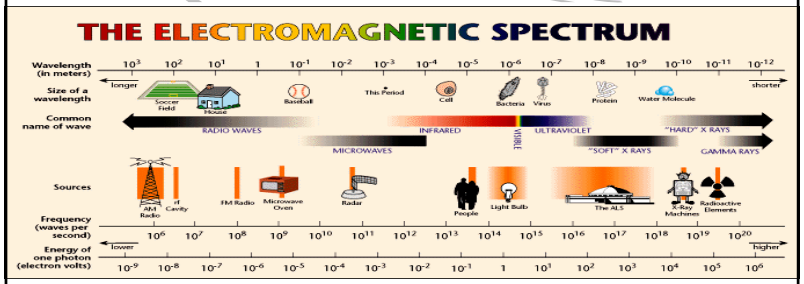
Spectroscopy



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

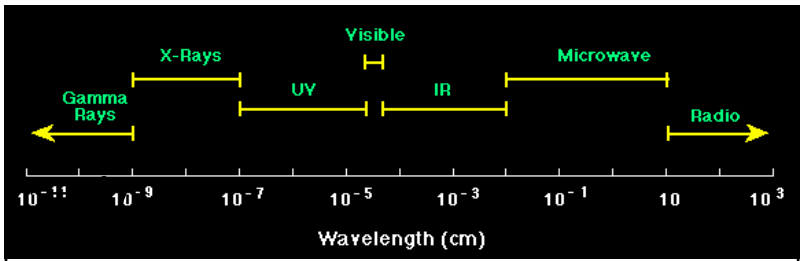
Interactions of "Light" with matter (Absorption / Scattering)

Frank-Condon Principle

Electronic transitions

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

Excitation Transfer / FRET



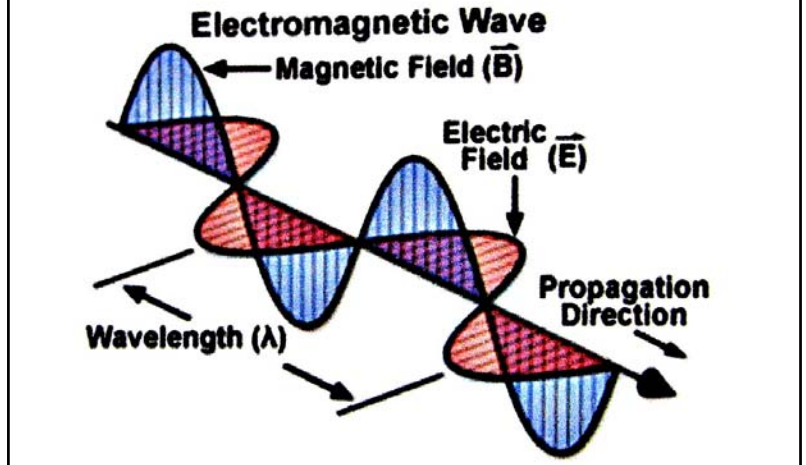
Speed of light (v) = **wavelength (λ)** x **frequency ($\bar{\upsilon}$)**

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

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

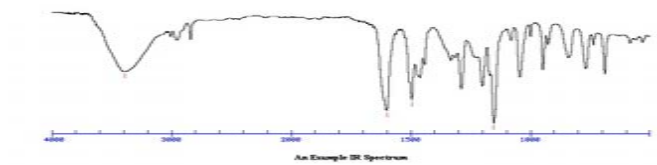
Velocity of light changes in different substances.

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



Light and Matter: Absorption (spectroscopy)

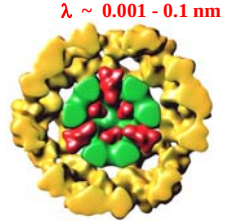
Scattering (image formation)



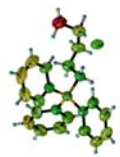
• Light Photography
λ ~ 400 - 700 nm

Abbe (~1878): Limit Res. ~ λ/2

• Electron Microscopy
λ ~ 0.001 - 0.1 nm

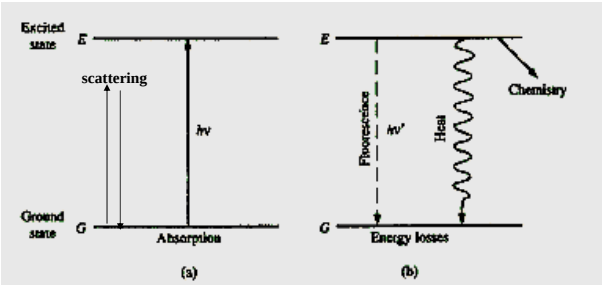


• X-Ray or NMR
λ ~ 0.1 nm



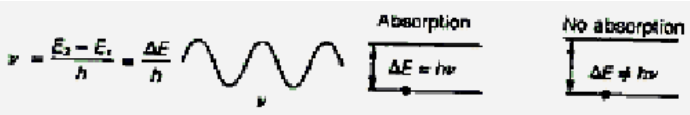
When **light interacts with matter**, there are **two possibilities**:

1. **Scattering** - the light is transmitted but velocity changes.
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**:

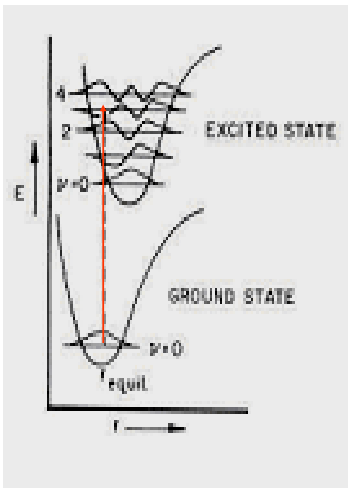
The **absorption usually occurs in the time it takes one wavelength to pass the molecule**.

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**

Frank-Condon Principle

- "The nuclear motion (10^{-13} s) is much slower as compared with electronic motion in 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".

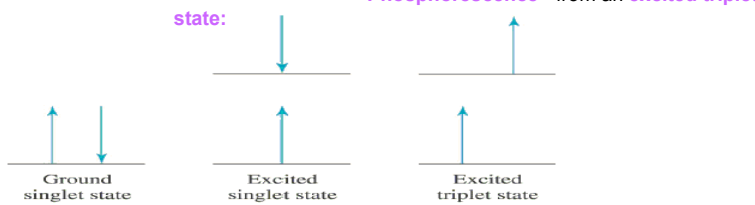


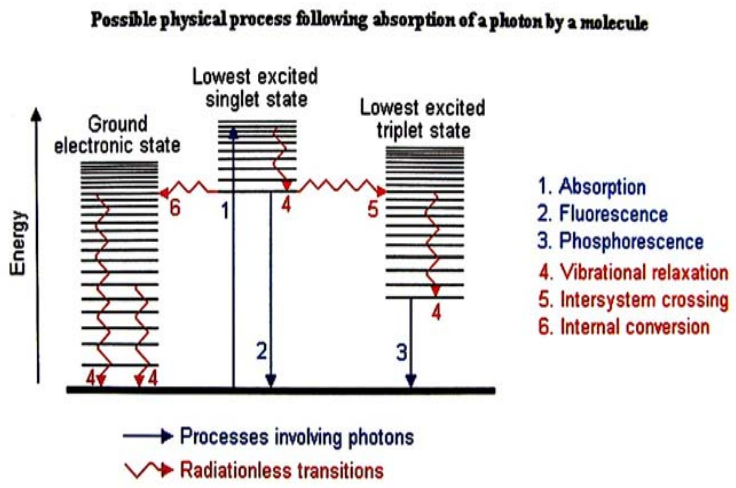
Electronic transitions

- **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:**





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

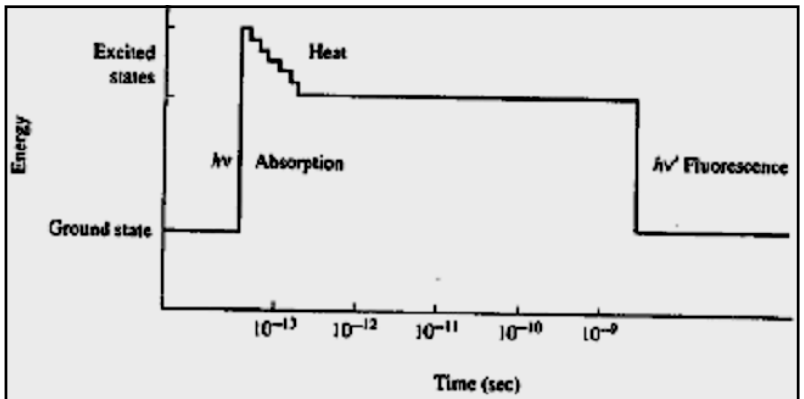
vibrational relaxation: 10^{-11}-10^{-10} s

internal conversion: 10^{-12} s

luminescence processes

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

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



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

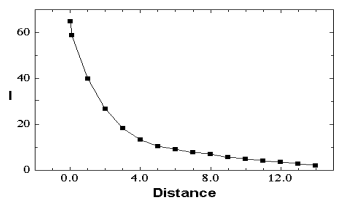
Because of the loss of energy, the emitted photon will have a lower energy than the absorbed. This means a lower frequency and longer wavelength.

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 or 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* is the fractional decrease in light intensity, α is a constant and c is the concentration. We can integrate this to get:

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

where I_0 is the initial intensity and I_t is the intensity of the light transmitted 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%), εM = 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 mg/mL)

Environmental Effects

$I_{nonpolar} > I_{polar}$ (folding / unfolding effect)
DNA – Helix-Coil Transitions ($\varepsilon_{\text{free base}} > \varepsilon_{ss} > \varepsilon_{ds}$) follow denaturation

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**.

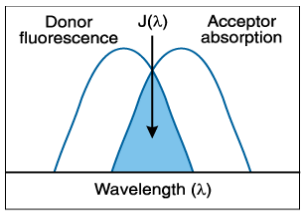
Consider an excited donor, **D*** and an acceptor that can be excited to a fluorescent state, **D***.

D → D* (absorption of light, hv, by donor)
D* → D + hv' (donor fluorescence)
D* + A → D + A* (excitation transfer)
D*→ D (other deexcitation)
A* → A + h v'' (acceptor fluorescence)

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 :

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

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

where ϵ_A = extinction coefficient of acceptor
F_D = fluorescence emission intensity of donor as a fraction of the total integrated intensity