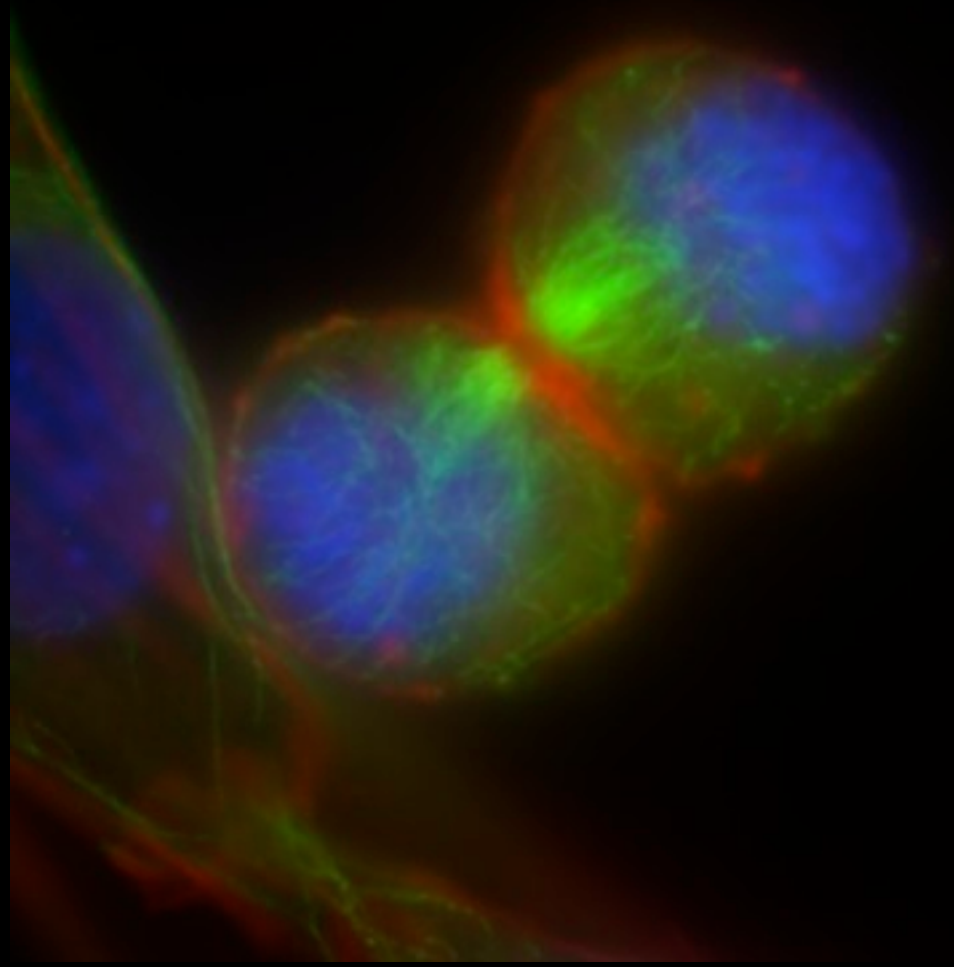
Super-resolution fluorescence microscopy



Typical widefield image...

- high specificity
- high sensitivity
- non-invasive
- multi-D (x, y, z, λ, t,...)
- rel. localisation & dynamics

Optical resolution is diffraction limited!

Magnification alone does not give more details!

...warmup:

"What determines the resolution of an optical microscope ?"

63x/1.25

€ 5.557,56

100x/1.25

€ 693,68

63x/1.4

€ 6.370,72

„.... what objective would you take..."

„... a bit more difficult...?“

25x/1.05	40x/1.0	40x/1.1
€ 15.000,00	€ 3.786	€ 11.110,48

What's the difference in brightness ?

„... what objective would you take...“

Numerical aperture determines ...

Brightness	$B = NA^4 / Mag^2$	(epifluorescence)
Lateral Resolution	$d_{x,y} = 0.61\ \lambda / NA$	(200-300 nm)
Axial Resolution	$d_z = 2\ \lambda / NA^2$	(500-700 nm)

Only applies under optimal conditions! BUT ...

spherical aberrations
chromatic aberrations
straylight
out-of-focus blur
noise
sample
...

Effective resolution is worse!
(max. 250 nm lateral and ≤ 1 µm axial)

...improved to some extent by confocal imaging or deconvolution

Super-resolution fluorescence microscopy

How Structured Illumination (SI) improves not only resolution ...

& how it is realized in OMX system

Comparison of super-resolution methods (Pros & Cons)

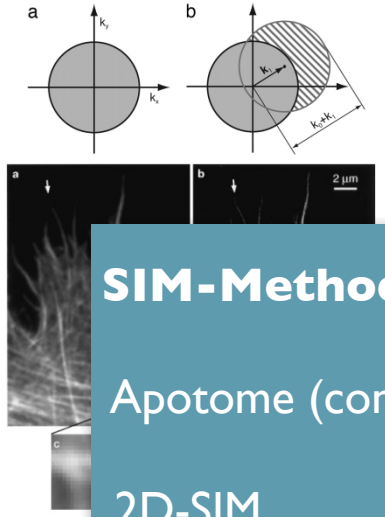
3D structured illumination microscopy

Superresolution microscopy - three major concepts

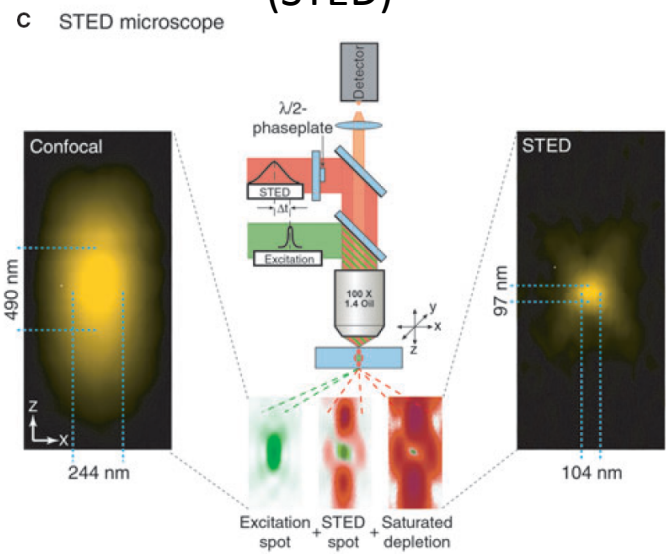
Super-resolution light microscopy:
Imaging beyond Abbe's diffraction limit

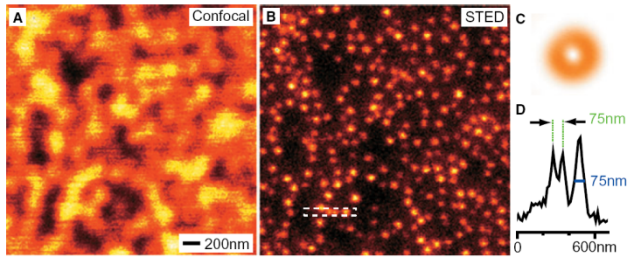
$$\Delta x, \Delta y = \frac{\lambda}{2n \sin \alpha}$$

Structured illumination



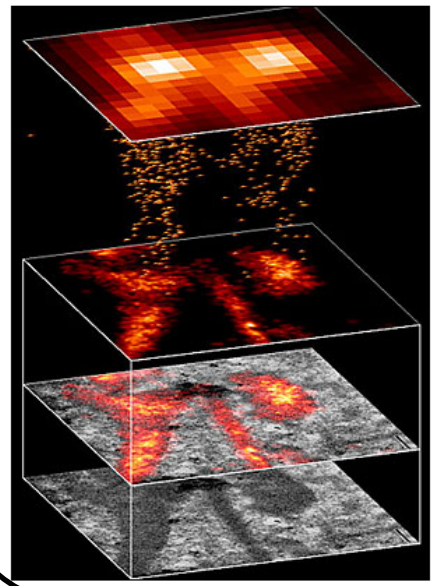
Stimulated emission depletion (STED)

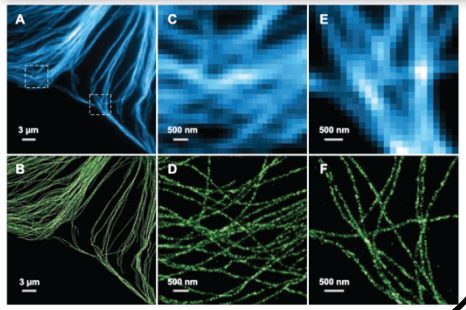




Localization

Photoactivation localization microscopy (PALM)





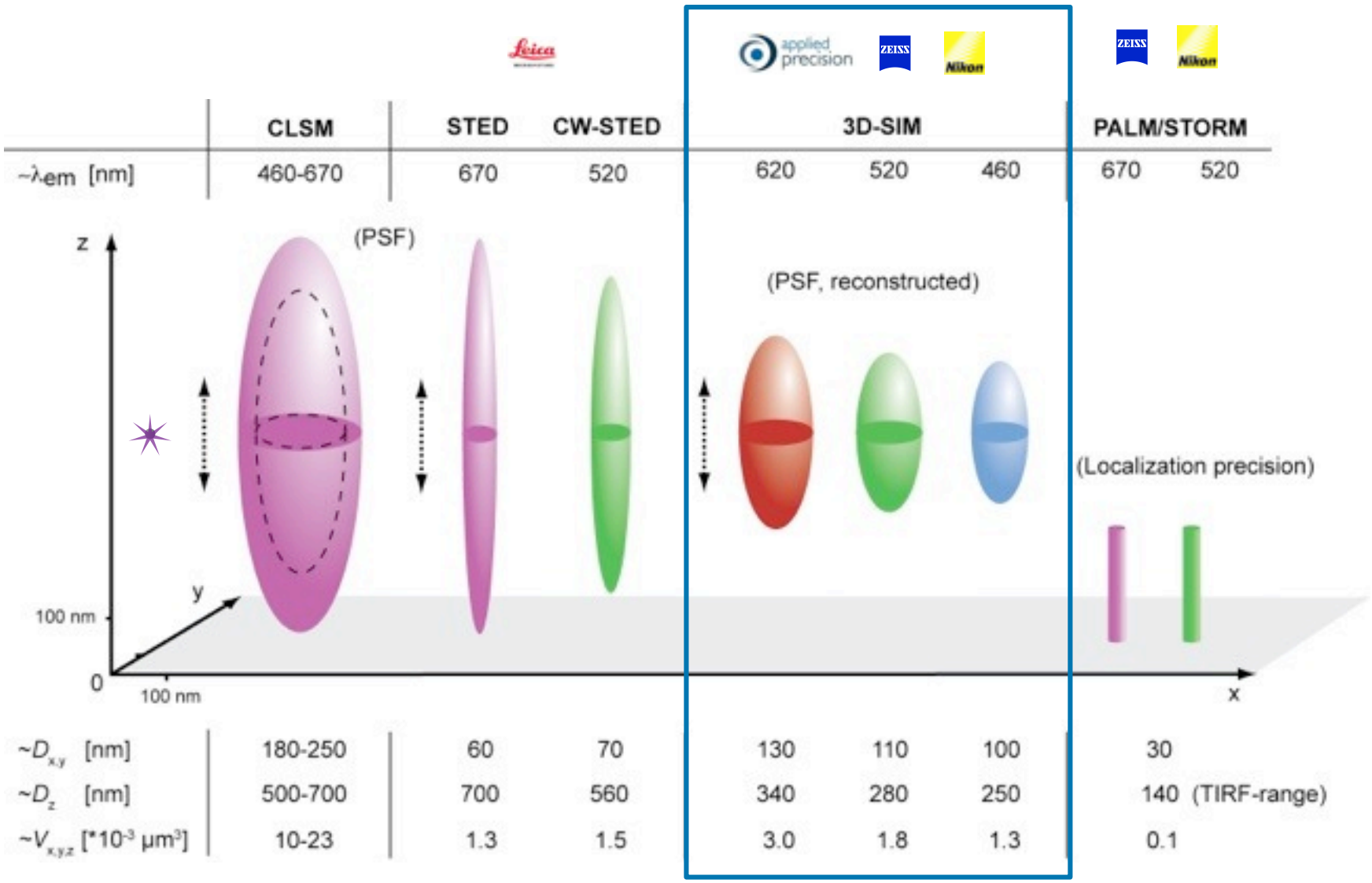
SIM-Methods:

Apotome (conventional SIM)

2D-SIM
3D-SIM (linear SIM)
TIRF-SIM

SSIM (non-linear SIM)
NL-SIM

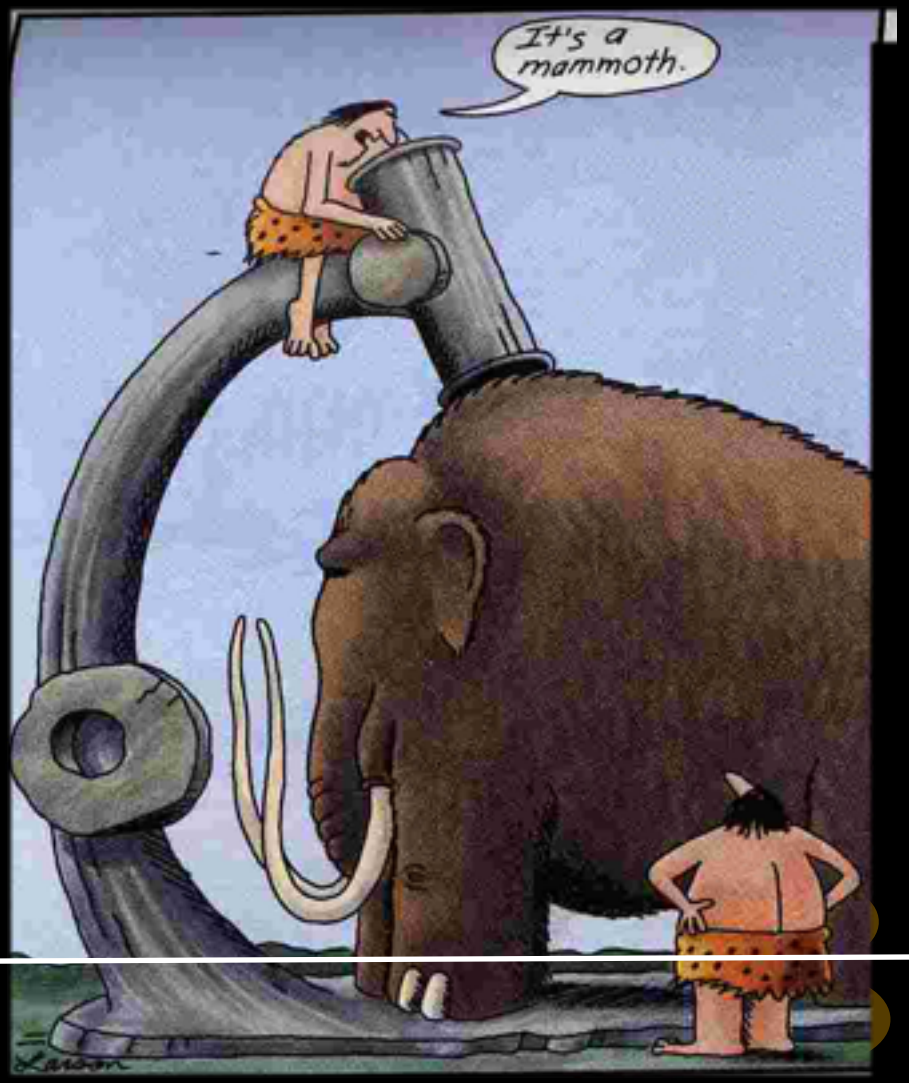
Resolving power of commercial super-resolution systems



3D-SIM resolves ~8-fold smaller volumes than conventional (confocal) microscopes

Not only resolution matters, ...

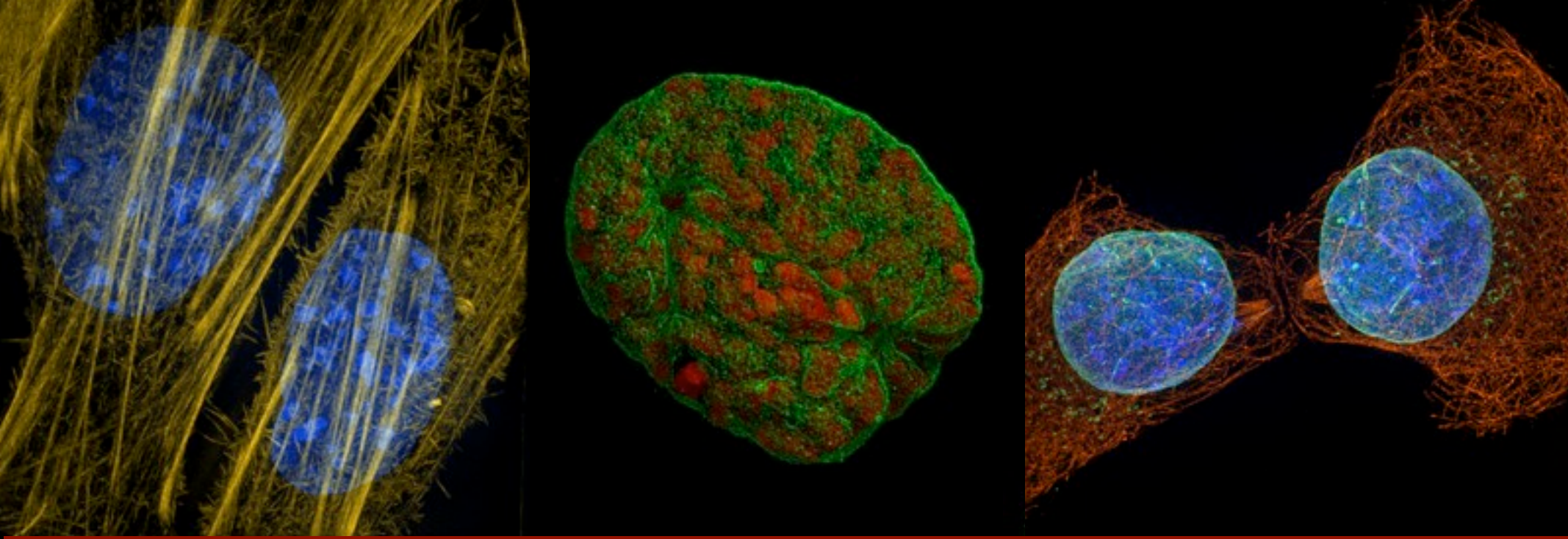
What could this be?



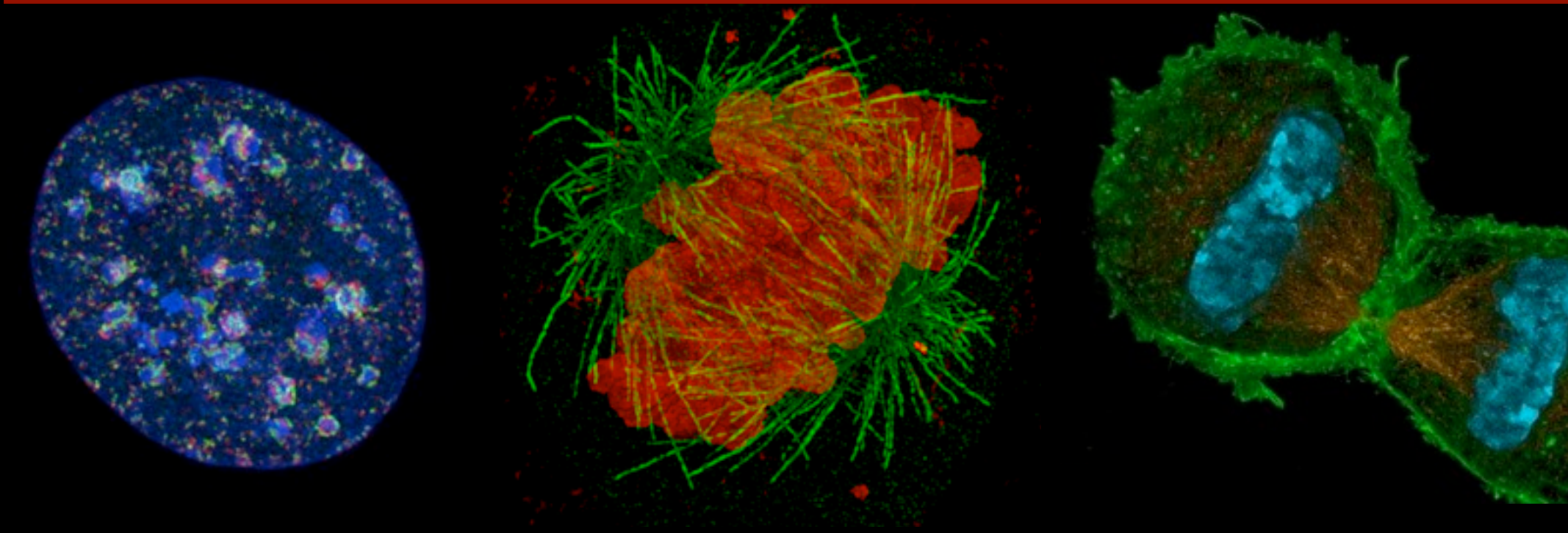
3D information (z-res., optical sectioning, z-depth) !

Not only resolution matters, but also context (II)

Prague National Museum



3D-SIM: 3-color • 3D optical sectioning • 8x enhanced volumetric resolution • 10-20 µm depth

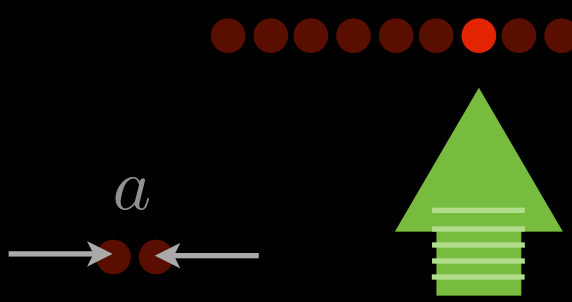


How does it work?

The basic principle: Abbe's view

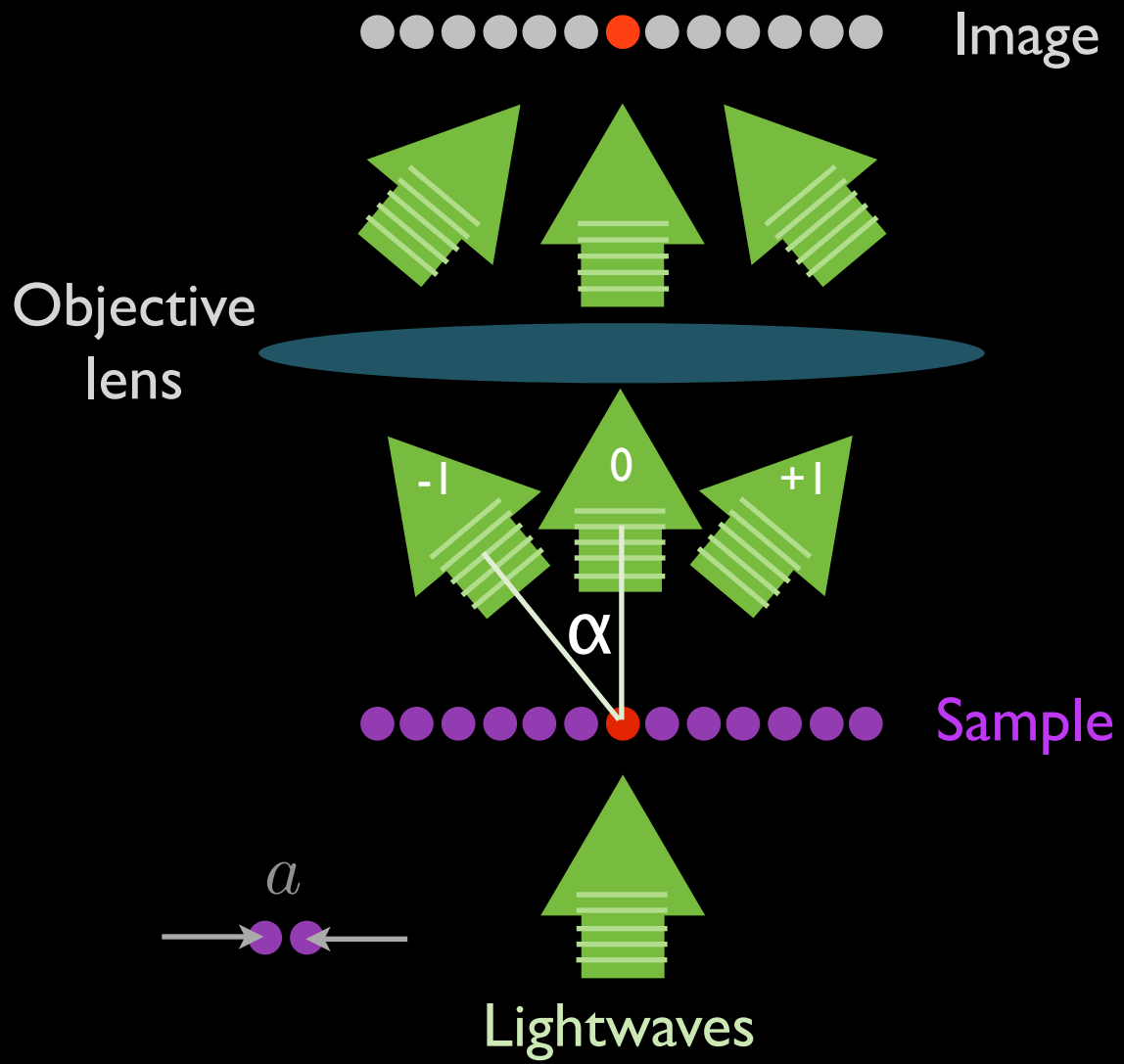
Sample = Structure

Periodicity

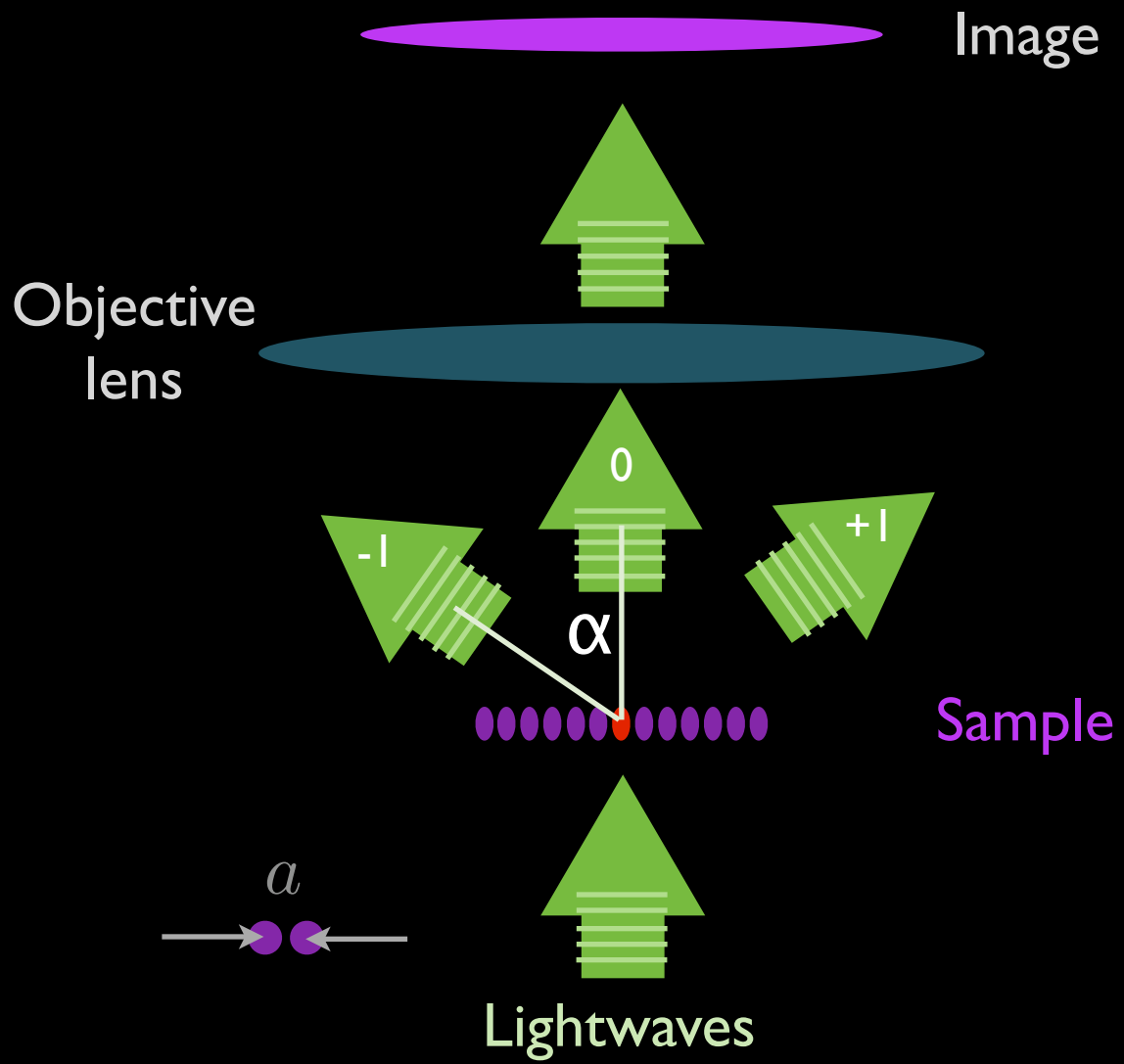


http://de.wikipedia.org/wiki/Ernst_Abbe

The basic principle: Abbe's view



The basic principle: Abbe's view



highest frequencies
(biggest α)
→
smallest structures

Image = superposed periodicities

Real space (xy) —FFT→ Frequency space (k_x, k_y)

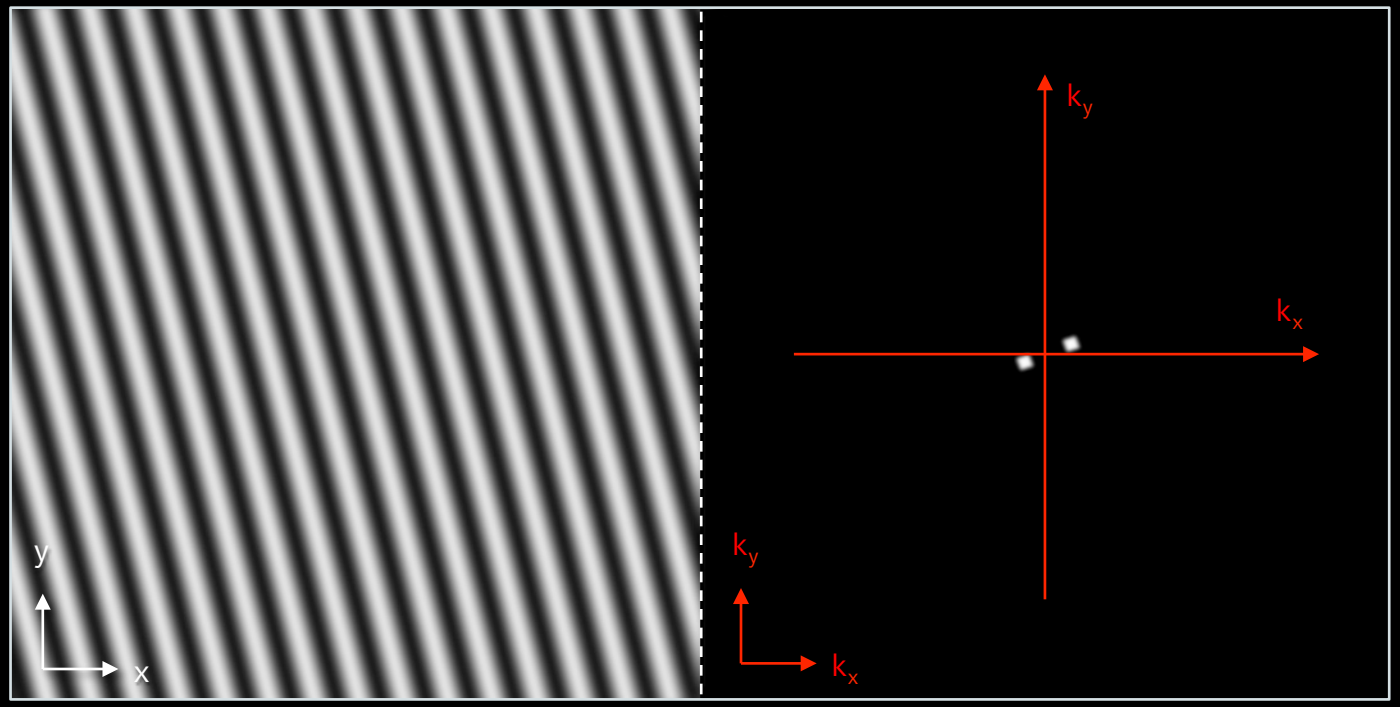


Image = superposed periodicities

Real space (xy) —FFT→ Frequency space (k_x, k_y)

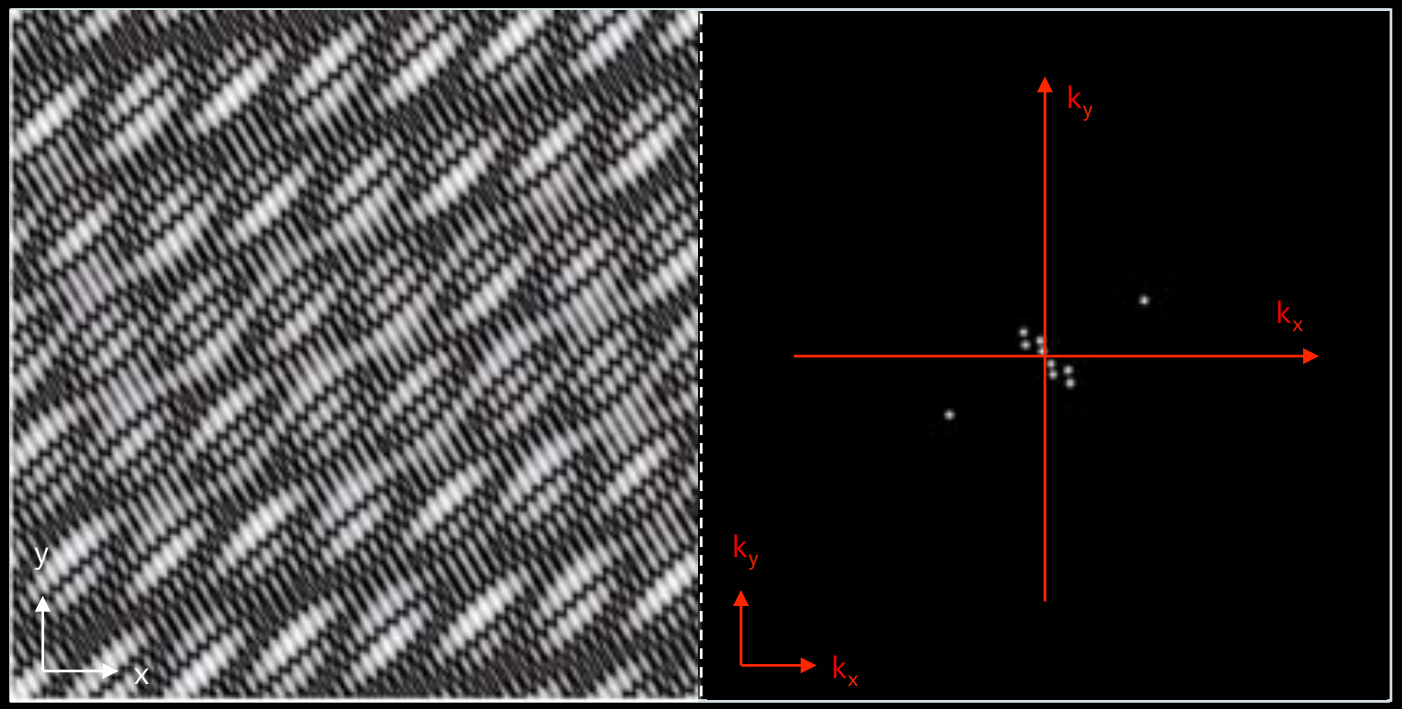


Image = superposed periodicities

Real space (xy) —FFT→ Frequency space (k_x, k_y)

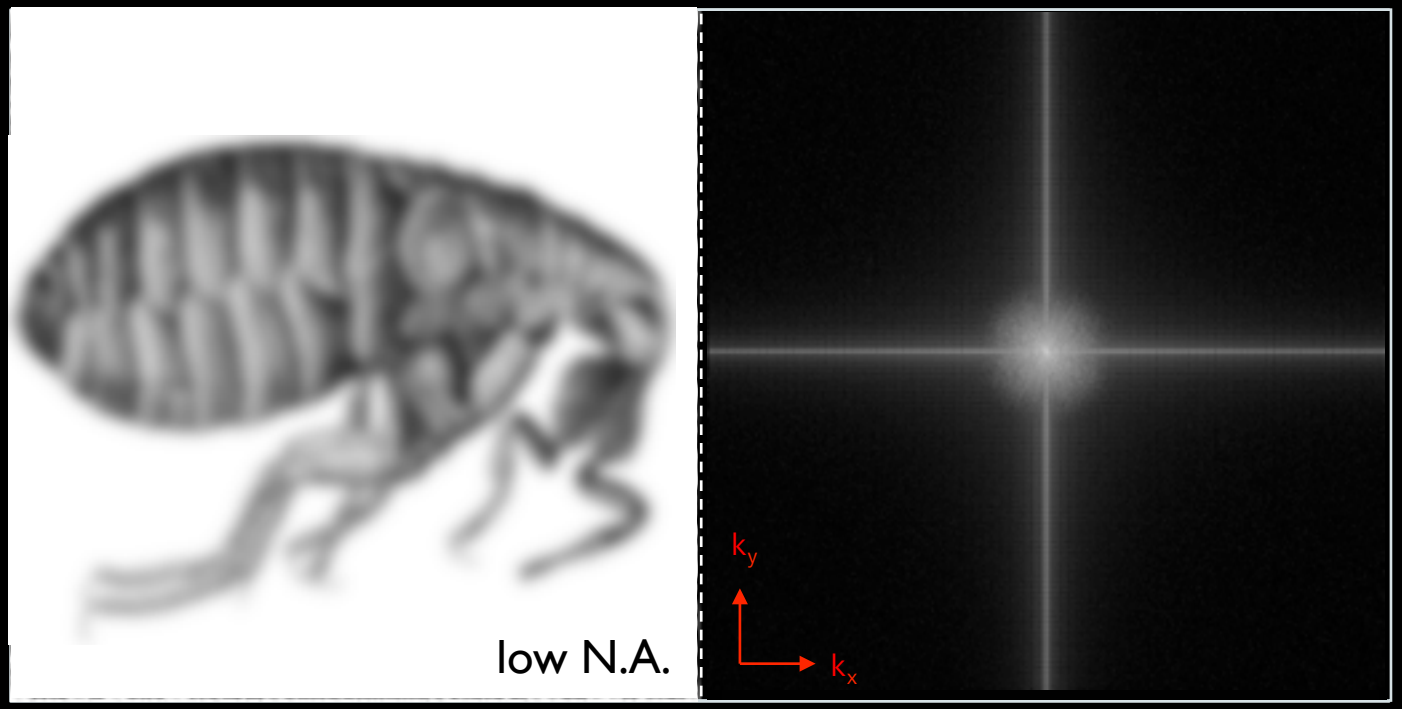
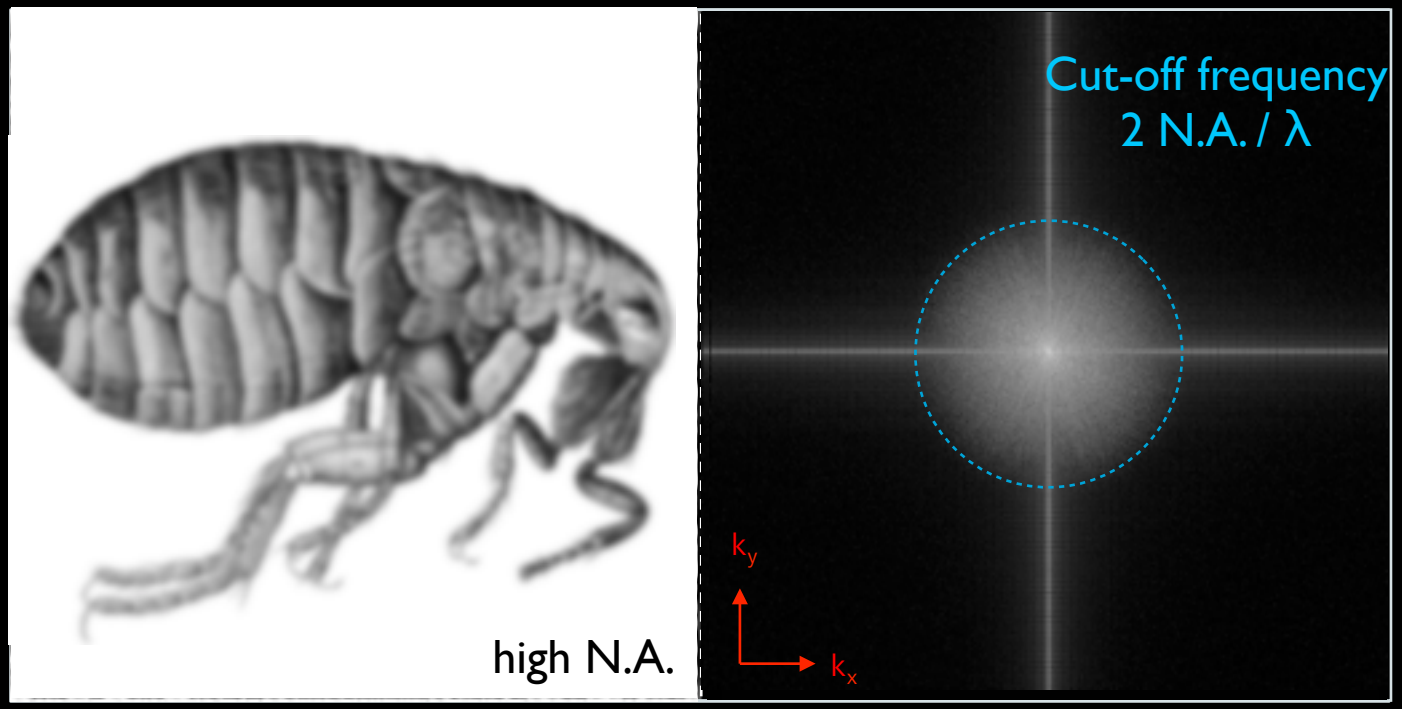
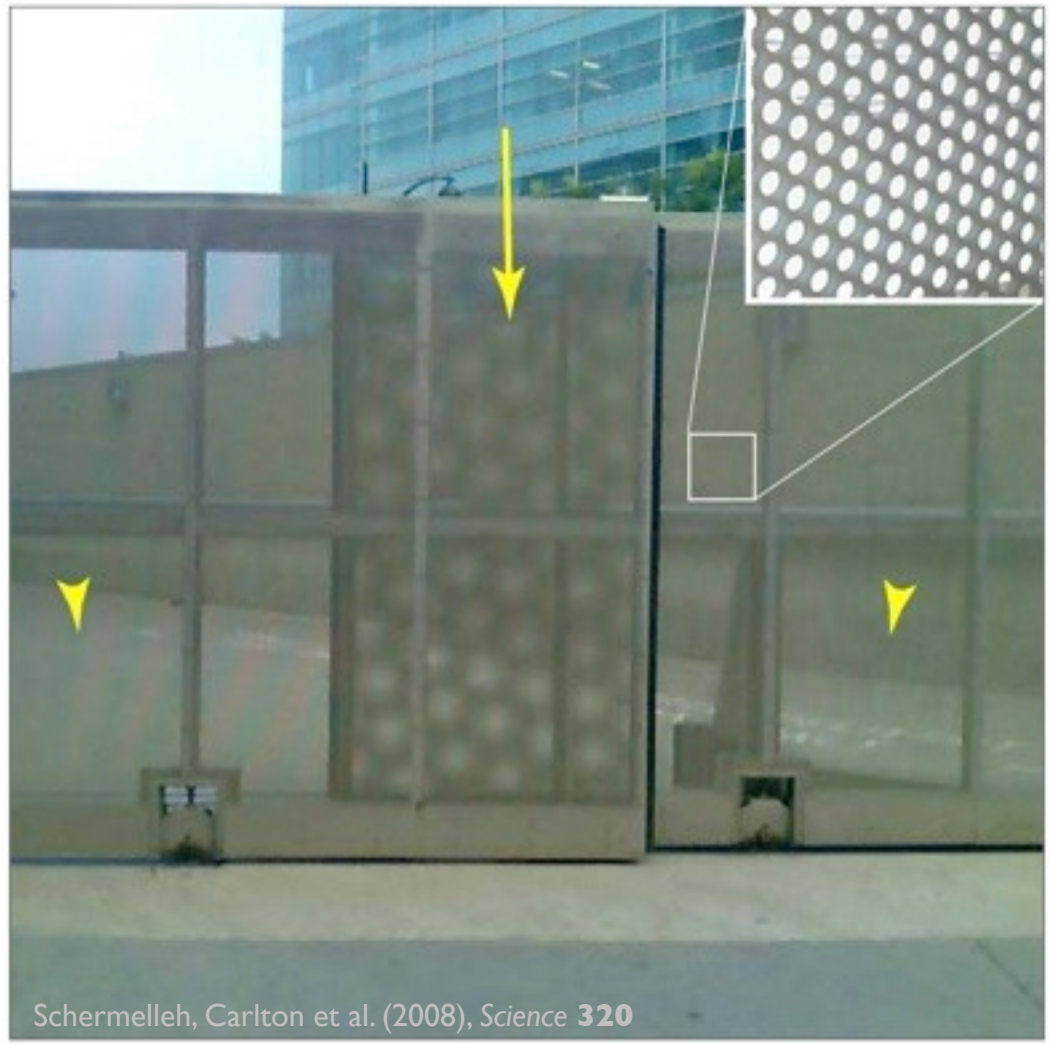


Image = superposed periodicities

Real space (xy) —FFT→ Frequency space (k_x, k_y)



SIM principle:
Moiré interference encoding high frequency information



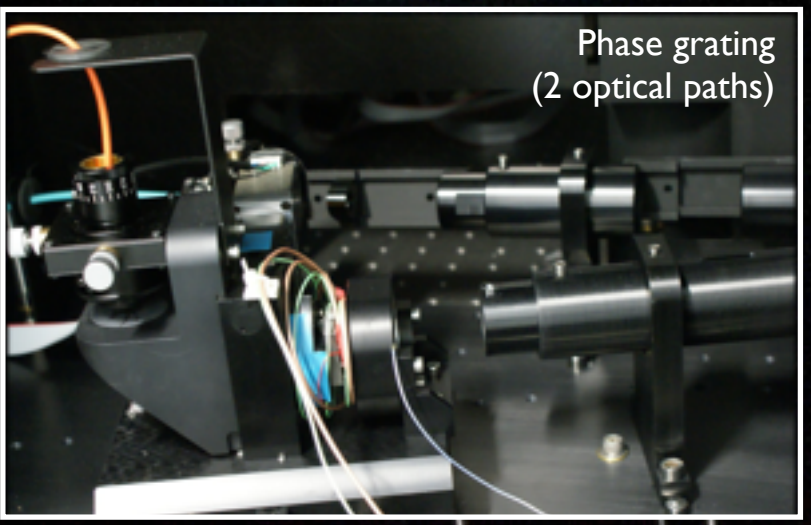
Fourier transform of the measured image

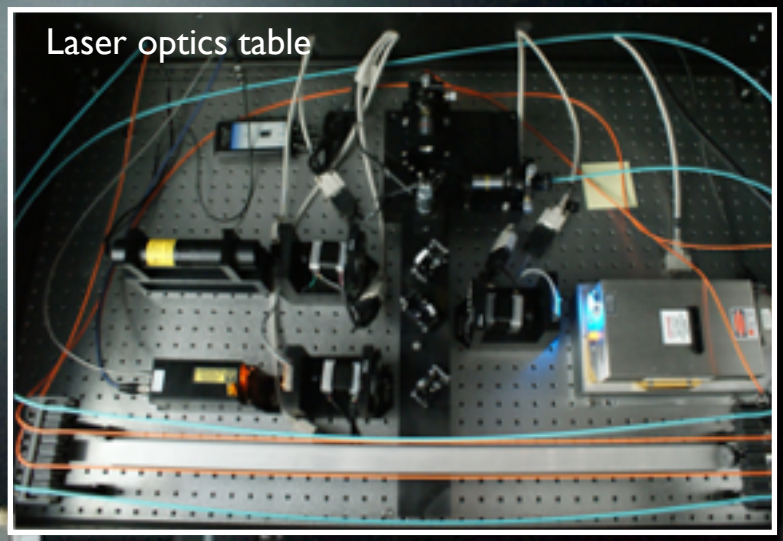
unknown structure

$$F\{f \times g\} = F\{f\} \otimes F\{g\} \rightarrow F\{f\} = F\{f \times g\} \otimes^{-1} F\{g\}$$

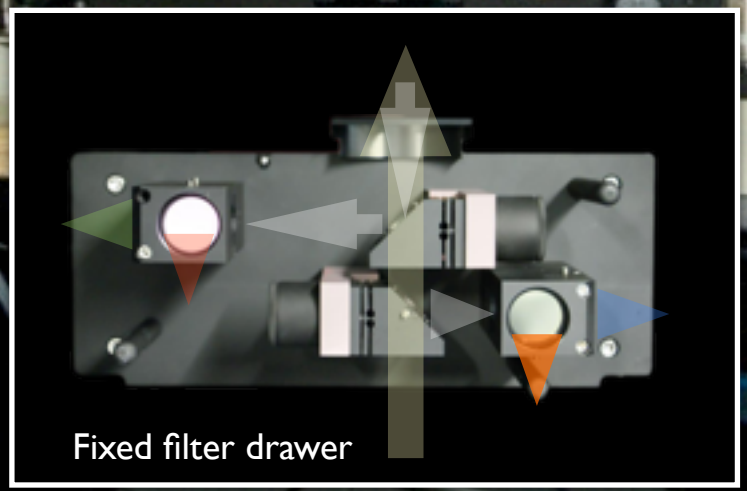
known illumination function

OMX V2 3D-SIM (early adopter)



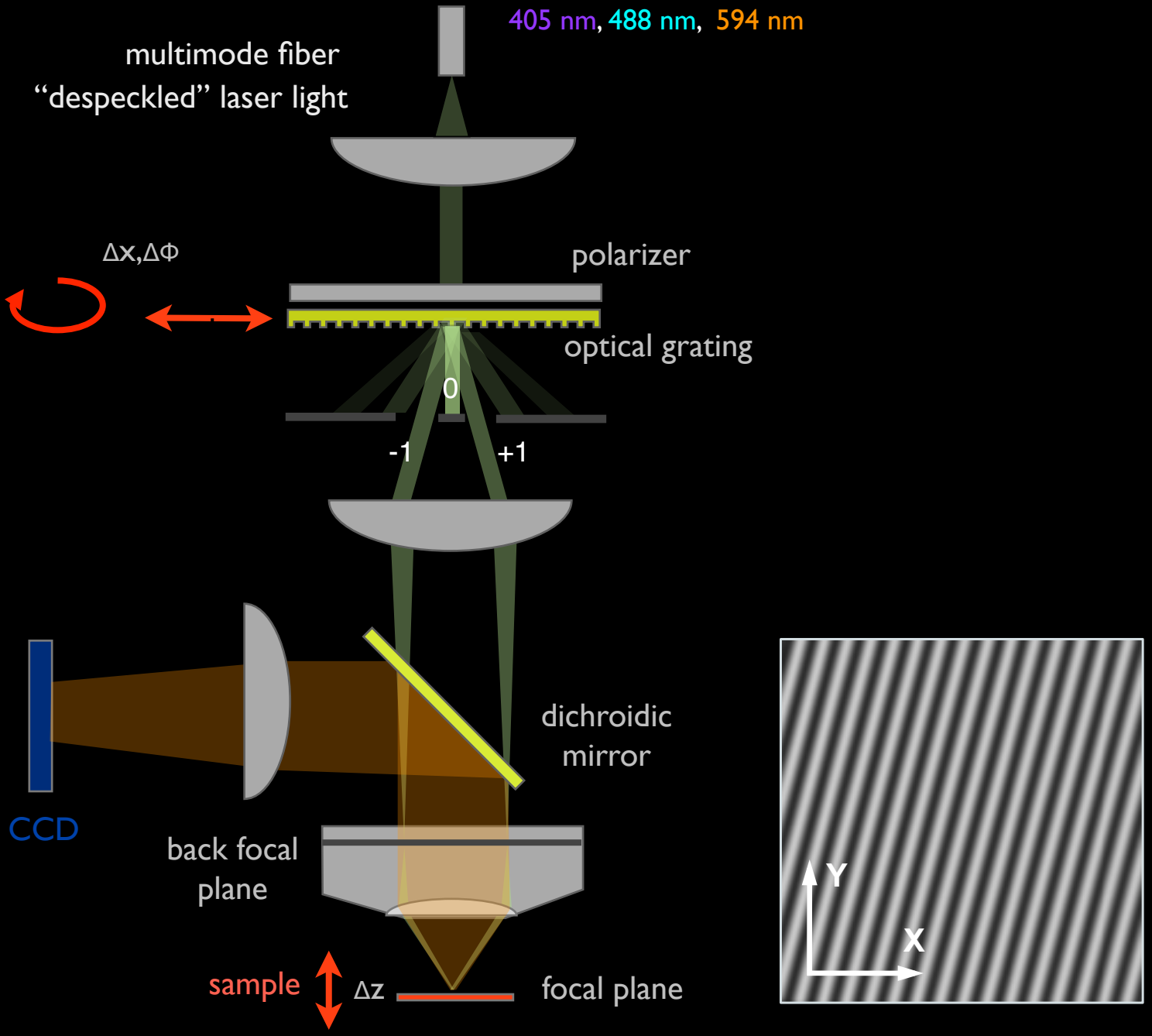


max. mech. stability
highest sensitivity



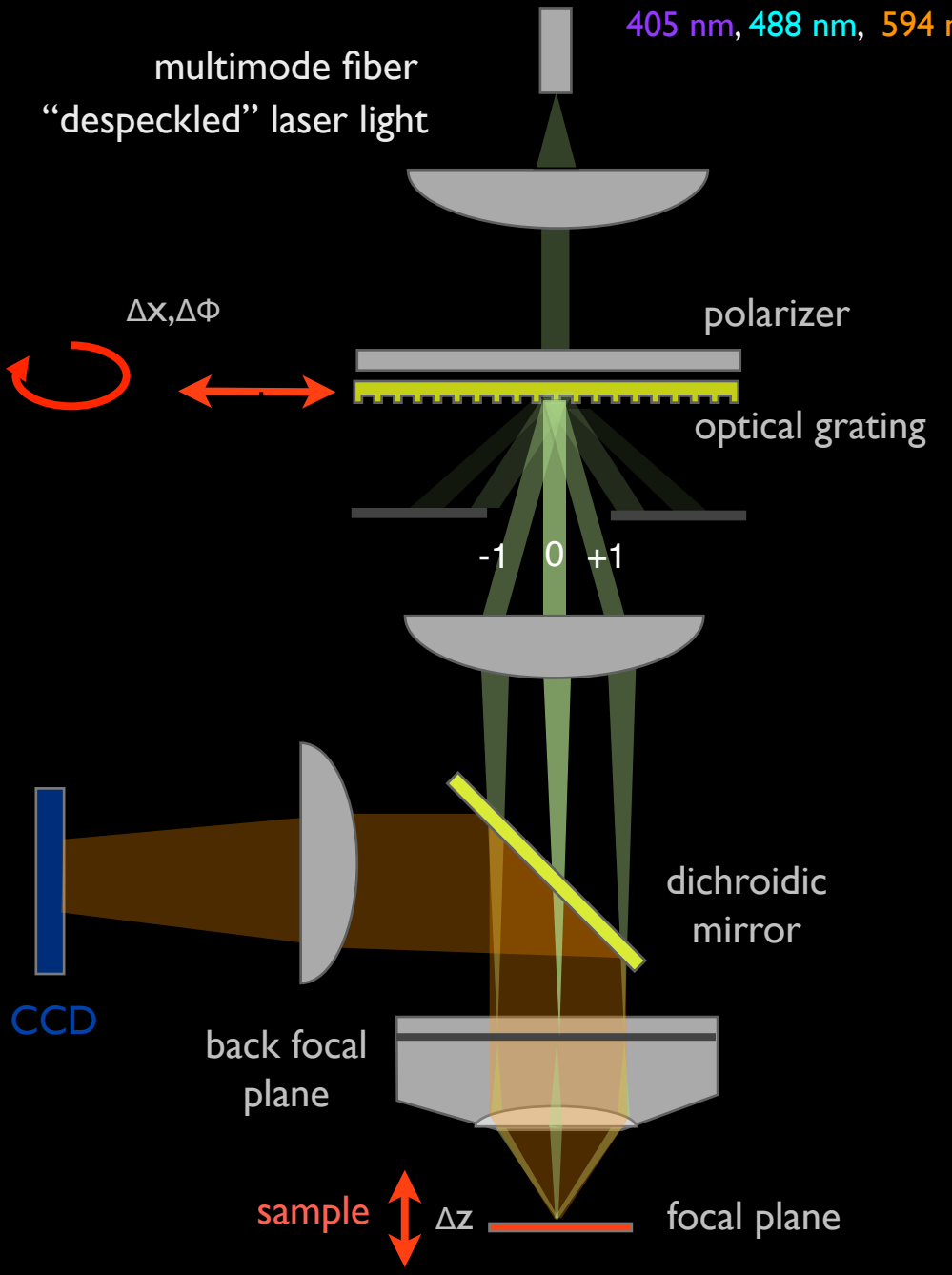
...demo!

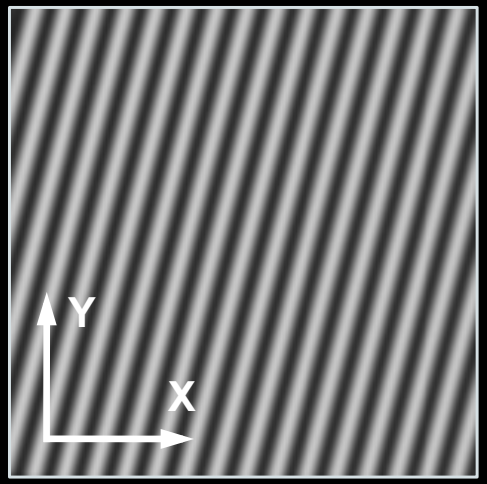
Generating 2D-structured illumination



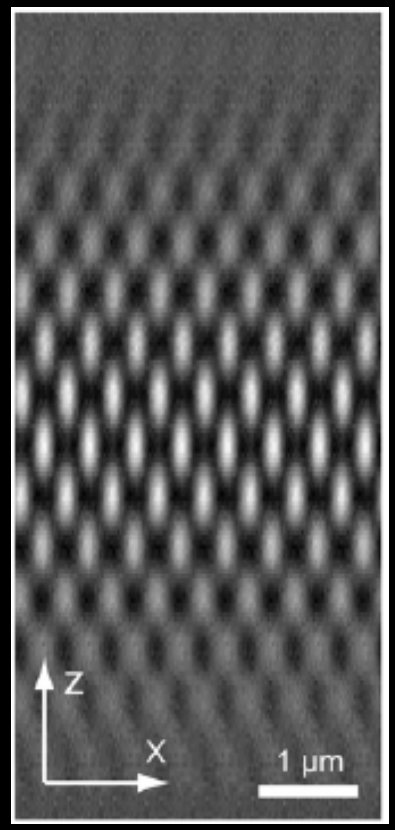
adapted from Gustafsson et al. (2008) *BiophysJ*, 94

Generating 3D-structured illumination



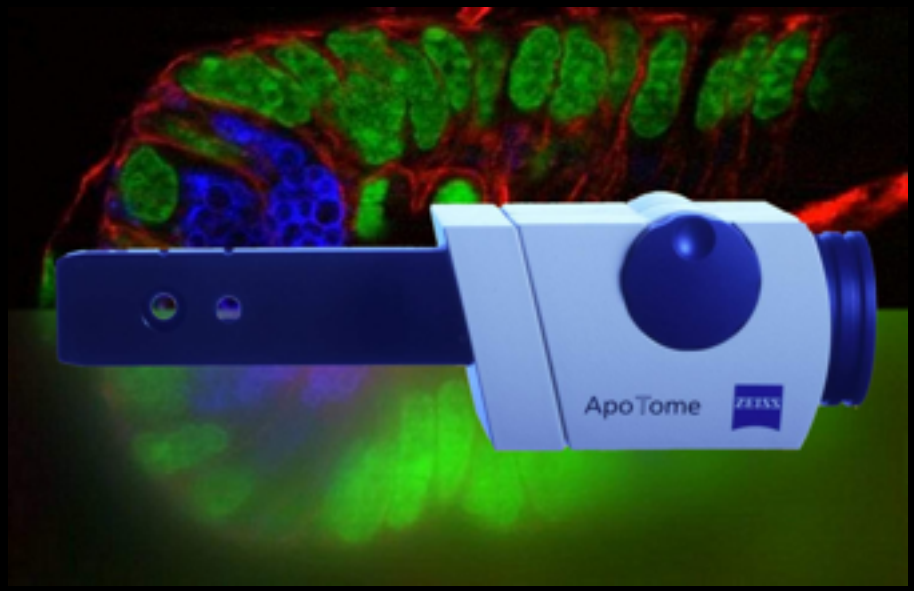


axial modulation



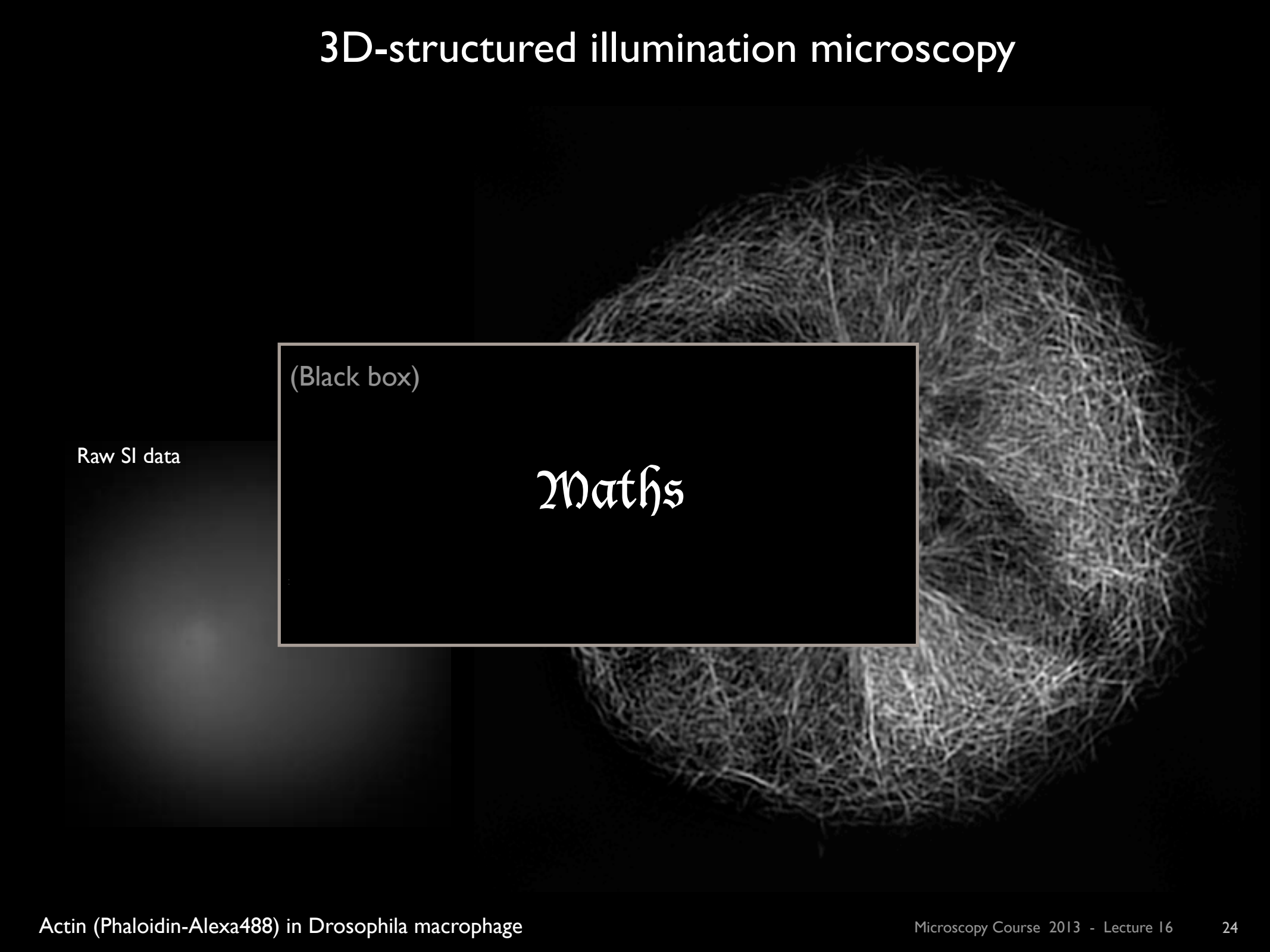
adapted from Gustafsson et al. (2008) *BiophysJ*, 94

Conventional SIM: Apotome uses coarse SI to remove out-of-focus blur



„Poor man's confocal"
No super-resolution!

3D-structured illumination microscopy

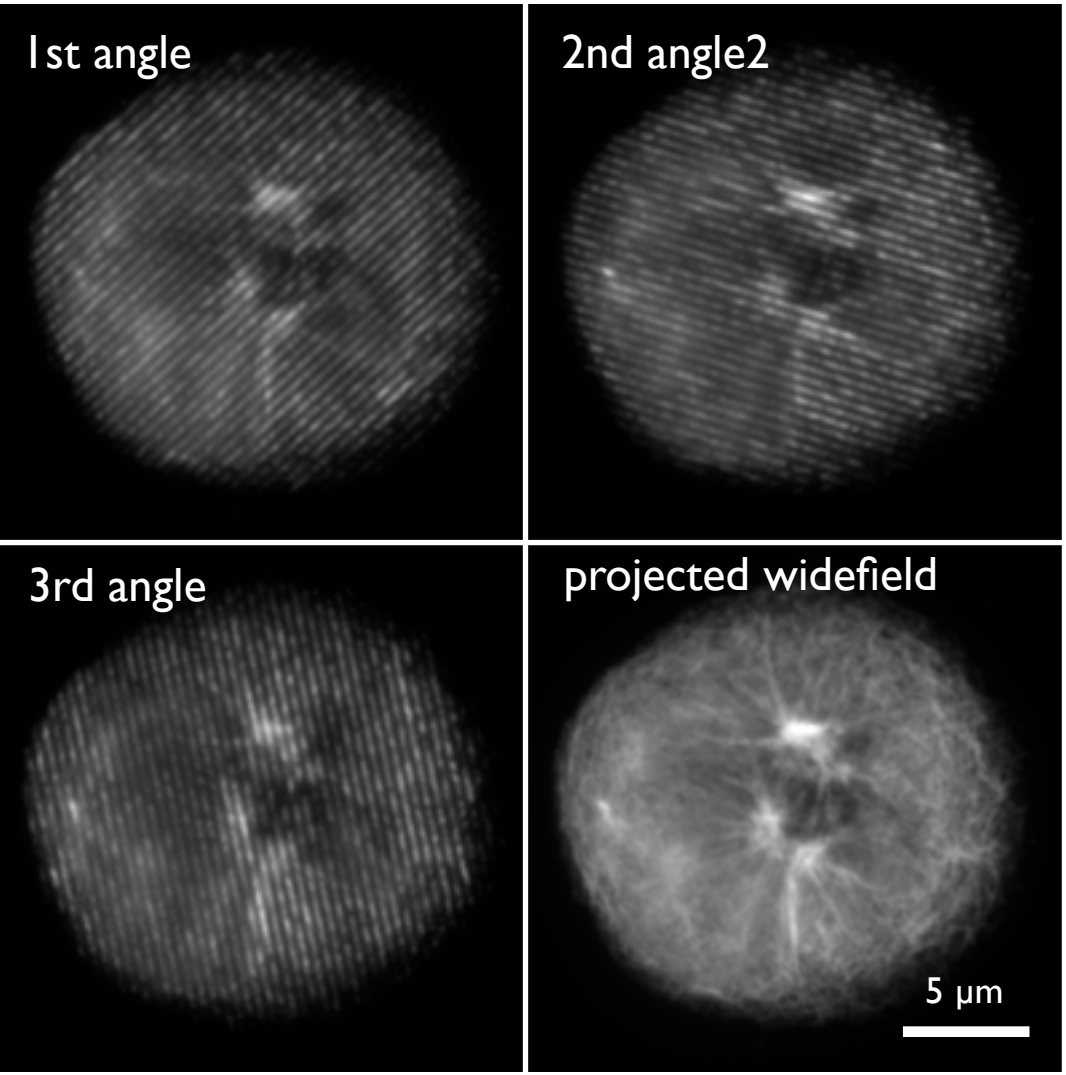


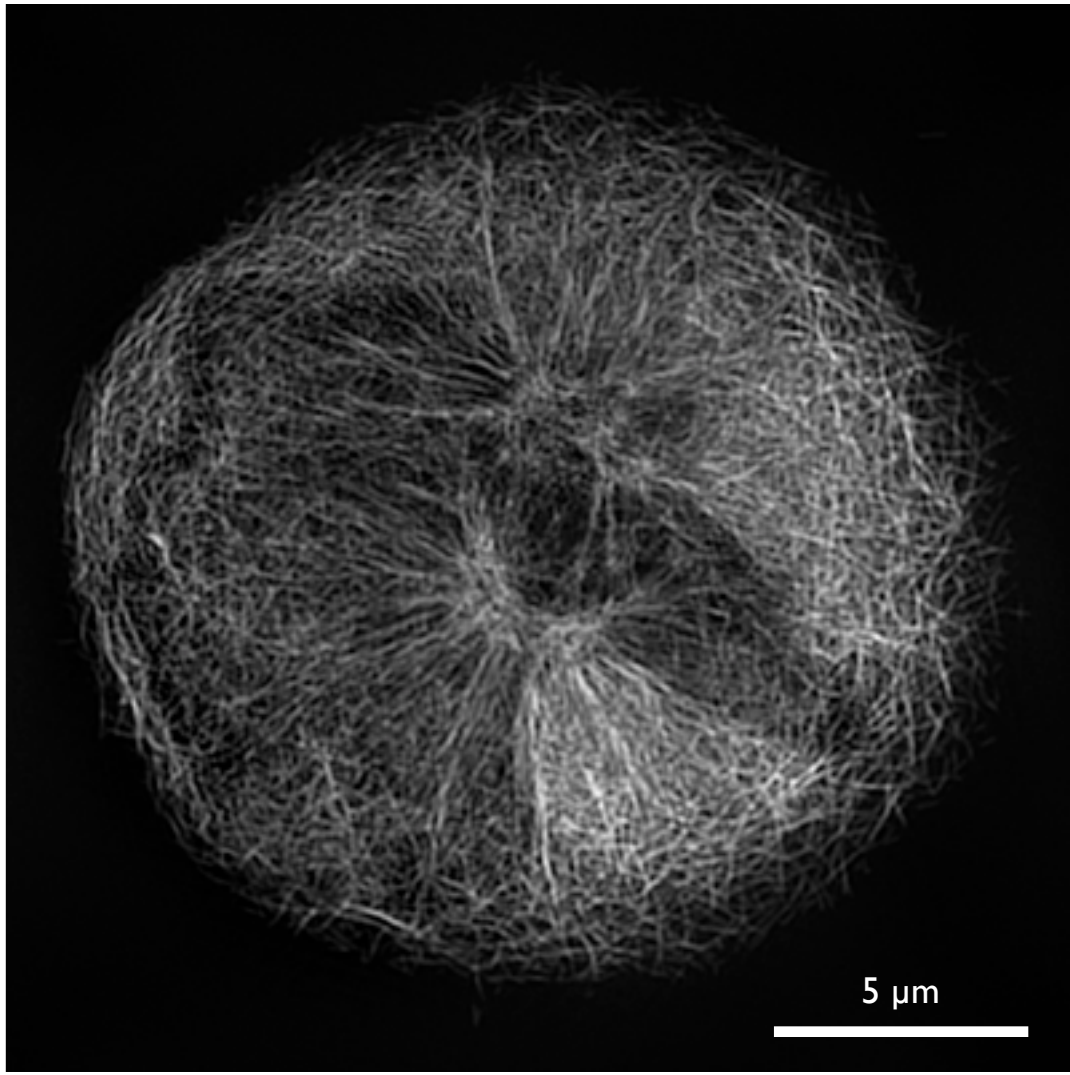
Actin (Phaloidin-Alexa488) in Drosophila macrophage

Doubled frequency support = 2-fold resolution in xy and z

SI raw

SI reconstructed





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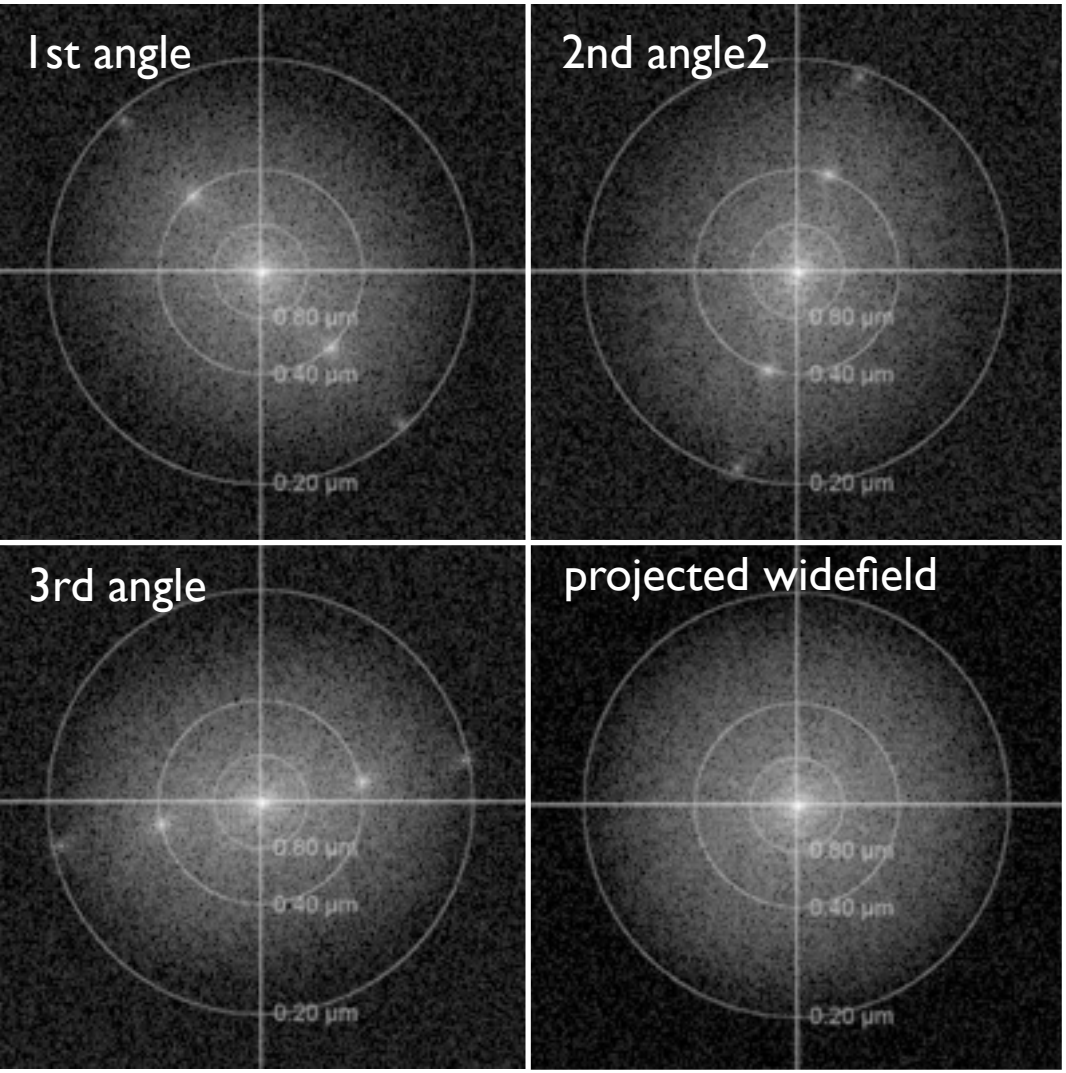
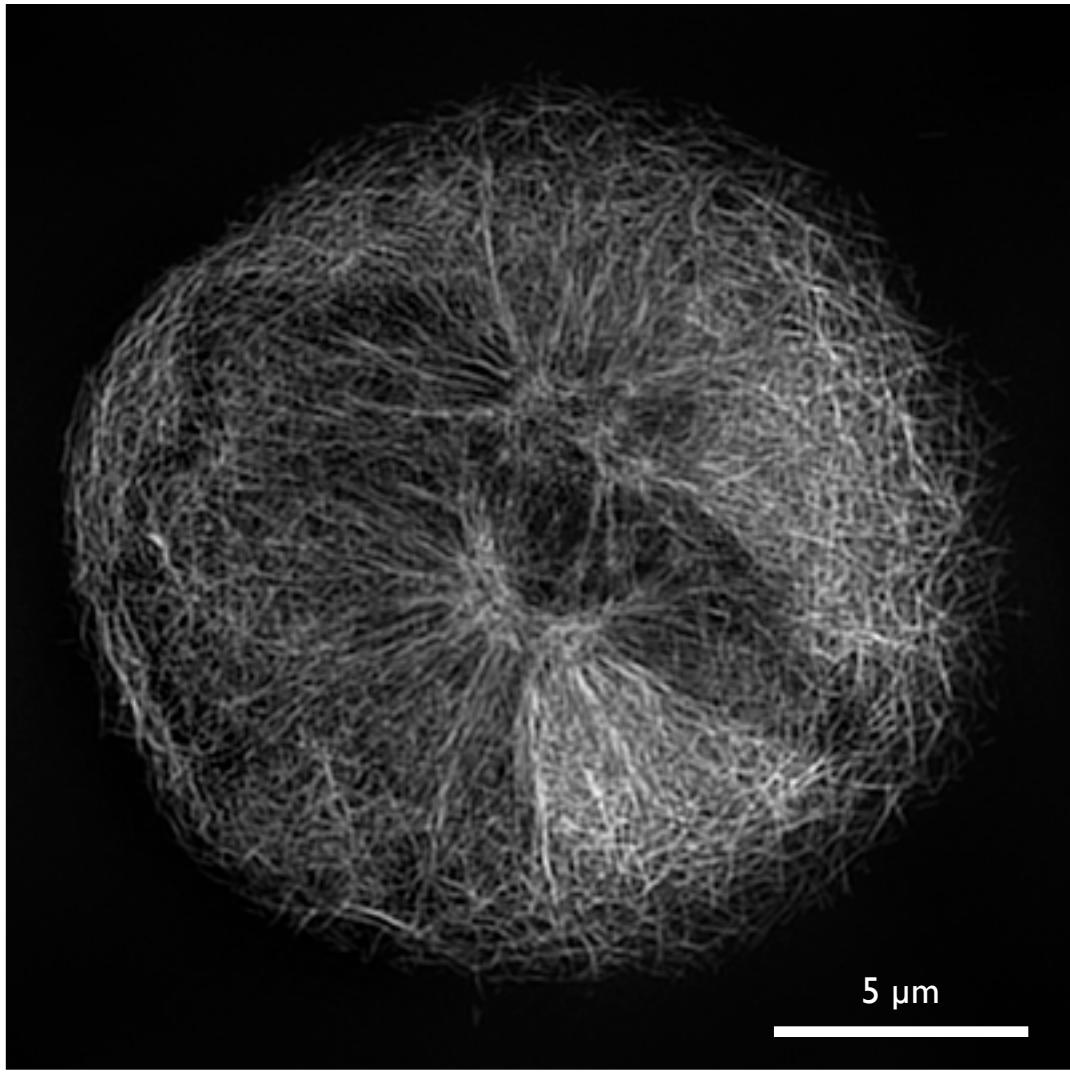


SI reconstructed



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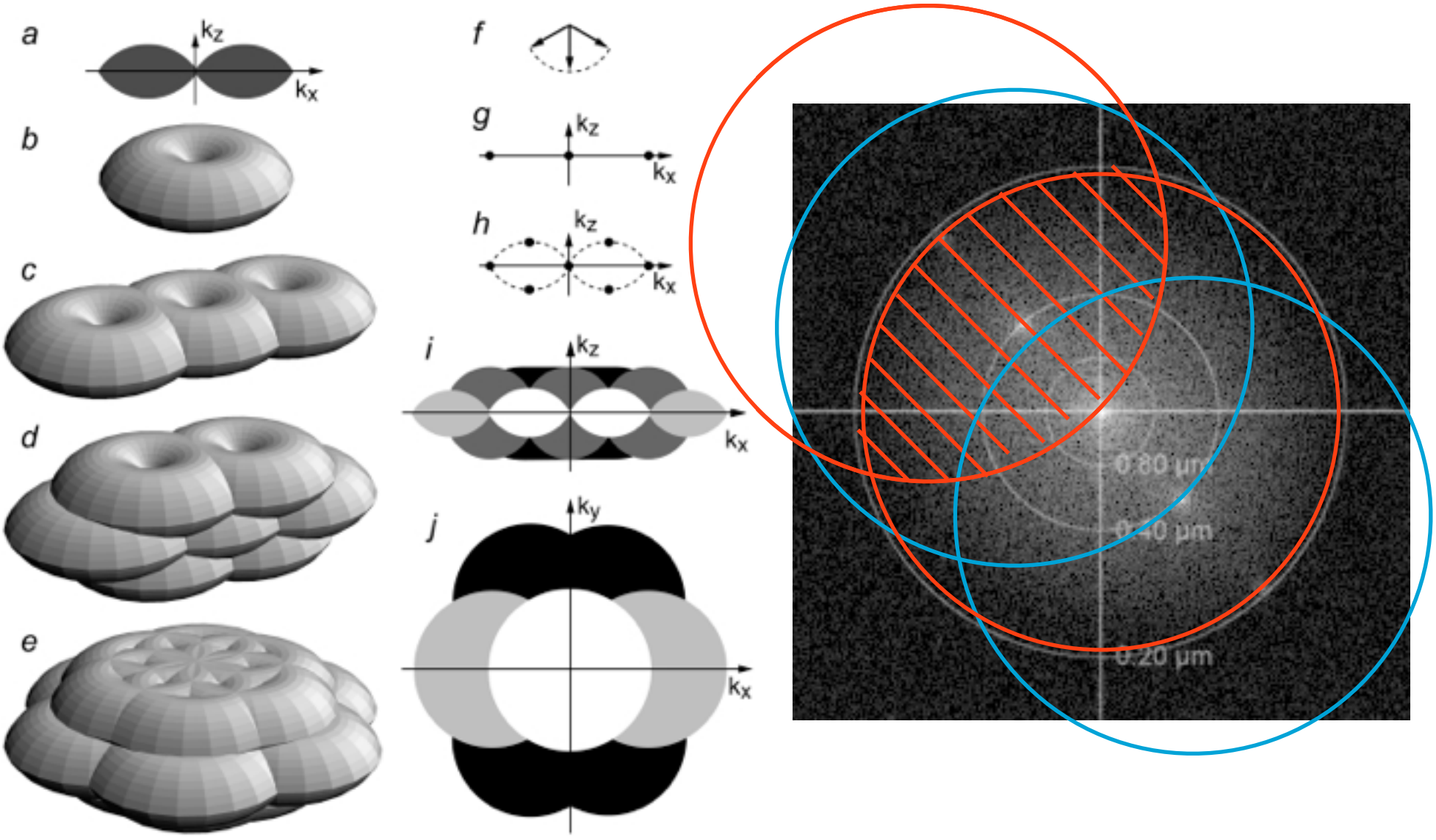
Doubled frequency support = 2-fold resolution in xy and z

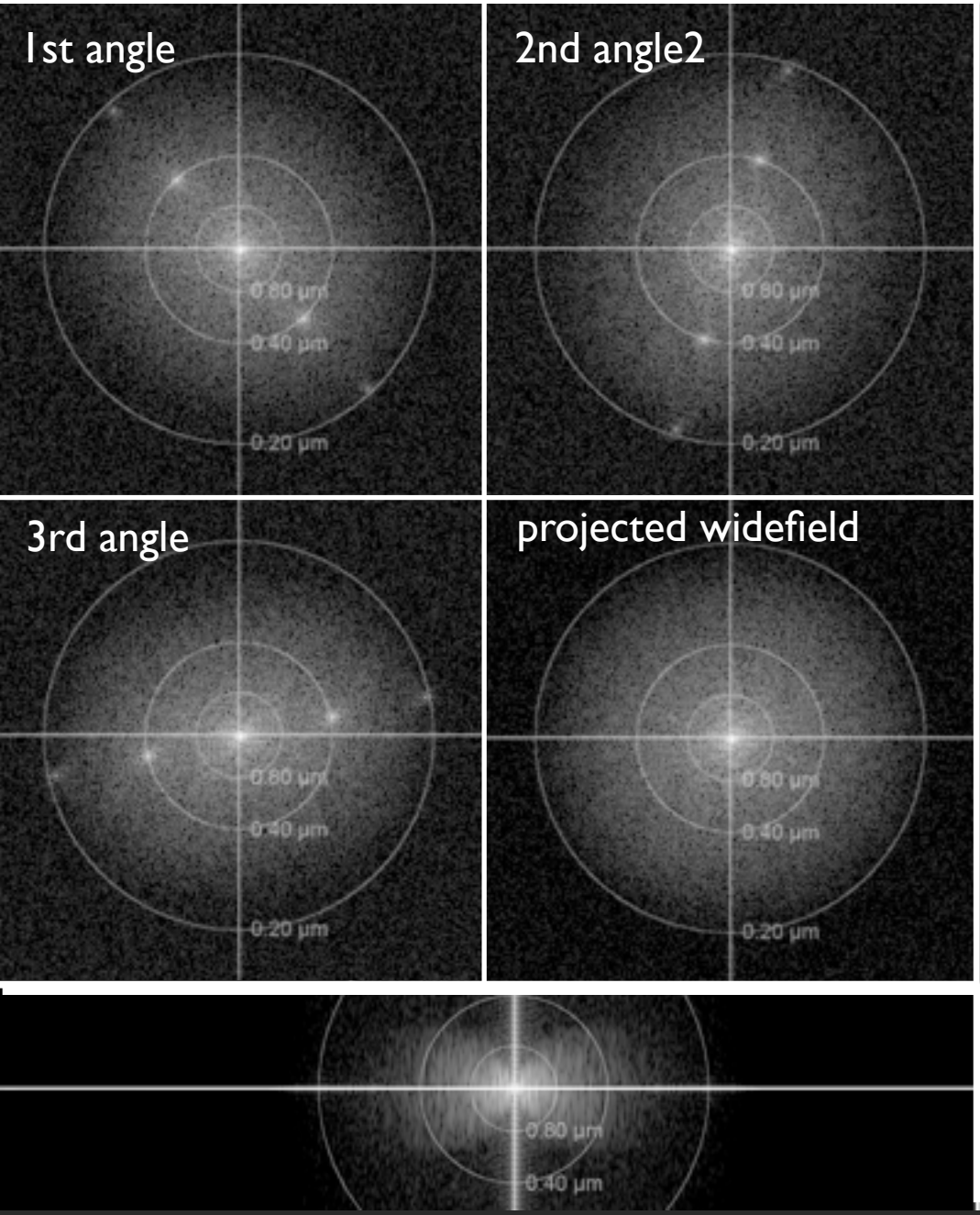


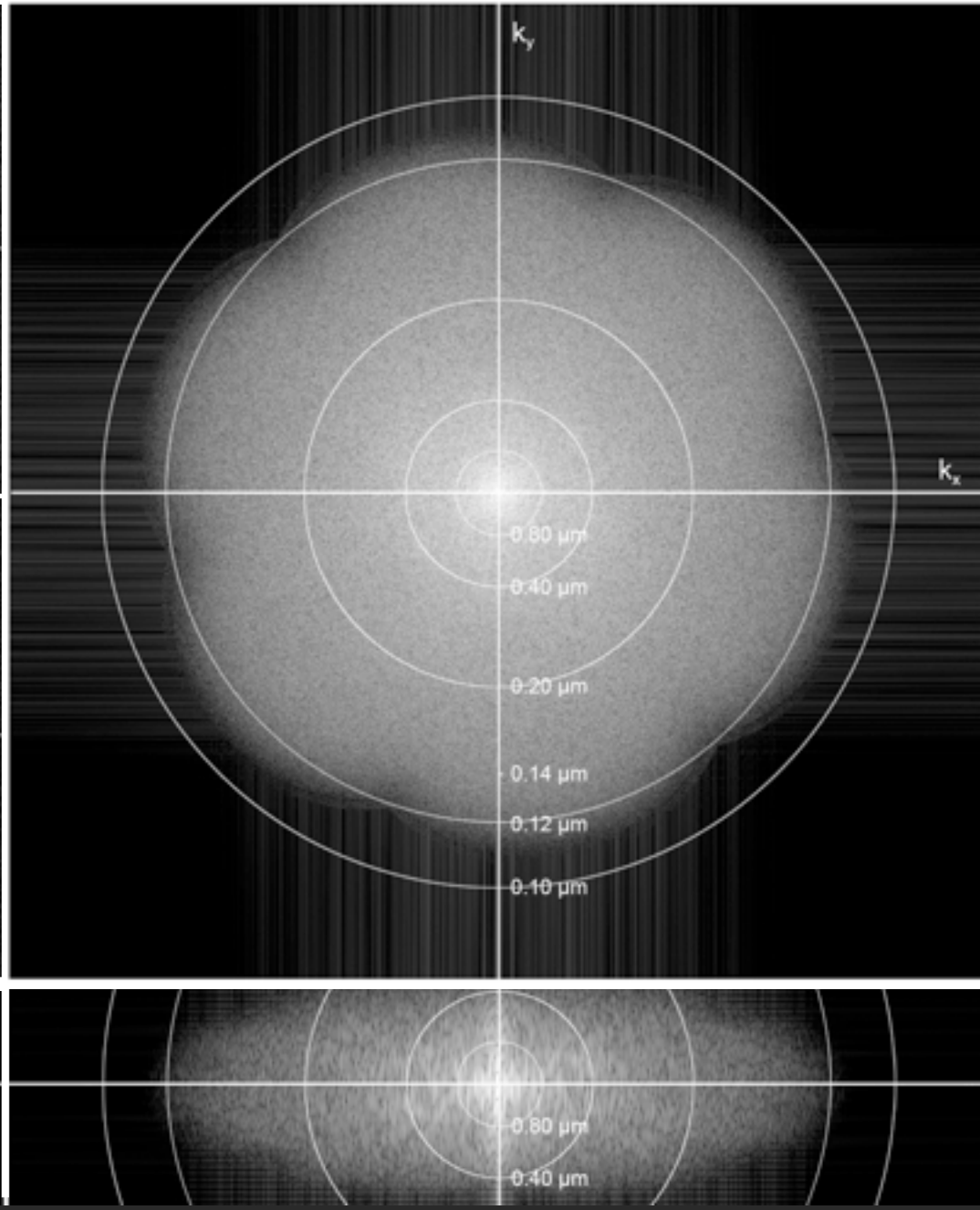
Gustafsson et al. (2008), *Biophys J* **94**

Doubled frequency support = 2-fold resolution in xy and z

SI raw

SI reconstructed

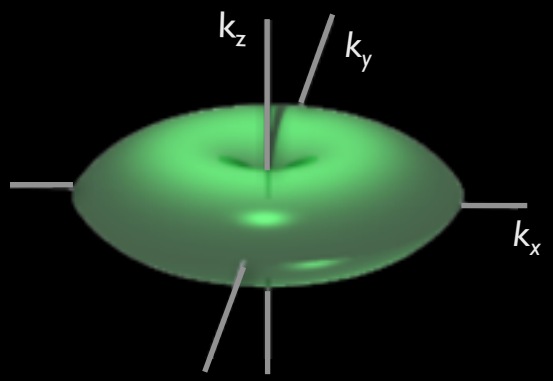




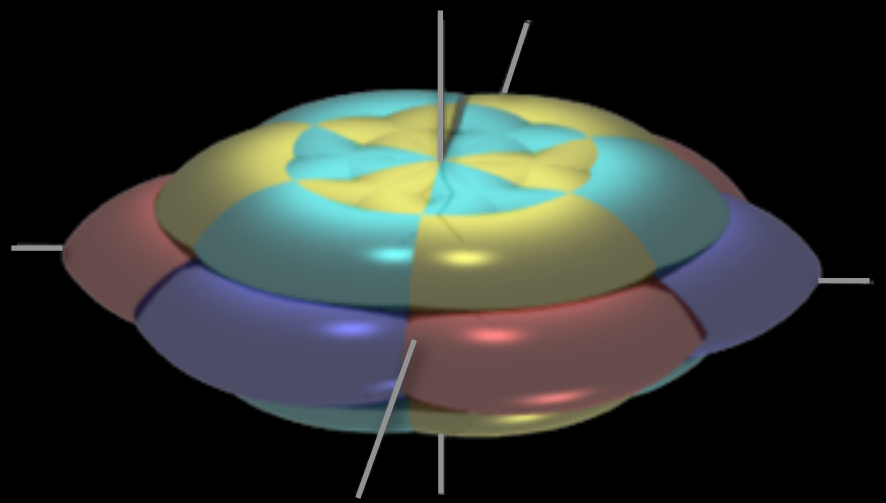
Doubled frequency support = 2-fold resolution in xy and z

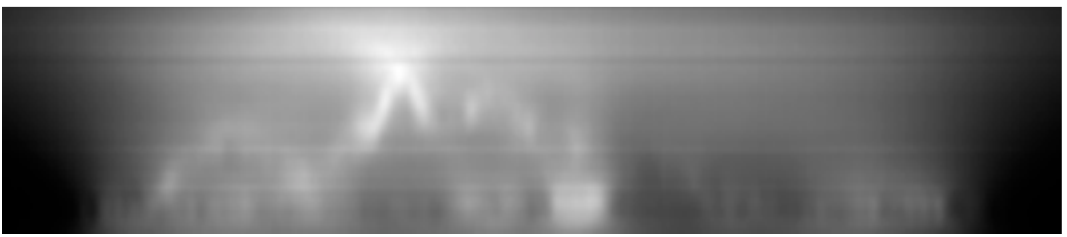
Widefield

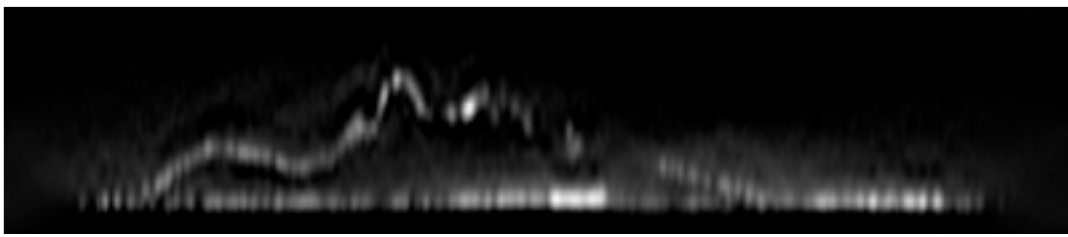
SI reconstructed



5 phases, 3 angles → optical sectioning
with 2x2x2-fold frequency support



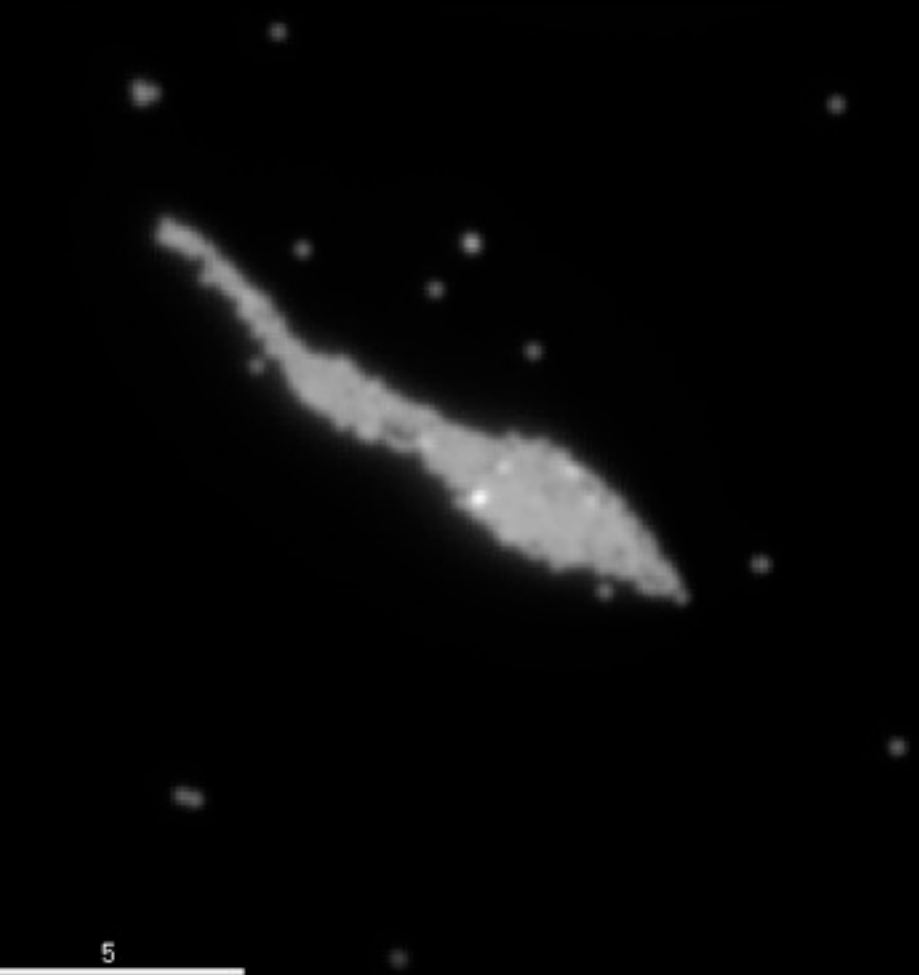


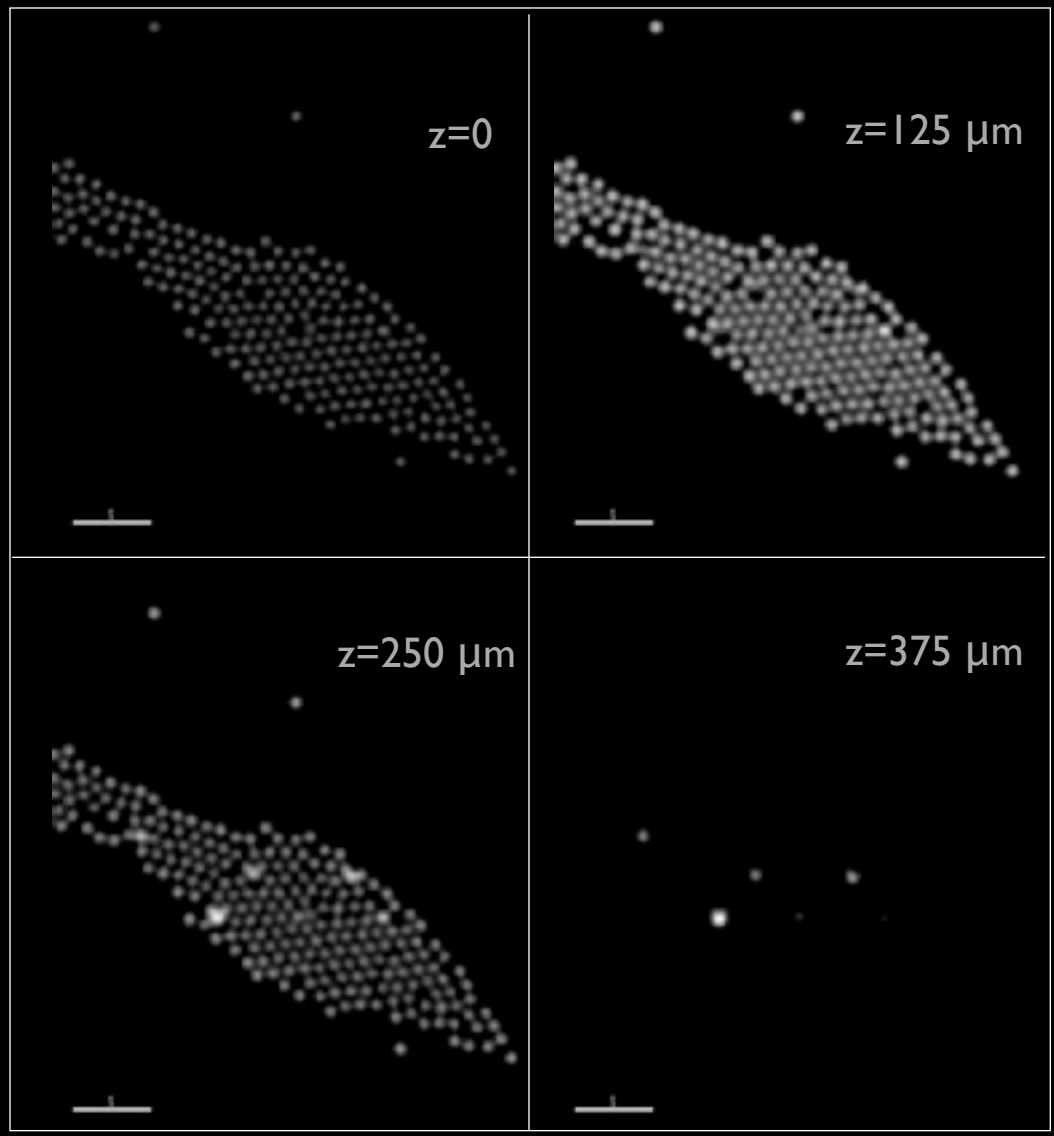


Actin (Phaloidin-Alexa488) in Drosophila macrophage

3D optical sectioning capacity

Example: 170 nm Fluospheres

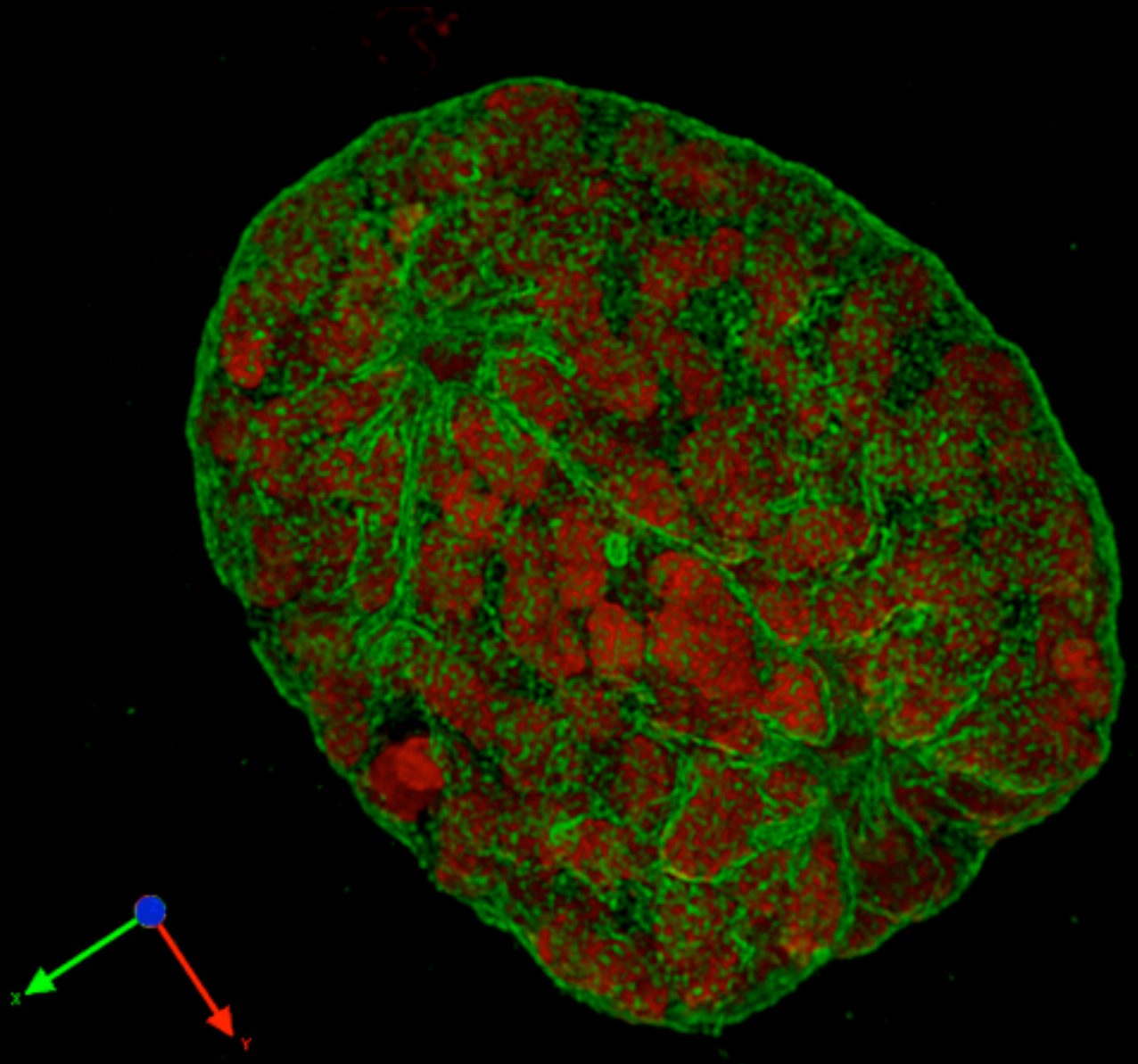




3D SIM example: Prophase

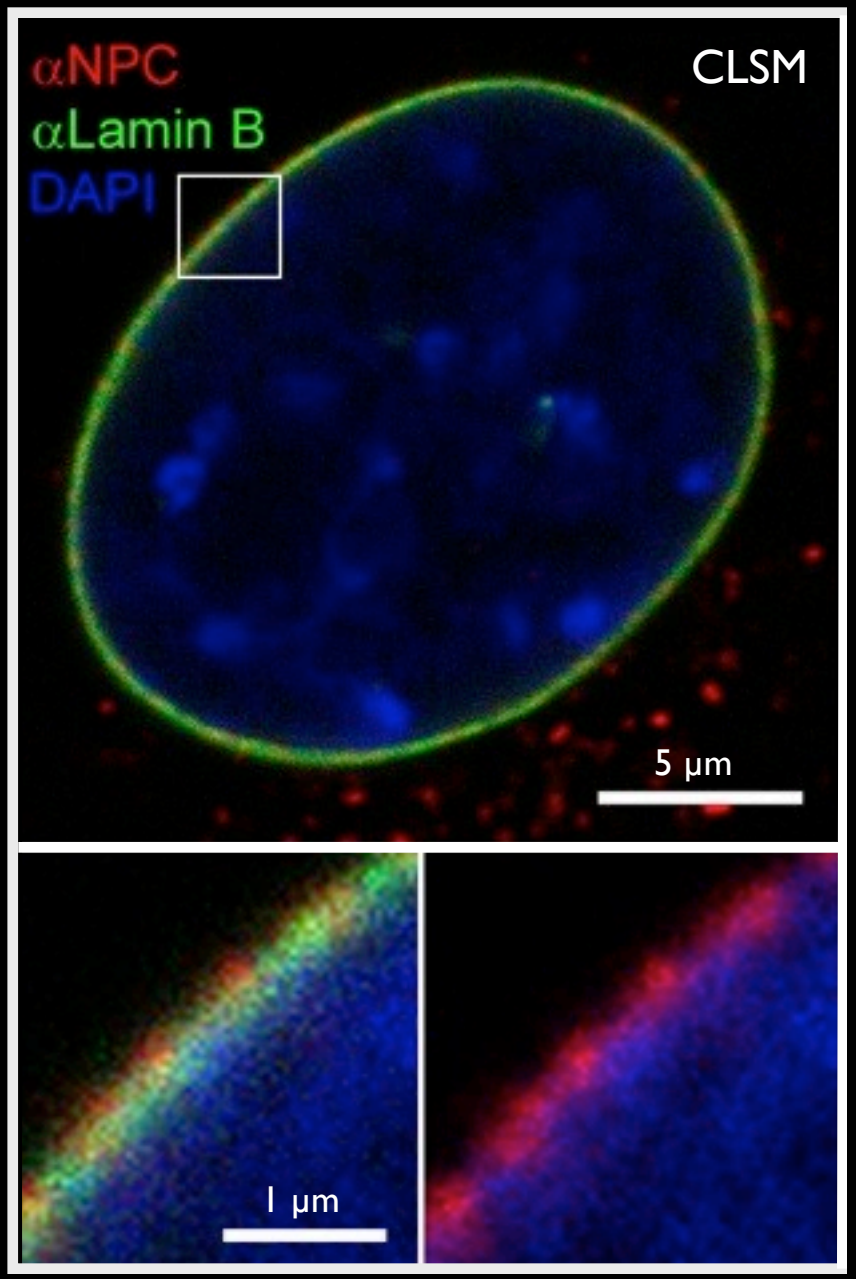
Lamin B
DAPI

3D volume rendering



Schermelleh, Carlton et al. (2008), Science **320**

3D-SIM resolves chromatin domains and interchromatin channels, leading towards nuclear pores



Schermelleh, Carlton et al. (2008), *Science* **320**

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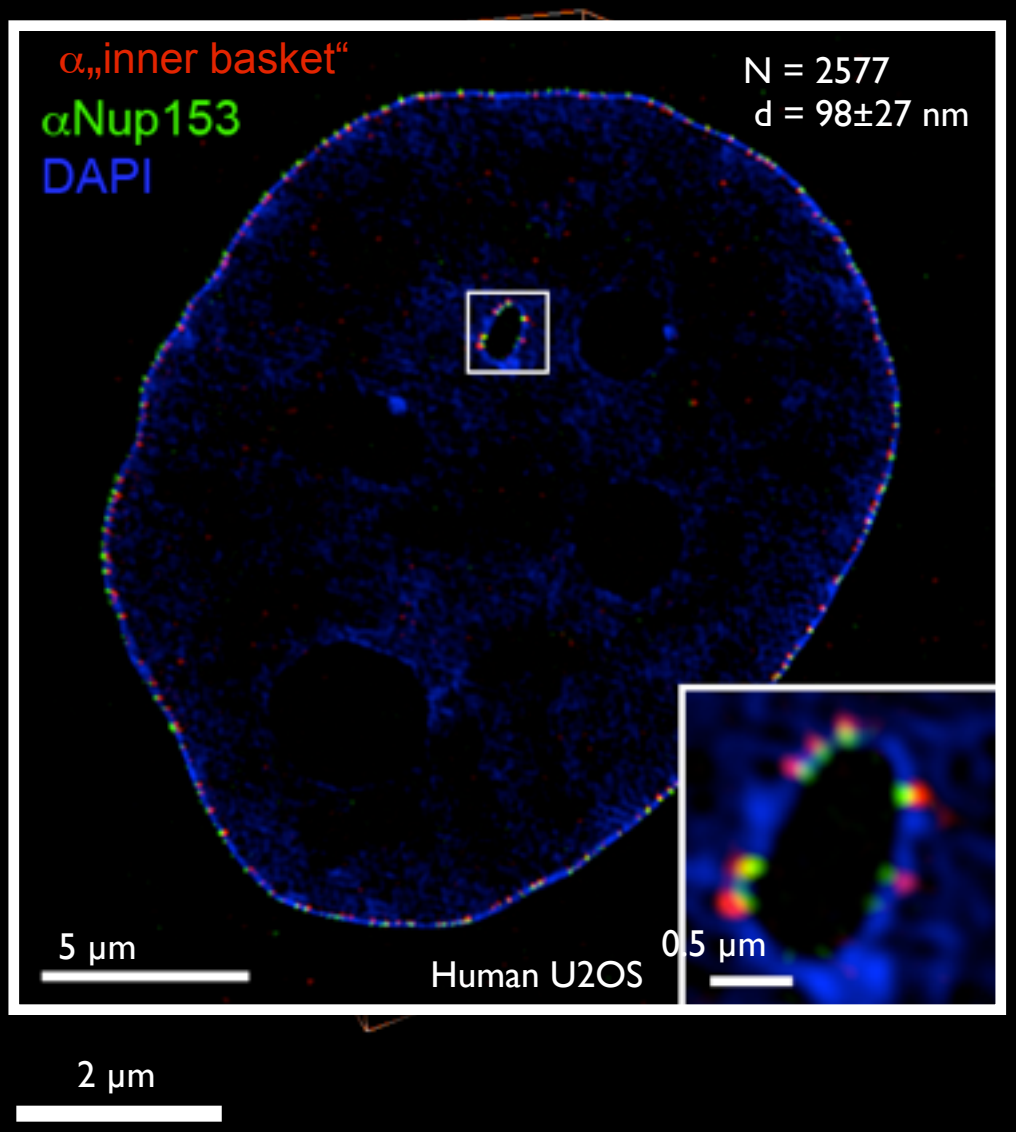
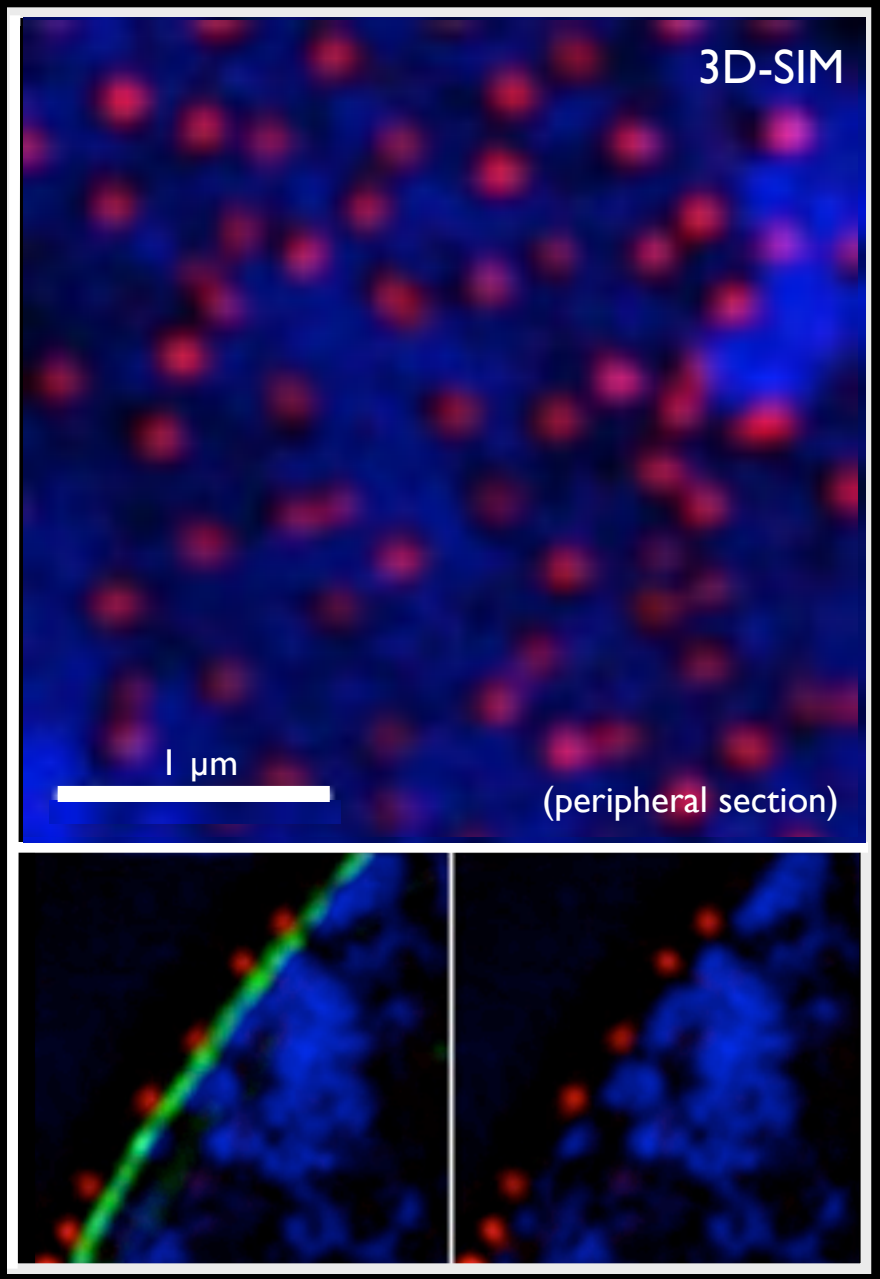
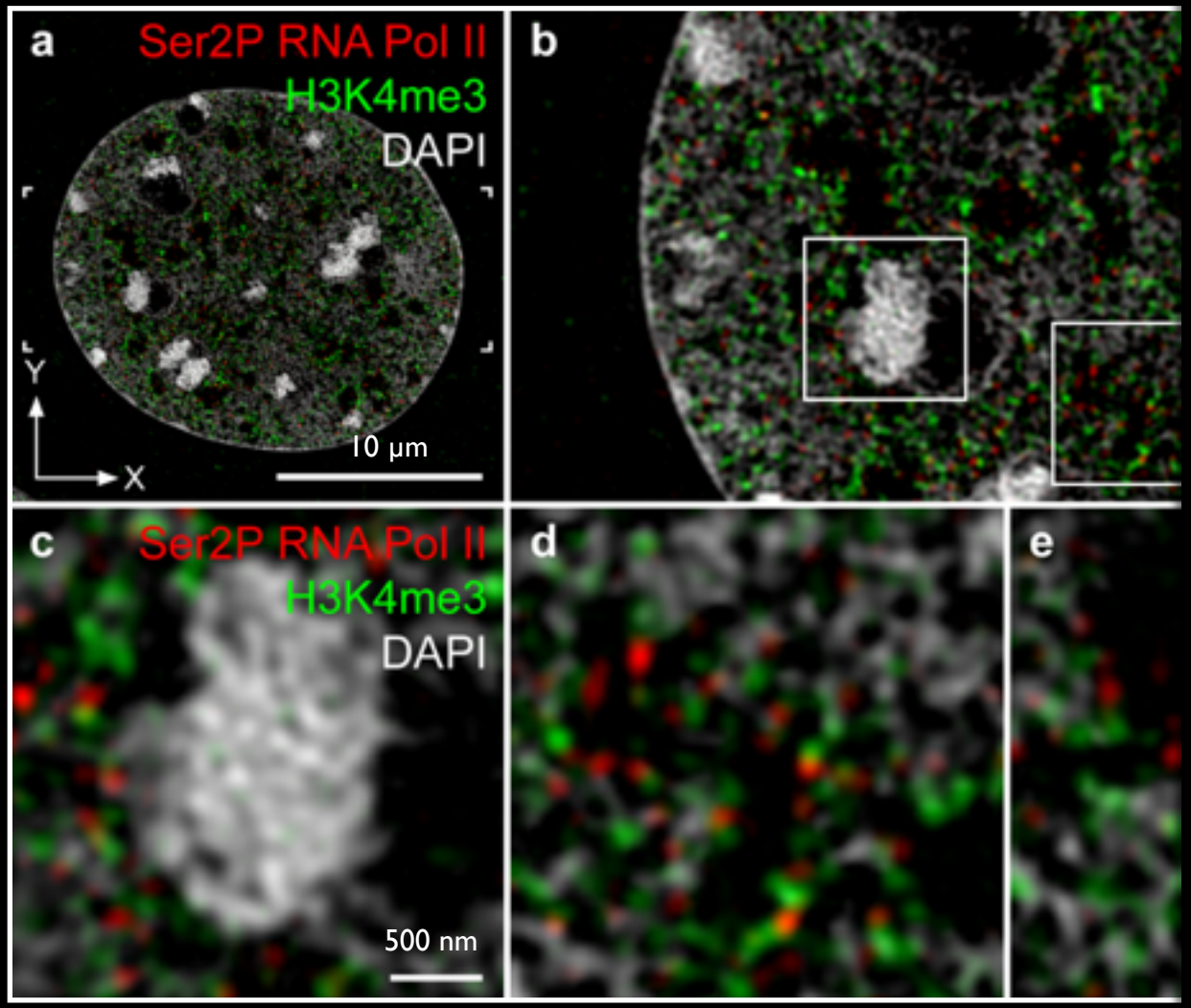
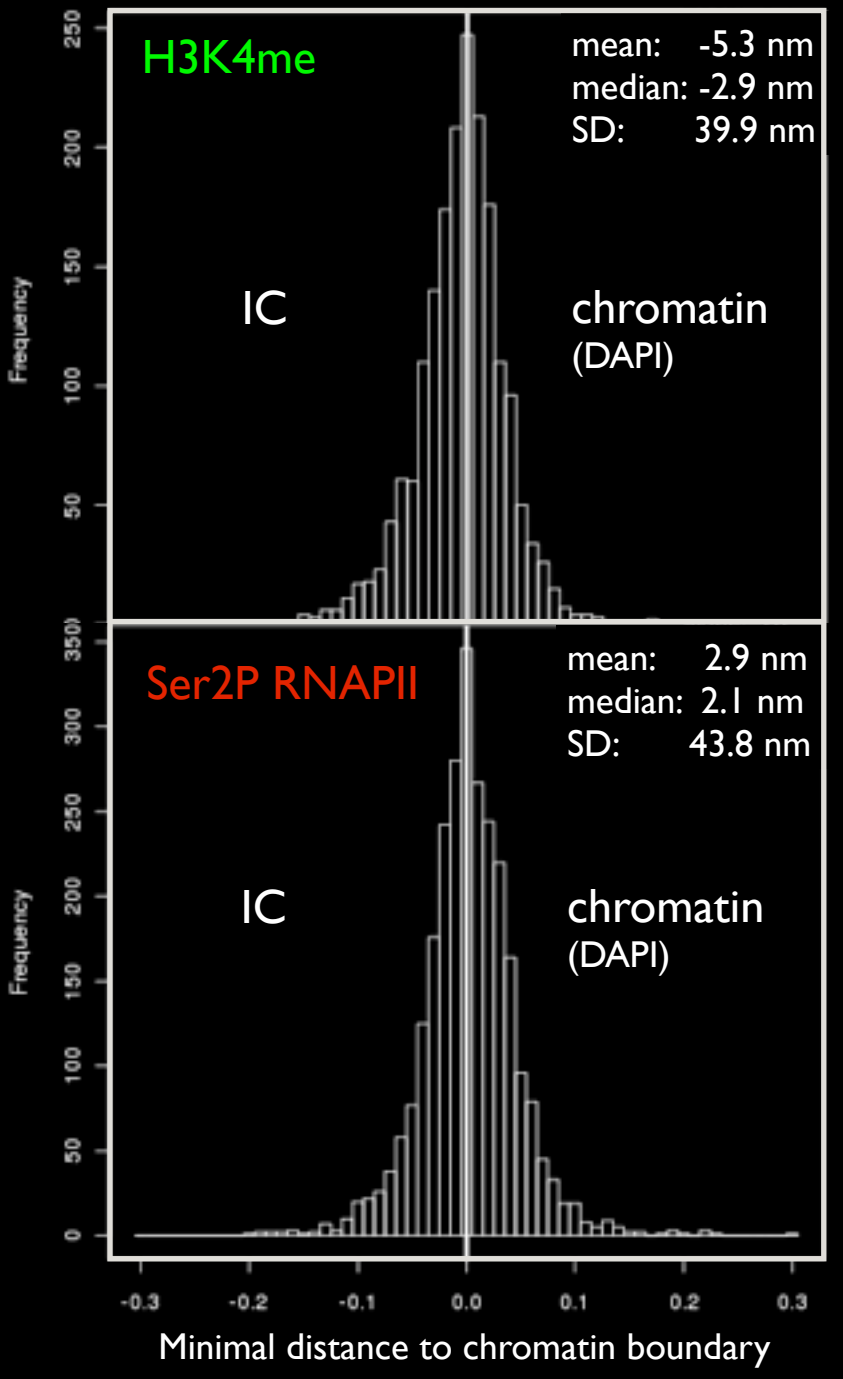




Active transcription marks enriched at chromatin borders

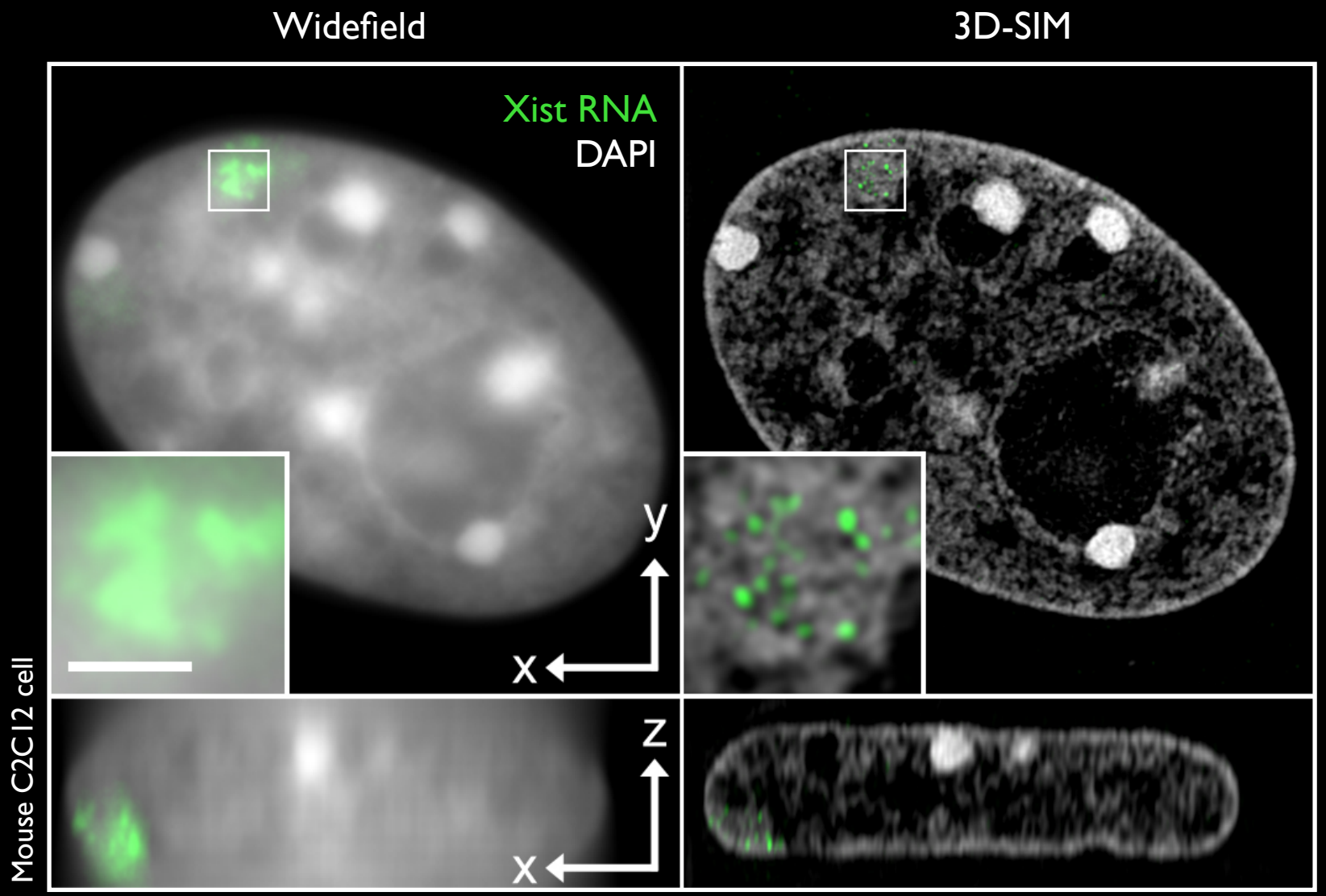


Mouse C127 cell



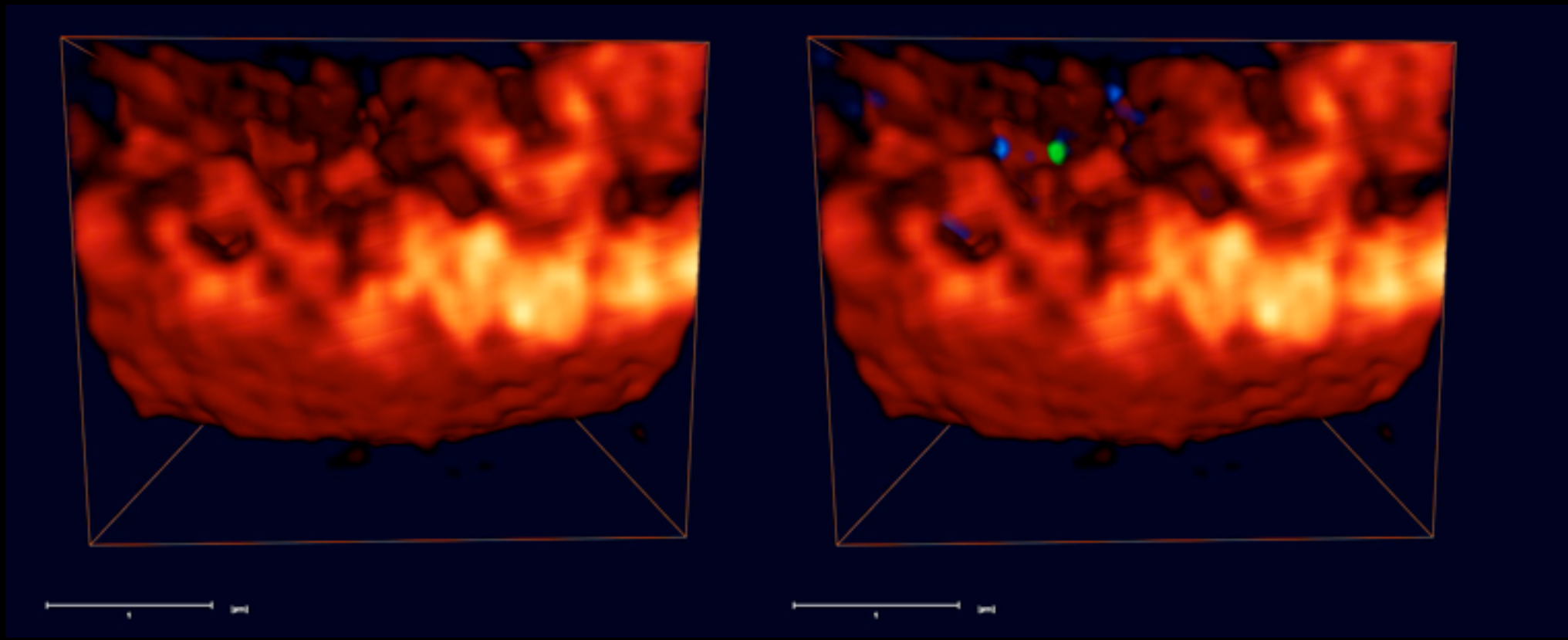
Markaki et al., 2011, *Cold Spring Harb Perspect Biol*, 75

Topology of the inactive X chromosome



Xist preferential localizes in decondensed interchromatin compartment within the Xi

Topology of the inactive X chromosome



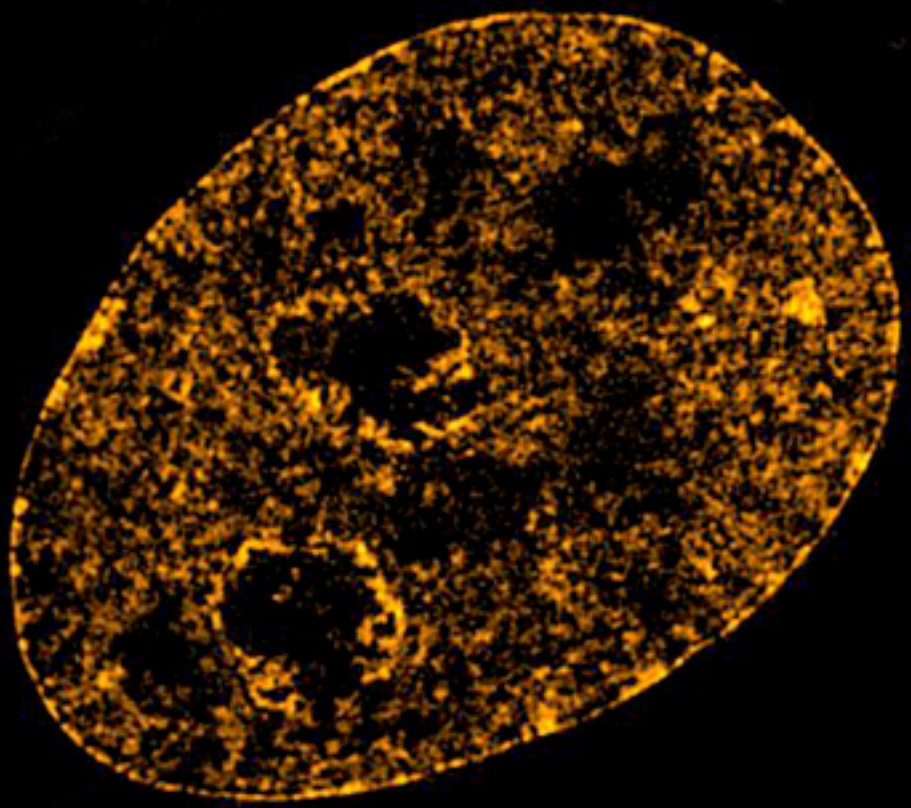
DAPI Xist-RNA RNAPII

Can we go live?

Live cell 3D-SIM with OMX Blaze

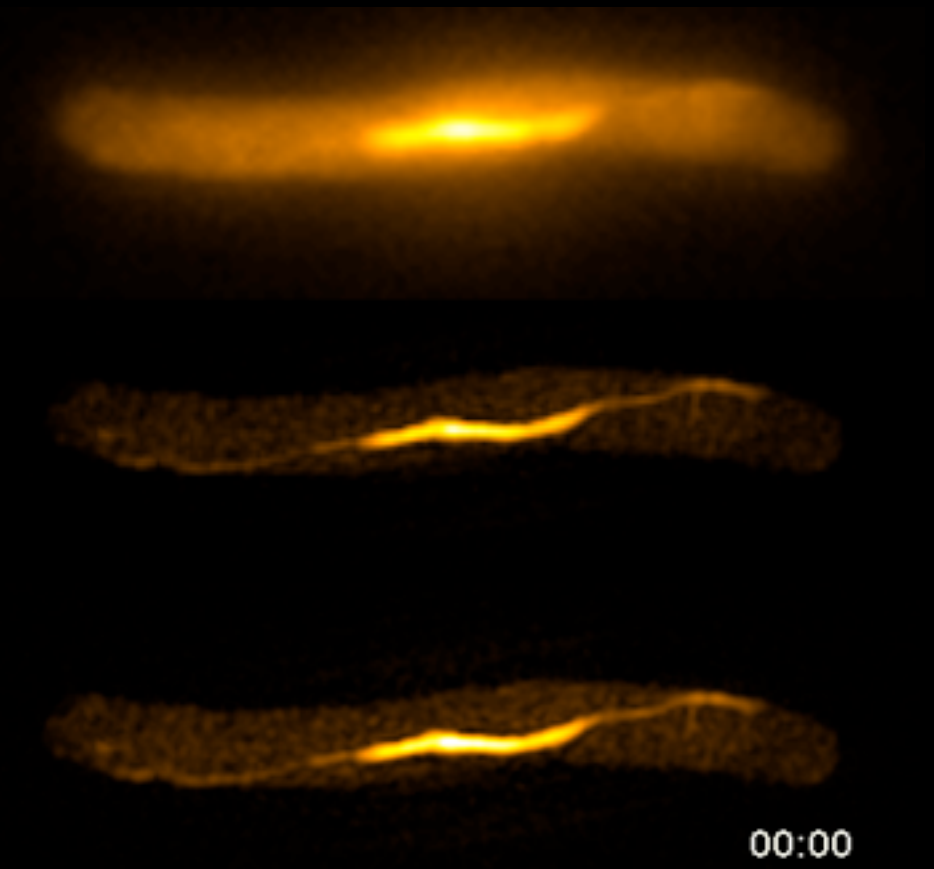
Inferometric SI generation + sCMOS cameras => 10 x faster imaging

H2B-GFP (unfixed)



7 µm z-stack (56 sections, 5 ms exposure)

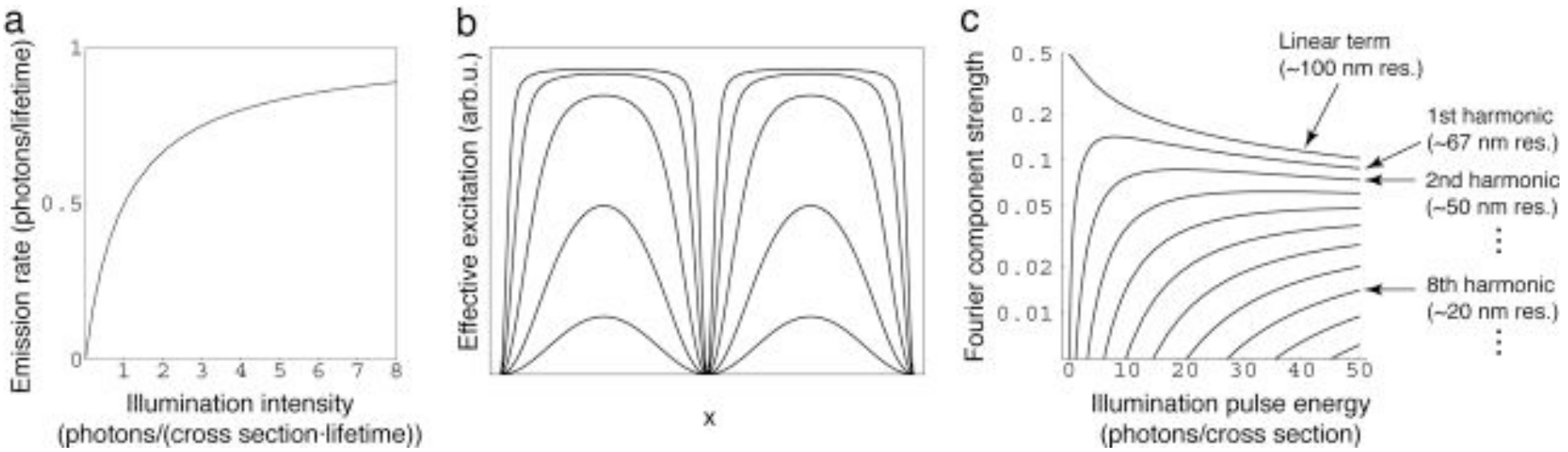
RecA-GFP (*E.coli*)

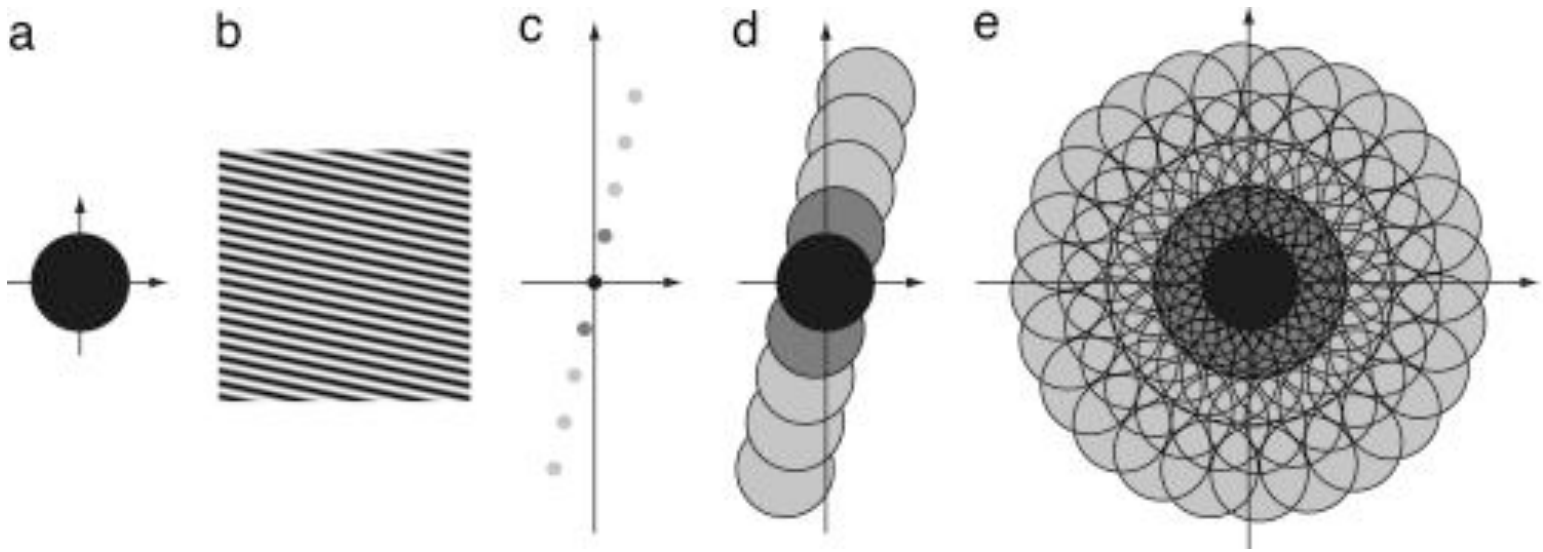


2 s / 3D-frame (1 µm z-stack = 120 images ; 100 ime points)

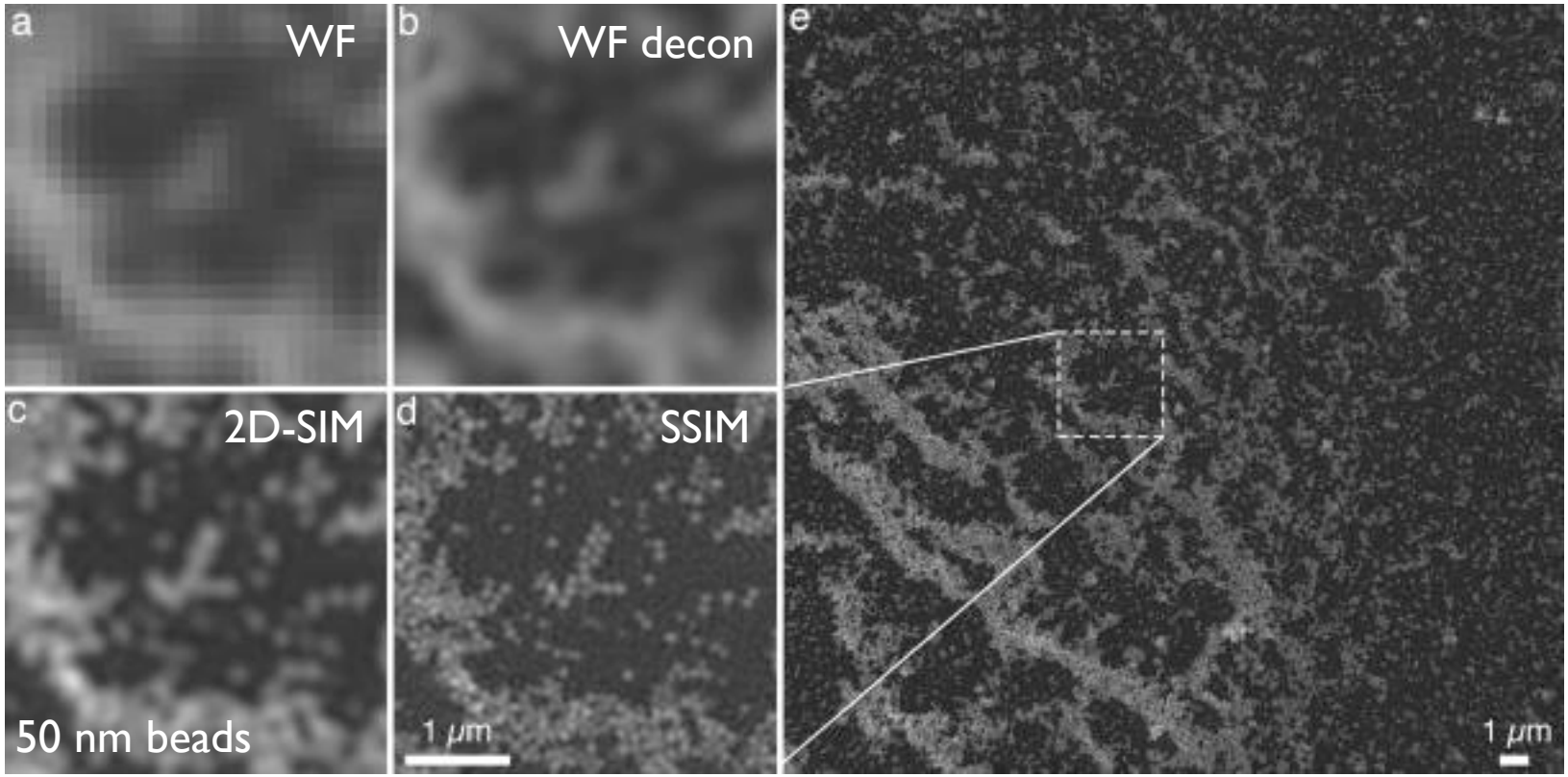
2D/3D-SIM is still resolution limited!
Can we go beyond ?

Non linear SIM - Saturated structured illumination microscopy (SSIM)





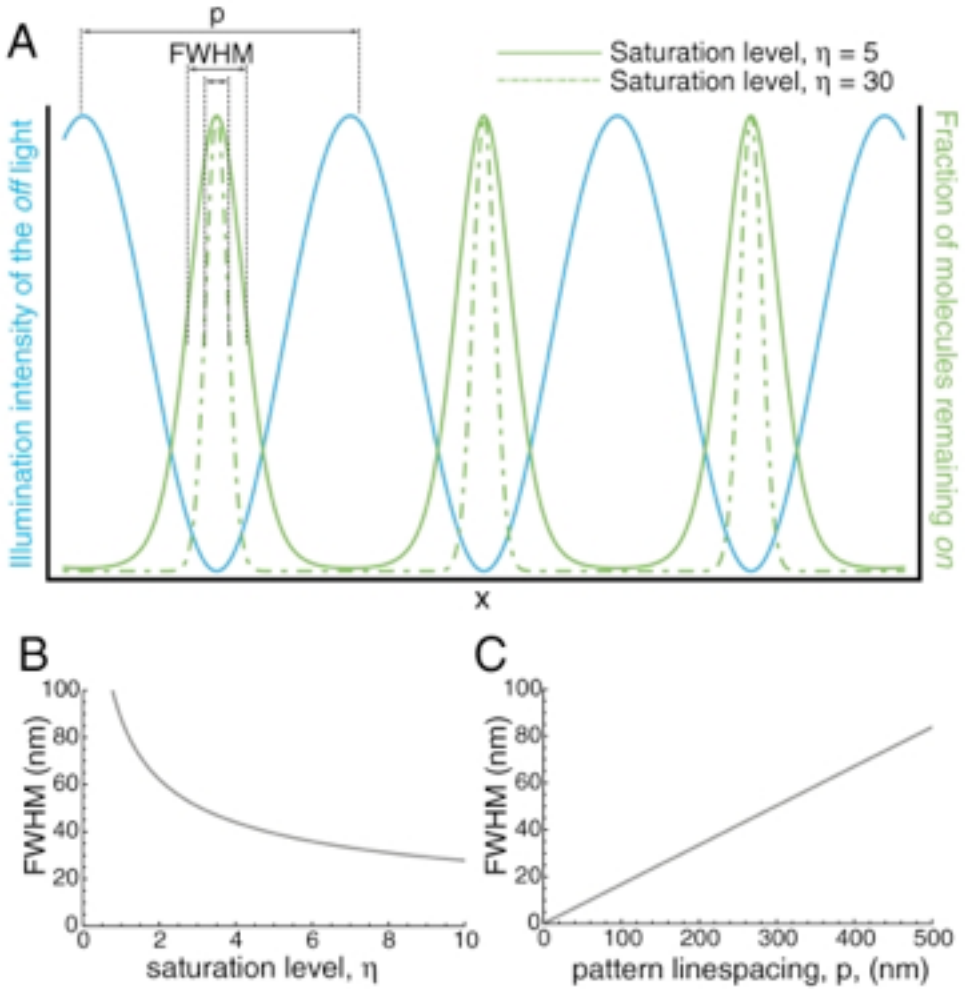
Non linear SIM - Saturated structured illumination microscopy (SSIM)

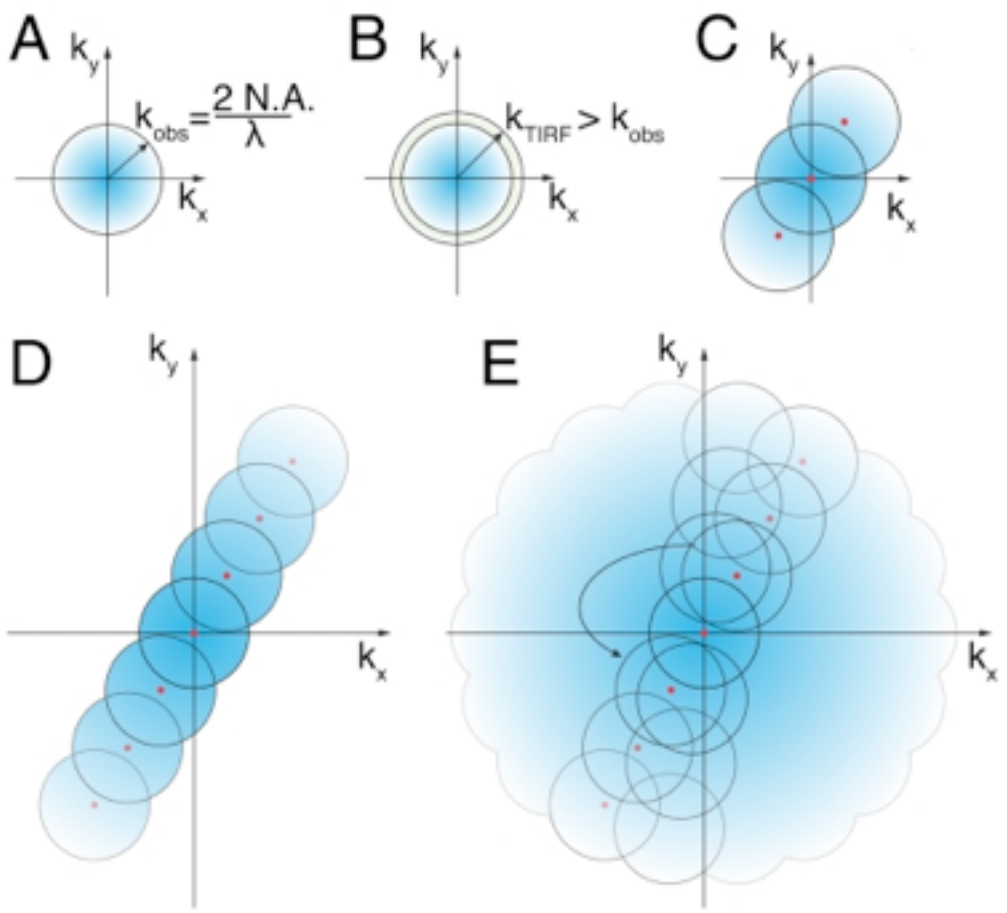


Resolution is theoretically unlimited!!!

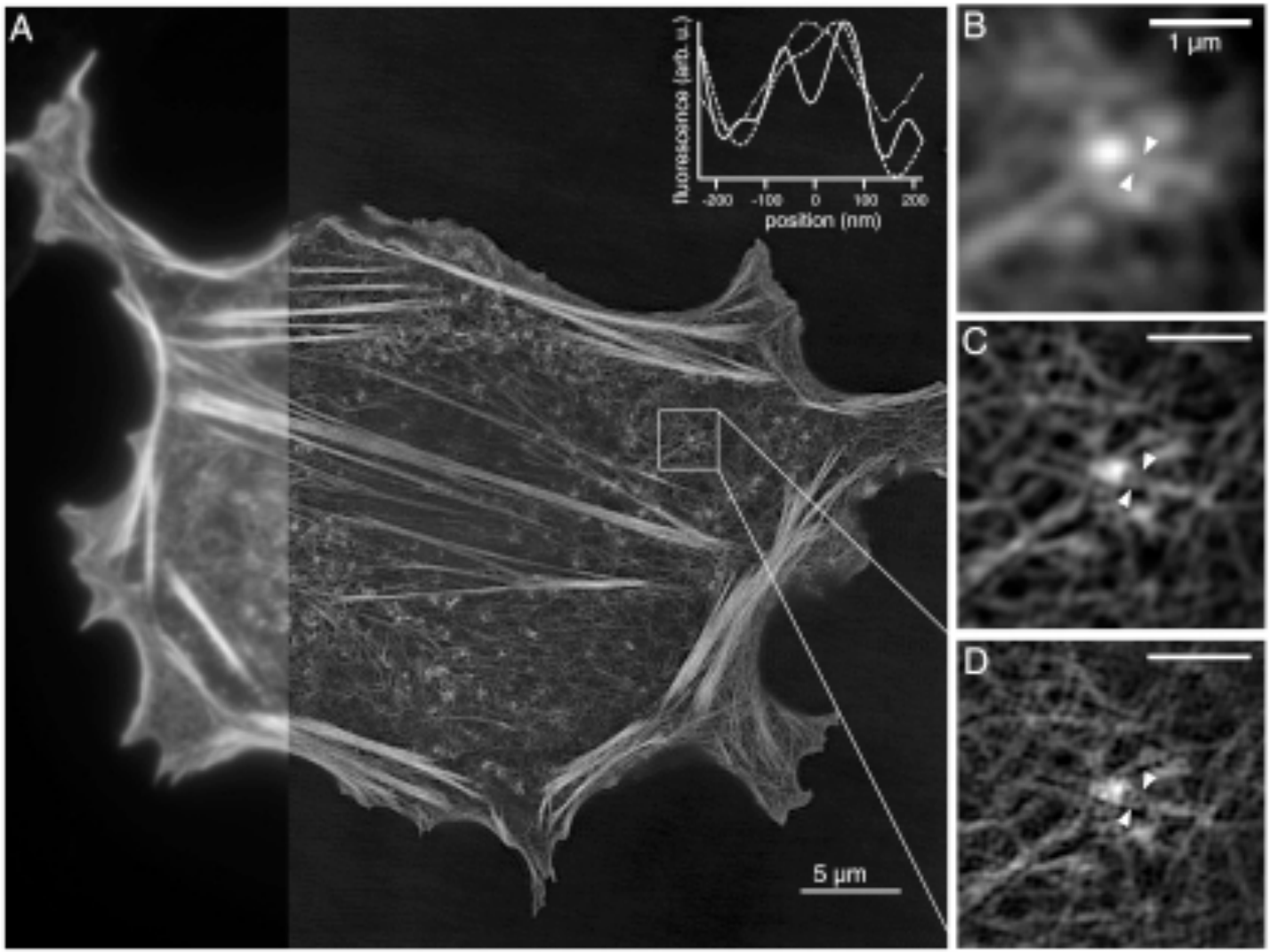
Problem: photostability of the dye
=> works on beads but not on biological samples

Non linear SIM with switchable fluorophores (Dronpa)





Non linear SIM with switchable fluorophores (Dronpa)



Biological imaging is possible!
but limited on the number of switching cycles
Only xy enhanced, requires TIRF

3D-SIM,
just another tool in the repertoire ?

It‘s not that simple!

The untold story

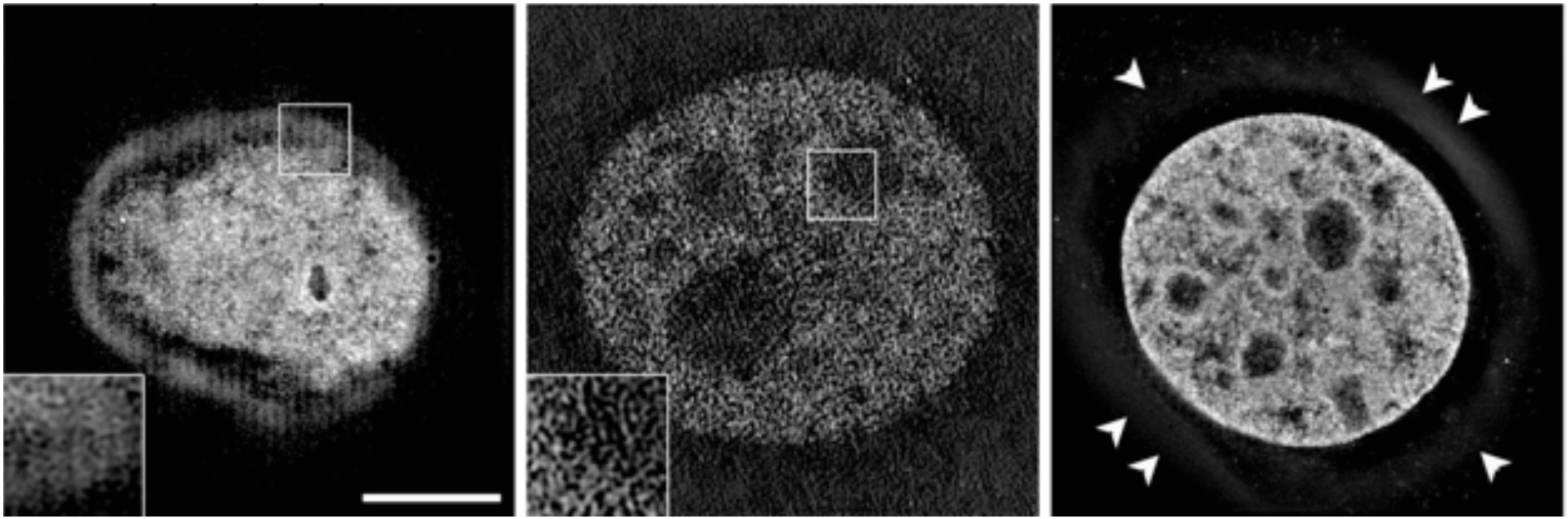
SI reconstruction artifacts

Stripes

High frequency noise

Halo / Doubling

C127 cell nuclei, chromatin staining

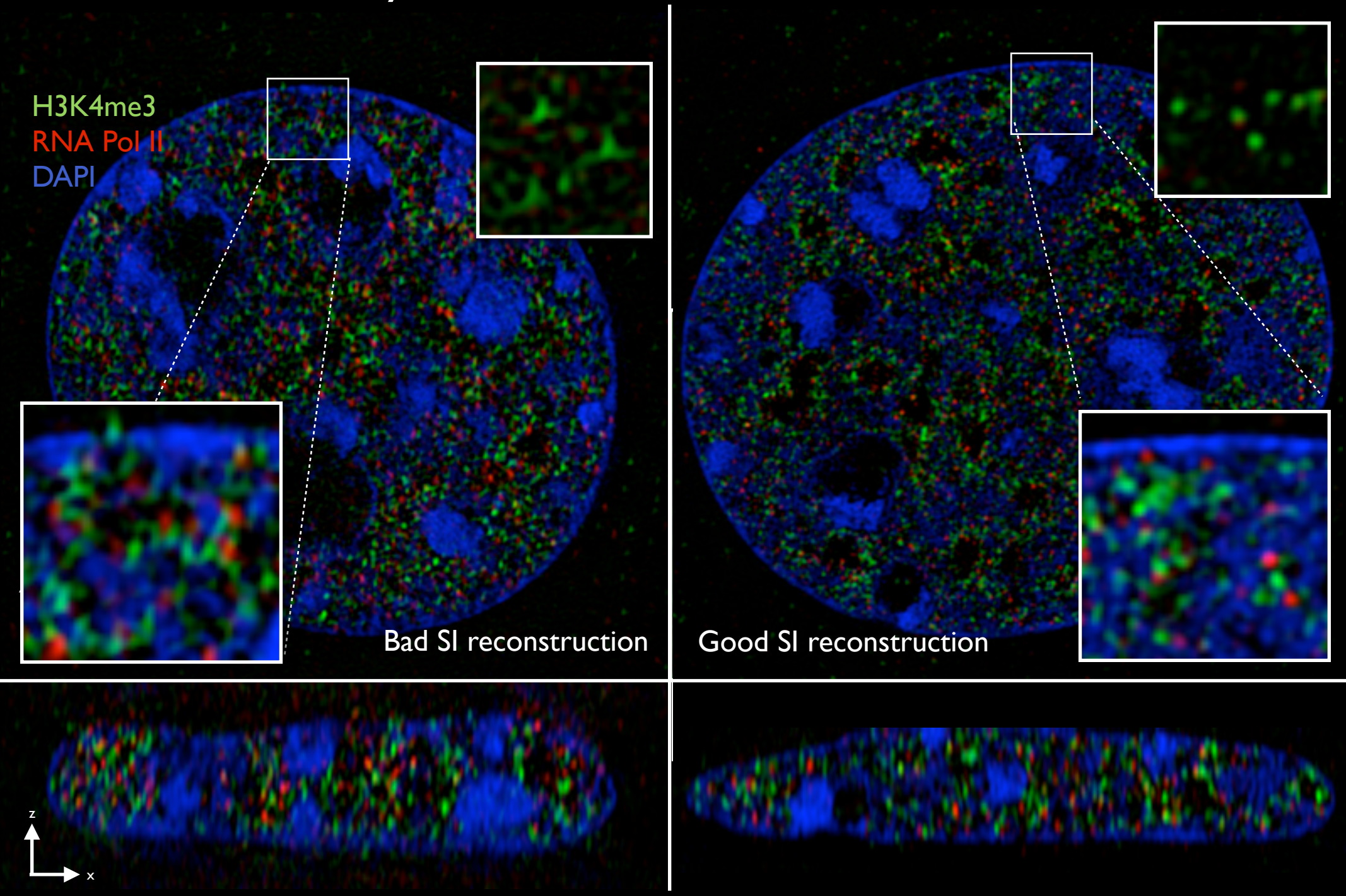


Bleaching,
Drift or vibrations
Moving particles
(locally constrained)

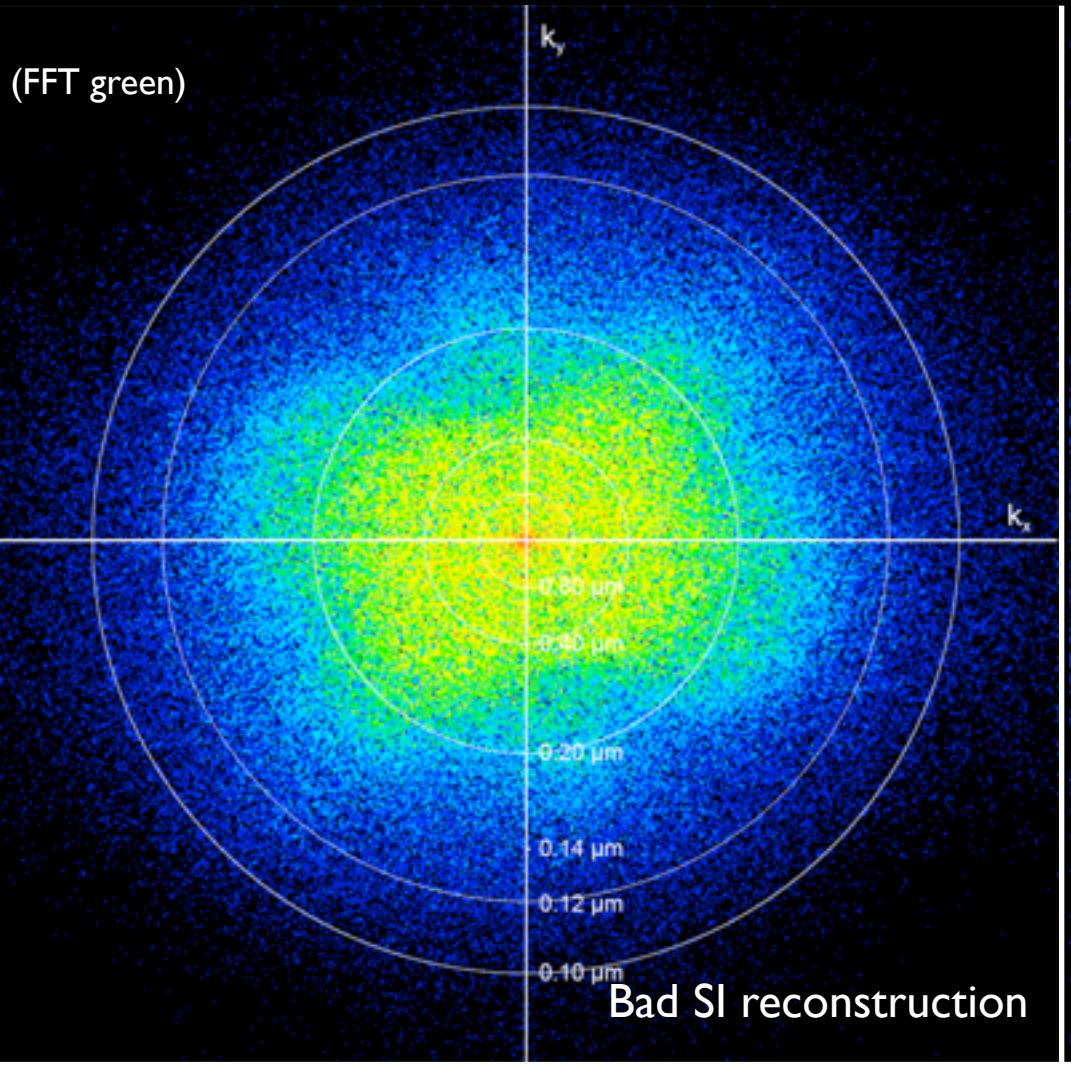
Low contrast-to-noise,
Low modulation contrast

Spherical aberration,
Refractive index mismatch

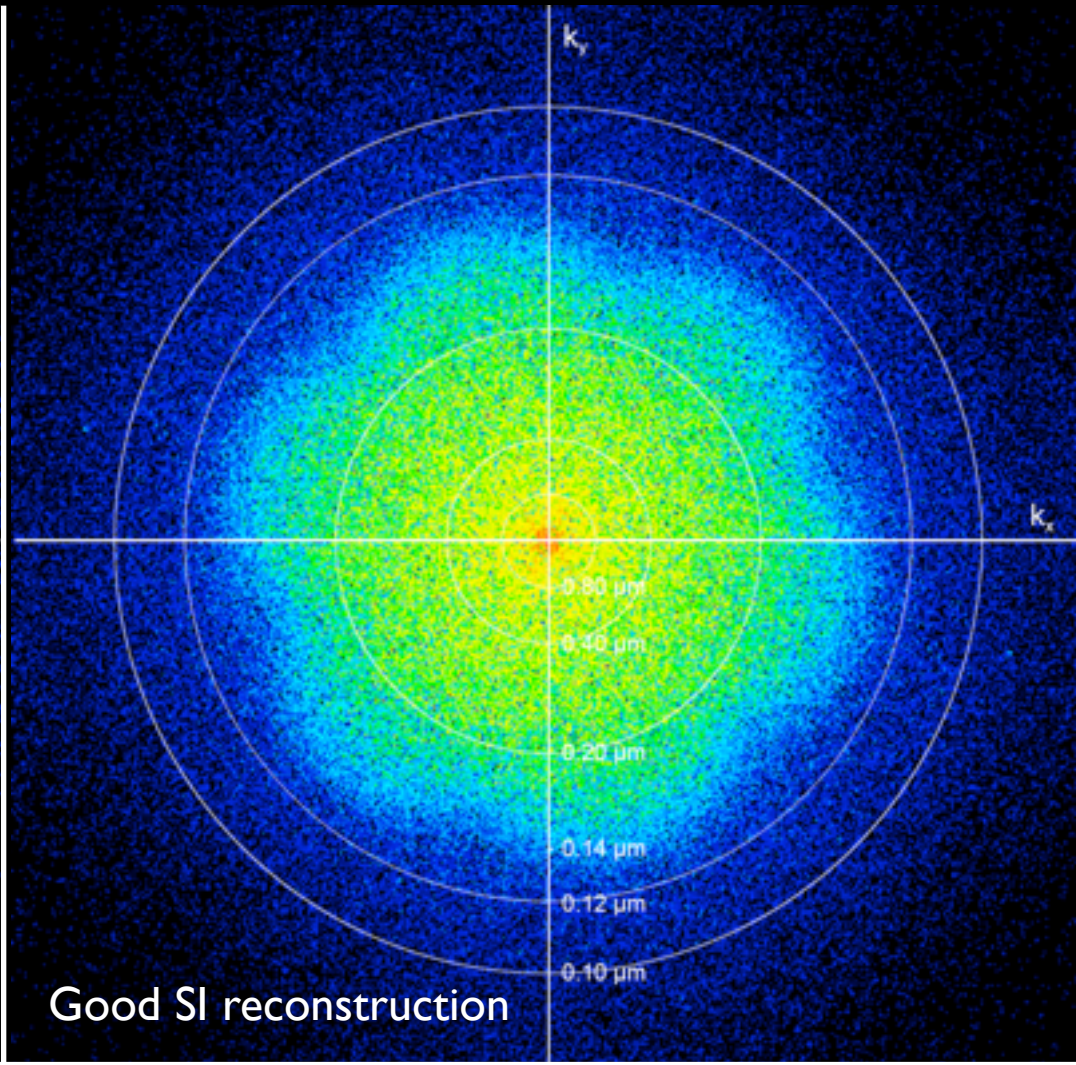
Quality control: SI-Reconstruction artifacts



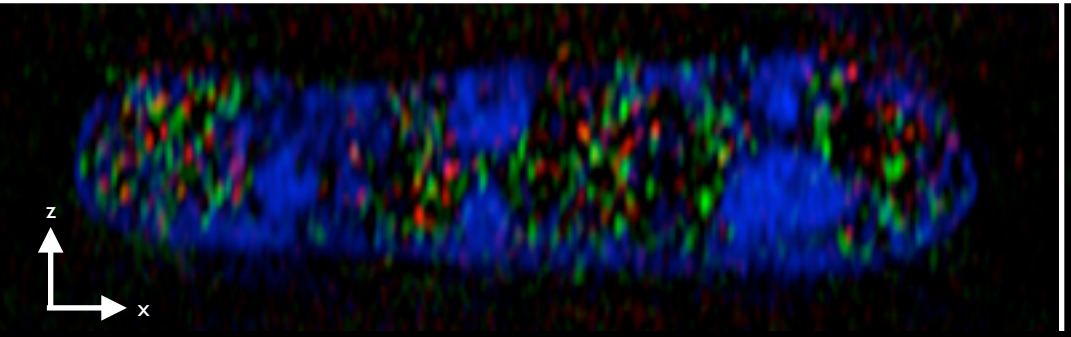
Quality control by Fourier analysis

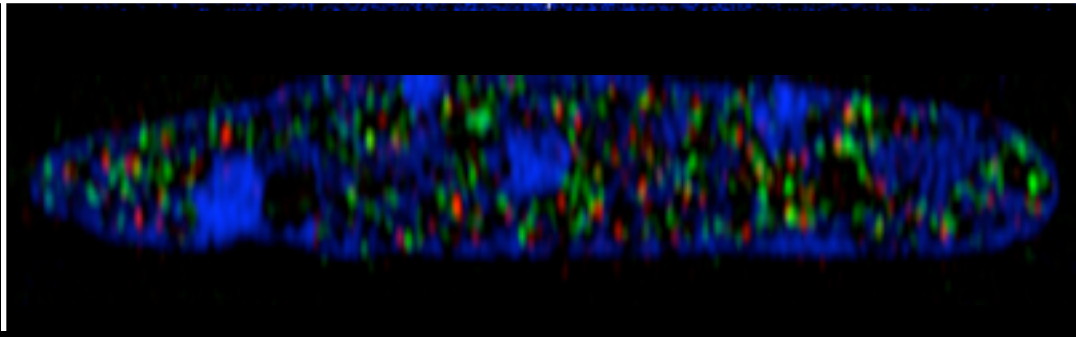


Bad SI reconstruction

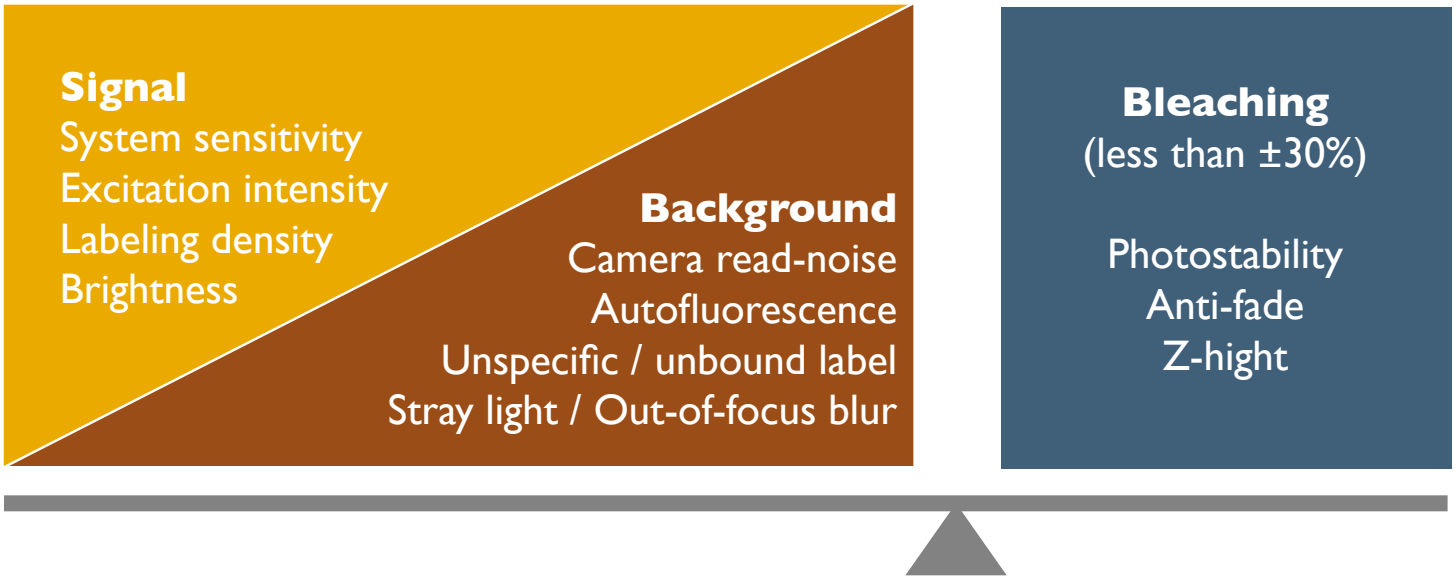


Good SI reconstruction

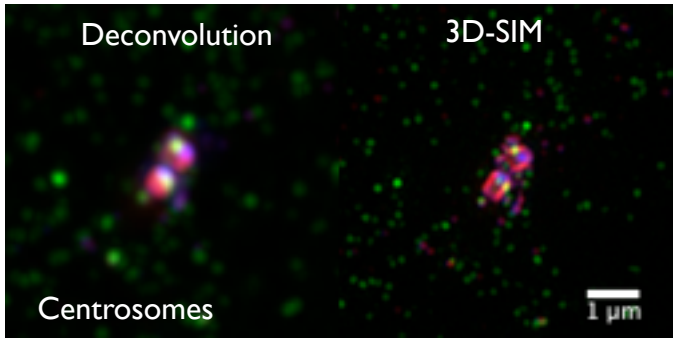




Balance between contrast and bleaching

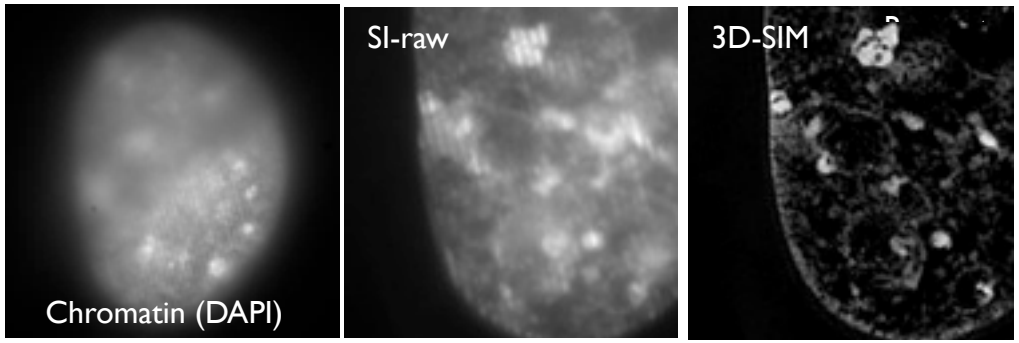


Discrete, isolated structures
Restricted z-hight, low background



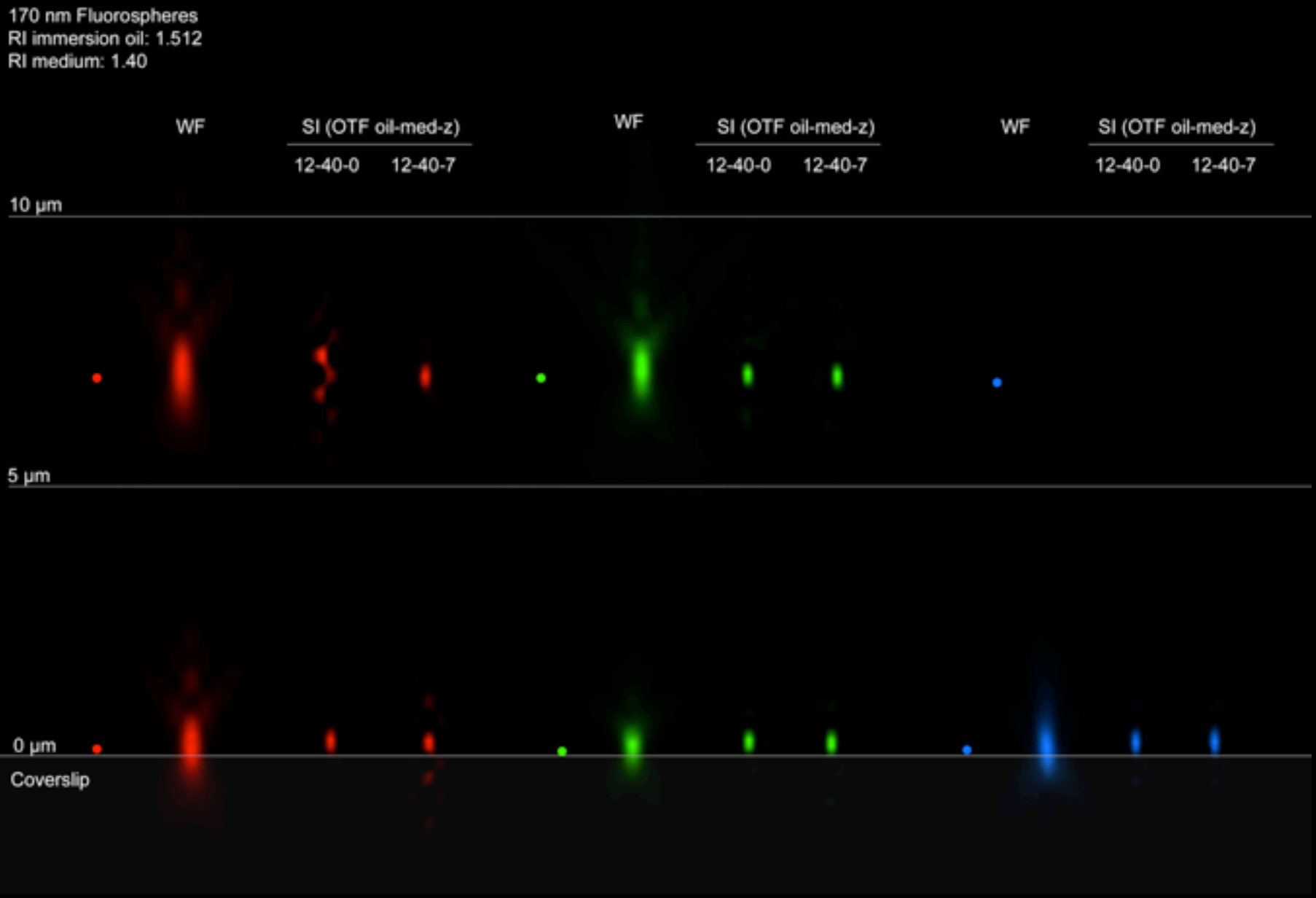
Tolerant to low intensities (>1.000 gray levels)
EM 5MHz (gain 3000)

Complex structures
Extended z-hight, out-of-focus blur contribution

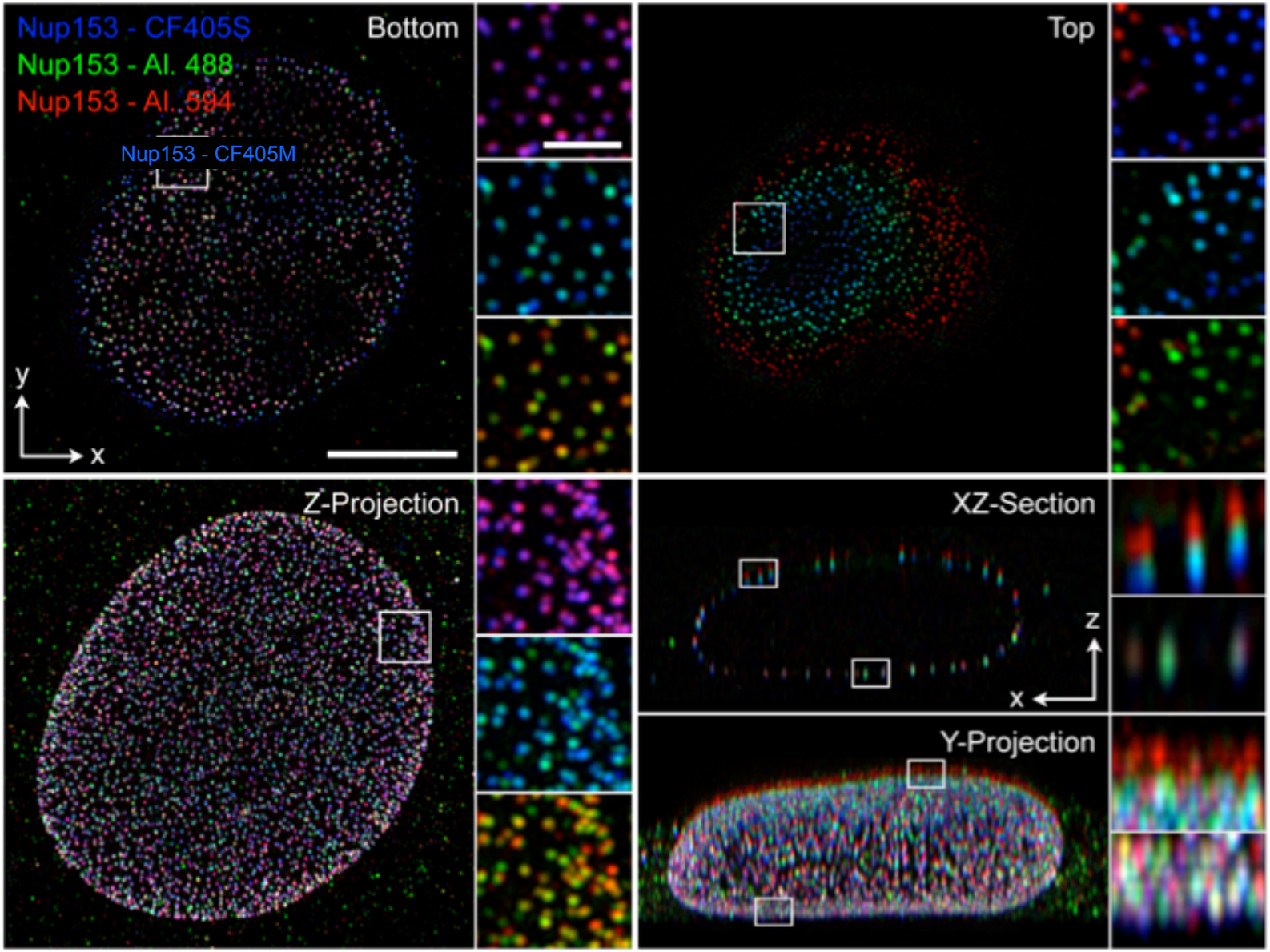


High intensities required (>12.000 gray levels)
Conventional 5MHz

Spherical aberration:
PSF changes with depth and wavelength



Sample specific channel alignment, chromatic aberration



- Biological 3D calibration sample to determine alignment parameter
- Adjust z-shift to optimally match in the center of the sample

Smeets et al., *Neuromethods,* in press

How to obtain the perfect 3D-SIM data? Quality is paramount

Labeling

Dyes (spectra, photostability)
Labeling method (FPs, IF, FISH,....)
Labeling specificity (antibodies)
Signal-to-noise / background

Sample

Optical quality (coverslip, cleaness)
Refractive index mismatch
Embedding medium, RI immersion
Imaging depth

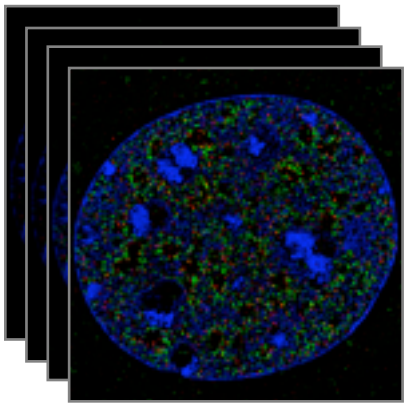
OMX Hardware

Mechanical stability
Photon efficiency
SI modulation contrast
Camera (CCD, EMCCD, sCMOS)

SI Postprocessing

PSF/OTF (λ-, depth-, RI-dependent)
Channel alignment
(...)

Dataset
x, y, z, λ,(t)



Statistical analysis

Quality control
Colocalization (?)
Segmentation
Distance measurements
(...)

3D-SIM (pros & cons)

+ up to 4 colors, standard dyes (e.g., Alexa, GFP...)

+ 3D with 2x resolution in XY and Z (8x volumetric)

+ Optical sectioning over larger volumes (10 µm in z)

+ Sensitive (EMCCD/sCMOS) and fast (OMX Blaze)
  → live cell imaging

o Only moderate xy-resolution improvment

- Mathematical reconstruction → artifact proness

- High requirements on sample quality and system calibration

C2C12 cell, 3D-SIM section, DAPI, RNA PolII, H3K4me3

STED (pros & cons)

+ What you see is what you get → no math required!
+ High xy-resolution (50-70 nm, Leica TCS STED)
+ > 20 µm depth

o Maximum 2 colors (no UV!)
o Special dyes required for optimal performance
o Speed scales with size → full frame rather slow!
o PMT/APD detectors less sensitive

- Only confocal axial resolution (600-800 nm)
- Relativ high energy load → photodamage
- Not ideal for 3D and live cell imaging
- Complex instrumentation, price tag

Localization Microscopy (pros & cons)

+ High localization precision (± 20 nm)

+ Quantification of single molecules
  (e.g., cluster analysis, single particle tracking)

+ Instrumentation relatively simple

o Localization precision ≠ structural resolution
  (± 50 nm, dependent on labeling density)

o Only single plane, best with TIRF, fxed samples

o Dye/embedding restrictions (photophysics)

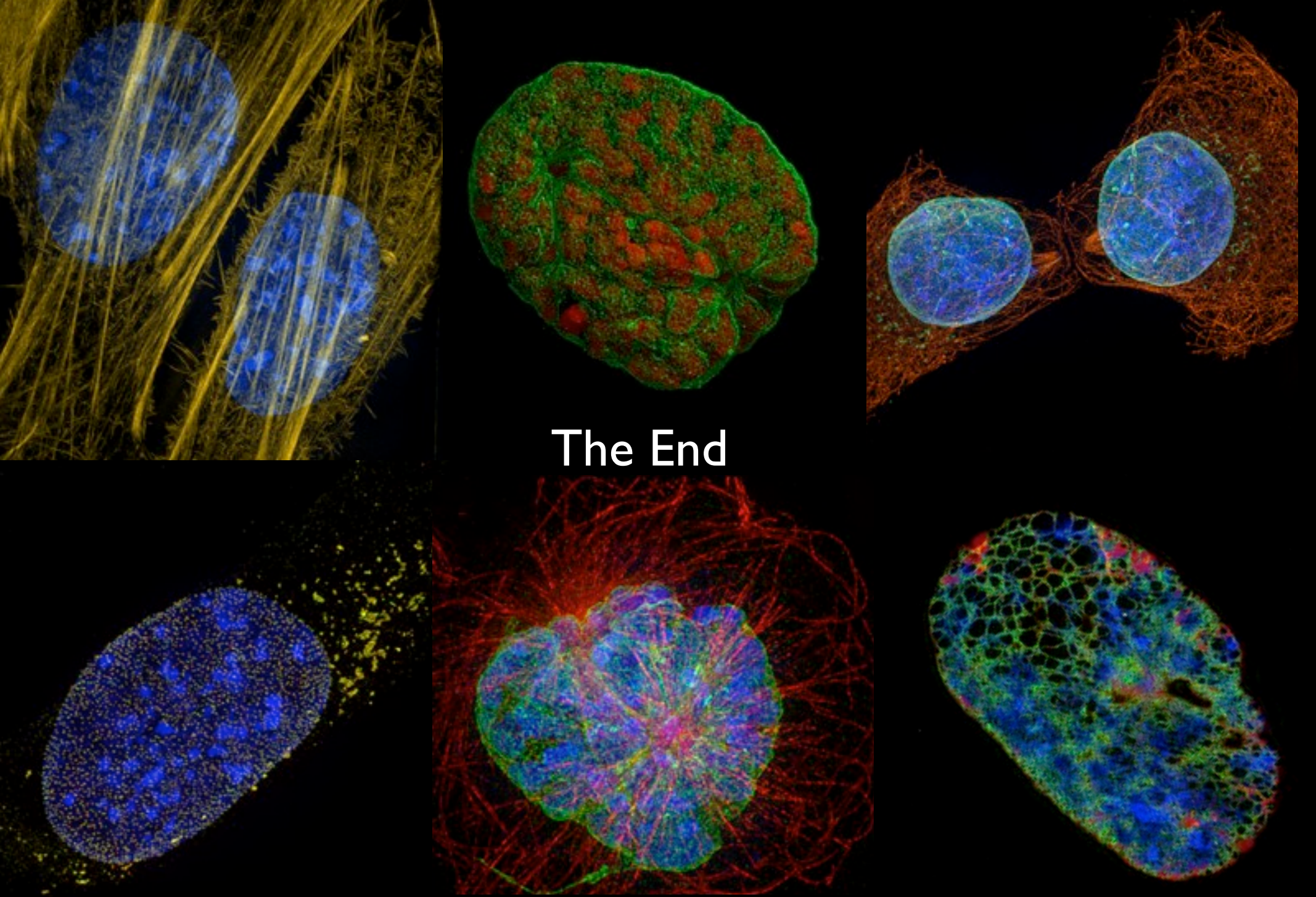
- Slow

- Not suited for z-extended 3D structures

Super-resolution microscopy comes with costs!

Photon budget

The best SR-technique will be determined by the demands of the application!



The End

Credits to Jürgen Neumann, Pete Carlton and Timo Zimmermann for sharing slides