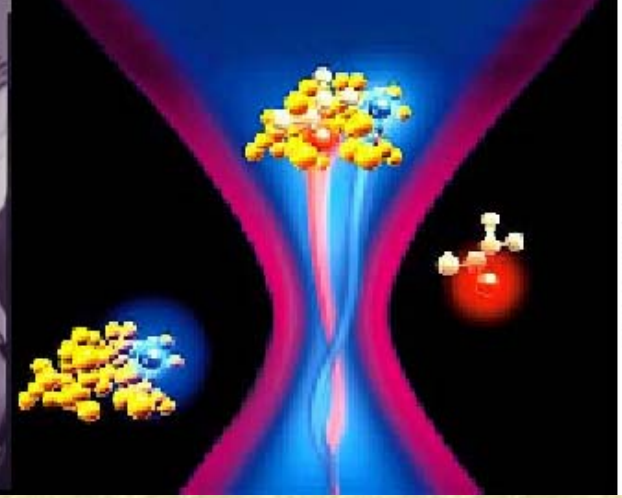
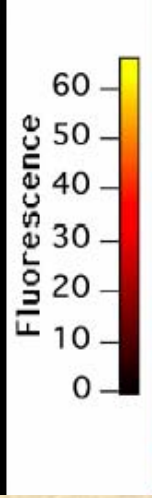
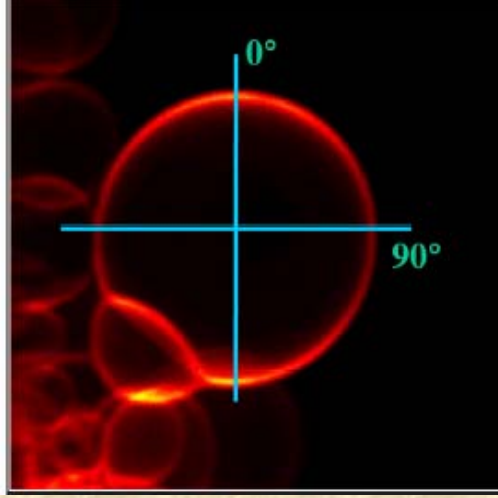





# *Fluorescence Workshop*
# *UMN Physics*
## *June 8-10, 2006*

**Quenching and FRET**
**Joachim Mueller**

**Static and dynamic quenching, FRET, efficiency, transfer rate, overlap integral, orientation factor**

University of Minnesota
schoolofphysics &astronomy
NSF

# Quenching

A number of processes can lead to a reduction in fluorescence intensity, which is referred to as 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

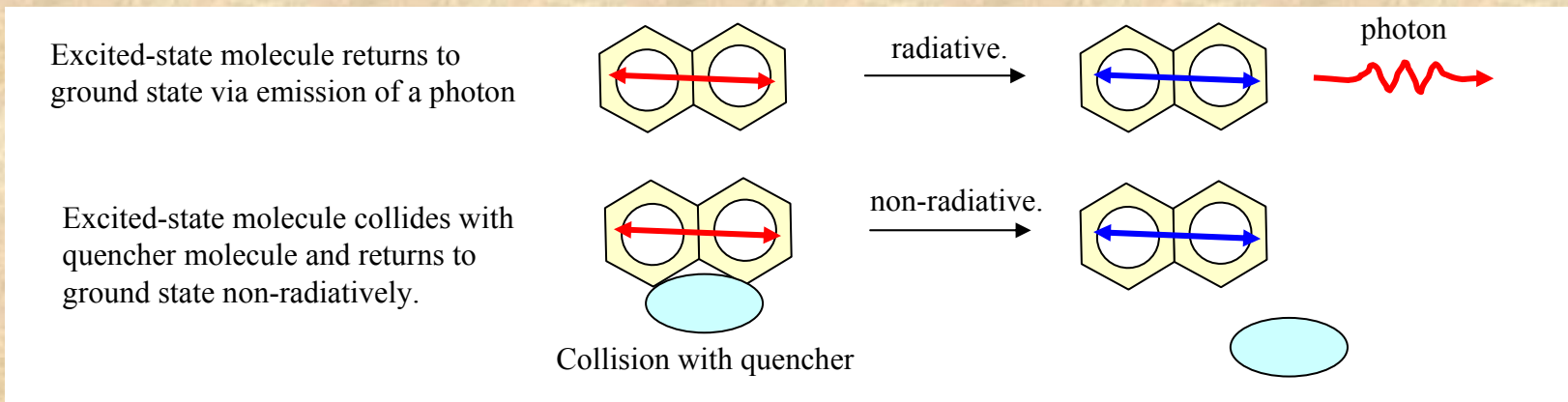
We focus on the two quenching processes usually encountered

(1) collisional (dynamic) quenching

(2) static (complex formation) quenching

## (1) 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.



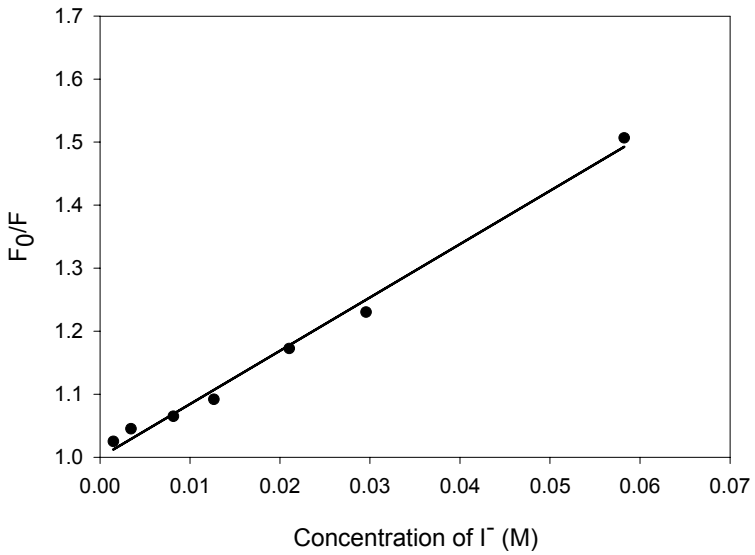
# Collisional Quenching: Stern-Volmer Plot

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

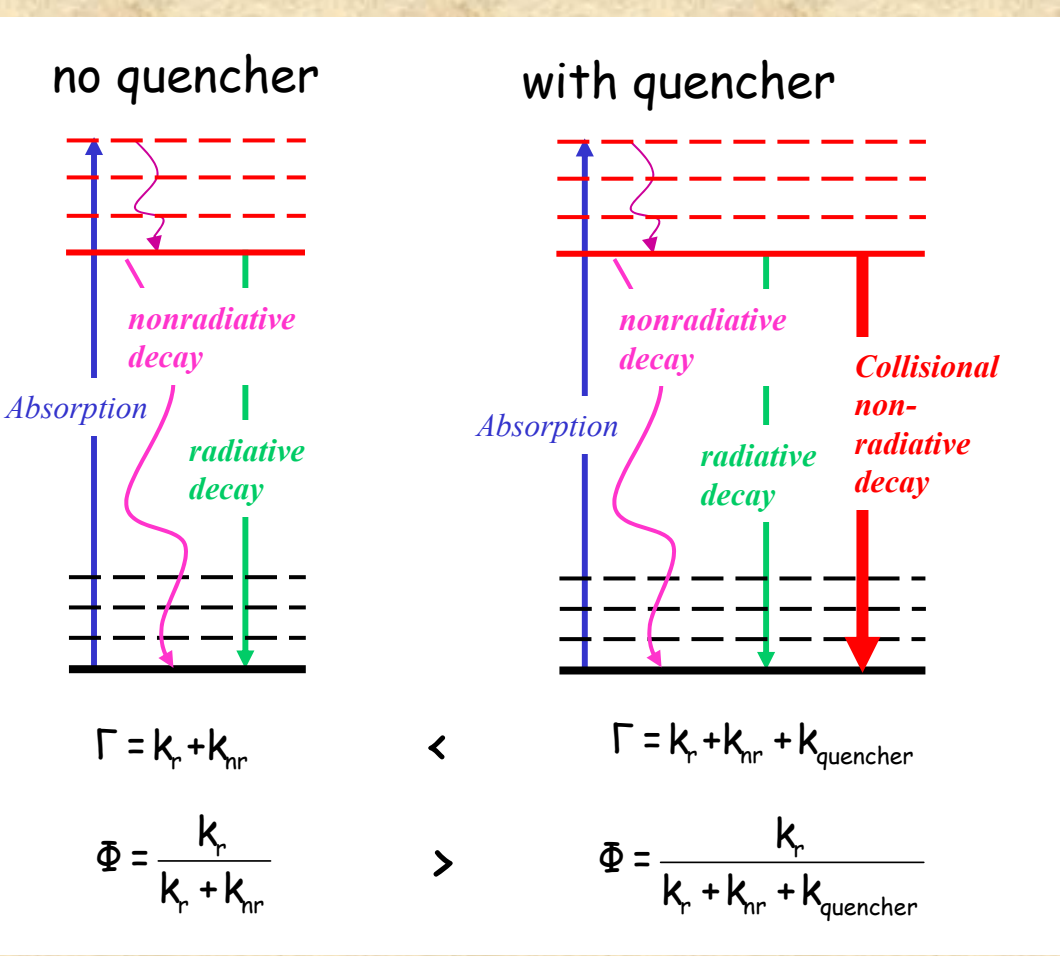
Thus, a plot of $F_0/F$ versus $[Q]$ should yield a straight line with a slope equal to $K_{SV}$. Such a plot, known as a Stern-Volmer plot, is shown below for the case of fluorescein quenched by iodide ion ($I^-$).




for 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 $\tau_0$ is the excited state lifetime in the absence of quencher.

# Collisional quenching also shortens the lifetime of fluorophore




## Stern-Volmer equation:

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

with $K_{SV} = k_q \tau_o$

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

For purely collisional quenching, also known as *dynamic* quenching:

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

→ $$\tau_0 / \tau = 1 + k_q \tau [Q]$$

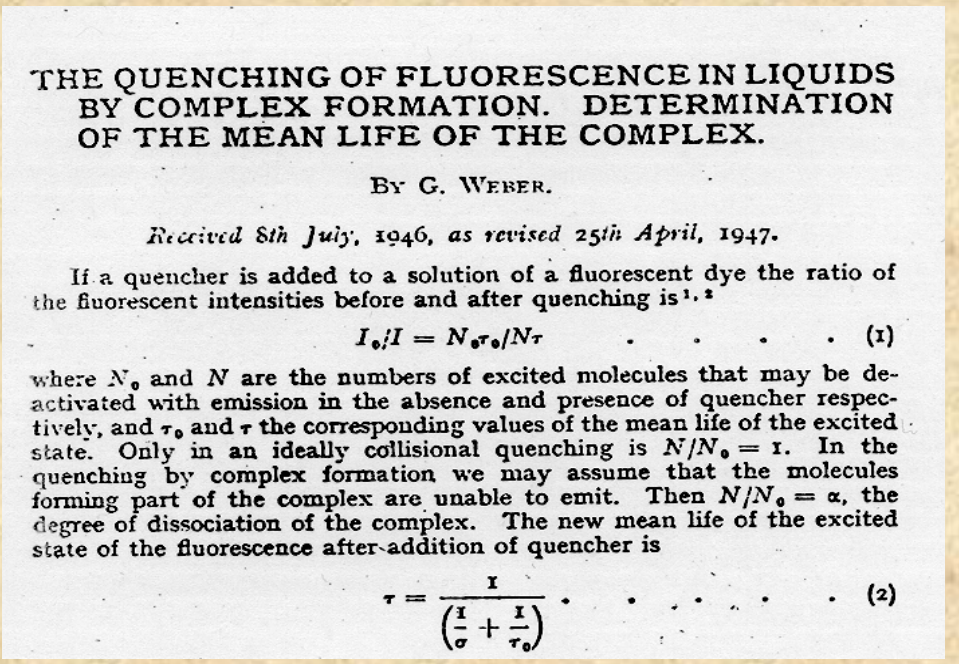
In the fluorescein/iodide system (previous slide), $\tau_o$ = 4ns and $k_q \sim 2 \times 10^9$ $M^{-1}$ $sec^{-1}$

## (2) 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:

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



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.*

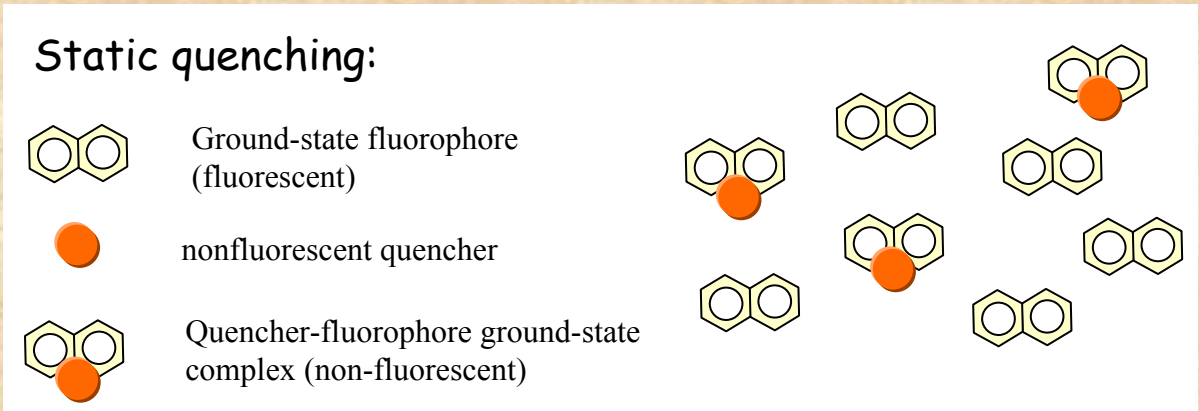
If a quencher is added to a solution of a fluorescent dye the ratio of the fluorescent intensities before and after quenching is[1,2]

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

where $N_0$ and $N$ are the numbers of excited molecules that may be deactivated with emission in the absence and presence of quencher respectively, and $\tau_0$ and $\tau$ 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 $K_a$ is the association constant of the complex. Such cases of quenching via complex formation were first described by Gregorio Weber.

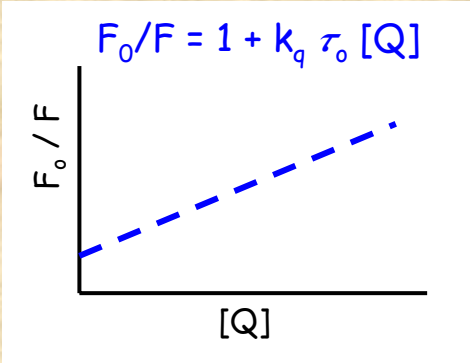


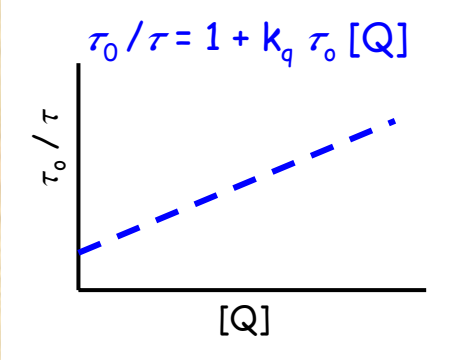

Static quenching only affects the complexed fluorophores. The properties of the uncomplexed fluorophores are not changed.

# Quenching and Lifetime of Fluorophores

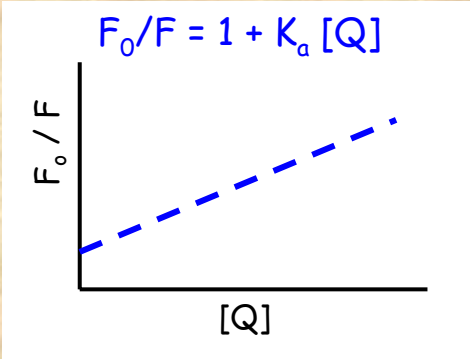
Static quenching will not reduce the lifetime of the sample since those fluorophores which are not complexed – and hence are able to emit after excitation – will have normal excited state properties. The fluorescence of the sample is reduced since the quencher is essentially reducing the number of fluorophores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

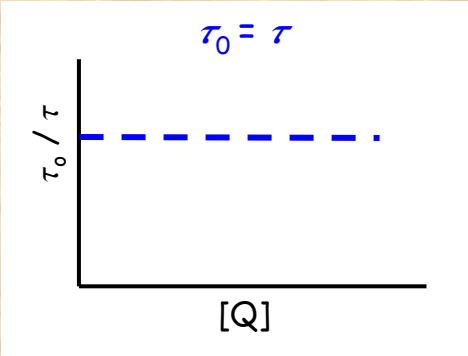
(1) Dynamic Quenching




(2) Static Quenching

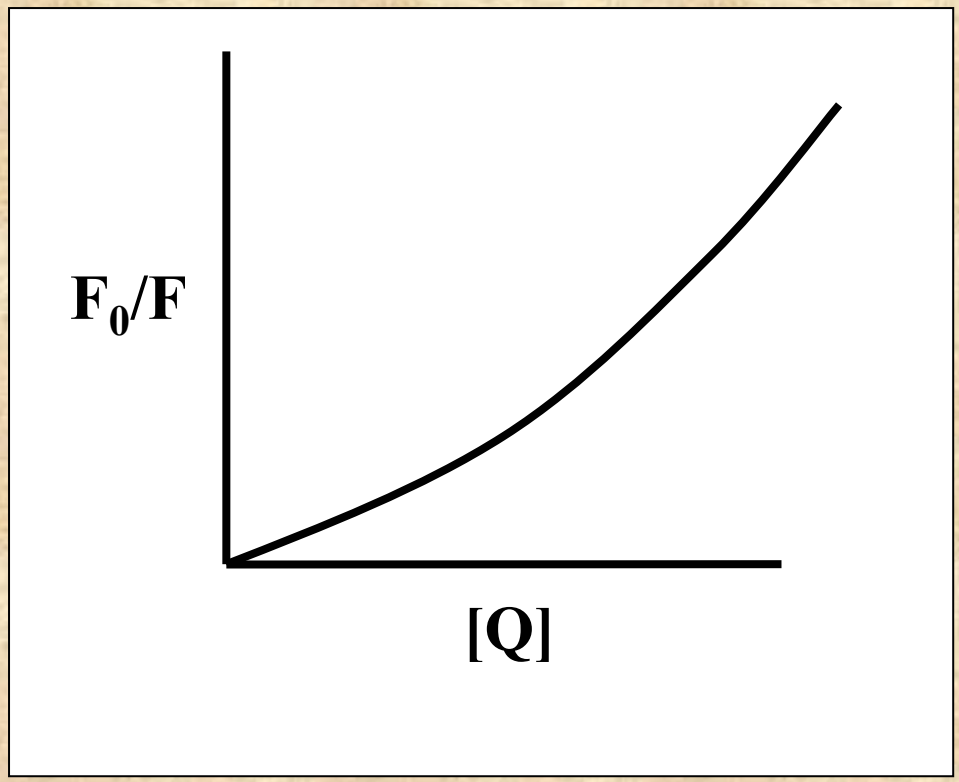


If both static and dynamic quenching occurs in the sample the following relation holds:

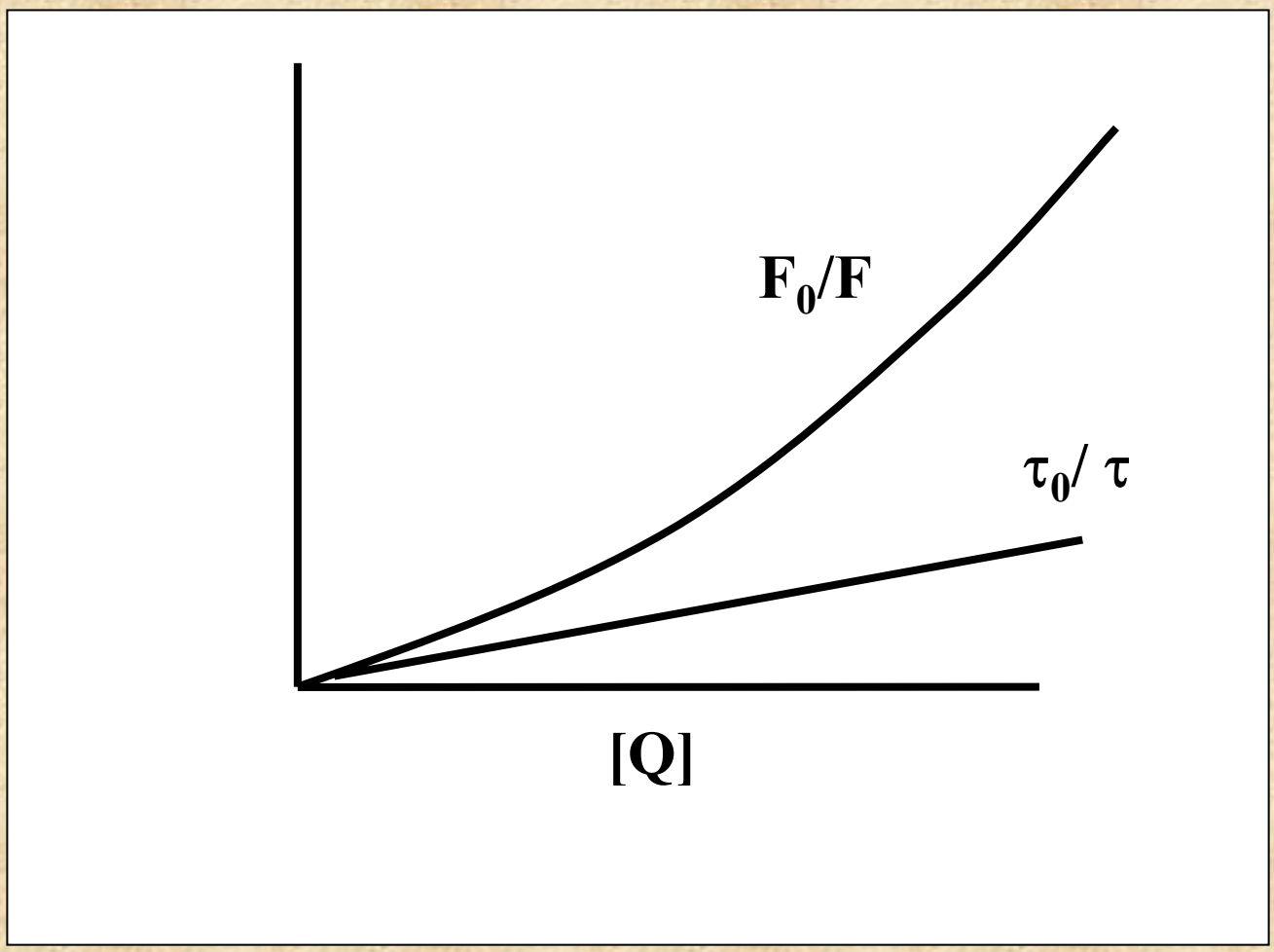
$$F_0/F = (1 + k_q \tau_o [Q]) (1 + K_a[Q])$$

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 $\tau_0/\tau$ versus [Q] would give a straight line



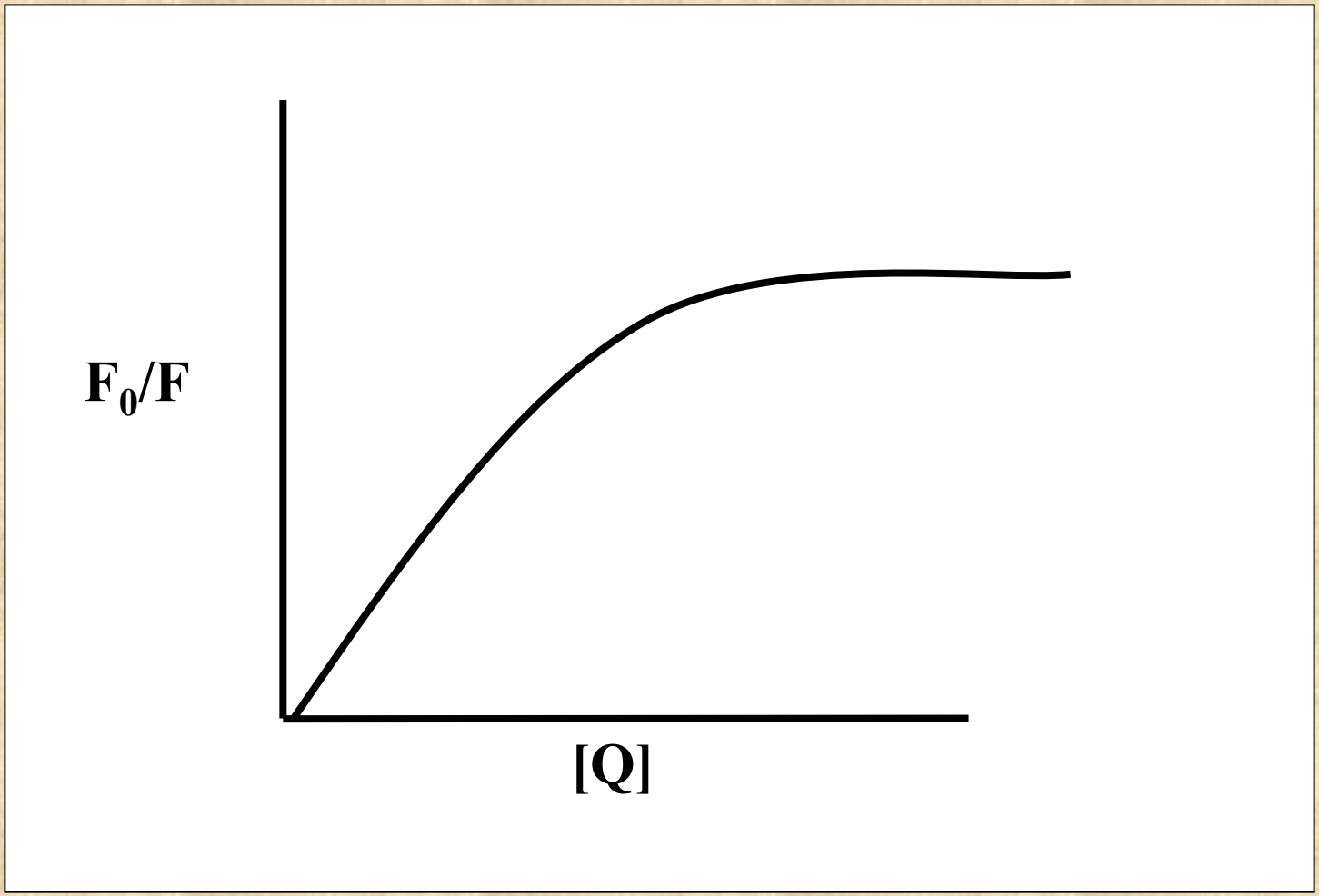
Sometimes you will see the equation for simultaneous static and dynamic quenching given as:

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

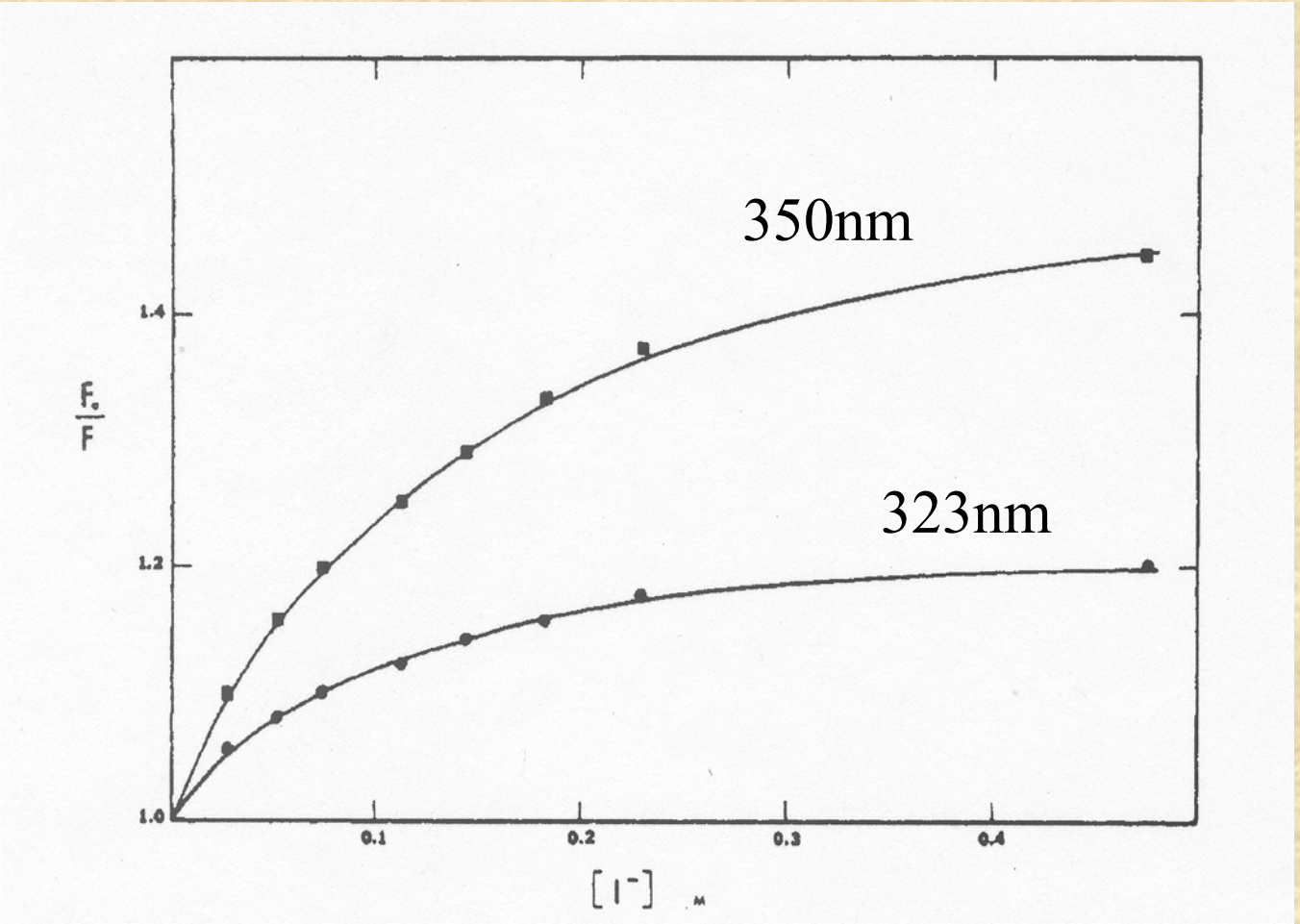
where the term $\mathbf{e^{V[Q]}}$ is used as a **phenomological** descriptor of the quenching process. The term V in this equation represents an *active volume* element around the fluorophore such that any quencher within this volume at the time of fluorophore excitation is able to quench the excited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



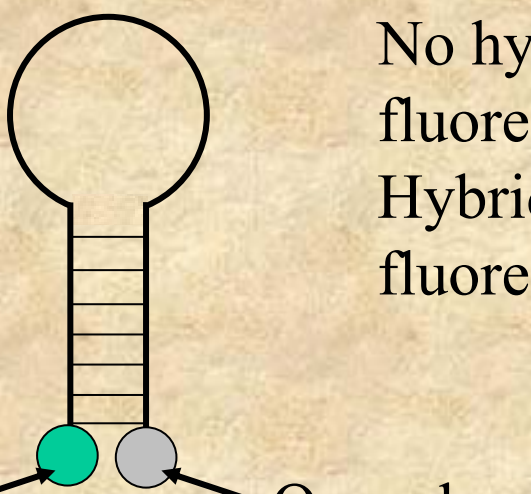
The quenching of LADH intrinsic protein fluorescence by iodide gives just such a plot. LADH is a dimer with 2 tryptophan residues per identical monomer. One residue is buried in the protein interior and is relatively inaccessible to iodide while the other tryptophan residue is on the protein's surface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

# Molecular beacons

- Invented in 1996 by Tyagi S. and Kramer F.
- Consist of ssDNA with:
  - an internal complementary sequence (stem)
  - a loop that anneals to a target



Probe
(fluorescein)

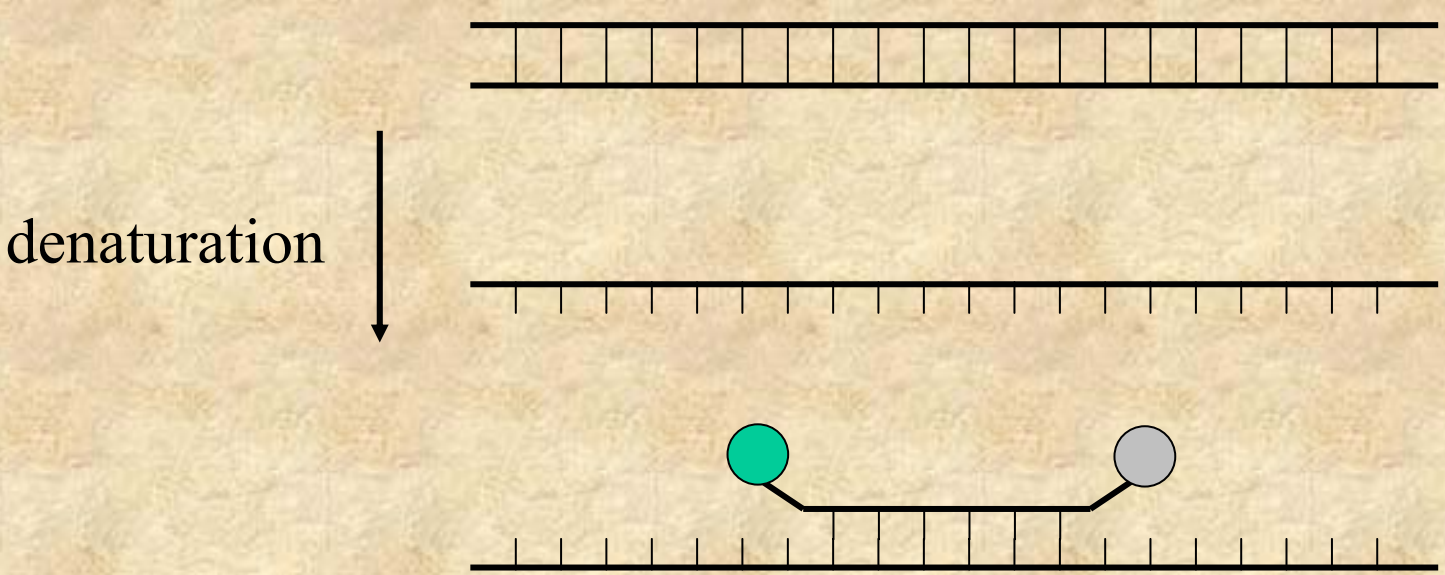
Quencher
(Dabcyl)

No hybridization: stem-loop closed, fluorescence is quenched.
Hybridization: stem-loop opened, fluorescence.

Max ratio: $F_0/F = 50$

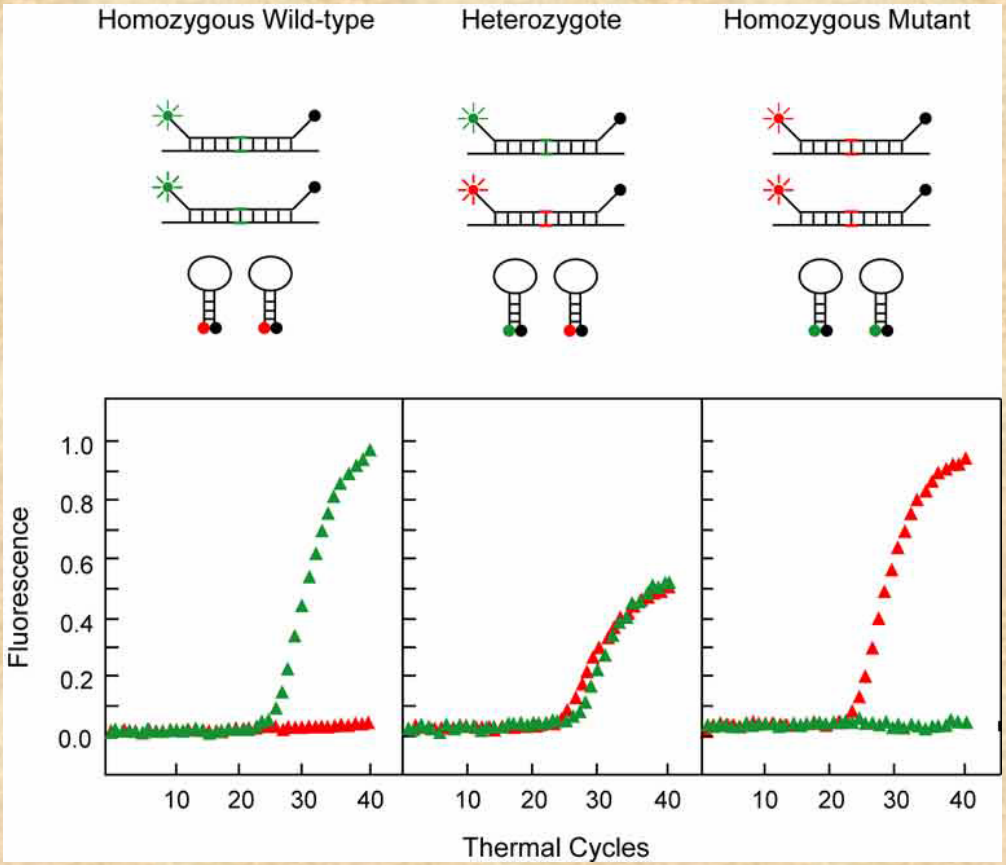
# Molecular beacons: applications

- real time PCR: following denaturation, beacon anneals to template separating dyes and quencher, yielding fluorescence Proportional to PCR product.



# Molecular beacons: applications

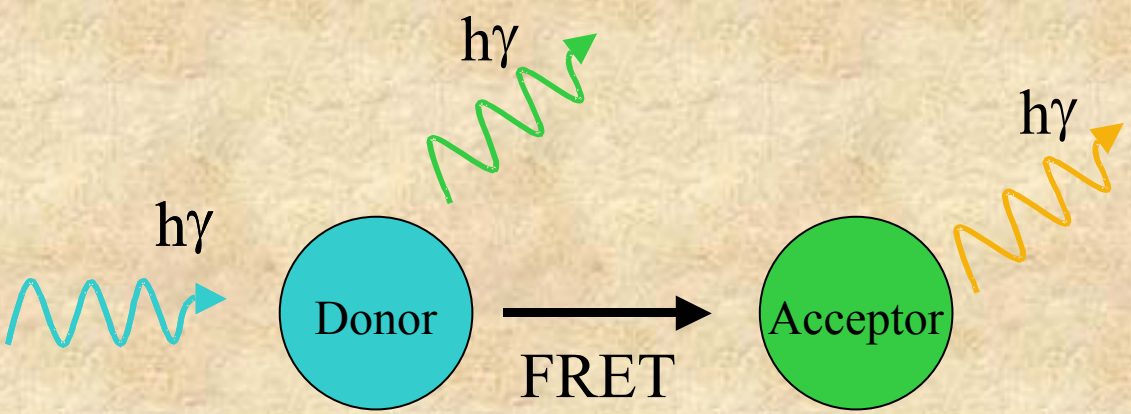
- diagnostic assay, genetic screening



# Fluorescence Resonance Energy Transfer (FRET)

(or **Förster Resonance Energy Transfer)**

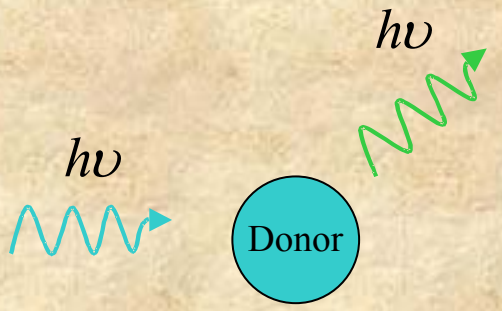
What is FRET ?




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.

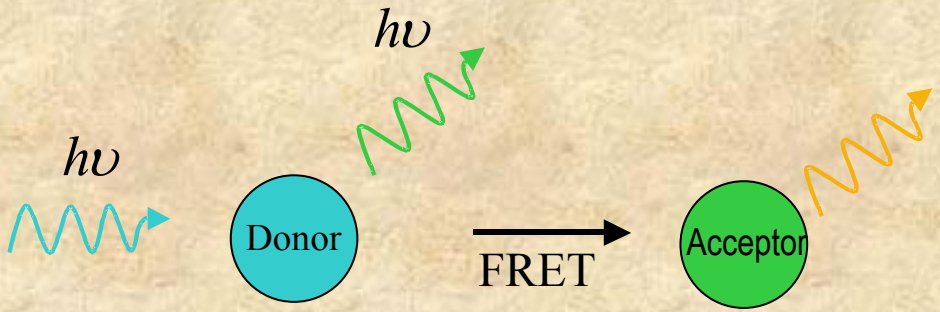
## Donor fluorophore alone




Donor fluorophore absorbs blue photon and emits a green photon.

Donor Lifetime: $\tau_d$
Donor Quantum Yield: $\Phi_d$

## Donor fluorophore with acceptor molecule close by




Donor fluorophore absorbs blue photon. The excited donor has two choices. It may emit a green photon or transfer its energy to the acceptor (which may be another fluorescent molecule). The probability for each of these two processes depends on the efficiency of the transfer.

In the presence of FRET
Donor Lifetime: $\tau^*_d < \tau_d$
Donor Quantum Yield: $\Phi^*_d < \Phi_d$

# **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.

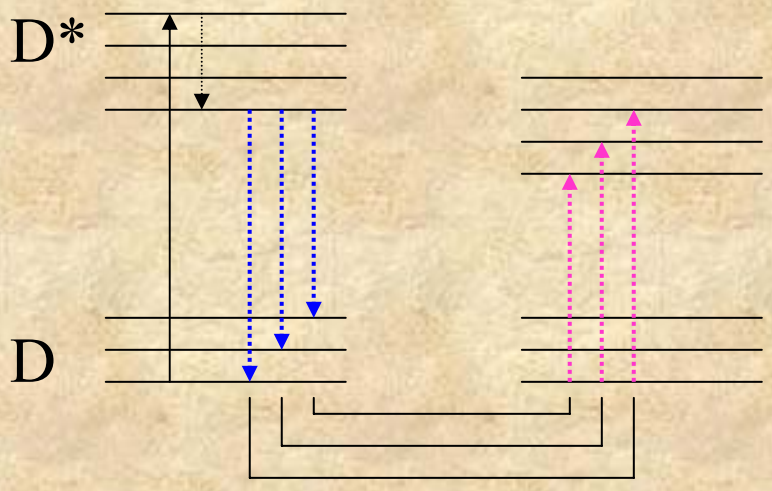
1924 E. Gaviola and P. Pringsham observed that an increase in the concentration of fluorescein in viscous solvent was accompanied by a progressive depolarization of the emission.

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

PM

# Efficiency of Energy transfer (*E*)

$E$ = Quantum yield of energy transfer = $\dfrac{\text{number of quanta transferred from D to A}}{\text{number of quanta absorbed by A}}$

FRET competes with all other pathways of deactivation from the excited state of the donor:

$$E = \frac{k_T}{k_T + k_{nr} + k_r} = \frac{k_T}{1/\tau_d^*}$$

$k_T$ is the rate of energy transfer
$k_{nr}$ is the rate of non-radiative transitions
$k_r$ is the rate of radiative transitions

$\tau_d = 1/(k_{nr} + k_r)$ is the donor lifetime

$\tau_d^* = 1/(k_T + k_{nr} + k_r)$ is the donor lifetime in the presence of FRET

$$\Rightarrow \quad E = 1 - \frac{\tau_d^*}{\tau_d} = 1 - \frac{F_d^*}{F_d}$$

$F_d$ is the fluorescence intensity of the donor
$F_d^*$ is the fluorescence intensity of the donor iin the presence of FRET

*E* is experimentally determined from the fluorescence lifetimes or intensities of the donor determined in absence and presence of the acceptor.

# The rate of transfer ($k_T$)

To calculate the FRET efficiency we need to know the rate of energy transfer $k_T$. This requires a lengthy derivation. We only summarize the salient features:

The rate of transfer $k_T$ is :

$$k_T = \frac{1}{\tau_d}\left(\frac{R_0}{R}\right)^6$$

$\tau_d$ is the fluorescence lifetime of the donor in the absence of FRET, $R$ is the distance between the the donor and acceptor molecules, and $R_0$ is defined by:

$$R_0 = 0.0211(n^{-4}\,\Phi_d\,\kappa^2\,J)^{1/6}\ \text{nm}$$

where $n$ is the refractive index of the medium (usually between 1.2-1.4), $\Phi_d$ is the fluorescence quantum yield of the donor in absence of FRET, $\kappa^2$ is the orientation factor for the dipole-dipole interaction and $J$ is the normalized spectral overlap integral (in $M^{-1}\,cm^{-1}\,(nm)^4$).

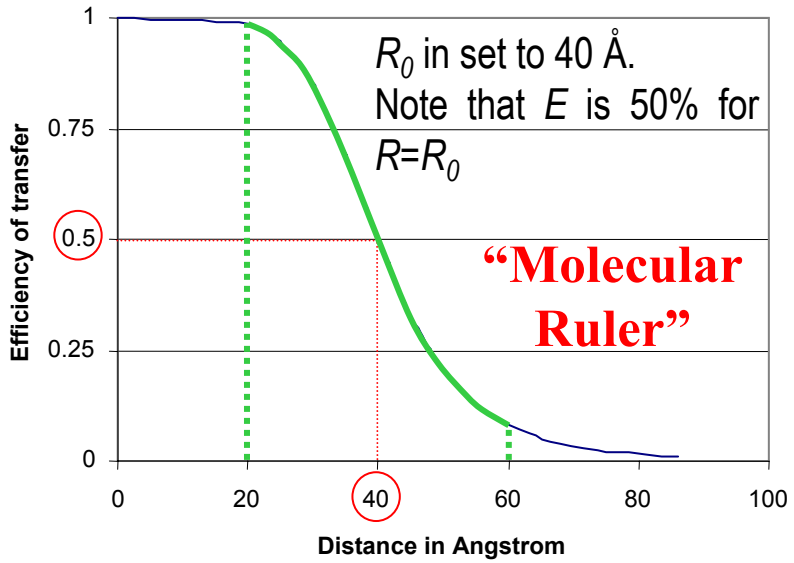
# Molecular Ruler

The FRET transfer rate $k_T$ depends on distance: $k_T = \frac{1}{\tau_d}\left(\frac{R_0}{R}\right)^6$

The FRET efficiency E is directly related to $k_T$: $E = \frac{k_T}{k_T + k_{nr} + k_r} = \frac{k_T}{k_T + 1/\tau_d}$

Thus the FRET efficiency E varies with the inverse sixth power of the distance between donor and acceptor

$$E = \frac{1}{1 + \left(\frac{R}{R_o}\right)^6}$$




$R_0$ is the Förster critical distance at which 50% of the excitation energy is transferred to the acceptor.

**From FRET studies we can get molecular distances**

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

# Spectral Overlap Integral *J*

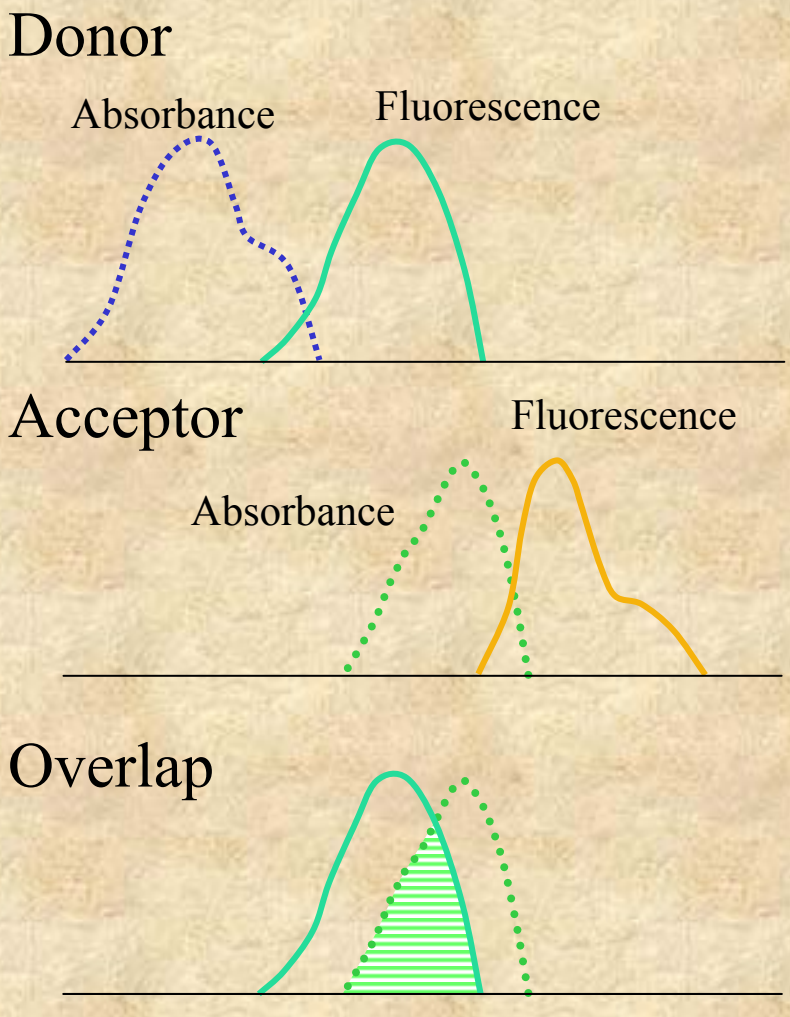
The overlap integral *J* is defined by:

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

Where $\lambda$ 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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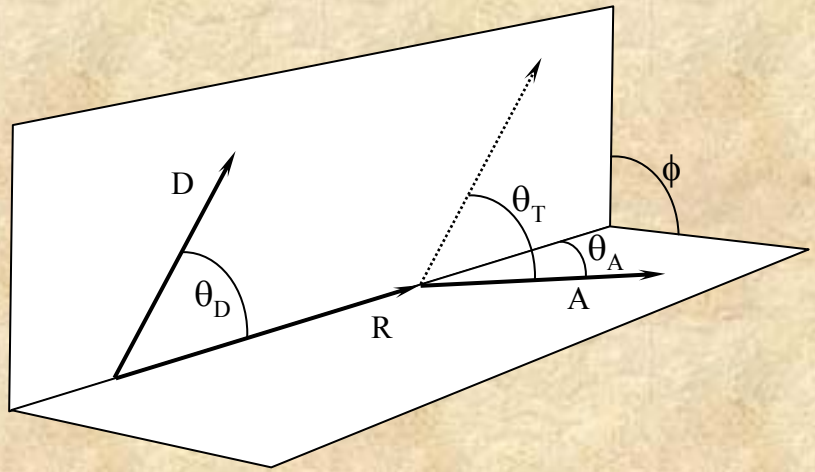
# The orientation factor $\kappa^2$

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




Where $\theta_T$ 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 $\theta_D$, $\theta_A$ are the angles between the separation vector R, and the D and A moment, respectively, and $\phi$ is the azimuth between the planes (D,R) and (A,R)

The limits for $\kappa^2$ 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, $\kappa^2$ can not be uniquely determined in solution. What value of $\kappa^2$ should be used ?

1. We can **assume** 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 $\kappa^2$ 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 $\kappa^2$ is equal to 2/3 is probably valid.

2. We can **calculate** the lower and upper limit of $\kappa^2$ using polarization data (Dale, Eisinger and Blumberg: 1979 Biophys. J. 26:161-93).

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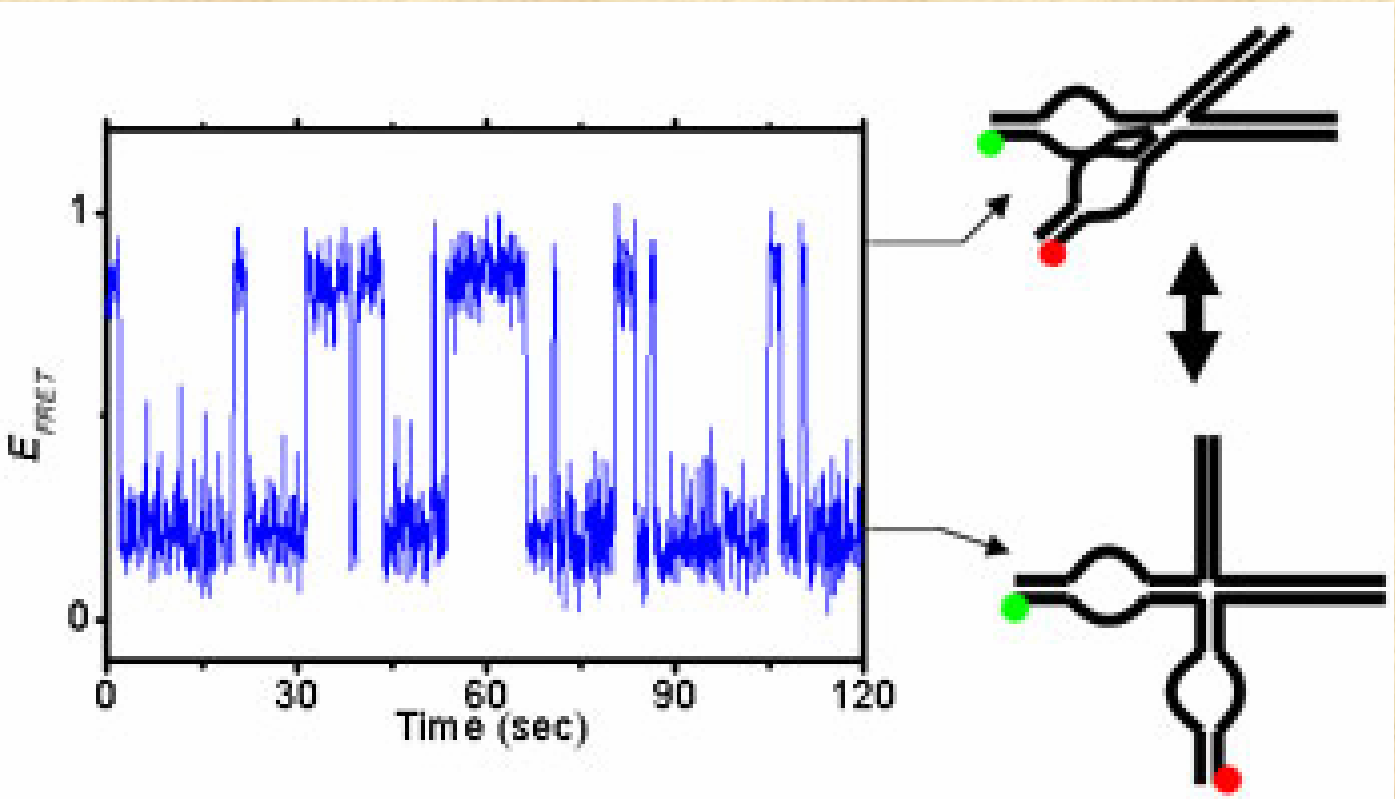
# Example: Single-molecule FRET

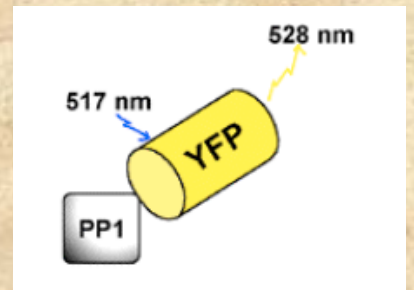
The example shown below is a time record of folding and unfolding of an RNA molecule hairpin ribozyme. We attach the donor (green) and acceptor (red) dyes to the RNA so that the folded state has high FRET and the unfolded state has low FRET. We can see this beautiful two-state fluctuations in FRET values as a function of time.

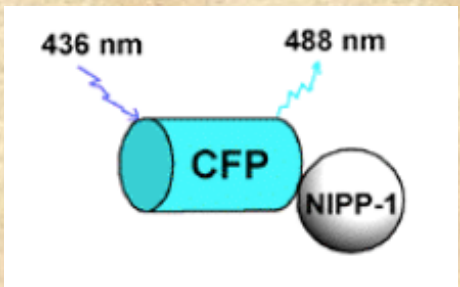


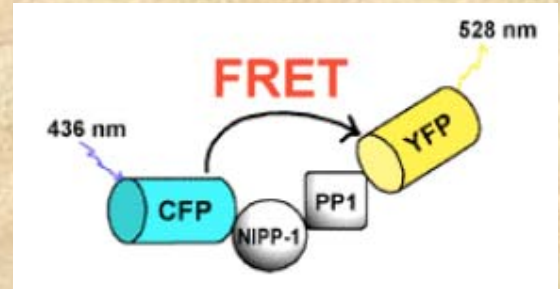
# Example: Single-molecule FRET

The example shown below is an in vivo measurement of proteins interaction using CFP and YFP.

For quantification purpose you need to measure crosstalk from yellow channel into FRET channel in cell expressing only the yellow fusion, then only the cyan fusion and then both yellow and cyan.



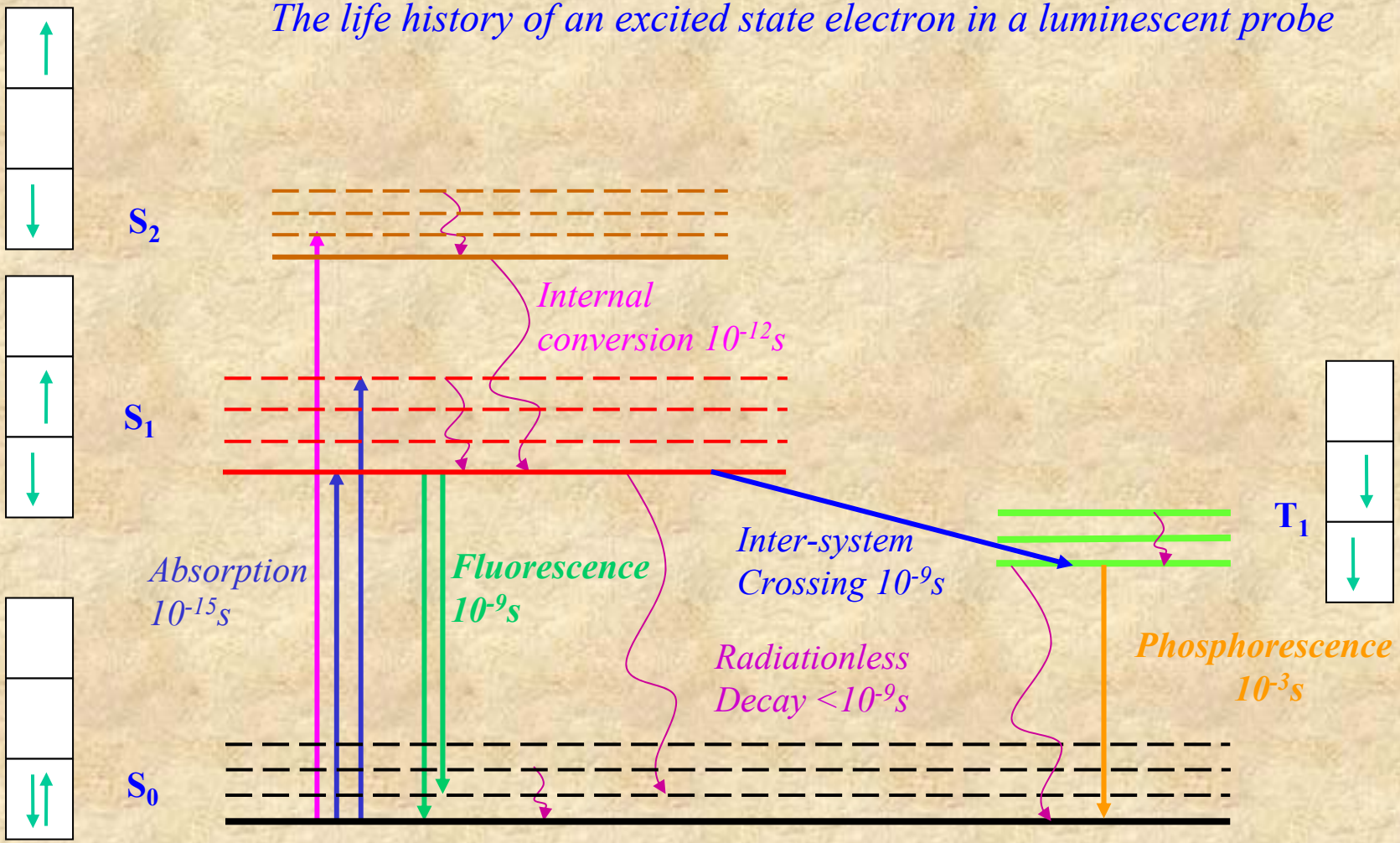




"Dynamic targeting of protein phosphatase 1 within the nuclei of living mammalian cells"; Trinkle-Mulcahy, L., Sleeman, J. and Lamond, A.I. (2001) *J. Cell Sci.*114:4219-4228.

# Phosphorescence

*The life history of an excited state electron in a luminescent probe*

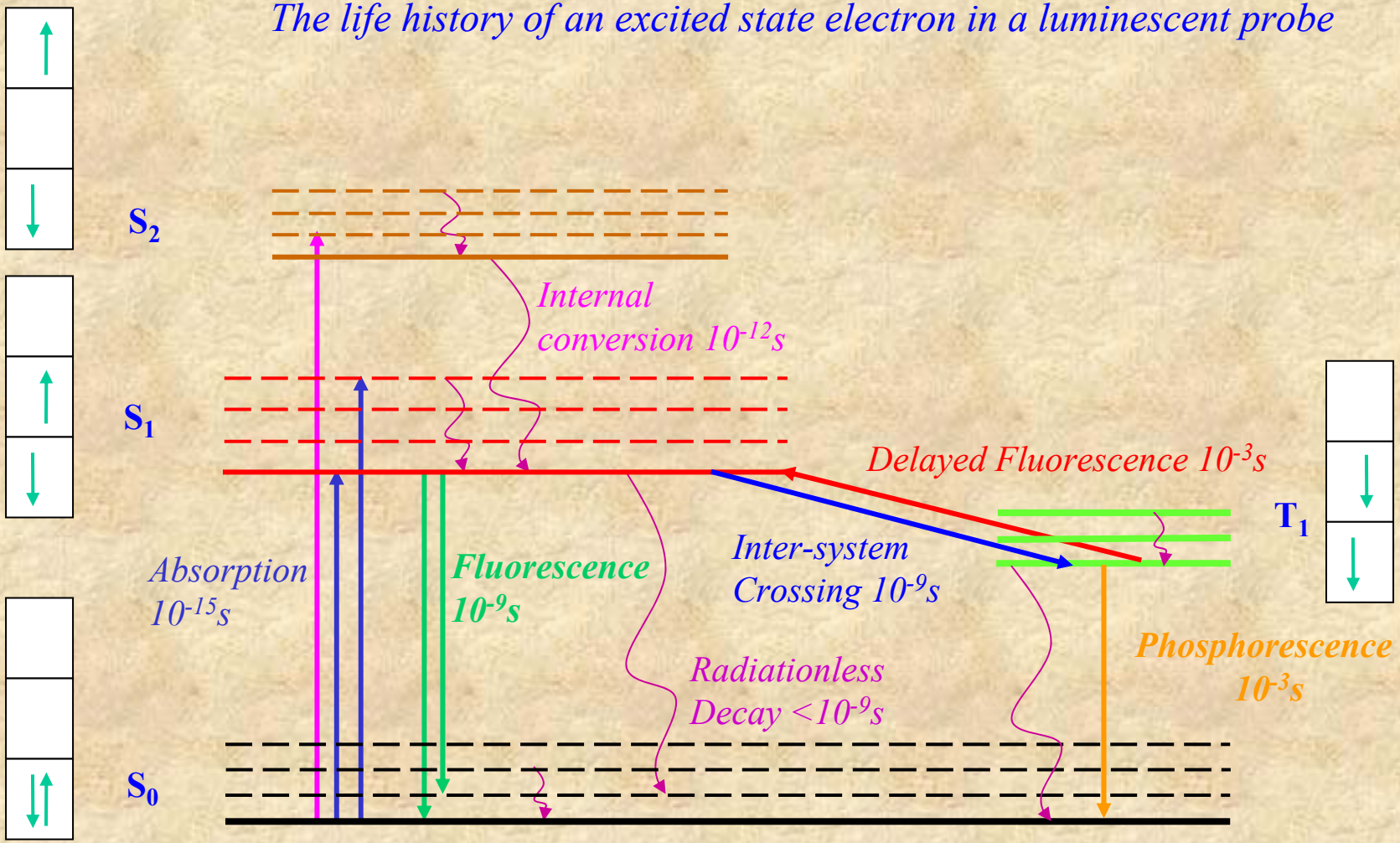



S : singlet state (all electrons in the molecule are spin-paired)

T : triplet state (the total spin quantum number is 1), two unpaired and parallel electron spins.

# Delayed Fluorescence

*The life history of an excited state electron in a luminescent probe*




S : singlet state (all electrons in the molecule are spin-paired)

T : triplet state (the total spin quantum number is 1), two unpaired and parallel electron spins.

***Triplet emission is lower in energy compared to singlet emission***