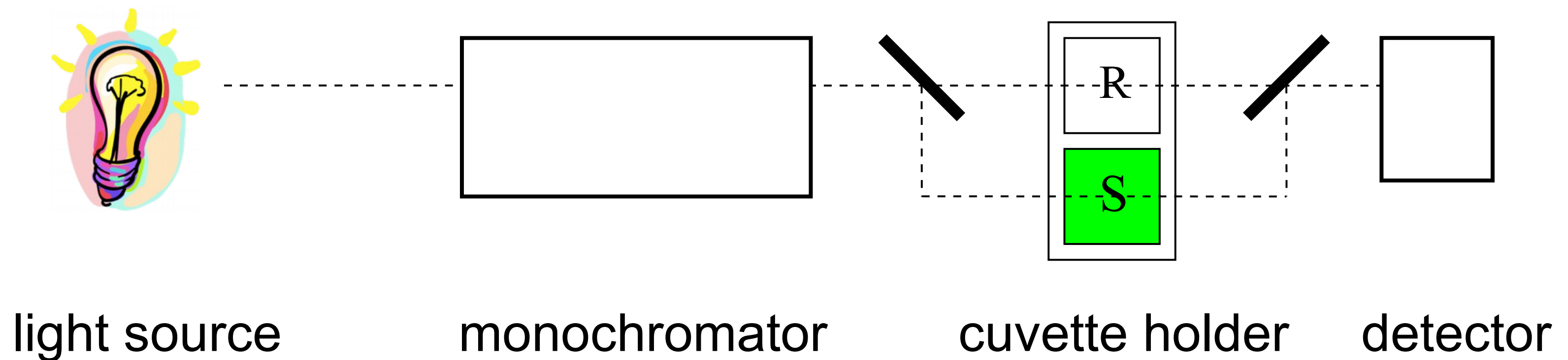
Spectroscopy

Absorption

Reported by:

1) percent transmission ($\%T_\lambda = 100 \times I_\lambda / I_{0,\lambda}$)
2) absorbance ($A_\lambda = \log \{I_{0,\lambda}/I_\lambda\} = \varepsilon_\lambda c l$)

Measured by: single or double beam spectrophotometer

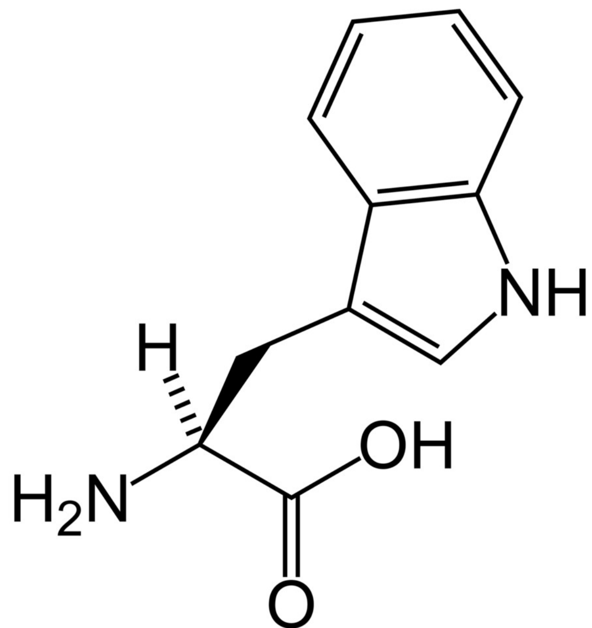


Significance of deviations from the Beer-Lambert Law:

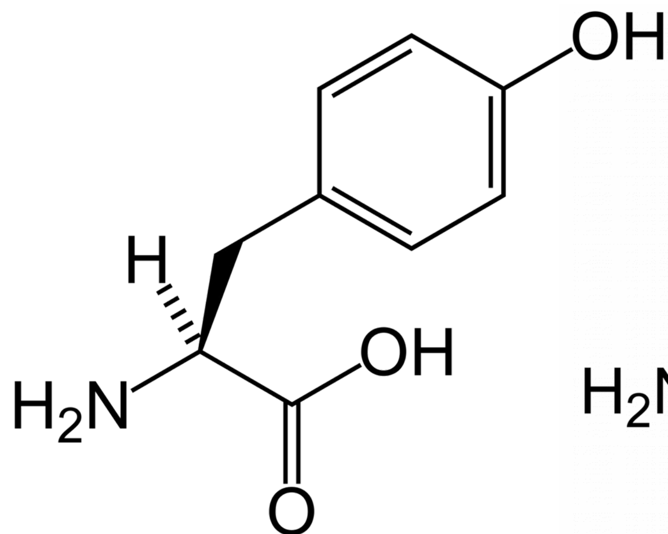
If nothing happens in a solution as a function of concentration of the component solutes, that is there are no concentration-dependent interactions, then the OD should increase linearly with increases in concentration of the components (this requires that all solute components have proportional increases in concentration or that different components have equivalent extinction coefficients.

Deviations mean interactions. The change in absorbance versus concentration will follow the thermodynamics of the system, reflecting cooperativity, positive or negative, or lack thereof.

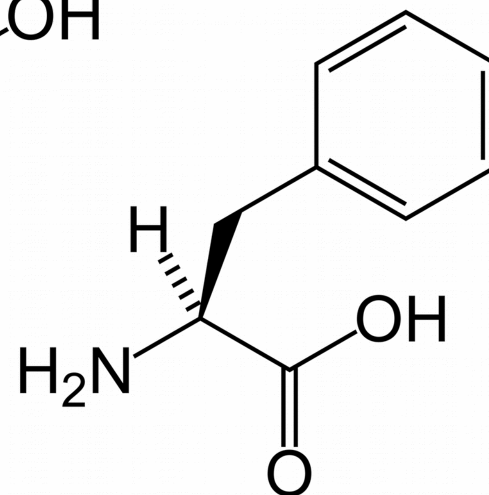
Aromatic Amino Acids



Trp (W)



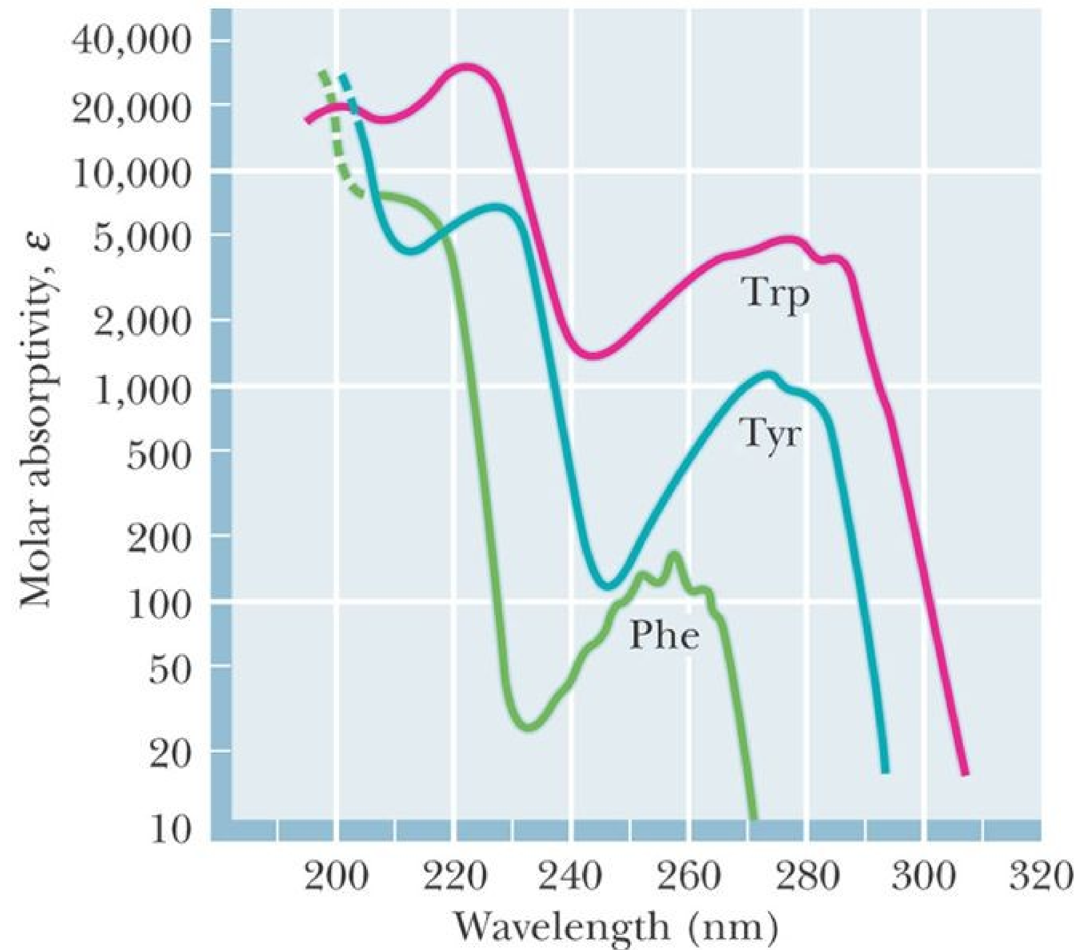
Tyr (Y)



Phe (F)

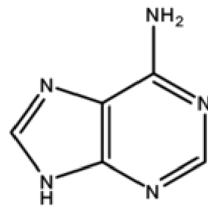
Spectroscopy

The ultraviolet absorption spectra of the aromatic amino acids at pH 6. (*From Wetlaufer, D.B., 1962. Ultraviolet spectra of proteins and amino acids.* Advances in Protein Chemistry **17:***303–390.*)

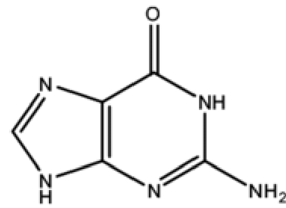


© 2005 Brooks/Cole - Thomson

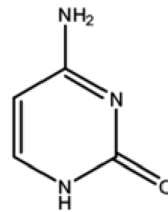
Spectroscopy



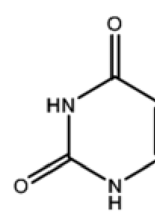
Adenine
A



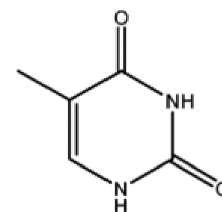
Guanine
G



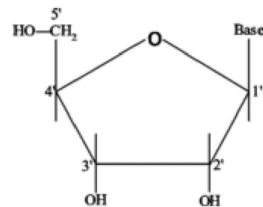
Cytosine
C



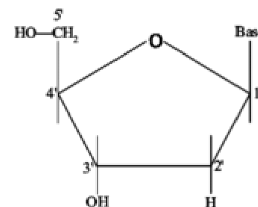
Uracil
U



Thymine
T



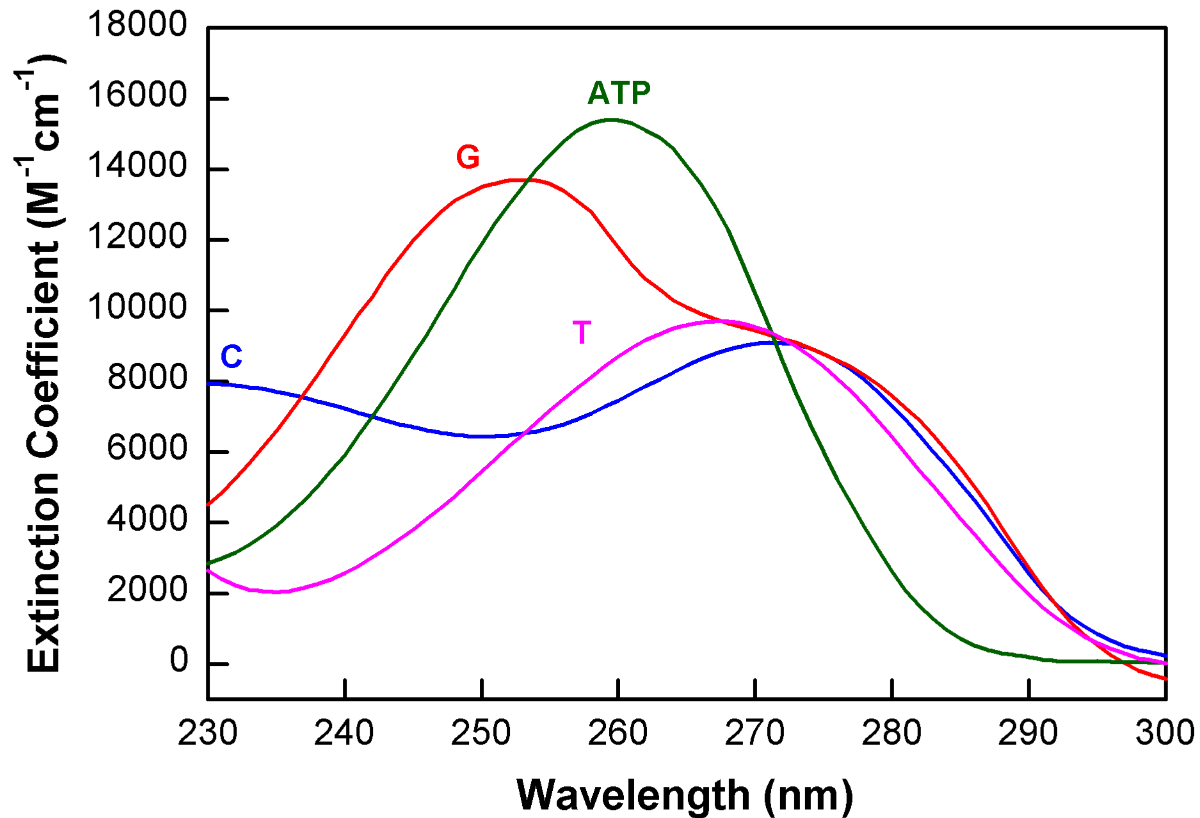
Ribose



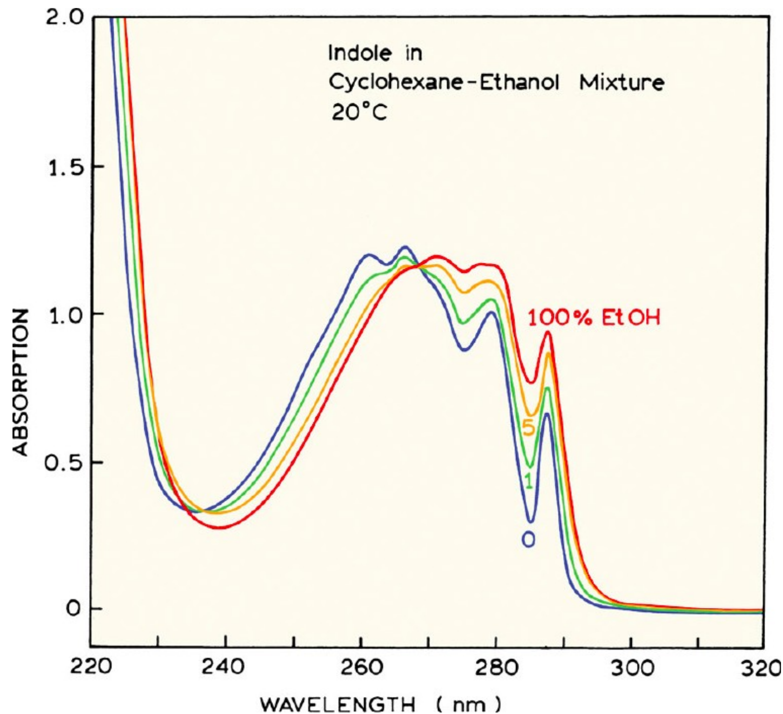
Deoxyribose

Figure 1.1. Structures of nucleic acid constituents

Absorption Spectra of the Nucleic Acids



Spectroscopy



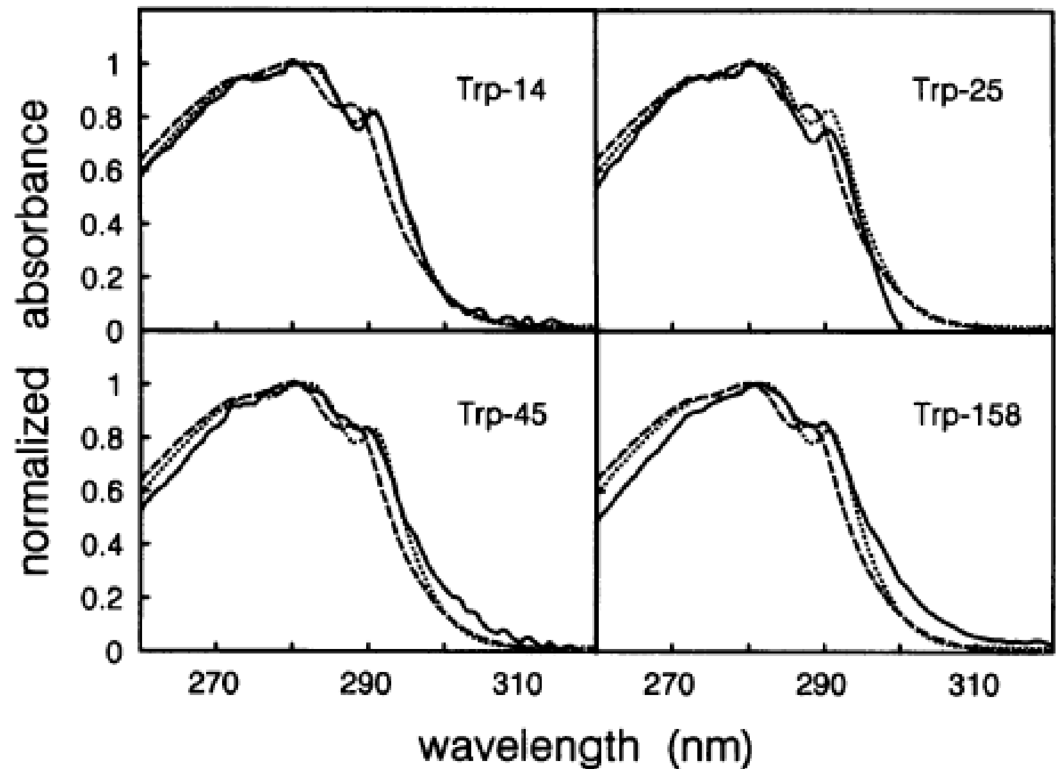
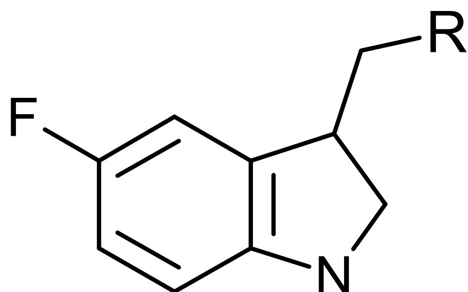


FIGURE 4 Comparison of normalized absorbance spectra of the four Trp in sTF (–) with that of NATA in aqueous buffer (- - -) and in dioxane (····).

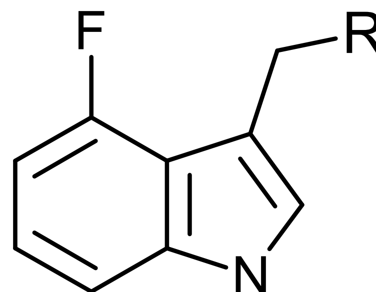
Lakowicz, Principles Fluorescence Spectroscopy 3rd Ed., 2006

Hasselbacher et, al., Biophys J 60, 20 (1995)

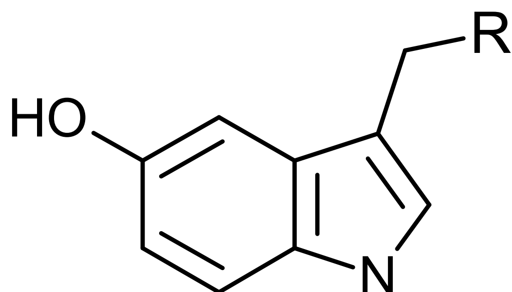
Tryptophan Analogs



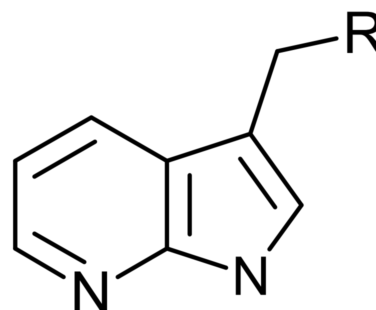
5-fluorotryptophan



4-fluorotryptophan



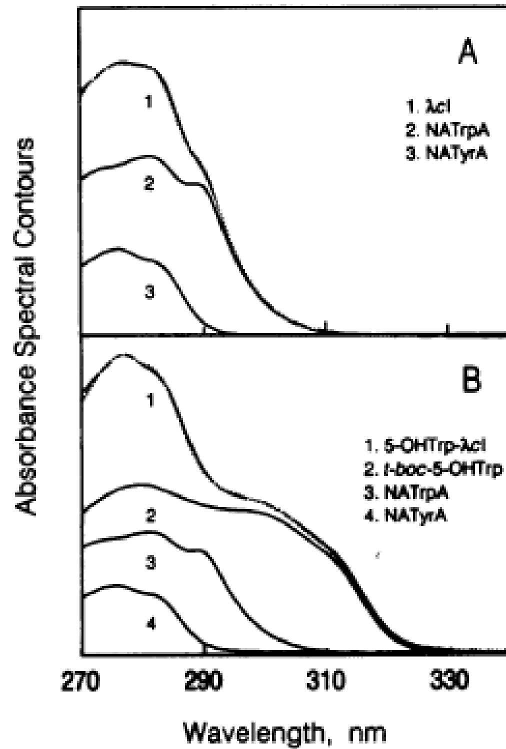
5-hydroxytryptophan



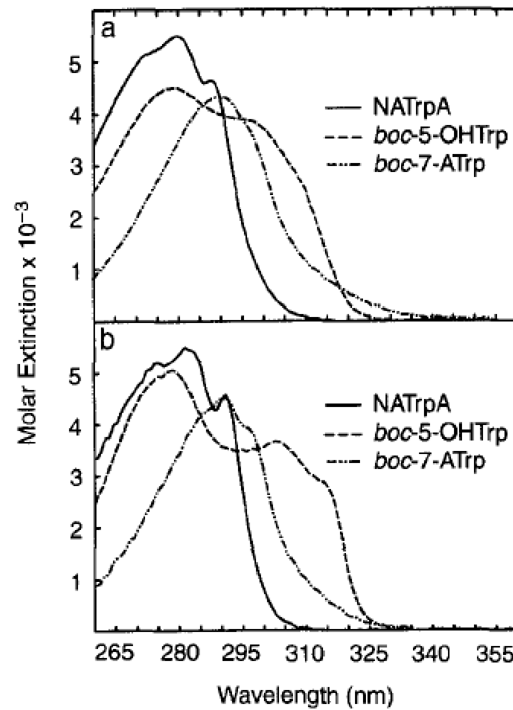
7-azatryptophan

Ross et al.,Topics in Fluorescence Spectroscopy 6, Lakowicz ed. 2000

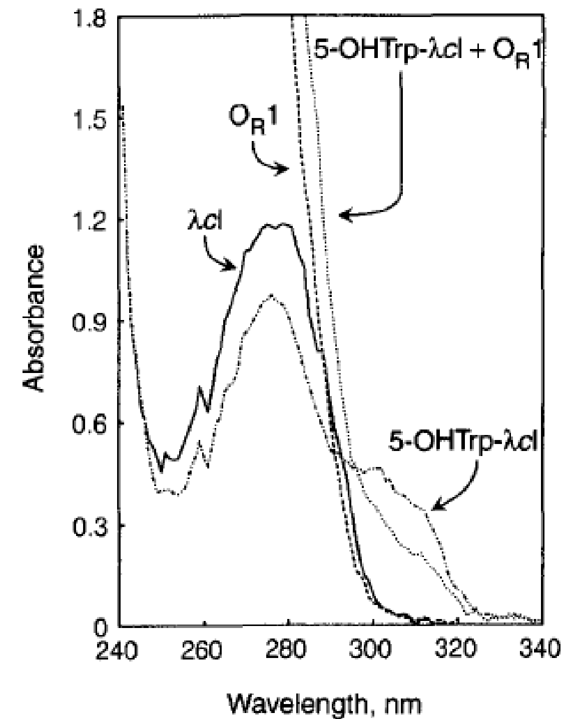
Tryptophan Analogs



Ross et al., Proc Natl Acad Sci USA 89, 12923 (1992)



Ross et al., Meth Enzymol 278, 151 (1998)



Ross et al., Meth Enzymol 278, 151 (1998)

Tryptophan Analogs

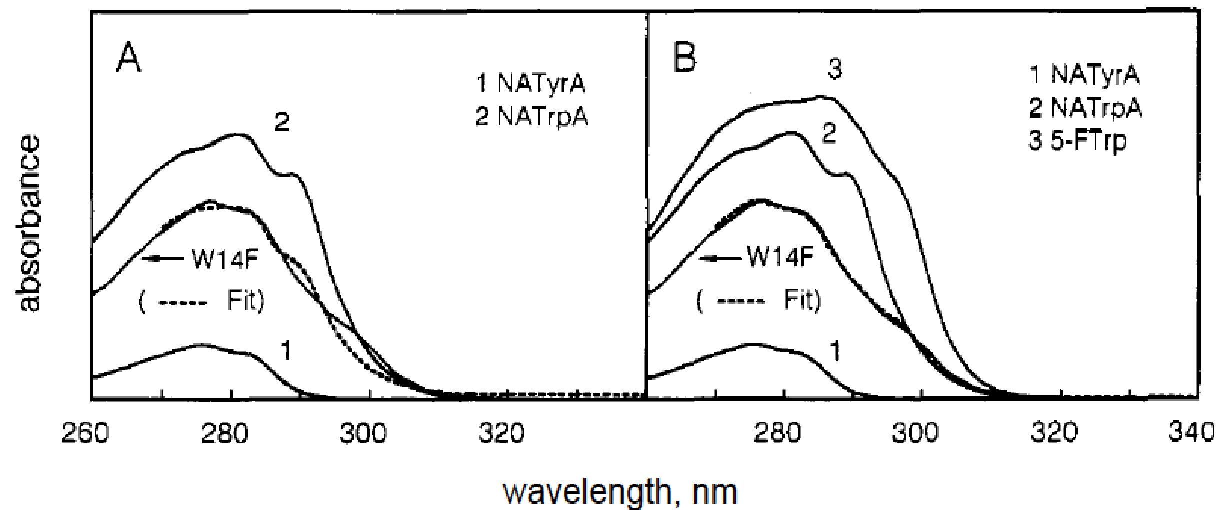
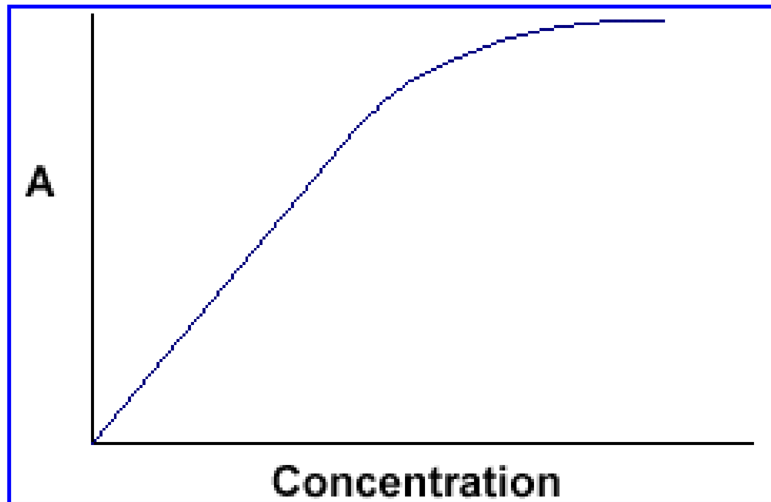


Figure 2.2. LINCS analysis of W14F sTF expressed in the presence of 5-FTrp. Panel A shows the fit from 270 to 340nm (dashed line) of the protein absorbance spectrum (solid line) using the NATyrA and NATrpA basis sets. Panel B shows the corresponding fit (dashed line) when 5-FTrp is included as a third basis set.

Ross et al., Topics in Fluorescence Spectroscopy 6, 17 (2000)

Relationship between Absorbance and Concentration



Why is this plot not linear for the entire range?

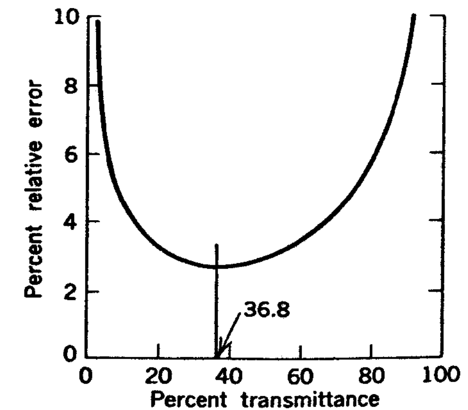
At some point the absorbance is so high that not sufficient light passes through to the detector, and linearity is no longer satisfied.

The absorbance at which an instrument becomes non-linear depends on the following factors:

1. Concentration of the analyte
2. Lamp intensity at the measured wavelength
3. Extinction coefficient of the analyte at measured wavelength
4. Sensitivity of detector at the measured wavelength

- **For best accuracy, always measure between 0.1 – 1.0 OD**

Spectroscopy



- Concentration

  Beer-Lambert Law (Beer's Law): $A_\lambda = \varepsilon_\lambda c l$

  most accurate range to obtain *c*: between 15 and 65 %T
  which is A between 0.2 and 0.8

- Identification of Chromophore(s) by Spectra

  Are spectra from multiple chromophores in a macromolecule additive?

  $$A(\lambda,\text{sample}) = \alpha A(\lambda,a) + \beta A(\lambda,b) + \gamma A(\lambda,c) + \delta A(\lambda,d) + \ldots..$$

  LINCS: LINear Combination of Spectra

  approximately linear in proteins

  but not linear in DNA or RNA

- Investigate Interacting Systems by Difference Spectra

  Quantification of Binding

  Evaluation of Conformational Changes

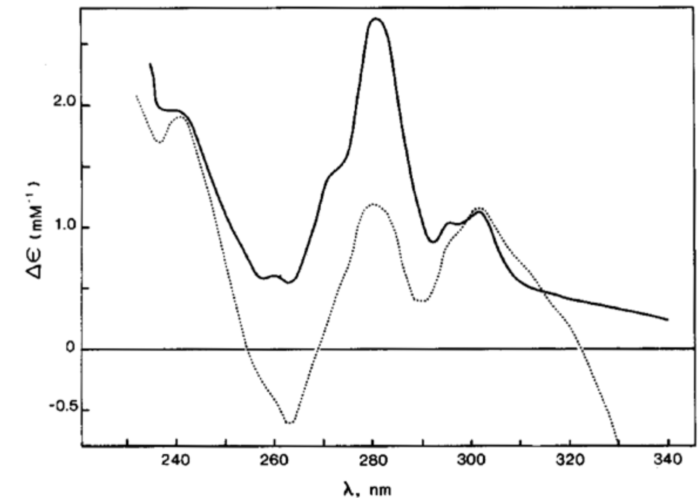


FIG. 2. Absorption difference spectra due to ternary complex formation with liver alcohol dehydrogenase: ——, enzyme·NAD⁺·trifluoroethanol complex; · · · ·, enzyme·NADH·isobutyramide complex.

Laws and Shore, J. Biol Chem 254, 2582 (1979)

PRINCIPLES OF FLUORESCENCE SPECTROSCOPY

Sir George Stokes
1819-1903

Born in County Sligo, Ireland, Stokes was the Lucasian Professor of Mathematics at Cambridge from 1849 until his death in 1903.

Some major contributions:

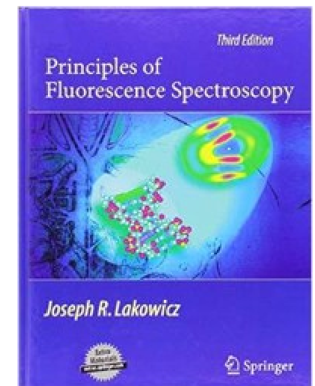
Fluid dynamics (Stokes' Law)

Wave theory of light

Polarization of light

Fluorescence of minerals

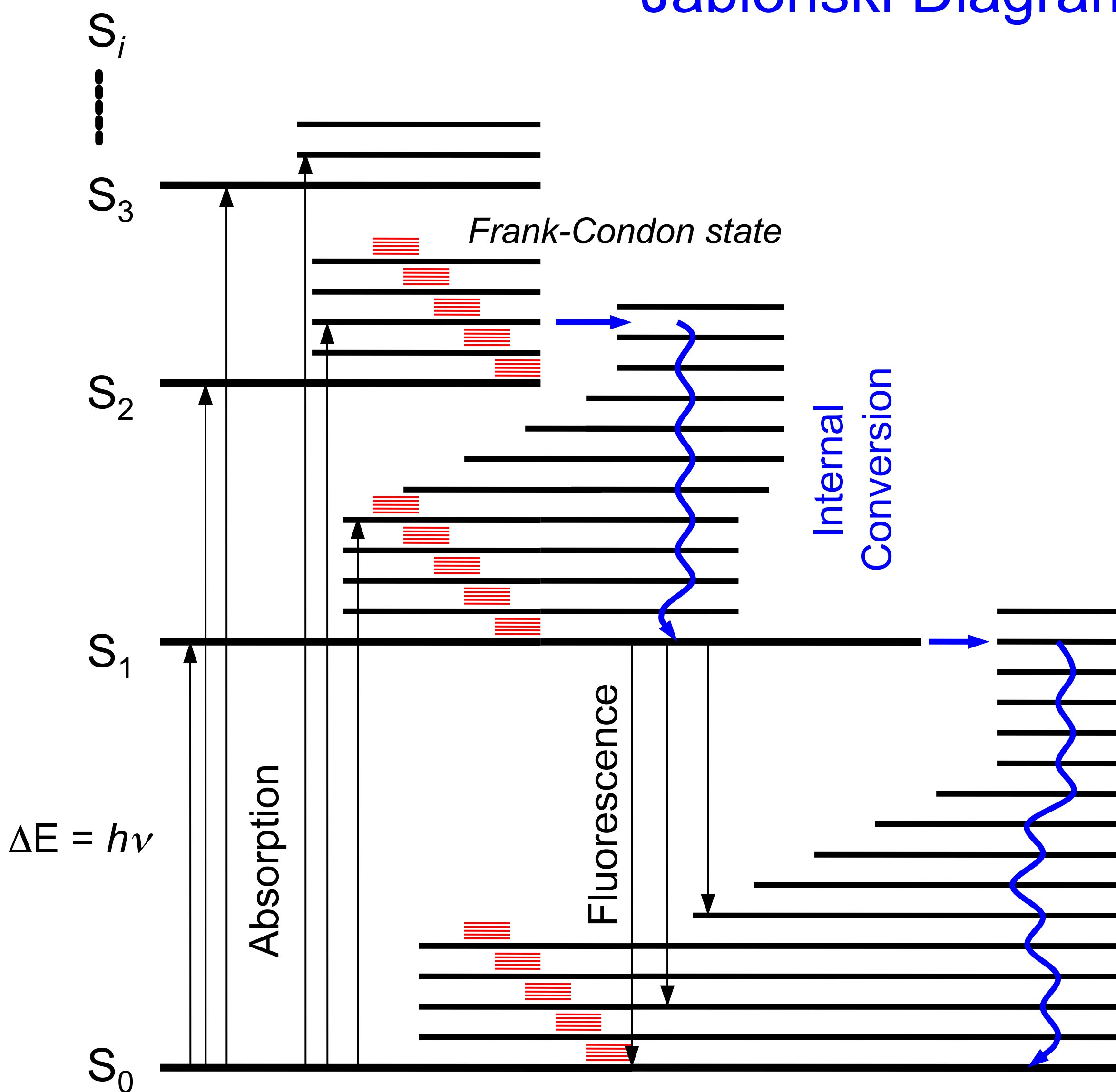
Stokes' line (Raman scatter)



Lakowicz, Principles Fluorescence Spectroscopy 3rd Ed., 2006

Spectroscopy

Jabłonski Diagram



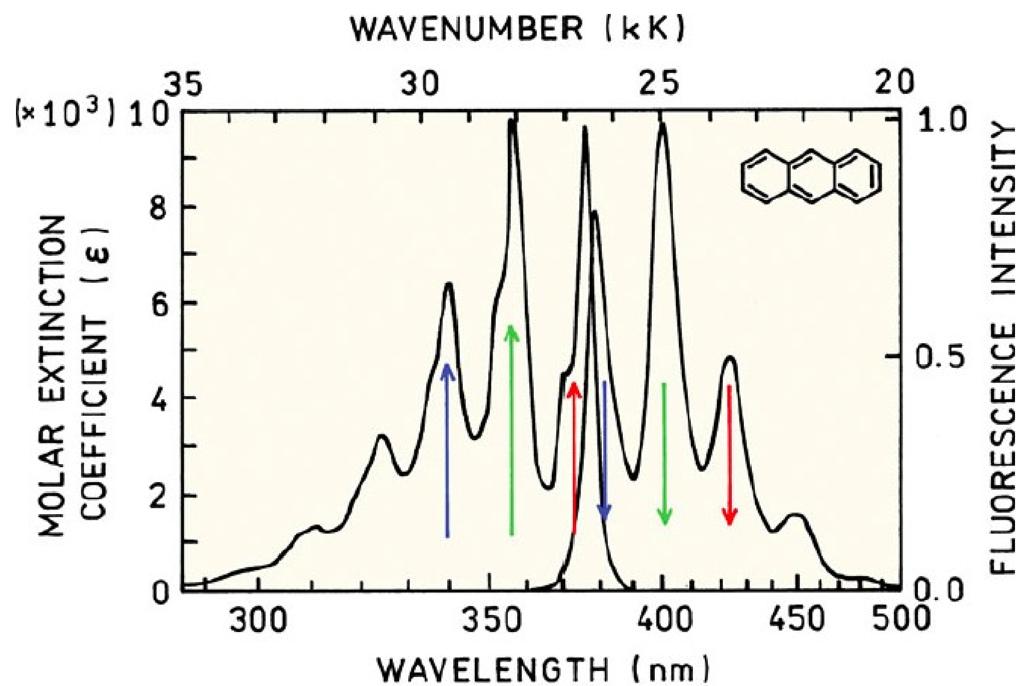
Time Scales

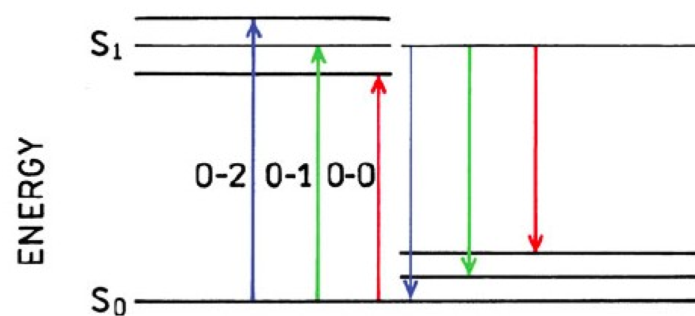
Abs.: ~ 10^{-15} s

IC.: < 10^{-12} s

Fluor: ~ 10^{-9} s

Spectroscopy





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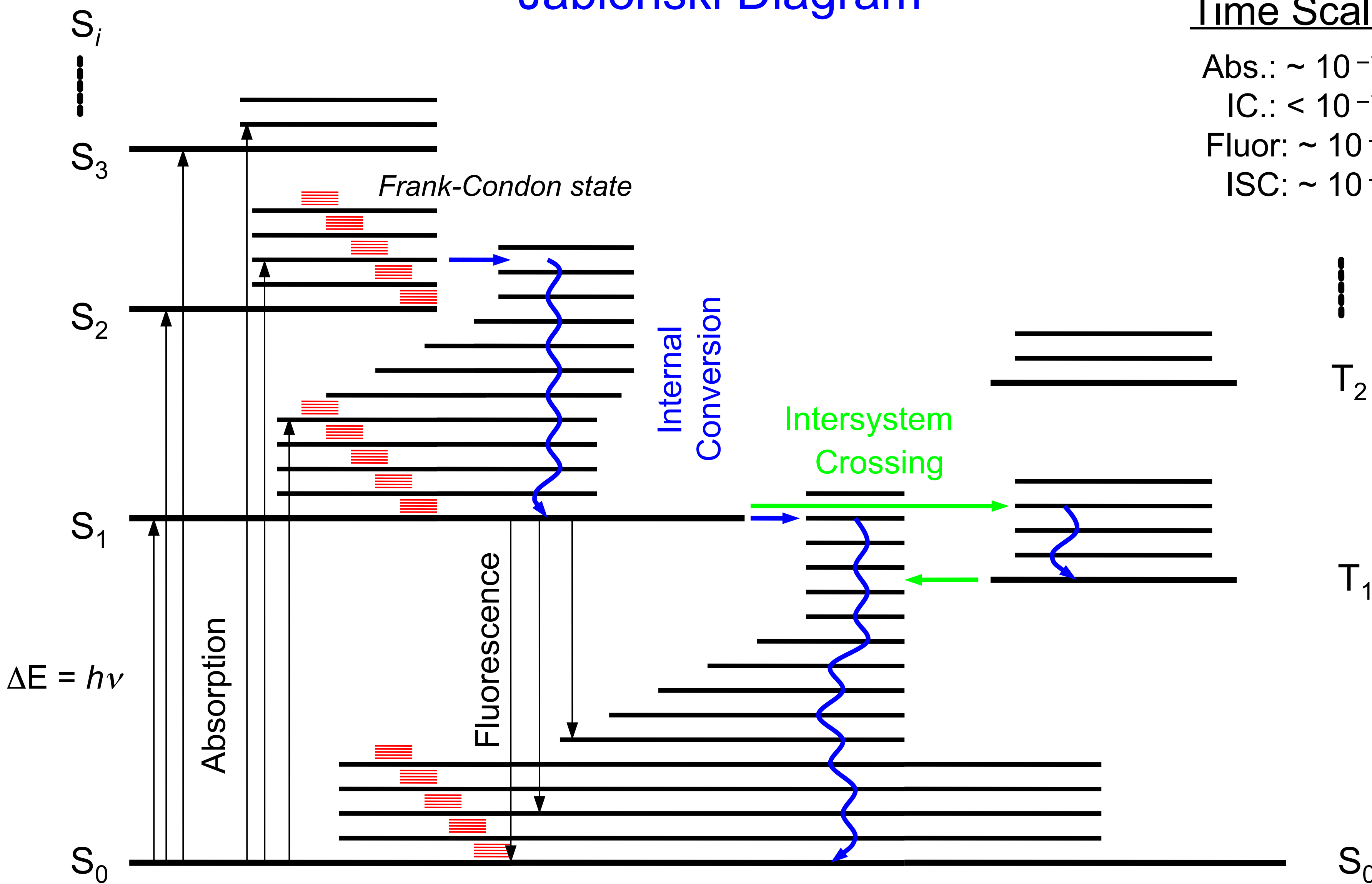
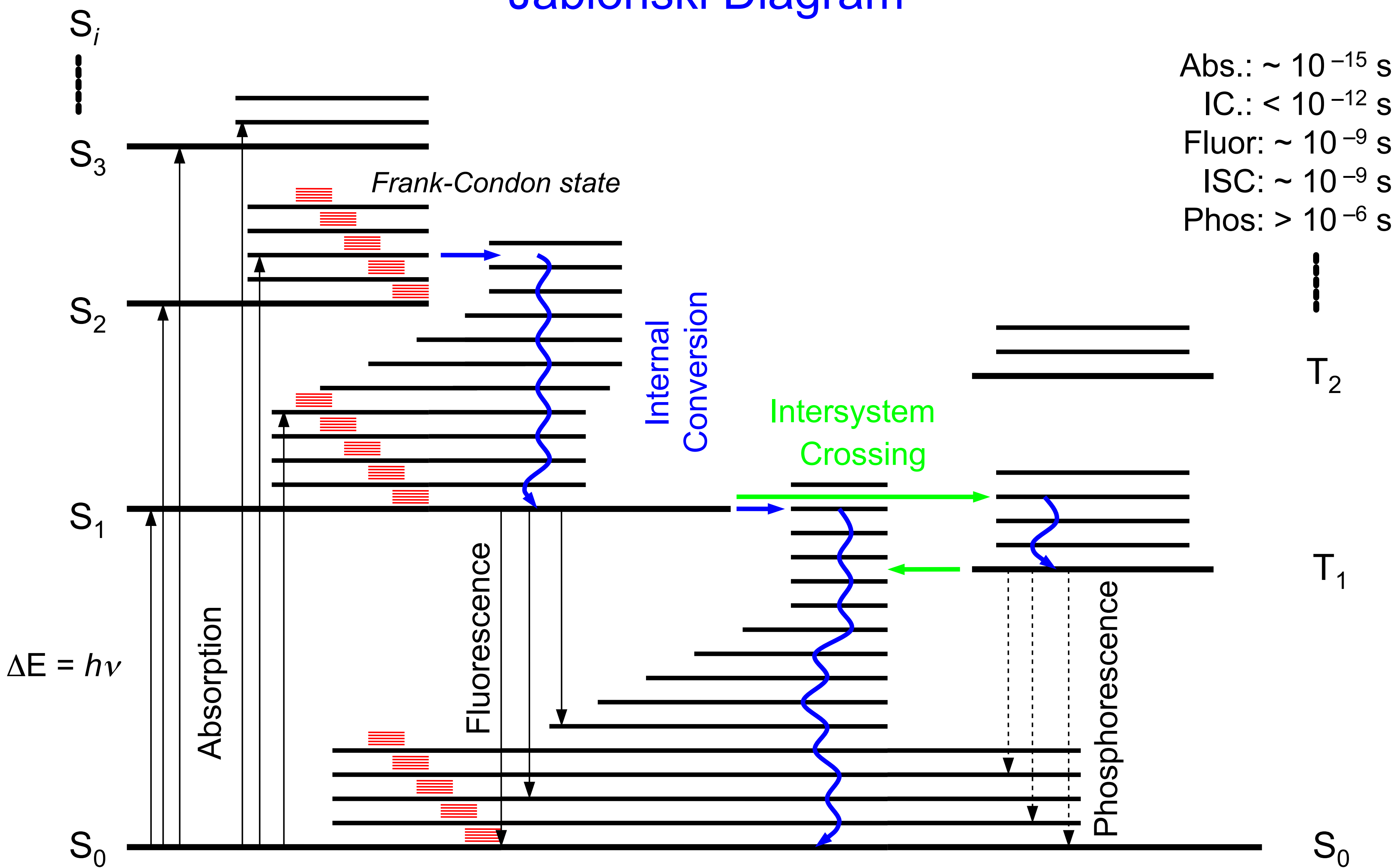
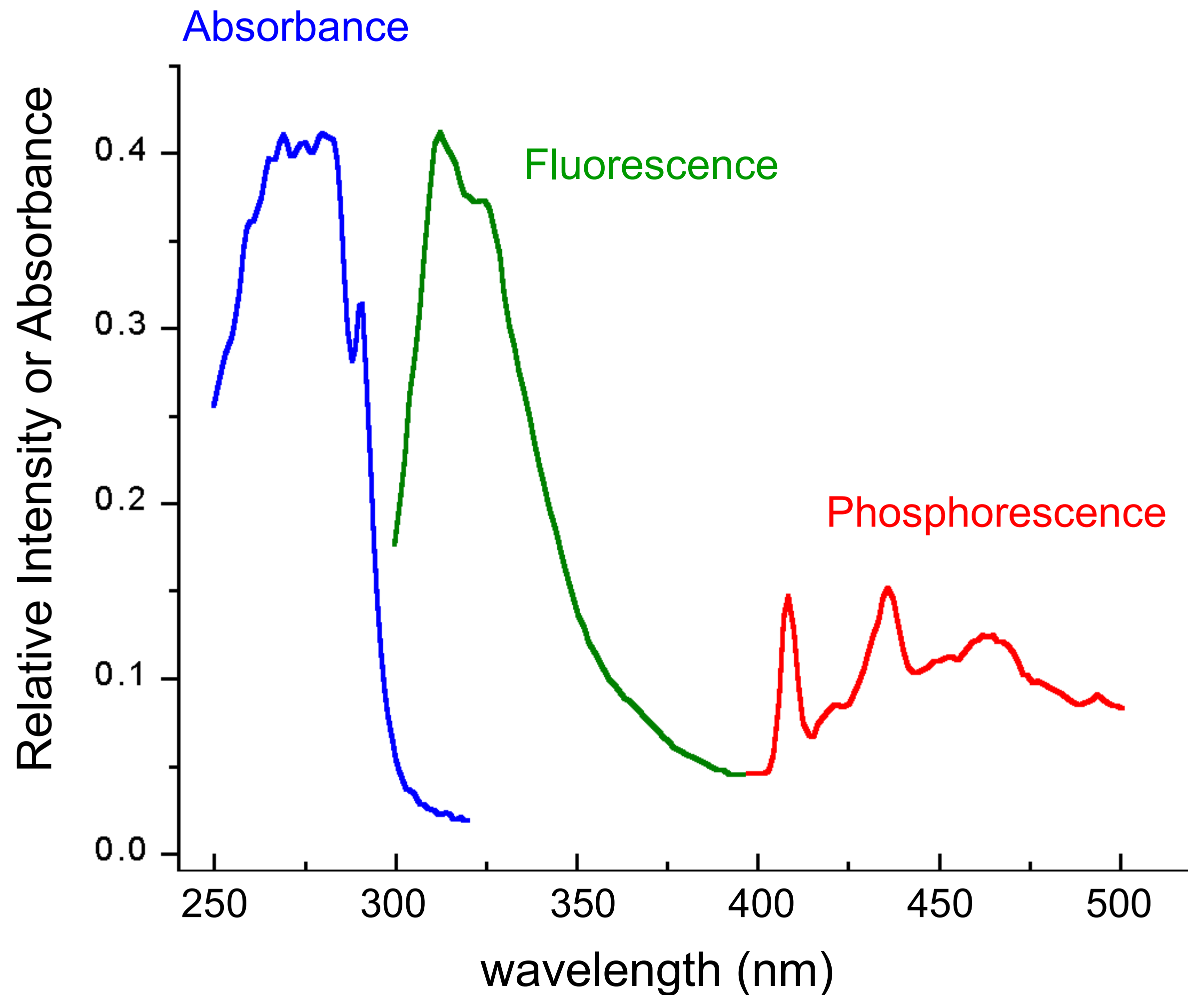
Spectroscopy



Spectroscopy



Single Trp Residue in Cod Parvalbumin; 77 K



Loss of Energy from Excited State back to Ground State

- Internal Conversion (IC; mainly through vibrational relaxation)
- Quenching: collisions with solvent, solutes, or groups of chromophore
- Intersystem Crossing (ISC)
  phosphorescence from long-lived triplet state
- Förster Resonance Energy Transfer (FRET)
- Emission of a photon
  fluorescence from lower energy than from initial Frank-Condon state; Stoke's shift
- Excited-State Reactions

Excited-State Reactions

- Bond Breaking (UV, x-ray)
- Bleaching
  - reactions with O_2, etc.
  - photorecovery experiments
- Labeling Reactions
- Generation of New Emitters
  - proton transfer ($A^* \leftrightarrow B^{-*} + H^+$)
  - excimer formation (excited-state dimer: $A^* + A \leftrightarrow A\,A^*$)
- Solvent (dipolar) Relaxation
  - $S_1 \rightarrow S_1' \rightarrow \rightarrow S_1'' \rightarrow \rightarrow \rightarrow S_1''' \rightarrow \rightarrow \rightarrow \rightarrow \ldots..$

Lifetime and quantum yield

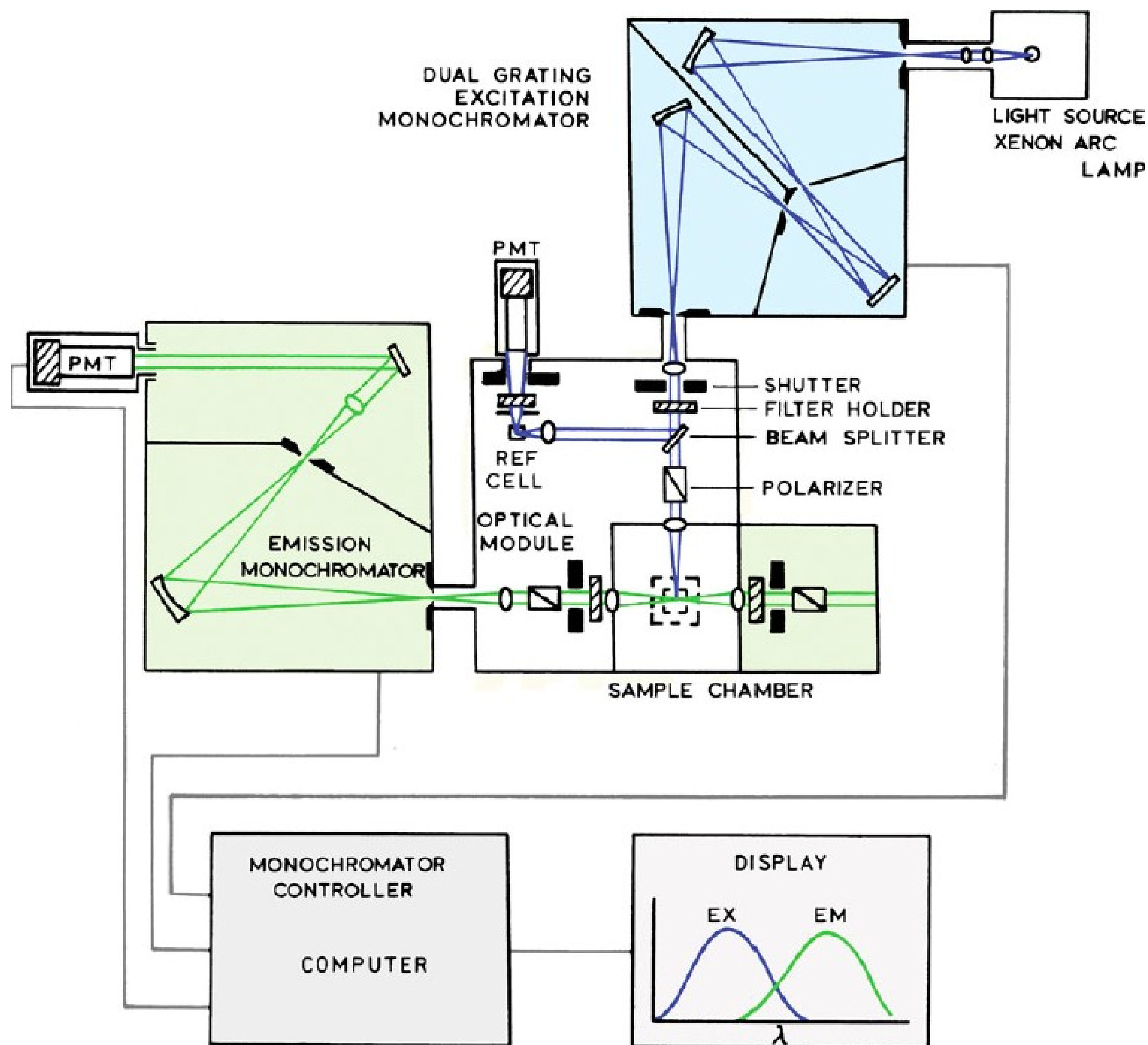
$\tau = 1 / (K_f + \Sigma K_{nr})$ excited-state lifetime

$\varphi = K_f / (K_f + \Sigma K_{nr})$ excited-state quantum yield

$F_\lambda = \varphi A_\lambda = \varphi \varepsilon_\lambda c l$ fluorescence intensity

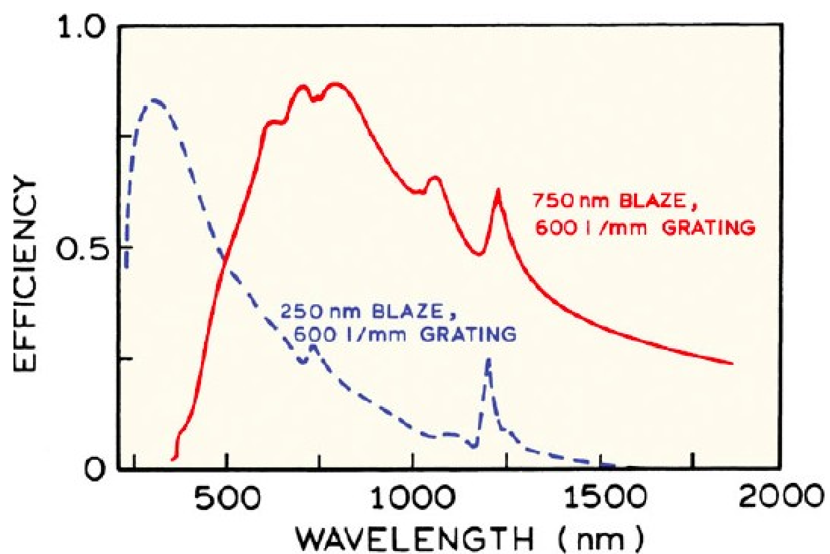
ΣK_{nr} = IC + ISC + e^- transfer + ….. other dynamic processes

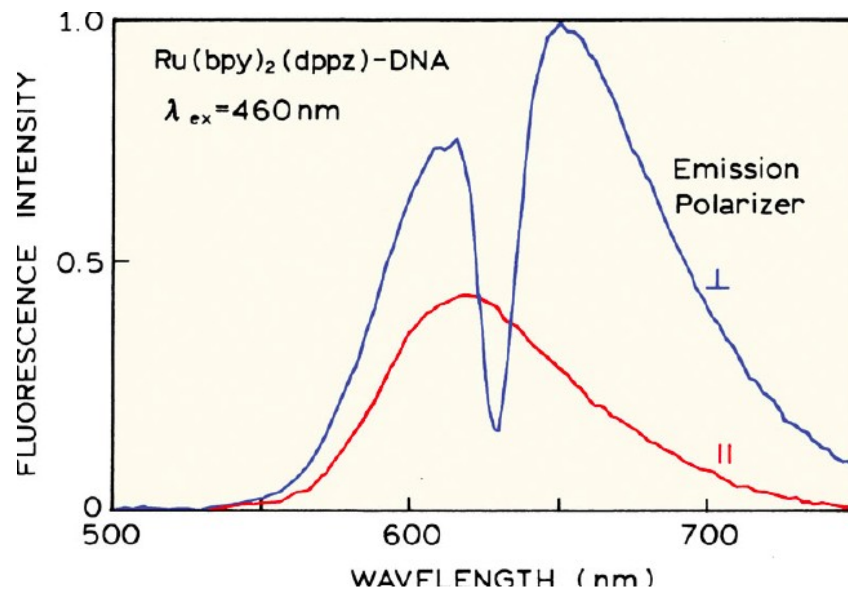
Spectroscopy



Lakowicz, Principles Fluorescence Spectroscopy 3rd Ed., 2006

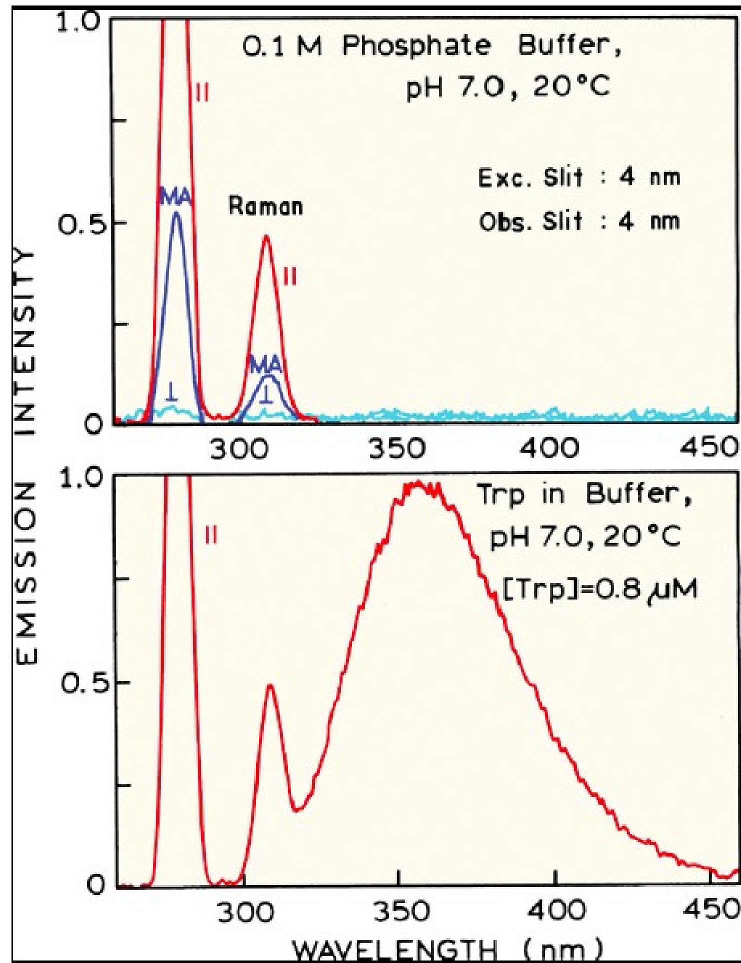
Spectroscopy

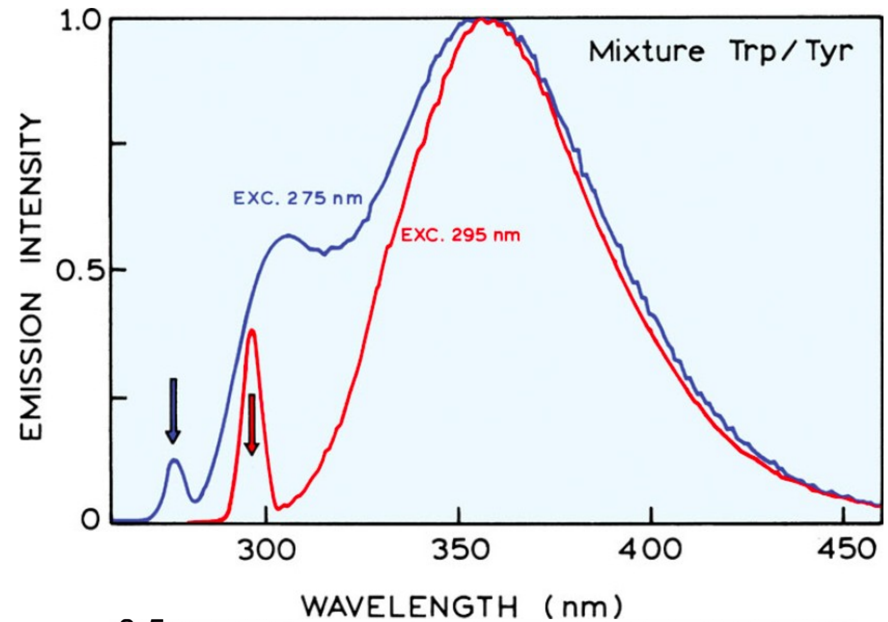


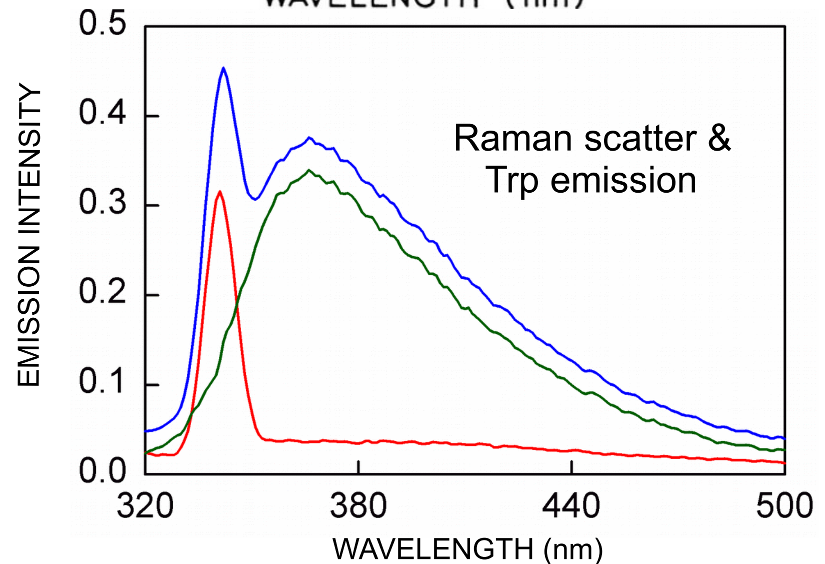


Lakowicz, Principles Fluorescence Spectroscopy 3rd Ed., 2006

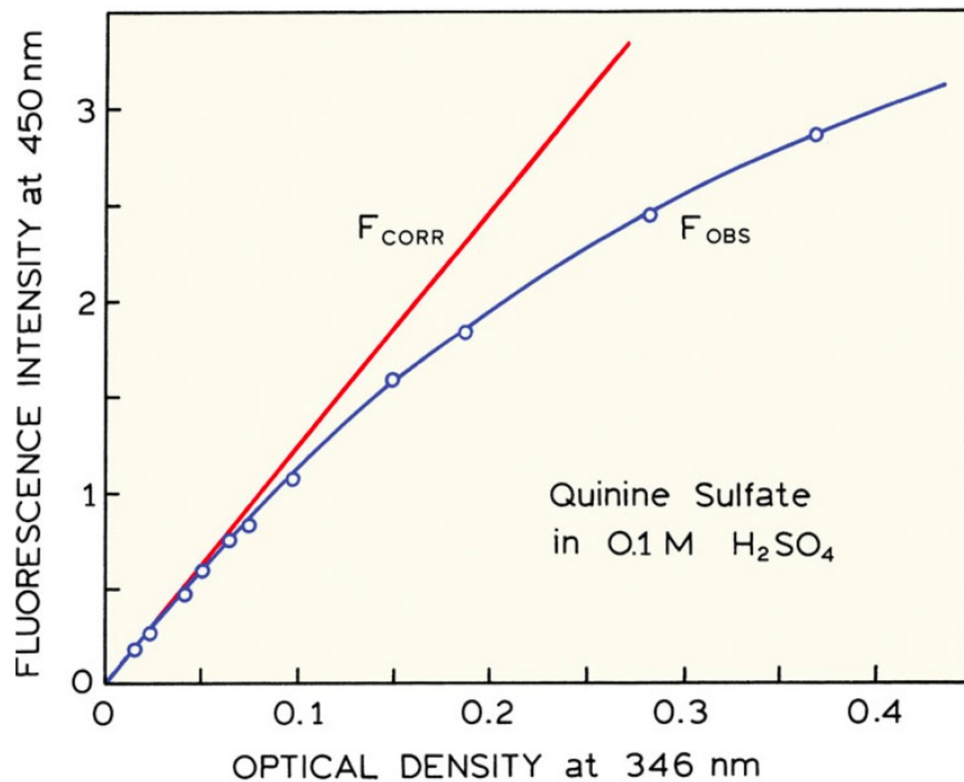
Spectroscopy

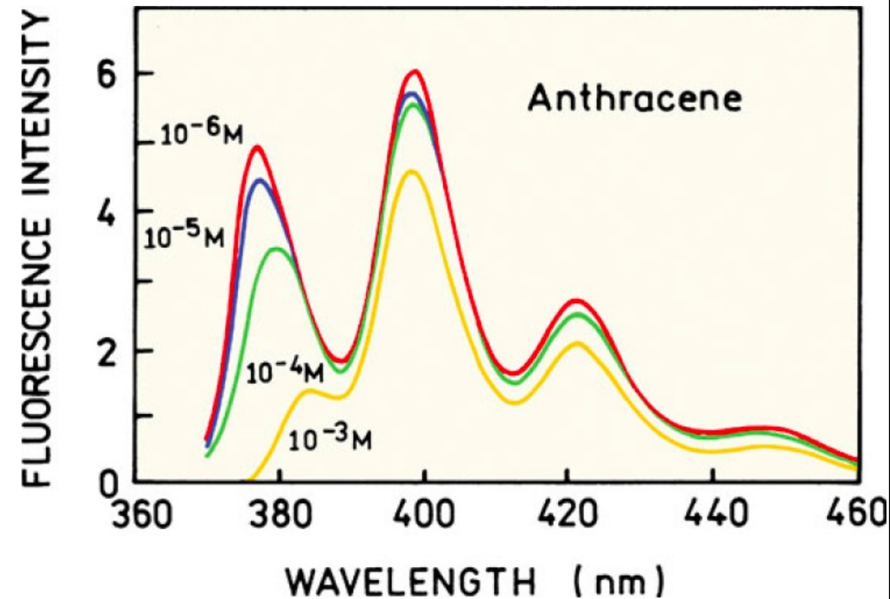






Lakowicz, Principles Fluorescence Spectroscopy 3rd Ed., 2006





Primary and secondary inner filter errors:

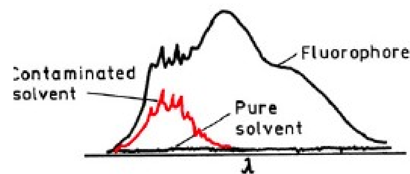
$F_{corr} = F_{obs} \log((OD_{ex} + OD_{em})/2)$

Lakowicz, Principles Fluorescence Spectroscopy 3rd Ed., 2006

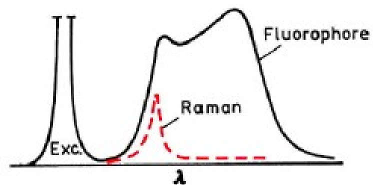
Spectroscopy

Fluorophore concentration too high

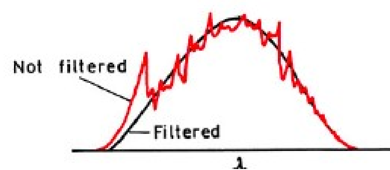
Contaminated solvent and/or cuvette

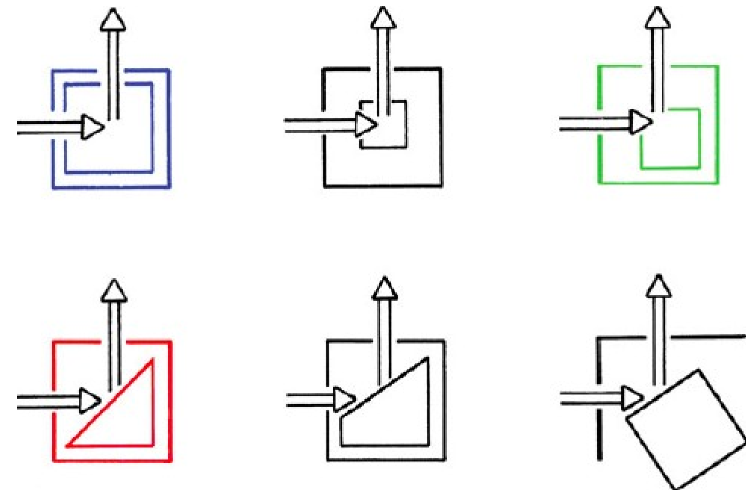


Scattered light



Particles in solution





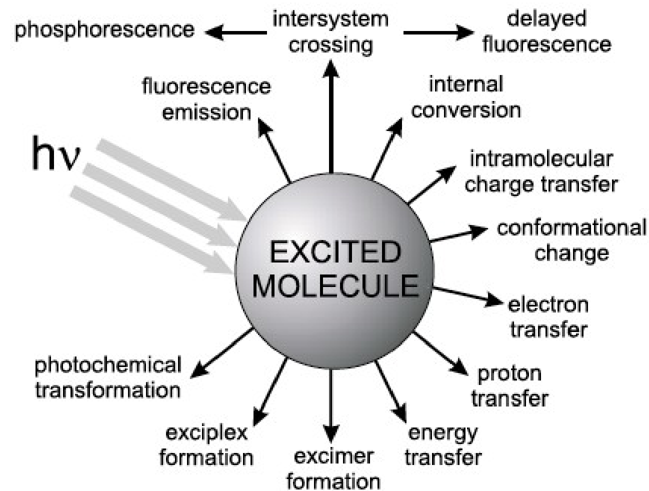
Geometry considerations:

most common is center focus (upper left and upper middle)

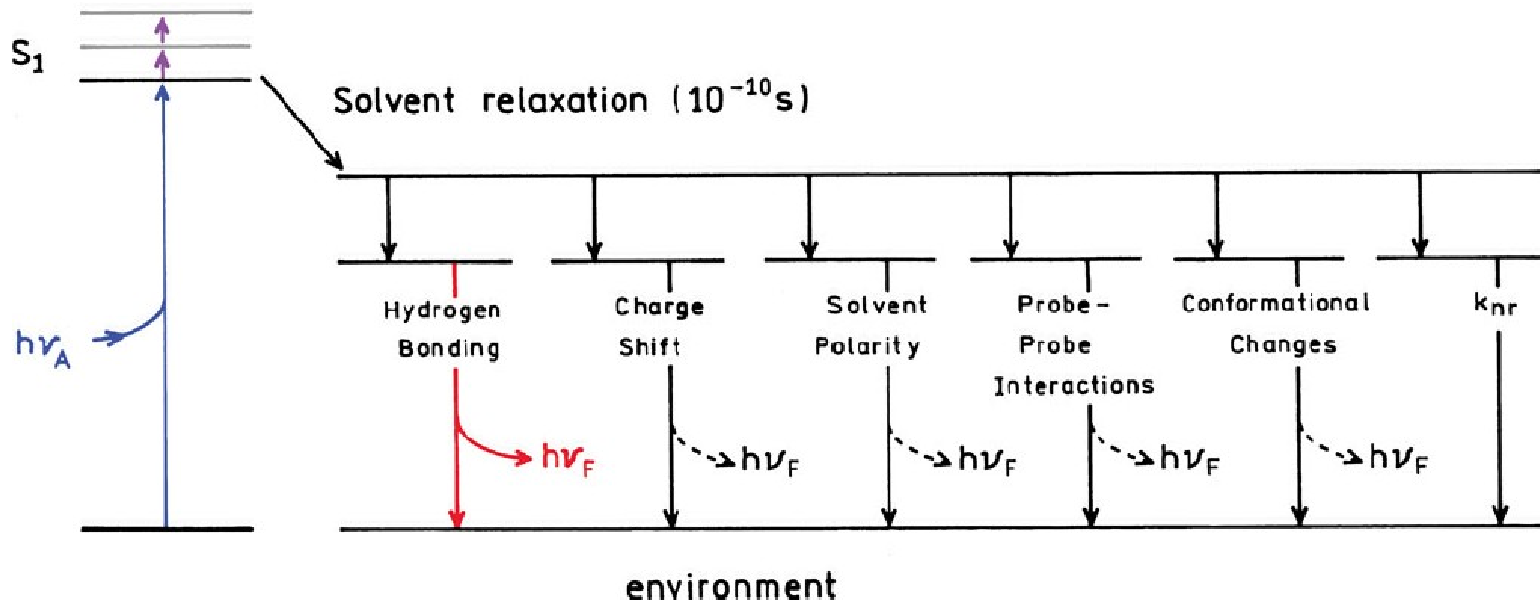
front-face illumination, used for optically thick samples, should be either at 30° or 60°, not 45°. Excitation reflection angle makes this obvious.

Lakowicz, Principles Fluorescence Spectroscopy 3rd Ed., 2006

Spectroscopy

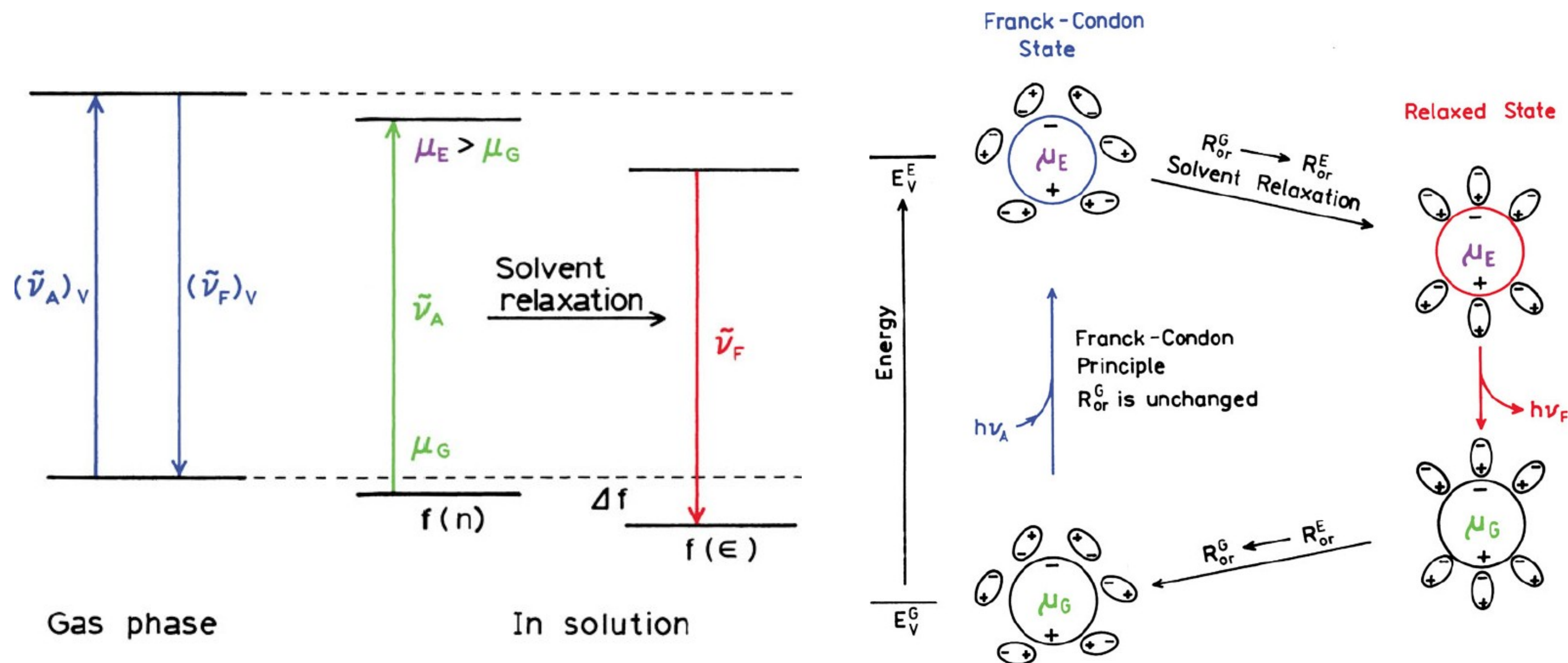


Valeur and Berbaran-Santos, Molecular Fluorescence, 2nd Ed., 2012



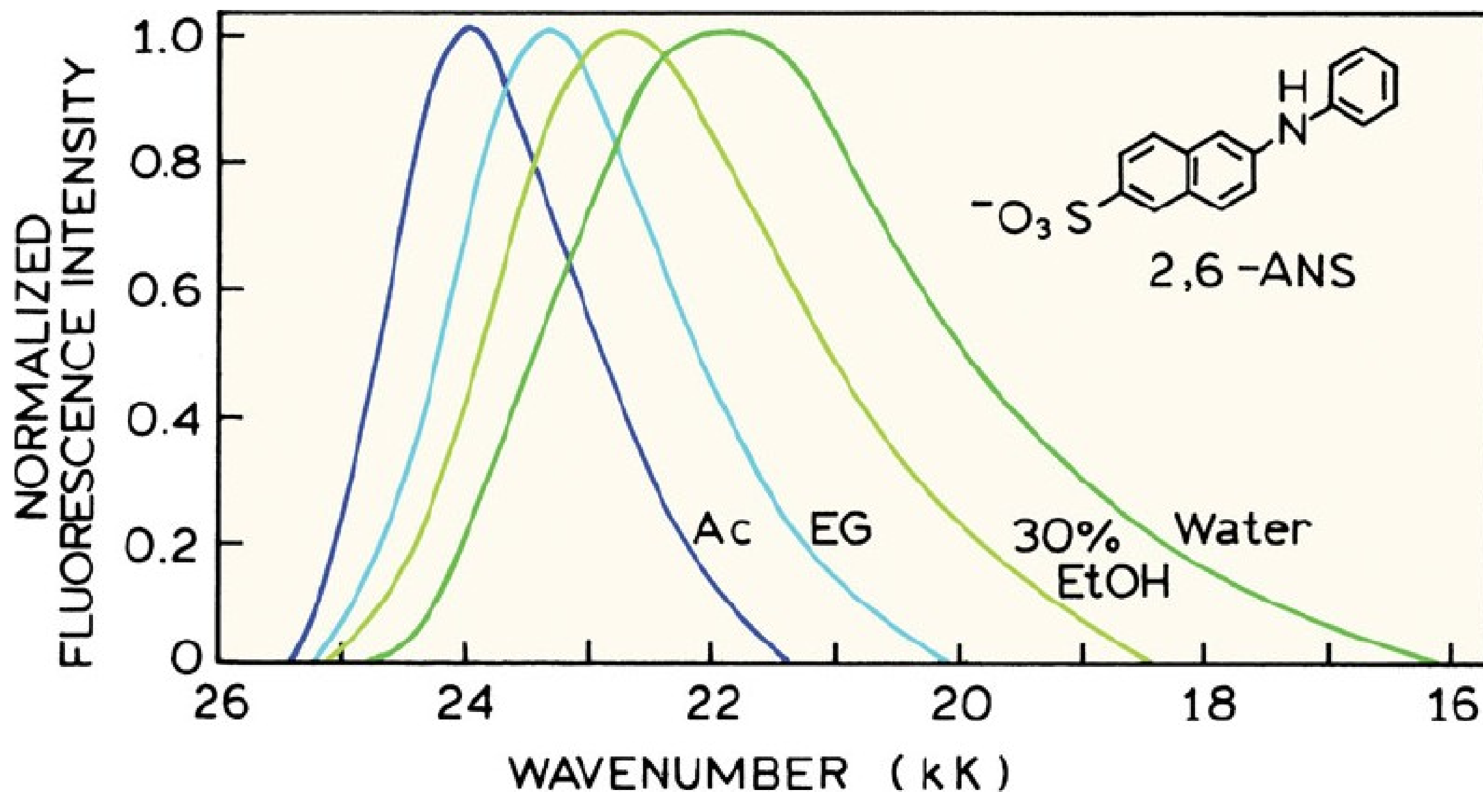
Lakowicz, Principles Fluorescence Spectroscopy 3rd Ed., 2006

Excited-State Reactions: Dipolar Relaxation



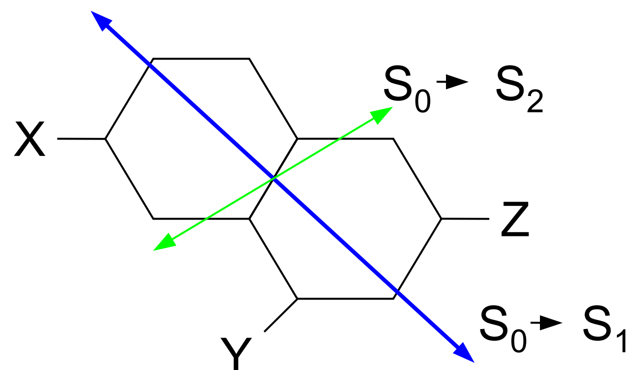
Lakowicz, Principles Fluorescence Spectroscopy 3rd Ed., 2006

Excited-State Reactions: Dipolar Relaxation

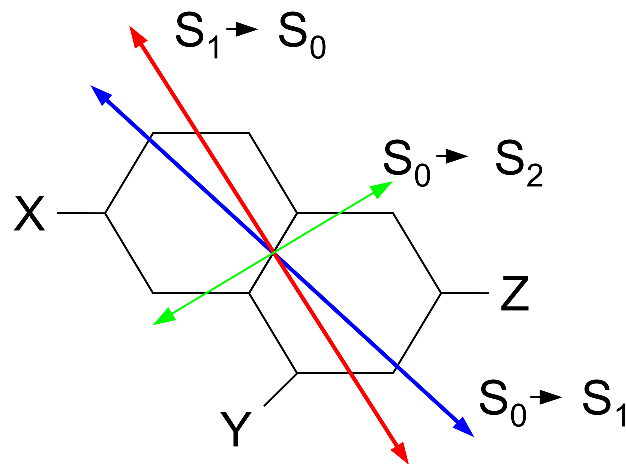


Lakowicz, Principles Fluorescence Spectroscopy 3rd Ed., 2006

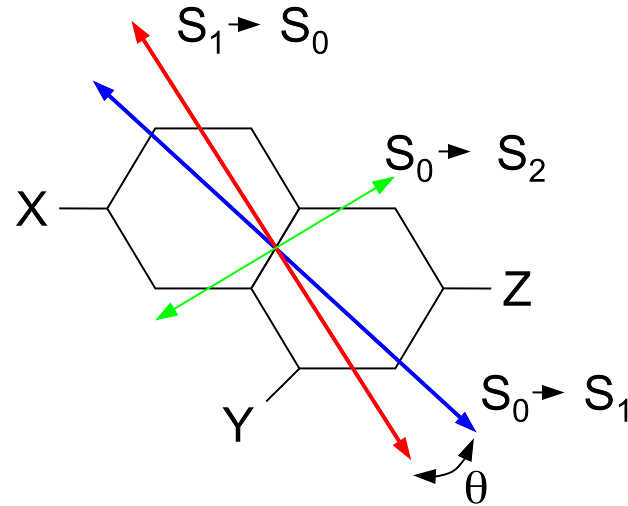
Absorption and Emission Transition Dipole Moments



Absorption and Emission Transition Dipole Moments



Absorption and Emission Transition Dipole Moments



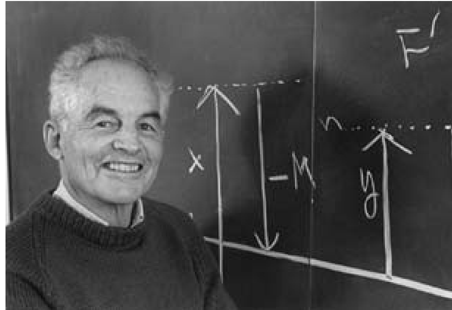
Anisotropy (Jabłonski, 1960):

$$r = (I_V - I_H)/(I_V - 2\ I_H) = (I_V - I_H)/I_{total})$$

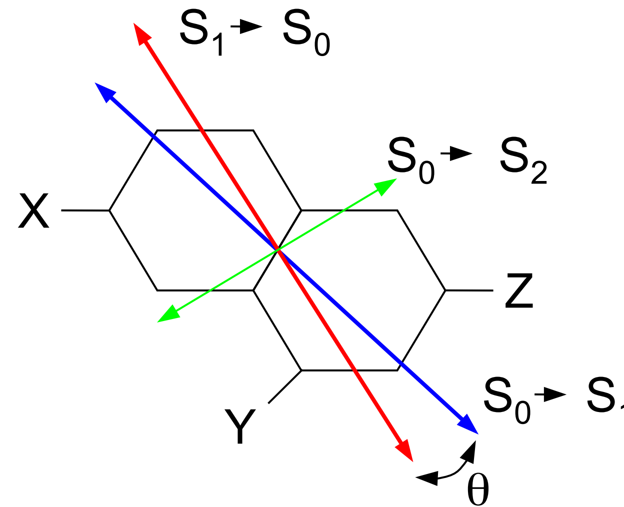
depends on the angle, θ, between absorption and emission transition moments

Absorption and Emission Transition Dipole Moments

Principle of Photoselection (Albrecht, 1961)



Andreas Albrecht, 1927-2002



Anisotropy (Jabłonski, 1960):

$$r = (I_V - I_H)/I_{total})$$

depends on the angle, θ, between absorption and emission transition moments

with random molecular orientation ⟶ $r_0 = (3\cos^2\theta - 1)/5$

if $\theta = 0°$ (parallel), then $r_0 = 0.4$

or if $\theta = 90°$ (perpendicular), then $r_0 = -0.2$

Principle Polarization Spectrum

Determination of r_0 as a function of excitation wavelength

at a constant λ_{em}

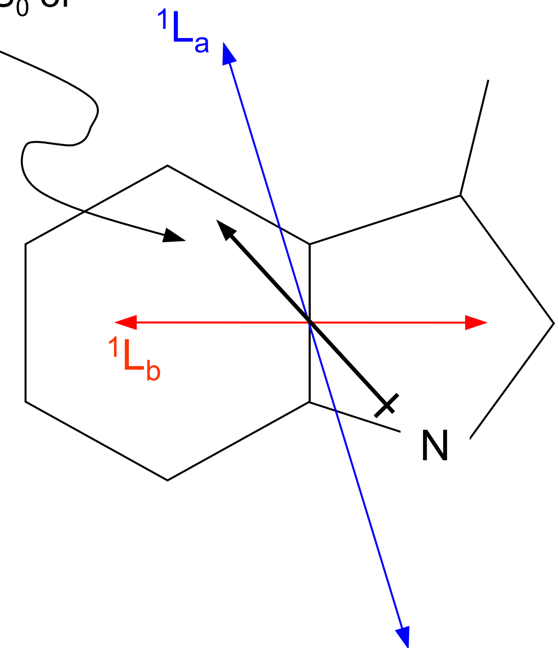
prevent depolarizing motions

scan λ_{ex} for all 4 sets of polarizer angles

calculate and plot r_0 vs λ_{ex}

see Figs. 10.6, 10.7, 10.29 in Lakowicz (2nd ed.)

permanent dipole moment in S_0 of ~ 2 debye



Information obtained

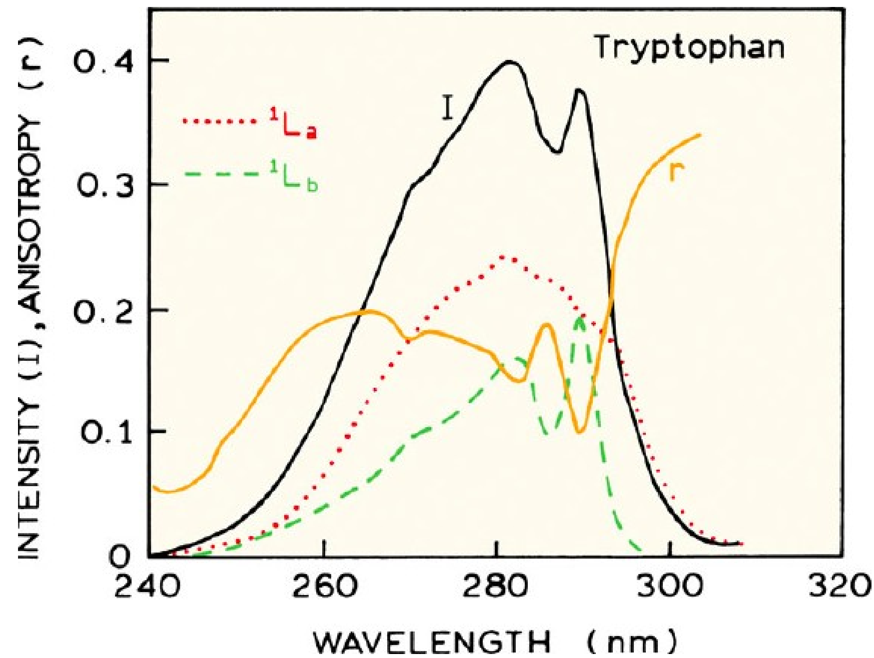
r_0 for different electronic transitions

thus calculate θ between abs. and em. dipole moments

as in indole, find 'hidden' transitions →

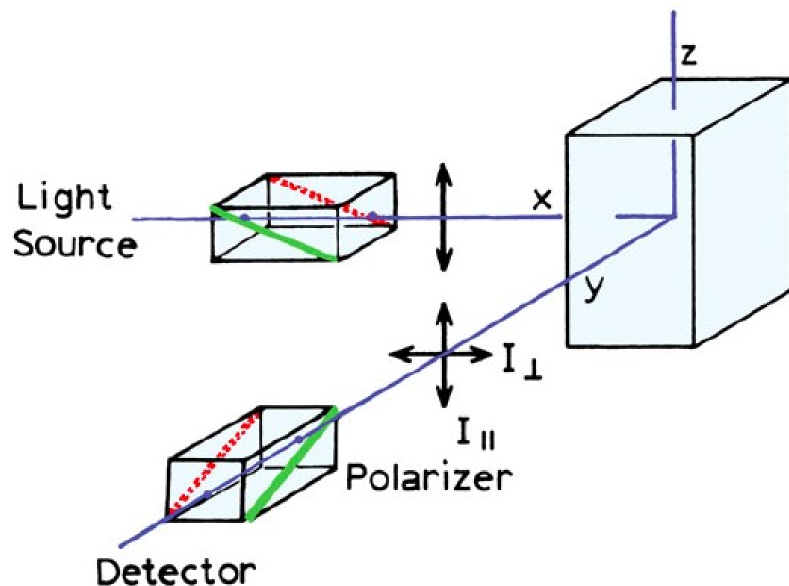
for indole spectrum, see Fig. 10.8 in Lakowicz (2nd ed.)

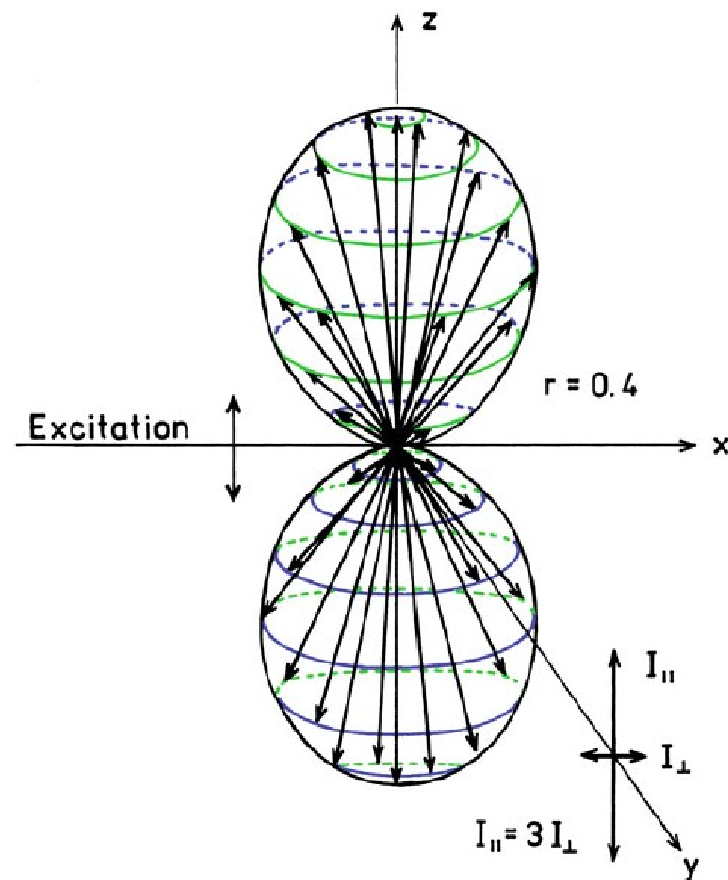
Principle Polarization Spectrum



Lakowicz, Principles Fluorescence Spectroscopy 3rd Ed., 2006

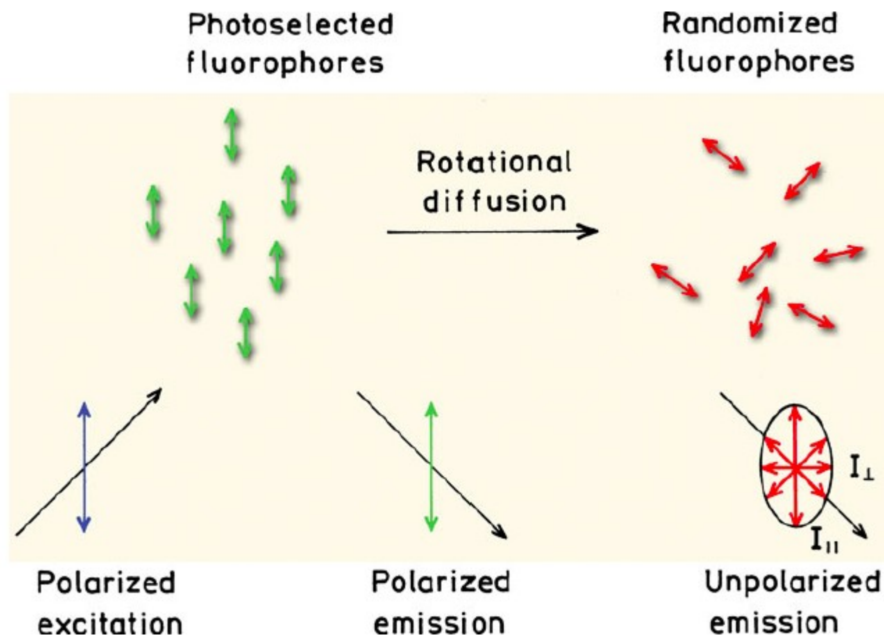
Anisotropy and Photoselection

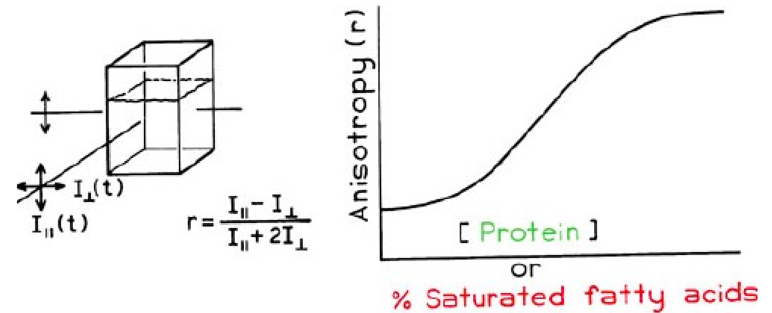


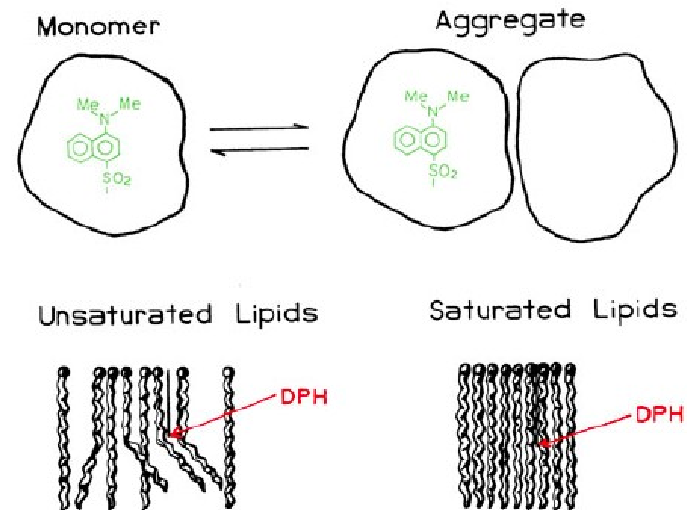


Lakowicz, Principles Fluorescence Spectroscopy 3rd Ed., 2006

Anisotropy and Photoselection







Lakowicz, Principles Fluorescence Spectroscopy 3rd Ed., 2006

Anisotropy and Photoselection

β (deg)	r_0	p_0
0	0.4	0.5
45	0.1	0.143
54.7	0	0
90	–0.2	–0.333

Perrin equation for spherical rotor: $r = r_0/(1 + \tau/\theta)$

Anisotropy and Photoselection

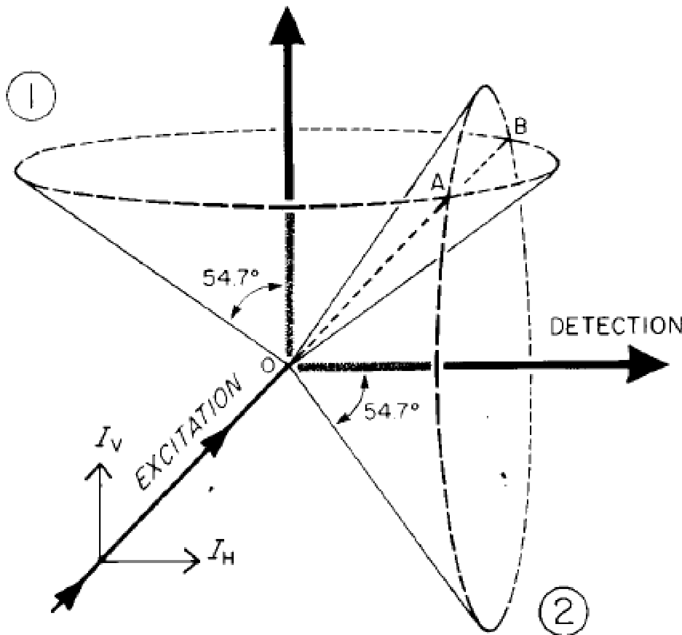


FIG. 15. "Magic" angle cones. Viewing the emission originating from O along the lines BO or AO eliminates the polarization related artifacts for any kind of excitation. See text for details.

Magic Angle conditions

$I_{total} = I_x + I_y + I_z = I_x + 2\ I_y$

$I_{viewed} = I_x \cos^2 \theta + I_y \cos^2 (90° - \theta)$

I_{viewed} will be proportional to I_{total} if

$[\cos^2 (90° - \theta)]/(\cos^2 \theta) = \tan^2 \theta = 2$

then $\theta = 54.7°$

Badea and Brand, Meth Enzymol 61, 378 (1979)

Anisotropy and Fluorescence

Interactions (binding) and addition laws

$F_{total} = F_V + 2\,F_H$

$F_{total} = \sum f_i = \sum \varphi_i\, a_i = \sum \varphi_i\, \varepsilon_i\, c_i\, l$

$FR_{total} = \sum f_i\, r_i = \sum \varphi_i\, a_i\, r_i$ leads to the non-addition law:

$R_{total} \neq \sum r_i$ (except under special circumstances)

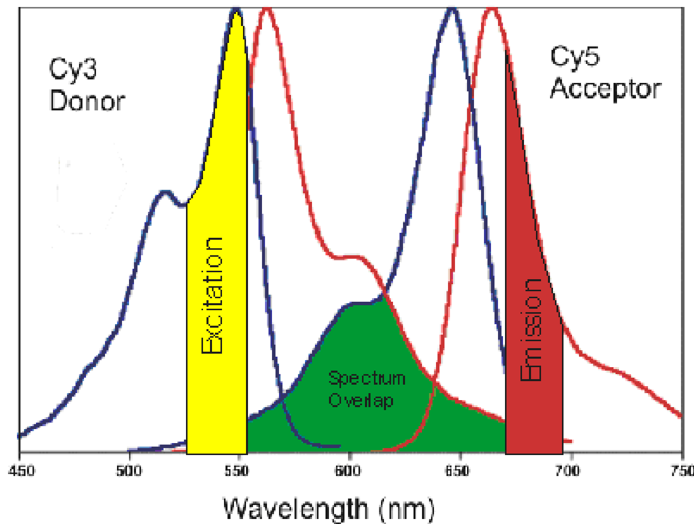
Spectroscopy

Jean B. Perrin
1870-1942

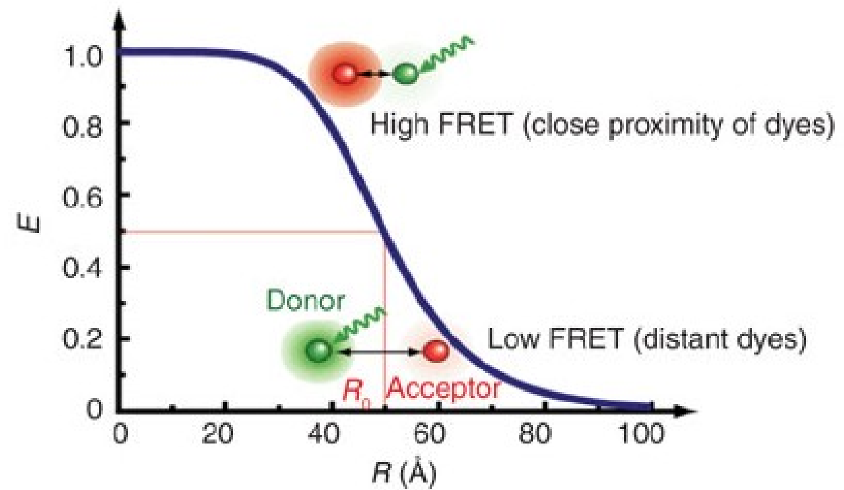
Francis Perrin
1901-1992

J. Robert Oppenheimer
1904-1967

Theodor Förster
1910-1974

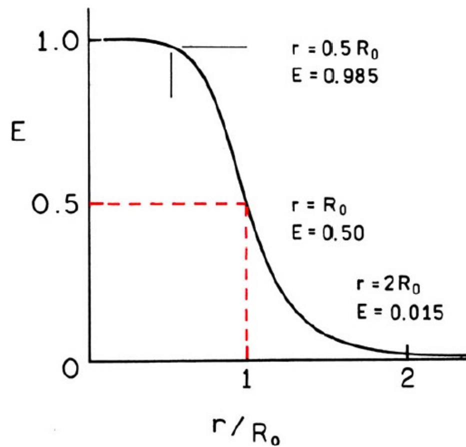


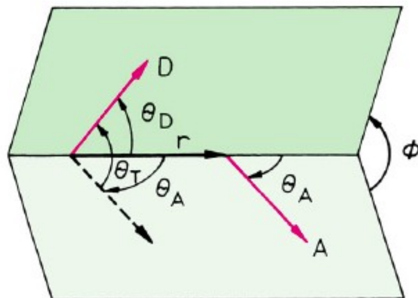
Held, BioTek.com, 2005

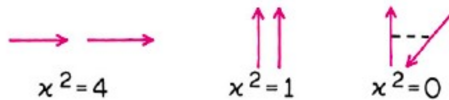


Lakowicz, Principles Fluorescence Spectroscopy 3rd Ed., 2006

Resonance Energy Transfer

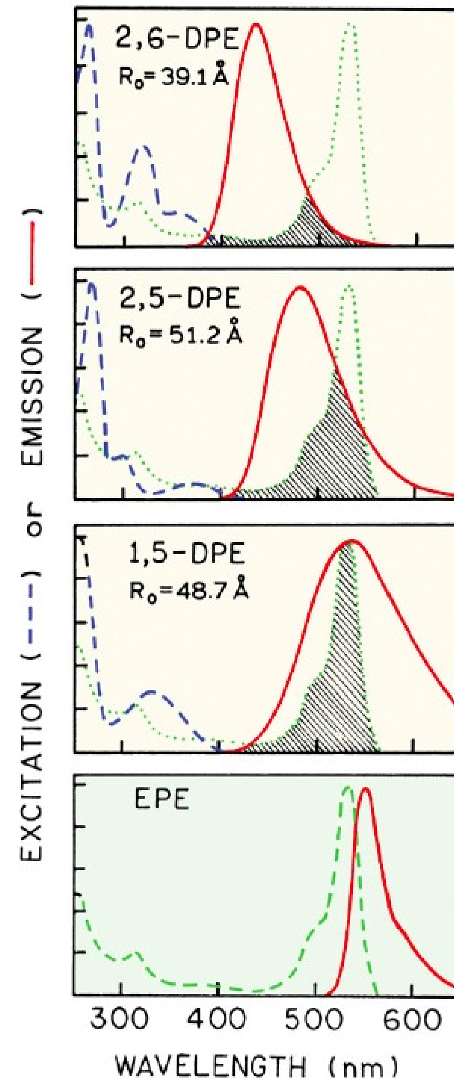






$$\kappa^2 = (\cos\theta_T - 3\cos\theta_D\cos\theta_A)^2$$

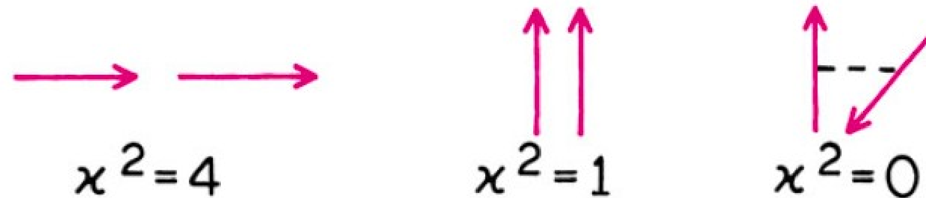
$$\kappa^2 = (\sin\theta_D\sin\theta_A\cos\phi - 2\cos\theta_D\cos\theta_A)^2$$



Lakowicz, Principles Fluorescence Spectroscopy 3rd Ed., 2006

Resonance Energy Transfer

The orientation factor, κ^2



The orientation factor, κ^2 can be calculated from the projections of the 9 combinations of donor and acceptor axes (draw projection of donor axes on acceptor axes):

1 orientation where $\kappa^2 = 4$

2 orientations where $\kappa^2 = 1$

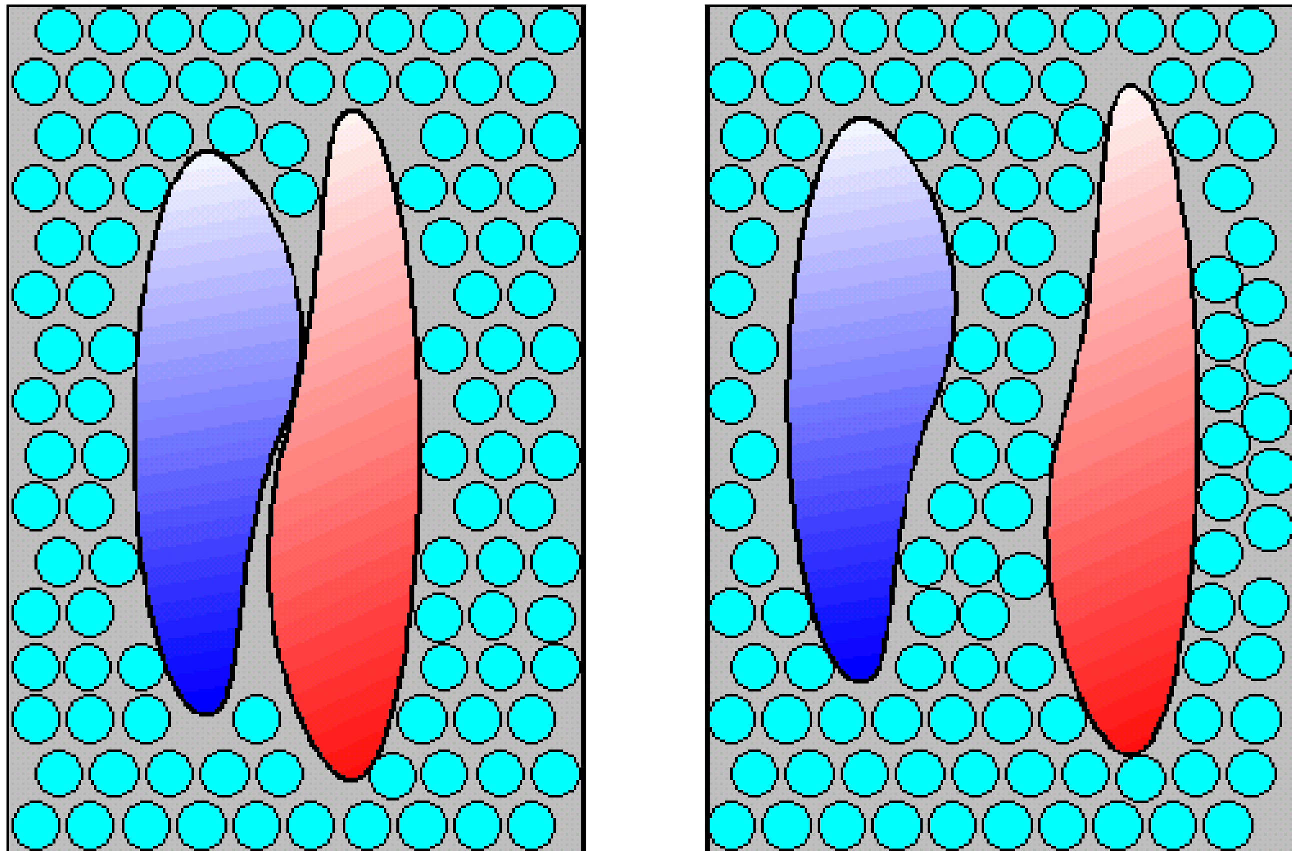
6 orientations where $k^2 = 0$

3 of the 9 combinations contribute to FRET: $\sum\kappa^2 = (1 \times 4) + (2 \times 1) = 6$

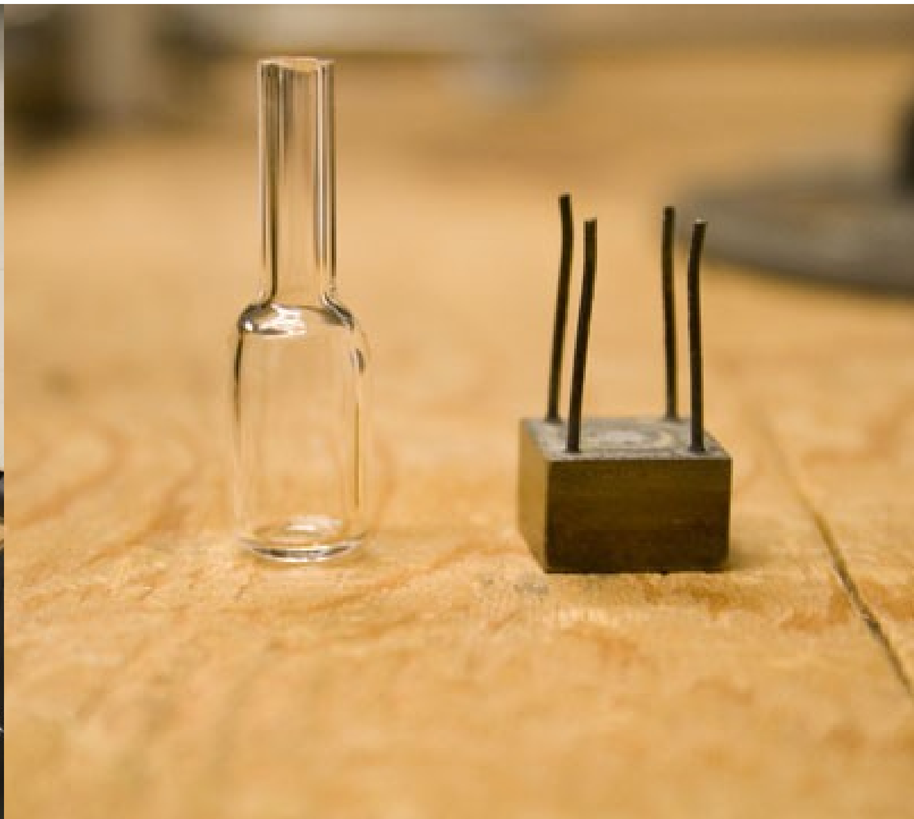
6 of 9 total combinations do not contribute to FRET: $\sum\kappa^2 = 0$

So the average κ^2 for all combinations is 6/9 = 2/3

Water Packs Better Against a Protein Surface Than Another Protein (or DNA) Does

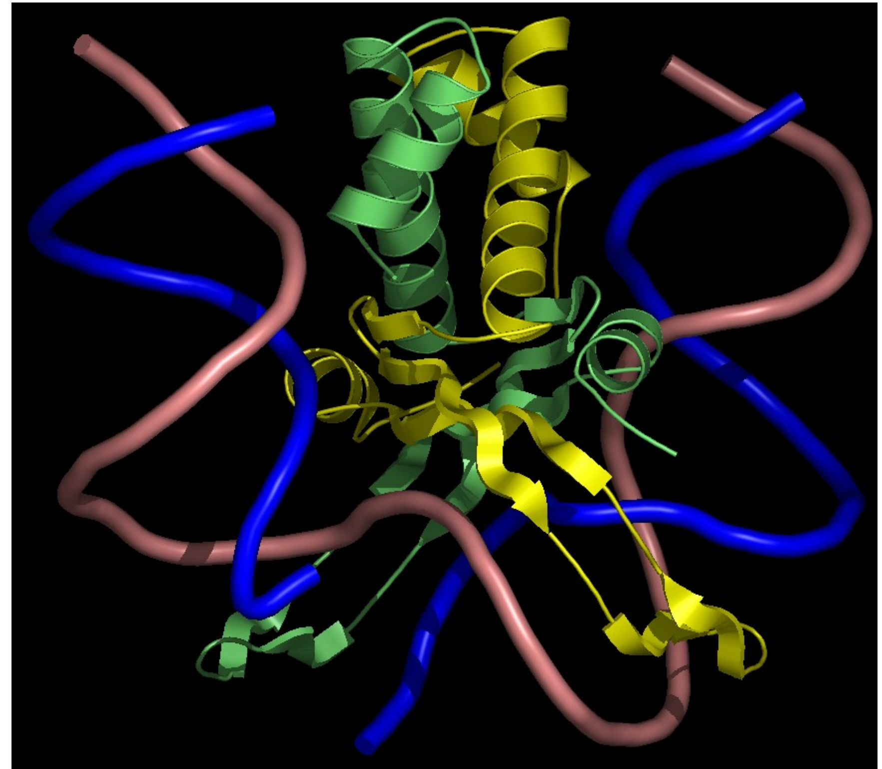


Spectroscopy



IHF : sequence-specific interaction

Figure 2. Electrophoretic mobility-shift assay of IHF binding to oligonucleotide A.2. IHF concentrations in Lanes 1–10 are 0, 20, 40, 60, 81, 99, 120, 165, 201 and 240 nM, respectively. This pseudo-color image was generated by coloring the emission collected through a 520-nm band pass filter green (FAM fluorescence) and coloring the emission collected through a 580-nm band pass filter red (TAMRA fluorescence). With excitation at 488 nm, the unliganded oligonucleotide is green, reflecting only FAM fluorescence. The yellow color of the mobility-shifted band results from a combination of green and red fluorescence, indicating efficient FRET due to the wrapped DNA in the bound complex.



Senear, et al, (2007) Nucleic Acid Res 35: 1761

Rice, P.A., et al, (1996) Cell 87: 1295-1306