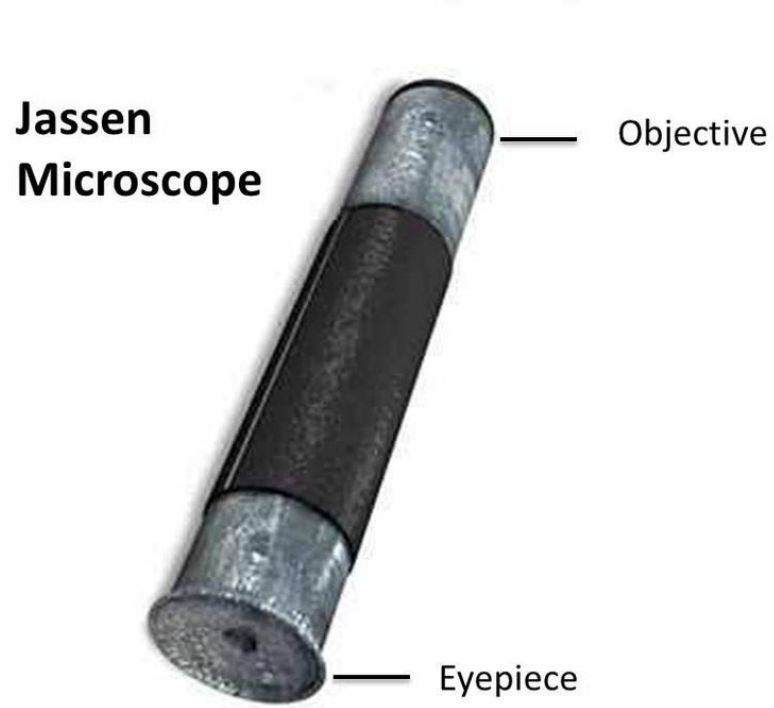
Special microscopic techniques

Dora Haluszka

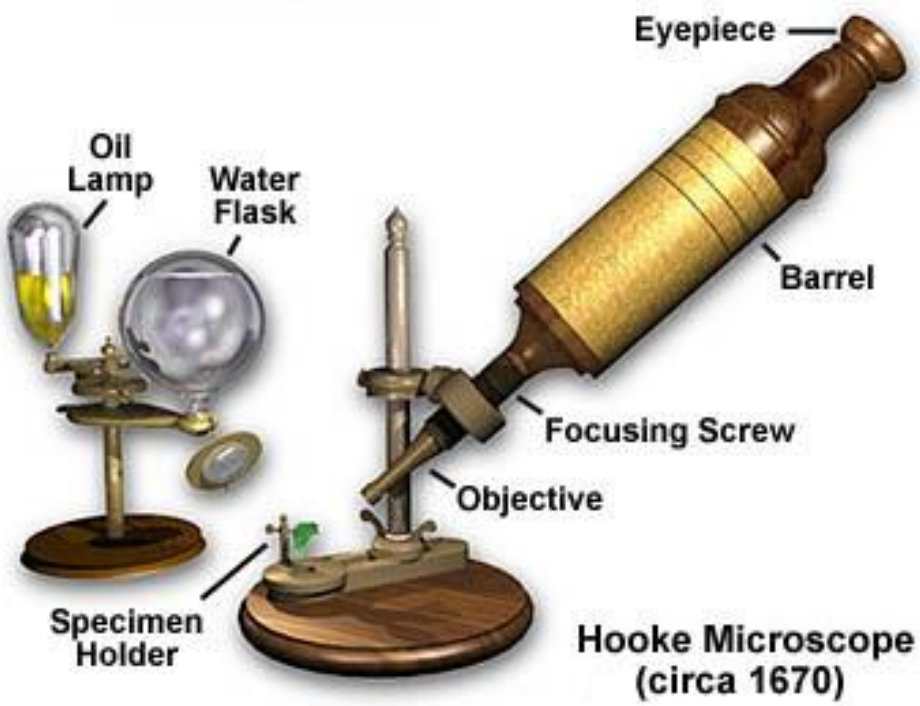
5/11/2021

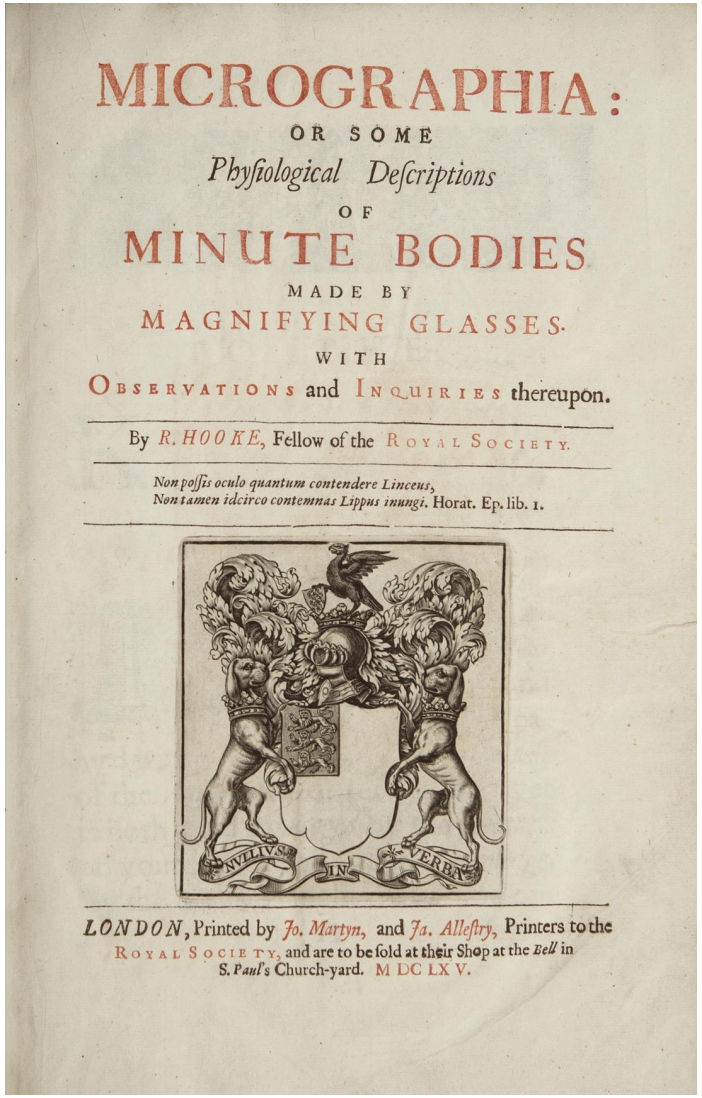
A brief history of microscopes

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



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





MICROGRAPHIA:

OR SOME

Physiological Descriptions

OF

MINUTE BODIES

MADE BY

MAGNIFYING GLASSES.

WITH

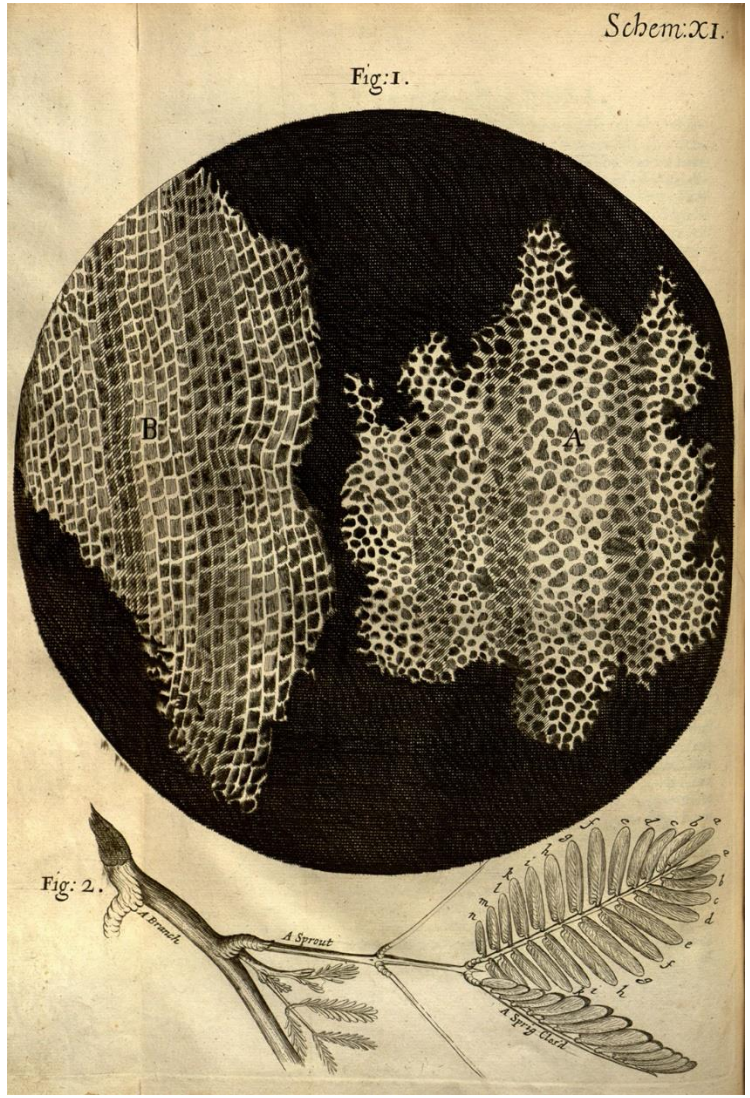
OBSERVATIONS and INQUIRIES thereupon.

By *R. HOOKE*, Fellow of the ROYAL SOCIETY.

Non possis oculo quantum contendere Linceus,
Non tamen idcirco contemnas Lippus inungi. Horat. Ep. lib. 1.

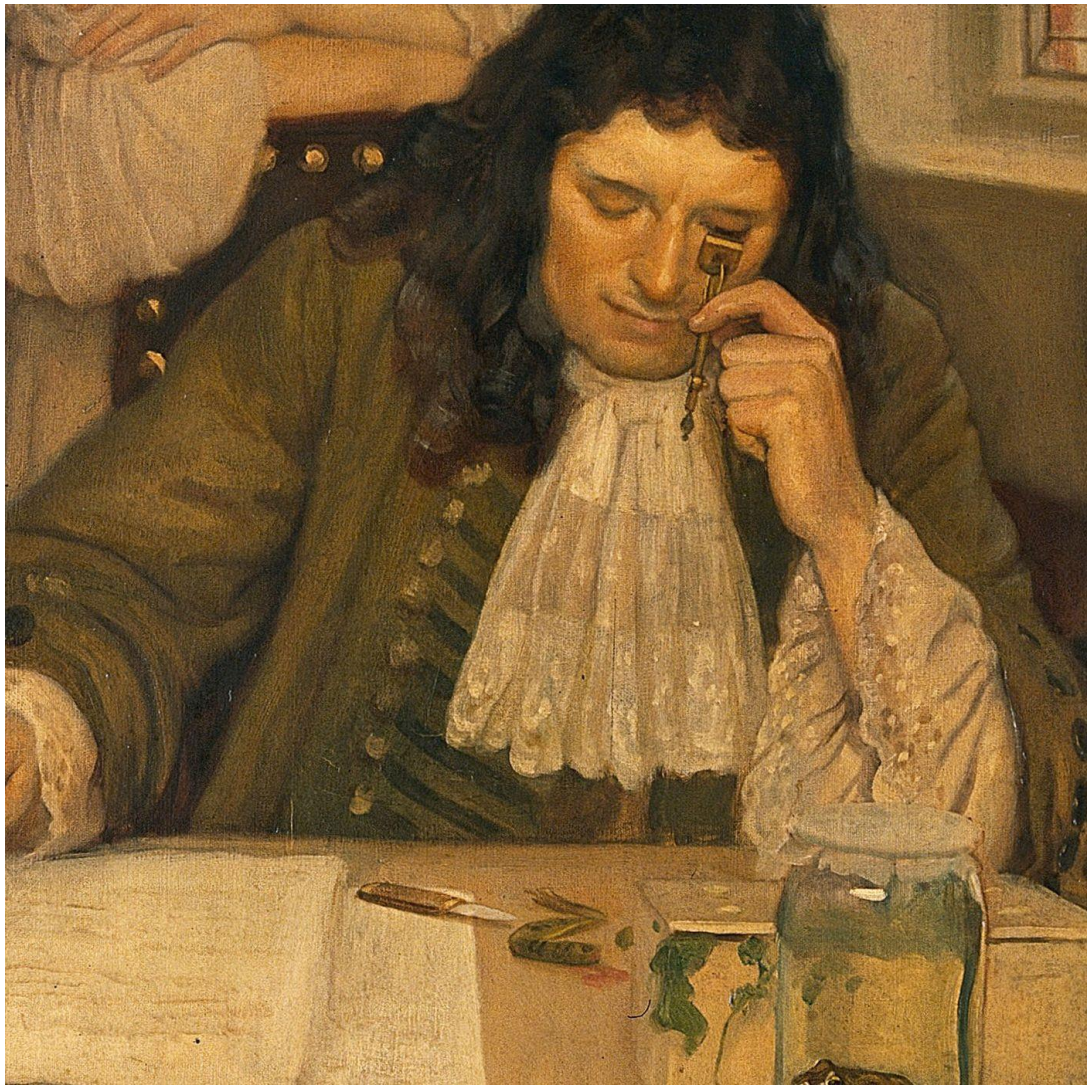
NULLIUS IN VERBA

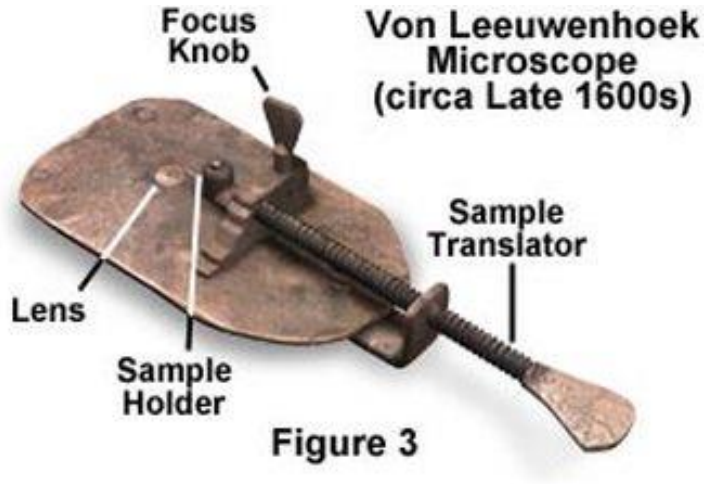
LONDON, Printed by *Jo. Martyn*, and *Ja. Allestry*, Printers to the ROYAL SOCIETY, and are to be sold at their Shop at the *Bell* in S. *Paul's* Church-yard. M DC LX V.

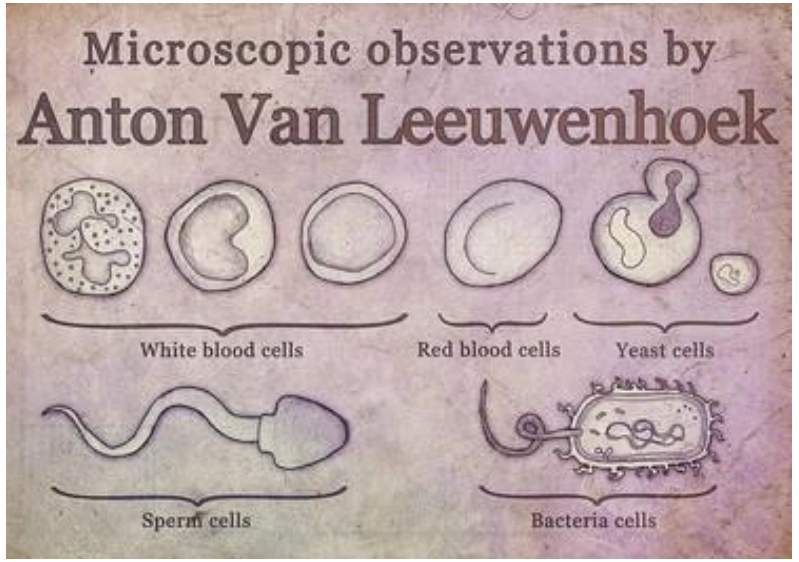


„The open universe”

- 1674: Antonie van Leeuwehoek – make simple microscopes, 270 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

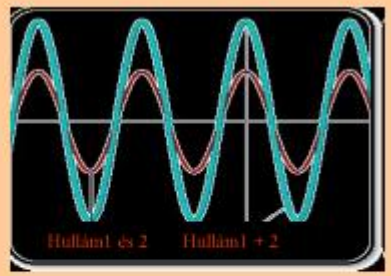
Figure 2

Ernst Abbe (1840-1905)

Carl Zeiss (1816-1888)

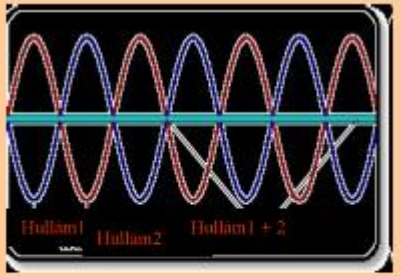
Microscope by Carl Zeiss (1879) with optics by Abbe

Fundamentals of wave optics



Similar phase

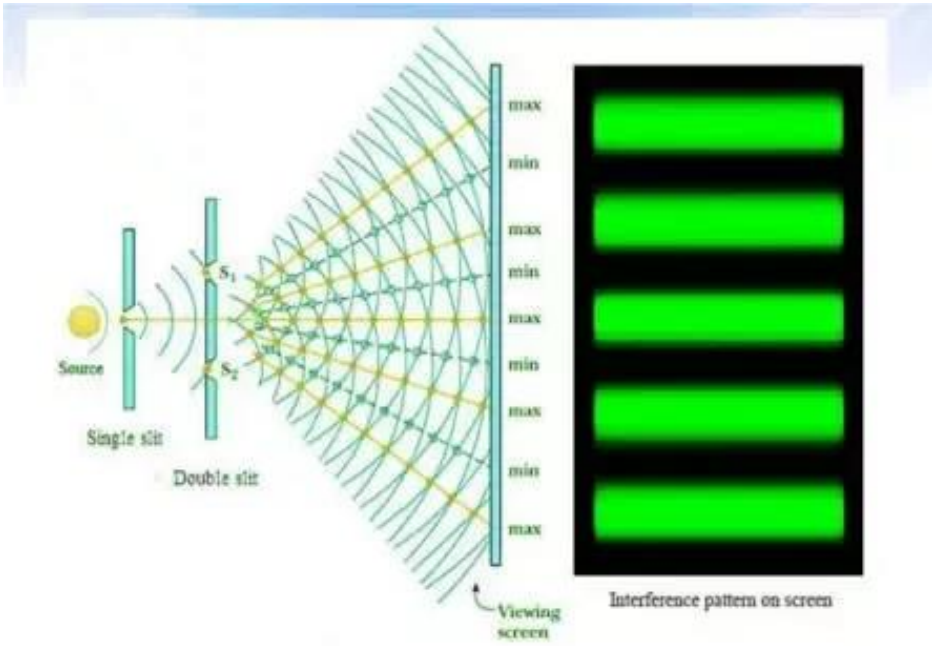
Constructive interference



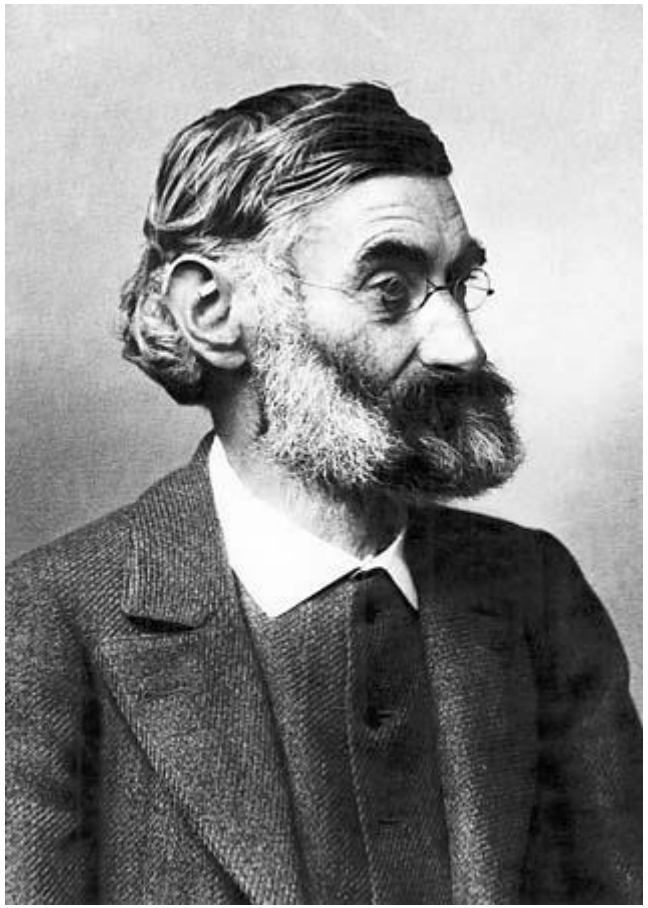
Opposite waves

Destructive interference

Young's experiment



Resolution limit of microscope



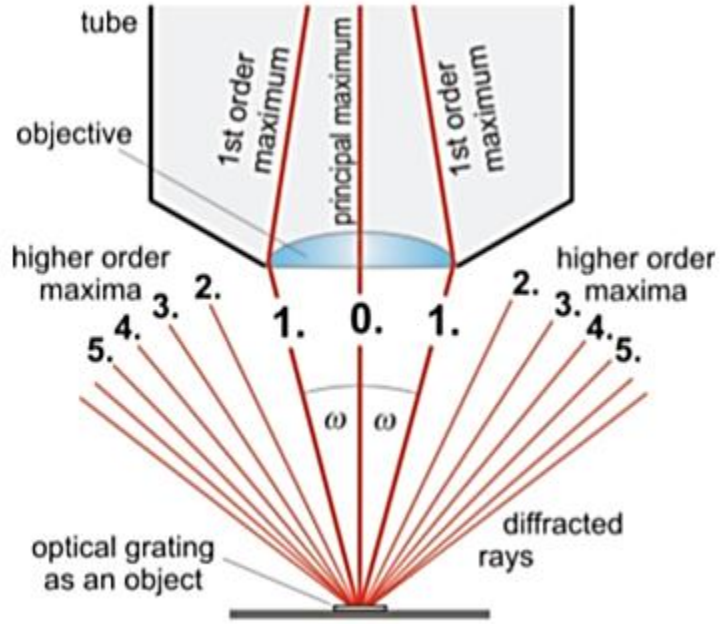
Ernst Abbe (1840-1905)

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.

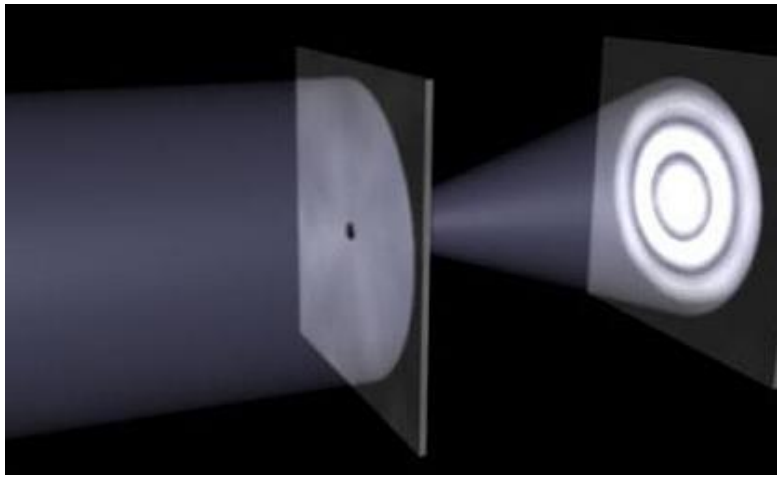
$$\delta = 0{,}61\frac{\lambda}{n \sin \omega}$$

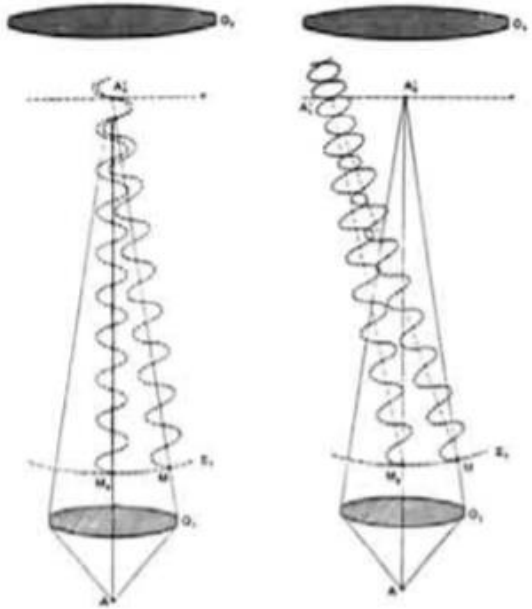
δ limit of resolution – distance between two object details which can be just resolved (200 nm)



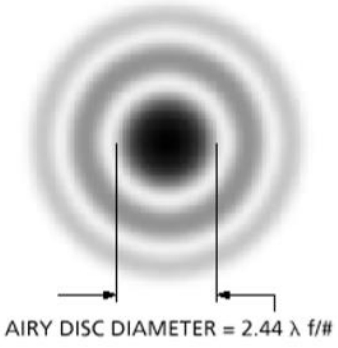
Airy disks – the evidence of wave character of light

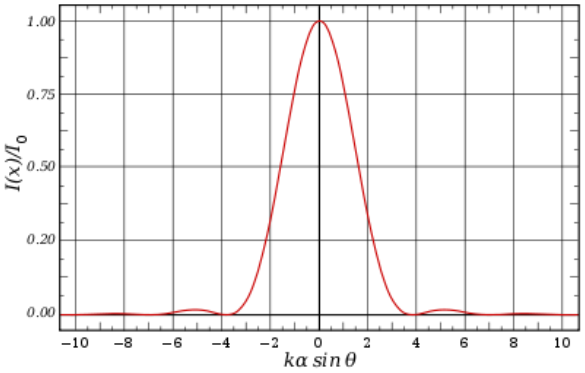
The Airy disk is descriptions of the best-focused spot of light that a perfect lens with a circular aperture can make, limited by the diffraction of light.





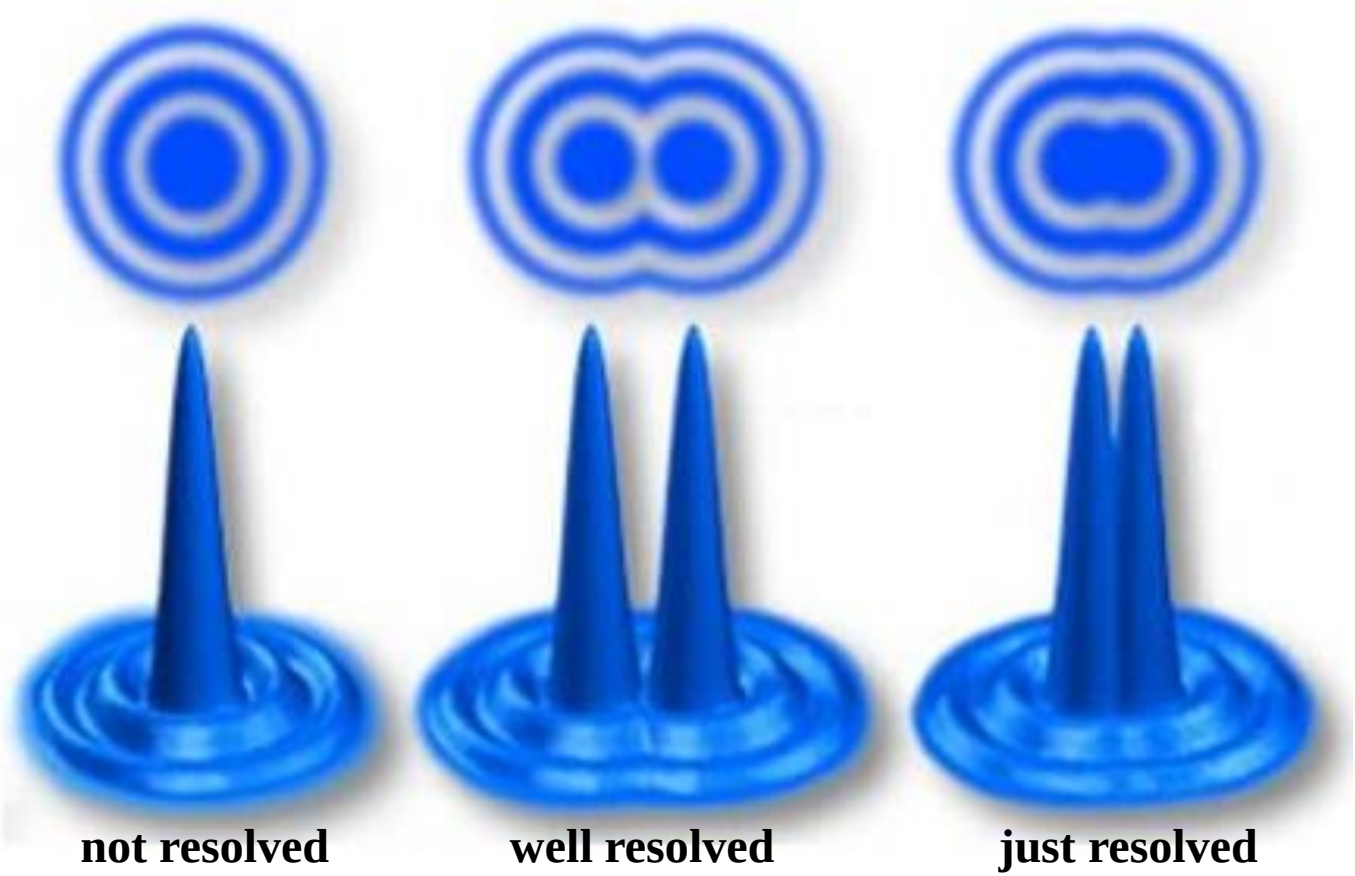
Formation: the waves in same phase produce diffraction maximum (left) while the waves shifted by 180° produce diffraction minimum (right).





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

How can we distinguish two image points?

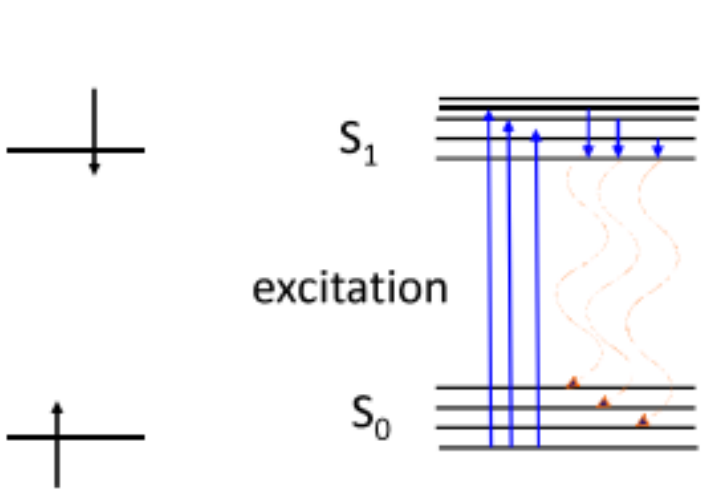


Rayleigh criterion:

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

S_1

excitation

S_0

Non-radiant transitions

Light emission

Fluorescence

First excited singlet state

the spin states of the electrons

sum of all e^- spin quantum numbers=0

De-excitation by photon emission between singlet states

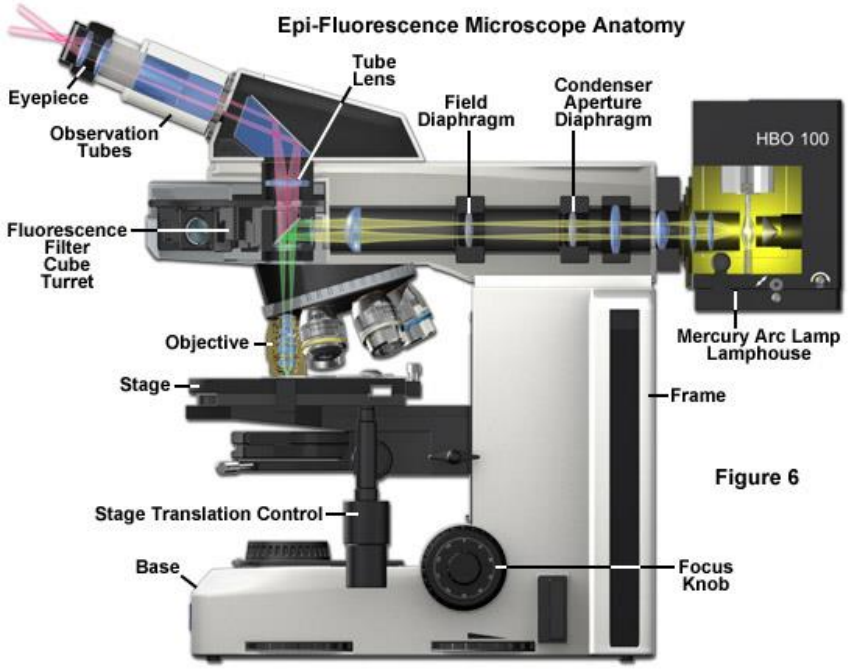
$E_{excitation} \geq E_{fluorescence}$

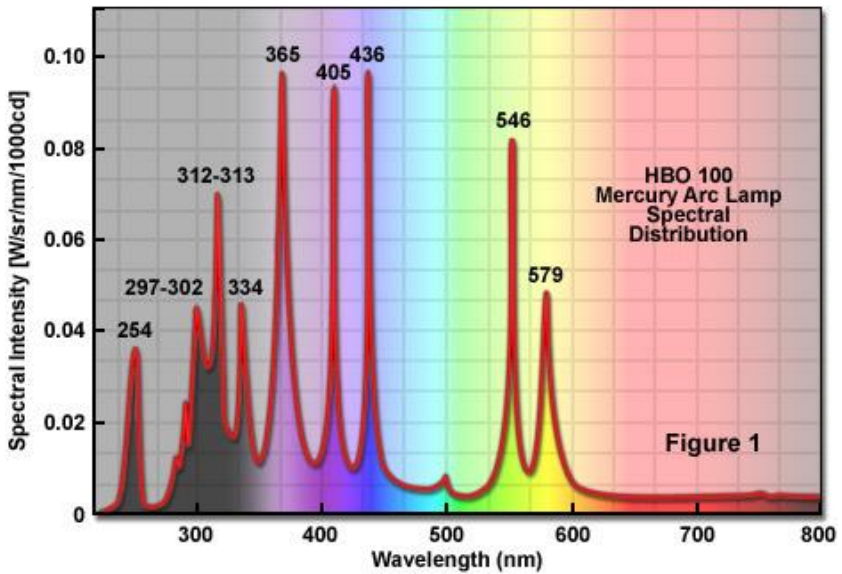
$\lambda_{excitation} \leq \lambda_{fluorescence}$

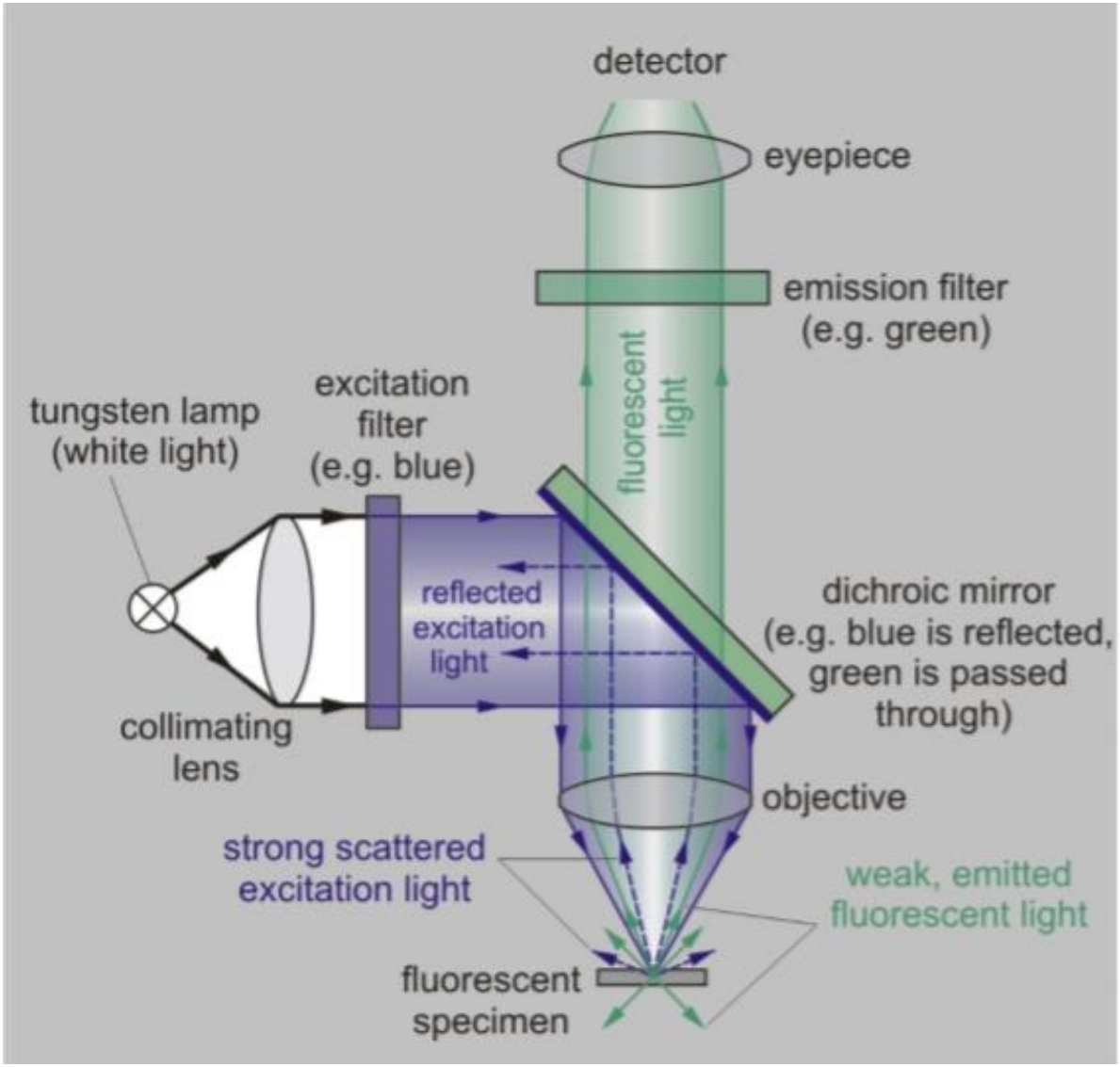
Stokes-shift

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

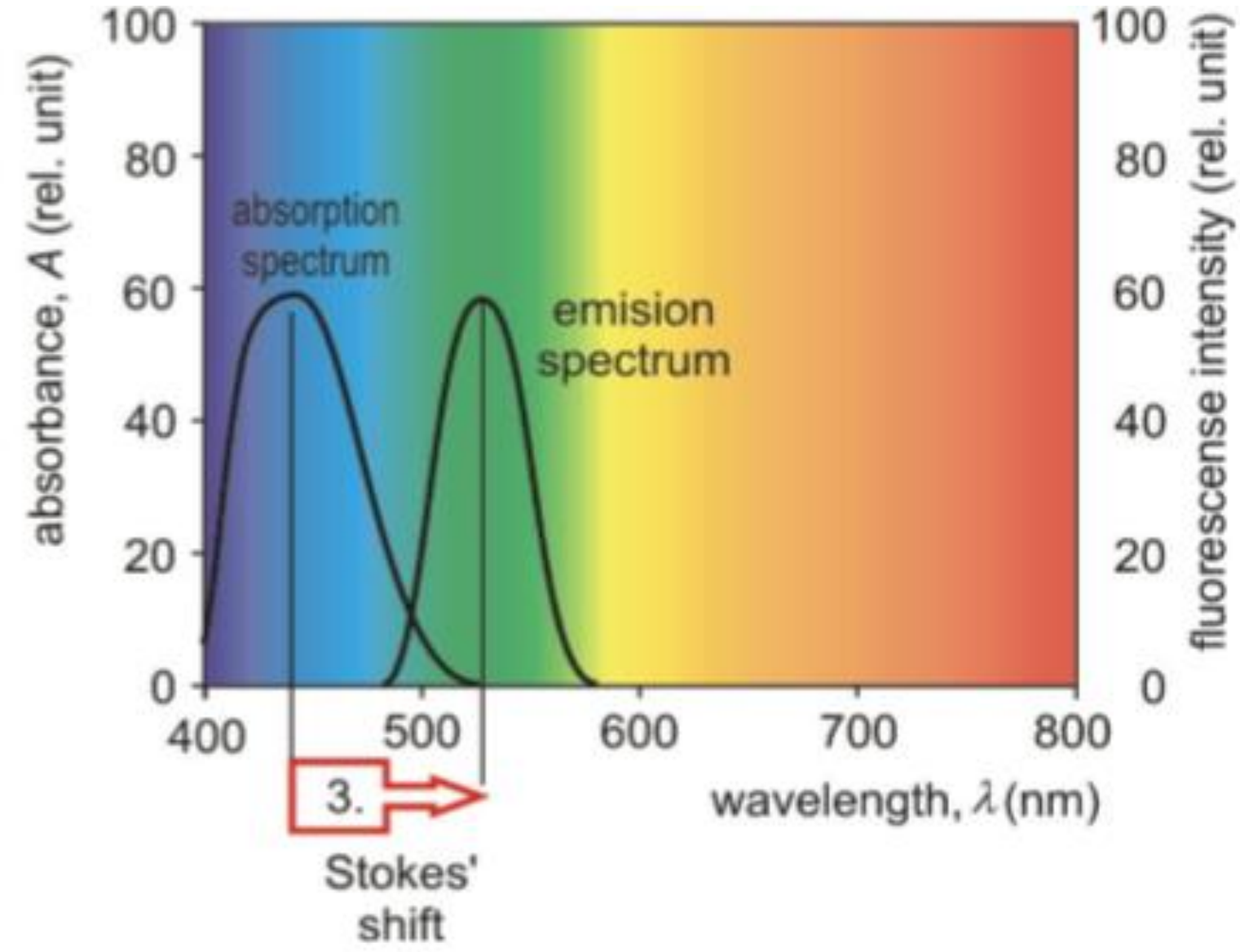
Fluorescence microscope







Absorption and emission spectrum



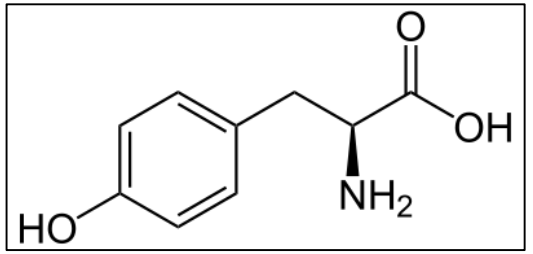
Source of fluorescence

- **Intrinsic** fluorophores:

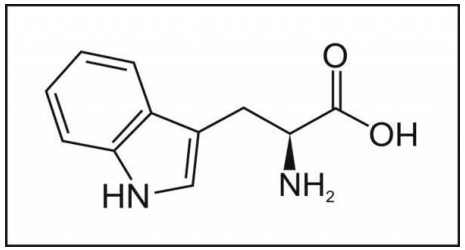
tryptophan, tyrosine aminoacids (aromatic) porphyrins

- **Extrinsic** fluorophores:

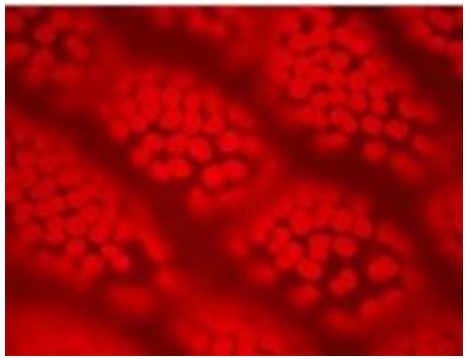
fluorescent dyes

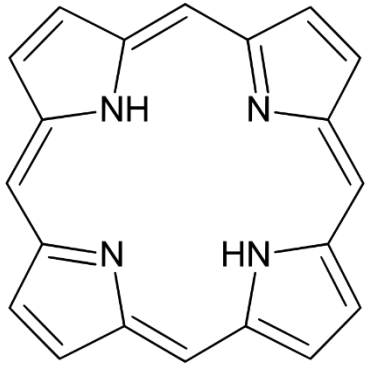


tyrosine



tryptophan

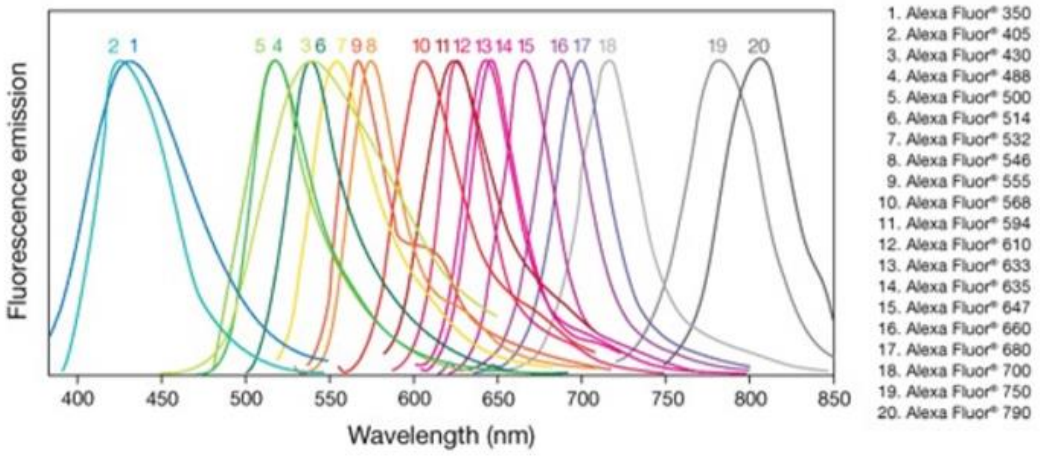

porphyrin fluorescence



porphyrin

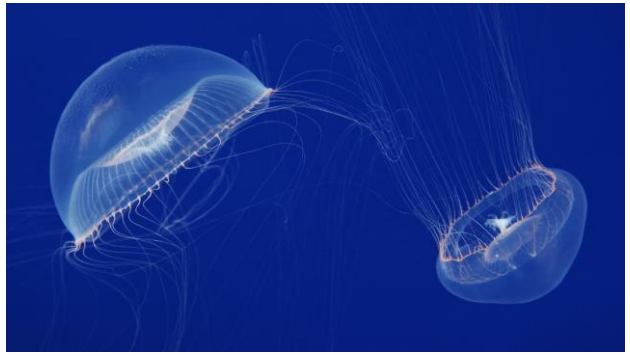
The perfect fluorescent dye:

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

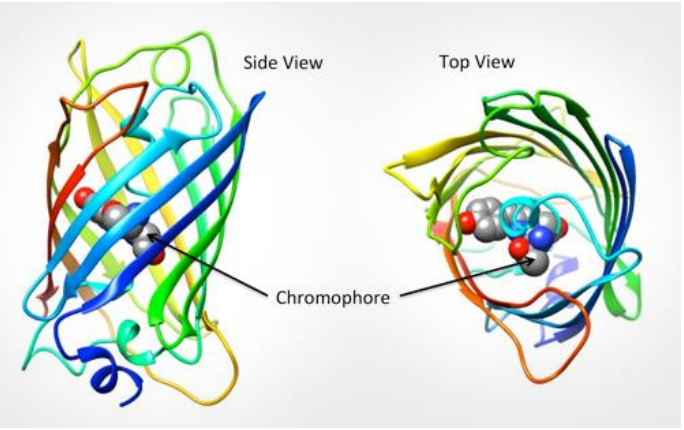


Fluorescent proteins

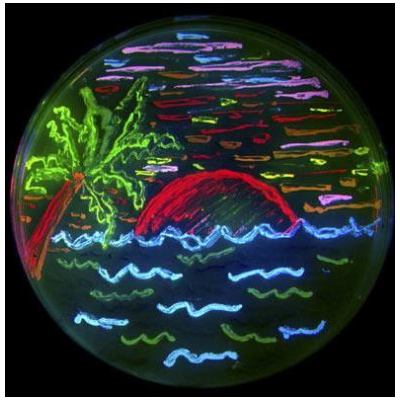
- Green Fluorescent Protein (GFP)
- first isolated from jellyfish (1960s)
- ~27 kDa, 238 aa, 11 strands β-barrel structure
- the central alpha helix contains the chormophore: Ser-65, Tyr-66, and Gly-67
- excitation: blue (475 nm) and UV (396 nm) light
- emission: 508 nm



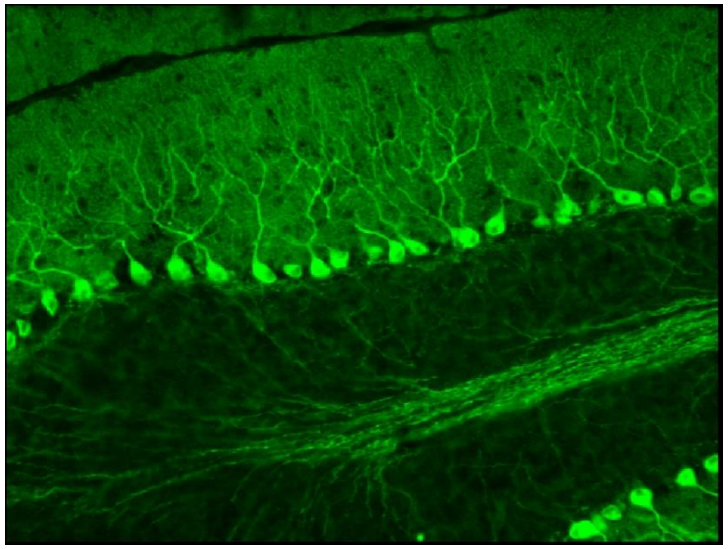
Aequorea victoria



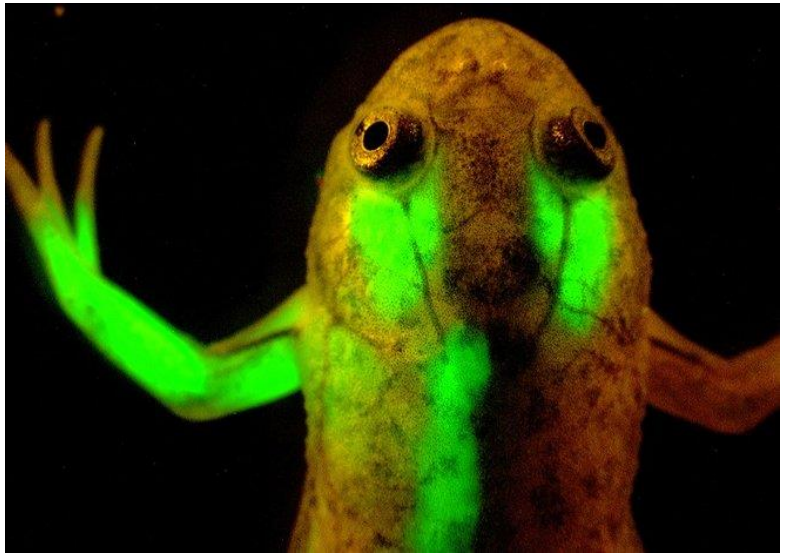
- Used as tagging protein
- Small size – has no effect on the funcion of examined protein
- Transfected cells
- Transgene animals: all cell express the GFP



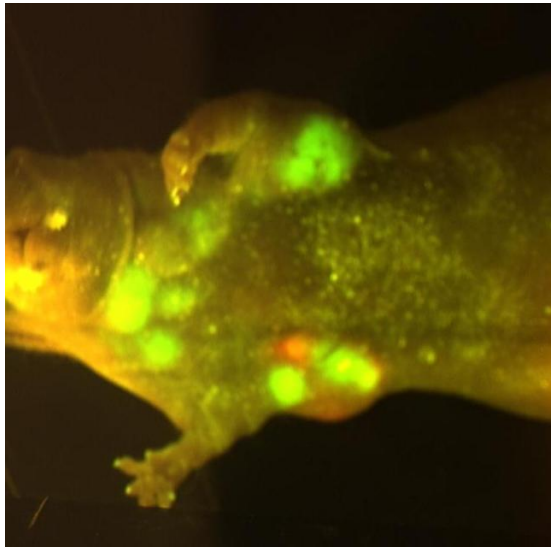
Transgene mice



Purkinje cells



Frog muscle cells



Tumor cells

2008. Nobel prize in chemistry

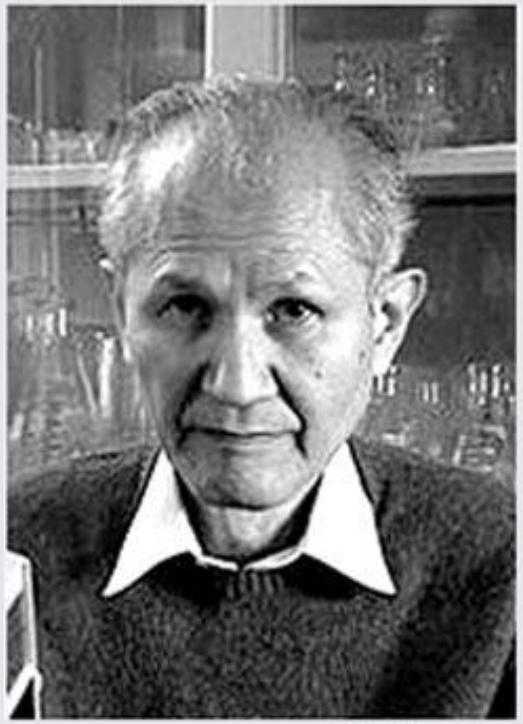


Photo: J. Henriksson/SCANPIX

Osamu Shimomura

Photo: J. Henriksson/SCANPIX

Martin Chalfie

Photo: UCSD

Roger Y. Tsien

General properties of lasers

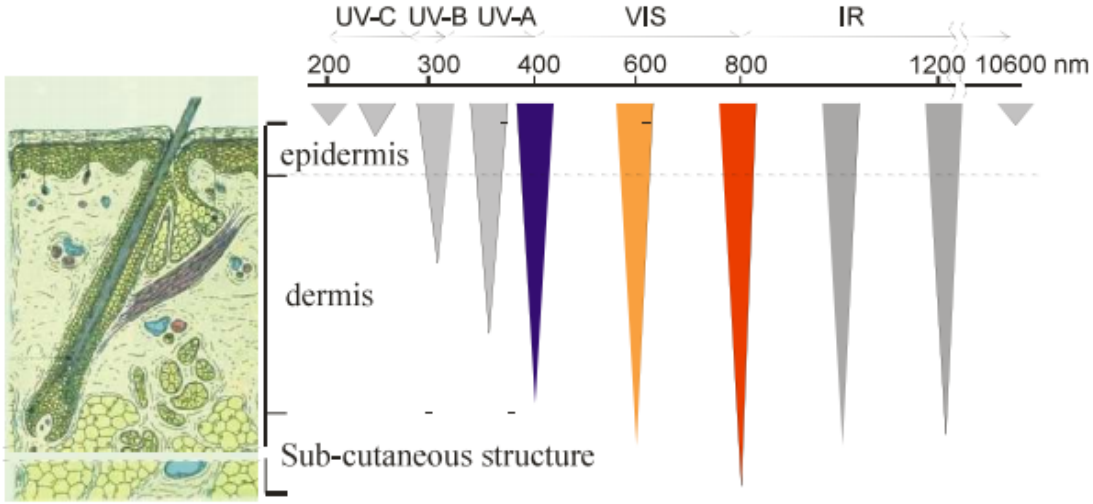
light amplification by stimulated emission of radiation

- 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

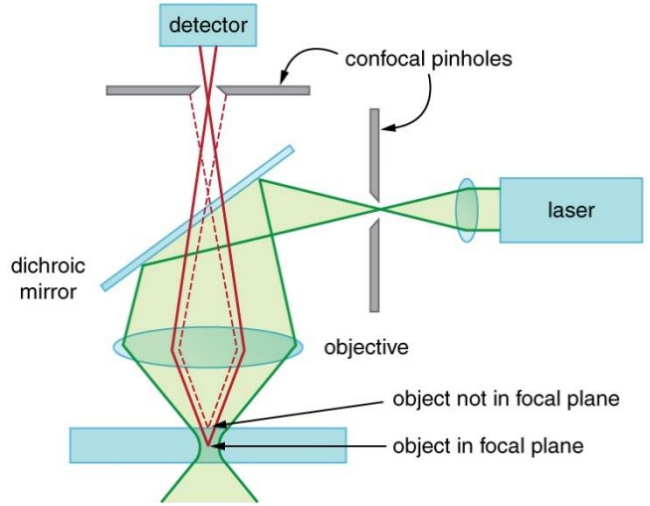


Light intensity is attenuated due to absorption, reflection, refraction.

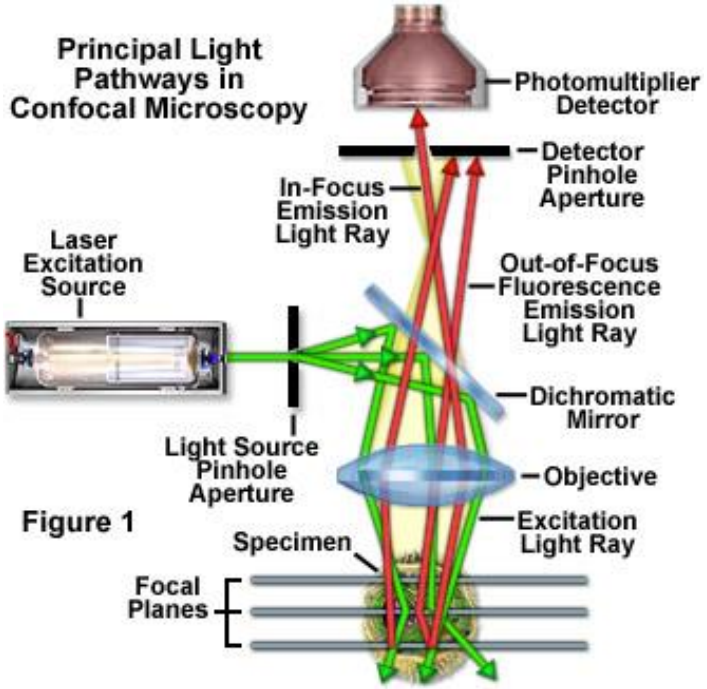
Penetration depth depends on the wavelength.

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



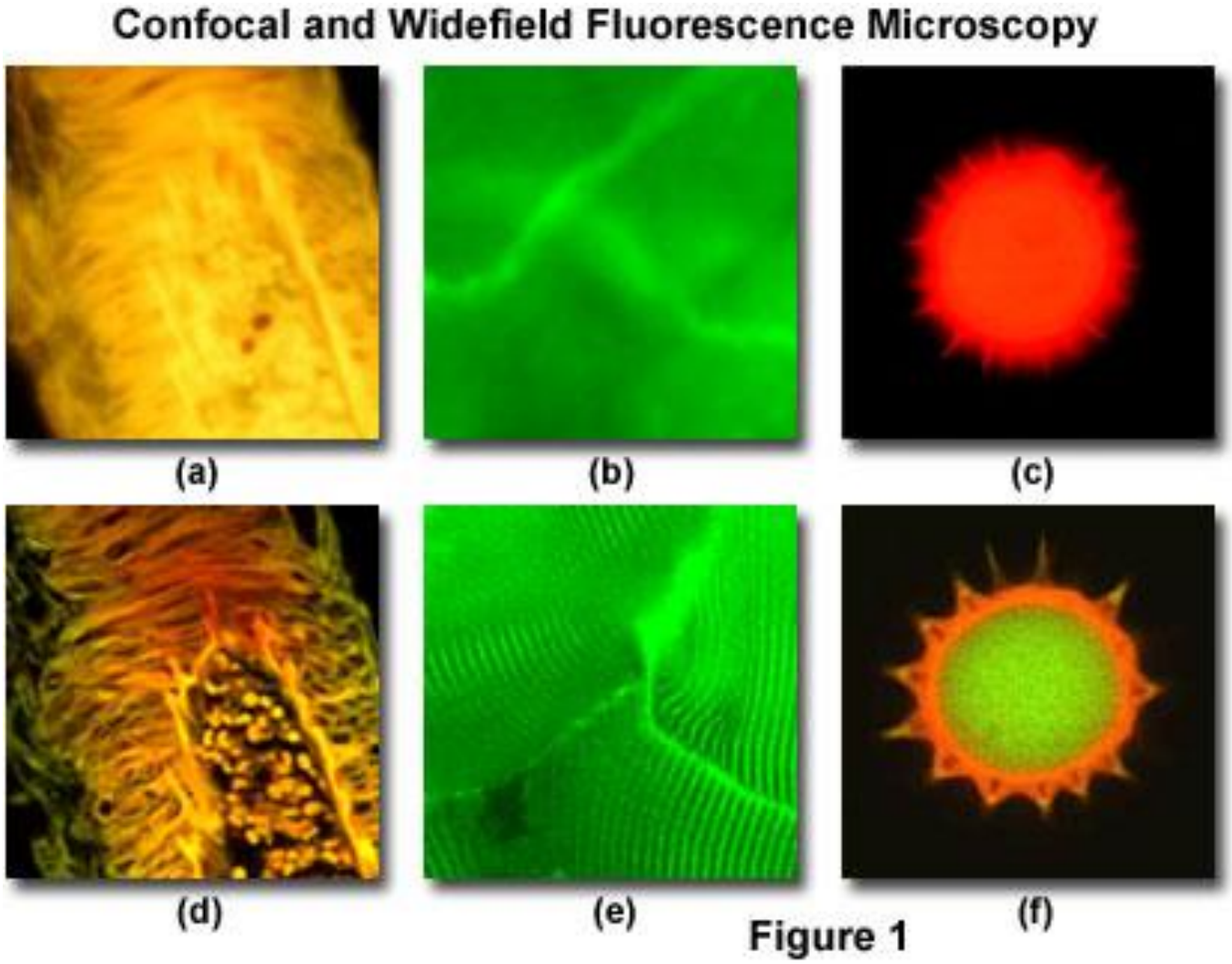
- laser beam – focused illumination
- excitation filter – selected wavelenght
- point-by-point scanning
- motorized XY scanning
- „optical sectioning”
- 3D imaging



Comparison the imaging of fluorescence and confocal microscopes

fluorescence

confocal



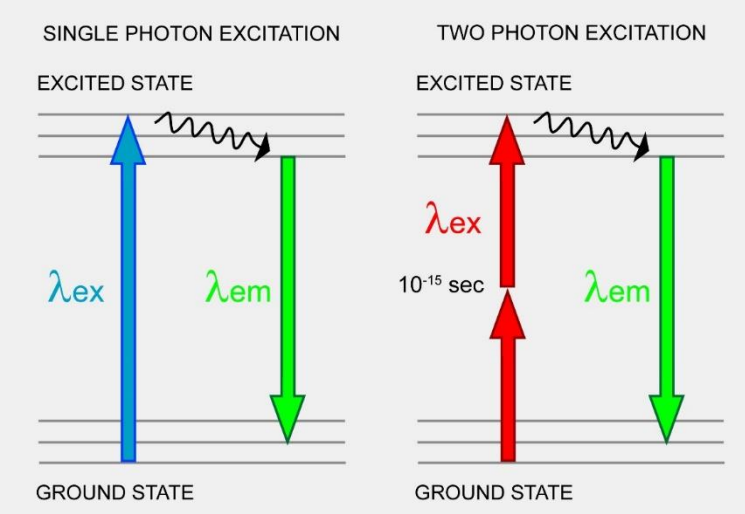
human medulla rabbit muscle pollen

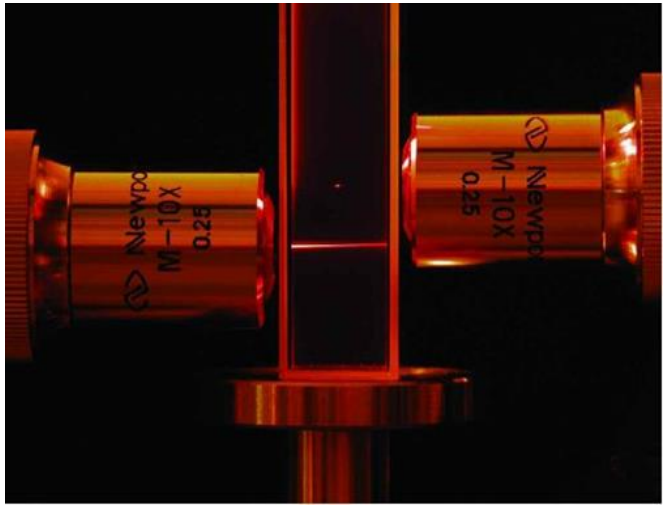
Two-photon microscopy

- 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

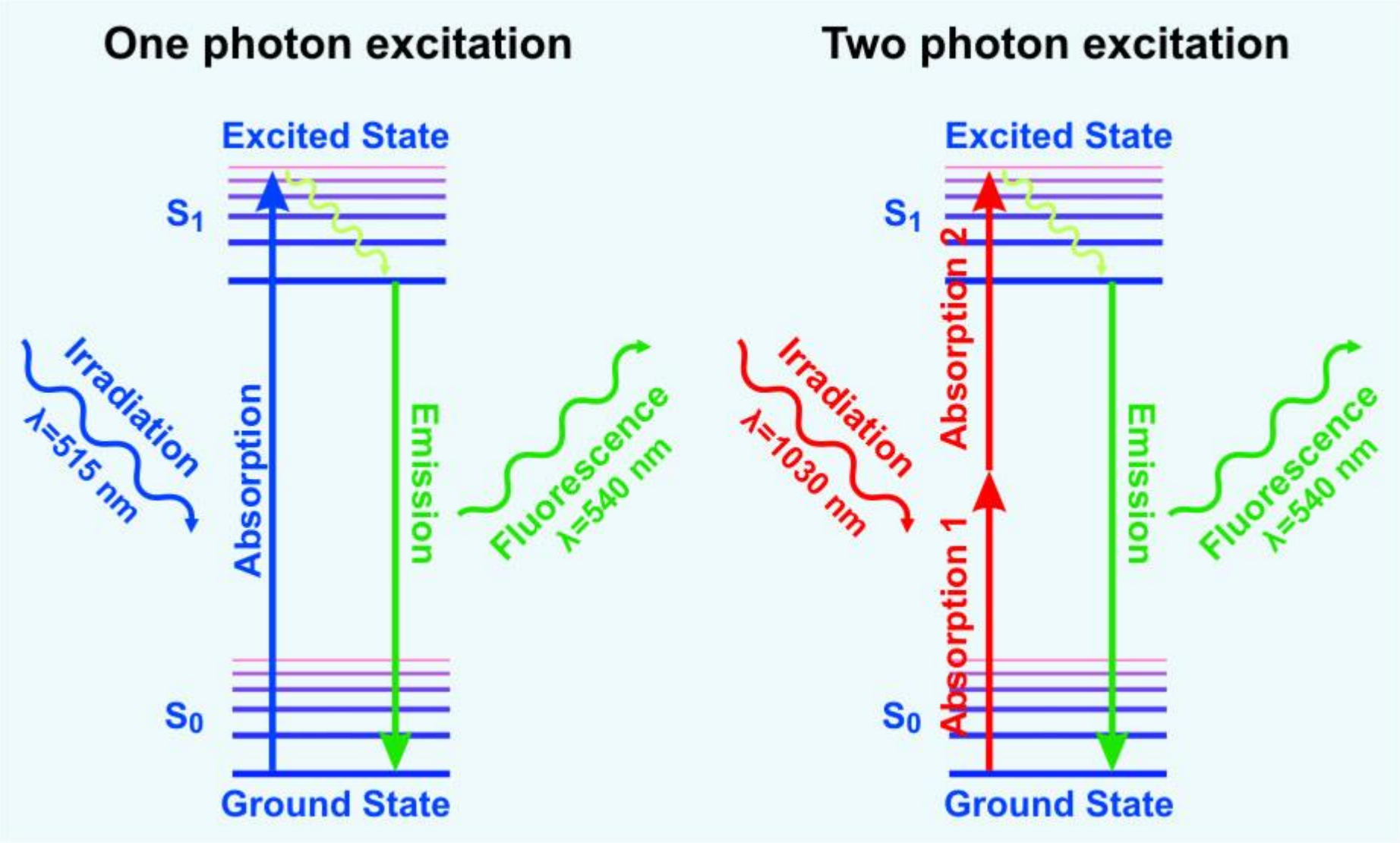
Maria Göppert-Mayer (1906-1972)

Wienfried Denk (1957-)



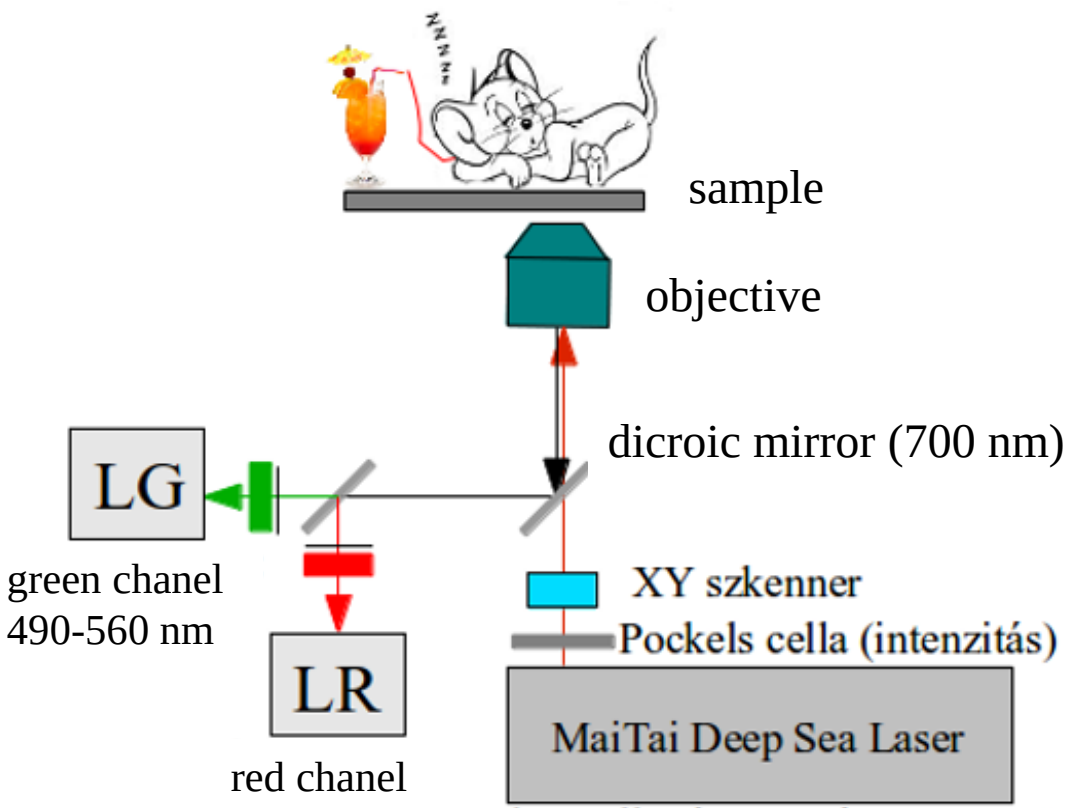


Light absorption and emission spectrum



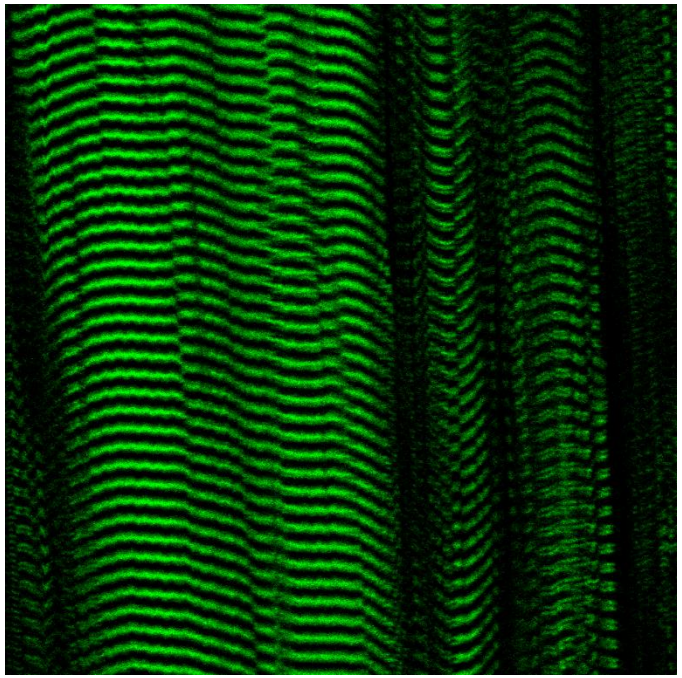
Advantages

- 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
- effective signal detection
- optical sectioning – 3D imaging
- imaging without labeling

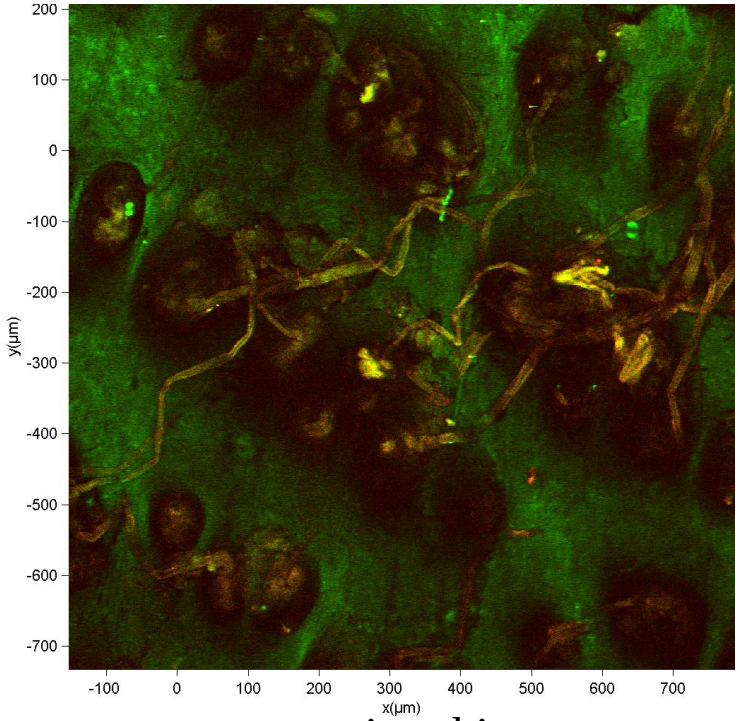


Tunable range: 700-1400 nm
Frequency: 80 MHz
Impulse lenght: 100 fs
Average power: 3 W

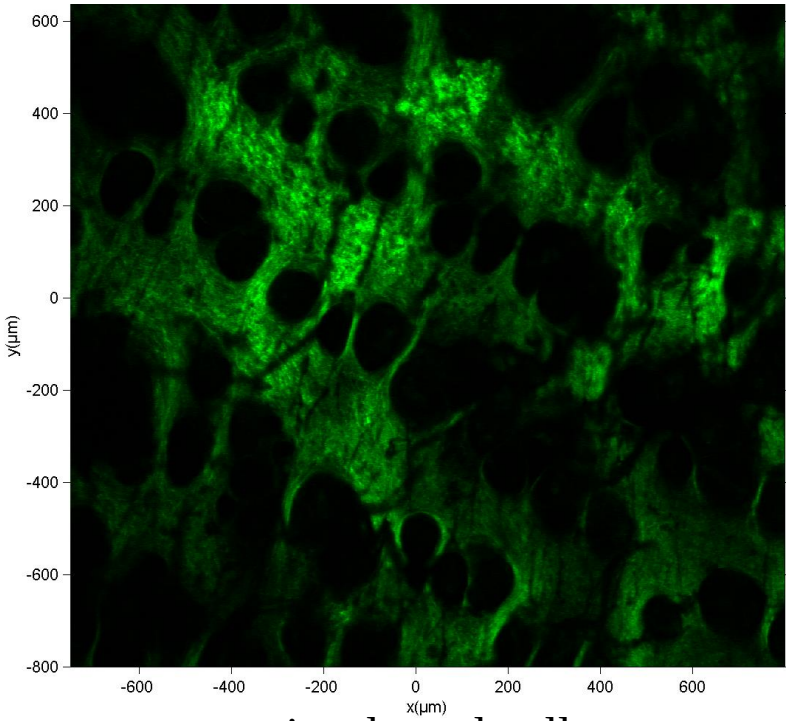
Label-free imaging



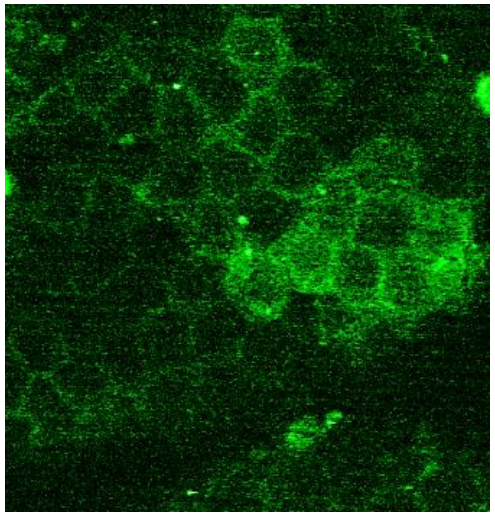
myosin



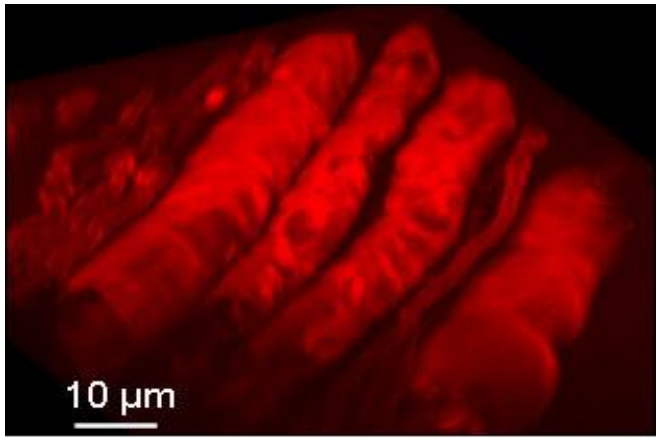
mice skin



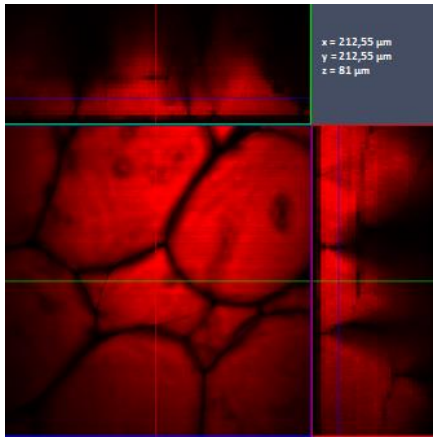
mice dermal collagen



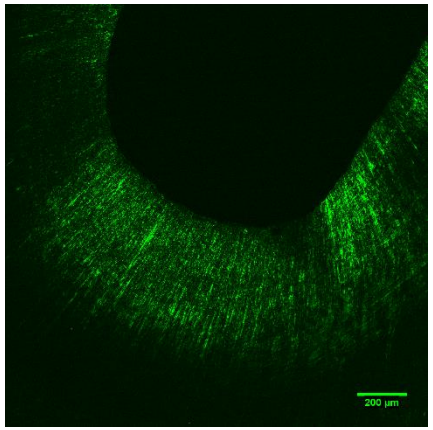
mice skin - keratin



myelin sheats



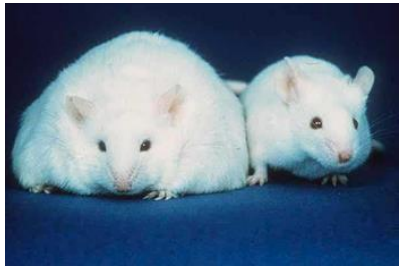
adipocytes

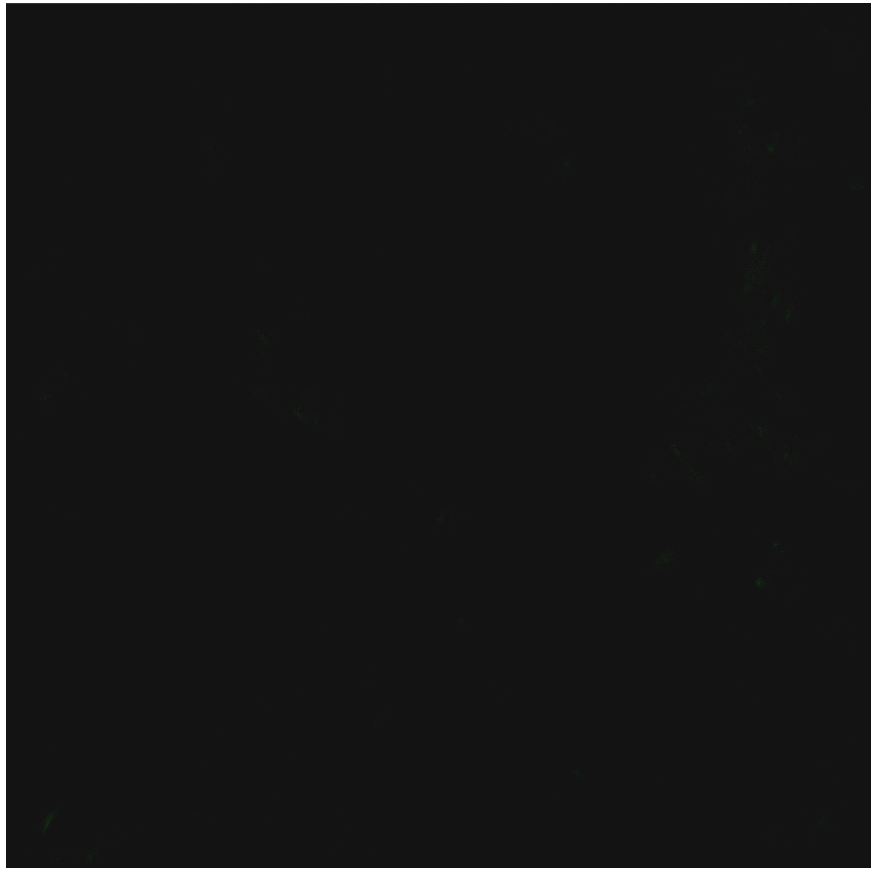


dental tubules

3D imaging

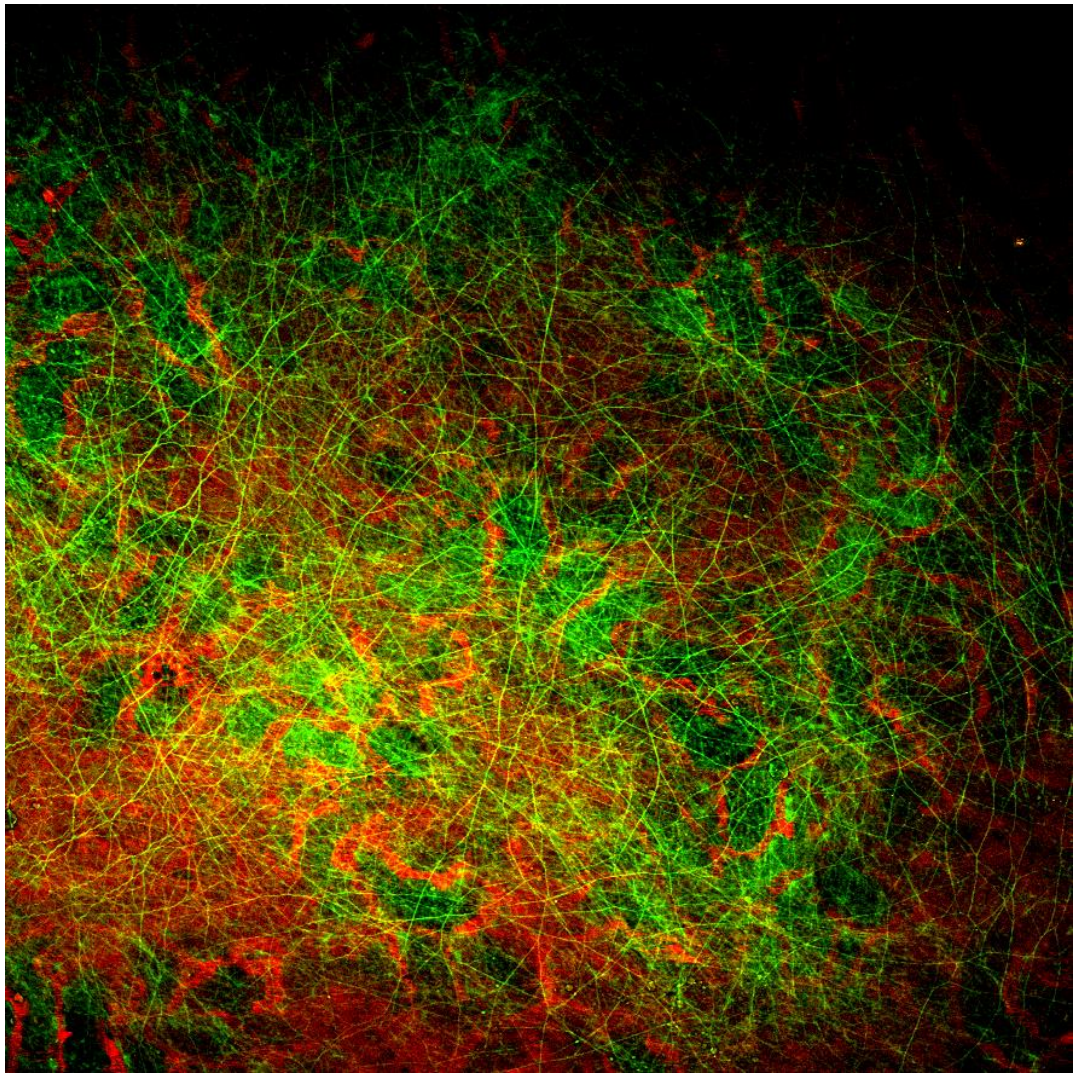
Comparison the dermal collagen structure of a control and type 2 diabetes affected mice



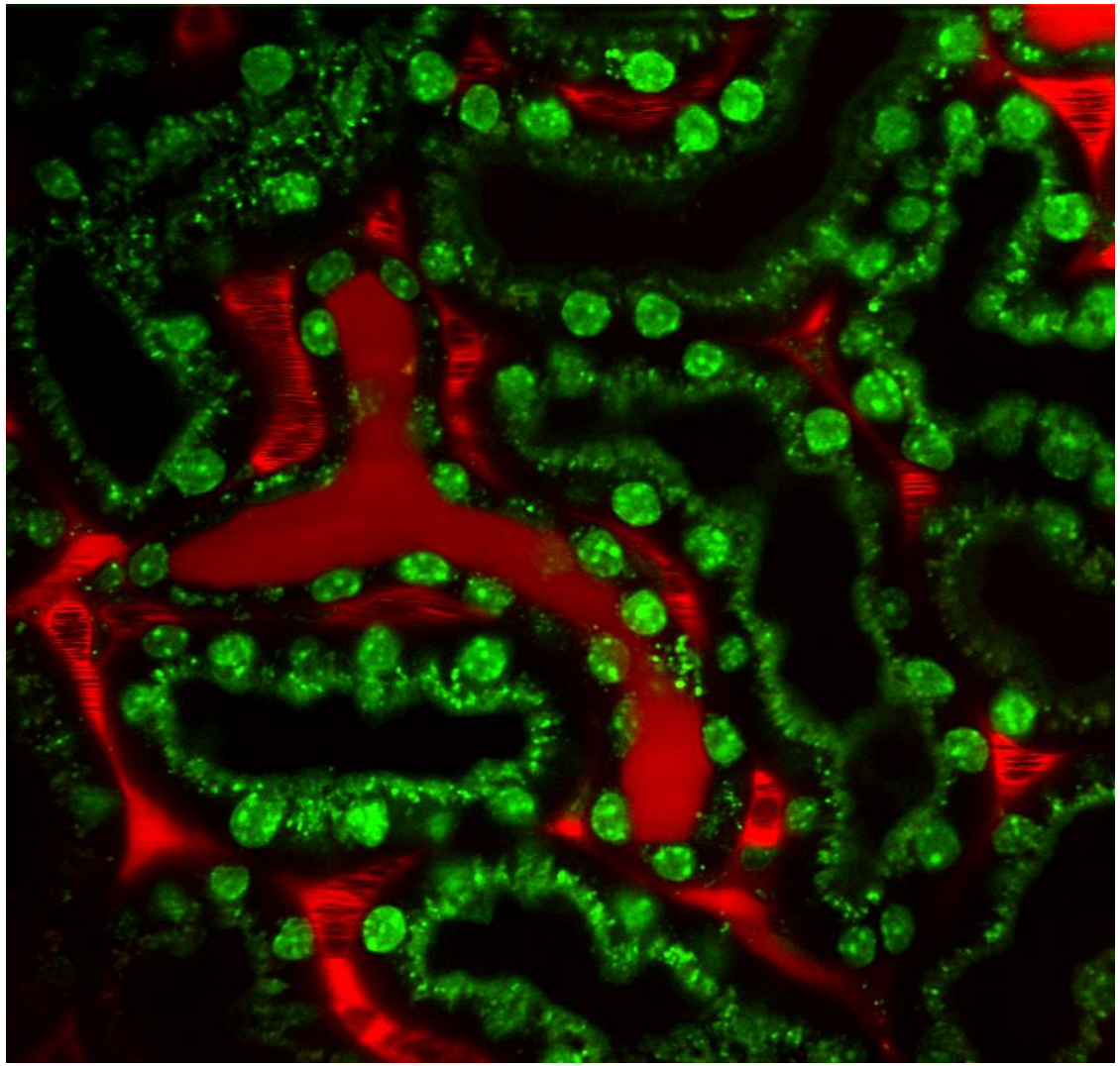


Optical sectioning,
z = 80 μm
200 μm x 200 μm
exc: 990 nm

Multiple fluorescent labeling



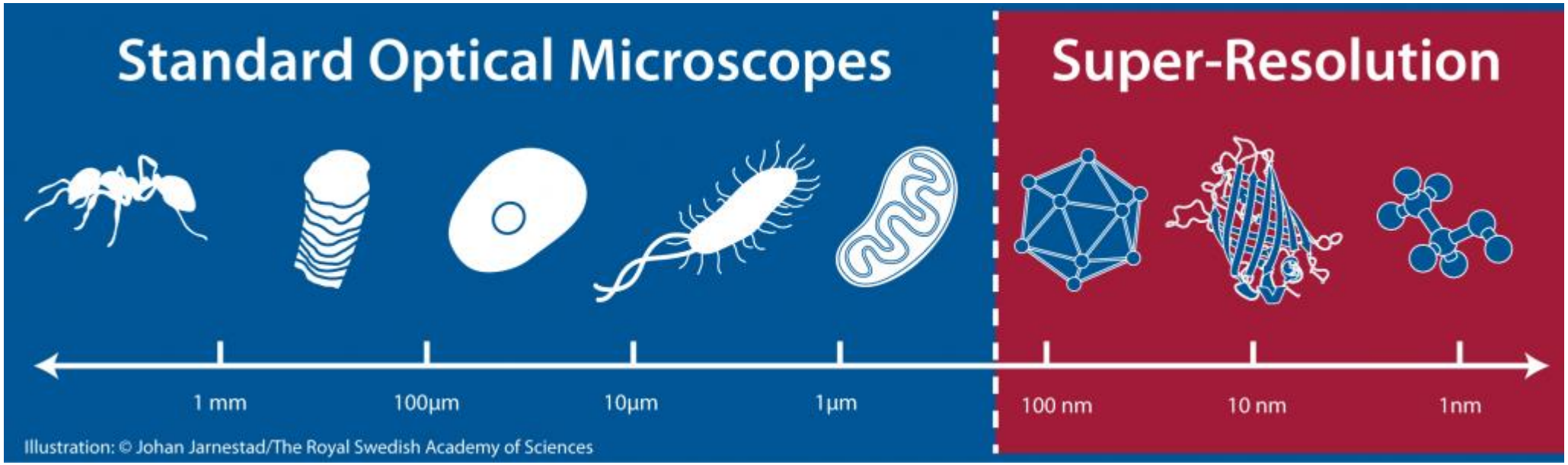
renal cortex



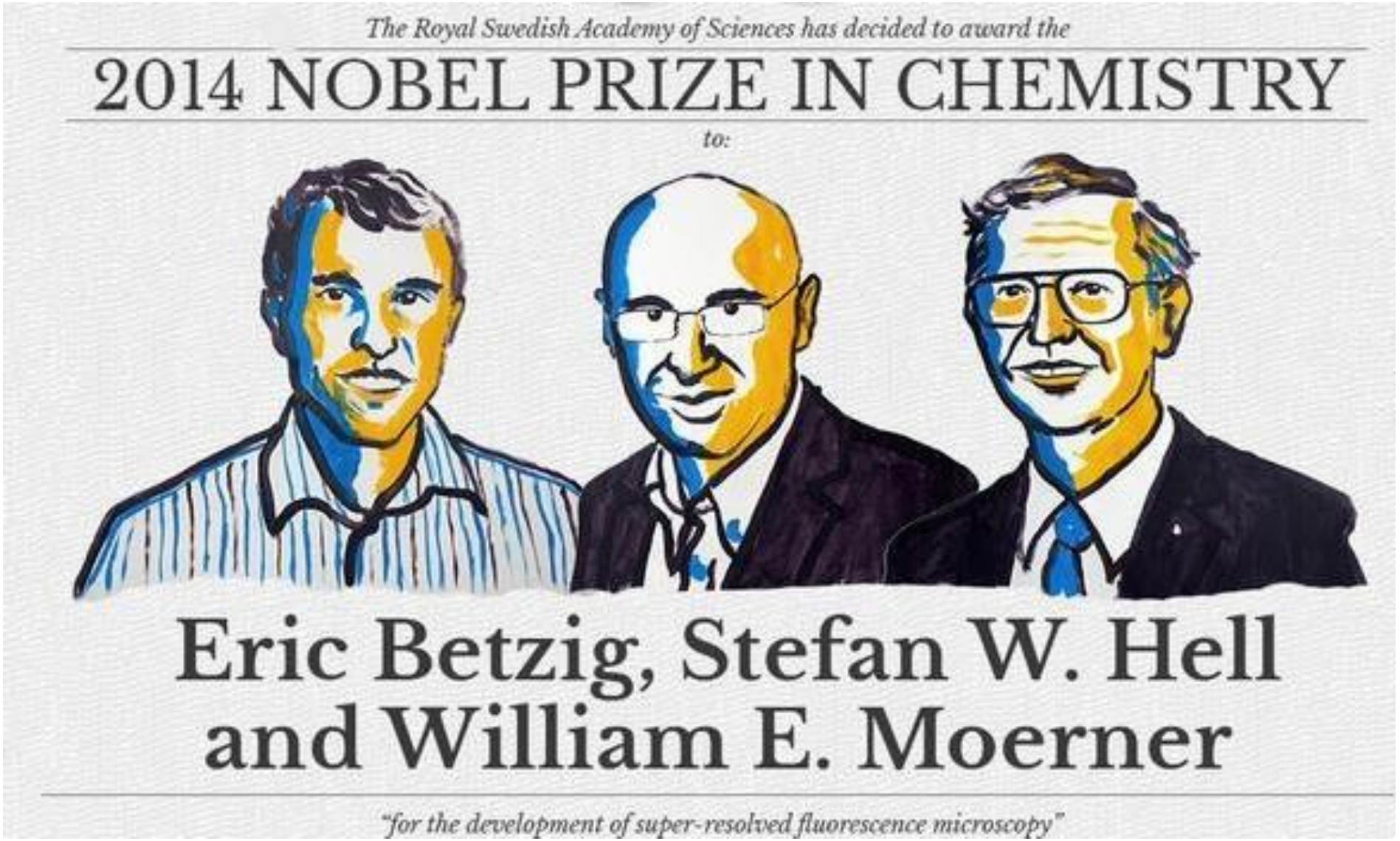
collecting ducts and JGA cells

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

How big are things?

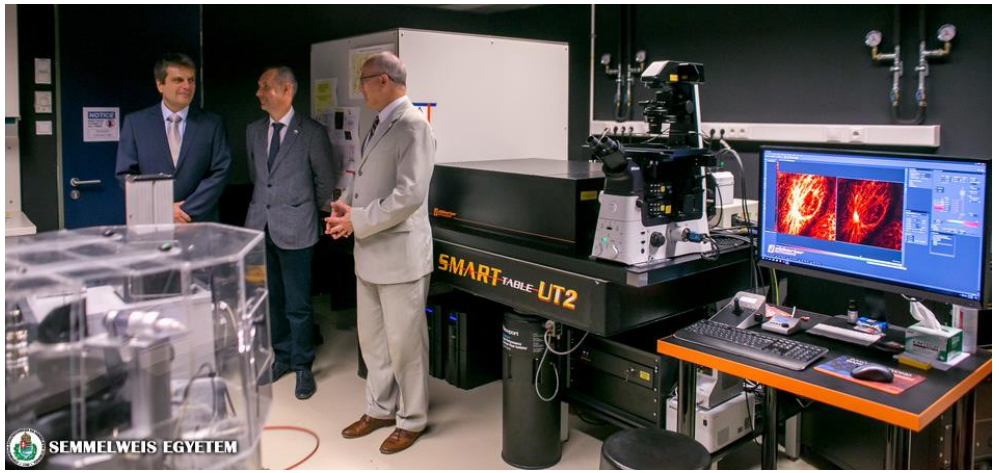


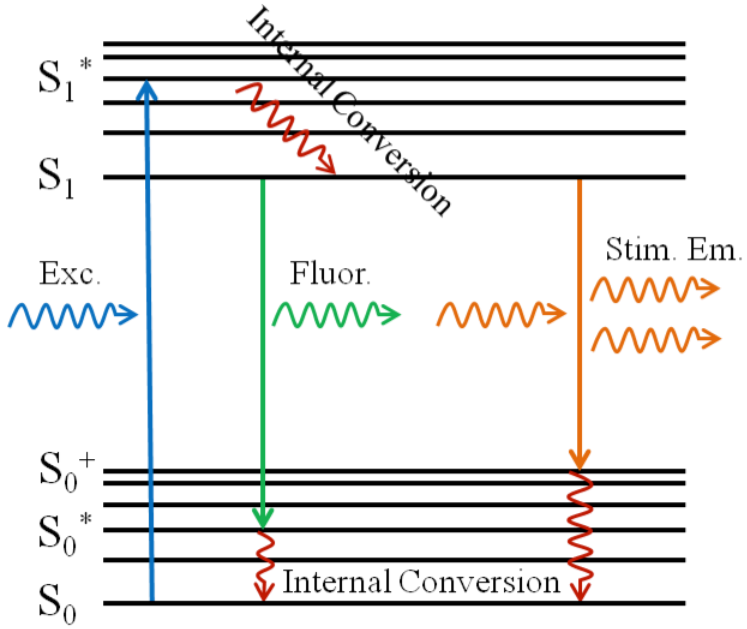
Superresolution microscopy



Superresolution microscope

- 2014. Eric Betzig, Stefan W. Hell és William E. Moerner were awarded 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
- STED selectively deactivates the fluorescence
- The excited electron is forced to relax into a higher vibration state than the fluorescence transition would enter

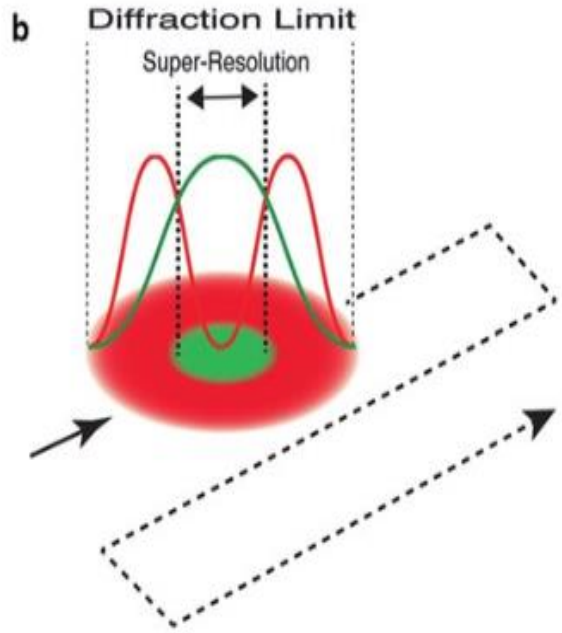


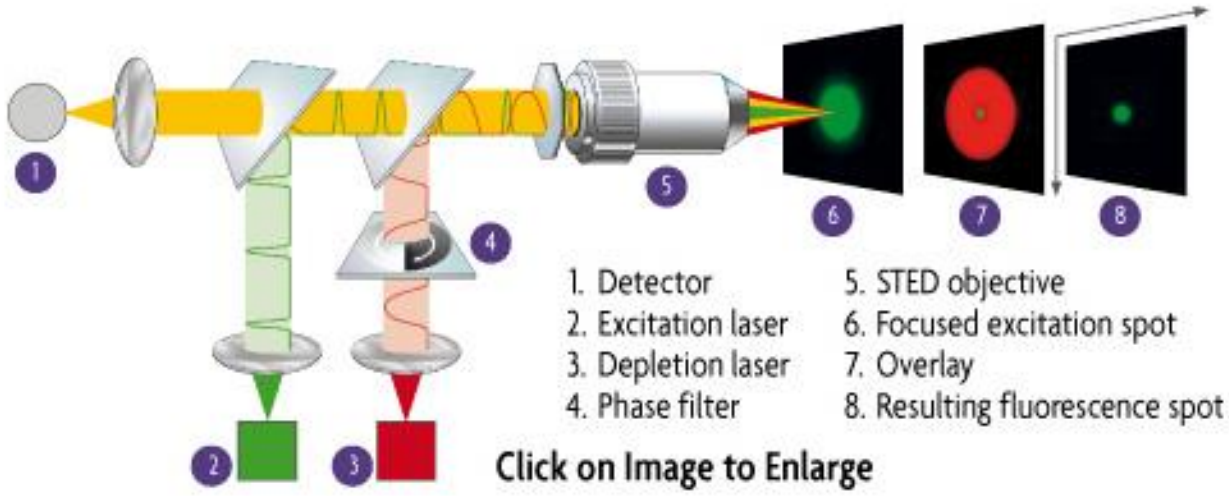


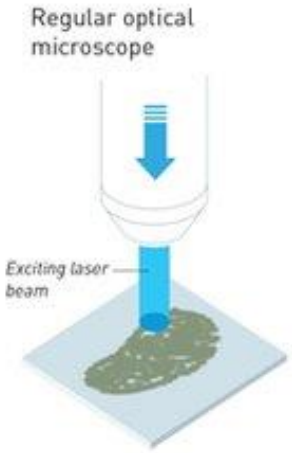
- excitation laser: produces an ordinary diffraction limited focus

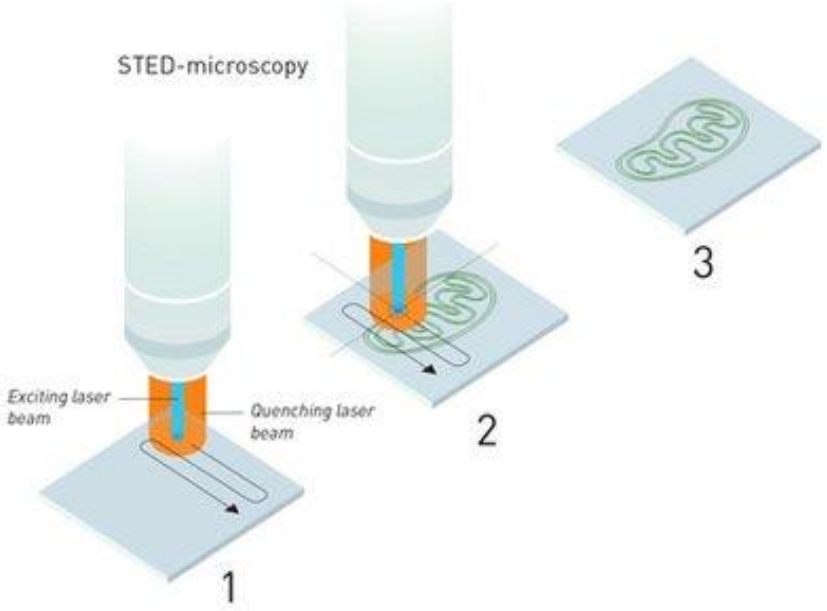
- depletion laser: doughnut shape, fluorescence from the molecules is quenched via stimulated emission

- point-by-point scanning





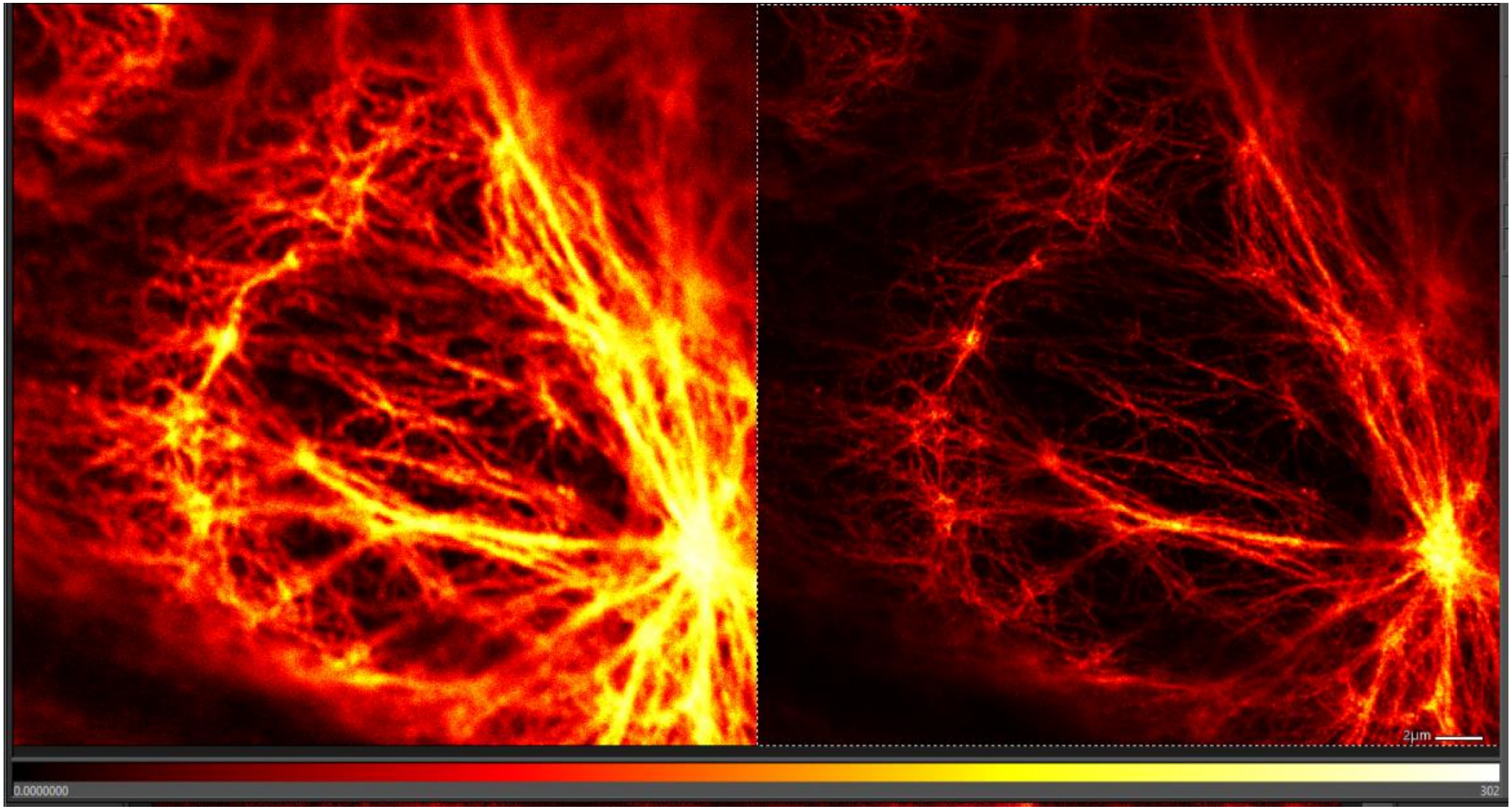




Vimentin: flexible intermediate filaments in the cytosceleton

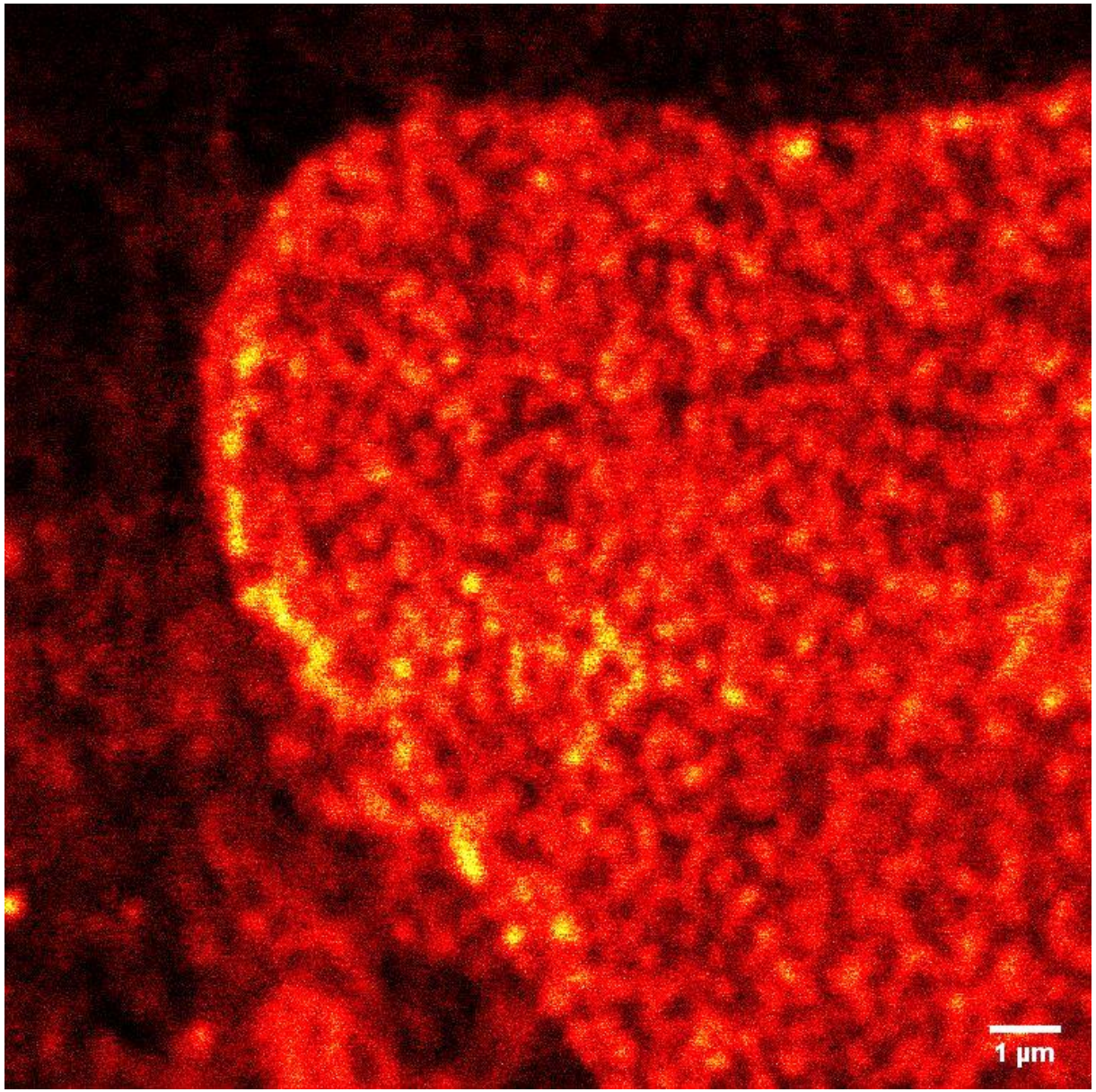
confocal

STED

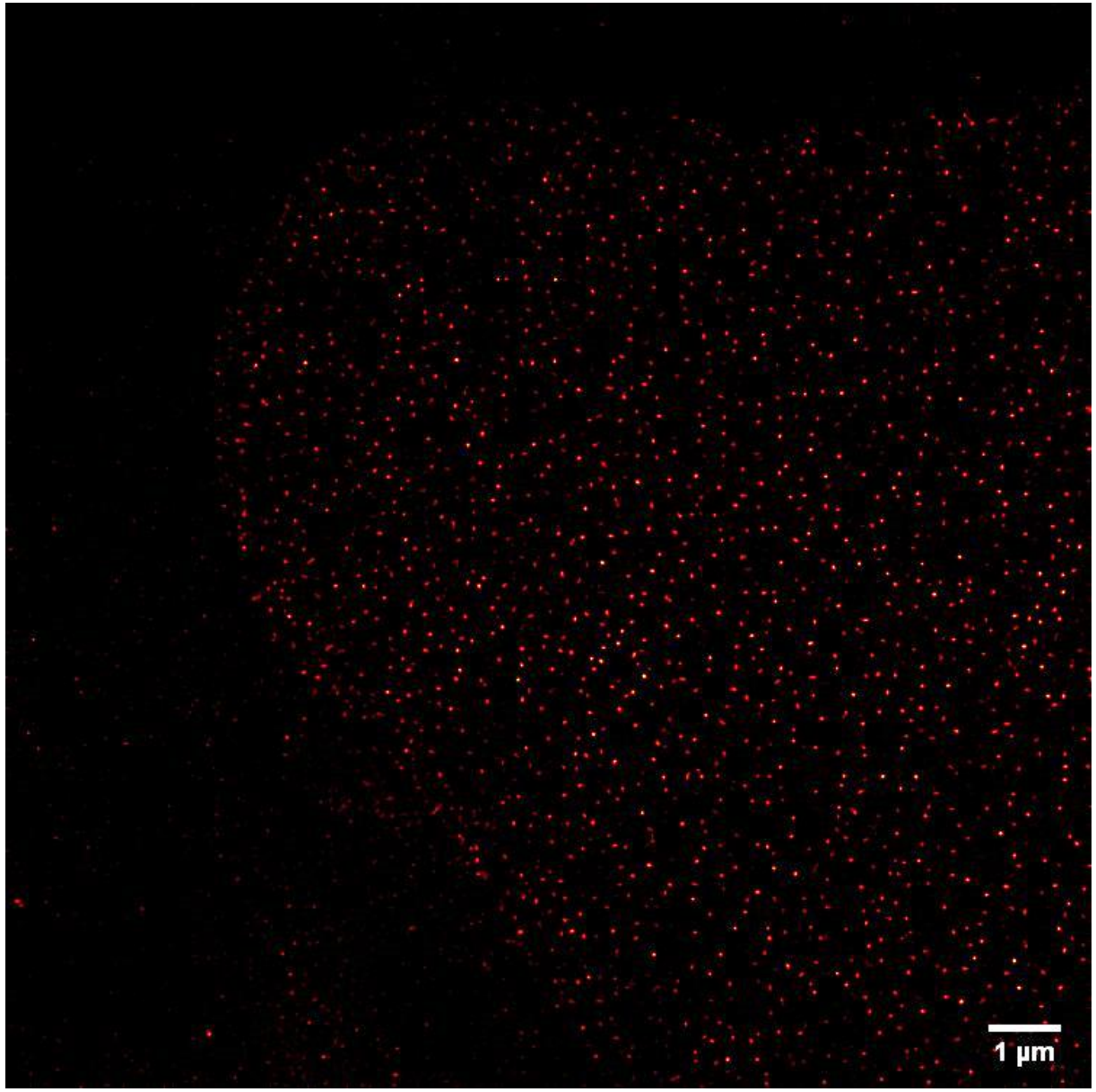


Nuclear pores of HeLa cells

confocal



STED



Checklist

- ✓ resolution limit of image formation
- ✓ Abbe’s principle
- ✓ working principle of fluorescence microscope: illumination, excitation/emission spectra, Stokes-shift, function of dicroic 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