SAPIENZA Università di Roma
Laurea magistrale in Ingegneria delle Nanotecnologie
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Biophotonics Laboratory Course

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Applications of optics and photonics

Microscopic Techniques
- Conventional Wide-Field Fluorescence
- TIRF
- FLIM
- FRET, FRAP
- Confocal
- Two-Photon
- Second Harmonic
- Super-resolution (SNOM, STED, PALM, STORM)

Non-Microscopic Label-free
- Surface plasmon Polaritons (SPP)
- Photonic crystals (PC)
- Raman , CARS
- Quantum dots

Non-Microscopic Techniques
- Citofluorimetry
- ELISA
- DNA-Chip
- Cycle-sequencing
- SOLID

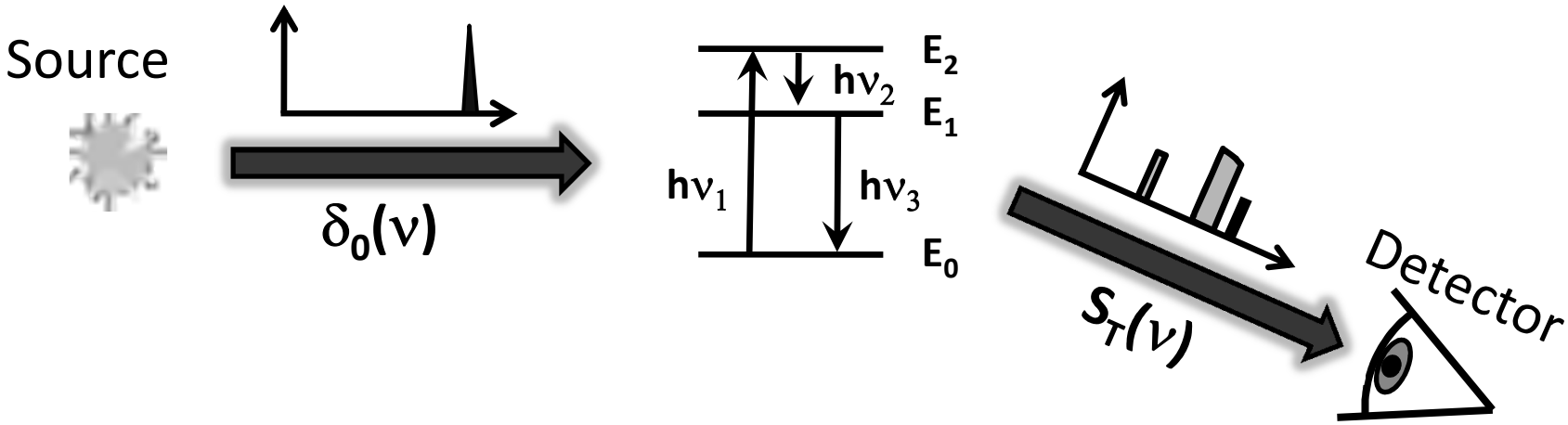
Other non Microscopic Techniques
- Southern
- Western
- Northern

All of them make use of the emission of luminescent markers (labels)

LECTURE 3
Organic Molecules Luminescence Spectroscopy
and
most used fluorophores in the field of Life Sciences

Luminescence Spectroscopy

Investigates the emission of e.m.r. from a molecular system as a function of the wavelength.

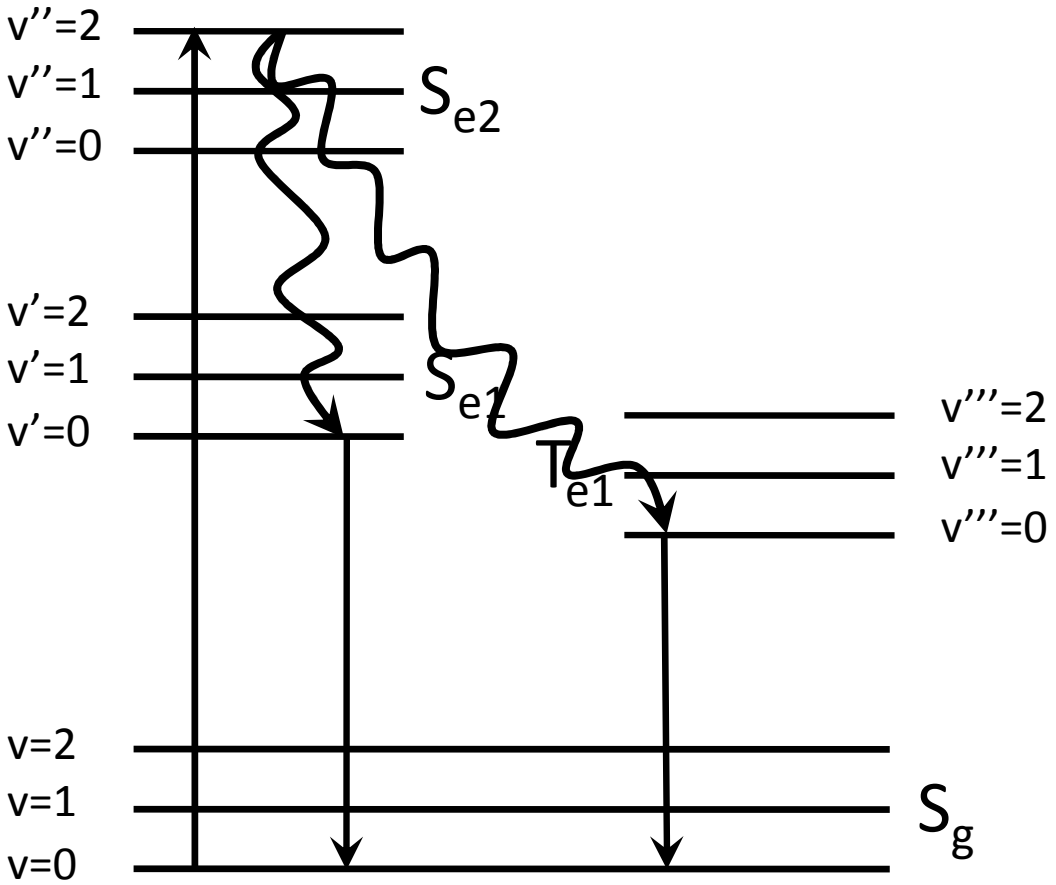


For the emission we have the same selection rules than for the absorption:

- The transition dipole moment must not be zero
- Spin conservation must be satisfied

Generally the excitation is carried out at one single wavelength λ (***lamp + monochromator***, ***laser***) and the detection is at an angle that is different from the excitation angle (off-axis).

Luminescence Spectroscopy



1) In general the excited singlet level $\mathbf{S_{e2}}$ can decay:

- non radiatively to the singlet level $\mathbf{S_{e1}}$
- non radiatively to the triplet level $\mathbf{T_{e1}}$

2) The singlet level $\mathbf{S_{e1}}$ can decay radiatively to the singlet level $\mathbf{S_g}$

3) The triplet level $\mathbf{T_{e1}}$ in principle could not decay to $\mathbf{S_g}$ but there is a small probability due to a weak spin-orbit interaction that mixes the spin states. The transition is very weak.

The transition $S_{e1} \rightarrow S_g$ gives rise to FLUORESCENCE

The transition $T_{e1} \rightarrow S_g$ gives rise to PHOSPHORESCENCE

Luminescence Spectroscopy

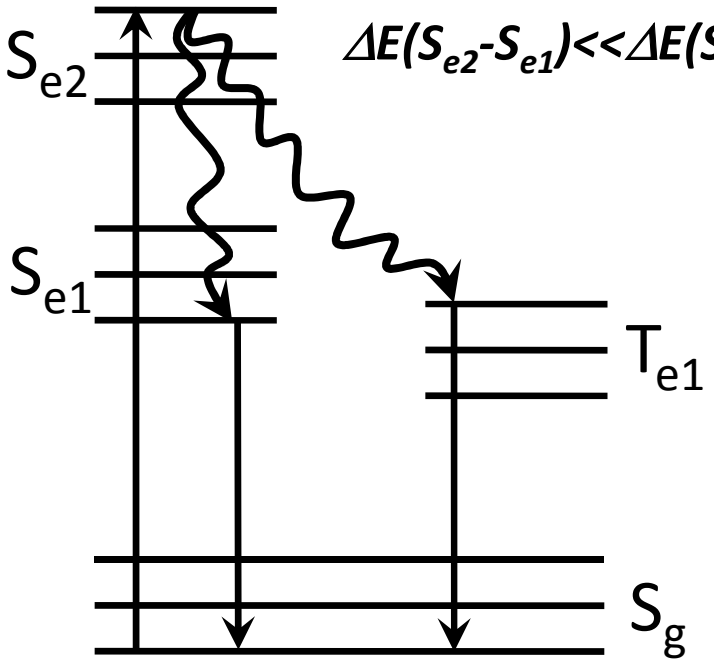
The intensity of the emission process is characterized by the ***fluorescence quantum yield*** defined as follows

$$\Phi = \frac{N_{ph}(\text{emitted})}{N_{ph}(\text{absorbed})}$$

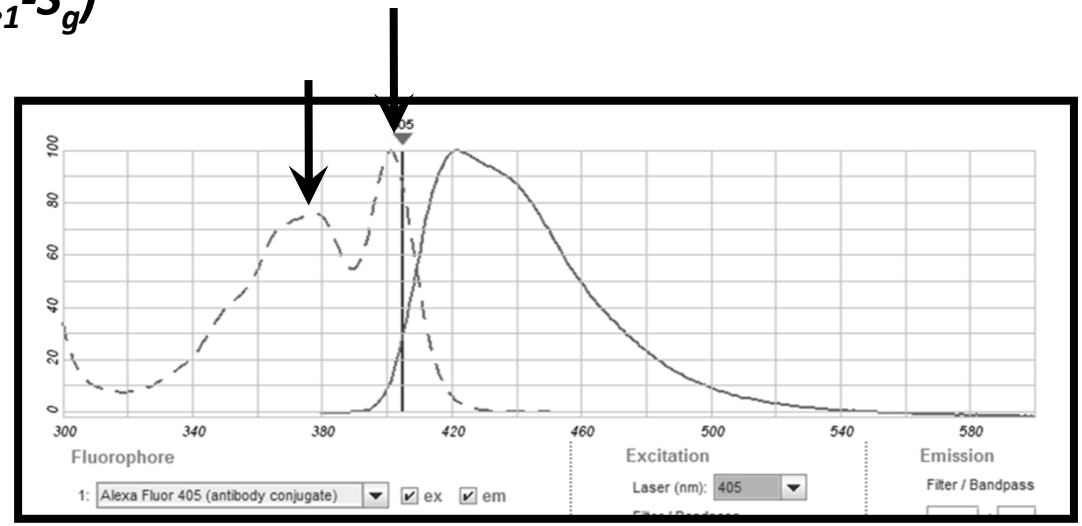
The intensity of the emission process will also depend on the:

- absorbance of the molecule at the excitation wavelength λ
- intensity of the e.m.r. at the excitation wavelength λ

Luminescence Spectroscopy - Fluorescence



$\Delta E(S_{e2}\text{-}S_{e1}) << \Delta E(S_{e1}\text{-}S_g)$



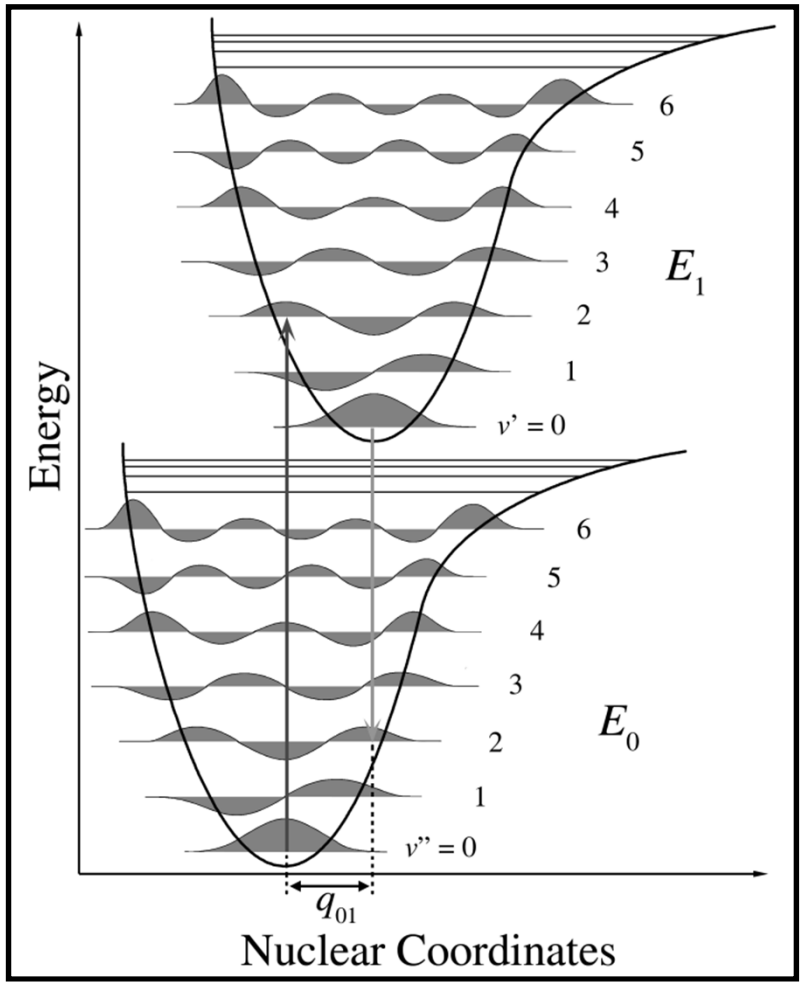
Alexa Fluor 405

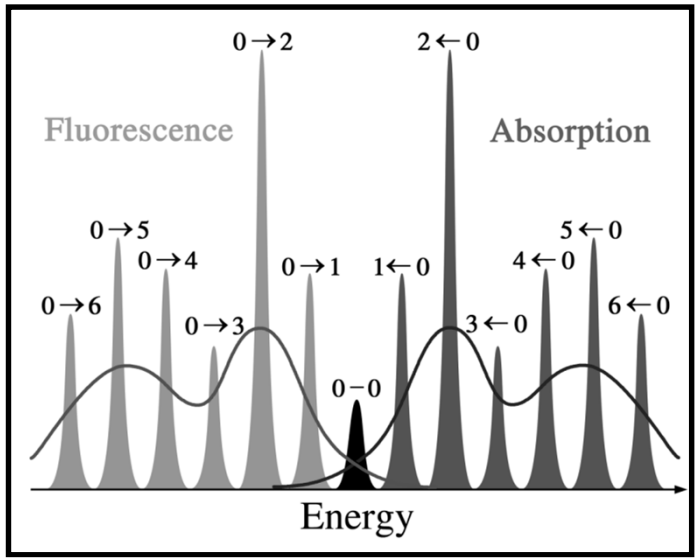
- For almost all molecules it is possible to have absorption from the ground level S_g to different excited singlet levels S_{e1}, $S_{e2,}$ …
- Radiative emission takes place only between S_{e1} and S_g (***Kasha Rule***) since the larger it is the energy distance between the two singlet electronic states the stronger it is the transition probability. As a consequence the emission spectrum does not change when we change the excitation wavelength λ.

Luminescence Spectroscopy - Fluorescence

Absorption of the e.m.r. brings the molecule in the vibrational states of the same excited electronic state. The decay takes place by means of two sequential processes:

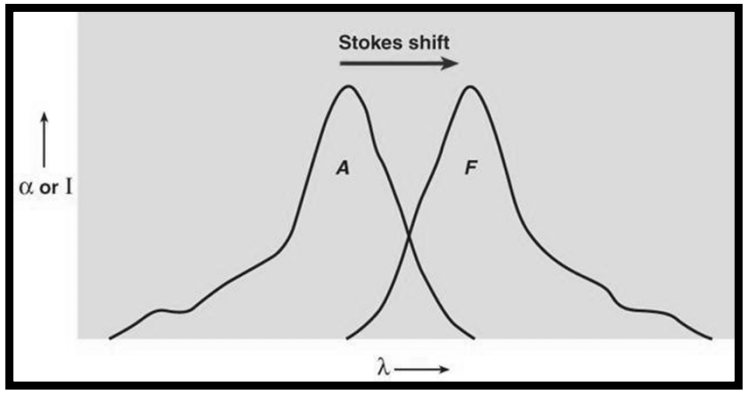
1) Non-radiative vibrational relaxation to the lowest vibrational level with energy loss via thermal heating
2) Radiative emission from the fundamental vibrational level of S_{e1} to any vibrational level of S_g.

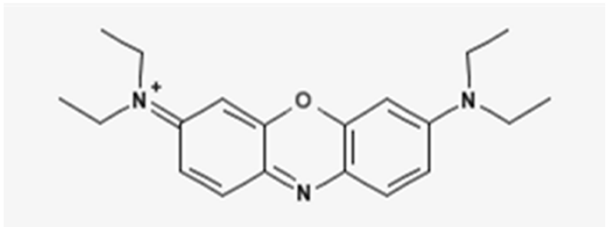




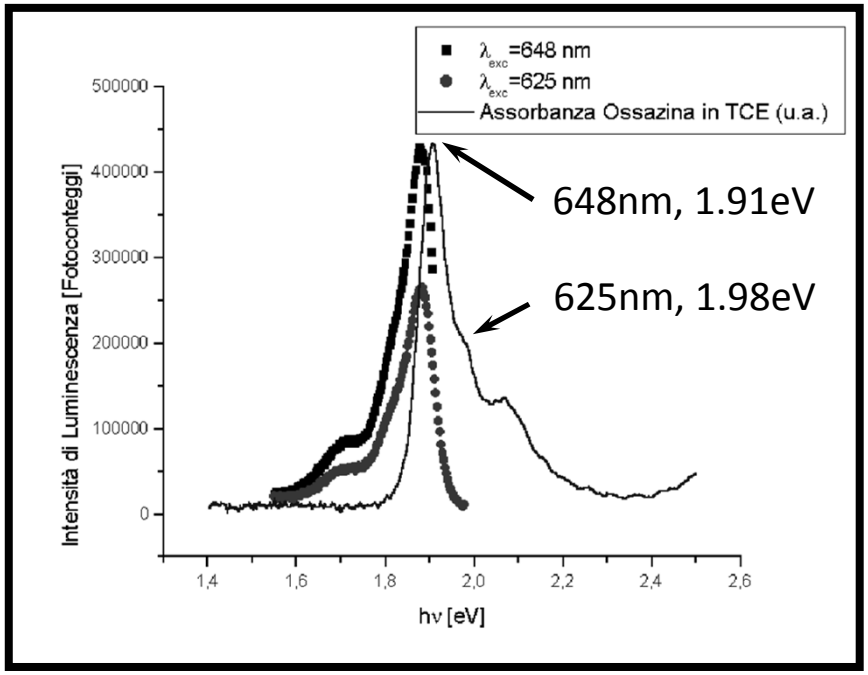
Luminescence Spectroscopy - Fluorescence

The Franck and Condon principle reveals itself through the specular symmetry of the absorption and emission spectra. The shift between the emission and absorption spectrum peaks is named Stokes shift.



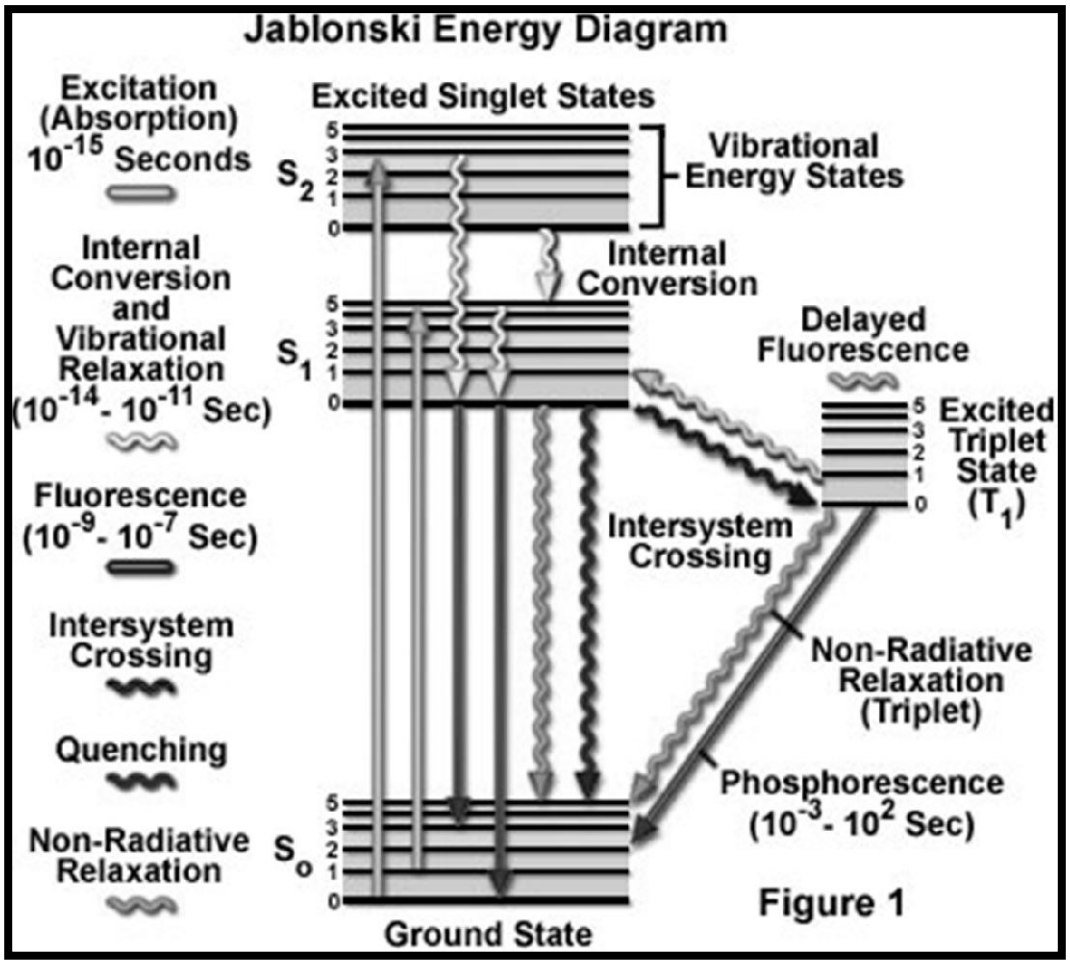


Oxazine 1



Other transition mechanisms between molecular states

The ***absorption***, ***fluorescence***, ***non-radiative decay*** processes between molecular states that we described are only some among those possible. The ***Jablonski Diagram*** resumes such processes, together with the others:

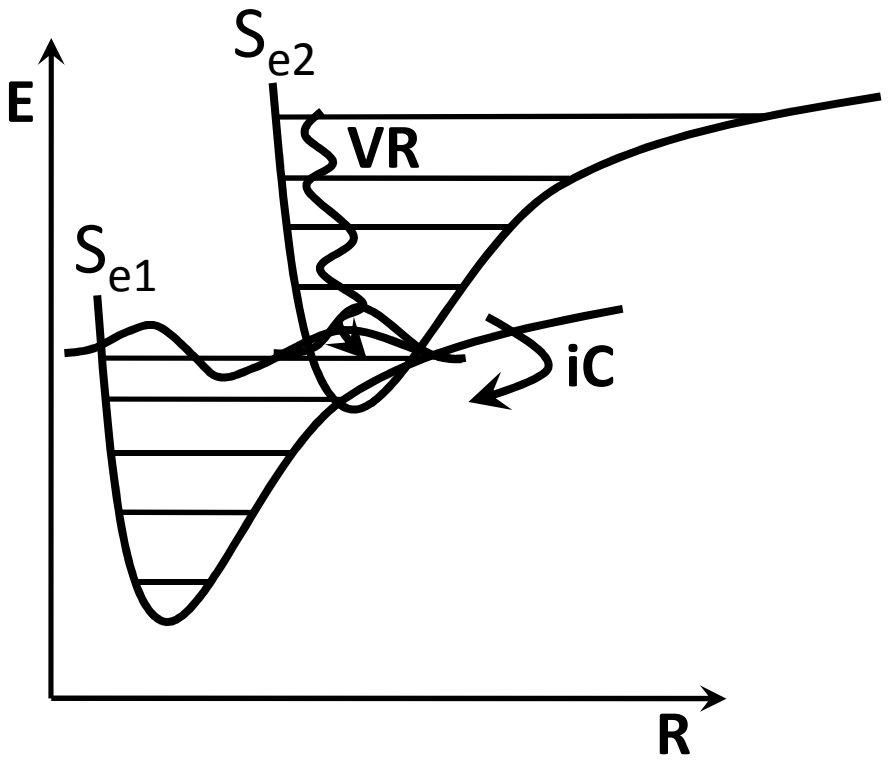


- ***Internal Conversion***
- ***Intersystem crossing***
- ***Phosphorescence***
- ***Quenching***

The characteristic time scales are inversely proportional to the probability of each phenomenon.

Other mechanisms – Internal conversion

Process in which a molecule releases its excess energy through two cascaded processes. The process involves the energy transfer to the vibrational sub-levels of the lower electronic state and their subsequent relaxation. Gives rise to heat dissipation in the surrounding of the molecule. Can be divided in two processes:



VR – Vibrational Relaxation

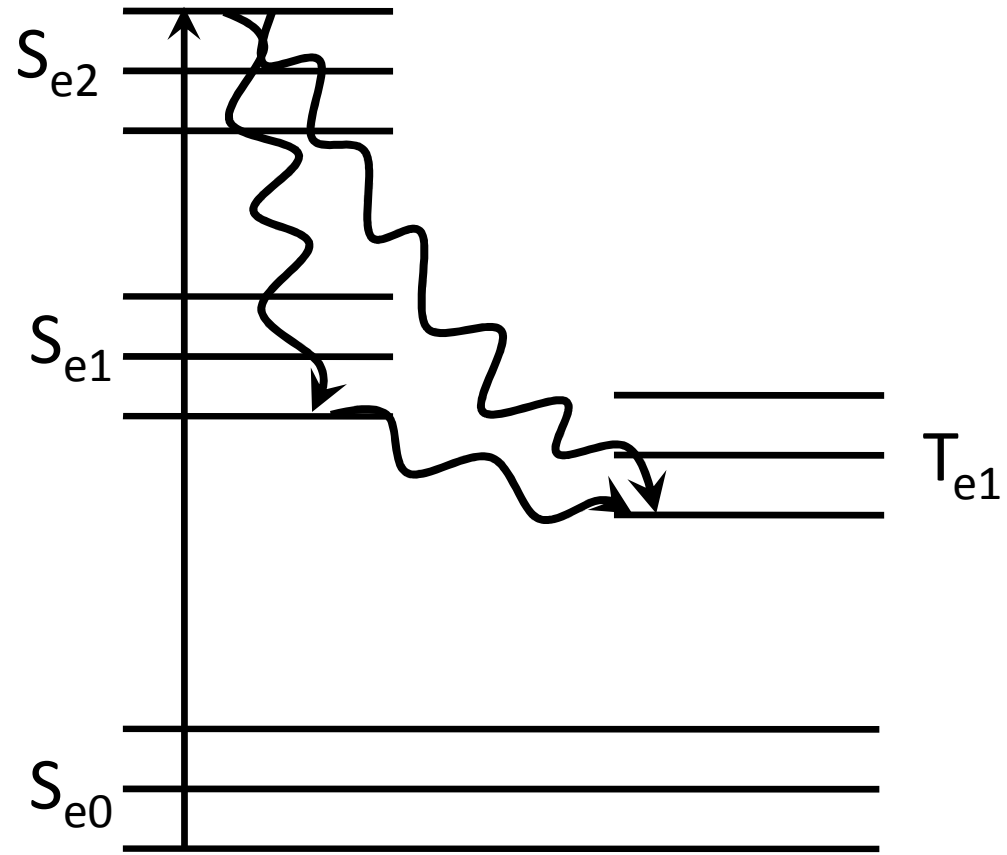
Decay between vibrational levels of the excited level

IC – Internal conversion

Decay to the vibrational levels of another electronic state. In order to have internal conversion it is necessary that there is a superposition between the vibrational wave functions in the two electronic states involved in the process.

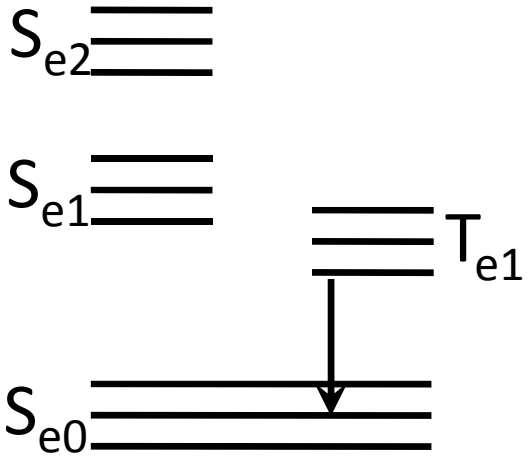
Other mechanisms – Inter system Crossing

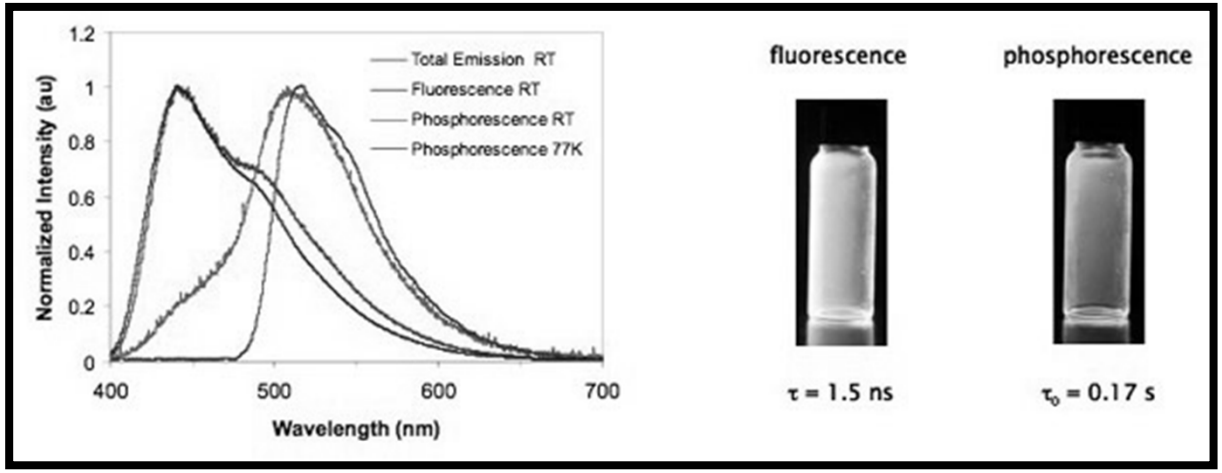
There can be a non-radiative transition between singlet (S) and triplet (T) states with a consequent change of the total spin.



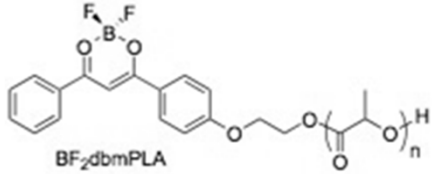
Other mechanisms – Phosphorescence

The excited triplet state can decay to the fundamental singlet state by means of a radiative process. he selection rule that imposes that transitions can take place only between states with the same total spin can be violated if there is a sufficiently strong spin-orbit interaction that couples such states. The process has a very small probability therefore it takes long time (seconds). Since the energy difference between the T_{e1} and S_{eo} states is smaller than the difference between the S_{e1} and S_{e0} states, the phosphorescence spectrum is shifted to smaller (larger) energies (wavelengths).





Difluoroboron Dibenzoylmethane-Polylactide

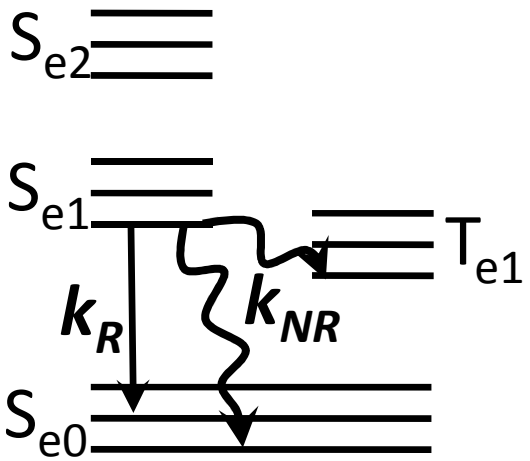


Other mechanisms – Quenching

The fluorescence emission probability from the state S_{e1} to S_{e0} can depend on other mechanisms that lead to a decrease of the fluorescence quantum yield. Such mechanisms can indeed provide additional non-radiative relaxation pathways. The fluorescence quantum yield can be expressed as function of the transition probabilities as follows:

$$\Phi = \frac{N_{ph}(\text{emitted})}{N_{ph}(\text{absorbed})} = \frac{k_R}{k_R + k_{NR}} \leq 1 \qquad \text{with} \qquad I_{FL} \propto \Phi$$

Where k_R and k_{NR} are the radiative and non-radiative transition probabilities, respectively. The non-radiative channels ''quench'' the radiative processes.



There are several mechanisms that gìve rise to quenching:

- Chemical reactions in the excited state
- Inter system crossing
- Formation of complexes
- Collisions
- Förster energy transfer

The quenching mechanism that is most used in fluorescence imaging applications is the ***Förster Transfer***.

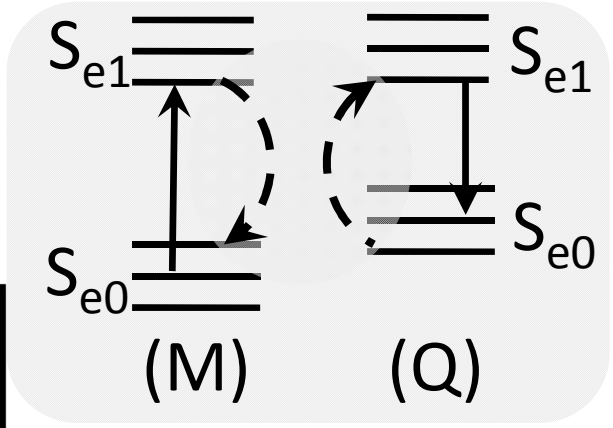
Other mechanisms – Förster transfer (Quenching)

An excited molecule (M or D as donor) can decay transferring its excess energy to a second molecule (Q or A as acceptor) by means of the non-radiative process named ***Forster transfer***. Such a process can give rise to an intense relaxation pathway and cause a strong decrease of the fluorescence quantum yield. The second molecule is named ***quencher***. ***Typically, the process takes place if the distance is less than 10nm***.

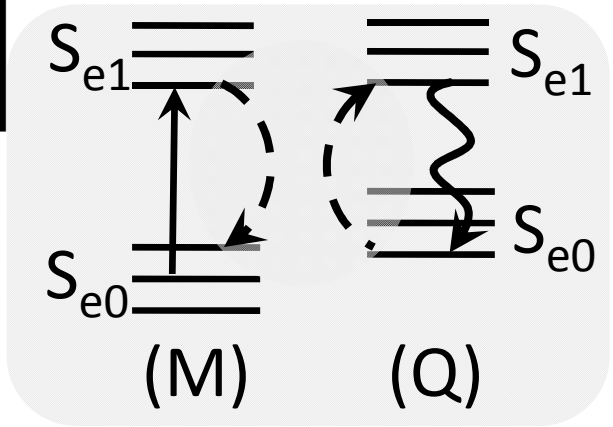
$$S_0 \xrightarrow{h\nu} S_{e1}$$

$$S_{e1} + Q \longrightarrow S_0 + Q^{exc}$$

$$Q^{exc} \longrightarrow Q$$



- If the quencher (Q) is luminescent it can re-emit with a spectrum that is different from the spectrum of M



- If the quencher is not luminescent it can dissipate the energy through non-radiative paths

Other mechanisms – Förster transfer (Quenching)

The Forster transfer process involves the exchange of a virtual photon between the donor (M) and the quencher/acceptor (Q). By means of such a photon the two molecules exchange the energy.
If we isolate the Förster transfer mechanism from the other non-radiative processes, we get:

$$\Phi^{FT} = \frac{N_{ph}(\text{emitted})}{N_{ph}(\text{absorbed})} = \frac{k_R}{k_R + k_{NR} + k_{FT}} \qquad \text{with} \qquad I_{FL}^{FT} \propto \Phi^{FT}$$

We therefore get the ratio of the fluorescence intensities without and with Forster transfer:

$$\frac{I_{FL}}{I_{FL}^{FT}} = \frac{\Phi}{\Phi^{FT}} = \frac{k_R + k_{NR} + k_{FT}}{k_R + k_{NR}} = 1 + \frac{k_{FT}}{k_R + k_{NR}}$$

Other mechanisms – Förster transfer (Quenching)

We can define the efficiency of the Förster transfer process η_{FT} as:

$$\eta_{FT} = 1 - \frac{\Phi_{FT}}{\Phi} = \frac{k_{FT}}{k_R + k_{NR} + k_{FT}}$$

η_{FT} depends on some parameter that can be grouped in:

- donor-acceptor distance
- superposition of the emission spectrum of the donor and absoprtion spectrum of the acceptor
- mutual orientation of the donor and acceptor

η_{FT} depends on the donor-acceptor distance (dipole-dipole interaction) as:

$$\eta_{FT} = 1 - \frac{\Phi_{FT}}{\Phi} = \frac{1}{1 + (r/R_0)^6}$$

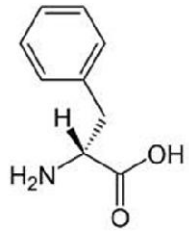
$$R_0^6 = \frac{9\Phi(\ln 10)\kappa^2 J}{128\pi^5 n^4 N_A}$$

Where the Förster distance $\boldsymbol{R_0}$ depends on the superposition integral of the spectra ***J***, on a factor related to the mutual orientation of the dipoles $\boldsymbol{\kappa}$, and on th erefractive index of the host medium ***n*** (N_A Avogadro number).

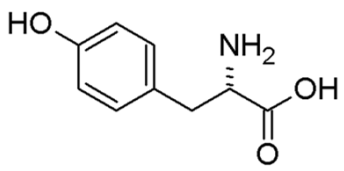
Fluorescence spectra of some endogenous chromophores

Example

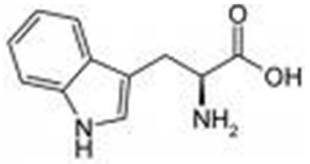
- Amino acids



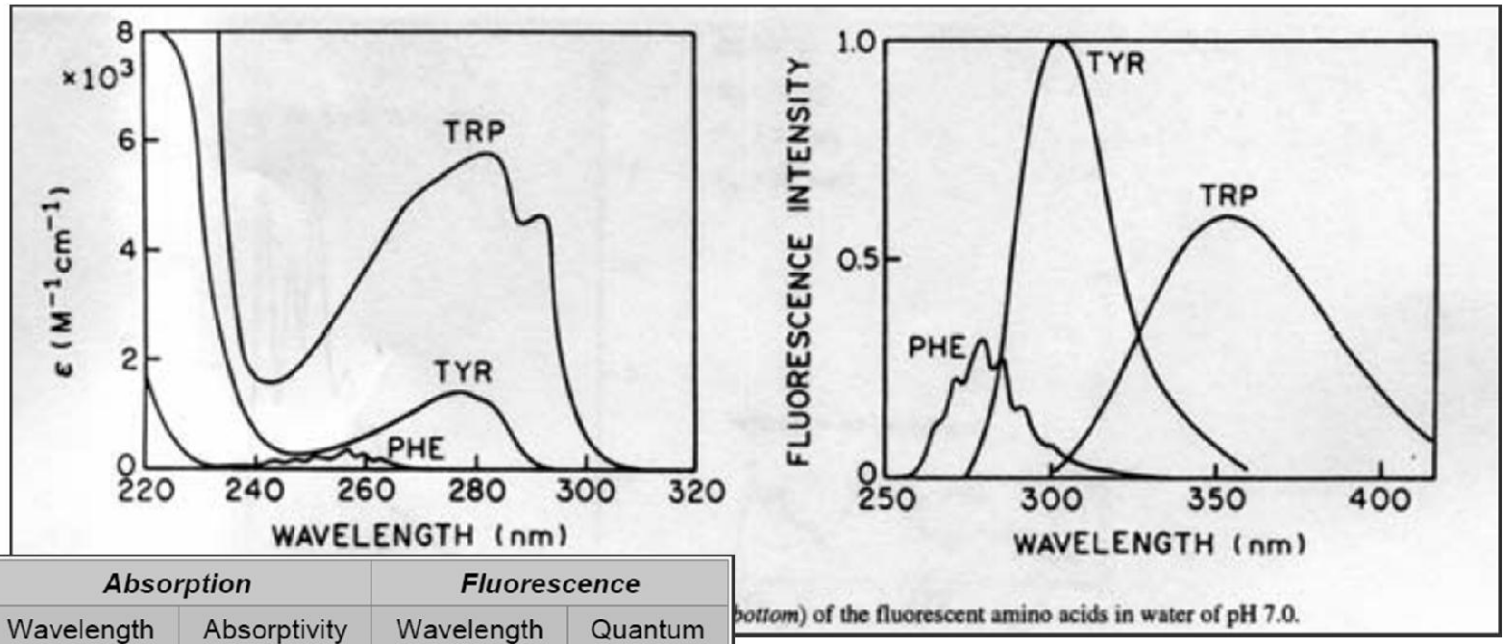
Phenylalanine (Phe)



Tyrosine (Tyr)



Triptophan (Trp)



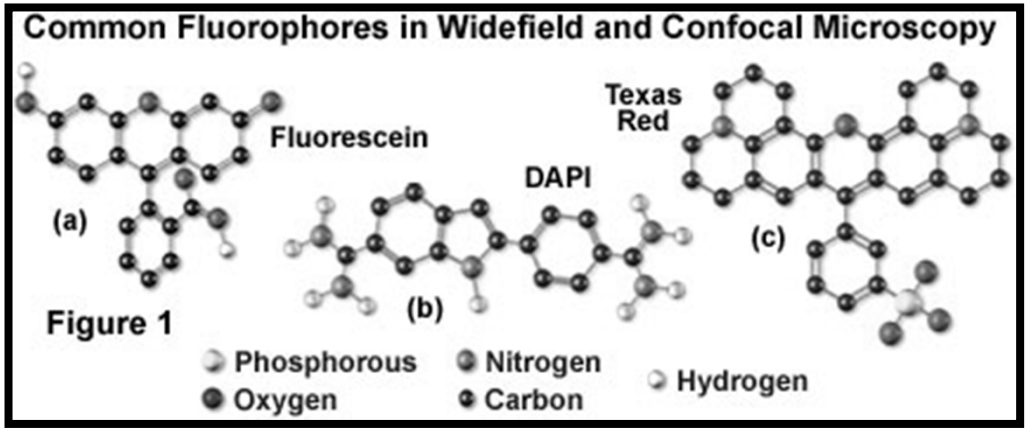
bottom) of the fluorescent amino acids in water of pH 7.0.

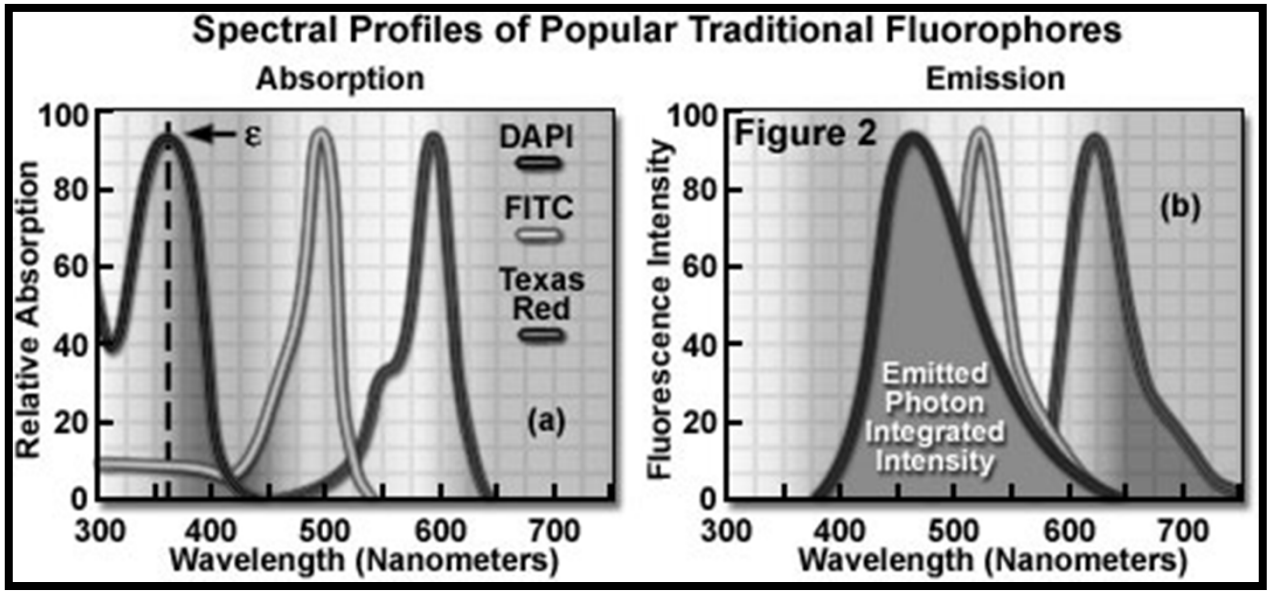
Amino acid	*Lifetime*	*Absorption*		*Fluorescence*	
		Wavelength	Absorptivity	Wavelength	Quantum
Tryptophan	2.6 ns	280 nm	5600	348 nm	0.20
Tyrosine	3.6 ns	274 nm	1400	303 nm	0.14
Phenylalanine	6.4 ns	257 nm	200	282 nm	0.04

Luminescence Spectra of exogenous chromophores

There exist a large variety of organic chromophores that are used to label the basic biochemical compounds. They possess peculiar absorption and luminescence properties.

EXAMPLE ***Traditional dyes***





Fluorescence Spectra of exogenous chromophores

EXAMPLE ***Cyanine***

- Based on the partially saturated indol group with two aromatic units.
- Connection via a variable length alkenic bridge
- Absorption and emission spectra similar to those of the traditional dyes
- Better water solubility
- Less sensible to pH and to an organic environment
- Large photostability
- Large quantum yield

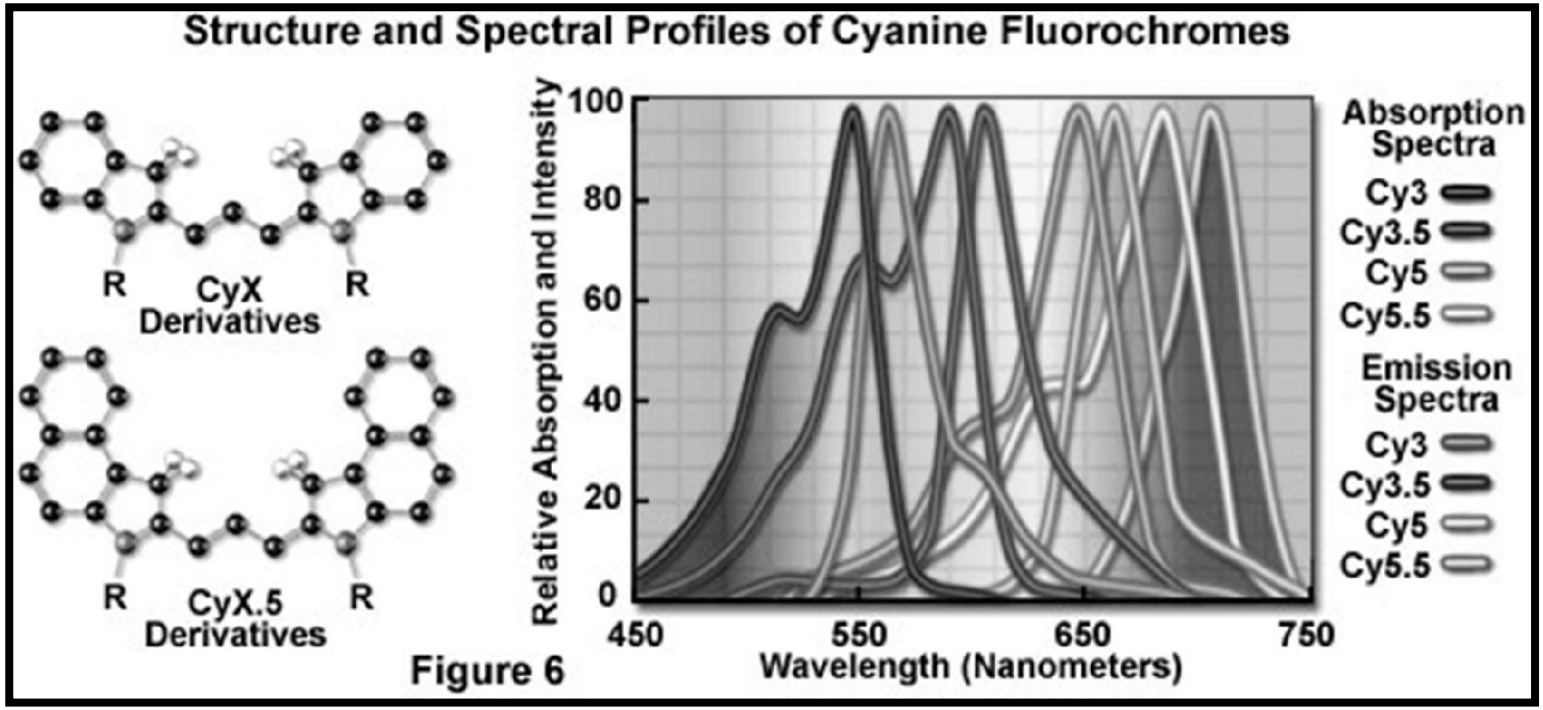
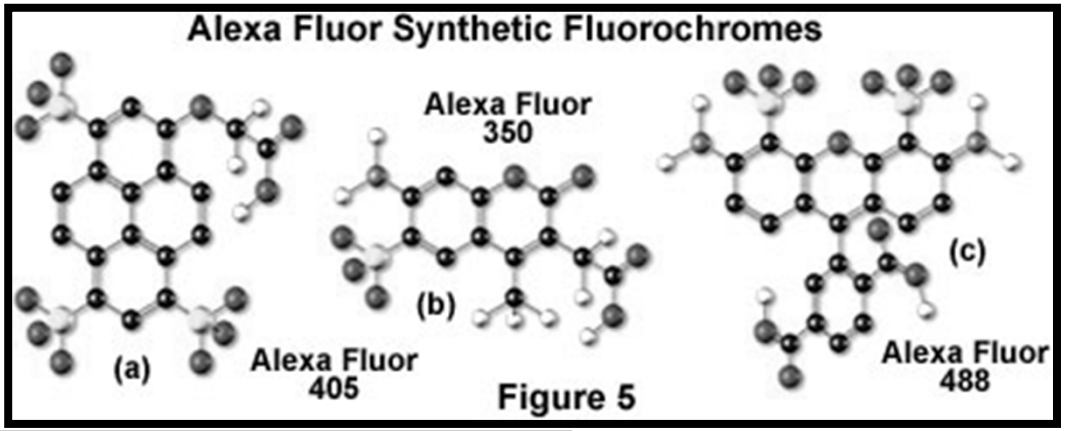


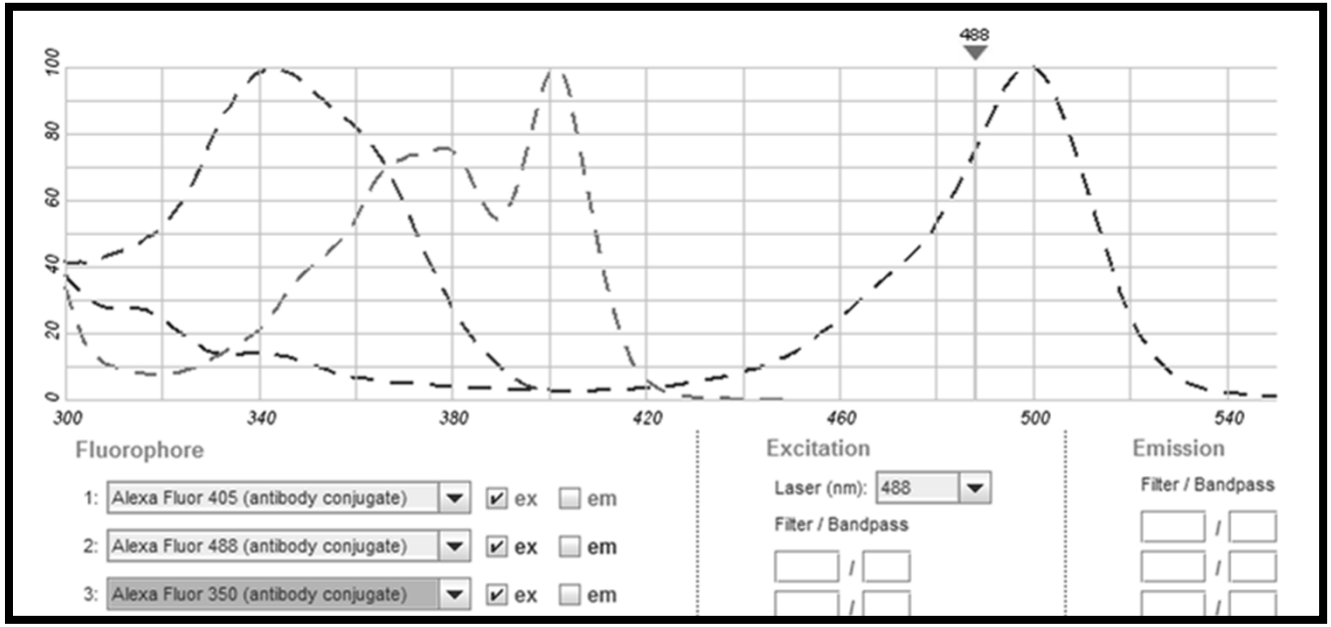
Figure 6

Luminescence Spectra of exogenous chromophores

EXAMPLE ***Alexa Fluor***

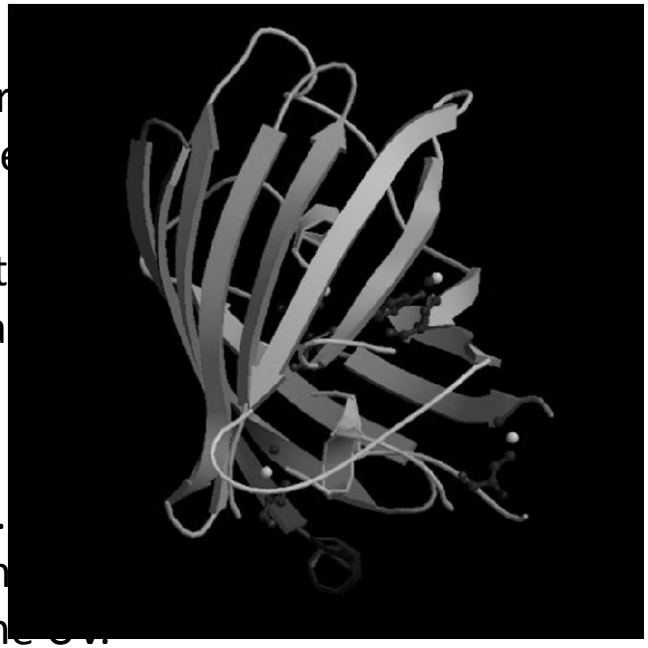
- Derived from the most common organic dye, Rhodamine
- Larger quantum yield
- Excellent solubility in water
- pH insensitive
- Very high photostability





Luminescence Spectra of exogenous chromophores

EXAMPLE ***Green Fluorescent Protein (GFP)***



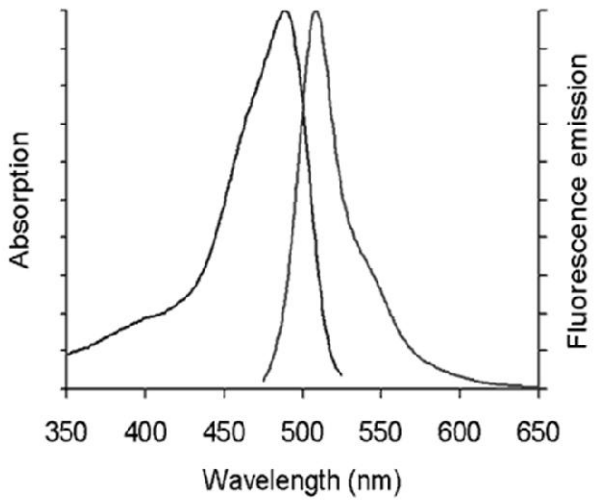
It is a protein that is expressed by the jellyfish Aequor
Victoria. Thanks to its fluorescence properties, to its mode
dimensions and to the possibility to modify to some extent
spectroscopic characteristics, the GFP has become during t
last ten years a well established label for experiments a
techniques in molecular biology.

Absoprtion is peaked at the wavelengths 395 nm and 475 nm.
emission is peaked around 505 nm. Usually they are excited in
blu (475nm), in order to avoid risks connected to the use of the

The GFP is formed by 238 amino acids and its molecular weight is 27000 Dalton. It is constituted by 11 beta sheets arranged to form a cylindrical structure that is named β-barrel o β-can. There are two more alpha-helical segments, one at the base of the barrel, the other along its central axis. Such last helix contains the chromophore (i.e. the portion that can emit light), which starts from the tri-peptide Ser 65-Tyr 66-Gly 67. In all the structure is very compact to prevent the chromophore from reacting with other molecules that could make it inactive.

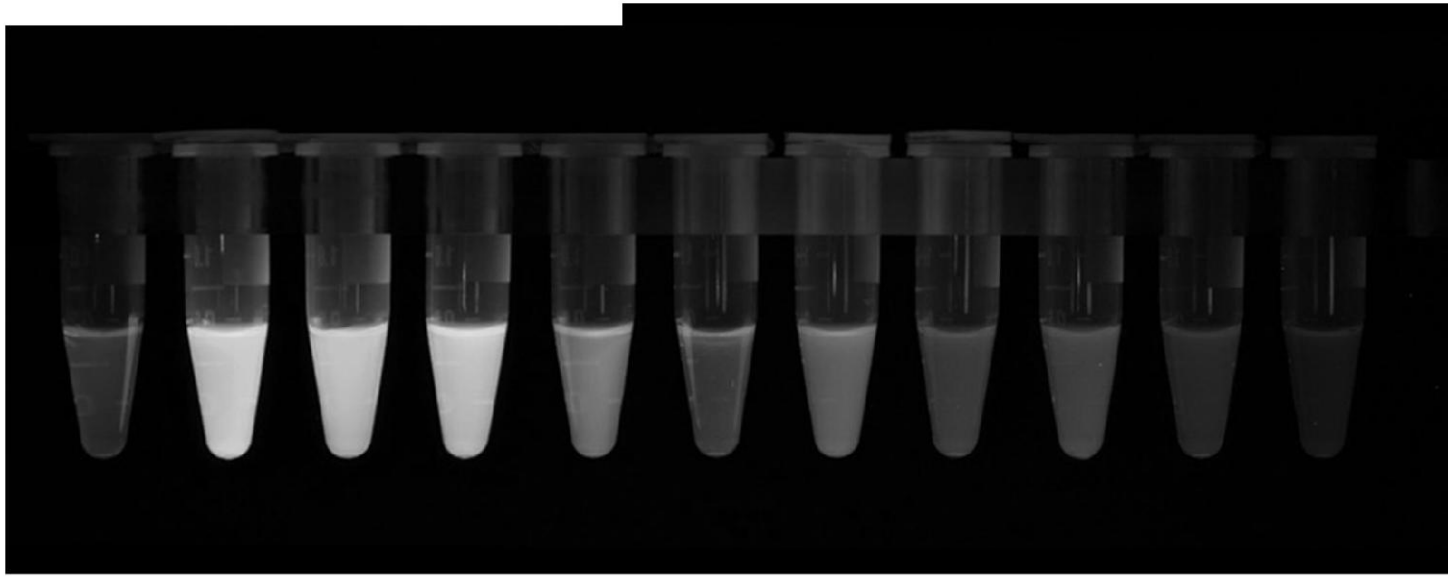
Luminescence Spectra of exogenous chromophores

EXAMPLE ***Green Fluorescent Protein (GFP)***



Due to its high potential for a wide range applications and to meet the needs of biomolecular research labs many mutants of GFP were developed, each characterized by a different absorption/emission spectrum.

Nobel for Chemistry 2008, M.Chalfie, O.Shimomura, R.Y.Tsien



Temporal decay of luminescence

- ***Lifetime*** – The time interval a molecule remains in an excited state before decaying, either radiatively or non-radiatively. It depends on the characteristics of the levels and on the interaction with the environment (bath). T is inversely proportional to the sum of the transition probabilities.

- In experiments, it is measured by monitoring the temporal decay of the luminescence signal for a large number N of molecules after a very fast optical excitation (non-stationary photoluminescence measurement).

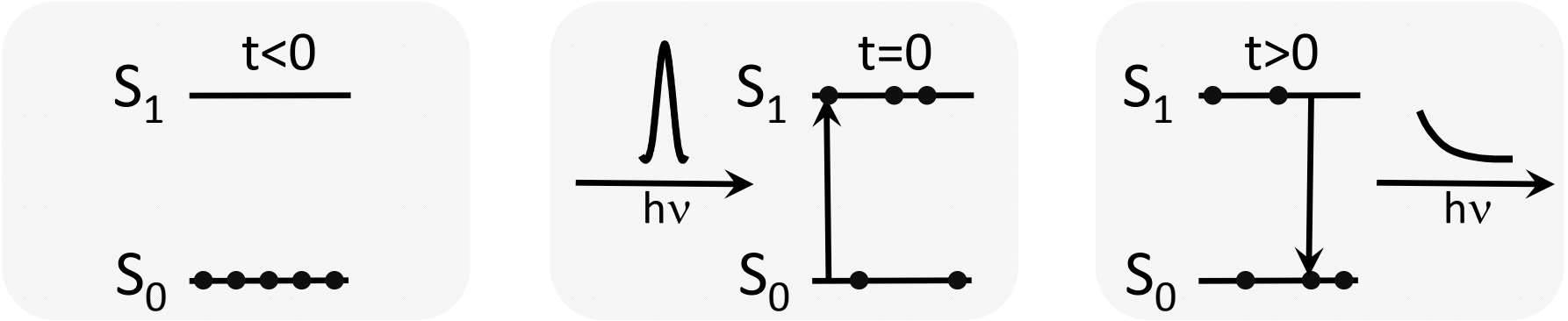
- Assuming a simple two-level system (ground and excited) the number of molecules in the excited state is given by:

$$N_e(t) = N_e(0)e^{-t/\tau_e} \qquad \tau_e := \text{lifetime}$$

Temporal decay of luminescence

The fluorescence intensity is proportional to the temporal variation of the number of excited molecules:

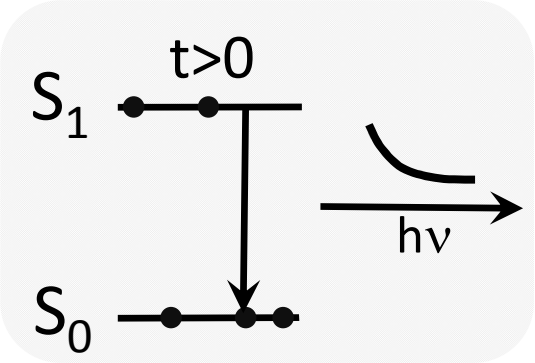
$$I_{FL} \propto -\frac{dN_e(t)}{dt} = \frac{N_e(0)}{\tau_e} e^{-t/\tau_e}$$



Temporal decay of luminescence

- The ***lifetime*** depends on all possible decay pathways from the excited to the ground state.
- In case of no quenching we get:

$$\tau_e = \frac{1}{k_R + k_{NR}}$$



- We define the radiative lifetime τ_R:

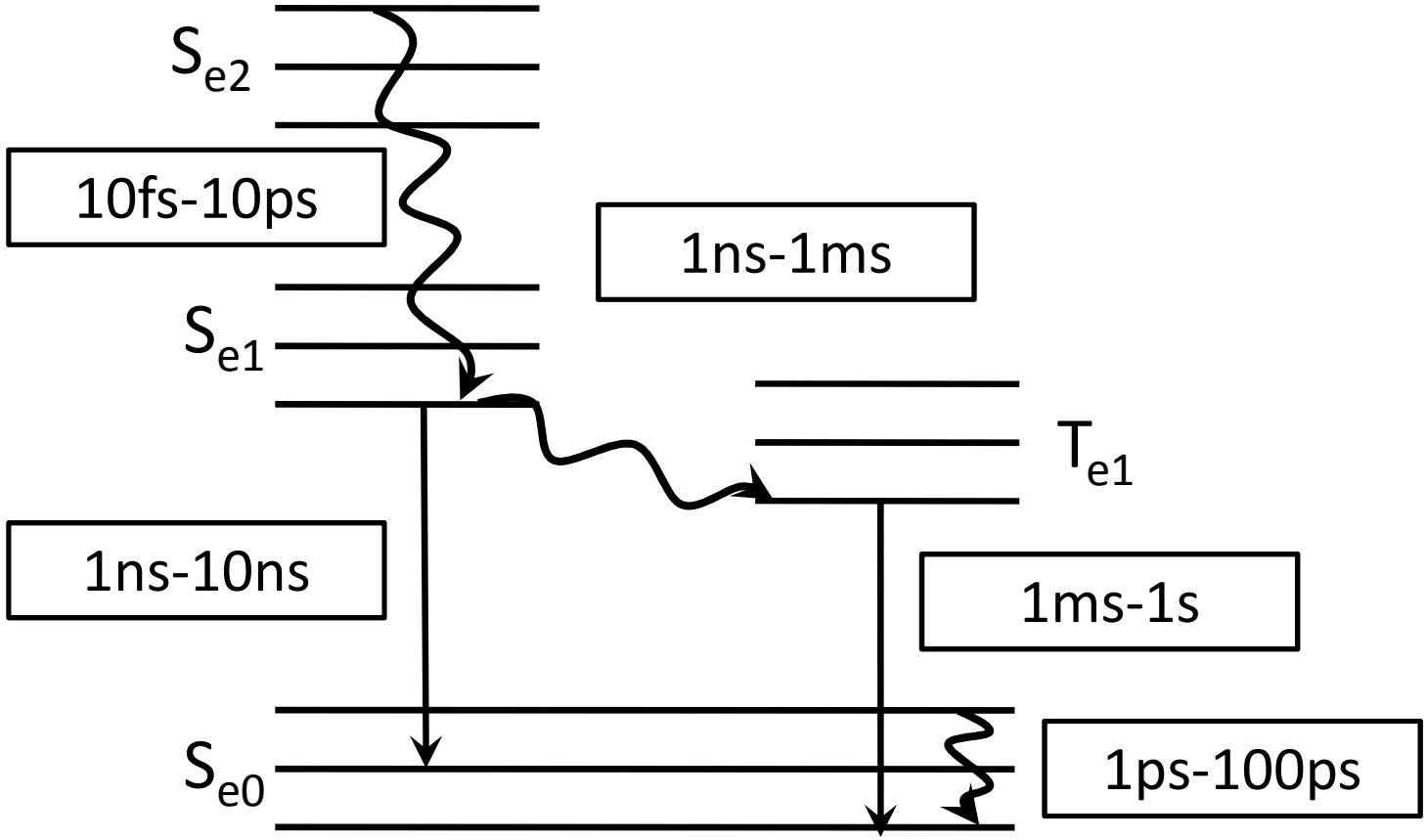
$$\tau_R = \frac{1}{k_R}$$

- The radiative lifetime can be retrieved from a simultaneous measurement of the quantum yield and of the lifetime:

$$\Phi = \frac{k_R}{k_R + k_{NR}} = \frac{\tau_e}{\tau_R} \quad \Rightarrow \quad \tau_R = \frac{\tau_e}{\Phi}$$

Temporal decay of luminescence

The characteristic lifetimes of the excited levels are typically of the order of:



Temporal decay of luminescence

1) In the presence of quenchers the lifetime depends on the concentration of quenchers:

$$\tau_e^Q = \frac{1}{k_R + k_{NR} + k_{NR}^Q [Q]}$$

It decreases when the concentration of quenchers increases:

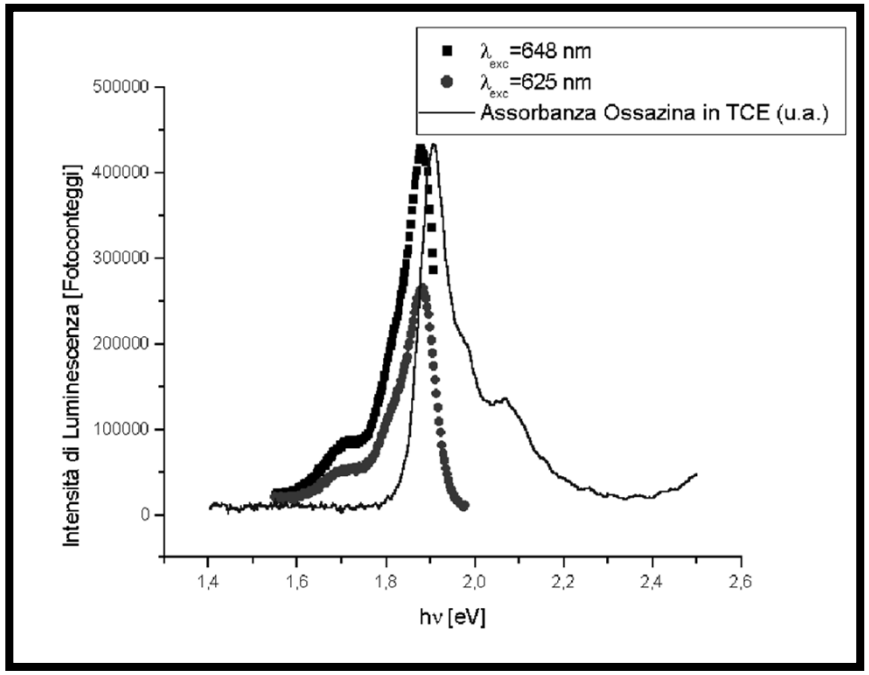
$$\frac{\tau_e}{\tau_e^Q} = \frac{k_R + k_{NR} + k_{NR}^Q [Q]}{k_R + k_{NR}} = 1 + \frac{k_{NR}^Q}{k_R + k_{NR}} [Q]$$

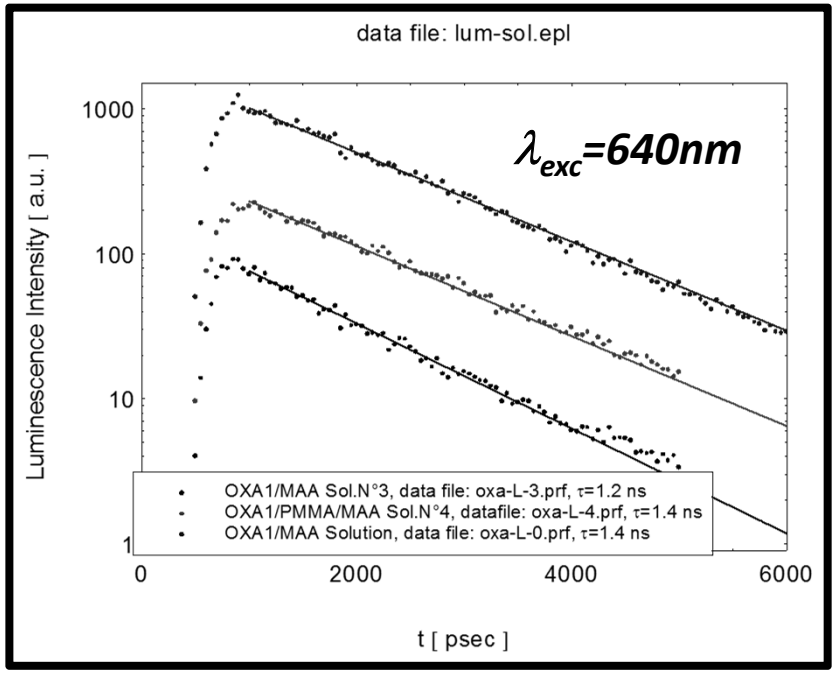
2) For a molecule with a system of levels that is more complicate than the simple two-level, the luminescence decay can be multi-exponential. As examples, if there are more molecular emitting species, if the quenching mechanism influences only some emitting sites, if there are aggregation phenomena.

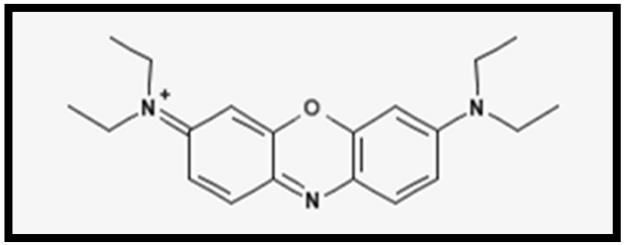
$$I_{FL}(t) = \sum_i A_i e^{-t/\tau_{e,i}} \qquad \tau_{e,i} := \text{lifetime of the i - th specie}$$

Temporal decay of luminescence

EXAMPLE Exponential decay of the luminescence of Oxazine 1







Oxazine 1

Temporal decay of luminescence

EXAMPLE Variation of the GFP lifetime as a function of the external local environment

Probing the local environment of green fluorescent protein (GFP) with fluorescence lifetime imaging (FLIM) and time-resolved fluorescence anisotropy imaging (tr-FAIM)

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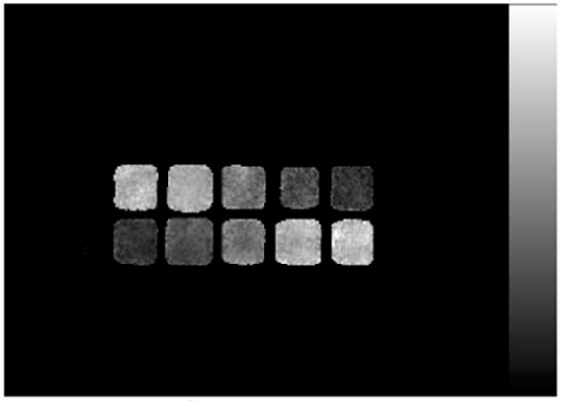
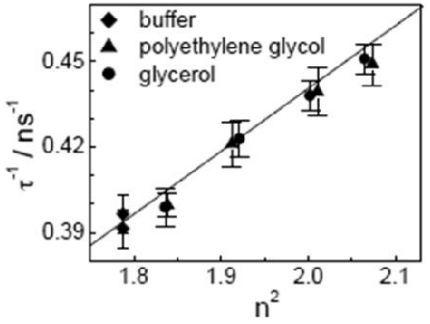
Abstract: Wide-field time-doma
imaging of green fluorescent pro

OCIS codes: (170.2520) Medical



a)



b)

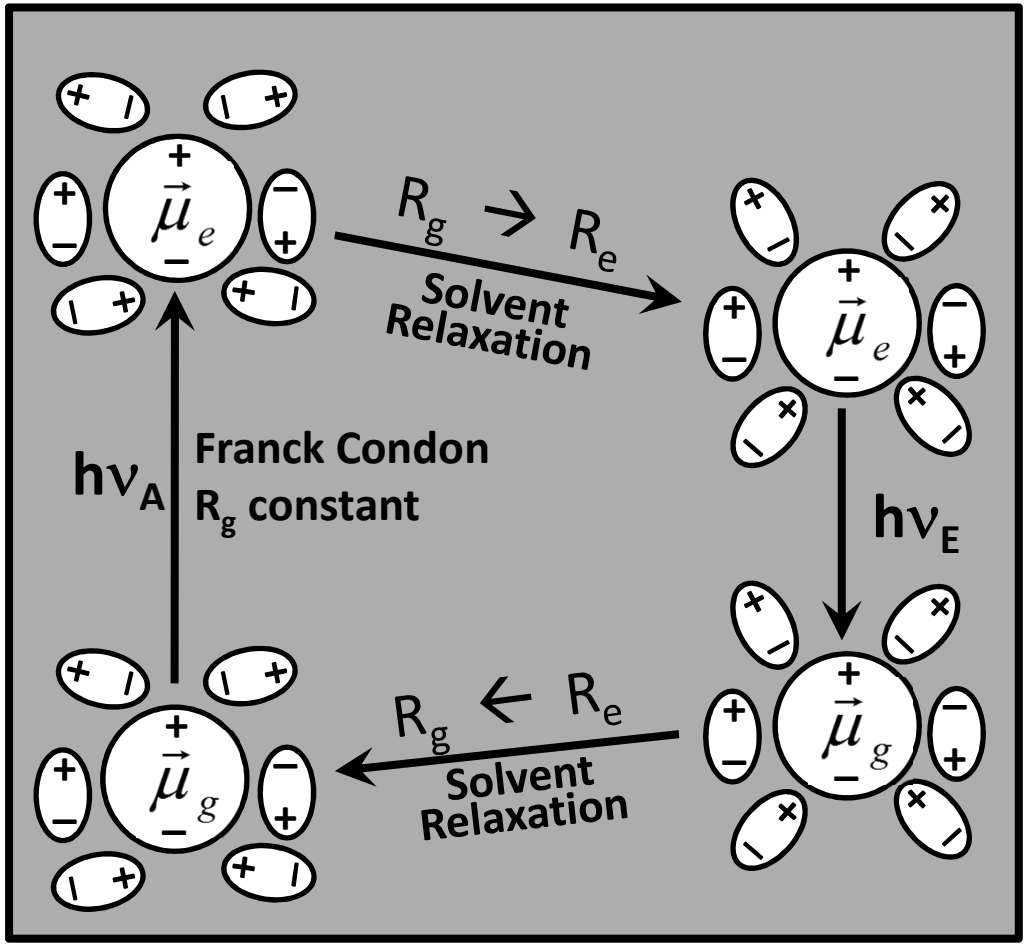
Fig 1. Multiwell plate FLIM of GFP. a) The fluorescence lifetime image. Each square represents a 4 mm by 4 mm well with a capacity of 125 µl. Top row: GFP in mixtures of aqueous buffer and polyethylene glycol, from left to right: buffer, 10% polyethylene glycol , 30% polyethylene glycol , 50% polyethylene glycol , 70% polyethylene glycol. Bottom row: GFP in mixtures of aqueous buffer and glycerol, from left to right: 70% glycerol, 50% glycerol, 30% glycerol, 10% glycerol, buffer. The grayscale represents the fluorescence lifetime of GFP over a range from 2.1 ns (dark) to 2.7 ns (light). A gradual decrease of the GFP fluorescence lifetime is evident as the glycerol and polyethylene glycol content, and thus the refractive index, is increased. Therefore, our time-domain FLIM system is sufficiently accurate to detect the GFP lifetime differences due to the refractive index. b) To demonstrate the applicability of the Strickler Berg formula, equation 1, the corresponding inverse fluorescence lifetimes of GFP are plotted versus the square of the refractive index.

Solvatochromism

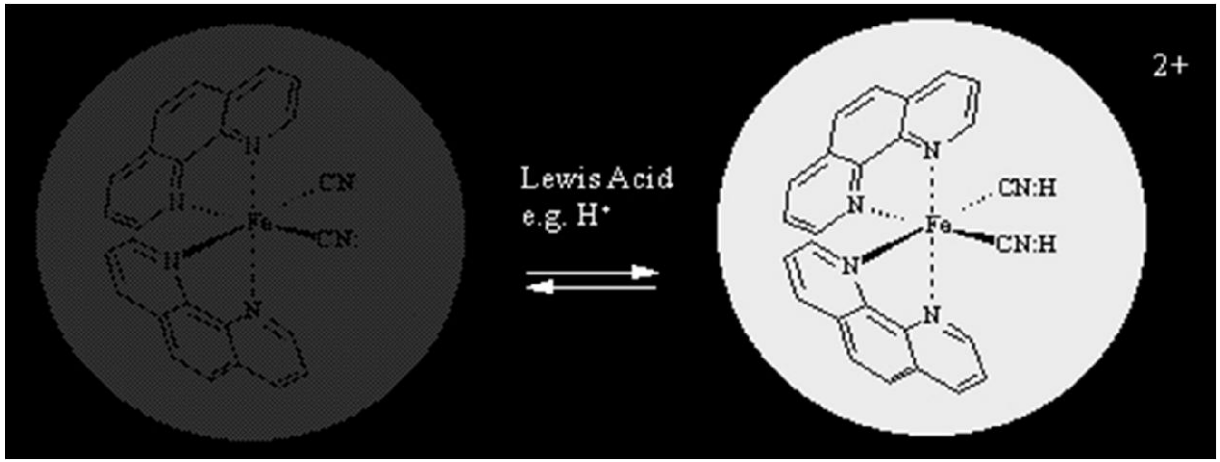
- The absorption and emission spectra of a molecule can change according to the polarity of the solvent.
- Since the polarity of the ground and excited states are different, the change of the polarity of the solvent leads to a change of the HOMO-LUMO energy gap.

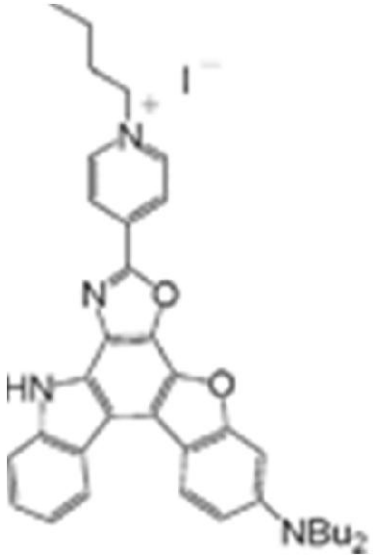
- During excitation the molecule changes instantaneously its dipole moment. The molecules of the solvent can take a short time (0.1-100ps) to rearrange themselves around the excited molecules.

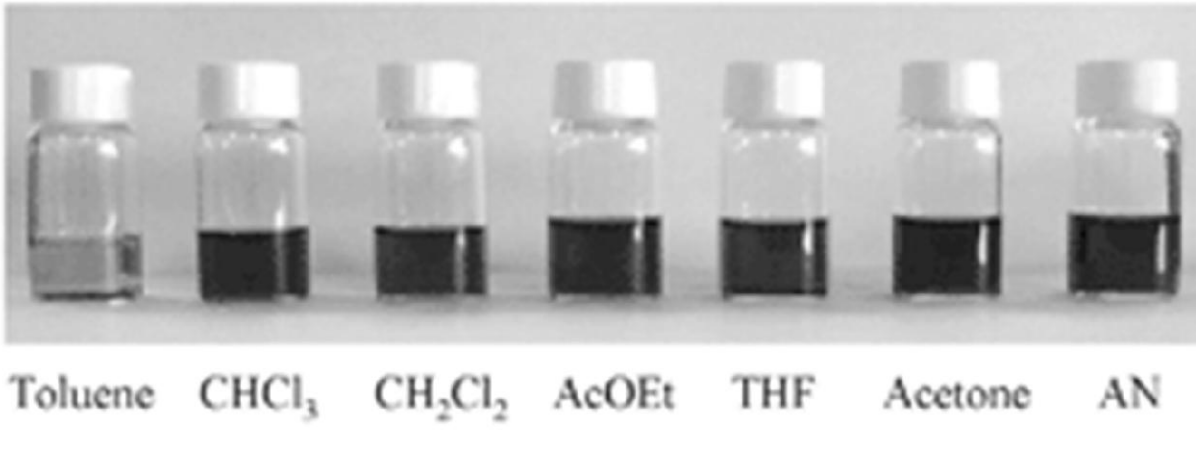
- The changes of the shape, intensity and position of the spectra can be a drect measurement of the interaction between the solute and the solvent.



Solvatochromism







Photobleaching

Photobleaching is the photo-chemical destruction of a chromophore. In microscopy it can complicate the observation of fluorescent molecules since these can be destroyed by the large light intensity that is necessary for the observation.

Chromophores can perform a limited number of excitation/decay cycles before losing their properties. Depending on the particular material, the chromophores can absorb/emit a maximum number of photons that differs from type to type and show a different ***lifetime***.

EXAMPLES (assuming the rate of 10^5 photons/s)

- Green fluorescent protein: 10^4-10^5 cycles; 0.1-1 s
- Typical organic dye: 10^5-10^6; 1-10 s
- CdSe / ZnS Quantum dot: 10^8; > 1000 min

NOTE The term ***lifetime*** used here must not be confused with the lifetime of the molecular levels that is measured in time resolved luminescence experiments.

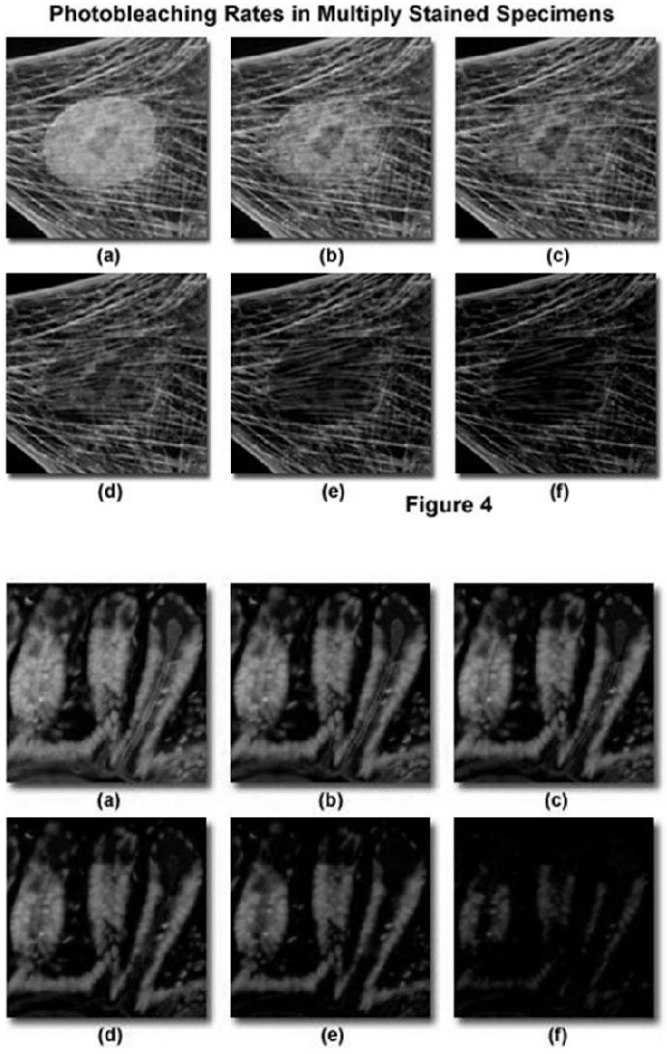


Figure 4