Physical methods in bio-molecular studies

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Final exam questions ED 2015-2016
14. Application fields of luminescence
 a) Light sources based on luminescence
 b) Medical and laboratory use of luminescence

65. Modern microscopic techniques
 a) confocal laser-microscopy
 b) two-photon excitation

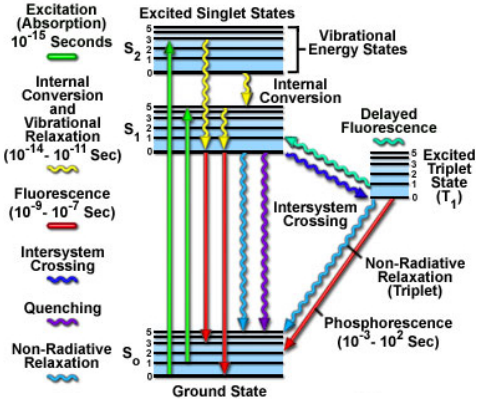
66. Scanning techniques in modern microscopy
 a) Concept of scanning
 b) Atomic force microscope

Further readings:
Damjanovich-Fidy-Szöllősi:
X/1, X/2., X/3.

March 6th, 2017

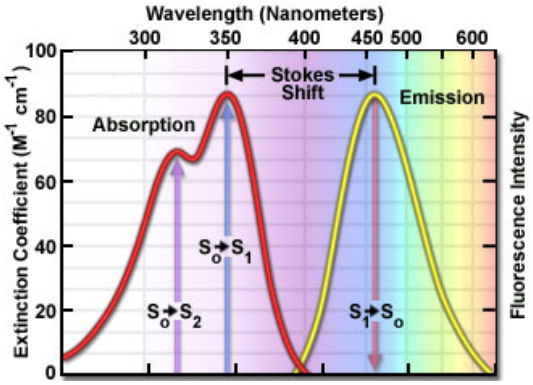
Light emission and absorption – Jablonski diagram

The Jablonski diagram is an energy diagram that illustrates the electronic states of a molecule and the transitions between them.



Light emission and absorption spectra

Stokes shift is the difference (in wavelength or frequency units) between the positions of the absorption and emission maxima.



Autofluorescence

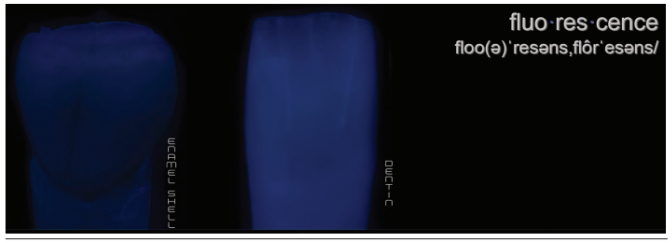
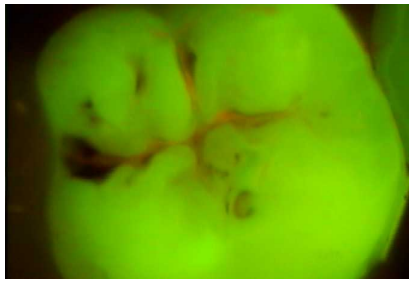


Figure 3: Enamel versus dentin. Dentin is more fluorescent than enamel under UV light. This greater fluorescence results from the higher organic content of dentin.

Dentin is more fluoresvent in UV light, than enamel.

Quantitative Light-induced Fluorescence (QLF)



information about caries and bacterial activity

UVA excitation fluorescence

tooth enamel GREEN: 430-560 nm

bacteria (protoporfirin-IX) RED: 590-700 nm

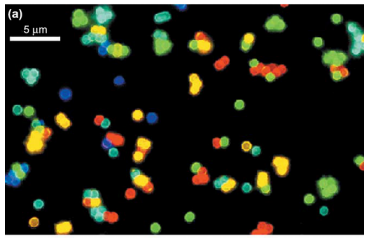
The perfect chromophore (fluorescent dye)

- small
- hydrophyllic
- can be excited in the visible range (two-photon: in the IR)
- large Stokes shift
- specific
  (biotin/avidin, His-tag/Ni, antibody/antigene, NH2, SH)
- Bright (abs*fluor quantum efficiency is high)
- not easily photobleached (except: FRAP, STORM)
- no photoreactions (except: un-cageing)
- no blinking (except: STORM)
- ...

Fluorescent quantum dots

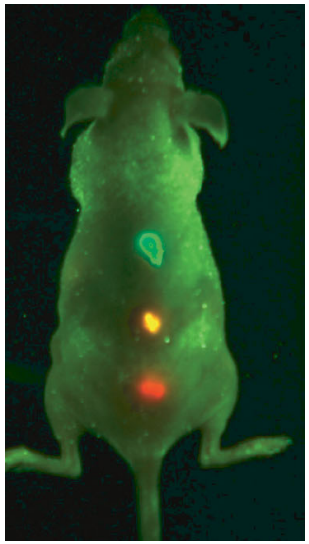
(a) fluorescence microscopic image of quantum dots made of CdSe with ZnS coating

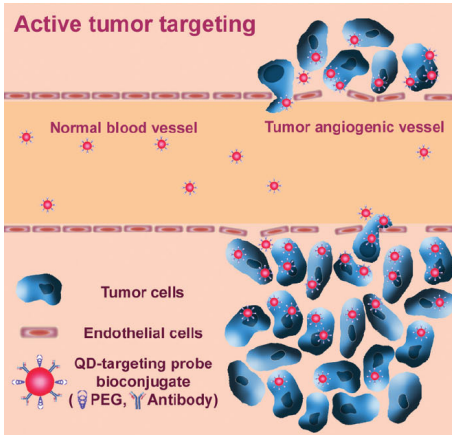
The size of the quantum dots determines their color.



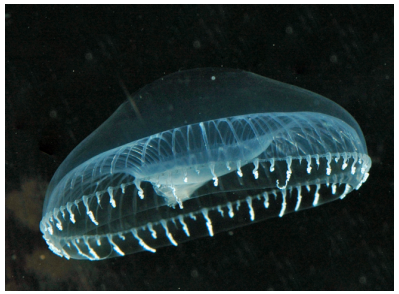
(b) ten solution containing CdSe/ZnS quantum dot of ten different sizes, thus fluorescing in ten different colors

Tumors labeled in vivo with fluorescent quantum dots

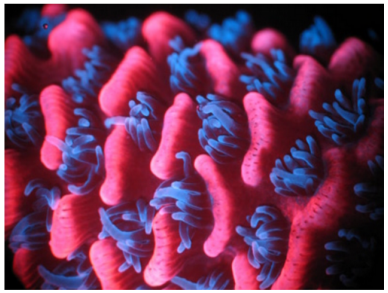




Fluorescent proteins



Aequorea victoria
(jellyfish)



Acropora millepora
(coral)

GFP (Green Fluorescent Protein)

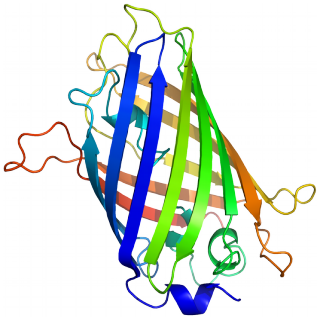
2008 Nobel price in chemistry

Osamu Shimomura – '60 isolated GFP

Douglas Prasher – 1992 cloned and sequenced GFP

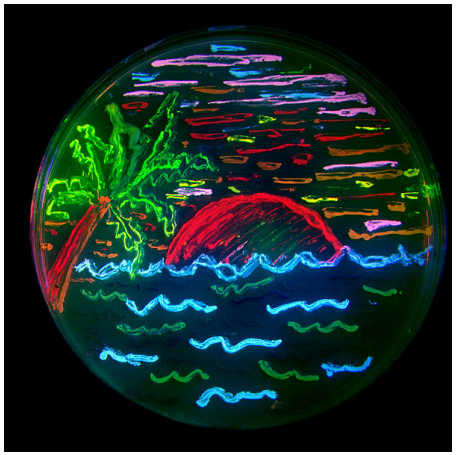
Martin Chalfie – 1994 used GFP to indicate gene expression

Roger Y. Tsien – 1995 constructed enhanced versions of GFP



A large variety of fluorescent proteins is available

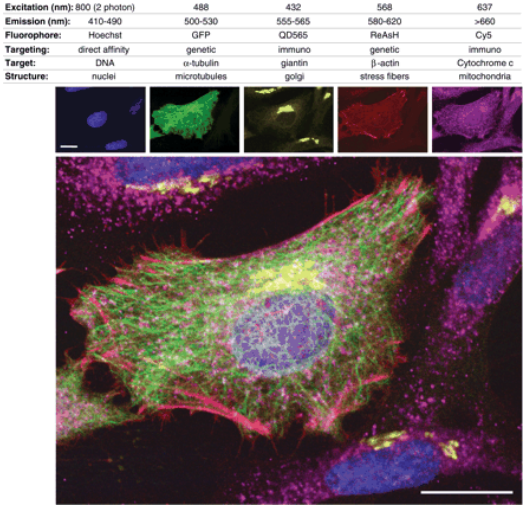
A picture painted entirely using bacteria expressing fluorescent proteins.



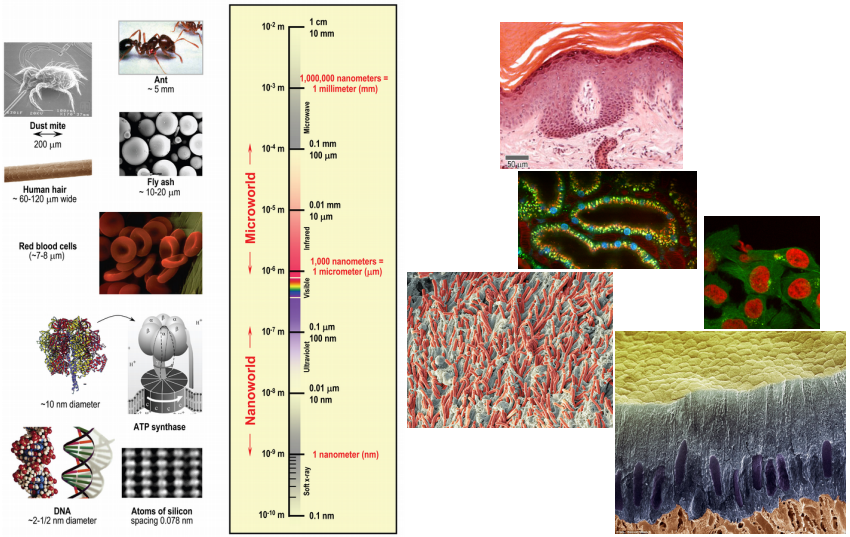
Parallel use of several fluorescent labels

HeLa cells stained with five different fluorescent dyes.

The bar is 20 µm long.



How big are things?



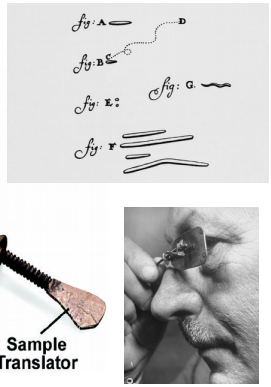
Hans Jansen and Zacharias Jansen construct a compound microscope in 1590

3x-10x magnification

Antoni van Leeuwenhoek (1632-1723) constructs a simple microscope in 1674

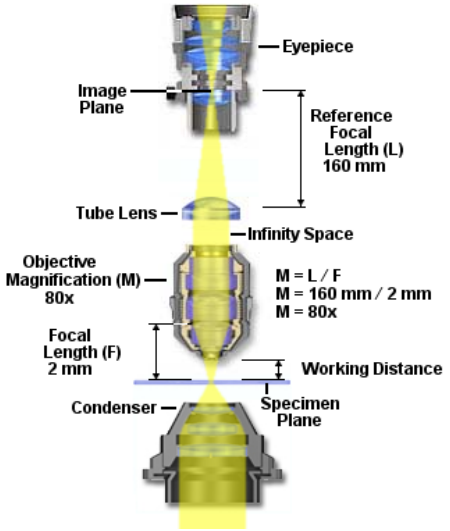
He was courious and could produce good lenses.

He discovered bacteria while viewing scrapings from his teeth and the teeth of others. He also discovered blood cells and was the first to see living sperm cells in animals.



70x-250x depending on the lens

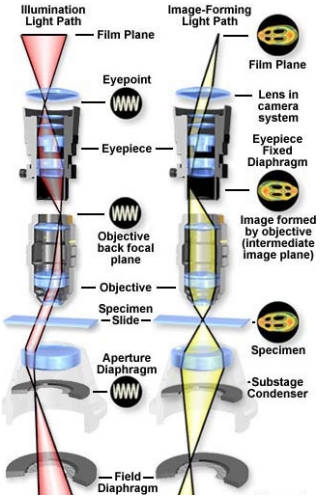
Infinity corrected optics



Köhler illumination

August Köhler
(1866-1948)

invented in 1893



Ernst Karl Abbe (1840-1905)

Carl Friedrich Zeiss
(1816-1888)

He put the production of optical devices on scientific bases.

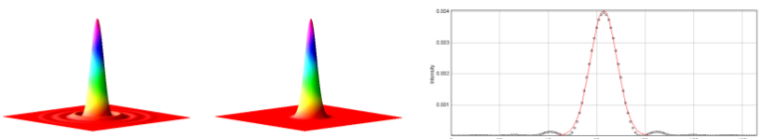
$$d = \frac{\lambda}{2n\sin\alpha}$$

$\delta = 0{,}61 \cdot \lambda / (n \cdot \sin\omega)$

Point Spread Function (PSF)

The image of a single point of an (fluorescent) object is not a point but a spot. This effect is the consequence of the wave nature of light.

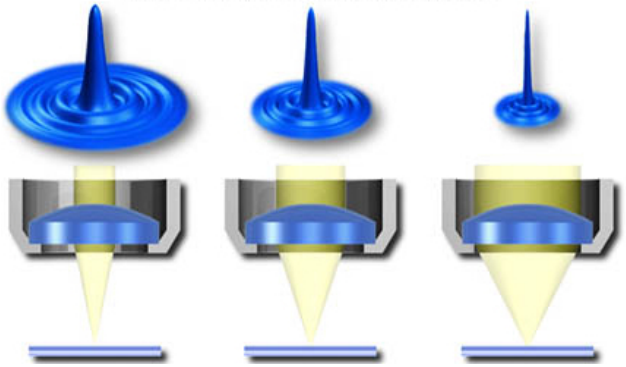
The objective focuses light in a volume and not into one point.



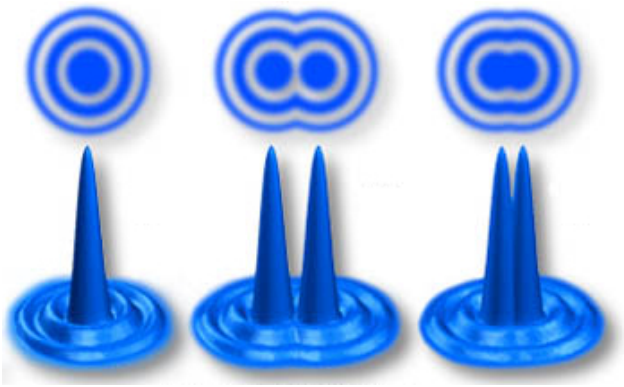
(a) Airy disk (b) 2D Gaussian (c) Profile through the centre of the Airy disk (black) and Gaussian fit (red)

Figure 15.7: Comparison of an Airy disk (taken from a theoretical PSF) and a Gaussian of a similar size, using two psychedelic surface plots and a 1D cross-section. The Gaussian is a very close match to the Airy disk.

The effect of the numeric aperture on the PSF



The effect of the wave nature of light on the image



Fluorescence microscope (widefield)

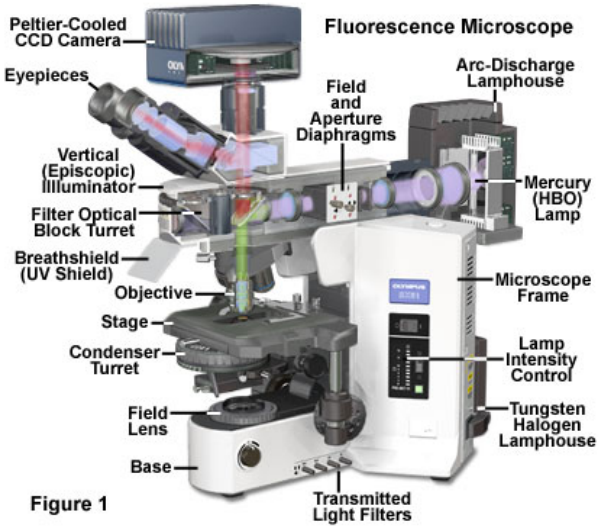


Figure 1

Fluorescence microscope

How to separate excitation from emission?

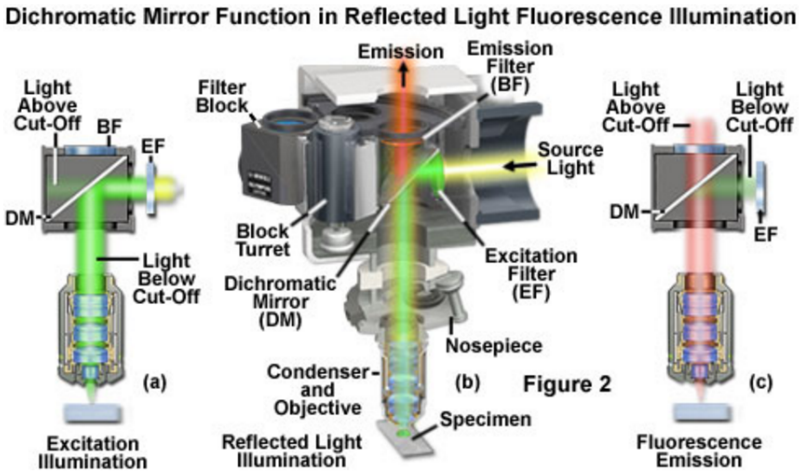
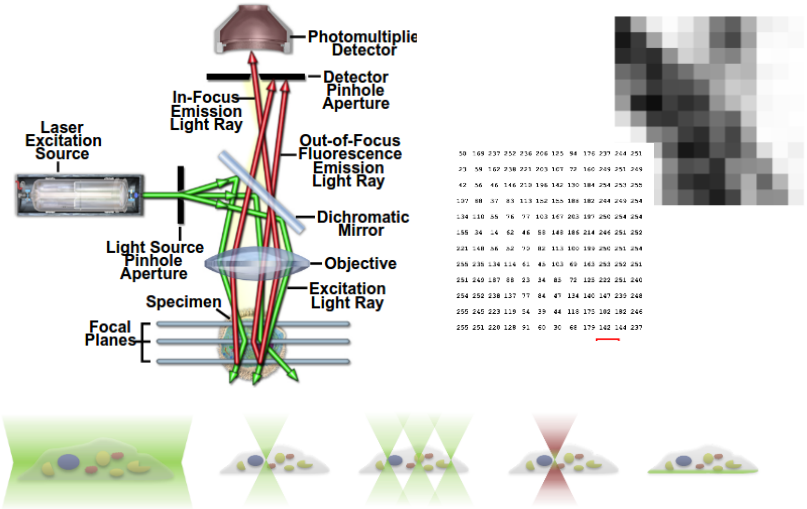


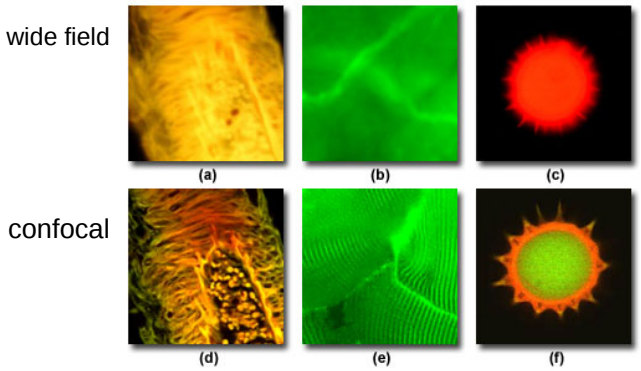
Figure 2

The working principle of the confocal microscope



(a) Widefield (b) LSCM (c) SDCM (d) 2-Photon (e) TIRF

Comparing the wide field and confocal images

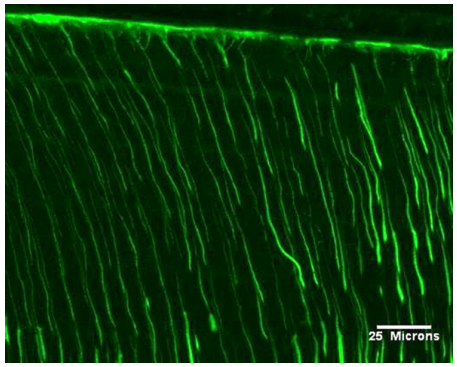


human medulla rabbit muscle pollen

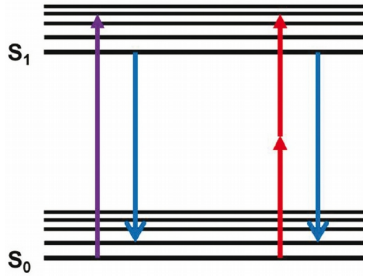
optical sectioning, stack-3D reconstruction, time-lapse, tracking

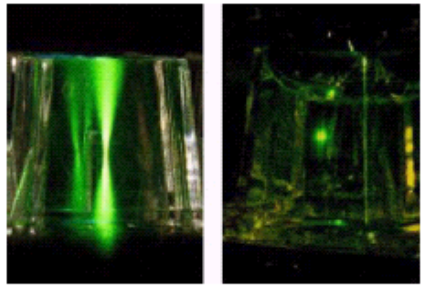
http://micro.magnet.fsu.edu/primer/index.html

Dentinal tubules of intact human tooth



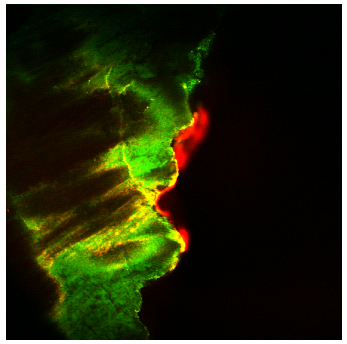
The working principle of the two-photon microscope



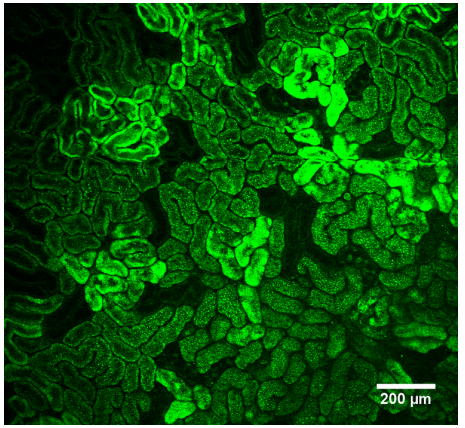


Why is it more effective for thick specimen imaging (in vivo) ?
-excitation: pulsed laser (high energy in one pulse)
-no out-of-focus 2P absorption-optical sectioning without pinhole
-scattering of the excitation is less for red-IR
-scattering of the fluorescence does not matter-every light is collected
-less photodamage
-autofluorescence
-simultaneous excitation of dyes

Two-photon microscope



tooth section



kidney in vivo - Ca map

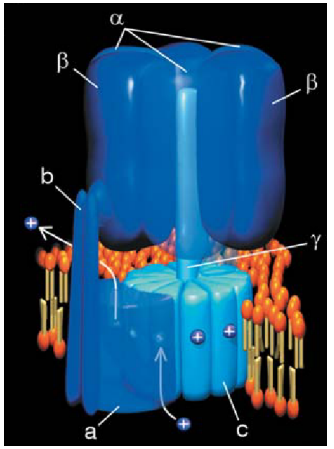
Study of single molecules

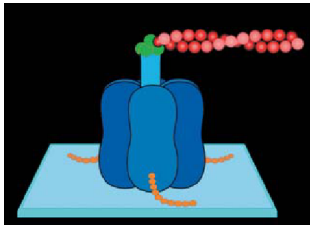
“Plenty of Room at the Bottom”

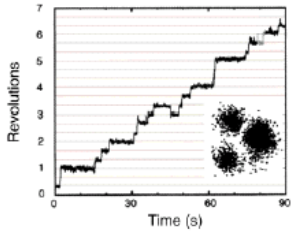
" The principles of physics, as far as I can see, do not speak against the possibility of maneuvering things atom by atom. It is not an attempt to violate any laws; it is something, in principle, that can be done; but in practice, it has not been done because we are too big."

Richard Feynman, 1959

Rotating movement of single ATP sintase molecules







Super-resolved microscopy

The Nobel Prize in Chemistry 2014

Photo: A. Mahmoud
Eric Betzig
Prize share: 1/3

Photo: A. Mahmoud
Stefan W. Hell
Prize share: 1/3

Photo: A. Mahmoud
William E. Moerner
Prize share: 1/3

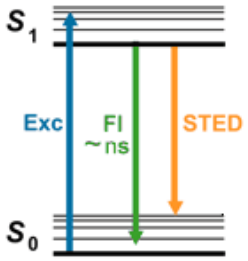
The Nobel Prize in Chemistry 2014 was awarded jointly to Eric Betzig, Stefan W. Hell and William E. Moerner *"for the development of super-resolved fluorescence microscopy"*.

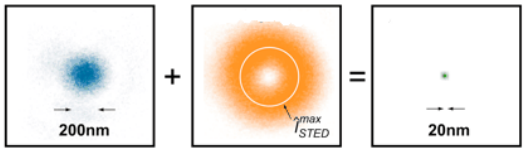
STED

PALM

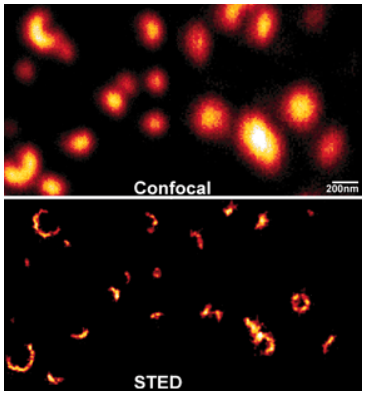
STORM

STimulated Emission Depletion (STED) microscope



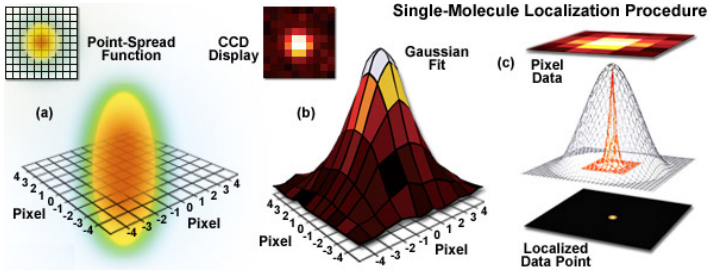


STimulated Emission Depletion (STED) microscope



Recycled material in the synaptic vesicles

Lokalizáció



STORM

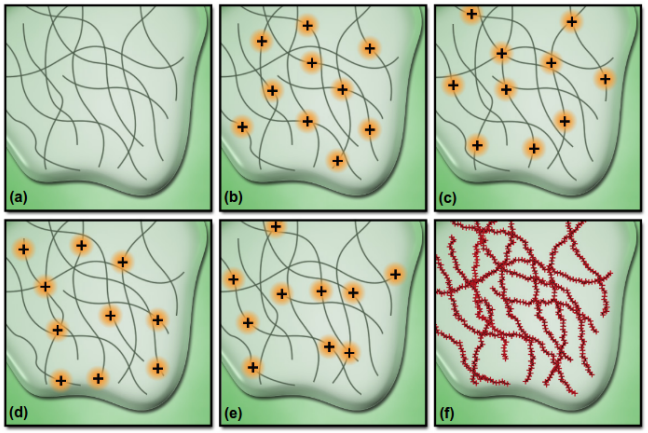
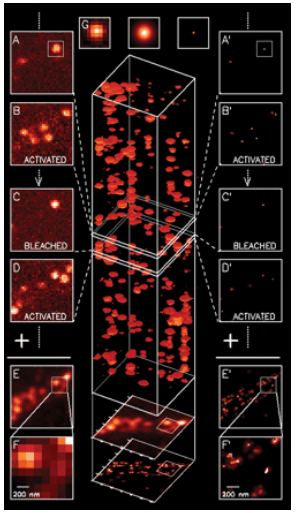


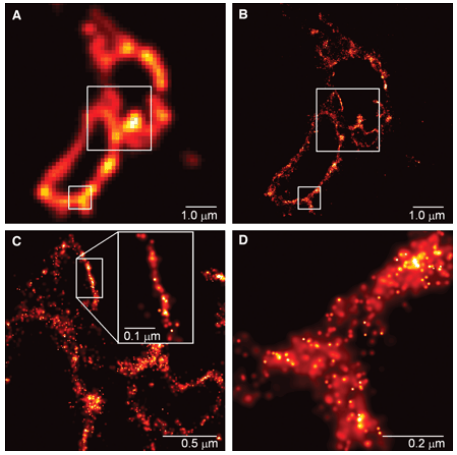
Photo-Activated Localization Microscopy (PALM)



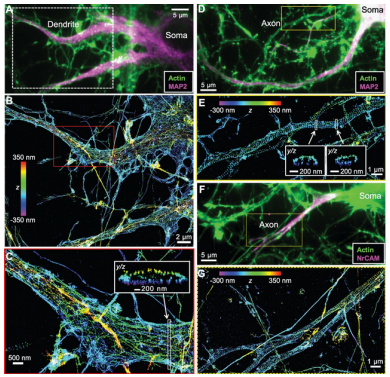
Based on the technology developed by Eric Betzig and Harald Hess

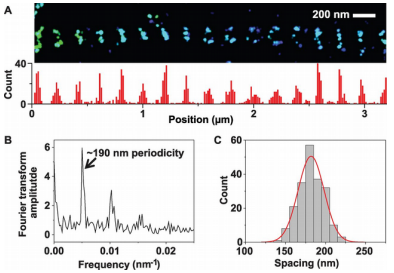
Photo-Activated Localization Microscopy (PALM)

CD63, lizosome transmembrane protein



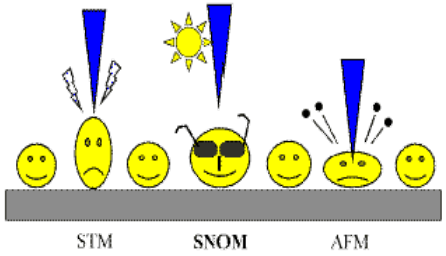
Cytoskeletal Structure in Axons





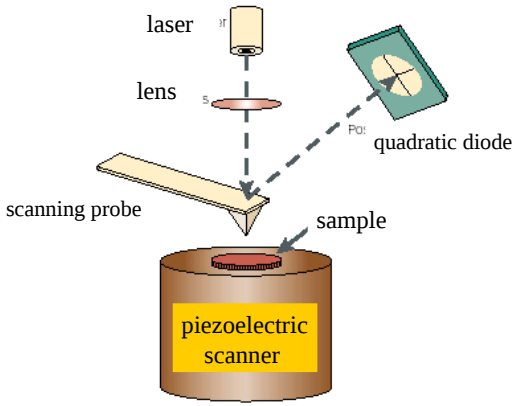
Scanning Probe Microscopy (SPM)

A topographic image of the surface of the sample is created by scanning the sample surface with a sharp probe and detecting interaction with the surface.



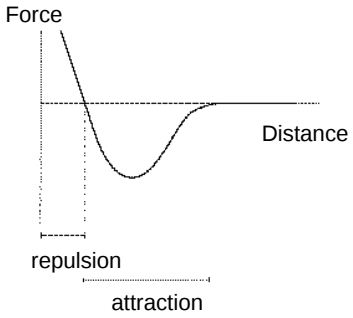
Atomic Force Microscopy - AFM

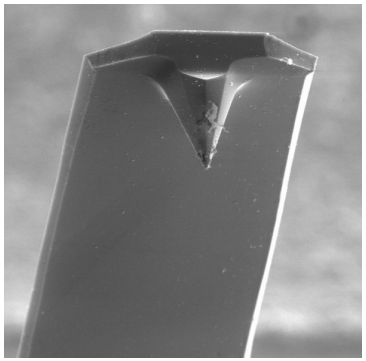
AFM: the detected interaction is the force between the sample surface and probe



Force between the probe tip and the sample

Properties of the probe:
- typically 100 μm long, 1μm thick, V shaped
- small spring constant
- high resonance frequency
- silicon (-oxide, -nitride)





Contact Mode AFM

The probe and the sample are is permanent contact.
Works in the repulsive region.
Keeps the interaction force constant and follows the surface.
The vertical deflection of the cantilever is detected.
Local force spectroscopy: record the interaction force as a function of displacement in one point of the surface.

Tapping Mode AFM

The probe oscillates with 20-100 nm amplitude touching the surface in every oscillation.
The oscillation frequency and amplitude and phase changes according to the topography of the surface.

Advantages and drawbacks

Contact Mode AFM

Advantage:
fast scanning
atomic resolution
good for hard surfaces

Drawback:
horizontal forces distort the image
water on the surface distorts the image
scratches soft biological samples

Tapping Mode AFM

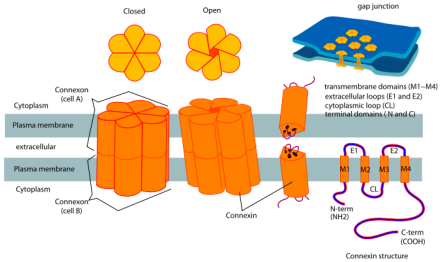
Advantage:
large lateral resolution (1 – 5nm)
less damage to soft samples

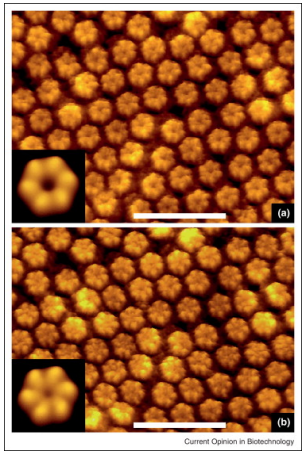
Drawback:
slow scanning

AFM image of the extracellular connexon surface

Calcium induced conformational changes of the extracellular connexon surface.

Scale bars represent 250 Å





Dentin collagene fibrils

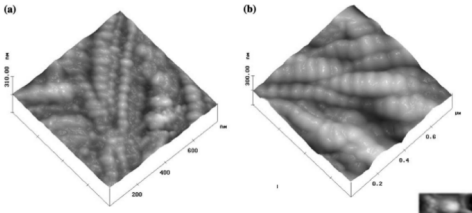
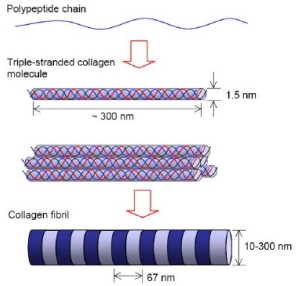


Fig. 3. Mixed-mode surface plots of tapping and phase mode AFM images of dentin collagen fibrils, obtained (a) in repeat pattern is still present after dehydration.



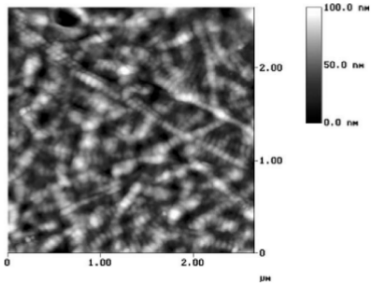


Fig. 2. Tapping mode AFM image of dentin collagen fibrils, obtained from demineralized specimen (15 s citric acid) treated with 6.5 vol% $NaOCl_{aq}$ for 130 s and in air, showing the repeat pattern and the random distribution of fibrils in intertubular dentin.

Question

Calculate the smallest resolvable distance in a microscope,
if 580 nm wavelength light is used for imaging,
and the following objective was used?
We would like to see individual labeled proteins in the cell,
that are supposedly at 70 nm distance. What would be the necessary N. A. of the required objective? Is there such an objective available?
Is it possible to image these proteins with some special method?