# 4. Time-resolved Fluorescence II

- 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 will change as a function of time.

Changes can be due to:

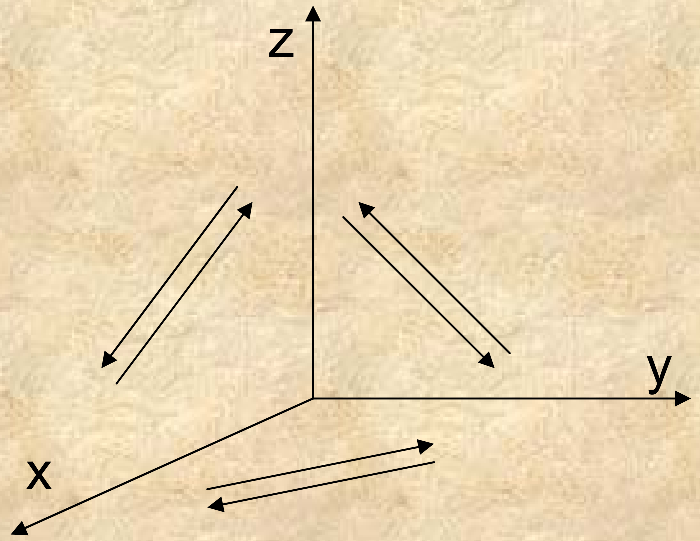
- Internal conversion to different electronic states
- changes in spatial orientation of the molecule
- energy transfer to a fluorescence acceptor with different orientation

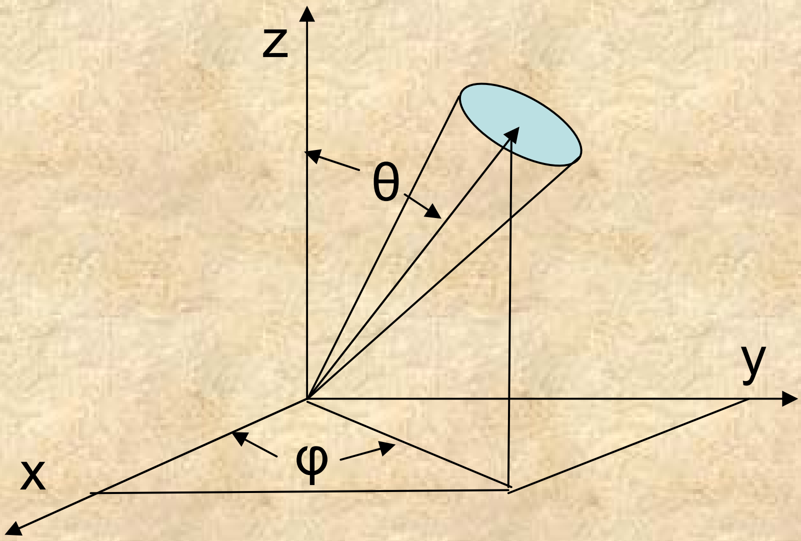
# Anisotropy Decay

Transfer of emission from one direction of polarization to another

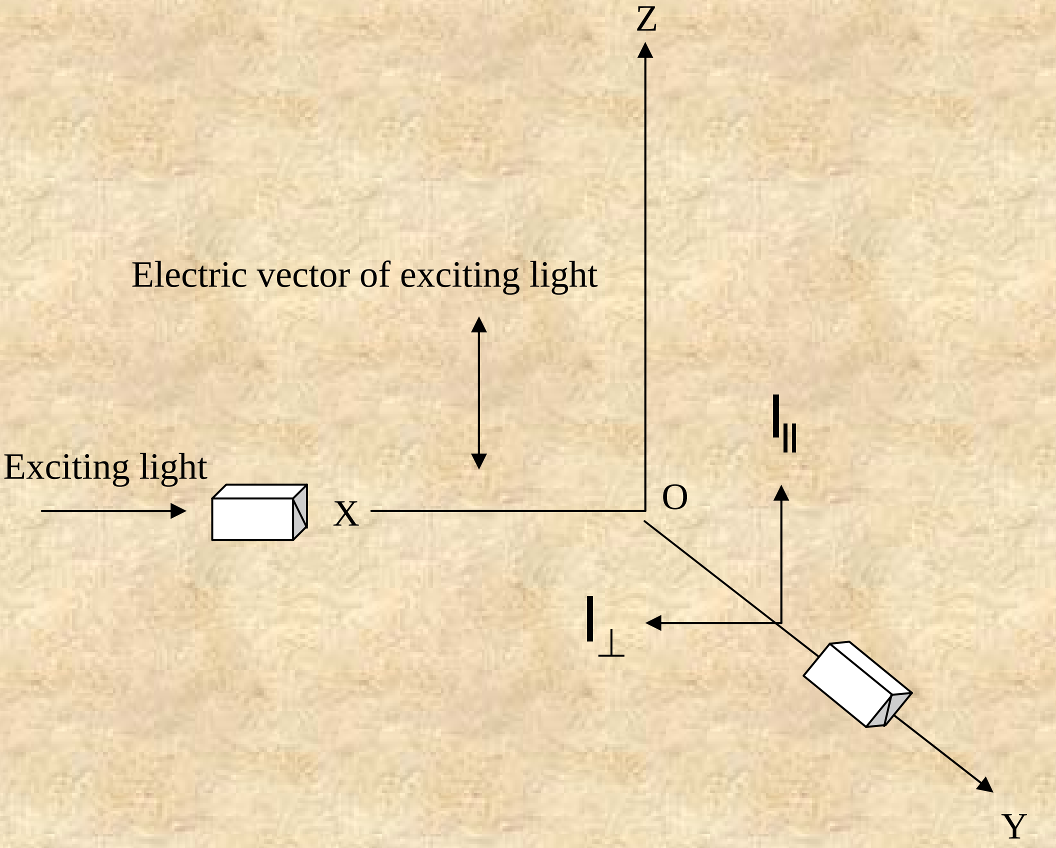
Two different approaches

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





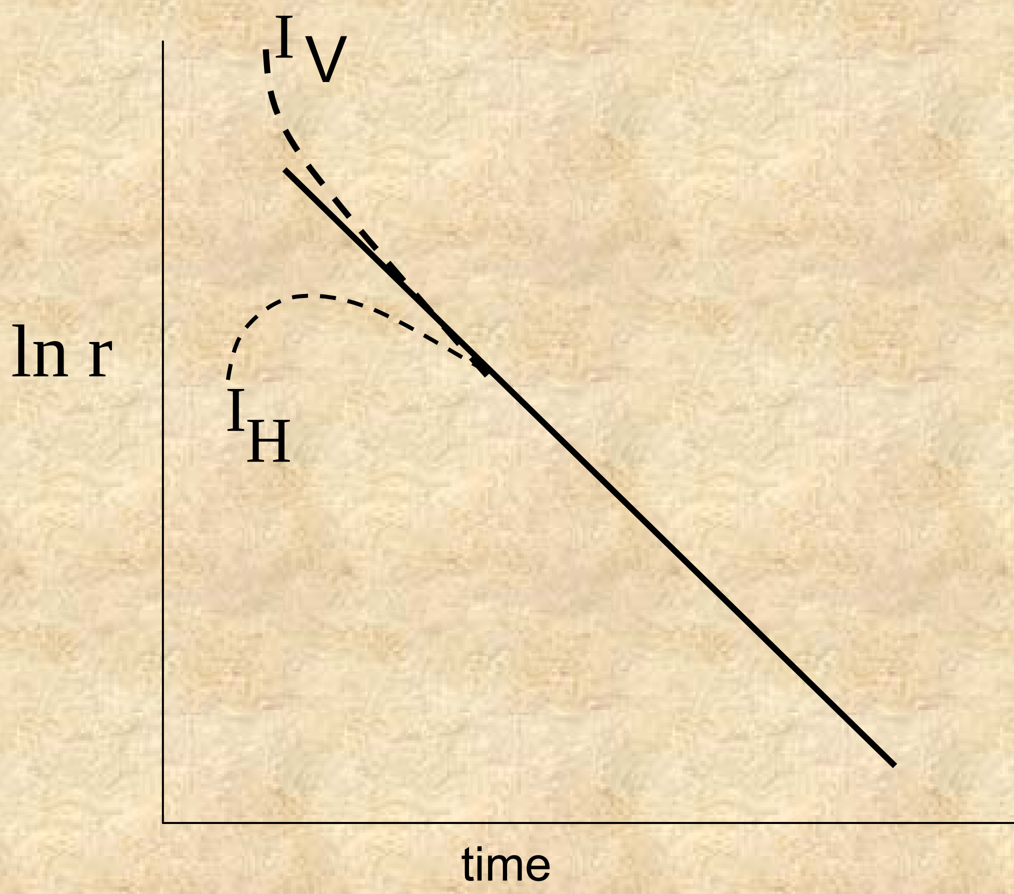
# Geometry for excitation and emission polarization



**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.**

Time-resolved methodologies provide information on 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**. In principle both methods yield the same information.

In the time-domain anisotropy 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:

$$r_t = r_o e^{-(t/\tau_c)}$$

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.

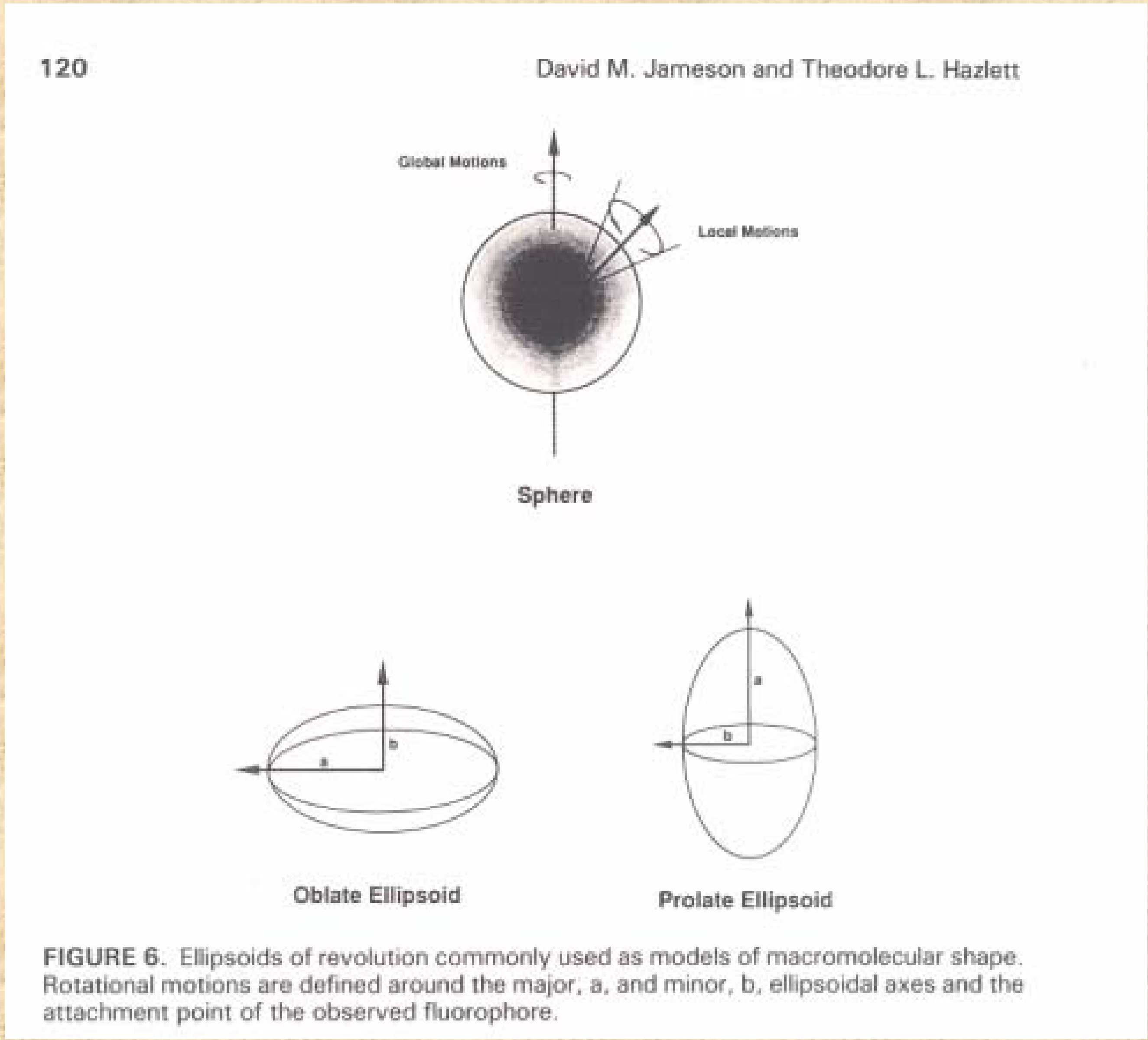


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**FIGURE 6.** Ellipsoids of revolution commonly used as models of macromolecular shape. Rotational motions are defined around the major, a, and minor, b, ellipsoidal axes and the attachment point of the observed fluorophore.

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 $\phi_1$ and $\phi_2$ are the angles between the absorption and emission dipoles, respectively, with the symmetry axis of the ellipsoid and $\varphi$ 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 (or relaxation) 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 $\sigma_1$ represents the "local" probe motion, $\sigma_2$ represents the "global" rotation of the macromolecule and $r_1 = r_0(1-\theta)$ and $\theta$ is the cone angle of the local motion

In dynamic polarization measurements, the sample is illuminated with vertically polarized modulated light and the phase delay 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

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

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

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

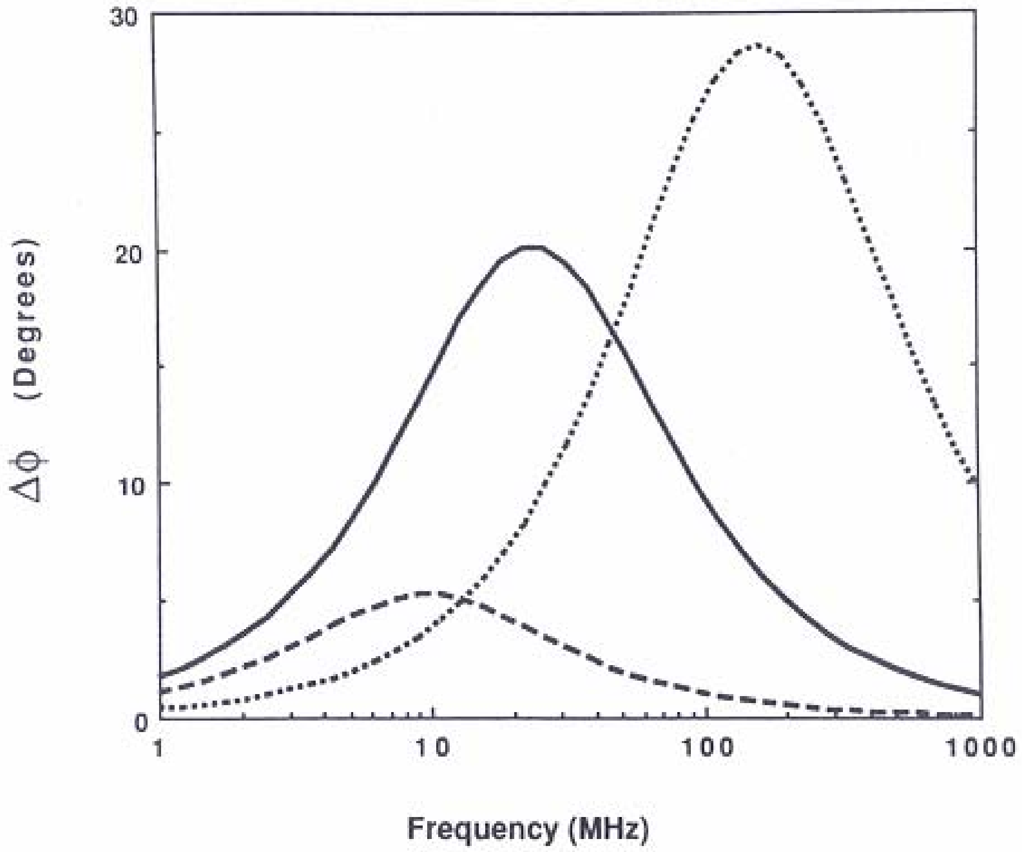

**FIGURE 7.** Differential phase data for an isotropic rotator with a 3-nsec (dotted line), 30-nsec (solid line), or 300-nsec (dashed line) rotational relaxation time. In each case a lifetime of 20 nsec was used and colinear excitation and emission dipoles were assumed.

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.

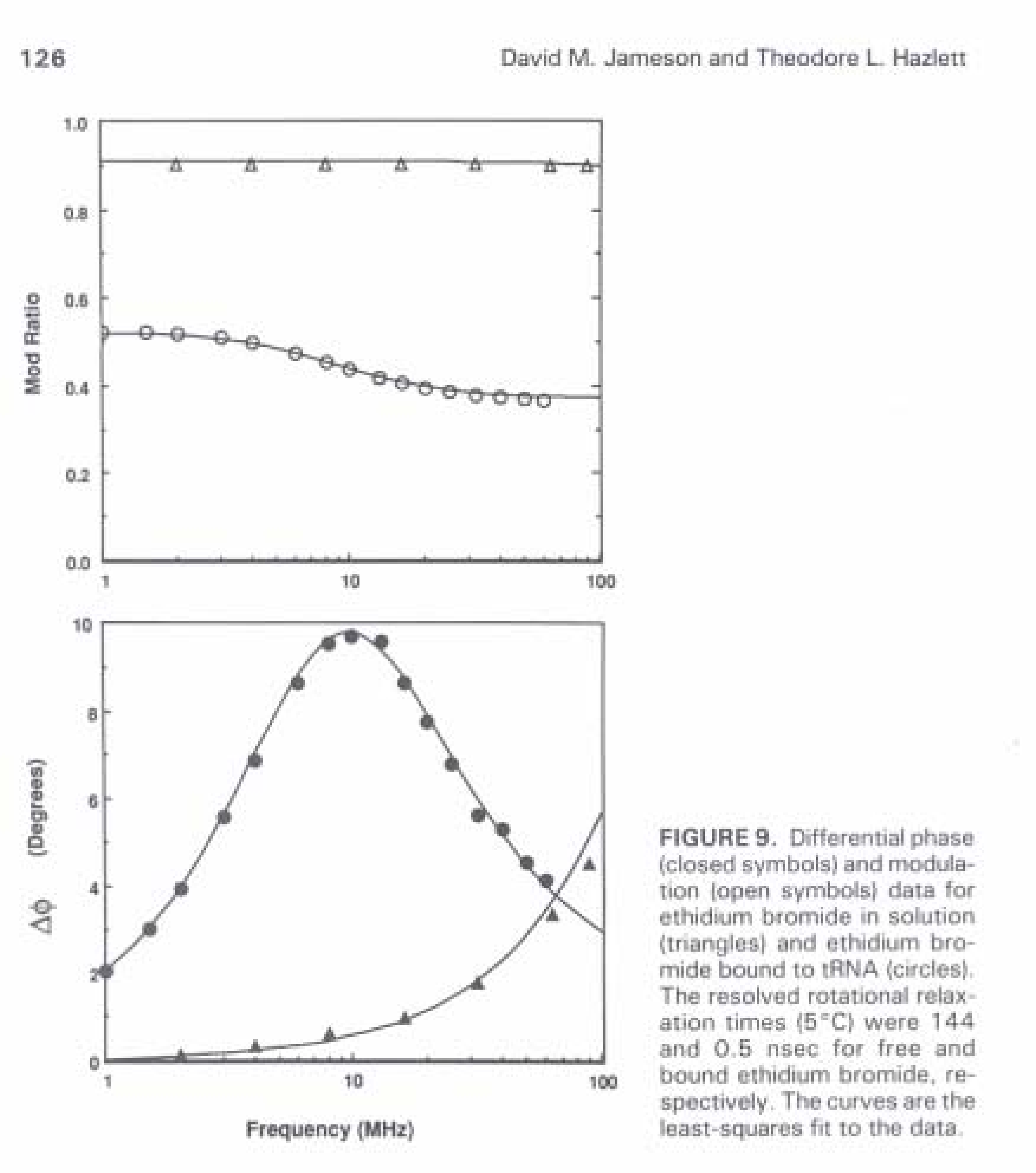

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**FIGURE 9.** Differential phase (closed symbols) and modulation (open symbols) data for ethidium bromide in solution (triangles) and ethidium bromide bound to tRNA (circles). The resolved rotational relaxation times (5°C) were 144 and 0.5 nsec for 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.

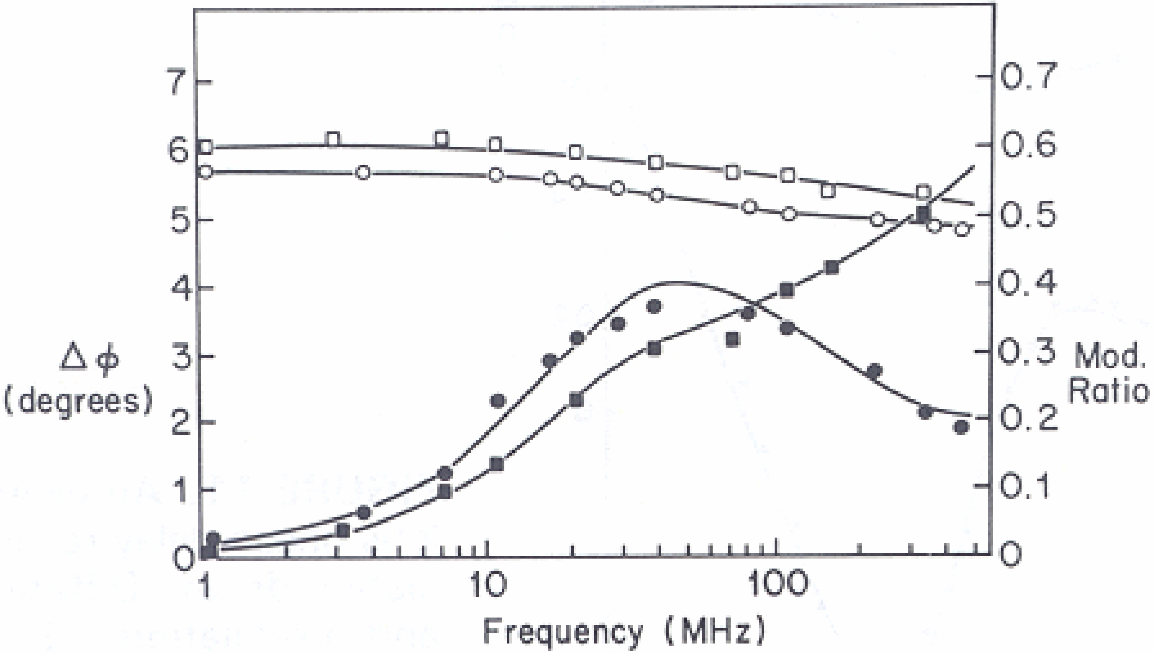
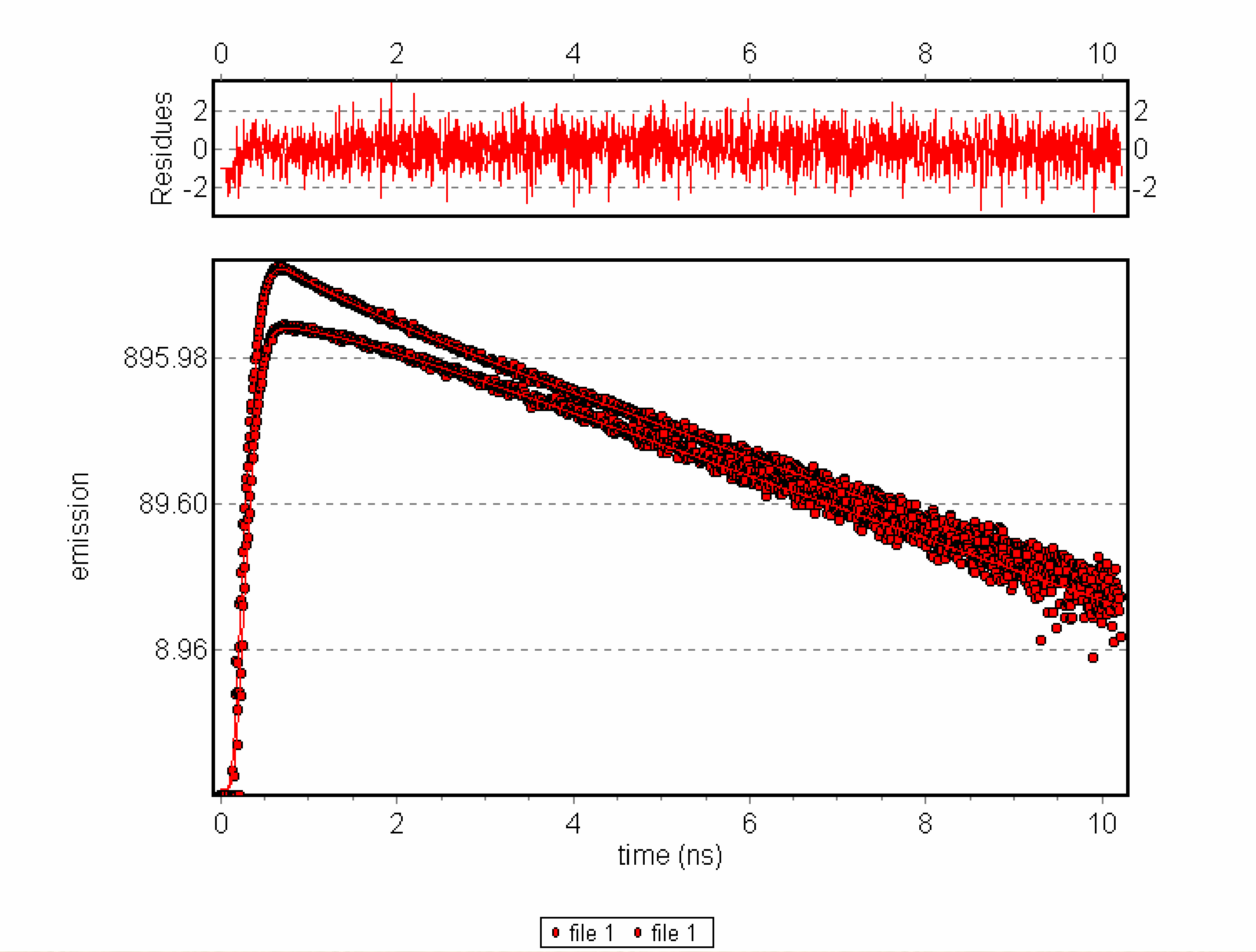
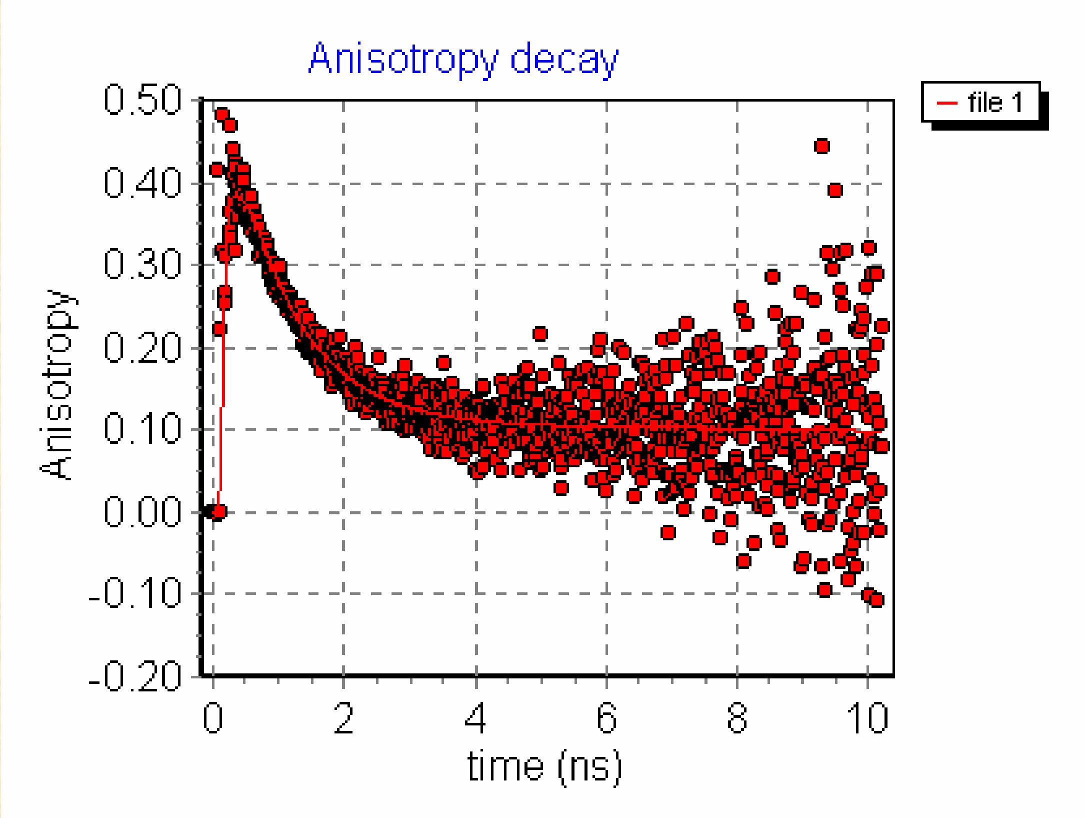


**FIGURE 10.** Multifrequency differential phase (closed symbols) and modulation (open symbols) data for elongation factor Tu complexed with GDP (circles) and elongation factor Ts (squares). Curves represent the least-squares fit to the data.

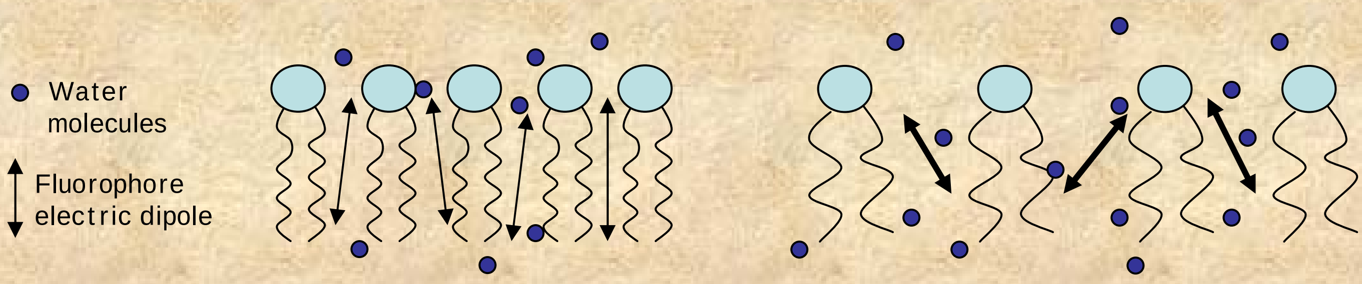
# Time decay anisotropy in the time domain



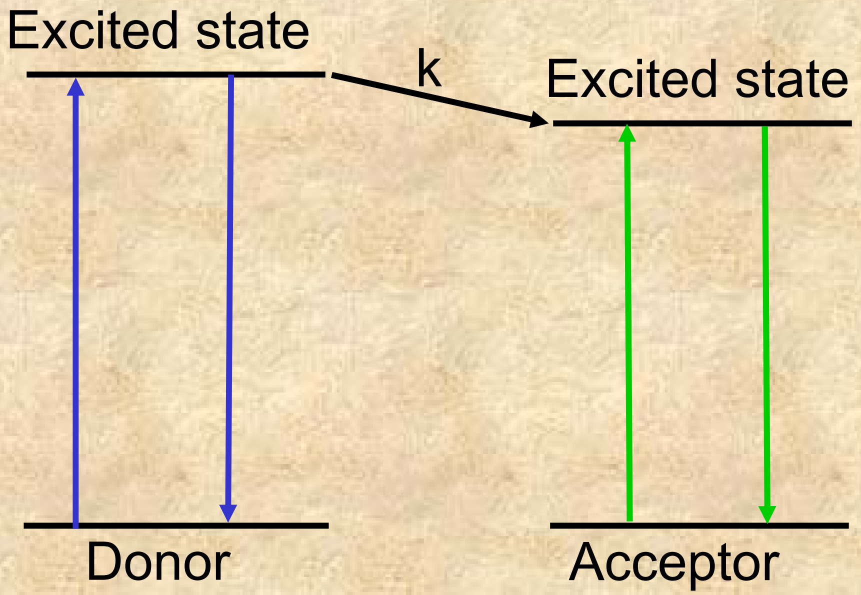
# Anisotropy decay of an hindered rotator



```
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 $\tau_d$ 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:

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

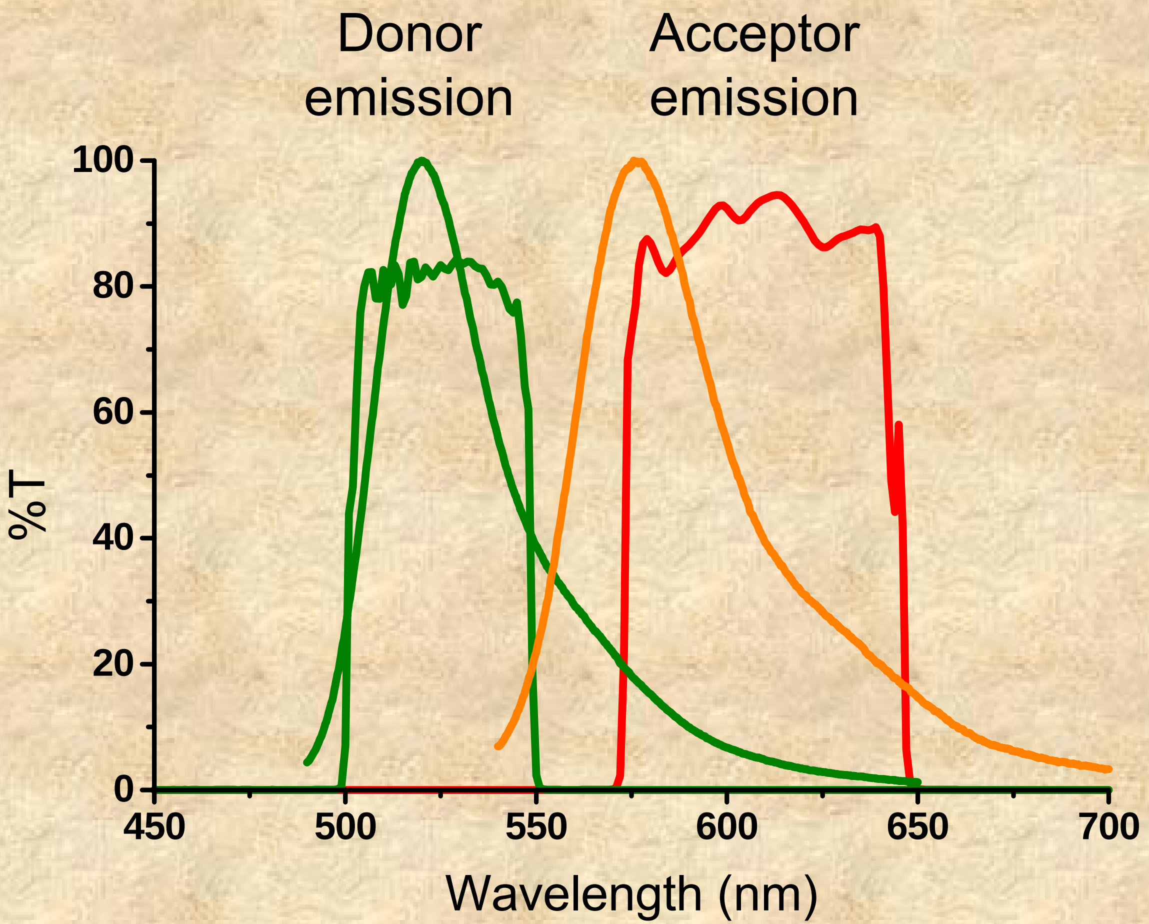
Where $n$ is the refractive index of the medium (usually between 1.2-1.4), $Q_d$ is the fluorescence quantum yield of the donor in absence of acceptor, $\kappa^2$ is the orientation factor for the dipole-dipole interaction and $J$ is the normalized spectral overlap integral. [$\varepsilon(\lambda)$ is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

From Dave Jameson, lecture 2

# 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

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

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

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

$\Gamma_d$ and $\Gamma_a$ 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

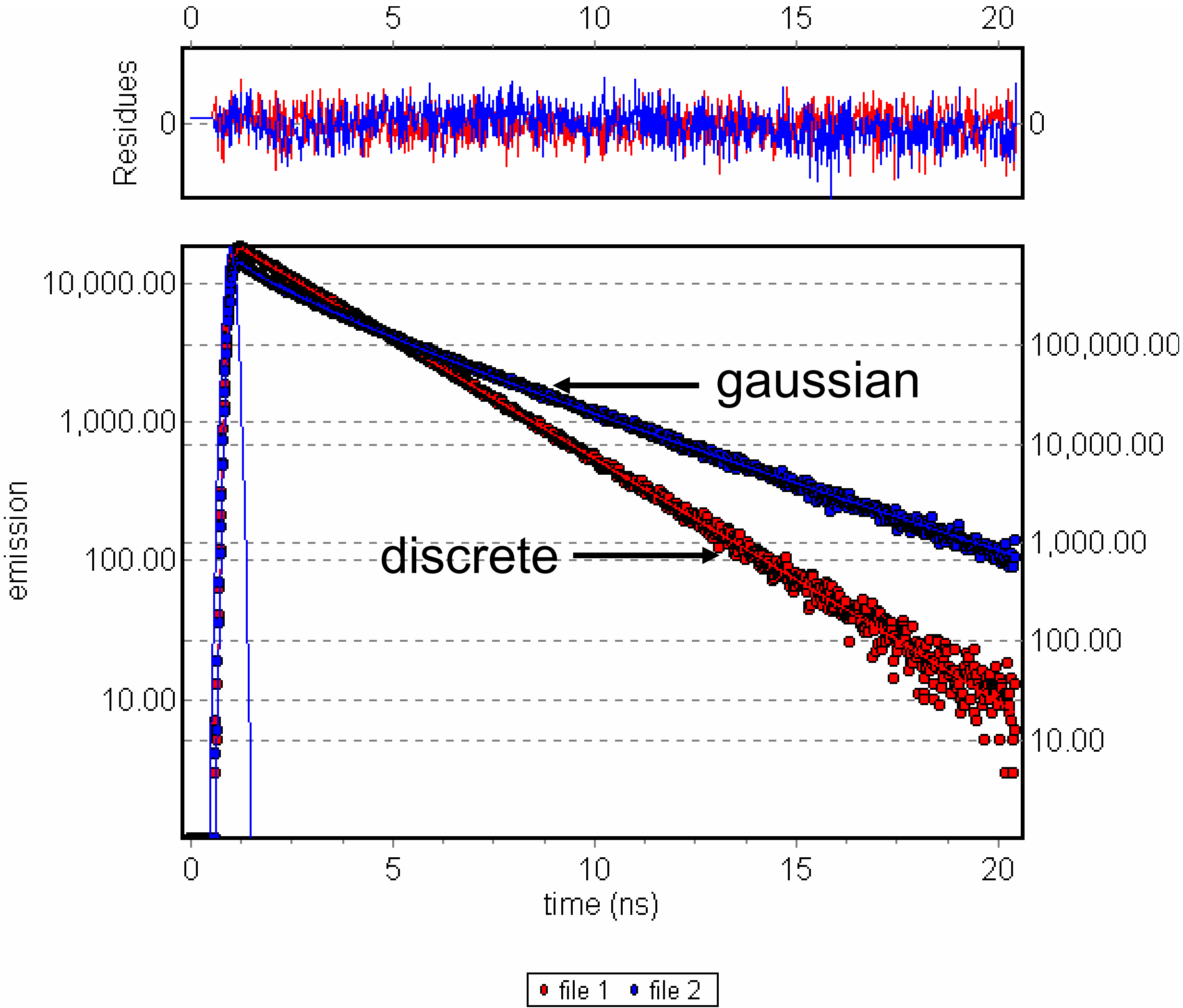
(Next figure)

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?

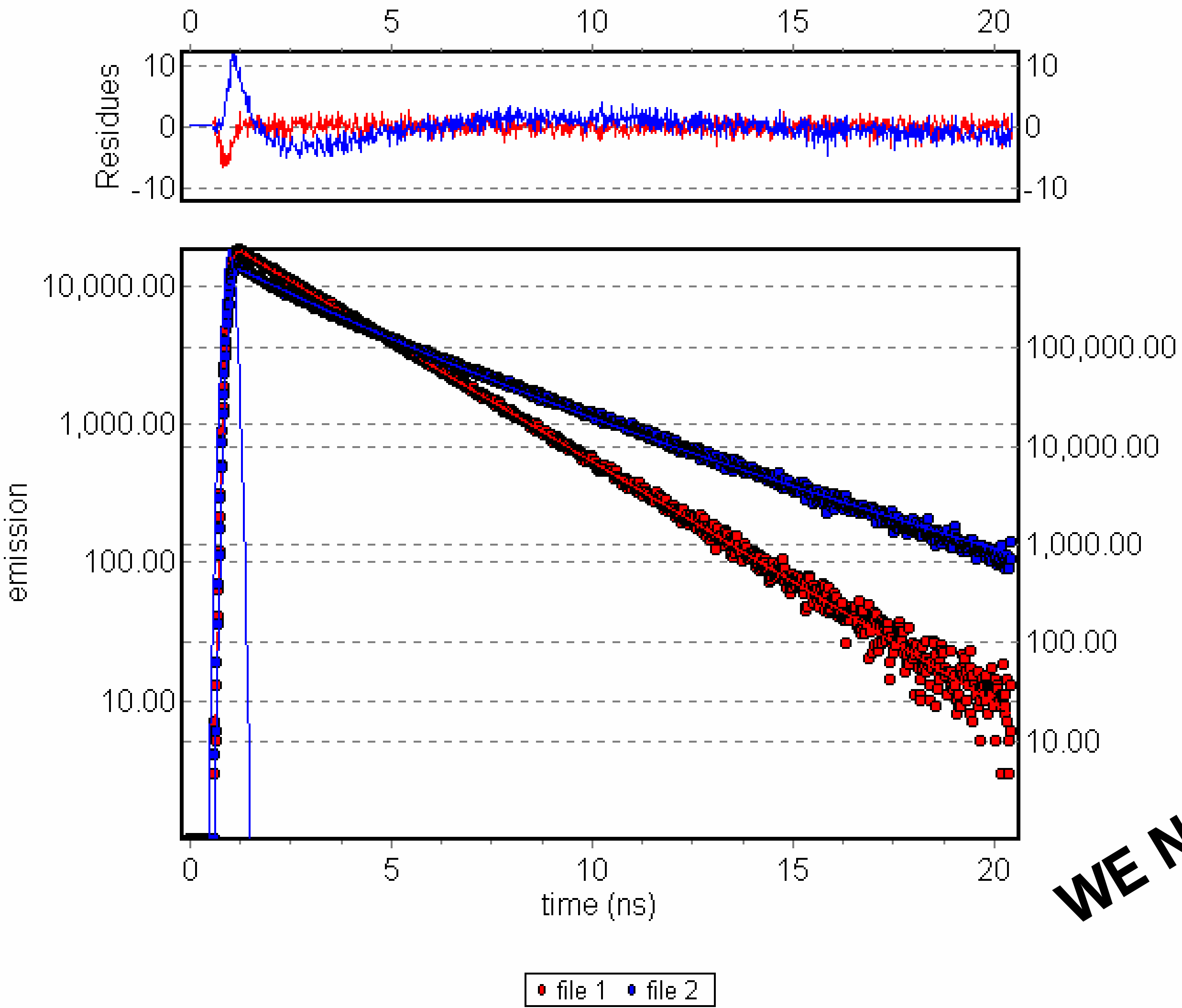
Clearly the fit distinguishes the two cases if we ask the question: what is the width of the length distribution?



```
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



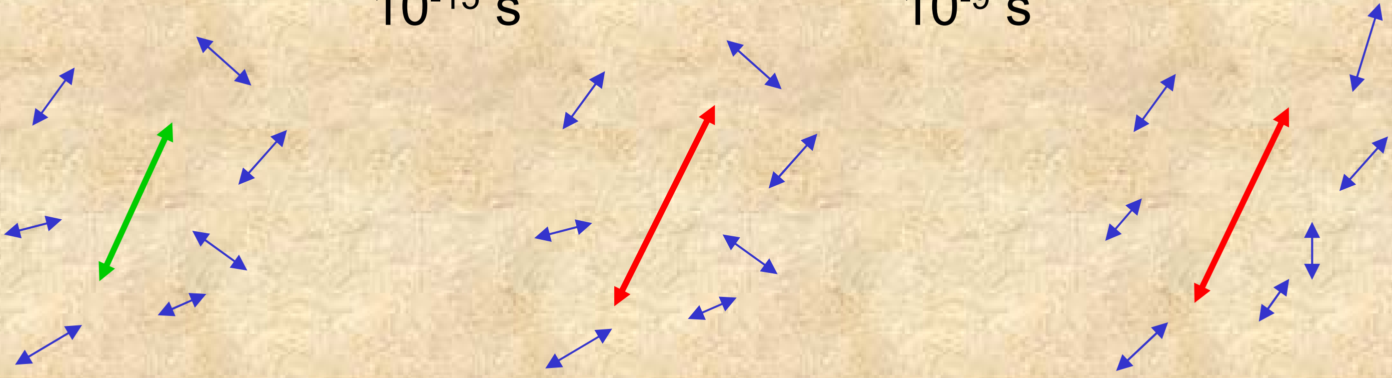
```
Discrete distance:
Local chisquare = 1.422
     sas 1->0 = V  0.00
discrete 1->0 = V  5.10
     sas 2->0 = V  0.99
discrete 2->0 = L  2.49
Gaussian distr distances

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

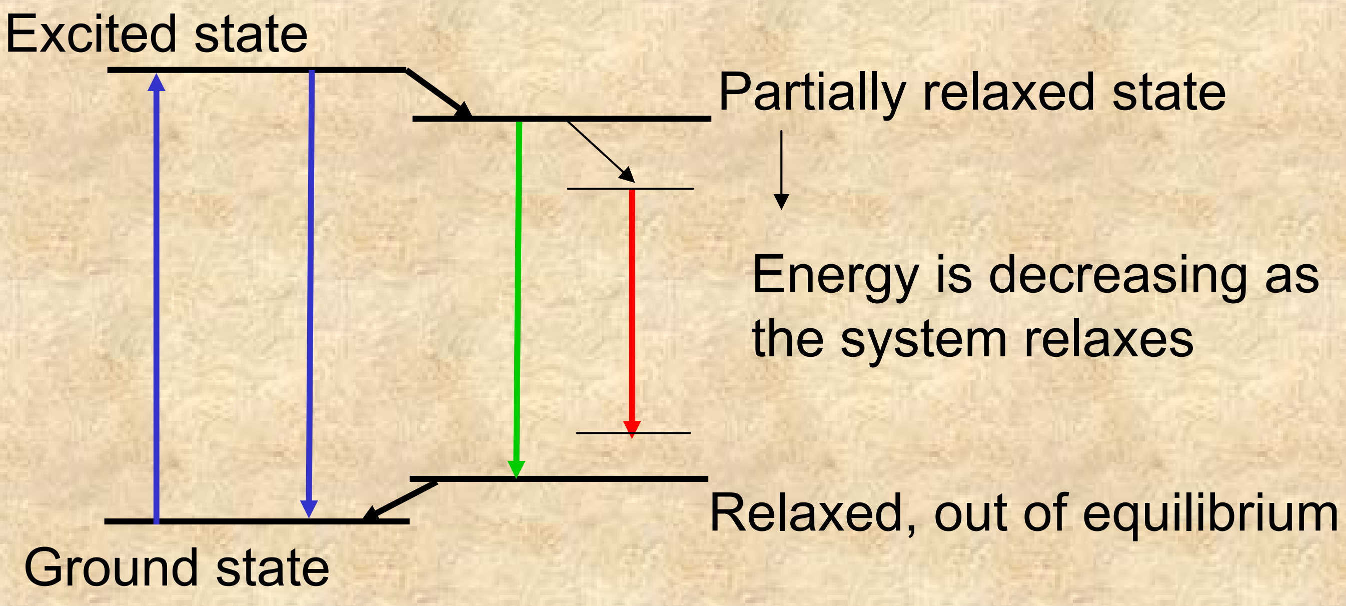
Long time after excitation

Equilibrium

Out of Equilibrium

Equilibrium

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



Excited state

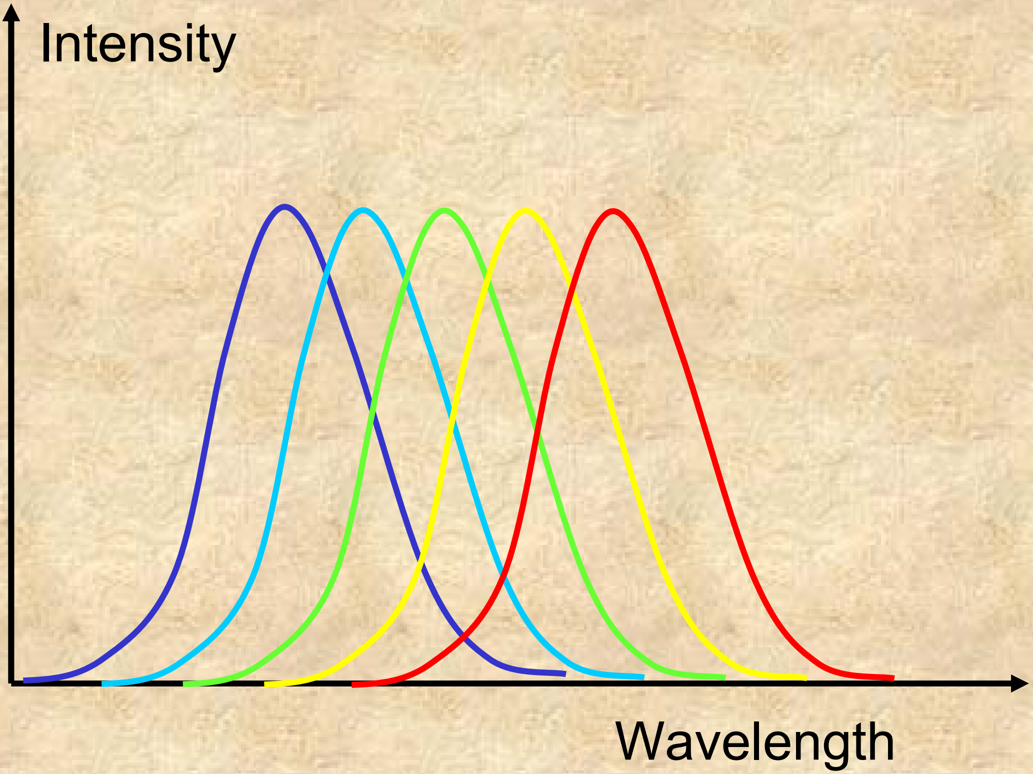
Partially relaxed state

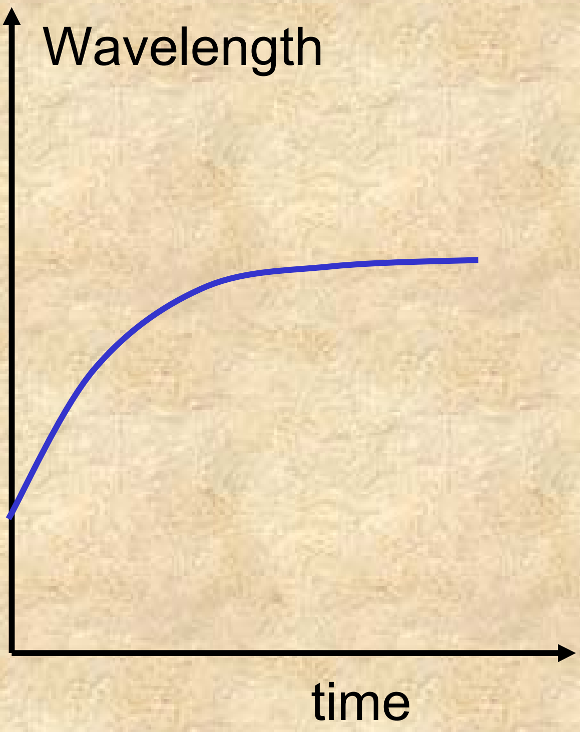
Energy is decreasing as the system relaxes

Relaxed, out of equilibrium

Ground state

# The emission spectrum moves toward the red with time

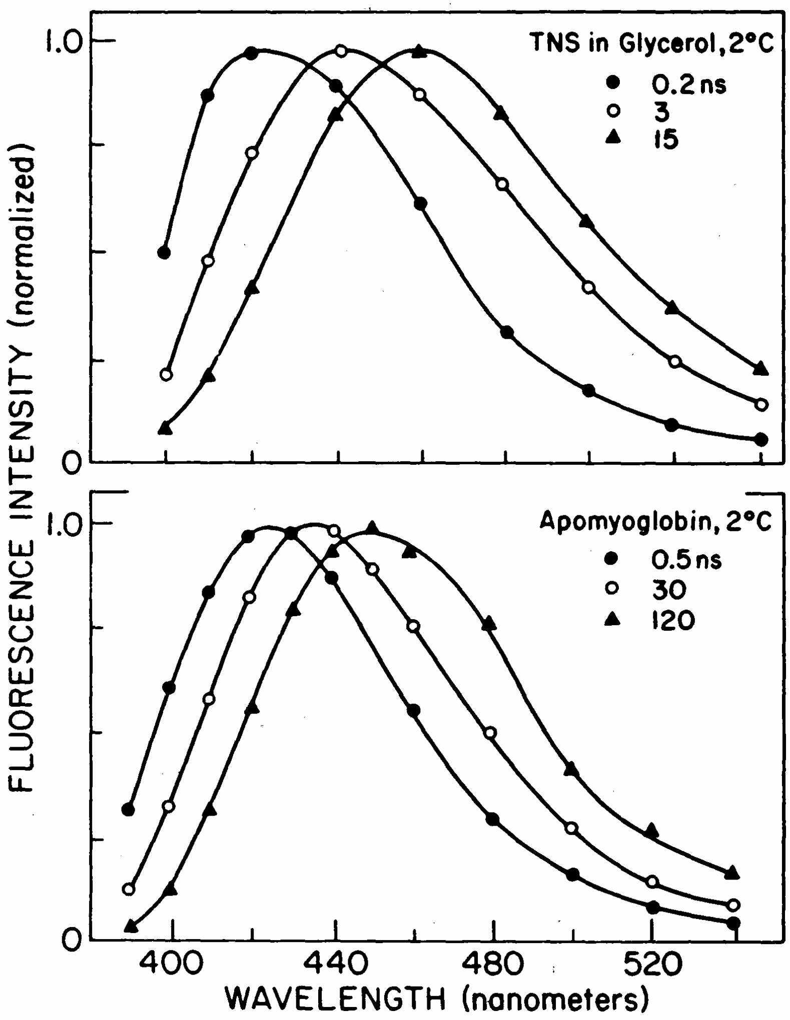


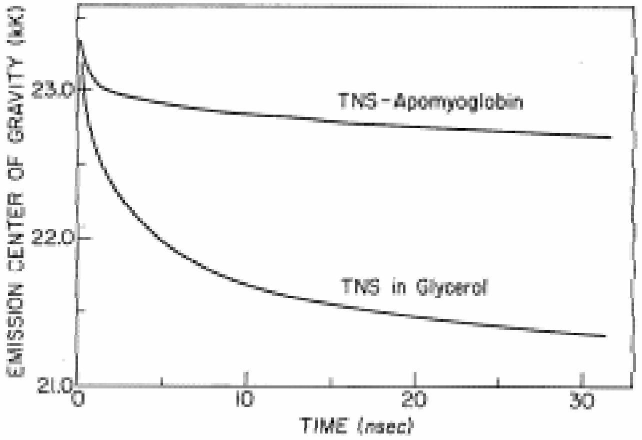


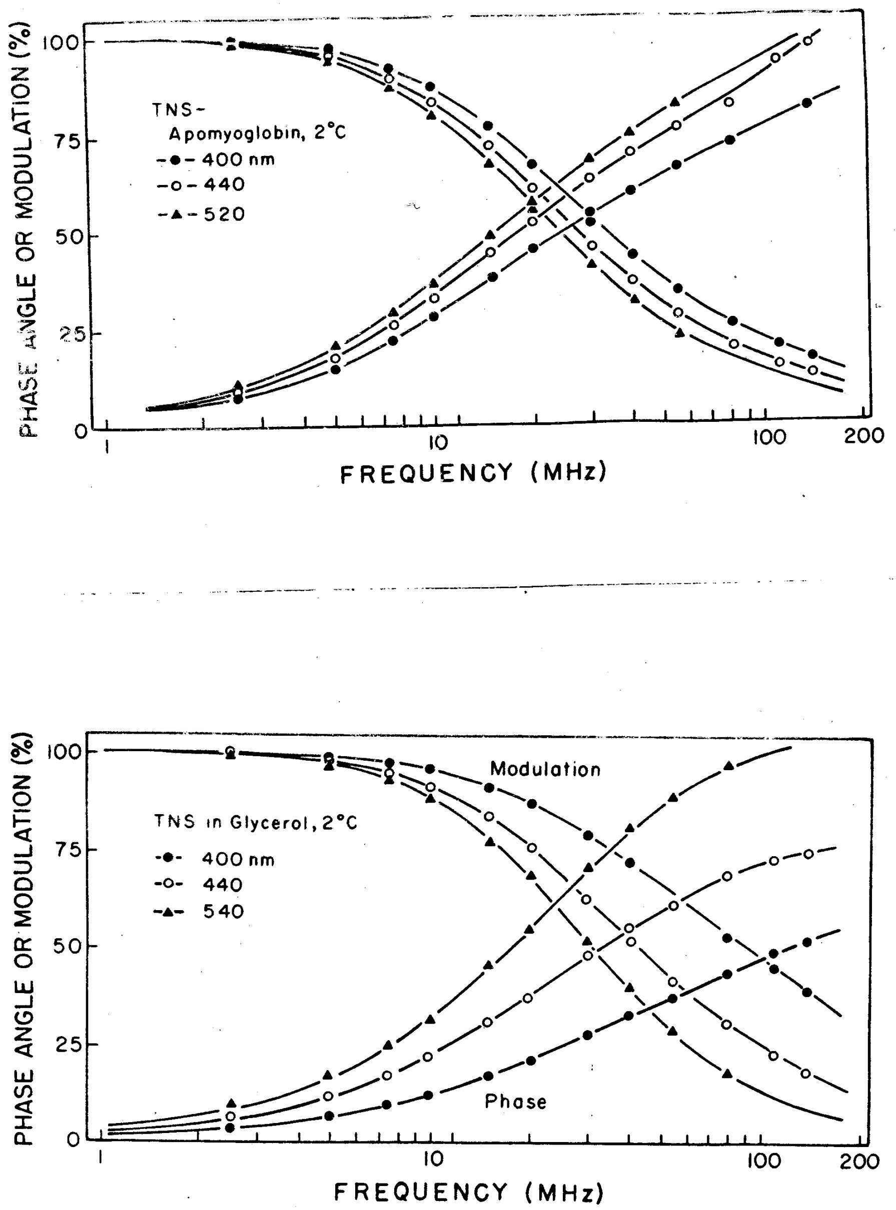
Time resolved spectra

What happens to the spectral width?

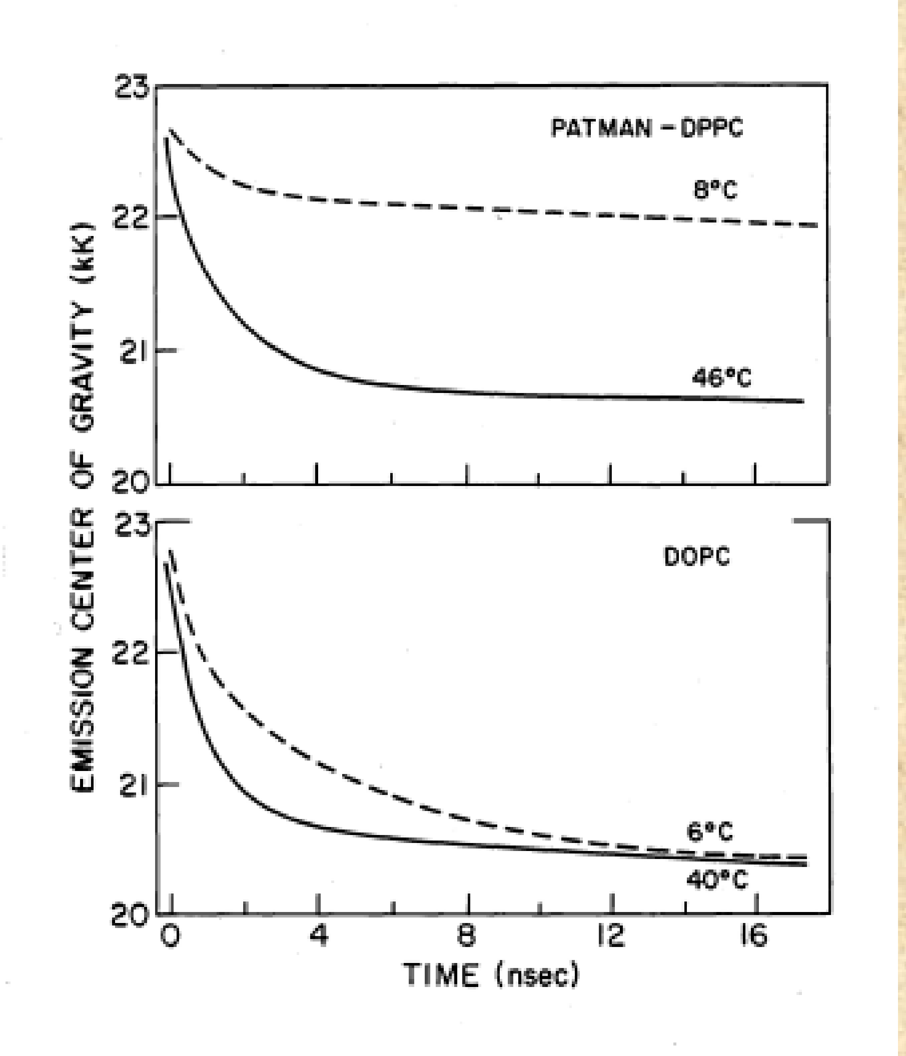
# Time resolved spectra of TNS in a Viscous solvent and in a protein

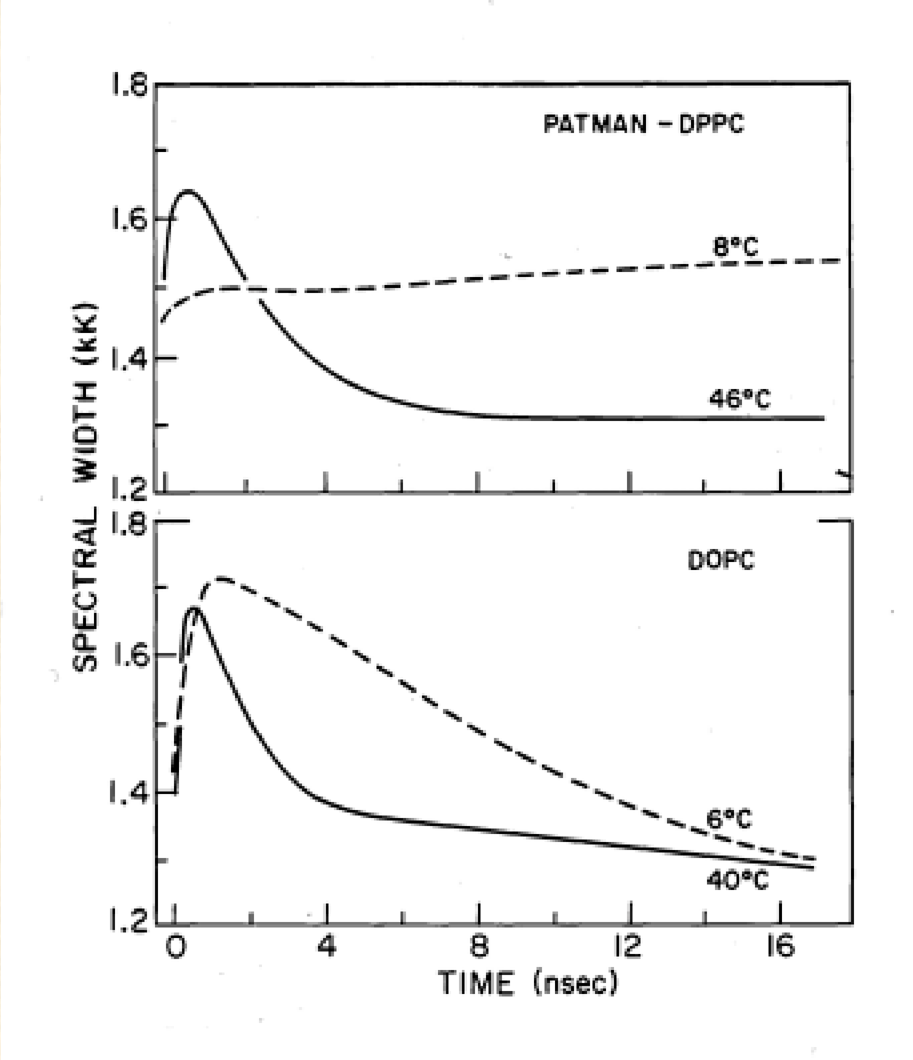


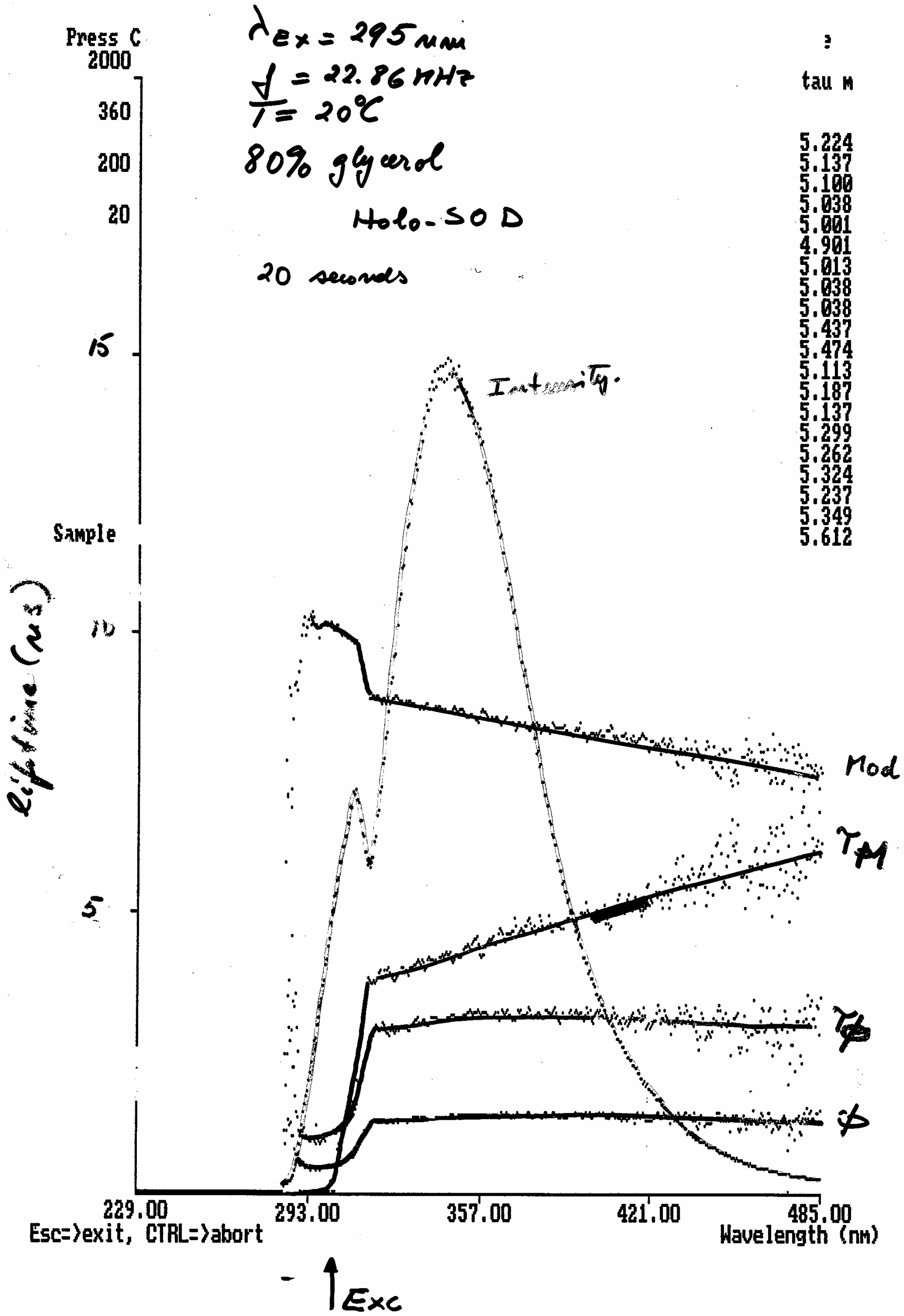




Time resolved spectra are built by recording of individual decays at different wavelengths


PATMAN – DPPC
8°C
46°C
DOPC
6°C
40°C
EMISSION CENTER OF GRAVITY (kK)
23
22
21
20
0
4
8
12
16
TIME (nsec)


PATMAN – DPPC
8°C
46°C
DOPC
6°C
40°C
SPECTRAL WIDTH (kK)
1.8
1.6
1.4
1.2
0
4
8
12
16
TIME (nsec)

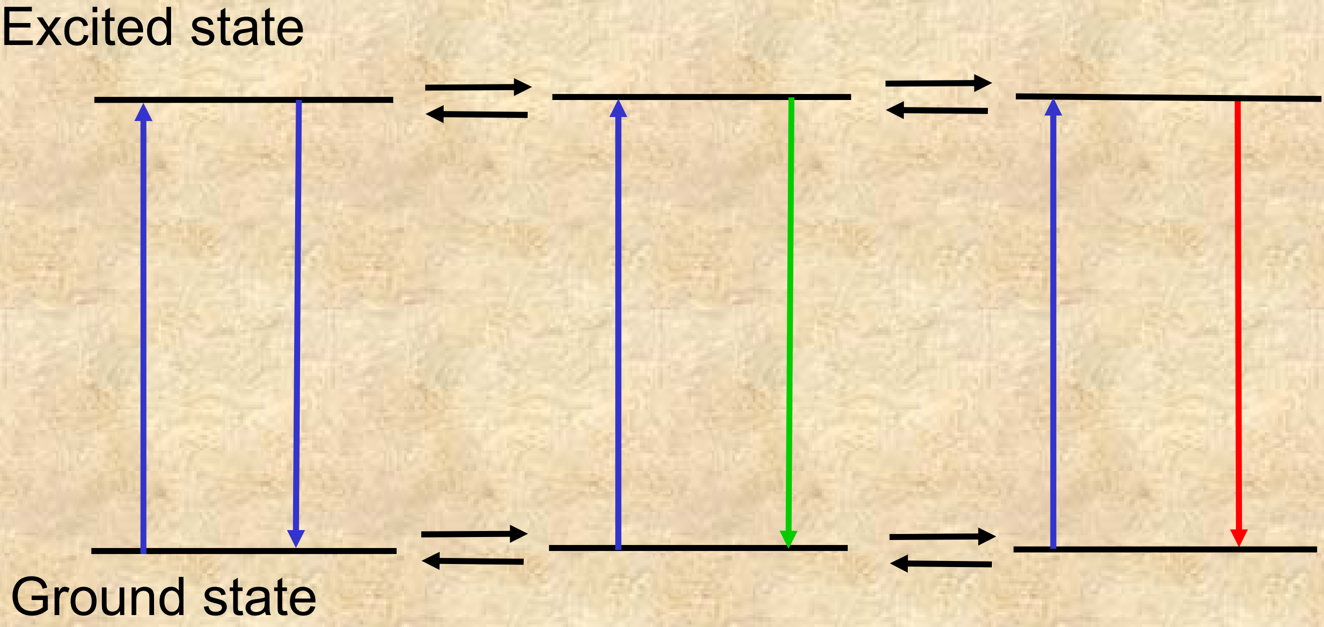


Time resolved spectra can also be recoded at once using time-resolved optical multichannel analyzers

# 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



Excited state

Ground state

Reactions can be either sequential or branching

If the reaction rate 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

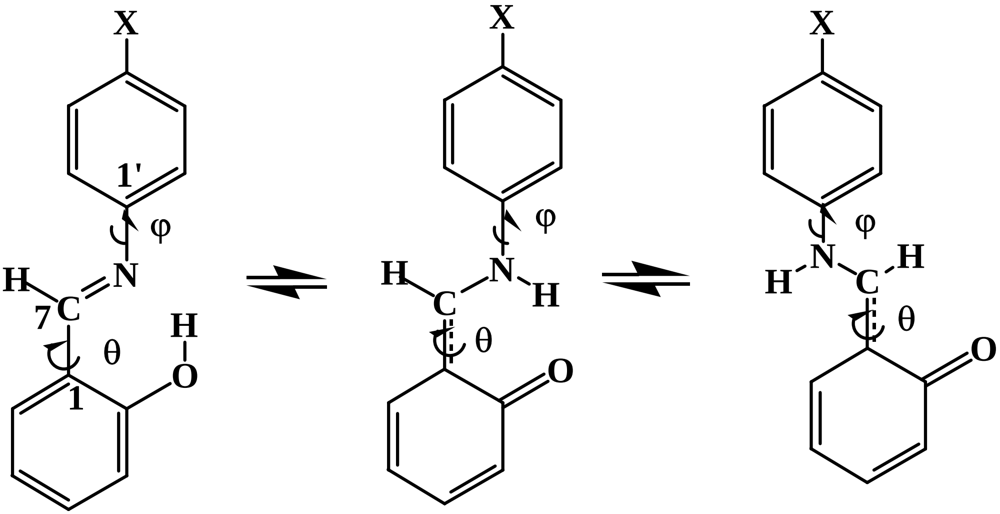
If the system has two states, the decay is doubly exponential

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

Example of excited-state reaction from V. Vargas (in press)

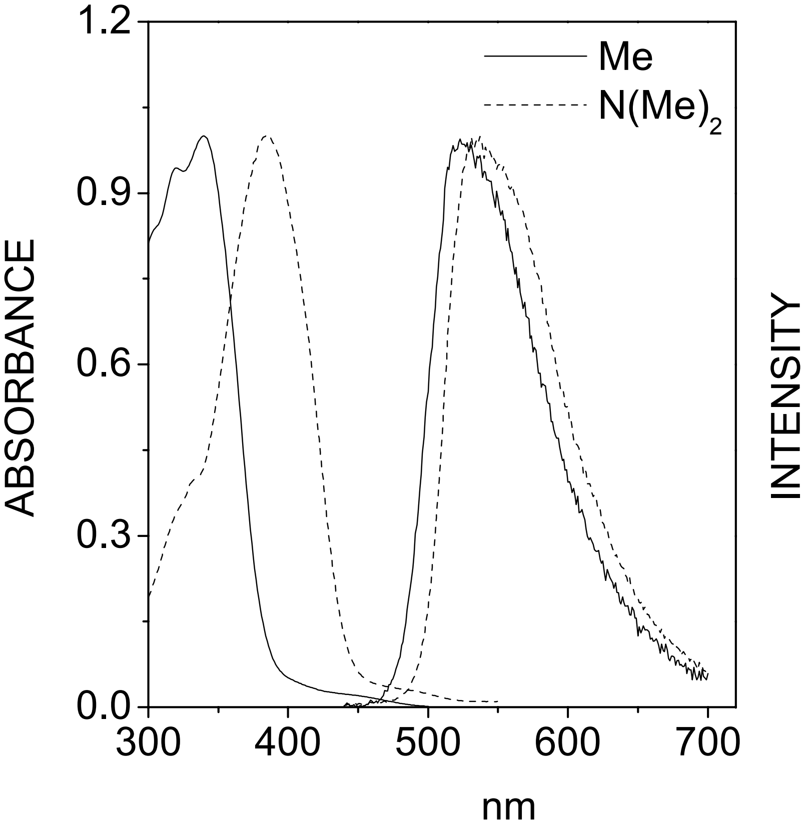
# Upon excitation, there is a cis-trans isomerization

N-salicylidene-*p*-X-aniline

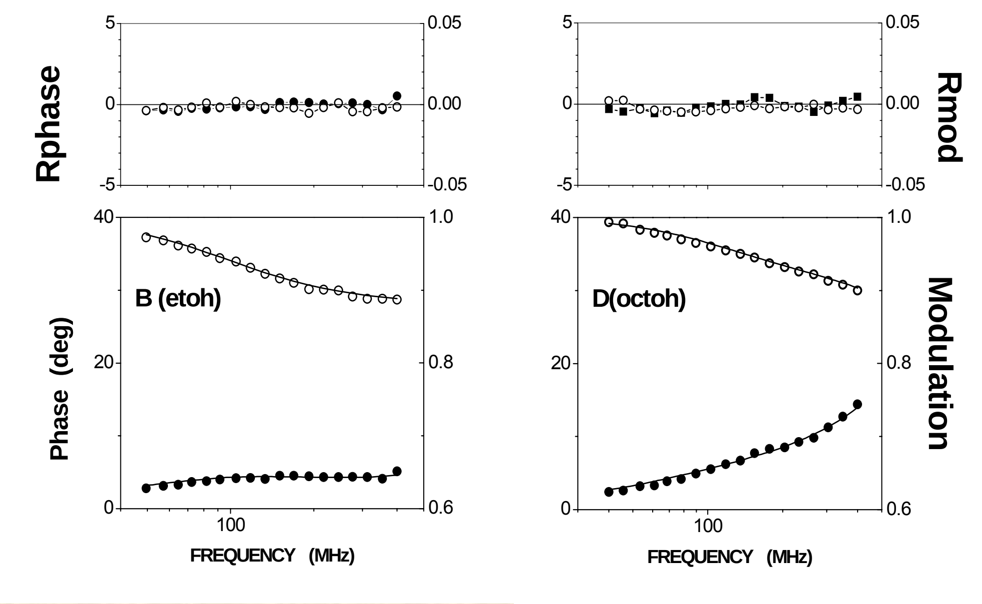


enol-imine *cis*-keto-amine *trans* keto-amine

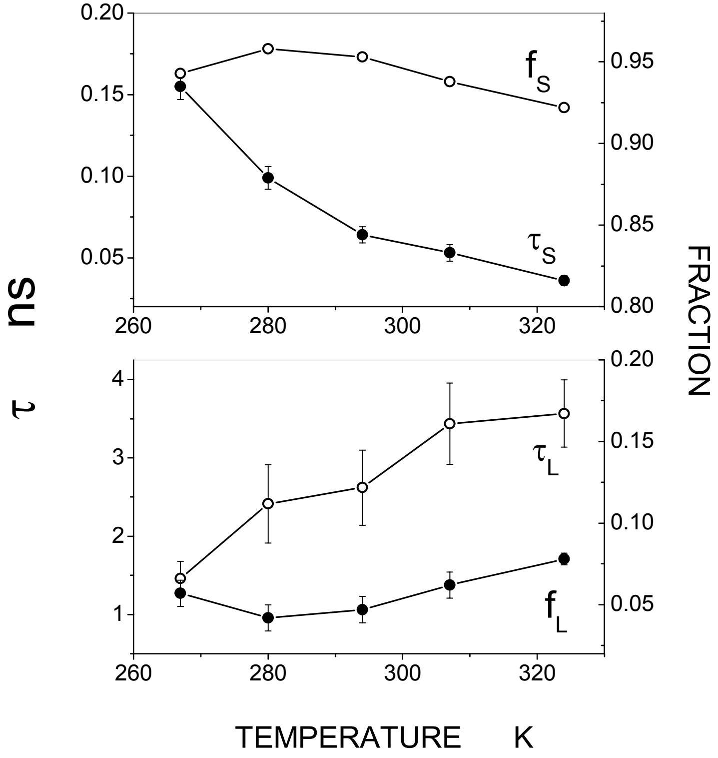
**1 X= $NO_2$**
**2 X= CN**
**3 X= H**
**4 X= Me**
**5 X= OMe**
**6 X= $NMe_2$**



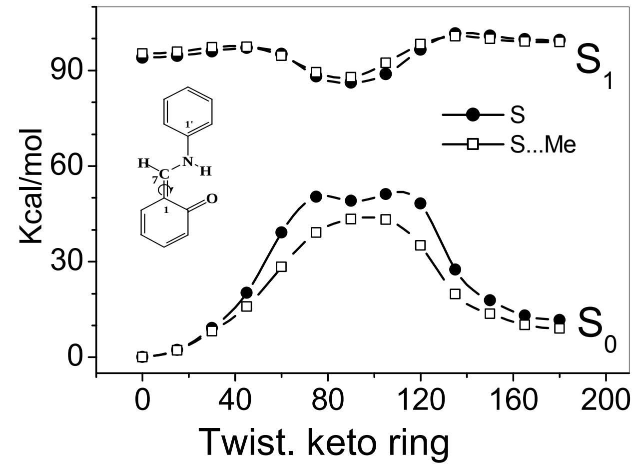
# Experimental data in the frequency-domain

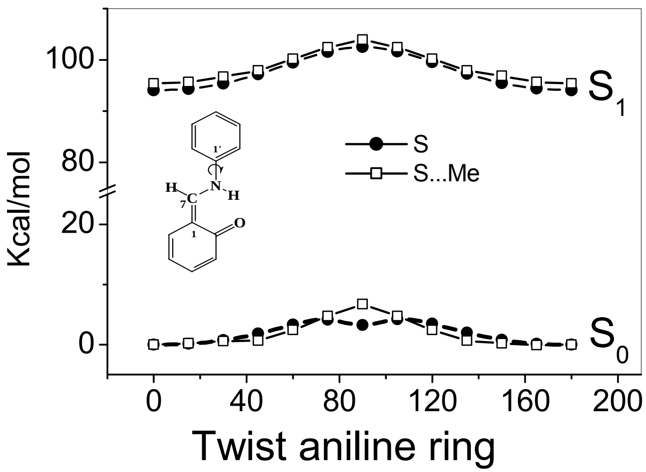


# Parameters from data fit



# The Model


90
60
30
0
Kcal/mol
0
40
80
120
160
200
Twist. keto ring
S1
S0
S
S...Me
H
C
7
N
H
1'
1
O


100
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Kcal/mol
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Twist aniline ring
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N
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1'
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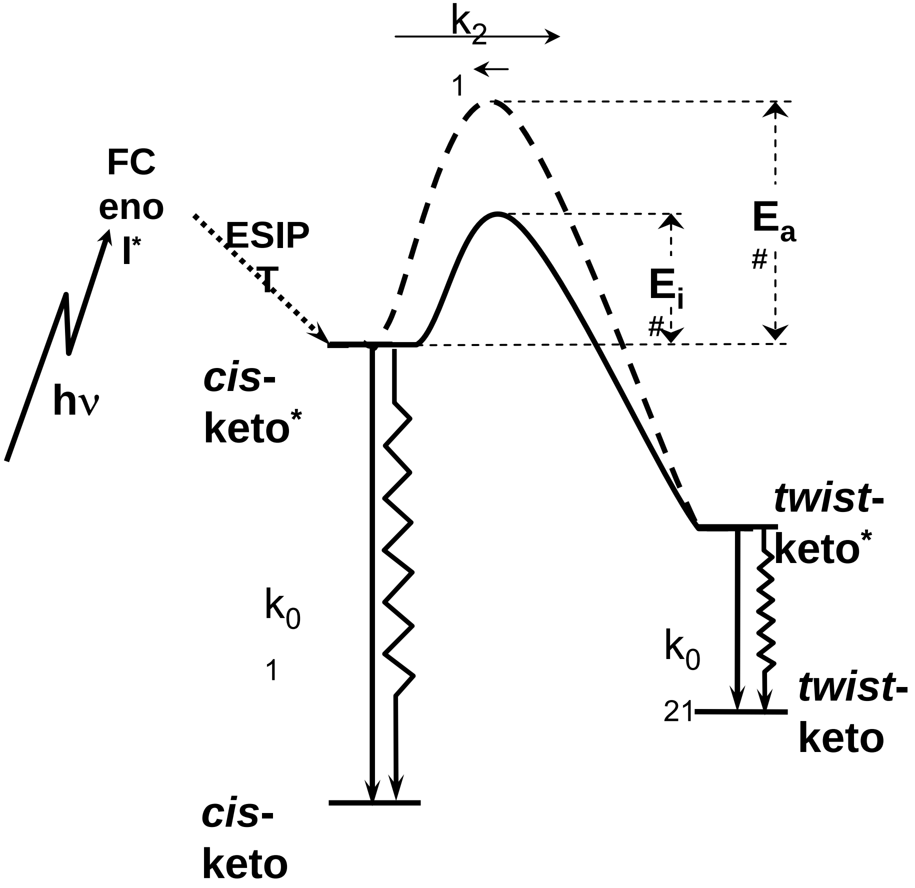
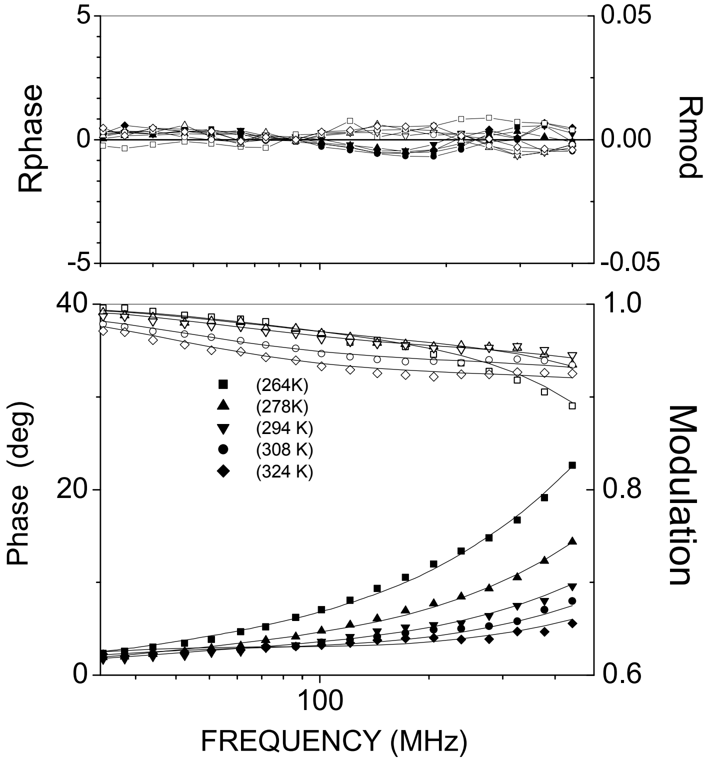
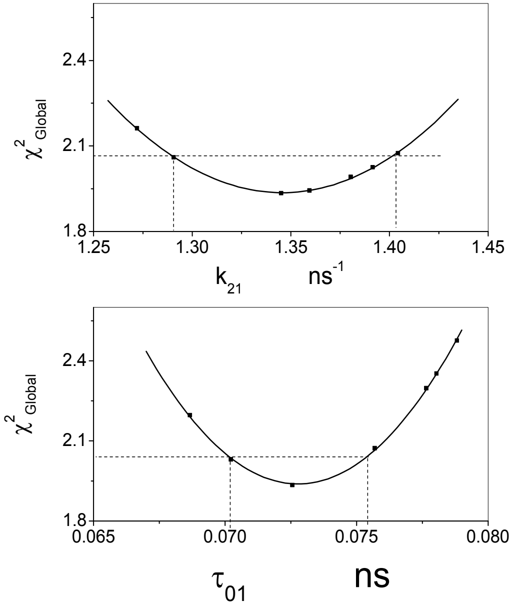

k2
1
FC
enol*
ESIPT
hν
cis-
keto*
Ea
#
Ei
#
twist-
keto*
k0
1
k0
21
twist-
keto
cis-
keto

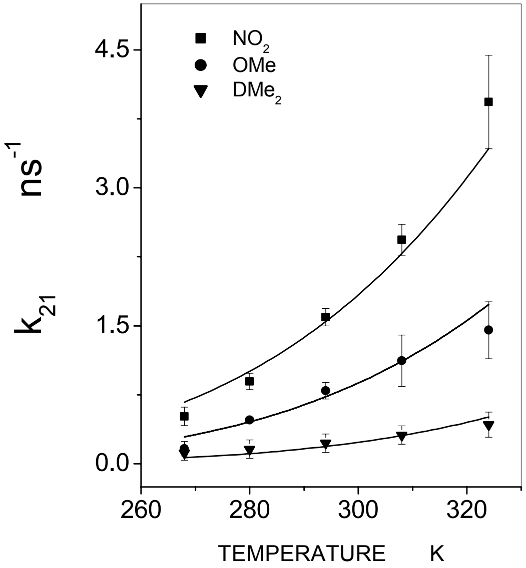
## Global Fit



## Error analysis



## Temperature dependence of rates



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)