

# Special microscopic techniques

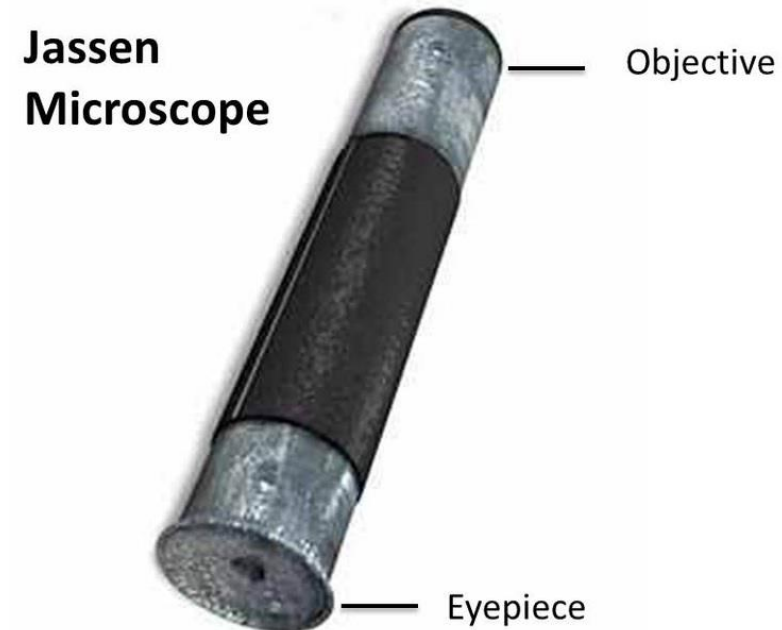
Dora Haluszka

10/11/2023



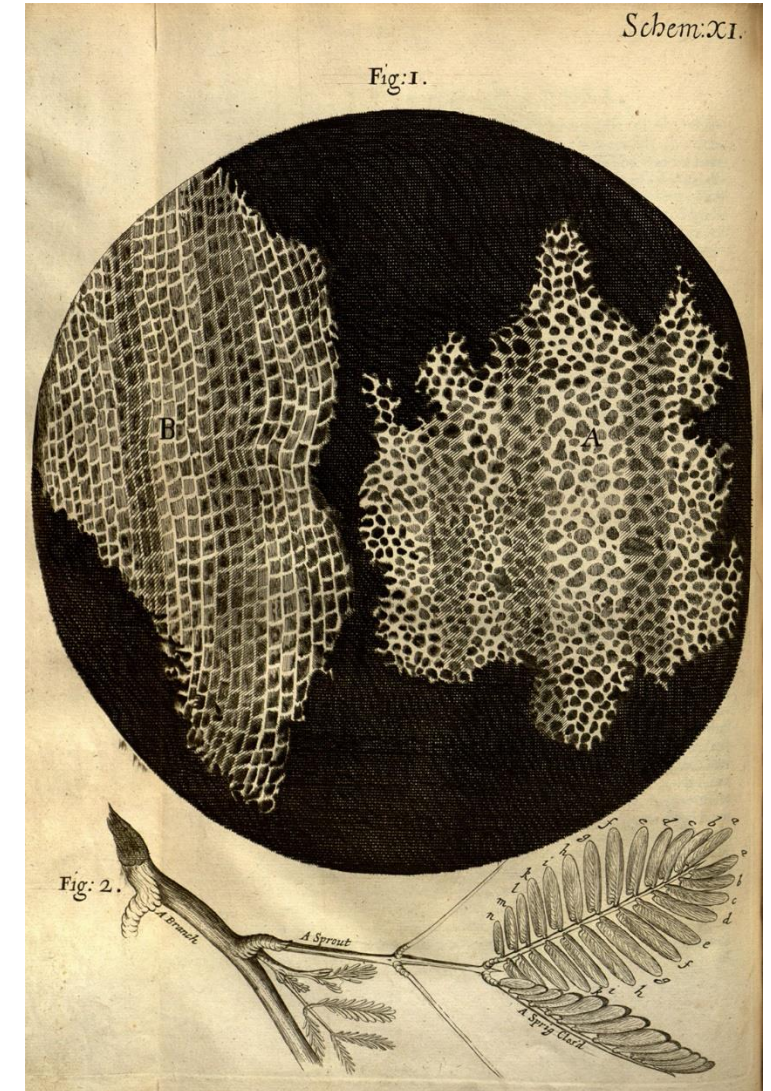
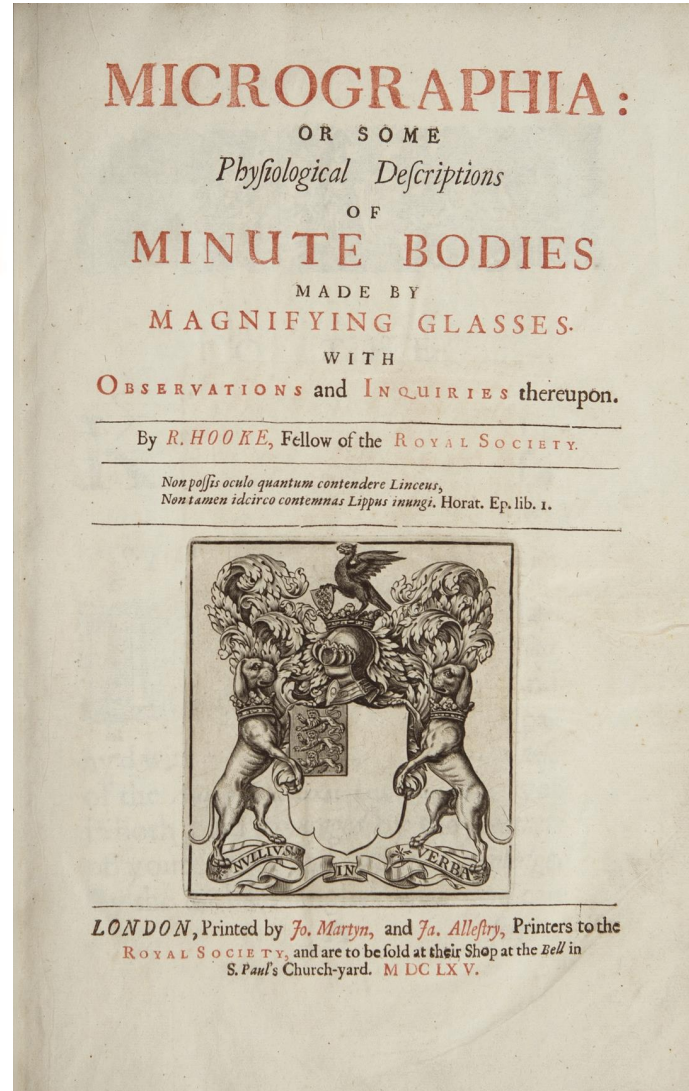
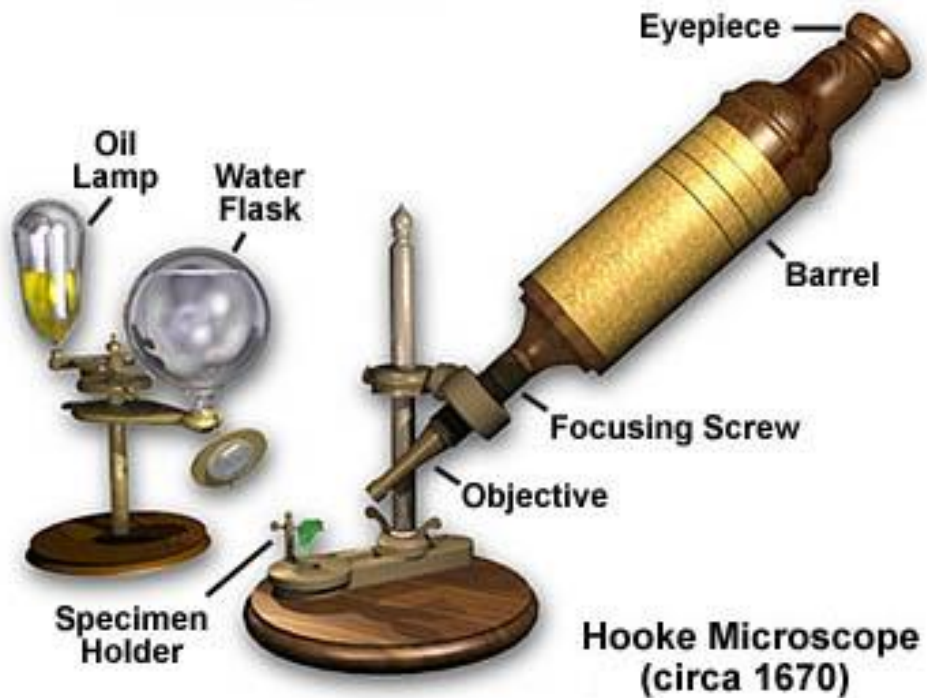
# Brief history of microscopes

- 1st century: Romans were looking through glass and testing it
- 1600s: Zacharias Jansen – first telescope/compound microscope





- 1667: Robert Hooke – „Micrographia” , cells of cork



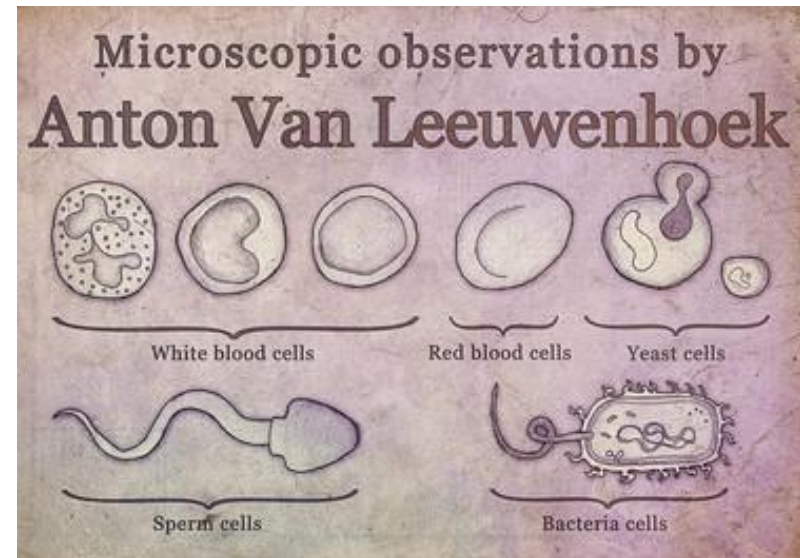
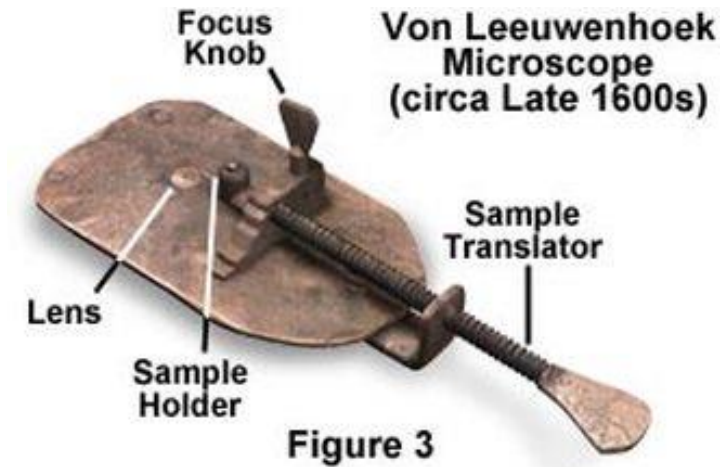
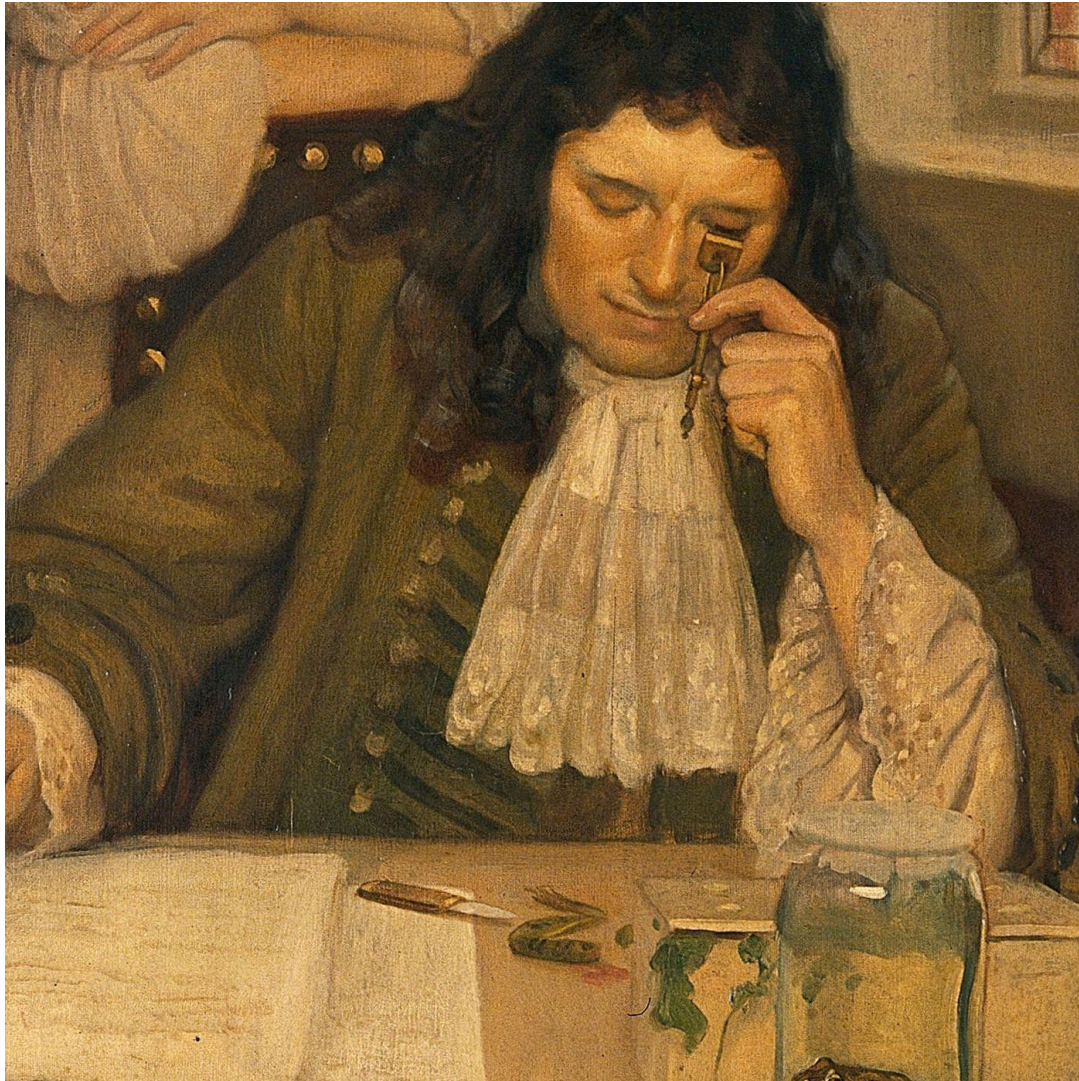




„The open universe”



- 1674: Antonie van Leeuwenhoek – make simple microscopes, 270 x magnification





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

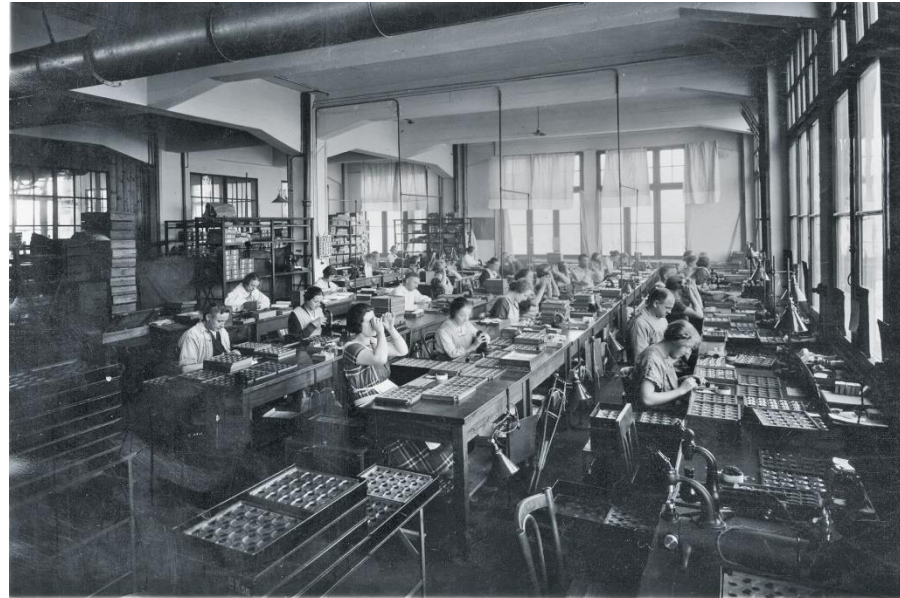
Figure 2



Ernst Abbe (1840-1905)



Carl Zeiss (1816-1888)



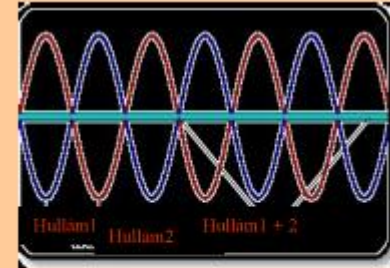
Microscope by Carl Zeiss (1879) with optics by Abbe

# Fundamentals of wave optics



Similar phase

Constructive interference

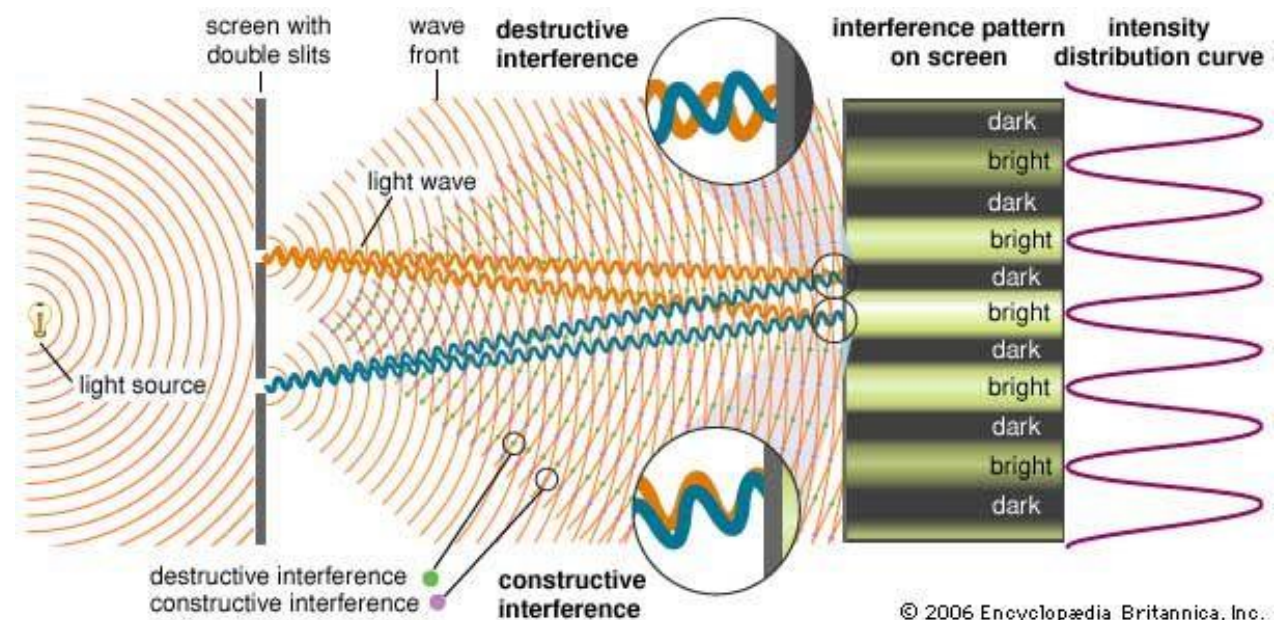


Opposite waves

Destructive interference



Young's experiment





# Resolution limit of microscope



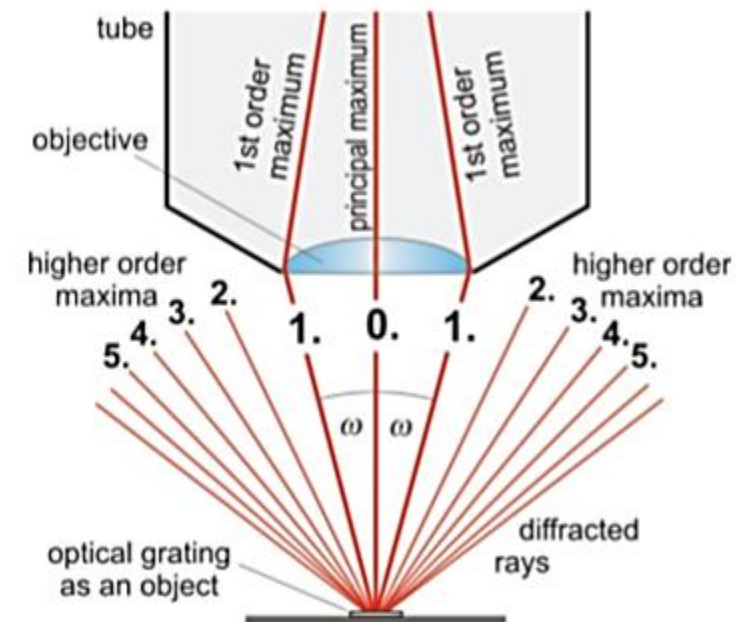
Ernst Abbe (1840-1905)

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.

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

$\delta$  limit of resolution – distance between two object details which can be just resolved  
(200 nm)



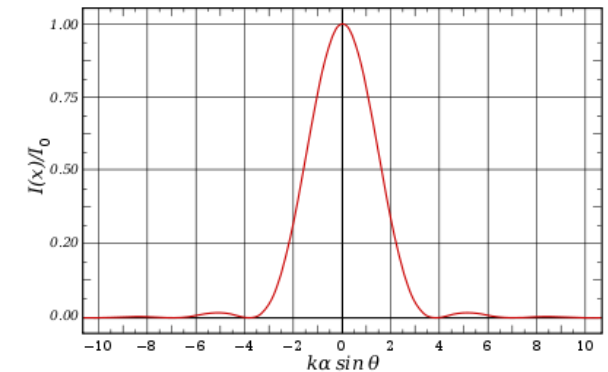
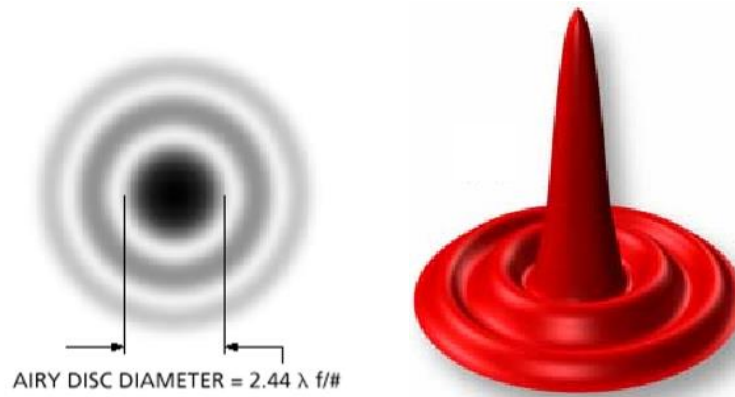
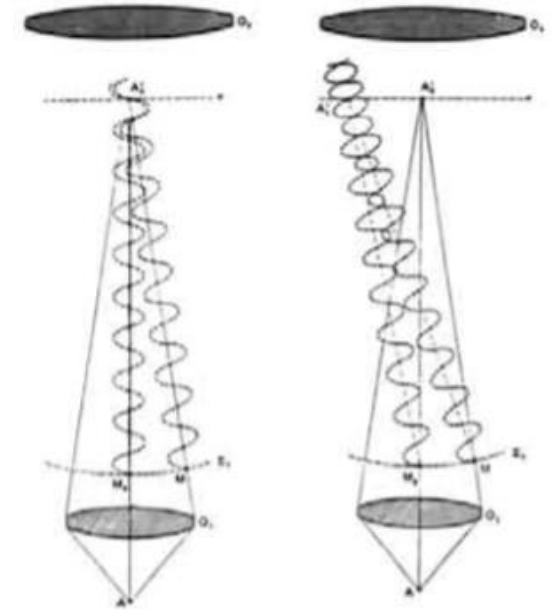
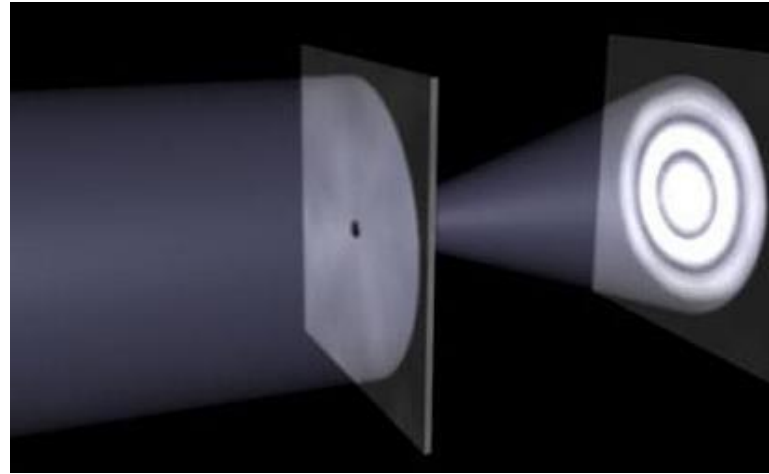


# Airy disks – the evidence of wave character of light

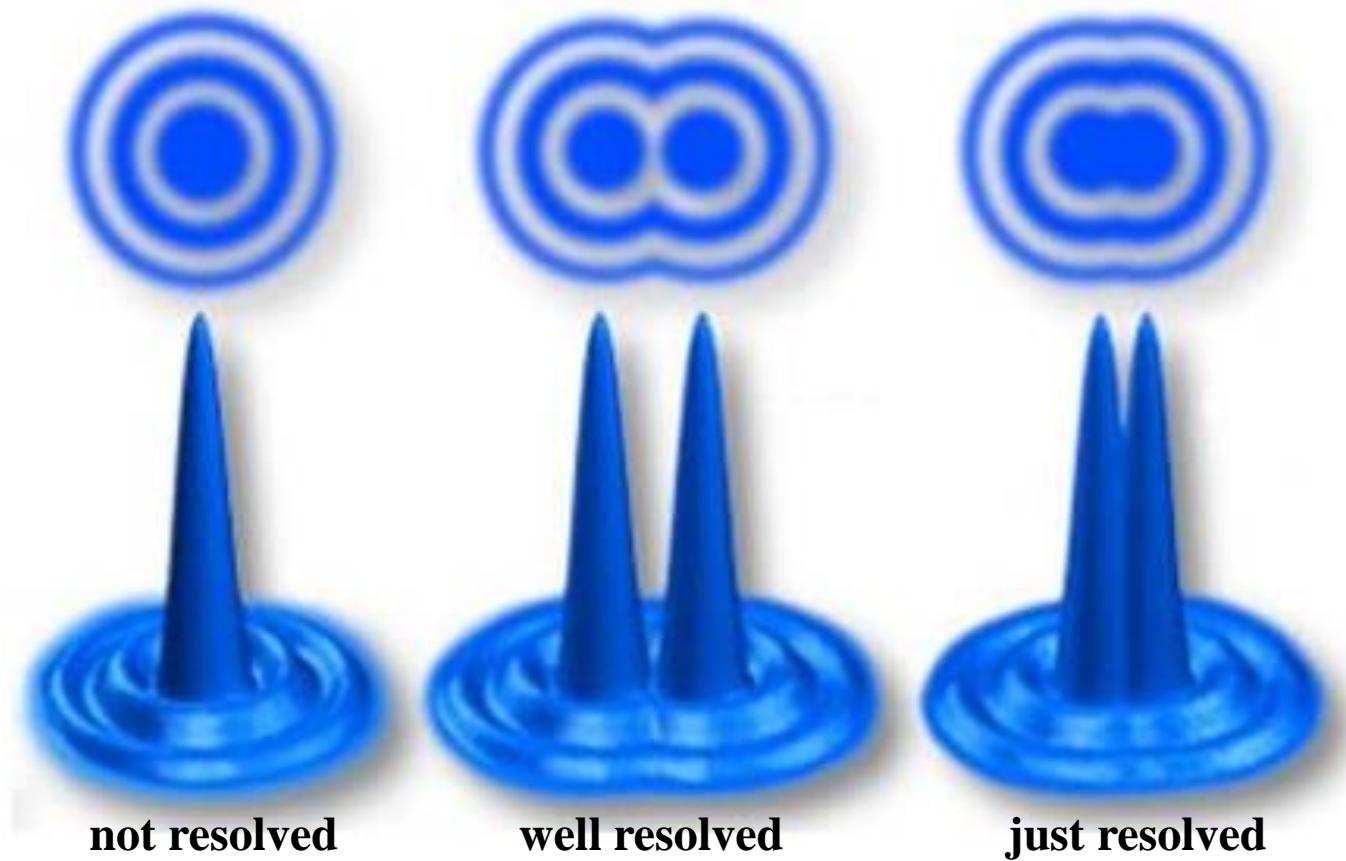
The Airy disk is descriptions of the best-focused spot of light that a perfect lens with a circular aperture can make, limited by the diffraction of light.

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

Point Spread Function (PSF):  
intensity distribution of diffracted waves



# How can we distinguish two image points?



## Rayleigh criterion:

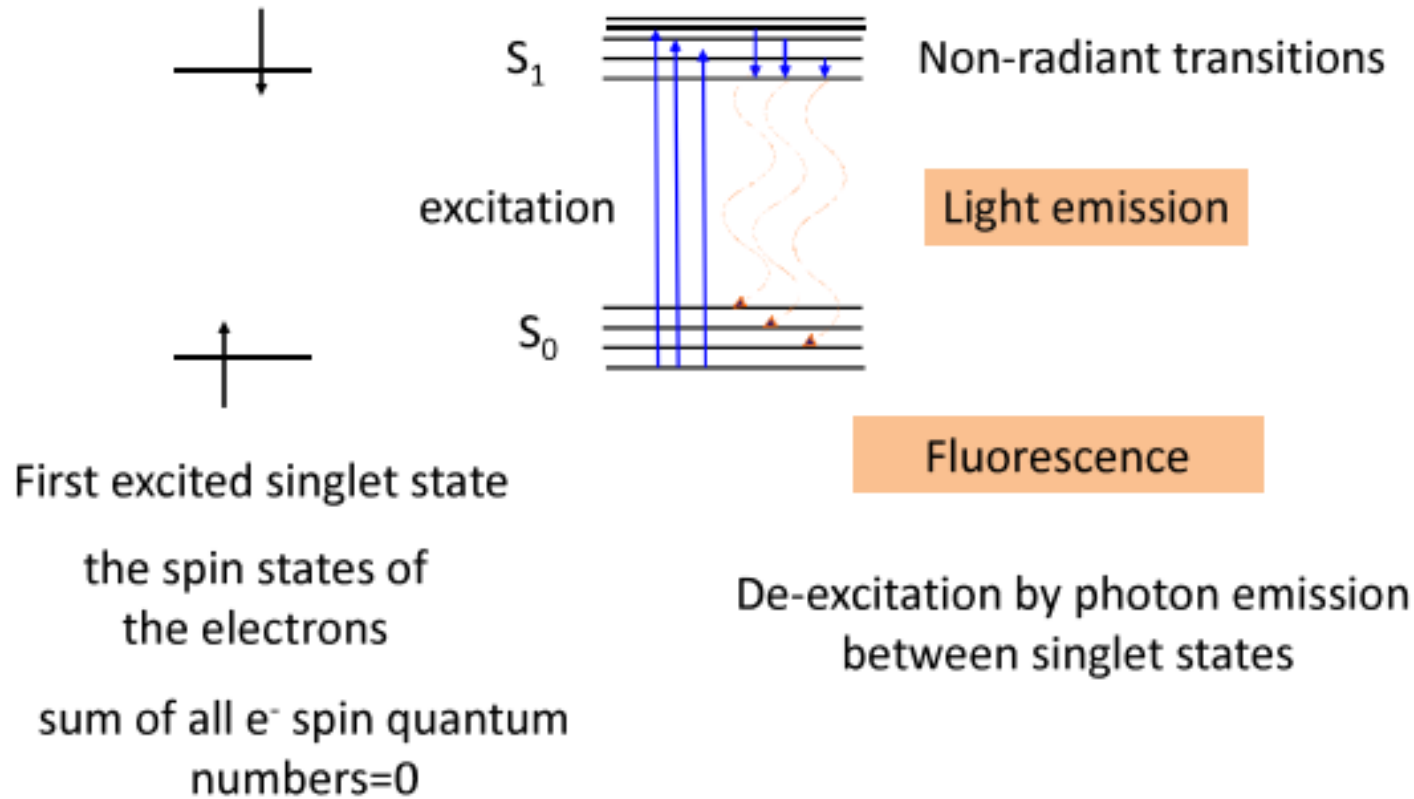
Objects may be resolved if their corresponding Airy disk do not overlap.

Just resolved: principal maximum of one image point coincides with the first order minimum of the other



# Fluorescence microscope

*Jablonski diagram*



$$E_{\text{excitation}} \geq E_{\text{fluorescence}}$$

$$\lambda_{\text{excitation}} \leq \lambda_{\text{fluorescence}}$$

Stokes-shift



Kasha's rule: light emission emanates from the relaxation of the lowest vibrational level of the first excited state to ground state.

# Fluorescence microscope

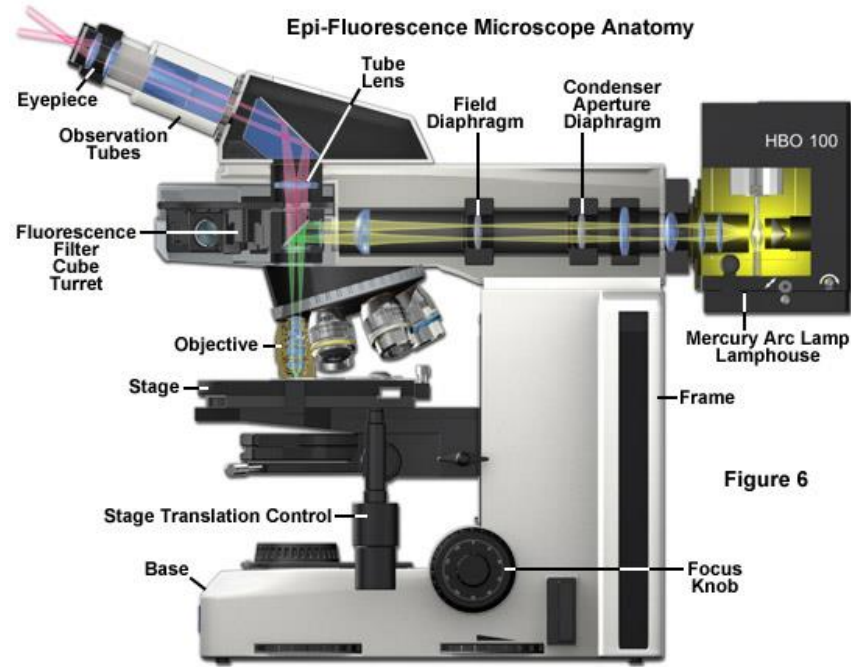


Figure 6

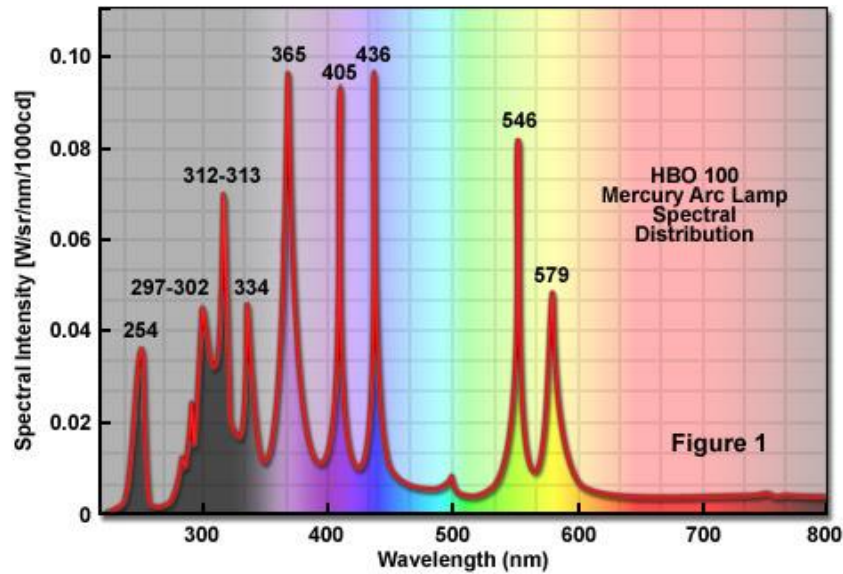
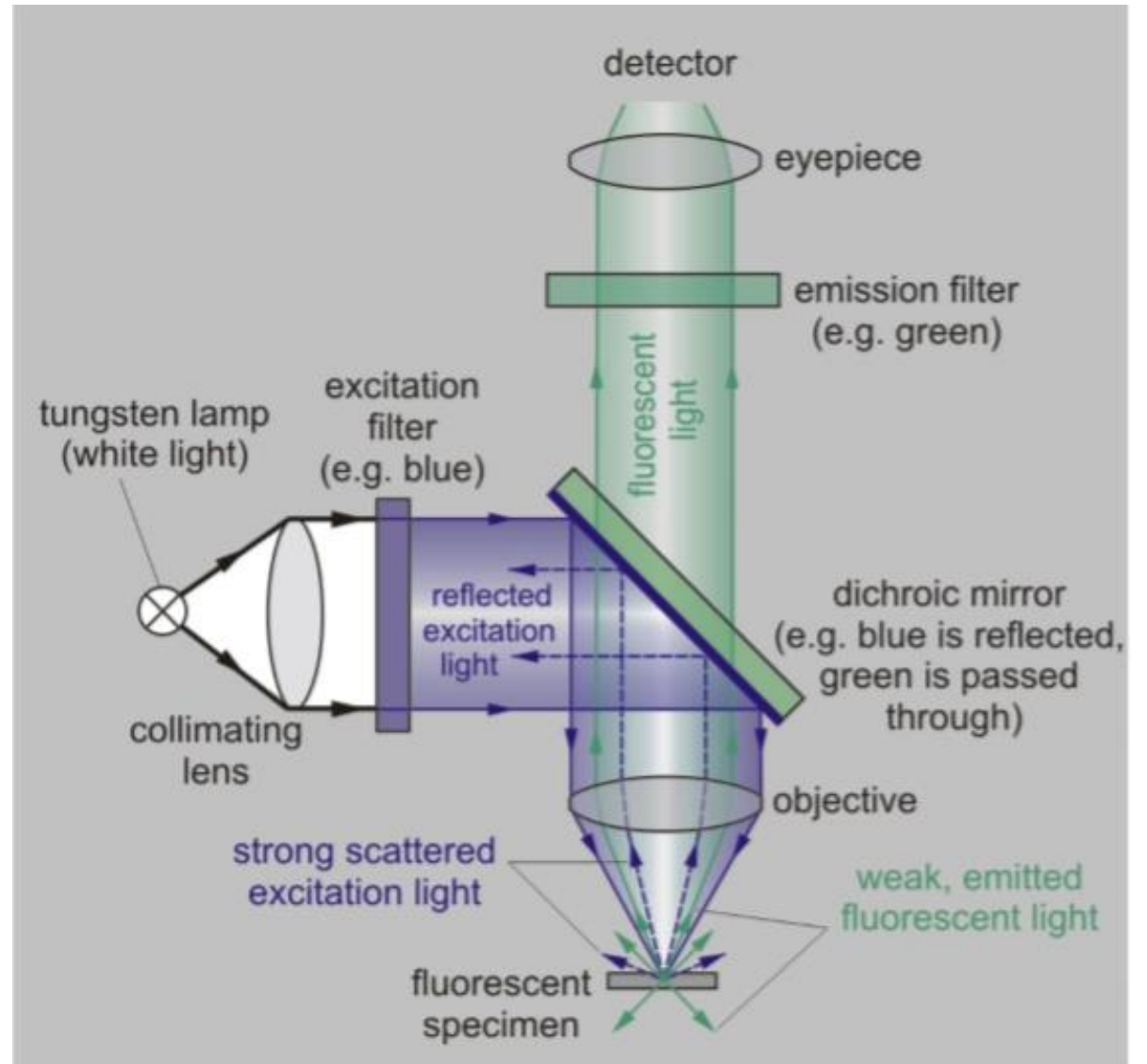
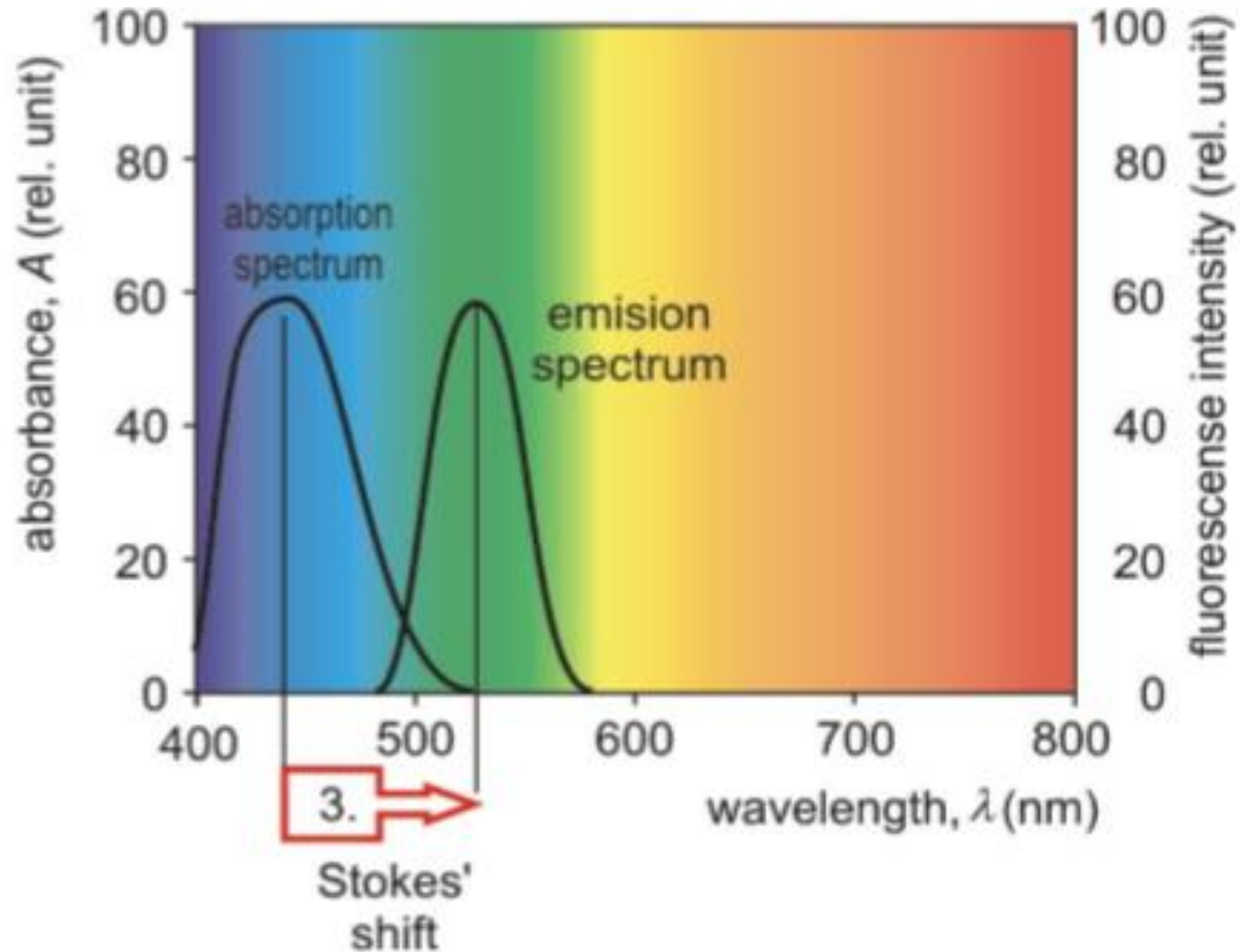


Figure 1





# Absorption and emission spectrum



# Source of fluorescence

- **Intrinsic** fluorophores:

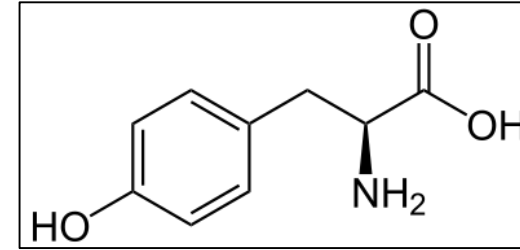
tryptophan, tyrosine aminoacids (aromatic)  
porphyrins

- **Extrinsic** fluorophores:

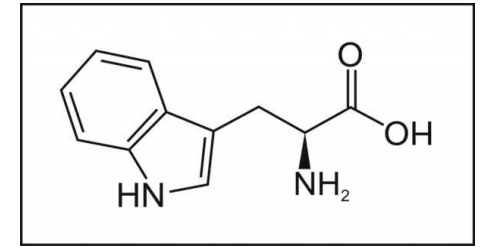
fluorescent dyes

## The perfect fluorescent dye:

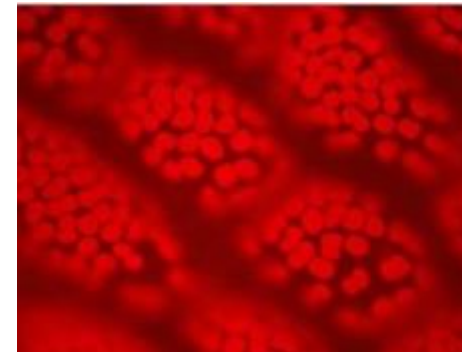
- Small
- Hydrophil
- Can be excited in the visible range
- Large Stokes-shift
- Specific
- No photoreactions



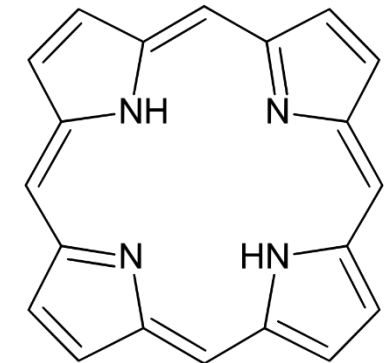
tyrosine



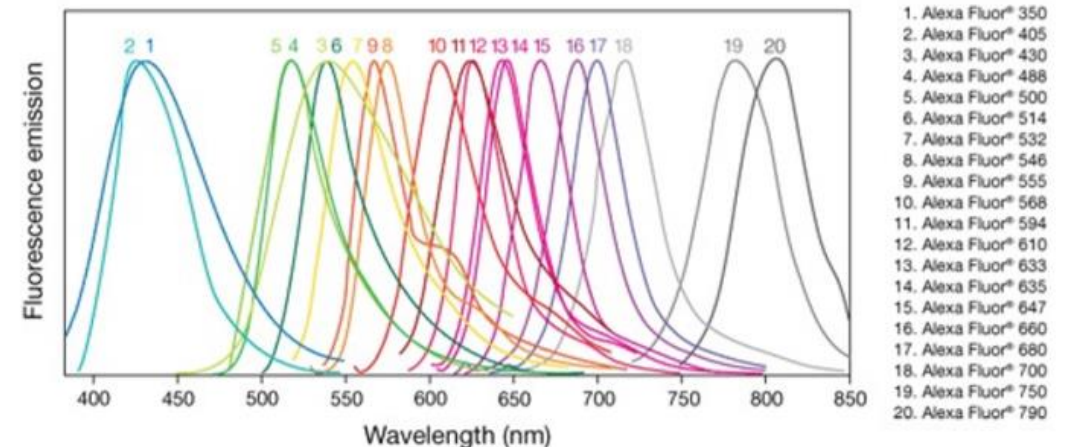
tryptophan



porphyrin fluorescence



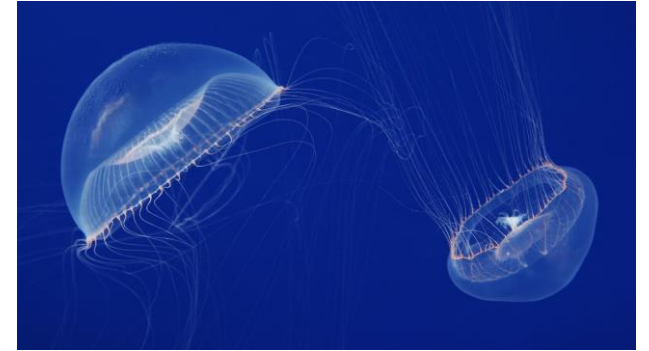
porphyrin



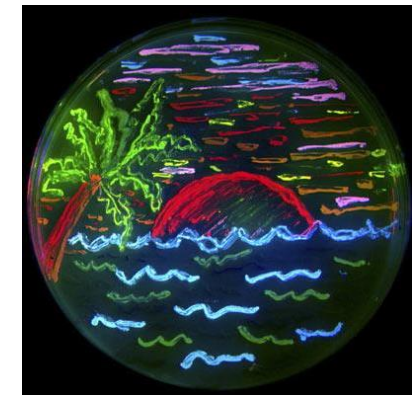
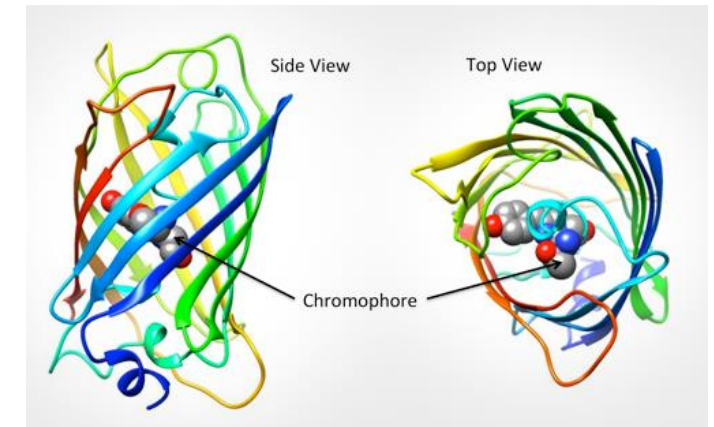


# Fluorescent proteins

- Green Fluorescent Protein (GFP)
- first isolated from jellyfish (1960s)
- ~27 kDa, 238 aa, 11 strands  $\beta$ -barrel structure
- the central alpha helix contains the chromophore: Ser-65, Tyr-66, and Gly-67
- excitation: blue (475 nm) and UV (396 nm) light
- emission: 508 nm
- Used as tagging protein
- Small size – has no effect on the function of examined protein
- Transfected cells
- Transgene animals: all cell express the GFP

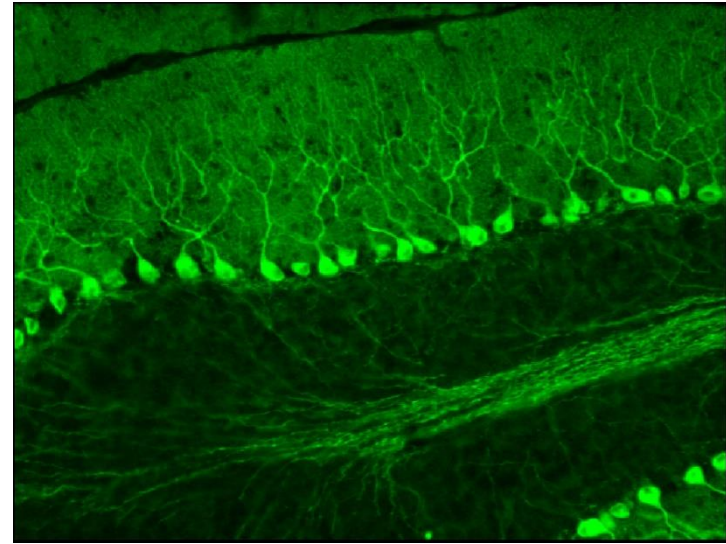


*Aequorea victoria*

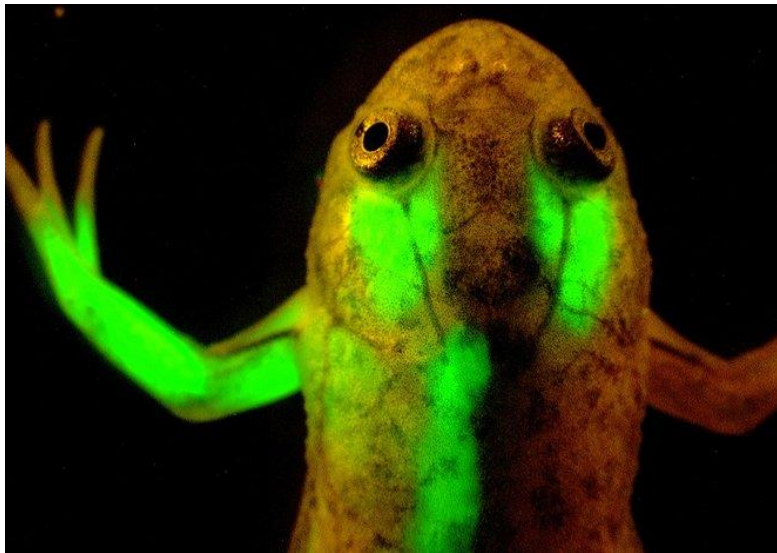




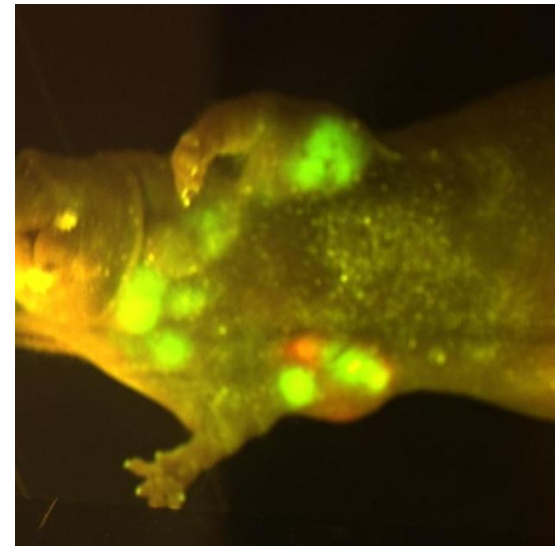
Transgene mice



Purkinje cells



Frog muscle cells



Tumor cells



## 2008. Nobel prize in chemistry

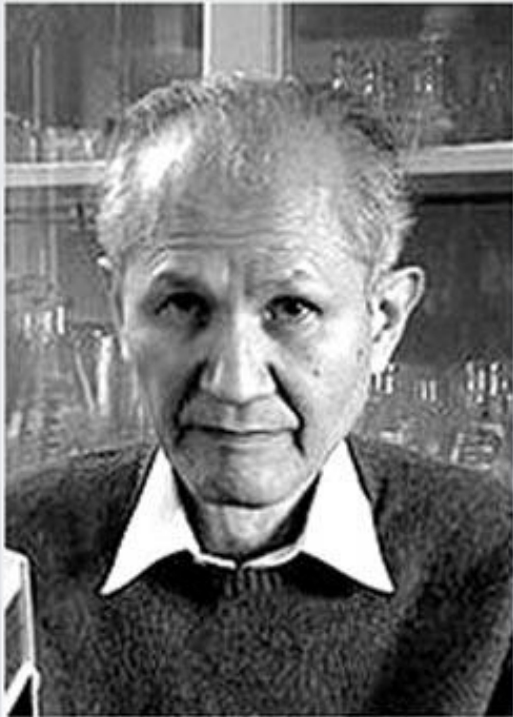


Photo: J.  
Henriksson/SCANPIX

**Osamu Shimomura**



Photo: J.  
Henriksson/SCANPIX

**Martin Chalfie**



Photo: UCSD

**Roger Y. Tsien**

# General properties of lasers

light **a**mplification by **s**timulated **e**mission of **r**adiation



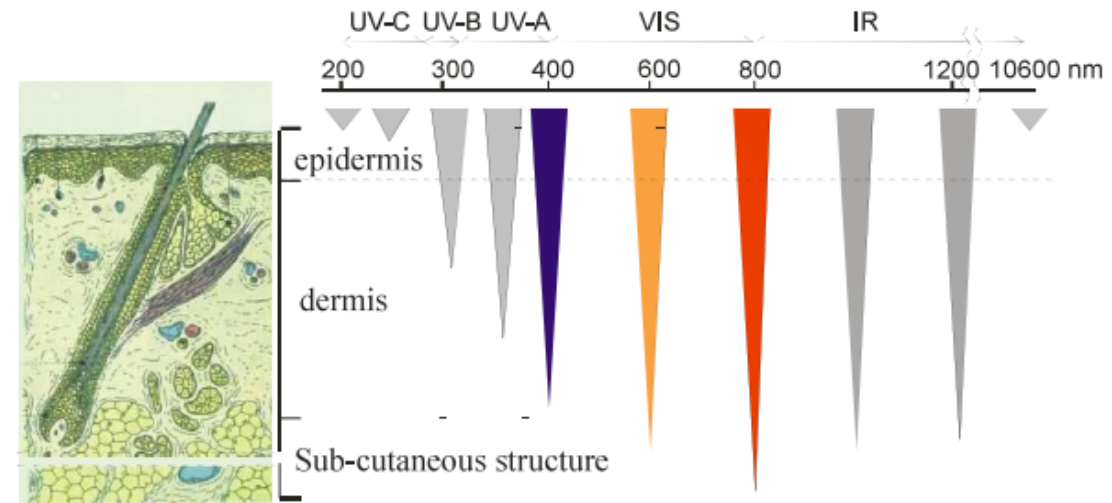
- monochromatic
- coherent
- polarized
- parallel, collimated beam

Possibility of very short pulses – *ps*, *fs*

Possibility of high power – *kW* - *GW*



## Penetration of light into the skin



Light intensity is attenuated due to absorption, reflection, refraction.

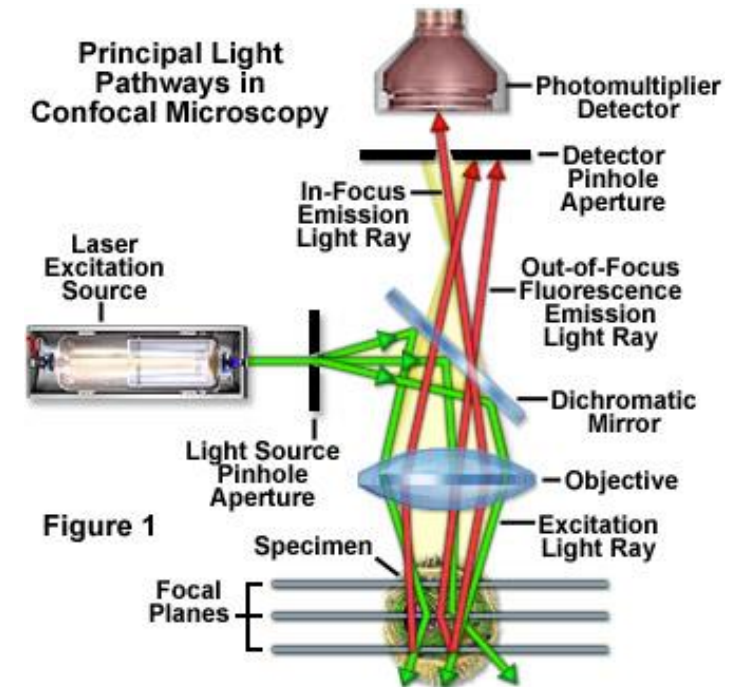
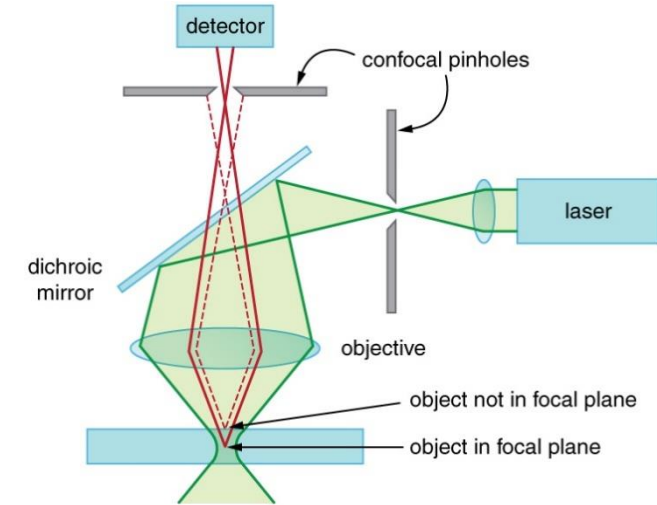
Penetration depth depends on the wavelength.



# 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

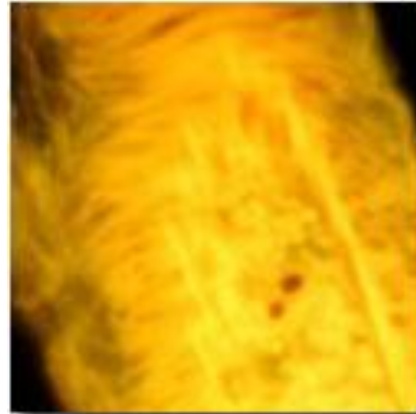
- laser beam – focused illumination
- excitation filter – selected wavelength
- point-by-point scanning
- motorized XY scanning
- „optical sectioning”
- 3D imaging



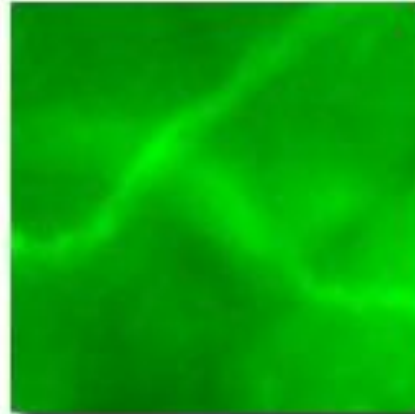
# Comparison the imaging of fluorescence and confocal microscopes

Confocal and Widefield Fluorescence Microscopy

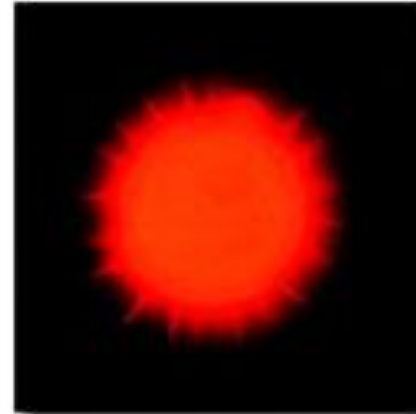
fluorescence



(a)

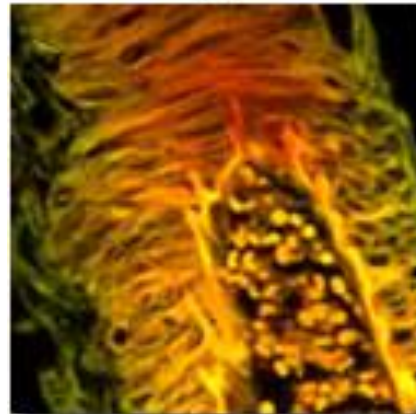


(b)

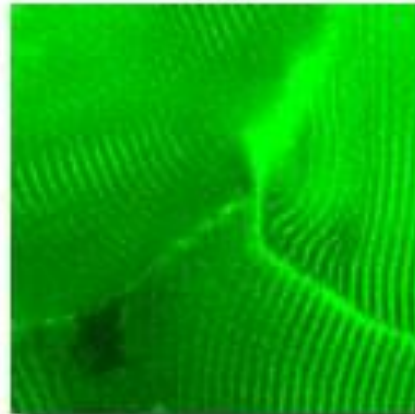


(c)

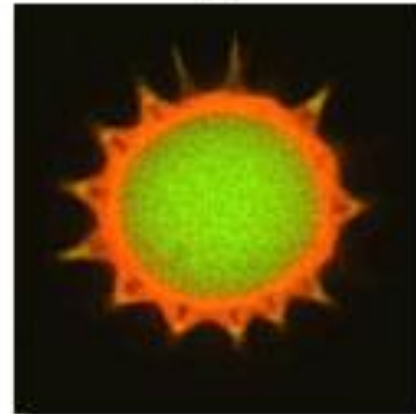
confocal



(d)



(e)



(f)

Figure 1

human medulla

rabbit muscle

pollen

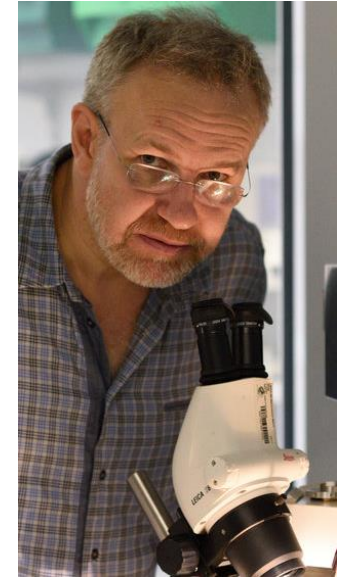


# Two-photon microscopy

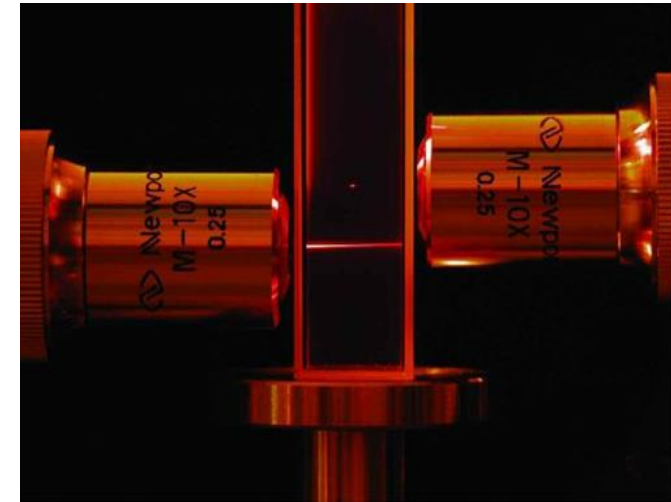
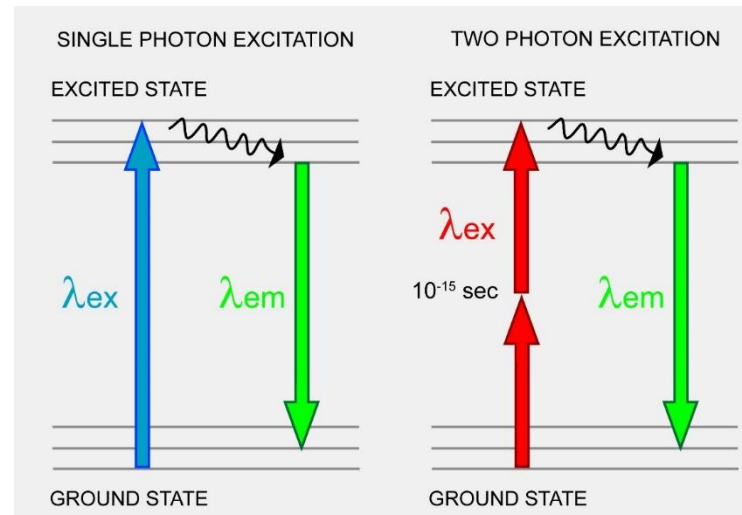
- 1931. Maria Göppert-Mayer
- in the excited molecule two photons absorb simultaneously
- femtosecond laser source ~ high flux of excitation photons
- 1990. first two-photon excitation microscope
- Wienfried Denk, Cornell University



Maria Göppert-Mayer (1906-1972)

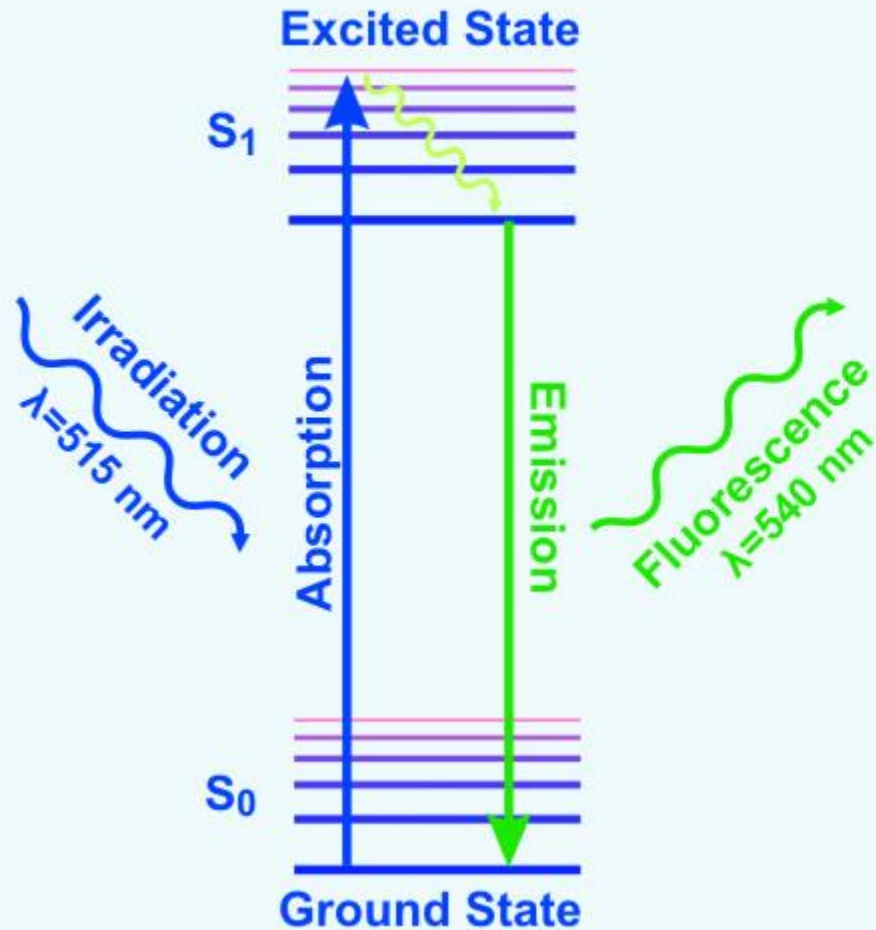


Wienfried Denk (1957-)

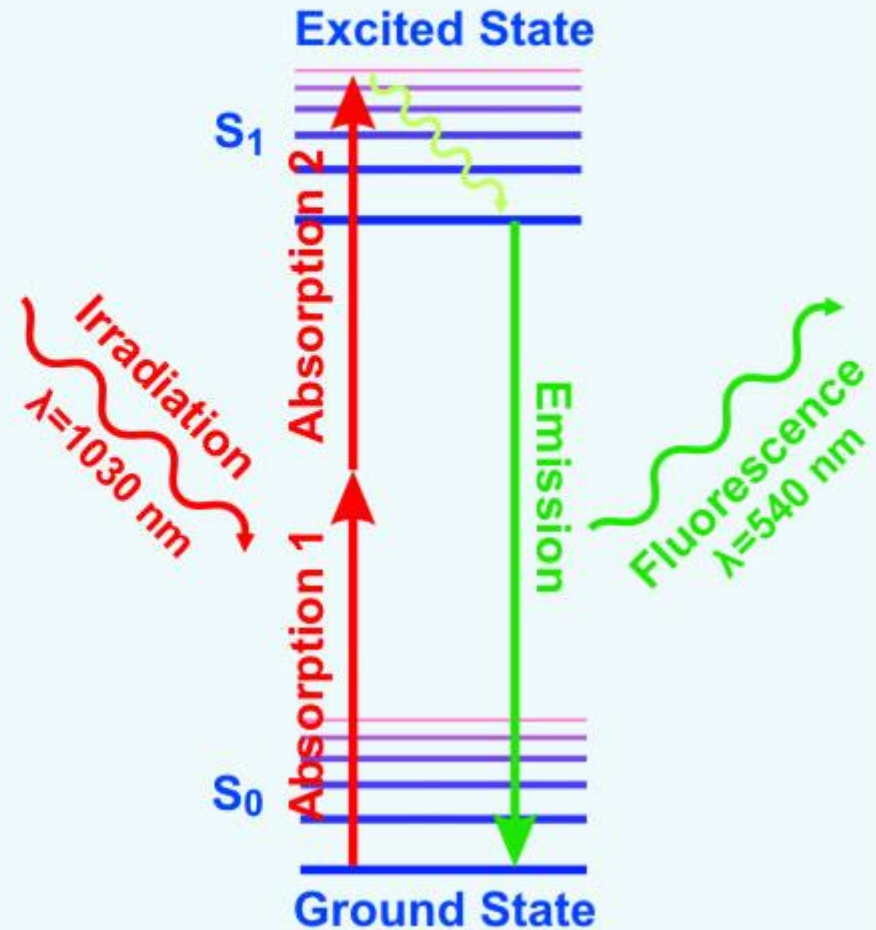


# Light absorption and emission spectrum

One photon excitation



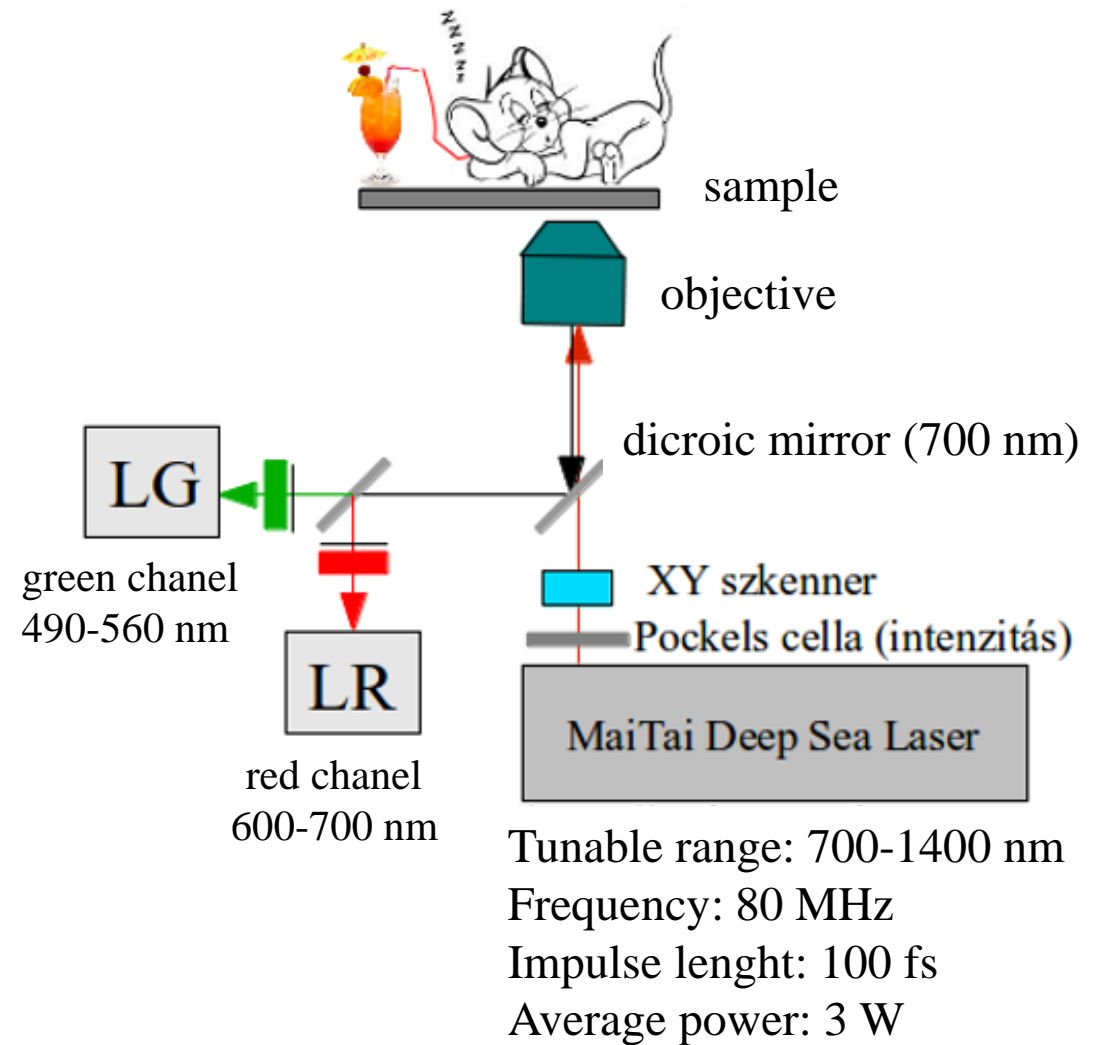
Two photon excitation



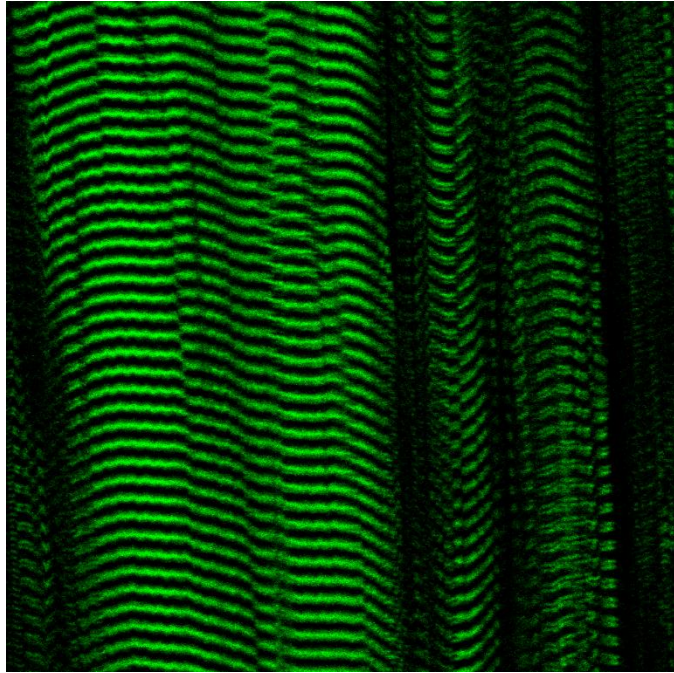


# Advantages

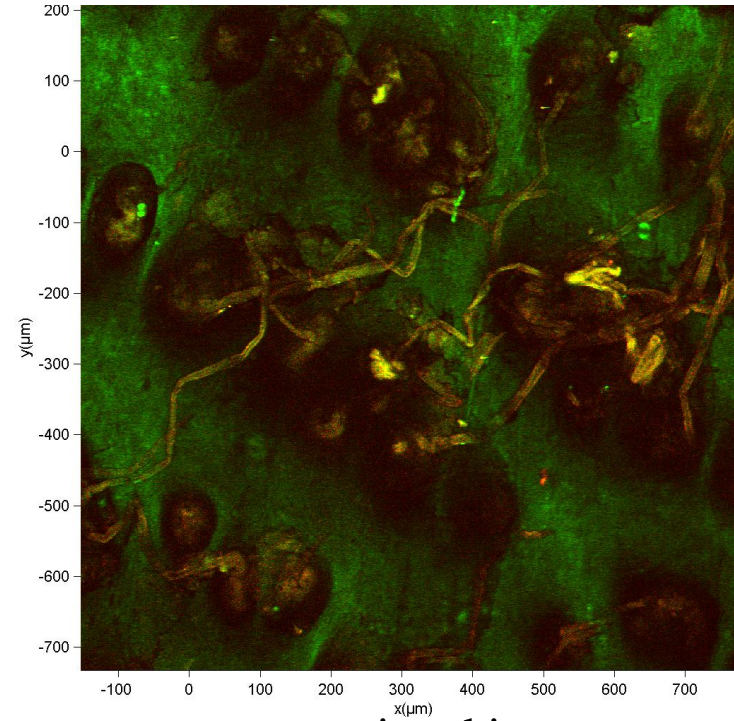
- excitation only in a tiny focal volume – reject out-of-focus
- low laser power – *in vivo* imaging
- infrared spectral range of laser source (700-1300 nm) – reduced scattering
- deep penetration
- effective signal detection
- optical sectioning – 3D imaging
- imaging without labeling



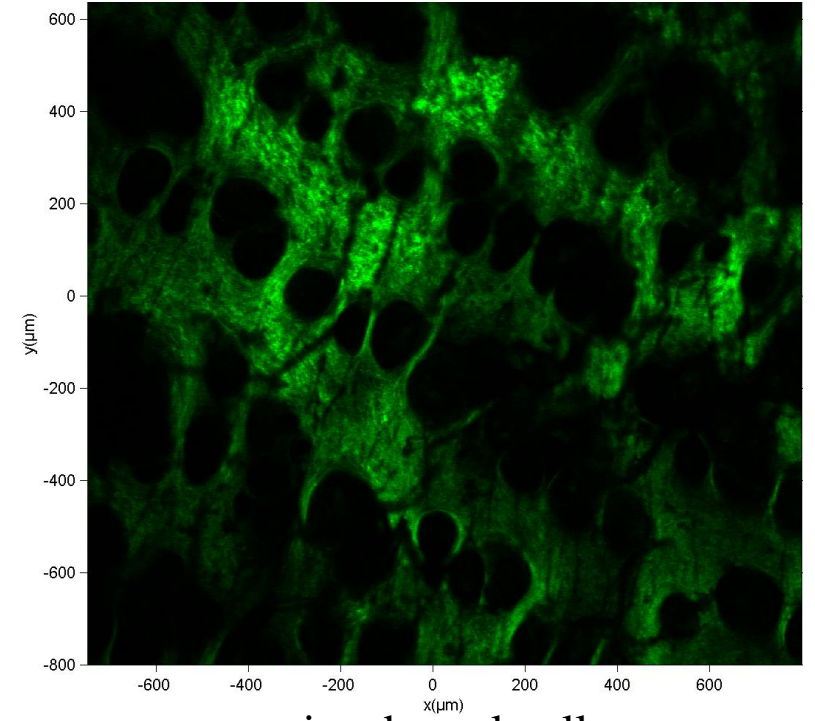
# Label-free imaging



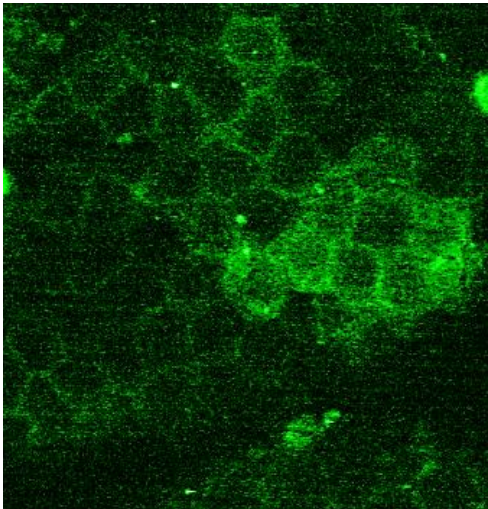
myosin



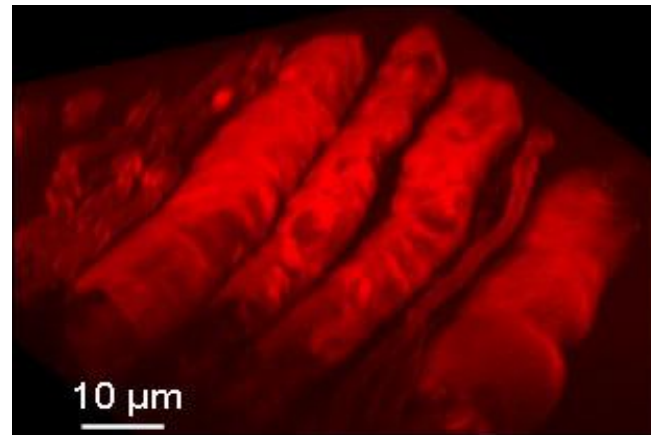
mice skin



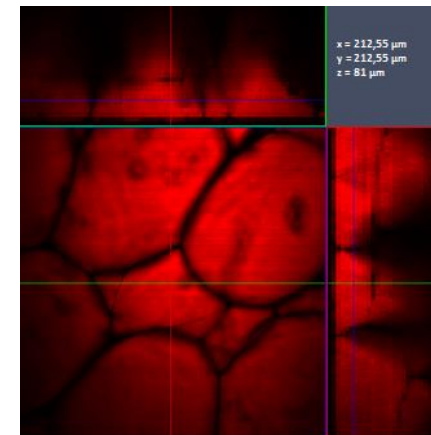
mice dermal collagen



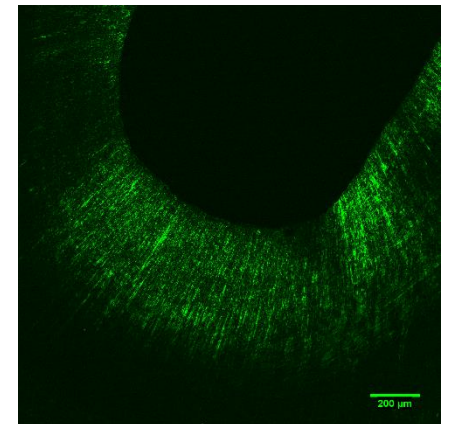
mice skin - keratin



myelin sheaths



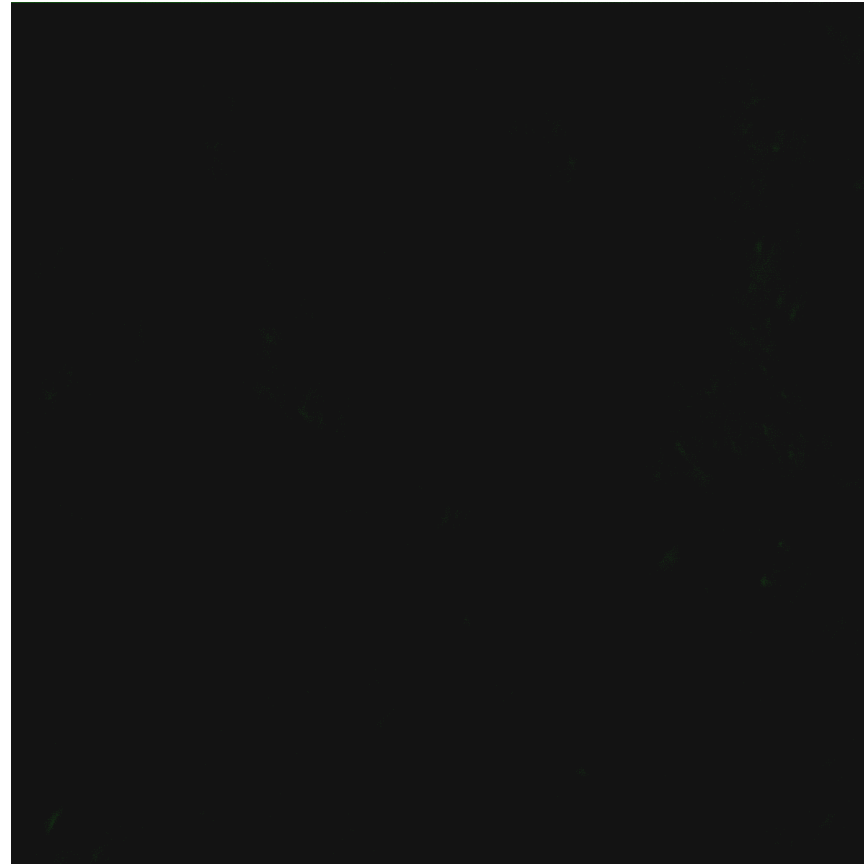
adipocytes



dental tubules

# 3D imaging

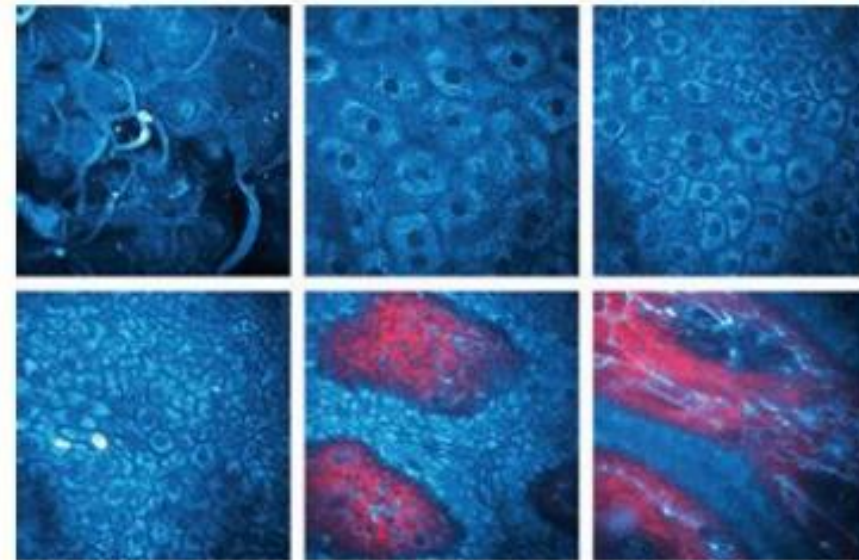
Comparison the dermal collagen structure of a control and type 2 diabetes affected mice



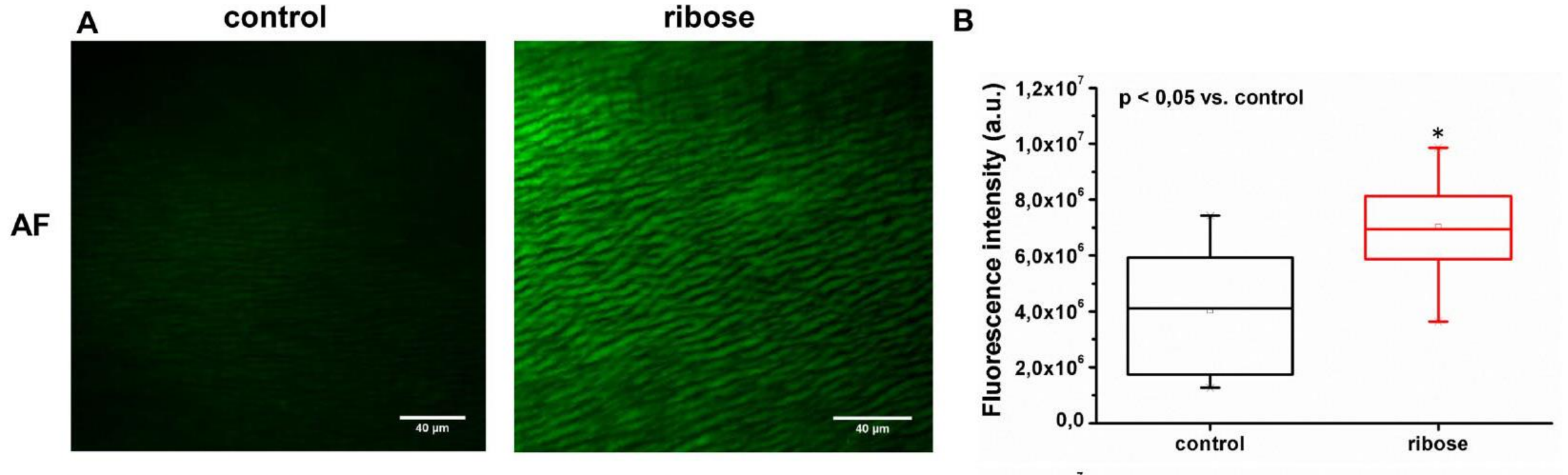
Optical sectioning,  
 $z = 80 \mu\text{m}$   
 $200 \mu\text{m} \times 200 \mu\text{m}$   
exc: 990 nm



# *In vivo* clinical application

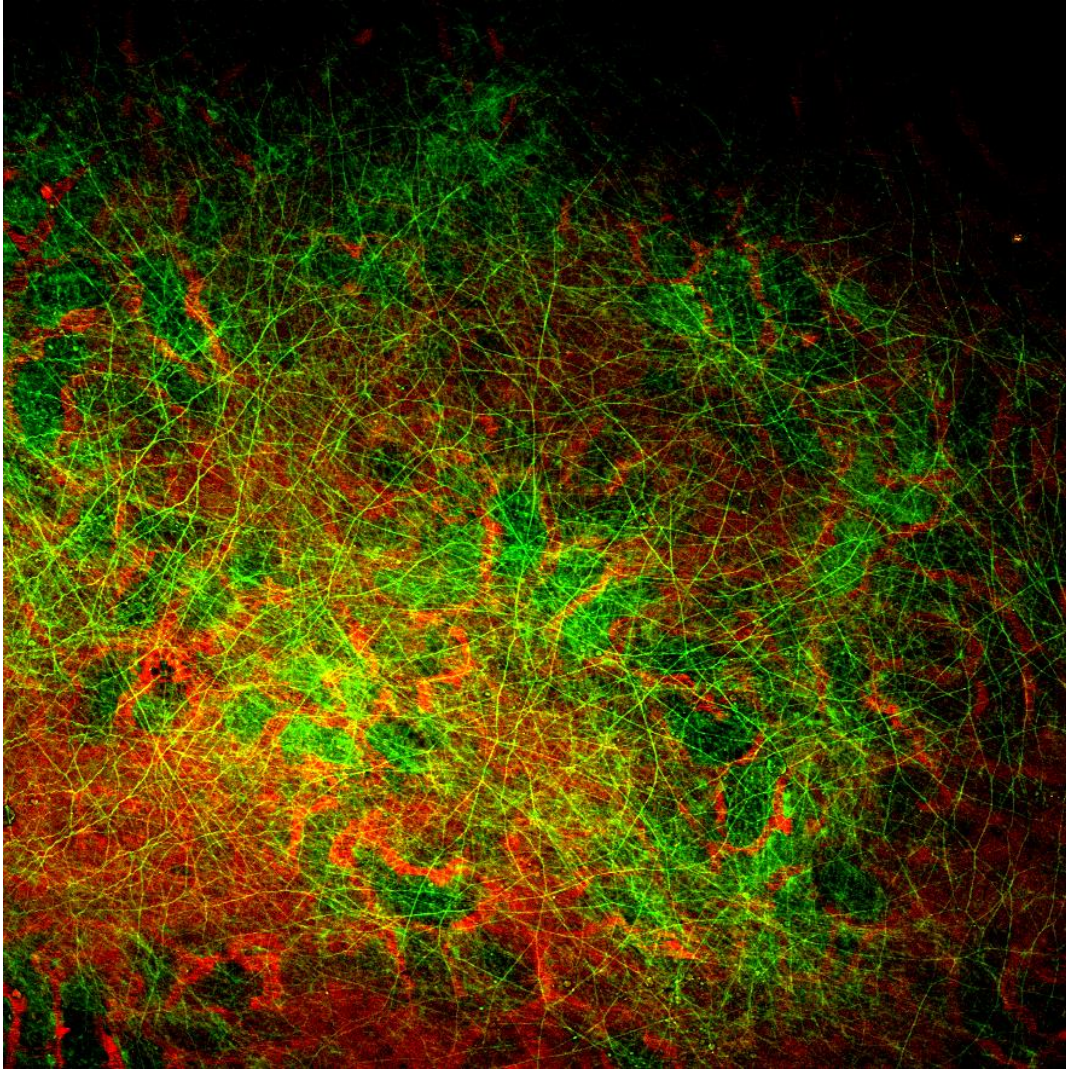


# Dental science

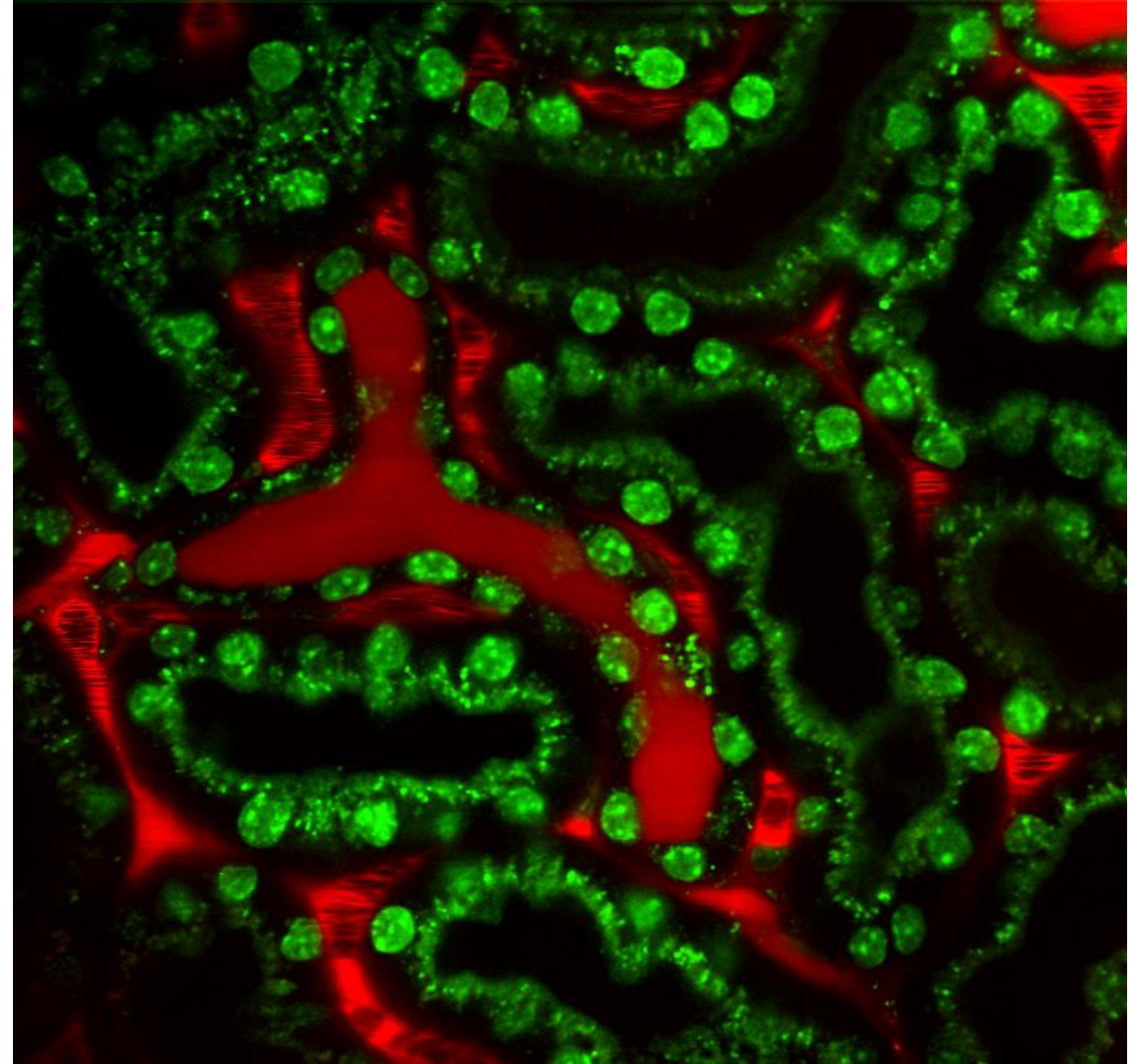




# Multiple fluorescent labeling



renal cortex



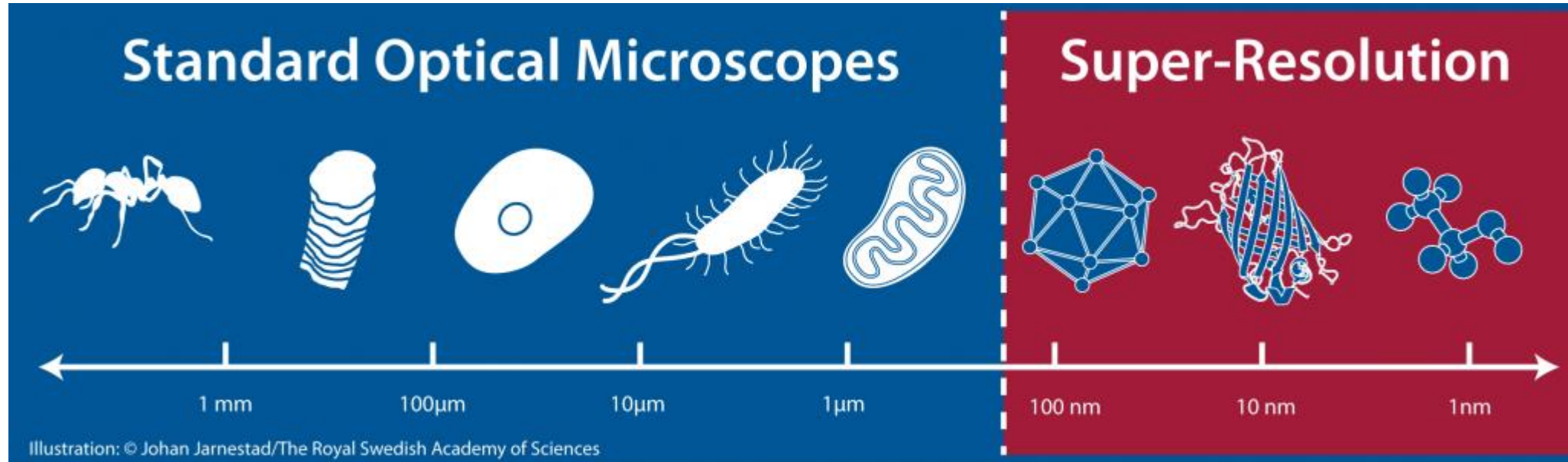
collecting ducts and JGA cells

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

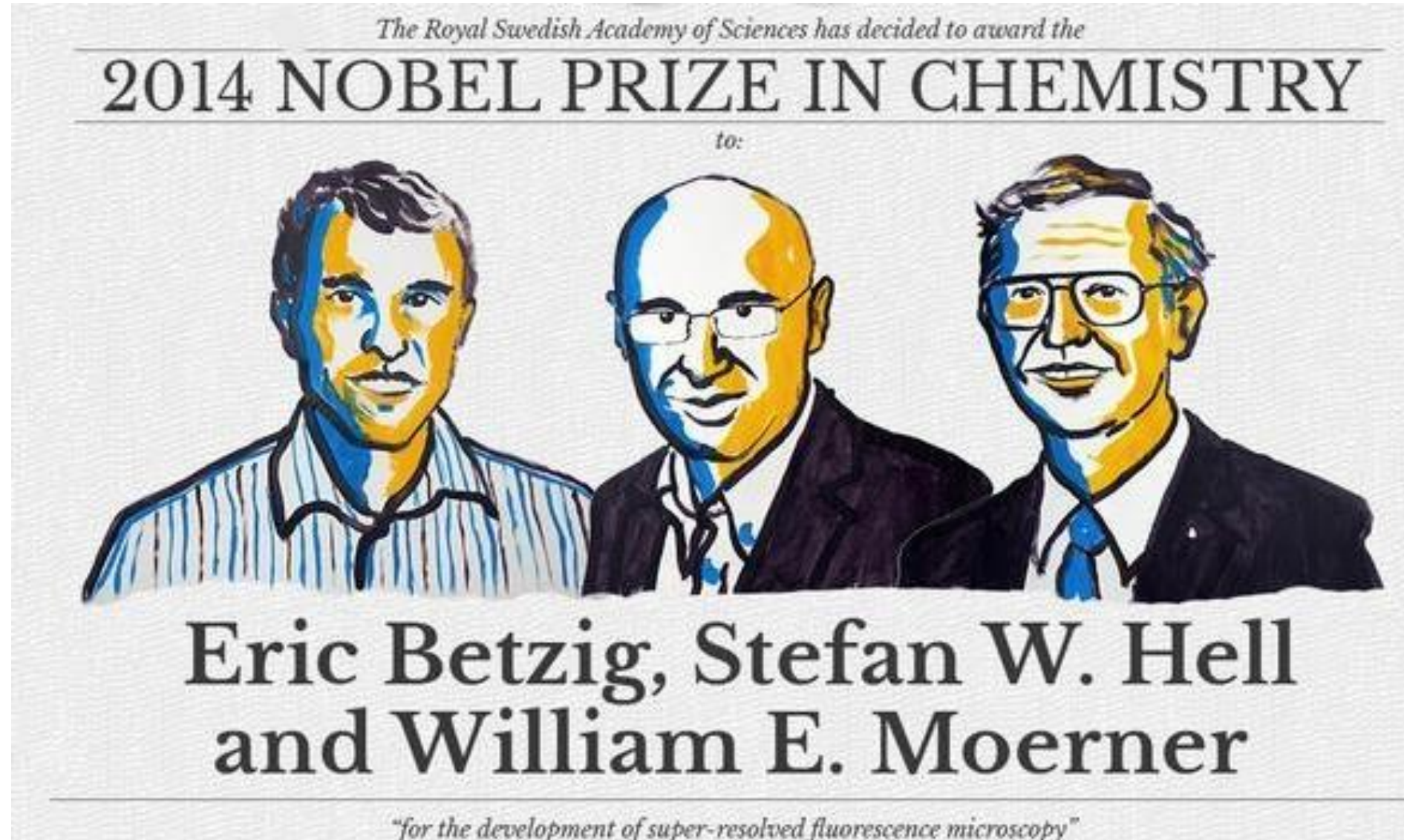


# How big are things?

**resolution limit: 200 nm**

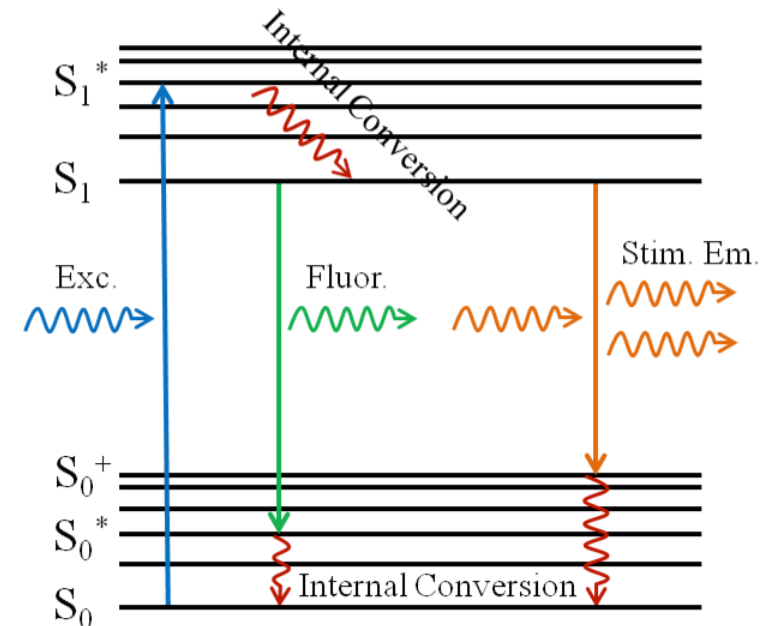
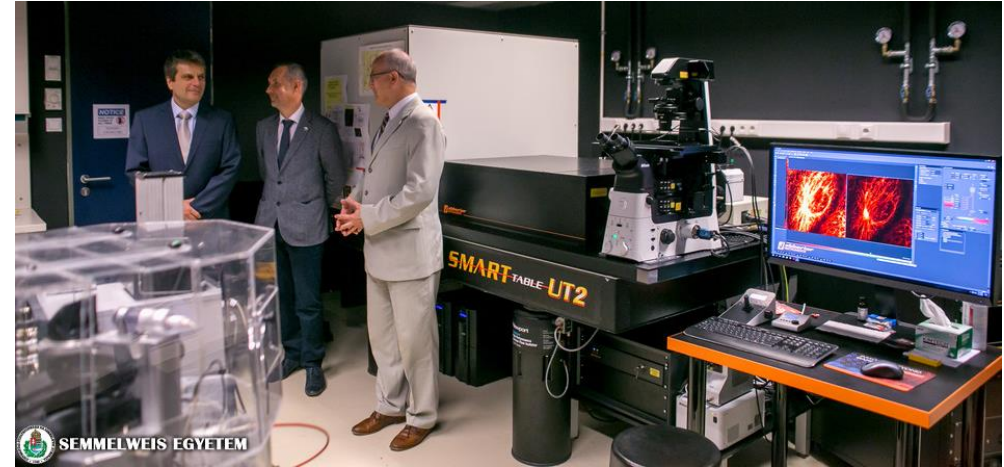


# Superresolution microscopy



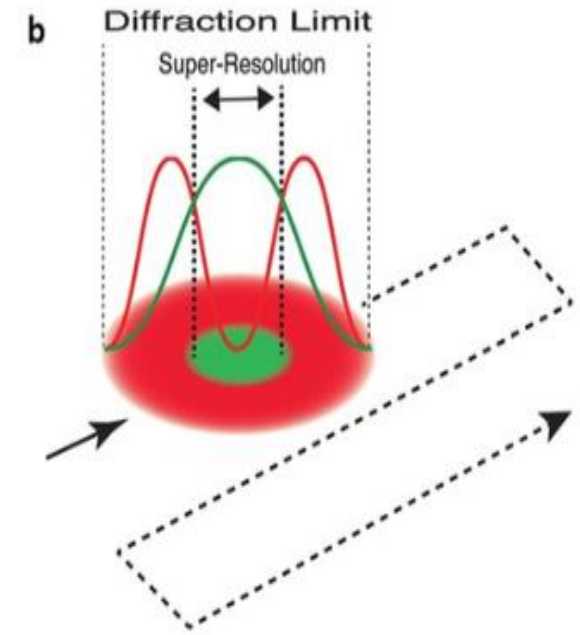
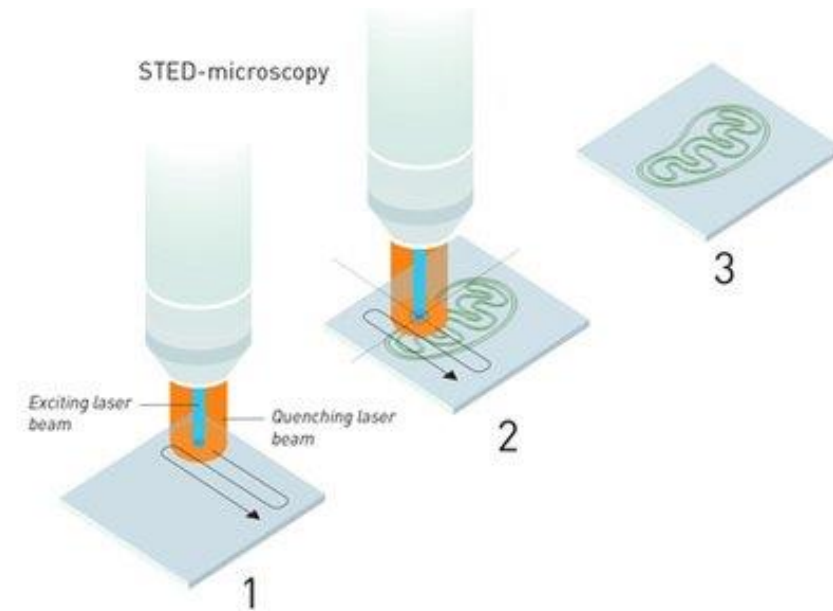
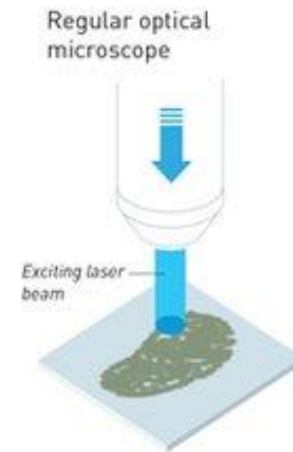
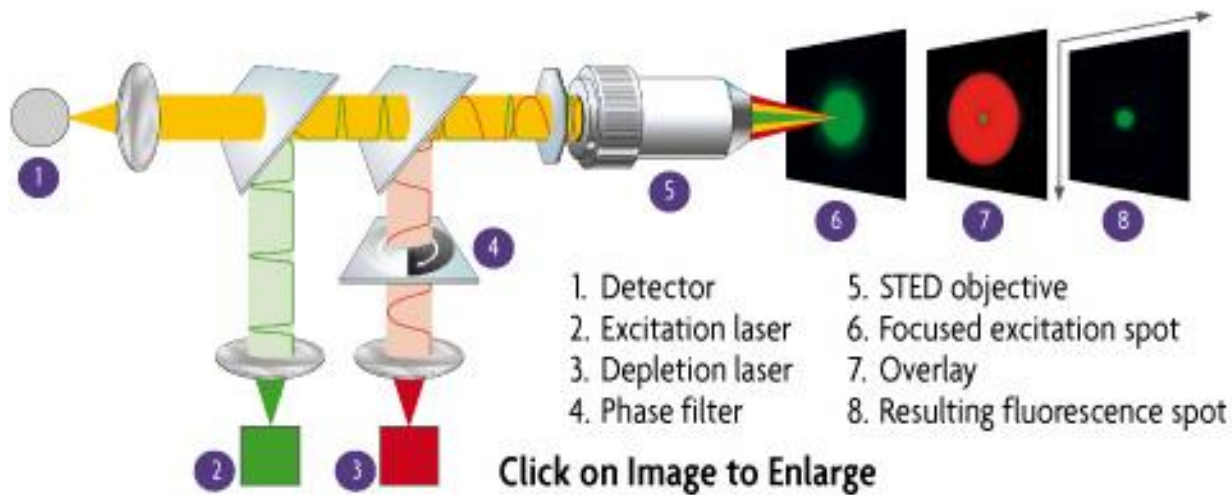
# Superresolution microscope

- 2014. Eric Betzig, Stefan W. Hell és William E. Moerner were awarded Nobel-prize in chemistry
- STED: stimulated emission depletion microscopy
- 2018. August – STED device arrived in our Institute
- allows for images to be taken at resolutions below the diffraction limit
- Stimulated emission: this is a process by which an incoming photon of a specific wavelength can interact with an excited atomic electron causing it to drop a lower energy level
- Phase, frequency, polarization, and direction of the photons emitted with the stimulated emission are identical with that of induced the stimulation → no contribution in image formation





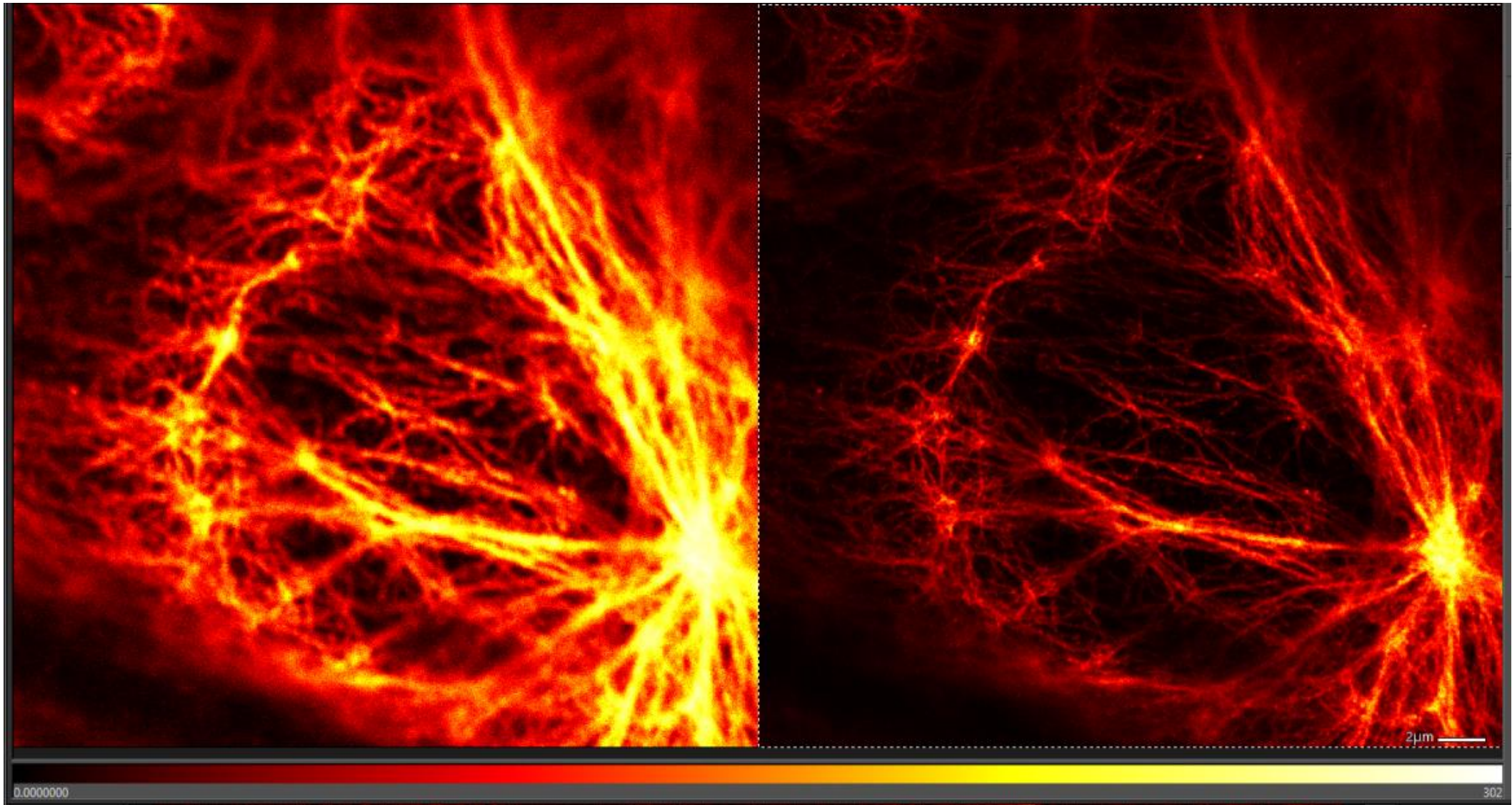
- excitation laser: produces an ordinary diffraction limited focus
- depletion laser: doughnut shape, fluorescence from the molecules is quenched via stimulated emission
- point-by-point scanning



# Vimentin: flexible intermediate filaments in the cytoskeleton

confocal

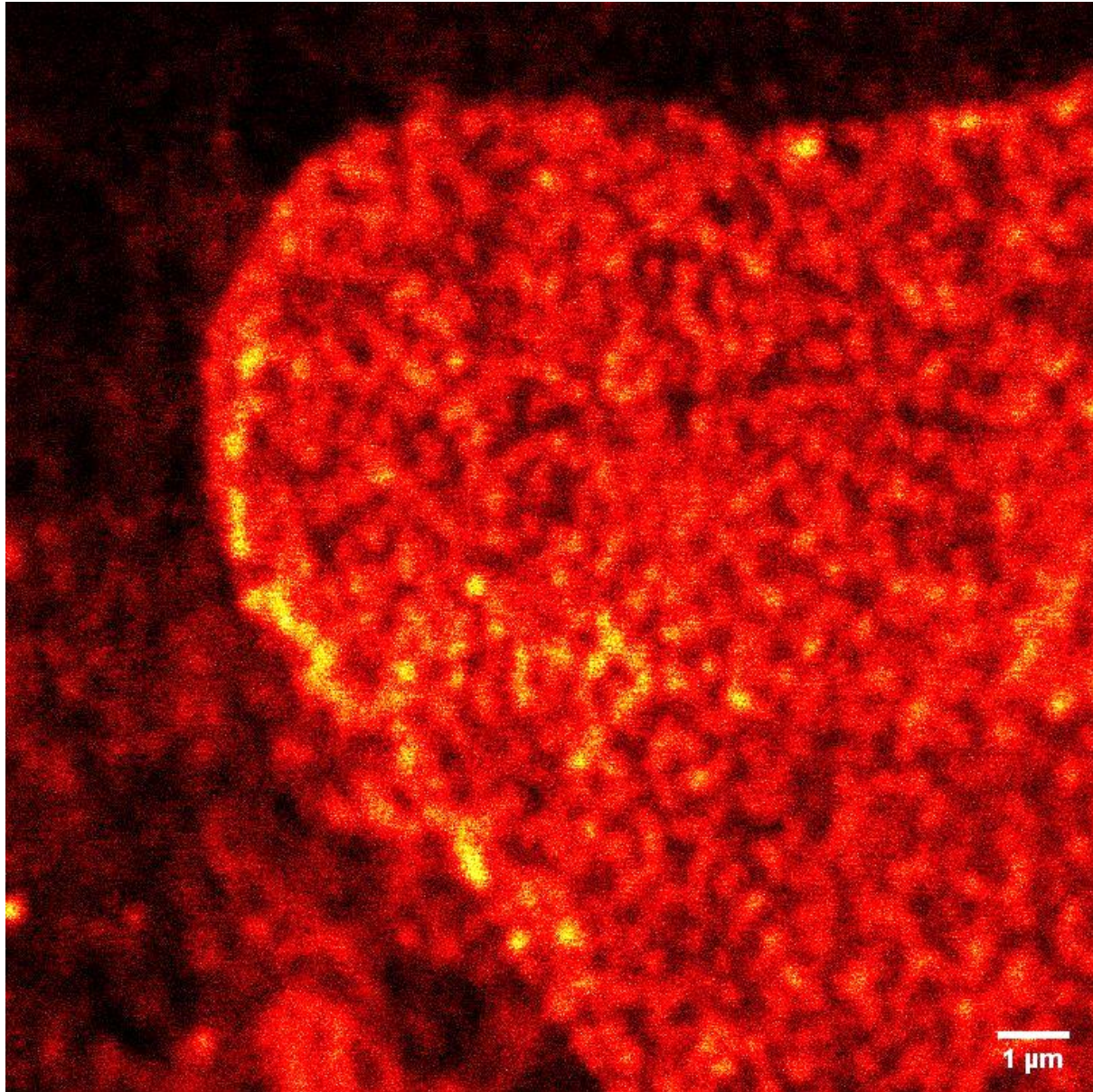
STED



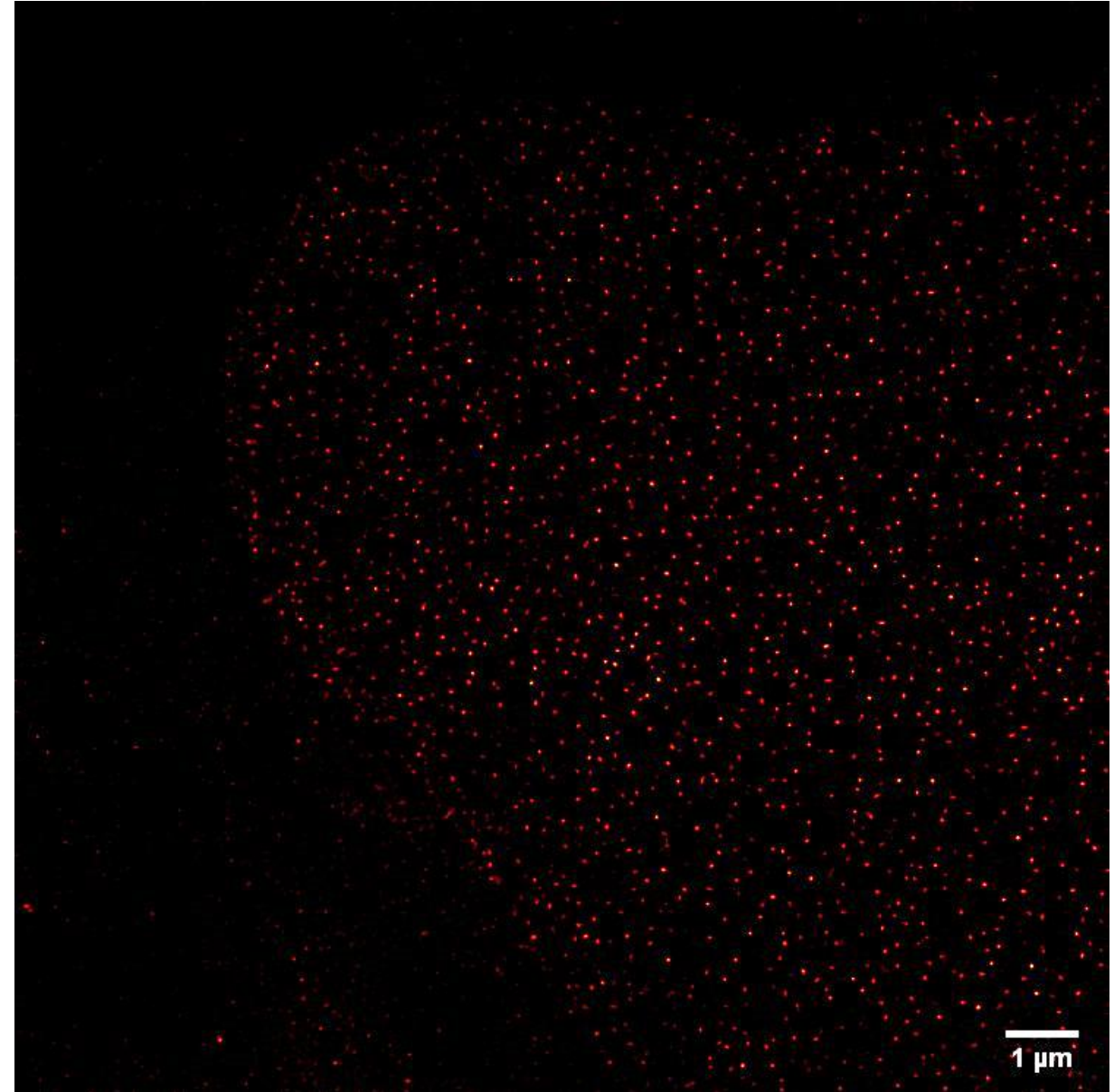


# Nuclear pores of HeLa cells

confocal



STED





# Checklist

- ✓ resolution limit of image formation
- ✓ Abbe's principle
- ✓ working principle of fluorescence microscope: illumination, excitation/emission spectra, Stokes-shift, function of dichroic mirror
- ✓ sources of fluorescence: intrinsic, extrinsic
- ✓ GFP protein
- ✓ working principle of confocal microscope: illumination, function of pinhole
- ✓ working principle of two-photon microscope: properties of laser source, excitation/emission spectra, penetration ability, advantages
- ✓ superresolution microscopy: principle of STED imaging