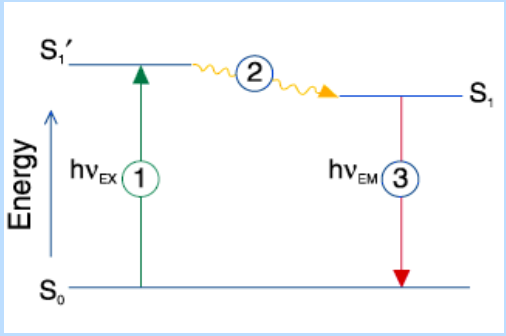
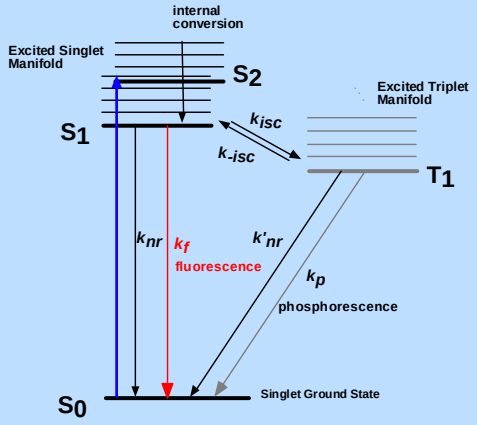
Introduction to Fluorescence



Jablonski Diagram



Intersystem crossing

a method for populating the triplet state

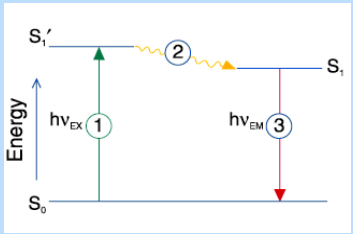
Internal conversion

Kasha rule

Triplet state

phosphorescence; significantly longer lifetimes than fluorescence

- 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



Jablonski Diagram

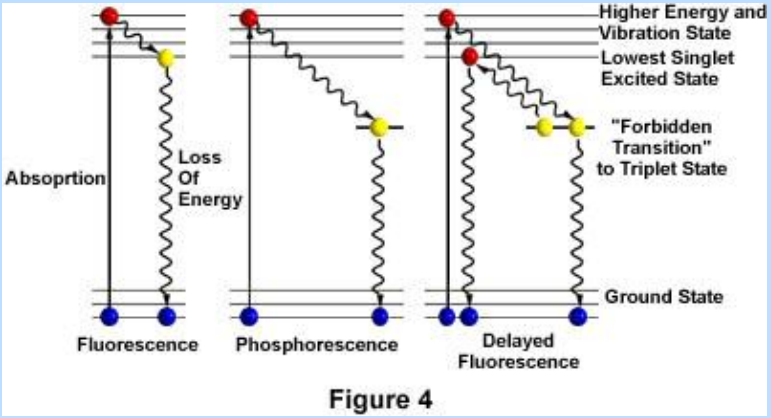
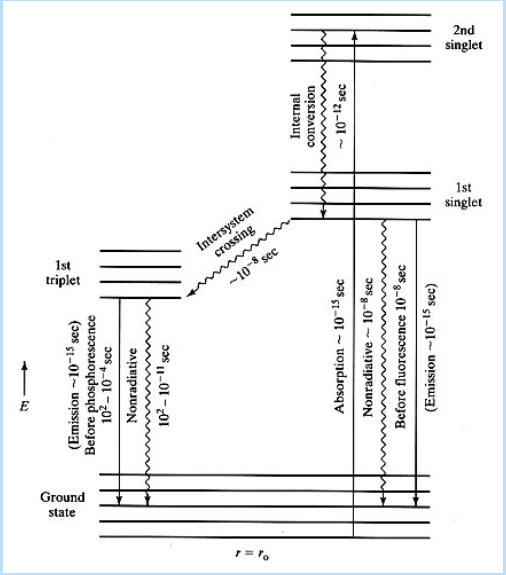
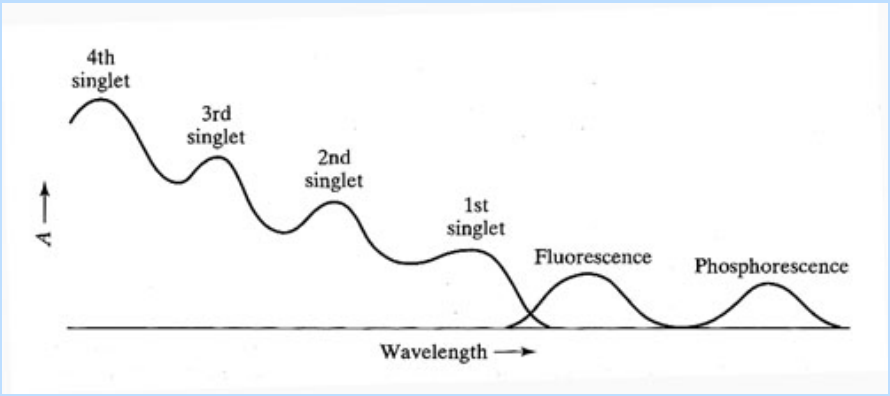


Figure 4

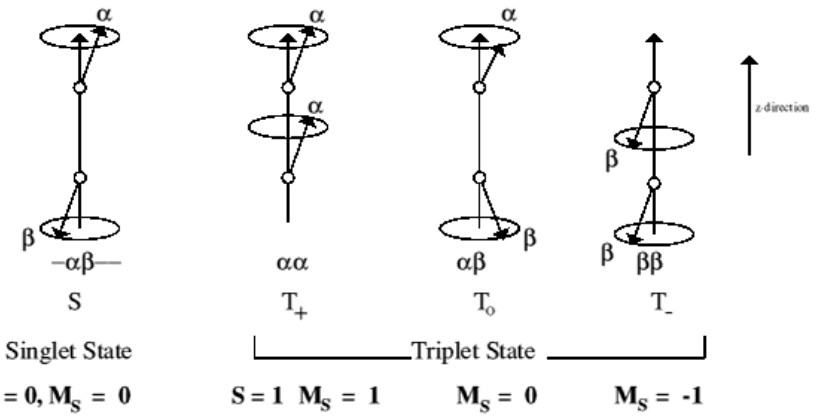
Alternative pathways for relaxation of excited molecule

Lifetimes of different processes

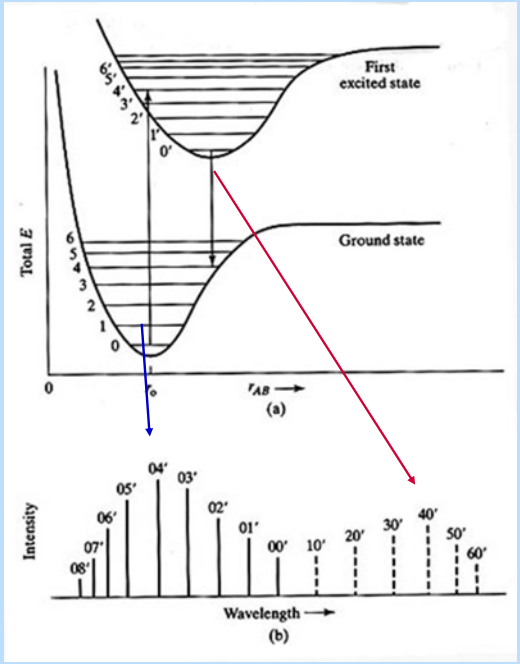




Diagrams to illustrate the Singlet and Triplet spin states



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 αβ 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.

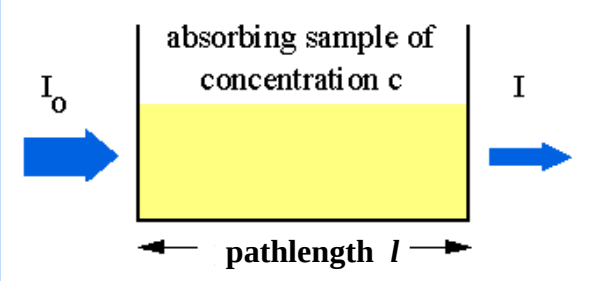


Critical Fluorescence Parameters

- "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 wavlengths; usually independent of excitation wavelength
  - Excitation spectra: Fix emission wavelength and scan through excitation spectra; usually same as absorption spectrum

Absorption spectroscopy



$$I = I_0 10^{-\varepsilon(\lambda)cl}$$

Lambert-Beer

$$T = \frac{I}{I_0}$$

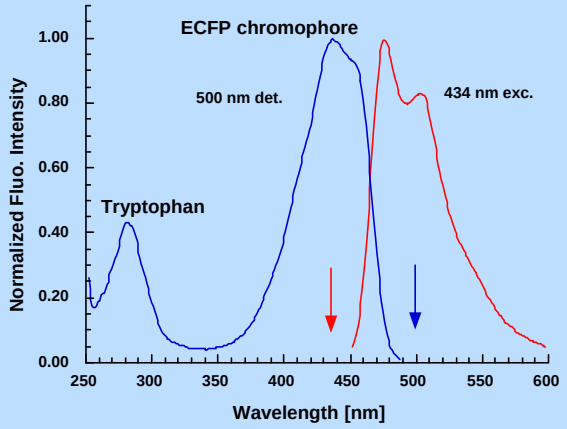
Transmittance

$$A = -\log T = -\log \frac{I}{I_0}$$

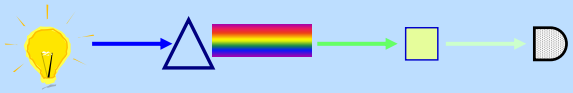
Absorbance

$$A = -\log T = \varepsilon(\lambda)cl$$

Spectral distribution

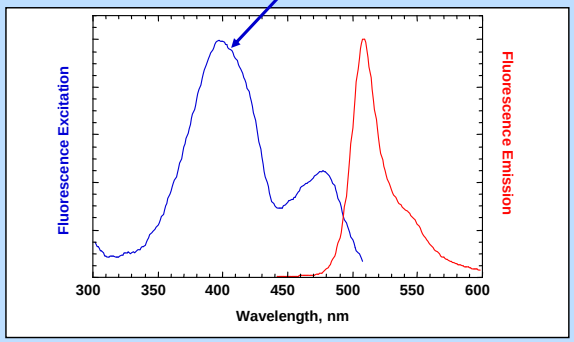


Determination of spectral distribution

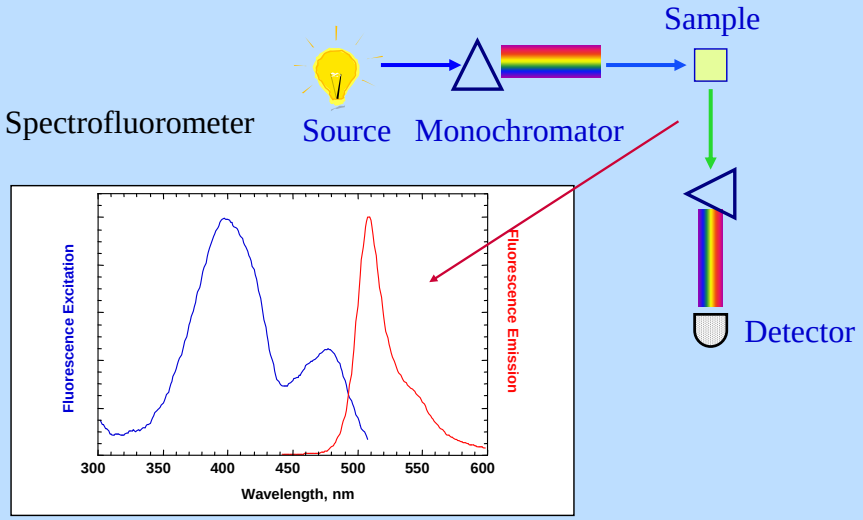


Source Monochromator Sample Detector

Spectrophotometer



Measuring Fluorescence

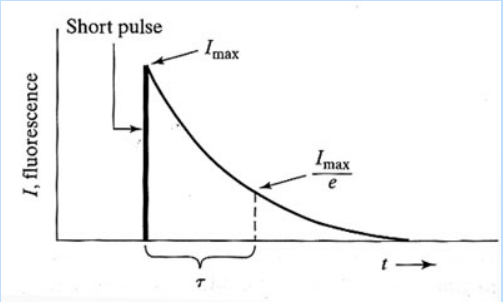


Fluorescence Lifetime(s)

all competing processes affect the fluorescence lifetime

Measured lifetime

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



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

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

$$Q = k_f \tau$$

Critical Fluorescence Parameters

- Photon Yield/Quantum Efficiency
  - a measure of the emission efficiency of the fluorophore

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

- Brightness
  - proportional to ability to absorb light (extinction coefficient, ε) AND
  - Quantum Yield, Q
- Total Fluorescence

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

  - where I_0 is incident light intensity,
  - l is pathlength
  - [c] is fluorophore concentration

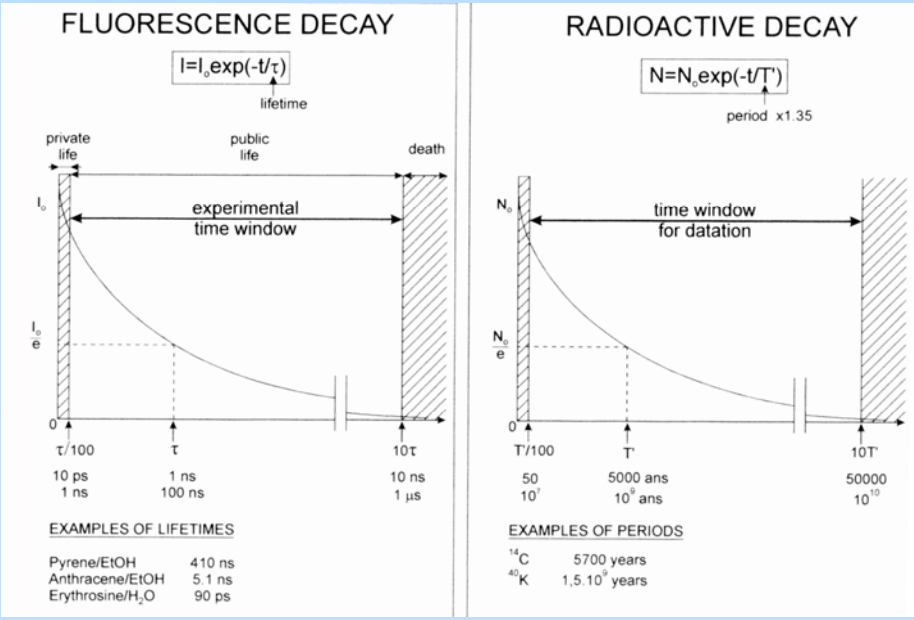
Fluorescence Lifetime(s)

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

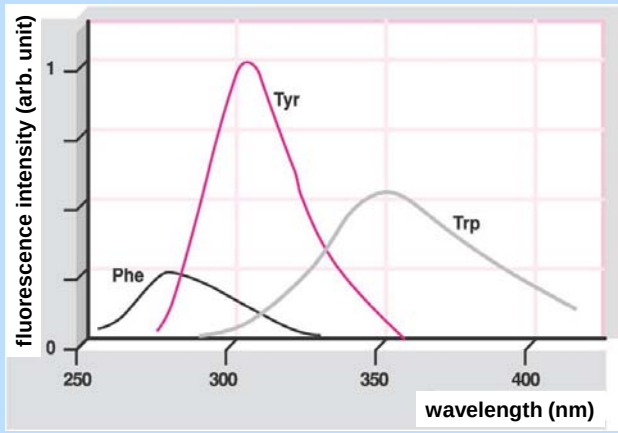
$$\tau_{int} = 1/(k_f + k_{ic} + k_{isc})$$

- 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

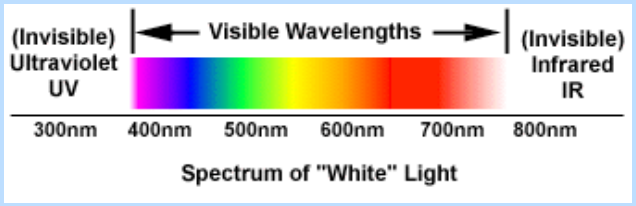


Fluorescence spectra of different amino acids



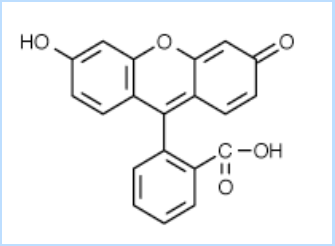
Fluorophores

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



Fluorophores

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

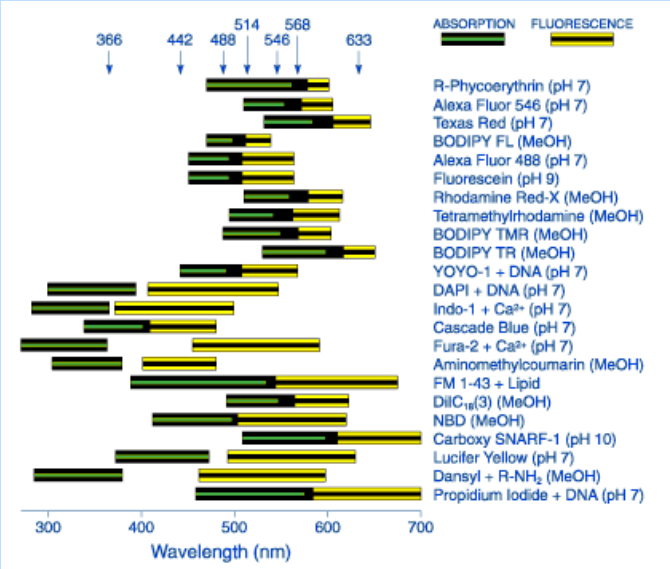


FLUORESCEIN

Molecular Formula: $C_{20}H_{12}O_5$

Molecular Weight: 332.31

Common Fluorophores



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

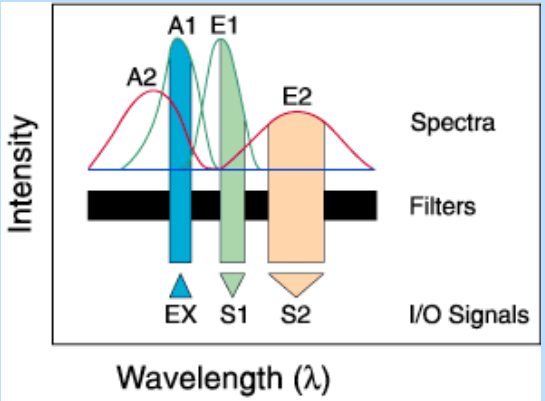
Some applications of fluorophores

- Immunofluorescence
- ion sensitive dyes

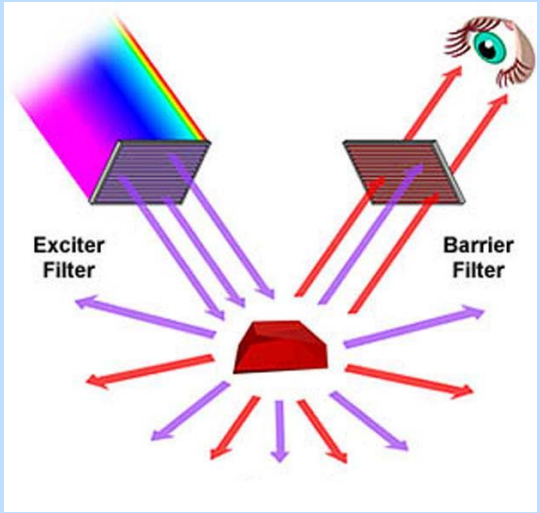
  -K^+, Na^+, Ca^{2+} specific markers

  -pH indicators
- membrane potential
  - increased intracellular fluorescence
- DNA dyes
- determination of protein fluorescence

Measuring Fluorescence



Measuring Fluorescence



Filters

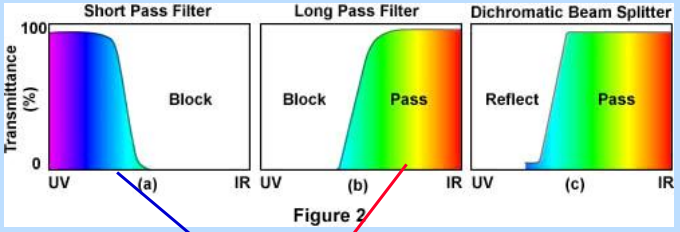


Figure 2

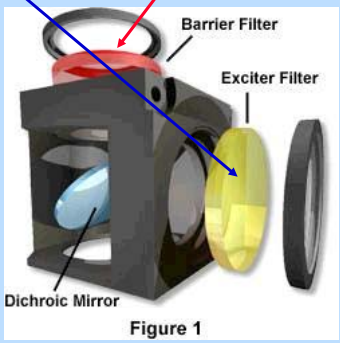


Figure 1

Filters

emitted light

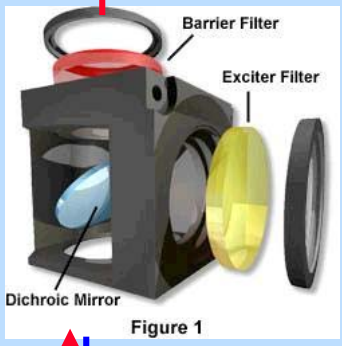


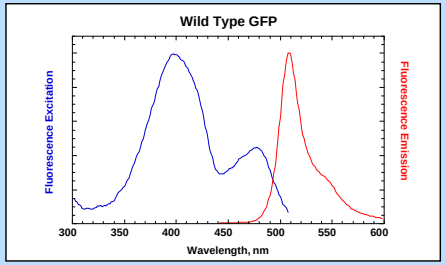
Figure 1

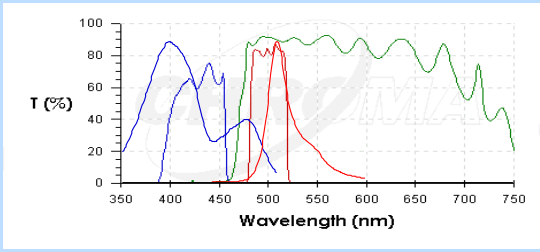
Typical Filter Cube
in a Microscope

Excitation light

sample

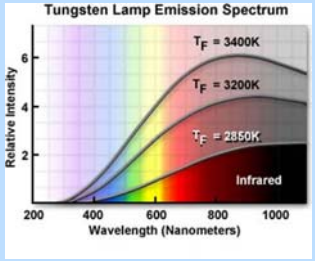
Selecting Filters

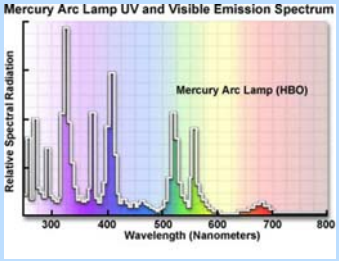


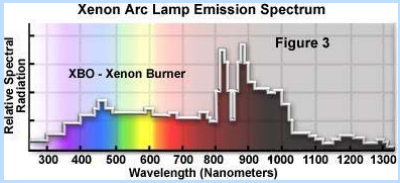


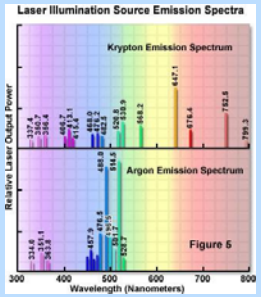
Chroma Technology 32000 WTGFP Bandpass Filter Set

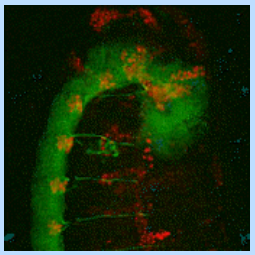
Light sources

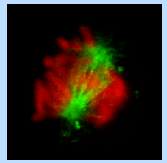


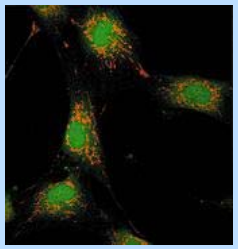


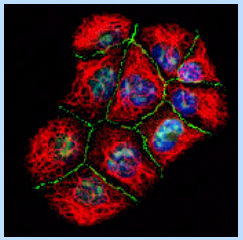


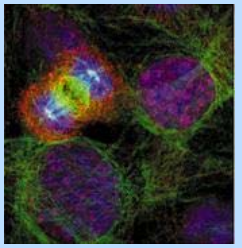




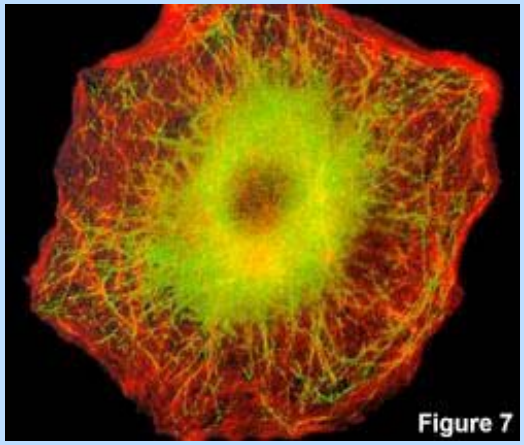






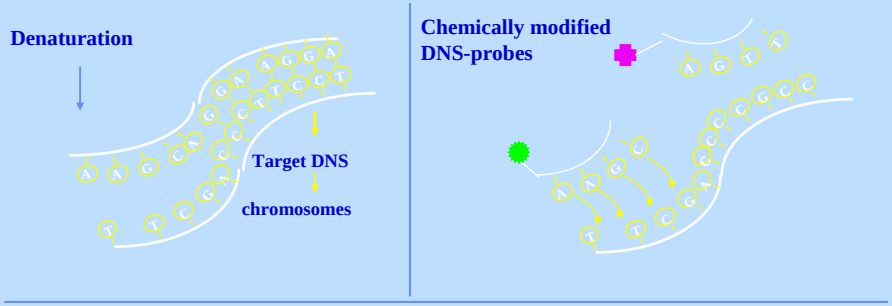


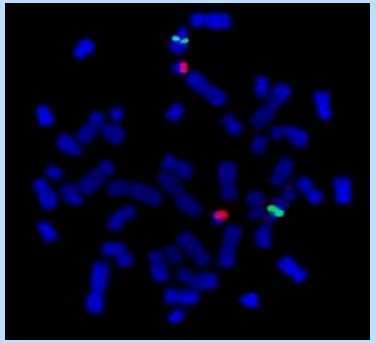
Fluorescence imaging

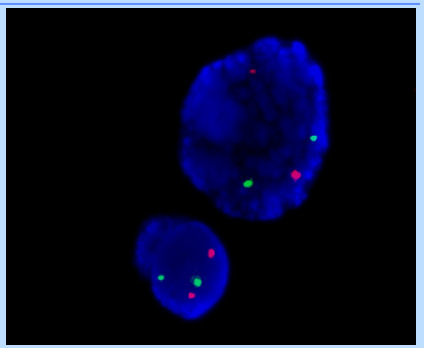


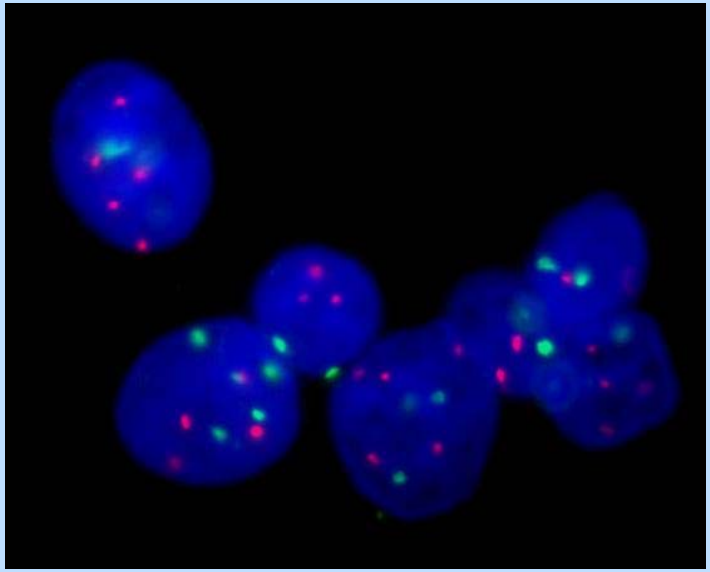
- The sample is stained with FITC (fluorescein isothiocyanate) and Rhodamine-phalloidin to selectively highlight microtubules and actin filaments.

Fluorescence in situ hibridization

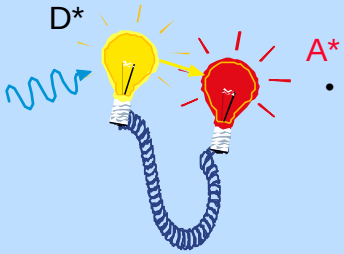








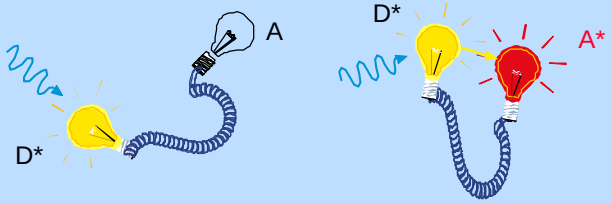
Photophysical Consequences of FRET



- 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

Fluorescence Resonance Energy Transfer (FRET)



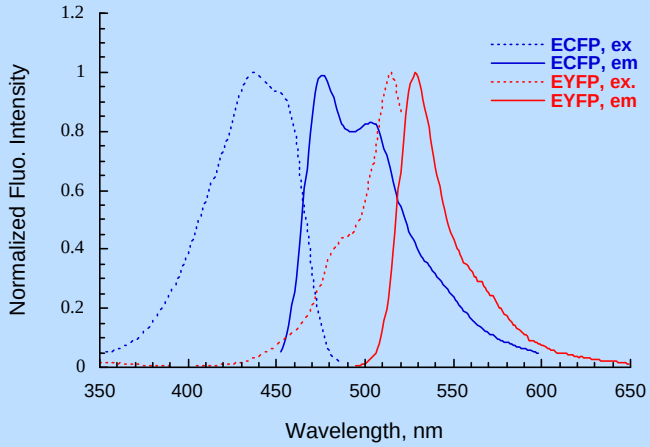
Donor and acceptor far apart - No FRET Donor and acceptor close together - FRET

- 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

FRET Pairs (GFP)

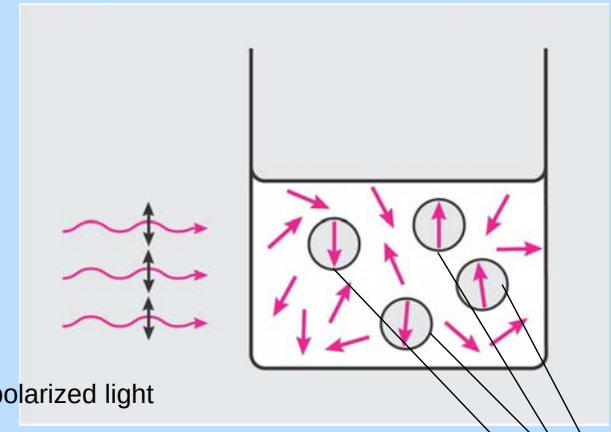
ECFP/EYFP



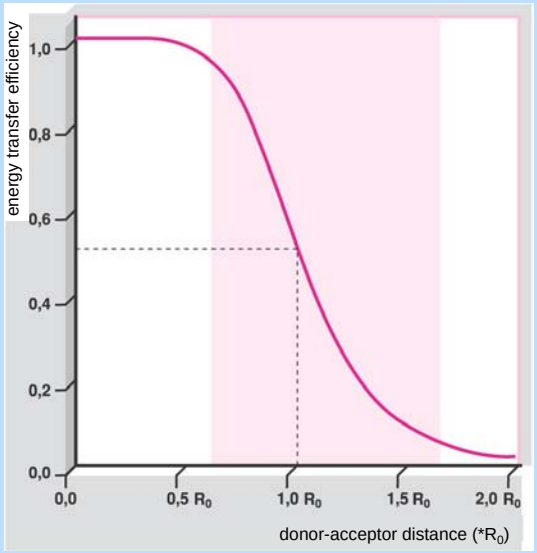
Detecting FRET

- **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

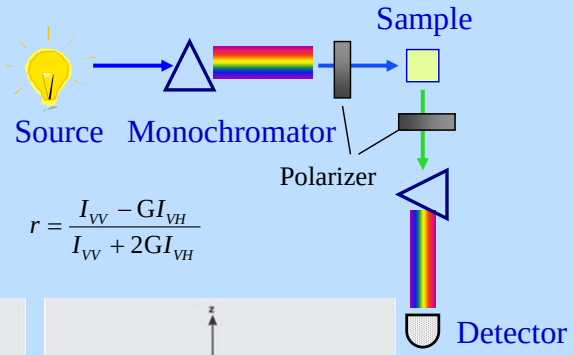
Photoselection of fluorophores



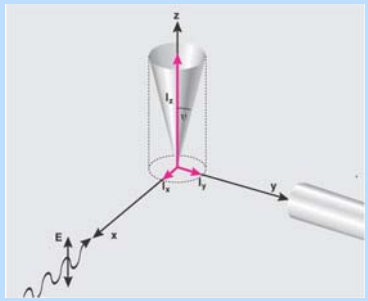
Distance dependence of FRET efficiency values

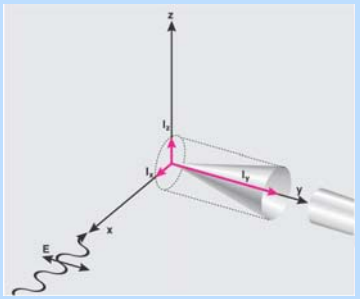


$$E = \frac{R_0^6}{R_0^6 + R^6}$$

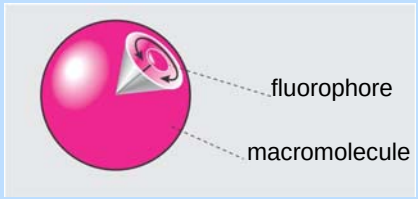


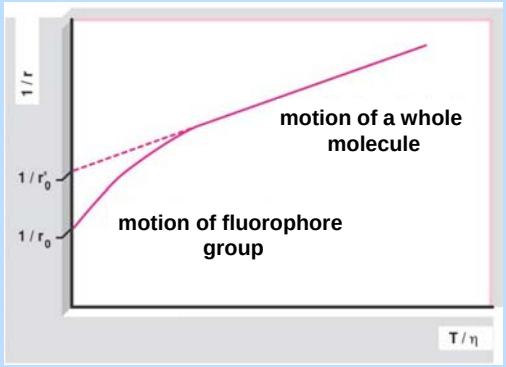
$$p = \frac{I_{VV} - GI_{VH}}{I_{VV} + GI_{VH}} \qquad r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}}$$





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





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

Time distribution of first photon upon excitation