

# Use of iClicker - bonus points for participation

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Each iClicker question is worth 10 pts.

Score: 10 pts for an answer  
12 pts for a correct answer  
0 pts for no answer

Note 1: most of these questions should be relatively easy to check comprehension

Note 2: these are all bonus points for participation

Note 3: thus, correct responses can compensate for a missed question.

# Steps to register your iClicker (roll call)

Step 0: Turn on your iClicker

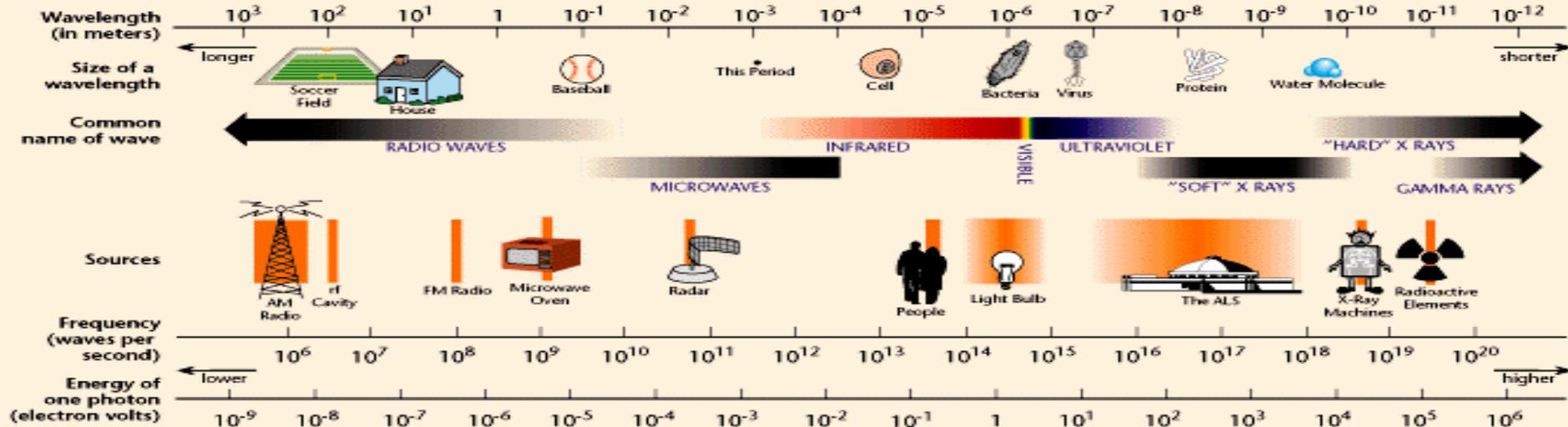
Step 1: Watch for your name and UTelD to scroll across the screen.

Step 2: Enter A/B/C/D or E as shown next to your name.

Step 3: Confirm/complete your registration by entering A/B/C/D or E shown next to your name.

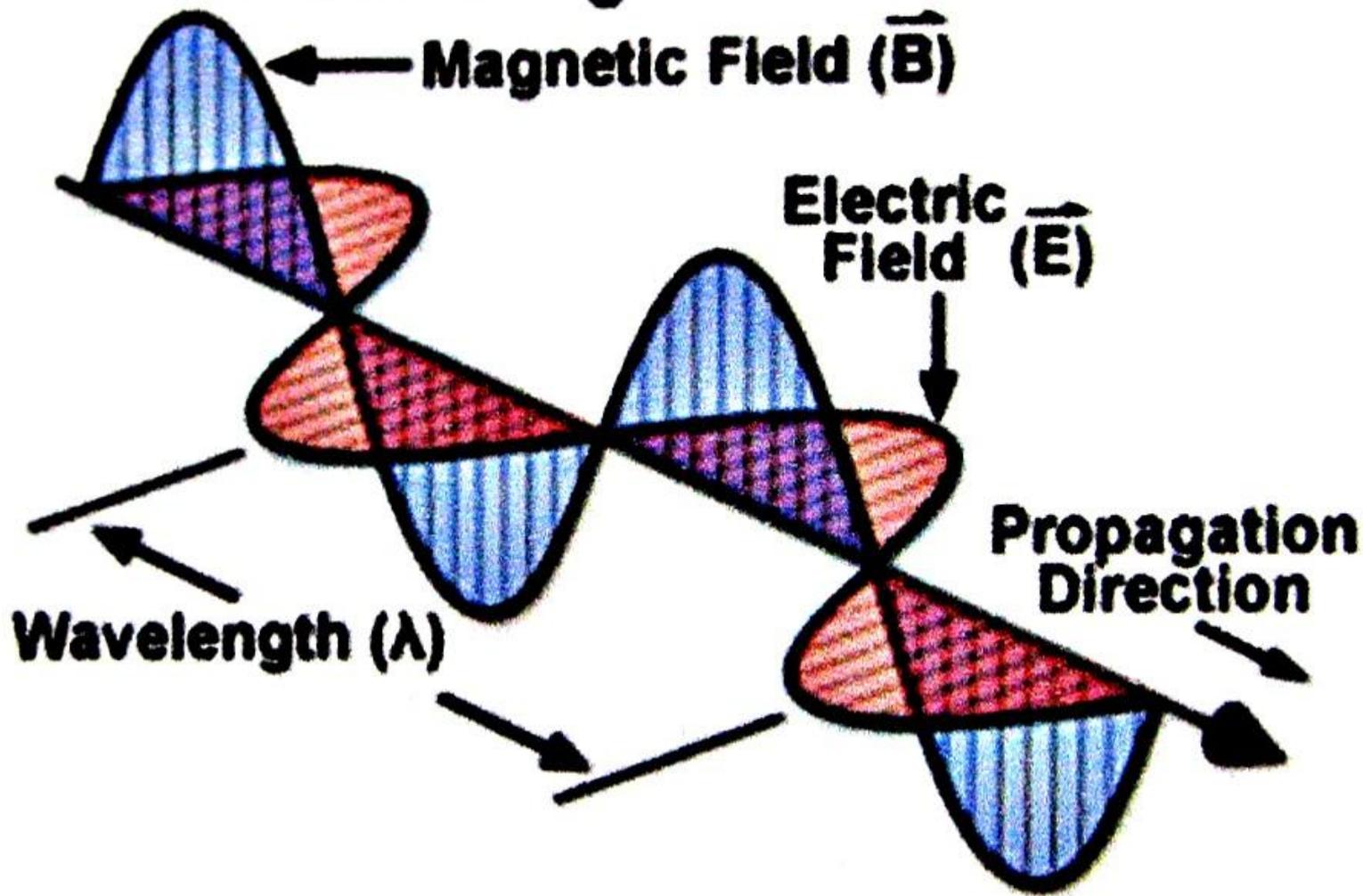
# Spectroscopy

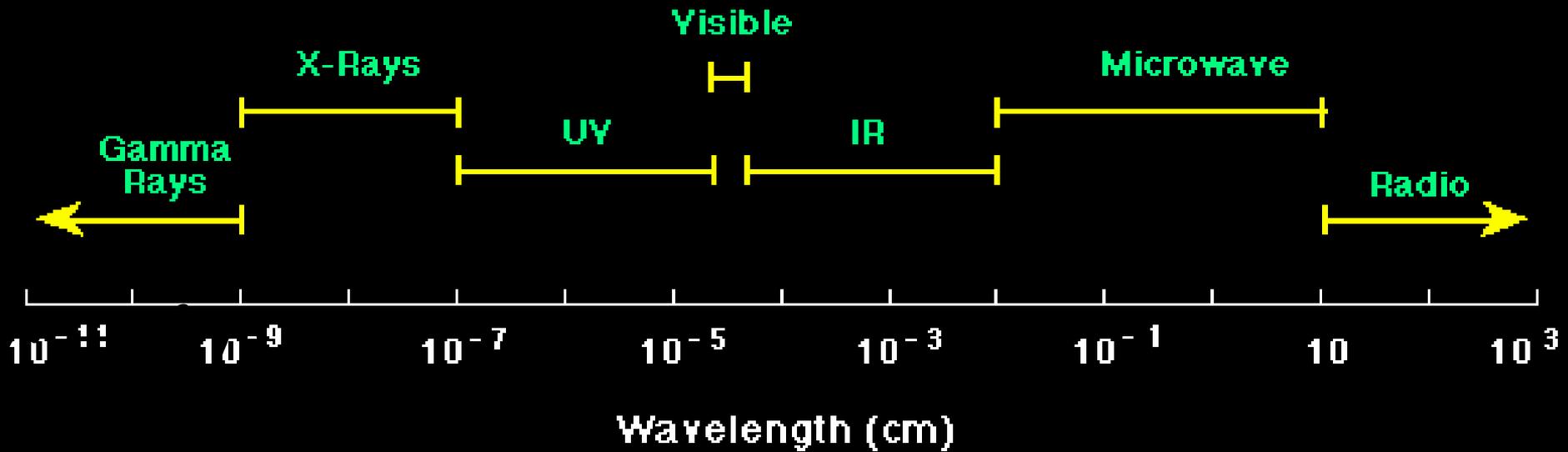
## THE ELECTROMAGNETIC SPECTRUM



- Objectives:** Review nature of electromagnetic radiation ( $\lambda / \nu / c$ )
- Interactions of "Light" with matter (Absorption / Scattering (n))
  - Frank-Condon Principle
  - Electronic transitions (Abs, Fluor, Phosphor.)
- Beer Lambert Law ( $A = O.D. = -\log(T) = \epsilon \cdot [c] \cdot l$ )
- Excitation Transfer / FRET

# Electromagnetic Wave





**Speed of light ( $v$ ) = wavelength ( $\lambda$ ) x frequency ( $\bar{\nu}$ )**

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

Units are  $\text{cm}^{-1}$ .  
 700 nm **red light** =  $1.43 \times 10^4 \text{ cm}^{-1}$   
 420 nm **violet light** =  $2.38 \times 10^4 \text{ cm}^{-1}$

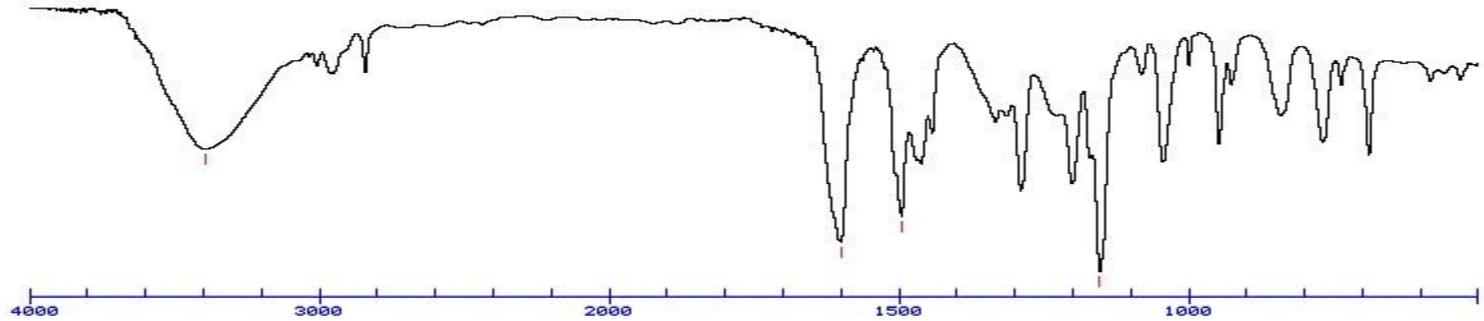
Velocity of light changes in different substances.

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

# Light and Matter: Absorption (spectroscopy)

## Scattering (image formation)

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An Example IR Spectrum

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- Light Photography

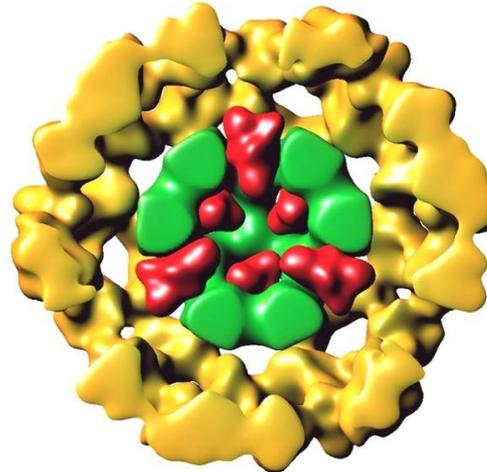
$\lambda \sim 400 - 700 \text{ nm}$



Abbe (~1878): Limit Res.  $\sim \lambda/2$

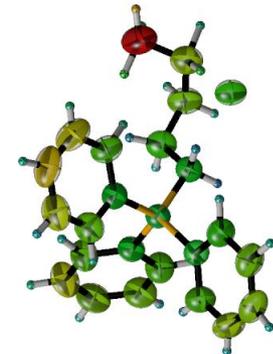
- Electron Microscopy

$\lambda \sim 0.001 - 0.1 \text{ nm}$



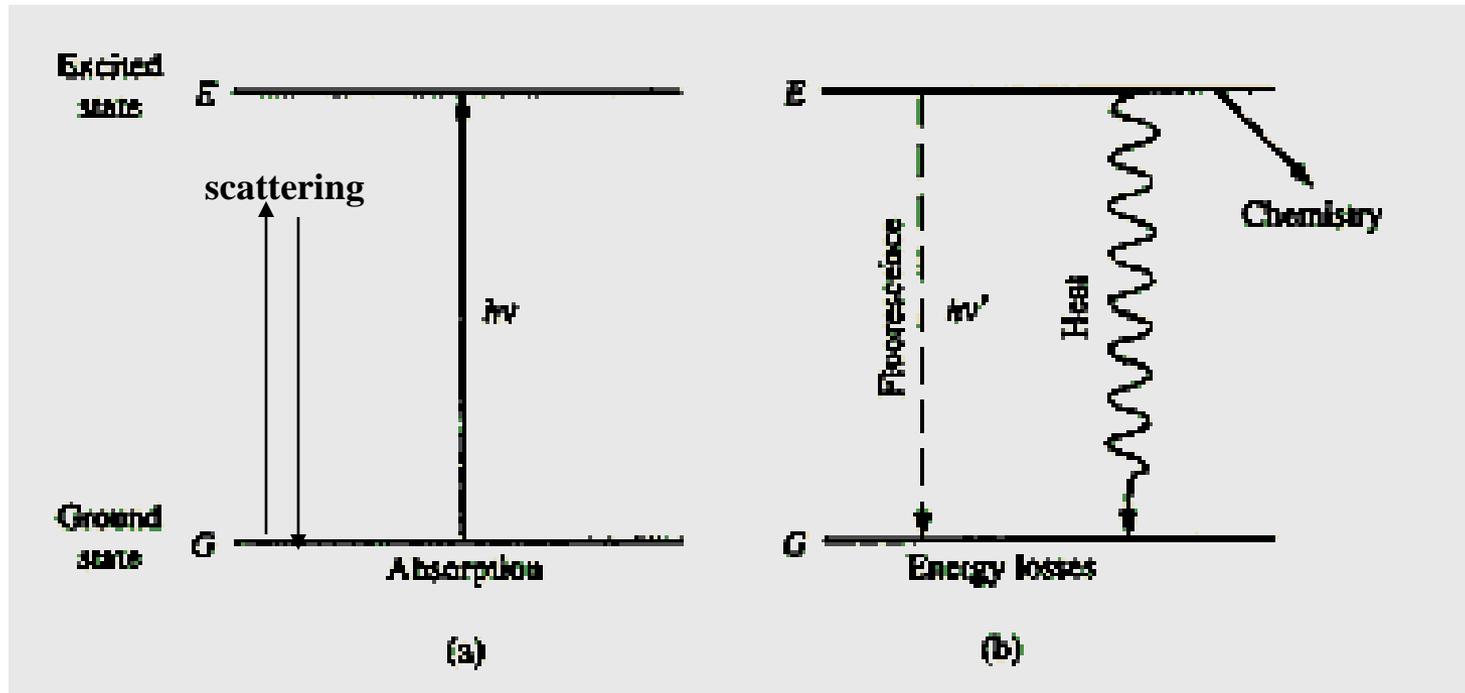
- X-Ray or NMR

$\lambda \sim 0.1 \text{ nm}$



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**:

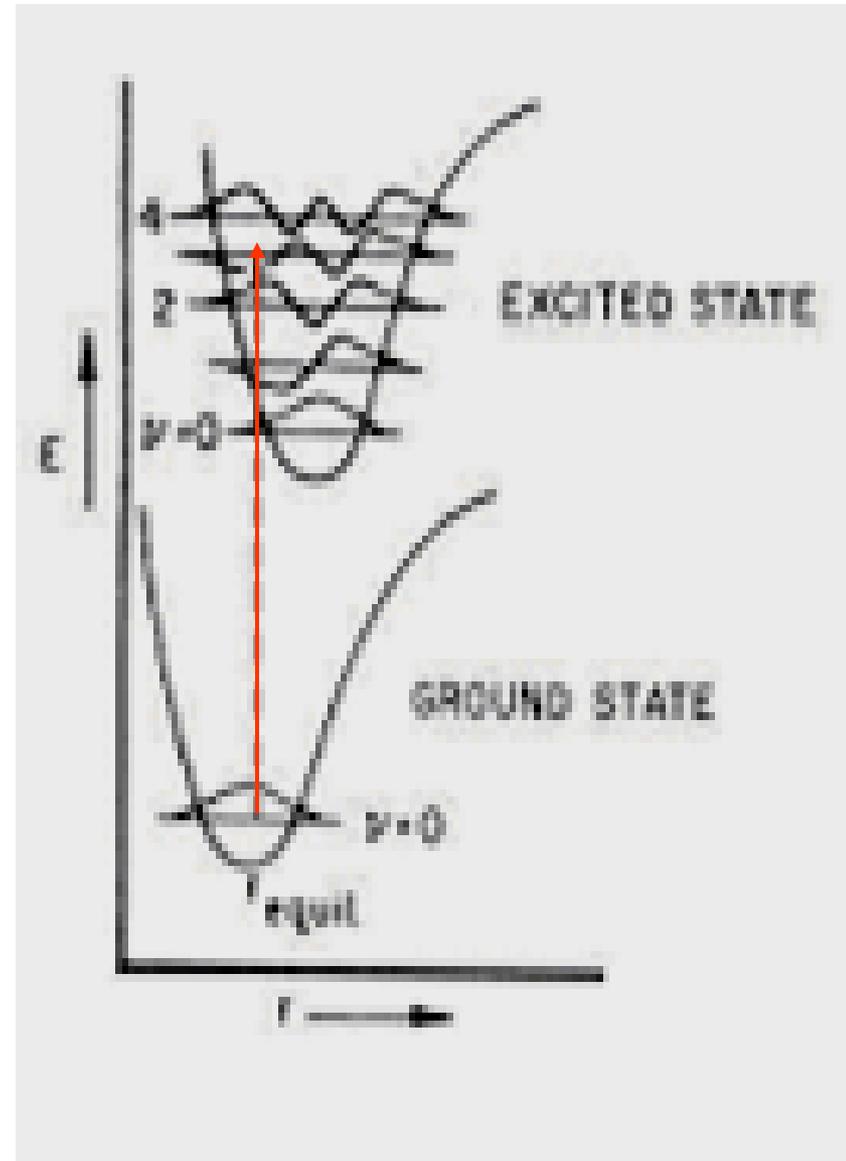
The **absorption usually occurs in the time it takes one wavelength to pass the molecule.**

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

$$3 \times 10^2 \text{ nm sec} / 3 \times 10^{17} \text{ nm} = \mathbf{10^{-15} \text{ sec}}$$

# 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**”.

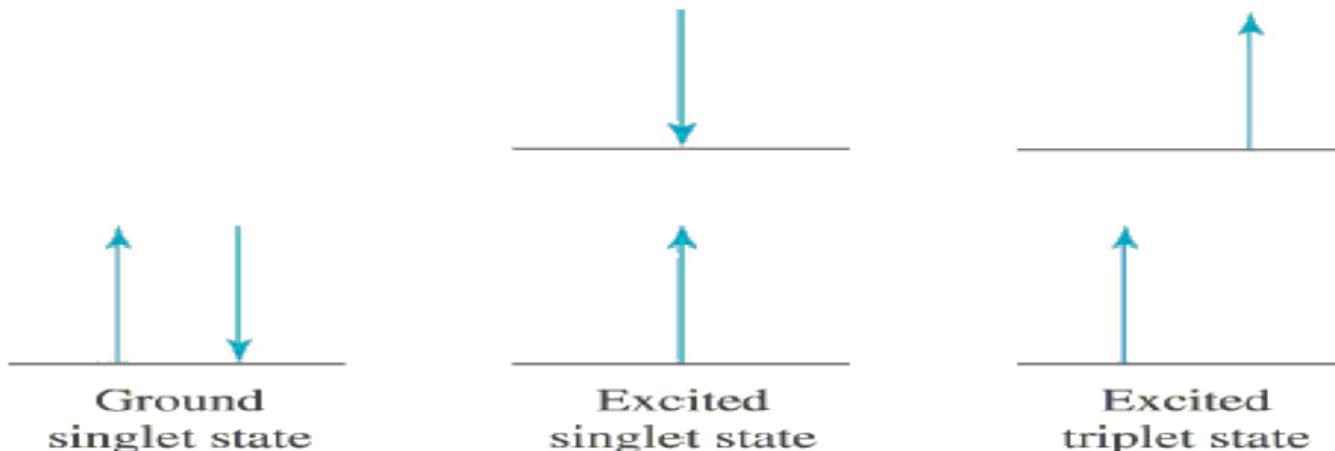


# Electronic transitions

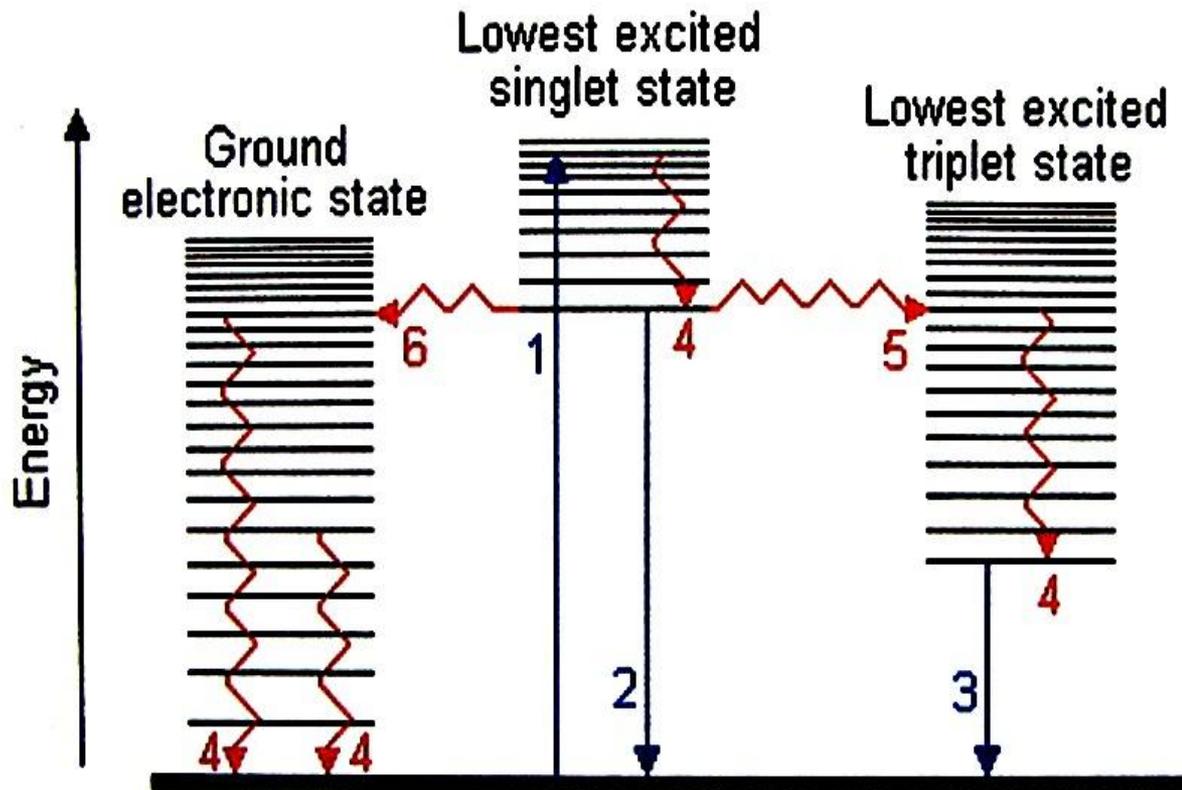
- **Selection rules:** allow **S**→**S**, and **T**→**T** processes but **not S**→**T** and **T**→**S**. Ground states are usually singlets; thus most excitations are to singlet excited states, like  $S_0 \rightarrow S_1$ ,  $S_0 \rightarrow S_2$ , ...
- **Triplet states are usually formed by intersystem 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:**



## 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

—→ Processes involving photons

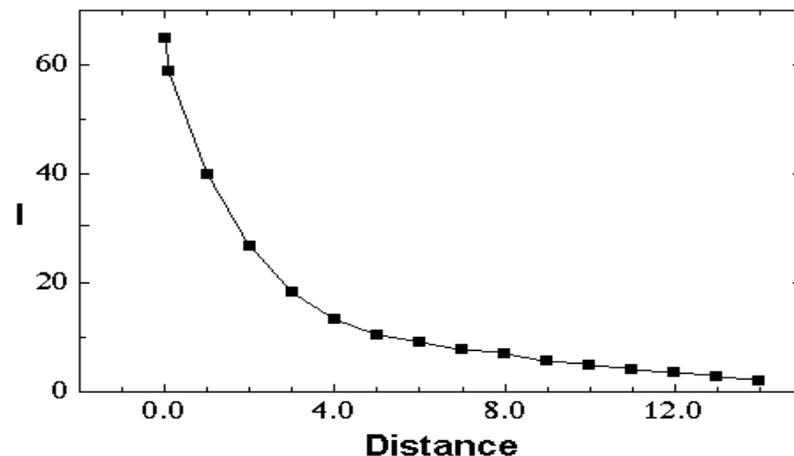
∩→ Radiationless transitions

# 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,

$\alpha$  = a constant

$c$  = concentration. Integrate to get:

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

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

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

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

Units on  $\epsilon$ :  $M^{-1} \text{ cm}^{-1}$

where **A** is "**absorbance**" or "**optical density**" and  $\epsilon$  is the "**molar absorptivity**" or "**molar extinction coefficient**" and  $\epsilon = \alpha / 2.303$

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### Absorption Spectrum – “fingerprint”

Beer-Lambert Law: Intensity ( $I$ ,  $I_0$ ); Transmittance ( $T = I / I_0$ )

$$\text{Absorbance (A): } A = \log (I_0 / I) = \log (1/T) = -\log(T)$$

Extinction Coefficient –  $E$  (1%),  $\epsilon(M)$  = Molar extinction coeff.

$$A = \text{O.D.} = \epsilon \cdot c \cdot l \quad \text{also} \quad [ E1\% \cdot \text{MW} = 10 \cdot \epsilon_M ]$$

Proteins:  $A_{280}$  ;  $E$  (1%)  $\sim 10$  (or O.D. of 1 for 1 mg/mL)

Nucleic Acids:  $A_{260}$  ;  $E$  (1%)  $\sim 200$  (or O.D. of 1 for 50 microg/mL)

### Environmental Effects

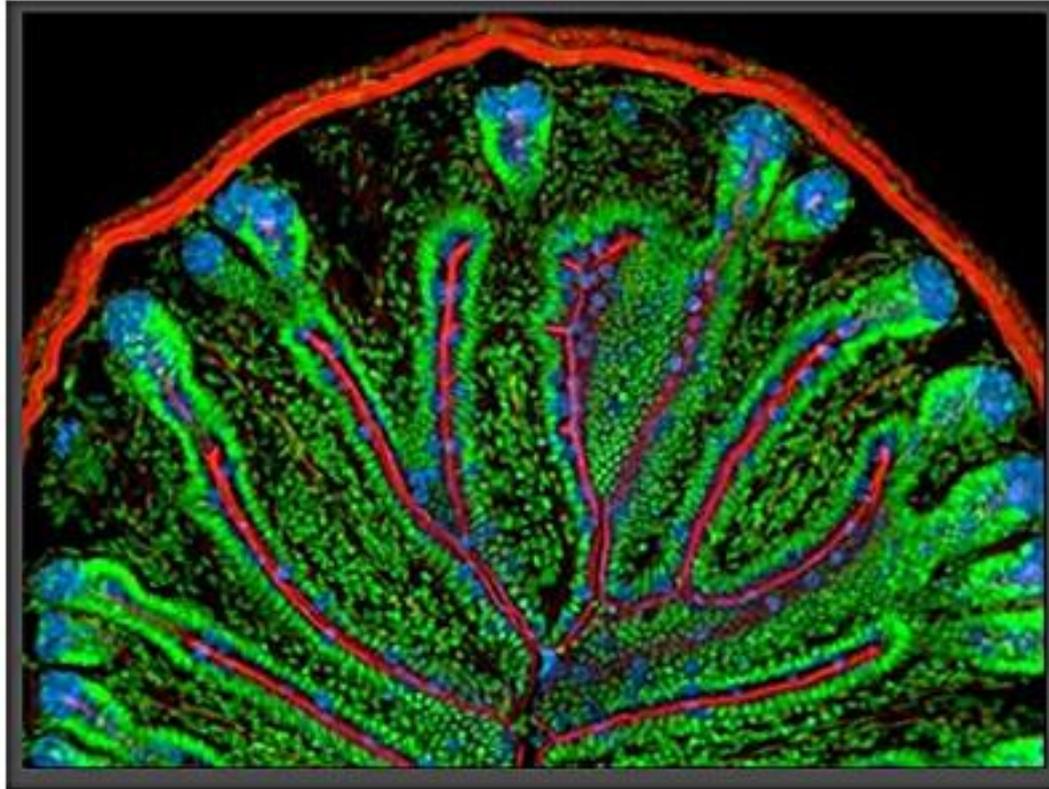
$I_{\text{nonpolar}} > I_{\text{polar}}$  (folding / unfolding effect)

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



# Definition of Fluorescence

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Goblet Cells and Secretions

Actin Cytoskeleton

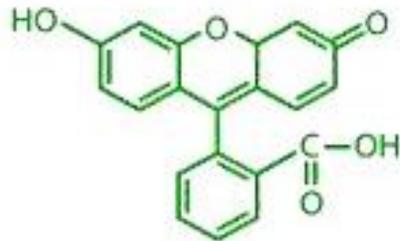
Nuclei

Mouse intestine

**Fluorescence usually occurs with rigid, conjugated polyaromatic hydrocarbons or heterocycles.**

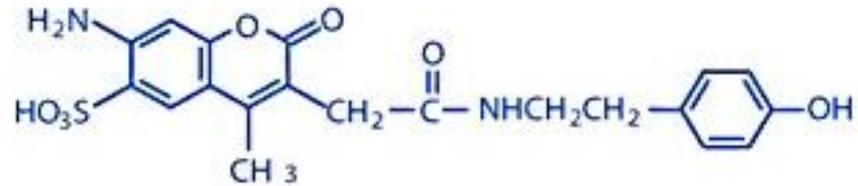


**Ethidium bromide**



**Fluorescein**

**Alexa Fluor 350**



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

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# Fluorescence

When 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

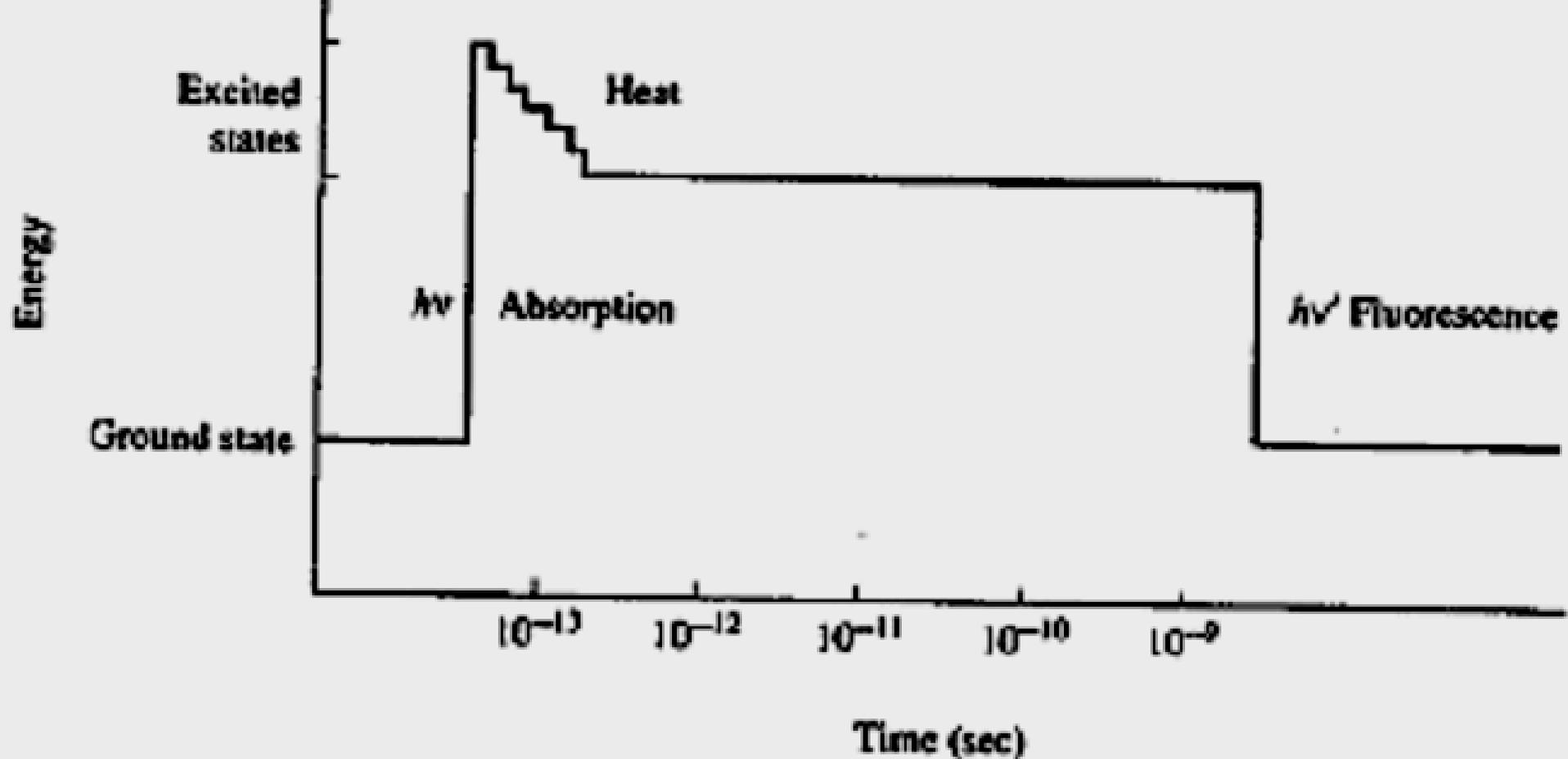
**vibrational relaxation:**  $10^{-11}$ - $10^{-10}$  s

**internal conversion:**  $10^{-12}$  s

luminescence processes

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

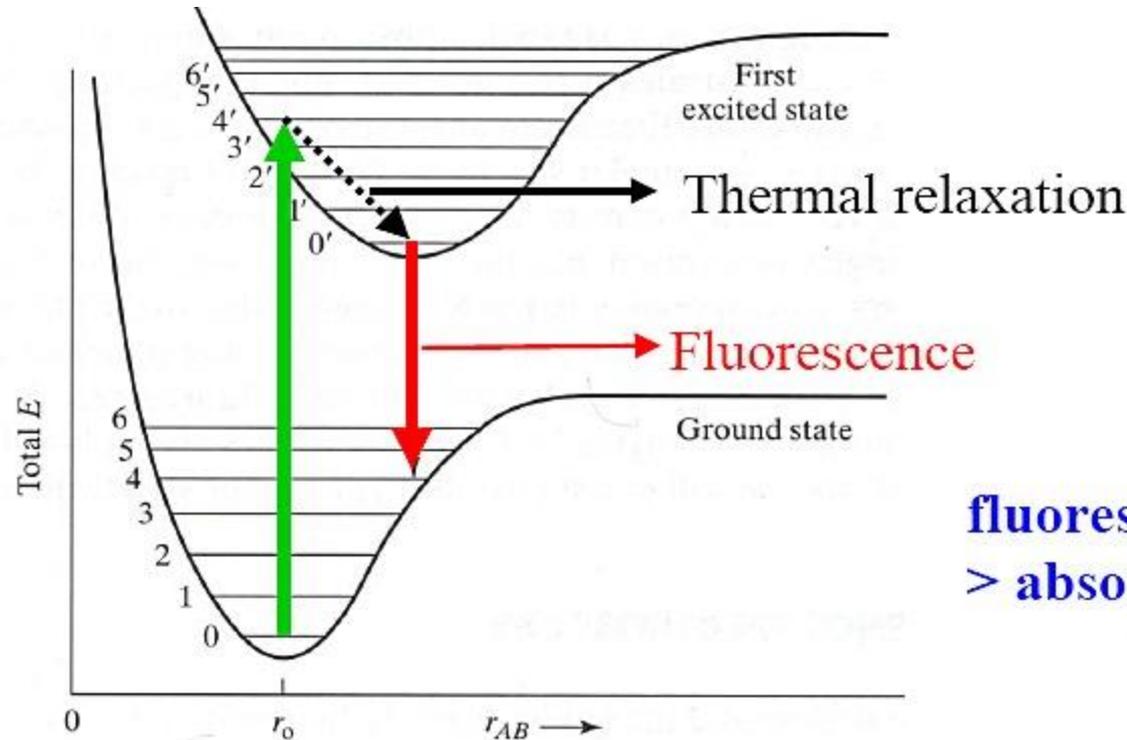
**phosphorescence:**  $10^4$ - $10^{-4}$  s



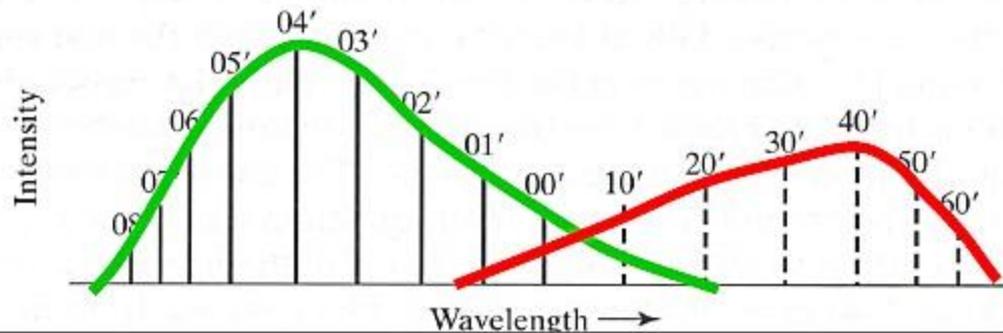
The molecule can relax from the ground vibrational state of the excited electronic state by fluorescence.

**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.

# Fluorescence spectra of proteins



**fluorescence wavelength  
> absorption wavelength**



# Fluorescence Measurements

Instrument / Advantages

## Fluorescence parameters / applications

Average fluorescence wavelength (shifts)

Fluorescence Intensity - quantum yield  $Q = F / A$

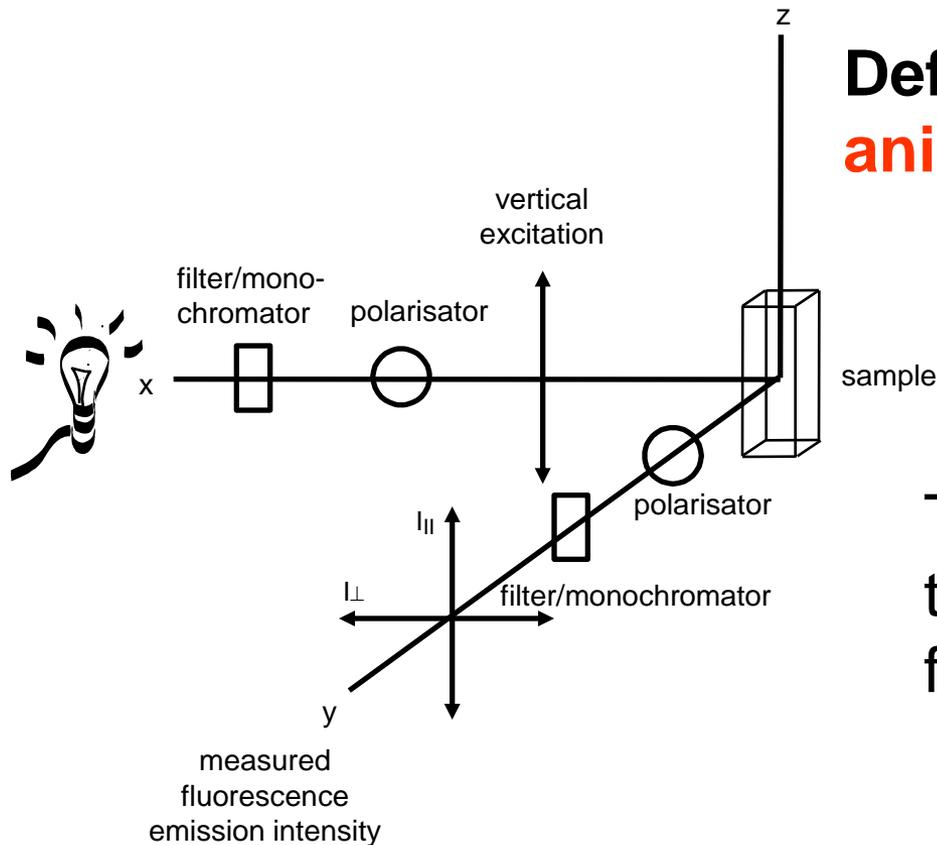
Fluorescence Lifetime ( $T$ ): average value of the **time** a fluorophore spends **in the excited state** before it returns to the ground state.  $N$  to  $N/e$   $F = F_0 e^{-t/T}$

Fluorescence polarization anisotropy  $r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}}$

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FRET

# Fluorescence Anisotropy



Definition of **fluorescence anisotropy**  $r$

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}}$$

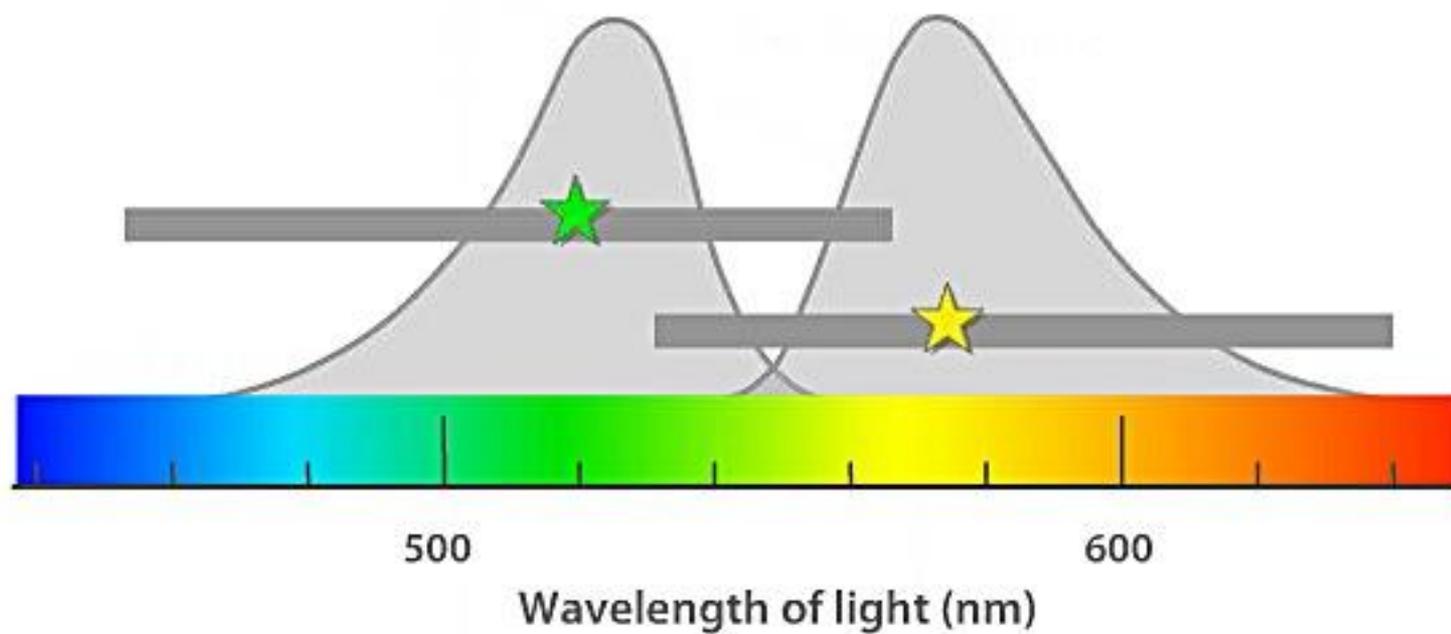
The **anisotropy**  $r$  reflects the rotational diffusion of a fluorescent species

# Summary

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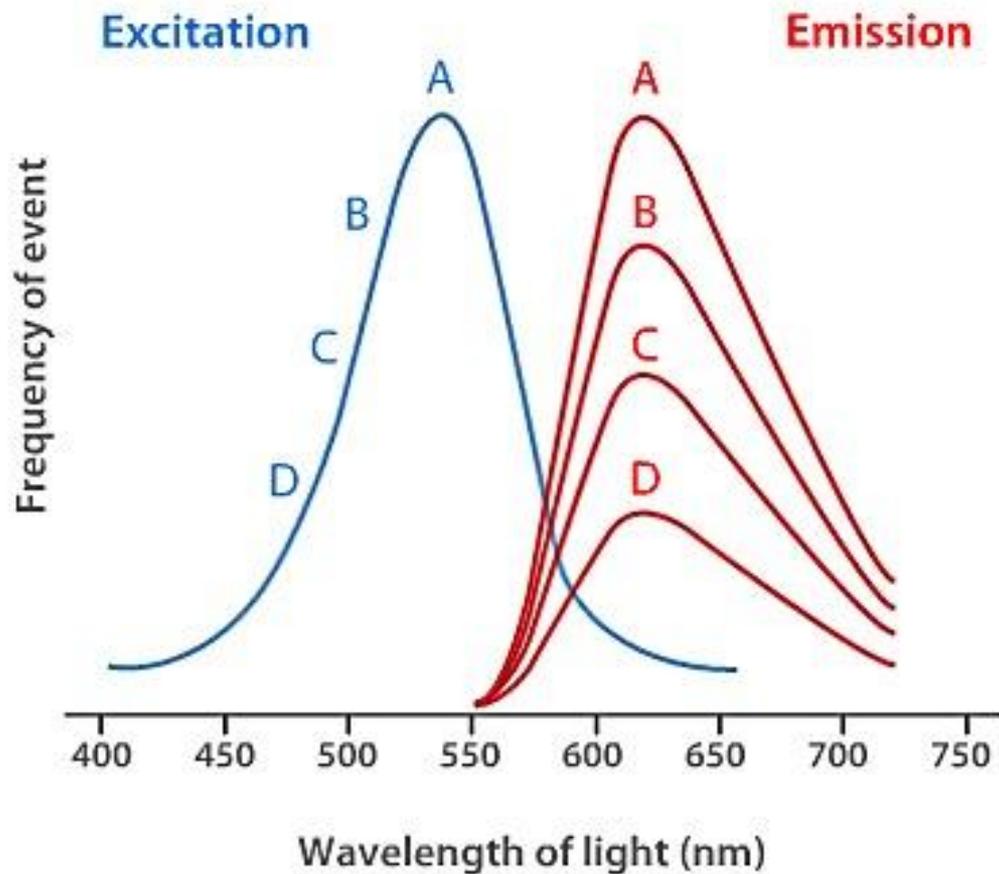
Excitation maximum

Emission maximum



# Fluorescence Emission

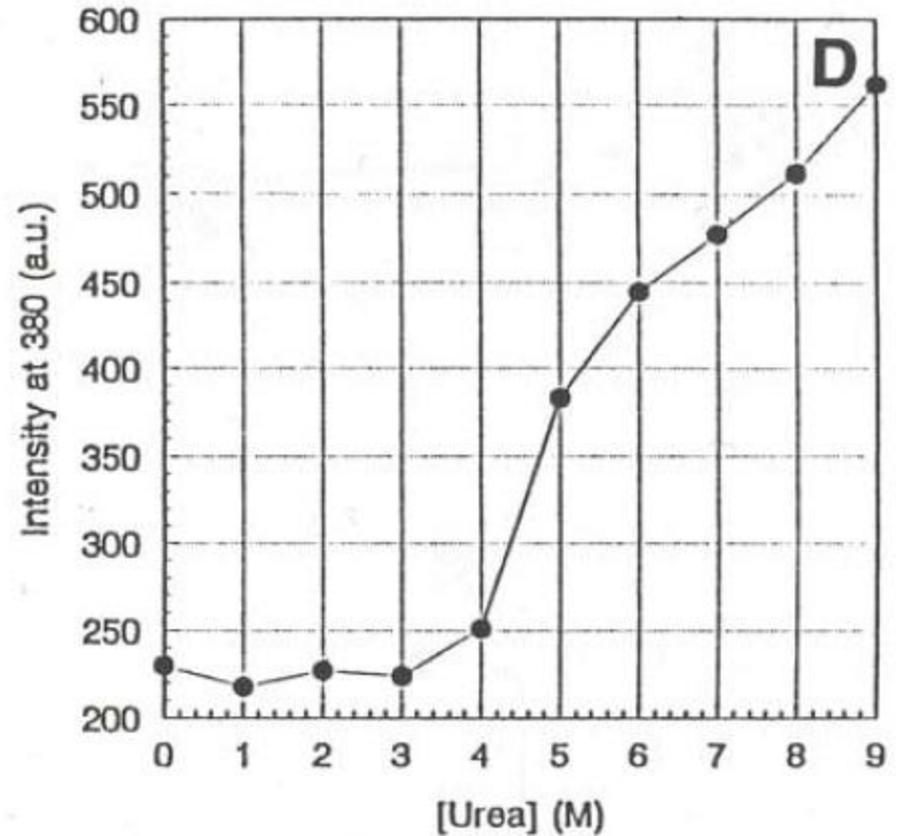
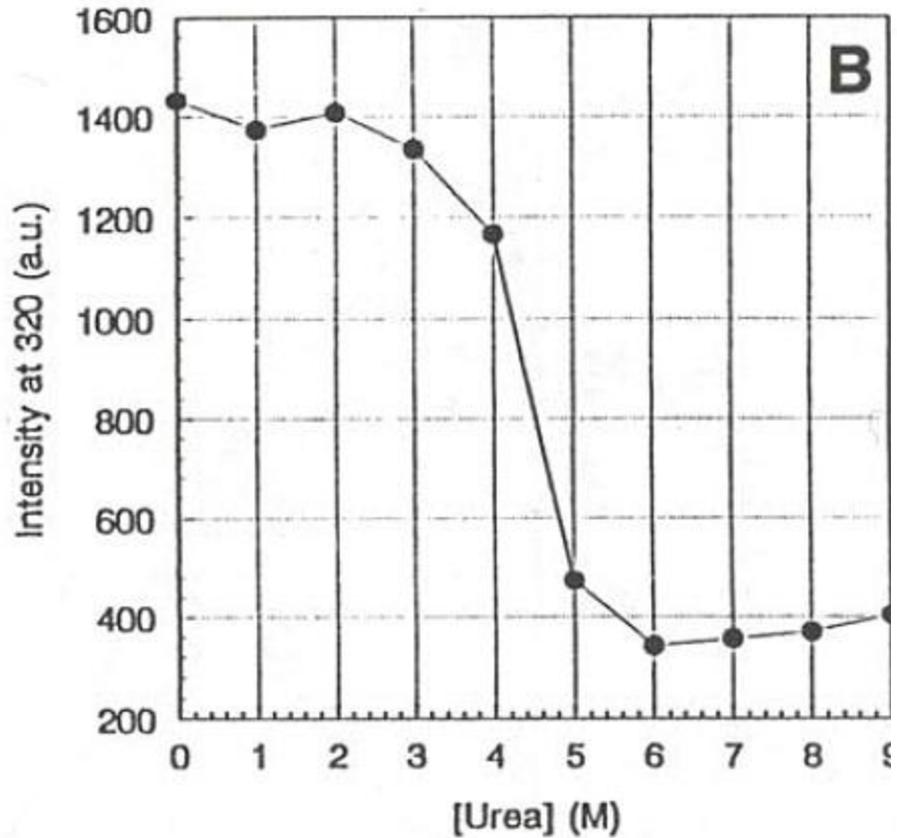
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Illumination at lower or higher wavelengths affects only the intensity of the emitted light

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# Application: Tryptophan Fluorescence and Protein Folding



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

# Excitation Transfer

**F**luorescence **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**.

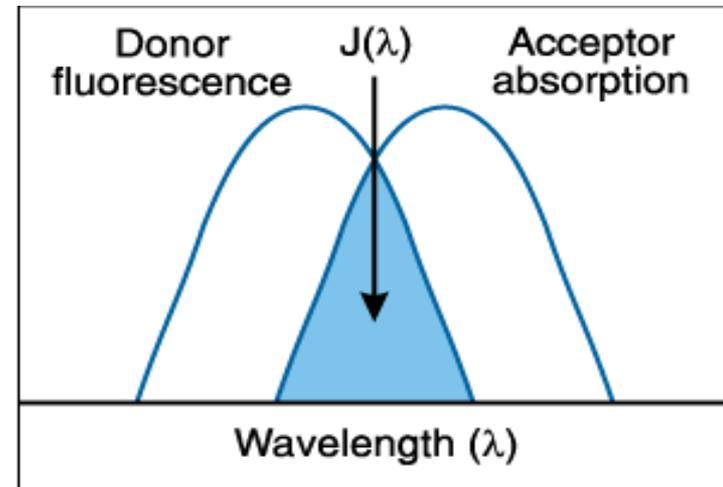
Consider an excited donor,  $D^*$  and an acceptor that can be excited to a fluorescent state,  $D^*$ .



## Primary Conditions for FRET

- Donor and acceptor molecules must be close ( $\sim 10\text{--}100 \text{ \AA}$ ).
- Absorption spectrum of acceptor must overlap the fluorescence emission spectrum of the donor (**spectral overlap**).
- Donor and acceptor transition dipoles must be  $\sim$  parallel.

FRET spectral overlap.



## Experimental Measurements

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

# Förster Radius, $R_0$

The distance at which energy transfer is 50% efficient is defined by the Förster radius ( $R_0$ ). The magnitude of  $R_0$  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.

$$R_0 = [8.8 \times 10^{-23} \cdot \kappa^2 \cdot n^{-4} \cdot \phi_d \cdot J(\lambda)]^{1/6} \text{ \AA}$$

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

$\phi_d$  = fluorescence quantum yield of the donor in the absence of the acceptor

$n$  = refractive index of the medium

$J(\lambda)$  = spectral overlap integral

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

where  $\epsilon_A$  = extinction coefficient of acceptor

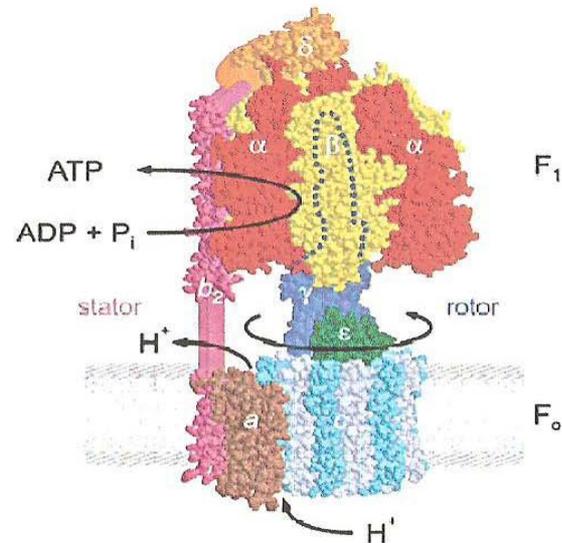
$F_D$  = fluorescence emission intensity of donor

as a fraction of the total integrated intensity

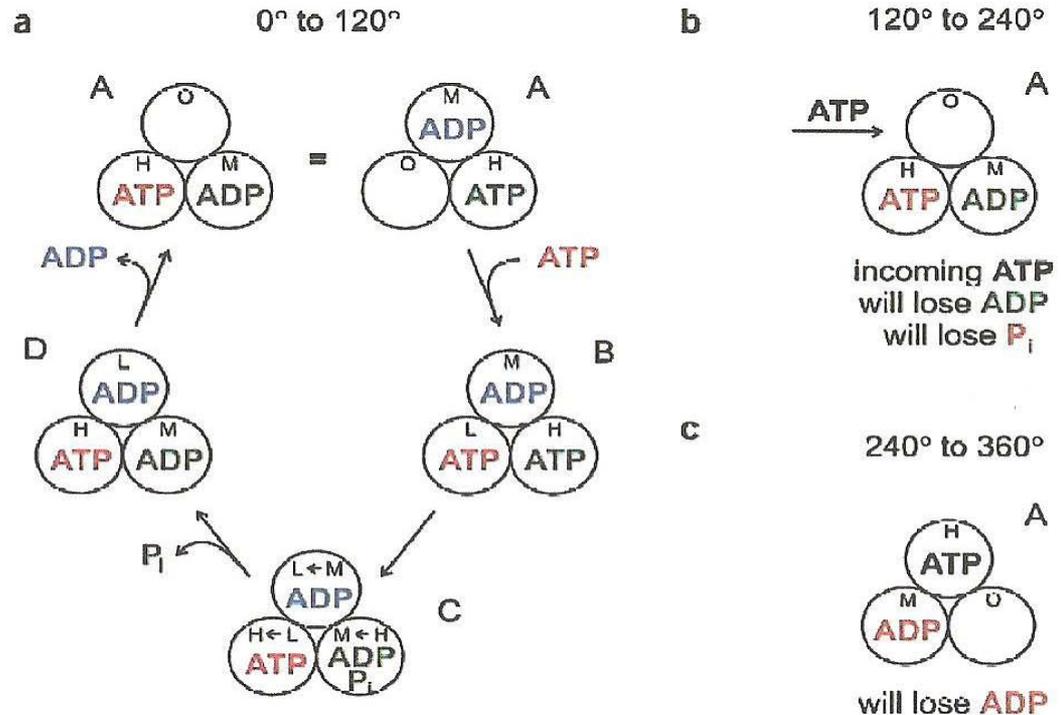
**Table.** Typical Values of  $R_0$ .

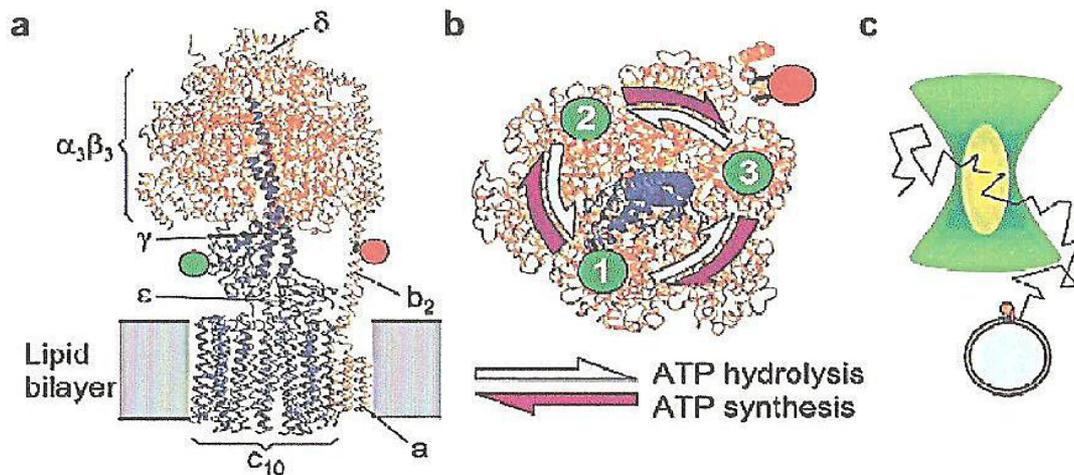
<b>Donor</b>	<b>Acceptor</b>	<b><math>R_0</math> (Å)</b>
Fluorescein	Tetramethylrhodamine	55
IAEDANS	Fluorescein	46
EDANS	Dabcyl	33
Fluorescein	Fluorescein	44
BODIPY FL	BODIPY FL	57
Fluorescein	QSY 7 and QSY 9 dyes	61

**Figure 1** Structure of ATP synthase. The enzyme from *E. coli*, with subunit stoichiometry  $\alpha_3\beta_3\gamma\delta\epsilon ab_2c_n$ . 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.

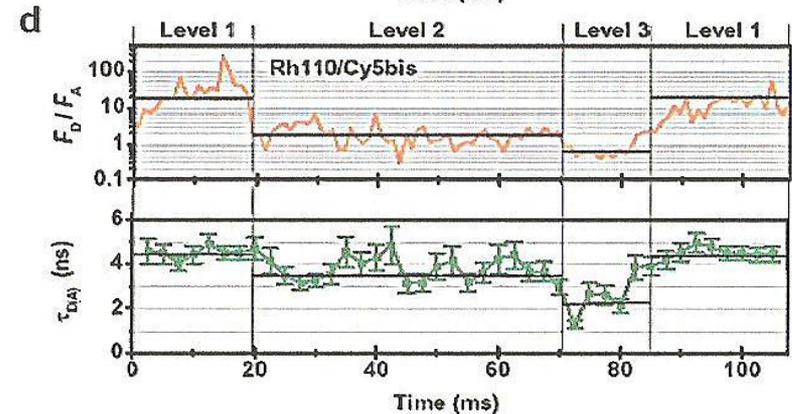
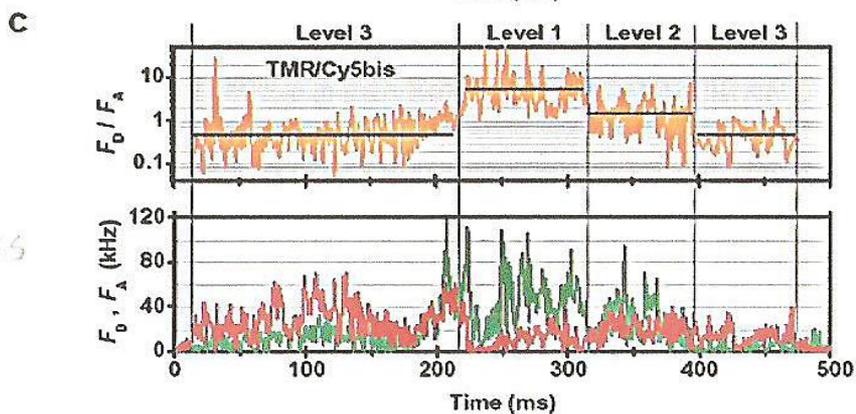
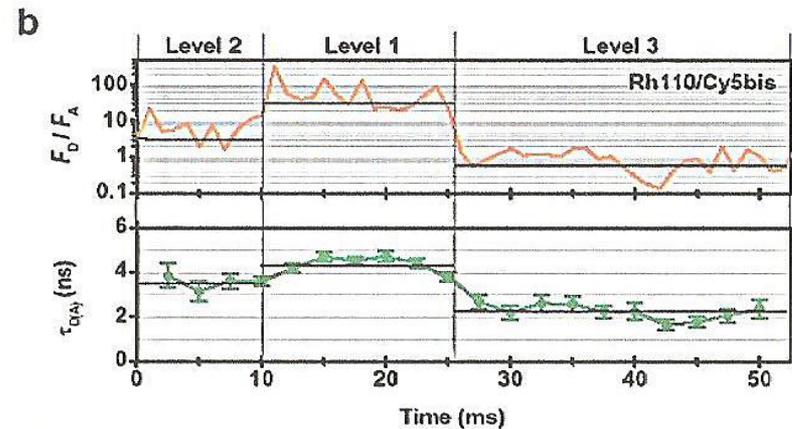
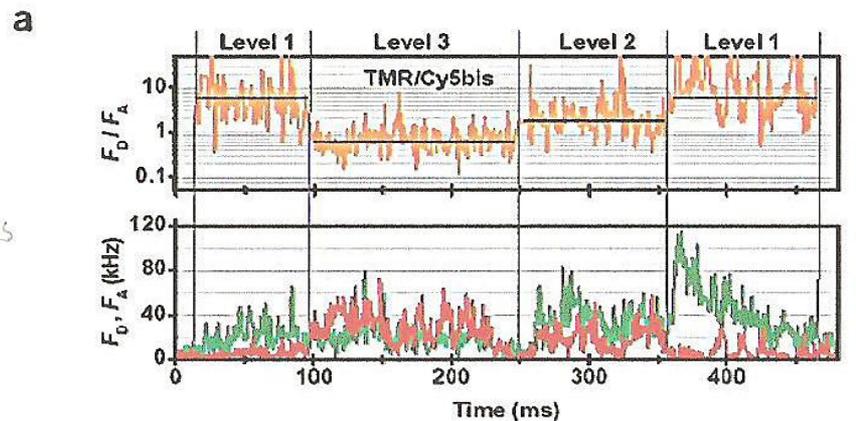


**Figure 2** Enzymatic mechanism of ATP hydrolysis by ATP synthase. (a) Each circle represents one of the three catalytic sites. O, open (unoccupied); L, lowest affinity for ATP; M, medium affinity; H, highest affinity. The series of enzyme states ABCDA describes what happens during consumption of one molecule of ATP and one 120° step of  $\gamma$  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,  $\gamma$  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  $\gamma$  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 rotation  $\gamma$ -T106C is farthest away from  $b$ -Q64C. Rotation of the  $\gamma$  subunit by  $120^\circ$  and  $240^\circ$  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).



# Review Questions: Sept. 21, 2010

3. What is the dependence between fluorescence transfer efficiency and distance between the donor and acceptor chromophores? \_\_\_\_\_
- A)  $r$     B)  $r^{**2}$     C)  $r^{**6}$     D)  $1/r^{**2}$     E)  $1/r^{**6}$
4. A pair of chromophores have a radial separation of 3.0 nm and an  $R_0 = 2.5$  nm. The best estimate for the distance between the two chromophores would be?
- \_\_\_\_\_
- A) 0.5 nm    B) 1.0 nm    C) 2.5 nm    D) 3.0 nm    E) Can not predict