Colorful principles of absorption and fluorescence

Advanced methods of biophysics in experimental biology

Ctirad Hofr

Light is electromagnetic waves

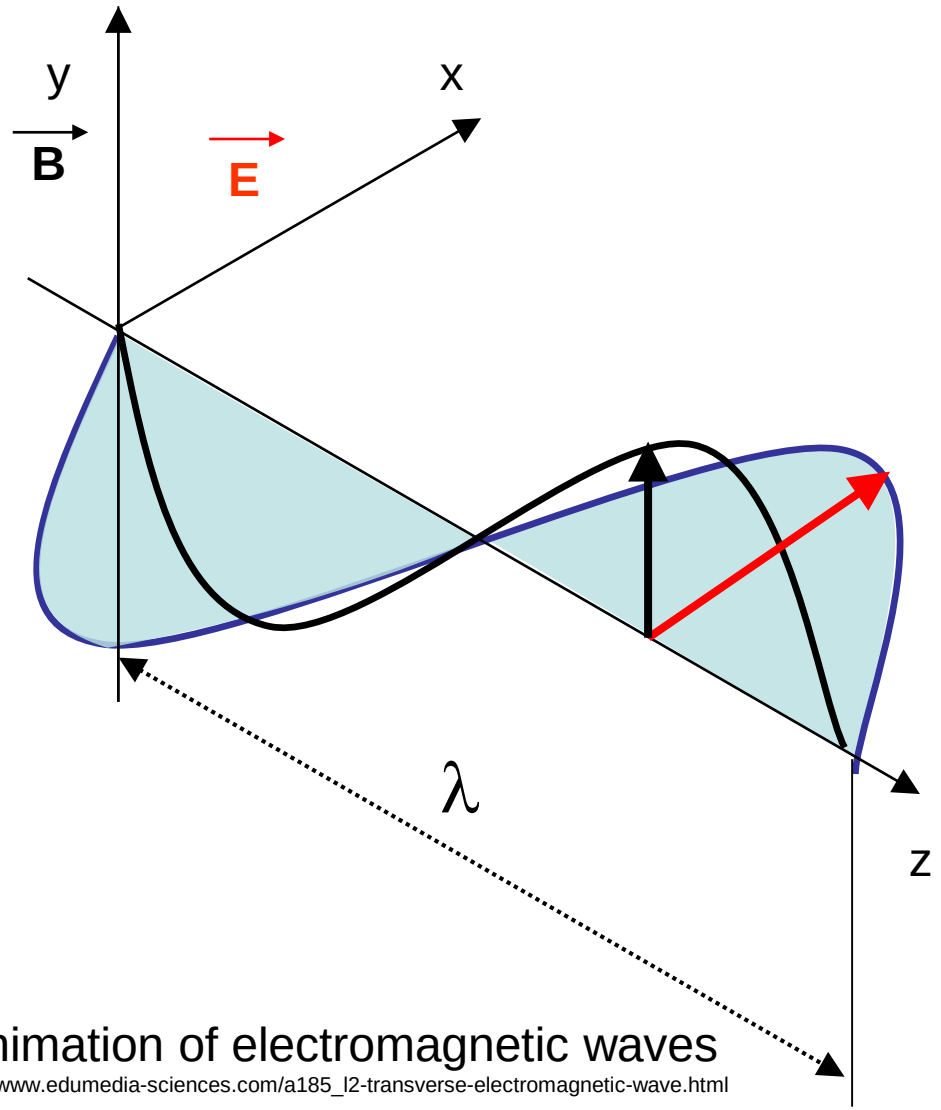
- Light consists of an electric component and a magnetic component, which oscillate in phase in perpendicular planes
- Light is characterized by frequency f and wavelength λ
- Frequency **f** determines how many times per second wave oscilates, unit is Hertz Hz = s^{-1}
- Wavelength determines the **spatial period** of the wave - the distance over which the wave's shape repeats, expressed in nanometers nm = 10^{-9} m
- Frequence f and wavelength λ is given by

$$\mathbf{c = \lambda f}$$

where c is the speed of light (c=299 792 458 m s^{-1} in vacuum)

- Energy E = h f, where h Planck's constant (6.626 10^{-34} J s)

Electromagnetic wave



Animation of electromagnetic waves
http://www.edumedia-sciences.com/a185_l2-transverse-electromagnetic-wave.html

$$c = \lambda f$$

c is constant, if wavelength increases, frequency must be reduced to get constant product.

Wavelength λ is inversely proportional to the frequency f

$$E = h f$$

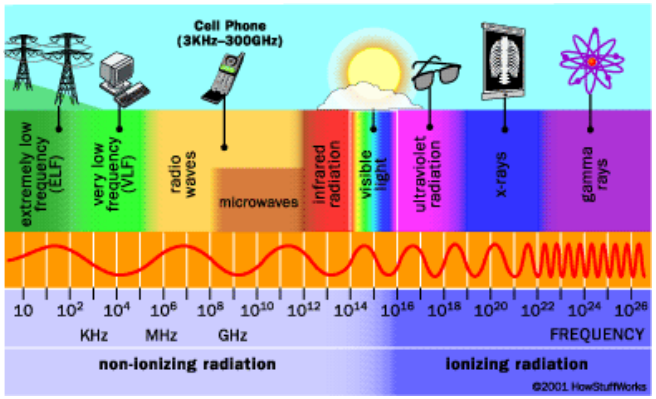
The greater the frequency, the greater the energy of the radiation.

The greater the wavelength λ, the lower the energy of the radiation.

Visible spectrum

Only a small portion of entire spectrum of radiation is visible.

The visible spectrum is bordered by wavelength of 400 nm and 700 nm.

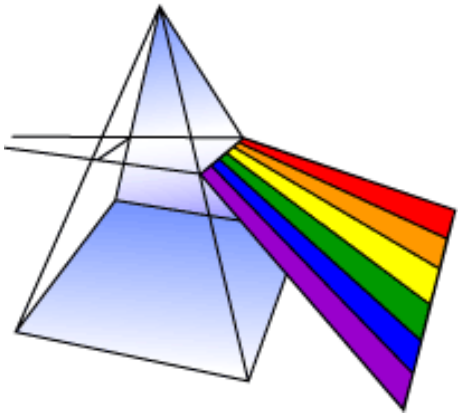


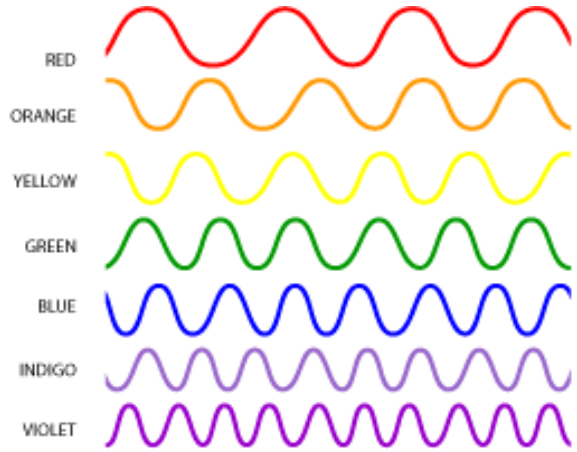
400 nm

$7.5\ 10^{14}$ Hz

700 nm

$4.3\ 10^{14}$ Hz

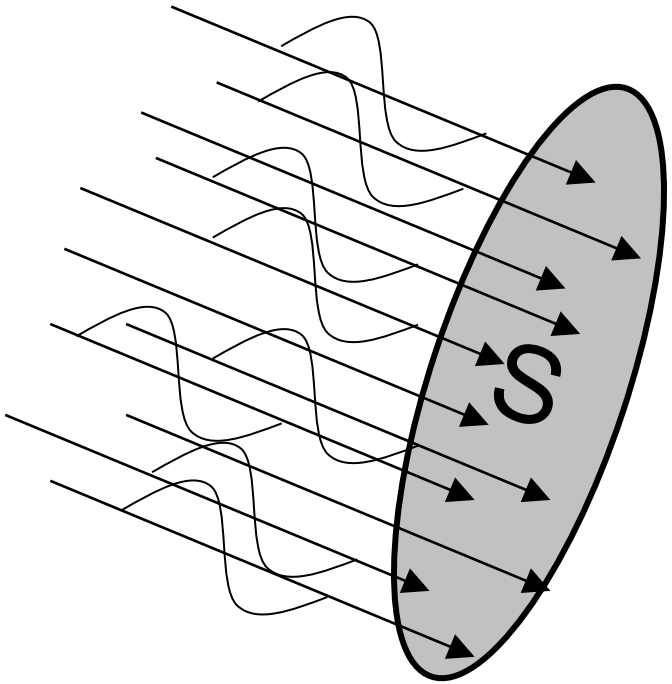




http://science.hq.nasa.gov/kids/imagers/ems/visible.html

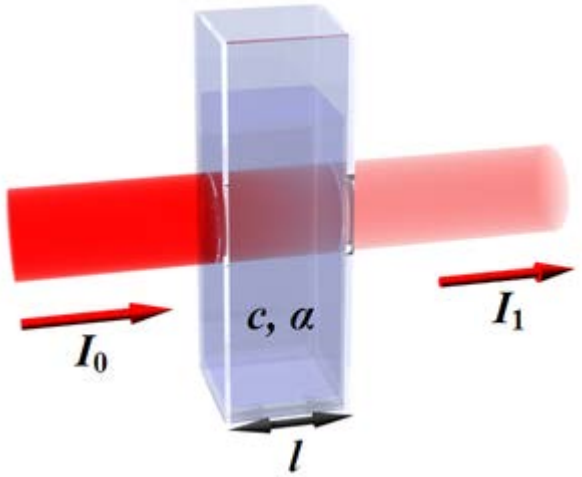
Intensity

Intensity – the number of photons passing through an unit area in a given direction per unit time



Absorpion

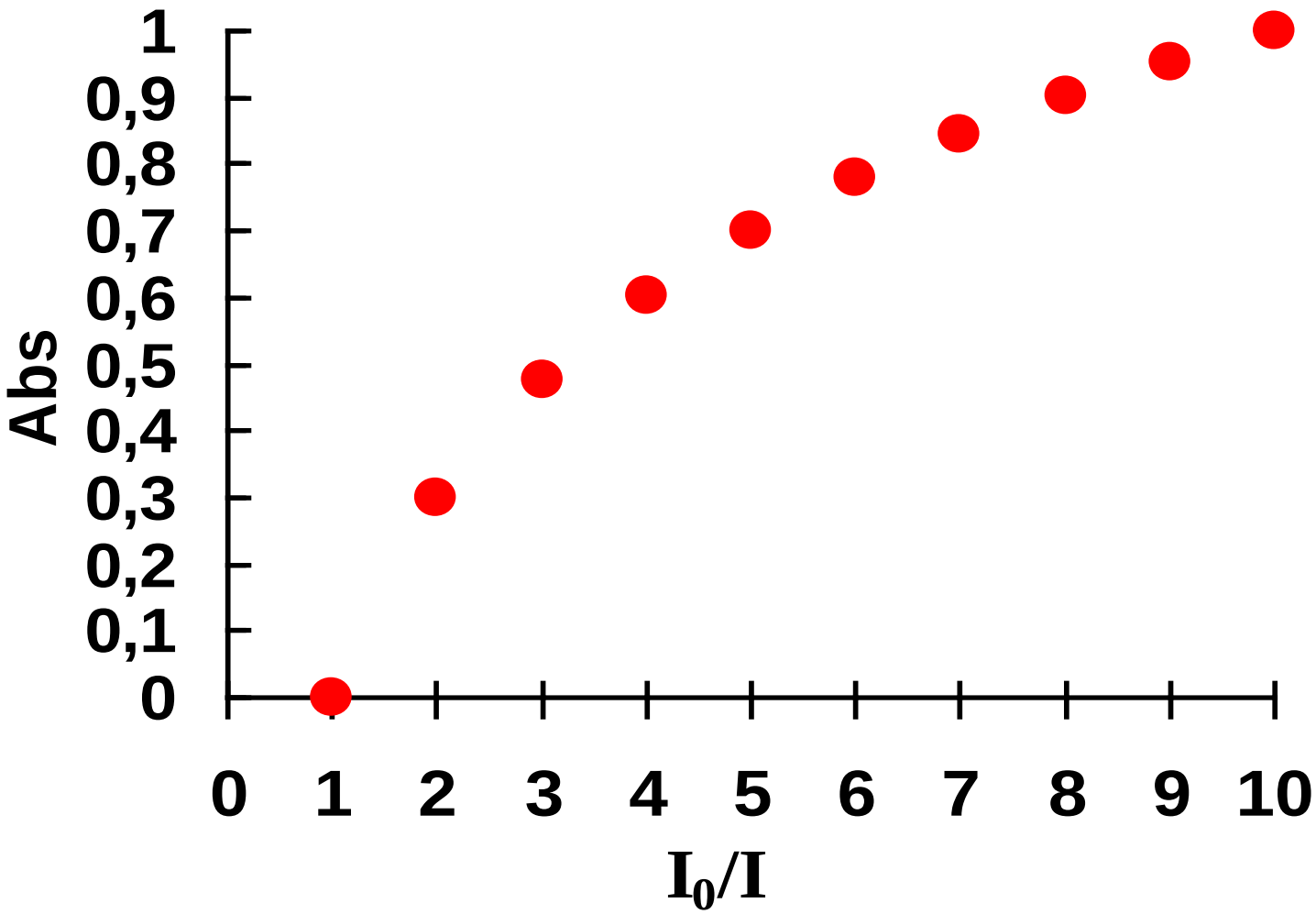
- A substance absorbs light
- For absorption of monochromatic light
- **Beer-Lamber Law:**
  Absorbance is directly proportional to the concentration and thickness of the solution layer



$$I = I_0 \cdot 10^{-\varepsilon \cdot c \cdot l} \quad A = \varepsilon \cdot c \cdot l = \log_{10} \frac{I_0}{I}$$

ε=molar extinction coefficient, c-concentration, l-length of optical path

Absorbance dependence on the relative intensity of the incident and transmitted light



Luminiscence

- Light emission from a substance; occurs from the electron excited states

According to the origin, luminiscence is divided to
1. photoluminescence 2. chemiluminiscence

Luminiscence is divided to:

1. fluorescence
2. phosphorescence

Fluorescence

- Emission from excited singlet states
- Practically: fluorescence is observed during excitation and disappears quickly after the shutdown
- Time decay τ (Lifetime) is the average time that elapses from the excitation to emission - the order of **1 to 10 nanoseconds**
- note: light traveles 30 cm in 1 ns

Phosphorescence

- Emission from excited (prohibited) triplet states
- Practically: the lifetime of phosphorescence is much longer than the lifetime of fluorescence

  Lifetime in order of

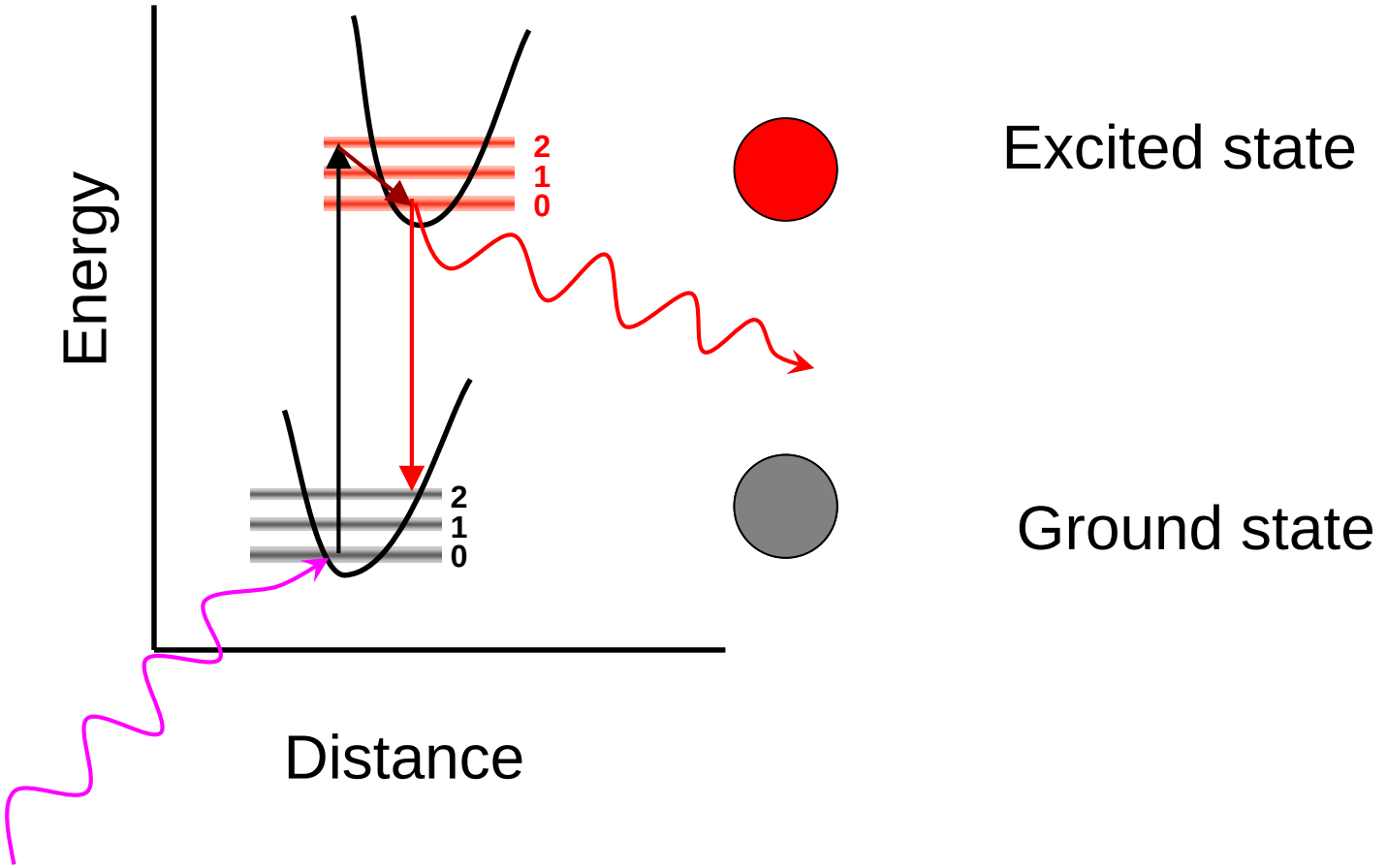
  milliseconds to seconds

note: light traveles 300 až 300 000 km in that time

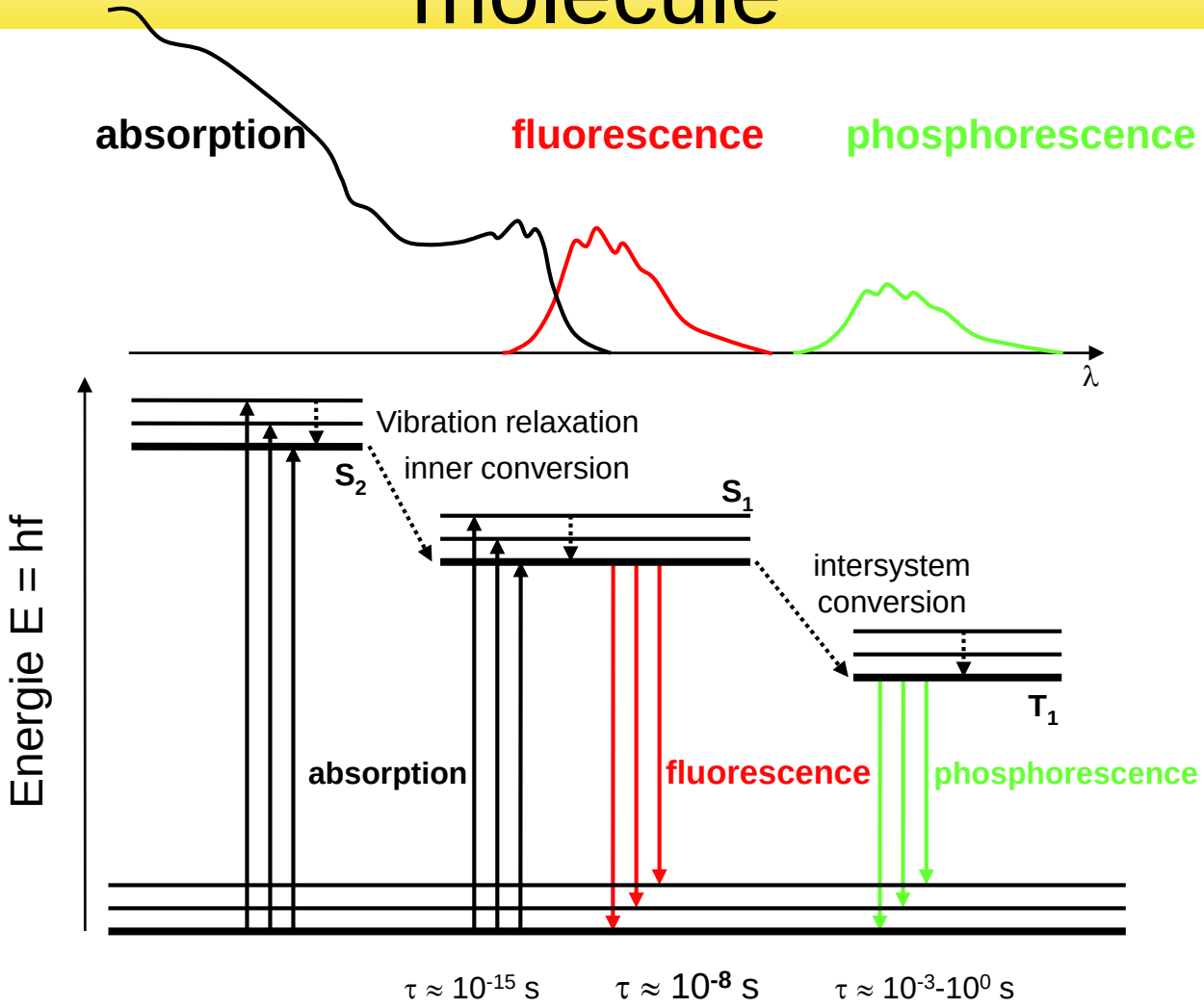
Frank-Condon principle of laziness of nuclei during absorption

Absorption of a photon by an electron (excitation of a molecule) is a very quick process in the order of femtoseconds (10^{-15}s). Because the atomic nucleus is much heavier than the electron, it doesn't move during photon absorption. After absorption of a photon – excitation, the whole molecule is in an unstable state („is hot") and vibrates to get rid of energy (to "cool").

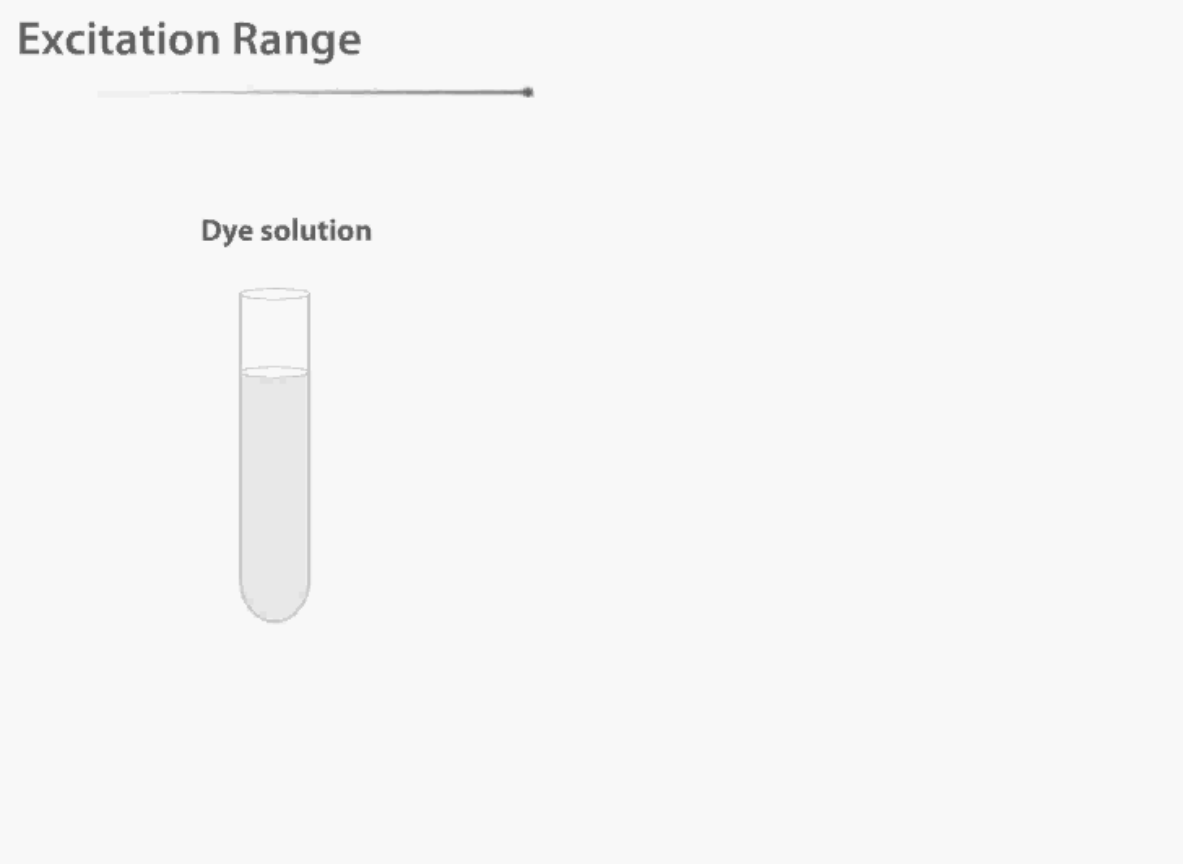
Absorption and emission of energy by the molecule



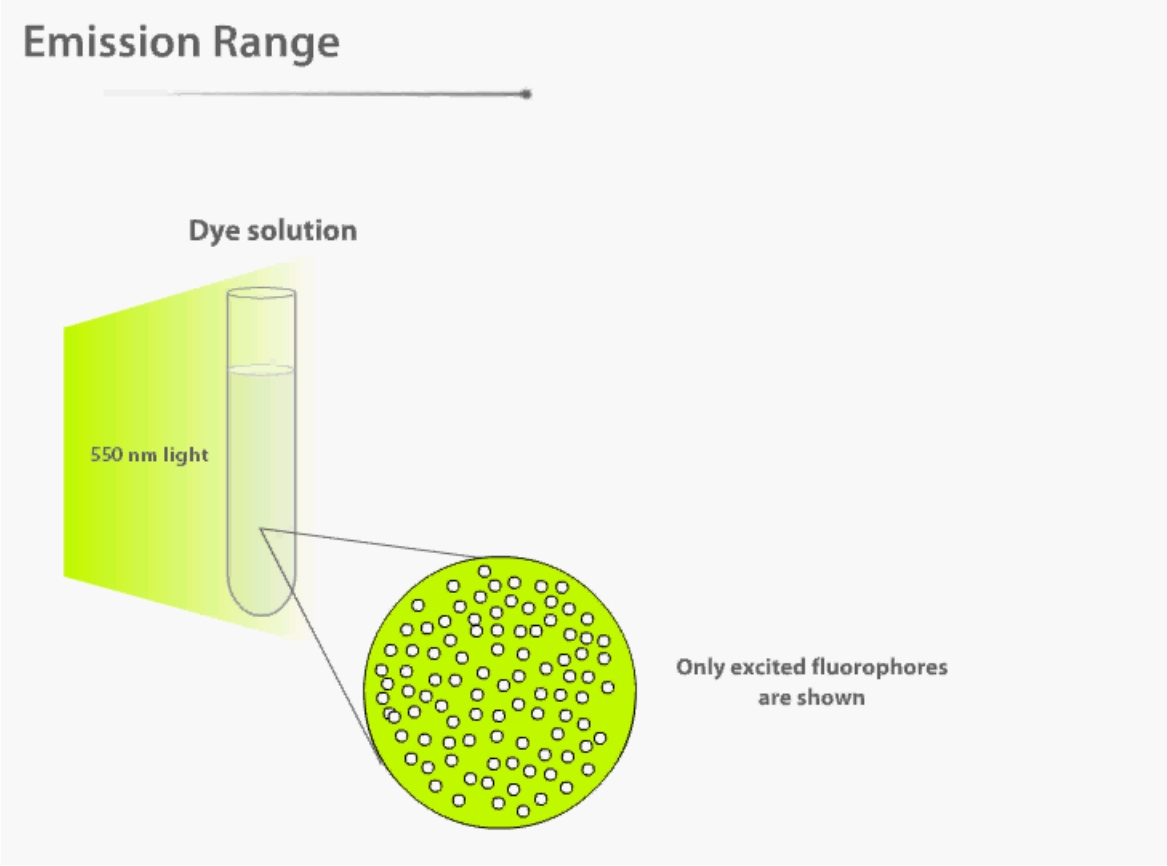
Radiative and non-radiative transitions between electronic-vibrational states of a molecule



Formation of the absorption = excitation spectrum

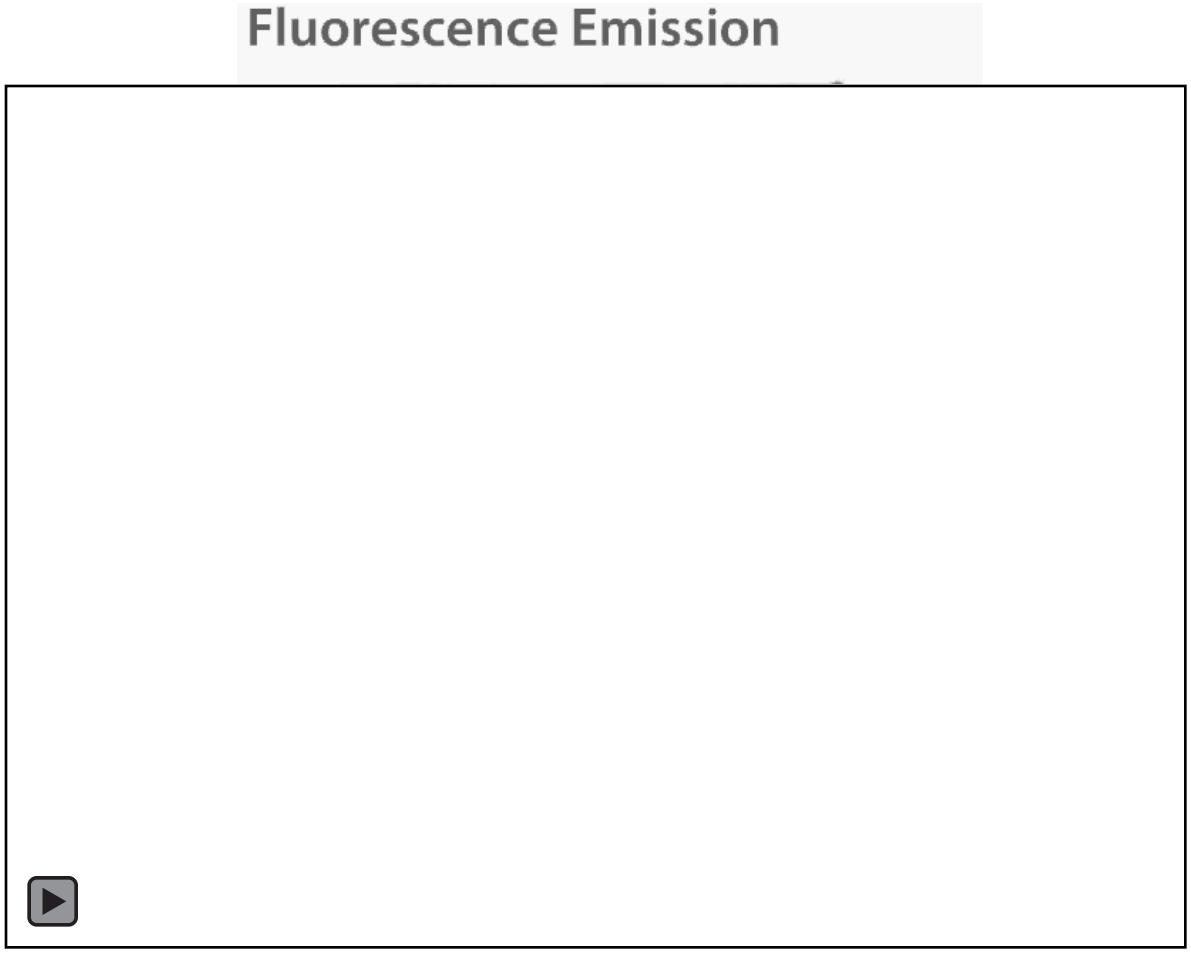


Formation of the emission spectrum



Výukový materiál společnosti **Invitrogen**

The dependence of the emission spectrum on the excitation light



Stokes shift

The emitted light has always lower energy (longer wavelength) than the energy of the absorbed light (smaller λ).

Difference between absorption maximum and fluorescence emission maximum of the spectrum is given by specific characteristic of the fluorophore.

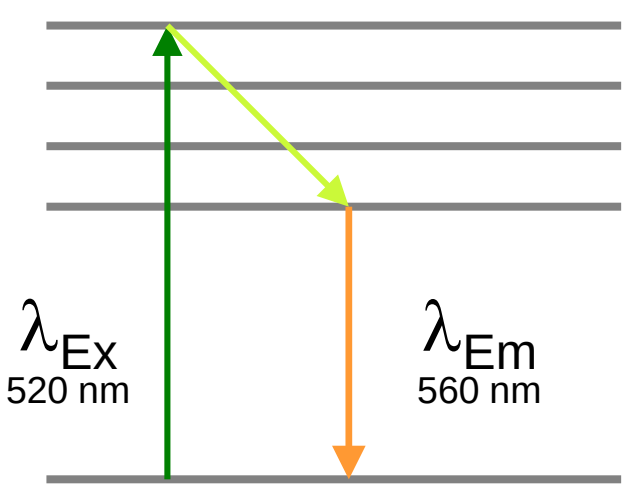
Formation of Stokes shift

Educational material of Invitrogen

Stokes law

The wavelength of the emitted light is greater than or equal to the wavelength of the excitation light

$$\lambda_{em} \geq \lambda_{ex}$$



This is due to the fact that after the light absorption, a partial loss of energy (heat) often occurs during transition from higher excited electron states to the lowest excited metastable state.

Stokes shift

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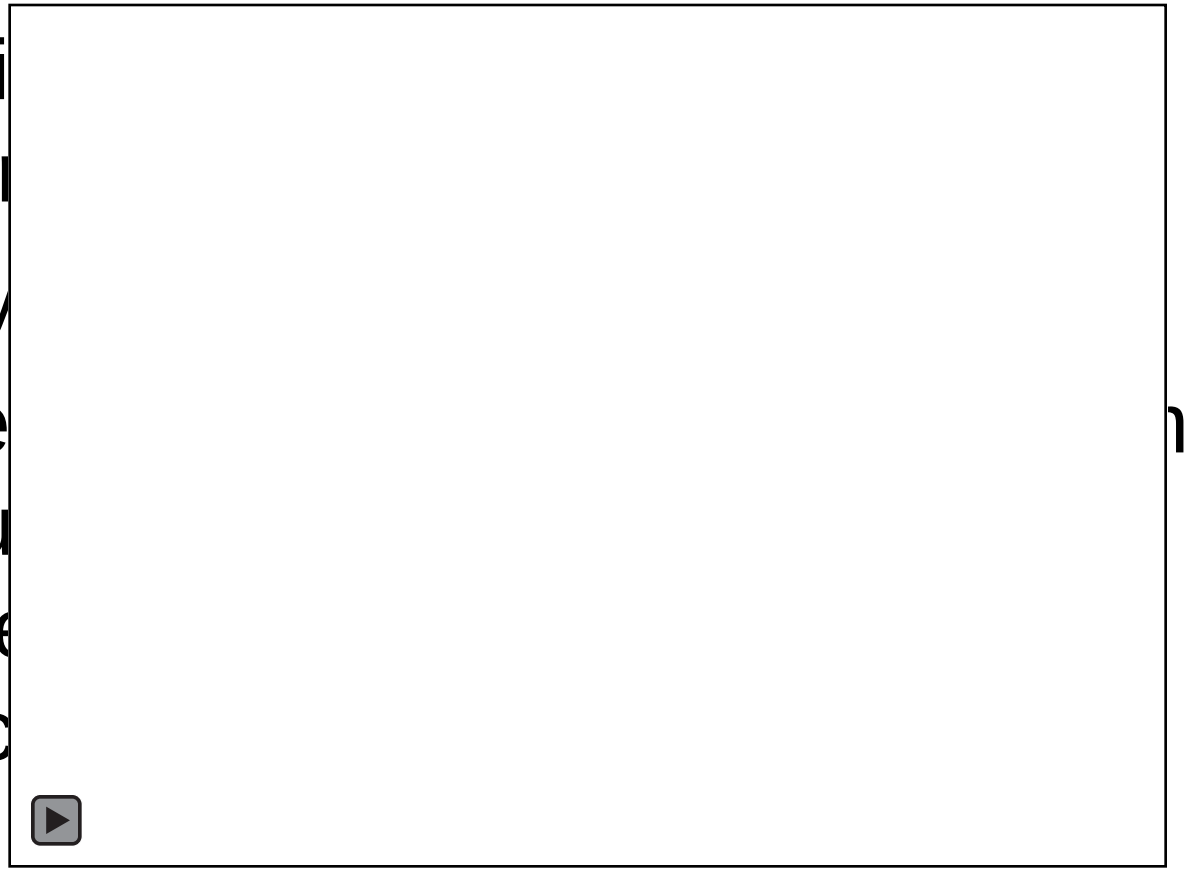
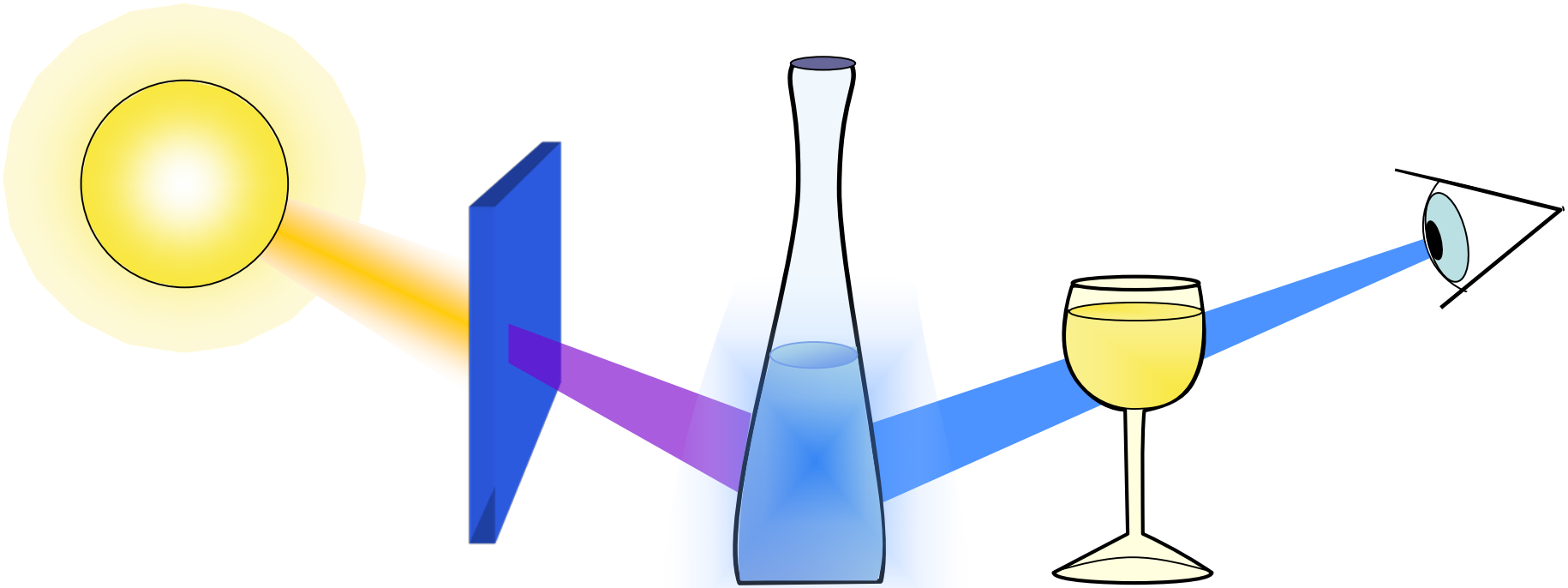


Experiment G. G. Stokese

1852, Cambridge



Sun

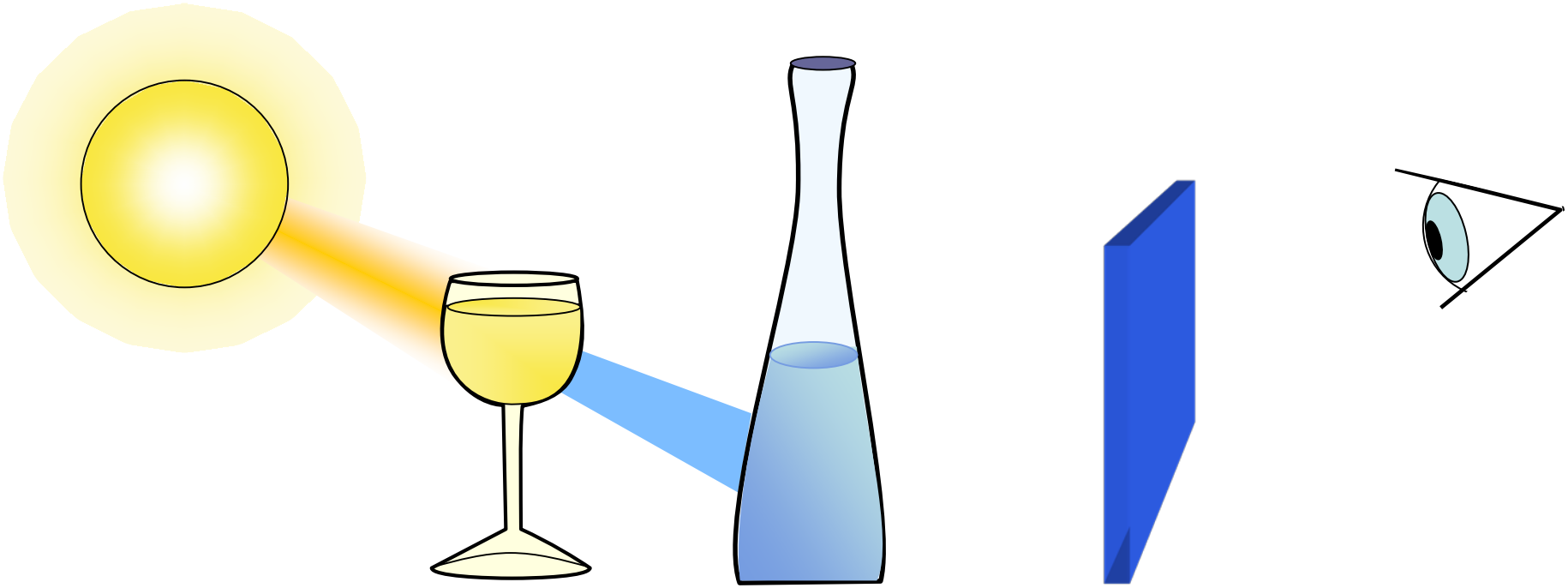
Blue glass
windows in the church
Transmits light with
$\lambda < 400$ nm
Excitation filtr

Quinine solution

Glass of wine
Transmits light with
$\lambda > 400$ nm
Emission filtr

G.G. Stokes

After filtr exchange – fluorescence disappears



After filter exchange, ie. if we put a glass of wine in the path of the sun's rays, transmitted light can no longer excite the solution of quinine.

Colorful animated introduction to the principle of fluorescence

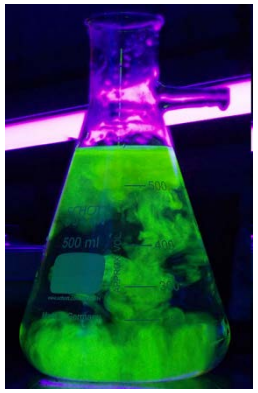
http://probes.invitrogen.com/resources/education/tutorials/1Intro/player.html

Typical fluorophores

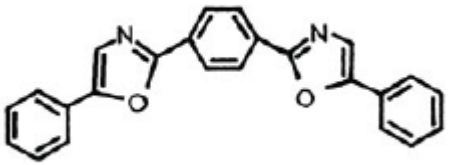
Fluorophores or fluoreoscent dyes are molecules, that emit fluorescence. Fluorescence is exhibited especially by aromatic compounds (polyaromatic hydrocarbons or heterocycles)..

Typical flourophores are for example:

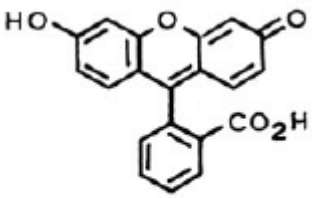
- quinine (tonic)
- fluorescein, rhodamine B (antifreeze, fluorescent labeling)
- POPOP (scintillators)
- Acridine orange, ethidium bromide (DNA)
- umbelliferone (ELISA)
- anthracene, perylene (environmental pollution by oils)



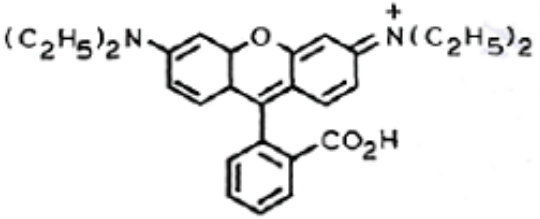
The use of fluorescence in geography



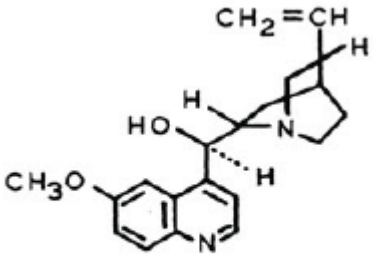
POPOP



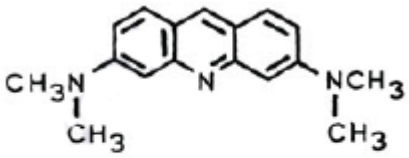
Fluorescein



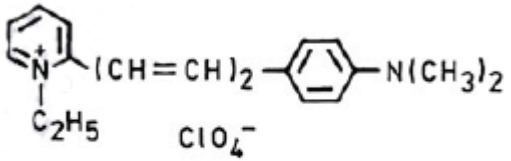
Rhodamine B



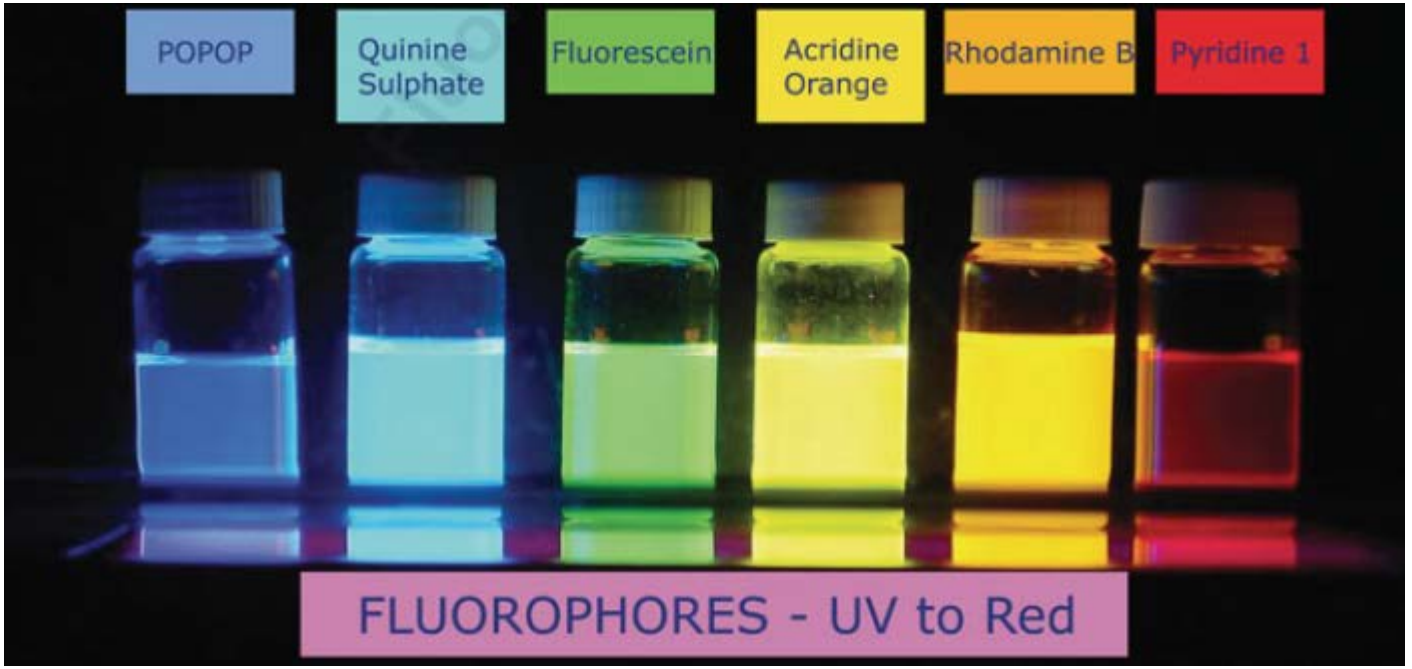
Quinine



Acridine Orange



Pyridine 1



Quantum yield

Quantum yield Q is the ratio of emitted and absorbed photons.

Indicates the efficiency with which photons excite fluorescence.

Quantum yield can be up 1.

In fact, it is lower thanks to the non-radiative transitions of molecules from the excited state.

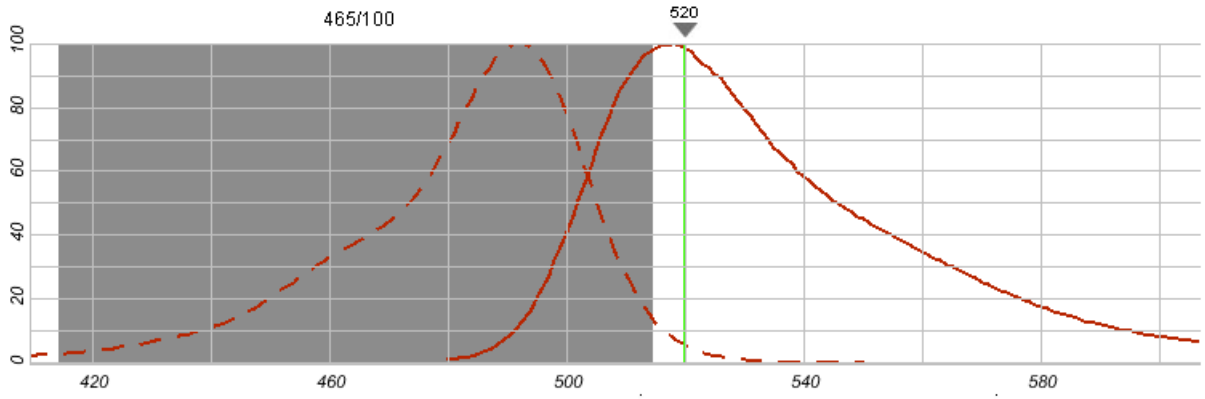
Rhodamine flourophores (~1) and fluorescein (0.95) has the highest quantum yields
http://www.iss.com/resources/reference/data_tables/FL_QuantumYieldStandards.html

Reduction of the quantum yield with temperature-**thermal quenching of luminescence** – is characteristic

Excitation spectrum

The dependence of fluorescence intensity on the excitation wavelength at the constant wavelength of the emitted light

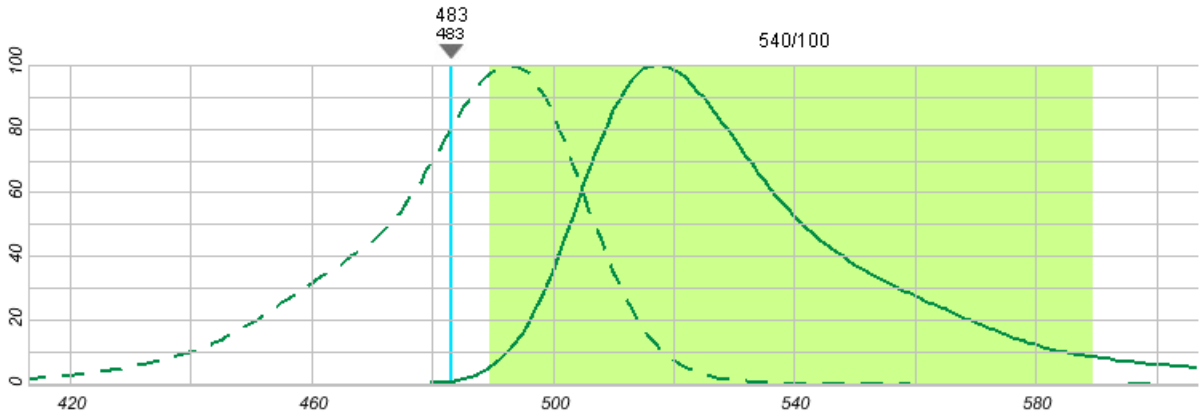
λ_{Ex} scan λ_{Em}= const.



Emission spectrum

The dependence of fluorescence intensity on the wavelength at the constant excitation wavelength

λ_{Ex}= const. λ_{Em} scan



Spectraviewer

Unchanged shape of the emission spectrum

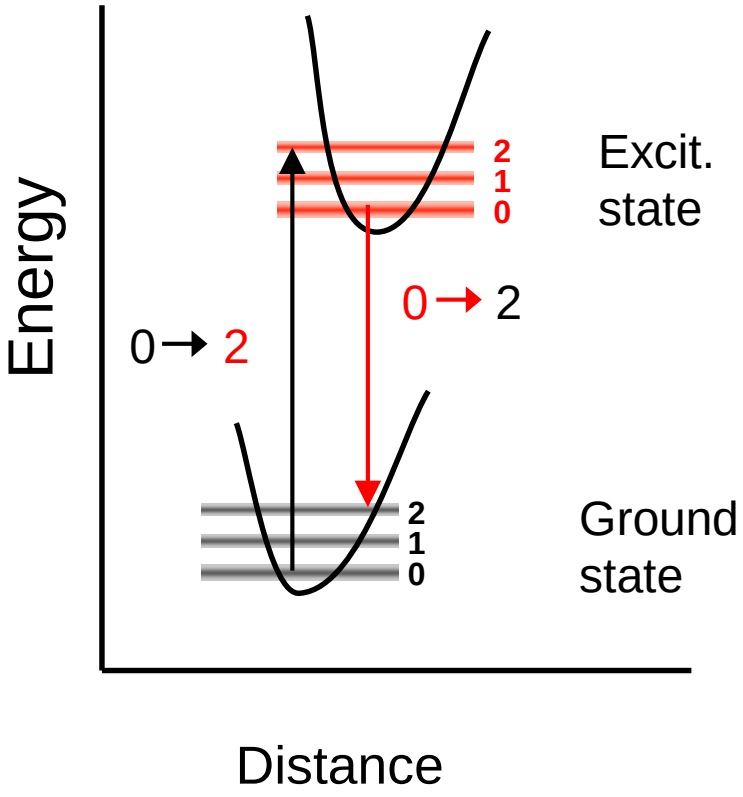
The shape of the emission spectrum is independent of the excitation wavelength.

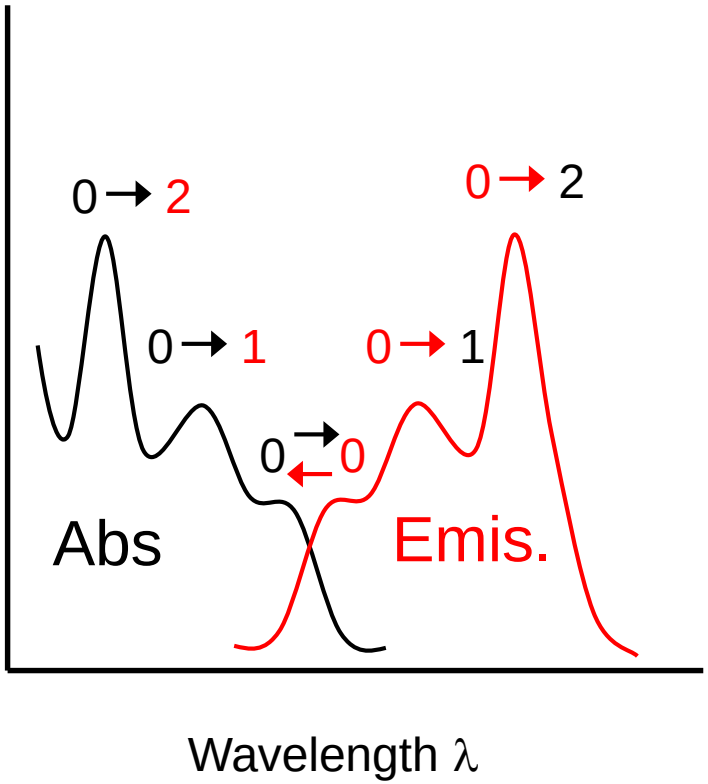
This phenomenon is due to the fact that the duration of the excited state and the quantum yield of the complex molecules in solution does not depend on the wavelength of the excitation light

The shape of the emission spectrum is unchanged at different excitation light

http://probes.invitrogen.com/resources/education/tutorials/2Spectra/player.html

Mirror symmetry of absorption and excitation spectrum



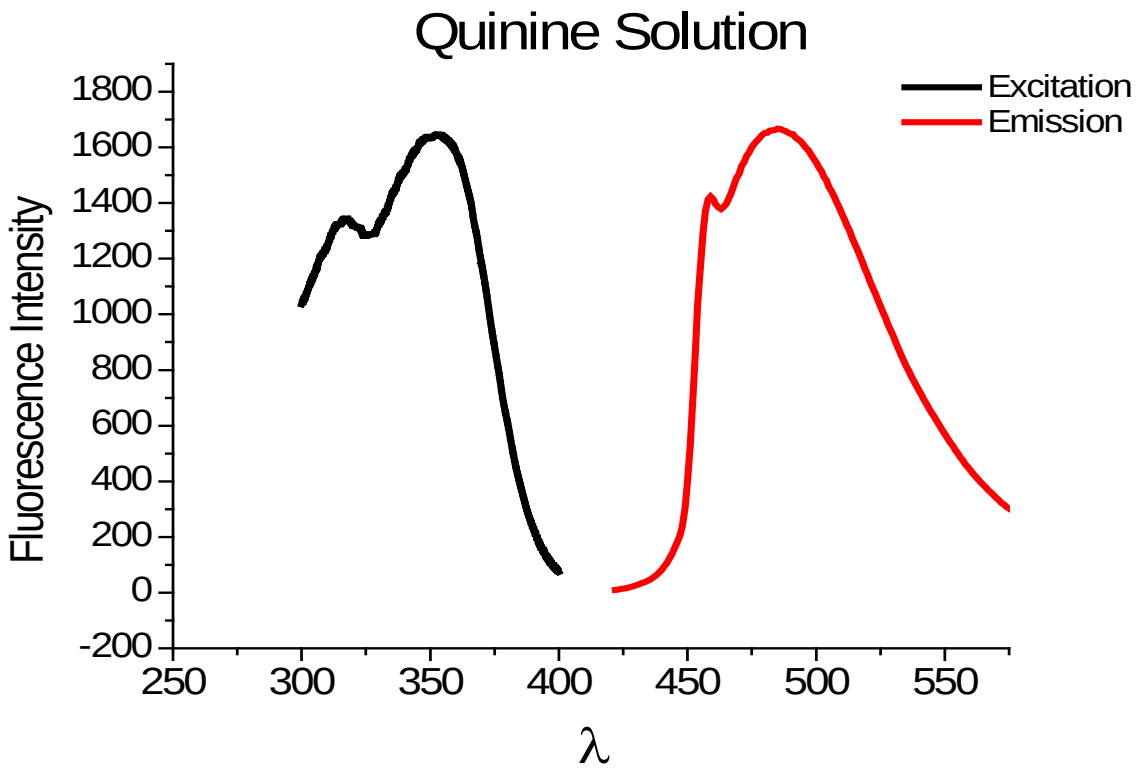


The law of mirror symmetry between absorption and emission spectrum

Structure of vibration levels is the same in ground and excited state, therefore the absorption and emission of corresponding vibration levels may occur with equal probability. This results in a mirror symmetry of absorption spectrum and fluorescence emission spectrum.

Practically: at very low concentrations of the sample, we can determine the shape of absorption spectrum from flourescent emission spectrum without using the amount of the samples higher in several orders of magnitude.

Fluorescent excitation and emission spectrum of the real solution



Mirror symmetry distorts during measurements of real samples due to fluorophore ionization at different pH, fluorophore complexation with other molecules in solution, or by simple contribution of other non-fluorescent molecules to the absorption (excitation) spectrum.

Next:

- What is needed to be able to measure the spectrum of the fluorophore?
- How can we detect fluorescent molecules in the gel?