Special microscopic techniques

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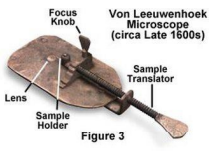
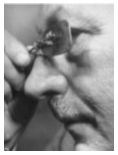
5/11/2018

„The open universe”

A brief history of the microscope

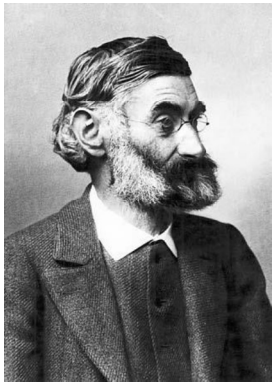
- Romans were looking through glass and testing it
- 1600s: Zacharias Jansen – first telescope/compound microsope
- 1667: Robert Hooke – „Micrographia” , cells of cork
- 1674: Antonie van Leeuwehoek – make simple microscopes, 300 x magnification





- Early 1800s
- Carl Zeiss – buisnessman in Jena – development of high quality microscope
- Ernst Abbe – He put the production of optical devices on scientific bases

Resolution limit of microscope



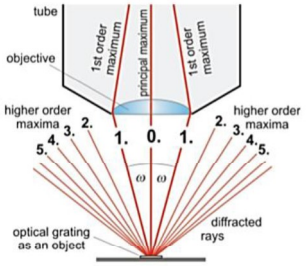
Ernst Abbe (1840-1905)

1873: Ernst Abbe – resolution limit of light microscope

Abbe's principle: An optical system can resolve only those details of the specimen, which diffract light rays in a way that besides the principal maximum at least the first order diffraction rays are allowed to contribute to the image formation.

$$\delta = 0{,}61\frac{\lambda}{n \sin \omega}$$

δ limit of resolution – distance between two object details which can be just resolved

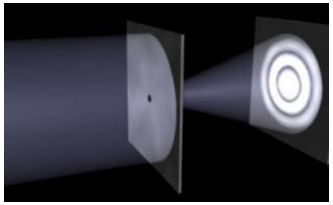


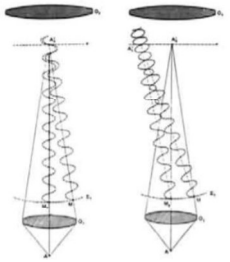
Airy disks – the evidence of wave character of light

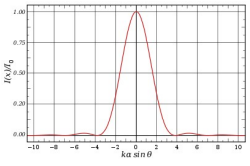
The Airy disk is descriptions of the best-focused spot of light that a perfect lens with a circular aperture can make, limited by the diffraction of light.

Formation: the waves in same phase produce diffraction maximum (left) while the waves shifted by 180° produce diffraction minimum (right).

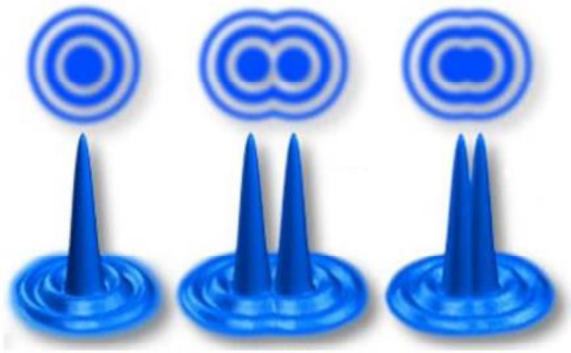
Point Spread Function (PSF): The objective focuses light in a volume and not into one point.







How can we distinguish two image points?



Rayleigh criterion:

Objects may be resolved if their corresponding Airy disk do not overlap.

Fluorescence microscope

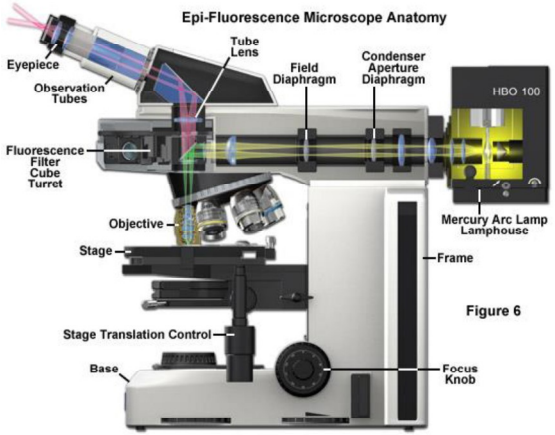
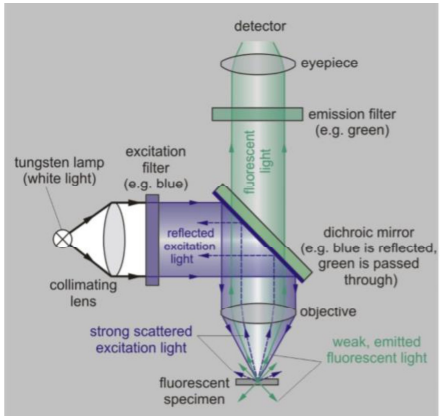
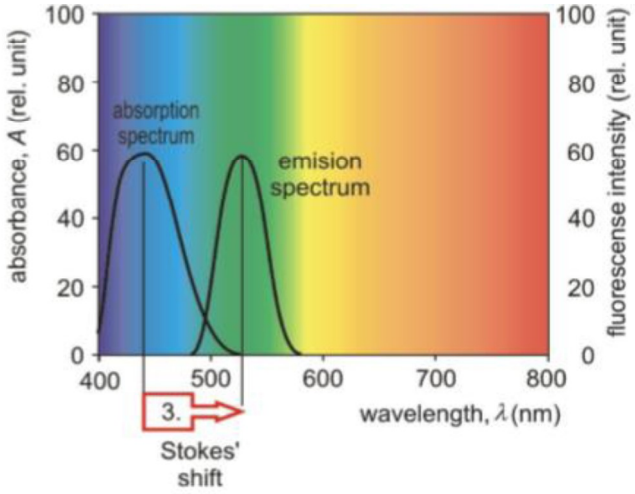


Figure 6



Light absorption and emission spectrum



Source of fluorescence

- **Intrinsic** fluorophores:

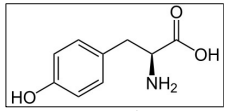
pl: tryptophan, tyrosine aminoacids, porphyrins

- **Extrinsic** fluorophores:

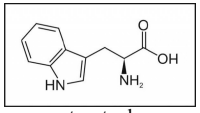
pl: fluorescent dyes

The perfect fluorescent dye:

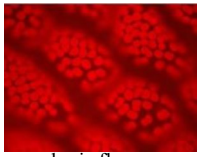
- Small
- Hydrofil
- Can be excited in the visible range
- Large Stokes-shift
- Specific
- No photoreactions

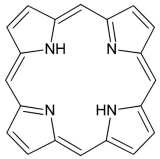


tyrosine

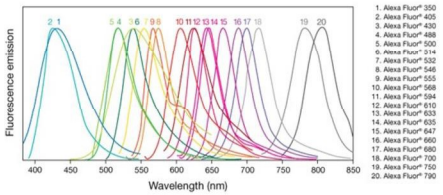


tryptophan


porphyrin fluorescence

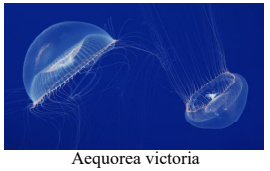


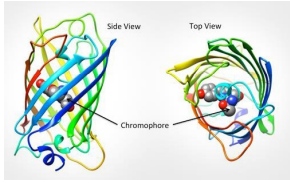
porphyrin

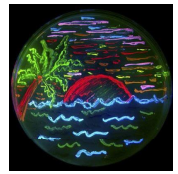


Fluorescent proteins

- Green Fluorescent Protein (GFP)
- first isolated from jellyfish (1960s)
- ~27 kDa, 238 aa, 11 strands β-barrel structure
- the central alpha helix contains the chormophore: Ser-65, Tyr-66, and Gly-67
- excitation: blue (475 nm) and UV (396 nm) light
- emission: 508 nm

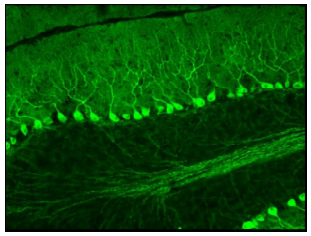

Aequorea victoria

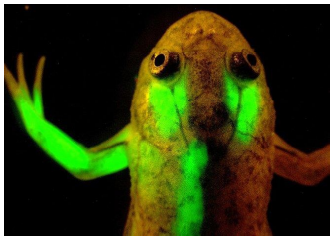


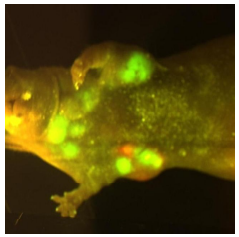


- Used as tagging protein
- Small size – has no effect on the funcion of examined protein
- Transfected cells
- Transgene animals: all cell express the GFP


Transgene mice


Purkinje cells


Frog muscle cells

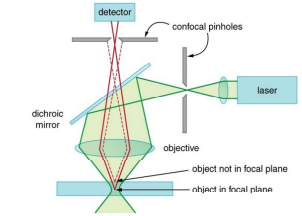

Tumor cells

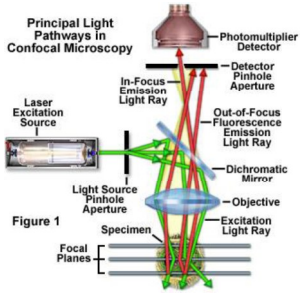
2008. Nobel-prize in chemistry

Confocal laser scanning microscope

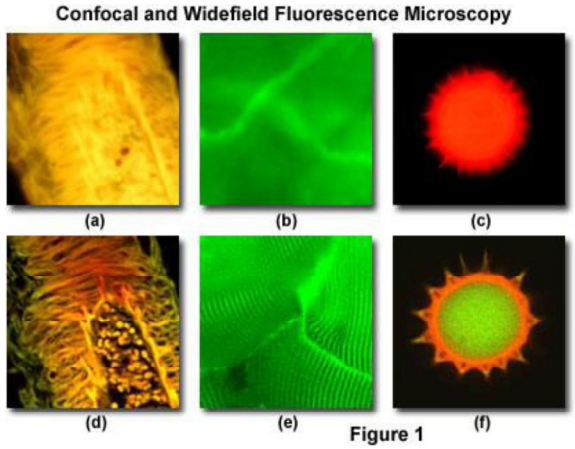
Confocal concept: a focused laser beam is used to produce a small spot illumination on the specimen, and a pinhole in front of the detector eliminates out-of-focus signal.

- laser beam – focused illumination
- excitation filter – selected wavelenght
- point-by-point scanning
- motorized XY scanning
- „optical sectioning”
- 3D imaging





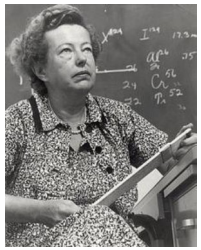
Comparison the imaging of flurescence and confocal microscpes



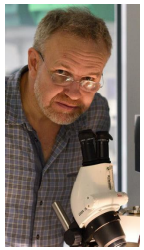
human medulla rabbit muscle pollen

Two-photon microscopy

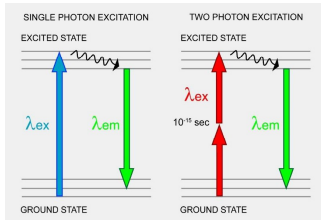
- 1931. Maria Göppert-Mayer
- in the excited molecule two photon absorb simultaneously
- femtosecond laser source ~ high flux of excitation photons
- 1990. first two-photon excitation microscope
- Wienfried Denk, Cornell University



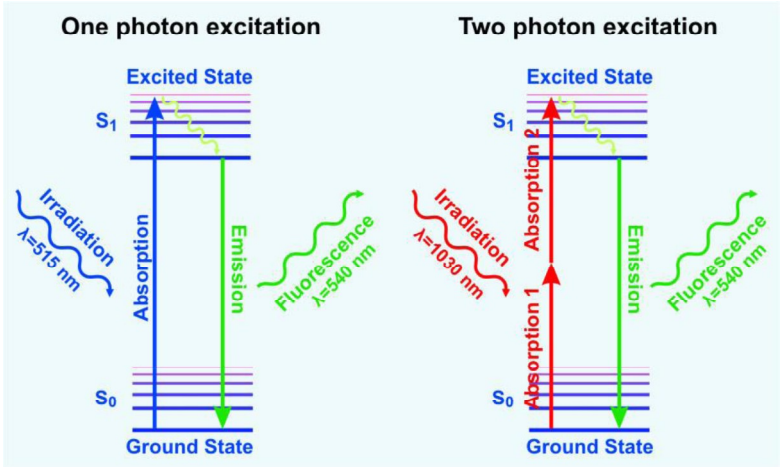
Maria Göppert-Mayer (1906-1972)



Wienfried Denk (1957-)

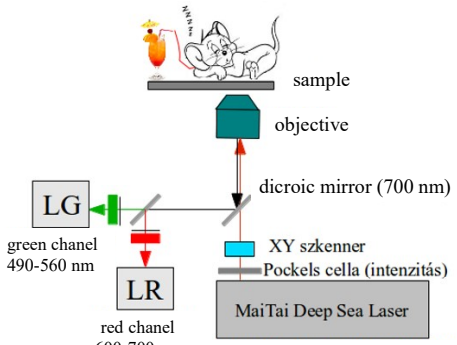


Light absorption and emission spectrum



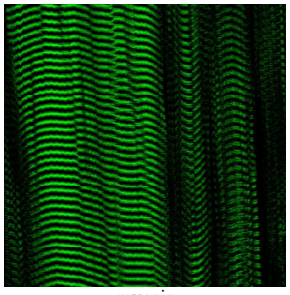
Advantages

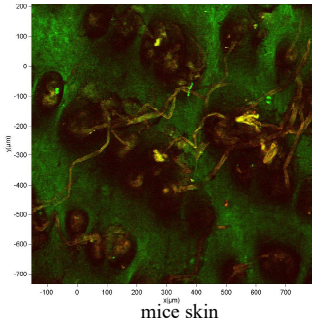
- excitation only in a tiny focal volume – reject out-of-focus
- low laser power – *in vivo* imaging
- tunable laser source – infrared spectral range (700-1300 nm) – reduced scattering
- deep penetration
- multicolor labeling
- effective signal detectation
- optical sectioning
- Imaging without labeling

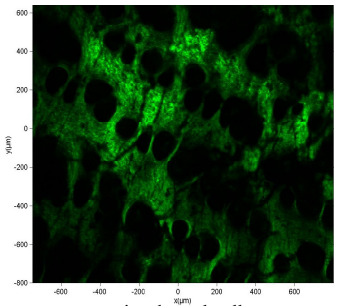


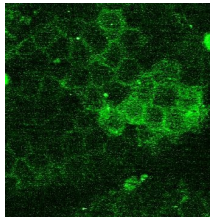
Tunable range: 700-1400 nm
Frequency: 80 MHz
Impulse lenght: 100 fs
Average power: 3 W

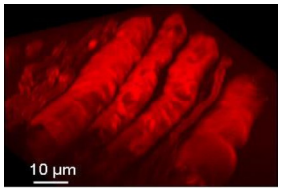
Label-free imaging


myosin

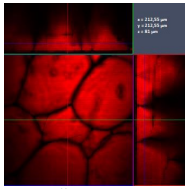

mice skin

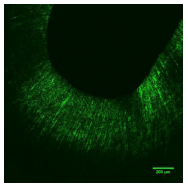

mice dermal collagen


mice skin - keratin



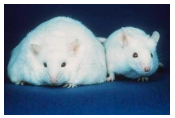
myelin sheats

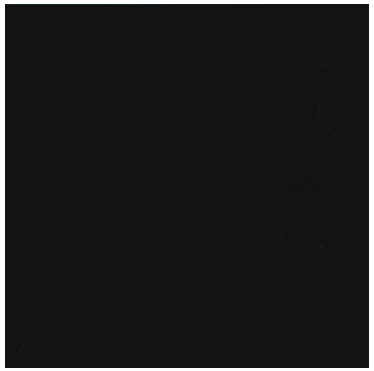

adipocytes


dental tubules

3D imaging

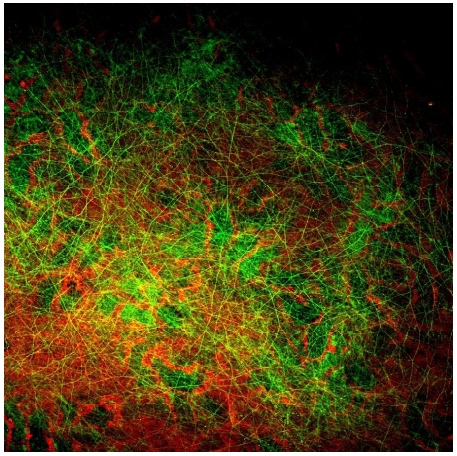
Comparison the dermal collagen structure of a control and type 2 diabetes affected mice

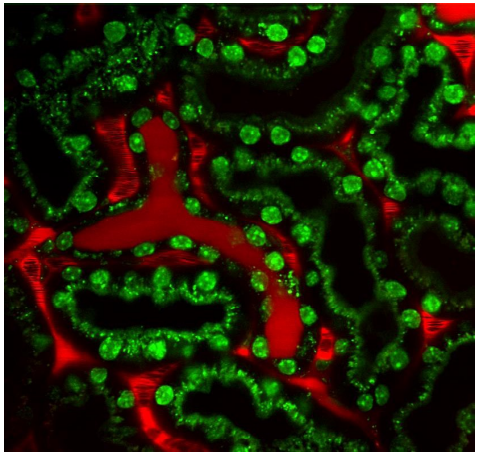




Optical sectioning,
z = 80 µm
200 µm x 200 µm
exc: 990 nm

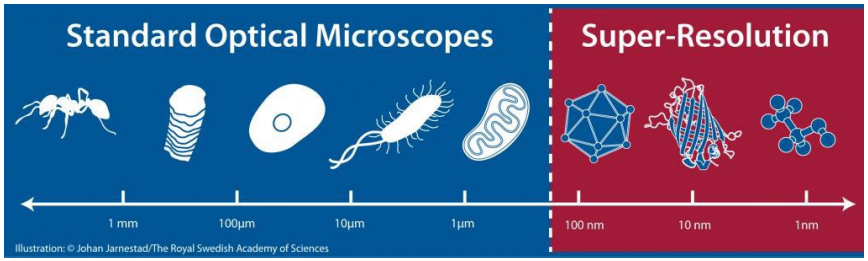
Multiple fluorescent labeling


renal cortex

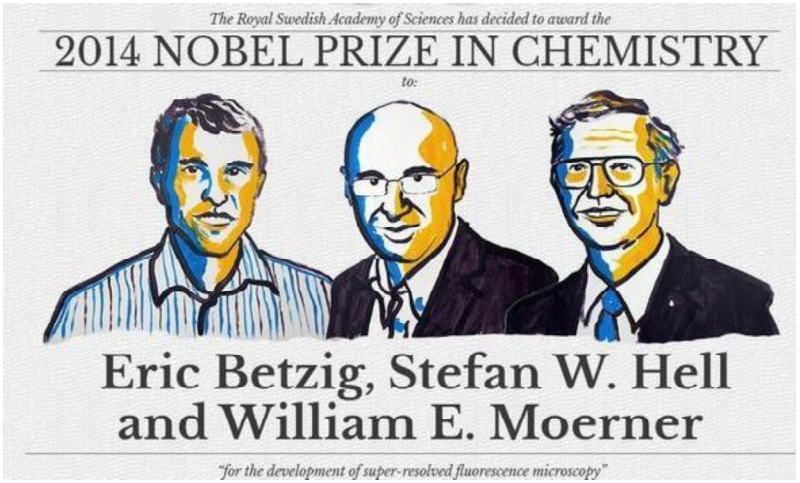

collecting ducts and JGA cells

green: quinacrine (renin-positive granules), Hoechst 33342 (nuclei), and autofluorescence; red: 70 kDa rhodamine dextran (vasculature).

How big are things?

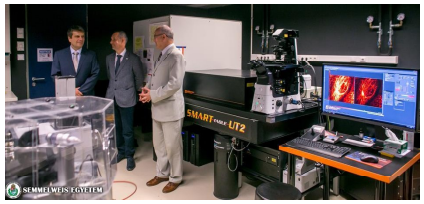


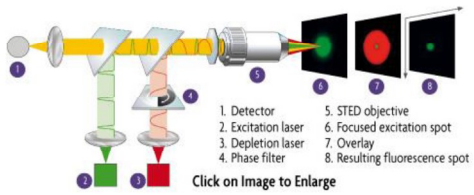
Superresolution microscopy

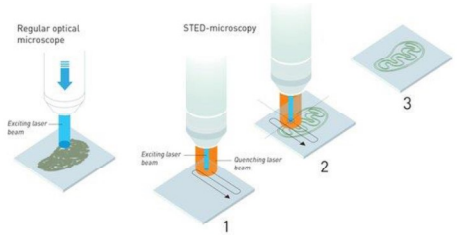


Superresolution microscope

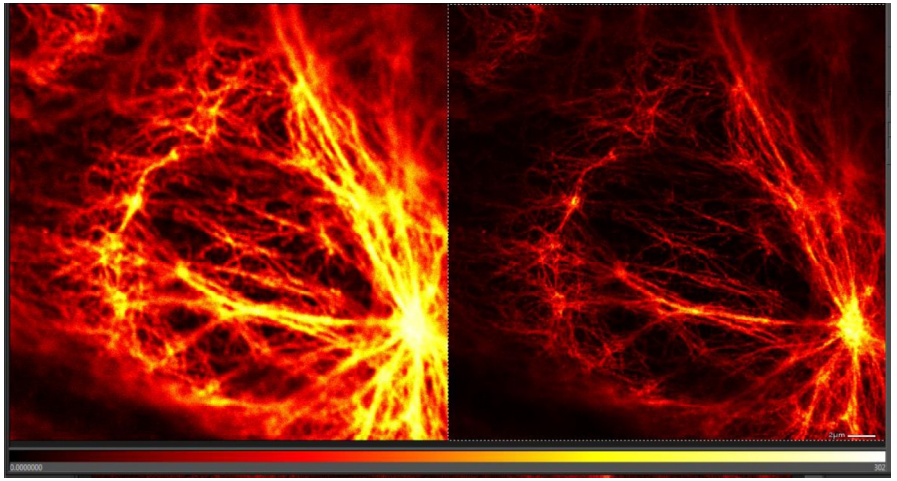
- 2014. Eric Betzig, Stefan W. Hell és William E. Moerner were awarded in Nobel-prize
- STED: stimulated emission depletion microscopy
- 2018. August – STED device arrived in our Instutue
- allows for images to be taken at resolutions below the diffraction limit
- excitation laser + depletion laser
- point-by-point scanning







confocal STED



Question:

What is the difference between the excitation and emission spectrum in fluorescence and two-photon microscopy?