# SAPIENZA Università di Roma Laurea magistrale in Ingegneria delle Nanotecnologie A.A. 2020-2021

# **Biophotonics Laboratory Course**

Prof. Francesco Michelotti
SAPIENZA Università di Roma
Facoltà di Ingegneria Civile e Industriale
francesco michelotti Quniroma 1. it

#### 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** 

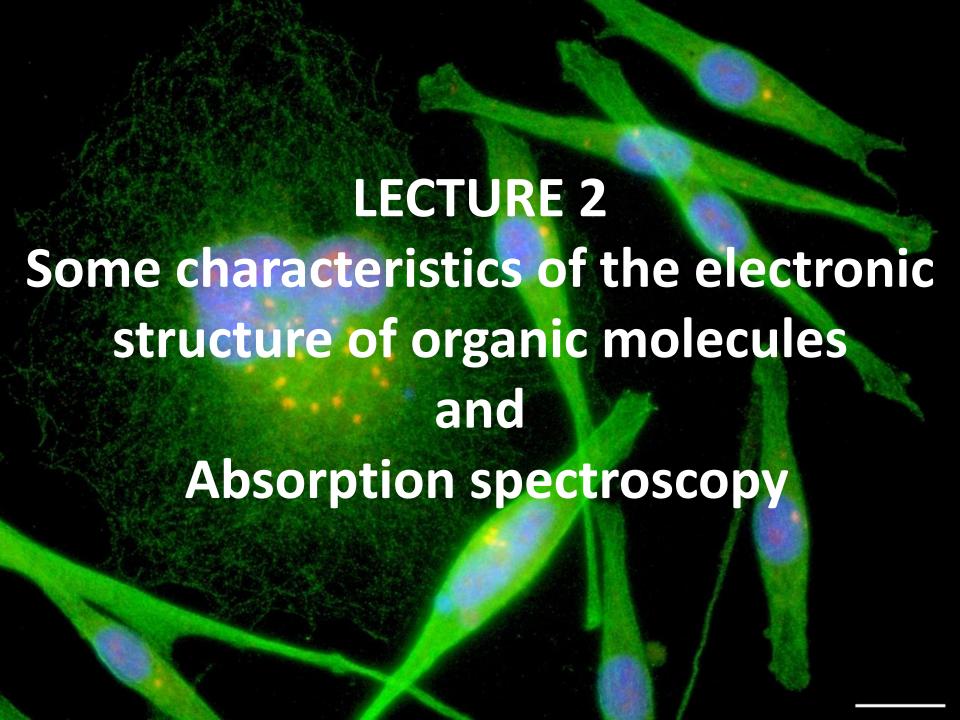
#### **Techniques**

- Southern
- Western
- Northern

# Non-Microscopic Label-free

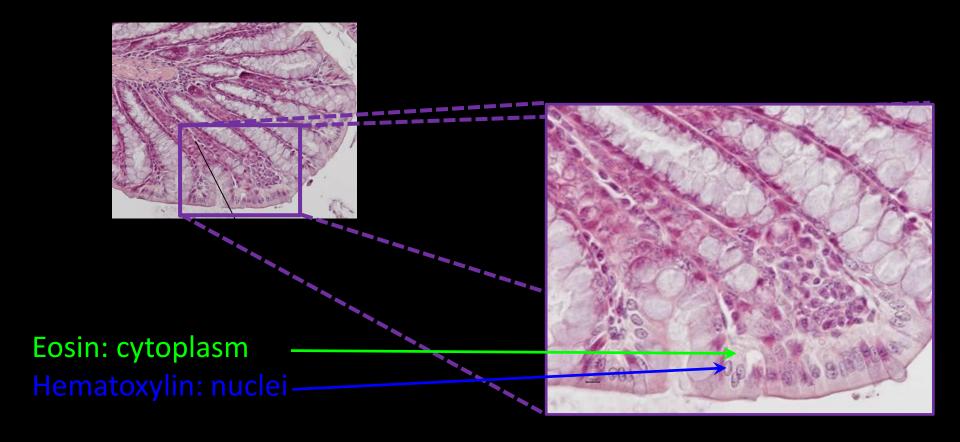
- Surface plasmon Polaritons (SPP)
- Photonic crystals (PC)
- Raman, CARS
- Quantum dots

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



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

**Example** Conventonial microscopy with stained tissues



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

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

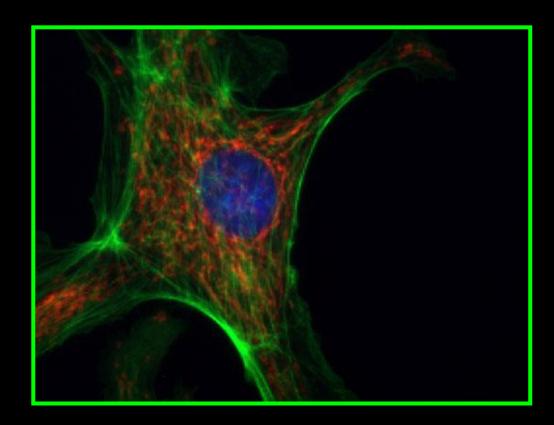
Endothelial cells stained with fluorescent molecules that bind selectively only to some cellular compartments

Red Mitochondria

**Green** F-Actin

cytoskeleton

Blue Nucleus



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

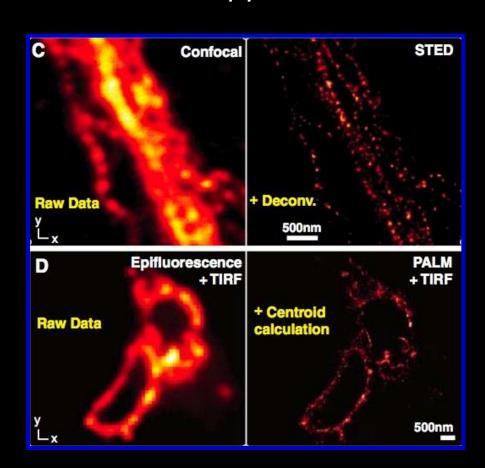
Example Confocal microscopy on cells labelled with fluorescent molecules Conventional Fluorescence Microscopy **Confocal Microscopy Human Marrow** Rabbit Muscle (Medulla) Sunflower Pollen

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

**Example** Super-resolution fluorescence microscopy

Comparison Confocal vs STED

Comparson Conv.Micr. vs PALM



- 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.

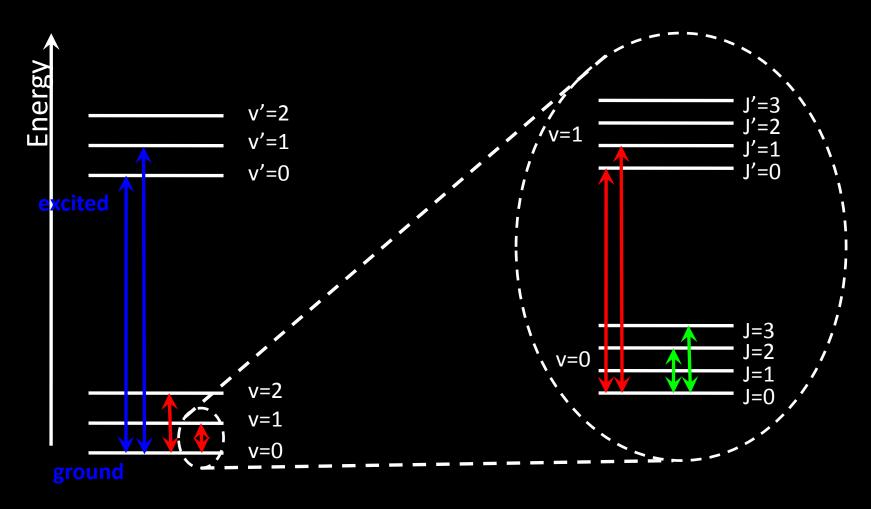
Every quantum state is associated to an energy, which is approximately given by the sum of such contributions:

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

with

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

#### Concepts on the energy levels of organic molecules



Transitions electronic vibrational rotational spin

 $\overline{E_{el}} \sim 2 \div 3 \text{ eV}$   $E_{vib} \sim 10 \div 300 \text{ meV}$   $E_{rot} \sim 0.3 \div 1 \text{ meV}$ 

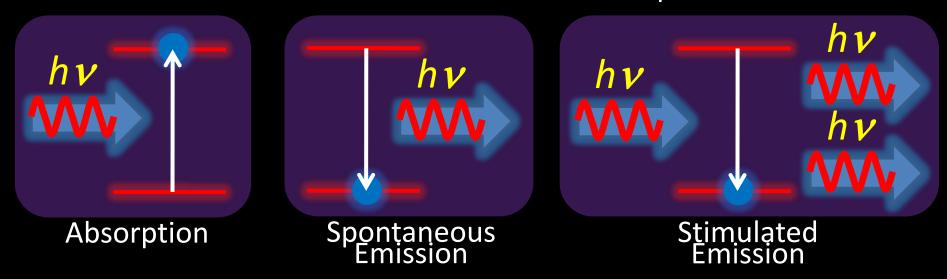
 $E_{rot} \sim 0.3 \div 1 \text{ meV}$  $E_{spin} \sim 0.4 \div 2 \mu eV$  1 eV= 1.602·10<sup>-19</sup> J= 8065 cm<sup>-1</sup>

#### 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:

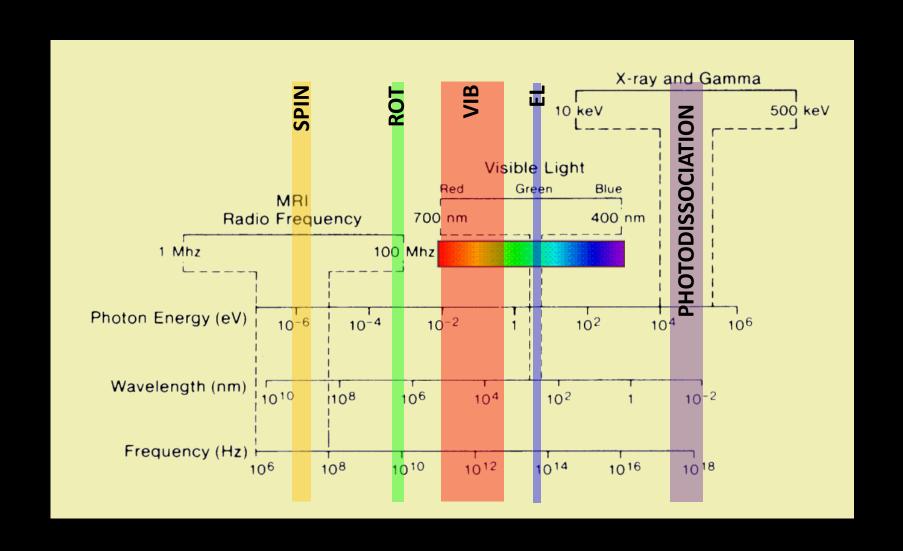
$$hv = \Delta E = E_2 - E_1$$

There exist three fundamental linear interaction processes:



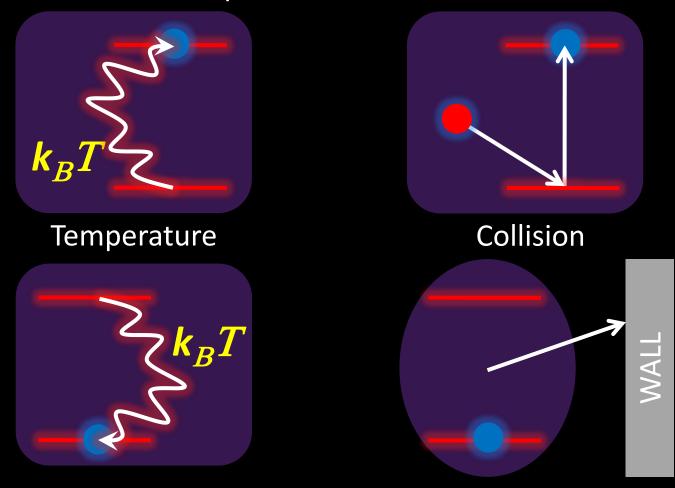
In the dipole interaction approximation <u>not</u> all transitions are permitted but there are some <u>Selection Rules</u>

#### 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.

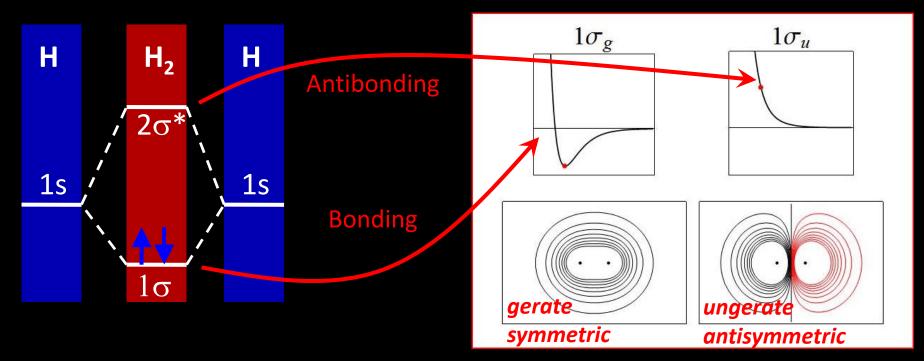


For such processes there is no selection rule

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

Homonuclear Biatomic Molecules:

H<sub>2</sub> case

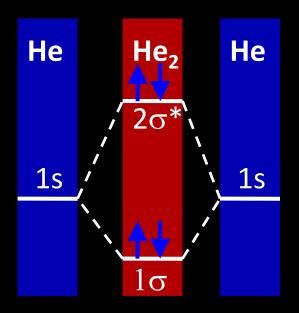


Interaction energy:

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)

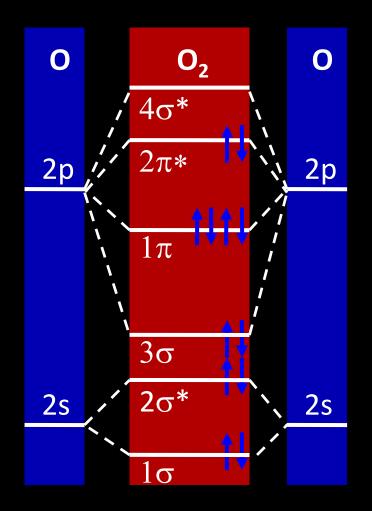
Homonuclear Biatomic Molecules: He<sub>2</sub> case



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.

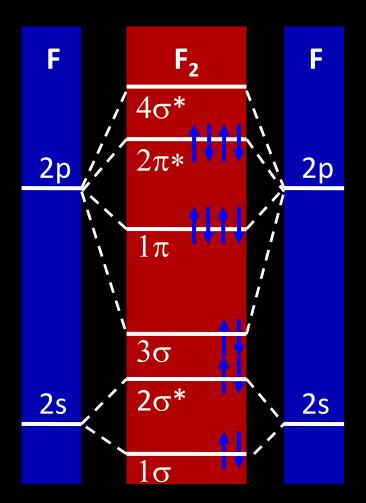
The He<sub>2</sub> molecule therefore is not stable and does not exist..

Homonuclear Biatomic Molecules:  $caso O_2$  and  $F_2$  case



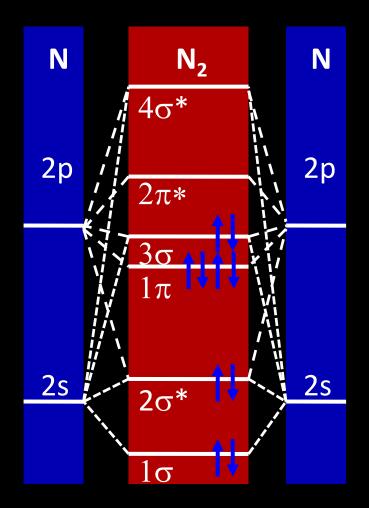
- The atomic orbitals that pertain to the inner shells do not combine to generate molecular orbitals.
- Only the orbitals with the same energy can combine
- Only couples of orbitals that have the same symmetry can combine
- In the case of O<sub>2</sub> and F<sub>2</sub> the 2s and
   2p atomic orbitals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

Homonuclear Biatomic Molecules:  $caso O_2$  and  $F_2$  case



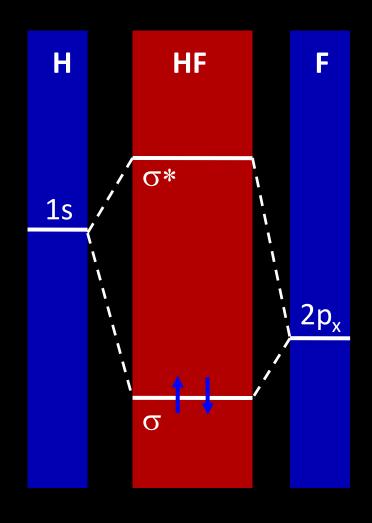
- The atomic orbitals that pertain to the inner shells do not combine to generate molecular orbitals.
- Only the orbitals with the same energy can combine
- Only couples of orbitals that have the same symmetry can combine
- In the case of O<sub>2</sub> and F<sub>2</sub> the 2s and
   2p atomic orbitals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

Homonuclear Biatomic Molecules: Li<sub>2</sub>, Be<sub>2</sub>, B<sub>2</sub>, C<sub>2</sub>, N<sub>2</sub> 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

Heteronuclear Biatomic Molecules: HF case



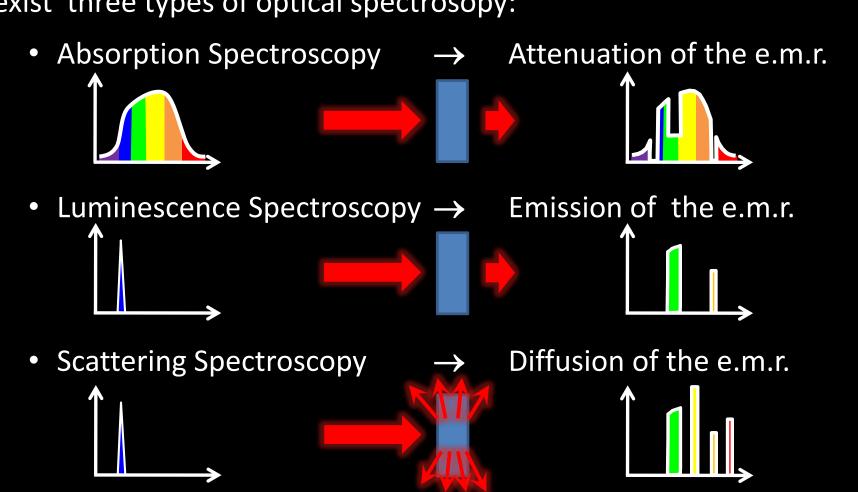
- One must take into consideration only the atomic orbitals of the most external shell, therefore 1s for the hydrogen and 2p<sub>x</sub> for the fluorine.
- Wet get two molecular orbitals of the σ type, one is bonding and the other antibonding

#### Polyatomic molecules

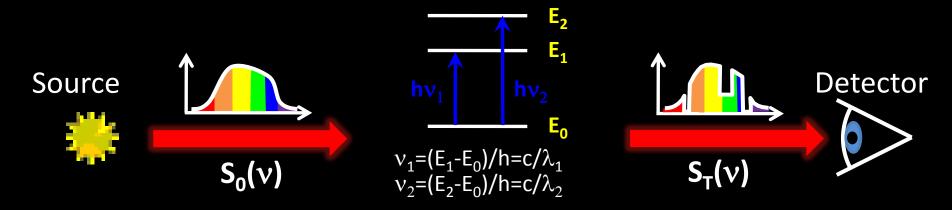
• For polyatomic and heteronuclear molecules the energy levels can be found by the same approach used for the biatomic molecules by using a larger number of orbitals, which are extended all over the whole molecule.

#### 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:



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



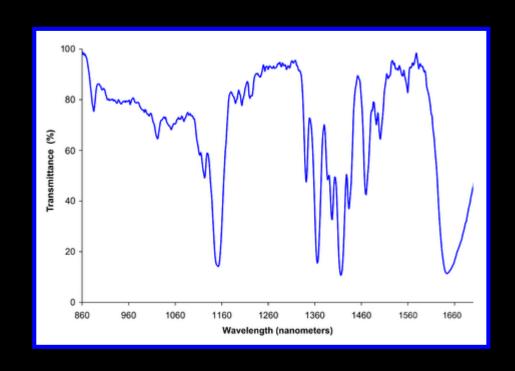
The spectrometers provide a measurement of the normalized spectral transmittance, removing the effects due to specular reflection at the interfaces of the sample:

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

Generally, the transmission spectrum does not show infinitesimally sharp lines, rather finite width bands.

The principal cause of the line broadening are:

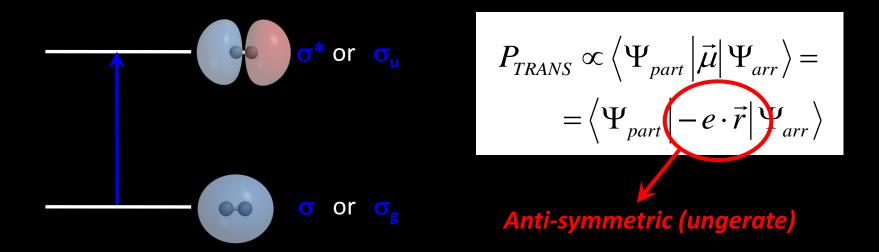
- Lifetime of the excited state τ (intrinsic broadening)
- Doppler
- Interactions with the external environment (collisions)
- Superposition of several different bands (example: vibrational structure)



Transmission spectrum of liquid Dichloromethane

#### Selection Rule

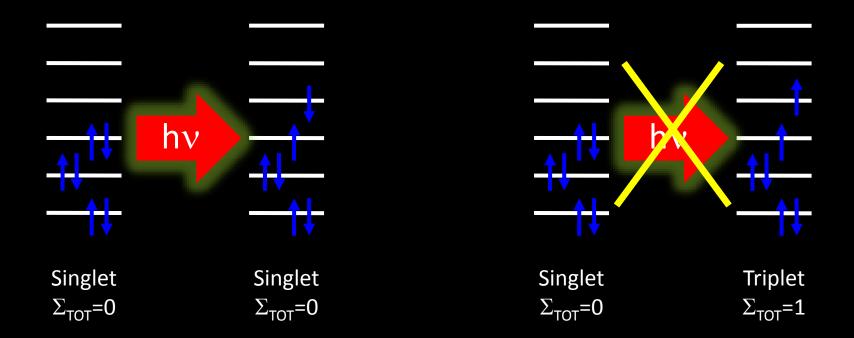
The transition between two energy levels can be induced by e.m.r. only if there is a change of the molecular dipole moment when transiting form a level to the other. In QM the dipole moment operator must have a non-zero matrix element between the starting and arrival states.



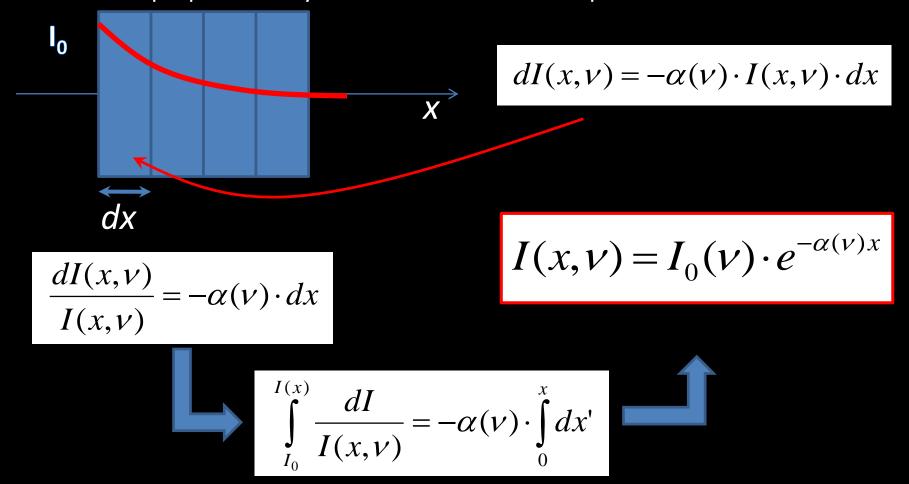
The starting and arrival states must have opposite parity.

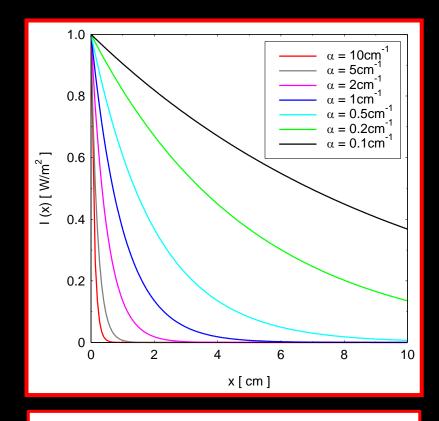
#### Selection Rule

The transition between two energy levels can be induced by e.m.r. only if there is no change of the total spin  $\Sigma$  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 ( $\Sigma_{TOT}=1$ )



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  $\alpha$  is the absorption coefficient.





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

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

We define absorbance the quantity:

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

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

where  $\varepsilon(\nu)$  is the molar extinction coefficient (L mol<sup>-1</sup> cm<sup>-1</sup>) and C is the concentration (mol L<sup>-1</sup>).

Therefore we have:

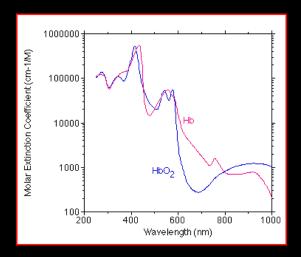
$$A = \log_{10} \frac{1}{T} = -\log_{10} \left[ 10^{-\varepsilon(v)Ch} \right] = \varepsilon(v)Ch$$

And A is proportional to the concentration.

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

#### **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:

$$C = 135 - 170 g/L$$

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

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

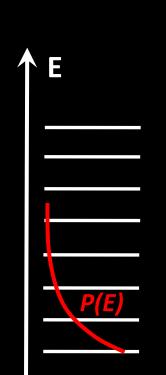
Considering that  $\epsilon_{Hb}$ = 5·10<sup>5</sup> L mol<sup>-1</sup> cm<sup>-1</sup>, for example at  $\lambda$  = 420nm, we get:

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

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

Some basic issue concerning the **Boltzmann Statistics** 

In stationary conditions (thermal equilibrium), the probability that an energy level is occupied depends on temperature ( $\mathbf{T}$ ) and on the energy separation between levels ( $\mathbf{\Delta F}$ ).



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

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

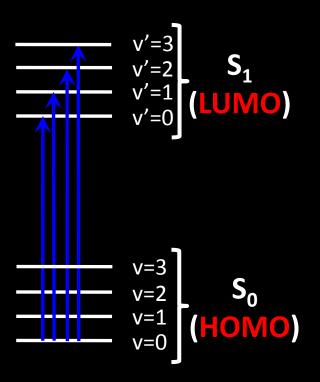
$$\frac{N(E)}{N(E_0)} = \frac{g(E)}{g(E_0)} e^{-\frac{\Delta E}{k_B T}}$$

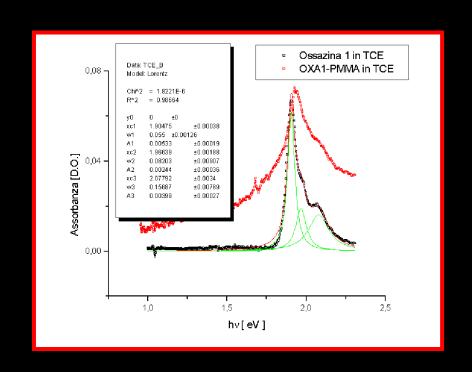
where g is the degeneraration of the energy level E.

Levels	<b>ΔE (eV)</b>	P(E <sub>1</sub> )/P(E <sub>0</sub> ) @ 300K
Electronic	2.5	10 <sup>-42</sup>
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.

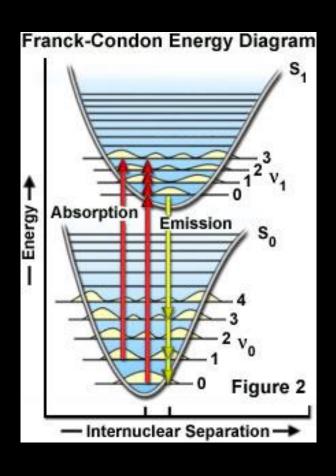
If we analyze the shape of the single bands we can notice a substructure that depends on the molecular vibrational levels. Such subbands are named vibronic bands.





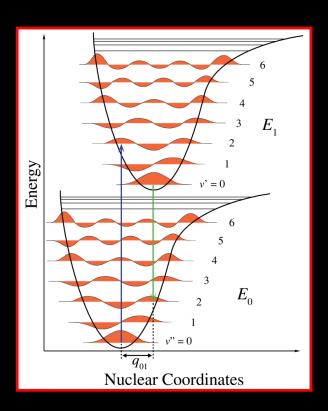
HOMO - Highest Occupied Molecular Orbital LUMO - Lowest Unoccupied Molecular Orbital

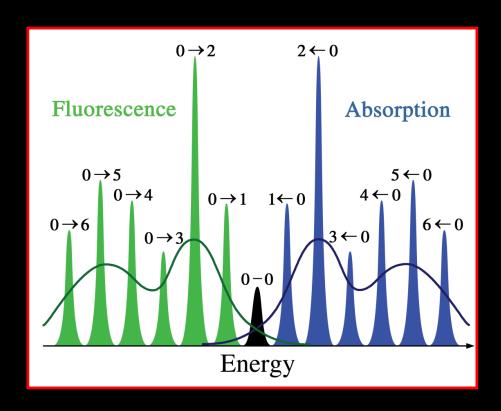
Biatomic Molecule We have only one vibrational mode along the main axis of the molecule



- 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<sub>0</sub> electronic level is occupied
- The relative intensity of the vibronic bands depends on the superposition of the vibrational wavefunctions in the two electronic states  $S_0$  and  $S_1$ .

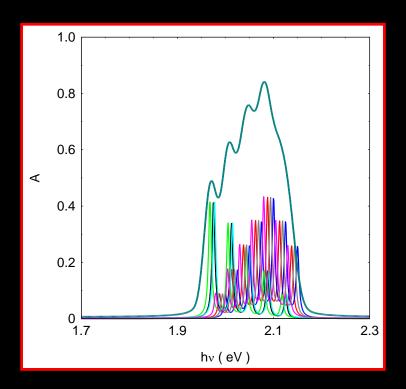
The intensity of the absorption (and emission) lines is proportional to the superposition integral of the vibrational wave-functions for the levels of the HOMO and LUMO states.





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)

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.



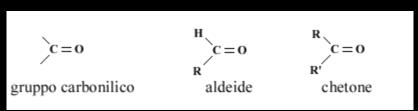
It is therefore very difficult to carry out a detailed analysis of the vibrational modes.

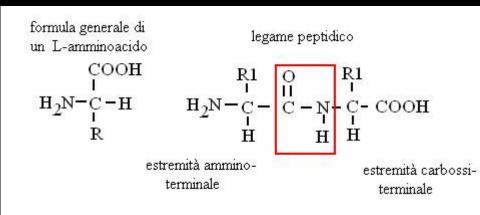
#### Absorption Spectra of endogenous chromophores

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

Carbonyl Group

Peptidic Group



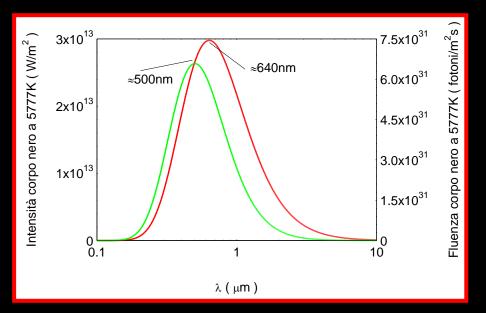


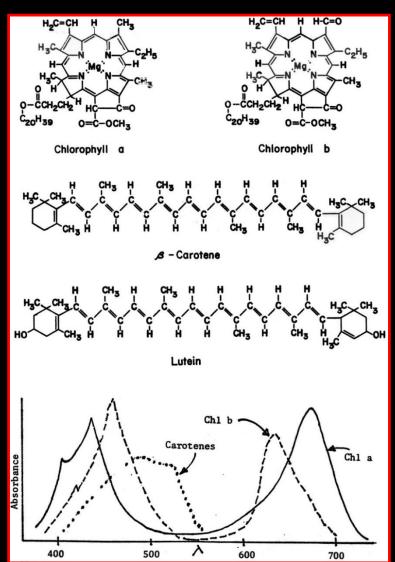
- Conjugated double bonds in linear polymers (carotenoids, retinol) and cyclic polymers (porphyrin derivates, aromatic amino acids, bases of the nucleic acids)
- lons of transiton metals in metallo-proteins: Mn, Fe, Co, Cu, Mo, V

#### Absorption Spectra of endogenous chromophores

#### **Examples**

- Chlorophylls
- Carotenoids

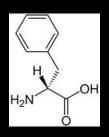




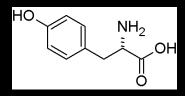
#### Absorption Spectra of endogenous chromophores

#### **Examples**

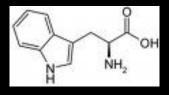
Amino Acids



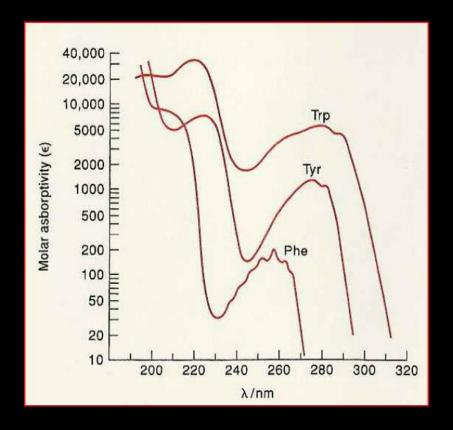
Phenylalanine (Phe)



Tyrosine (Tyr)



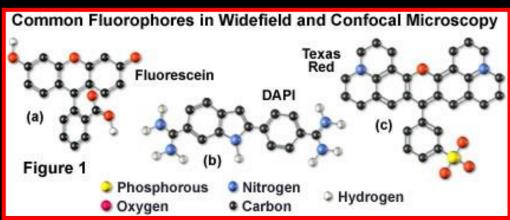
Tryptophan (Trp)

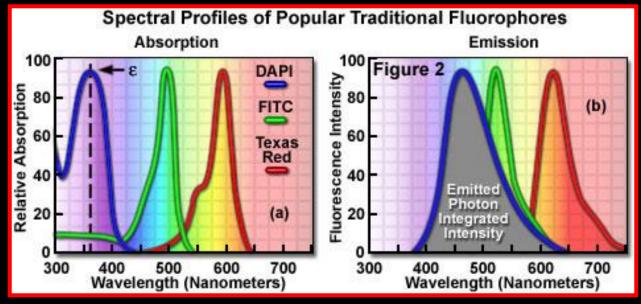


#### Absorption Spectra of exogenous chromophores

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* 

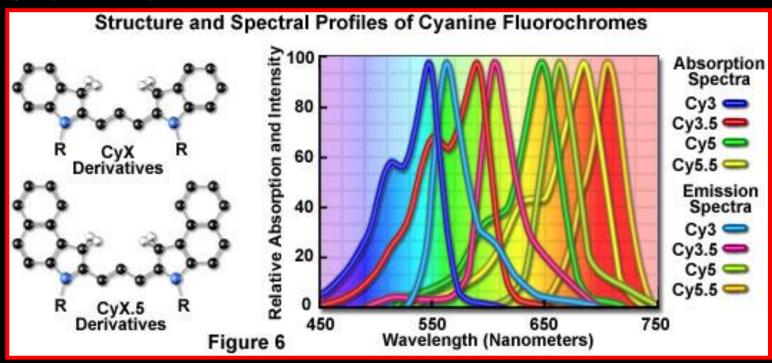




#### Absorption Spectra of exogenous chromophores

#### 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



#### 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

