SAPIENZA Università di Roma
Laurea magistrale in Ingegneria delle Nanotecnologie
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Biophotonics Laboratory Course

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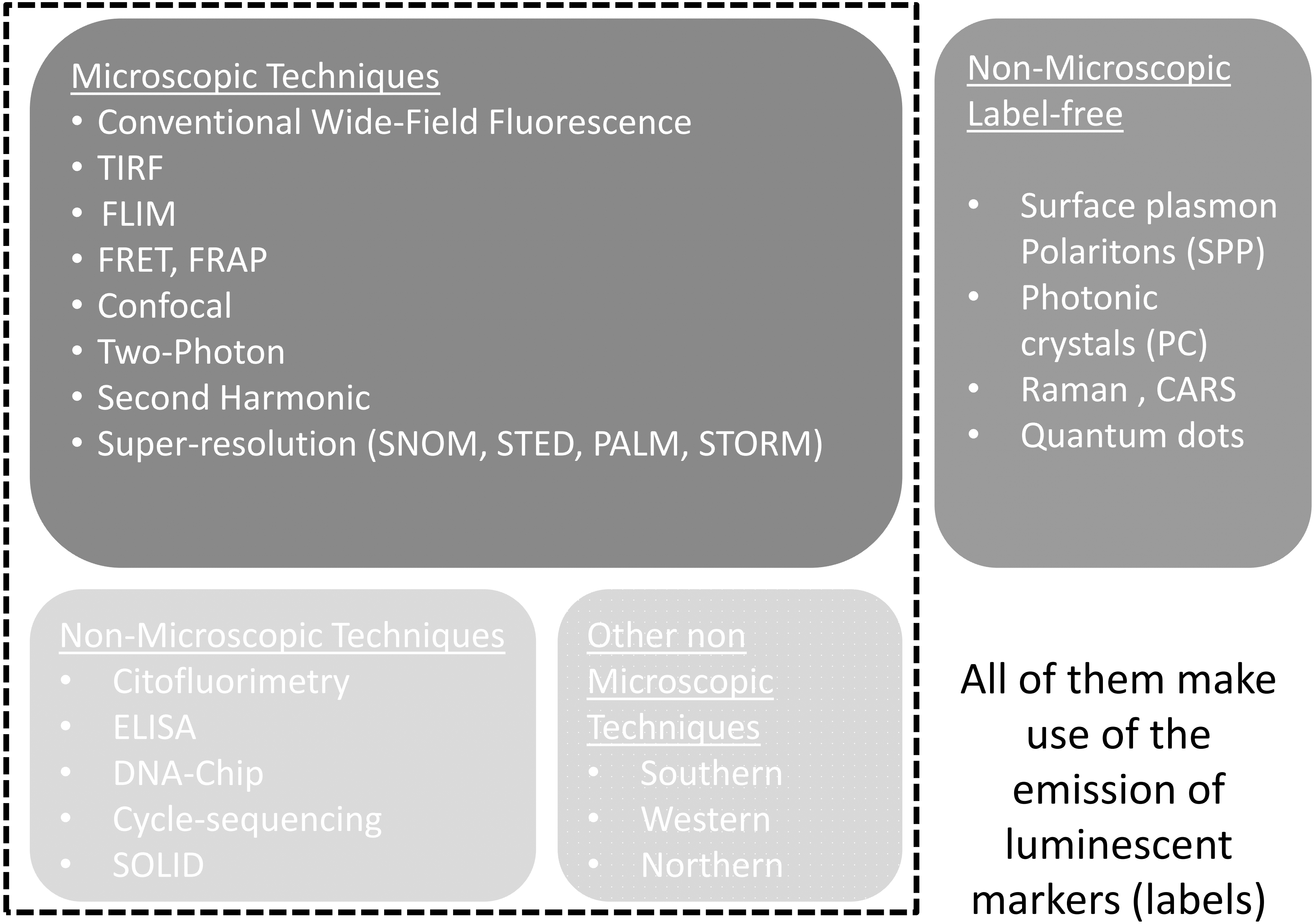
Applications of optics and photonics



Microscopic Techniques

- Conventional Wide-Field Fluorescence
- TIRF
- FLIM
- FRET, FRAP
- Confocal
- Two-Photon
- Second Harmonic
- Super-resolution (SNOM, STED, PALM, STORM)

Non-Microscopic Techniques

- Citofluorimetry
- ELISA
- DNA-Chip
- Cycle-sequencing
- SOLID

Other non Microscopic Techniques

- Southern
- Western
- Northern

Non-Microscopic Label-free

- Surface plasmon Polaritons (SPP)
- Photonic crystals (PC)
- Raman , CARS
- Quantum dots

All of them make use of the emission of luminescent markers (labels)

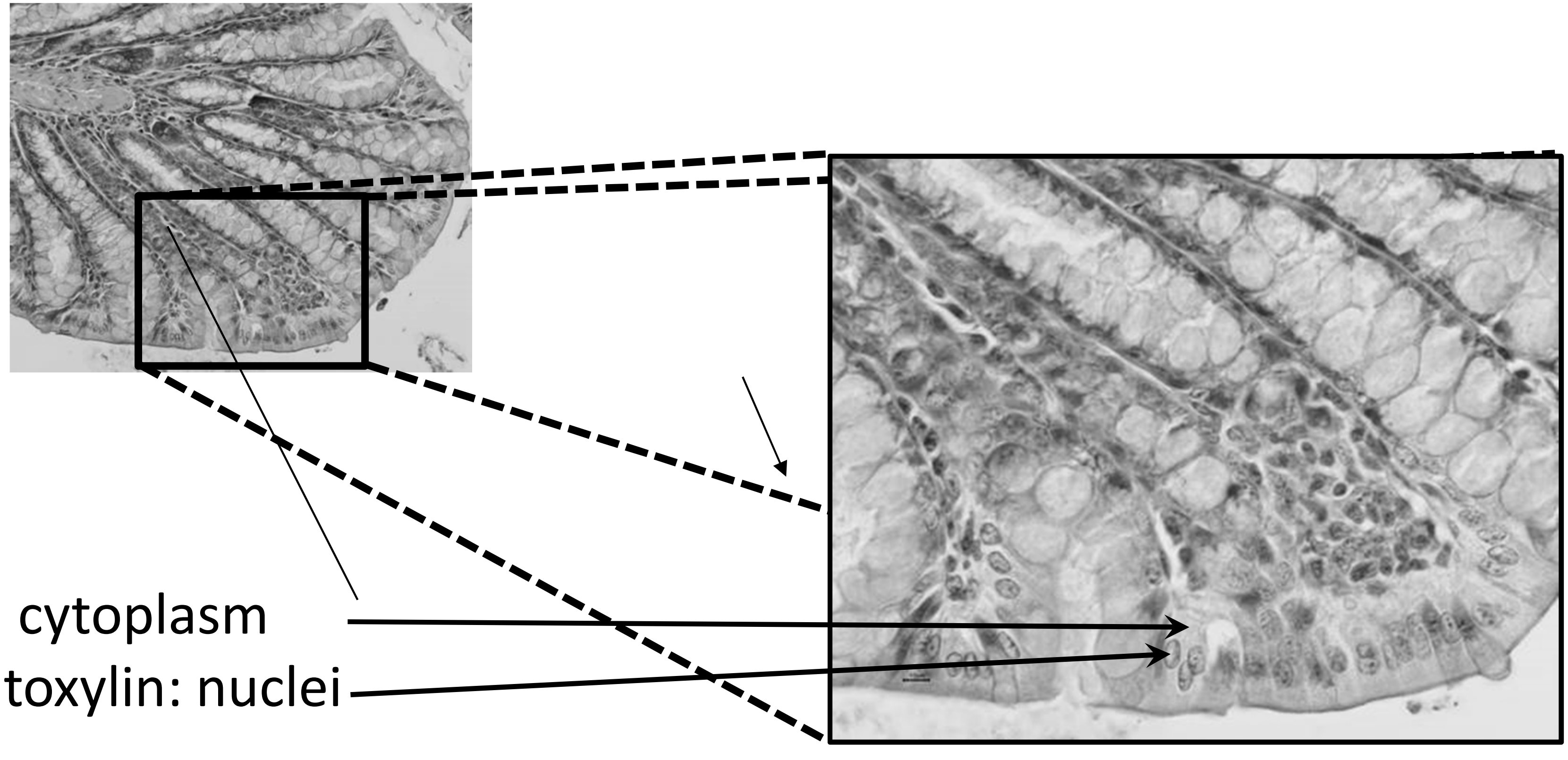
LECTURE 2

Some characteristics of the electronic structure of organic molecules
and
Absorption spectroscopy

Applications of optics and photonics

Labelling of biological tissues and molecules with coloured dyes is the basis of almost all cited techniques.

Example Conventonial microscopy with stained tissues



Eosin: cytoplasm

Hematoxylin: nuclei

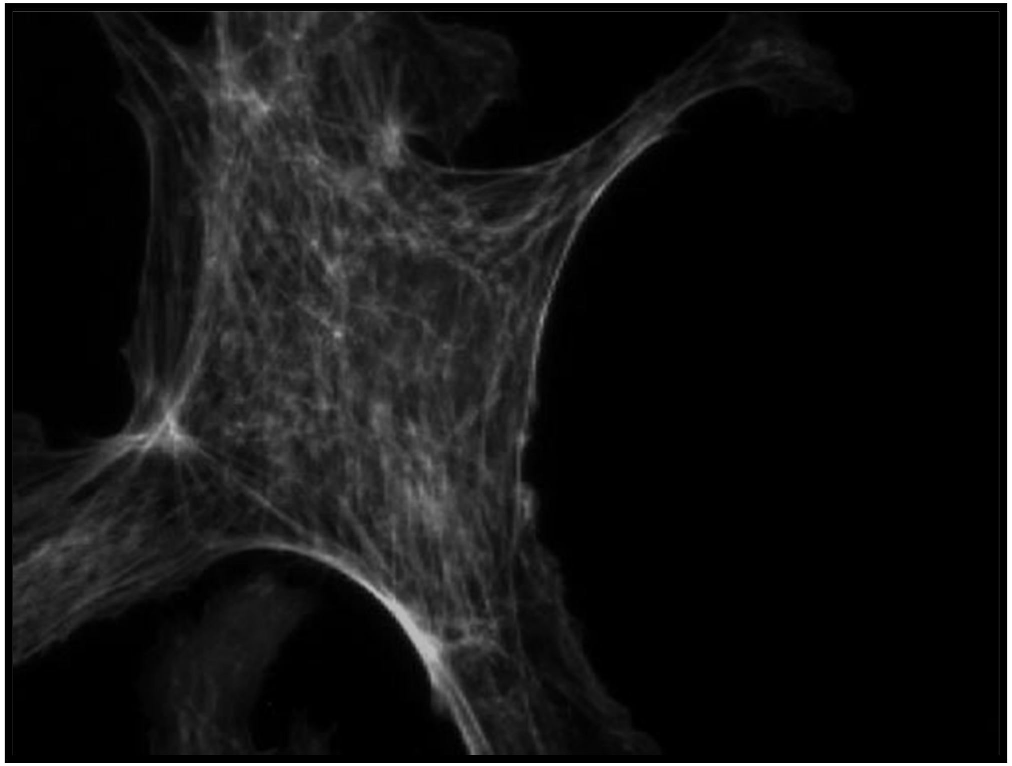
Applications of optics and photonics

Labelling of biological tissues and molecules with coloured dyes is the basis of almost all cited techniques.

Example Fluorescence microscopy on cells stained (labelled) with fluorescent molecules.

Endothelial cells stained with fluorescent molecules that bind selectively only to some cellular compartments

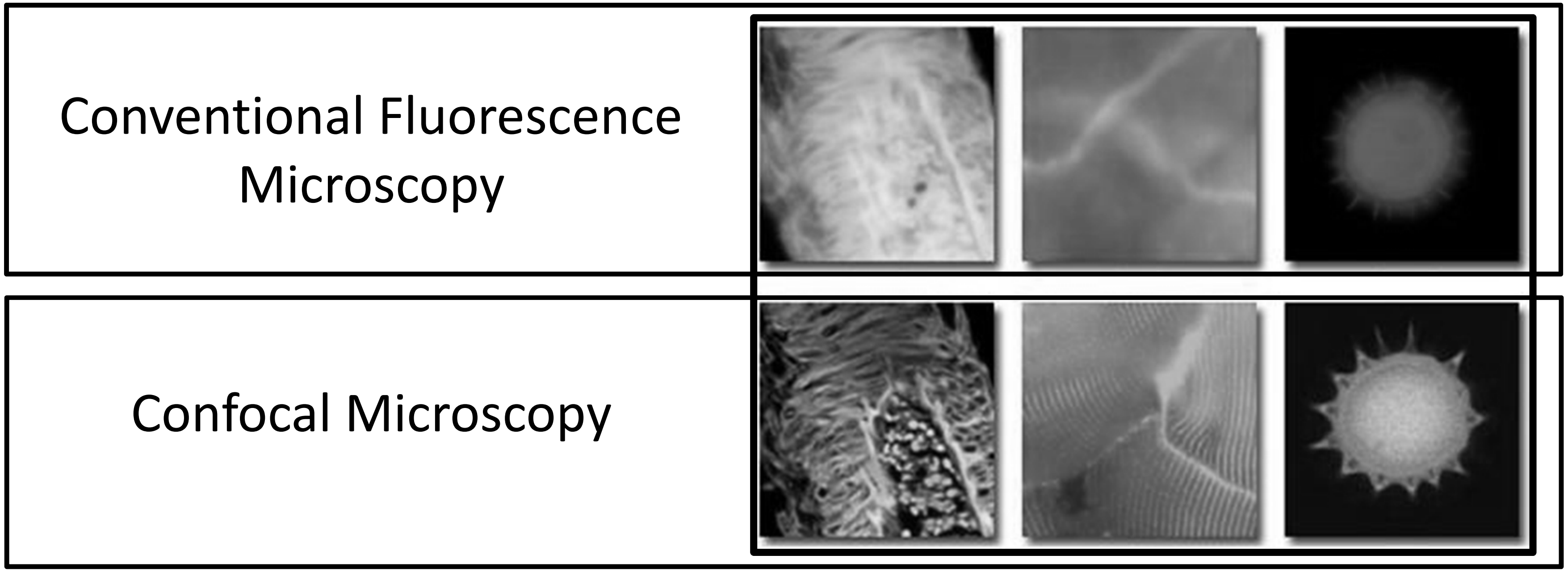
Red	Mitochondria
Green	F-Actin cytoskeleton
Blue	Nucleus



Applications of optics and photonics

Labelling of biological tissues and molecules with coloured dyes is the basis of almost all cited techniques.

Example Confocal microscopy on cells labelled with fluorescent molecules



Conventional Fluorescence Microscopy

Confocal Microscopy

Human Marrow (Medulla)

Rabbit Muscle

Sunflower Pollen

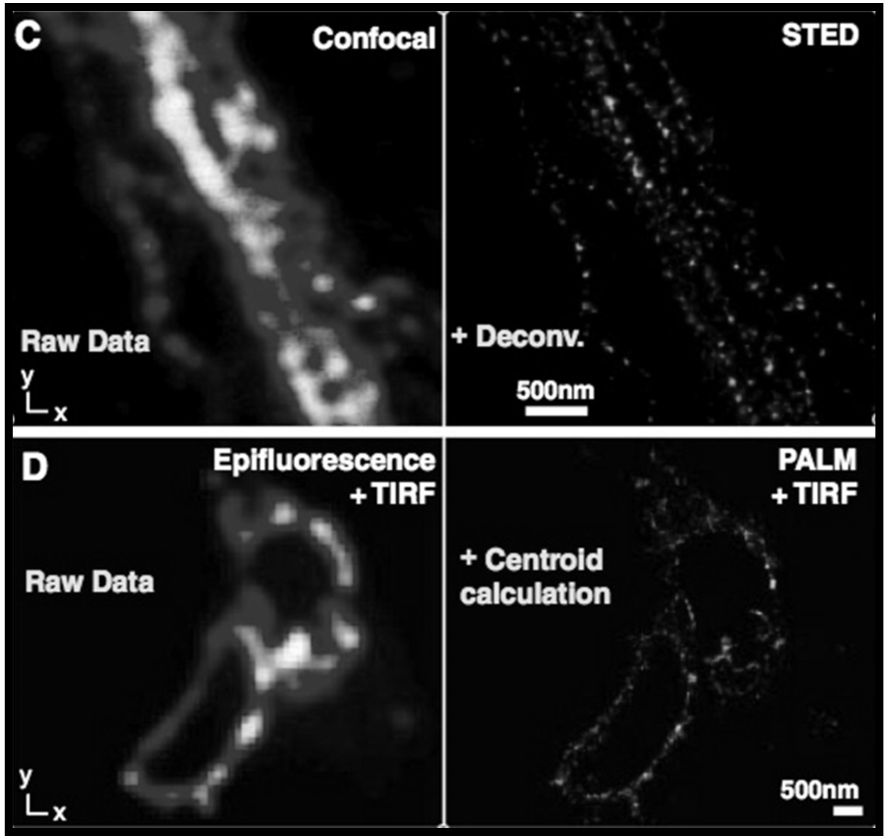
Applications of optics and photonics

Labelling of biological tissues and molecules with coloured dyes is the basis of almost all cited techniques.

Example Super-resolution fluorescence microscopy

Comparison Confocal vs STED

Comparson Conv.Micr. vs PALM



Applications of optics and photonics

- Usually the molecules that are used to stain (label) the biological tissues, proteins or DNA fragments are organic dyes.
- As a consequence this part of the course will be devoted to describe the mechanisms that govern the interaction of an organic molecule with the electromagnetic radiation (light), in particular absorption and emission of light.
- By the end of the course we shall also address emission and asborption of metal and semiconducting nanoparticles.

Concepts on the energy levels of organic molecules

Molecules are constituted by nuclei and electrons. They are characterized by quantum states that are associated to the motion and interaction of charges, to their vibration and to their rotation. We therefore have electronic, vibrational, rotational and spin contributions to the energy of the levels.

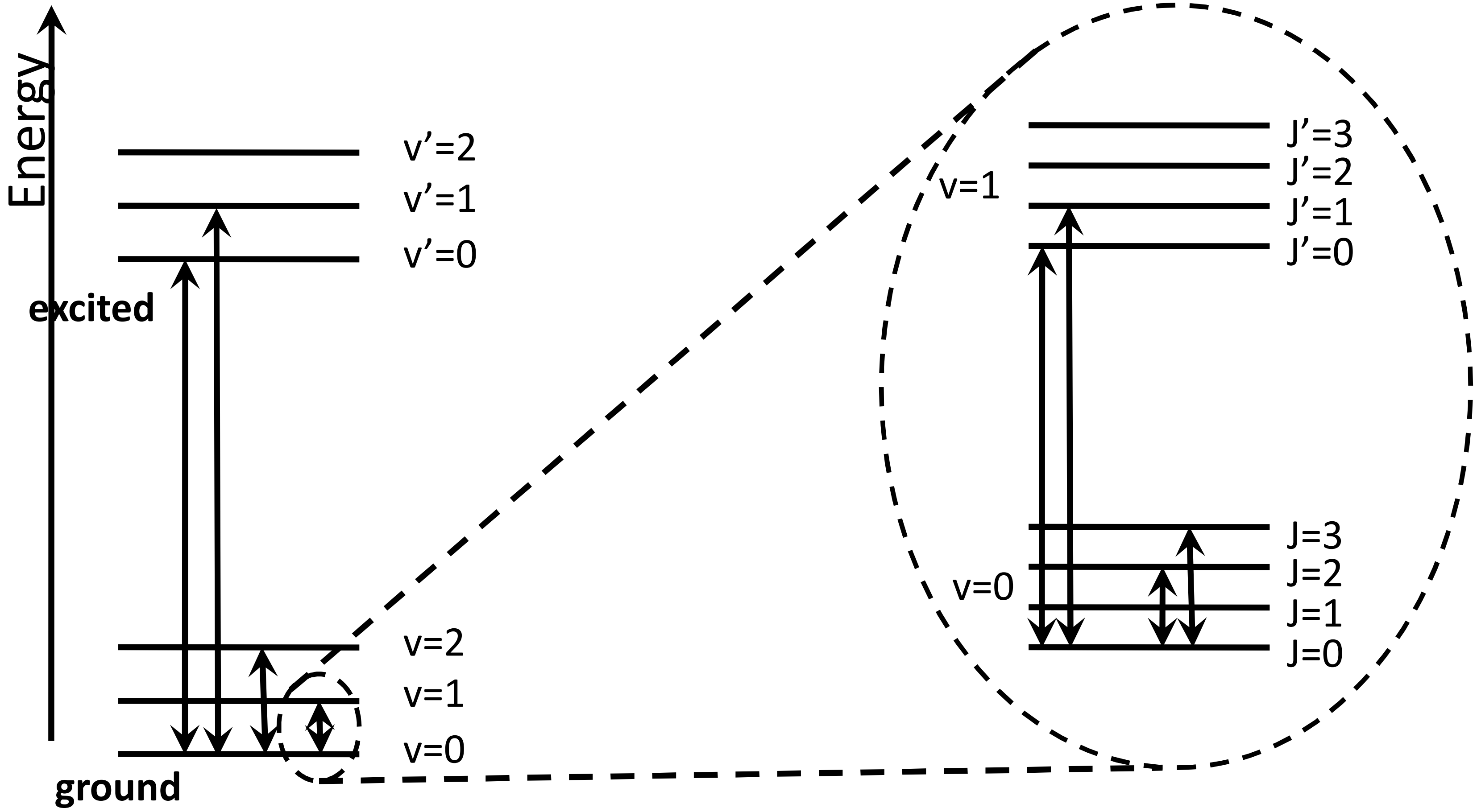
Every quantum state is associated to an energy, which is approximately given by the sum of such contributions:

$$\mathbf{E_{mol} \sim E_{el} + E_{vib} + E_{rot} + E_{spin}}$$

with

$$E_{el} > E_{vib} > E_{rot} > E_{spin}$$

Concepts on the energy levels of organic molecules



Transitions	electronic	E_{el}	$\sim 2\div3$ eV
	vibrational	E_{vib}	$\sim 10\div300$ meV
	rotational	E_{rot}	$\sim 0.3\div1$ meV
	spin	E_{spin}	$\sim 0.4\div2$ μeV

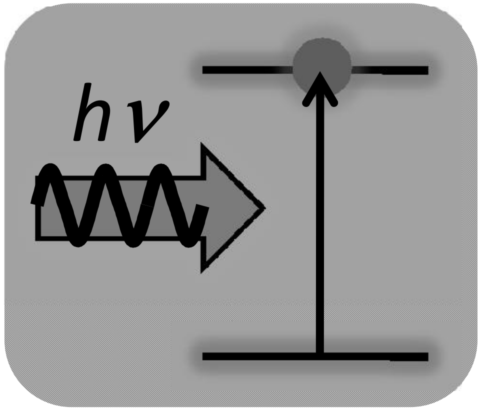
1 eV= $1.602\cdot10^{-19}$ J= 8065 cm^{-1}

Linear interaction betwen organic molecules and e.m. radiation

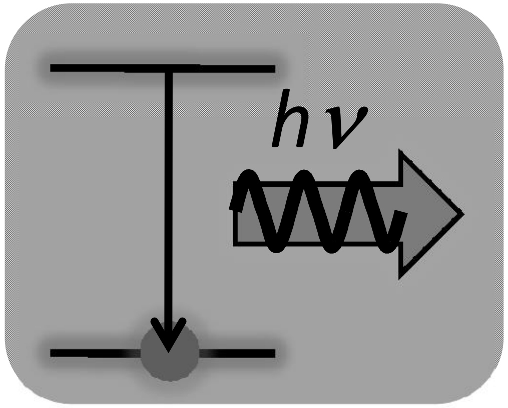
The transition between two energy levels can be associated to the absorption/emission of a photon with energy given by the difference of the energies of the two levels:

$$h\nu = \Delta E = E_2 - E_1$$

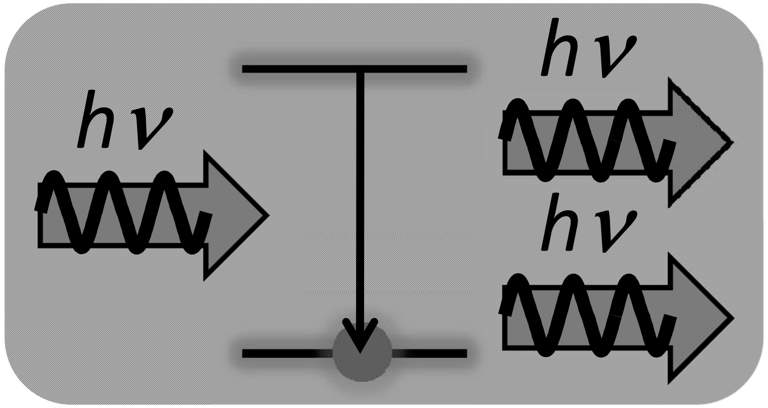
There exist three fundamental linear interaction processes :



Absorption



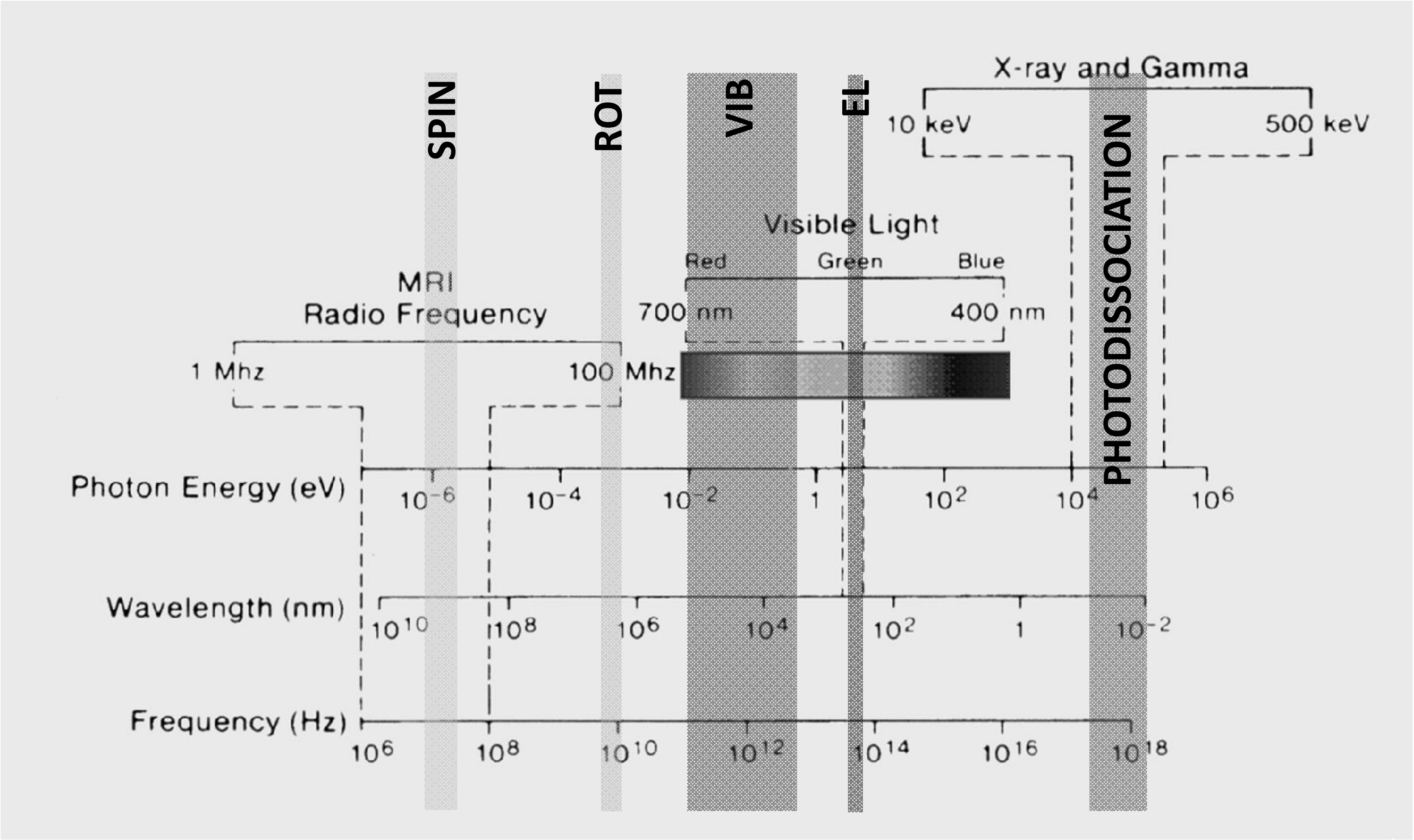
Spontaneous Emission



Stimulated Emission

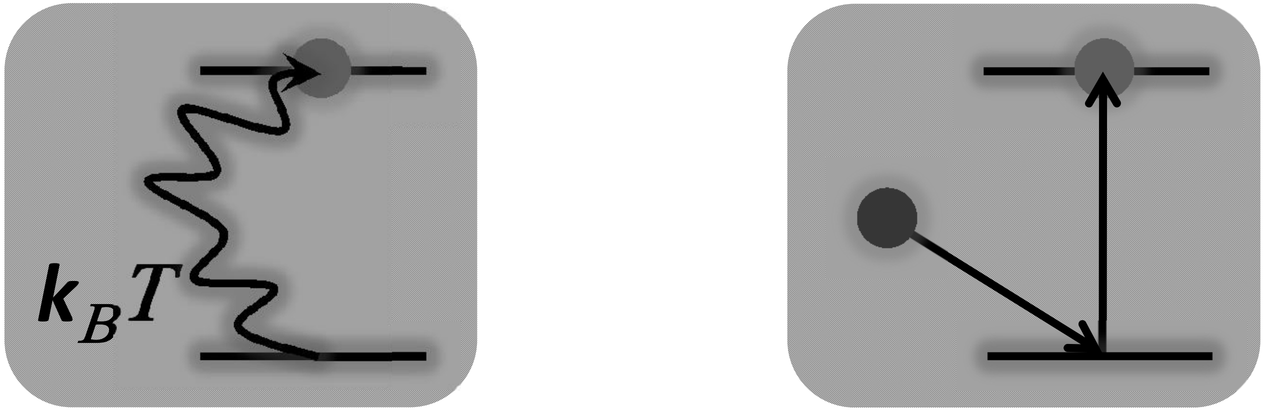
In the dipole interaction approximation <u>not</u> all transitions are permitted but there are some *Selection Rules*

Linear interaction betwen organic molecules and e.m. radiation



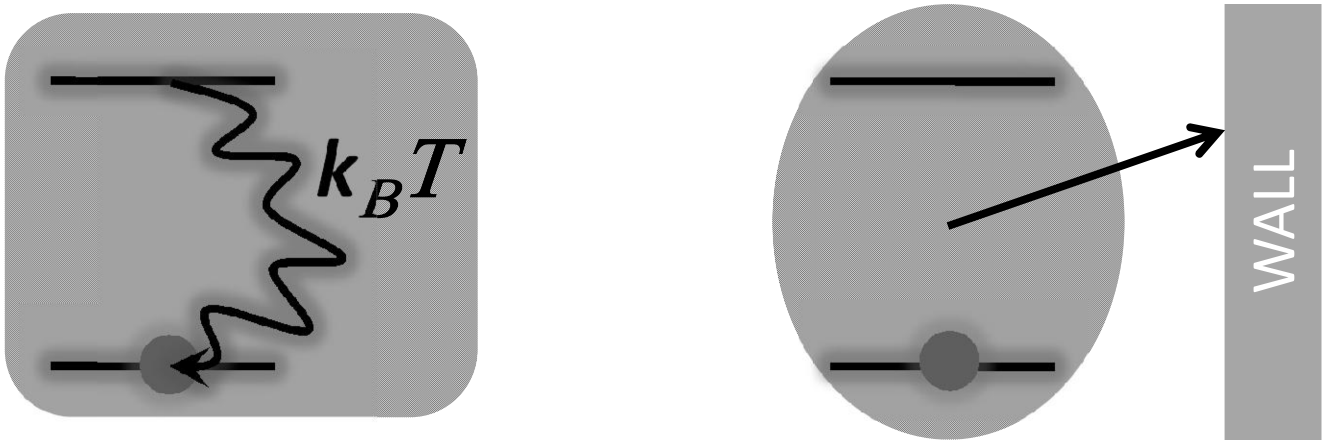
Linear interaction betwen organic molecules and e.m. radiation

The transition between two energy levels can take place also without absorption/emission of a photon.



Temperature

Collision

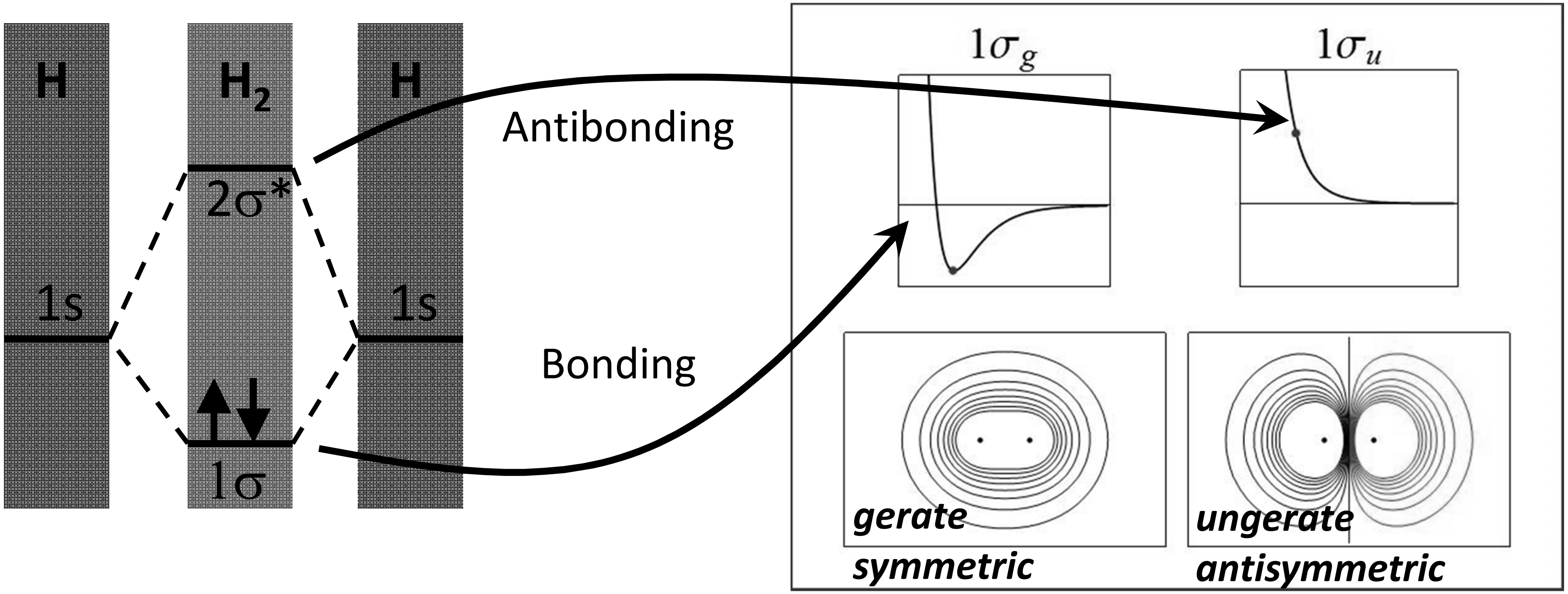


For such processes there is no selection rule

More concepts concerning the levels of organic molecules

The levels diagram of a molecule depends on the levels of the atomic constituents.(LCAO)

Homonuclear Biatomic Molecules: H_2 case

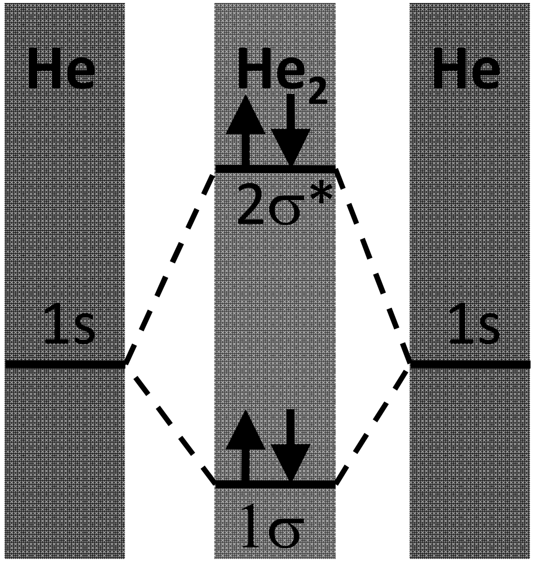


Interaction energy: Coulomb repulsion between nuclei
Coulomb repulsion between electrons
Coulomb attraction between electrons & nuclei

The energy of a level depends on the internuclear distance R (Morse curve)

More concepts concerning the levels of organic molecules

Homonuclear Biatomic Molecules : He$_2$ case

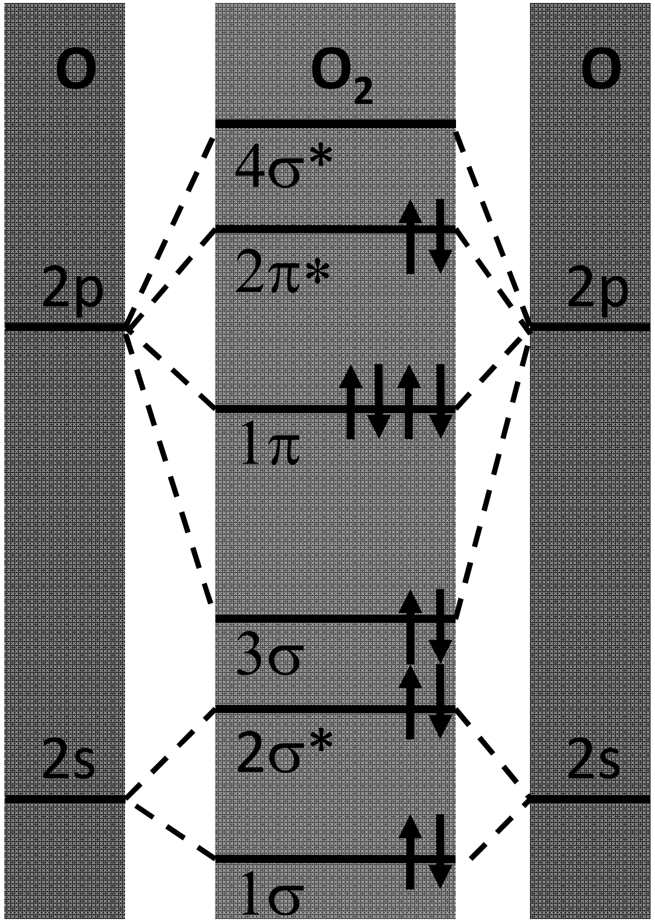


The sum of the energies of the electrons in the bonding and antibonding orbitals is greater than the sum of the energies of the single and separated atoms.

The He$_2$ molecule therefore is not stable and does not exist..

More concepts concerning the levels of organic molecules

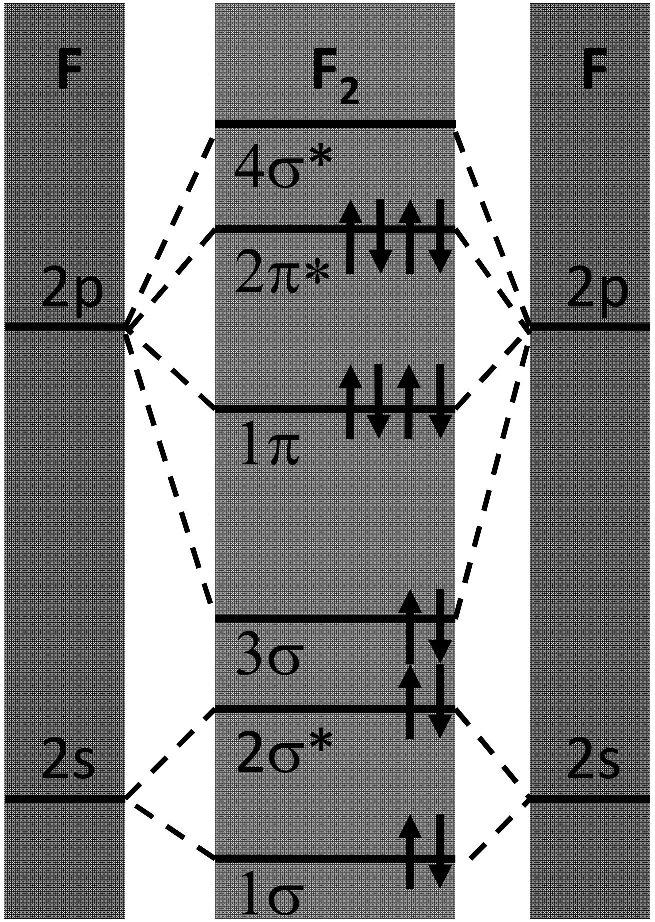
Homonuclear Biatomic Molecules : caso O_2 and F_2 case



- The atomic orbitals that pertain to the inner shells do not combine to generate molecular orbitals.
- Only the orbitals with the same energy can combine
- Only couples of orbitals that have the same symmetry can combine
- In the case of O_2 and F_2 the 2s and 2p atomic orbitals do not overlap

More concepts concerning the levels of organic molecules

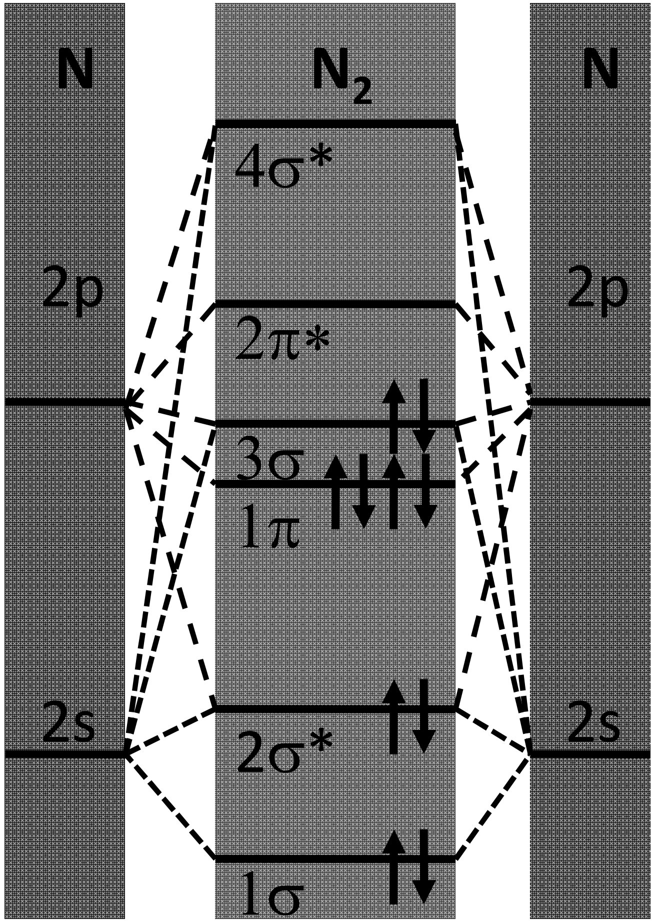
Homonuclear Biatomic Molecules : caso O_2 and F_2 case



- The atomic orbitals that pertain to the inner shells do not combine to generate molecular orbitals.
- Only the orbitals with the same energy can combine
- Only couples of orbitals that have the same symmetry can combine
- In the case of O_2 and F_2 the 2s and 2p atomic orbitals do not overlap

More concepts concerning the levels of organic molecules

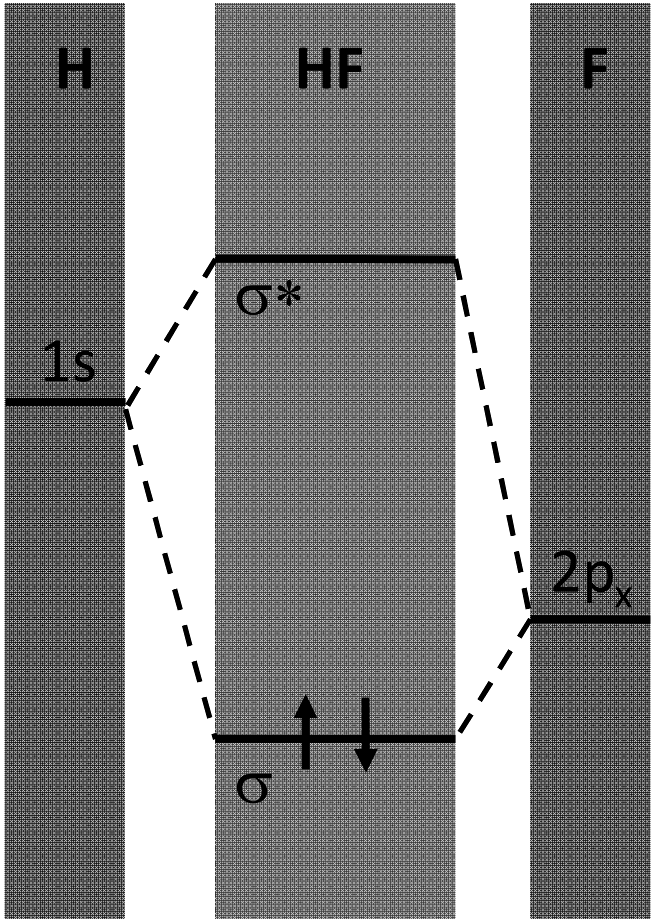
Homonuclear Biatomic Molecules : Li_2 ,Be_2, B_2, C_2 , N_2 case



- The atomic orbitals that pertain to the inner shells do not combine to generate molecular orbitals.
- Only the orbitals with approximately the same energy can combine
- Only couples of orbitals that have the same symmetry can combine
- In this case the 2s and 2p atomic orbitals are overlapped

More concepts concerning the levels of organic molecules

Heteronuclear Biatomic Molecules : HF case



- One must take into consideration only the atomic orbitals of the most external shell, therefore 1s for the hydrogen and $2p_x$ for the fluorine.

- Wet get two molecular orbitals of the σ type, one is bonding and the other antibonding

More concepts concerning the levels of organic molecules

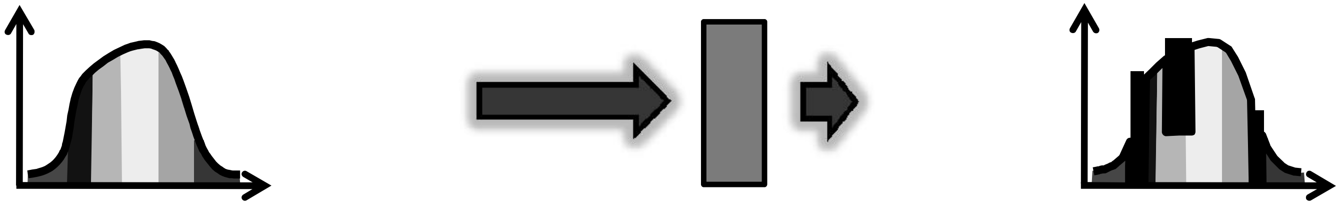
Polyatomic molecules

- For polyatomic and heteronuclear molecules the energy levels can be found by the same approach used for the biatomic molecules by using a larger number of orbitals, which are extended all over the whole molecule.

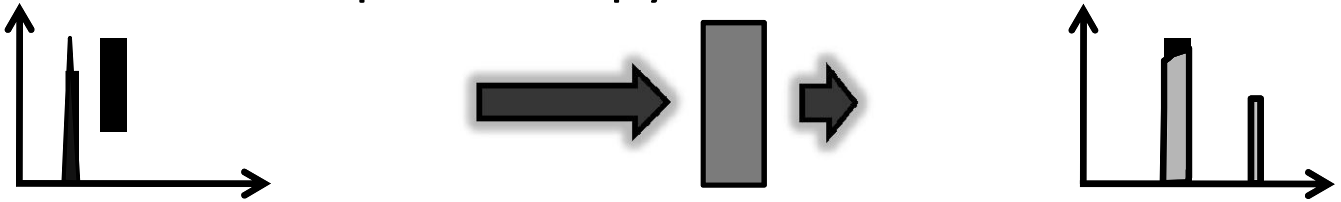
Optical spectroscopy

The energy levels of a molecule can be investigated by means of optical spectroscopy, therefore studing the interaction of molecular charges with the electromagnetic radiation (e.m.r.). Basically, there exist three types of optical spectrosopy:

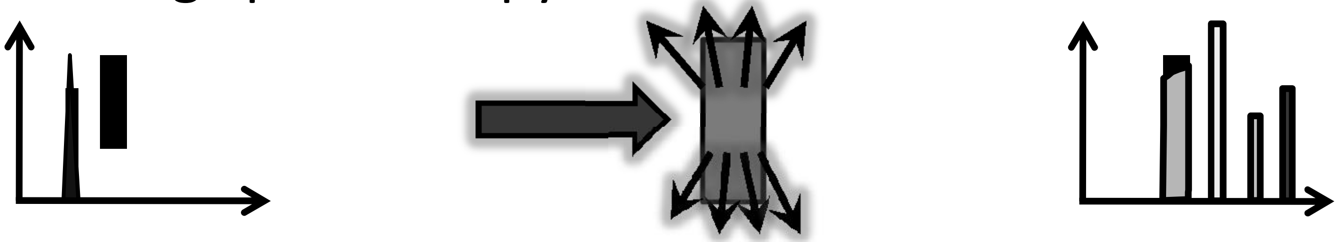
- Absorption Spectroscopy → Attenuation of the e.m.r.



- Luminescence Spectroscopy → Emission of the e.m.r.

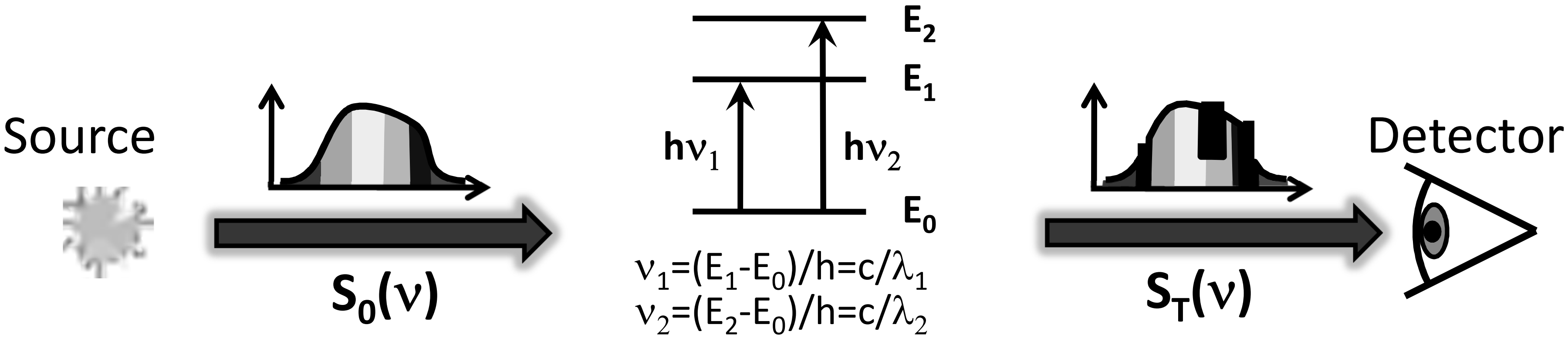


- Scattering Spectroscopy → Diffusion of the e.m.r.



Absorption Spectroscopy

It studies the attenuation of the e.m.r. due to absorption in a molecular system as a function of the wavelength.



Source

$S_0(\nu)$

E_2

E_1

E_0

$h\nu_1$ $h\nu_2$

$\nu_1=(E_1-E_0)/h=c/\lambda_1$

$\nu_2=(E_2-E_0)/h=c/\lambda_2$

$S_T(\nu)$

Detector

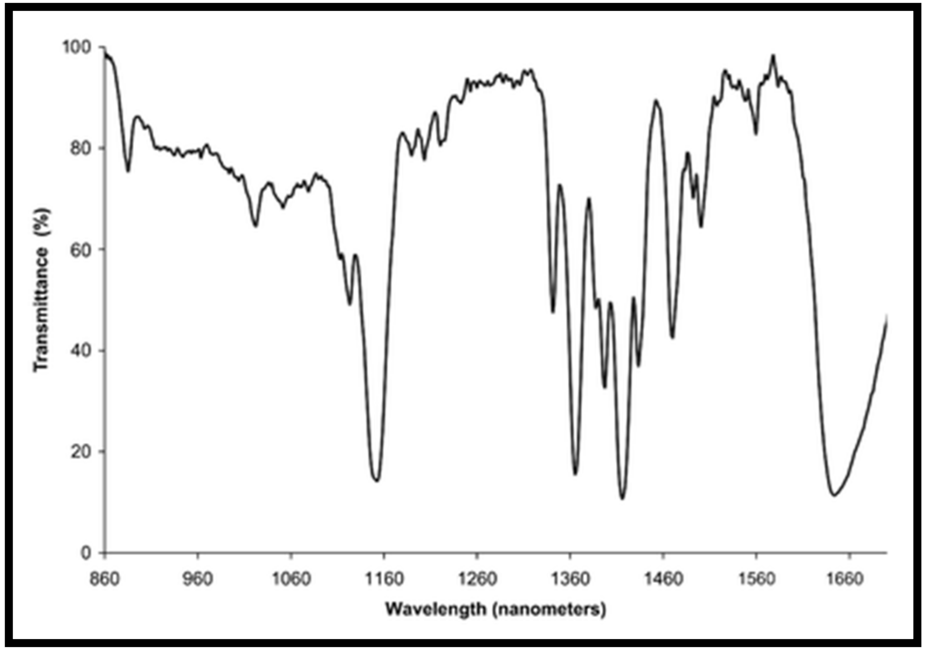
The spectrometers provide a measurement of the normalized spectral transmittance, removing the effects due to specular reflection at the interfaces of the sample:

$$T(\nu)=\frac{S_T(\nu)}{S_0(\nu)}$$

Absorption Spectroscopy

Generally, the transmission spectrum does not show infinitesimally sharp lines, rather finite width bands.
The principal cause of the line broadening are:

- Lifetime of the excited state τ (intrinsic broadening)
- Doppler
- Interactions with the external environment (collisions)
- Superposition of several different bands (example: vibrational structure)

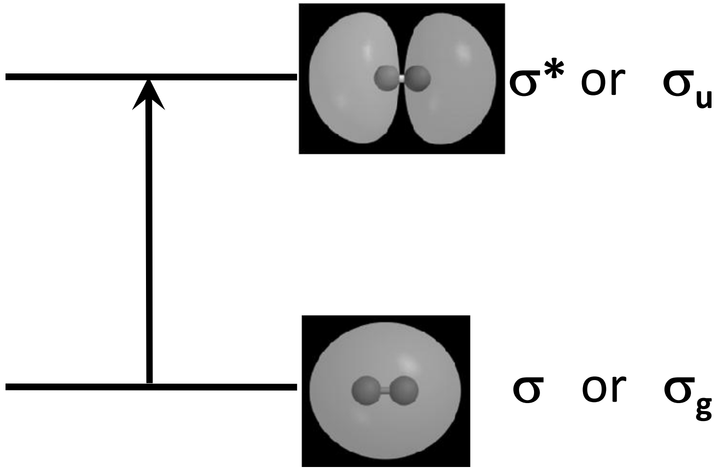


Transmission spectrum of liquid Dichloromethane

Absorption Spectroscopy

Selection Rule

The transition between two energy levels can be induced by e.m.r. only if there is a change of the molecular dipole moment when transiting form a level to the other. In QM the dipole moment operator must have a non-zero matrix element between the starting and arrival states.



$$P_{TRANS} \propto \left\langle \Psi_{part} \middle| \vec{\mu} \middle| \Psi_{arr} \right\rangle =$$

$$= \left\langle \Psi_{part} \middle| -e \cdot \vec{r} \middle| \Psi_{arr} \right\rangle$$

Anti-symmetric (ungerate)

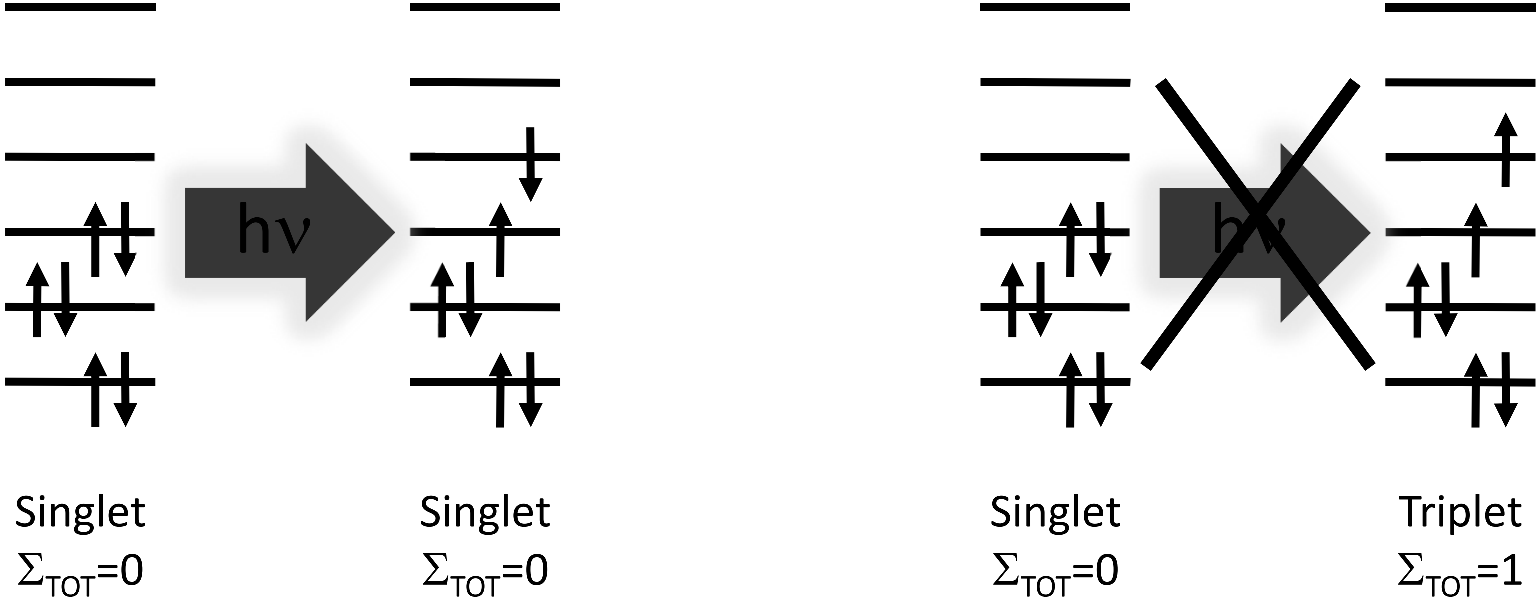
The starting and arrival states must have opposite parity.

Absorption Spectroscopy

Selection Rule

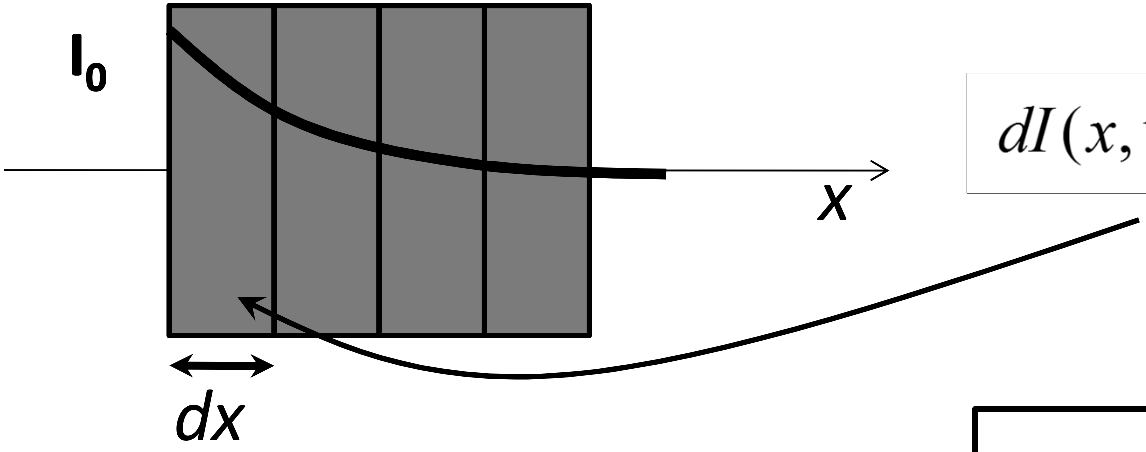
The transition between two energy levels can be induced by e.m.r. only if there is no change of the total spin Σ when transiting form a level to the other (in the approximation of weak spin-orbit coupling)

The states are classified in singlet states ($\Sigma_{TOT}=0$) and triplet states ($\Sigma_{TOT}=1$)



Absorption Spectroscopy

Macroscopically, the material absorption is described by the ***Lambert-Beer*** law. Such a law is derived from the assumption that the luminous intensity ***I***, when passing through an infinetly thin layer with thickness ***dx***, decreases by a quantity ***dI*** that is proportional to the thickness dx and to the local intensity itself. The proportionality coefficient α is the absorption coefficient.



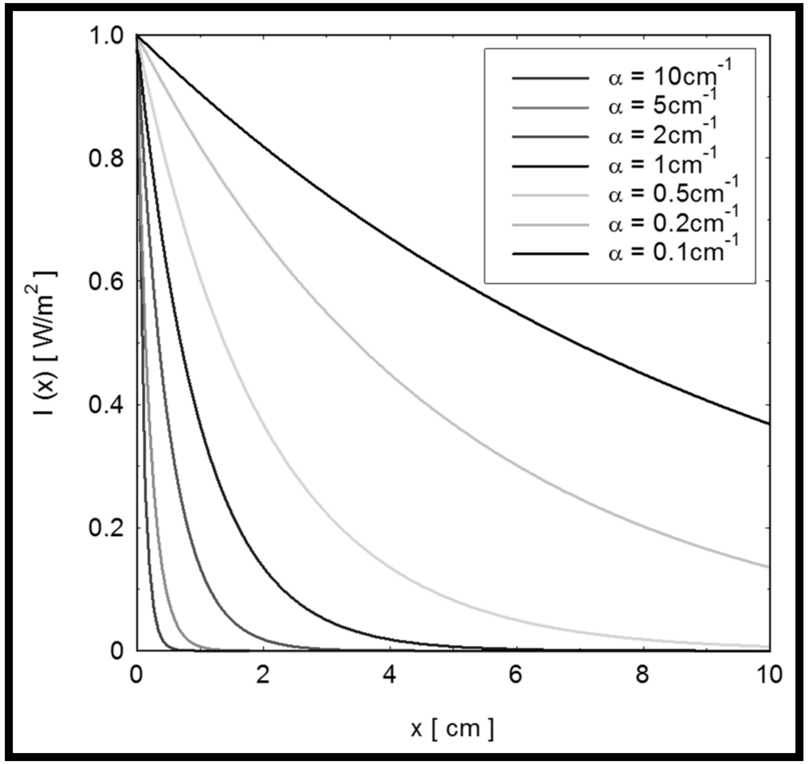
$$dI(x,\nu) = -\alpha(\nu) \cdot I(x,\nu) \cdot dx$$

$$\frac{dI(x,\nu)}{I(x,\nu)} = -\alpha(\nu) \cdot dx$$

$$\int_{I_0}^{I(x)} \frac{dI}{I(x,\nu)} = -\alpha(\nu) \cdot \int_0^x dx'$$

$$\boxed{I(x,\nu) = I_0(\nu) \cdot e^{-\alpha(\nu)x}}$$

Absorption Spectroscopy



$$I(x,\nu) = I_0(\nu) \cdot e^{-\alpha(\nu)x}$$

- The intensity decreases exponentially inside an absorbing material
- α is the *absorption coefficient* and is measured in *cm*$^{-1}$
- ***L=1/α*** is the *absorption lenght* and corresponds to the thickness that is necessary to reduce the intensity by a factor 1/e.

Absorption Spectroscopy

We define ***absorbance*** the quantity:

$$\boxed{A = \log_{10} \frac{1}{T}}$$

In chemistry, for a solution of organic molecules in a transparent (non absorbing) solvent the transmittance of a sample with thickness h is defined as:

$$\boxed{T(\nu) = 10^{-\varepsilon(\nu)Ch}}$$

where $\varepsilon(\nu)$ is the molar extinction coefficient (L mol^{-1} cm^{-1}) and C is the concentration (mol L^{-1}).

Therefore we have:

$$\boxed{A = \log_{10} \frac{1}{T} = -\log_{10}\left[10^{-\varepsilon(\nu)Ch}\right] = \varepsilon(\nu)Ch}$$

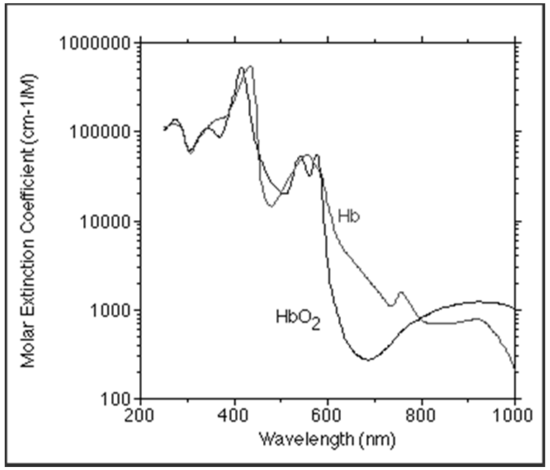
And A is proportional to the concentration.

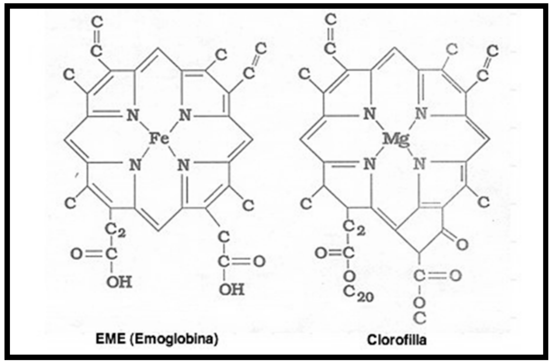
NOTE $\boxed{\alpha(\nu) = \ln 10 \cdot \varepsilon(\nu)C = 2.3 \cdot \varepsilon(\nu)C}$

Absorption Spectroscopy

EXAMPLE – LIVING TISSUES

In the visible range, the absorption of soft living tissues is principally caused by the hemoglobin that is present in blood in water solution.





In an adult, hemoglobin (Hb) normally is present at the concentration :

$$C = 135 - 170 \text{ g/L}$$

Taking into account the molecular weight of Hb MW= 68000 dalton, we have:

$$C = 2.2 \text{ mM} = 2.2 \text{ mmol/L}$$

Considering that $\varepsilon_{Hb} = 5 \cdot 10^5$ L mol^{-1} cm^{-1}, for example at λ = 420nm, we get:

$$\boldsymbol{\alpha = 2.3\varepsilon C = 2.3 \times 5 \cdot 10^5 \times 2.2 \cdot 10^{-3} \cong 2500 \text{ cm}^{-1}}$$

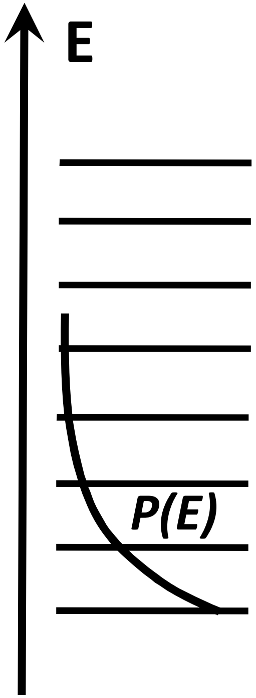
$$\mathbf{L = 1/\alpha \cong 4\mu m = 4 \cdot 10^{-6} m}$$

Absorption Spectroscopy

Some basic issue concerning the ***Boltzmann Statistics***

In stationary conditions (thermal equilibrium), the probability that an energy level is occupied depends on temperature (***T***) and on the energy separation between levels (***∆E***).

$$\boxed{\frac{P(E)}{P(E_0)} = e^{-\frac{\Delta E}{k_B T}} \quad \text{con} \quad k_B = 1.38 \cdot 10^{-23} J/K}$$



The number of molecules in an excited state at energy E is given by:

$$\boxed{\frac{N(E)}{N(E_0)} = \frac{g(E)}{g(E_0)} e^{-\frac{\Delta E}{k_B T}}}$$

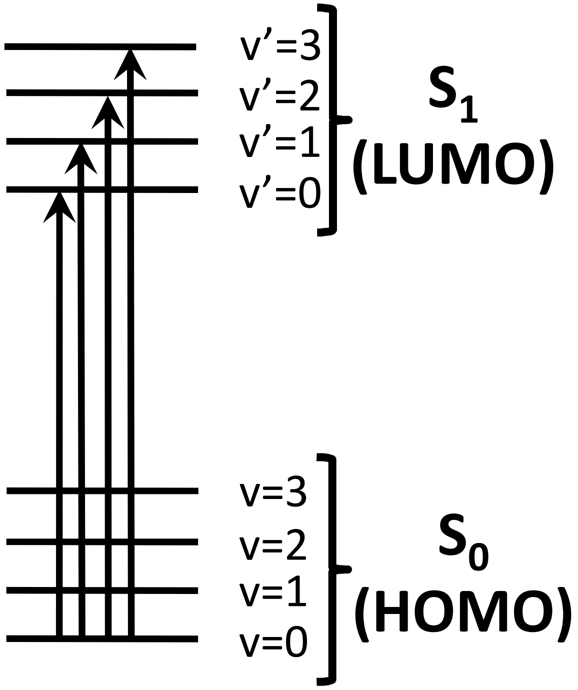
where ***g*** is the ***degenerararation*** of the energy level E.

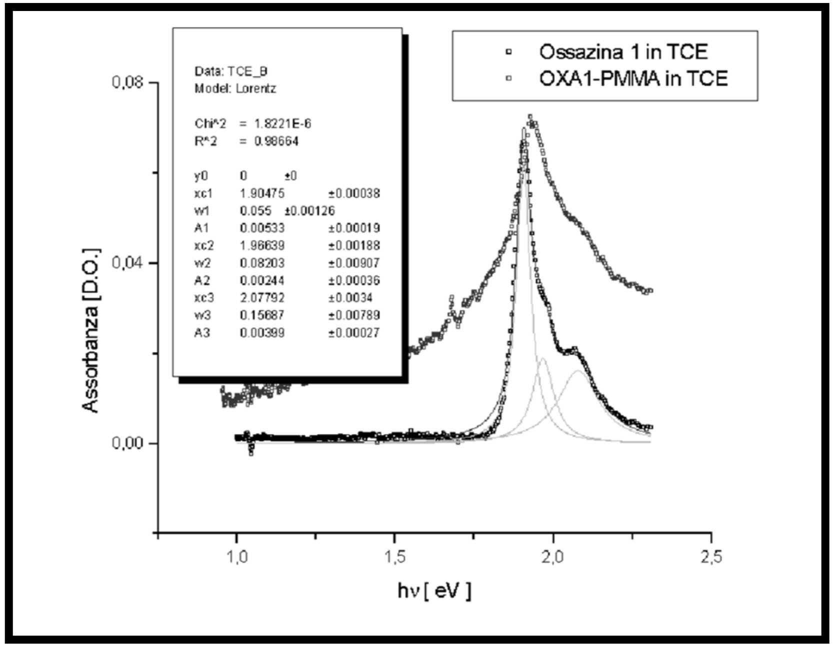
Levels	∆E (eV)	$P(E_1)/P(E_0)$ @ 300K
Electronic	2.5	10^{-42}
Vibrational	0.12	0.009
Rotational	0.0005	0.98

At room temperature (RT) all molecules are in the ground electronic and vibrational state. The rotational levels are possibly populated.

Structure of the absorption spectra

If we analyze the shape of the single bands we can notice a sub-structure that depends on the molecular vibrational levels. Such sub-bands are named vibronic bands.



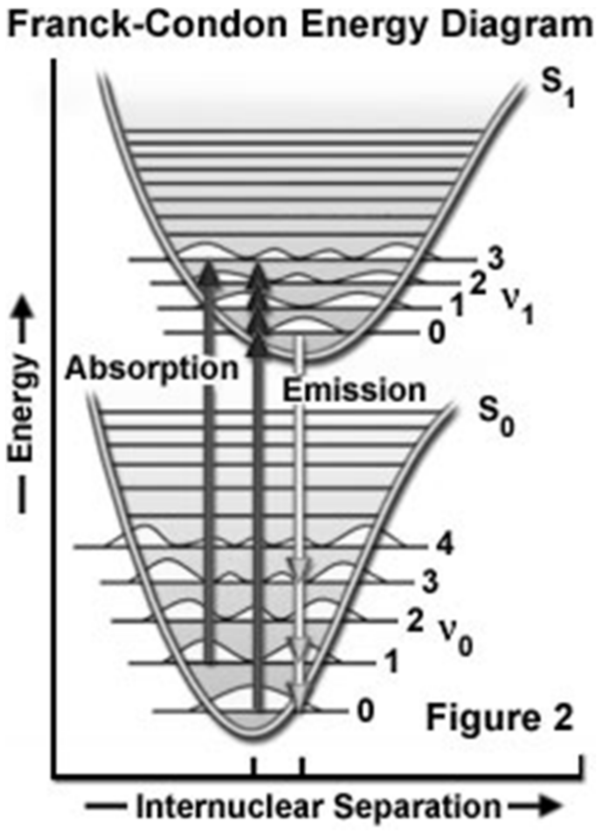


```
Data: TCE_B
Model: Lorentz

Chi^2   =  1.8221E-6
R^2     =  0.98664

y0      0          ±0
xc1     1.90475             ±0.00038
w1      0.055   ±0.00126
A1      0.00533             ±0.00019
xc2     1.96639             ±0.00188
w2      0.08203             ±0.00907
A2      0.00244             ±0.00036
xc3     2.07792             ±0.0034
w3      0.15687             ±0.00789
A3      0.00399             ±0.00027
```

HOMO - Highest Occupied Molecular Orbital
LUMO - Lowest Unoccupied Molecular Orbital

Structure of the absorption spectra

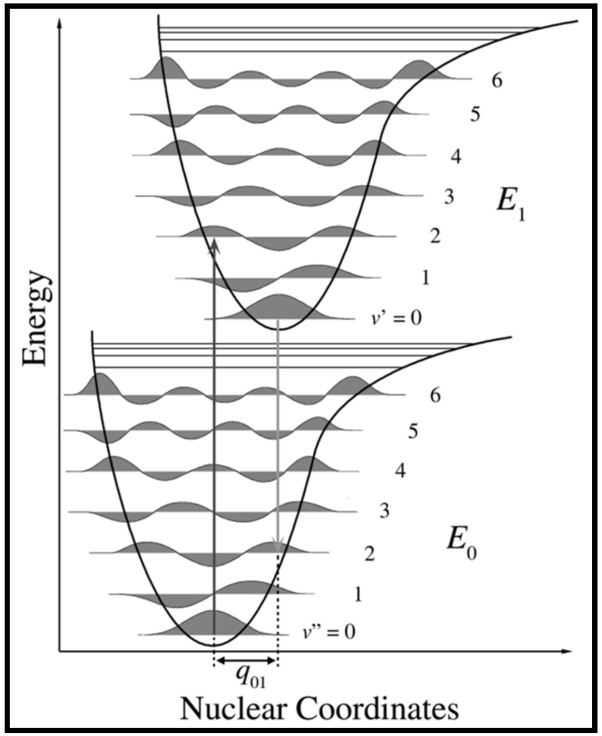
Biatomic Molecule We have only one vibrational mode along the main axis of the molecule

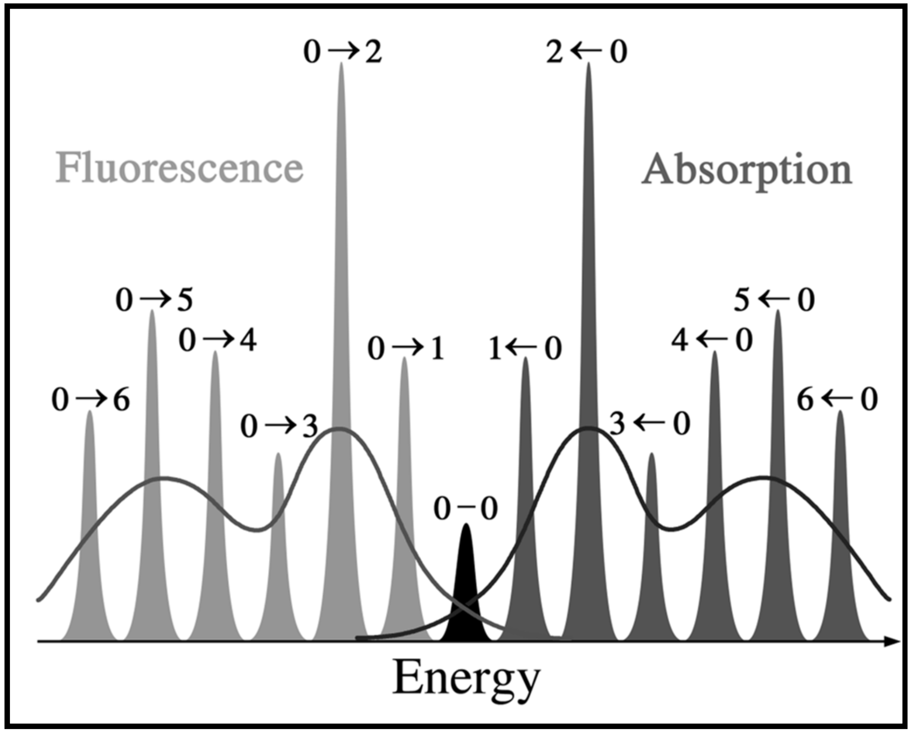


- The Morse energy curve can be approximated by that of a harmonic oscillator (***parabola***). The vibrational levels are equispaced.
- The electrons move much faster than the nuclei do (***Born-Oppenheimer Approximation***)
- The electronic transitions take place without any change of the internuclear distance (***Franck and Condon principle***)
- At room temperature only the lowest vibrational level of the S_0 electronic level is occupied
- The relative intensity of the vibronic bands depends on the superposition of the vibrational wave-functions in the two electronic states S_0 and S_1.

Structure of the absorption spectra

The intensity of the absorption (and emission) lines is proportional to the superposition integral of the vibrational wave-functions for the levels of the HOMO and LUMO states.

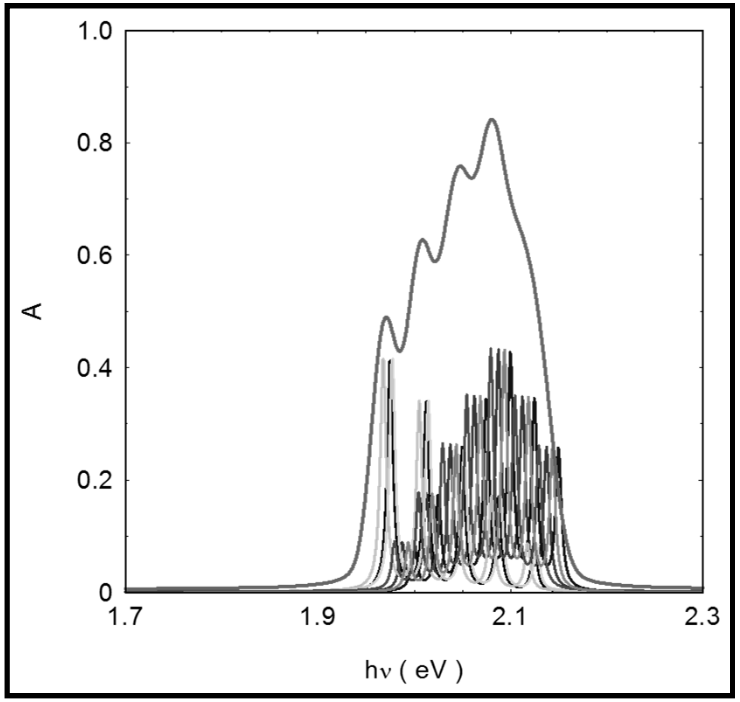




The electronic transitions between the lowest vibrational levels of the two electronic levels are characterized by the same energy (frequency) both in absorption and in emission (***zero-phonon line***)

Structure of the absorption spectra

Polyatomic Molecules We have more than a vibration mode. All vibration modes can contribute simultaneously to the shape of the absorption spectrum. The spectrum appears as a single asymmetric band with vibrational contributions that are not easily distinguished.

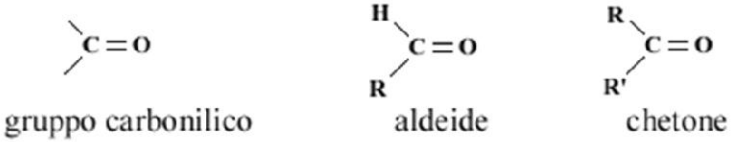


It is therefore very difficult to carry out a detailed analysis of the vibrational modes.

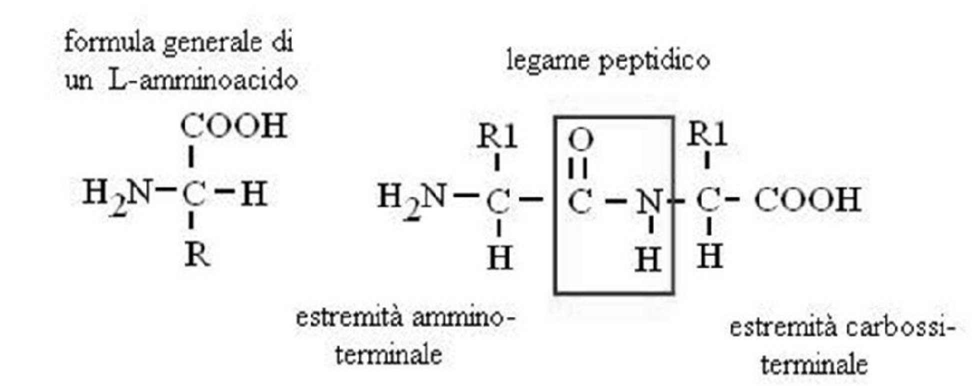
Absorption Spectra of endogenous chromophores

The basic biochemical compounds are characterized by either bonds or molecular groups with peculiar spectroscopic features:

- ***Carbonyl Group***



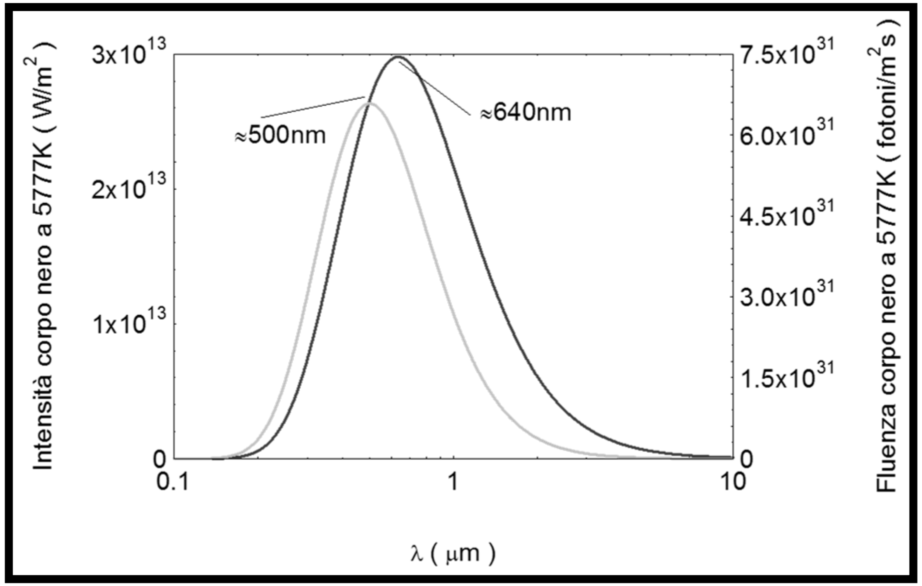
- ***Peptidic Group***

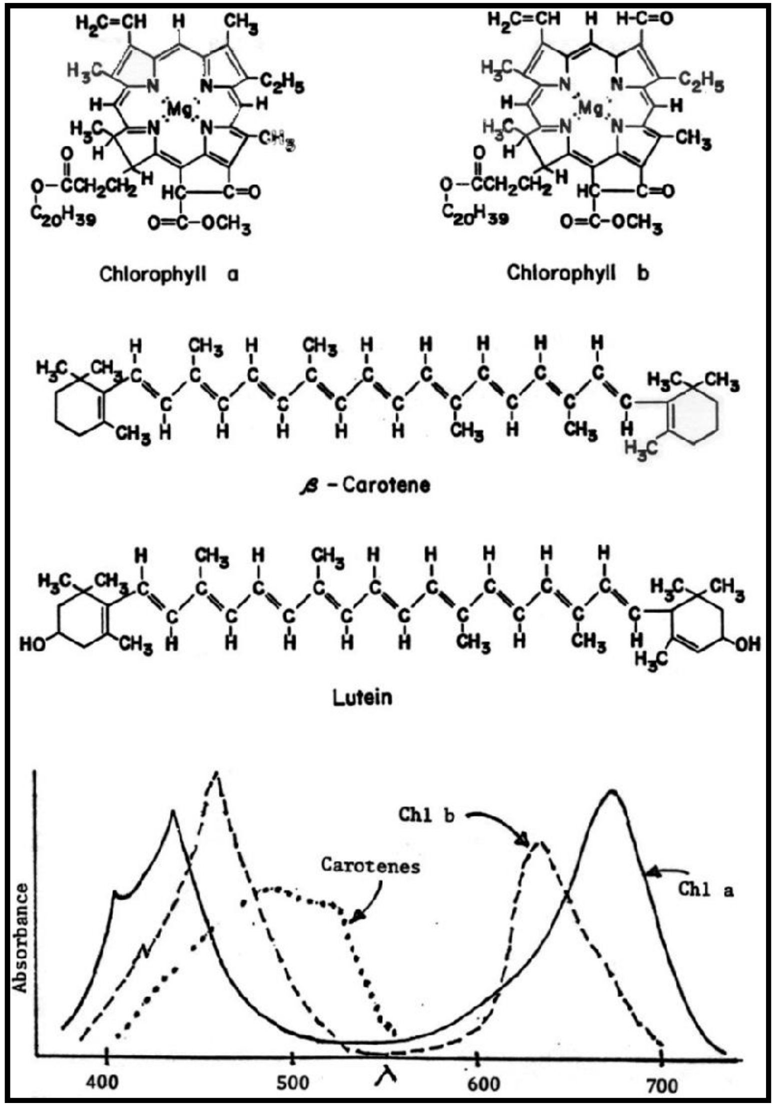


- ***Conjugated double bonds*** in linear polymers (carotenoids, retinol) and cyclic polymers (porphyrin derivates, aromatic amino acids, bases of the nucleic acids)
- ***Ions of transiton metals*** in metallo-proteins: Mn, Fe, Co, Cu, Mo, V

Absorption Spectra of endogenous chromophores

Examples
- Chlorophylls
- Carotenoids

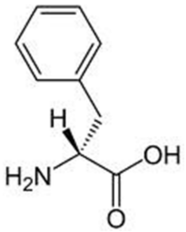




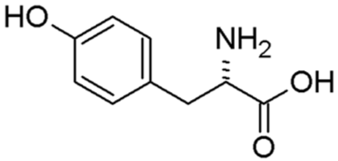
Absorption Spectra of endogenous chromophores

Examples

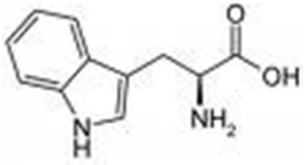
- Amino Acids



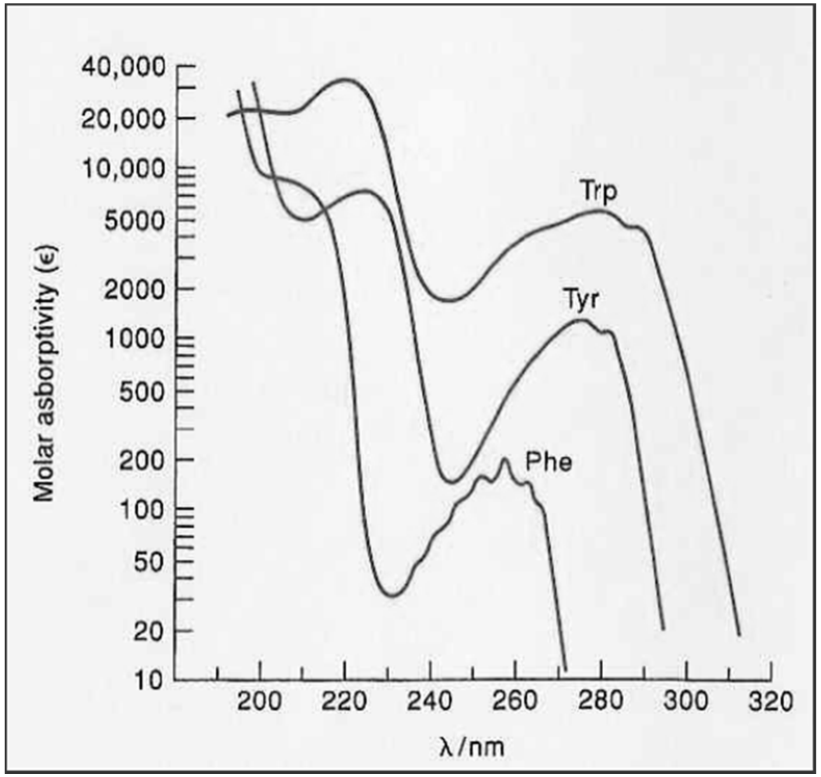
Phenylalanine (Phe)



Tyrosine (Tyr)



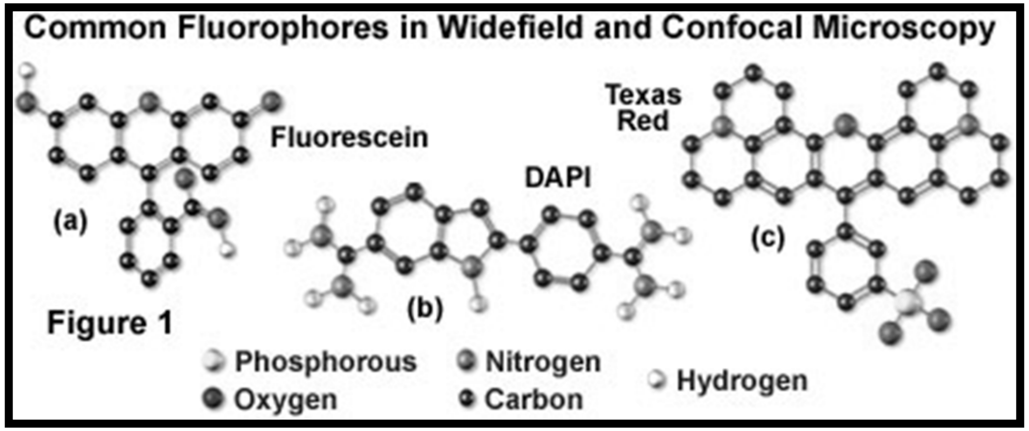
Tryptophan (Trp)

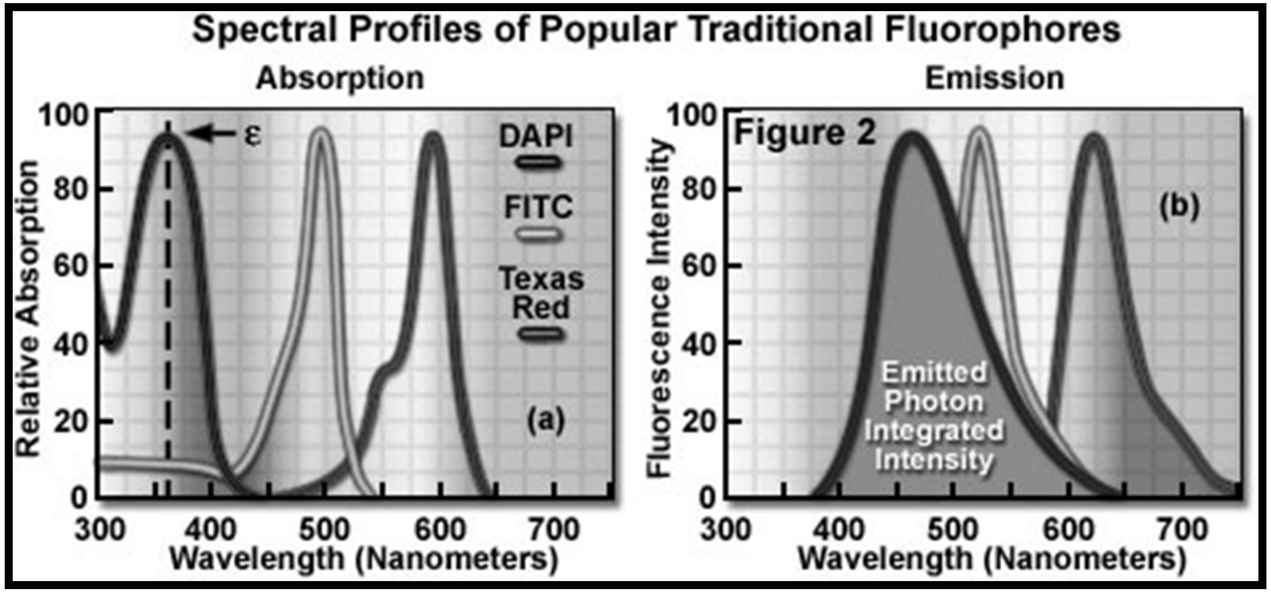


Absorption Spectra of exogenous chromophores

There exist a large variety of organic chromophores that are used to label the basic biochemical compounds. They possess peculiar absoprtion and luminescence properties..

EXAMPLE ***Traditional dyes***

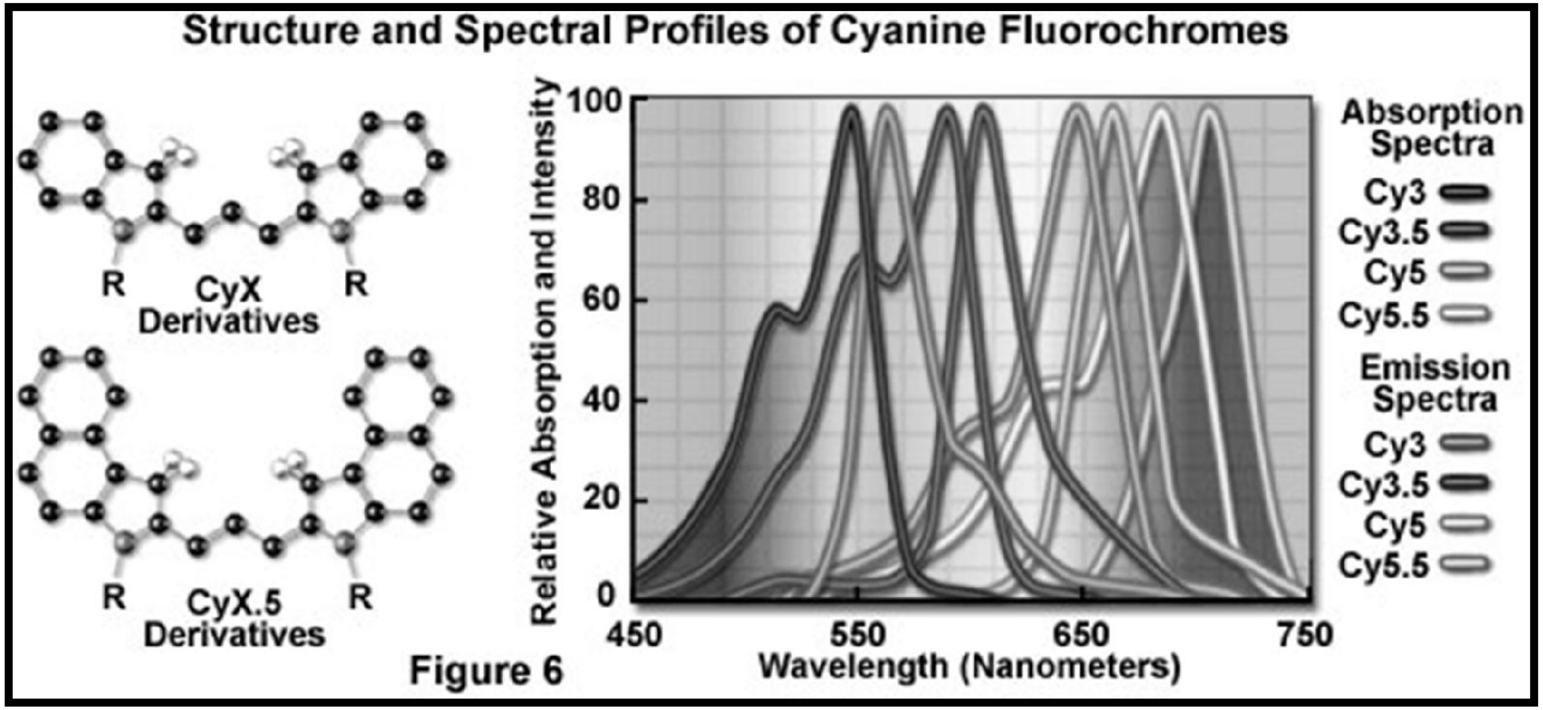




Absorption Spectra of exogenous chromophores

EXAMPLE ***Cyanine***

- Based on the partially saturated indol group with two aromatic units.
- Connection via a variable length alkenic bridge
- Absoprtion and emission spectra similar to those of the traditional dyes
- Better water solubility
- Less sensible to pH and to an organic environment
- Large photostability
- Large quantum yield



Structure and Spectral Profiles of Cyanine Fluorochromes

Figure 6

Absorption Spectra of exogenous chromophores

EXAMPLE ***Alexa Fluor***

- Derived from the most common organic dye, Rhodamine
- Larger quantum yield
- Excellent solubility in water
- pH insensitive
- Very high photostability

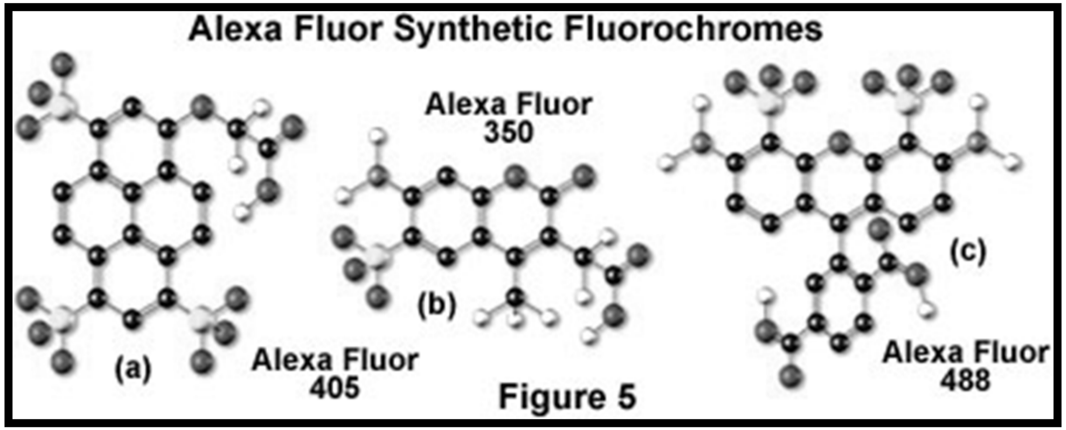
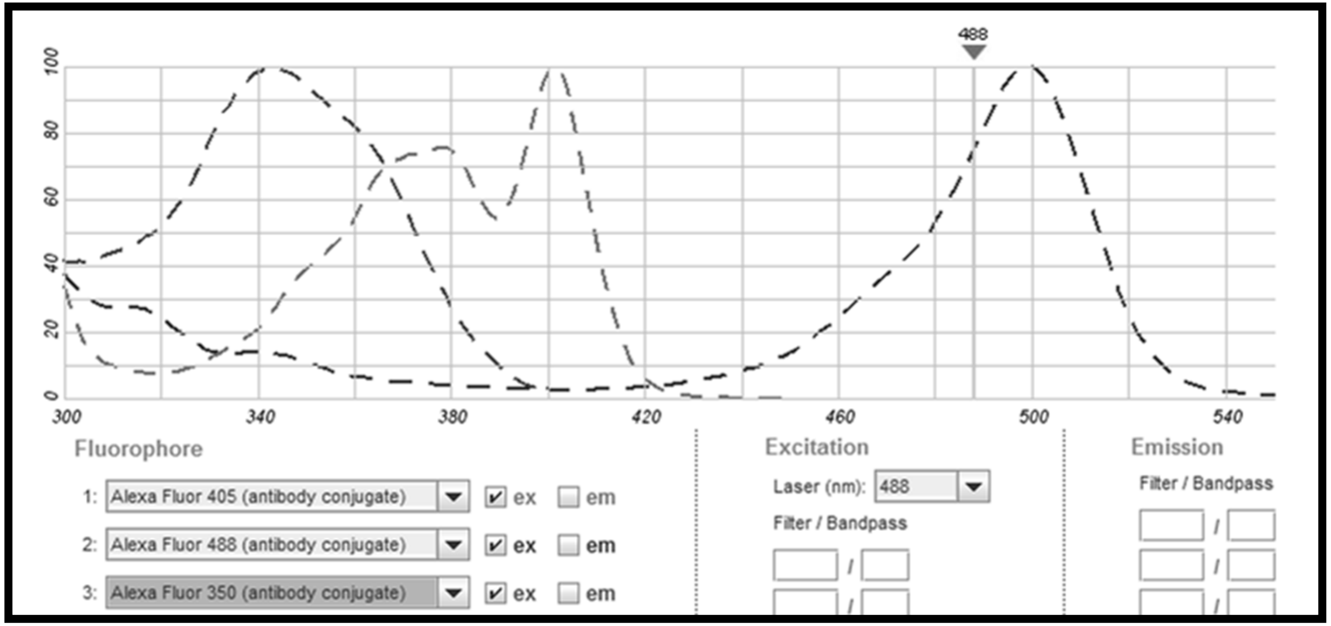


Figure 5



Fluorophore

1: Alexa Fluor 405 (antibody conjugate) ☑ ex ☐ em

2: Alexa Fluor 488 (antibody conjugate) ☑ ex ☐ em

3: Alexa Fluor 350 (antibody conjugate) ☑ ex ☐ em

Excitation

Laser (nm): 488

Filter / Bandpass

Emission

Filter / Bandpass