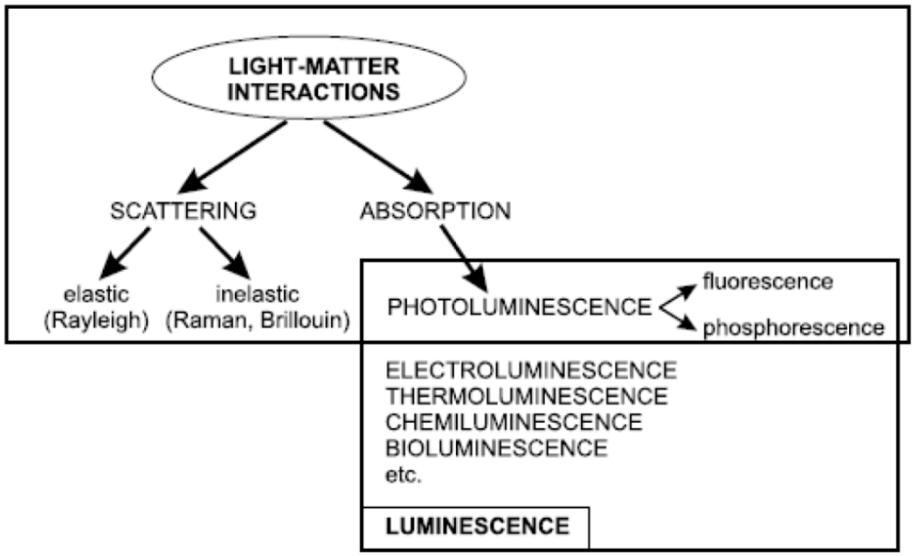
SPECTROSCOPY-1
UNIT 3

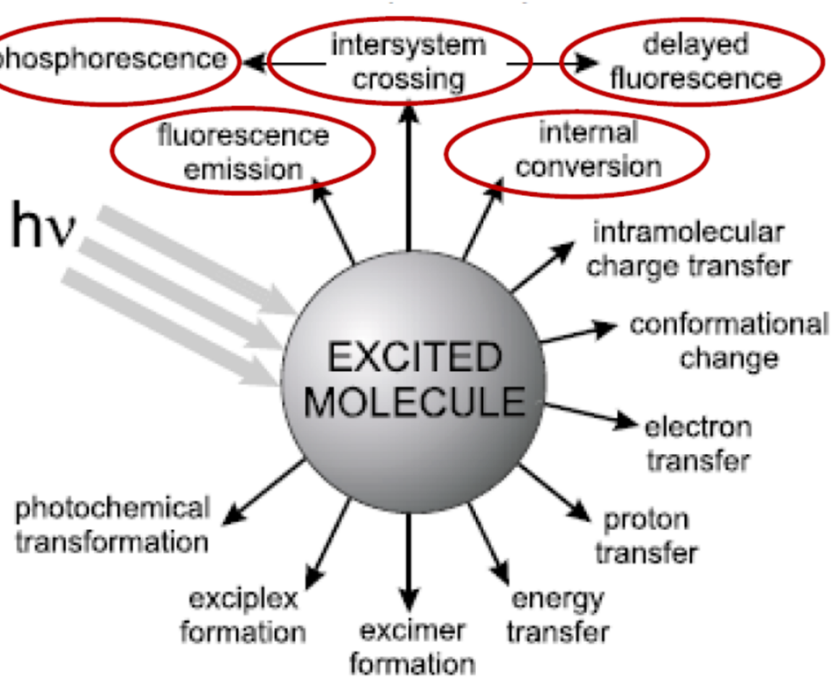
PG 1ST SEMESTER

PRIYANKA BARUA

Fluorescence and phosphorescence



Possible de-excitation pathways of excited molecules.



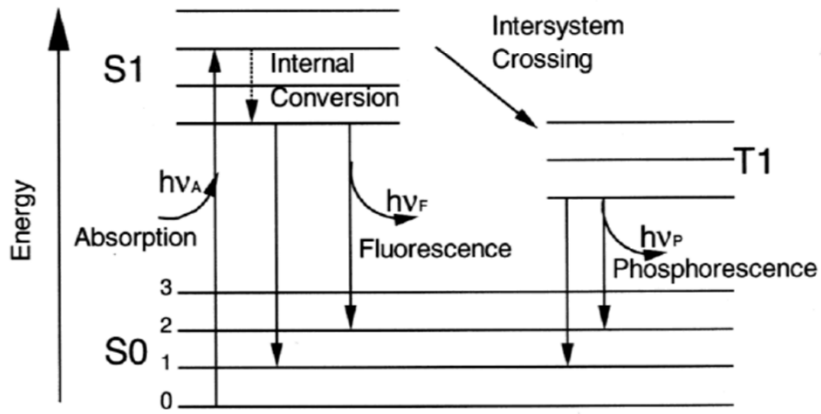
Luminescence: emission of photons from electronically excited states of atoms, molecules and ions.

Fluorescence: **a process in which a part of energy (UV, Visible) absorbed by a substance is released in the form of light** as long as the stimulating radiation is continued.

The fluorescence emission took place from a **singlet excited states** (average lifetime: from $<10^{-10}$ to 10^{-7} sec).

Phosphorescence: a process in which energy of light absorbed by a substance is released relatively slowly in the form of light.

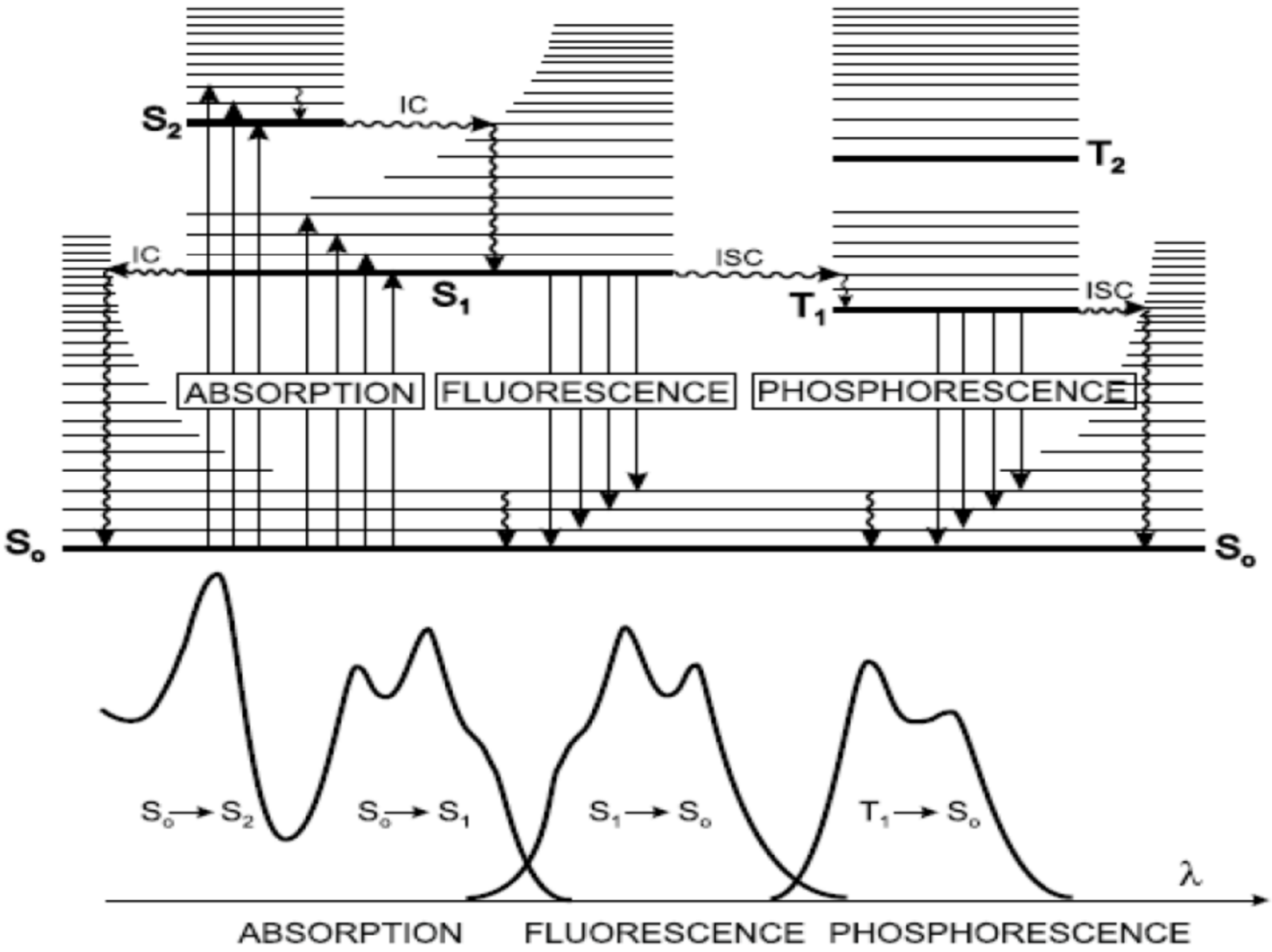
The phosphorescence emission took place from a **triplet excited states** (average lifetime: from 10^{-5} to $>10^{+3}$ sec).



The Perrin–Jablonski diagram

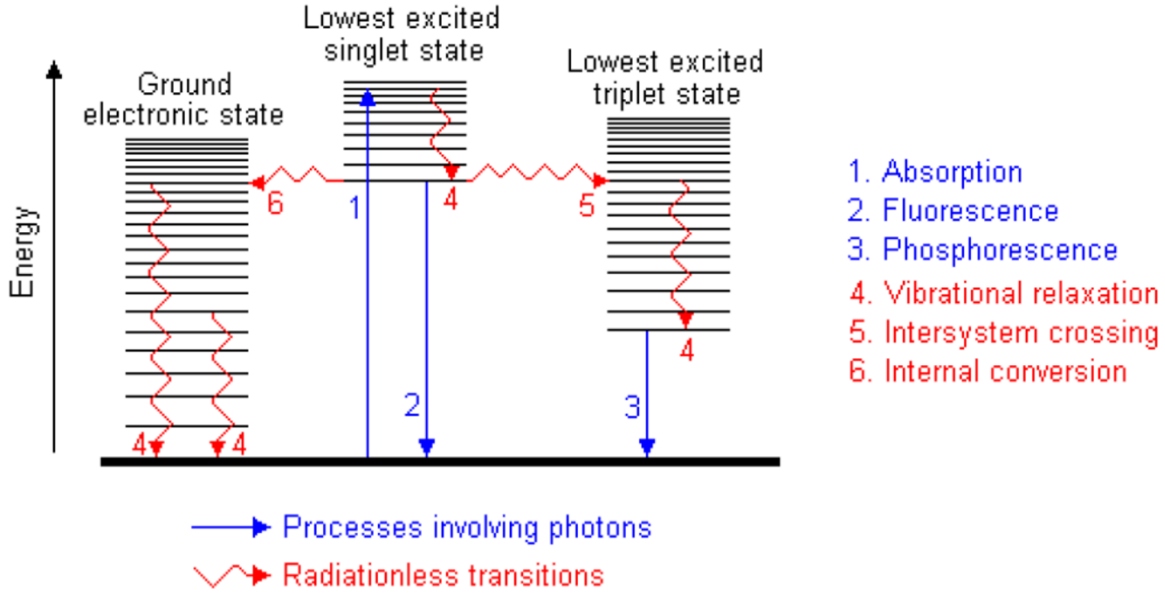
Possible processes:

- photon absorption
- vibrational relaxation
- internal conversion
- intersystem crossing
- fluorescence
- phosphorescence
- delayed fluorescence
- triplet–triplet transitions



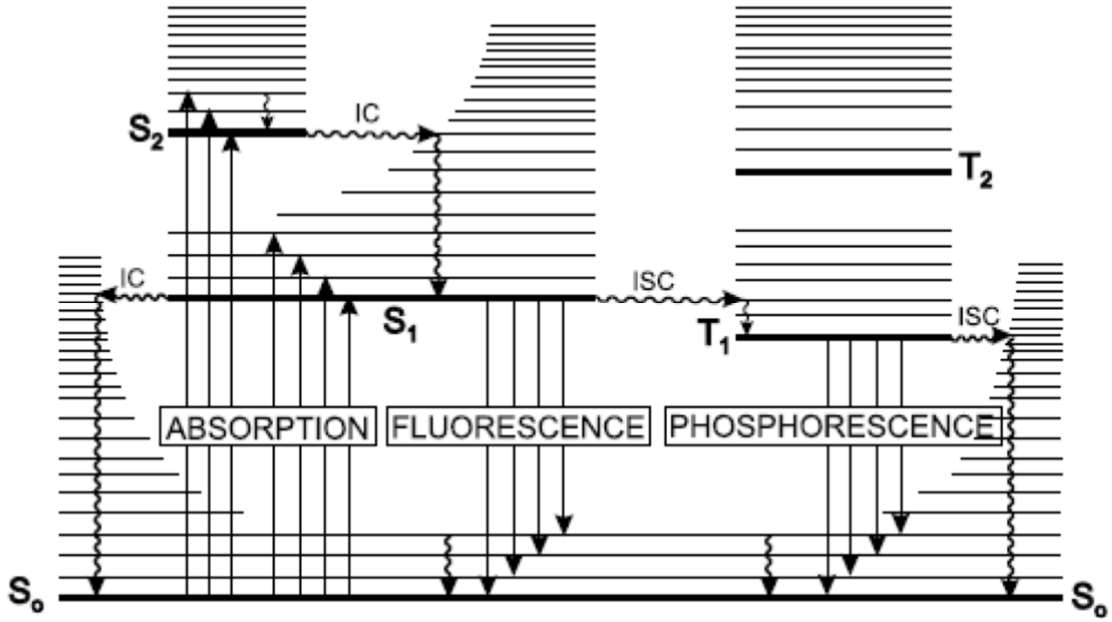
Vibrational relaxation (non-radiative process):

- The energy deposited by the photon into the electron is given away to other vibrational modes as *kinetic energy.*
- This kinetic energy may stay within the same molecule, or it may be transferred to other molecules around the excited molecule during collisions of the excited molecule with the surrounding molecules (solvent).
- When a molecule is excited to an energy level higher than the lowest vibrational level (v>0) of the first electronic state, **vibrational relaxation** leads the excited molecule towards the **lowest vibrational level (v = 0) of the S_1 singlet state**.
- Since this is a very fast transition, it is extremely likely to occur immediately following absorbance



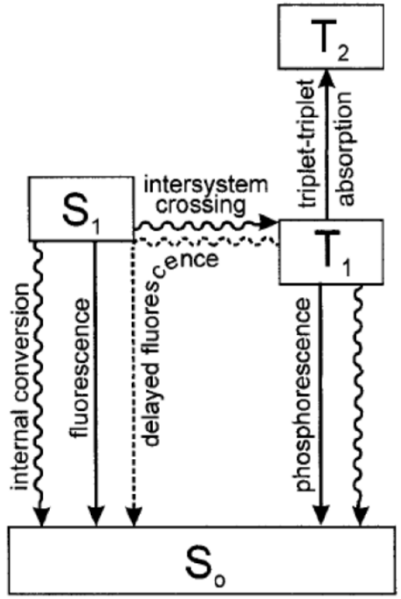
Internal conversion *is a non-radiative and isoenergetic transition between two electronic states of the same spin multiplicity*.

- Internal conversion occurs because of the overlap of vibrational and electronic energy states.
- From S_1, internal conversion to S_0 is possible but is less efficient than conversion from S_2 to S_1, because of the much larger energy gap between S_1 and S_0.
- Internal conversion occurs in the *same time frame as vibrational relaxation*, therefore, is a very likely way for molecules to dissipate energy from light perturbation.



Intersystem crossing *is a non-radiative transition between two isoenergetic vibrational levels belonging to electronic states of* ***different multiplicities***

- For example, an excited molecule in the 0 vibrational level of the S_1 state can move to the isoenergetic vibrational level ($v > 0$) of the T_1 triplet state. Then vibrational relaxation brings it into the lowest vibrational ($v = 0$) level of T_1.
- For some molecules, intersystem crossing may be fast enough (10^{-7}–10^{-9} s) to compete with other pathways of de-excitation from S_1 (fluorescence and internal conversion $S_1 \rightarrow S_0$).
- Crossing between states of different multiplicity is in principle **forbidden**, but for some molecules spin–orbit coupling can be large enough to make it possible!
- The probability of intersystem crossing depends on the singlet and triplet states involved.
- If the electronic transition $S_0 \rightarrow S_1$ is $n \rightarrow \pi^*$ type, intersystem crossing is often efficient.

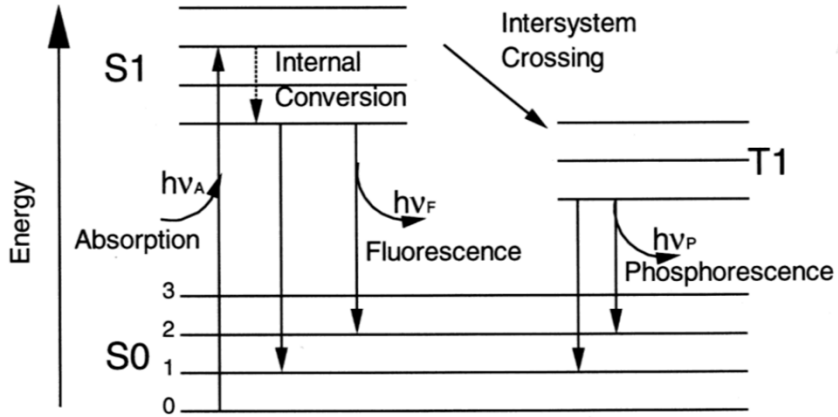


Triplet–triplet absorption *(non-radiative transition)*

- Once a molecule is excited and reaches its triplet state T_1, it can absorb another photon at a different wavelength because triplet–triplet transitions are spin allowed.
- Triplet-triplet transitions can be observed if the population of molecules in the triplet state is large enough, which can be achieved by illumination with an intense pulse of light.

Phosphorescence *is the radiative transition from the triplet state T_1 to S_0.*

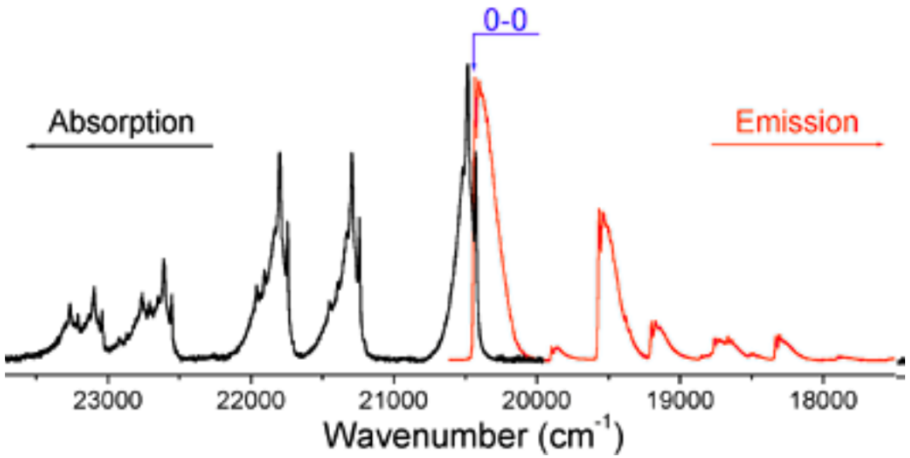
- In solution at room temperature, non-radiative de-excitation from the triplet state T_1, is predominant over phosphorescence (e.g., external conversion: the energy transfer between molecules through molecular collisions).
- The transition $T_1 \rightarrow S_0$ is forbidden (but it can be observed because of spin–orbit coupling), the radiative rate constant is very low.
- During this process, the numerous collisions with solvent molecules favor intersystem crossing and vibrational relaxation in S_0.
- At low temperatures and/or in a **rigid medium (eg. Frozen solvent)**, phosphorescence can be observed. The lifetime of the triplet state may, under these conditions, be long enough to observe phosphorescence on a time-scale up to seconds, even minutes or more.
- The phosphorescence spectrum is located at wavelengths higher than the fluorescence spectrum because the energy of the lowest vibrational level of the triplet state T_1 is lower than that of the singlet state S_1.

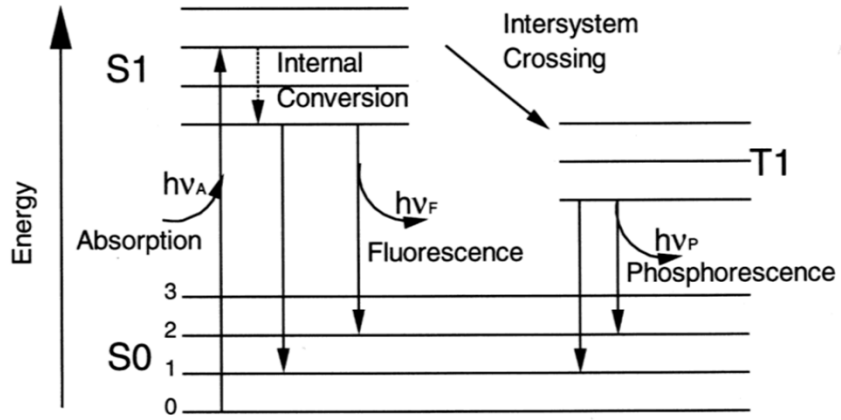


Fluorescence (radiative transition): *the emission of photons accompanying the $S_1 \rightarrow S_0$ relaxation.*

- Fluorescence emission occurs from S_1 and therefore its characteristics (except polarization) do not depend on the excitation wavelength.
- The 0–0 transition is usually the same for absorption and fluorescence.
- The fluorescence spectrum is located at higher wavelengths (lower energy) than the absorption spectrum because of the energy loss in the excited state due to vibrational relaxation.

***Stokes Rule*:** the wavelength of a fluorescence emission should always be higher than that of absorption.

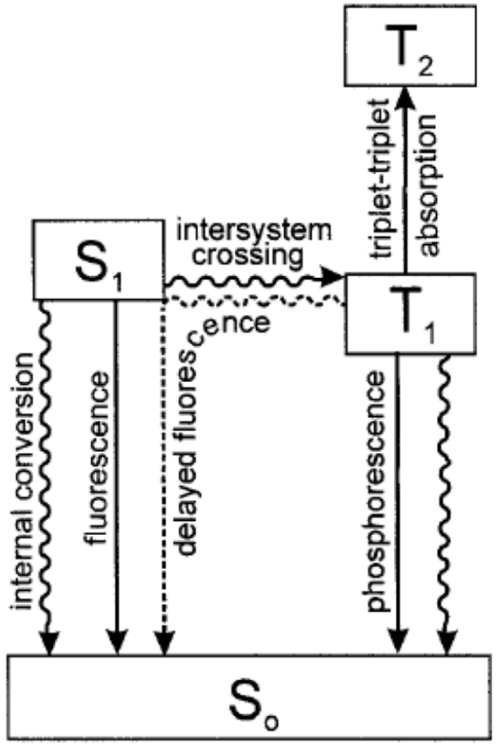




Delayed fluorescence

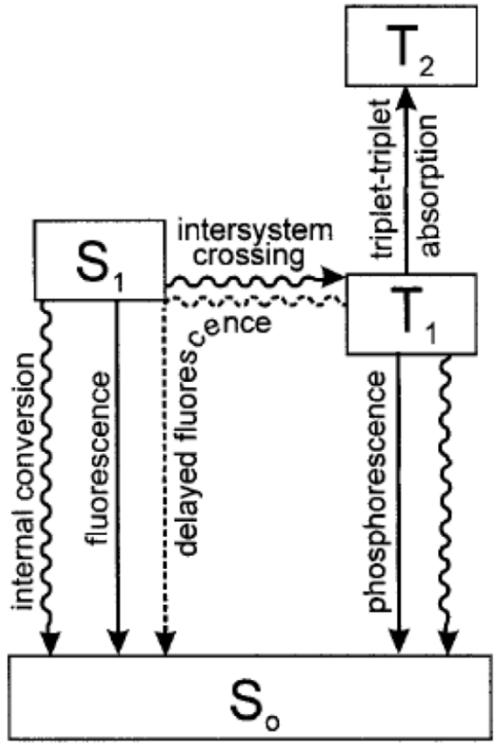
Thermally activated delayed fluorescence *(radiative)*

- Reverse intersystem crossing $T_1 \rightarrow S_1$ can occur when the energy difference between S_1 and T_1 is small and when the lifetime of T_1 is long enough.
- Results an emission with the same spectral distribution as normal fluorescence but with a much longer decay time constant because the molecules stay in the triplet state before emitting from S_1.
- This delayed fluorescence emission is thermally activated; consequently, its efficiency increases with increasing temperature.
- It is also called ***delayed fluorescence of E-type*** because it was observed for the first time with Eosin.
- Delayed fluorescence is very efficient in fullerenes.
- Delayed fluorescence does not normally occur in aromatic hydrocarbons because of the relatively large difference in energy between S_1 and T_1.



Delayed fluorescence (due to triplet–triplet annihilation)

- In concentrated solutions, a collision between two molecules in the T_1 state (triplet–triplet annihilation - *non-radiative*) can provide enough energy to allow one of them to return to the S_1 state.
- The triplet–triplet annihilation could leads to a delayed fluorescence emission, called ***delayed fluorescence of P-type*** (it was observed for the first time with Pyrene).
- The decay time constant of the delayed fluorescence process is half the lifetime of the triplet state in dilute solution.
- The intensity has a characteristic quadratic dependence with excitation light intensity.



De-excitation rate constants

After excitation of a molecule population by a very short pulse of light, the de-excitation processes (radiative or nonradiative) determine the decreasing of excited population.

The time a molecule spends in the excited state is determined by the sum of the rate constants (kinetic constants) of all de-excitation processes

The fluorescence is observed if $k_f > k_i + k_x$

The ***rate constants*** for the various processes are denoted as follows:

k_f (k^S_r):**fluorescence** emission ($S_1 \rightarrow S_0$)

k_{ph} (k^T_r): **phosphorescence** emission ($T_1 \rightarrow S_0$)

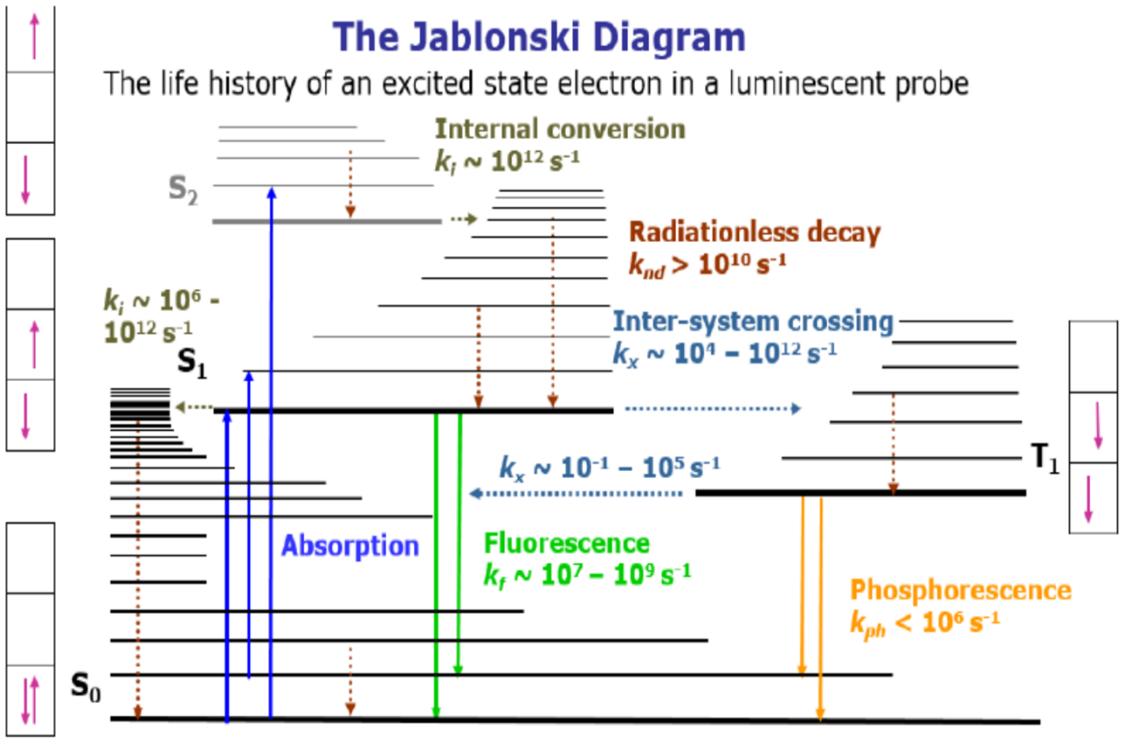
k_i (k^S_{ic}): internal conversion ($S_1 \rightarrow S_0$)

k_x (k_{isc}): intersystem crossing ($S_1 \rightarrow T_1$)

k_{nr} (k^S_{nr}): the overall non-radiative rate constant

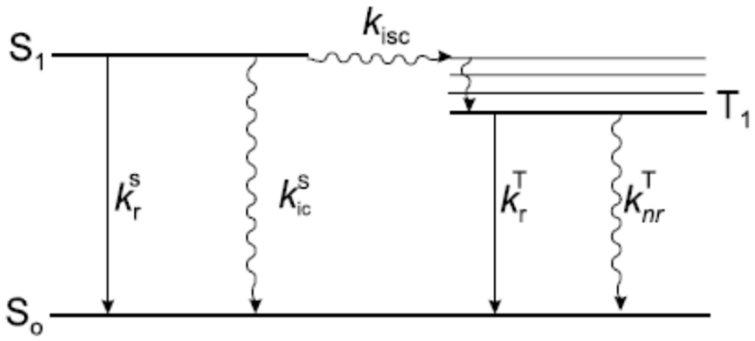
($k^S_{nr} = k^S_{ic} + k_{isc}$)

(k^T_{nr}): intersystem crossing ($T_1 \rightarrow S_0$)



If the only way of de-excitation from S_1 to S_0 is fluorescence emission, the lifetime (called ***the radiative lifetime***) is:

$$\tau_r = \frac{1}{k_f}$$



The ***lifetime of singlet excited state*** S_1, is given by:

$$\tau_f = \frac{1}{k_f + k_{nr}} = \frac{1}{k}$$

The de-excitation rate (k) is the sum of the rates of all possible de-excitation pathways: $k = k_f + k_i + k_x + k_{ET} + \ldots = k_f + k_{nr}$

Nonradiative processes:

- isolated molecules in "gas-phase" only internal conversion (k_i) and intersystem crossing (k_x).
- in condensed phase additional pathways due to interaction with molecular environment: excited state reactions, intermolecular energy transfer (k_{ET}), ...

Quantum yield

The **fluorescence *quantum yield*** (QY_f) :

$$QY_f = \frac{\#\ photon\ emitted}{\#\ photon\ absorbed} = \frac{k_f}{k_f + k_{nr}}$$

- the fraction of excited molecules that return to the ground state S_0 with emission of fluorescence photons
- the ratio of the number of emitted photons (over the whole duration of the decay) to the number of absorbed photons.

Fluorescence **quantum yield** is proportional to fluorescence lifetime:

$$QY = \frac{k_f}{k_f + k_{nr}} = \frac{k_f}{k} = \frac{\tau}{\tau_r} \approx \tau$$

The radiation lifetime $\tau_r = k_f^{-1}$ is practically **constant** for a given molecule.
The fluorescence lifetime $\tau = k^{-1} = (k_f + k_{nr})^{-1}$ **depends** on the environment of the molecule through k_{nr}.

Addition of another radiationless pathway increases k_{nr}, and, those, decreases τ and QY

► **Quenching:** any process which decreases the **fluorescence** intensity of a given substance.
A number of processes can lead to a **reduction in fluorescence intensity** (excited state reactions, energy transfer, complex-formation and collisional quenching.)
Excited molecule returns to the ground state by radiationless transition (without emitting light) as a result of a **collision with quenching molecule** (analyte directly quenches the fluorophore – change in lifetime or intensity).

Common quenchers:
O2, halogens (Br, I), nitrocompounds, acrylamide

► **Effect of temperature**
Generally, an increase in temperature results in a decrease in the fluorescence quantum yield and the lifetime because the non-radiative processes related to thermal agitation (collisions with solvent molecules, intramolecular vibrations and rotations, etc.) are more efficient at higher temperatures.

In general, the presence of heavy atoms as substituents of aromatic molecules (e.g. Br, I) results in **fluorescence quenching** (internal heavy atom effect) because of the increased probability of intersystem crossing.
In fact, intersystem crossing is favored by spin–orbit coupling whose efficiency has a Z^4 dependence (Z is the atomic number).

The heavy atom effect on emissive properties of naphthalene:

	Φ_F	k_{isc}/s^{-1}	Φ_P	τ_T/s
Naphthalene	0.55	1.6×10^6	0.051	2.3
1-Fluoronaphthalene	0.84	5.7×10^5	0.056	1.5
1-Chloronaphthalene	0.058	4.9×10^7	0.30	0.29
1-Bromonaphthalene	0.0016	1.9×10^9	0.27	0.02
1-Iodonaphthalene	<0.0005	$>6 \times 10^9$	0.38	0.002

The heavy atom effect can be small for some aromatic hydrocarbons if:
(i) the **fluorescence quantum yield** is large so that de-excitation by fluorescence emission dominates all other de-excitation processes;
(ii) there is no triplet state energetically close to the singlet state (e.g. perylene).

- Phosphorescence is observed only under certain conditions because the triplet states are very efficiently deactivated by collisions with solvent molecules (or oxygen and impurities) because their lifetime is long.
- These effects can be reduced and may even disappear when the molecules are in a frozen solvent, or in a rigid matrix (e.g. polymer) at room temperature.
- The increase in phosphorescence quantum yield by cooling can reach a factor of 10^3. (this factor is generally no larger than 10 for fluorescence quantum yield)

Quenching

A number of processes can lead to a reduction in fluorescence intensity, i.e., quenching

These processes can occur during the excited state lifetime – for example collisional quenching, energy transfer, charge transfer reactions or photochemistry – or they may occur due to formation of complexes in the ground state

We shall focus our attention on the two quenching processes usually encountered – namely collisional (dynamic) quenching and static (complex formation) quenching

Collisional Quenching

Collisional quenching occurs when the excited fluorophore experiences contact with an atom or molecule that can facilitate **non-radiative transitions** to the ground state. Common quenchers include O_2, I^-, Cs^+ and acrylamide.

$$F + h\nu \longrightarrow F^*$$
$$F^* \longrightarrow F + h\nu$$
$$F^* + Q \longrightarrow F + Q^*$$

Here, the interaction results in the dissipation of excitation energy by a non radiative energy transfer from F^* to Q without or, less fluorescence.

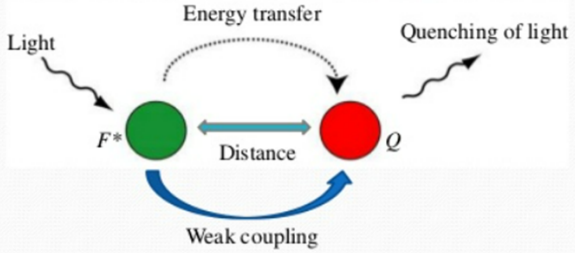


Fig : Simple mechanism of collisional quenching

Halides ions such as chlorides or, iodides are well known collisional quenchers. For example, quenching of quinine drug by chloride ion or, quenching of tryptophan by iodide ion follow collisional quenching process.

Static Quenching

In some cases, the fluorophore can form a stable complex with another molecule. If this *ground-state* is non-fluorescent then we say that the fluorophore has been statically quenched.

$$F + Q \longrightarrow F:Q$$
$$F:Q + h\nu \longrightarrow Q^* + F$$
$$Q^* \longrightarrow Q + \text{energy}$$

Here, a complex formation occurs between the fluorescing molecule at the ground state (F) and the quencher molecule (Q) through a strong coupling. Such complex may not undergo excitation or, may be excited to a little extent reducing the fluorescence intensity of the molecule.

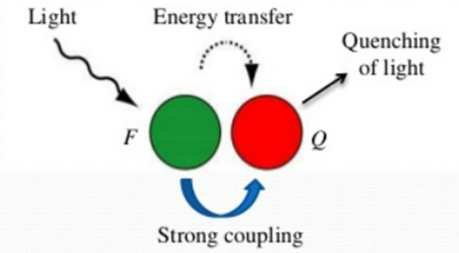


Fig: Static quenching

Caffeine and related xanthines and purines reduce intensity of riboflavin by static mechanism. Quenching that occurs due to oxygen also follows this mechanism.

In such a case, the dependence of the fluorescence as a function of the quencher concentration follows the relation:

$$F_0/F = 1 + K_a[Q]$$

where K_a is the association constant of the complex. Such cases of quenching via complex formation were first described by Gregorio Weber.

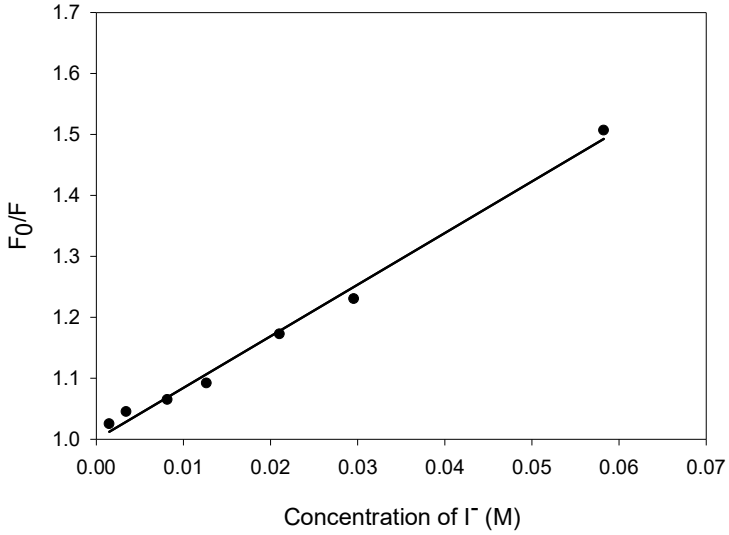
In the simplest case of collisional quenching, the following relation, called the **Stern-Volmer equation**, holds:

$$F_0/F = 1 + K_{SV}[Q]$$

where F_0 and F are the fluorescence intensities observed in the absence and presence, respectively, of quencher, [Q] is the quencher concentration and K_{SV} is the **Stern-Volmer quenching constant.**
In case of purely dynamic quenching, K_{SV} is written as K_D.

In the simplest case, then, a plot of F_0/F versus [Q] should yield a straight line with a slope equal to K_{SV}.

Such a plot, known as a Stern-Volmer plot, is shown below for the case of fluorescein quenched by iodide ion (I^-).



In this case, $K_D \sim 8$ L-mol^{-1}

$K_D = k_q \tau_0$
where **k_q is the bimolecular quenching rate constant** (proportional to the sum of the diffusion coefficients for fluorophore and quencher) and **τ_0 is the excited state lifetime** in the absence of quencher.

In the case of purely collisional quenching, also known as ***dynamic*** quenching,:

$$\mathbf{F_0/F = \tau_0/\tau.}$$

Hence in this case: $\mathbf{\tau_0/\tau = 1 + k_q \tau[Q]}$
In the fluorescein/iodide system, τ = 4ns and $k_q \sim 2 \times 10^9$ M^{-1} sec^{-1}

For static quenching the dependence of the fluorescence intensity upon quencher concentration is easily derived by consideration of the association constant for complex formation. This constant is given by

$$K_S = \frac{[F-Q]}{[F][Q]} \qquad (1)$$

where $[F - Q]$ is the concentration of the complex, $[F]$ is the concentration of uncomplexed fluorophore, and $[Q]$ is the concentration of quencher

total concentration of fluorophore $[F]_0$ is given by

$$[F]_0 = [F] + [F - Q] \qquad (2)$$

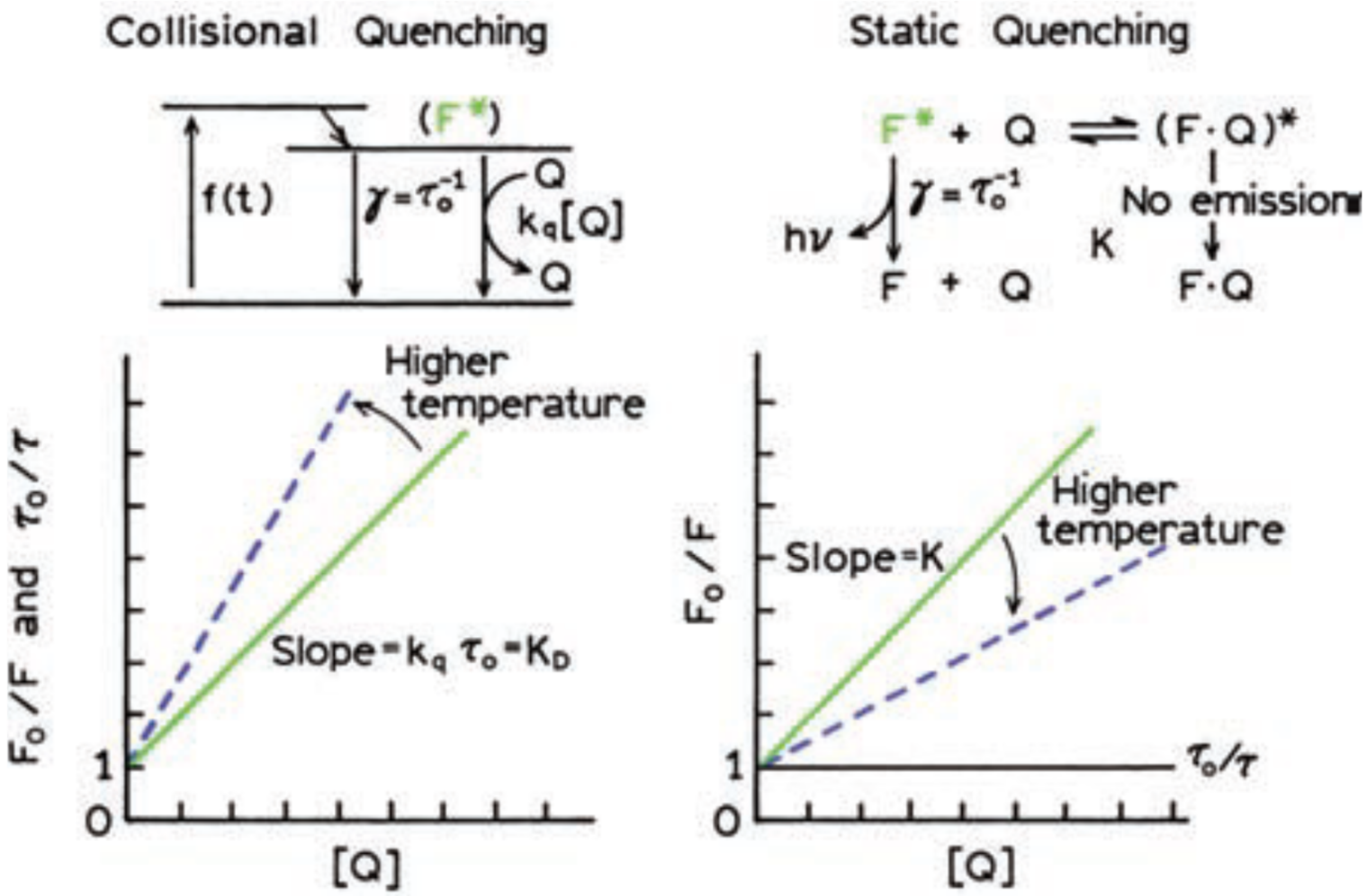
Substitution of eq 2 into eq. 1 yields

$$K_S = \frac{[F]_0 - [F]}{[F][Q]} = \frac{[F]_0}{[F][Q]} - \frac{1}{[Q]} \qquad (3)$$

Rearranging eq. 3 and replacing concentrations by fluorescence intensities, we get

$$\frac{F_0}{F} = 1 + K_S\,[Q]$$

For static quenching, $\tau_0/\tau = 1$


Collisional Quenching
(F*)
f(t)
γ = τ₀⁻¹
Q
k_q[Q]
Q
Static Quenching
F* + Q ⇌ (F·Q)*
hν
γ = τ₀⁻¹
K
No emission
F + Q
F·Q
Higher temperature
Slope = k_q τ₀ = K_D
F₀/F and τ₀/τ
1
0
[Q]
Higher temperature
Slope = K
F₀/F
τ₀/τ
1
0
[Q]

Concentration quenching

Concentration quenching is a kind of self quenching.

It occurs when the concentration of the fluorescing molecule increases in a sample solution. The fluorescence intensity is reduced in highly concentrated solution (>50 µg/ml).

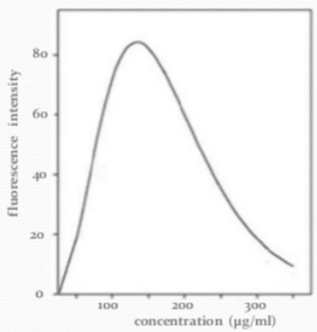


Fig: Influence of concentration on the fluorescence of phenol solutions.

Chemical quenching

Chemical quenching is due to various factors like change in pH, presence of oxygen, halides and electron withdrawing groups, heavy metals etc.

- **Change in pH :** Aniline at pH (5-13) gives fluorescence when excited at 290 nm. But pH <5 or, pH >13 it does not show any fluorescence.
- **Oxygen :** Oxygen leads to the oxidation of fluorescent substance to non fluorescent substance and thus, causes quenching.
- **Halides and electron withdrawing groups :** Halides like chloride ions, iodide ions and electron withdrawing groups like $-NO_2$, -COOH , -CHO groups lead to quenching.

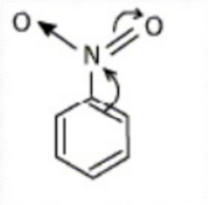
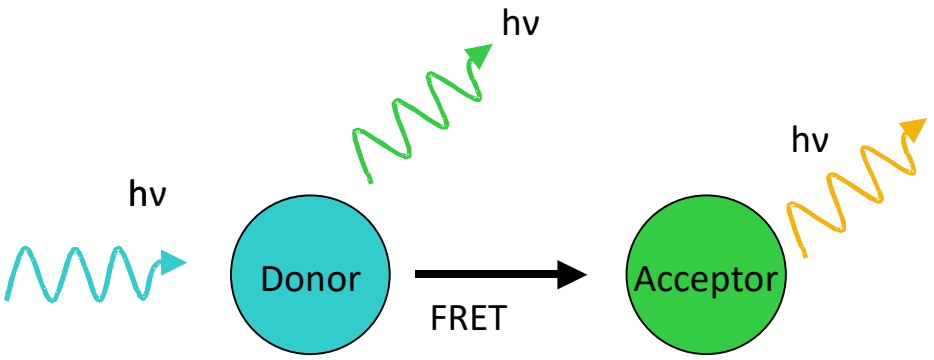


Fig: electron withdrawal process by nitro group

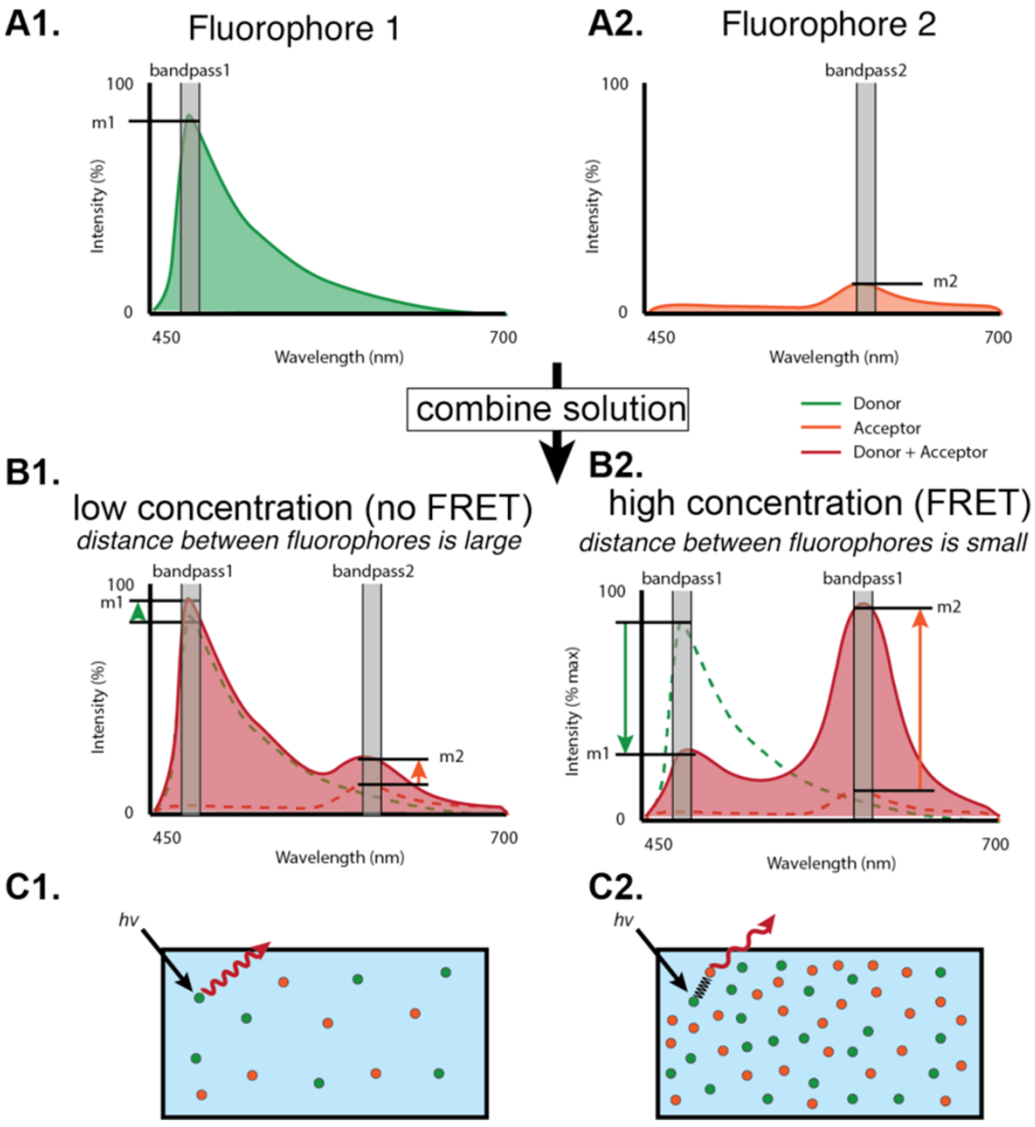
- **Heavy metals :** presence of heavy metals also lead to quenching because of collision and complex formation.

Fluorescence Resonance Energy Transfer (FRET)
(or **Förster Resonance Energy Transfer)**

What is FRET ?



When the donor molecule absorbs a photon, and there is an acceptor molecule close to the donor molecule, radiation-less energy transfer can occur from the donor to the acceptor.
FRET results in a decrease of the fluorescence intensity and lifetime of the donor probe, It enhance the fluorescence of the acceptor probe when the acceptor is fluorescent.



Fluorometer

The fluorescence spectrometers provide a tool for analyzing the spectral distribution of the emitted light from the sample. **Generally, the fluorescence spectrometers use double-beam optics to compensate the inherent power fluctuations in the source**. The technique includes measurement of the fluorescent emission at a right angle to the incident beam. The emitted radiation passes through a second filter or monochromator to isolate the fluorescent peak for measurement.

The optical paths of the excitation and detection of light are along the orthogonal axis. The orthogonal arrangement ensures minimal leakage of excitation light into the detection side to be captured by the high sensitivity photodetectors such as photomultipliers or charge-coupled device cameras.

The light from an excitation source passes through a filter or monochromator, and strikes the sample. A proportion of the incident light is absorbed by the sample, and some of the molecules in the sample fluoresce. **The fluorescent light is emitted in all directions.** Some of this fluorescent light passes through a second filter or monochromator and reaches **a detector, which is usually placed at 90° to the incident light beam to minimize the risk of transmitted or reflected incident light reaching the detector**.

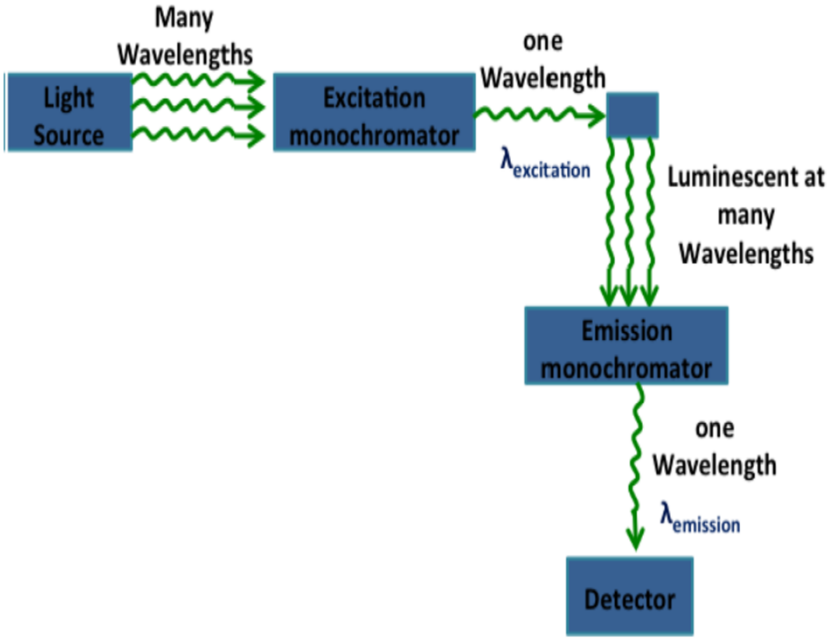


Figure: Schematic diagram of spectrofluorometer

Spectrofluorometer: Most spectrofluorometers record both the excitation and emission spectra. Resolution is obtained with changeable fixed slits. The advantage of spectrofluorometers is that they provide variations in the wavelength selection and allow the operator to scan over a range of wavelengths. The disadvantage of spectrofluorometer includes, it is several times costly compared to the filter fluorometer and can only provide moderate sensitivity and specificity. For research purposes, the spectrofluorometer with optimal sensitivity and specificity, and monochromators with continuous variable slits and a broad wavelength range (200 - 1000 nm), have been preferred. Such instruments have dual excitation or dual emission monochromators for increasing the sensitivity and reducing stray light.

Fluorometer Instrument

The typical fluorometer instruments include three basic items: *source of light*, *specimen chamber* with integrated optical components and *sample holder,* and high sensitivity *detector.*

4.1 Light Source: The first component of the fluorescence spectroscopy is light source. It is used to excite the molecule to higher excited state from which they lose their energy in the form of fluorescence. Generally, the source must be more intense than that required for UV-Visible absorption spectroscopy; magnitude of the emitted radiation is directly proportional to the power of the source.

A number of light sources can be used to excite the molecule:

4.1.1 Incandescent lamp: Incandescent lamps have the advantages that they are low cost, easy to use, long lasting and air cooled. Their disadvantage is that they are not very intense in their light output, not easily focused, and not easily pulsed for short time temporal measurements.

4.1.2 Arc lamps: Arc lamps have much more intense output but are less stable, costly, and cumbersome to use and often require water cooling.

4.1.3 Laser: laser sources are capable of generating monochromatic light with extremely high intensities and are sometimes used as excitation sources. Laser can either be continuous wave type that could emit relatively steady output intensity or pulsed, where the light output is discretely emitted with times. The main disadvantages of laser source are that of their high cost and limited availability of wavelength. Since the radiation produced is monochromatic, there is no need for an excitation monochromator.

Filter fluorometers often employ a low-pressure mercury vapour lamp. This source produces intense lines at certain wavelengths. One of these lines could be suitably utilized for excitation onto a fluorescent sample. The most common light sources for fluorometers are lamp sources, such as xenon arc lamps. These lamps provide a relatively uniform intensity over a broad spectral range from the ultraviolet to the near infrared. Spectrofluorometers need a continuous radiation source, are often equipped with a 75-450 Watt high pressure xenon arc lamp.

4.1.4 Mercury Vapor: Mercury vapor produces ultraviolet radiation when current flows through them. This type of source can be used for the materials that can produces fluorescence through ultraviolet light.

4.1.5 Photodiodes: **PIN (p-i-n)** photodiodes can also be used as a low-power light source for fluorescence but are having less sensitive.

Different light sources can be used because they provide different irradiation for fluorescence properties because of different wavelength of radiated energies. Such as laser can provide light of wavelength under 0.01 nm that provides an immense amount of energy for fluorescence. There is a big challenge to control the wavelength provided by laser. In the other way, the wavelength provided by the other sources such as lamps, mercury vapor and xenon has a range of 200-800+ nanometer and serves different functions for fluorescence spectroscopy.

4.2 Dispersive Elements: To make fluorescence spectroscopy as a most useful analyzing technique, it is necessary to separate their constituent elements for the excitation energy from the source and emitted fluorescence from the sample. Fluorometers use either interference or absorption filters. Spectrofluorometers are usually fitted with grating monochromators.

There are two types of dispersive elements: ***Filters*** *and* ***Monochromators***

Monochromators are used to disperse white light consisting of various colors or wavelength. This dispersion can be achieved with the help of prism or diffraction gratings. The diffraction grating makes light that enters the grating change the angle depending on the wavelength. This change makes it possible to get the necessary wavelength with the proper adjustment.

Usage of the multiple types of filters or monochromators depends on the type of fluorescence spectroscopy. The primary filters that excite the sample provide the appropriate wavelength necessary, while the secondary filters monochromate the emitted light when sent to the detector.

4.3 Detectors: Fluorescence signals are usually of low intensity, and photomultiplier tubes and diode-array detectors are the commonly used detectors for this purpose. The fluorescence spectroscopy measures optical signals using two types of detectors, single and multiple channel detectors. Single channel detector only detects a single wavelength at a time from the sample and multiple channel detectors can detect multiple wavelengths at a specific time.

4.4 Read-out devices: The output after getting from a detector is amplified or displayed on a read out device. The read out devices may be a meter or digital display.

4.5 Sample holder: To hold the sample of interest the sample cell or cuvette is used with an precaution that the material on the sample cuvette must allow the excitation or the emission light to pass through it.