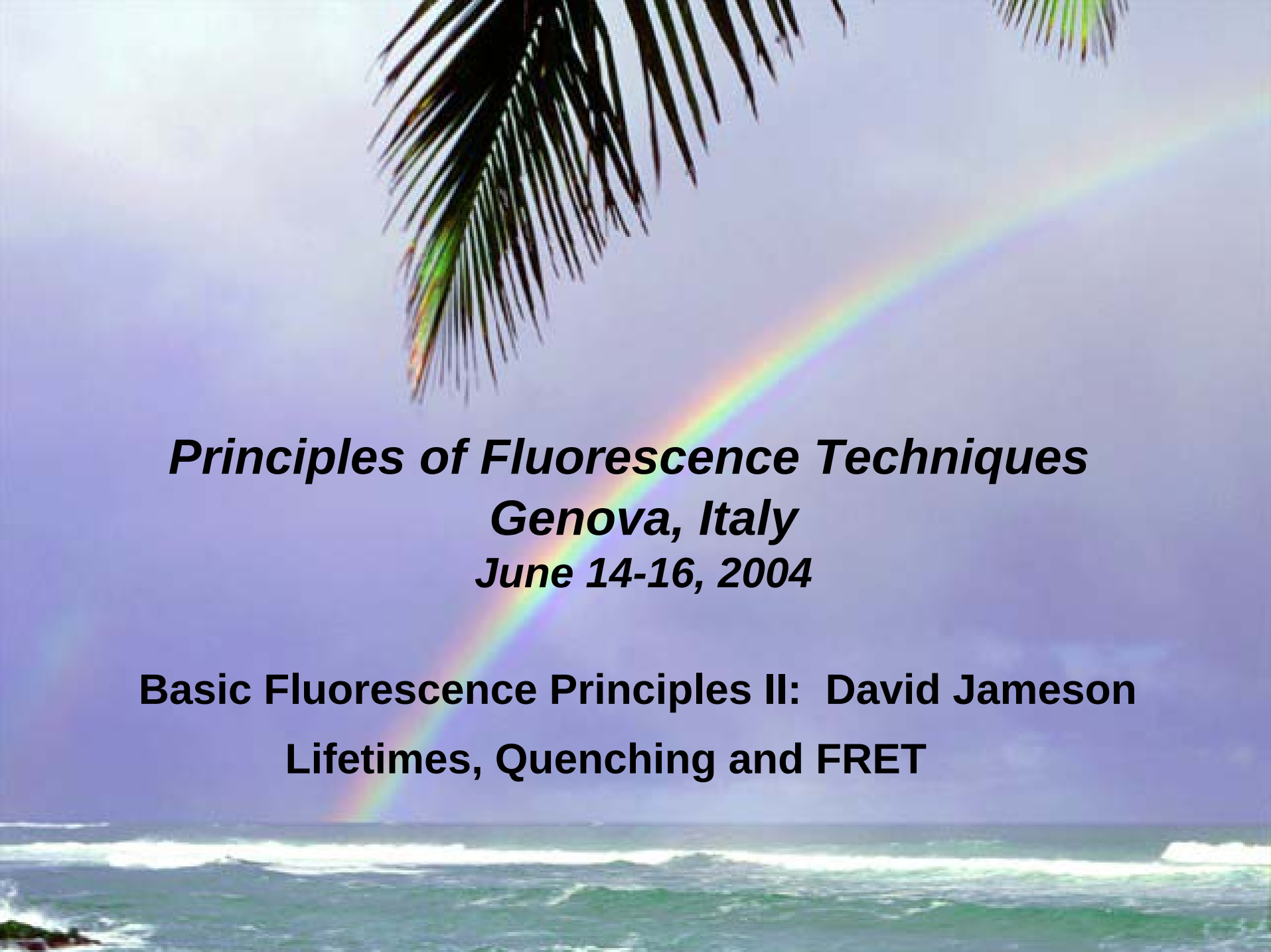


Principles of Fluorescence Techniques
Genova, Italy
June 14-16, 2004

Basic Fluorescence Principles II: David Jameson

Lifetimes, Quenching and FRET

What is meant by the “lifetime” of a fluorophore???

Although we often speak of the properties of fluorophores as if they are studied in isolation, such is not usually the case.

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

In general, the behavior of an excited population of fluorophores is described by a familiar 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 last equation, takes the form:

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

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

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

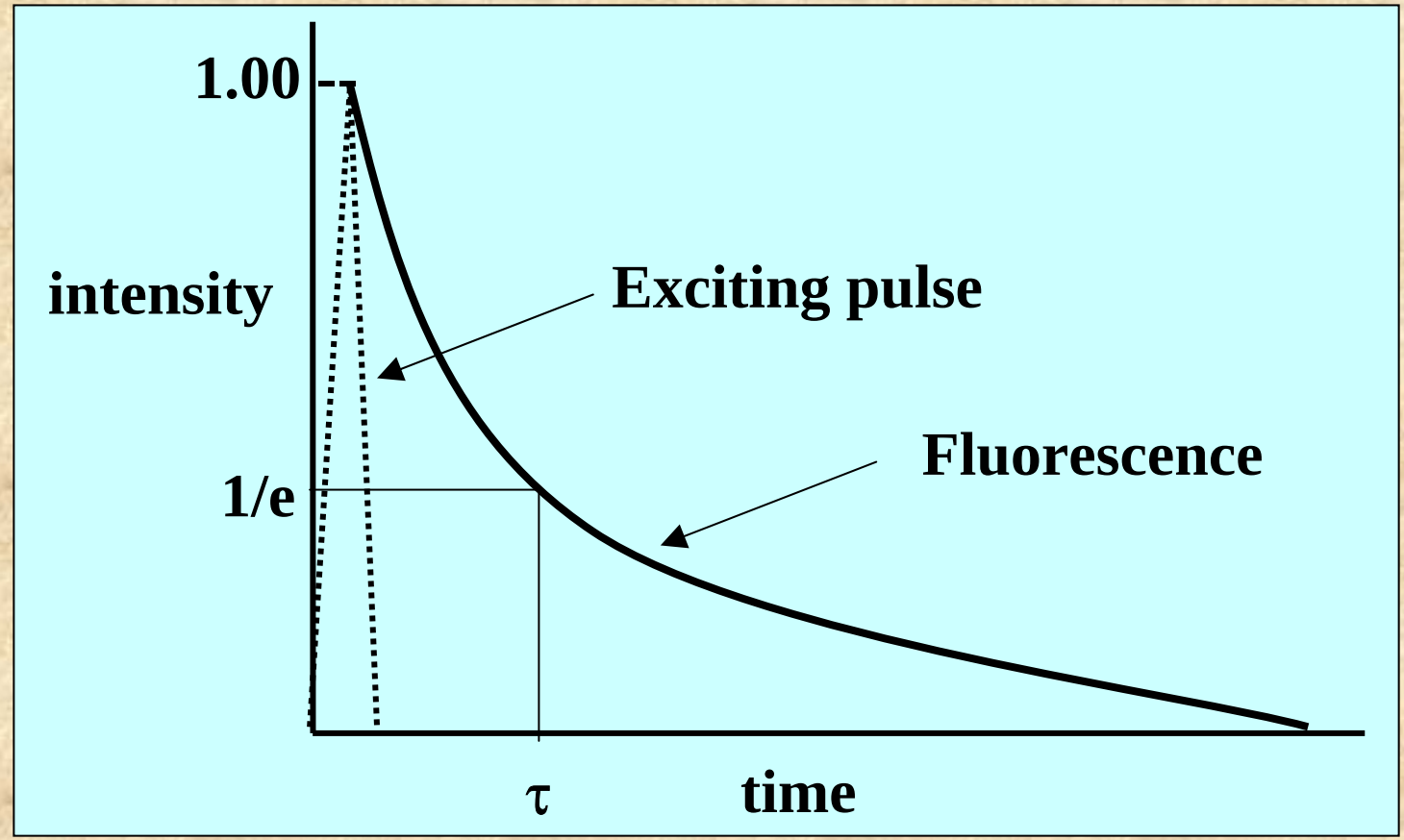
The lifetime, τ, is equal to Γ^{-1}

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:

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



Knowledge of a fluorophore's excited state lifetime is crucial for quantitative interpretations of numerous fluorescence measurements such as quenching, polarization and FRET.

In most cases of interest, it is virtually impossible to predict *a priori* the excited state lifetime of a fluorescent molecule. The true molecular lifetime, i.e., the lifetime one expects in the absence of any excited state deactivation processes – can be approximated by the Strickler-Berg equation (1962, J. Chem. Phys. 37:814).

$$\tau_m^{-1} = 2.88 \times 10^{-9} n^2 \left\langle \nu_f^{-3} \right\rangle \int_{\Delta\bar{\nu}_a} \varepsilon(\bar{\nu}) d\ln\bar{\nu}$$

where

$$\left\langle \overline{\nu_f^{-3}} \right\rangle = \frac{\int_{\Delta\bar{\nu}_e} F(\bar{\nu}) d\nu}{\int_{\Delta\bar{\nu}_a} F(\bar{\nu}) \overline{\nu^{-3}} d\nu}$$

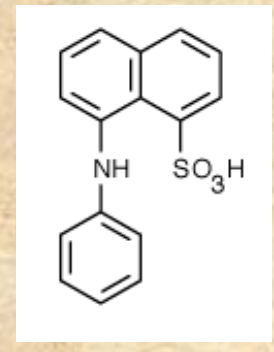
τ_m is the molecular lifetime, n is the refractive index of the solvent, $\Delta\nu_e$ and $\Delta\nu_a$ correspond to the experimental limits of the absorption and emission bands (S_0 - S_1 transitions), ε is the molar absorption and $F(\nu)$ describes the spectral distribution of the emission in photons per wavelength interval.

How well do these equations actually work?

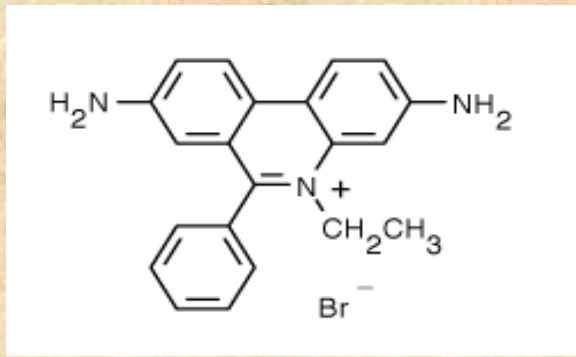
Not very well – usually off by factors of 2 – 5 fold.

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

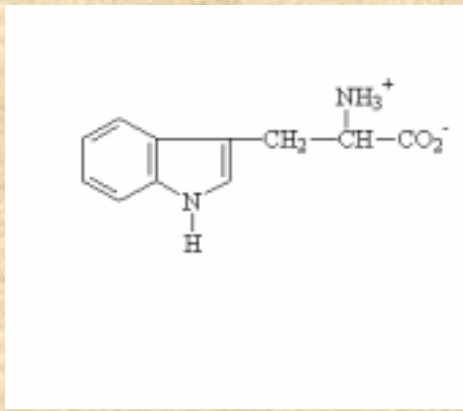
Examples of this fact would be NADH, which in water has a lifetime of ~0.4 ns but bound to dehydrogenases can be a long as 9 ns.



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



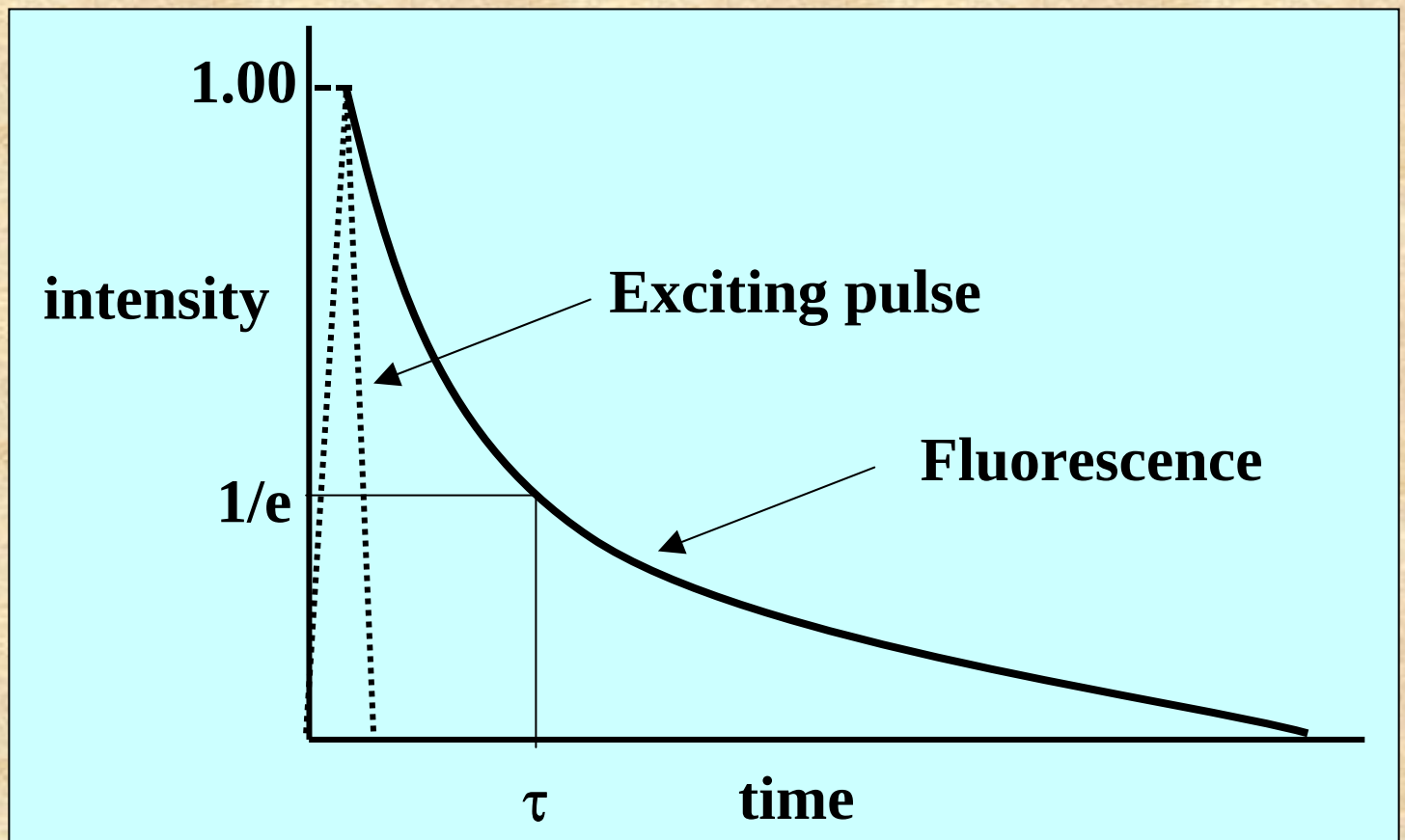
Ethidium bromide is 1.8 ns in water, 22 ns bound to DNA and 27ns bound to tRNA



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.

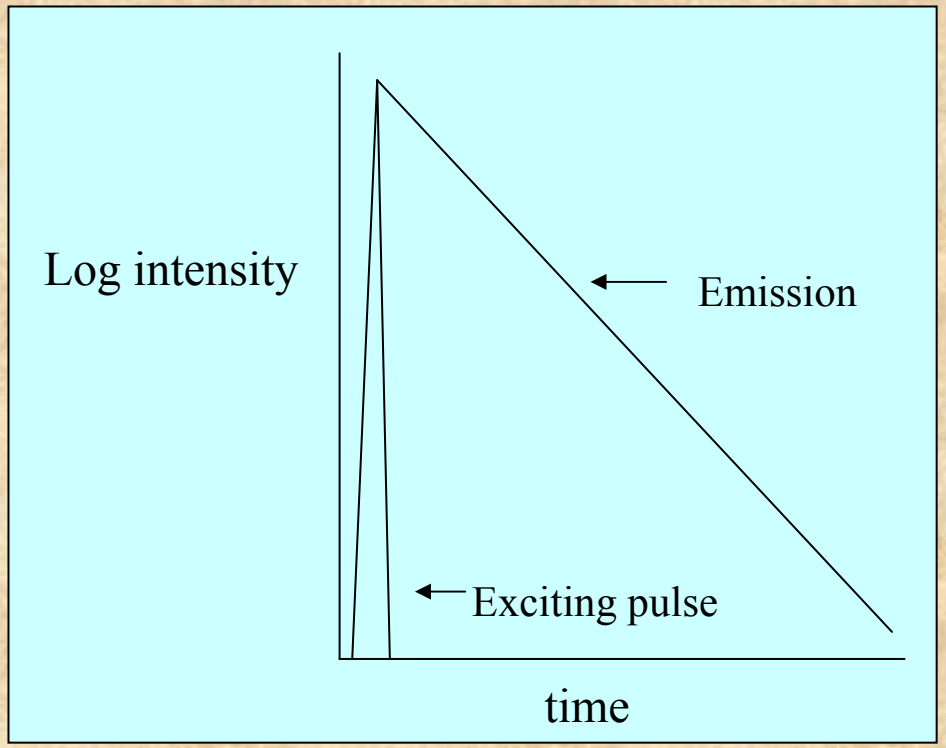


As shown in the intensity decay figure, the *fluorescence* lifetime, t , is the time at which the intensity has decayed to 1/e of the original value. The decay of the intensity with time is given by the relation:

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

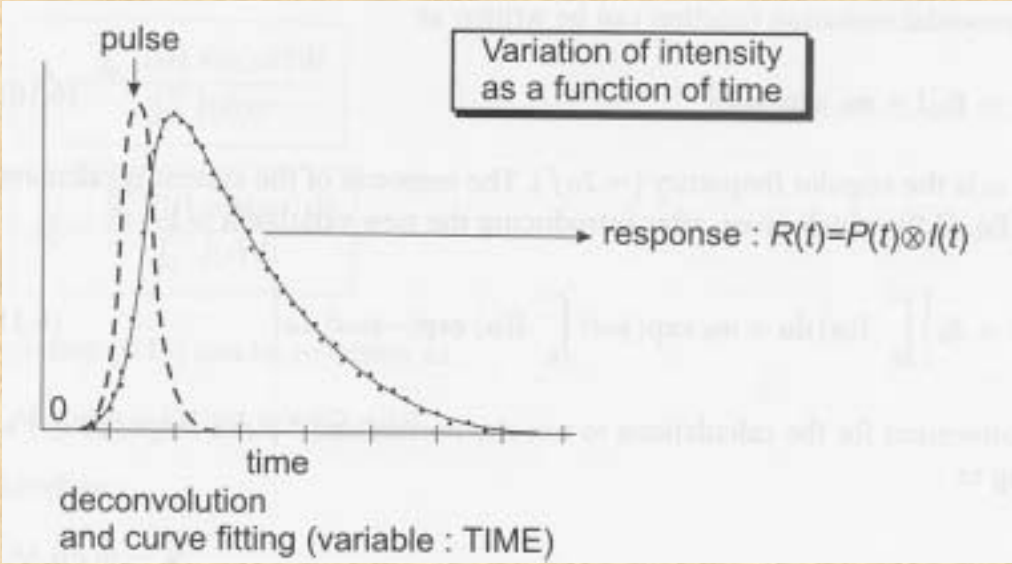
Where I_t is the intensity at time t, α is a normalization term (the pre-exponential factor) and τ is the lifetime.

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



If the decay is a single exponential and if the lifetime is long compared to the exciting light then the lifetime can be determined directly from the slope of the curve.

If the lifetime and the excitation pulse width are comparable some type of *deconvolution* method must be used to extract the lifetime.



Great effort has been expended on developing mathematical methods to "deconvolve" the effect of the exciting pulse shape on the observed fluorescence decay.

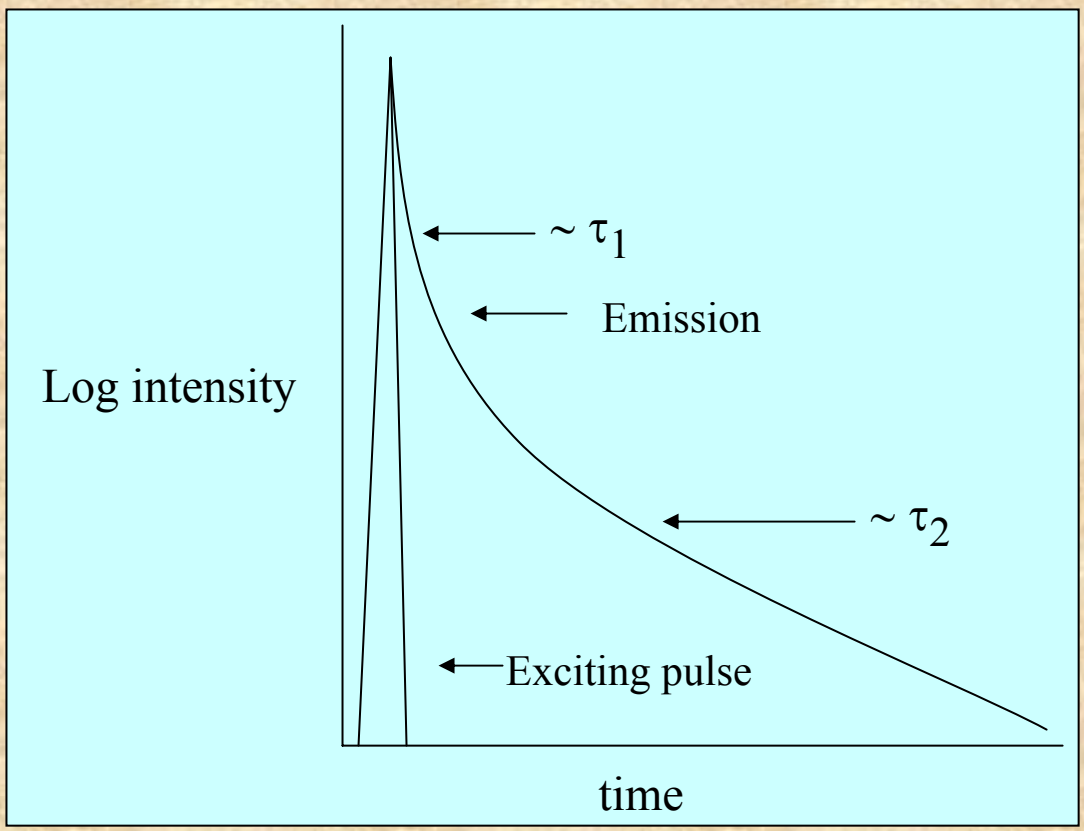
With the advent of very fast laser pulses these deconvolution procedures became less important for most lifetime determinations, although they are still required whenever the lifetime is of comparable duration to the light pulse.

If the decay is multiexponential, the relation between the intensity and time after excitation is given by:

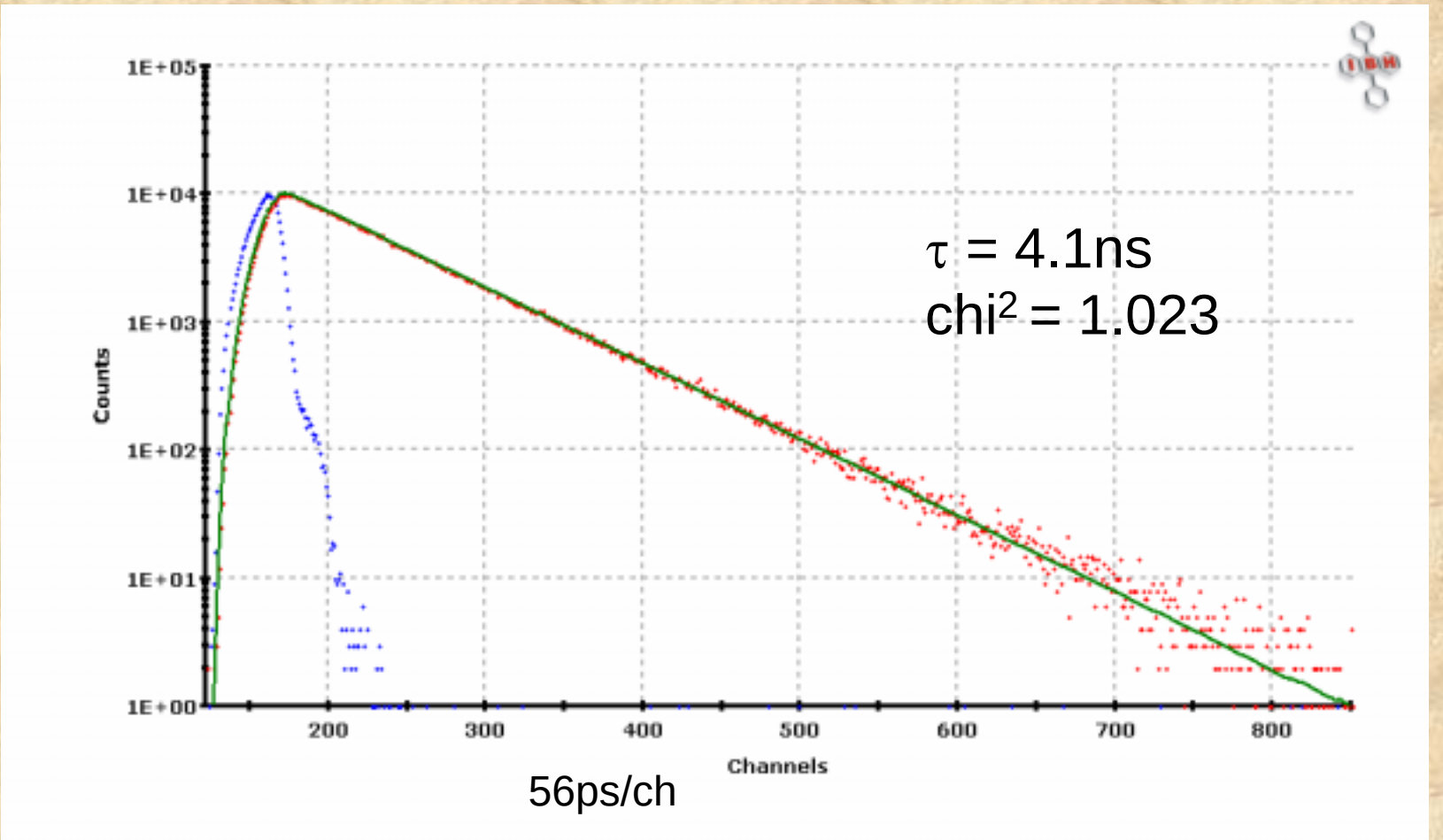
$$I(t) = \sum_i \alpha_i e^{-t/\tau_i}$$

One may then observe data such as those sketched below:

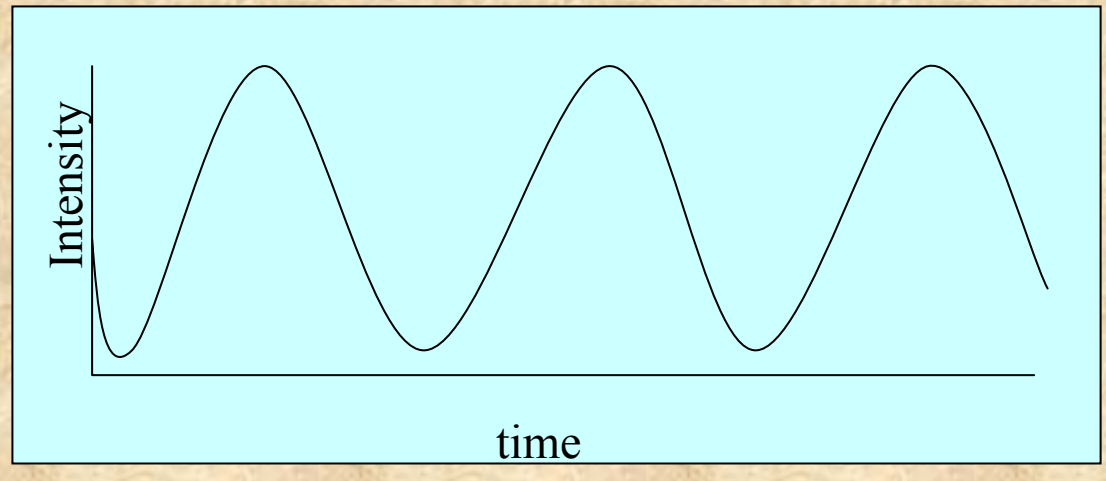
Here we can discern at least two lifetime components indicated as τ_1 and τ_2 . This presentation is oversimplified but illustrates the point.



Here are pulse decay data on anthracene in cyclohexane taken on an IBH 5000U Time-correlated single photon counting instrument equipped with an LED short pulse diode excitation source.



In the harmonic method (also known as the phase and modulation or frequency domain method) a continuous light source is utilized, such as a laser or xenon arc, and the intensity of this light source is modulated sinusoidally at high frequency as depicted below. Typically, an *electro-optic* device, such as a *Pockels cell* is used to modulate a continuous light source, such as a CW laser or a xenon arc lamp. Alternatively, LEDs or laser diodes can be directly modulated.



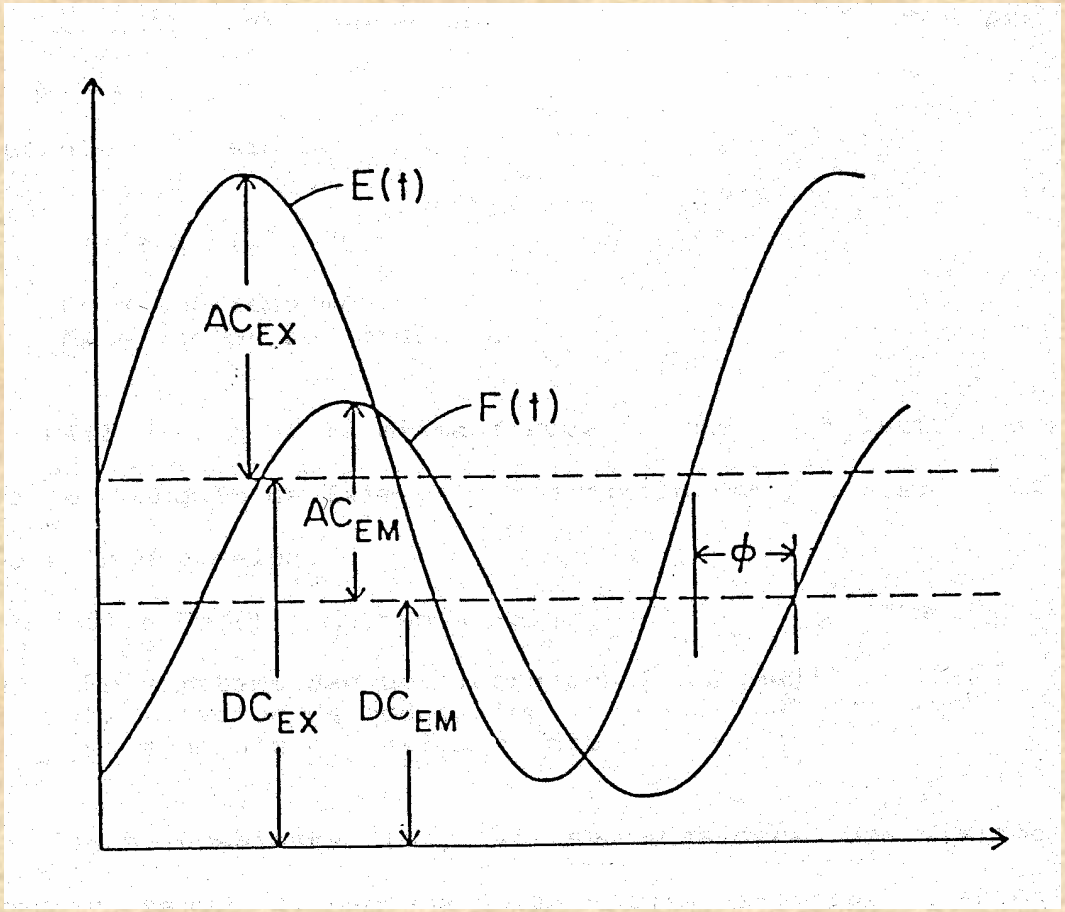
In such a case, the excitation frequency is described by:

$$E(t) = Eo\,[1 + M_E \sin \omega t]$$

E(t) and Eo are the intensities at time t and o, M_E is the modulation factor which is related to the ratio of the AC and DC parts of the signal and ω is the angular modulation frequency.

$\omega = 2\pi f$ where f is the linear modulation frequency

Due to the persistence of the excited state, fluorophores subjected to such an excitation will give rise to a modulated emission which is shifted in phase relative to the exciting light as depicted below.



This sketch illustrates the 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.

One can demonstrate that:

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

This relationship signifies that measurement of the phase delay, ϕ, forms the basis of one measurement of the lifetime, τ. In particular one can demonstrate that:

$$\mathbf{\tan \phi = \omega\tau}$$

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.,

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

To get a feeling for typical phase and modulation data, consider the following data set.

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

These differences between τ_P and τ_M and their frequency dependence form the basis of the methods used to analyze for lifetime heterogeneity, i.e., the component lifetimes and amplitudes.

In the case just shown, the actual system being measured was a mixture of two fluorophores with lifetimes of 12.08 ns and 1.38 ns, with relative contributions to the total intensity of 53% and 47% respectively.

Here must must be careful to distinguish the term *fractional contribution to the total intensity* (usually designated as f) from α, the pre-exponential term referred to earlier. The relation between these two terms is given by:

$$f_i = \frac{\alpha_i \tau_i}{\sum_j \alpha_j \tau_j}$$

where j represents the sum of all components. In the case just given then, the ratio of the pre-exponential factors corresponding to the 12.08 ns and 1.38 ns components is approximately 1/3. In other words, there are three times as many molecules in solution with the 1.38 ns lifetime as there are molecules with the 12.08 ns lifetime.

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

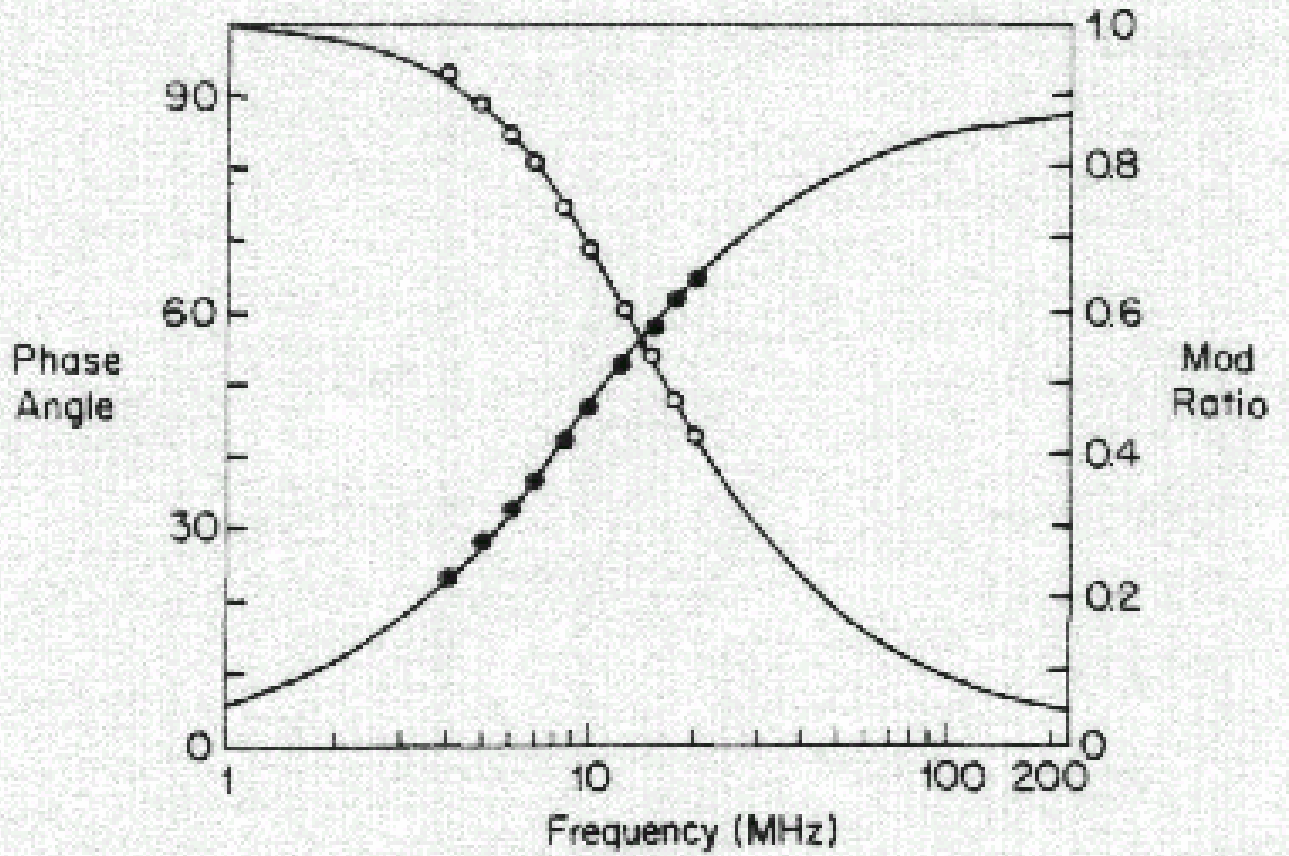
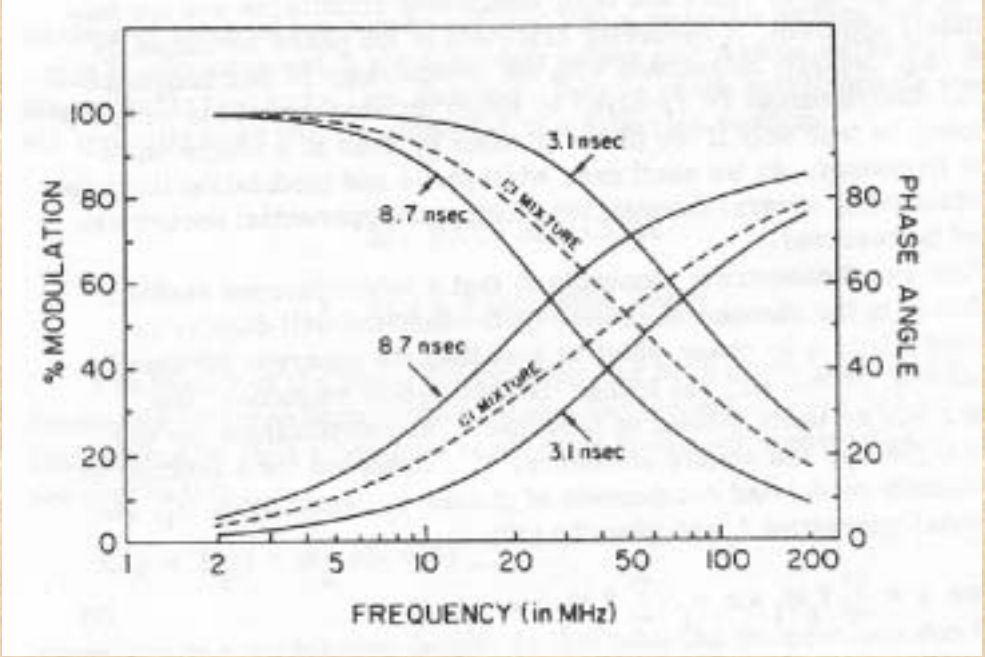


Fig. 2. Multifrequency phase (●) and modulation (○) lifetime data for HRP(desFe) in 0.1 M phosphate buffer (pH 7.4). Solid curves correspond to a single exponential decay of 16.87 ns. Excitation wavelength was 514.5 nm; emission was observed through a Schott OG 570 cuton filter which passes $\lambda > 560$ nm.

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 intenisties)



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

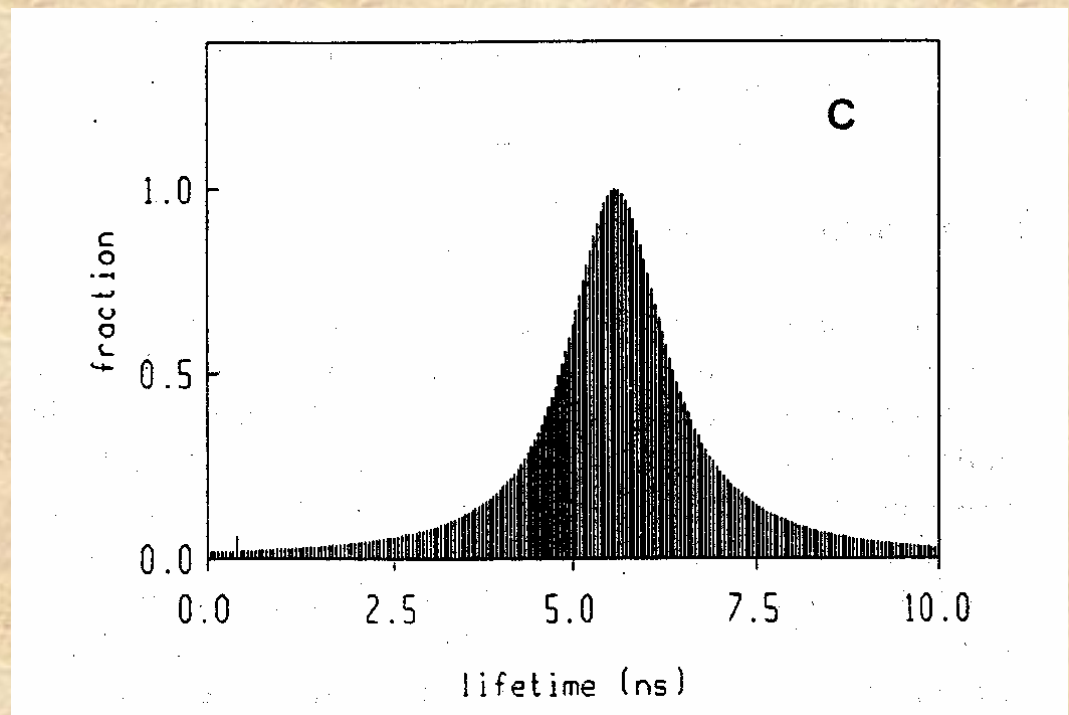
The quality of the fit is then judged by the *chi-square value* (χ^2) which is given by:

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

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.

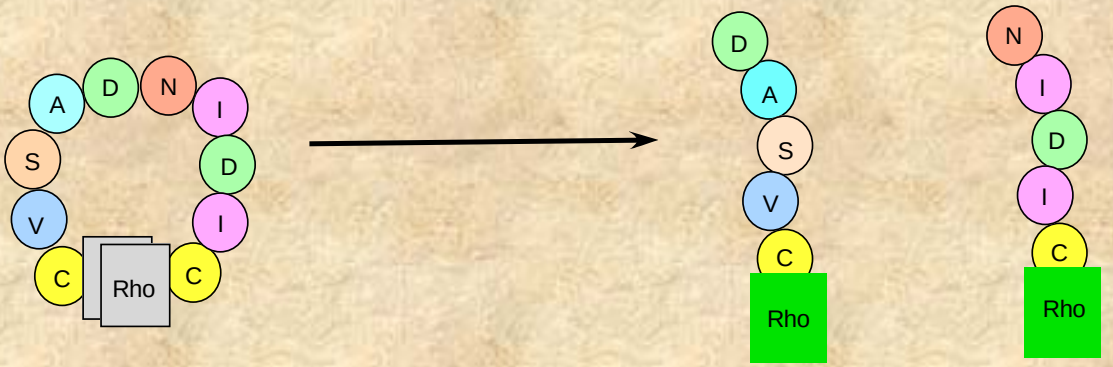
In addition to decay analysis using discrete exponential decay models, one may also choose to fit the data to *distribution* models. In this case, it is assumed that the excited state decay characteristics of the emitting species actually results in a large number of lifetime components. Shown below is a typical lifetime distribution plot for the case of single tryptophan containing protein – human serum albumin.

The distribution shown here is Lorentzian but depending on the system different types of distributions, e.g., Gaussian or asymmetric distributions, may be utilized. This approach to lifetime analysis is described in: Alcala, J. R., E. Gratton and F. G. Prendergast. Fluorescence lifetime distributions in proteins. *Biophys. J. 51*, 597-604 (1987).



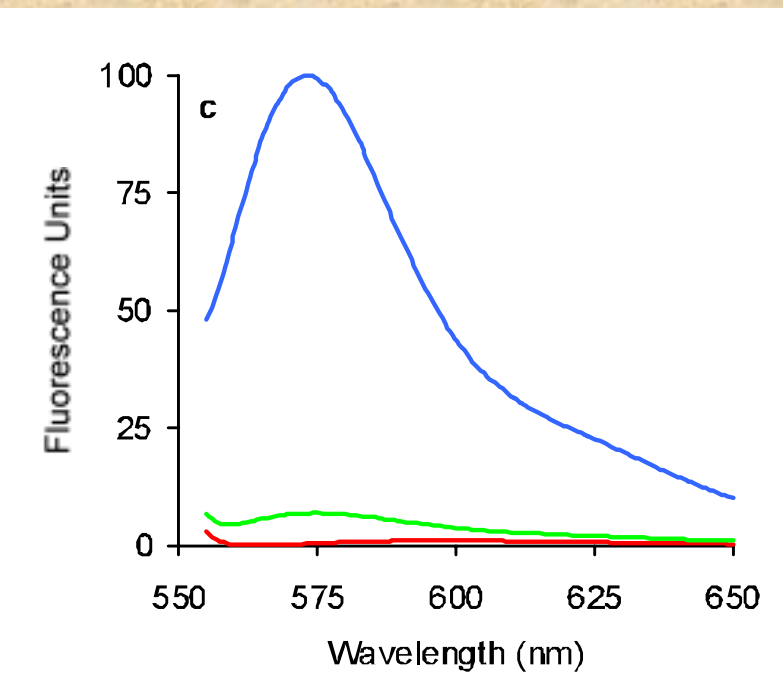
Another popular lifetime analysis method is the *Maximum Entropy Method* (MEM). In this method no *a priori* intensity decay model is assumed.

An example of the use of lifetime data is given by a study of a rhodamine labeled peptide which can be cleaved by a protease (from Blackman et al. (2002) Biochemistry *41*:12244)



Weak fluorescence

Strong fluorescence



In the intact peptide the rhodamine molecules form a ground-state dimer with a low quantum yield (green curve). Upon cleavage and of the peptide the rhodamine dimer breaks apart and the fluorescence is greatly enhanced (blue curve).

Lifetime data allow us to better understand the photophysics of this system

Lifetime data for two rhodamine isomers (5' and 6') linked to the peptide

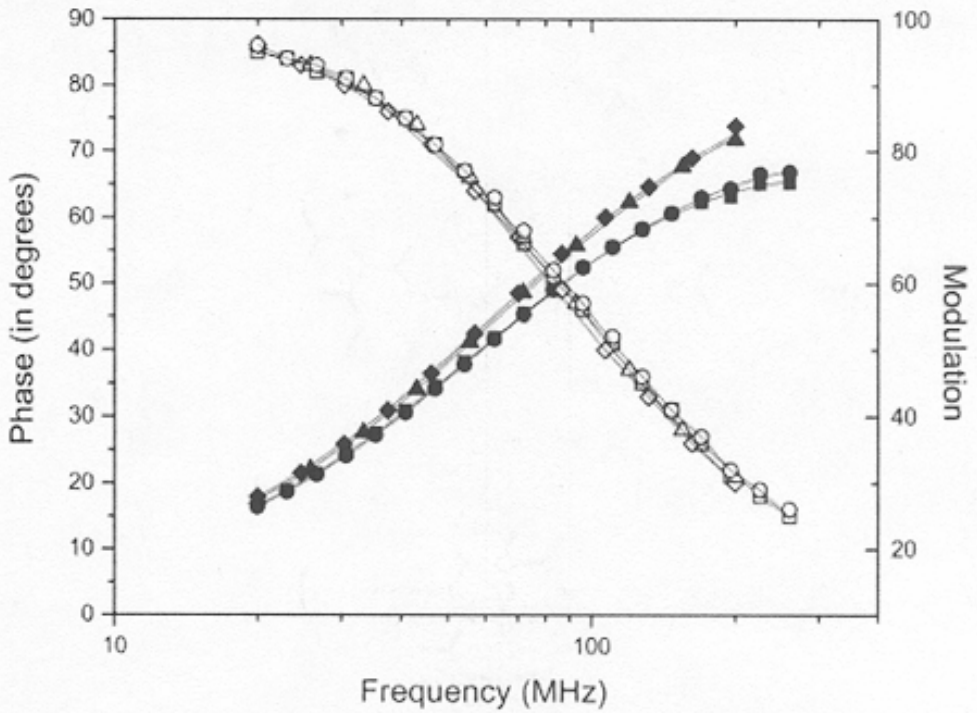


FIGURE 6: Typical phase (filled symbols) and modulation (open symbols) data for pepF1-5R before (circles) and after (triangles) Pronase treatment and for pepF1-6R before (squares) and after (diamonds) Pronase treatment.

Table 3: Fluorescence Lifetime Parameters of Intact and Cleaved Labeled Peptides[a]

sample	τ_1 (ns)	f_1	α_1	τ_2 (ns)	f_2	α_2
pepF1-5R	2.44	0.95	0.52	0.14	0.05	0.48
pepF1-5R + Pronase	2.43	1.00				
pepF1-6R	2.50	0.95	0.37	0.076	0.05	0.63
pepF1-6R + Pronase	2.50	1.00				

As the lifetime data indicate, before protease treatment the rhodamine lifetime was biexponential with 95% of the intensity due to a long component and 5% due to a short component. Assuming that the quantum yields and lifetimes are linked, however, one can calculate that molar ratios of the long and short components are nearly equal. Hence one can argue that the intact peptide exists in an equilibrium between open (unquenched) and closed (quenched) forms.

Quenching

A number of processes can lead to a reduction in fluorescence intensity, i.e., quenching

These processes can occur during the excited state lifetime – for example collisional quenching, energy transfer, charge transfer reactions or photochemistry – or they may occur due to formation of complexes in the ground state

We shall focus our attention on the two quenching processes usually encountered – namely collisional (dynamic) quenching and static (complex formation) quenching

Collisional Quenching

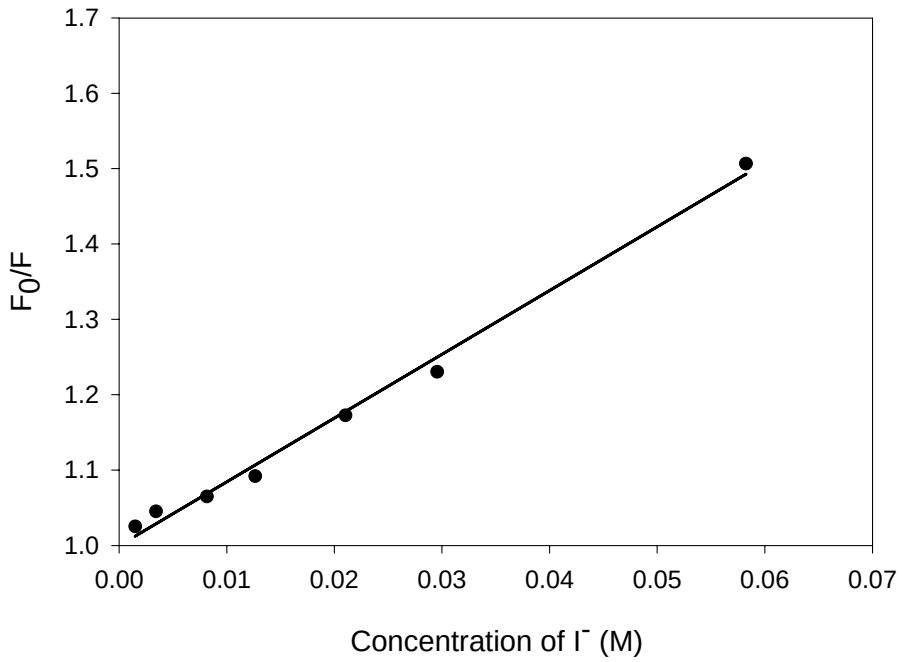
Collisional quenching occurs when the excited fluorophore experiences contact with an atom or molecule that can facilitate non-radiative transitions to the ground state. Common quenchers include O_2, I^-, Cs^+ and acrylamide.

In the simplest case of collisional quenching, the following relation, called the Stern-Volmer equation, holds:

$$F_0/F = 1 + K_{SV}[Q]$$

where F_0 and F are the fluorescence intensities observed in the absence and presence, respectively, of quencher, [Q] is the quencher concentration and K_{SV} is the Stern-Volmer quenching constant

In the simplest case, then, a plot of F_0/F versus [Q] should yield a straight line with a slope equal to K_{SV}. Such a plot, known as a Stern-Volmer plot, is shown below for the case of fluorescein quenched by iodide ion (I^-).



In this case, $K_{SV} \sim 8$ L-mol^{-1}

$K_{SV} = k_q \tau_0$ where k_q is the bimolecular quenching rate constant (proportional to the sum of the diffusion coefficients for fluorophore and quencher) and τ_0 is the excited state lifetime in the absence of quencher.

In the case of purely collisional quenching, also known as *dynamic* quenching,:

$$F_0/F = \tau_0/\tau.$$

Hence in this case: $\tau_0/\tau = 1 + k_q \tau[Q]$

In the fluorescein/iodide system, $\tau = 4$ns and $k_q \sim 2 \times 10^9$ M^{-1} sec^{-1}

Static Quenching

In some cases, the fluorophore can form a stable complex with another molecule. If this *ground-state* is non-fluorescent then we say that the fluorophore has been statically quenched.

In such a case, the dependence of the fluorescence as a function of the quencher concentration follows the relation:

$$F_0/F = 1 + K_a[Q]$$

where K_a is the association constant of the complex. Such cases of quenching via complex formation were first described by Gregorio Weber.

THE QUENCHING OF FLUORESCENCE IN LIQUIDS BY COMPLEX FORMATION. DETERMINATION OF THE MEAN LIFE OF THE COMPLEX.

By G. Weber.

Received 8th July, 1946, as revised 25th April, 1947.

If a quencher is added to a solution of a fluorescent dye the ratio of the fluorescent intensities before and after quenching is[1, 2]

$$I_0/I = N_0\tau_0/N\tau \quad . \quad . \quad . \quad . \quad (1)$$

where N_0 and N are the numbers of excited molecules that may be de-activated with emission in the absence and presence of quencher respectively, and τ_0 and τ the corresponding values of the mean life of the excited state. Only in an ideally collisional quenching is $N/N_0 = 1$. In the quenching by complex formation we may assume that the molecules forming part of the complex are unable to emit. Then $N/N_0 = \alpha$, the degree of dissociation of the complex. The new mean life of the excited state of the fluorescence after addition of quencher is

$$\tau = \frac{1}{\left(\frac{1}{\sigma} + \frac{1}{\tau_0}\right)} \quad . \quad . \quad . \quad . \quad . \quad (2)$$

where σ is the mean free life of the fluorescent molecules. In general, the degree of dissociation of the complex cannot be directly determined, but α can be eliminated from eqn. (1). Calling the mean life of the complex Σ, and the total concentration of dye [F], at a definite concentration of quencher an equilibrium exists of the form

$$1/\sigma \,.\, \alpha[F] = 1/\Sigma \,.\, (1 - \alpha)[F] \quad . \quad . \quad . \quad (3)$$

where the left-hand side represents the number of complexes formed in unit time and the right-hand side the number broken down in the same period. Replacing $1/\sigma$ by its value given in eqn. (2), solving for $1/\alpha$ and introducing this in (1), we have

$$I_0/I = \tau_0/\tau[1 + \Sigma/\tau_0(\tau_0/\tau - 1)] \quad . \quad . \quad . \quad (4)$$

It is evident that if $\Sigma \ll \tau_0$

$$I_0/I = \tau_0/\tau \quad . \quad . \quad . \quad . \quad . \quad (5)$$

a rule proposed by Perrin[1] for the quenching by collisions. Eqn. (4) shows that Σ/τ_0 can be determined if the ratios I_0/I and τ_0/τ are known. The first can be easily obtained by photometric measurements while the second can be determined from the polarisation of the fluorescent light. According to Perrin's theory of the polarisation of the fluorescence in liquids[3, 4]

$$\frac{\tau_0}{\tau} = \frac{\frac{1}{\bar{p}_i} - \frac{1}{\bar{p}_0}}{\frac{1}{\bar{p}} - \frac{1}{\bar{p}_0}} \quad . \quad . \quad . \quad . \quad . \quad (6)$$

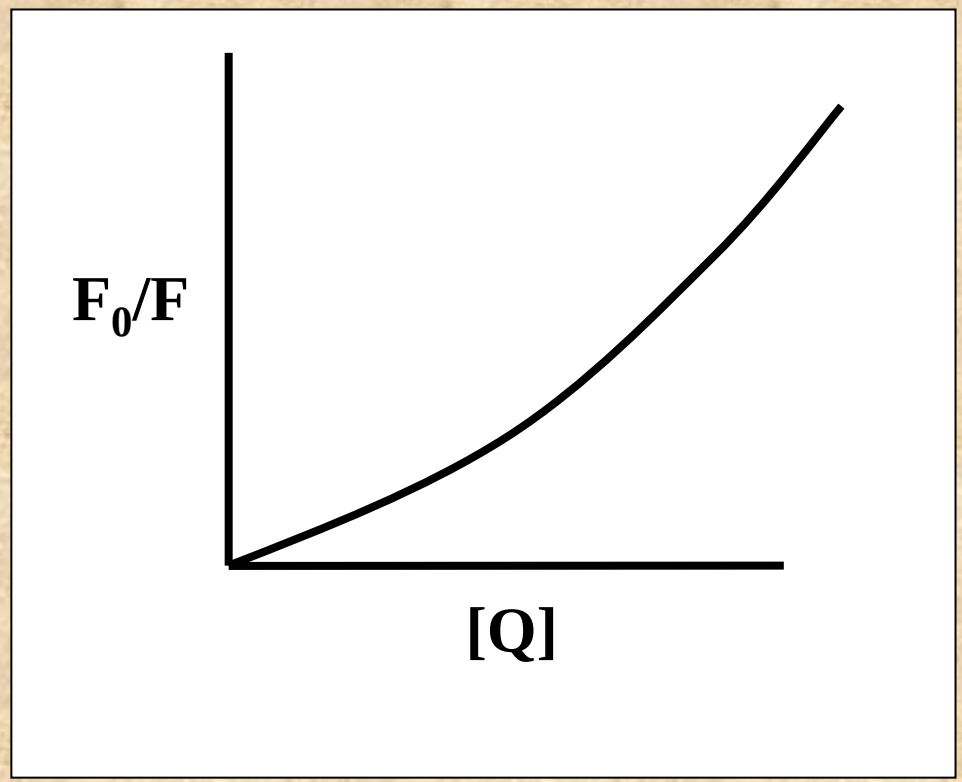
[1] Perrin, *Ann. Physique*, 1929, **12**, 169.
[2] Wawilov and Franck, *Z. Physik.*, 1931, **69**, 100.
[3] Perrin, *J. Physique*, 1926, **7**, 390.
[4] Sveshnikoff, *Acta Physicochim.*, 1936, **4**, 453.

In the case of static quenching the lifetime of the sample will not be reduced since those fluorophores which are not complexed – and hence are able to emit after excitation – will have normal excited state properties. The fluorescence of the sample is reduced since the quencher is essentially reducing the number of fluorophores which can emit.

If both static and dynamic quenching are occurring in the sample then the following relation holds:

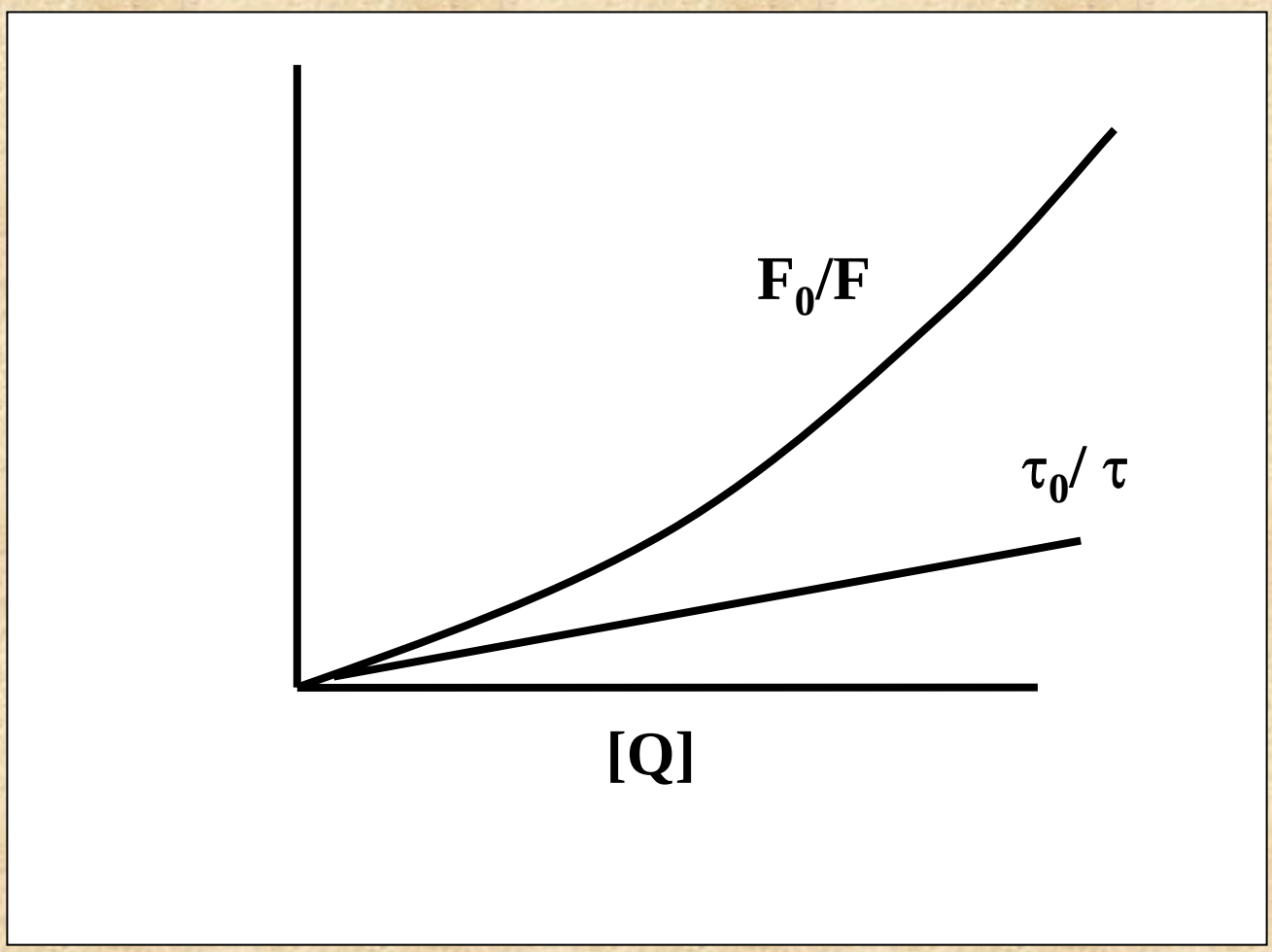
$$F_0/F = (1 + k_q \tau[Q])\,(1 + K_a[Q])$$

In such a case then a plot of F_0/F versus [Q] will give an upward curving plot



The upward curvature occurs because of the $[Q]^2$ term in the equation

However, since the lifetime is unaffected by the presence of quencher in cases of pure static quenching, a plot of τ_0/τ versus [Q] would give a straight line



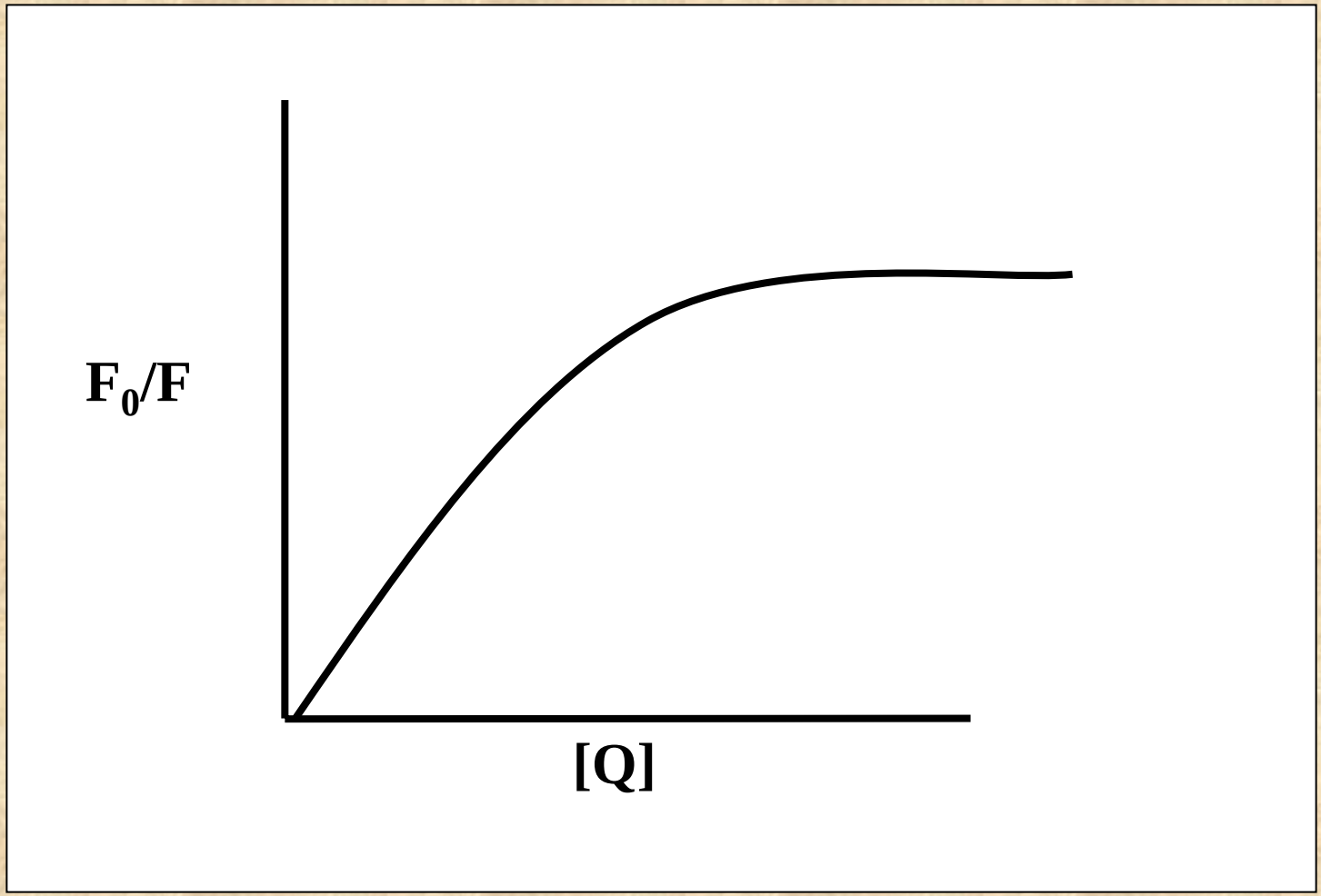
Sometimes you will see the equation for simultaneous static and dynamic quenching given as:

$$F_0/F = (1 + K_{SV}[Q])\mathbf{e}^{\mathbf{V[Q]}}$$

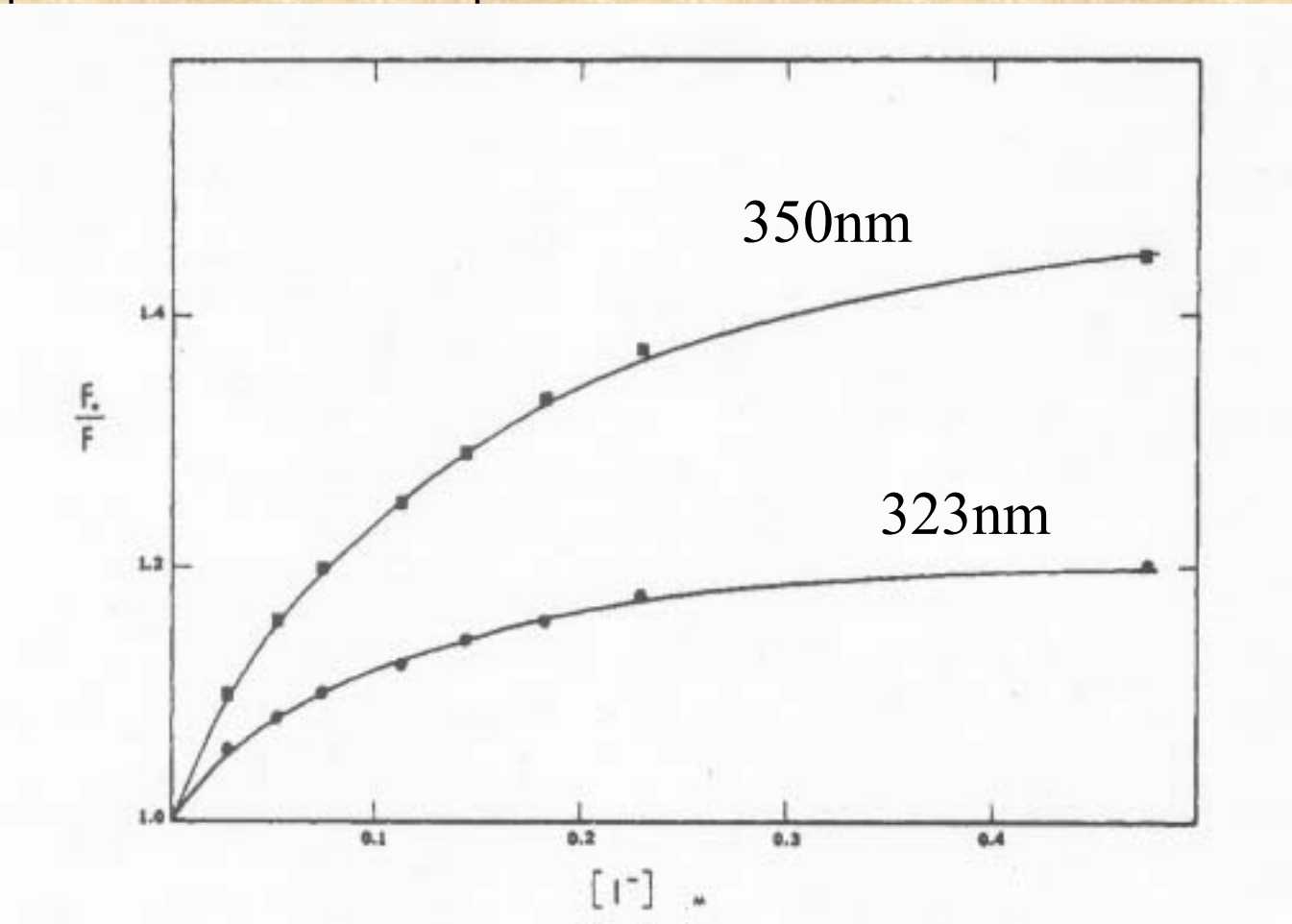
where the term $\mathbf{e}^{\mathbf{V[Q]}}$ is used as a phenomological descriptor of the quenching process. The term V in this equation represents an *active volume* element around the fluorophore such that any quencher within this volume at the time of fluorophore excitation is able to quench the excited fluorophore.

Non-linear Stern-Volmer plots can also occur in the case of purely collisional quenching if some of the fluorophores are less accessible than others. Consider the case of multiple tryptophan residues in a protein – one can easily imagine that some of these residues would be more accessible to quenchers in the solvent than other.

In the extreme case, a Stern-Volmer plot for a system having accessible and inaccessible fluorophores could look like this:



The quenching of LADH intrinsic protein fluorescence by iodide gives, in fact, just such a plot. LADH is a dimer with 2 tryptophan residues per identical monomer. One residue is buried in the protein interior and is relatively inaccessible to iodide while the other tryptophan residue is on the protein's surface and is more accessible.

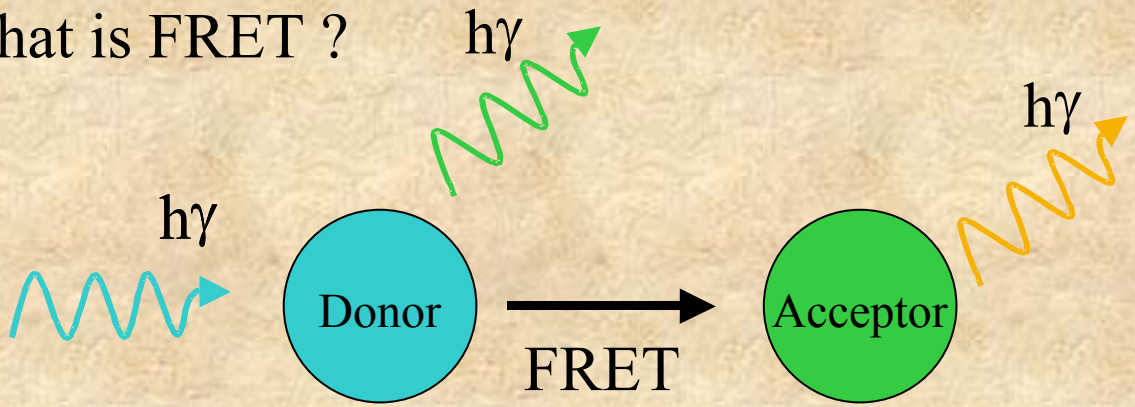


In this case (from Eftink and Selvidge, Biochemistry 1982, 21:117) the different emission wavelengths preferentially weigh the buried (323nm) or solvent exposed (350nm) trytptophan.

Fluorescence Resonance Energy Transfer (FRET)

(or **Förster Resonance Energy Transfer)**

What is FRET ?



When the donor molecule absorbs a photon, and there is an acceptor molecule close to the donor molecule, radiationless energy transfer can occur from the donor to the acceptor.

FRET results in a decrease of the fluorescence intensity and lifetime of the donor probe, It enhance the fluorescence of the acceptor probe when the acceptor is fluorescent.

PM

FRET - Fluorescence (Förster) Resonance Energy Transfer

Milestones in the Theory of Resonance Energy Transfer

1918 J. Perrin proposed the mechanism of resonance energy transfer

1922 G. Cario and J. Franck demonstrate that excitation of a mixture of mercury and thallium atomic vapors with 254nm (the mercury resonance line) also displayed thallium (sensitized) emission at 535nm.

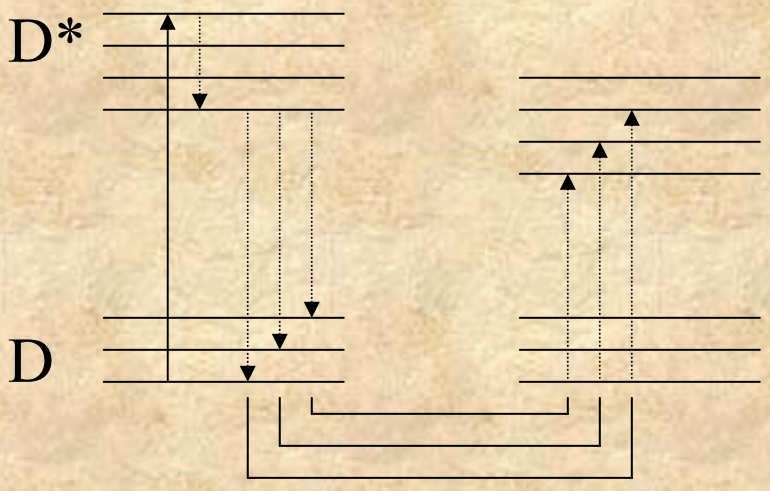
1924 E. Gaviola and P. Pringsham observed that an increase in the concentration of fluorescein in viscous solvent was accompanied by a progressive depolarization of the emission.

1928 H. Kallmann and F. London developed the quantum theory of resonance energy transfer between various atoms in the gas phase. The dipole-dipole interaction and the parameter R_0 are used for the first time

1932 F. Perrin published a quantum mechanical theory of energy transfer between molecules of the same specie in solution. Qualitative discussion of the effect of the spectral overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor

1946-1949 T. Förster develop the first quantitative theory of molecular resonance energy transfer

Simplified FRET Energy Diagram



Coupled transitions

Suppose that the energy difference for one of these possible deactivation processes in the donor molecule matches that for a possible absorption transition in a nearby acceptor molecule. Then, with sufficient energetic coupling between these molecules (overlap of the emission spectrum of the donor and absorption spectrum of the acceptor), both processes may occur simultaneously, resulting in a transfer of excitation from the donor to the acceptor molecule

The interaction energy is of a dipole-dipole nature and depends on the distance between the molecules as well as the relative orientation of the dipoles

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The rate of transfer (k_T) of excitation energy is given by:

$$k_T = (1/\tau_d)(R_0/R)^6$$

Where τ_d is the fluorescence lifetime of the donor in the absence of acceptor, R the distance between the centers of the donor and acceptor molecules and R_0 is defined by:

$$R_0 = 0.211(n^{-4} Q_d \kappa^2 J)^{1/6} \quad \text{Å}$$

Where n is the refractive index of the medium (usually between 1.2-1.4), Q_d is the fluorescence quantum yield of the donor in absence of acceptor, κ^2 is the orientation factor for the dipole-dipole interaction and J is the normalized spectral overlap integral. [$\varepsilon(\lambda)$ is in $M^{-1}\,cm^{-1}$, λ is in nm and J are $M^{-1}\,cm^{-1}\,(nm)^4$]

R_0 is the Förster critical distance at which 50% of the excitation energy is transferred to the acceptor and can be approximated from experiments independent of energy transfer.

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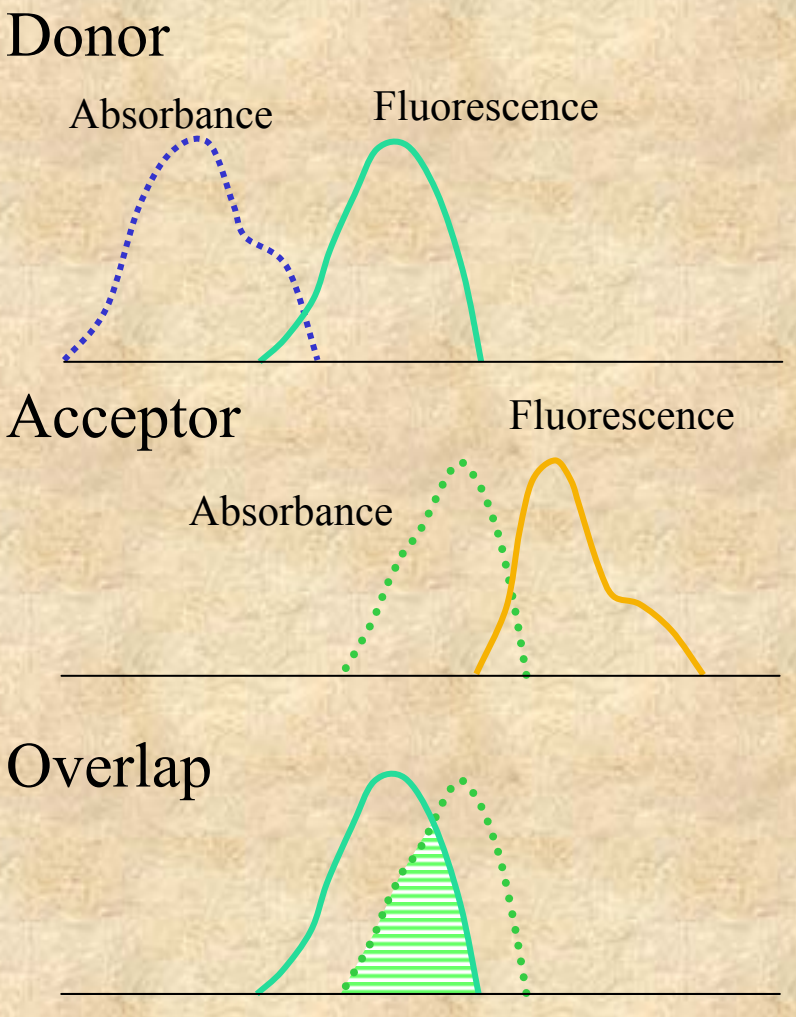
The overlap integral *J* is defined by:

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

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



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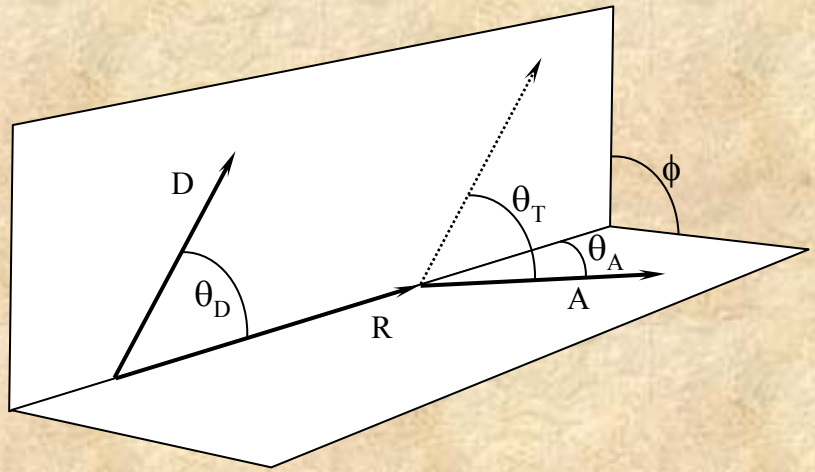
The orientation factor κ^2

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



Where θ_T is the angle between the D and A moments, given by

$$\cos\theta_T = \sin\theta_D \sin\theta_A \cos\phi + \cos\theta_D \cos\theta_A$$

In which θ_D, θ_A are the angles between the separation vector R, and the D and A moment, respectively, and ϕ is the azimuth between the planes (D,R) and (A,R)

The limits for κ^2 are 0 to 4, The value of 4 is only obtained when both transitions moments are in line with the vector R. The value of 0 can be achieved in many different ways.

If the molecules undergo fast isotropic motions (dynamic averaging) then $\kappa^2 = 2/3$

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Except in very rare case, κ^2 can not be uniquely determined in solution. What value of κ^2 should be used ?

1. We can **<u>assume</u>** isotropic motions of the probes and a value of $\kappa^2 = 2/3$, and verify experimentally that it is indeed the case.

***By swapping probes*:** The environment of the probe will be different and if κ^2 is not equal to 2/3, because orientations of the probes are not dynamically average (during the lifetime of the probe) due to restricted motions of the fluorophores, then the distance measured by FRET will be different.

By using different probes: If the distance measured using different probe pairs are similar (taking into account the size of the probes) then the assumption that κ^2 is equal to 2/3 is probably valid.

2. We can **<u>calculate</u>** the lower and upper limit of κ^2 using polarization data (Dale, Eisinger and Blumberg: 1979 Biophys. J. 26:161-93).

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Determination of the energy transfer efficiency (*E*)

$$E = \frac{k_T}{k_T + \sum_{i \neq T} k_i}$$

Where k_T is the rate of transfer and k_i are all other deactivation processes.

Experimentally, *E* can be calculated from the fluorescence lifetimes or intensities of the donor determined in absence and presence of the acceptor.

$$E = 1 - \frac{\tau_{da}}{\tau_d} \quad \text{or} \quad E = 1 - \frac{F_{da}}{F_d}$$

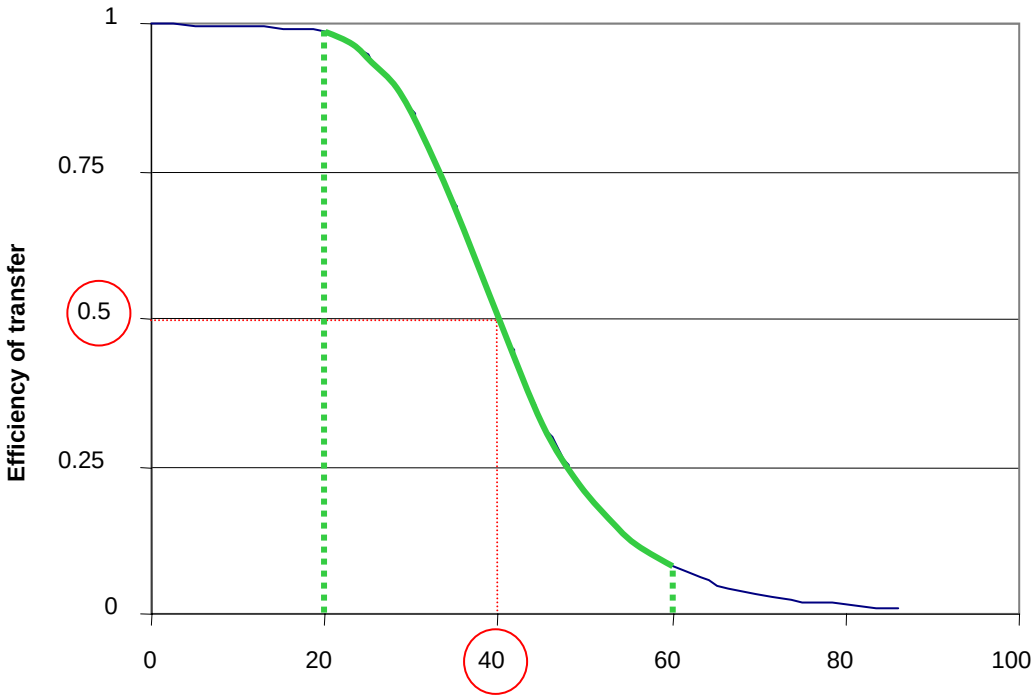
PM

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

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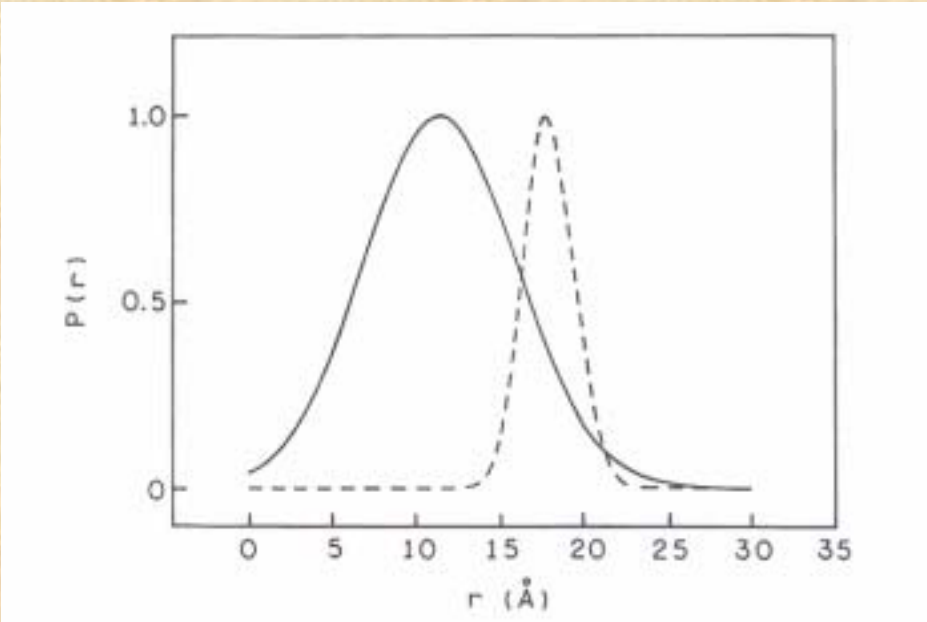
Distance Distribution Analysis

If the biomolecule of interest is flexible, one may imagine that the distance between two "target" points on the molecule, appropriately labelled with donor and acceptor groups, will not be fixed but will instead experience a distribution of separation distances which reflect the solution dynamics of the system. The observed efficiency of energy transfer will then be directly related to this distribution of distances.

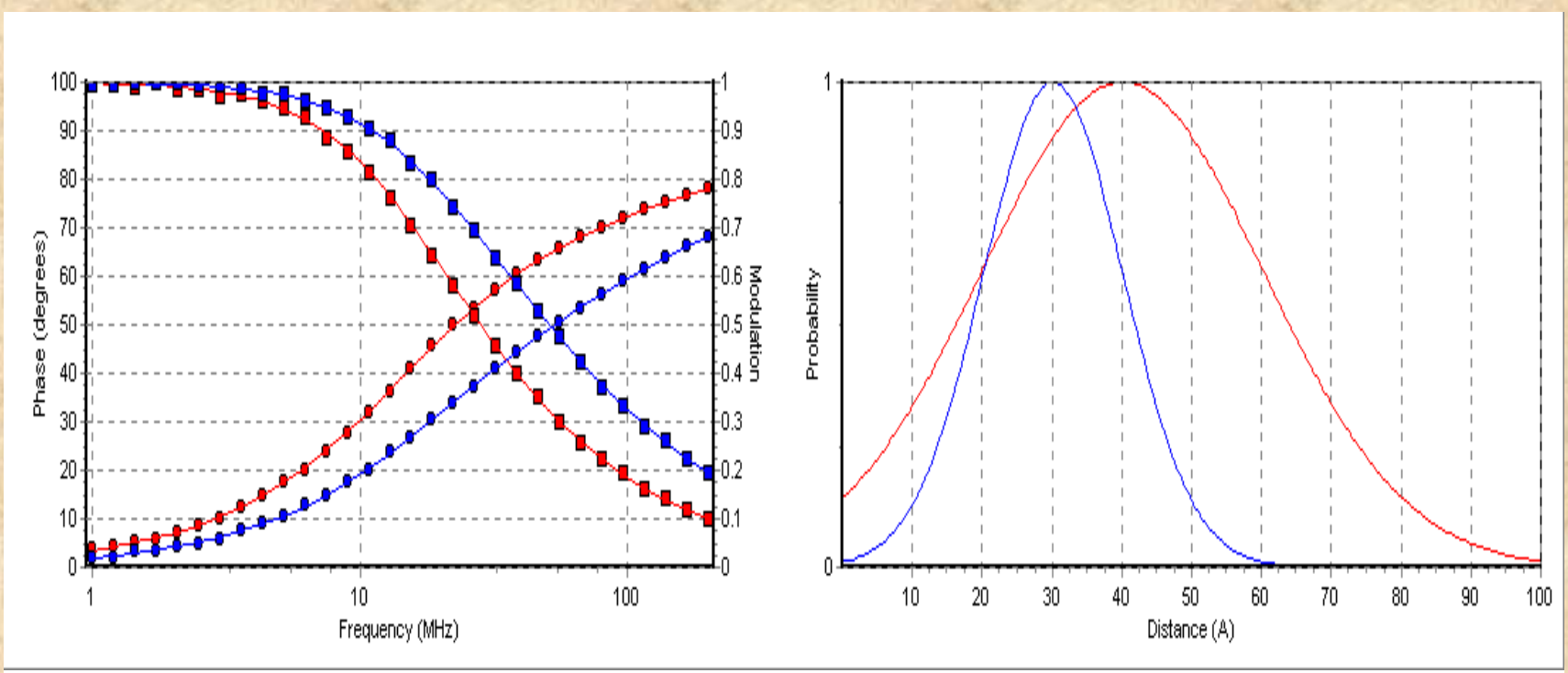
Such a distribution cannot be determined using steady-state methodologies in a single donor/acceptor experiment, however, methodologies based on lifetime procedures do permit recovery of a distribution and the applicability of these methods, using both time and frequency domain techniques, have been demonstrated in a number of model and unknown systems.



Distance distribution functions between tryptophan 22 and AEDANS-Cys52 in troponin in the presence (dashed line) and absence (solid line) of calcium.

This approach was first suggested by Haas, et al., (1975) *Proc Natl Acad Sci USA* **72**, 1807. An example of this analysis is shown here, from the work of She et al. 1998 J Mol Biol. **281:**445-52.

Simulations of phase and modulation data for two distance distributions are shown here.



Phase (circles) and modulation (squares) values expected for the donor lifetime (13 ns) in the presence of acceptor for distance distributions centered at 40Å (red) and 30Å (blue). In both cases the R_0 value was fixed at 40Å and the width of the distance distribution was fixed to 20Å in one case and 10Å in the other case. Random phase (0.2°) and modulation (0.004) noise was superimposed on the initial simulation and the distance distribution curves were calculated.

An elegant example of the use of FRET methodologies to study protein systems is given by the work of Lillo et al. ("Design and characterization of a multisite fluorescence energy-transfer system for protein folding studies: a steady-state and time-resolved study of yeast phosphoglycerate kinase" Biochemistry. 1997 Sep 16;36(37):11261-72 and "Real-time measurement of multiple intramolecular distances during protein folding reactions: a multisite stopped-flow fluorescence energy-transfer study of yeast phosphoglycerate kinase" Biochemistry. 1997 Sep 16;36(37):11273-81)

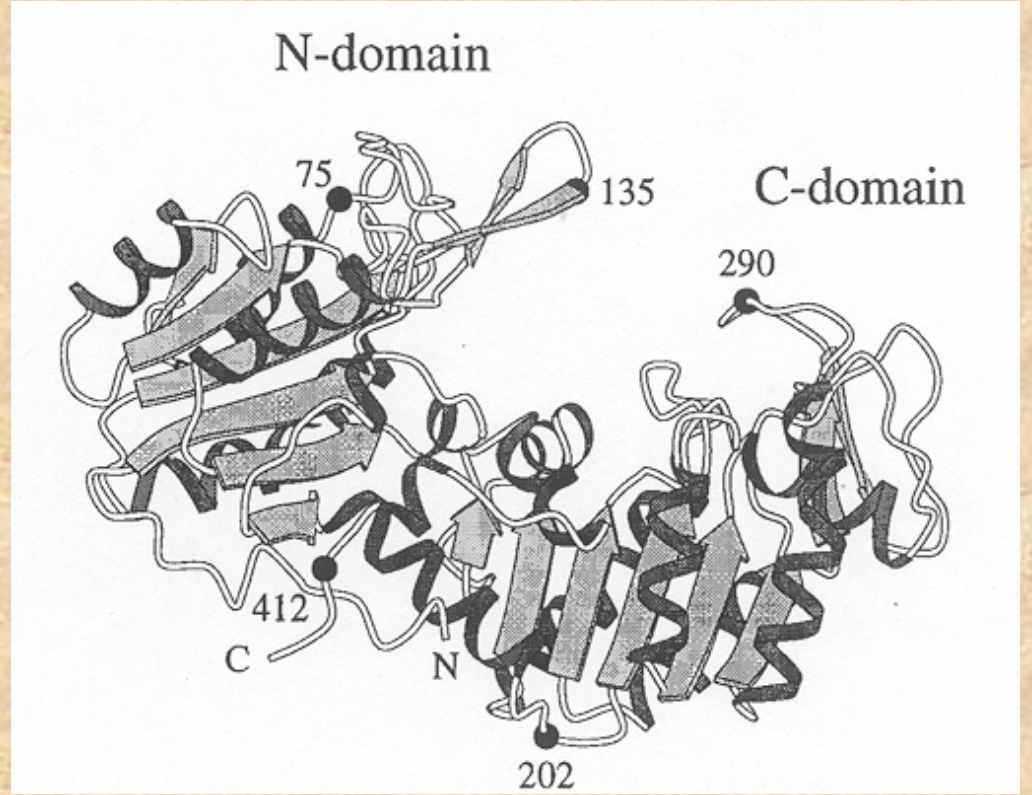
Site-directed mutagenesis was used to introduce pairs of cysteine residues in the protein at the positions shown

The pairs studied were:

135 – 290; 75 – 290

290 – 412; 412 – 202

135 – 412; 412 - 75



The donor was IAEDANS and the acceptor was IAF (iodoacetamindo-fluorescein). The various labeled protein products were separated by chromatography!

Table 1: Summary of the Labeled Proteins Examined for the Photophysical Characterization of Each Energy-Transfer Pair Cys*i* → Cys*j*

sample	name	Cys*i* → Cys*j*	no. of cysteines	fluorophore
donor only (D-PGK)	*i*-single cysteine	D- - -	1 (*i*)	AEDANS (*i*)
	j-single cysteine	- - -D	1 (*j*)	AEDANS (*j*)
	i-two cysteines	D- - -Cys	2 (*i*, *j*)	AEDANS (*i*)
	j-two cysteines	Cys- - -D	2 (*i*, *j*)	AEDANS (*j*)
	(*i*, *j*)-two cysteine average	D- - -Cys + Cys- - -D	2 (*i*, *j*)	AEDANS (*i*) + AEDANS (*j*)
	(*i*, *j*)-two cysteine "double donor"	D- - -D	2 (*i*, *j*)	AEDANS (*i*, *j*)
acceptor only	*i*-single cysteine	A- - -	1 (*i*)	AF (*i*)
	j-single cysteine	- - -A	1 (*j*)	AF (*j*)
donor-acceptor (D-PGK-A)	*i*, *j* specific label	D → A	2 (*i*, *j*)	AEDANS (*i*) and AF (*j*)
	j, *i* specific label	A ← D	2 (*i*, *j*)	AEDANS (*j*) and AF (*i*)
	i, *j* average label	D → A + A ← D	2 (*i*, *j*)	AEDANS (*i*) and AF (*j*) and + AEDANS (*j*) and AF (*i*)

Lifetime measurements were carried out on all samples

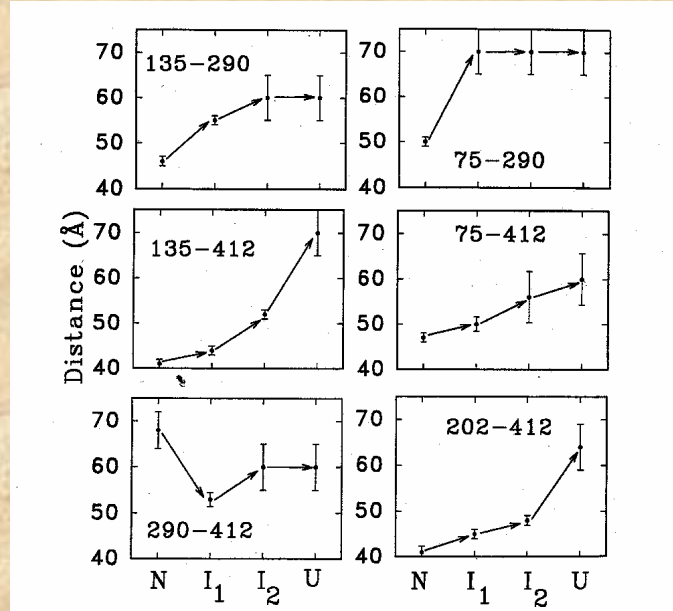
Table 5: Comparison of the Measured FRET Distances with That Predicted from the Crystal Structure[e]

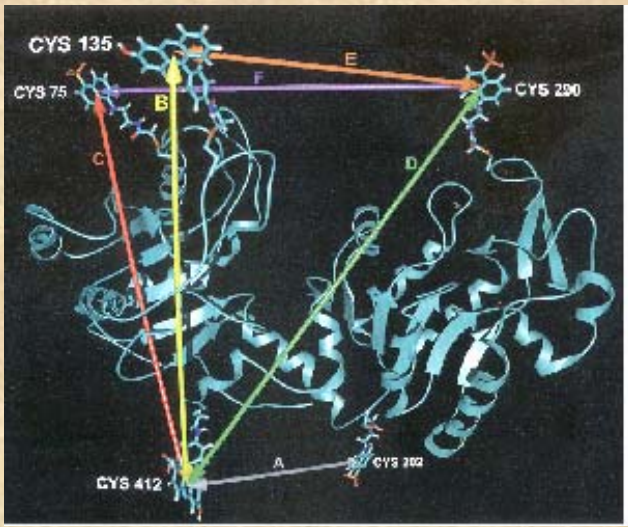
energy-transfer pair	measured steady-state distance (Å)	measured time-resolved discrete distance		measured time-resolved distance distribution			crystal structure $C_\alpha \rightarrow C_\alpha$ (Å)[a]	estimated dye-to-dye distances (Å)[b]
		R (Å)	χ^2	R_c (Å) [±2]	σ (Å)	χ^2		
135 ↔ 290	43	43.3	2.7	39.4	7.3	1.3	39	39
		40.3[c]	1.6	38.8[c]	6.1	1.2		
135 ↔ 412	40	40.4	2.7	39.5	3.8	1.3	40	46
412 → 135	40	39.5	2.1	38.0	3.9	1.2		
		38.7	1.4	38.1[c]	3.4	1.3		
290 ↔ 412	69	63.6	1.4	64.8	13.5	1.3	48	56
		56.6[c]	1.8	58.6[c]	13.2	1.4		
75 ↔ 290	50	51.7	4.3	46.6	13.5	1.2	40	46
202 ↔ 412	39	41.7	1.5	37.8	6.6	1.1	26	34
412 → 75	47	48.2	3.1	44.8	13.5	1.4	32	46
all[d]	–	60–70	1.1	60–80	15–30	1.1	–	–

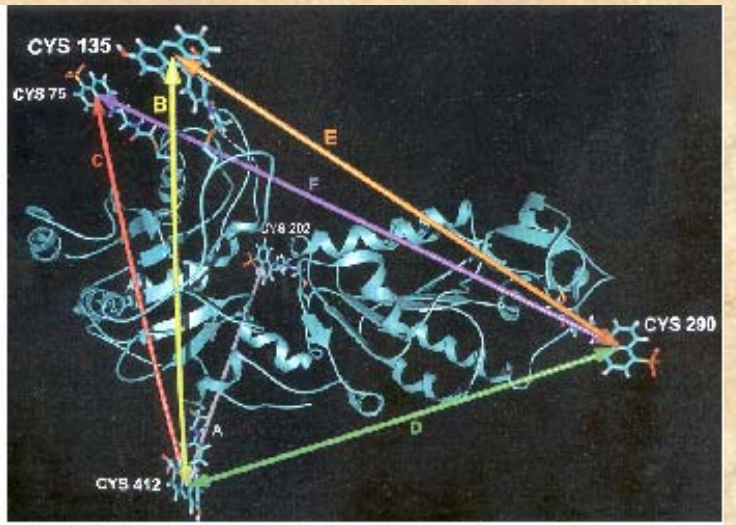
[a] Watson et al. (1982). [b] Donor-to-acceptor distance from MD simulations based on Watson et al. (1982) crystal structure. [c] Acceptor-side FRET measurements. [d] Unfolded samples (MOPS buffer at pH 7.5 and 25 °C and 2 M GuHCl). [e] MOPS buffer at pH 7.5 and 25 °C. D ↔ A: average labeled samples (donor distributed between the two Cys sites). D → A: specific labeled samples. Unless otherwise indicated, distance determinations are from donor-side experiments. The errors on the recovered distances are dominated by "nonfitting" sources and are estimated to be ±3 Å (see the text).

The intramolecular distances for the six energy transfer pairs are recovered for the each intermediate formed during the GuHCL induced unfolding of PGK

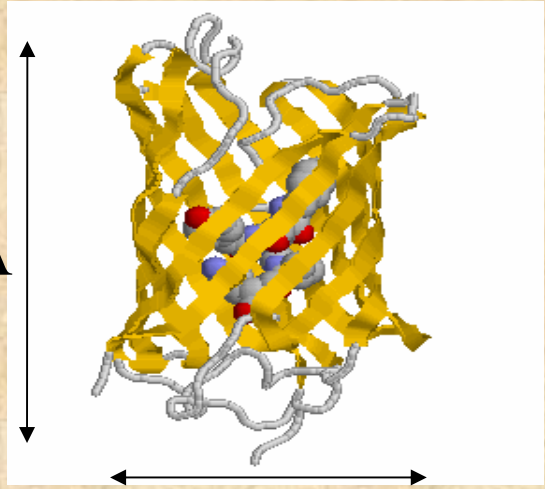
The authors proposed a specific structural transition associated with the unfolding of PGK from the native state (left) to the first unfolded state (right).







The C terminal domain (on the right of the monomer) is twisted by approximately 90º relative to the N-terminal domain resulting in an increase in the distances A,E and F and a shortening of the distance D. PM



FRET experiments have been done using the green fluorescent protein (GFP)

GFP was originally isolated from the jellyfish *Aequorea victoria*. It is composed of 11 β-sheets, forming a barrel like structure called b-can, surrounding an a-helix containing the chromophore

The GFP is fused to the protein of interest and expressed in the organism under study.

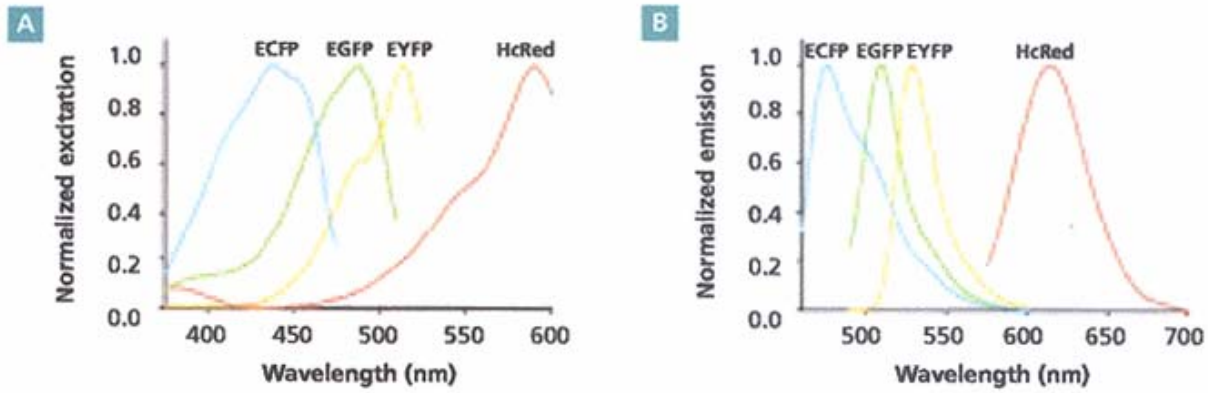


Figure 1. Excitation and emission spectra of BD Living Colors™ ECFP, EGFP, EYFP, and HcRed. HcRed's excitation maximum = 588 nm; emission maximum = 618 nm. EGFP's excitation maximum = 489 nm; emission maximum = 508 nm. EYFP's excitation maximum = 514 nm; emission maximum = 527 nm. ECFP's excitation maximum= 434 nm; emission maximum = 477 nm.

Mutations in the amino acids surrounding the chromophore results in GFP with different spectral properties.

Examples of the use of GFP and FRET *in vivo* can be found in: Tramier et al., 2003 "Homo-FRET versus hetero-FRET to probe homodimers in living cells" Methods Enzymol. 360:580-97

Homo-transfer of electronic excitation energy

So far, we considered the donor and acceptor molecules to be different. However, if the probe excitation spectrum overlaps its emission spectrum, FRET can occur between identical molecules.

« Il suffit qu'un transfert d'activation puisse se produire entre deux molécules voisines d'orientation différentes, c'est a dire portant des oscillateurs non parallèles, pour qu'il en résulte en moyenne une diminution de l'anisotropie de distribution des oscillateurs excites et par suite de la polarisation de la lumière émise. »
(F. Perrin *Ann de Phys.* 1929)

It suffices that a transfer of activation can occur between two neighboring molecules with different orientations, that is with non-parallel oscillators, in order to have, on average, a decrease in the anisotropy of the distribution of excited oscillators, and therefore a decrease of the polarization of the emitted light.

« …L'existence de transferts d'activation est expérimentalement prouvée pour de telles molécules par la décroissance de la polarisation de la lumière de fluorescence quand la concentration croit… »
(F. Perrin *Ann de Phys*. 1932)

…The existence of transfer of activation is proven experimentally for such molecules by the decrease in polarization of the fluorescent light when the concentration is increased…

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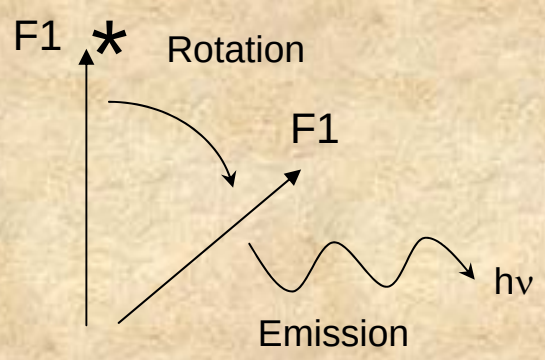
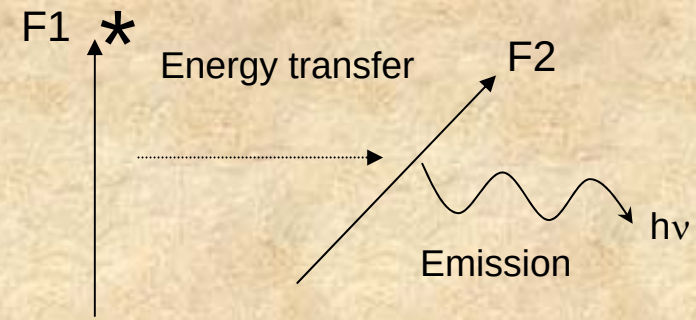
"…Excitation transfer between alike molecules can occur in repeated steps. So the excitation may *migrate* from the absorbing molecule over a considerable number of other ones before deactivation occurs by fluorescence or other process. Though this kind of transfer cannot be recognized from fluorescence spectra, it may be observed by the decrease of fluorescence polarization…" (Förster, 1959)

A.



B.



A. Depolarization resulting from rotational diffusion of the fluorophore. The excited fluorophore (F1*) rotates then emits light.

B. The excited fluorophore (F1*) transfer energy to another fluorophore F2 which in turn emits light.

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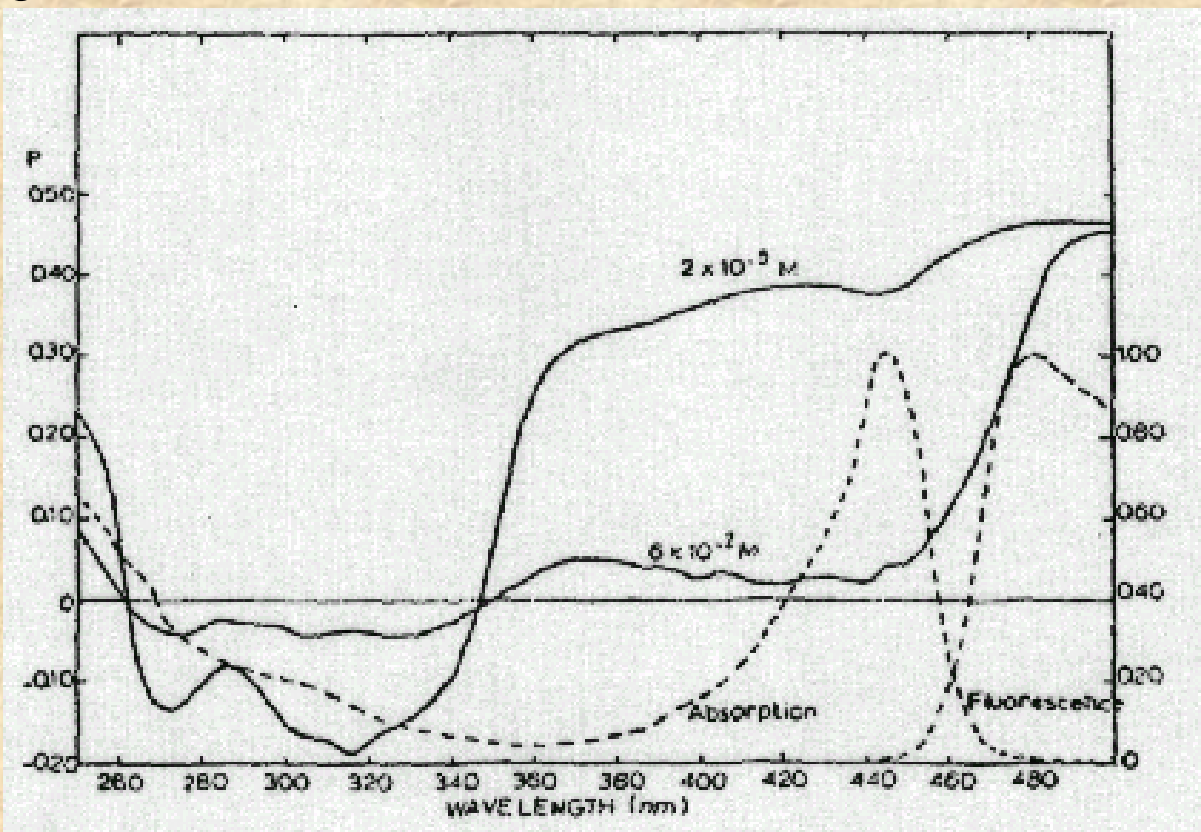
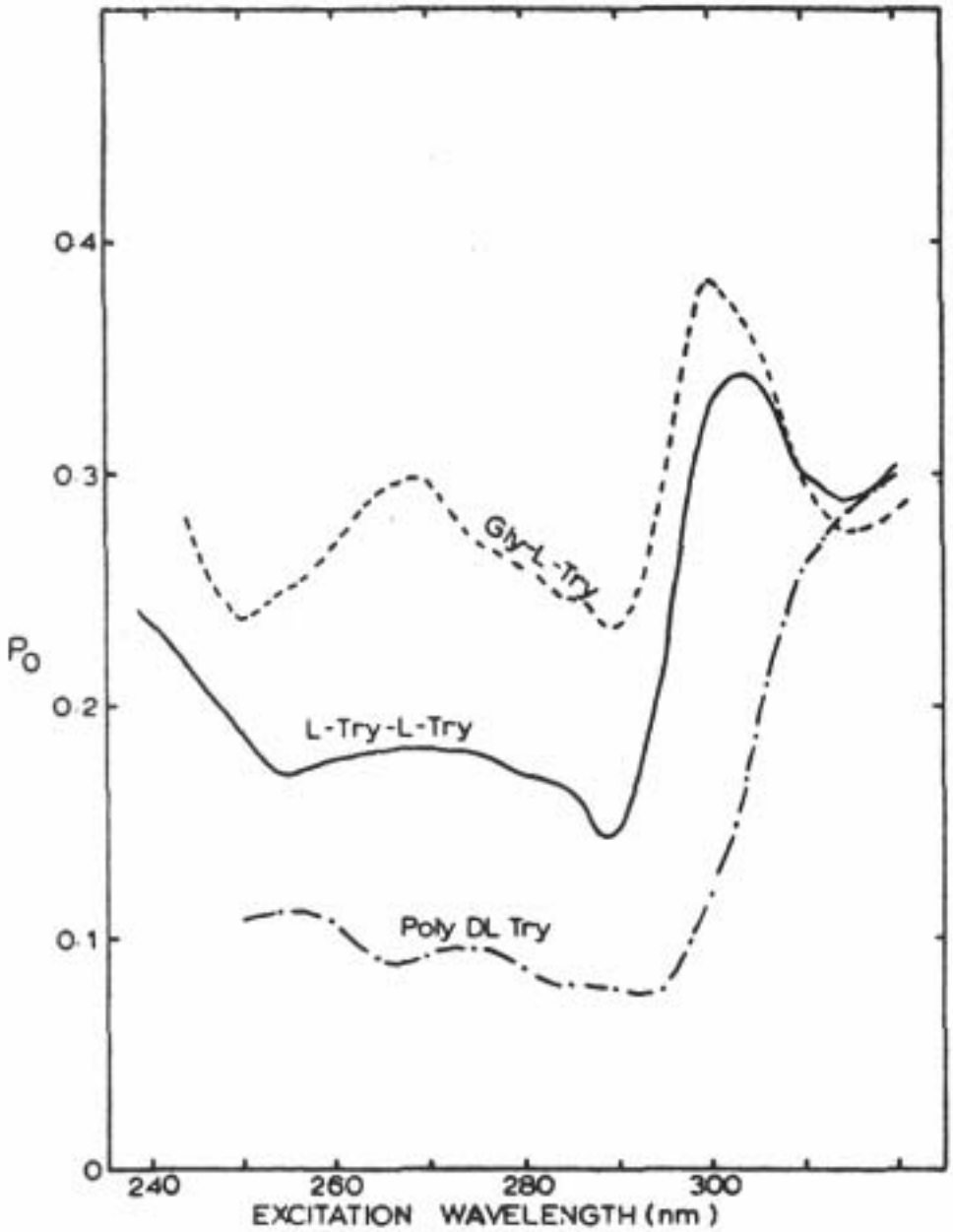
Weber's Red-Edge Effect

Electronic energy transfer between identical fluorophores was originally observed by Gaviola and Pringsheim in 1924. In 1960 Weber was the first to report that homotransfer among indole molecules disappeared upon excitation at the red-edge of the absorption band - this phenomenon is now known as the "Weber red-edge effect".

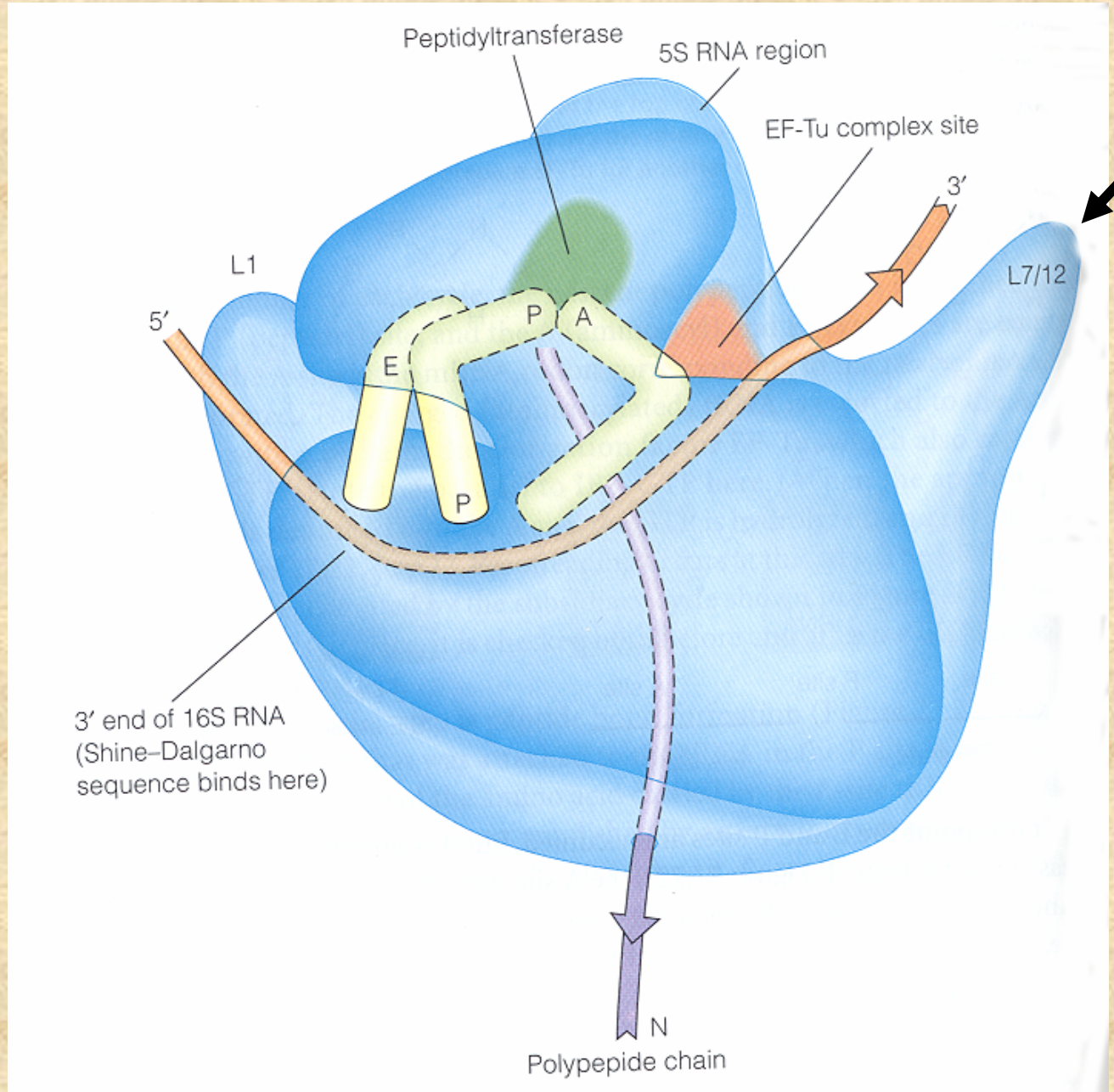
In 1970 Weber and Shinitzky published a more detailed examination of this phenomenon. They reported that in the many aromatic residues examined, transfer is much decreased or undetectable on excitation at the red edge of the absorption spectrum .



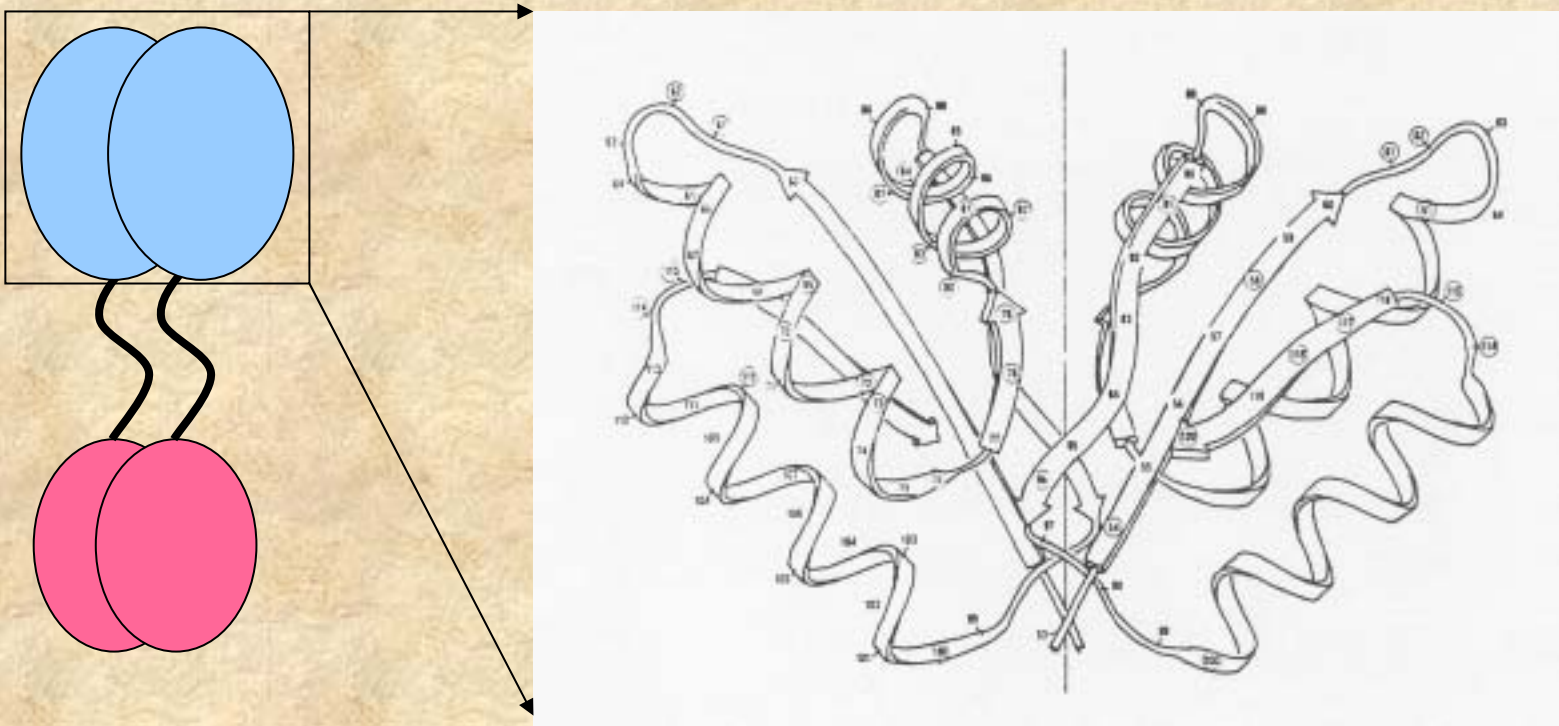


Fluorescence polarization spectra of glycyl L-tryptophan and L-tryptophyl-L-tryptophan in propylene glycol at –50ºC, and of poly DL-tryptophan in dimethylformamide-propylene glycol (1:9) at –50ºC from Weber and Shinitzky (1970).

An example of homo-FRET used to study protein interactions is the work by Hamman et al (Biochemistry *35*:16680) on a prokaryotic ribosomal protein



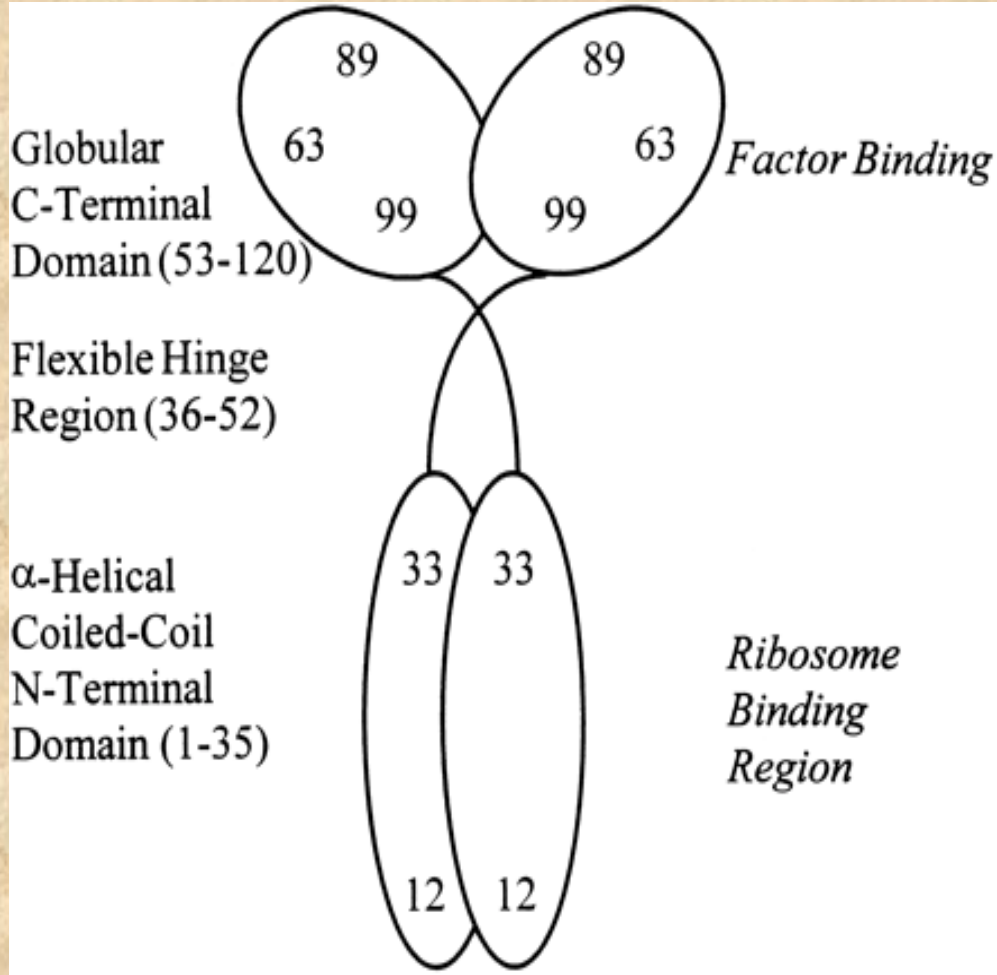
L7/L12 is present as two dimers in the ribosome. An X-ray structure of monomeric C-terminal domains led to the speculation that the C-terminal domains of L7/L12 interacted through hydrophobic surfaces as shown below



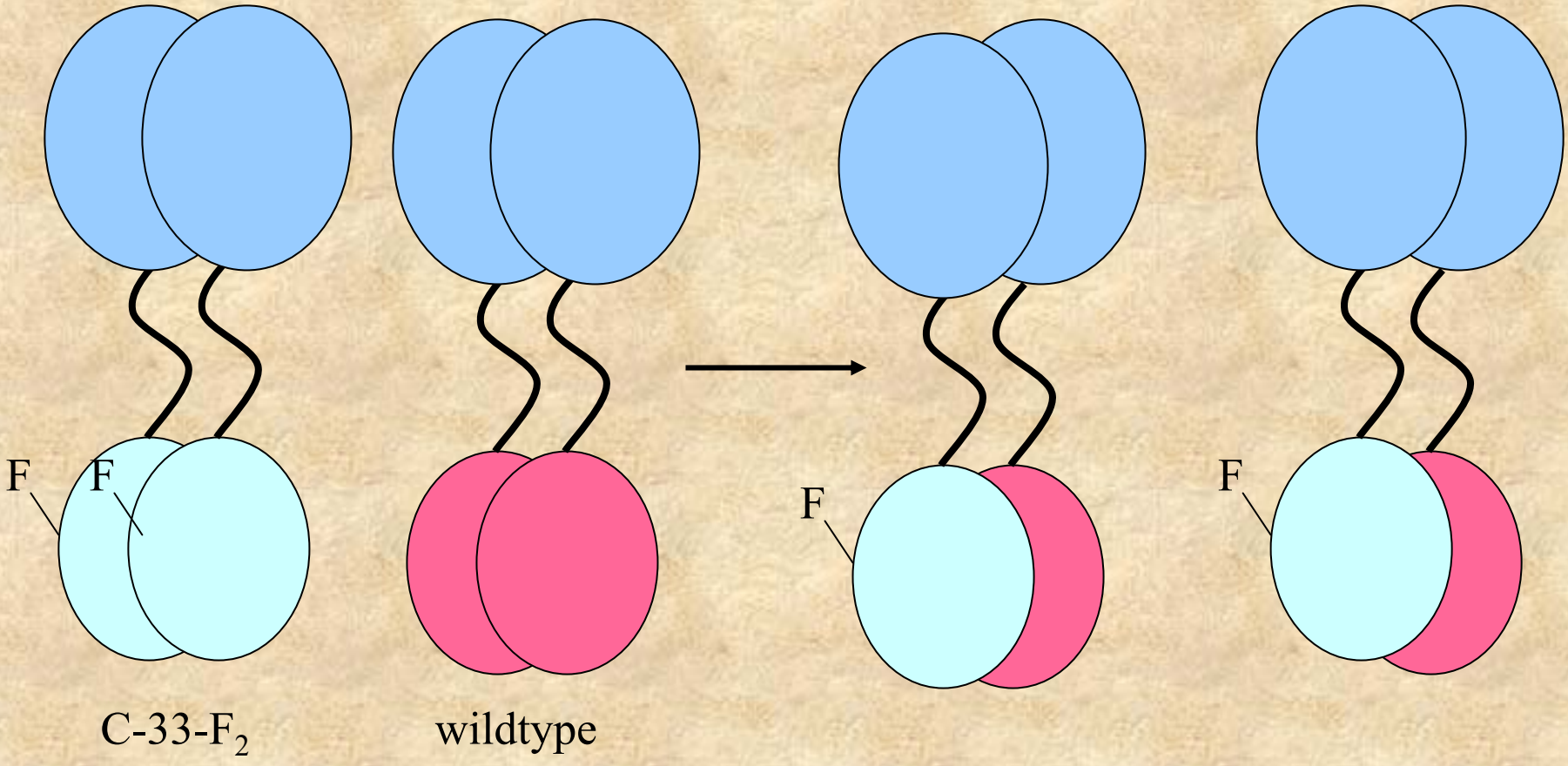
To study this protein fluorescence probes were introduced at specific locations along the L7/L12 peptide backbone.

To introduce these probes at specific locations site-directed mutagenesis was used to place cysteine residues in different locations

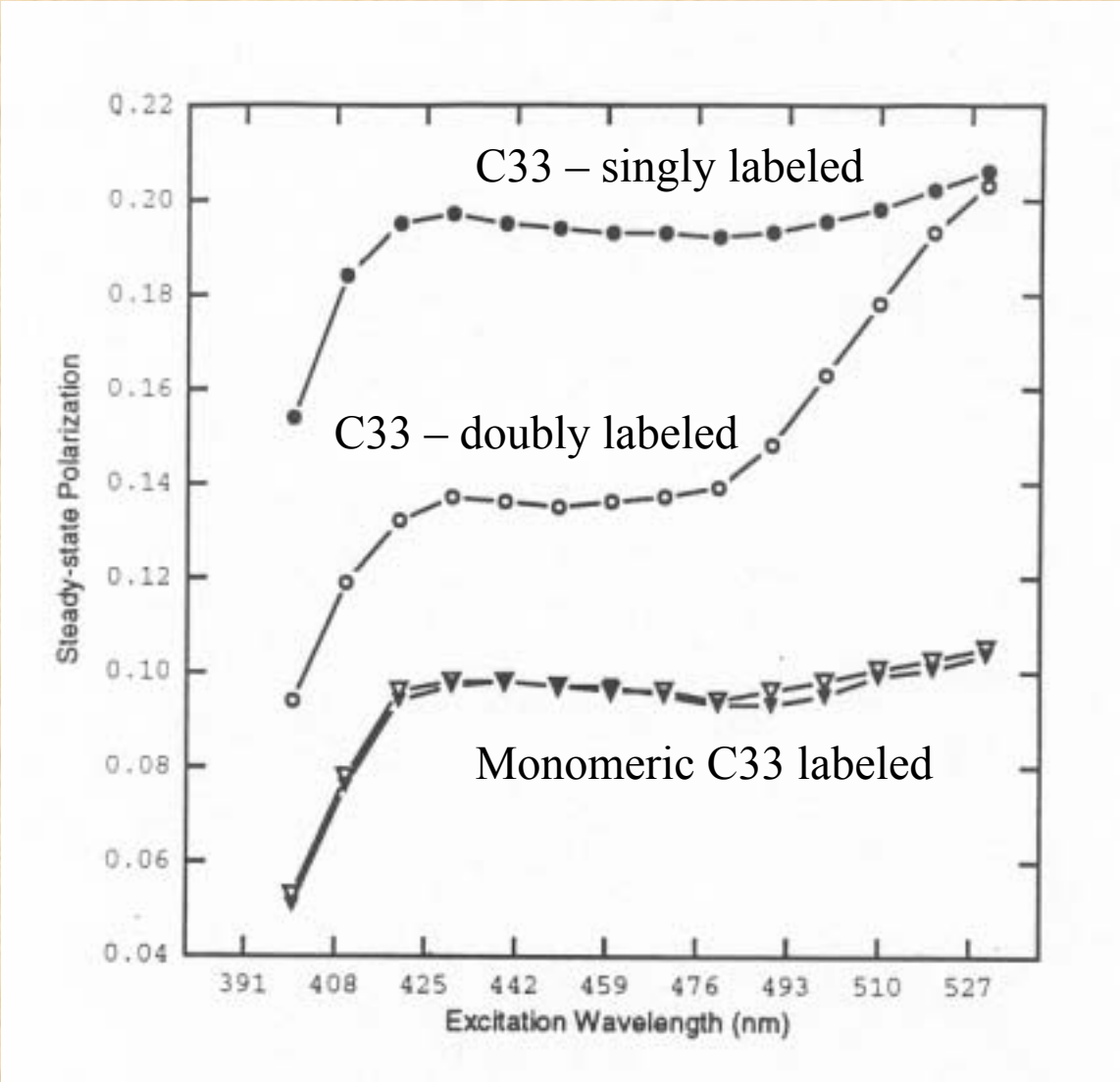
Sulfhydryl-reactive fluorescence probes were then covalently attached to these cysteine residues



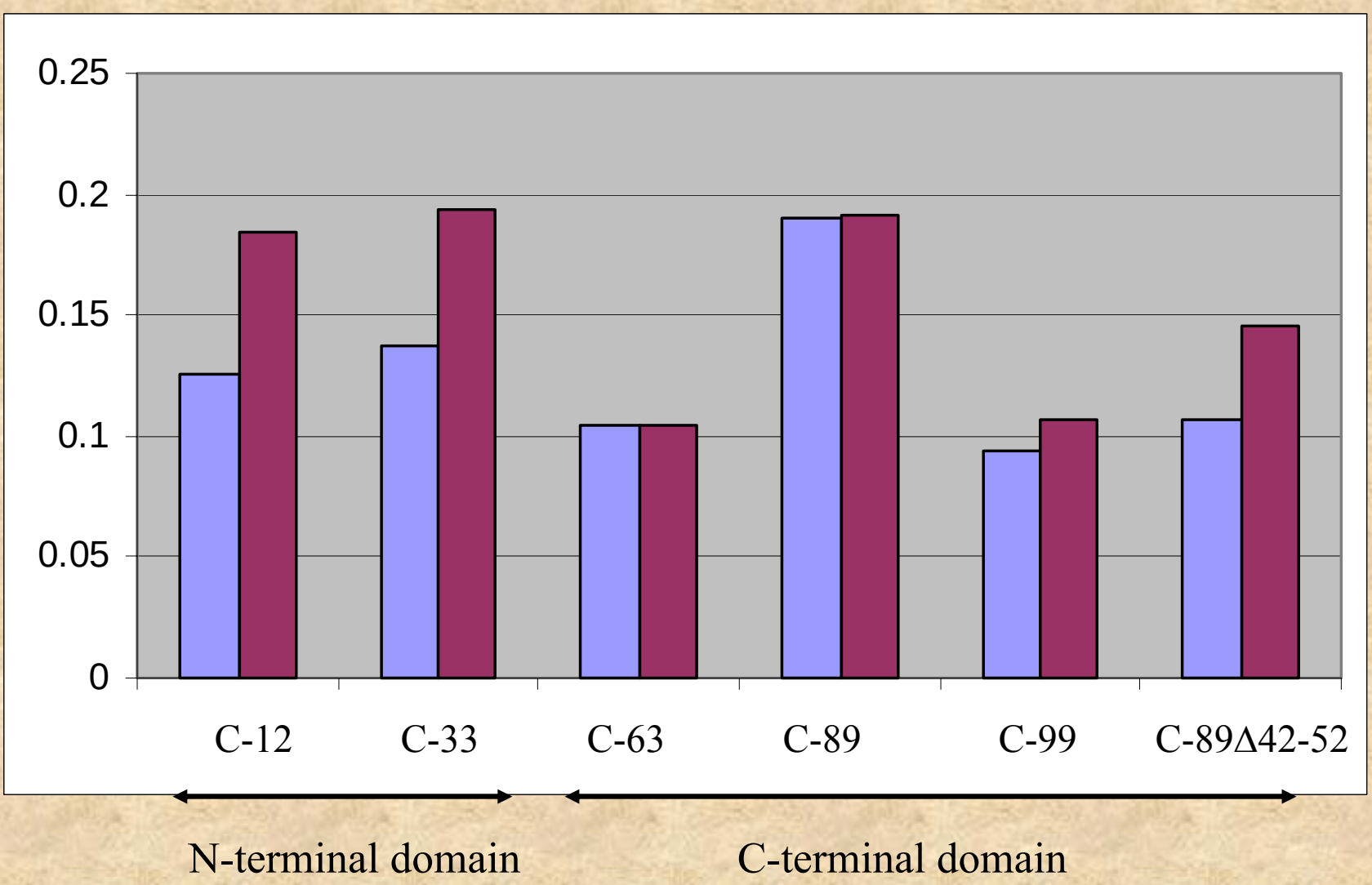
Subunit exchange experiments allowed the preparation of singly labeled dimers



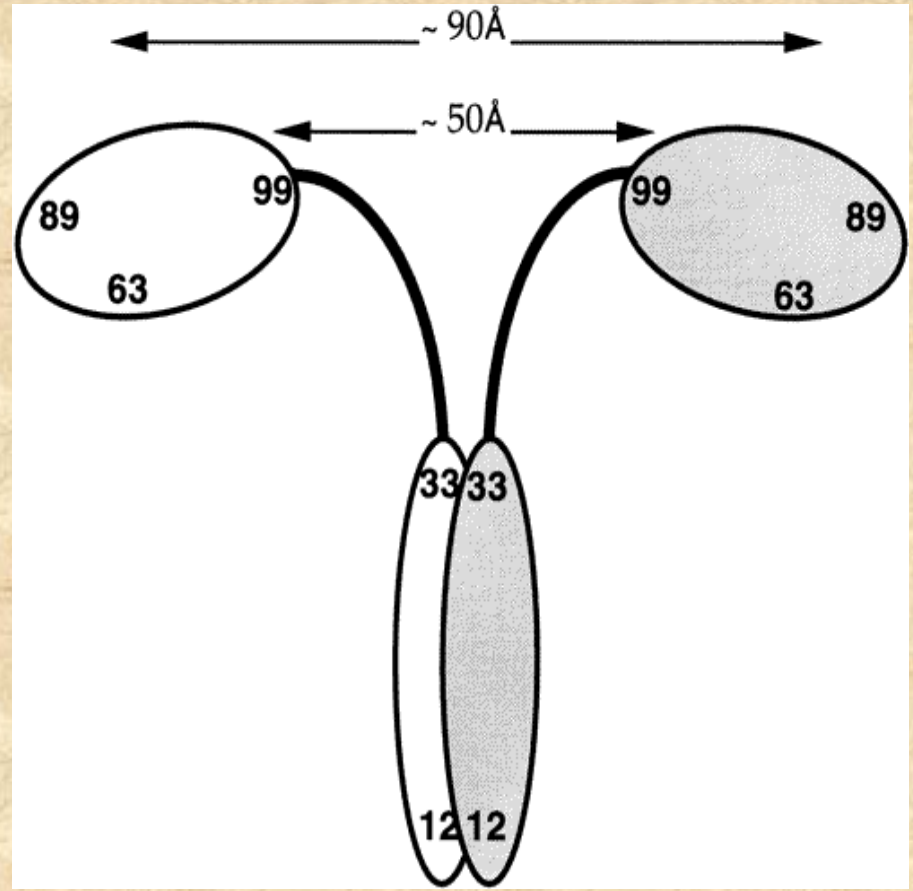
The presence of homoFRET was evident in the excitation polarization spectrum as shown by the Weber Red-Edge Effect.



The polarization values, before and after subunit exchange, indicate which residues undergo homoFRET. The polarization data below are for fluorescein labeled constructs before (violet) and after (magenta) subunit exchange



These changes in polarization due to homoFRET allow us to assign maximum proximity values for the C-terminal domains.



The conclusion is that the C-terminal domains are well-separated – contrary to the original model from the X-ray studies and the usual depictions in the literature

Sources on fluorescence theory and practice:

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