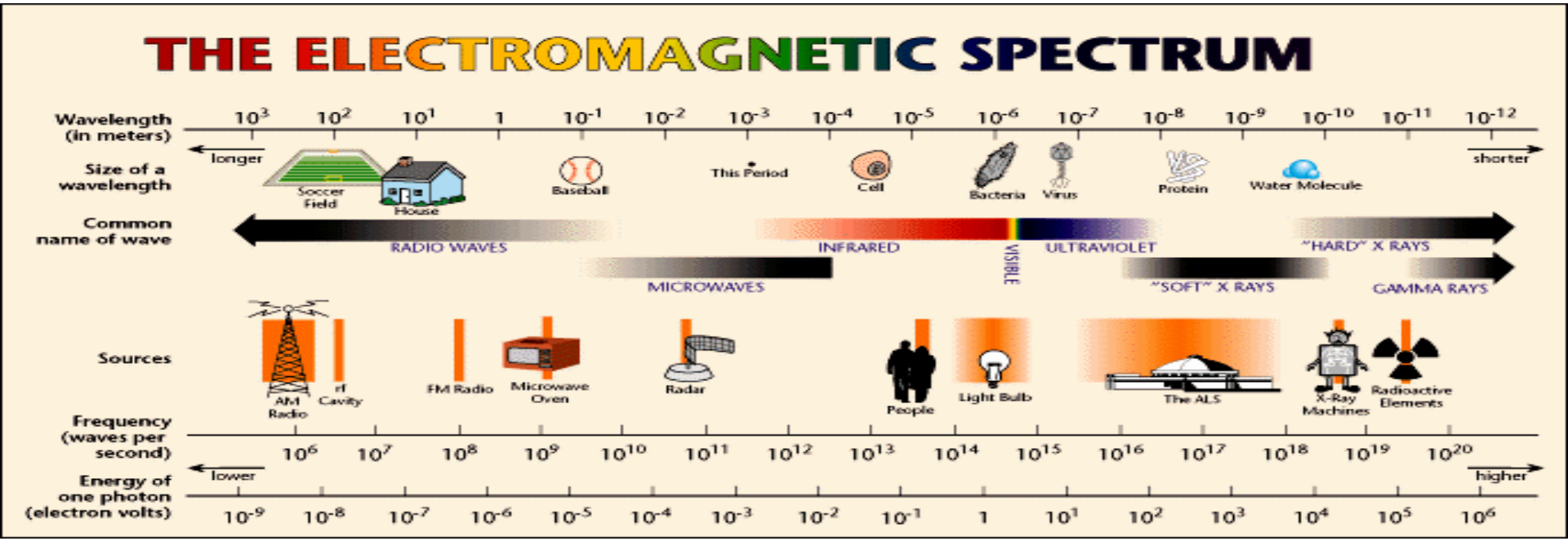
Spectroscopy



Objectives: **Review nature of electromagnetic radiation (λ / ν / c)**

Interactions of "Light" with matter (Absorption / Scattering (n))

Frank-Condon Principle

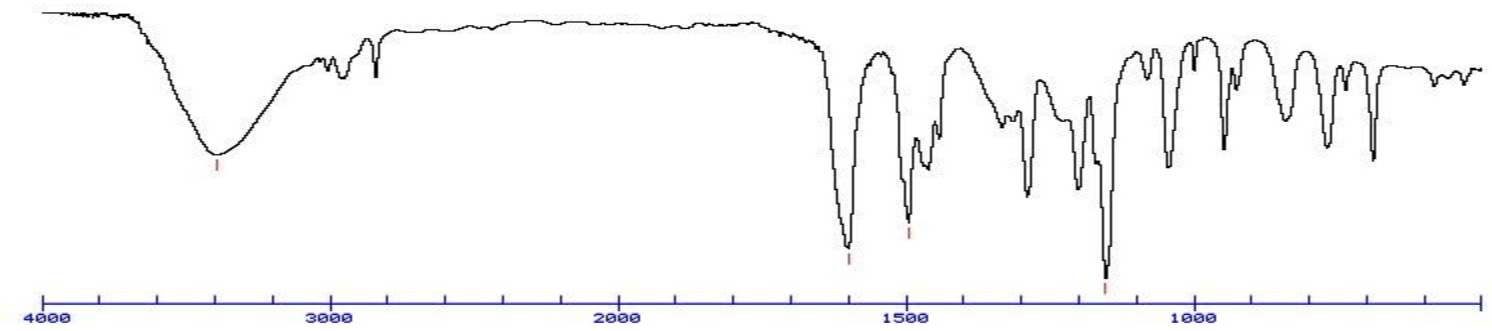
Electronic transitions (Abs, Fluor, Phosphor.)

Beer Lambert Law (A = O.D. = - log(T) = ε • [c] • l)

Excitation Transfer / FRET

Light and Matter: Absorption (spectroscopy)

Scattering (image formation)



An Example IR Spectrum

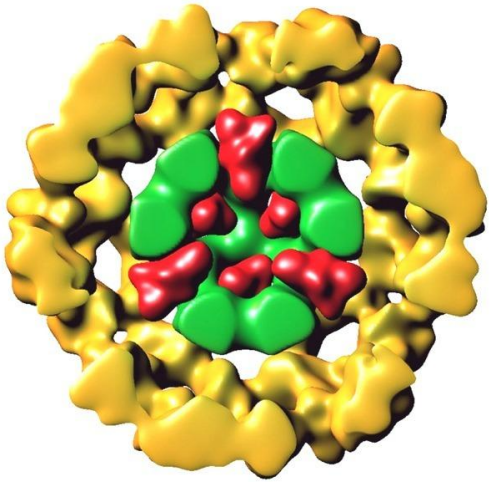
Abbe (~1878): Limit Res. ~ λ/2

- Light Photography

  λ ~ 400 - 700 nm

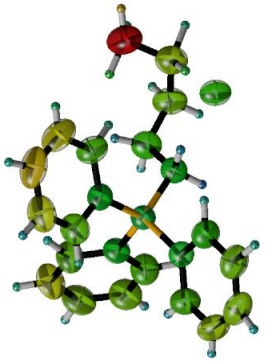
- Electron Microscopy

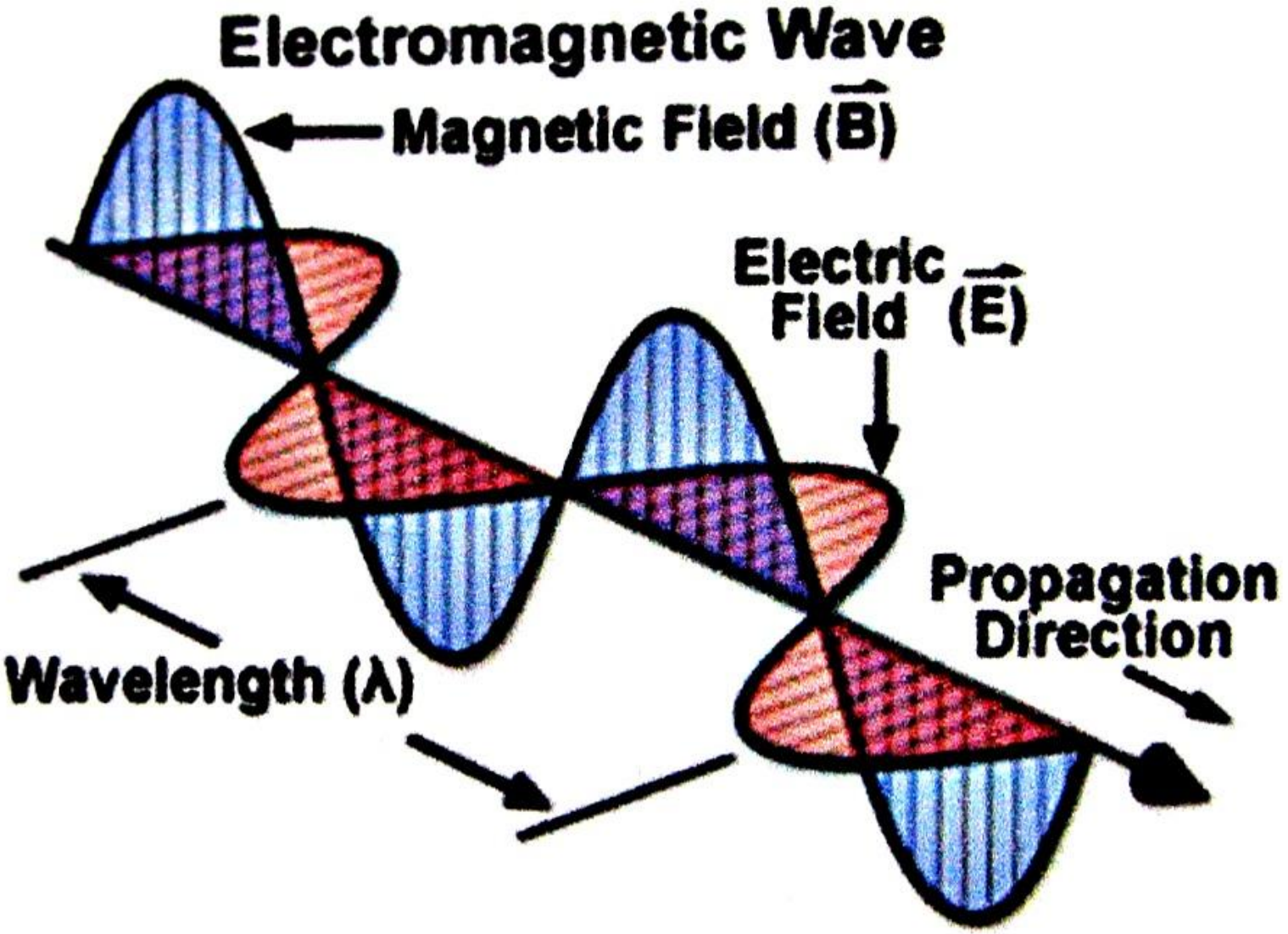
  λ ~ 0.001 - 0.1 nm

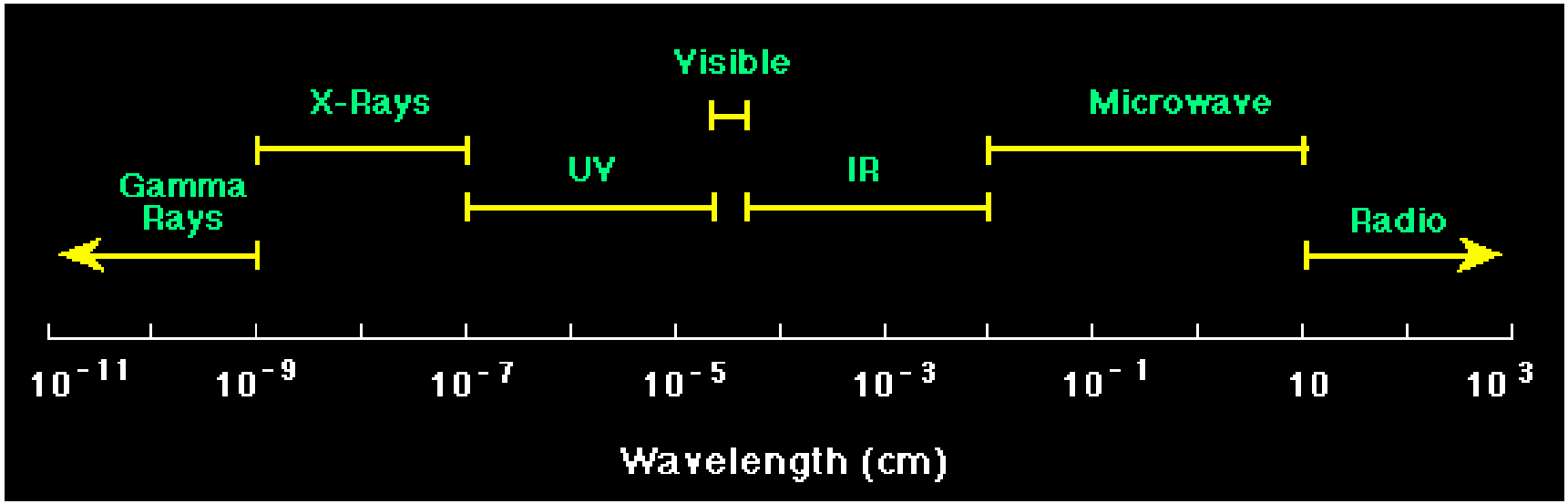


- X-Ray or NMR

  λ ~ 0.1 nm




Electromagnetic Wave
Magnetic Field (B)
Electric Field (E)
Propagation Direction
Wavelength (λ)



$\upsilon = c/\lambda$ υ=frequency, λ=wavelength, c=velocity of light ($c = 3 \cdot 10^{10}$ cm/sec)

$\Delta E = h\upsilon$ E=energy, υ=frequency, h=Planck's constant ($h = 6.6 \cdot 10^{-27}$ erg sec)

$$c = \lambda\upsilon$$

Frequency (υ) = (**Speed of light (v) / Wavelength (λ)**)

Wavenumber, $\bar{\upsilon} = \frac{1}{\lambda}$, is the number of wave maxima per cm.

700 nm **red light** = 1.43 x 10^4 cm^{-1} units on $\bar{\upsilon}$ are cm^{-1}

420 nm **violet light** = 2.38 x 10^4 cm^{-1}

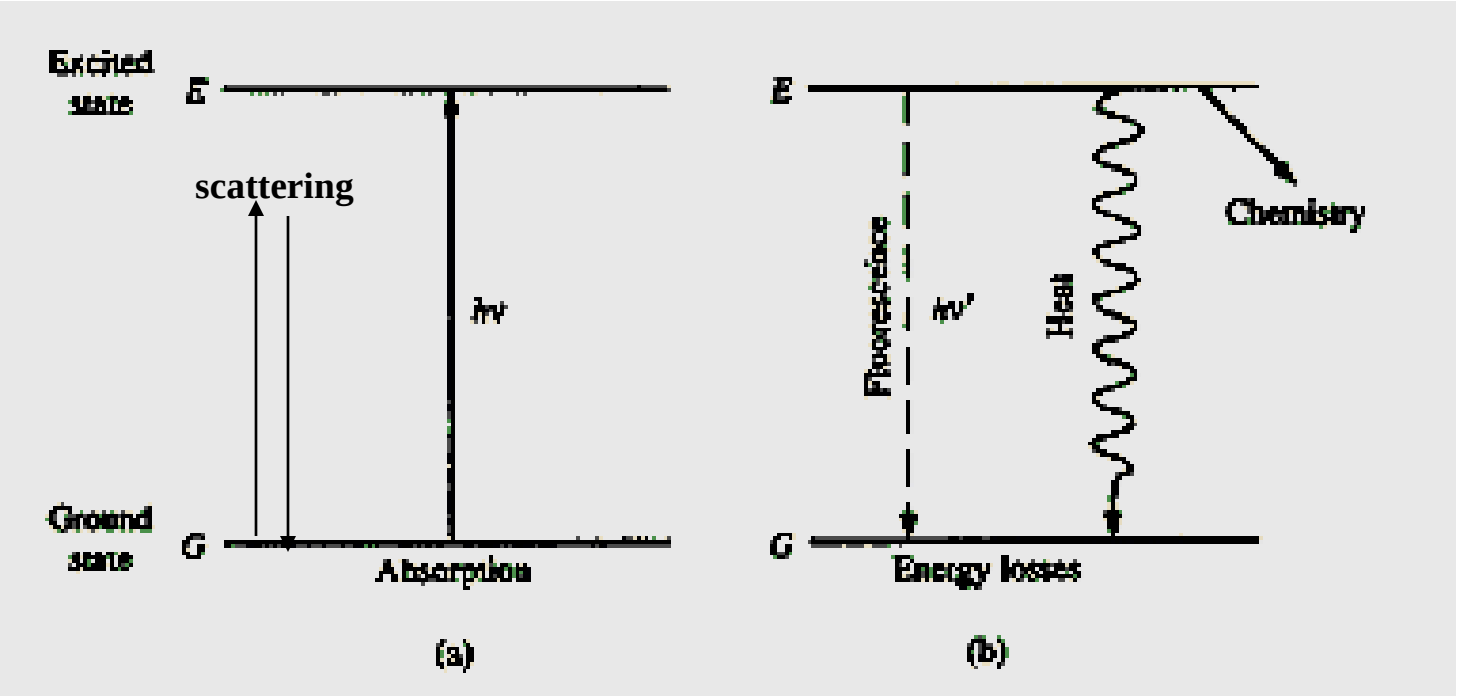
Velocity of light changes in different substances.

→ **Index of refraction** of a substance, n = c / v

n = 1	vac
n = 1.0+	air
n = 1.33	water
n = 3.5	Si

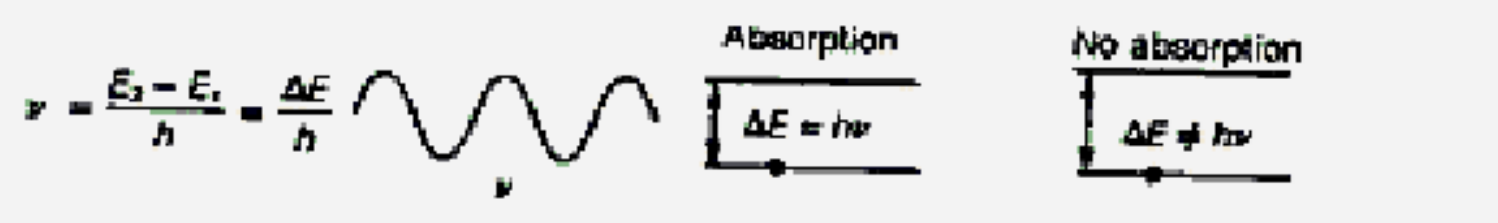
When **light interacts with matter**, there are **two possibilities**:

1. **Scattering** - the light is transmitted but velocity changes (n).
2. **Absorption** (photons are absorbed)
    a. they produce heat
    b. the cause a chemical change
    c. they are reemitted (**fluorescence**, **phosphorescence**)



The oscillating electric field induces a force on the charges particles (electrons, protons).

If the **frequency of oscillation corresponds to an energy-level difference, the photon will be absorbed** - its energy will change form into electron or nuclear motion.



Time frame for **absorption**:

The **absorption usually occurs in the time it takes one wavelength to pass the molecule**.

the speed of light, $c = 3 \times 10^{17}$ nm sec^{-1}
for uv light, the wavelength, $\lambda \approx 300$ nm.

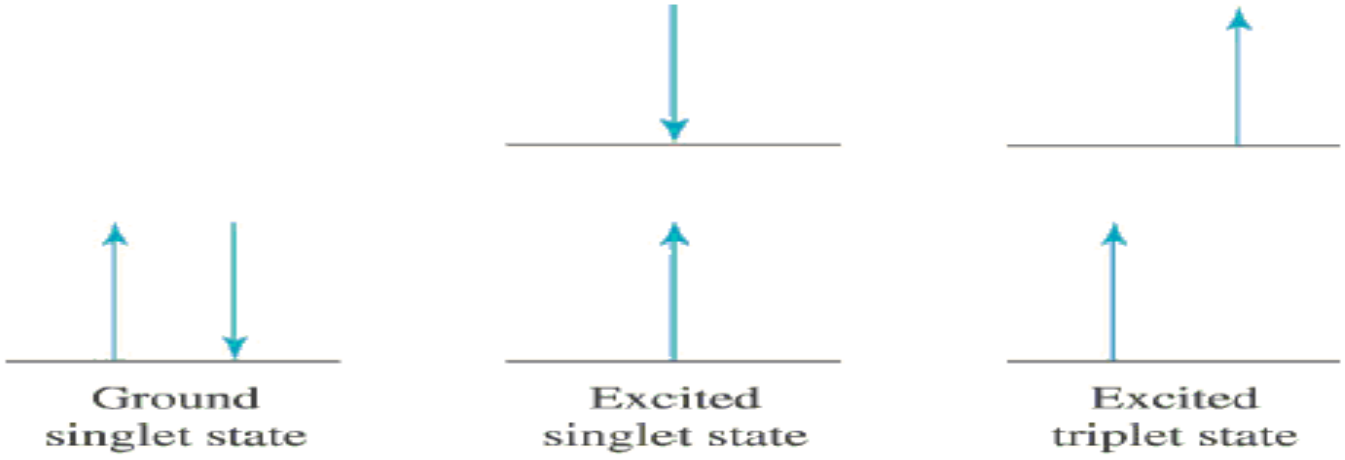
3×10^{2} nm sec / 3×10^{17}nm = **10^{-15} sec**

Electronic transitions

- **Selection rules**: allow **S→S**, and **T→T** processes but **not S→T and T→S**. Ground states are usually singlets; thus most excitations are to singlet excited states, like $S_0 \rightarrow S_1$, $S_0 \rightarrow S_2$, …
- **Triplet states are usually formed by intersystem crossing** from an excited singlet state, such as S_1, rather than by direct excitation from the S_0 ground state.

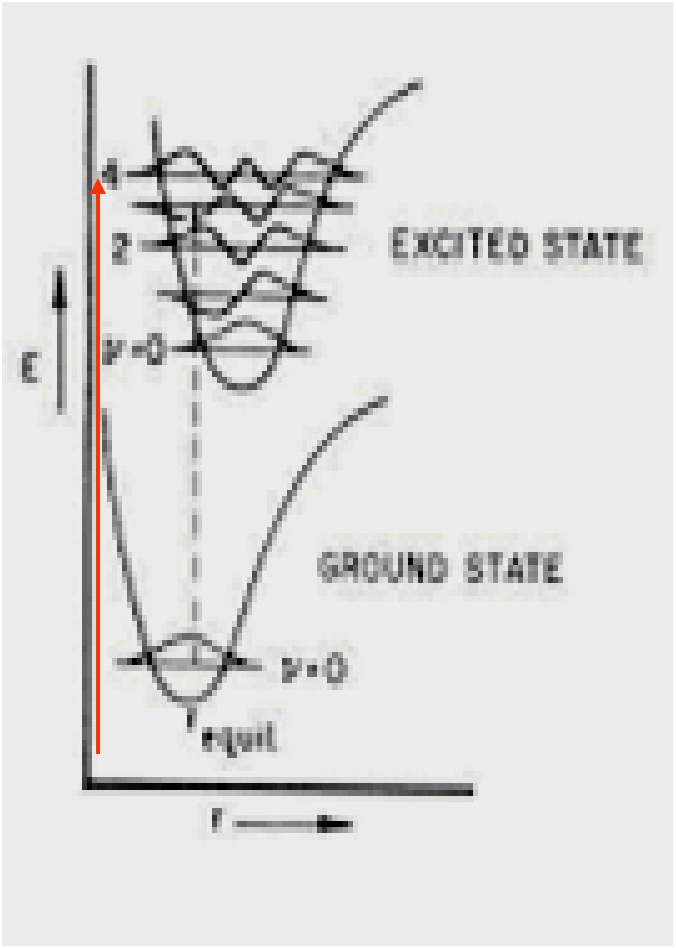
Fluorescence - from an **excited singlet state**

Phosphorescence - from an **excited triplet state:**

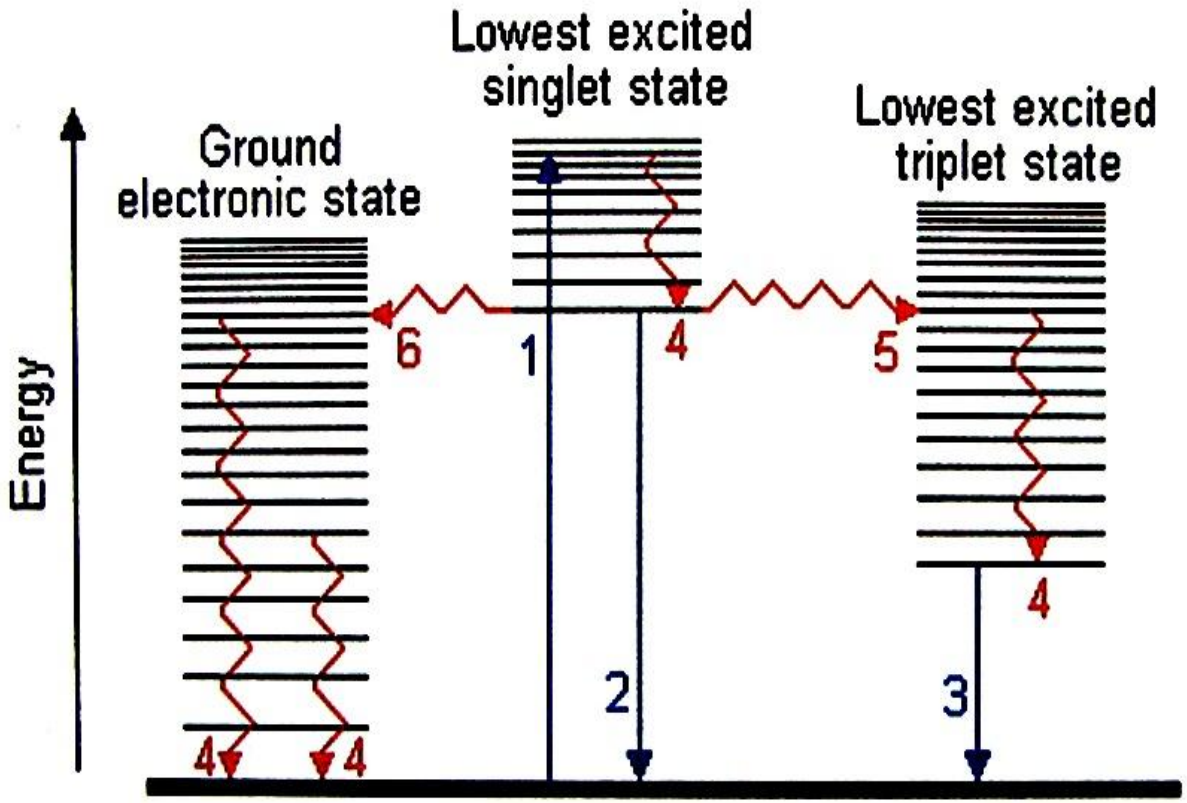


Frank-Condon Principle

- "The nuclear motion (10^{-13} s) is much slower as compared with electronic transition (10^{-15} s), so it is negligible during the time required for an electronic excitation."
- Since the nucleus does not move during the excitation, the internuclear distance keeps the same, and "the most probable component of a electronic transition involves only the vertical transitions".



Possible physical process following absorption of a photon by a molecule



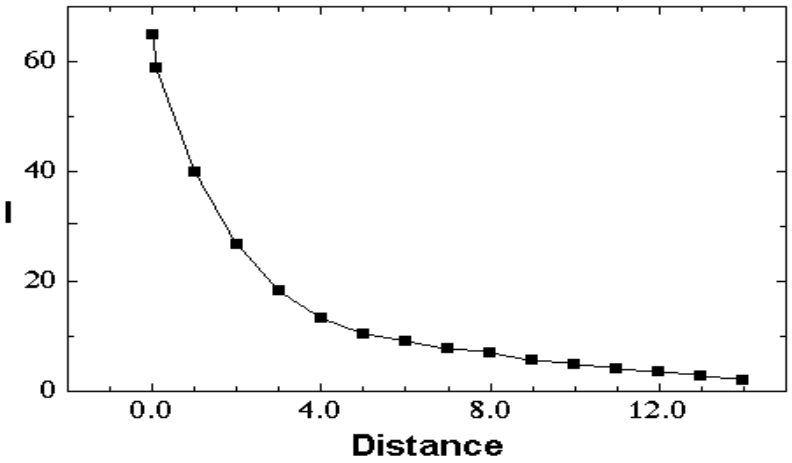
1. Absorption
2. Fluorescence
3. Phosphorescence
4. Vibrational relaxation
5. Intersystem crossing
6. Internal conversion

Absorbance: Beer-Lambert Law

When light passes through a homogeneous sample, the *fractional* decrease *(not absolute)* in light intensity is the same across any interval, dx. This resembles **1st order kinetics ~ radioactive decay**. The change in light intensity, I, with distance is

$$\frac{dI}{dx} = -I\alpha c$$

rearranges to: $$-\frac{dI}{I} = \alpha c dx$$



where *dI/I* = fractional decrease in light intensity,

α = a constant

c = concentration. Integrate to get:

$\ln(I_0/I_t) = \alpha c l$ or $I_t = I_0 e^{-\alpha c l}$

where I_0 = initial intensity, I_t = intensity at a distance *l*.

It follows that the **transmitted intensity decreases exponentially with concentration**.

$$A = \log \frac{I_0}{I_t} = \varepsilon c l$$

Units on ε: M^{-1} cm^{-1}

where **A** is "**absorbance**" or "**optical density**" and **ε** is the "**molar absorptivity**" or "**molar extinction coefficient**" and ε = α / 2.303

Absorption Spectrum – "fingerprint"

Beer-Lambert Law: Intensity (I, Io); Transmittance ($T = I / I_o$)

Absorbance (A): $A = \log (I_o / I) = \log (1/T)$

Extinction Coefficient – E (1%), ε(M) = Molar extinction coeff.

$A = O.D. = \varepsilon \bullet c \bullet l$ also $[\ E1\% \bullet MW = 10 \bullet \varepsilon_M\ ]$

Proteins: A280 ; E (1%) ~ 10 (or O.D. of 1 for 1 mg/mL)

Nucleic Acids: A260 ; E (1%) ~ 200 (or O.D. of 1 for 50 microg/mL)

Environmental Effects

$I_{nonpolar} > I_{polar}$ (folding / unfolding effect)

DNA – Helix-Coil Transitions ($\varepsilon_{\text{free base}} > \varepsilon_{ss} > \varepsilon_{ds}$) follow denaturation

Absorbance (A): $A = \log (I_o / I) = \log (1/T)$

Extinction Coefficient – E (1%), ε(M) = Molar extinction coeff.
$A = O.D. = \varepsilon \bullet c \bullet l$ also [$E1\% \bullet MW = 10 \bullet \varepsilon_M$]

---

5. The absorbance of UV light at 280 nm by proteins is mostly due to the aromatic amino acids tyrosine and tryptophan. Lactate DH is a tetramer with each subunit having 332 a.a. (36,507 Da) and containing 6 residues of tryptophan ($\varepsilon = 5.6 \times 10^3\ M^{-1}cm^{-1}$), 5 residues of tyrosine ($\varepsilon = 1.4 \times 10^3\ M^{-1}cm^{-1}$) and 8 residues of phenylalanine ($\varepsilon = 0.2 \times 10^3\ M^{-1}cm^{-1}$).

a) Estimate the **molar extinction coefficient** for this protein at 280 nm. (ε = 168,800 $M^{-1}cm^{-1}$)

(3) For 1 M of Tetramer: $|A| = |\varepsilon|$: $A = 5600(24)(1) + 1400(20)(1) + 200(32)(1)$

b) Estimate the $E^{1\%}$ for this protein at 280 nm. ($E^{1\%}$ = 11.5 $(g/dL)^{-1}cm^{-1}$)

(2) $|E^{1\%}| \cdot M = 10 \cdot |\varepsilon| \Rightarrow E^{1\%} = \frac{10 \cdot 168{,}800}{146028} = 11.5\ (g/dL)^{-1}cm^{-1}$

c) Calculate the **absorbance** and **percent transmission** for a solution of this protein at a concentration of **0.60 mg/mL** from a cell with a path length of **0.50 cm** measured at **280 nm.**

(2) (A = 0.345 ; %T = 45.2)

$A = E^{1\%} \cdot c \cdot l = 11.5\ (g/dL)^{-1}cm^{-1} \cdot \frac{0.06\ g}{dL} \cdot 0.50\ cm$

Technical Resources

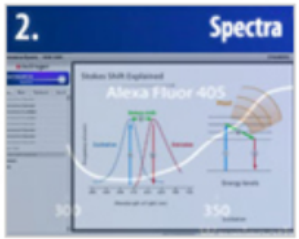
- Contact Product Support
- Technical Reference Library
- Product FAQs
- Manuals & Protocols
- Instrument Repair Request
- MSDS, COA & other Support Documents
- Software Downloads
- Contact Order Support
- Ordering & Web FAQs
- Events
- Training
- Newsletters & Journals

Fluorescence Tutorials

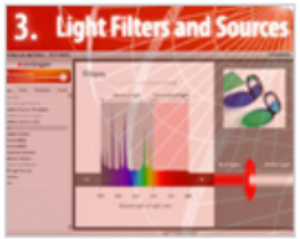
Fluorescence-based techniques are valuable tools for studying cellular structure and function, and interactions of molecules in biological systems. Fluorescence is also important in the detection and quantitation of nucleic acids and proteins in gel electrophoresis, microarrays, and fluorescence spectroscopy.

You are probably already familiar with fluorescence as a property of some substances that "glow in the dark". A large variety of fluorescent chemicals have been synthesized and modified to specifically interact with cellular structures in order to make them detectable in many different colors. With sophisticated microscopes and instruments, it is possible to detect, image, and measure the amount of fluorescence in samples as small as individual cells, and with multiple fluorescent colors. The combination of specialized fluorescent chemicals and instruments has given us an unprecedented detailed view of cells.

Get a basic understanding of fluorescence

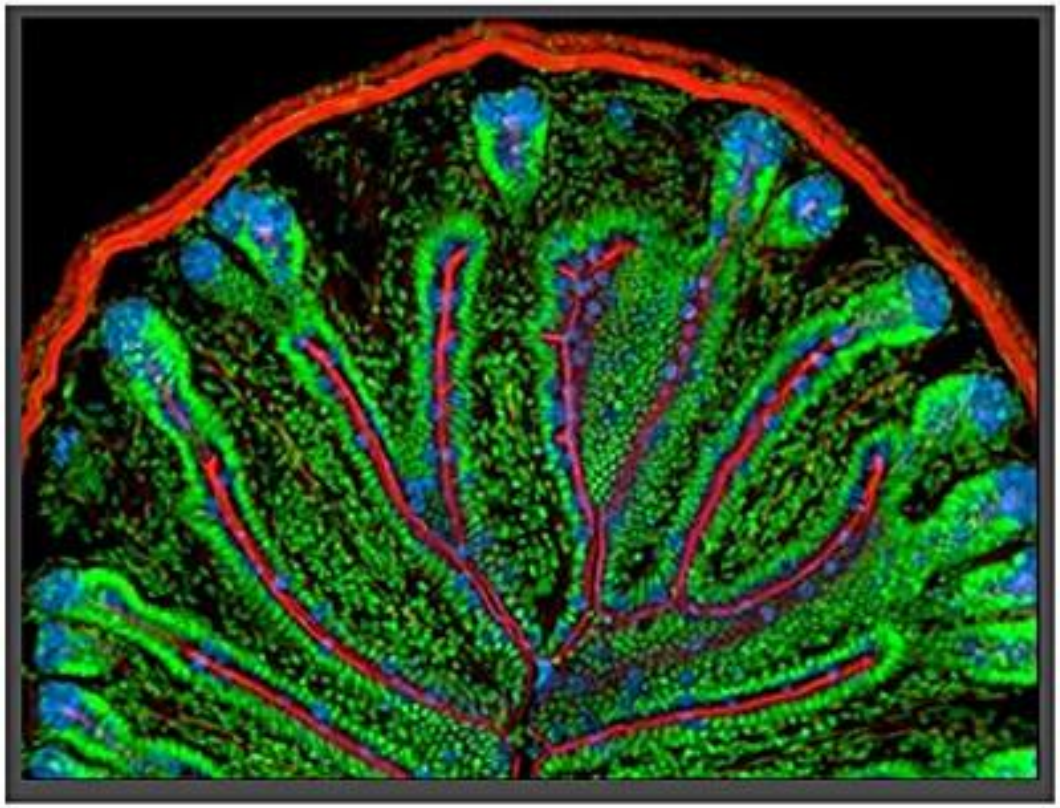


Learn how to interpret excitation/emission spectra



Understand the differences between

Definition of Fluorescence



Mouse intestine

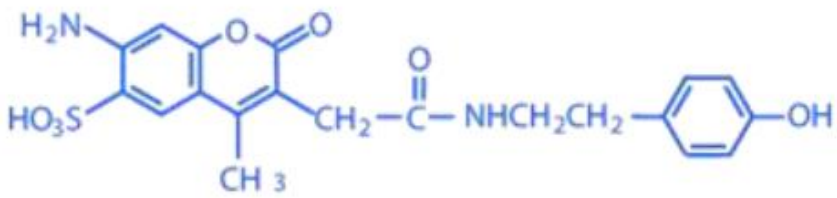
Goblet Cells and Secretions

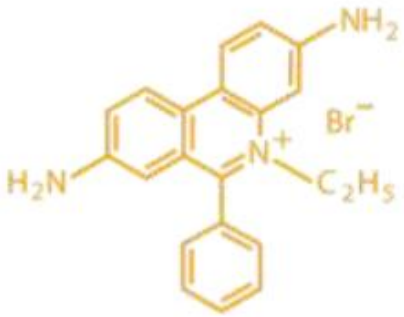
Actin Cytoskeleton

Nuclei

Definition of Fluorescence

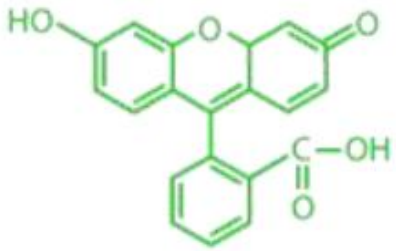
Alexa Fluor 350





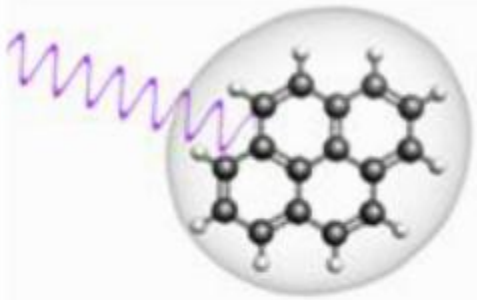
Ethidium bromide

How and why do these dyes and stains emit different colors of light?

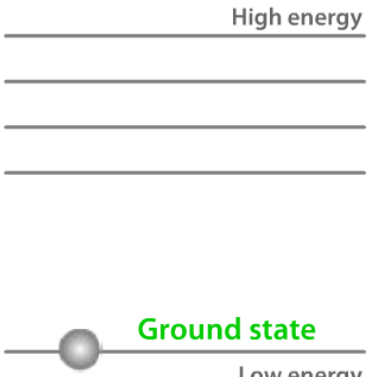


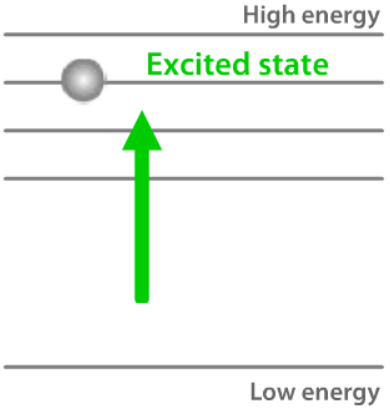
Fluorescein

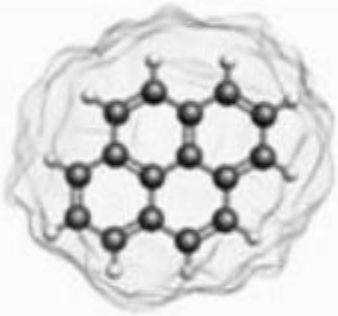
Definition of Fluorescence



Absorption



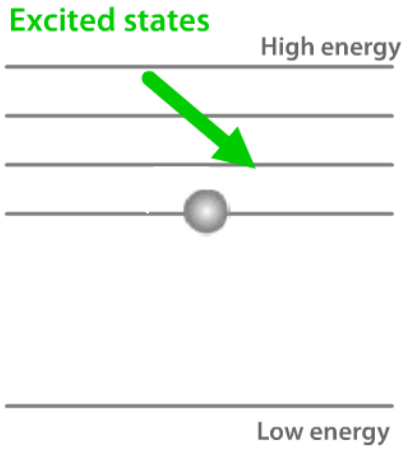


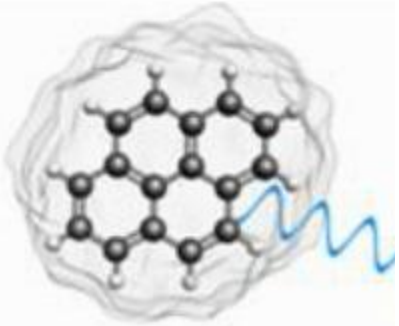


Excitation

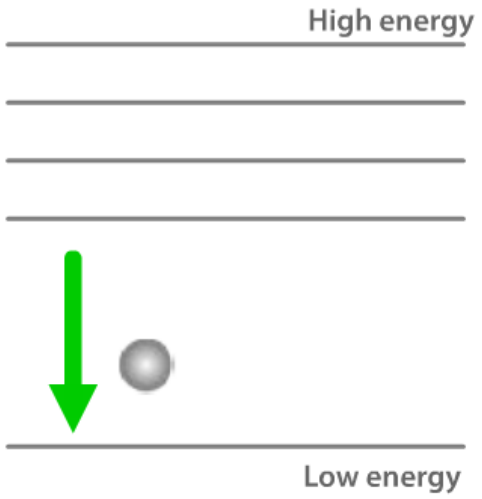
Excited lifetime

10^{-15} to 10^{-9} seconds

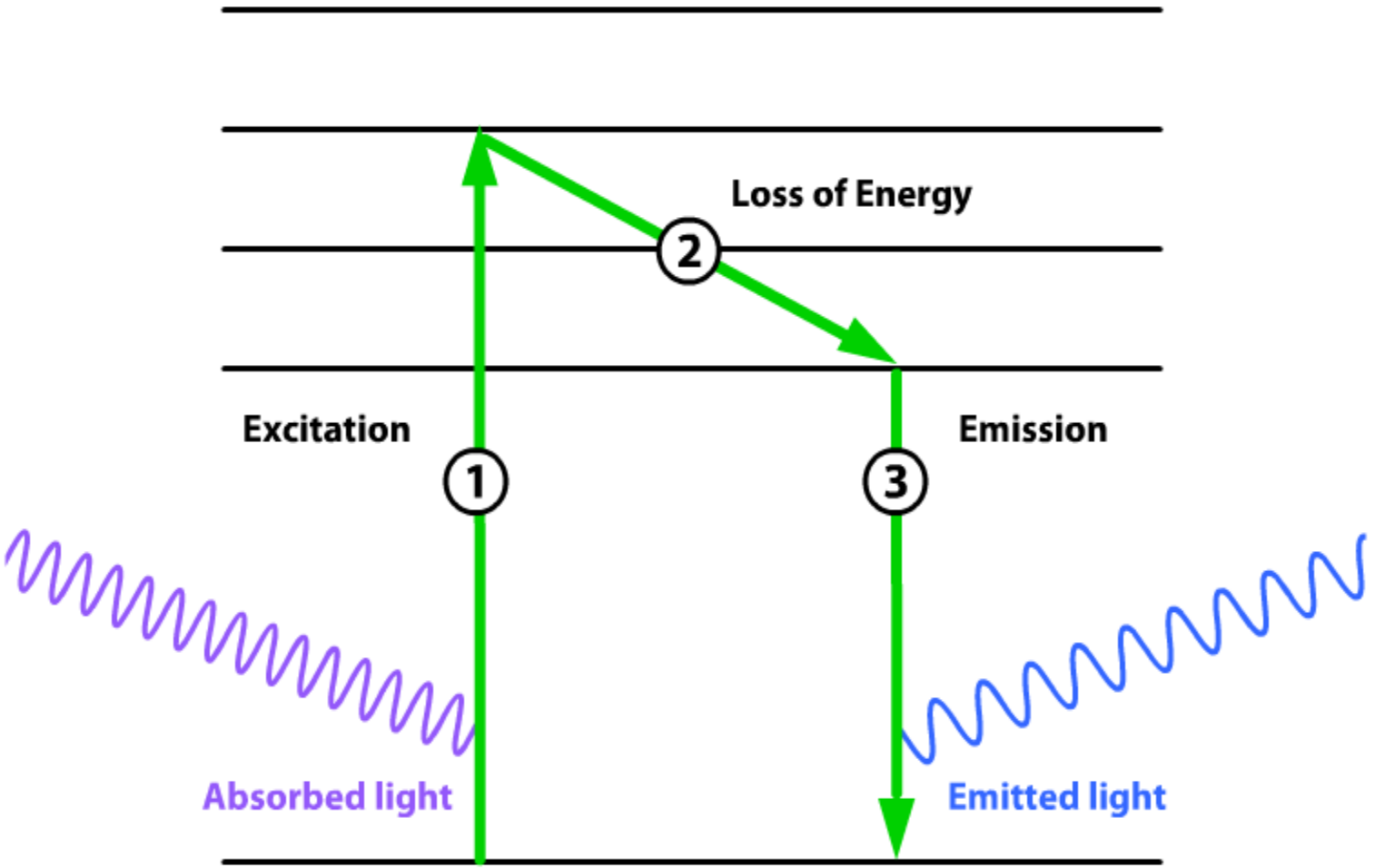


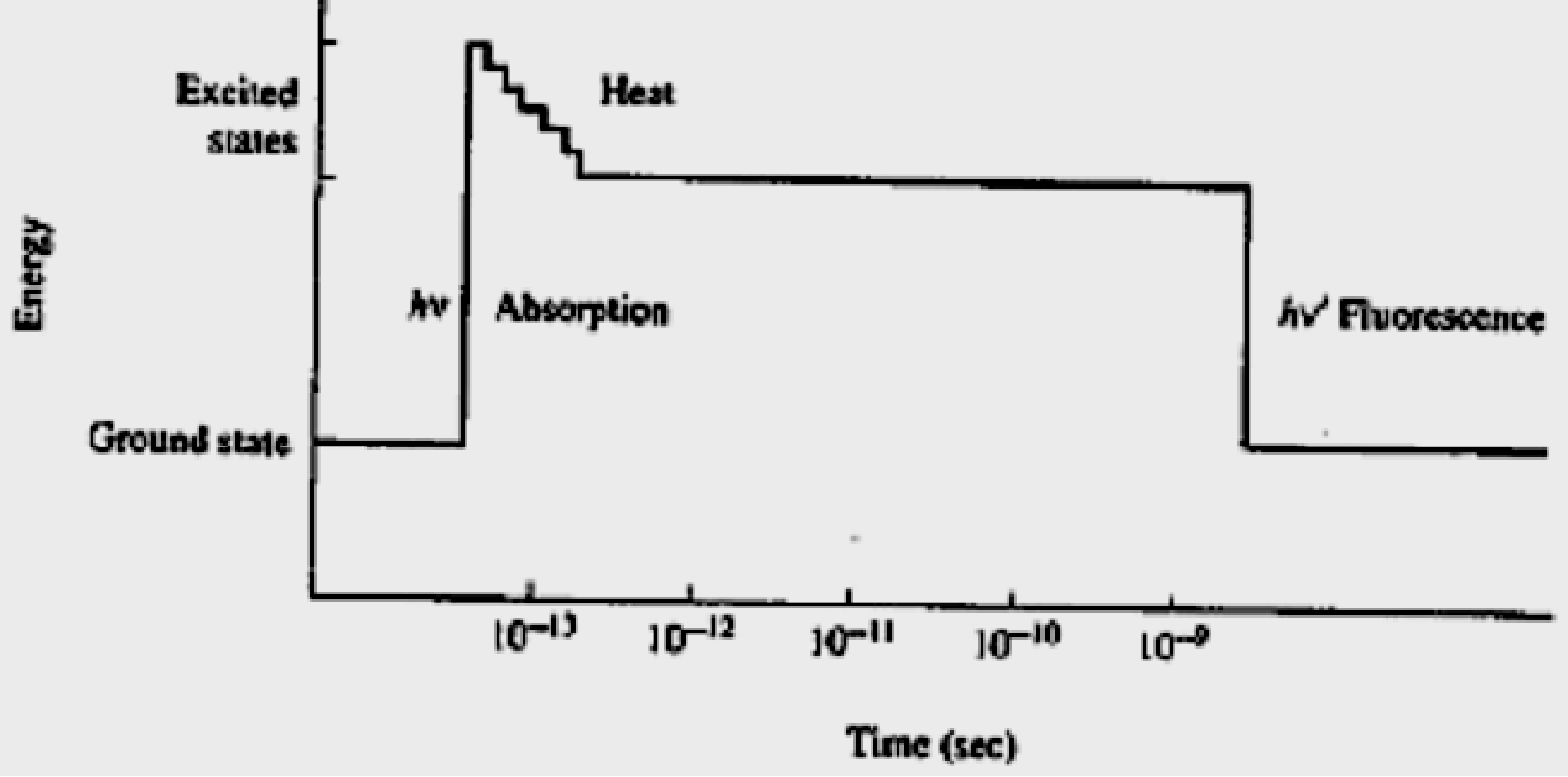


Emission



Summary

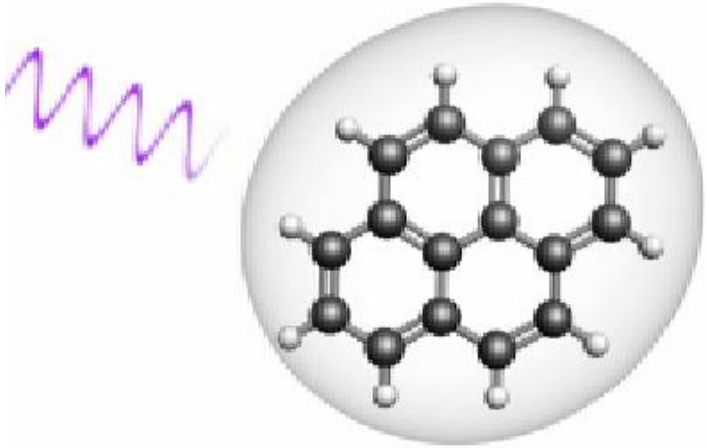

Loss of Energy
2
Excitation
1
Emission
3
Absorbed light
Emitted light

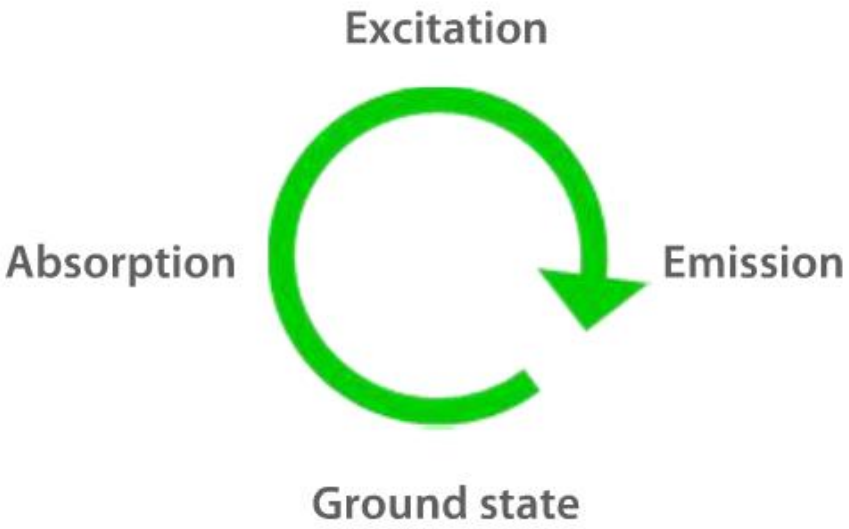


The molecule can relax from the ground vibrational state of the excited electronic state by fluorescence.

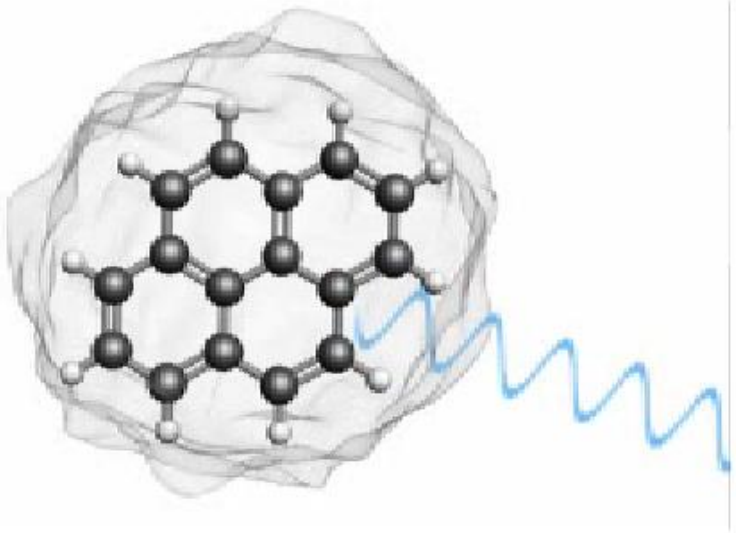
Because of the loss of energy, the emitted photon will have a lower energy than the absorbed. This means a lower frequency and longer wavelength.

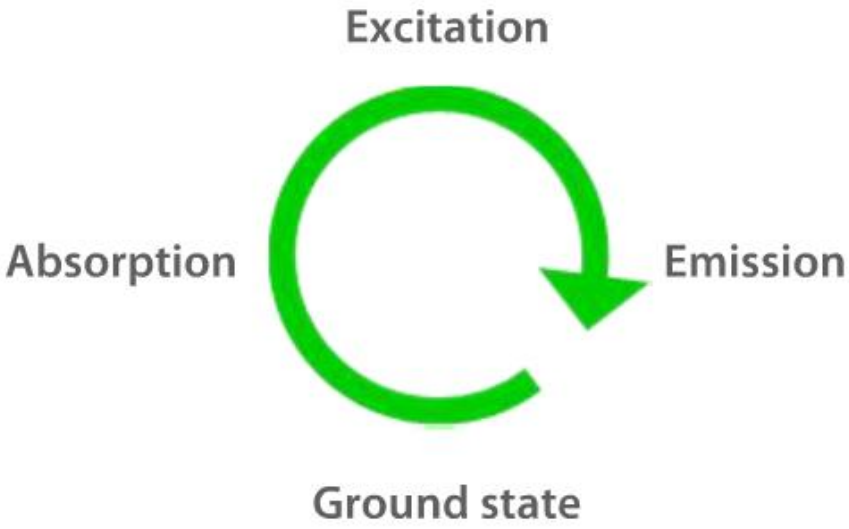
Photobleaching



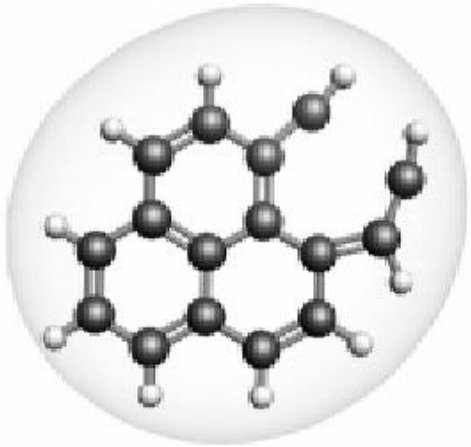


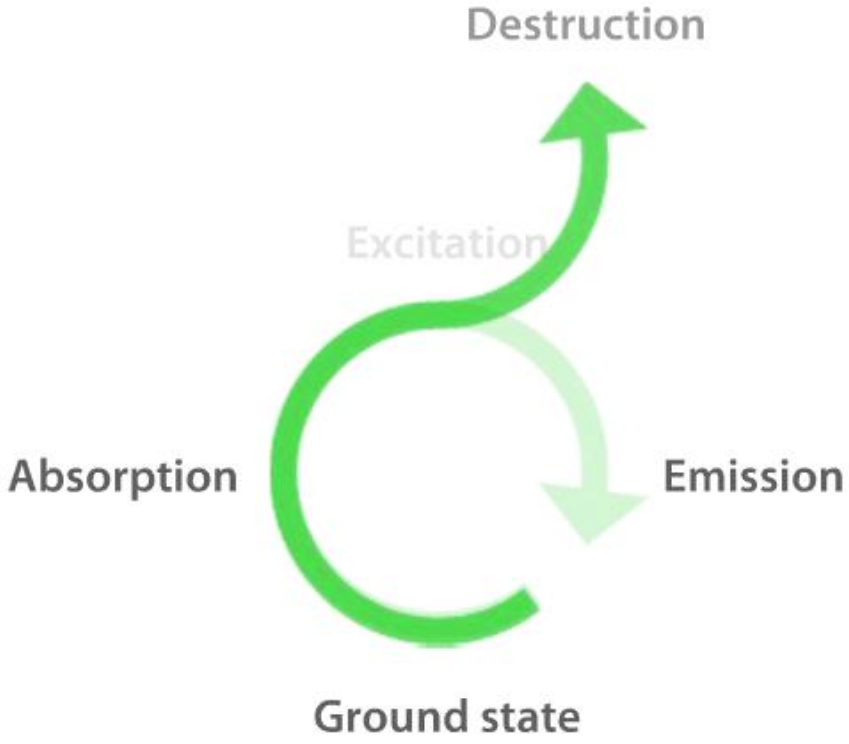
Photobleaching



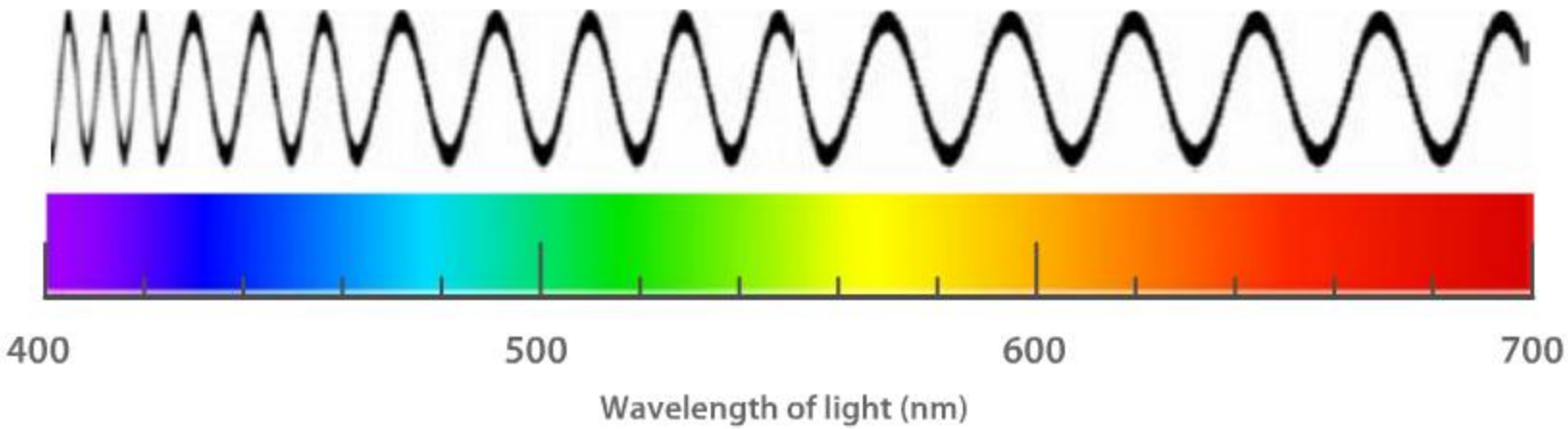


Photobleaching





The Visible Light Spectrum



Shorter wavelength

Higher frequency

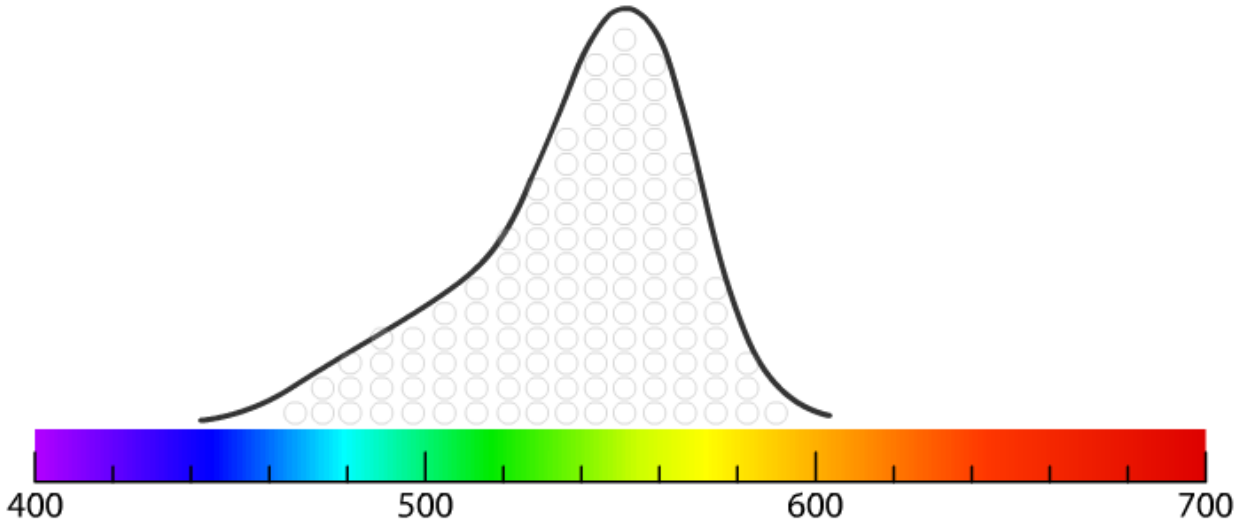
Higher energy

Longer wavelength

Lower frequency

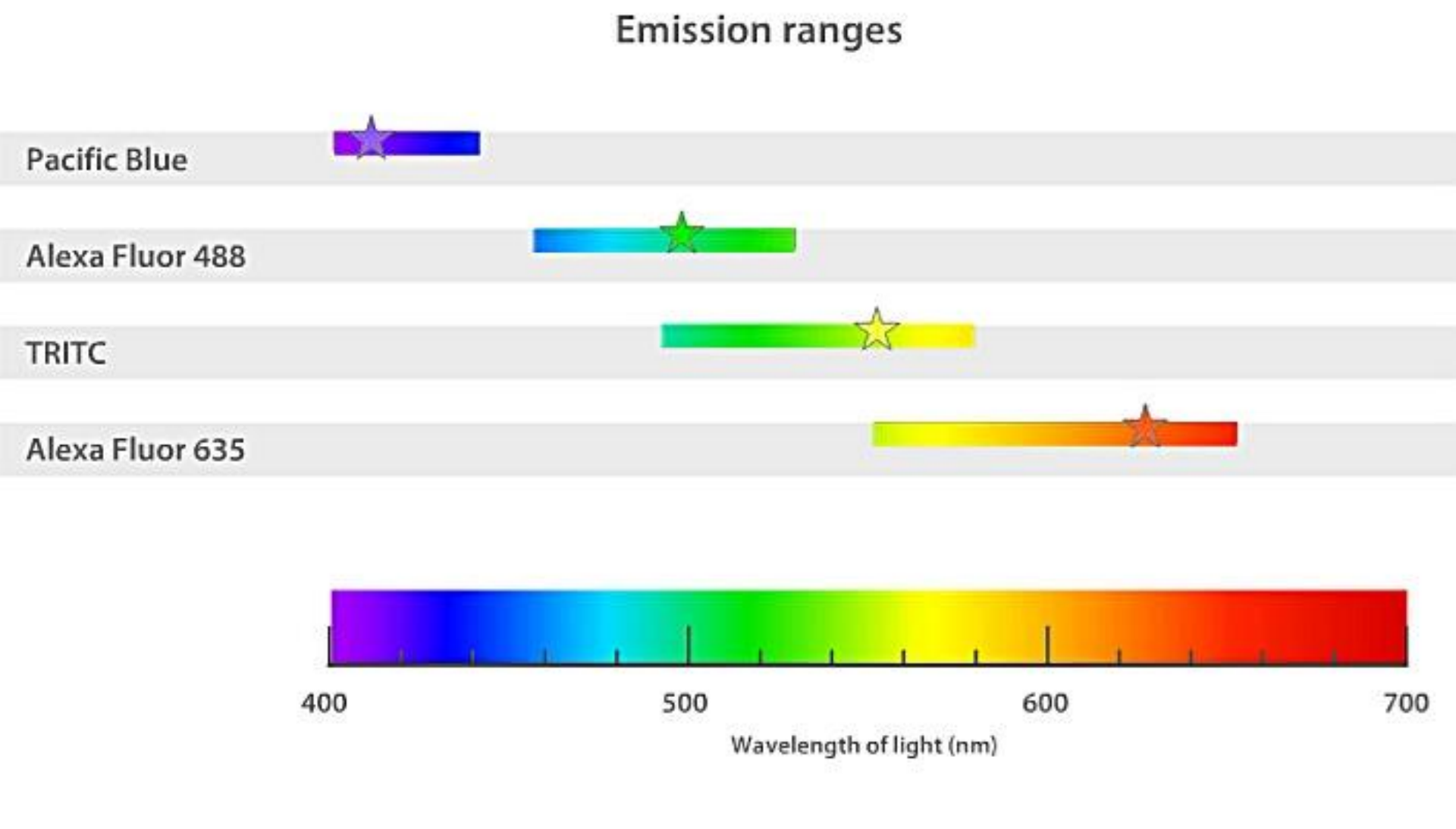
Lower energy

Fluorescence Excitation Spectrum

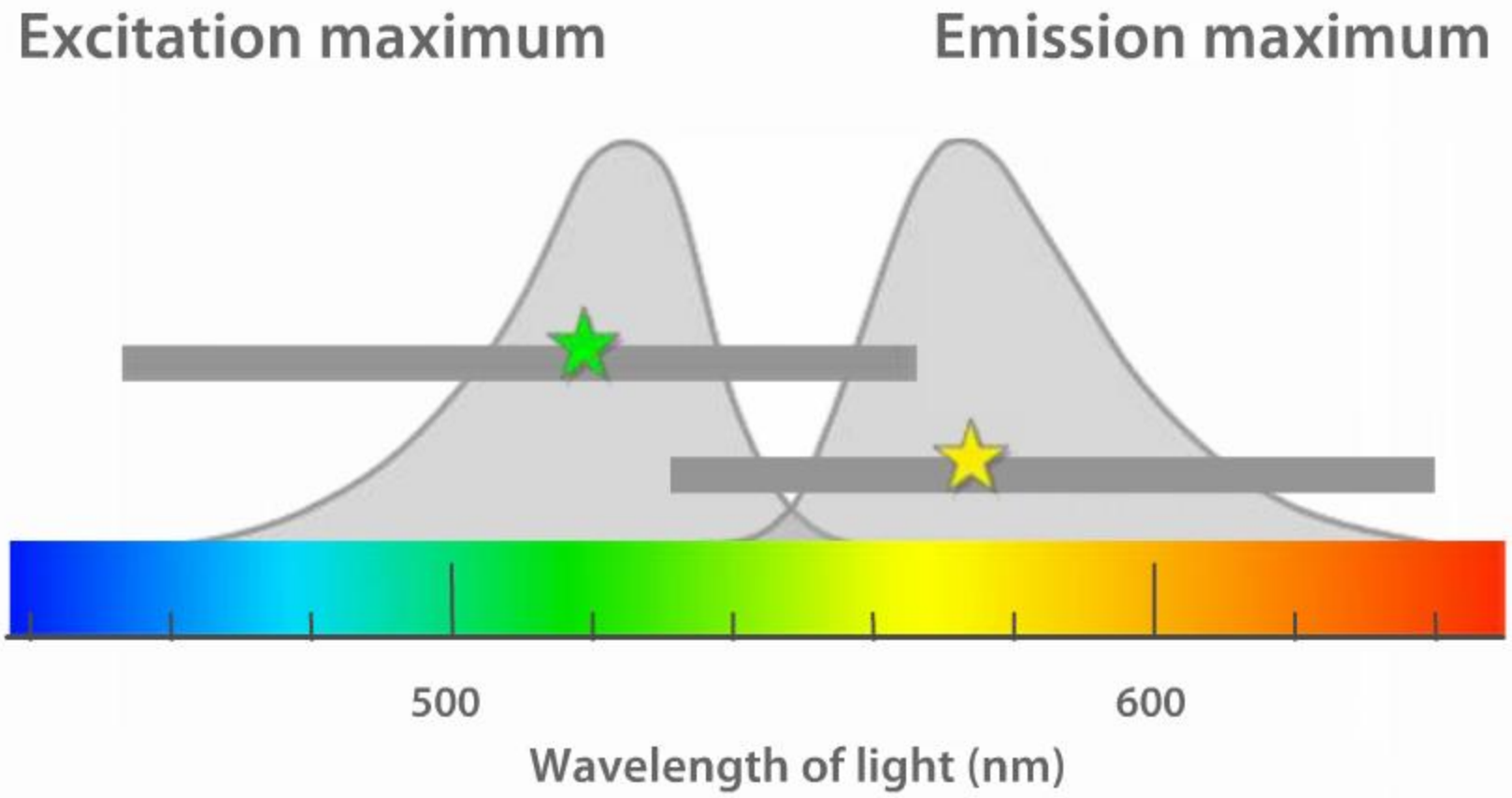


Fluorescence excitation spectrum

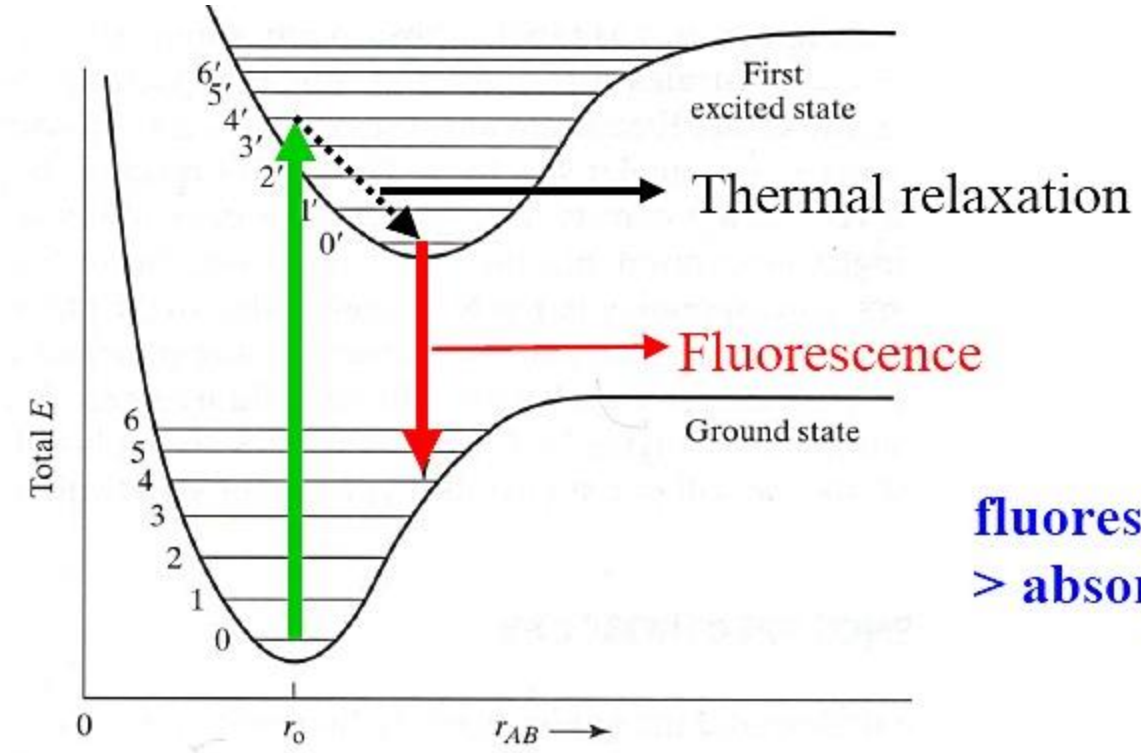
Emission Range



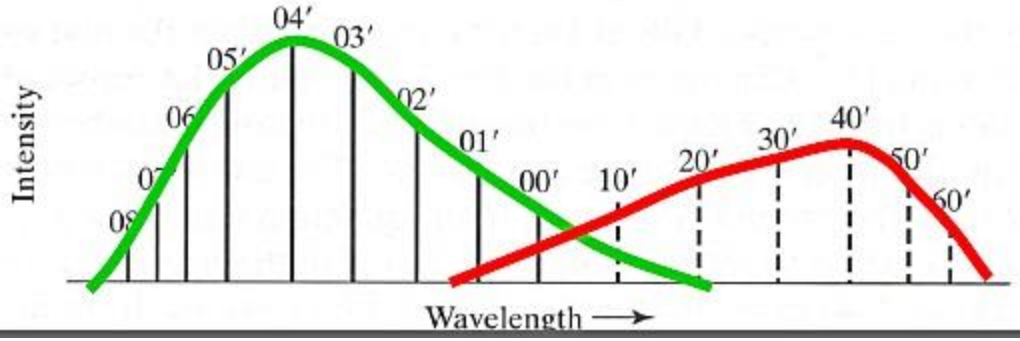
Summary


Excitation maximum
Emission maximum
500
600
Wavelength of light (nm)

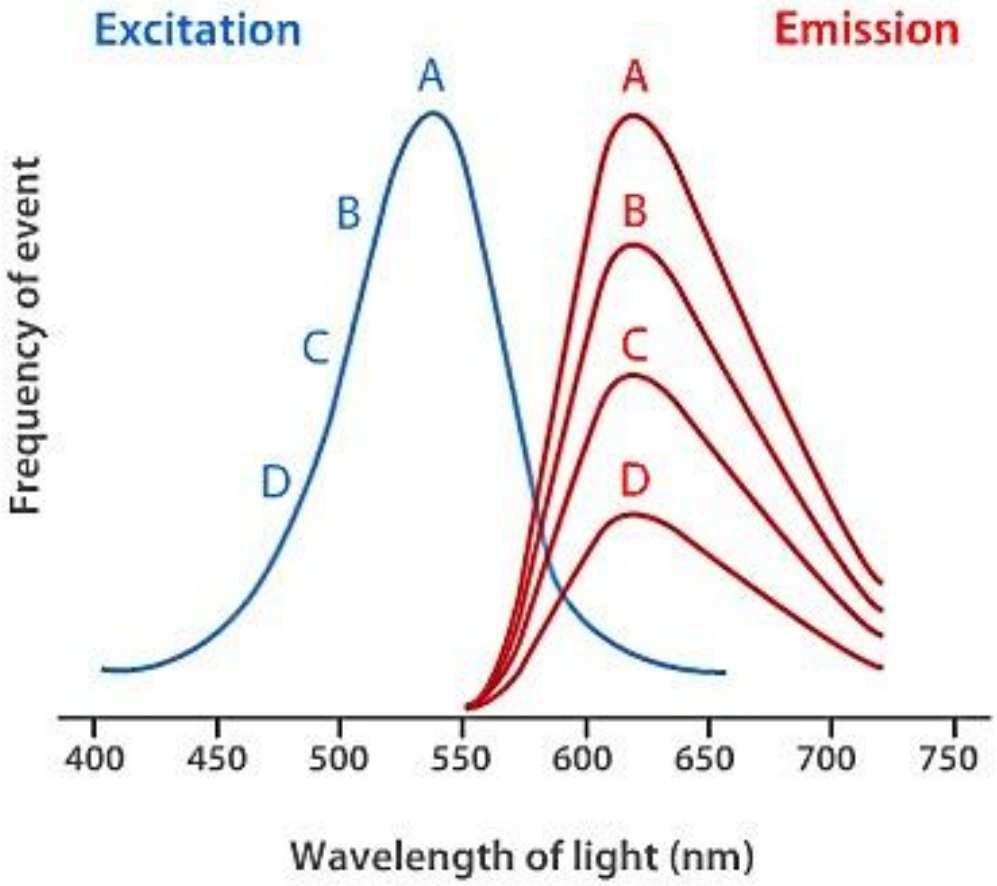
Fluorescence spectra of proteins



fluorescence wavelength > absorption wavelength



Fluorescence Emission



Illumination at lower or higher wavelengths affects only the intensity of the emitted light

For more in-depth information:

Handbook: Introduction to Fluorescence Techniques
http://probes.invitrogen.com/handbook/sections/0001.html

Fluorescence Tutorials: Fluorescence Spectra
http://probes.invitrogen.com/resources/education/

Fluorescence Spectra Viewer
http://probes.invitrogen.com/resources/spectraviewer/

Fluorescence

When atoms and molecules absorb UV/vis radiation, electrons are promoted to higher energy states. Various processes lead to relaxation of the excited atoms or molecules. In the case of molecules, this involves vibrational relaxation, internal conversion, and emission (**fluorescence** and **phosphorescence**).

Typical time frames:

absorption: 10^{-15} s

vibrational relaxation: 10^{-11}-10^{-10} s

internal conversion: 10^{-12} s

luminescence processes

fluorescence: 10^{-5}-10^{-10} s

phosphorescence: 10^{-4}-10^{4} s

Fluorescence Measurements

Instrument

Advantages

Fluorescence parameters / applications

Fluorescence Intensity - quantum yield

Average fluorescence wavelength (shifts)

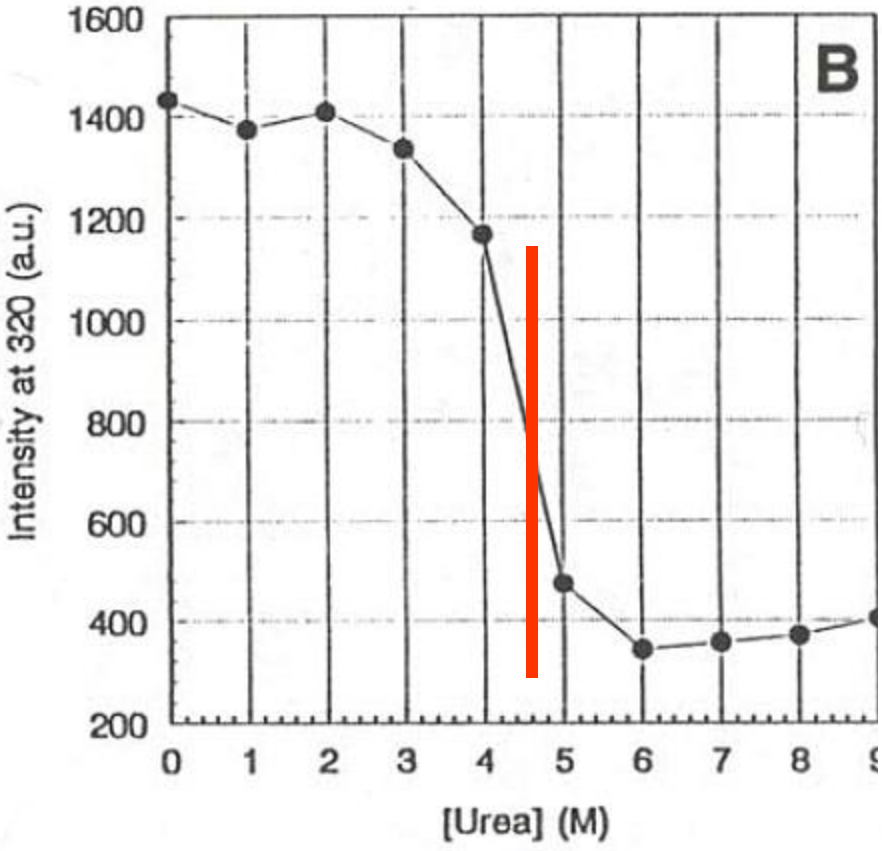
Fluorescence Lifetime

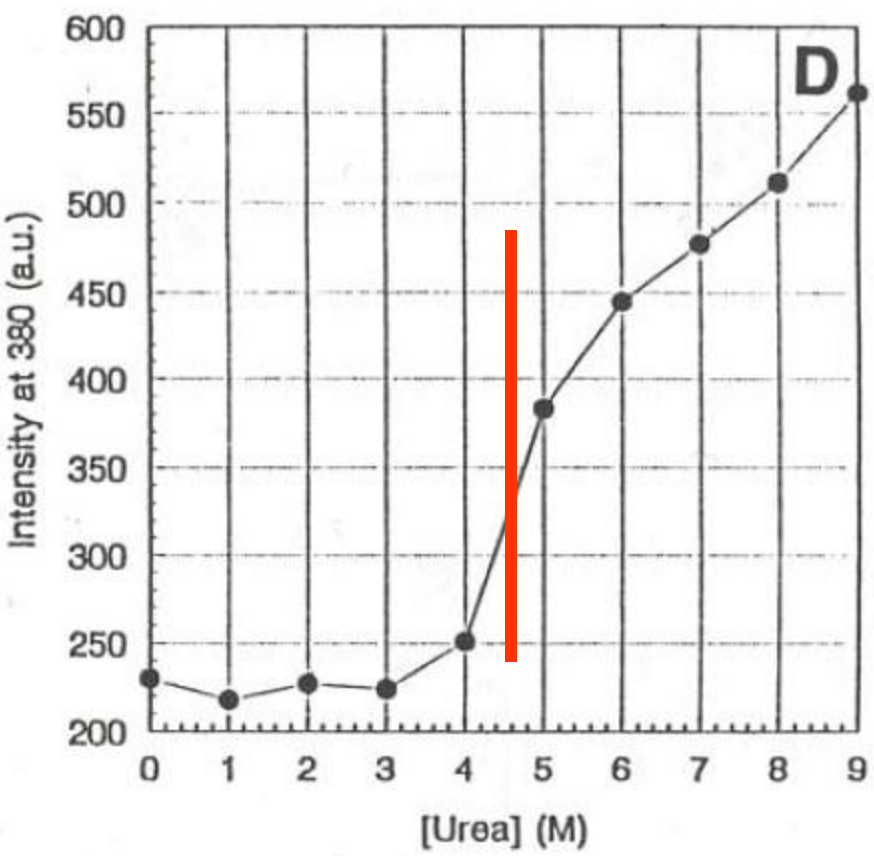
Fluorescence polarization anisotropy (binding)

FRET

Application: Tryptophan Fluorescence and Protein Folding

fluorescence wavelength (shifts)





Trp excitation at 295 nm – emission at 320 and 350 nm vs. [Urea]

Excitation Transfer

Fluorescence **R**esonance **E**nergy **T**ransfer (**FRET**) is an important tool for studying macromolecular structure and dynamics in solution. Some amino acids fluoresce and the **energy transfer strongly depends on distance between donor and acceptor** making it a valuable tool to study protein **folding** and other **dynamics**.

Consider an excited donor, **D*** and an acceptor that can be excited to a fluorescent state, **D***.

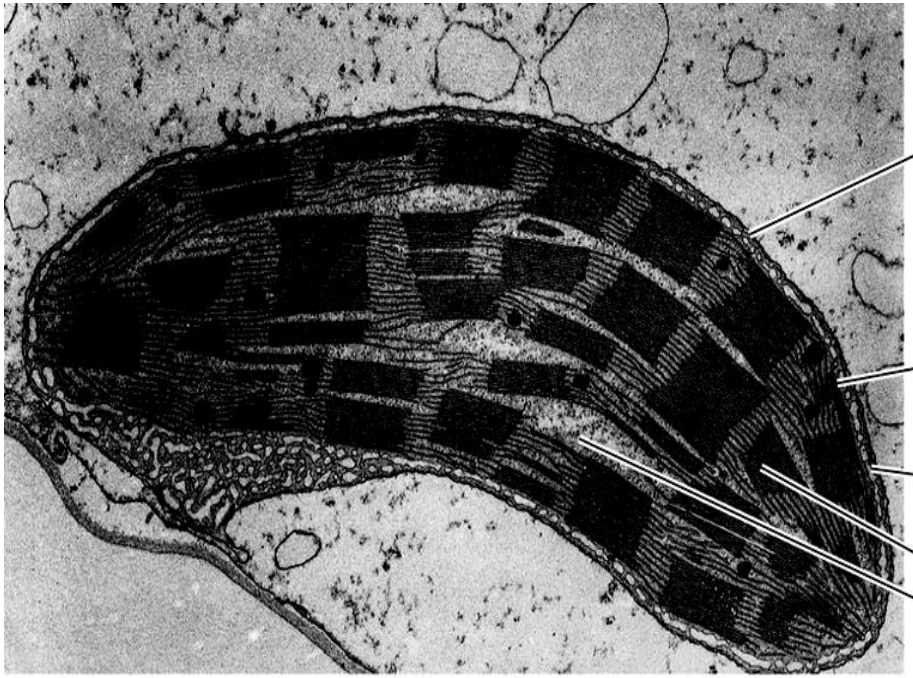
$D \rightarrow D^*$ (absorption of light, hv, by donor)

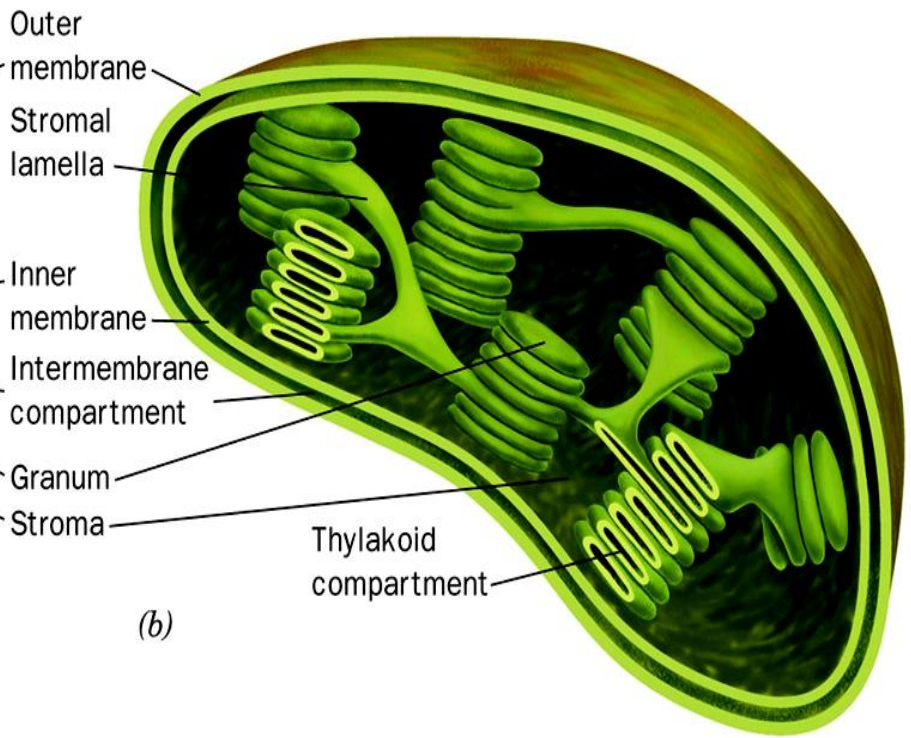
$D^* \rightarrow D + hv'$ (donor fluorescence)

$D^* + A \rightarrow D + A^*$ (excitation transfer)

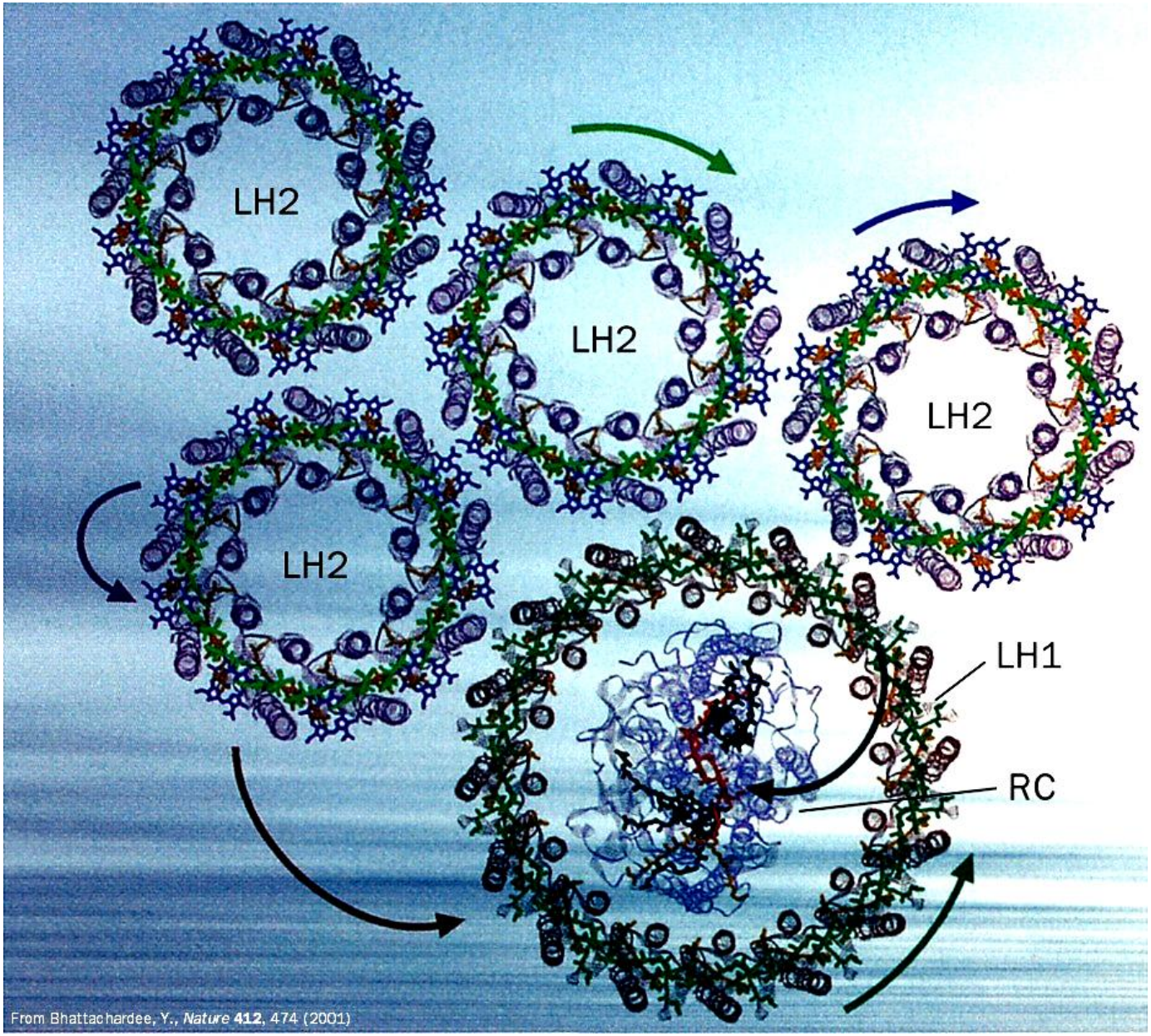
$D^* \rightarrow D$ (other deexcitation)

$A^* \rightarrow A + h\,v''$ (acceptor fluorescence)



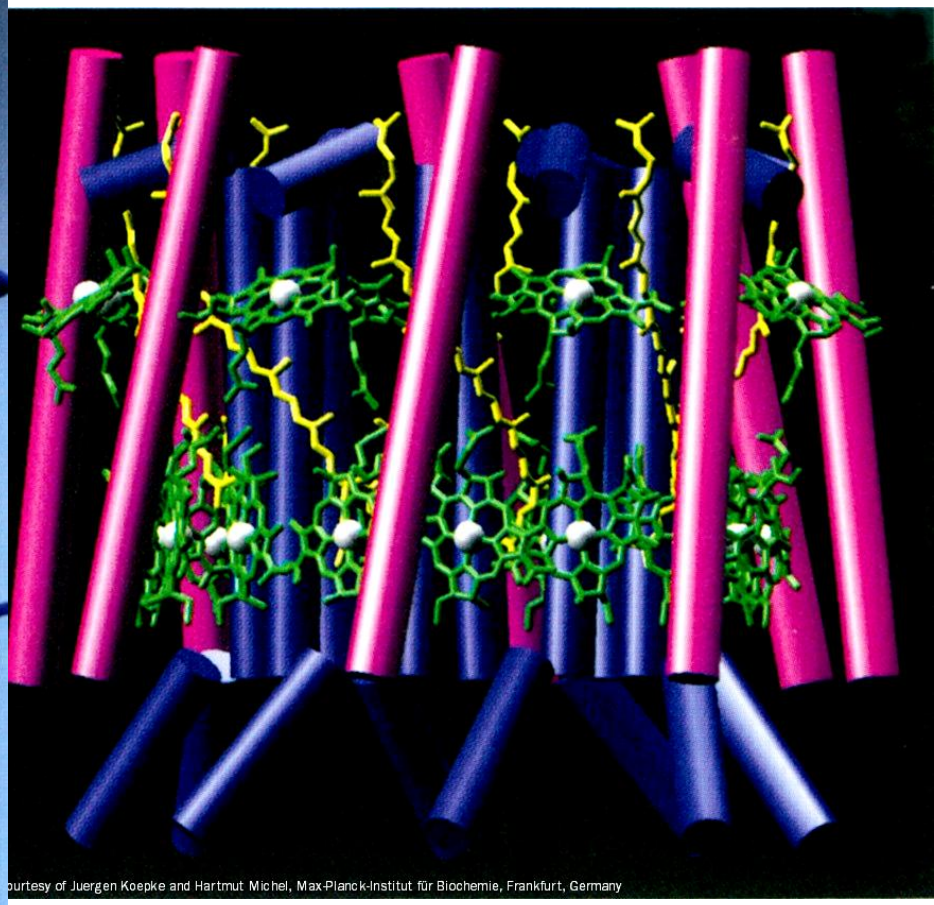


Chloroplast from corn.

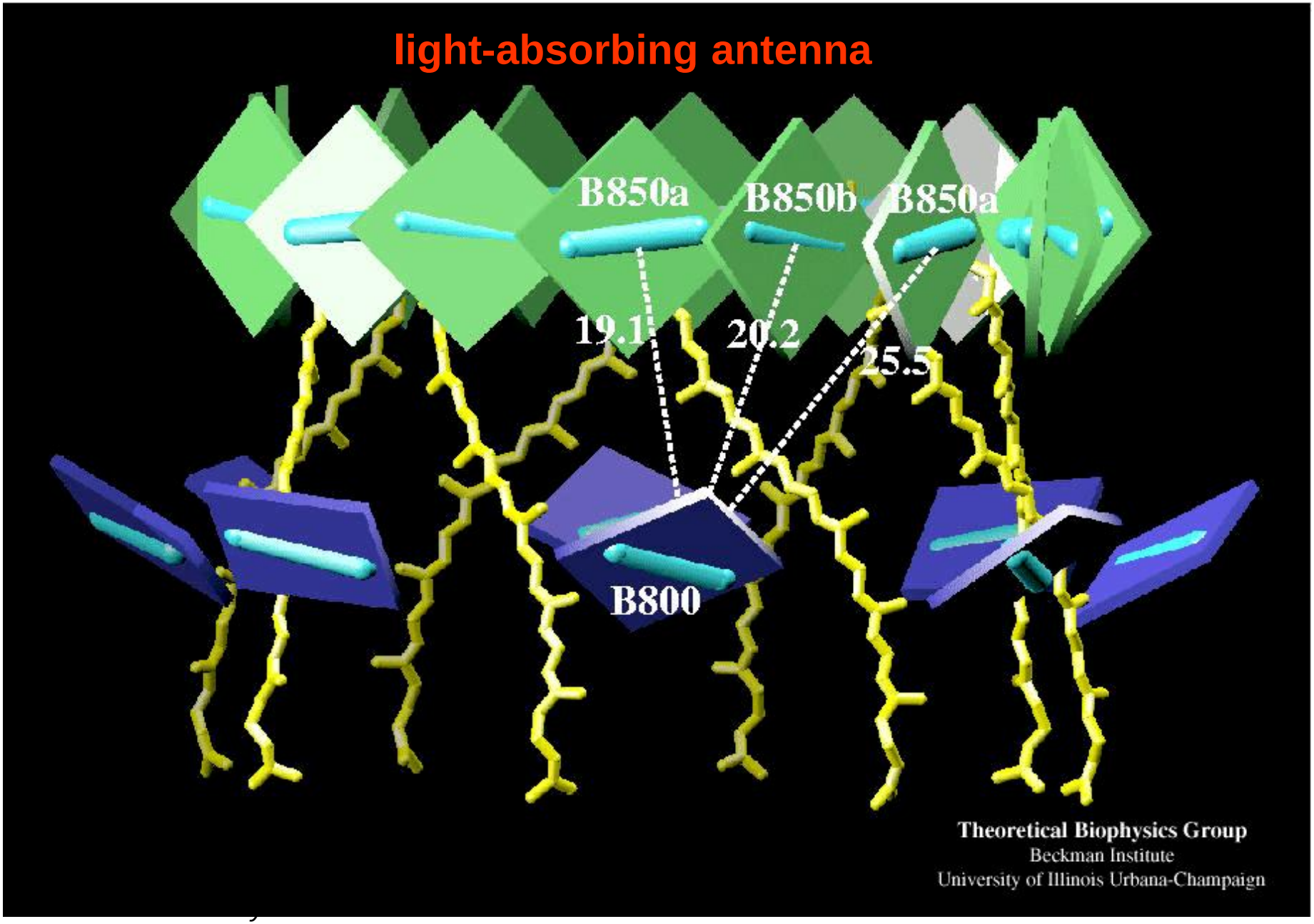


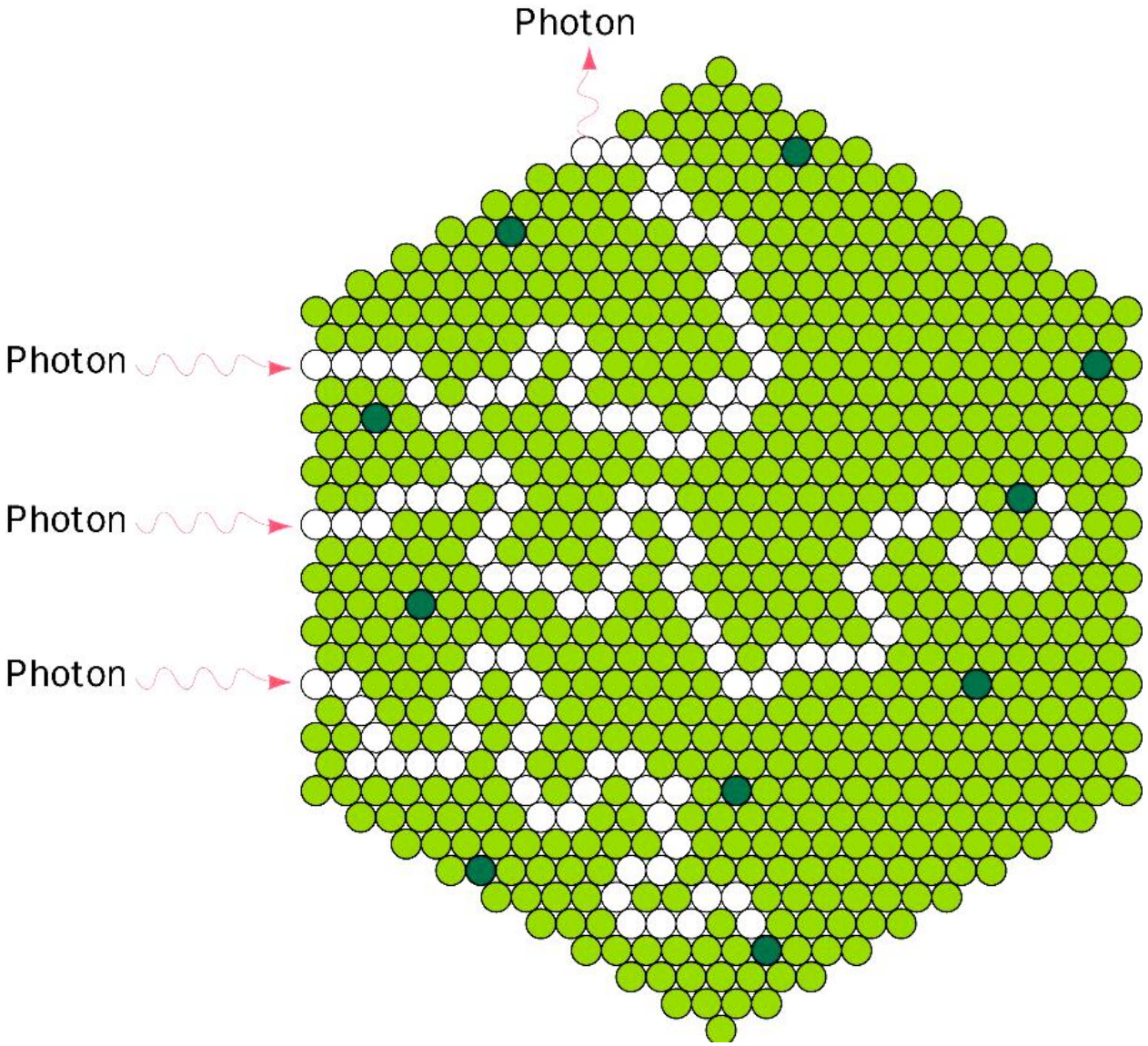
Model of the **light-absorbing antenna** system of purple photosynthetic bacteria.

light-absorbing antenna

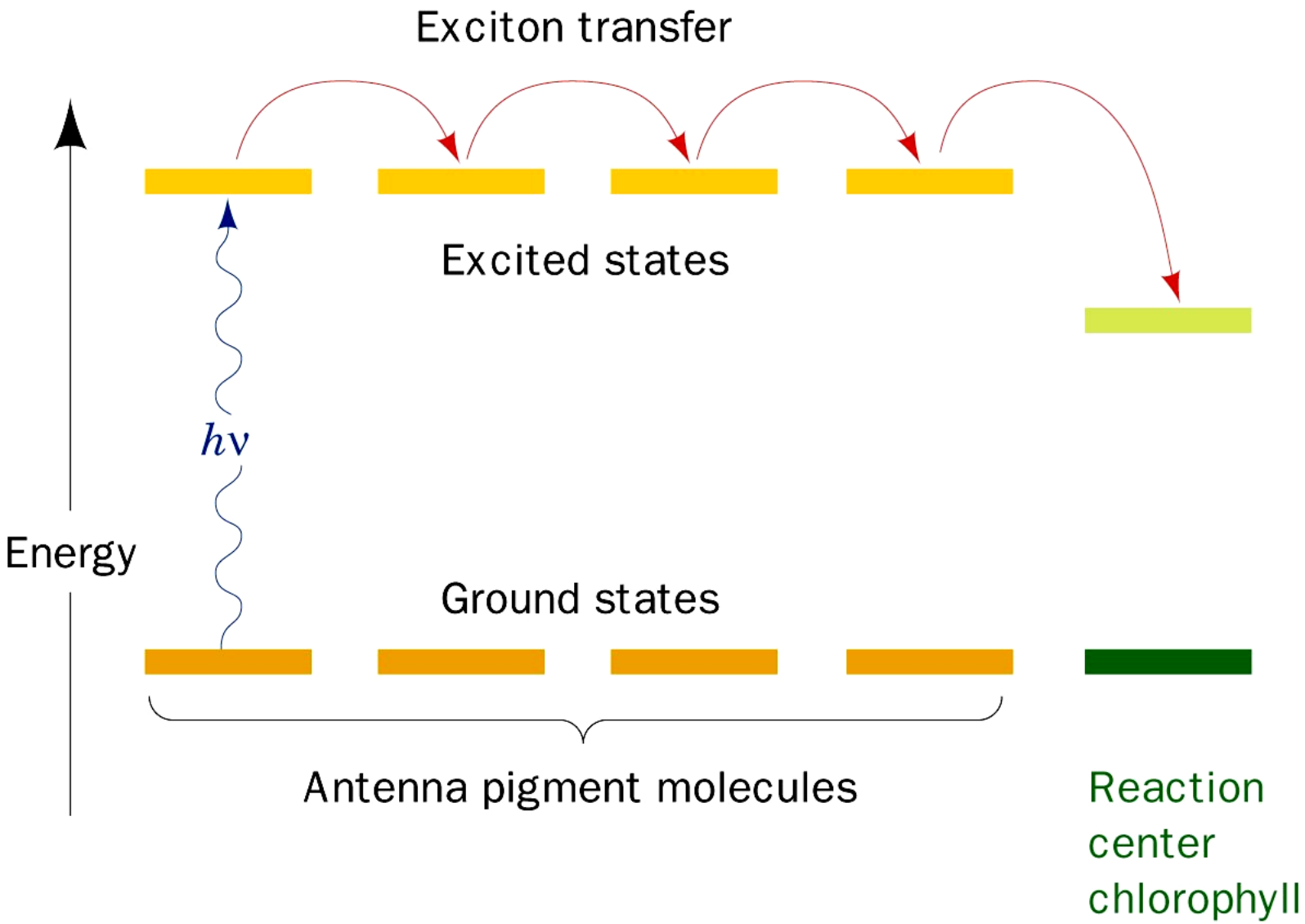


X-Ray structure of **LH2** from *Rs. molischianum.* View perpendicular (a) and parallel (b) to the bacterial membrane from the cytoplasm.





Flow of energy through a photosynthetic antenna complex. (*a*) The excitation resulting from photon absorption randomly migrates by **exciton transfer**.

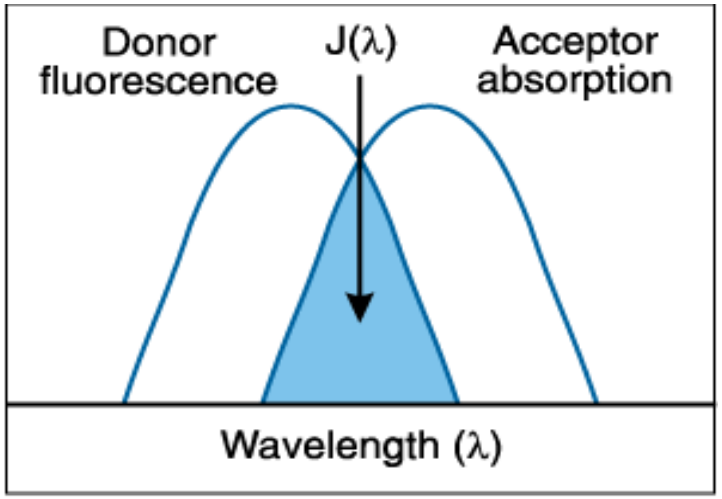


Flow of energy through a **photosynthetic antenna complex**. The excitation is trapped by the RC chlorophyll.

Primary Conditions for FRET

- Donor and acceptor molecules must be close (~10–100 Å).
- Absorption spectrum of acceptor must overlap the fluorescence emission spectrum of the donor.
- Donor and acceptor transition dipoles must be ~ parallel.

FRET spectral overlap.



Experimental Measurements

1. **Decrease in fluorescence quantum yield** of donor in the presence of acceptor.
2. **Decrease in fluorescence lifetime of acceptor** in the presence of donor.
3. **Increase in the fluorescence of D** in the presence of A.

Förster Radius, R_0

The distance at which energy transfer is 50% efficient is defined by the Förster radius (R_o). The magnitude of R_o is dependent on the spectral properties of the donor and acceptor dyes and the efficiency depends on the inverse sixth power of intermolecular distance :

$$\text{Efficiency} = k_T / (k_T + k_d) = r_0^6 / (r_0^6 + r^6) = 1/(1 + (r^6/r_0^6))$$

where k_d is the rate constant for de-excitation,
and k_T is the rate constant for transfer.

$$R_O = [8.8 \times 10^{23} \bullet \kappa^2 \bullet n^{-4} \bullet \phi_d \bullet J(\lambda)]^{1/6} \text{ Å}$$

where κ^2 = dipole orientation factor (range 0 to 4; $\kappa^2 = 2/3$ for randomly oriented donors and acceptors)

ϕ_d = fluorescence quantum yield of the donor in the absence of the acceptor

n = refractive index of the medium

$J(\lambda)$ = spectral overlap integral

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

where ϵ_A = extinction coefficient of acceptor

F_D = fluorescence emission intensity of donor as a fraction of the total integrated intensity

Efficiency = $k_T / (k_T + k_d) = r_0^6 / (r_0^6 + r^6) = 1/(1 + (r^6/r_0^6))$

where k_d is the rate constant for de-excitation,
and k_T is the rate constant for transfer.

Example of using FRET to estimate separation distance:

6. Consider a FRET experiment where the measured efficiency of energy transfer between two chromophores is 20.0%. If R_0 = 40.0 Å for these two chromophores, **estimate the separation** of the two chromophores. (R = 50.4 Å)

(2) $Eff = 0.20 = \frac{r_0^6}{r_0^6 + r^6} = \frac{1}{(1 + (r/r_0)^6}$

$0.20 + 0.20x^6 = 1.0$ where $x = r/r_0$

$x^6 = \frac{.80}{.20} = 4$

$x = 1.26$ and $r = 1.26 r_0$

$= 50.4$ Å

I hereby declare that I did this work independently: MM

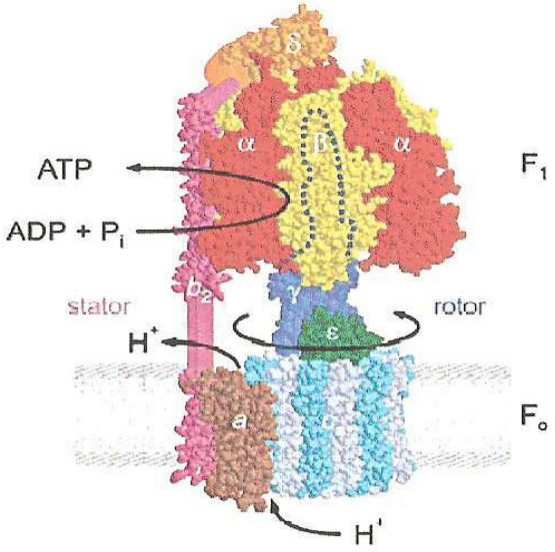


Figure 1 Structure of ATP synthase. The enzyme from *E. coli*, with subunit stoichiometry $\alpha_3\beta_3\gamma\delta\varepsilon ab_2c_n$. In mitochondria and chloroplasts additional subunits are present. Stoichiometry of the *c* ring (*n*) varies (it is believed to be 10–12 in *E. coli* and 10, 11 or 14 in other organisms). See ref. 5 for further details.

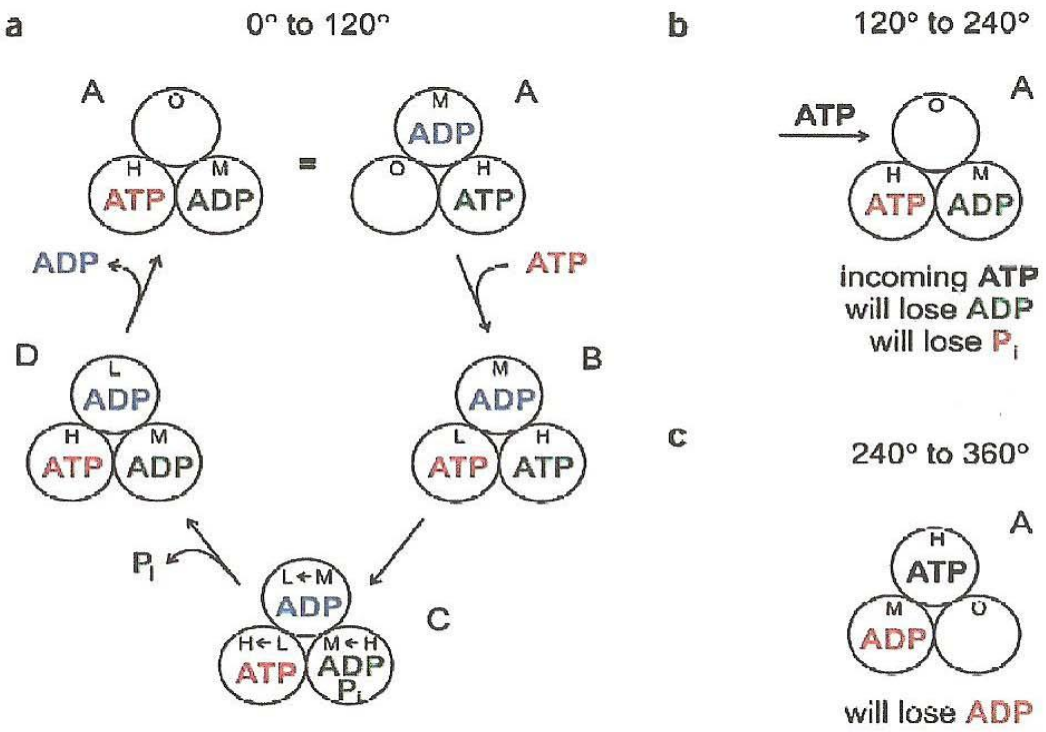


Figure 2 Enzymatic mechanism of ATP hydrolysis by ATP synthase. (a) Each circle represents one of the three catalytic sites. O, open (unoccupied); L, lowest affinity for ATP; M, medium affinity; H, highest affinity. The series of enzyme states ABCDA describes what happens during consumption of one molecule of ATP and one 120° step of γ rotation. Binding of an incoming ATP (red) to the ATP-waiting state (A) brings about hydrolysis of already-resident ATP (green), with the chemical reaction transition state occurring between states B and C. Concomitantly, γ rotation is initiated, leading to a switch in site conformations (arrows in C). P_i derived from the already-resident ATP (green) is released (the exact timing is unsolved; see text and ref. 7). Release of already-resident ADP (blue) occurs from state D. Note that ATP binding (red), ATP hydrolysis (green) and ADP release (blue) occur at three different sites, and that at the end of the first 0°-to-120° rotation step, incoming ATP (red) is still bound intact. (b) The ATP-waiting state at the beginning of the second (120° to 240°) step. In this step the red ATP will be hydrolyzed. (c) The ATP-waiting state at the beginning of the third (240° to 360°) rotation step. In this step the red ADP will be released.

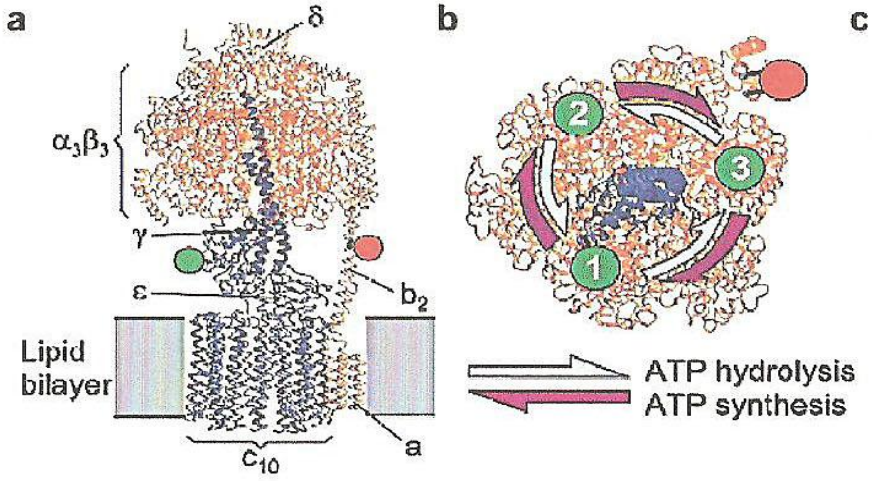


Figure 1 Model of F_0F_1 from *E. coli* (see Methods). (a) Side view. The FRET donor is bound to the γ subunit (green circle), the FRET acceptor Cy5bis to the b subunits (red circle). 'Rotor' subunits are blue, 'stator' subunits are orange. (b) Cross-section at the fluorophore level, viewed from F_0. Cy5bis (red) crosslinks the b subunits. Donor position 1 (green) of cysteine γ-T106C is farthest away from b-Q64C. Rotation of the γ subunit by 120° and 240° results in donor positions 2 and 3, respectively. (c) Photon bursts are observed when a freely diffusing single liposome with a single FRET-labeled F_0F_1 traverses the confocal detection volume (yellowish) within the laser focus (green).