Fluorescence Spectroscopy

03/12/02: Marcia Levitus

- absorption of light.
- radiative and non-radiative processes in molecules.
- properties of fluorescence.
- experimental determinations.
- fluorescence of macromolecules
    - > intrinsic and extrinsic fluorophores
    - > solvent effects
    - > quenching

03/14/02: Carlos Bustamante

Energy transfer and fluorescence polarization

03/19/02: Marcia Levitus

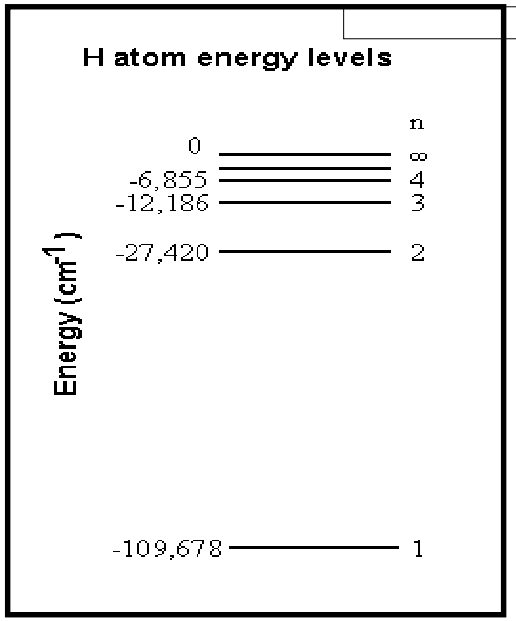
Single molecule fluorescence

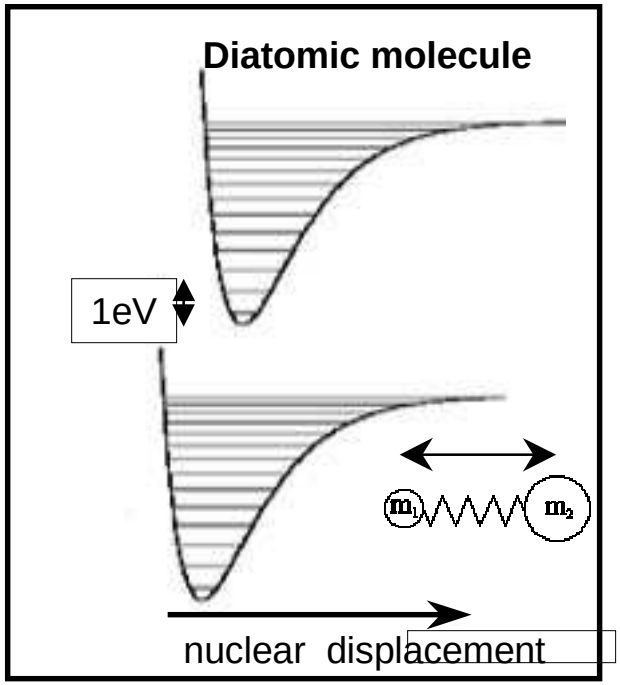
Interaction of light with matter

The interaction of radiation with matter can cause transitions between the energy levels of the atoms or molecules. The possible energy of an electronic orbital, molecular vibration, or rotation is restricted to a well-defined energy.

Type of Radiation	Wavelength Range	Type of Transition
gamma-rays	<1 pm	nuclear
X-rays	1 nm-1 pm	inner electron
ultraviolet	400 nm-1 nm	outer electron
visible	750 nm-400 nm	outer electron
near-infrared	2.5 μm-750 nm	outer electron molecular vibrations
infrared	25 μm-2.5 μm	molecular vibrations
microwaves	1 mm-25 μm	molecular rotations, electron spin flips
radio waves	>1 mm	nuclear spin flips

$k_B T$ (300K)=25meV → ≈ 50 μm

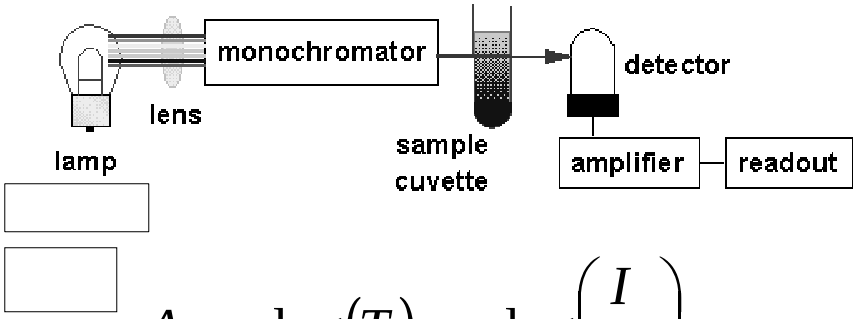


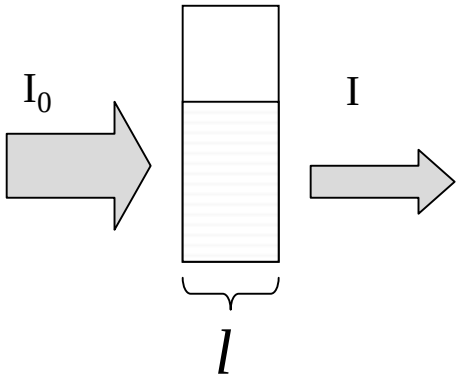


Electronic levels

Vibrational levels

Absorption of light





$$A = -\log(T) = -\log\left(\frac{I}{I_0}\right)$$

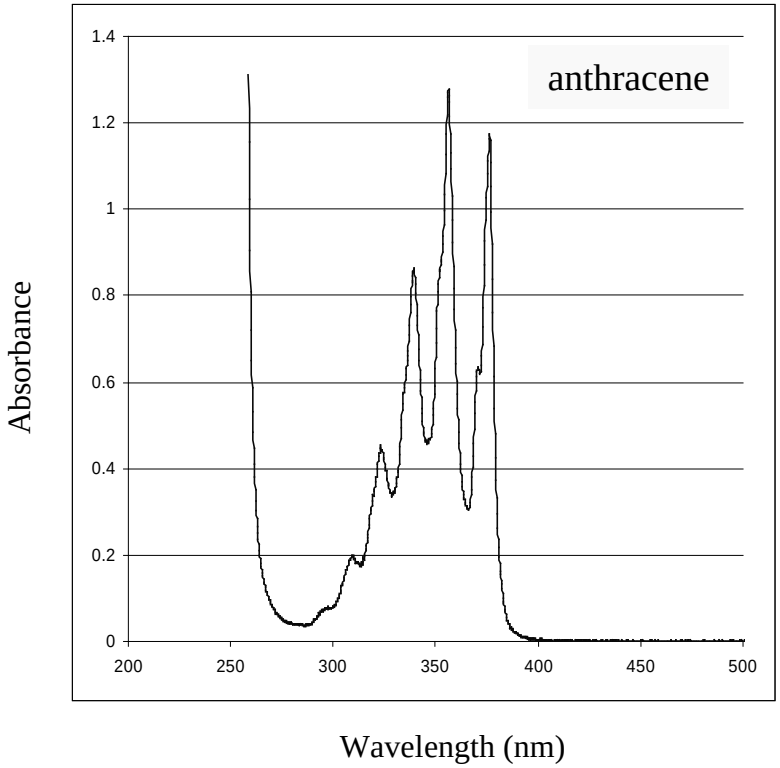
$$I_0 = I + I_{abs}$$ (no reflected light)

$$I_{abs} = I_0\left(1 - 10^{-A}\right)$$

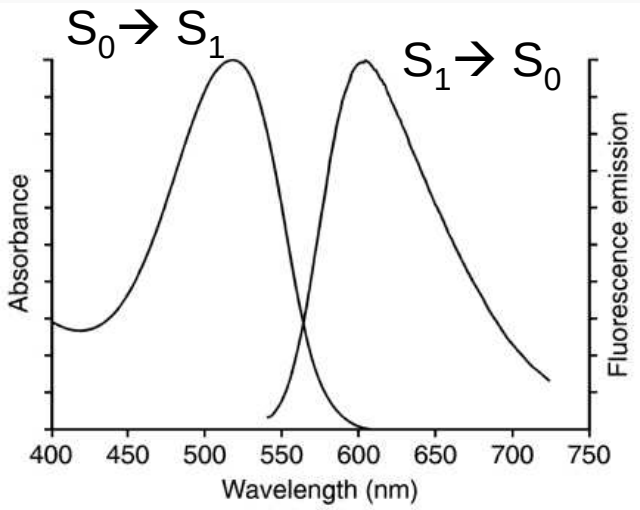
$$A = \varepsilon.l.C$$

ε: extinction coefficient ($M^{-1}.cm^{-1}$)
l: path length (cm)
C: concentration (M)

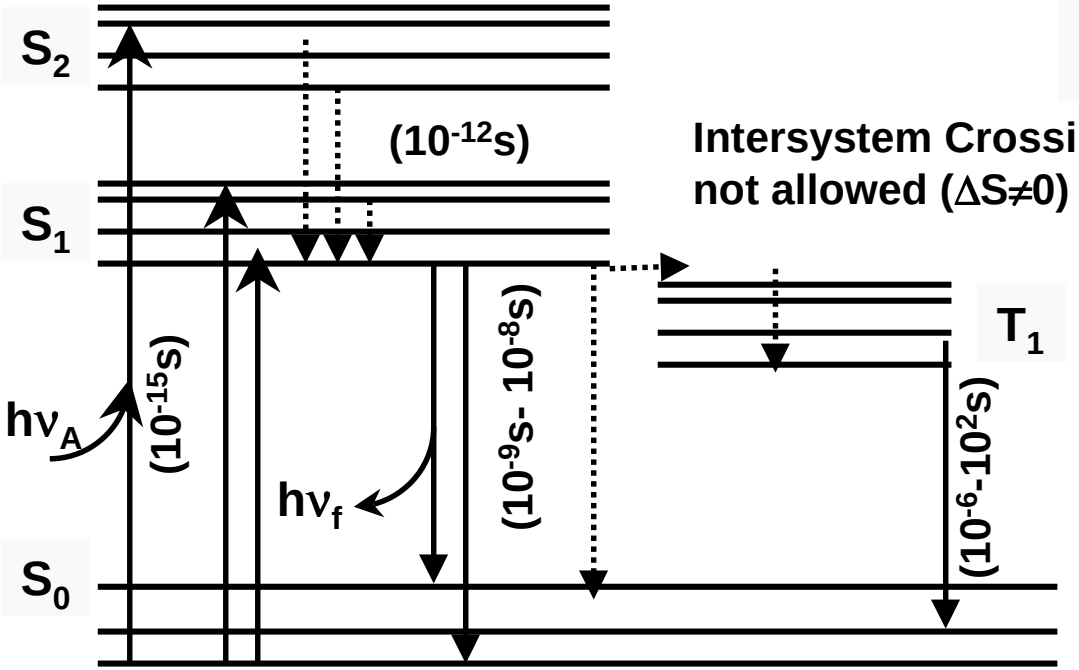
A=Absorbance, T= Transmittance



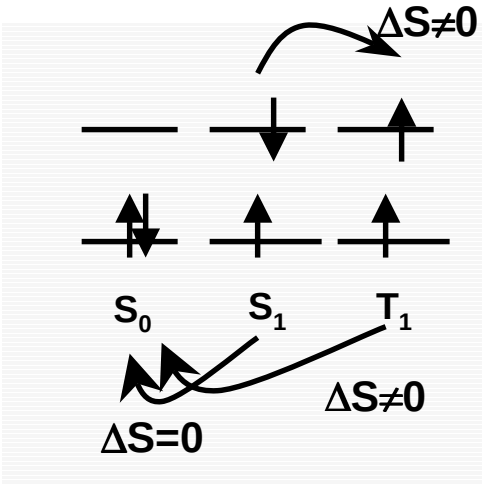
Jablonski diagram



Internal conversion: in condensed phases molecules rapidly relax to the ground vibrational state of the first excited electronic state



Intersystem Crossing: not allowed ($\Delta S \neq 0$)

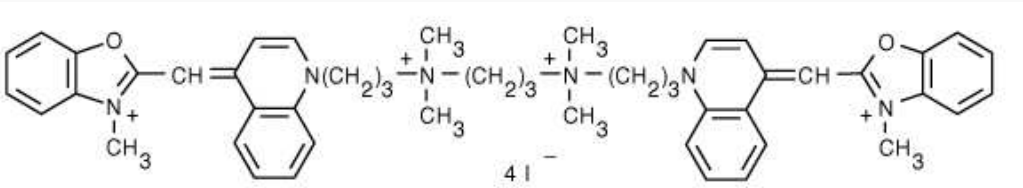


absorption

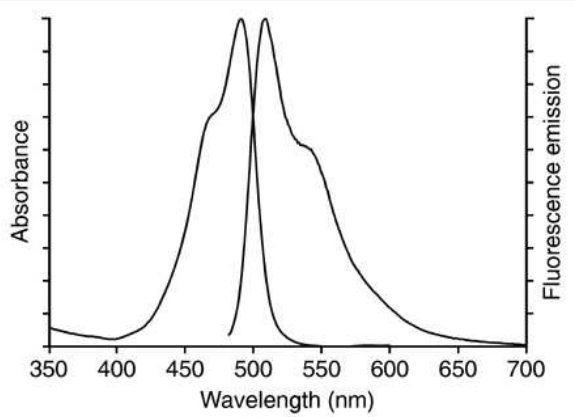
Fluorescence
(no change in spin)

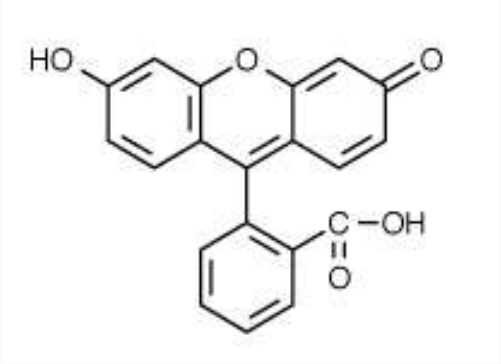
phosphorescence
(not allowed ($\Delta S \neq 0$))

Examples of fluorophores

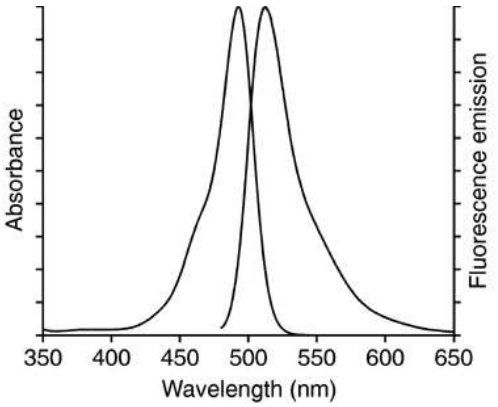


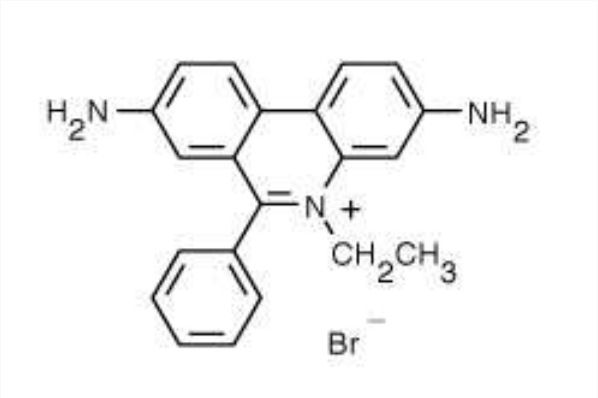
YOYO-1 bound to DNA.



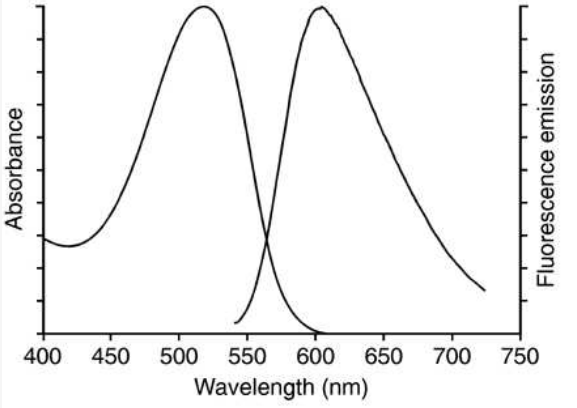


fluorescein





ethidium bromide bound to DNA.



Properties of Fluorescence Emission:

1- The absorption spectrum reflects the vibrational levels of the excited electronic state. The fluorescence spectrum reflects the vibrational levels of the ground state.

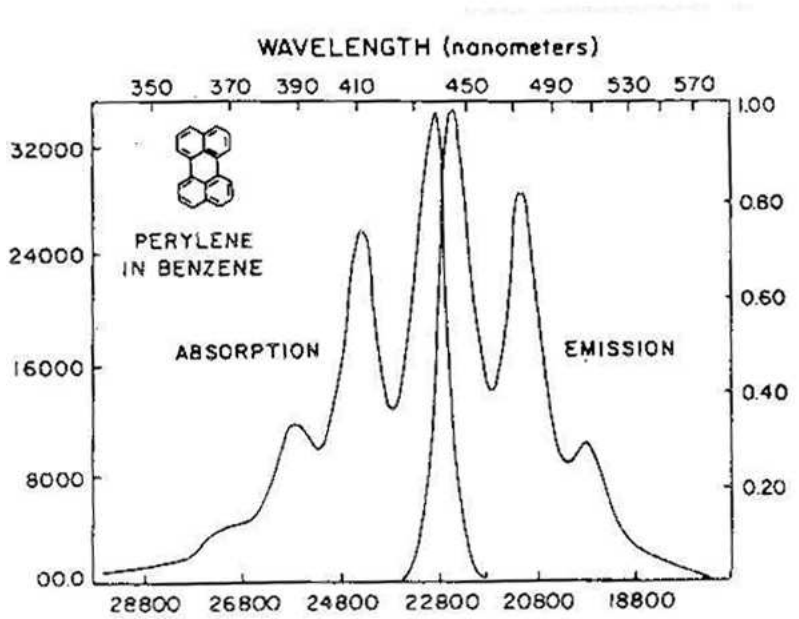
2- Stokes shift:

λemission > λabsorption

(even for the 0→0 transition)

3- The spectrum of the emitted light is invariant with the excitation wavelength.
because of the fast relaxation to the lowest vibrational level of S_1

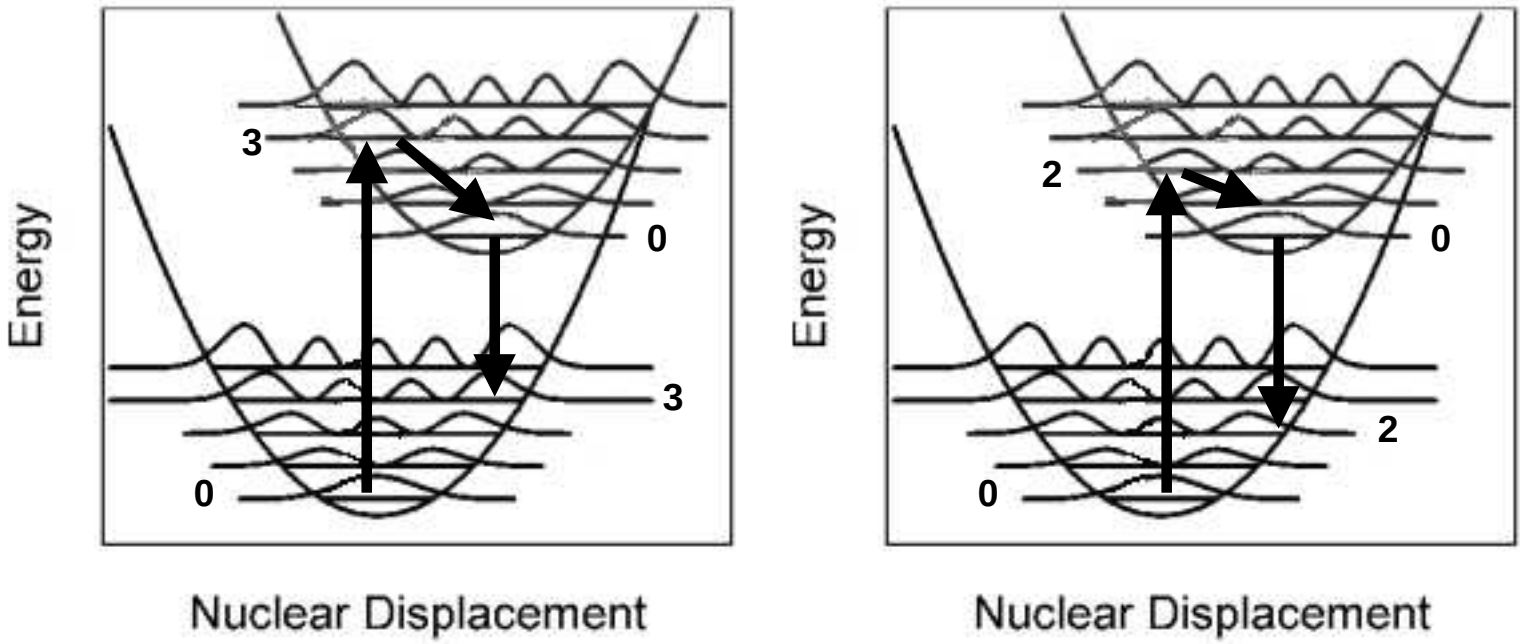
Exception: azulene, it can emit from both S_1 and S_2.



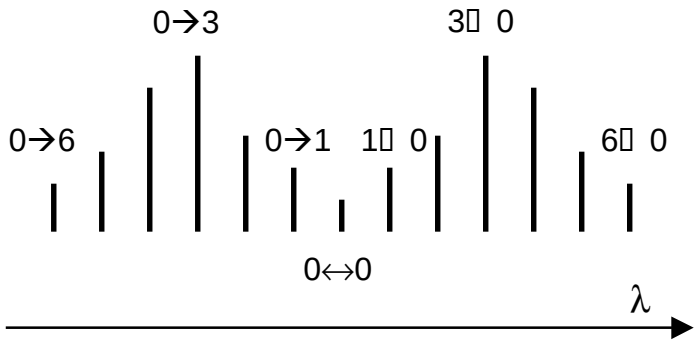
4- The mirror image rule.

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

The Franck-Condon principle: transitions are vertical in both absorption and emission



The Franck-Condon factor is the same for absorption and fluorescence

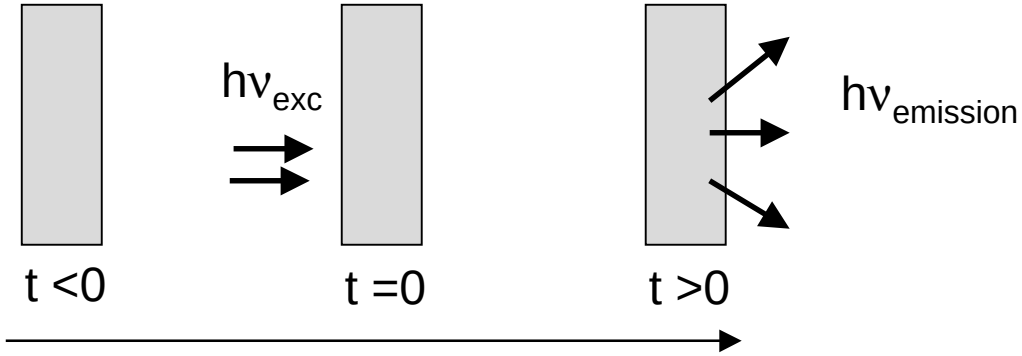


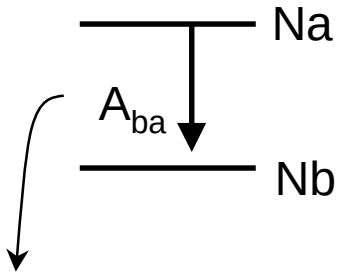
Exceptions:

- very long lived S_1 state : emission occurs from a different geometry.

- Reactions from the excited state.

Fluorescence lifetime and quantum yield

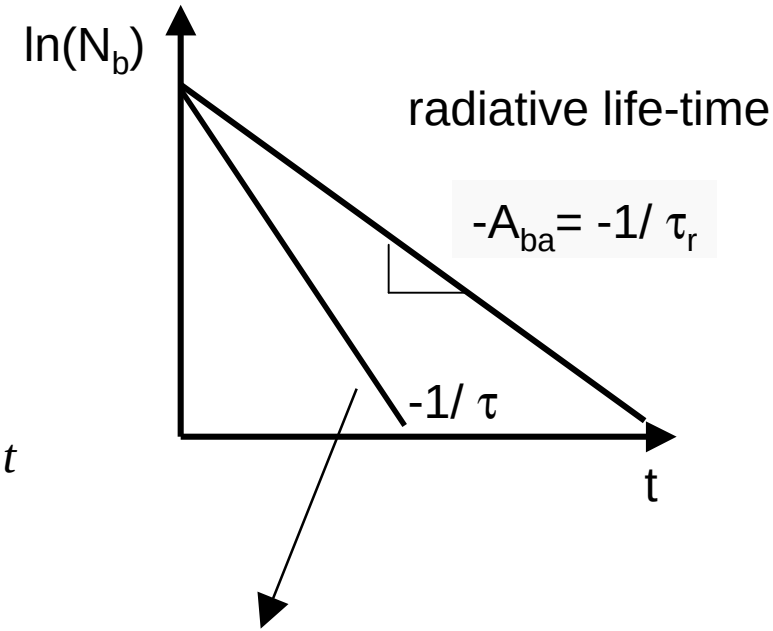




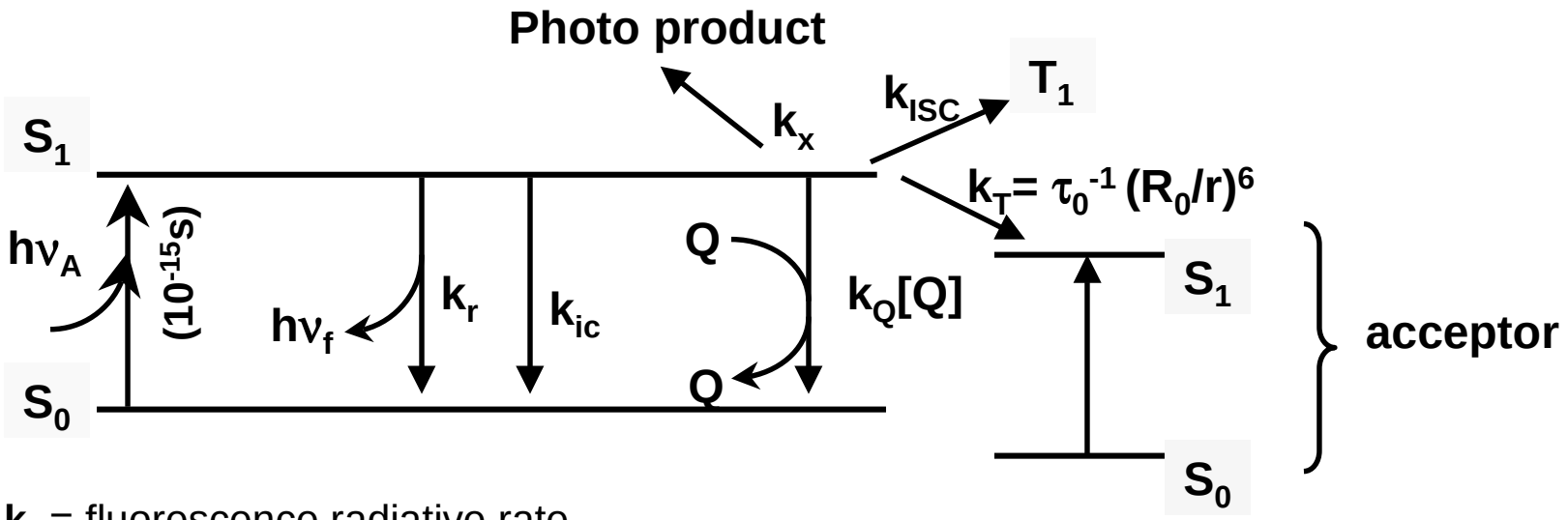
Fluorescence radiative rate

$$-\frac{dN_b}{dt} = A_{ba} N_b$$

$$N_b(t) = N_b(0) e^{-A_{ba} t}$$



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



k_r = fluorescence radiative rate

k_{ic} = internal conversion (collision with solvent + internal vibrational modes) → increases with T

k_Q = quenching (collision with solute molecules, such as O_2)

k_T = energy transfer (FRET)

k_x = reaction from excited state

k_{ISC} = intersystem crossing (forbidden)

$$k = (k_r + k_{IC} + k_{ISC} + k_x + k_q[Q] + k_{FRET} \ldots)$$

$$-\frac{d[S_1]}{dt} = k[S_1] \rightarrow [S_1](t) = [S_1](0)e^{-t/\tau_f}$$

$$\tau_f = \left(k_r + k_{IC} + k_{ISC} + k_x + k_Q[Q] + k_{FRET} \ldots\right)^{-1}$$

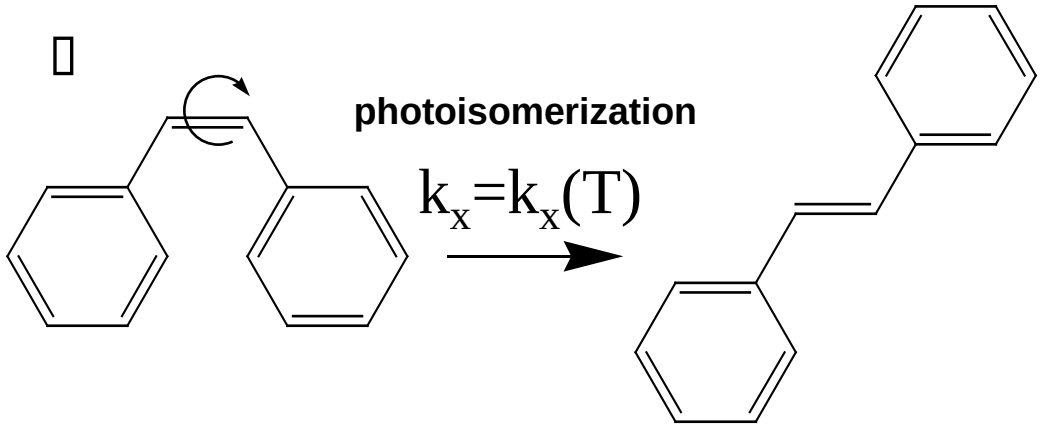
$$\phi = \frac{\text{photons emitted}}{\text{photons absorbed}} = \frac{k_r}{k} = \tau_f . k_r = \frac{\tau_f}{\tau_r}$$

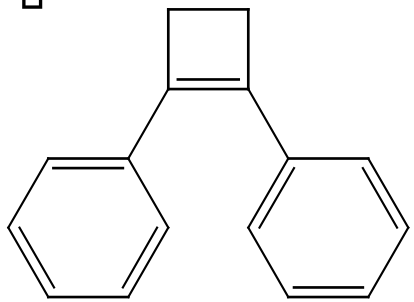
ϕ= fluorescence quantum yield;

τ_f = fluorescence life-time

τ_r = radiative fluorescence life-time

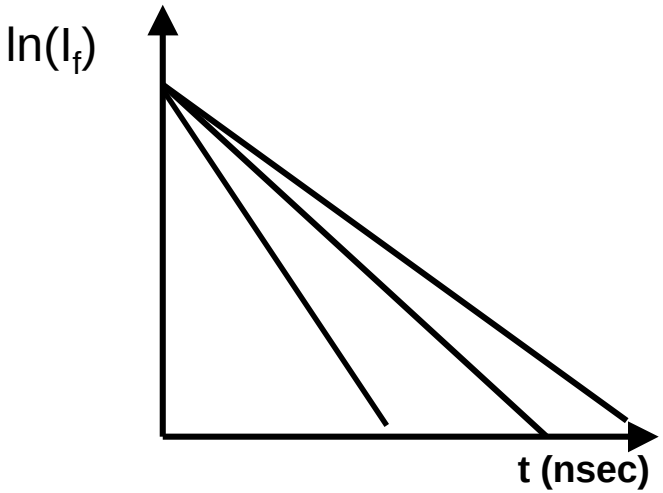
Effect of structure and environmental rigidity





ϕ (25°C, organic solvent)= 0.05
ϕ (25°C, glycerol)= 0.15
ϕ (77K, organic solvent) = 0.75

ϕ(25°C)= 1
ϕ(77K) = 1

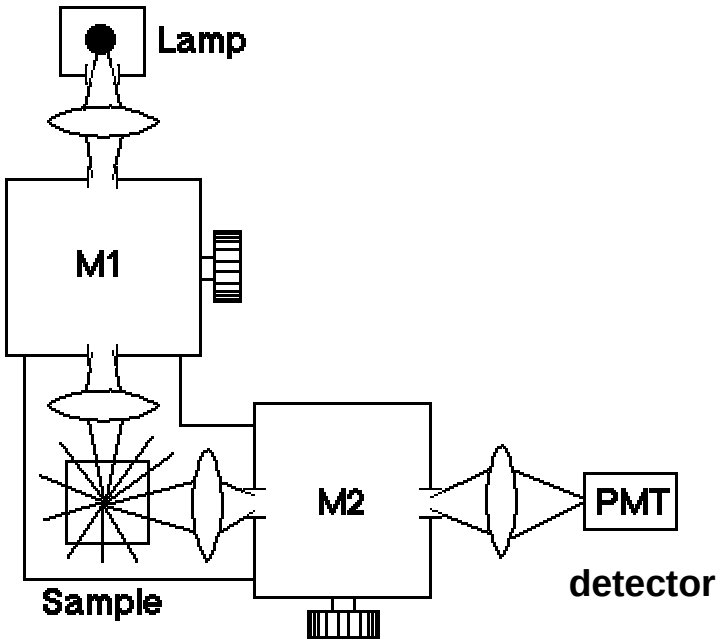


$$\phi = \frac{\text{photons emitted}}{\text{photons absorbed}} = \frac{k_r}{k} = \tau_f . k_r = \frac{\tau_f}{\tau_r}$$

77K

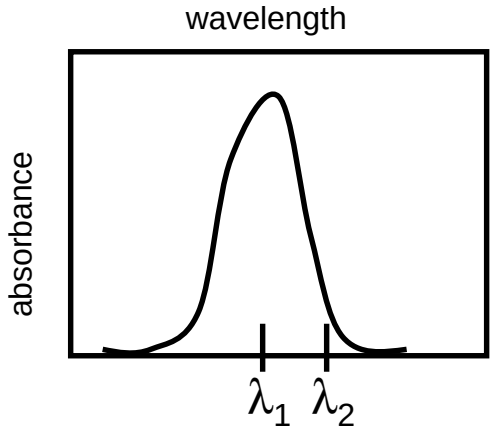
25°C

Measurement of fluorescence spectra

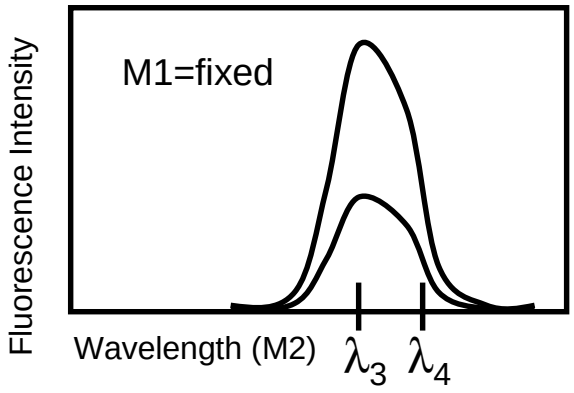


M1: excitation monochromator
M2: emission monochromator

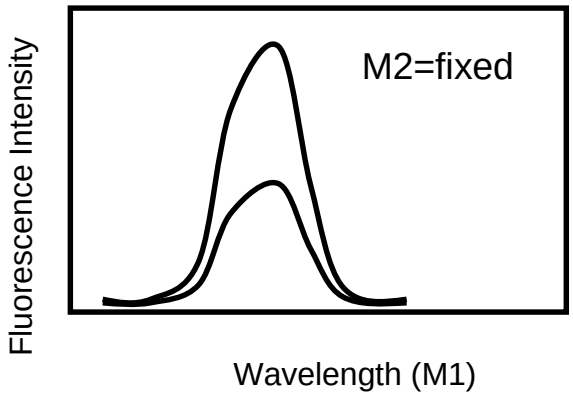
Absorption spectrum



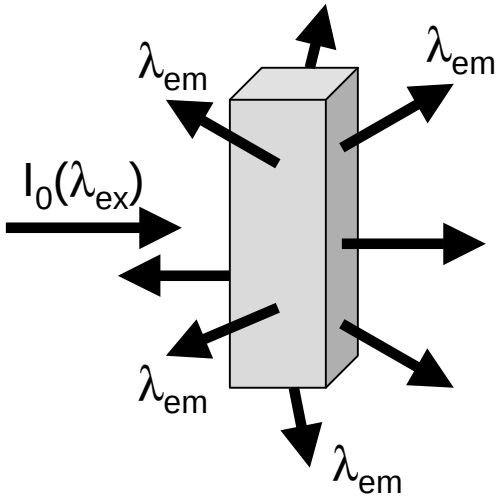
Fl. Emission spectrum



Fl. Excitation spectrum



Measurement of fluorescence quantum yields



fraction of intensity emitted at that particular wavelength

fraction of total fluorescence that is detected

$$I_f(\lambda_{em}) = I_{Abs}(\lambda_{ex}).\ \phi_f\ .\ f(\lambda_{em}).\ K$$

ϕ_f: fluorescence quantum yield

$I_{Abs}(\lambda_{ex})$: absorbed intensity at λ_{ex}

$I_f(\lambda_{em})$: measured intensity of fluorescence at λ_{em}

$$I_{abs}(\lambda_{ex}) = I_0(\lambda_{ex})\left(1-10^{-A(\lambda_{ex})}\right)$$

If A→0 $\quad I_{abs}(\lambda_{ex}) \cong 2.303.A(\lambda_{ex}).I_0(\lambda_{ex})$

$$\square_{\lambda em} I_f = I_{abs}(\lambda_{ex}).\phi_f.K \cong 2.303.A(\lambda_{ex}).I_0(\lambda_{ex}).\phi_f.K$$

If we measure the sample and a standard under the same experimental conditions, keeping λex constant:

$$\frac{\square_{\lambda em} I_f^{\text{sample}}}{\square_{\lambda em} I_f^{\text{standard}}} = \frac{A^{\text{sample}}(\lambda_{ex})}{A^{\text{standard}}(\lambda_{ex})} \cdot \frac{\phi_f^{\text{sample}}}{\phi_f^{\text{standard}}}$$

Standards:

Quinine sulfate in H_2SO_4 1N: ϕ_f =0.55

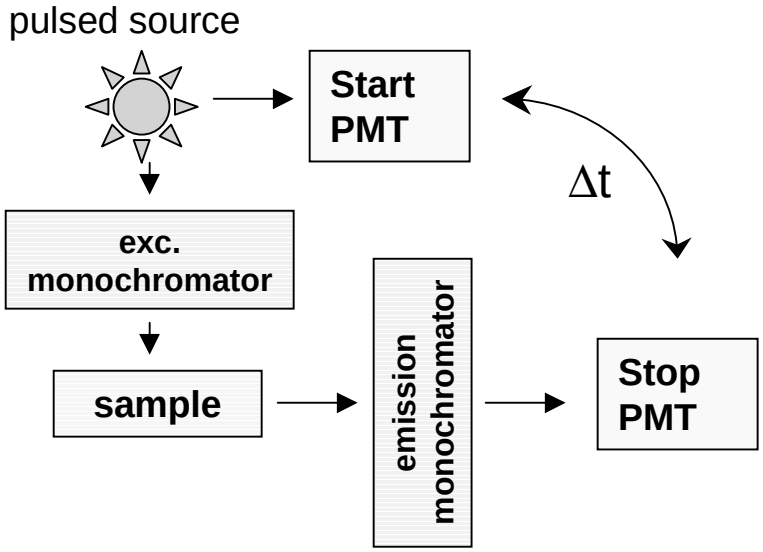
Fluorescein in NaOH 0.1N: ϕ_f =0.93

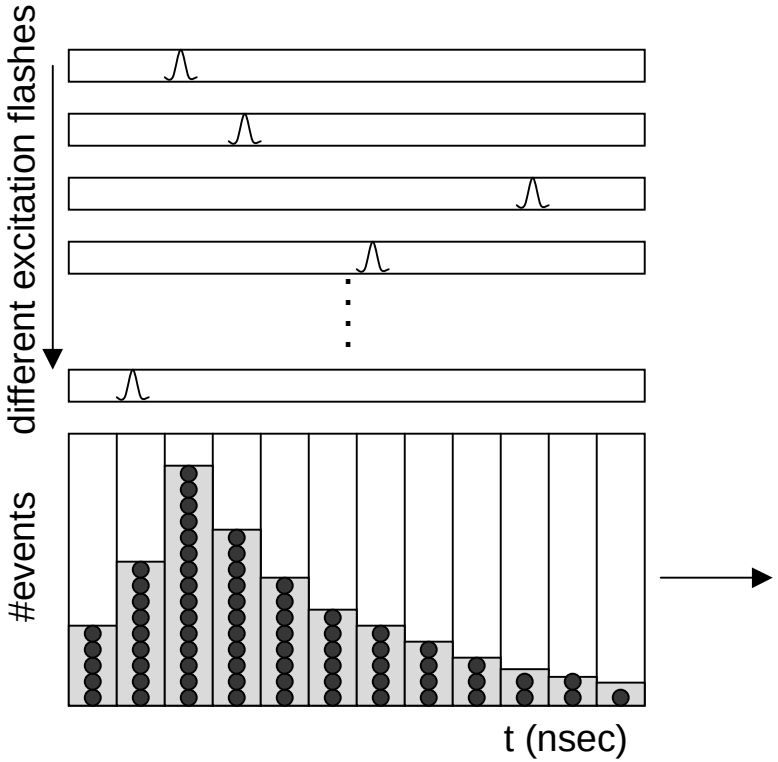
Important : the index of refraction of the two solvents (sample and standard) must be the same

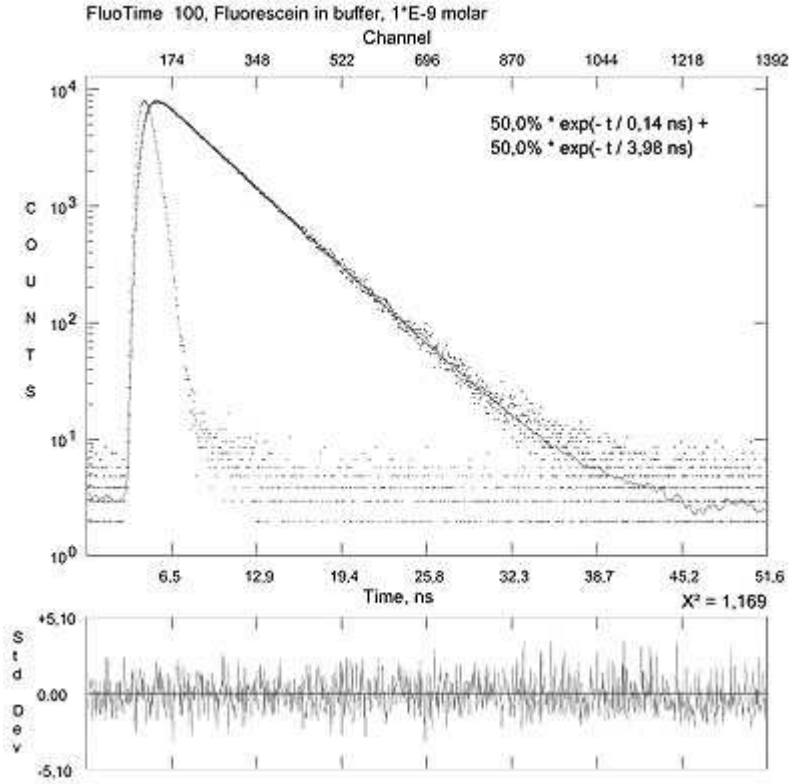
Measurement of fluorescence lifetimes

Time correlated single photon counting:

The TCSPC measurement relies on the concept that the probability distribution for emission of a single photon after an excitation yields the actual intensity against time distribution of all the photons emitted as a result of the excitation. By sampling the single photon emission after a large number of excitation flashes, the experiment constructs this probability distribution.







Intrinsic fluorescence of macromolecules

Substance	Conditions	Absorption λ_{max} (nm)	Absorption $\varepsilon_{max} \times 10^{-3}$	Fluorescence[1] λ_{max} (nm)	Fluorescence[1] ϕ_F	τ_F (nsec)	Sensitivity $\varepsilon_{max}\phi_F \times 10^{-2}$
Tryptophan	H_2O, pH 7	280	5.6	348	0.20	2.6	11.
Tyrosine	H_2O, pH 7	274	1.4	303	0.14	3.6	2.0
Phenylalanine	H_2O, pH 7	257	0.2	282	0.04	6.4	0.08
Y base	Yeast $tRNA^{Phe}$	320	1.3	460	0.07	6.3	0.91
Adenine	H_2O, pH 7	260	13.4	321	2.6×10^{-4}	<0.02	0.032
Guanine	H_2O, pH 7	275	8.1	329	3.0×10^{-4}	<0.02	0.024
Cytosine	H_2O, pH 7	267	6.1	313	0.8×10^{-4}	<0.02	0.005
Uracil	H_2O, pH 7	260	9.5	308	0.4×10^{-4}	<0.02	0.004
NADH	H_2O, pH 7	340	6.2	470	0.019	0.40	1.2

$\blacktriangleright \varepsilon_{max}.\phi_f$

Emission maxima and lifetimes of single **tryptophan** proteins

Protein	λ_{max}(nm)	τ_0(nsec)
Azurin	308	4.0
RNaseT1	324	3.5
HAS	342	6.0
Nuclease	334	5.0
Monellin	342	2.6
Glucagon	352	2.8
ACTH	352	3.1

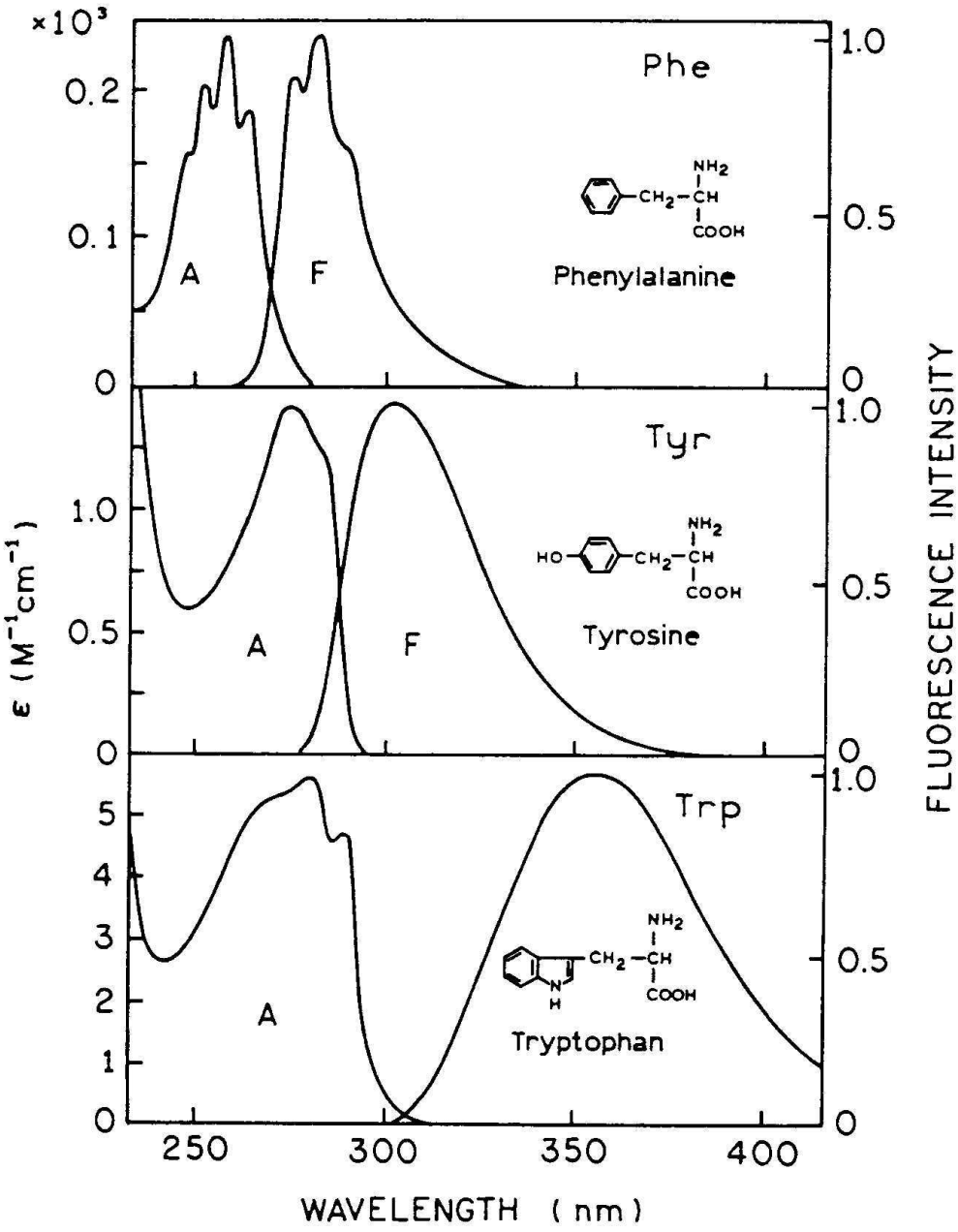
Usually determines the fluorescence of proteins.
Tyr often is quenched by nearby tryptophanes

Protein fluorescence

Tryptophan, the dominant intrinsic fluorophore, is generally present at about 1mol% in proteins. A protein may possess just one or a few Trp residues, which facilitates interpretation of the spectral data.

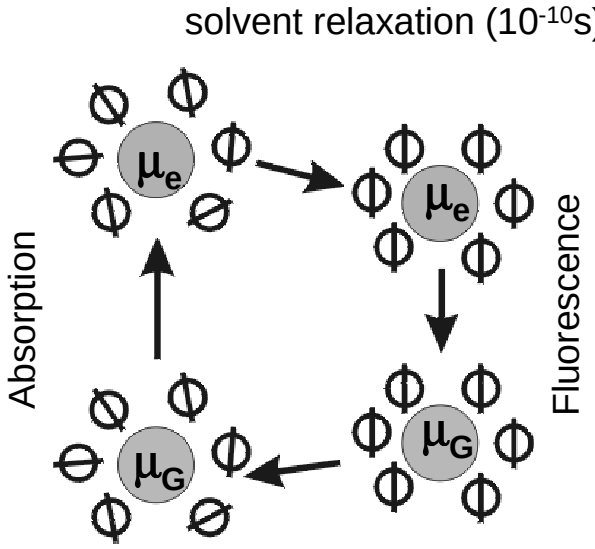
Tryp is very sensitive to its local environment. It is possible to see changes in emission spectra in response to conformational changes, subunit association, substrate binding, denaturation, and anything that affects the local environment surronding the indole ring. Also, Trp appears to be uniquely sensitive to collisional quenching, either by externally added quenchers, or by nearby groups in the protein.

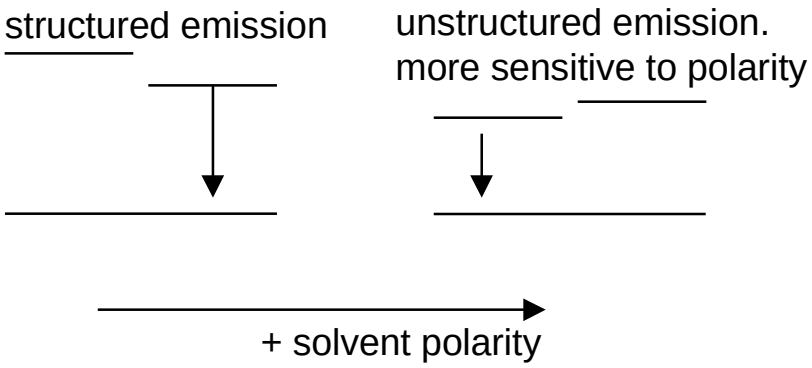
Trp fluorescence can be selectively excited at 295-305 nm. (to avoid excitation of Tyr)



Protein fluorescence: solvent effects.

General solvent effects:





Lippert equation:

$$(\overline{\nu}_A - \overline{\nu}_F) = \frac{2}{hC}\left(\frac{\varepsilon - 1}{2\varepsilon + 1} - \frac{n^2 - 1}{2n^2 + 1}\right)\frac{(\mu_E - \mu_G)^2}{a^3} + \text{constant}$$

Trp displays complex spectral properties due to the presence of two nearly isoenergetic excited states. These electronic transitions display distinct absorption, and emission spectra, and are differently sensitive to solvent polarity

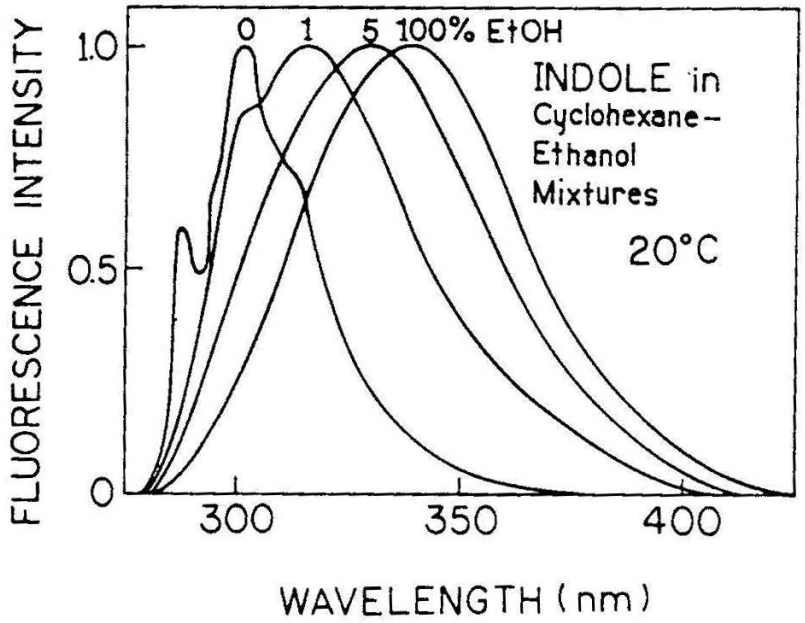
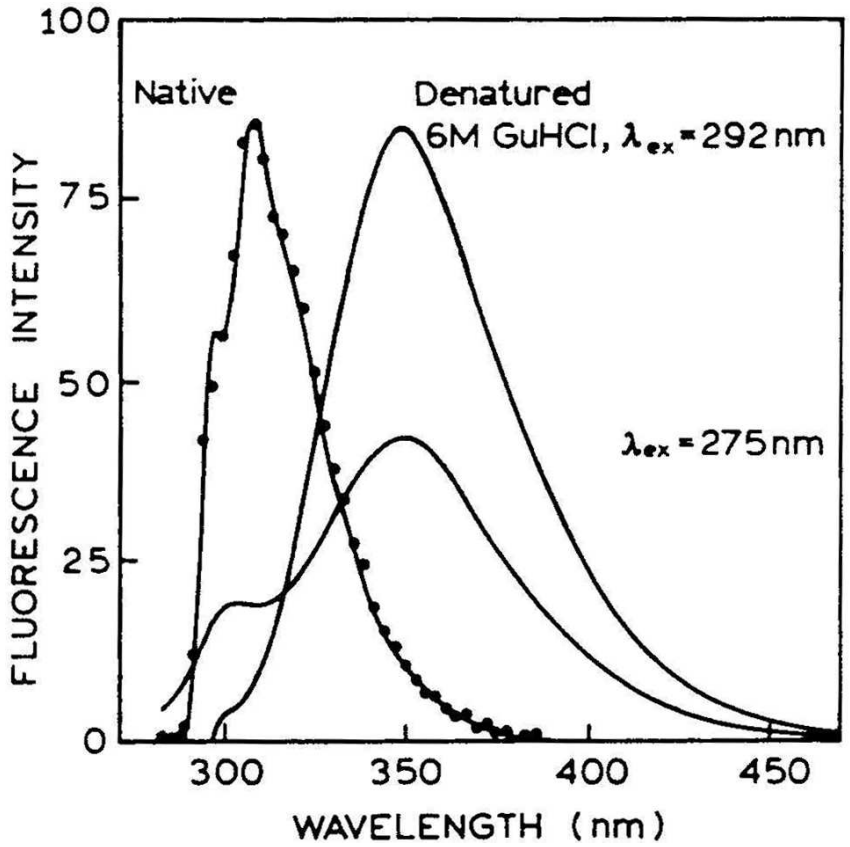


Figure 16.5. Emission spectra of indole in cyclohexane, ethanol, and their mixtures at 20 °C. From Ref. 23.



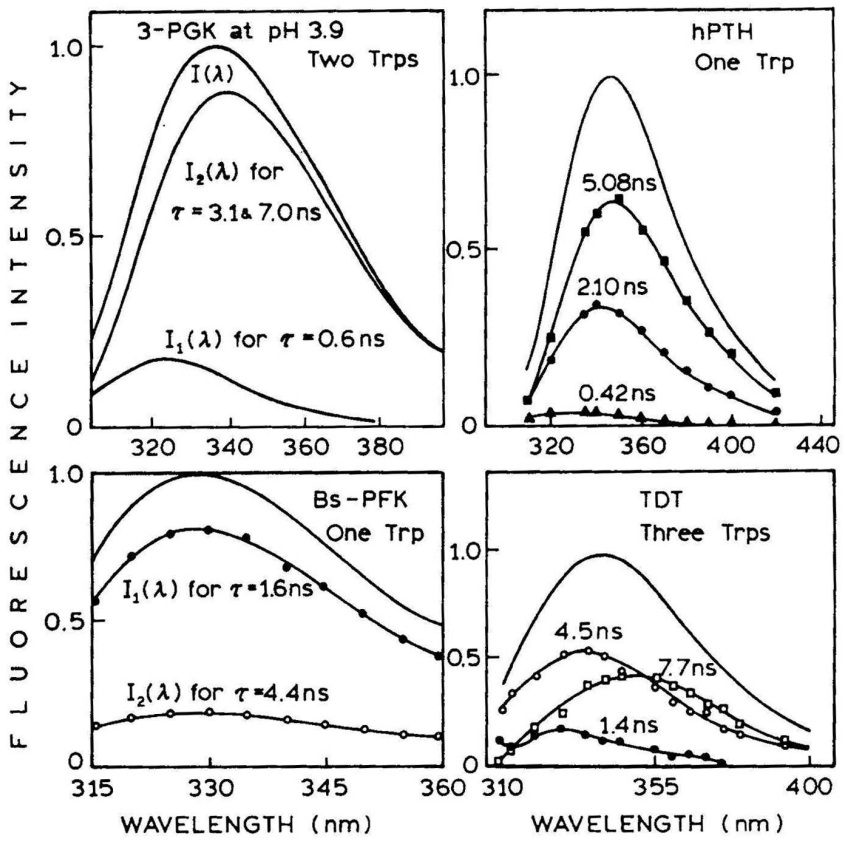
Emission spectra of *Pseudomonas fluorescens* azurin Pfl.
For 275-nm excitation, a peak is observed due to the tyrosine residue(s)

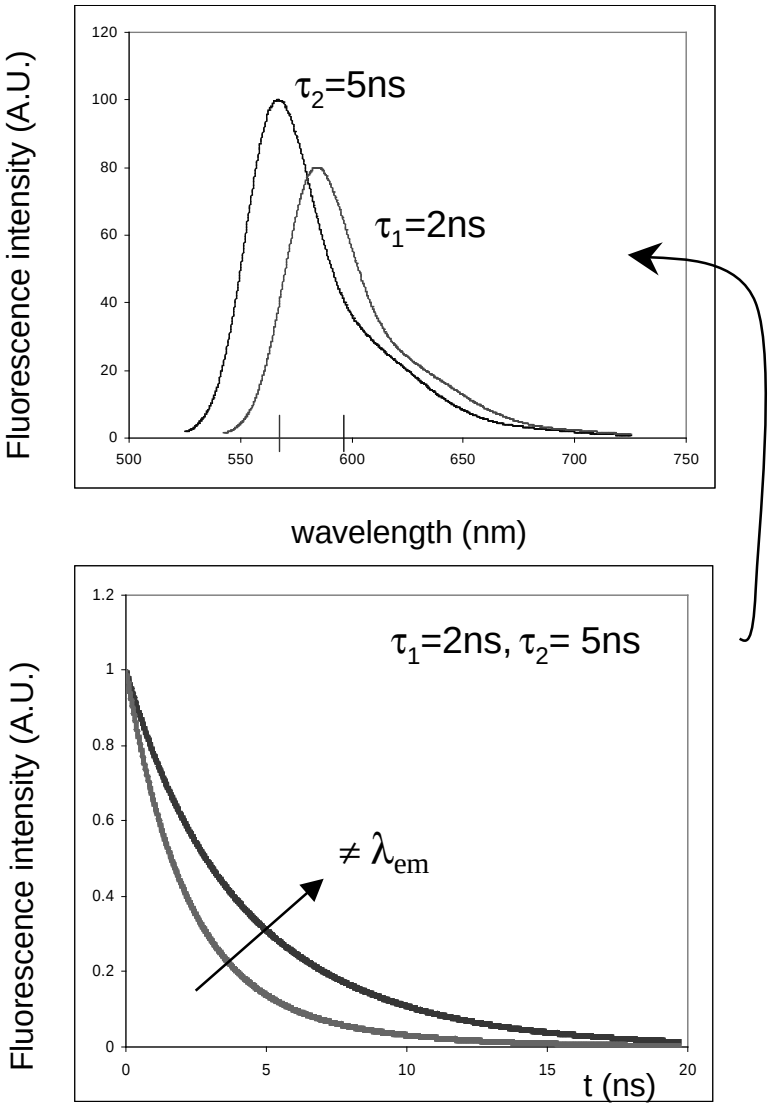
The position and structure of the fluorescence suggests that the indole residue is located in a completely nonpolar region of the protein. These results agree with X-ray studies, which show that the indole group is located in the hydrophobic core of theprotein.
In the presence of a denaturing agent, the TrpP emission loses its structure and shifts to 351nm, characteristic of a fully exposed Trp residue.
Changes in emission spectra can be used to follow protein unfolding

Time-resolved protein fluorescence:

Resolution of the contributions of individual tryptophan residues in multi-tryptophan proteins.

$$I(\lambda,t)=\sum_i \alpha_i(\lambda)\exp(-t/\tau_i)$$





Collisional quenching of proteins has been extensively utilized to determine the extent of Trp exposure to the aqueous phase.

Collisional quenching requires the fluorophore and quencher to be in molecular contact. A water-soluble quencher will not easily penetrate the protein matrix.

k_Q reflects the efficiency of quenching, or the accessibility of the fluorophores to the quencher.
Diffusion-controlled quenching typically results in values of k_O near $10^{10}\ M^{-1}s^{-1}$.

$$F \xrightarrow{h\nu_{exc}} F^*$$

$$F^* \xrightarrow{k_r} F + h\nu_{em} \quad \text{Fluorescence}$$

$$F^* + Q \xrightarrow{k_Q[Q]} F + Q \quad \text{Quenching}$$

$$F^* \xrightarrow{k_{IC}} F + \text{heat} \quad \text{Internal conversion}$$

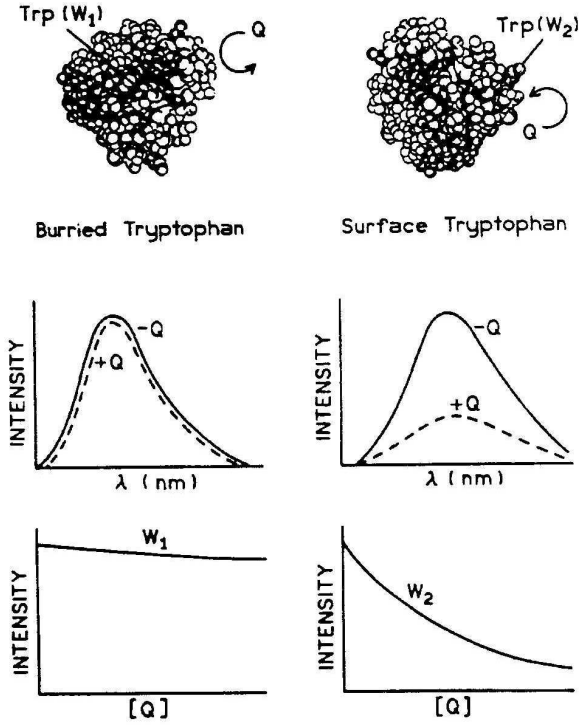
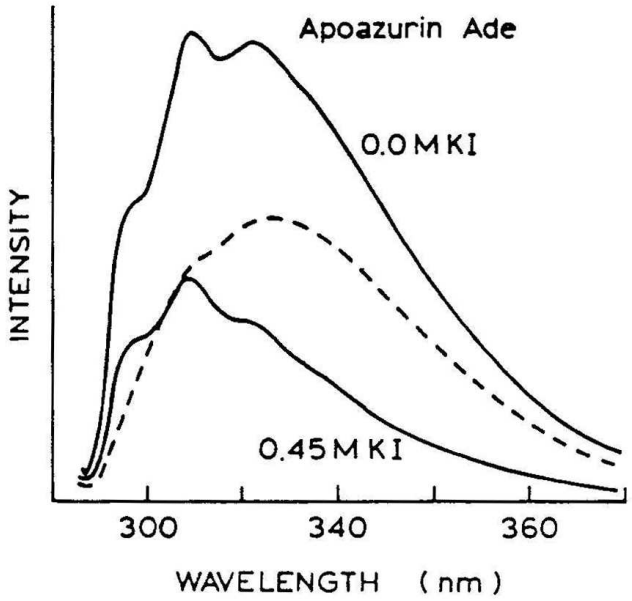


Figure 16.26. Collisional quenching of surface-accessible (W_1) and buried (W_2) tryptophan residues in proteins.

$$\frac{\phi}{\phi_0} = \frac{k_r / k_r + k_{IC} + k_Q[Q]}{k_r / k_r + k_{IC}}$$

without quencher

$$\frac{\phi_0}{\phi} = 1 + \frac{k_Q}{k_r + k_{IC}}[Q] = 1 + \tau_0 k_Q[Q] = \frac{I_0}{I} = \frac{\tau_0}{\tau}$$



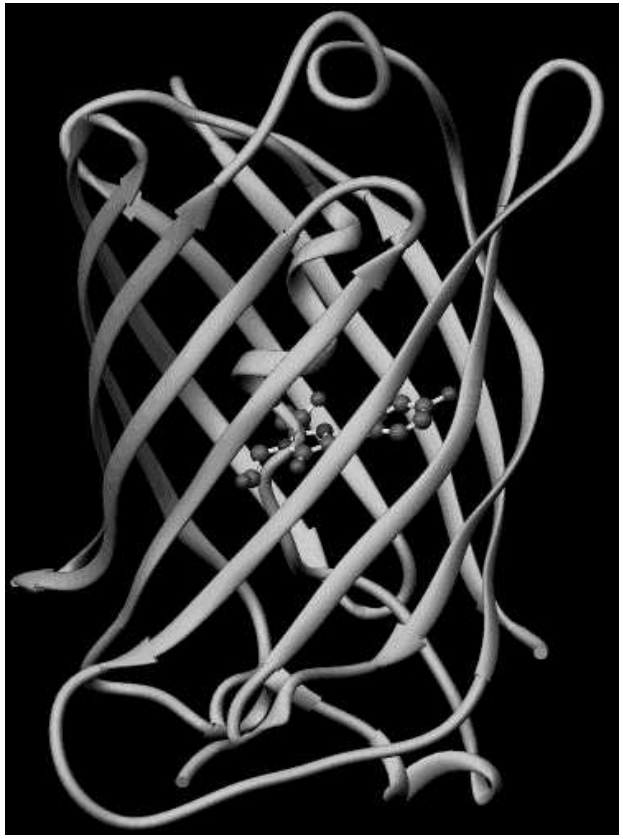
Apoazurin Ade has two Trp residues, one surface residue and one buried residue.

In the presence of iodide (collisional quencher), the spectrum resembles the structured emission seen in non-polar environments.
The spectrum of the quenched Trp can be seen from the difference spectrum (dashed line), and is characteristic of an exposed residue in a partially hydrophobic environment.

Green fluorescent protein (abbreviated GFP) is isolated from the Pacific jellyfish *Aequorea victoria* and now plays central roles in biochemistry and cell biology due to its widespread use as an *in vivo* reporter of gene expression, cell lineage, protein - protein interactions and protein trafficking

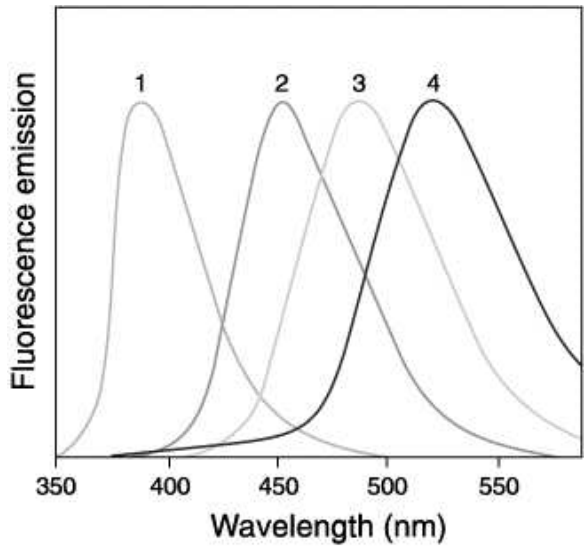
The structure of GFP has been solved by several groups and was found to be an eleven-strand beta-barrel wrapped around a central alpha-helix core. This central core contains the chromophore which is spontaneously formed from a chemical reaction involving residues Ser 65, Tyr 66, and Gly 67 wherein there is cyclization of the polypeptide backbone between Ser 65 and Gly 67 to form a 5-membered ring, followed by oxidation of Tyr 66. The high quantum yield of GFP fluorescence probably arises from the nearly complete protection of the fluorophore from quenching water or oxygen molecules by burial within the beta-barrel. One of the most important attributes of GFP which makes it so useful in the life sciences is that the luminescent chromophore is formed *in vivo*, and can thus generate a labeled cellular macromolecule without the difficulties of labeling with exogenous agents.

GFP can be fused to another protein either N- or C-terminally. This is due to the fact that both termini of GFP appear rather flexible on the surface of the beta-can, so that GFP's structure is not significantly distorted or destroyed by the fused protein.



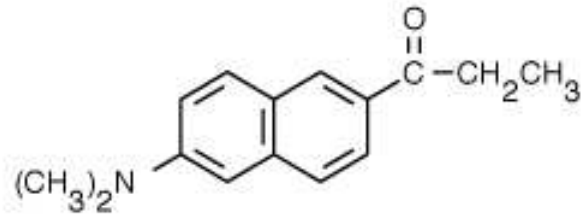
Ribbon diagram of the Green Fluorescent Protein (GFP) drawn from the wild-type crystal structure. The buried chromophore, which is responsible for GFP's luminescence, is shown in full atomic detail.

Extrinsic fluorophores:



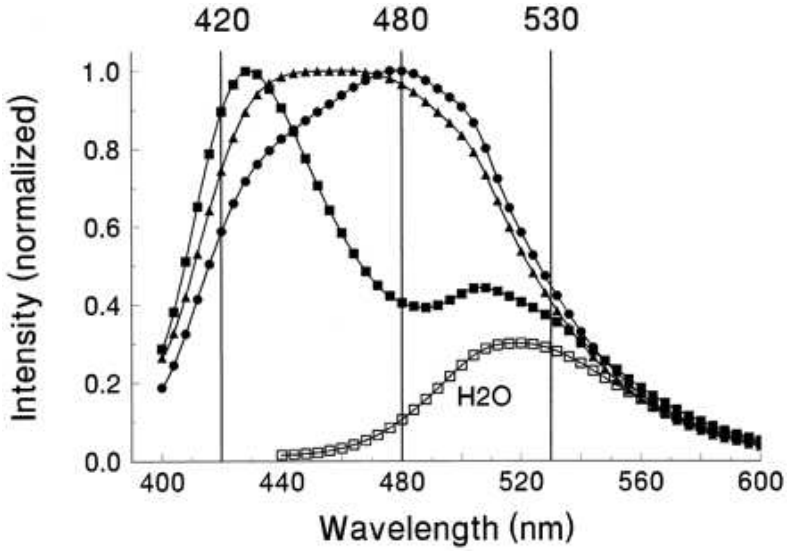
Normalized emission spectra of prodan in **1)** cyclohexane, **2)** dimethylformamide, **3)** ethanol, and **4)** water

Prodan has both an electron-donor and an electron-acceptor substituent, resulting in a large excited-state dipole moment and extensive solvent polarity–dependent fluorescence shifts



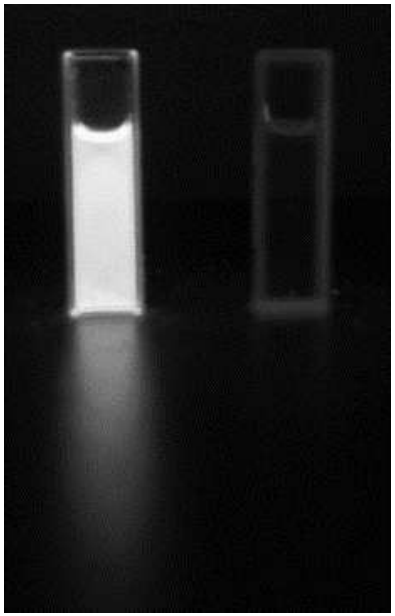
www.probes.com

The spectral sensitivity of Prodan fluorescence excitation and emission spectra to the phase state of phospholipid multilamellar vesicles has been compared in vesicles composed of pure gel, pure liquid-crystalline, and an equimolar mixture of the two phases

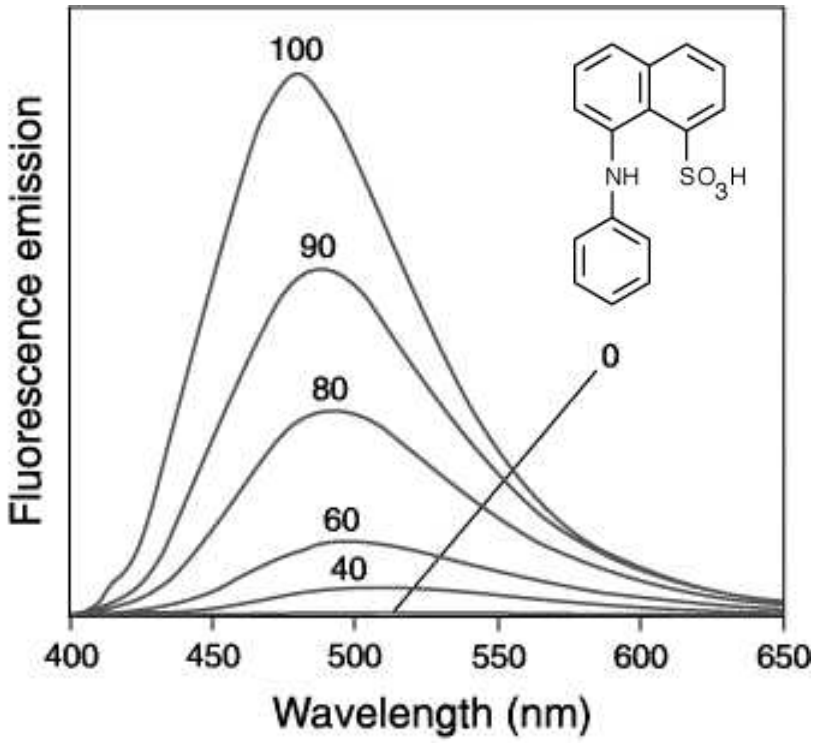


Biophys J, April 1998, p. 1984-1993, Vol. 74, No. 4

1,8-ANS has proved to be a sensitive probe for partially folded intermediates in protein-folding pathways. The basis of these applications is the strong fluorescence enhancement exhibited by these amphiphilic dyes when their exposure to water is lowered. Consequently, fluorescence of ANS increases substantially when proteins to which it is bound undergo transitions from unfolded to fully or partially folded states that provide shielding from water. Molten globule intermediates are characterized by particularly high ANS fluorescence intensities due to the exposure of hydrophobic core regions that are inaccessible to the dye in the native structure.



Fluorescence enhancement of 1,8-ANS (1-anilinonaphthalene-8-sulfonic acid, upon binding to protein. The image shows aqueous solutions of 1,8-ANS excited by ultraviolet light. Addition of protein (bovine serum albumin) to the solution in the cuvette on the left results in intense blue fluorescence. The fluorescence of uncomplexed free dye in the cuvette on the right is negligible in comparison.



Fluorescence emission spectra of equal concentrations of 1,8-ANS in ethanol:water mixtures. The labels adjacent to each curve indicate the percentage of ethanol in the solvent mixture.

Nucleic acid fluorescence:

Intrinsic fluorescence is too wee and too deep in the UV for practical applications.
There are numerous probes which spontaneously bind to DNA and display enhanced emission.

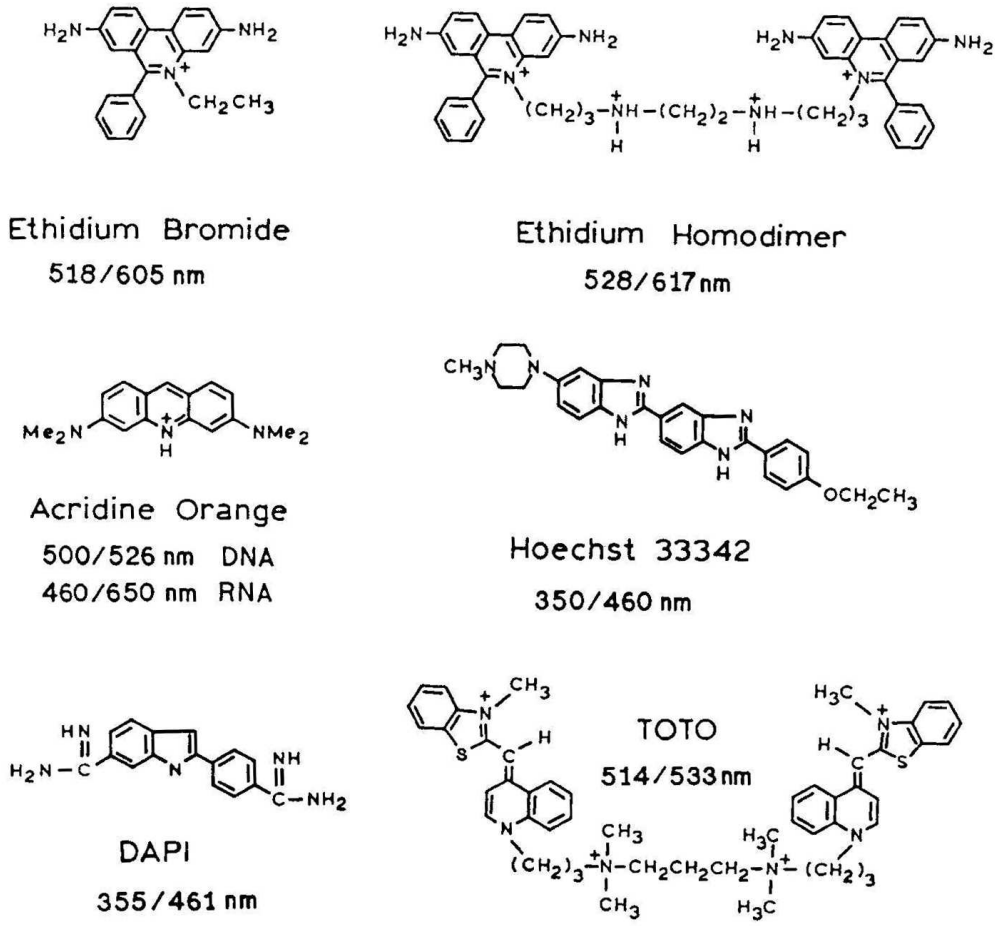
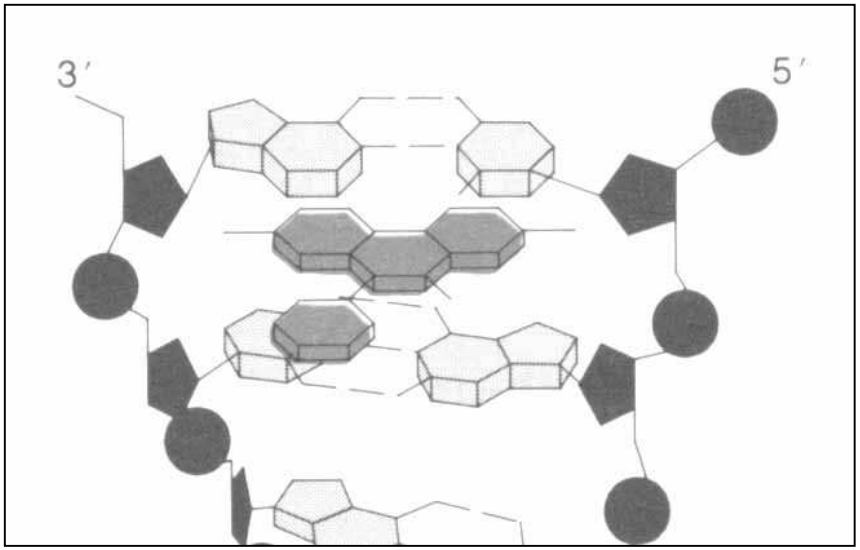
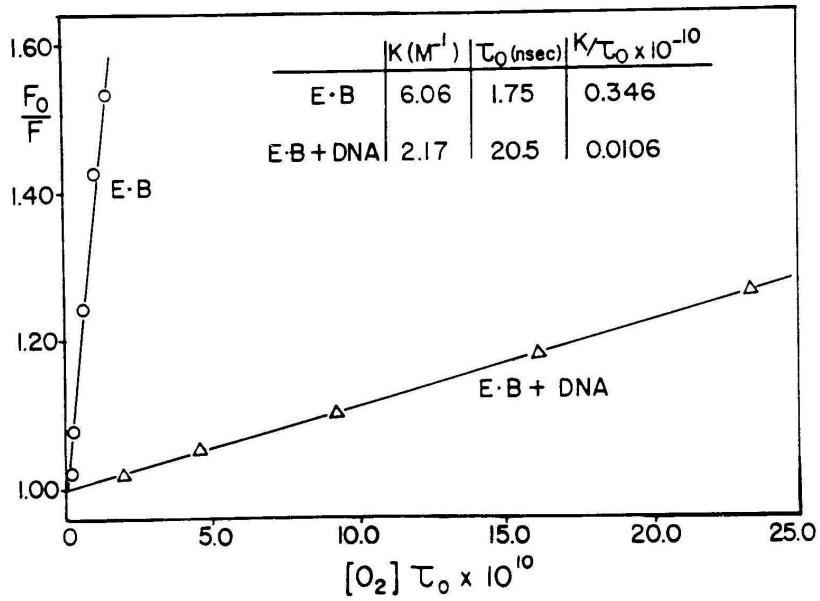


Figure 3.21. Representative DNA probes. Excitation and emission wavelengths refer to DNA-bound dye.

Ethidium bromide is one of the most widely used dyes. The mode of binding appears to be due to intercalation of the planar aromatic ring between the base pairs of double-helical DNA.
EB is weakly fluorescent in water, and its intensity increases about 30-fold upon binding to DNA.
Other dyes, such as acridine orange, also bind by intercalation.

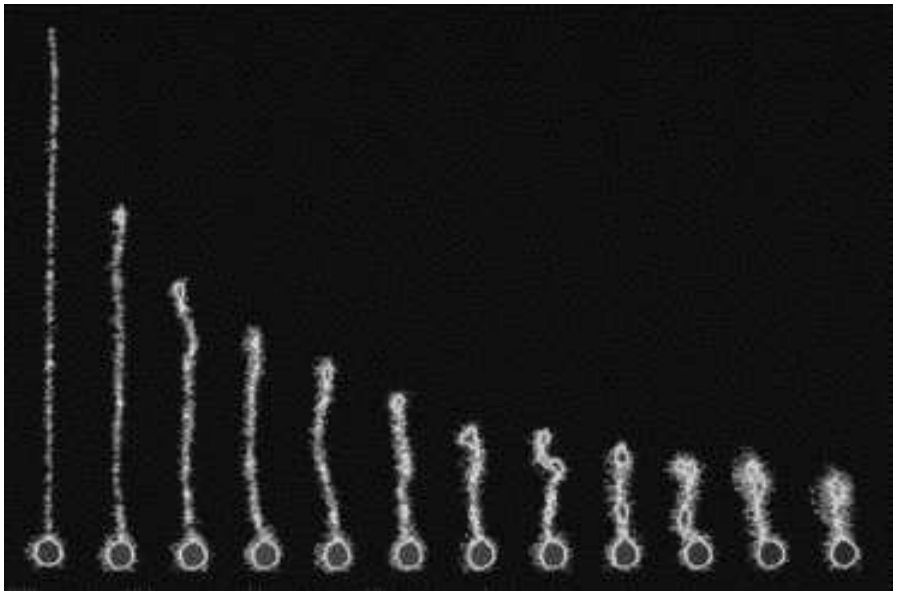




	$K\,(M^{-1})$	τ_0 (nsec)	$K/\tau_0 \times 10^{-10}$
E·B	6.06	1.75	0.346
E·B + DNA	2.17	20.5	0.0106

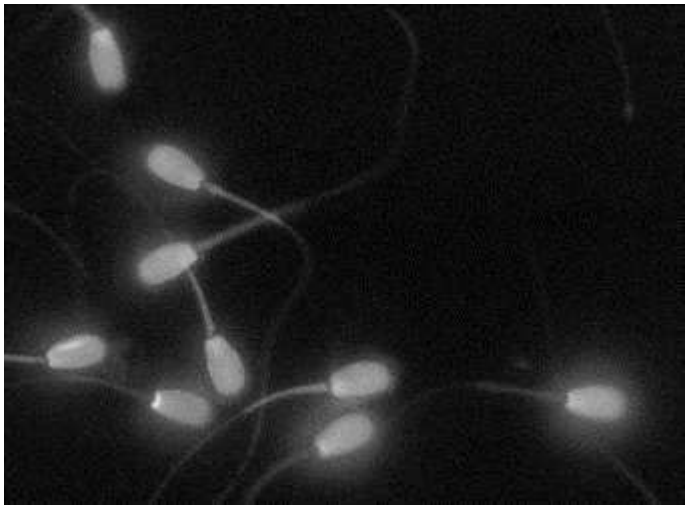
Figure 9.10. Oxygen quenching of ethidium bromide in solution and when intercalated in double helical DNA. Reprinted with permission from the American Chemical Society. (Fro Ref. 28.)

Dye Name	Ex/Em *	Application
Dyes for Ultrasensitive Solution Quantitation		
PicoGreen	502/523	Ultrasensitive reagent for solution quantitation of dsDNA
OliGreen	498/518	Ultrasensitive reagent for solution quantitation of ssDNA and oligonucleotides
RiboGreen	500/520	Ultrasensitive reagent for solution quantitation of RNA
Dyes for Sensitive Detection of Nucleic Acids in Gels and on Blots		
SYBR Gold stain	495/537	Ultrasensitive gel stain for single- or double-stranded DNA or RNA post-electrophoresis
SYBR Green I stain	494/521	Ultrasensitive gel stain for double-stranded DNA and oligonucleotides post-electrophoresis Also useful for real-time PCR assays
SYBR Green II stain	492/513	Sensitive stain for RNA and ssDNA post-electrophoresis
SYBR DX DNA blot stain	475/499	Sensitive stain for DNA on blots (not recommended for staining RNA)



The relaxation of a single, 39 µm–long DNA molecule stained with YOYO-1 iodide imaged at 4.5 second intervals. After the 1 µm polystyrene sphere was trapped with optical tweezers, the attached DNA was stretched to its full extension in a fluid flow and then allowed to relax upon stoppage of fluid flow due to its entropic elasticity. The YOYO-1 iodide–DNA complex is excited with the 488 nm spectral line of the argon-ion laser and visualized through a 515 nm longpass optical filter using a Hamamatsu SIT camera with image processing.

Perkins TT, Quake SR, Smith DE, Chu S.
Science 1994 May 6;264(5160):822-6

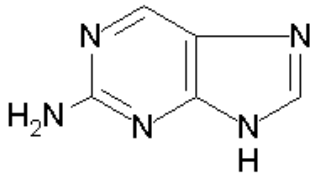


mixture of live and dead bovine sperm cells. Live sperm with intact membranes are labeled with nucleic acid stain, SYBR 14, and fluoresce green. Dead sperm, which have been killed by unprotected freeze-thawing, are labeled with propidium iodide and fluoresce red-orange.

Molecular Probes: www.probes.com

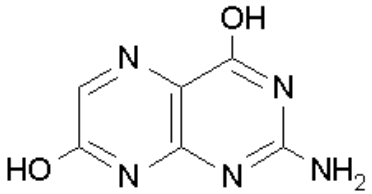
DNA Base Analogs:

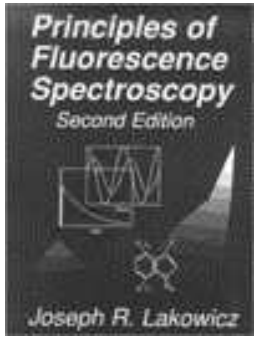
2 – Aminopurine (analog of adenine)



In solution, 2-AP has a high quantum yield and a single exponential decay.
Upon incorporation into ds-DNA, its fluorescence is partially quenched and its decay becomes complex.
The sensitivity of 2-AP to its environment makes it a useful probe for studies of DNA conformation and dynamics.

isoxanthopterin (analog of guanine)





Principles of Fluorescence Spectroscopy
Joseph R. Lakowicz J. Lakowicz

Format: Textbook Hardcover, 2nd ed., 698pp.
ISBN: 0306460939
Publisher: Kluwer Academic Publishers
Pub. Date: January 1999

Topics in Fluorescence Spectroscopy Joseph R. Lakowicz (Editor)
(Kluwer Academic/Plenum Publishers)

Volume 1: Techniques January 1992
Volume 2: Principles Volume 3: Biochemical Applications
Volume 4: Probe Design and Chemical Sensing
Volume 5: Nonlinear and Two-Photon Induced Fluorescence
Volume 6: Protein Fluorescence November 2000

Biophysical Chemistry. C. R. Cantor and P.R. Schimmel. Vol. II, (!980), Freeman, New York.

Applications of Fluorescence in the Biomedical Sciences. Eds. D. L. Taylor, A. S. Waggoner, F. Lanni, R. F. Murphy, and R. R. Birge. (1986), Alan R. Liss, Inc., New York.

1. L. Stryer and R.P. Haugland. Energy Transfer: A spectroscopic ruler. Proc. Natl. Acad. Sci. USA **58**, 719 - 726 (1967).
2. L. Stryer. Fluorescence energy transfer as a spectroscopic ruler. Ann Rev. Biochem. **47**, 819 - 846 (1978).
3. Murchie, A.I. H. *et al.* Fluorescence Energy Transfer shows that the four-way DNA junction is a right -handed cross of antiparallel molecules. *Nature*, **341**, 763 - 766 (1989).
4. Tuschl, T *et al*. A three-dimensional model for the hammerhead ribozyme based on fluorescence measurements. Science, **266**, 785 - 789, (1994).
5. T. Heyduk and J. C. Lee. Solution studies on the structure of bent DNA in the cAMP receptor protein-lac DNA complex. Biochemistry, **31**, 5165 - 5171 (1992).
6. T. Heyduk and J.C. Lee. Application of fluorescence energy transfer and polarization to monitor *E. coli* cAMP receptor protein and *lac* promoter interaction. Proc. Natl. Acad. Sci. USA **87**, 1744 - 1748 (1990).
7. T. Heyduk *et al.* CAP interacts with RNA polymerase in solution in the absence of promoter DNA. Nature, **364**, 548 - 549 (1993)

EFTINK MR
THE USE OF FLUORESCENCE METHODS TO MONITOR UNFOLDING TRANSITIONS IN PROTEINS
BIOPHYS J 66 (2): 482-501 FEB 1994

ROSS JBA, SCHMIDT CJ, BRAND L
TIME-RESOLVED FLUORESCENCE OF THE 2 TRYPTOPHANS IN HORSE LIVER ALCOHOL-DEHYDROGENASE
BIOCHEMISTRY-US 20 (15): 4369-4377 1981