The Power of SIM

How Structured Illumination improves
not only resolution ...

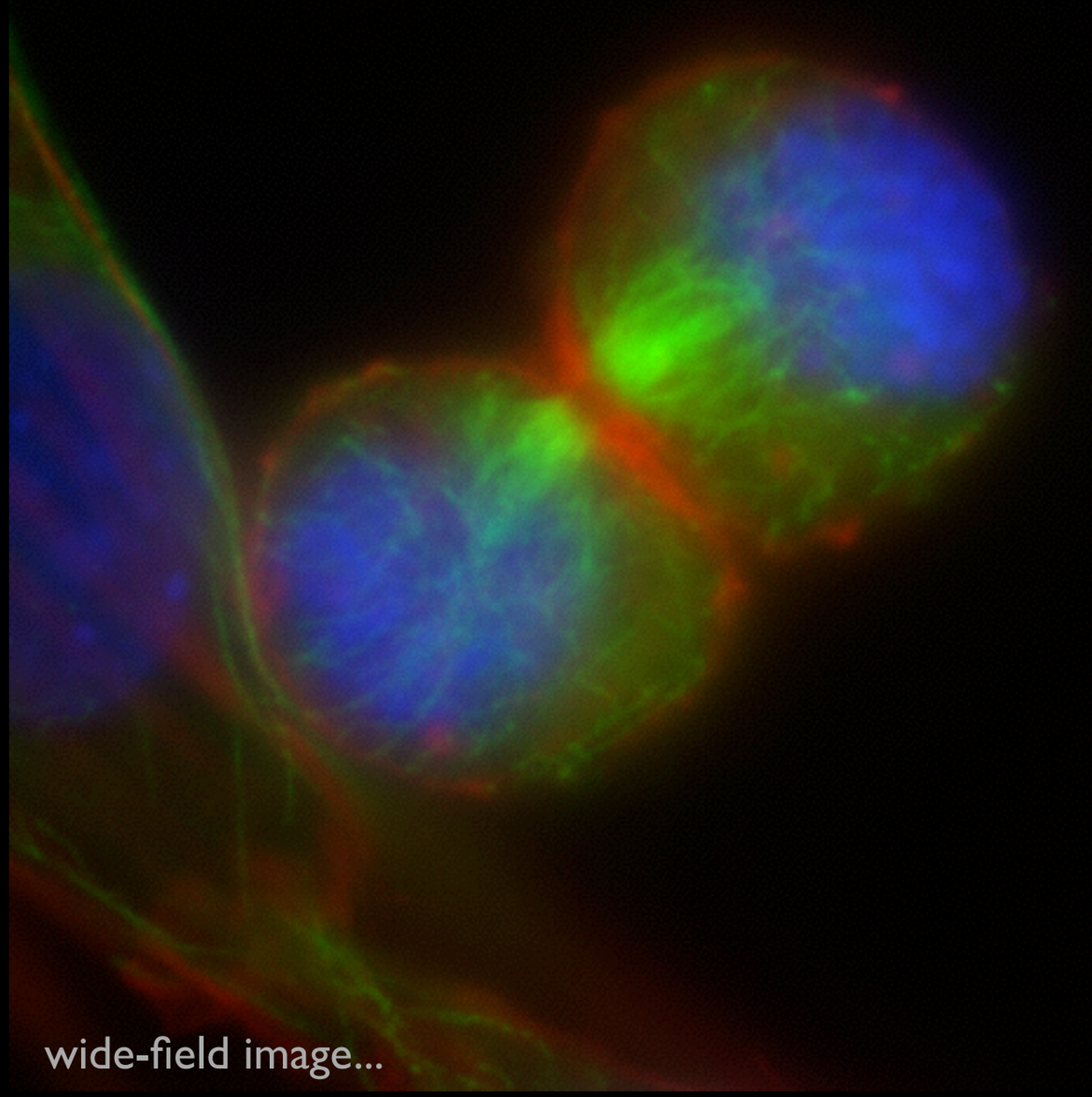
& how it is realized in
OMX system

Comparison with other
SR methods (Pros & Cons)

3D structured illumination microscopy of a mouse cell nucleus

Micron OXFORD
lothar.schermelleh@bioch.ox.ac.uk

Super-resolution fluorescence microscopy



wide-field 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!

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...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
chromatic aberrations
straylight
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

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& how it is realized in
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Comparison with other
SR methods (Pros & Cons)

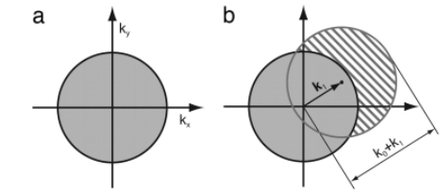
3D structured illumination microscopy

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



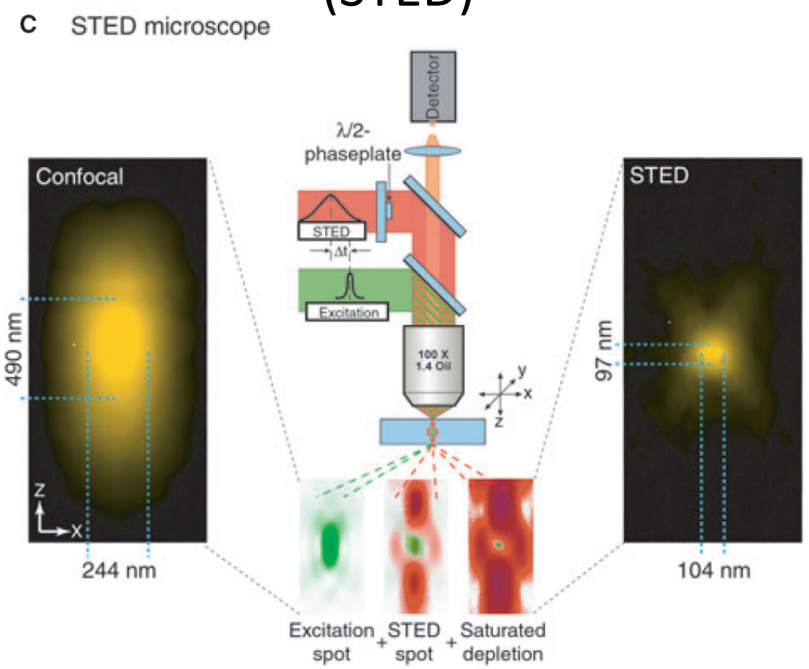
SIM-Methods:

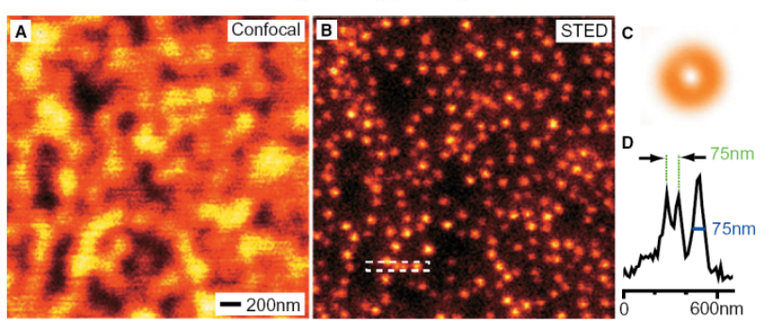
Apotome (conventional SIM)

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

SSIM (non-linear SIM)
NL-SIM

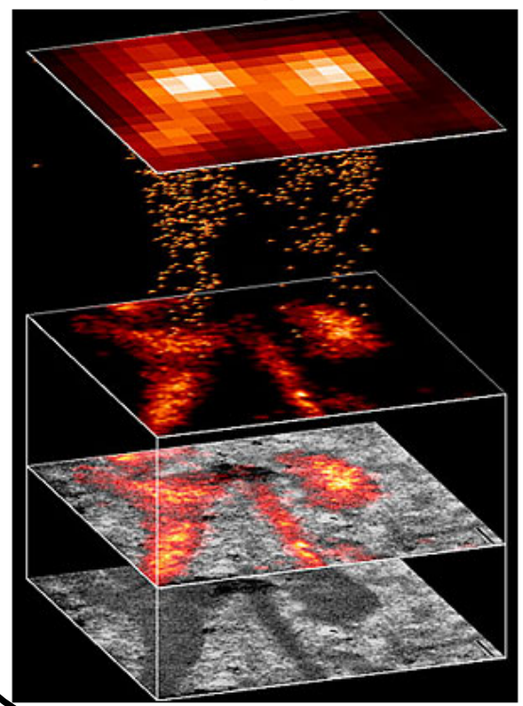
Stimulated emission depletion (STED)

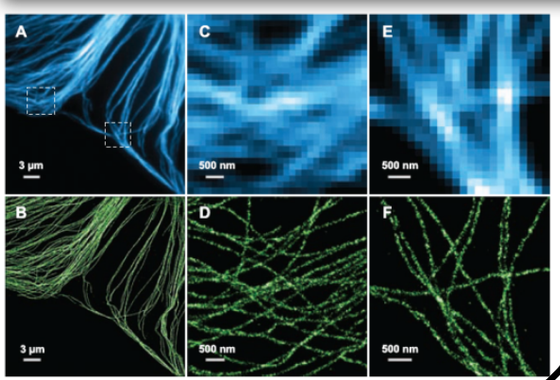




Localization

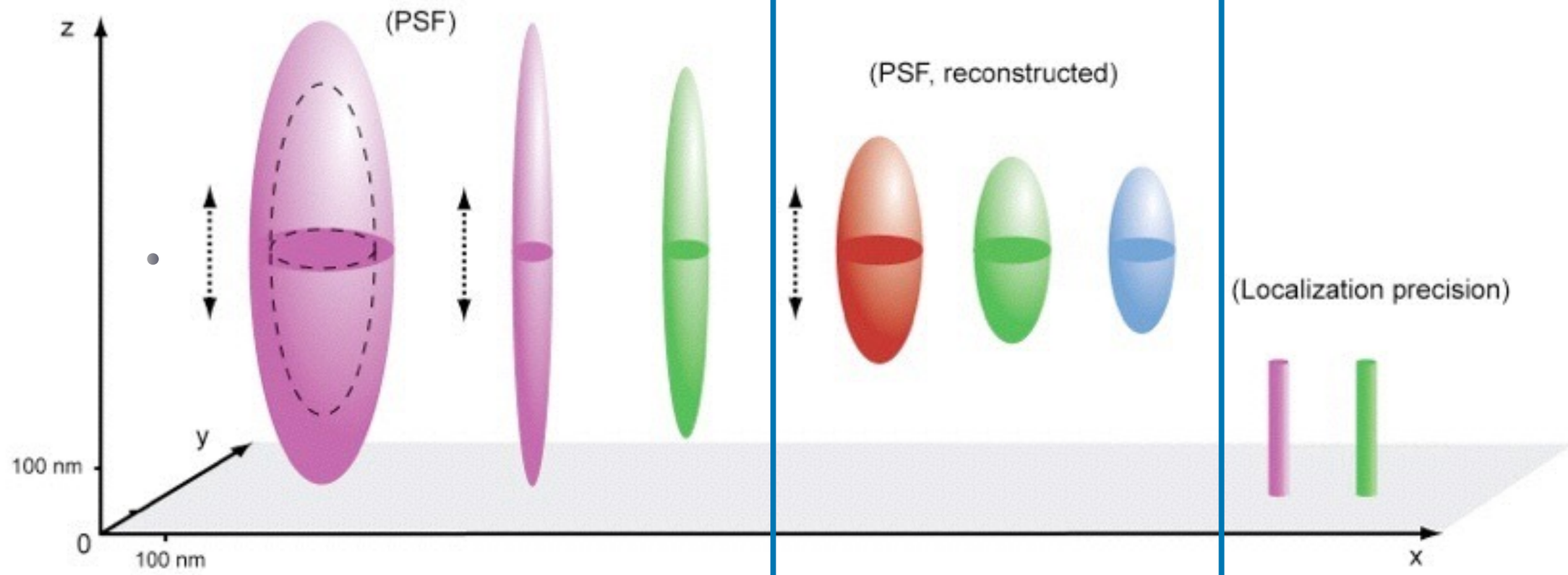
Photoactivation localization microscopy (PALM)





Resolving power of commercial super-resolution systems

	CLSM	STED	CW-STED	3D-SIM			PALM/STORM	
~λem [nm]	460-670	670	520	620	520	460	670	520
~$D_{x,y}$ [nm]	180-250	60	70	130	110	100	30	
~D_z [nm]	500-700	700	560	340	280	250	140 (TIRF-range)	
~$V_{x,y,z}$ [*10^{-3} µm³]	10-23	1.3	1.5	3.0	1.8	1.3	0.1	

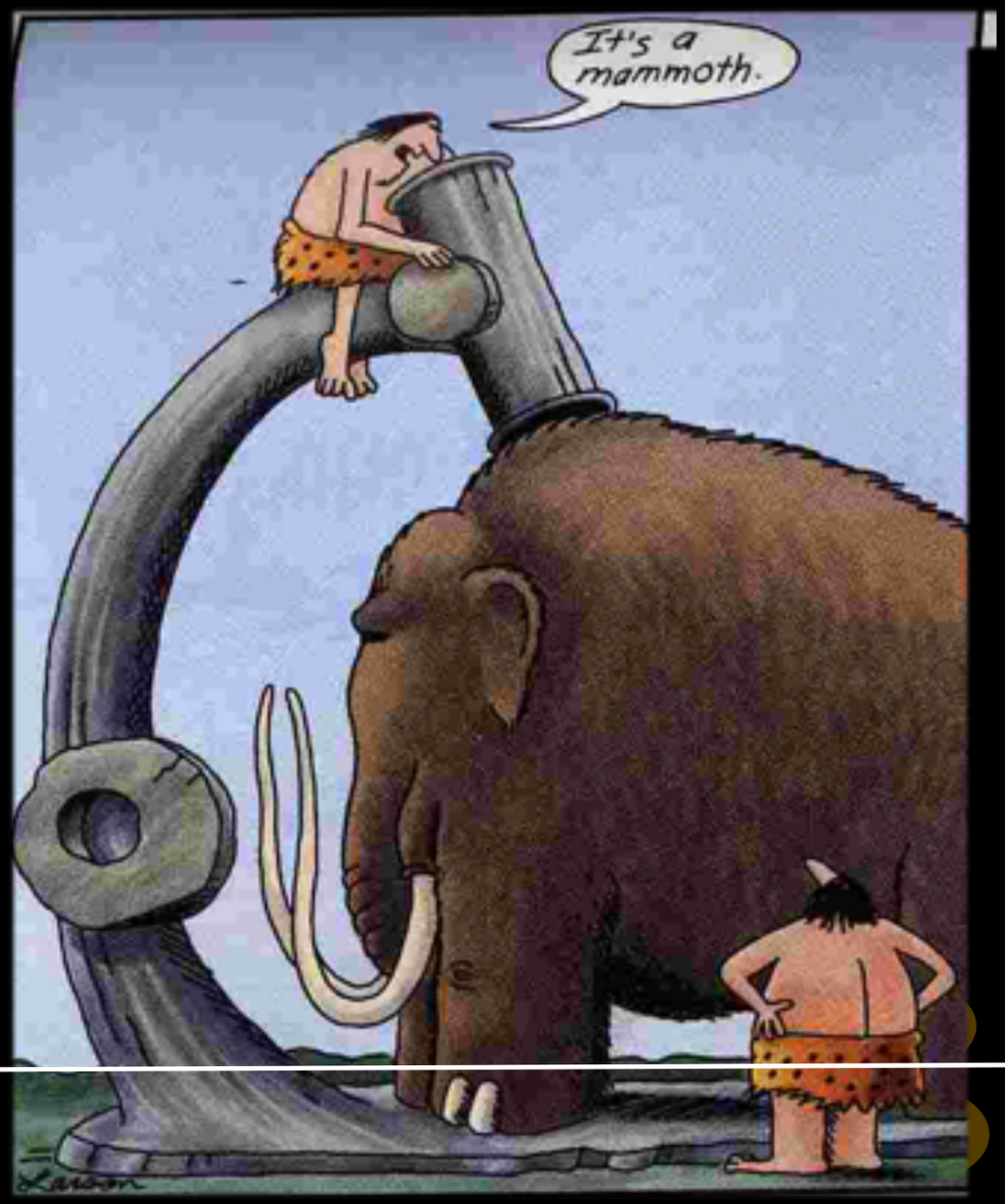


3D-SIM resolves ~8-fold smaller volumes than conventional microscopy

Schermelleh, Heintzmann, Leonhardt, *JCB*, 2010

Not only resolution matters, ...

What could this be?



3D information (z-resolution, optical sectioning, imaging depth)

Not only resolution matters, but also context (II)

Multicolour (λ) information

To understand the game you need to see the player move

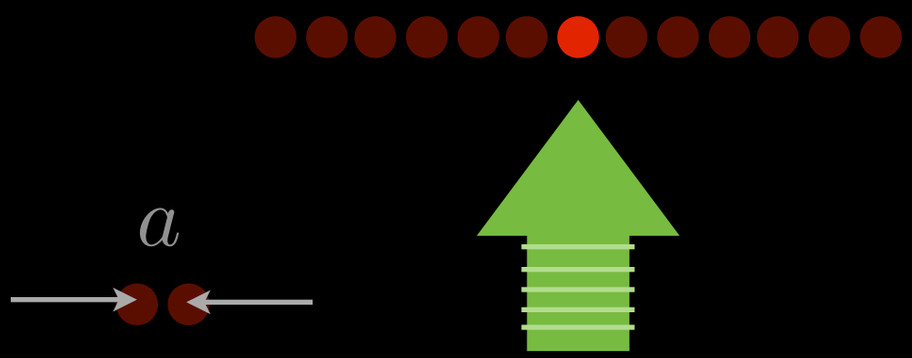
Temporal information (live cell imaging)

How does SIM work?

The basic principle: Abbe‘s view

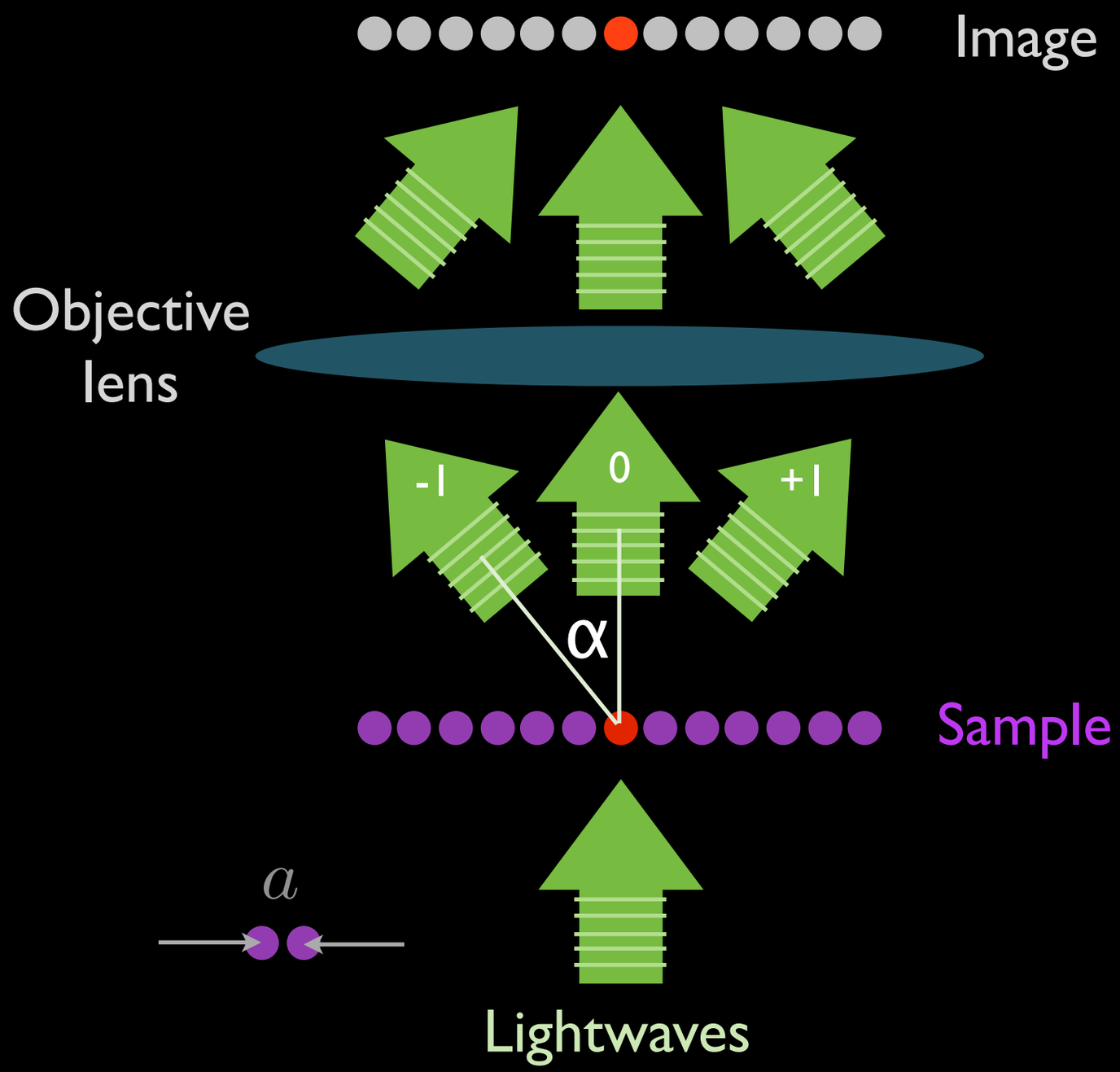
Sample = Structure

→ Periodicity



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

The basic principle: Abbe's view



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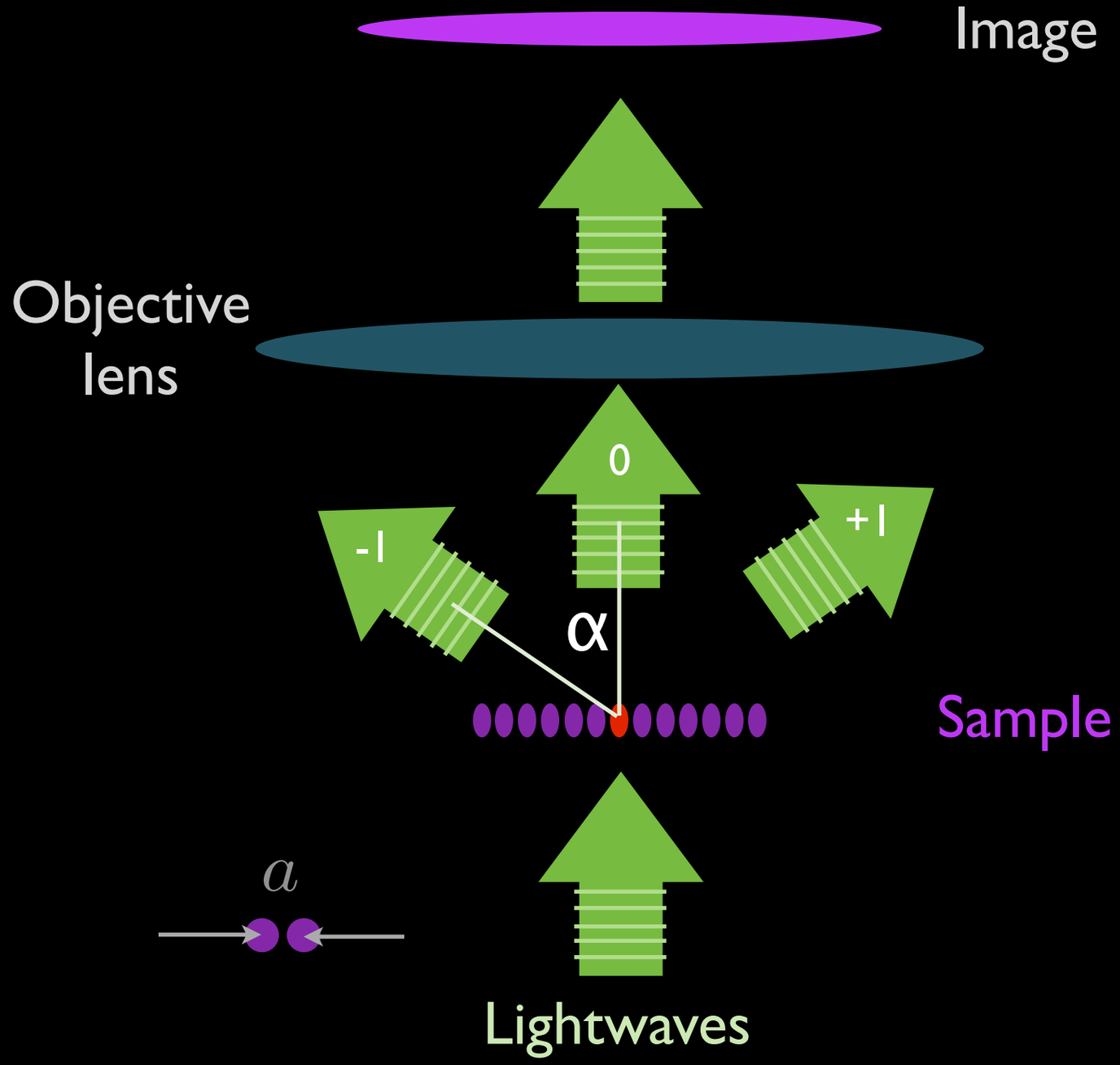
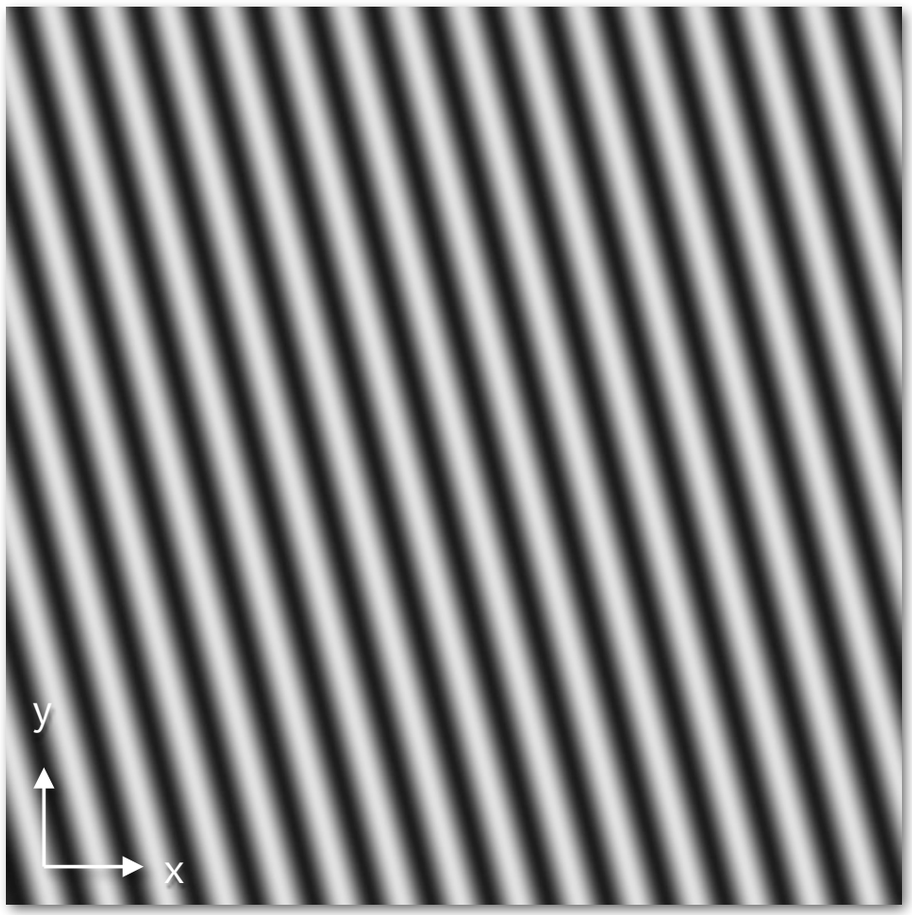


highest frequencies
(biggest α)
→
smallest structures

Fourier transformation in a nutshell

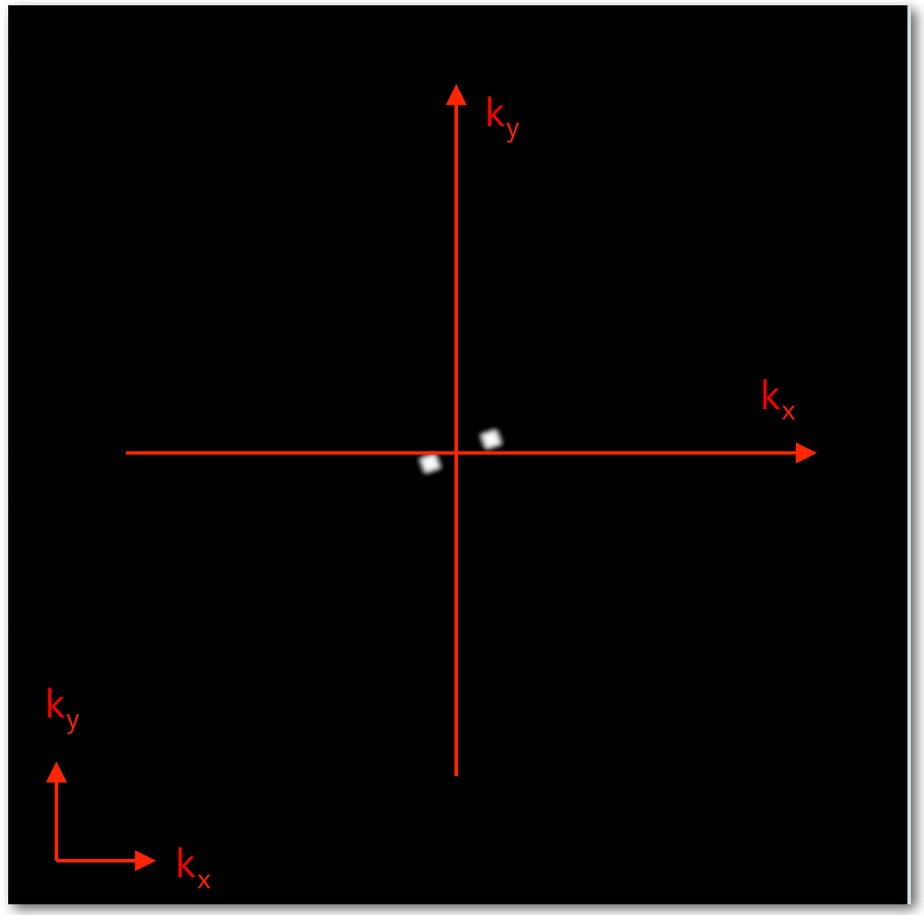
Real space (xy)



Fourier Transform

Inverse
Fourier Transform

Frequency space (k_x, k_y)
(a.k.a. Fourier space, reciprocal space)



Alternative representation of information
Low-resolution: near the origin
High-resolution: farther out
kx, ky: Spatial frequencies, periods/μm

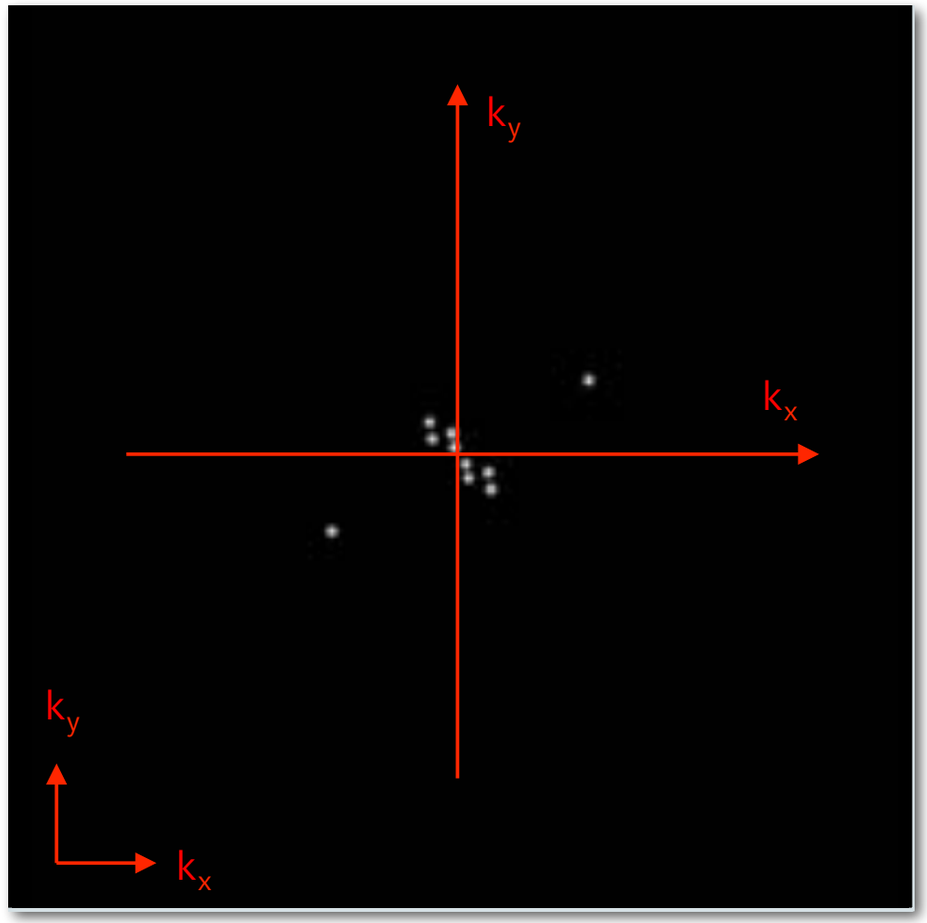
Image = superposed periodicities

Real space (xy)

Fourier Transform

Inverse
Fourier Transform

Frequency space (k_x, k_y)
(a.k.a. Fourier space, reciprocal space)



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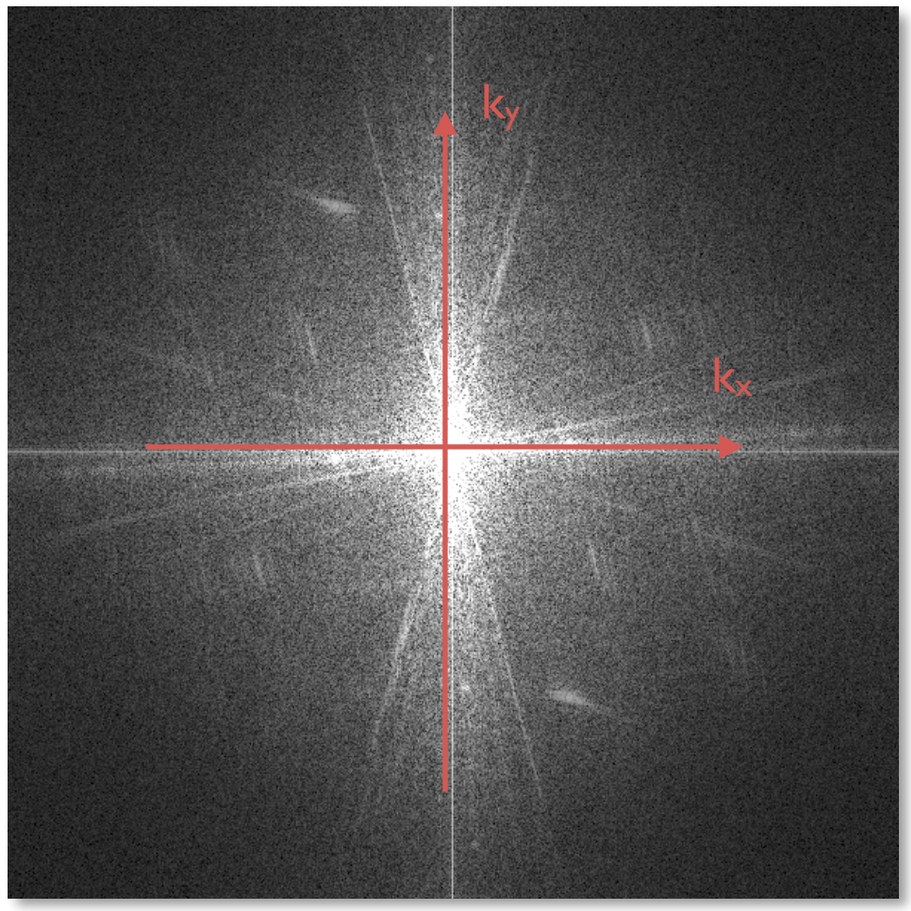

Alternative representation of information
Low-resolution: near the origin
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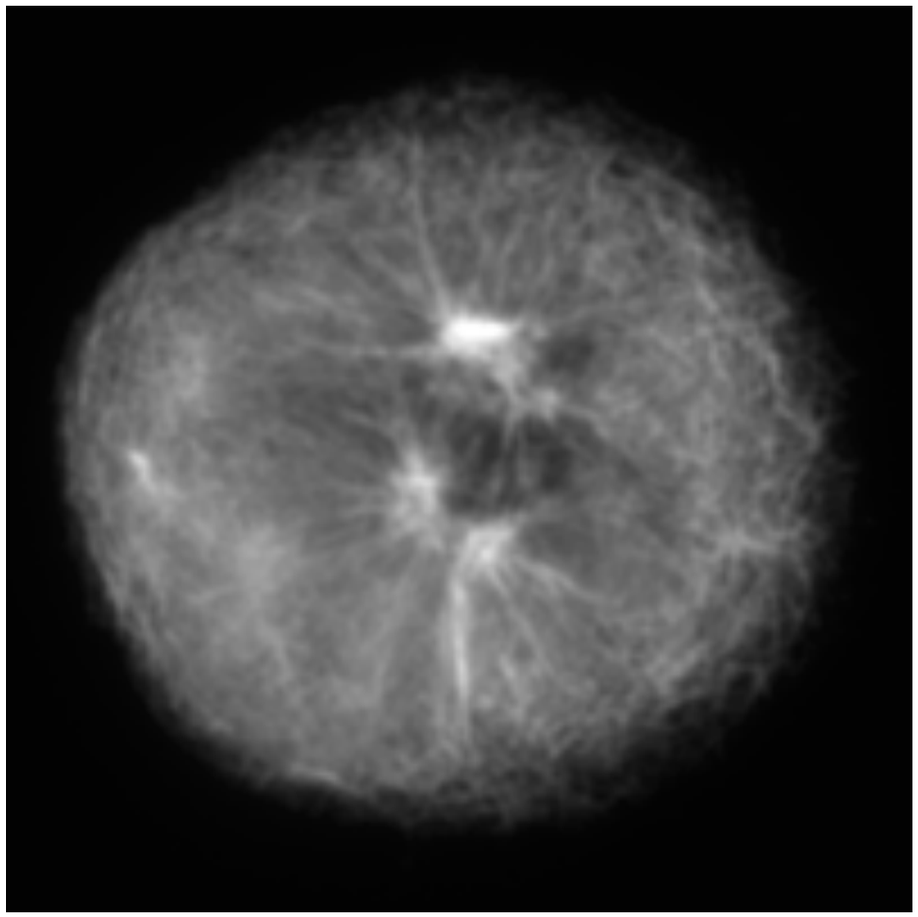
kx, ky: Spatial frequencies, periods/μm

Frequency support in a wide-field microscope

Real space (xy)



Fourier Transform

Inverse
Fourier Transform

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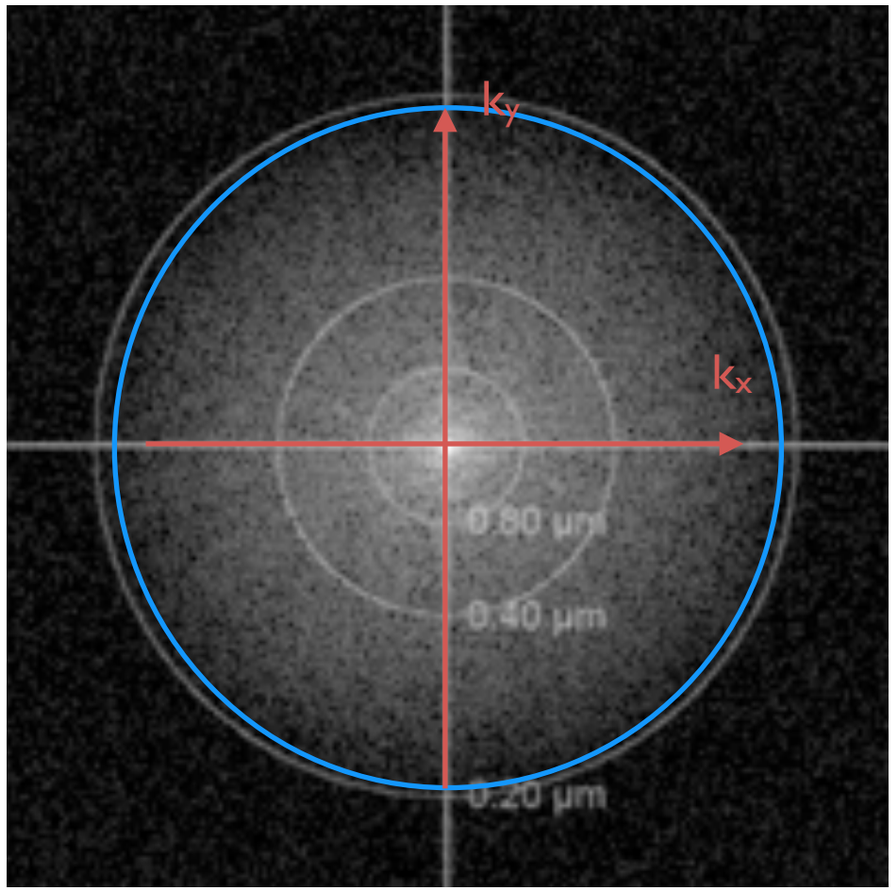
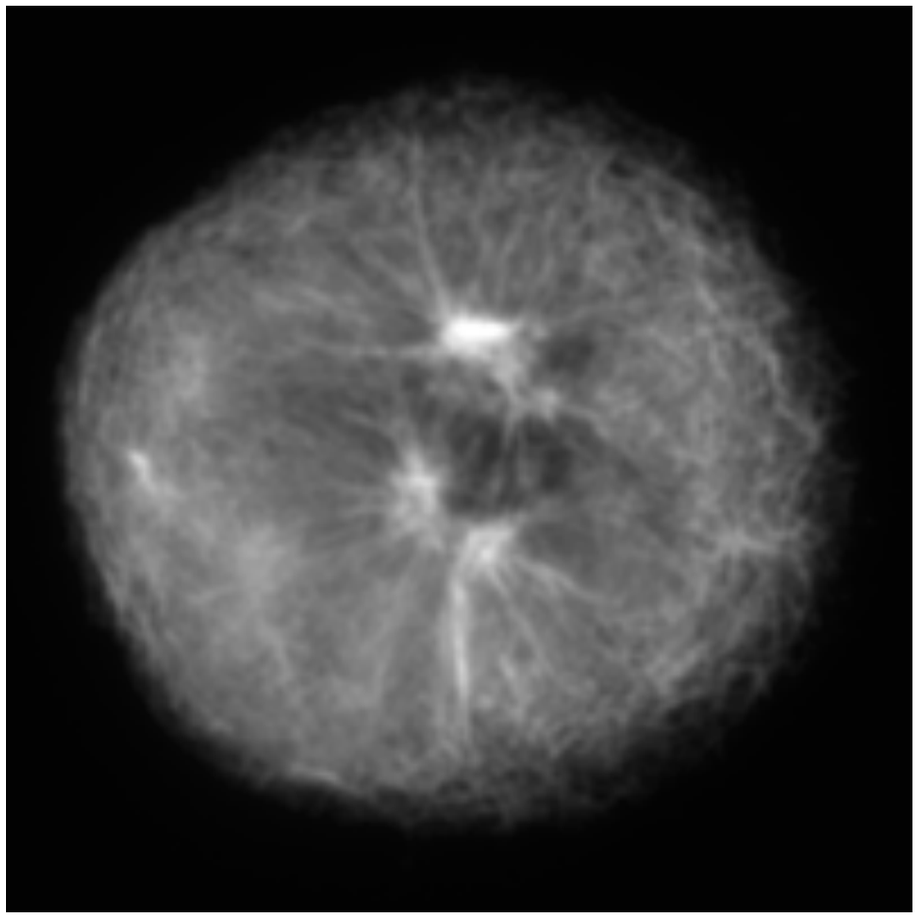


observable region (limited by NA & λ)

Frequency support in a wide-field microscope

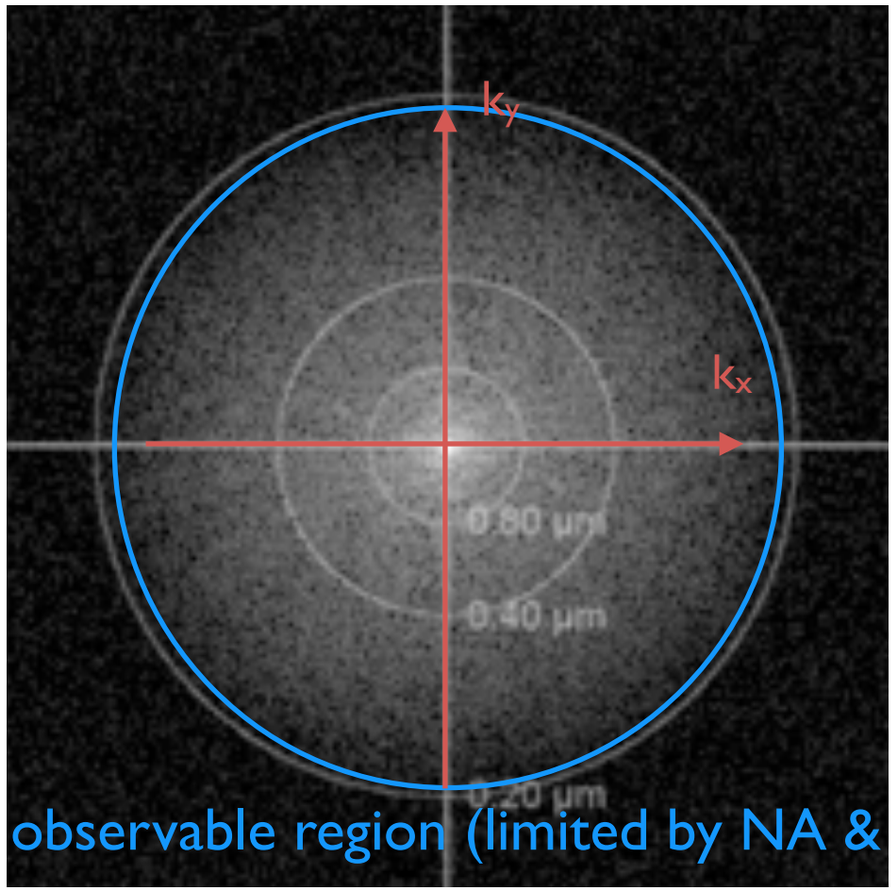
Wide-field image
(= real object convolved with PSF)



Fourier Transform

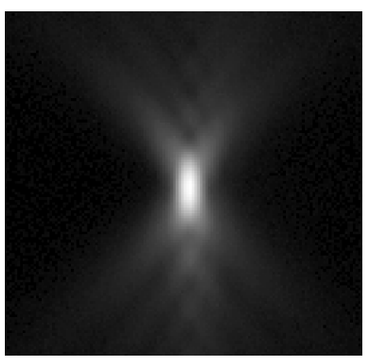
Inverse
Fourier Transform

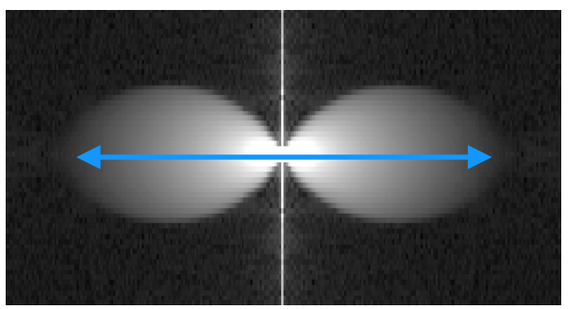
Wide-field frequency space
(= all frequencies multiplied with OTF)



observable region (limited by NA & λ)

Point spread function
PSF
(orthogonal, x-z)

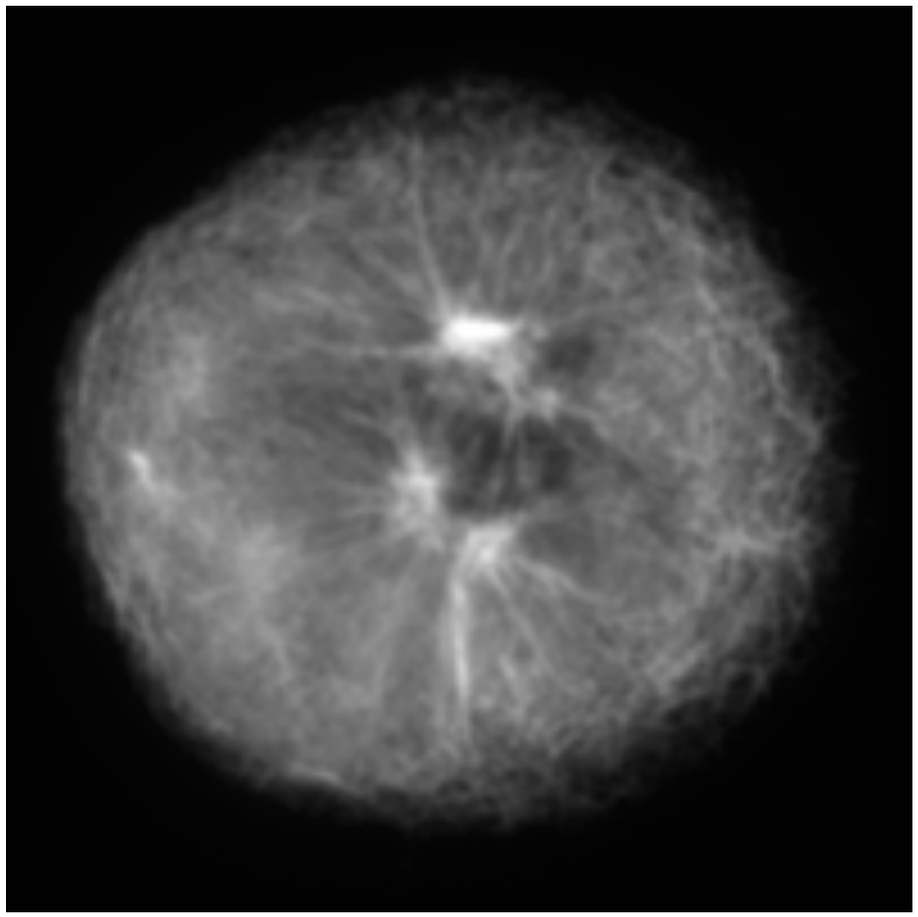




Optical
transfer function
OTF
(orthogonal, k_x,-k_z)

Frequency support in a wide-field microscope

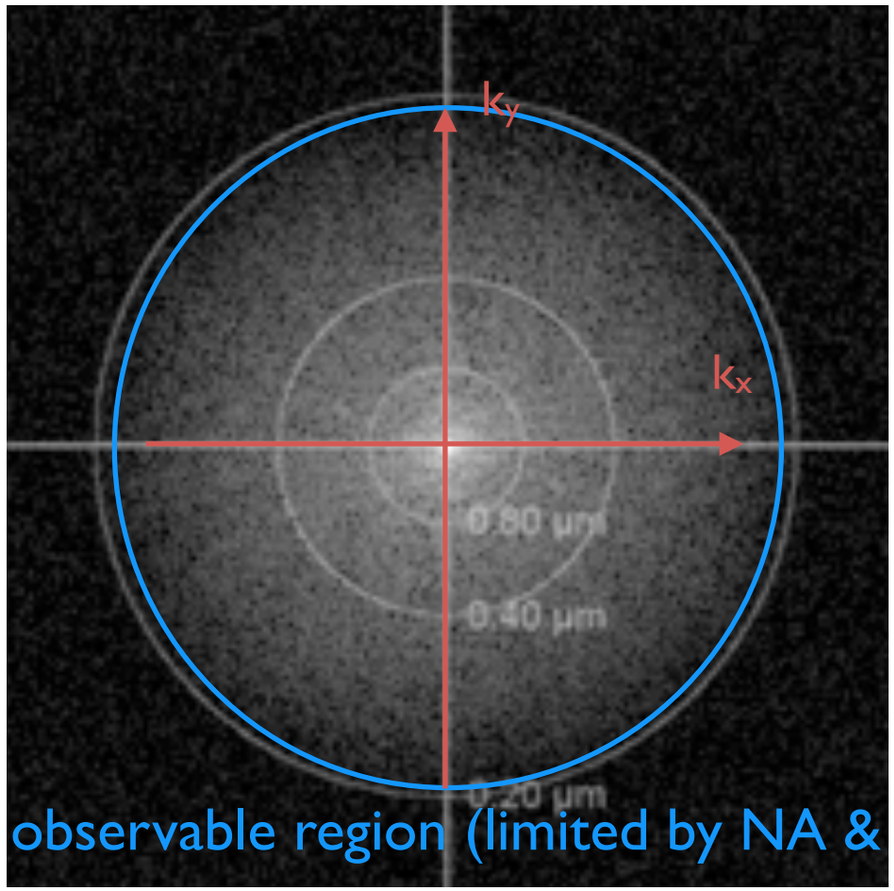
Wide-field image
(= real object convolved with PSF)



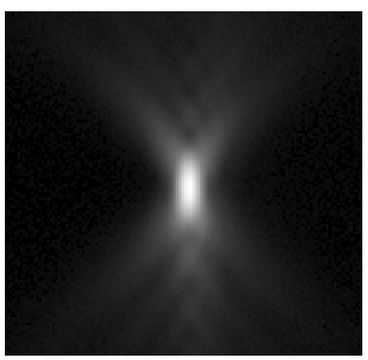
Fourier Transform

Inverse
Fourier Transform

Wide-field frequency space
(= all frequencies multiplied with OTF)

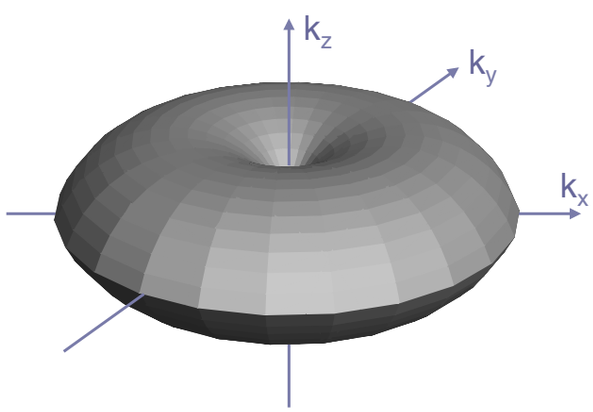


Point spread function
PSF
(orthogonal, xz)



Fourier Transform

Inverse
Fourier Transform



Optical
transfer function
OTF
(3D rendering)

SIM principle: Moiré interference

Fourier transform of the measured image

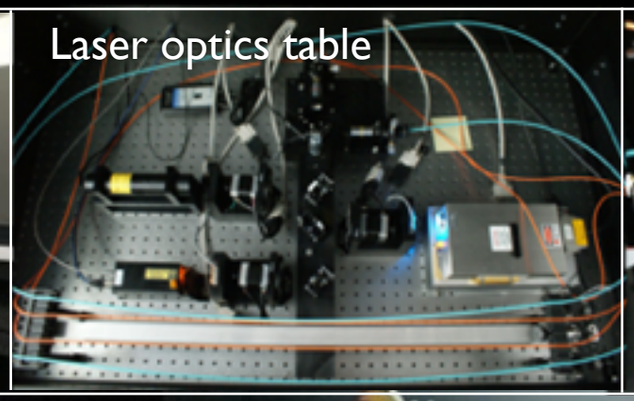
unknown structure

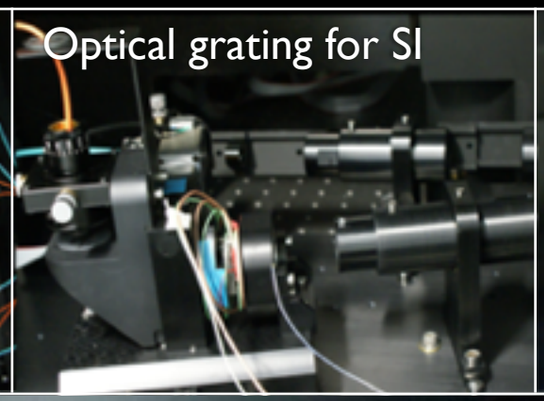
$$F\{f \times g\} = F\{f\} \otimes F\{g\} \quad \longrightarrow \quad F\{f\} = F\{f \times g\} \otimes^{-1} F\{g\}$$

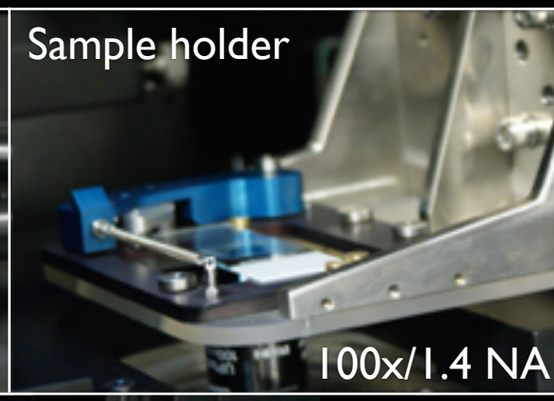
known illumination function

How to generate an illumination pattern in a microscope?

OMX 3D-SIM microscope system

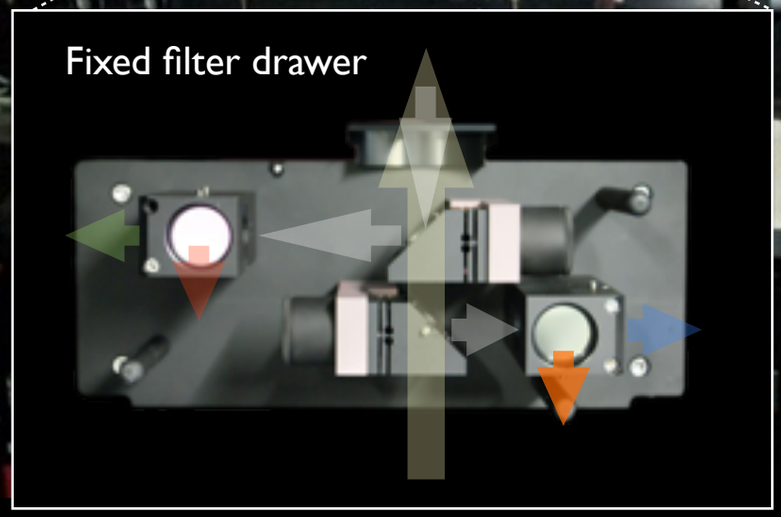






Max. mech. stability
Highest sensitivity

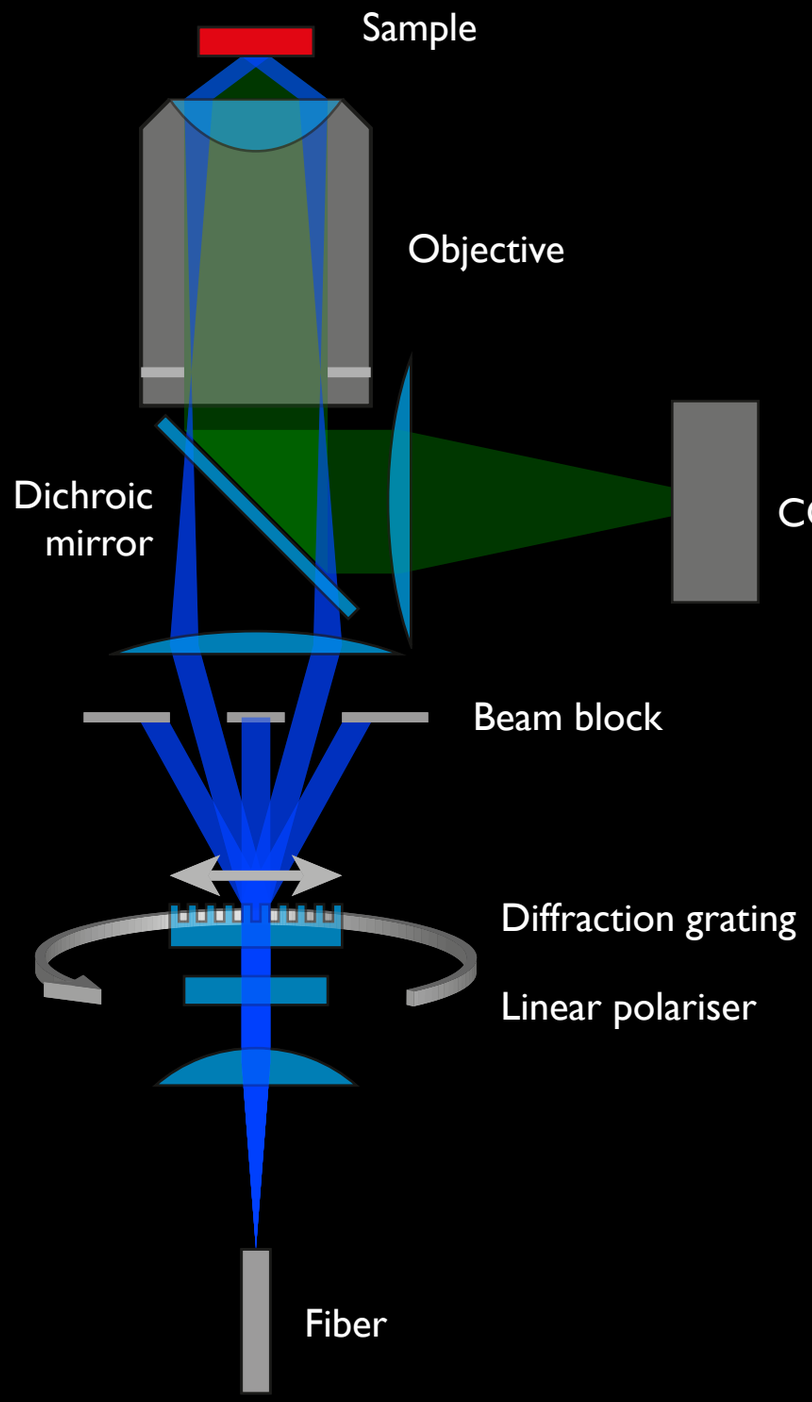
High-sensitive
EMCCD camera
(3x)



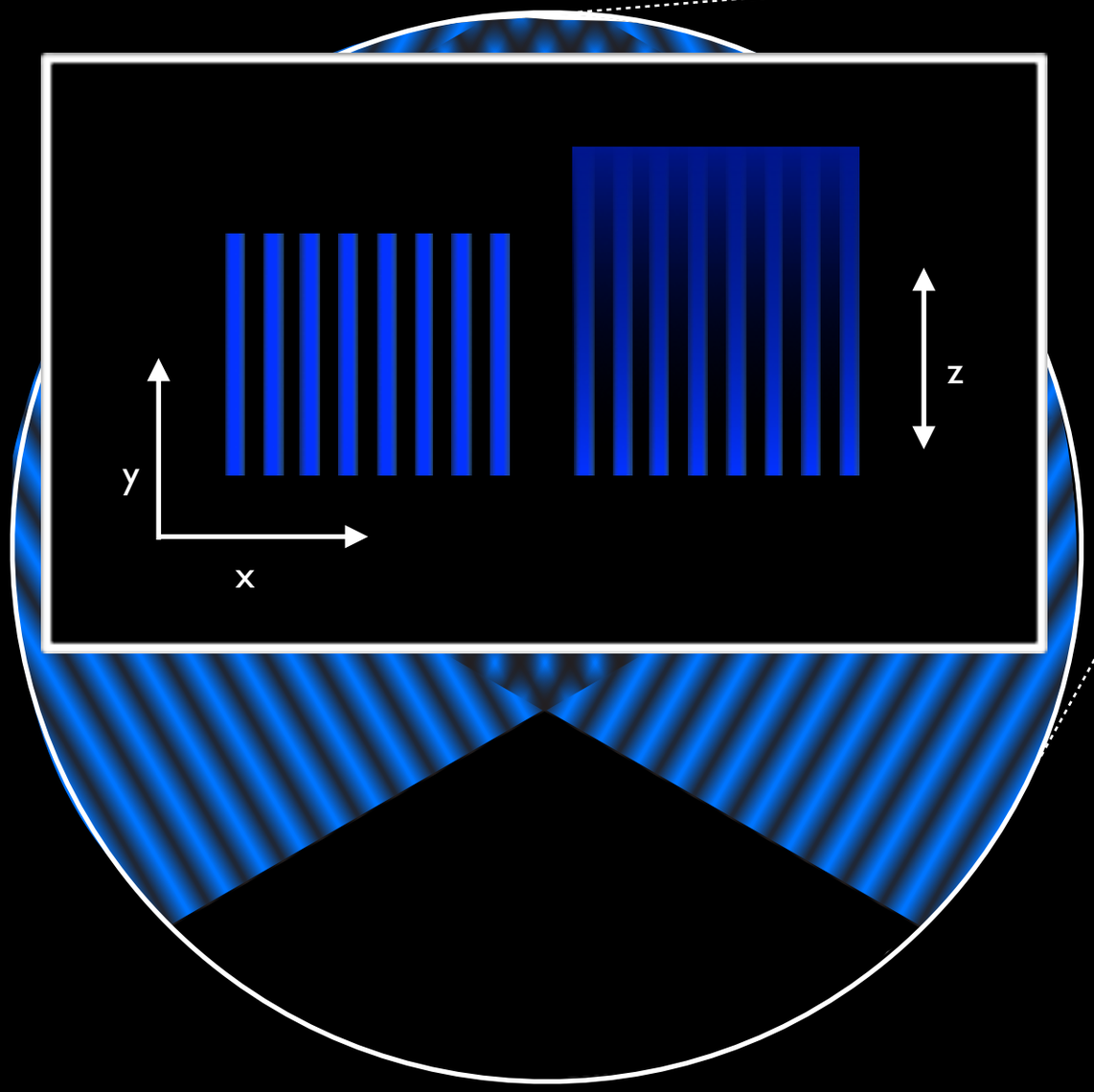
Mess

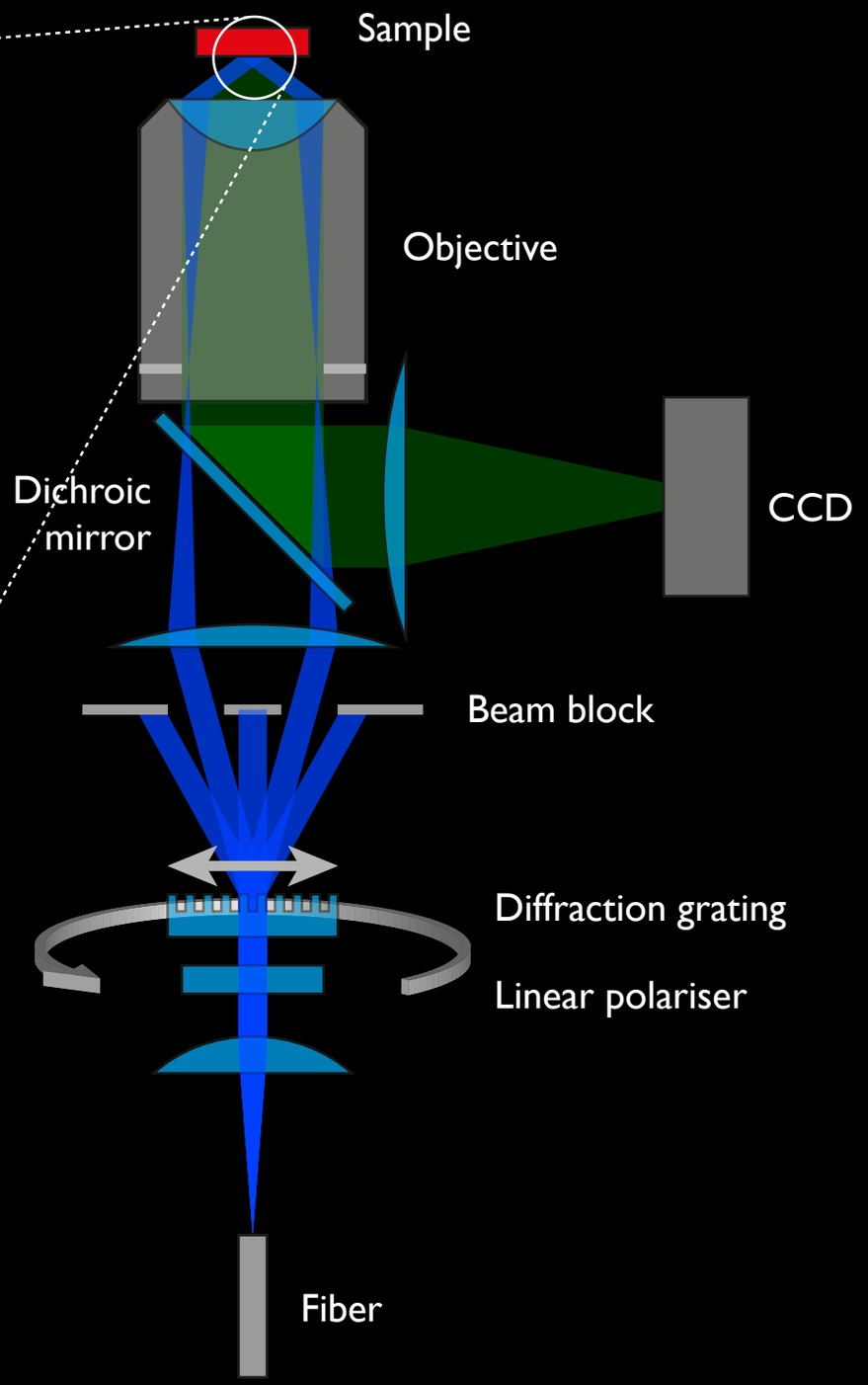
OMX V2

2D-SIM: microscope design



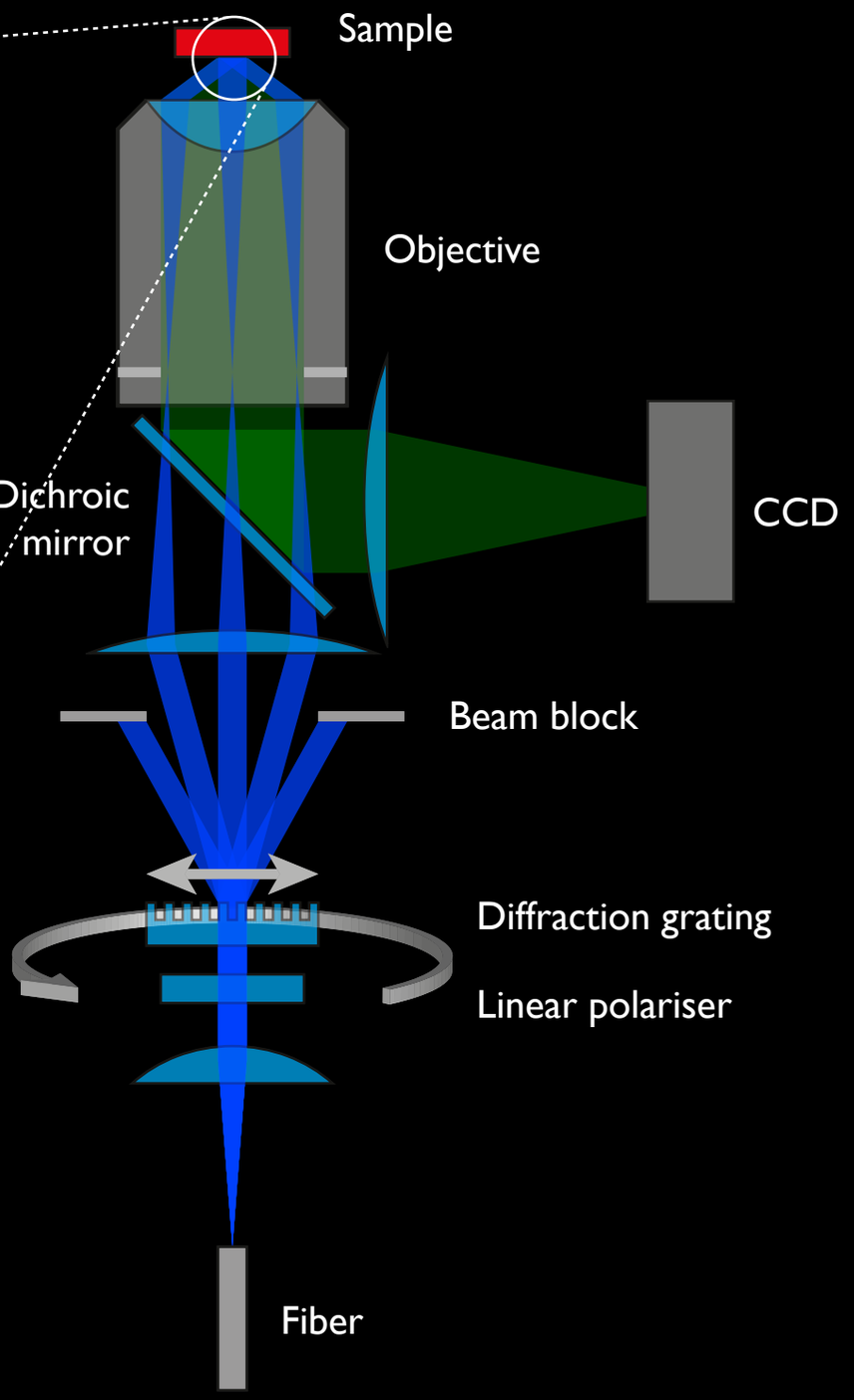
2D-SIM: microscope design



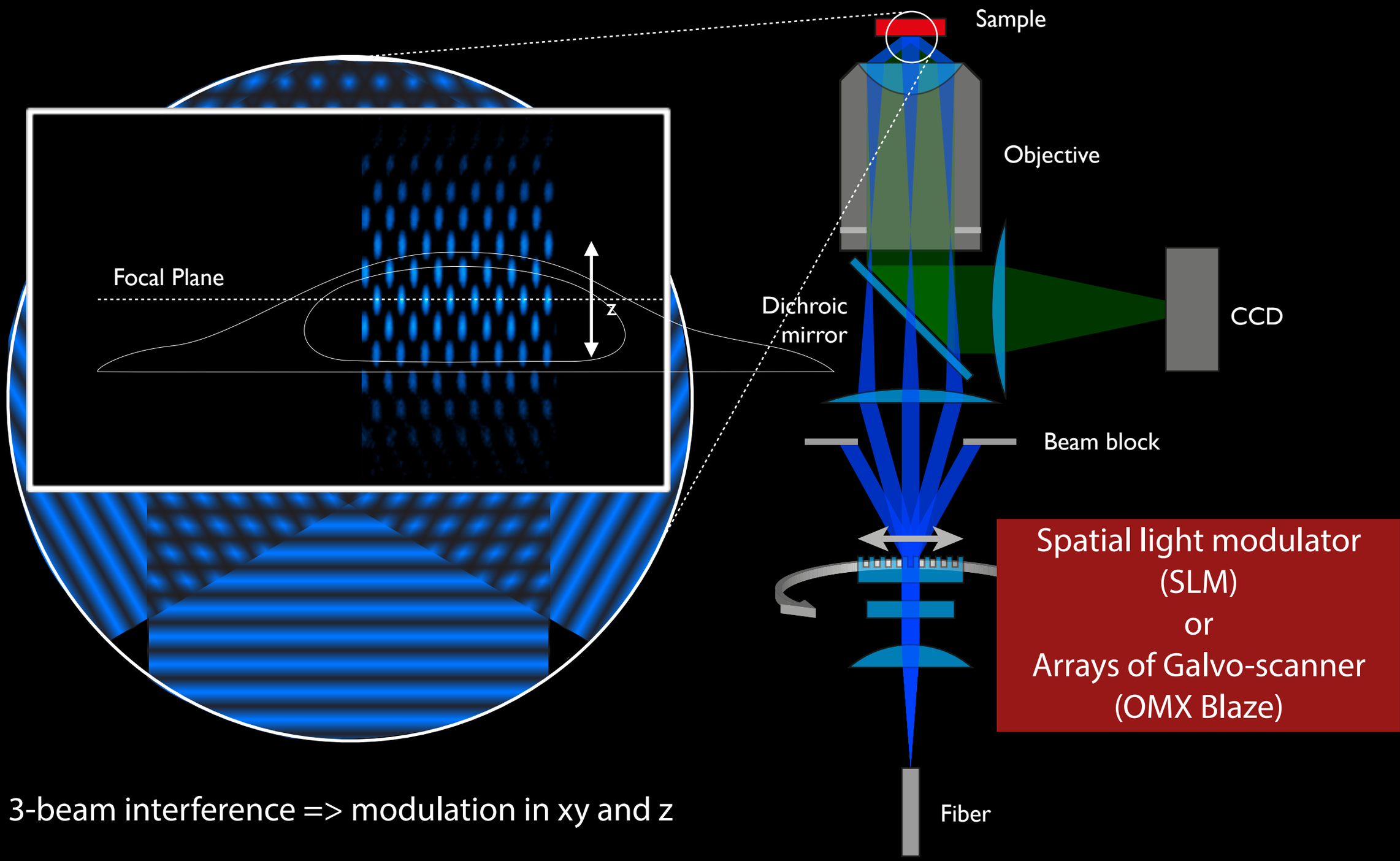


2-beam interference => modulation (stripes) in xy

3D-SIM: microscope design



3D-SIM: microscope design



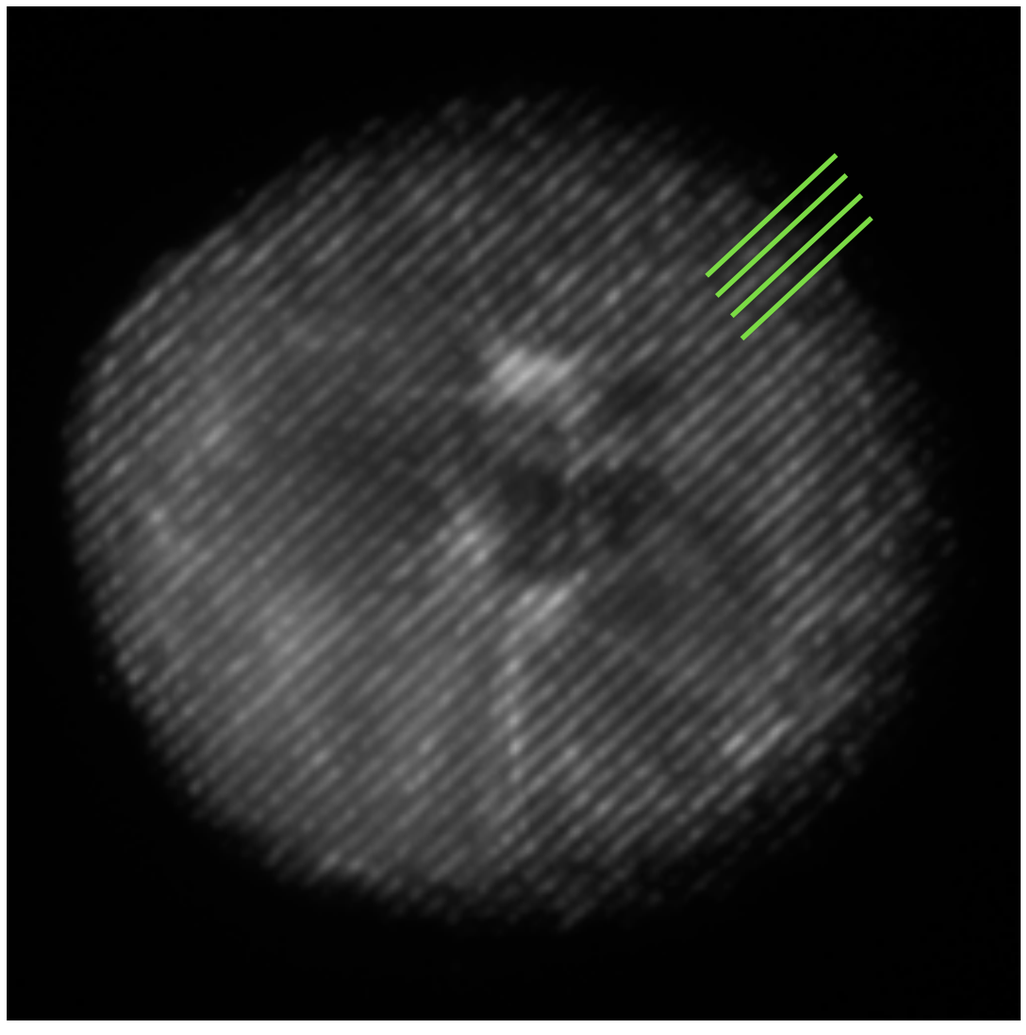
3-beam interference => modulation in xy and z

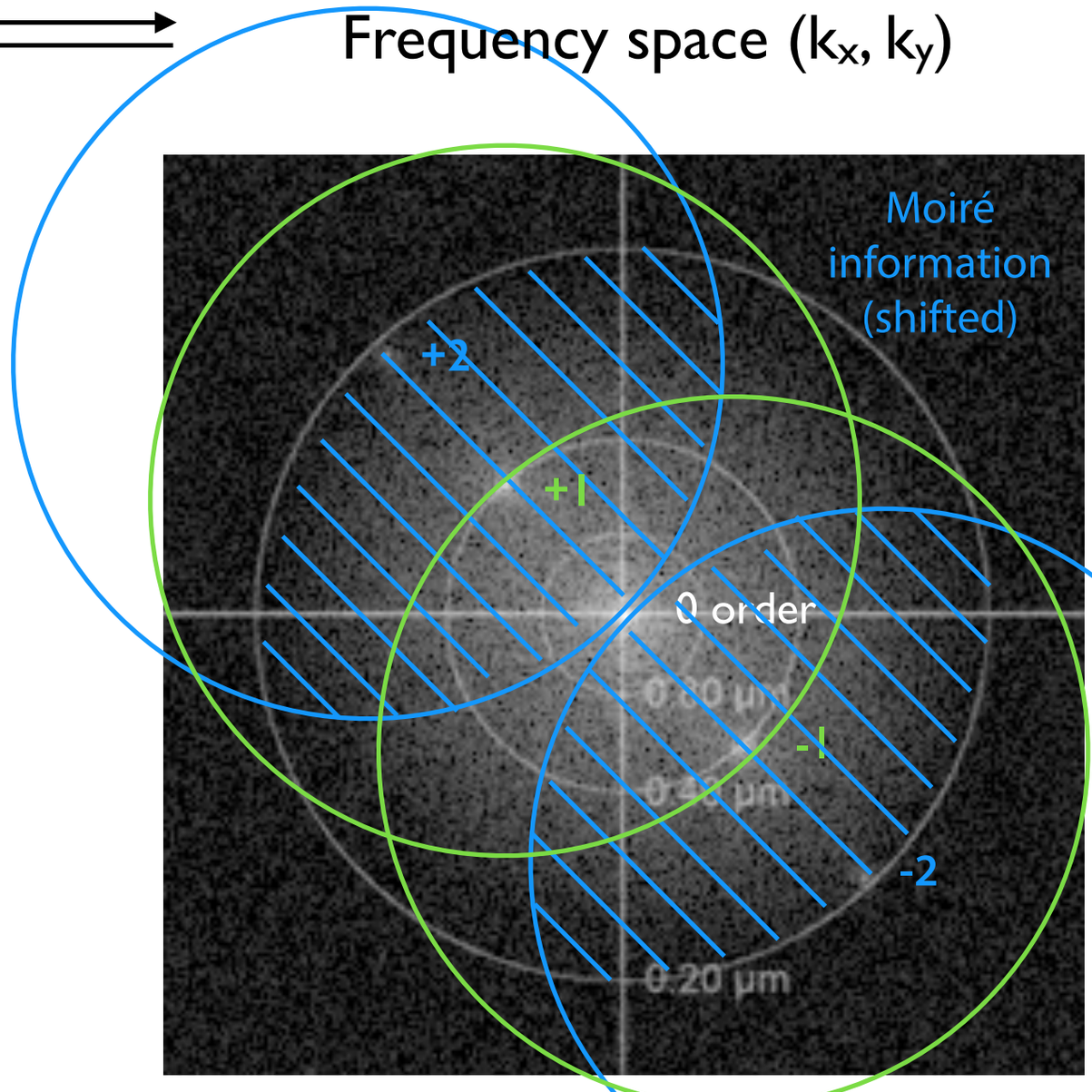
Doubling frequency support in x-y and z

Real space (xy)

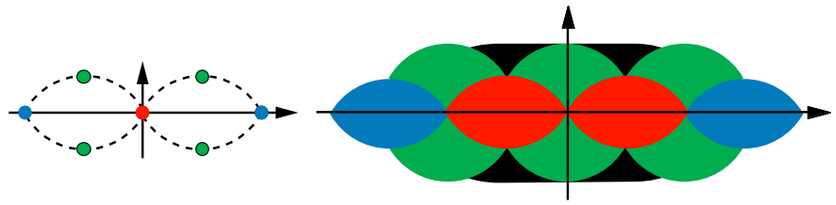
FFT

Frequency space (k_x, k_y)

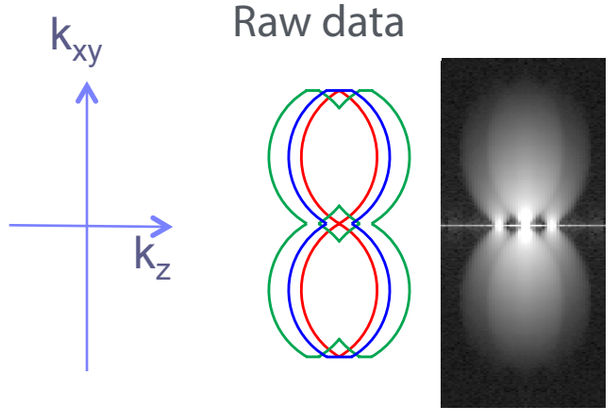




Doubling frequency support in x-y and z

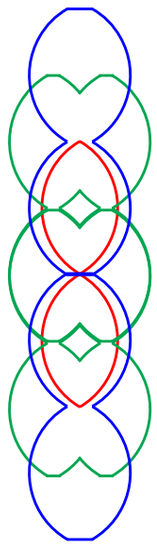


Raw data



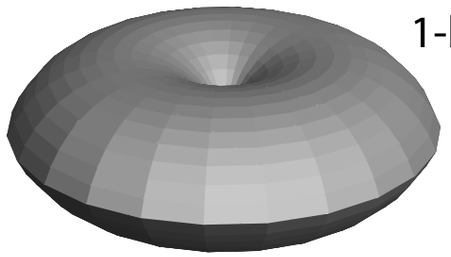
Separated bands

Shifted and reassembled

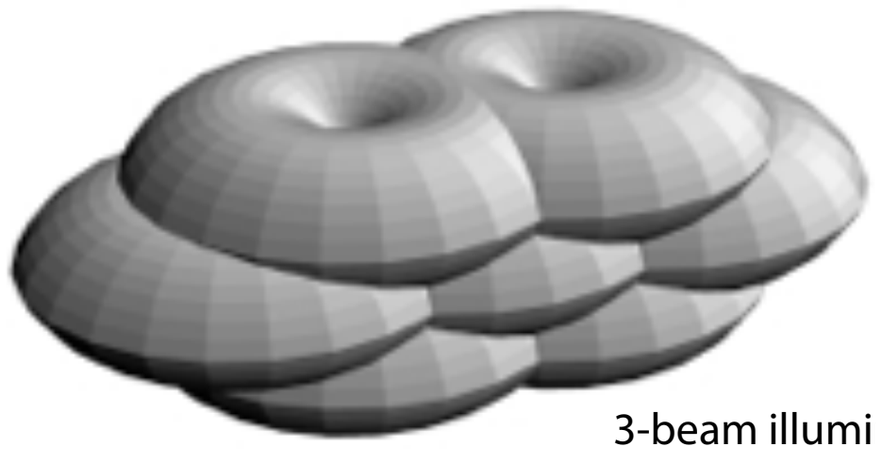


5 phase shifts
(2π/5)

Observable region (3D rendering)



1-beam illumination
(wide-field)



3-beam illumination

3-beam illumination
3 orientations

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

3D-SIM: How does it work in practice?

Raw SI data

Tubulin in Drosophila macrophage

3D-SIM: How does it work 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 times 10-100 ms

Tubulin in Drosophila macrophage

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

1st angle

2nd angle

SI raw

3rd angle

projected wide-field

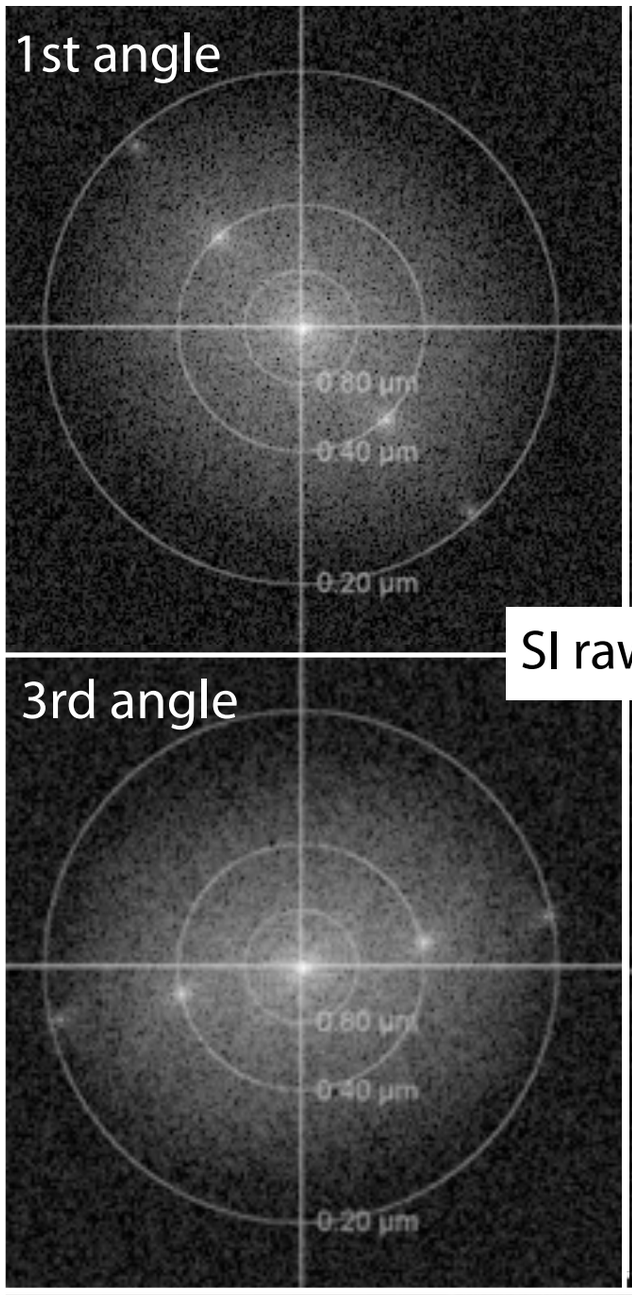
5 µm

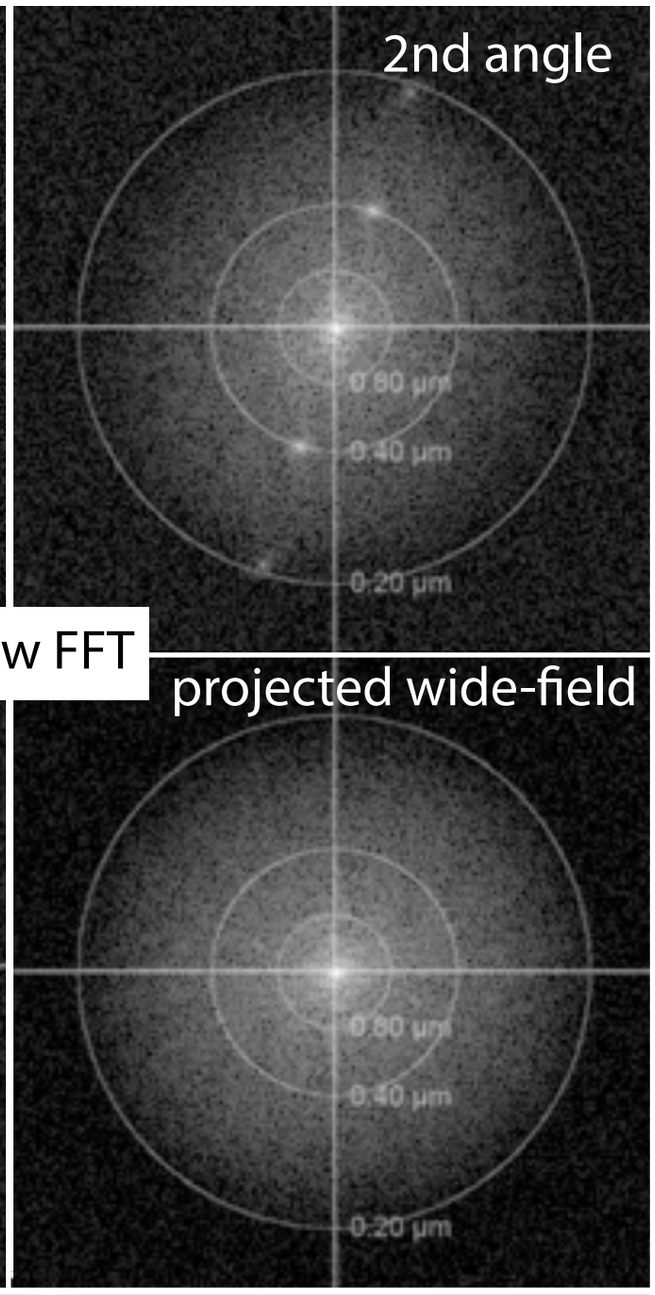
SI reconstructed

5 µm

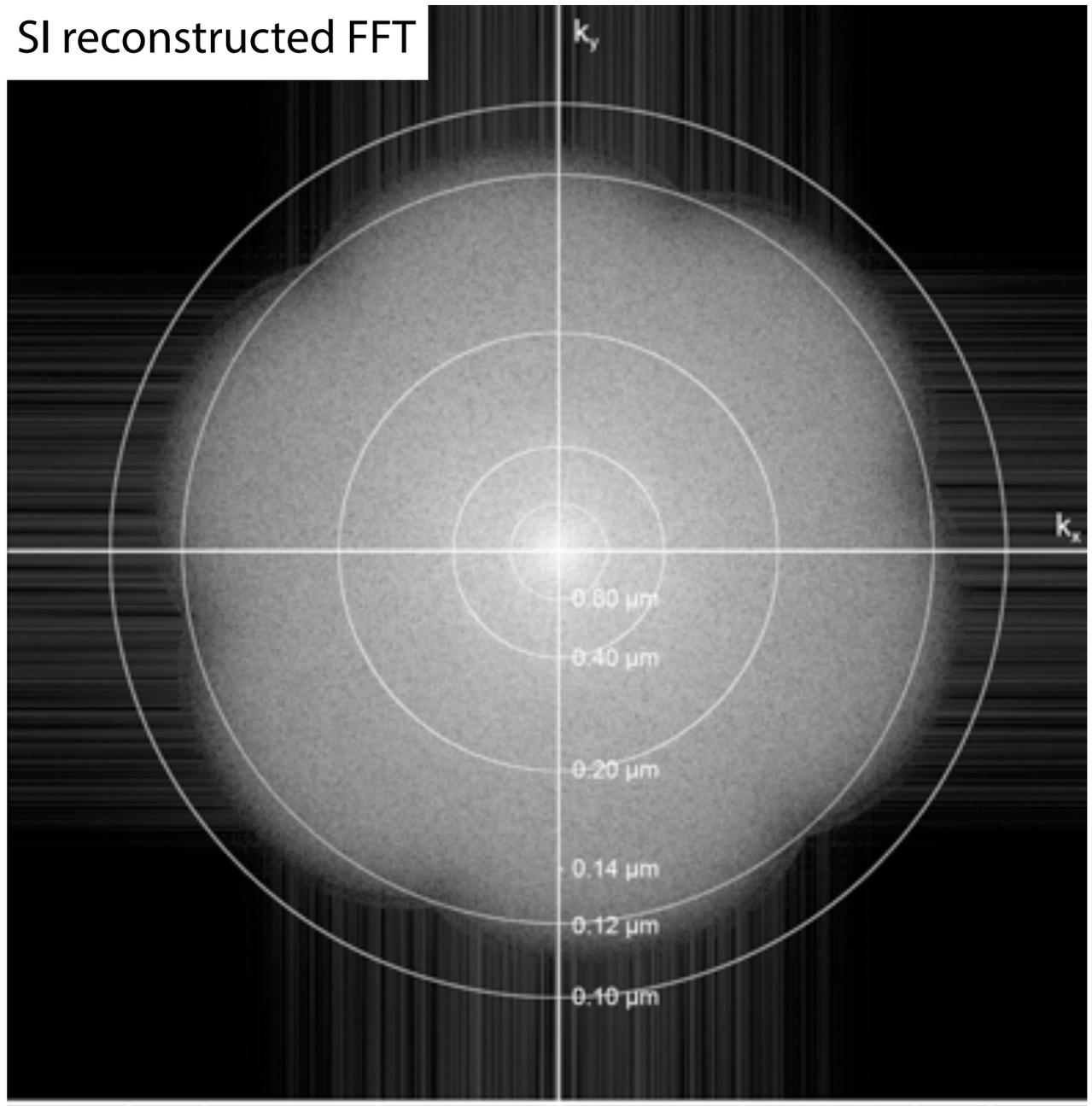
orthogonal view

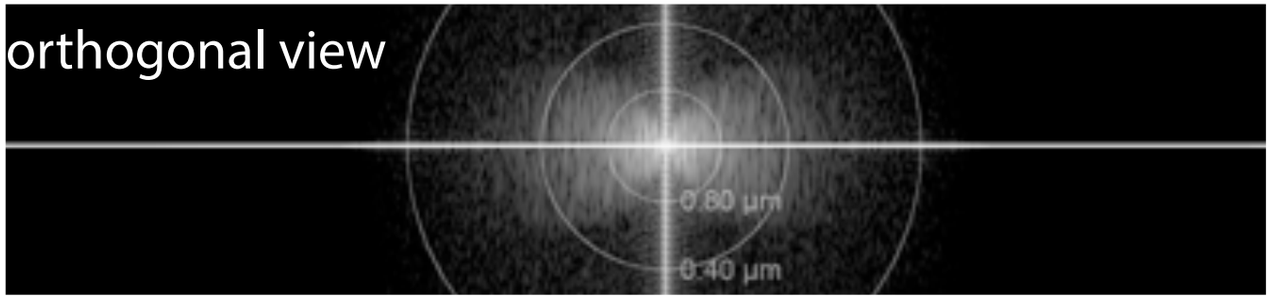
Doubled frequency support = 2-fold resolution in xy and z

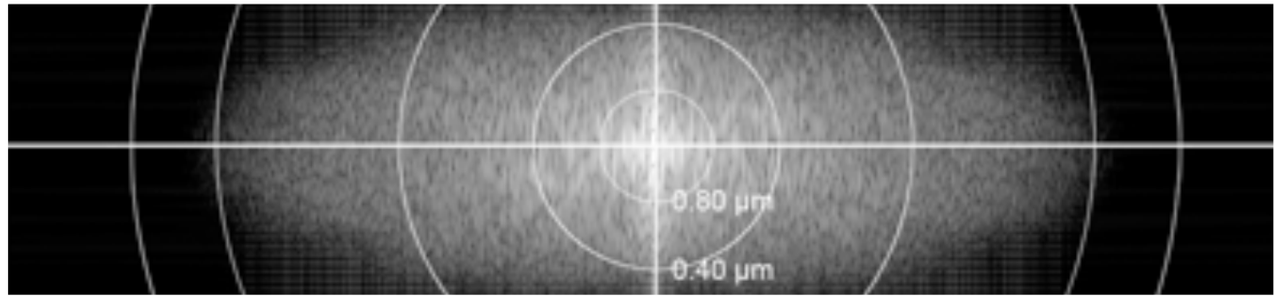




SI raw FFT

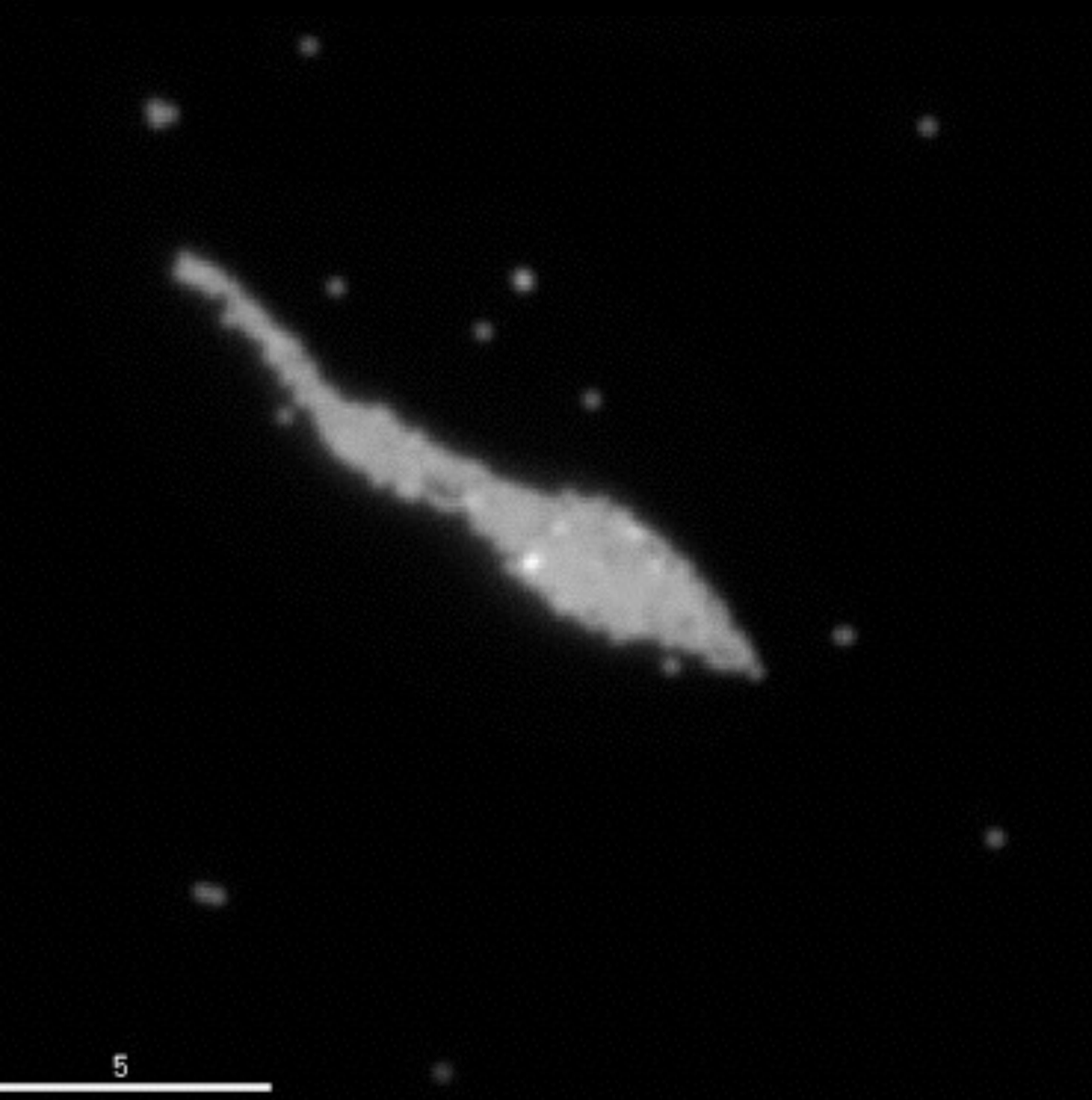


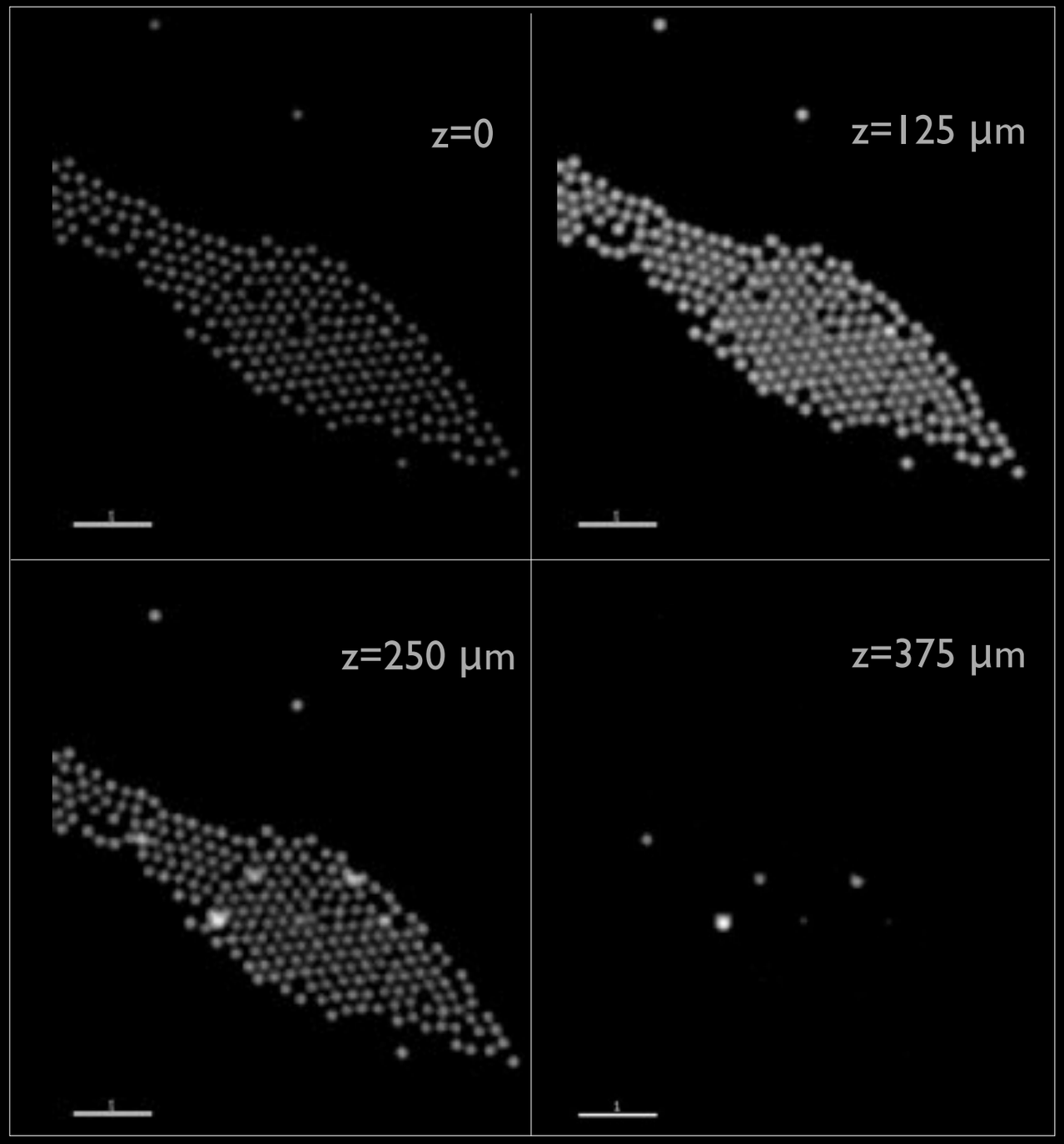




3D optical sectioning capacity

Example: 170 nm Fluospheres

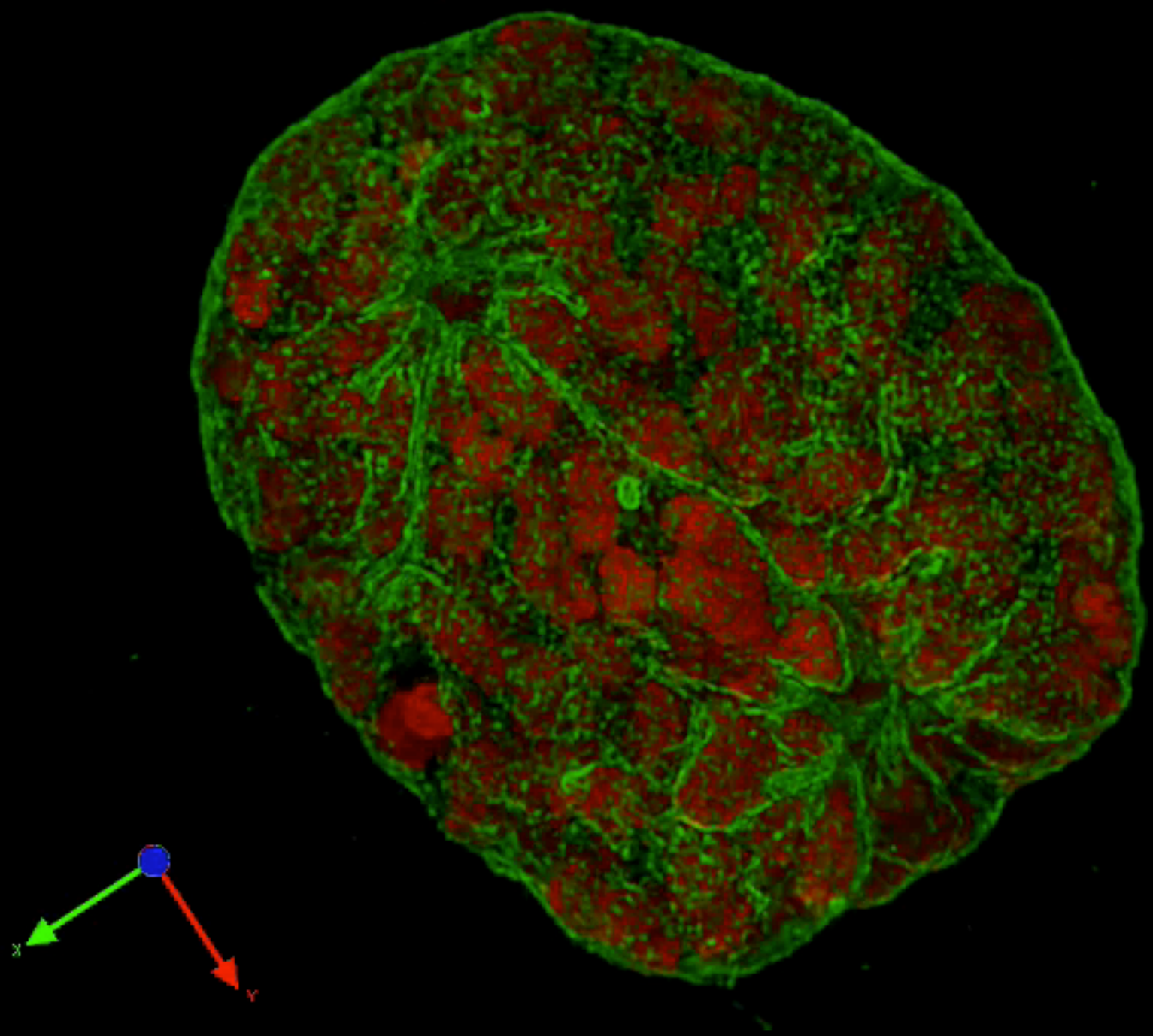




3D SIM example: Prophase

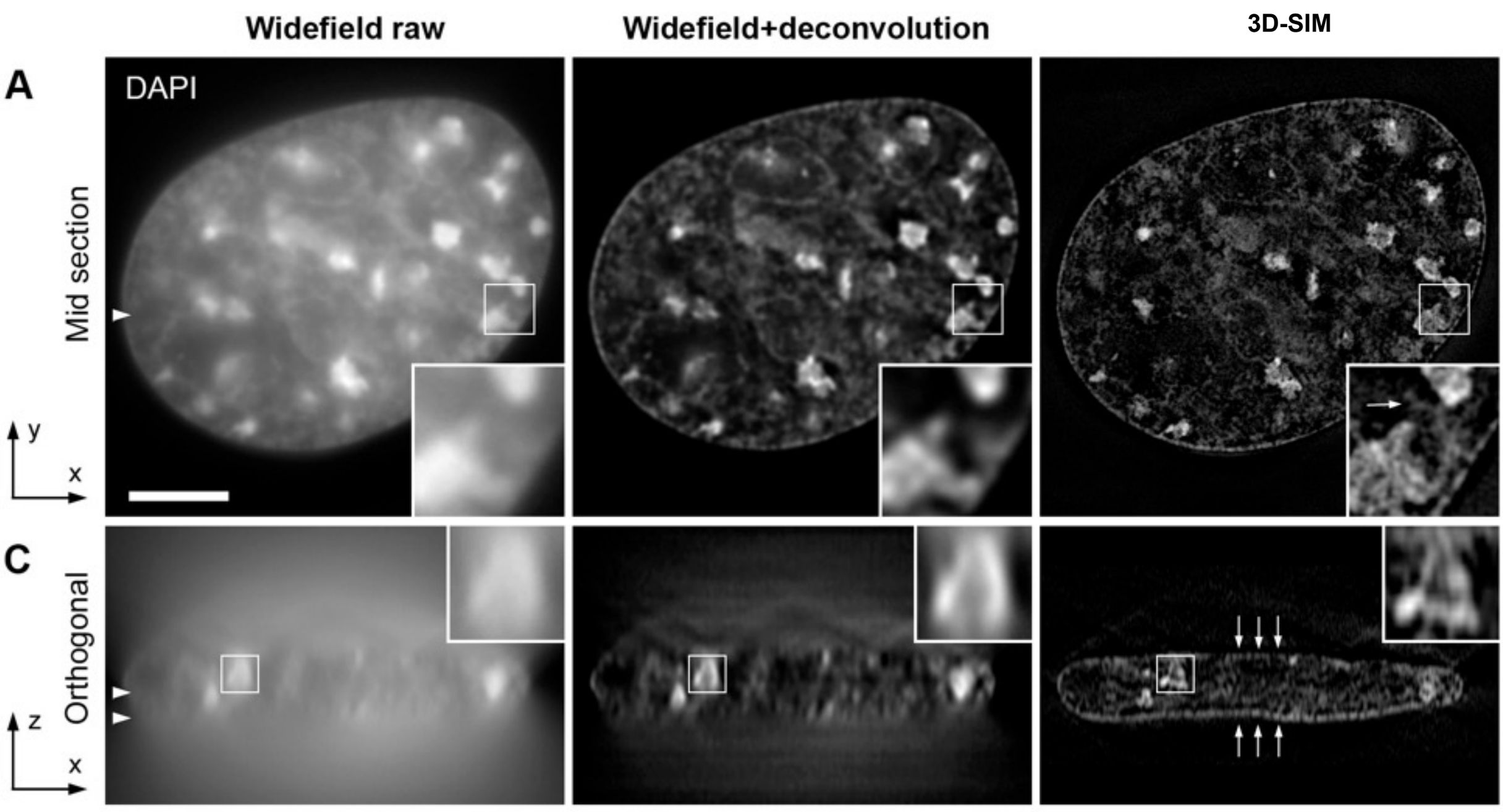
Lamin B
DAPI

3D volume rendering



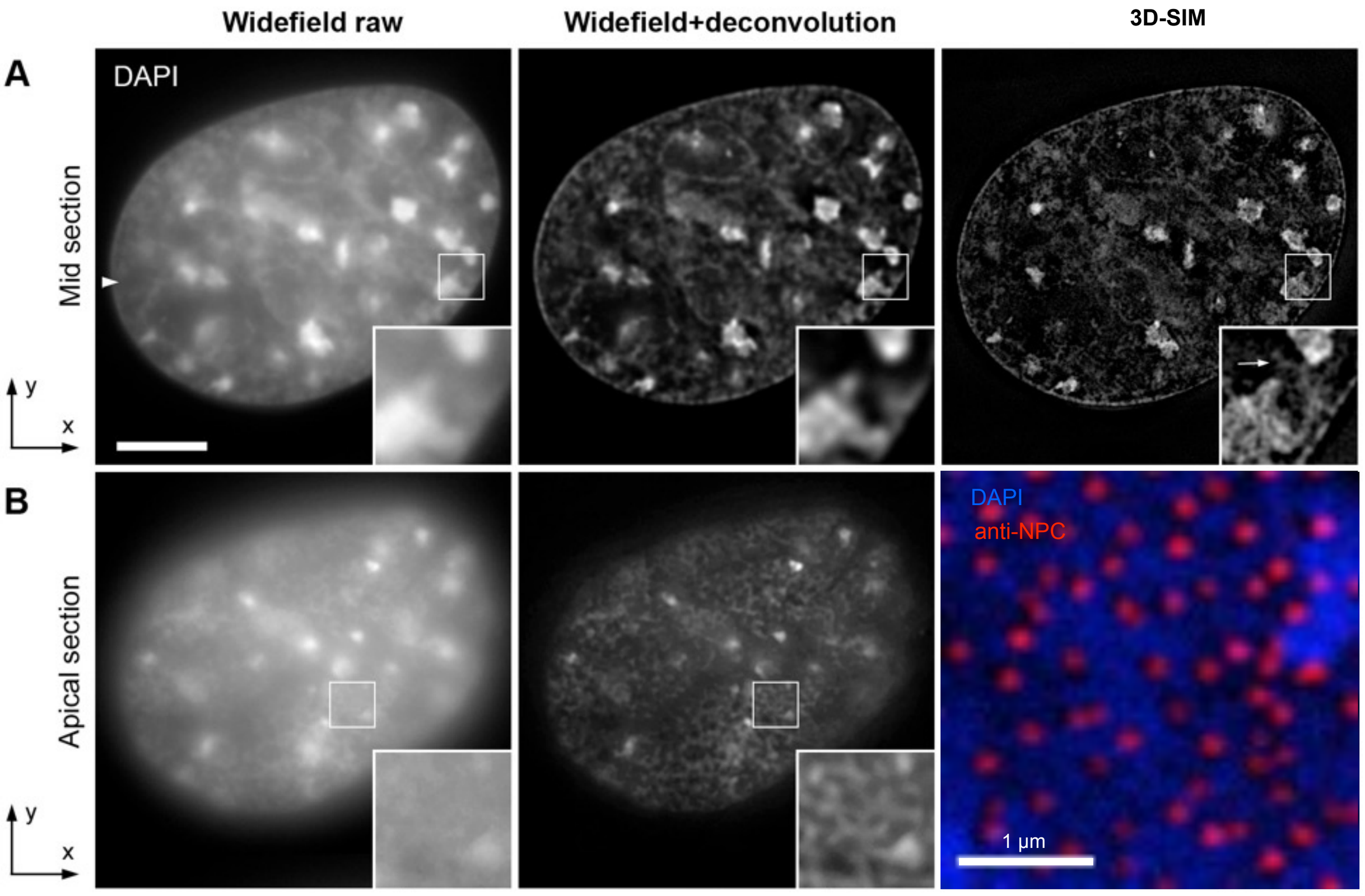
Schermelleh, Carlton et al. (2008), *Science* **320**

3D SIM imaging of chromatin



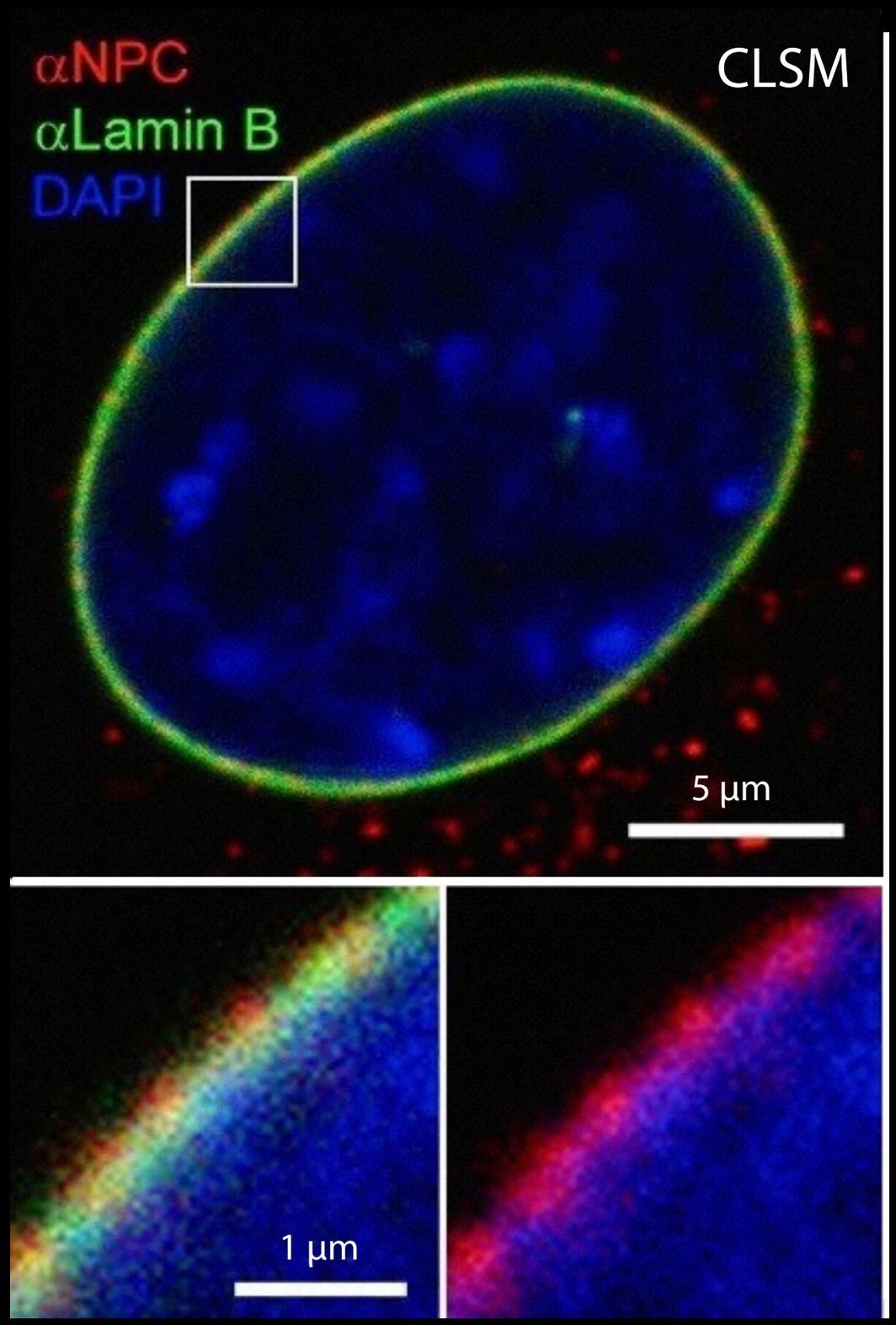
Schermelleh, Carlton et al. (2008), *Science* **320**

3D SIM imaging of chromatin



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

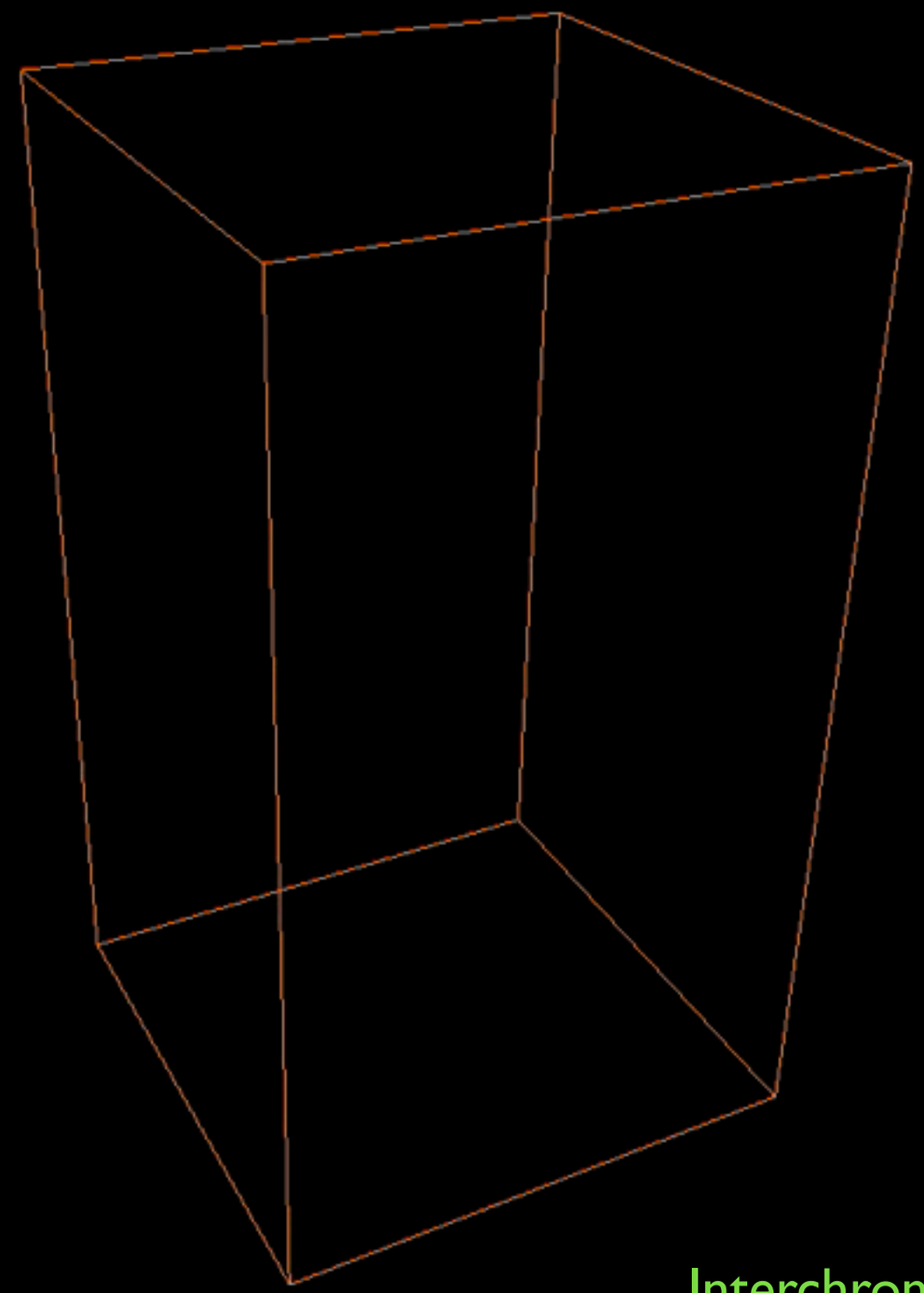
3D-SIM resolves chromatin domains and interchromatin channels



Mouse C2C12 cells

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

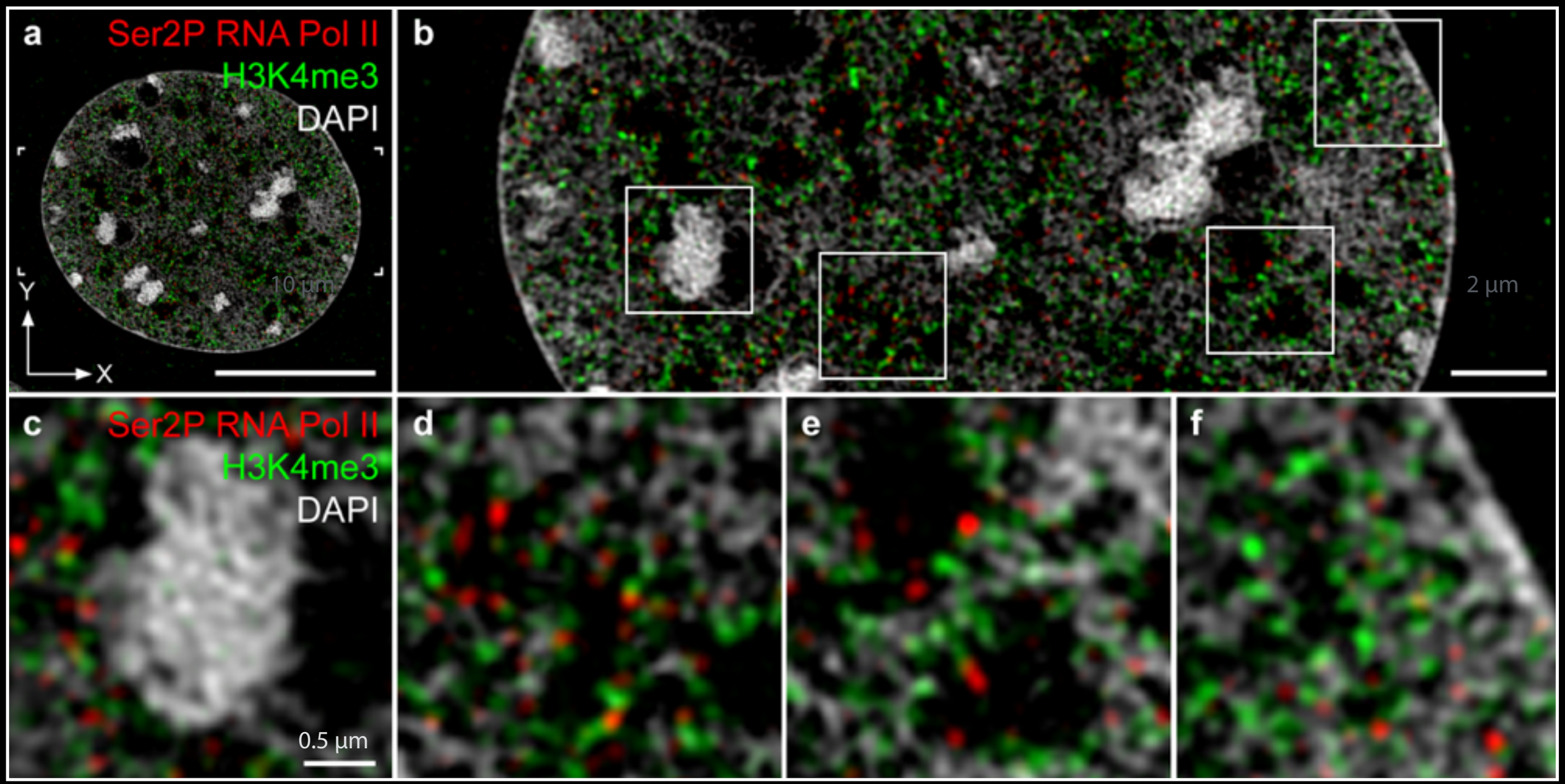
Compartmentalisation of the 3D nuclear space



Chromatin
Interchromatin compartment (IC)

D. Smeets

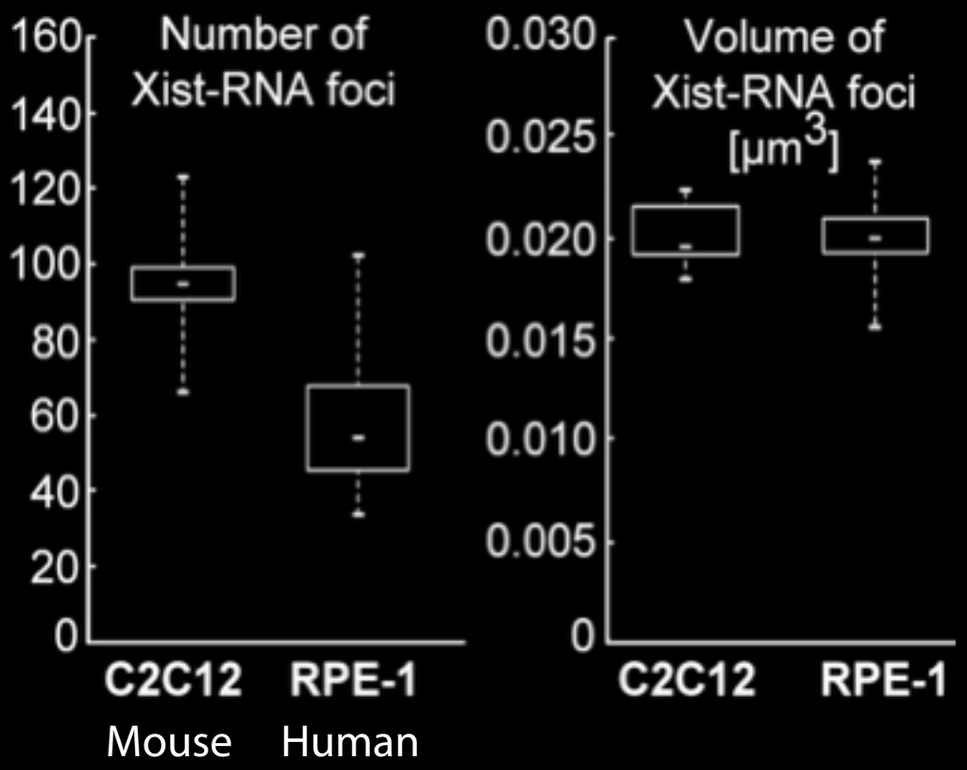
Active marker are constrained to chromatin domain boundaries



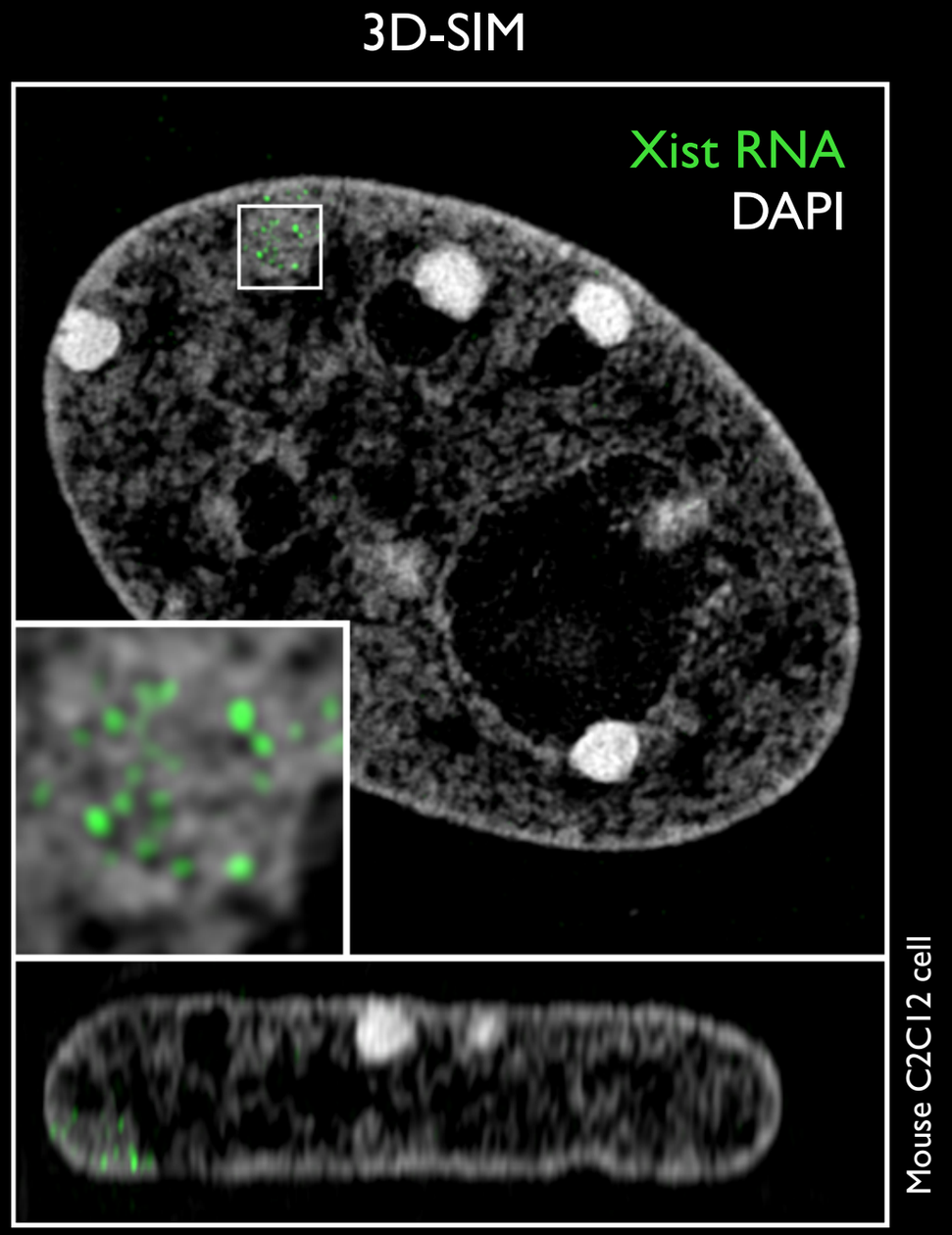
Mouse C127 cell

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

Super-resolution topology inactive X-chromosome



Smeets et al. (2014), *Epigenetics & Chromatin*



Markaki et al., (2013) *Methods Mol Biol*

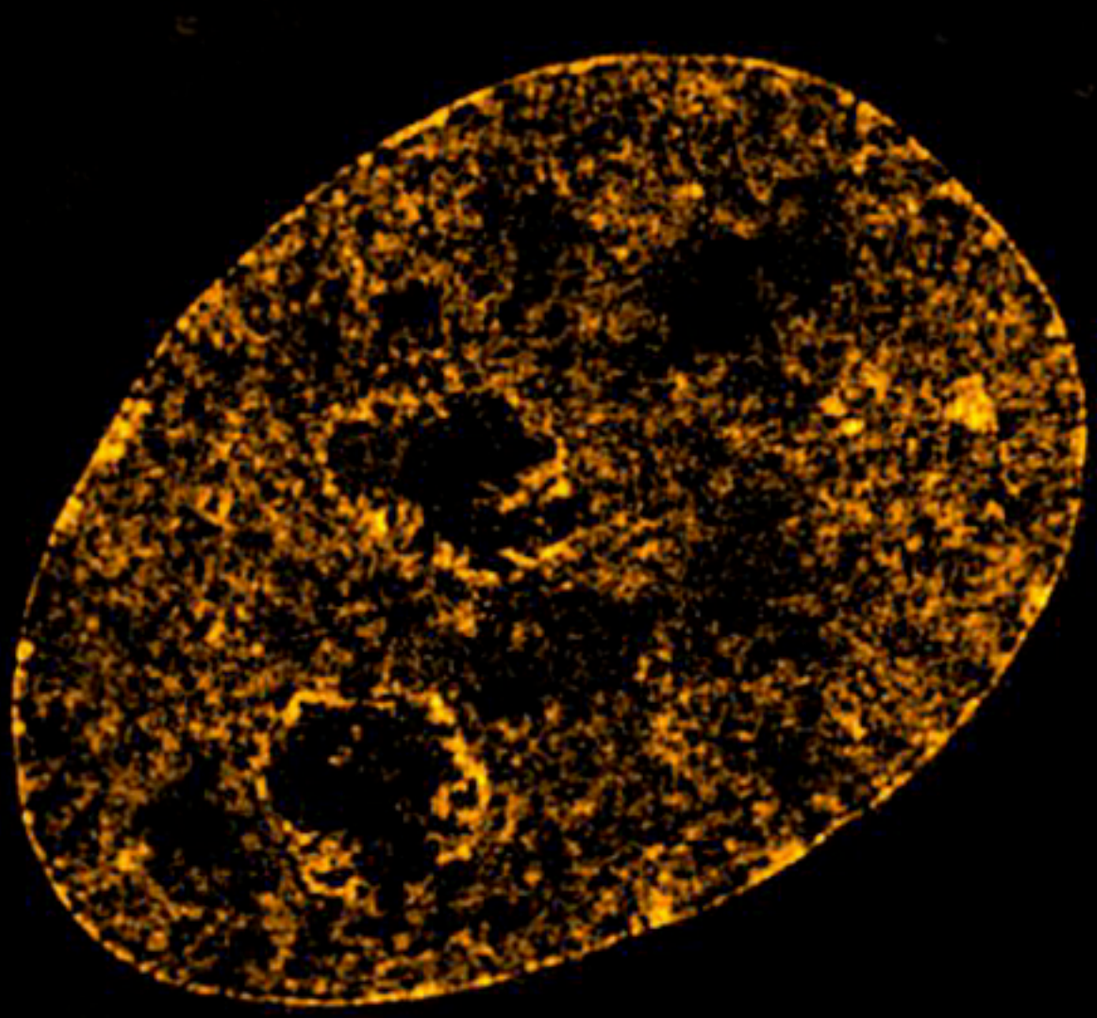
Xist RNA forms distinct domains within the Barr Body
Evidence for multimerisation (3-10 Xist RNAs/focus)

Can we go live?

Live cell 3D-SIM with OMX Blaze

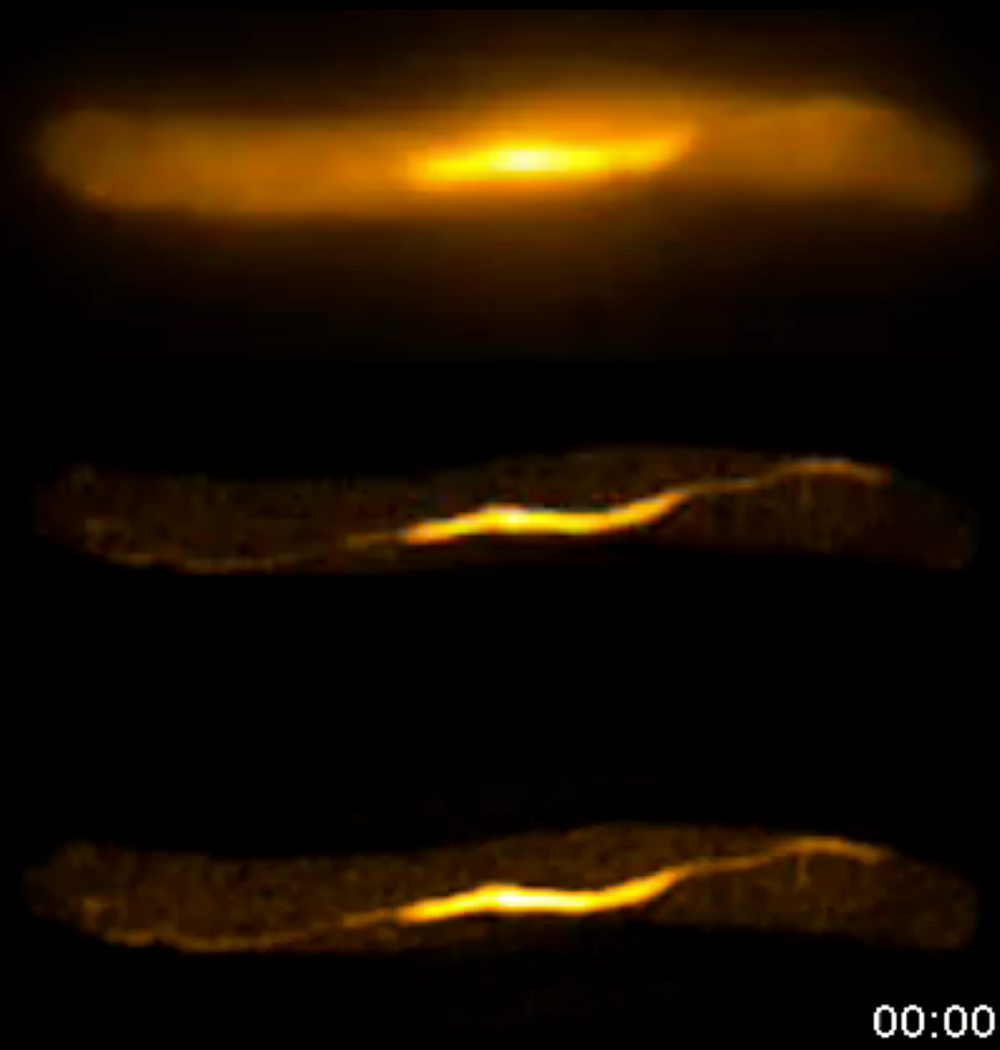
Inferometric pattern generation + sCMOS cameras → 10 x faster imaging

H2B-GFP (unfixed)



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

RecA-GFP (*E.coli*)



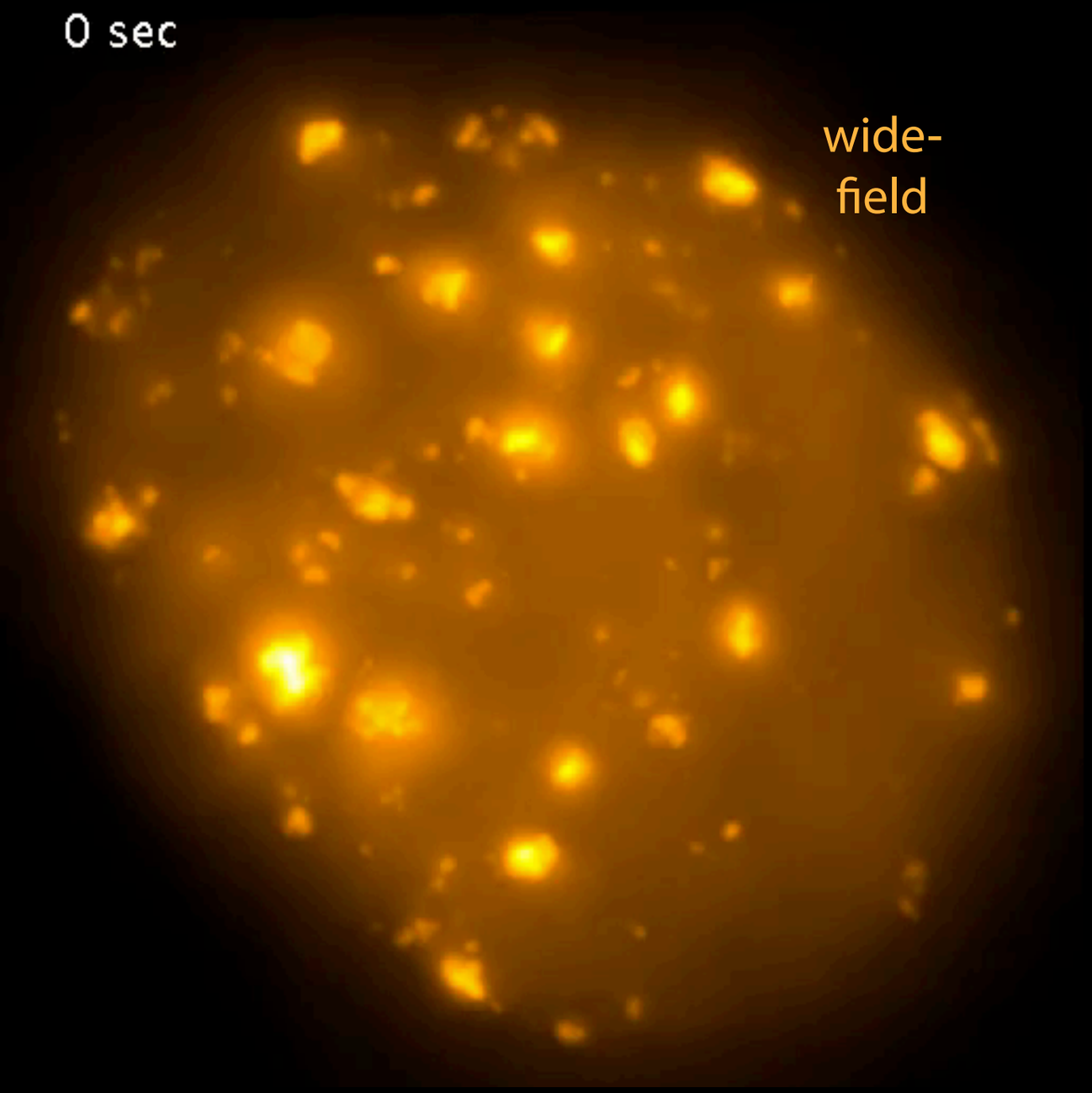
2 s / 3D-frame (1 µm z-stack = 120 images ; 100 ime points)

Christian Lesterlin (D. Sherratt Lab)

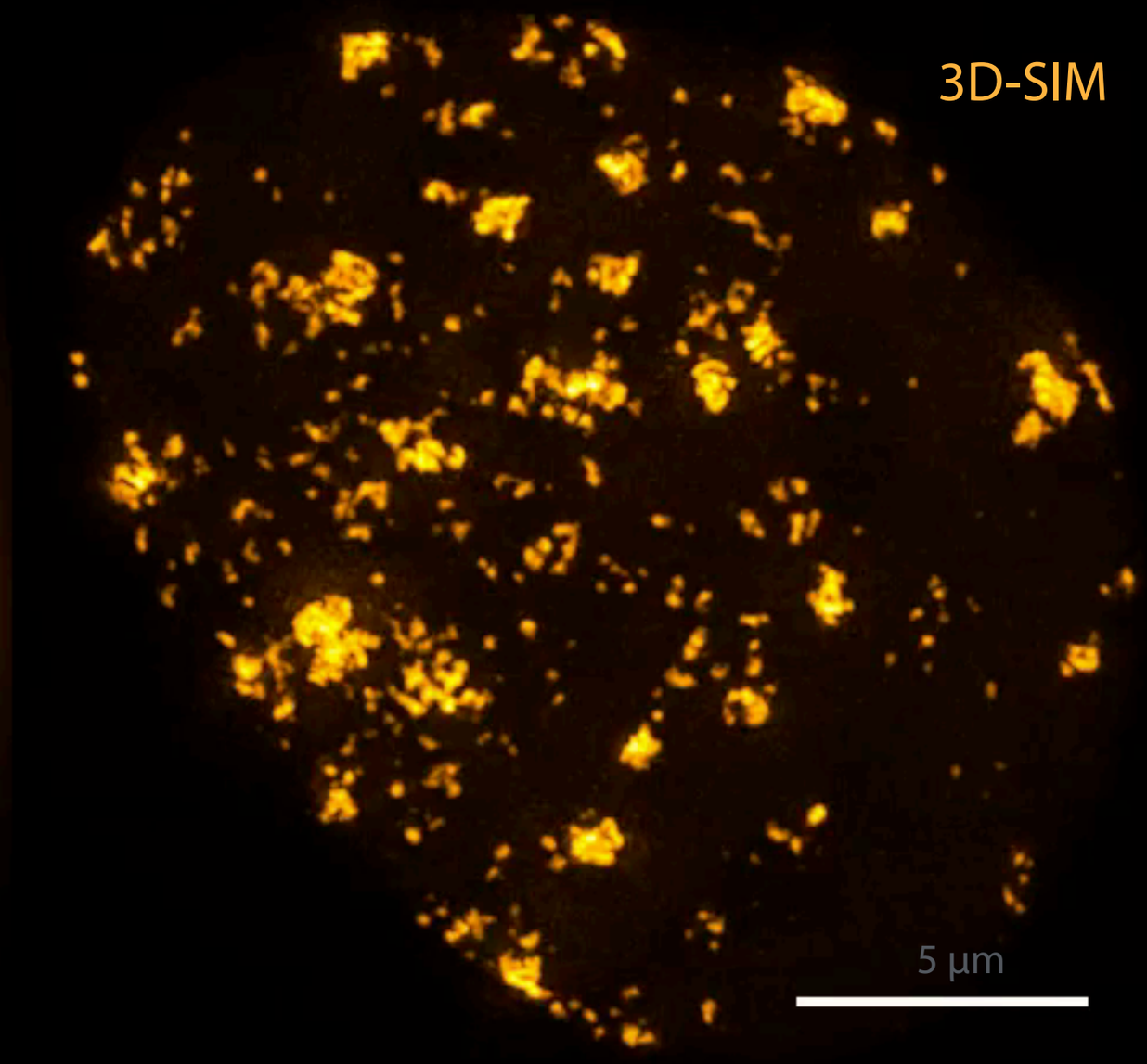
Live cell 3D super-resolution imaging of replication sites

DNA replication foci
(GFP-PCNA in mouse C2C12 myoblast cell)

(OMX Blaze)



wide-field



3D-SIM

10 s / frame (5 µm z-stack = 600 images / frame)

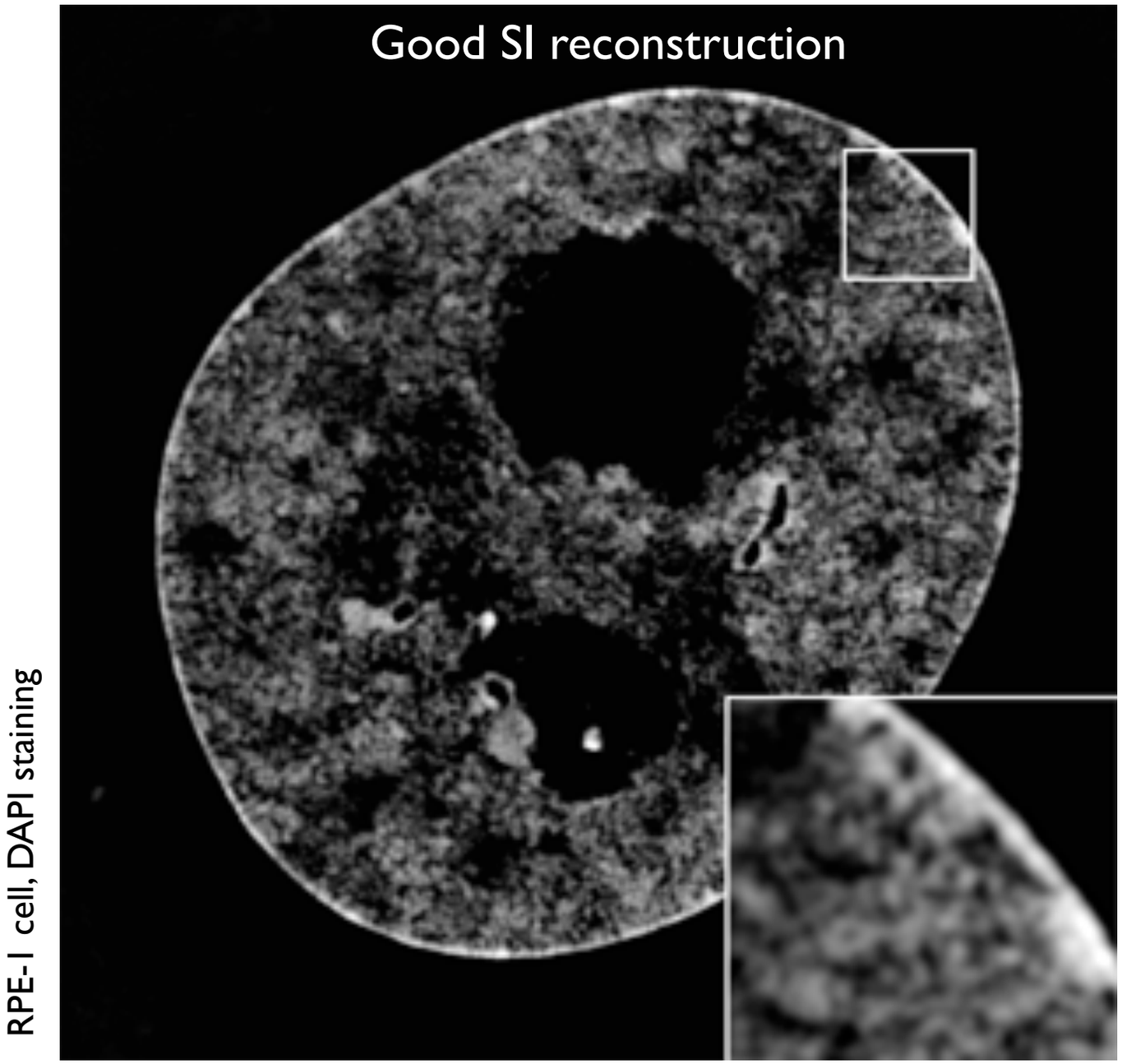
max. projection

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

It‘s not that simple!

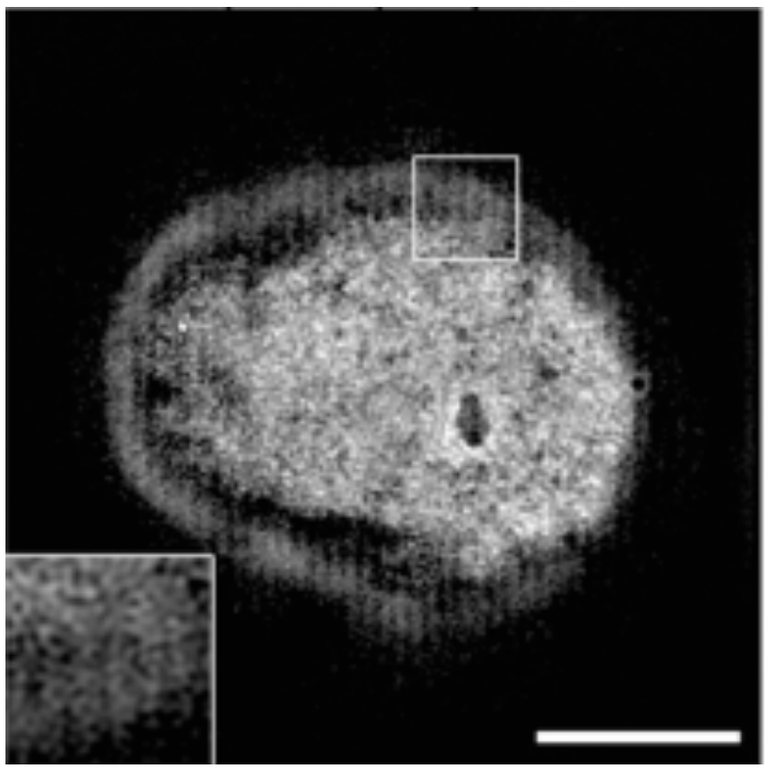
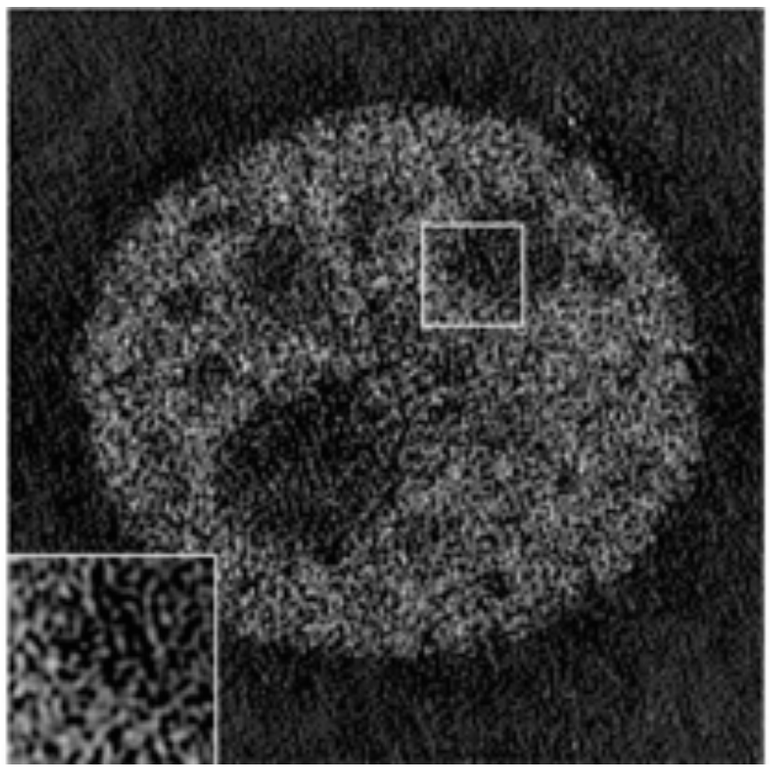
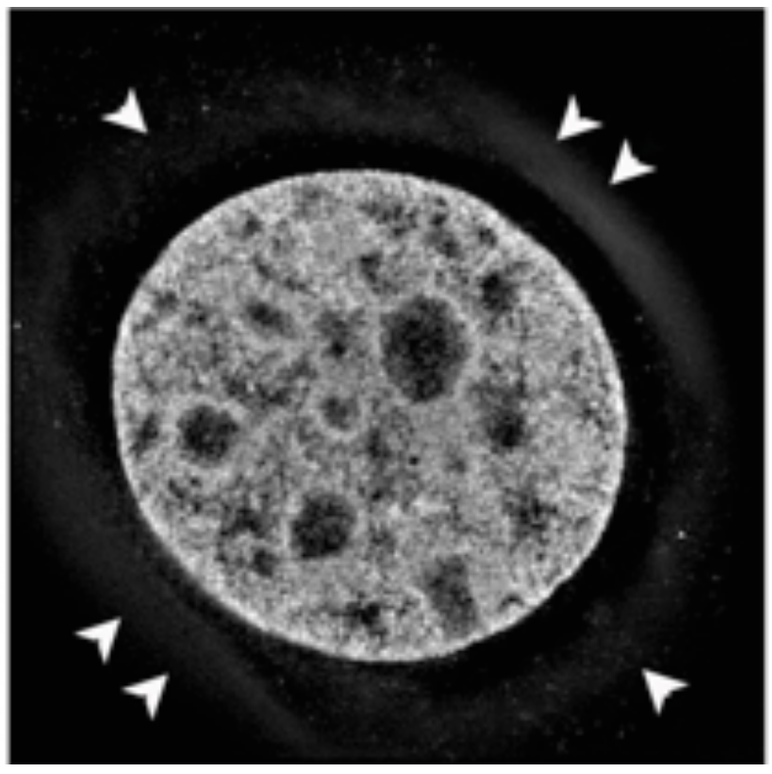
The untold story

SI reconstruction artifacts



SI reconstruction artifacts

HeLa clell nuclei, chromatin staining

Stripes	High frequency noise	Halo / Doubling
		
Bleaching, Drift or vibrations Moving particles (locally constrained)	Low contrast-to-noise, Low modulation contrast	Spherical aberration, Refractive index mismatch

SI reconstruction artifacts

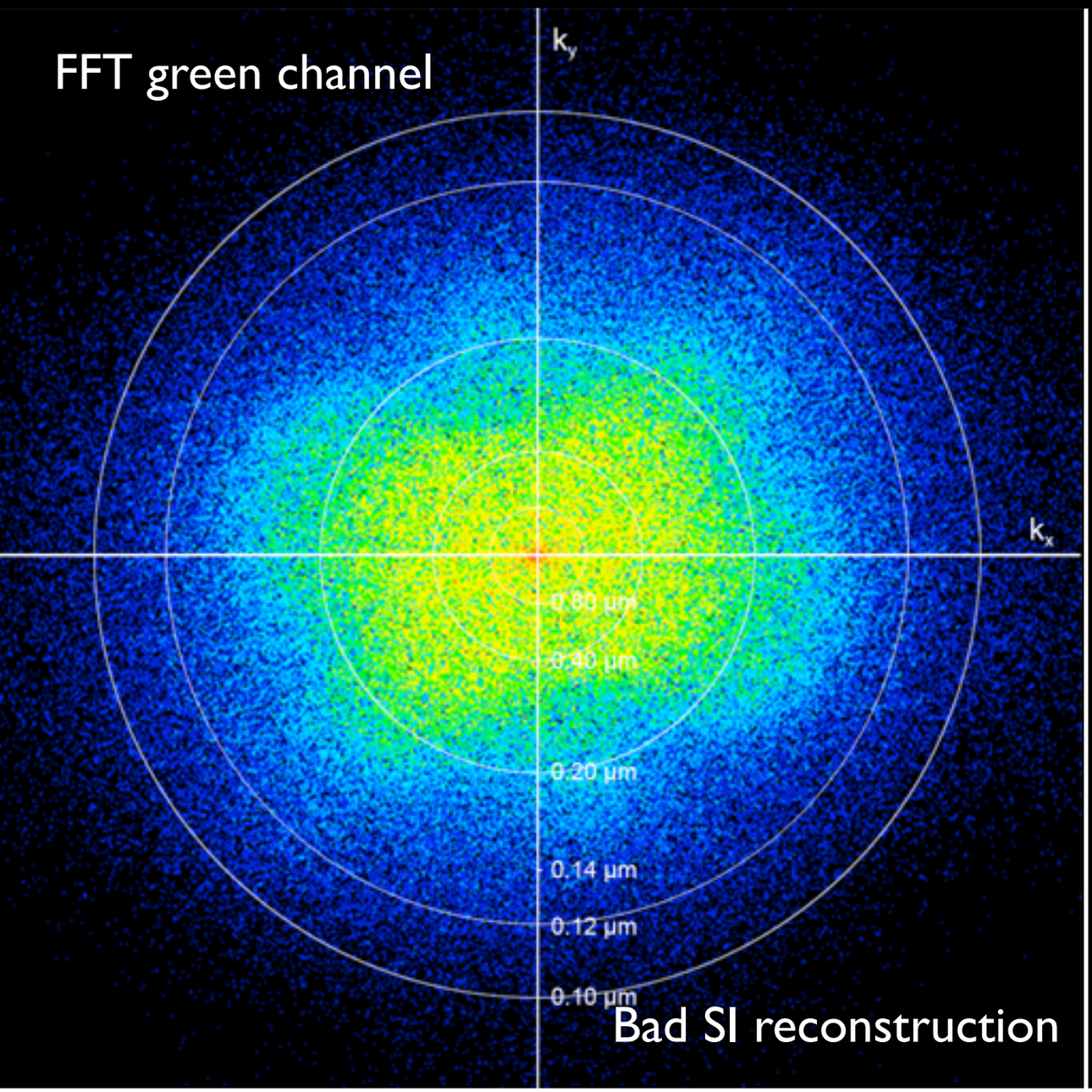
H3K4me3
RNA Pol II
DAPI

Bad SI reconstruction

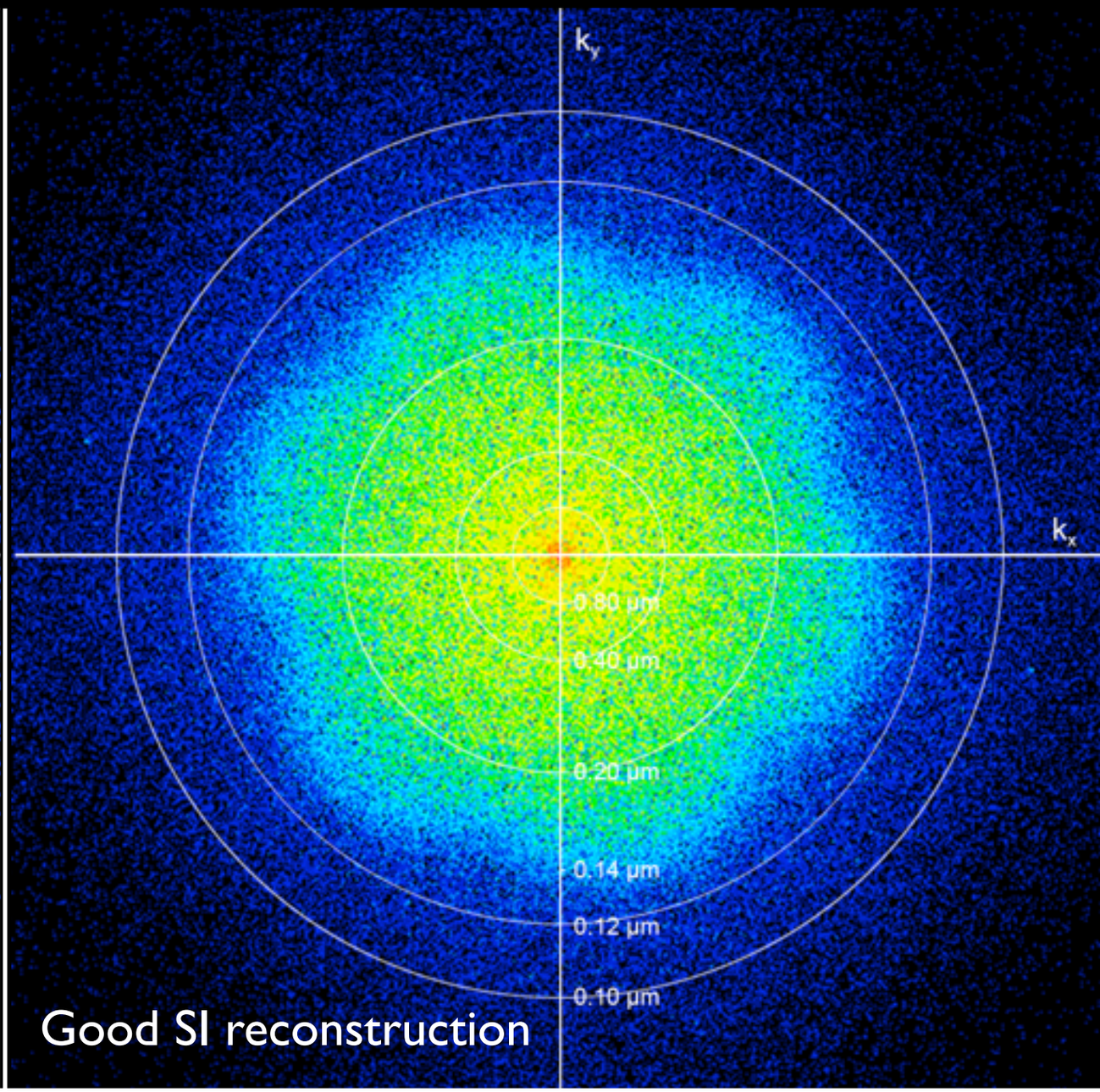
Good SI reconstruction

Quality control by Fourier analysis

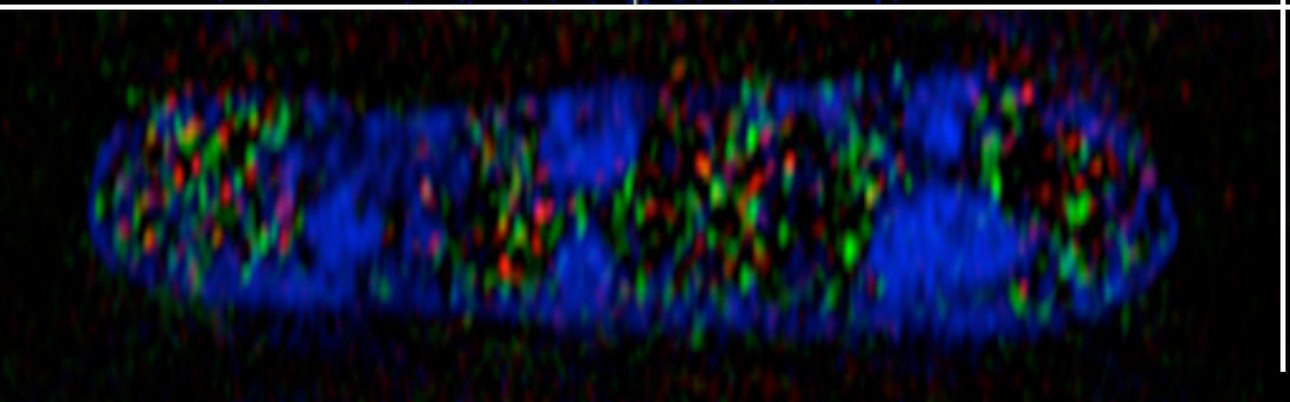
FFT green channel

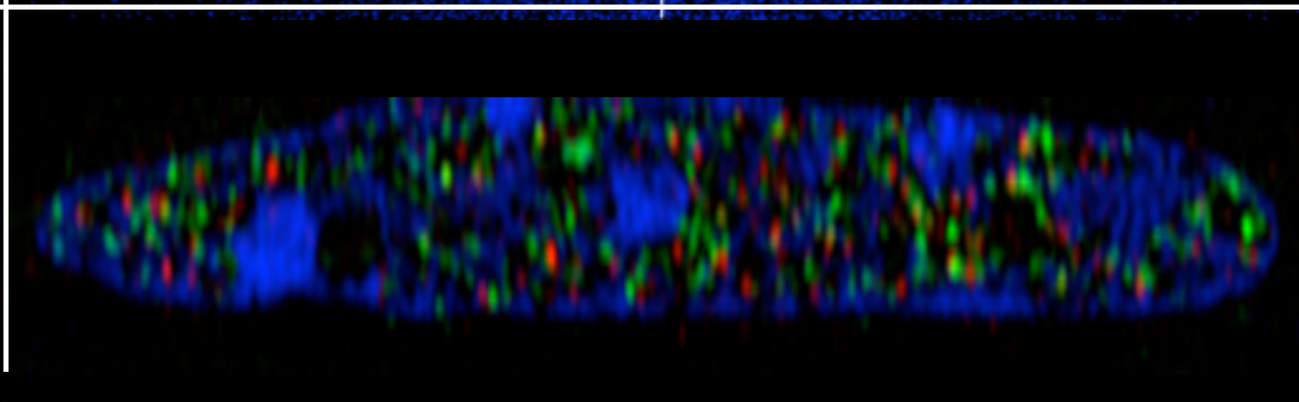


Bad SI reconstruction

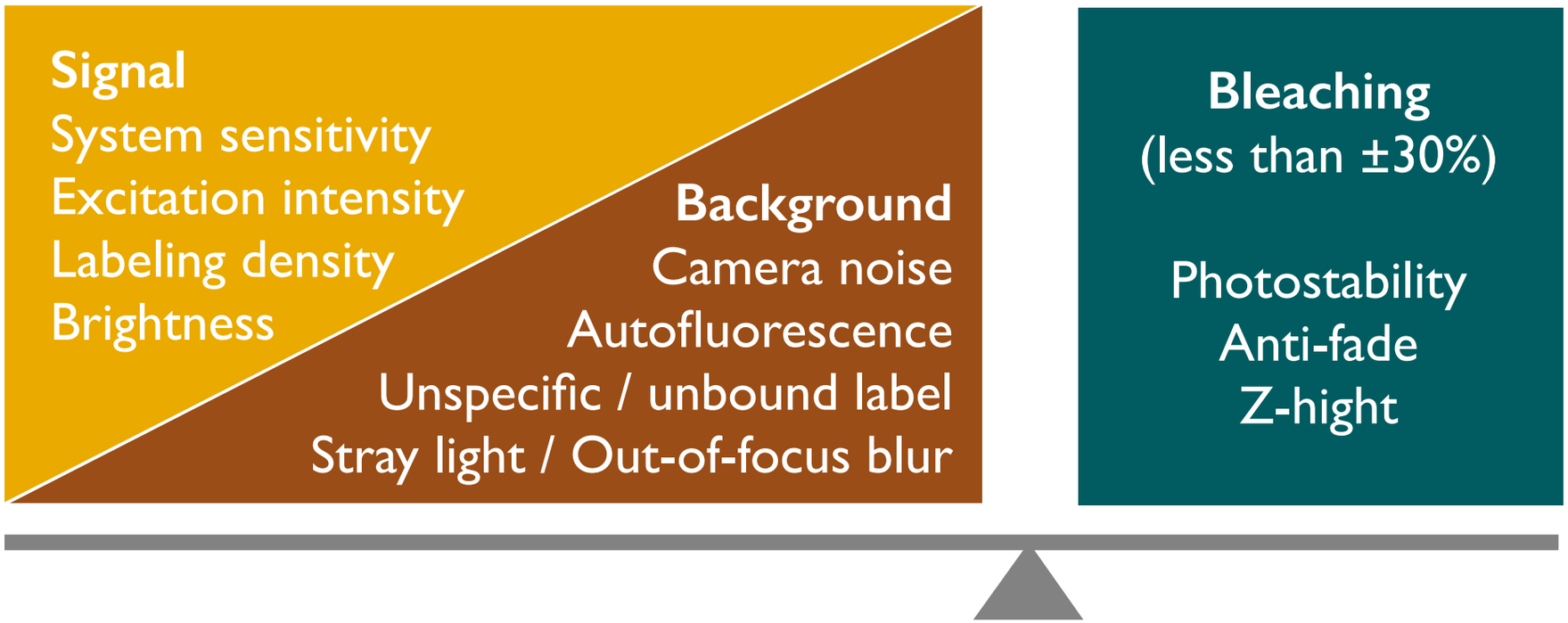


Good SI reconstruction



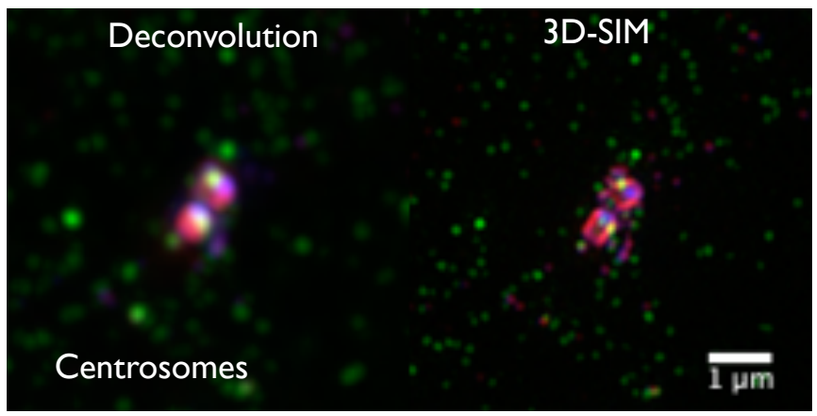


Balance between (modulation) contrast and bleaching



Discrete structures

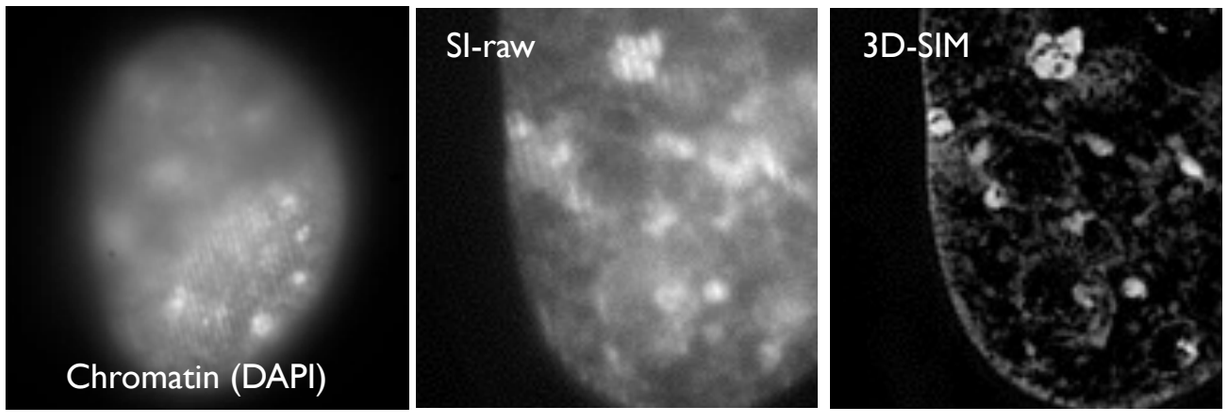
Restricted z-hight, low background



Tolerant to low intensities
>1.000 dynamic range (EMCCD/sCMOS)

Complex structures

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



High intensities required
>12.000 dynamic range (CCD) >40.000 (EMCCD)

3D-SIM workflow: quality is paramount !!!

Labelling

- Dyes (spectra, photo-stability)
- Labelling method (FPs, IF, FISH,....)
- Labelling specificity (antibodies)
- Signal-to-noise / background

Sample

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

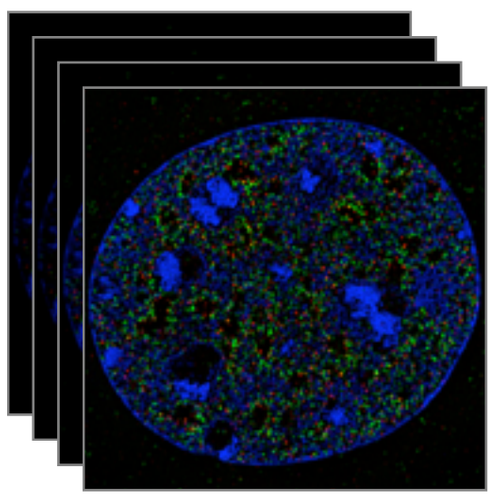
Microscope

- Mechanical stability
- Photon efficiency
- Modulation contrast / calibration
- Camera: (EM)CCD / sCMOS

Postprocessing

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

Dataset
x, y, z, λ,(t)



Quantitative Analysis

- Quality control
- Co-localisation
- Segmentation
- Distances

3D-SIM - pros & cons

Context

+ **Multi-color** with standard dyes
+ Lateral and **axial resolution** improvement
+ **3D optical sectioning** with enhanced **contrast**

Versatility

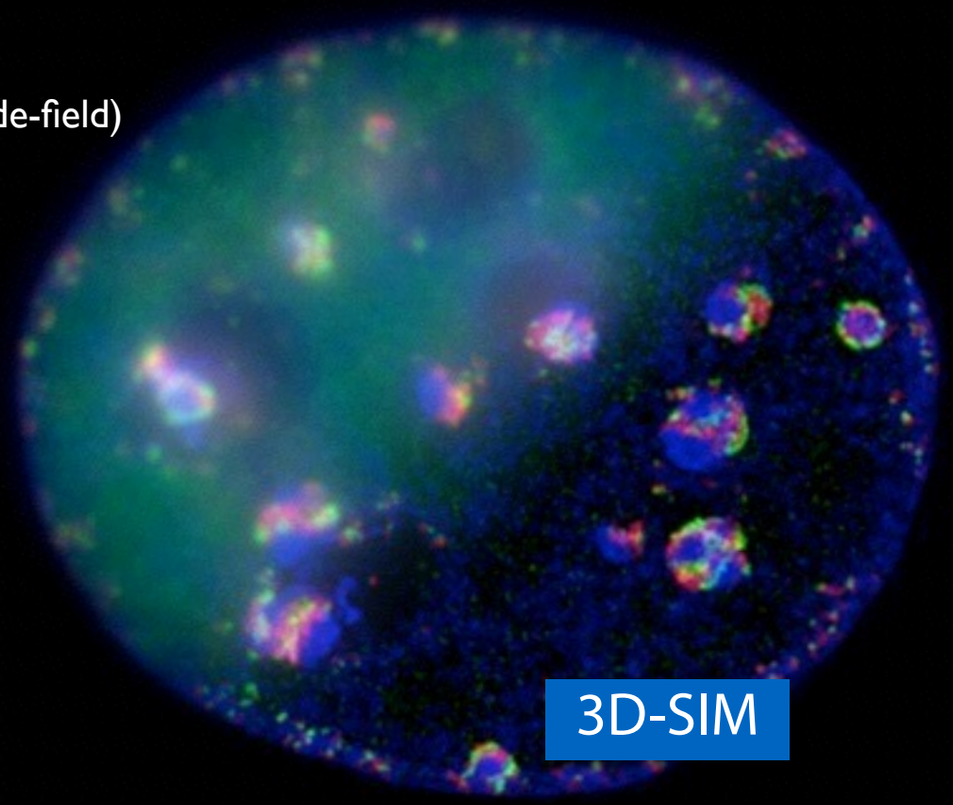
+ **Energy load / photodamage** lower than other SR-techniques
+ Relative large **imaging depth** (few 10 µm, w/ Silicon)
+ S**ensitivity** and **speed** (OMX Blaze) → live cell imaging

Challenges

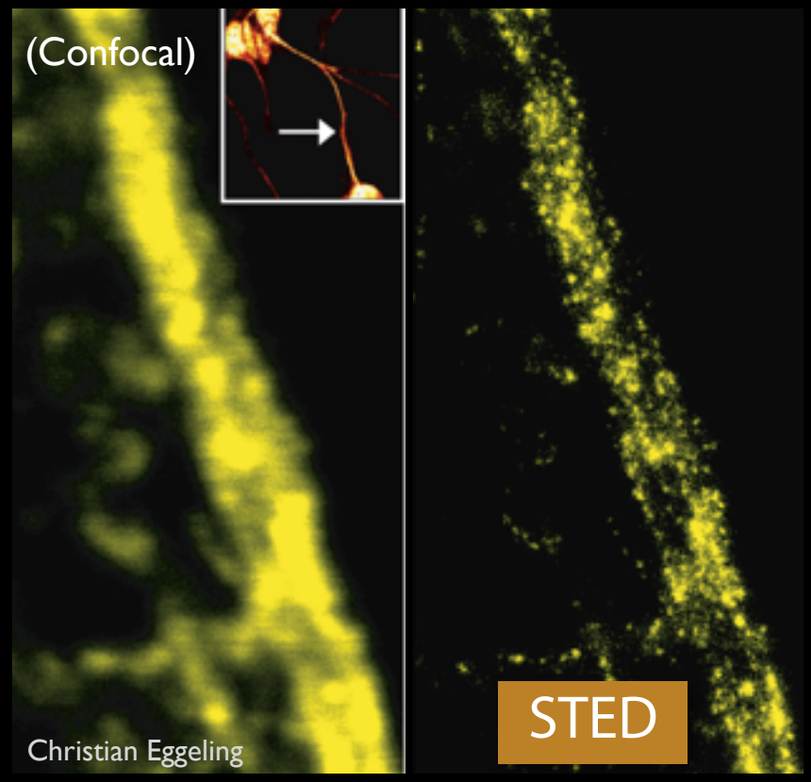
- Only moderate lateral resolution improvement
- Mathematical reconstruction → artifacts
- High requirements on sample quality and system calibration

Super-resolution techniques to surpass the diffraction limit

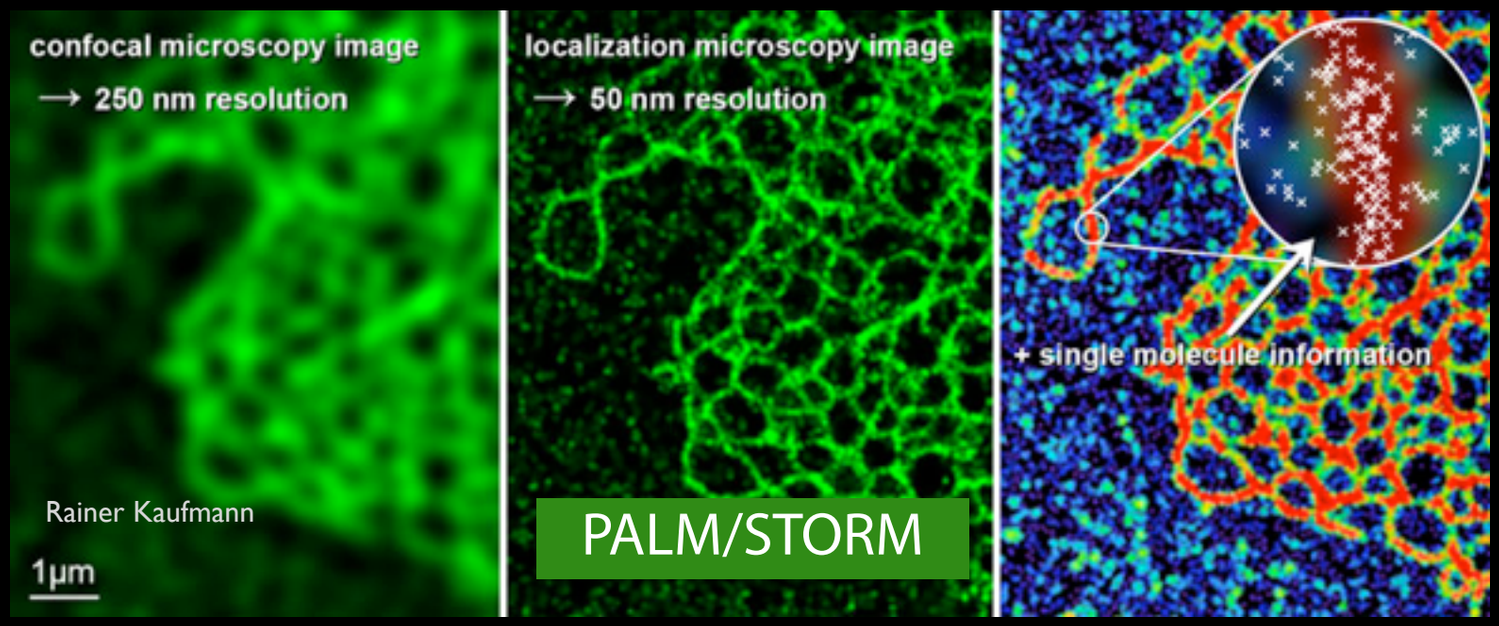
(Wide-field)



120 nm (xy); ± 300 nm (z)



± 60 nm (xy)

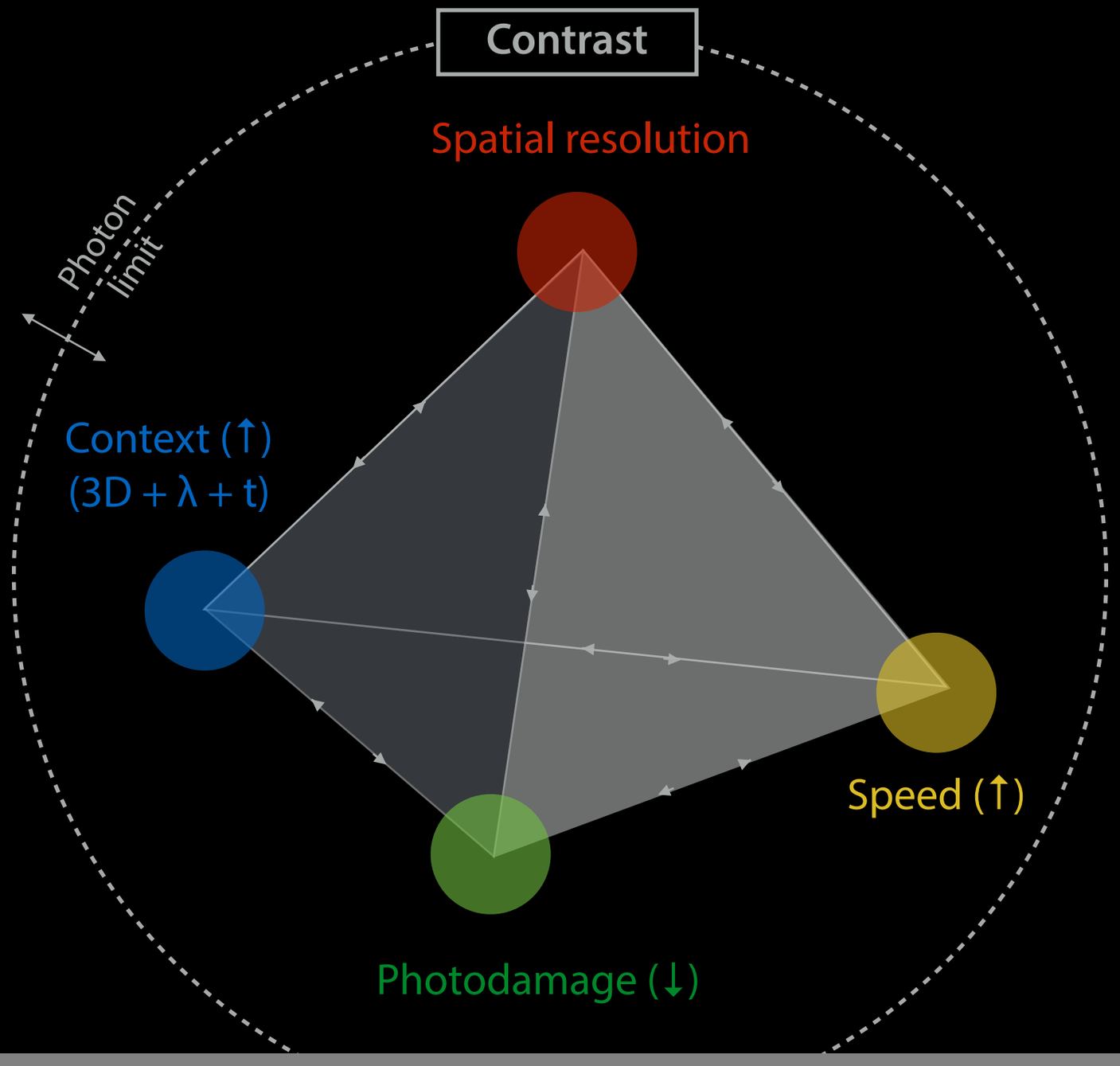


± 20 nm (xy localisation precision); ± 50 nm (structural resolution)

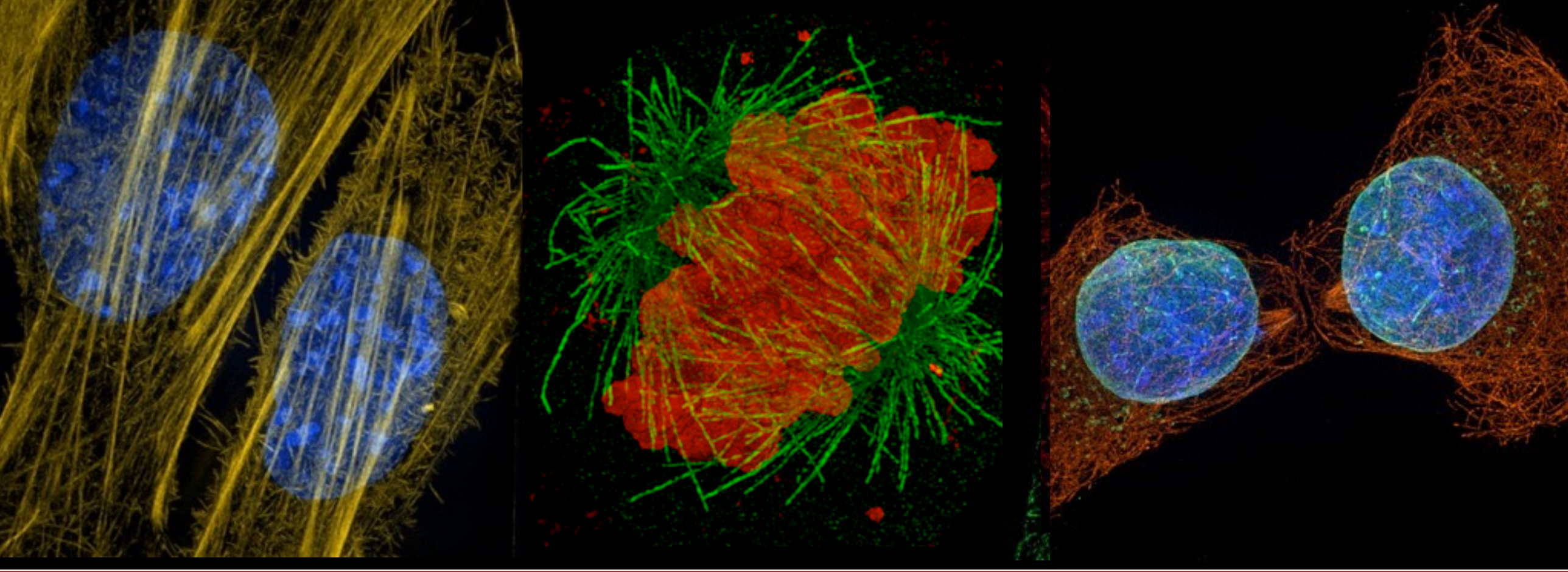
Trade-offs in super-resolution microscopy (no free lunch !!)

Conversion
quantum efficiency,
optics, detector
algorithm …

Input
sample,
labeling,
dyes…



The optimal technique is determined by demands of the application!
Spatial resolution is only part of the equation!



SIM rocks!

Thanks to Jürgen Neumann, Lin Shao, Julio Mateo Langerak for sharing slides