

# Advanced light microscopy

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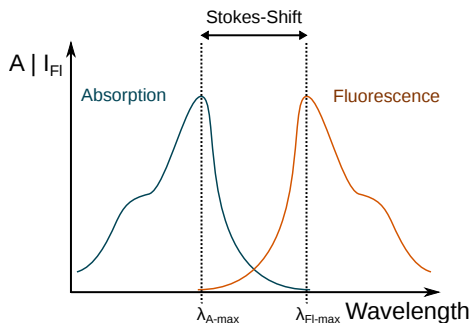
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## 1 Recapitulation

- Absorption- and emission spectra of fluorophores
- Stokes shift
- Franck-Condon Principle
- Quantum Yield
- Concurring Processes
- Fluorescence Lifetime
- Photobleaching and Quenching

# Absorption- and emission spectra of fluorophores

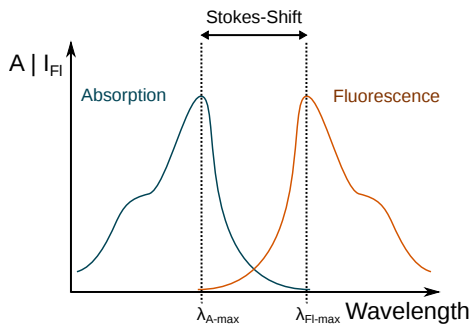
**Absorption and emission** of fluorescing molecules as a function of wavelength.



- Parameters to extract: **Central wavelength** of absorption and emission; **Stokes shift**
- **Mirror rule**: Spectra look like mirror images of one another

## Stokes shift

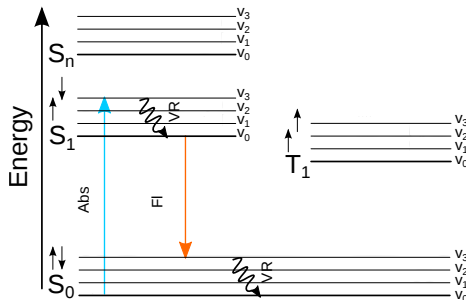
The difference between the **central wavelength of absorption** and the **central wavelength of emission** is termed the **Stokes shift**.



- Larger Stokes shifts allow for better chromatic filtering of signals.
- Dichroic Mirrors can separate the excitation light pathway from the emission light pathway.

## Stokes shift explained

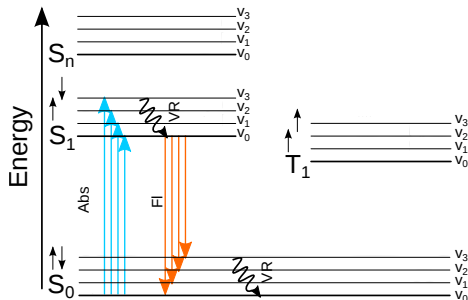
The **Stokes shift** can easily be explained on a **Jablonski diagramm** considering **Kasha's Rule**.



- A Jablonski diagramm displays different possible energy levels of a molecule and the transitions between them.
- **Kasha's Rule:** Fluorescence occurs from the lowest vibrational energy level of the first excited singlet state  $S_1$ .

## Stokes shift explained

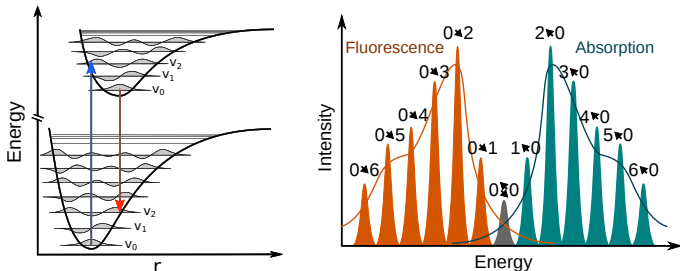
The **Stokes shift** can easily be explained on a **Jablonski diagramm** considering **Kasha's Rule**.



- **Kasha's Rule:** Fluorescence occurs from the lowest vibrational energy level of the first excited singlet state  $S_1$ .
- The **Mirror Rule** can be explained by transitions to different vibrational energy levels, considering the **Franck-Condon Principle**.

# Franck-Condon Principle

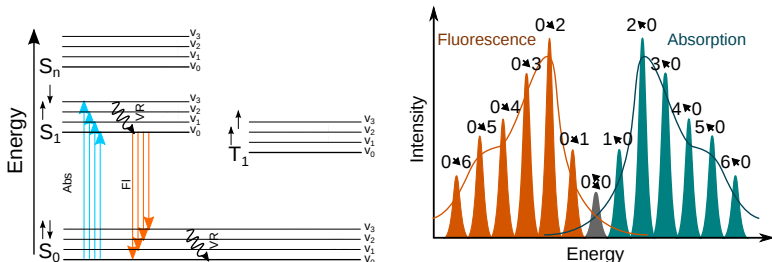
The **Franck-Condon Principle** explains that mirrored transitions have similar probabilities.



- Electronic transitions occur instantly in relation to the movement of nuclear movement inside the molecule.
- Maximum overlap indicates the most probable transition.

## Mirror Rule

**Franck-Condon Principle** describes the probabilities of electronic transitions to different vibrational energy levels.

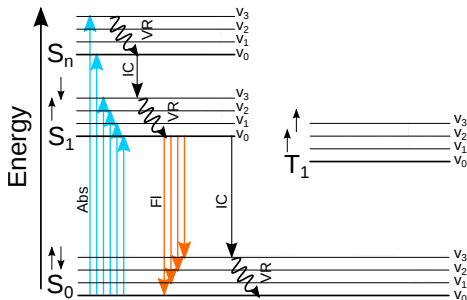


- Transitions with a high probability contribute to higher intensities in absorption and emission spectra.
- In liquid or solid state materials the sharp bands of the spectrum are broadened inhomogeneously. Well known shape of fluorophore spectra.



## Quantum Yield

The **Quantum Yield** is the ratio of emitted photons and absorbed photons of a fluorescent molecule



$$\Phi_f = \frac{k_r}{k_r + k_{nr}}$$

- $\Phi_f$  is calculated by the rate constant  $k_r$  for **radiative processes** and the rate constant  $k_{nr}$  for **non radiative processes**.
- Inner conversion (IC) is a non radiative process concurring to fluorescence.

# Concurring Processes

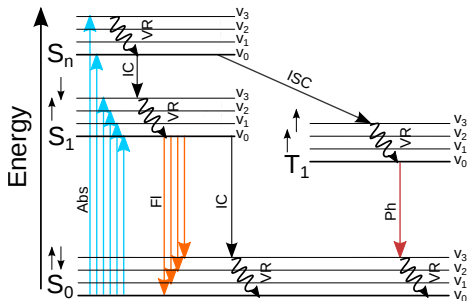
There are several **concurring processes to fluorescence**.

Rate constants of different transition pathways

Fluorescence	$S_1 \rightarrow S_0$	$k_f$	$10^7 - 10^9$	$s^{-1}$
Internal Conversion	$S_n   T_n \rightarrow S_1   T_1$	$k_{ic}$	$10^{10} - 10^{14}$	$s^{-1}$
	$S_1 \rightarrow S_0$		$10^6 - 10^7$	$s^{-1}$
Vibrational Relaxation	$S_{1;v=n} \rightarrow S_{1;v=0}$	$k_{vr}$	$10^{10} - 10^{12}$	$s^{-1}$
Singlet-Singlet-Absorption	$S_1 \rightarrow S_n$	$k_{exc}$	$10^{15}$	$s^{-1}$
Intersystem Crossing	$S_1 \rightarrow T_1$	$k_{isc}$	$10^5 - 10^8$	$s^{-1}$
	$S_n \rightarrow T_n$			
	$T_n \rightarrow S_n$			
Phosphorescence	$T_1 \rightarrow S_0$	$k_p$	$10^2 - 10^3$	$s^{-1}$
Triplet-Triplet-Absorption	$T_1 \rightarrow T_n$	$k_{exc}$	$10^{15}$	$s^{-1}$

## Fluorescence Lifetime

The average time a molecule spends in its first excited singlet state before spontaneous fluorescence emission occurs is termed **fluorescence lifetime**



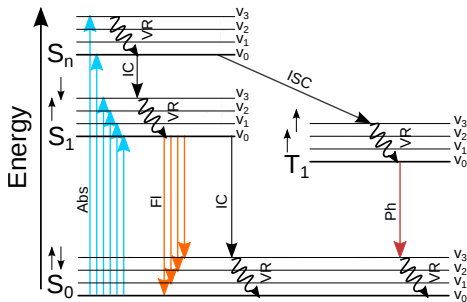
$$\tau_f = \frac{1}{k_r + k_{nr}}$$

$$I(t) = I_0 \exp\left(\frac{-t}{\tau_f}\right)$$

- The fluorescence lifetime  $\tau_f$  is the inverse sum of rate constants for radiative and non radiative relaxations ( $k_r$ ;  $k_{nr}$ )
- The initial fluorescence intensity of fluorescent molecules  $I_0$  exhibits an exponential decay, with the lifetime as decay constant.

# Photobleaching

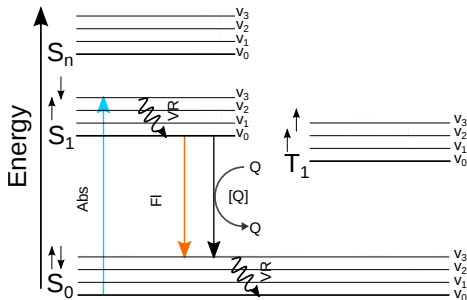
**Photobleaching** is a non reversible process in which the fluorescent molecule loses its ability to emit fluorescence photons



- There are several pathways for photobleaching i.e.:
- Ionization of the molecule.
- Population of the triplet state.

# Quenching

## Collisional quenching



## Stern-Volmer-Equation

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

- The Stern-Volmer-Equation describes the dependency of quenched fluorescence intensity  $F$  and the quencher concentration  $[Q]$ .
- If the molecule is sensitive to the quencher the Stern-Volmer constant  $K$  takes large values.
- A possible quencher in aqueous solutions is molecular oxygen.