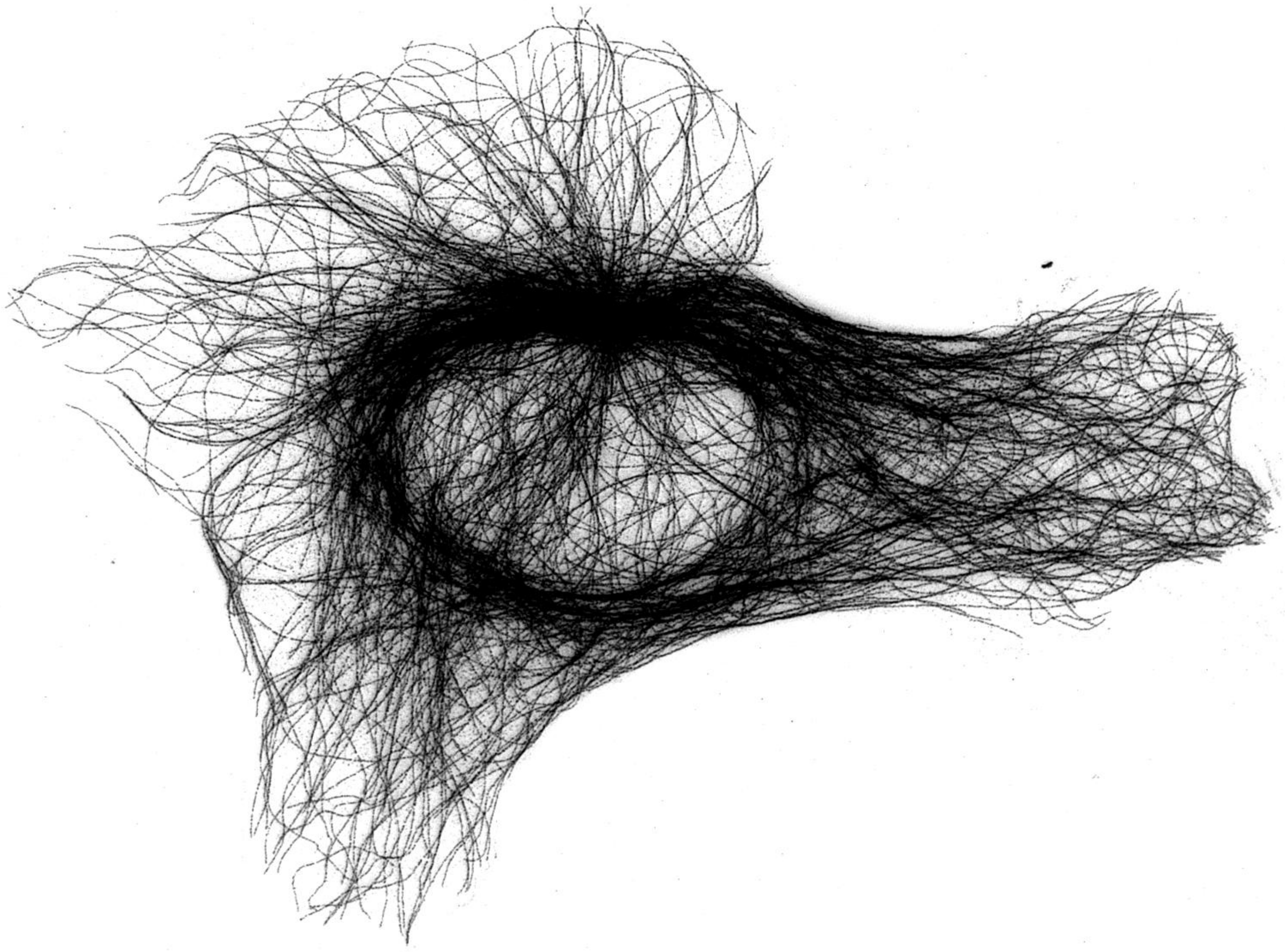
MMIB 2025

Super-resolution Methods in LM

STORM
SIM
STED

Ivan Novotny
LM IMG CAS

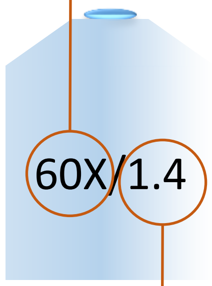
IMG

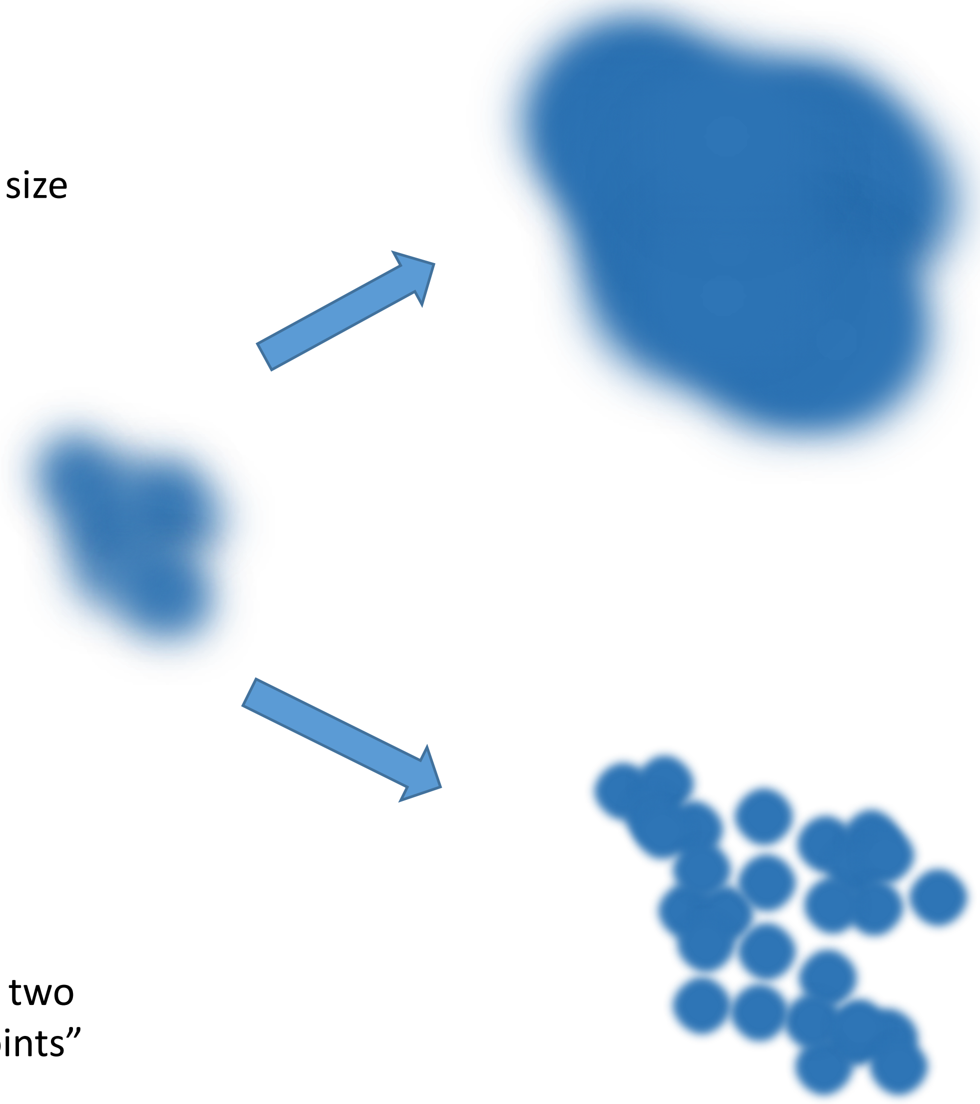


Magnification

"How many times the apparent size of the object is increased"

TO SEE THINGS BIGGER





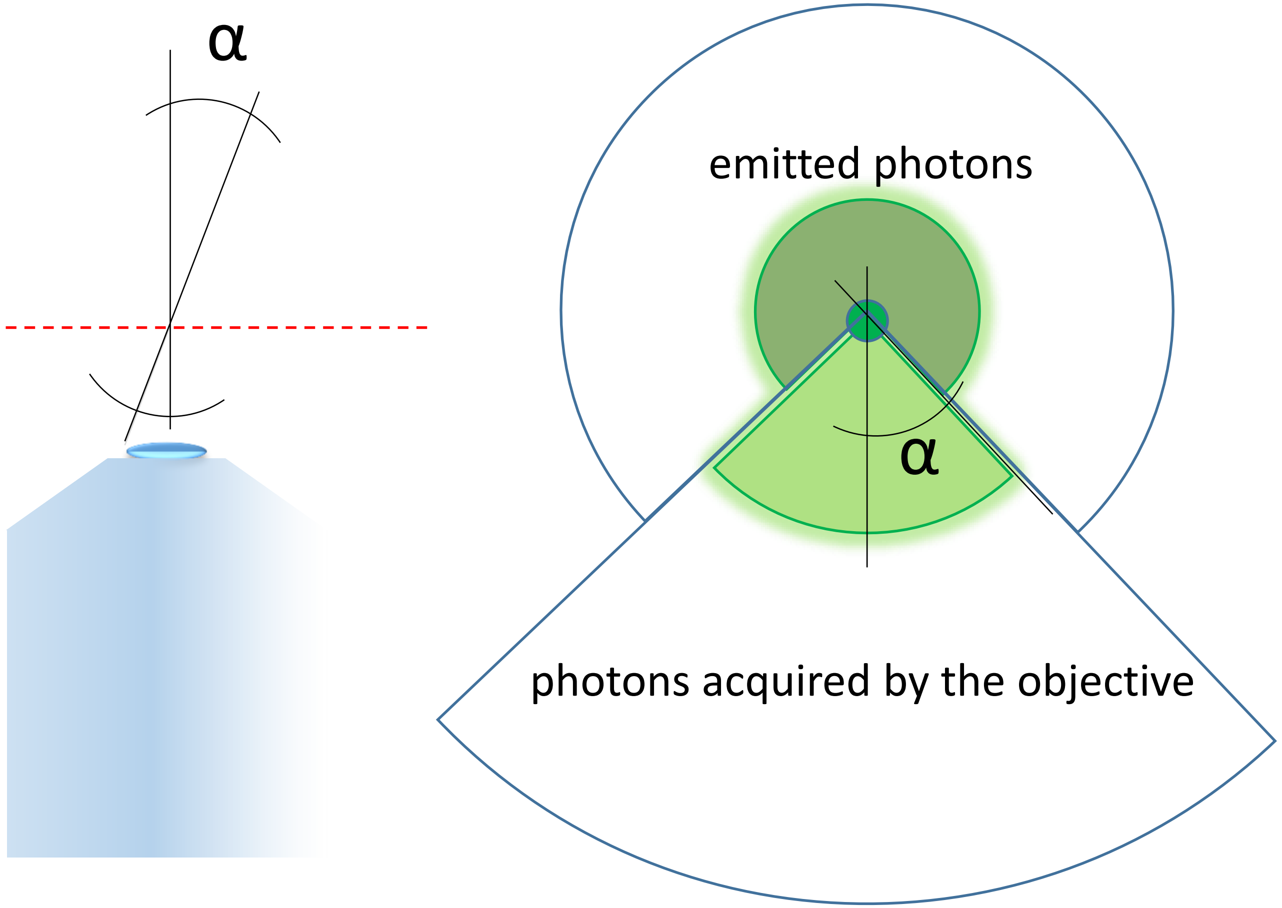
Resolution

"The smallest distance at which two points can be identify as two points"

TO SEE MORE DETAILS

Resolution

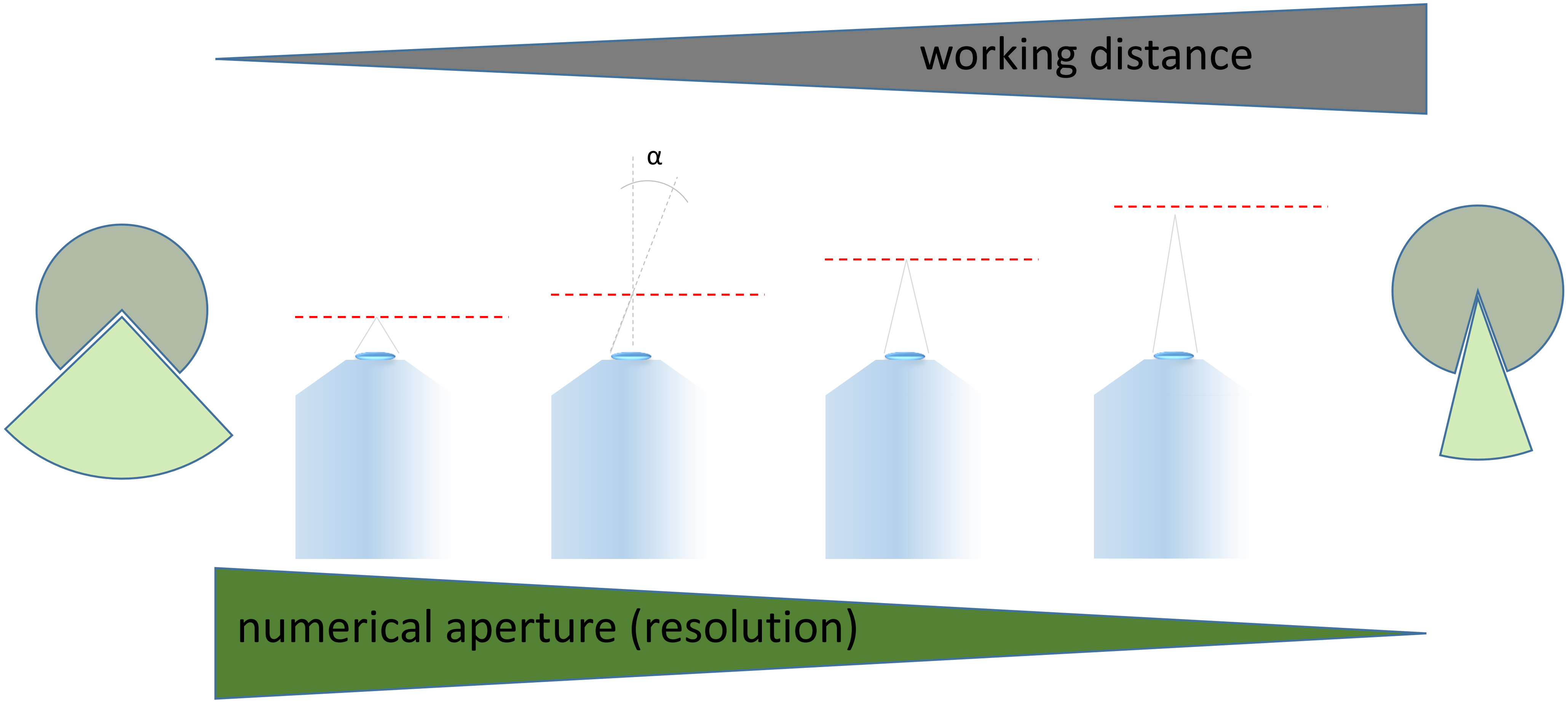
NUMERICAL APERTURE corresponds to the resolution of the objective



$$NA = n \sin\alpha$$

$$d = \frac{\lambda}{2NA}$$

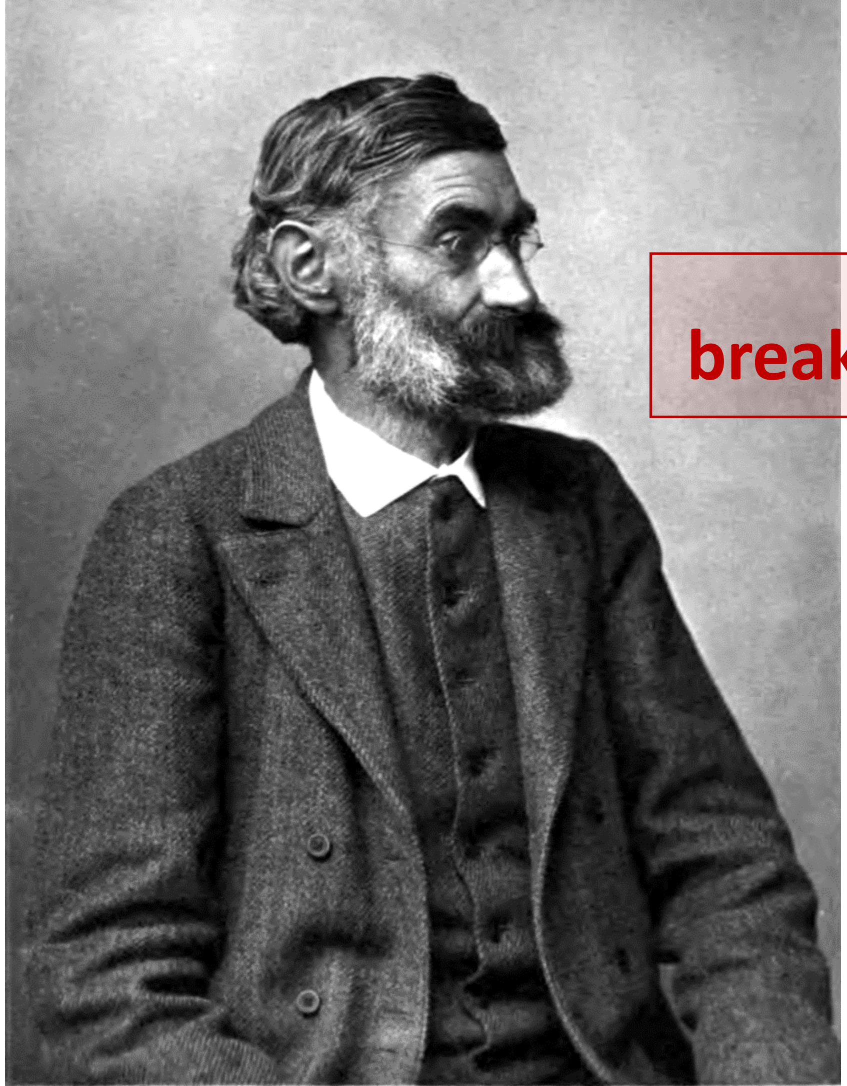
Photon = information ➔ resolution



$$d = \frac{\lambda}{2NA} \quad , \qquad NA = n\,sin\alpha$$

$$d = \frac{\lambda}{2NA}$$

Ernst Abbe



break through the resolution barrier

Stephan Hell

Erick Betzig

Mats Gustafsson

- How to understand the SUPER-resolution:

  Localization microscopy

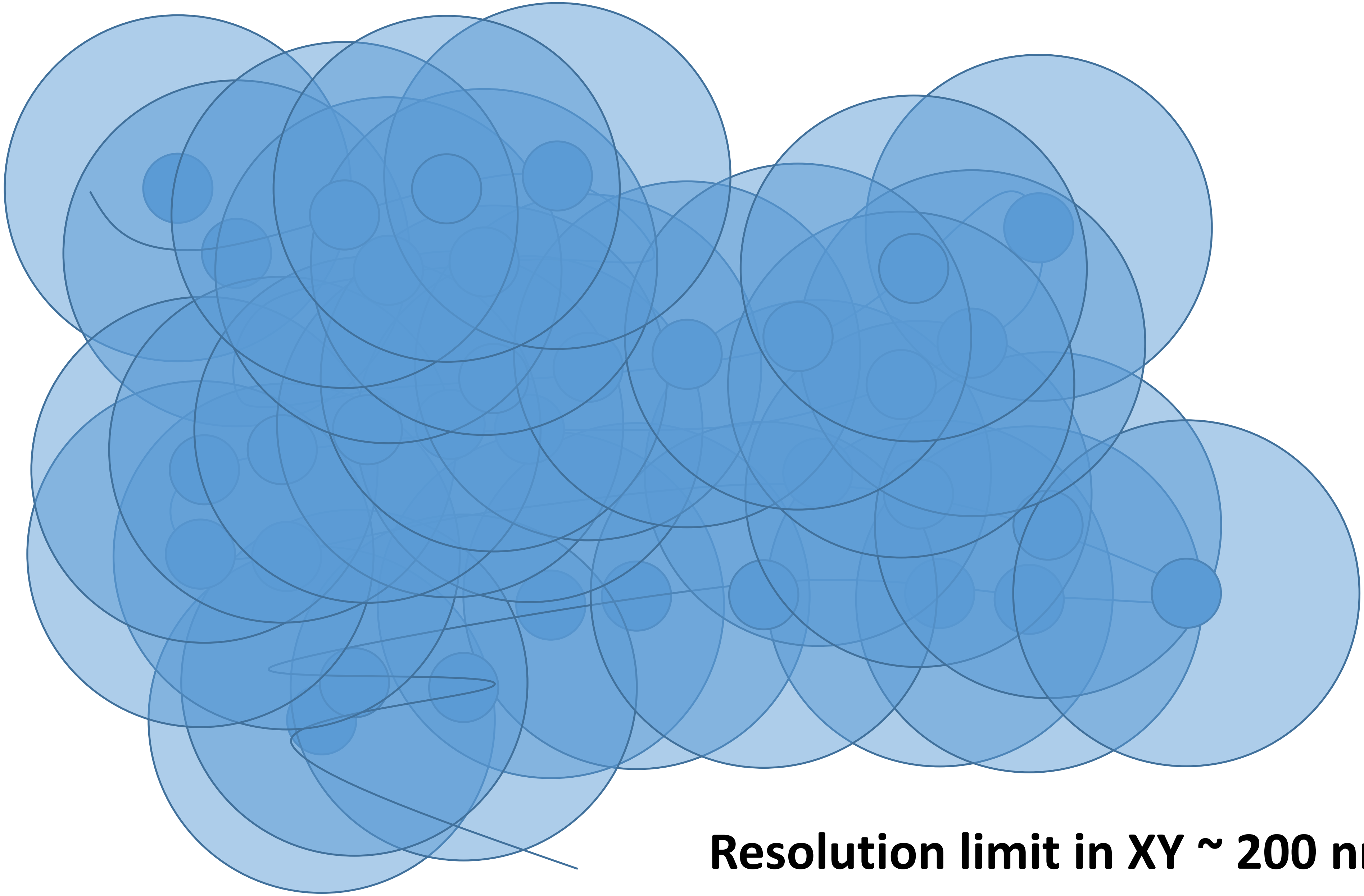
  SIM

  STED

- Advantages and disadvantages and sample preparation requirements of SR methods
- Limitations of SR methods

- How to understand principles of
  - Localization microscopy
  - SIM
  - STED

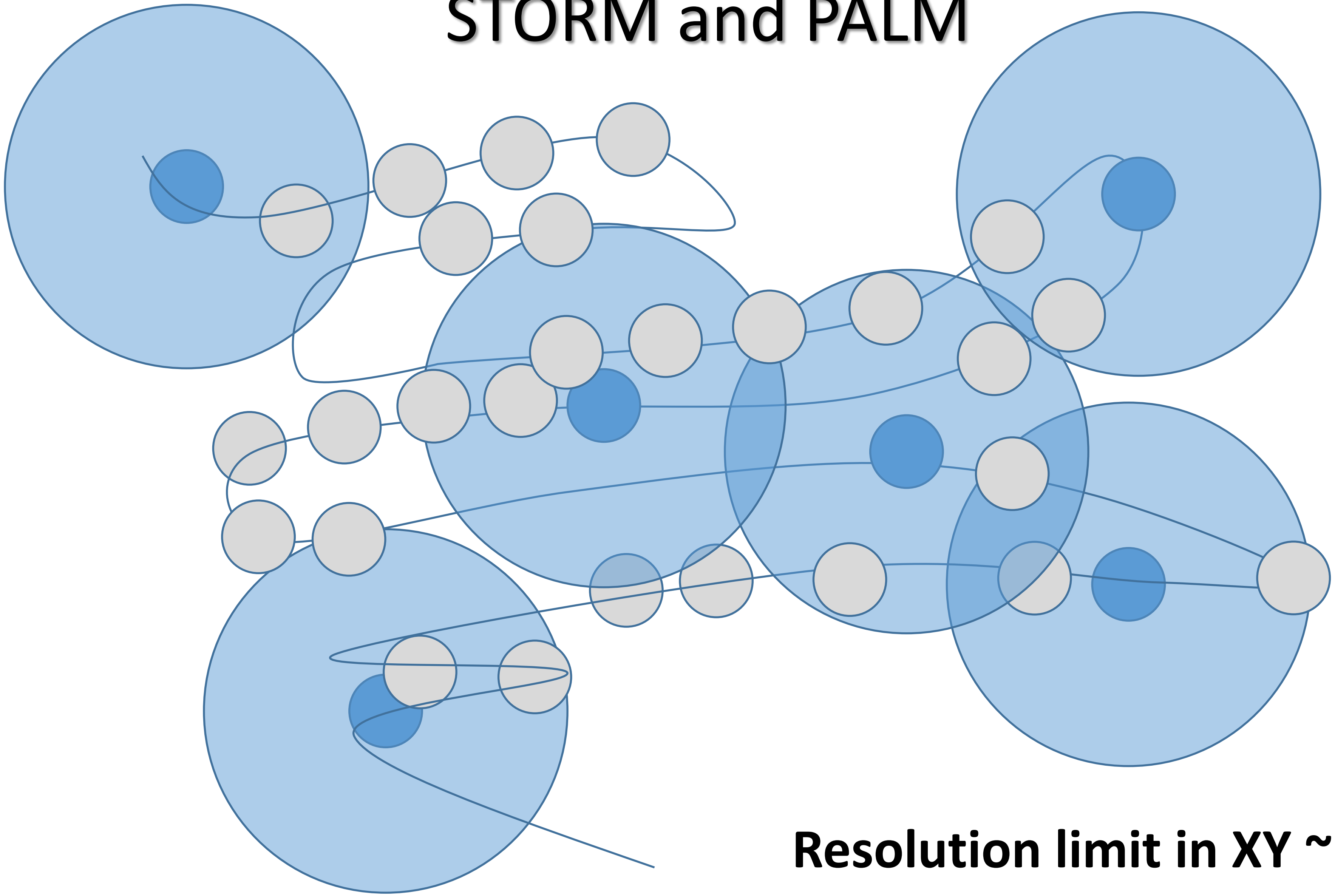
Superresolution microscopy



Resolution limit in XY ~ 200 nm

Superresolution microscopy – single molecule localization microcopy

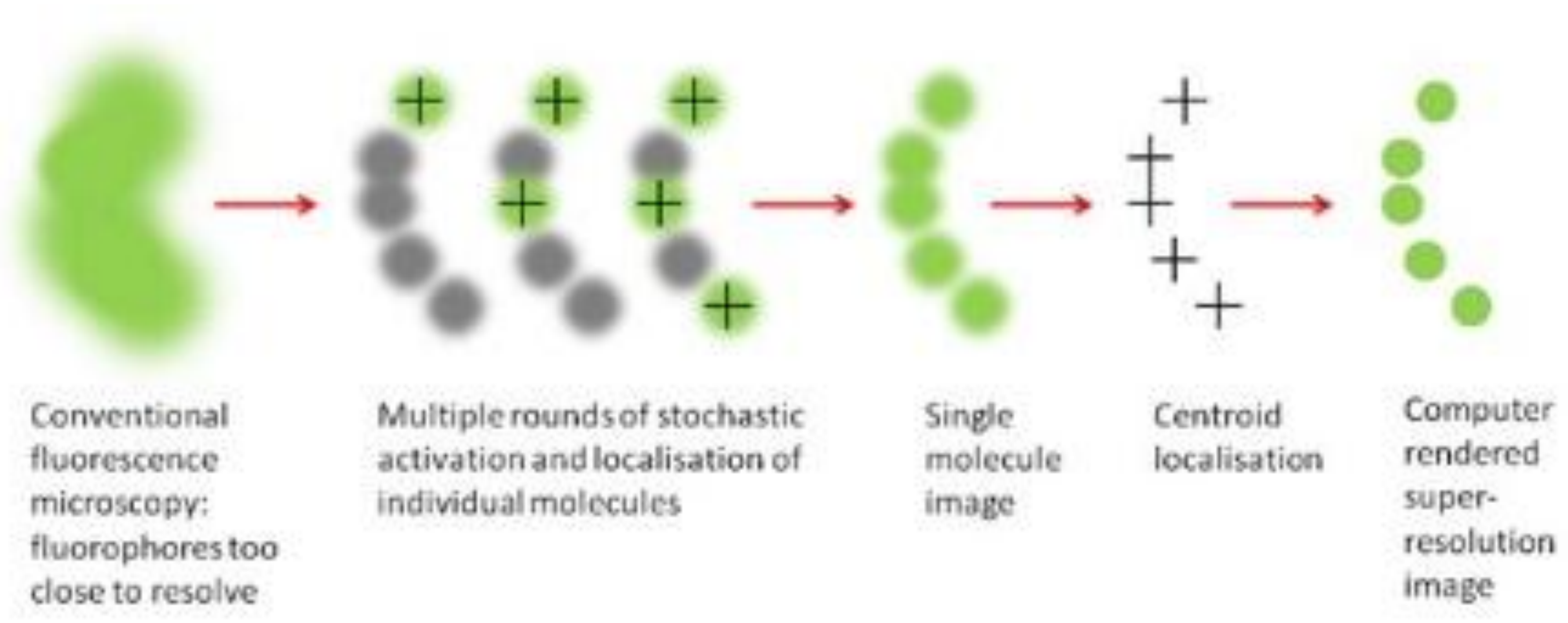
STORM and PALM



Resolution limit in XY ~ 20 nm

Superresolution microscopy – single molecule localization microcopy

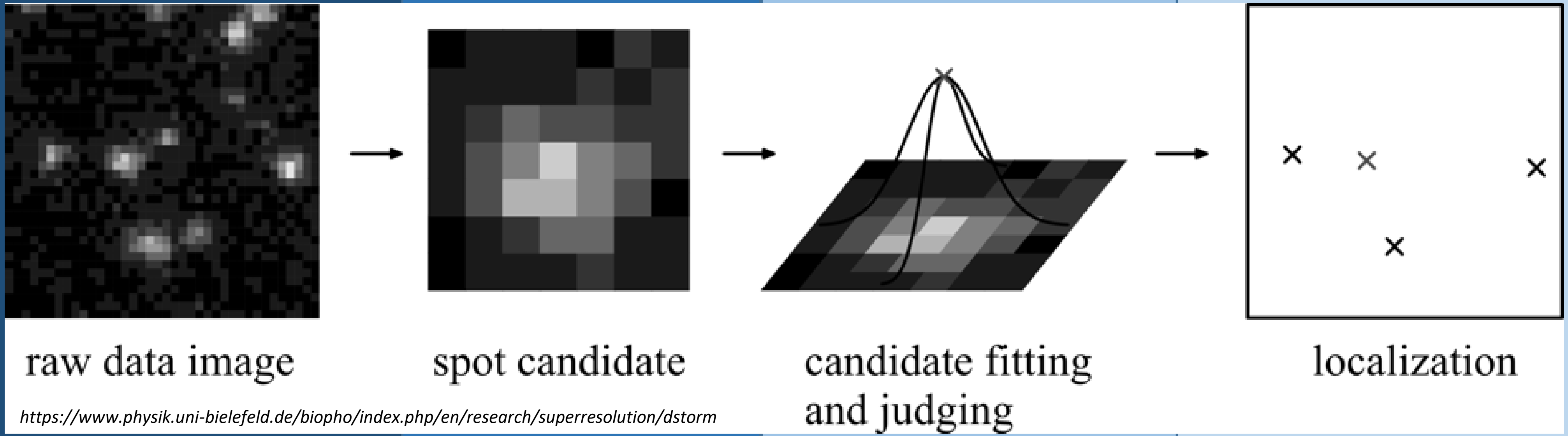
STORM and PALM



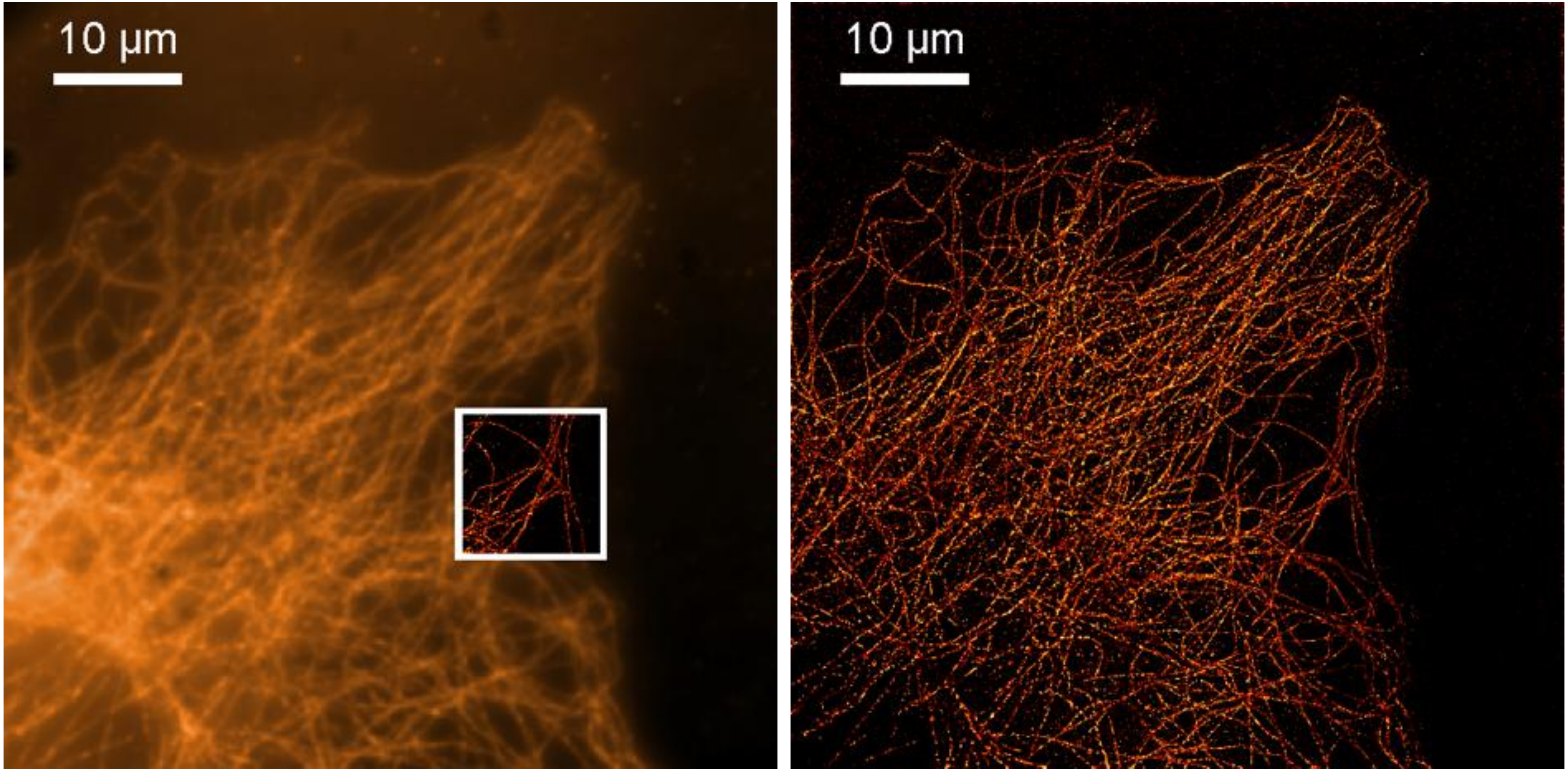
https://cam.facilities.northwestern.edu/588-2/single-molecule-localization-microscopy/

localization microcopy workflow

Acquisition of **thousands** images	Select **single molecule** flashes	Calculation of **centroids**	**Localization** of individual signals



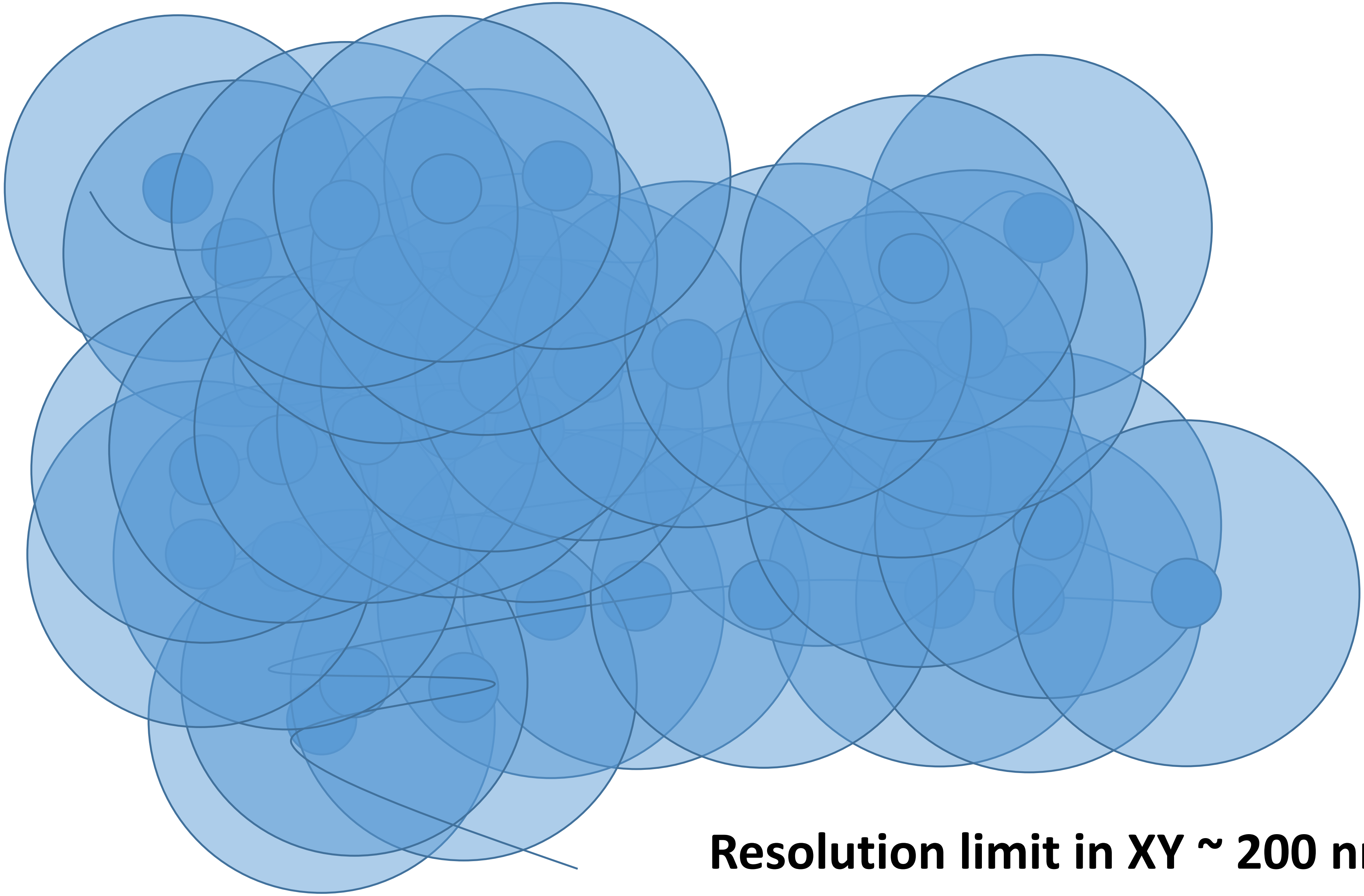
https://www.physik.uni-bielefeld.de/biopho/index.php/en/research/superresolution/dstorm



https://www.physik.uni-bielefeld.de/biopho/index.php/en/research/superresolution/dstorm

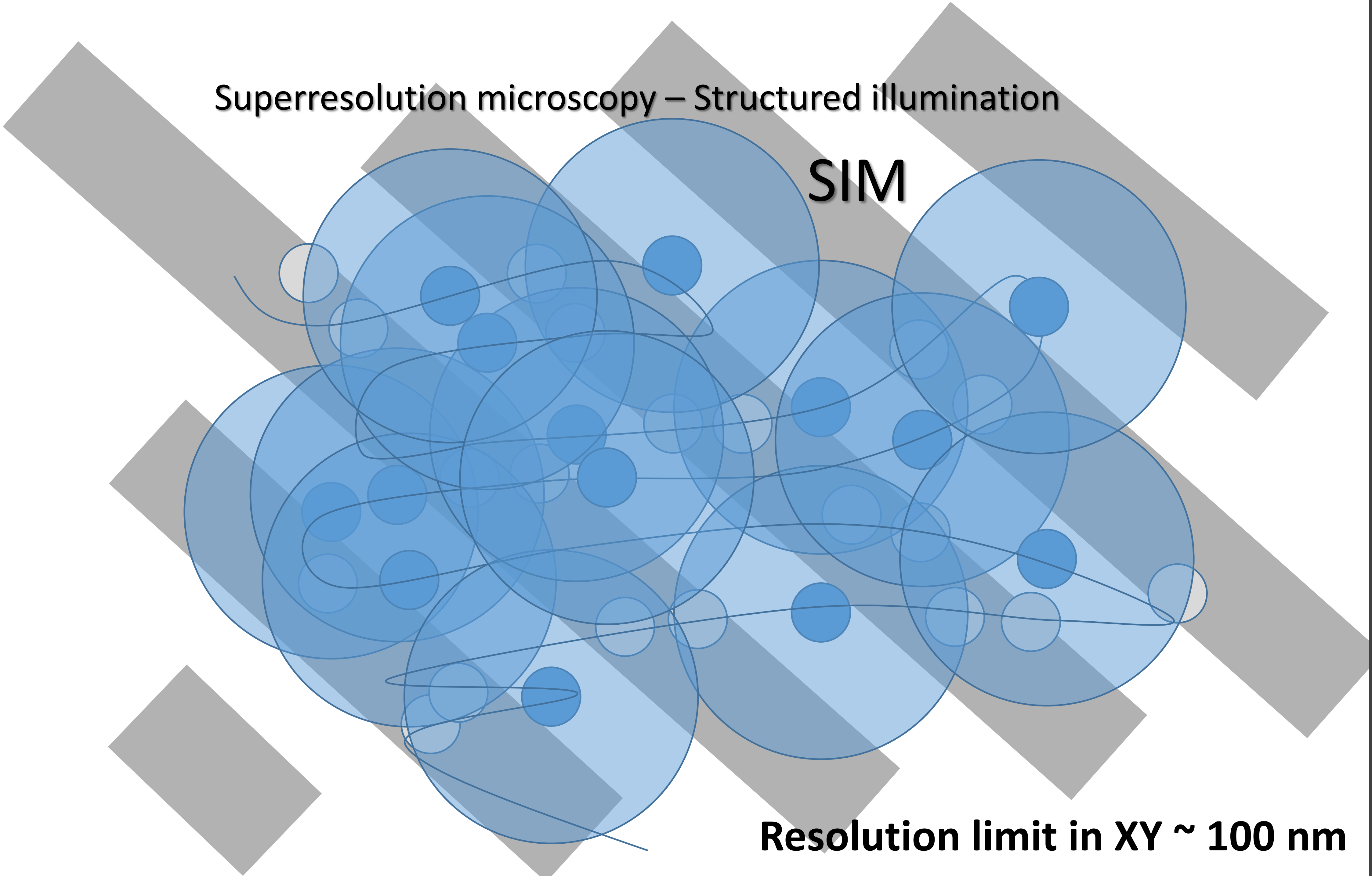
- How to understand principles of
  - Localization microscopy
  - SIM
  - STED

Superresolution microscopy



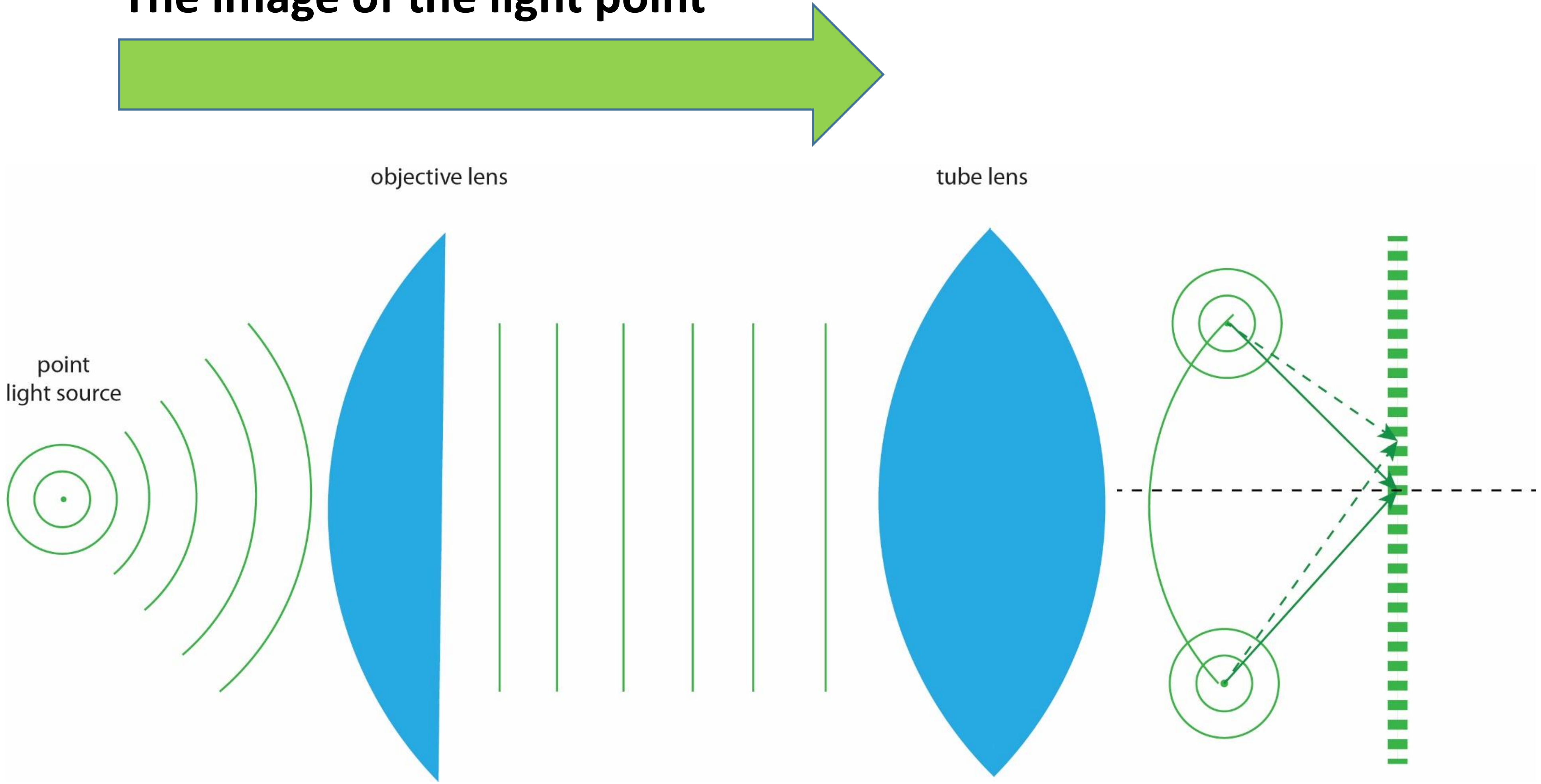
Resolution limit in XY ~ 200 nm

Superresolution microscopy – Structured illumination

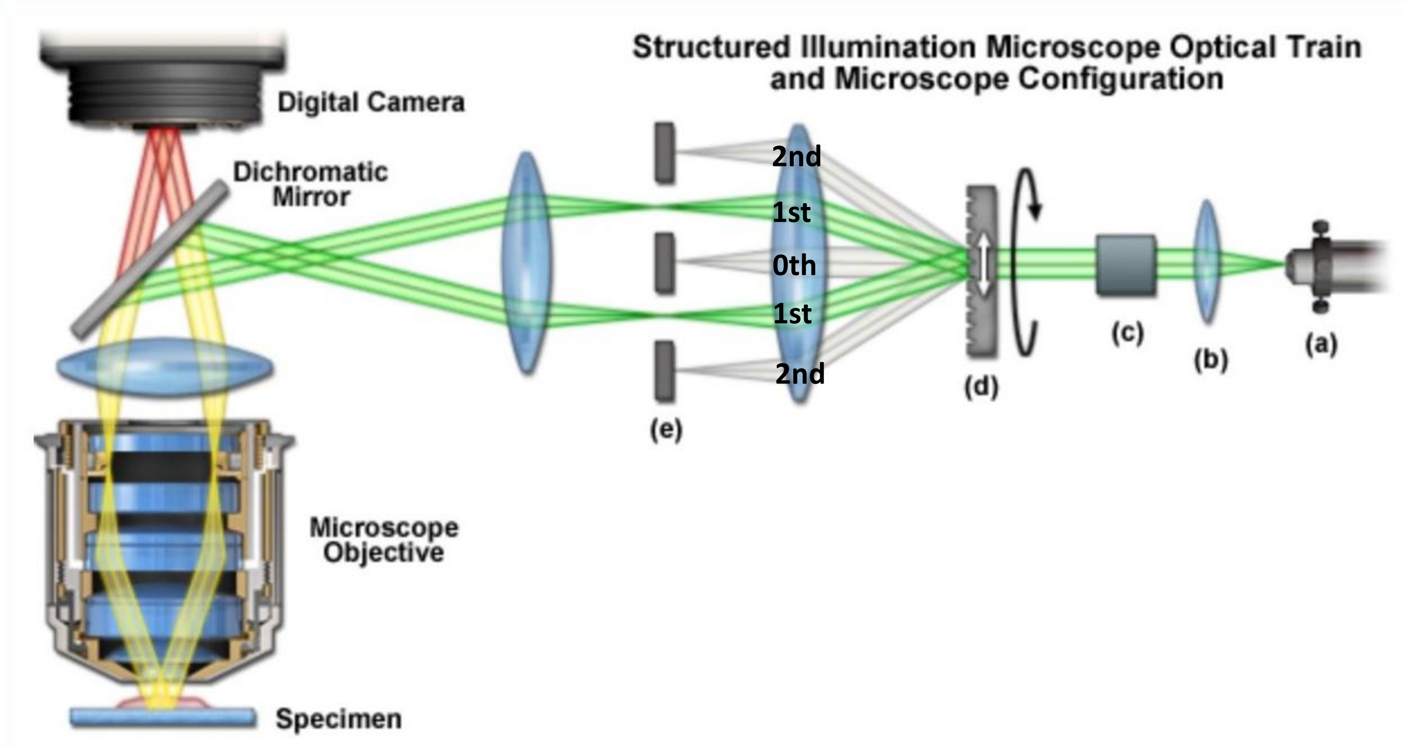


Resolution limit in XY ~ 100 nm

The image of the light point



2D-SIM

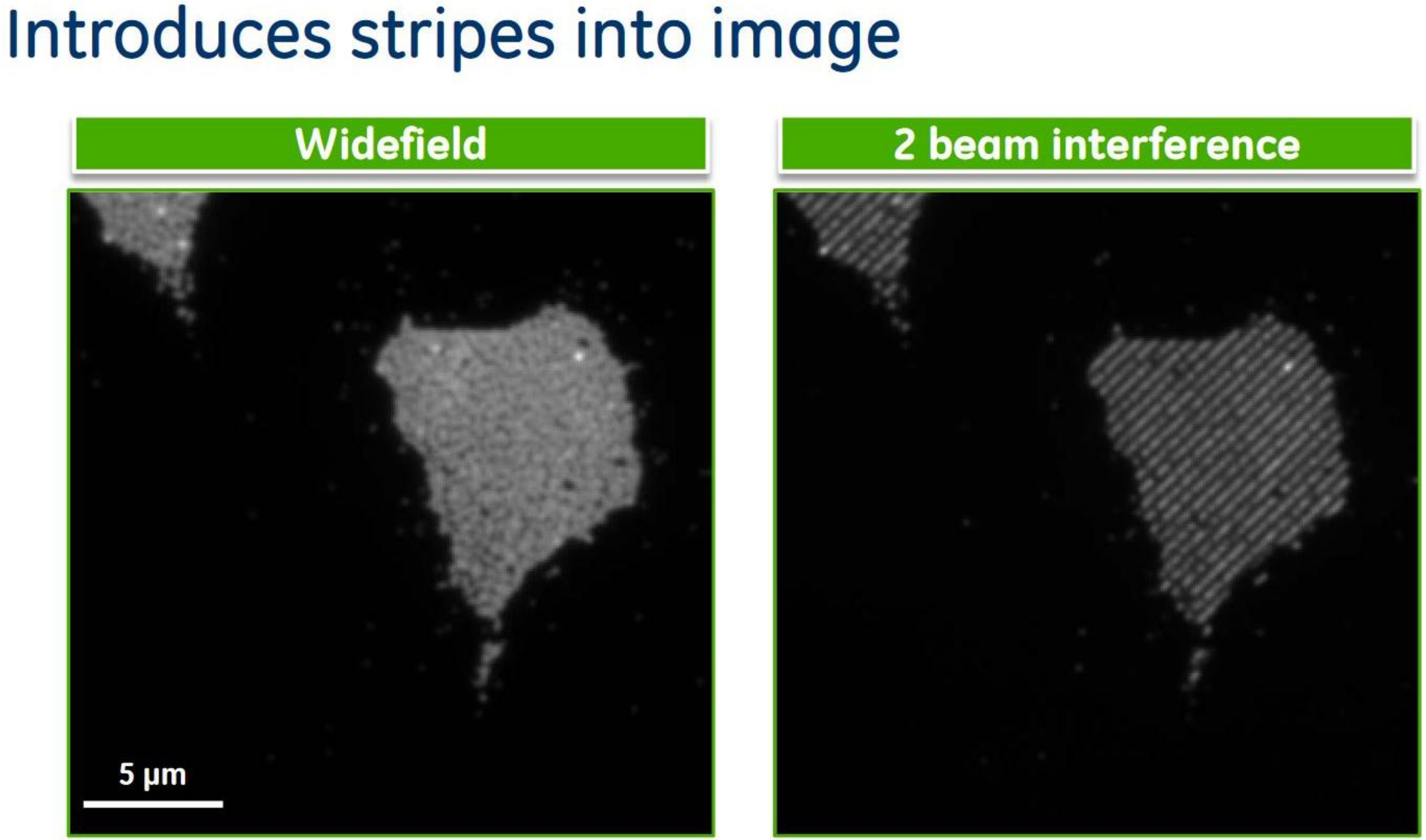


a) laser – a source of excitation; b) collimator; c) polarizer;

d) **diffraction grating**; e) beam block

source: zeiss-campus.magnet.fsu.edu/articles/superresolution/supersim.html

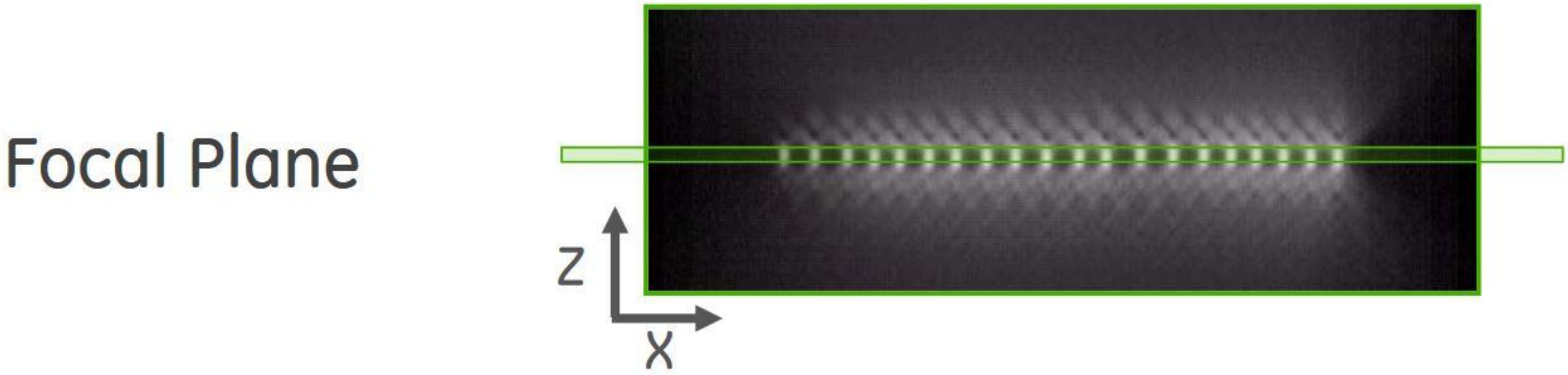
2-Beam Interference Grid Pattern illuminates the specimen



source: gelifesciences.com

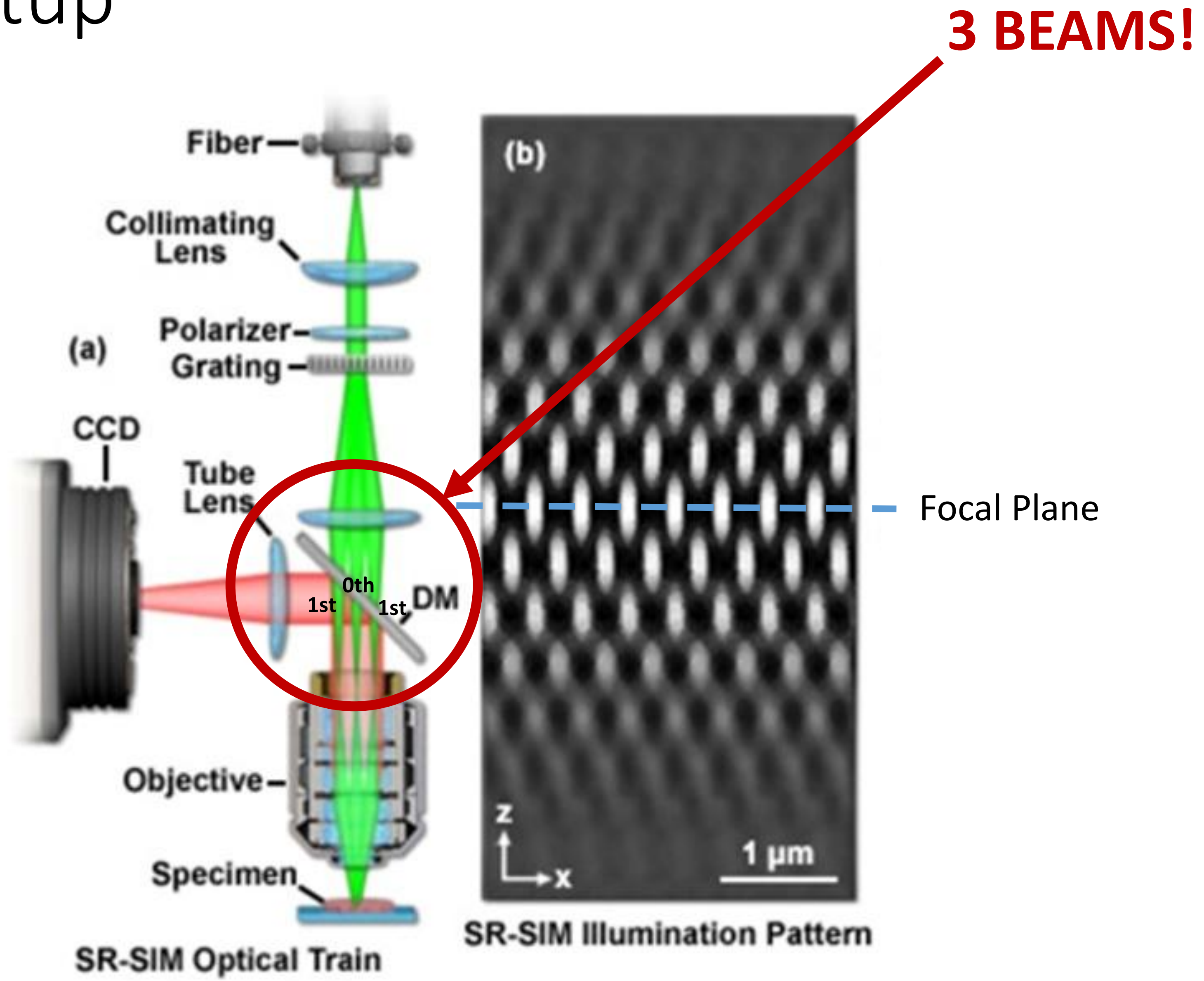
2-Beam Interference Grid Pattern

- **No increase** in contrast or resolution in Z-axis!



source: gelifesciences.com

3D-SIM setup

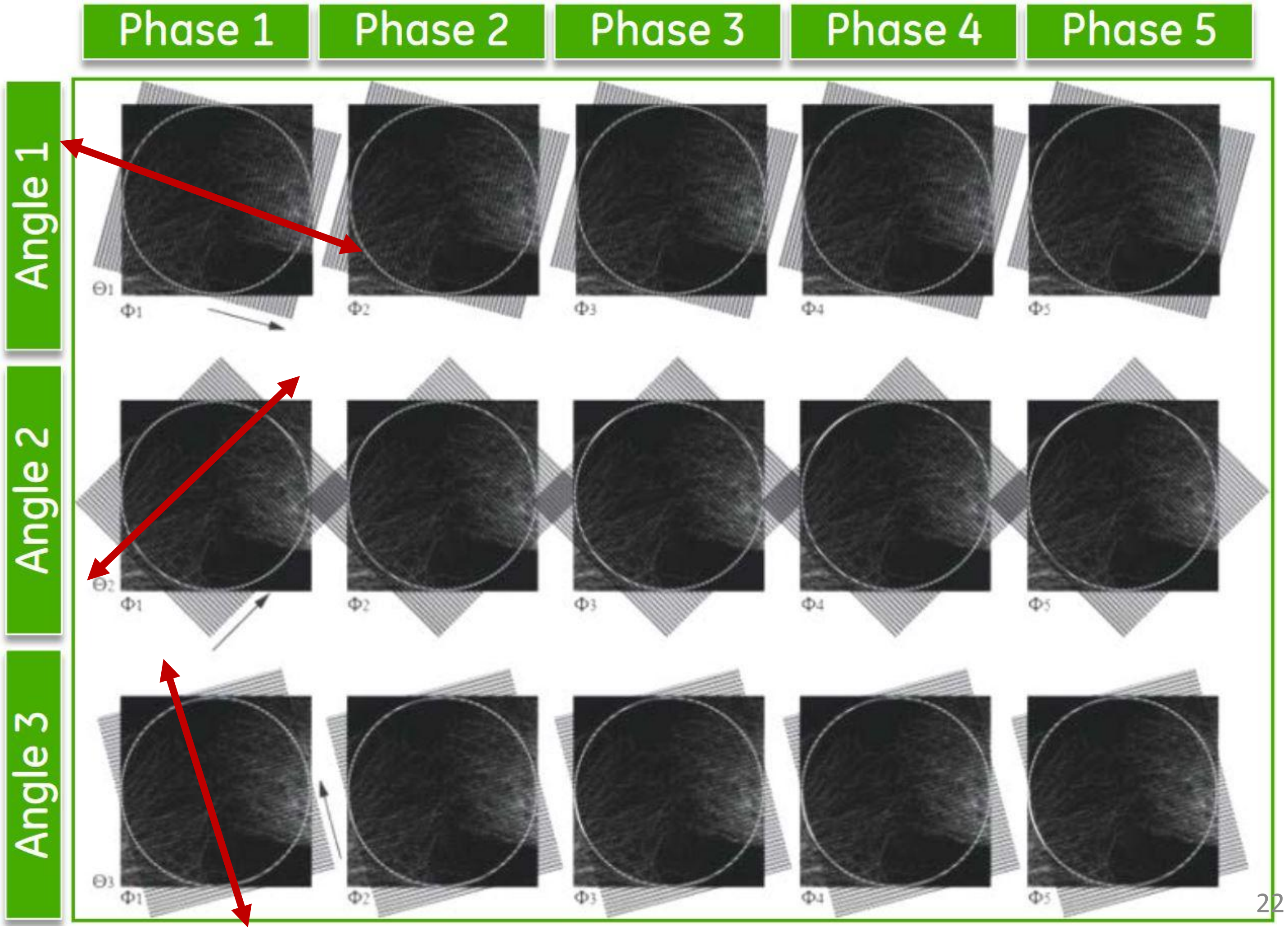


source: zeiss-campus.magnet.fsu.edu/articles/superresolution/supersim.html

3D-SIM Imaging

Image with striped pattern at **3 angles** & **5 phases**

15 stripped widefield images per one optical layer

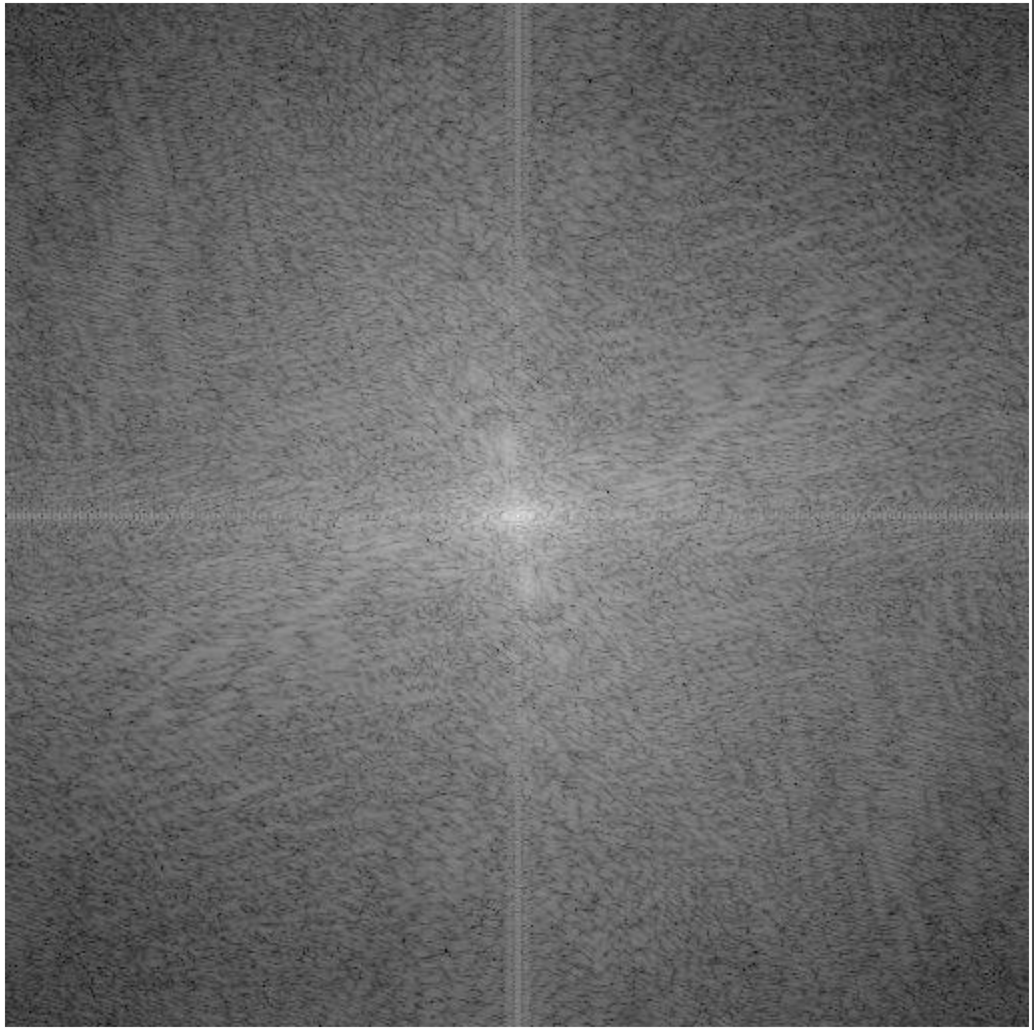


source: gelifesciences.com

SIM – reconstruction

- **Transformation of acquired pictures into Fourier space** (Fourier transform)
- Separation of components
- Alignment of separated components
- Inverse Fourier transform of the reconstructed Fourier image

Fourier transformation of the image

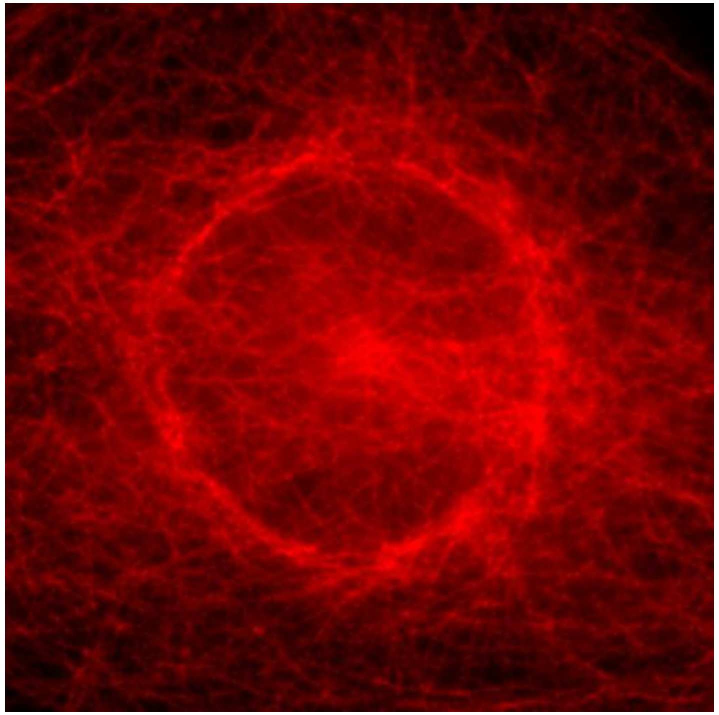


SIM – reconstruction – Fourier transform

Transformation of a **spatial image** into a **Fourier image!**

[0, 0] x

y

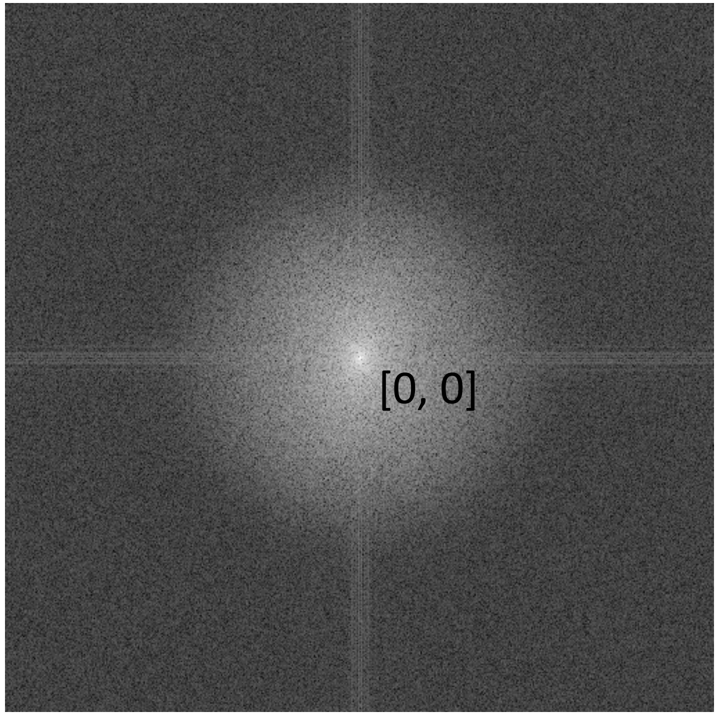


[512, 512]

Fourier transform

ky

-kx



[0, 0]

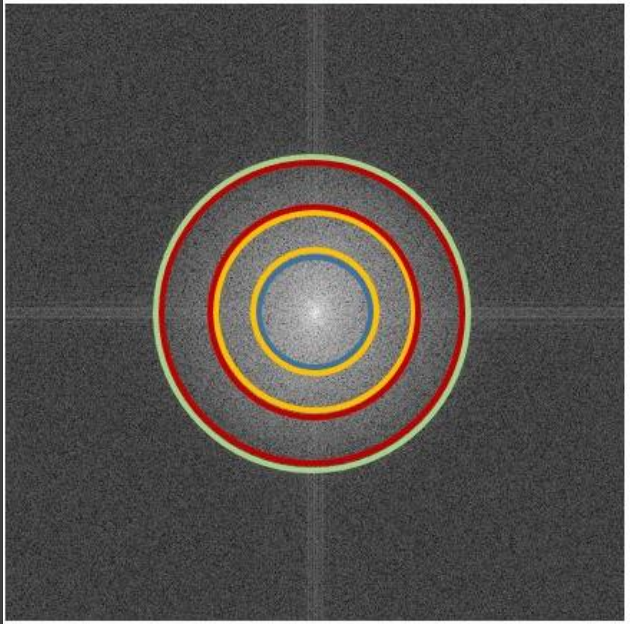
kx

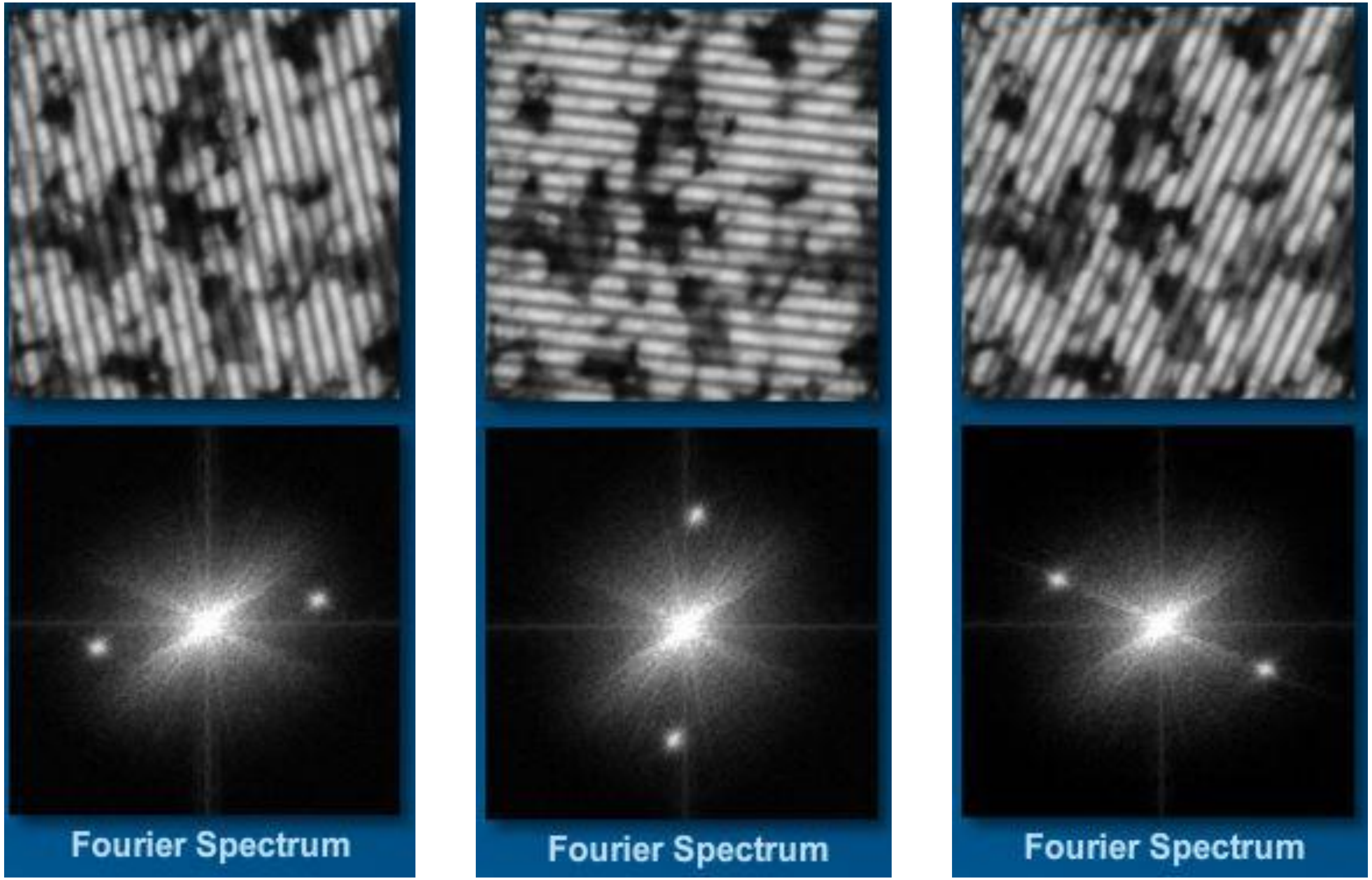
-ky

Spatial image
x, **y** – spatial coordinates
intensity – amount of fluorescence emission at [x, y]

Fourier image
kx, ky – spatial frequences
intensity – occurrence of spatial frequence at [kx, ky]

SIM – reconstruction – **Transformation into Fourier space** (grids rotated by 60°)





source: zeiss-campus.magnet.fsu.edu

SIM – reconstruction – **summary**

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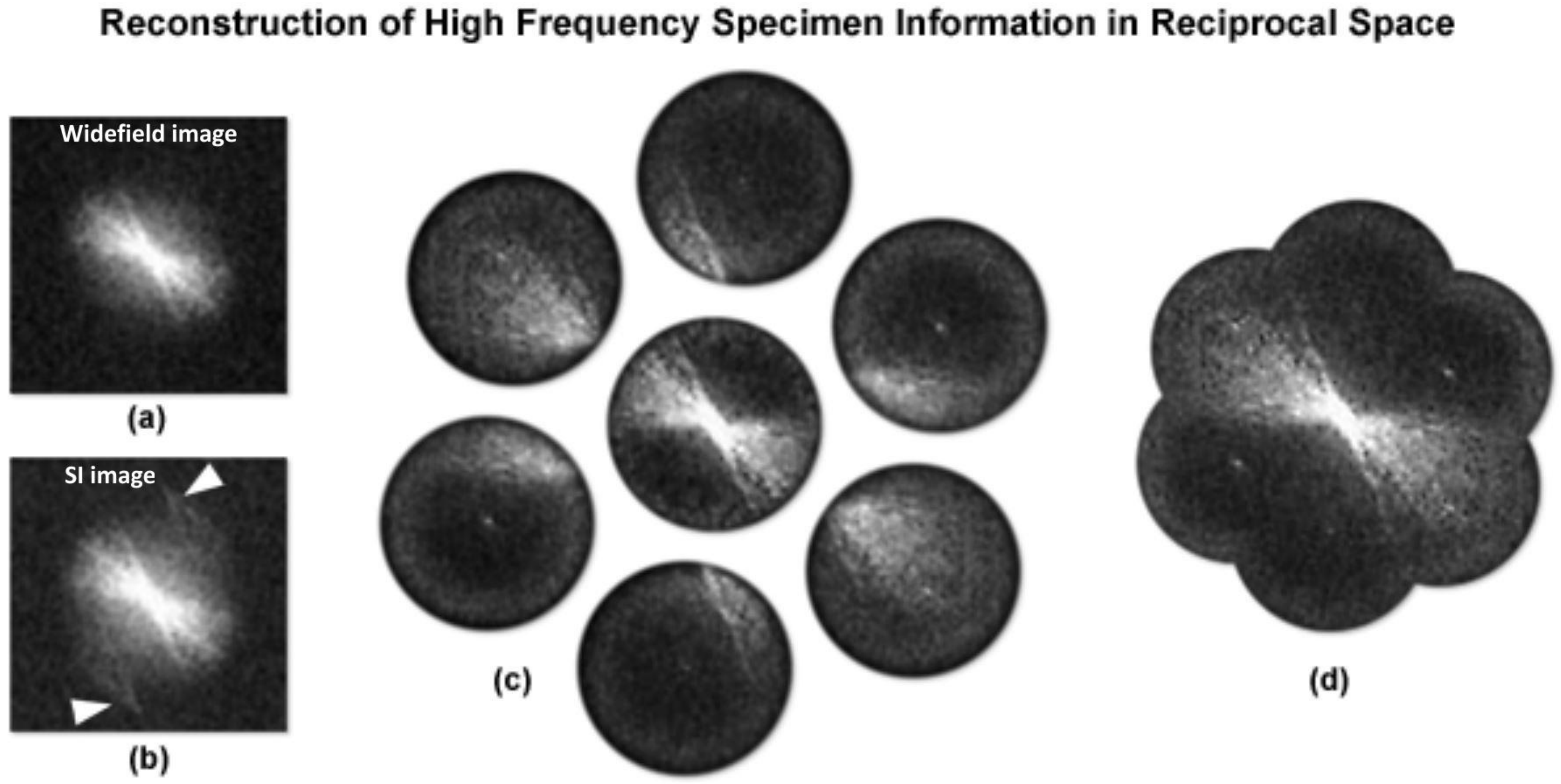
- **Separation of components**
- Alignment of separated components
- Inverse Fourier transform of the reconstructed Fourier image

SIM – reconstruction – Separation of components



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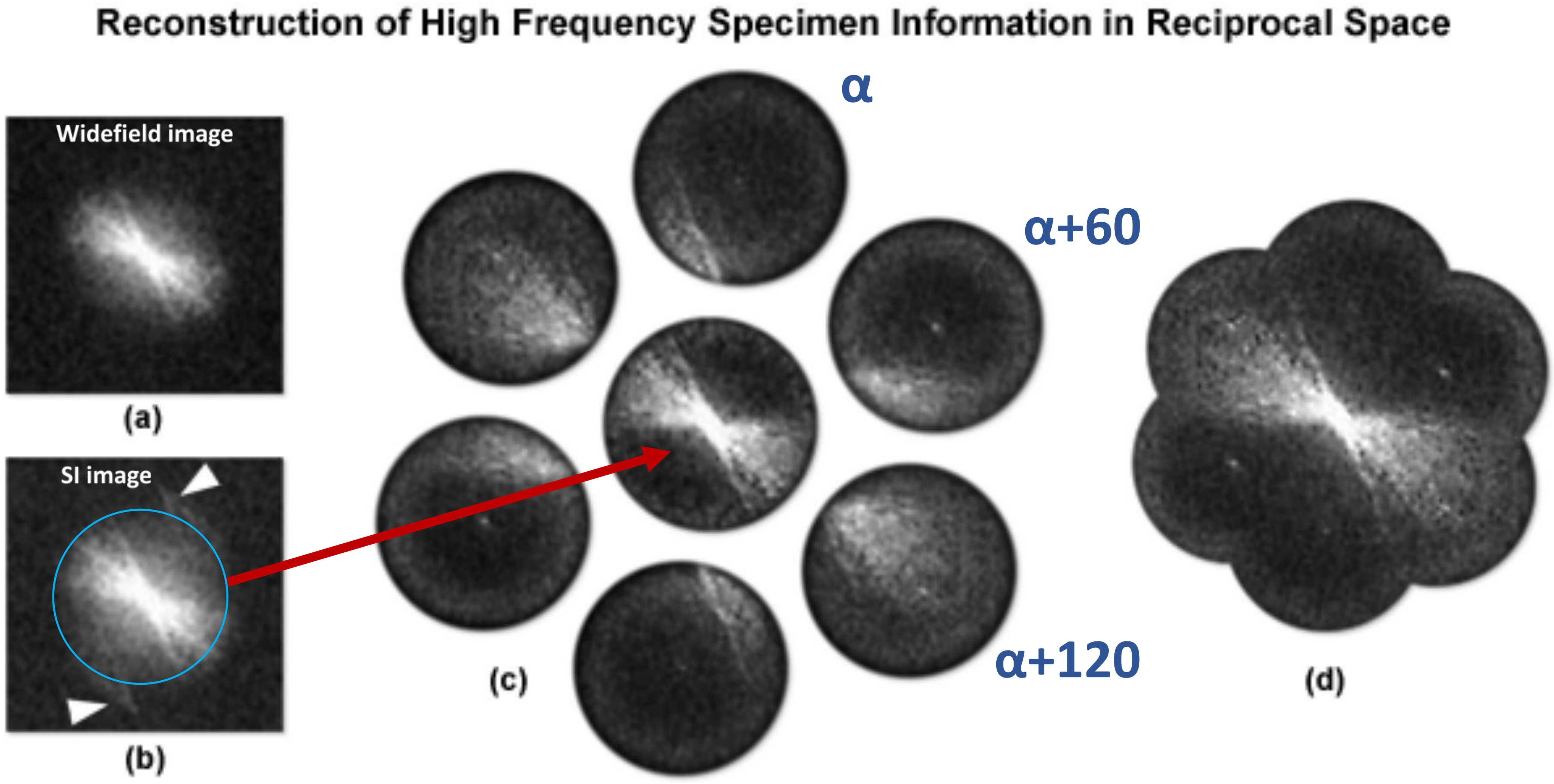
SIM – reconstruction – Separation of components



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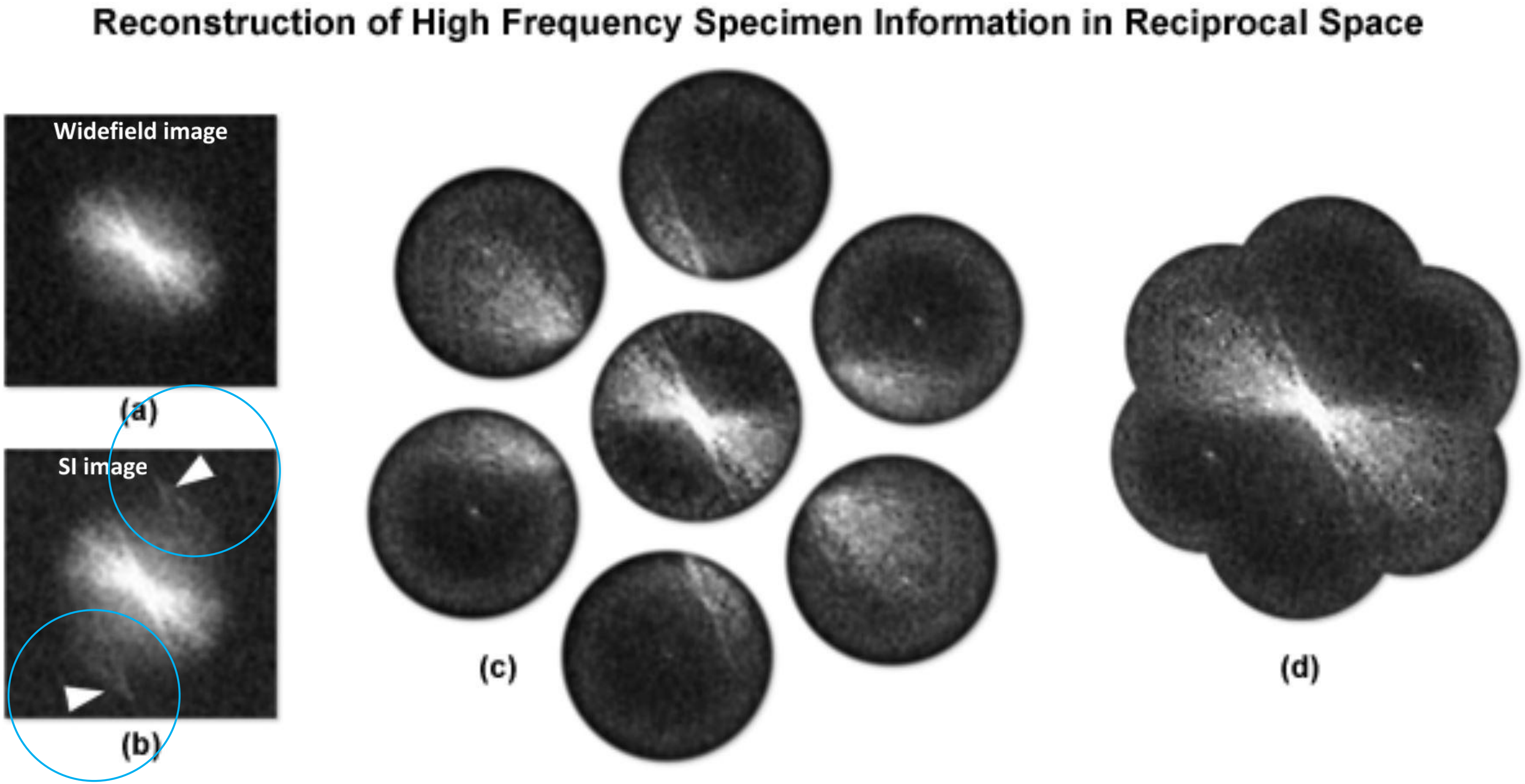
SIM – reconstruction – Separation of components



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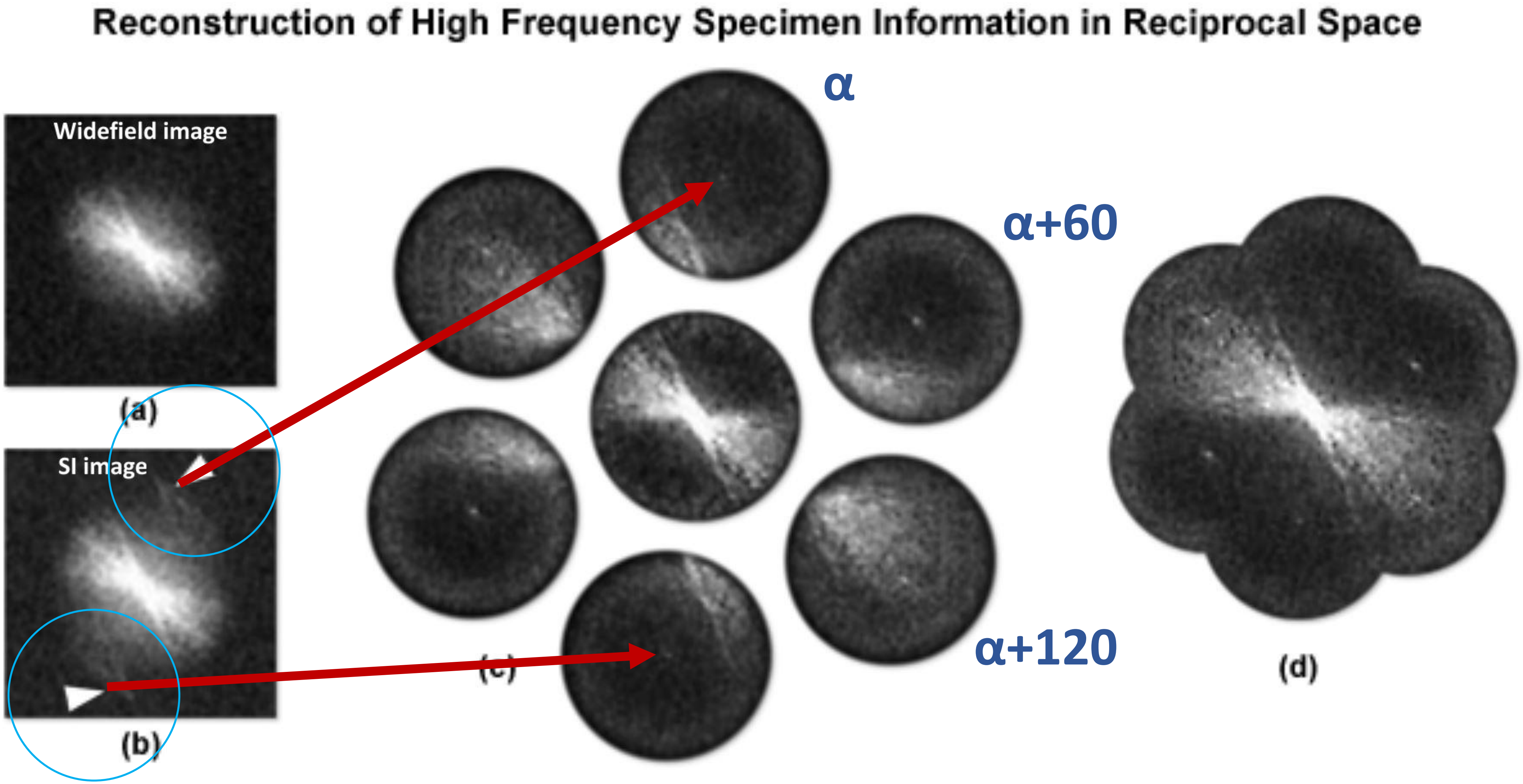
SIM – reconstruction – Separation of components



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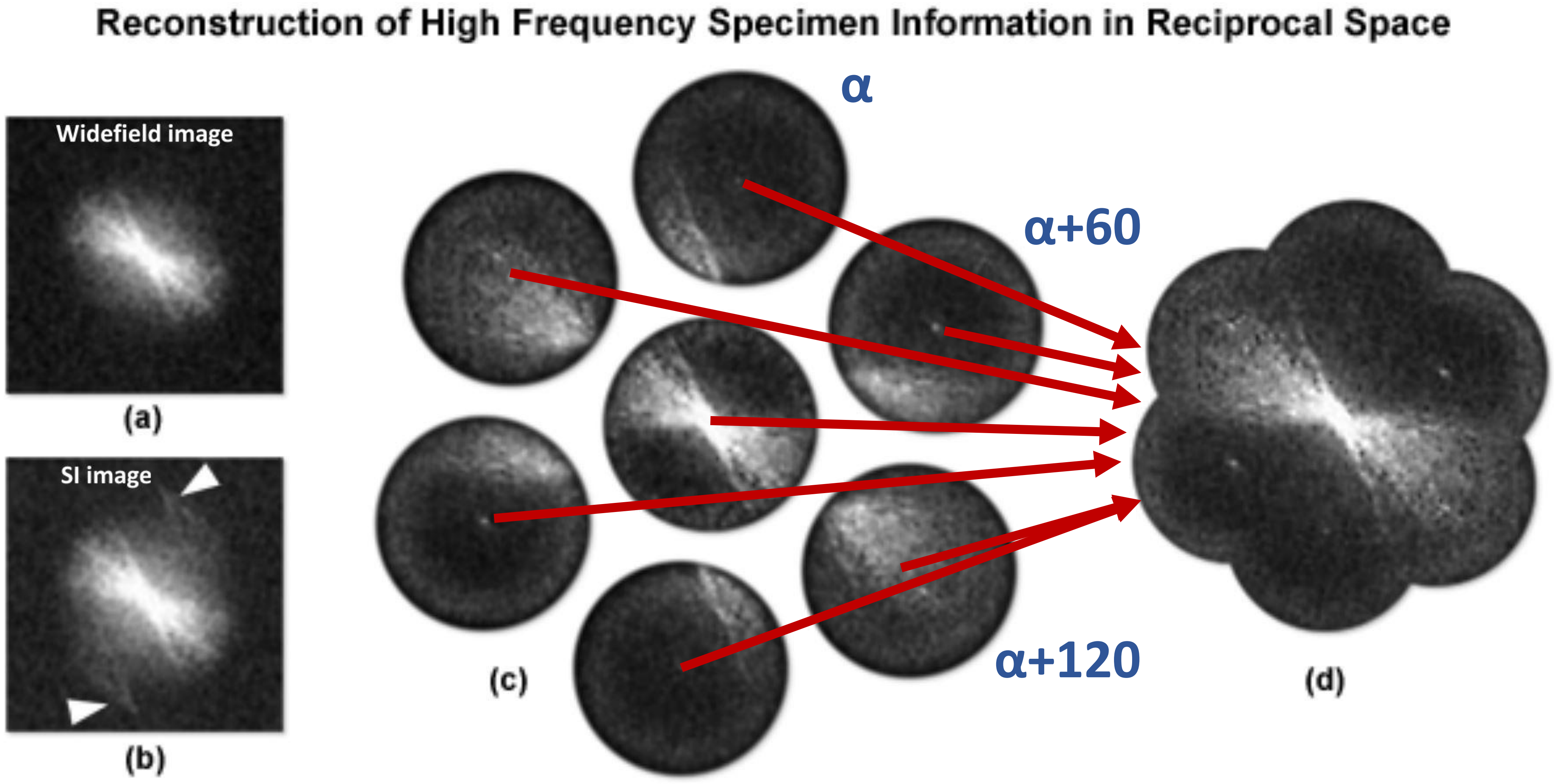
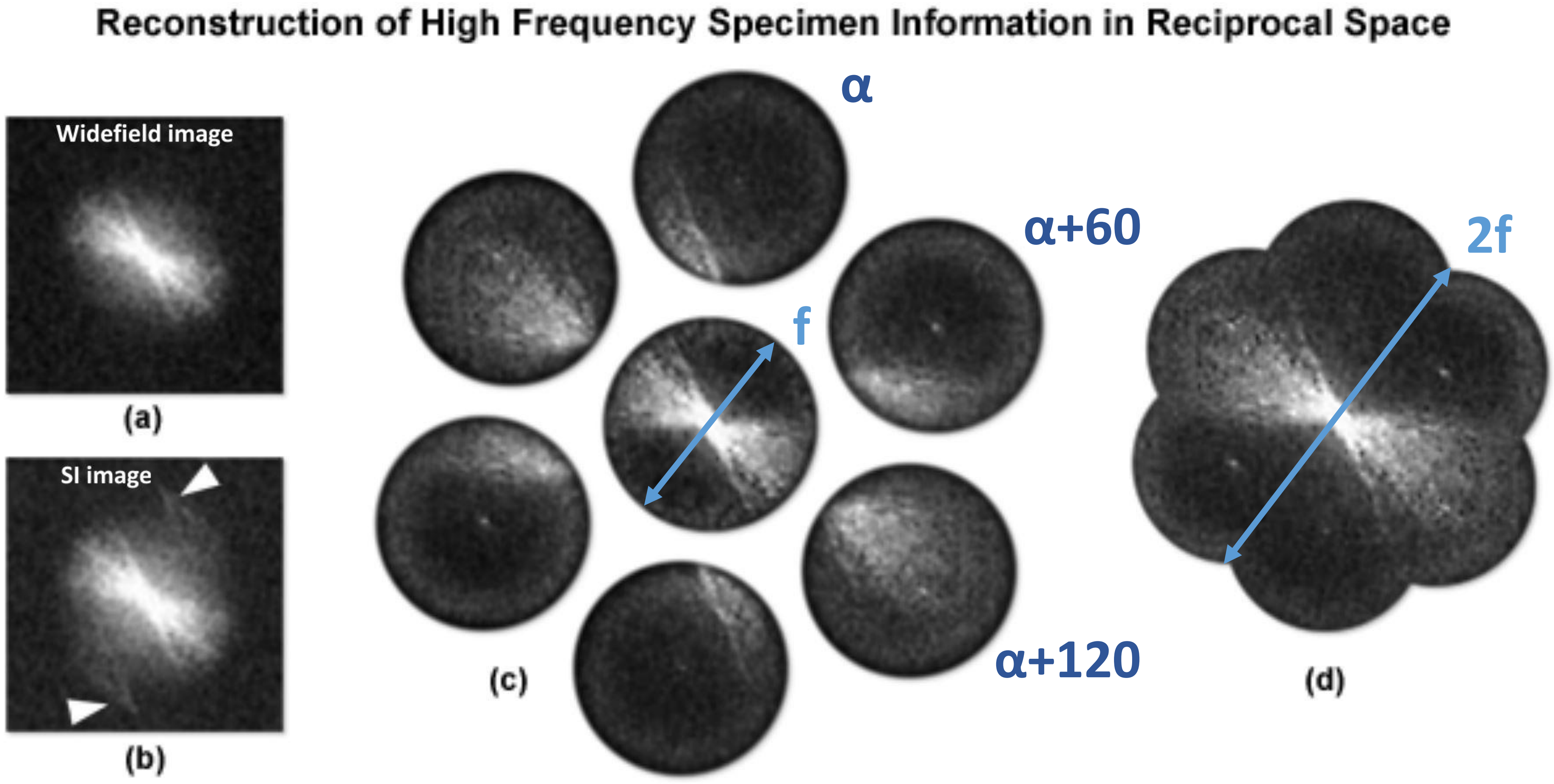
SIM – reconstruction – Alignment of separated components



source: zeiss-campus.magnet.fsu.edu

SIM – reconstruction – Alignment of separated components

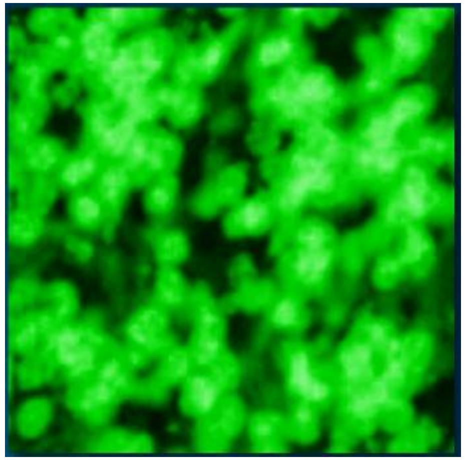


source: zeiss-campus.magnet.fsu.edu

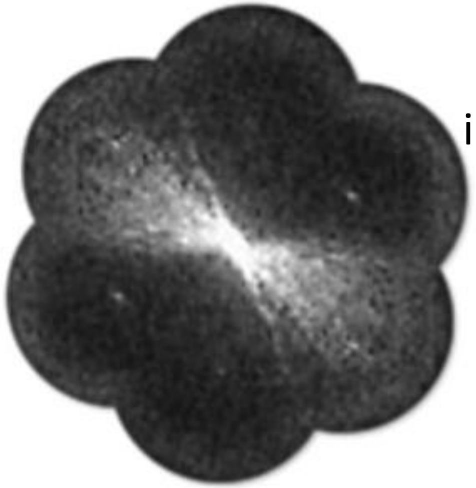
SIM – reconstruction – Inverse Fourier transform of the reconstructed image

Fourier images **spatial images**

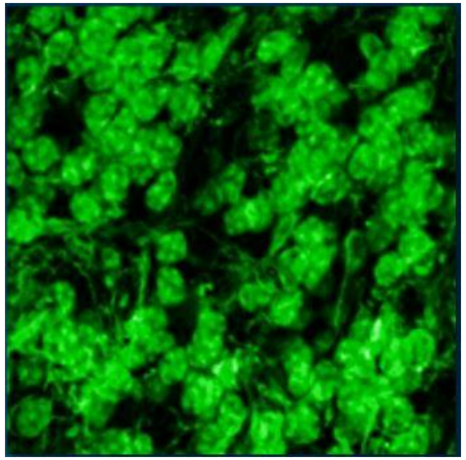
inverse Fourier transform



Widefield image



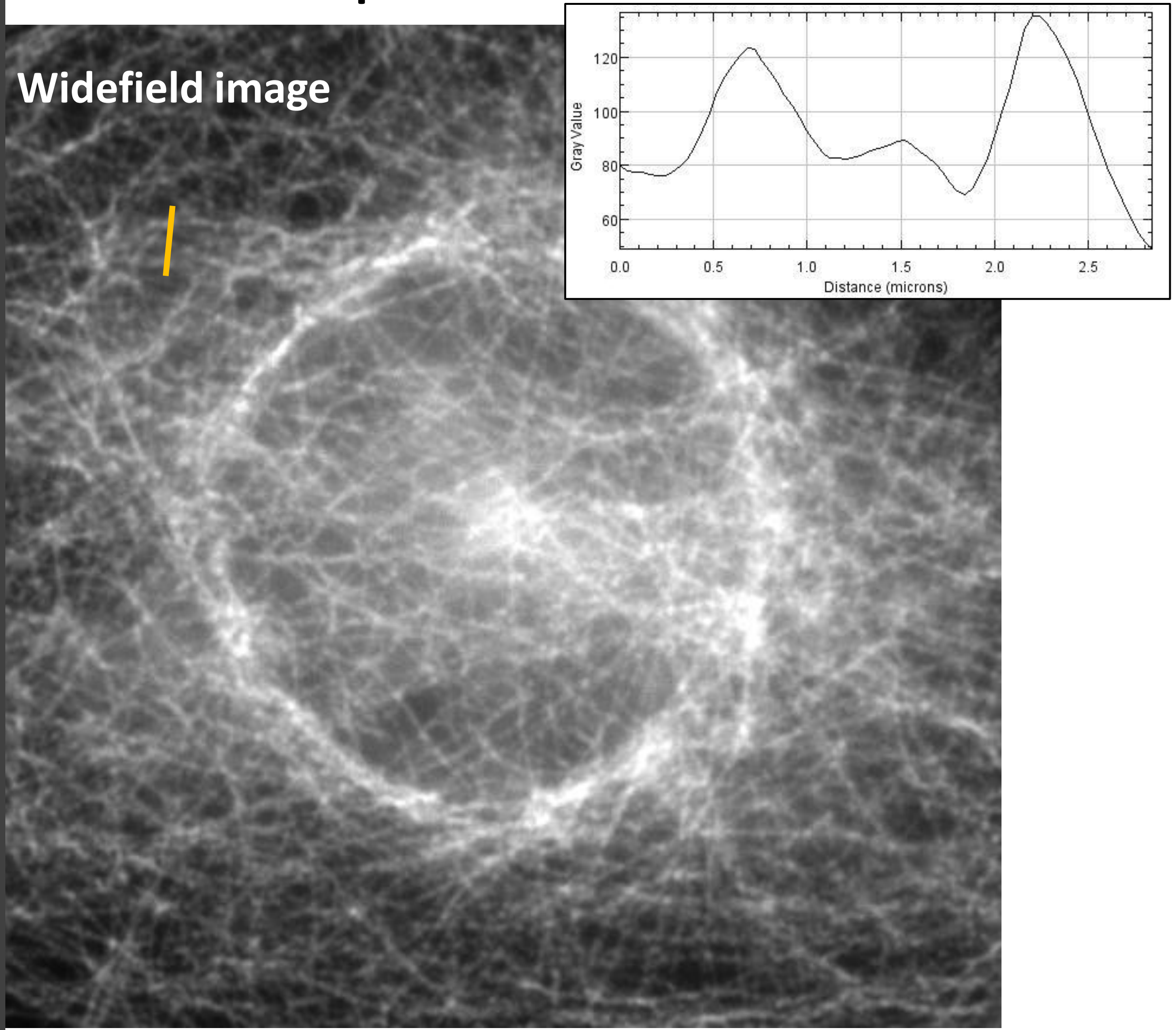
inverse Fourier transform

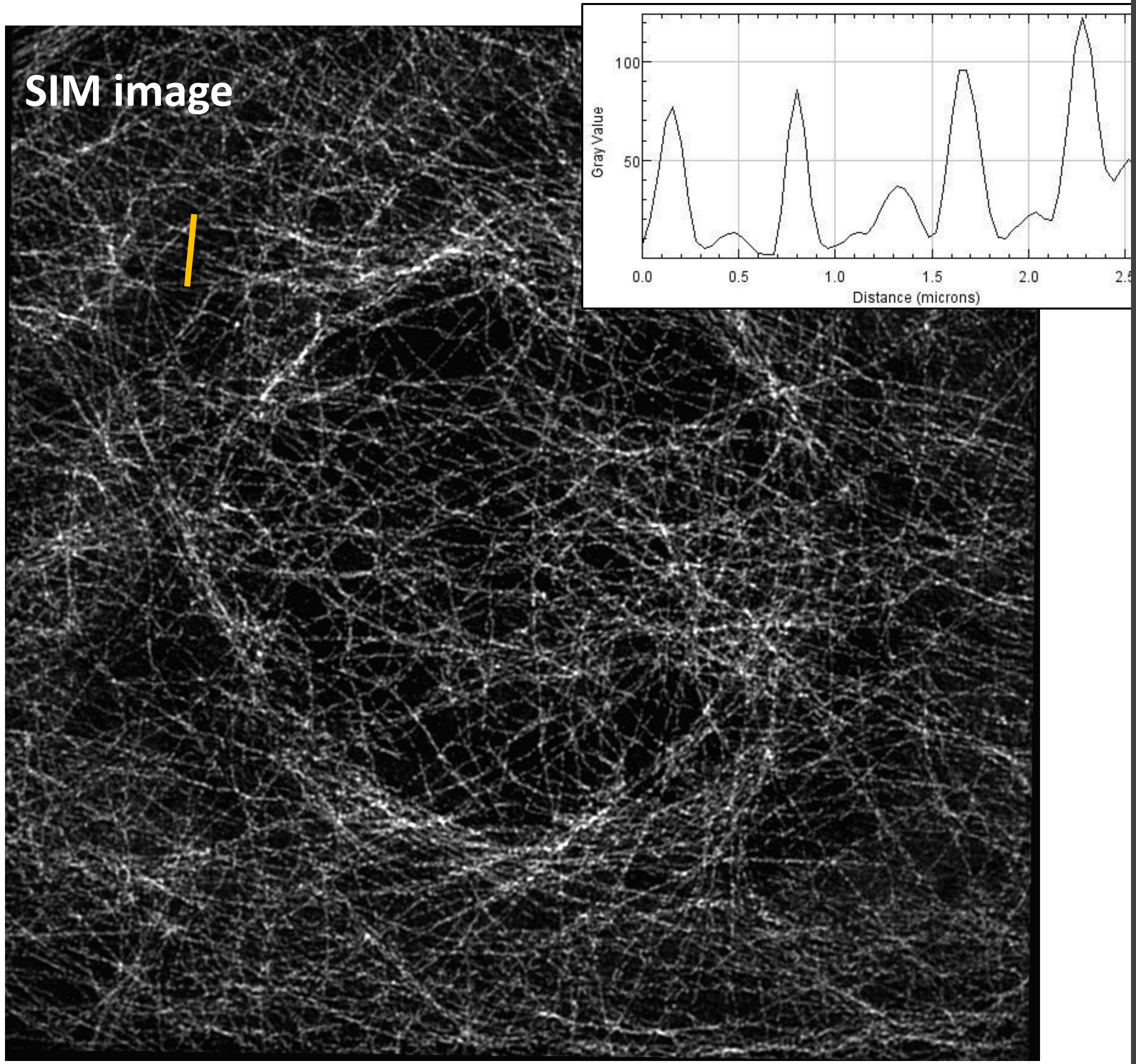


Super-resolution SIM image

source: zeiss-campus.magnet.fsu.edu

Example data from WF and SIM

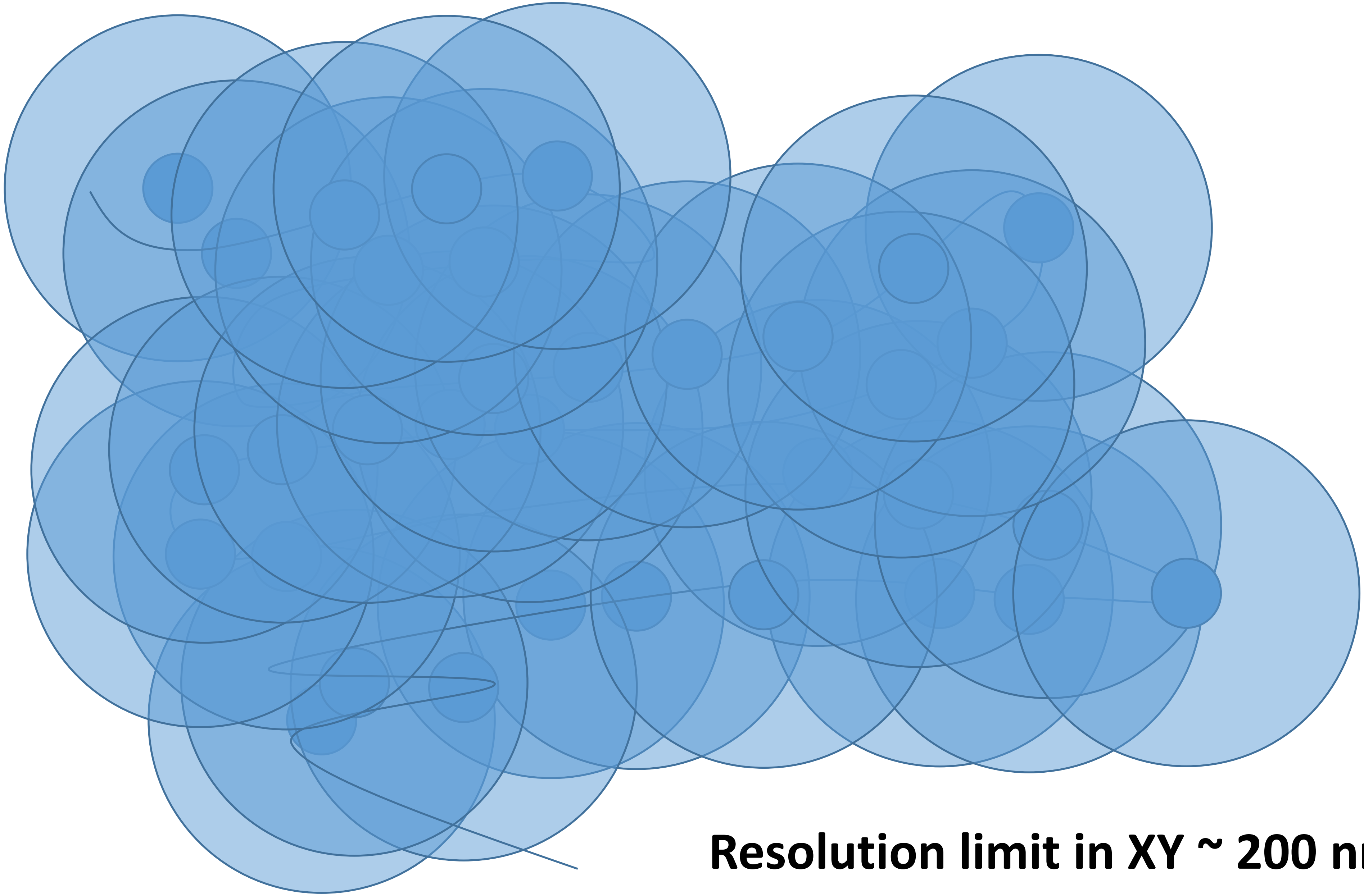




Tubulin in He-La cells; exc. 555nm, em. 609nm; OMX SR-SIM Deltavision

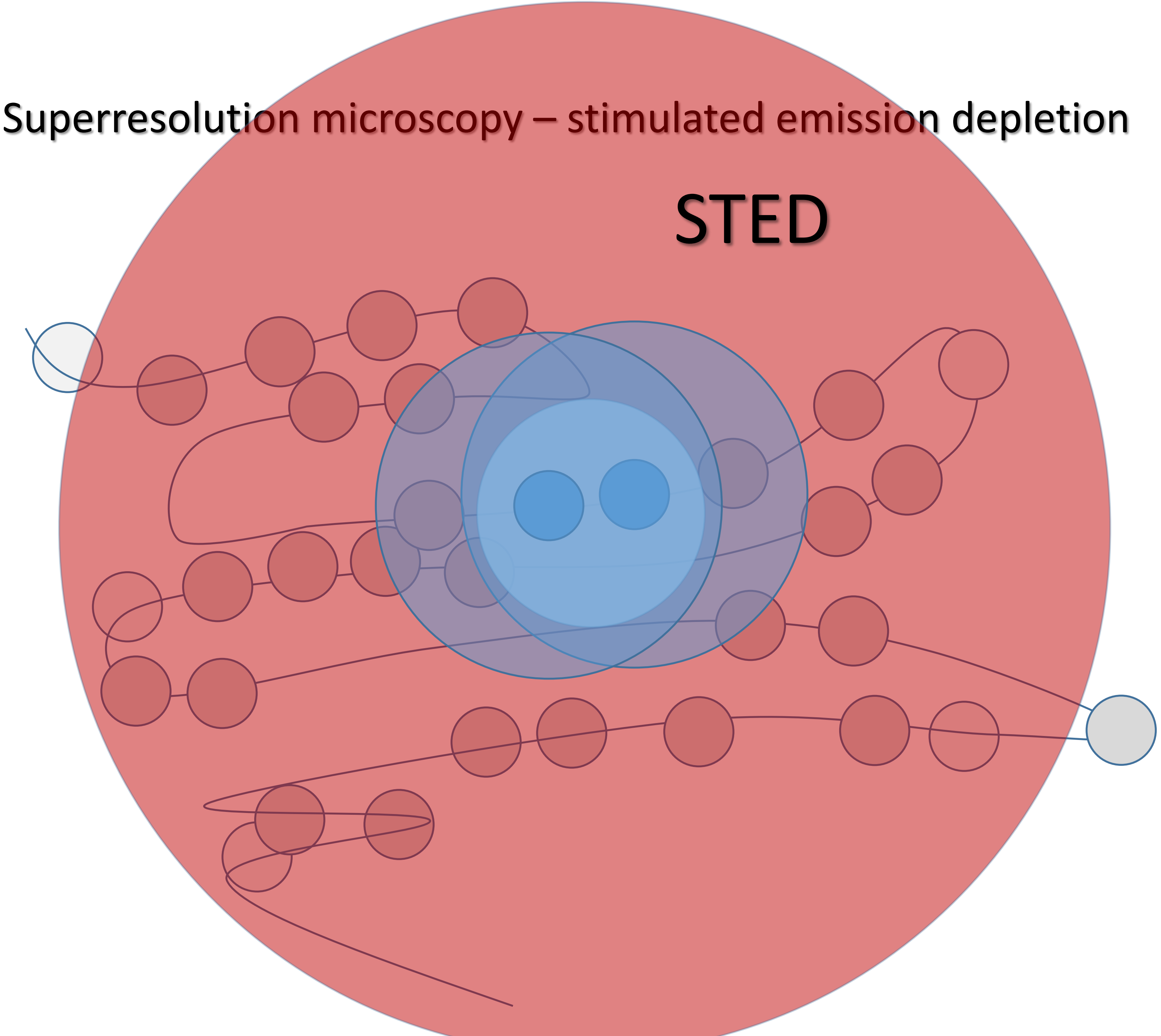
- How to understand principles of
    - Localization microscopy
    - SIM
    - STED

Superresolution microscopy



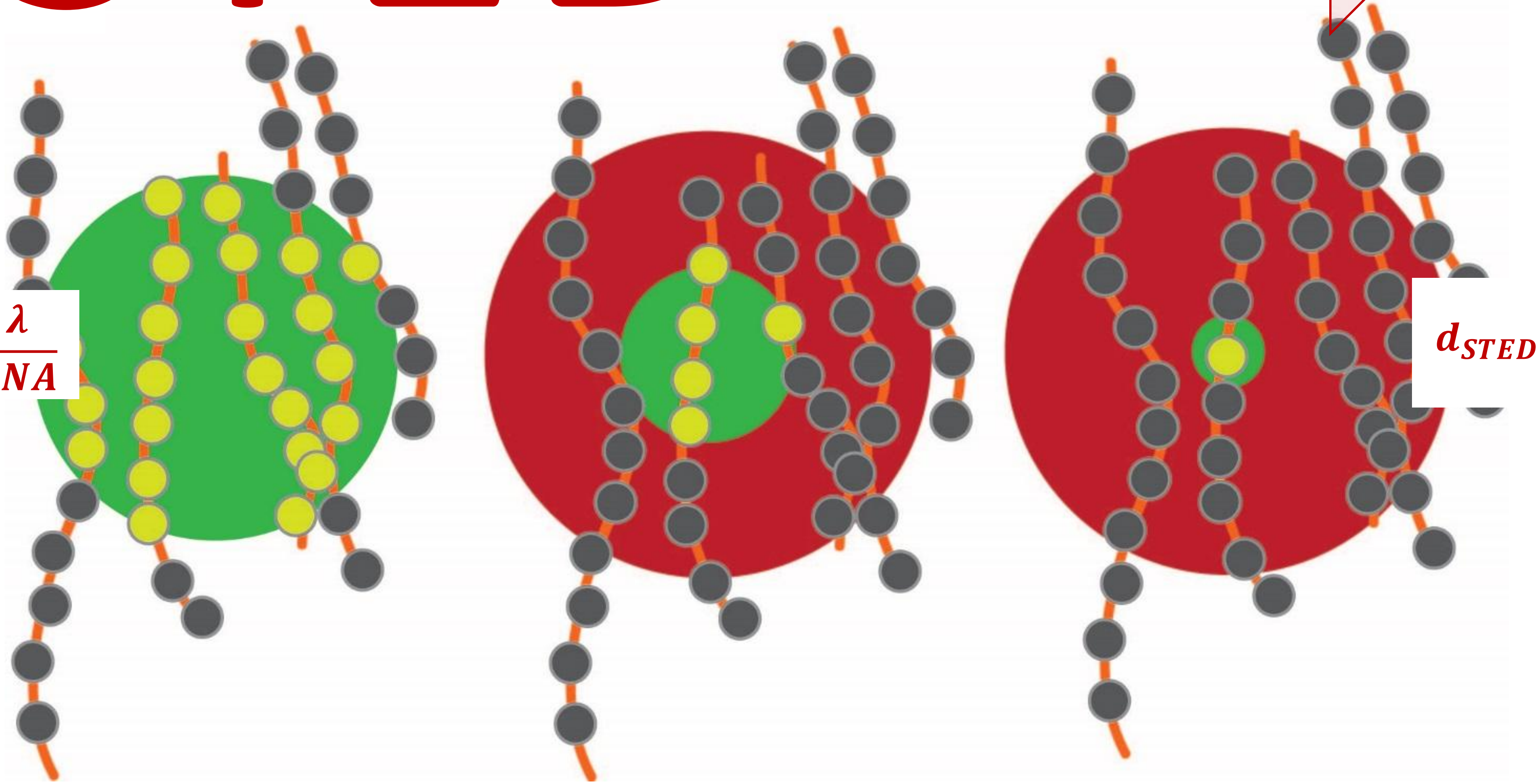
Resolution limit in XY ~ 200 nm

Superresolution microscopy – stimulated emission depletion



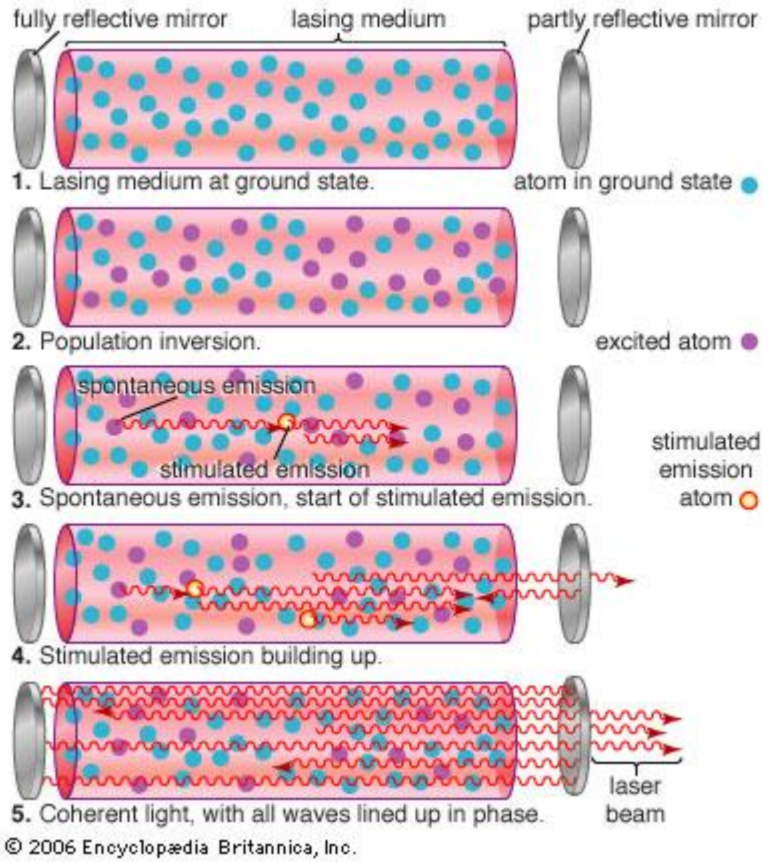
STED

$$d = \frac{\lambda}{2NA}$$

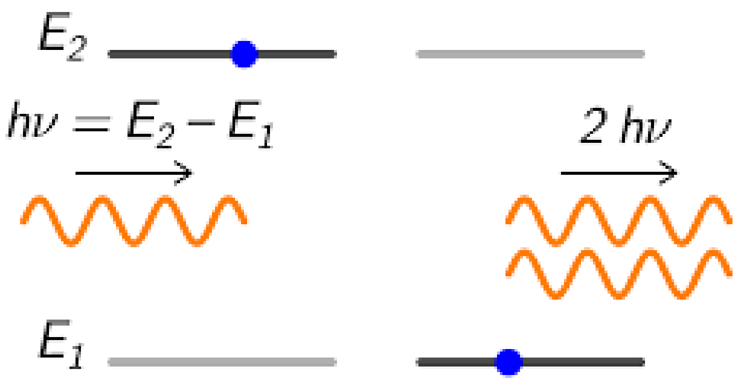


$$d_{STED} = \frac{\lambda}{2NA\sqrt{1+\xi}}$$

Principle of the LASER: stimulated emission

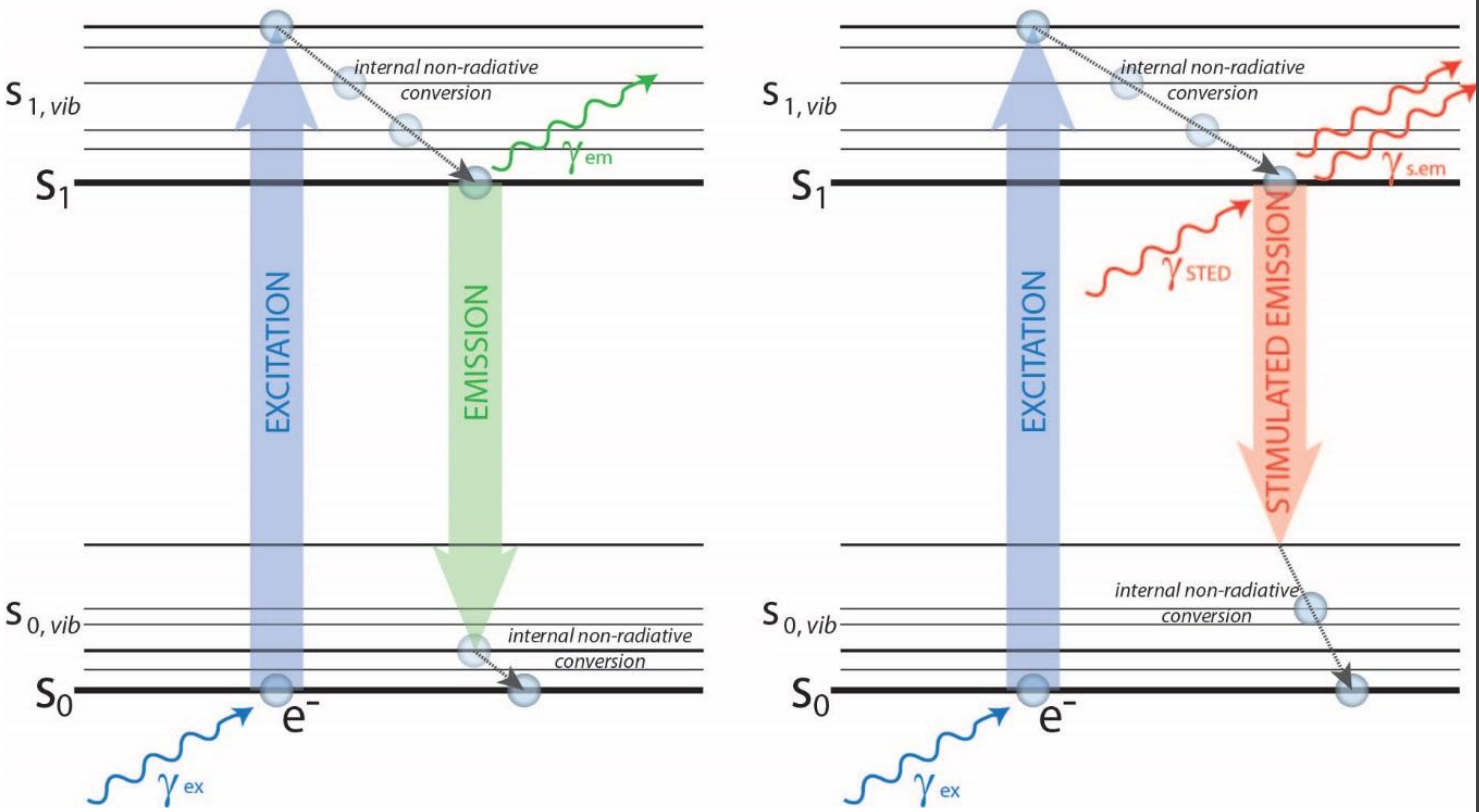


LASER = light amplification by stimulated emission of radiation.

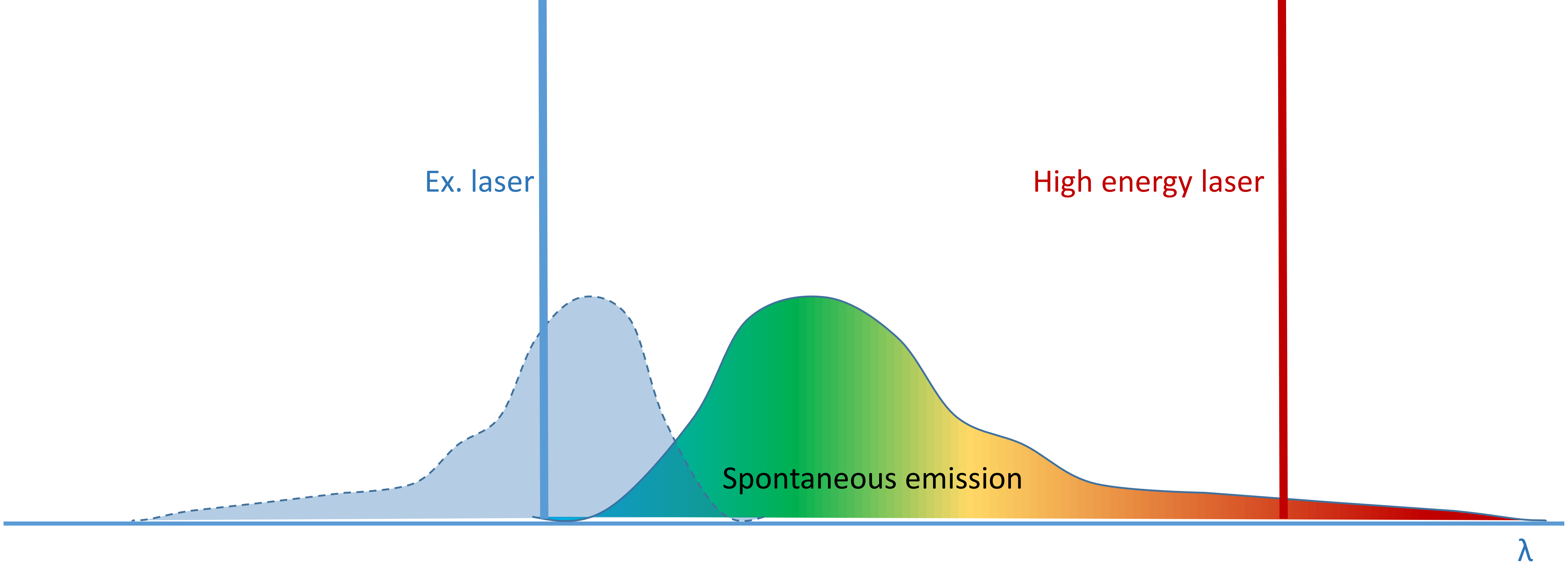


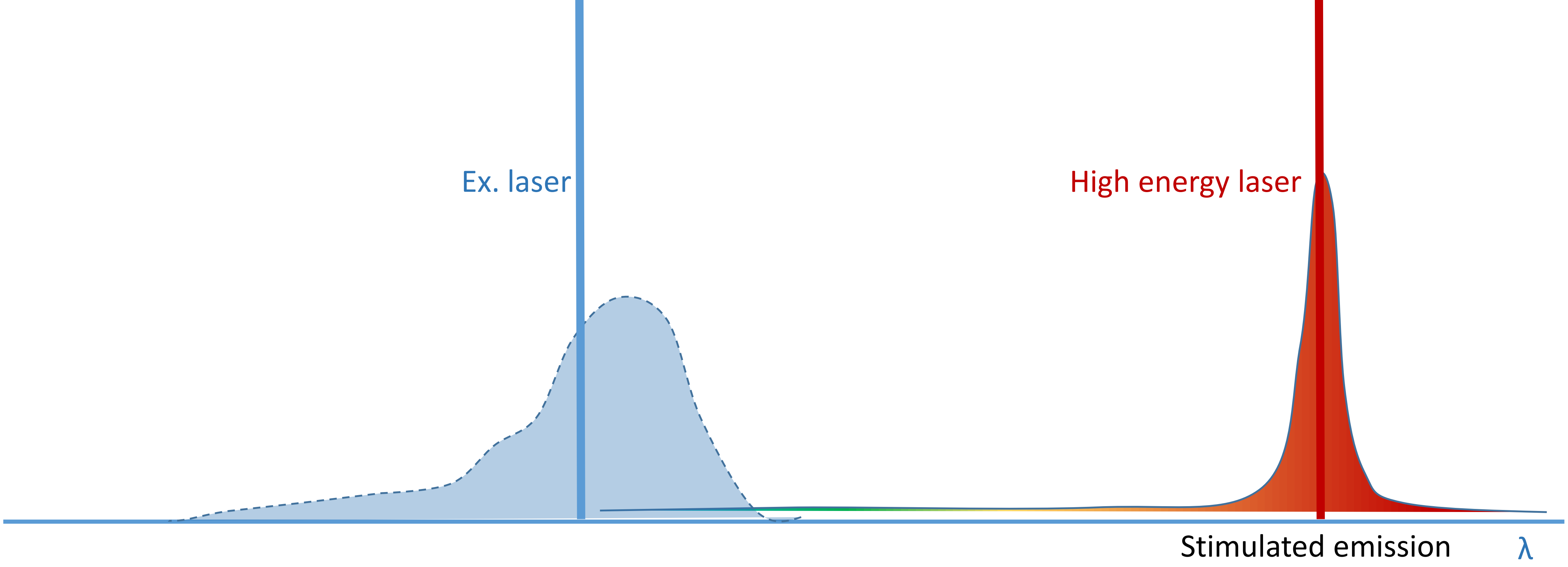
https://www.britannica.com/technology/stimulated-emission

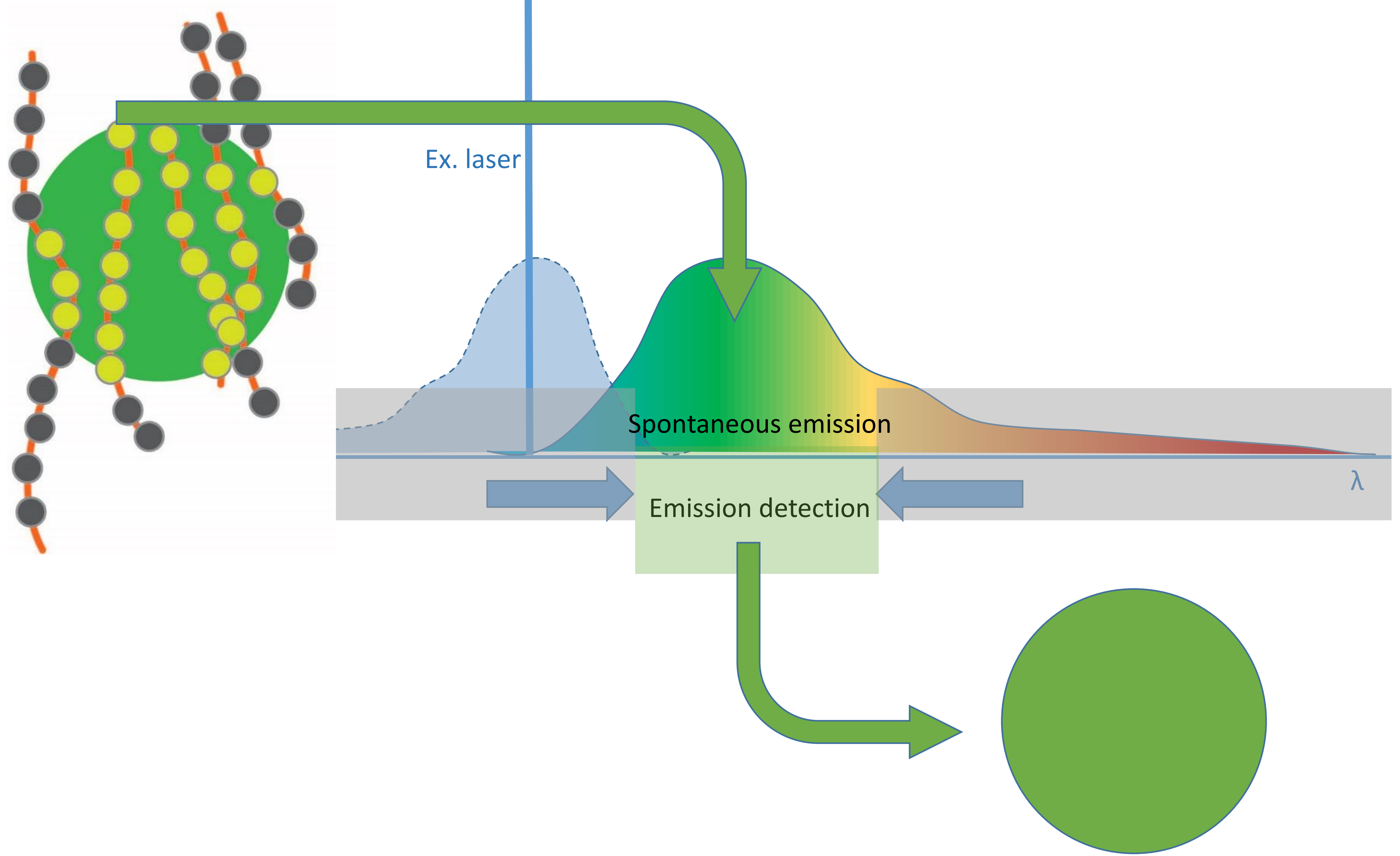
https://web2.ph.utexas.edu/~coker2/index.files/xrayslasers.htm



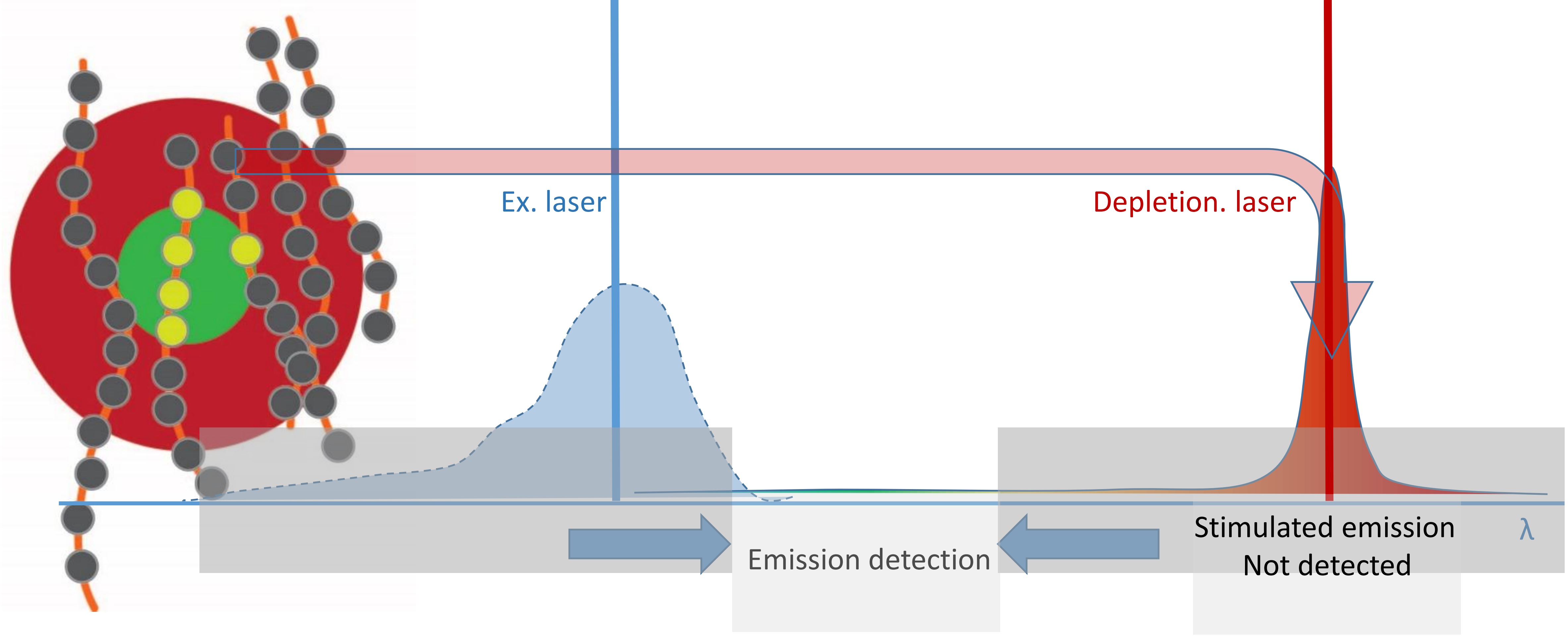
$S_{1, vib}$
S_1
$S_{0, vib}$
S_0
EXCITATION
EMISSION
internal non-radiative conversion
γ_{em}
internal non-radiative conversion
e^-
γ_{ex}
$S_{1, vib}$
S_1
$S_{0, vib}$
S_0
EXCITATION
internal non-radiative conversion
$\gamma_{s.em}$
γ_{STED}
STIMULATED EMISSION
internal non-radiative conversion
e^-
γ_{ex}

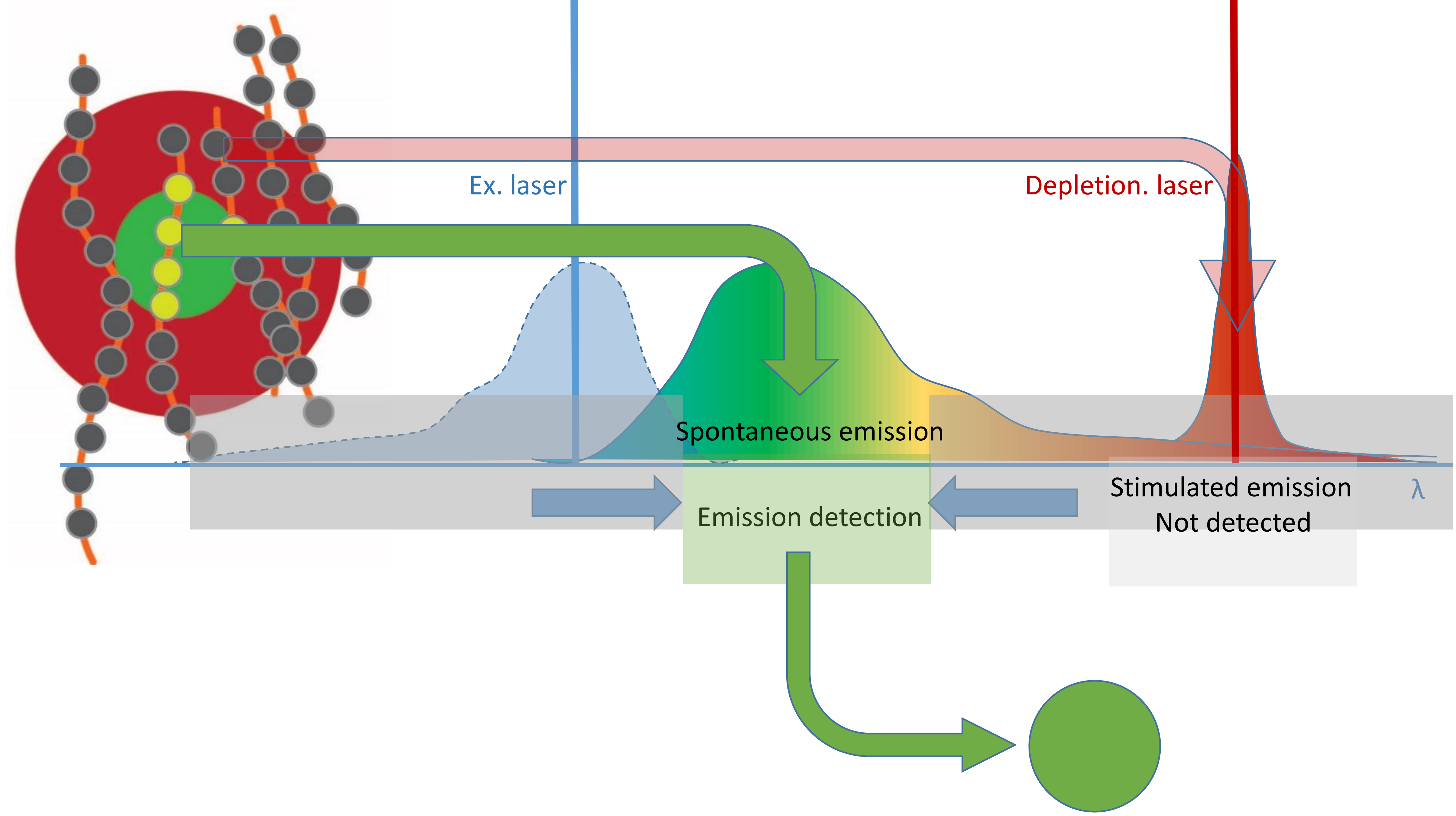

Ex. laser
High energy laser
Spontaneous emission
λ


Ex. laser
High energy laser
Stimulated emission
λ



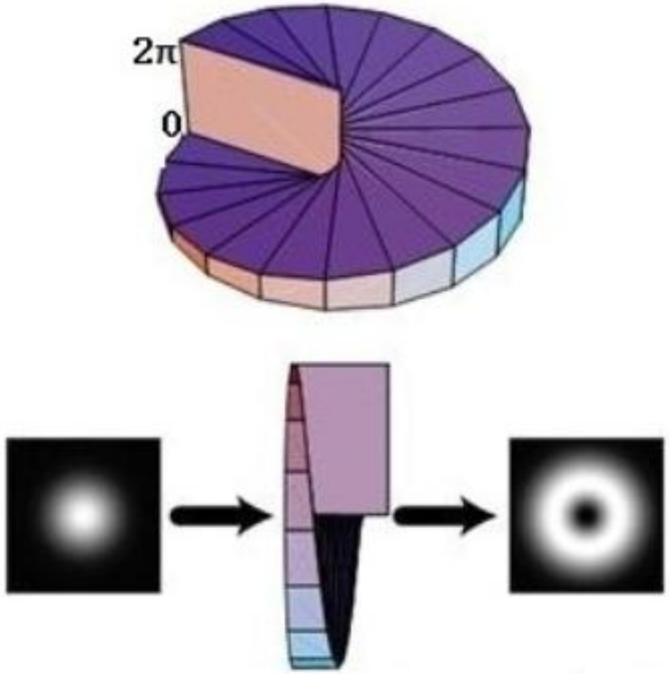
Ex. laser
Spontaneous emission
λ
Emission detection


Ex. laser
Depletion. laser
Emission detection
Stimulated emission
Not detected
λ

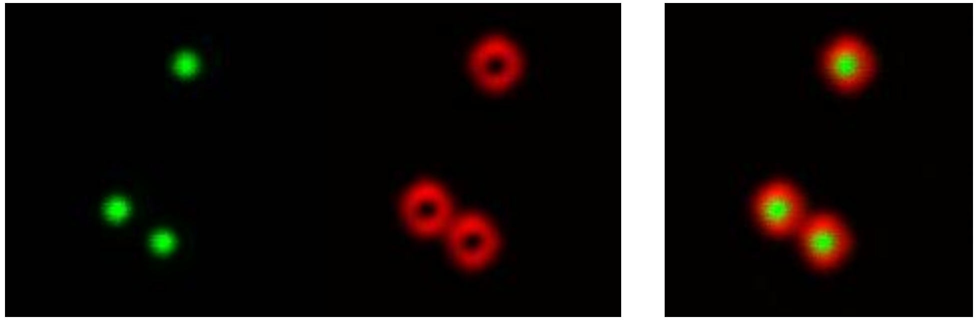

Ex. laser
Depletion. laser
Spontaneous emission
Emission detection
Stimulated emission
Not detected
λ

The donut is created via optical vortex

Courtesy of Courtial and O'Holleran, 2007

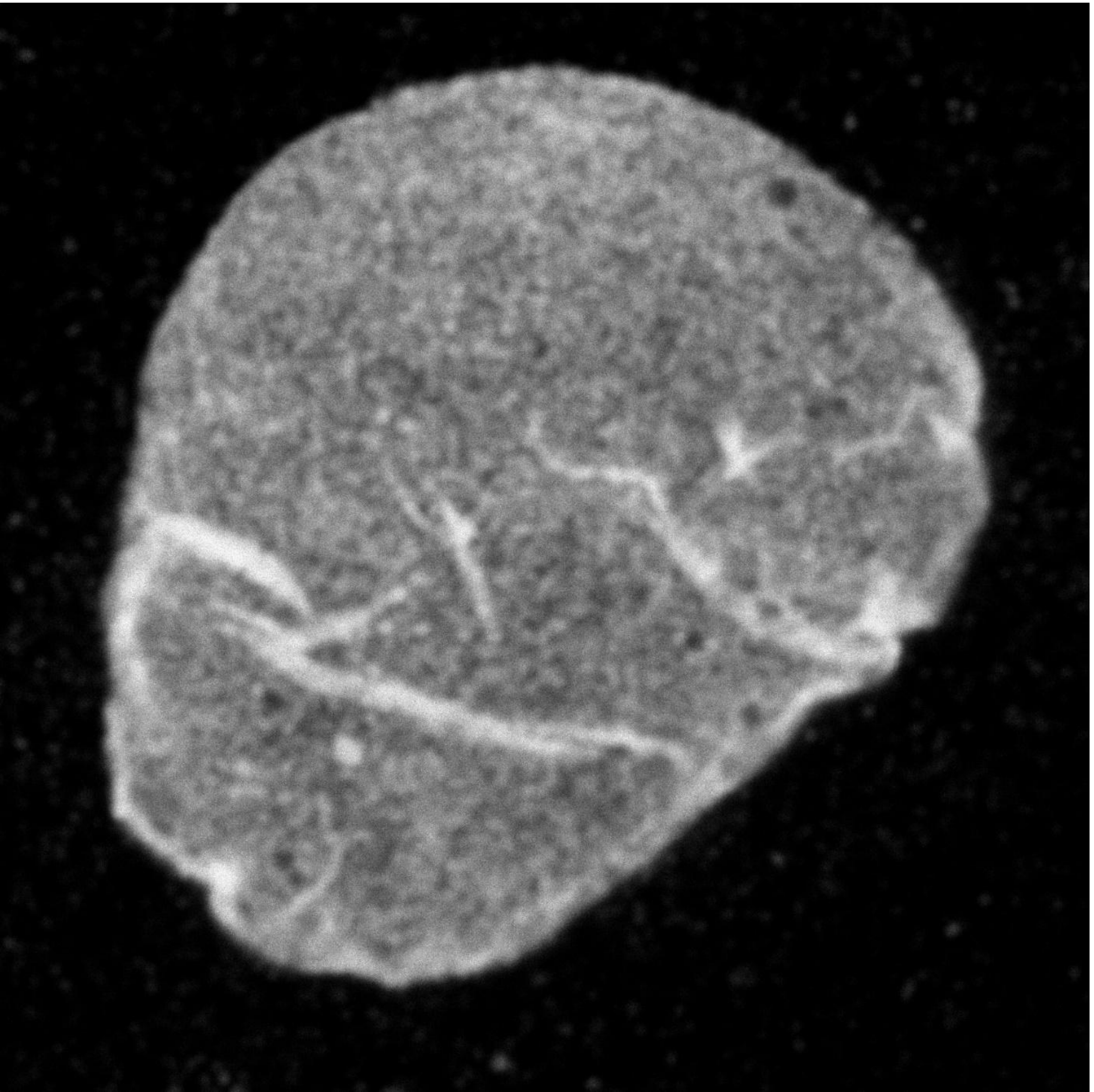


Excitation beam Depletion beam Overlay

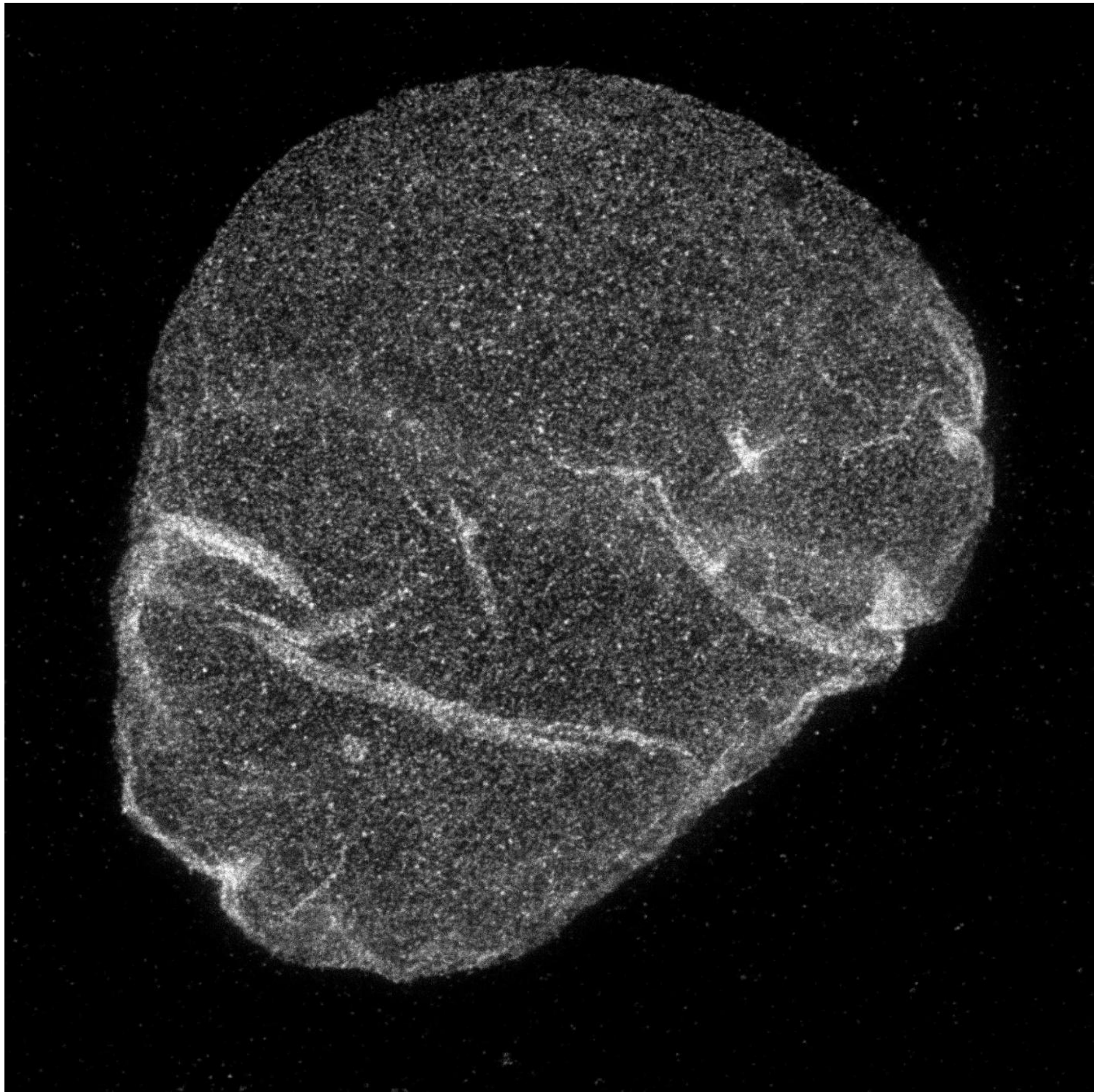


80 nm gold beads, 550 nm excitation, 660 nm depletion

Nuclear lamina, confocal



Nuclear lamina, STED – cw 660 nm



- **Localization microscopy** – widefield method, final image is calculated
- **SIM** – widefield microscopy based method - final image is calculated
- **STED** – confocal method, final image is "real"

- How to understand the SUPER-resolution:

  STORM

  SIM

  STED

- Advantages and disadvantages and sample preparation requirements of SR methods
- Limitations of SR methods

SMLM (STORM or PALM)

- High resolution ~20 nm
- Not difficult hardware equipment
- Precise localization of the structures

- Fluorophores must be able to blink
- Blinking buffers optimization
- Long acquisition time (thousands images per channel)
- Not suitable with thick samples

Best synthetic dyes for STORM

DAPI, Hoechst 33342, Hoechst 33258

AlexaFluor488, Atto488, Atto520 | live: Oregon Green, PA-GFP, dronpa, mGeos

Cy3B, AlexaFluor568 | live: TMR, PA-TagRFP, PA-mCherry1

Cy5, AlexaFluor647, Atto655 (live), Atto680

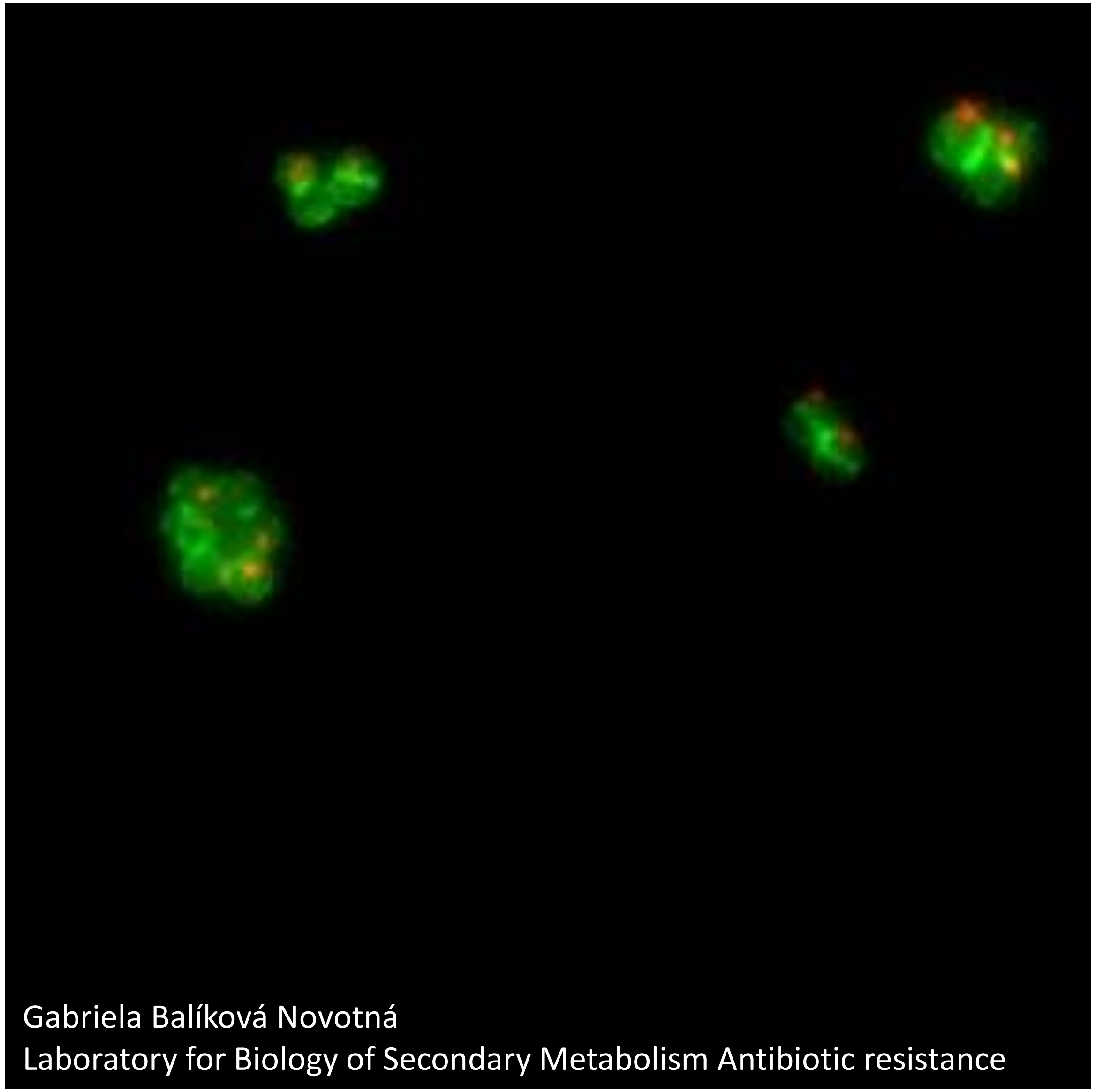
AlexaFluor750

SIM

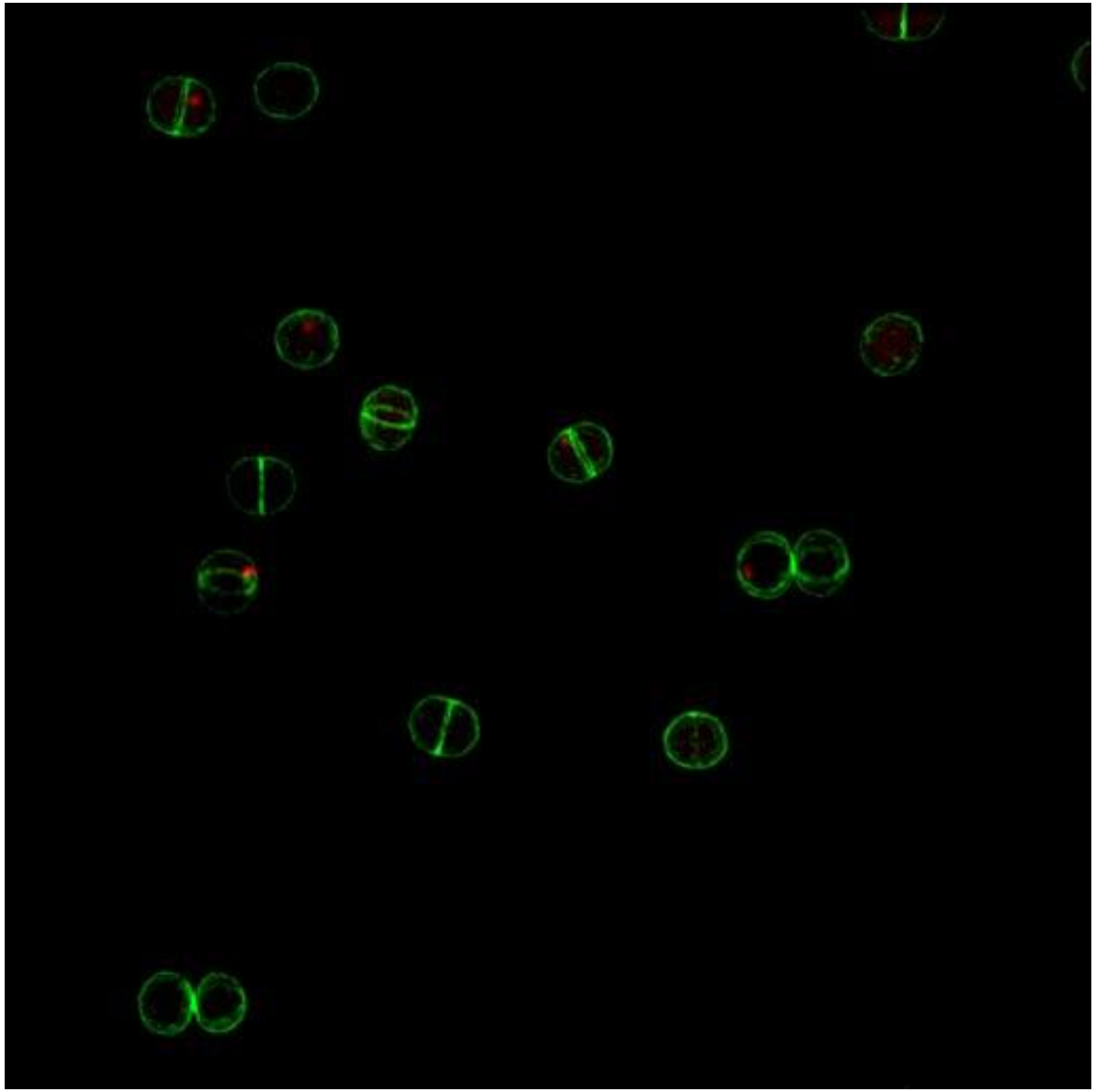
- No special requirements for fluorophores
- Relatively gentle acquisition
- Multi-channel acquisition

- Just twice better resolution
- Reconstruction could introduce artifacts
- Up to 12 um sample thickness

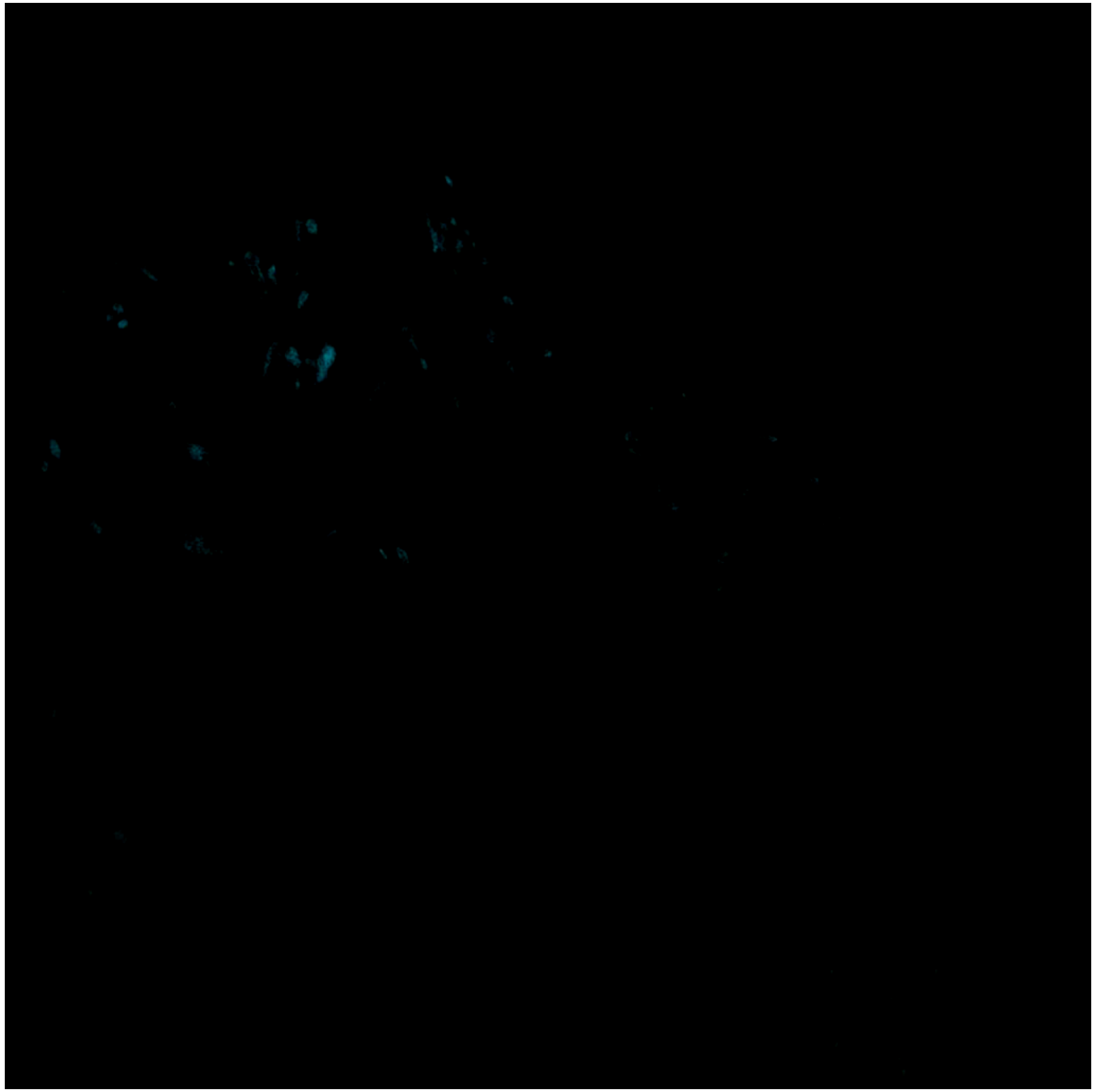
Confocal image

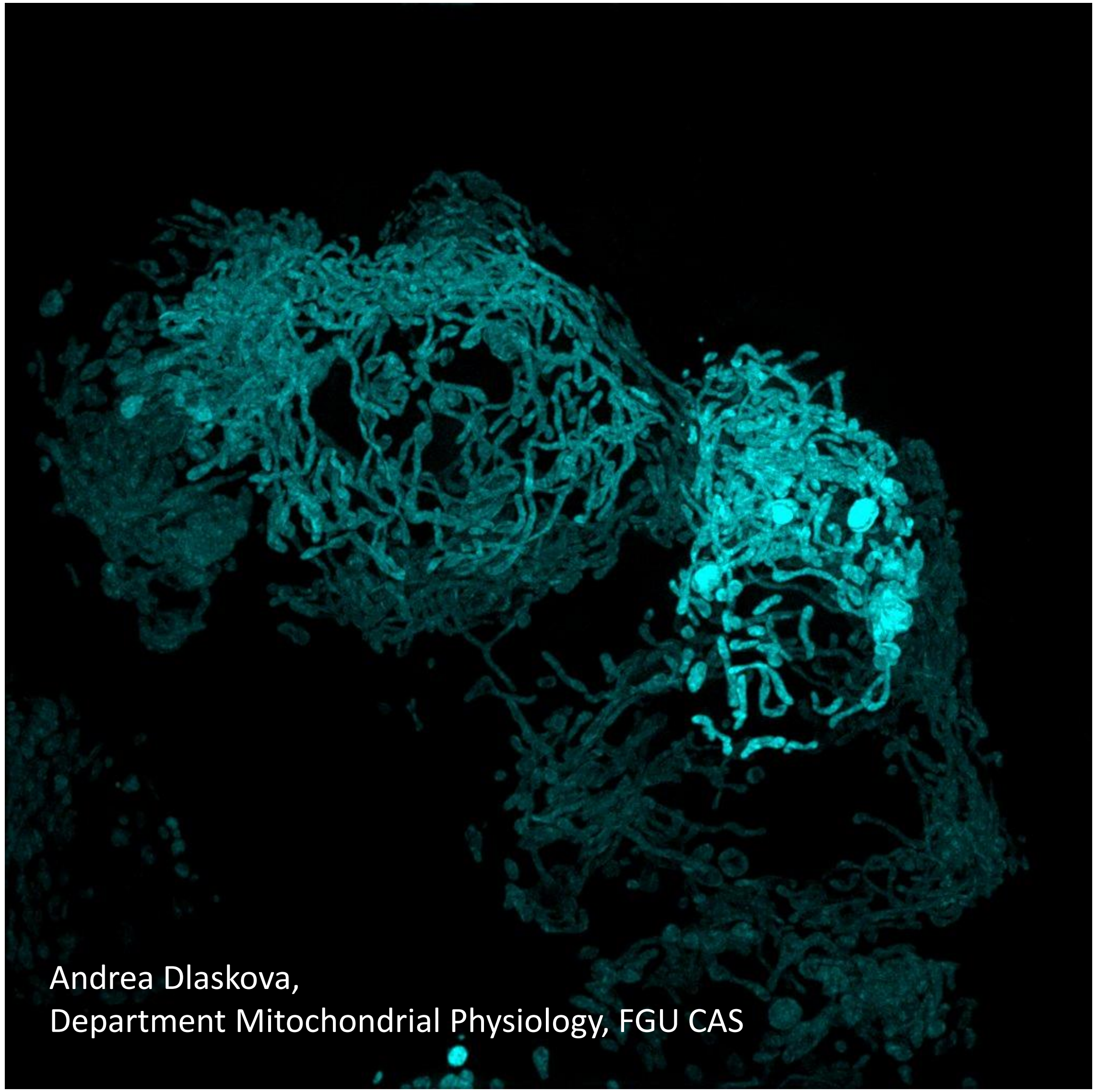


SIM image

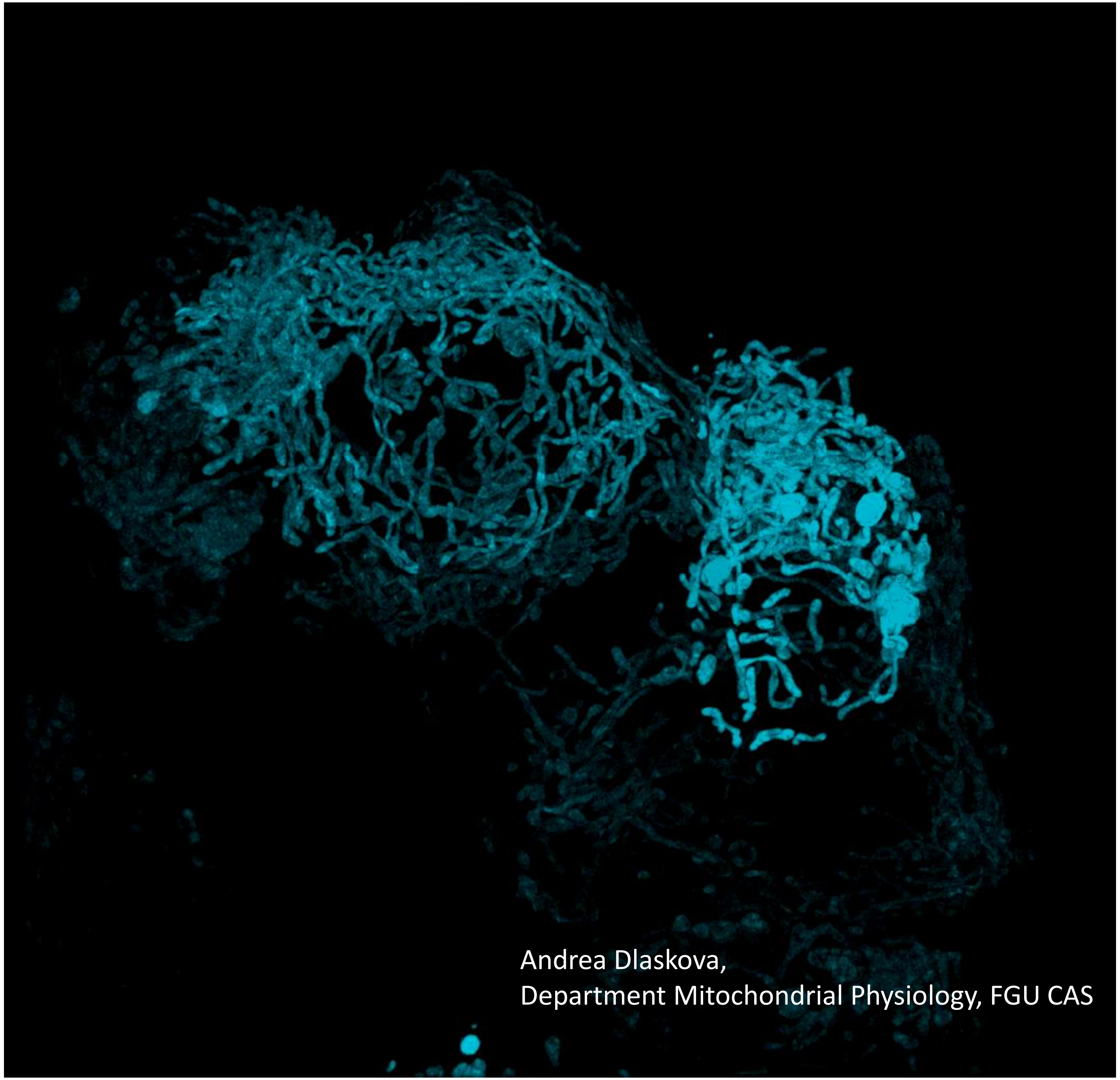


SIM image, live cells, full z-stack and projection





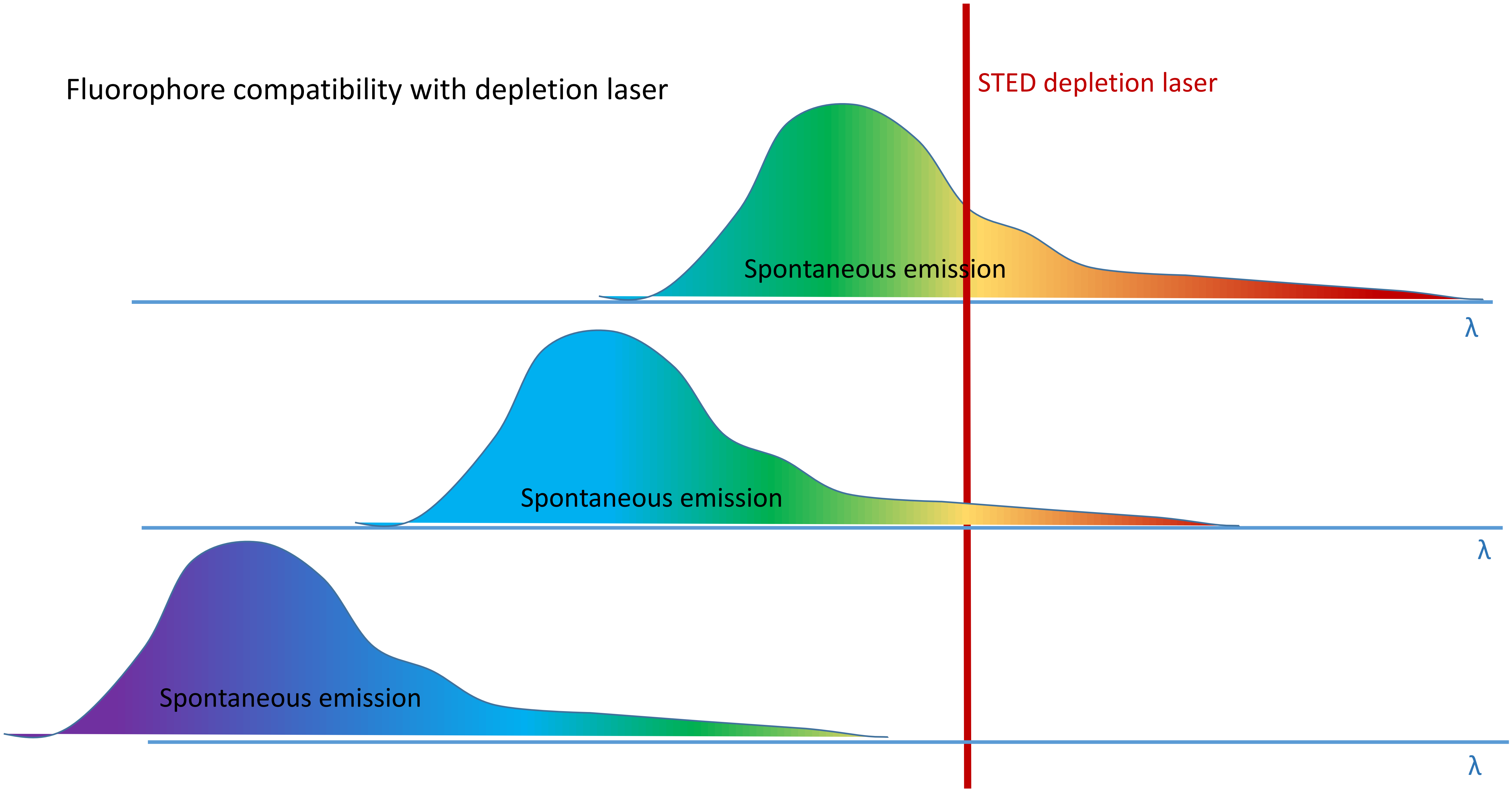
SIM image, live cells, 3D projection



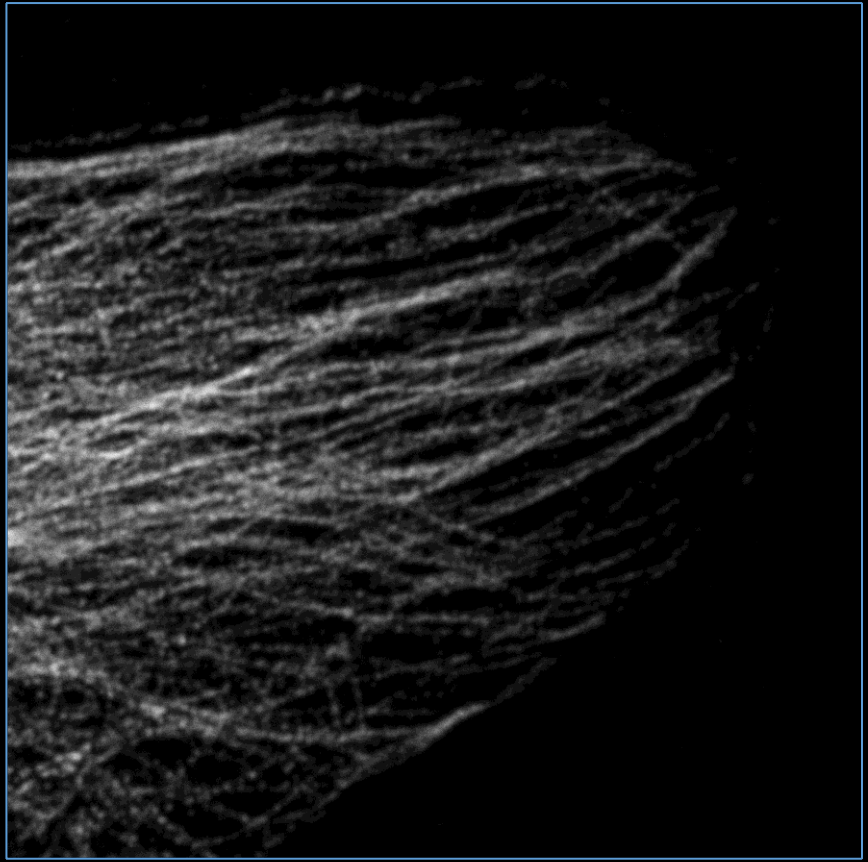
STED

- Instantly acquired “real” image, no computation
- High resolution (~40 nm)
- Thick samples possible

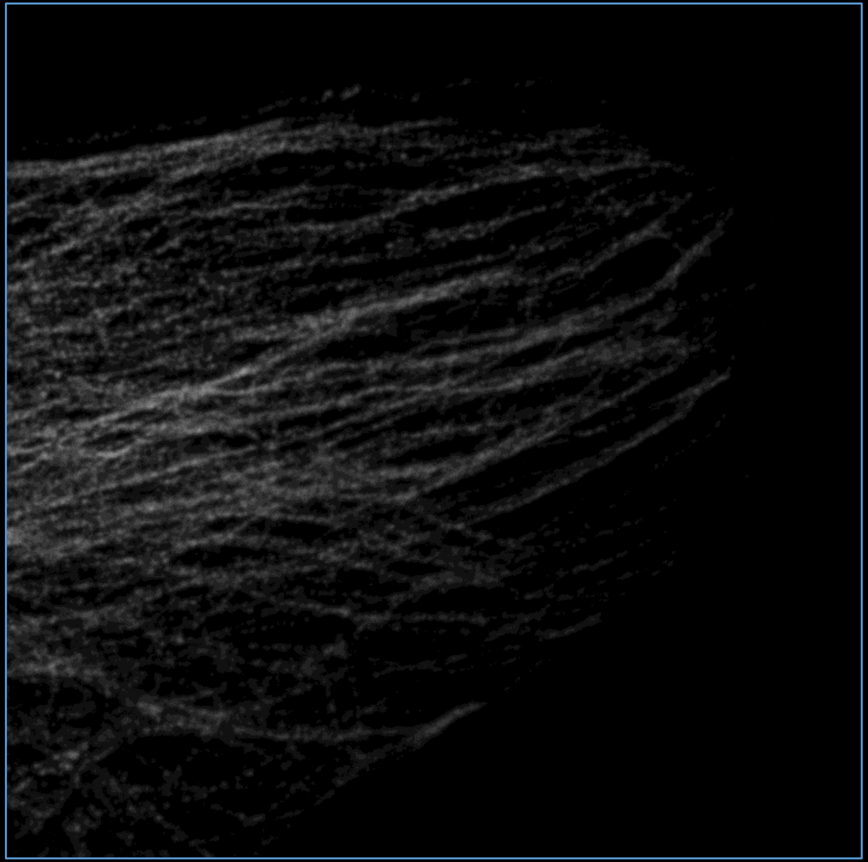
- High power of depletion laser Low signals, deconvolution needed
- Fluorophores must be suitable for depletion laser (2-channels per 1 depletion laser max)


Fluorophore compatibility with depletion laser
STED depletion laser
Spontaneous emission
λ
Spontaneous emission
λ
Spontaneous emission
λ

AlexaFluor 488 - cw660

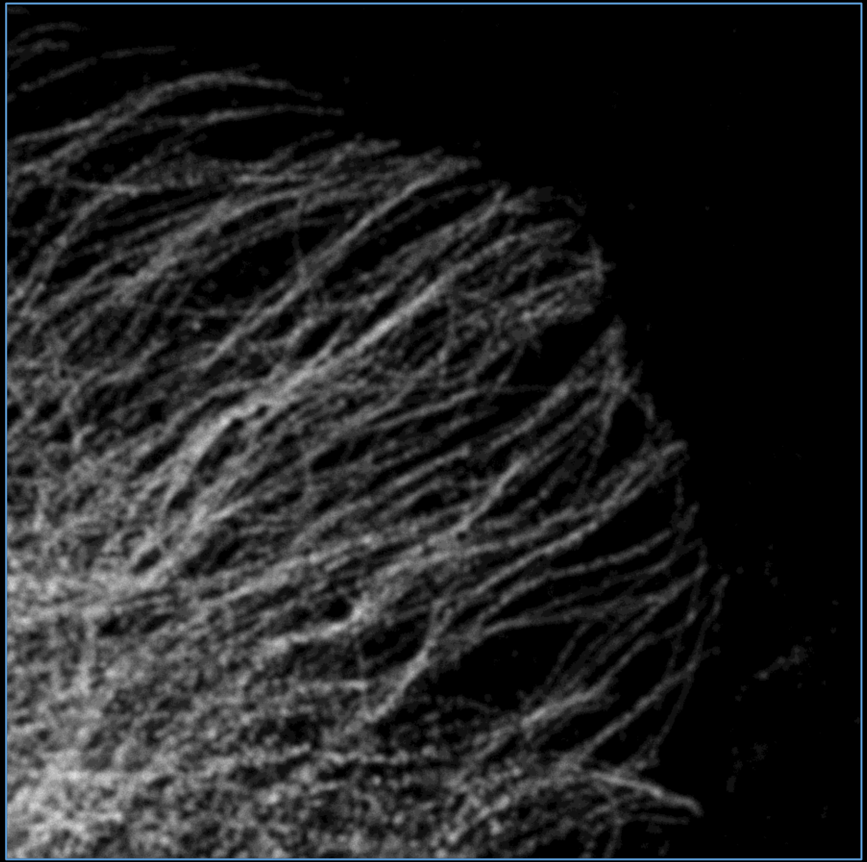


Confocal



gSTED

AlexaFluor 555 - cw660



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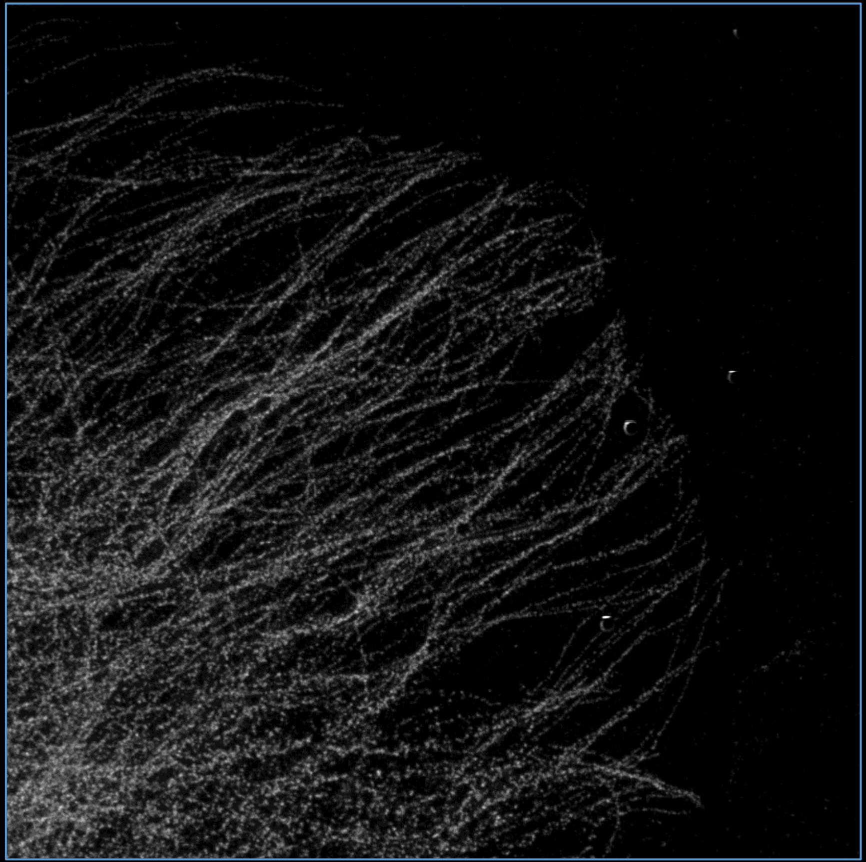
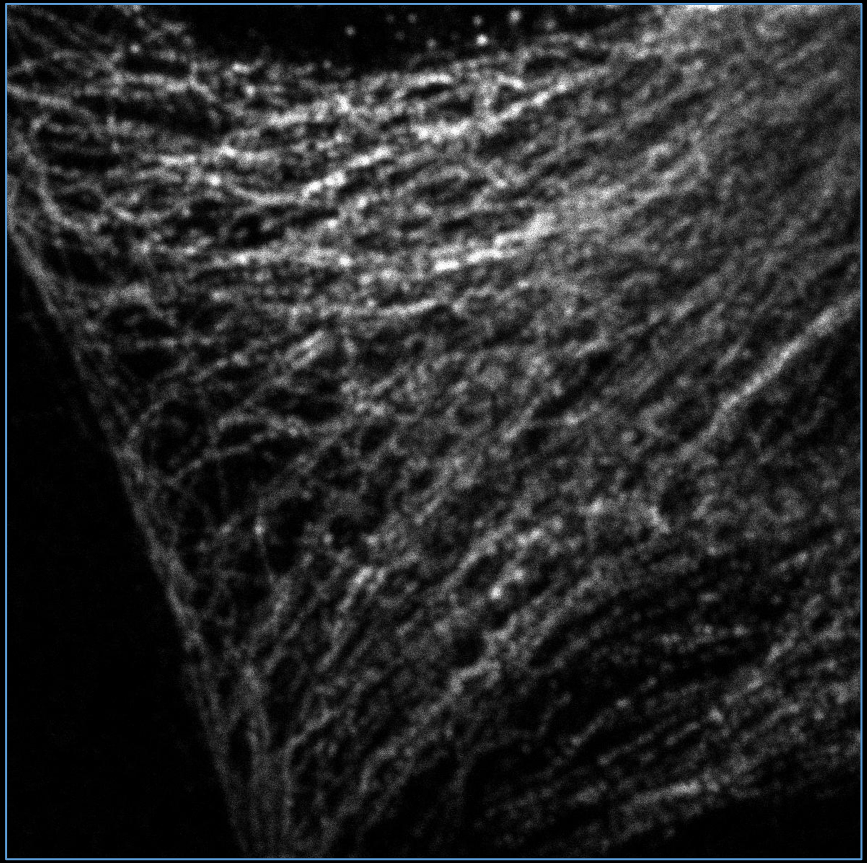


gSTED

AlexaFluor 594 - cw660



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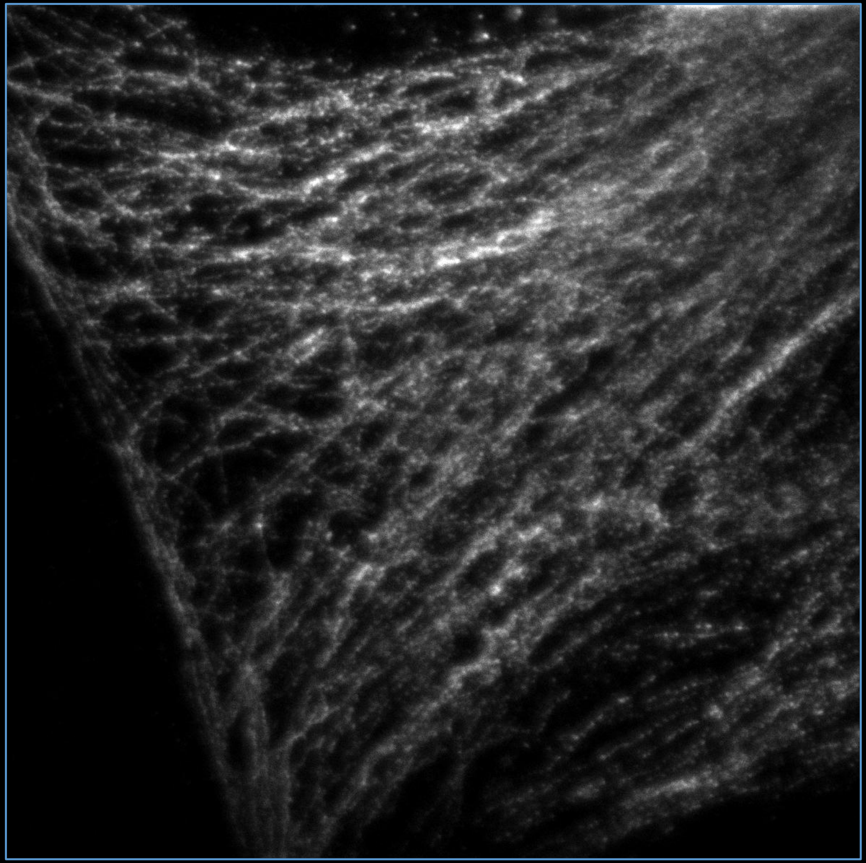
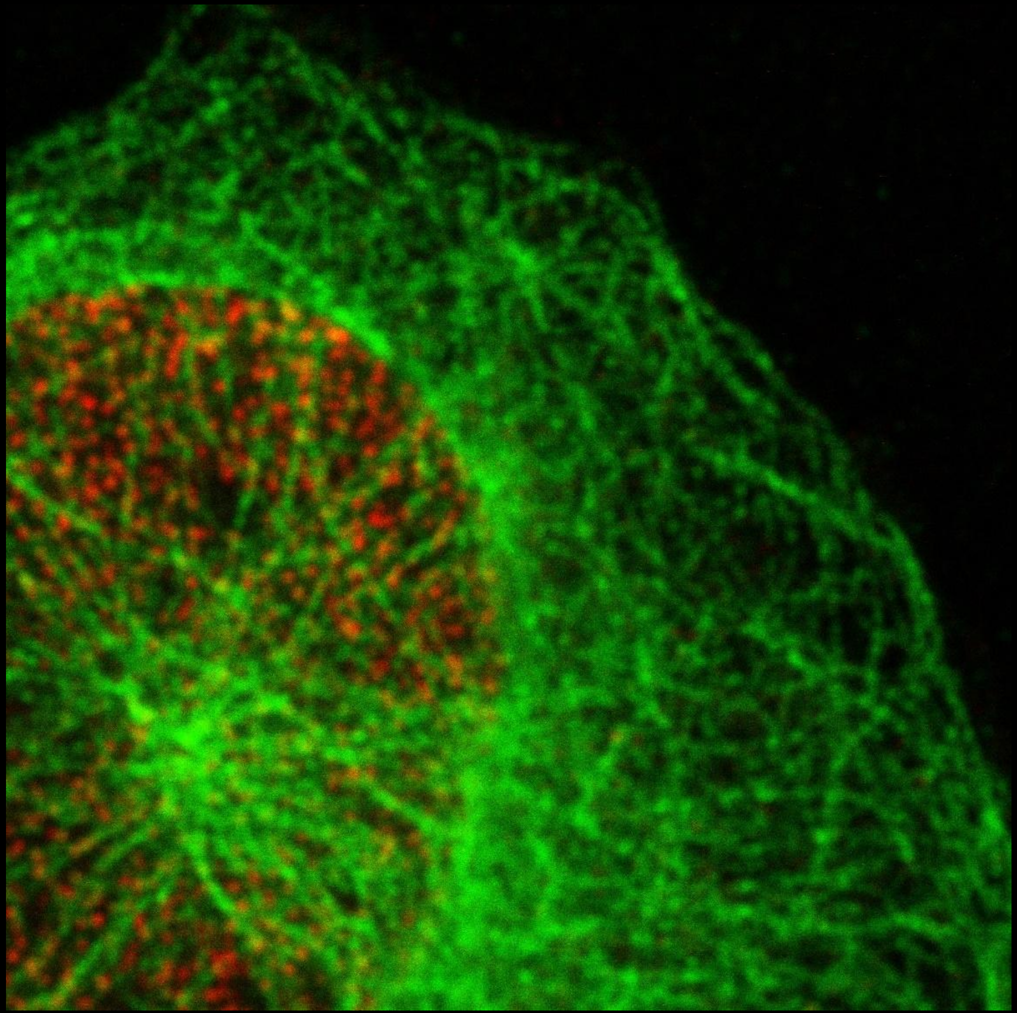
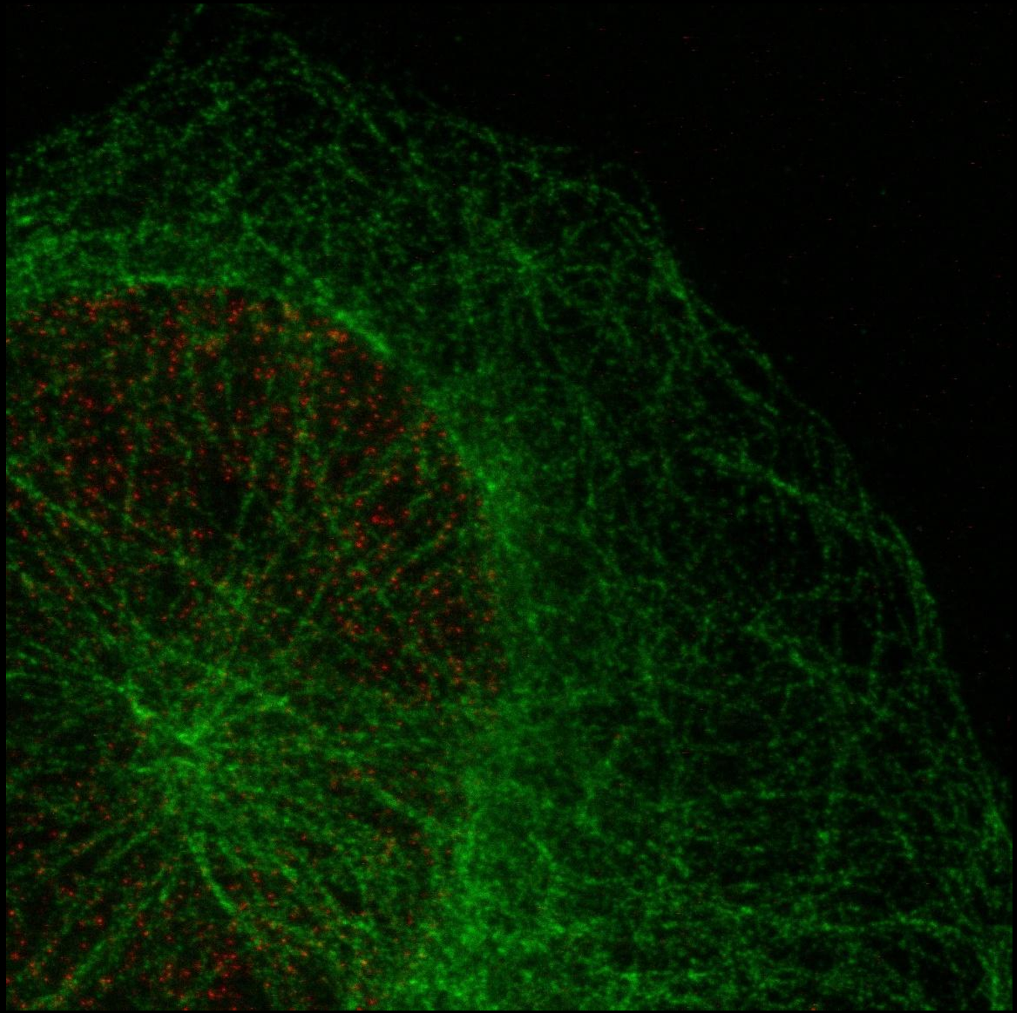


gSTED

AlexaFluor 594/ Abberior Star RED – pulse 775



Confocal



STED

Thank you for your attention