Analytical chemistry

Lecture №28

Emission spectroscopy
Part I

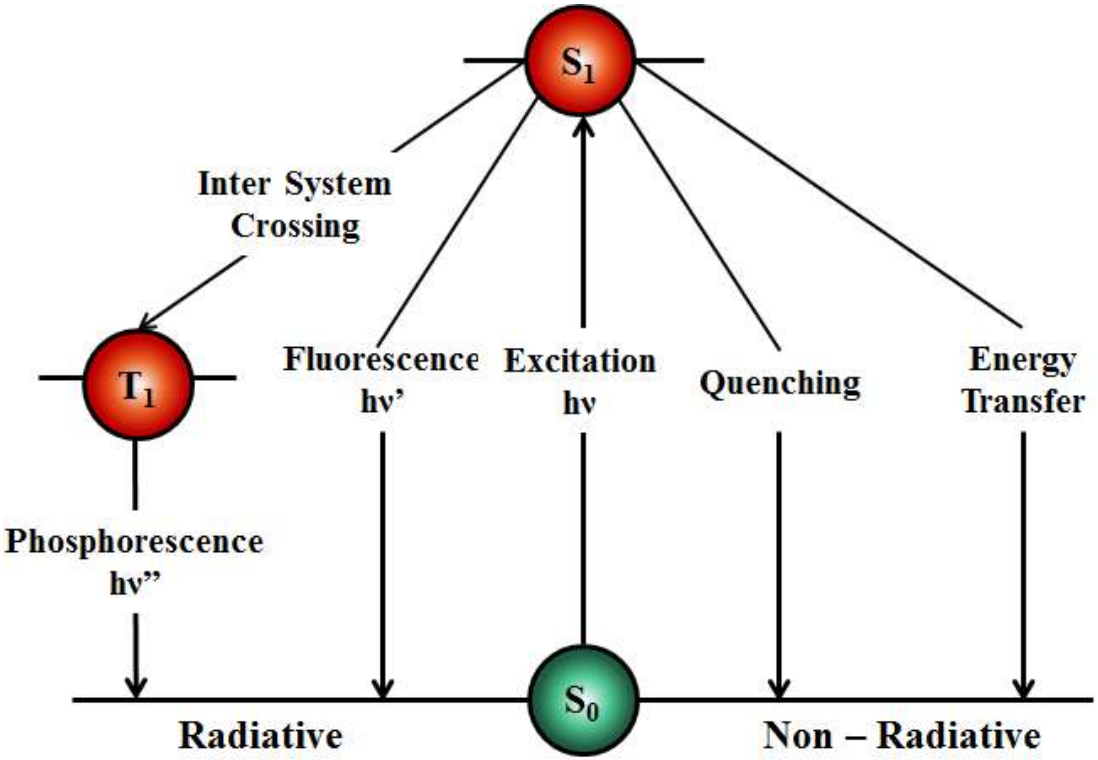
Introduction

When a molecule absorbs light, an electron is promoted to a higher excited state (generally a singlet state, but may also be a triplet state). The excited state can get depopulated in several ways.

• The molecule can lose its energy non–radioactively by giving its energy to another absorbing species in its immediate vicinity (**energy transfer)** or by collisions with other species in the medium.

• If an excited state triplet overlaps with the exited state singlet, the molecule can cross over into this triplet state. This is known as **inter system crossing.** If the molecule then returns to the ground state singlet ($T_1 \rightarrow S_0$) by emitting light, the process is known as **phosphorescence.**

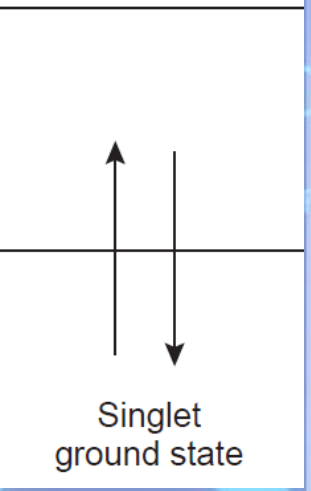
• The molecule can partially dissipate its energy by undergoing conformational changes and relaxed to the lowest vibrational level of the excited state in a process called **vibrational relaxation.** If the molecule is rigid and cannot vibrationally relax to the ground state, it then returns to the ground state ($S_1 \rightarrow S_0$) by emitting light, the process is known as **fluorescence.**



Fluorescence and phosphorescence are both subclasses of the general phenomena of ***luminescence***; specifically they are classified as *photoluminescence, emission of light* following excitation by photons. **Radioluminescence** involves excitation by energetic radiation (*γ -rays, etc.);* ***electroluminescence*** involves direct electrical excitation***;*** **piezoluminescence** involves luminescence created by pressure**.** **Thermoluminescence** is somewhat of a misnomer; it does not really involve excitation by heat. **Triboluminescence** is generated when material is pulled apart, ripped, scratched, crushed, or rubbed from energy released due to the breaking of chemical bonds. **Chemiluminescence** provides electronic excitation by energy derived from a chemical reaction; such reactions may be brought about in natural biological systems (bioluminescence, as in fireflies). **Electrogenerated chemiluminescence** (ECL) is typically also considered a special subclass.

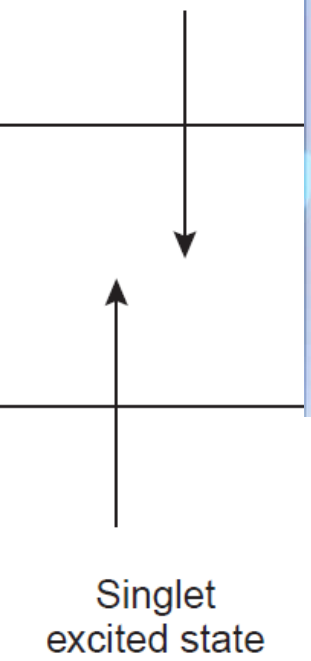
Luminescence - Radiation resulting from photoexcitation (photoluminescence), chemical excitation (chemiluminescence), or thermal excitation (thermoluminescence).

Molecular Photoluminescence Spectroscopy



Photoluminescence is divided into two categories: ***fluorescence and phosphorescence.***

Absorption of an ultraviolet or visible photon promotes a valence electron from its *ground state* to an *excited state* with conservation of the electron's spin. For example, a pair of electrons occupying the same electronic ground state have opposite spins and are said to be in a singlet spin state. Absorbing a photon promotes one of the electrons to a ***singlet excited state*. Emission** of a photon from a singlet excited state to a singlet ground state, or between any two energy levels with the same spin, is called ***fluorescence*.** The probability of a fluorescent transition is very high, and the average lifetime of the electron in the excited state is only 10^{-5}–10^{-8} s. Fluorescence, therefore, decays rapidly after the excitation source is removed.



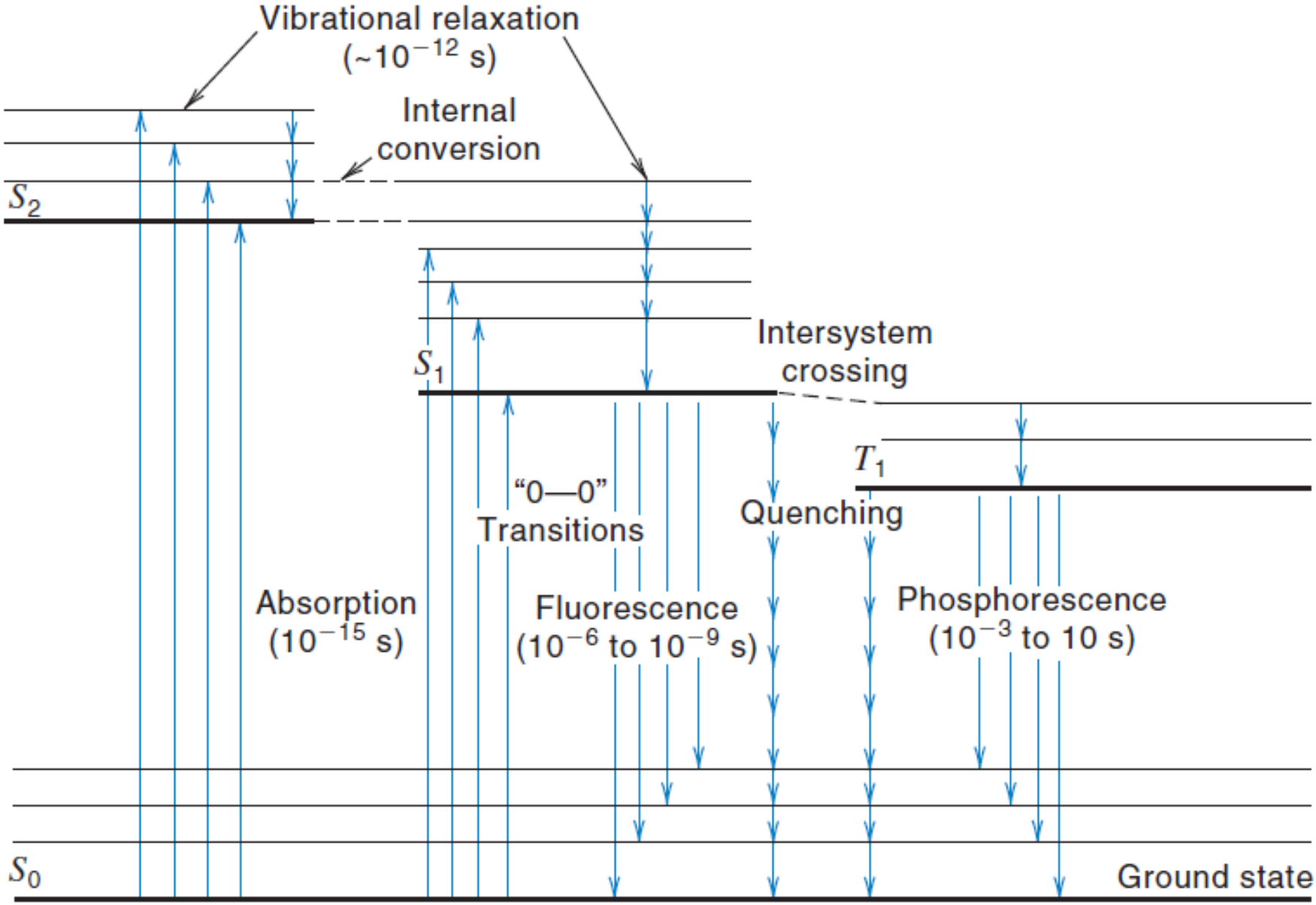
In some cases an electron in a singlet excited state is transformed to a **triplet excited state** in which its spin is no longer paired with that of the ground state. Emission between a triplet excited state and a singlet ground state, or between any two energy levels that differ in their respective spin states, is called ***phosphorescence*.** Because the average lifetime for phosphorescence ranges from 10^{-4} to 10^{4} s, phosphorescence may continue for some time after removing the excitation source.

Relaxation Processes

- **internal conversion -** A form of radiationless relaxation in which the analyte moves from a higher electronic energy level to a lower electronic energy level.
- **external conversion -** A form of radiationless relaxation in which energy is transferred to the solvent or sample matrix.
- **intersystem crossing -** A form of radiationless relaxation in which the analyte moves from a higher electronic energy level to a lower electronic energy level with a different spin state.
- **Vibrational relaxation** involves transfer of the excess energy of a vibrationally excited species to molecules of the solvent. This process takes place in less than 10^{-15} s and leaves the molecules in the lowest vibrational state of an electronic excited state.

Fluorescence bands consist of a large number of closely spaced lines.

Relaxation Processes



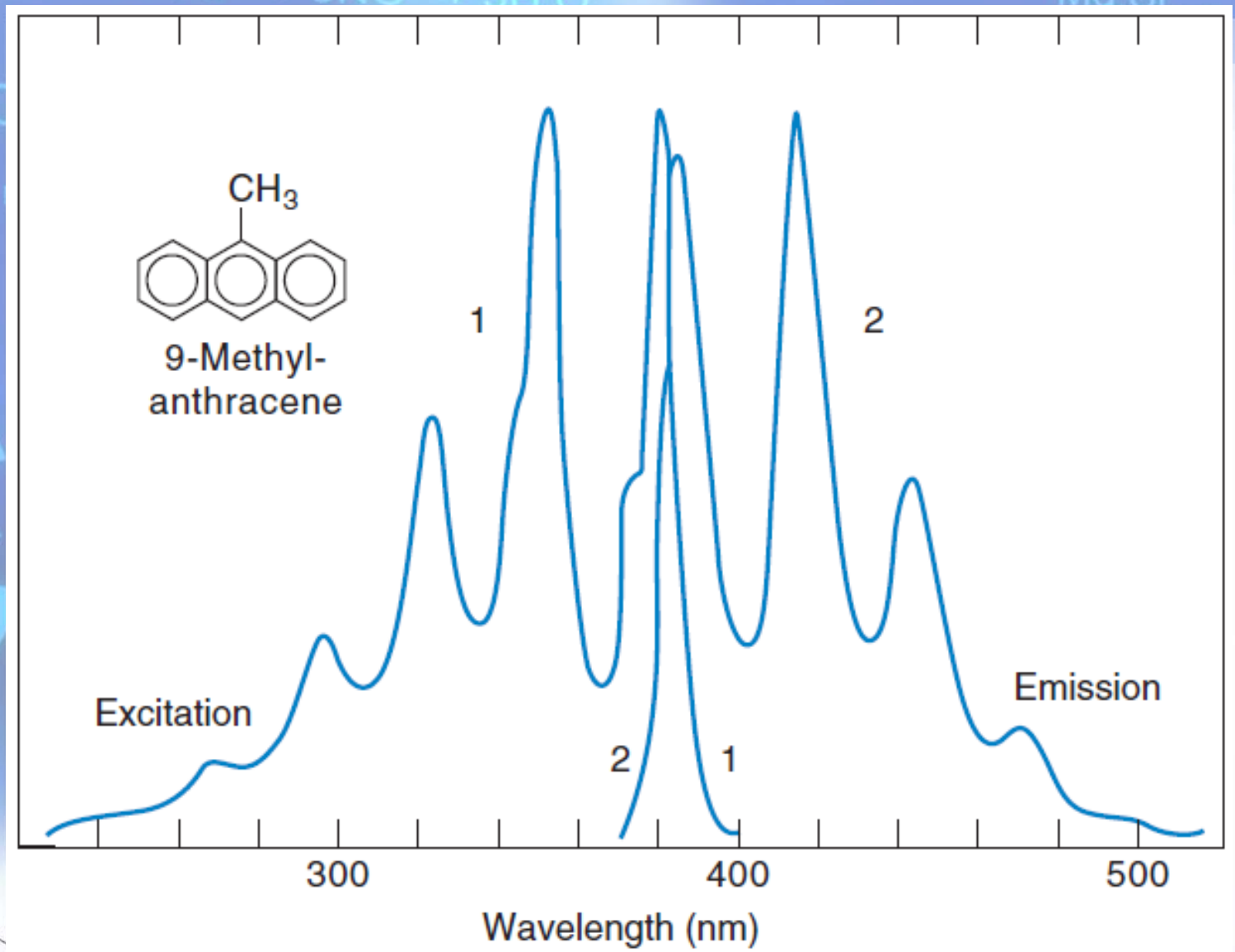
Energy level diagram (sometimes referred to as a Jablonski diagram) showing absorption processes, relaxation processes, and their rates.

Excitation Versus Emission Spectra

• The excitation spectrum usually corresponds closely in shape to the absorption spectrum of the molecule. There is frequently (but not necessarily) a close relationship between the structure of the excitation spectrum and the structure of the emission spectrum. In many relatively large molecules, the vibrational spacings of the excited states, especially S_1, *are very similar to those in* S_0. *Thus, the form of the* emission spectrum resulting from decay to the various S_0 *vibrational levels tends to* be a "***mirror image***" of the excitation spectrum arising from excitation to the various vibrational levels in the excited state, such as S_1. Substructure, of course, results also from different rotational levels at each vibrational level. An **excitation spectrum** is obtained by monitoring emission at a fixed wavelength while varying the excitation wavelength.

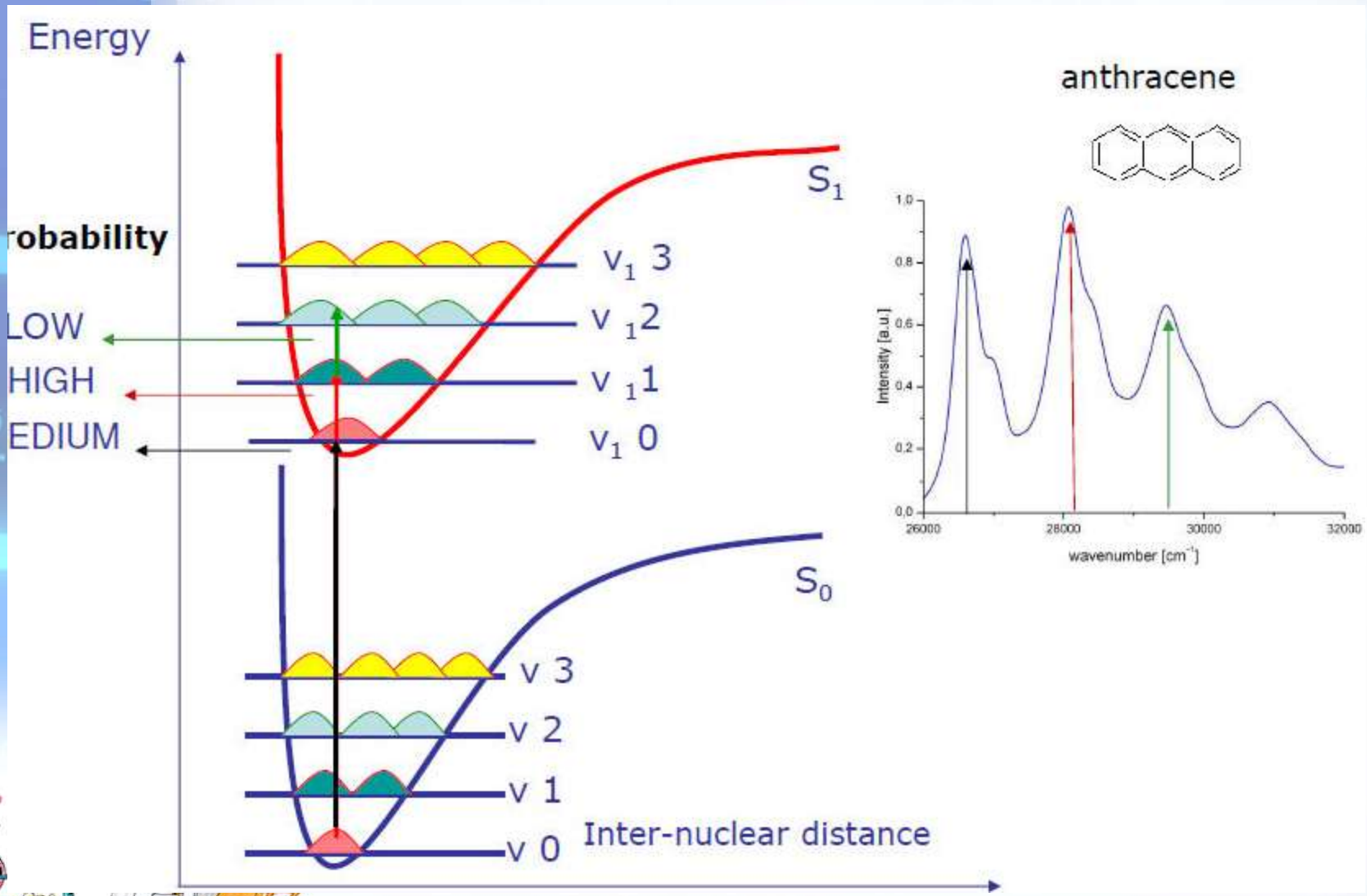
• Molecular fluorescence bands are mostly made up of lines that are longer in wavelength, higher in frequency, and thus lower in energy than the band of absorbed radiation responsible for their excitation. This shift to longer wavelength is called the **Stokes shift.**

• **Kasha's Rule**: The same fluorescence emission spectrum is generally observed irrespective of excitation wavelength. This happens since the internal conversion is rapid.

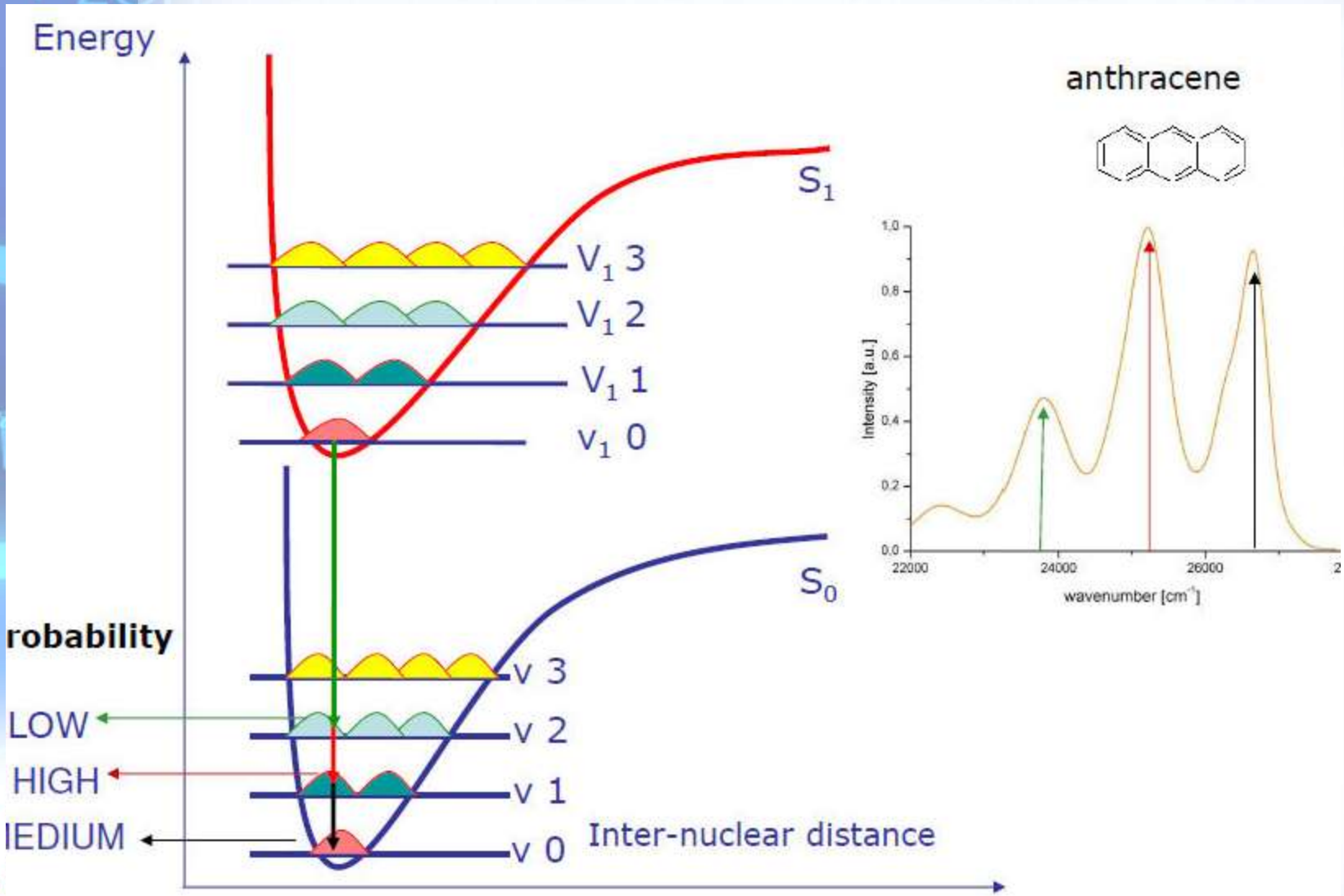


Excitation and emission spectra of fluorescing molecule

Fluorescence excitation spectrum



Fluorescence emission spectrum

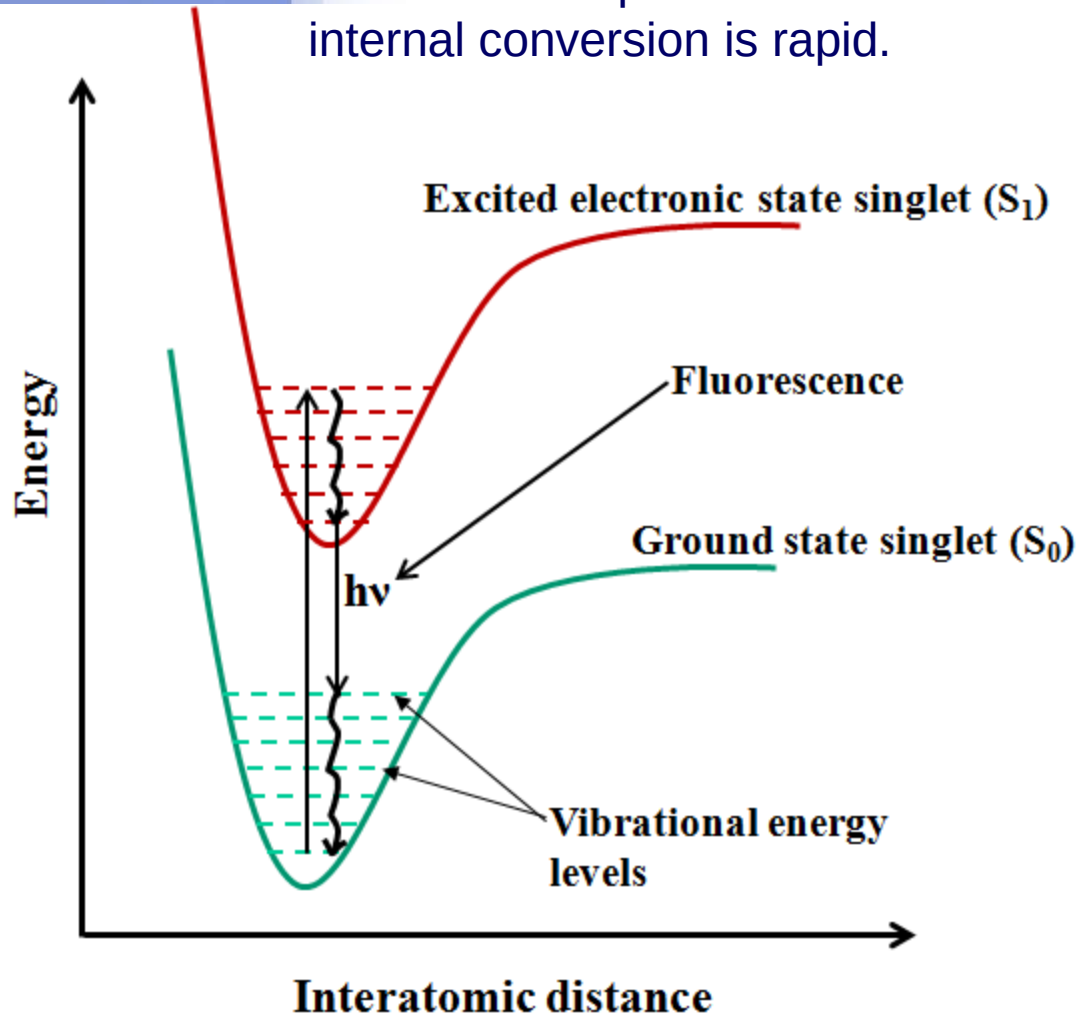


Characteristic of a fluorescence spectra

- ***The Stokes Shift***

The energy of emission is typically less than that of absorption. Fluorescence typically occurs at lower energies or longer wavelength.

- ***Kasha's Rule:*** The same fluorescence emission spectrum is generally observed irrespective of excitation wavelength. This happens since the internal conversion is rapid.



- Upon return to the ground state the fluorophore can return to any of the ground state vibrational level.
- The spacing of vibrational energy levels of the excited states is similar to that of the ground state.
- The consequence of above two is that the emission spectrum is typically a mirror image of the absorption spectrum of the S_0 to S_1 transition.

Fluorescence life times and quantum yield

• Quantum yield is the ratio of the number of photons emitted to the number absorbed.

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

Γ= the emissive rate of fluorophore.
k_{nr}= rate of non-radiative decay

• The lifetime of the excited state is defined by the average time the molecule spends in the excited state prior to the return to the ground state.

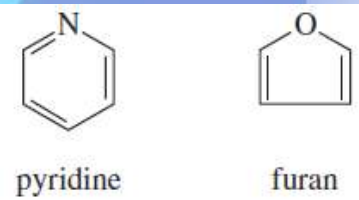
$$\tau = \frac{1}{\Gamma + k_{nr}}$$

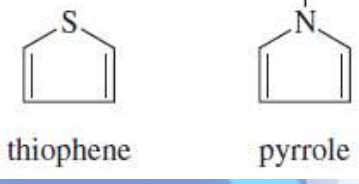
• The lifetime of the fluorophore in the absence of radiative process is called the intrinsic or natural life time.

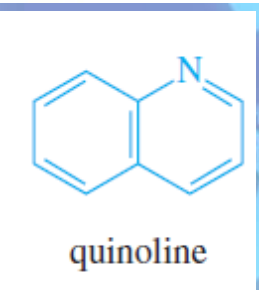
$$\tau_n = \frac{1}{\Gamma}$$

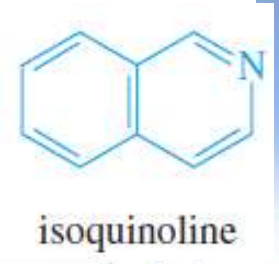
• Fluorescence lifetimes are near 10 ns.
• Scintillators have large Γ value. Hence they have large Q and lifetime.
• The fluorescence emission of aromatic substances containing nitro group are generally weak due to large k_{nr} value.

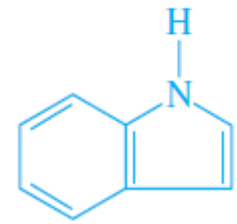
CHEMICAL STRUCTURE AND FLUORESCENCE











First of all, the greater the absorption by a molecule, the greater its fluorescence intensity. Many aromatic and heterocyclic compounds fluoresce, particularly if they contain certain substituted groups.

Compounds containing aromatic rings give the most intense and most useful molecular fluorescence emission. While certain aliphatic and alicyclic carbonyl compounds as well as highly conjugate double-bonded structures also fluoresce, there are very few of these compared to the number of fluorescent compounds containing aromatic systems.

Most unsubstituted aromatic hydrocarbons fluoresce in solution, with the quantum efficiency increasing with the number of rings and their degree of condensation. The simplest heterocyclics, such as pyridine, furan, thiophene, and pyrrole, do not exhibit molecular fluorescence, but fused-ring structures containing these rings often do. **Substitution on an aromatic ring** causes shifts in the wavelength of absorption maxima and corresponding changes in the fluorescence bands. In addition, substitution frequently affects the fluorescence efficiency.

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• Multiple conjugated double bonds favor fluorescence. One or more electron-donating groups such as —OH, —NH_2, and —OCH_3 enhances the fluorescence. Polycyclic compounds such as vitamin K, purines, and nucleosides and conjugated polyenes such as vitamin A are fluorescent. Groups such as —NO_2, —COOH, —CH_2COOH, —Br, —I, and azo groups tend to ***inhibit*** *fluorescence.*

• The nature of other substituents may alter the degree of fluorescence. The fluorescence of many molecules is greatly **pH** dependent because only the ionized or un-ionized form may be fluorescent. For example, phenol, C_6H_5OH, is fluorescent but its anion, $C_6H_5O^-$, is not.

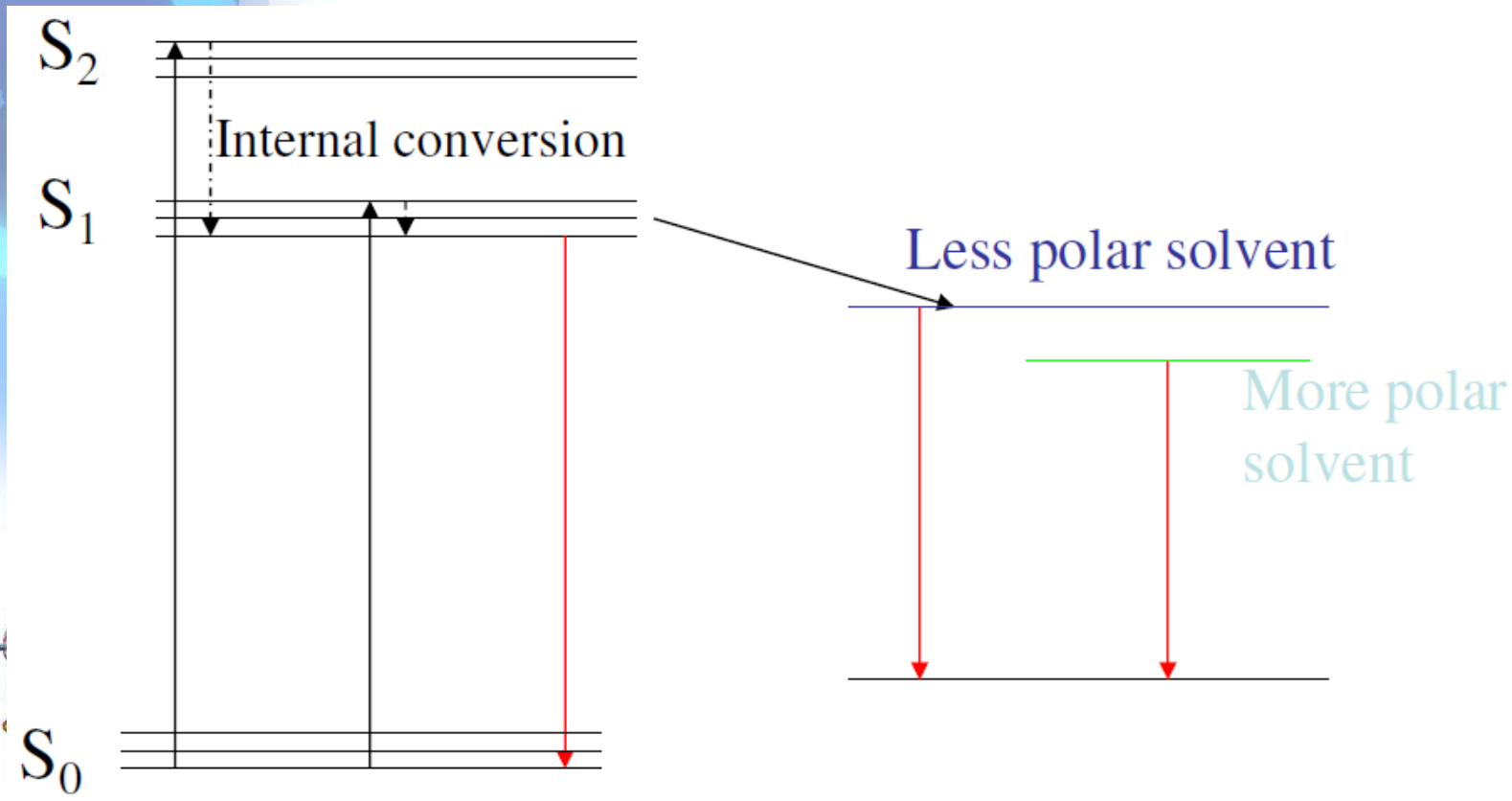
• Most unsubstituted aromatic hydrocarbons fluoresce in solution, with the quantum efficiency increasing with the number of rings and their degree of condensation.

If a compound is nonfluorescent, it may be converted to a fluorescent derivative. For example, nonfluorescent steroids may be converted to fluorescent compounds by dehydration with concentrated sulfuric acid. These cyclic alcohols are converted to phenols. Similarly, dibasic acids, such as malic acid, may be reacted with *β-naphthol* in concentrated sulfuric acid to form a fluorescing derivative.

Temperature and Solvent Effects

In most molecules, the quantum efficiency of fluorescence *decreases* with *increasing* temperature because the increased frequency of collision at elevated temperatures increases the probability of collisional relaxation. A decrease in solvent viscosity leads to the same result.

Typically, the fluorophore has a larger dipole moment in the excited state than the ground state. Solvent shifts the emission to lower energy due to stabilization of the excited state by the polar solvent molecule. As the solvent polarity is increased , this effect becomes larger.

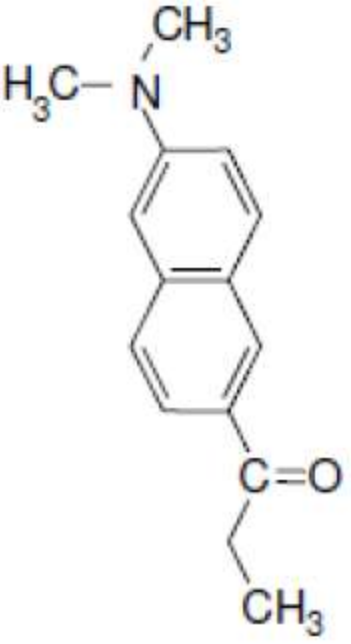


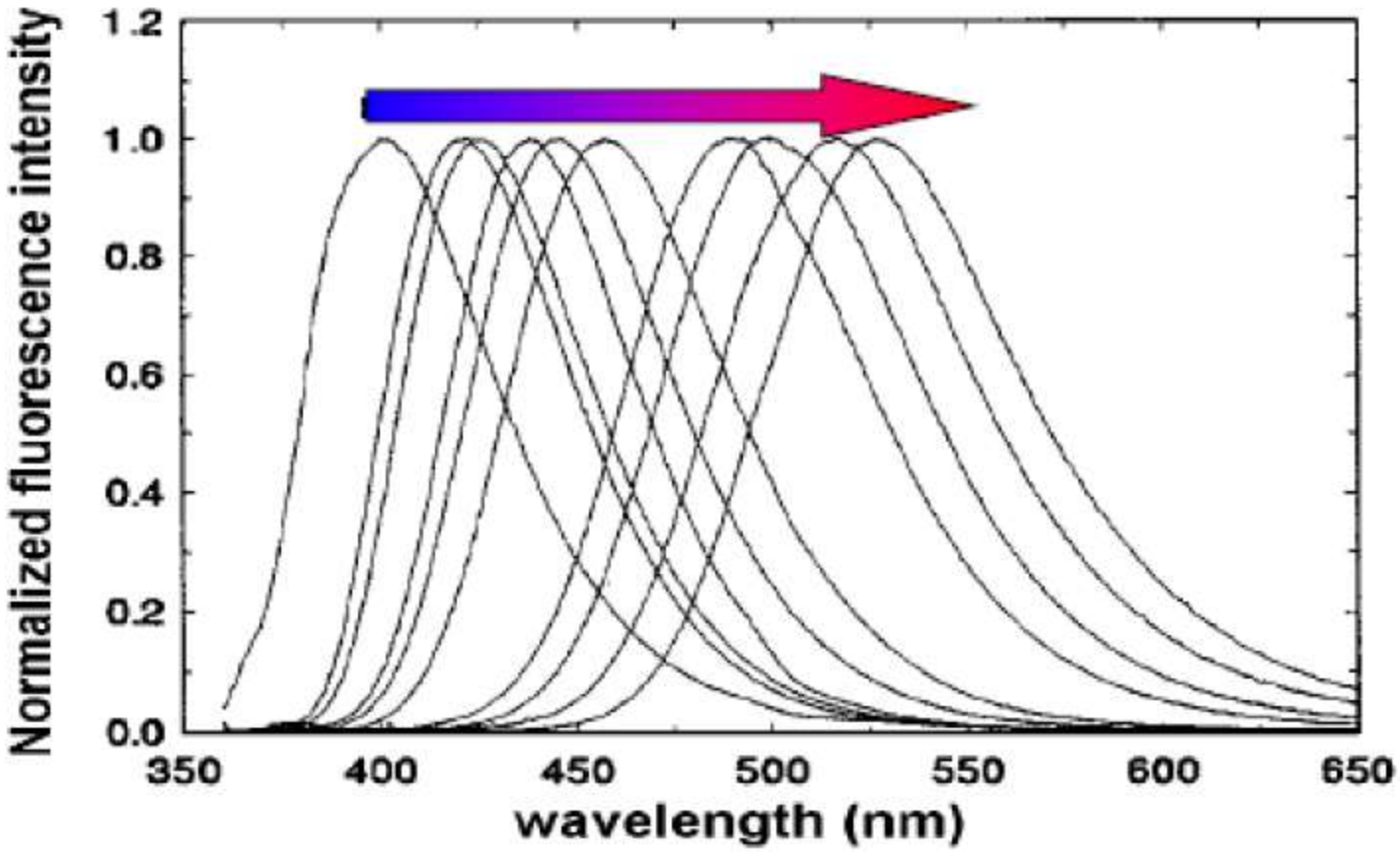
Fluorescence spectra of Prodan

Emission spectra of prodan in different solvents:

Heptane
water

Increase of solvent polarity leads to larger red-shift

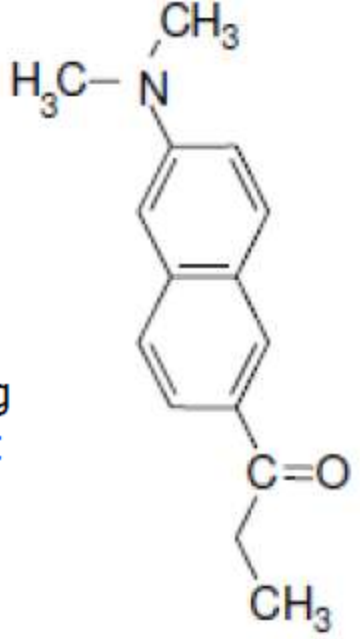




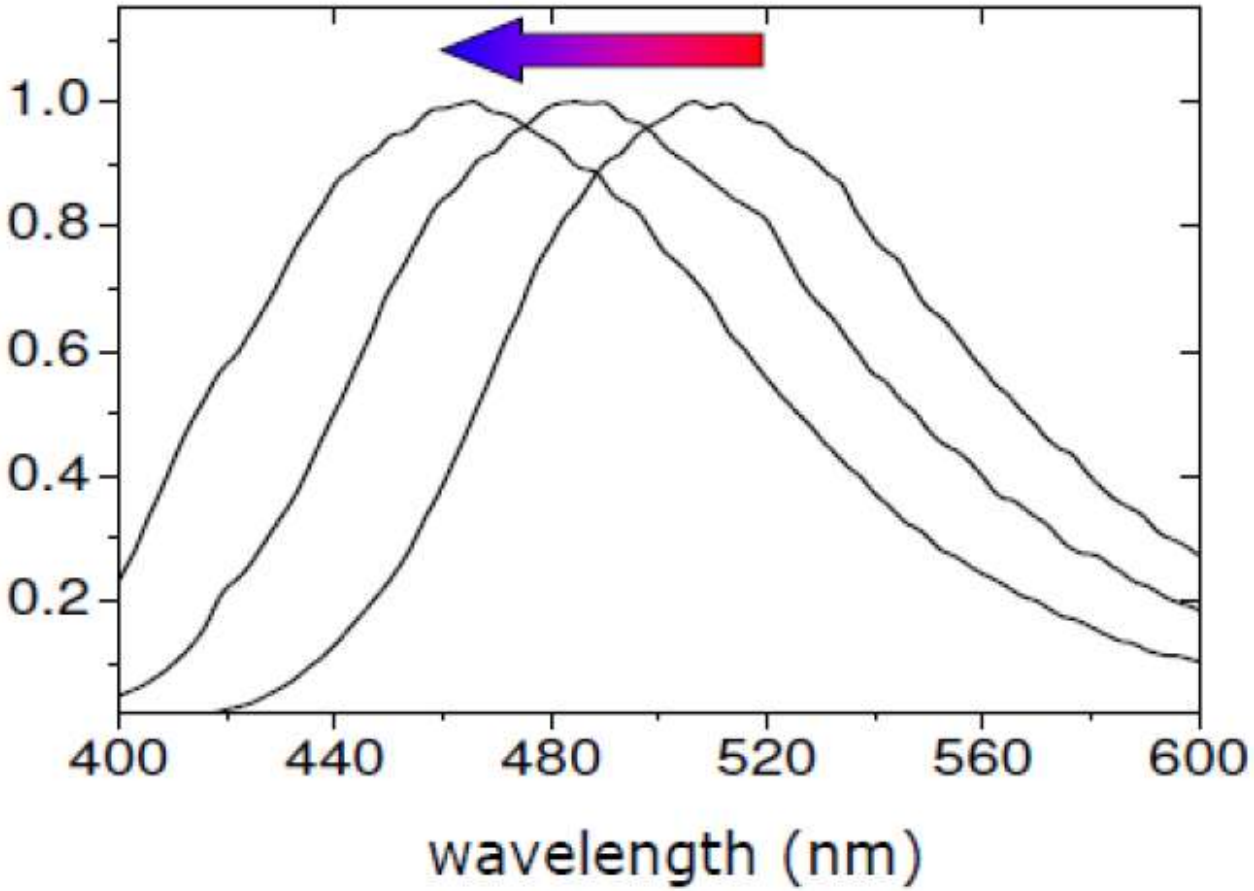
Emission spectra of prodan at different temperatures:

100 K

300 K



Decrease of temperature → increase of viscosity → increasing fluorescence contributions of non-relaxed states → blue-shift



RELATIONSHIP BETWEEN CONCENTRATION AND FLUORESCENCE INTENSITY

Fluorescence intensity is proportional to the intensity of the source. Absorbance, on the other hand, is independent of it.

The radiant power of fluorescence emitted *F is proportional to the radiant power of* the excitation beam absorbed by the system:

$$F = K'(P_0 - P)$$

where P_0 *is the radiant power of the beam incident on the solution and P is its power* after it passes through a length *b of the medium. The constant K r depends on the* quantum efficiency of the fluorescence. In order to relate *F to the concentration c of* the fluorescing particle, we write Beer's law in the form

$$\frac{P}{P_0} = 10^{-\varepsilon bc}$$

where ε is the molar absorptivity of the fluorescing species and *εbc is the absorbance.*

$$F = K'P_0(1 - 10^{-\varepsilon bc})$$

When *εbc = A < 0.05, the first term inside the brackets, 2.3 εbc, is much larger than* subsequent terms, and we can write

$$F = 2.3K'\varepsilon bcP_0$$

or when the incident power P_0 *is constant,*

$$F = Kc$$

Thus, a plot of the fluorescence power emitted versus the concentration of the emitting species should be linear at low concentrations. When *c becomes large* enough that the absorbance exceeds about 0.05 (or the transmittance is smaller than about 0.9), the relationship represented by Equation become nonlinear, and *F lies below an extrapolation of the linear plot.*

Quenching

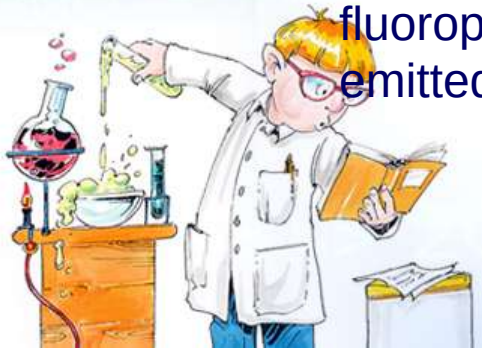
• Fluorescence quenching refers to any process that decreases the fluorescence intensity of a sample.

• A variety of molecular association can result in quenching. These include excitedstate reactions, molecular rearrangements, energy transfer, groundstate complex formation, and collisional quenching.

• A wide variety of substances act as quenchers of fluorescence. Quenching by oxygen is due to its paramagnetic nature causes the fluorophore to undergo intersystem crossing to the triplet state.

Iodine and bromine substituents decrease the quantum yield.

A colored species in solution with a fluorescing analyte may interfere by absorbing the excitation radiation or the emitted fluorescent radiation or both. This is the so-called **inner-filter effect.** For example, in sodium carbonate solution, potassium dichromate exhibits absorption peaks at 245 and 348 nm. These overlap with the excitation and emission peaks for tryptophan and would interfere. The inner-filter effect can also arise from too high a concentration of the fluorophore itself. Some of the analyte molecules will reabsorb the emitted radiation of others.



Scope of quenching and energy loss during fluorescence

• Energy may be lost in vibrational transition, collision with the solvent, heat transfer etc. Only a part of the light absorbed is emitted. It's because of this that the quantum yield in most practical cases is not equal to one.

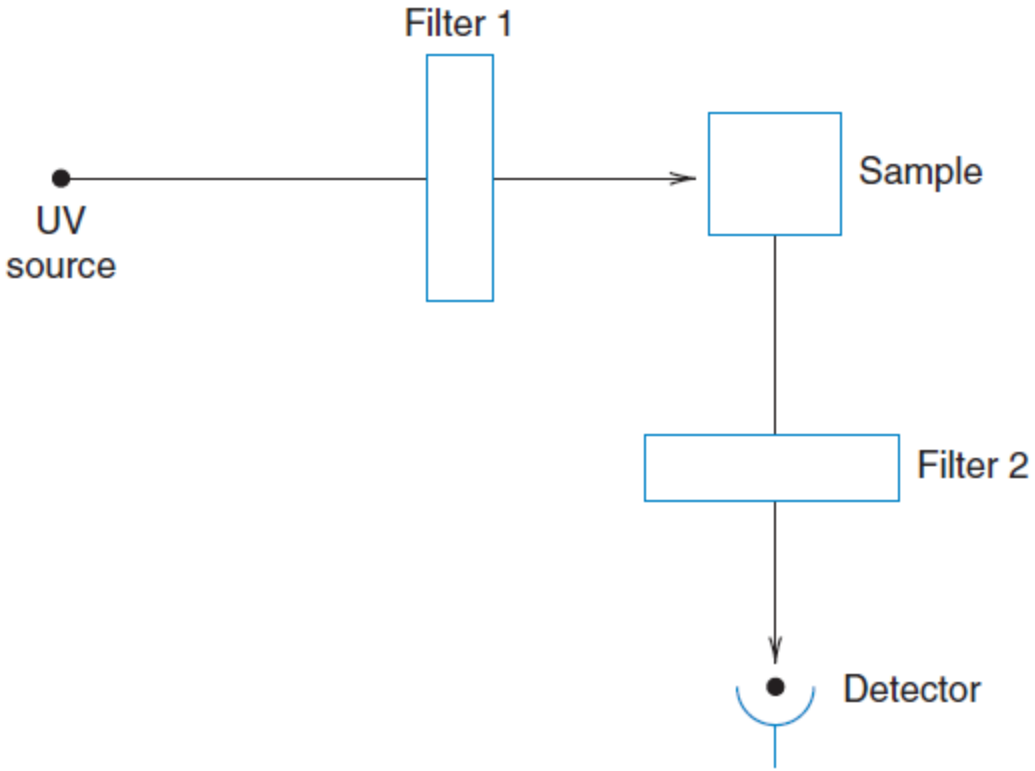
• Quenching of fluorescence may also occur due to the presence of some foreign molecule in the solution which is acting as a quencher, or due to some structural rearrangement in the molecule (say protein), which drives the fluorophore to a conformation where it is in vicinity of a quencher (any amino acid residue or disulphide bond).

• Fluoresence intensity may also decrease due to the transfer of the emitted energy to some other chromophore, which absorbs at that energy. This phenomena is called FRET. However, FRET and quenching should not be treated synonymously

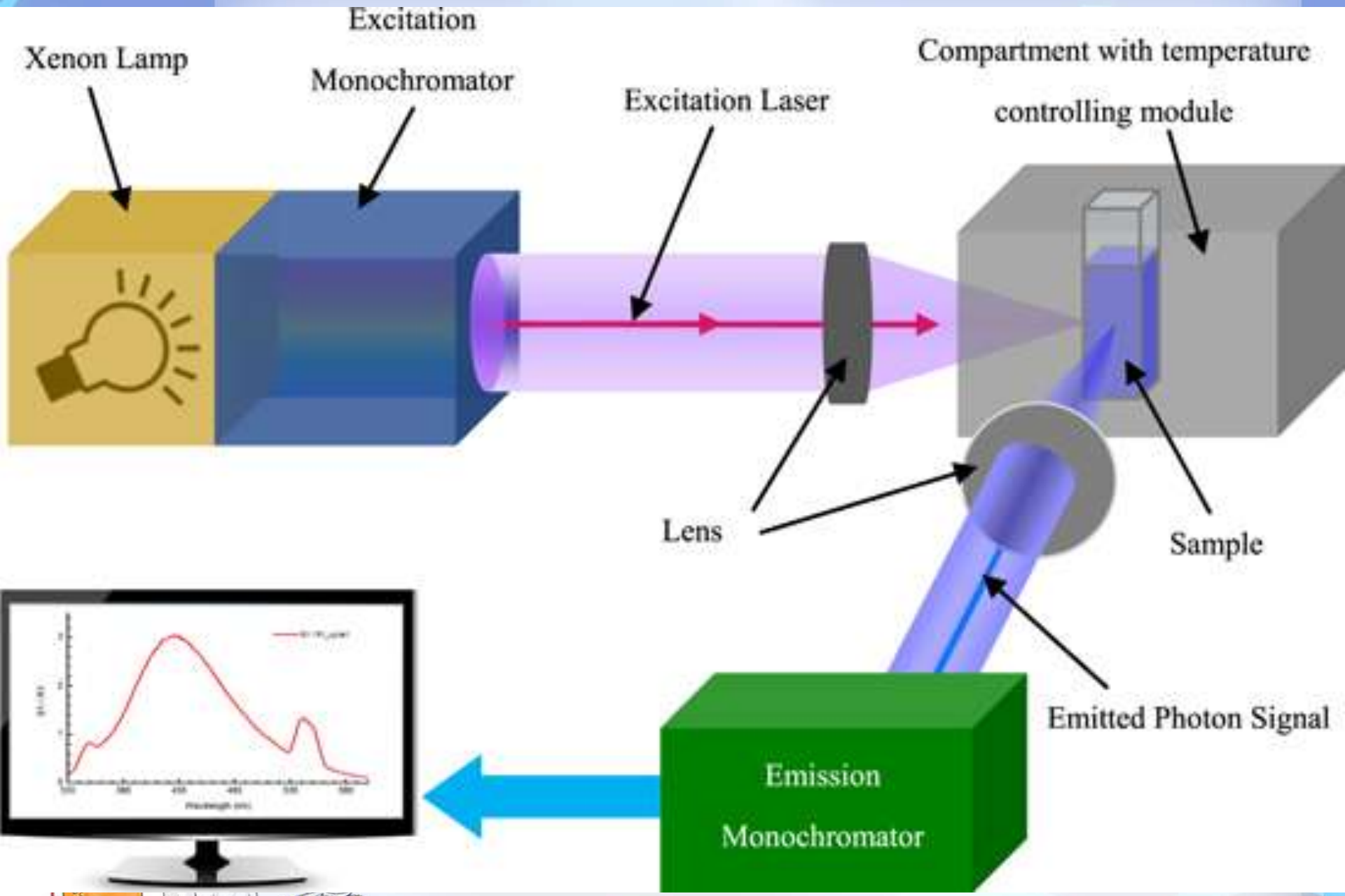
FLUORESCENCE INSTRUMENTATION

For fluorescence measurements, it is necessary to separate the emitted radiation from the incident radiation. This is most easily done by measuring the fluorescence at right angles to the incident radiation. The fluorescence radiation is emitted in all directions, but the incident radiation passes straight through the solution.

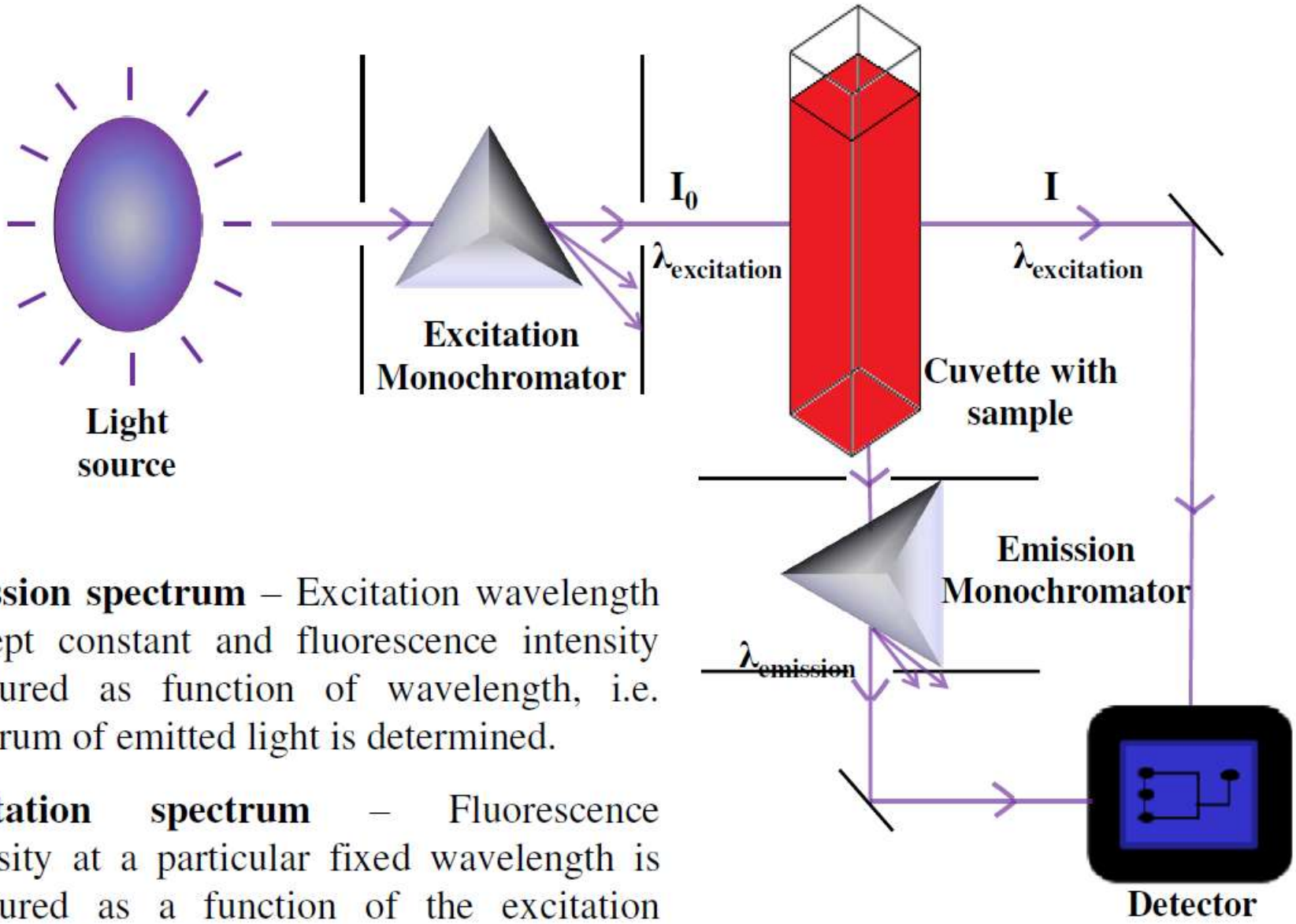
Two basic instrumental designs are used for measuring molecular fluorescence. In a ***fluorometer*** the excitation and emission wavelengths are selected with absorption or interference *filters*.



When a monochromator is used to select the excitation and emission wavelengths, the instrument is called a ***spectrofluorometer.*** *With a monochromator,* the excitation source is usually a high-pressure Xe arc lamp, which has a continuum emission spectrum.

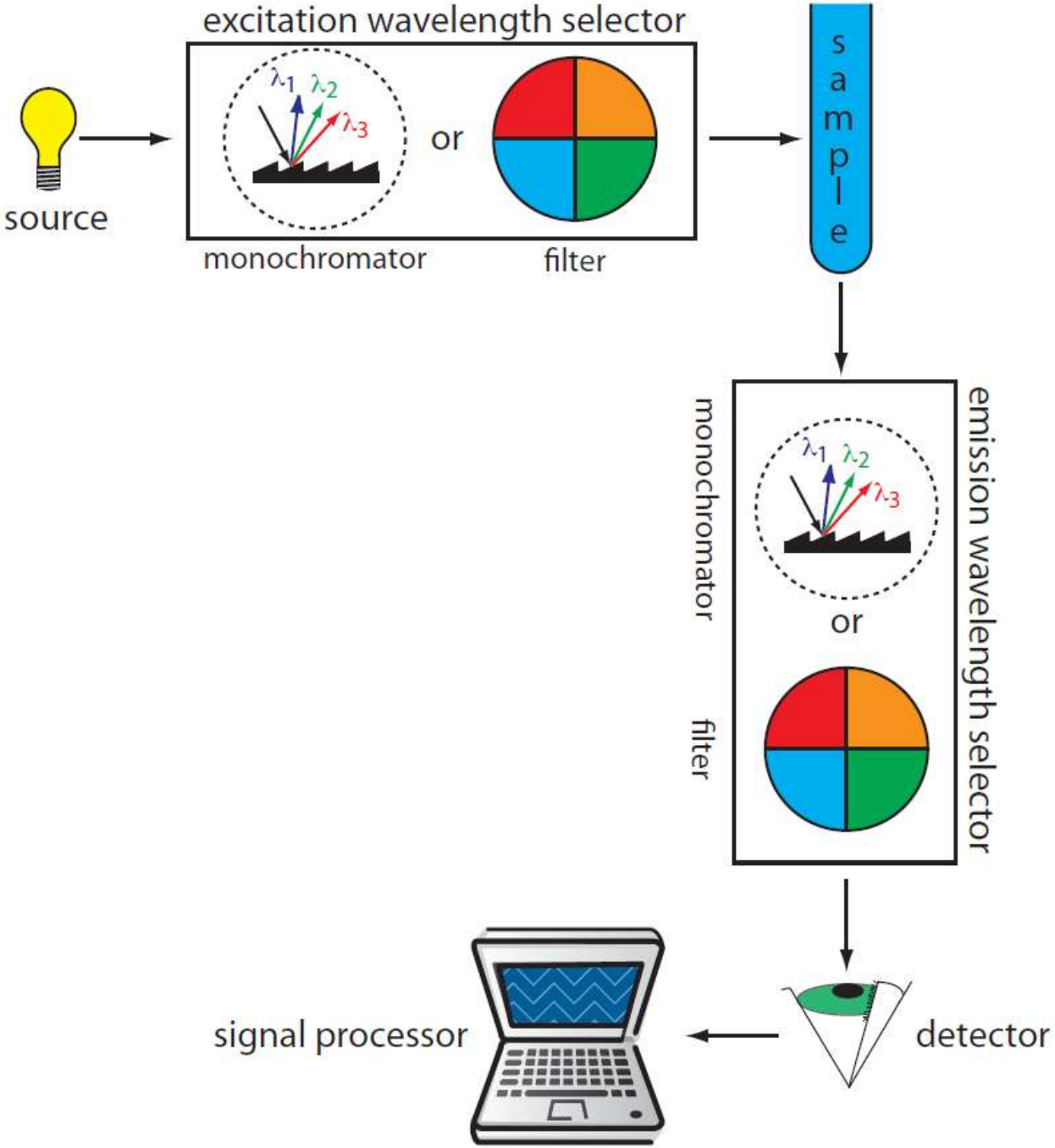

Excitation
Xenon Lamp
Monochromator
Excitation Laser
Compartment with temperature
controlling module
Lens
Sample
Emitted Photon Signal
Emission
Monochromator

Experimental Set Up



Emission spectrum – Excitation wavelength is kept constant and fluorescence intensity measured as function of wavelength, i.e. spectrum of emitted light is determined.

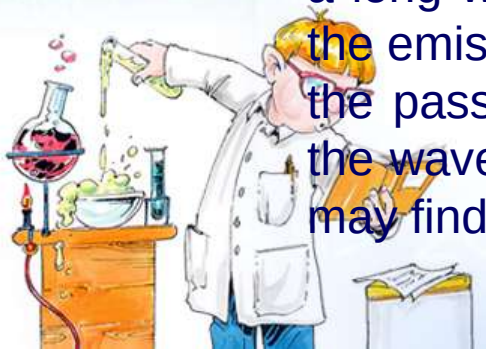
Excitation spectrum – Fluorescence intensity at a particular fixed wavelength is measured as a function of the excitation wavelength.



Schematic diagram for measuring fluorescence showing the placement of the wavelength selectors for excitation and emission. When a filter is used the instrument is called a fluorimeter, and when a monochromator is used the instrument is called a spectrofluorimeter.

Radiation sources for fluorescence are usually more powerful than typical absorption sources. As a result of these different dependencies on source intensity, fluorescence methods are generally one to three orders of magnitude more sensitive than methods based on absorption. Mercury arc lamps, xenon arc lamps, xenon-mercury arc lamps, and lasers are typical fluorescence sources. Monochromators and transducers are typically similar to those used in absorption spectrophotometers. Photomultipliers are still widely used in high-sensitivity spectrofluorometers, but CCDs and photodiode arrays have become popular in recent years. The sophistication, performance characteristics, and cost of fluorometers and spectrofluorometers vary widely as with absorption spectrophotometers. Generally, fluorescence instruments are more expensive than absorption instruments of corresponding quality.

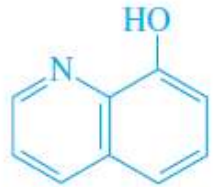
In the simple filter fluorometer instrument a excitation filter (filter 1) is used to select wavelengths that efficiently excite the analyte fluorescence. This filter is typically a short-pass or bandpass filter with a long wavelength cutoff that is shorter than the cut-on wavelength of the emission filter (filter 2), usually a long-pass filter. Thus filter 1 allows the passage of only the wavelength of excitation while filter 2 passes the wavelength of emission but not the wavelength of excitation, which may find its way to the detector by scattering.



Most fluorescing molecules absorb ultraviolet radiation over a band of wavelengths, and so a simple line source is sufficient for many applications. Such a source is a medium-pressure mercury vapor lamp. A spark is passed through mercury vapor at low pressure, and principal lines are emitted at 253.7, 365.0, 520.0 (green), 580.0 (yellow), and 780.0 (red) nm. *Wavelengths shorter than 300 nm are harmful to the eyes, and one must never look directly at a short-wavelength UV source.*

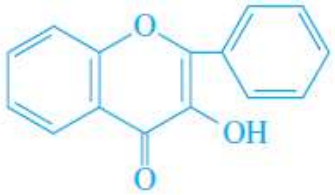
The mercury vapor itself absorbs most of the 253.7-nm radiation (self-absorption), and a blue filter in the envelope of the lamp may be added to remove most of the visible light. The 365-nm line is thus the one used primarily for the activation. A high-pressure xenon arc (a continuum source) is usually used as the source in more sophisticated instruments that will scan the spectrum (spectrofluorometers) because it has a more uniform disribution of energy throughout the UV-Visible spectrum. The lamp pressure is 7 atm at 25◦C and 35 atm at operating temperatures and is typically housed in a protective but well-vented housing.

Applications of Fluorescence Methods



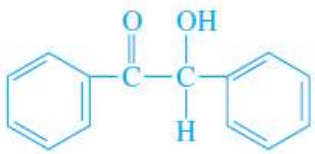
8-hydroxyquinoline
(reagent for Al, Be, and other metal ions)

Fluorescence spectroscopy is not a major structural or qualitative analysis tool because molecules with subtle structural differences often have similar fluorescence spectra. Also, fluorescence bands in solution are relatively broad at room temperature. However, fluorescence has proved to be a valuable tool in oil spill identification. The source of an oil spill can often be identified by comparing the fluorescence emission spectrum of the spill sample to that of a suspected source. The vibrational structure of polycyclic hydrocarbons present in the oil makes this type of identification possible.



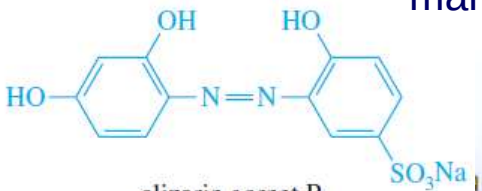
flavanol
(reagent for Zr and Sn)

Quantitative fluorescence methods have been developed for inorganic, organic, and biochemical species. Inorganic fluorescence methods can be divided into two classes: direct methods and indirect methods. Direct methods are based on the reaction of the analyte with a complexing agent to form a fluorescent complex. Indirect methods depend on the decrease in fluorescence, also called **quenching,** as a result of interaction of the analyte with a fluorescent reagent. Quenching methods are primarily used for the determination of anions and dissolved oxygen.



benzoin
(reagent for B, Zn, Ge, and Si)

The number of applications of fluorescence methods to organic and biochemical problems is impressive. Among the compound types that can be determined by fluorescence are amino acids, proteins, coenzymes, vitamins, nucleic acids, alkaloids, porphryins, steroids, flavonoids, and many metabolites.



alizarin garnet R
(reagent for Al, F^-)

In addition to methods that are based on measurements of fluorescence intensity, there are many methods involving measurements of fluorescence lifetimes. Several instruments have been developed that provide microscopic images of specific species based on fluorescence lifetimes.

Examples of Naturally Photoluminescent Organic Analytes

aromatic amino acids	phenylalanine (F) tyrosine (F) tryptophan (F, P)
vitamins	vitamin A (F) vitamin B2 (F) vitamin B6 (F) vitamin B12 (F) vitamin E (F) folic acid (F)
catecholamines	dopamine (F) norepinephrine (F)
pharmaceuticals and drugs	quinine (F) salicylic acid (F, P) morphine (F) barbiturates (F) LSD (F) codeine (P) caffeine (P) sulfanilamide (P)
environmental pollutants	pyrene (F) benzo[a]pyrene (F) organothiophosphorous pesticides (F) carbamate insecticides (F)

Evaluation

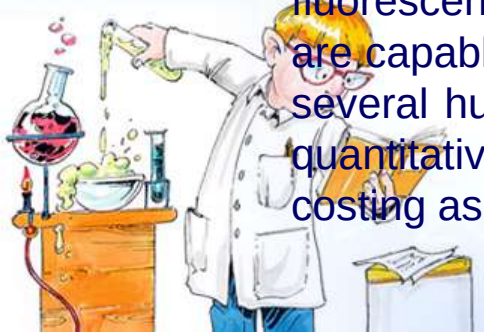
• **Accuracy** The accuracy of a fluorescence method is generally 1–5% when spectral and chemical interferences are insignificant. Accuracy is limited by the same types of problems affecting other spectroscopic methods. In addition, accuracy is affected by interferences influencing the fluorescent quantum yield. The accuracy of phosphorescence is somewhat greater than that for fluorescence.

•**Precision** When the analyte's concentration is well above the detection limit, the relative standard deviation for fluorescence is usually 0.5–2%. The limiting instrumental factor affecting precision is the stability of the excitation source. The precision for phosphorescence is often limited by reproducibility in preparing samples for analysis, with relative standard deviations of 5–10% being common.

• **Sensitivity** the sensitivity of a fluorescent or phosphorescent method is influenced by a number of parameters. The importance of quantum yield and the effect of temperature and solution composition have been considered.

• **Selectivity** The selectivity of molecular fluorescence and phosphorescence is superior to that of absorption spectrophotometry for two reasons: first, not every compound that absorbs radiation is fluorescent or phosphorescent, and, second, selectivity between an analyte and an interferant is possible if there is a difference in either their excitation or emission spectra.

• **Time, Cost, and Equipment** As with other optical spectroscopic methods, fluorescent and phosphorescent methods provide a rapid means of analysis and are capable of automation. Fluorometers are relatively inexpensive, ranging from several hundred to several thousand dollars, and often are very satisfactory for quantitative work. Spectrofluorometers are more expensive, with some models costing as much as $50,000.



Molecular Phosphorescence Spectroscopy

Phosphorescence materials and pigments, called **phosphors,** find many uses, including the markings of safety-related signs, such as highway exit and stop signs. Luminous watches contain a phosphor consisting of alkaline earth metal aluminates doped with rare earth elements, such as europium. Cathode-ray tubes, used in some oscilloscopes, computer monitors, and older television sets have solid-state phosphors coated on the screen, allowing actions of the electron beam to be visualized.

Transitions from an excited singlet state to the ground singlet state produce molecular fluorescence. This singlet-singlet transition is highly probable, and thus, the lifetime of an excited singlet state is very short (10–5 s or less). On the other hand, transitions from an excited triplet state to the ground singlet state produce molecular *phosphorescence*. Because the triplet-singlet transition produces a change in electron spin, it is much less probable. As a result, the triplet state has a much longer lifetime (typically 10^{-4} to 10^{4} s).

The long lifetime of phosphorescence is also one of its drawbacks. Because the excited state is relatively long-lived, nonradiational processes have time to compete with phosphorescence for deactivation. Therefore, the efficiency of the phosphorescence process, as well as the corresponding phosphorescence intensity, is relatively low. To increase the efficiency, phosphorescence is commonly observed at low temperatures in rigid media, such as glasses. Another approach is to adsorb the analyte on a solid surface or enclose it in a molecular cavity (micelle or cyclodextrin cavity), which protects the fragile triplet state. This technique is known as **room temperature phosphorescence.**

Because of its weak intensity, phosphorescence is much less widely applicable than fluorescence. However, molecular phosphorescence has been used for the determination of a variety of organic and biochemical species, including nucleic acids, amino acids, pyrine and pyrimidine, enzymes, polycyclic hydrocarbons, and pesticides. Many pharmaceutical compounds exhibit measurable phosphorescence signals. The instrumentation for phosphorescence is also somewhat more complex than that for fluorescence. Phosphorescence instruments usually discriminate phosphorescence from fluorescence by delaying the phosphorescence measurement until the fluorescence has decayed to nearly zero. Many fluorescence instruments have attachments, called **phosphoroscopes,** that allow the same instrument to be used for phosphorescence measurements.