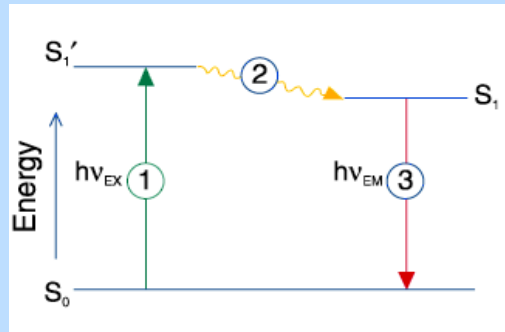
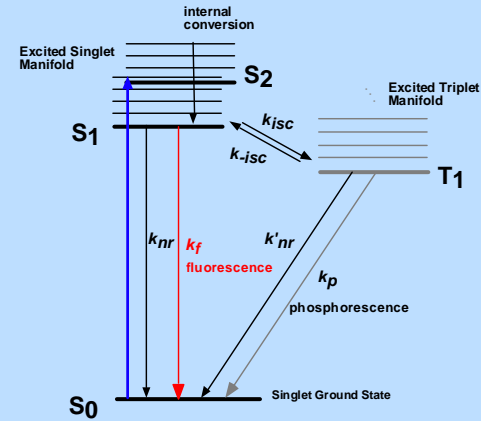


Introduction to Fluorescence



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Jablonski Diagram



Intersystem crossing

a method for populating the triplet state

Internal conversion

Kasha rule

Triplet state

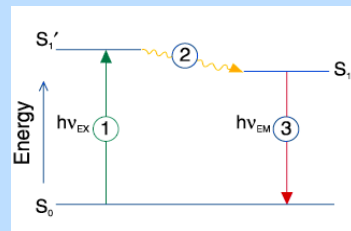
phosphorescence; significantly longer lifetimes than fluorescence

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•What is Fluorescence?

- defined as the decay from an excited singlet state of a fluorophore
- the result of **Absorption(1)** of a photon
- leading to an excited singlet state, S_1
- followed by a **decay (2)** from S_1 (timescale of nanoseconds; other processes can occur in this time)
- yielding emitted light of lower energy, i.e. **redshifted (3)** in wavelength (Stokes' shift)

•the Stokes' shift allows efficient discrimination of the excitation, making fluorescence a very sensitive technique



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Jablonski Diagram

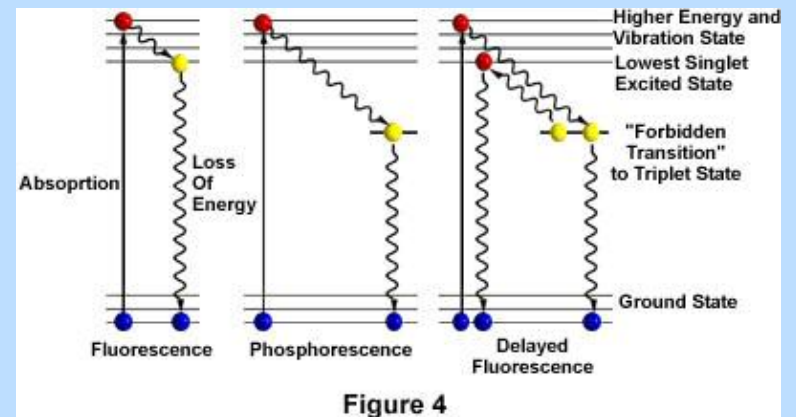
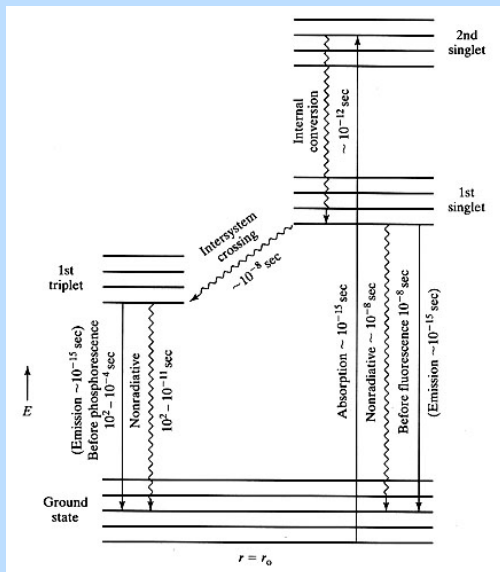


Figure 4

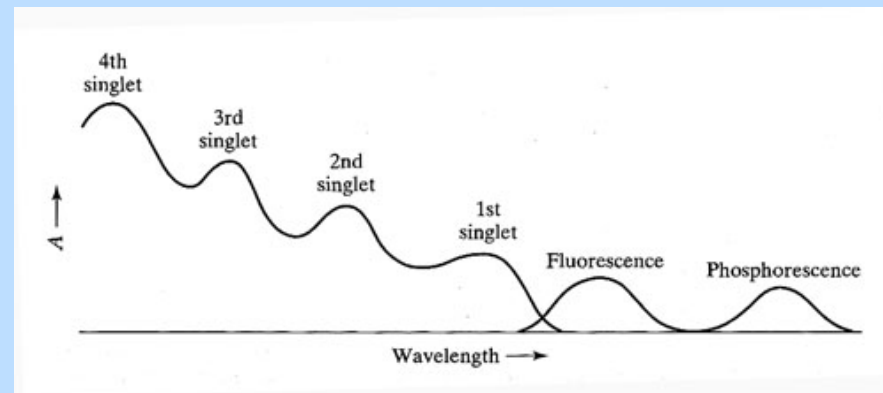
Alternative pathways for relaxation of excited molecule

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Lifetimes of different processes

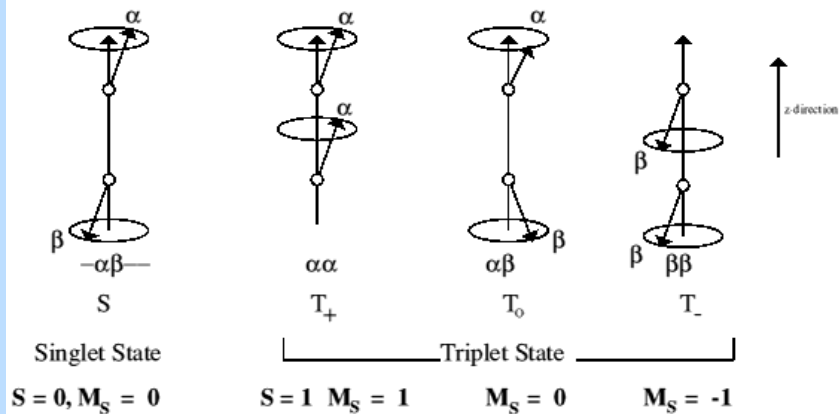


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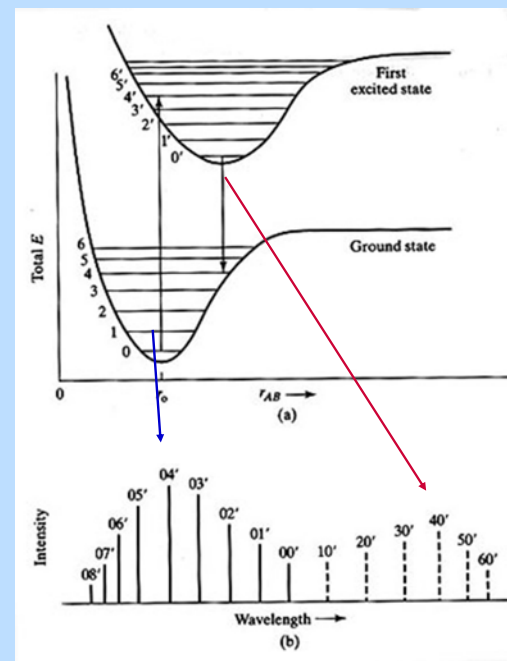
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Diagrams to illustrate the Singlet and Triplet spin states



In the singlet states the two spins are co-planar but point in different x-directions. Thus there is no resultant z-component or x-component. In the triplet state the $\alpha\beta$ component has spins which are also co-planar but now the spins point in the same (x) direction. In this case there is no z-component but there is an x-component.

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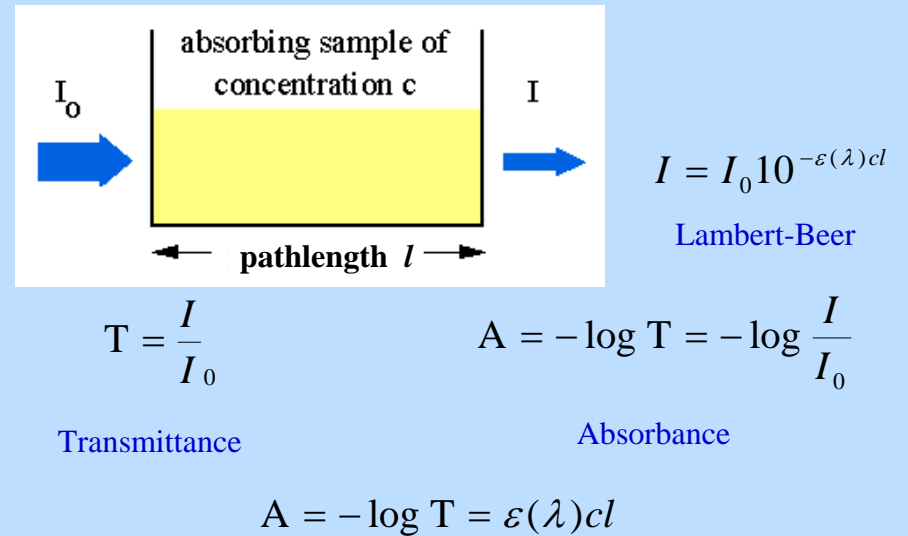
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Critical Fluorescence Parameters

- “To characterize the photoexcited emission from molecules in a system of unknown complexity, we should determine the spectral distribution, photon yield, lifetime of the excited state, and polarization of the fluorescence emission, as a function of the wavelength of emission.”
 - Gregorio Weber, Meth. Enzym. 278, p. 1 (1997)
- Spectral distribution
 - Emission spectra: Fix the excitation wavelength and scan through emission wavelengths; usually independent of excitation wavelength
 - Excitation spectra: Fix emission wavelength and scan through excitation spectra; usually same as absorption spectrum

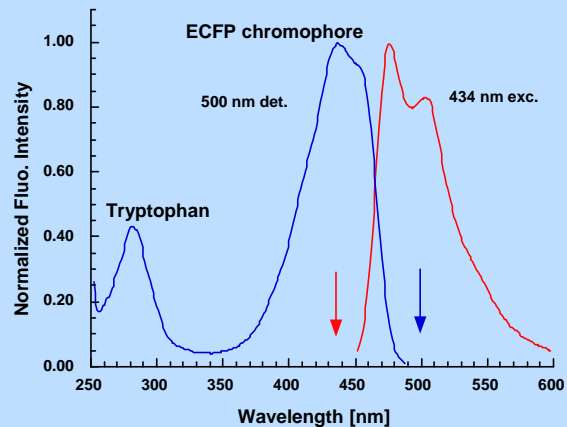
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Absorption spectroscopy



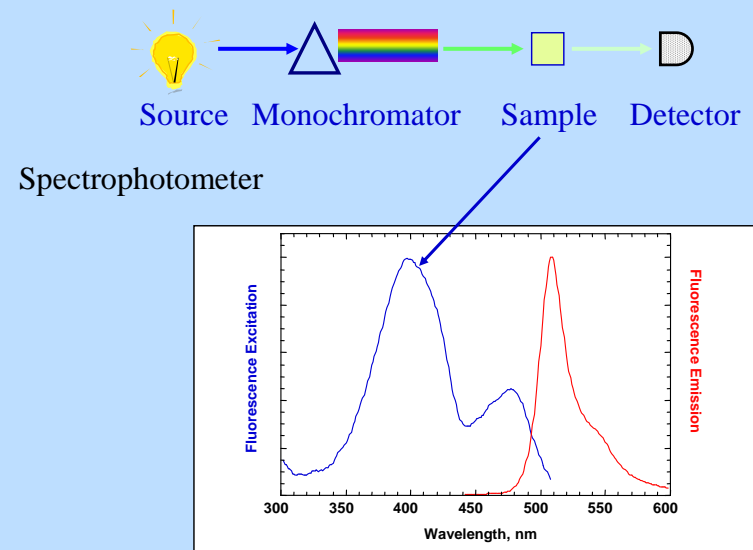
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Spectral distribution



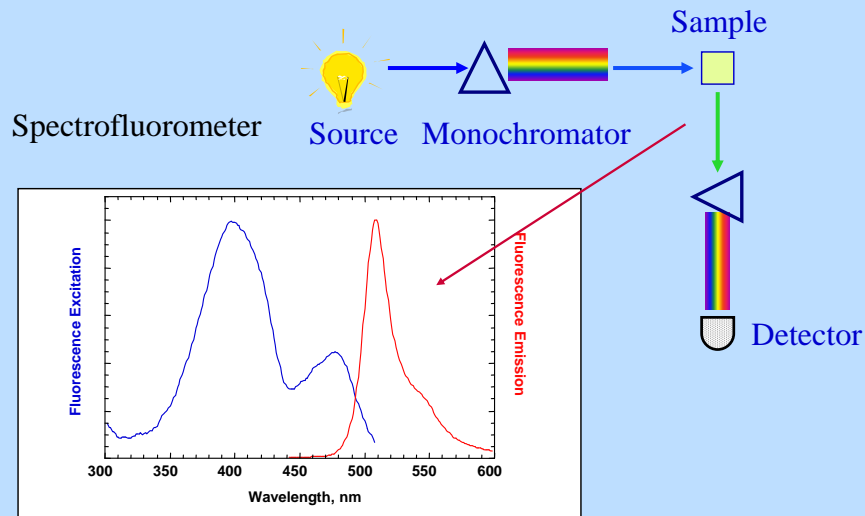
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Determination of spectral distribution



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Measuring Fluorescence



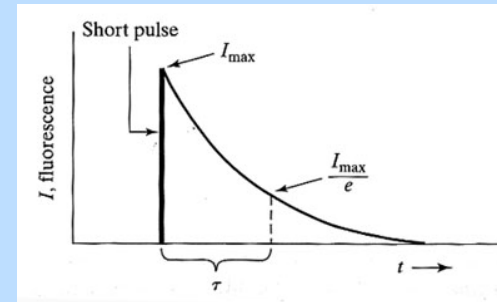
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Fluorescence Lifetime(s)

all competing processes affect the fluorescence lifetime

Measured lifetime

$$\frac{dN}{dt} = -N(k_f + k_{ic} + k_{isc})$$



$$N = N_0 e^{-(k_f + k_{ic} + k_{isc})t}$$

$$\tau_{\text{int}} = 1/(k_f + k_{ic} + k_{isc})$$

$$Q = k_f \tau$$

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Critical Fluorescence Parameters

- **Photon Yield/Quantum Efficiency**

– a measure of the emission efficiency of the fluorophore

$$Q = \frac{\text{\# of photons emitted}}{\text{\# of photons absorbed}} \quad Q = \frac{k_f}{k_f + k_{ic} + k_{isc}}$$

- **Brightness**

– proportional to ability to absorb light (extinction coefficient, ε)
AND

– Quantum Yield, Q

- **Total Fluorescence**

$$F = I_0 \epsilon [c] l Q$$

– where I₀ is incident light intensity,

– l is pathlength

– [c] is fluorophore concentration

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Fluorescence Lifetime(s)

- **Fluorescence lifetime (τ)** is the characteristic time that the fluorophore spends in the excited state.

$$\tau_{\text{int}} = 1/(k_f + k_{ic} + k_{isc})$$

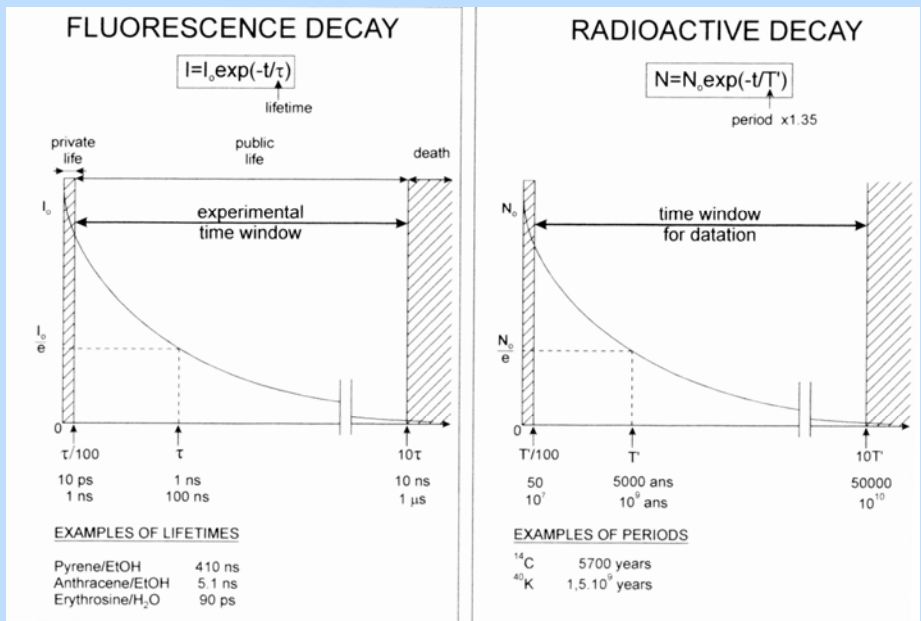
- **During this time in the excited state, the fluorophore undergoes multiple interactions with the environment**

- collisional quenching
- fluorescence energy transfer
- intersystem crossing
- rotational motion

- A homogeneous system (fluorophore+uniform solvent) should, in principle, exhibit a single lifetime

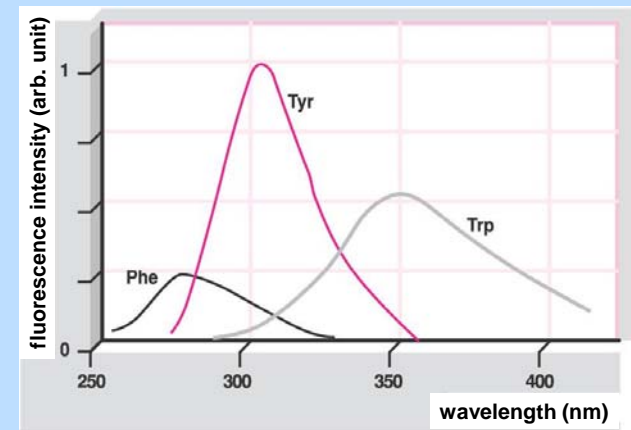
- Heterogeneous systems (most real systems) such as cells typically show multiple lifetimes

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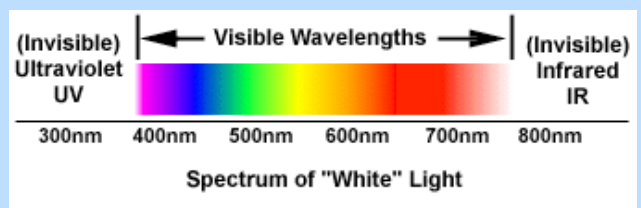
Fluorescence spectra of different amino acids



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Fluorophores

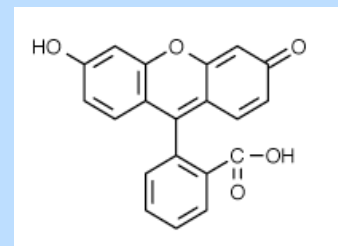
- What is a fluorophore?
 - any molecule that fluoresces is called a fluorophore
 - typically polyaromatic hydrocarbons
 - some amino-acids, in particular Trp, Tyr and Phe



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Fluorophores

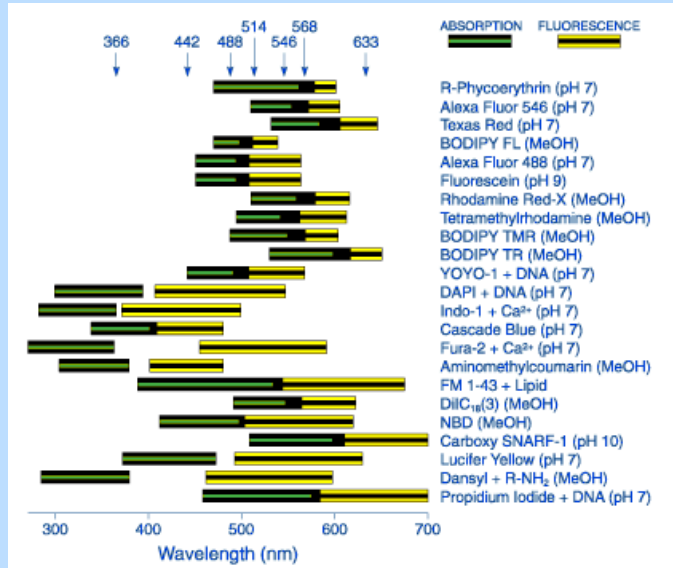
- Common fluorophores
 - exogenous fluorophores - dyes such as Fluorescein, Rhodamine, Acridine Orange, Ethidium Bromide, Cy dyes
 - endogenous fluorophores - NADH autofluorescence, e.g.



FLUORESCIEIN
 Molecular Formula: C₂₀H₁₂O₅
 Molecular Weight: 332.31

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Common Fluorophores



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Measuring Fluorescence

- Spectrofluorometer
 - excitation and emission spectra
 - usually based on diffraction gratings
 - usually for bulk solutions (cuvette experiments)
- Fluorescence microscope
 - spatially resolved fluorescence
 - cellular samples, e.g.
 - typically filter-based

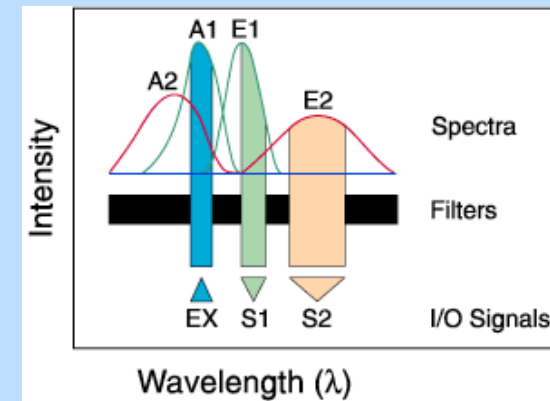
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Some applications of fluorophores

- Immunofluorescence
- ion sensitive dyes
 - K^+ , Na^+ , Ca^{2+} specific markers
 - pH indicators
- membrane potential
 - increased intracellular fluorescence
- DNA dyes
- determination of protein fluorescence

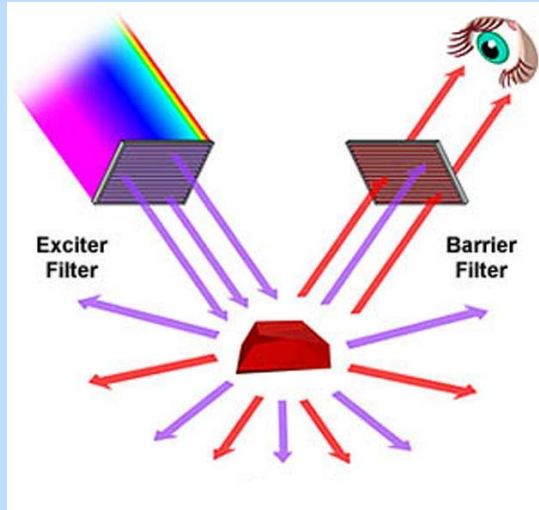
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Measuring Fluorescence



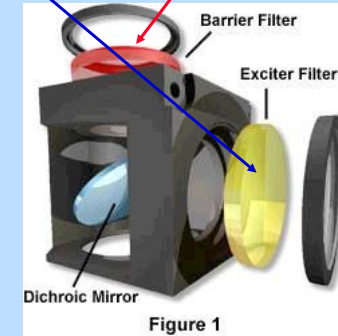
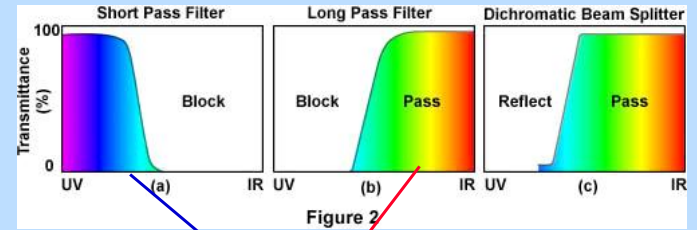
A. Jenei (2007-08)

Measuring Fluorescence



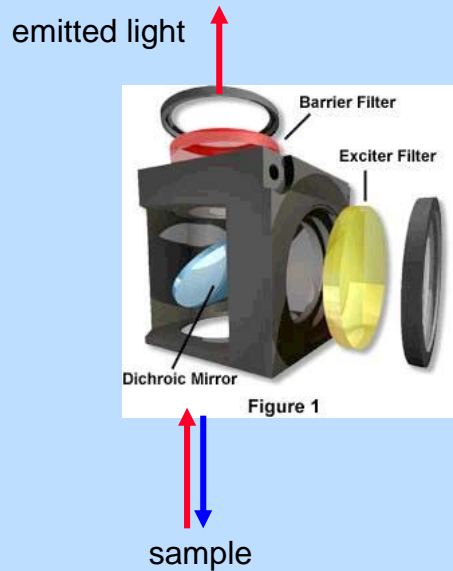
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Filters



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Filters



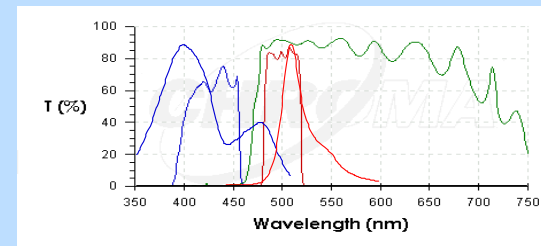
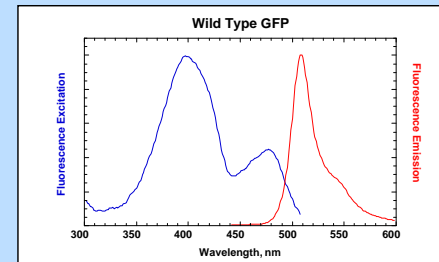
Typical Filter Cube in a Microscope

Excitation light



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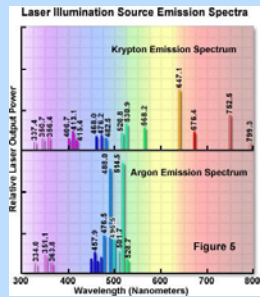
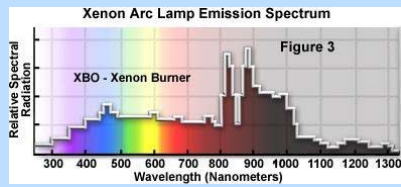
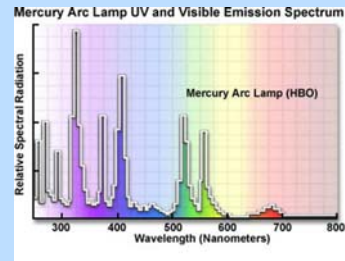
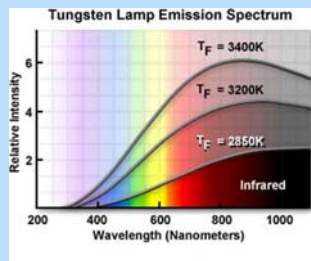
Selecting Filters



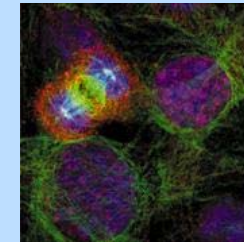
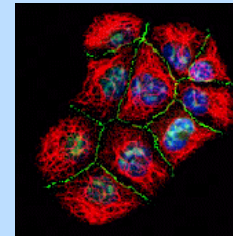
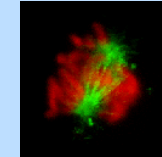
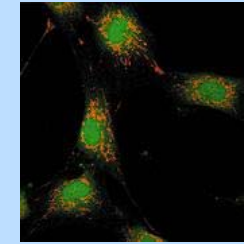
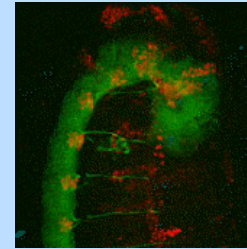
Chroma Technology 32000 WTGFP Bandpass Filter Set

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Light sources

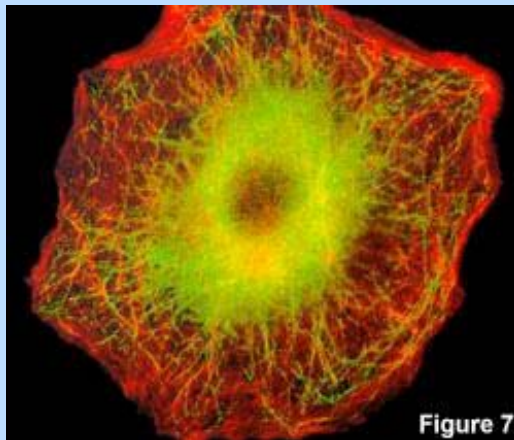


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A. Jenei (2007-08)

Fluorescence imaging

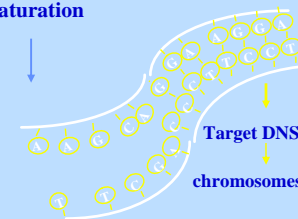


- The sample is stained with FITC (fluorescein isothiocyanate) and Rhodamine-phalloidin to selectively highlight microtubules and actin filaments.

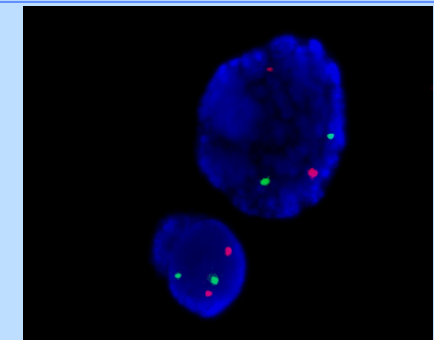
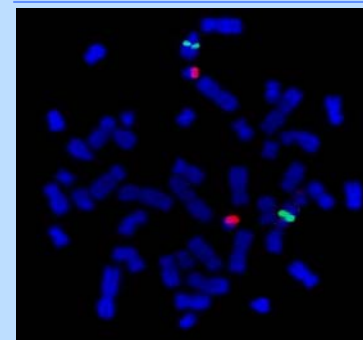
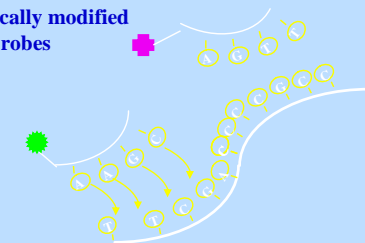
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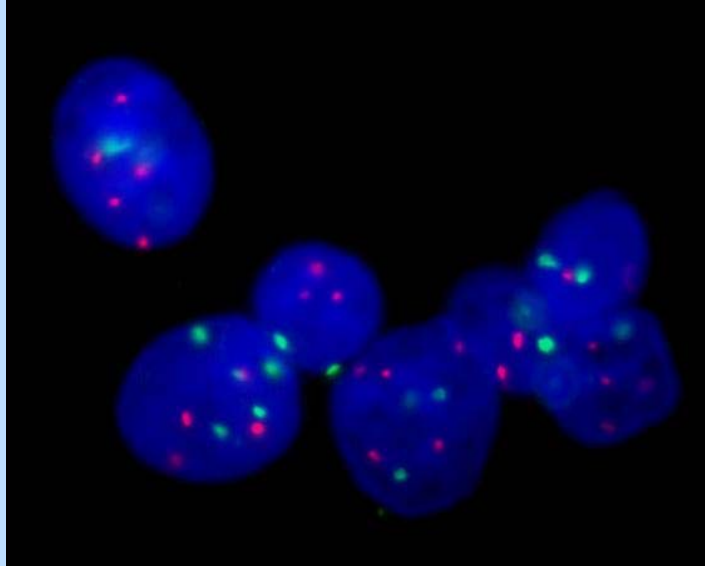
Fluorescence in situ hybridization

Denaturation



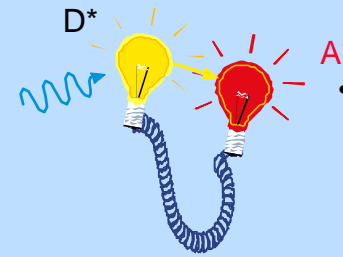
Chemically modified DNS-probes





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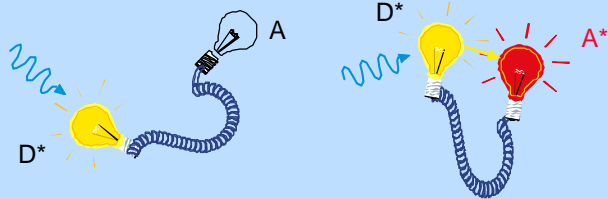
Photophysical Consequences of FRET



- FRET introduces an additional deactivation pathway for the excited donor molecule
- Upon energy transfer
 - Donor excited state (D^*) lifetime decreases
 - Donor fluorescence intensity decreases
 - Donor photobleaching rate decreases
 - Acceptor fluorescence intensity (if the acceptor is fluorescent) increases

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Fluorescence Resonance Energy Transfer (FRET)



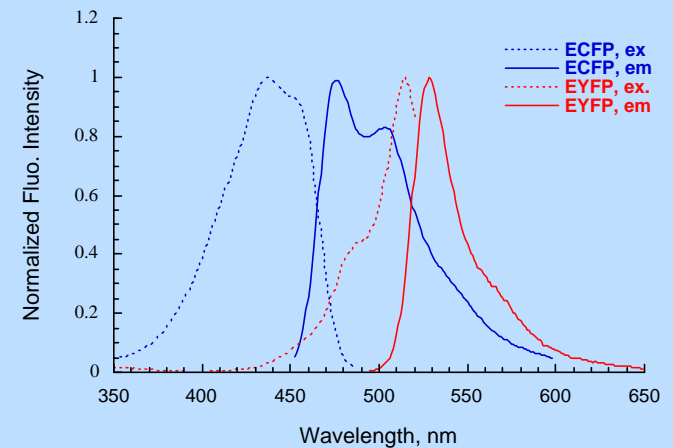
Donor and acceptor far apart - No FRET Donor and acceptor close together - FRET

- non-radiative (electromagnetic) transfer from excited chromophore (donor) to receptor molecule (acceptor) by dipole-dipole coupling
- dynamic Förster transfer process
- strongly distance dependent, rate constant $\propto 1/R^6$
- powerful method for looking at molecule association, protein-protein interactions, receptor-ligand interactions

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FRET Pairs (GFP)

ECFP/EYFP



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Detecting FRET

- Spectral

- increase of acceptor fluorescence
- decrease of donor fluorescence

- Lifetime

- decrease of donor fluorescence lifetime

- Donor Photobleaching

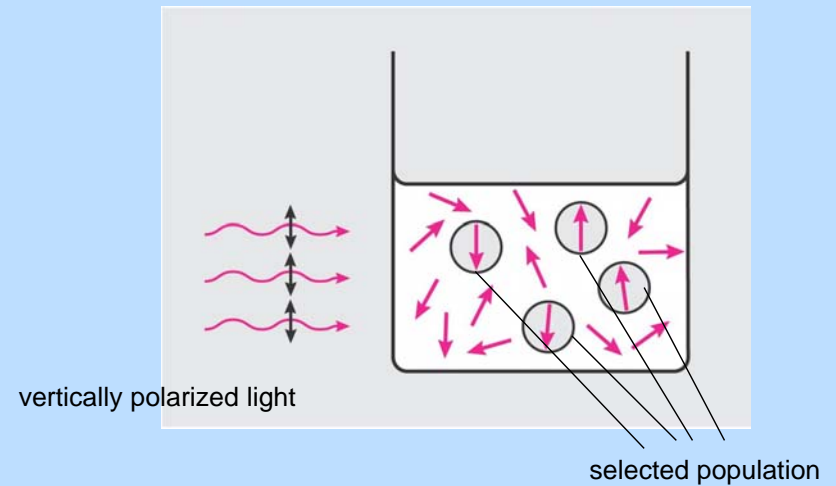
- decrease in donor photobleaching rate in the presence of acceptor (FRET)

- Acceptor Photobleaching

- create an area free of acceptor by photobleaching
- increase in donor fluorescence

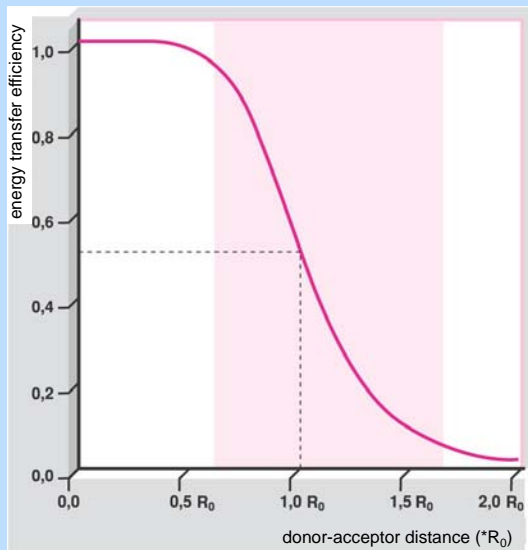
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Photoselection of fluorophores



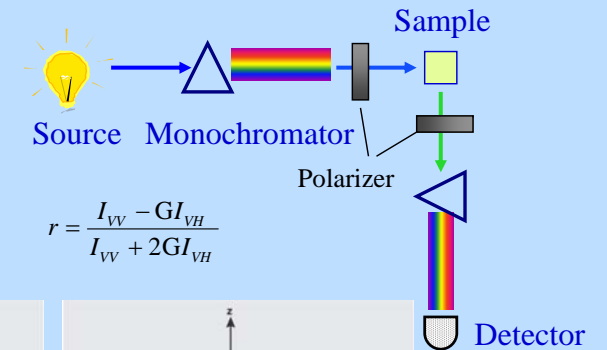
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Distance dependence of FRET efficiency values



$$E = \frac{R_0^6}{R_0^6 + R^6}$$

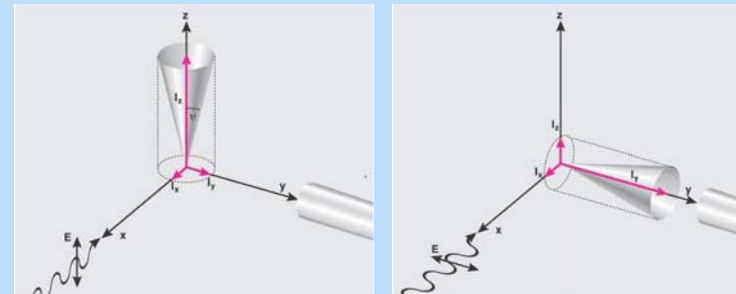
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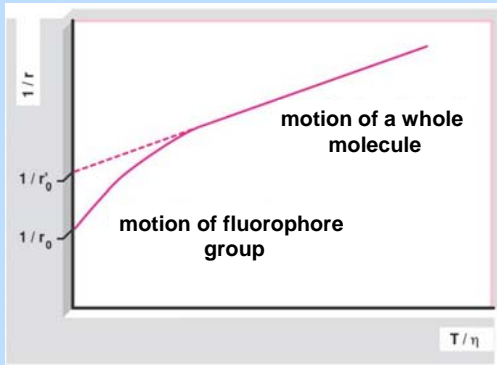
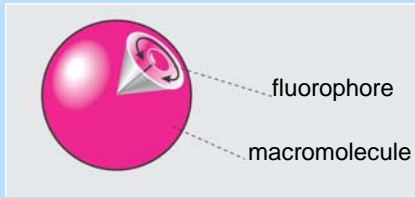
$$p = \frac{I_{VV} - GI_{VH}}{I_{VV} + GI_{VH}}$$

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}}$$

$$G = \frac{I_{HV}}{I_{HH}}$$



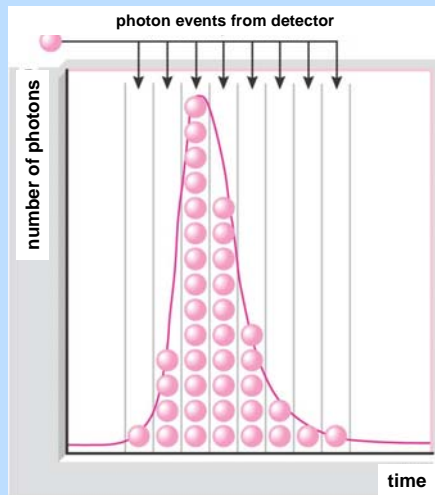
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$$\frac{1}{r} = \frac{1}{r_0} \left(1 + \frac{kT}{V\eta} \tau \right)$$

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Time distribution of first photon upon excitation



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