Part A.8
Fluorescence Spectroscopy I

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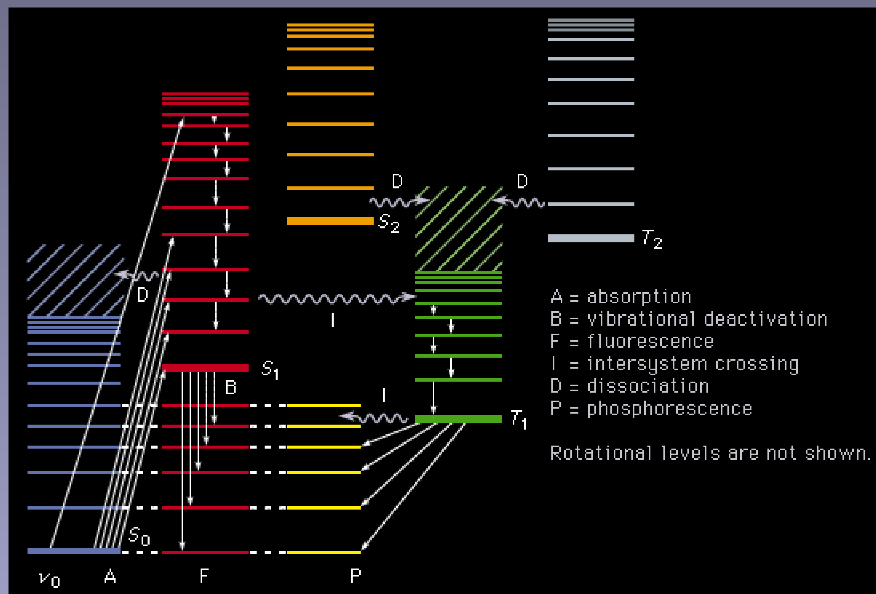


Fluorescence: absorption and emission processes
Fluorescence life time
Fluorescence quantum yield
Solvent effects
Time-resolved fluorescence
Fluorescence anisotropy

Fluorescence spectroscopy: Introduction

Fluorescence spectroscopy is a valuable technique that allows insights into structural/flexible properties of macromolecules, including proteins. The use of dyes enhances applicability to monitoring and visualisation tasks.

Intrinsic protein fluorescence

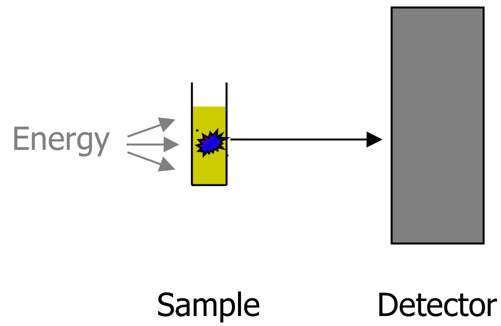
- Conclusions on location of fluorophores (inside/outside, close together/far apart)
- Monitoring of protein denaturation
- Measurement of distances at molecular level
- Resolution of distinct conformations of a fluorophore
- Rotational correlation times: rotational movements, oligomerisation behaviour

Extrinsic fluorescence

- Surface mapping of proteins
- Fluorimetric assays: ligand binding, membrane binding, ion flux
- Determination of diffusion coefficients
- Fluorescent molecules as reporters: visualisation on the molecular level

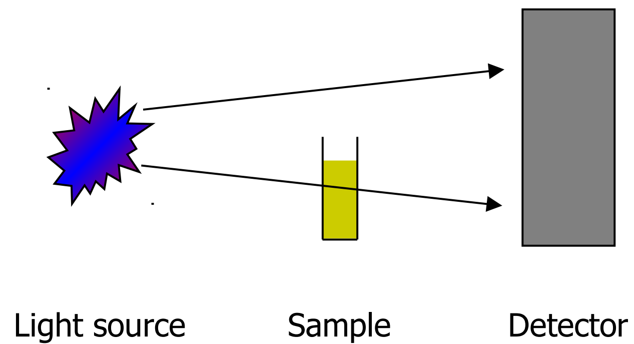
Light emission and absorption

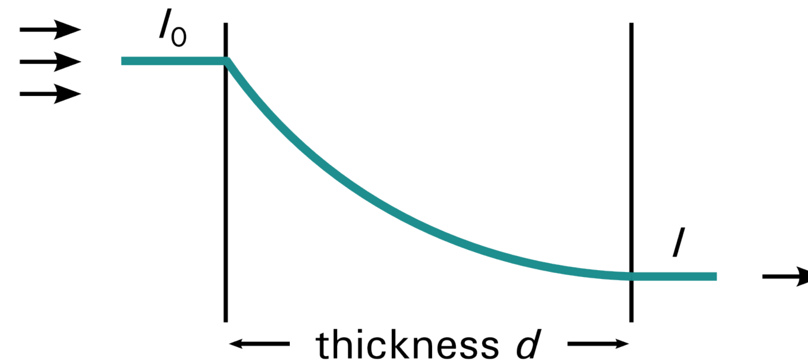
Emission



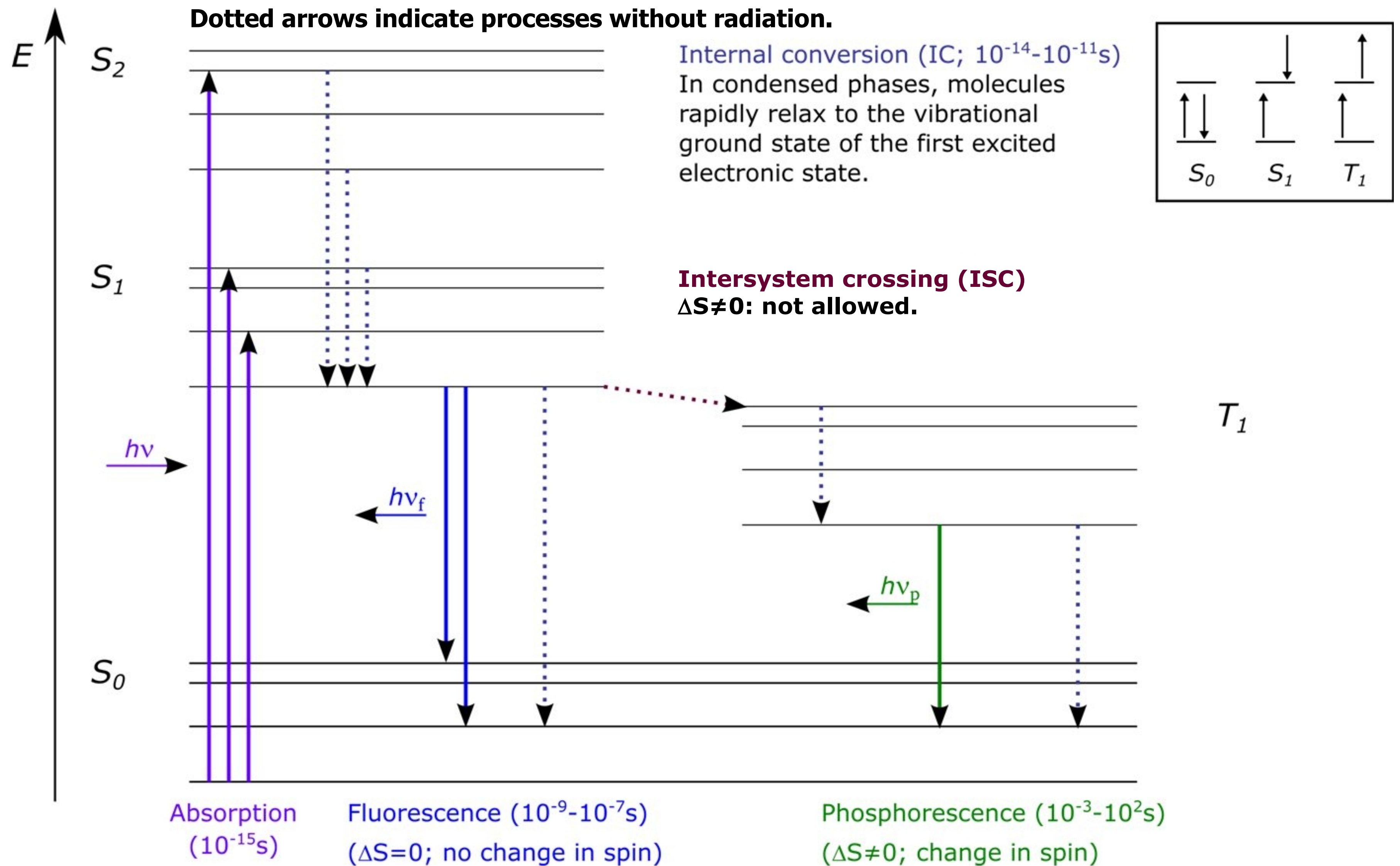
Fluorescence spectroscopy is an emission spectroscopy

Absorption





Jablonski diagram



Fluorescence absorption/emission

Properties of fluorescence emission

The absorption spectrum reflects the vibrational levels of the excited electronic state.

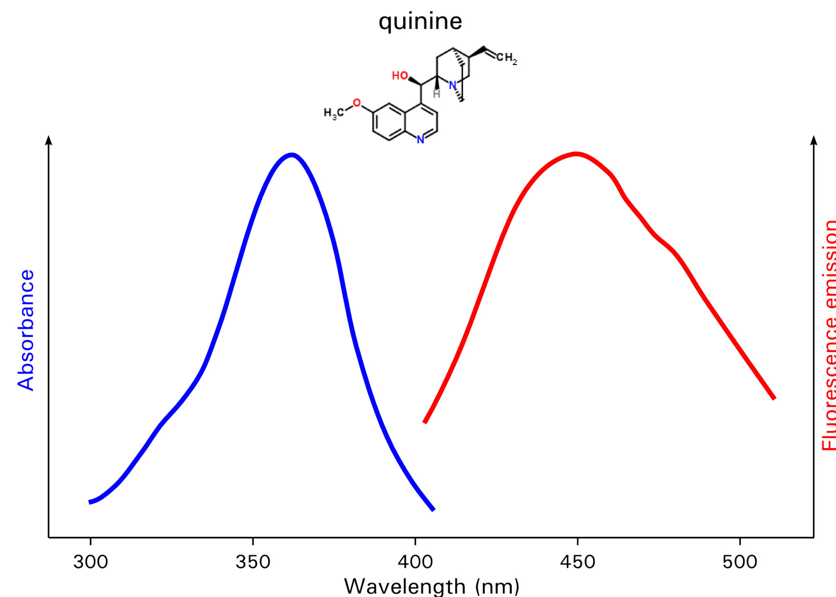
The fluorescence spectrum reflects the vibrational levels of the ground state.

Stokes shift: $\lambda_{emission} > \lambda_{absorption}$

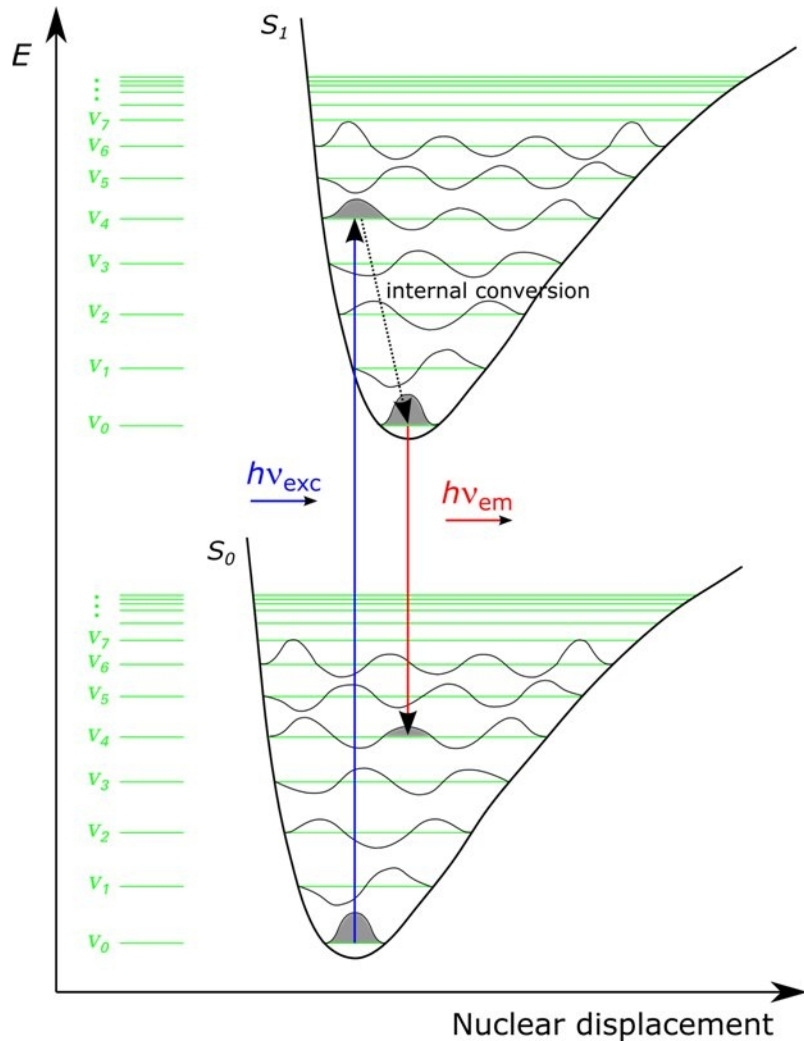
The spectrum of the emitted light is invariant with respect to the excitation wavelength, because of the fast relaxation to the lowest vibrational level of S_1.
(Exception: azulene, it can emit from both S_1 and S_2.)

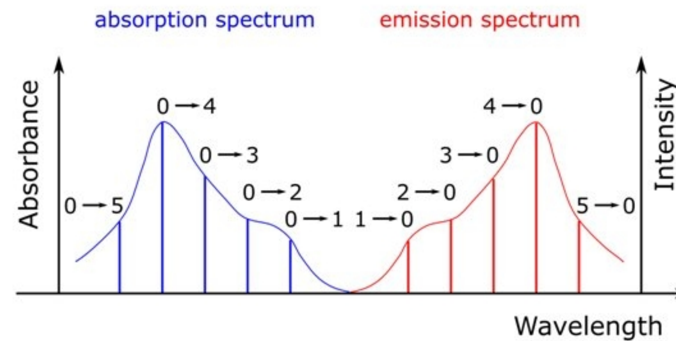
The mirror image rule

The fluorescence emission often appears as the mirror image of the absorption spectrum.



The Franck-Condon principle



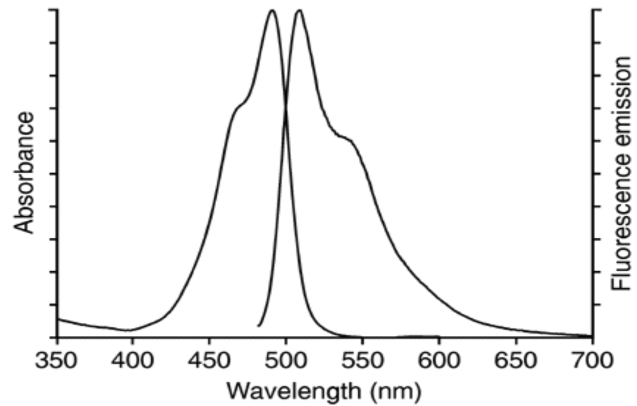


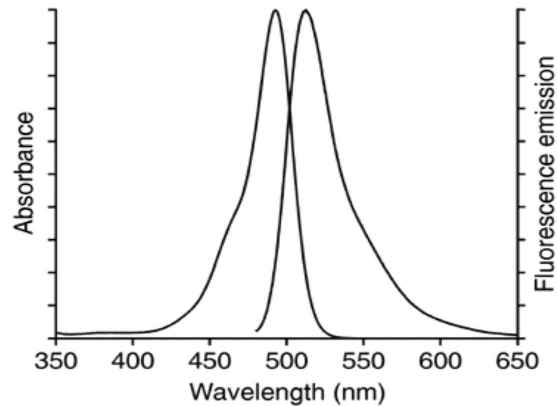
The **Franck-Condon factor** (vibrational overlap integral) is the same for absorption and fluorescence.

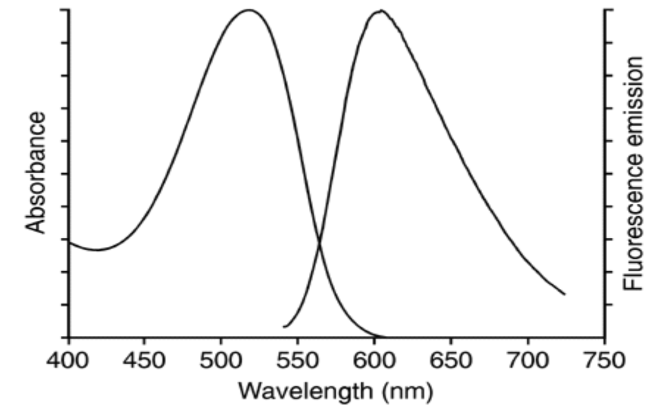
Exceptions:

- very long lived S_1 state: emission occurs from a different geometry
- reactions from the excited state

Fluorescence absorption/emission: Examples



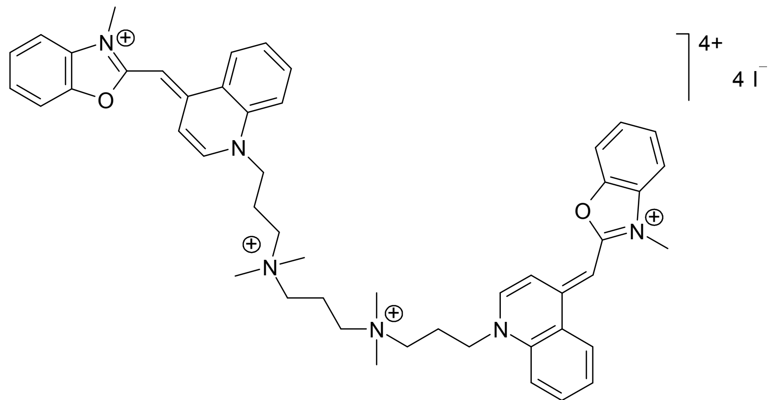


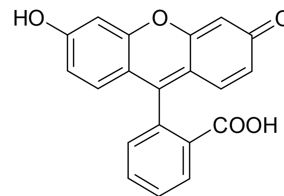


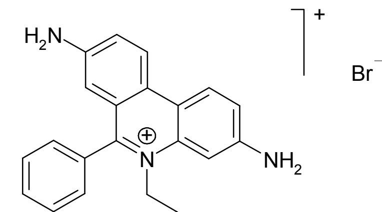
YOYO-1 bound to DNA

fluorescein

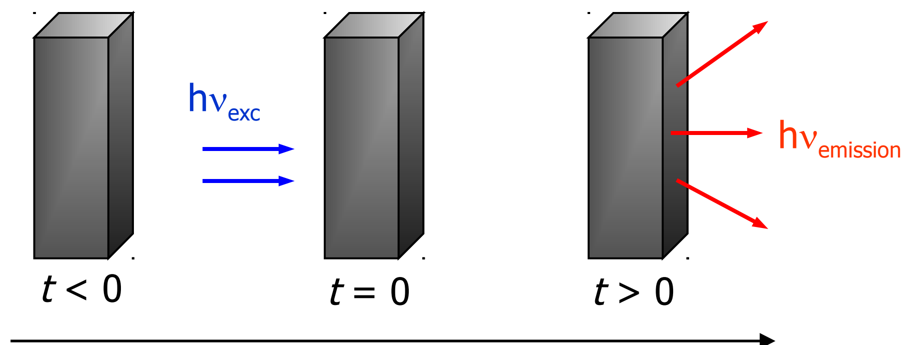
ethidium bromide bound to DNA



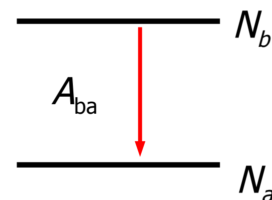




Fluorescence life time

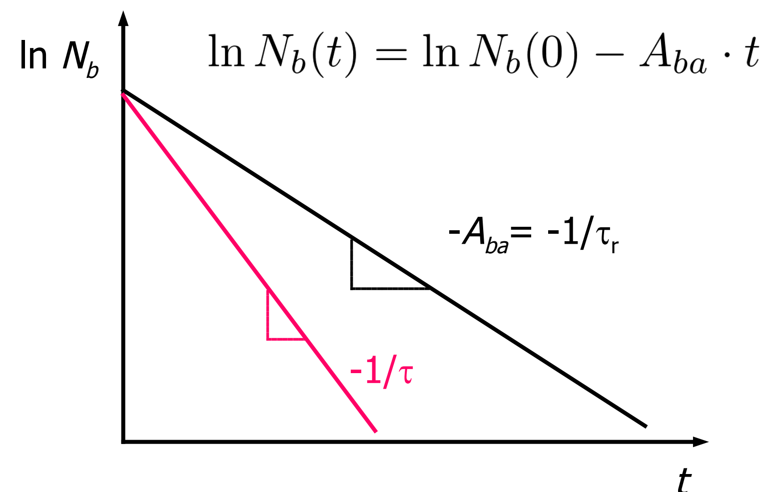


Fluorescence radiative rate A_{ba}



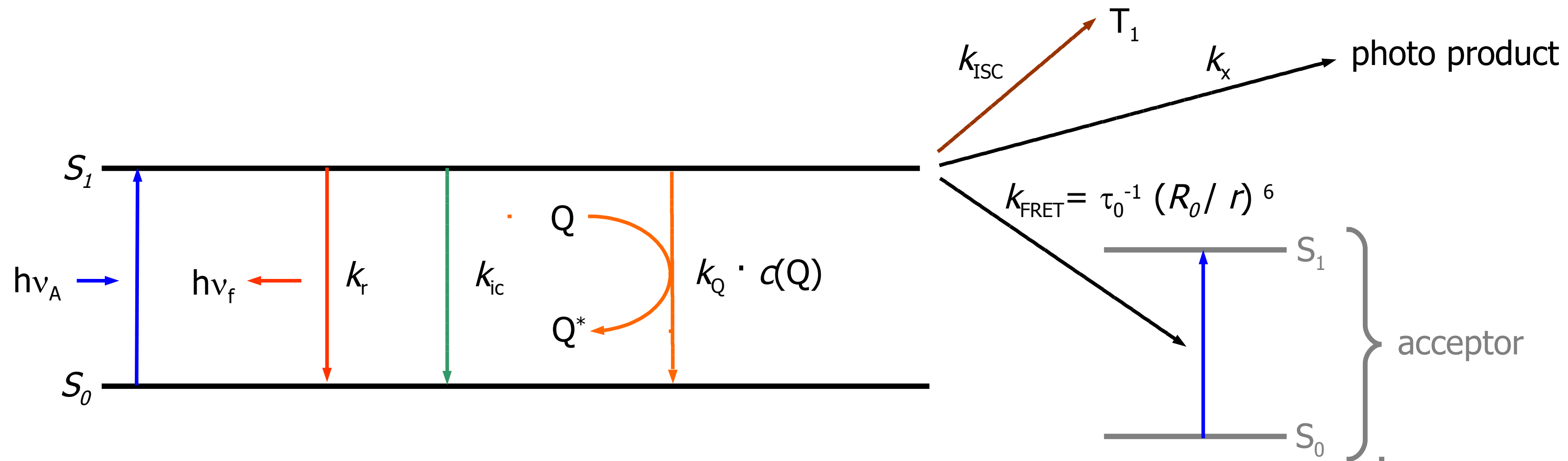
$$-\frac{dN_b}{dt} = A_{ba} \cdot N_b \;\text{=>}\; N_b(t) = N_b(0) \cdot e^{-A_{ba} \cdot t}$$

Radiative life time



In general, the life time of the excited state is shorter than τ_r due to other deactivation pathways from the excited state S_1.

Fluorescence quantum yield: Deactivation processes



Rate constants for different processes:

$\boldsymbol{k_r}$	fluorescence radiative rate
$\boldsymbol{k_{IC}}$	internal conversion (collision with solvent and internal vibrational modes); increases with temperature
$\boldsymbol{k_Q}$	quenching (collision with solute molecules, H_2O, O_2)
$\boldsymbol{k_{FRET}}$	fluorescence resonance energy transfer (FRET)
$\boldsymbol{k_x}$	reaction from excited state
$\boldsymbol{k_{ISC}}$	intersystem crossing (forbidden)

Fluorescence quantum yield

Apparent fluorescence rate constant:

$$k = k_r + k_{IC} + k_{ISC} + k_x + k_Q \cdot c(Q) + k_{FRET} + \ldots$$

Life time of excited state/fluorescence:

$$-\frac{dc_{S_1}}{dt} = k \cdot c_{S_1} \quad \Rightarrow \quad c_{S_1}(t) = c_{S_1}(0) \cdot e^{-\frac{t}{\tau_f}}$$

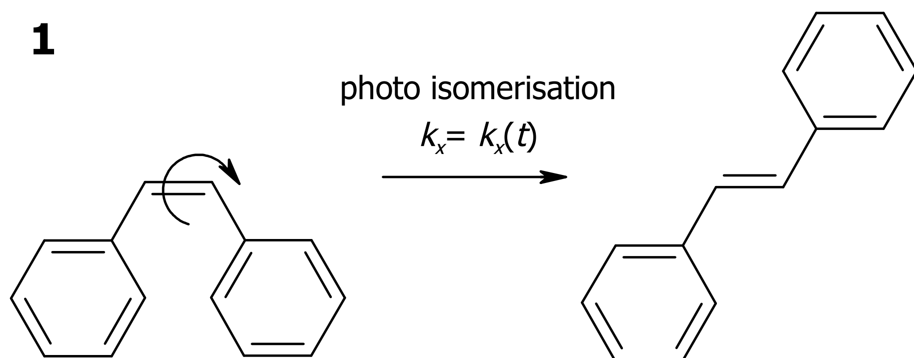
$$\tau_f = \frac{1}{k_r + k_{IC} + k_{ISC} + k_x + k_Q \cdot c(Q) + k_{FRET} + \ldots}$$

Quantum yield:

$$\Phi = \frac{photons\ emitted}{photons\ absorbed} = \frac{k_r}{k} = \tau_f \cdot k_r = \frac{\tau_f}{\tau_r}$$

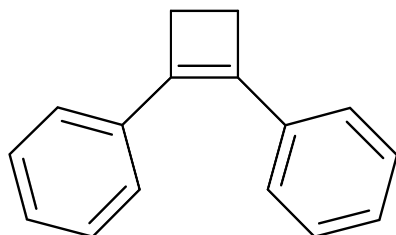
Fluorescence life time and quantum yield: Effects of rigidity and environment

1



ϕ(298 K, organic solvent) = 0.05
ϕ(298 K, glycerol) = 0.15
ϕ(77 K, organic solvent) = 0.75

2

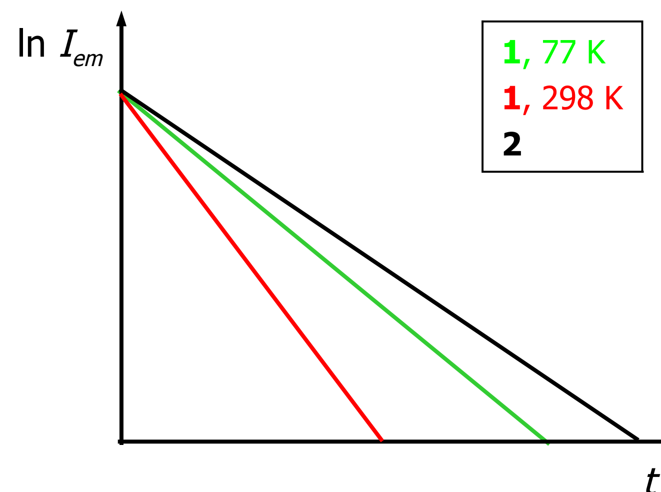


ϕ(298 K) = 1
ϕ(77 K) = 1

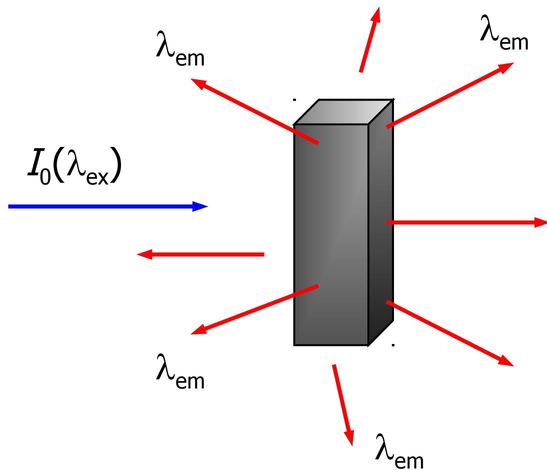
Quantum yield

$$\Phi = \frac{photons\ emitted}{photons\ absorbed} = \frac{k_r}{k} = \tau_f \cdot k_r = \frac{\tau_f}{\tau_r}$$

Radiative life time



Measurement of fluorescence quantum yields



fluorescence quantum yield — fraction of intensity emitted at that particular wavelength

$$I_f(\lambda_{em}) = I_{abs}(\lambda_{exc}) \cdot \Phi_f \cdot f(\lambda_{em}) \cdot K$$

measured intensity of fluorescence at λ_{em} — absorbed intensity at λ_{ex} — fraction of total fluorescence that is detected

$$I_{abs}(\lambda_{exc}) = I_0(\lambda_{exc}) \cdot (1 - 10^{-A(\lambda_{exc})})$$

If $A \rightarrow 0$:

$$I_{abs}(\lambda_{exc}) \approx 2.303 \cdot I_0(\lambda_{exc}) \cdot A(\lambda_{exc})$$

=>

$$\int_{\lambda_{em}} I_f = I_{abs}(\lambda_{exc}) \cdot \Phi_f \cdot K \approx 2.303 \cdot I_0(\lambda_{exc}) \cdot A(\lambda_{exc}) \cdot \Phi_f \cdot K$$

If we measure the sample and a standard under the same experimental conditions, at constant λ_{ex}:

$$\frac{\int_{\lambda_{em}} I_f^{sample}}{\int_{\lambda_{em}} I_f^{standard}} = \frac{A^{sample}(\lambda_{exc})}{A^{standard}(\lambda_{exc})} \cdot \frac{\Phi_f^{sample}}{\Phi_f^{standard}}$$

Important: the index of refraction of the two solvents (sample and standard) must be the same!

Standards:

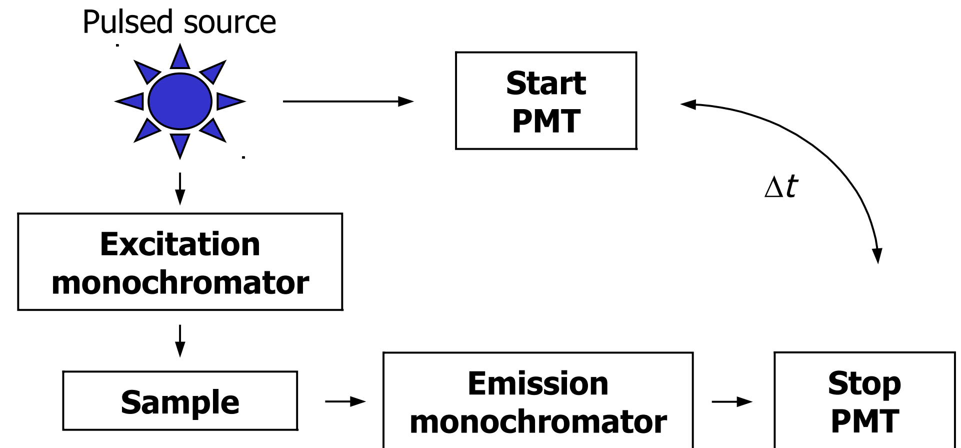
Quinine sulphate in H_2SO_4 (1 N): ϕ_f =0.55

Fluorescein in NaOH (0.1 N): ϕ_f =0.93

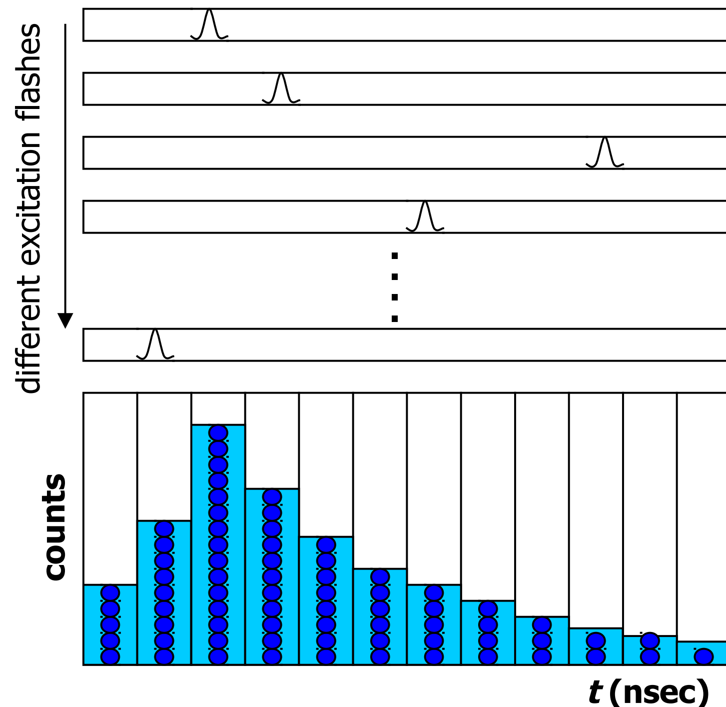
Measurement of fluorescence life times

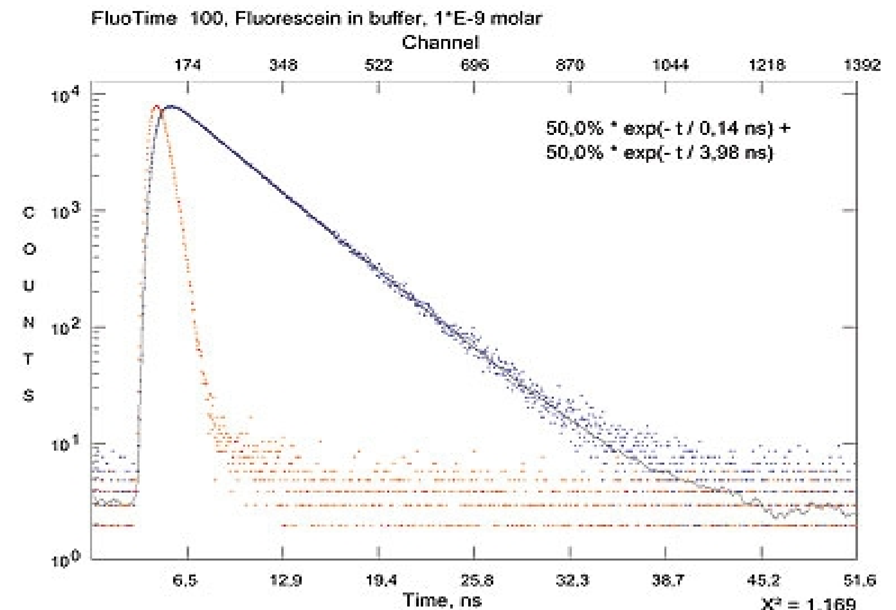
Time correlated single photon counting

- single photon emission follows probability distribution
- a large number of excitation flashes generate multiple single photon emission events
- counting the single photons in a time-dependent manner leads to construction of a probability distribution



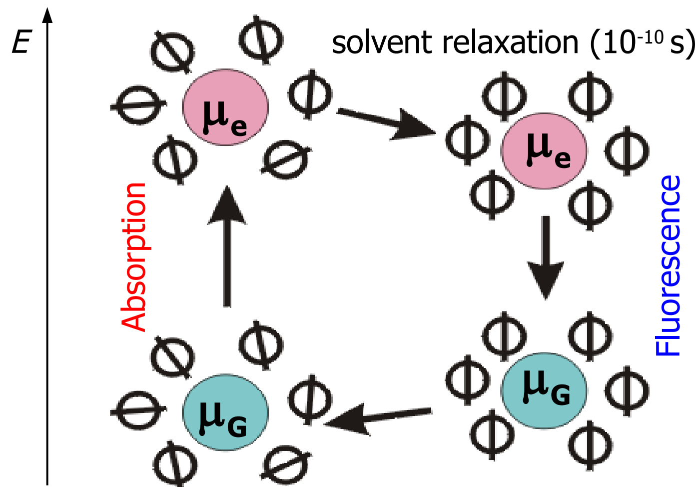
Probability distribution





Protein fluorescence: Solvent effects

General solvent effects



General, empirical rules to interpret fluorescence spectra of proteins:

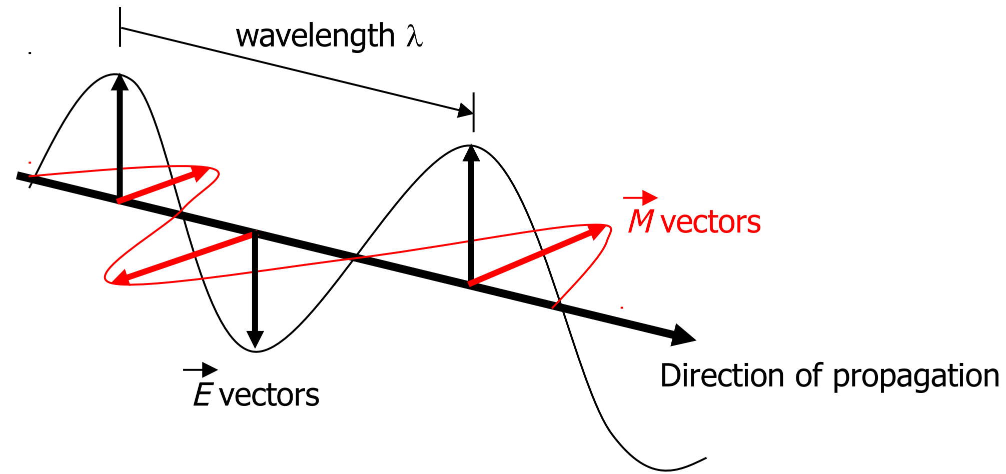
- The changing exposure of a fluorophore from hydrophobic to hydrophilic environment causes a **bathochromic (red) shift** of the emission peak.
- This is accompanied by a decrease in intensity.
- The changing exposure of a fluorophore from hydrophilic to hydrophobic environment causes a **hypsochromic (blue) shift** of the emission peak.
- Latter process is accompanied by an increase in emission intensity.

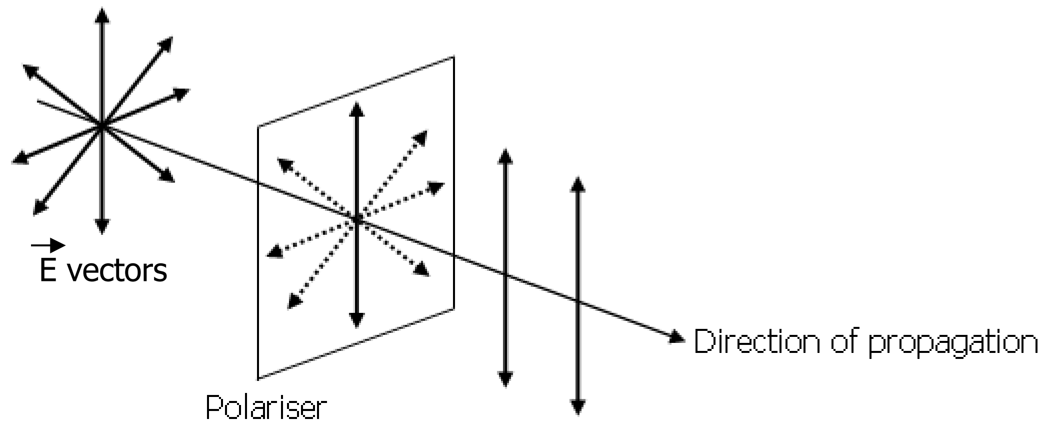
Similar to absorption, emission processes might also show spectral shifts (bathochromic or hypsochromic shifts) depending on variation of the solvent.

The variation might be due to different polarity, dielectric constant or polarisability of the solvent.

Fluorescence anisotropy

Polarised light

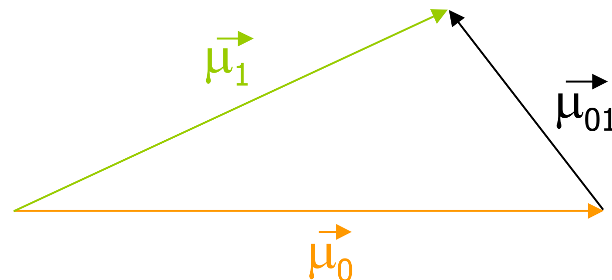




The transition dipole momentum

Absorption of light excites the chromophore and changes distribution of electric charge; the dipole momentum changes accordingly.

The **transition dipole momentum** is the vector difference between the momentum in the ground and the excited state.

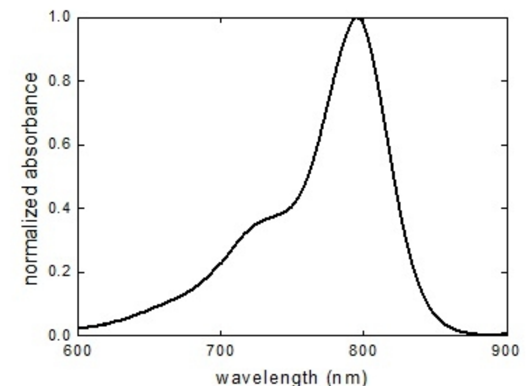


Dipole strength: $D_{01} = |\vec{\mu}_{01}|^2$

The transition dipole momentum is a measure for transition probability.

$D_{01} \rightarrow 0$: forbidden transitions

$D_{01} \rightarrow 1$: allowed transitions



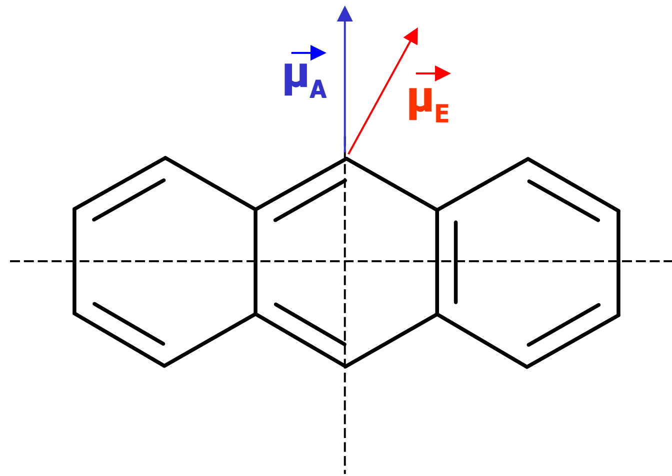
The plot of absorption probability against wavelength is called **absorption spectrum**.

Fluorescence anisotropy

Fluorescence emission of a fluorophore is characterised by wavelength and direction of polarisation.

In analogy to absorption, an emission transition dipole momentum $\vec{\mu}_E$ can be defined.

The absorption and emission transition dipole momentum each have a defined orientation within a certain molecule; $\vec{\mu}_E$ is usually tilted against $\vec{\mu}_A$ by 10° – 40°.

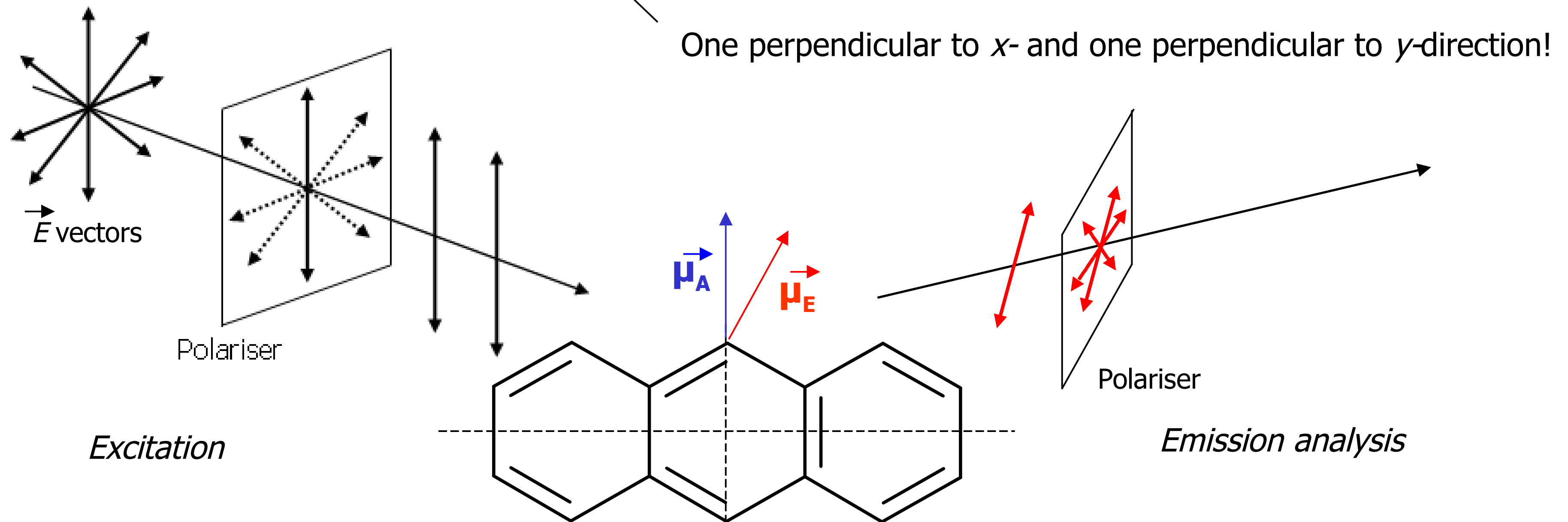


Fluorescence anisotropy

An incident linearly polarised beam allows for measurement of fluorescence emission perpendicular and parallel to the plane of polarisation (of the incident beam).

Polarisation and **Anisotropy**:

$$P = \frac{I_{\updownarrow} - I_{\leftrightarrow}}{I_{\updownarrow} + I_{\leftrightarrow}} \qquad A = \frac{I_{\updownarrow} - I_{\leftrightarrow}}{I_{\updownarrow} + 2I_{\leftrightarrow}}$$



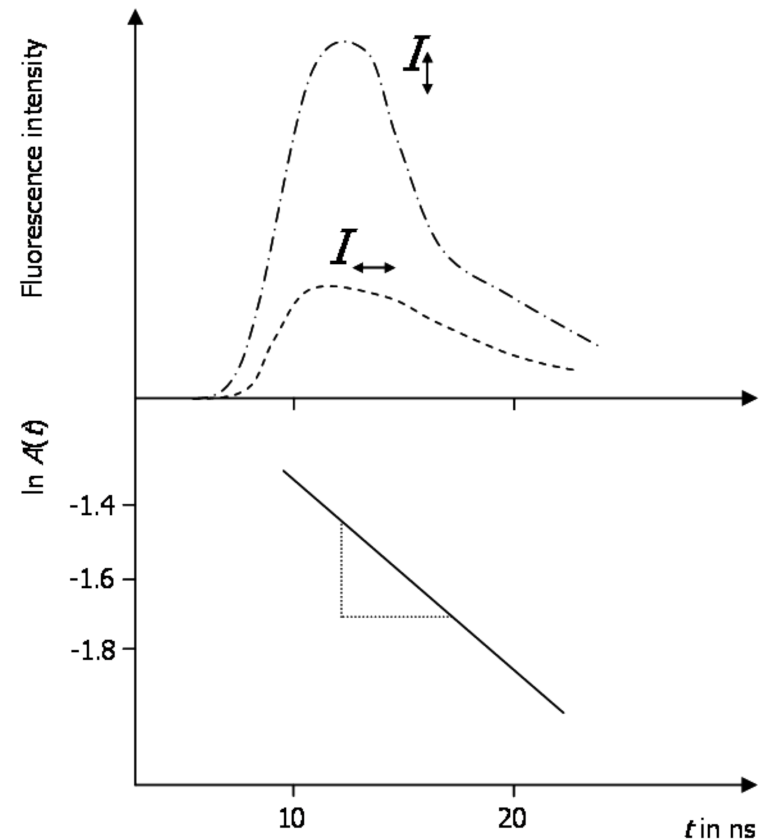
Fluorescence anisotropy

Analysis of fluorescence polarisation enables conclusions as to:

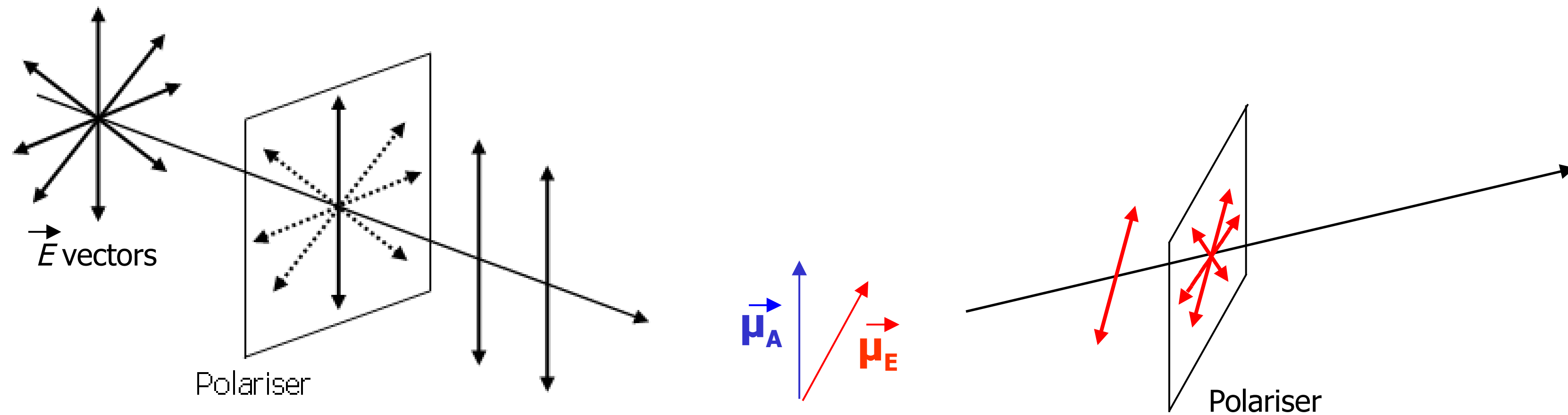
- Rotation mobility (rotation correlation time)
- Orientation
- Viscosity of environment

Time-resolved measurement of fluorescence in an orientation parallel and perpendicular to the linearly polarised excitation beam: Anisotropy decreases exponentially with time. From the slope of ln*A*, the rotation correlation time ϕ is accessible.

$$A = \frac{I_{\updownarrow} - I_{\leftrightarrow}}{I_{\updownarrow} + 2 \cdot I_{\leftrightarrow}} \qquad A(t) = A_0 \cdot e^{\frac{-t}{\phi}}$$



Fluorescence anisotropy



The intrinsic anisotropy (A_0) is usually measured by embedding the fluorophore in a frozen polyol.

If the fluorophores are free to move (e.g. in solution), the degree of polarisation of the emitted light is less, since the molecules can change their orientation before re-emitting photons.

The ratio of polarisation of incident and emitted light thus depends on how quickly the fluorophore orientation changes (rotational life time ϕ) compared to the fluorescence life time (τ).

$$\frac{A_0}{A} = 1 + \frac{\tau}{\phi} \qquad A = \frac{I_{\updownarrow} - I_{\leftrightarrow}}{I_{\updownarrow} + 2 \cdot I_{\leftrightarrow}} \qquad A(t) = A_0 \cdot e^{\frac{-t}{\phi}}$$

The rotational life time comprises the tumbling of the entire molecule, as well as orientation changes of the fluorophore itself.

Fluorescence anisotropy

Binding constants
Preferably, the monitored fluorophore should be bound to the smaller molecule, since the rate at which that molecule tumbles can decrease significantly when it is bound tightly to a larger molecule.

Folding kinetics
If the fluorophore is bound to a relatively large molecule like a protein, RNA or DNA, the change in the mobility accompanying folding can be used to study the dynamics of folding.

Kinetics of reactions that cause a change in the rotational time.

Viscosity
When combining fluorescence anisotropy with microscopy, the local viscosity of cytosol or membranes can be studied (membrane microstructure, relative concentrations of various lipids, etc).

Further reading

Text books and reviews
Brown M.P. & Royer C. (1997) Fluorescence spectroscopy as a tool to investigate protein interactions. Current Opinion in Biotechnology 8, 45-49.

Groemping Y. & Hellmann N. (2005) Spectroscopic methods for the determination of protein interactions. Current Protocols in Protein Science, Chapter 20, Unit 20.8.

Hofmann A., Simon A., Grkovic T. & Jones M. (2014) Methods of Molecular Analysis in the Life Sciences. Cambridge University Press.

Hwang L.C. & Wohland T. (2007) Recent advances in fluorescence cross-correlation spectroscopy. Cell Biochemistry and Biophysics 49, 1-13.

Jameson D.M. & Ross J.A. (2010) Fluorescence polarisation /anisotropy in diagnostics and imaging. Chemical Reviews 110, 2685-2708.

Lakowicz J.R. (1999) Principles of Fluorescence Spectroscopy. 2nd edition, Kluwer Academic / Plenum Publishers, New York. (An authoritative text book on fluorescence spectroscopy.)

Roy R., Hohng S. & Ha T. (2008) A practical guide to single-molecule FRET. Nature Methods 5, 507-516.

Wilson, K. & Walker, J. (2010) Principles and Techniques of Biochemistry and Molecular Biology. 7th ed, Cambridge University Press. Chapter 12.

Web sites
Fluorescence Tutorials
http://www.invitrogen.com/site/us/en/home/support/Tutorials.html

Fluorescence Microscopy: Excitation Balancer for multiply labelled specimen
http://www.microscopyu.com/tutorials/java/fluorescence/excitationbalancer/index.html