

BIOMEDICAL APPLICATIONS OF FLUORESCENCE

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Emission by luminescence: everywhere



Photoluminescence

Luminescence everywhere

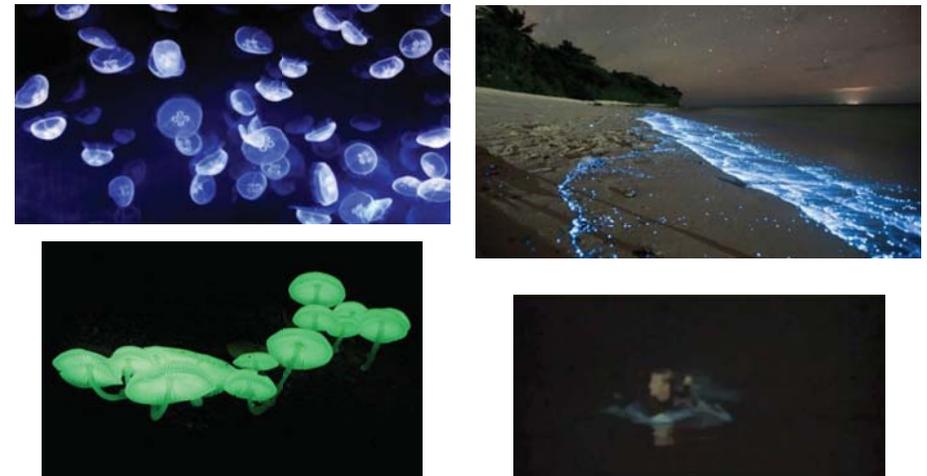


Display lights

Aurora borealis

Radioluminescence

Luminescence everywhere



Bioluminescence

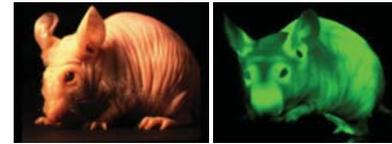
Luminescence everywhere



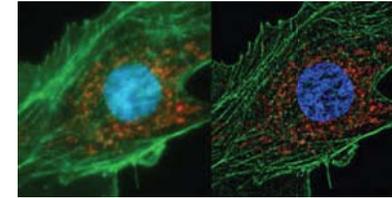
Bioluminescence
Firefly



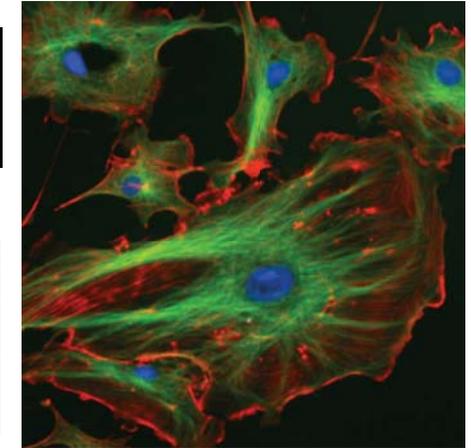
Luminescence everywhere



GFP-mouse (green nude mouse)



Superresolution microscopy (Nobel-prize 2014)



Epifluorescence microscopy (cytoskeletal system)

Fluorescence

Definitions of luminescence

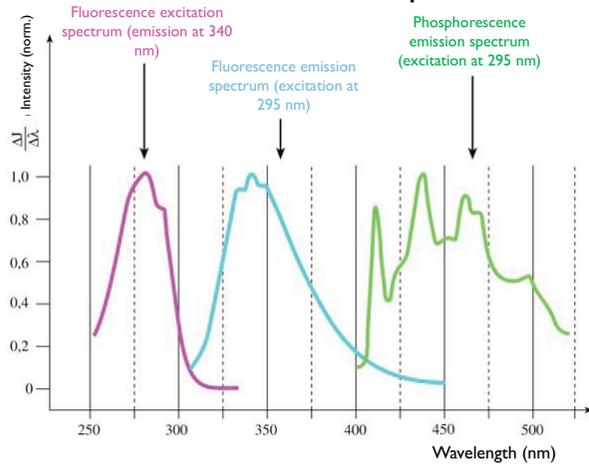
- Relaxation from excited state followed by light emission
- Radiation emitted by matter in excess of thermal emission
- “Cold light”
- Processes of fluorescence and phosphorescence

Types of luminescence

(a) Excitation Mode	Luminescence Type
absorption of radiation (UV/VIS)	photoluminescence
chemical reaction	chemiluminescence, bioluminescence
thermally activated ion recombination	thermoluminescence
injection of charge	electroluminescence
high energy particles or radiation	radioluminescence
friction	triboluminescence
sound waves	sonoluminescence
(b) Excited State (Assuming Singlet State)	Luminescence Type
first excited singlet state	fluorescence, delayed fluorescence
lowest triplet state	phosphorescence

Characterization of luminescence

Luminescence spectra



- Band spectra
- Fluorescence excitation and emission spectra are symmetric
- Stokes shift



George Stokes (1819-1903)

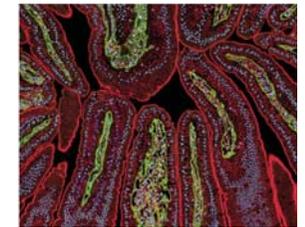
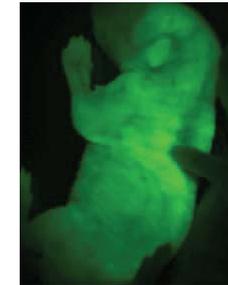
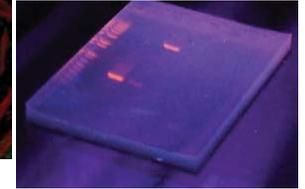
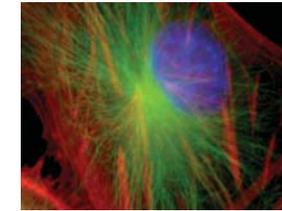
Fluorescent dyes: "fluorophores"

By the specific attachment fluorophores, non-fluorescent molecules may also be studied (fluorescent labeling)

Biomedical applications of fluorescence

A few examples:

- Fluorescence microscopy
- DNA sequencing (chain termination method)
- DNA detection (EtBr)
- DNA microarray
- Immunofluorescence
- Fluorescence-activated cell sorting (FACS)
- Förster resonance energy transfer (FRET)
- Fluorescence recovery after photobleaching (FRAP)
- Fluorescent protein conjugation technologies
- Quantum dots
- etc...



Fluorescence applications

- Source of fluorescence
Intrinsic, extrinsic (labeling, conjugation)
- Mechanisms of relaxation from excited state
- Spectroscopic applications
FRET
- Special applications
FACS, FRAP
- Microscopy
TIRF, confocal, multiphoton fluorescence, super resolution

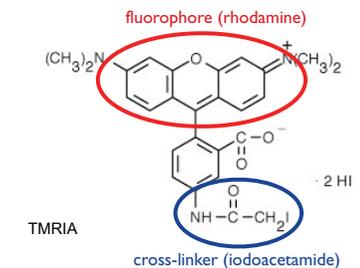
Source of fluorescence I.

Intrinsic fluorophores:

E.g., tryptophan, tyrosine in proteins (amino acids containing conjugated double bonds)

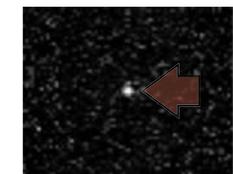
Extrinsic fluorophores:

Externally added dye molecules
Method - "fluorescent labeling"
Problems: chemical specificity and addressability, spatial specificity and mobility



Aspects of fluorescent labeling:

1. spectral properties (e.g., location and width of excitation spectrum, its distance from the emission maximum, location of the emission spectrum in the visible range)
2. quantum efficiency
3. stability (tendency for photobleaching)
4. blinking



Single quantum dot blinking

Source of fluorescence II.

Conjugation with fluorescent proteins

I. Green Fluorescent Protein, GFP



Size: ~27 kDa, 238 aa

Structure: 11-strand β -barrel

Chromophore: from the Ser65-Tyr66-Gly67 residues of the central helix

Fluorescence depends on intact 3D structure

Tandem fusion constructs from the genes of GFP and the explored protein

Advantages: *in vivo* measurements, spectral variants of mutants which enable the simultaneous investigation of several constructs.

Disadvantages: blinking, only terminal (N or C) labeling, GFP might sterically interfere with the function of the targeted protein.



GFP-mouse

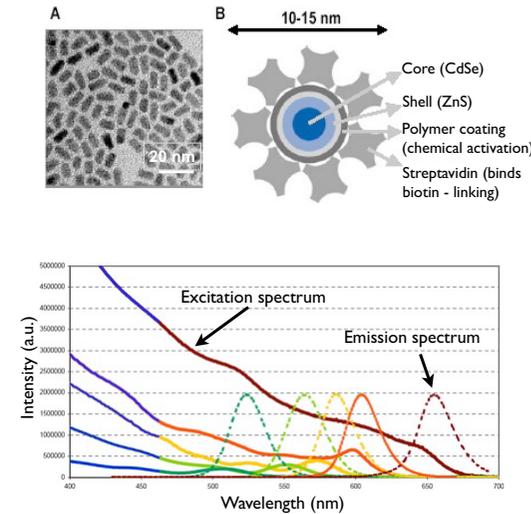
2. Color mutants of GFP (yellow, red, cyan, etc.)

3. Photoactivatable GFP analogue

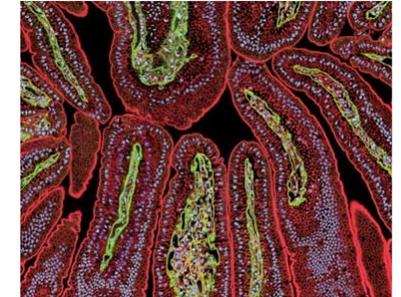
4. Kaede: fluorescent protein from corals with green-red photoconversion induced by UV light.

Source of fluorescence III.

Labeling with quantum dots (semiconductor nanocrystals)



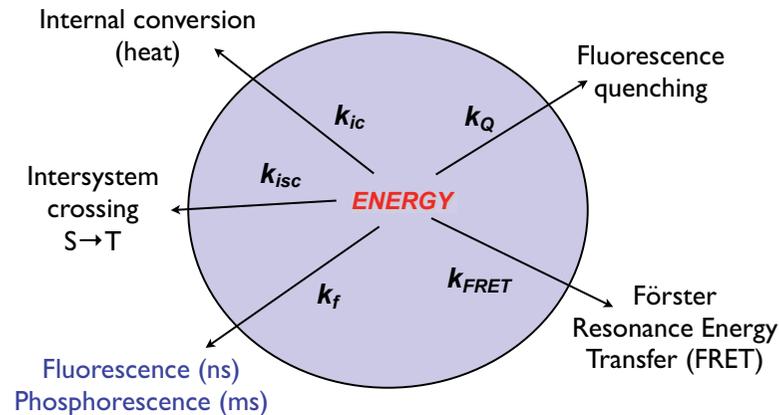
- Wide excitation spectrum
- Tunable emission spectrum (depends on particle size)
- Low tendency of photobleaching
- Tendency of blinking



Mouse intestinal epithelium labeled with quantum-dot-conjugated antibodies (red: actin, green: laminin, blue: nucleus)

Spectroscopic applications

Fate of absorbed energy



Radiative and non-radiative transitions!

Förster Resonance Energy Transfer (FRET)

In general:

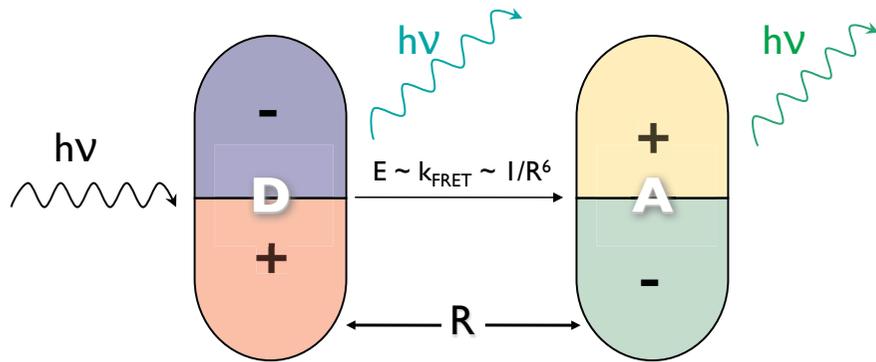
- Occurs by non-radiative dipole-dipole interaction between an excited **donor** and an proper **acceptor** molecule under certain conditions (spectral overlap and close distance).
- **Fluorescence Resonance Energy Transfer (FRET):** if the participants of the transfer are fluorophores.



Theodor Förster (1910-1974)

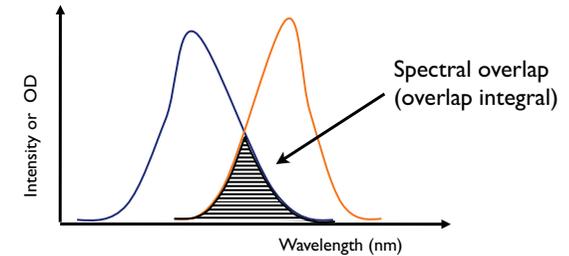
Mechanism of FRET

- Acceptor (A) emission contributes to the relaxation of the excited donor (D) molecule.

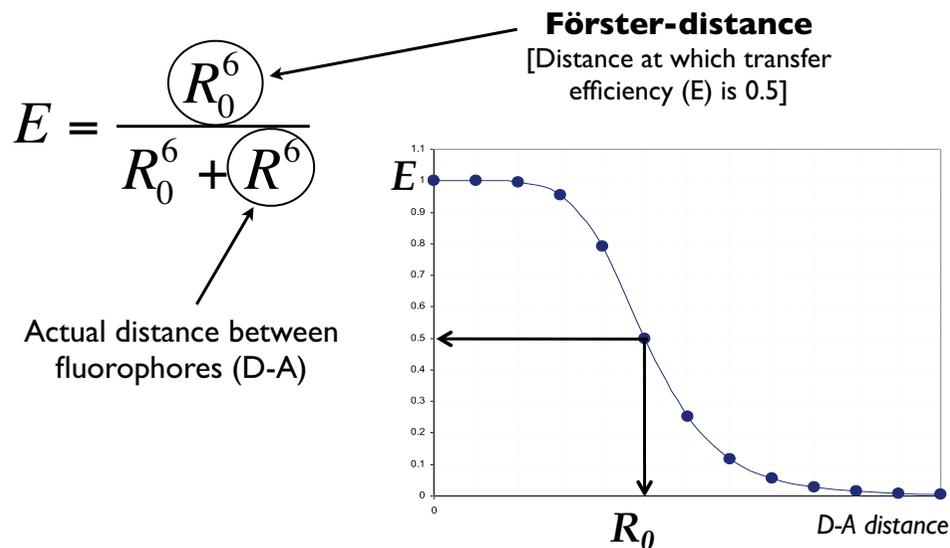


Conditions of FRET

- **Fluorescent** donor and acceptor molecules.
- The distance (**R**) between donor and acceptor molecules is 2-10 nm!
- **Overlap** between the emission spectrum of the **donor** and the absorption spectrum of the **acceptor**.

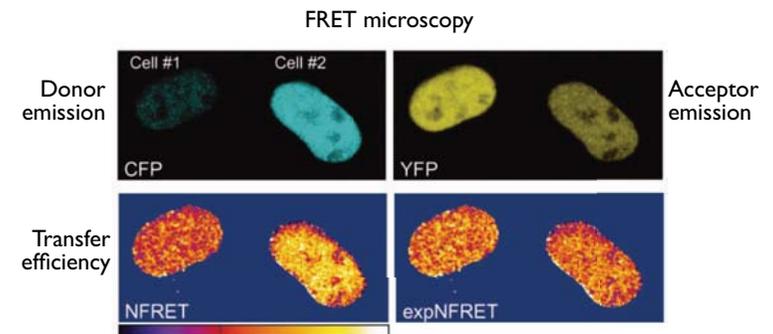


Distance dependence of FRET



Applications of FRET

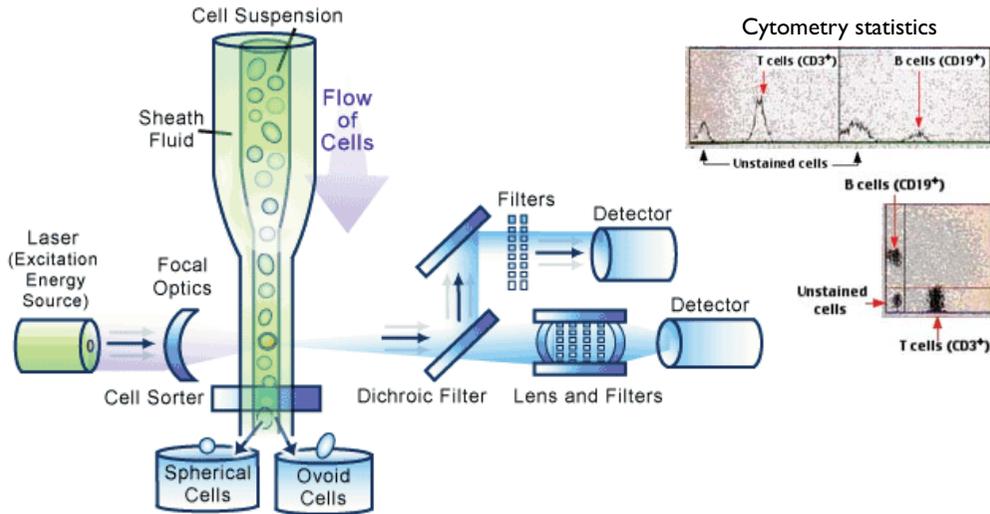
- **Molecular ruler:** distance measurement on the nm (10^{-9}m) scale.
- High sensitivity (see sixth-power dependency)!
- **Applications:**
 - Measurement of **interactions** between molecules.
 - Measurement of **structural** changes on molecules.



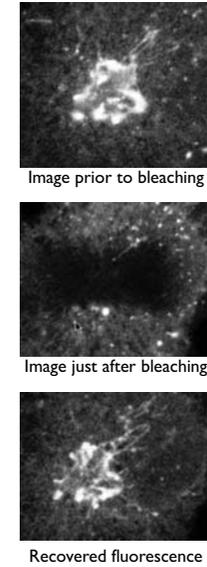
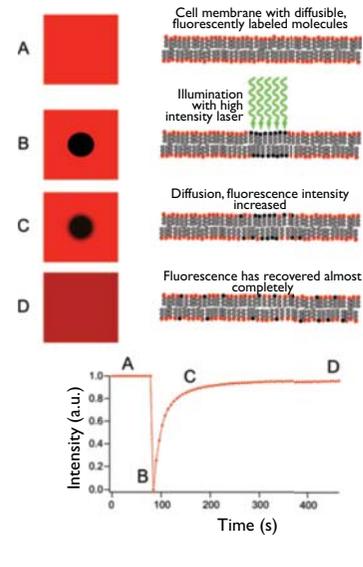
Fluorescence activated cell sorter (FACS)

Flow cytometry

- A cell suspension, fluorescently labeled by using specific antibodies, is analyzed cell by cell
- Numerous parameters are measured simultaneously (fluorescence intensity at several wavelengths, small- and large-angle scatter)
- Statistical analysis
- If needed, cells can be separated according to their fluorescence



Fluorescence Recovery After Photobleaching (FRAP)

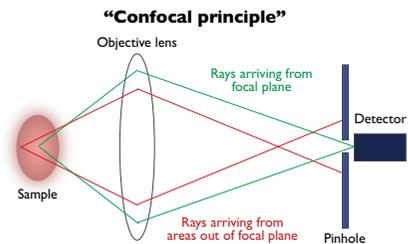
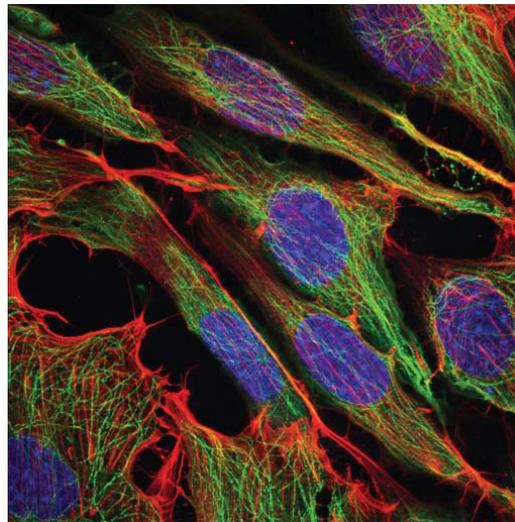
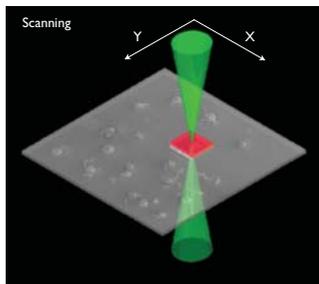


The diffusion coefficient can be determined from the time-dependent recovery of fluorescence as:

$$D = \frac{w^2}{4t_D}$$

D = diffusion coefficient
w = width of bleached area
t_D = time constant

Laser scanning confocal microscopy

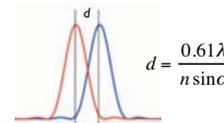


Super-resolution microscopy

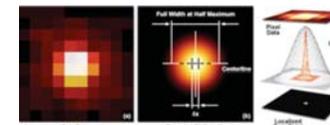
Chemistry Nobel-prize, 2014

Resolution problem is converted into position-determination problem

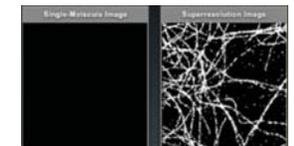
Resolution problem (Abbé)



Position determination problem (precision depends on photon count)



“Stochastic” data collection, single fluorophores



STORM: “stochastic optical reconstruction microscopy”

