# Fluorescence microscopy – general overview

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IMG
Light Microscopy Core Facility

- Transmitted light microscopy
- Fluorescence light microscopy

- Resolution
- PSF
- Widefield microscope
- Confocal microscope
- Super-resolution microscopy

# Transmitted light microscopy

- light is passed from the illumination source on the opposite side of the specimen to the objective (illumination is **transmitted** through the specimen)
- **contrast-enhancing techniques** - sample preparation as well as optical tricks that generate intensity changes which are useful for observation and imaging

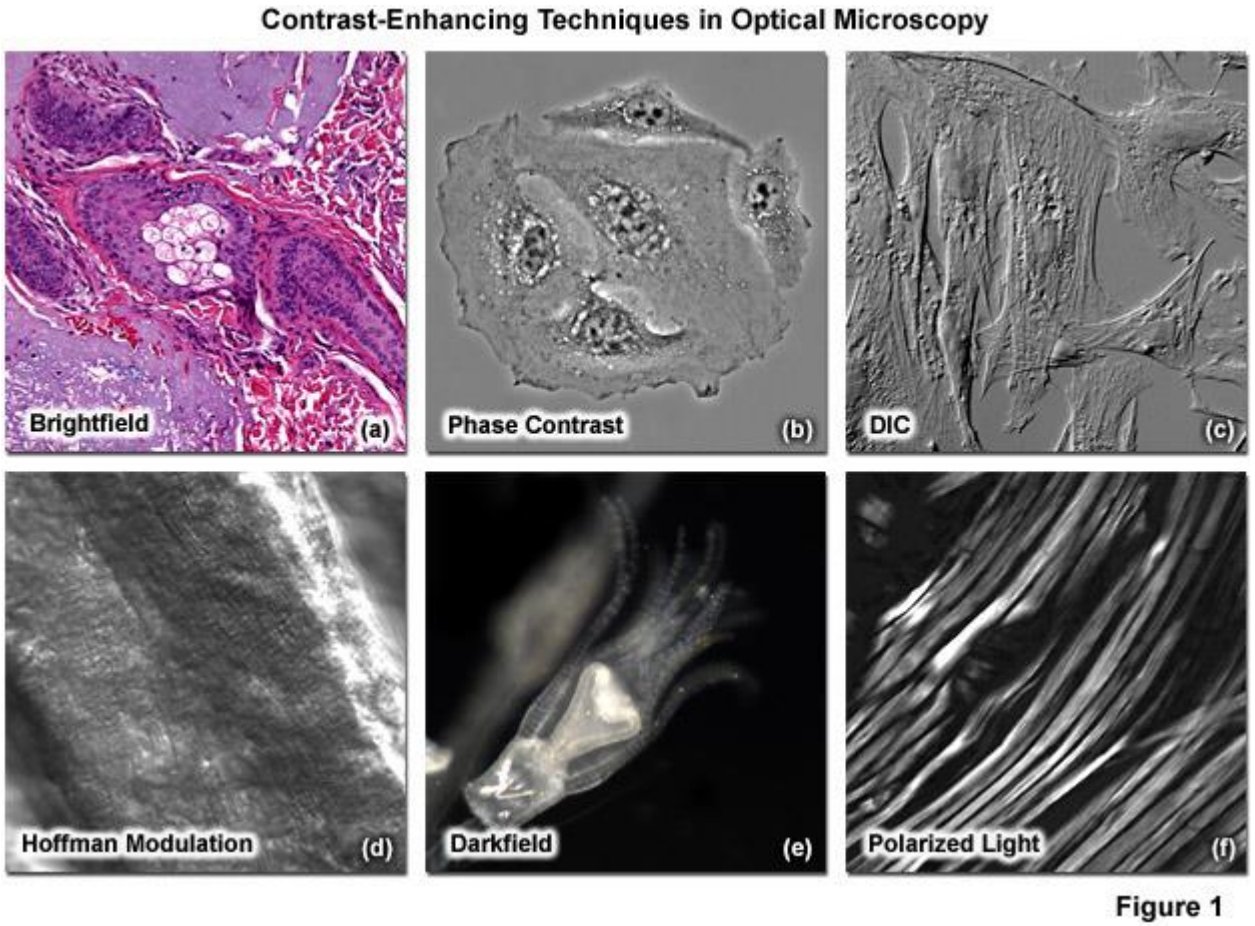
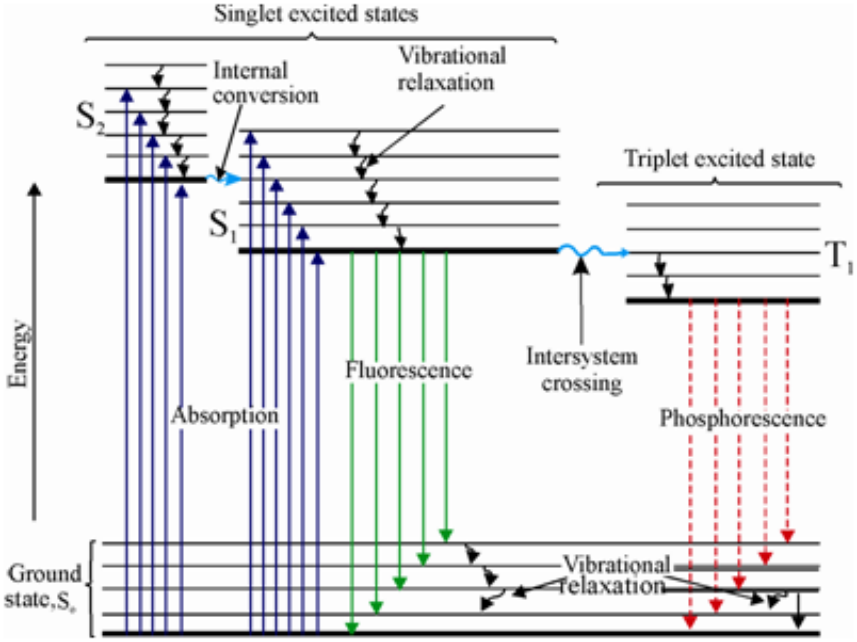



Figure 1

http://zeiss-campus.magnet.fsu.edu/articles/basics/contrast.html

# Fluorescence light microscopy

- **Luminiscence** - emission of light by a substance not resulting from heat, as a result of chemical reaction, electric current, absorption of photons, etc.
  - **Fluorescence** – a result of singlet-singlet electronic relaxation




Jablonski diagram

https://www.quora.com/What-is-the-Jablonski-diagram

# Fluorescence light microscopy

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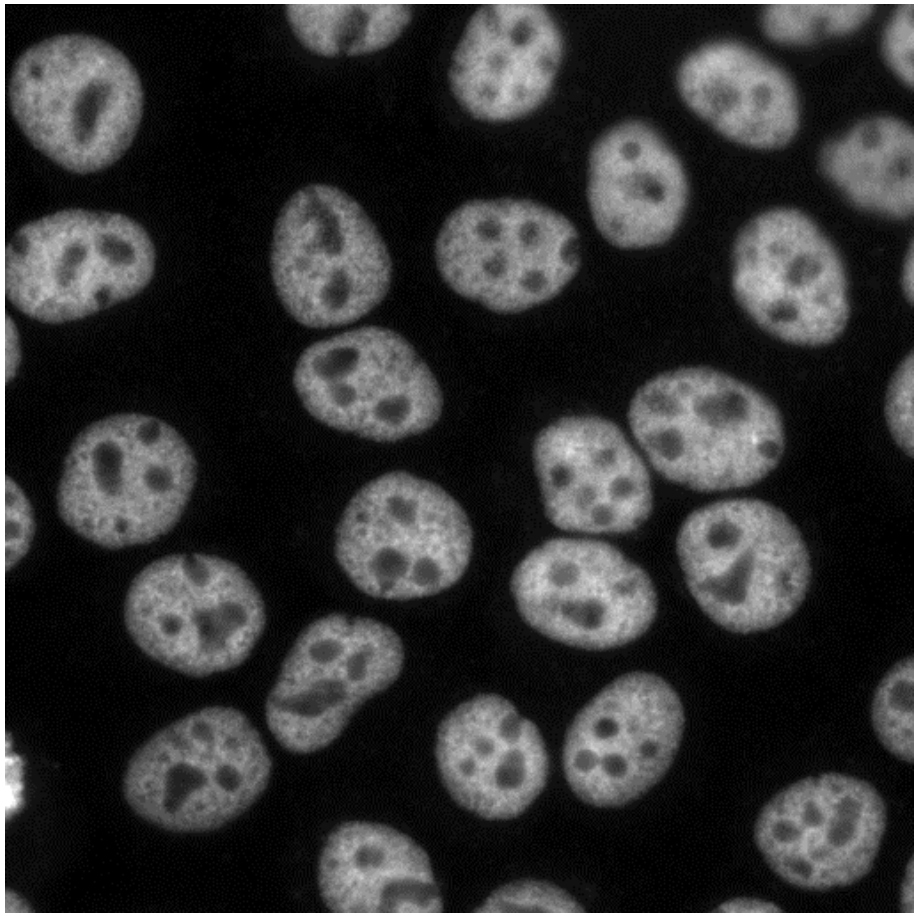
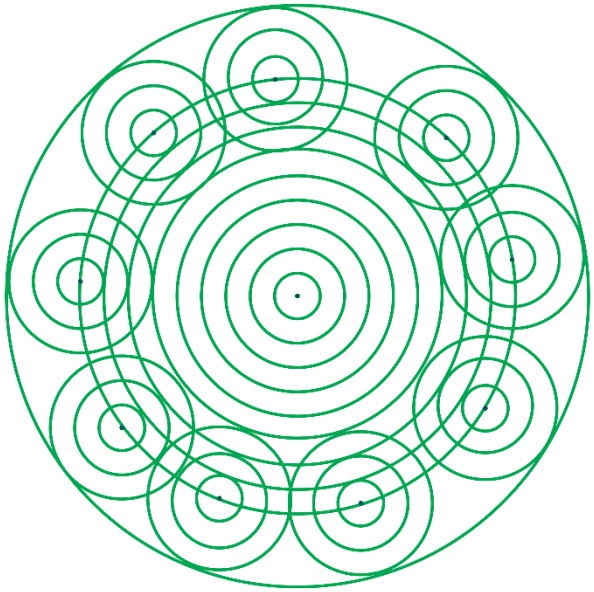
  - **Fluorescence** – a result of singlet-singlet electronic relaxation



# Image formation



Huygens principle

- every point on a wavefront is itself the source of spherical wavelets -> secondary sources of radiation

- wavefront of a propagating wave of light at any instant conforms to the envelope of spherical wavelets emanating from every point on the wavefront at the prior instant

# Image formation

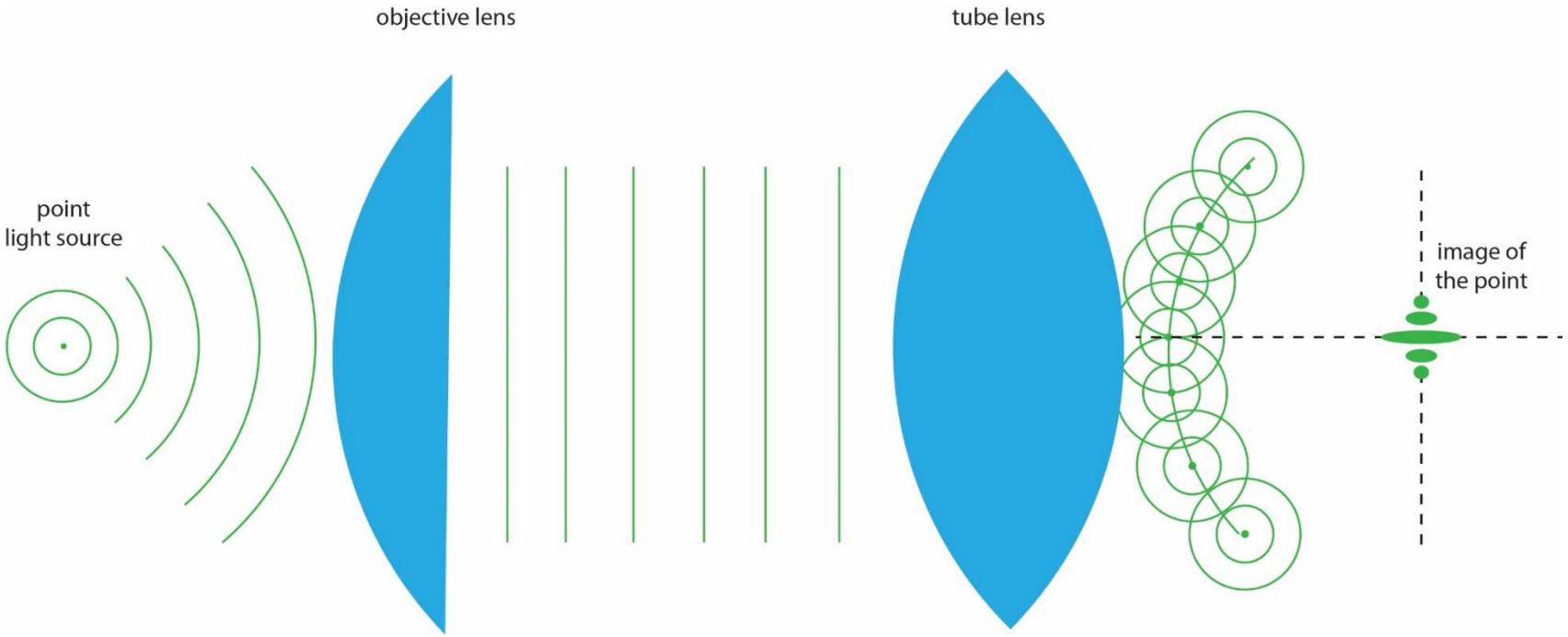
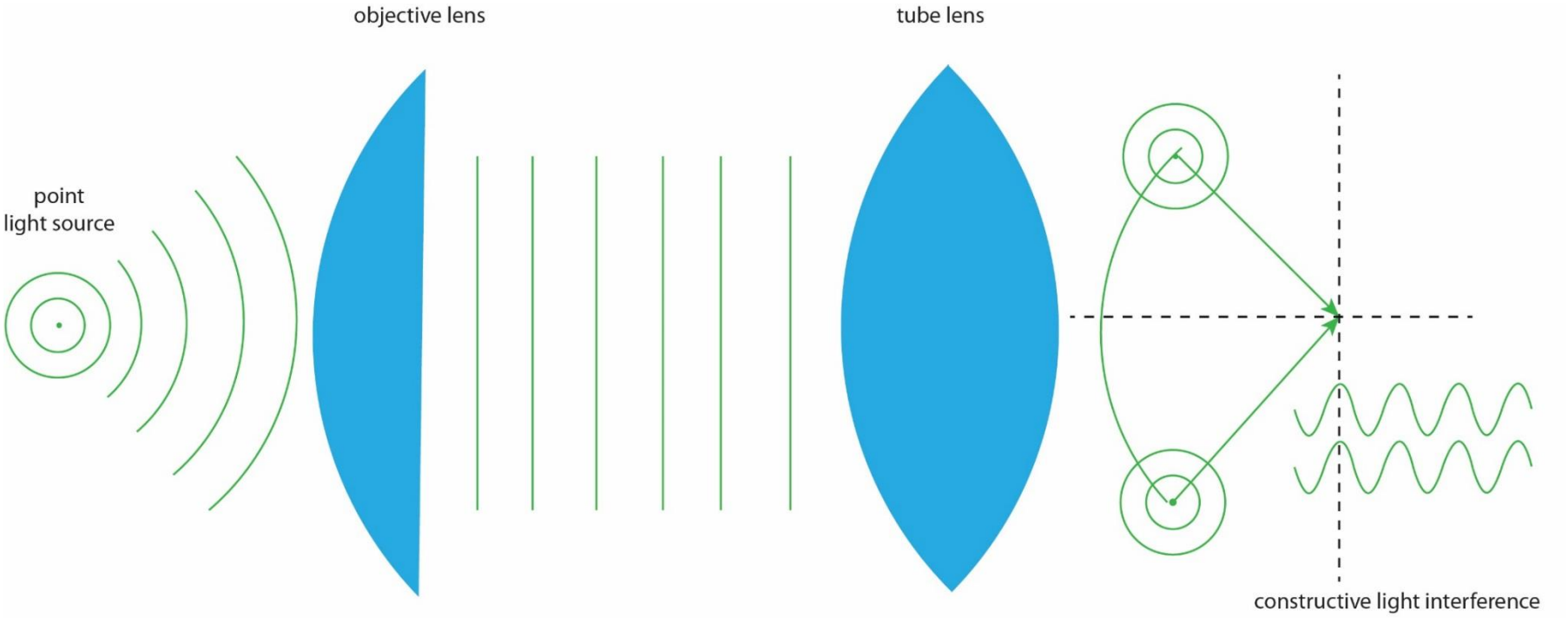



Image of a point light source

- focusing lens introduces the appropriate delays in the light paths (more delay in the center, and less in the borders)
- secondary sources

Image courtesy of Ivan Novotný

# Image formation




## In-phase contributions

- focus is at equal distance from all the secondary sources, all wave fronts from the secondary sources arrive in phase

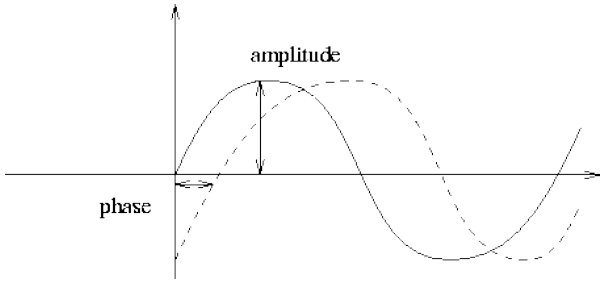
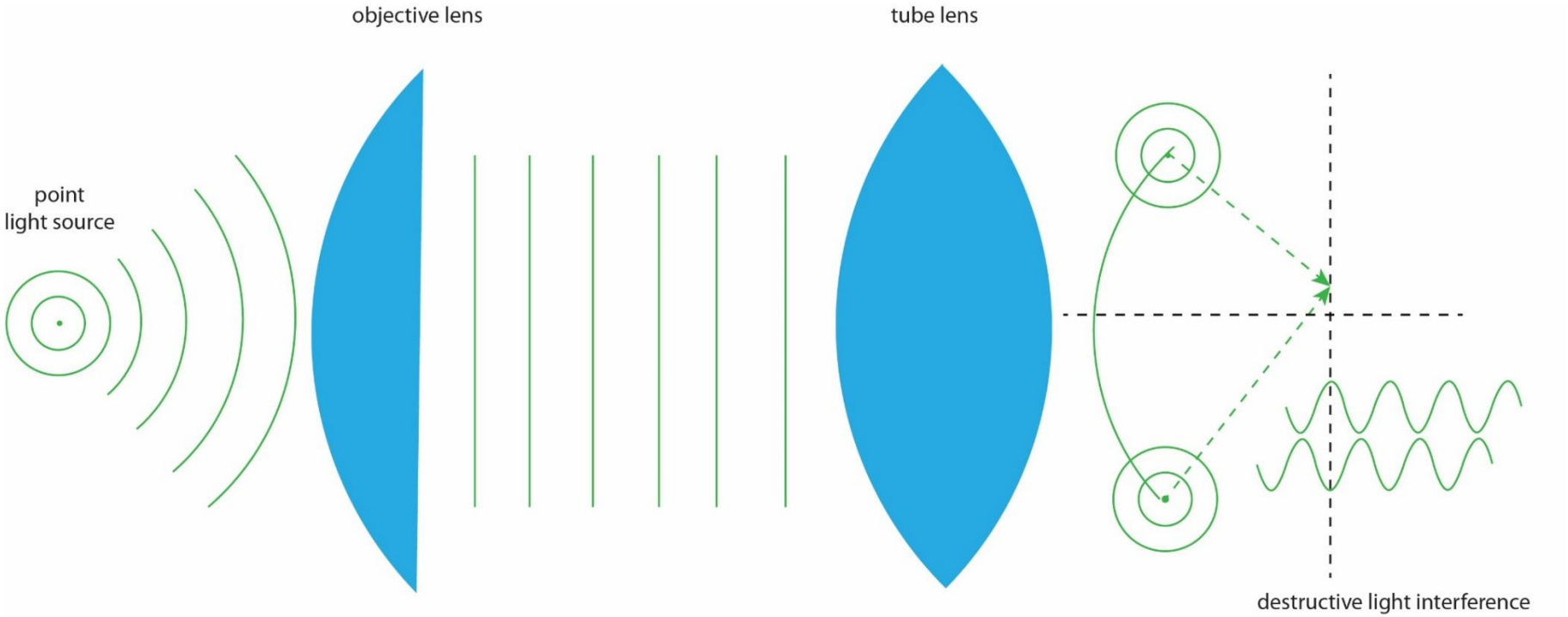



Image courtesy of Ivan Novotný

# Image formation




Out-of-phase contributions

- resulting intensity decreases

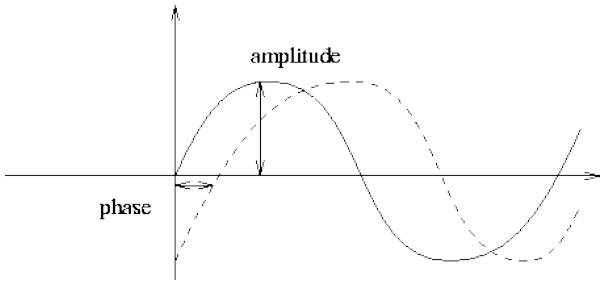
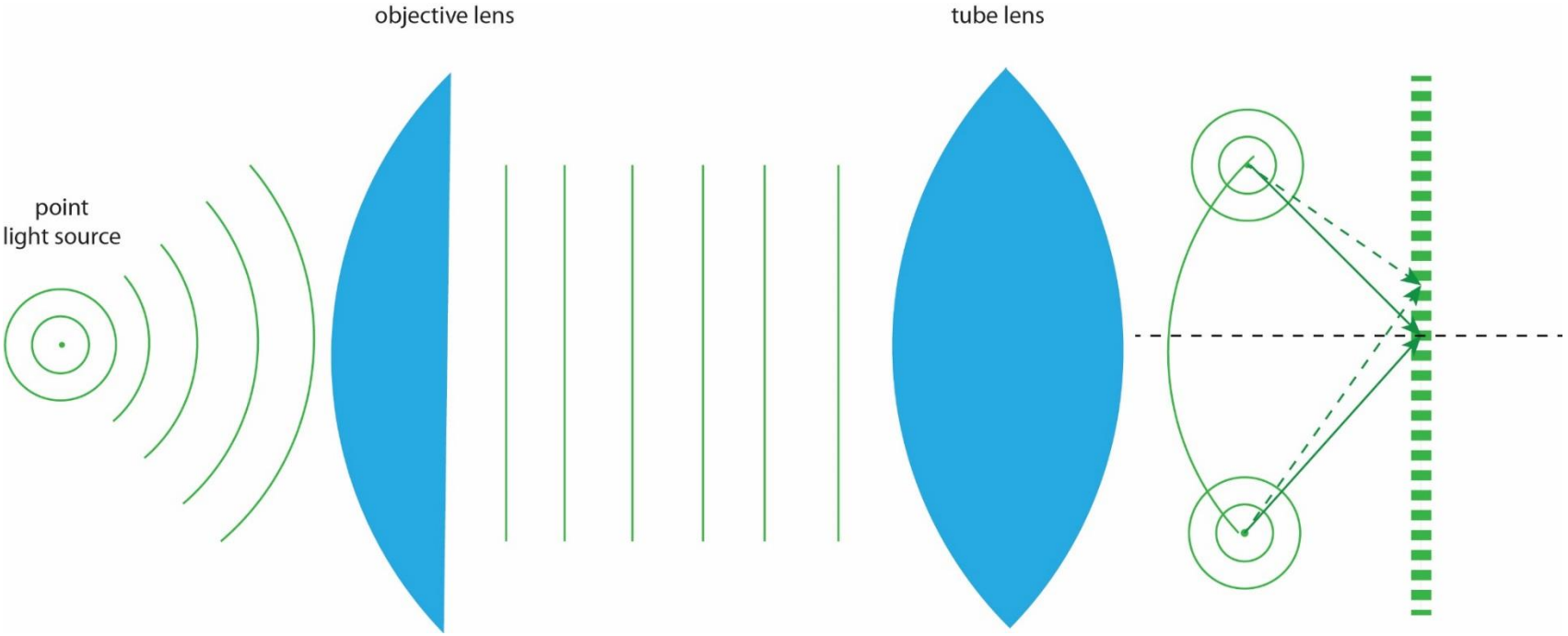


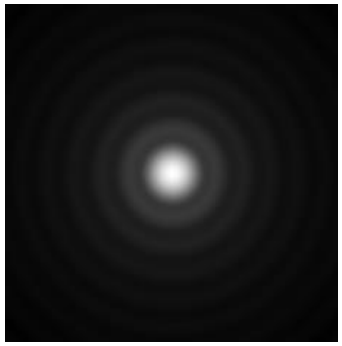

Image courtesy of Ivan Novotný

# Image formation




- constructive interference -> local maxima
- destructive interference -> local minima

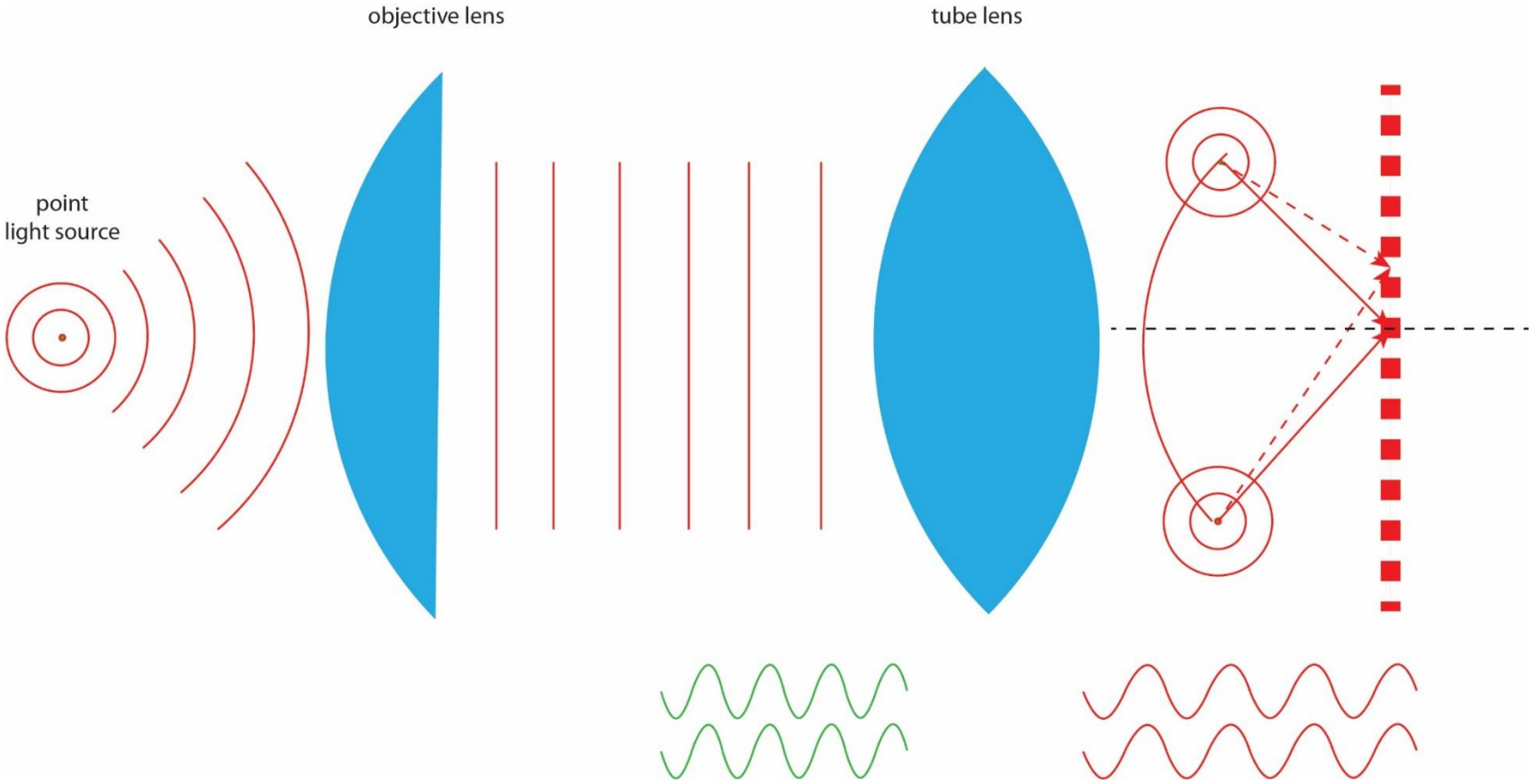
- interference behavior described above is obtained similarly also along the optical axis (z)



- central Airy disk

Image courtesy of Ivan Novotný

# Image formation




- intensity changes depend on the wavelength

Image courtesy of Ivan Novotný

# Image formation

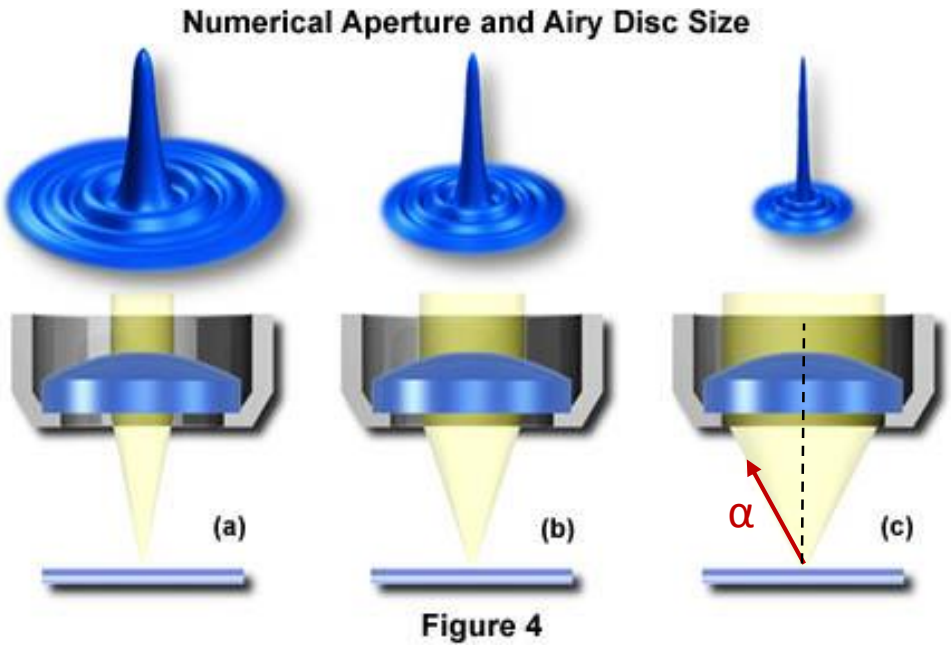


Figure 4

The amount of light that a lens can collect is measured by its ***numerical aperture***

*maximal half-angle of the cone of light*

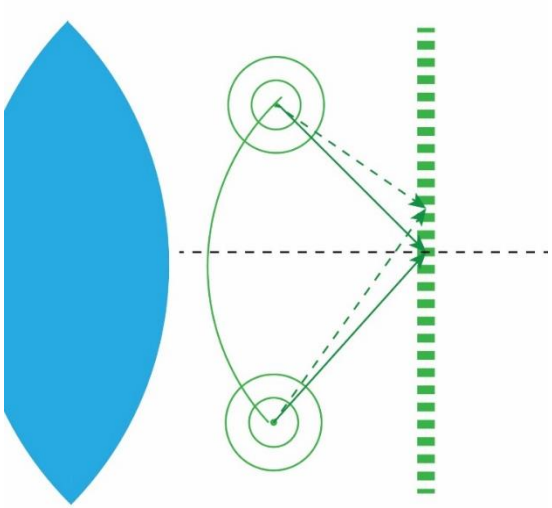
$$NA = n \sin\alpha$$

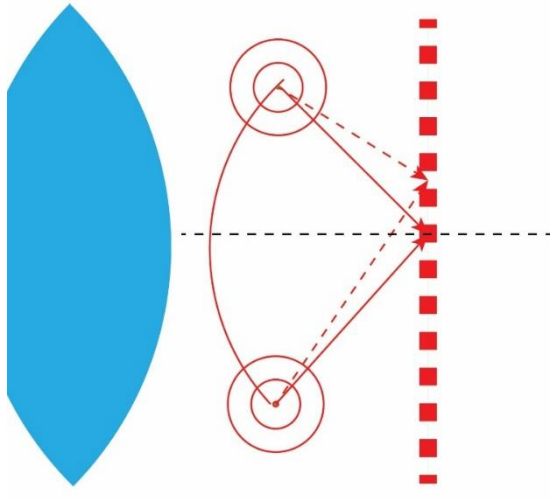
*refractive index*

https://micro.magnet.fsu.edu/primer/anatomy/numaperture.html

# Resolution

$$r \sim \frac{\lambda}{NA}$$





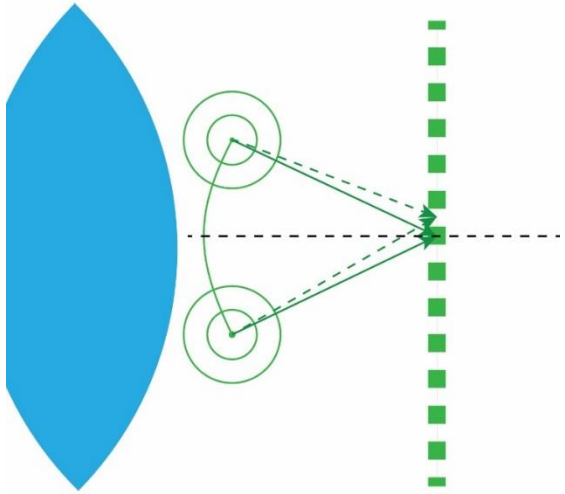


Image courtesy of Ivan Novotný

# Resolution

- *minimum resolvable distance* - the minimum distance between two distinguishable objects in an image

Abbe criterion

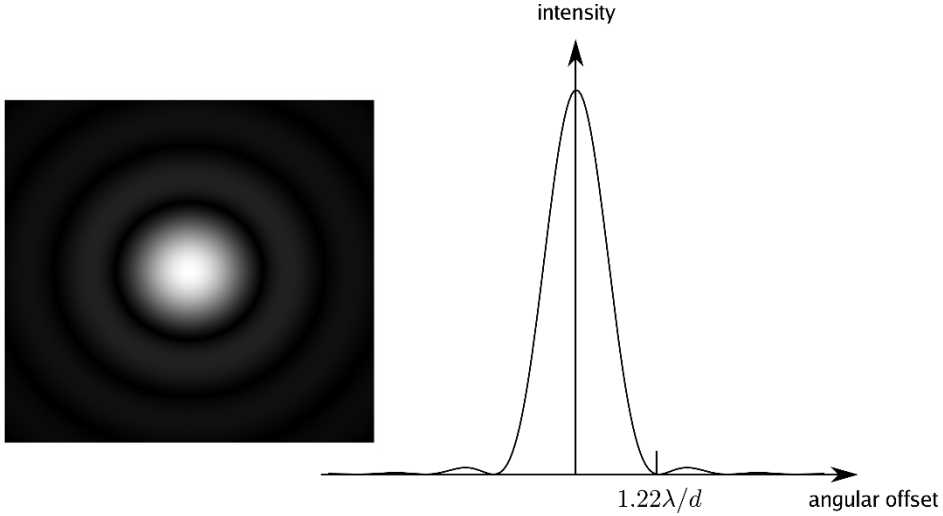
(radius of Airy disk)

$$r = \frac{0.5\lambda}{NA} = \frac{0.5\lambda}{n\,sin\alpha}$$

Rayleigh criterion

(principal diffraction maximum from one of the point sources overlaps with the first minimum from the other point source)

$$r = \frac{0.61\lambda}{NA} = \frac{0.61\lambda}{n\,sin\alpha}$$



# Resolution

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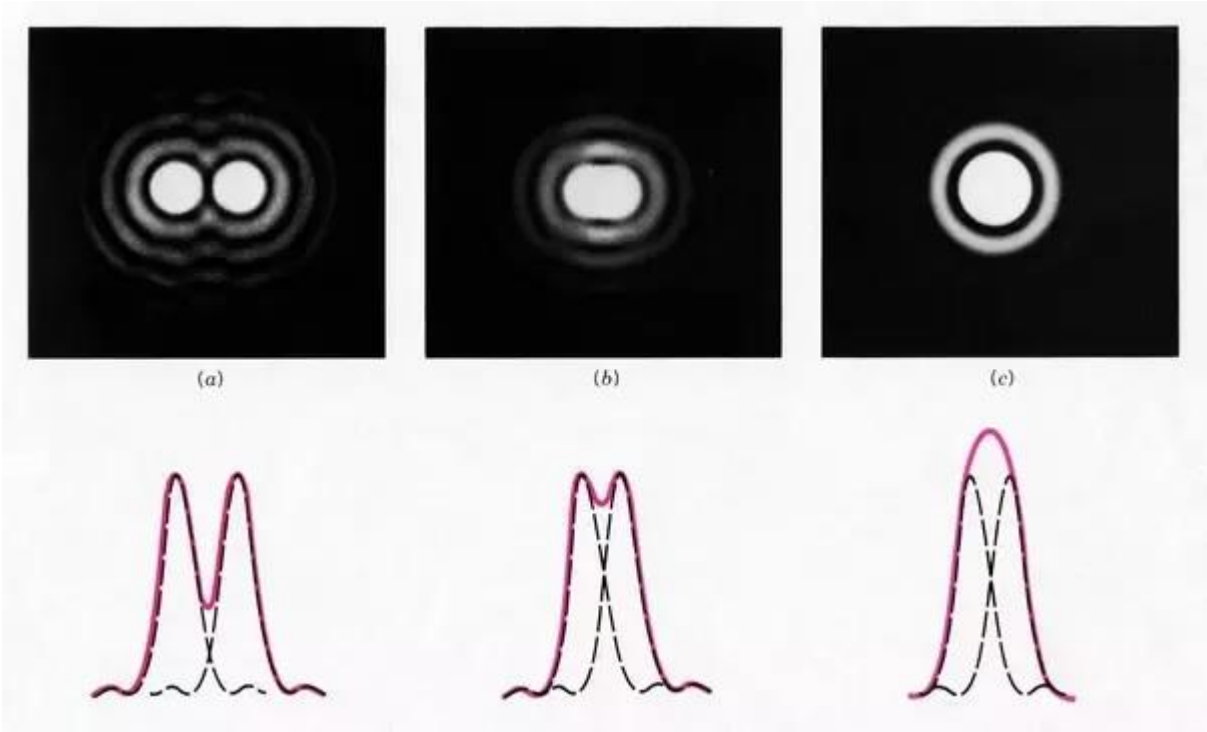



*resolved* *Rayleigh limit* *not resolved*

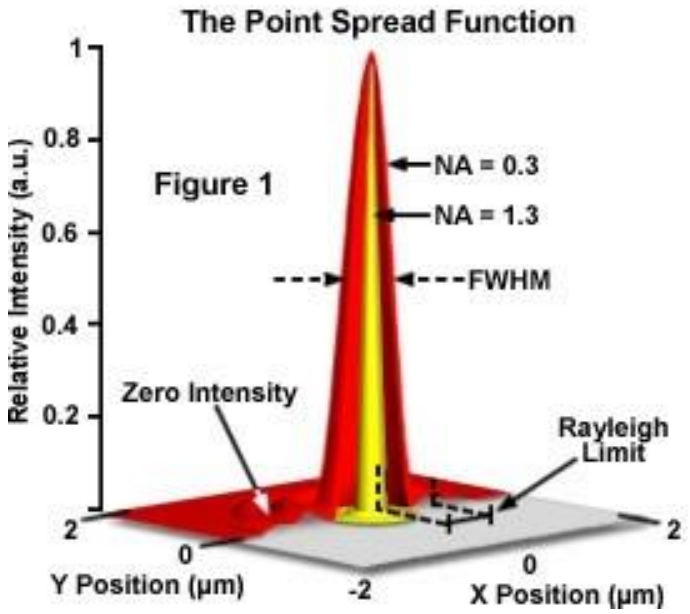
*e.g. for GFP (emission 510 nm), NA=1.4 r=222 nm*

*-> limited by diffraction*

https://www.quora.com/What-does-it-mean-by-resolving-power-of-a-microscope-and-telescope

# PSF

**Point spread function** (**PSF**) describes the response of an imaging system to a point source or point object

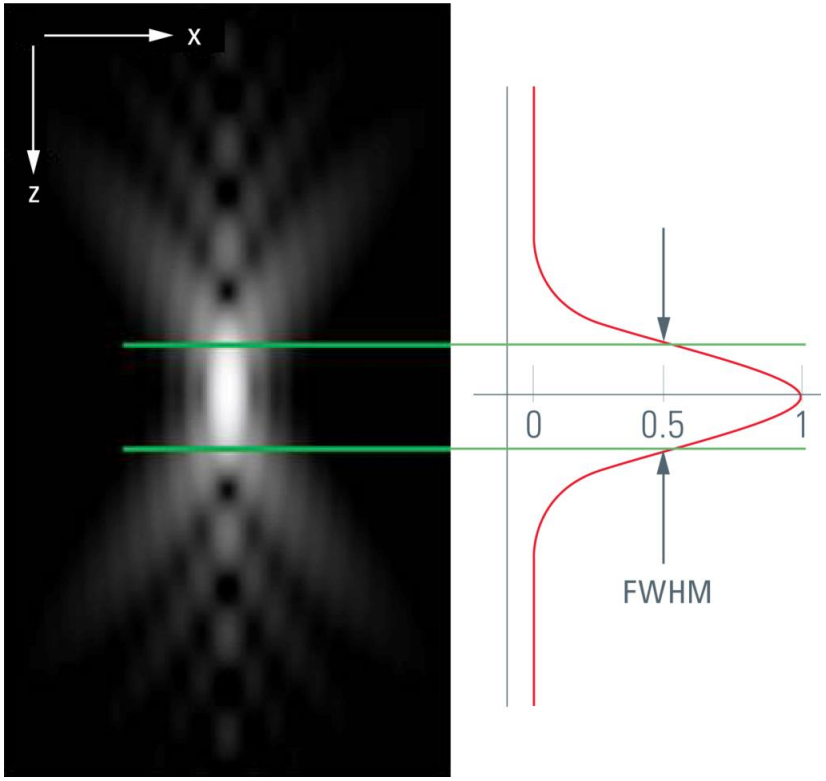


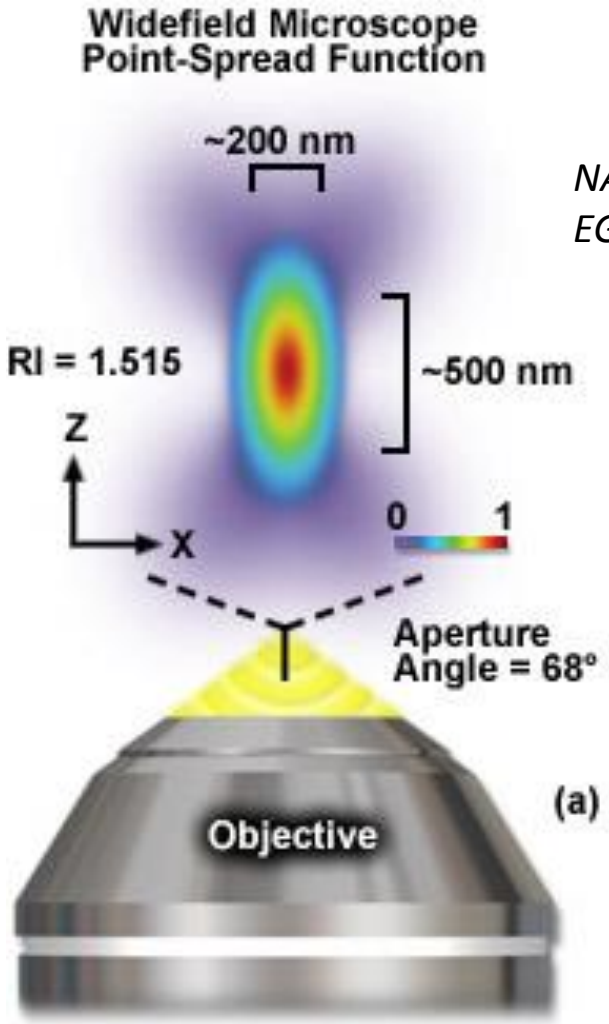

- lateral <u>resolving power</u> of an objective lens can be evaluated by measuring the size of the Airy disk (FWHM)

- different shapes in *z* depending on the instrument used -> ***axial resolution***

- viewed in the x-y plane

# PSF



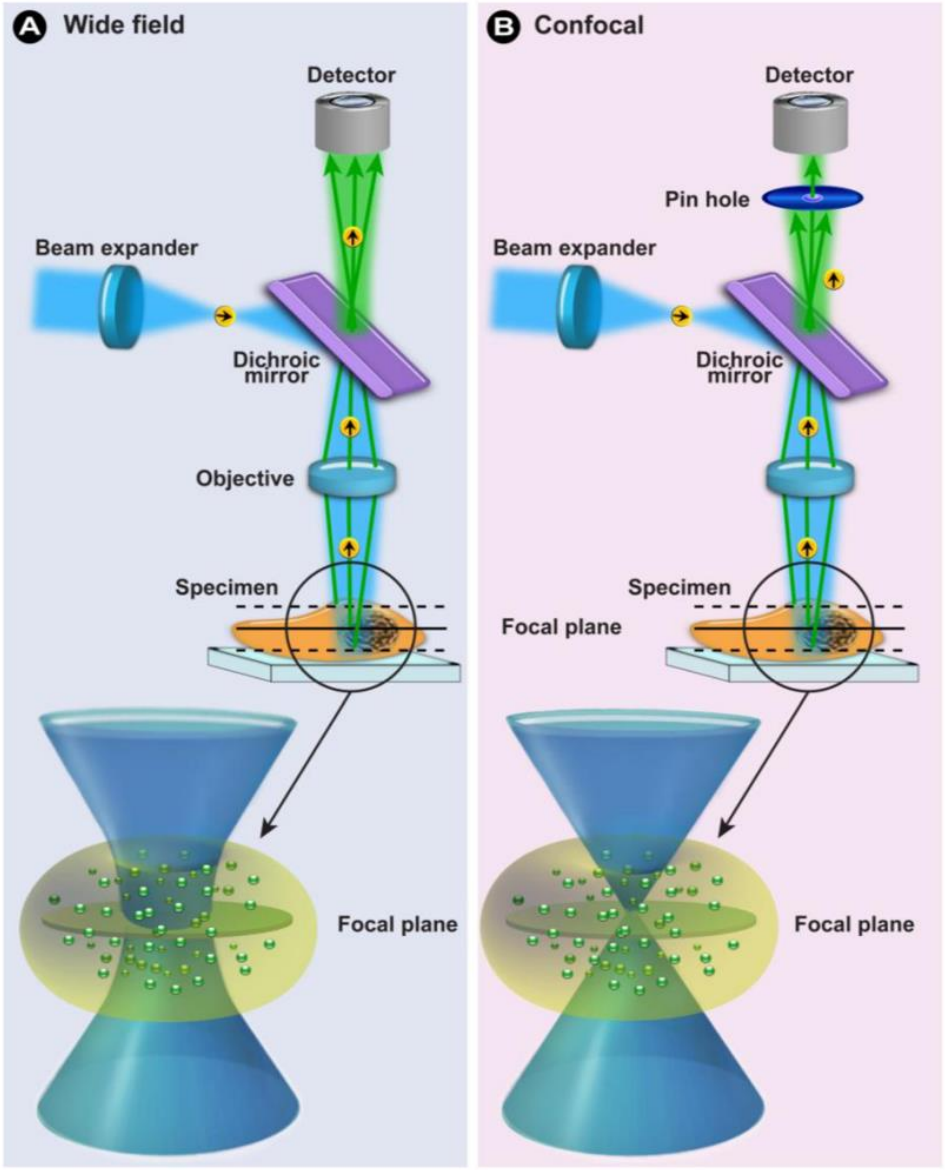



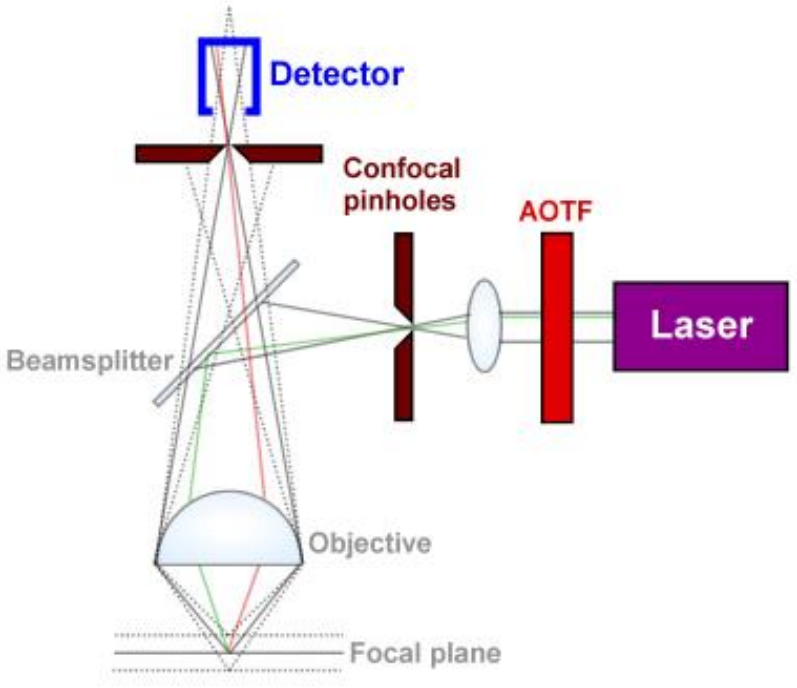

Axial resolution (Abbe):

$$r_{axial} = \frac{2\lambda n}{NA^2}$$

https://www.leica-microsystems.com/science-lab/confocal-optical-section-thickness/,
http://zeiss-campus.magnet.fsu.edu/print/superresolution/introduction-print.html

# Widefield vs. Confocal microscope

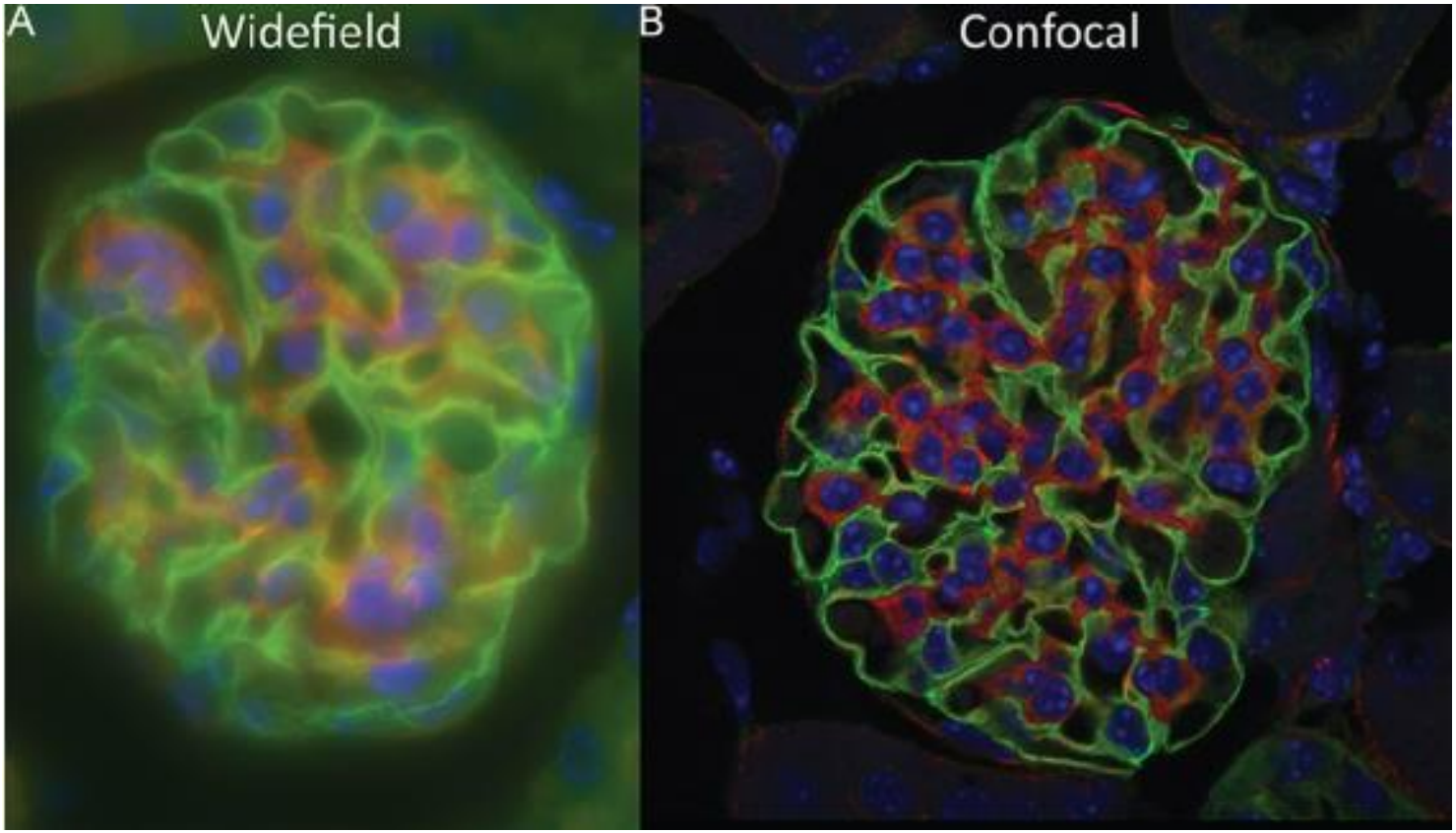



- dramatically increased contrast by removal *of out-of-focus* haze
- optical sectioning by the spot-scanning laser confocal microscope

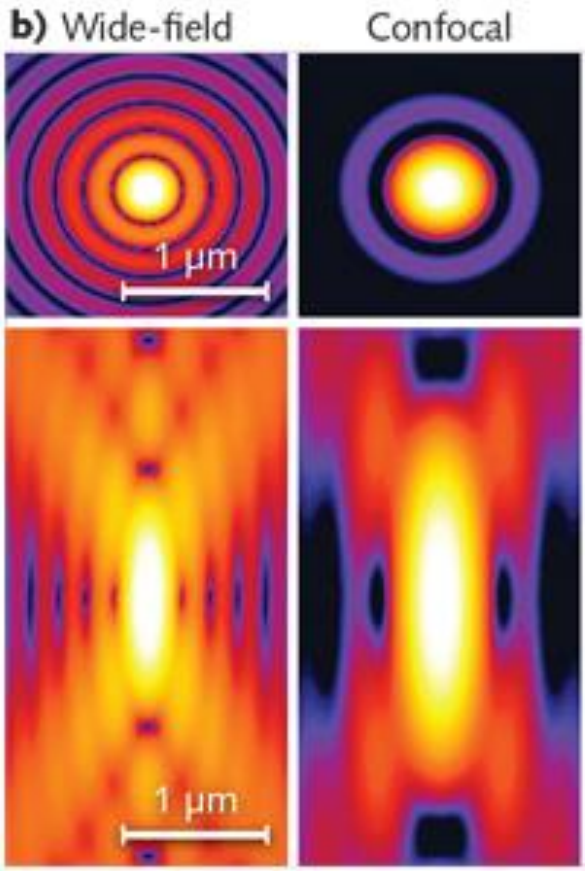
http://www.mdpi.com/1420-3049/17/4/4047/htm

# Widefield vs. Confocal microscope




https://microscopysolutions.ca/2012/05/31/any_way_you_slice_it/

# Widefield vs. Confocal microscope




Confocal resolution:

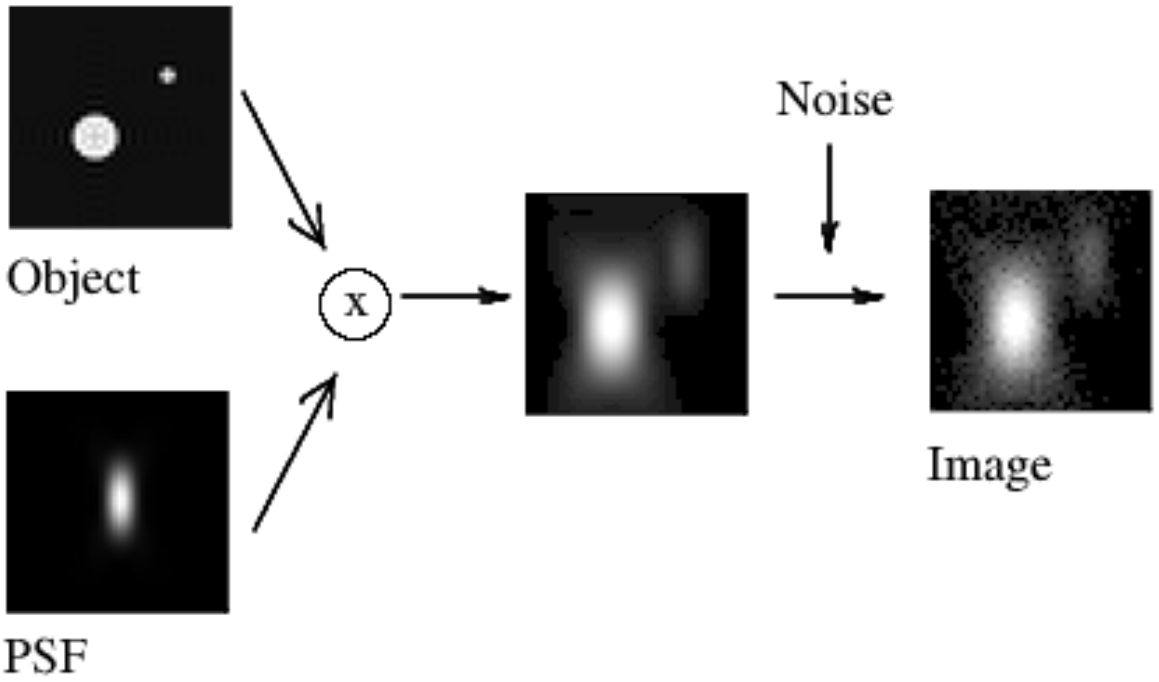
$$r_{lateral} = \frac{0.4\lambda}{NA}$$

$$r_{axial} = \frac{1.4\lambda n}{NA^2}$$

- axial intensity distributions for a typical widefield and confocal fluorescence microscope

https://www.leica-microsystems.com/science-lab/confocal-optical-section-thickness/

# Deconvolution



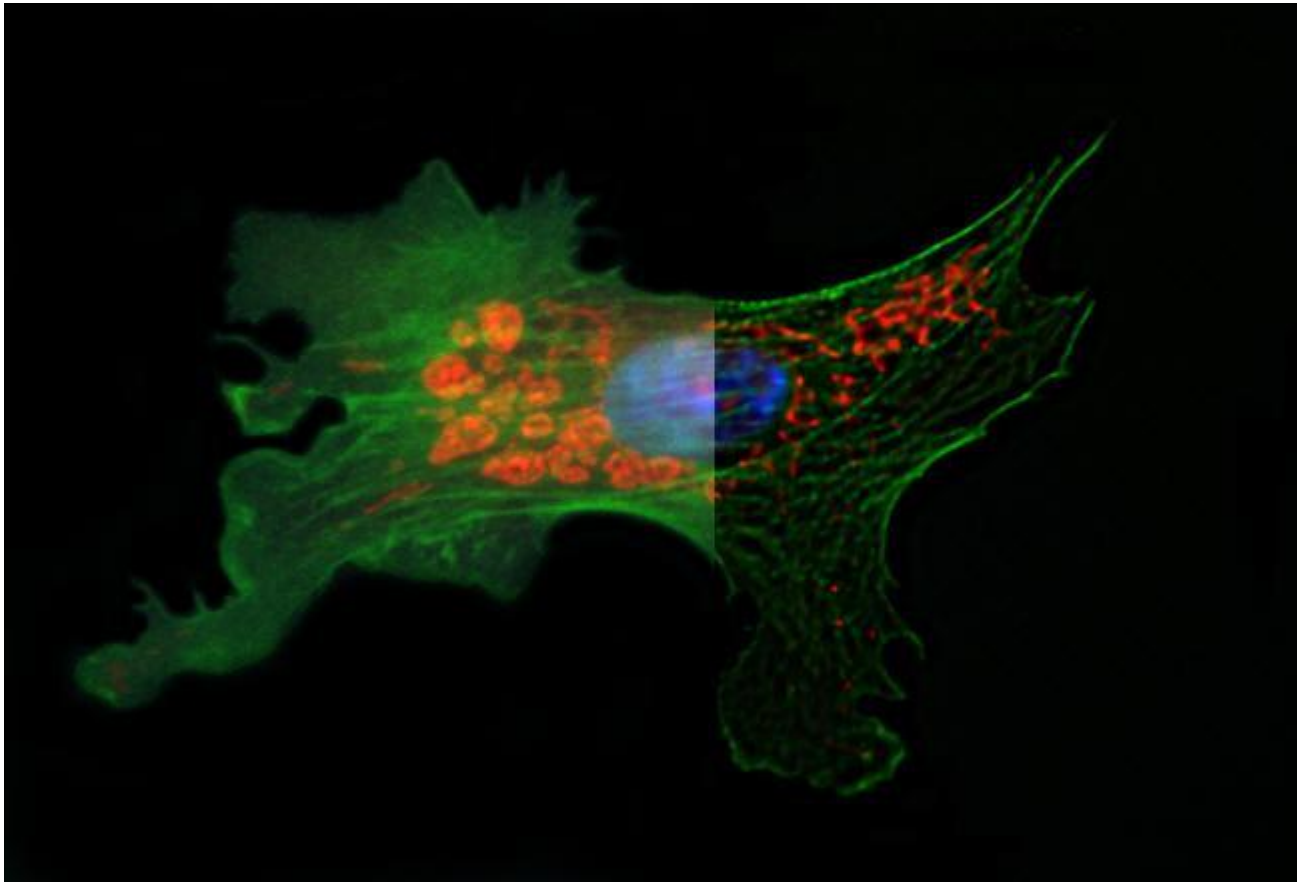

- **convolution** implies replacing every original (sub-resolution) light source by its correspondent PSF to produce a blurry image

- **deconvolution** (image restoration) would go the opposite way, collecting all this spread light and *putting it back* to its original location

-> recover an image degraded by processes described by convolution

https://svi.nl/Deconvolution

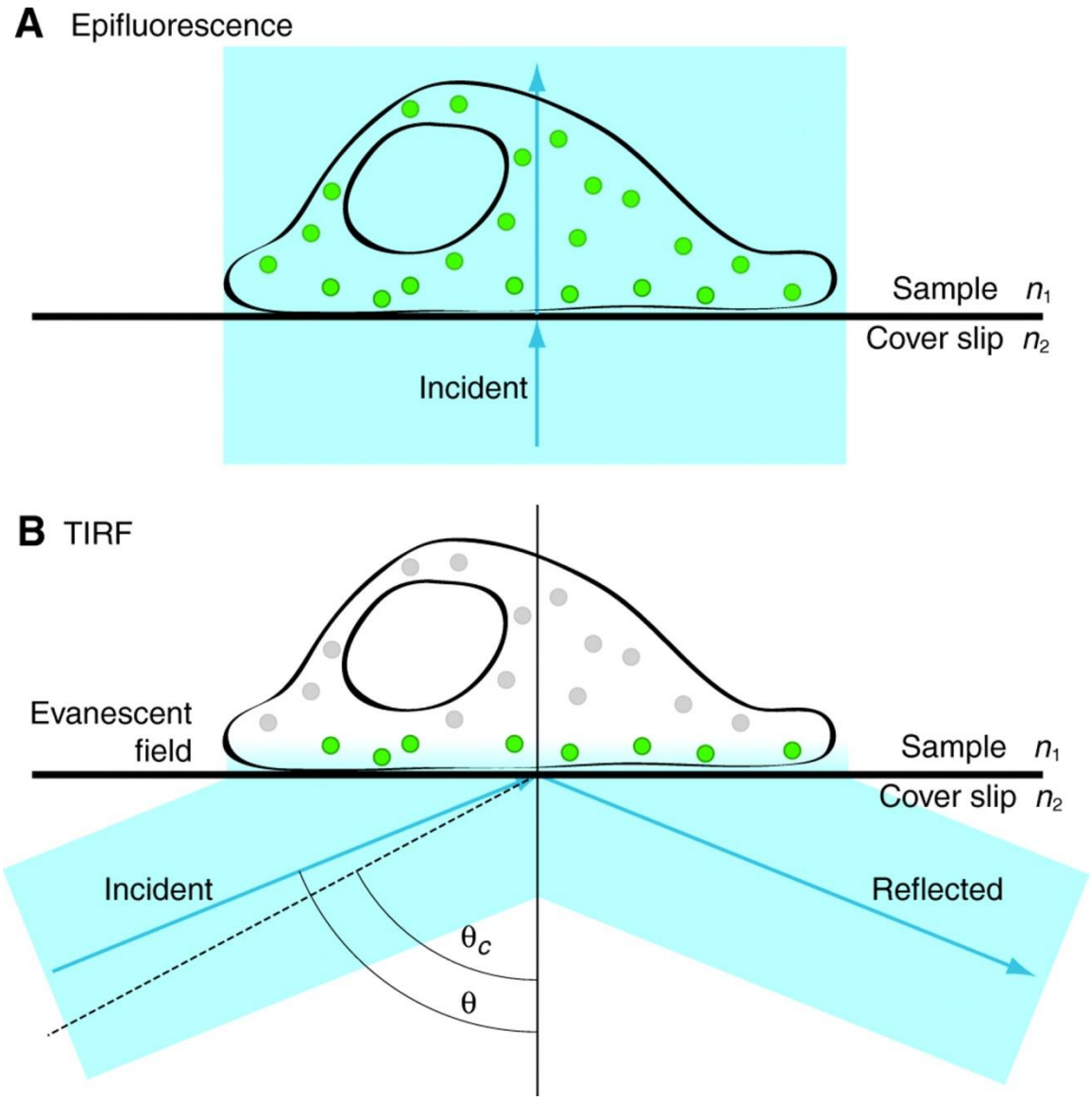
# Deconvolution



Widefield microscopy image -> *deconvolution* with experimental PSFs

https://svi.nl/Deconvolution

# TIRF microscope

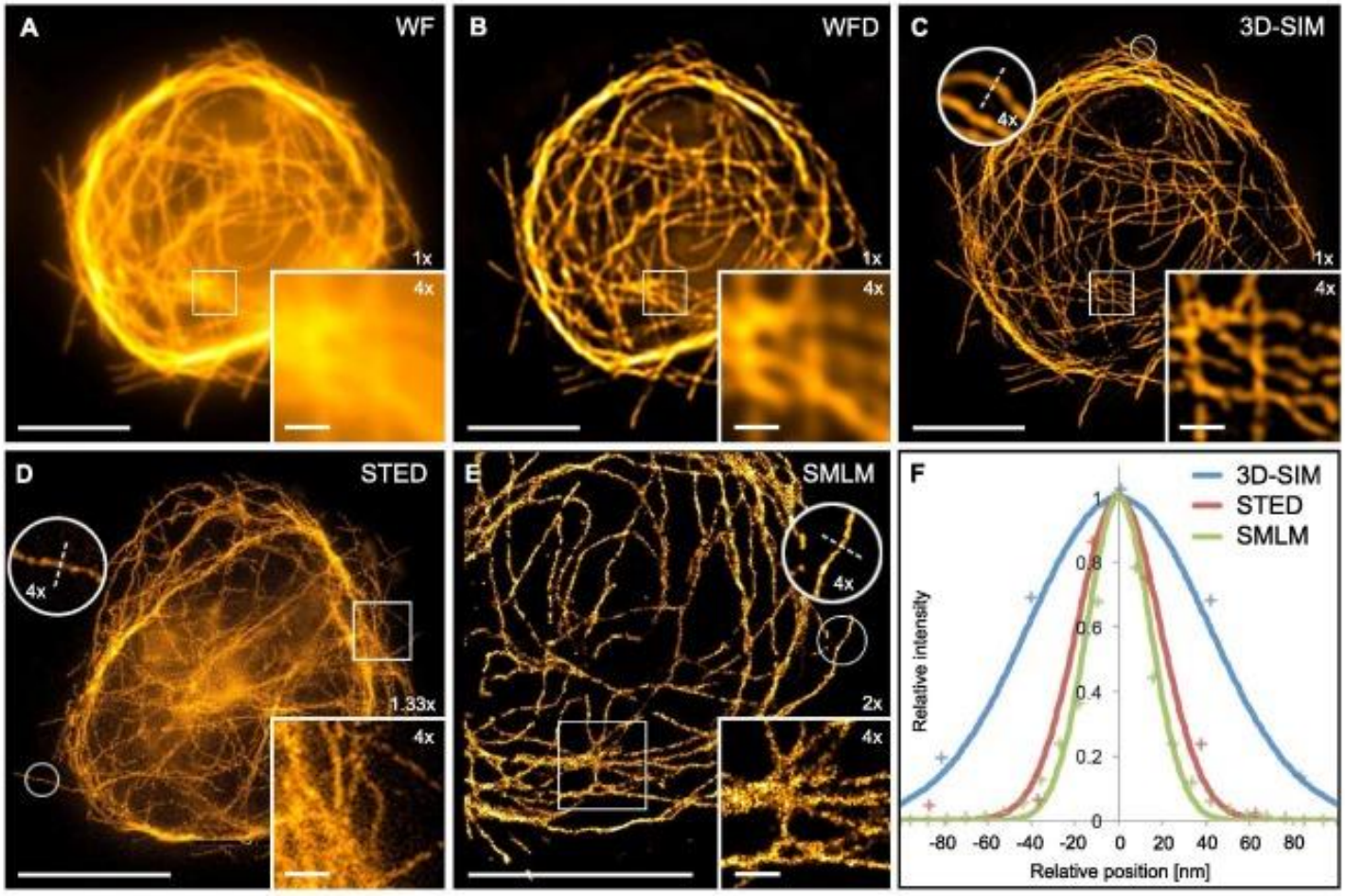



- a thin region of a specimen, usually less than **100 nm** can be observed (axial resolution)
- boundary to a medium of lower refractive index
- at larger angles than **critical angle** the light is reflected entirely back into the first medium -> *total internal reflection*
- **evanescent wave** selectively illuminates and excites fluorophores in a restricted region of the specimen

http://jcs.biologists.org/content/123/21/3621

# Super-resolution microscopy

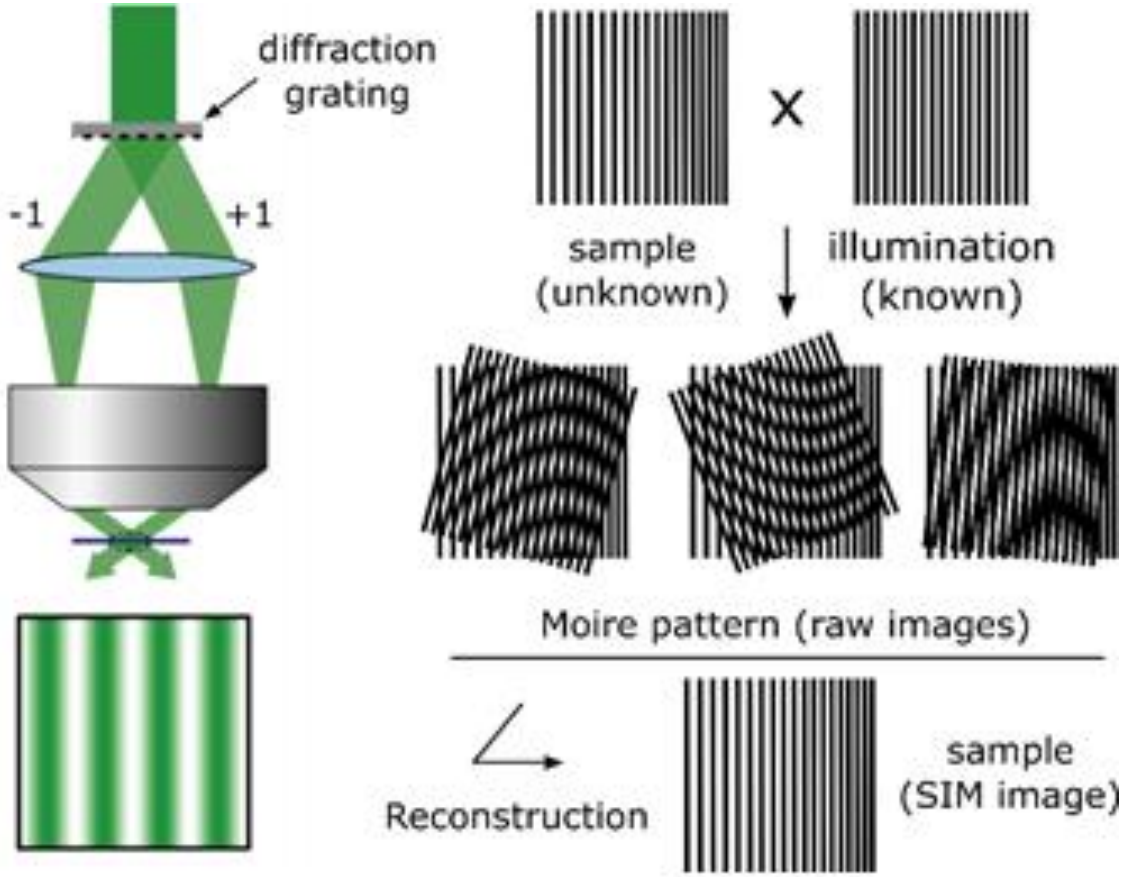
Breaking the diffraction barrier:




http://www.sciencedirect.com/science/article/pii/S1046202315300190

# SIM

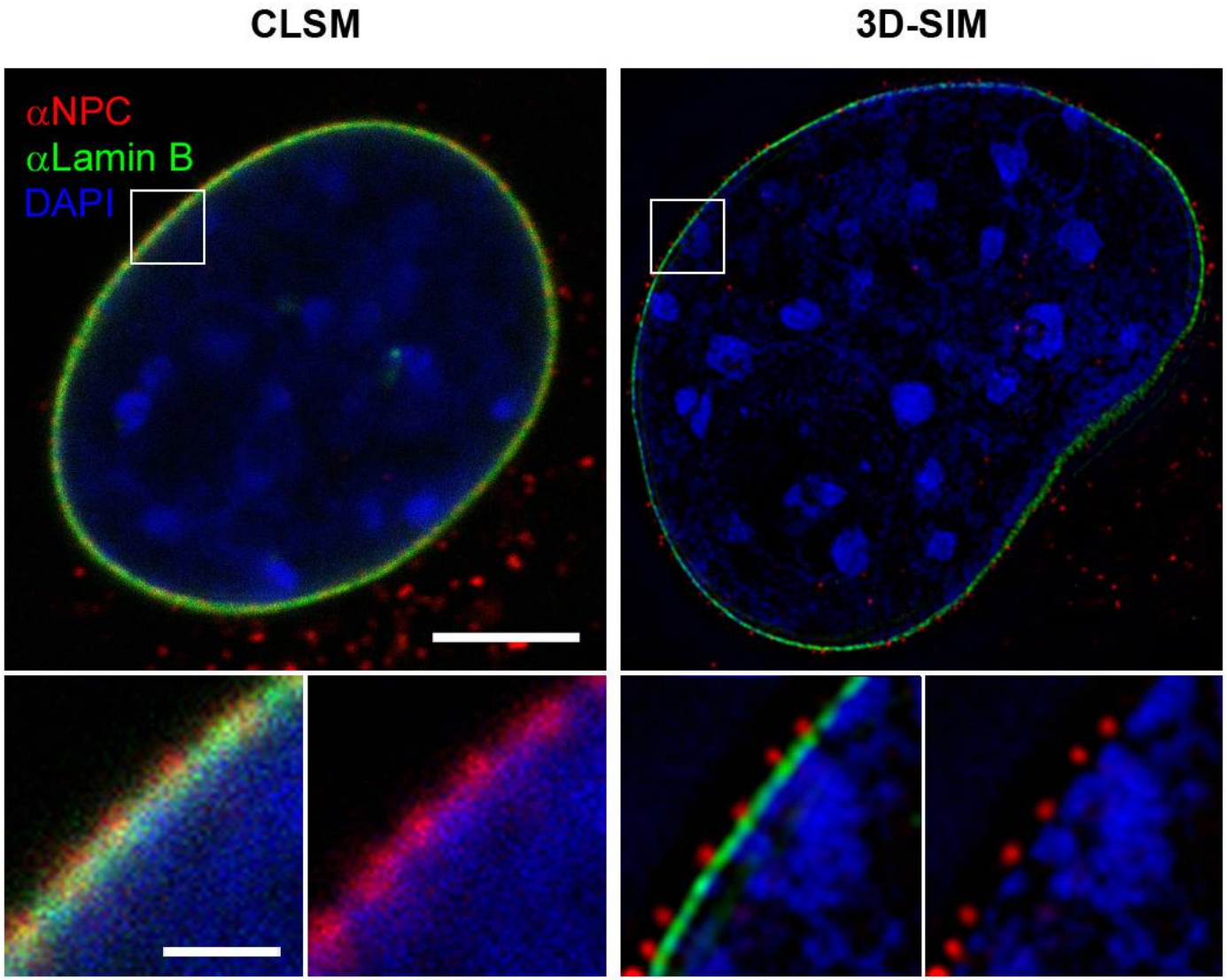
- **Structured Illumination Microscopy** - patterned illumination to spatially modulate the fluorescence behavior of molecules within a diffraction-limited region, such that not all of them emit simultaneously, thereby achieving subdiffraction limit resolution




- final image is then computationally *reconstructed* from multiple snapshots collected by scanning and rotating the pattern

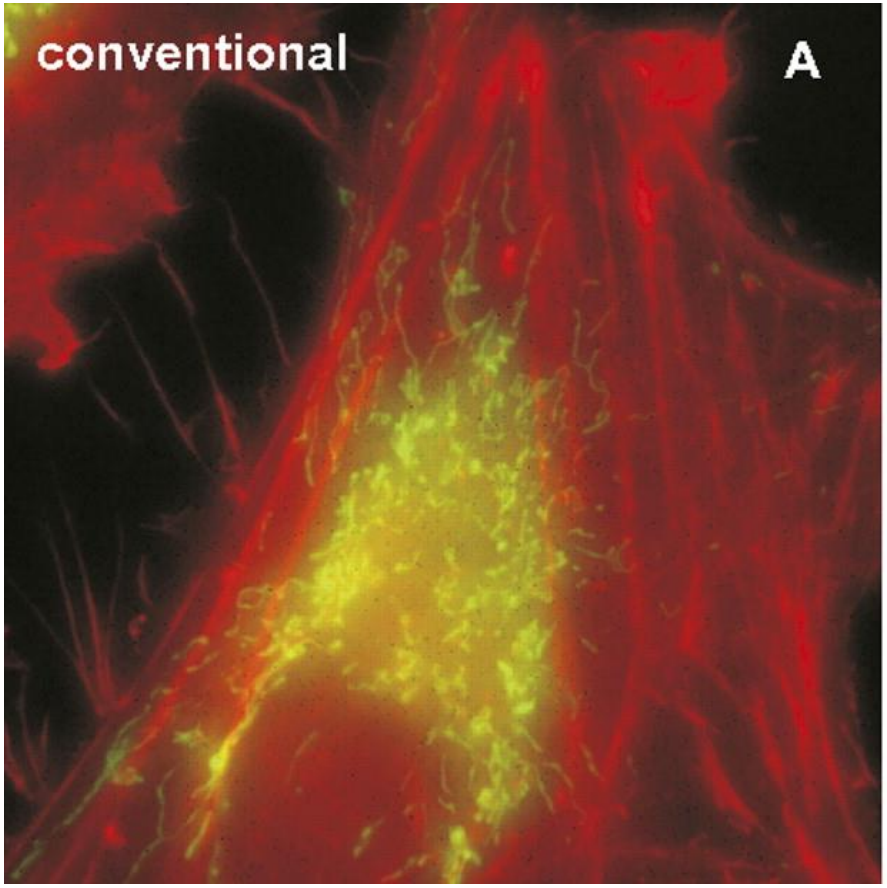
http://www.andor.com/learning-academy/super-resolution-imaging-structured-illumination-microscopy-application-note

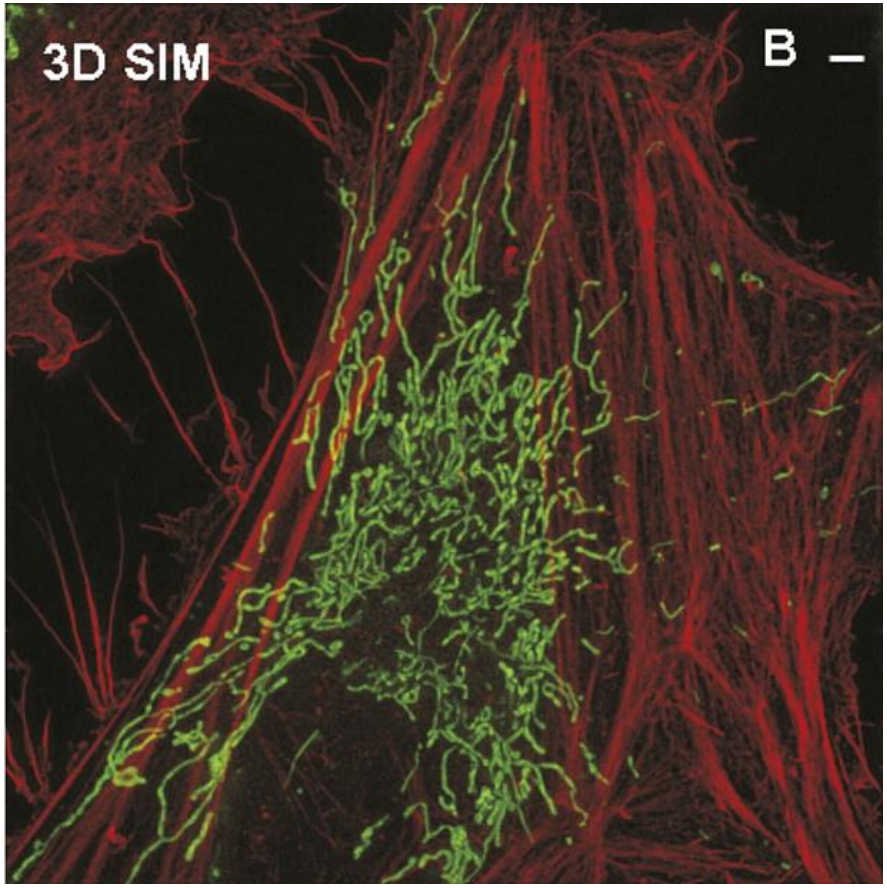
# SIM




**Resolution ~120 nm** in xy and **~250 nm** in z

# Super-resolution microscopy



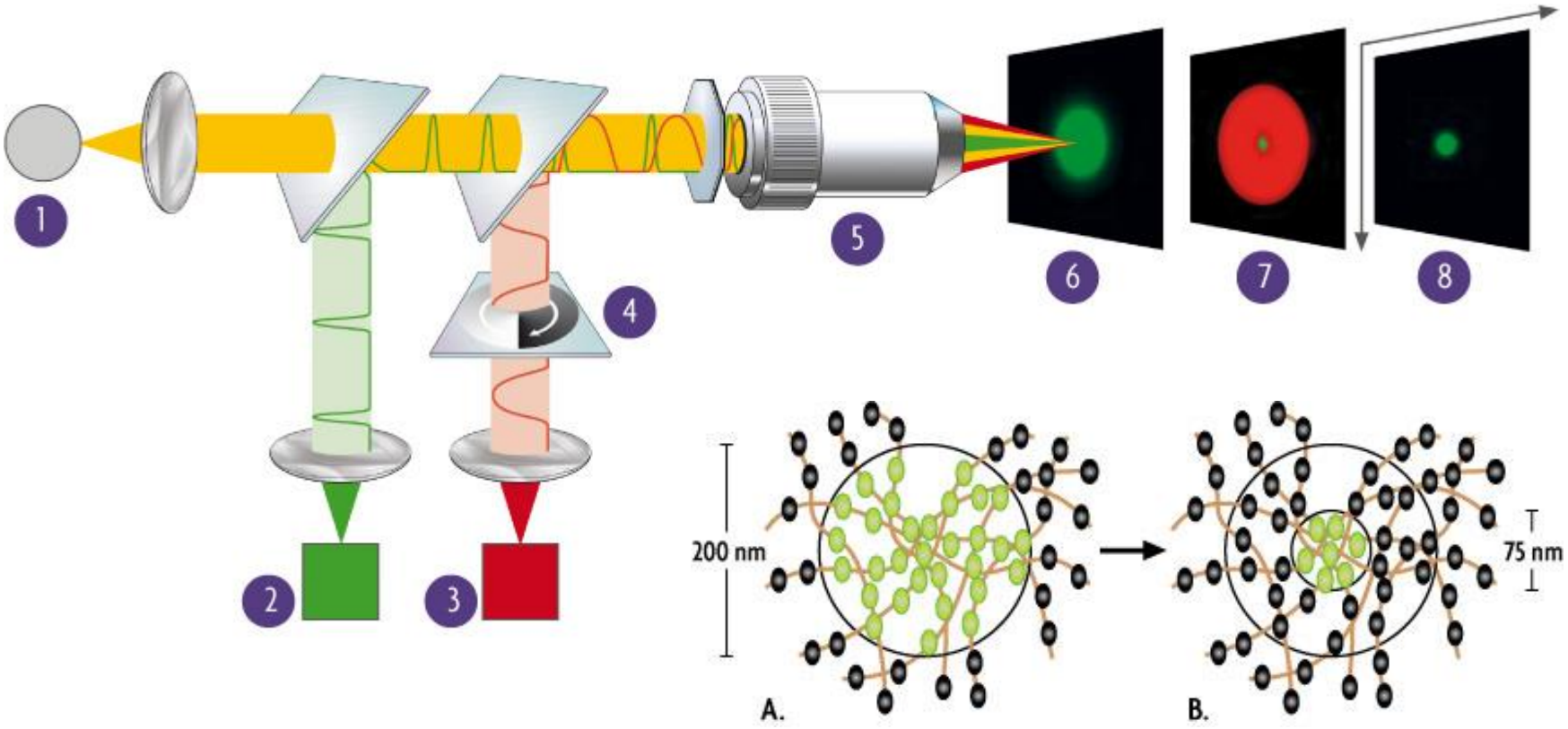

Maximum intensity projection

**Resolution ~120 nm** in xy and **~250 nm** in z

http://www.pnas.org/content/109/14/5311/

# STED

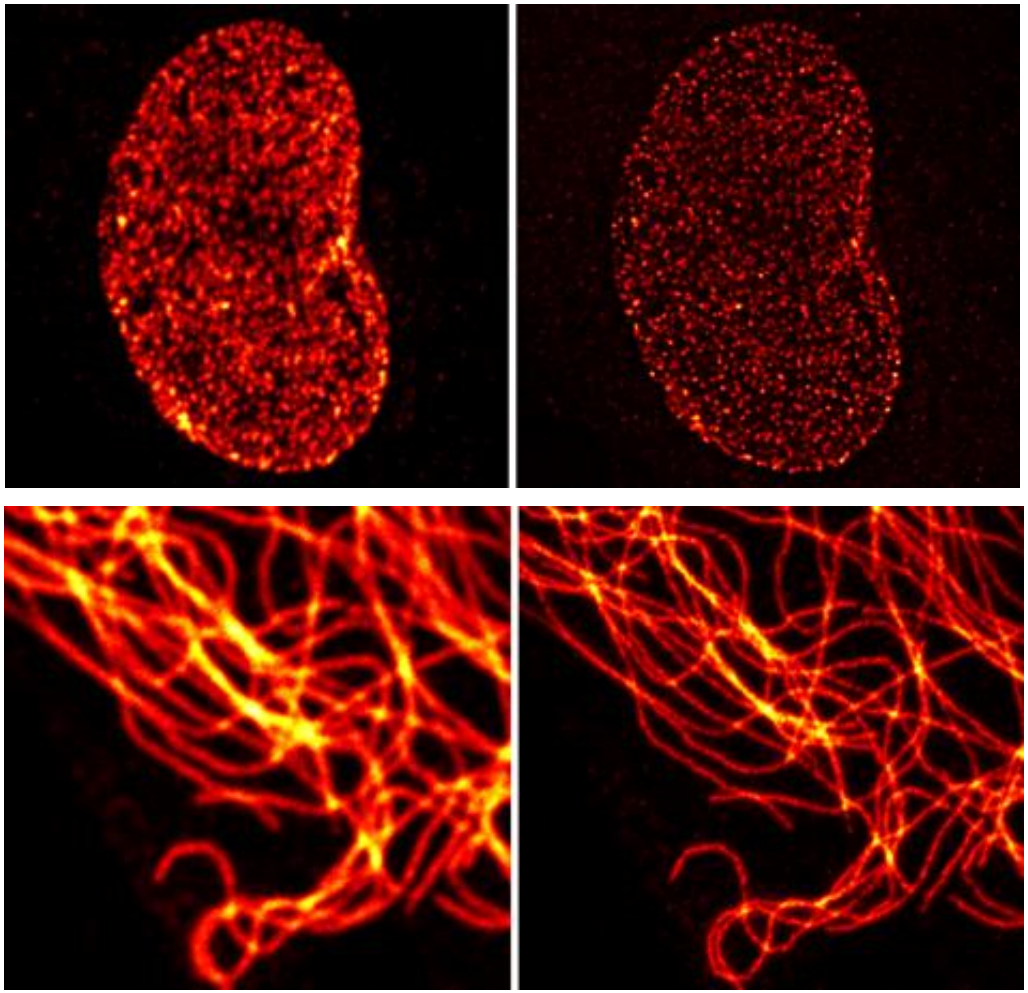
- **Stimulated Emission Depletion**- uses two laser pulses, the excitation pulse for excitation of the fluorophores to their fluorescent state and the STED pulse for the de-excitation of fluorophores by means of stimulated emission, minimizing the area of illumination




https://www.activemotif.com/catalog/627/sted-microscopy-products

# STED

Confocal

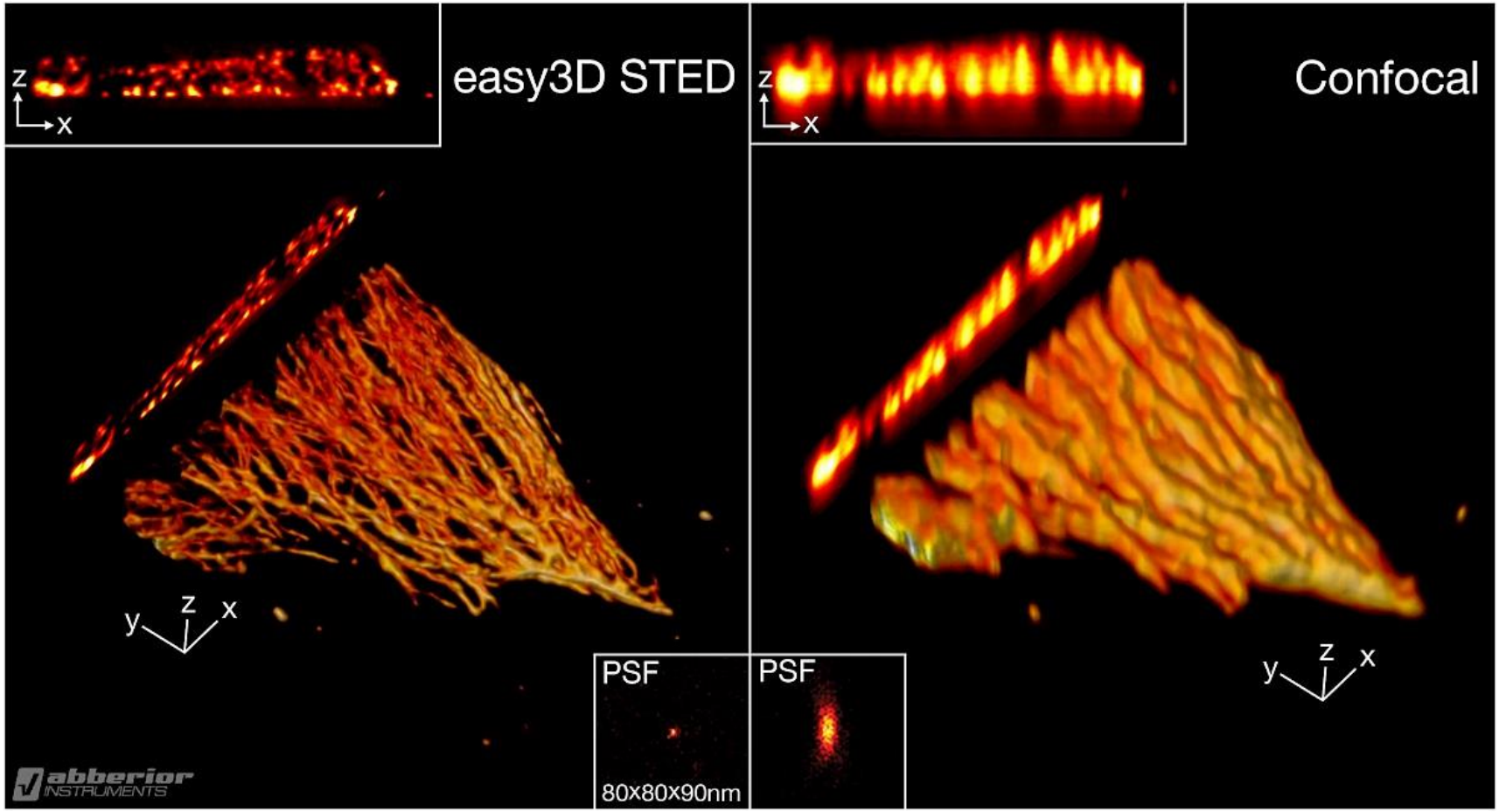


STED

**Resolution ~40 nm** in xy

http://www.activemotif.com/catalog/595/chromeo-488

# STED

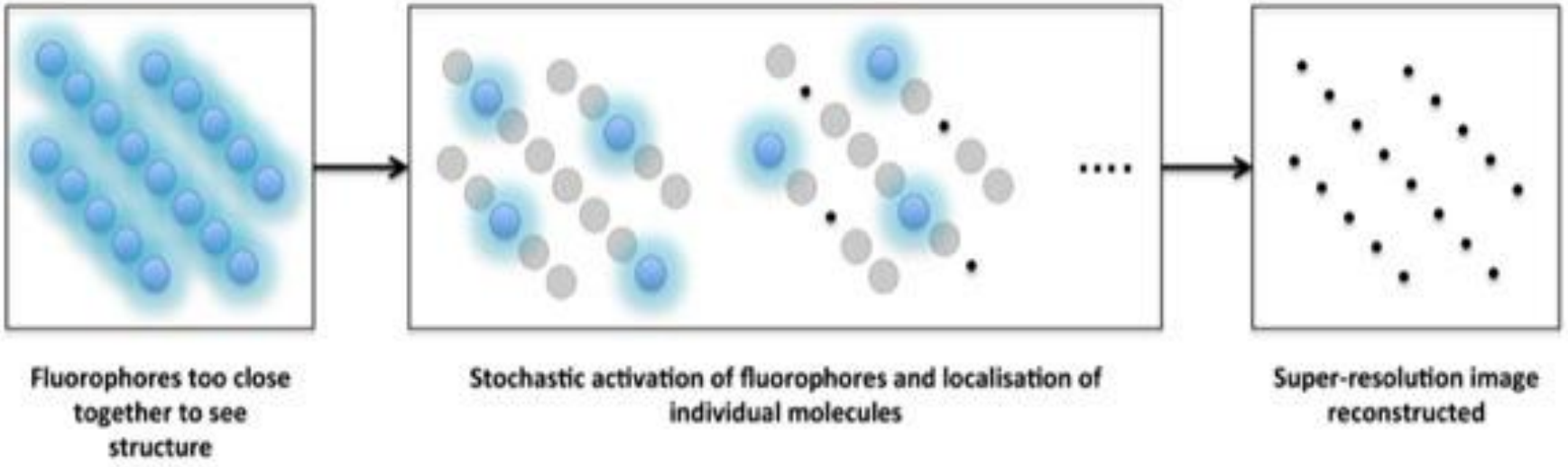



**Resolution ~100 nm** in z

http://www.abberior-instruments.com/products/expert-line/easy3d-module/

# SMLM

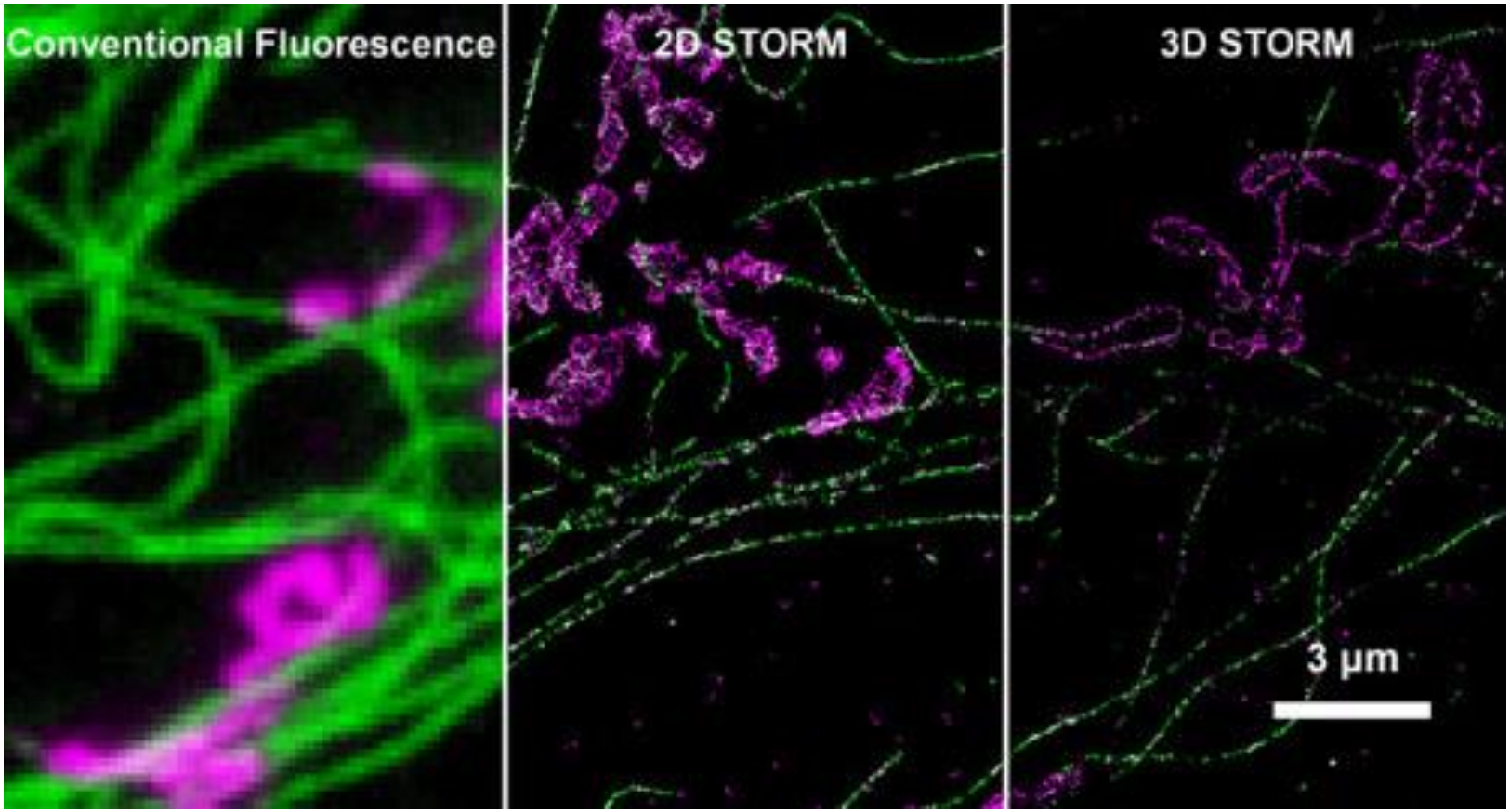
- **Single Molecule Localization Microscopy** - super-resolution is achieved by isolating emitters and fitting their images with the point spread function (PSF)




- Stochastic optical reconstruction microscopy *(STORM)*
- Photo activated localization microscopy *(PALM)*
- Fluorescence photo-activation localization microscopy *(FPALM)*
  - sequential activation and time-resolved localization of photoswitchable or photoactivatable fluorophores to create high resolution images

https://bitesizebio.com/21069/

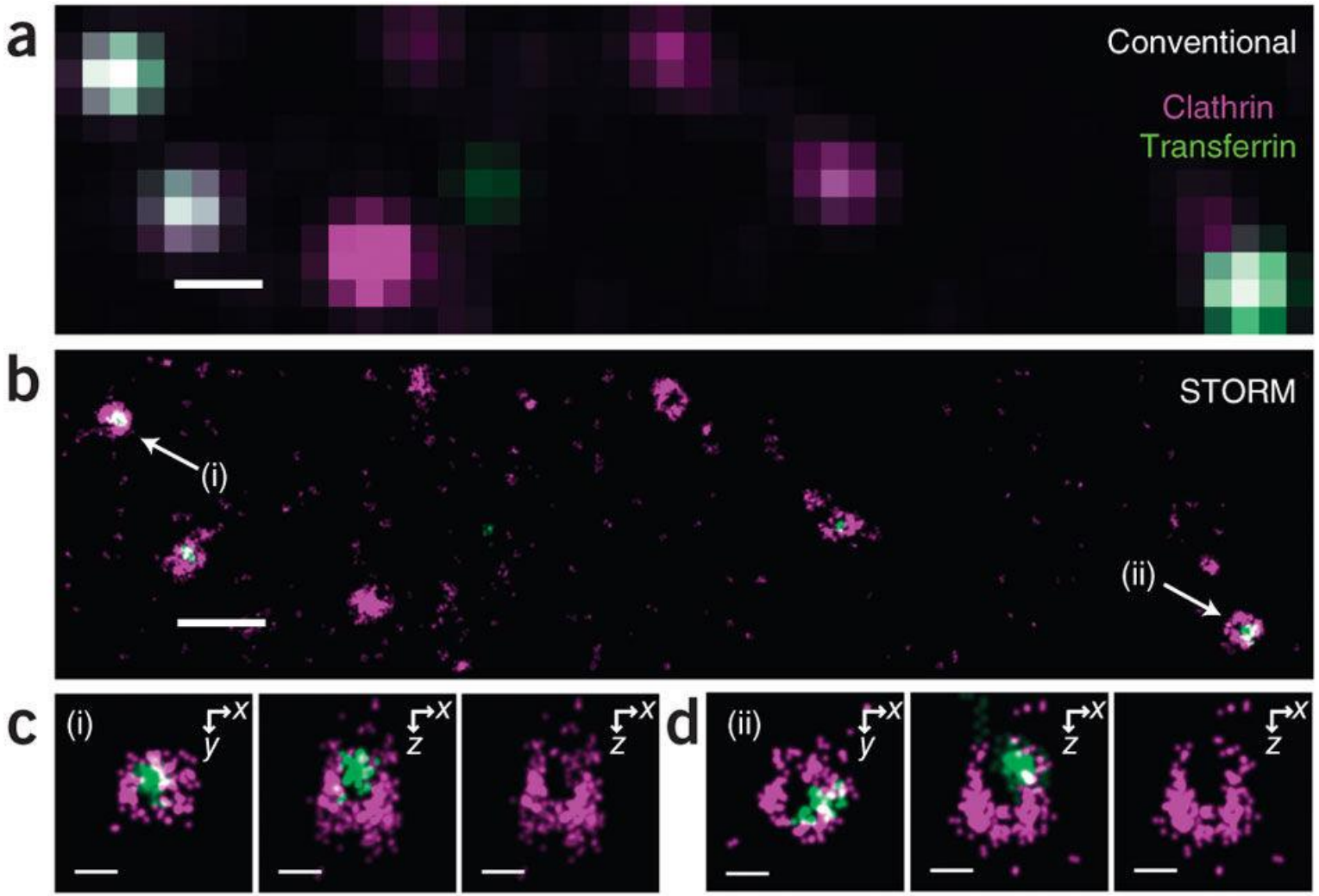
# SMLM




**Resolution ~25 nm** in xy, often in TIRF setup

http://huanglab.ucsf.edu/STORM.html

# SMLM




dSTORM (direct STORM) – Alexa647, Alexa568

**Resolution ~30 nm** in xy, **~50 nm** in z

http://www.nature.com/nmeth/journal/v8/n6/abs/nmeth.1605.html

Thank you for your attention!