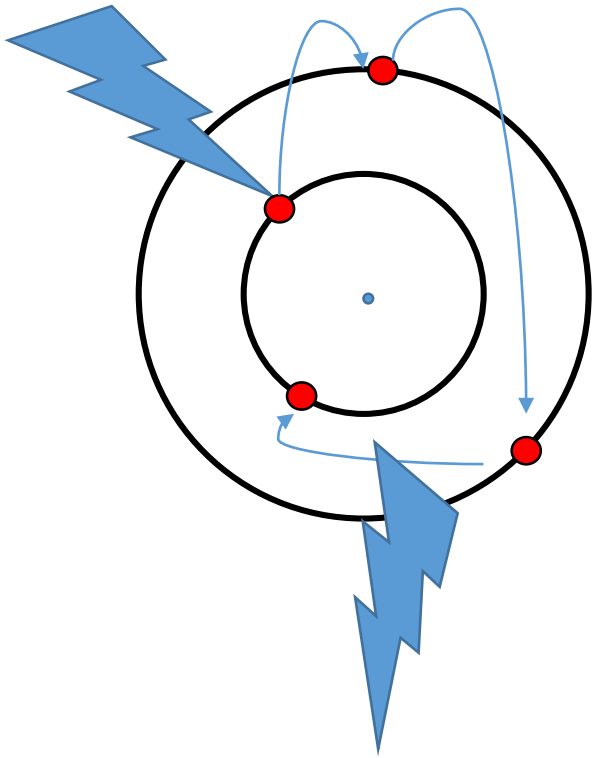
Fluorescence

- Last class
  - Polarization microscopy
  - DIC
- This class
  - Intro to fluorescence
  - Fluorophores

Fluorescence – another way to generate contrast in biological samples

Easiest to think about a single atom. Nucleus surrounded by electrons

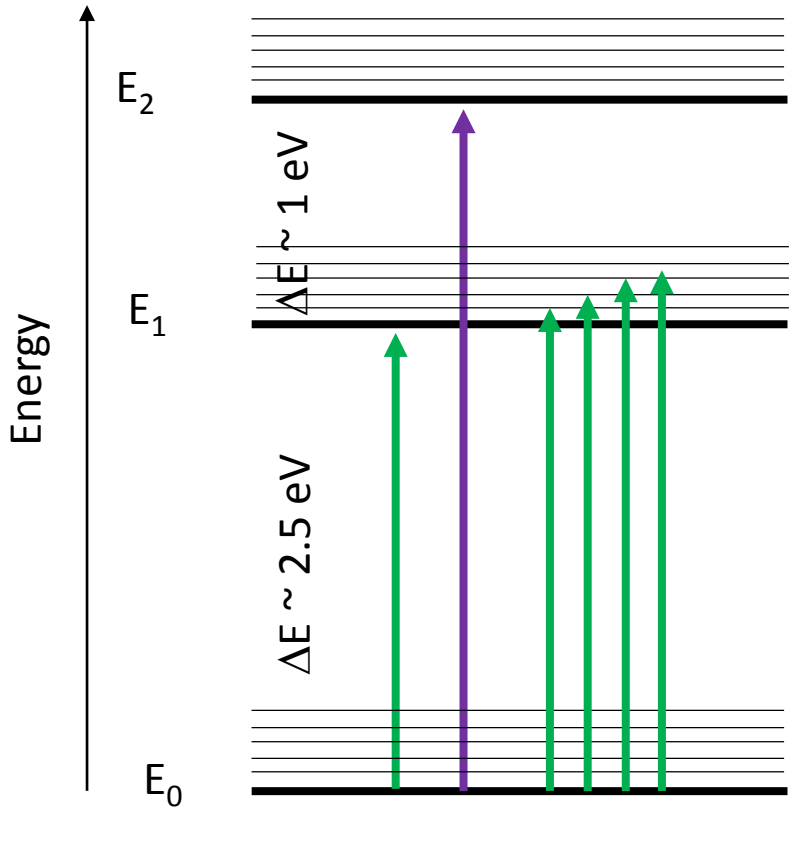


1. Absorption of a photon – photon energy used to promote electron
2. Electron stays in the excited state for a little while ~.1 – 100 nanoseconds
3. Electron falls back down – energy is given off as a photon
4. Photon moves away

Result is a photon that is emitted in a new direction

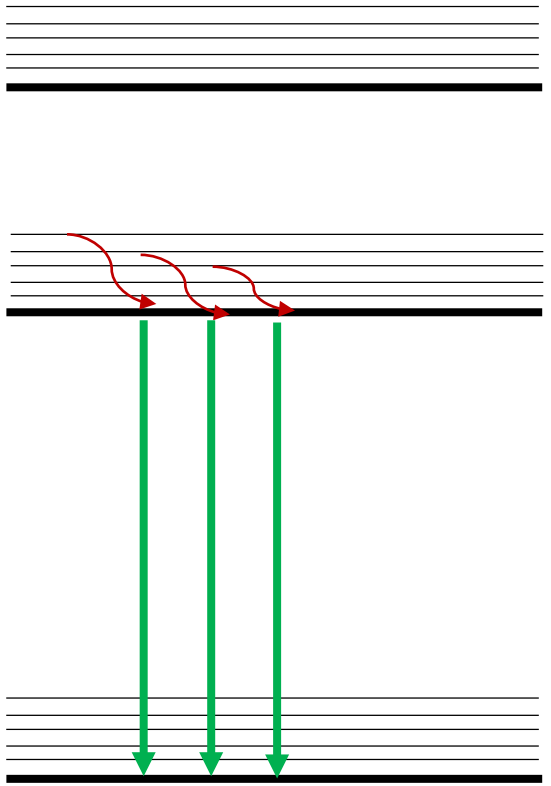
Jablonski diagram

Many vibrational states in each electronic state

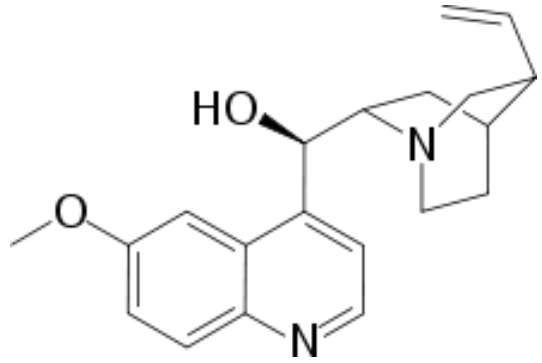


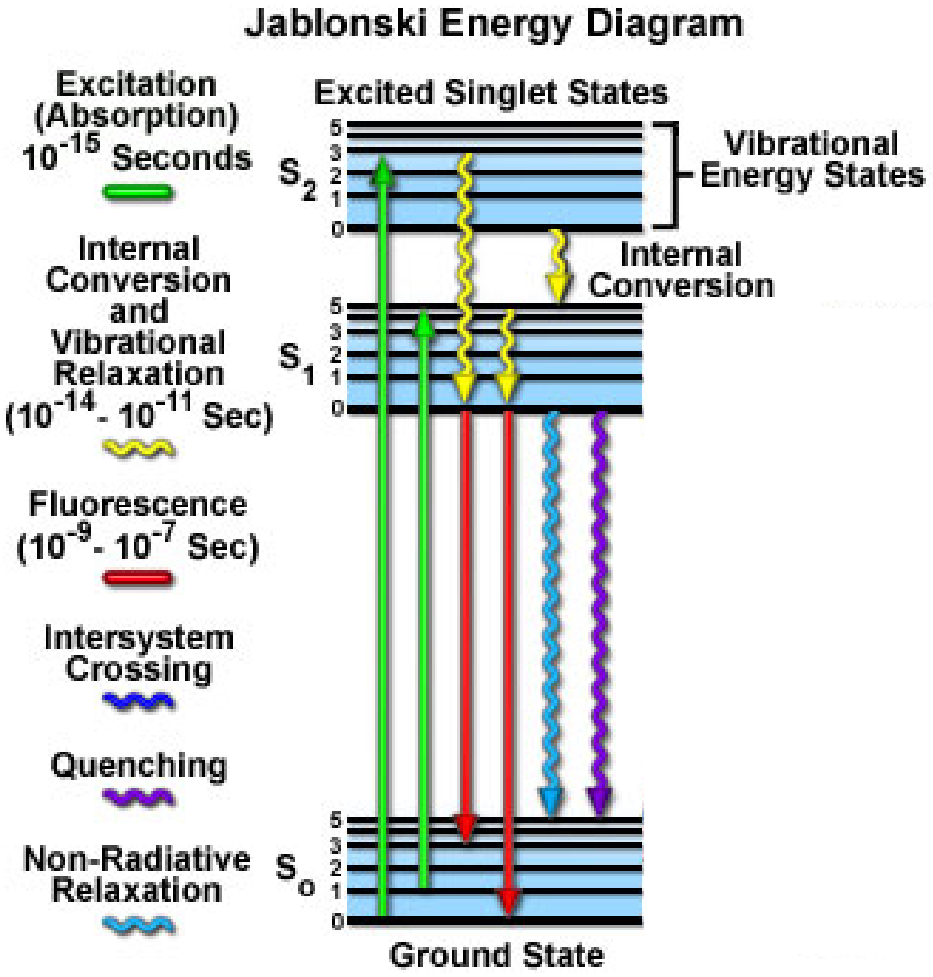
$E_{photon} = hc/\lambda$

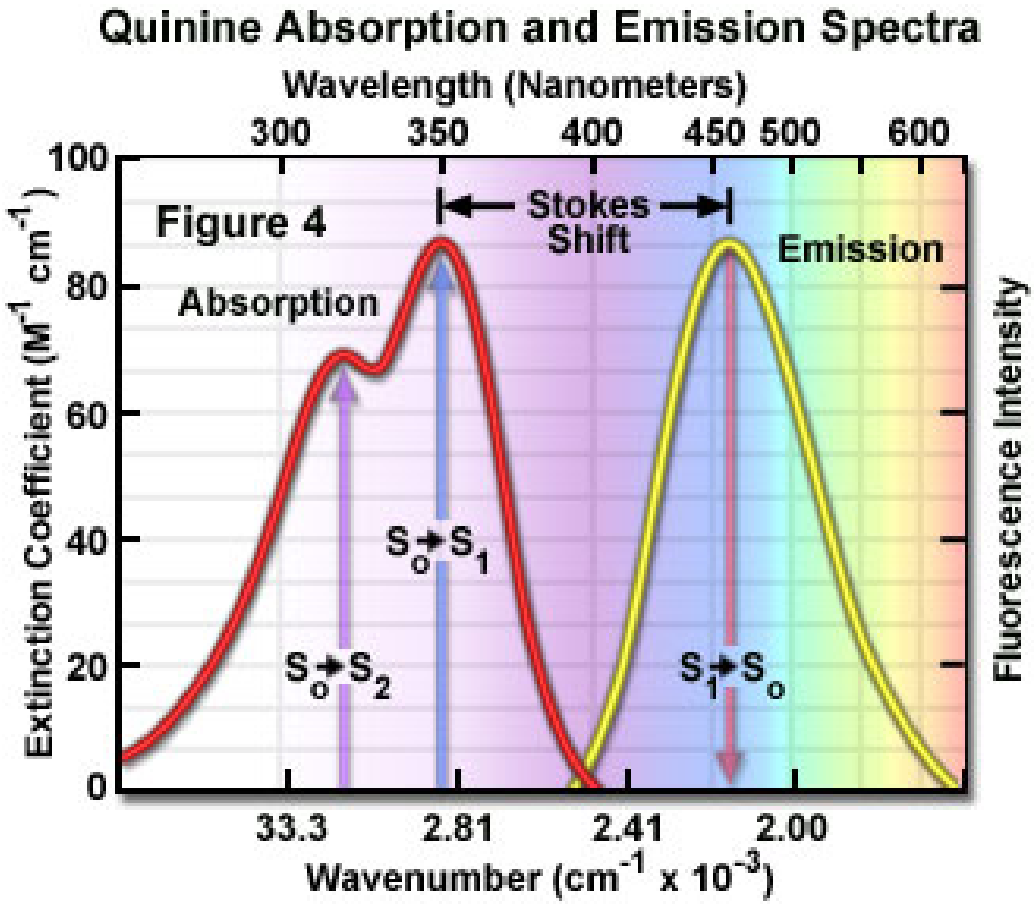
It is the difference in wavelength that gives fluorescence its utility



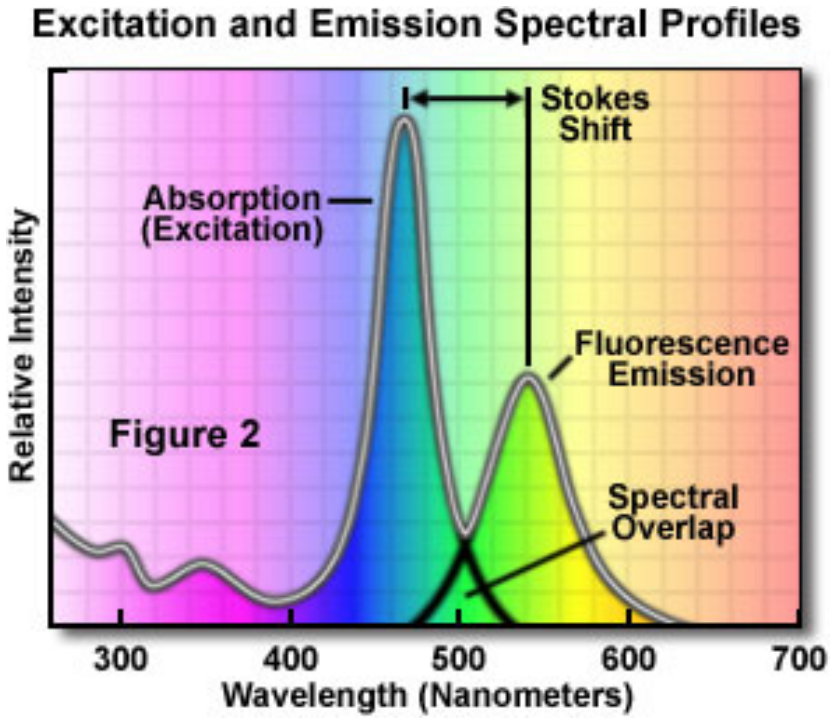
Jablonski diagrams to spectra







Absorption and emission



Absorption spectrum is broad. Local motion, environment, and vibrational level affect spectrum

Absorption peak is set by likelihood of absorbing a given photon – extinction coefficient

Emission spectrum is independent of absorption wavelength

Emission is also broad, for the same reasons

Stokes shift is the difference in wavelength between the absorbed and emitted photon

Normal image made up of many many fluorophores, all undergoing this cycle. We don't see the states because everything gets smoothed out

Non-radiative transfer

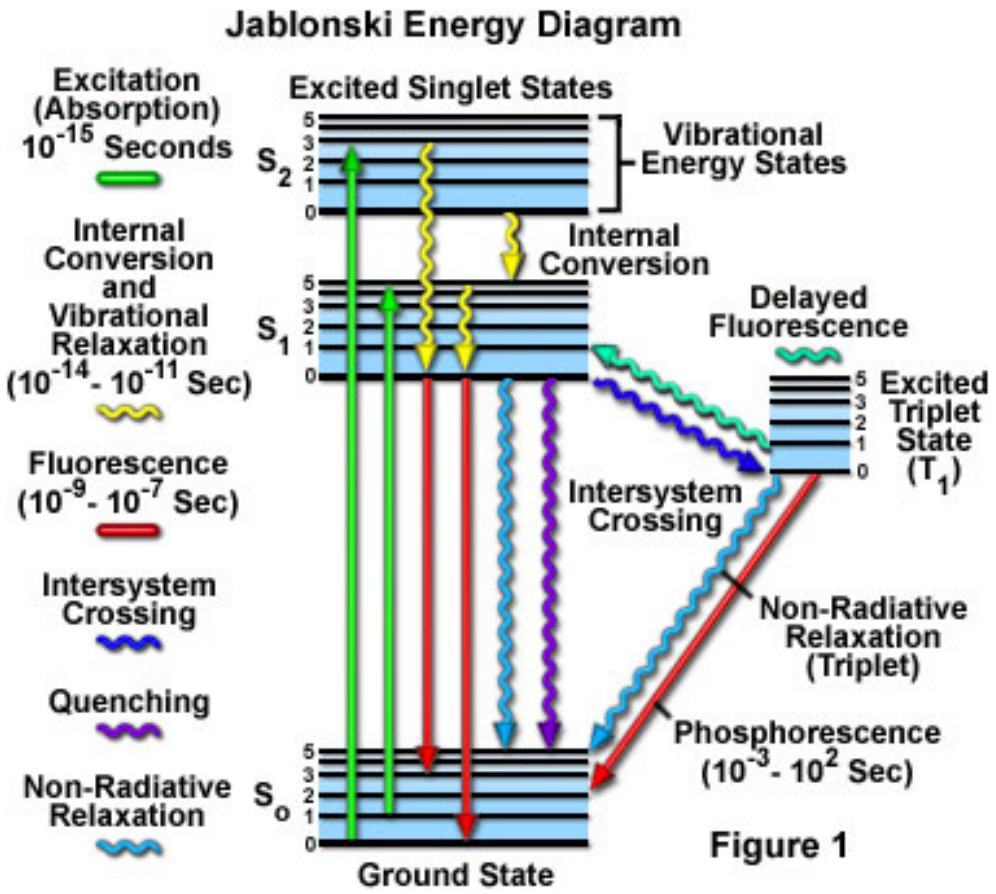


Figure 1

Sometimes electron can be moved into a triplet state – A "long" lived state that can not easily decay to ground state

Phosphorescence

Triplet state is highly active, very likely to excite some other molecule, create a free radical

Quantum Yield: Photons emitted/photons absorbed

QY <= 1

$$Q = k_f/(k_f + k_{nr})$$

k_f = rate of fluorescence decay

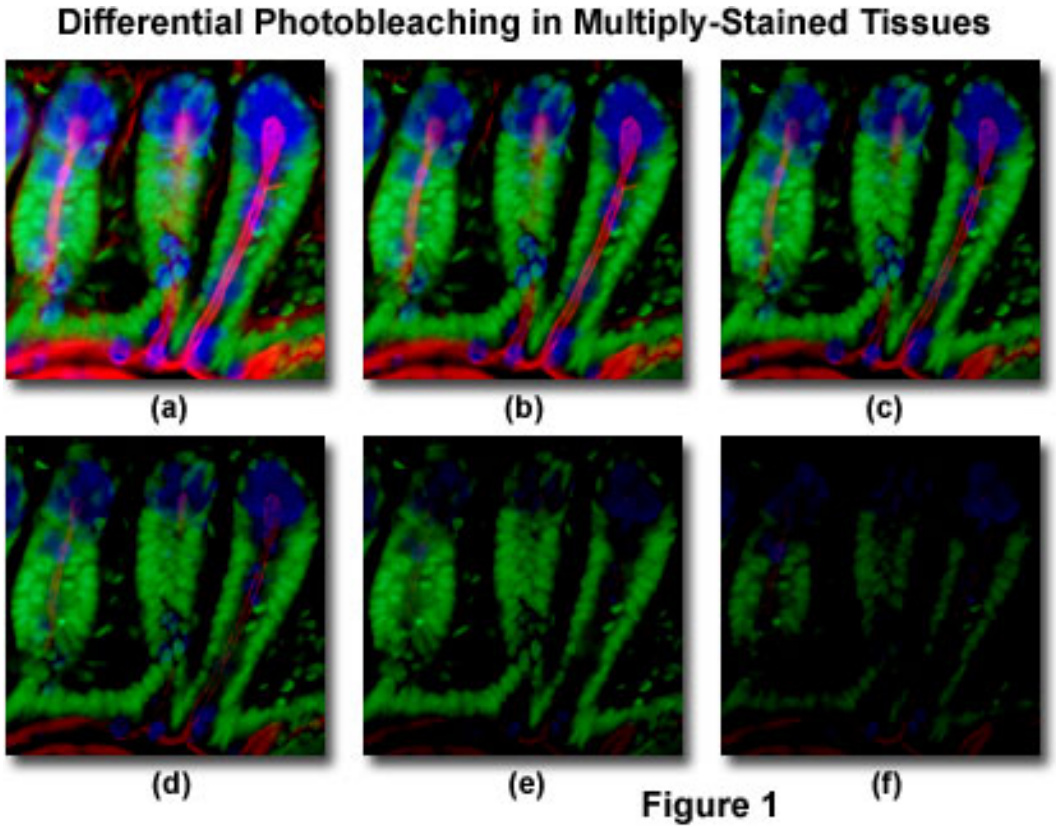
k_{nr} = combined non-radiative decay

Lifetime: Average amount of time in excited state

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

Photobleaching

- Gradual loss of fluorescent intensity
- Unavoidable, but can be reduced
- Highly dependent on oxygen concentration and electron acceptors
- Often occurs out of triplet state which is highly reactive
- Reduce excitation intensity
- Use oxygen and free radical scavengers (vitamin C, glucose oxidase)
- Triplet state quenchers (BME, Trolox)



Properties of fluorophores

- Excitation max
- Emission max
- Spectrum breadth
- Molar extinction coefficient
- Quantum yield
- Photostability
- Photons per molecule
- Solubility

Compound	Solvent	Excitation Wavelength (nm)	Emission Wavelength (nm)	Quantum Yield
Acridine Orange	Ethanol	493	535	0.46
Benzene	Ethanol	248	300-350	0.04
Chlorophyll-A	Ethanol	440	685	0.23
Eosin	Water	521	544	0.16
Fluorescein	Water	437	515	0.92
Rhodamine-B	Ethanol	555	627	0.97

Extinction coefficient = Amount of light that can be absorbed => 1/(M*cm)

Quantum Yield = Photons emitted/photons absorbed

Brightness = ε * QY/1000 (Although often given in relative amounts)

Photostability = #photons before photobleaching (Although usually given in very strange units)

Fluorescence intensity = $QY * I_o(1 - e^{-\varepsilon bc})$

I_0 = incident intensity

ε = absorption coefficient

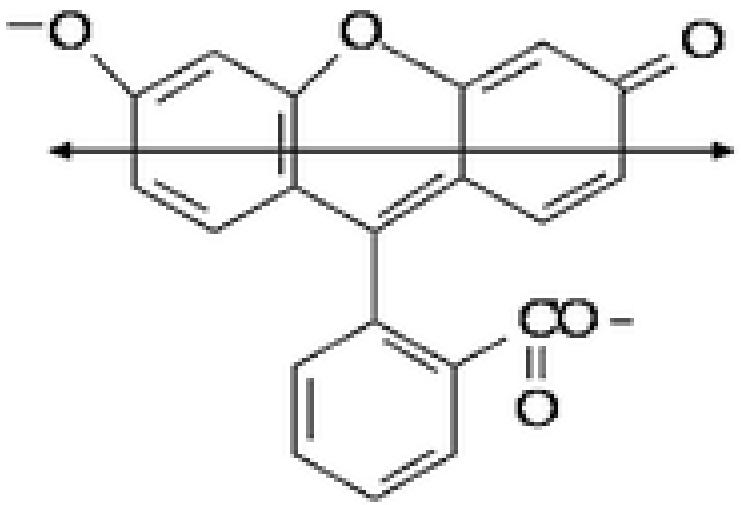
b = path length

c = molar concentration

Fluorophores MUST have dipole absorber

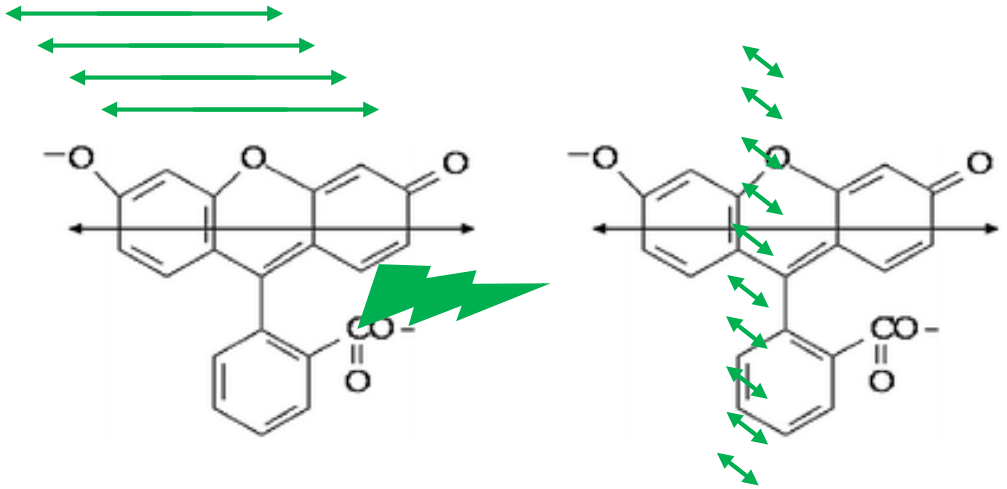
Dipole = Charge separated in space

+ ----------→ -

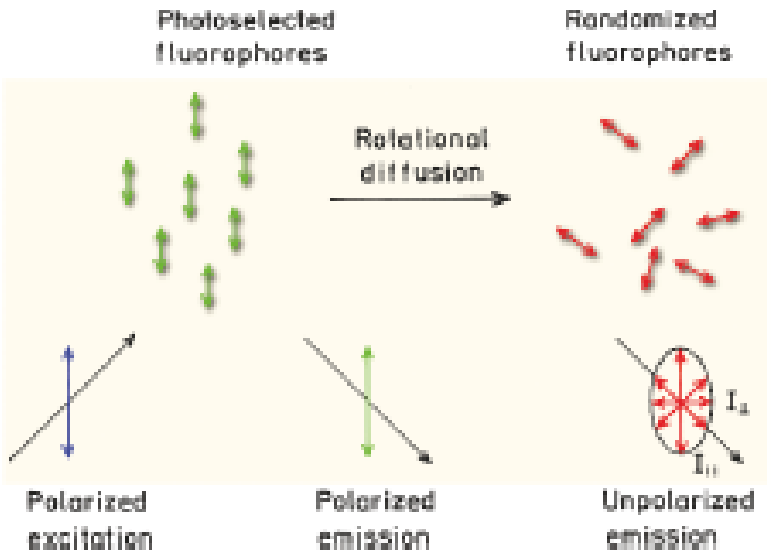


Fluorescein

Absorption must occur from photon polarized in the same direction as the dipole

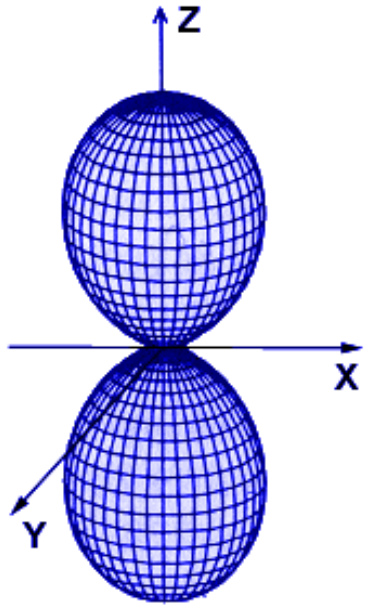


Dipole emission



Fluorescence emission is not isotropic.

Assuming no movement, the emission is most likely to be emitted in the same orientation as the absorption

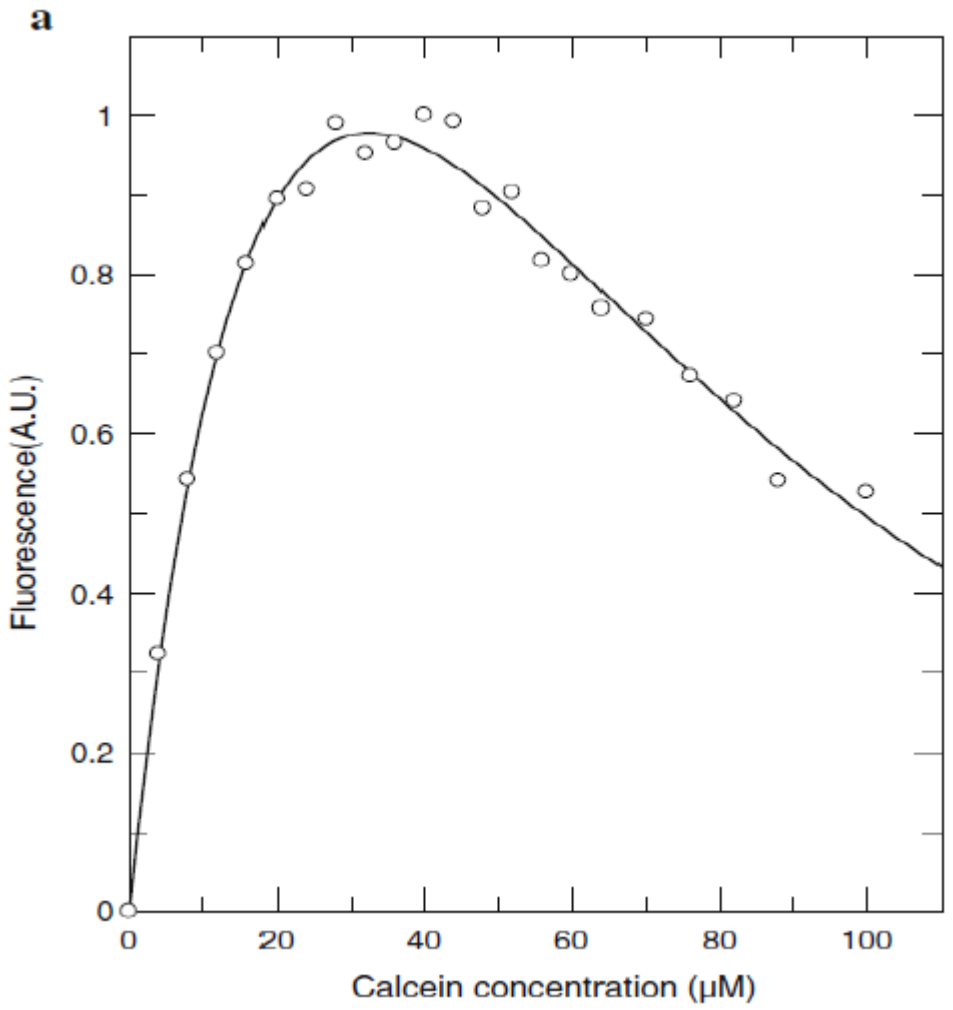


One-Photon

$r_{01} = 2/5$

Self quenching

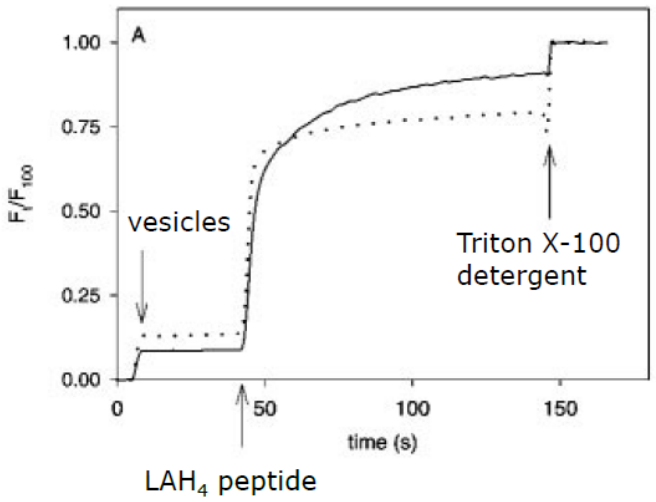
- At high enough concentration, dye will begin to quench each other
- Care must be taken when measuring concentration to ensure you are not quenching

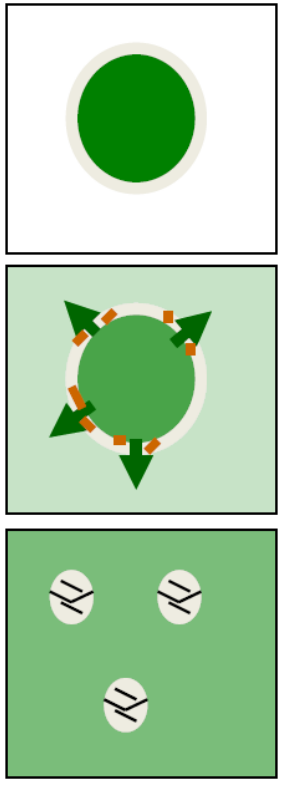


$$QY = \frac{k_f}{k_f + k_{nr}} \longrightarrow QY = \frac{k_f}{k_f + k_{nr} + k_q[Q]}$$

Utilizing self quenching

Vesicle pore formation and dynamics





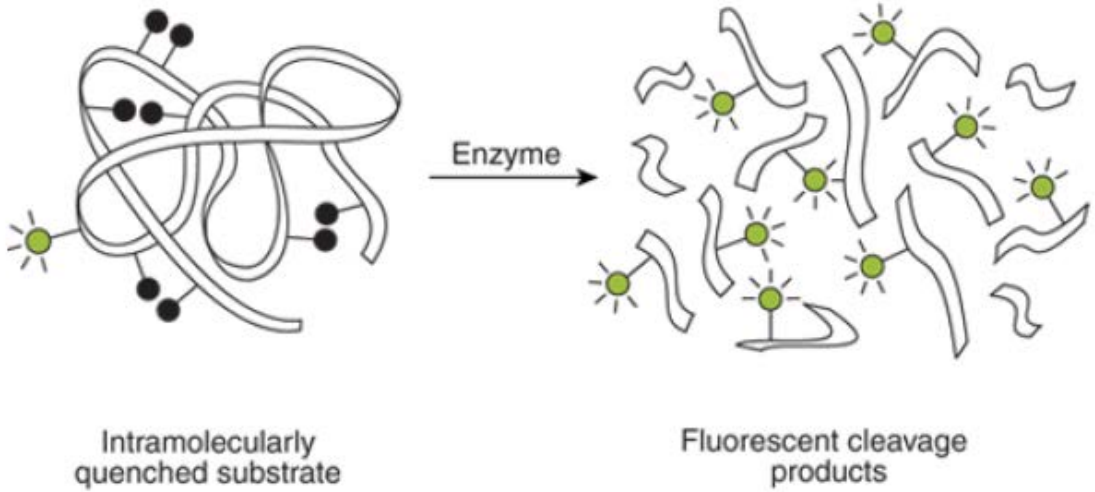
vesicles loaded with 60 mM **calcein**

peptide LAH_4 creates pores in the lipid bilayer, through which the dye can leak out

detergent Triton X-100 micellizes the vesicles

final calcein concentration ~ 5 μM

Vogt and Bechinger, J Biol Chem 1999, 274: 29 115



Protease kinetics

And on to Matlab…