single-molecule fluorescence resonance energy transfer

(1) introduction

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06/05/2004

topics

- light-matter interaction
- absorbance, luminescence, fluorescence
- fluorophores, quantum dots, GFP`s
- spectra, intensity, quantum yield,
- lifetime
- anisotropy / polarization
- fluorescence resonance energy tranfer **FRET**
- single-molecule detection

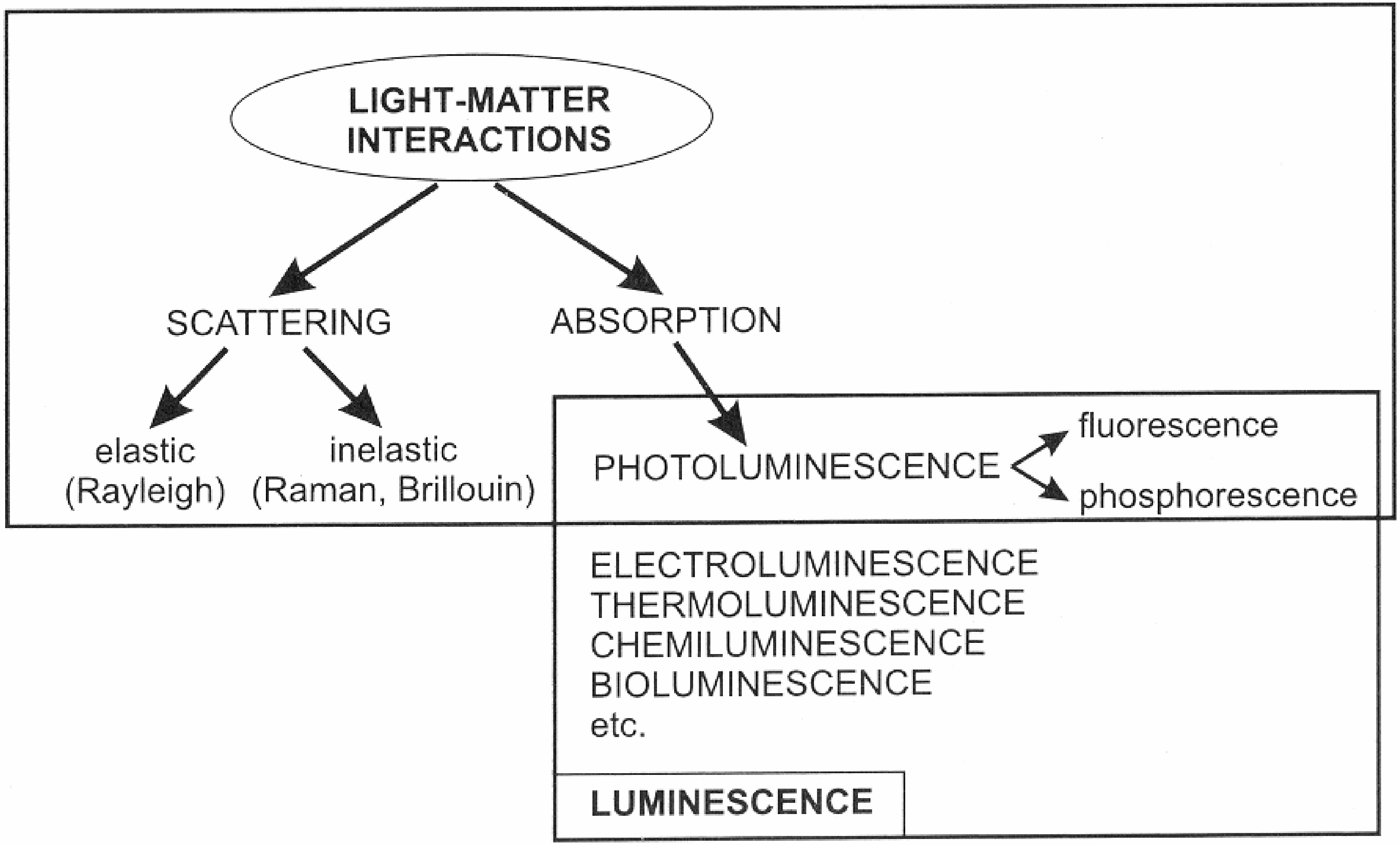


Fig. 1.1. Position of fluorescence and phosphorescence in the frame of light–matter interactions.

Born-Oppenheimer approximation

- the state of a molecule is characterized by its electronic state, S, its vibrational state, n, and its rotational state, L:

$$| S,n,L > \quad cq \quad \Psi(r,R)$$

- *because of the large mass difference between electrons and nuclei* ($m_p/m_e = 1860$) *the electrons will immediately follow any rearrangement of the nuclei. The total state can be written as*

$$| S,n,L > \; = \; | S_{nL} > \cdot \; | n,L > \quad cq \quad \Psi(r,R) = \psi_R(r) \cdot \Phi(R)$$

Franck-Condon Principle (1)

- each electronic state is split into its vibrational levels
- the (vibrational) wavefunctions are described by the harmonic oscillator

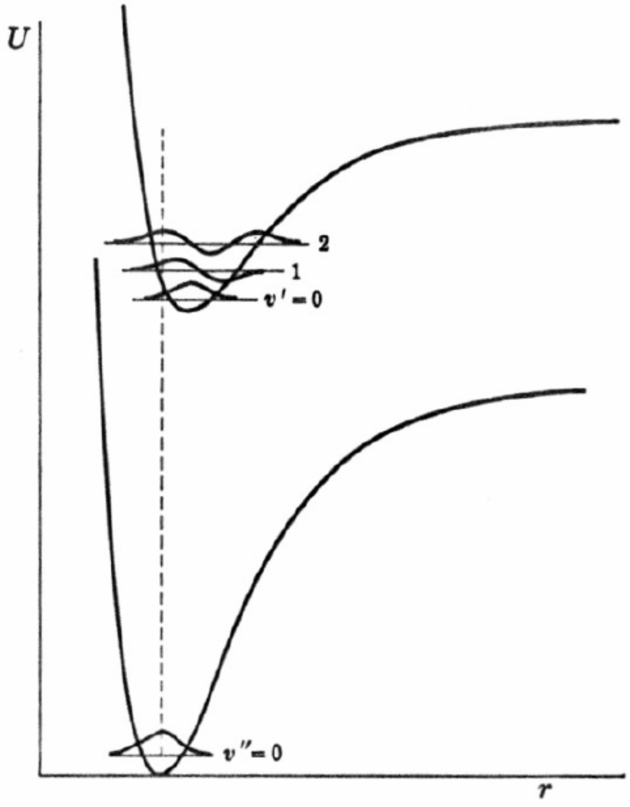


Fig. 91. **Franck-Condon Principle According to Wave Mechanics.** The potential curves are so drawn that the "best" overlapping of the eigenfunctions occurs for $v' = 2$, $v'' = 0$ (see the broken vertical line).

Franck-Condon Principle (2)

- adiabatic approximation
  - the electrons are instantaneously in the final state
  - transitions vertical
  - the highest intensity is where the vertical line crosses the potential of the final state

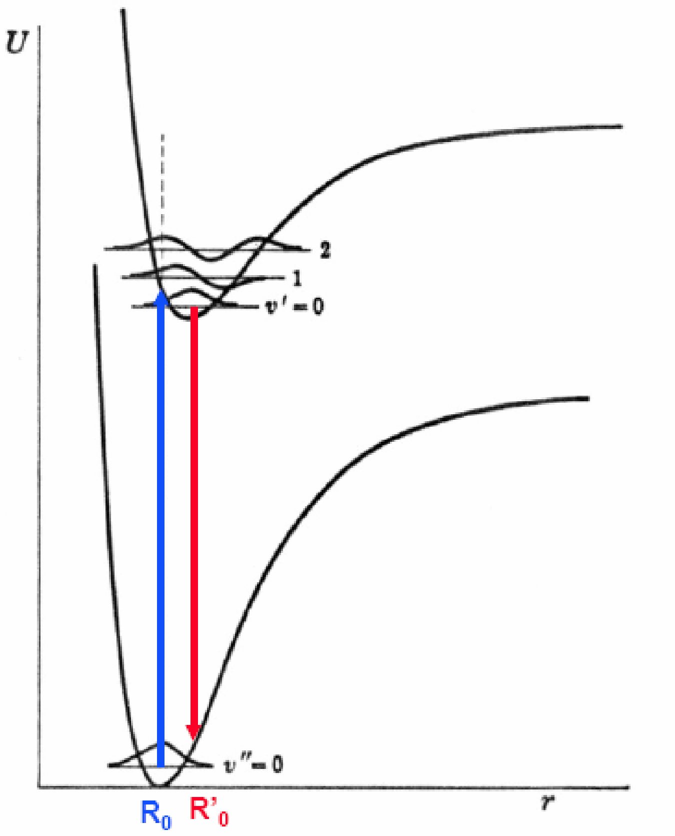


FIG. 91. **Franck-Condon Principle According to Wave Mechanics.** The potential curves are so drawn that the "best" overlapping of the eigenfunctions occurs for $v' = 2$, $v'' = 0$ (see the broken vertical line).

Fermi's Golden Rule

- probabilitity for a transition from an initial state $|\, i >$ to a final state $|\, f >$

$$p_{if} = \frac{\pi}{2\hbar^2} \left| \langle f | V | i \rangle \right|^2 \delta\left(E_f - \left(E_i + E_V\right)\right)$$

- *specifically interaction of a dipole with an electromagnetic field*

$$p_{if} = \frac{\pi}{2\hbar^2} \mu_{if}^2 E_0^2 \quad with \quad \mu_{if} = \langle f | er | i \rangle$$

transition dipole-moment

Frank-Condon Factor

- transition from |0,n,L> to |1,n‘,L‘>
- transition dipole-moment

$\mu_{if} = \langle 1, n', L' |\, er \,| 0, n, L \rangle$

- adiabatic approximation

$\mu_{if} = \langle 1 | er | 0 \rangle \cdot \langle n', L' | n, L \rangle$

- Franck-Condon factors

$S_{n',n} = \langle n', L' | n, L \rangle$

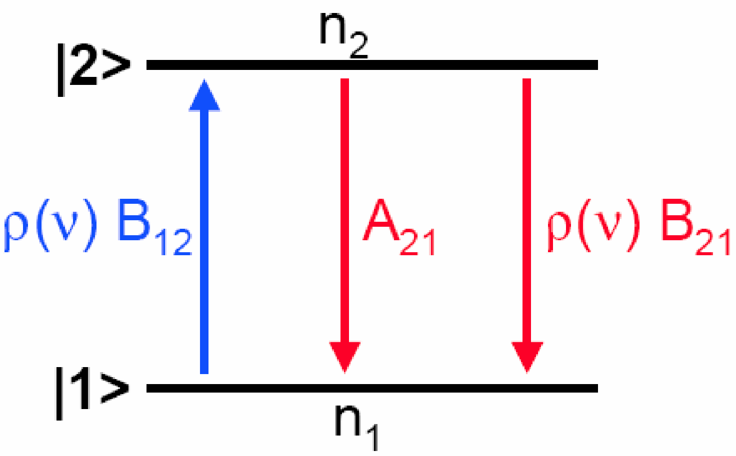
calculate the overlap integral between the two vibronic wavefunctions

- for harmonic oszillator

$$S^2_{0,n} = \frac{p^n}{n!} e^{-p}$$

$$p = \frac{m\omega}{2}(R_0 - R_0')^2 \quad \textit{Stokes loss}$$

absorption and emission (1)

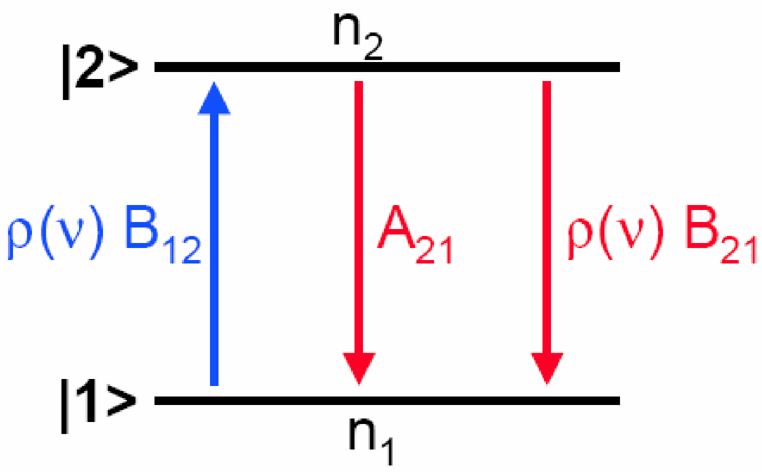


- **A_{21}** spontaneous emission
- **B_{12}, B_{21}** stimulated absorption cq emission

Einstein coefficients for the probability of transitions

$\rho(\nu)$: radiation density

absorption and emission (1)



- **A_{21}** spontaneous emission
- **B_{12}, B_{21}** stimulated absorption cq emission

- rate equations

$$\dot{n}_1 = -B_{12}\rho(\nu)\,n_1 + \left(A_{21} + B_{21}\rho(\nu)\right)n_2$$
$$\dot{n}_2 = +B_{12}\rho(\nu)\,n_1 - \left(A_{21} + B_{21}\rho(\nu)\right)n_2$$

- detailled balance

$$\frac{n_2}{n_1} = \exp\left(-\frac{h\nu}{k_B T}\right)$$

- steady-state

$$\rho(\nu) = \frac{A_{21}/B_{21}}{B_{12}/B_{21}\left(\exp\left(\frac{h\nu}{k_B T}\right) - 1\right)}$$

absorption and emission (2)

- Einstein coefficients

$$\rho(\nu) = \frac{A_{21}/B_{21}}{B_{12}/B_{21}\left(\exp\left(\frac{h\nu}{k_BT}\right)-1\right)}$$

- Planck radiation

$$\rho(\nu)\,d\nu = \frac{8\pi h\nu^3}{c^3}\frac{1}{\exp(h\nu/k_BT)-1}\,d\nu$$

- comparison yields

$$B_{12} = B_{21}$$

$$A_{21} = \frac{8\pi h\enclose{circle}{\nu^3}}{c^3}B_{21}$$

VIS: all emission is spontaneous

energy - frequency - wavelength

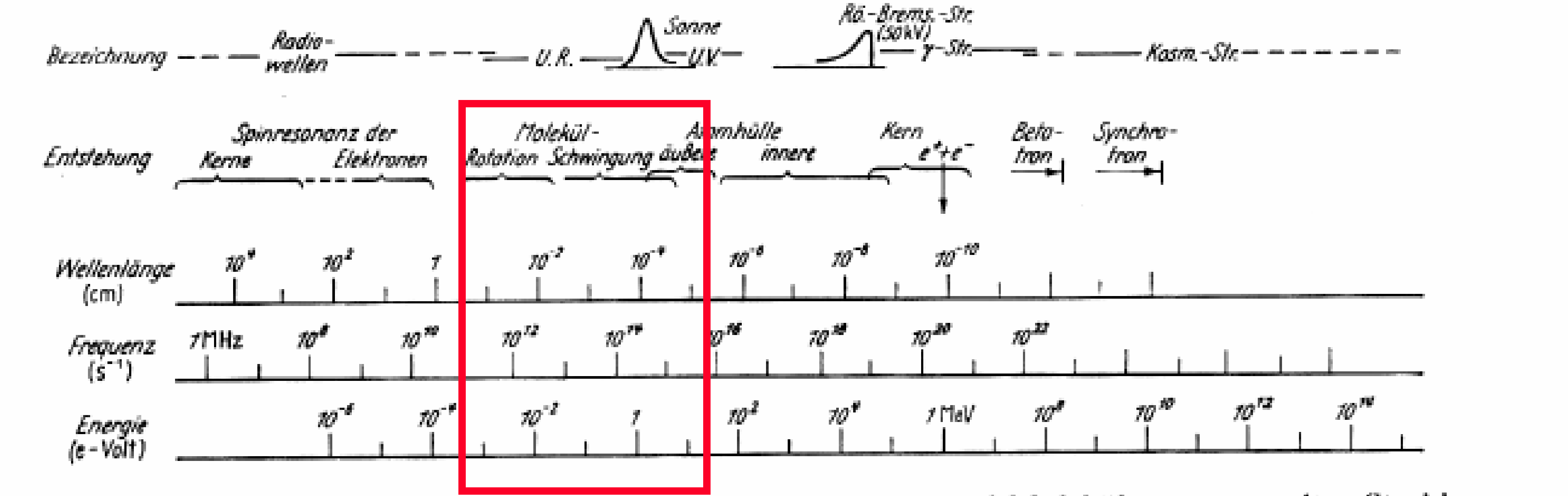
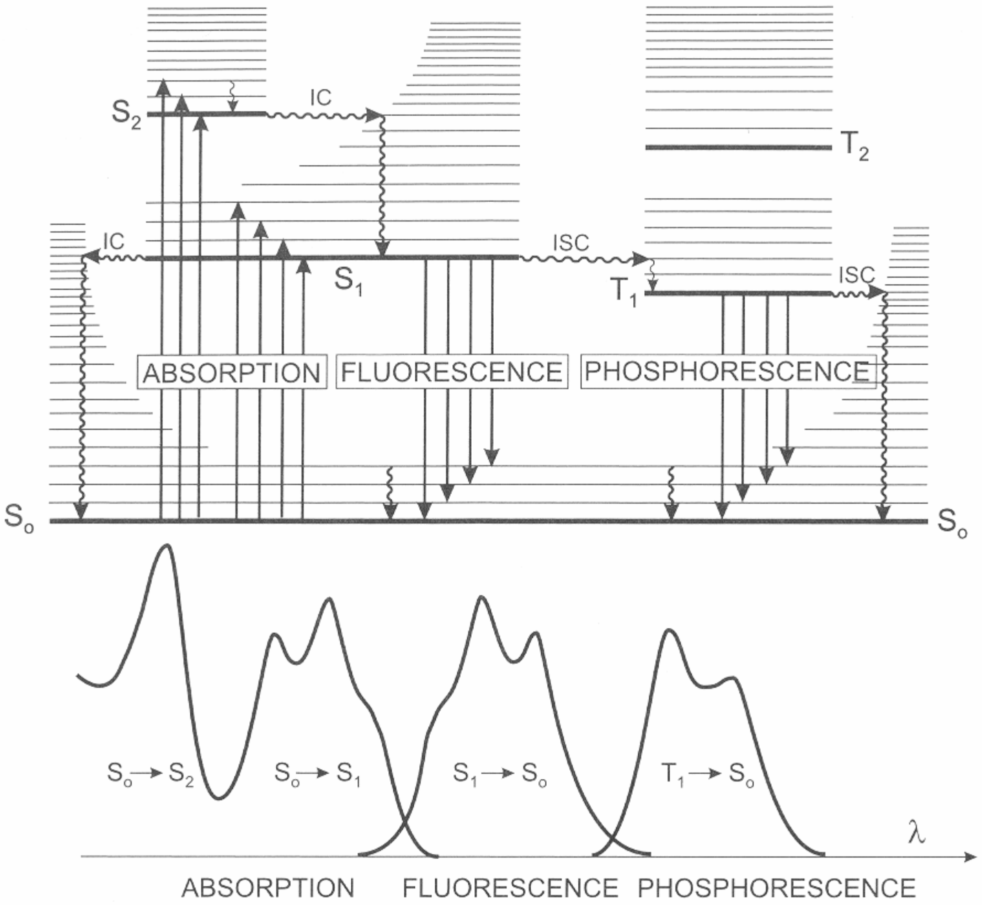


Abb. 11.2. Wellenlänge, Frequenz und Energie der von Atomen und Molekülen ausgesandten Strahlungen

Jablonski diagram



CHARACTERISTIC TIMES

absorption 10^{-15} s
vibrational relaxation 10^{-12}-10^{-10} s
lifetime of the excited state S_1 10^{-10}-10^{-7} s → fluorescence
intersystem crossing 10^{-10}-10^{-8} s
internal conversion 10^{-11}-10^{-9} s
lifetime of the excited state T_1 10^{-6} -1 s → phosphorescence

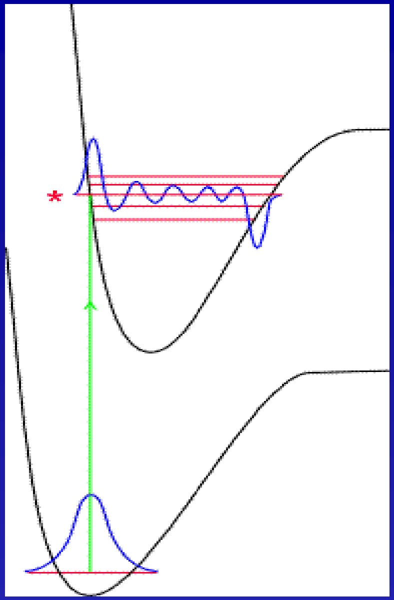
Fig. 3.1. Perrin–Jablonski diagram and illustration of the relative positions of absorption, fluorescence and phosphorescence spectra.

- electronic transitions
  - $\tilde{\nu} \sim 10^4\ \mathrm{cm}^{-1}$
  - $\tau_s \sim 10$ ns
  - $\tau_T \sim 1\ \mu s - 1$ ms
- vibrational transitions
  - $\tilde{\nu} \sim 10^2 - 10^3\ \mathrm{cm}^{-1}$
  - $\tau \sim 1$ ps
- rotational transitions
  - $\tilde{\nu} \sim 1$-$10\ \mathrm{cm}^{-1}$
  - $\tau \sim 100$ ps

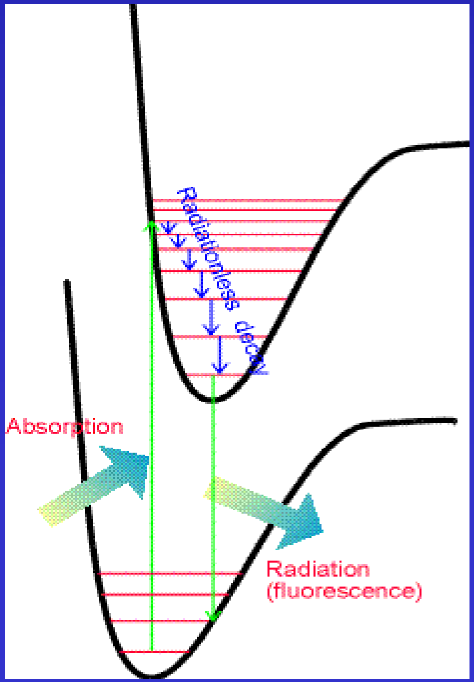
fluorescence

(1) absorbance of one photon (fs)

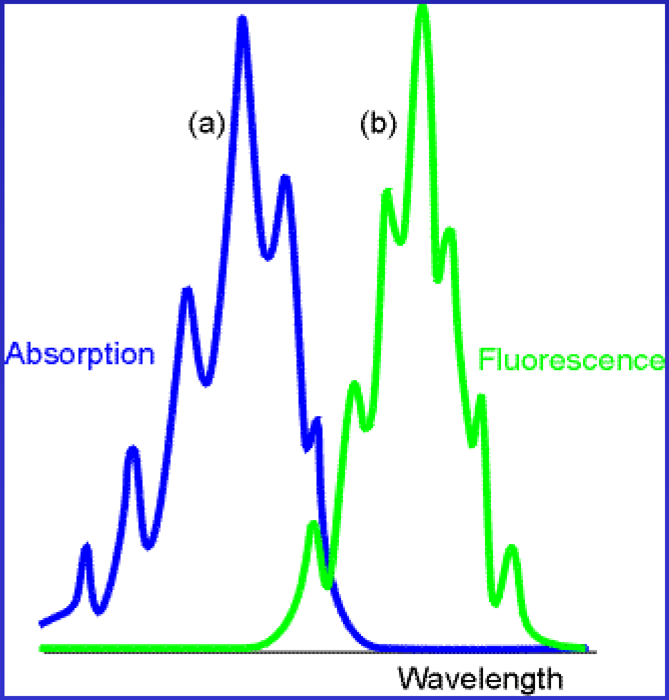
(2) vibrational relaxation (ps)



(3) fluorescence parameters:



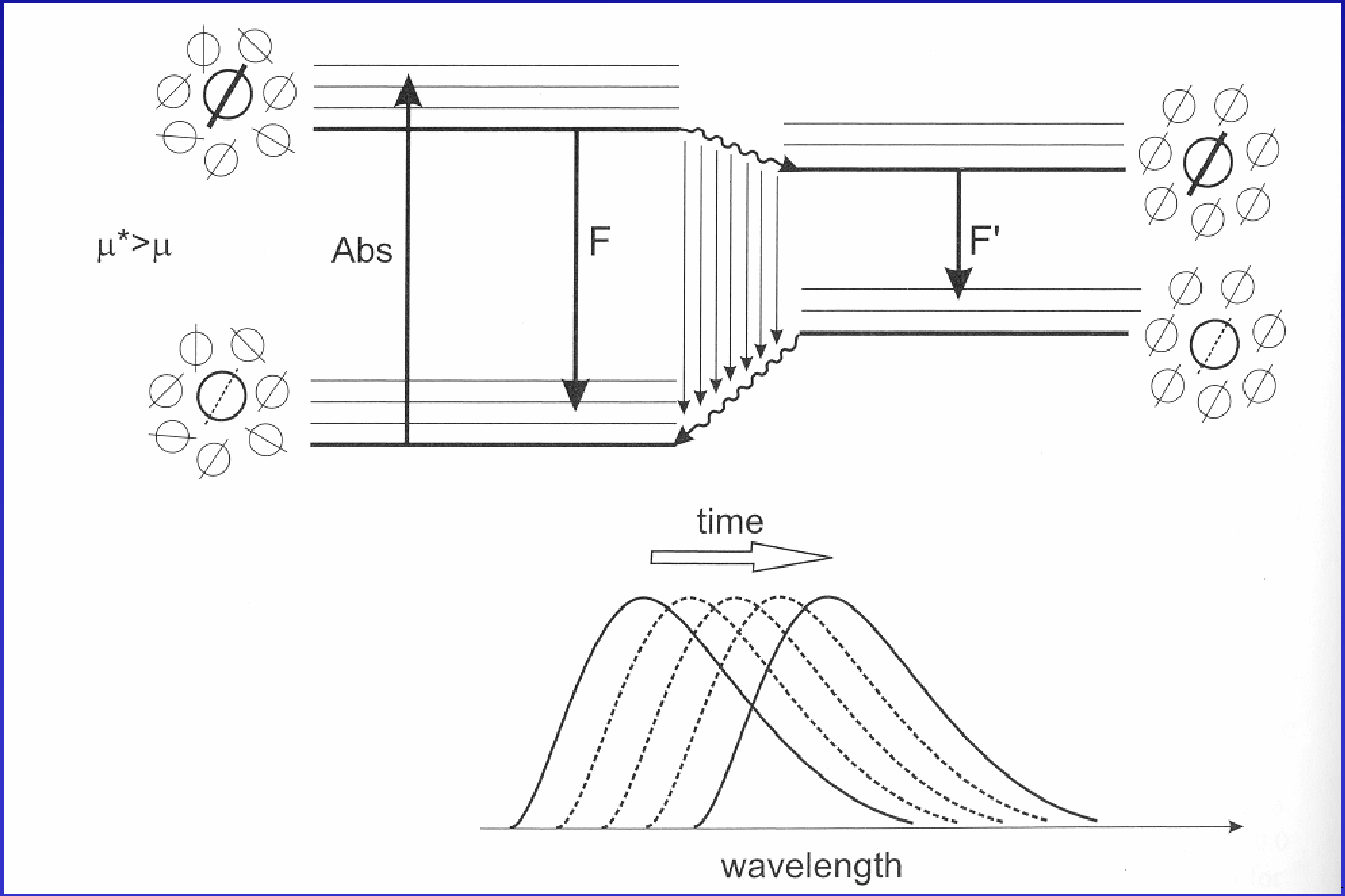
spectrum
quantum yield
lifetime
anisotropy



spectrum and linewidth

- Heisenberg uncertainty principle
- Doppler effect
- solvent effects
- ensemble

solvent relaxation



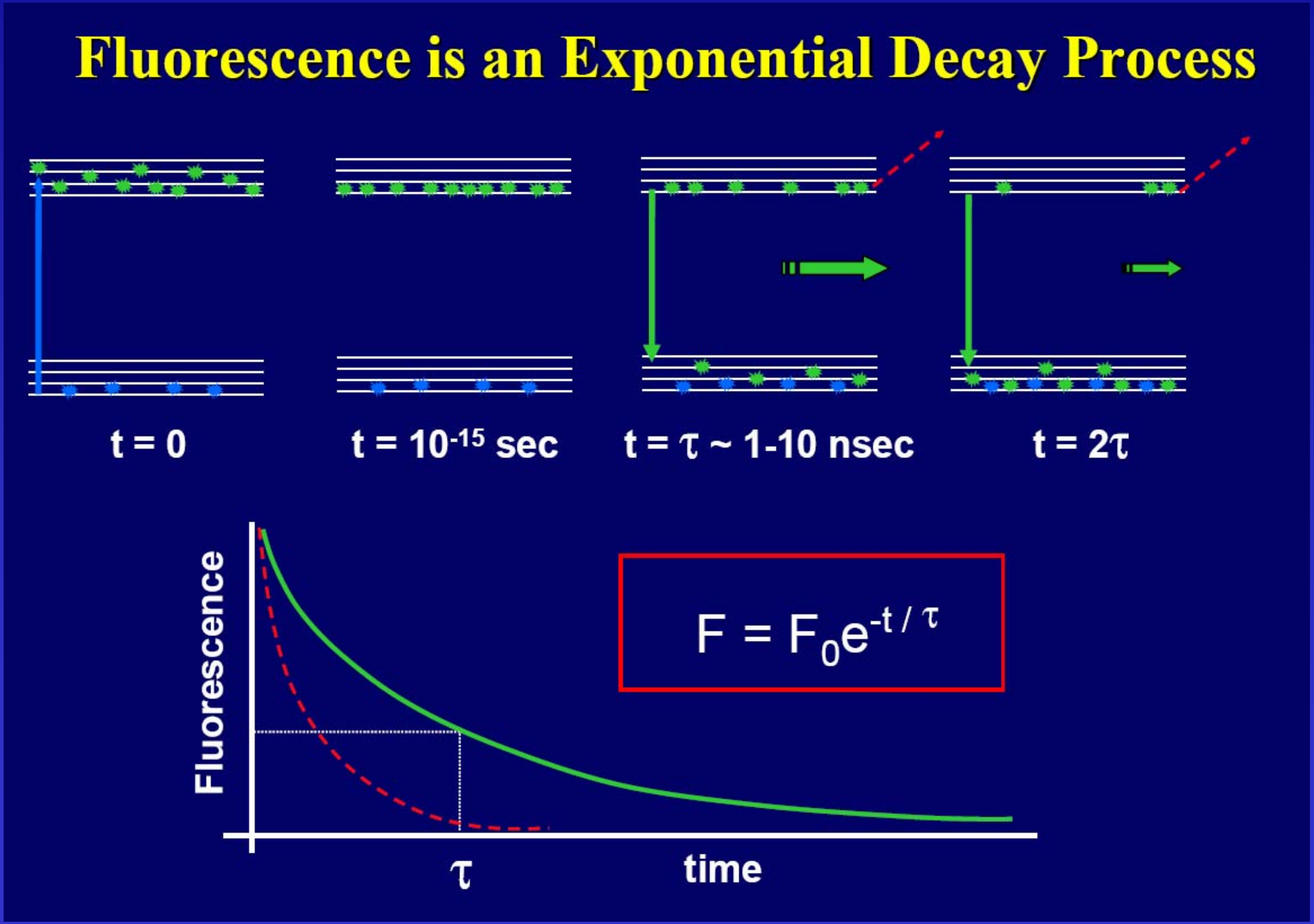
influencing fluorescence

- pressure, temperature
- viscosity
- polarity
- hydrogen bonds, pH
- ions
- quenching
- electric potential

quantum yield (for fluorescence)

- ratio of the number of emitted photons to the number of absorbed photons
- ratio of the rate constant for radiative deactivation ($S_1 \rightarrow S_0$) to the sum of rate constants for all deactivation processes
- ratio of the lifetime of S_1 (fluorescence lifetime) to the radiative lifetime (in absence of any non-radiative deactivation)

fluorescence lifetime



Fluorescence is an Exponential Decay Process

$$F = F_0 e^{-t/\tau}$$

anisotropy

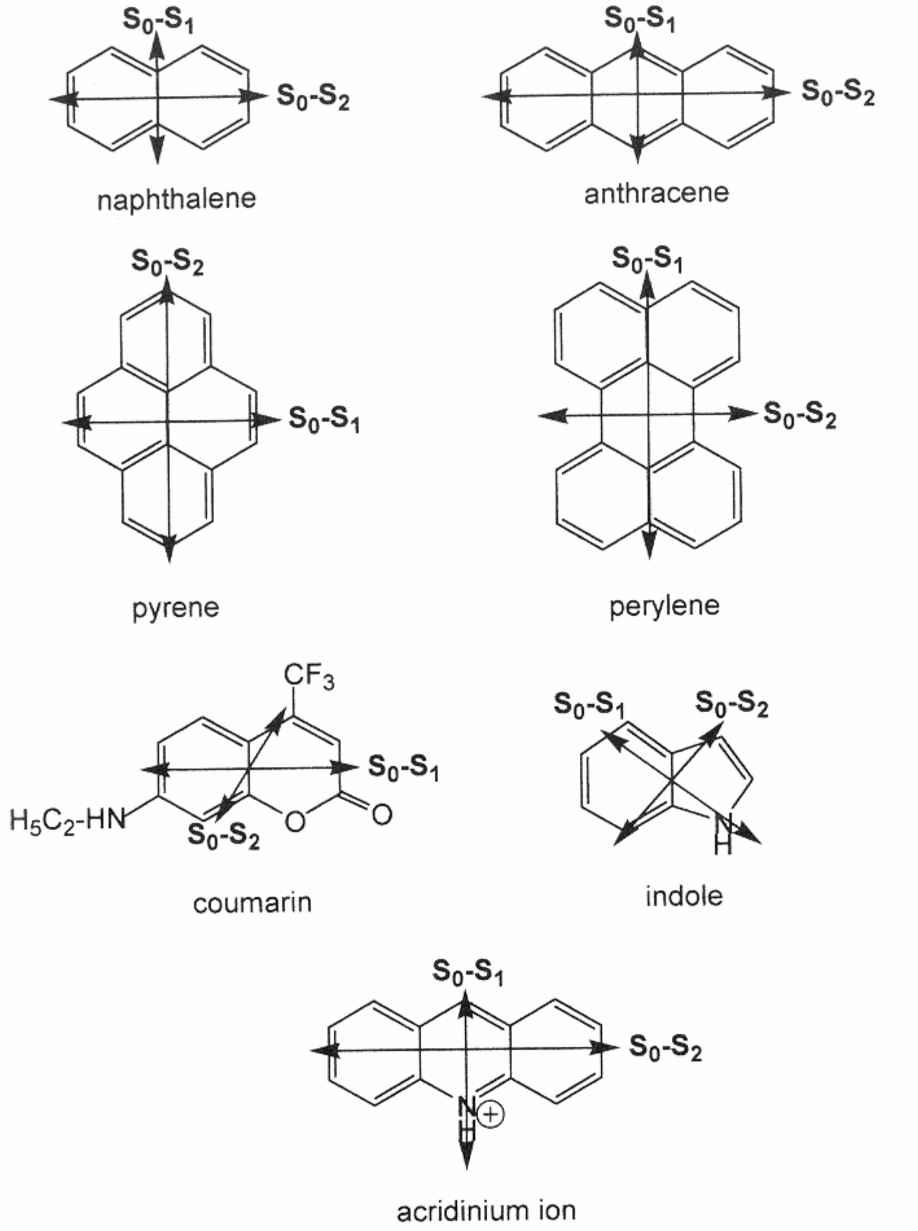
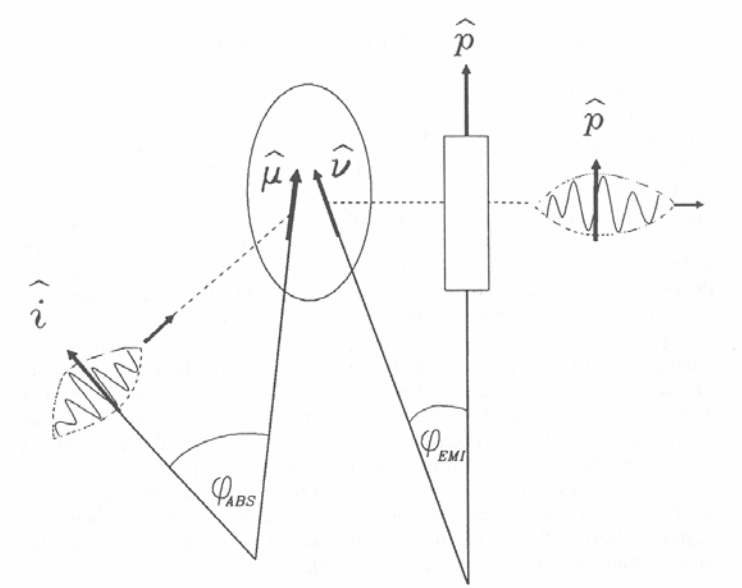


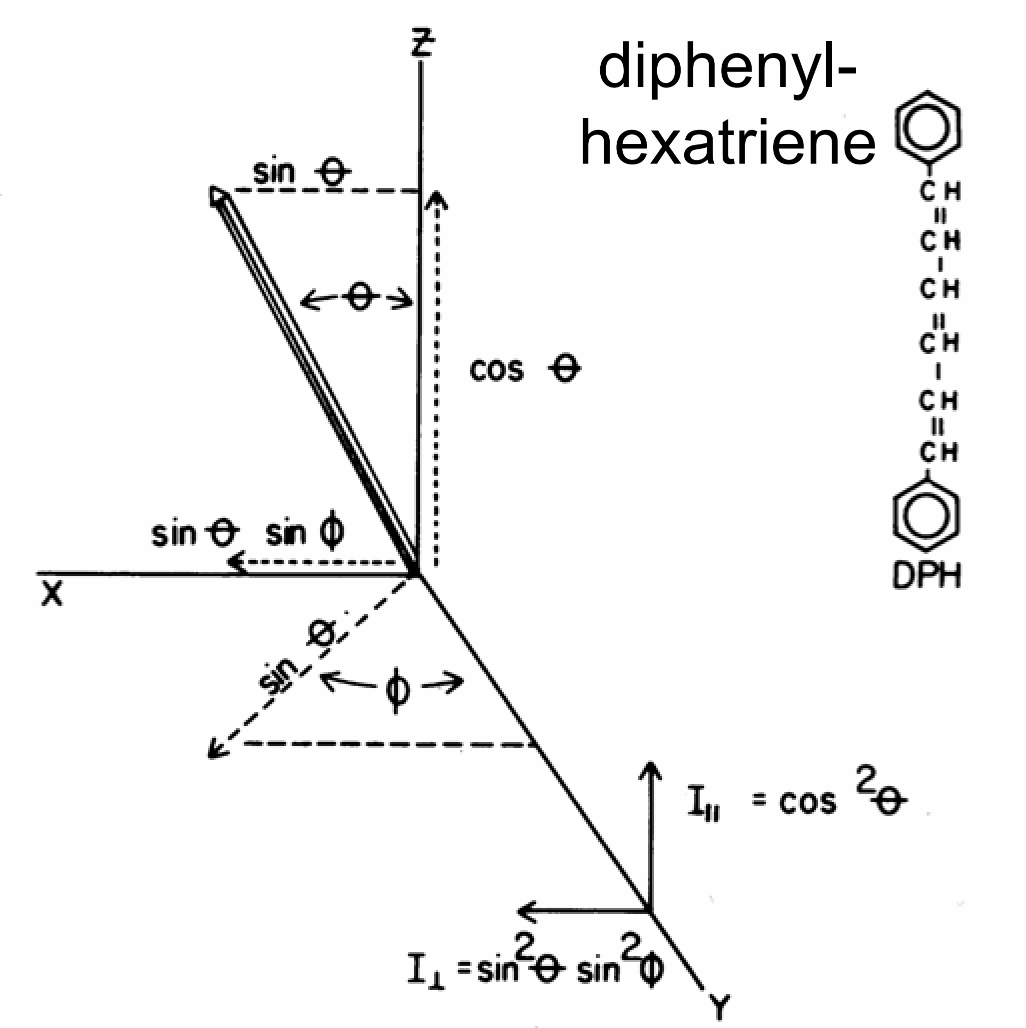
Fig. 2.3. Examples of molecules with their absorption transition moments.



î : exciting photon

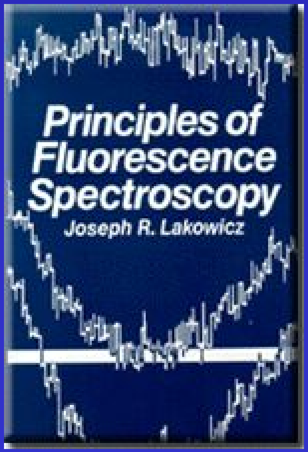
p : polarized fluorescence photon

transition dipole moment



⇒ transition dipole moments are oriented in the geometry of the fluorophore for excitation and fluorescence

from:


Principles of Fluorescence Spectroscopy
Joseph R. Lakowicz

definition by intensities

polarization [P]

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$$

anisotropy [r]

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}}$$

1. in isotropic solution: EX with linearly polarized light

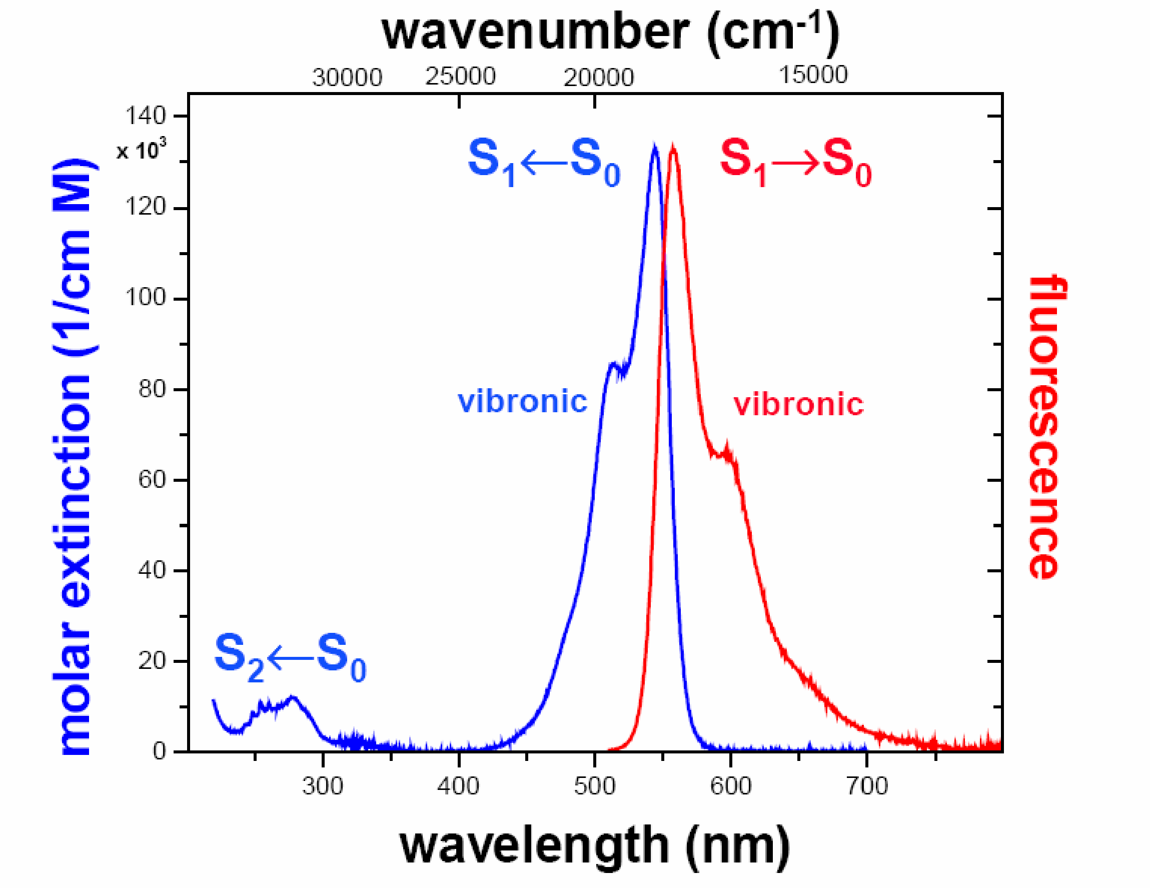
⇒ selective excitation of absorption dipoles parallel to the electric vector (photoselection).

2. rotational diffusion during fluorescence lifetime

⇒ depolarization

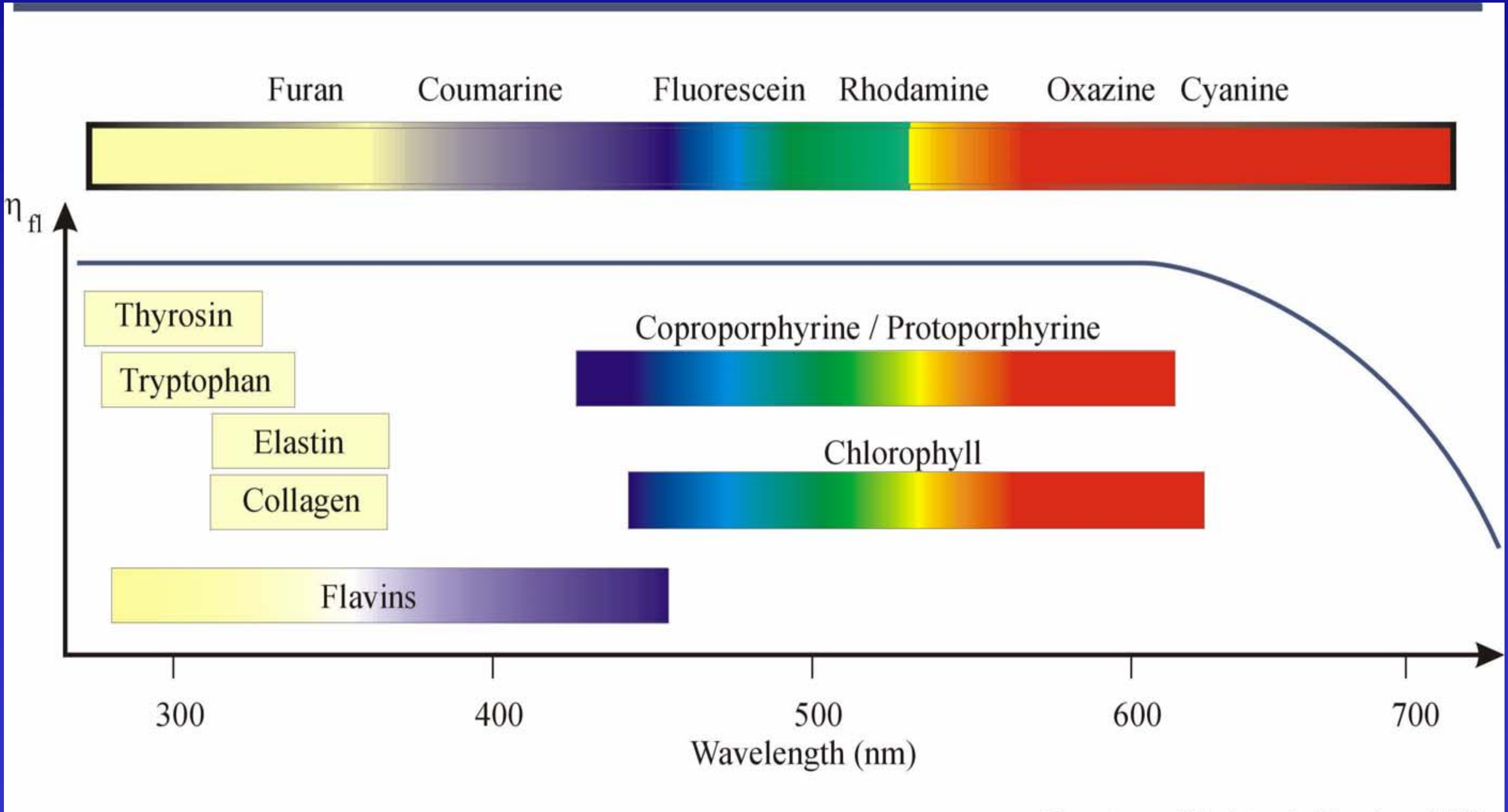
absorption and emission (3)

example:
dye
Cy3



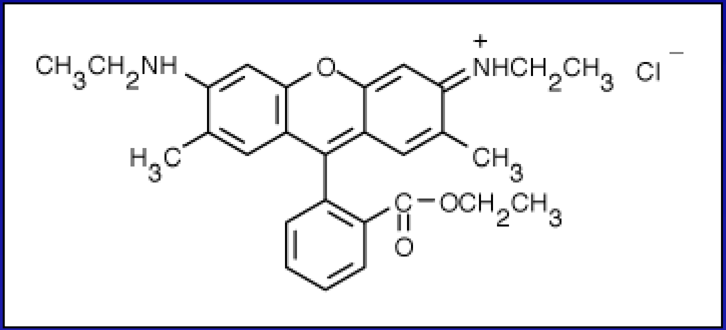
- frequency $E = h\nu$ $[\nu]$ = Hz
- wavelength $E = hc/\lambda$ $[\lambda]$ = nm
- **wavenumber $E = hc\tilde{\nu}$** **$[\tilde{\nu}]$ = 1/cm**

fluorophores

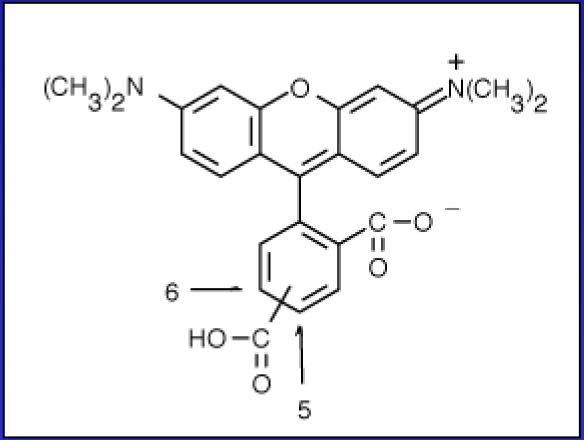


Courtesy: Christoph Zander. 1999
Uni GH Siegen

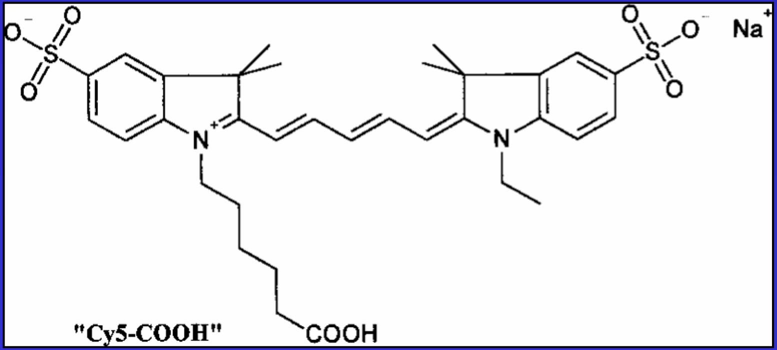
fluorophores II



rhodamine 6 G



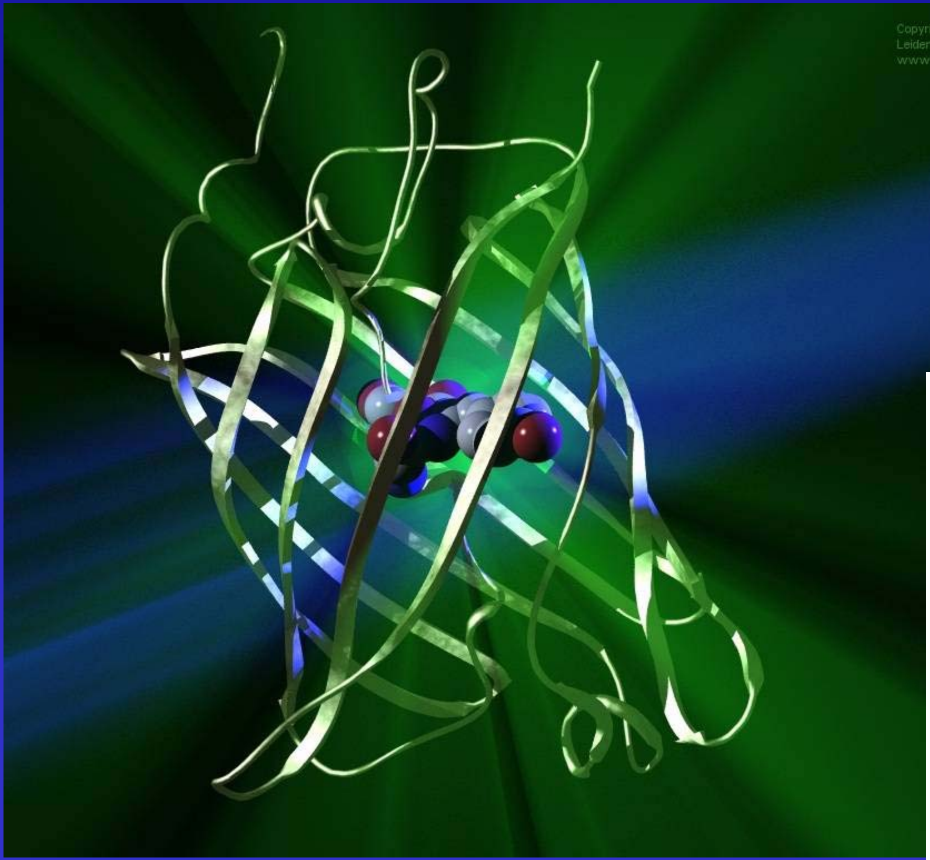
tetramethylrhodamine



cyanine 5

fluorophores III

green fluorescent protein GFP (Uni Leiden; spectra: Clontech)



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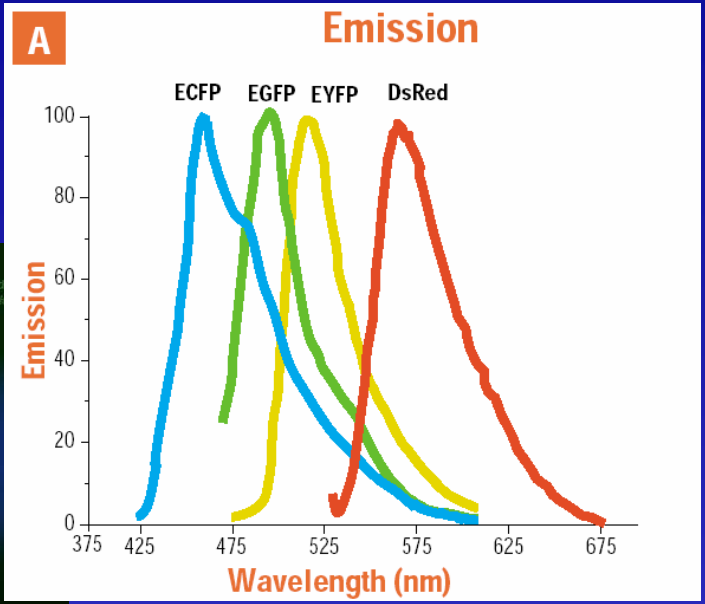
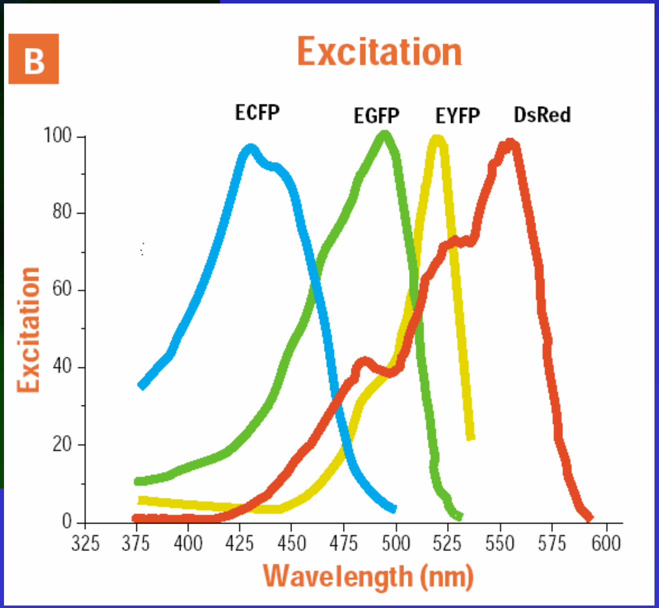
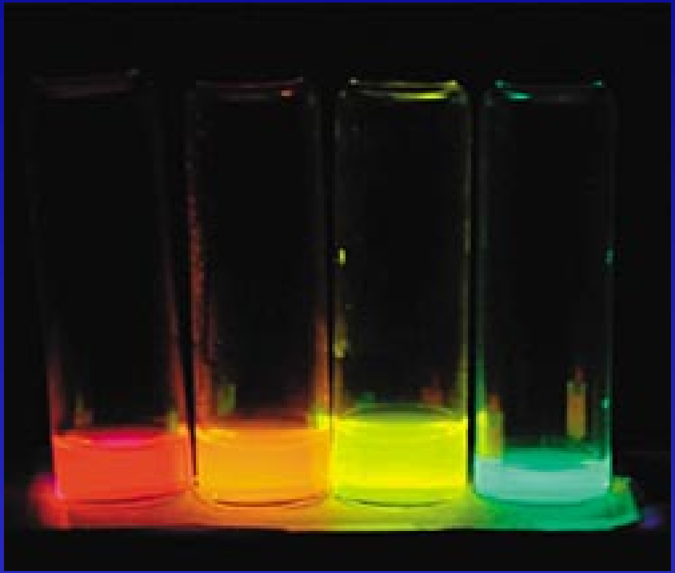
www.chem.leidenuniv.nl/metprot/

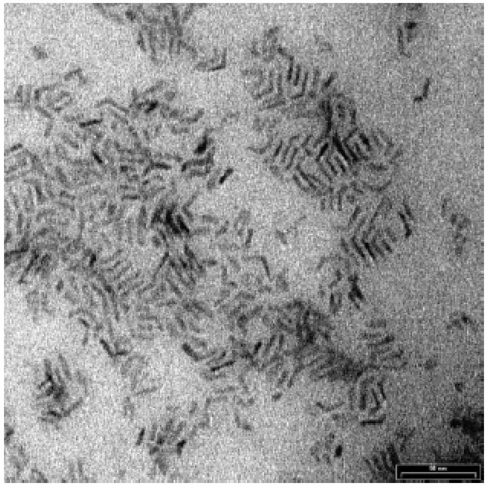




fluorophores IV

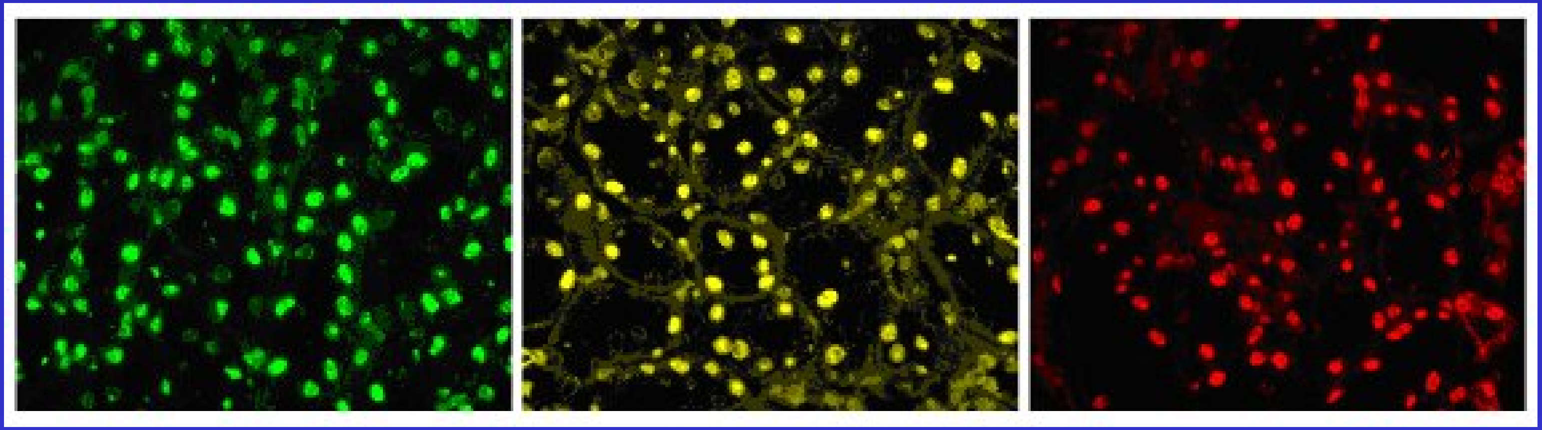


nanocrystals
semiconductor
quantum dots, CdSe
with capping



Nanorods
T. Nann
Uni Freiburg

QuantumDotCorp 565-585-605-streptavidin: kidney cells



fluorescence observables

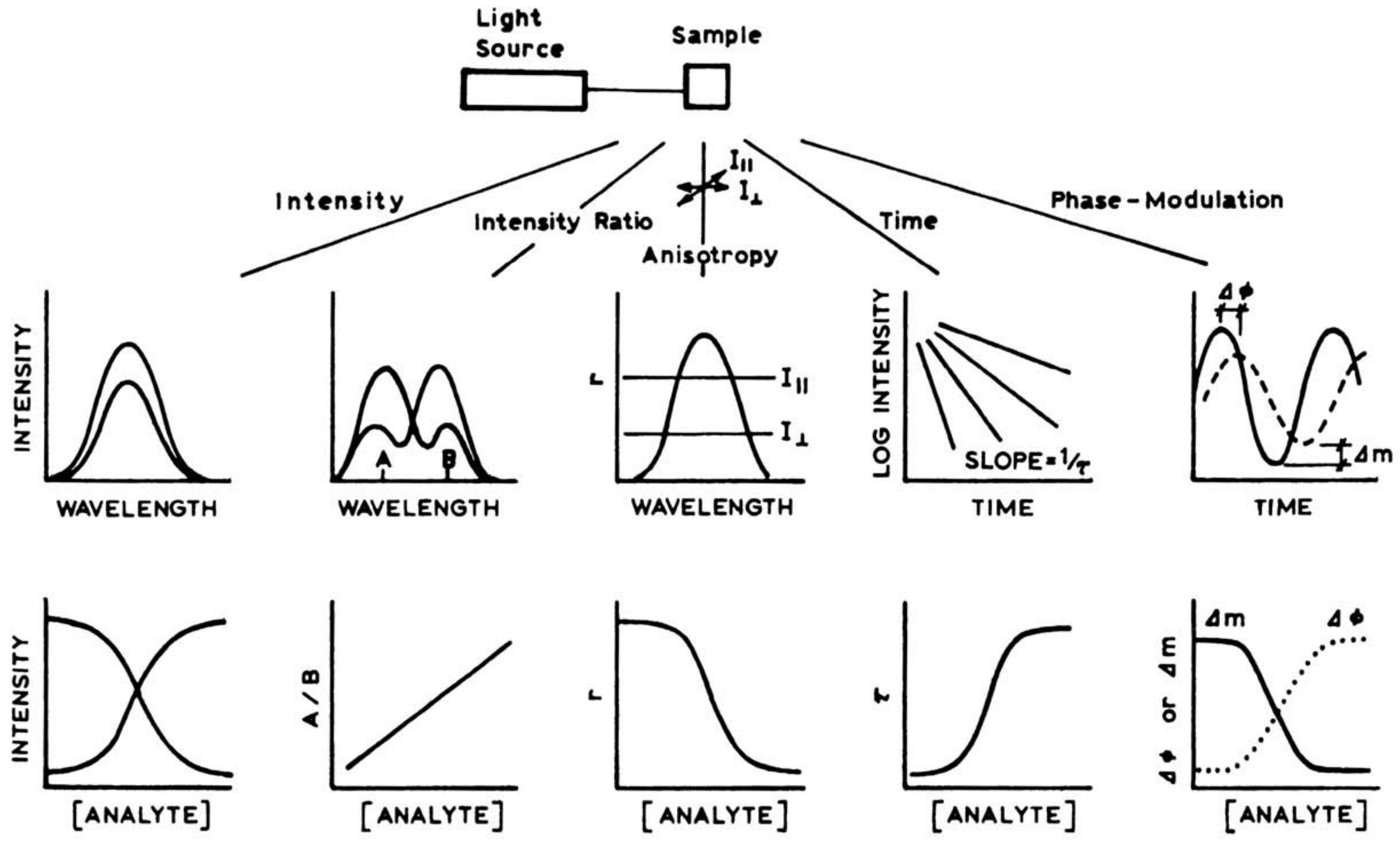


Figure 19.3. Spectral observables for fluorescence sensing. From left to right: Intensity, intensity ratio, anisotropy, time-domain lifetime, and phase-modulation lifetime.

fluorescence spectrometer

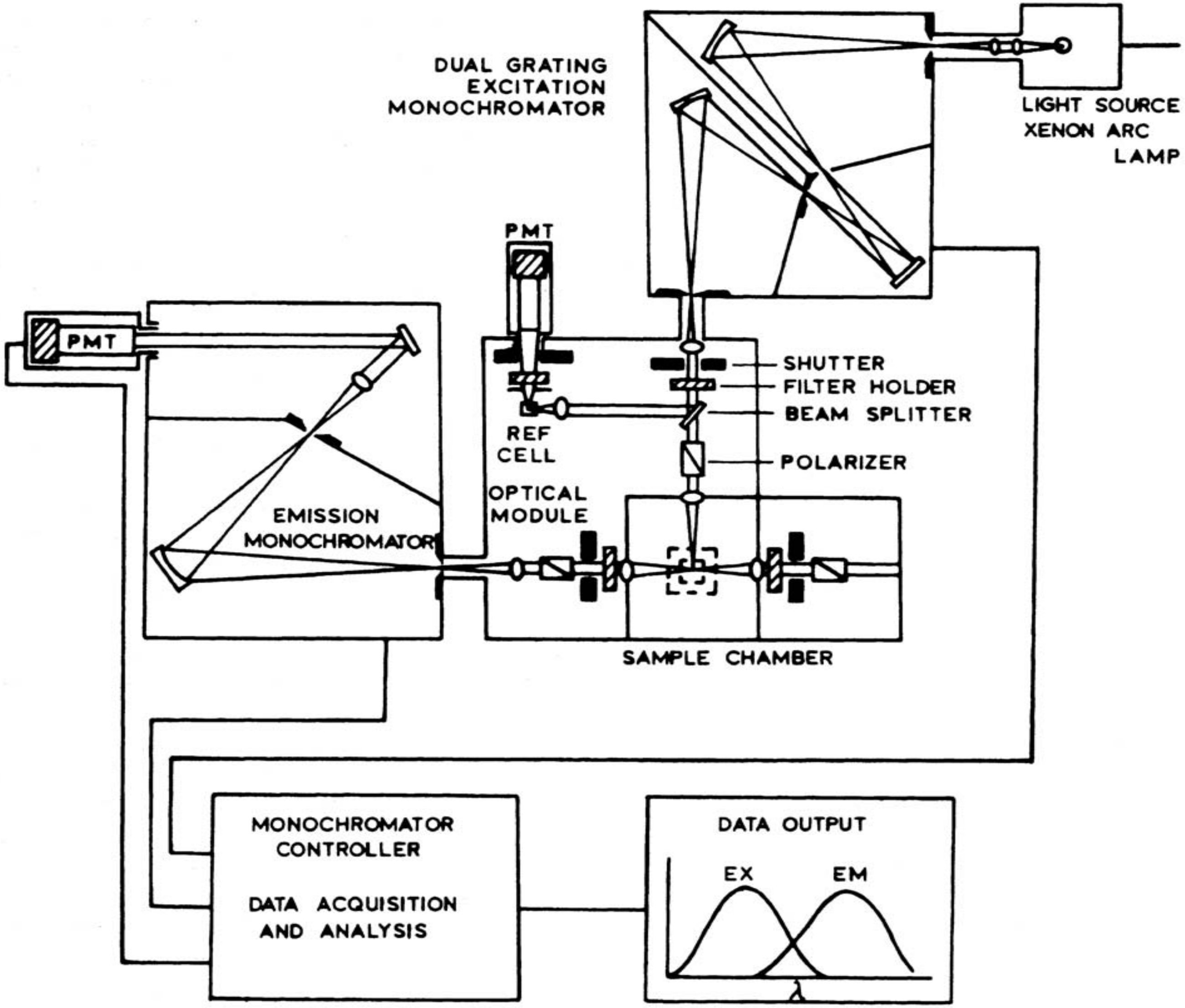
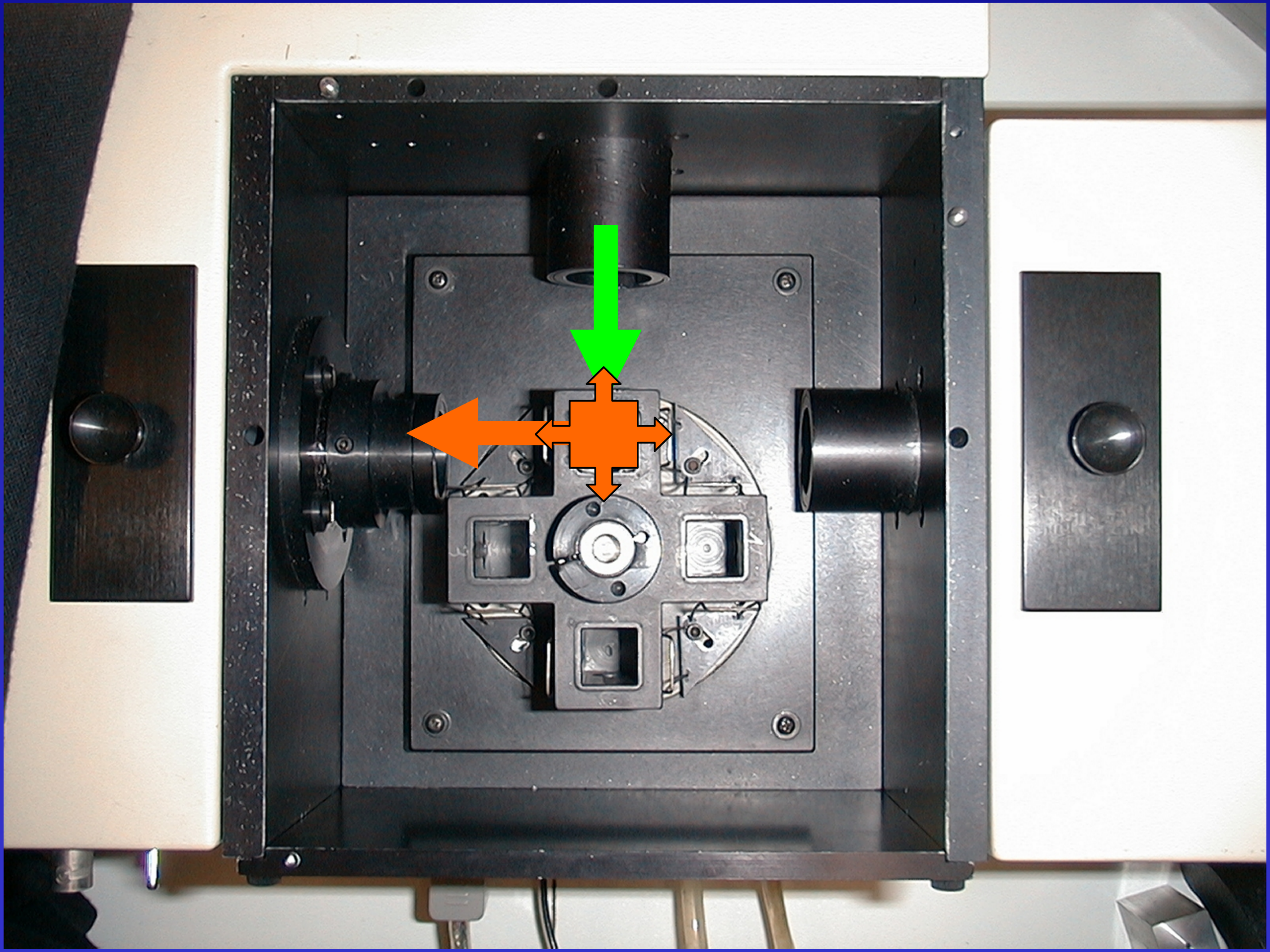
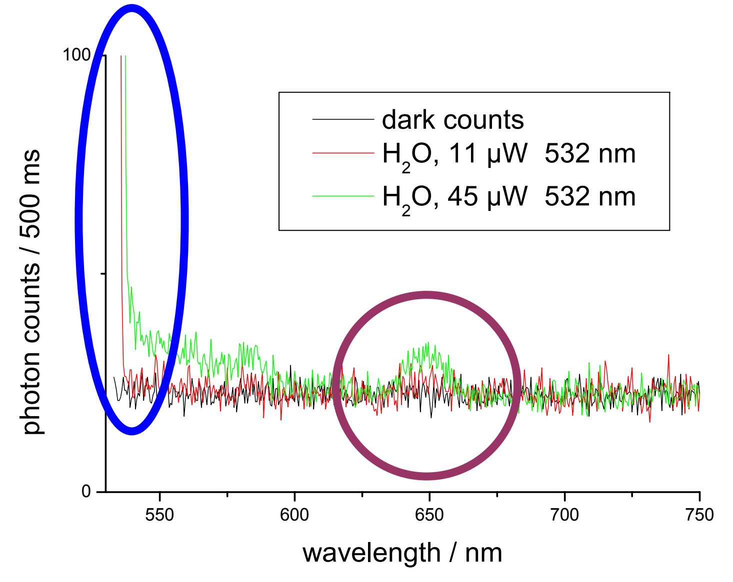


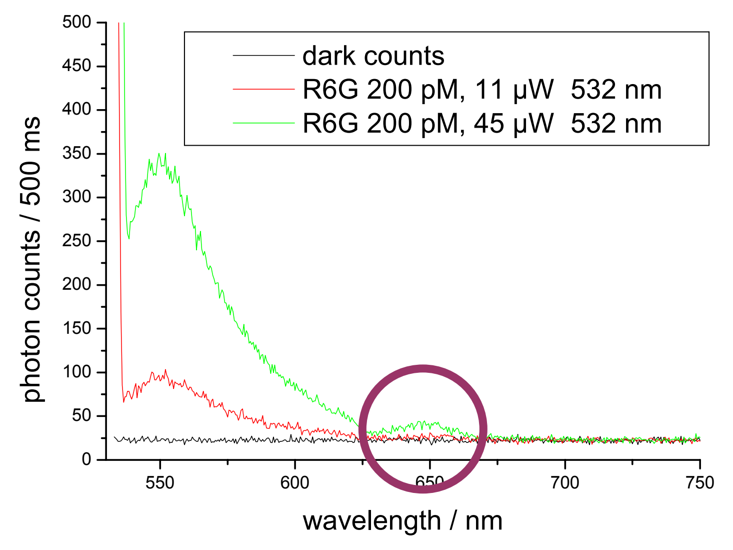
Figure 2.1. Schematic diagram of a spectrofluorometer. PMT, Photomultiplier tube. Revised from commercial literature from SLM Instruments.

sample chamber SLM 8100



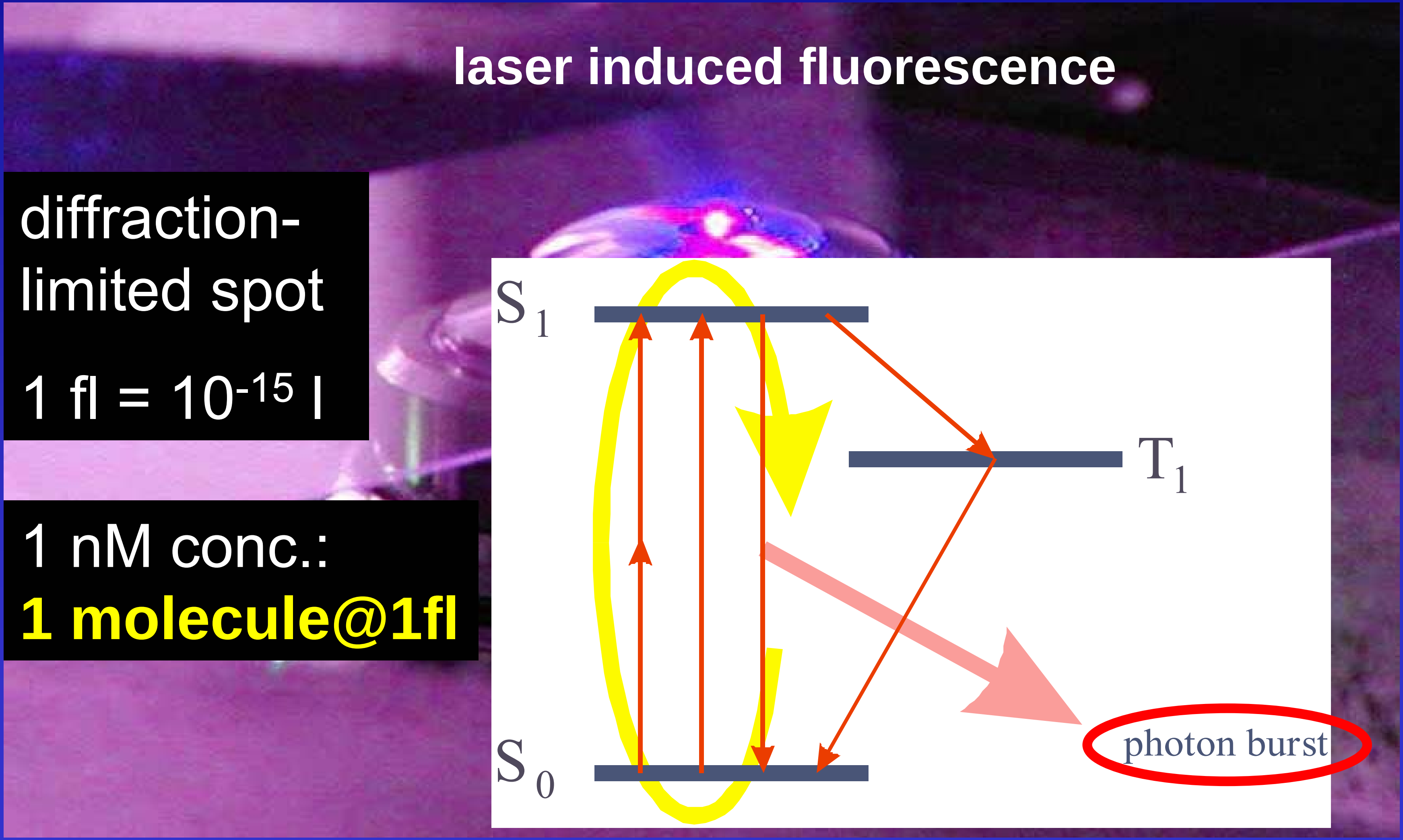
fluorescence of R6G in H_2O





- excitation volume: 5mm x 2mm x 2mm = 20 µl
- R6G 200 pM ⇒ $2.4 \cdot 10^{9}$ Molecules
- H_2O 55 M ⇒ $6.6 \cdot 10^{20}$ Molecules
- ratio 1 : $2.8 \cdot 10^{11}$

principle of
single-molecule fluorescence detection



laser induced fluorescence

diffraction-limited spot

1 fl = 10^{-15} l

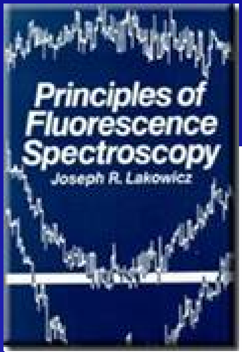
1 nM conc.:

1 molecule@1fl

relaxation modes

- internal conversion (IC)
- intersystem crossing (ISC)
- fluorescence
- phosphorescence
- delayed fluorescence (after reverse ISC)
- triplet-triplet annihilation
- triplet-triplet transition
- photoinduced intramolecular charge transfer (TICT)
- intermolecular de-excitation (quenching or **FRET**)
- photochemical transformation
- excimer or exciplex formation

fluorescence resonance energy transfer : FRET (1)



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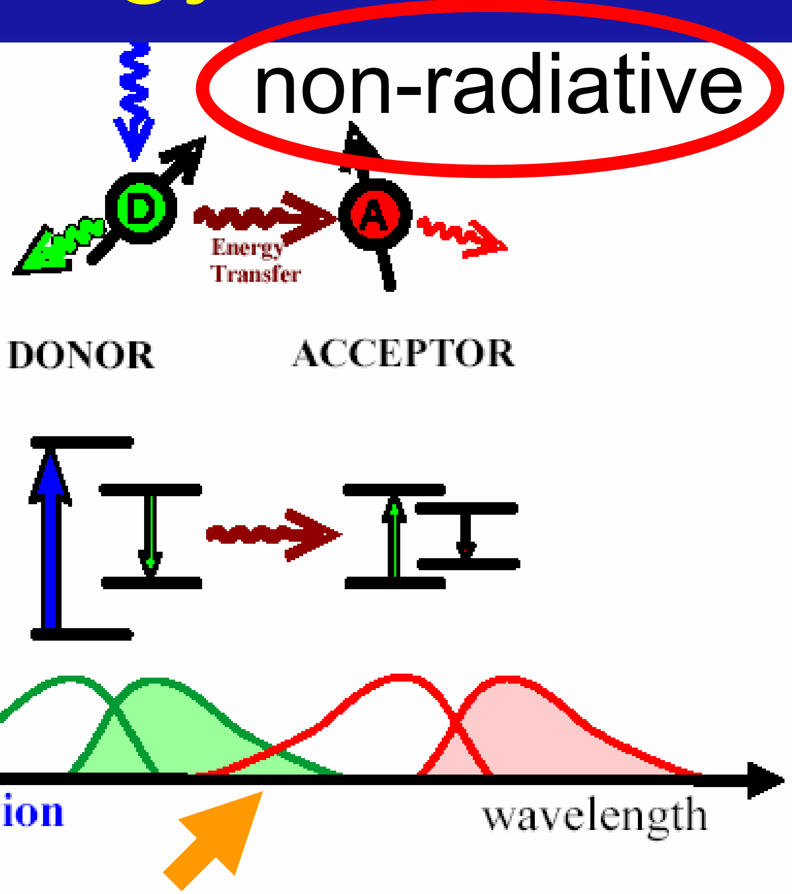
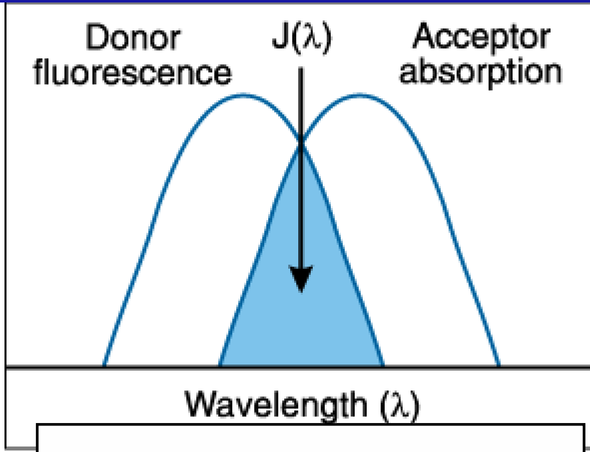
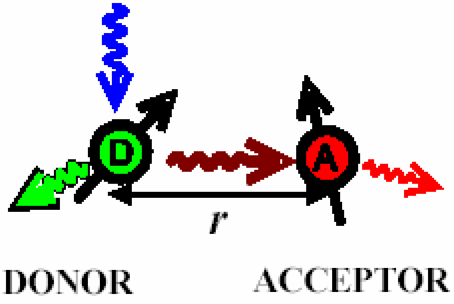


FRET requires overlap of the emission band of the donor and the absorption band of the acceptor



$$k_T = \frac{1}{\tau_d}\left(\frac{R_0}{r}\right)^6$$



efficiency of transfer E

$$\begin{tikzcd}
& D + A & D + A & \\
D + A + h\nu_{ex} \arrow[r] & D^* + A \arrow[u, "k_{Di}"] \arrow[d, "k_D"'] \arrow[r, "k_T"] & D + A^* \arrow[u, "k_{Ai}"] \arrow[r] & A + D + h\nu_A \\
& D + A + h\nu_D & &
\end{tikzcd}$$

$$E = \frac{k_T}{k_T + k_D + k_{Di}}$$

fluorescence resonance energy transfer : FRET (2)

$$k_T = \frac{9000(\ln 10)\kappa^2\phi_d}{128\pi^5 n^4 N r^6 \tau_d} \int_0^\infty \frac{F_d(\bar{\nu})\varepsilon_a(\bar{\nu})}{\bar{\nu}^4}\, d\bar{\nu}$$

$$k_T = (r^{-6} J \kappa^2 n^{-4} \lambda_d) \times 8.71 \times 10^{23}\ \mathrm{sec}^{-1}$$

spectral overlap (depends on λ):

→

$$J = \int_0^\infty F_d(\lambda)\varepsilon_a(\lambda)\lambda^4\, d\lambda$$

fluorescence resonance energy transfer : FRET (3)

$$k_T = \frac{1}{\tau_d}\left(\frac{R_0}{r}\right)^6$$

distance dependence and

Förster radius R_0

$$k_T = \frac{9000(\ln 10)\kappa^2\phi_d}{128\pi^5 n^4 N r^6 \tau_d}\int_0^\infty \frac{F_d(\bar{\nu})\varepsilon_a(\bar{\nu})}{\bar{\nu}^4}\,d\bar{\nu}$$

$$k_T = (r^{-6}J\kappa^2 n^{-4}\lambda_d) \times 8.71 \times 10^{23}\ \mathrm{sec}^{-1}$$

$$R_0^6 = \frac{9000(\ln 10)\kappa^2\phi_d}{128\pi^5 N n^4}\int_0^\infty \frac{F_d(\bar{\nu})\varepsilon_a(\bar{\nu})\,d\bar{\nu}}{\bar{\nu}^4}\,d\bar{\nu}$$

Förster radius R_0 and Donor quantum yield

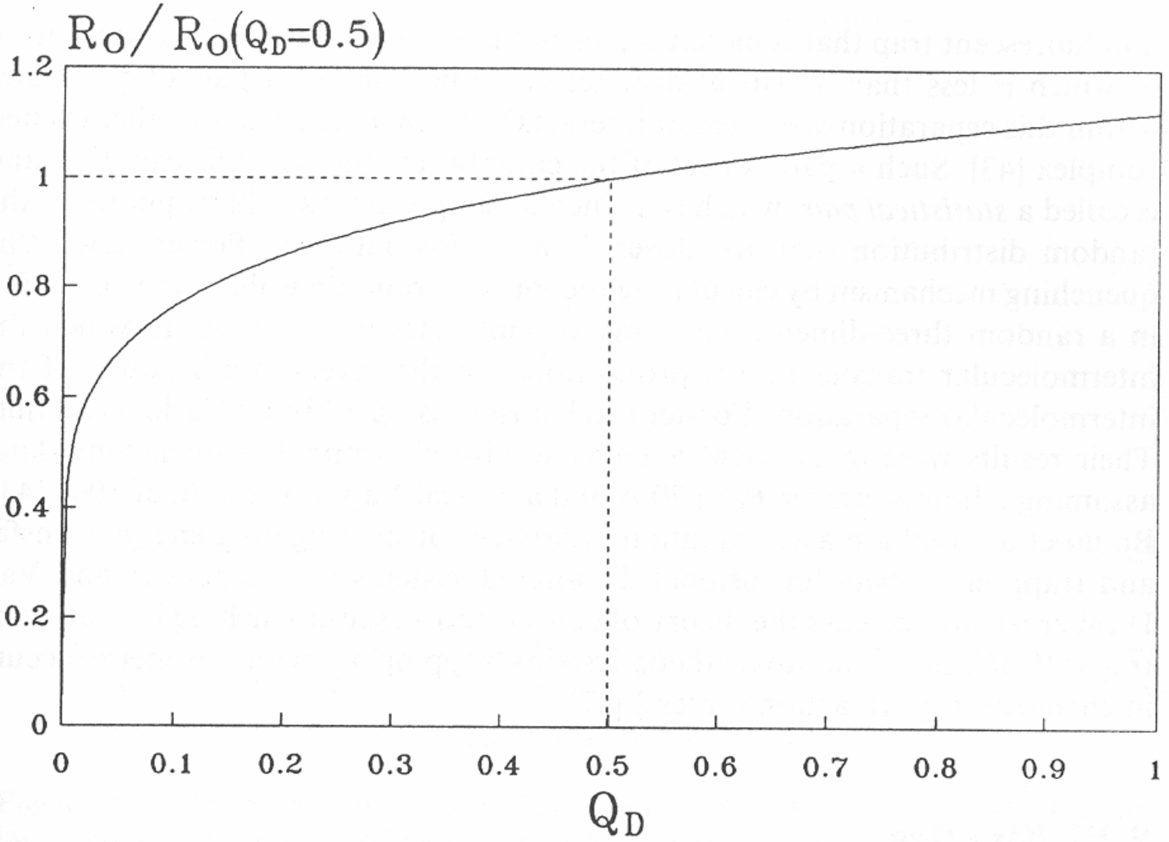


Figure 2.10. The relative Förster distance versus Q_D, the quantum yield of the donor in the absence of acceptor. R_0 denotes the Förster distance and $R_0(Q_D = 0.5)$ is the Förster distance at $Q_D = 0.5$. Q_D varies between 0 and 1 (see Chapter 7 for data).

Förster radius R_0 and index of refraction n

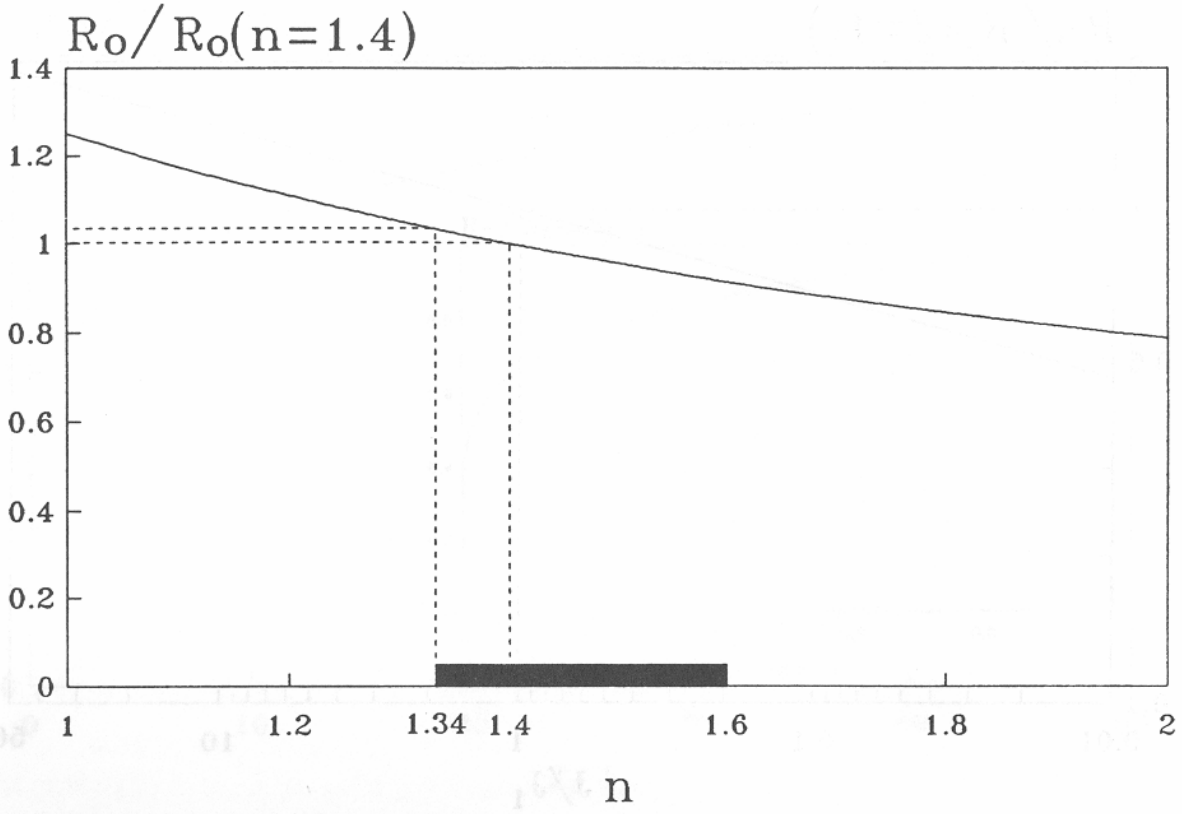
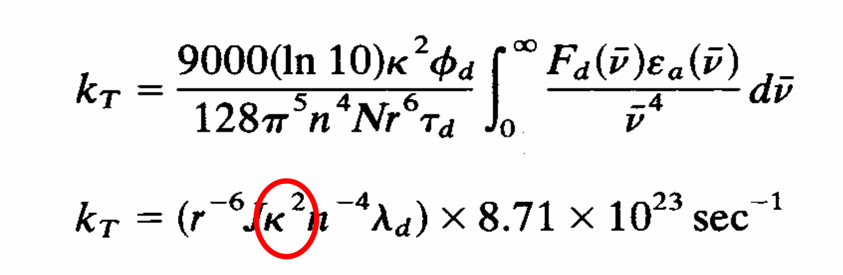


Figure 2.9. The relative Förster distance versus *n*, the refractive index of the medium in which the donor and acceptor are embedded. R_0 denotes the Förster distance and $R_0(n = 1.4)$ is the Förster distance at $n = 1.4$. All n-values in the literature are in the 1.34–1.6 range. The values 1.34 and 1.4 are the ones that are most frequently used.

fluorescence resonance energy transfer : FRET (4)

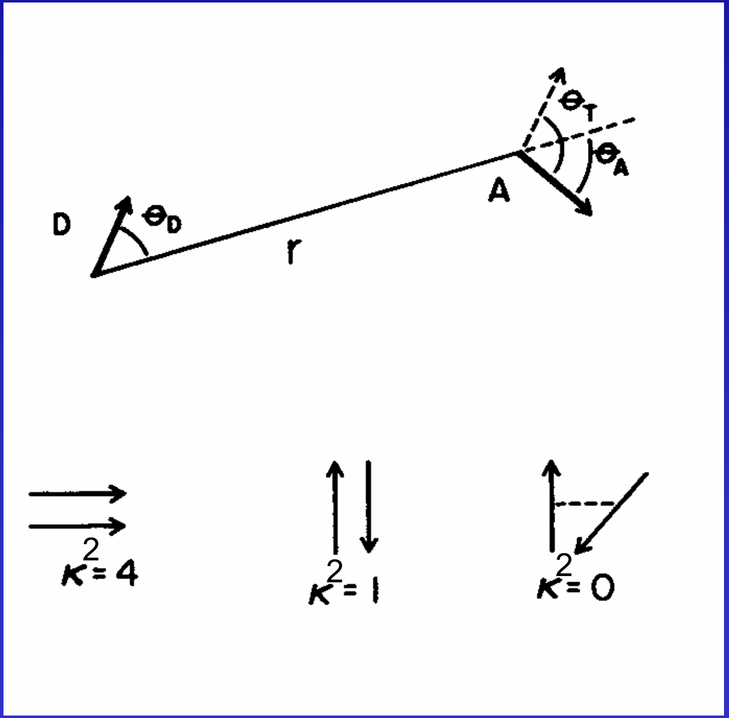
orientational factor κ^2

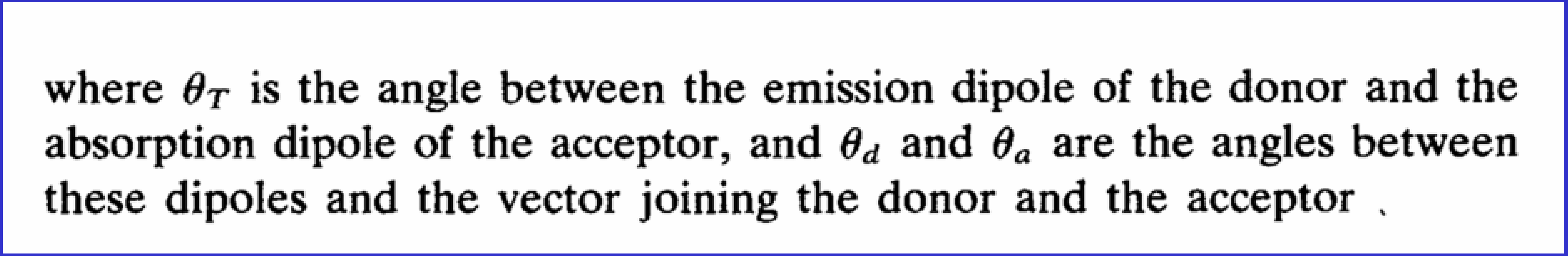
freely rotating: $\kappa^2 = 2/3$



$$k_T = \frac{9000(\ln 10)\kappa^2\phi_d}{128\pi^5 n^4 N r^6 \tau_d} \int_0^\infty \frac{F_d(\bar{\nu})\varepsilon_a(\bar{\nu})}{\bar{\nu}^4}\, d\bar{\nu}$$

$$k_T = (r^{-6} J \kappa^2 n^{-4} \lambda_d) \times 8.71 \times 10^{23}\ \text{sec}^{-1}$$





where θ_T is the angle between the emission dipole of the donor and the absorption dipole of the acceptor, and θ_d and θ_a are the angles between these dipoles and the vector joining the donor and the acceptor .

fluorescence resonance energy transfer : FRET (5)

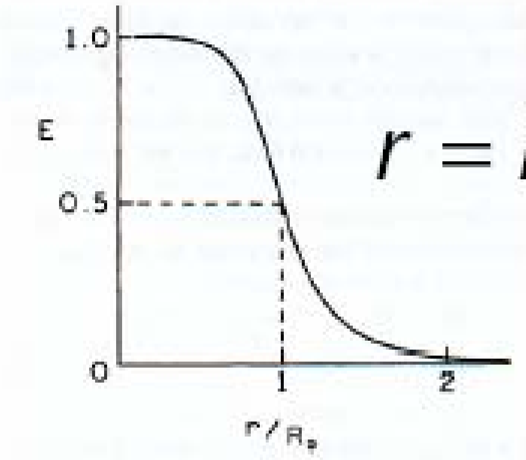
transfer efficiency and distance measurement

$$E = \frac{k_T}{\tau_d^{-1} + k_T} = \frac{k_T}{\Gamma_d + k_T}$$

⇨

$$E = 1 - (\tau_{da}/\tau_d)$$

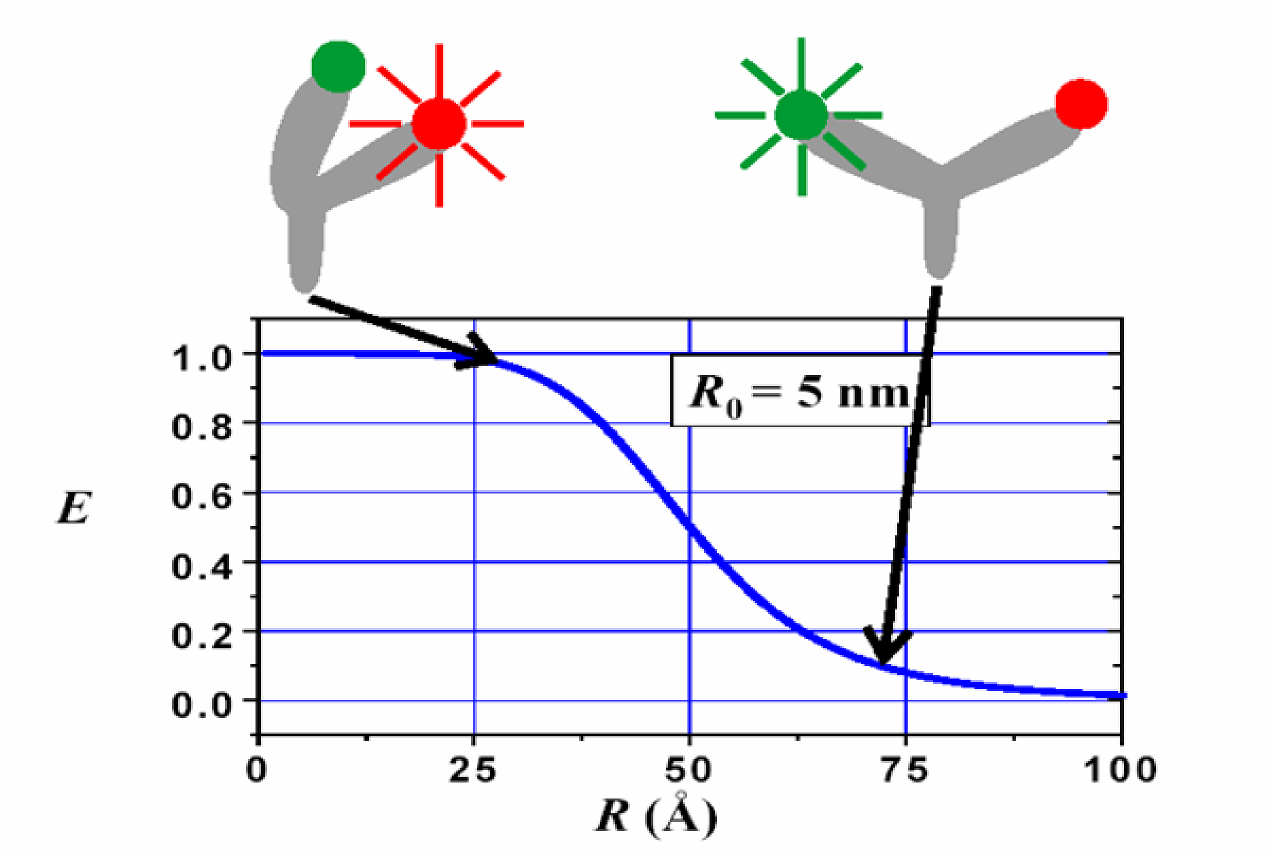
$$E = 1 - (F_{da}/F_d)$$



$$r = R_0^{\ 6}/(1/E - 1)$$

$$E = \frac{R_0^6}{R_0^6 + r^6}$$

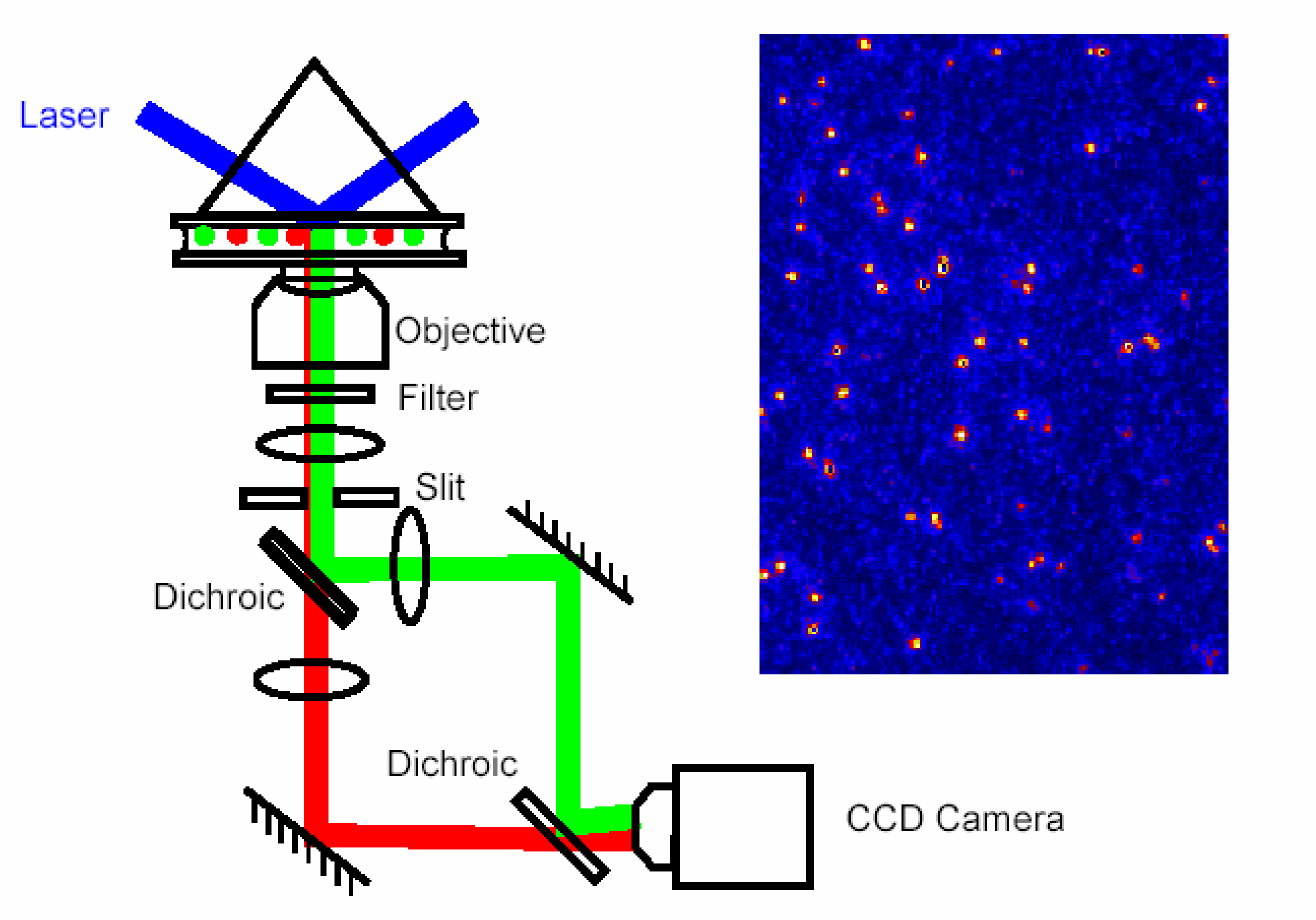
fluorescence resonance energy transfer : FRET (6)



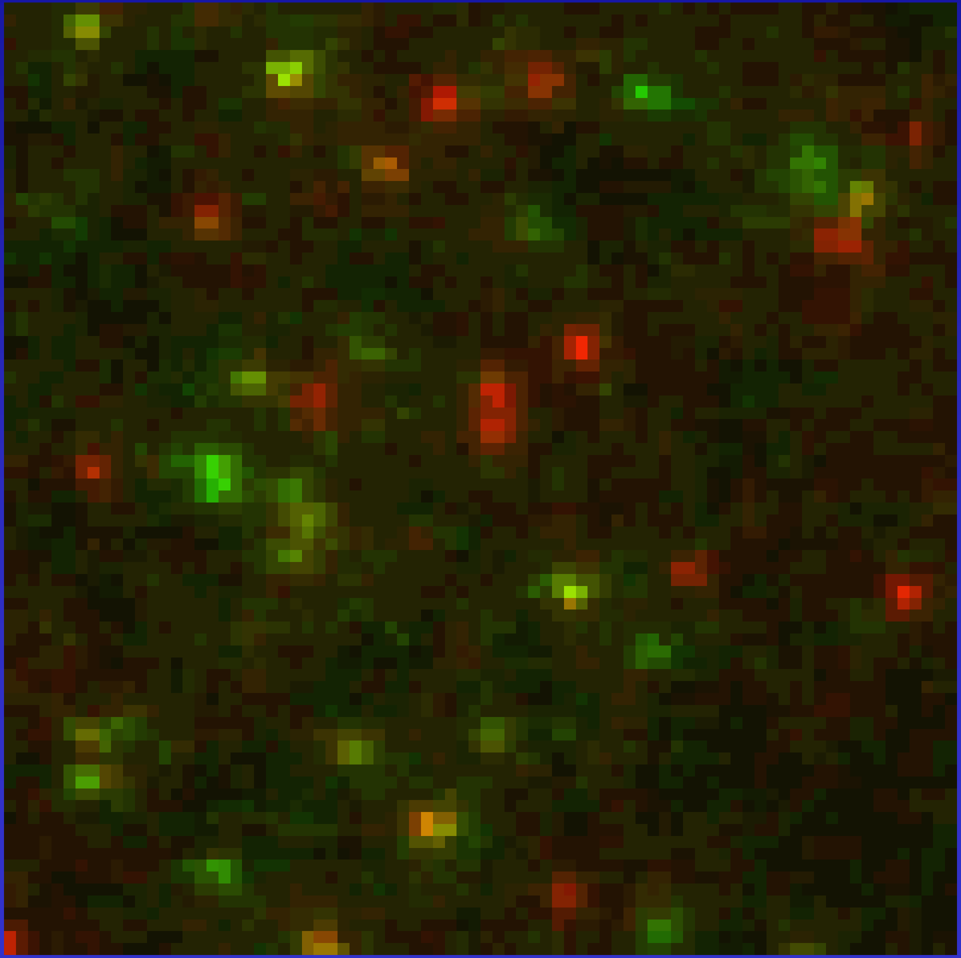
fluorophore interaction: energy, symmetry, distance

single-molecule FRET imaging

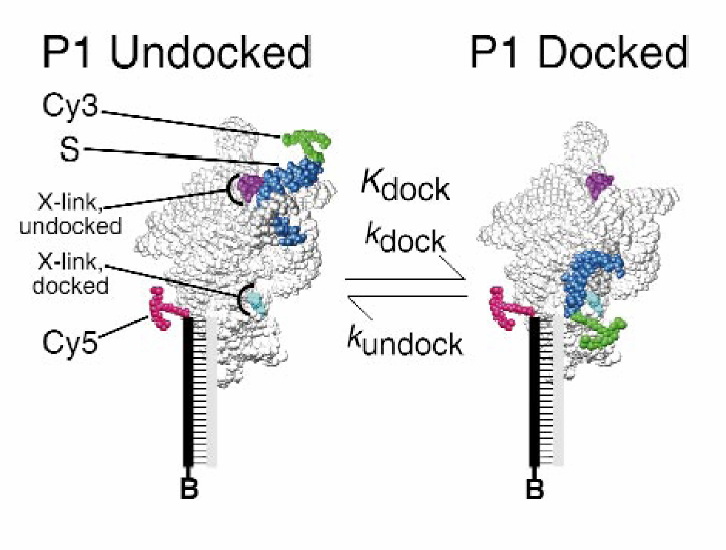
TIRFM with CCD

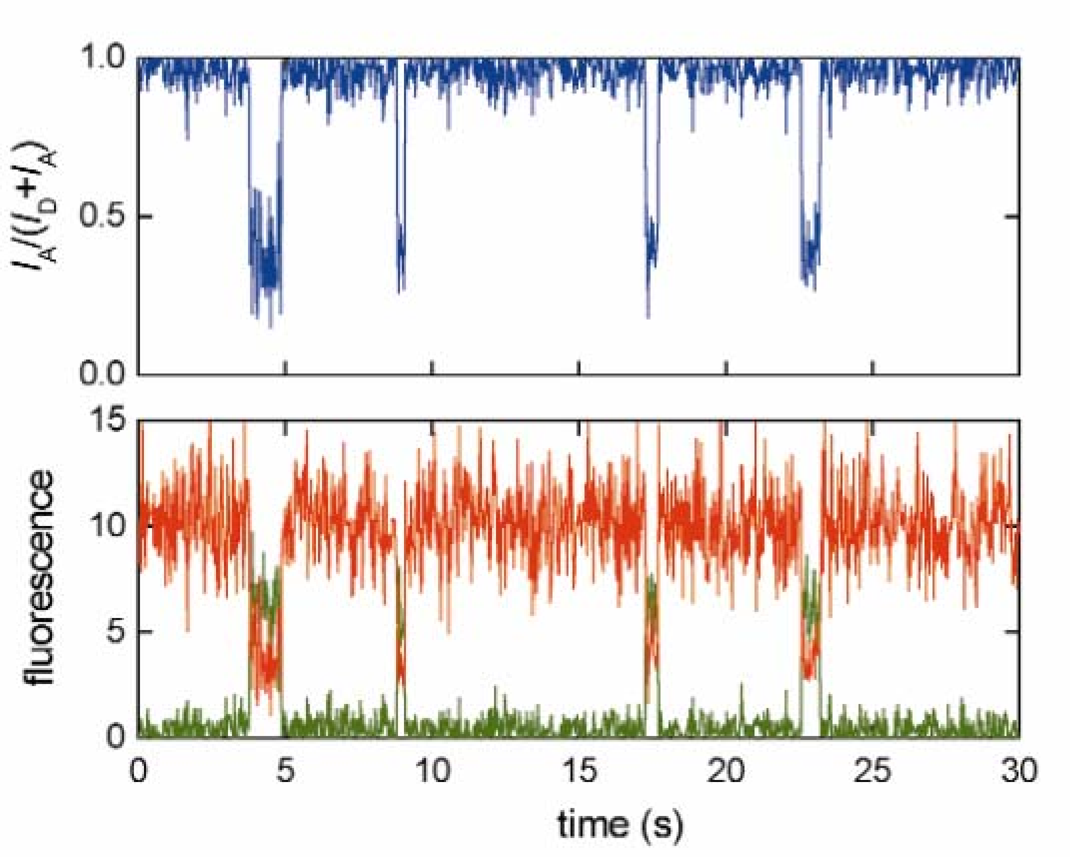


Ribozyme folding movie



reverible docking / dissociation of P1 site @*Tetrahymena* ribozyme





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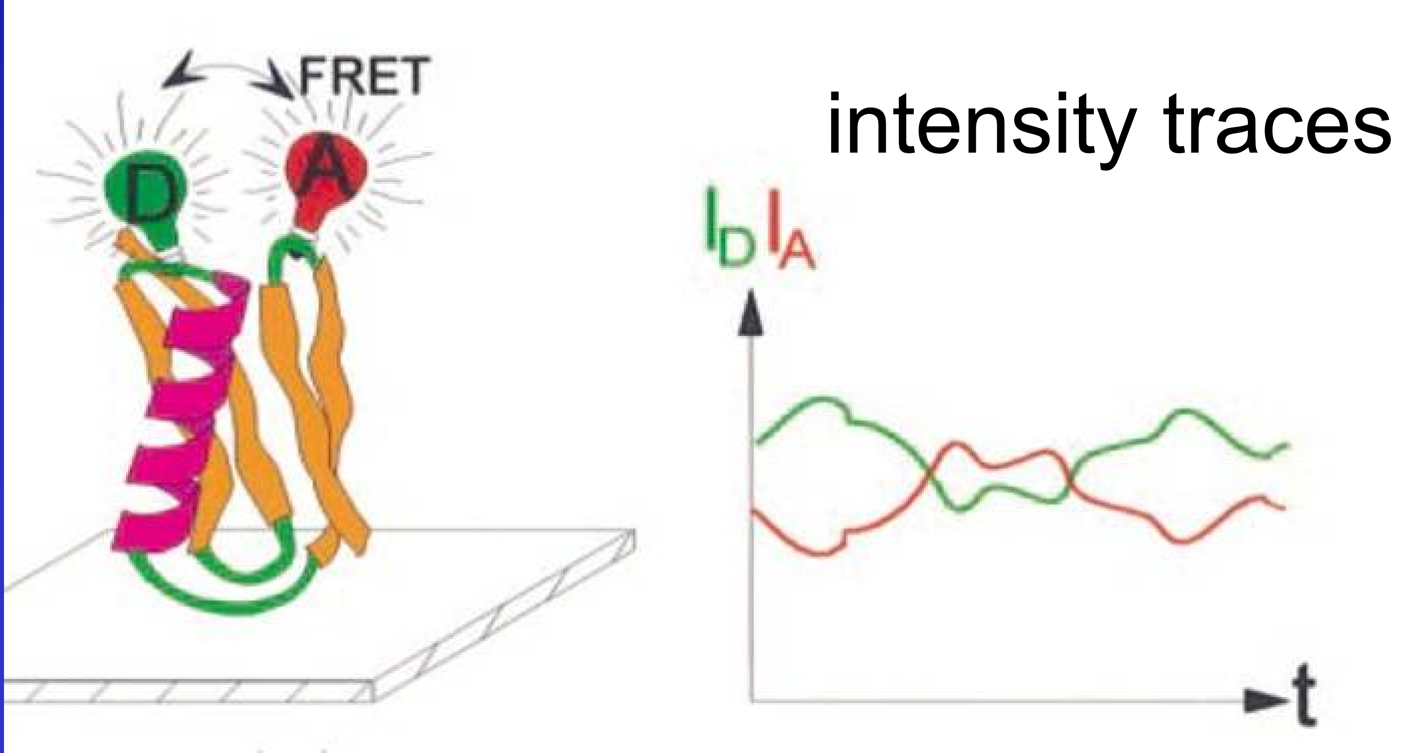
fluorescence resonance energy transfer : FRET (7)

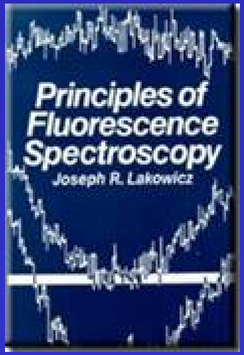
distance dependence on nanometer scale: 1-10



intensity traces

literature

- http://www.biophys.leidenuniv.nl/Teaching/Spectroscopy/Fluorescence.pdf
  (T.Schmidt)
- Bernard Valeur, Molecular Fluorescence, Wiley-VCH
- Joseph Lakowicz, Principles of Fluorescence Spectroscopy, Plenum Press



Principles of Fluorescence Spectroscopy
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next 26/05/2003

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