CEITEC

Central European Institute of Technology

BRNO | CZECH REPUBLIC

S5015

Light microscopy methods in biology

# Lecture 3: **Superresolution Microscopy**

Jakub Pospíšil

Cellular Imaging Core Facility, Ceitec MU

jakub.pospisil@ceitec.muni.cz

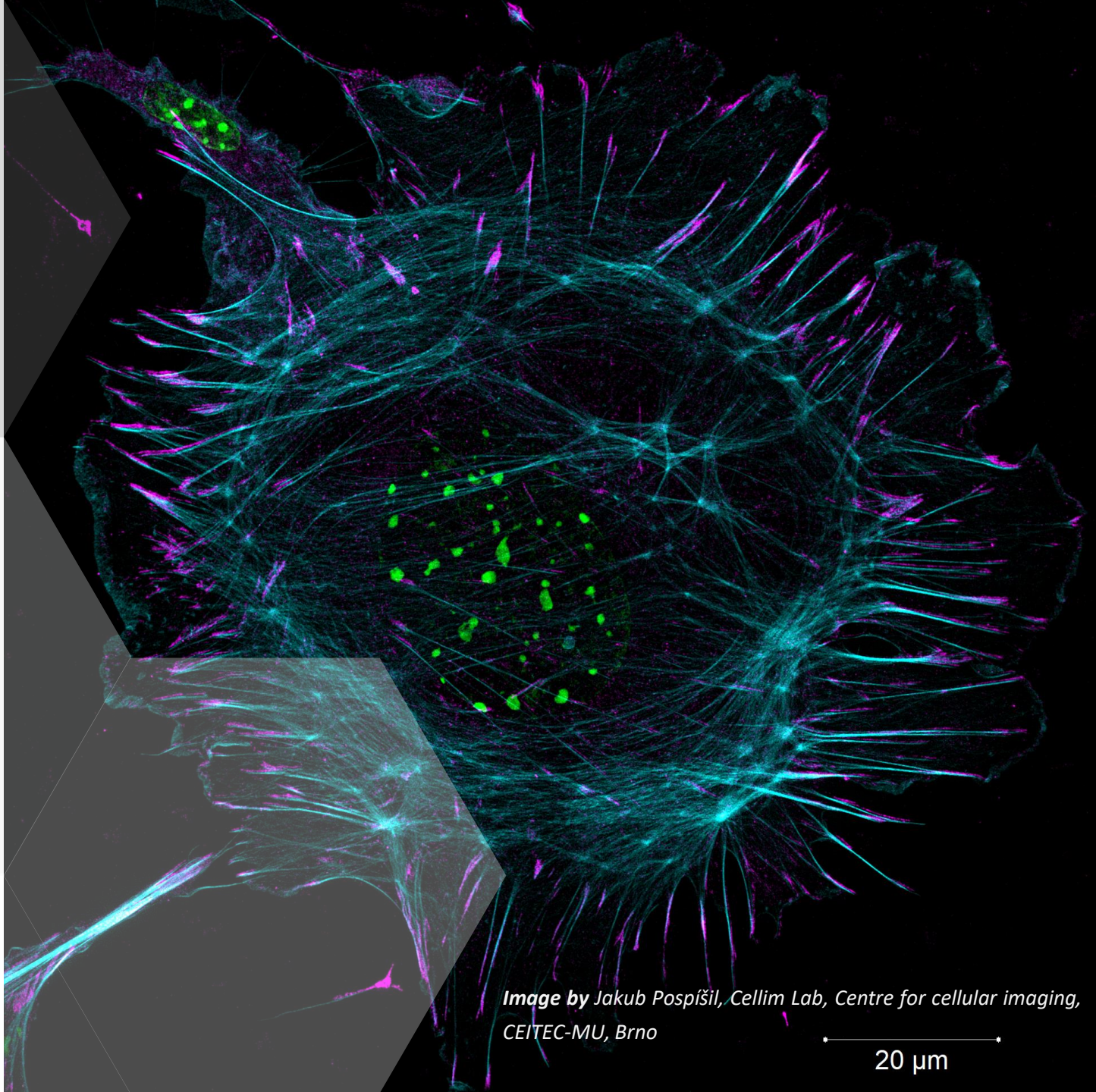
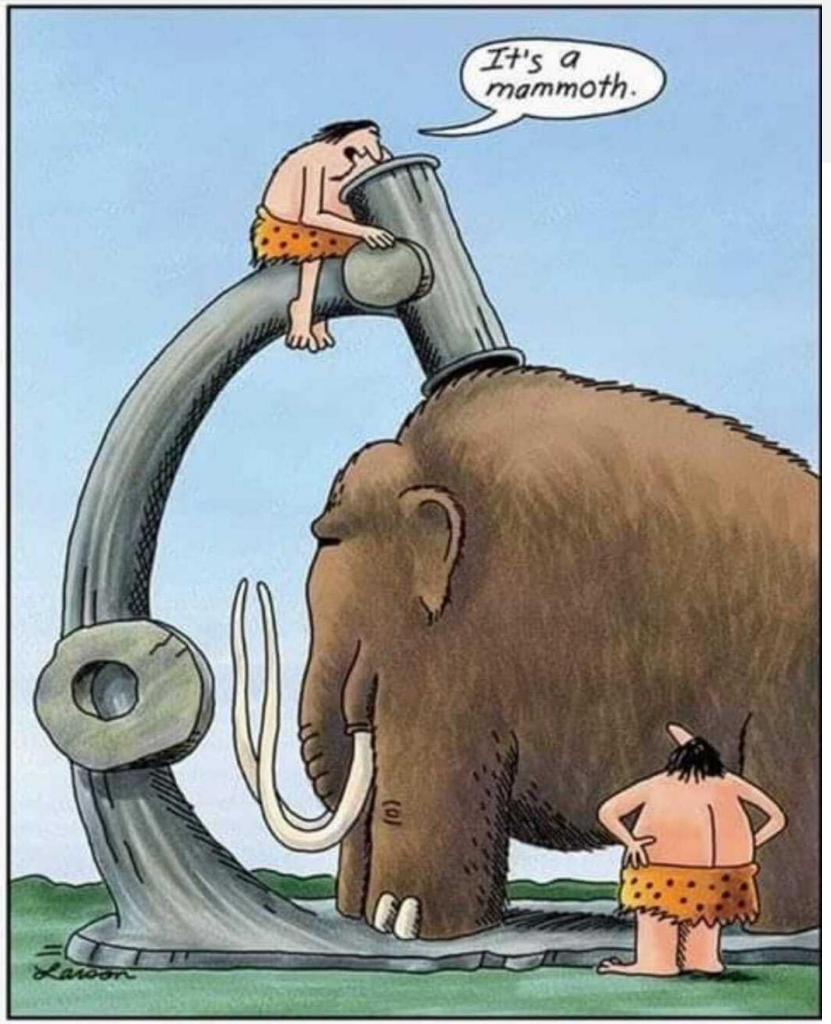
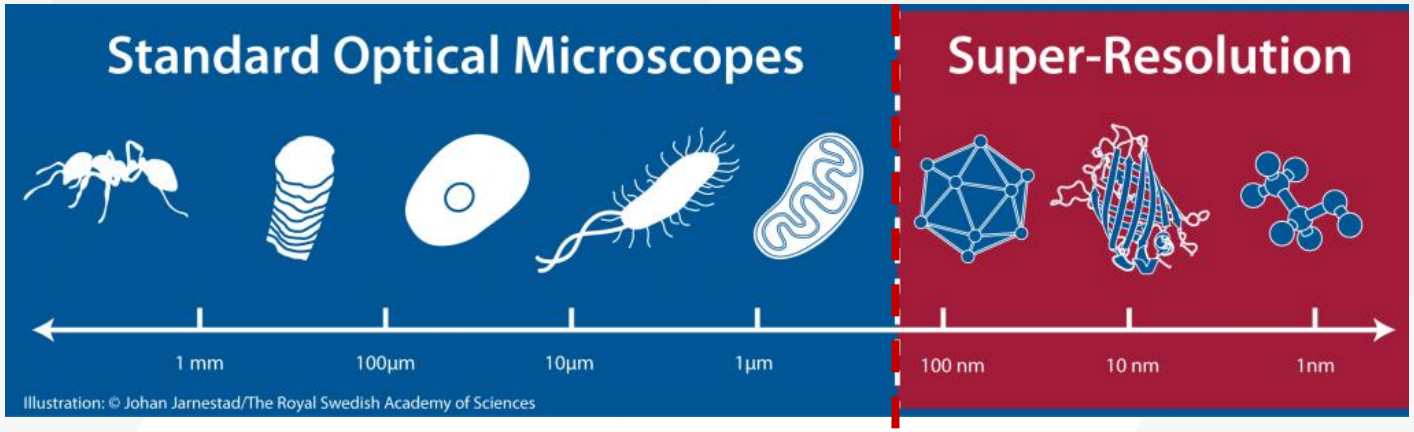



***Image by** Jakub Pospíšil, Cellim Lab, Centre for cellular imaging, CEITEC-MU, Brno*




Early microscope

# Resolution ranges of Biological Imaging techniques




PET, MRI and Ultrasound

**Fluorescence microscopy**

- Widefield and TIRF fluorescence micrscopy
- Confocal Microscopy

Abbe`s diffraction limit (200 nm)

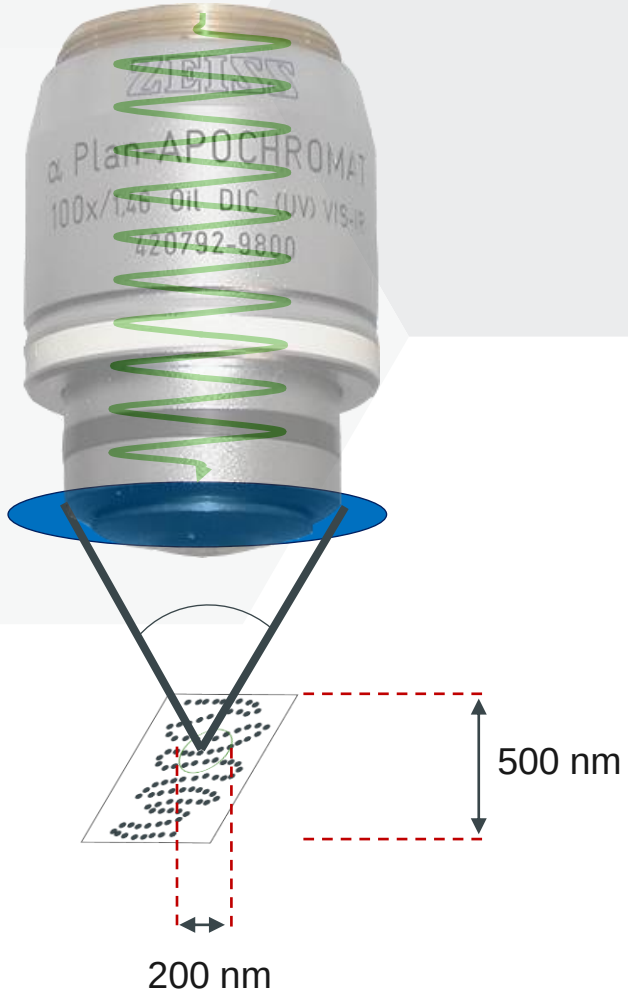
Electron micrscopy

# Difraction limit

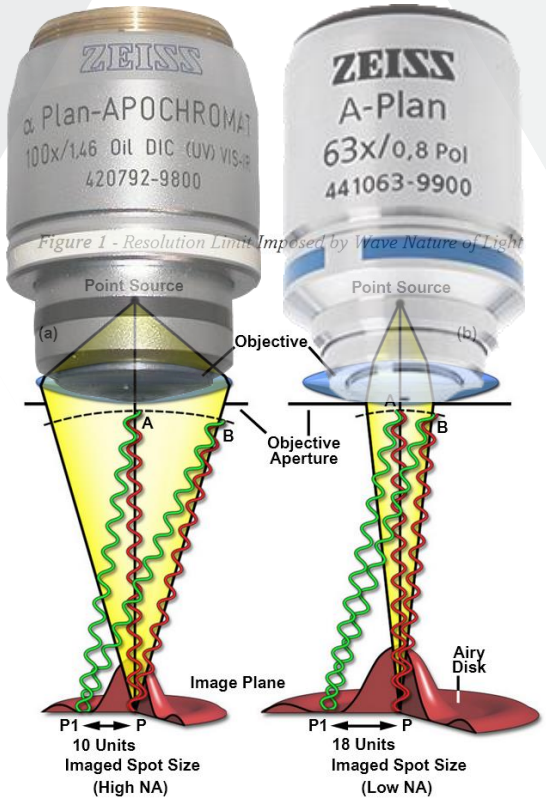
The diffraction limit defined by Abbe corresponds to the radius of the spot where the light is diffracted.

**Wavelength**
(λ)

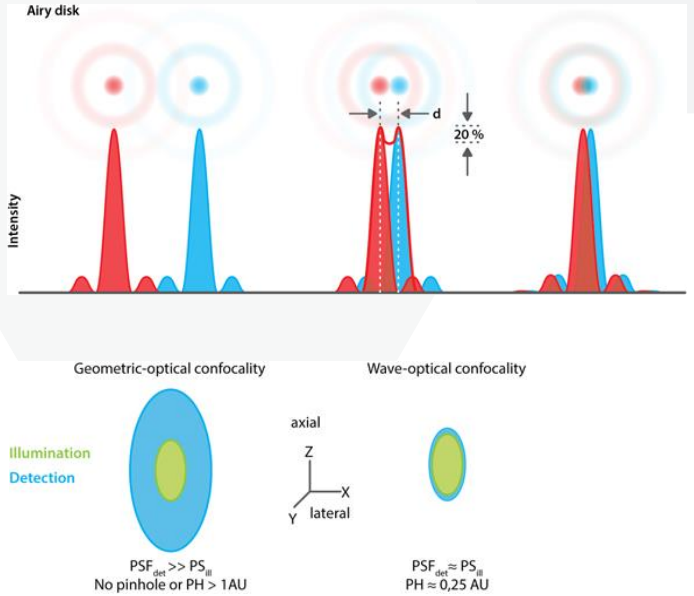
**Lens**
(NA)




*Verdet (1869)*
*Abbe (1873)*
*Helmholtz (1874)*
*Rayleigh (1874)*




$$d = \frac{\lambda}{2[\eta \bullet \sin(\alpha)]}$$



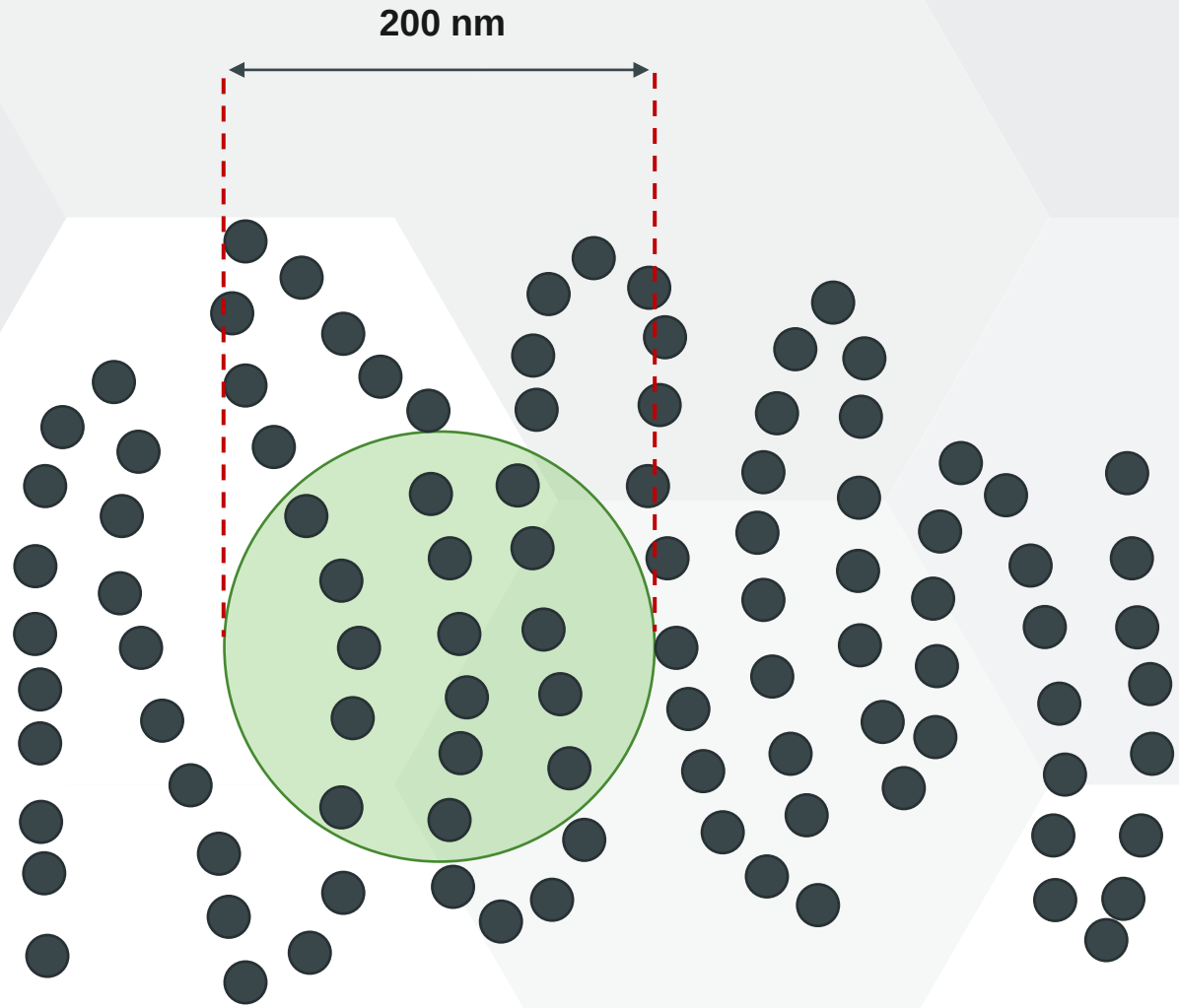

$Resolution_z = 2\lambda / [\eta \bullet \sin(\alpha)]^2$

# Difraction limit

**The Abbe diffraction limit depends on:**

- The light wavelength (λ)
- refractive index of the medium (n)
- half-angle of the converging spot (Θ)

$$\frac{\lambda}{2nsin\alpha}$$




**Problem:** molecules (features) within <200 nm not recognizable

***"Even though the classical resolution limits are imposed by physical law, they can in fact, be exceeded and the limitations are true only under certain assumptions.***

*1) Observation takes place in the conventional geometry in which light is collected by a single objective lens;*

*2) That the excitation light is uniform throughout the sample;*

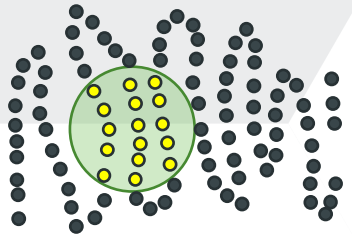
*3) Fluorescence takes place through normal, linear absorption and emission of a single photon"*



*Gustafsson M G 1999 Extended resolution fluorescence microscopy Curr. Opin. Struct. Biol.* ***9*** *627–34*

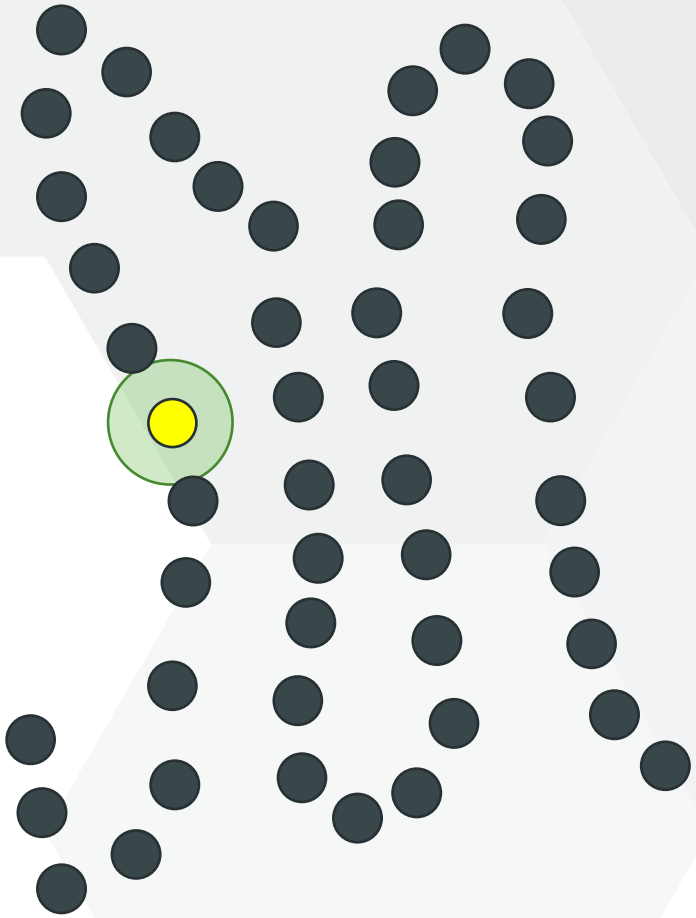
# Expansion Microscopy (ExM)

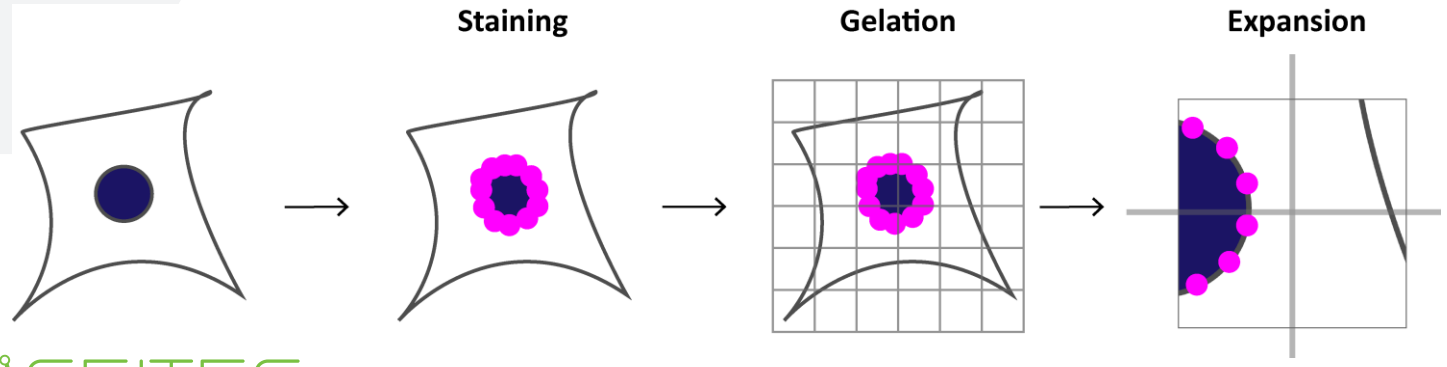

**Ed Boyden**, the leader of the Synthetic Neurobiology Group at the Massachusetts Institute of Technology
http://syntheticneurobiology.org/videos

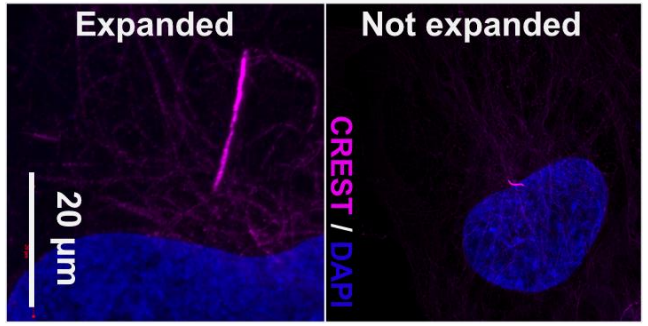


ExM

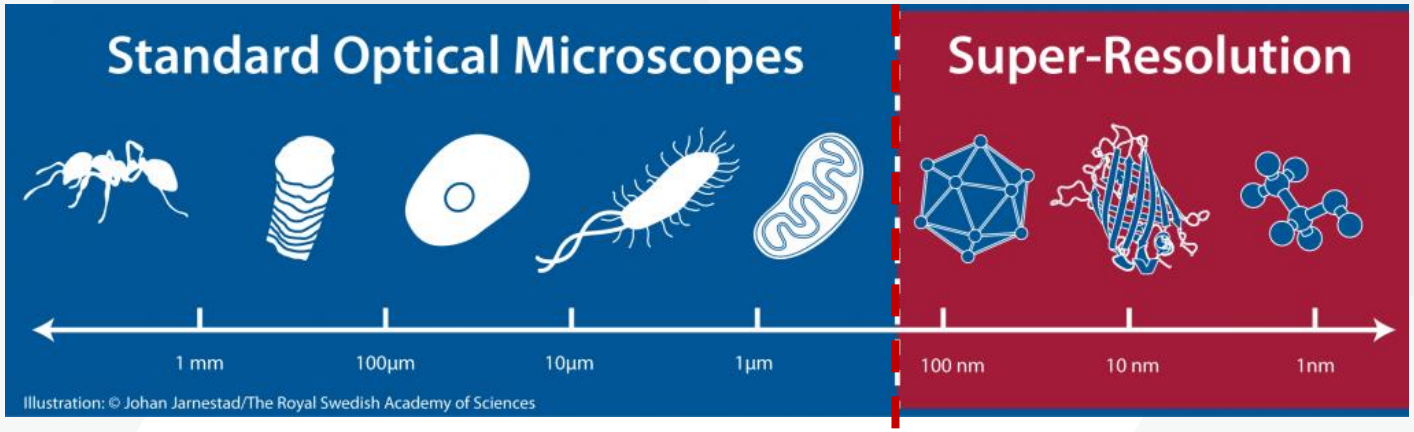
4,5x





# Resolution ranges of Biological Imaging techniques




PET, MRI and Ultrasound

**Fluorescence microscopy**

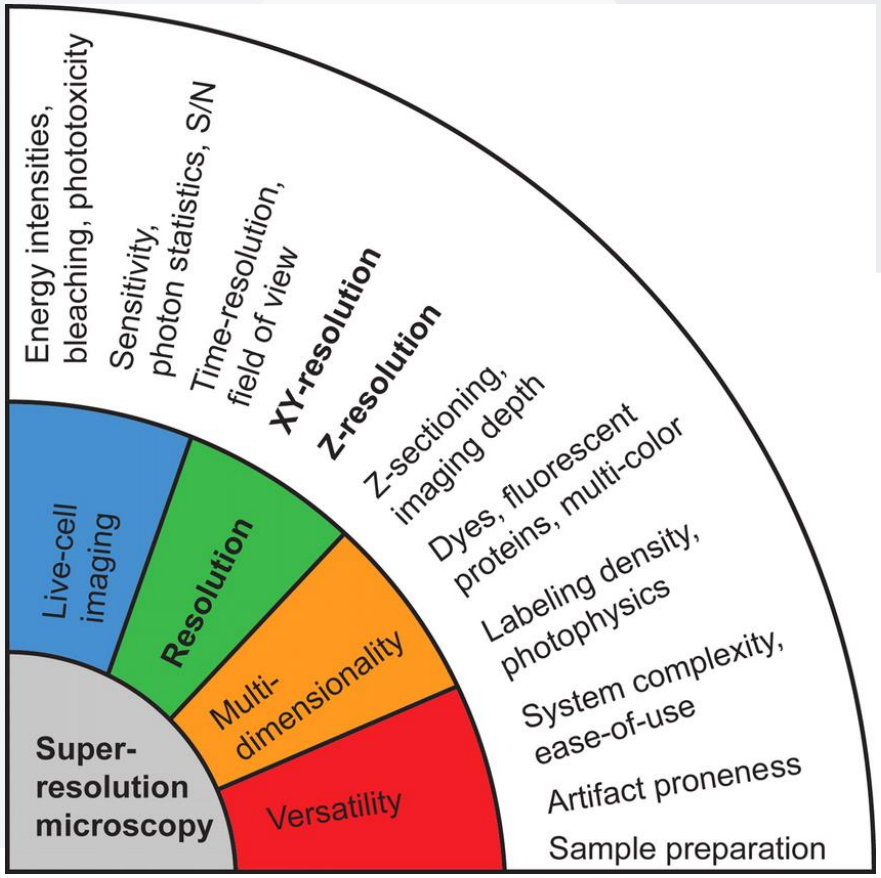
- Widefield and TIRF fluorescence micrscopy
- Confocal Microscopy
- Superresolution micrscopy

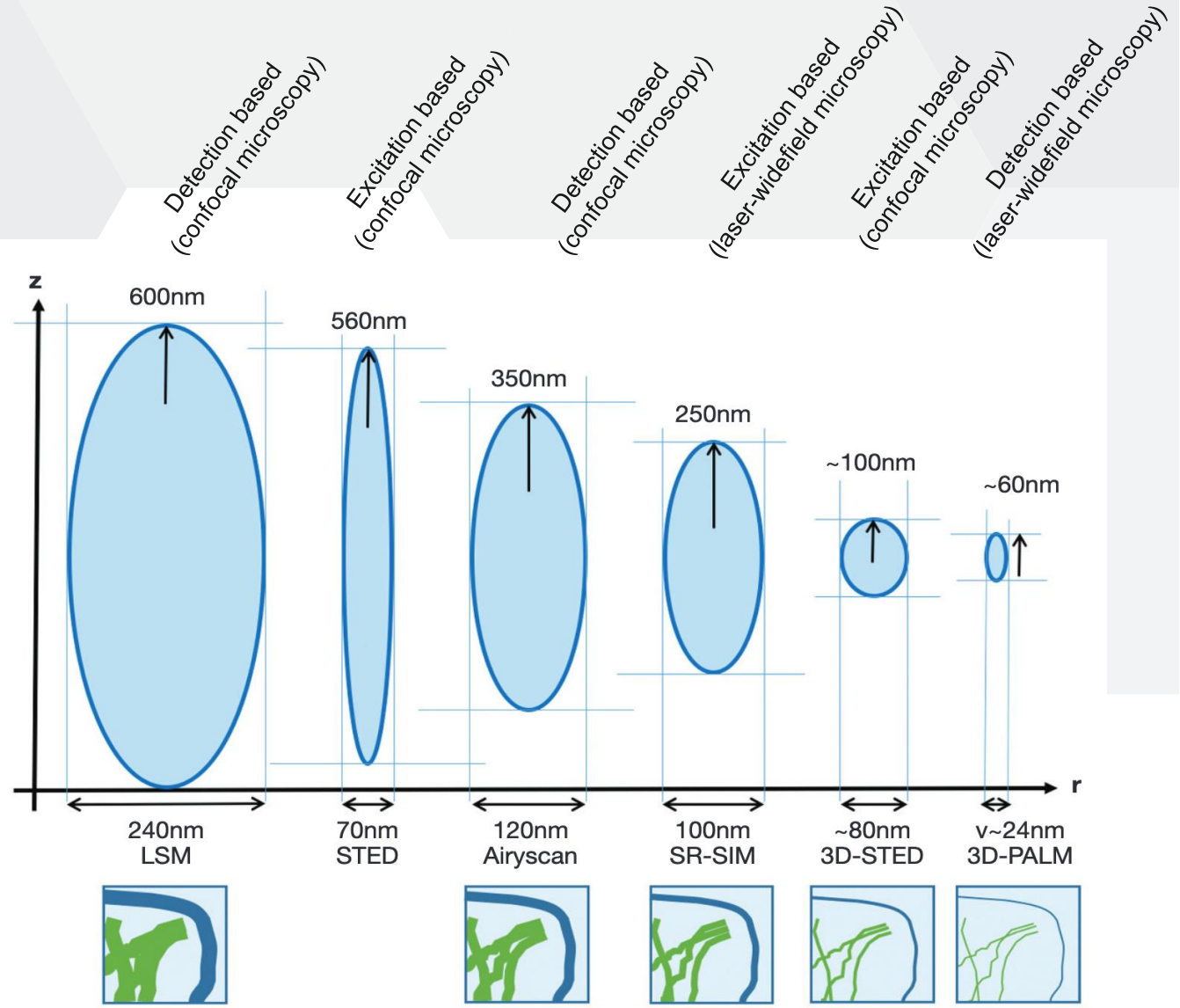
Abbe`s diffraction limit (200 nm)

Electron micrscopy

# SR microscopy clasification

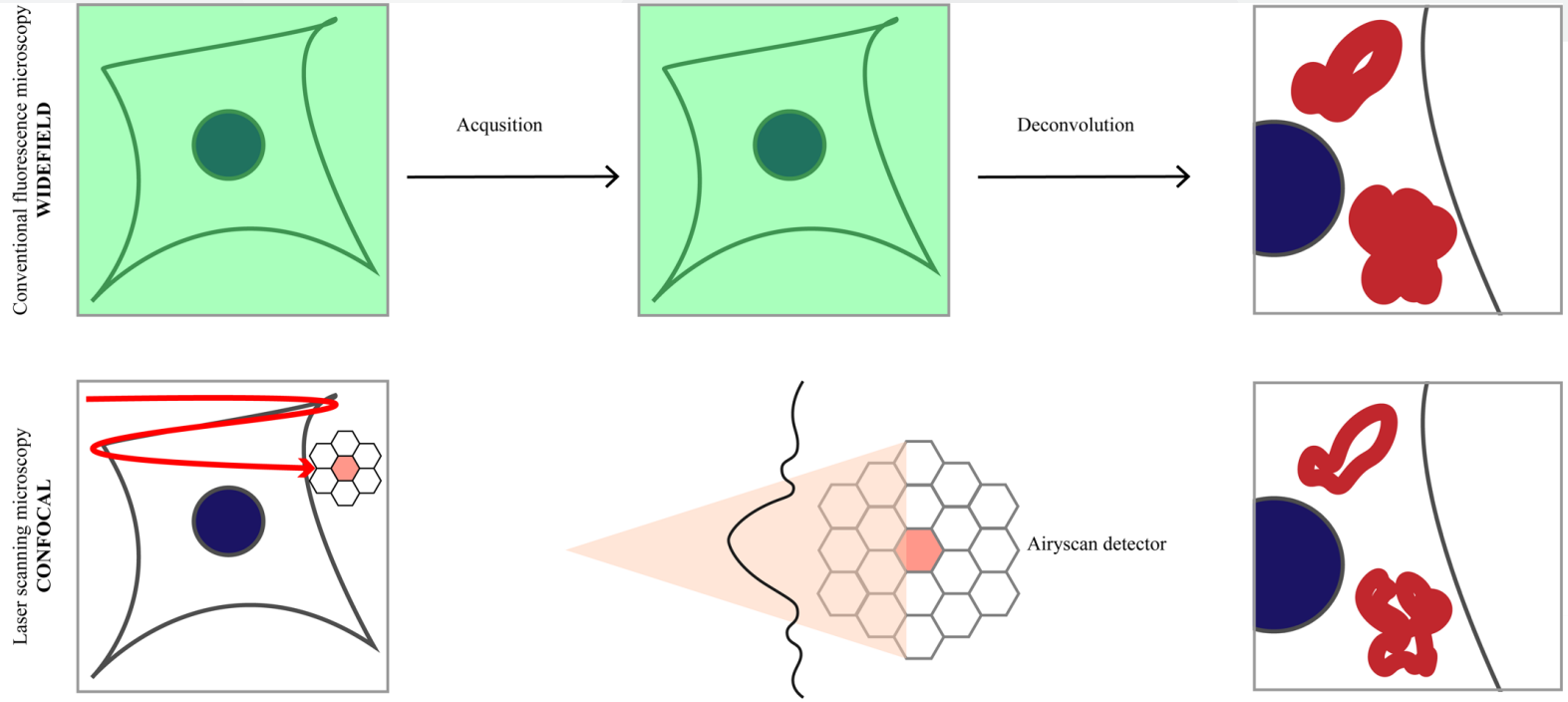
**Challenges and trade-offs in super-resolution fluorescence microscopy.**



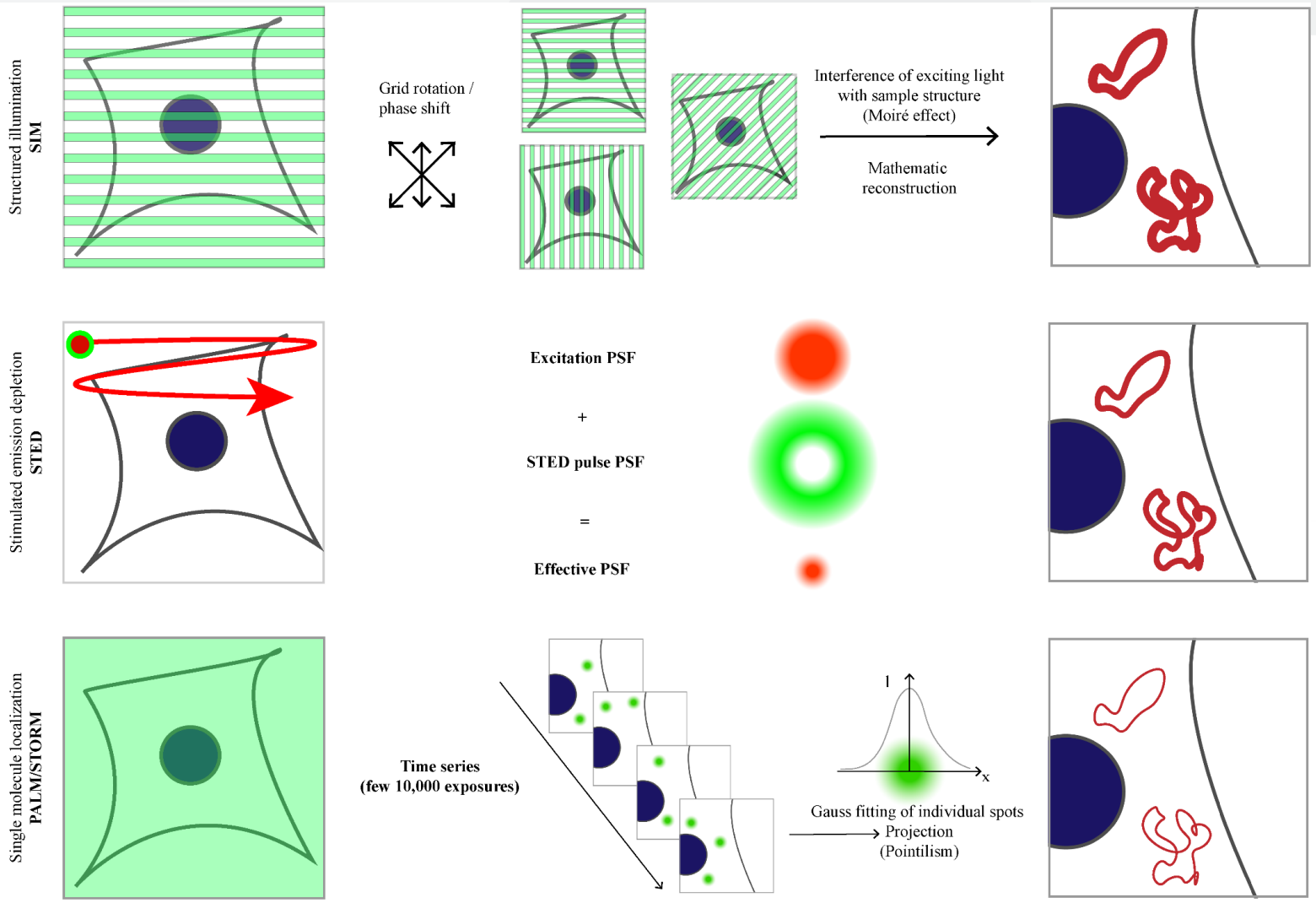



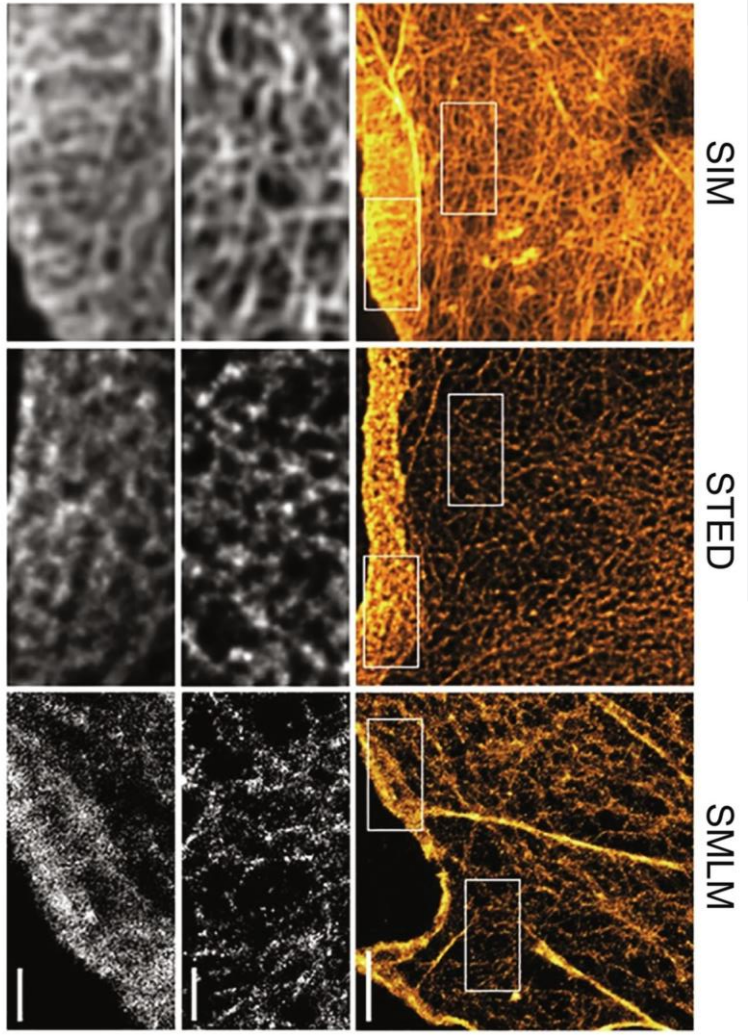

https://rupress.org/jcb/article/190/2/165/35915/A-guide-to-super-resolution-fluorescence

# Superresolution microscopy strategies



# Superresolution microscopy strategies



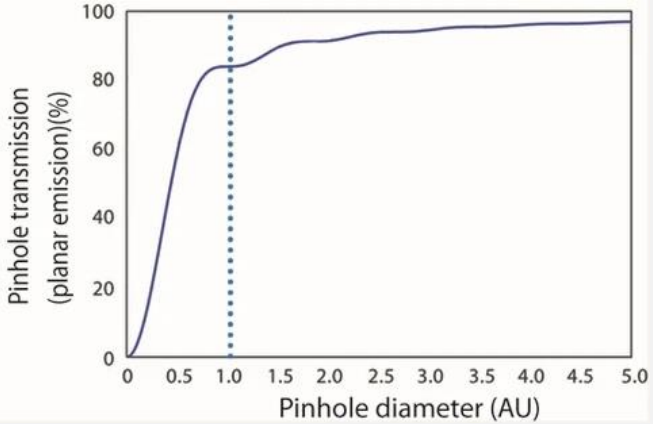

https://www.sciencedirect.com/science/article/pii/S106358231830019X#f0020

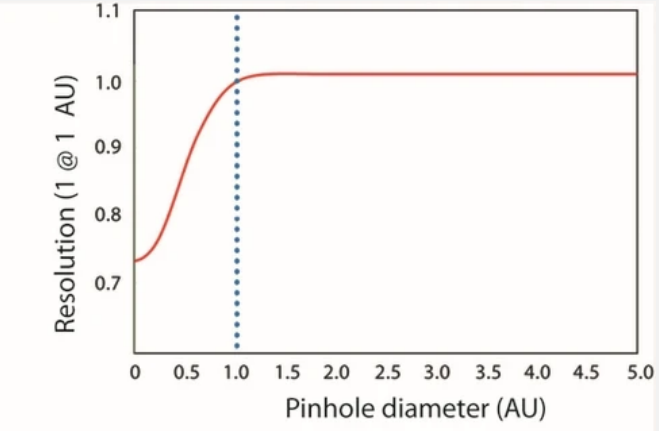
# Airyscan microscopy

## Confocal imaging with improved signal-to-noise ratio and super-resolution

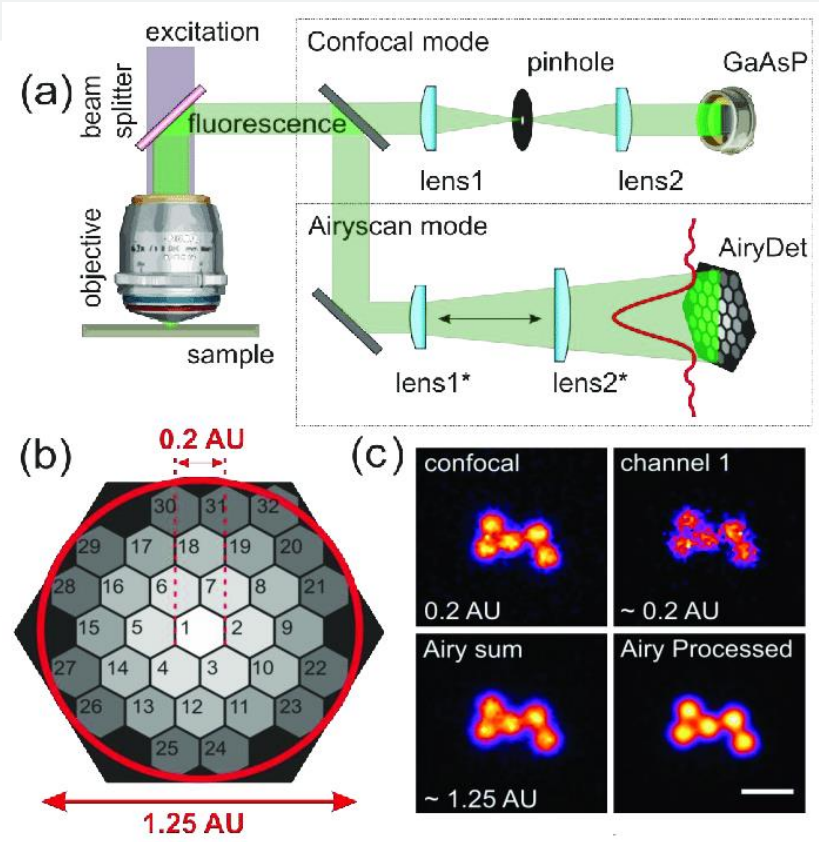
Seeing beyond

**Characterization of the effect of pinhole size on resolution and SNR in confocal microscopy**




Axial and lateral resolution of a confocal microscope improves with smaller pinhole (below 1 AU). But the signal decreases quickly!




Airyscan consists of an array of detectors where each element acts as a small pinhole. Each detector element provides its own signal, and the software builds the image from a combination of these signals. The array is able to collect more light from the microscope's open pinhole. This greatly improved light efficiency even comes with higher resolution.

https://www.researchgate.net/publication/318287874_Exploring_the_Potential_of_Airyscan_Microscopy_for_Live_Cell_Imaging

# Airyscan microscopy

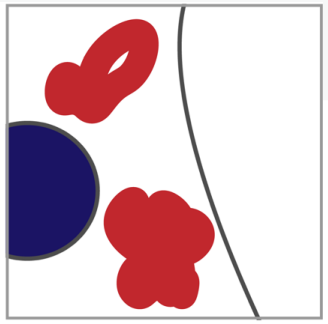
## Sample preparation

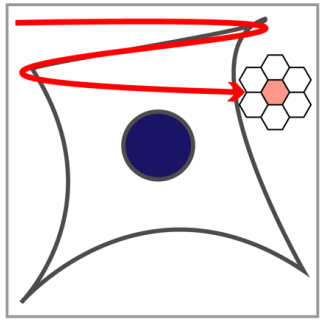
Standard fixation and sample handling

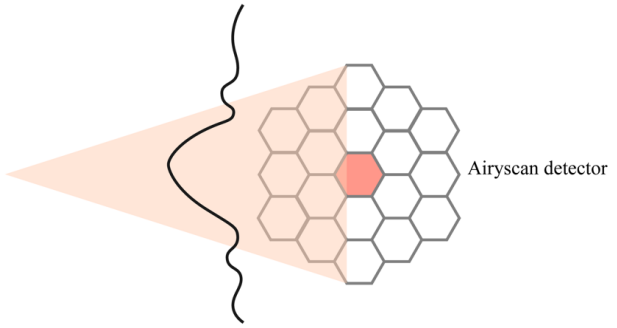
Common fluorescence labelling

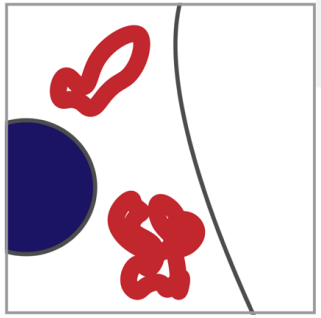
Airyscan detector acquisition

Ariscan processing







# Airyscan **advantages and challanges**

## Advantages

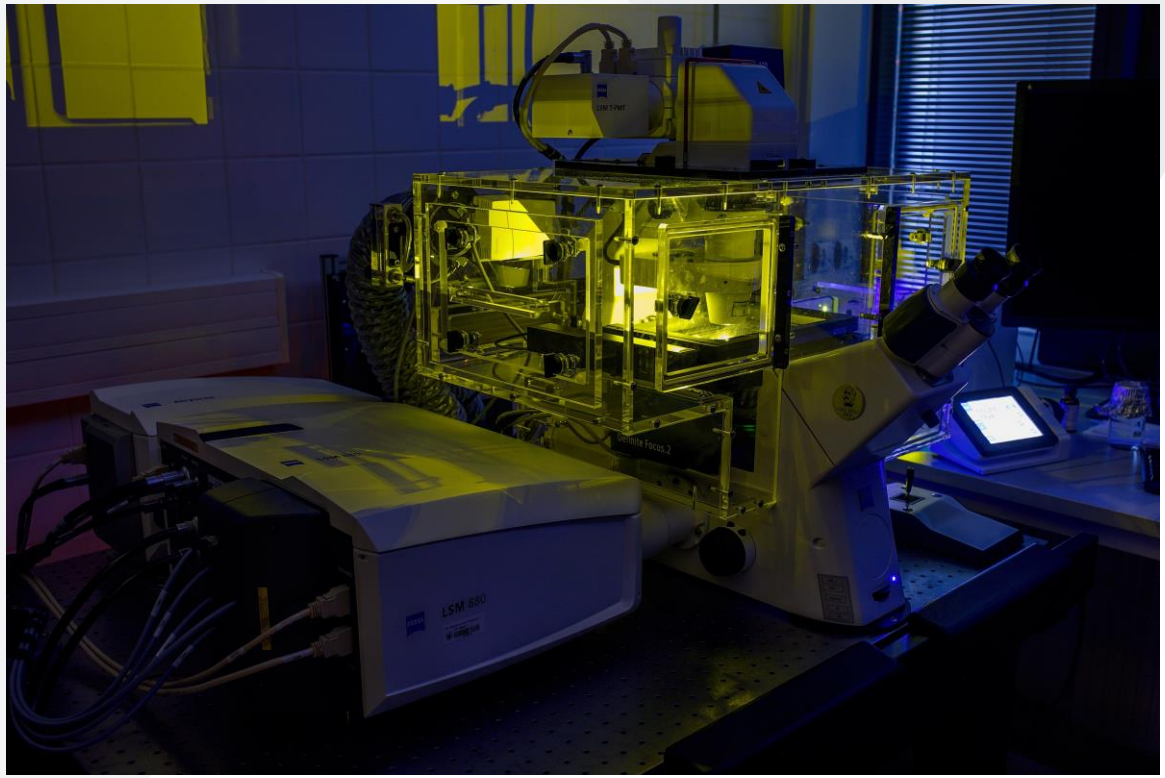
- Compatibility with various samples
- Useful for any photostable fluorophore
  - Little adaption for sample preparation
- Good live cell imaging condition
- Resolution improvement
- Low phototoxicity

## Challenges

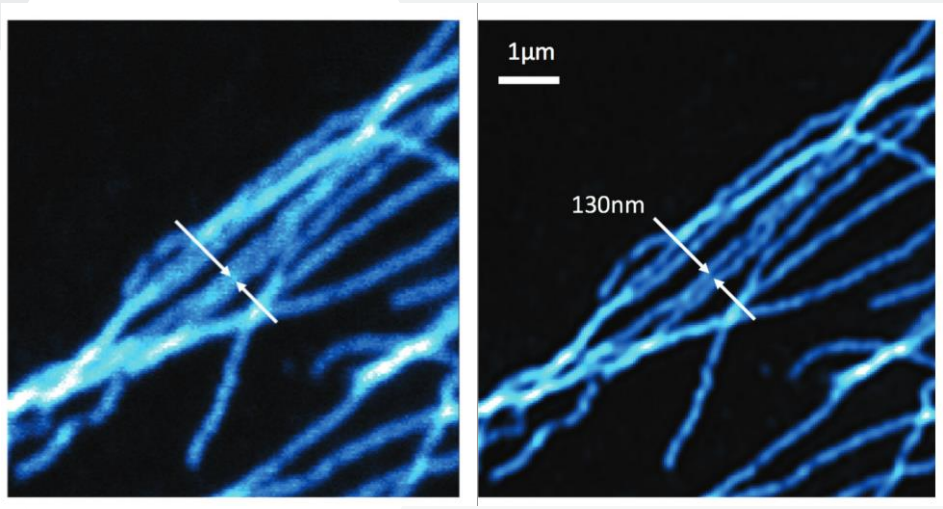
- Speed
- Limited sample thickness
- Subject to algorithmic effects due to required mathematical post-processing

# Airyscan microscopy
## Applications

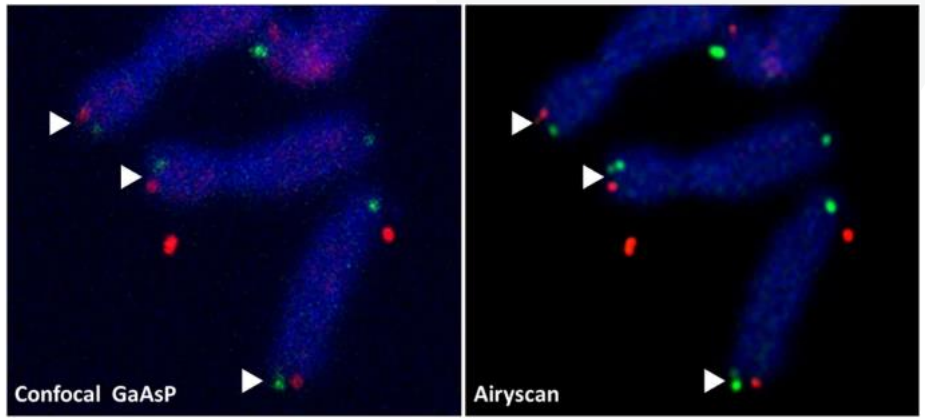
Zeiss LSM880 (Cellim-CEITEC)



Comparison of confocal (left) and Airyscan (right) microscopy
***Microtubules labled with Alexa 561, (Zeiss)***



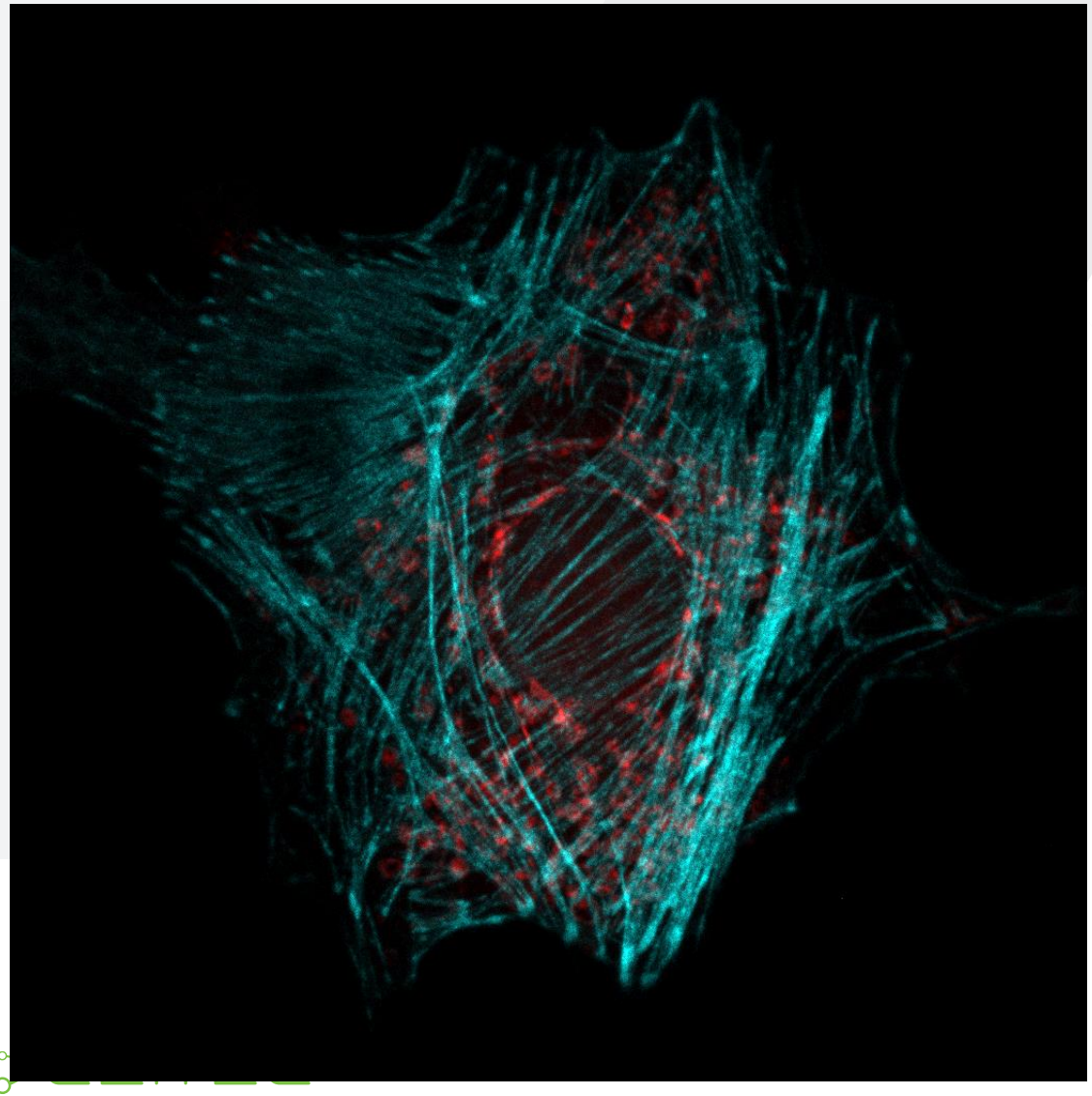

Comparison of Airyscan (left) and confocal (right) microscopy
***Stalled forks and telomere breakage, (J. Karlseder, Molecular and Cell Biology laboratory)***



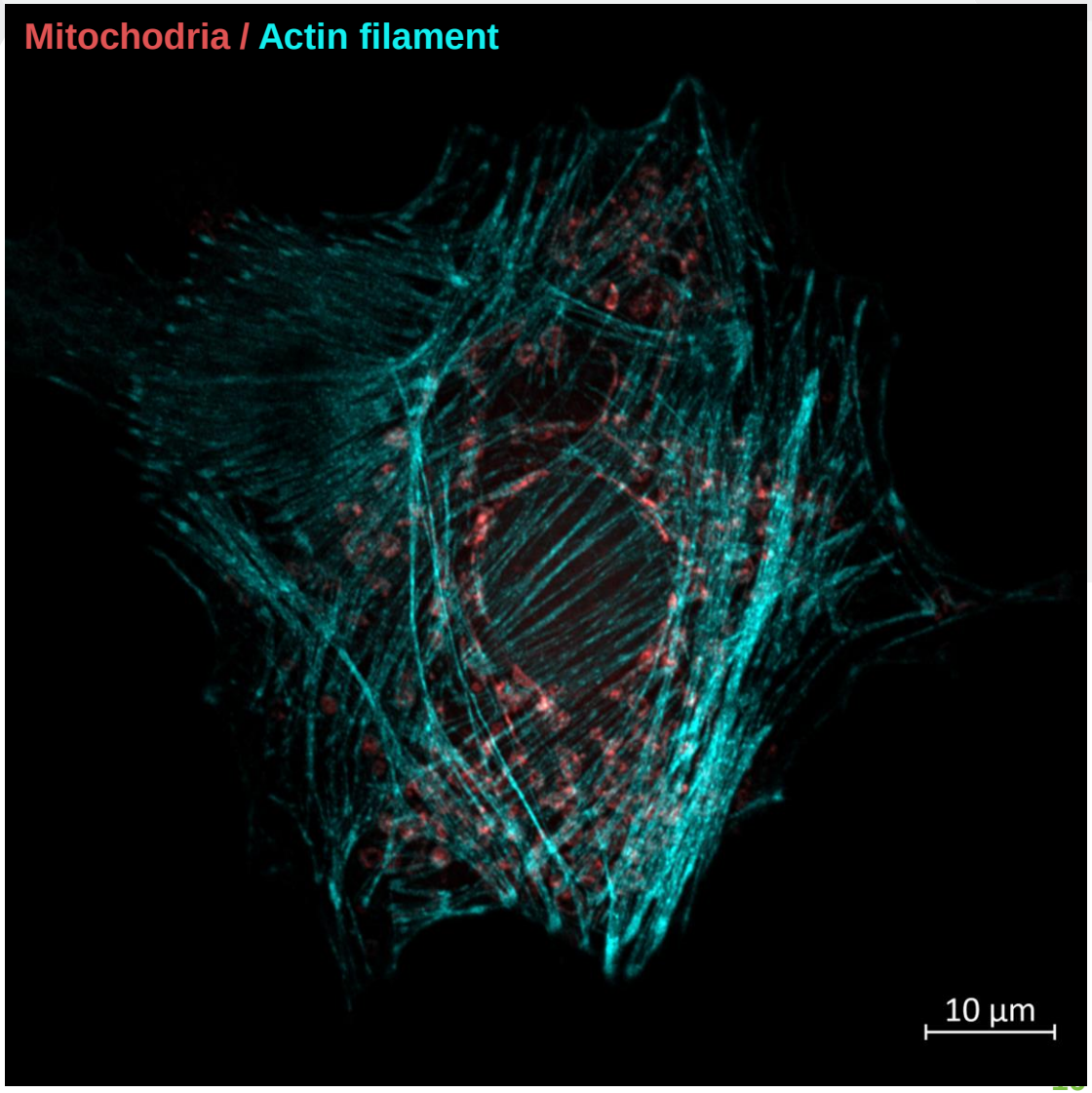
# Airyscan microscopy

## Applications – LSM880 airyscan/confocal (CELLIM)

Confocal



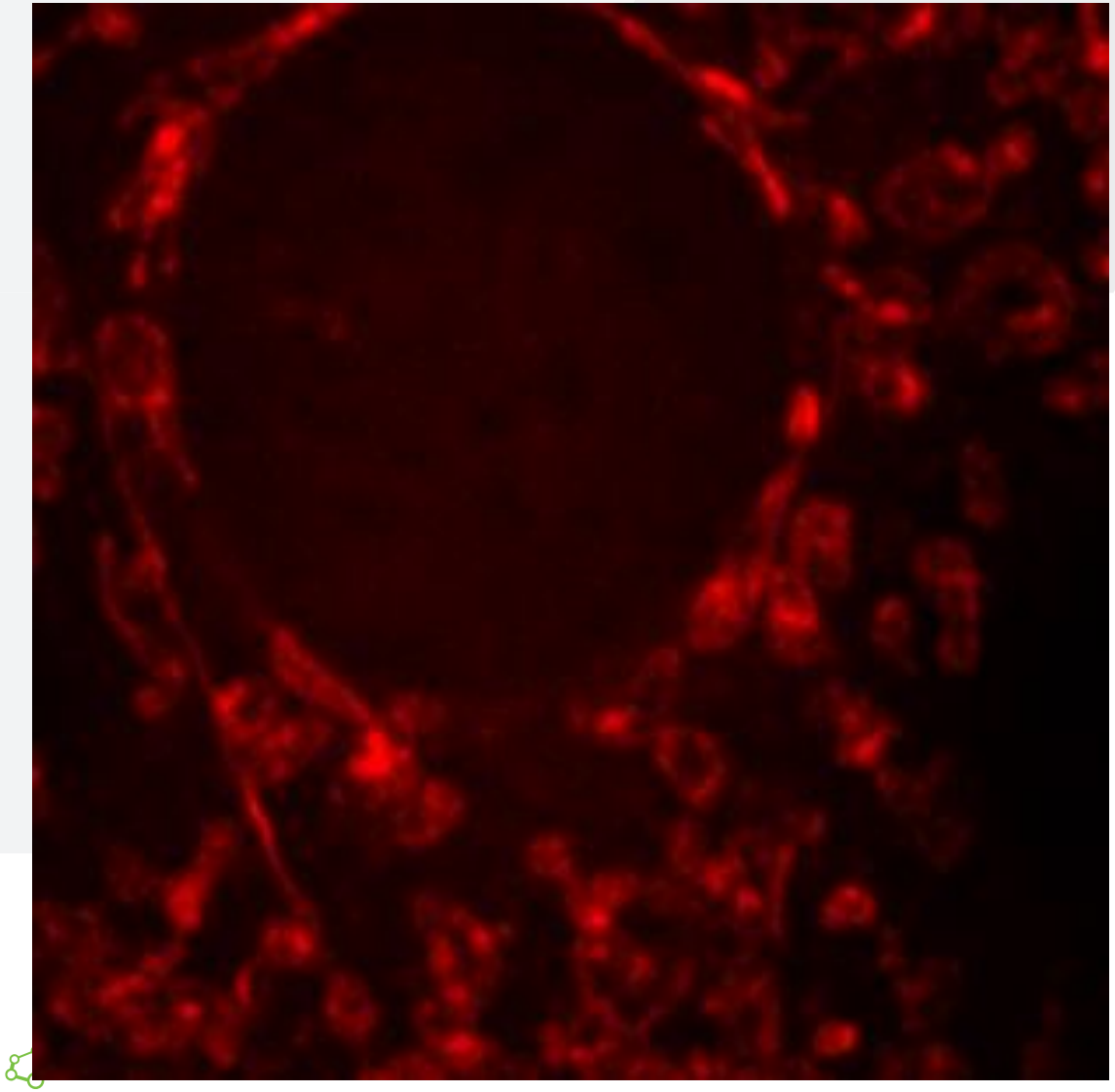
Airyscan module



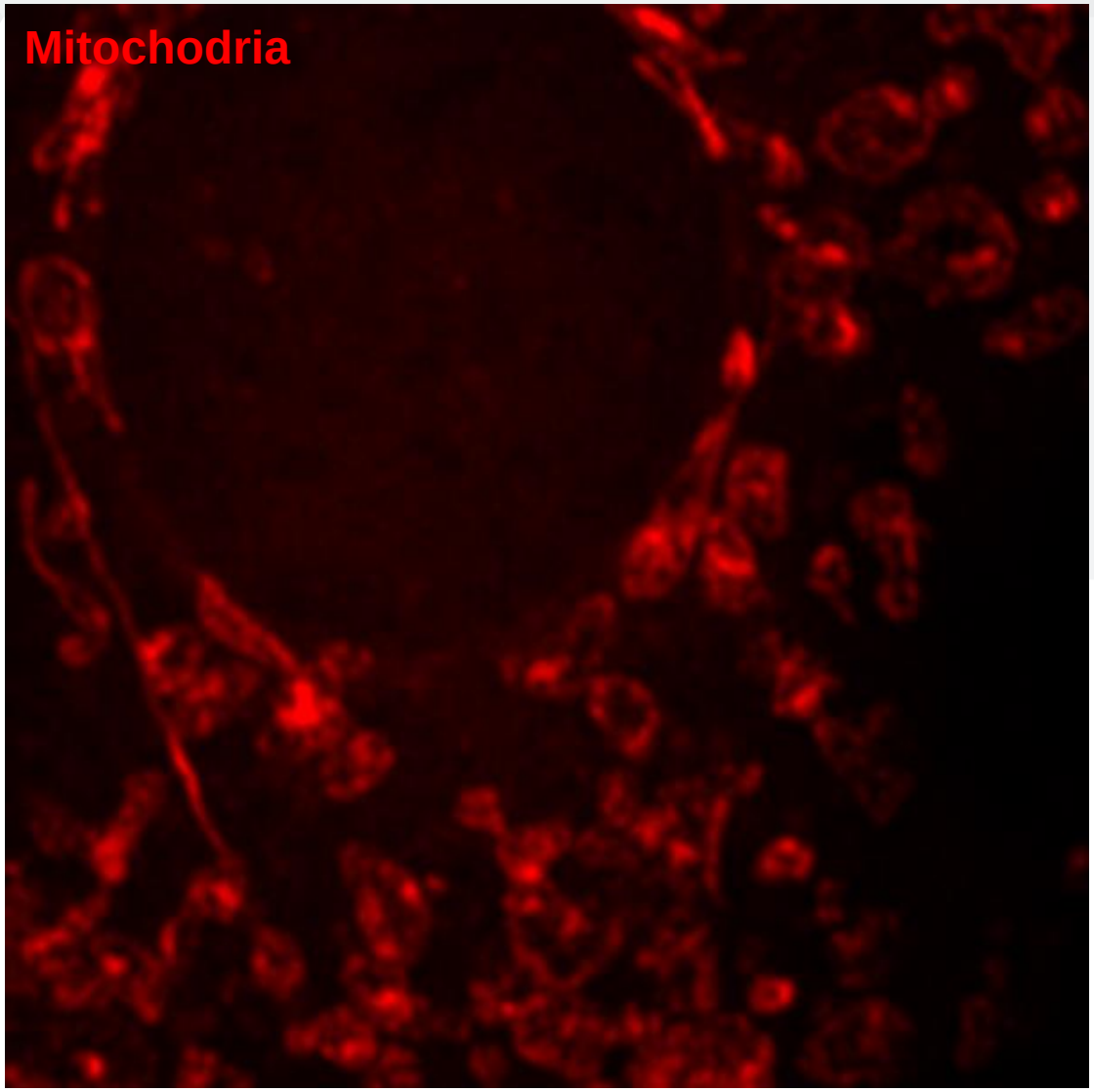
# Airyscan microscopy

## Applications – LSM880 airyscan/confocal (CELLIM)

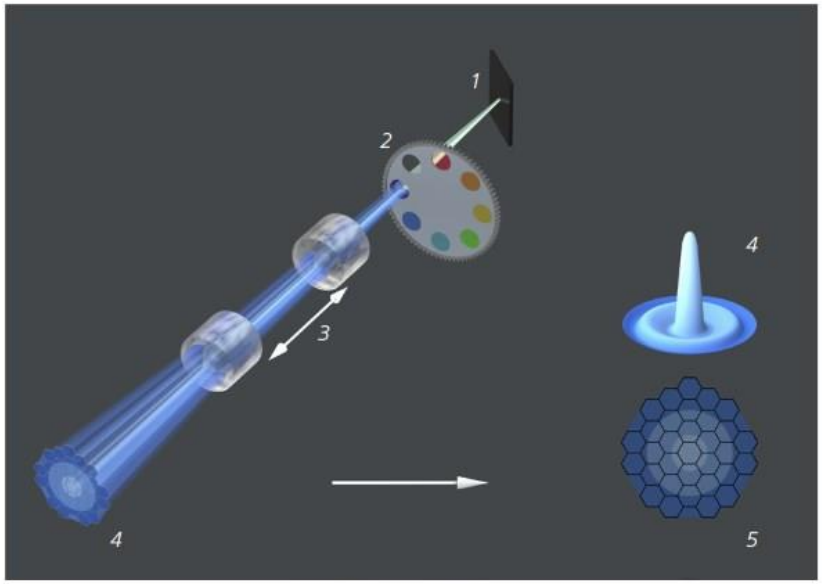
Confocal module



Airyscan module

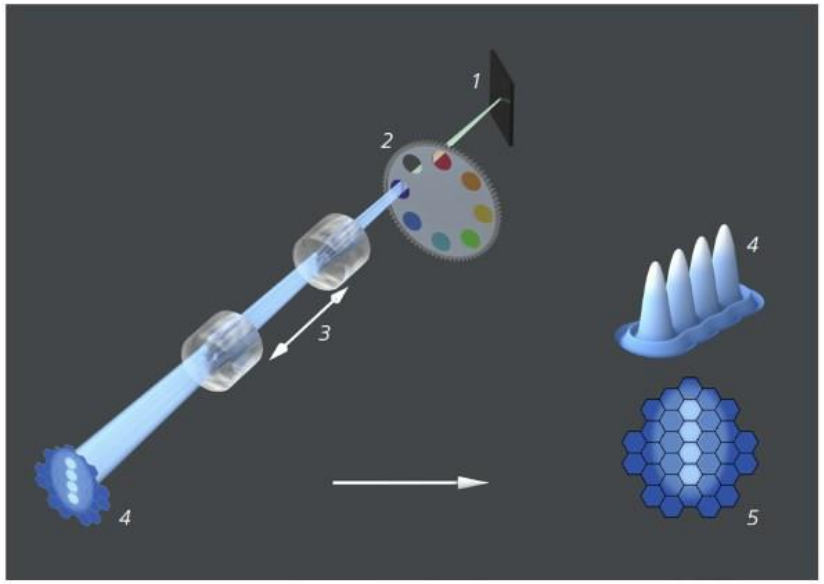


# Airyscan microscopy
## Modalities: Airyscan FAST module




1. Mirror
2. Emission filters
3. Zoom optics
4. Airy disk
5. Airyscan detector

**Figure 1** Beampath Airyscan




1. Mirror
2. Emission filters
3. Zoom optics
4. Airy disk
5. Airyscan detector

**Figure 2** Airyscan Fast mode

*The Fast module for AiryScan shapes the excitation spot into an ellipse, the AiryScan array detector is then used to detect 4 pixels simultaneously,* ***increasing scan speed 4-fold (27 fps for 480x480)*** *while still* ***improving resolution significantly*** *compared to conventional confocal (170 nm lateral).*

Users can capture more structural information about **highly dynamic processes**. This is the highest speed of any linear scanning confocal microscope. It also has superresolution and sensitivity modes, which increase its flexibility.



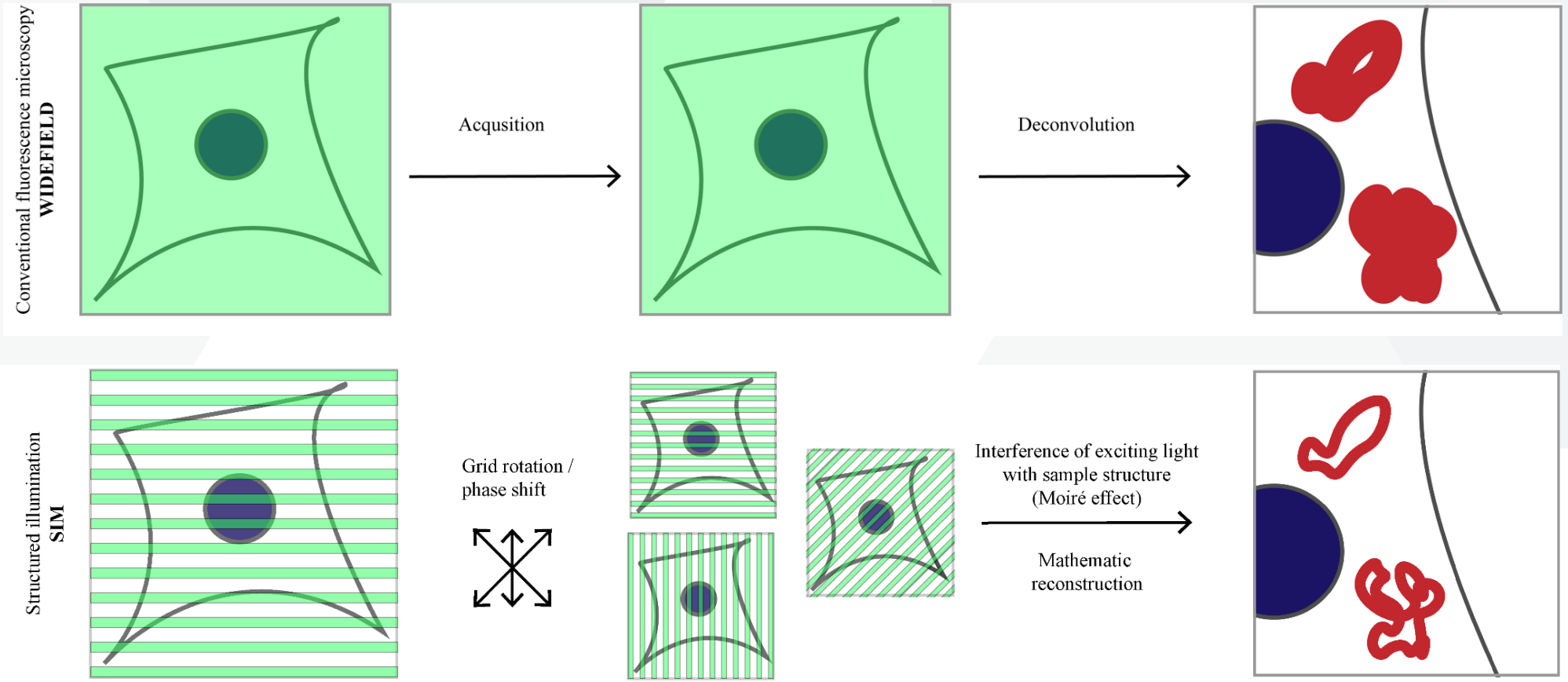
https://www.nature.com/articles/nmeth.f.398

# Structured ilumination microscopy (SIM)

## SIM combines fluorescence, widefield-based structured illumination and digital image reconstruction (2002)

Rainer Heintzmann

Mats Gustafsson

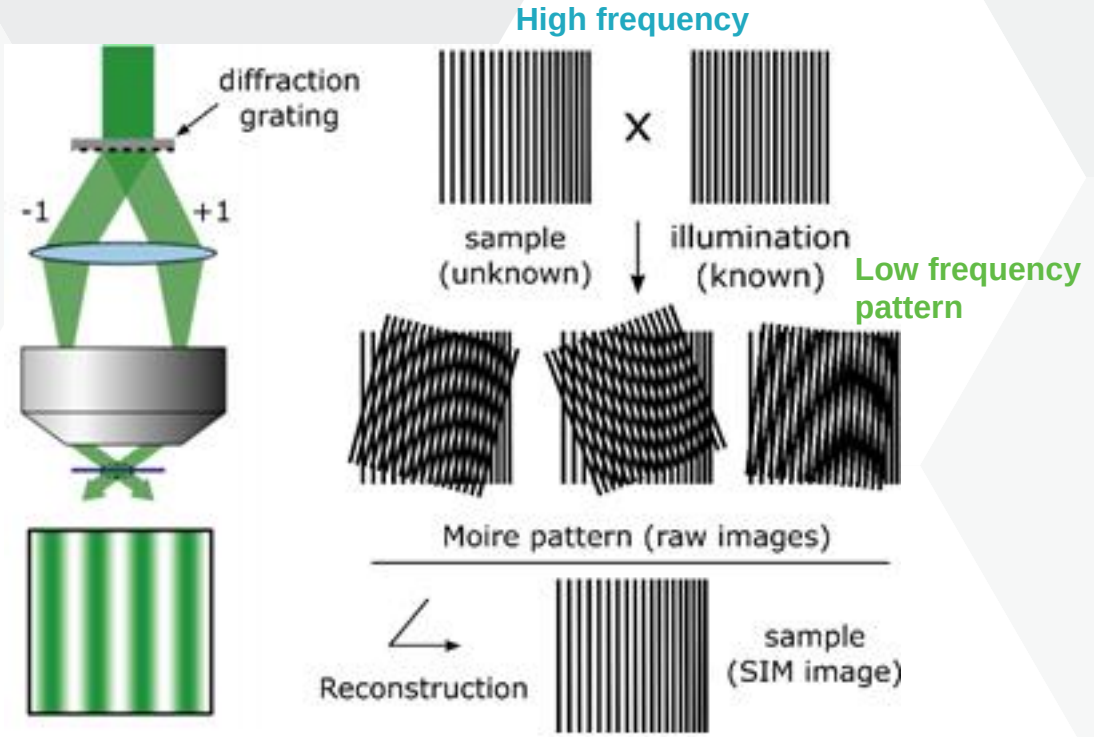


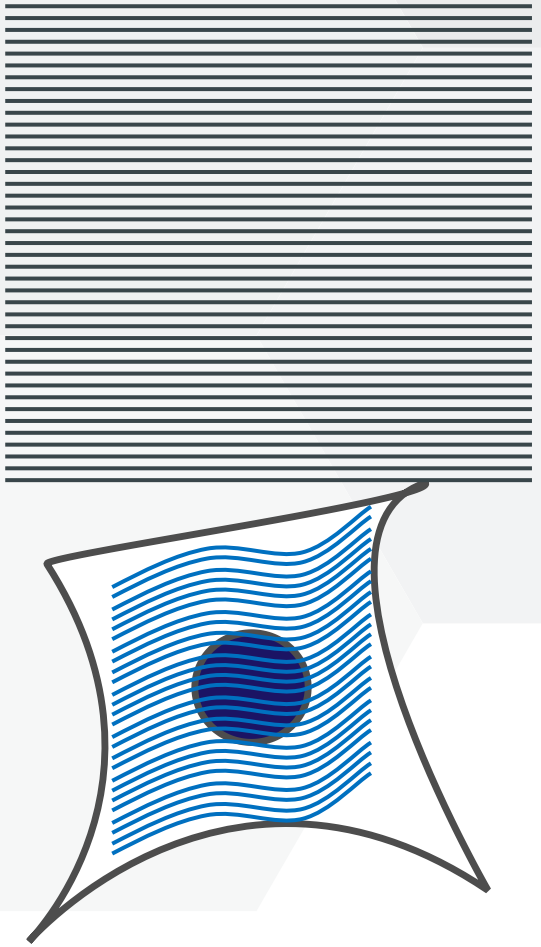

SR-SIM uses the information contained in the known illumination pattern

# Structured ilumination microscopy (SIM)

**The Super-Resolution SIM technique principle is to use interference-generated light patterns to create a Moiré effect**

This allows to extract information with higher resolution.





https://onlinelibrary.wiley.com/doi/full/10.1046/j.1365-2818.2000.00710.x

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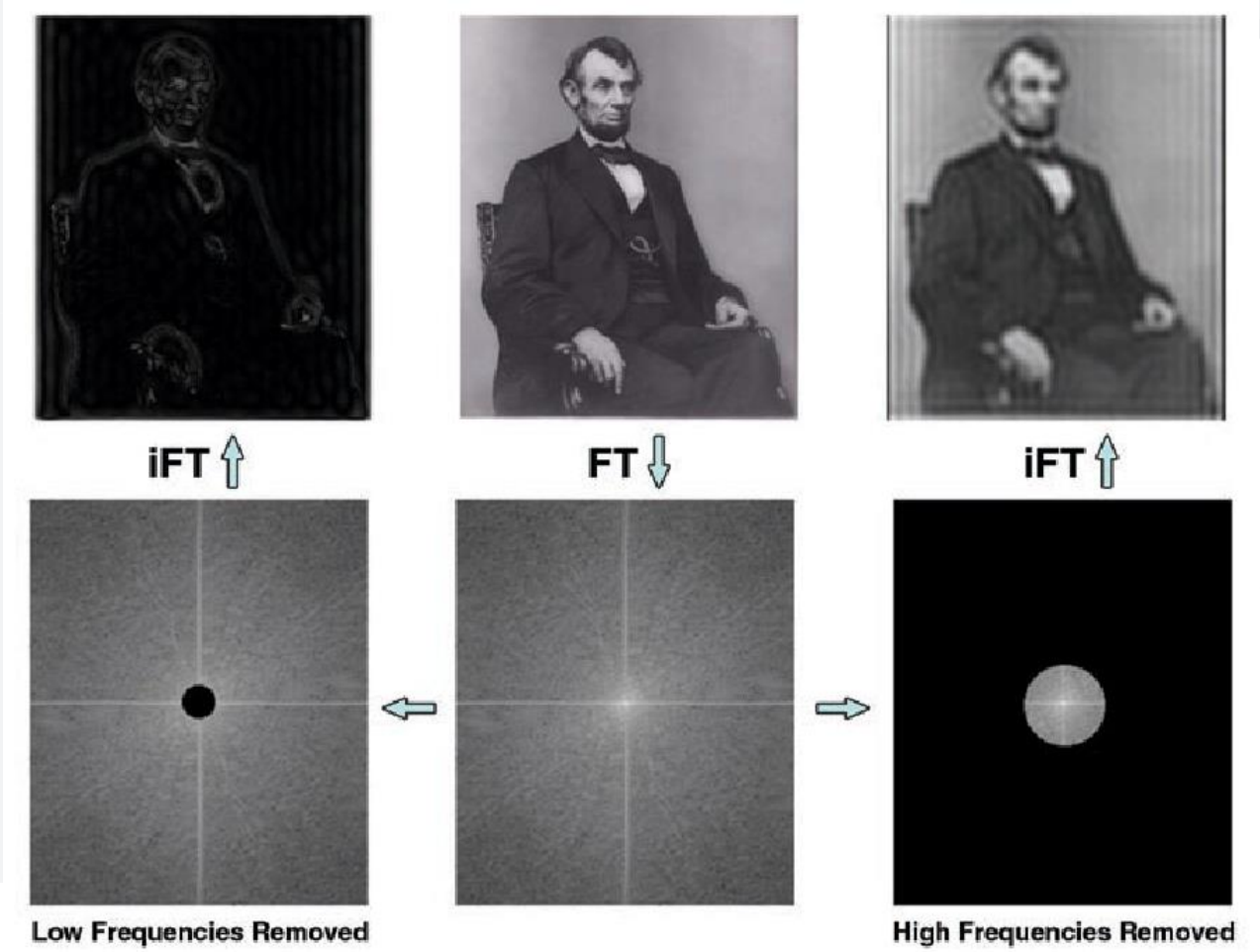
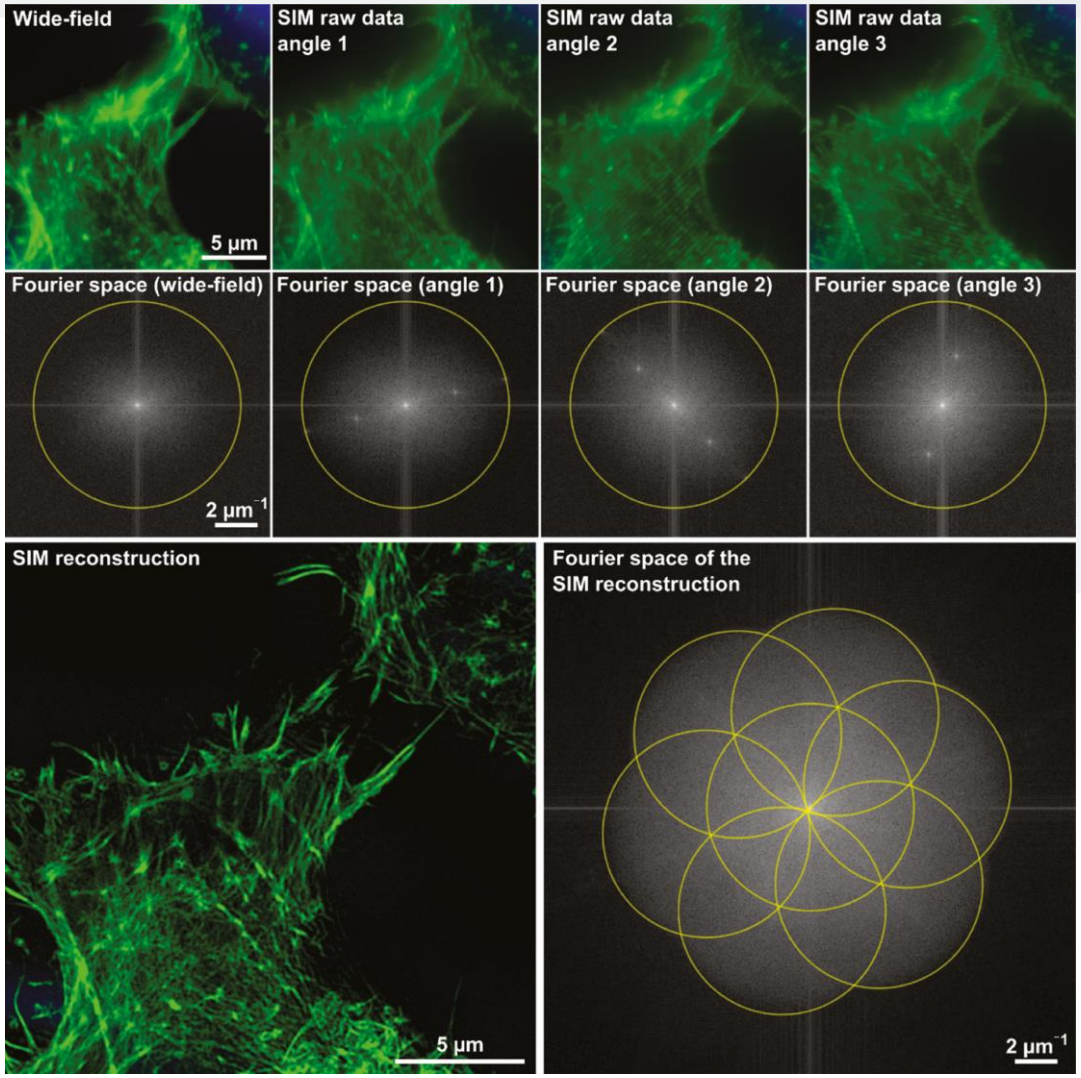
## The Super-Resolution SIM technique principle is to use interference-generated light patterns to create a Moiré effect




https://www.semanticscholar.org/paper/An-introduction-to-the-Fourier-transform%3A-to-MRI.-Gallagher-Nemeth/8c65f1e10b198149ac5c8fd2c4d6888b1769ffe6/figure/7

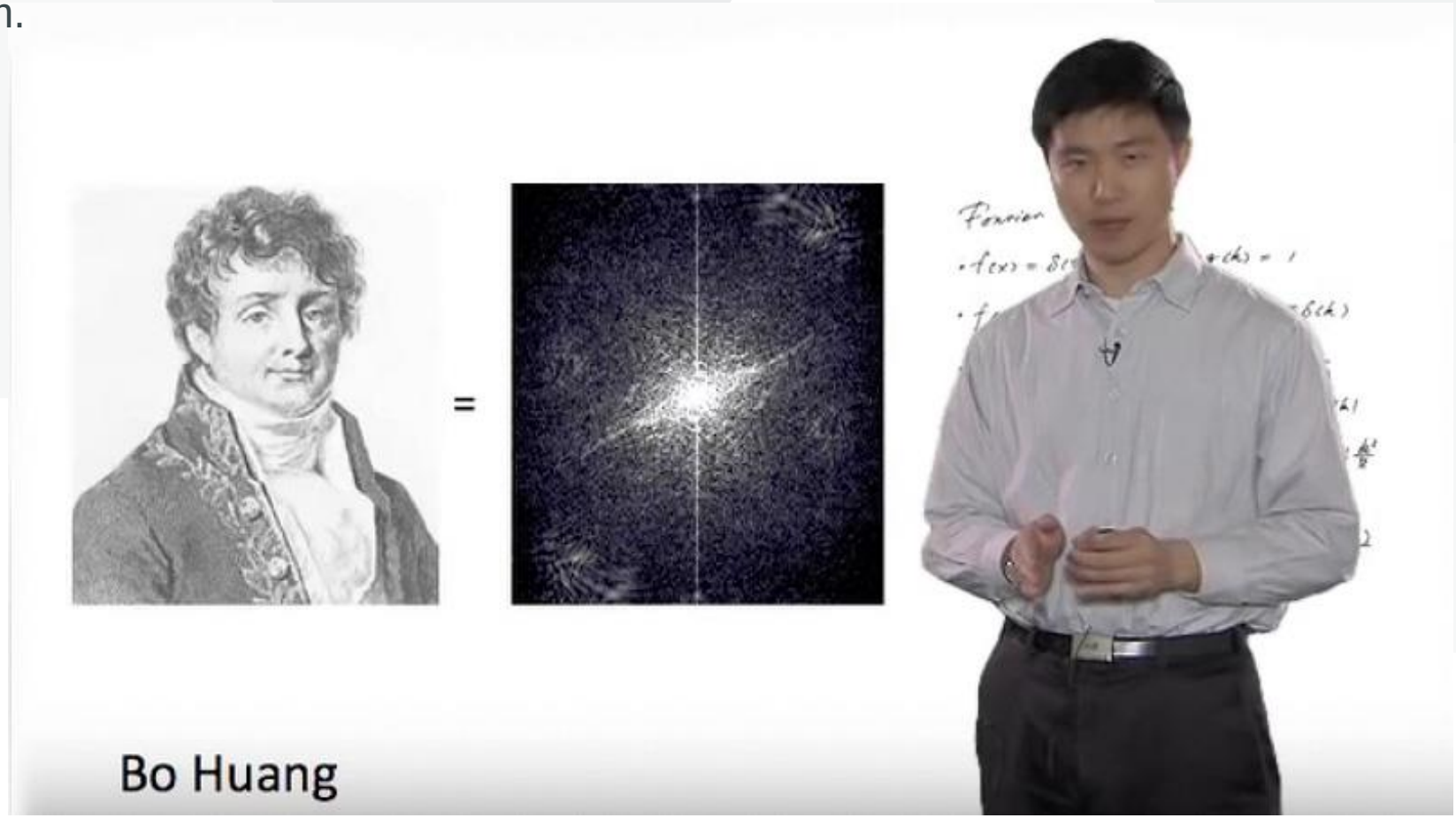



https://www.degruyter.com/document/doi/10.1515/nanoph-2017-0055/html

# Fourier Space: Fourier Transform

**This lectures explains the Fourier transform in terms understandable to non-mathematicians, and explains the relations with microscopy.**

Fourier transform is intimately associated with microscopy, since the alternating planes occurring in the microscope (focal plane – back-focal plane, etc.) are related to each other by a function very similar to the Fourier transform.

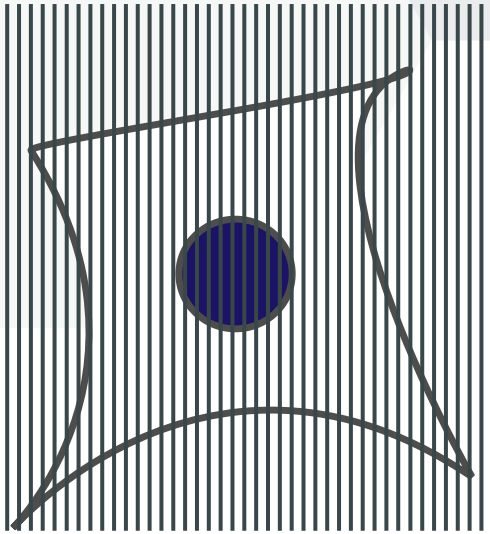



https://www.ibiology.org/talks/fourier-transform/

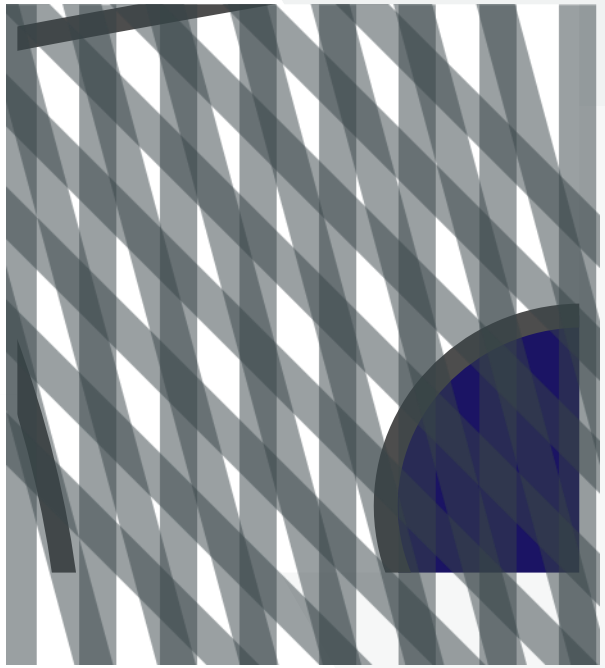
# Structured ilumination microscopy (SIM)

## SIM combines fluorescence, widefield-based structured illumination and digital image reconstruction

### Classic SIM



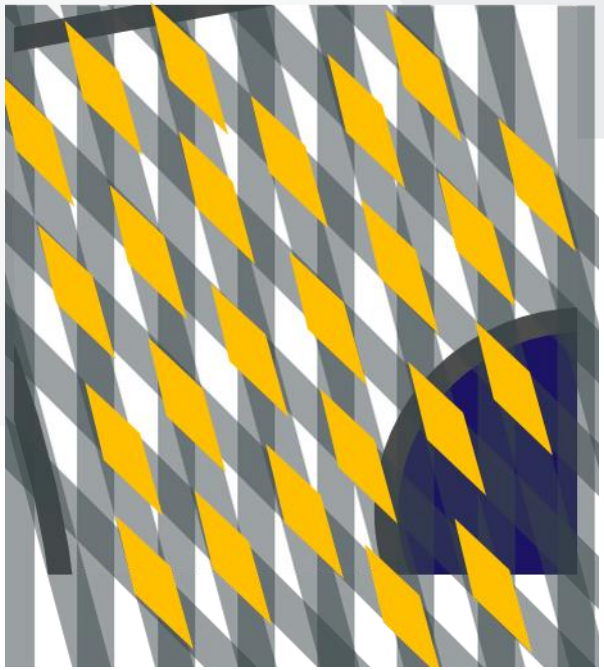
Redundant light exposure



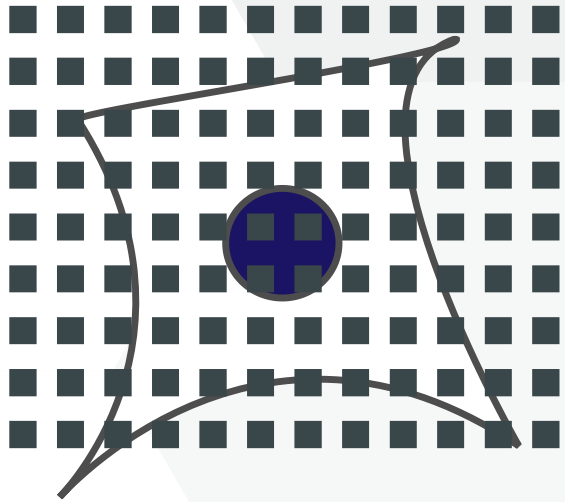
# Structured ilumination microscopy (SIM)

## Widefield imaging at super-resolution

### Classic SIM



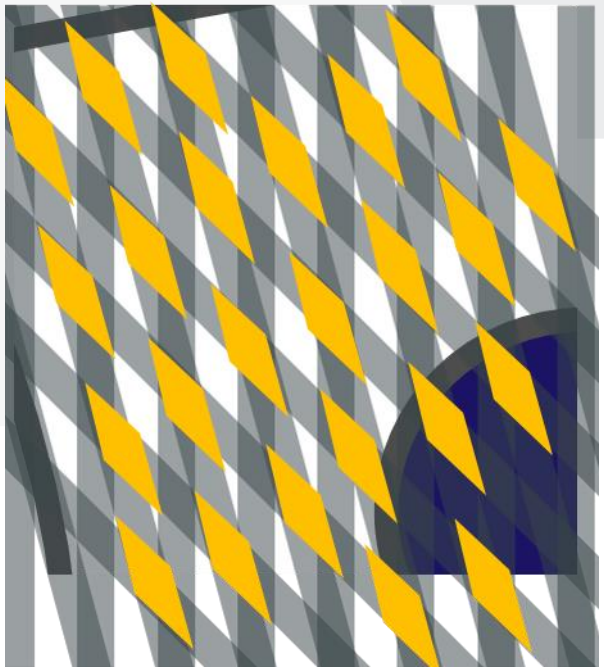
### Lattice SIM



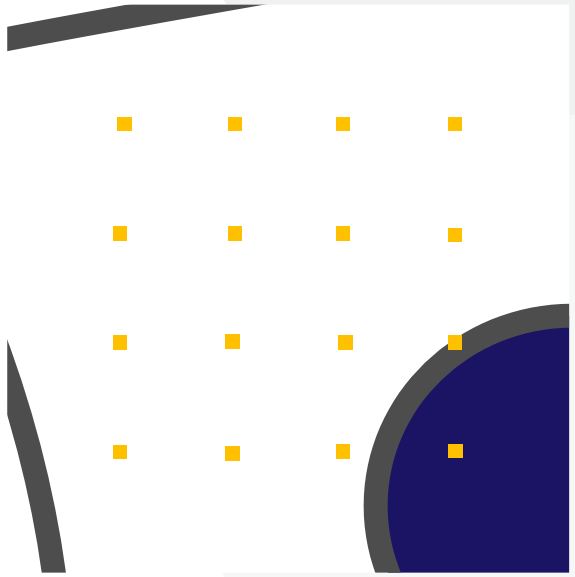
# Structured ilumination microscopy (SIM)

## Widefield imaging at super-resolution

### Classic SIM



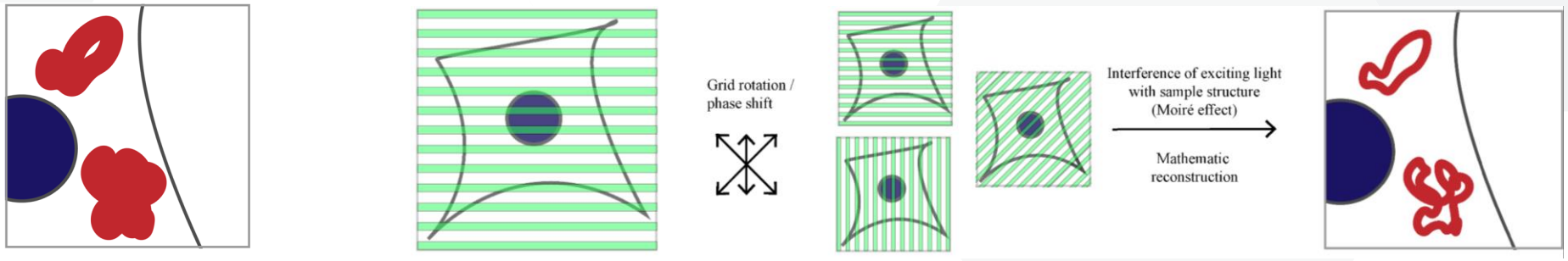
### Lattice SIM



- **Image faster** with high image quality and low bleaching
- **Better image quality** at the same speed and low bleaching
- **Image more gently** with high speed and image quality

# Lattice SIM
## Sample preparation

Standard fixation and sample handling → common fluorescence labelling → SIM acquisition → SIM processing



# SIM **advantages and challanges**

## Advantages

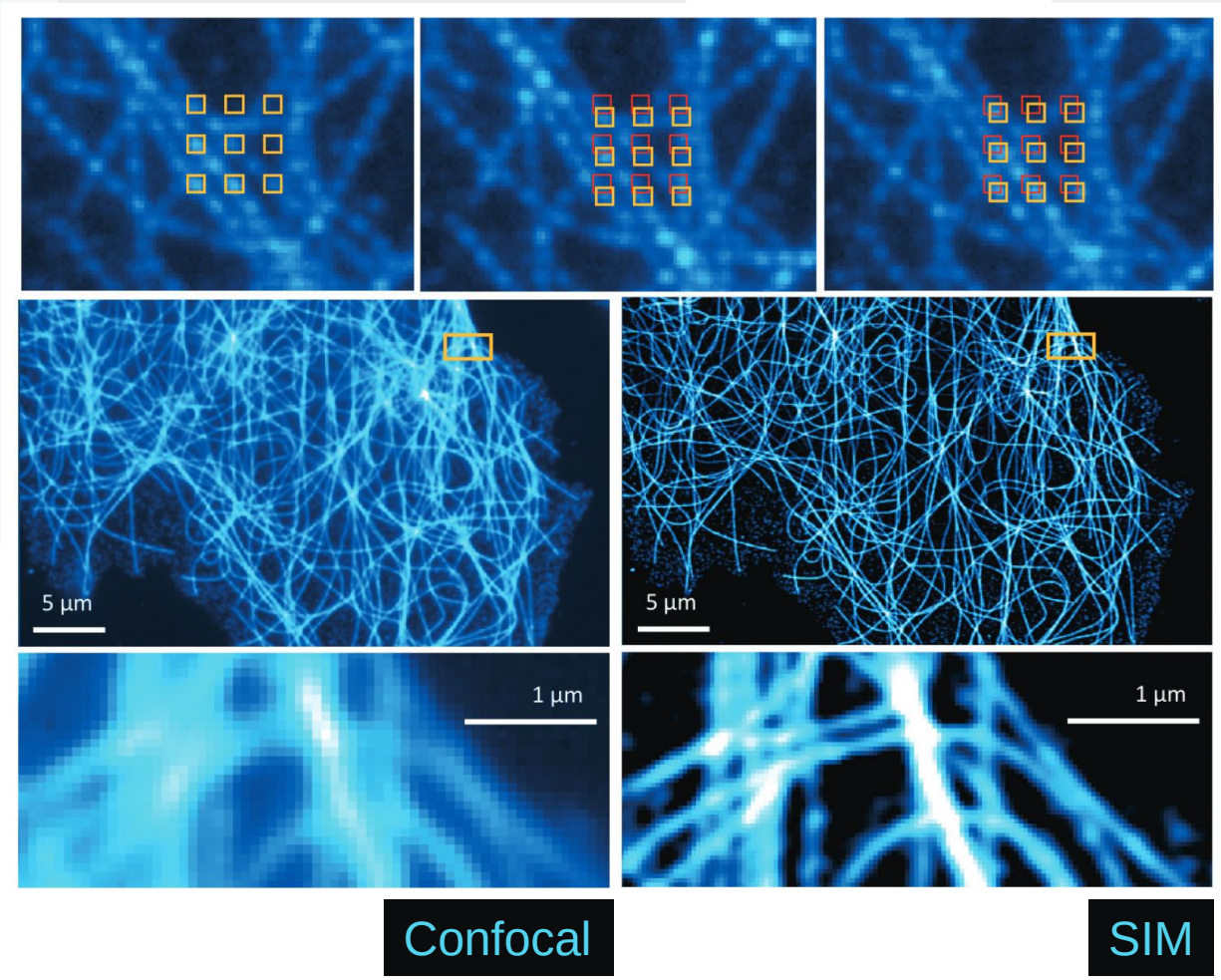
- Compatibility with various samples
- Useful for any photostable fluorophore
  - Little adaption for sample preparation
- Good live cell imaging condition
- High throughput and fast acquisition
- Resolution improvement
- Lattice SIM up to 100 µm distance from cpverslip surface
- Straightforward data analysis

## Challenges

- Limited sample thickness
- Phototoxicity (depends on sample type)
- Subject to algorithmic effects due to required mathematical post-processing

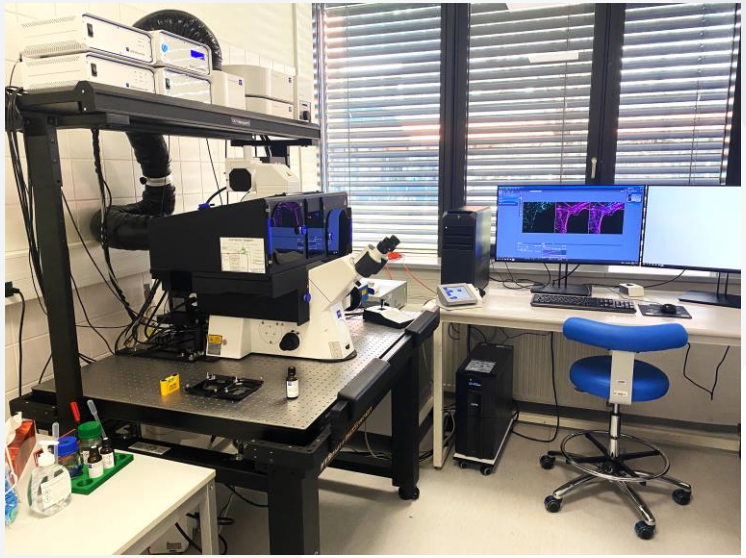
# Lattice SIM
## Applications

Lattice SIM acquisiotion process
***Tubulin structures labled with Alexa 561, (Elyra7, Zeiss)***

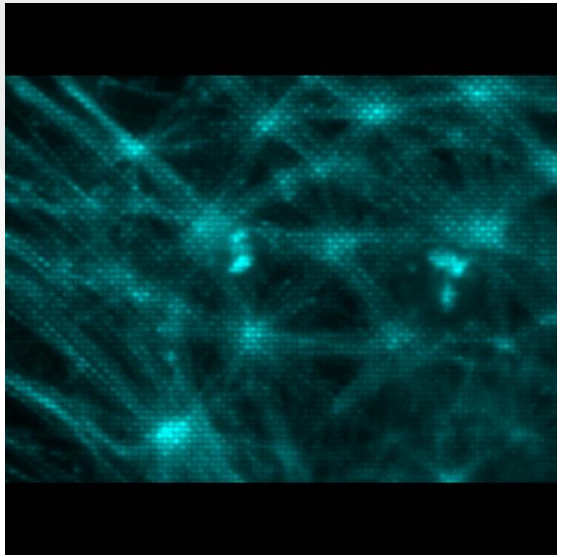


# Elyra7 Lattice SIM
## Applications (CELLIM)

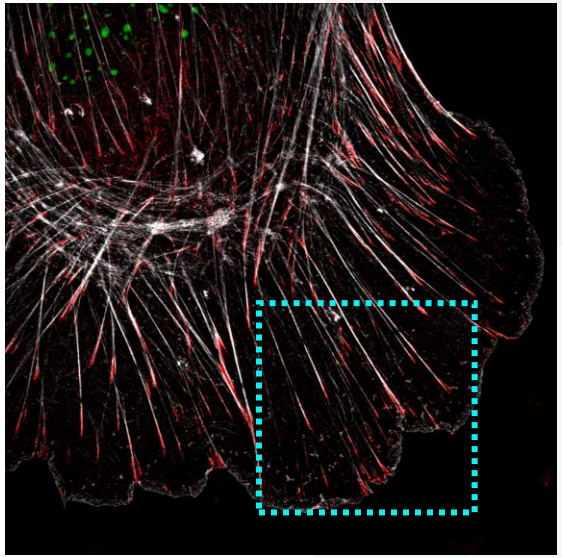
Zeiss Elyra7 (CELLIM)

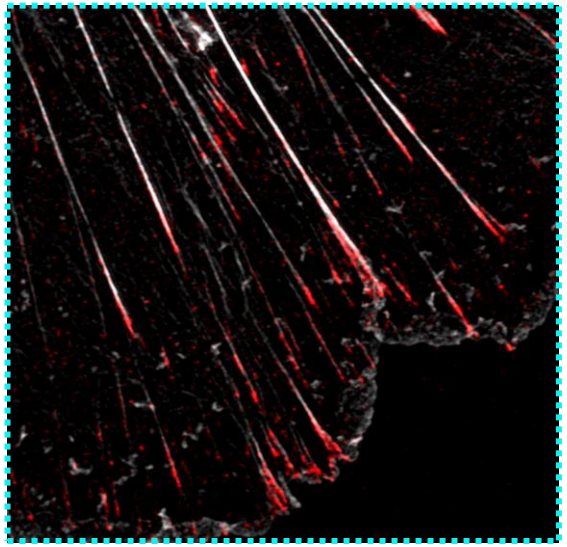


Lattice SIM demonstration (CELLIM)

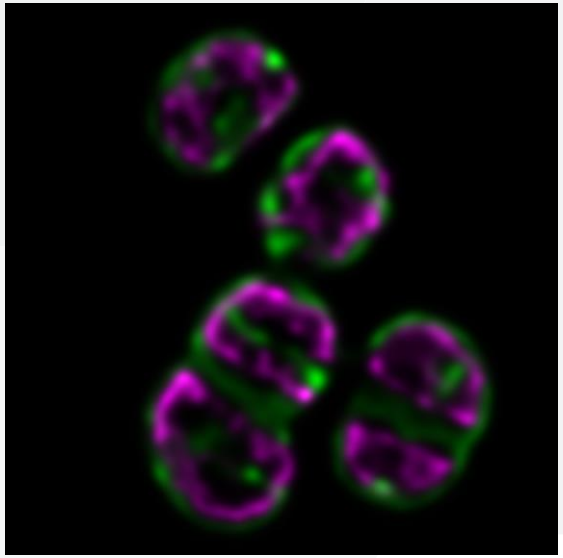


Growth cone of MEFs cells (Elyra7, CELLIM)
***Actin filament (white), FAKs (red)***

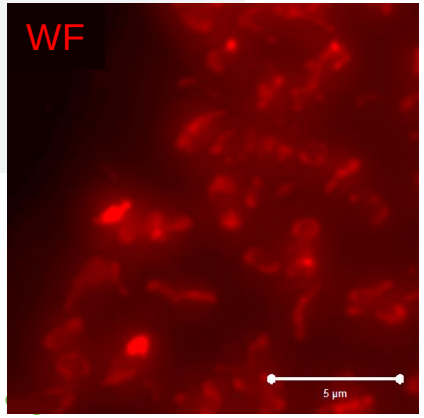


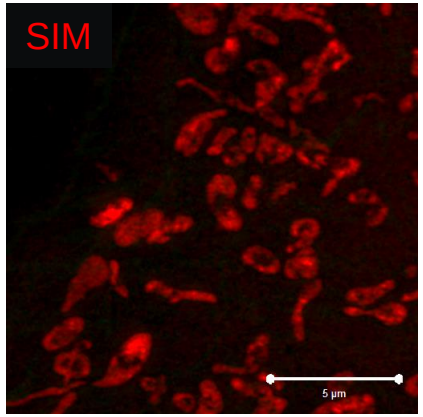


Staphyloccocus (Michaela Procházková, Pavel Plevka, Ceitec MU Brno)
***Cell mebrane (green), Nuclei (purple)***



Mitochodrial membrane stained with anti-TOM20 antibody, (Elyra7, CELLIM)



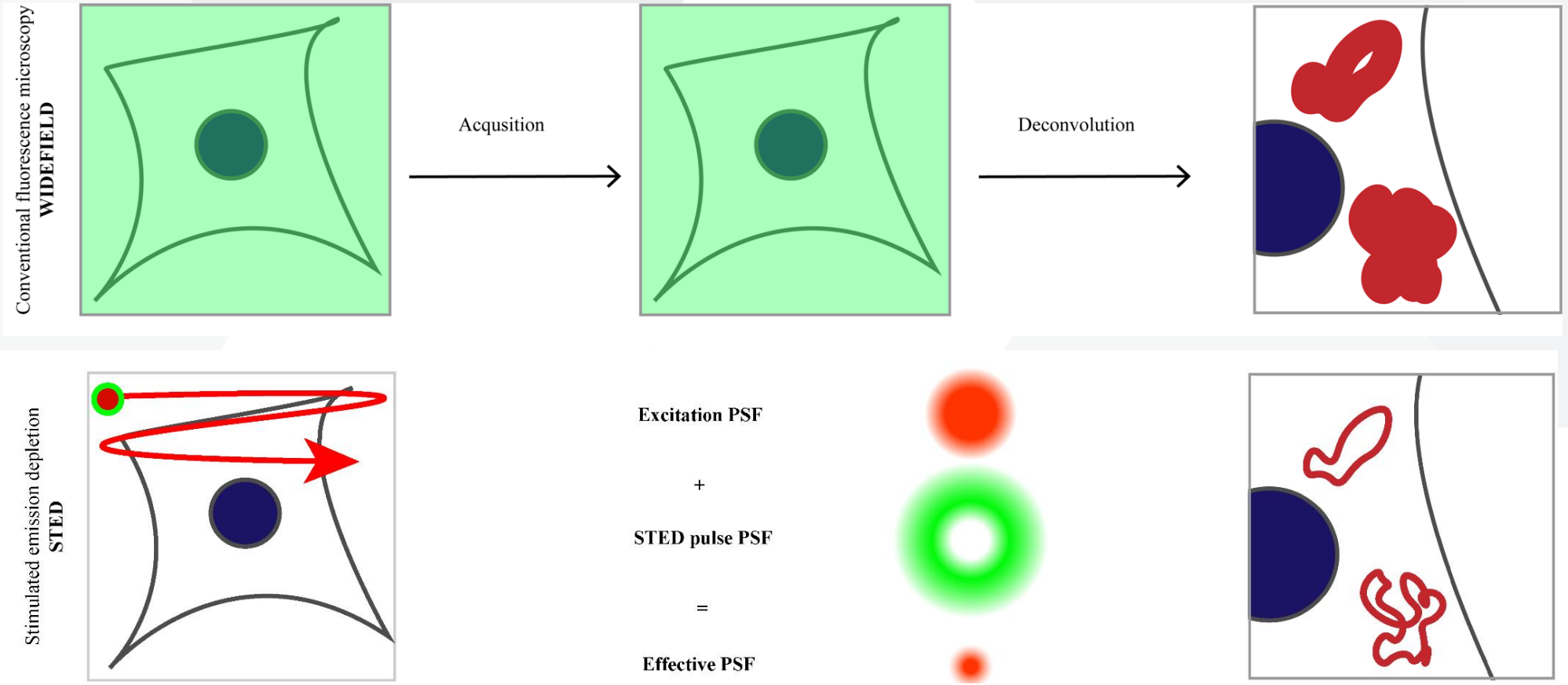
# Stimulated emission depletion microscopy (STED)

## PSF shaping with saturated emission depletion

Stephan Hell

Jan Wichmann (1994)
**Stimulated Emmision**

M. Kroug (1995)
**ground state Depletion**

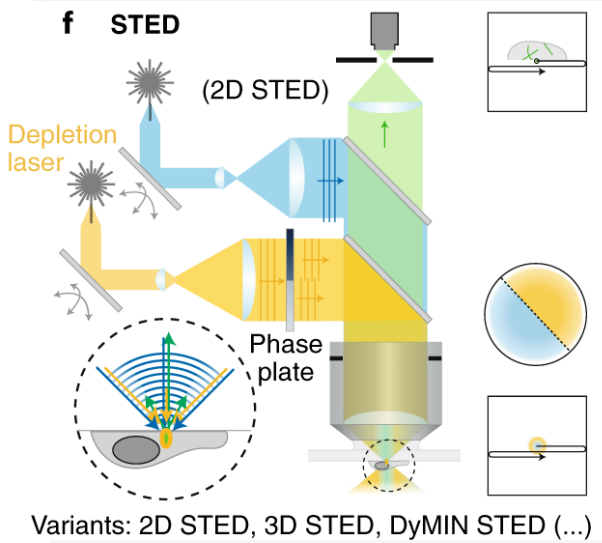


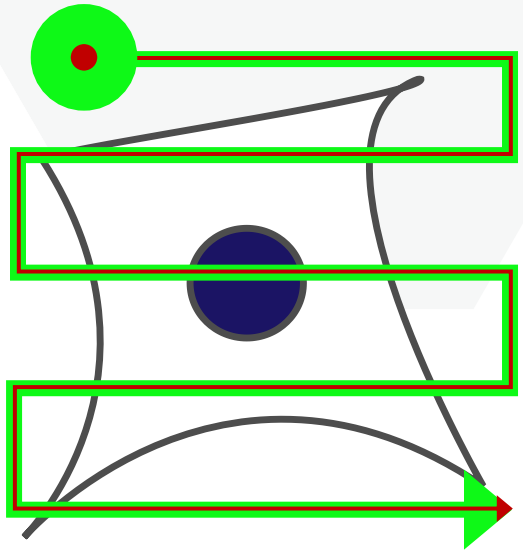
# Stimulated emission depletion microscopy (STED)

## PSF shaping with saturated emission depletion

**In STED microscopy:**

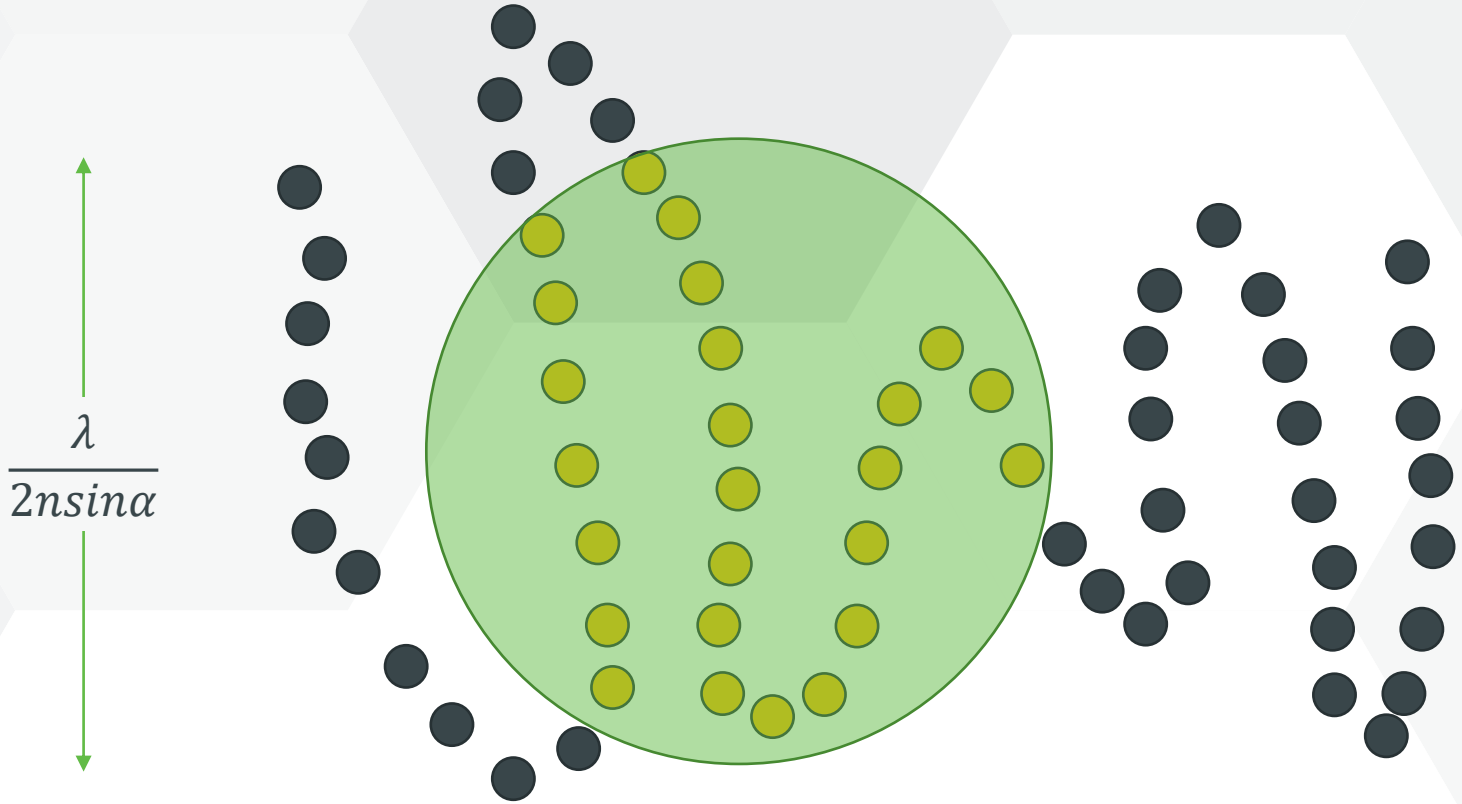
- Focal plane is scanned with two overlapping laser beams
- Typically being pulsed with a mutual deley
- The first laser excitates the flourophores
- The second longer wavelength laser drives the fluorophores back to the ground state by the process of stimulated emmision.
- A phase plate (phase mask) in the light path of the depletion laser generates a donut-shaped energy distribution, leaving only a small volume from which light can be emitted that is then being detetcted.
- Thus, the PSF is shaped to a volume smaller than the diffraction limit

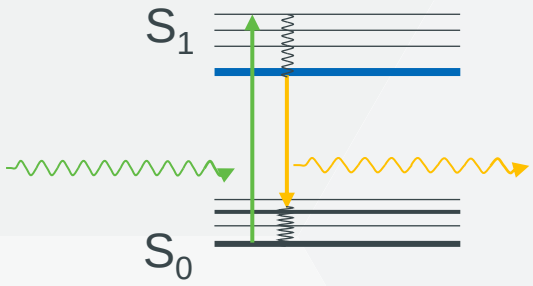


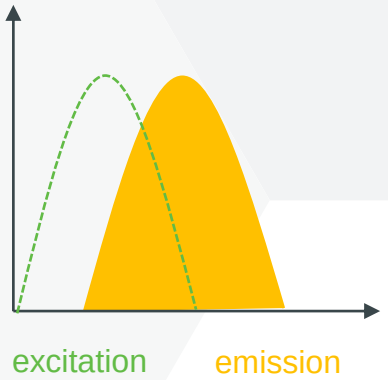



# Stimulated emission depletion microscopy (STED)

## PSF shaping with saturated emission depletion



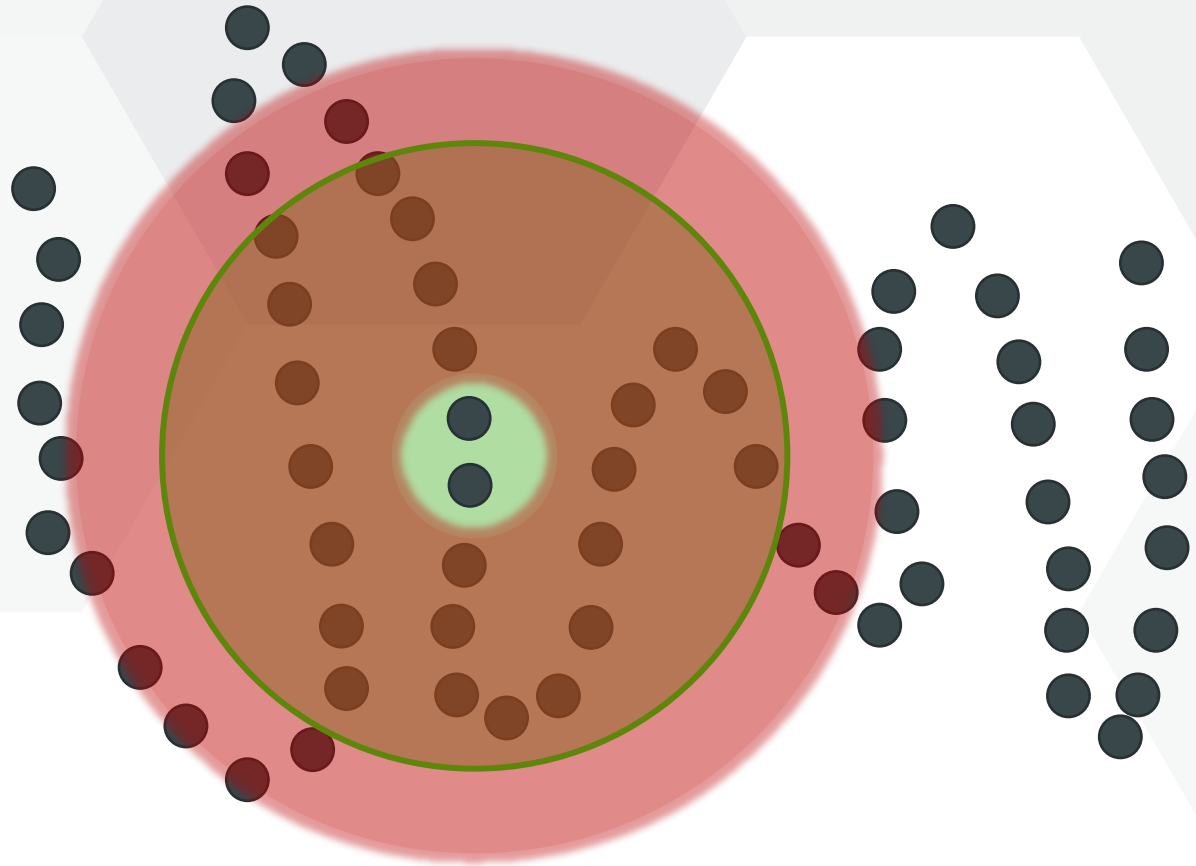



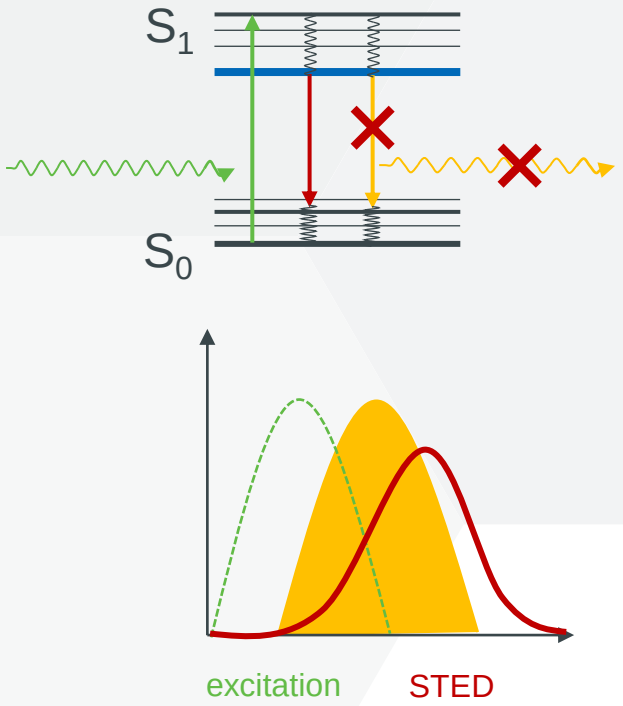

**Problem:** molecules (features) within <200 nm not recognizable

# Stimulated emission depletion microscopy (STED)

## PSF shaping with saturated emission depletion

$$\frac{\lambda}{2nsin\alpha}$$



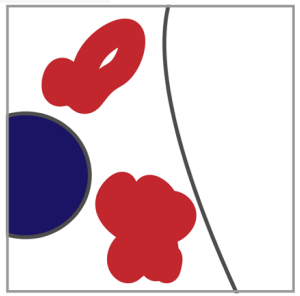


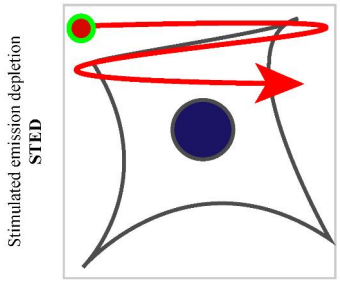

**Problem:** molecules (features) within <200 nm not recognizable

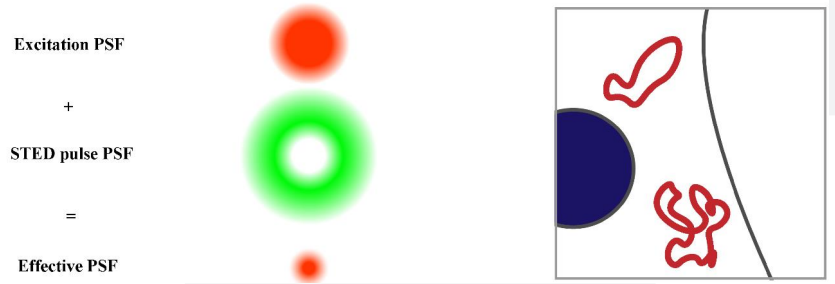
**Solution:** keep some molecules (features) dark

# STED microscopy
## Sample preparation

Standard fixation → Specific requirement for IF labeling → STED acquisition → ~~STED processing~~





# STED **advantages and challanges**

## Advantages

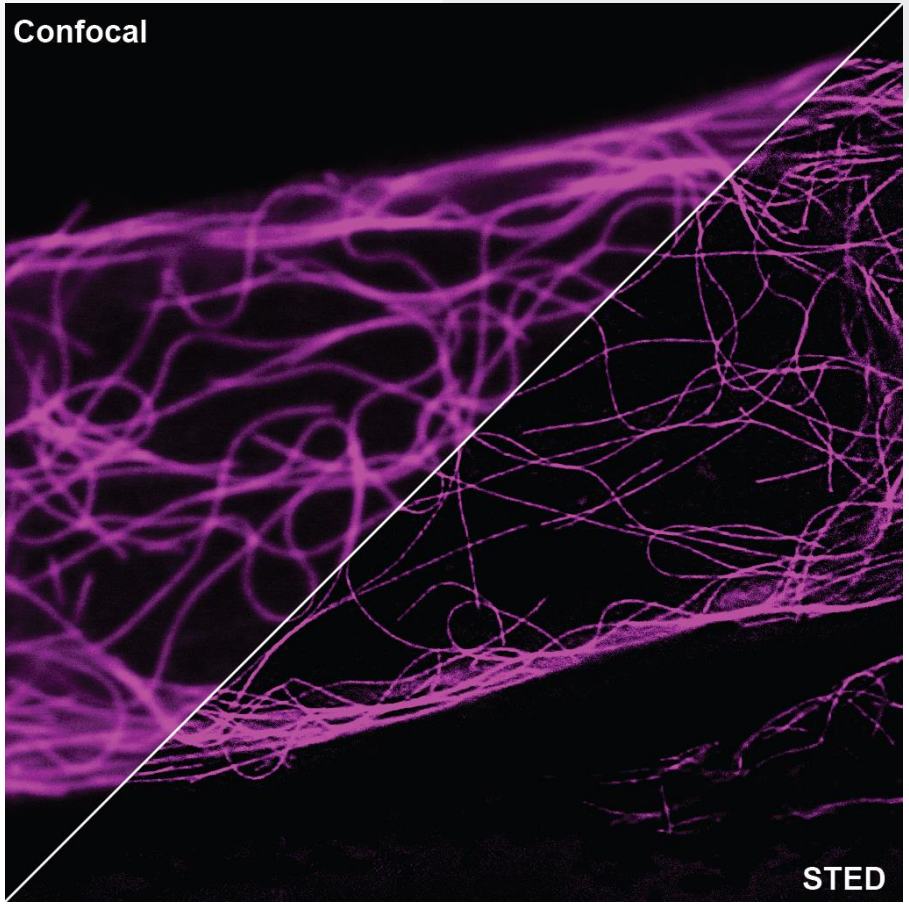
- Imaging resolution improved by directly optimizing the point spread function, not during post-procesing
- Multicolor imaging
- Applications when biological question requires <100 nm resolution, but cells must be fixed to achieve this
- The depletion beam can also be shaped along the z-axis, giving resolution in z of about 80 nm (at a slight expense of lateral resolution)

## Challenges

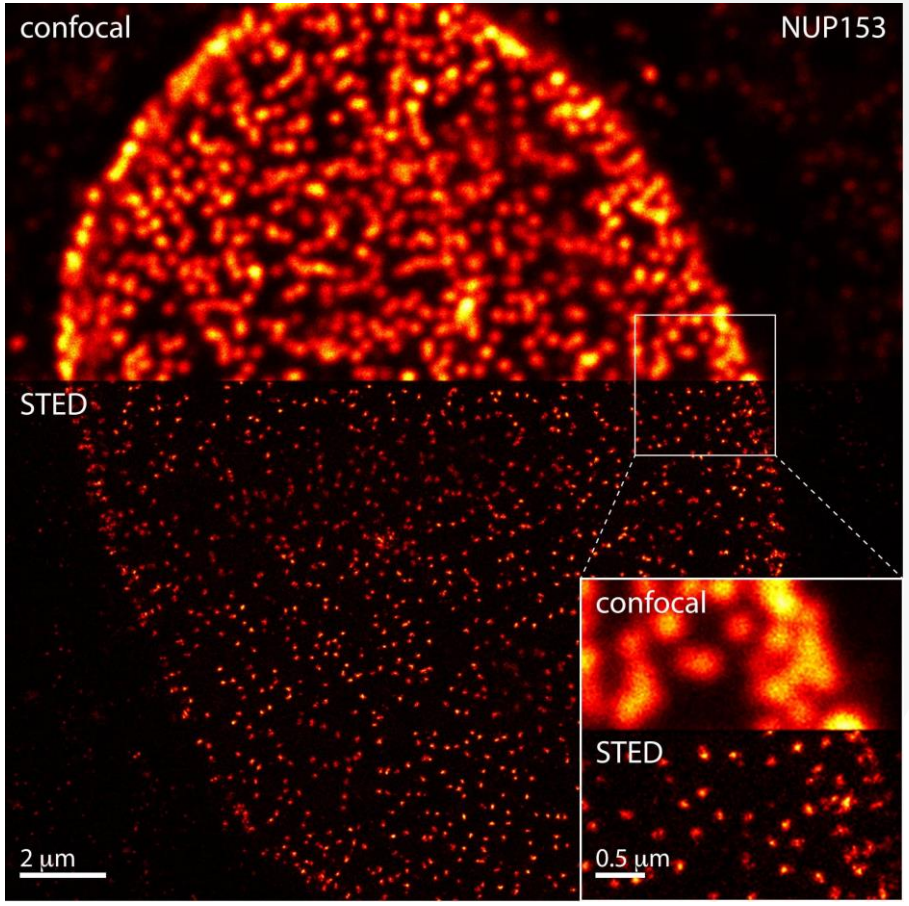
- Not suitable for live cell measurment
- Point scanning methods = lower scan speed (depends on FOV)
- Difficult laser alignment
- Intense (5W) depletion laser -> expensive
- Very phototoxic, high photobleaching
- Photostable fluorophores required
- Deconvolution may need to be applied for low signal particularly if sample has high background

# STED microscopy
## Applications

Comparison of confocal (upper) and STED (lower) microscopy
**SPY555-tubulin labeled HeLA cells**
(courtesy of Spirochrome)

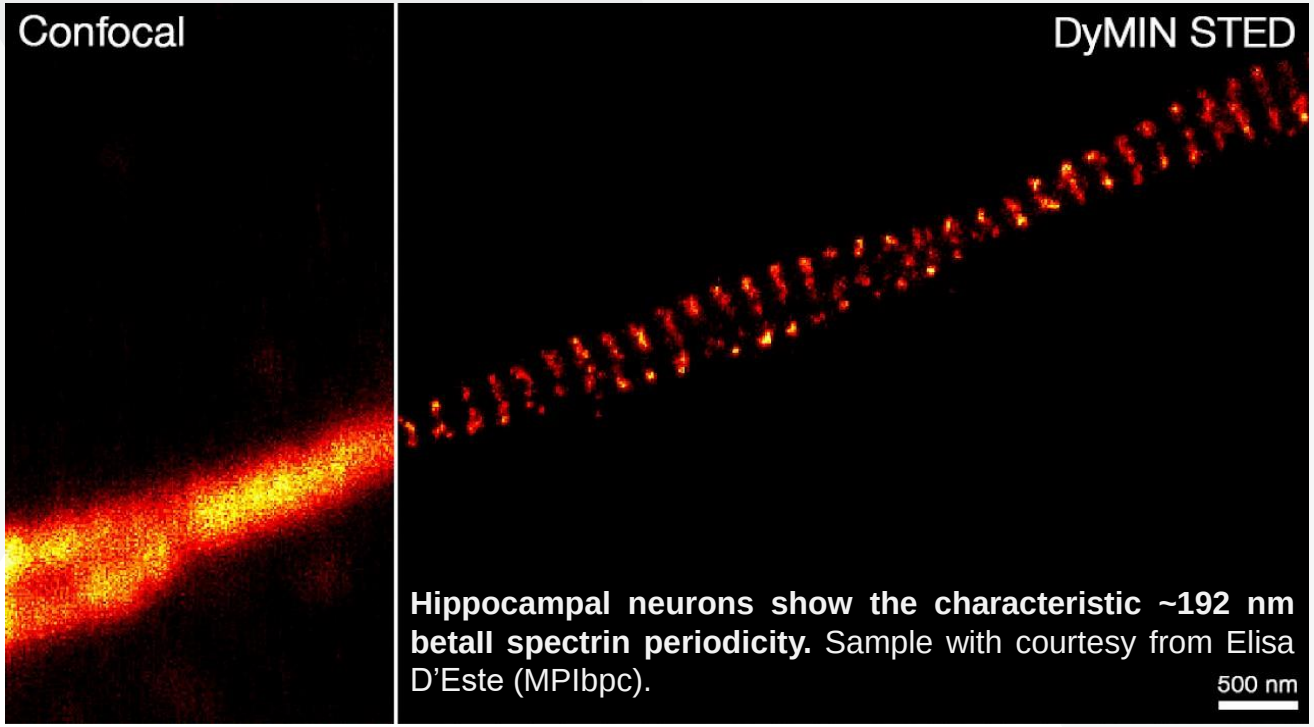



Comparison of confocal (upper) and STED (lower) microscopy
**HeLA cells stained against nuclear pore complex protein NUP153,** *(http://jcb.rupress.org/content/190/2/165.fu)*



# STED microscopy

## Modalities: 3D STED with Dynamic Minimum (DyMIN STED)




**DyMIN STED** is a co-development between Stefan Hell and coworkers and Abberior Instruments.

**Live-cell superresolution microscopy with resolutions down to 25 nm*** - DyMIN STED dramatically reduces the light irradiation on your sample (up to two orders of magnitude).

**Resolution truly down to 25 nm** - As demonstrated by separating two fluorescent point-structures being 30 nm apart.

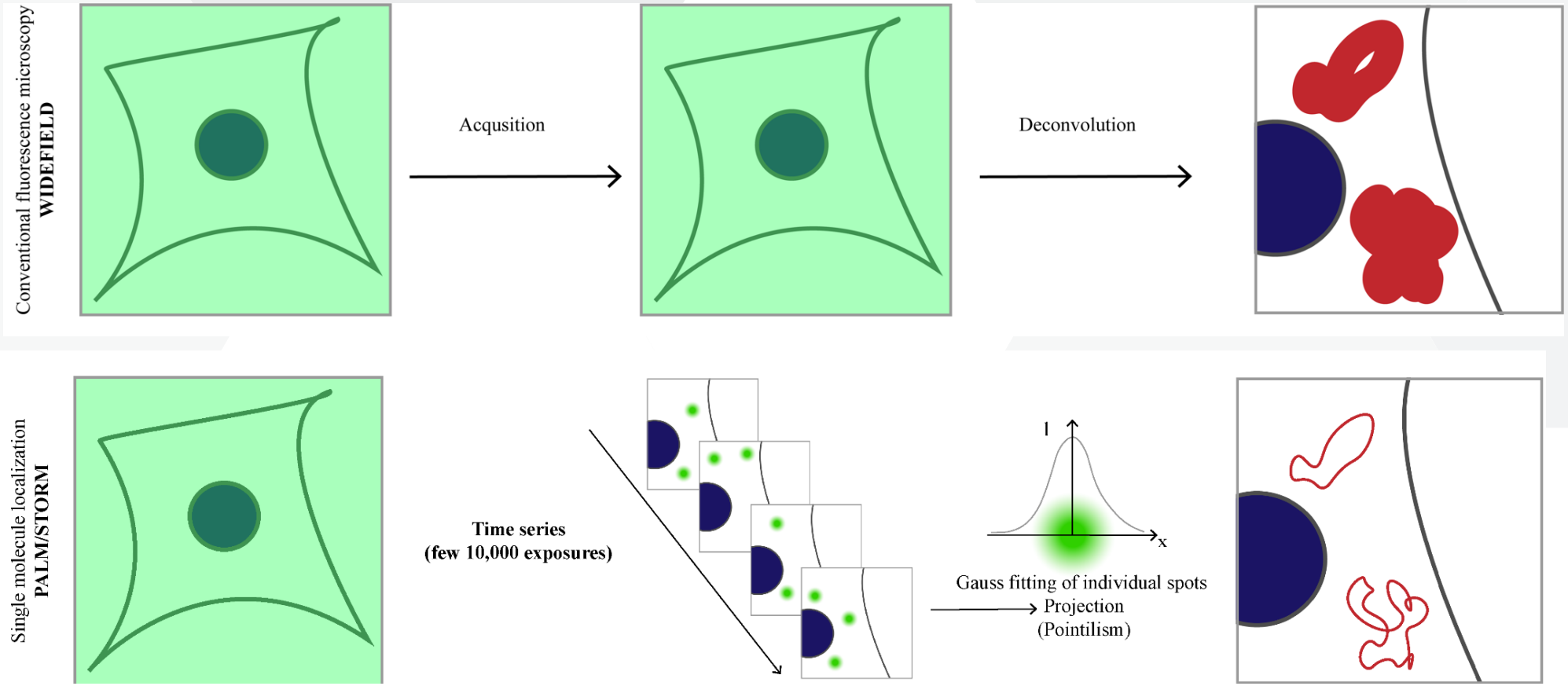
**Volume / time-lapse imaging with easy3D STED resolution** - DyMIN STED substancially reduces photobleaching and enables long term measurements over volumes or over dozens and dozens of frames.

# Single molecule localization microscopy (dSTORM/PALM)

## PSF optically recostructed

Eric Betzig

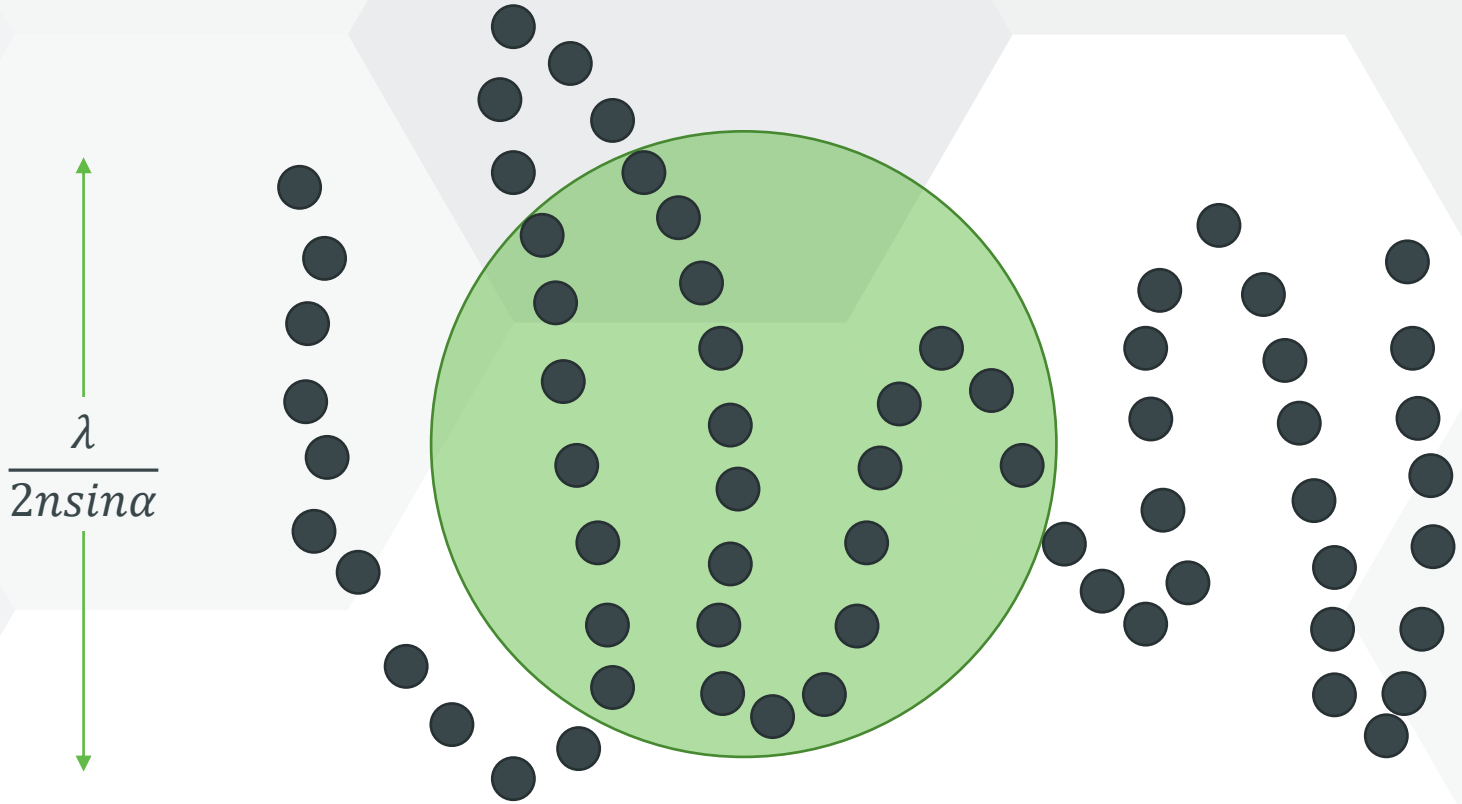
William Moerner

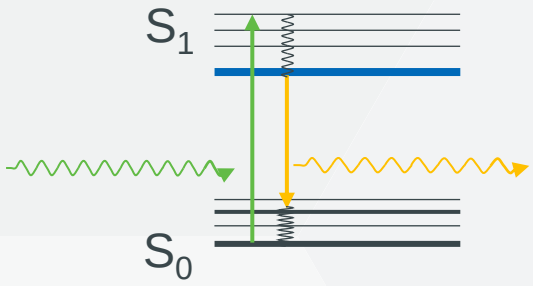


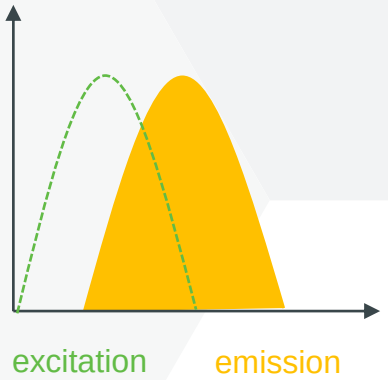

**Stochastic Optical Reconstruction Microscopy (dSTORM)**
**Photo Activated Localization Microscopy (PALM)**

# SMLM (dSTORM/PALM)

## PSF optically recostructed



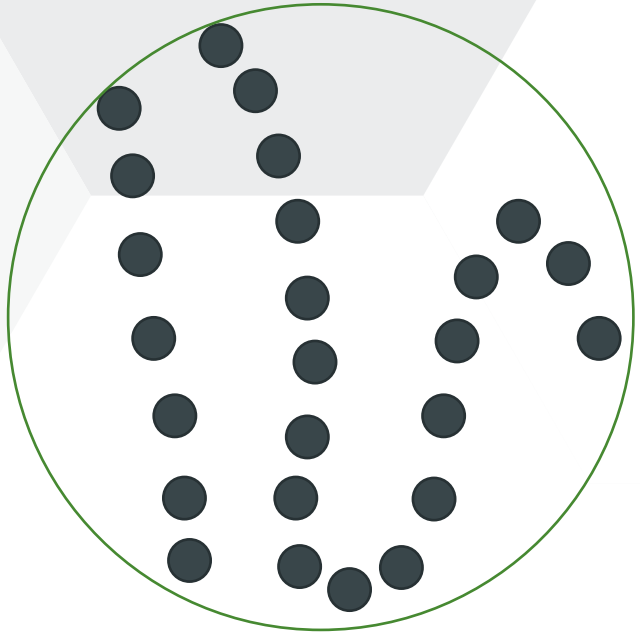




**Problem:** molecules (features) within <200 nm not recognizable

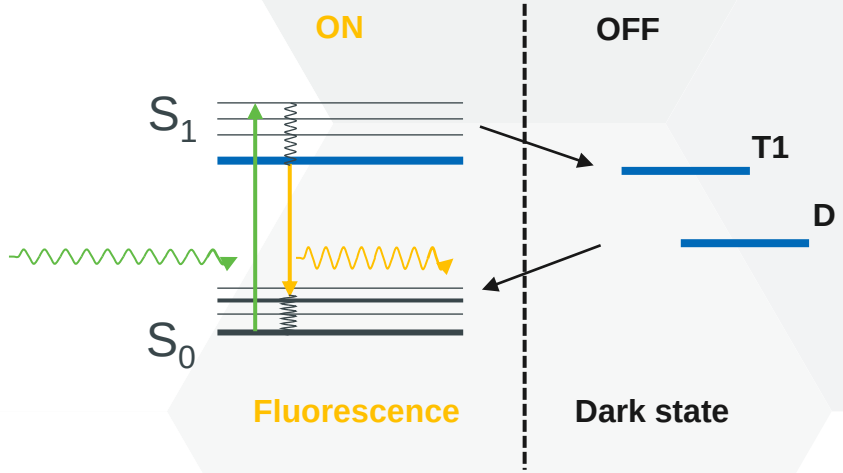
# SMLM (dSTORM/PALM)

## PSF optically recostructed

$$\frac{\lambda}{2nsin\alpha}$$



Simplified Jablonski Diagram




**Problem:** molecules (features) within <200 nm not recognizable

# Single molecule localization microscopy (dSTORM/PALM)

## PSF optically recostructed

- Determines the position of individual fluorescent molecules located at a structure of interest, rather than resolving them optically.
- The positions can be determined with a precision of the order of 10 nm
- The resolution depends on the size and density of molecules and the obtainable signal-to-noise ratio (theoreticaly unlimited).
- Typical images, however, provide 10-fold improved resolution in comparison to conventional microscopy (20 nm in xy and 60 nm in z).

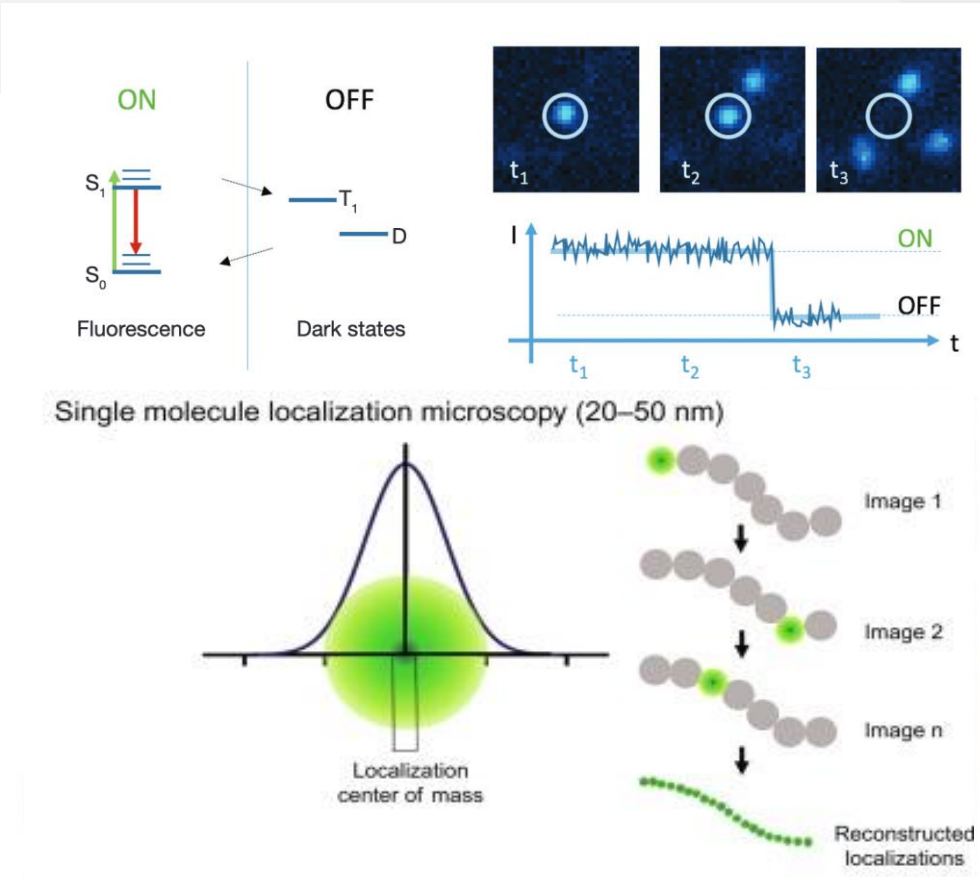
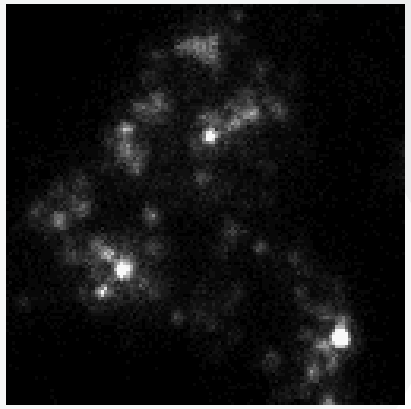
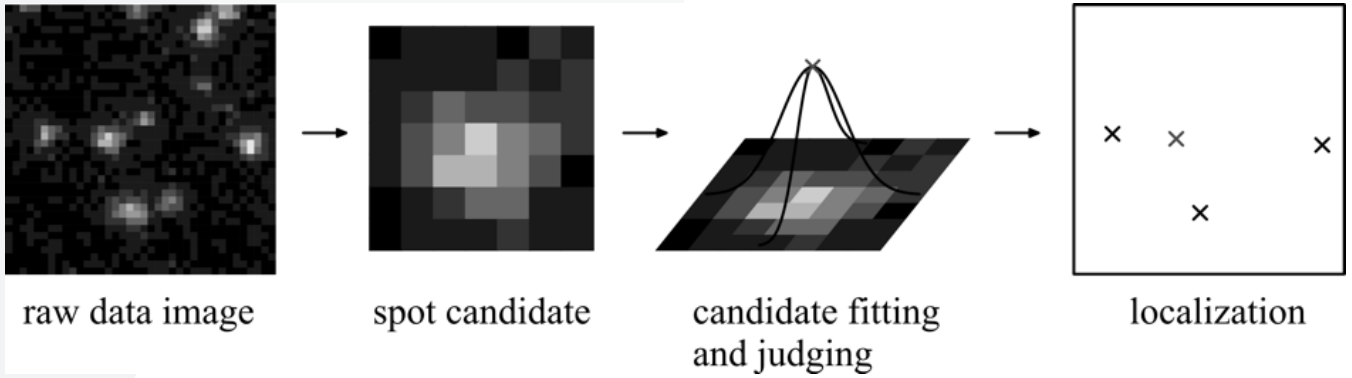



**FIG 4: Localization microscopy. Upper row illustrates the typical on/off behavior of single molecules as seen in a time-series (left a simplified Jablonski-Perrin Fluorescence diagram). Individually molecules are visible as diffraction limited patterns that switch on and off in time (the last off step is usually irreversible). The graph shows a typical intensity transient for a single molecule. The two images below show segments of microtubules as reconstructed from the individual molecule positions (left) with around 20 nm resolution and the corresponding widefield image (right) for comparison (Sample using standard Alexa 561 immunolabeling and embedding).**

# Single molecule localization microscopy (dSTORM/PALM)

## PSF optically recostructed





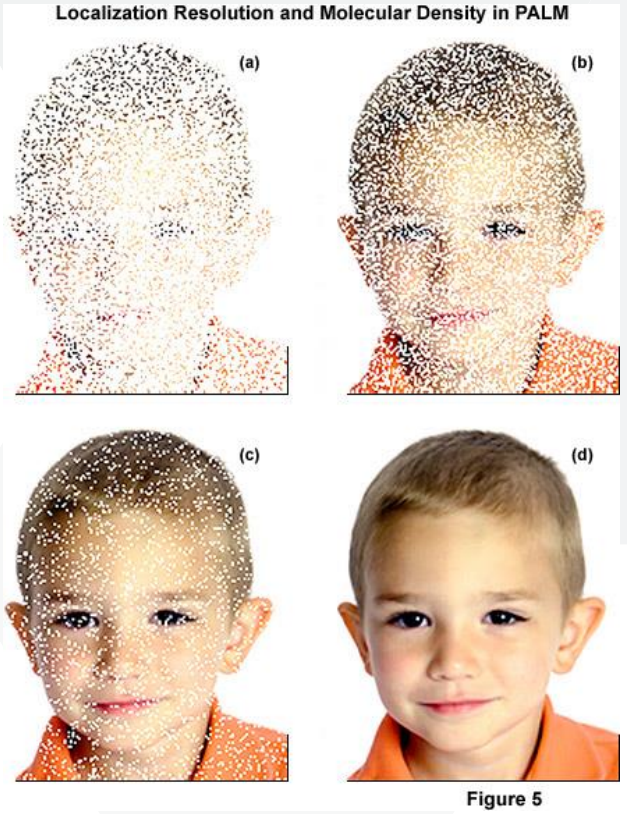



Figure 5

**Thousands of such positions are gathered and superimposed, then it is possible to generate an image of a structure with improved resolution.**

# (dSTORM/PALM)

## COMPARISON

### dSTORM

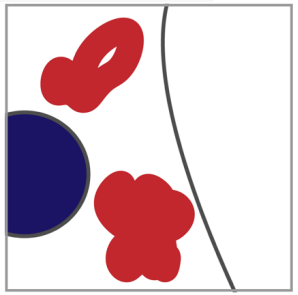
- blinking **passively**
- interaction of fluorescent molecules with its **blinking buffer** which cause the molecules to switch ON and OFF (hence the term “stochastic”).
- Under **proper conditions** (e.g. pH val-ue, redox states, etc.) only few molecules are ON during the acquisition of each frame and therefore easily distinguishable
- label molecules emit at random times due to chemical reactions or interactions in their immediate vicinity
- Fixed samples

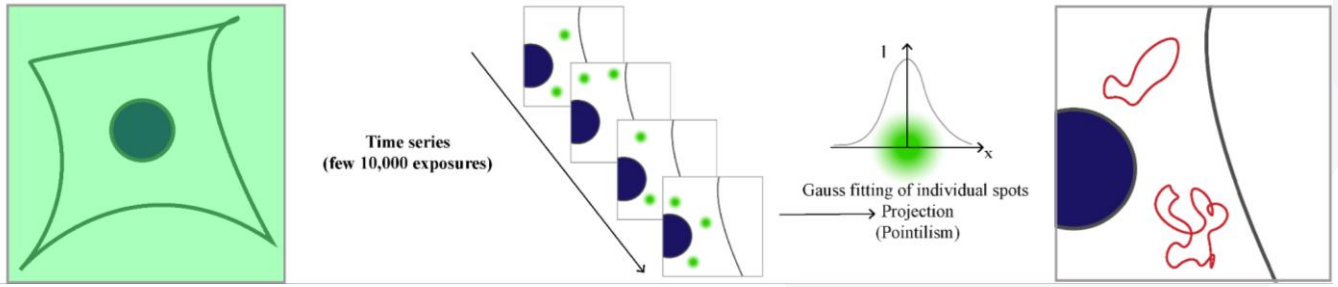
### PALM

- photoactivated (blinking **actively**) - uses **photoactivation** to switch the molecules.
- employs **photoactivatable dyes** (predominantly switchable fluorescent proteins, like photoswitchable GFP, tdEOS, etc
- The switching of the individual molecules is still **random**, but the rate with which the molecules switch on or off can be controlled by increasing or decreasing the intensity of the **switching laser** (e.g. 405 nm).
- They can be used in vivo, have a higher specificity and do not require fixation and permeabilization of the specimen
- Live samples

# SMLM microscopy
## Sample preparation

Standard fixation → Specific requirement for IF labeling → dSTORM/PALM acquisition → dSTORM/PALM processing






https://hcbi.fas.harvard.edu/files/hcbidoug/files/en_41_011_065_elyra_sample-prep-quickguide.pdf

# SMLM **advantages and challanges**

## Advantages

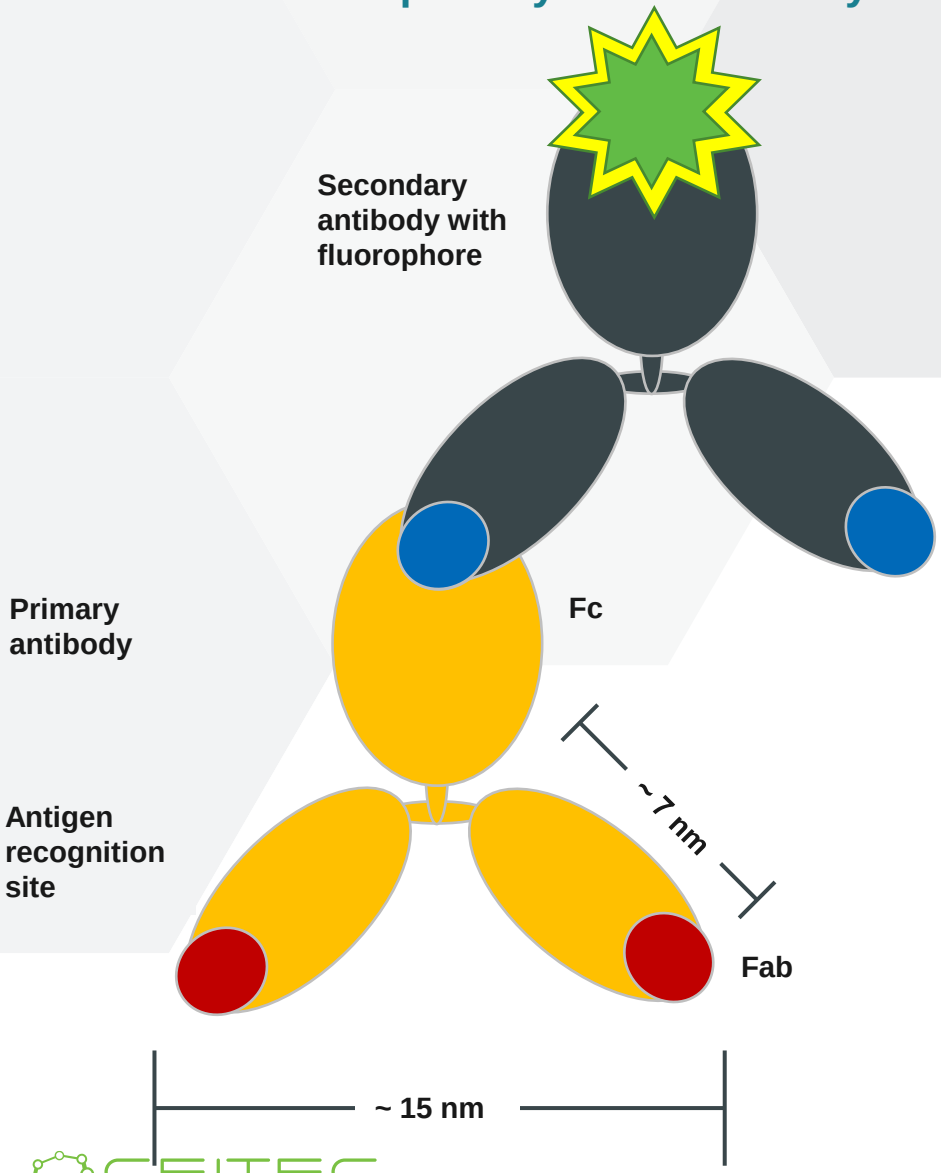
- PALM can be used for live cell imaging
- twocolour imaging
- PALM / dSTORM deliver the highest resolution of all presented super-reso-lution methods (theoretically unlimited - typically 20 nm in xy / 60 nm in z) and can deliver molecular detail.
- Best results are obtained from transparent and well-prepared specimens near the coverslide surface (ca. 10 µm from the coverslip surface).

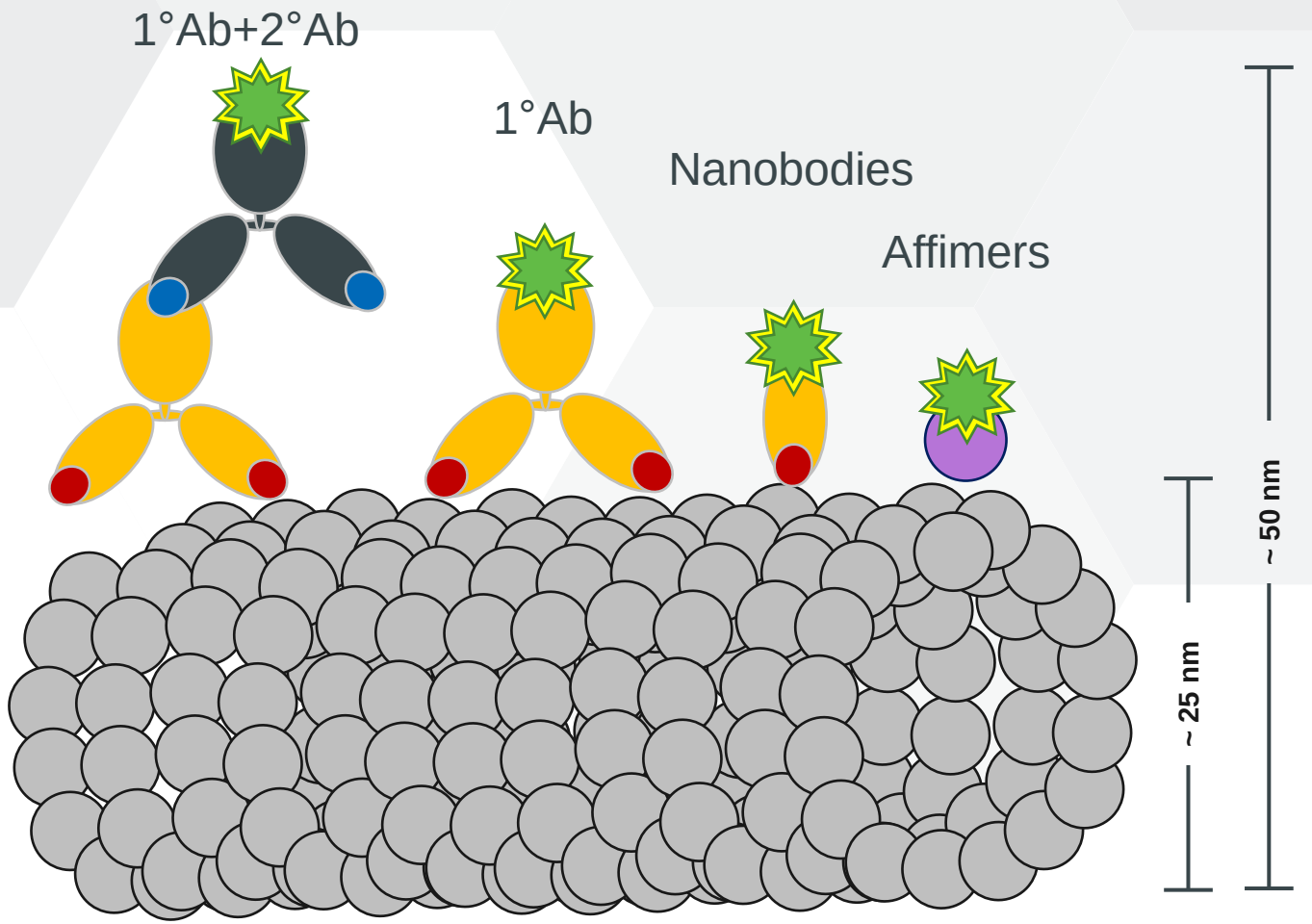
## Challenges

- dSTORM generally not suitable for live cell imaging
- PALM and dSTORM are considered slow because collection of a typical image sequence (>1000 frames) takes upward of 10s, typically minutes.
- Phototoxicity and photobleaching has to be considered
- Data analysis possibilities that are not easily accessible via other methods
- **The biggest challenge for PALM/dSTORM is the need for photoswitchable molecules or addition of chemistry to bring the labels into an adequate “blinking” regime. Also, PALM and dSTORM have limited in vivo applications. Long term stability is a crucial concern for PALM/dSTORM equipment.**

# Antibody challenge

## Combination of primary and secondary antibody: large `linkage` error (~20nm)

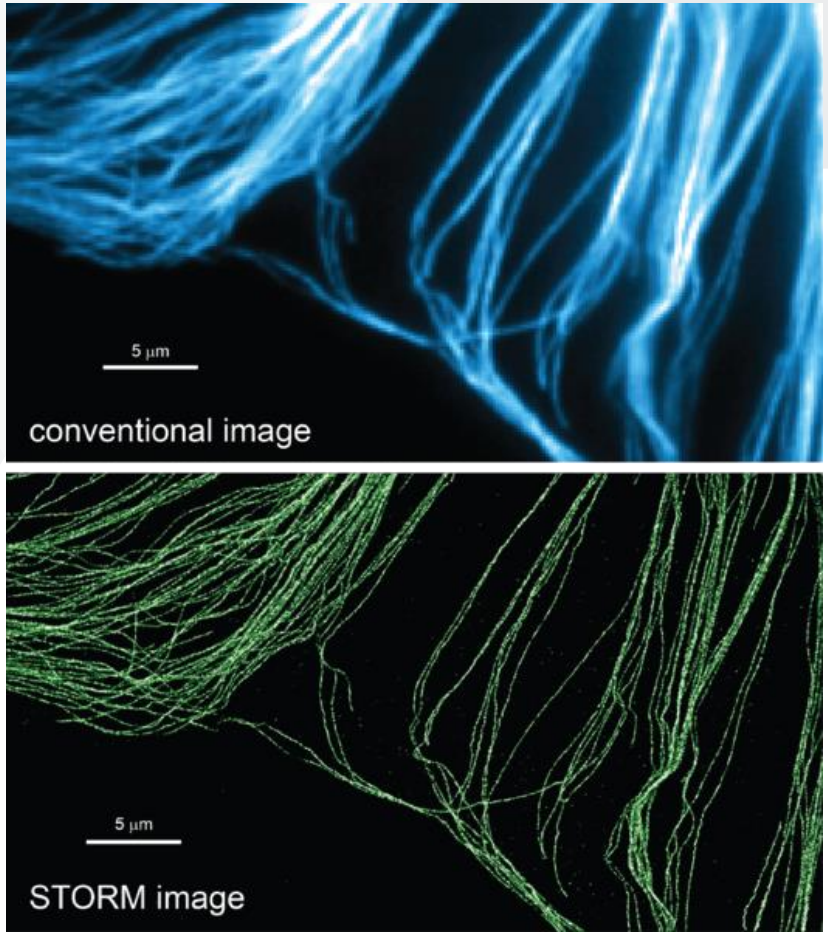


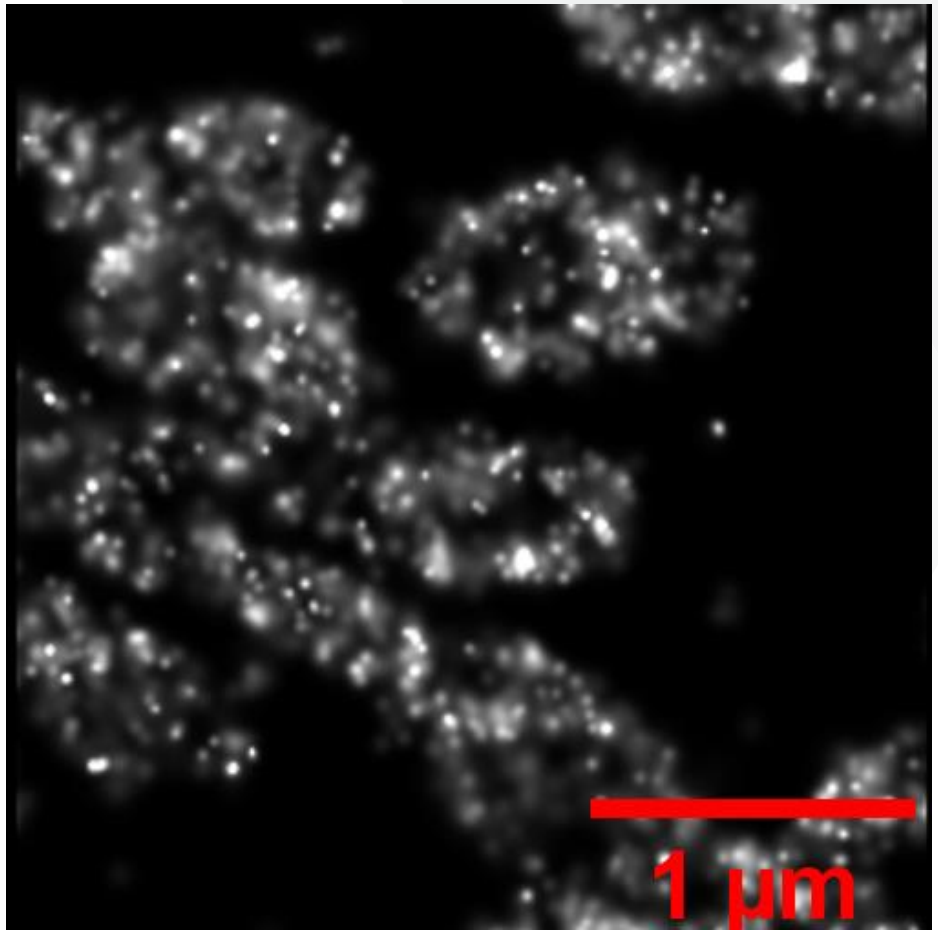

If we consider the size of antibodies it is preferable to do direct antibody labeling without a primary and secondary Ab. Smaller antibodies as **nanobodies** with a size in range of 2 nm may be preffered. Nowadays new **affimers** (small ptoeins, with antibody specificity) being produced, which are even smaller than nanobodies.

# dSTORM/PALM microscopy
## Applications

Microtubules in a cell (Multicolor Super-Resolution Imaging with Photo-Switchable Fluorescent Probes, Science),***Confocal microscopy (upper), dSTORM (lower)***




Mitochodrial membrane stained with anti-TOM20 antibody and **photoswitchable Alexafluor-647**, (Elyra7, CELLIM)

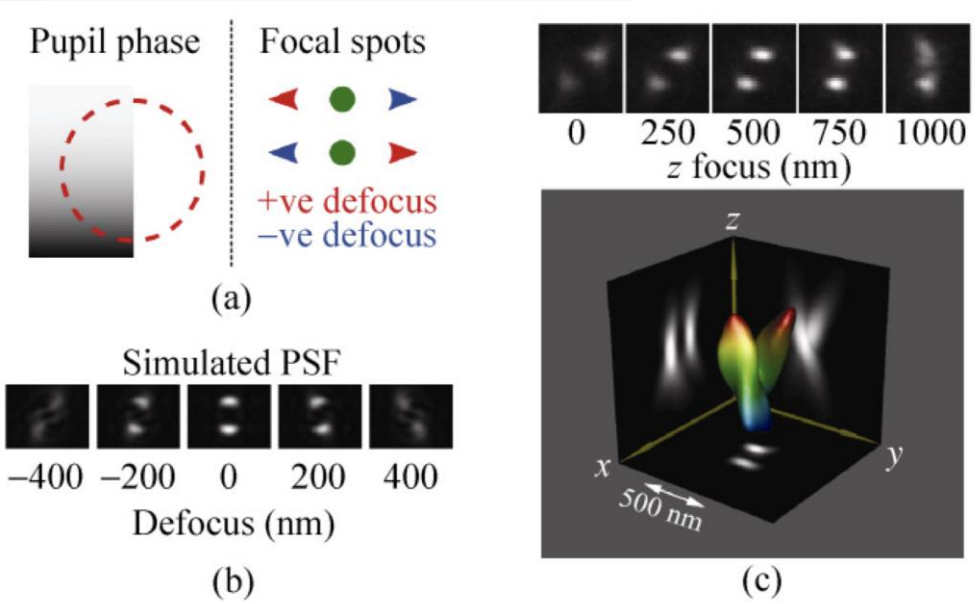


# dSTORM microscopy

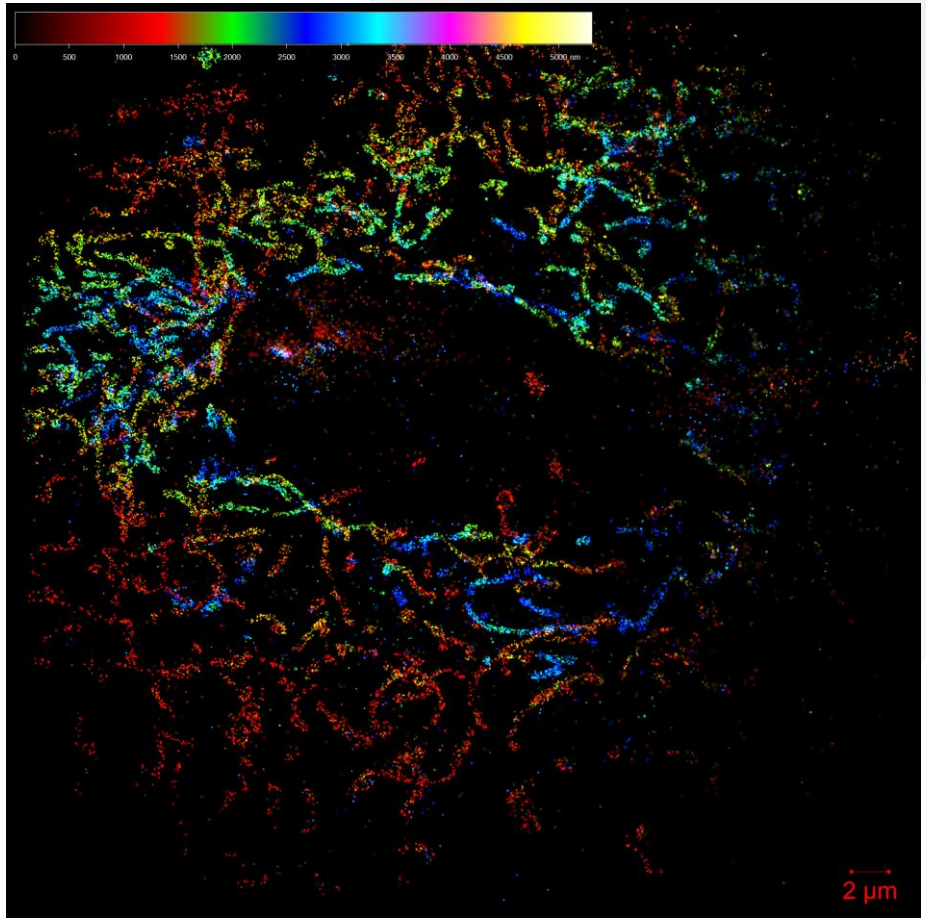
## Modalities: 3D dSTORM

**3D STORM** – point spread function distortion based on Z position

Special optical element to shape point spread function of emitters




**3D dSTORM** of Mitochodrial membrane stained with anti-TOM20 antibody and **photoswitchable Alexafluor-647**, (CELLIM)



# Conclusion

# OVERALL

- Super-resolution microscopy requires thorough sample preparation
- Image quality is rapidly affected by impurities (dust grains, bubbles, unspecific staining, etc.)
- Care must be taken that all parts of the system, from the cover glass to the mounting or embedding medium are clean and well-defined (e.g. uniform thickness, clean mounting, labeling specificity, etc.).

# Versatility / live cell imaging

| | Airyscan | SIM | PALM/dSTORM | STED |
|---|---|---|---|---|
| **Live cell imaging** | Compatible | Compatible | Not considered | Not compatible |
| **Laser wavelengths** | No restrictions | No restrictions | High laser power / limited dyes | the highest irradiation dosage / limited dyes |
| **Specific objectives** | No restrictions | No restrictions | Specific objectives | Specific objectives |
| **Speed** | Limited by FOV – up to 30 fps | Highest acquisition speed – over 100 fps | Thousends of images neccesity | Limited by FOV – up to 30 fps |
| **Depth of samples** | Thick specimens (less sensitive towards changes in RI) | Depends on accurate projection of the illumination pattern - thickness up to 20 um distance from | more challenging when going into thick, over-labeled, noise-rich, scattering specimens | - quality rapidly decays with penetration depth<br>- laser dosage also on the image |

# What next?

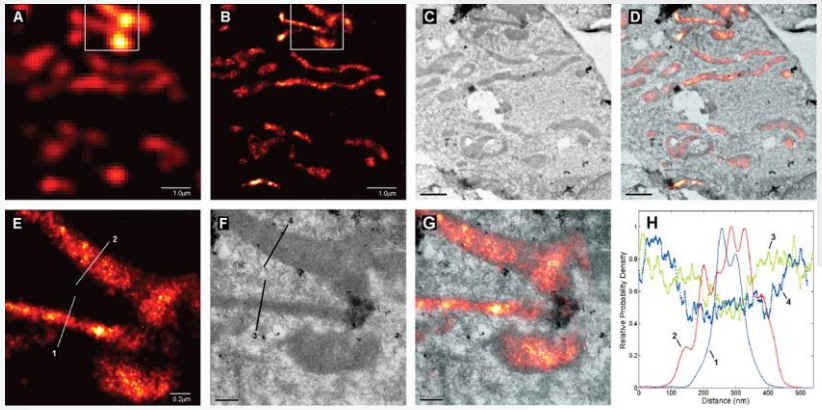
- Highest possible resolution
- High contrast
- Fast acquisition
- Lower laser power
- Thick samples / live samples
- Minimal labelling
- phototoxicity
- Online functional data processing

- Economical viability
- Easy to use by non-experts

# What next?

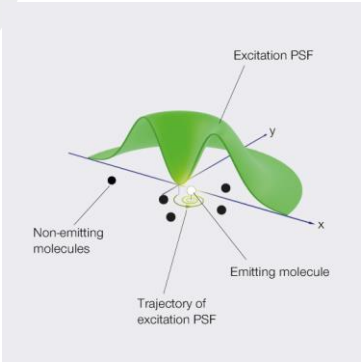
## 1) Correlative TEM/PALM microscopy

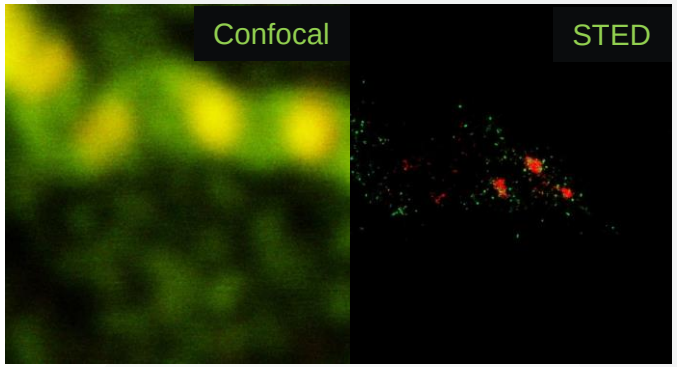
https://www.science.org/doi/10.1126/science.1127344




## 3) MINFLUX (abberior)

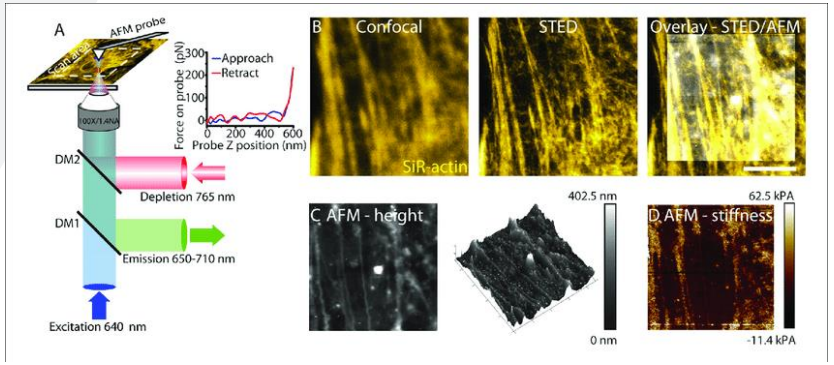
https://abberior-instruments.com/products/minflux/




## 2) Correlative AFM/STED

https://www.frontiersin.org/articles/10.3389/fncel.2017.00104/full



# Cellular Imaging Core Facility - CELLIM

https://www.czech-bioimaging.cz

https://www.eurobioimaging.eu

http://cellim.ceitec.cz

CORE FACILITY CELLULAR IMAGING

CEITEC Masaryk University
Kamenice 753/5
625 00 Brno CZ

cellim@ceitec.muni.cz
+420 54949 7085

Edit profile

**Cellular Imaging Core Facility - CEITEC**
@Ceitec_CellimCF

Light microscopy facility of @CEITEC_Brno @muni_cz. Part of #CzechBioimaging and @EuroBioimaging research infrastructures.

https://twitter.com/Ceitec_CellimCF

Milan Ešner, Ph.D.
*Head of Core Facility*
**Phone:** +420 54949 6133
**Email:** milan.esner@ceitec.muni.cz
**Office:** Kamenice 753/5, Brno, 625 00, office A2/118

Petra Macháčková, Ph.D.
*Widefield and confocal microscopy*
**Phone:** +420 54949 7085
**Email:** petra.machackova@ceitec.muni.cz
**Office:** Kamenice 753/5, Brno, 625 00, office A2/118

Jakub Pospíšil
*Widefield and confocal microscopy, advanced methods*
**Phone:** +420 54949 7202, +420 54949 7085
**Email:** jakub.pospisil@ceitec.muni.cz
**Office:** Kamenice 753/5, Brno, 625 00, office A2/118

Wojciech Jesionek, Ph. D.
*Image and data analysis*
**Phone:** +420 54949 7085
**Email:** wojciech.jesionek@ceitec.muni.cz
**Office:** Kamenice 753/5, Brno, 625 00, office A2/118