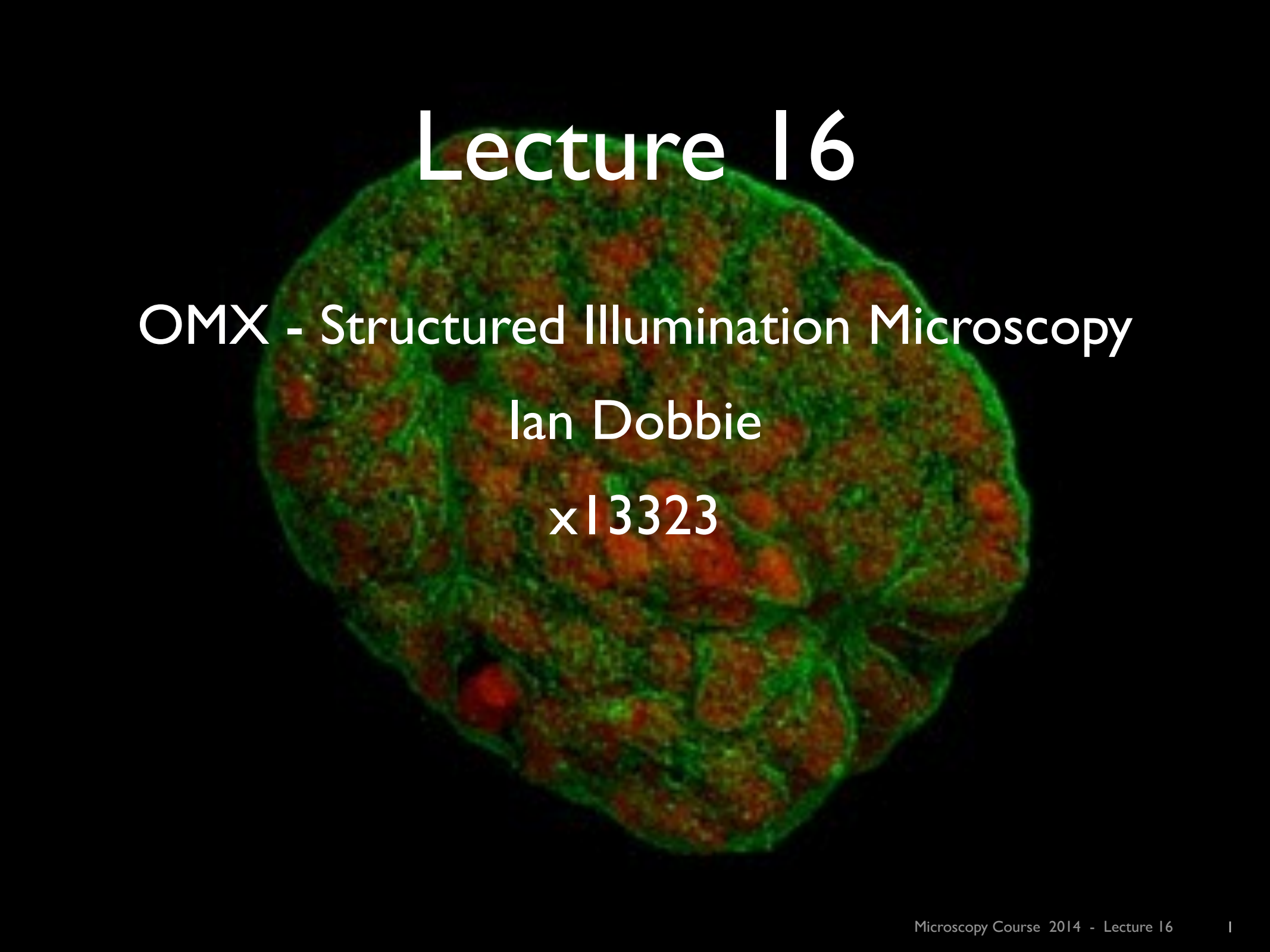
Lecture 16

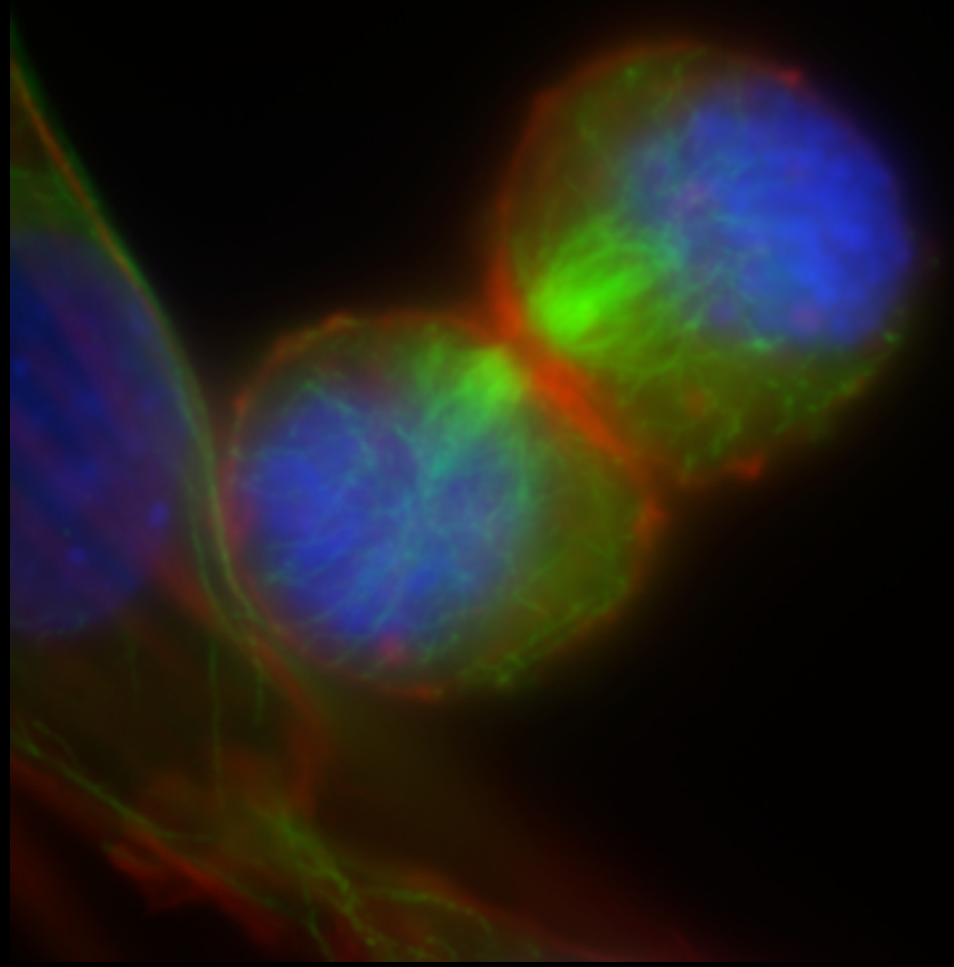
OMX - Structured Illumination Microscopy

Ian Dobbie

x13323



Super-resolution fluorescence microscopy



Typical widefield image...

- Specificity
- Sensitivity
- Non-invasive (*in situ* & *in vivo*)
- Multi-dimension (x, y, z, λ, t,...)
- Relative localisation & dynamics
- "Single cell" to "high throughput"

Spatial resolution is diffraction limited!

Magnification alone does not give more details!

...warmup:

"What determines the resolution of an optical microscope ?"

1	2	3
		
63x/1.25	100x/1.25	63x/1.4
£ 3 618.00	£ 550.00	£ 5 055.00

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

„... a bit more difficult...?“

1	2	3
		
25x/1.05	40x/1.0	40x/1.1
£ 12,800	£ 3,004	£ 8,816

What's the difference in brightness ?

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

Numerical aperture determines ...

Brightness	$F_{epi} = 10^4 \, NA^4 / Mag^2$	(epifluorescence)
Lateral Resolution	$d_{x,y} = 0.61 \, \lambda / NA$	(200-300 nm)
Axial Resolution	$d_z = 2 \, \lambda / NA^2$	(500-700 nm)

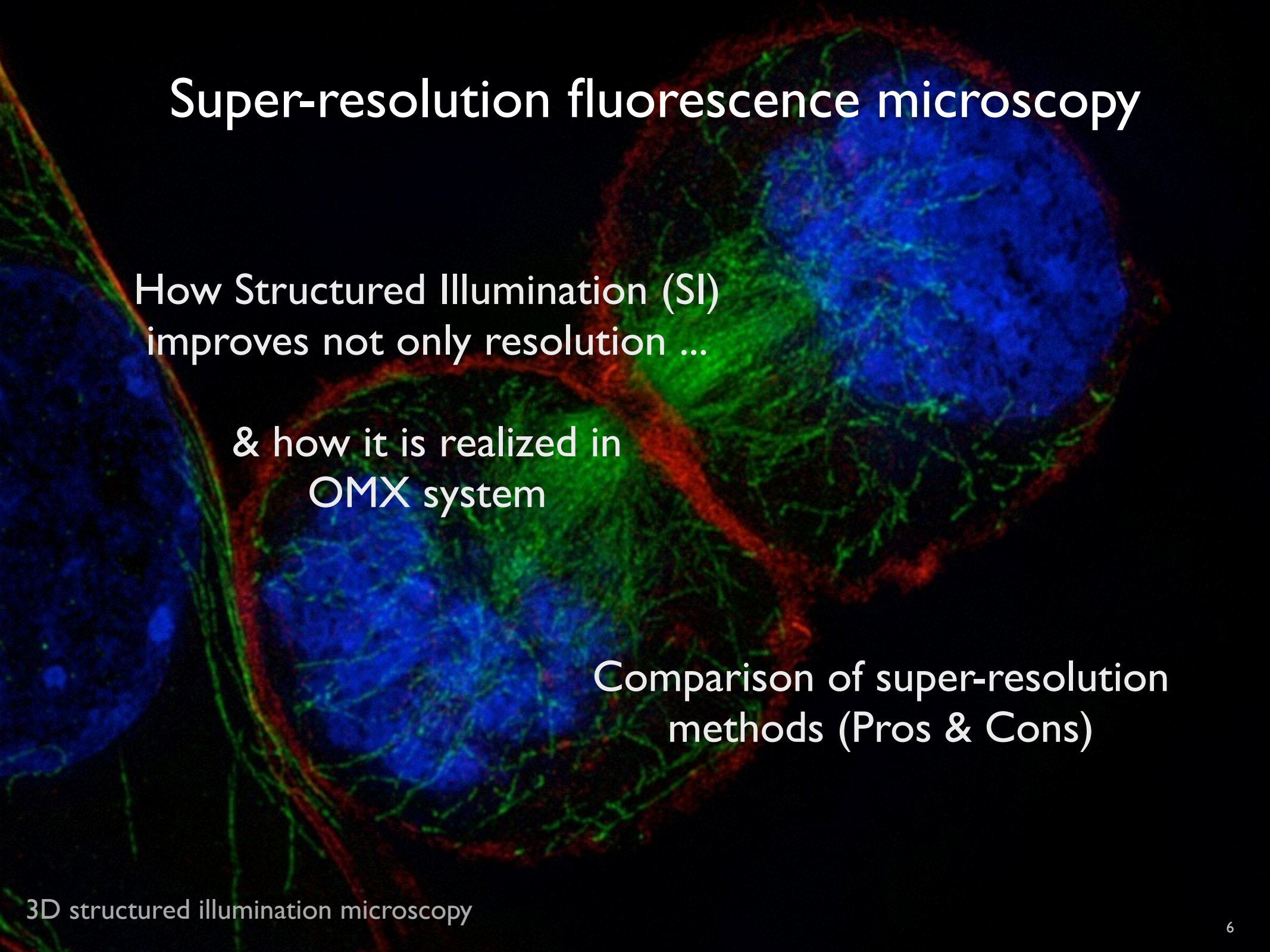
Only applies under optimal conditions! BUT ...

spherical aberrations (refractive index mismatch, sample)
chromatic aberrations
stray light
out-of-focus blur
detector noise

...
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

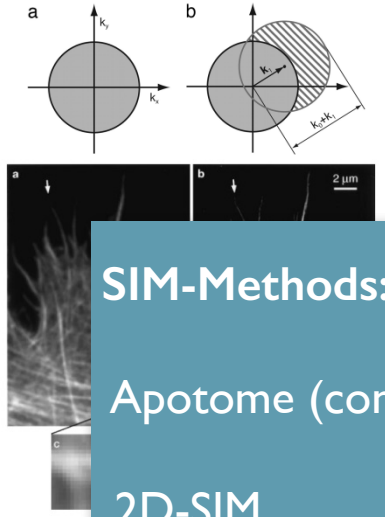
Comparison of super-resolution methods (Pros & Cons)

Superresolution microscopy - three major concepts

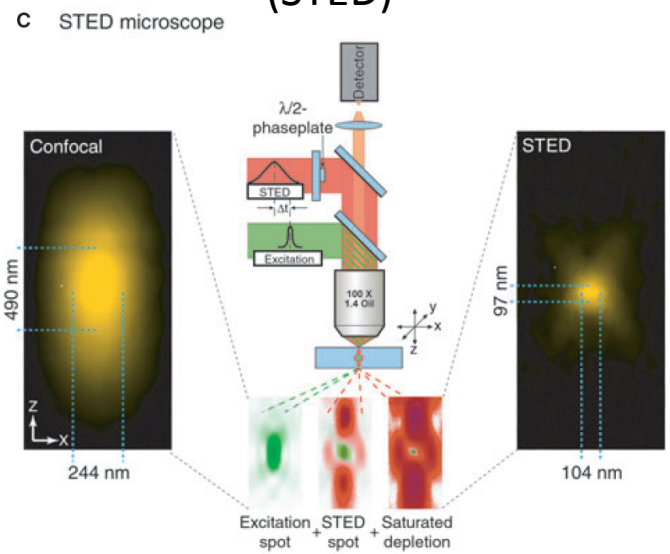
Super-resolution light microscopy:
Imaging beyond Abbe's diffraction limit

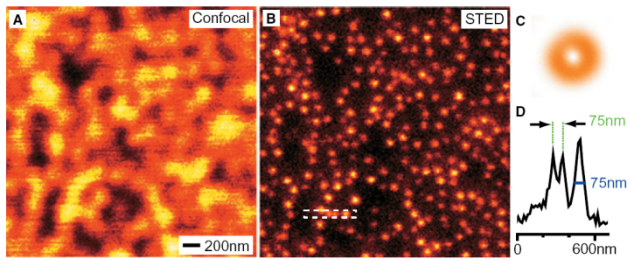
$$\Delta x, \Delta y = \frac{\lambda}{2n \sin \alpha}$$

Structured illumination



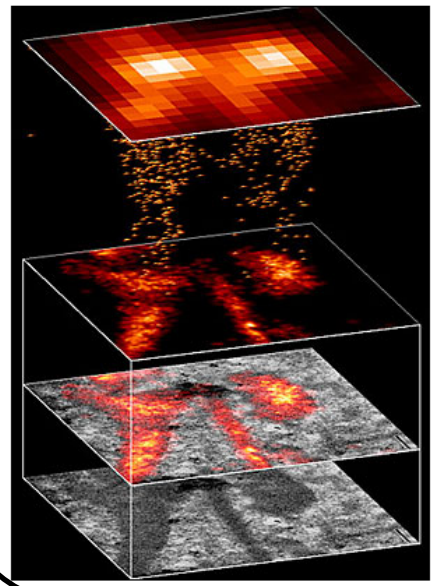
Stimulated emission depletion (STED)

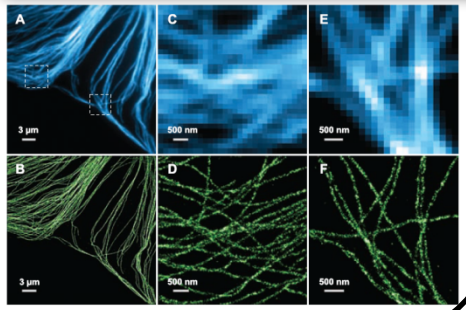




Localization

Photoactivation localization microscopy (PALM)





SIM-Methods:

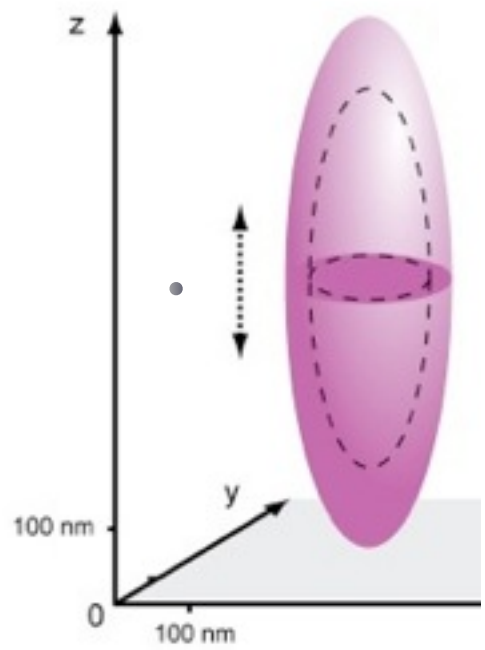
Apotome (conventional SIM)

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

SSIM (non-linear SIM)
NL-SIM

Resolving power of commercial super-resolution systems

	CLSM
~λem [nm]	460-670

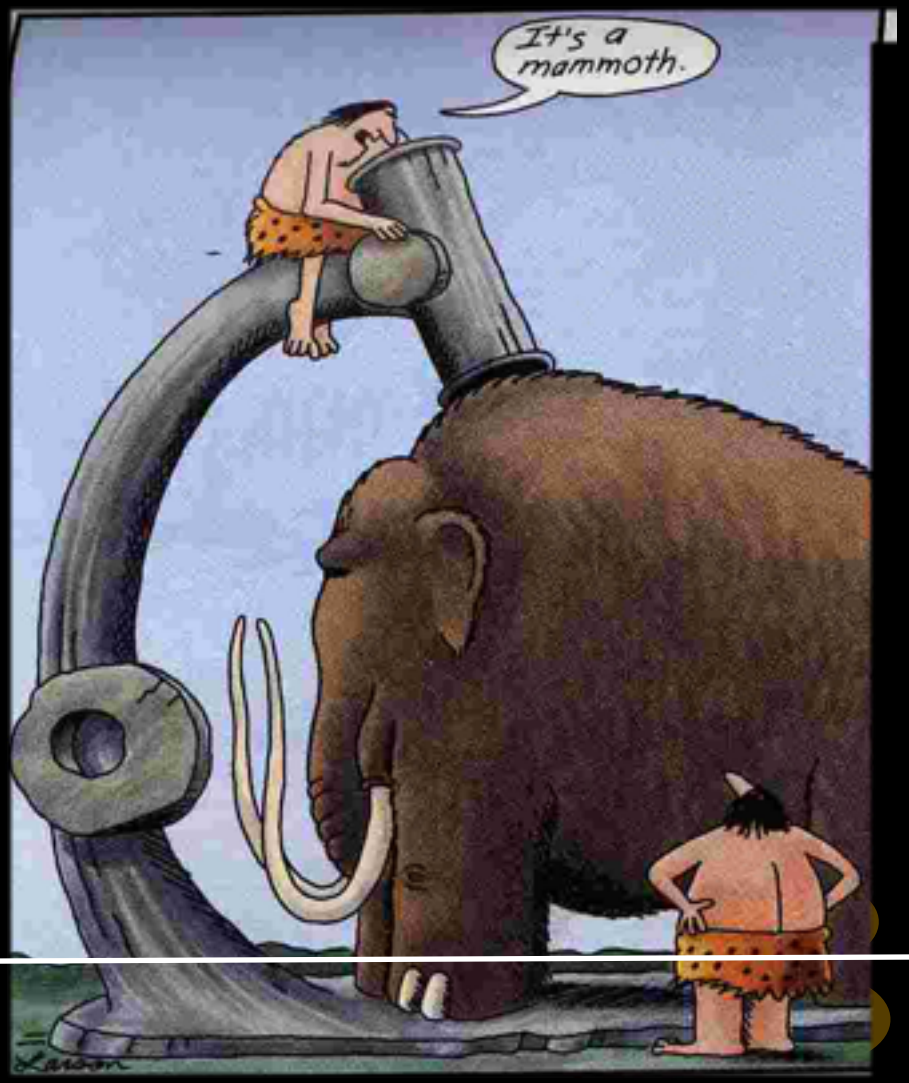


~$D_{x,y}$ [nm]	180-250
~D_z [nm]	500-700
~$V_{x,y,z}$ [*10^{-3} µm^3]	10-23

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

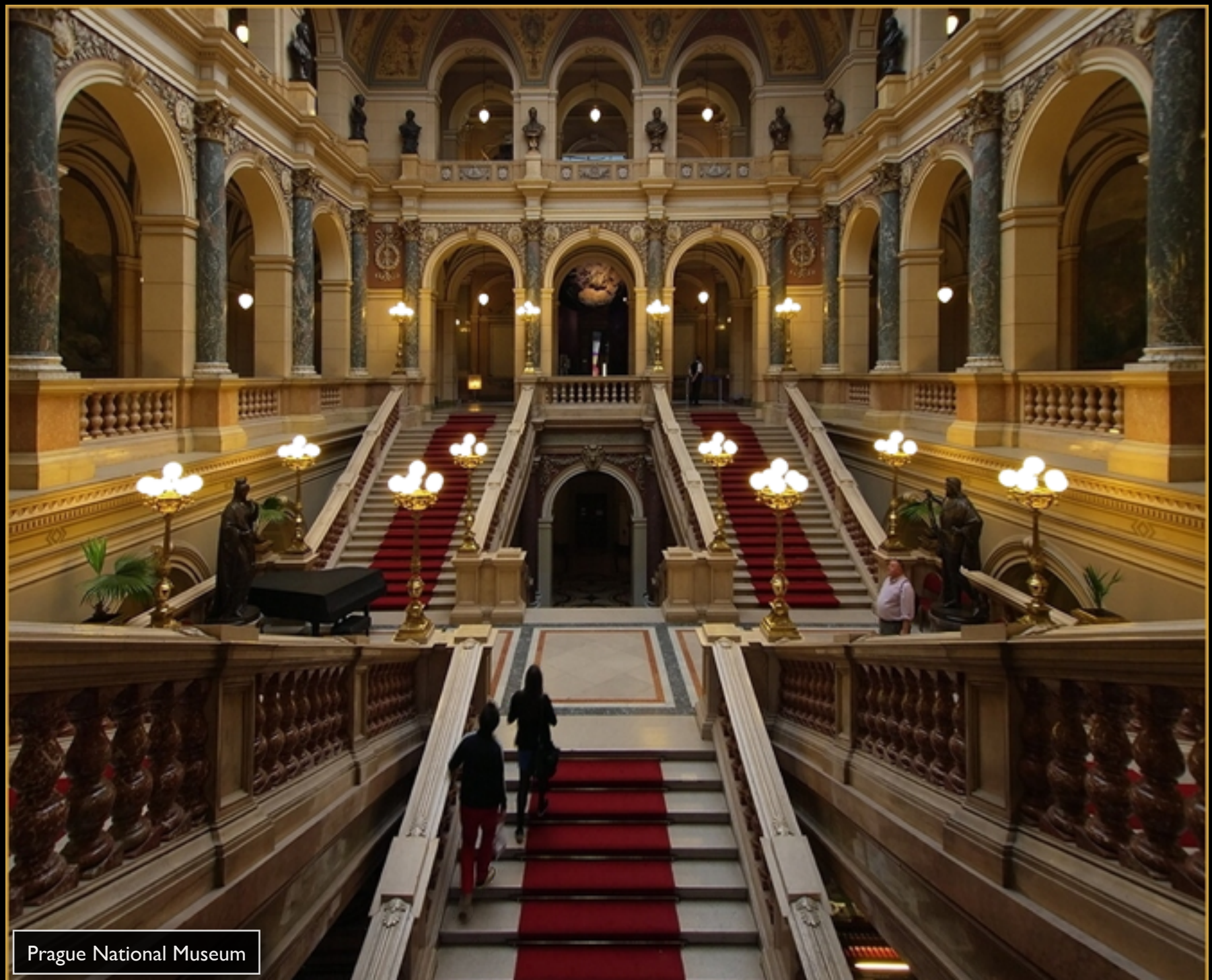
Not only resolution matters, ...

What could this be?

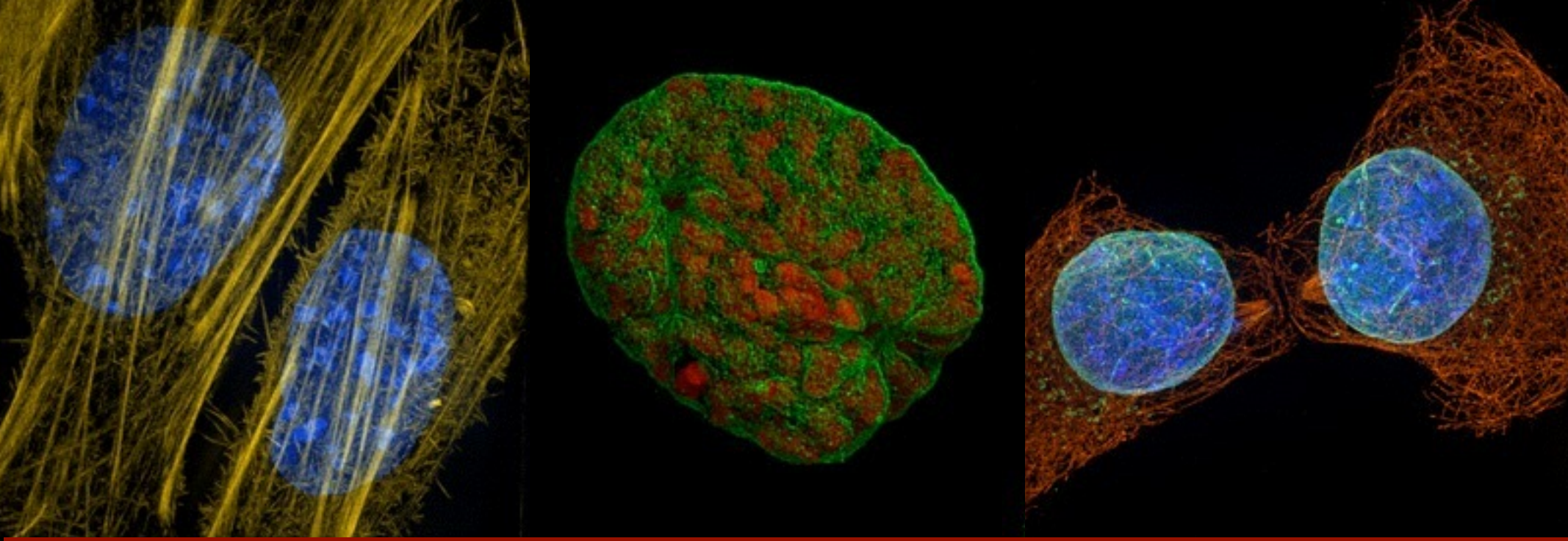


3D information (z-res., optical sectioning, z-depth) !

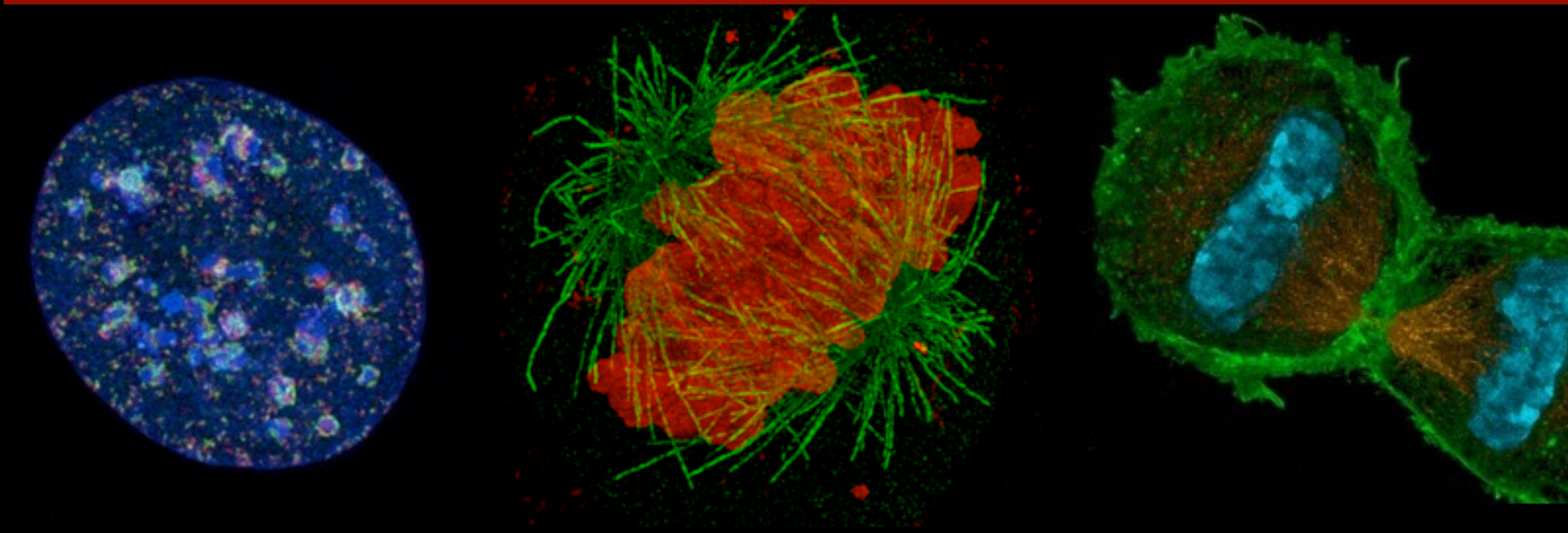
Not only resolution matters, but also context (II)



Prague National Museum



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

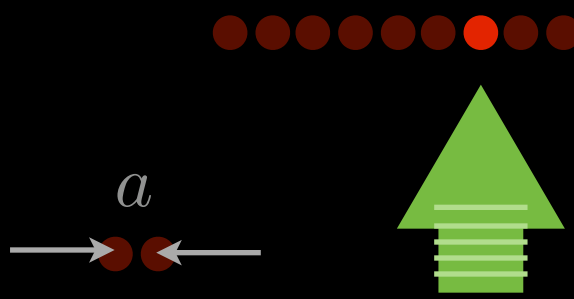


How does it work?

The basic principle: Abbe's view

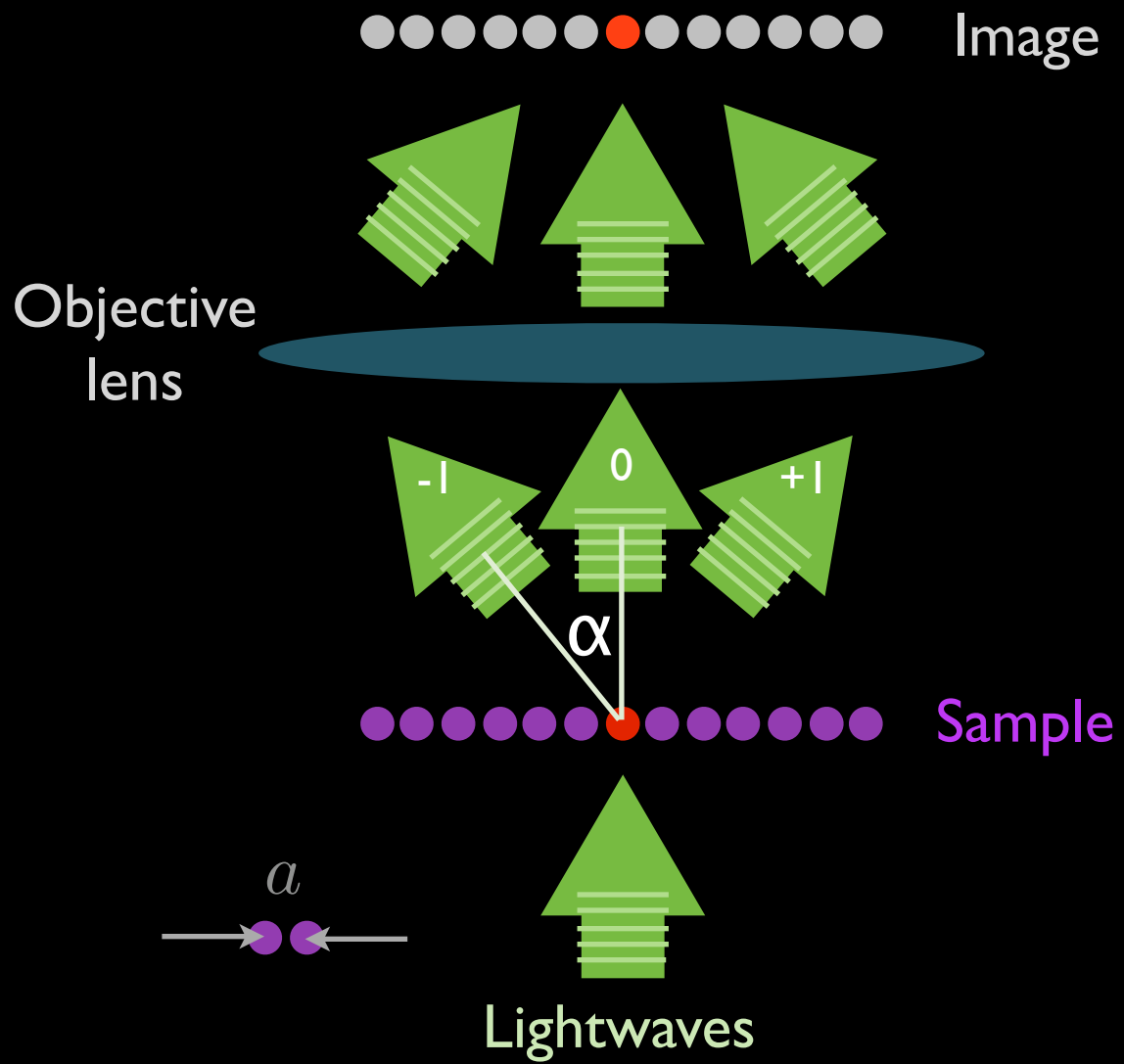
Sample = Structure

Periodicity

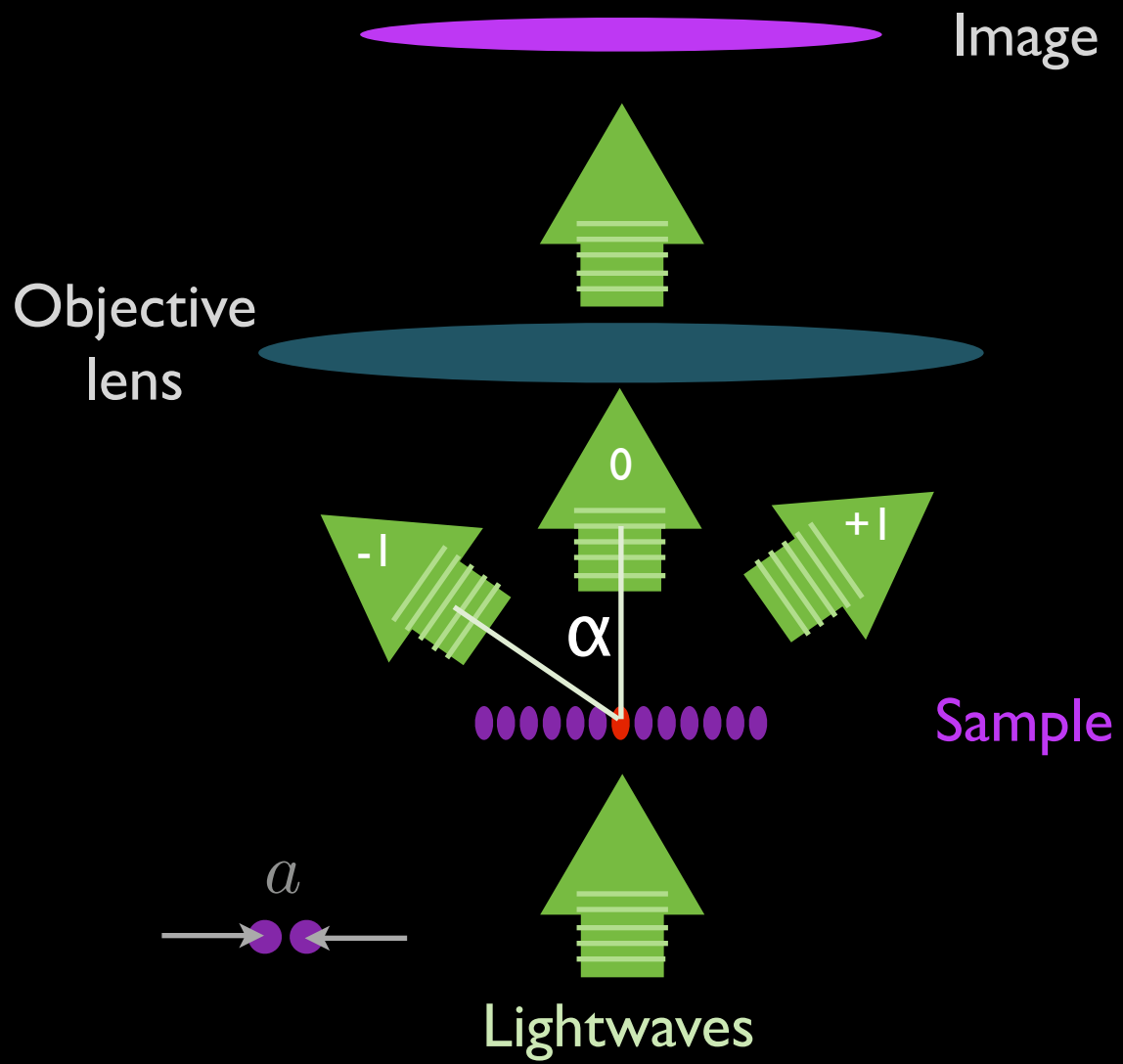


http://de.wikipedia.org/wiki/Ernst_Abbe

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)

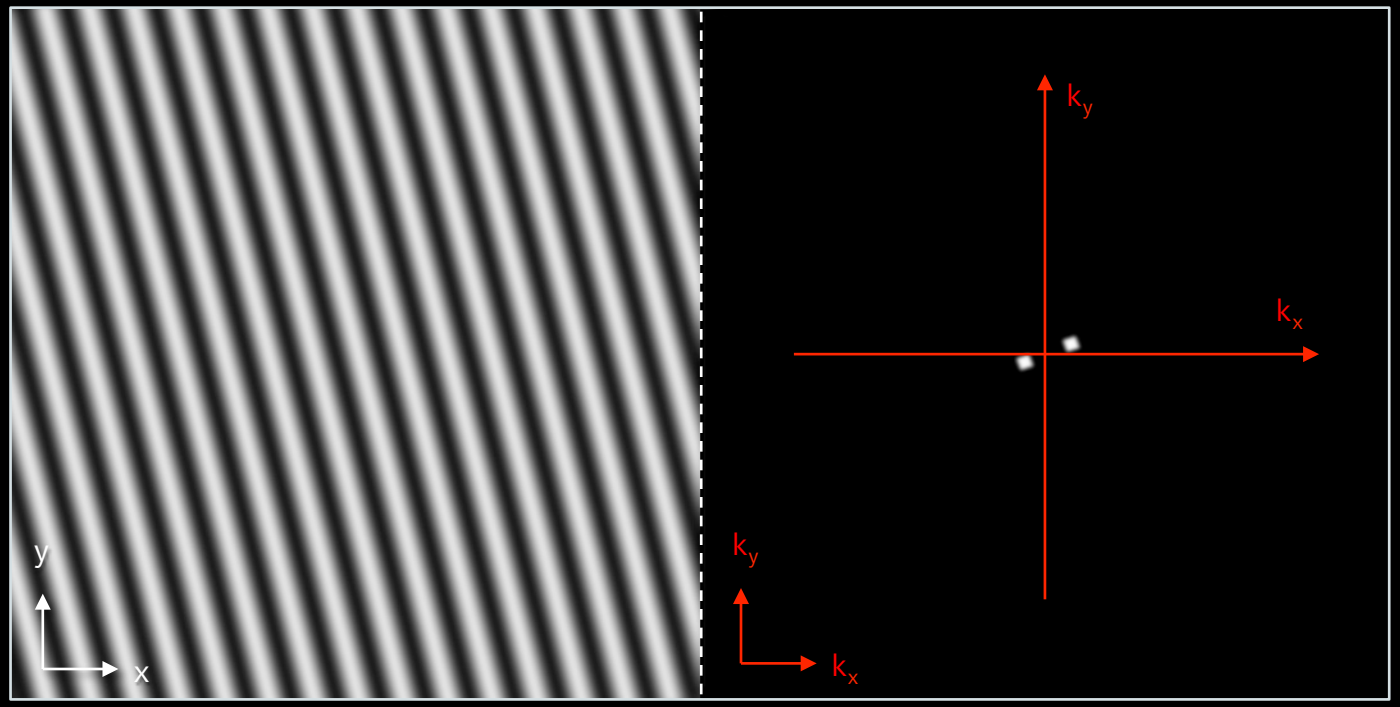


Image = superposed periodicities

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

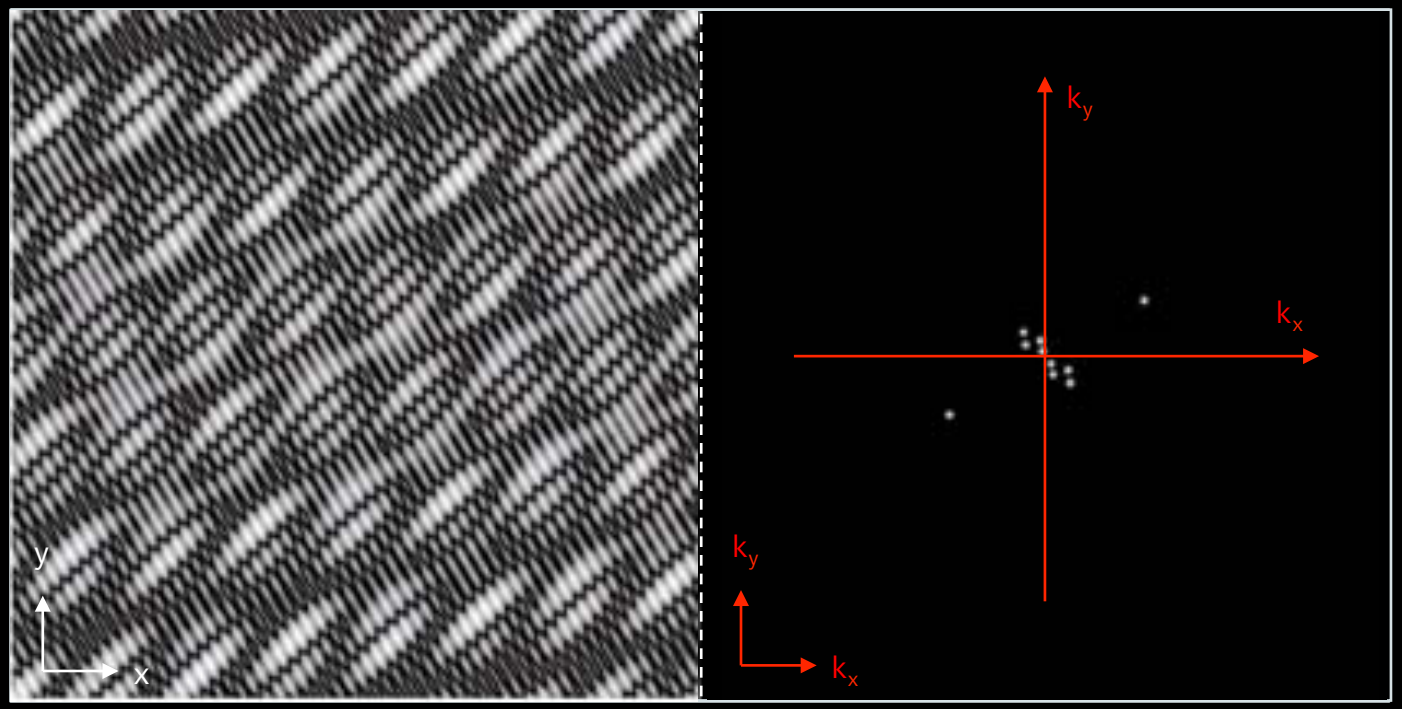


Image = superposed periodicities

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

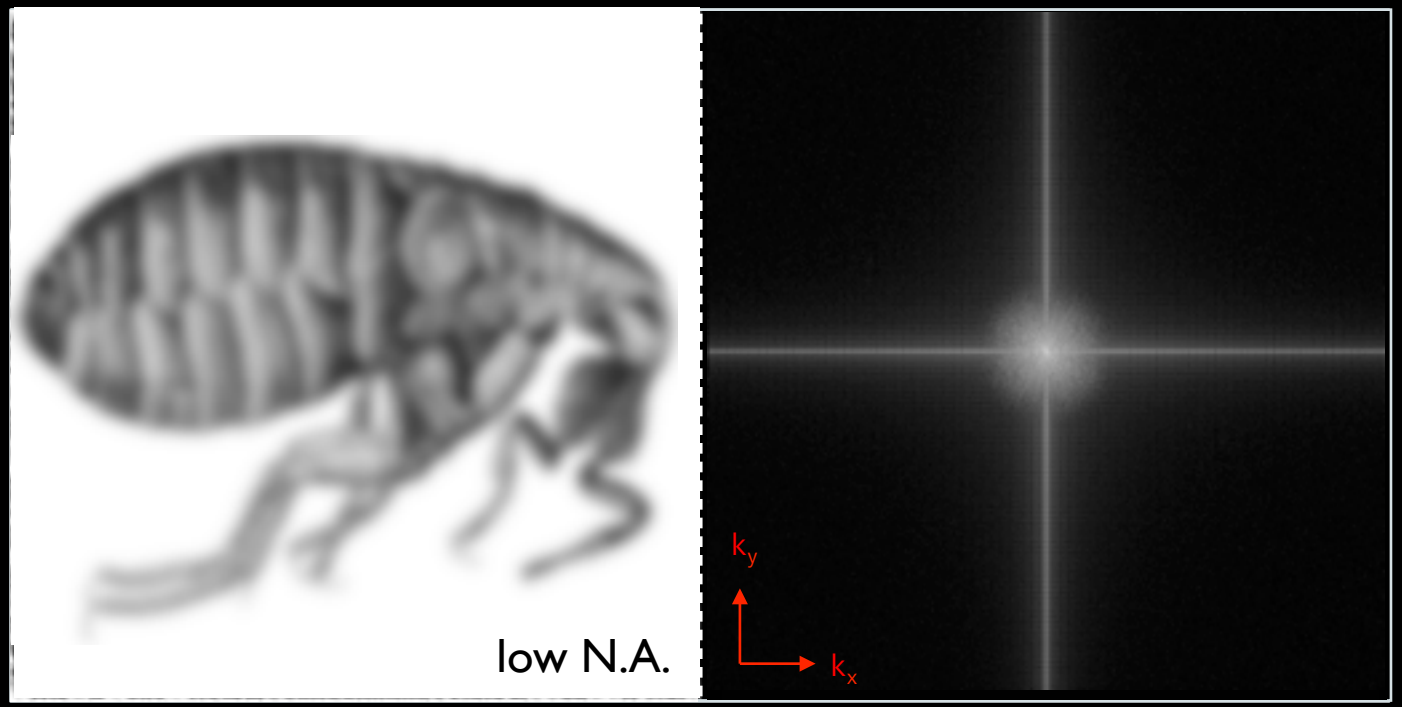
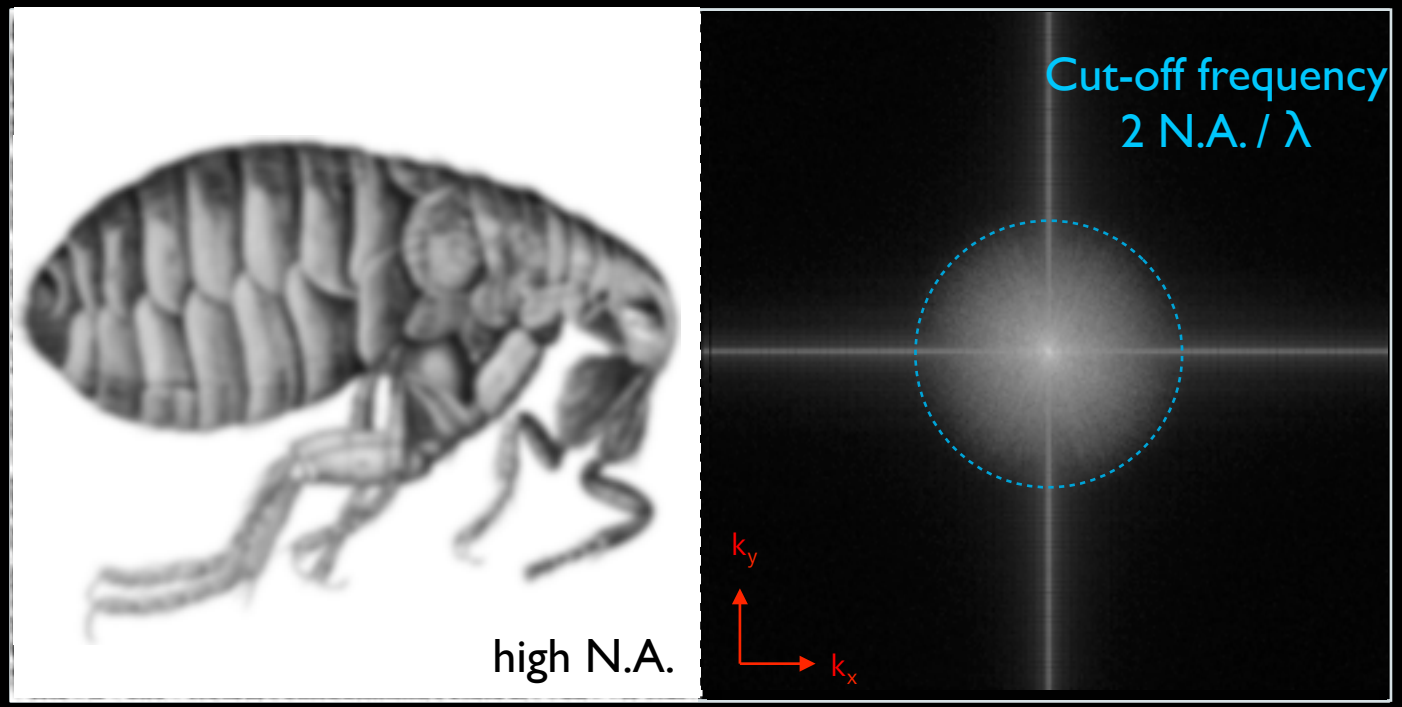


Image = superposed periodicities

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



SIM principle - super-resolution by Moiré interference

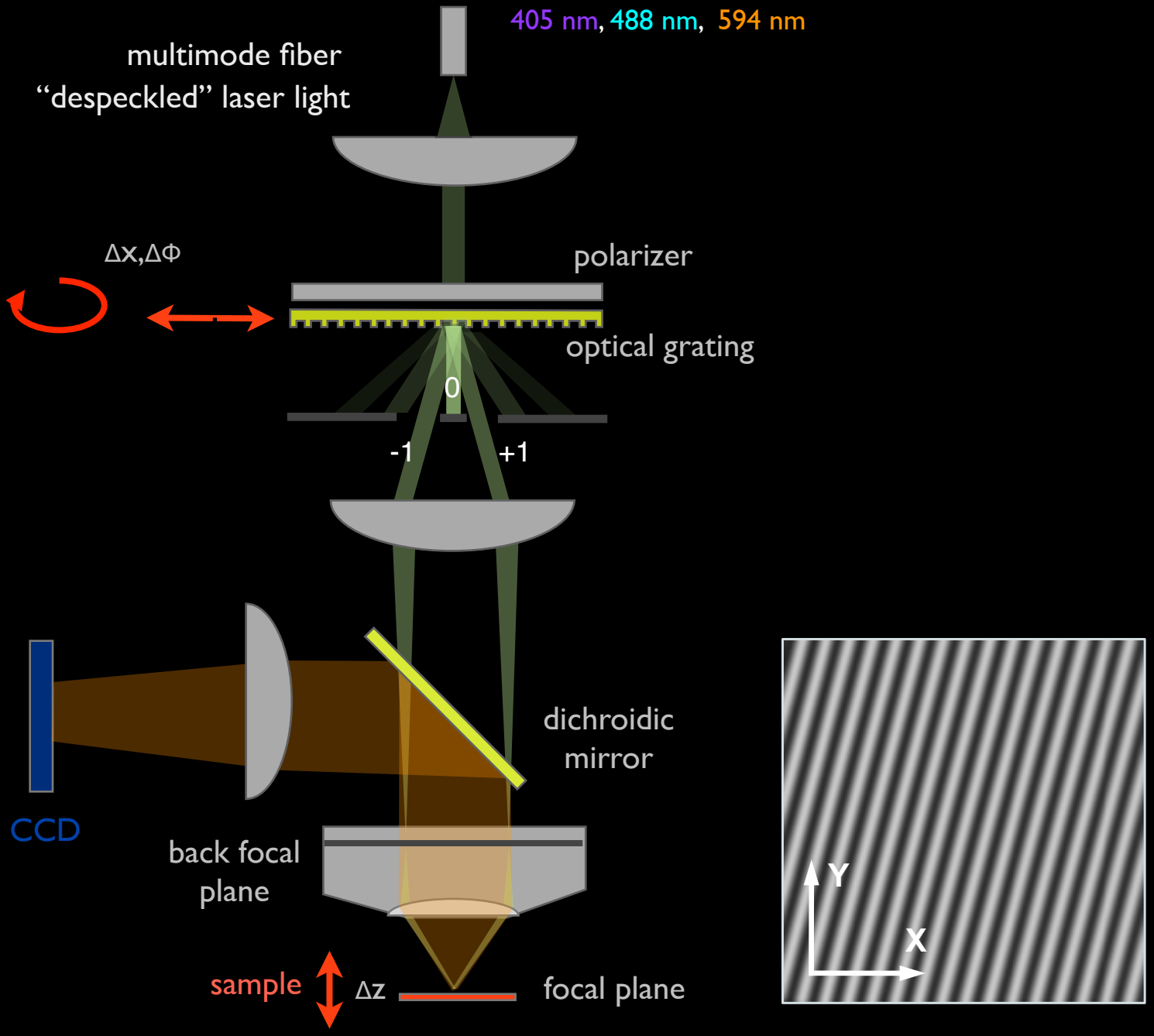
unknown structure

Fourier transform of the measured image

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

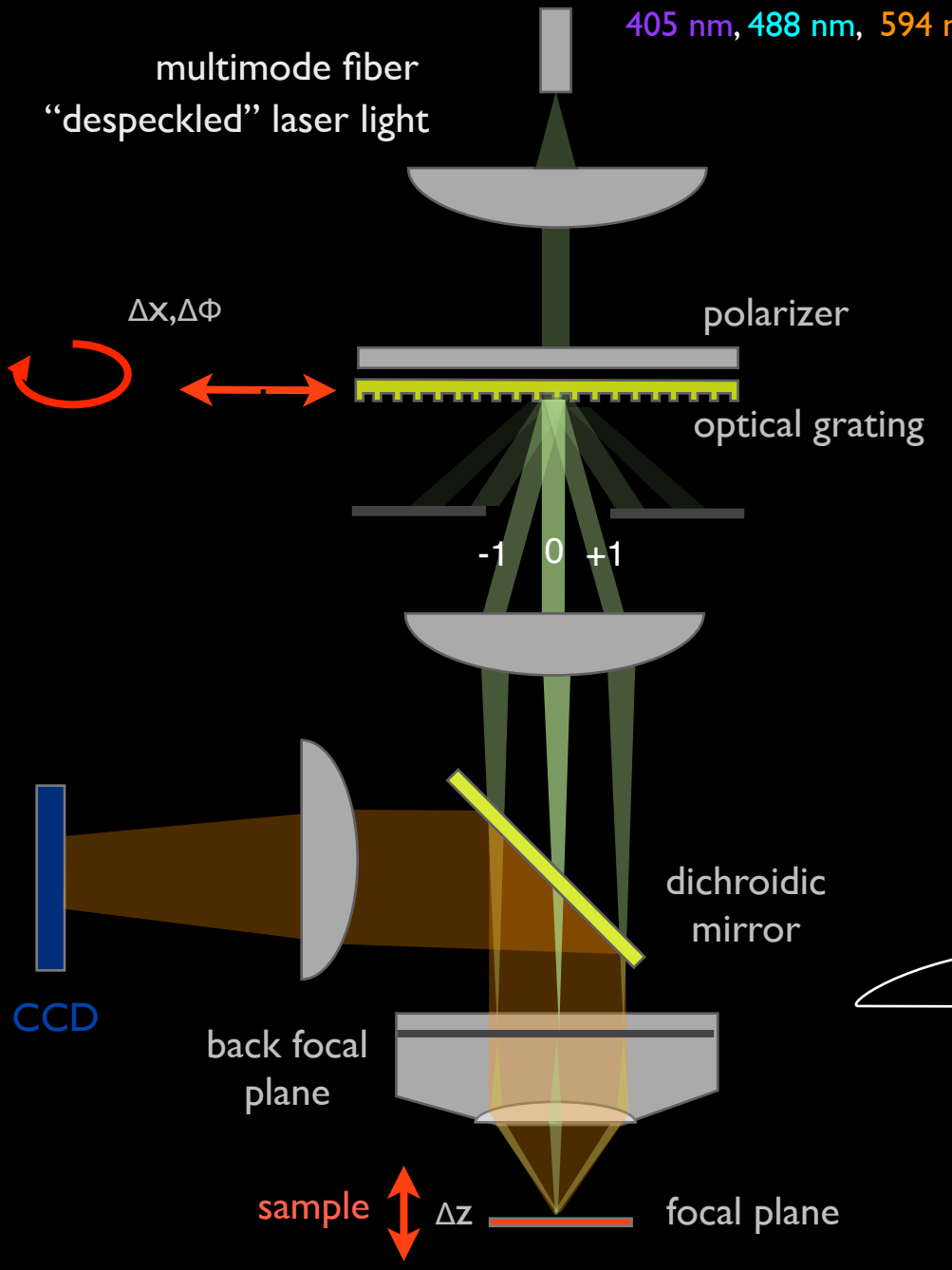
known illumination function

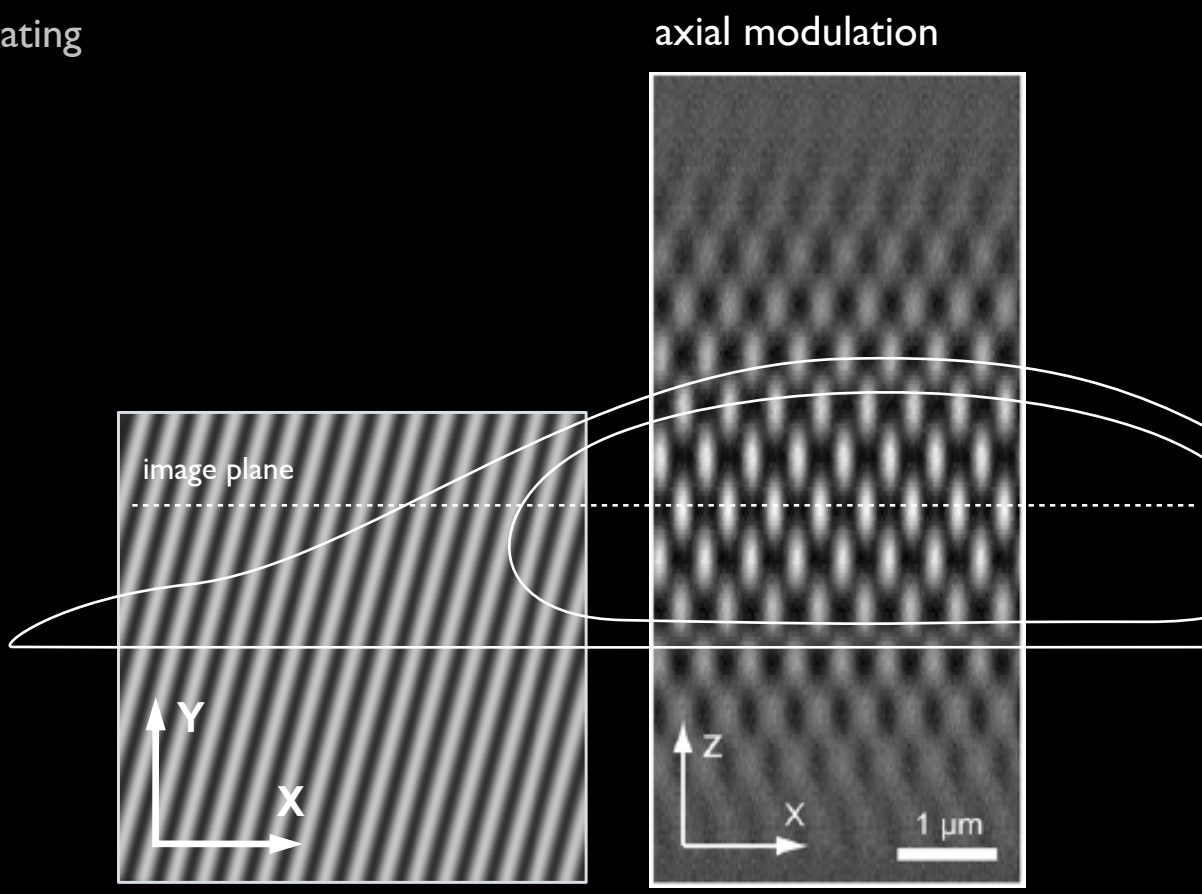
Generating 2D-structured illumination



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

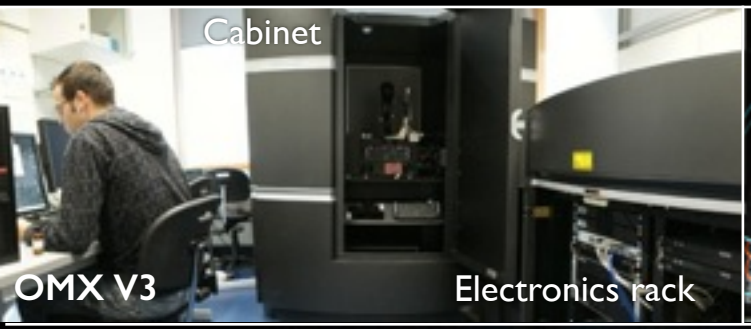
Generating 3D-structured illumination

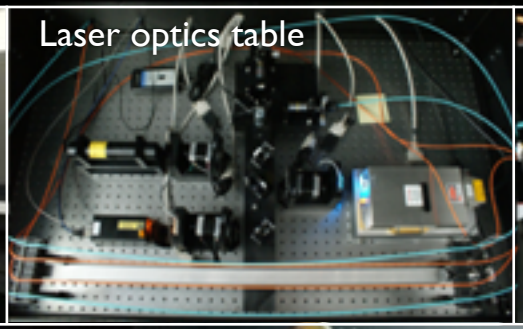


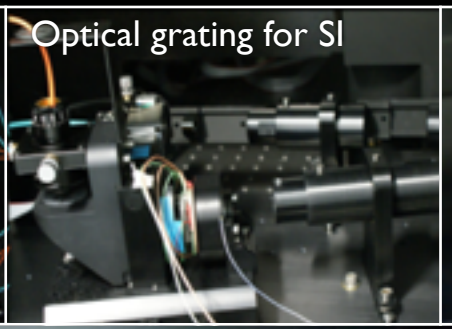


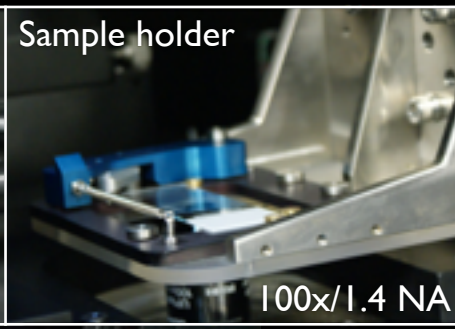
adapted from Gustafsson et al. (2008) *BiophysJ*, 94

OMX 3D-SIM microscope system









Max. mech. stability
Highest sensitivity

High-sensitive
EMCCD camera
(3x)

Fixed filter drawer

Mess

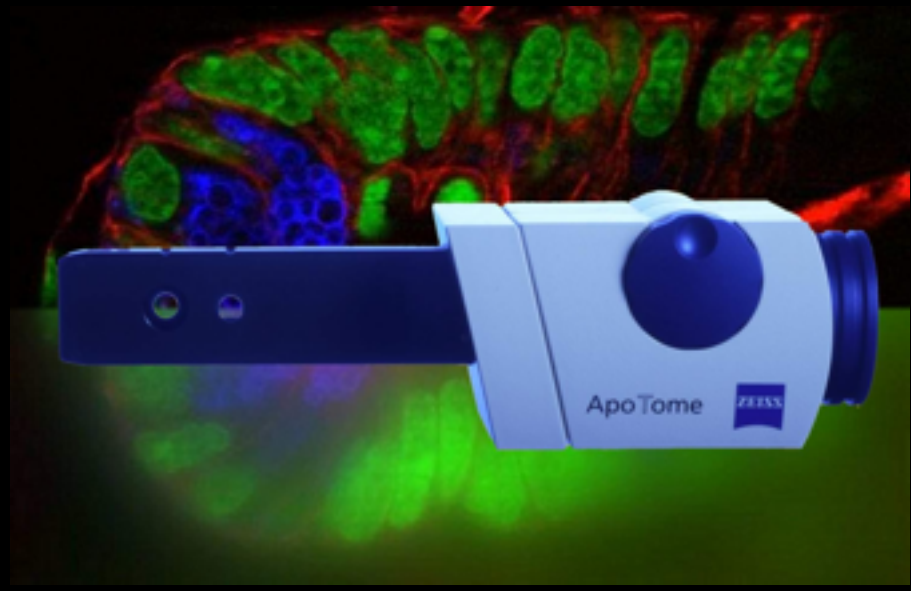
OMX V2

3D-SIM in practice

Raw SI data

Actin (Phaloidin-Alexa488) in Drosophila macrophage (Eva Wegel)

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

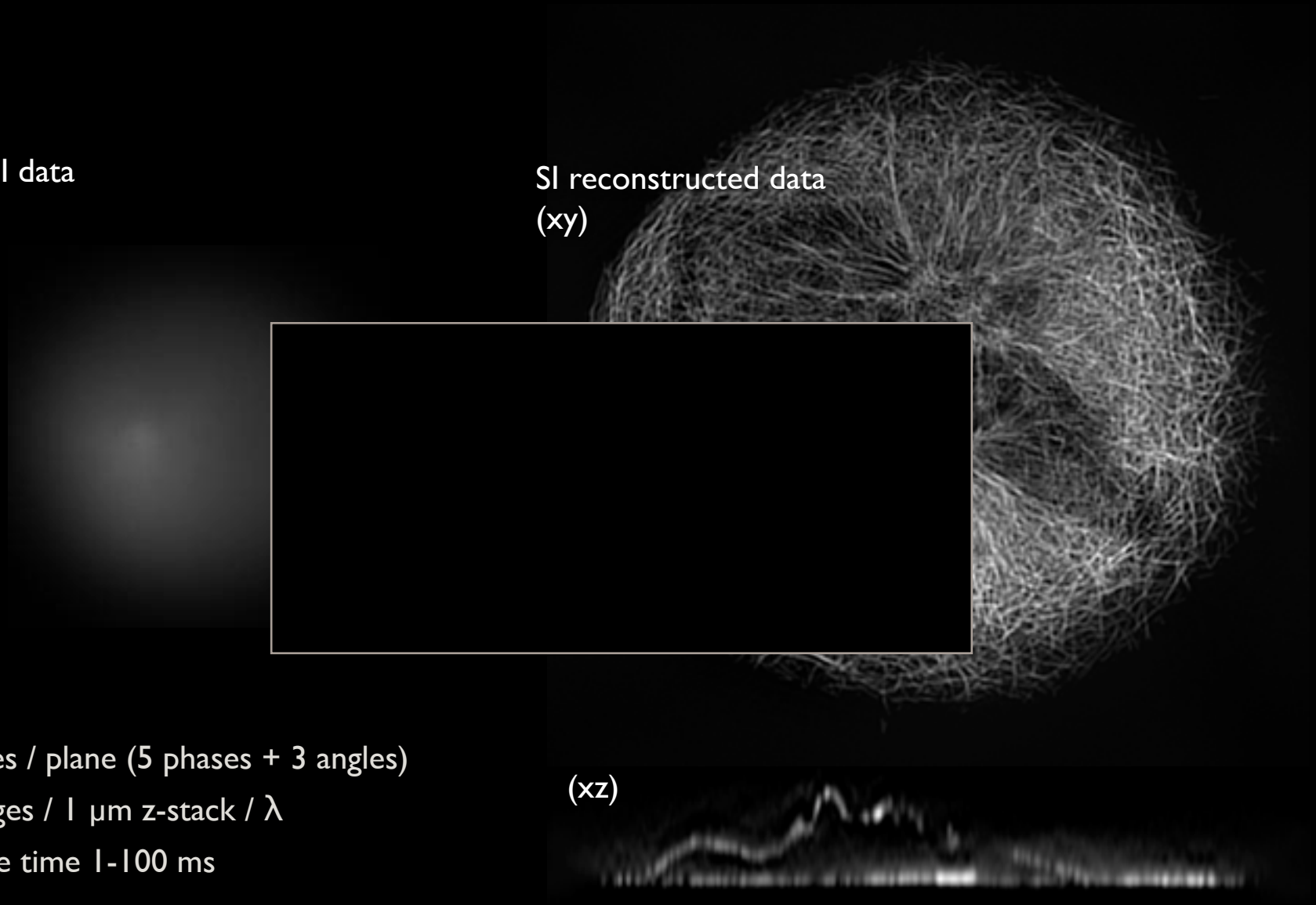


„Poor man‘s confocal“
No super-resolution!

3D-SIM in practice

Raw SI data

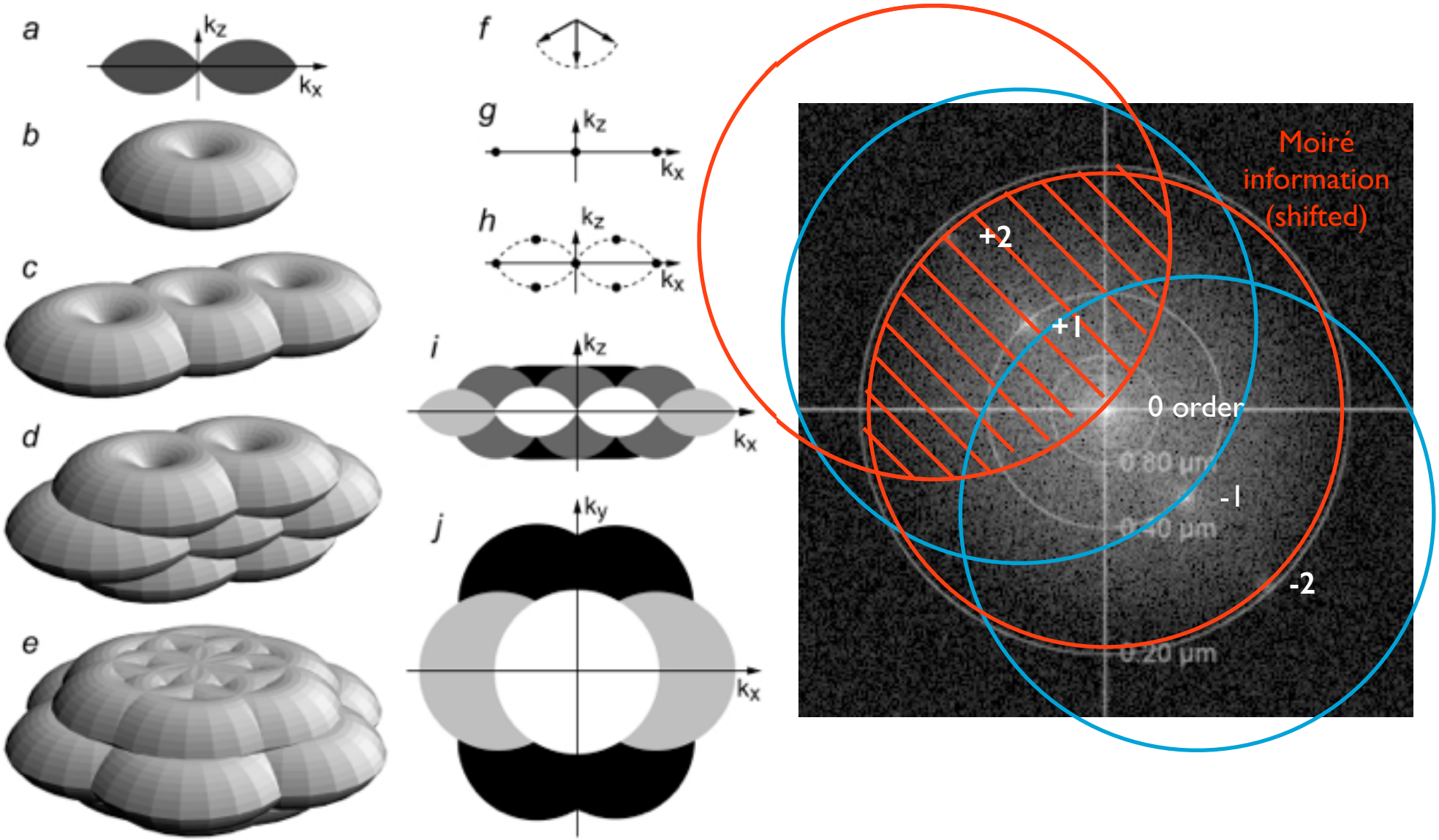
SI reconstructed data
(xy)



(xz)

- 15 images / plane (5 phases + 3 angles)
- 135 images / 1 µm z-stack / λ
- Exposure time 1-100 ms

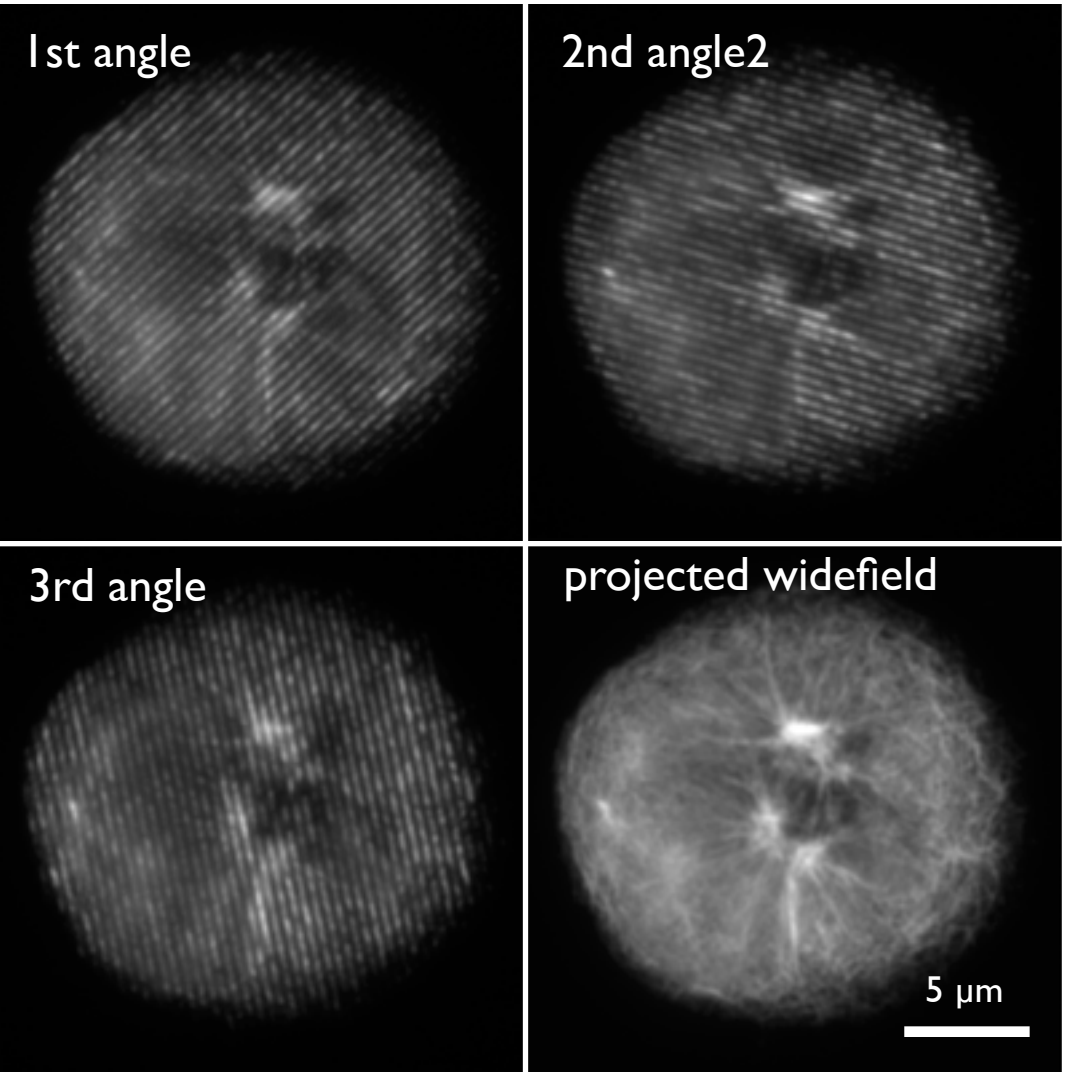
Doubling frequency support by SI

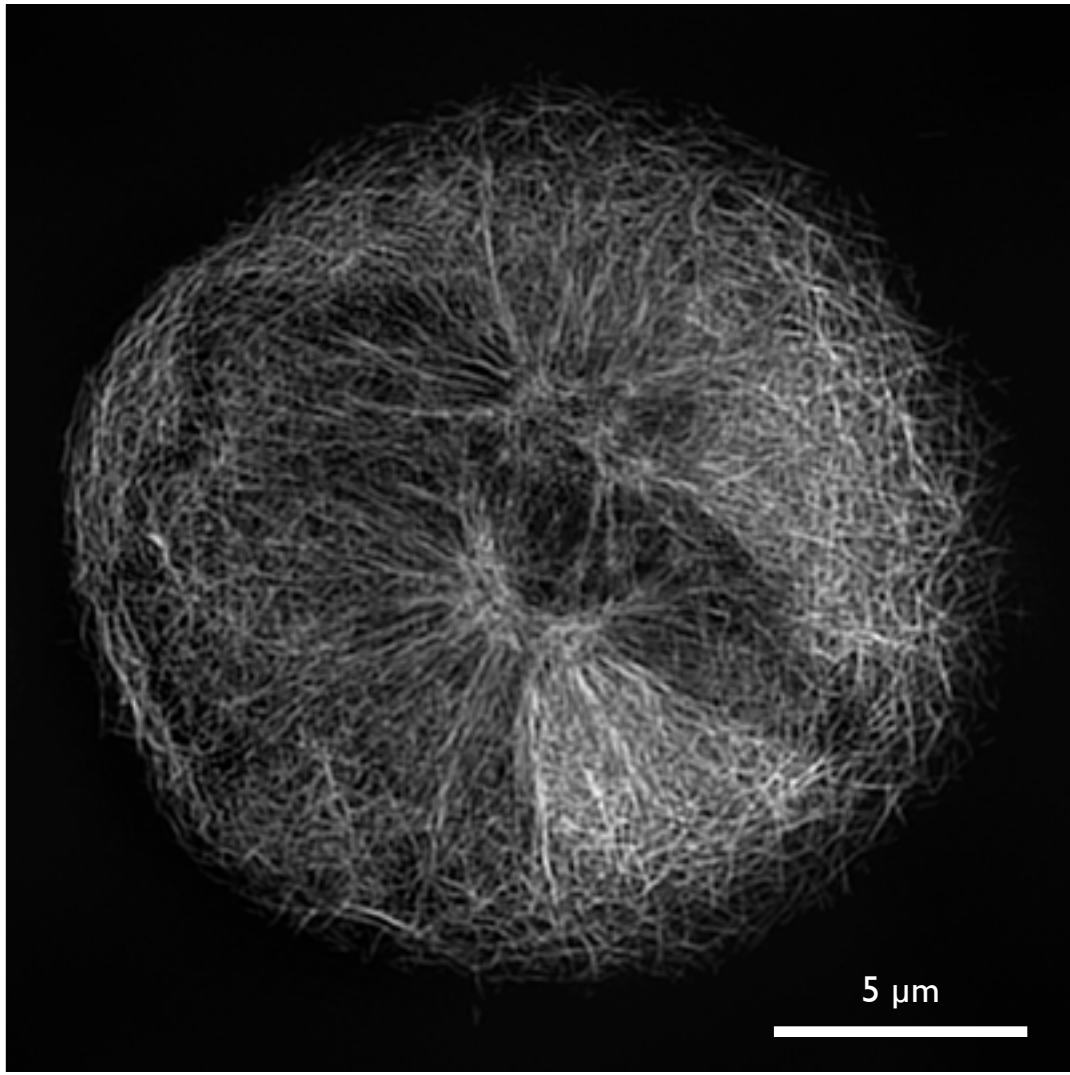


Doubling frequency support by SI

SI raw

SI reconstructed

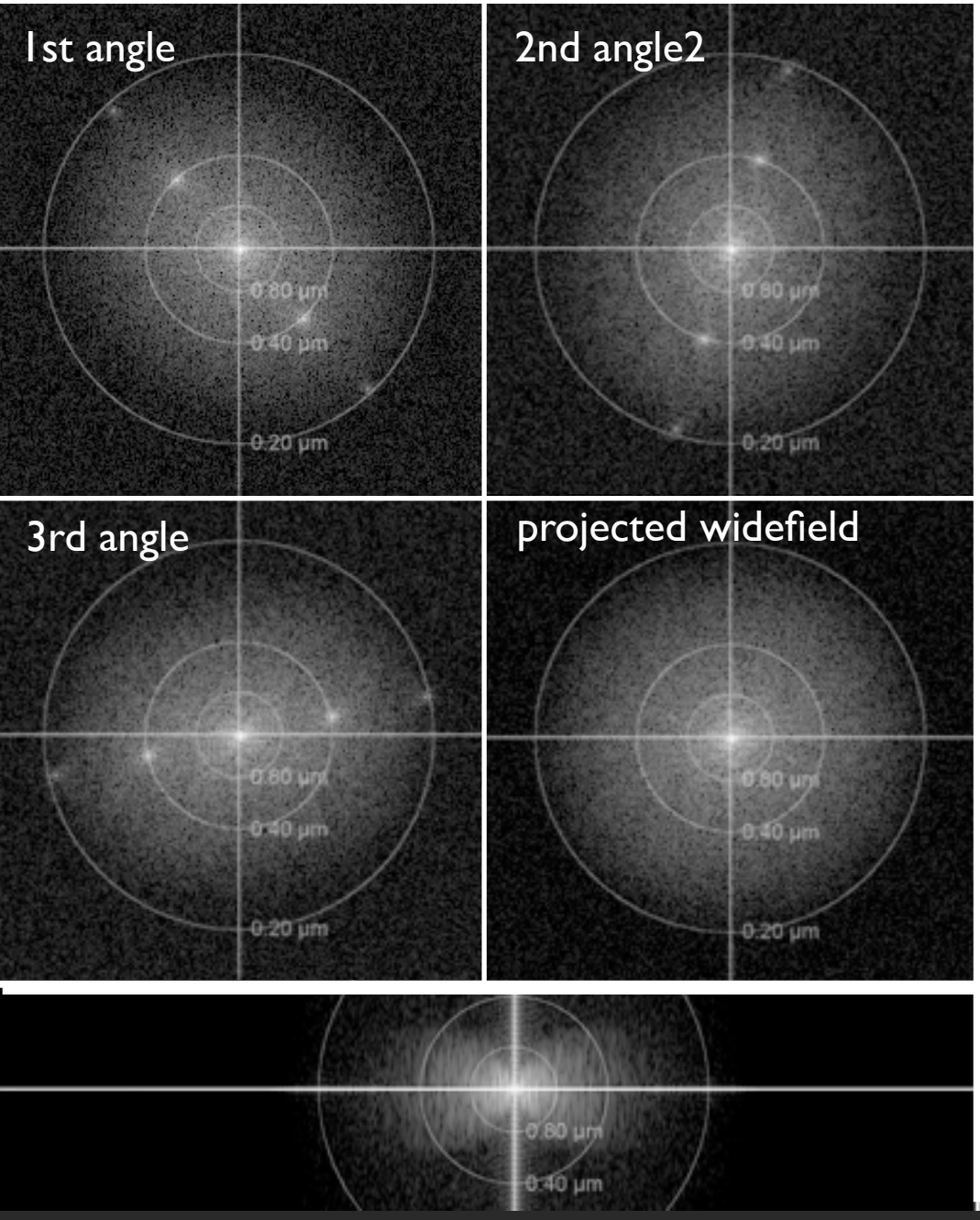


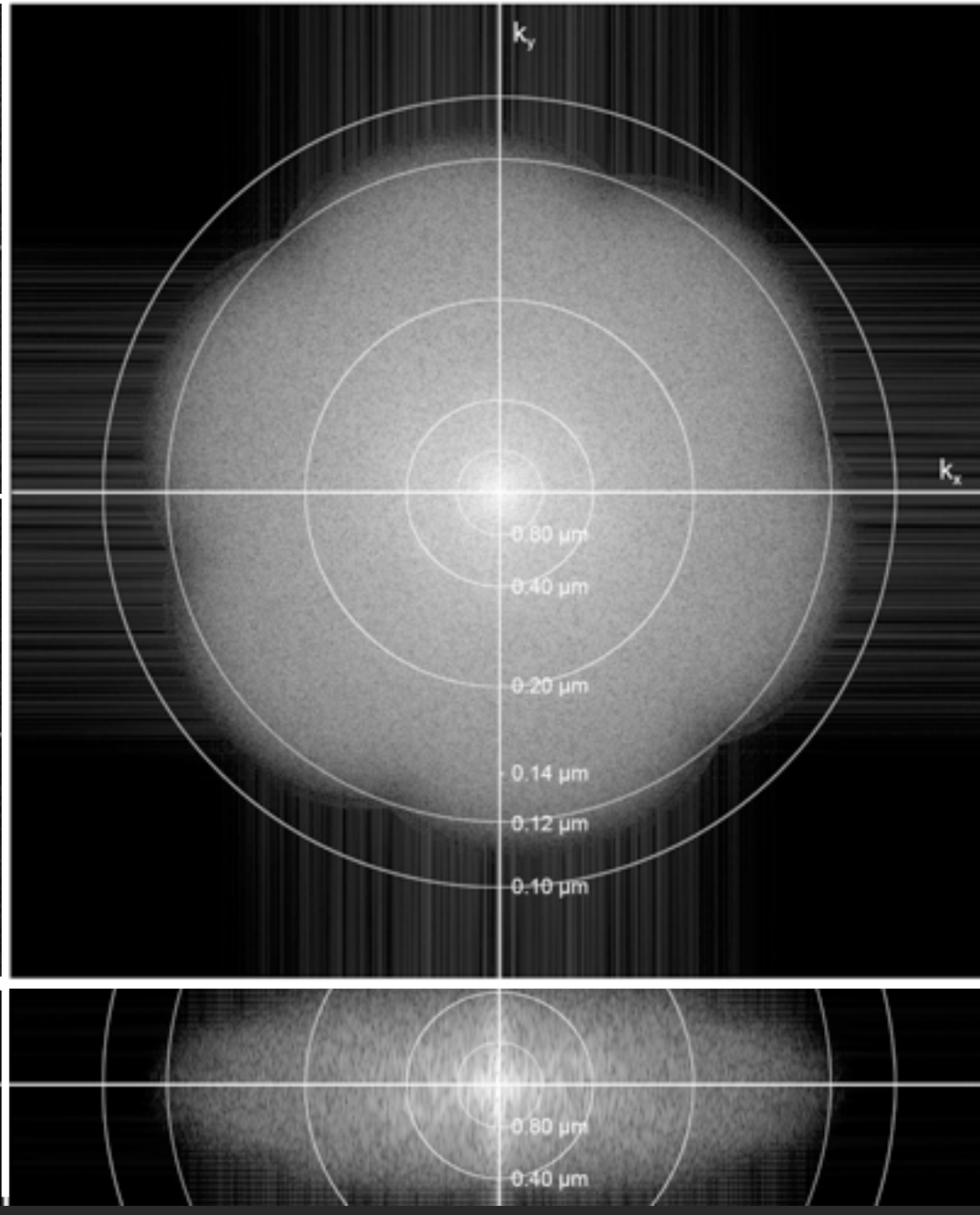


Doubled frequency support = 2-fold resolution in xy and z

SI raw

SI reconstructed

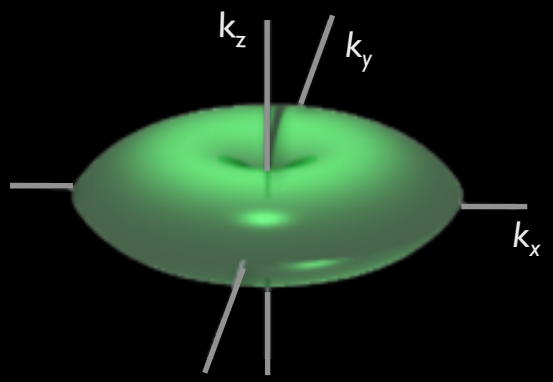




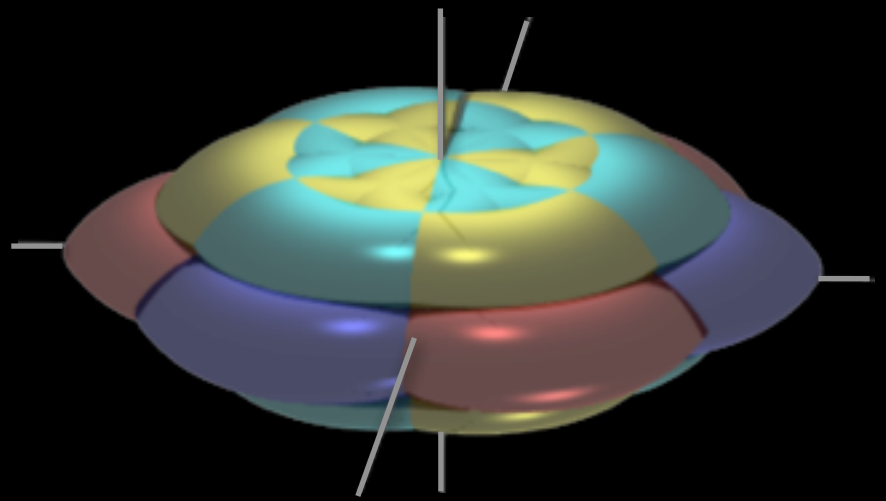
Doubled frequency support = 2-fold resolution in xy and z

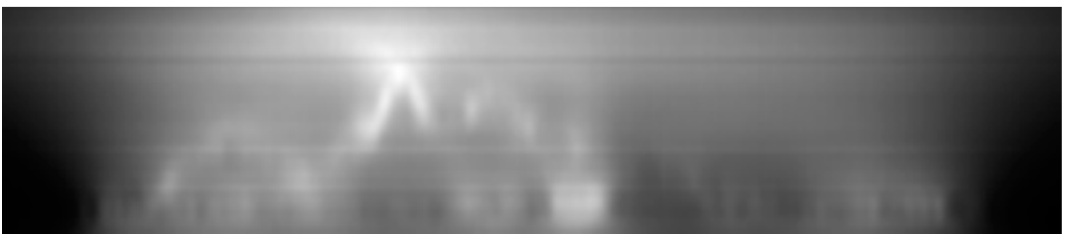
Widefield

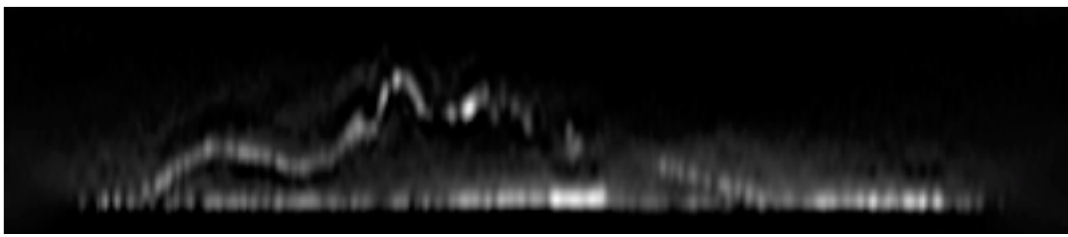
SI reconstructed



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

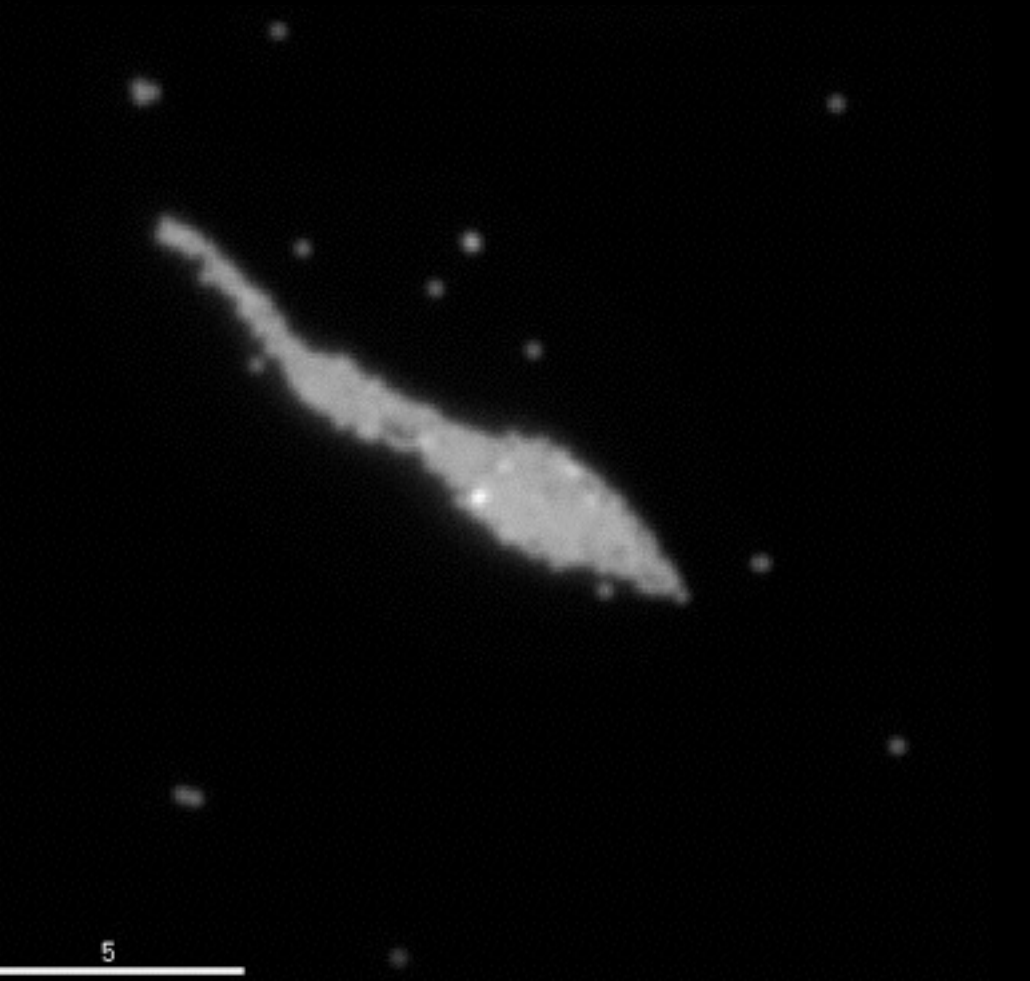


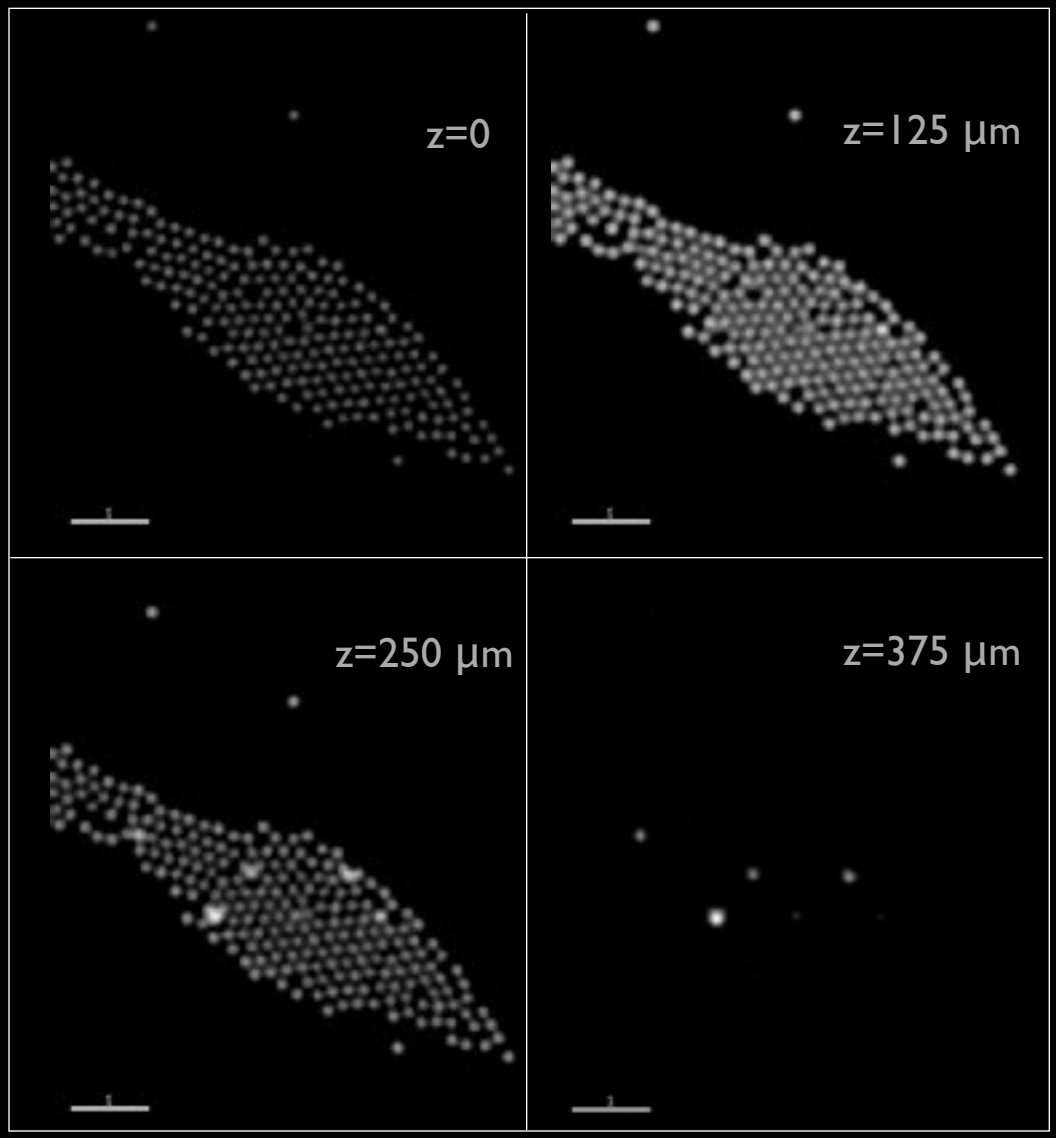




3D optical sectioning capacity

Example: 170 nm Fluospheres

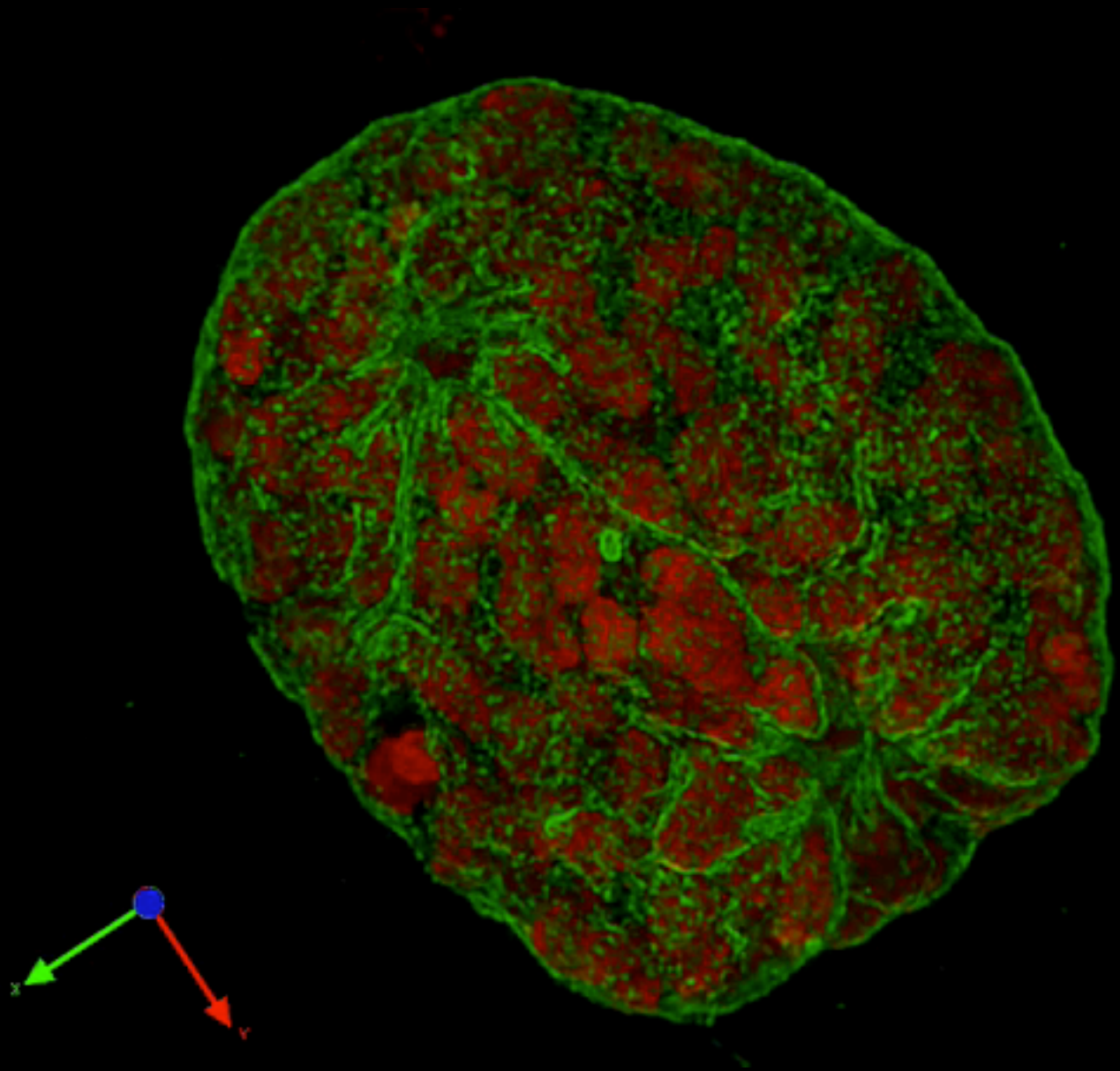




3D SIM example: Prophase

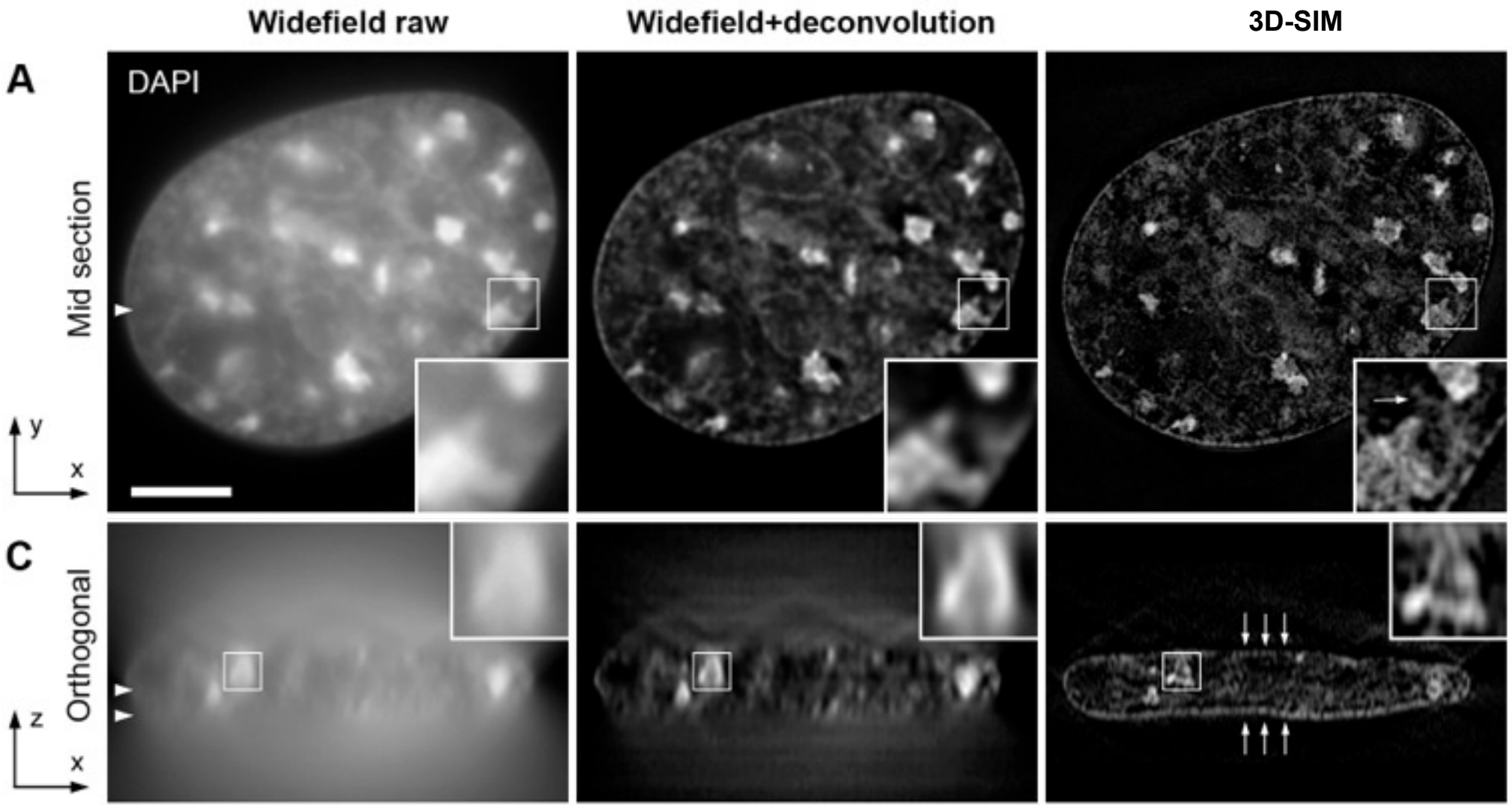
Lamin B
DAPI

3D volume rendering



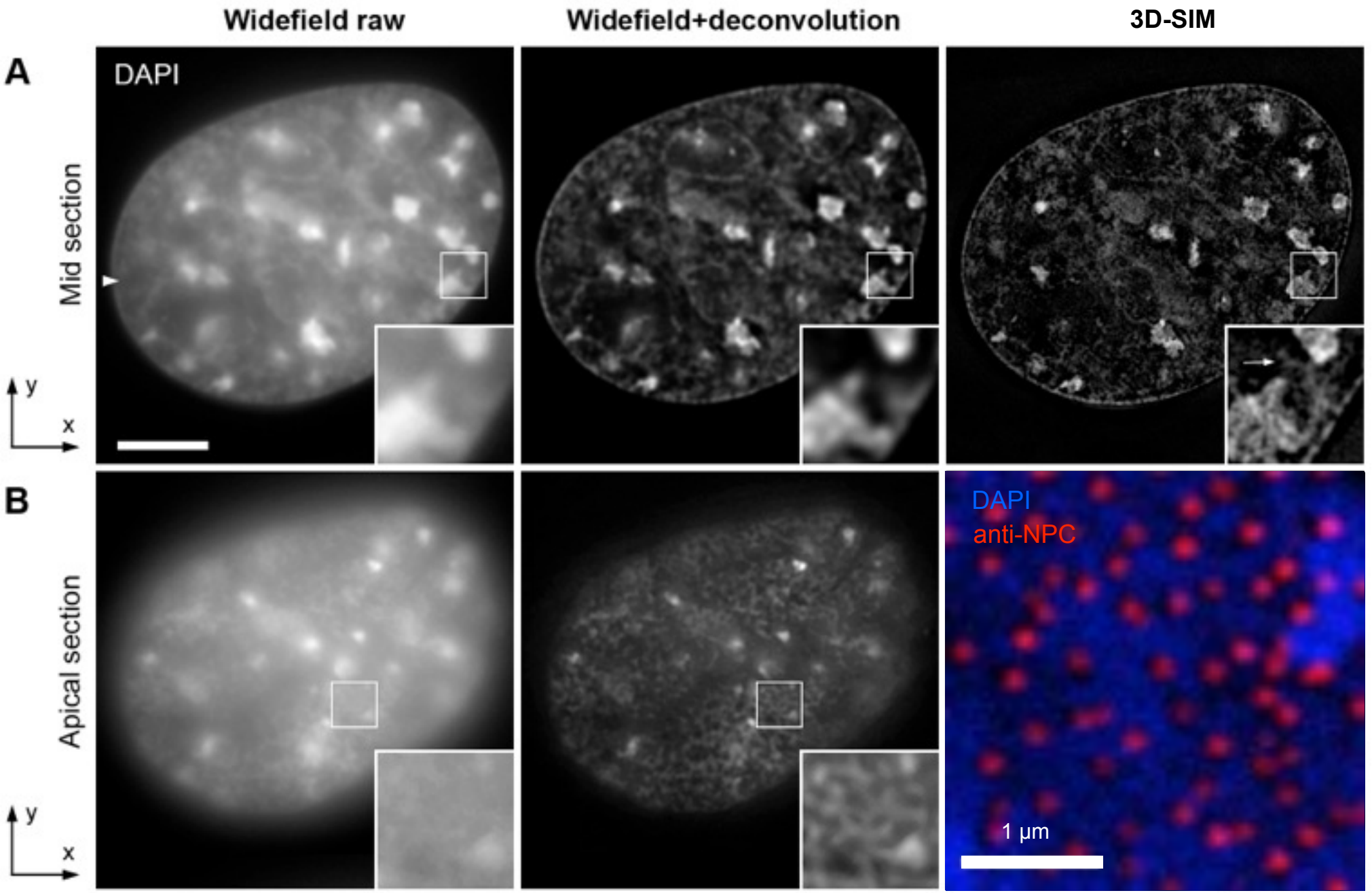
Schermelleh, Carlton et al. (2008), *Science* **320**

3D SIM example: chromatin

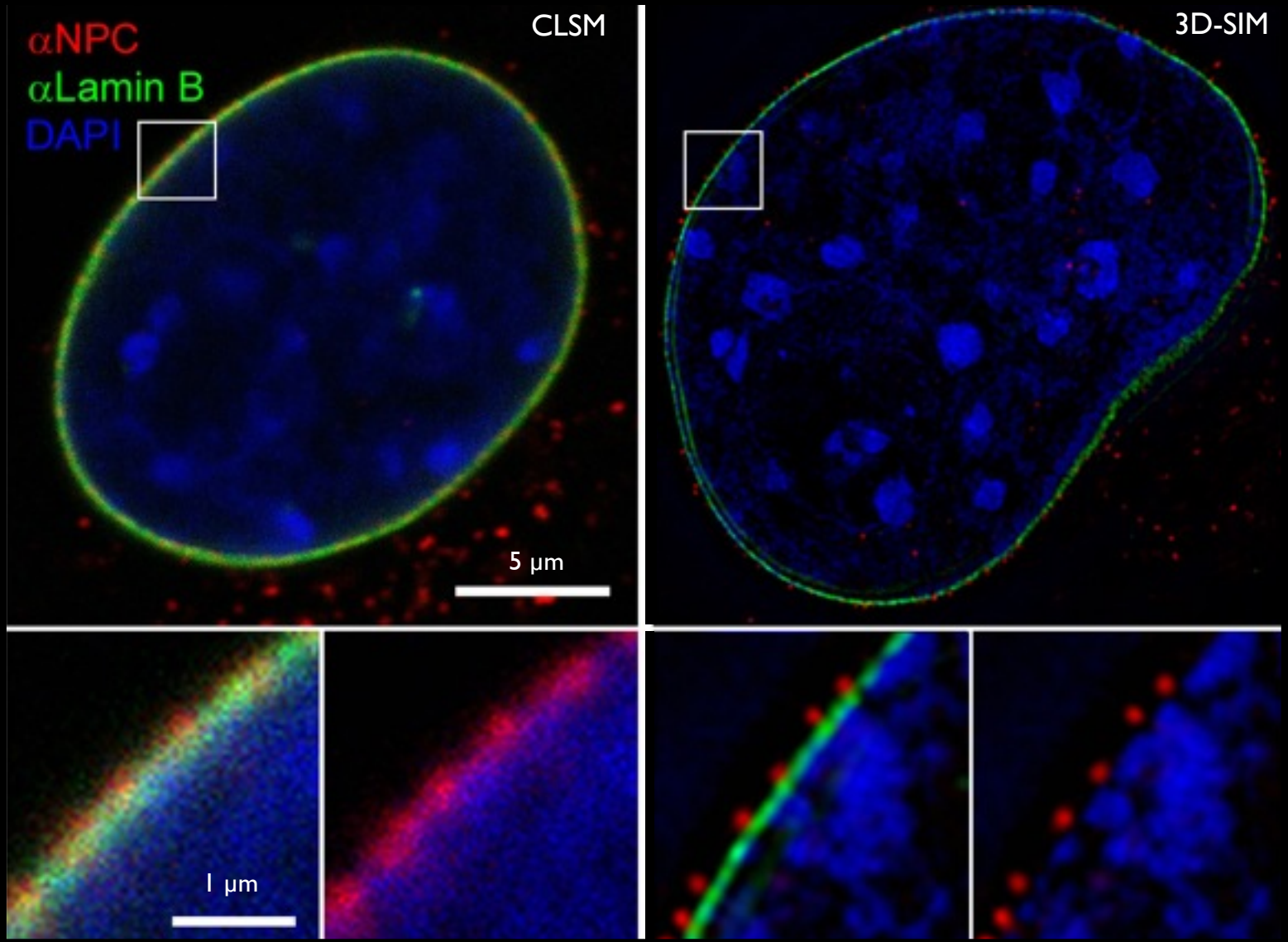


mouse C2C12 cell (DAPI staining)

3D SIM example: chromatin



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



Mouse C2C12 cell

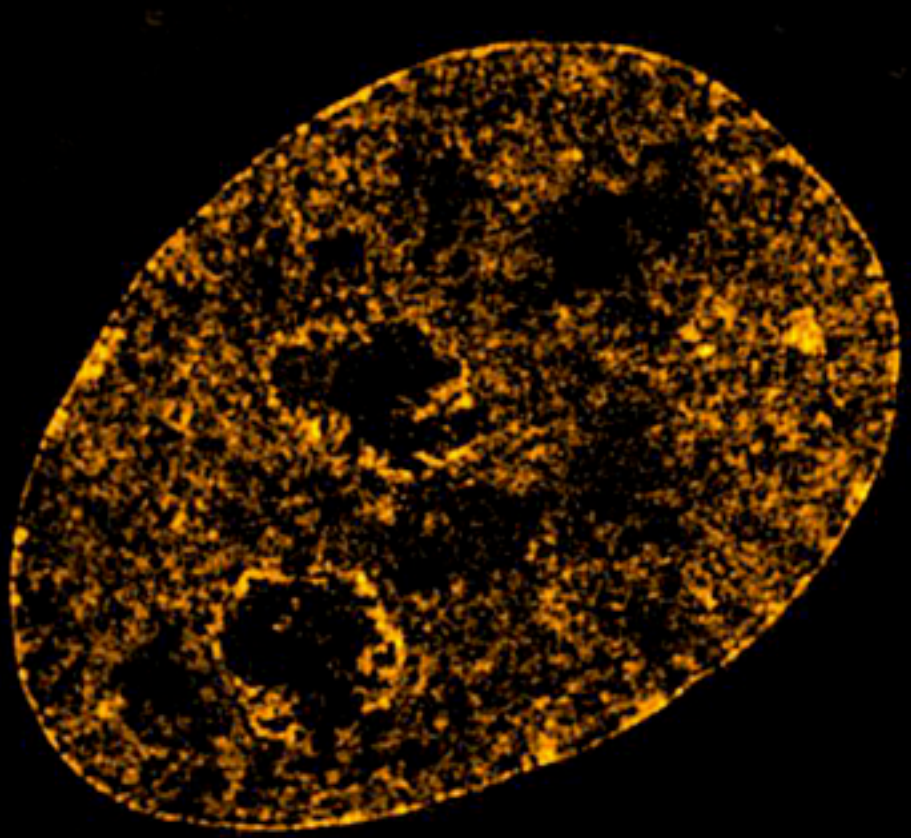
Schermelleh et al. (2008), *Science* **320**

Can we go live?

Live cell 3D-SIM with OMX Blaze

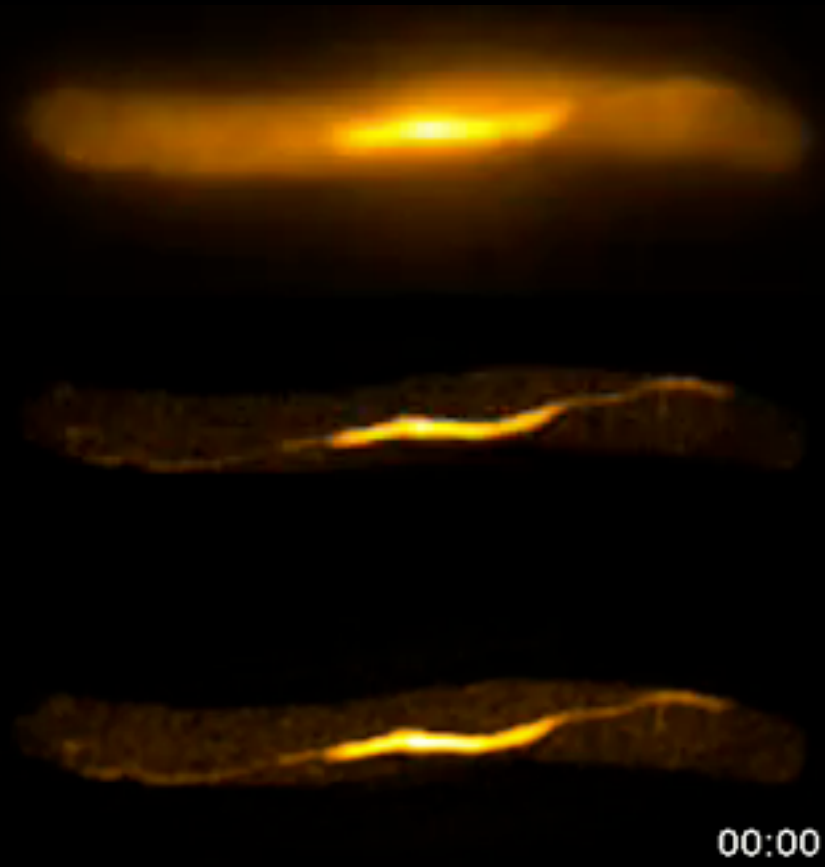
Inferometric pattern generation + sCMOS cameras → 10 x faster imaging

H2B-GFP (unfixed)



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

RecA-GFP (*E.coli*)



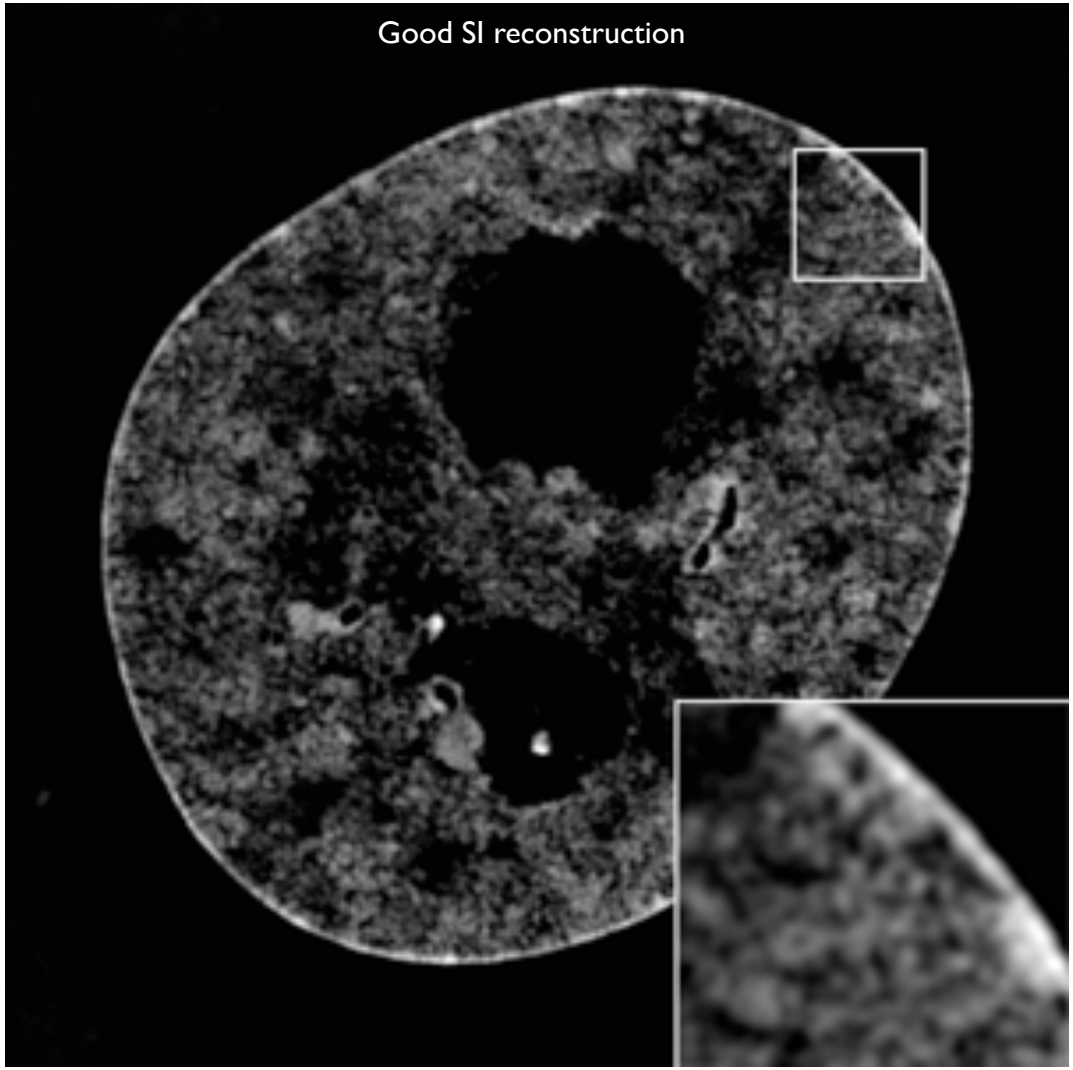
2 s / 3D-frame (1 µm z-stack = 120 images ; 100 ime points)

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

It‘s not that simple!

The untold story

SI reconstruction artifacts

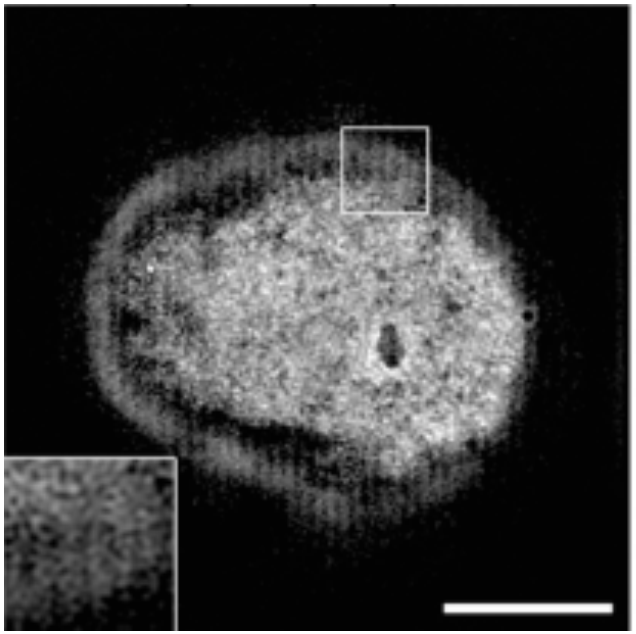


RPE-1 cell, DAPI staining

SI reconstruction artifacts

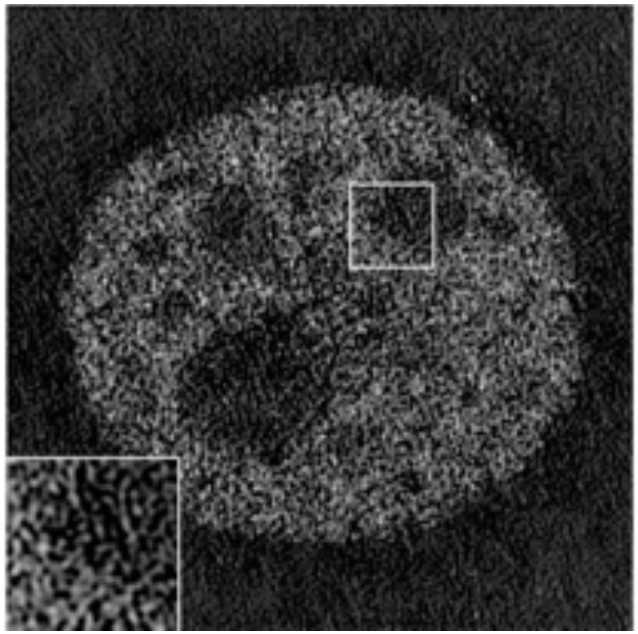
C127 cell nuclei, chromatin staining

Stripes



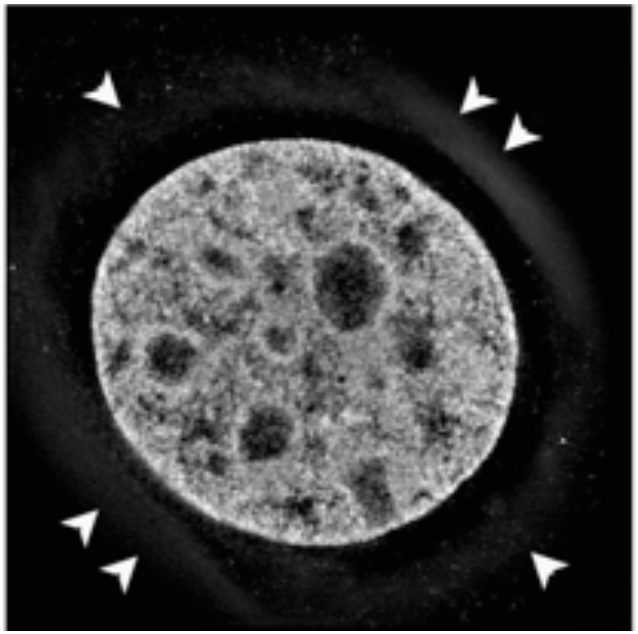
Bleaching,
Drift or vibrations
Moving particles
(locally constrained)

High frequency noise



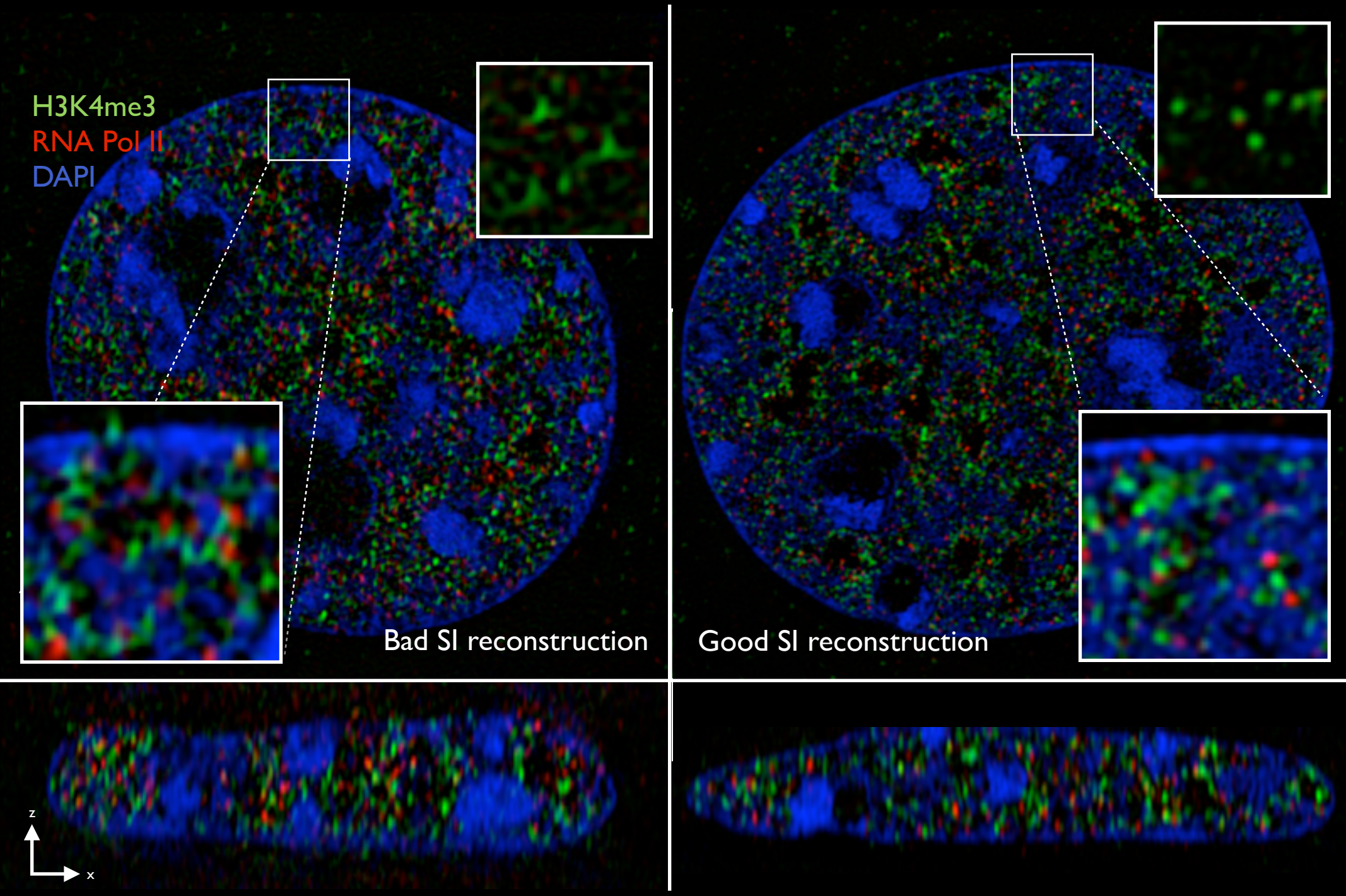
Low contrast-to-noise,
Low modulation contrast

Halo / Doubling



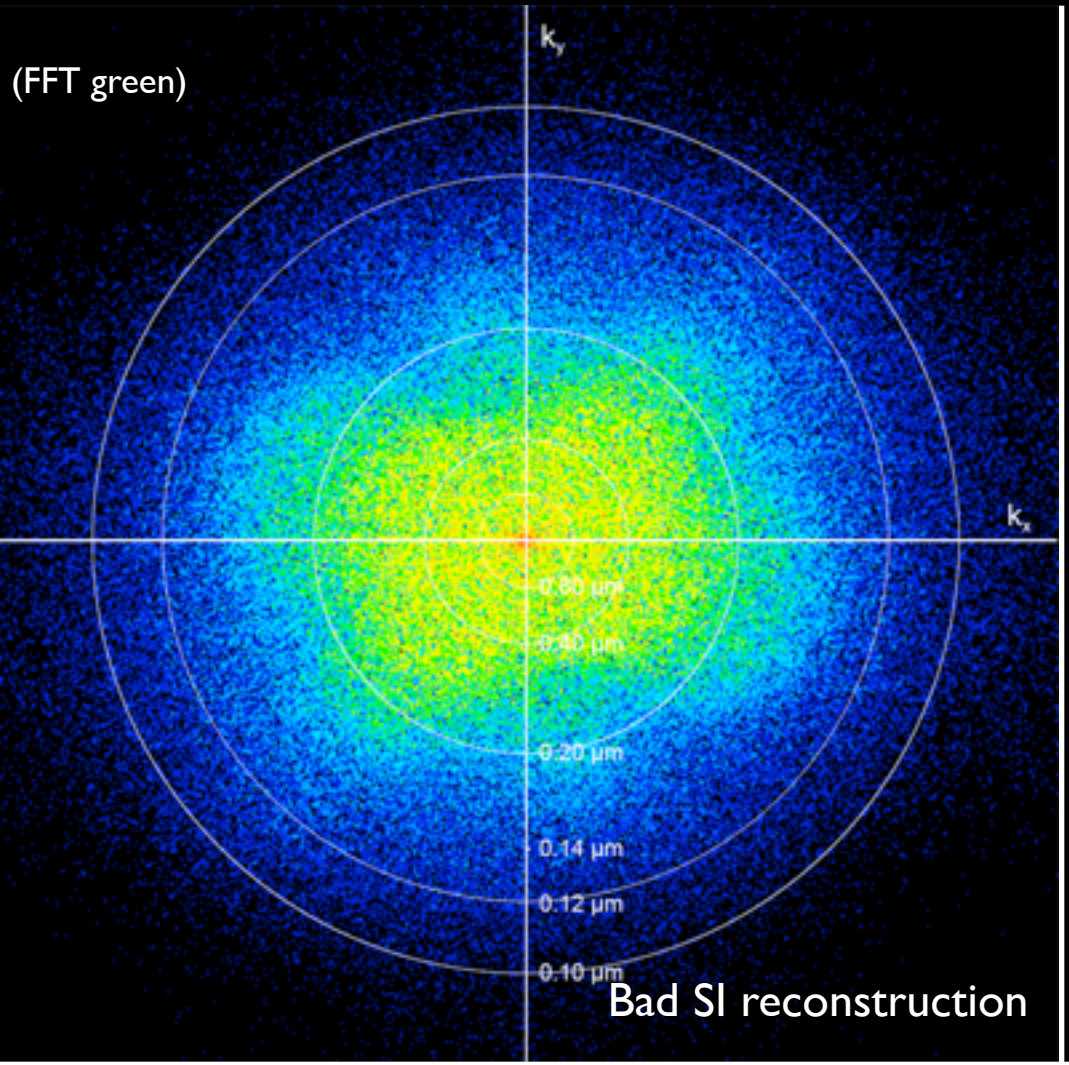
Spherical aberration,
Refractive index mismatch

Quality control: Reconstruction artifacts

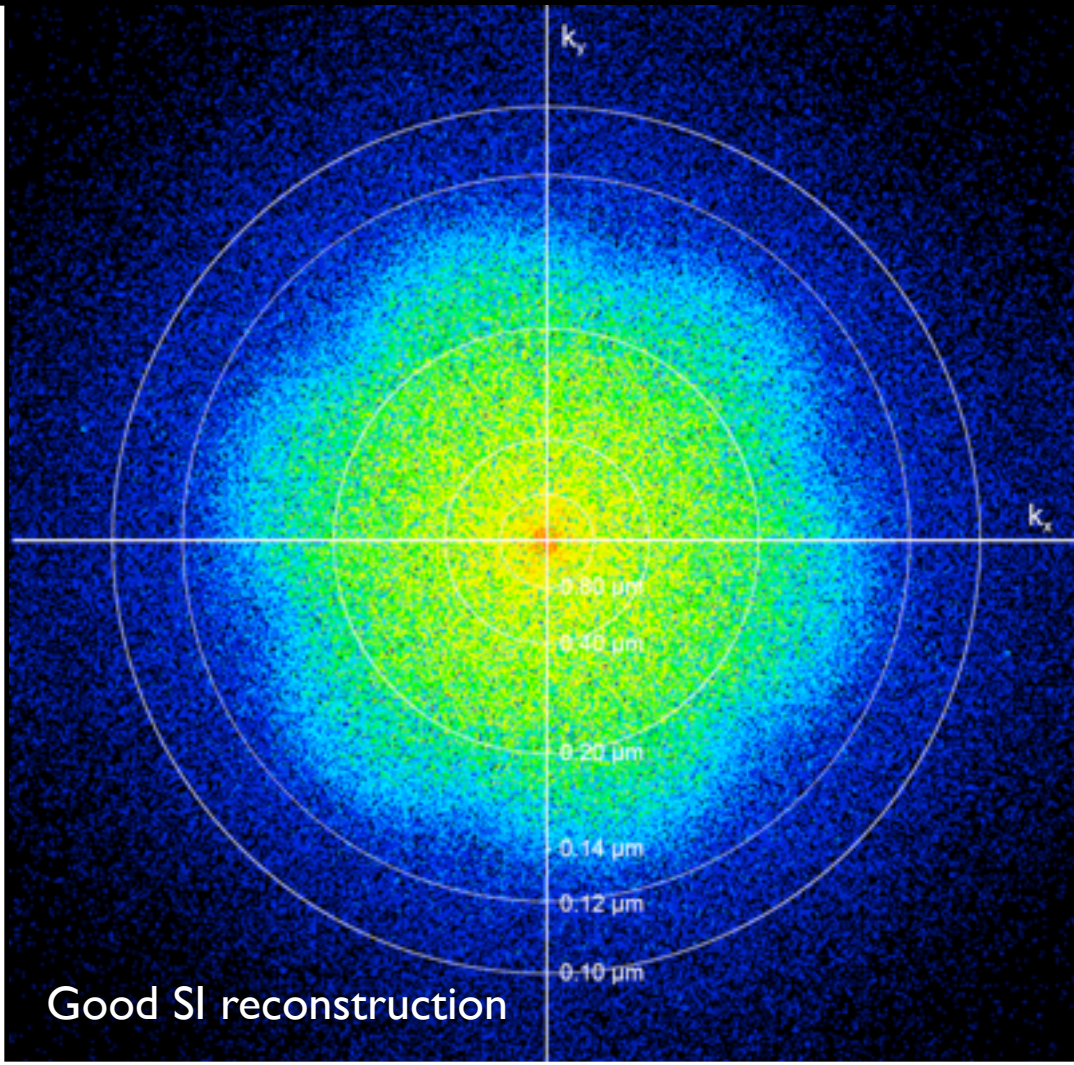


Quality control by Fourier analysis

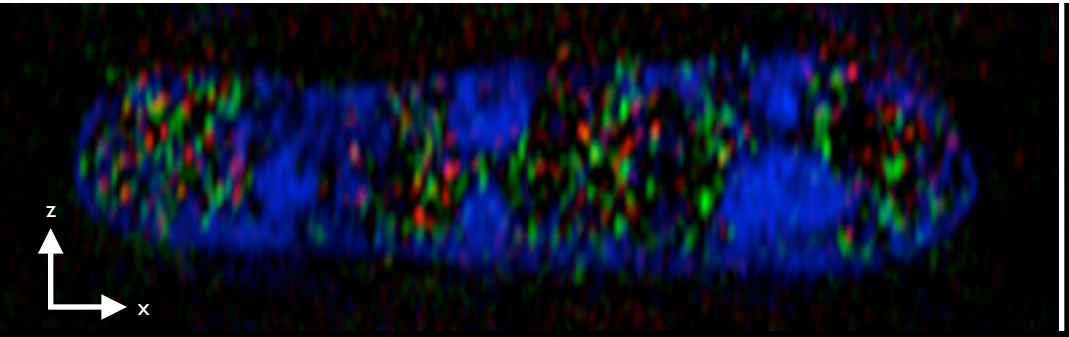
(FFT green)

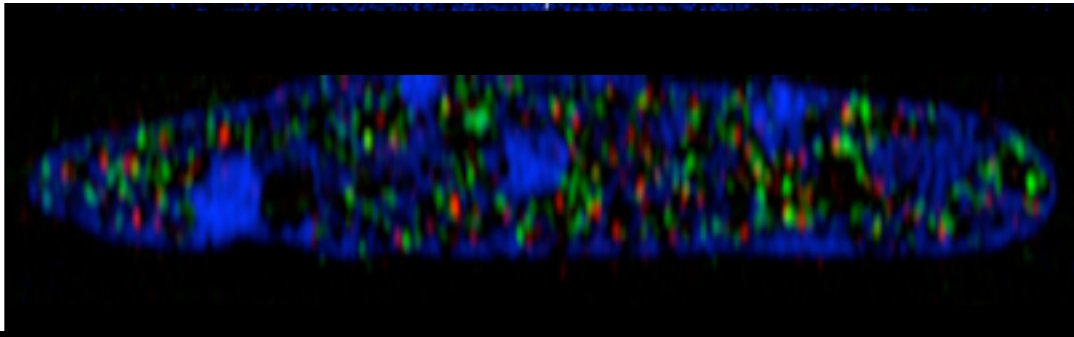


Bad SI reconstruction

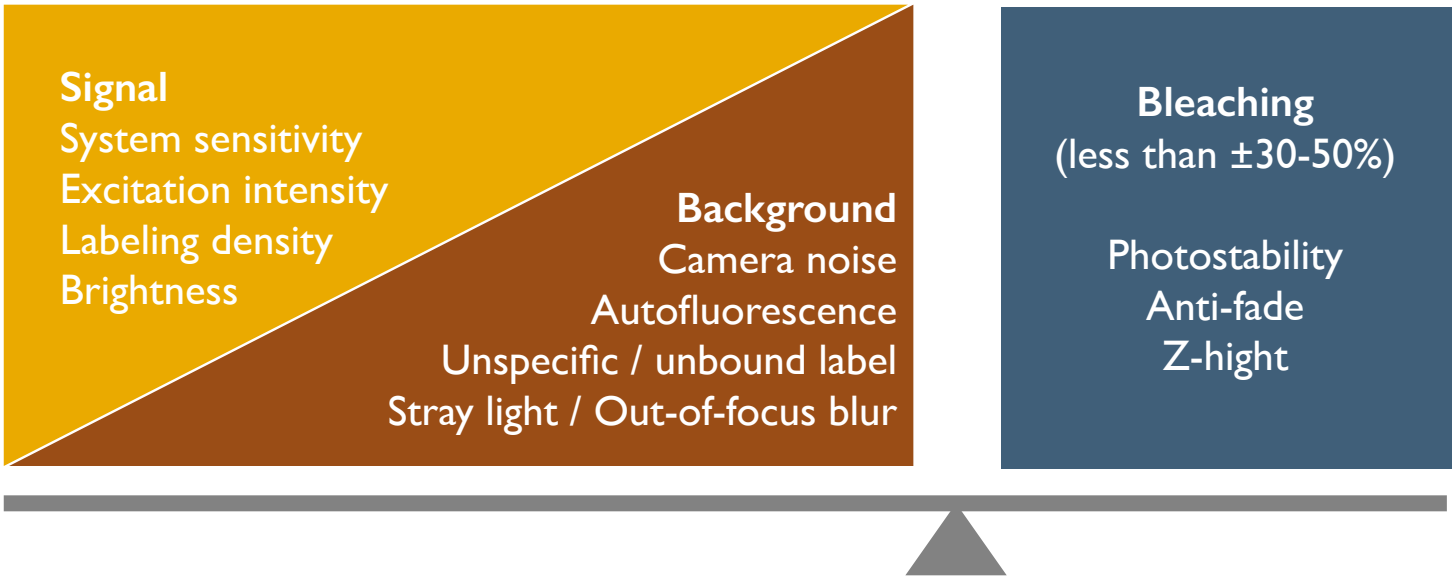


Good SI reconstruction

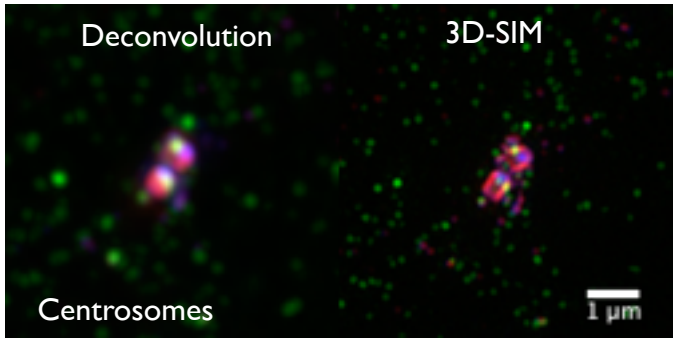




Balance between contrast and bleaching

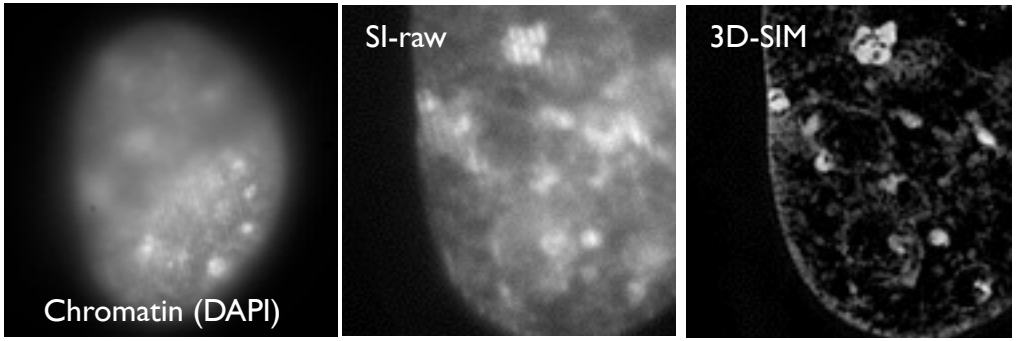


Discrete, isolated structures
Restricted z-hight, low background



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

How to get the best image? Quality is paramount

Labeling

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

Sample

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

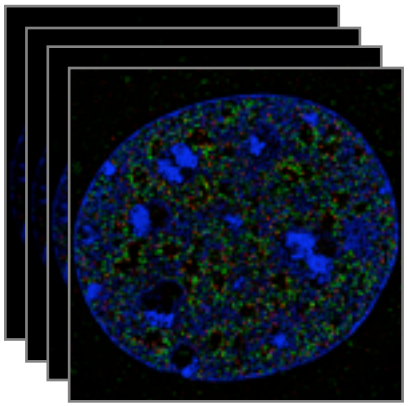
OMX Hardware

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 - the pros & cons

+ Multi-color, standard dyes (e.g., DAPI, Alexa, GFP...)
+ 3D with 2x resolution in XY and Z (8x volumetric)
+ Massive contrast improvement / high dynamic range
+ Z-sectioning over larger volumes (10 µm in z)
+ Sensitive (EMCCD/sCMOS) and fast (OMX Blaze) → live cell imaging
+ Fast imaging of a large field of view (40 x 40 µm)

Context

Versatility

o Only moderate lateral resolution improvement

- Mathematical reconstruction → artifacts
- High requirements on sample quality and system calibration

No free lunch! - trade-offs in super-resolution microscopy

Photon budget

(Wide-field)

3D-SIM

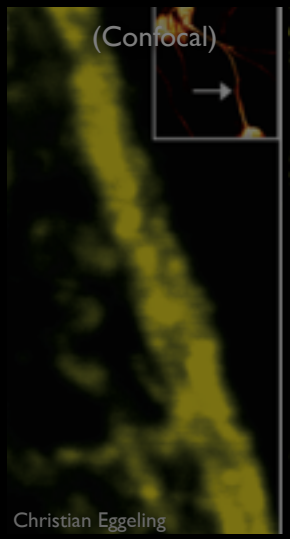
± 120 nm (xy); ± 300 nm (z)

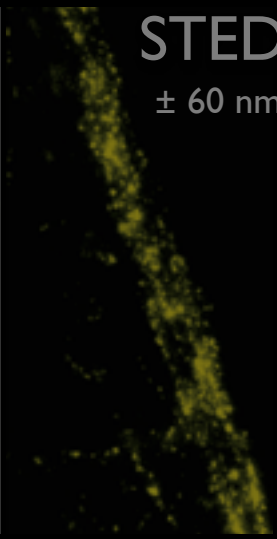
Spatial resolution (Contrast)

Multi-D: z-depth, λ, t

Low Photodamage (Bleaching)

Temporal Resolution (Speed)

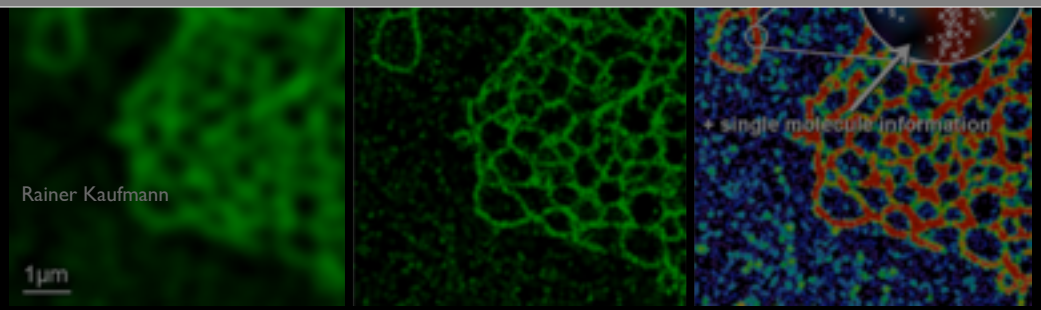




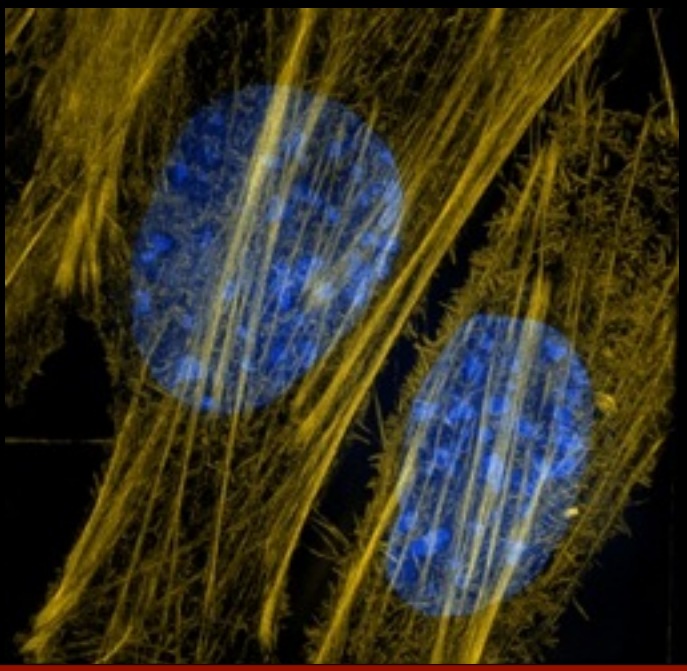
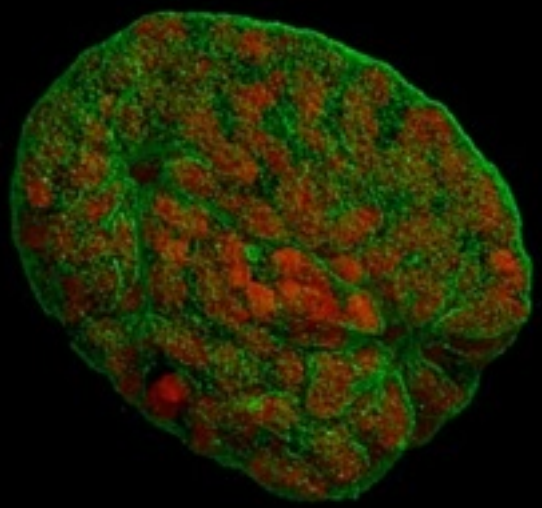
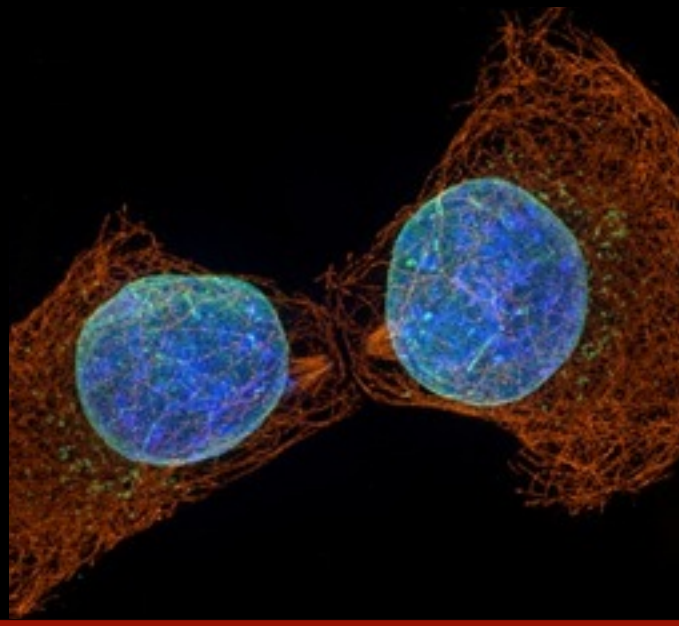
STED

± 60 nm (xy)

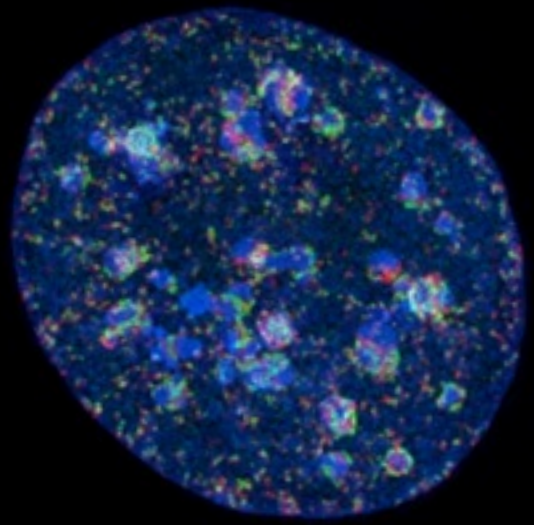
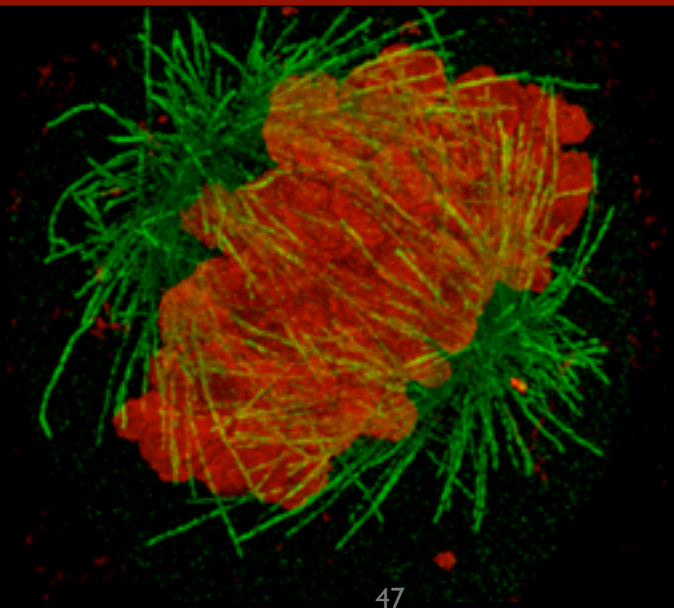
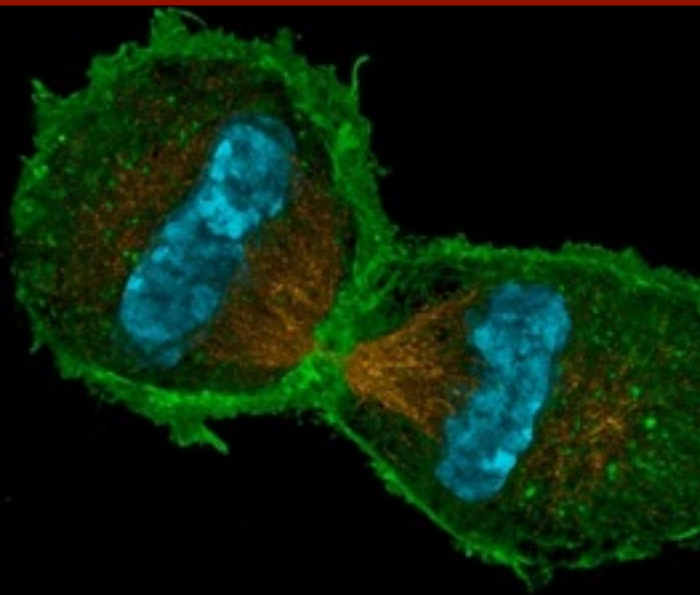
The best SR-technique is determined by demands of the biological application!
Spatial resolution is only part of the equation !



± 20 nm (xy accuracy);
≥ 50 nm (xy, structural res.)

SIM rocks!

s to Jürgen Neumann, Pete Carlton, Lothar Schermelleh for sharing slides