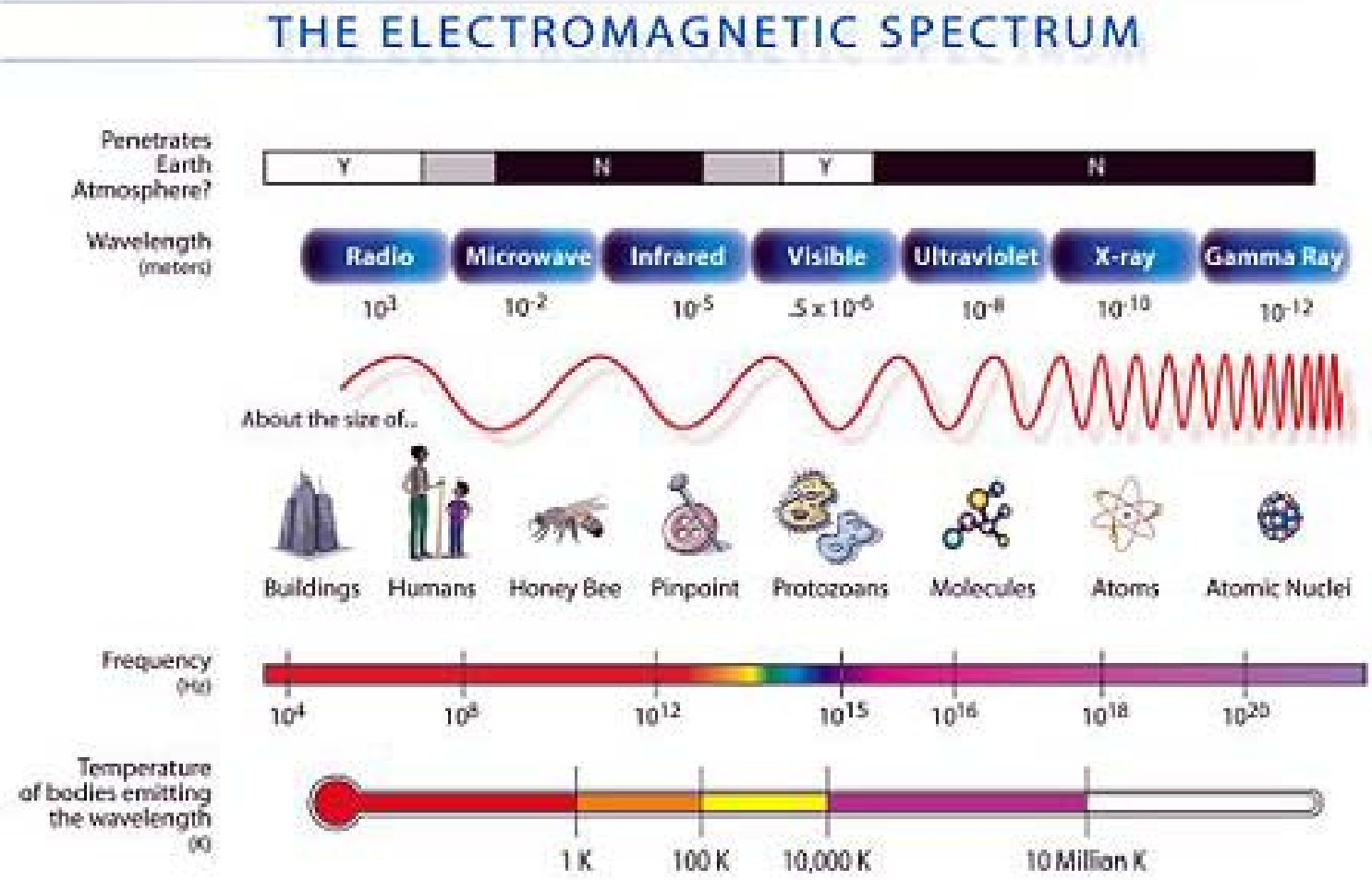
Lecture 15

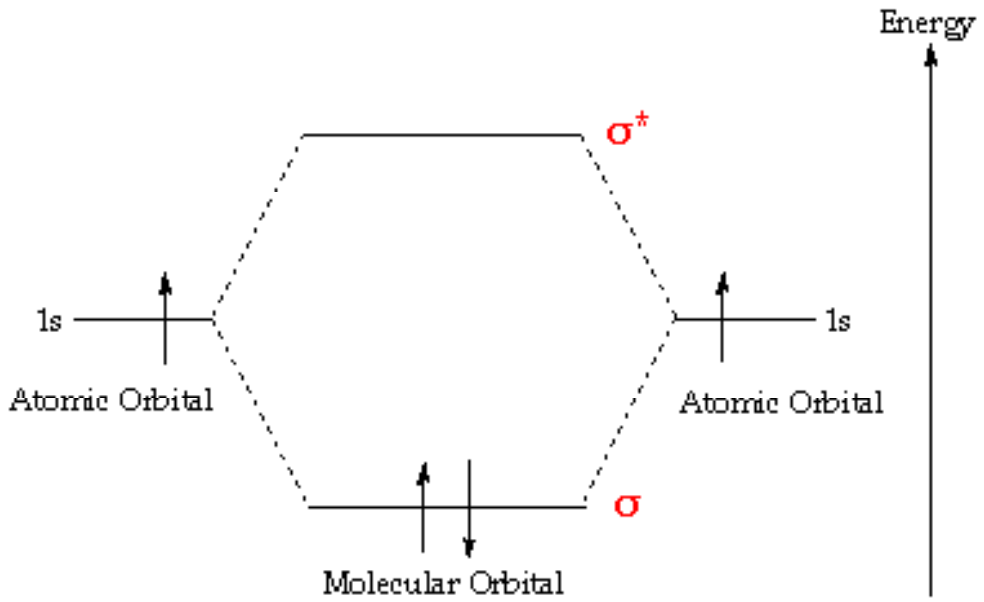
Absorption and Emission of Light by Molecules



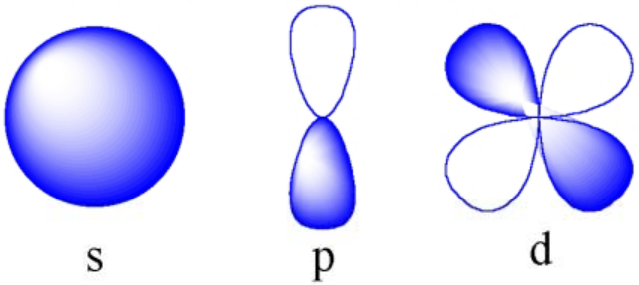
The energy of visible light (30-100 kcal/mol) is close to the energies required for chemical reactions in biomolecules – biomolecules absorb visible light. X-ray and γ (gamma) rays are ionizing radiation – break molecule apart. Lower - IR radiation – excite vibration and rotation in molecules (MW)

Molecular orbitals

- Atoms have electrons which occupy atomic orbitals with discrete energy levels
- When atoms form a molecule – atomic orbitals interact and form a molecular orbital, which also has a discrete energy levels

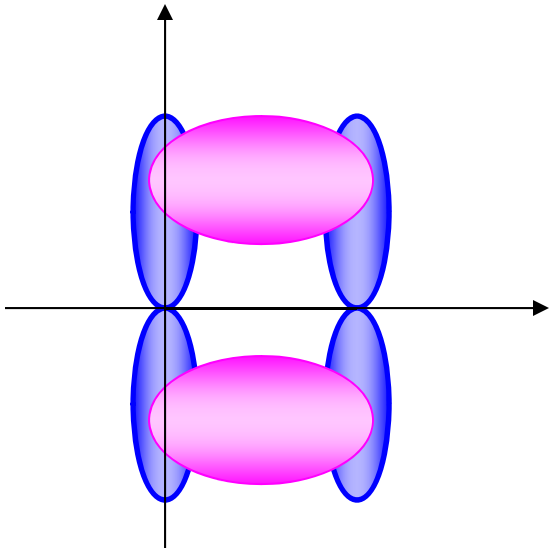


Atomic orbitals



These are the angular parts of the wavefunction. The radial part decays exponentially with distance from the nucleus of an atom.

Molecular orbital Pi (π)



Simple examples:

Molecular orbitals can be:

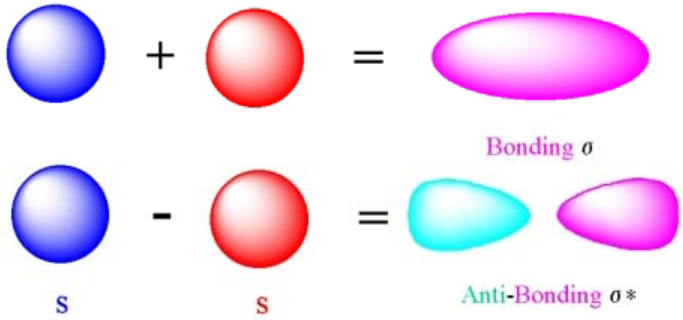
Sigma (σ) – single bond

Pi (π) – double or triple bond

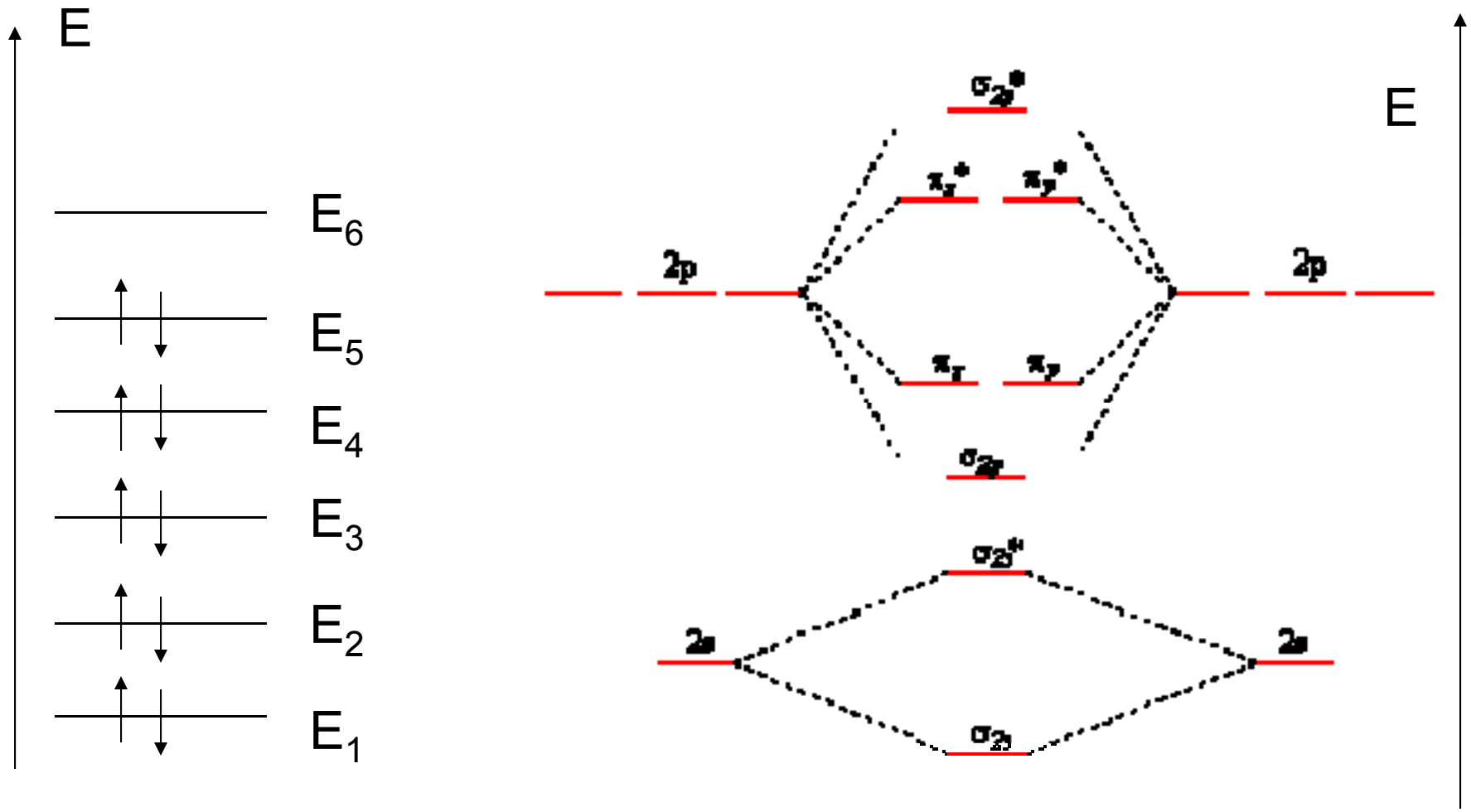
bonding and anti-bonding (*)

Molecular orbital Sigma (σ)

The molecular orbitals in a diatomic molecule are formed from linear combinations of atomic orbitals

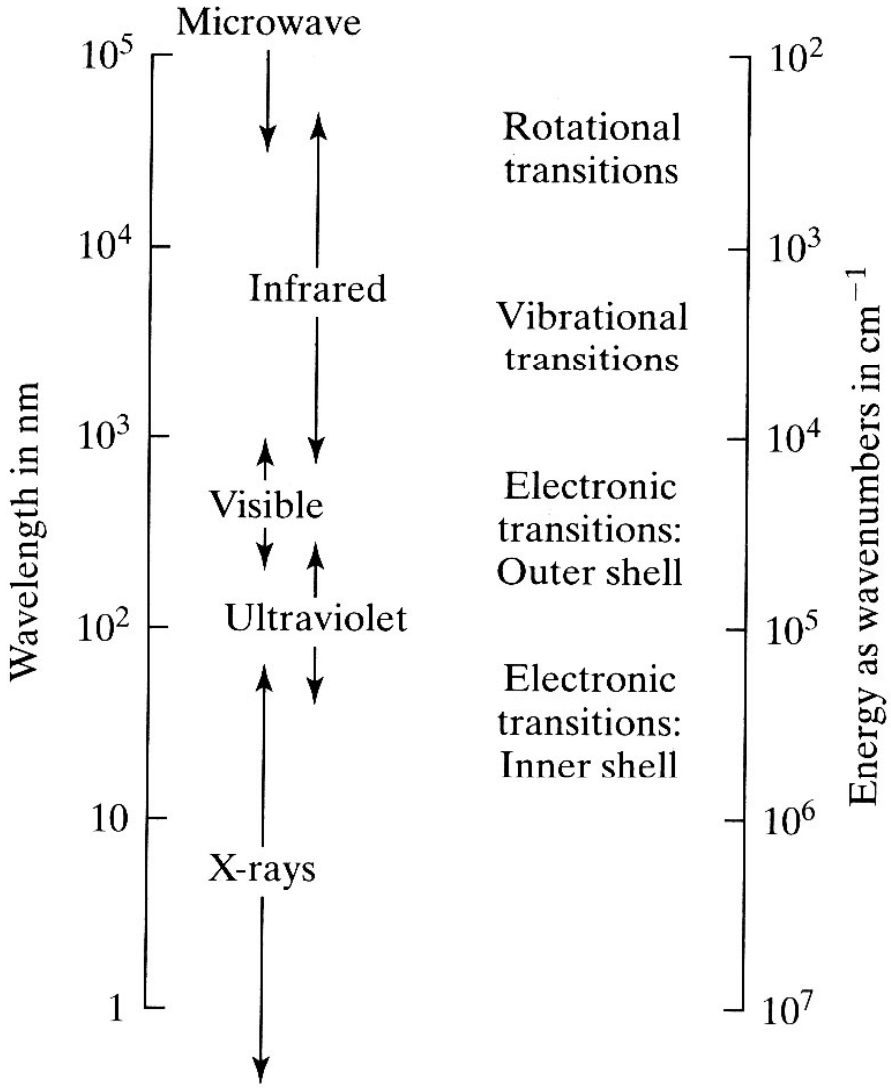


Discrete Energy levels in molecule

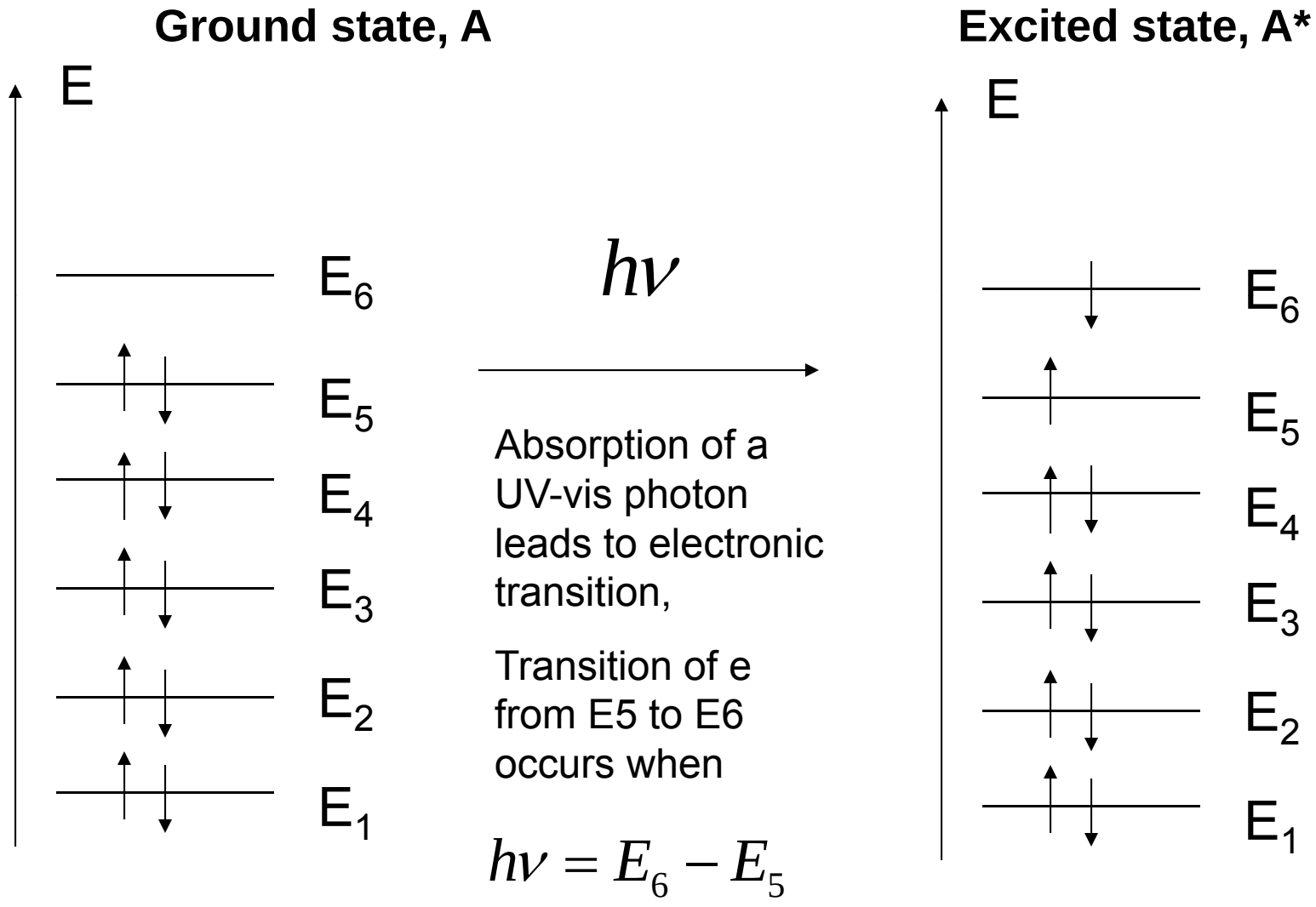


Absorption

- Absorption of a electromagnetic radiation raises the molecule from ground state to an excited state
- Total energy is the sum of all components (electronic, vibrational, rotational, translations, spin orientation energies) (vibrational energies are quite small)



Absorption of UV-Visible light



- **Electronic Energy levels and Electronic energy states**

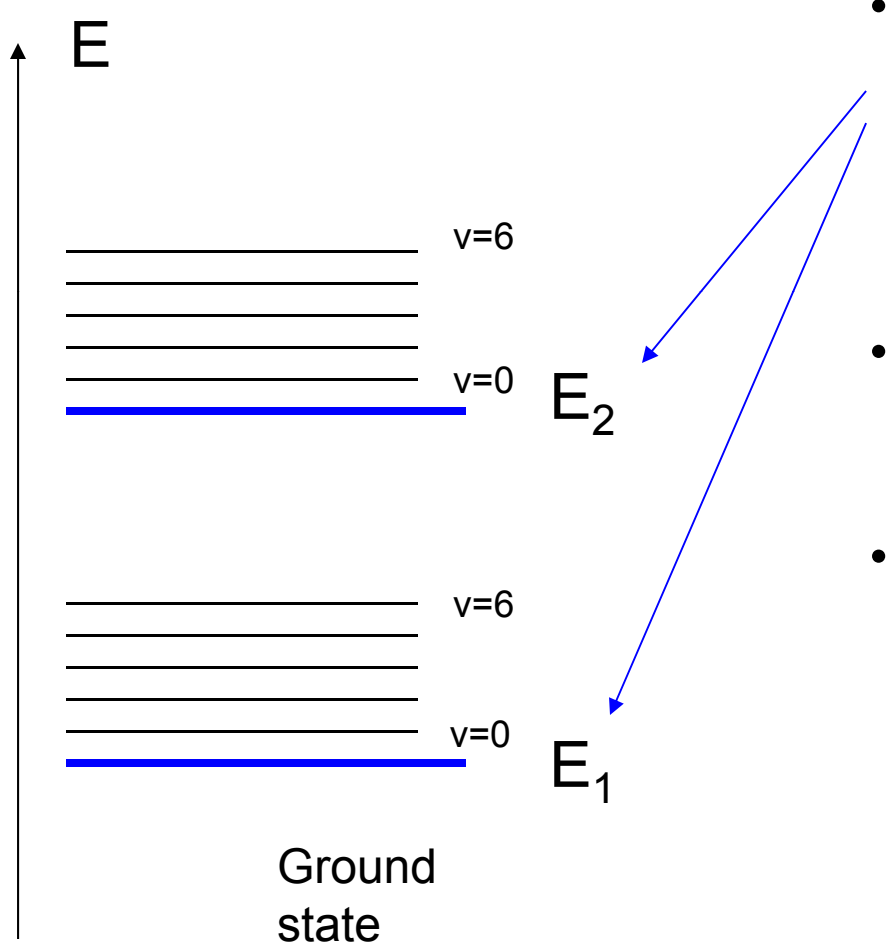
- Electronic energy levels : E_1 to E_5 …specify the energy levels of a particular electron

- Electronic energy state refers to a total electronic energy of the molecule = the sum of all the energies of individual electrons present

- Ground state – where all electrons in the lowest energy levels possible

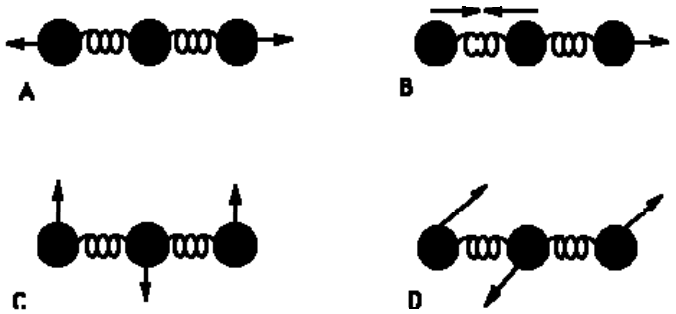
- When one electron moved to the higher level – the total energy becomes higher – the molecule is in the excited state – can more easily participate in reactions

Absorption of Infrared Radiation



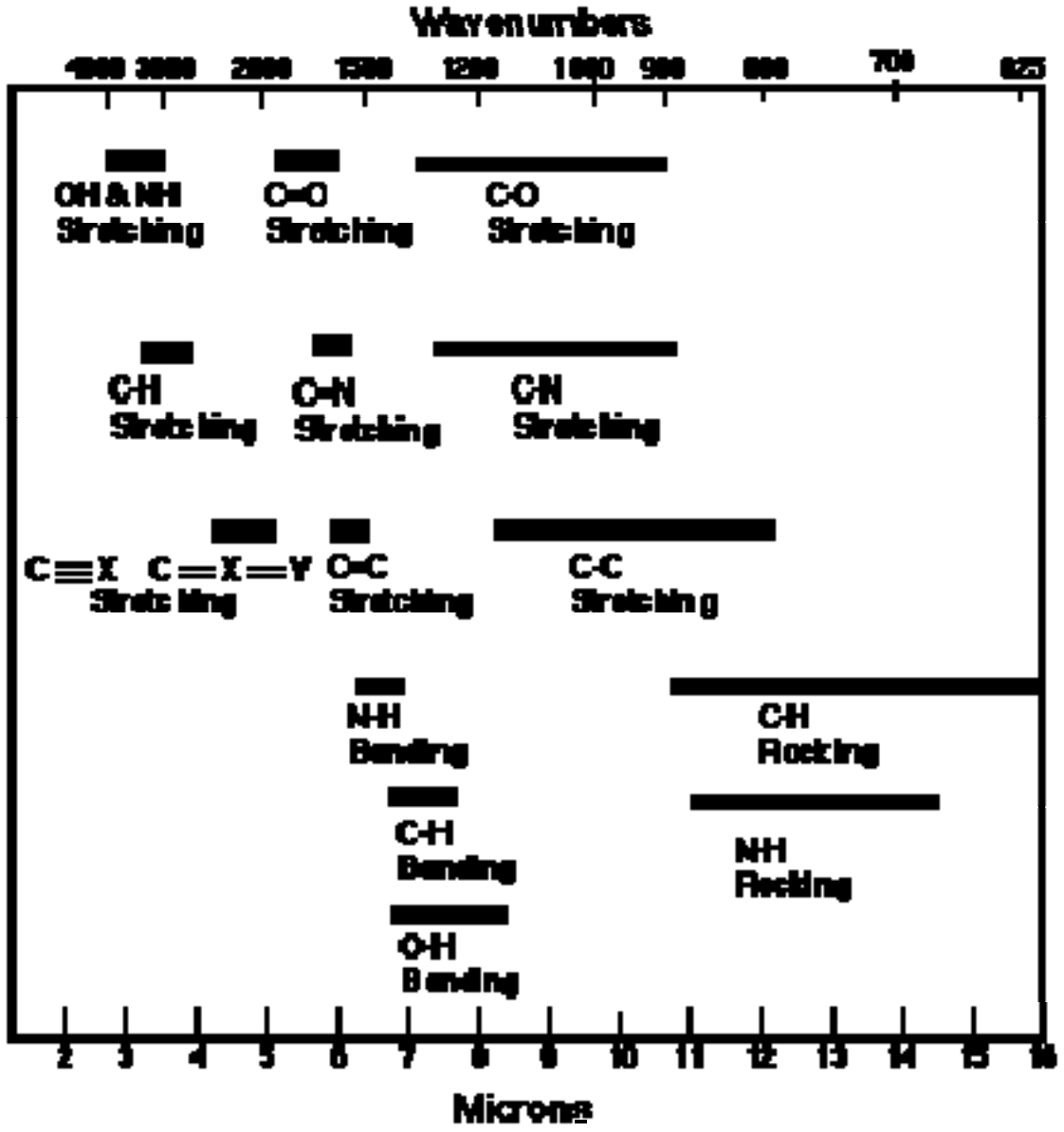
- UV-visible light causes electron transfer from one electron energy level to another

- IR – transition between different vibrational energy levels v=0,1,2,3,4,5,6
- This causes vibration of molecule



IR spectroscopy

- Vibration energy is a specific characteristic of the bond. This is used in IR spectroscopy
- Characteristic IR absorption bands of some chemical bonds



Characteristic Infrared Bands of the Peptide Linkage

Designation	Approximate frequency (cm^{-1})	Description
A	~3300	NH stretching in resonance with (2 × amide II) overtone
B	~3100	
I	1600–1690	C=O stretching
II	1480–1575	CN stretching, NH bending
III	1229–1301	CN stretching, NH bending
IV	625–767	OCN bending, mixed with other modes
V	640–800	Out-of-plane NH bending
VI	537–606	Out-of-plane C=O bending
VII	~200	Skeletal torsion

From H. Susi, *Methods Enzymol.* 26:455–472 (1972).

Luminescence

- Luminescence is the emission of light by a substance. It occurs when an electron
- returns to the electronic ground state from an excited state and loses it's excess energy as a photon.

- Luminescence is a collective name given to three related emission types:

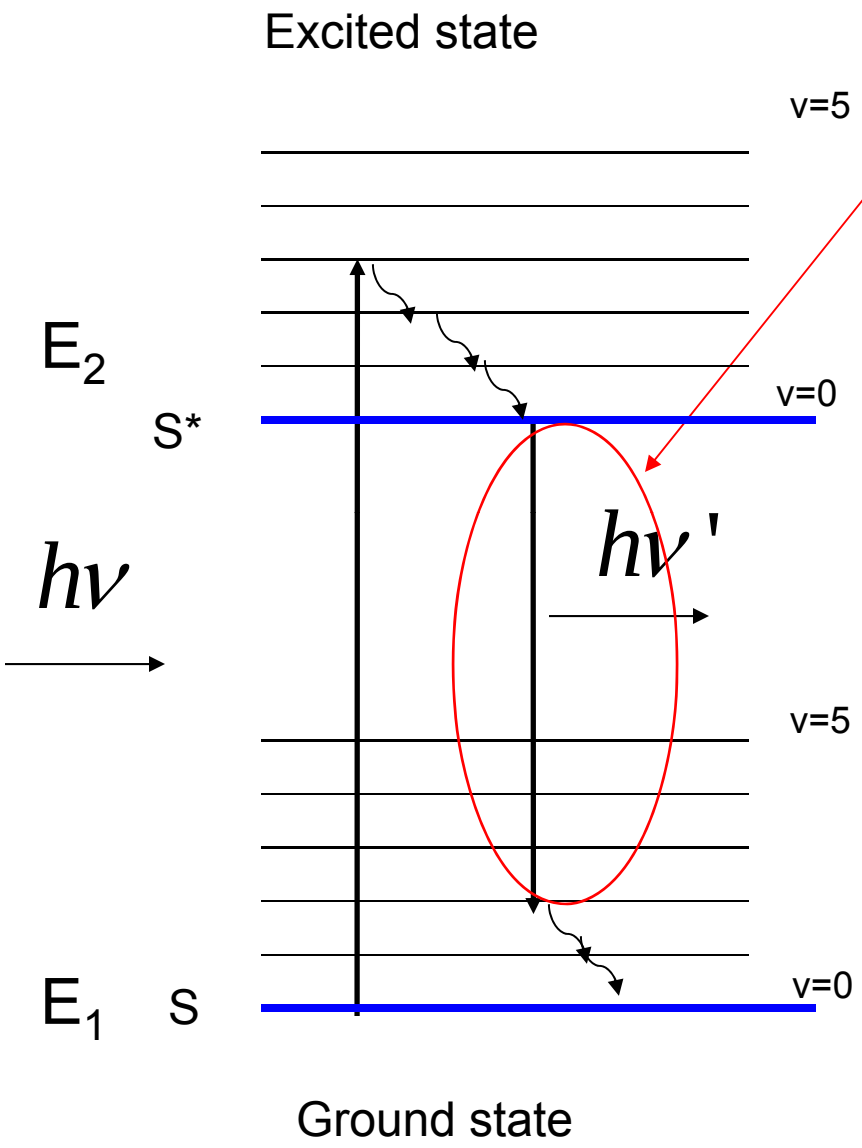
- •fluorescence
- •phosphorescence
- •chemiluminescence

Fluorescence

- Fluorescence occurs when the molecule returns to the *electronic* ground state, from *electronic* excited singlet state, by emission of a photon.

- If a molecule which absorbs UV radiation does not fluoresce it means that it must have lost its energy some other way.

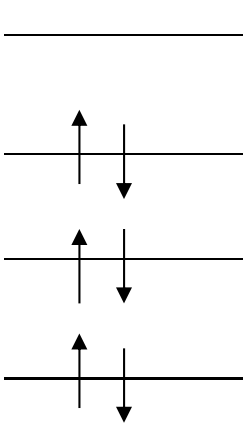
Fluorescence



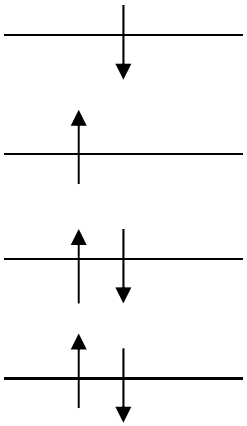
When the molecule absorbs light the electron moves to the next electronic level (excited state) and also to higher vibrational levels (hot), the molecules is hot and excited, if vibrational level =0 molecule is cold and excited,

The molecule collides with other molecules (solvent) and goes to the lowest vibrational level – **vibrational relaxation**. Next, molecule returns to the ground electronic state by radiating photon – **fluorescence,** it may be still not the lowest vibrational level, the molecule then undergoes additional vibrational relaxation and returns to the ground state with original energy

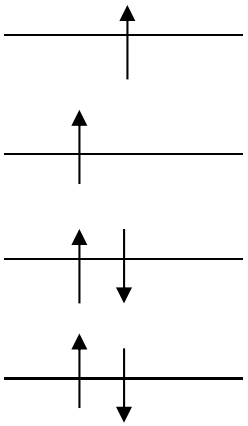
Singlet and triplet states



Ground state
singlet



First excited
state singlet

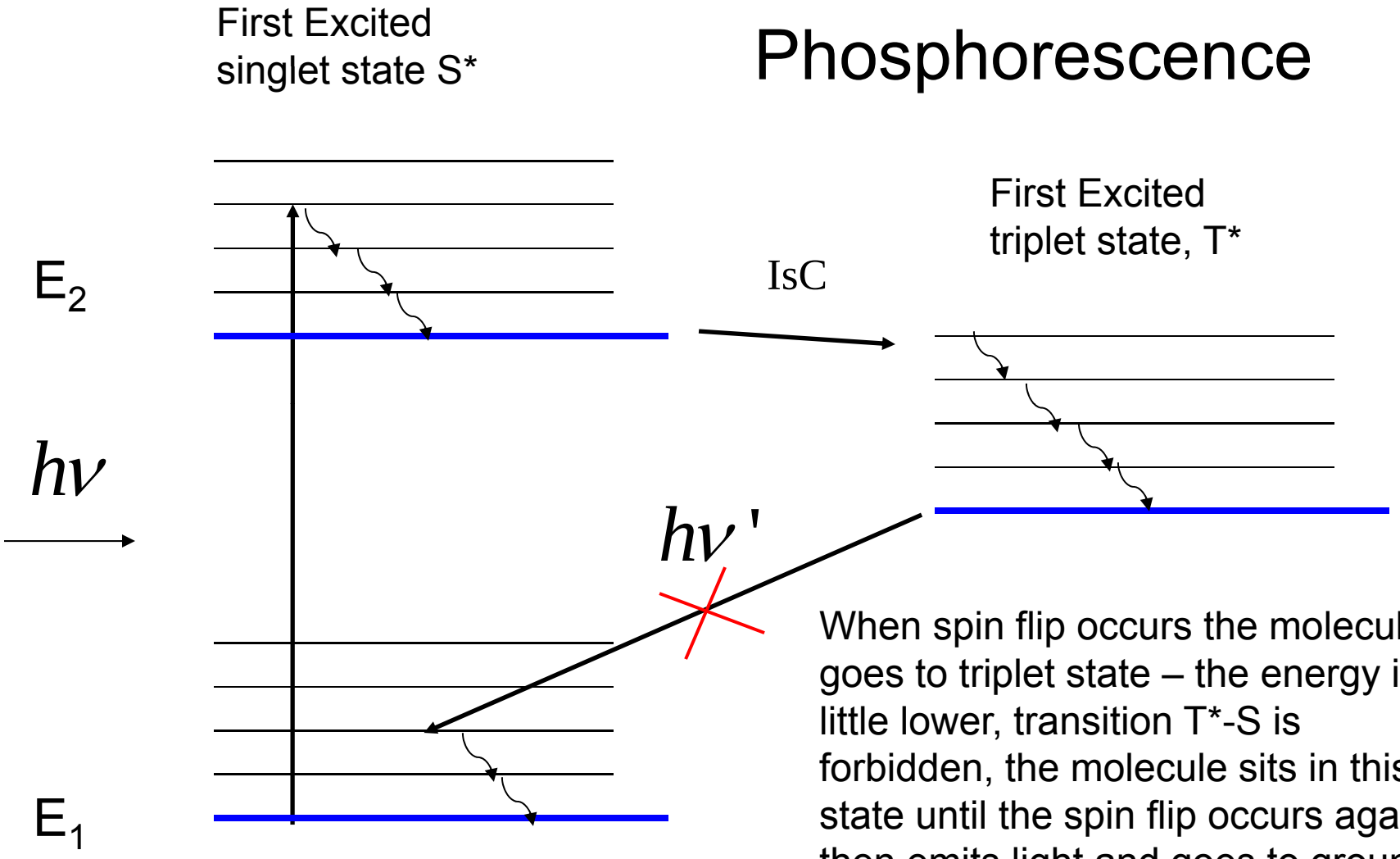


First excited
state triplet

Multiplicity M=2S+1,

transitions between states with different multiplicity are forbidden

Phosphorescence



When spin flip occurs the molecule goes to triplet state – the energy is a little lower, transition T*-S is forbidden, the molecule sits in this T* state until the spin flip occurs again, then emits light and goes to ground state. Transition T*-S with light emission is called **phosphorescence**

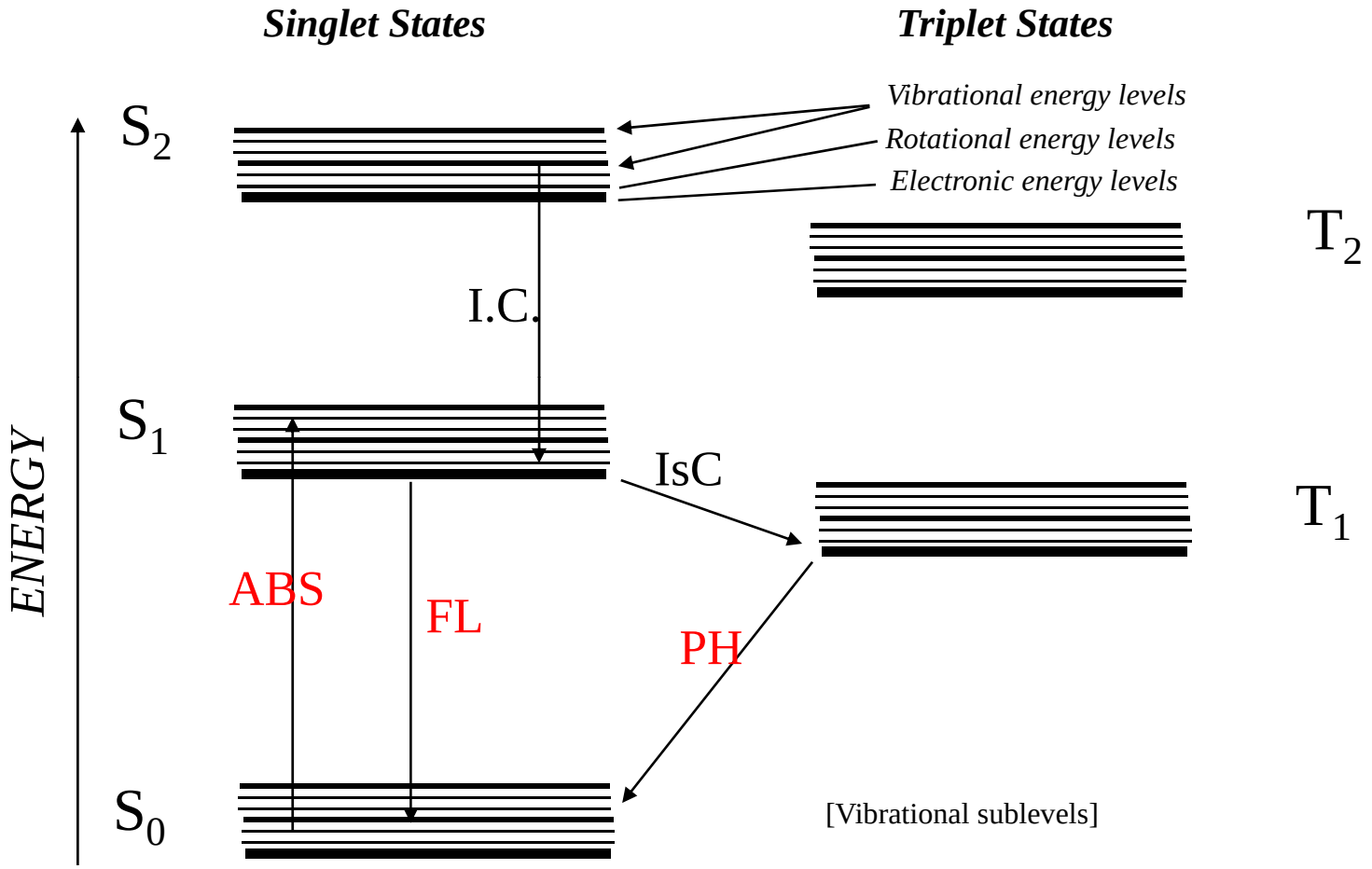
Phosphorescence

- Following absorption, molecules can relax via a non-radiative transition to the T_1 rather than the S_1 state - this is called an intersystem crossing

- Phosphorescence is emission of light during forbidden transfer from excited triplet state to the ground singlet state. Because it is forbidden, it has a low probability, and takes a longer time

- Phosphorescence has a longer lifetime than fluorescence (milliseconds rather than femtoseconds
- Phosphorescence generally occurs at longer wavelengths than fluorescence because the energy difference between S_0 and T_1 is lower

Jablonski Diagram



ABS - Absorbance *S 0.1.2 - Singlet Electronic Energy Levels*

FL - Fluorescence *T 1,2 - Corresponding Triplet States*

I.C.- Nonradiative Internal Conversion *IsC - Intersystem Crossing* *PH - Phosphorescence*

summary

Absorption: $S_0 \rightarrow S_1$ very fast 10^{-15} -10^{-13} s

External Conversion: radiationless transition to lower state by collisional deactivation

Internal Conversion: radiationless transition to lower state when vibrational energy levels "match"

Fluorescence: emission not involving spin change ($S_1 \rightarrow S_0$) efficient, short-lived 10^{-9} -10^{-5} s

Intersystem Crossing: transition with spin change (S to T)

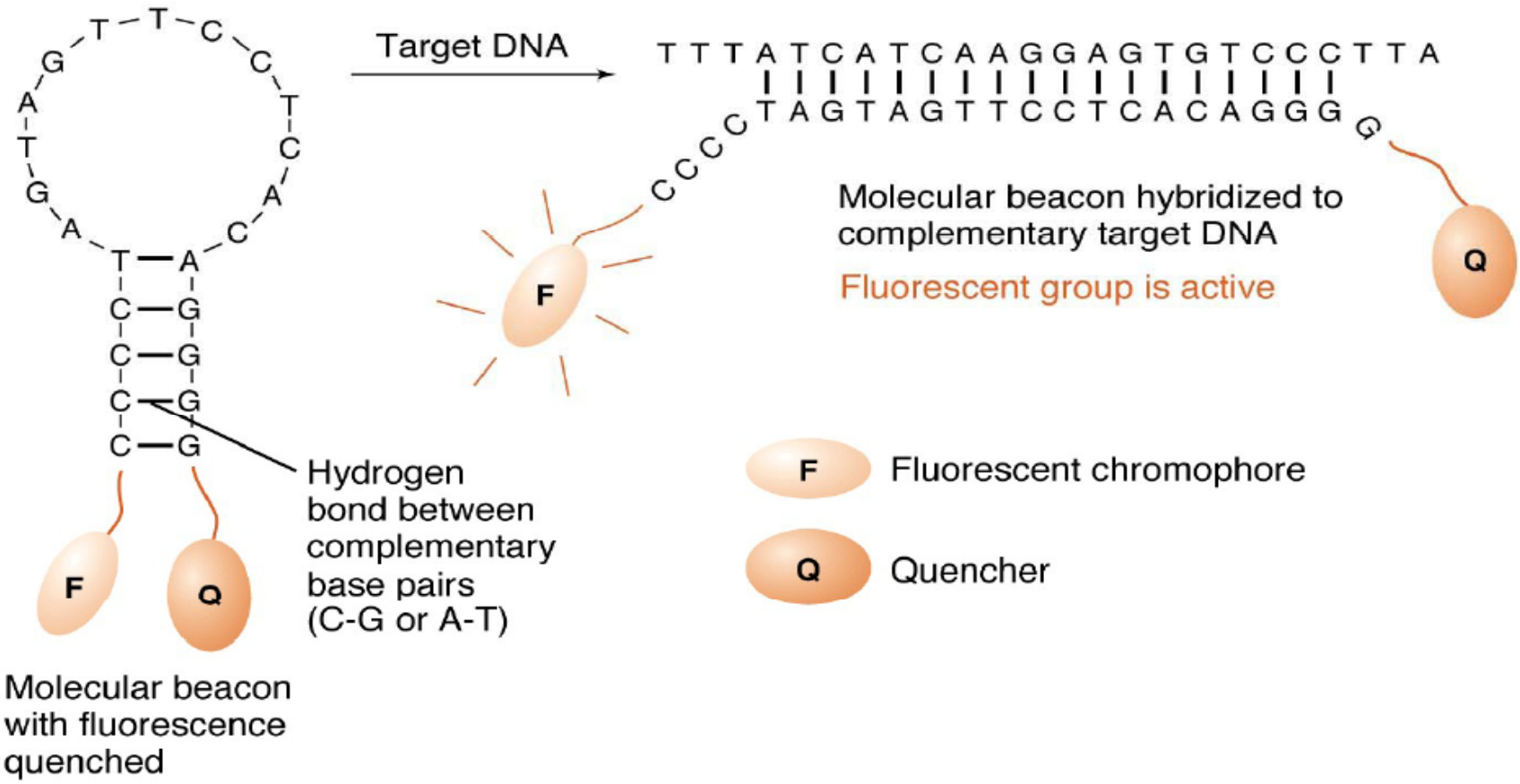
Phosphorescence: emission involving spin change ($T_1 \rightarrow S_0$) inefficient, long-lived 10^{-3} -10 s. Transitions between states of different multiplicities are improbable "forbidden" (e.g. T→S or T→S)

Quenching and Bleaching

- **Quenching** is when excited molecules relax to ground states via nonradiative pathways avoiding fluorescence emission (vibration, collision, intersystem crossing)
- Molecular oxygen quenches by increasing the probability of intersystem crossing
- Polar solvents such as water generally quench fluorescence by orienting around the excited state dipoles

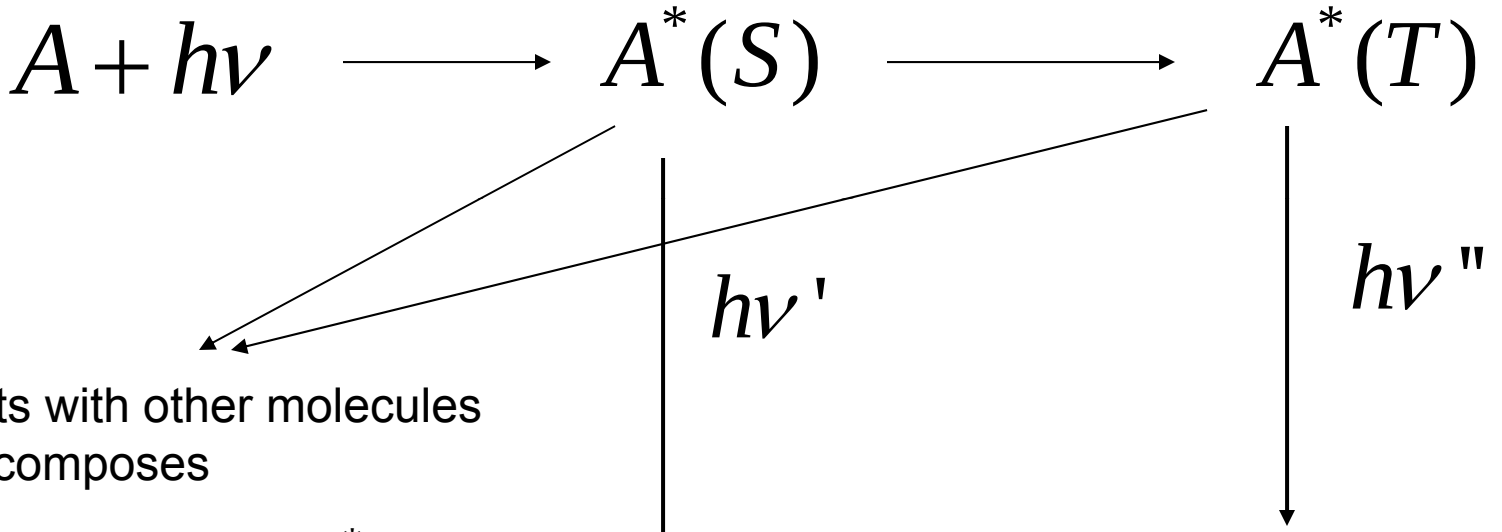
- **Photobleaching** is defined as destruction of an excited fluorophore

Using quenching to probe DNA hybridization



In general, excited state has higher energy and is not stable

- When the molecule is in its excited state it returns to the ground state through several pathways:



Reacts with other molecules or decomposes

$$A^* + B \rightarrow A + B^*$$

$$A^* \rightarrow B + C$$

Fluoresces,

$$A^* \rightarrow A + h\nu'$$

Phosphoresces,

$$A^* \rightarrow A + h\nu''$$

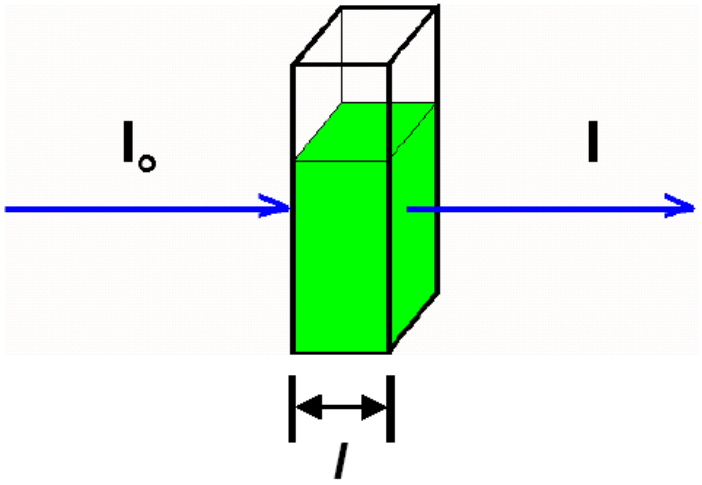
Absorption and fluorescence spectra

- Absorption and fluorescence spectra are good characteristics of the molecule, because they characterize absorption of and emission of light by a molecule, which are unique to the molecule due to specific arrangement of molecular orbitals and distribution of energy levels in the molecule

- Spectrophotometry (absorption spectroscopy) records:
- Absorption (absorbance) spectra –dependence of absorbance on wavelength

- Fluorescence spectroscopy records:
- Fluorescence spectra - dependence of fluorescence emission on wavelength

Spectrophotometry. Beer-Lambert Law



- for light travelling through a cuvette thickness ***l*** cm containing absorbing molecules with concentration **C**

Transmittance $\%T = \frac{I}{I_0} \times 100$

Absorbance

(optical density) $A = \log \frac{I_0}{I}$

Beer-Lambert Law

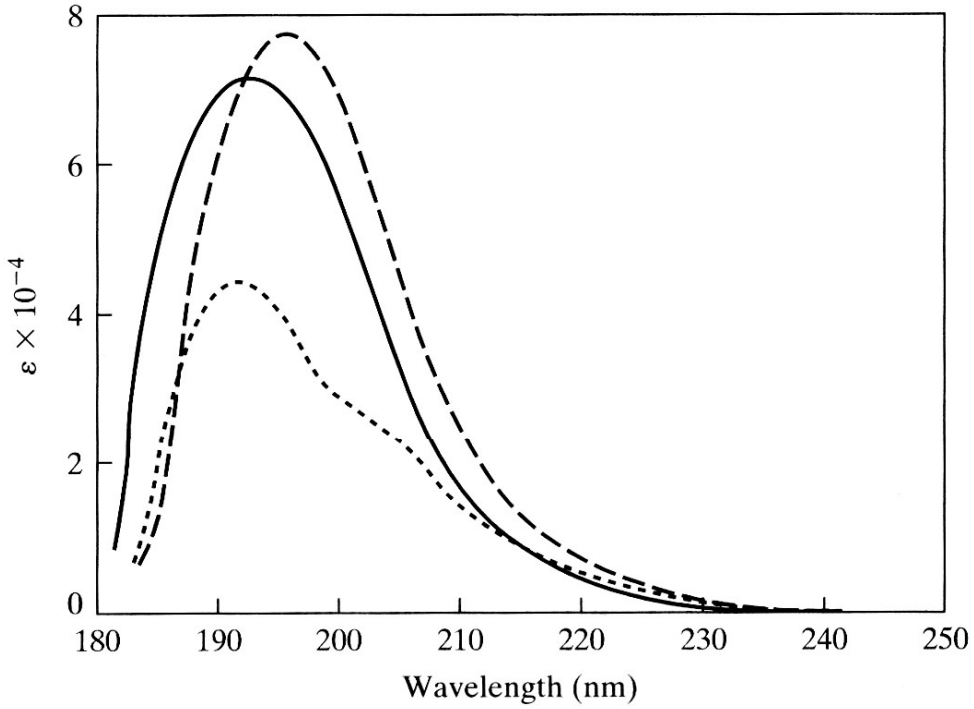
$$A = \log(I_0 / I) = \varepsilon C l$$

Extinction coefficient, different for every chromophore, and depends on the wavelength, units: $dm^3 mol^{-1} cm^{-1}$.

$$\varepsilon = 0.4343\sigma \qquad \ln \frac{I_0}{I} = \sigma C l$$

σ is absorption cross section, characterizes the probability of photon to be absorbed

Absorption by proteins

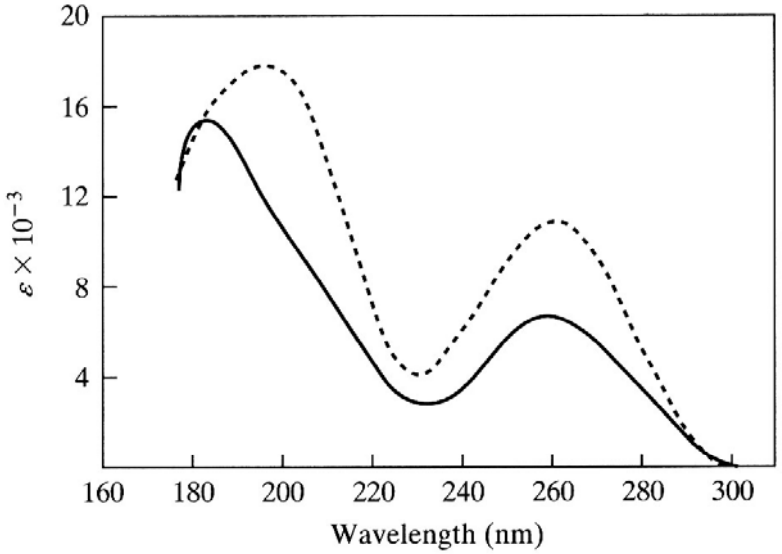


The electronic absorption spectra for poly-L-lysine hydrochloride in aqueous solution as a random coil at pH 6.0, 25°C (———); α-helix at pH 10.8, 25°C (- - -); β-strand at pH 10.8, 52°C (— —). [Adapted from K. Rosenheck and P. Doty (1961) *Proc. Natl. Acad. Sci. USA* **47**, 1775–1785.]

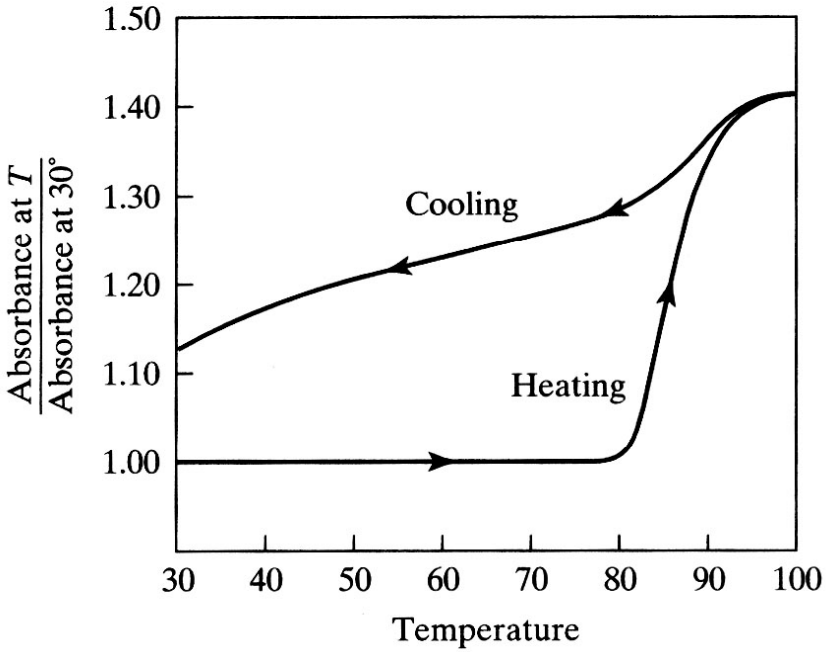
EXTINCTION COEFFICIENT AT 190 nm FOR SOME PROTEINS

Protein	$\varepsilon(190) \times 10^{-3}$
α-Chymotrypsin	9.69
Cytochrome *c*	9.72
Elastase	10.29
Hemoglobin	9.62
Lactate dehydrogenase	8.51
Lysozyme	11.46
Myoglobin	9.15
Papain	10.10
Ribonuclease	9.64

DNA absorption



The absorption spectrum of native *E. coli* DNA (———) and the average spectrum for the four component deoxynucleotides in aqueous solution (-----).



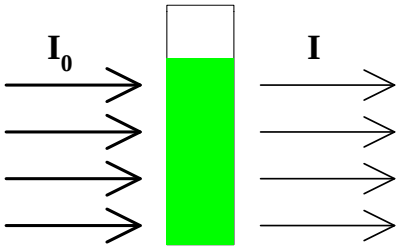
DNA melting registered by electronic absorption

Fluorescence characteristics

Fluorescence Intensity F

$$F = \left(I_0 - I\right) \cdot \Phi$$

here Φ is the fluorescence quantum efficiency
I_0 and I are the incident and transmitted intensities



$$\left(I_0 - I\right) = I_0\left(1 - 10^{-\varepsilon C l}\right)$$

and therefore

$$F = I_0\left(1 - 10^{-\varepsilon C l}\right) \cdot \Phi$$

Fluorescence Lifetime (τ)

- is the time delay between the absorbance and the emission

Fluorescence quantum efficiency

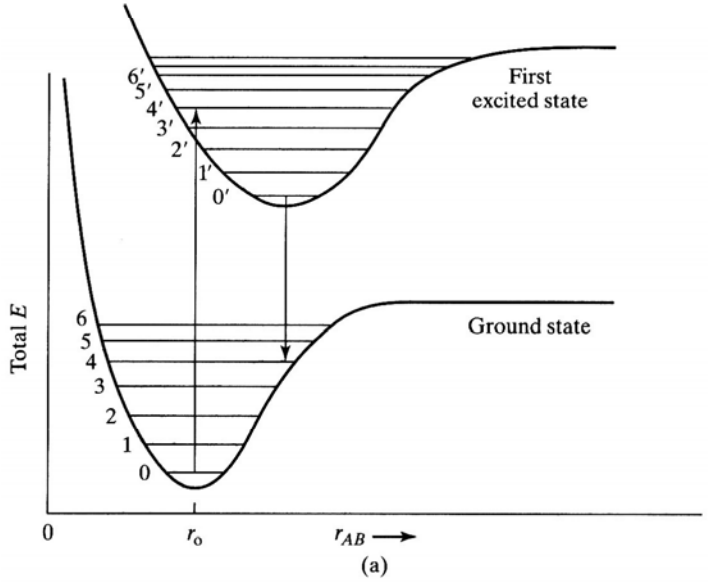
- Fluorescence quantum efficiency Φ

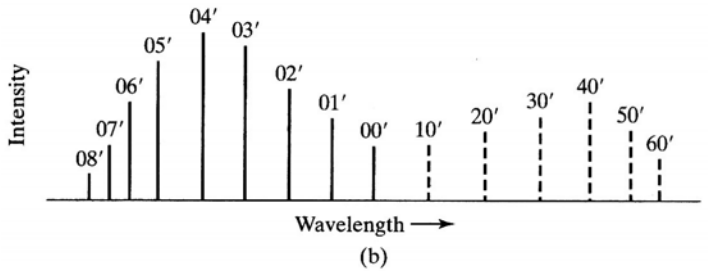
$$\Phi = \frac{N\_photons\_emitted}{N\_photons\_absorbed}$$

$$\Phi = \frac{k_F}{k_F + k_{IC} + k_{IS} + k_Q [Q]}$$

- Here k_F is the rate constant of fluorescence; k_{IC} and k_{IS} are the rates of internal conversion and intersystem crossing, k_Q is the rate of external quenching reaction and ***[Q]*** is the concentration of the quencher.

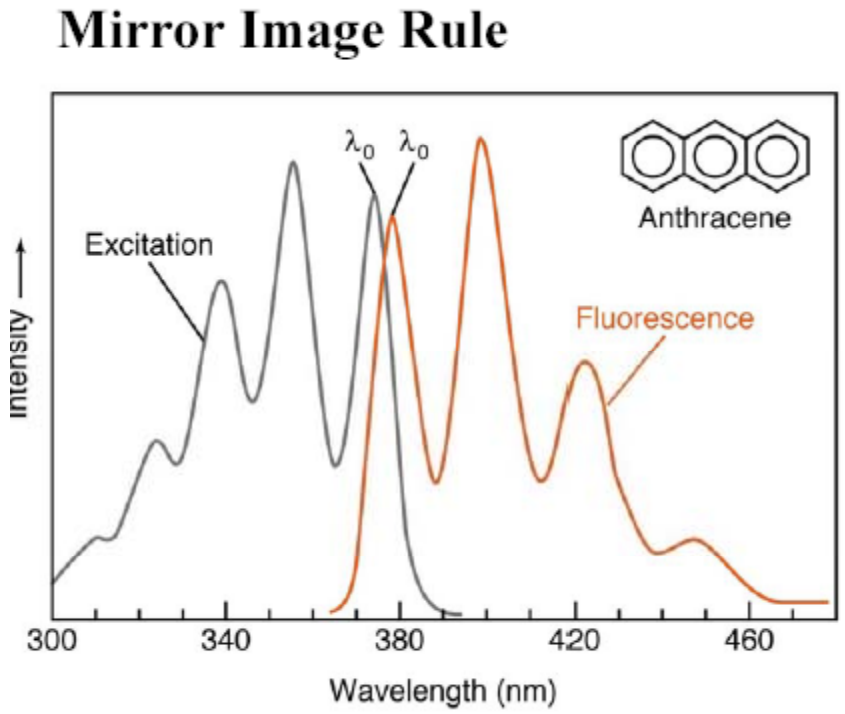
Fluorescence spectrum



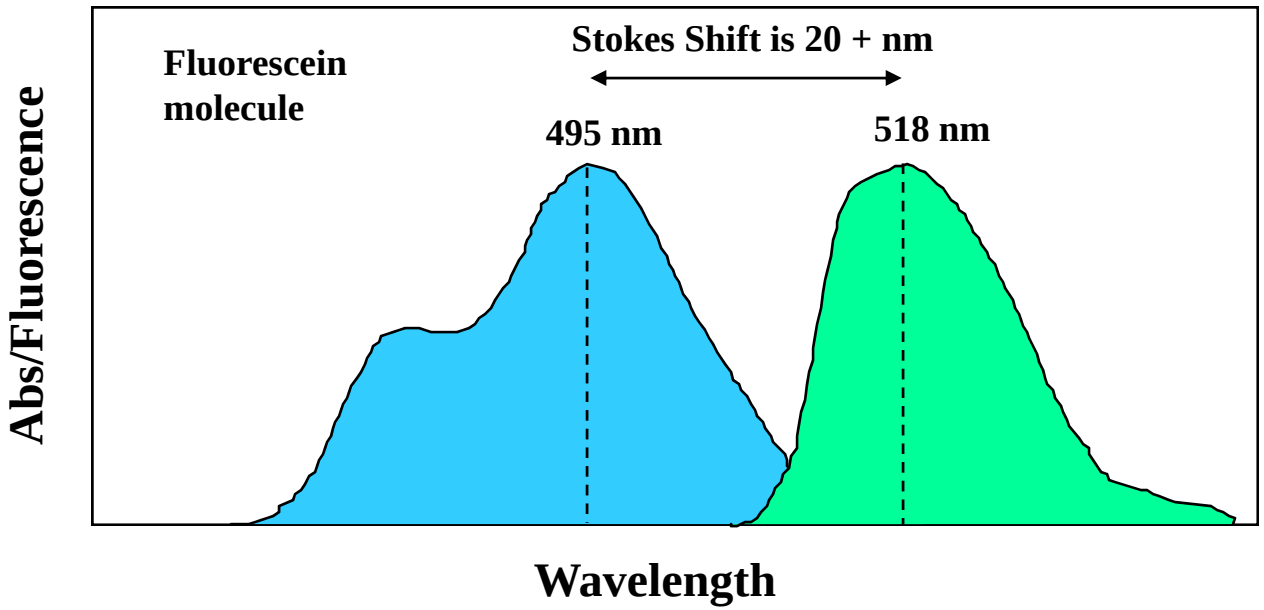


The energy differences between the bands in the emission spectrum are similar to those in the absorption spectrum. – because of this the emission spectrum is a mirror image of the absorption spectrum.

The fine structure of the absorption spectrum is characterized by the vibrational modes of the excited state (S1), whereas the fine structure of the fluorescence spectrum is characterized by the vibrational modes of the electronic ground state (S0).



- Stokes Shift
  - is the energy difference between the max peak of absorbance and the max peak of emission



Fluorescing molecules
Native molecules

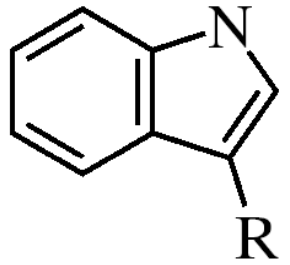
Fluorescence characteristics of protein and nucleic acid constituents and coenzymes

Substance	Conditions	Absorption		Fluorescence[§]			Sensitivity
		λ_{max} (nm)	$\varepsilon_{max} \times 10^{-3}$	λ_{max} (nm)	ϕ_F	τ_F (nsec)	$\varepsilon_{max}\phi_F \times 10^{-2}$
Tryptophan	H_2O, pH 7	280	5.6	348	0.20	2.6	11.
Tyrosine	H_2O, pH 7	274	1.4	303	0.14	3.6	2.0
Phenylalanine	H_2O, pH 7	257	0.2	282	0.04	6.4	0.08
Y base	Yeast $tRNA^{Phe}$	320	1.3	460	0.07	6.3	0.91
Adenine	H_2O, pH 7	260	13.4	321	2.6×10^{-4}	<0.02	0.032
Guanine	H_2O, pH 7	275	8.1	329	3.0×10^{-4}	<0.02	0.024
Cytosine	H_2O, pH 7	267	6.1	313	0.8×10^{-4}	<0.02	0.005
Uracil	H_2O, pH 7	260	9.5	308	0.4×10^{-4}	<0.02	0.004
NADH	H_2O, pH 7	340	6.2	470	0.019	0.40	1.2

[§] Values shown for ϕ_F are the largest usually observed. In a given case actual values can be considerably lower.

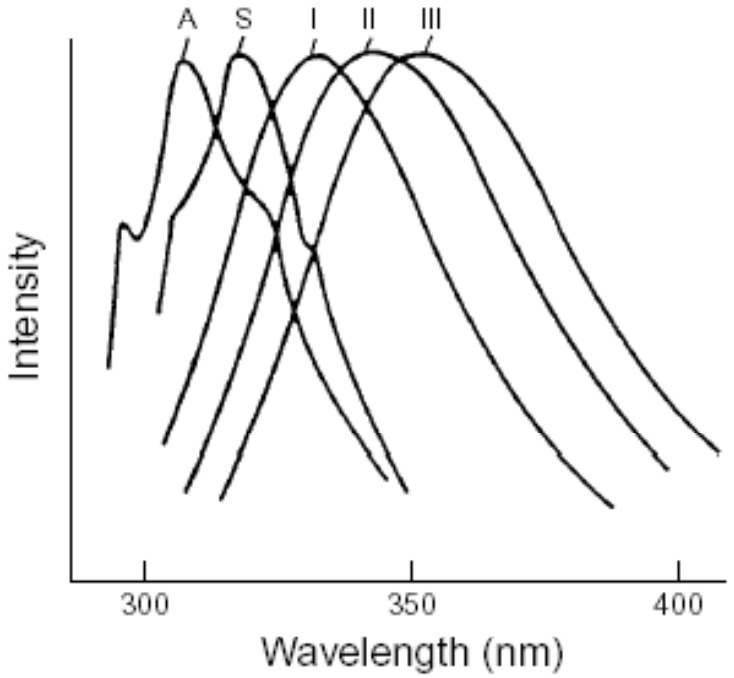
Tryptophan fluorescence

- Aromatic amino acids



- Electronic transitions of the tryptophan's indole ring is the strongest source of amino-acid fluorescence.
- Absorption @ 260-290 nm. Emission strongly depends on environment:

- A – strongly nonpolar environment (inside protein)
- S – nonpolar environment but close to polar group
- I – polar environment inside globule
- II – at the surface of protein in contact with bound water
- III – at the surface of protein in contact with free water



Excitation - Emission Peaks of typical fluorescence probes

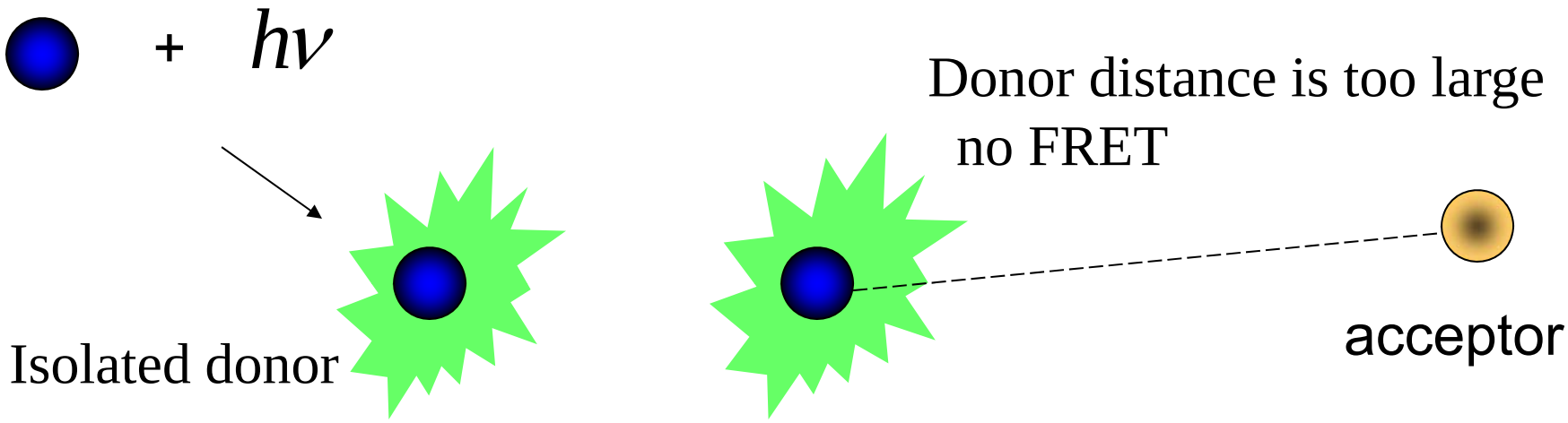
Fluorophore	EX_{peak}	EM_{peak}	% Max Excitation at 488	568	647 nm
FITC	496	518	87	0	0
Bodipy	503	511	58	1	1
Tetra-M-Rho	554	576	10	61	0
L-Rhodamine	572	590	5	92	0
Texas Red	592	610	3	45	1
CY5	649	666	1	11	98

Note: You will not be able to see CY5 fluorescence under the regular fluorescent microscope because the wavelength is too high.

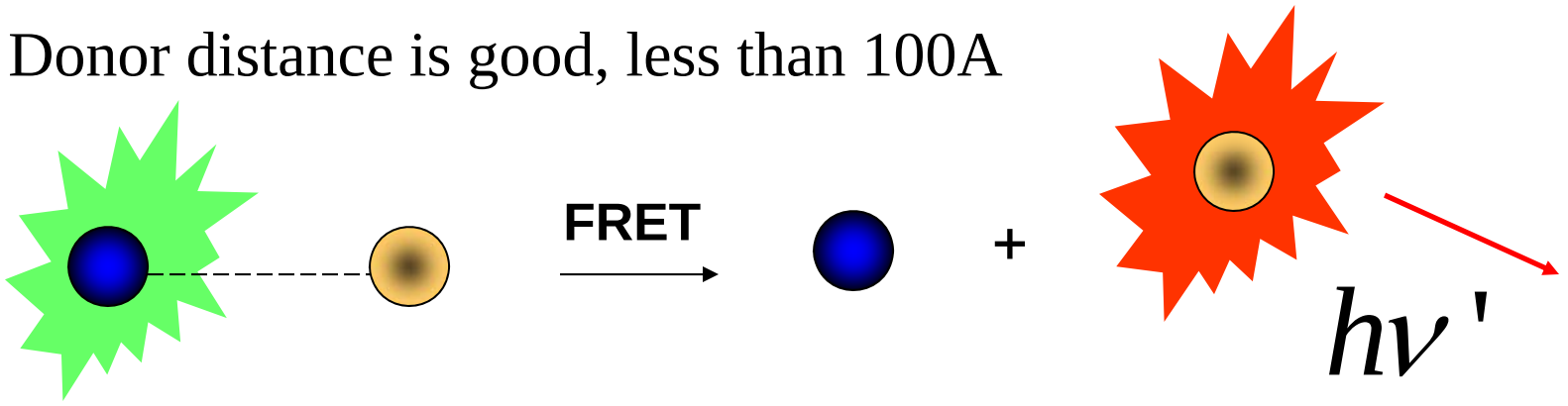
Fluorescence Resonance Energy Transfer FRET

- Resonance energy transfer can occur when one molecule (donor) transfers energy to another (acceptor) without radiation. Donor and acceptor molecules are less than 100 Å of one another (preferable 20-50 Å)
- Energy transfer is non-radiative which means the donor is not emitting a photon which is absorbed by the acceptor
- The acceptor becomes excited and then fluoresces
- Fluorescence RET (FRET) can be used to study the dynamics and binding of biomolecules.

FRET

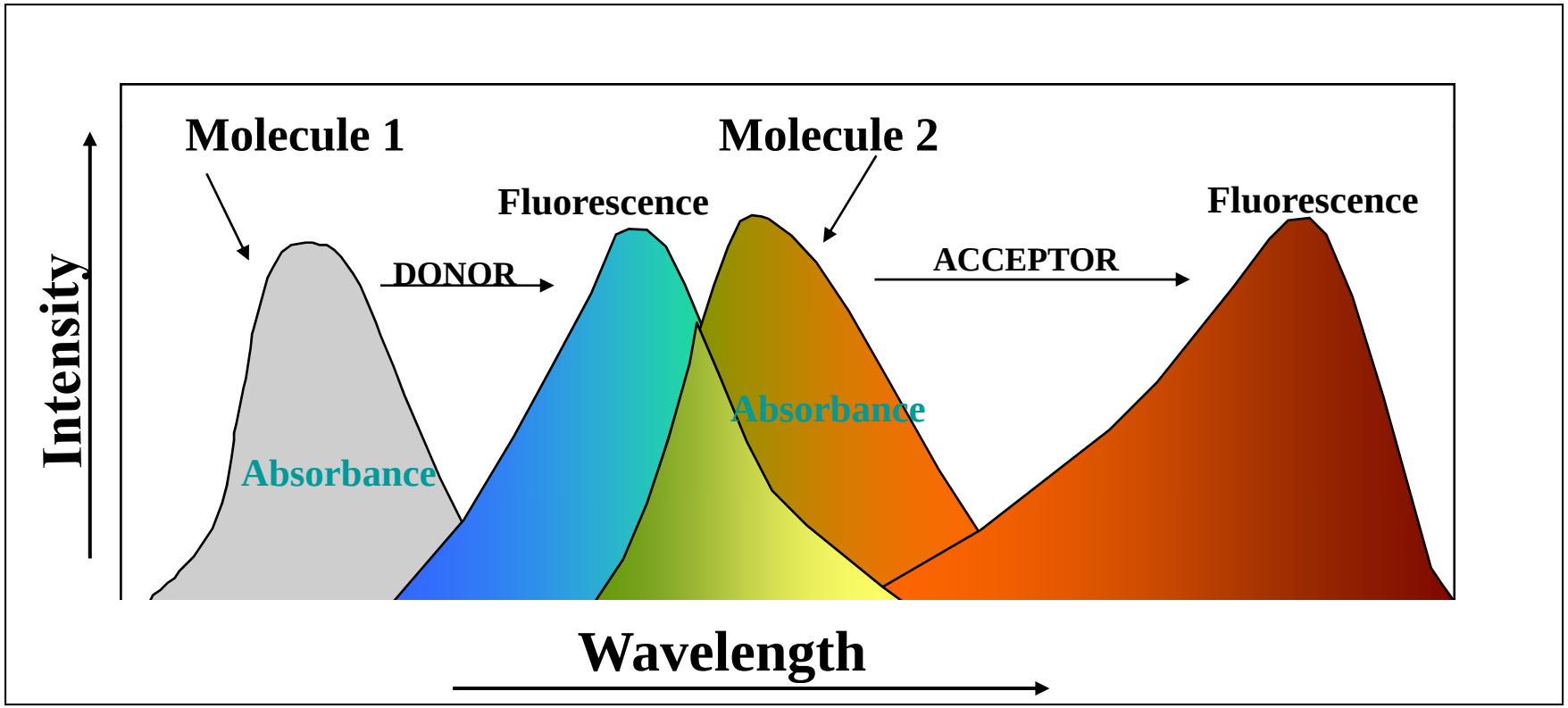


Donor distance is good, less than 100A



FRET requires that emission spectrum of donor overlaps with absorption spectrum of acceptor

The rate of this transfer process is very sensitive to the distance ***R*** between the donor and acceptor:



FRET

FRET requires that emission spectrum of donor overlaps with absorption spectrum of acceptor

The rate of this transfer process is very sensitive to the distance ***R*** between the donor and acceptor:

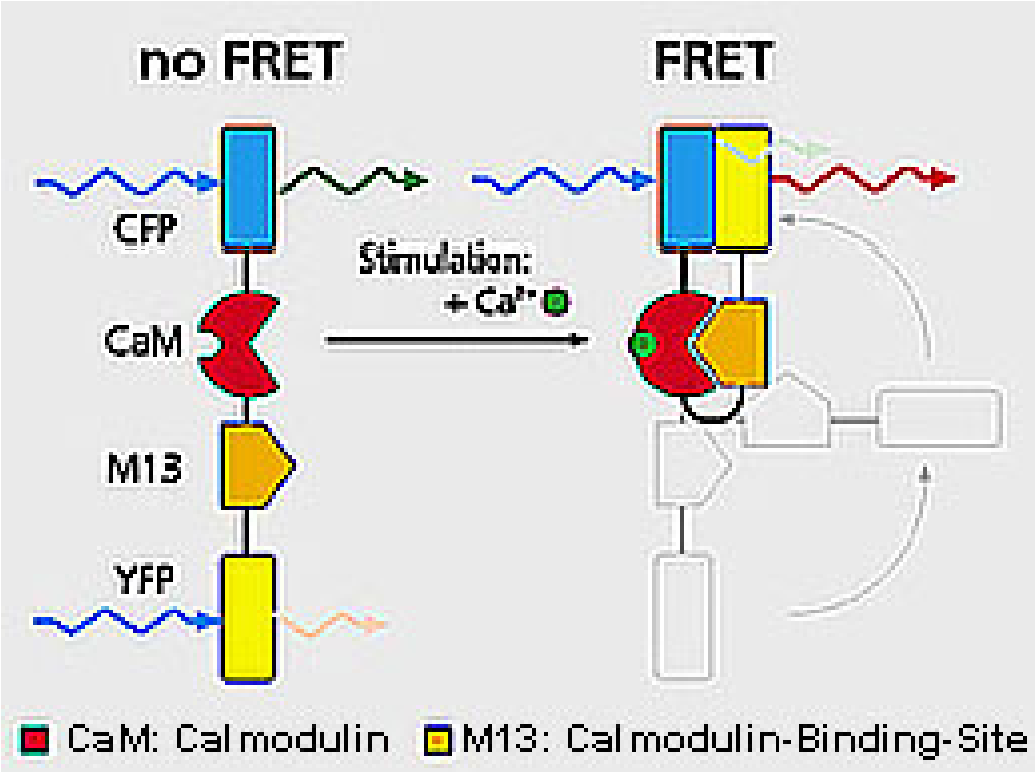
$$k_t = \frac{1}{\tau_d}\left(R_0/R\right)^6$$

Life time of a donor

Distance when FRET occurs is </=100A, max when R< or =R_0, R_0=Forster distance =100 A

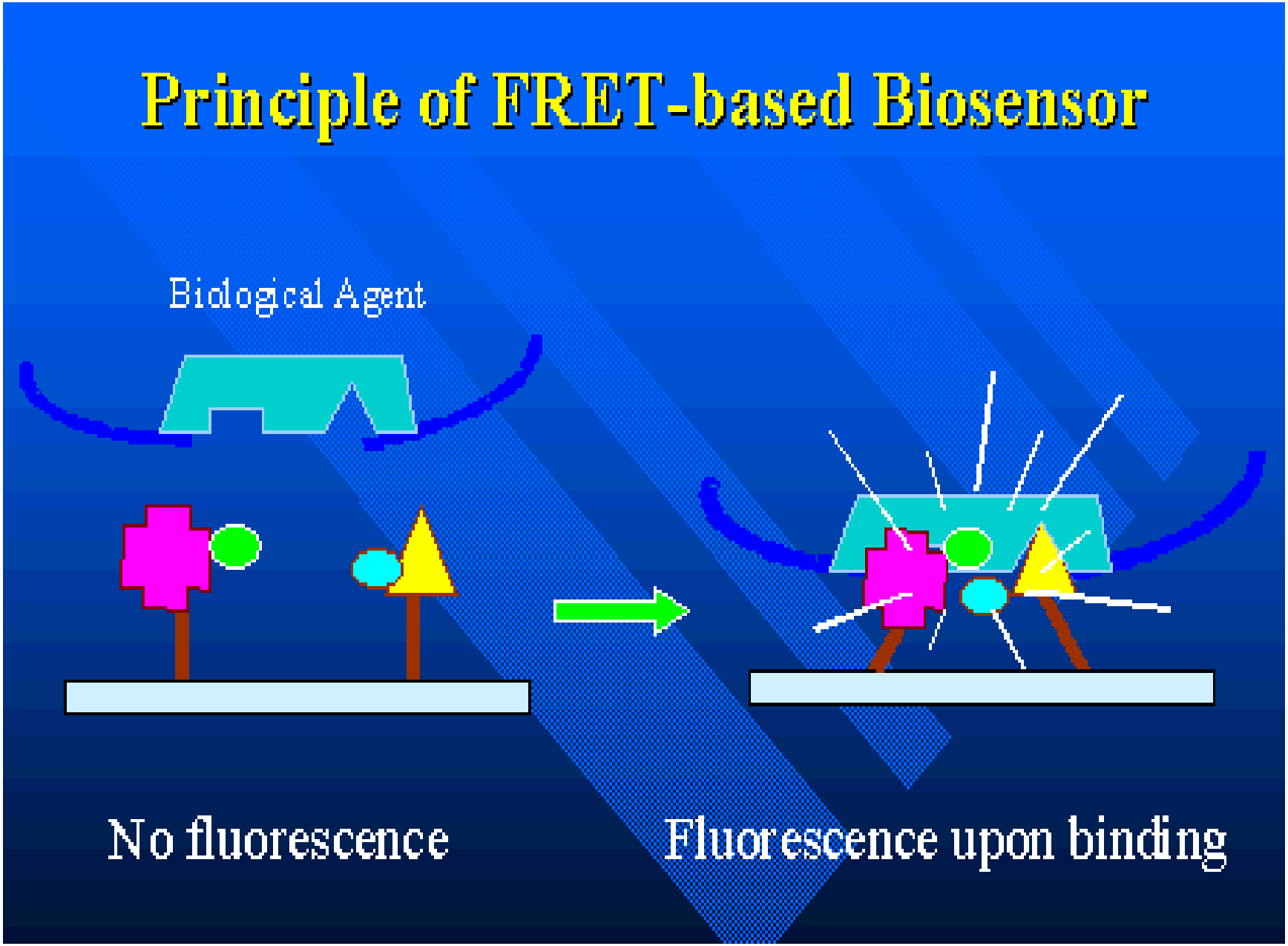
FRET can be used to measure donor-acceptor distance variation from ~1.5 nm to ~6 nm

Ca2+ dependent binding



http://www.zeiss.de/C12567BE00472A5C/GraphikTitelIntern/FRET_EMFP/$File/FRET-pic05.JPG

Principle of FRET-based Biosensor

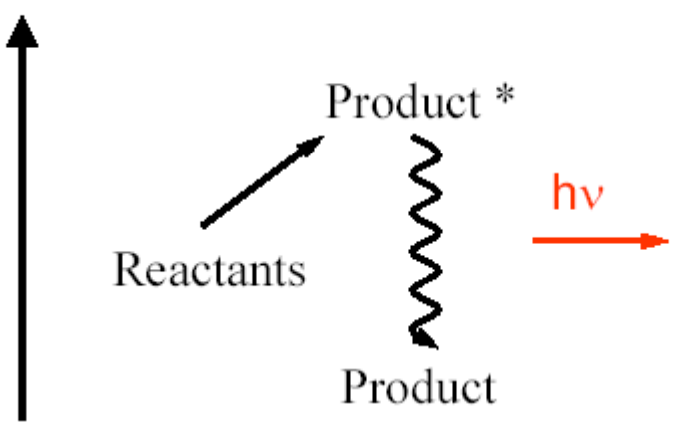


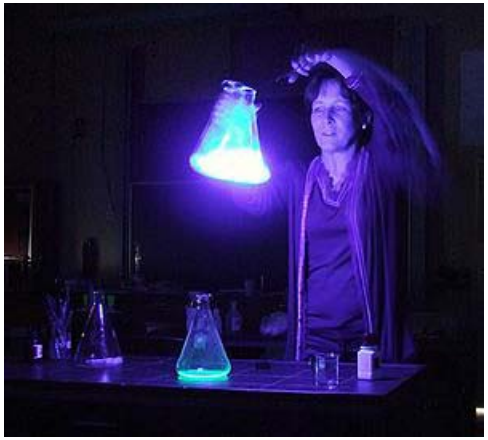
Chemiluminescence (CL)

Emission of light as a result of a chemical reaction

Requirements: 1)Energy must be sufficient to produce electronically excited state

2) The reaction pathway must favour the formation of an electronically excited state. The excited state must luminesce or transfer its energy to another molecule that then luminesces.

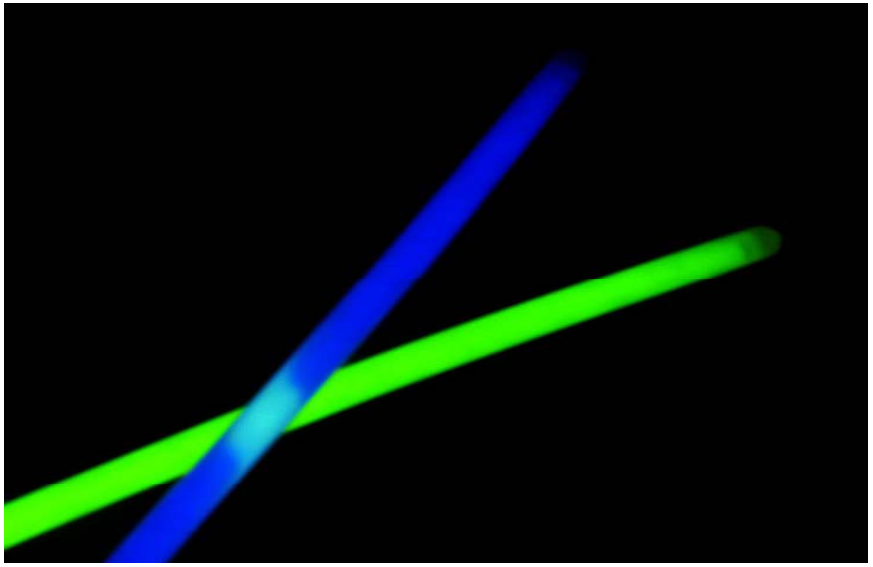




CL highly specific – very few reactions result in light emission
Very sensitive >> fluorescence (no stray light)

Bioluminescence – is example of chemiluminescence in biological systems:
Glowing Fireflies –
produce the chemicals luciferin (a pigment) and luciferase (an enzyme). The luciferin reacts with oxygen to create light, luciferase catalyzes the reaction.

Chemiluminescence application example
Glowing sticks
give off light when two solutions are mixed. The sticks consist of a small, brittle container within a flexible outer container. Each container holds a unique solution. When the outer container is flexed, the inner container breaks, allowing the solutions to combine, causing the necessary chemical reaction. After breaking, the tube is shaken to thoroughly mix the two components. Example – luminol reacts with hydrogen peroxide and the product produced is fluorescent. It is advisable to keep the mixture away from skin and to prevent accidental ingestion if the glow stick case splits or breaks. If spilled on skin, the chemicals could cause slight skin irritation, swelling, or, in extreme circumstances, vomiting and nausea



Sources:

Physics for biological sciences F.R. Hallett

Light and Fluorescence
J.Paul Robinson, PhD
Professor of Immunopharmacology and Bioengineering
Purdue University
www.cyto.purdue.edu
http://tinyurl.com/2wkpp

Probes for Proteins

Probe	*Excitation*	*Emission*
FITC	488	525
PE	488	575
APC	630	650
PerCP™	488	680
Cascade Blue		
Coumerin-phalloidin	350	450
Texas Red™	610	630
Tetramethylrhodamine-amines		
CY3 (indotrimethinecyanines)	540	575
CY5 (indopentamethinecyanines)		

Specific Organelle Probes

Probe	*Site*	*Excitation*	*Emission*
BODIPY	Golgi	505	511
NBD	Golgi	488	525
DPH	Lipid	350	420
TMA-DPH	Lipid	350	420
Rhodamine 123	Mitochondria	488	525
DiO	Lipid	488	500
dil-Cn-(5)	Lipid	550	565
diO-Cn-(3)	Lipid	488	500

BODIPY - borate-dipyrromethene complexes
NBD - nitrobenzoxadiazole
DPH - diphenylhexatriene
TMA - trimethylammonium