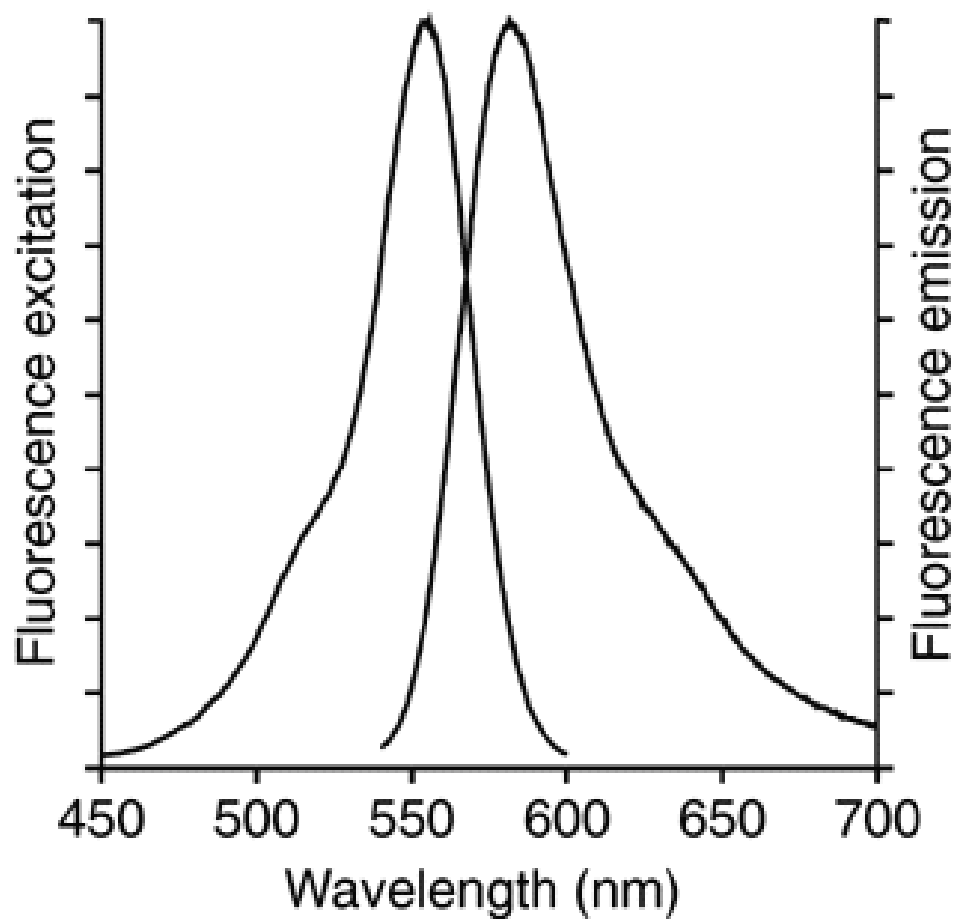
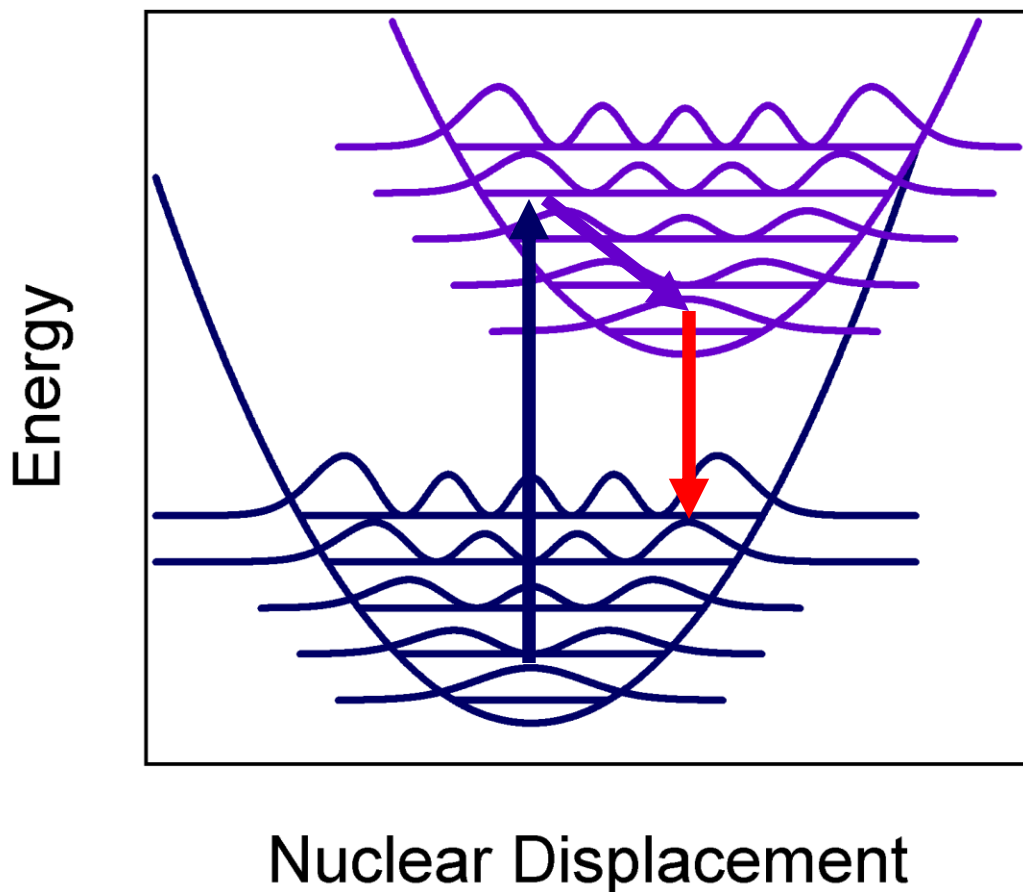


Fluorescence spectroscopy



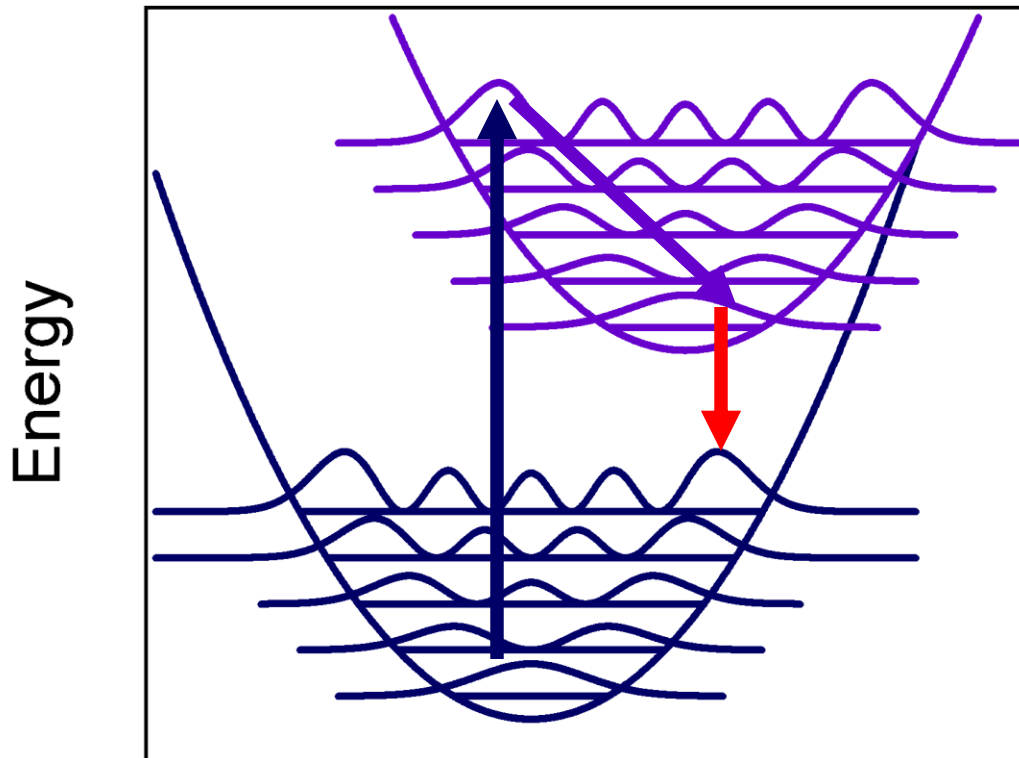
Fluorescence usually occurs after vibrational relaxation



1. Absorption
2. Vibrational relaxation
3. Fluorescence



The Franck-Condon principle: Transitions are vertical in both absorption and emission

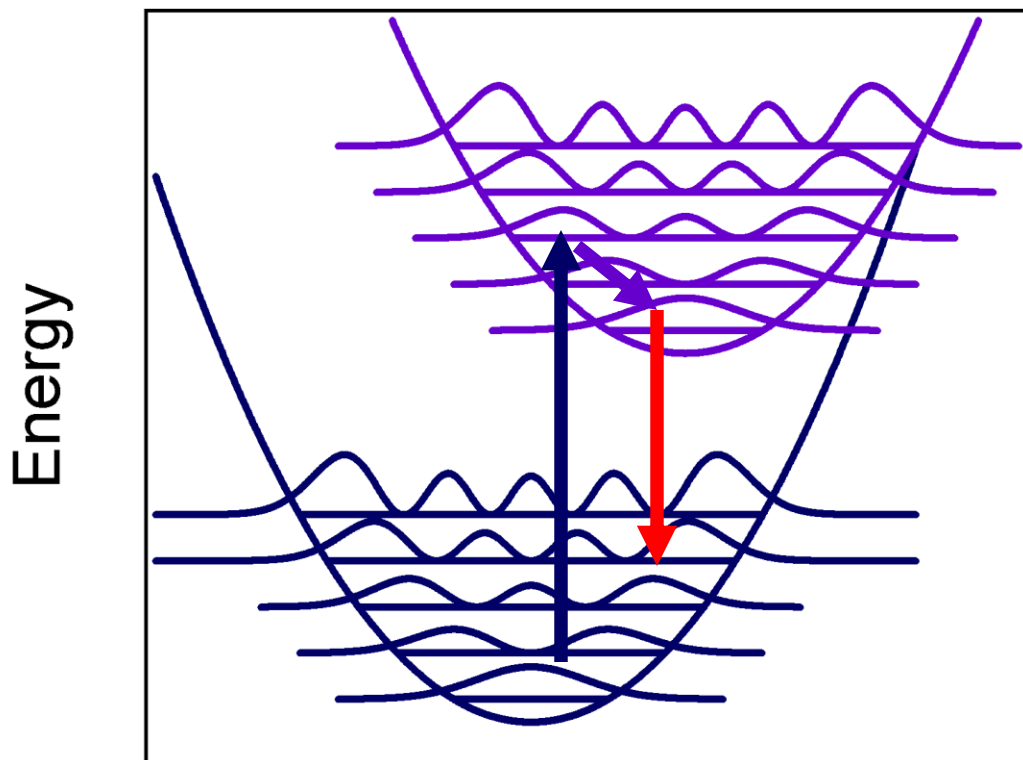


1. Absorption
2. Vibrational relaxation
3. Fluorescence

Nuclear Displacement



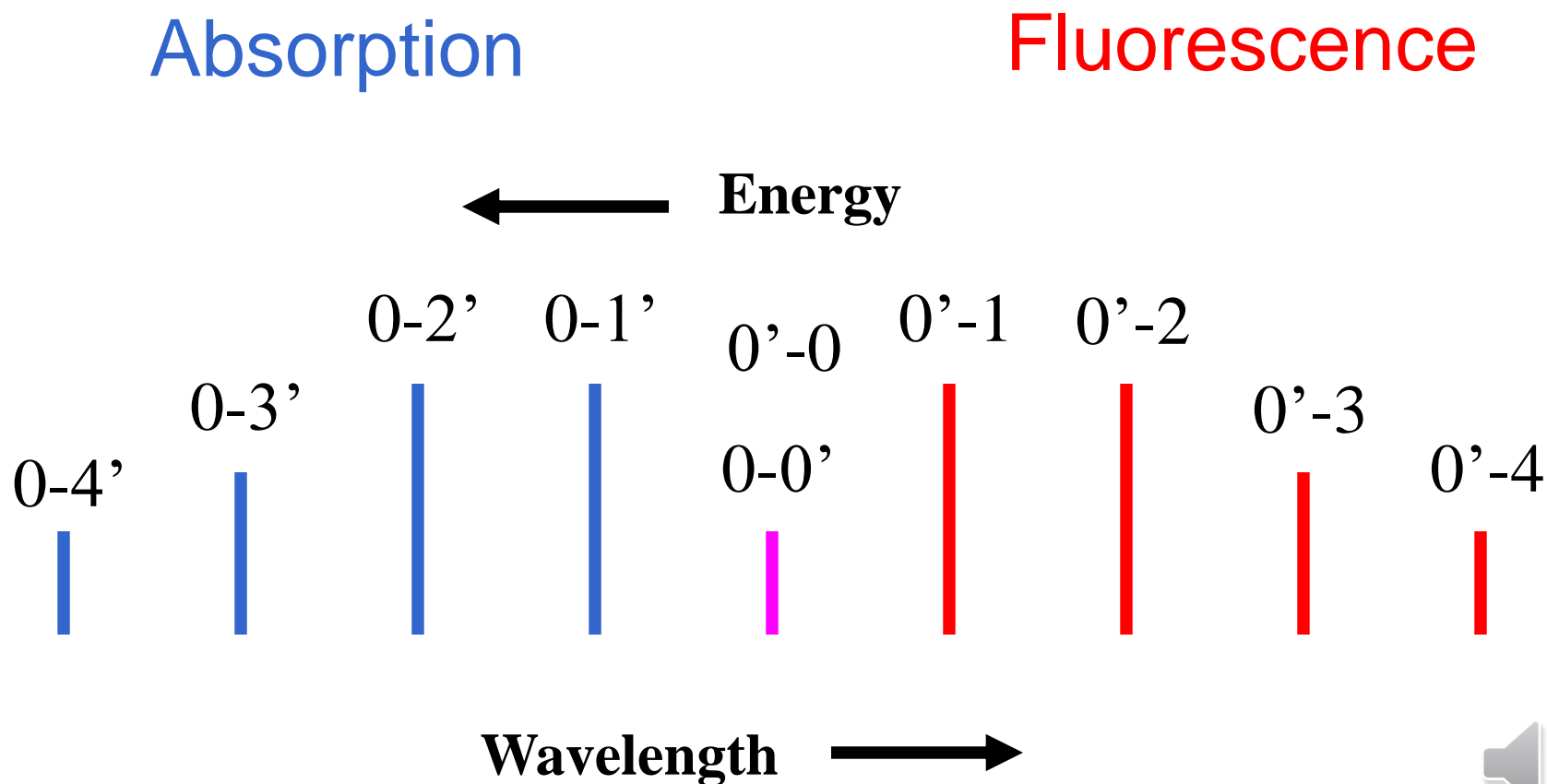
The Franck-Condon factor is the same for absorbance and fluorescence



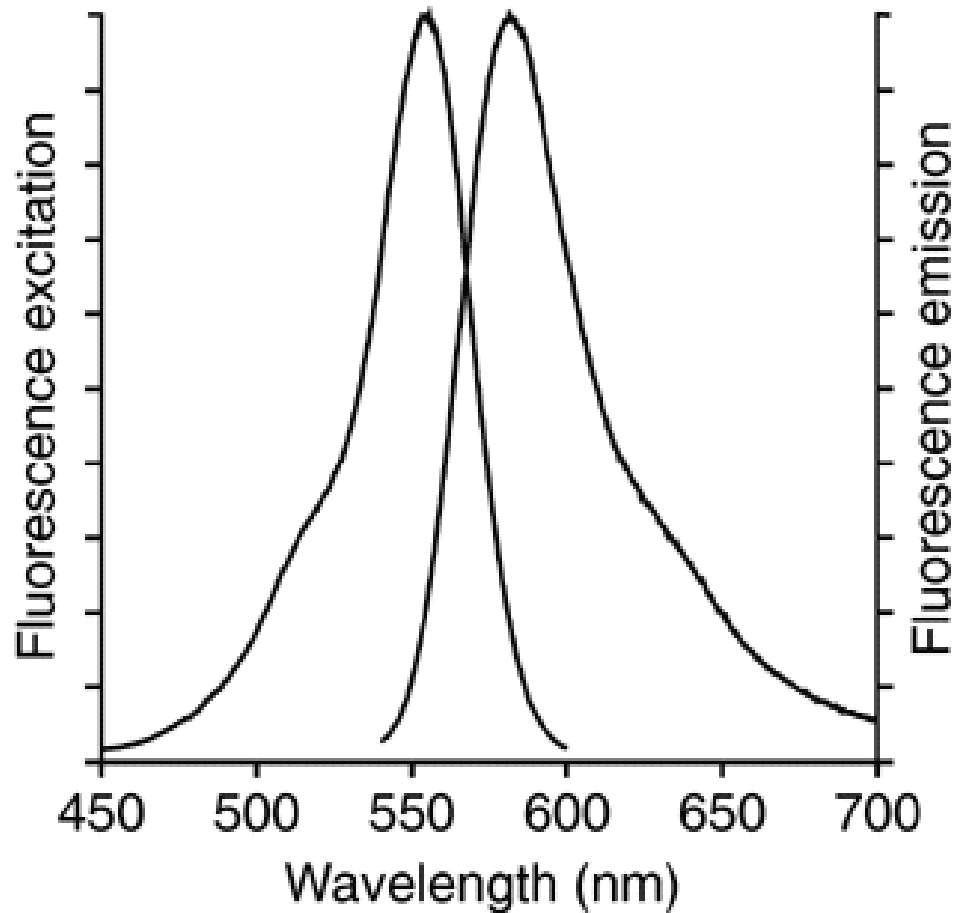
1. Absorption
2. Vibrational relaxation
3. Fluorescence



This leads to a “mirror image” relationship between absorption and fluorescence bands



Rhodamine is an example of a mirror image relationship



Fluorescence lifetime and quantum yield

The intrinsic lifetime for a single state is given by a single exponential with time constant τ_0 :

$$N(t) = N(0)e^{-t/\tau_0}$$

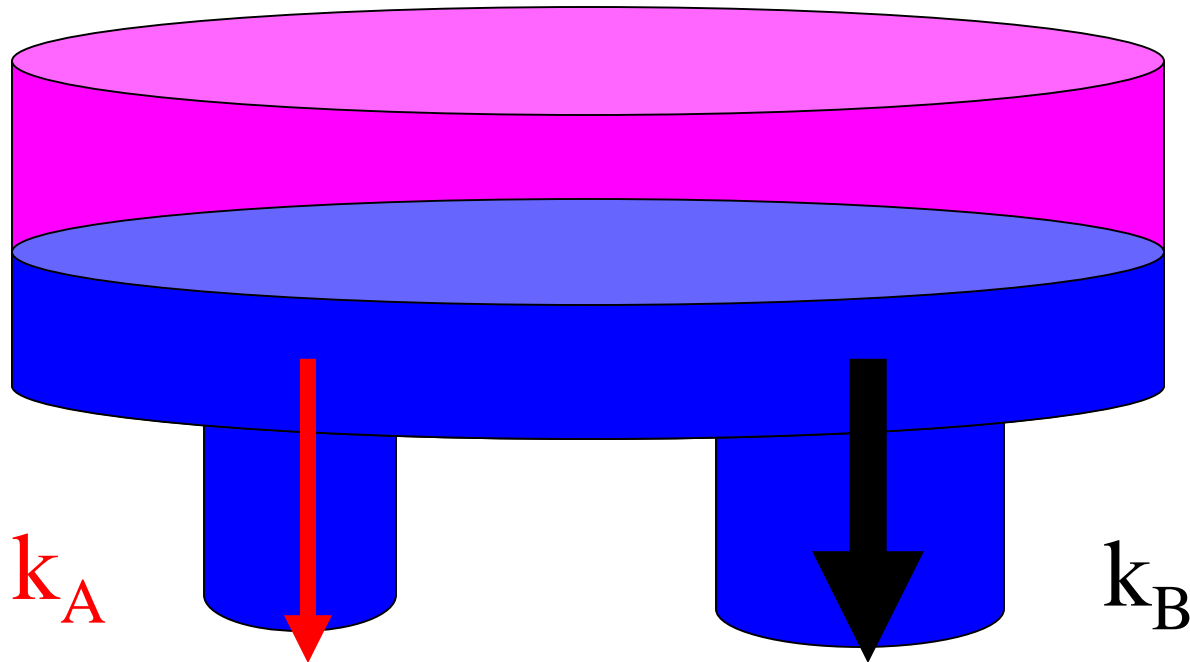
The quantum yield is the ratio of the molecules that decay by the fluorescent pathway to the total:

$$\phi = \frac{k_f}{k_f + k_{nr} + k_{other}} = \frac{\tau_0}{\tau_f}$$

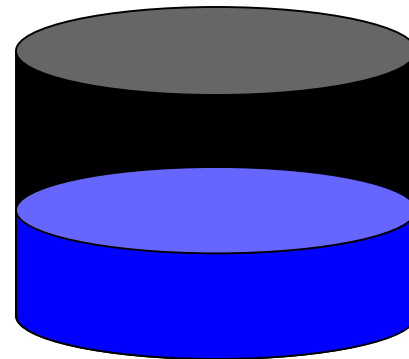
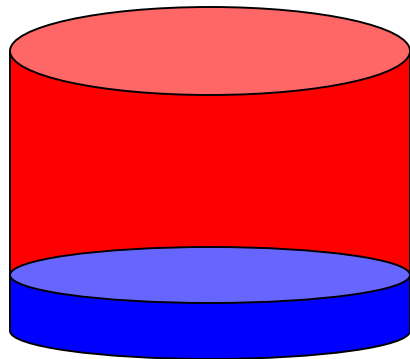


Hydraulic analogy for quantum yield

Tank with
two drains



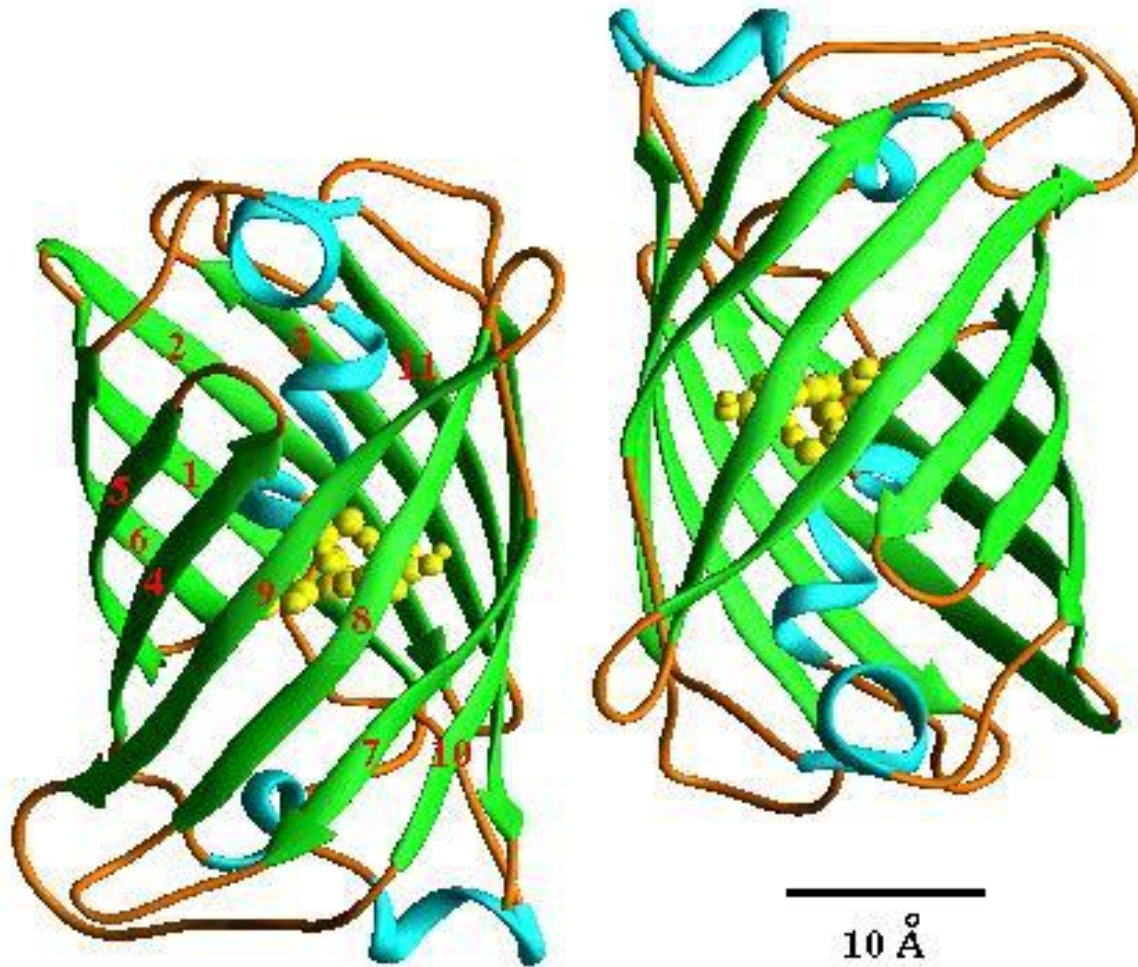
$$\Phi_A = \frac{k_A}{k_A + k_B}$$



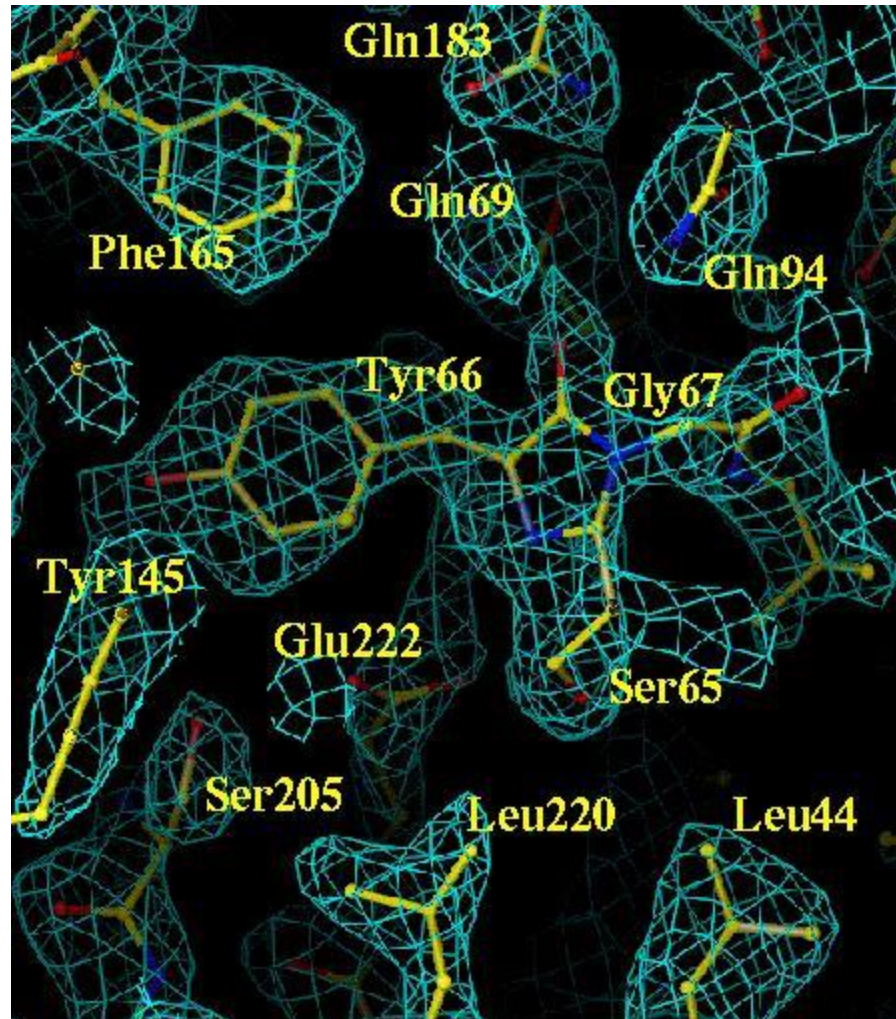
$$\Phi_B = \frac{k_B}{k_A + k_B}$$



GFP Structure



The chromophore in 3D



GFP as a reporter system

- Green fluorescent protein is particularly useful as a reporter in living cells and organisms. *GFP* gene fusions provide a "window" on to the mechanisms that regulate the activity of specific genes, in specific, living cells.



- As the message is transcribed for the gene of interest,
- GFP is made. Detection of GFP activity is a
- measure of gene expression.



Fluorescence quenching

The quantum yield gives the intrinsic fraction of the molecules that decay by emitting light. In addition, the fluorescence emission can be further quenched by:

1. Collisional quenching – molecular collisions in solution
2. Intersystem crossing – conversion from singlet to triplet
3. Electron transfer – ${}^1\text{DA} \rightarrow \text{D}^+\text{A}^-$
4. Energy transfer – emission is transferred to an acceptor

Diffusional quenching

The Stern-Volmer equation is: $\Phi_0/\Phi = 1 + k_q\tau_0 [Q]$
 k_q is the quenching rate constant, τ_0 is the lifetime in the absence of quencher, $[Q]$ is the quencher concentration.



Stern-Volmer Equation for fluorescence quenching in solution

$$\Phi_0 = k_f / (k_f + k_{nr})$$

$$\Phi = k_f / (k_f + k_{nr} + k_q[Q])$$

$$\Phi_0 / \Phi = (k_f + k_{nr} + k_q[Q]) / (k_f + k_{nr})$$

$$\tau_0 = 1 / (k_f + k_{nr})$$

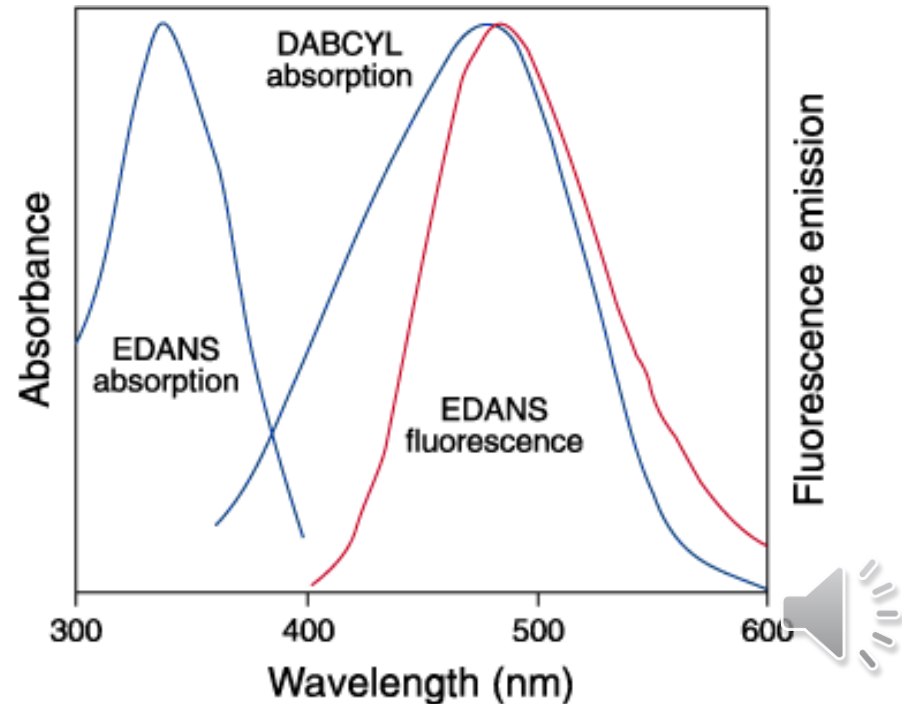
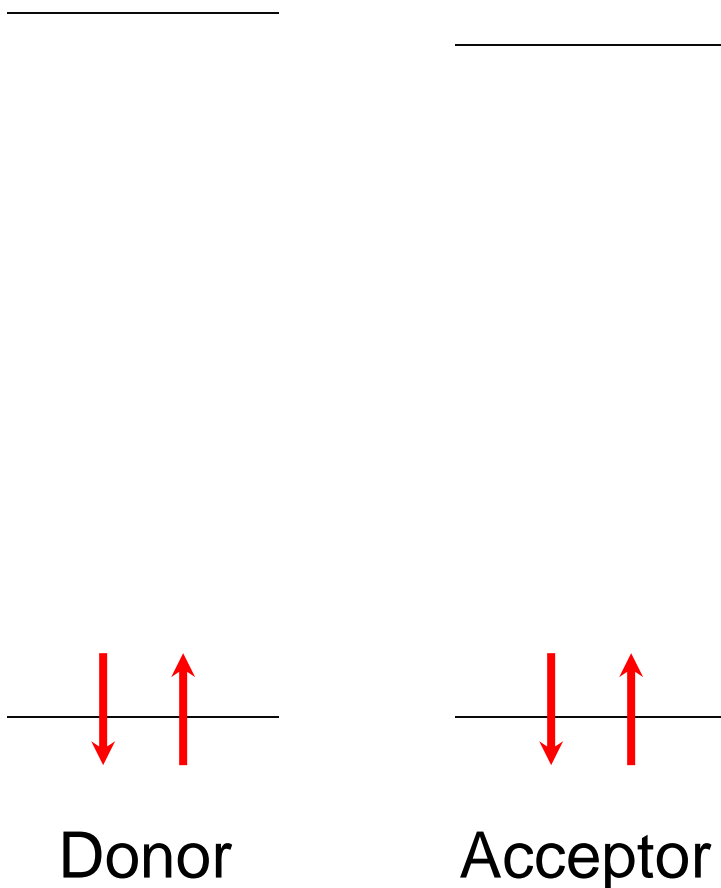
$$\Phi_0 / \Phi = (1 / \tau_0 + k_q[Q]) / (1 / \tau_0)$$

$$\Phi_0 / \Phi = 1 + k_q \tau_0 [Q]$$



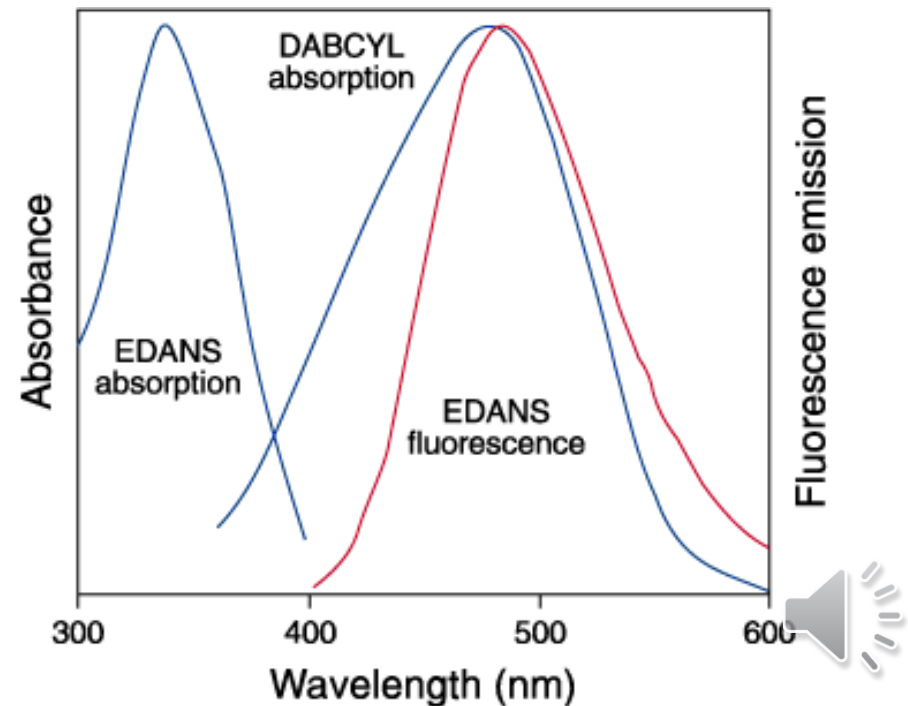
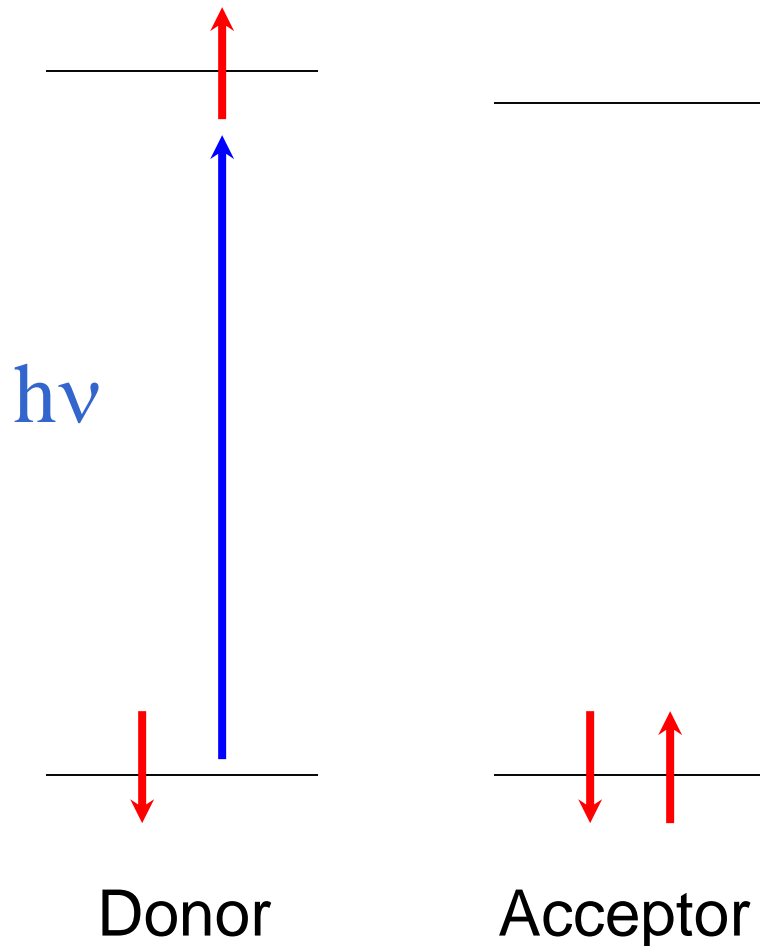
Spectral overlap permits quenching

Spectral overlap between EDANS fluorescence and DABCYL absorption permits efficient quenching of EDANS fluorescence by resonance energy transfer to nonfluorescent DABCYL.



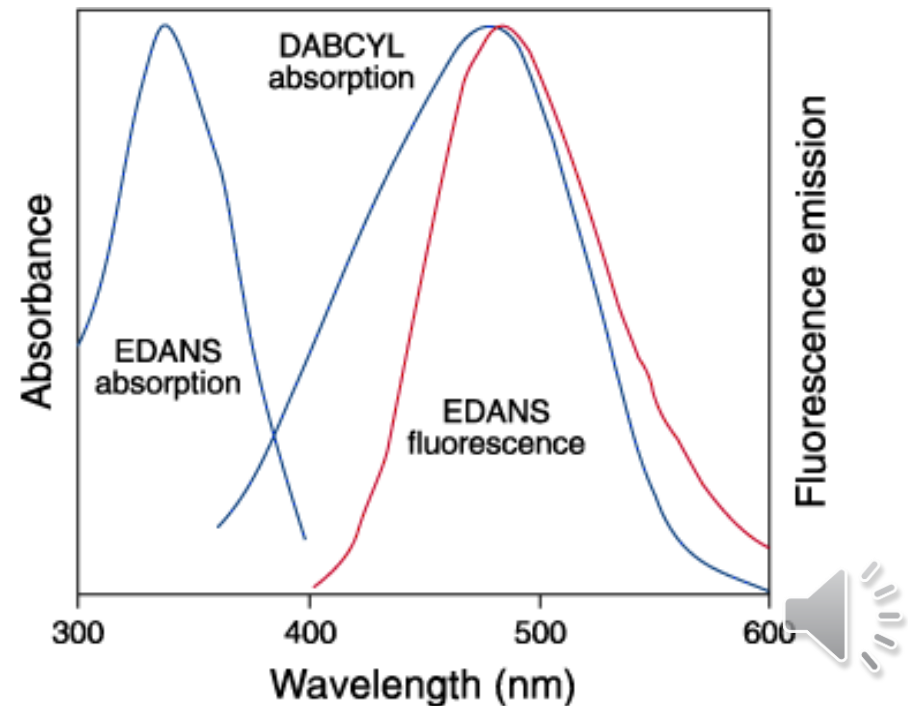
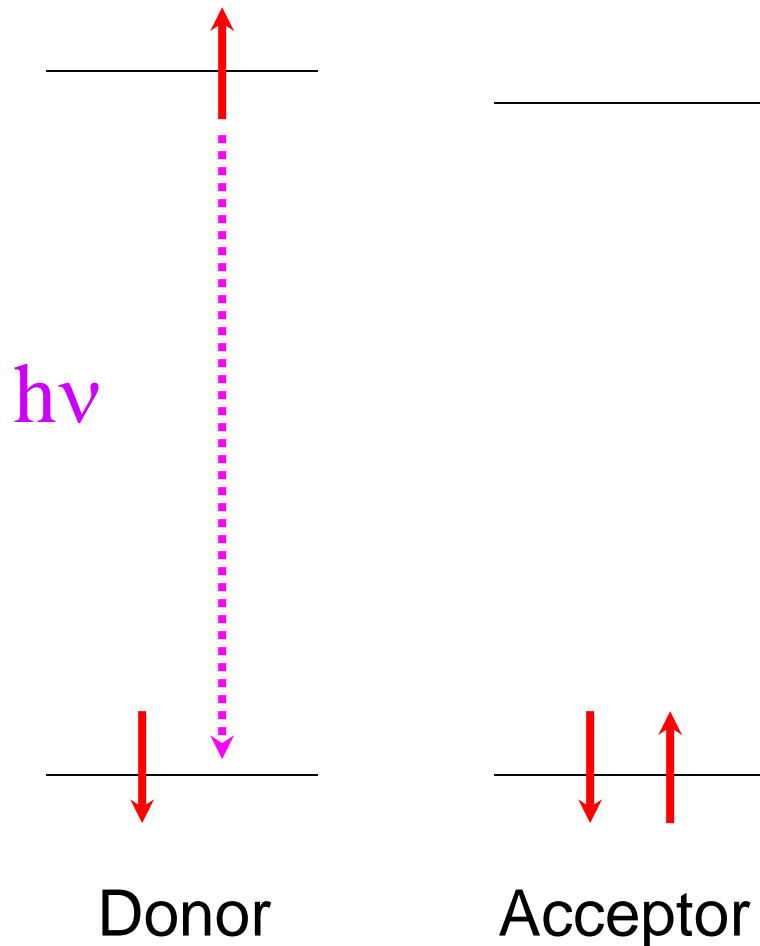
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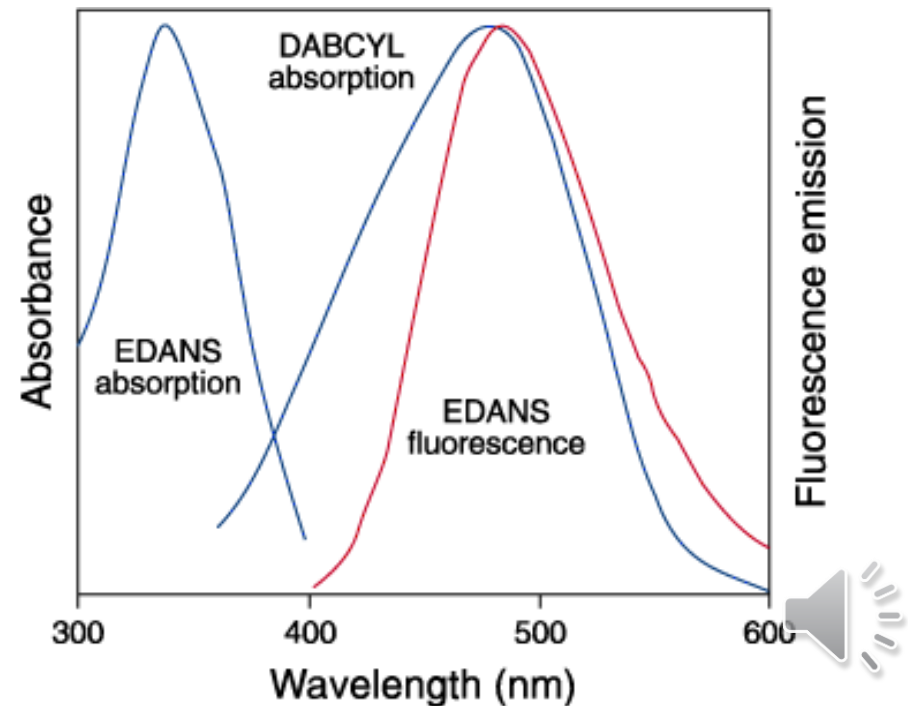
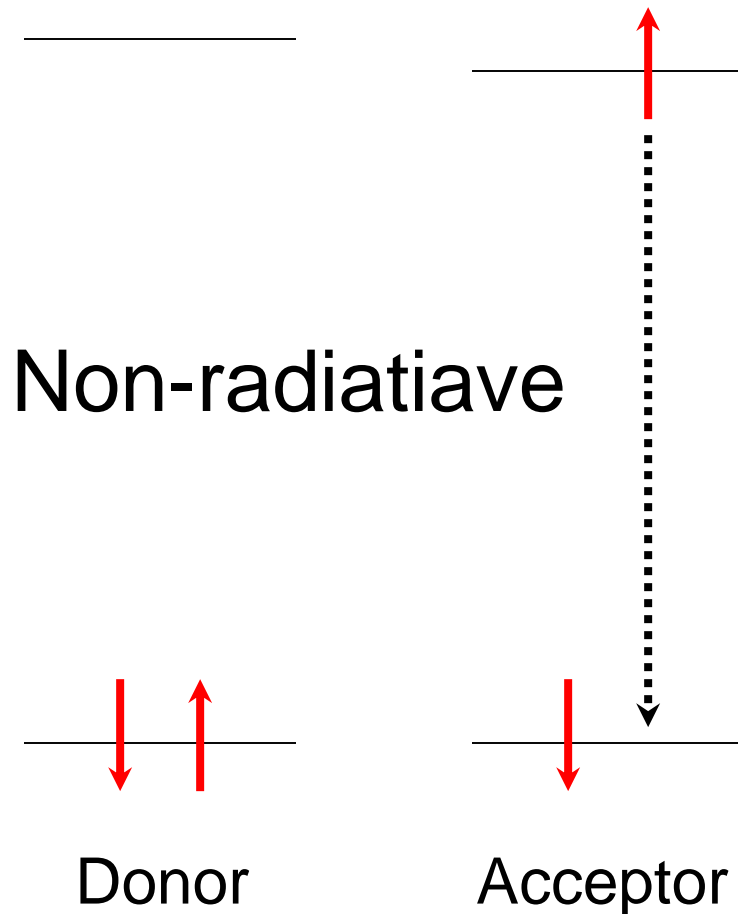
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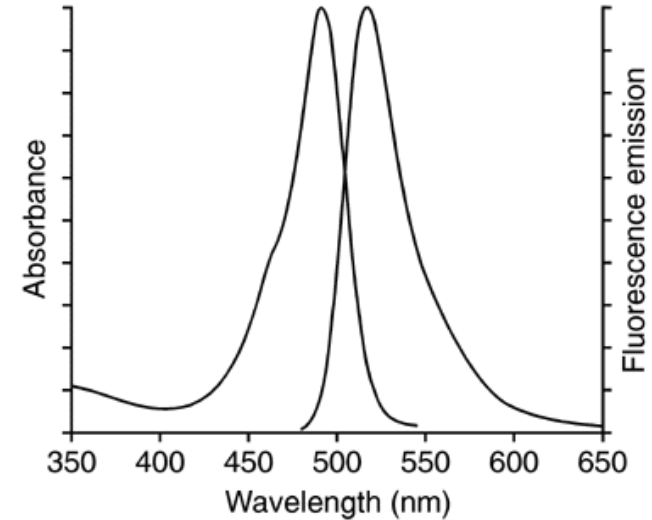
Fluorescent resonant energy transfer (FRET)

Fluorescence resonance energy transfer (FRET) is a distance-dependent interaction between the electronic excited states of two dye molecules in which excitation is transferred from a donor molecule to an acceptor molecule without emission of a photon. FRET is dependent on the inverse sixth power of the intermolecular separation, making it useful over distances comparable with the dimensions of biological macromolecules. Thus, FRET is an important technique for investigating a variety of biological phenomena that produce changes in molecular proximity.

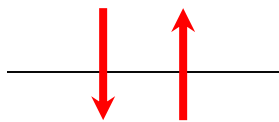


Energy transfer mechanism

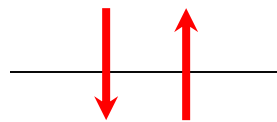
Fluorescein



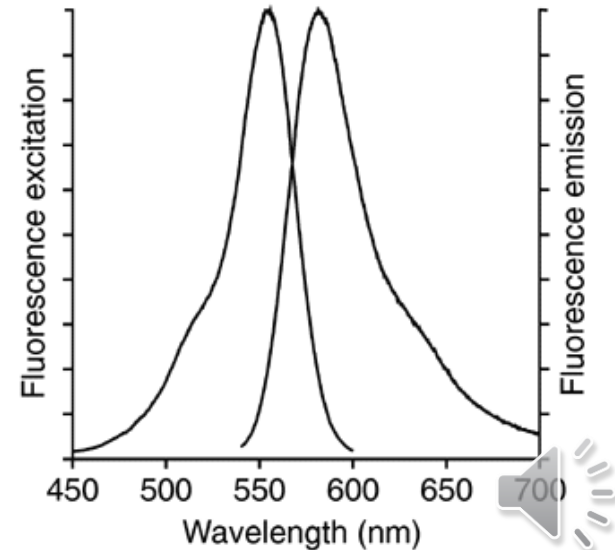
Rhodamine



Donor

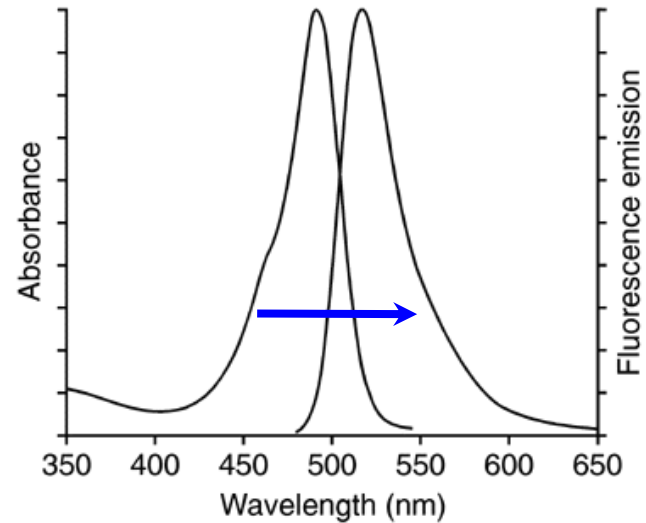
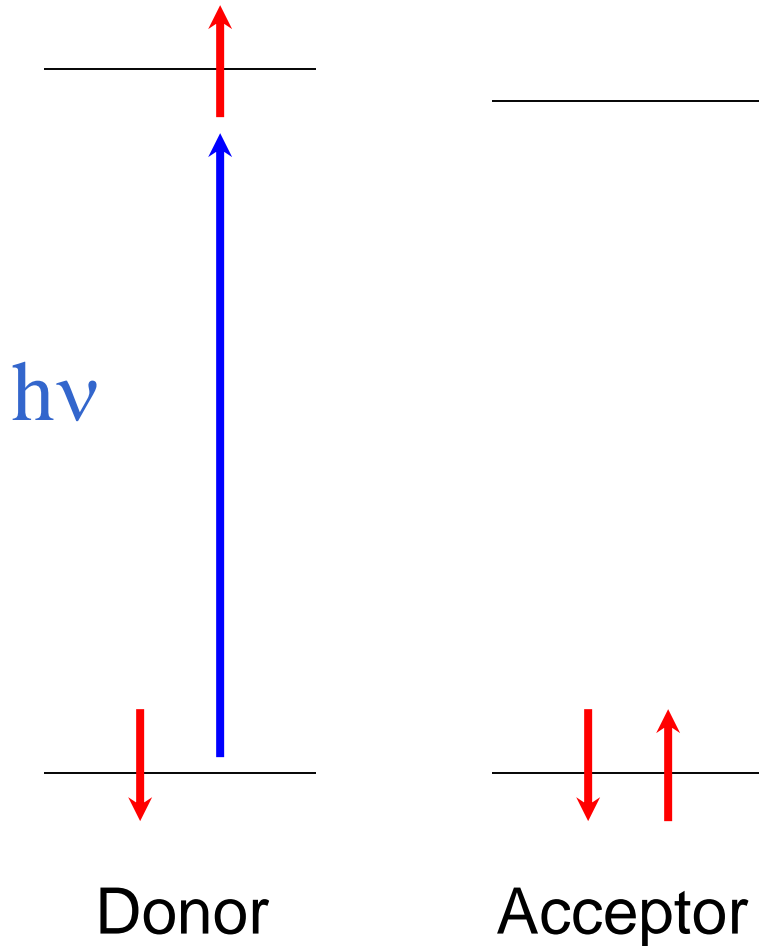


Acceptor

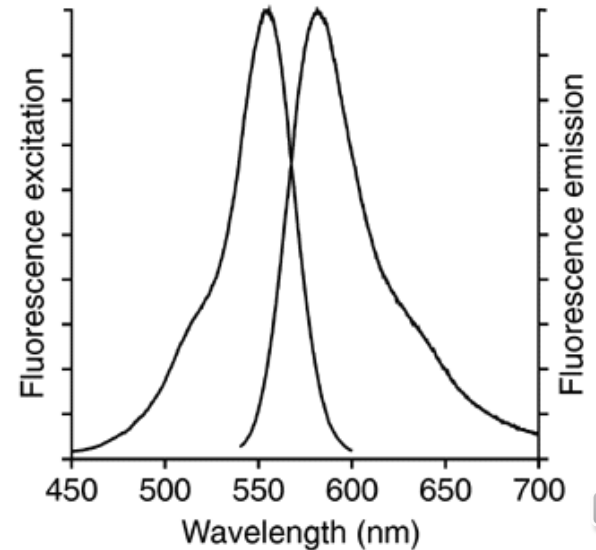


Energy transfer mechanism

Fluorescein

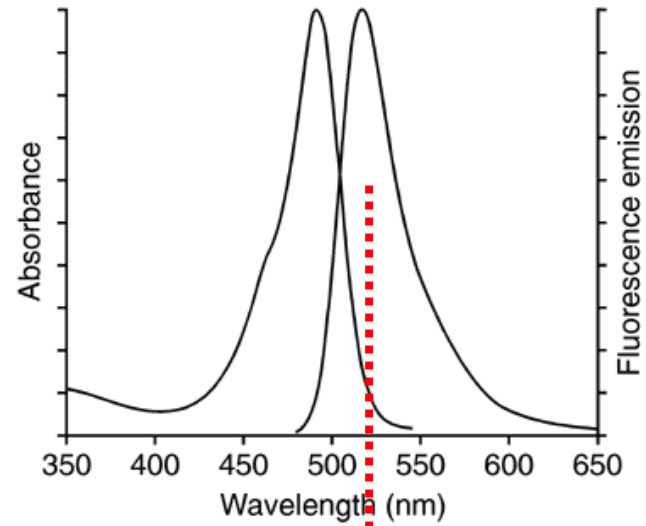


Rhodamine

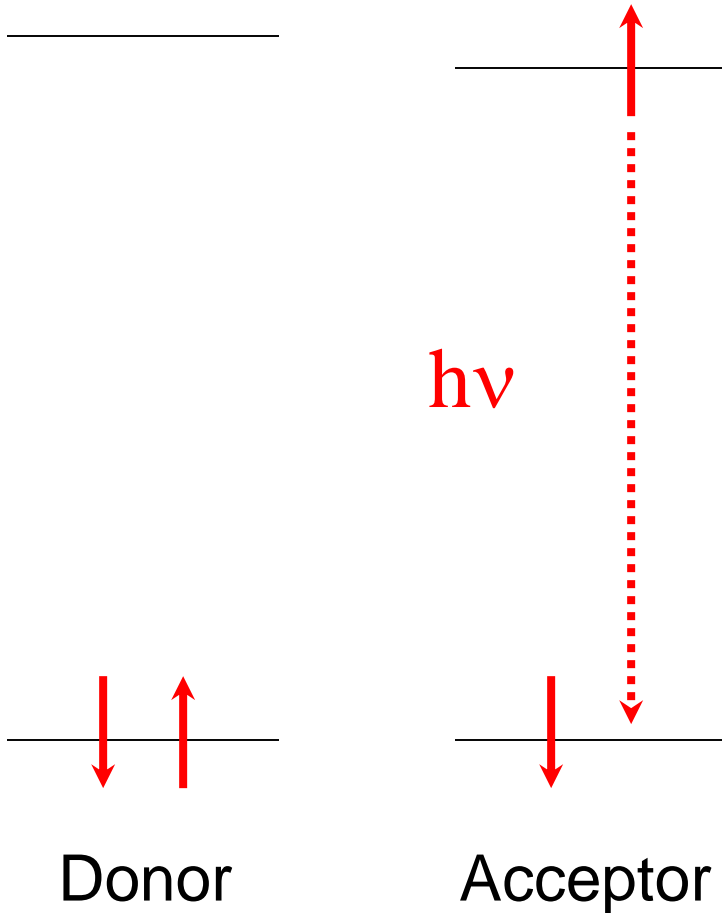
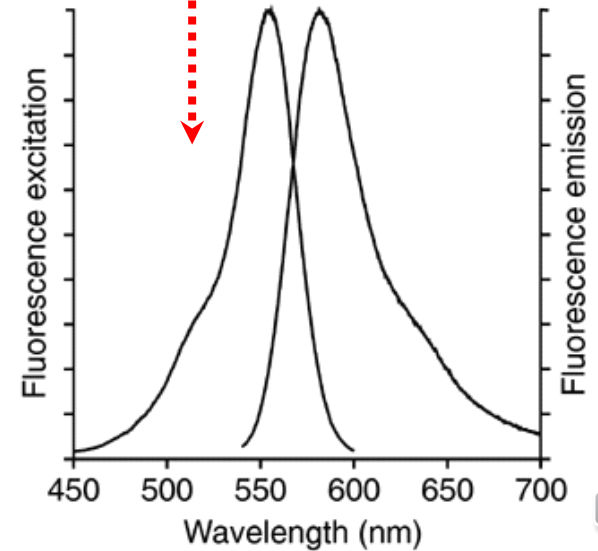


Energy transfer mechanism

Fluorescein



Rhodamine



Primary Conditions for FRET

- Donor and acceptor molecules must be in close proximity (typically 10–100 Å).
- The absorption spectrum of the acceptor must overlap fluorescence emission spectrum of the donor.
- Donor and acceptor transition dipole orientations must be approximately parallel.



The Förster Radius

The rate constant for energy transfer is:

$$k_{DA} = (R_0/R)^6$$

The distance at which energy transfer is 50% is:

$$R_0 = [8.8 \times 10^{-28} \kappa^2 \tau_0^{-1} n^{-4} \Phi J(\lambda)]^{1/6}$$

κ - orientation factor (2/3 for an isotropic sample)

n - index of refraction

Φ - quantum yield of the donor

Spectral overlap integral is

$$J(\lambda) = \int \varepsilon(\lambda) F_D(\lambda) \lambda^4 d\lambda \text{ cm}^3 \text{ M}^{-1}$$

Donor	Acceptor	R_0 (Å)
Fluorescein	Tetramethylrhodamine	55
IAEDANS	Fluorescein	46