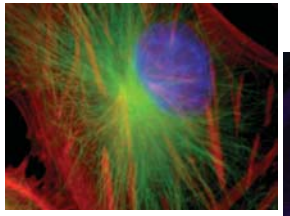
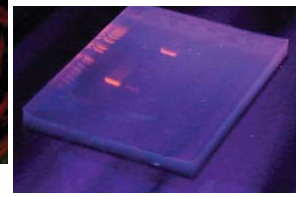
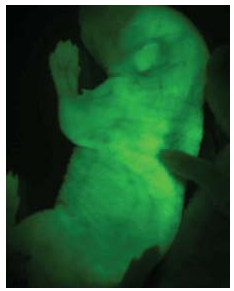
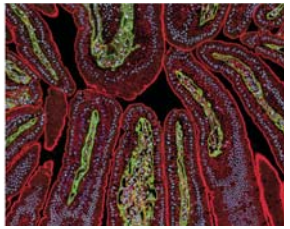
Biomedical applications of fluorescence

Zsolt Mártonfalvi

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 photoleaching (FRAP)
- Fluorescent protein conjugation technologies
- Quantum dots
- etc...

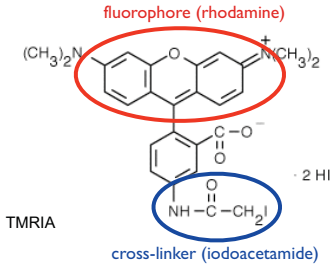
Fluorescence applications

- 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

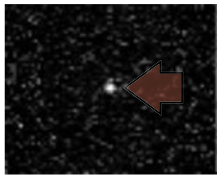
Source of fluorescence I.

Intrinsic fluorophores:
E.g., tryptophan, tyrosine in proteins (amino acids contaning 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

1. 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

Fluoresence 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.

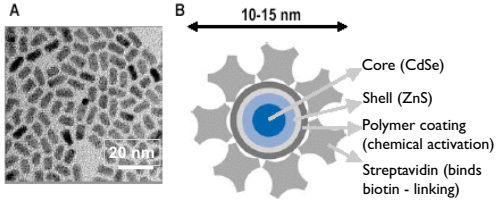
Disadvantages: blinking, only terminal (N or C) labeling, GFP might sterically interfere with the function of the targeted protein.

GFP-mouse

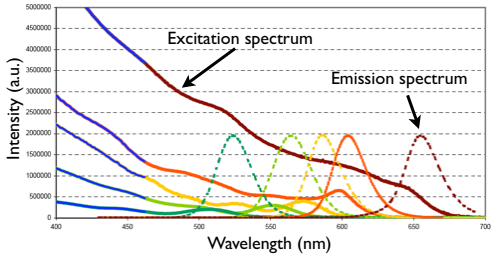
2. Color mutants of GFP (yellow, red, cyan, etc.)
3. Photoactivatable GFP analogue
4. Kaede: fluorescent protein from corals with green-red photoconversion induced by UV light.

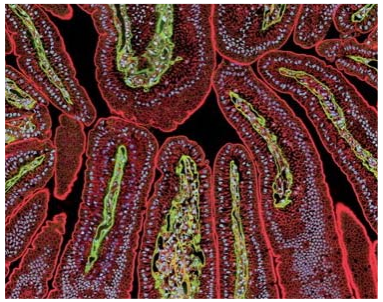
Source of fluorescence III.

Labeling with quantum dots (semiconductor nanocrystals)



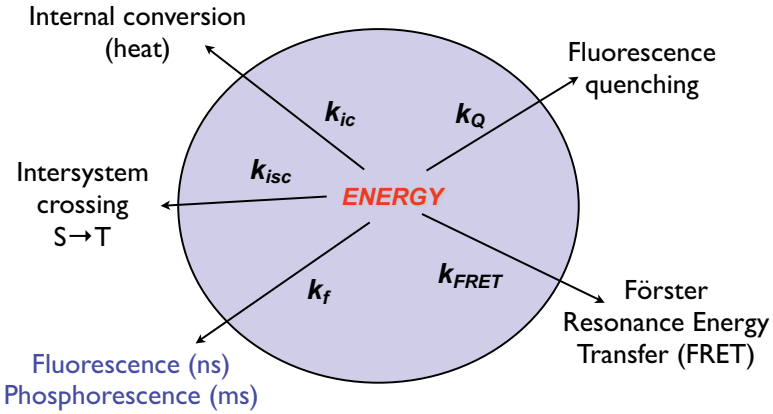
- Wide excitation spectrum
- Tunable emission spectrum (depends on particle size)
- Low tendency of photobleaching
- Tendency of blinking





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

Fate of absorbed energy



Radiative *and* non-radiative transitions!

Förster Resonance Energy Transfer (FRET)

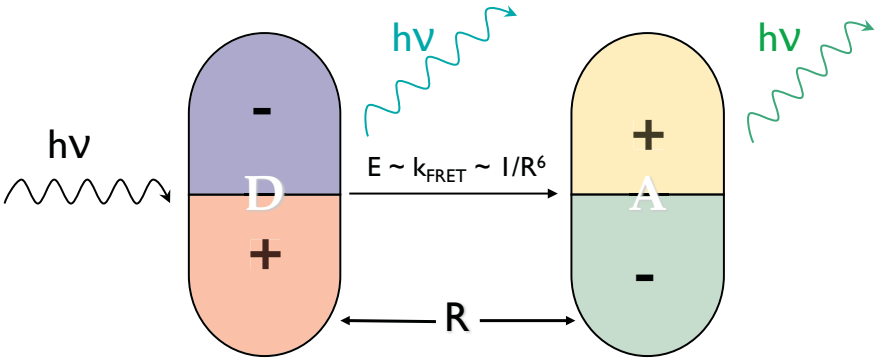
In general:

- 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)

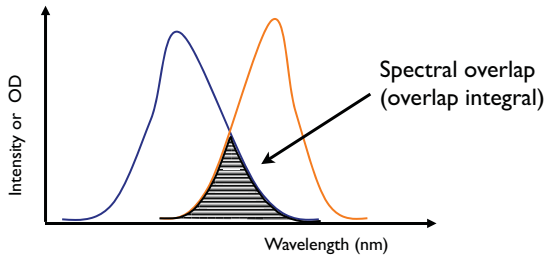
Mechanism of FRET

- 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**.

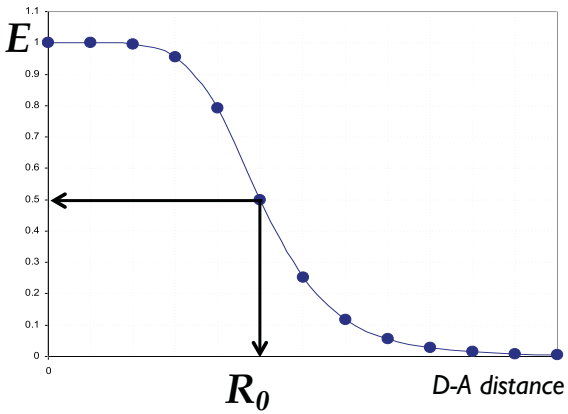


Distance dependence of FRET

$$E = \frac{R_0^6}{R_0^6 + R^6}$$

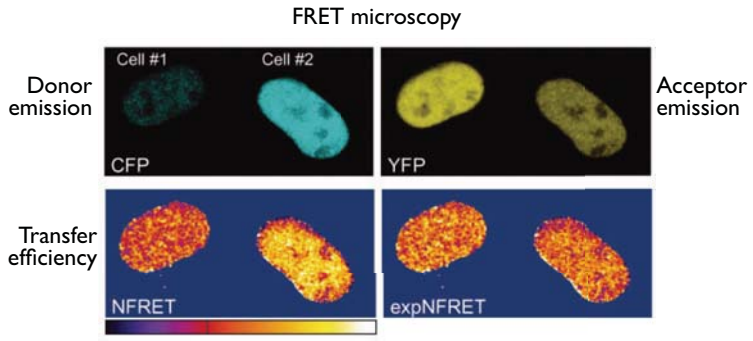
Förster-distance
(Distance at which transfer efficiency (E) is 0.5)

Actual distance between fluorophores (D-A)



Applications of FRET

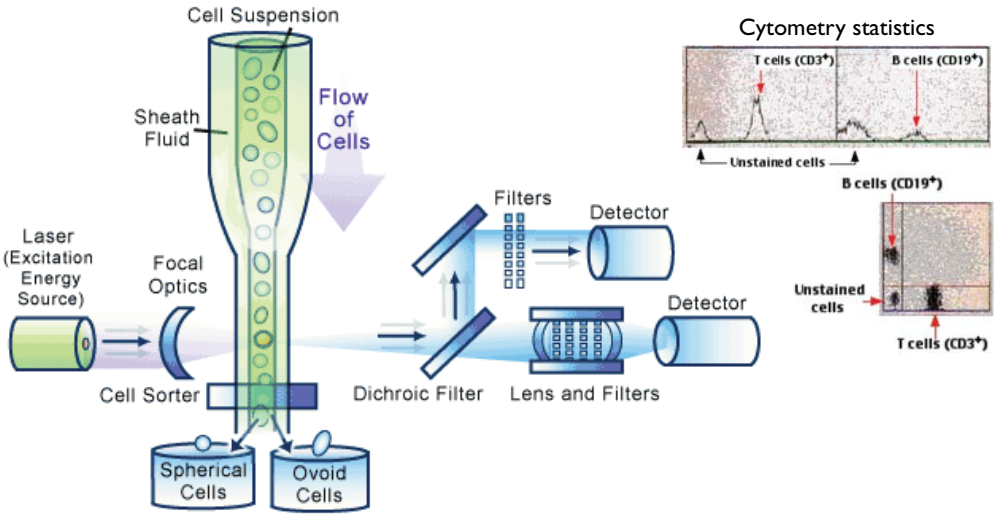
- **Molecular ruler:** distance measurement on the nm (10^{-9}m) scale.
- High sensitivity (see sixth-power dependency)!
- **Applications:**
  - Measurement of ***interactions*** between molecules.
  - Measurement of ***structural*** changes on molecules.



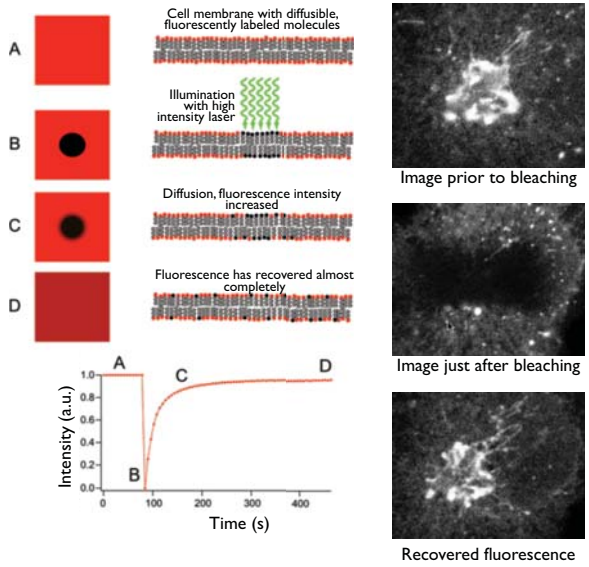
Fluorescence activated cell sorter (FACS)

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)

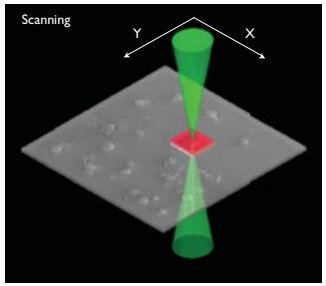


The diffusion coefficient can be determined from the time-dependent recovery of fluorescence as:

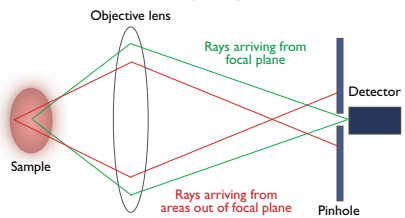
$$D = \frac{w^2}{4t_D}$$

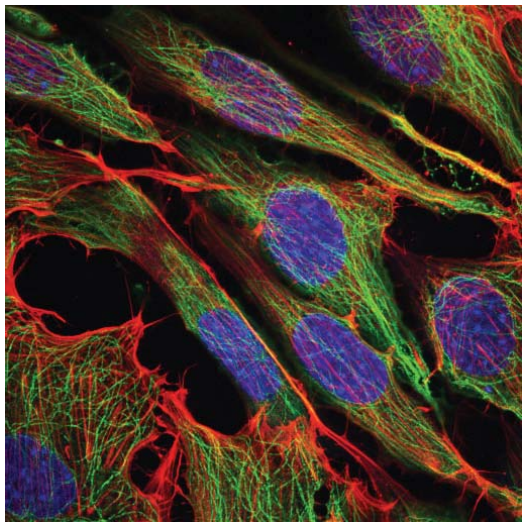
D = diffusion coefficient
w = width of bleached area
t_D = time constant

Laser scanning confocal microscopy



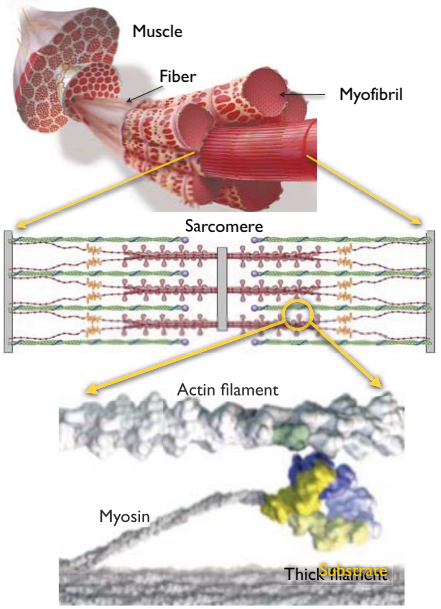
"Confocal principle"

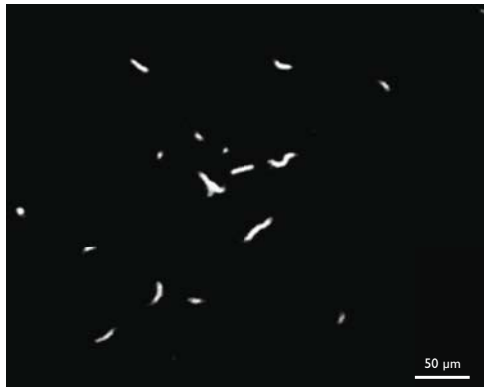




Green: microtubules; Red: actin; Blue: nuclei

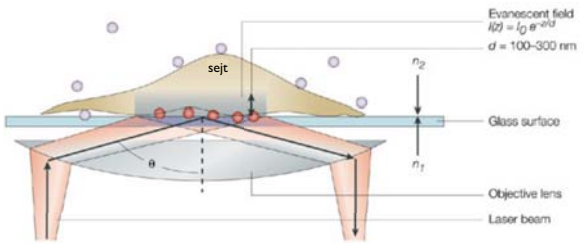
Intensified video microscopy

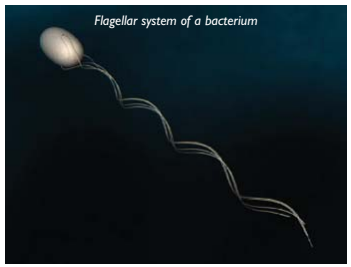




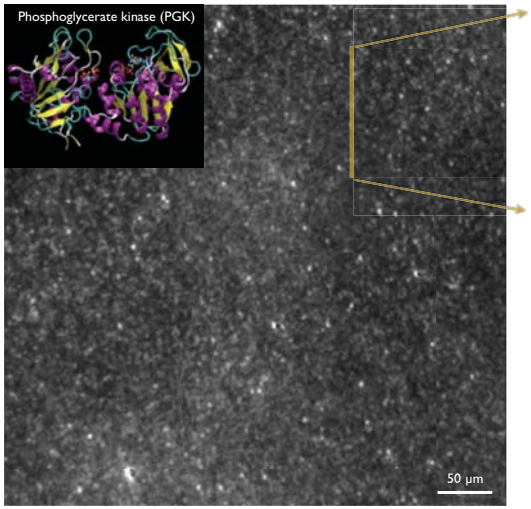
Rhodamine-labeled actin filament motility on HMM

Total Internal Reflection Fluorescence microscopy (TIRFM)

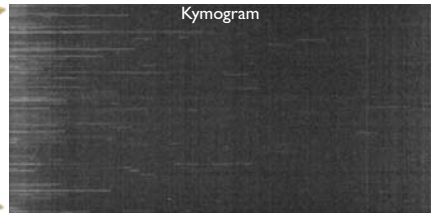




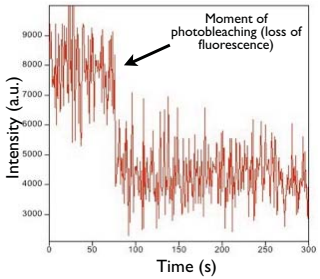
TIRFM image of single enzyme



PGK molecules labeled with Alexa488, TIRF microscopy

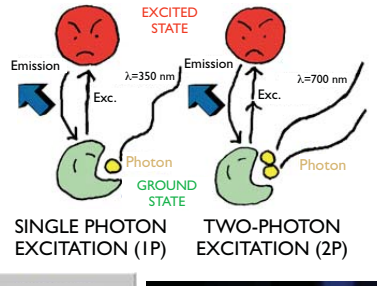


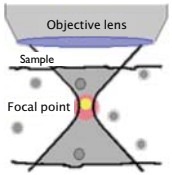
Single-step photobleaching (exponential function in ensemble!)

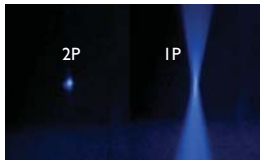


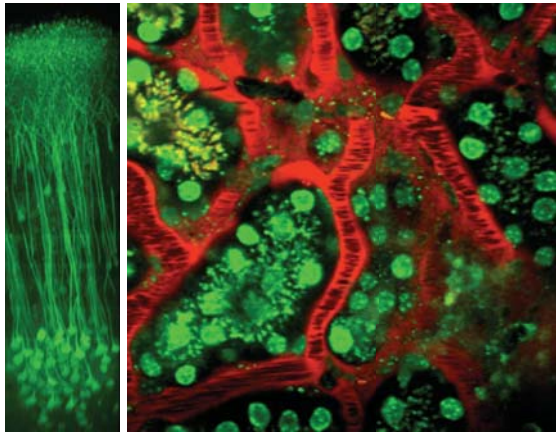
Two-photon fluorescence

- The energy of two (or more) photons is summed.
- Excitation (hence emission) only in the focal point (limited photodamage)
- Excitation with large-wavelength (near IR), short (fs) light pulses
- Because of large wavelength, deep optical penetration (~2 mm)









Cortical pyramid cells

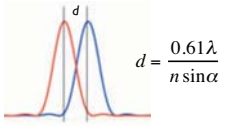
Focusing through the kidney cortex: proximal tubular system and blod vessels (red: rhodamine albumin in plasma)

Super-resolution microscopy

Chemistry Nobel-prize, 2014

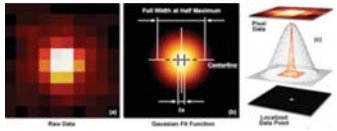
Resolution problem is converted into position-determination problem

Resolution problem (Abbé)

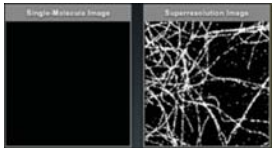


$$d = \frac{0.61\lambda}{n \sin\alpha}$$

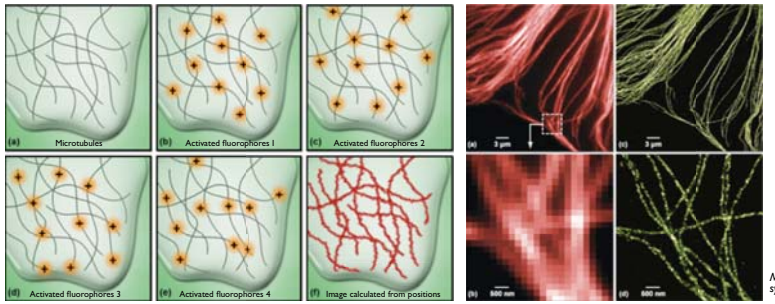
Position determination problem
(precision depends on photon count)



"Stochastic" data collection, single fluorophores



STORM: "stochastic optical reconstruction microscopy"



Data collection process

Microtubular system