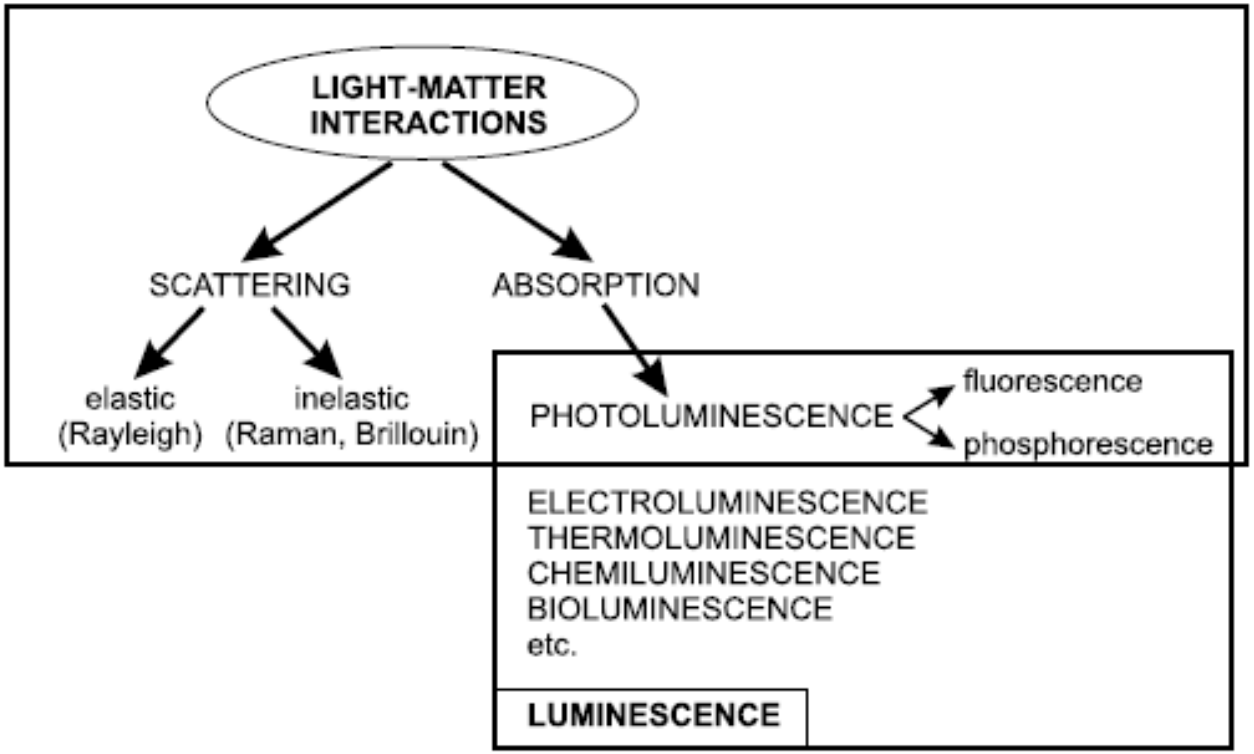
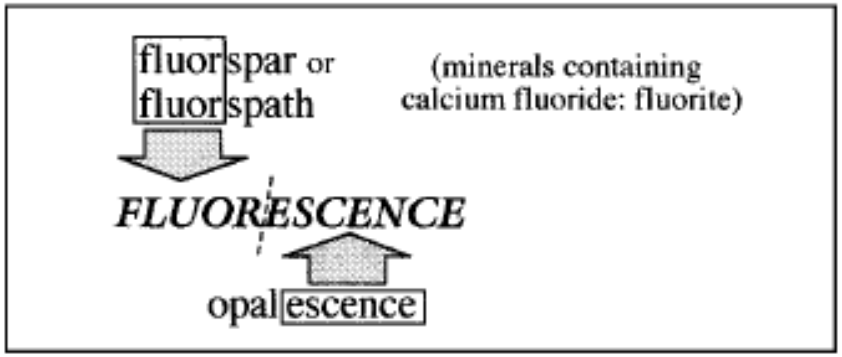
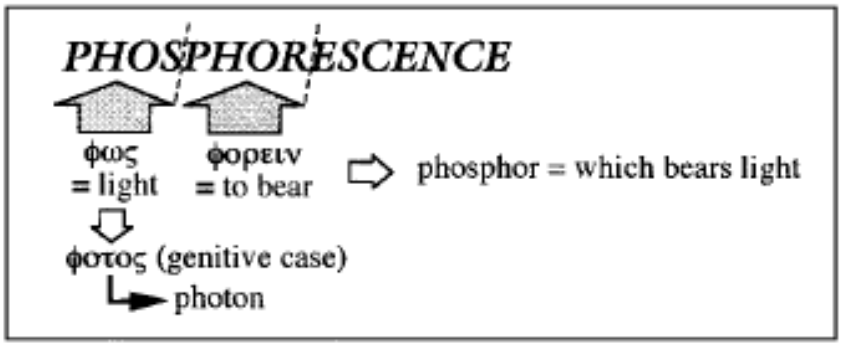
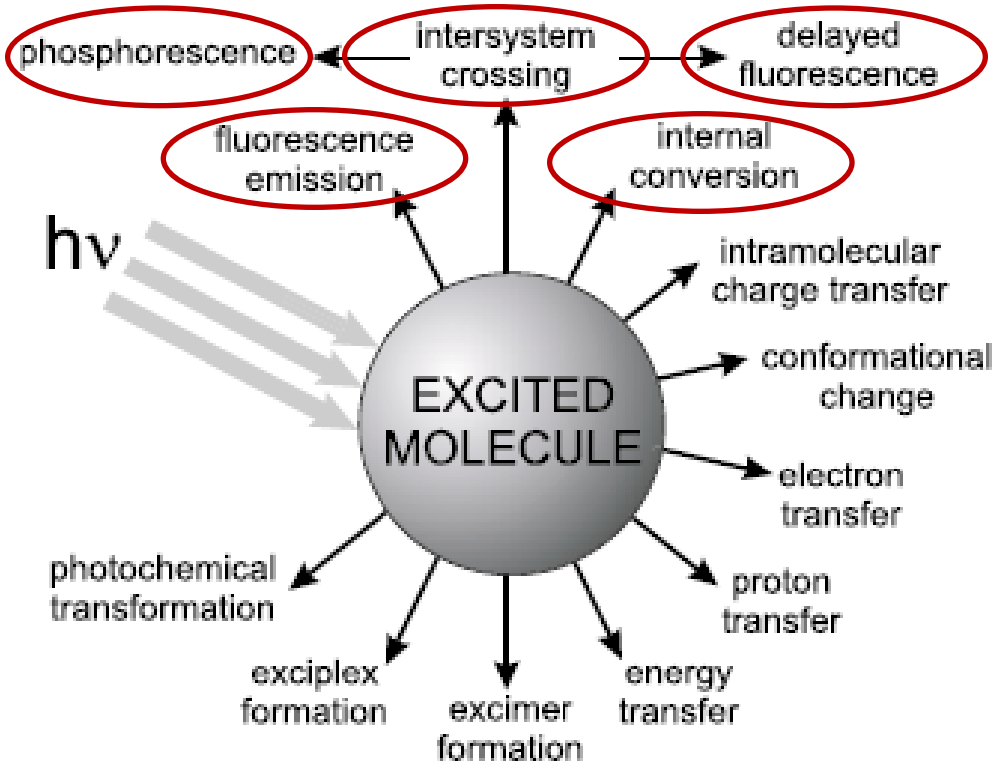
Fluorescence and phosphorescence

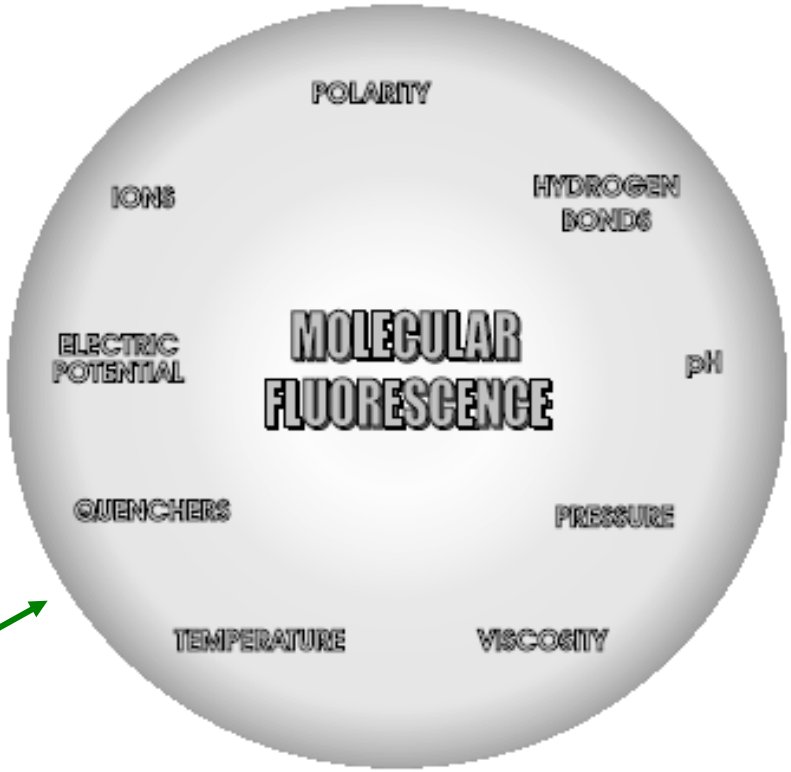






Possible de-excitation pathways of excited molecules.





Various parameters influence the emission of fluorescence.

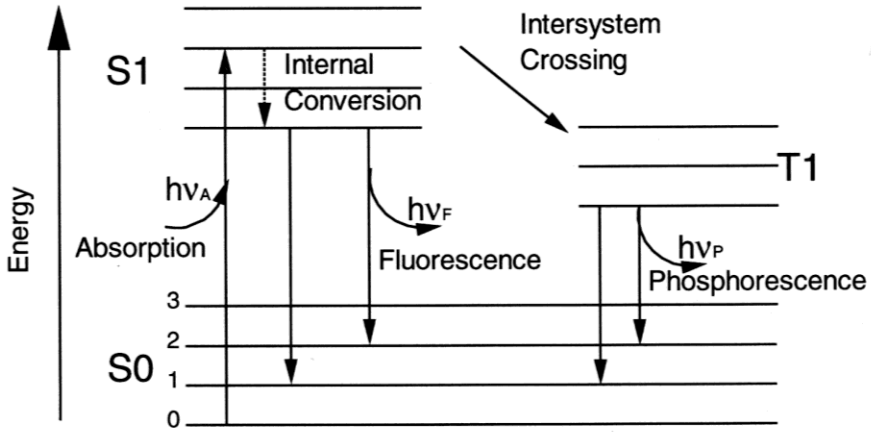
Luminescence: emission of photons from electronically excited states of atoms, molecules and ions.

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).

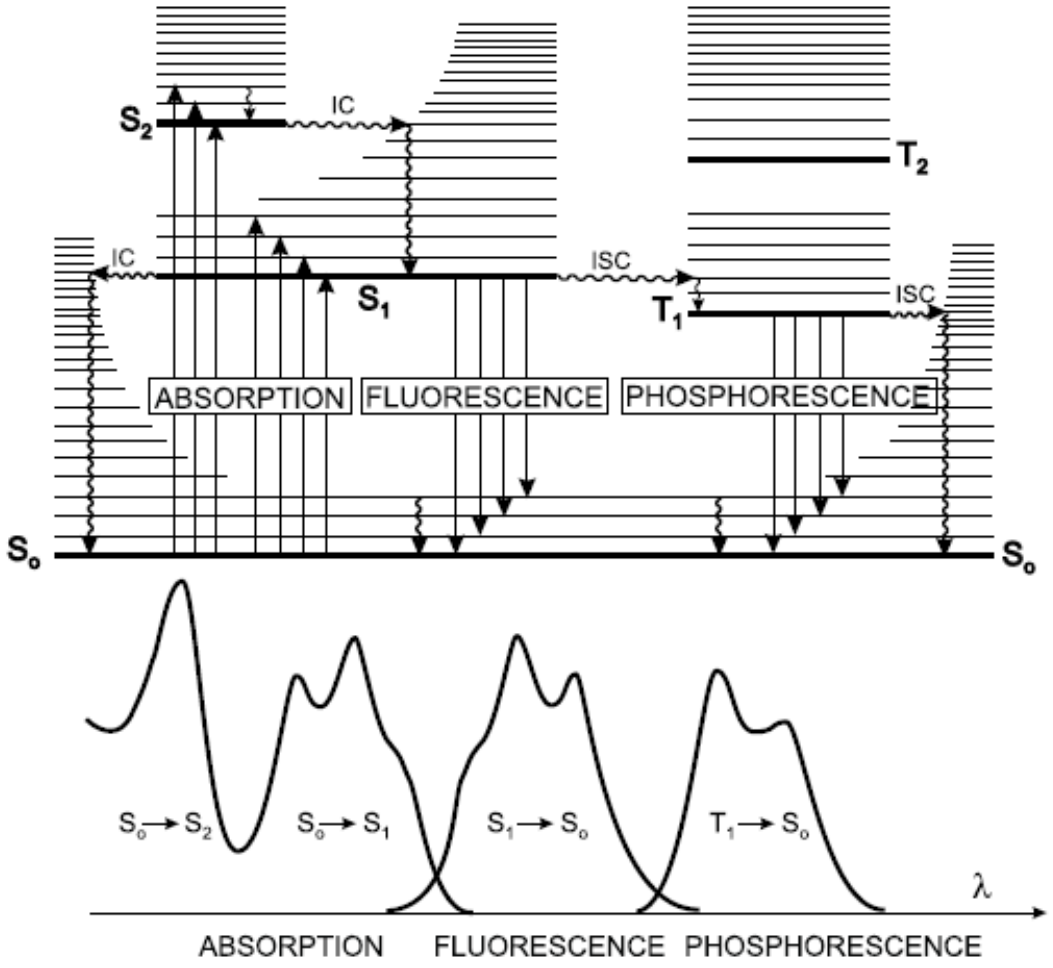


S_0: fundamental electronic state
S_0, S_1, S_2: singlet electronic states
T_1, T_2: triplet electronic states

Possible processes:

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

The Perrin–Jablonski diagram



Caracteristic times:

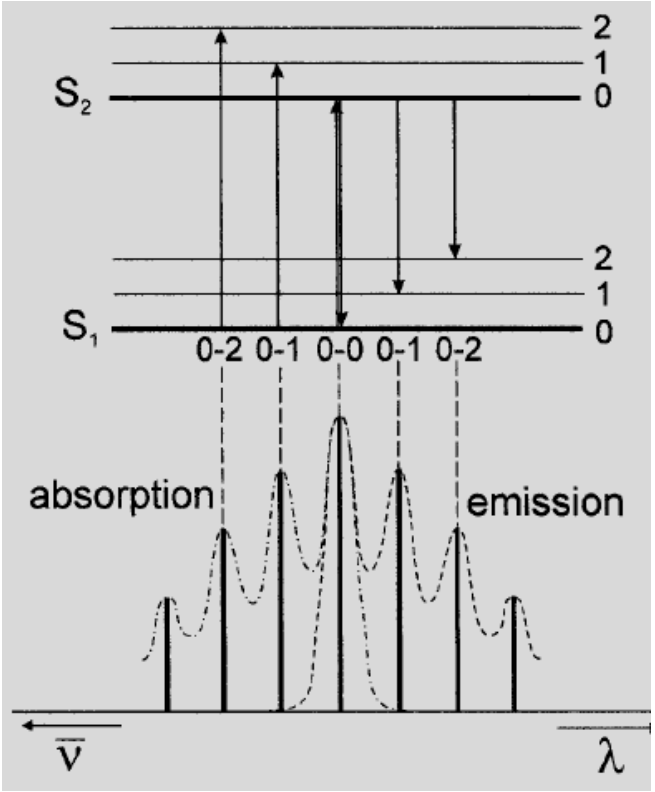
absorption: 10^{-15} s
vibrational relaxation: 10^{-12}-10^{-10} s
internal conversion: 10^{-11}-10^{-9} s
intersystem crossing: 10^{-10}-10^{-8} s
lifetime of the excited states: 10^{-10}-10^{-7} s (fluorescence)
lifetime of the excited states:10^{-6} s (phosphorescence)

The vertical arrows corresponding to absorption start from the 0 (lowest) vibrational energy level of S_0 because the majority of molecules are in this level at room temperature.

Absorption of a photon can bring a molecule to one of the vibrational levels of S_1; S_2; . . . :

For **some aromatic hydrocarbons** (naphthalene, anthracene and perylene) the absorption and **fluorescence spectra exhibit vibrational bands**. The energy spacing between the vibrational levels are *almost similar* in S_0 and S_1 so that the emission spectrum often appears to be symmetrical to the absorption spectrum (mirror image rule).

However, most fluorescent molecules exhibit broad and structure less absorption and emission bands, which means that each electronic state consists of an almost continuous manifold of vibrational levels.



In some cases *the absorption spectrum can partially overlap the fluorescence spectrum:* in this case, the excitation occurs from a vibrational excited level of the S_0 state (the energy difference between the 0 and 1 vibrational levels of S_0 is low (~ 500 cm^{-1}))

The energy gap between S_0 and S_1 is much larger than between the vibrational levels, so the probability of finding a molecule in S_1 at room temperature as a result of thermal energy is nearly zero.

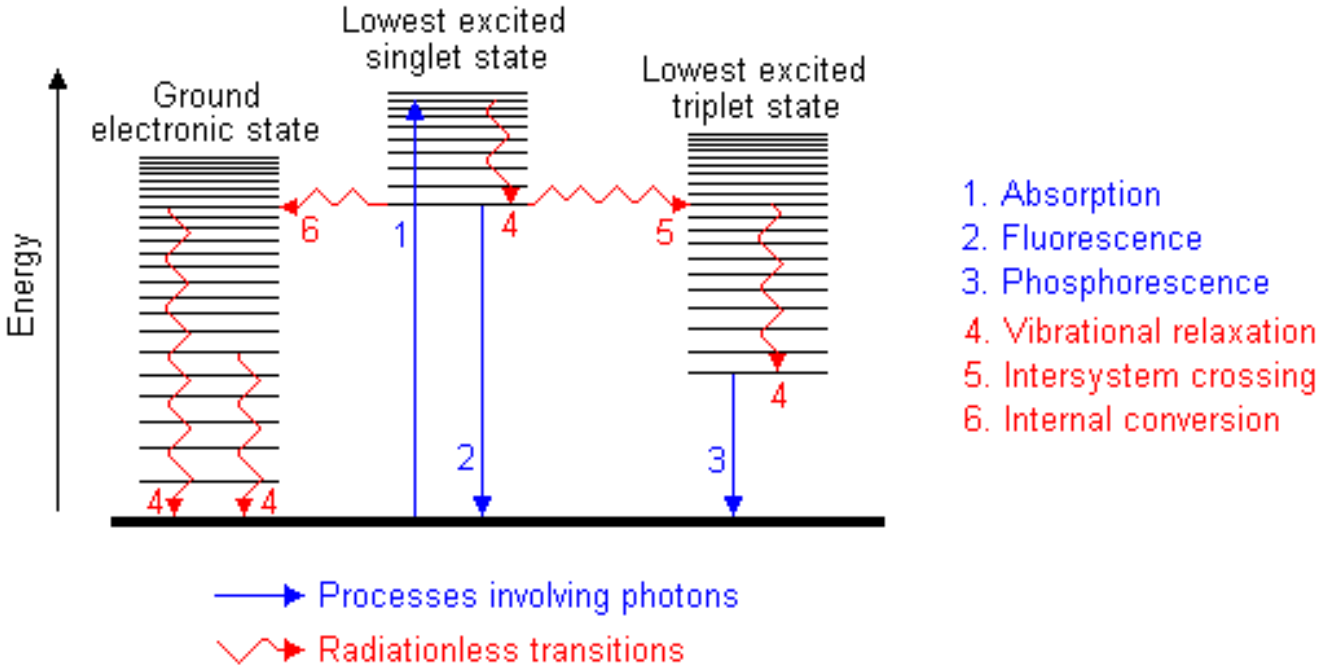
($E_{S1} - E_{S0} \sim 4 \cdot 10^{-19}$ J, compared with $k \cdot T \sim 4\ 10^{-21}$ J)

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

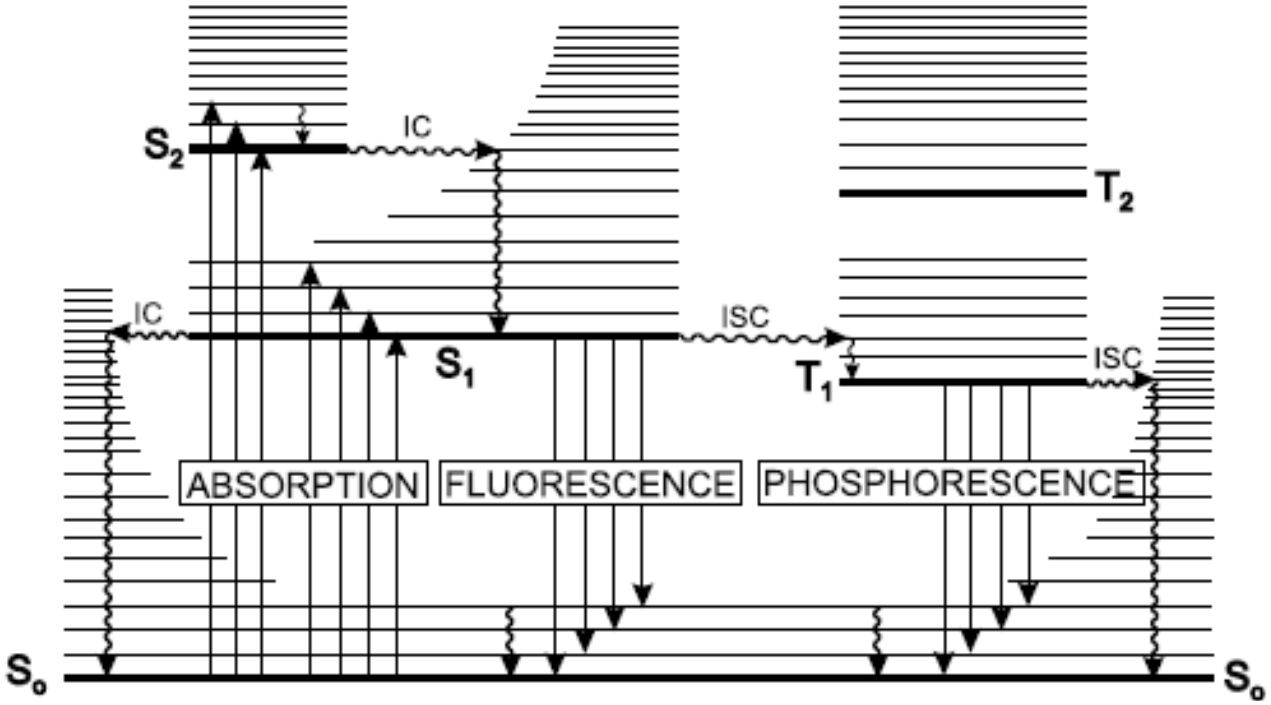


Internal conversion *is a non-radiative and isoenergetic transition between two electronic states of the same spin multiplicity*.

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.

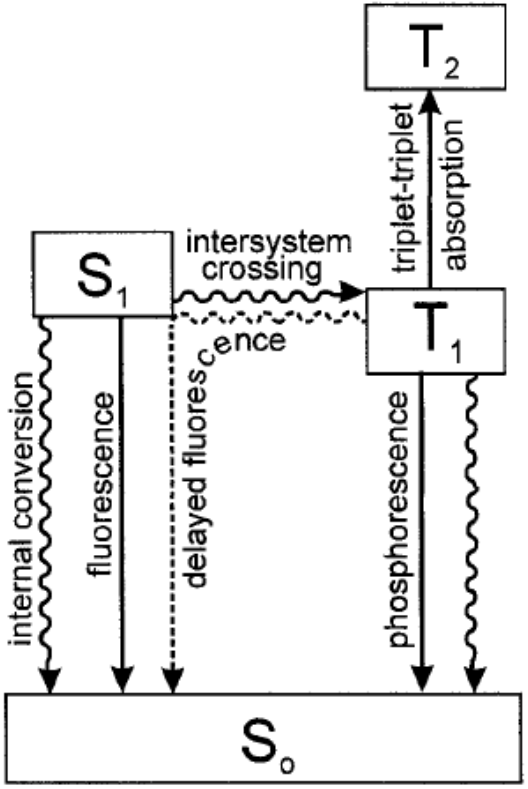
Internal conversion occurs in the *same time frame as vibrational relaxation*, therefore, is a very likely way for molecules to dissipate energy from light perturbation.



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 molecule (phosphorus) 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.

The presence of heavy atoms increases spin–orbit coupling and thus favors intersystem crossing.

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).

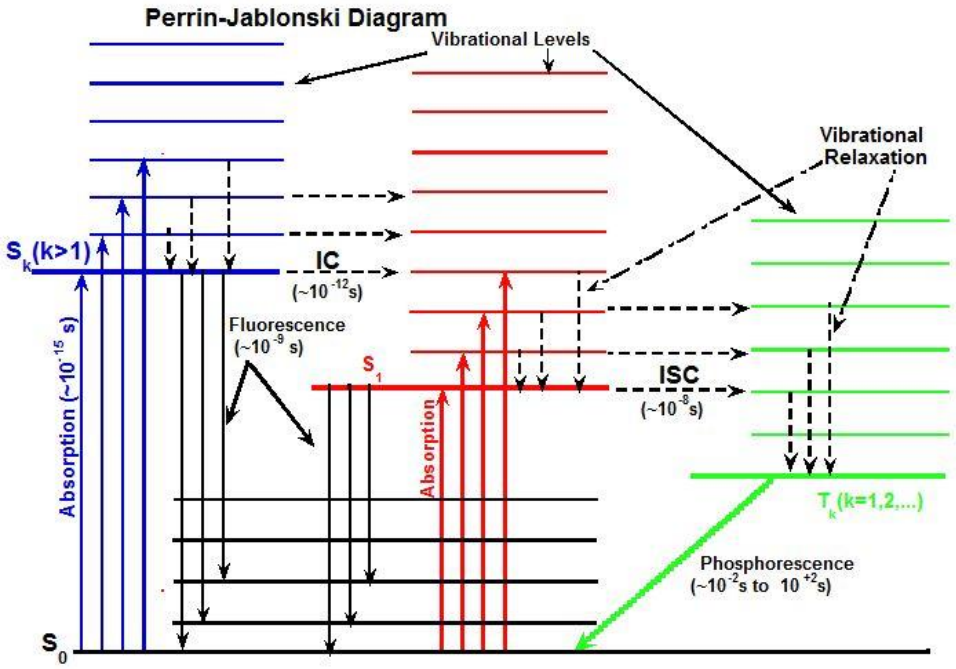
The transition $T_1 \rightarrow S_0$ is forbidden (but it can be observed because of spin–orbit coupling), the radiative rate constant is very low.

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**, 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.

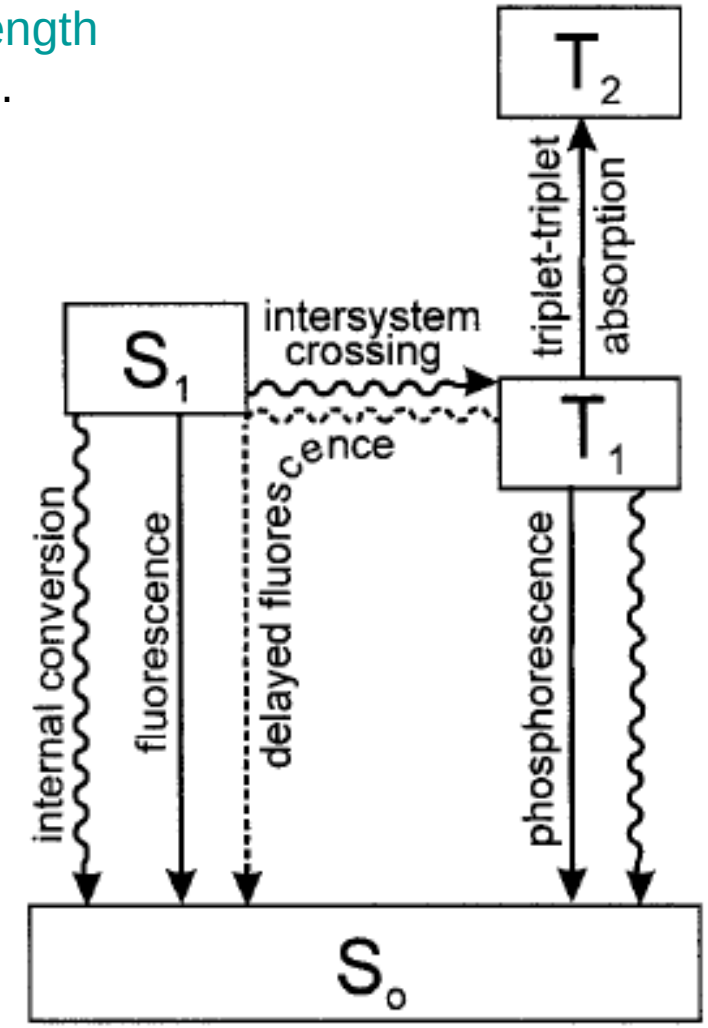
The phosphorescence spectrum is located at wavelengths higher than the fluorescence spectrum because the energy of the lowest vibrational level of the triplet state T_1 is lower than that of the singlet state S_1.



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.



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

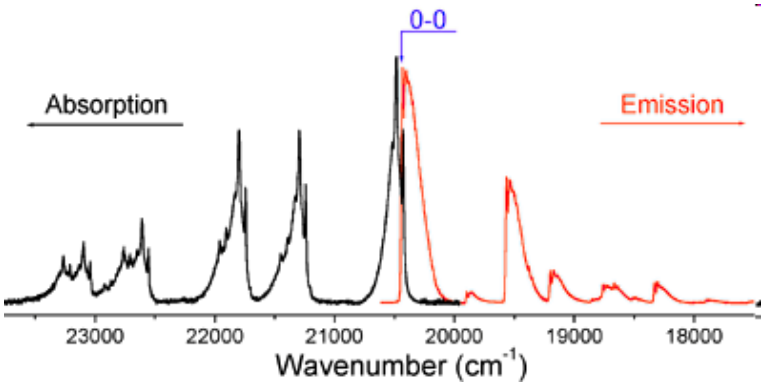
Fluorescence emission occurs from S_1 and therefore its characteristics (except polarization) do not depend on the excitation wavelength.

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.

(In most cases, the absorption spectrum partly overlaps the fluorescence spectrum, i.e. a fraction of light is emitted at shorter wavelengths than the absorbed light because at room temperature, a small fraction of molecules is in a vibrational level higher than level 0 in the ground state as well as in the excited state. At low temperature, this departure from the Stokes Law should disappear)



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.

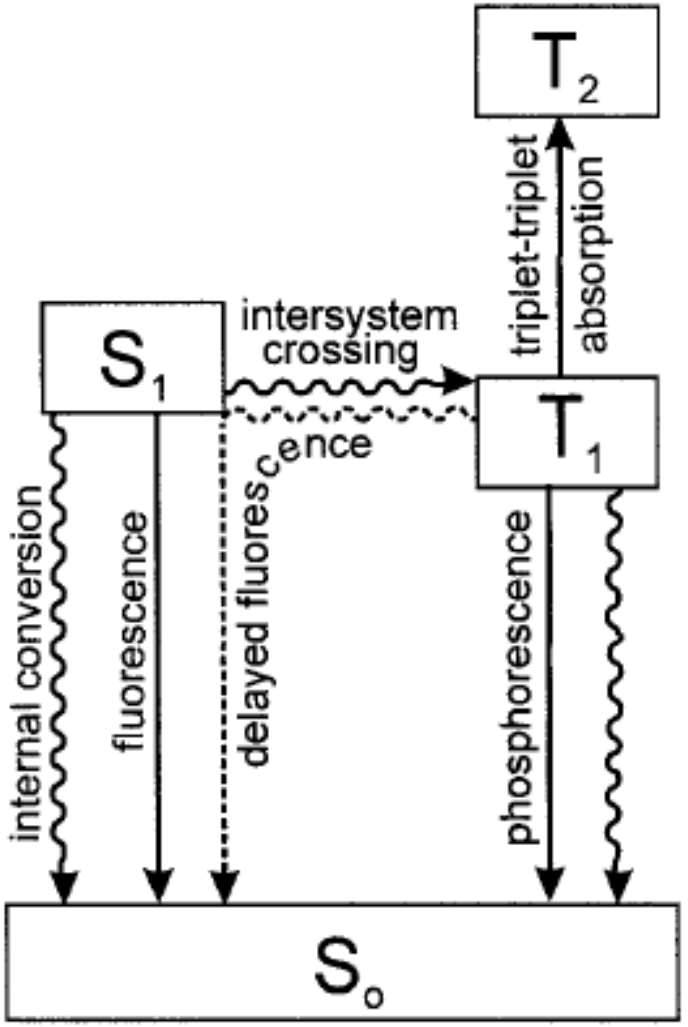
Results an emission with the same spectral distribution as normal fluorescence but with a much longer decay time constant because the molecules stay in the triplet state before emitting from S_1.

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.

Fluorescence features

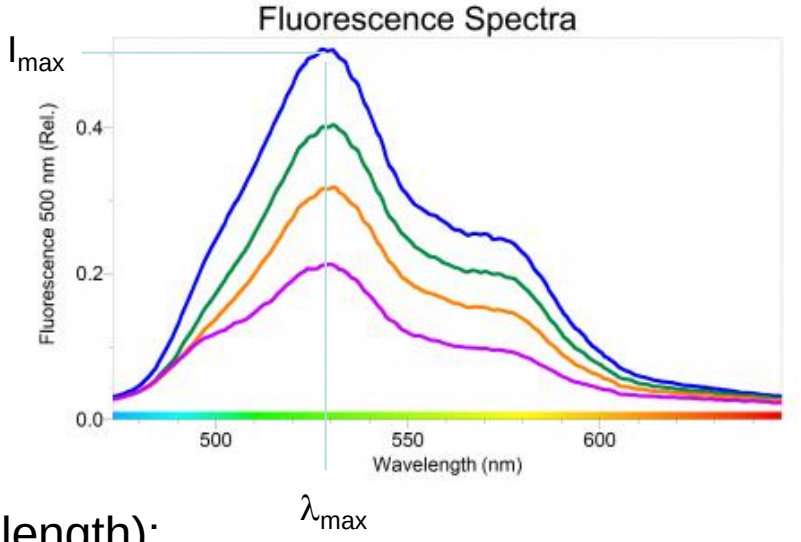
Fluorescence:

- involves the emission of light.
- is the result of the absorption of light.
- occurs after a finite duration subsequent to the absorption
- an outside source of energy is required.

The emission of fluorescence photons is a ***spontaneous process***.
Under certain conditions, *the* *stimulated fluorescence emission* can occur!

The **fluorescence emission** can be describe using several **parameters**:

- rate constant,
- life time,
- quantum yield,
- intensity,
- wavelength of maximum intensity



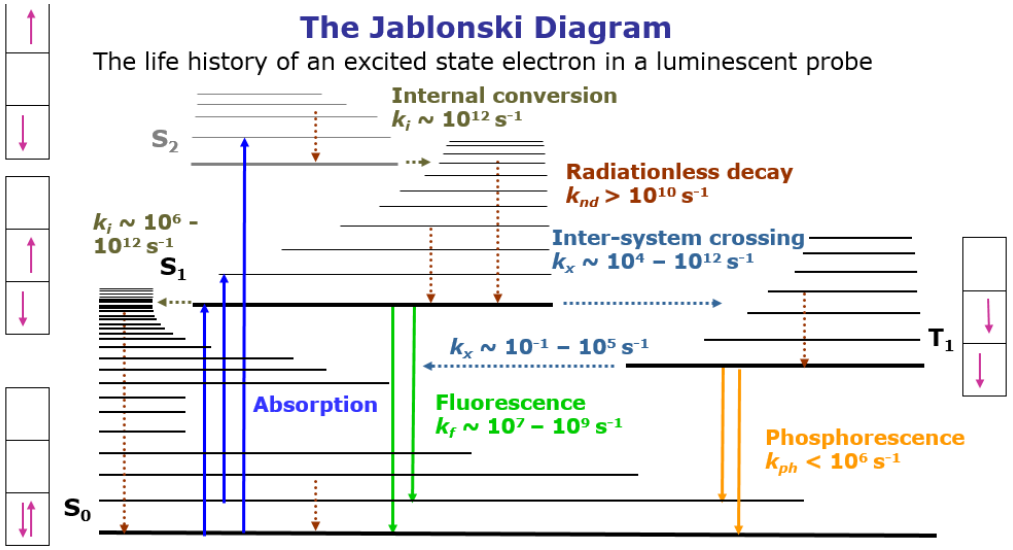
The fluorescence spectrum (intensity versus wavelength):

λ_{max} = wavelength corresponding to maximum fluorescence intensity is achieved

I_{max} = the intensity of fluorescent peak

De-excitation rate constants

After excitation of a molecule population by a very short pulse of light, the deexcitation 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

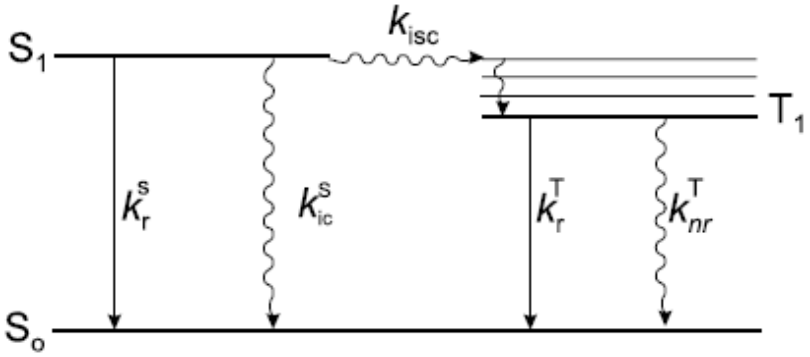
The ***rate constants*** for the various processes are denoted as follows:

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 ($T_1 \rightarrow S_0$)

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

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 **fluorescence lifetime (τ_s)** (fluorescence decay time) defines the time window of observation of dynamic phenomena.

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

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

The behavior of an excited population of fluorophores is described by the rate equation:

$$\frac{dn^*(t)}{dt} = -k \cdot n^*(t) + f(t)$$

where n* is the number of excited molecule at a time t, k is the rate constant of all deexcitation processes $k = k_f + k_{nr}$ and f(t) is an arbitrary function of the time, describing the time course of the excitation. ([k] = s^{-1} (transitions per molecule per unit time)

If excitation is switches off at t = 0, the last equation describes the decrease in excited molecule at all further times, and takes the form:

$$\frac{dn^*(t)}{dt} = -k \cdot n^*(t)$$

Integration gives: $n^*(t) = n^*(0) \cdot e^{-kt}$

The fluorescence **lifetime** τ is equal to k^{-1}

If a population of fluorophores are excited, the **lifetime** (τ) is the time it takes for **the number of excited molecules to decay to 1/e** (36.8%) of the original population:

$$\frac{n^*(t)}{n^*(0)} = e^{-t/\tau}$$

The ***fluorescence intensity*** (I_f) is defined as **the amount of photons emitted** per unit time and per unit volume of solution.

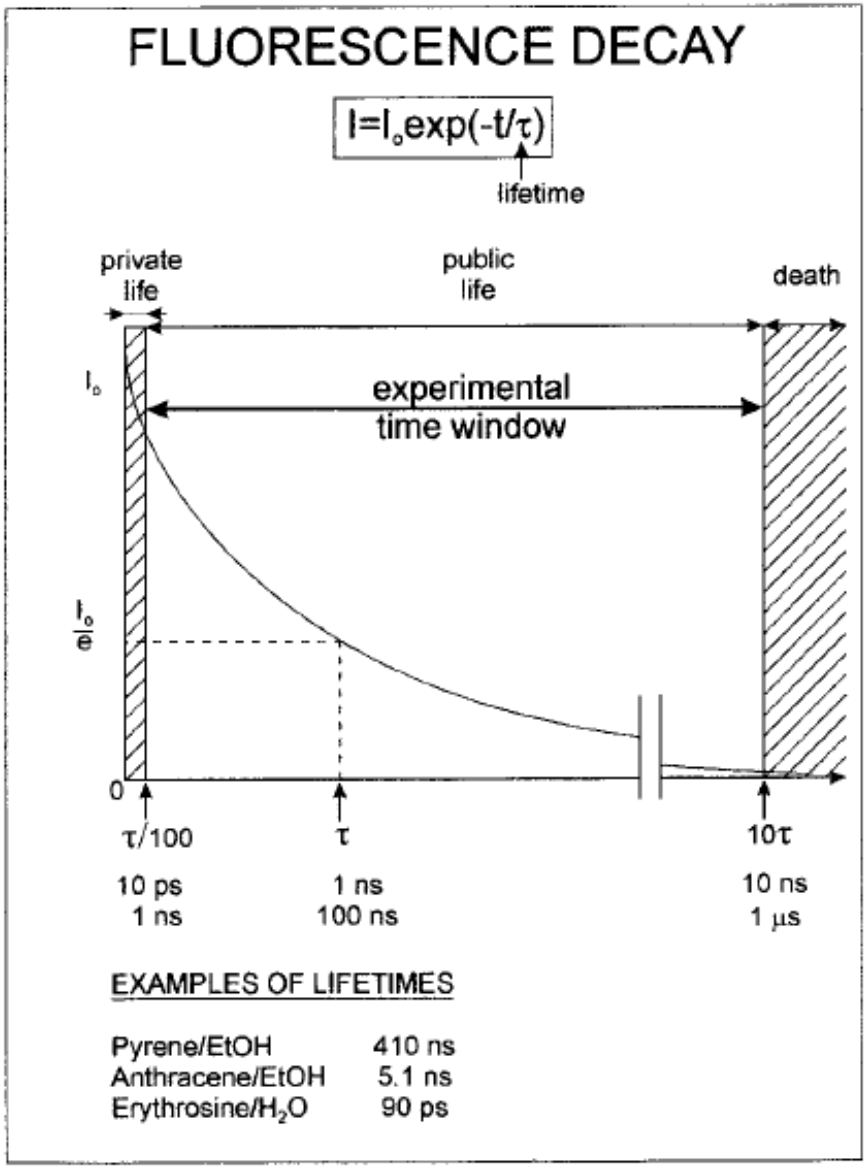
The fluorescence intensity $i_f(t)$ at time t after excitation (by a very short pulse of light at time 0) is proportional (at any time) to the instantaneous concentration of molecules still excited n*(t).

$$i_f(t) = k \cdot n^*(t)$$

The proportionality factor is related the rate of all deexcitation processes ($k = k^S_r + k^S_{nr}$):

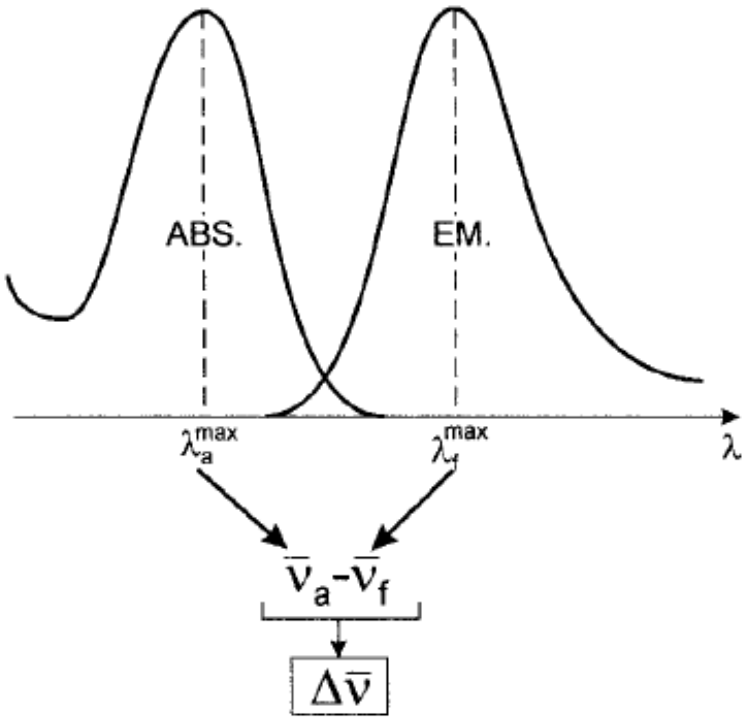
$$I_f(t) = I(0) \cdot e^{-t/\tau}$$

The lifetime τ is the time needed for the concentration of molecular entities to decrease to 1/e of its original value.

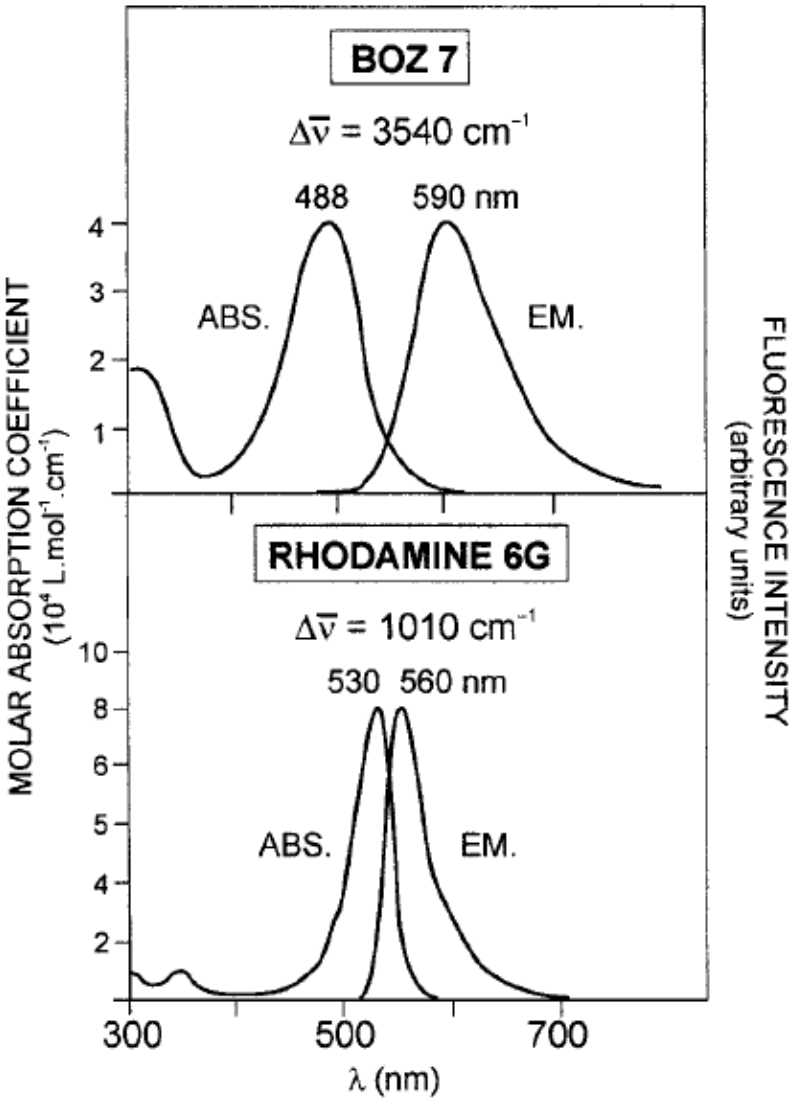


The **fluorescence emission decreases exponentially** with a **characteristic time,** reflecting the average lifetime of the molecules in the S_1 excited state (excited-state lifetime).

The ***Stokes shift*** ($\Delta\nu$): the distance (*in wavenumbers*) between the maximum of the first absorption band and the maximum of fluorescence.



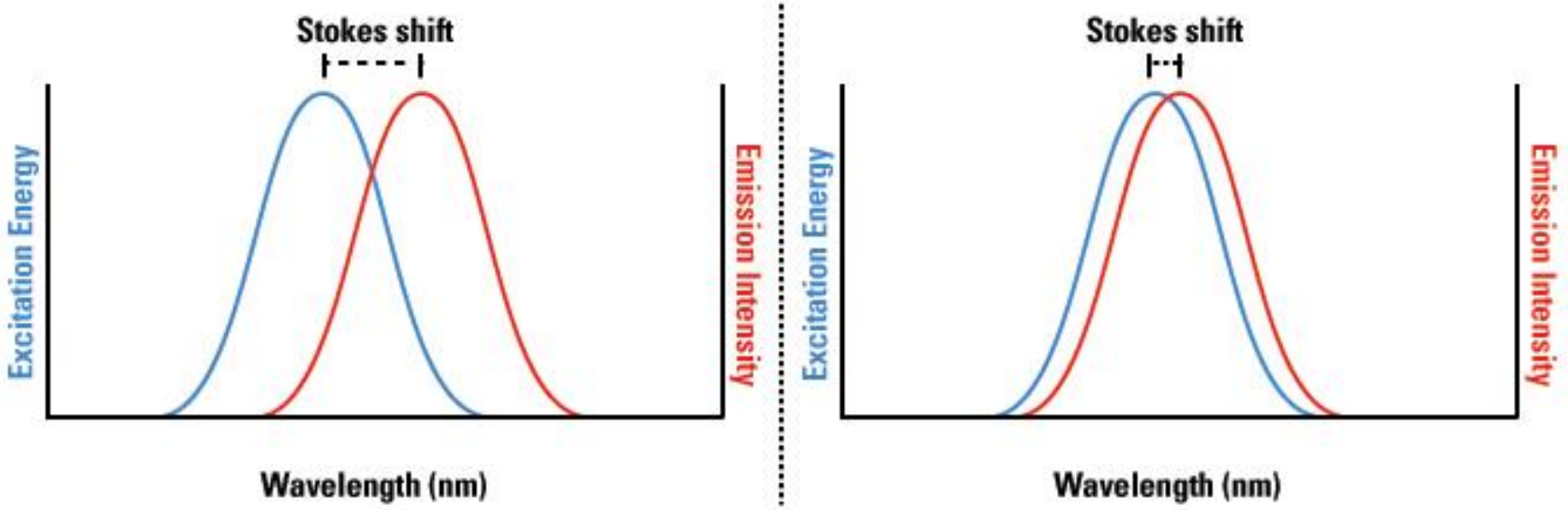
benzoxazinone derivative BOZ7



The Stokes Shift
- is a key aspect in the detection of the emitted fluorescence in biological applications.
- is a distinct characteristic of each fluorophore.

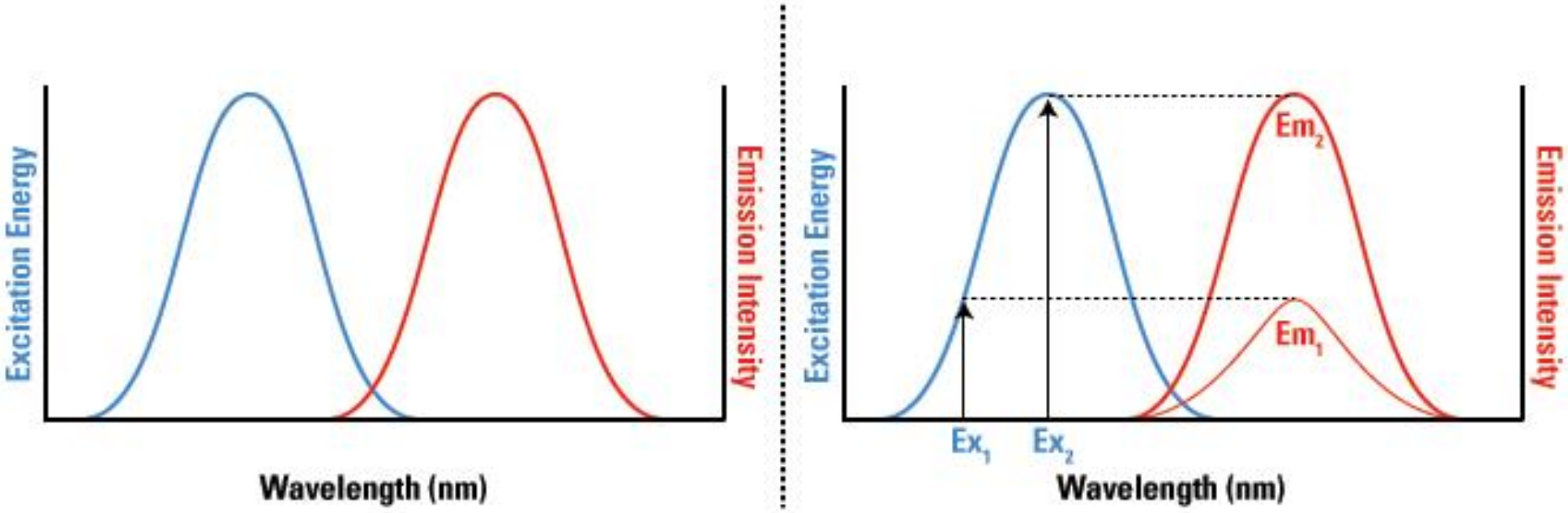
The detection of emitted fluorescence can be difficult to distinguish from the excitation light when using fluorophores with very small Stokes shifts, because the excitation and emission wavelengths greatly overlap.

Fluorophores with large Stokes shifts are easy to distinguish because of the large separation between the excitation and emission wavelengths.



The Stokes shift is especially critical in multiplex fluorescence applications, because the emission wavelength of one fluorophore may overlap, and therefore excite, another fluorphore in the same sample.

The fluorescence emission **wavelength is independent of the excitation wavelength** due to partial loss of energy prior to emission,



The fluorescence emission **intensity is proportional to the amplitude of the excitation wavelength**.

The intensity of the emitted light (Em_1, Em_2) is directly proportional to the energy required to excite a fluorophore, at any excitation wavelength (Ex_1 , Ex_2).

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

The measurement of fluorescence lifetime is more robust than measurement of fluorescence intensity (from which the QY is determined) because it depends on the intensity of excitation nor on the concentration of the fluorophores.

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).

Fluorescence intensity and Quantum yield

Fluorescence intensity (I_f) can be calculated from Beer-Lambert law considering some of the absorbed light is emitted as radiation (fluorescence), the rest being non-radiative emitted (thermal effect).

The amount of light emitted by fluorescence (I_f) is proportional to the fluorescence quantum yield QY:

$$I_{abs} = I_0 - I_1 \quad I_1 = I_o e^{-\varepsilon bc}$$

$$I_f = QY \cdot I_{abs} = QY \cdot I_0 (1-e^{-\varepsilon \cdot l \cdot c}) \approx \Phi_F \cdot I_0 . \varepsilon \cdot c \cdot l$$

$$(e^x \approx 1 + x + 1/2x^2 + \ldots)$$

$$I_f = QY \cdot I_0 \cdot \varepsilon \cdot c \cdot l = k \cdot c$$

Fluorescence intensity (I_f) is proportional to the concentration **c** (like absorbance!).

For high concentrations linear relationship **no** longer holds due to self quenching.

- if the quantum yield is higher, the majority of light absorbed is found in fluorescent emission,
- if the quantum yield is small, most absorbed energy is lost by thermal effect.

Measuring Fluorescence Quantum Yields

The QY of a fluorophore is determined relative to a reference compound of known QY. If the same excitation wavelength, gain and slit bandwidths are applied for the two samples then the QY is calculated as

$$QY = QY_{ref}\frac{\eta^2}{\eta_{ref}^2}\frac{I}{A}\frac{A_{ref}}{I_{ref}}$$

where: QY_{ref} is the quantum yield of the reference compound, η is the refractive index of the solvent, *I* is the integrated fluorescence intensity (area under the plot), *A* is the absorbance at the excitation wavelength.

In order to avoid inner filter effects and ensure linear response on the intensity, the absorbances (*A)* at the wavelength of excitation must be between {0.02 - 0.05}

It is of uttermost importance that the baseline of the absorption spectrum is near perfect when calculating fluorescence quantum yields.

In order to choose the reference compound:

- The lowest energy absorption band of the reference and dye should overlap as much as possible.
- The QY of the reference should match the expected QY of the dye.

A Table of Standard Materials and Their Literature Quantum Yield Values

Compound	Solvent	Literature Quantum yield	Emission range / nm	Reference
Cresyl violet	Methanol	0.54	600-650	*J. Phys. Chem.*, 1979, **83**, 696
Rhodamine 101	Ethanol + 0.01% HCl	1.00	600-650	*J. Phys. Chem.*, 1980, **84**, 1871
Quinine sulfate	0.1M H_2SO_4	0.54	400-600	*J. Phys. Chem.*, 1961, **65**, 229
Fluorescein	0.1M NaOH	0.79	500-600	*J. Am. Chem. Soc.*, 1945, 1099
Norharmane	0.1M H_2SO_4	0.58	400-550	*J. Lumin.*, 1992, **51**, 269-74
Harmane	0.1M H_2SO_4	0.83	400-550	*J. Lumin.*, 1992, **51**, 269-74
Harmine	0.1M H_2SO_4	0.45	400-550	*J. Lumin.*, 1992, **51**, 269-74
2-methylharmane	0.1M H_2SO_4	0.45	400-550	*J. Lumin.*, 1992, **51**, 269-74
Chlorophyll A	Ether	0.32	600-750	*Trans. Faraday Soc.*, 1957, **53**, 646-55
Zinc phthalocyanine	1% pyridine in toluene	0.30	660-750	*J. Chem. Phys.*, 1971, **55**, 4131
Benzene	Cyclohexane	0.05	270-300	*J. Phys. Chem.*, 1968, **72**, 325
Tryptophan	Water, pH 7.2, 25C	0.14	300-380	*J. Phys. Chem.*, 1970, **74**, 4480
2-Aminopyridine	0.1M H_2SO_4	0.60	315-480	*J. Phys. Chem.*, 1968, **72**, 2680
Anthracene	Ethanol	0.27	360-480	*J. Phys. Chem.*, 1961, **65**, 229
9,10-diphenyl anthracene	Cyclohexane	0.90	400-500	*J. Phys. Chem.*, 1983, **87**, 83

Phosphorescence lifetime

A fraction of excited molecules can reach the triplet state, from which they return to the ground state either radiatively or nonradiatively.

The concentration of molecules in the triplet state decays exponentially with a time constant τ_{ph} representing *the* ***lifetime of the triplet state*** **(phosphorescence lifetime)**:

$$\tau_{ph} = \frac{1}{k_r^T + k_{nr}^T}$$

For organic molecules, the lifetime of the singlet state ranges from tens of picoseconds to hundreds of nanoseconds, whereas the triplet lifetime is much longer (microseconds to seconds).

However, such a difference cannot be used to make a distinction between fluorescence and phosphorescence because some inorganic compounds (for instance, uranyl ion) or organometallic compounds may have a long lifetime.

Monitoring of phosphorescence or delayed fluorescence enables us to study much slower phenomena.

Phosphorescence process can be describe using same parameters: rate constant, quantum yield, intensity, life time.

$$\tau_{ph} = \frac{1}{k_r^T + k_{nr}^T}$$

phosphorescence rate constant (k^T_r)
phosphorescence quantum yield (QY_{ph})
phosphorescence life time (τ_{ph})
phosphorescence intensity (I_{ph})

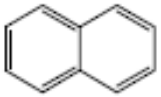
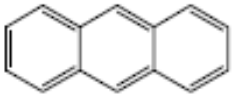
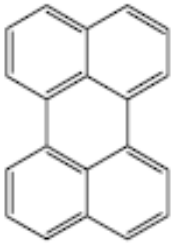
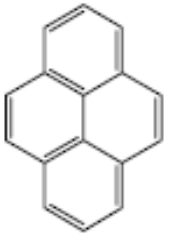
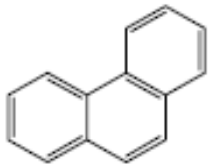
$$I_p h = I_0 e^{-\frac{t}{\tau_T}}$$

$$QY_{ph} = \frac{k_r^T}{k_r^T + k_{nr}^T} QY_{isc}$$

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)

Quantum yields (Φ) and lifetimes (τ) of some aromatic hydrocarbons:

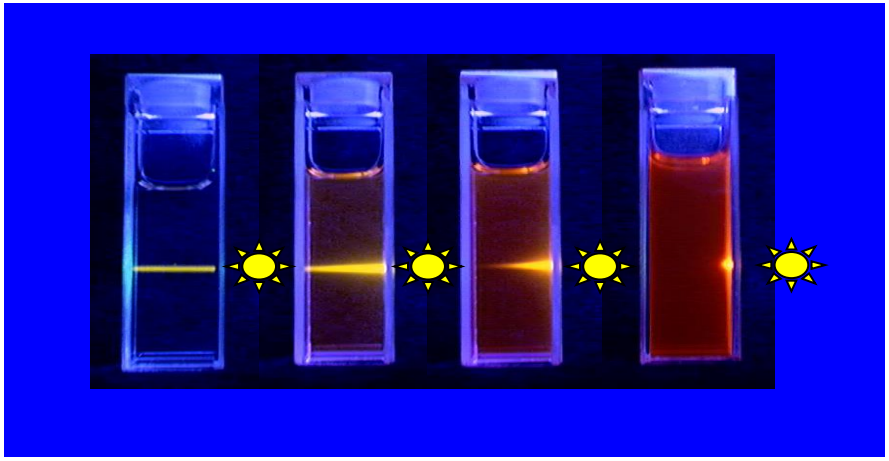
Compound	*Formula*	*Solvent (temp.)*	Φ_F	τ_S *(ns)*	Φ_{isc}	Φ_P	τ_T *(s)*
Benzene		Ethanol (293 K)	0.04	31			
		EPA[a] (77 K)				0.17	7.0
Naphthalene		Ethanol (293 K)	0.21	2.7	0.79		
		Cyclohexane (293 K)	0.19	96			
		EPA (77 K)				0.06	2.6
Anthracene		Ethanol (293 K)	0.27	5.1	0.72		
		Cyclohexane (293 K)	0.30	5.24			0.09
		EPA (77 K)					
Perylene		n-Hexane	0.98		0.02		
		Cyclohexane (293 K)	0.78	6			
Pyrene		Ethanol (293 K)	0.65	410	0.35		
		Cyclohexane (293 K)	0.65	450			
Phenanthrene		Ethanol (293 K)	0.13		0.85		
		n-Heptane (293 K)	0.16	0.60			
		EPA (77 K)				0.31	3.3
		Polymer film	0.12		0.88		0.11

Fluorescence sources of interference

In the condensed phase, many parameters can affect the quantum yields and lifetimes: *to high concentration, presence of quenchers, temperature*, pH, polarity, viscosity, hydrogen bonding, etc.

► Inner filter effect:

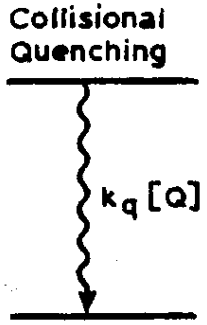
Intensity of excitation light isn´t constant because each layer of the sample absorbs some of the incident radiation (*intensity* of exciting light is higher in the front part of cuvette and lower in the rear part of cuvette).



Rhodamine B: Sample concentration & the *inner filter effect*

► **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 collisionalquenching.)



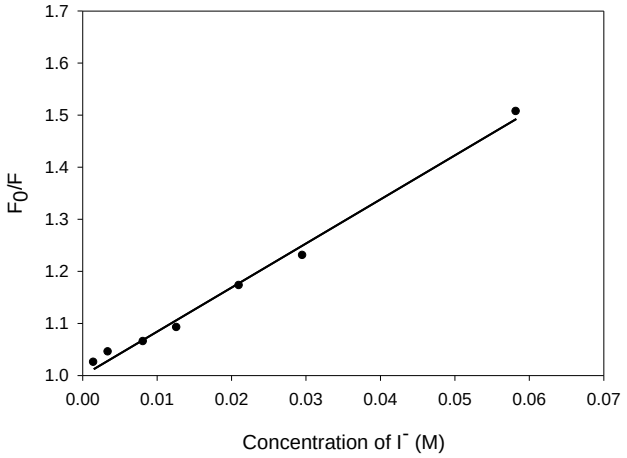
Excited molecule returns to the ground state by radiationless transition (without emitting light) as a result of a **collision with quenching molecule** (analyte directly quenches the fluorophore – change in lifetime or intensity).

Stern-Volmer equation describe the collisional quenching: $I_0/I = 1 + K_{SV}[Q]$

I_0 and I are the fluorescence intensities observed in the absence and presence, respectively, of quencher

[Q] is the quencher concentration
K_{SV} is the Stern-Volmer quenching constant

A plot of I_0/I versus [Q] should yield a straight line with a slope equal to K_{SV}.



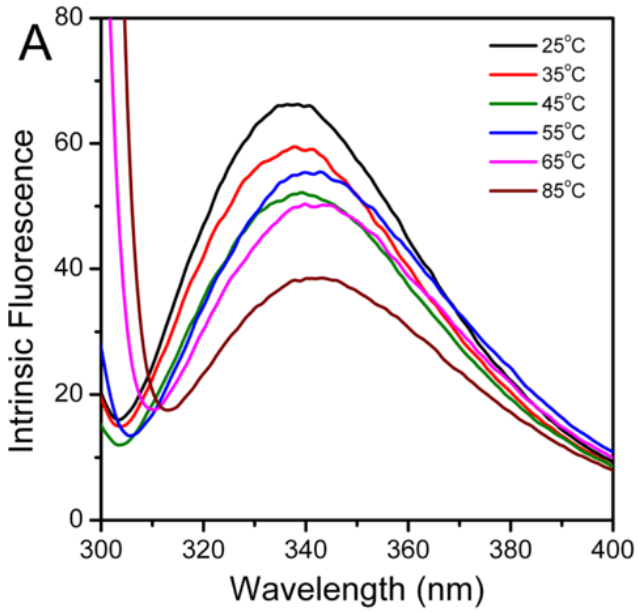
Common quenchers:

O_2, halogens (Br, I), nitrocompounds, acrylamide

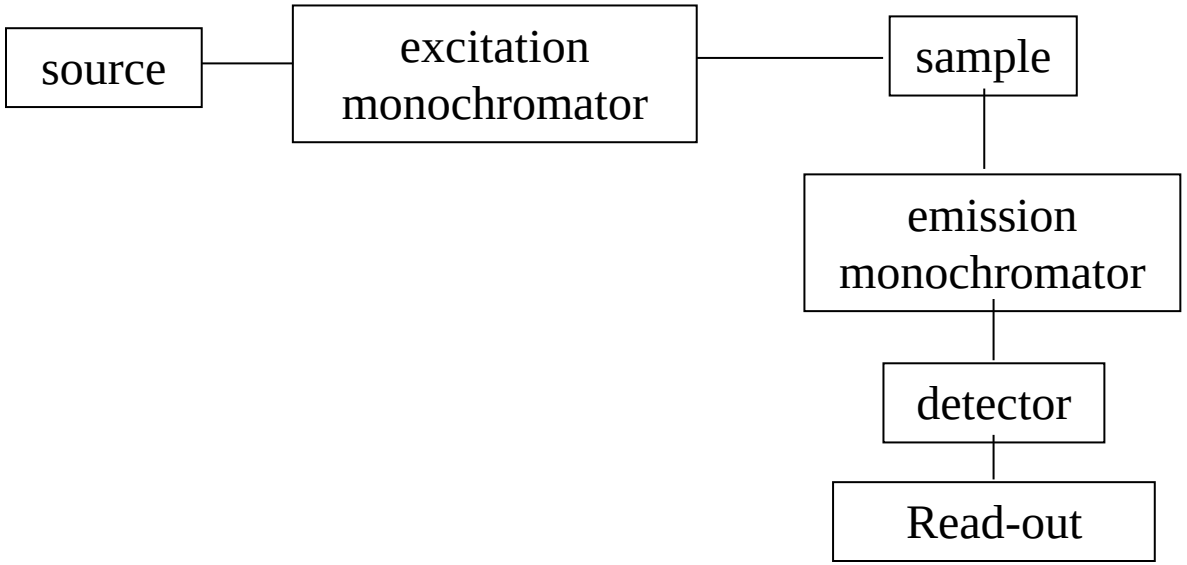
► Effect of temperature

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.

Experiments are often in good agreement with the empirical linear variation of $\ln(1/\Phi_F-1)$ versus $1/T$.



Fluorescence measurement



Filter fluorimeters

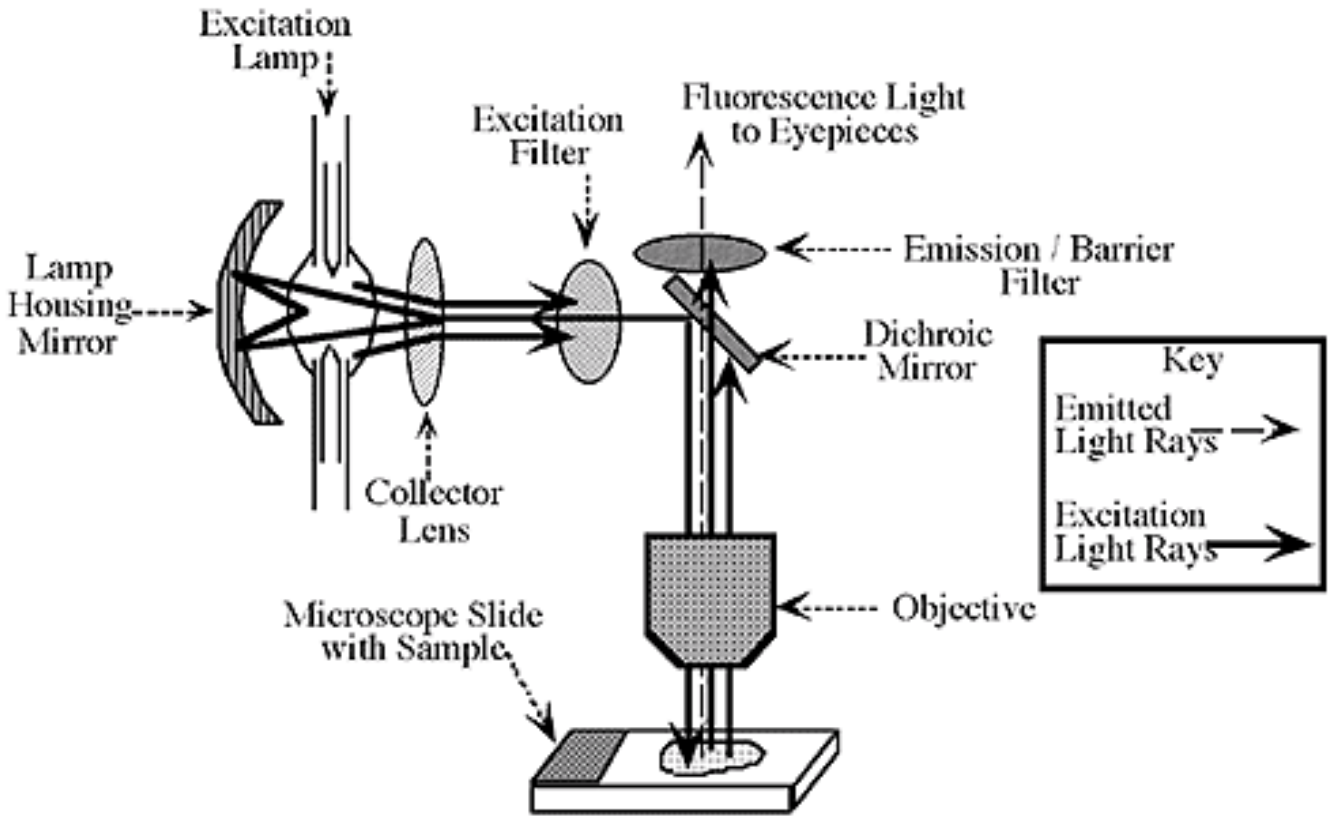
Spectrofluorometers

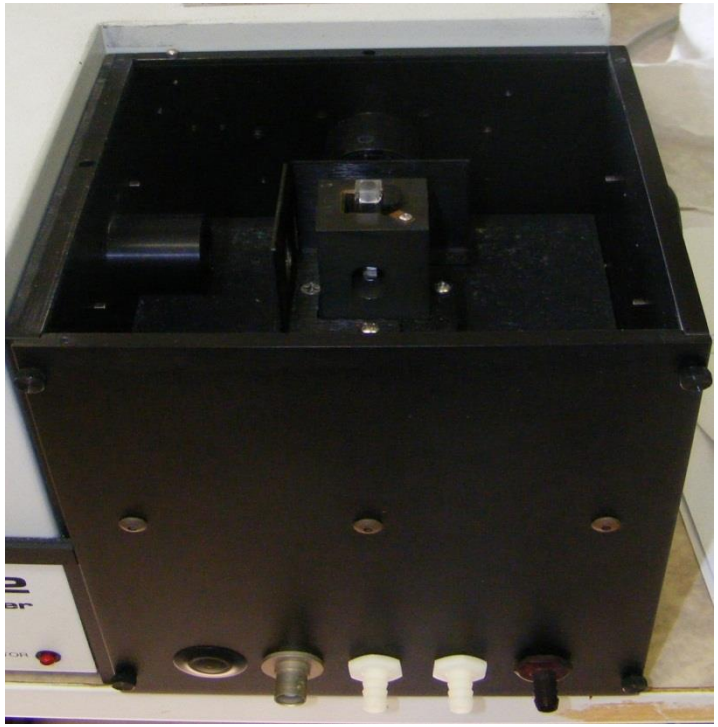
Fluorescent microscopes

Fluorescent scanners

Flow cytometry

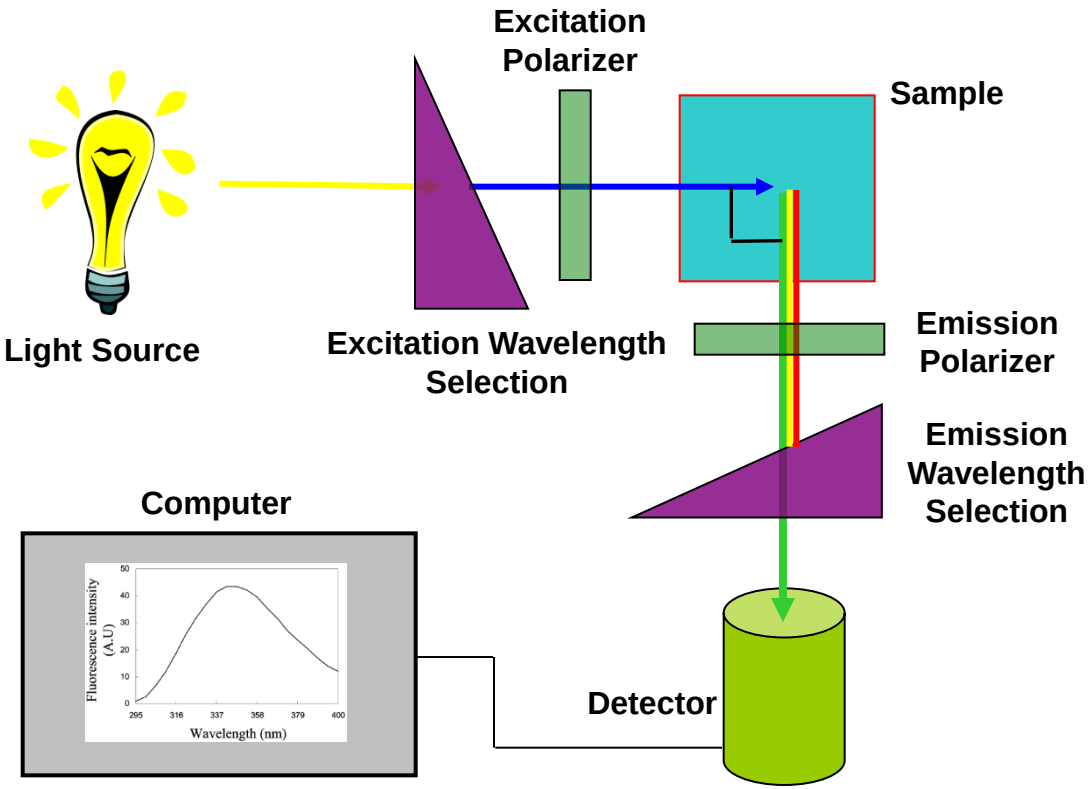
Fluorescent microscopes





Spectrofluorometer

Fluorimeter



Important considerations for fluorimeters:

1. Must have intense source, D_2 & W not intense enough so use Xe or Hg
2. Fluorescence is not as strong as absorbtion so need more sensitive detectors like PMT
3. Cells transparent on 4 sides detection usually at right angles of source
4. Phosphorescence measurements may be made on fluorimeters with 2 differences:
   a) Irradiation + time delay + measurement
   b) Cooled samples @ 77K to prevent collisional deactivation

Methods of fluorescence determination

Direct methods - natural fluorescence of the fluorecent sample is measured

Indirect methods - the nonfluorescent compound is converted into a fluorescent derivative by specific reaction or marked with fluorescent dye by attaching dye to the studied substance

Quenching methods - analytical signal is the reduction in the intensity of some fluorescent dye due to the quenching action of the measured sample.

Natural fluorophores:

- Polyaromatic hydrocarbons
- Vitamin A, E
- Coenzymes (FAD, FMN, NADH)
- Carotenes
- Quinine
- Steroids
- Aromatic aminoacids
- Nucleotides
- Fluorescent proteins –GFP (green fluorescent protein)

Fluorescent probes

Compounds whose fluorescence doesn´t change after their interaction with biological material:

acridine orange (DNA)

fluorescein (proteins)

rhodamine (proteins)

...

Compounds whose fluorescence change according to their environment:

ANS (1-anilinonaftalen-8- sulphonate) - polarity

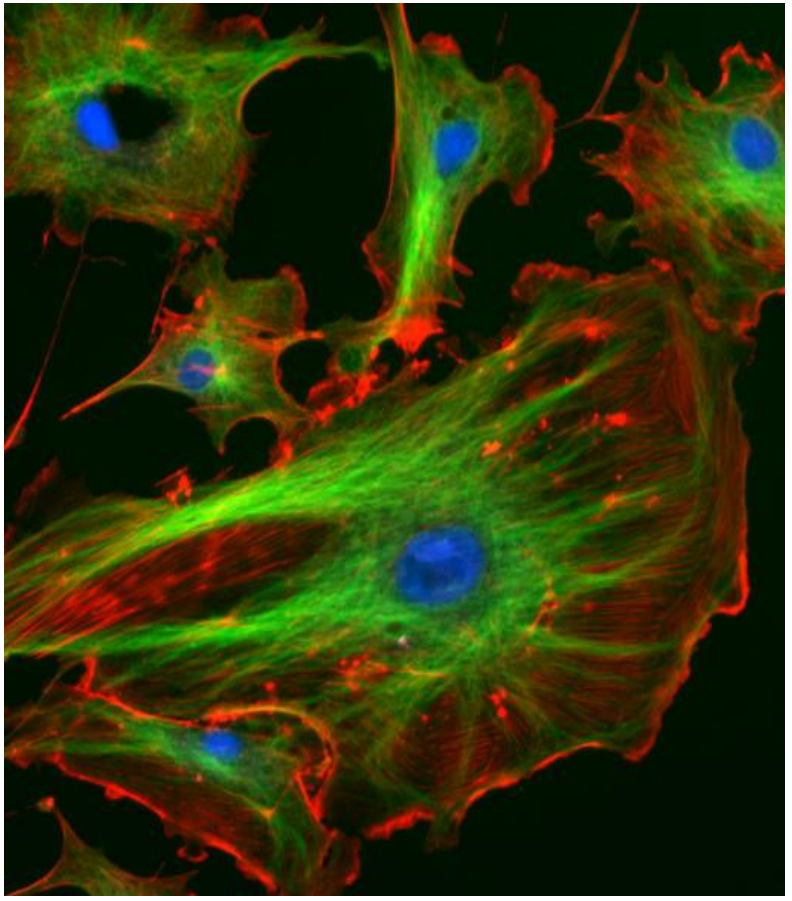
Fura-2 - tracking the movement of calcium within cells

...

The larger the fluorescence quantum yield, the easier it is to observe a fluorescent compound, especially a fluorescent probe.

Some applications of fluorescence detection

- Protein conformation
- Membrane potential
- Membrane transport
- Membrane viscosity
- Enzymatic reactions
- DNA analysis
- Genetic engineering (manipulations)
- Immunochemical methods
- Cell proliferation and apoptosis



Fluorescence and phosphorescence are one of the most sensitive techniques available!