (cm^{-1}). Two typical fluorescence emission spectra are shown in Figure 2. Emission spectra vary widely and are dependent upon the chemical structure of the fluorophore and the solvent in which it is dissolved. The spectra of some compounds, such as perylene, show significant structure due to the individual vibrational energy levels of the ground and excited states. Other compounds, such as quinine, show spectra devoid of vibrational structure.

- Jablonski diagram

The processes that occur between the absorption and emission of light are usually illustrated by the Jablonski diagram. Jablonski diagrams are often used as the starting point for discussing light absorption and emission. Jablonski diagrams are used in a variety of forms, to illustrate various molecular processes that can occur in excited states.

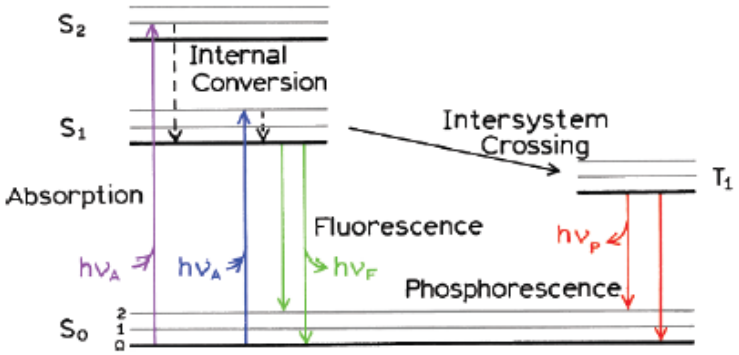


Figure 3. One form of a Jablonski diagram.

A typical Jablonski diagram is shown in Figure 3. The singlet ground, first, and second electronic states are depicted by S_0, S_1, and S_2, respectively. At each of these electronic energy levels the fluorophores can exist in a number of vibrational energy levels, depicted by 0, 1, 2, etc. In this Jablonski diagram we excluded a number of interactions, such as quenching, energy transfer, and solvent interactions. The transitions between states are depicted as vertical lines to illustrate the instantaneous nature of light absorption. Transitions occur in about 10^{-15} s, a time too short for significant displacement of nuclei. This is the Franck-Condon principle. (See Chapter 13 of Physical Chemistry Textbook, McQuarrie or the attached lecture note)

The energy spacing between the various vibrational energy levels is illustrated by the emission spectrum of perylene (Figure 2). The individual emission maxima (and hence vibrational energy levels) are about 1500 cm^{-1} apart. At room temperature thermal energy is not adequate to significantly populate the excited vibrational states. Absorption and emission

occur mostly from molecules with the lowest vibrational energy. The larger energy difference between the S_0 and S_1 excited states is too large for thermal population of S_1. For this reason we use light and not heat to induce fluorescence.

Following light absorption, several processes usually occur. A fluorophore is usually excited to some higher vibrational level of either S_1 or S_2. With a few rare exceptions, molecules surrounded by solvents and other molecules in condensed phases rapidly relax to the lowest vibrational level of S_1. This process is called the *internal conversion* and generally occurs within 10^{-12} s or less. Since fluorescence lifetimes are typically near 10^{-8} s, internal conversion is generally complete prior to emission. Hence, fluorescence emission generally results from a thermally equilibrated excited state, that is, the lowest energy vibrational state of S_1.

Return to the ground state typically occurs to a higher excited vibrational ground state level, which then quickly (10^{-12} s) reaches thermal equilibrium (Figure 4). Return to an excited vibrational state at the level of the S_0 state is the reason for the vibrational structure in the emission spectrum of perylene. An interesting consequence of emission to higher vibrational ground states is that the emission spectrum is typically a mirror image of the absorption spectrum of the $S_0 \rightarrow S_1$ transition. This similarity occurs because electronic excitation does not greatly alter the nuclear geometry. Hence the spacing of the vibrational energy levels of the excited states is similar to that of the ground state. As a result, the vibrational structures seen in the absorption and the emission spectra are similar.

Molecules in the S_1 state can also undergo a spin conversion to the first triplet state T_1. Emission from T_1 is termed *phosphorescence*, and is generally shifted to longer wavelengths (lower energy) relative to the fluorescence. Conversion of S_1 to T_1 is called the *intersystem crossing*. Transition from T_1 to the singlet ground state is forbidden because of spin selection rule ($\Delta S = 0$), and as a result the rate constants for triplet emission are several orders of magnitude smaller than those for fluorescence. Molecules containing heavy atoms such as bromine and iodine are frequently phosphorescent because of large spin-orbit coupling, which breaks the spin selection rule. The heavy atoms facilitate intersystem crossing and thus enhance phosphorescence quantum yields.

- Fluorescence lifetimes and quantum yields

The fluorescence lifetime and quantum yield are perhaps the most important characteristics of a fluorophore. Quantum yield is the number of emitted photons relative to the number of absorbed photons. Substances with the largest quantum yields, approaching unity, such as rhodamines, display the brightest emissions. The lifetime is also important, as it determines the time available for the fluorophore to interact with or diffuse in its environment, and hence the information available from its emission.

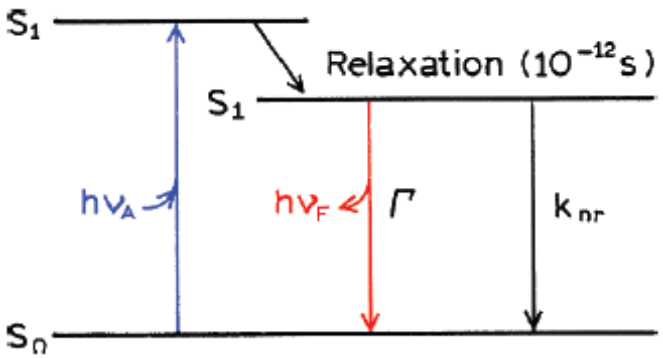


Figure 4. A simplified Jablonski diagram to illustrate the meaning of quantum yields and lifetimes.

The meanings of quantum yield and lifetime are best represented by a simplified Jablonski diagram (Figure 4). In this diagram we do not explicitly illustrate the individual relaxation processes leading to the relaxed S_1 state. Instead, we focus on those processes responsible for return to the ground state. In particular, we are interested in the emissive rate of the fluorophore (Γ) and its rate of nonradiative decay (typically through the interaction with its environment including other molecules or solvent) to S_0 (k_{nr}).

The fluorescence quantum yield is the ratio of the number of photons emitted to the number absorbed. The rate constants Γ and k_{nr} both depopulate the excited state. The fraction of fluorophores that decay through emission, and hence the quantum yield, is given by

$$Q = \frac{\Gamma}{\Gamma + k_{nr}}$$

The quantum yield can be close to unity if the radiationless decay rate is much smaller than the rate of radiative decay, that is $k_{nr} << \Gamma$. We note that the energy yield of fluorescence is always less than unity because of Stokes losses *e.g.* the internal conversion. For convenience we have grouped all possible non-radiative decay processes with the single rate constant k_{nr}.

The lifetime of the excited state is defined by the average time the molecule spends in the excited state prior to return to the ground state. Generally, fluorescence lifetimes are near 10 ns. For the fluorophore illustrated in Figure 4 the lifetime is

$$\tau = \frac{1}{\Gamma + k_{nr}}$$

Fluorescence emission is a random process, and few molecules emit their photons at precisely $t = \tau$. The lifetime is an average value of the time spent in the excited state. For a single exponential decay 63% of the molecules have decayed prior to $t = \tau$ and 37% decay at $t > \tau$.

- Fluorescence quenching

Fluorescence quenching refers to any process that decreases the fluorescence intensity of a sample. A variety of molecular interactions can result in quenching. These include,

i) Collisional quenching

Quenching by collisional encounters between the fluorophore and quencher is called collisional or dynamic quenching. Energy of an excited electron in a molecule can be converted into other forms or transferred to the other molecule, preventing from the recombination with fluorescence.

ii) Relaxation through nonradiative processes

Energy of an excited electron in a molecule can also be converted into heat of the molecule (vibration) rather than the energy of a photon (photoemission).

iii) Excited-state reactions such as bond dissociation

In some cases, such energy can be used to overcome a reaction barrier and thus triggers decomposition of the molecule or bond dissociation without photoemission.

iv) Electron transfer

The excited electron can also be transferred to another molecule (inter-molecular electron transfer) or to another part of the same molecule (intra-molecular electron transfer). The latter case is likely to be occurred if the molecule has both electron donor and acceptor moieties.

v) Energy transfer

The energy is itself able to be transferred to other molecules (inter-molecular energy transfer) or another moiety of the same molecule (intra-molecular transfer). The excited electron may emit a photon, but the released energy is used to excite another electron. This is a key process to understand the high efficiency of light-harvesting complexes in life.

Static quenching can be a valuable source of information about binding between the fluorescent sample and the quencher. Static quenching can also be a complicating factor in the data analysis. Fluorescence quenching has been widely studied both as a fundamental phenomenon, and as a source of information about biochemical systems. These biochemical applications of quenching are *due to the molecular interactions* that result in quenching.

Both static and dynamic quenching require molecular contact between the fluorophore and quencher. In the case of *collisional* quenching, the quencher must diffuse to the fluorophore during the lifetime of the excited state. Upon contact, the fluorophore returns to the ground state, without emission of a photon. In general, quenching occurs without any permanent change in the molecules, that is, without a photochemical reaction.

In *static* quenching a complex is formed between the fluorophore and the quencher, and this complex is nonfluorescent. For either static or dynamic quenching to occur the

fluorophore and quencher must be in contact. The requirement of molecular contact for quenching results in the numerous applications of quenching. For example, quenching measurements can reveal the accessibility of fluorophores to quenchers. Consider a fluorophore bound either to a protein or a membrane. If the protein or membrane is impermeable to the quencher, and the fluorophore is located in the interior of the macromolecule, then neither collisional nor static quenching can occur. For this reason quenching studies can be used to reveal the localization of fluorophores in proteins and membranes, and their permeabilities to quenchers.

Time-domain lifetime measurement

In time-domain or pulse fluorometry, the sample is excited with a pulse of light (Figure 5). The width of the pulse is made as short as possible, and is preferably much shorter than the decay time τ of the sample. The time-dependent intensity is measured following the excitation pulse, and the decay time τ is calculated from the slope of a plot of log I(t) versus t, or from the time at which the intensity decreases to 1/e of the intensity at t = 0.

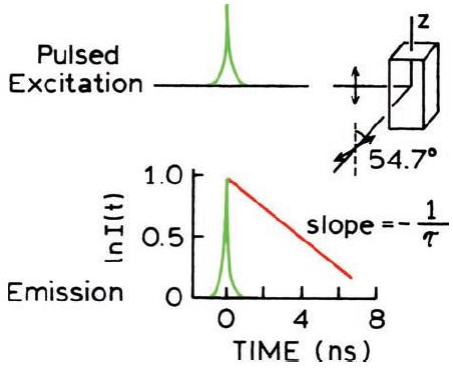


Figure 5. Pulse or time-domain lifetime measurements.

- Meaning of the lifetime or decay time

Prior to further discussion of lifetime measurements, it is important to have an understanding of the meaning of the lifetime τ. Suppose a sample containing the fluorophore is excited with an infinitely sharp (δ-function) pulse of light. This results in an initial population (n_0) of fluorophores in the excited state. The excited-state population decays with a rate $\Gamma + k_{nr}$ according to

$$\frac{dn(t)}{dt} = (\Gamma + k_{nr})\, n(t) \qquad (1)$$

where n(t) is the number of excited molecules at time t following excitation, Γ is the emissive rate, and k_{nr} is the nonradiative decay rate. Emission is a random event, and each excited fluorophore has the same probability of emitting in a given period of time. This results in an exponential decay of the excited state population, $n(t) = n_0 \exp(-t/\tau)$.

In a fluorescence experiment we do not observe the number of excited molecules, but rather fluorescence intensity, which is proportional to n(t). Hence, Eq. (1) can also be written in terms of the time-dependent intensity I(t). Integration of Eq. (1) with the intensity substituted for the number of molecules yields the usual expression for a single exponential decay:

$$\mathrm{I(t)} = I_0 \exp(-t/\tau) \qquad (2)$$

where I_0 is the intensity at time 0. The lifetime τ is the inverse of the total decay rate, $\tau = (\Gamma + k_{nr})^{-1}$. In general, the inverse of the lifetime is the sum of the rates which depopulate the excited state. The fluorescence lifetime can be determined from the slope of a plot of log I(t) versus t (Figure 5), but more commonly by fitting the data to assumed decay models.

The lifetime is the average amount of time a fluorophore remains in the excited state following excitation. This can be seen by calculating the average time in the excited state <t>. This value is obtained by averaging t over the intensity decay of the fluorophore:

$$< \mathrm{t} > = \frac{\int_0^\infty tI(t)dt}{\int_0^\infty I(t)dt} = \frac{\int_0^\infty texp\left(-\frac{t}{\tau}\right)dt}{\int_0^\infty exp\left(-\frac{t}{\tau}\right)dt} \qquad (3)$$

The denominator is equal to τ. Following integration by parts, one finds the numerator is equal to τ^2. Hence for a single exponential decay the average time a fluorophore remains in the excited state is equal to the lifetime:

$$< \mathrm{t} > = \tau \qquad (4)$$

It is important to note that Eq. (4) is not true for more complex decay laws, such as multi- or non-exponential decays. Using an assumed decay law, an average lifetime can always be calculated using Eq. (3). However, this average lifetime can be a complex function of the parameters describing the actual intensity decay. For this reason, caution is necessary in interpreting the average lifetime.

Another important concept is that the lifetime is a statistical average, and fluorophores emit randomly throughout the decay. The fluorophores do not all emit at a time delay equal to the lifetime. For a large number of fluorophores some will emit quickly following the excitation, and some will emit at times longer than the lifetime. This time distribution of emitted photons is the intensity decay.

Equipment

<u>- Diode laser</u>

* Principles of laser (Chapter 15 of ref. 3, Physical Chemistry Textbook by McQuarrie)

It is recommended to read chapter 15 of reference 3 to fully learn about the lasers, laser spectroscopy, and photochemistry. Below following is a part of chapter 15, explaining the real basics about principles of

laser.

The word *laser* is an acronym for light amplification by stimulated emission of radiation. Lasers are used in a variety of devices and applications such as supermarket scanners, optical disk storage drives, compact disc players, ophthalmic and angioplastic surgery, and military targeting. Lasers have also revolutionized research in physical chemistry. Their impact on the field of spectroscopy and light-initiated reactions, or *photochemistry*, has been tremendous. Using lasers, chemists can measure the spectra and photochemical dynamics of molecules with high spectral or time resolution. Furthermore, the techniques are so sensitive that a single molecule can be studied. Every chemist today should know how lasers work and understand the unique properties of the light they generate.

Lasers are composed of three essential elements (Figure 6): (1) a gain medium that amplifies light at the desired wavelength, (2) a pumping source that excites the gain medium, and (3) mirrors that direct the light beam back and forth through the gain medium. Each of these components will be discussed in turn.

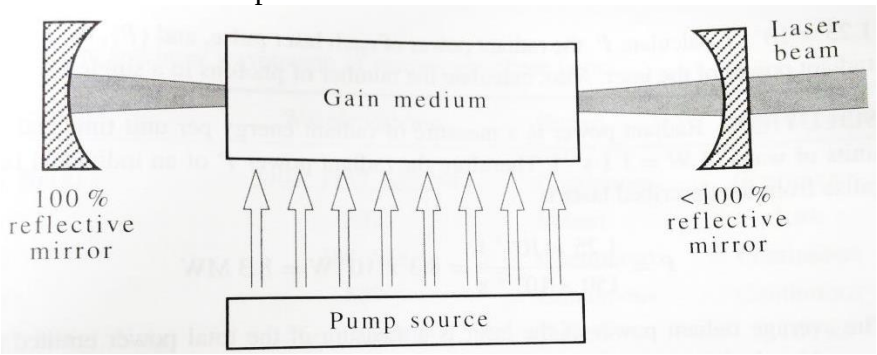


Figure 6. A diagram of the insides of a laser. The gain medium is placed between two mirrors; the arrangement of these components is called the laser cavity. A pump source excites the atoms, molecules, or ions that constitute the gain medium. The radiation that is emitted by the excited-state atoms is directed back and forth through the gain medium using the mirrors. One of the mirrors has a reflectivity that is less than 100%, which allows light to escape the cavity. This output light is the laser beam.

Gain medium:

The gain medium of a laser can be a solid-state material, a liquid solution, or a gas mixture. Since the report of the first laser in 1960, many different media have been used. The first laser used a solid ruby rod as a lasing medium. Ruby is a crystal of corundum, Al_2O_3, in which some of the Al^{3+} ions are replaced by impurity Cr^{3+} ions. There are many solid-state gain media, like ruby, in which the active ion (Cr^{3+} for ruby) is embedded in a host material (Al_2O_3 for ruby). Many commercially available lasers use Nd^{3+} as the gain medium. Laser output can be a continuous light beam or a short burst of light.

The active element in a gas-phase laser can be a noble-gas atom (e.g., the He-Ne laser), a positive ion(e.g., Ar^+ laser, K^+ laser), a metal atom (e.g., He-Cd laser, Cu vapor laser), a

neutral molecule (e.g., N_2 laser, CO_2 laser), or an unstable complex created by the pumping process (e.g., $XeCl^*$). Gas-phase lasers produce light in the ultraviolet, visible, and infrared regions of the spectrum. Some of these lasers are capable of generating light at several different frequencies. For example, the CO_2 laser involves population inversion (and therefore lasing) between different rotational-vibrational levels of the electronic ground state. Laser light can be generated in small, discrete frequency steps dictated by the energy separation of the rotational levels of CO_2.

Because the energy of laser light must correspond to an energy difference between two quantized stationary states of the gain medium, the laser light must be monochromatic (single color). The electric field of a monochromatic light source can be expressed as $E = A\cos(\omega t + \varphi)$, where A is the amplitude, ω is the angular frequency of the light ($\omega = 2\pi\nu$), and φ is the phase angle, which serves to reference the field to some fixed point in time. The phases of light waves from a lamp vary randomly ($0 \leq \varphi \leq 2\pi$). In contrast, the stimulated-emission process requires that the phases of the incident light wave and stimulated light wave have the same phase. Thus, the light waves emitted from a laser are all in phase. This property of laser light is called *coherence*. Many modern spectroscopic techniques take advantage of the coherence of laser light.

Pumping sources: There are two common approaches for pumping the gain medium: optical excitation and electrical excitation. In optical excitation, a high-intensity light source is used to excite the gain medium. Devices that use continuous lamps, flashlamps, and lasers as pumping sources are commercially available. Electrical excitation involves using intense electrical discharges to excite the gain material. This approach is commonly used in gas lasers. The discharge of a large current through the gain medium can be done continuously or in short pulses. Collisions between the high-energy electrons created in the discharge and the atoms or molecules in the gas container produce atoms, molecules, or ions in excited states.

Laser cavity design: Combining a gain medium with a pumping source does not make a laser. Once a population inversion is achieved, light of a specific frequency can be amplified. Unfortunately, a single pass of light through the gain medium generally does not produce much amplification in the light intensity. To generate high-intensity outputs, the light must be directed back and forth through the gain medium. Lasers accomplish this feat by having the gain medium placed inside an optical cavity called a *resonator*, which usually includes a pair of mirrors that direct the light back and forth through the gain medium. Only the light that travels back and forth along the path defined by the gain medium and the cavity mirrors can be amplified. If both mirrors were 100% reflective, the device would not create any output. In a laser, one of the mirrors is 100% reflective, and the other is less than 100% reflective, thereby allowing some of the light

to escape from the resonator.

* Light sources for TCSPC - Laser Diodes and Light-Emitting Diodes

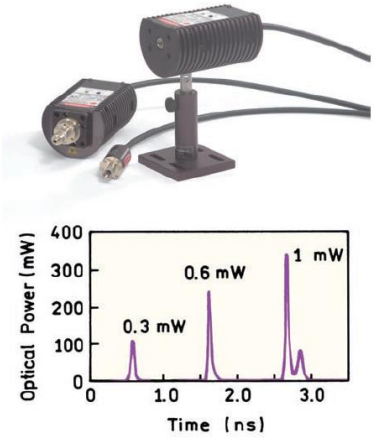


Figure 7. Pulsed picosecond laser diode emitting at 370 nm. The FWHM is less than 70 ps with a maximum repetition rate of 40 MHz.

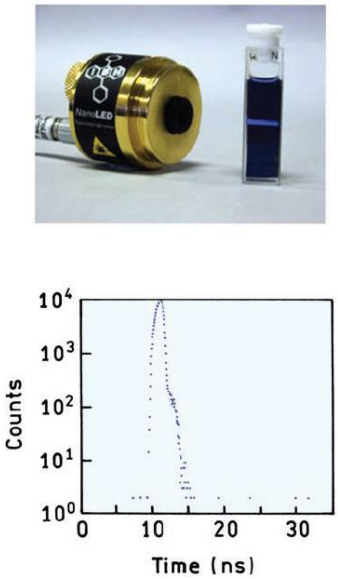


Figure 8. Pulsed light-emitting diode at 405 nm. FWHM = 1.4 ns.

- Time-Correlated Single-Photon Counting (TCSPC)

* Principles of TCSPC

At present most of the time-domain measurements are performed using time-correlated single-photon counting, but other methods can be used when rapid measurements are needed. Rather than present a history of the method, we will start by describing current state-of-the-art instrumentation. These instruments use high repetition rate mode-locked picosecond (ps) or femtosecond (fs) laser light sources, and high-speed microchannel plate (MCP) photomultiplier tubes (PMTs). For many applications, these expensive systems are being rapidly replaced by systems using pulsed-laser diodes (LDs), light-emitting diodes (LEDs), and small, fast PMTs.

The principle of TCSPC is somewhat unique (Figure 9). The sample is excited with a pulse of light, resulting in the waveform shown at the top of the figure. This is the waveform that would be observed when many fluorophores are excited and numerous photons are observed. However, for TCSPC the conditions are adjusted so that less than one photon is detected per laser pulse. In fact, the detection rate is typically 1 photon per 100 excitation pulses. The time is measured between the excitation pulse and the observed photon and stored in a histogram. The x-axis is the time difference and the y-axis the number of photons detected for this time difference. When much less than 1 photon is detected per excitation pulse, the histogram represents the waveform of the decay. If the count rate is higher the histogram is biased to shorter times. This is because with TCSPC only the first photon can be observed. At present the electronics are not fast

enough to measure multiple photons per pulse when the lifetimes are in the nanosecond range. Multiple photons per pulse can be measured for decay times near a microsecond or longer.

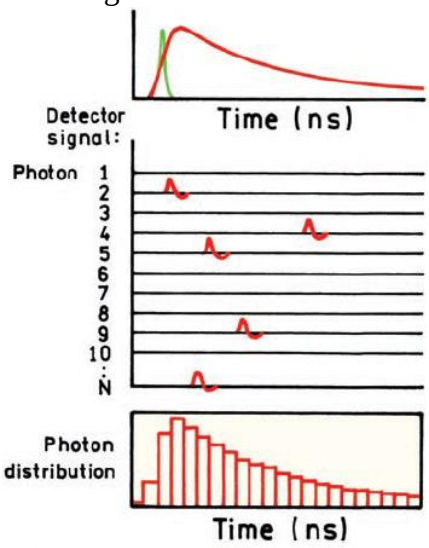


Figure 9. Principle of TCSPC. The pulses in the middle panel represent the output from a constant fraction discriminator.

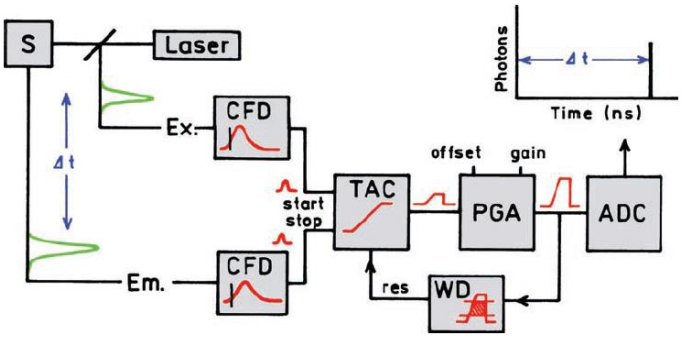


Figure 10. Electronic schematic for TCSPC.

Specialized electronics are used for measuring the time delay between the excitation and emission (Figure 10). The experiment starts with the excitation pulse that excites the samples and sends a signal to the electronics. This signal is passed through a constant function discriminator (CFD), which accurately measures the arrival time of the pulse. This signal is passed to a time-to-amplitude converter (TAC), which generates a voltage ramp that is a voltage that increases linearly with time on the nanosecond timescale. A second channel detects the pulse from the single detected photon. The arrival time of the signal is accurately determined using a CFD, which sends a signal to stop the voltage ramp. The TAC now contains a voltage proportional to the time delay (Δt) between the excitation and emission signals. As needed the voltage is amplified by a programmable gain amplifier (PGA) and converted to a numerical value by the analog-to-digital converter (ADC). To minimize false readings the signal is restricted to given range of voltages. If the signal is not within this range the event is suppressed by a window discriminator (WD). The voltage is converted to a digital value that is stored as a single event with the measured time delay. A histogram of the decay is measured by repeating this process numerous times with a pulsed-light source.

Pre-Laboratory Questions

1. What are the principles of fluorescence and the meaning of fluorescence lifetime? How are they determined?

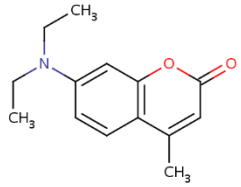
2. What are the principles of laser?

3. What are the roles of each apparatus system and the mechanisms of fluorescence measuring set-up?

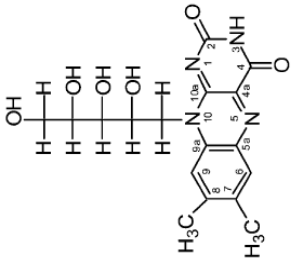
Materials

Reagents

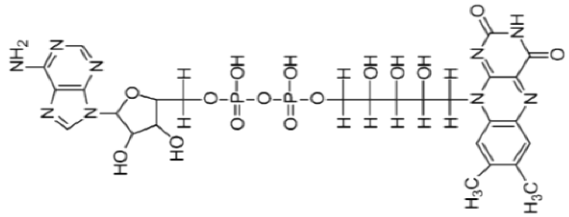
- Coumarin 460 (Coumarin 1) (7-Diethylamino-4-methylcoumarin), $C_{14}H_{17}NO_2$ [91-44-1]



- Riboflavin (Vitamin B_2), $C_{17}H_{20}N_4O_6$ [83-88-5]



- Flavin adenine dinucleotide (FAD) (Riboflavin 5′-adenosine diphosphate disodium salt), $C_{27}H_{31}N_9Na_2O_{15}P_2 \cdot xH_2O$ [84366-81-4], be careful for hydrolysis



- Ethanol
- Distilled water
- pH buffer solutions
- HCl
- KOH

Apparatus

- Diode laser (375 nm)
- Monochromator
- Photomultiplier tube (PMT) detector

- Time-correlated single-photon counting (TCSPC)
- Laptop PC
- pH meter
- Cuvette

Safety and Hazards
- Laser irradiation
- Careful handling acids and bases

Experimental Procedure

Preparation of sample
- Coumarin 460, EtOH solution

1. Dissolve proper amount of coumarin 460 sample in ethanol so that the concentration of the solution to be about $(1\sim5)\times10^{-5}$ M. Use minimum amount of sample. (e.g. 0.23 ~ 1.1 mg sample in 100 mL solution)
2. Record the weight of the used sample, the volume of the solution, and the concentration of the solution.

- Riboflavin, aqueous solutions

1. Dissolve proper amount of riboflavin sample in pH buffer solutions (pH = 4, 7, and 10) so that the concentration of the solution to be $(5\sim10)\times10^{-5}$ M. For exact concentration with respect to the pH of solution, refer to Table 3 below or reference 6. Use minimum amount of sample. (e.g. 0.95 ~ 1.9 mg sample in 50 mL solution)
2. In a similar way, prepare solution with different pHs, using pH buffer solutions and proper amount of HCl and KOH. Total 5 solutions should be made, with the values of pH to be about 1, 4, 7, 10, and 13.

* How to use pH meter
- Prepare pH electrode storage solution. pH electrode should be stored in that particular solution in between the measurements.
- Press ‘Measure’ to start your measurement. Measured pH value will be displayed, and it will stop when stabilized. Shake solution to dissolve the solute faster and to know the exact pH value.
- For solutions which are highly acidic or highly basic (pH ~ 1 or 13), use pH ~ 4 or 10 solutions you made, and put little amount of HCl or KOH while you monitor at the varying

pH value at the same time.

3. Record the pH of the solution, the weight of the used sample, the volume of the solution, and the concentration of the solution.

- FAD, aqueous solution

1. Dissolve proper amount of FAD sample in pH 7 buffer solution so that the concentration of the solution to be about 10^{-4} M. Use minimum amount of sample. (e.g. about 1.7 mg sample in 20 mL solution)
2. Record the pH of the solution, the weight of the used sample, the volume of the solution, and the concentration of the solution.
3. Due to the hydrolysis phenomenon of FAD, perform the fluorescence lifetime measurement as soon as the solution has made. FAD sample is stored in a freezer, and it should be re-stored as soon as you finish sample preparation process.

Measurements

1. Arrange the equipment. Please carefully handle the equipment only if you perfectly understood consequences for following procedures. If not, discussion with your TA about what you have not understood would be highly recommended.
2. Turn on TCSPC module and then turn on laptop PC.
3. Load a sample in cuvette.
4. Place the cuvette in front of the entrance slit of the monochromator.
5. Turn on the diode laser with key switch. For repetition rate control, 20 MHz is recommended for current experiment. Leave power/bias control as it is.

<u>Hazards: laser irradiation</u>

6. Arrange the position of the diode laser so that the cuvette loaded with sample solution is irradiated and the direction is perpendicular with monochromator entrance slit. Fluorescence of the sample should be observed.
7. Before starting the fluorescence measurement, block all the external lights as much as possible including light bulbs, so that you can reduce background signals in dark environment.
8. Turn on the monochromator. Execute the program “monoscan.exe”; Referring to the corresponding reference below (Table 1, Figure 11, and Figure 12), figure out at what

wavelength the sample solution has the maximum emission. Tune the monochromator wavelength using in program "monoscan.exe". Enter the desired wavelength and press 'Goto'.

Table 1 Fluorescence emission maxima for coumarin dyes[4]

Solvent	1	
	λ_f	ν_f
cyclohexane	395	25 3
ethyl acetate	416	24 0
acetonitrile	434	23 0
ethanol	451	22 2
ethanol–water	458	21 8
glycerol	463	21 6

a) λ_f in nm, ν_f in 1000 cm^{-1}, ethanol–water = 50 : 50 (V/V).

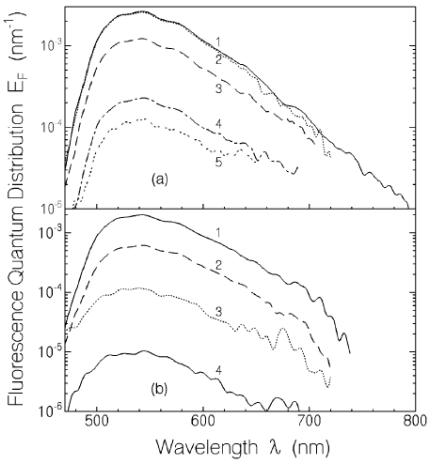


Figure 11. Fluorescence quantum distributions of riboflavin in aqueous solution.[6] The riboflavin concentration used in the measurements were in the region between 5×10^{-5} mol dm^{-3} and 1×10^{-4} mol dm^{-3} (see Table 3). (a) Neutral and acidic pH. Curve 1, pH=7; curve 2, pH=4.35; curve 3, pH=2.4; curve 4, pH=1.2; curve 5, pH=0.85. (b) Basic pH. Curve 1, pH=9.3; curve 2, pH=10.25; curve 3, pH=11.35; curve 4, pH=13.35.

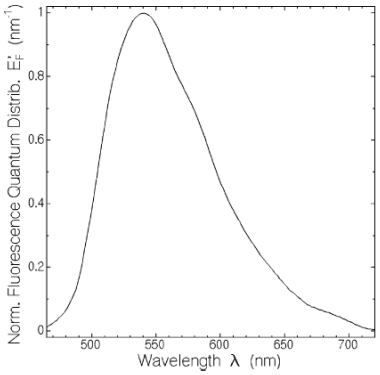


Figure 12. Normalized fluorescence quantum distribution, $E'_F(\lambda) = E_F(\lambda)/E_{F,max}$, of FAD in aqueous solution at pH=2.5.[7] No spectral difference was resolved over the pH range from pH=1 to pH=11.3.

9. Execute the program "spcm.exe". Press 'OK'. Please do not adjust other control parameters and just press 'Start!'. Slowly raise detector gain by clicking the arrow button in the 'DCC-100' control panel until the fluorescence decay signal appears. Caution: detector overload should not appear as a red light in the rear side of the PMT detector. This procedure should be checked by TA because any inappropriate handling may cause permanent damage to the detector.
10. Decay of fluorescence will be plotted in real time. Notice that the maximum value no longer changes if it already reached the maximum (saturated). Data of the plotted graph can be stored. For example, see Figure 13 and 14 below.

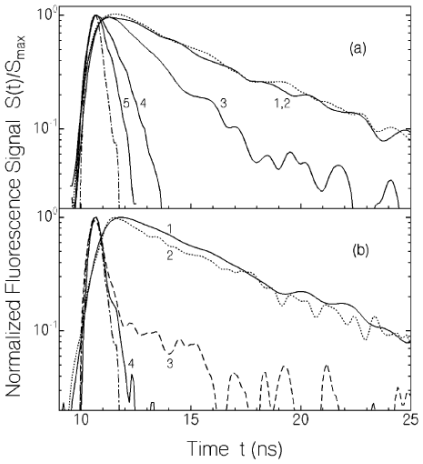


Figure 13. Experimental temporal fluorescence signal traces of riboflavin in aqueous solutions at various pH values. Dash-dotted curves show system response function. (a) Neutral and acidic solutions. Curve 1 (solid line), pH=7; curve 2 (dotted line), pH=4.35; curve 3, pH=2.4; curve 4, pH=1.2; and curve 5, pH=0.85. (b) Basic solutions. Curve 1, pH=9.3; curve 2, pH=11.35; curve 3, pH=11.7; curve 4: pH=13.35.[6]

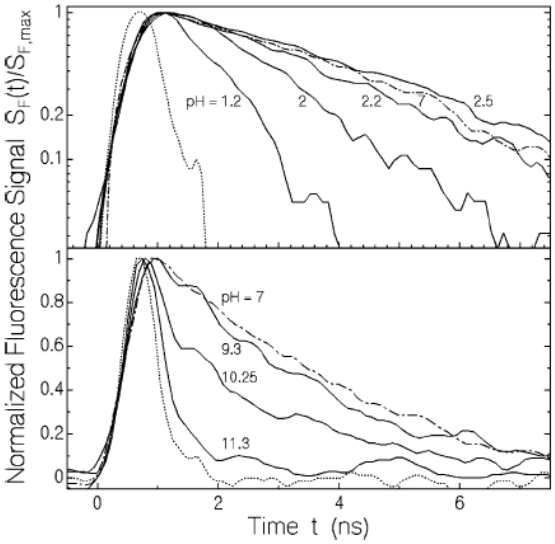


Figure 14. Fluorescence signal traces of FAD in aqueous solution at various pH values. Dotted curves show the system response function.[7]

11. You can tune the monochromator wavelength until finding the emission maximum. Record the value for each sample.
12. At fluorescence maximum wavelength, press 'Stop' and store the result. You can reprocess data (e.g. smoothing) if needed, providing that does not alter the original tendency.
13. Press 'Start' and slowly drop the detector gain down to 0 by clicking arrow button in the 'DCC-100' control panel. Then you may change sample solution and measure the fluorescence decay for it in the same way.
14. After finishing all measurements, turn off equipment and clear up the optical table. Be careful that you should turn off laptop PC first, and then TCSPC module.
15. Fitting process: use an appropriate software. (MS Excel does not provide data analysis for nonlinear curve fitting.) "Origin" provides various data analysis and graphing workspace for scientists and engineers. Its evaluation demo version is available on 'http://www.originlab.com'.

- Fit the measured data with an exponential decay function. The formula may be like this.

$$y = y_0 + A_1 e^{-(x-x_0)/t_1}$$

- Select data range. It should start from the maximum point of the curve.
- You may have to fix a parameter, such as x_0 or A_1, if the fitting process does not go well.
- If the above function does not fit well, try it with another exponential decay function with different time constant.
- Then, obtain the fitting parameters. The parameter of t_1 indicates the lifetime value. Figure 15 is an example of the fitting of a measured fluorescence decay curve of coumarin 460

solution.

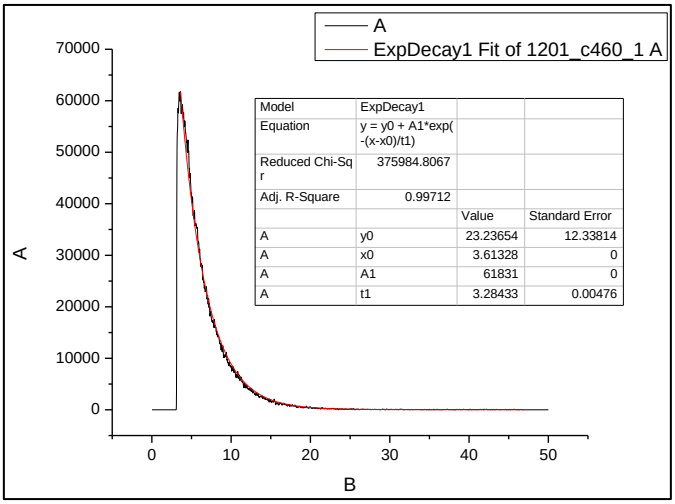


Figure 15. Measured fluorescence data of coumarin 460 solution with its exponentially fitted parameters

16. Data sheet that you have to fill is given below.

Sample solution			Concentration (10^{-5} M)	Max. emission wavelength (nm)	Lifetime (ns)	Literature value (ns)
Coumarin 460						
Riboflavin	pH					
					/	/
FAD	pH					

Post-Laboratory Data Evaluation

1. Calculate the fluorescence lifetime of each sample by fitting the data with exponential decay equations. Most of data will be fitted mono-exponentially, but there is an exception.

Table 2 Coumarin fluorescence quantum yields and lifetimes[4]

Solvent	1	
	Φ_f	τ_f
cyclohexane	0 32	2 8
ethyl acetate	0 93	3 1
acetonitrile	0 73	3 4
ethanol	0 59	3.1
ethanol–water b)	0 22	1.4
glycerol	0 53	3 8

a) τ_f in ns, dye concentration = $(1-5) \times 10^{-5}$ M.

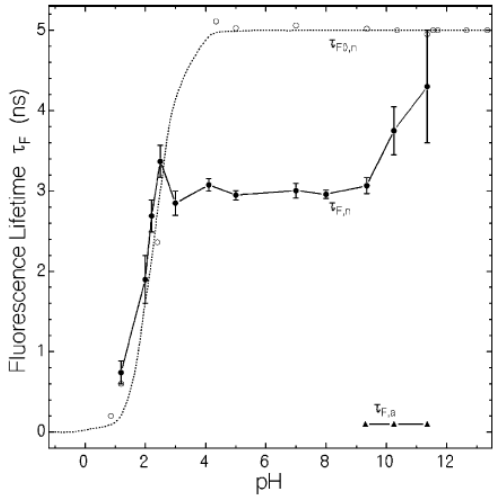


Figure 16. Fluorescence time constants, $\tau_{F,n}$ of fluorescent neutral form (filled circles), and $\tau_{F,a}$ of anionic form (filled triangles) of FAD in aqueous solution versus pH. For comparison the fluorescence time constants, $\tau_{F0,n}$ of riboflavin are included by open circles and dotted line.[6,7]

2. Observe pH dependence of fluorescence decay of riboflavin. You should notice that riboflavin has double exponential form at high pH. See Table 3.

Table 3 Experimental pH dependence of fluorescence quantum yield, Φ_F, and fluorescence lifetimes, τ_F, of riboflavin in aqueous solution[6]

pH	C (10^{-5} mol dm^{-3})	ϕ_F	τ_F (ns)		
			Single exponential	Double exponential	
−1.09	10.9	$<4 \times 10^{-5}$			
0.85	6.3	0.0132	0.20 ± 0.05		
1.20	9.8	0.0289	0.6 ± 0.05		
2.40	6.2	0.124	2.36 ± 0.05		
4.10	5.7	0.226			
4.35	5.9	0.255	5.11 ± 0.05		
5.00	8.5		5.03 ± 0.05		
7.00	5.9	0.267	5.06 ± 0.08		
9.35	7.8	0.201	5.02 ± 0.08		
10.25	10.1	0.0618	5.00 ± 0.05		
11.35	7.9	0.013	4.95 ± 0.15		
11.55	5.3			<0.35	≈5.0
11.70	6.6	0.00725		<0.35	≈5.0
12.65	7.0	0.0028		<0.35	≈5.0
13.35	9.2	0.00178		<0.35	

C is concentration of the riboflavin solutions.

3. What are the mechanisms of pH dependence of riboflavin? Hint: see Figure 17.

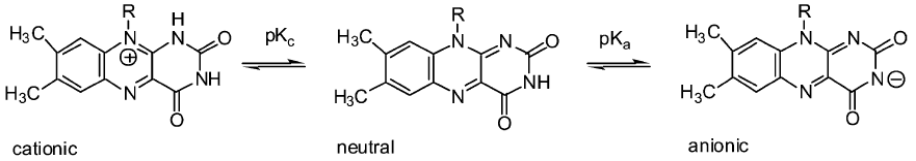


Figure 17. Molecular structure of riboflavin under different acidic conditions.

4. Explain the mechanisms of observed fluorescence quenching of FAD. Hint: Light absorption of a FAD molecule causes its isomerization and change in its electronic structure as shown in Figure 19.

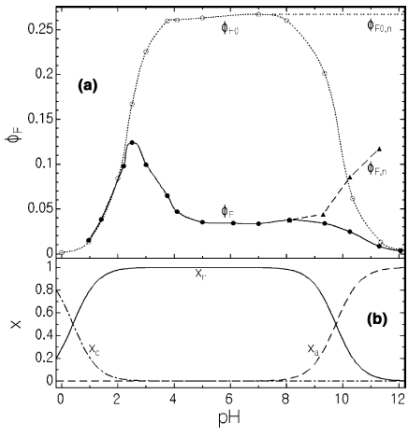


Figure 18. (a) Fluorescence quantum yield, Φ_F, of FAD in aqueous solution versus pH.[7] Experimental points are shown by line-connected filled circles. For comparison the dot-connected open circles show the fluorescence quantum yield, Φ_{F0}, of riboflavin in aqueous solution versus pH. Above pH=8 the expected fluorescence quantum yields, $\Phi_{F,n}$ and $\Phi_{F0,n}$ of the neutral and anionic forms of FAD and riboflavin are included. (b) Mole-fractions of cationic, x_c, neutral, x_n, and anionic, x_a, form of riboflavin. Curves are thought to be equal for FAD.

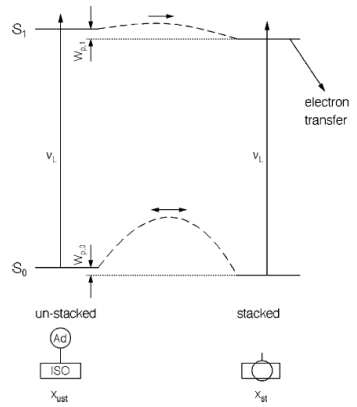


Figure 19. Illustration of isomerisation of FAD.[7] Potential energy diagram of un-stacked FAD (ISO-Ad) and stacked FAD in S_0 ground state and first excited S_1 state is shown. Vertical arrows indicate light absorption of frequency ν_L. There occurs bi-directional transfer on the ground-state potential surface, and there occurs unidirectional transfer on the excited-state potential surface from un-stacked to stacked conformation with immediate electron transfer.

5. Understand why these 3 samples are selected and name other possible sample materials for this experiment.

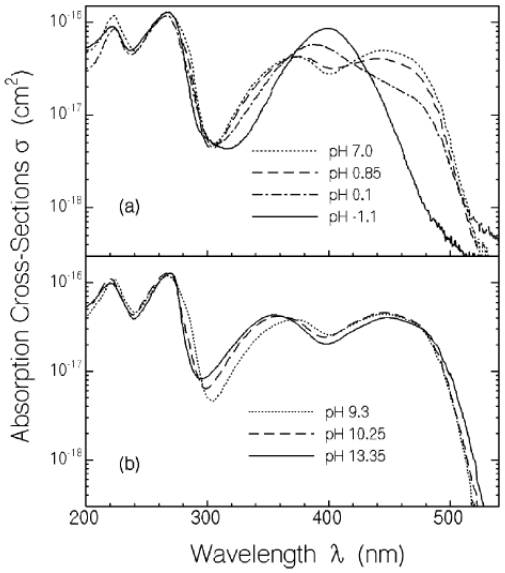


Figure 20. (a) and (b): Absorption cross-section spectra of riboflavin in aqueous solutions at various values of pH.[6] The riboflavin concentration used in the measurements was around 1×10^{-4} mol dm^{-3}.

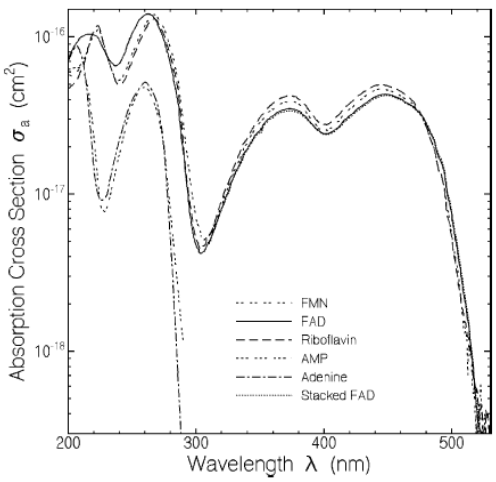


Figure 21. Absorption cross-section spectra in neutral aqueous solutions of FAD, FMN, riboflavin, AMP, and adenine.[7] The spectrum of FMN (double-dashed curve) is thought to be equal to the spectrum of un-stacked FAD. The dotted curve is thought to represent the spectrum of stacked FAD. It is nearly undistinguishable from the solid FAD curve.

6. Compare obtained results with the literature and explain the difference.

References

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