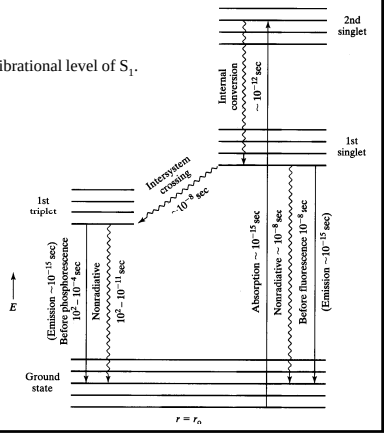
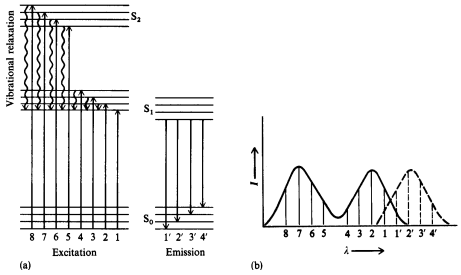
Fluorescence:

- Absorption
- Relaxation to ground vibrational level of S_1.
- Emission



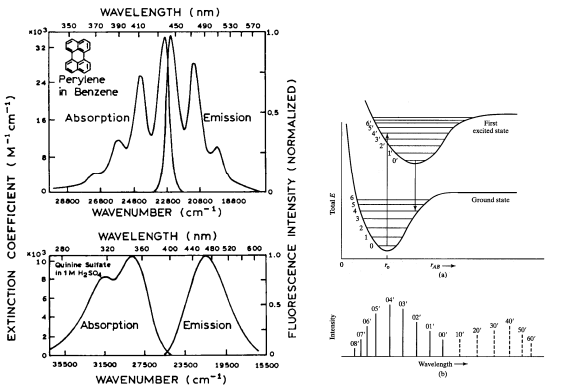
The lifetime of the excited state (~ 10^{-8} sec) makes fluorescence a valuable probe of biological macromolecules.

The emission spectrum is always displaced to lower energy.



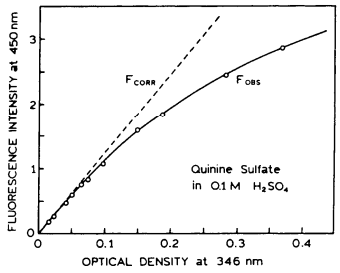
Vibrational relaxation prior to emission
Solvent rearrangement
The displacement of the emission spectrum to longer wavelengths - Stokes' Shift.

Emission spectra usually mirror images of the absorption spectrum



The inner filter effect

Fluorescence studies prone to self-absorption artifacts



$$F_{corr} = F_{obs}\ \text{antilog}\left(\frac{OD_{ex} + OD_{em}}{2}\right)$$

Excitation:
The flurophore exists in some ground state (**S_0**).
A photon of energy **$h\nu_{EX}$** is supplied by an external source such as an **incandescent lamp or a laser** and absorbed by the fluorophore.
This creates an excited electronic singlet state (**S_1'**).

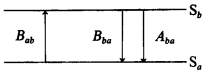
Excited-state lifetime:
The excited state exists for a finite time (typically **1^{-10} - 10^{-9}** seconds).
During this time, the fluorophore undergoes conformational changes and is also subject to interactions with its molecular environment.

The energy of **S_1'** is partially dissipated, yielding a relaxed singlet excited state (**S_1**) from which fluorescence emission originates.
Not all molecules initially excited by absorption (Stage 1) return to the ground state (**S_0**) by fluorescence emission.

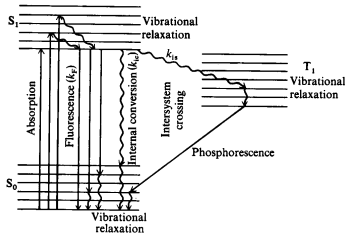
Other processes such as **collisional quenching, fluorescence energy transfer and intersystem crossing** may also depopulate **S_1**.

Quantum Yield $\phi = \dfrac{\textit{Number of emitted photons}}{\textit{Number of absorbed photons}}$

Properties of a strongly fluorescing molecule
Large molar absorptivity (extinction coefficient, ε).
The excited state must be populated for fluorescence to occur.



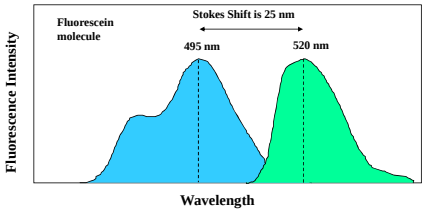
Einstein coefficients - probability of spontaneous emission of light from the excited state of a molecule is proportional to the molecule's ability to absorb light.



Stokes Shift

– is the energy difference between the lowest energy peak of absorbance and the highest energy of emission

The Stokes shift is fundamental to the sensitivity of fluorescence techniques because it allows **emission photons** to be detected isolated from excitation photons.



Quantum Yield

From Einstein's coefficients

$A_{ba} = 8\pi\nu^3 c^{-3} B_{ab}$

$A_{ba} = k_F \equiv$ fluorescence rate constant $= 1/\tau_R$

where τ_R is the "radiative lifetime"

The radiative lifetime is the lifetime that would be observed if all of the molecules returned to the ground state via fluorescence.

The actual, or observed, lifetime is symbolized τ:

$$\tau = \frac{1}{k} = \frac{1}{k_F + \sum_i k_i}$$

Having absorbed a photon, what is the likelihood of a photon being re-emitted?

This probability is given by the "quantum yield" (φ_F) for the system:

$$\varphi_F = \frac{k_F}{k_F + k_{ic} + k_{is} + k_q[Q]} = \frac{\tau}{\tau_F}$$

Biological fluorophores

Intrinsic fluorophores

Proteins

Tryptophan dominates protein fluorescence spectra

- high molar absorptivity
- moderate quantum yield
- ability to quench tyrosine and phenylalanine emission by energy transfer.

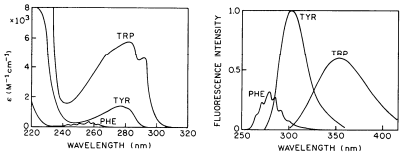


Table 3.1. Fluorescence Parameters of Aromatic Amino Acids in Water at Neutral pH[a]

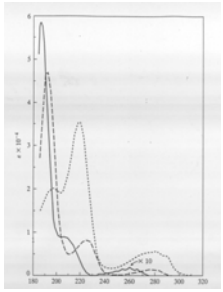
Species	λ_{ex} (nm)	λ_{em} (nm)	Bandwidth (nm)	Quantum yield	Lifetime (ns)
Phenylalanine	260	282	—	0.02	6.8
Tyrosine	275	304	34	0.14	3.6
Tryptophan	295	353	60	0.13	3.1[b]

Aromatic amino acids

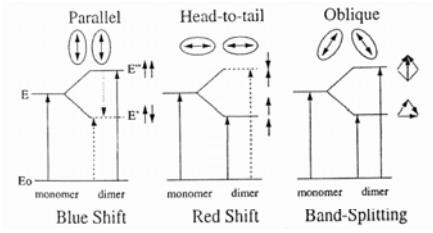
The amino acids phenylalanine, tyrosine, and tryptophan have π-π* transitions.

There is a pattern of weak bands from 240 - 300 nm and much more intense bands between 190 - 220 nm.

The weak bands are allowed by vibronic coupling (L).



Wavelength (nm)



Two dipoles brought **side by side**, relative orientation (parallel *vs.* antiparallel) - the energy of individual molecules, *E*, will split into two new states with the energies *E''* and *E'*

parallel dipoles repel make up an overall higher dipole moment *i.e.* stronger absorption - higher energy state - **blue shift of absorption**

antiparallel dipoles attract cancel each other to make a weak absorption - lowers the energy of that state - **red shift of absorption**

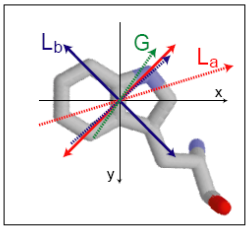
Tryptophan:

Described as a three level system: the ground state, and two excited states L_a, and L_b, with permanent and transition dipole moments given in the Table. The orientations of the dipole moments in the molecular frame are different.

Since the L_a and L_b levels are nearly degenerate, their transition energies are taken to be both at 280 nm

state/transition	notation	direction	magnitude (D)
$\|0\rangle$	$\vec{\mu}_0$	(0.690,-0.723,0.0)	2.0
L_a	$\vec{\mu}_a$	(0.956,-0.294,0.0)	3.5
L_b	$\vec{\mu}_b$	(0.751,-0.660,0.0)	3.5
$\|0\rangle \rightarrow L_a$	$\vec{\mu}_{0a}$	(0.707, -0.707,0.0)	5.5
$\|0\rangle \rightarrow L_b$	$\vec{\mu}_{0b}$	(0.707,0.707,0.0)	2.5

Unit vectors in the direction of the different dipole moments for the lowest transition in tryptophan.

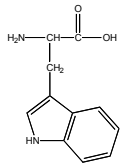


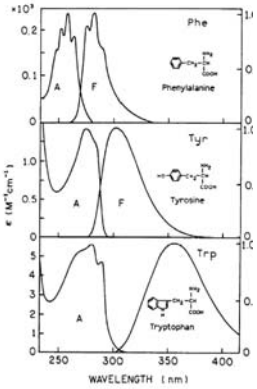
Dipole moments of tryptophan.

Full arrows represent the transition dipole moments, dashed ones represent the permanent dipole moments. G stands for ground state. Dipole moments belonging to excited state La (Lb) are depicted in red (blue).

The two lowest absorption bands in tryptophan are ascribed to the long and short axes of the indole ring.

The weak band at 280 nm is ^1L_b and the strong band at 220 nm is $^1B_{a,b}$. The weak band has a large orbital angular momentum change.

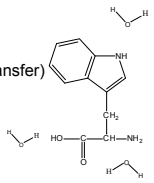


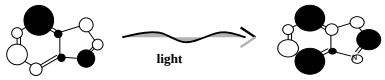


After a molecule absorbs light, it takes time for it to re-emit light.

During this time the molecule can undergo:

1) solvent rearrangement
2) rotation of the molecule)
3) loss of energy to neighboring molecule (energy transfer)
4) reaction (quenching)

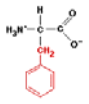




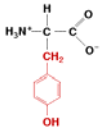
Ground state dipole moment: 2.1
Excited state dipole moment: 5.4

More polar in the excited state: electron density changed

Protein Fluorescence

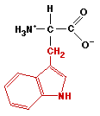


Phenylalanine (Phe)



Tyrosine (Tyr)

Proximity of aromatic groups in a folded protein results in efficient energy transfer between these groups.



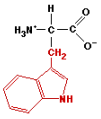
Tryptophan (Trp)

Absorbance Max

Phenylalanine	257.4 nm
Tyrosine	274.6 nm
Tryptophan	279.8 nm

Proteins containing all three aromatic residues emit fluorescence light typical of Trp

Proteins containing only Tyr and Phe emit light typical of Tyr.



Tryptophan (Trp)

Trp residues that are exposed to water fluoresce maximally at a wavelength of **350 nm**, while totally buried Trp residues emit at about **330 nm**.

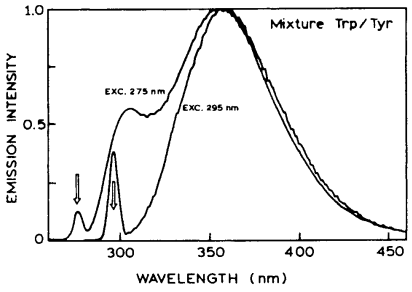
(Eftink et al., Biochemistry 26: 8338-8346 (1987).

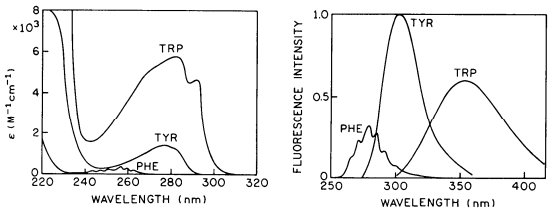
Fluorescence by a protein is complex when there is more than one aromatic side chain.

The proximity of aromatic groups in a folded protein results in efficient **energy transfer** between these groups.

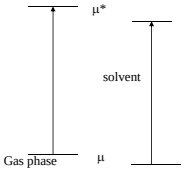
Light absorbed by one chromophore is transferred to another that absorbs at a **longer** wavelength, which can then emit the energy as fluorescence.

The emission spectrum of tryptophan containing a trace of tyrosine. Excitation at 275 nm and 295 nm.





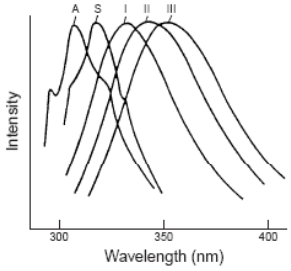
Local electric fields cause spectral shifts



Solvent can affect the ground state and excited state molecules causing spectral shift

Example: H bonding to tryptophan. Changes its absorption by about 10 nm

Normalized fluorescence spectra of tryptophan

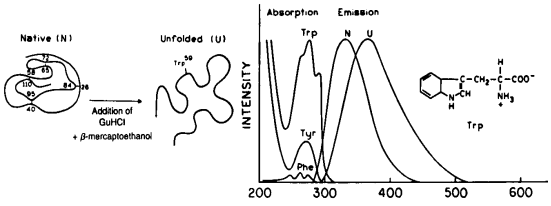


Residues belonging to five spectral classes A, S, I, II and III
A: Indole in Cyclohexane
III: 100% Ethanol

Protein Denaturation

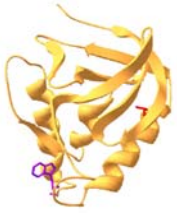
The fluorescence spectra of most proteins are dominated by the tryptophan contribution.

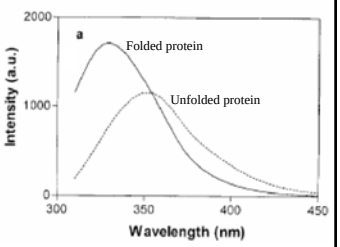
Emission spectrum of tryptophan undergoes a significant red shift upon exposure to a more polar environment.

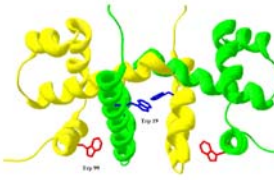


Tryptophan fluorescence indicates location

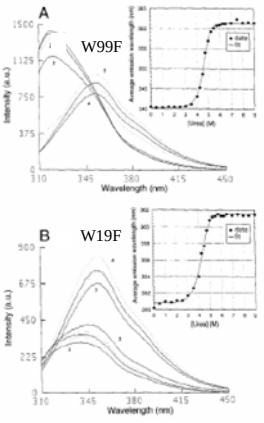
Nuclease



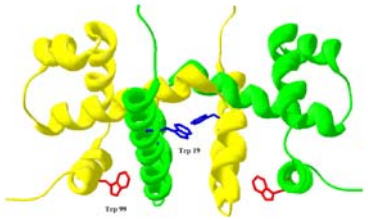




Trp Repressor



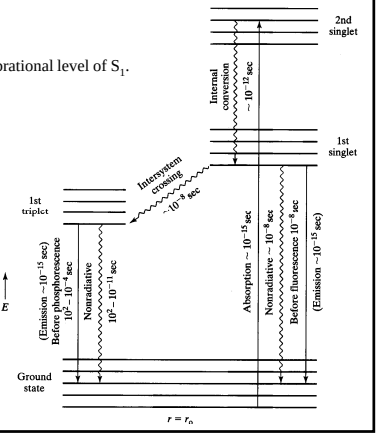
- Unfolding: Trp exposed to water
- Trp at position 19 in more stable part of the protein than Trp at position 99



WT	332nm	0.063
W99F	328nm	0.164
W19F	339nm	0.039

Fluorescence:

- Absorption
- Relaxation to ground vibrational level of S_1.
- Emission



The lifetime of the excited state (~ 10^{-8} sec) makes fluorescence a valuable probe of biological macromolecules.

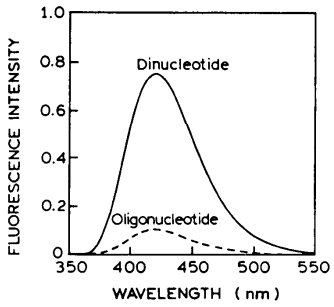
Nucleic acids - strongly absorbing molecules are non-fluorescent

Several other processes compete with fluorescent for returning the molecule to the ground state

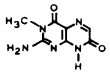
a) Internal conversion: conversion of electronic energy to vibrational energy. Especially effective if S_0 and S_1 vibrational levels overlap

b) Intersystem crossing. The "forbidden" (hence, low probability) of the excited state singlet to a triplet state.

Most nucleic acids are nonfluorescent. DNA base analogs, 2-aminopurine (an adenine analog) and isoxanthopterin (IXP, a guanine analog) are fluorescent and quenched by incorporation into double-stranded structures.

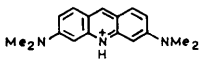


2-Aminopurine (2-AP)



Isoxanthopterin (IXP)

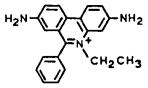
Noncovalent DNA probes.



Acridine Orange

500/526 nm DNA

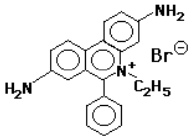
460/650 nm RNA



Ethidium Bromide

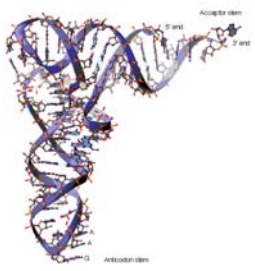
518/605 nm

Binding of Ethidium-Bromide to Transfer RNA

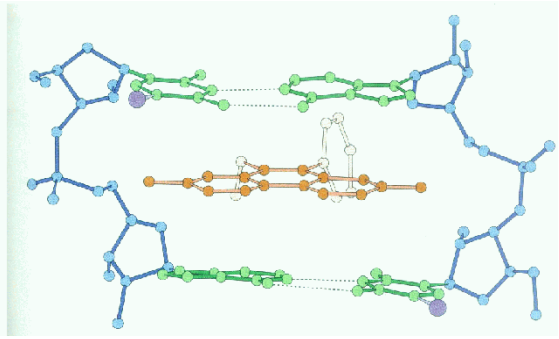


Ethidium Bromide

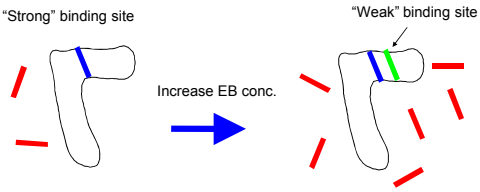
Yeast phenylalanine tRNA



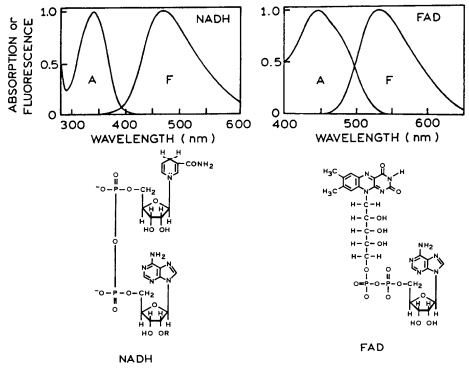
Ethidium bromide can intercalate into nucleic acid structures
It binds well to both DNA and RNA



Fluorescence investigations of EB - tRNA interactions have indicated a "strong" binding site and one or more "weak, non-specific" binding sites.



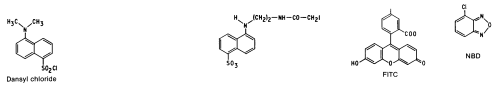
Enzyme cofactors NADH, FAD and pyridoxal/pyridoxamine are fluorescent.



Extrinsic probes: Protein labeling reagents

Table 5.2 Typical fluorescent probes[a]

Probe	Uses	Absorption λ_{max} (nm)	Absorption ϵ_{max} ($\times 10^{-3}$)	Emission[b] λ_{max} (nm)	Emission[b] ϕ_F	τ_F (ns)	Sensitivity $\epsilon_{max}\phi_F$ ($\times 10^{-3}$)
Dansyl chloride	Covalent attachment to protein: Lys, Cys	330	3.4	510	0.1	13	3.4
1,5-I-AEDANS	"	360	6.8	480	0.5	15	34
7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD)	Lys, Tyr	345	9.5	—	~1		
Fluorescein isothiocyanate (FITC)	Covalent attachment to protein: Lys	495	42	516	0.3	4	116
8-Anilino-1-naphthalene sulfonate (ANS)	Noncovalent binding to proteins	374	6.8	454	0.98	16	67
Pyrene and various derivatives	Polarization studies in membranes	342	40	383	0.25	100	100
Ethenoadenosine and various derivatives	Analogues of nucleotides bind to proteins. Incorporate into nucleic acids	300	2.6	410	0.40	26	10
Ethidium bromide	Noncovalent binding to nucleic acids	515	3.8	600	~1	26.5	38
Proflavine monosemicarbazide	Covalent attachment to RNA 3′-ends	445	15	516	0.02	—	30



Fluorogenic probes

Non-fluorescent compounds that become fluorescent, or release fluorescent species, upon chemical modification.

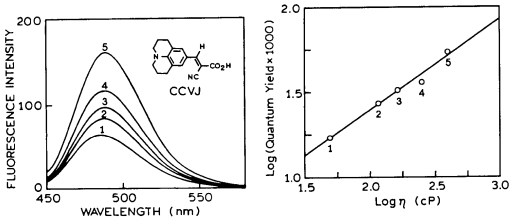
Table 3.3. Fluorogenic Amino Reagents[a]

Compound[b]	λ_{ex} max (nm)	ϵ (M^{-1} cm^{-1})	λ_{em} max (nm)
Fluorescamine	395	6,300	480
MDPF	290, 390	6,400	480
CBQCA	465	—	560
NBD	470	12,900	550

[a]From Ref. 21, p. 127 and Ref. 20 p. 42.
[b]MDPF, 2-methoxy-2,4-diphenyl-3 (2H)-furanone; CBQCA, 3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde; NBD, 7-Nitrobenz-2-oxa-1,3-diazol-4-yl.

Fluorogenic amino reagents provide sensitive detection of amino acids, peptides, and proteins.

Viscosity probes.
Fluorescence intensity influenced by viscosity

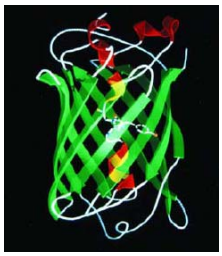


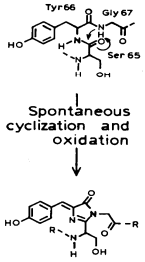
At high viscosity, internal motions are hindered, and the molecule is strongly fluorescent.

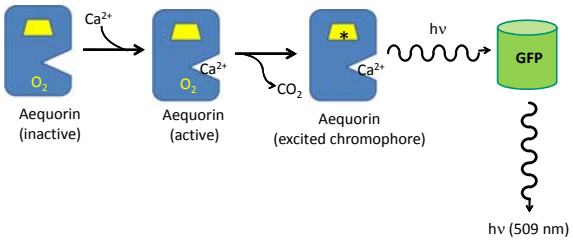
At low viscosity, however, the excited state is rapidly depopulated by charge transfer from the amino group to the vinyl group.

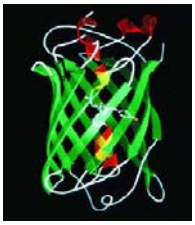
Green Fluorescent Protein (GFP) -bioluminescent jellyfish *Aequorea victoria*.

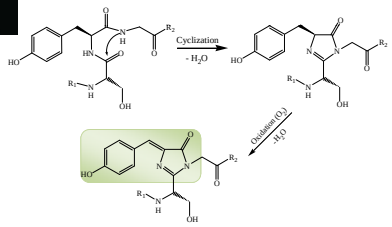
- Obvious β-barrel structure, with chromophore housed within the barrel.
- Remarkably, the chromophore is formed spontaneously (from Ser-65, Tyr-66, Gly-67) upon folding of the polypeptide chain, without the need for enzymatic synthesis.
- It is possible to insert the gene for GFP into cells and use the resulting protein as a reporter for a variety of applications.

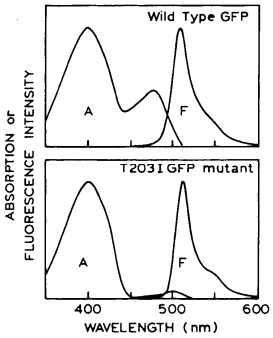








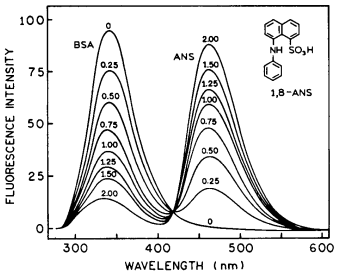




Binding of ANS (anilinonaphthalene sulfonic acid) by bovine serum albumin (BSA)

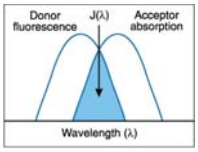
λ_{ex}=280 nm

Emission from ANS alone in aqueous solution is very weak. In the presence of BSA, the fluorescence of ANS is increased, and the wavelength maximum is shifted to lower wavelength ("blue shift"), the result of adsorption onto nonpolar regions of the BSA surface.



Tryptophan emission of serum albumin decreases with the binding of ANS.
Example of **resonant energy transfer.**

Fluorescence Resonance Energy Transfer (FRET)



Primary Conditions for FRET

Donor and acceptor molecules must be in close proximity (typically 10–100 Å).

The absorption spectrum of the acceptor must overlap fluorescence emission spectrum of the donor.

Donor and acceptor transition dipole orientations must be approximately parallel.

Förster Radius

The distance at which energy transfer is 50% efficient (i.e., 50% of excited donors are deactivated by FRET) is defined by the Förster radius (Ro).

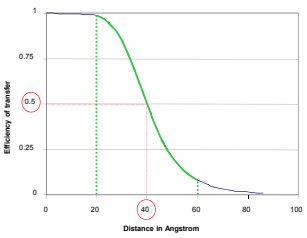
The magnitude of R_o is dependent on the spectral properties of the donor and acceptor dyes.

The distance dependence of the energy transfer efficiency (*E*)

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

Where *r* is the distance separating the centers of the donor and acceptor fluorophores, R_0 is the Förster distance.

The efficiency of transfer varies with the inverse sixth power of the distance.



R_0 in this example was set to 40 Å.

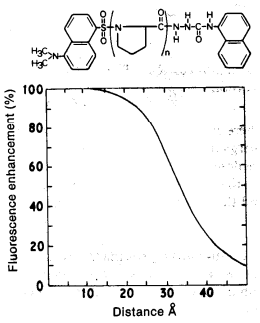
When the *E* is 50%, $R=R_0$

Distances can generally be measured between ~0.5 R_0 and ~1.5R_0

PM

Distance-dependence was demonstrated by Lubert Stryer

Energy is transferred between the naphthyl group (right) and the dansyl group



The proline spacers adopt a helical configuration, permitting calculation of the average distance between the donor and acceptor groups for a given length spacer.

The measured efficiencies (in this case the enhancement of the dansyl fluorescence) confirmed the expected $1/r^6$ dependence.

Fluorescence Resonant Energy Transfer (FRET, RET)

FRET is a process by which excitation energy is transferred from one chromophore (the donor chromophore) to another chromophore (the acceptor chromophore).

The donor must be fluorescent; the acceptor need not be.

If the emission spectrum of the donor overlaps the absorption spectrum of the acceptor, energy can be transferred from the donor to the acceptor

1) The energy transfer process is non-radiative dipole-dipole interaction.

2) The probability of energy transfer depends on the extent of overlap of the donor emission and acceptor absorption spectrum.

3) Energy transfer has two manifestations:
- The intensity of the donor emission spectrum is reduced.
- Fluorescence emission by the acceptor - called "sensitized emission."

4) Energy does not go back and forth from donor to acceptor.

5) The efficiency of energy transfer is strongly dependent on the distance between the donor and acceptor and the orientation of the donor and acceptor transition dipoles. E α $1/r^6$

Theory

In the absence of energy transfer, the lifetime is τ.

In the presence of acceptor, $\frac{1}{\tau_T} = \frac{1}{\tau} + k_T$, and $k_T = \frac{1}{\tau_T} - \frac{1}{\tau}$

The efficiency (E_T) of depopulation by RET is then

$$E_T = \frac{k_T}{1/\tau + k_T} = \frac{1/\tau_T - 1/\tau}{1/\tau_T}$$

So $\frac{\tau_T}{\tau} = 1 - E_T$

Since $\phi_D = \frac{1}{\tau}$ and $\phi_T = \frac{1}{\tau_T}$, then $\frac{\phi_T}{\phi_D} = \frac{I_T}{I_D} = 1 - E_T$

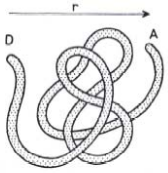
Define R_o as the distance at which energy transfer is 50% efficient.

At R_o, $k_T = \frac{1}{\tau}$

also, $k_T \alpha \left(\frac{1}{r^6}\right)$, $\therefore\ k_T = \frac{C}{R_o^{\ 6}}$

$\therefore\ \frac{1}{\tau} = \frac{C}{R_o^{\ 6}}$ so that $C = \frac{1}{\tau} R_o^6$

Thus, $k_T = \frac{1}{\tau}\left(\frac{R_o^6}{r^6}\right)$



Substituting back into the earlier expression for *E* :

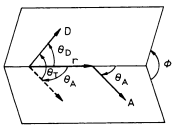
$$E = \frac{k_T}{\tau^{-1} + k_T} = \frac{R_o^6}{R_o^6 + r^6}$$

$$r = R_o\left(\frac{1-E}{E}\right)^{1/6}$$

The magnitude of R_o depends on the degree of overlap between the donor emission spectrum and the acceptor absorption spectrum, which is given by the overlap integral, *J*. For calculating *J*, the normalized emission spectrum is used

$$J = \int_0^\infty F_{D,norm}(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda = \frac{\int_0^\infty F_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda}{\int_0^\infty F_D(\lambda)d\lambda}$$

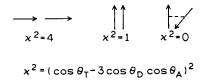
$$R_o^{\ 6} = \frac{9000(\ln 10)\kappa^2 Q_D}{128\pi^5 N n^4} \int_0^\infty F_{D,norm}(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda$$



If wavelength is expressed in cm and ε in M^{-1} cm^{-1}

$R_o = 9.78\times10^3[\kappa^2 n^{-4} Q_D J]^{1/6}$ (in Å)

$R_o^{\ 6} = 8.79\times10^{23}[\kappa^2 n^{-4} Q_D J]$ (in $Å^6$)



n is the refractive index, and Q_D is the donor quantum yield in the absence of acceptor.

κ^2 is the "orientation factor"

Depending on the geometry of the donor/acceptor transition dipoles, κ^2 can assume values from 0 to 4. It is usually assumed that κ^2 = 2/3, the value for donors and acceptors that randomize their orientations prior to energy transfer.

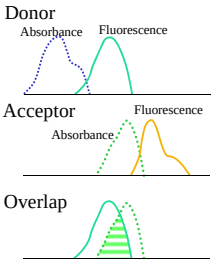
The overlap integral *J* is defined by:

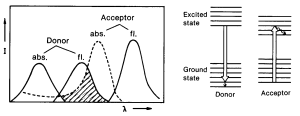
$$J = \int_0^\infty f_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda$$

Where λ is the wavelength of the light, $\varepsilon_A(\lambda)$ is the molar extinction coefficient at that wavelength and $f_D(\lambda)$ is the fluorescence spectrum of the donor normalized on the wavelength scale:

$$f_D(\lambda) = \frac{F_{D\lambda}(\lambda)}{\int_0^\infty F_{D\lambda}(\lambda)d\lambda}$$

Where $F_{D\lambda}(\lambda)$ is the donor fluorescence per unit wavelength interval





Applications

-Estimation of inter-chromophore distances in biological macromolecules, using intrinsic and extrinsic probes

-Strategy for assaying molecular proximity

-Biological phenomena that have been examined by FRET include:
- •protein-ligand interactions
- •protein-protein interactions
- •protein folding/unfolding
- •DNA denaturation/renaturation
- •protein-membrane interactions
- •membrane diffusion (both protein and lipid)

- Diffusion-enhanced energy transfer from long-lived donors (e.g., lanthanides) allows the distance of a chromophore from the surface of a macromolecule to be determined.

Quenching:

Certain moieties are particularly efficient at de-exciting the excited state - mostly through interaction with the electron in the excited state orbital.

High-molecular weight ions or triplet species can be effective quenchers. Examples include O_2, I^-, and acrylamide.

Quenching

Two general types: Dynamic and Static

Dynamic (collisional, Stern-Volmer)
Measures accessibility of fluorophore.

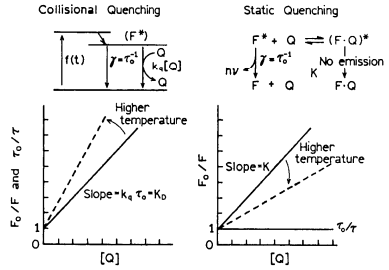
$$\frac{F_o}{F} = 1 + k_q\tau_o[Q] = 1 + K_D[Q]$$

$K_D \equiv$ Stern-Volmer constant

Static (complex formation)

$$\frac{F_o}{F} = 1 + K_S[Q]$$

$$K_S = \frac{[F\text{-}Q]}{[F][Q]}$$

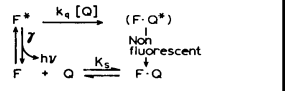


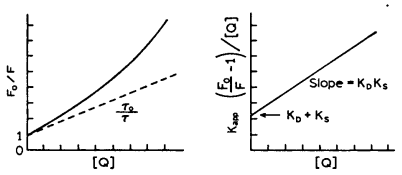
Combined dynamic and static quenching

$$\frac{F_o}{F} = (1 + K_D[Q])(1 + K_S[Q])$$

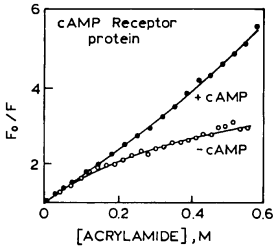
$$= 1 + (K_D + K_S)[Q] + K_D K_S[Q]^2$$

$$= 1 + K_{app}[Q], \text{ where } K_{app} = \left(\frac{F_o}{F} - 1\right)\frac{1}{[Q]}$$



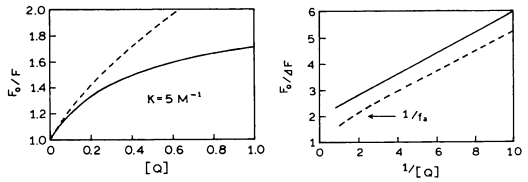


Quenching can also be used to recognize conformational changes that occur with substrate, ligand, or effector binding.



Data for the cAMP receptor protein with two Trp

In the absence of cAMP, only one of the two Trp residues are quenched by acrylamide. Addition of cAMP causes both chromophores to become sensitive to the quencher.

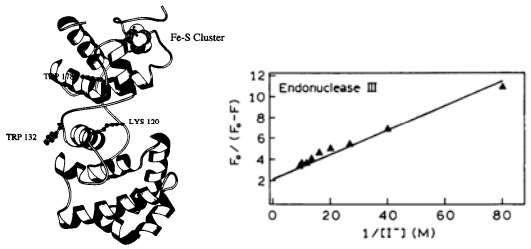


Dynamic quenching with fractional accessibility.
Downward curvature is observed in the S-V plot when only a fraction of the fluorophores is accessible to quencher.
In this case, a modified Stern-Volmer plot of ($F_o/\Delta F$) vs 1/[Q] will be linear.

The reciprocal of the vertical intercept is the fraction of the fluorophore accessible to quencher.

The dashed lines in the figures show the behavior observed when the "inaccessible" population has a K_D value one-tenth that of the accessible population.

Fractional accessibility of Trp residues in endonuclease III.



Fluorescence anisotropy.

Since excitation and emission dipoles have a particular orientation, fluorescence is anisotropic (i.e., has a preferred orientation).

If irradiated a perfectly oriented, immobilized sample were irradiated with light polarized parallel to the absorption transition dipole, the resulting fluorescence would have the same polarization as the incident light (provided that the emission transition dipole is parallel to the absorption transition dipole.

If the molecules in the sample were free to tumble prior to emission, the polarization of the emitted light could differ from the incident light.

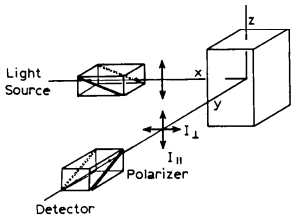
The extent to which the fluorescence anisotropy is decreased is strongly dependent on the rate at which the molecule rotates or tumbles
i.e., its *rotational correlation time*

The strategy for measuring steady-state fluorescence anisotropy is shown in the figure. A sample is irradiated with linearly polarized light.

Fluorescence intensity measurements are made with an emission polarizer oriented either parallel or perpendicular to the plane of polarization of the incident beam.

Anisotropy (symbolized either *r* or *A*) is defined as

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



In solution, the fundamental (or intrinsic) anisotropy of a fluorophore is given by the expression below, where β is the angle between the excitation and emission transition dipoles. If the transition dipoles are parallel, then r_o has a value of 0.40.

$$r_o = A_o = \frac{2}{5}\left(\frac{3\cos^2\beta - 1}{2}\right)$$

This is the value that is measured in viscous medium at low temperature, conditions under which rotational motions are largely frozen out.

Since rotation of the chromophore prior to emission decreases the observed anisotropy values, anisotropy measurements can be used to measure rotational correlation times. If instrumentation is available for time-resolved measurements, the anisotropy decay for a spherical protein molecule will be a single exponential:

$$r(t) = r_o \exp[-t/\theta] = r_o \exp[-6Dt]$$

In this equation, θ is the rotational correlation time, and *D* is the rotational diffusion coefficient.

A graph of *ln[r(t)]* vs *t* will have a slope of *–1/ θ.*

Rotational correlation times can also be estimated from steady-state polarization measurements, provided the fluorescent lifetime (τ) is known, via the *Perrin Equation:*

$$\frac{1}{r} = \frac{1}{r_o} + \frac{\tau}{r_o\theta}$$; for a globular protein,

$$\theta = \frac{\eta V}{RT} = \frac{\eta M}{RT}(\bar{v} + h)$$, so that

$$\frac{1}{r} = \frac{1}{r_o} + \frac{\tau RT}{r_o \eta V}$$ (Perrin Equation)

In the equation θ, $\bar{v}$ is the specific volume, and *h* is the degree of hydration (g H_2O per g protein).

- In these studies, the protein is labeled with an extrinsic fluorophore having a fluorescent lifetime similar to the rotational correlation time.
- The anisotropy of the protein is then measured over a range of T/η values.
- A plot of 1/r vs T/η will have a y-intercept of $1/r_o$ and slope equal to $\tau R/ r_o V$.

The method can be used even if the fluorophore is depolarized by segmental (i.e., local) motion, as well as by the overall tumbling of the protein molecule, provided that the segmental motions are much faster than the rotational diffusion.

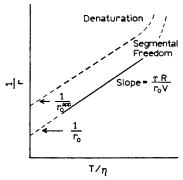


Table 10.4. Calculated Rotational Correlation Times for Proteins

T	Molecular weight (kDa)	Correlation time θ[a] (ns) h = 0	h = 0.2	h = 0.4
2 °C	10	5.5	6.9	8.4
	25	13.7	17.3	21.1
	50	27.4	34.6	42.0
	100	54.8	69.2	84.0
	500	274.0	346.0	420.0
20 °C	10	3.1	3.9	4.7
	25	7.0	9.7	11.8
	50	15.4	19.5	23.6
	100	30.8	39.0	47.2
	500	154.0	195.0	236.0
37 °C	10	2.0	2.5	3.1
	25	5.0	6.4	7.7
	50	10.0	12.7	15.4
	100	20.1	25.4	30.8
	500	100.5	127.0	154.0

[a]Calculated using θ = ηM (ν̄ + h)/RT with ν̄ = 0.75 ml/g and the indicated degree of hydration (h). The viscosities are η(2 °C) = 1.67 cP; η (20 °C) = 1.00 cP; and η(37 °C) = 0.69 cP.

Table 10.3. Rotational Correlation Times for Proteins[a]

Protein	Molecular weight	Observed θ (ns)	$\theta_{obs}/\theta_{calc}$[b]
Apomyoglobin	17,000	8.3	1.9
β-Lactoglobulin (monomer)	18,400	8.5	1.8
Trypsin	25,000	12.9	2.0
Chymotrypsin	25,000	15.1	2.3
Carbonic anhydrase	30,000	11.2	1.4
β-Lactoglobulin (dimer)	36,000	20.3	2.1
Apoperoxidase	40,000	25.2	2.4
Serum albumin	66,000	41.7	2.4

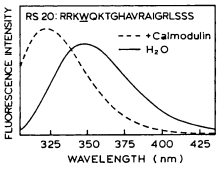
[a]From Ref. 30.

[b]θ_{obs} is the observed rotational correlation time, adjusted to the value of T/η corresponding to water at 25 °C. θ_{calc} is the rotational correlation time calculated for a rigid, unhydrated sphere with the molecular weight of the protein, assuming a partial specific volume of 0.73 ml/g.

Examples:

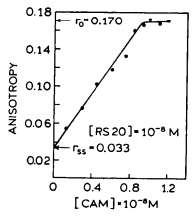
The figure shows the blue-shift that occurs when the peptide is bound by calmodulin.

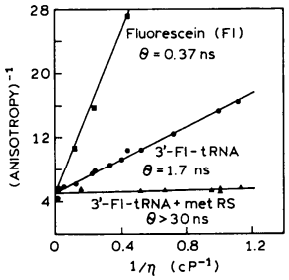
The emission maximum shift and the increase in the maximal fluorescence intensity suggest that the tryptophan residue is in a more apolar environment.



The figure to the right shows the changes in the anisotropy of a peptide fragment of myosin light-chain kinase upon titration with calmodulin, which binds the peptide.

- The increase in the anisotropy indicates that the peptide interacts tightly with calmodulin.
- The maximal anisotropy appears at a calmodulin:peptide ratio of 0.93, implying stoichiometry not greater than 1: 1
- The shape of the curve suggests that at a calmodulin concentration of 10^{-8} M (equivalent to the peptide concentration) there is no free peptide.
- The anisotropy of tryptophan emission of the free peptide is 0.033 and of the fully bound peptide 0.170.





The figure above shows the Perrin Plot for fluorescein, Fl-labeled-tRNA, and the Fl-tRNA bound to methionyl-tRNA synthetase.

Time-resolved fluorescence spectroscopy

Time-resolved data contain more information than is available from steady-state measurements. e.g., consider a protein containing two Trp residues.

- Because of spectral overlap, it is usually not possible to resolve the signals from the two residues.

-If the two Trp residues have different fluorescent lifetimes, their contributions to the overall fluorescence behavior can be determined from time-resolved measurements.

- One can then determine how each is influenced by the interactions of the protein with substrates, effectors, or other macromolecules.

Since the fluorescence lifetime of a molecule is very sensitive to its molecular environment, measurement of the fluorescence lifetime(s) reveals much about the state of the fluorophore.

Many macromolecular events, such as rotational diffusion, resonance-energy transfer, and dynamic quenching, occur on the same time scale as the fluorescence decay.

Thus, time-resolved fluorescence spectroscopy can be used to investigate these processes and gain insight into the chemical surroundings of the fluorophore.

Fluorescence lifetimes are generally on the order of 1-10 nsec, although they can range from hundreds of nanoseconds to the sub-nanosecond time scale.

What is the "lifetime" of a fluorophore?

Absorption and emission processes are almost always studied on *populations* of molecules and the properties of the typical members of the population are obtained from the macroscopic properties of the process.

The behavior of an excited population of fluorophores is described by a rate equation:

$$\frac{dn^*}{dt} = -n^* \Gamma + f(t)$$

where n^* is the number of excited elements at time t, Γ is the rate constant of emission and f(t) is an arbitrary function of the time, describing the time course of the excitation . The dimensions of Γ are sec^{-1} (transitions per molecule per unit time).

If excitation occurs at t = 0, the equation is:

$$\frac{dn^*}{dt} = -n^* \Gamma$$

and describes the decrease in excited molecules at all further times. Integration gives:

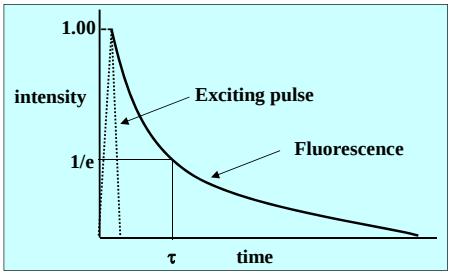
$$n^*(t) = n^*(0)\exp(-\Gamma t)$$

The lifetime, τ, is equal to Γ^{-1}

If a population of fluorophores are excited, the lifetime is the time it takes for the number of excited molecules to decay to 1/e or 36.8% of the original population according to:

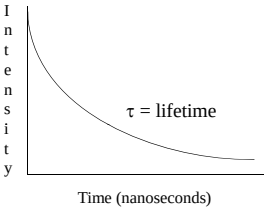
$$\frac{n^*(t)}{n^*(0)} = e^{-t/\tau}$$

In pictorial form: $\frac{n^*(t)}{n^*(0)} = e^{-t/\tau}$



Lifetime

- Fluorescence lifetime is the average time that an electron spends in the excited state before a photon is emitted
- Measurement of the fluorescence from a large number of molecules, following a short pulse excitation, will show an exponential decay
- The lifetime is given by the 1/e point of the decay

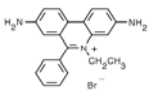


Exponential decay: $I = I_o e^{-t/\tau}$

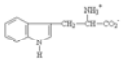
The lifetime and quantum yield for a given fluorophore is often dramatically affected by its environment.

Examples of this fact would be NADH, which in water has a lifetime of ~0.4 ns but bound to dehydrogenases can be a long as 9 ns.

ANS in water is ~100 picoseconds but can be 8 – 10 ns bound to proteins



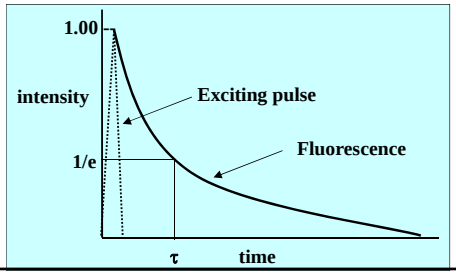
Ethidium bromide is 1.8 ns in water, 22 ns bound to DNA and 27ns bound to tRNA



The lifetime of tryptophan in proteins ranges from ~0.1 ns up to ~8 ns

Excited state lifetimes have traditionally been measured using either the *impulse* response or the *harmonic* response method. In principle both methods have the same information content. These methods are also referred to as either the "time domain" method or the "frequency domain" method.

In the *impulse (*or pulse) method, the sample is illuminated with a short pulse of light and the intensity of the emission versus time is recorded. Originally these short light pulses were generated using *flashlamps* which had widths on the order of several nanoseconds. Modern laser sources can now routinely generate pulses with widths on the order of picoseconds or shorter.

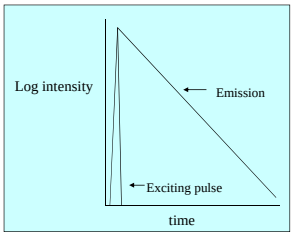


As shown in the intensity decay figure, the *fluorescence* lifetime, t , is the time at which the intensity has decayed to 1/e of the original value. The decay of the intensity with time is given by the relation:

$$I_t = \alpha e^{-t/\tau}$$

Where I_t is the intensity at time t, α is a normalization term (the pre-exponential factor) and τ is the lifetime.

It is more common to plot the fluorescence decay data using a logarithmic scale as shown here.



Quenching phenomena are best studied using time-resolved measurements, because one can readily distinguish static and dynamic quenching.

Formation of static ground-state complexes **does not decrease the decay time** of the uncomplexed fluorophores because only the unquenched fluorophores are observed in a fluorescence experiment.

Dynamic quenching is a rate process acting on the entire excited-state population and thus **decreases the mean decay time** of the excited-state population.

RET is also best studied by time-resolved measurements.

e.g. if a protein contains a donor and acceptor, and the steady-state measurements indicate that the donor is 50% quenched by the acceptor.

- the observation of 50% donor quenching can be due to 100% quenching for one-half of the donors or to 50% quenching of all the donors, or some combination of these two limiting cases.
- Steady-state data cannot distinguish between these two cases. However, very different donor intensity decays would be observed for each case.

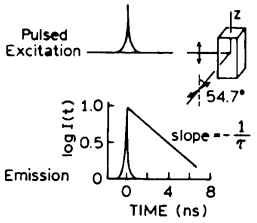
The down side to time-resolved measurements is that the instrumentation is highly specialized and costly.

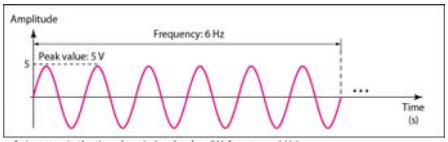
Two experimental strategies: **time-domain** and **frequency-domain**.

Time-domain measurements are conceptually simpler.

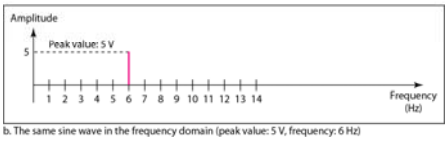
The sample is irradiated with a brief pulse of light, then subsequent (exponential) fluorescent decay is monitored.

The general technique is called "time-correlated single photon counting" (TCSPC)



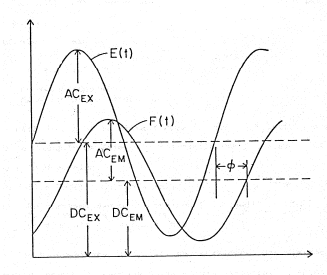


a. A sine wave in the time domain (peak value: 5 V, frequency: 6 Hz)



b. The same sine wave in the frequency domain (peak value: 5 V, frequency: 6 Hz)

Fluorophores subjected to such an excitation will give rise to a modulated emission which is shifted in phase relative to the exciting light
- This is due to the persistence of the excited state



phase delay (ϕ) between the excitation, E(t), and the emission, F(t). Also shown are the AC and DC levels associated with the excitation and emission waveforms.

It can be shown that:

$$F(t) = Fo\,[1 + M_F \sin(\omega t + \phi)]$$

This relationship signifies that measurement of the phase delay, ϕ, forms the basis of one measurement of the lifetime, τ.

$$\tan\phi = \omega\tau$$

The *modulations* of the excitation (M_E) and the emission (M_F) are given by:

$$M_E = \left(\frac{AC}{DC}\right)_E \quad \text{and} \quad M_F = \left(\frac{AC}{DC}\right)_F$$

The *relative modulation*, M, of the emission is then:

$$M = \frac{(AC/DC)_F}{(AC/DC)_E}$$

τ can also be determined from M according to the relation: $M = \dfrac{1}{\sqrt{1+(\omega\tau)^2}}$

Using the *phase shift* and *relative modulation* one can thus determine a *phase lifetime* (τ_P) and a *modulation lifetime* (τ_M).

If the fluorescence decay is a single exponential, then τ_P and τ_M will be equal at all modulation frequencies.

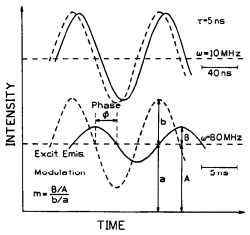
If, however, the fluorescence decay is multiexponential then $\tau_P < \tau_M$ and, moreover, the values of both τ_P and τ_M will depend upon the modulation frequency, i.e.,

$$\tau_P(\omega_1) < \tau_P(\omega_2) \quad \text{if} \quad \omega_1 > \omega_2$$

Typical phase and modulation data

Frequency (MHz)	τ_P (ns)	τ_M (ns)
5	6.76	10.24
10	6.02	9.70
30	3.17	6.87
70	1.93	4.27

Frequency-domain (FD) measurements. Also called phase modulation.



The sample is excited with intensity-modulated light. The intensity is varied at a high frequency comparable to the reciprocal of the decay time. When a fluorescent sample is excited in this way the emission is forced to respond at the same modulation frequency.

Since the excited state has a finite lifetime, the emission is delayed relative to the excitation. The delay is measured as a *phase shift*, ϕ, which is used to calculate the decay time.

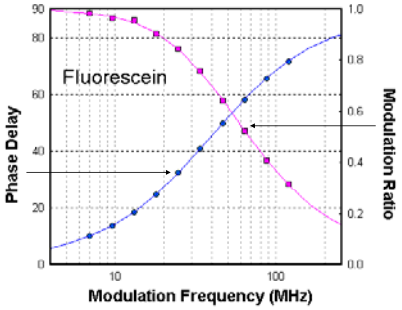
The peak-to-peak height of the emission is also decreased relative to that of the modulated excitation beam. This phenomenon, called *demodulation*, *m*, can also be used to calculate the decay time.

$$\tan\phi = \omega\tau; \quad \tau = \omega^{-1}\tan\phi;$$

ω is the angular modulation frequency

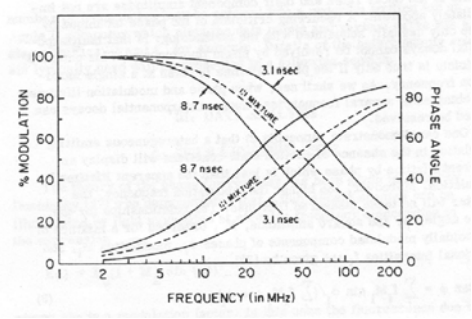
$$m = \frac{B/A}{b/a} = \frac{1}{\sqrt{1+\omega^2\tau^2}}; \quad \tau = \frac{1}{\omega}\left[\frac{1}{m^2}-1\right]^{1/2}$$

Multifrequency phase and modulation data are usually presented as shown below:



The plot shows the frequency response curve (phase and modulation) of Fluorescein in phosphate buffer pH 7.4. using a 470 nm LED. The emission was collected through a 530 high pass filter.
The data is best fitted by a single exponential decay time of 4 ns.

A case of multi-exponential decays is shown here for a system of two lifetime species of 8.7ns and 3.1ns and a 1 to 1 mixture (in terms of fractional intensities)



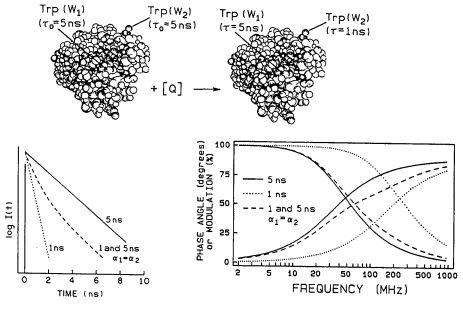
Multifrequency phase and modulation data is usually analyzed using a non-linear least squares method in which the actual phase and modulation ratio data (<u>not</u> the lifetime values) are fit to different models such as single or double exponential decays.

The quality of the fit is then judged by the *chi-square value* (χ^2) which is given by:

$$\chi^2 = \{[(Pc - Pm)/\sigma^P] + (Mc - Mm)/\sigma^M]\}/(2n - f - 1)$$

where P and M refer to phase and modulation data, respectively, c and m refer to calculated and measured values and σ^P and σ^M refer to the standard deviations of each phase and modulation measurement, respectively. n is the number of modulation frequencies and f is the number of free parameters.

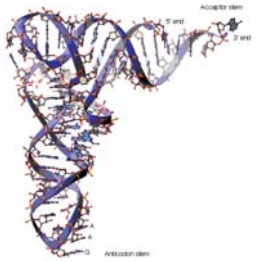
Consider a protein that contains two Trp residues – one buried, the other accessible. Assume both have 5 nsec lifetimes. Although intrinsically indistinguishable, they could be resolved in the presence of a quencher, which would shorten the excited-state lifetime of the accessible chromophore.
Simulated time- and frequency-domain data for this system are shown below:



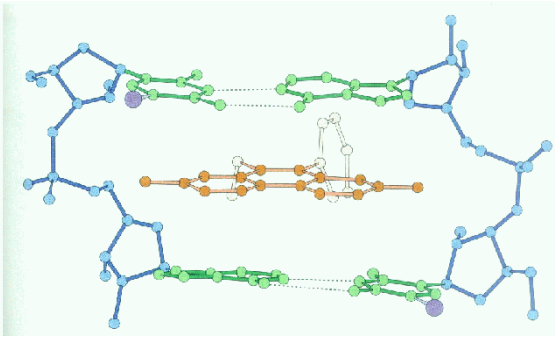
Binding of Ethidium-Bromide to Transfer RNA

Ethidium Bromide

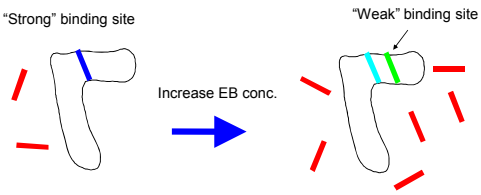
Yeast phenylalanine tRNA



Ethidium bromide can intercalate into nucleic acid structures It binds well to both DNA and RNA



Fluorescence investigations of EB - tRNA interactions, carried out for more than 30 years, have indicated a "strong" binding site and one or more "weak, non-specific" binding sites.

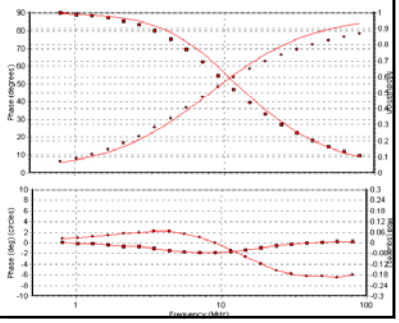


What are the lifetimes of the strong and the weak binding sites?

If the tRNA is in excess only one EB will bind to the "strong" binding site which has a K_d of around 1 micromolar (under these conditions a single exponential decay of 27ns is observed). If the EB/tRNA ratio is increased, one or more additional EB's will bind and the question is: What are the lifetimes of EB bound to different sites on tRNA?" Show below are phase and modulation data for a solution containing 124 μM yeast $tRNA^{phe}$ and 480 μM EB

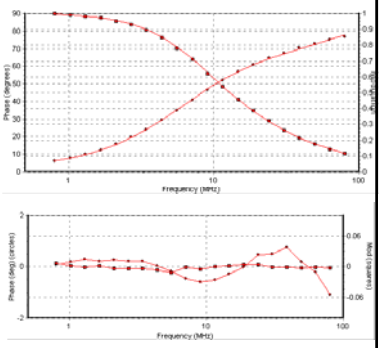
The phase and modulation data were first fit to a single exponential component shown as the solid lines in the top plot. The residuals for this fit are shown in the bottom plot.

In this case τ = 18.49 ns and the χ^2 value was 250.



The data were then fit to a 2-component model shown here In this case the two lifetime components were 22.71 ns with a fractional intensity of 0.911 and 3.99 ns with a fractional intensity of 0.089.

The χ^2 for this fit was 3.06 (note the change in scale for the residual plot compared to the first case shown).



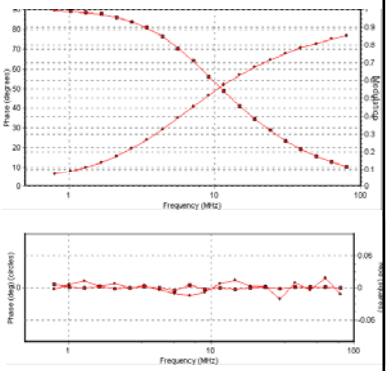
A 3-component model improves the fit still more. In this case

τ_1 = 24.25 ns, f_1 = 0.83

τ_2 = 8.79 ns, f_2 = 0.14

τ_3 = 2.09 ns, f_3 = 0.03

χ^2 = 0.39.

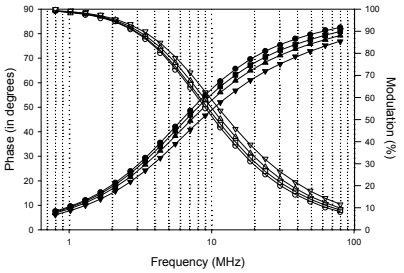


Adding a fourth component – with all parameters free to vary - does not lead to a significant improvement in the χ^2. In this case one finds 4 components of 24.80 ns (0.776), 12.13ns (0.163), 4.17 ns (0.53) and 0.88 ns (0.008).

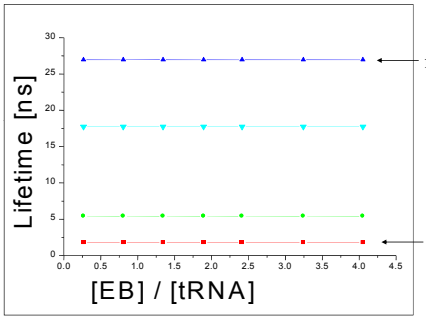
But we are not using all of our information! We can actually fix some of the components in this case. We know that **free EB** has a lifetime of **1.84 ns** and we also know that the lifetime of **EB bound to the "strong" tRNA binding site** is **27 ns**. So we can fix these in the analysis. The results are four lifetime components of 27 ns (0.612), 18.33 ns (0.311), 5.85 ns (0.061) and 1.84 ns (0.016). The χ^2 improves to 0.16.

We can then go one step better and carry out **"Global Analysis"**.
In Global Analysis, multiple data sets are analyzed simultaneously and different parameters (such as lifetimes) can be "linked" across the data sets. The important concept in this particular experiment is that the lifetimes of the components stay the same and only their fractional contributions change as more ethidioum bromide binds.

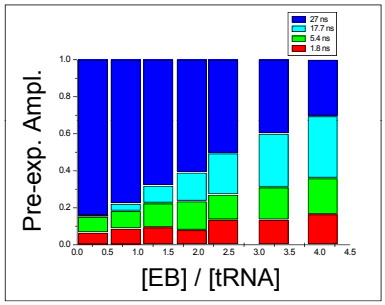
In this system, 8 data sets, with increasing EB/tRNA ratios, were analyzed. Some of the data are shown below for EB/tRNA ratios of 0.27 (circles), 1.34 (squares), 2.41 (triangles) and 4.05 (inverted triangles).



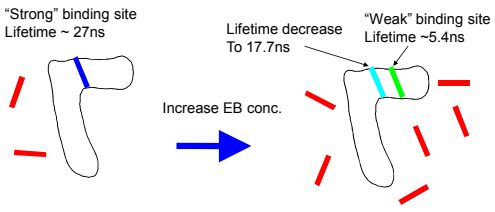
Global Analysis on seven data sets fit best to the 4 component model with two fixed components of 27ns and 1.84ns and two other components of 17.7ns and 5.4ns.



As shown in the plot below, as the EB/tRNA ratio increases the fractional contribution of the 27ns component decreases while the fractional contributions of the 17.7ns and 5.4ns components increase.



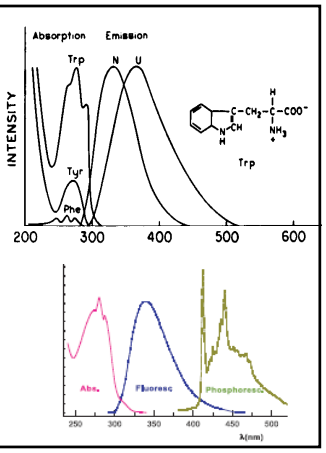
The Model



Radiative emission can also occur from the excited triplet state, in which case it is called "phosphorescence."

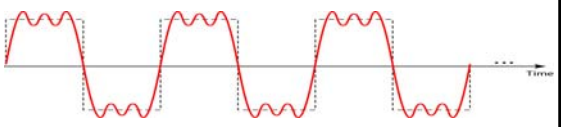
Phosphorescence is characterized by a very long lifetime (seconds or longer), because emission must be accompanied by the unlikely conversion of the triplet state back to a singlet.

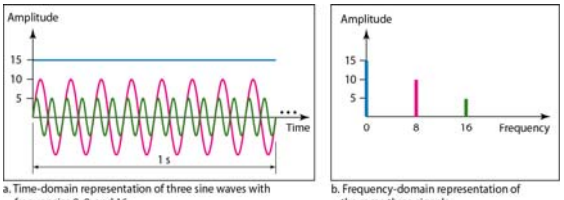
It is difficult to observe in solution at room temperature because of internal conversion and "quenching."



Fourier Series

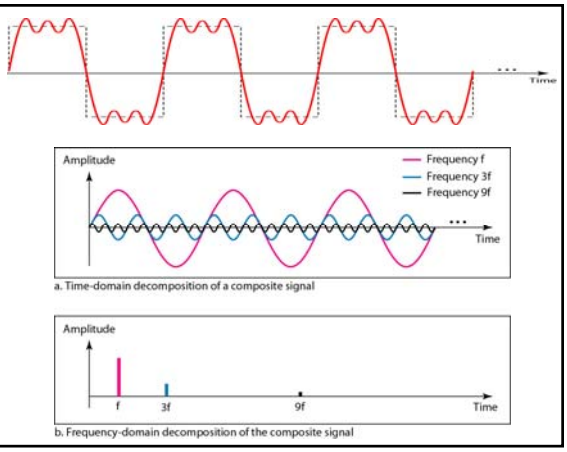
- Every composite periodic signal can be represented with a series of sine and cosine functions.
- The functions are integral harmonics of the fundamental frequency “f” of the composite signal.
- Using the series we can decompose any periodic signal into its harmonics.





a. Time-domain representation of three sine waves with frequencies 0, 8, and 16

b. Frequency-domain representation of the same three signals

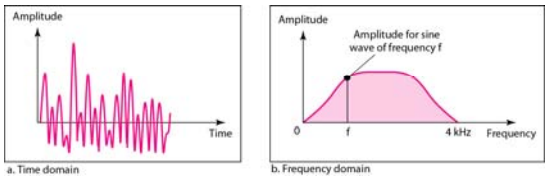


a. Time-domain decomposition of a composite signal

b. Frequency-domain decomposition of the composite signal

Fourier Transform

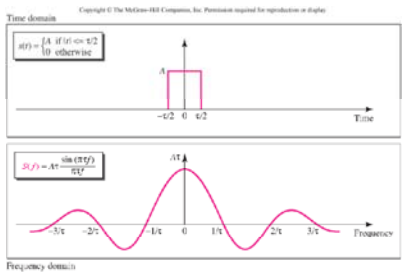
- Fourier Transform gives the frequency domain of a nonperiodic time domain signal.



a. Time domain

b. Frequency domain

Example of a Fourier Transform



Inverse Fourier Transform

$$S(f) = \int_{-\infty}^{\infty} s(t) e^{-j2\pi ft}\, dt$$

Fourier transform

$$s(t) = \int_{-\infty}^{\infty} S(f) e^{j2\pi ft}\, dt$$

Inverse Fourier transform

Fluorophore	*Absorption λ_{max} (nm)*	*Fluorescence λ_{max} (nm)*
Fluorescein	495	515
Tetramethylrhodamine	525	575

A protein uses ATP to convert CO to CO_2. A pair of fluorophores (fluorescein and tetramethylrhodamine) has been chemically attached to the ATP reaction sites. Three labeled protein samples are prepared:
Sample I with fluorescein attached to the ATP binding site
Sample II with tetramethylrhodamine attached to the CO/CO2 reaction site
Sample III with both fluorophores attached to both the ATP and reaction sites, respectively.
Their quantum yields are measured as follows: Φ_I **= 0.38**, Φ_{II} **= 0.33**, and Φ_{III} **= 0.22**. ***R_0 for the fluorescein –tetramethylrhodamine pair is 55 Å.***

a) Of this pair, which is the donor and which is the acceptor? Why?
b) In **Sample III**, which wavelength is used to excite the fluorophore for a FRET experiment, and which wavelength is used to monitor the fluorescence?
c) What is the energy transfer efficiency (*Eff*)?
d) What is the spatial separation (in Å) between the binding and reaction sites?