Lecture 5. Anisotropy decay/data analysis

Enrico Gratton

- Anisotropy decay
- Energy-transfer distance distributions
- Time resolved spectra
- Excited-state reactions

Basic physics concept in polarization

The probability of emission along the x (y or z) axis depends on the orientation of the transition dipole moment along a given axis.

If the orientation of the transition dipole of the molecule is changing, the measured fluorescence intensity along the different axes changes as a function of time.

Changes can be due to:

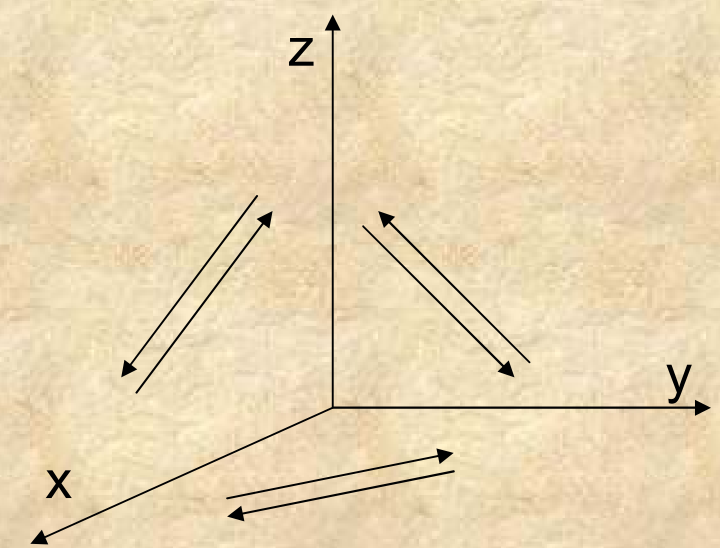
- Internal conversion to different electronic states
- changes in spatial orientation of the molecule
- energy transfer to a fluorescence acceptor with different orientation

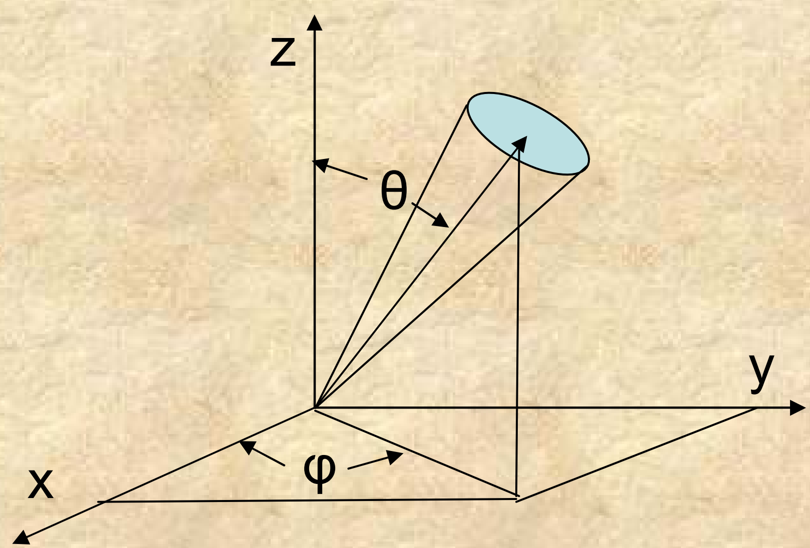
Anisotropy Decay

Transfer of emission from one direction of polarization to another

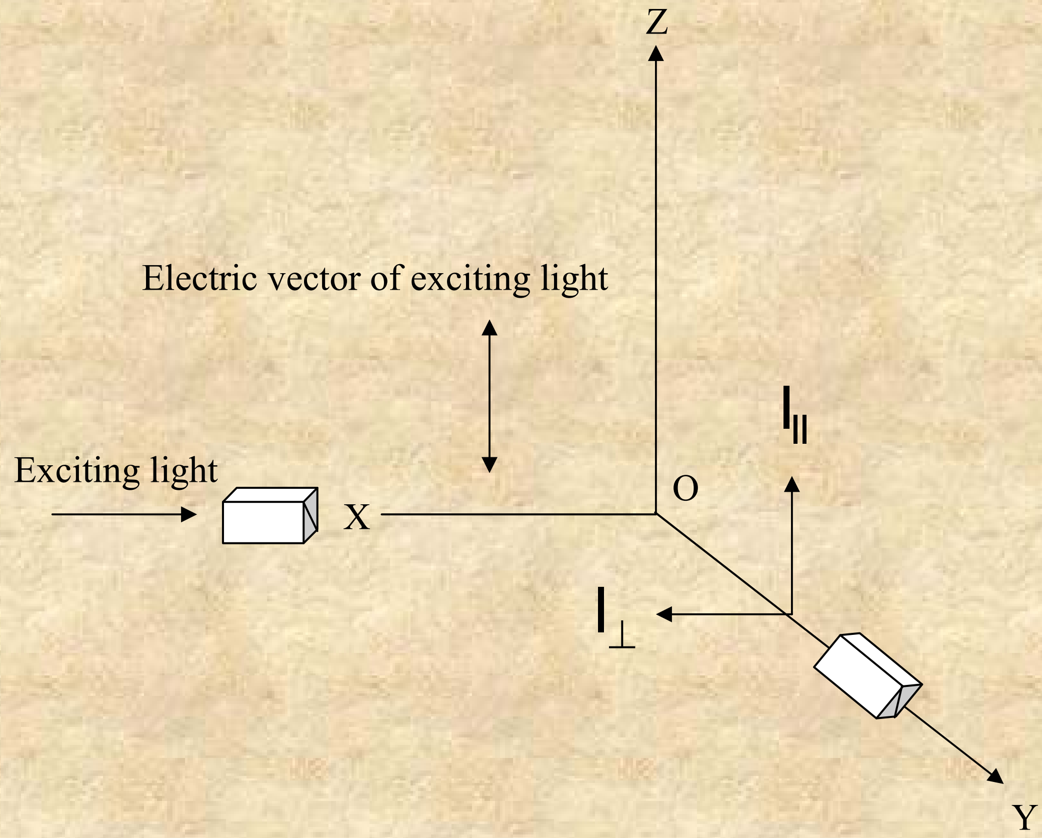
Two different approaches

- Exchange of orientation among fixed directions
- Diffusion of the orientation vector

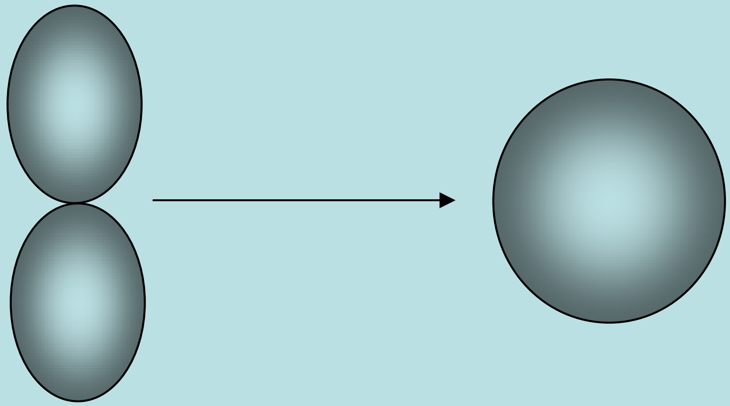




Geometry for excitation and emission polarization



In this system, the exciting light is traveling along the X direction. If a polarizer is inserted in the beam, one can isolate a unique direction of the electric vector and obtain light polarized parallel to the Z axis which corresponds to the vertical laboratory axis.



Photoselection

Return to equilibrium

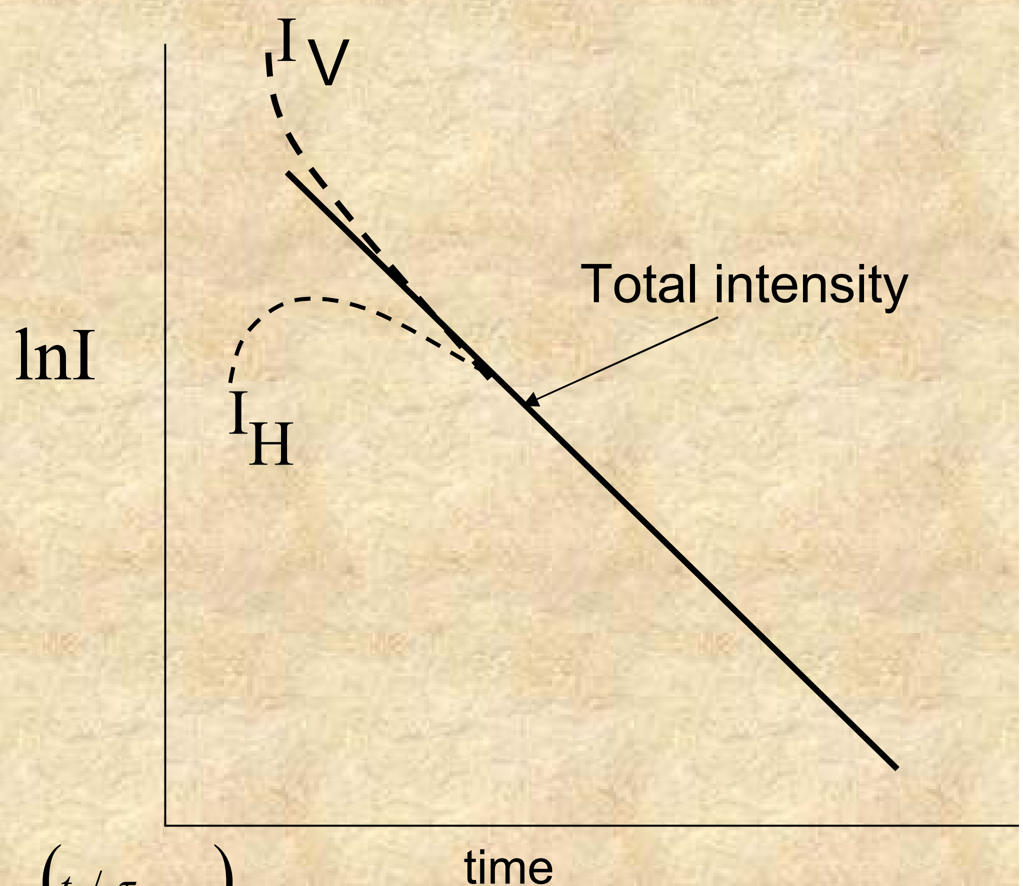
More light in the vertical direction

Same amount of light in the vertical and horizontal directions

Only valid for a population of molecules!

Time-resolved methodologies measure the changes of orientation as a function of time of a system. The time-domain approach is usually termed the **anisotropy decay** method while the frequency-domain approach is known as **dynamic polarization**. Both methods yield the same information.

In the time-domain method the sample is illuminated by a pulse of vertically polarized light and the decay over time of both the vertical and horizontal components of the emission are recorded. The anisotropy function is then plotted versus time as illustrated here:



The decay of the anisotropy with time (r_t) for a sphere is then given by:

$$r = \frac{Iv - Ih}{Iv + 2\,Ih} = r_o e^{-\left(t/\tau_c\right)}$$

In the case of non-spherical particles or cases wherein both “global” and “local” motions are present, the time-decay of anisotropy function is more complicated.

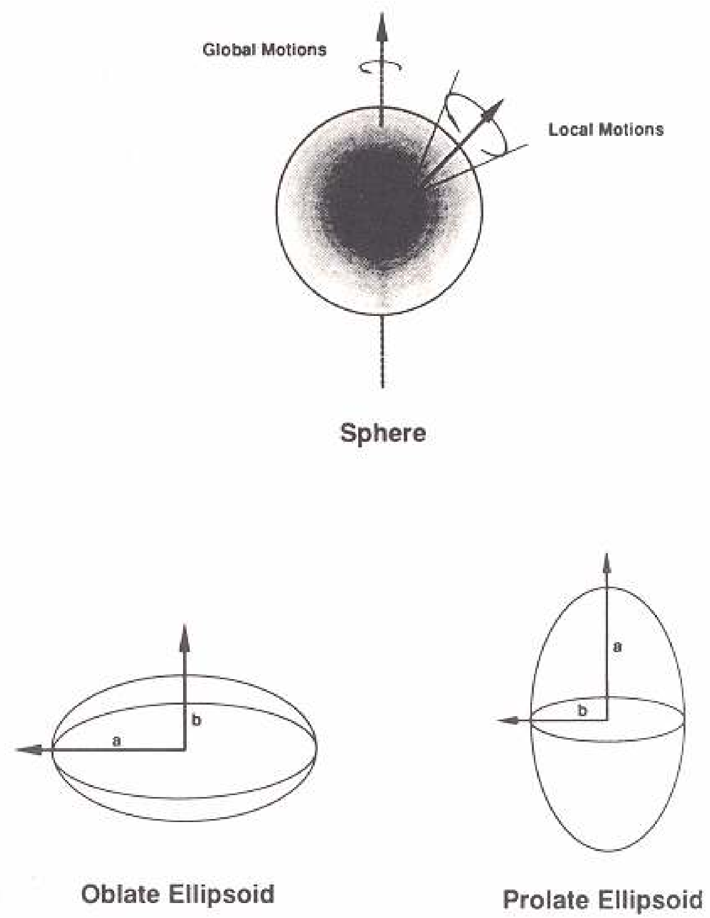


FIGURE 6. Ellipsoids of revolution commonly used as models of macromolecular shape. Rotational motions are defined around the major, a, and minor, b, ellipsoidal axes and the attachment point of the observed fluorophore.

For example, in the case of symmetrical ellipsoids of revolution the relevant expression is:

$$r_{(t)} = r_1 e^{-\left(t/\tau_{c1}\right)} + r_2 e^{-\left(t/\tau_{c2}\right)} + r_3 e^{-\left(t/\tau_{c3}\right)}$$

where: $\tau_{c1} = 1/6D_2$

$\tau_{c2} = 1/(5D_2 + D_1)$

$\tau_{c3} = 1/(2D_2 + 4D_1)$

where D_1 and D_2 are diffusion coefficients about the axes of symmetry and about either equatorial axis, respectively and:

$r_1 = 0.1(3\cos^2\phi_1 - 1)(3\cos^2\phi_2 - 1)$

$r_2 = 0.3\sin 2\phi_1 \sin 2\phi_2 \cos\varphi$

$r_3 = 0.3\sin^2\phi_1 \sin^2\phi_2 (\cos\varphi - \sin^2\varphi)$

where ϕ_1 and ϕ_2 are the angles between the absorption and emission dipoles, respectively, with the symmetry axis of the ellipsoid and φ is the angle formed by the projection of the two dipoles in the plane perpendicular to the symmetry axis.

Resolution of the rotational rates is limited in practice to two rotational correlation times which differ by at least a factor of two.

For the case of a **"local"** rotation of a probe attached to a spherical particle, the general form of the anisotropy decay function is:

$$r_{(t)} \cong r_1 e^{-\left(t/\sigma_1\right)} * r_2 e^{-\left(t/\sigma_2\right)}$$

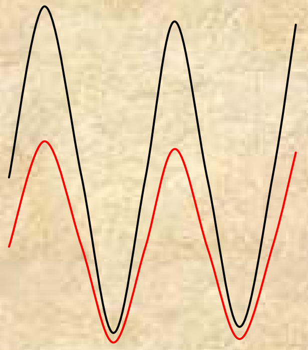
Where σ_1 represents the "local" probe motion, σ_2 represents the "global" rotation of the macromolecule, $r_1 = r_0(1-\theta)$ and θ is the "cone angle" of the local motion ($3\cos^2$ of the cone aperture)

In dynamic polarization measurements, the sample is illuminated with vertically polarized modulated light and the phase delay (dephasing) between the parallel and perpendicular components of the emission is measured as well as the modulation ratio of the AC contributions of these components. The relevant expressions for the case of a spherical particle are:

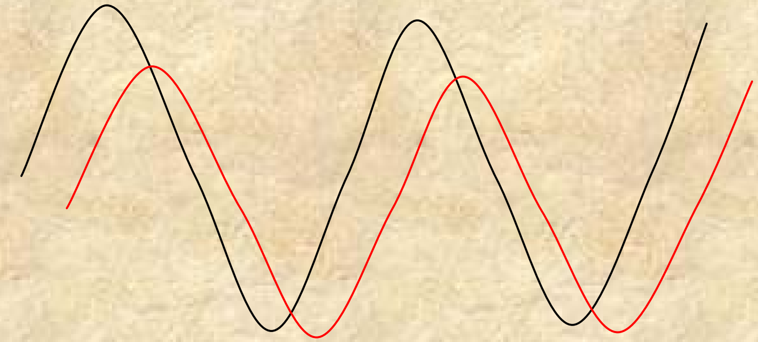
$$\Delta\phi = \tan^{-1}\left[\frac{18\omega r_o R}{\left(k^2+\omega^2\right)\left(1+r_o-2r_o^2\right)+6R\left(6R+2k+kr_o\right)}\right]$$

and $$Y_2 = \frac{\left(\left(1-r_o\right)k+6R\right)^2+\left(1-r_o\right)^2\omega^2}{\left[\left(1+2r_o\right)k+6R\right]^2+\left(1+2r_o\right)^2\omega^2}$$

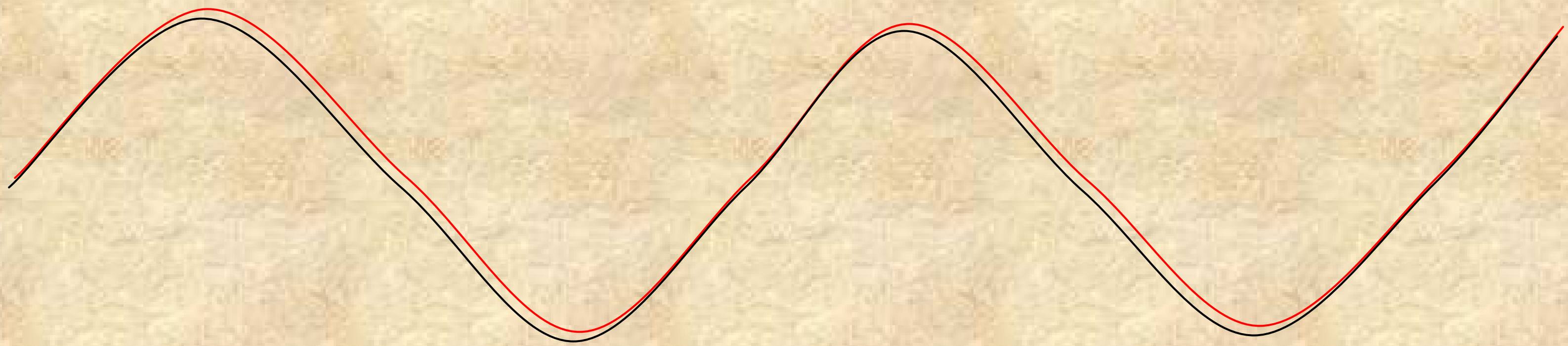
Where $\Delta\phi$ is the phase difference, Y the modulation ratio of the AC components, ω the angular modulation frequency, r_o the limiting anisotropy, k the radiative rate constant ($1/\tau$) and R the rotational diffusion coefficient.



At high frequency (short time) there is no dephasing because the horizontal component has not been populated yet



At intermediate frequencies (when the horizontal component has been maximally populated there is large dephasing



At low frequency (long time) there is no dephasing because the horizontal component and the vertical component have the same intensity

The illustration below depicts the $\Delta\phi$ function for the cases of spherical particles with different rotational relaxation times.

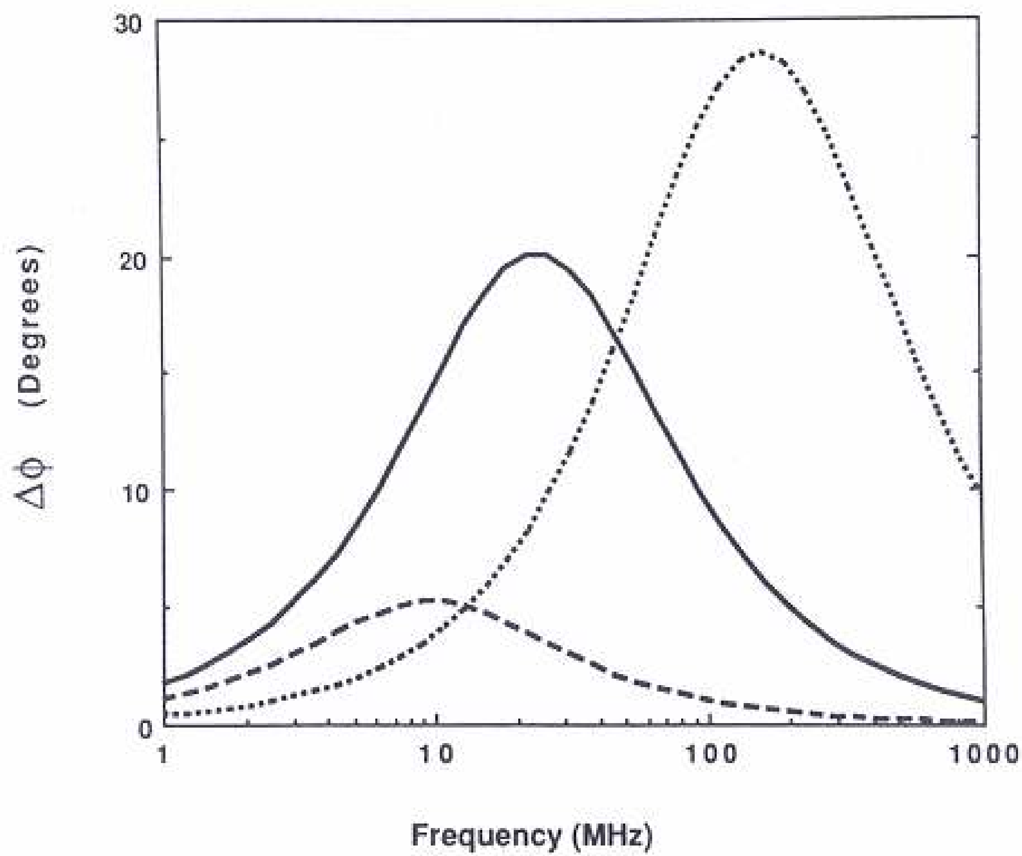


FIGURE 7. Differential phase data for an isotropic rotator with a 3-nsec (dotted line), 30-nsec (solid line), or 300-nsec (dashed line) rotational relaxation time. In each case a lifetime of 20 nsec was used and colinear excitation and emission dipoles were assumed.

The figures here show actual results for the case of ethidium bromide free and bound to tRNA - one notes that the fast rotational motion of the free ethidium results in a shift of the “bell-shaped” curve to higher frequencies relative to the bound case. The lifetimes of free and bound ethidium bromide were approximately 1.9 ns and 26 ns respectively.

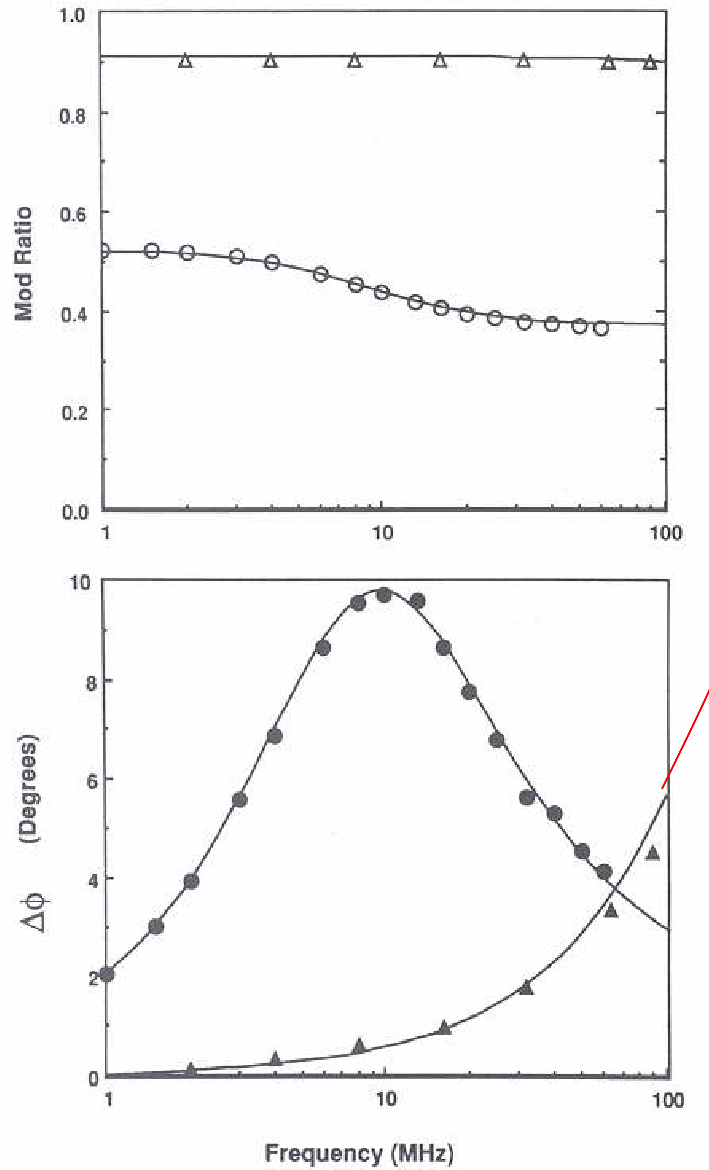


FIGURE 9. Differential phase (closed symbols) and modulation (open symbols) data for ethidium bromide in solution (triangles) and ethidium bromide bound to tRNA (circles). The resolved rotational relaxation times (5°C) were 144 and 0.5 nsec for free and bound ethidium bromide, respectively. The curves are the least-squares fit to the data.

In the case of local plus global motion, the dynamic polarization curves are altered as illustrated below for the case of the single tryptophan residue in elongation factor Tu which shows a dramatic increase in its local mobility when EF-Tu is complexed with EF-Ts.

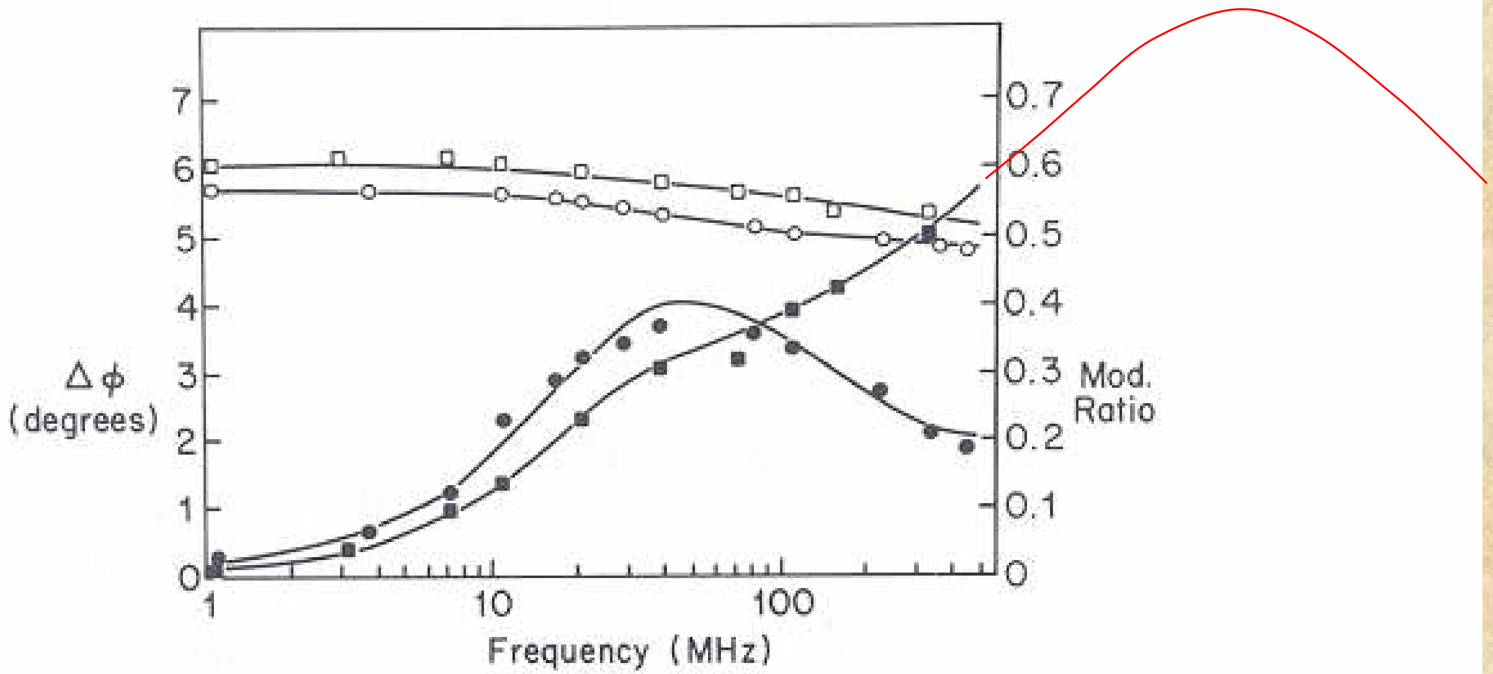
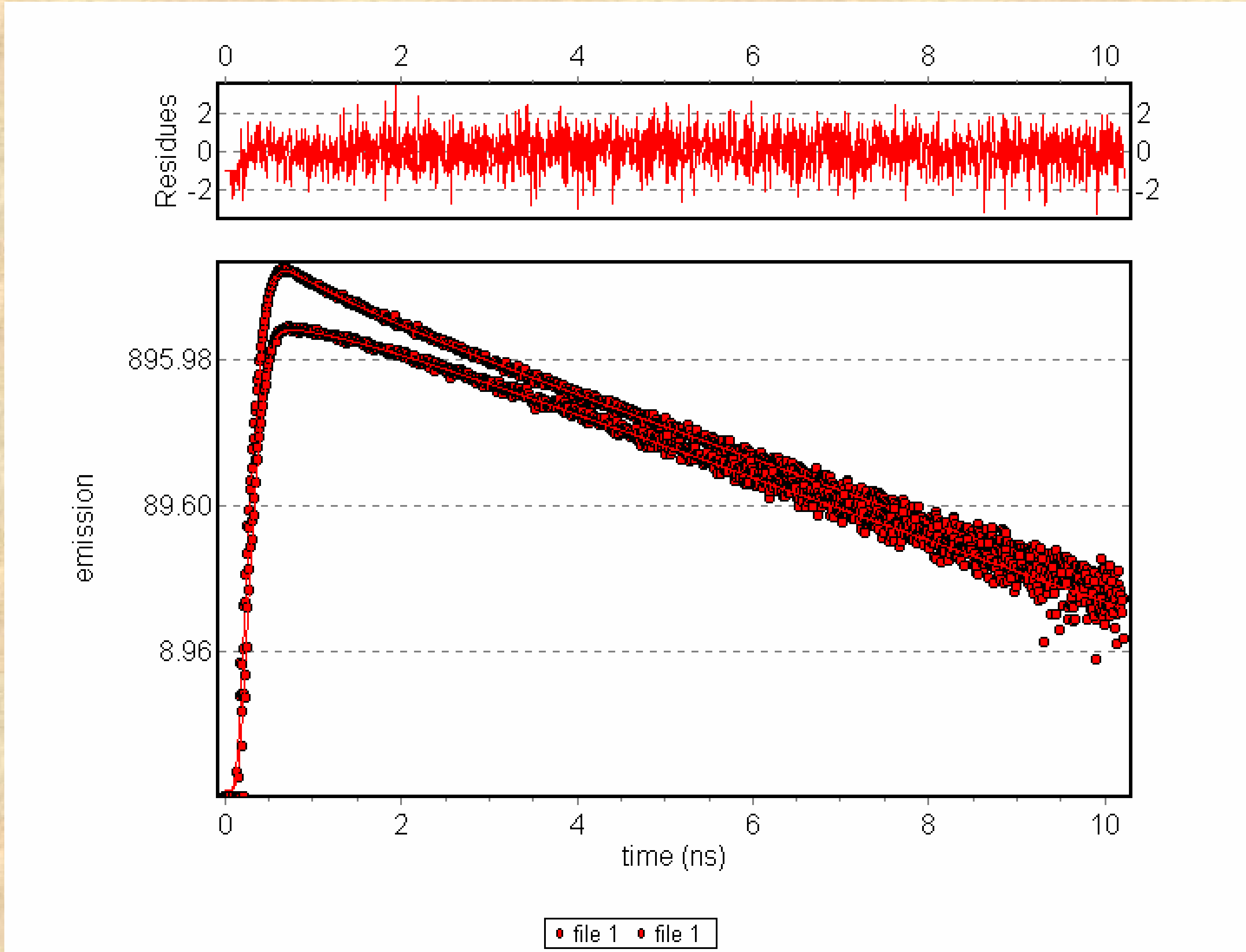
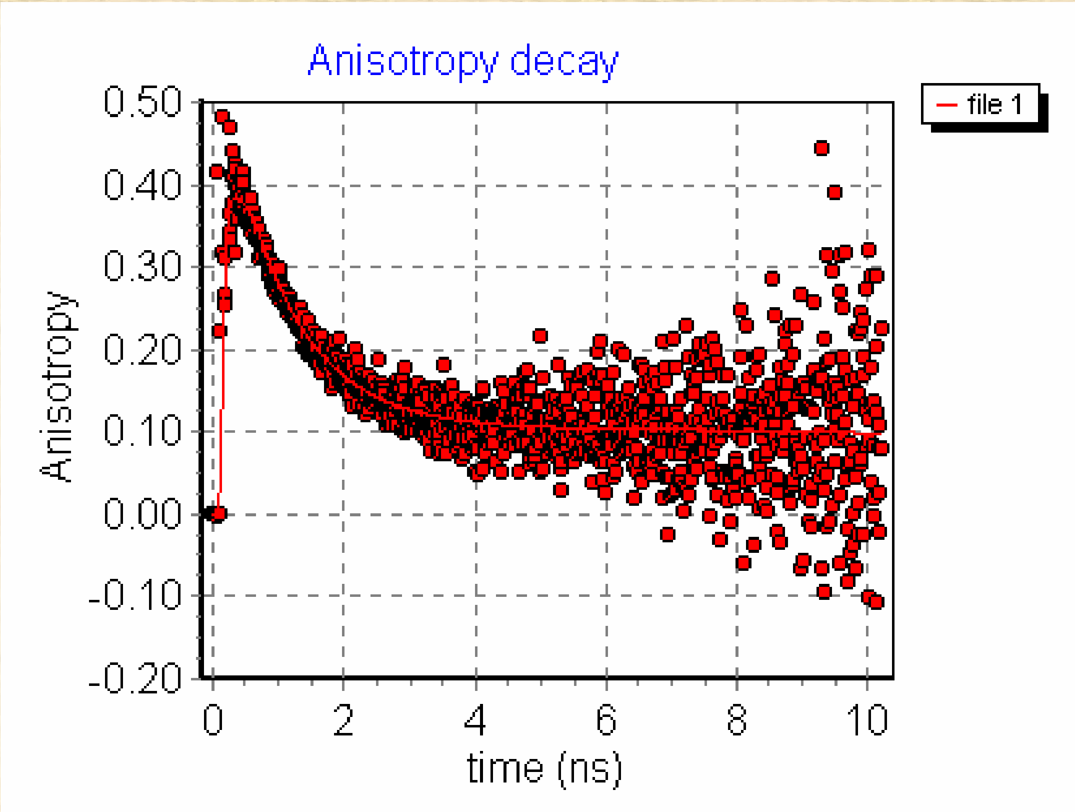


FIGURE 10. Multifrequency differential phase (closed symbols) and modulation (open symbols) data for elongation factor Tu complexed with GDP (circles) and elongation factor Ts (squares). Curves represent the least-squares fit to the data.

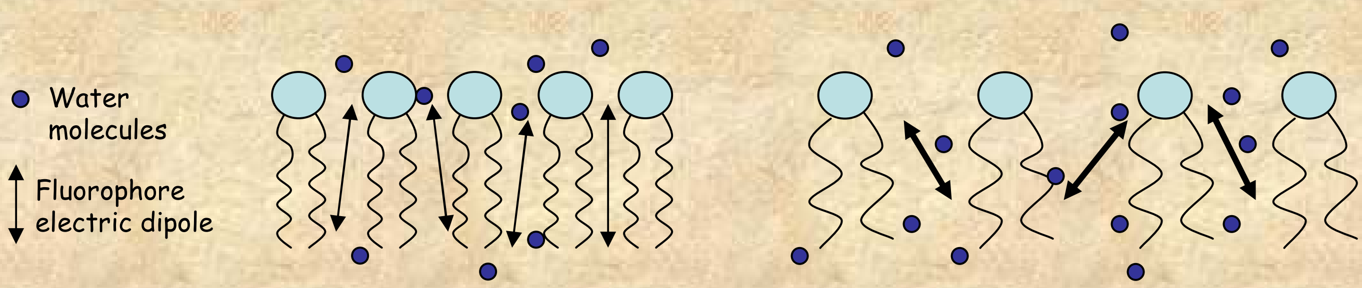
Time decay anisotropy in the time domain



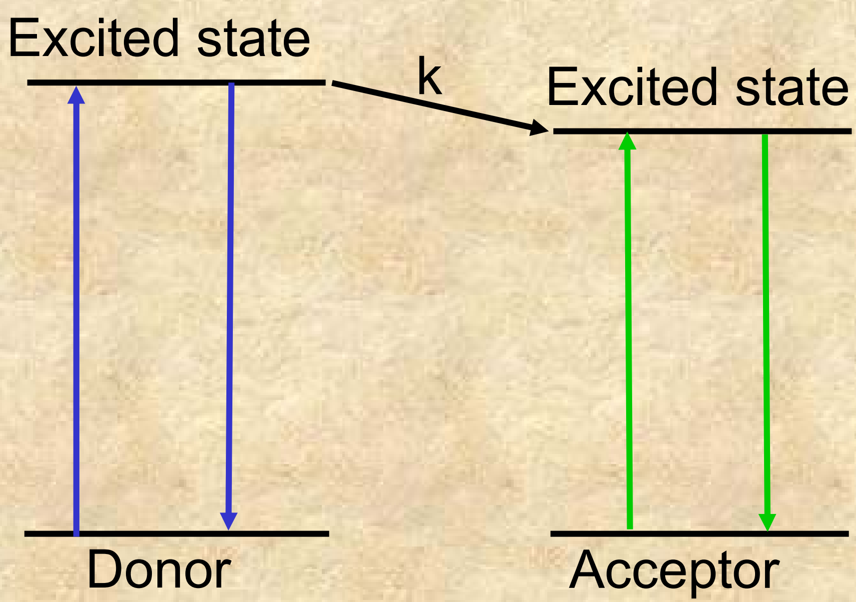
Anisotropy decay of an hindered rotator




```
Local chisquare =    1.11873
sas         1->0 = V   0.3592718
discrete 1->0 = V   1.9862748
r0          1->0 = V   0.3960686
r-inf       1->0 = V   0.1035697
phi 1       1->0 = V   0.9904623
qshift           = V   0.0087000
g_factor         = F   1.0000000
```



Energy transfer-distance distributions



Donor-acceptor pair

Simple excited state reaction
No back reaction for heterotransfer

All the physics is in the rate k

In general, the decay is double exponential both for the donor and for the acceptor if the transfer rate is constant

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:

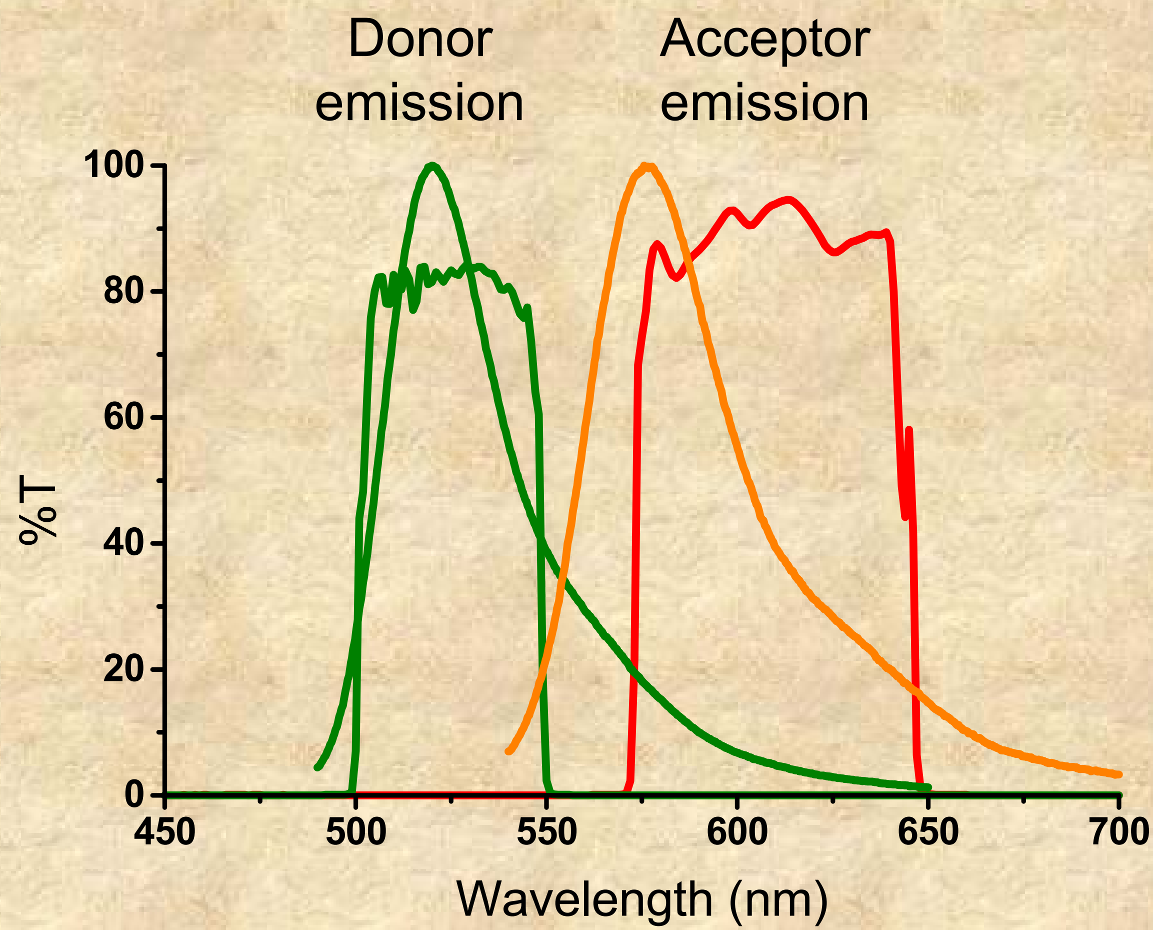
$$R_0 = 0.211(n^{-4}Q_d\kappa^2 J)^{1/6} \quad \text{Å}$$

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.

In principle, the distance R for a collection of molecules is variable and the orientation factor could also be variable

Analysis of the time-resolved FRET with constant rate



Fluorescein-rhodamine bandpasses

General expressions for the decay
Hetero-transfer; No excitation of the donor

$$I_D = a_d e^{-k_1 t} - b_d e^{-k_2 t}$$

Intensity decay as measured at the donor bandpass

$$I_A = a_a e^{-k_1 t} - b_a e^{-k_2 t}$$

Intensity decay as measured at the acceptor bandpass

$k_1 := \Gamma_a + k_t$ $\quad$ $k_2 := \Gamma_d$

$a_d = -B_a k_t$ $\quad$ $b_d = B_d(\Gamma_a - \Gamma_d - k_t)$

$a_a = B_d(\Gamma_a - \Gamma_d) - B_d k_t$ $\quad$ $b_a = -B_a(\Gamma_a - \Gamma_d)$

Γ_d and Γ_a are the decay rates of the donor and acceptor.
B_d and B_a are the relative excitation of the donor and of the acceptor.
The total fluorescence intensity at any given observation wavelength is given by

$$I(t) = SAS_d\, I_d(t) + SAS_a\, I_a(t)$$

where SAS_d and SAS_a are the relative emission of the donor and of the acceptor, respectively.

If the rate k_t is distributed, for example because in the population there is a distribution of possible distances, then we need to add all the possible values of the distance weighted by the proper distribution of distances

Example (in the time domain) of gaussian distribution of distances

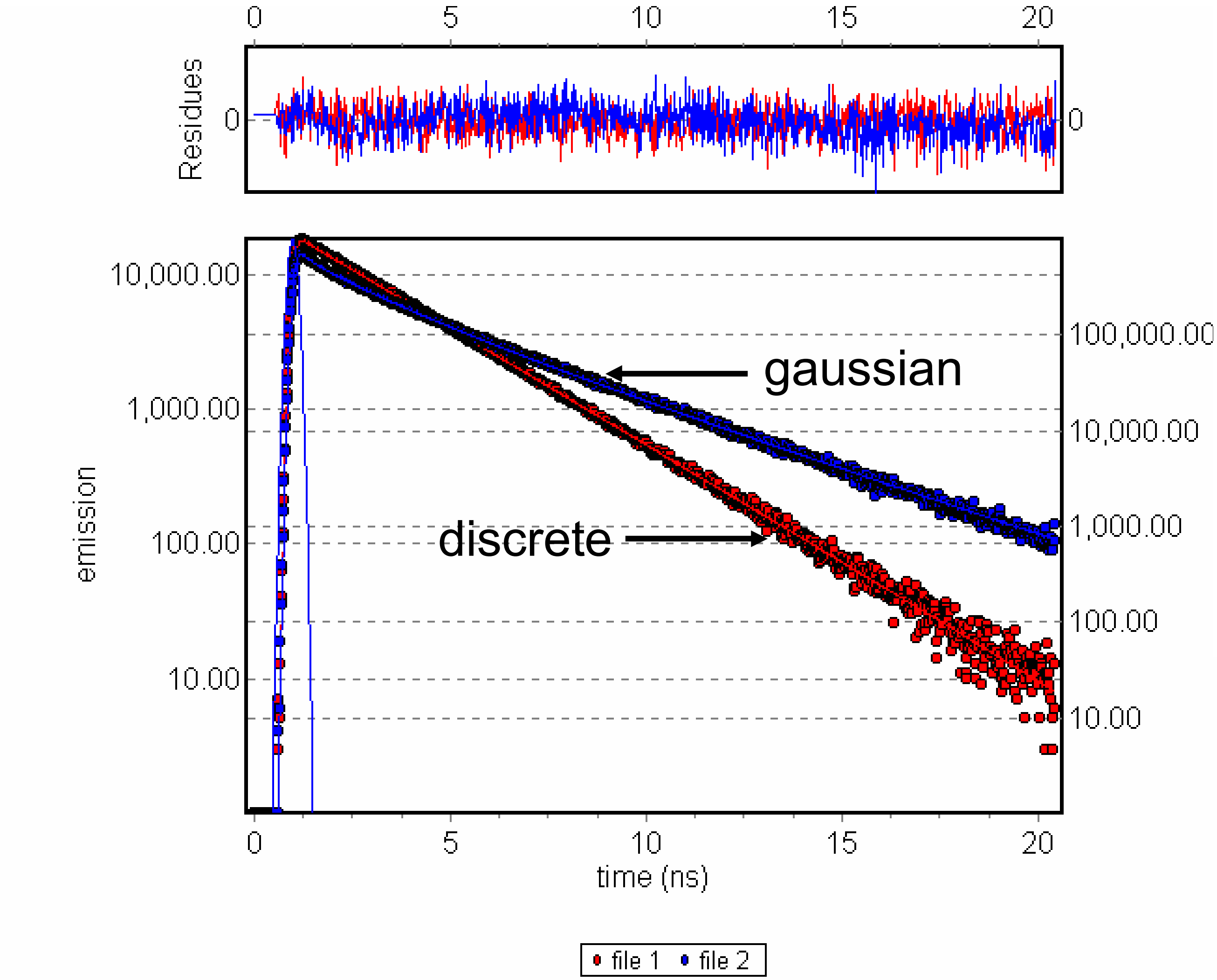
(Next figure)

If the distance changes during the decay (dynamic change) then the starting equation is no more valid and different equations must be used (Beechem and Hass)

FRET-decay, discrete and distance gaussian distributed

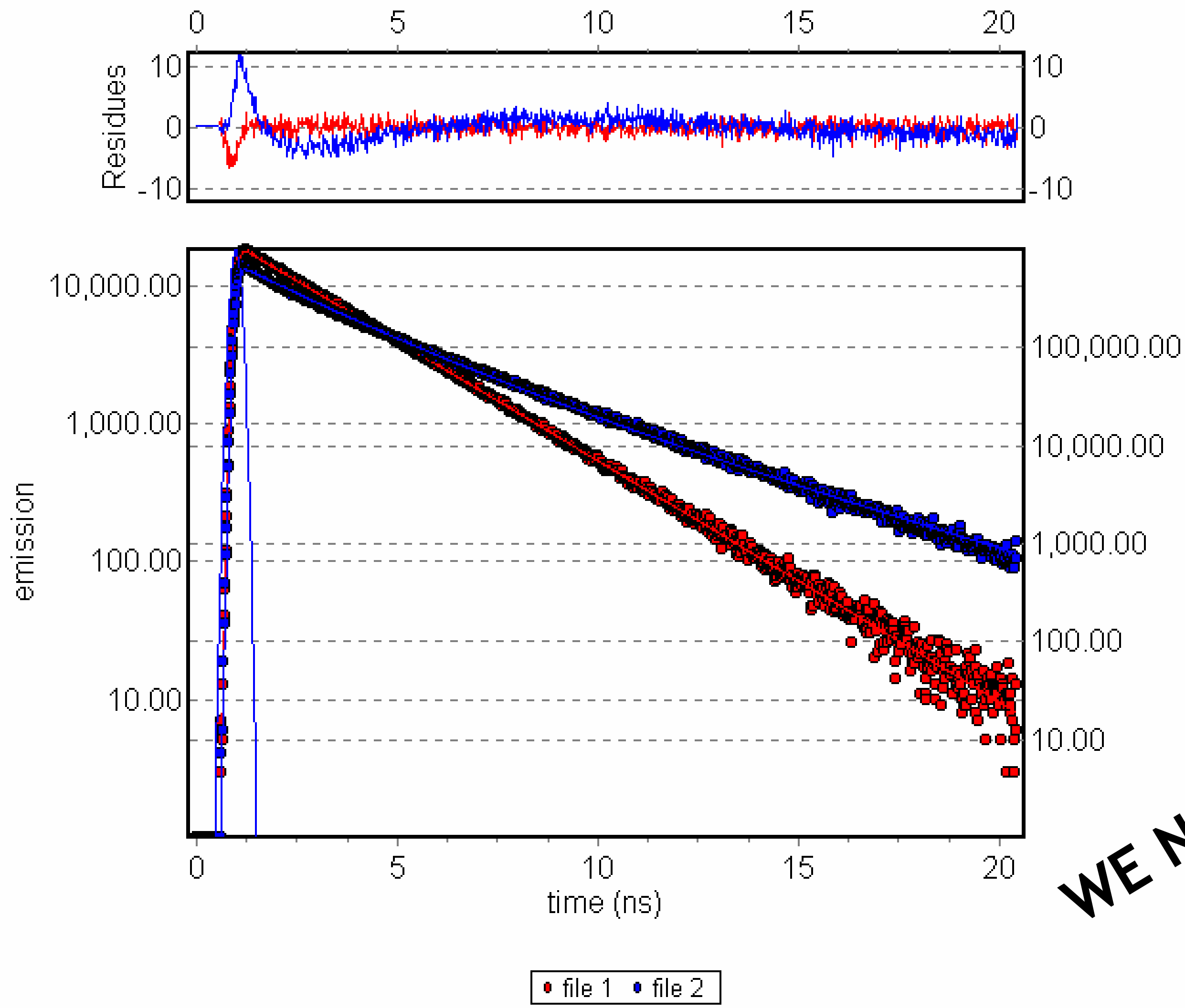
Question: Is there a "significant" difference between one length and a distribution of lengths?

Clearly the fit distinguishes the two cases if we ask the question: what is the width of the length distribution?




```
Discrete
Local chisquare =              1.080
    Fr_ex donor 1->0 = V   0.33
    Fr_em donor 1->0 = V   0.00
      Tau donor 1->0 = F   5.00
   Tau acceptor 1->0 = F   2.00
Distance D to A 1->0 = F 40.00
       Ro (in A) 1->0 = F 40.00
 Distance width 1->0 = V   0.58

Gaussian distributed
Local chisquare =              1.229
    Fr_ex donor 1->0 = V   0.19
    Fr_em donor 1->0 = V   0.96
      Tau donor 1->0 = L   5.00
   Tau acceptor 1->0 = L   2.00
Distance D to A 1->0 = L 40.00
       Ro (in A) 1->0 = L 40.00
 Distance width 1->0 = V 26.66
```

FRET-decay, discrete and distance gaussian distributed
Fit attempt using 2-exponential linked
The fit is “poor” using sum of exponentials linked. However, the fit is good if the exponentials are not linked, but the values are unphysical



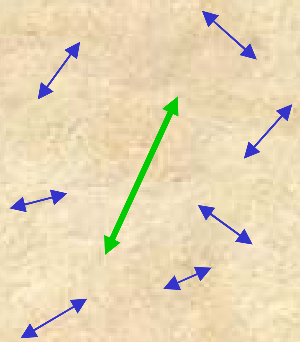

```
Discrete distance:
Local chisquare = 1.422
      sas 1->0 = V  0.00
discrete 1->0 = V  5.10
      sas 2->0 = V  0.99
discrete 2->0 = L  2.49

Gaussian distr distances
Experiment #   2 results:
Local chisquare =  4.61
      sas 1->0 = V  0.53
discrete 1->0 = L  5.10
      sas 2->0 = V  0.47
discrete 2->0 = L  2.49
```

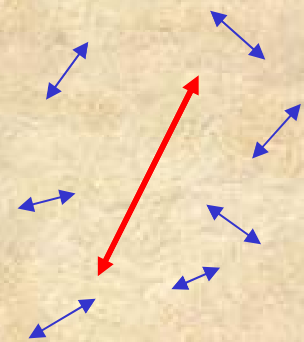
WE NEED GLOBALS!

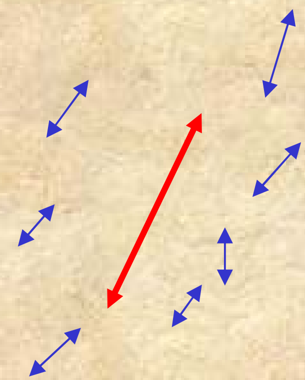
Time dependent spectral relaxations

Solvent dipolar orientation relaxation

10^{-15} s 10^{-9} s





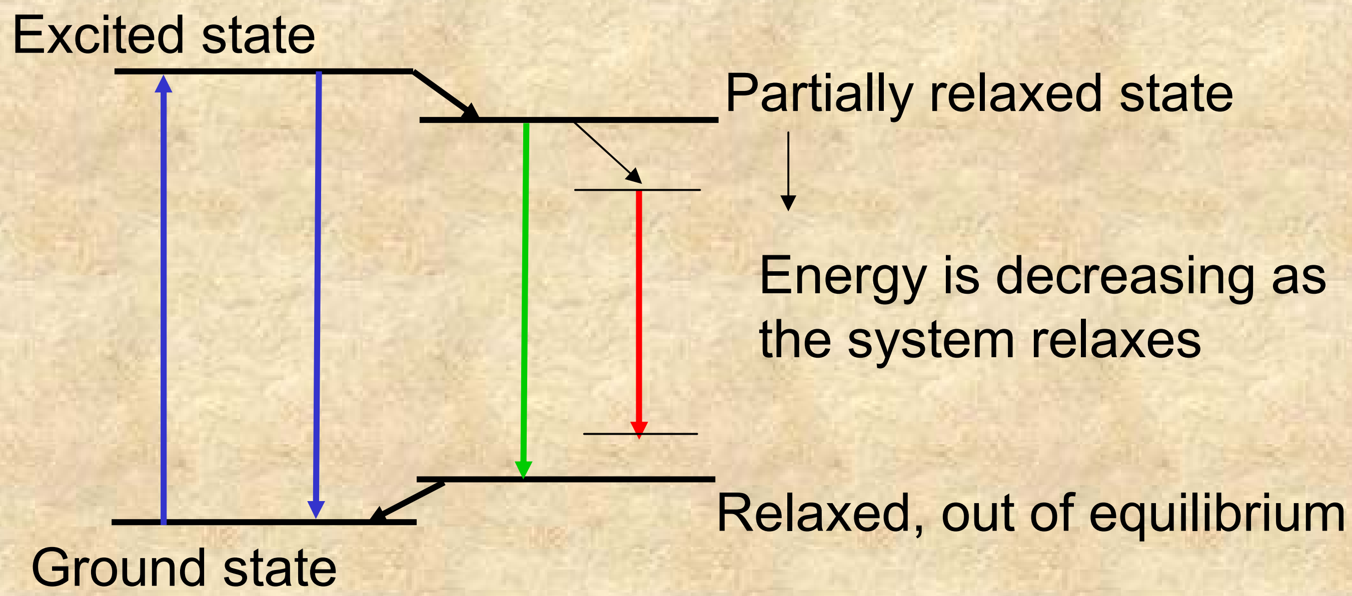


Ground state | Frank-Condon state | Relaxed state

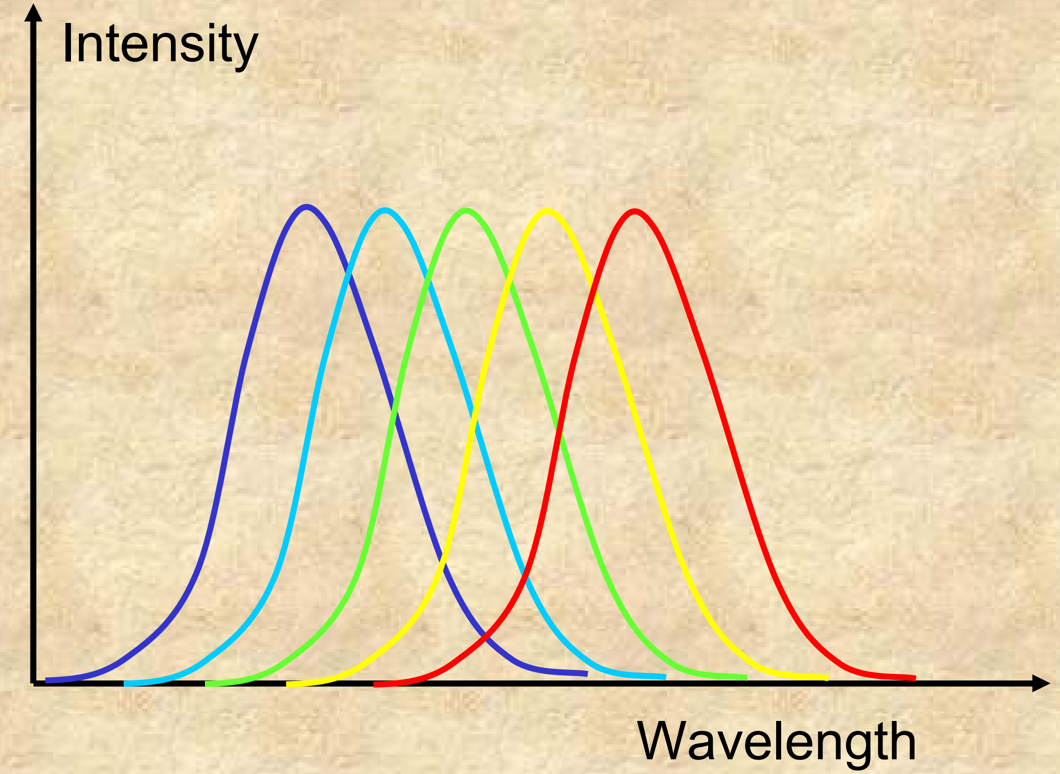
Immediately after excitation | Long time after excitation

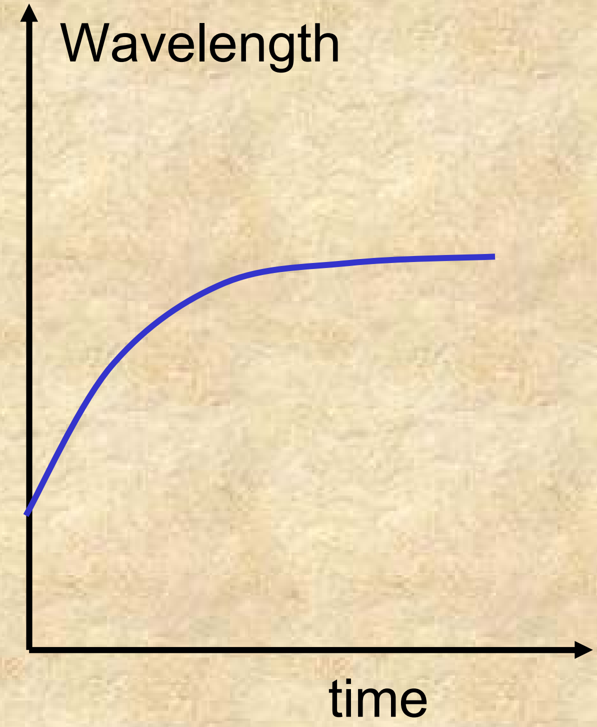
Equilibrium | Out of Equilibrium | Equilibrium

As the relaxation proceeds, the energy of the excited state decreases and the emission moves toward the red



The emission spectrum moves toward the red with time

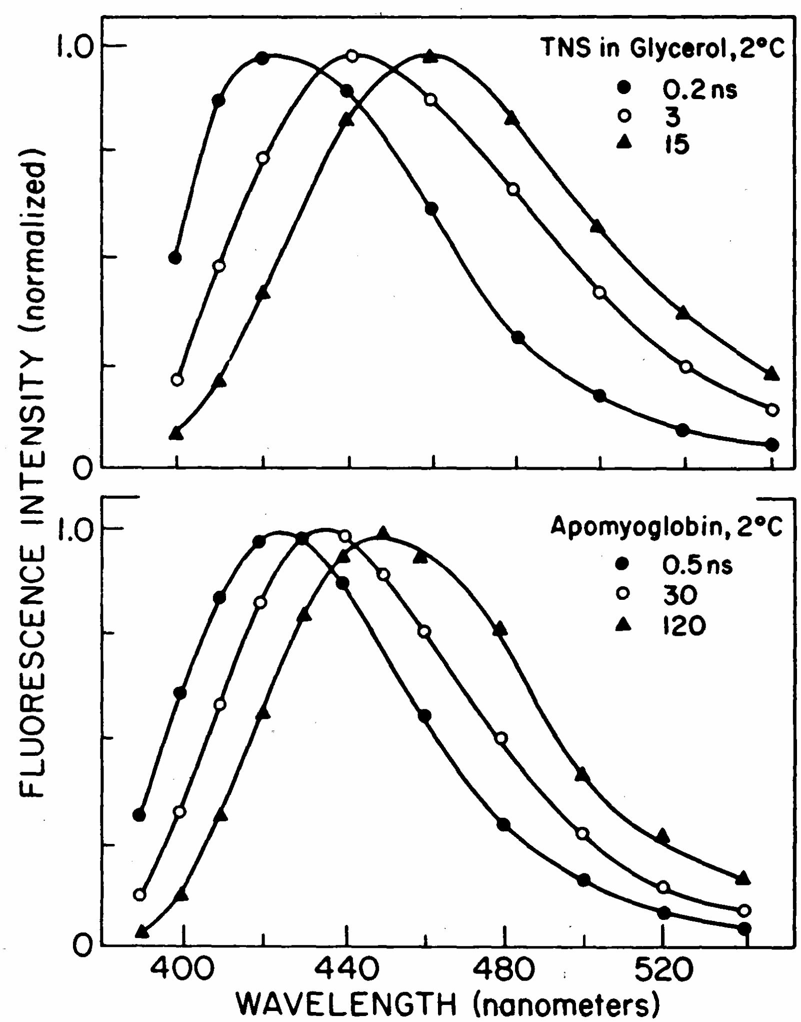


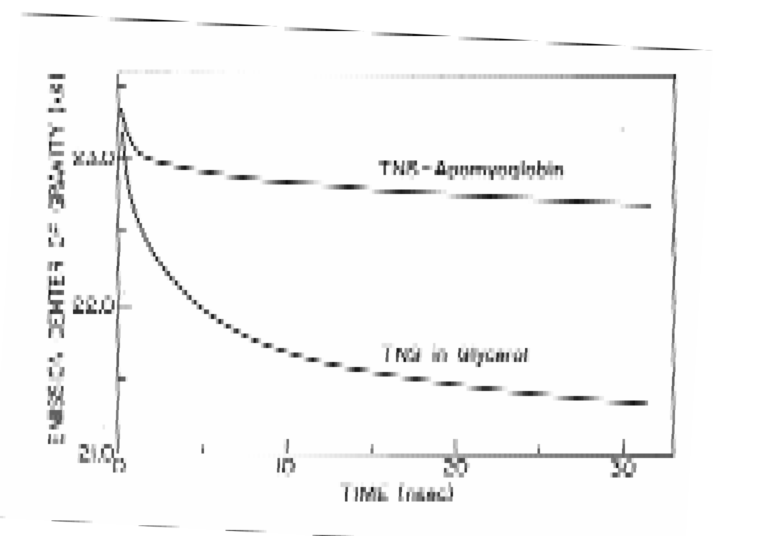


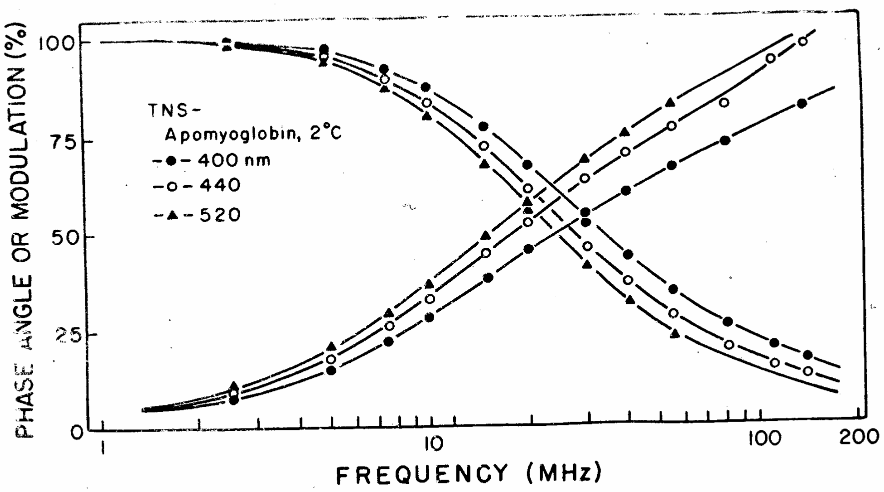
Time resolved spectra

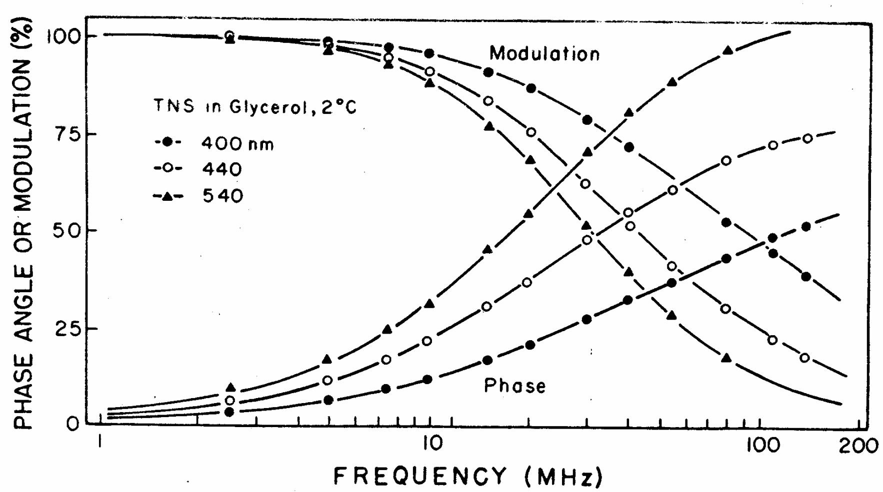
What happens to the spectral width?

Time resolved spectra of TNS in a Viscous solvent and in a protein

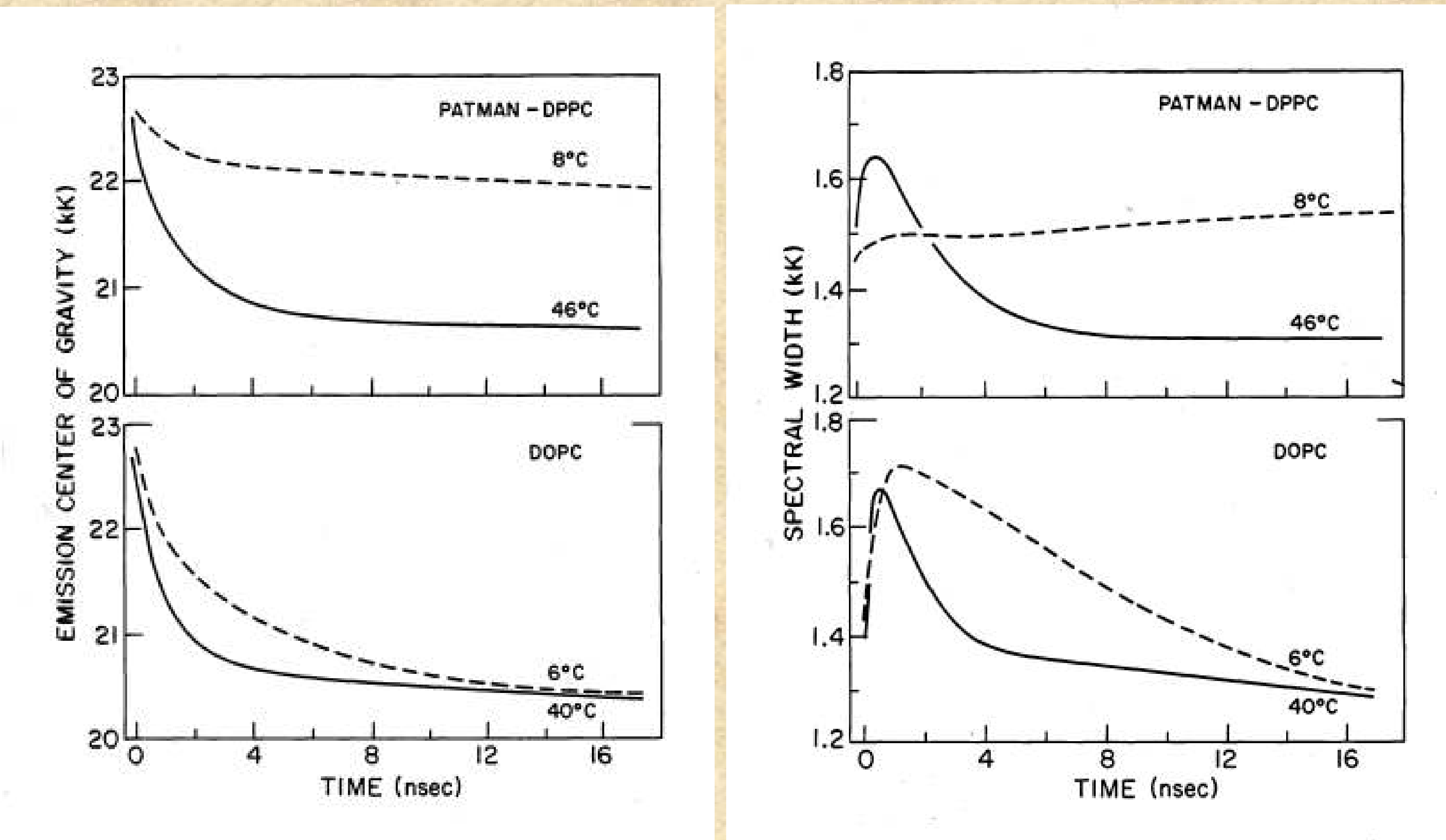


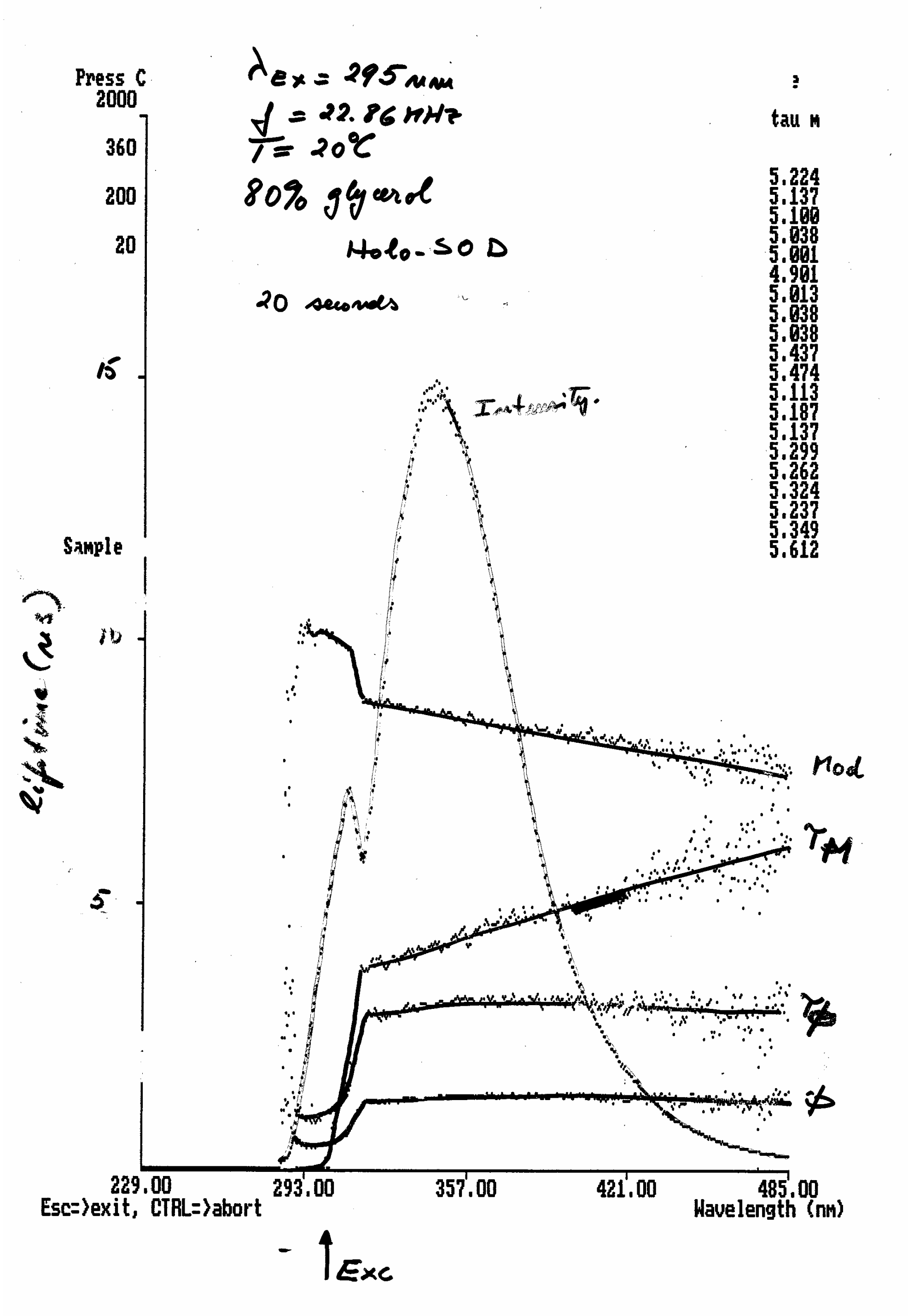






Time resolved spectra are built by recording of individual decays at different wavelengths


PATMAN - DPPC
8°C
46°C
DOPC
6°C
40°C
EMISSION CENTER OF GRAVITY (kK)
TIME (nsec)
PATMAN - DPPC
8°C
46°C
DOPC
6°C
40°C
SPECTRAL WIDTH (kK)
TIME (nsec)

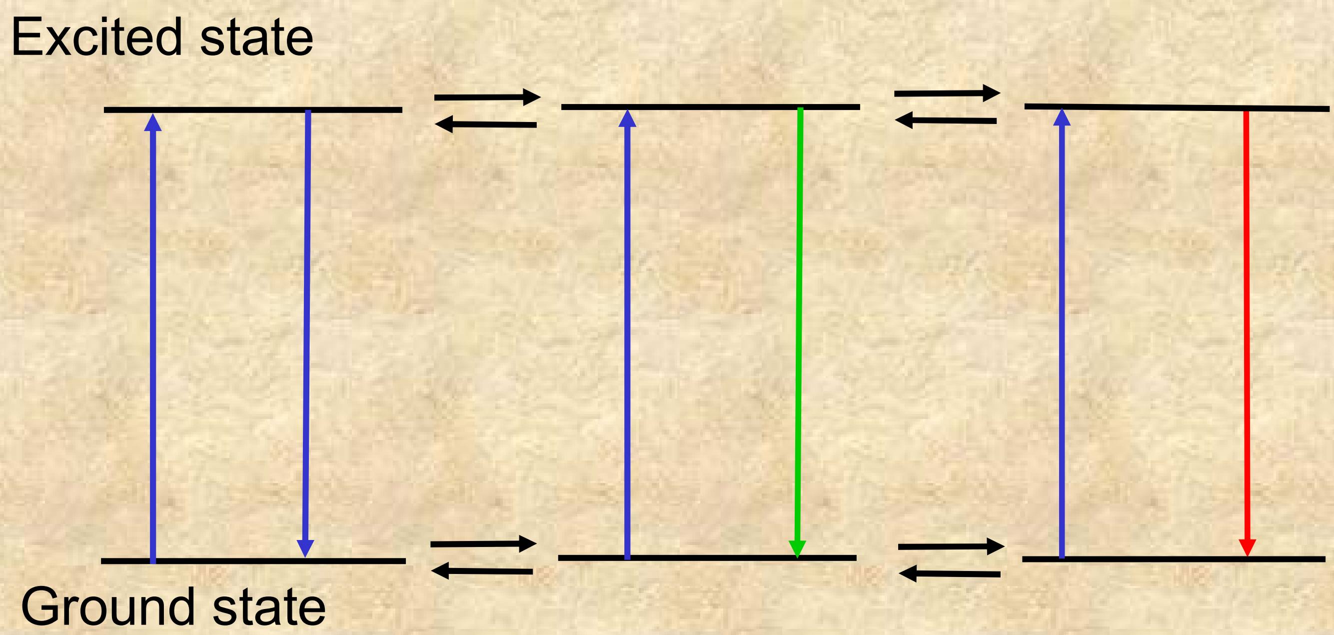


Time resolved spectra can also be recoded at once using time-resolved optical multichannel analyzers

Excited-state reactions

- Excited state protonation-deprotonation
- Electron-transfer ionizations
- Dipolar relaxations
- Twisting-rotations isomerizations
- Solvent cage relaxation
- Quenching
- Dark-states
- Bleaching
- FRET energy transfer
- Monomer-Excimer formation

General scheme



Reactions can be either sequential or branching

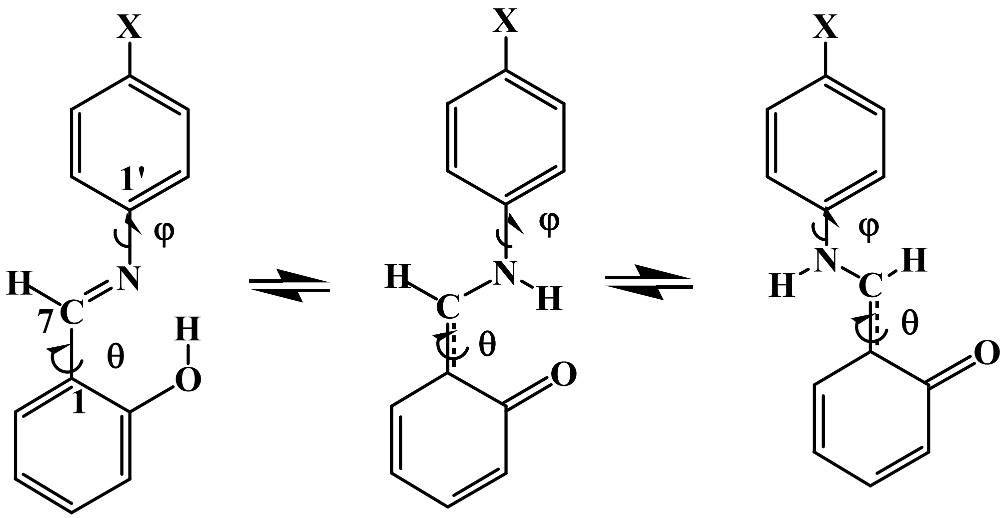
If the reaction rates are constant, then the solution of the dynamics of the system is a sum of exponentials. The number of exponentials is equal to the number of states

If the system has two states, the decay is doubly exponential

Attention: None of the decay rates correspond to the lifetime of the excited state nor to the reaction rates, but they are a combination of both

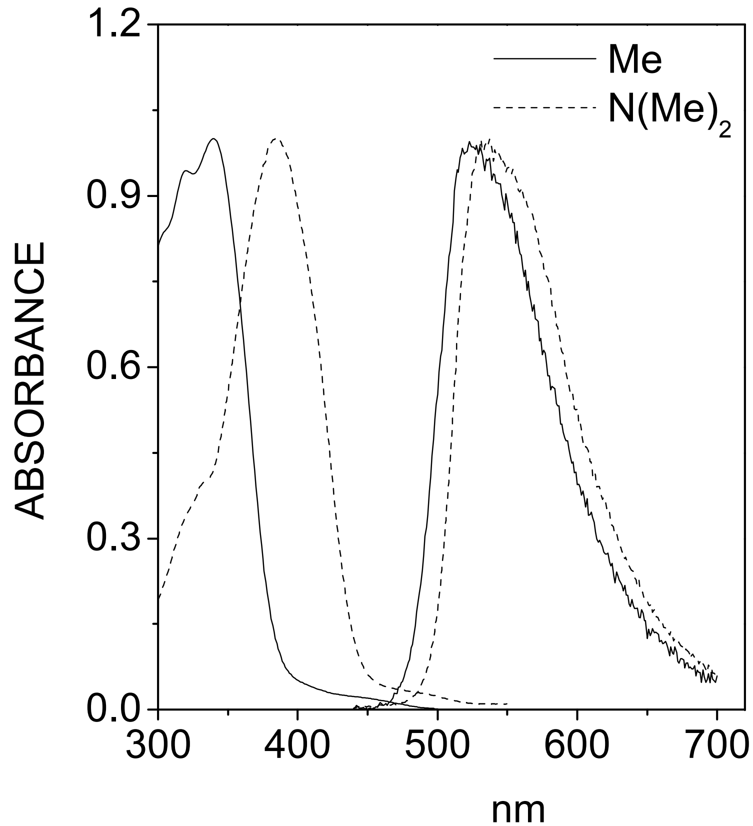
Upon excitation, there is a cis-trans isomerization

N-salicylidene-*p*-X-aniline

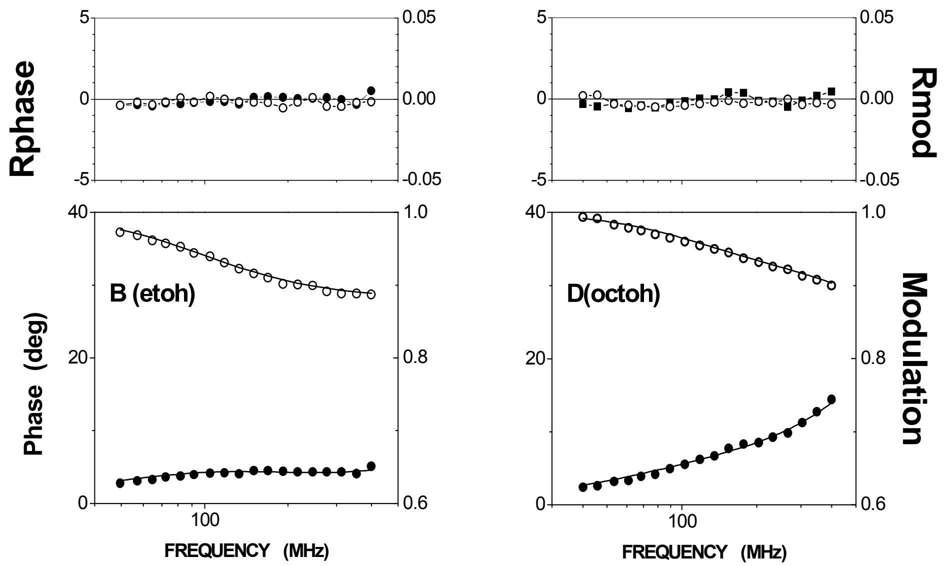


enol-imine　　*cis*-keto-amine　　*trans* keto-amine

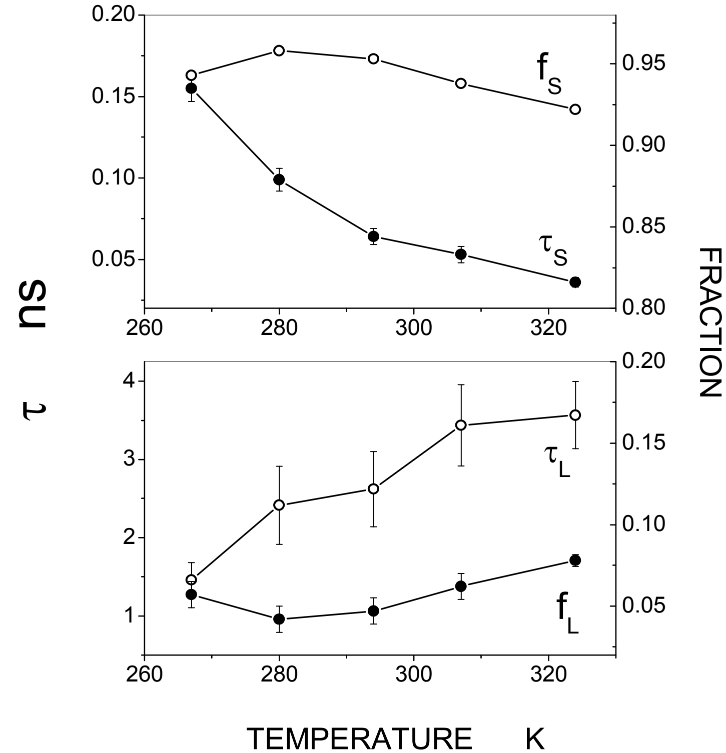
1 X= NO_2　　**4 X= Me**
2 X= CN　　**5 X= OMe**
3 X= H　　**6 X= NMe_2**



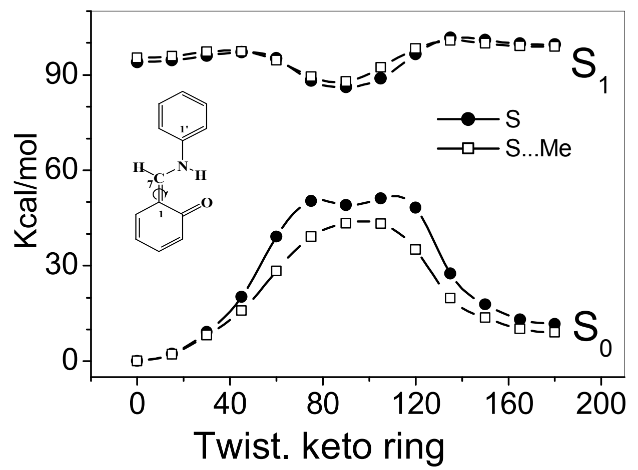
Experimental data in the frequency-domain



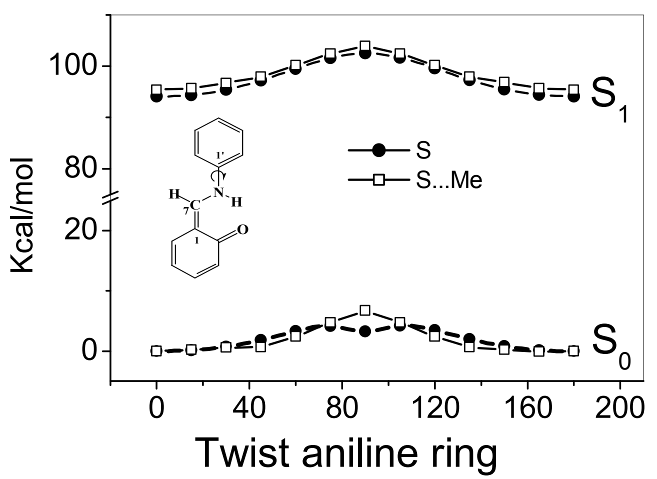
Parameters from data fit



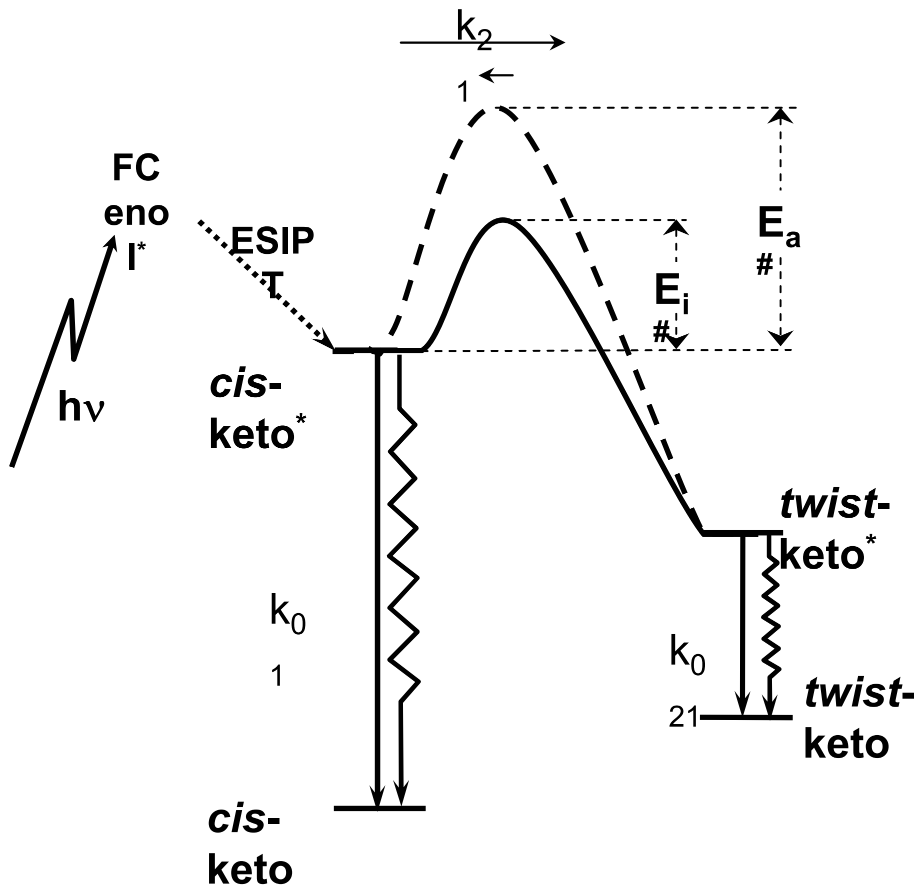
The Model



Kcal/mol
90
60
30
0
0 40 80 120 160 200
Twist. keto ring
S1
S0
S
S...Me

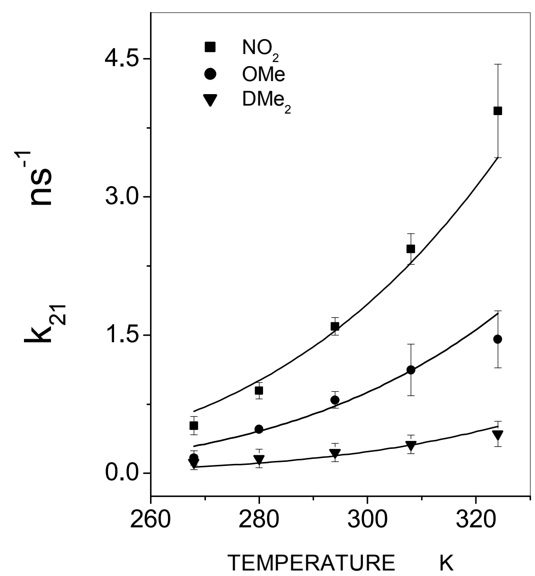


Kcal/mol
100
80
20
0
0 40 80 120 160 200
Twist aniline ring
S1
S0
S
S...Me

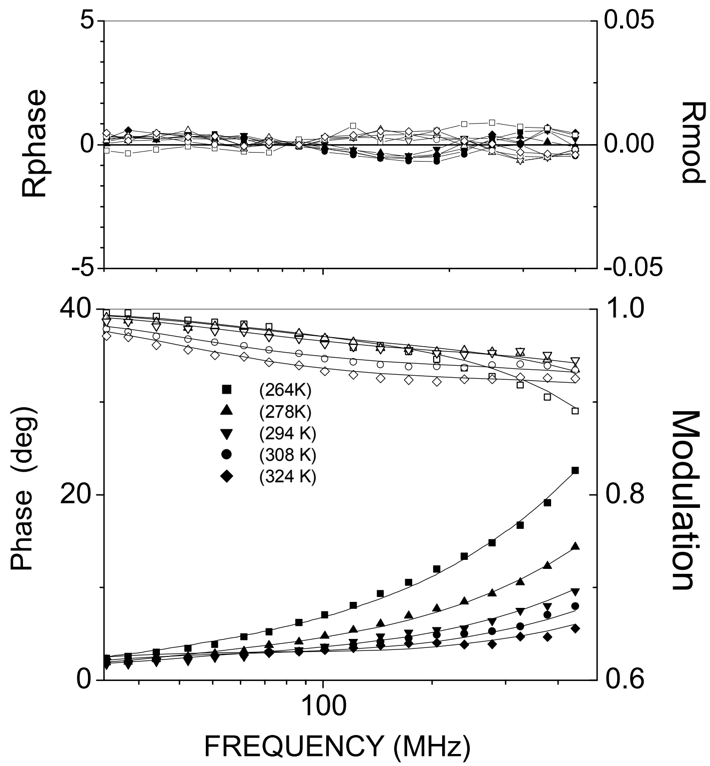


k2
1
FC
enol*
ESIPT
hν
cis-
keto*
Ei
#
Ea
#
twist-
keto*
k0
1
k0
21
twist-
keto
cis-
keto

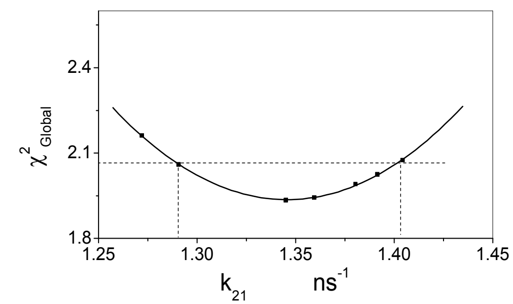
Temperature dependence of rates

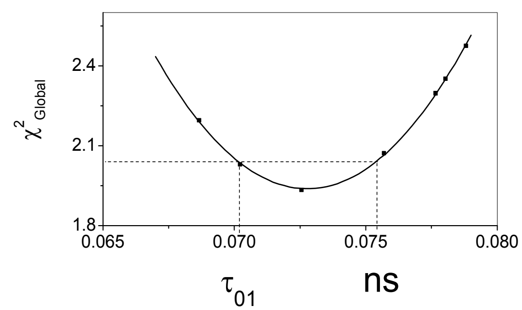


Global Fit



Error analysis





Ready for publication!

Sources on polarization and time-resolved theory and practice:

Books:

Molecular Fluorescence (2002) by Bernard Valeur
Wiley-VCH Publishers

Principles of Fluorescence Spectroscopy (1999) by Joseph Lakowicz
Kluwer Academic/Plenum Publishers

Edited books:

Methods in Enzymology (1997) Volume 278 Fluorescence Spectroscopy (edited by L. Brand and M.L. Johnson)

Methods in Enzymology (2003) Biophotonics (edited by G. Marriott and I. Parker)

Topics in Fluorescence Spectroscopy: Volumes 1-6
(edited by J. Lakowicz)