Physical methods in bio-molecular studies

Kis Petik Katalin

Related final exam questions

14. Application fields of **luminescence**
 b) Medical and laboratory use of luminescence

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

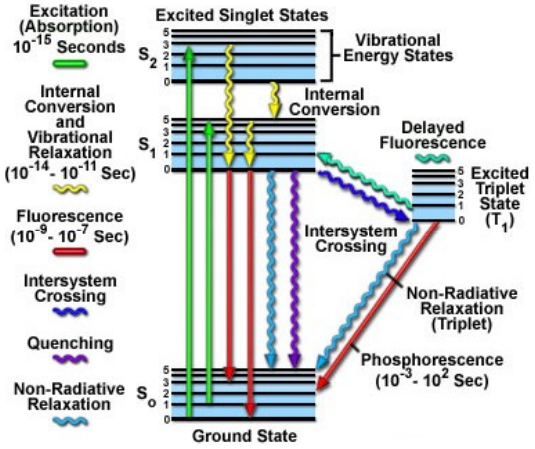
66. **Scanning** techniques in modern microscopy
 a) Concept of scanning
 b) Atomic force microscope

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

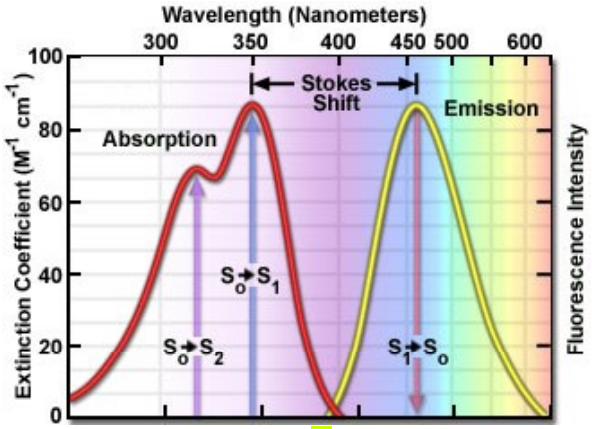
March , 2018

Light emission and absorption – Jablonski diagram

energy diagram that illustrates the electronic states of a molecule and the transitions between them.



Light emission and absorption (excitation) spectra

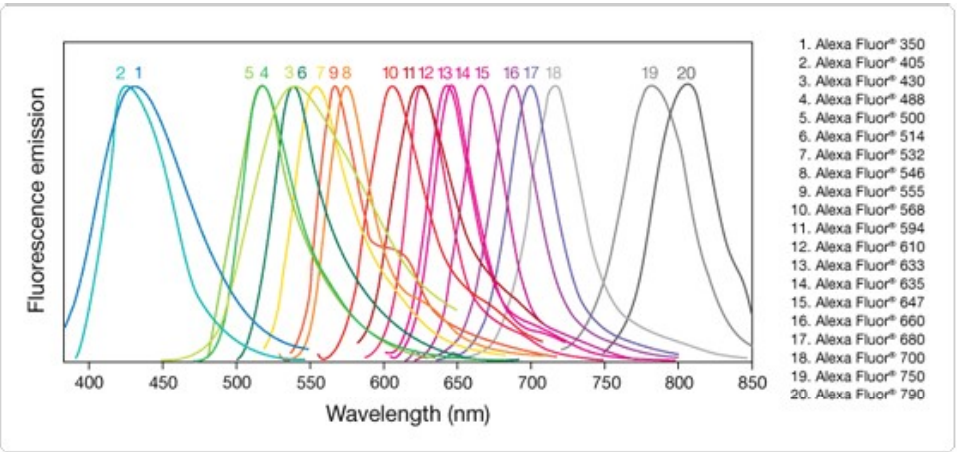


excitation at proper wavelength

detect emission at proper wavelength range

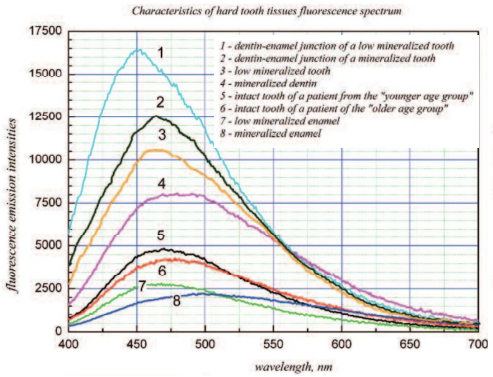
separate

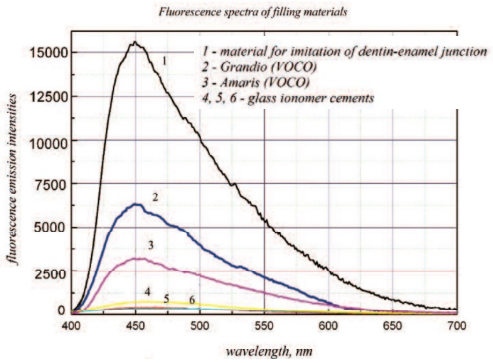
Fluorescent probes (dyes)



different dye molecules of different color can be connected to different places

Autofluorescence of tooth tissue and filling material





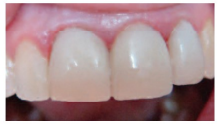


Figure 1: Facing work on central and lateral maxillary incisors.

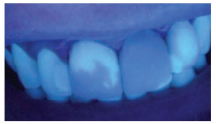
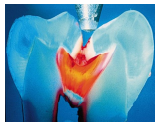
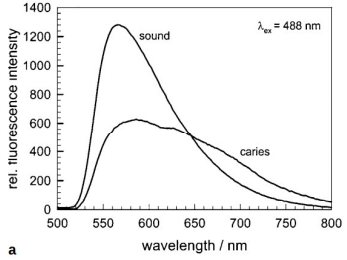


Figure 2: The restoratives differ in terms of the spectrum and intensity of fluorescence when teeth are exposed to short-wave light.

a crown made of porcelain with low fluorescent qualities will stand out in bright sunlight.

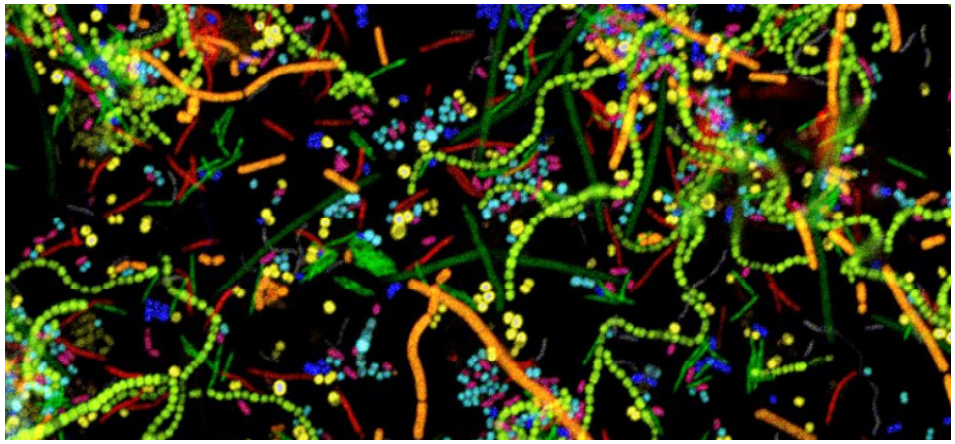


porphyrins in superficial carious lesions



a

Labeling by fluorescent probes



Fifteen different species of human oral microbes were labeled with **species-specific probes** using a technique called Combinatorial Labeling and Special Imaging combined with Fluorescent In Situ Hybridization (CLASI-FISH) and imaged with a confocal microscope.

Credit: Gary Borisy PhD, The Forsyth Institute, Cambridge MA

The perfect chromophore (fluorescent dye)

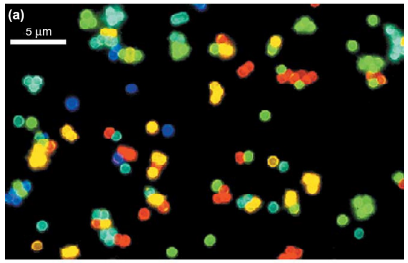
- small
- hydrophyllic
- can be excited in the visible range (two-photon: in the IR)
- large Stokes shift
- specific
  (biotin/avidin, His-tag/Ni, antibody/antigene, NH2, SH)
- bright (abs*fluor quantum efficiency is high)
- not easily photobleached (except: FRAP, STORM)
- no photoreactions (except: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

Fluorescent quantum dots

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

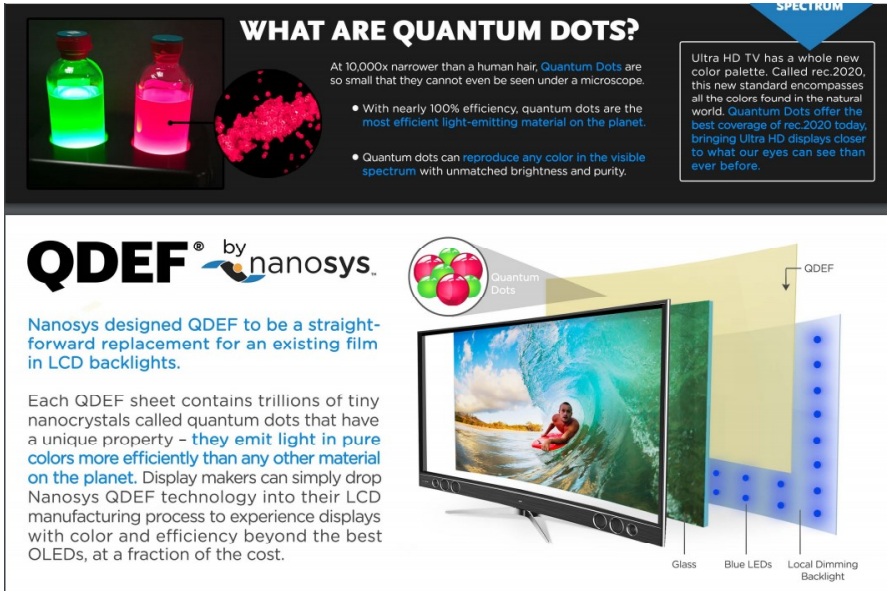
The size determines their color.

nanoscale semiconductor materials tightly confine either electrons or electron holes. artificial atoms: single objects with bound, discrete electronic states, as is the case with naturally occurring atoms or molecules

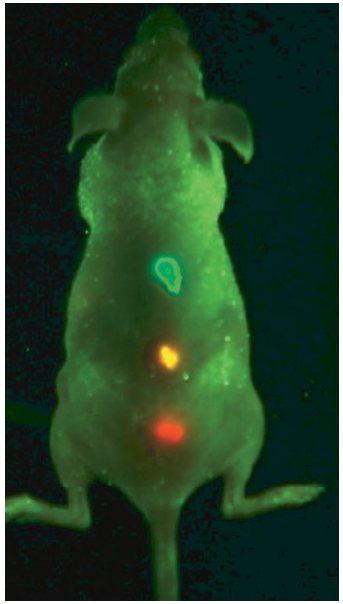


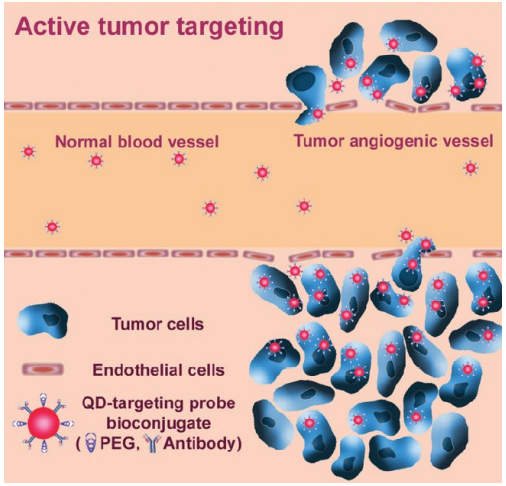
(b) ten solutions containing CdSe/ZnS quantum dot of ten different sizes, thus fluorescing in ten different colors

Fluorescent quantum dots



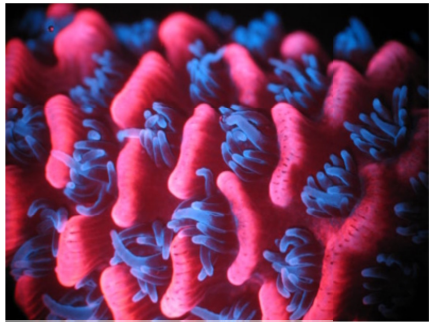
Tumors labeled in vivo with fluorescent quantum dots





Fluorescent proteins

Aequorea victoria
(jellyfish)



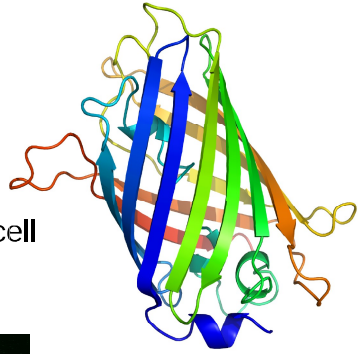
Acropora millepora
(coral)

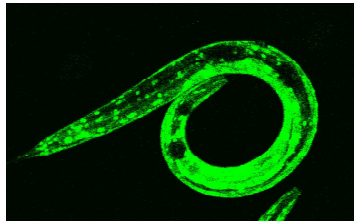
GFP (Green Fluorescent Protein)

protein encoded by a gene,
it can be expressed together
with a protein of interest

we can see,
where is the protein inside the body or cell

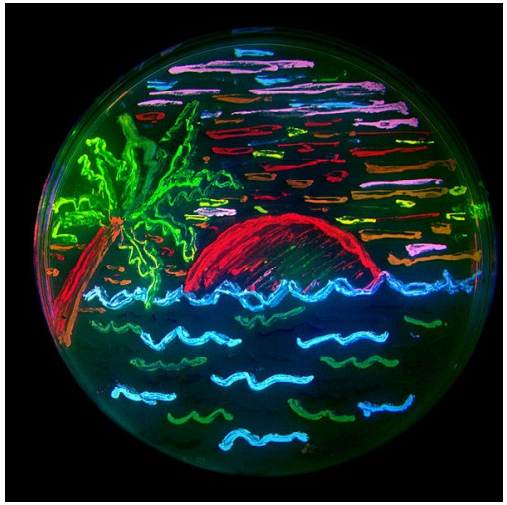
color variations





A large variety of fluorescent proteins is available

A picture painted entirely using bacteria expressing fluorescent proteins.

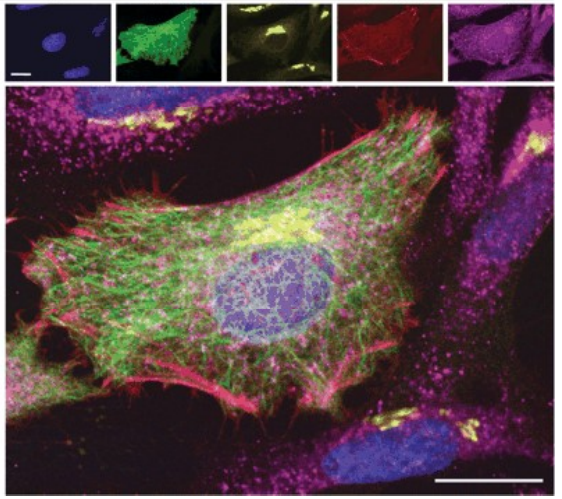


Parallel use of several fluorescent labels

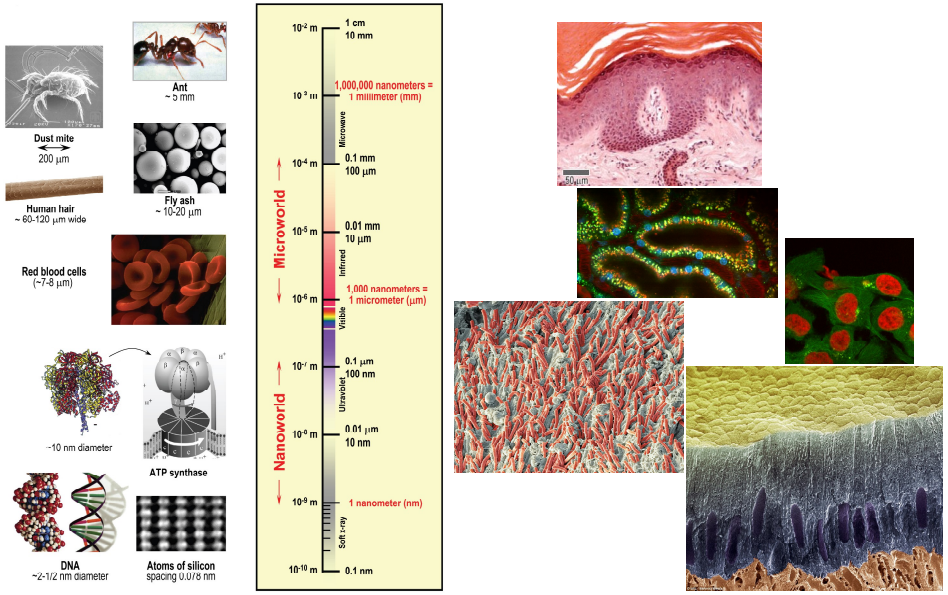
Excitation (nm):	800 (2 photon)	488	432	568	637
Emission (nm):	410-490	500-530	555-565	580-620	>660
Fluorophore:	Hoechst	GFP	QD565	ReAsH	Cy5
Targeting:	direct affinity	genetic	immuno	genetic	immuno
Target:	DNA	α-tubulin	giantin	β-actin	Cytochrome c
Structure:	nuclei	microtubules	golgi	stress fibers	mitochondria

HeLa cells stained wit five different fluoresce dyes.

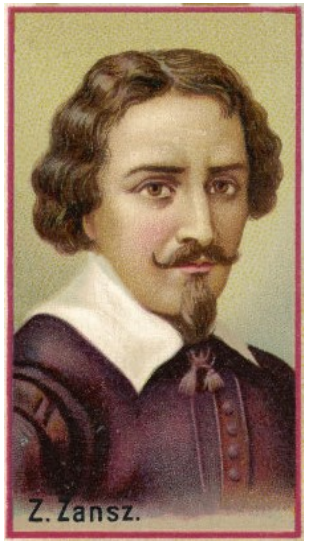
The bar is 20 µm long



How big are things?



Hans Jansen and Zacharias Jansen construct a compound microscope in 1590

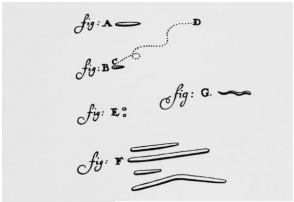


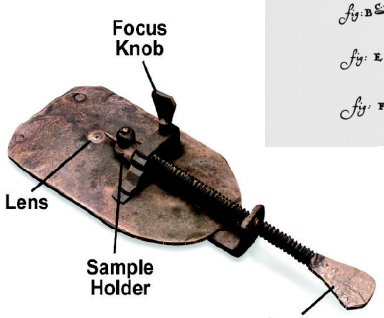
3x-10x magnification

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

He was courious and could produce good lenses.

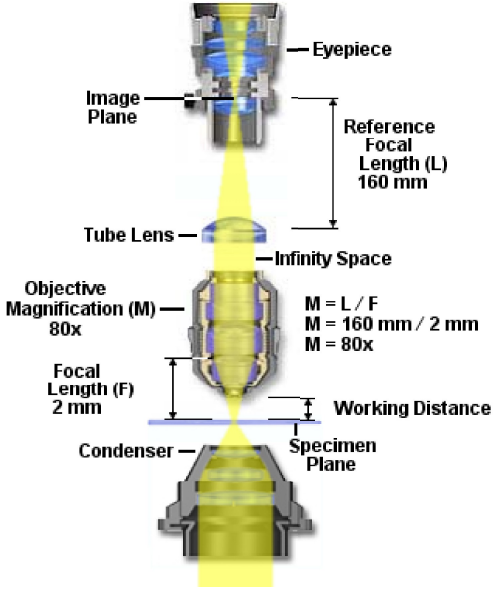
He discovered bacteria while viewing scrapings from his teeth and the teeth of others. He also discovered blood cells and was the first to see living sperm cells in animals.





70x-250x depending on the lens

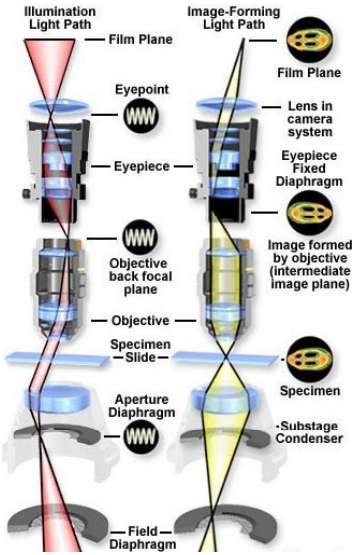
Infinity corrected optics



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.

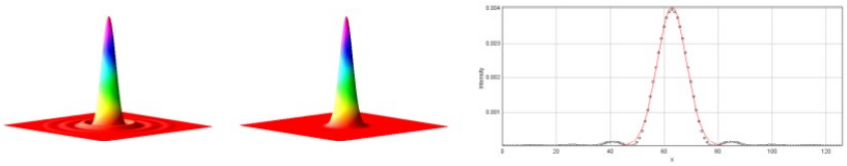
$$d = \frac{\lambda}{2n\sin\alpha}$$

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

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.

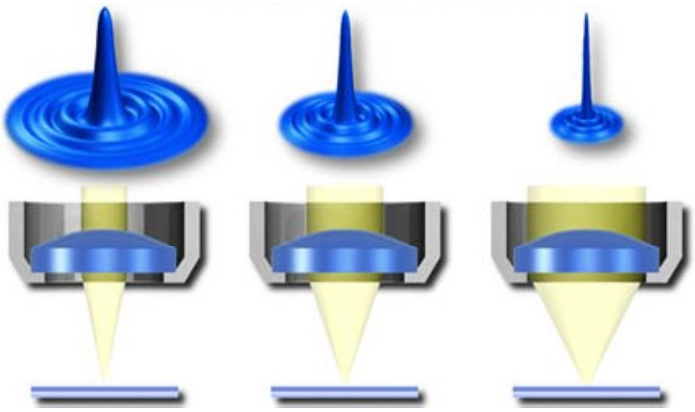
The objective focuses light in a volume and not into one point.



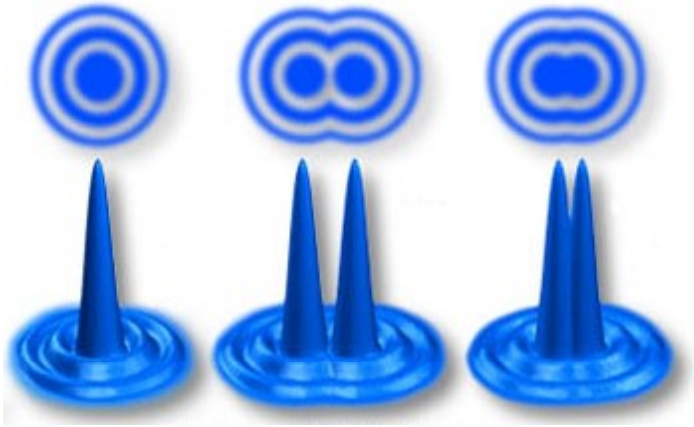
(a) Airy disk (b) 2D Gaussian (c) Profile through the centre of the Airy disk (black) and Gaussian fit (red)

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)

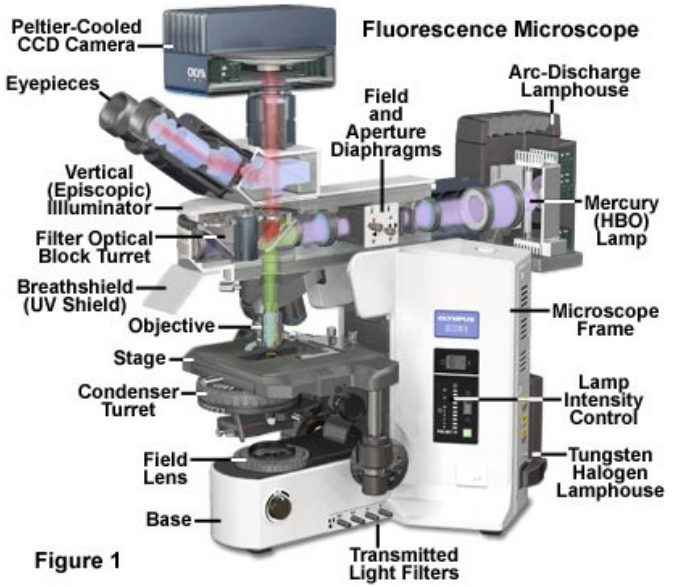
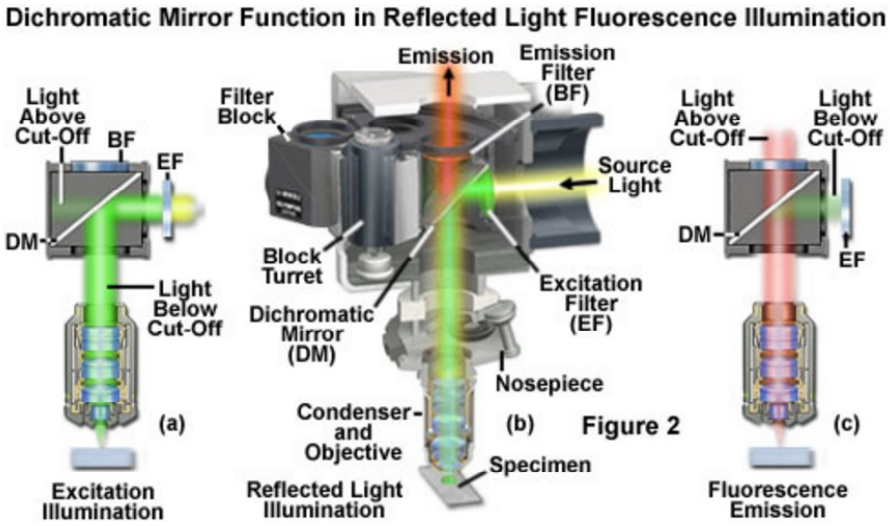


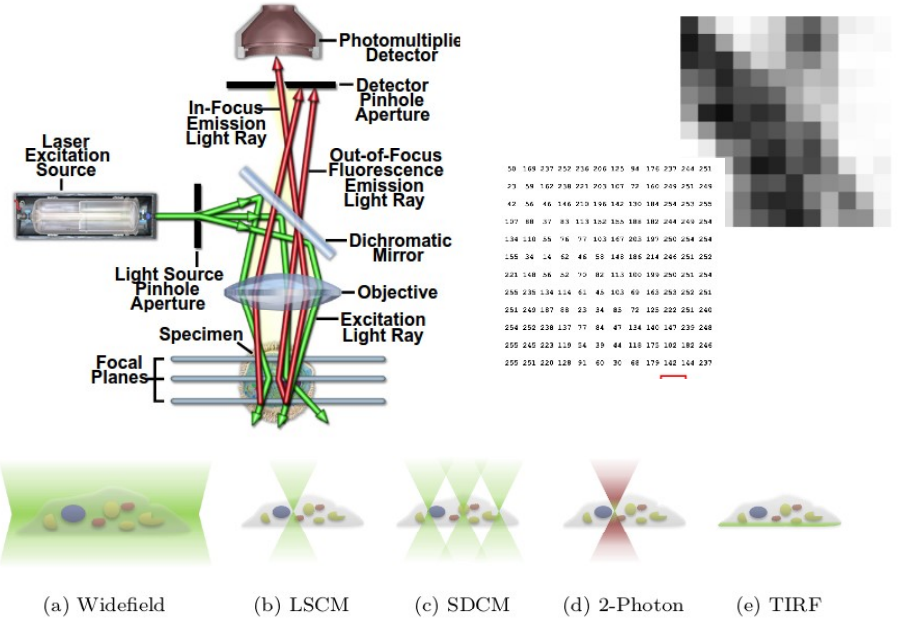
Figure 1

Fluorescence microscope

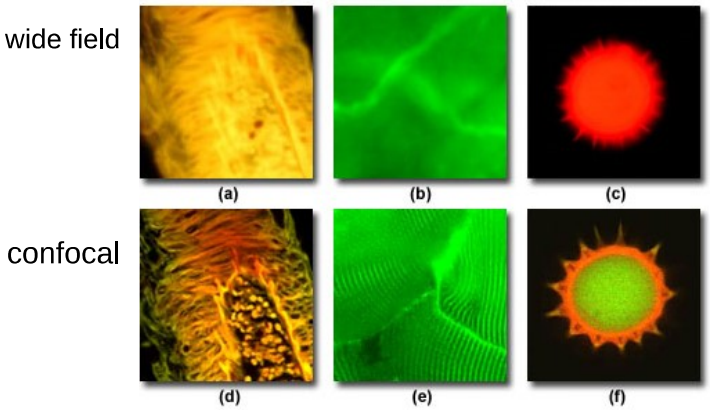
How to separate excitation from emission?



The working principle of the confocal microscope



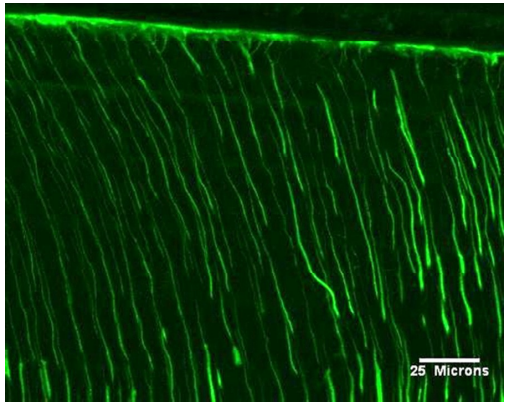
Comparing the wide field and confocal images



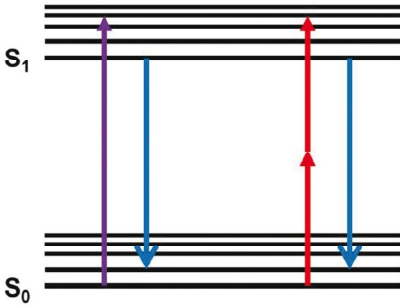
optical sectioning, stack-3D reconstruction, time-lapse, tracking

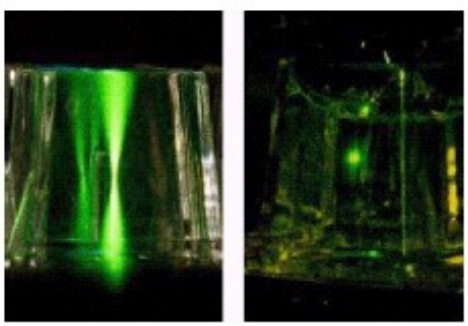
http://micro.magnet.fsu.edu/primer/index.html

Dentinal tubules of intact human tooth



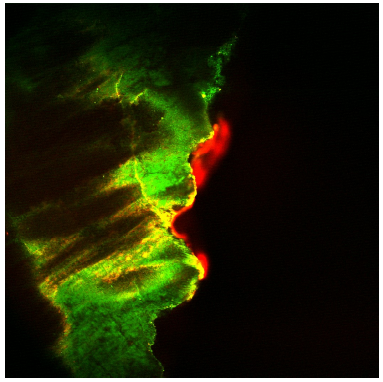
The working principle of the two-photon microscope



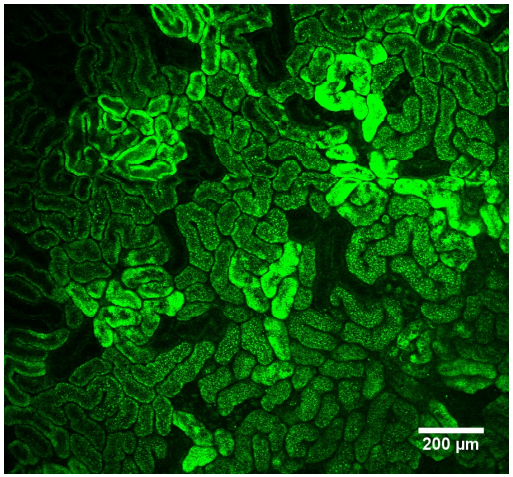


Why is it more effective for thick specimen imaging (in vivo) ?
-excitation: pulsed laser (high energy in one pulse)
-no out-of-focus 2P absorption-optical sectioning without pinhole
-scattering of the excitation is less for red-IR
-scattering of the fluorescence does not matter-every light is collected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

Two-photon microscope



tooth section



kidney in vivo - Ca map

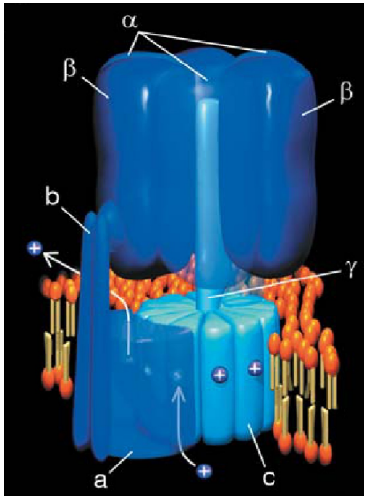
Study of single molecules

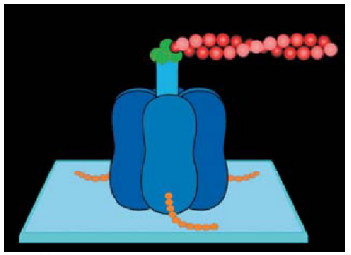
“Plenty of Room at the Bottom”

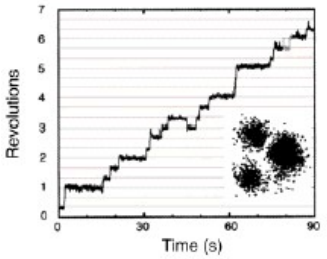
" The principles of physics, as far as I can see, do not speak against the possibility of maneuvering things atom by atom. It is not an attempt to violate any laws; it is something, in principle, that can be done; but in practice, it has not been done because we are too big."

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

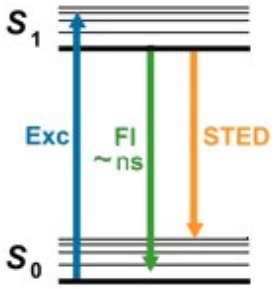
The Nobel Prize in Chemistry 2014 was awarded jointly to Eric Betzig, Stefan W. Hell and William E. Moerner *"for the development of super-resolved fluorescence microscopy"*.

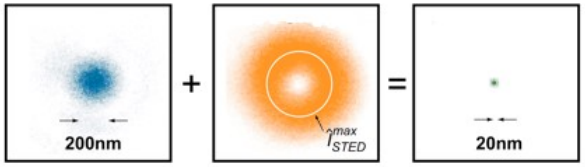
STED

PALM

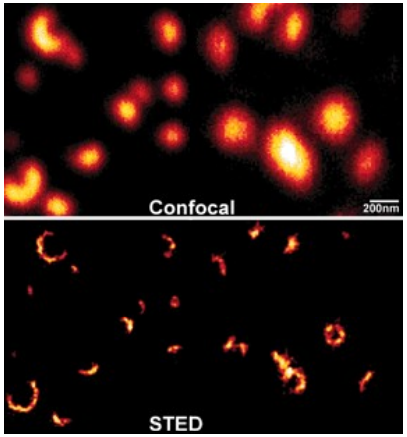
STORM

STimulated Emission Depletion (STED) microscope



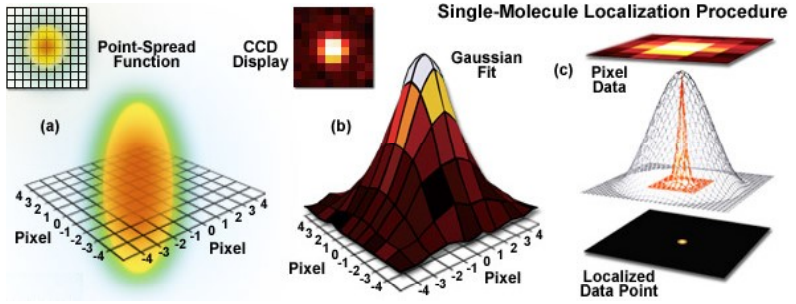


STimulated Emission Depletion (STED) microscope



Recycled material in the synaptic vesicles

Localization



STORM

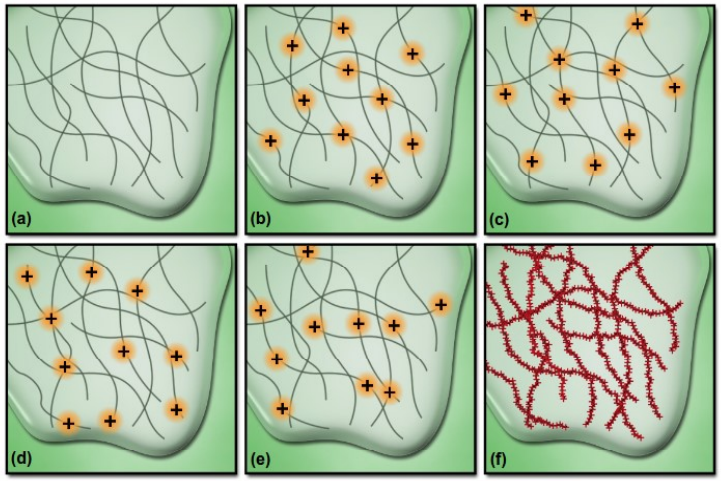
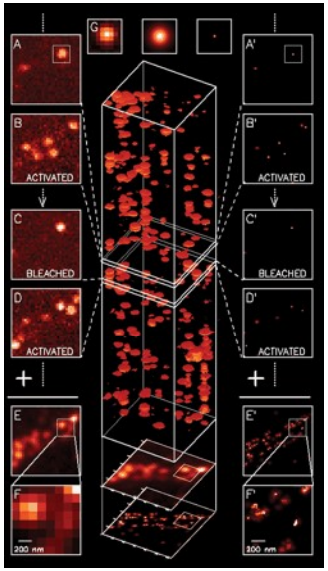


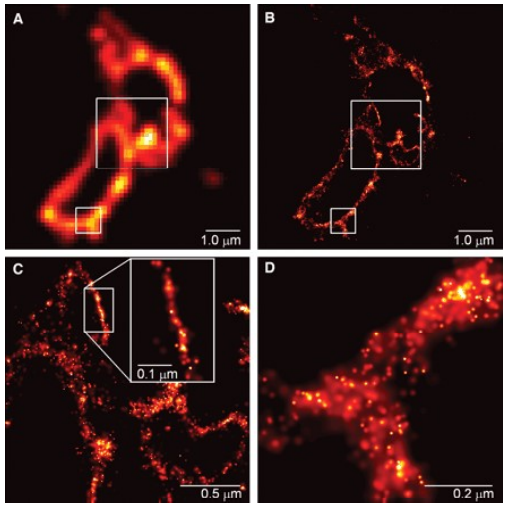
Photo-Activated Localization Microscopy (PALM)



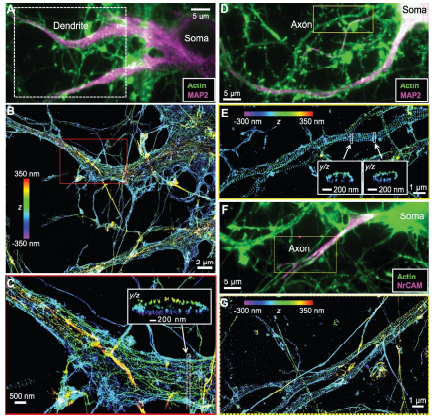
Based on the technology developed by Eric Betzig and Harald Hess

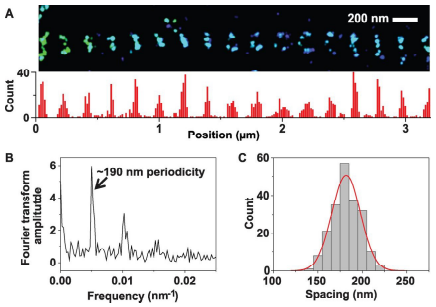
Photo-Activated Localization Microscopy (PALM)

CD63, lizosome transmembrane protein



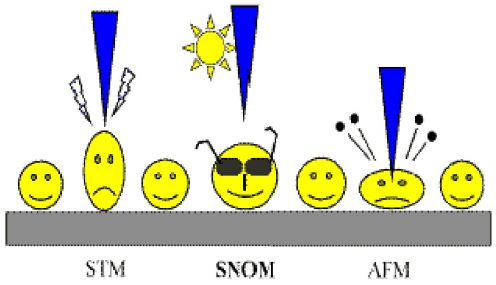
Cytoskeletal Structure in Axons





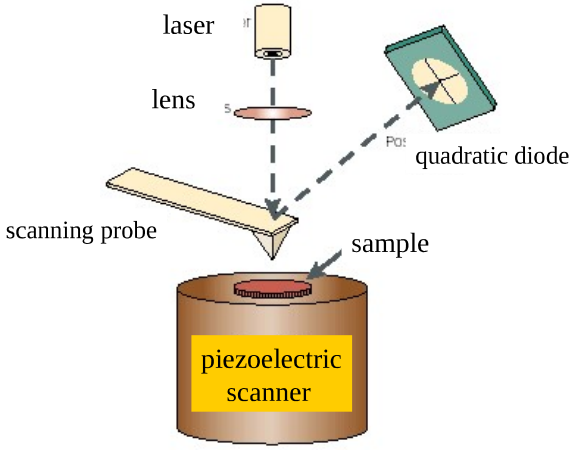
Scanning Probe Microscopy (SPM)

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.



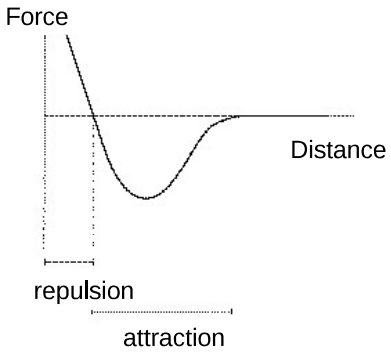
Atomic Force Microscopy - AFM

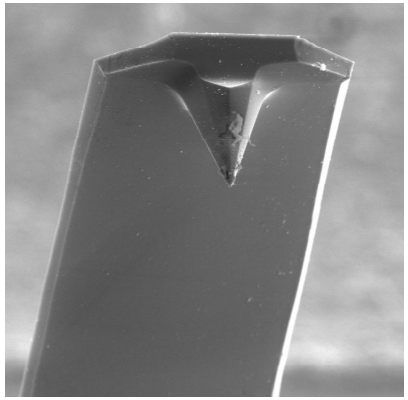
AFM: the detected interaction is the force between the sample surface and probe



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

Dentin collagene fibrils

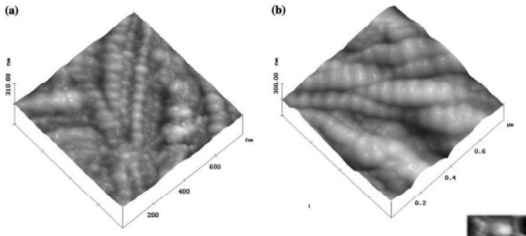
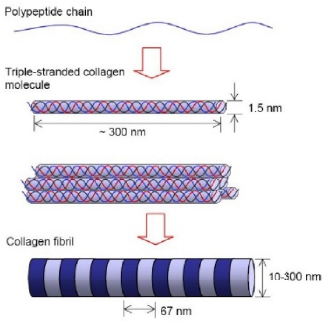


Fig. 3. Mixed-mode surface plots of tapping and phase mode AFM images of dentin collagen fibrils, obtained (a) in repeat pattern is still present after dehydration.



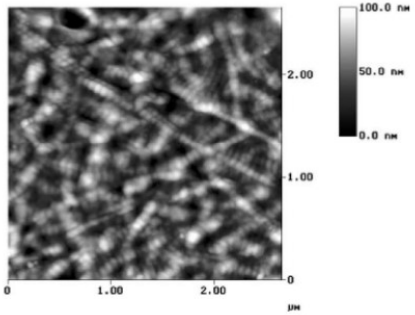
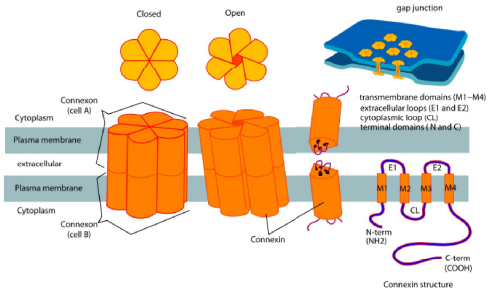


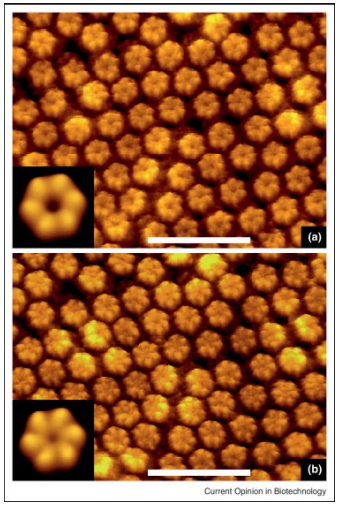
Fig. 2. Tapping mode AFM image of dentin collagen fibrils, obtained from demineralized specimen (15 s citric acid) treated with 6.5 vol% $NaOCl_{aq}$ for 130 s and in air, showing the repeat pattern and the random distribution of fibrils in intertubular dentin.

AFM image of the extracellular connexon surface

Calcium induced conformational changes of the extracellular connexon surface.

Scale bars represent 250 Å





Question

How can you separate the strong excitation light from the weak fluorescent emission in the wide field fluorescent microscope?