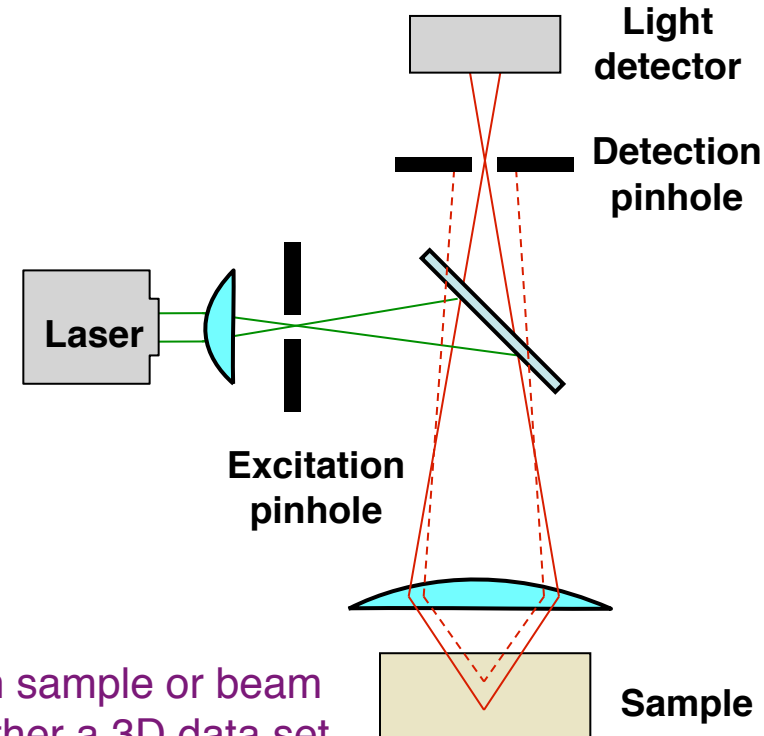
3D fluorescence microscopy

For 3D, acquire a "focal series" (stack) of images:
Take an image, refocus the sample, take another image, refocus, etc.

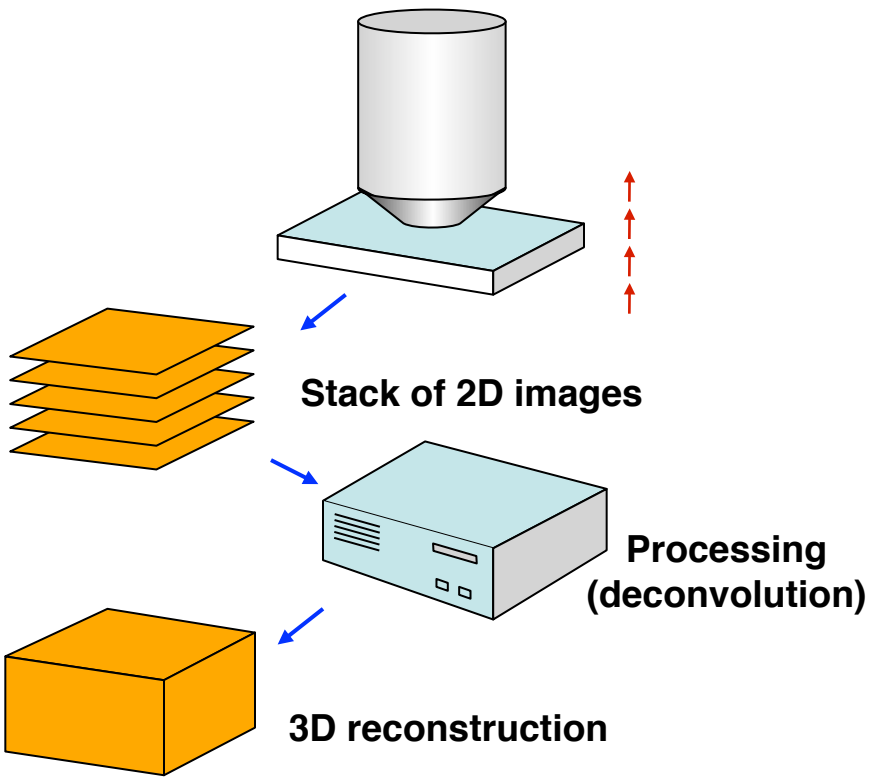
Problem: Each image contains out-of-focus blur from other focal planes

Approach 1:
Physically exclude the blur by confocal microscopy



Scan sample or beam to gather a 3D data set

Approach 2:
Remove the blur computationally

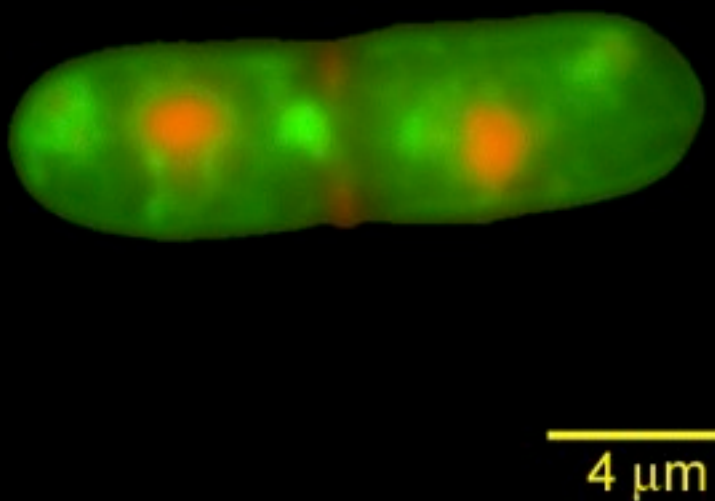


How well can this be done?

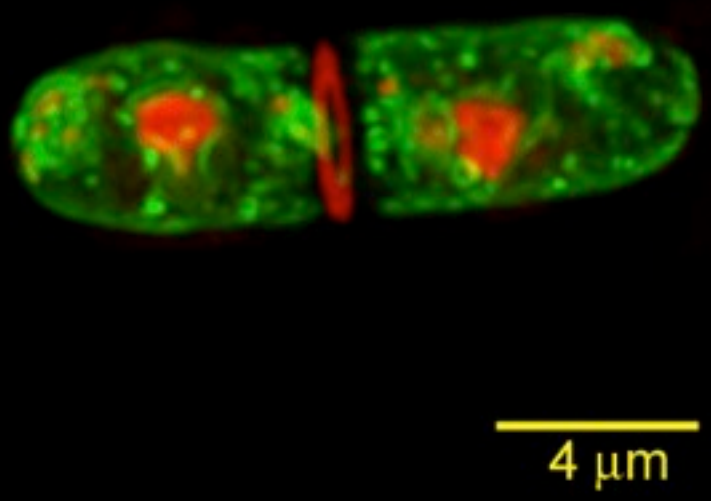
Deconvolution of 3D data

(Dividing fission yeast cell)

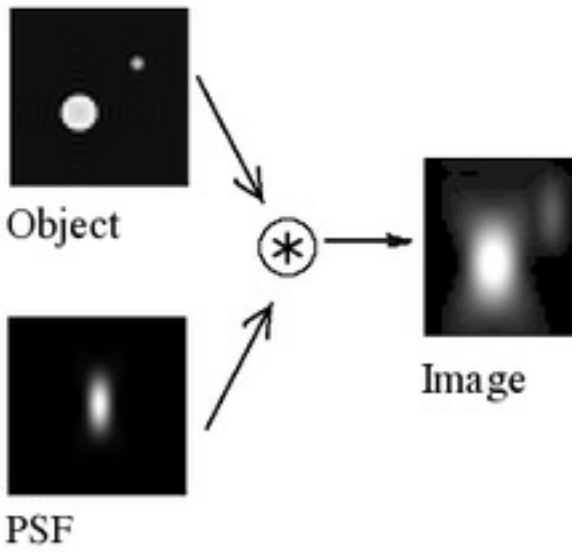
Raw data

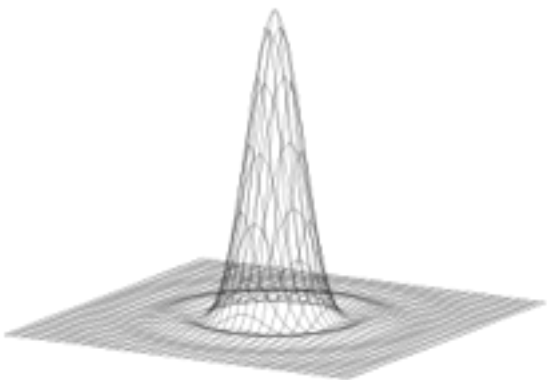


Deconvolved

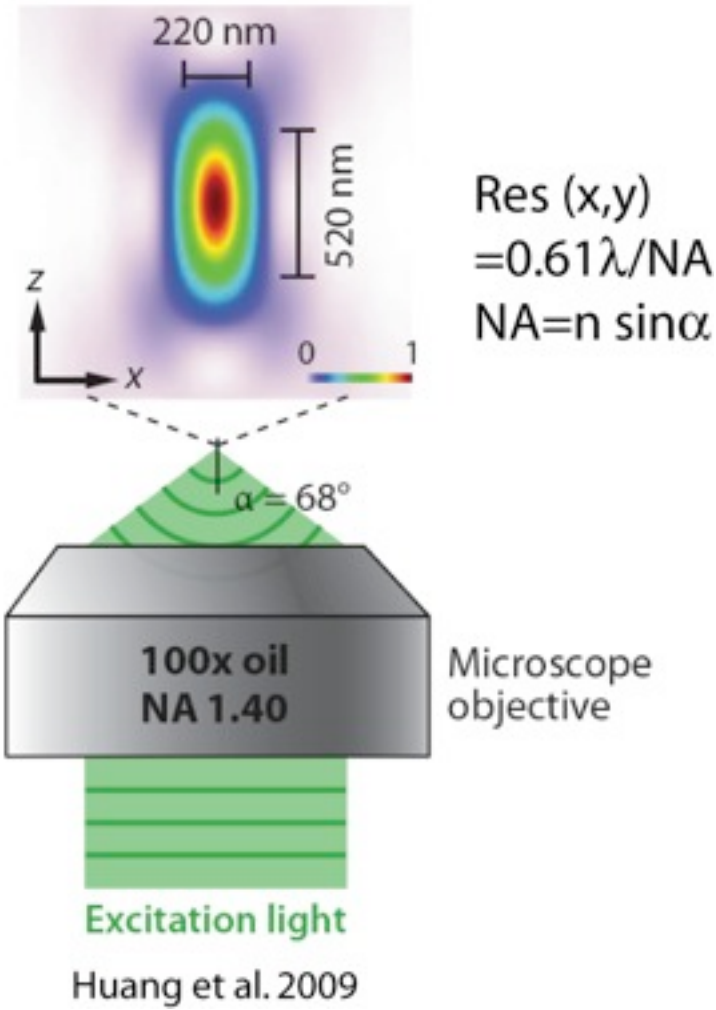


Light microscopy imaging is defined by the Point Spread Function





PSF



Res (x,y)
$=0.61\lambda/NA$
$NA=n \sin\alpha$

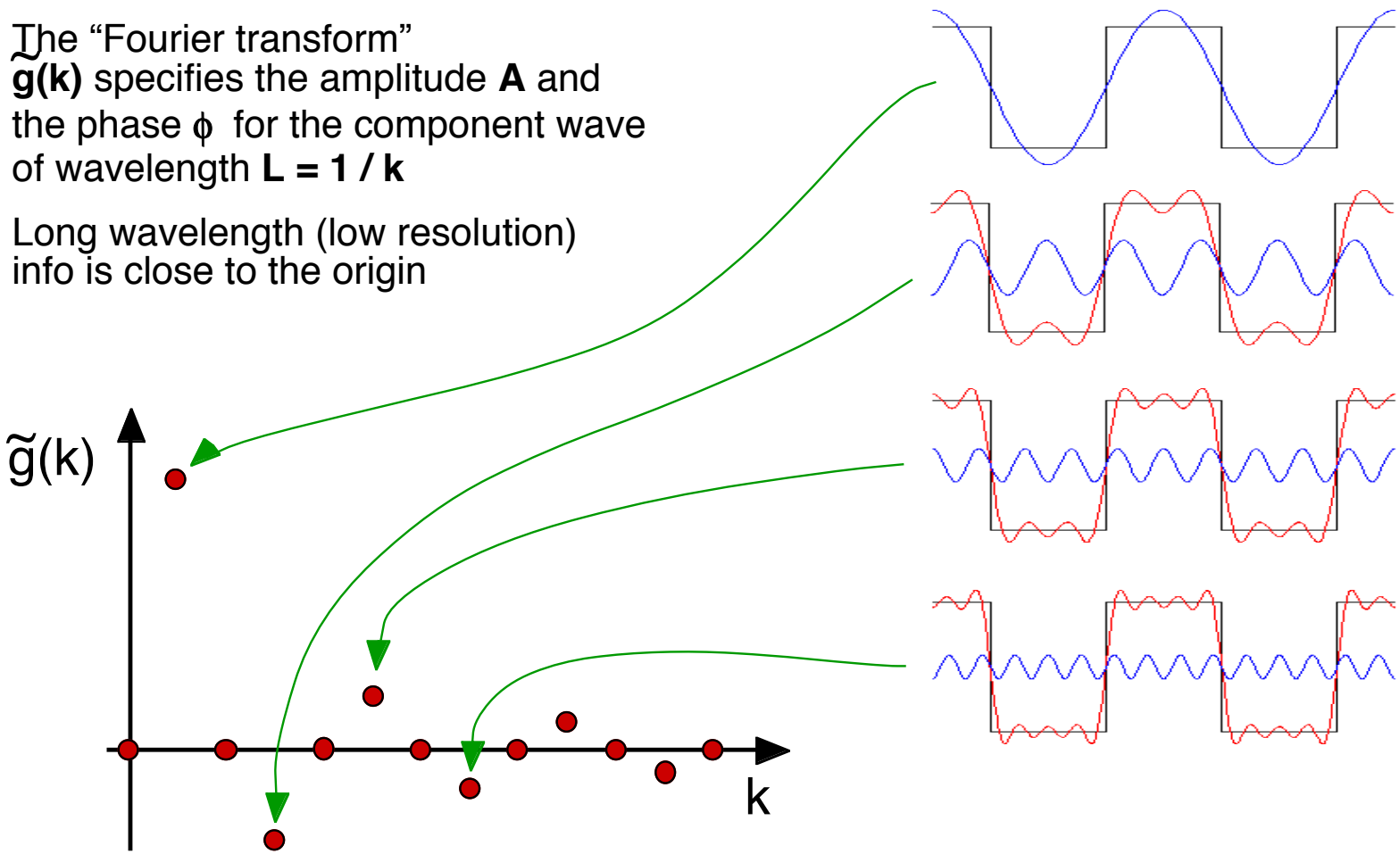
The Fourier Transform

$$\tilde{g}(k) = \int g(x)\, e^{-2\pi i k x}\, dx$$

Any (nice) function **g(x)** can be *equally well* described as a sum of waves.

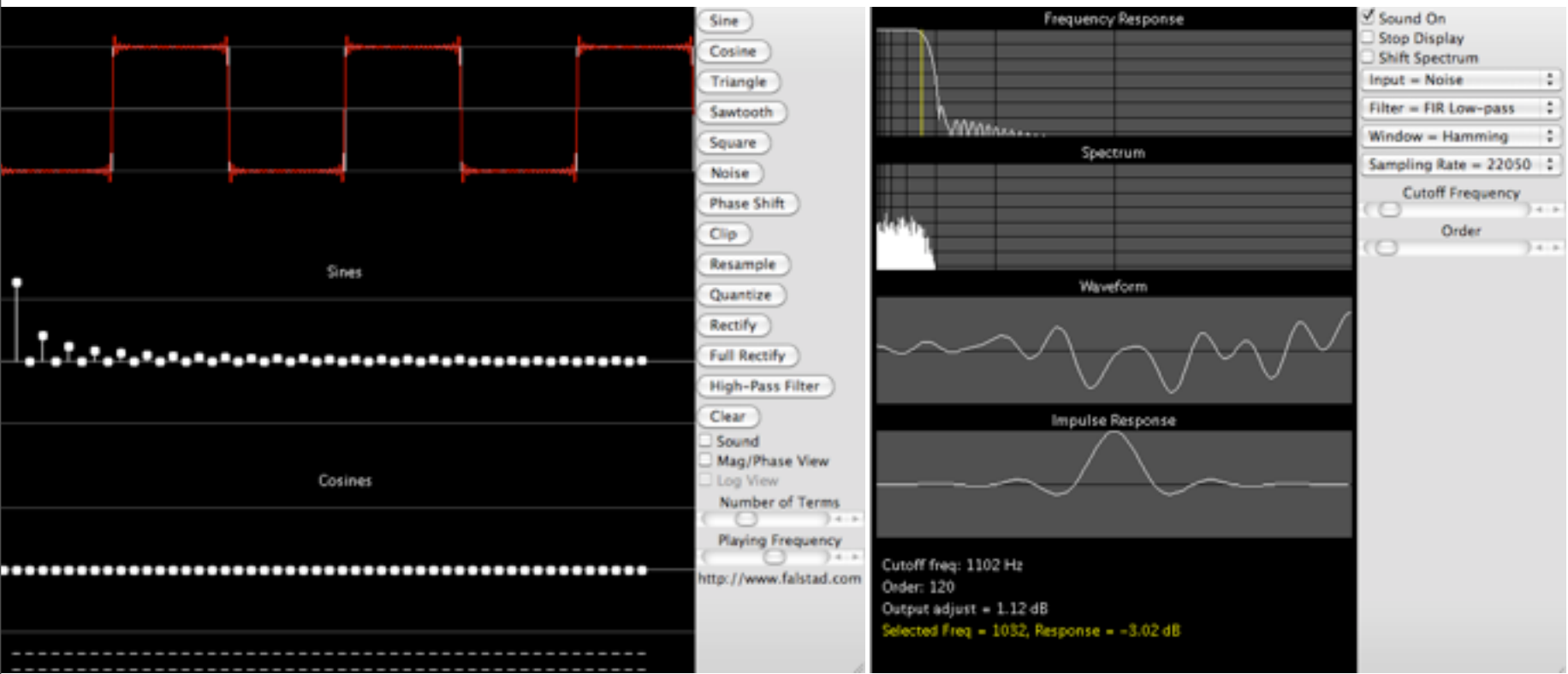
The "Fourier transform" $\tilde{g}(k)$ specifies the amplitude **A** and the phase ϕ for the component wave of wavelength **L = 1 / k**

Long wavelength (low resolution) info is close to the origin



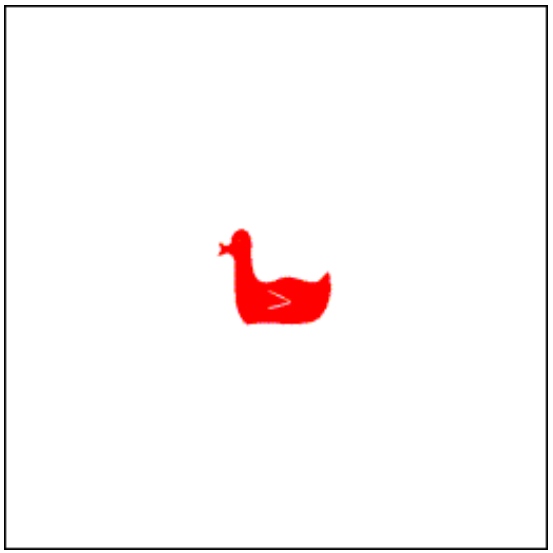
Fourier Transform and Digital Filter Applets

http://www.falstad.com/mathphysics.html

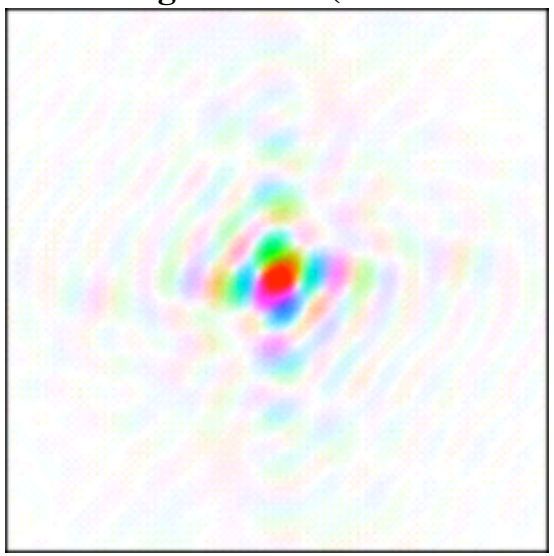


Low Resolution Duck

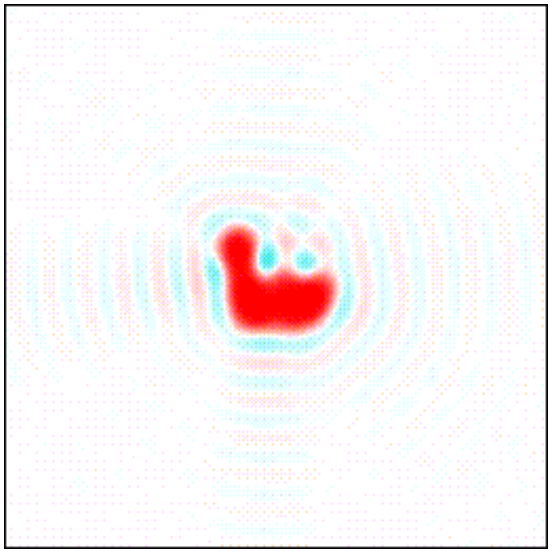
A Duck



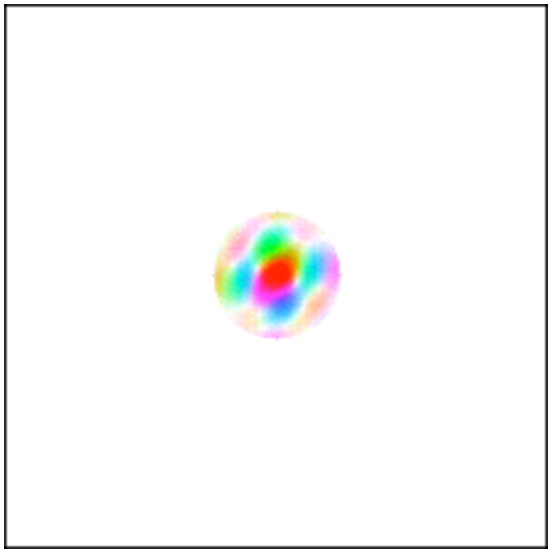
and its scattering function (Fourier Transform):



a blurred duck <=



Using only low resolution data



Convolutions

$$(f \otimes g)(r) = \int f(a)\ g(r-a)\ da$$

Why do we care?

- They are everywhere…
- The **convolution theorem**:

  If $h(r) = (f \otimes g)(r)$,

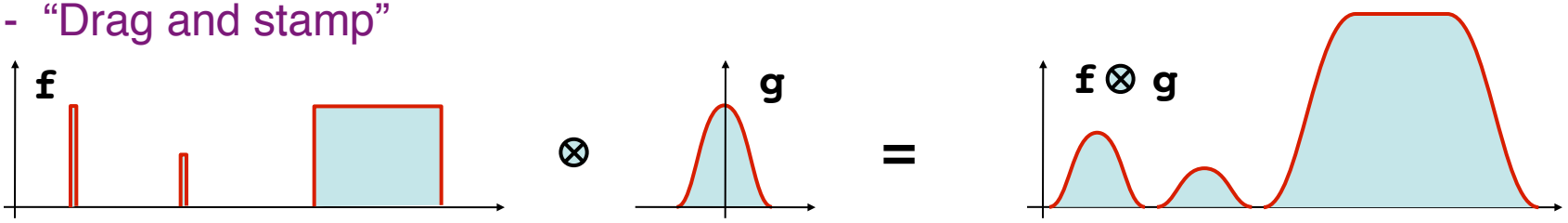
  then $\tilde{h}(k) = \tilde{f}(k)\ \tilde{g}(k)$

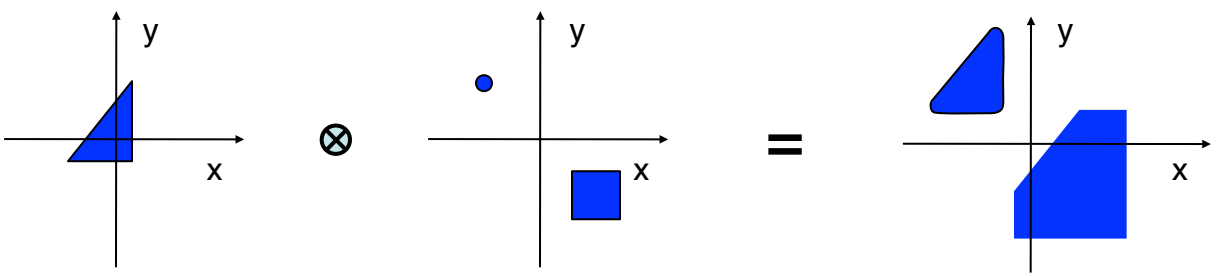
A convolution in real space becomes a product in reciprocal space & vice versa

Symmetry: $g \otimes f = f \otimes g$

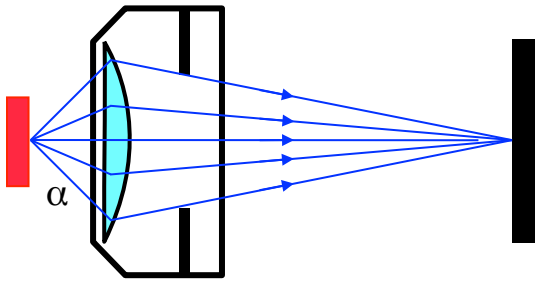
So what **is** a convolution, intuitively?

- "Blurring"
- "Drag and stamp"





2D PSF, OTF of an in-focus lens viewing a point



α = maximum angle

N.A. = η sin α,

the highest spatial frequency is then $f_c = (2\eta \sin \alpha)/\lambda$

$f_c =$ 0.178 μm for λ= 500nm, 1.4NA oil immersion lens

using Raleigh's criterion, the smallest separation between two points that can be resolved is 1/(1.22 •f_c)

$d_{min} = 0.146\ \mu$m

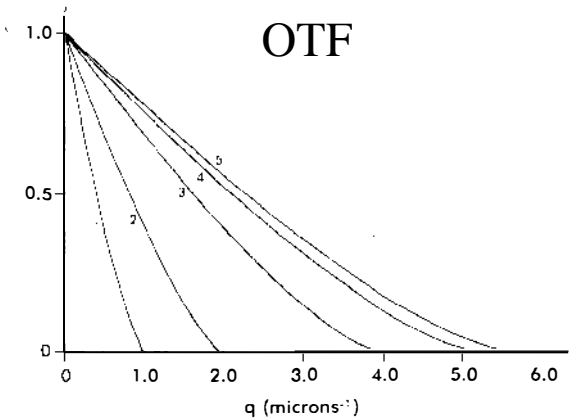


Figure 3 The in-focus contrast-transfer functions for several Zeiss lenses are compared. They are (*1*) 10 × 0.25 N.A., (*2*) 25 × 0.5 N.A., (*3*) 50 × 1.0 N.A. oil, (*4*) 100 × 1.25 N.A. oil, (*5*) 63 × 1.4 N.A. oil.

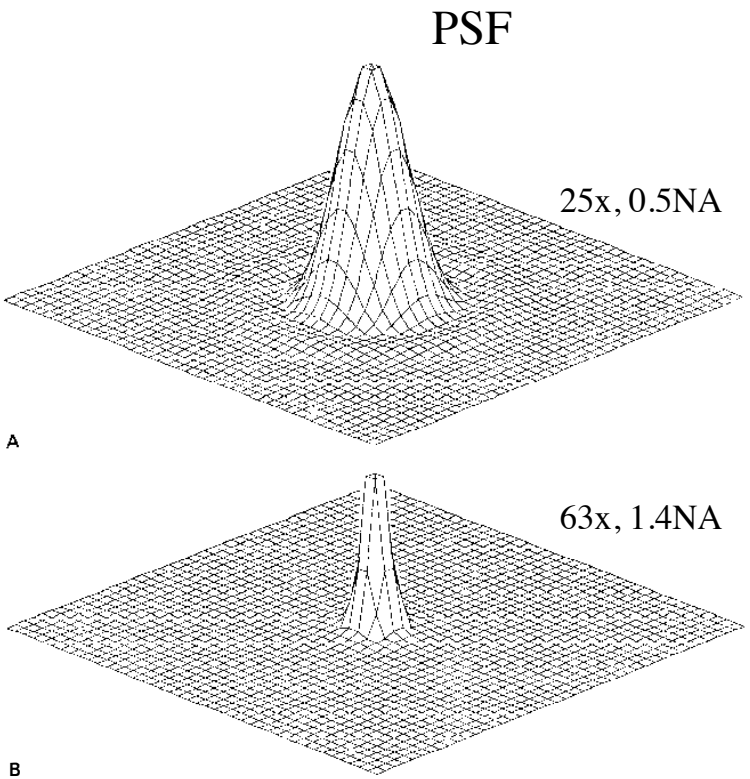
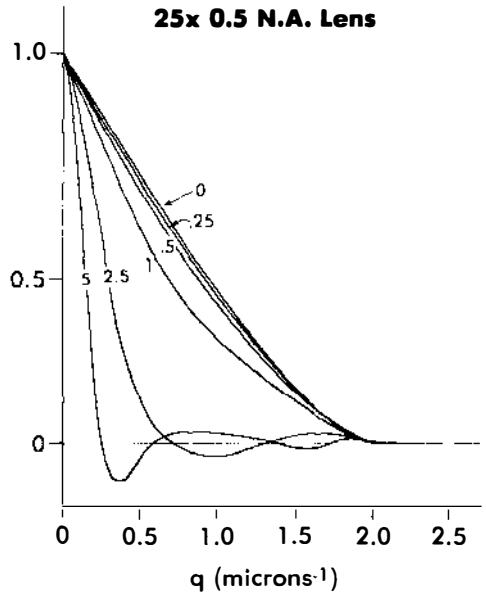
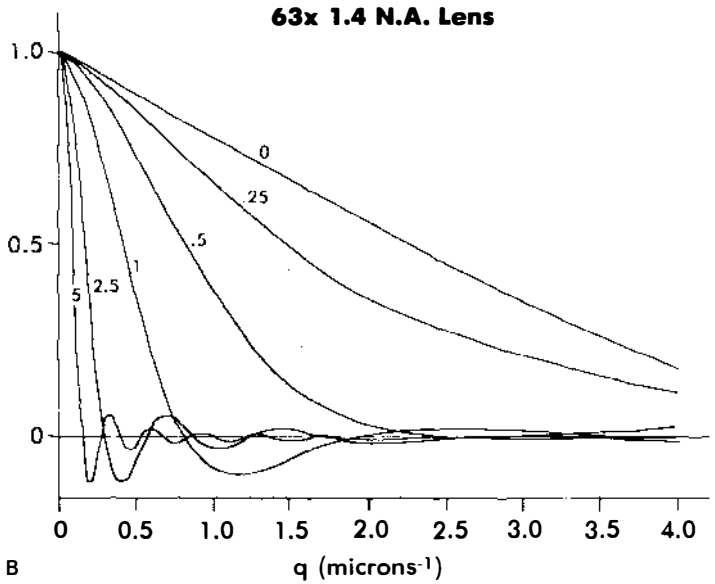
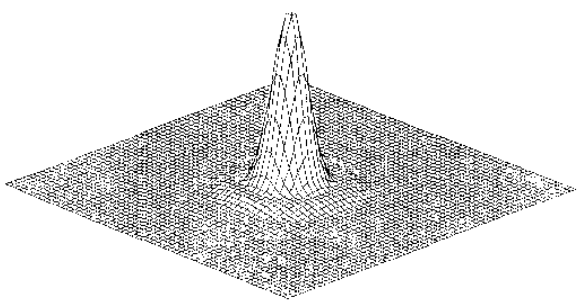


Figure 4 The point-spread functions calculated by Fourier transformation of the CTF's of Figure 3. (*A*) 25 × 0.5 N.A. lens (*# 2*); (*B*) 63 × 1.4 N.A. lens (*# 5*). Each grid square is λ/6 or 833 Å. Note how much sharper the 1.4 N.A. lens is than the 0.5 N.A. lens.

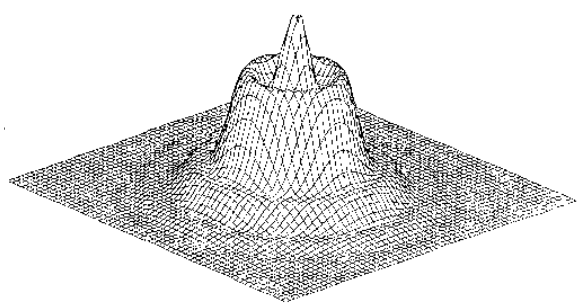
OTF, PSF of a defocused lens



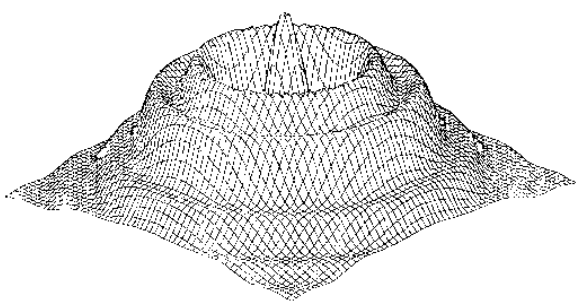




25x lens 0 μm defocus



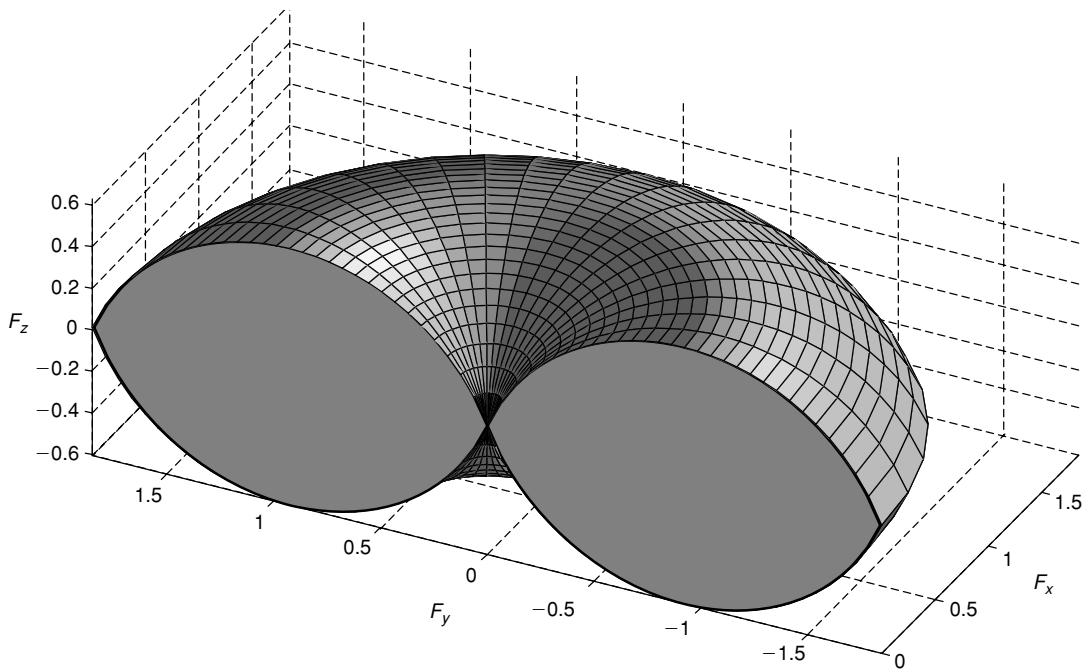
25x lens 2.5 μm defocus



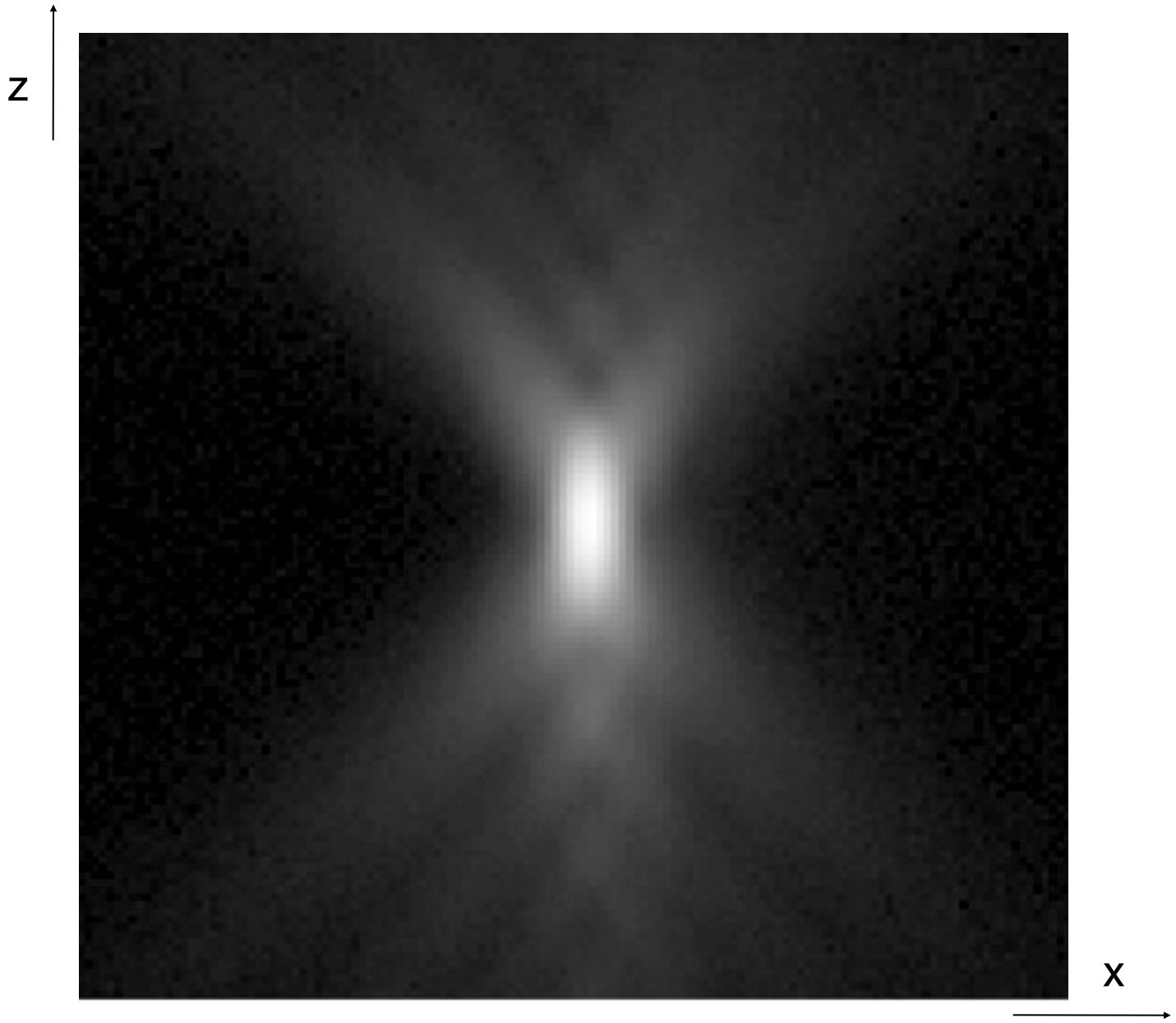
25x lens 5 μm defocus

3D OTF

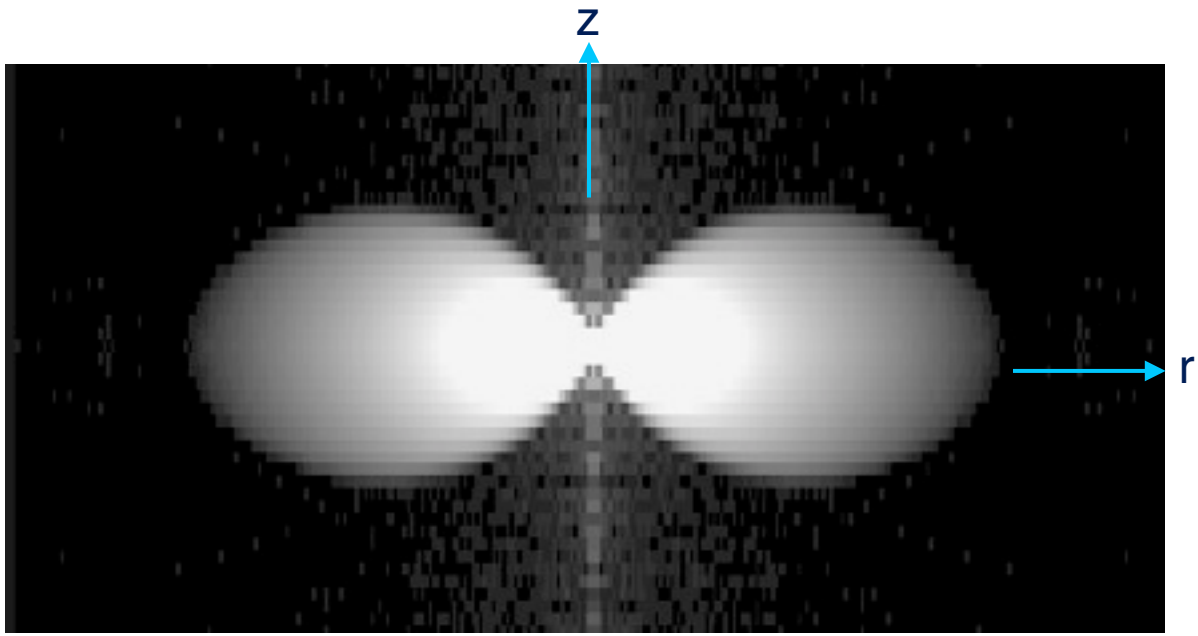
Combining different defocus OTFs from a stack of optical sections gives a donut shaped observable region (support) in Fourier Space



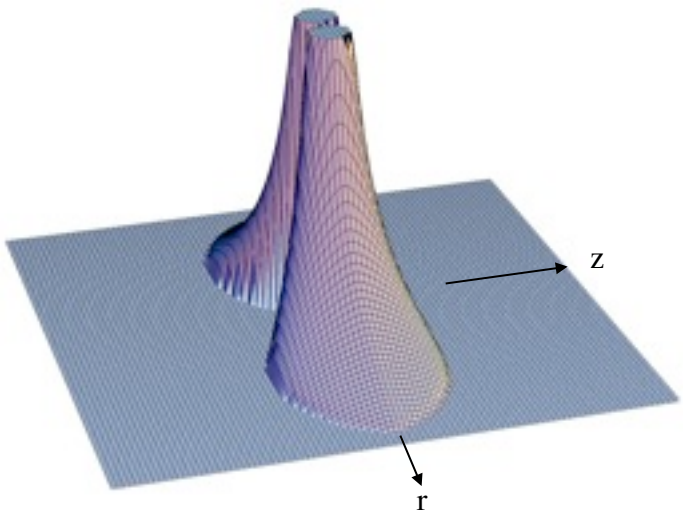
Experimentally measured PSF



Experimentally measured OTF

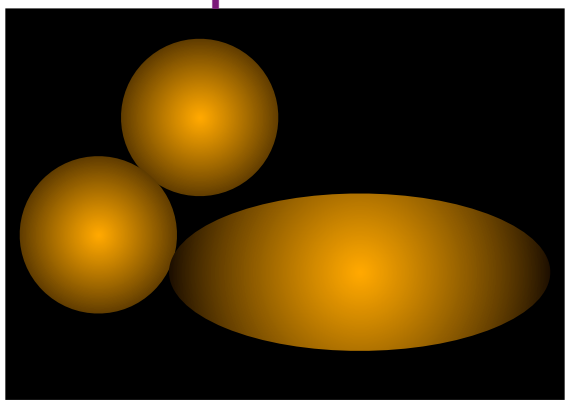


- Intensity is very peaked at origin
- data along Z direction is missing



PSF, OTF & deconvolution

In real space:

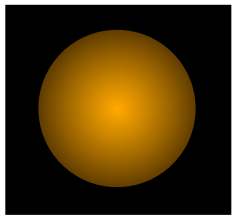


Observed Image(r)

=

True Object(r)

⊗



Point Spread Function, PSF(r)

In reciprocal space the convolution becomes a product:

$$\widetilde{\text{Image}} = \widetilde{\text{Object}} \cdot \widetilde{\text{PSF}}$$

$\widetilde{\text{PSF}}$ is called the "Optical Transfer Function", OTF

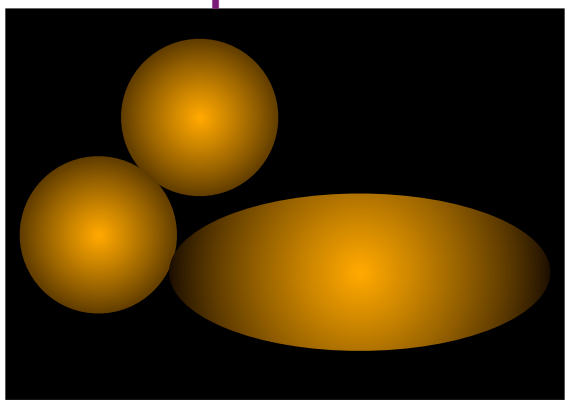
This suggests:

$$\widetilde{\text{Object}} = \frac{\widetilde{\text{Image}}}{\text{OTF}}$$

??? ("Deconvolution")

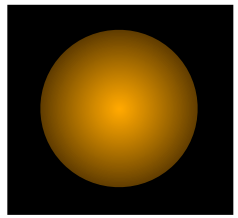
PSF, OTF & deconvolution

In real space:



=

⊗



Observed Image(r)

True Object(r)

Point Spread Function, PSF(r)

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This suggests:

$$\widetilde{\text{Object}} = \frac{\widetilde{\text{Image}}}{\text{OTF}}$$

??? ("Deconvolution")

What's the catch??

A: We can't divide by OTF(k) if it is **zero (**or small because of noise)

Deconvolution strategies

Nearest neighbor: simplest method, only takes into account adjacent sections
ie subtract out blurred version of adjacent sections from central section

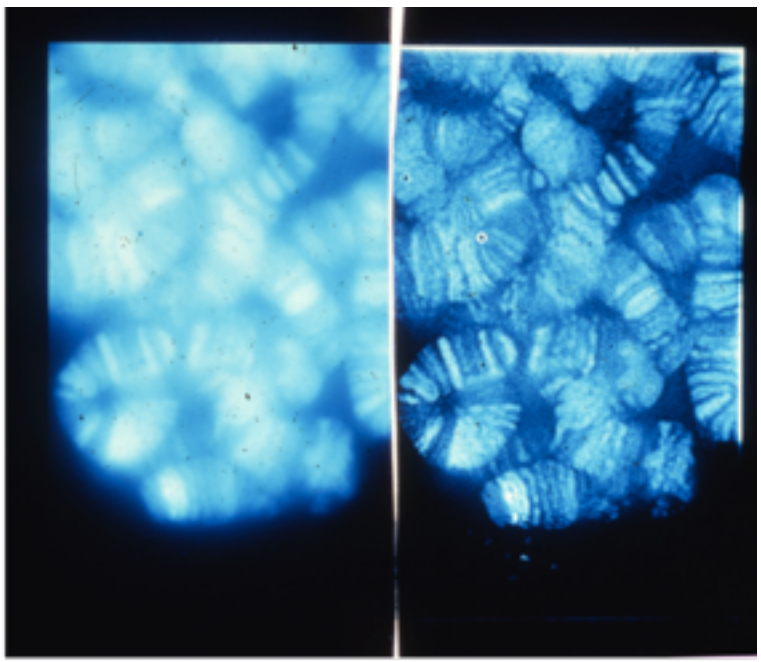
$$I_j \approx c_1[\, O_j - c_2(PSF_{\Delta z} \otimes O_{j+1} + PSF_{-\Delta z} \otimes O_{j-1}) \,]$$

for speed do the convolutions as Fast Fourier Transforms (FFT), multiplication, FFT^{-1}

$$PSF_{\Delta z} \otimes O_{j+1} = FFT^{-1}[\, FFT(O_{j+1}) \bullet OTF_{\Delta z}) \,]$$

optical section from a DAPI stained polytene nucleus

before and after nearest neighbor



Deconvolution strategies

much better to consider the contributions of all the sections to one another = 3D

Weiner filter: simplest 3D method, linear processing, takes care of zeros

$$\widetilde{\text{Object}} = \frac{\widetilde{\text{Image}}}{\text{OTF} + \gamma}$$

γ is related to the signal to noise, sets maximum amplification

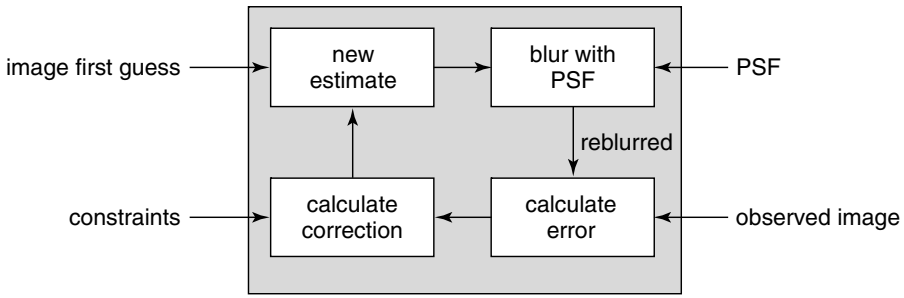
or if OTF is complex:

$$\frac{\widetilde{\text{Image} \cdot \text{OTF}^*}}{\text{OTF} \cdot \text{OTF}^* + \gamma}$$

Deconvolution strategies

even better to include *a priori* knowledge about solution such as positivity: Object ≥ 0

family of iterative constrained methods
work by calculating convolution, followed by update



arithmetic update (vanCittert's method)

start with $I^0 = O$

$I^{k+1} = I^k + (O - PSF \otimes I^k)$ with positivity constraint: if $I^{k+1} < 0$ then set $I^{k+1} = 0$

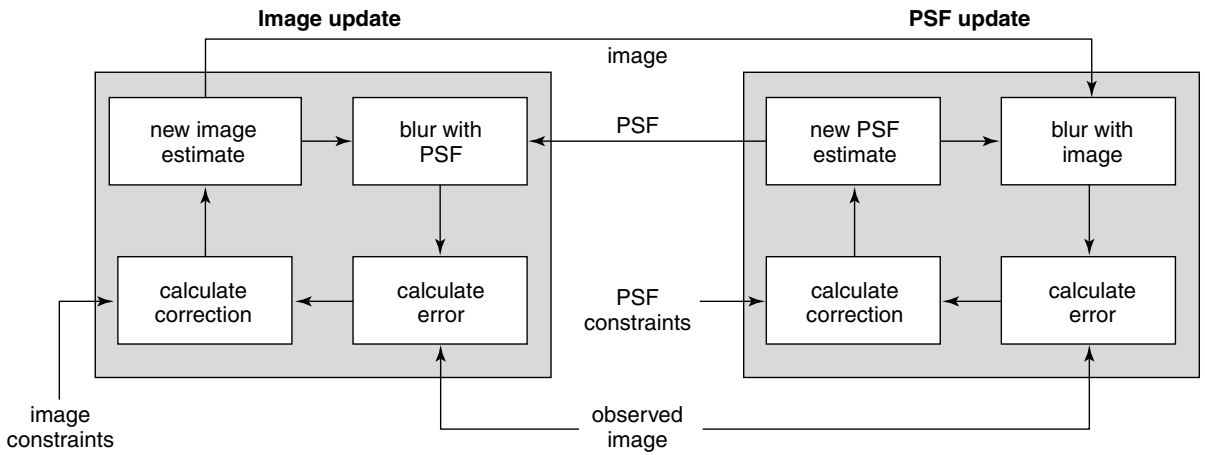
or use multiplicative update (Gold's method)

$$I^{k+1} = I^k \bullet \frac{O}{PSF \otimes I^k}$$

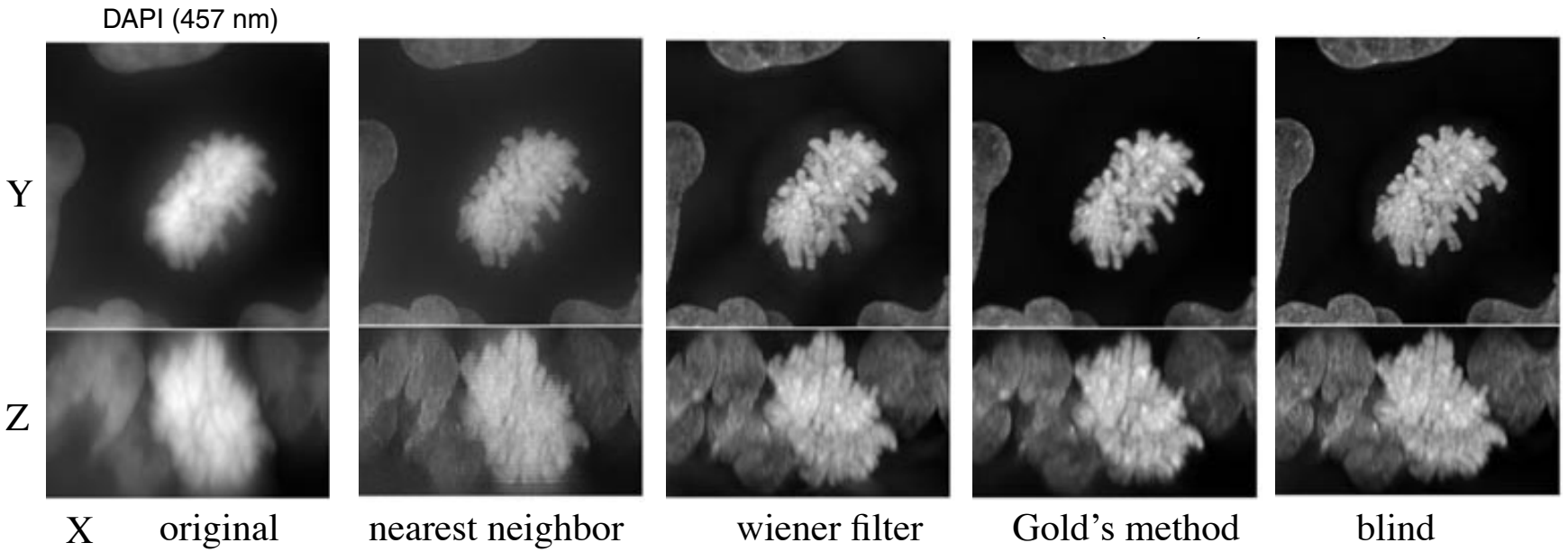
Deconvolution strategies

what if your PSF is not accurate?

blind deconvolution seeks to estimate both Image and PSF



Some comparisons: Hela cells, DAPI stained

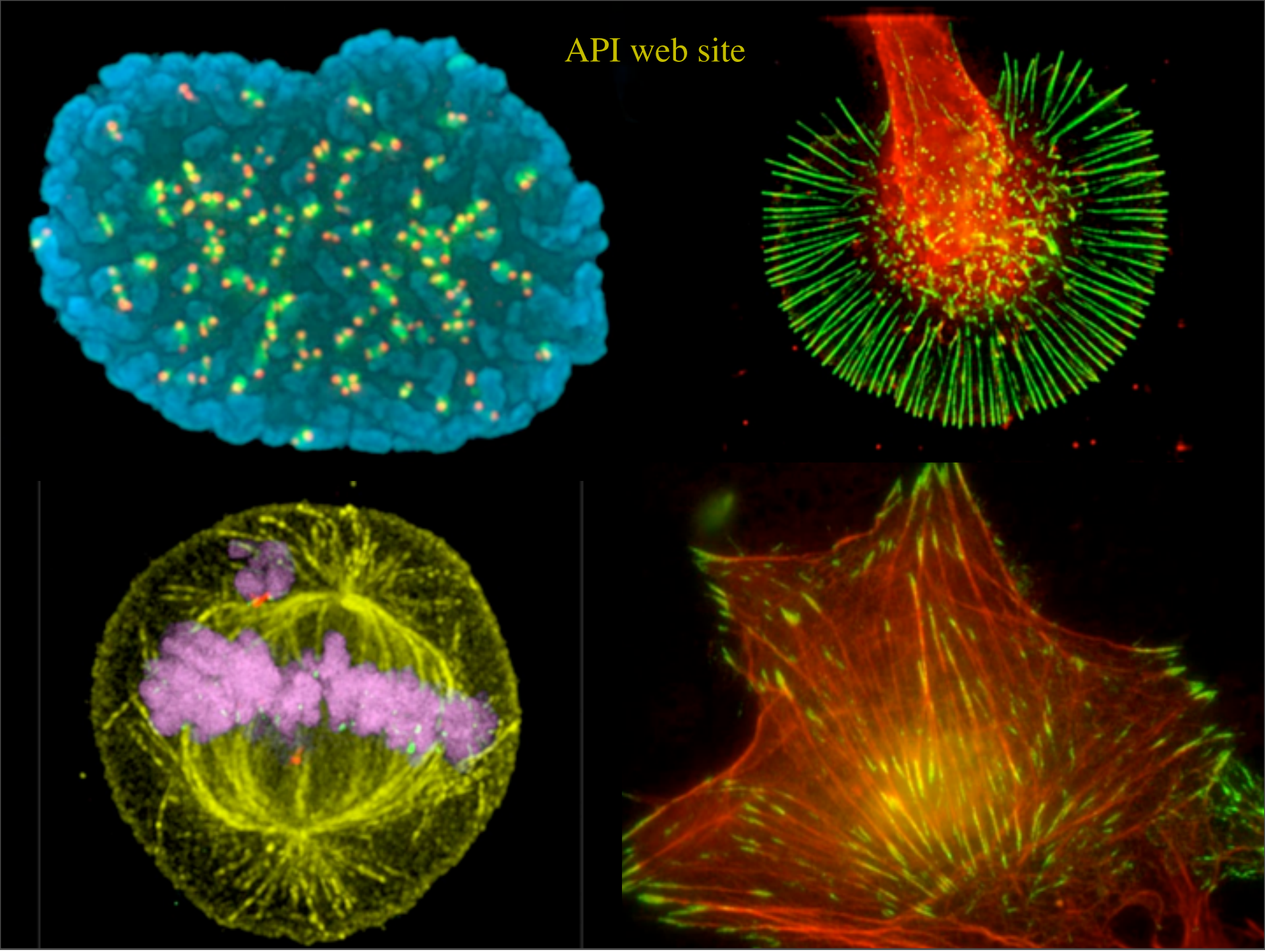


now many related variations

practical issues are signal to noise, accuracy of PSF

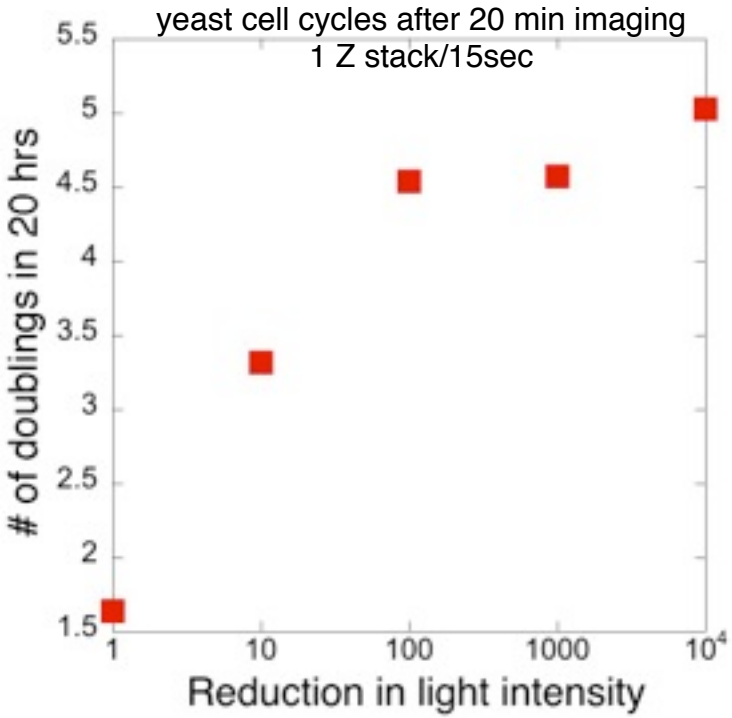
if sample is very thick then OSF can vary throughout sample
(minimize by matching index of refraction

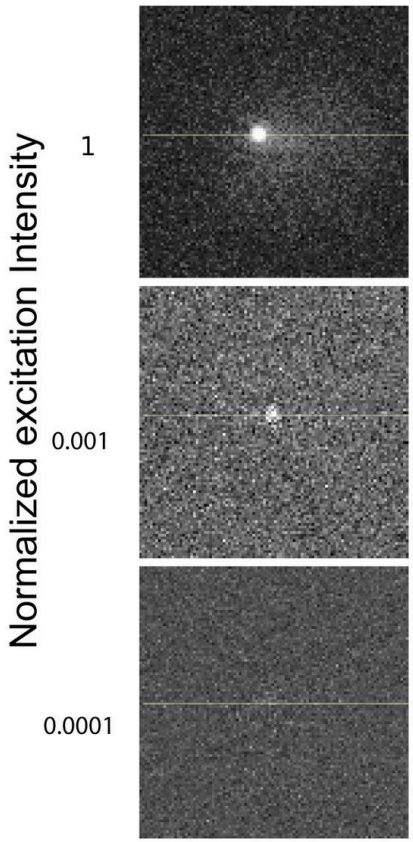
API web site



The in vivo imaging problem: phototoxicity

the challenge of live imaging:





maximum allowable photon flux that allows unperturbed viability is 4.8 nW/μm^2

"With this dose level, and with the current state-of-the-art deconvolution, the signal was insufficient for any quantitative measurement, even tracking"

Pete Carlton et al, PNAS, 2010

Formulation of the deconvolution problem

$$g_{opt}(r) = \arg\min_{g} \sum_{r} \left(h(r) * g(r) - f(r) \right)^2 + \lambda R(g)$$

Regularization functional
defines how noise is handled

$$R(g) = \sum_{r} \left[\log\left(1 + \alpha \left(g^2(r) + \sum_{i=1}^{6} (L_i(r) * g(r))^2 \right) \right) + \sum_{r} N(g(r)) \right]$$

use entropy based optimization (ER-Decon)

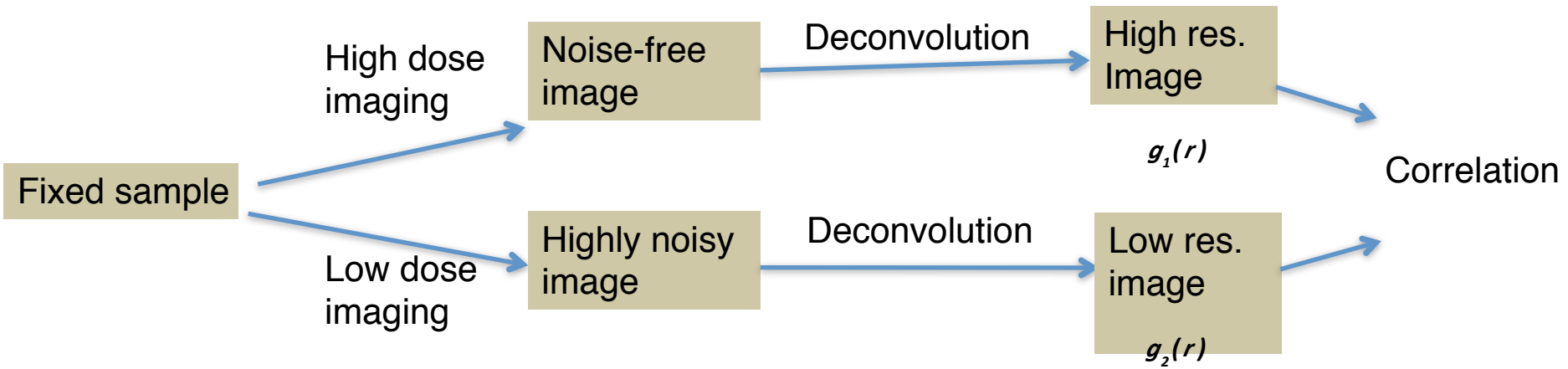
$\{L_1, L_2, \ldots, L_6\}$: Second derivative filters

α: Nonlinearity factor

$$N(s) = \begin{cases} s^2 & \text{for } s < 0 \\ 0 & \text{for } s \geq 0 \end{cases}$$

M. Arigovindan

Validate by quantitative comparison to high dose image



Compare our method (ER-Decon), with two existing methods

(1) **Huygens** – commercially available method

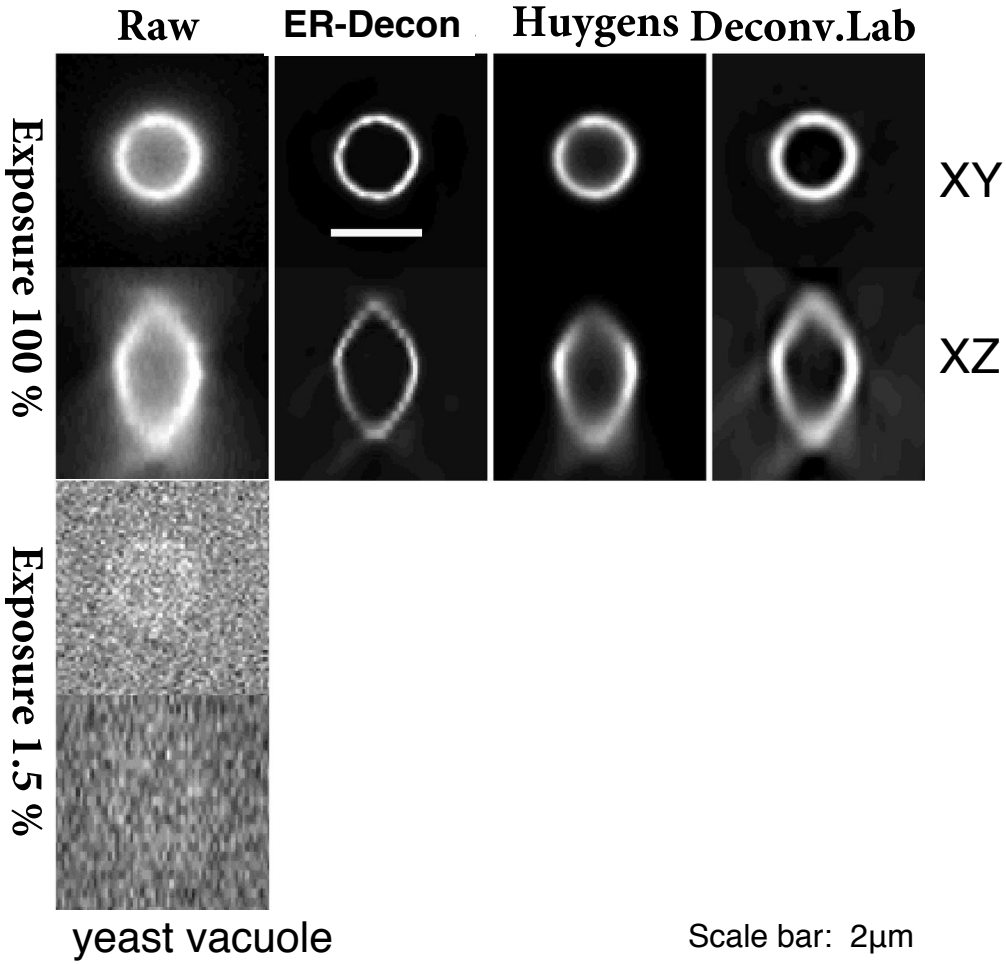
(uses MLE approach)

(2) **DeconvolutionLab (DeconLAB) from Unser's group at EPFL** -

best among free packages (uses wavelet approach)

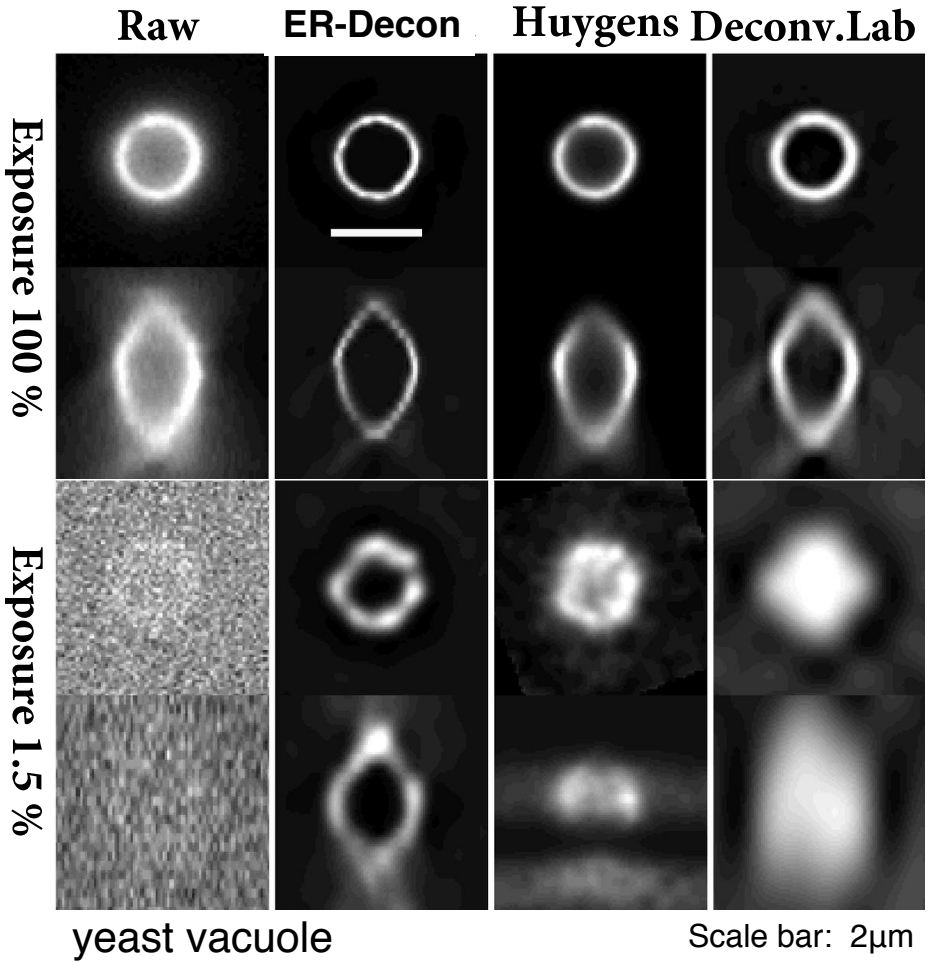
M. Arigovindan

Remarkable signal recovery simultaneous with deconvolution



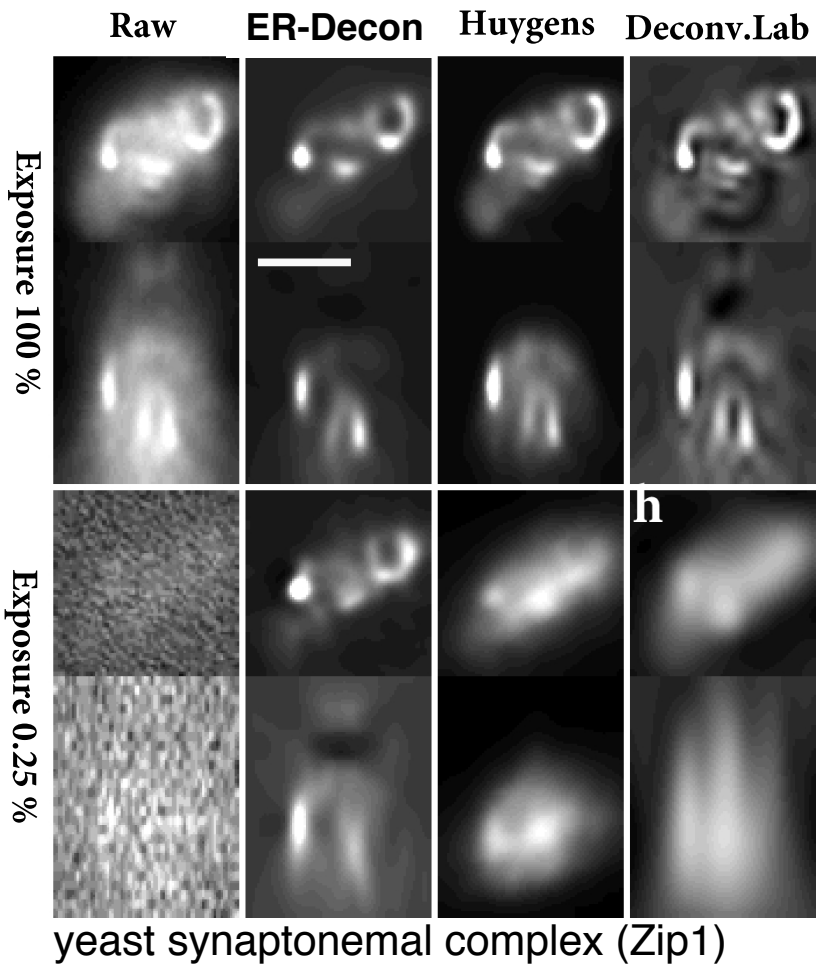
M. Arigovindan

Remarkable signal recovery simultaneous with deconvolution



yeast vacuole

Scale bar: 2µm



yeast synaptonemal complex (Zip1)

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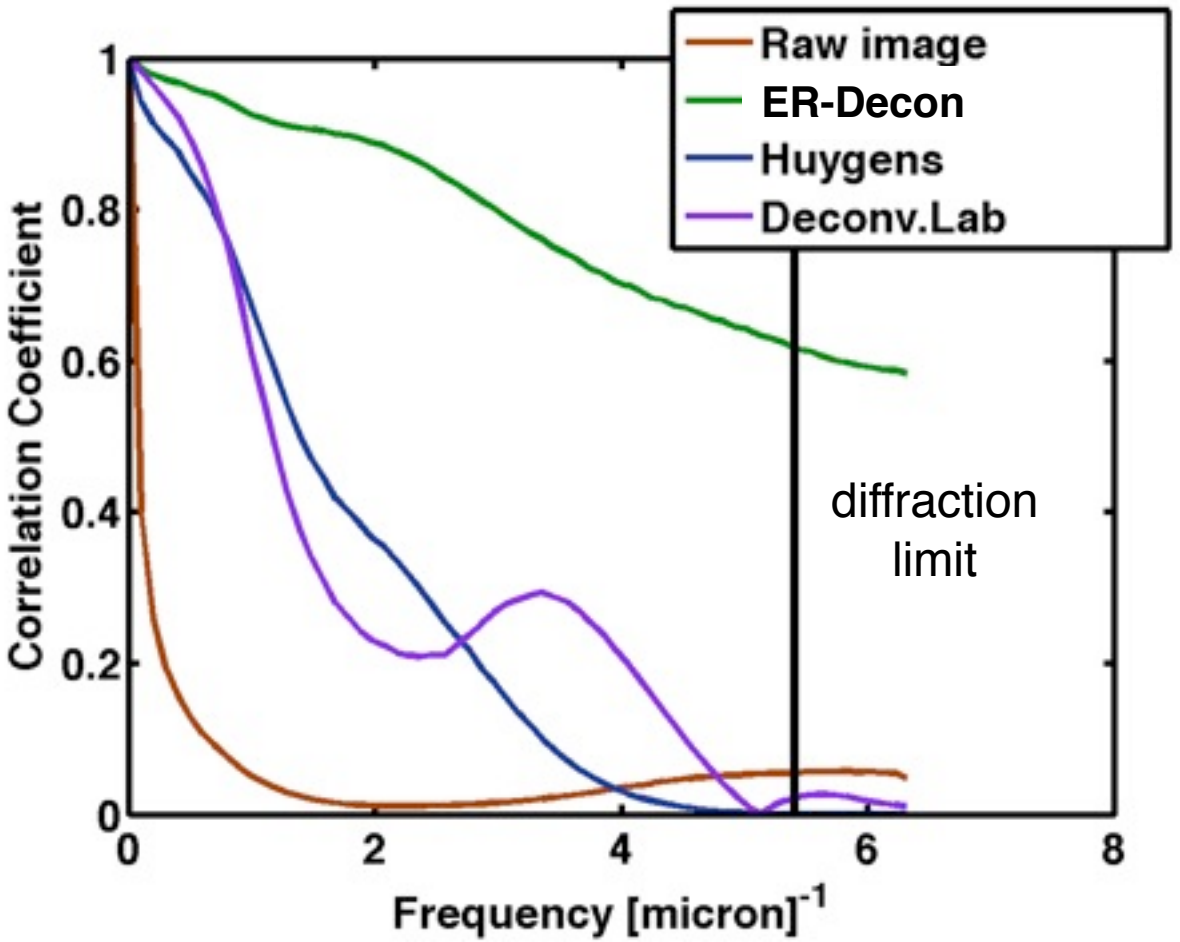
Fourier shell analysis confirms signal recovery
(400-fold reduced intensity)



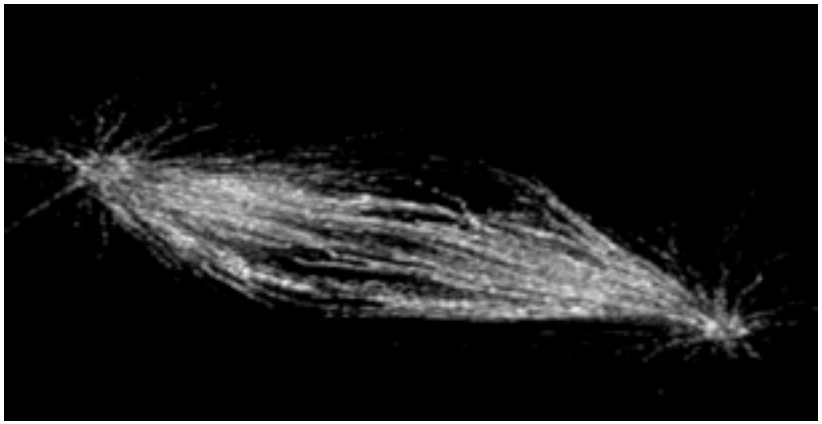
enabling technology for in vivo imaging

M. Arigovindan

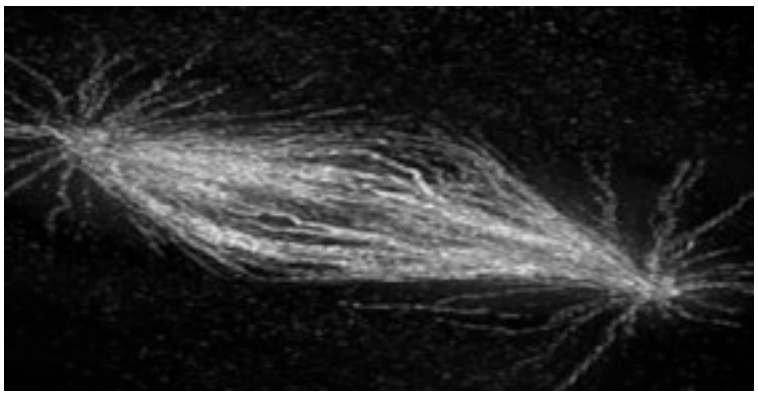
ER-Decon deconvolution of SIM data

Widefield

SIM-Wiener filtering

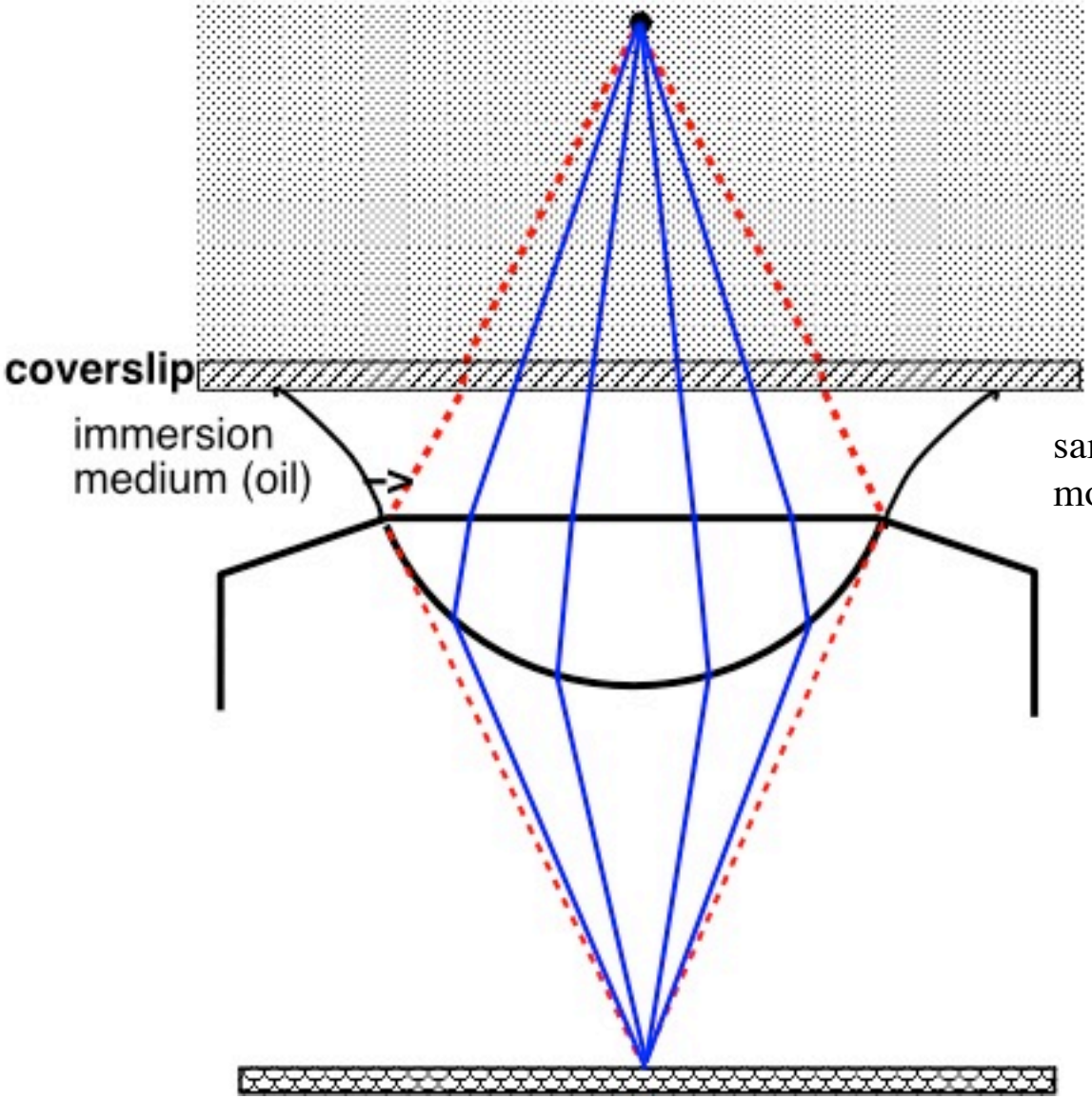


SIM + ER-Decon

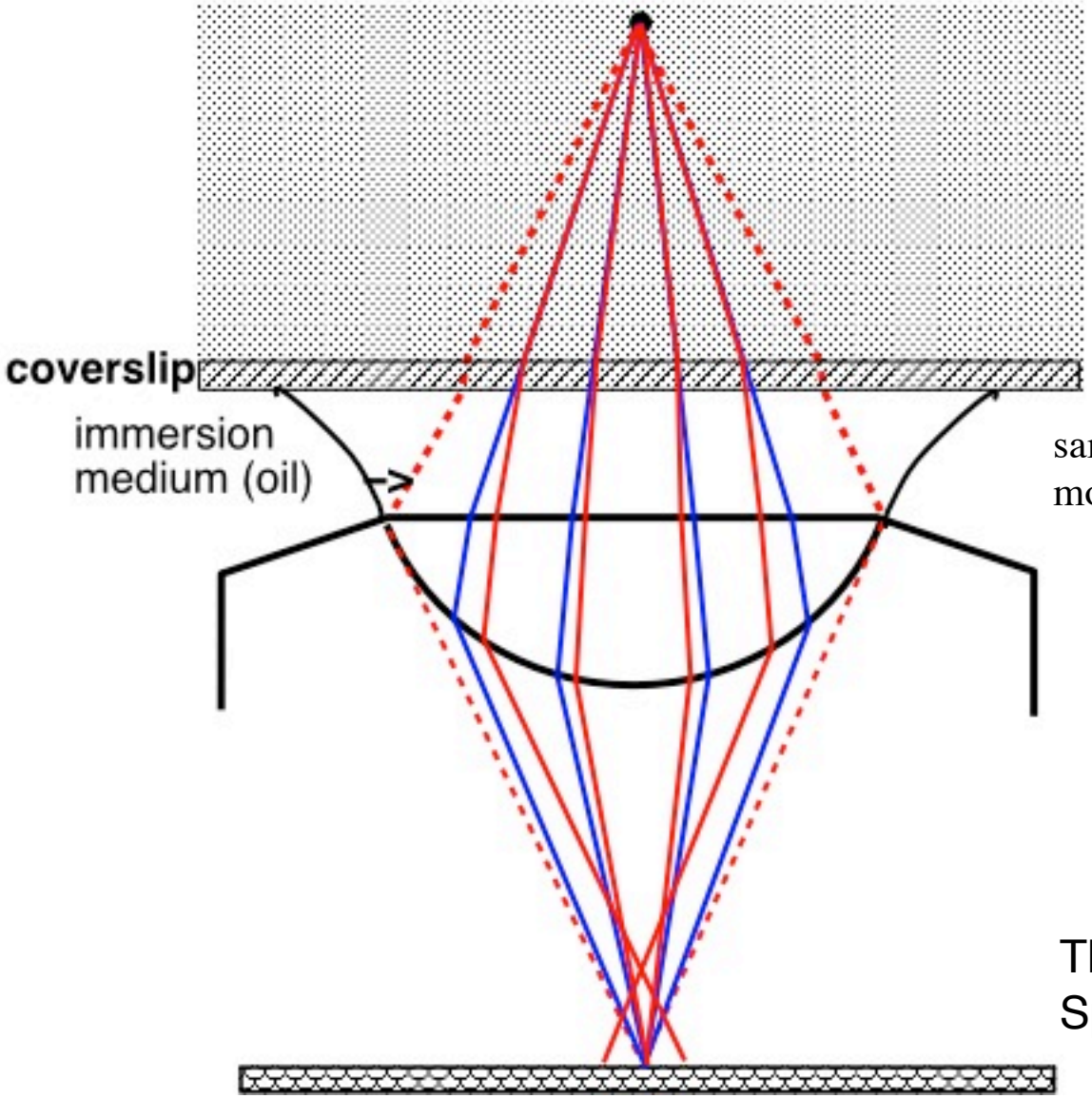


M. Arigovindan

Properties of the Sample Affect Image Formation in Microscopy

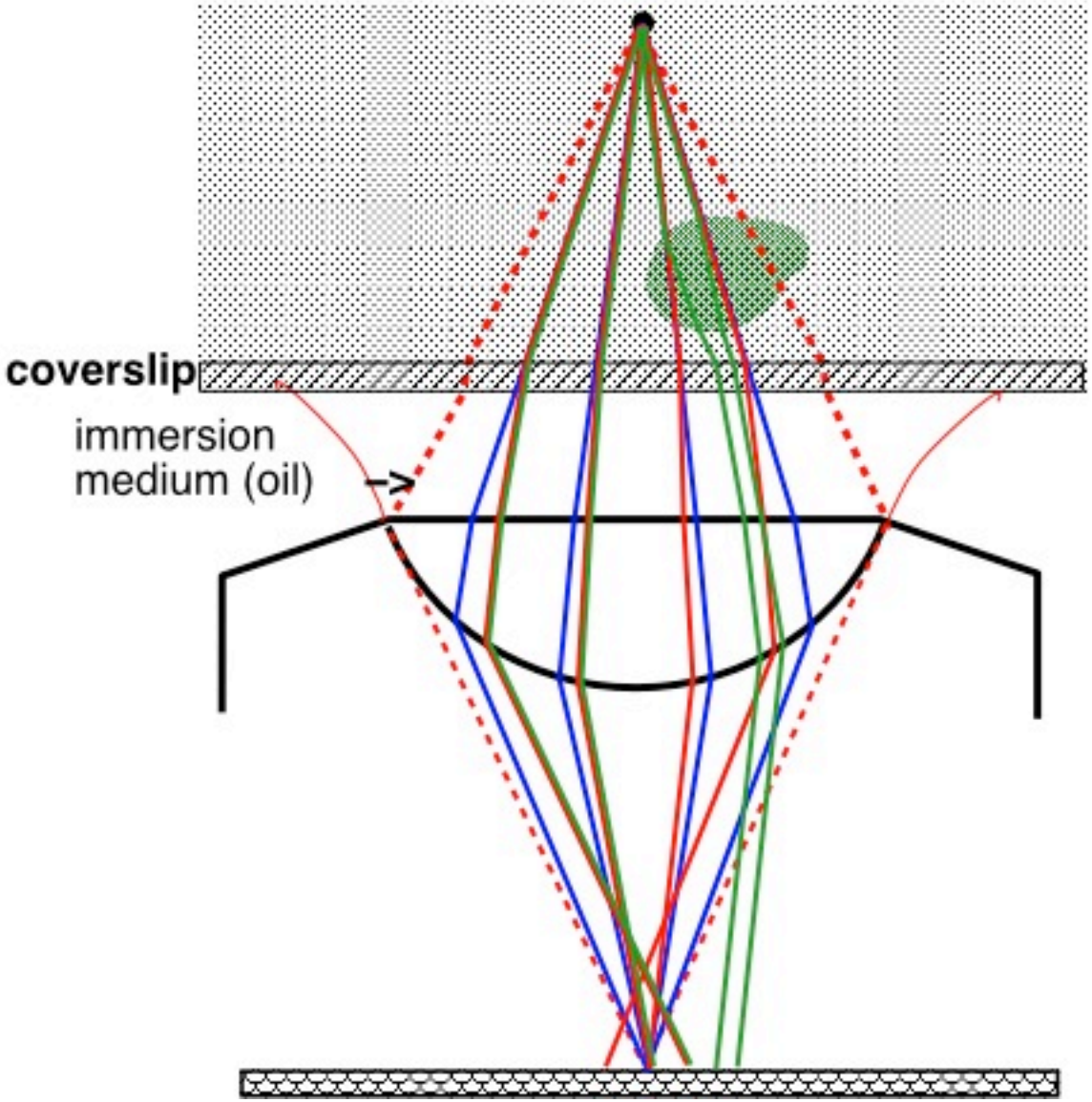


Properties of the Sample Affect Image Formation in Microscopy



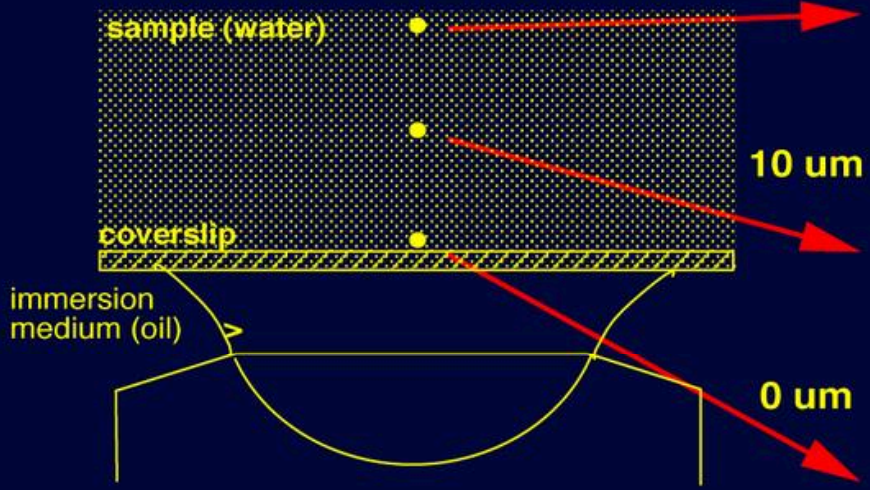
This causes
Spherical Aberration

Properties of the Sample Affect Image Formation in Microscopy



sample η not constant

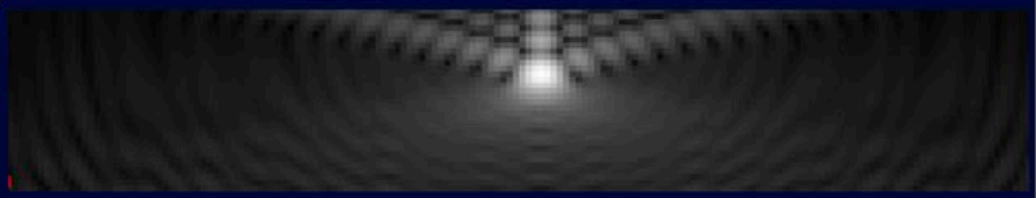
Depth-Dependent Effects in Water: Simulations of PSFs

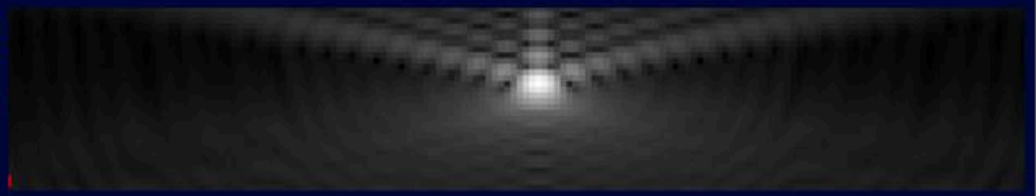


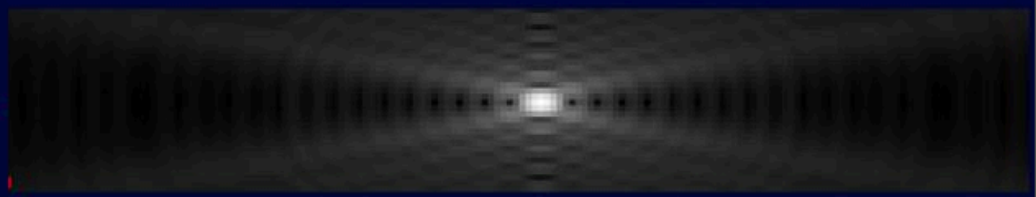
Depth:
20 um

10 um

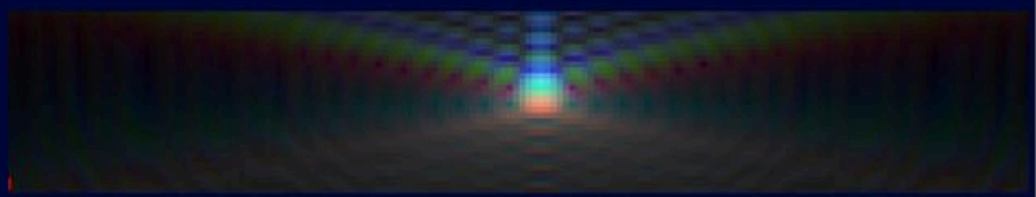
0 um







Overlay: 0 um (red)
10 um (green)
20 um (blue)
pixel spacing: xy 0.0566, z 0.25um

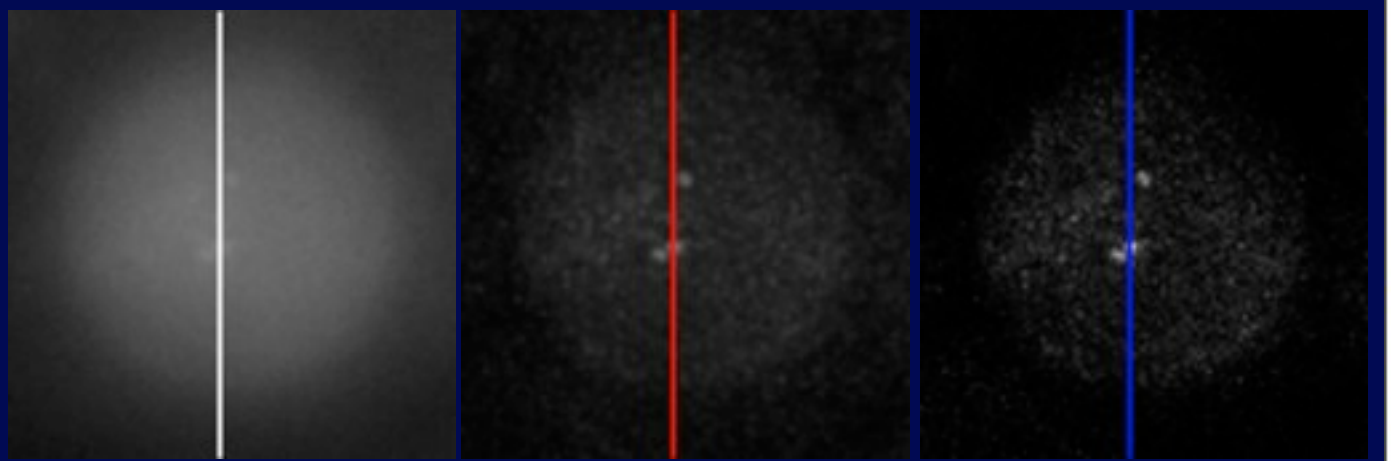


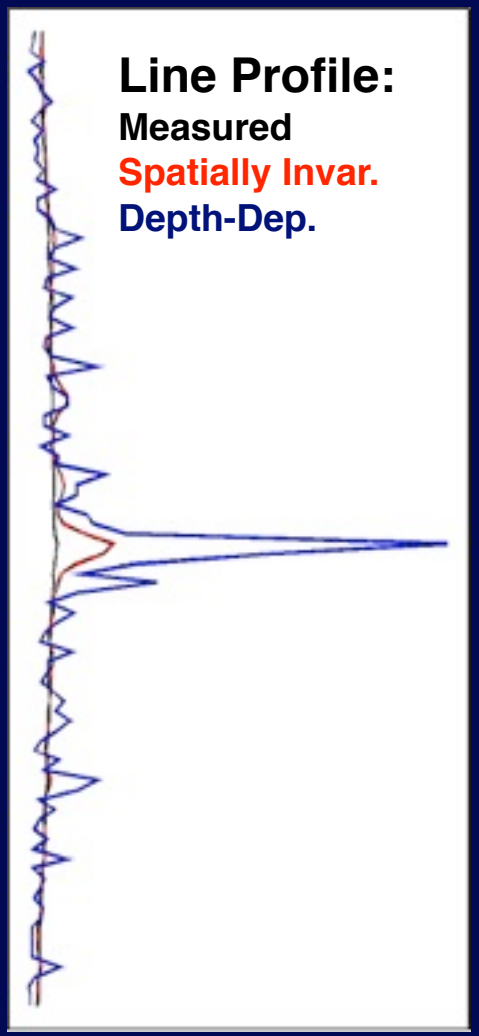
Hanse

Depth Dependent Deconvolution: Biological Data

Line Profile Through 1 Spot in Nucleus
Section ~7 .6 μm into sample

Measured Data | **Post-Deconvolution**
Spatially-Invariant | **Depth-Dependent**





Spot FWHM:

Measured = 0.28 μm
Spatially-Invariant = 0.20 μm
Depth-Dependent = 0.11 μm

Hanser

Adaptive optics can correct for depth dependent effects (reshape optical wavefront)

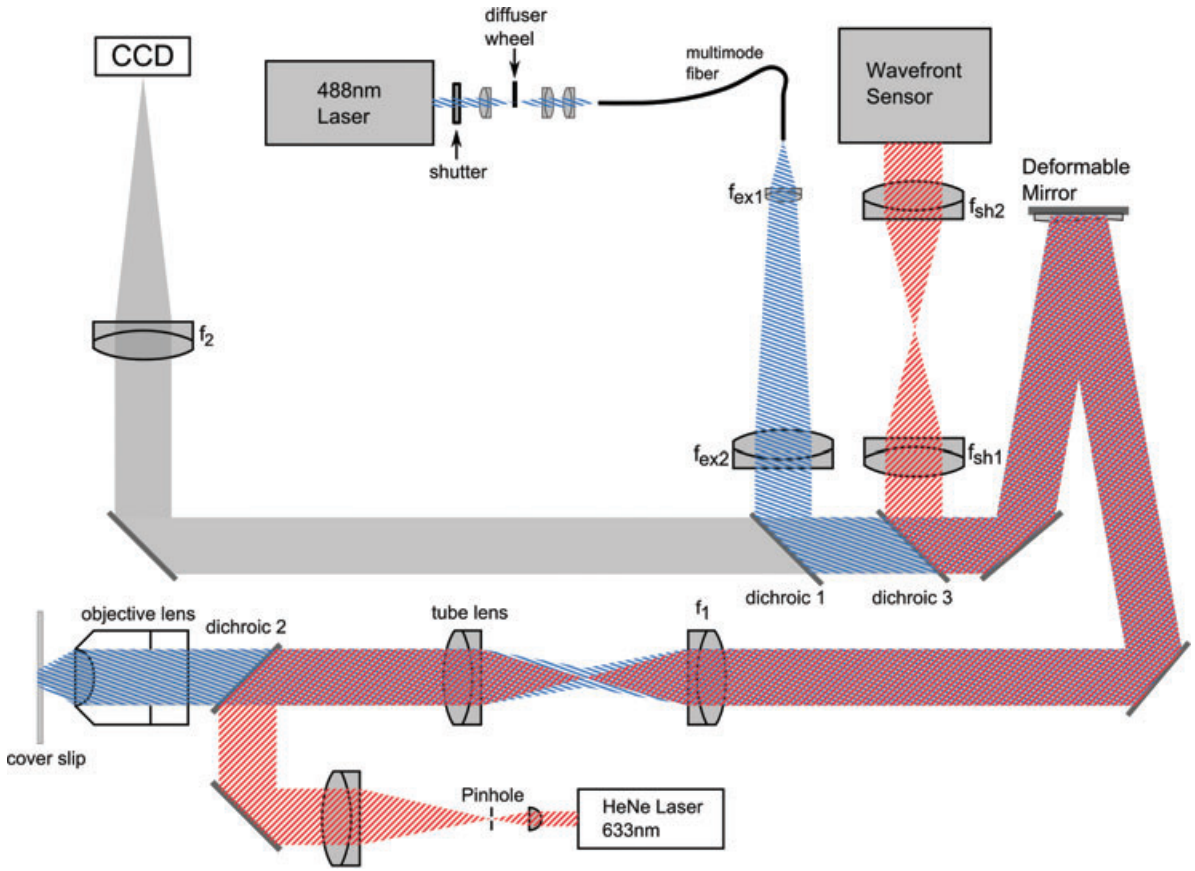
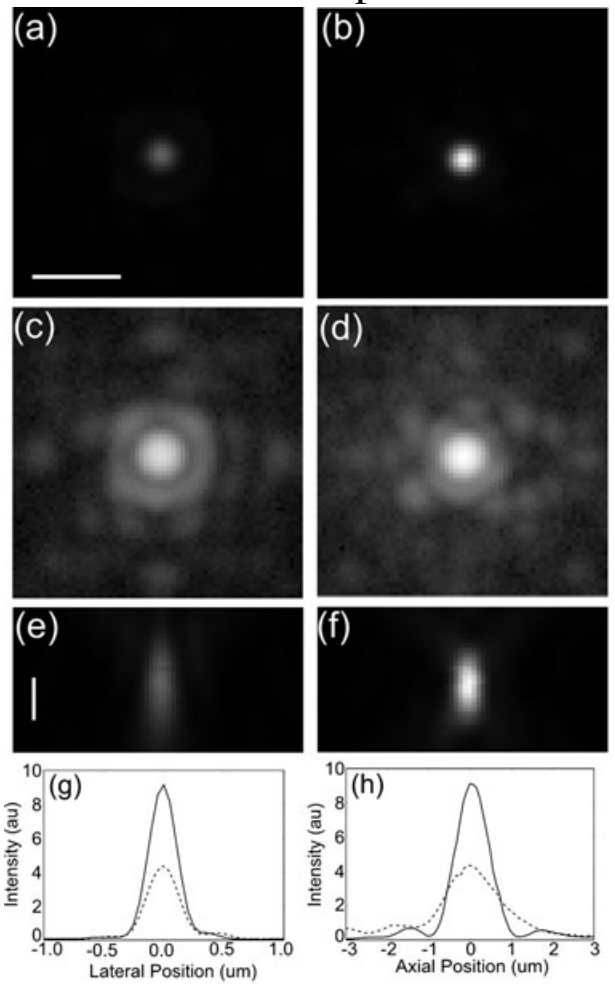


Fig. 2. Microscope layout. Grey represents the emission path. The blue striped beam is the excitation light and the red striped beam is the reference beam. See text for details.

Adaptive optics can correct for depth dependent effects

flat mirror

mirror shaped to correct spherical aberration



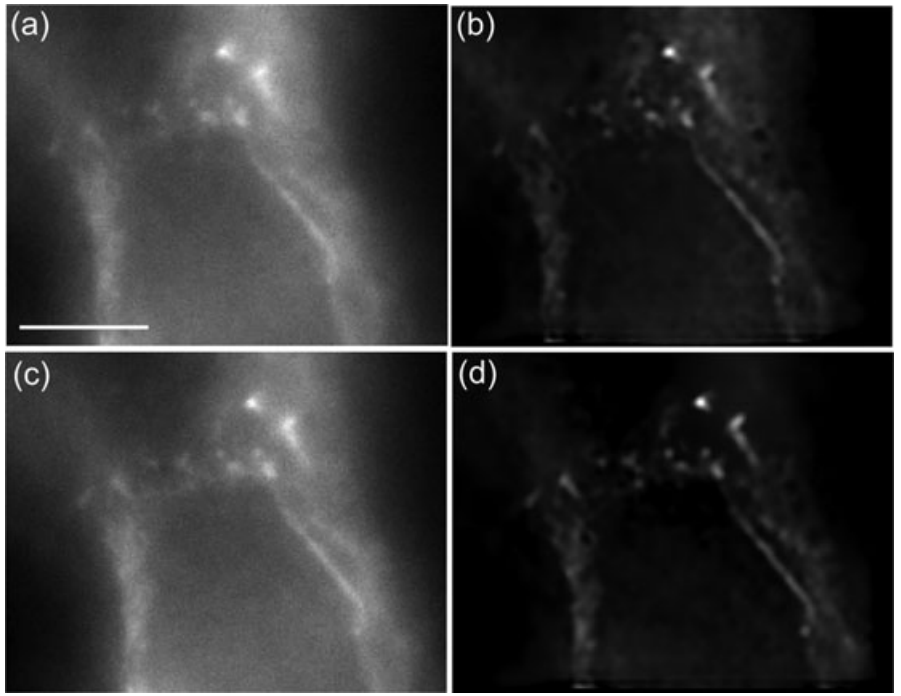
X-Y

X-Y log scale

X-Z

raw

decon



-AO

+AO

Alexa 488-phalloidin stained mouse cells: images at 4.4μm below cover slip

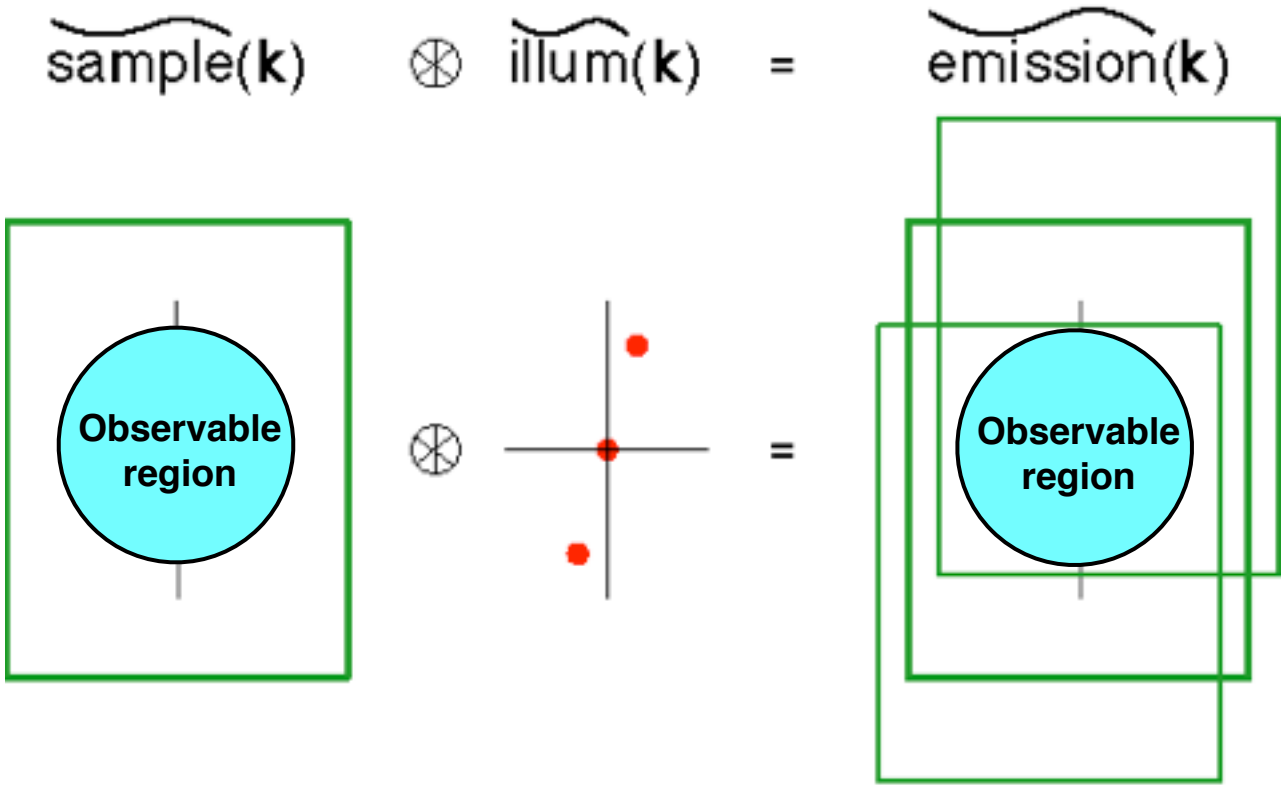
Structured illumination microscopy: the idea

Two patterns superposed multiplicatively give rise to **moiré fringes**

The moiré fringes may be coarse enough to resolve even if neither original pattern is

- *Illuminate the sample with a light pattern*
- *Observe moiré fringes between the pattern and the sample structure*
- *Deduce otherwise unresolvable information about the sample*

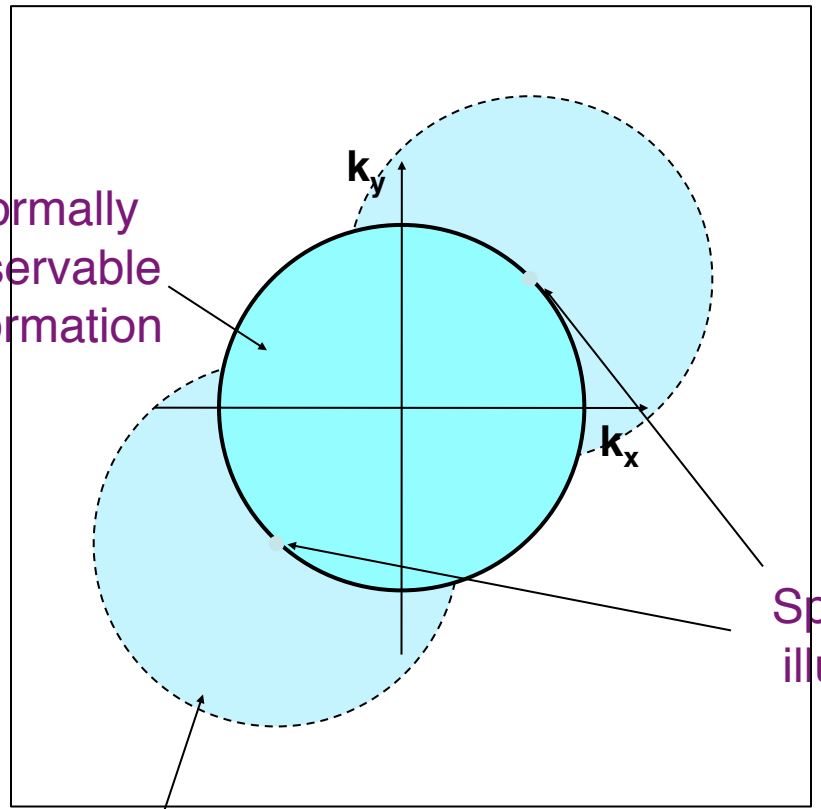
Illuminate sample with parallel stripes



The emitted light contains 3 superimposed information components shifted by 0, ± the inverse stripe spacing

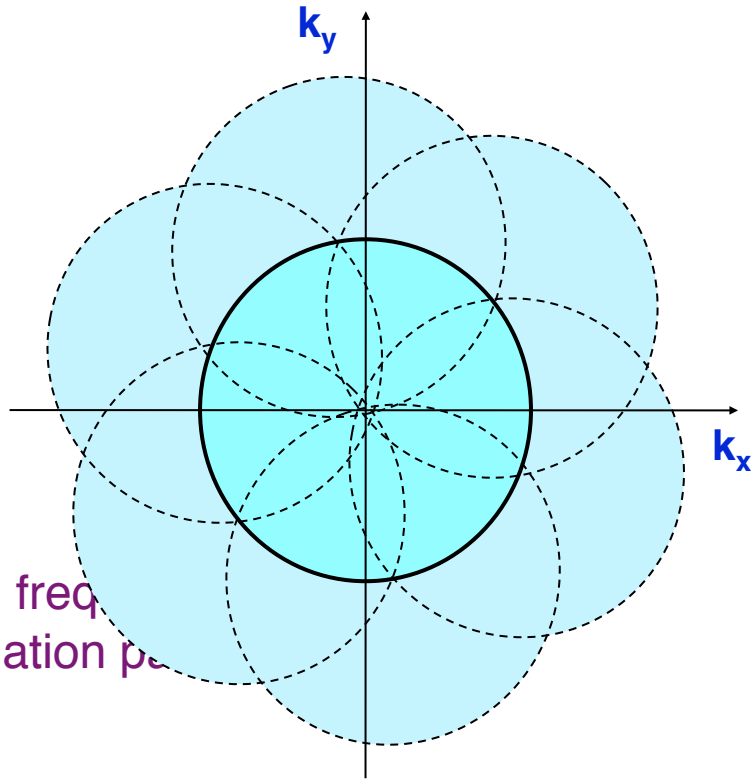
Record 3 images (0°, 120°, 240° shifts) to sort out 3 components

Resolution extension by Structured Illumination



Normally observable information

Spatial freq
illumination p



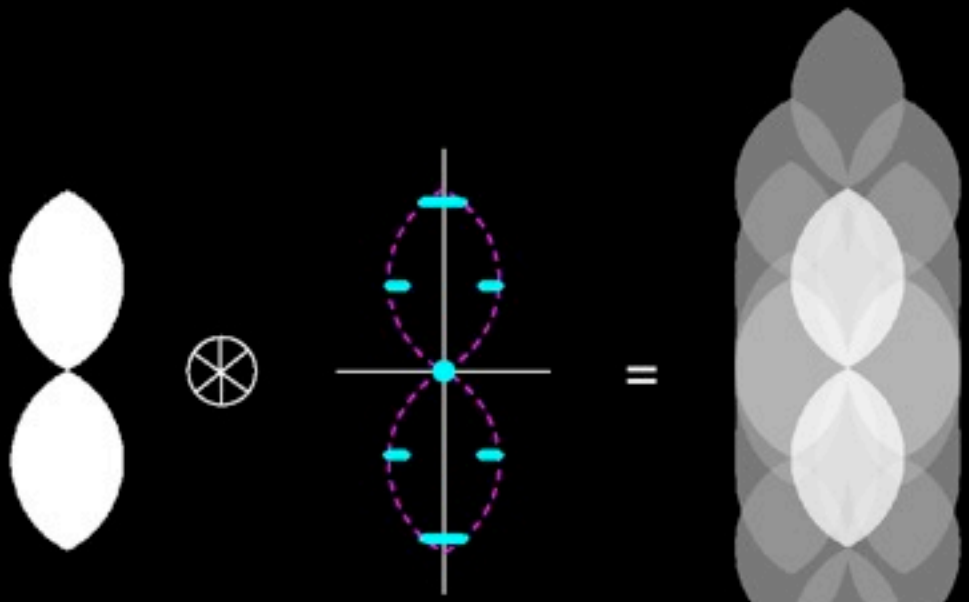
…and can repeat in many directions

Information observable as moiré fringes

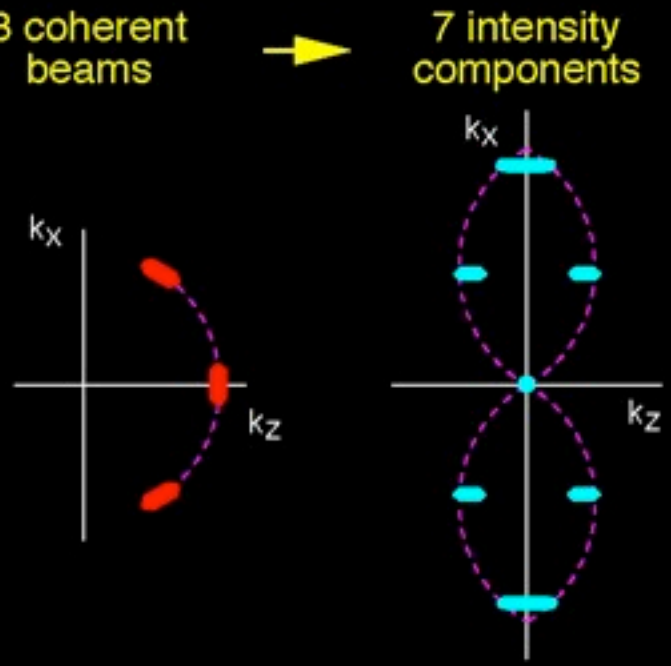
Q: What about 3D?

A: Use 3 beams

(admit diffraction orders 1, 0 and +1)



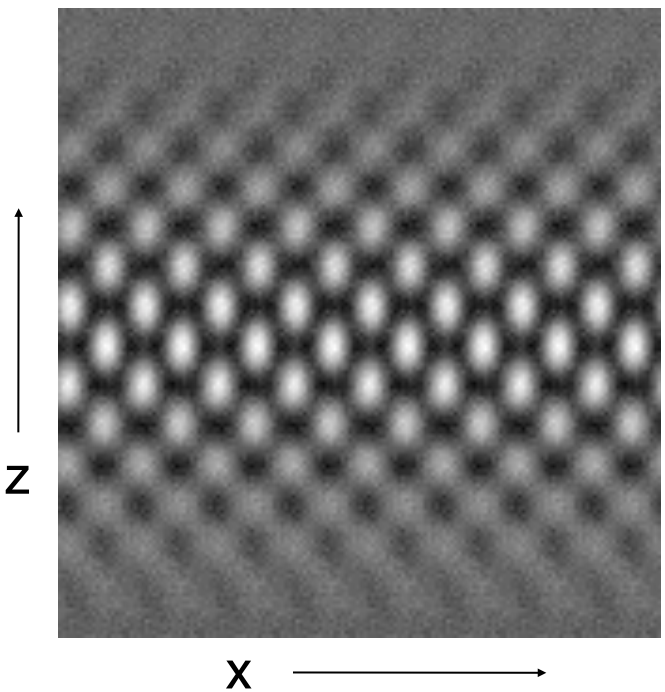
conventional observable region ⊛ illumination structure = new observable region

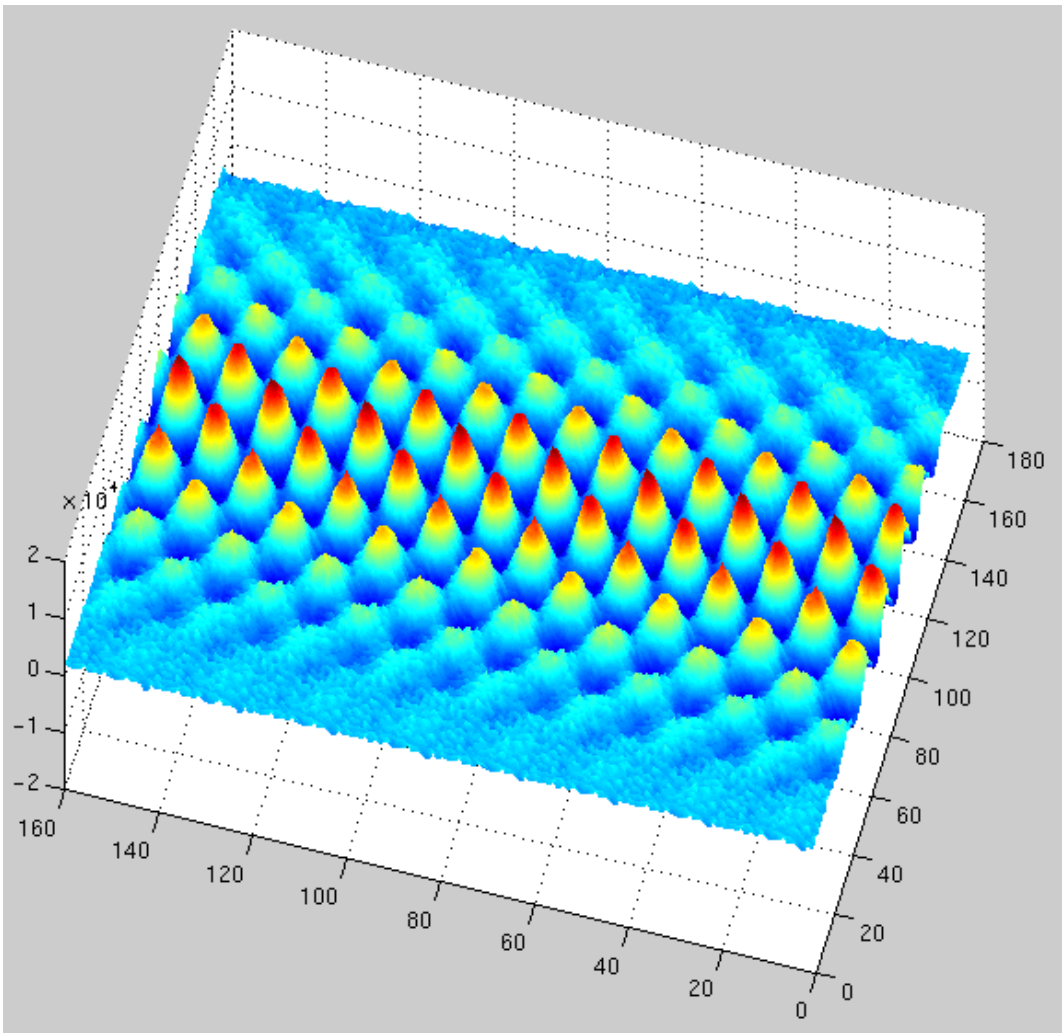


Covers full "confocal" OTF support without discarding light

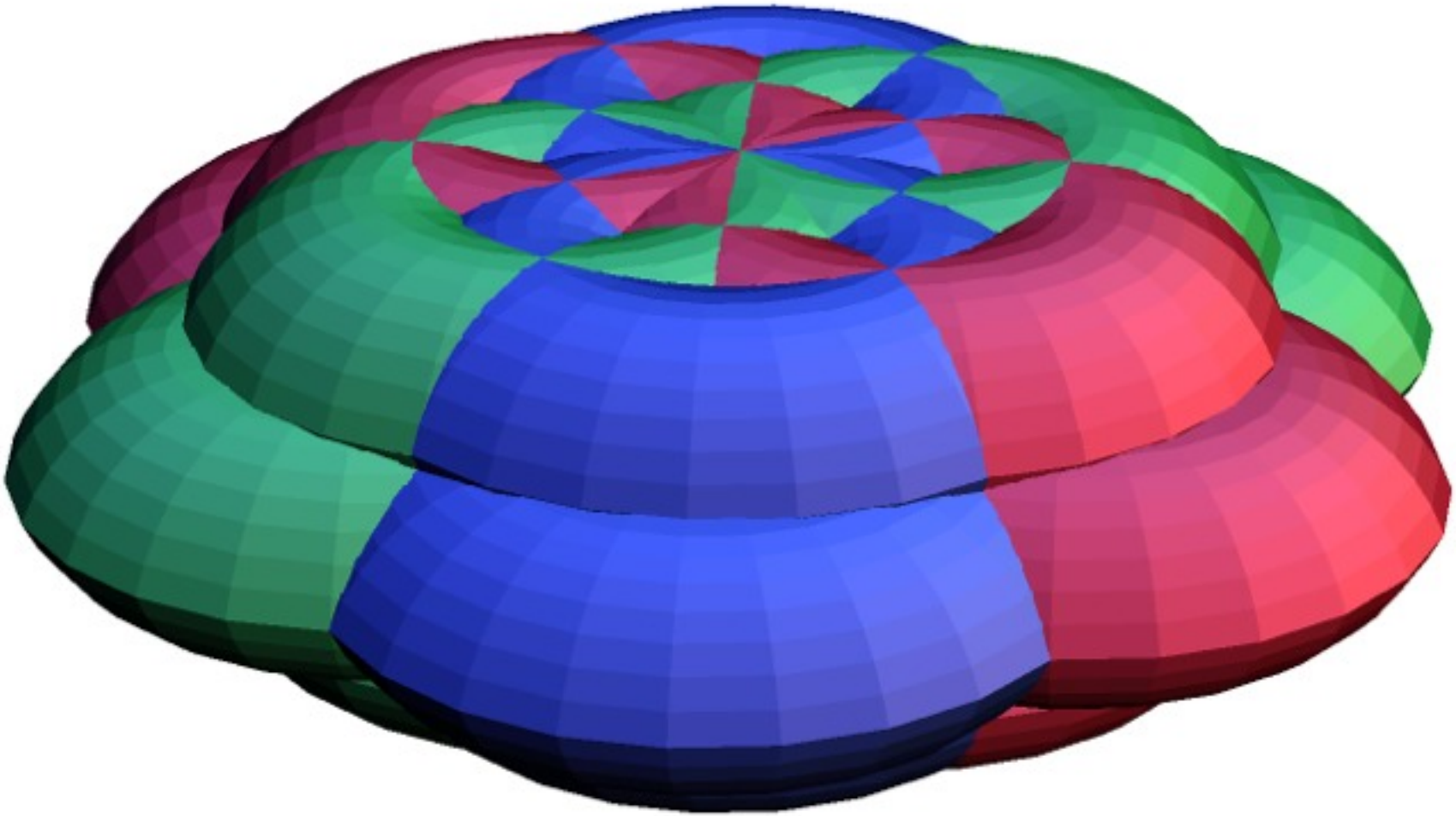
Same procedure as for 2D, except need 5 phases

illumination intensity in real space

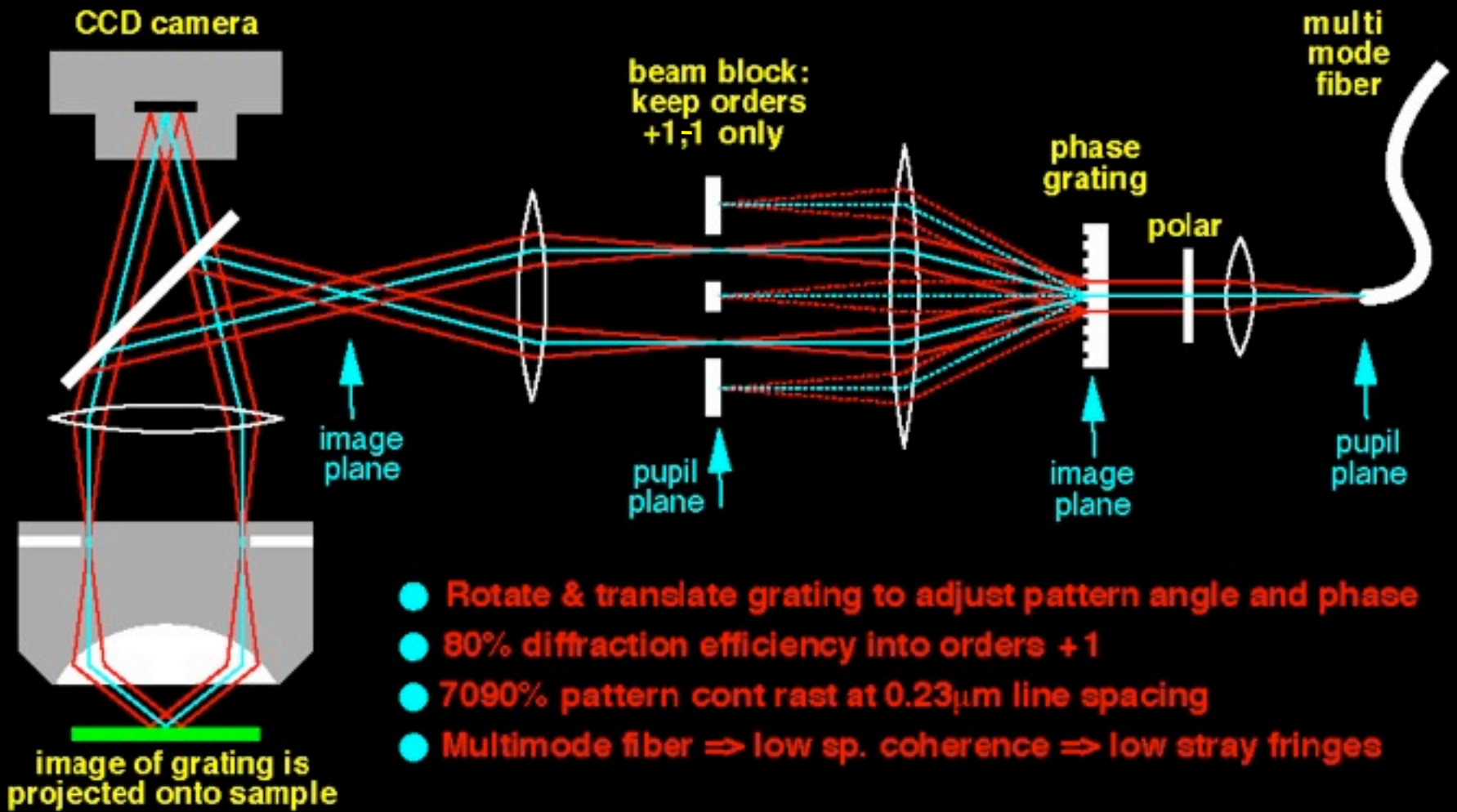




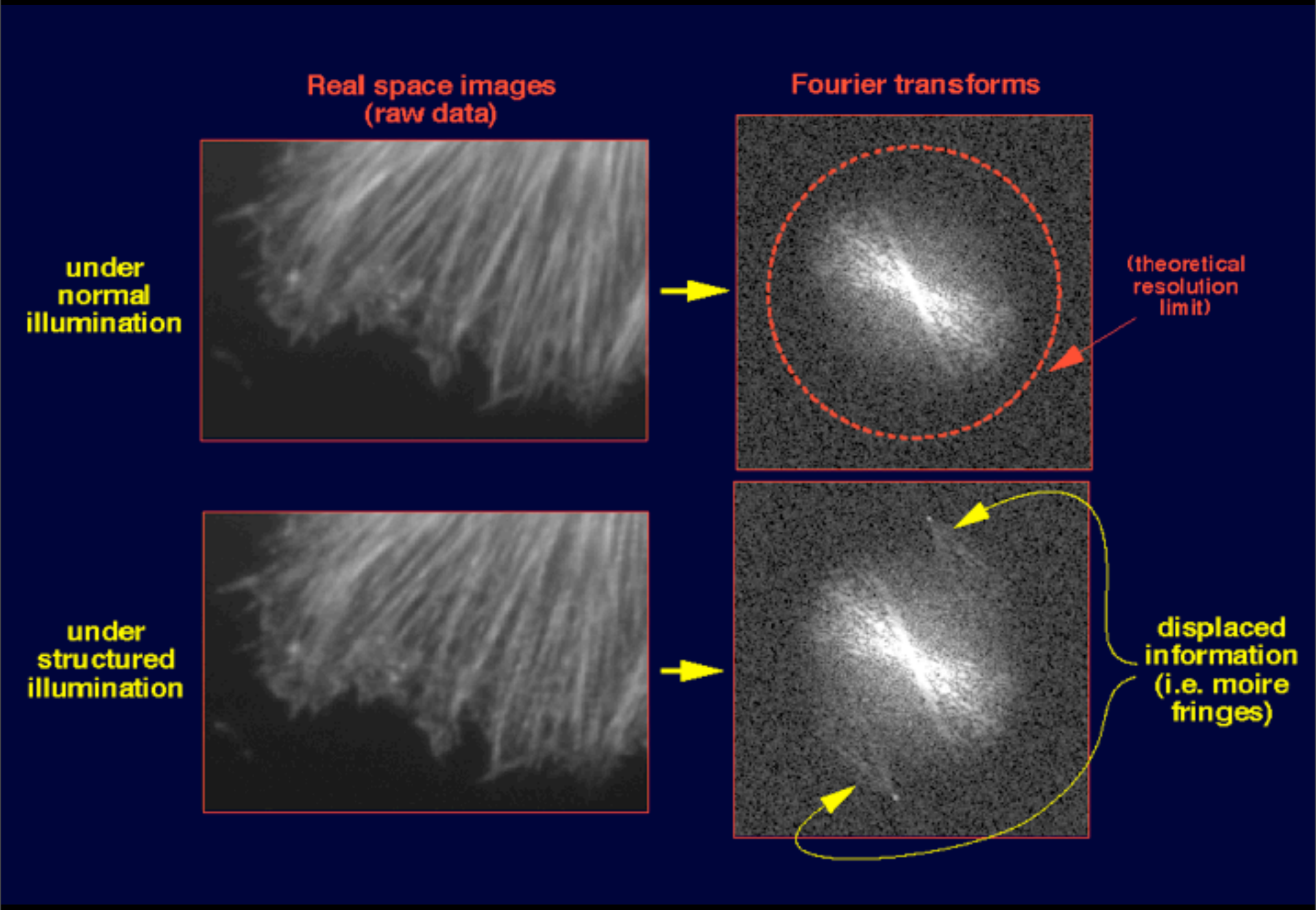
Observable Region
Structured Illumination (3 orientations)

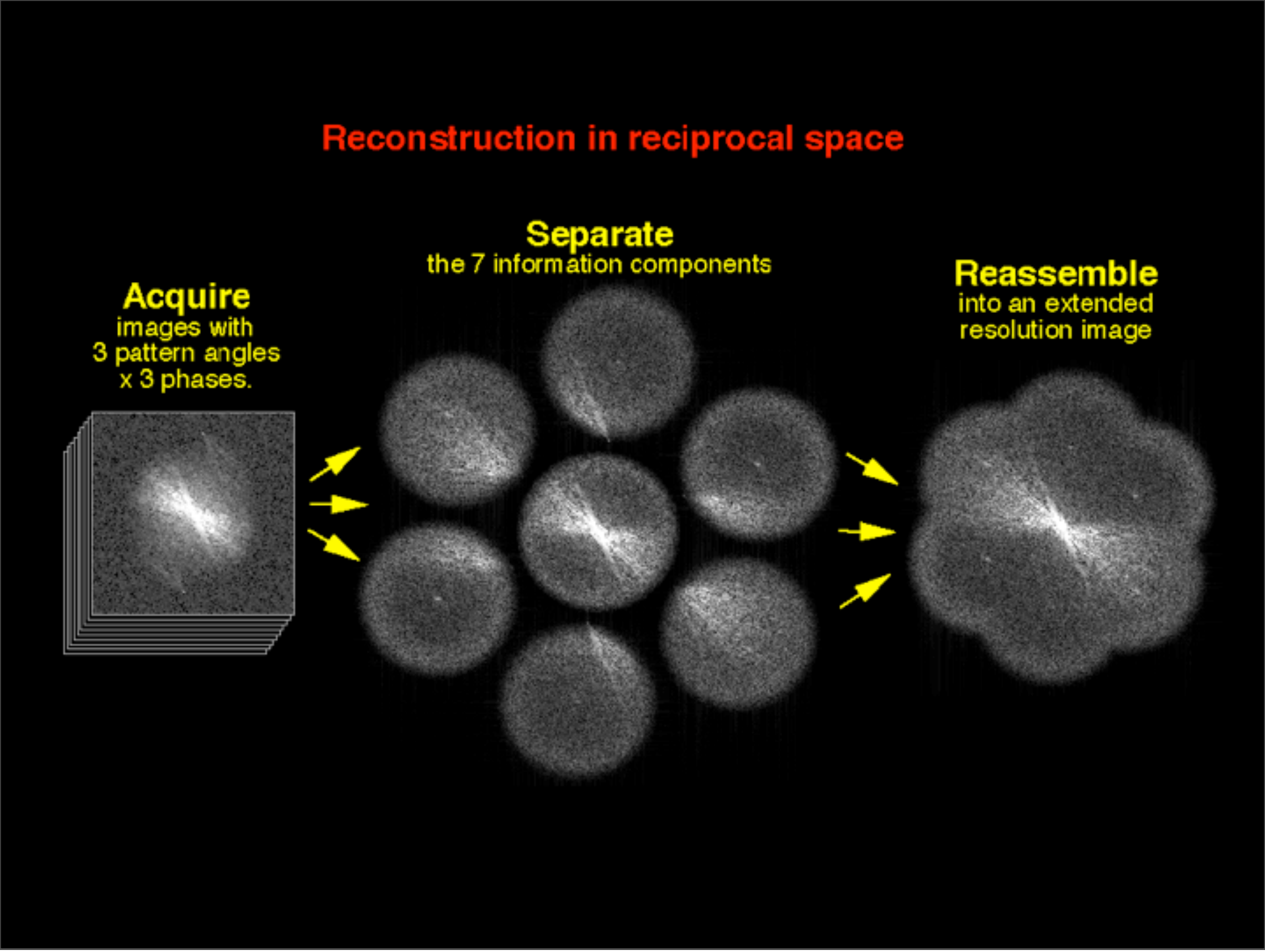


Structured illumination apparatus



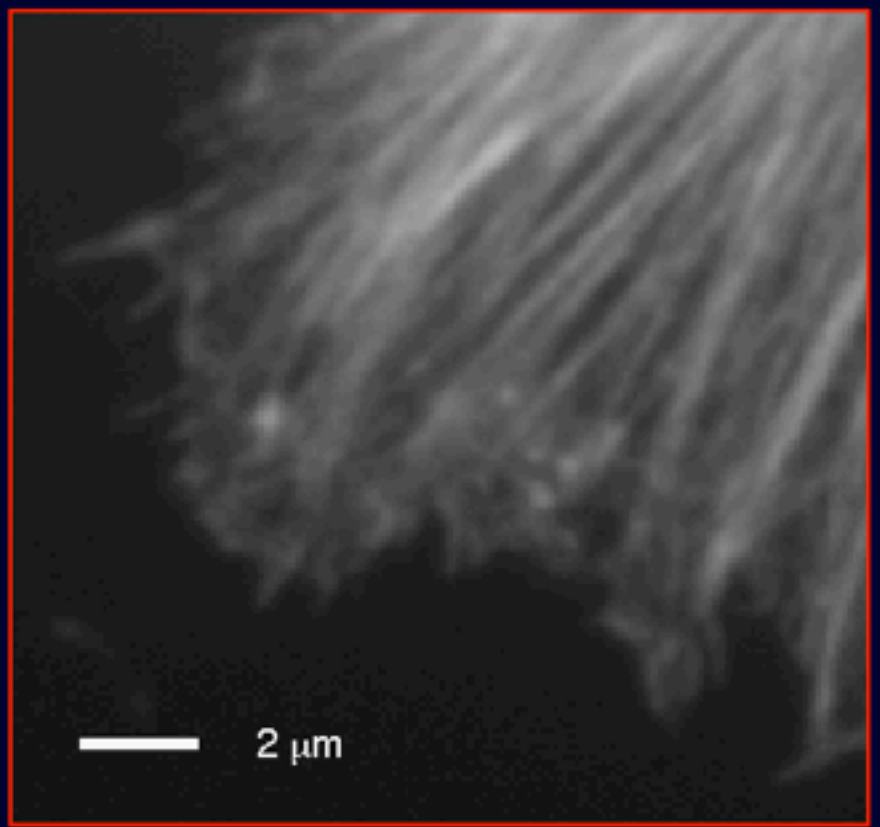
- Rotate & translate grating to adjust pattern angle and phase
- 80% diffraction efficiency into orders +1
- 7090% pattern cont rast at 0.23μm line spacing
- Multimode fiber ⇒ low sp. coherence ⇒ low stray fringes


Real space images
(raw data)
Fourier transforms
under
normal
illumination
(theoretical
resolution
limit)
under
structured
illumination
displaced
information
(i.e. moire
fringes)

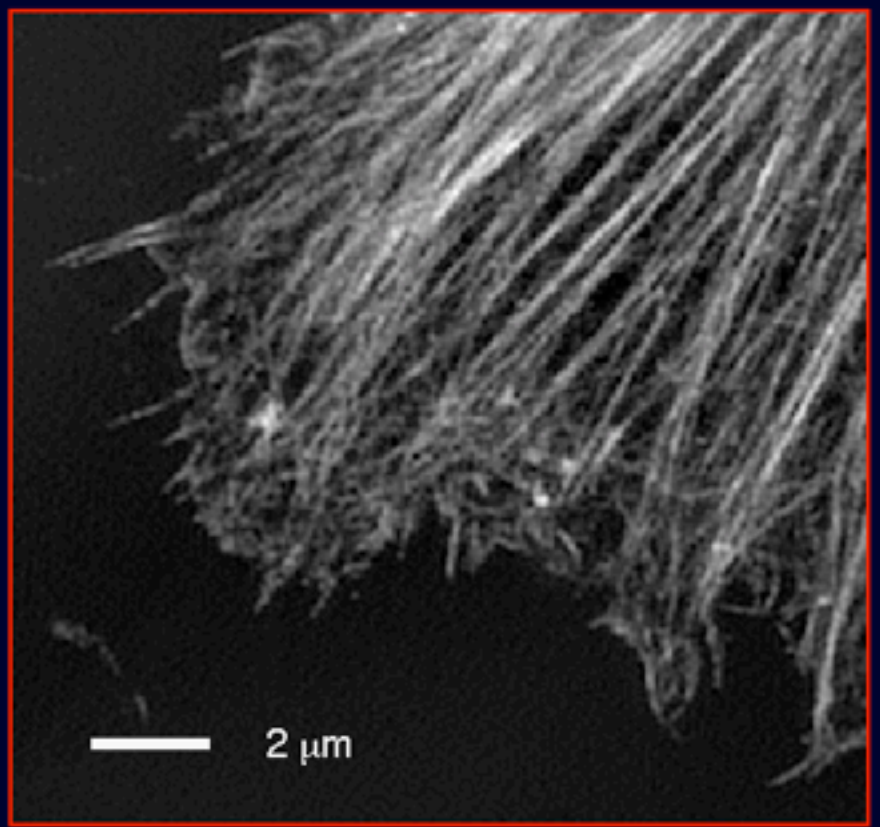

Reconstruction in reciprocal space
Acquire
images with
3 pattern angles
x 3 phases.
Separate
the 7 information components
Reassemble
into an extended
resolution image

Resolution comparison
Actin in a HeLa cell

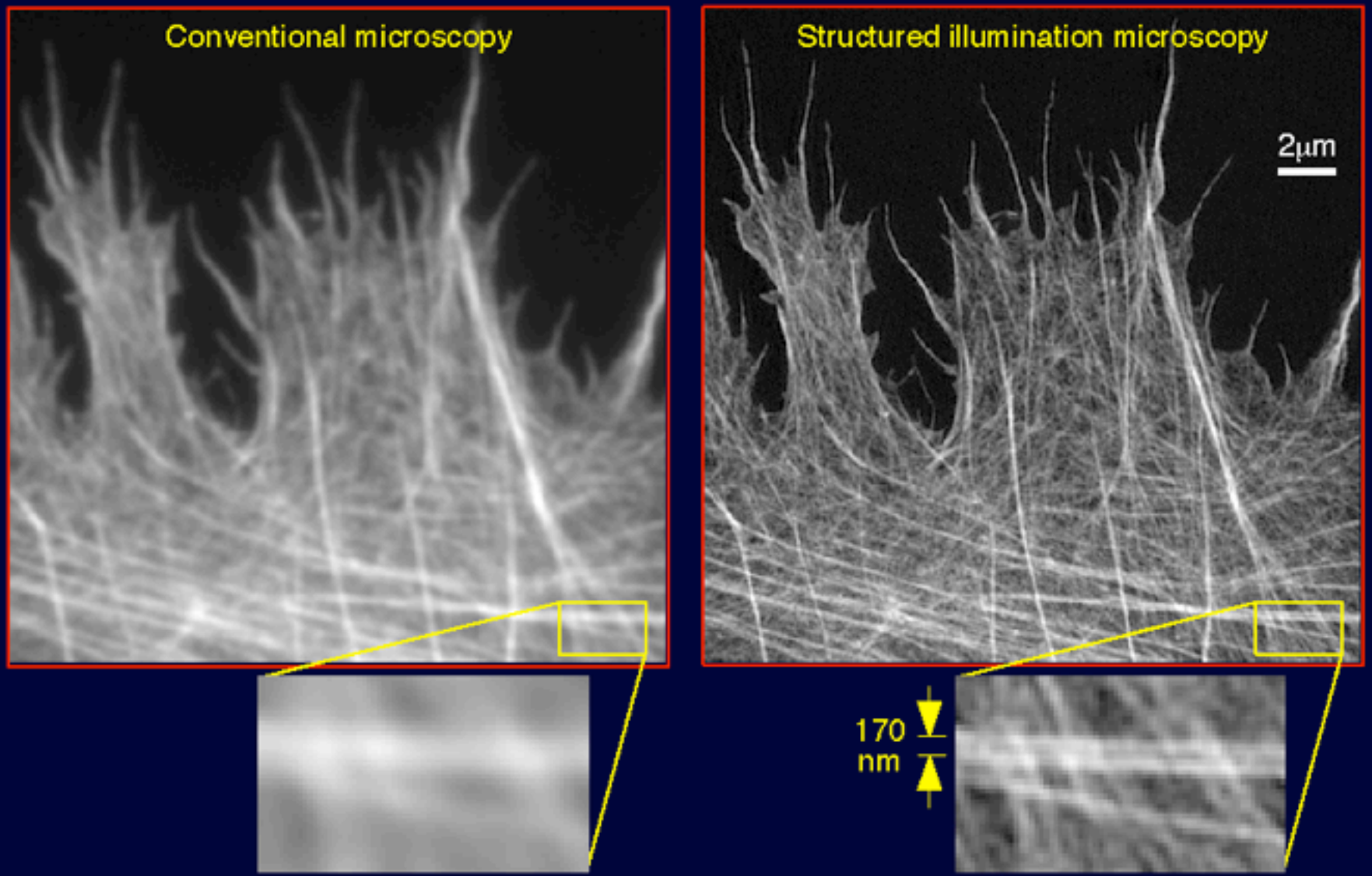
Conventional microscopy



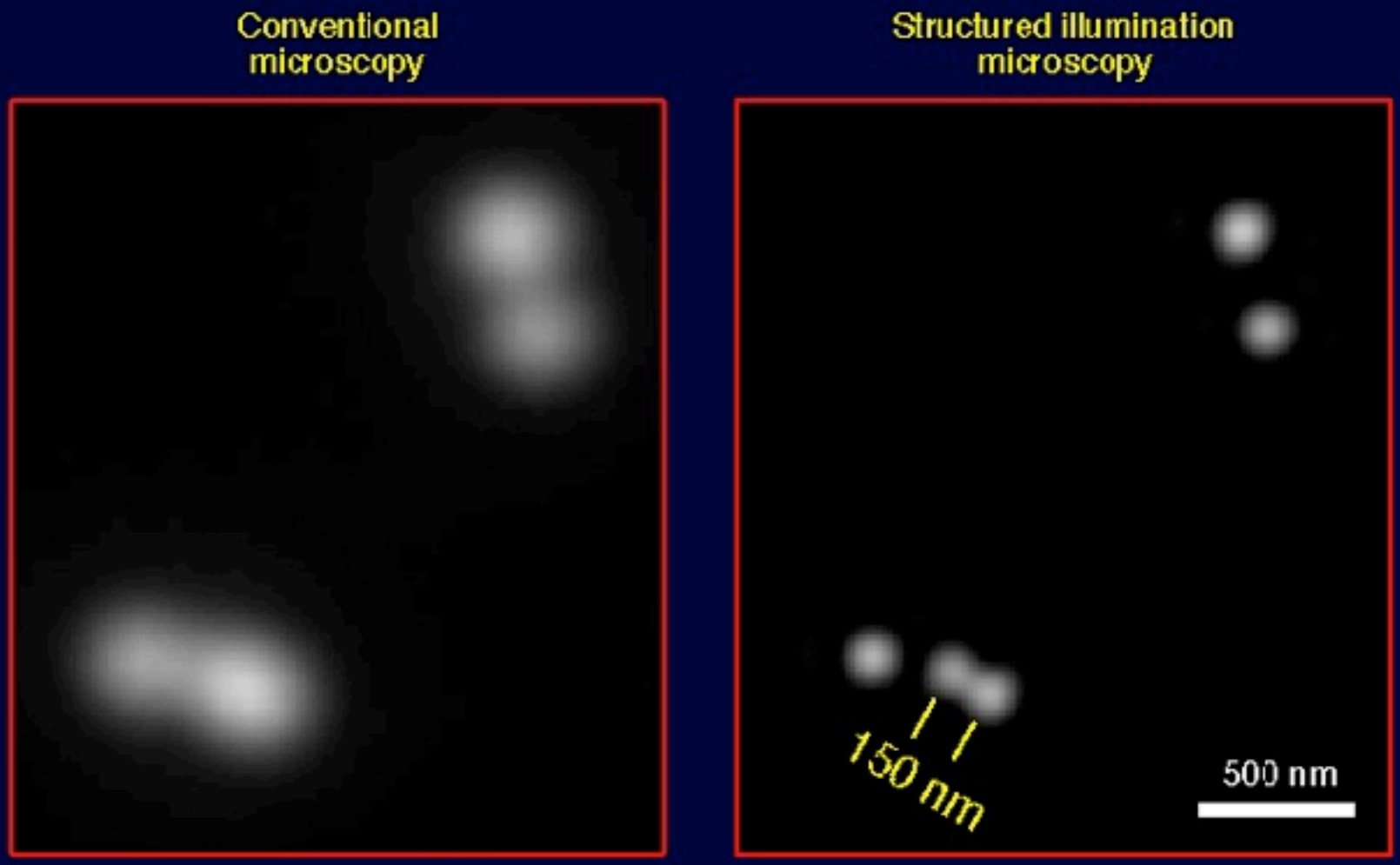
Structured illumination
final reconstruction

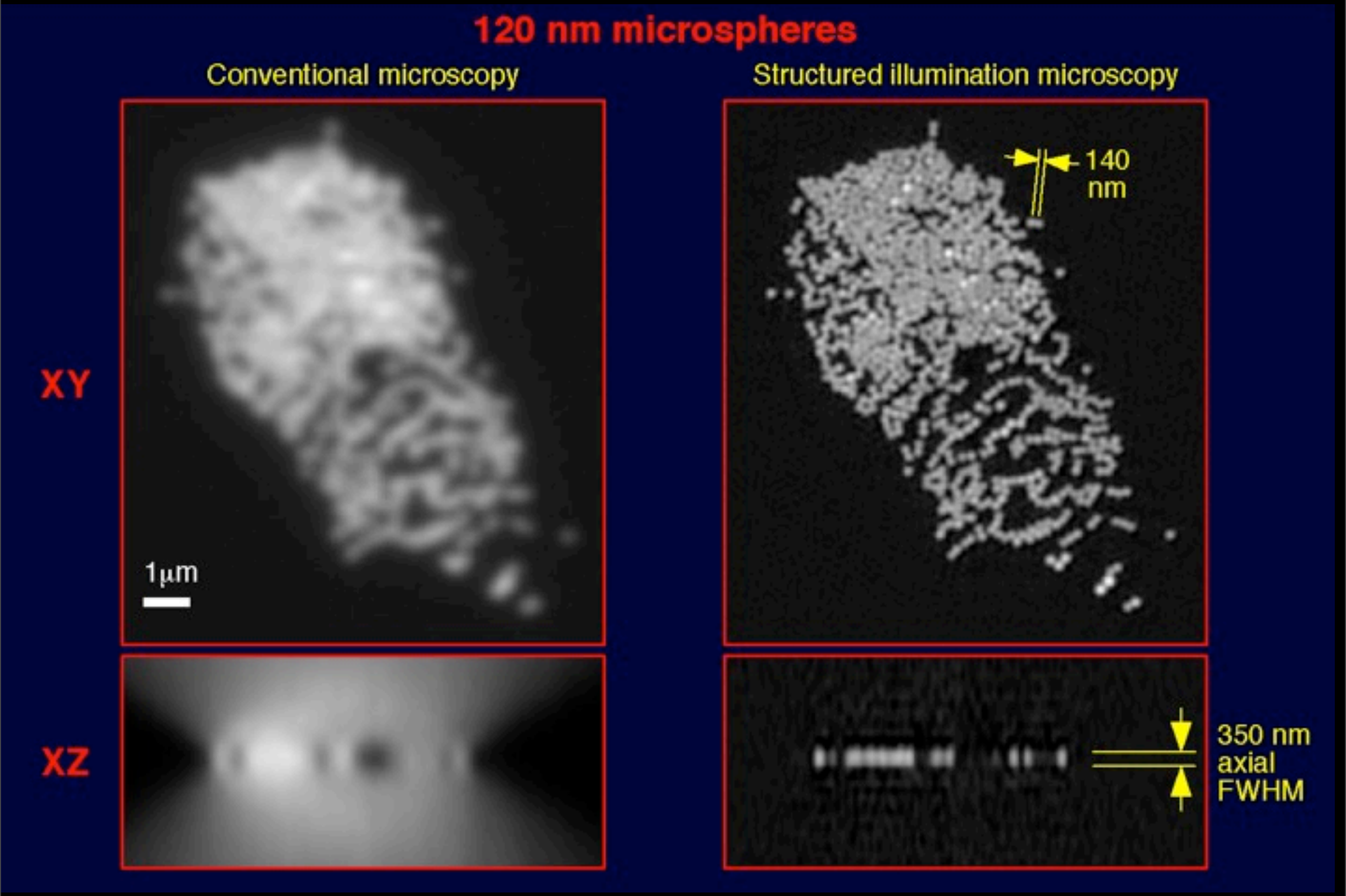


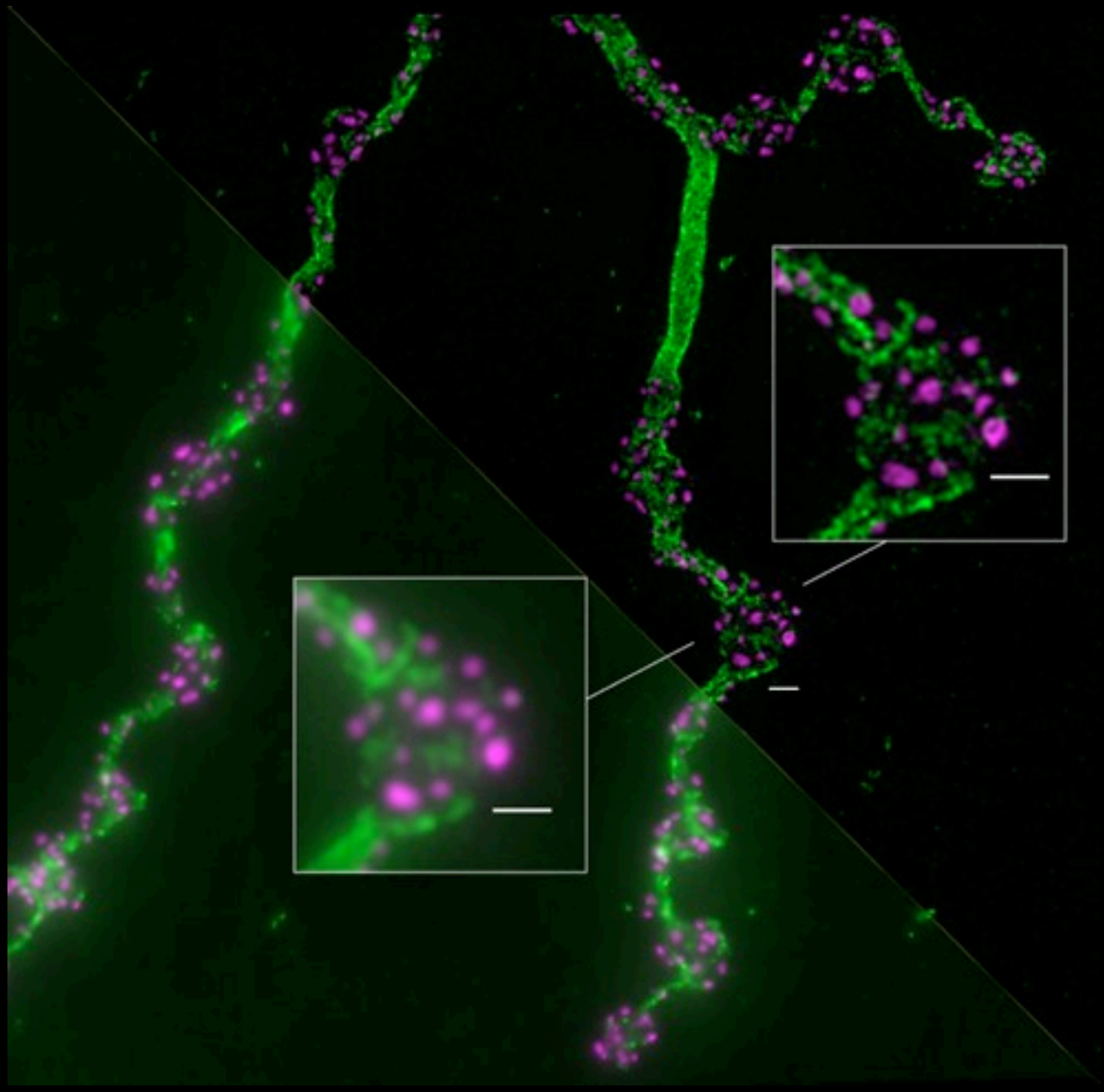
Actin in a HeLa cell

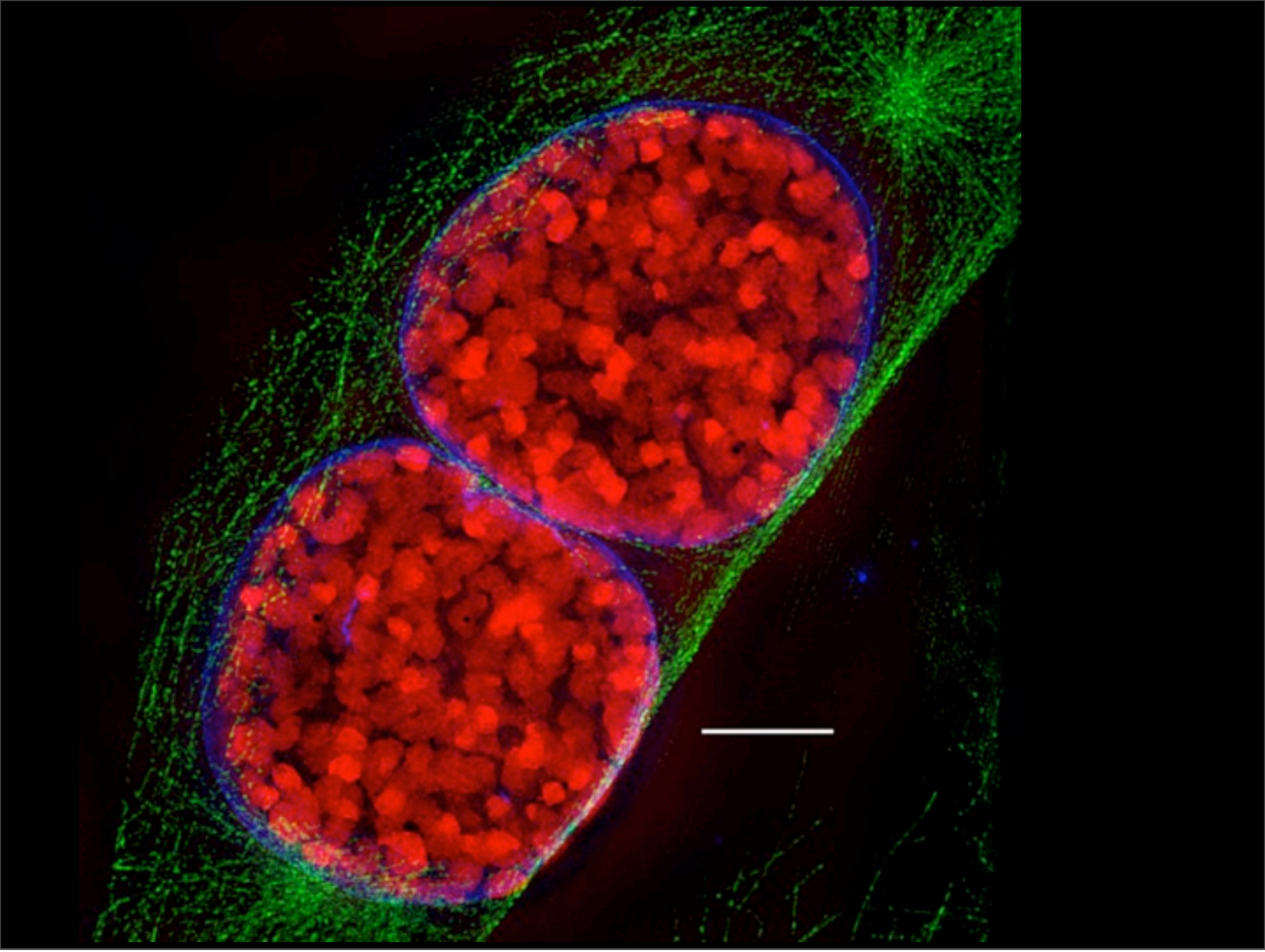


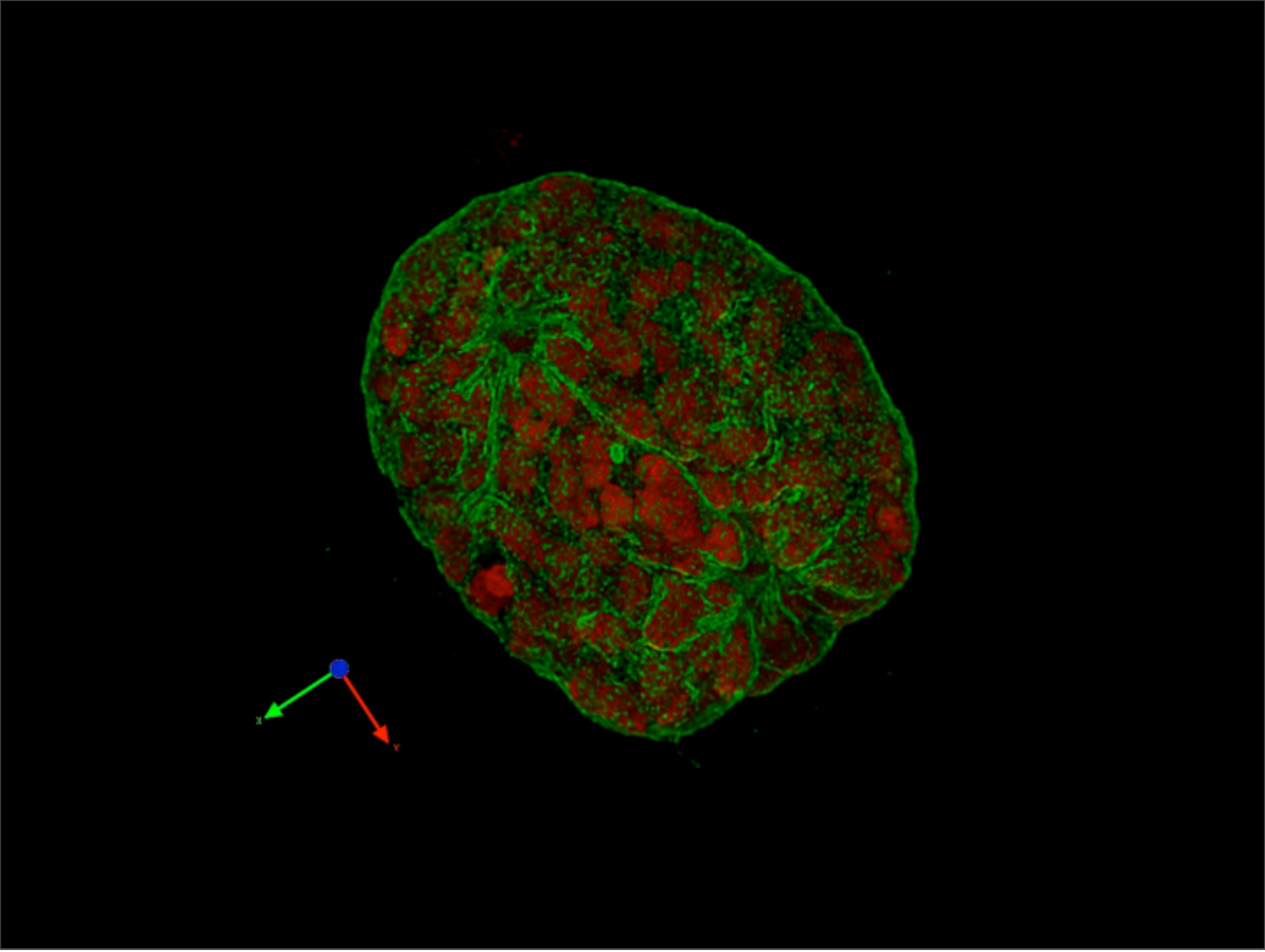
120 nm microspheres



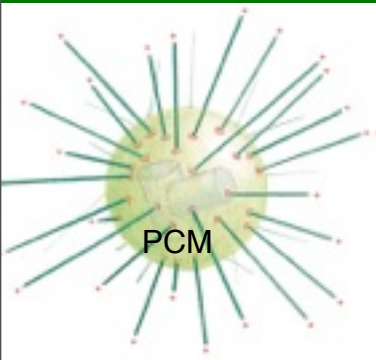

120 nm microspheres
Conventional microscopy
Structured illumination microscopy
XY
XZ
1μm
140 nm
350 nm axial FWHM





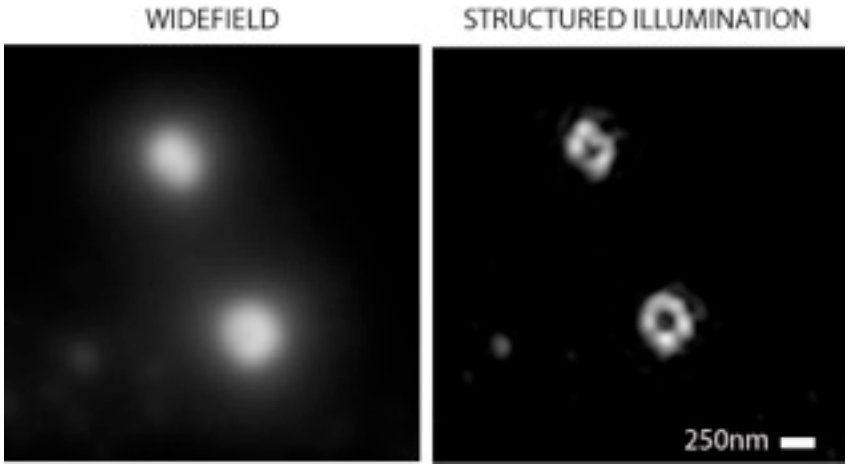

x
y

PCM long considered amorphous (decades of EM)



- systematically map key components in PCM
- light microscopy provides required selectivity
- challenge: small size (200 - 500nm)

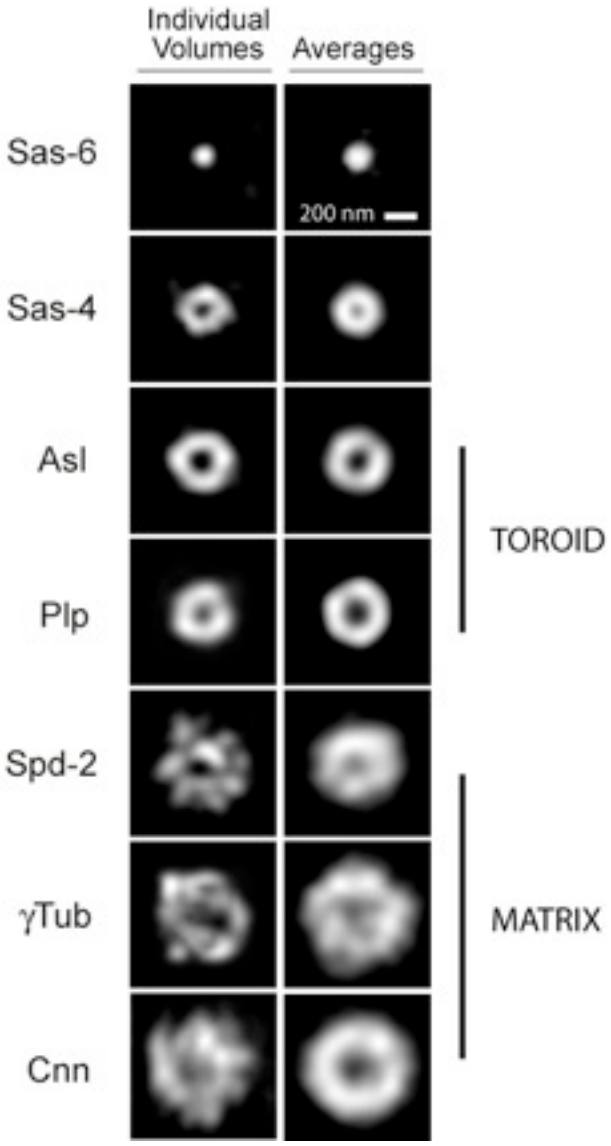
=> only tractable with sub-diffraction microscopy



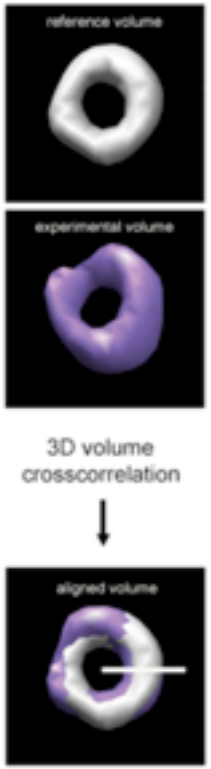
combination of SIM/STORM provides maximal confidence/resolution

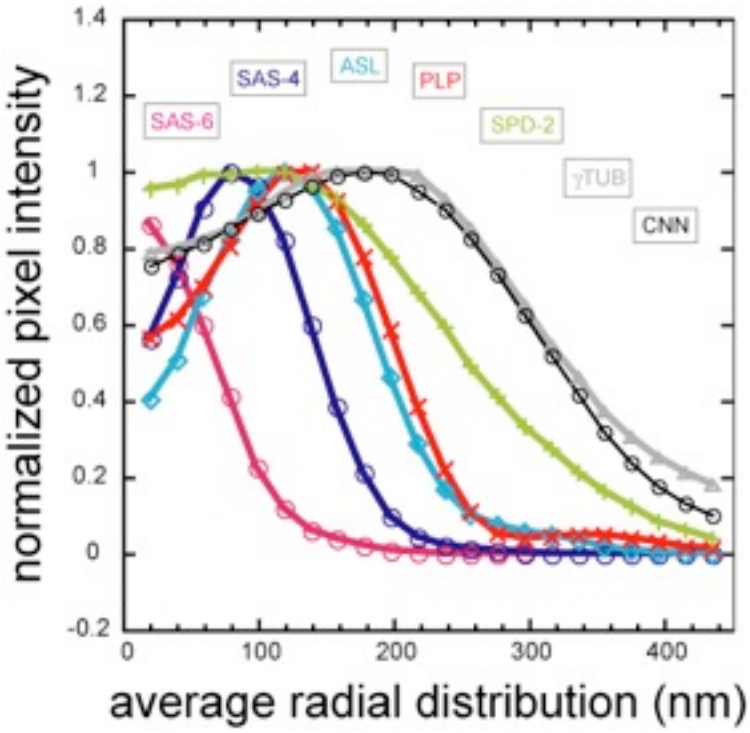
V. Mennella

3DSIM + 3D Averaging + Quantitative Analysis: PCM is organized in well-define architectural subdomains



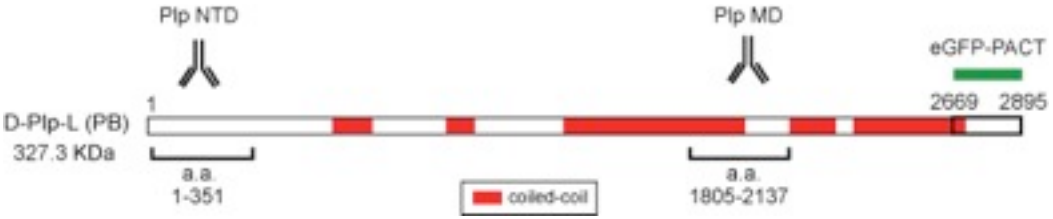
align 3D volumes and average

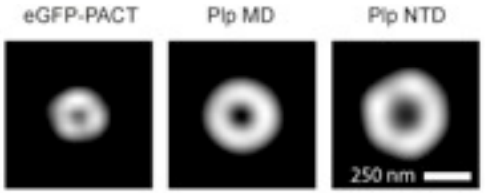


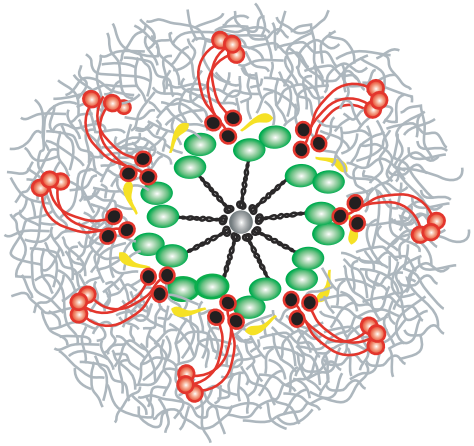


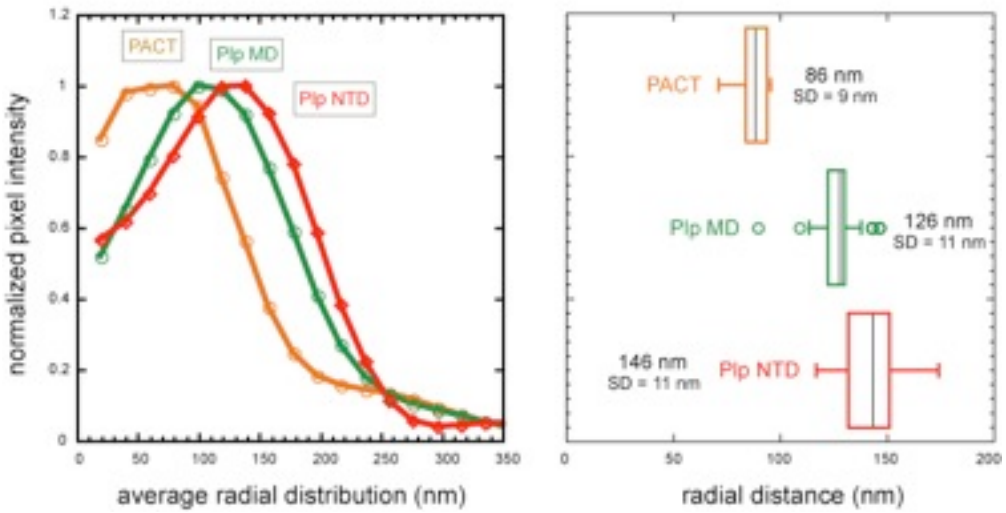
V. Mennella, B. Keszthrlyi

What is the molecular architecture of Plp?







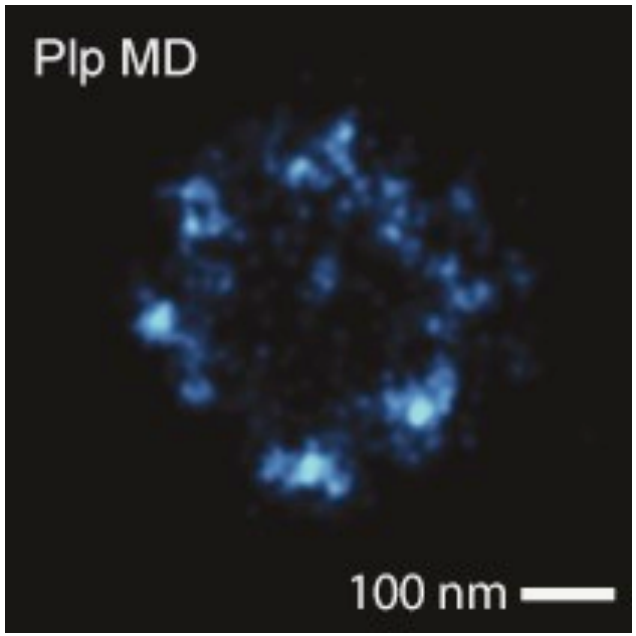


Plp radiates outwards from centriole wall

Molecular Structural information in cells!

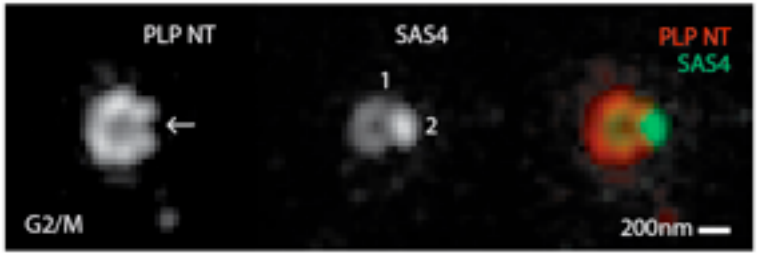
V. Mennella

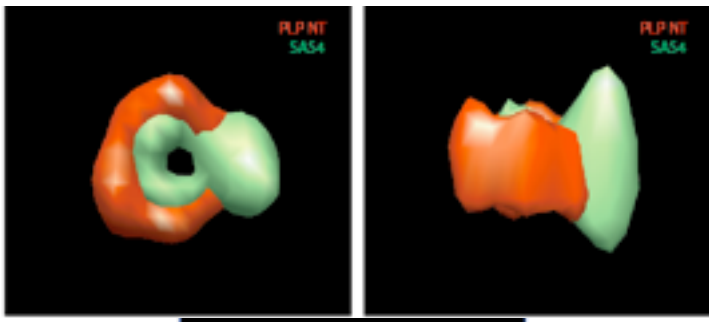
PLP organized in symmetrical clusters that open for new procentriole



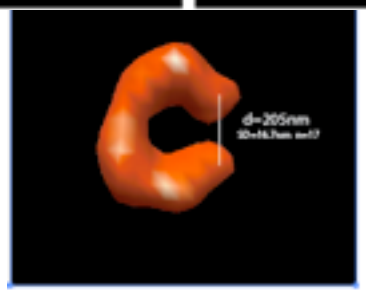
STORM ~ 9 fold symmetry

XYZ resolution:
32nm,37nm,59nm



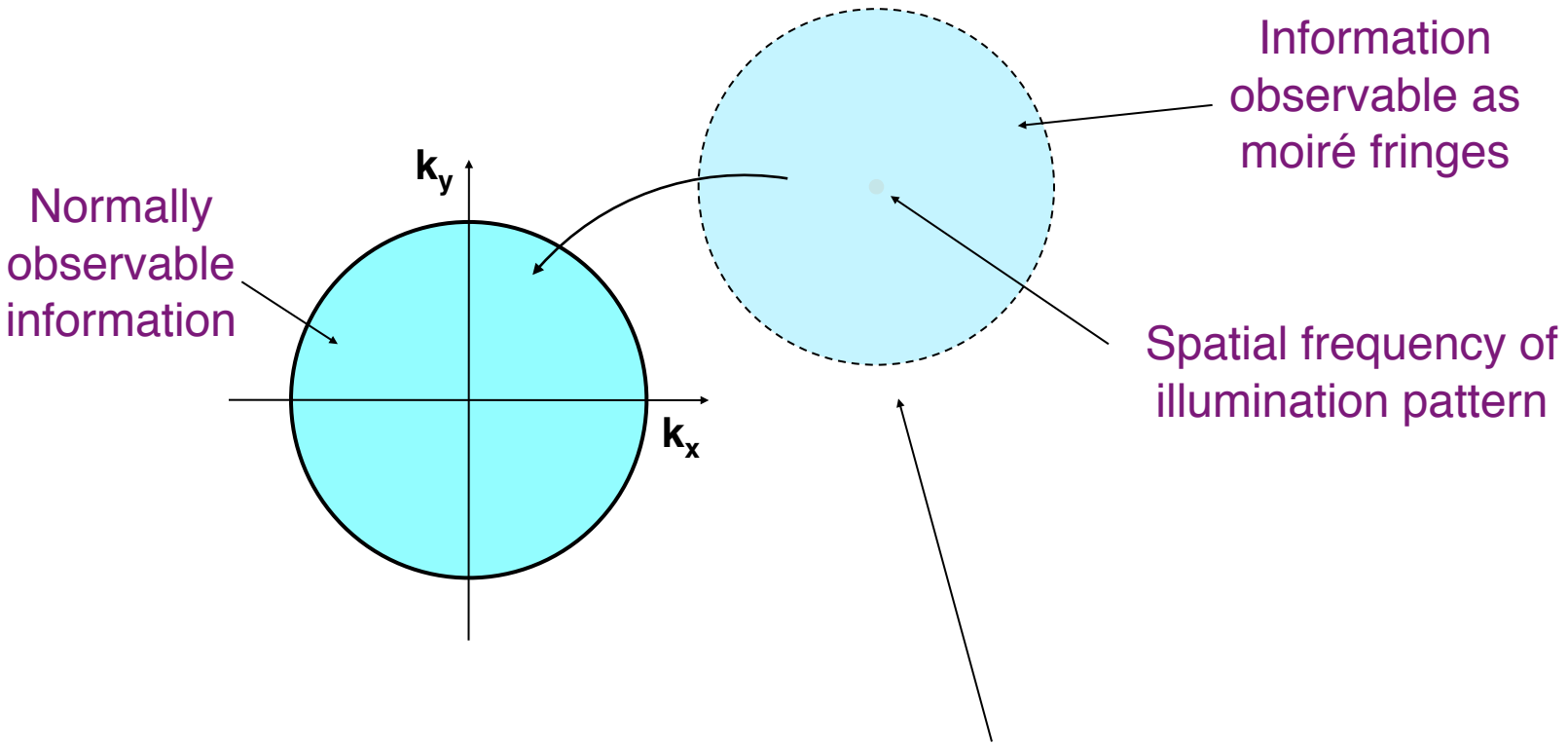


3D
SIM



V. Mennella, B. Huang

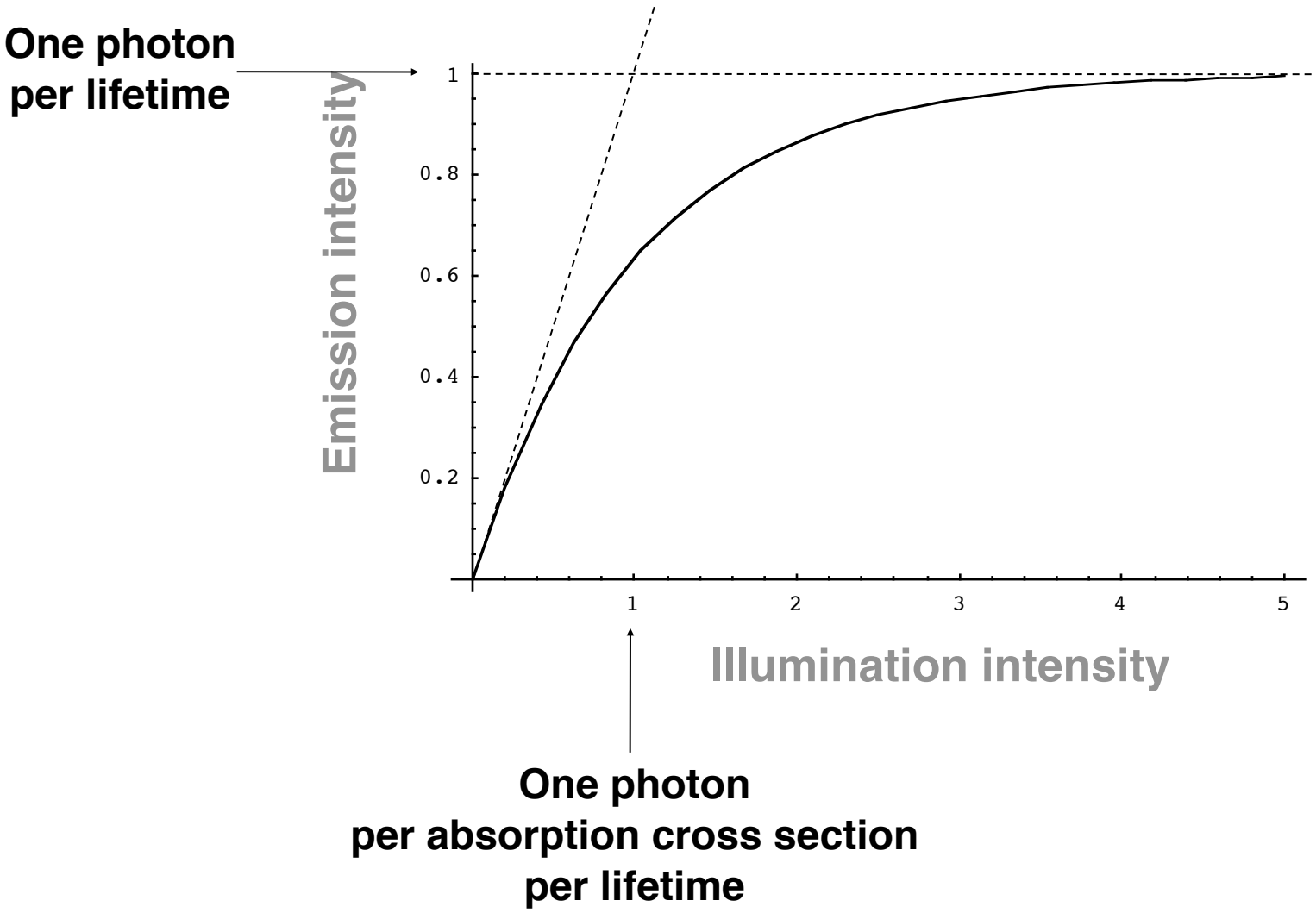
What are the limits of Structured Illumination?



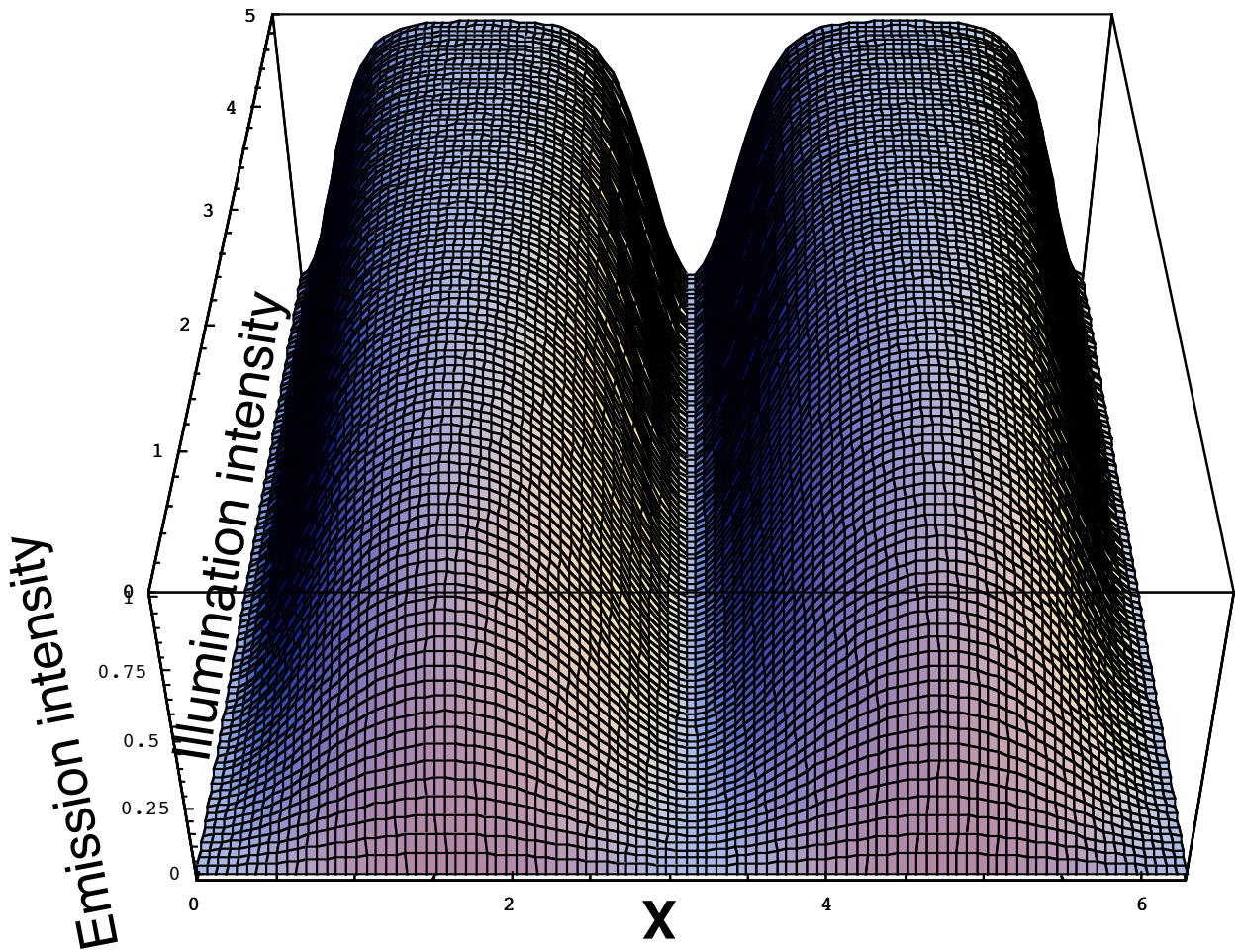
- Linear theory says this is impossible
- How about exploiting non-linear processes?

A simple source of nonlinearity: saturation

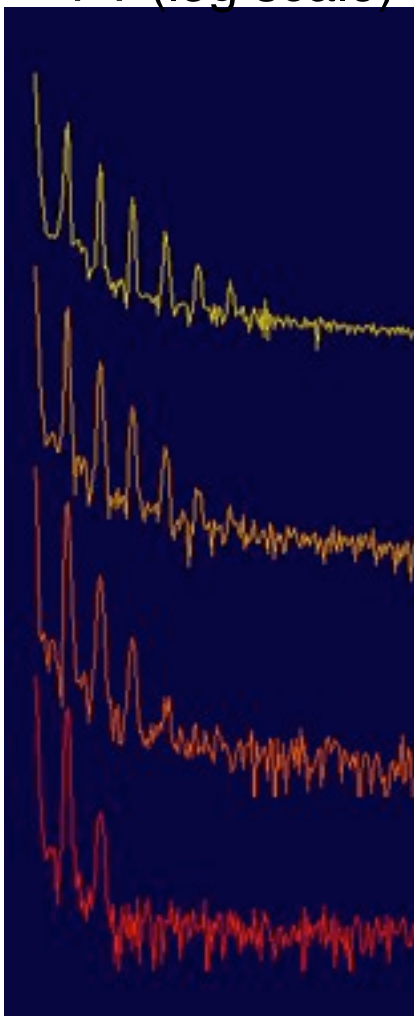
(R. Heintzmann Max-Planck Gottingen)



Saturated Structured Illumination



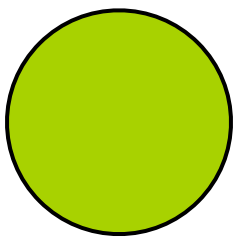
FT (log scale)



Resolution extension by nonlinear structured illumination

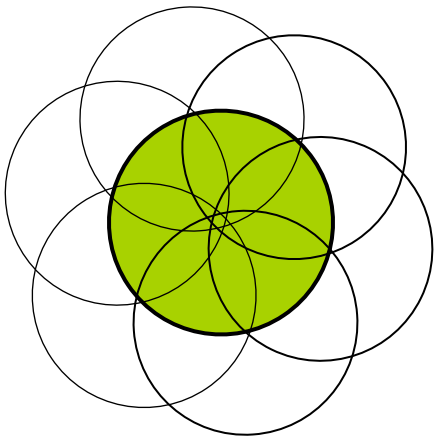
Effective observable regions

Conventional microscopy



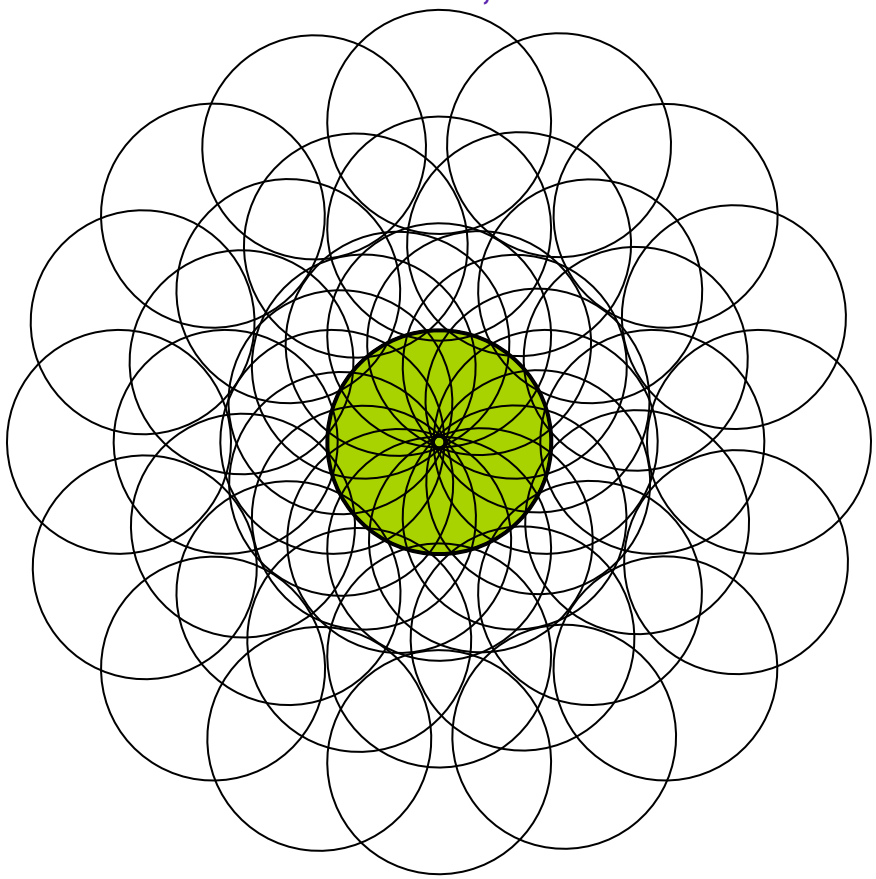
200 nm res.

Linear Structured illumination 3 directions



100 nm res.

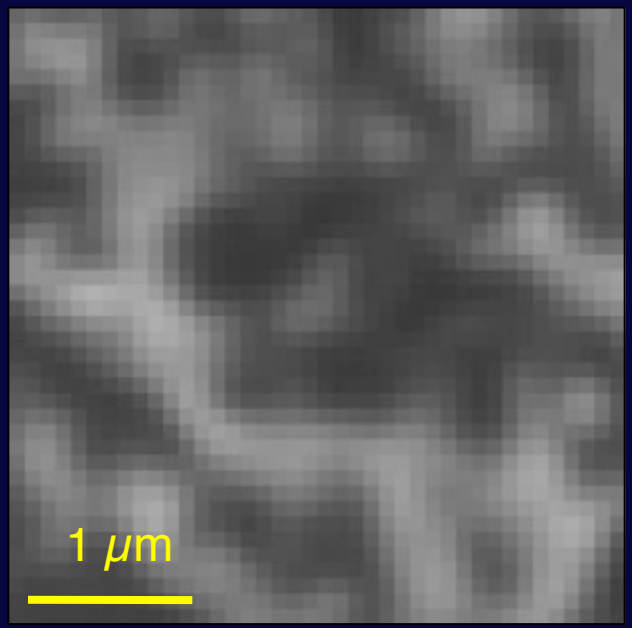
Nonlinear structured illumination 2 new harmonics, 8 directions



50 nm res.

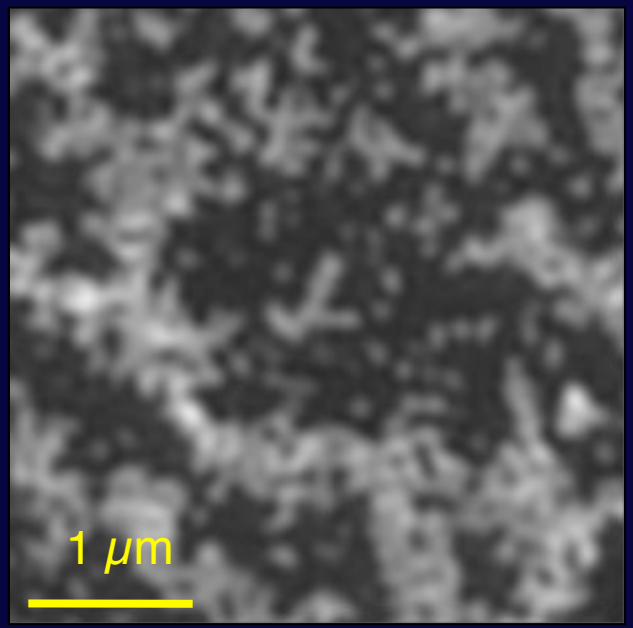
Non-linear structured illumination

Conventional microscopy



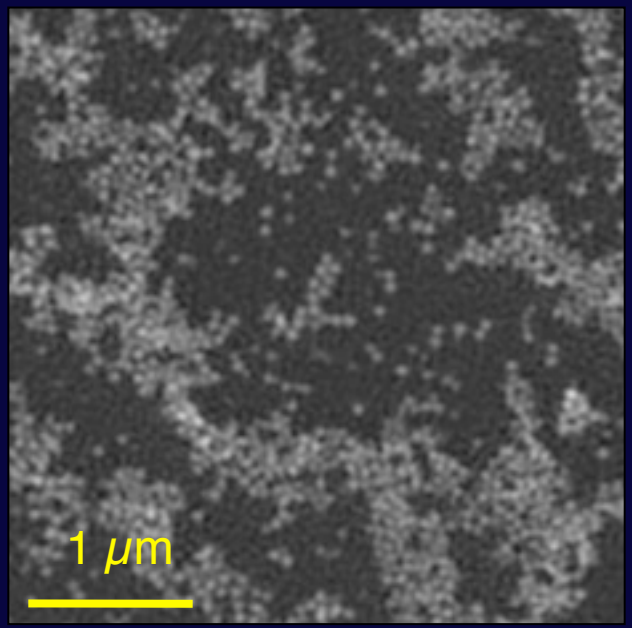
~250 nm resolution (diffraction limit)

Linear structured illumination



~120 nm resolution

Saturated structured illumination

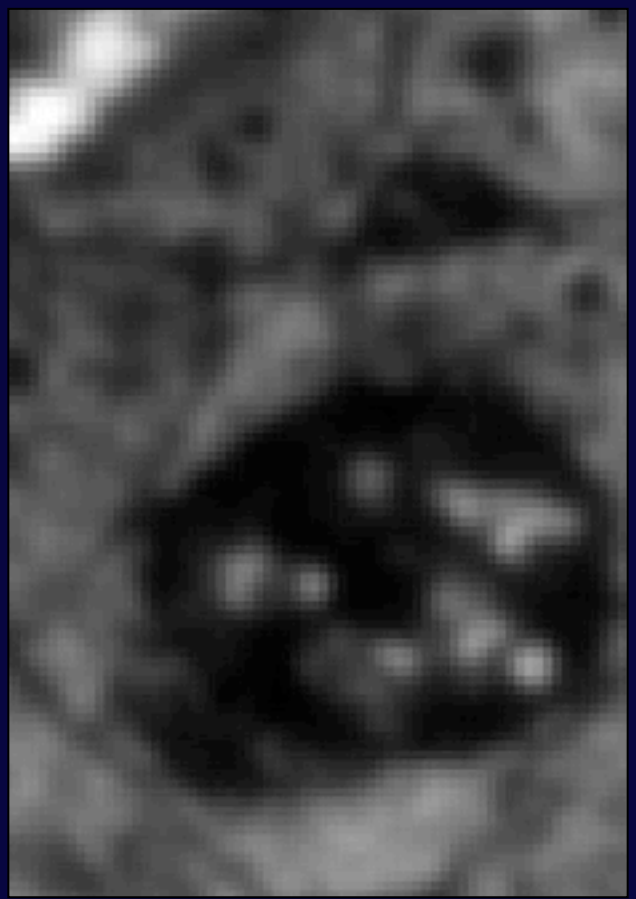


46 nm resolution!

50 nm microspheres

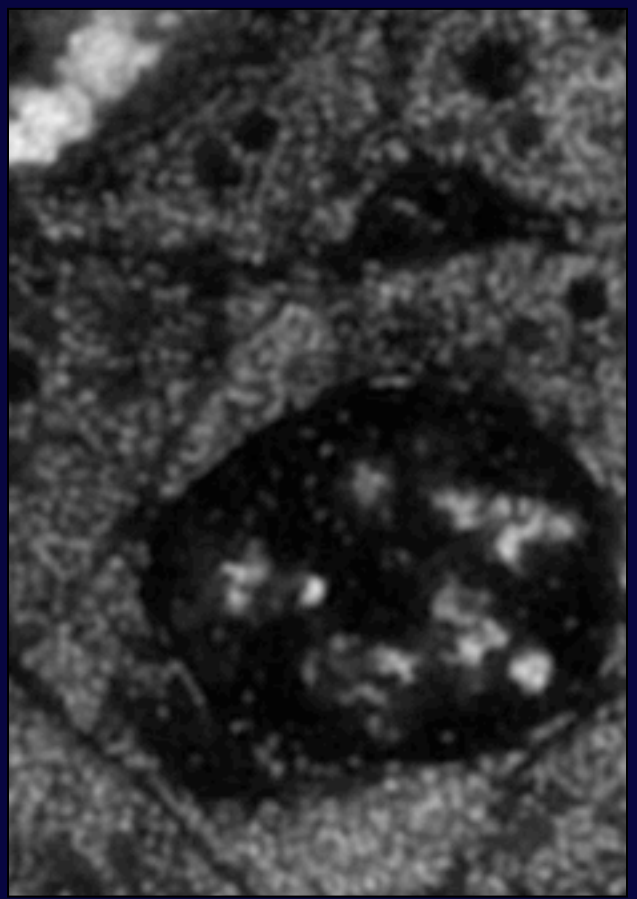
Drosophila embryo section DNA/RNA stain

Conventional microscopy



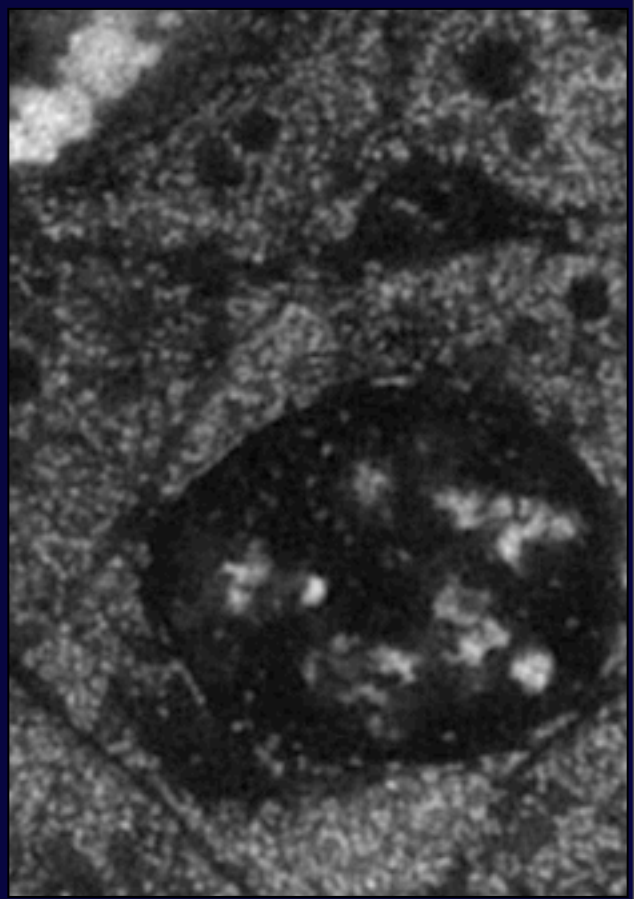
Min. FWHM ≈ 280 nm

Linear structured illumination



Min. FWHM ≈110 nm

Saturated structured illumination (1 new harmonic)



Min. FWHM ≈ 80 nm

Going beyond the diffraction limit: more light collection angles

We know:

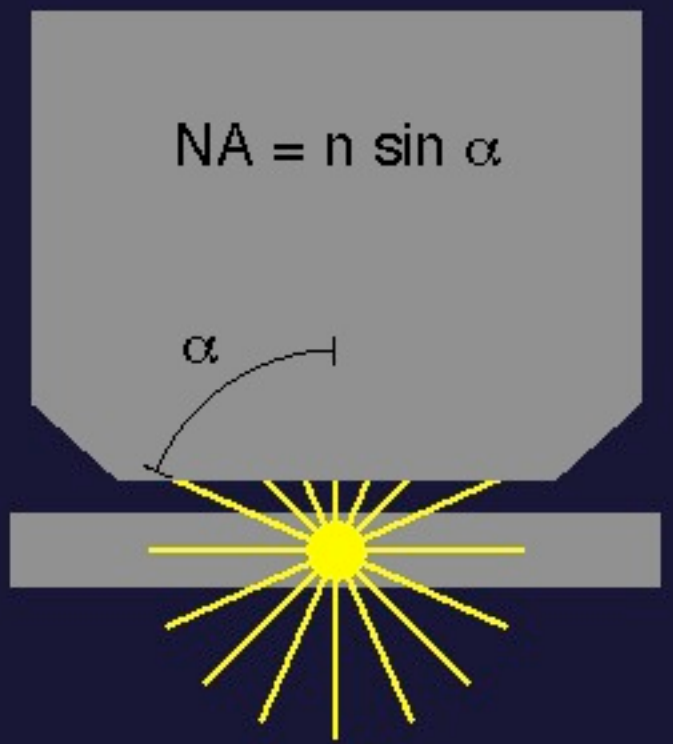
Higher NA

→

Gathering light over larger set of angles

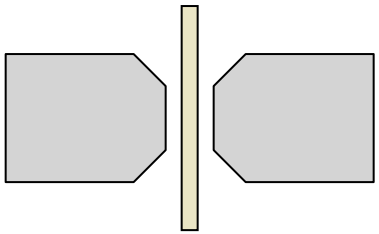
→

Higher resolution



…So what about gathering the light emitted toward the back side?

OTF when detecting through two lenses

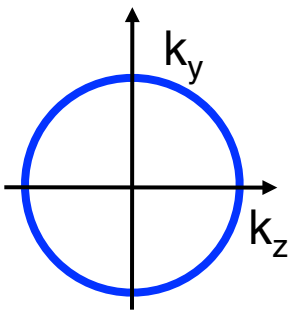


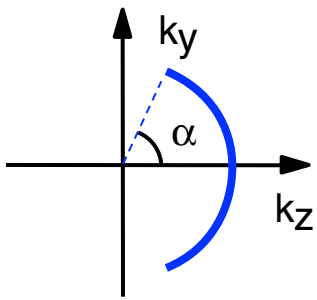
Free space

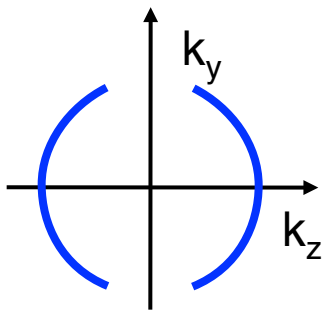
Through one objective

Through two objectives

support of $\tilde{A}(\mathbf{k})$





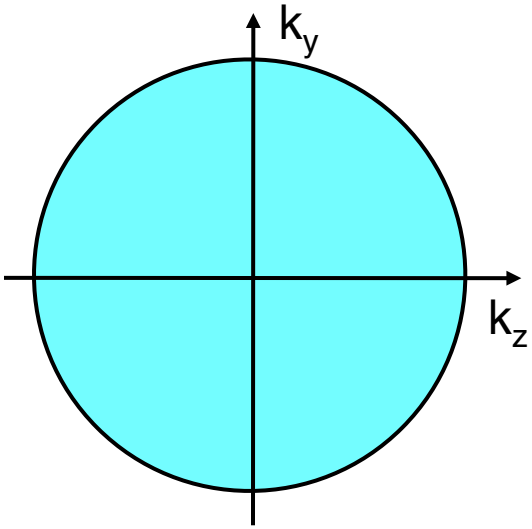


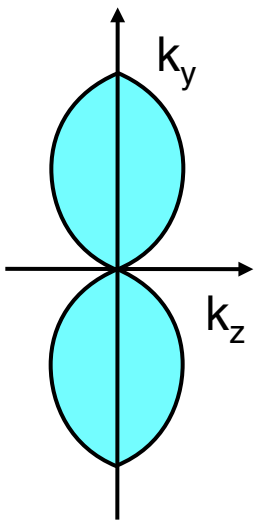
Convolved with itself:

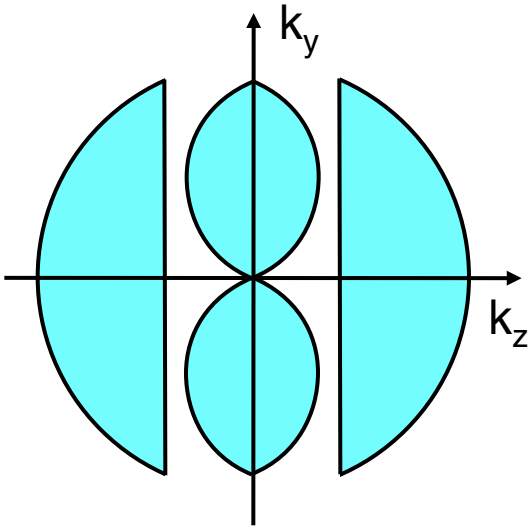
Convolved with itself:

Convolved with itself:

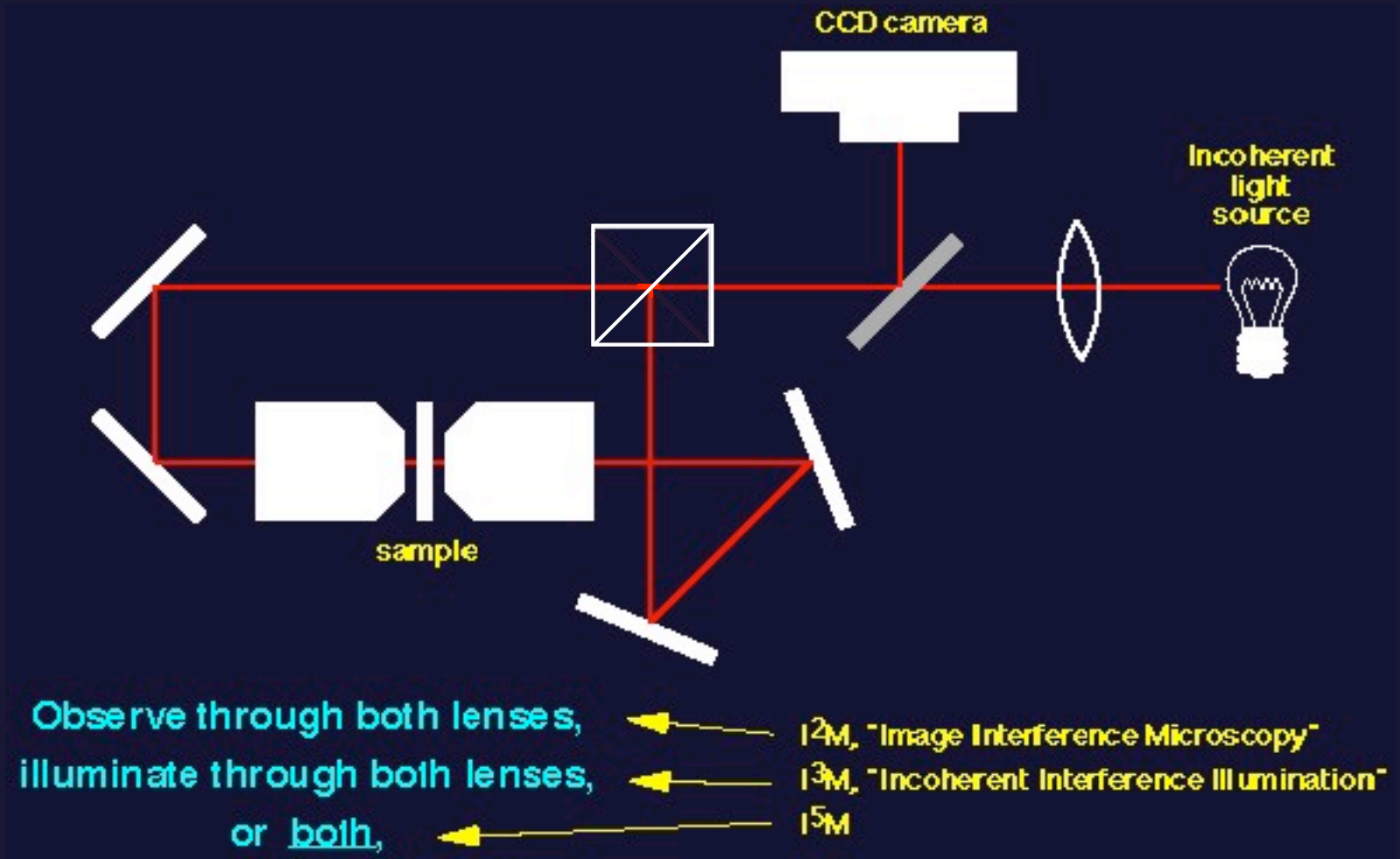
support of $OTF_{det}(\mathbf{k})$



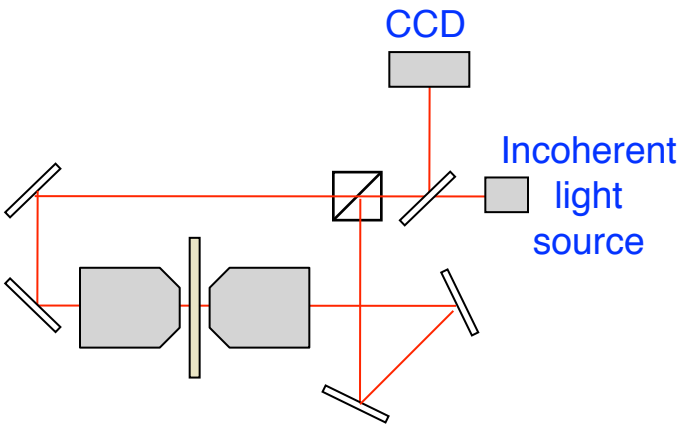


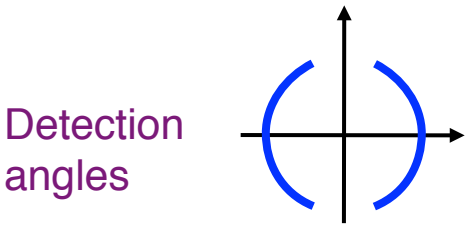


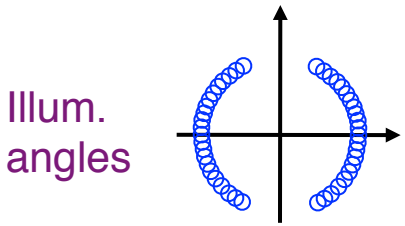
I5M concept

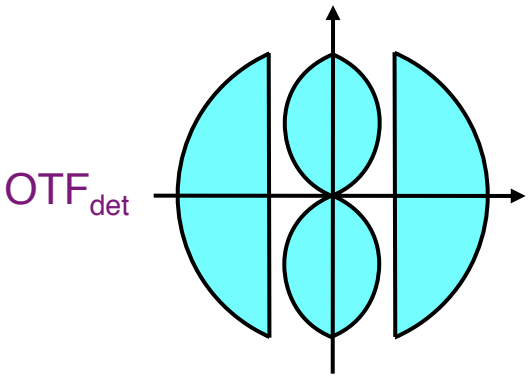

CCD camera
Incoherent light source
sample
Observe through both lenses,
illuminate through both lenses,
or both,
I2M, "Image Interference Microscopy"
I3M, "Incoherent Interference Illumination"
I5M

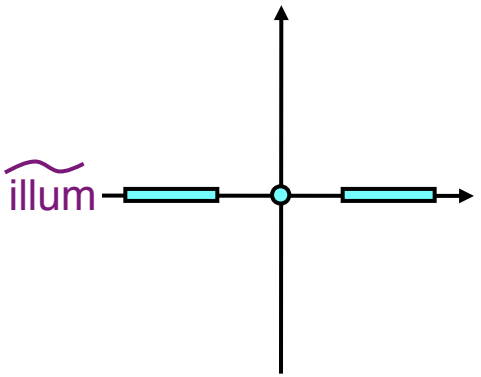
I5M OTF

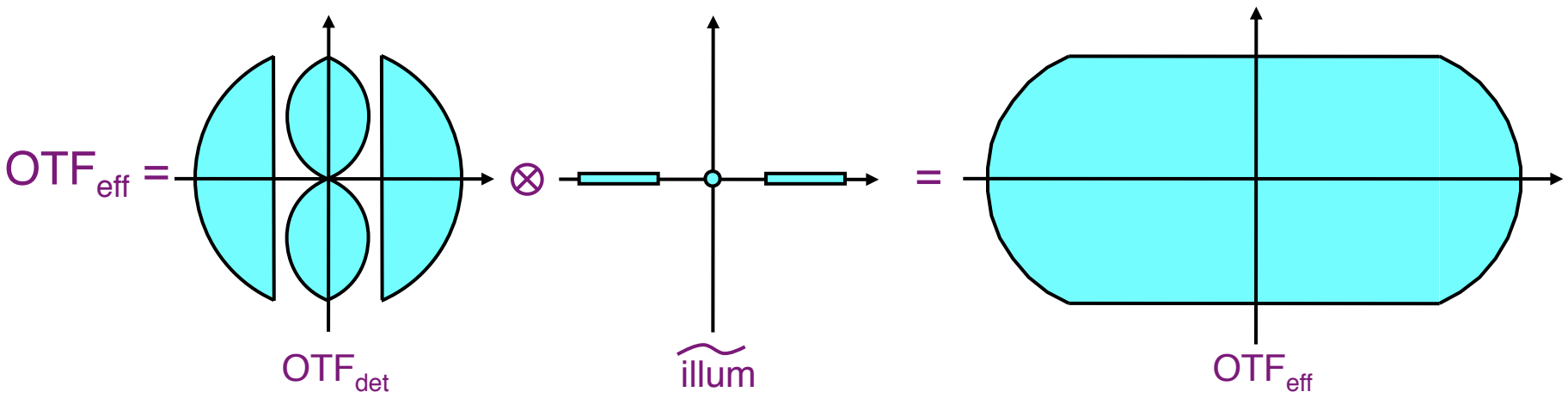

CCD
Incoherent
light
source


Detection
angles

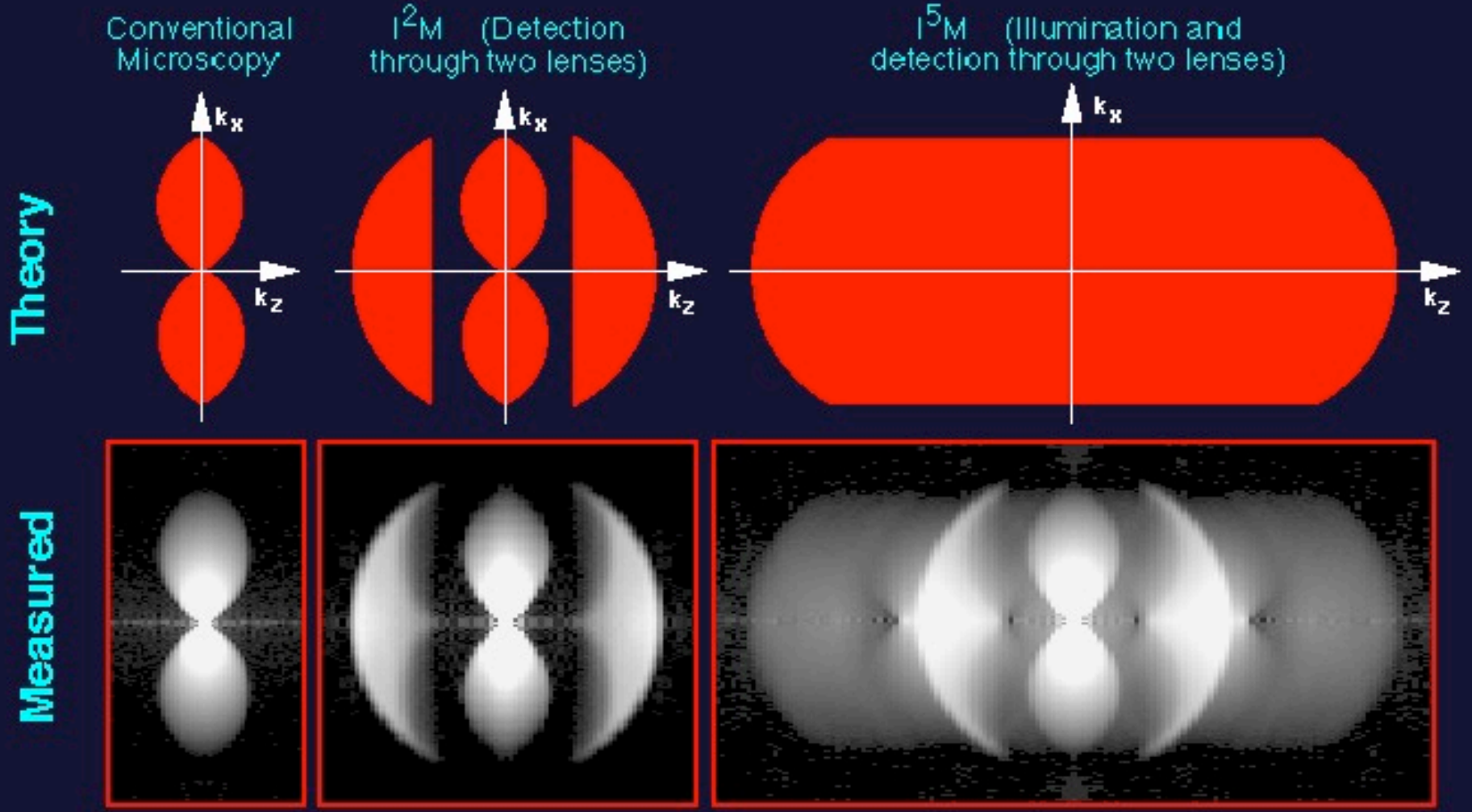

Illum.
angles


OTF det


illum

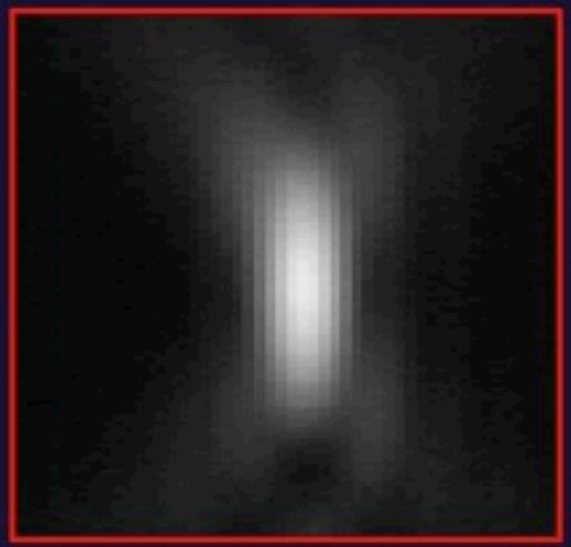

OTF eff =
OTF det
illum
=
OTF eff

Observable Information
[Optical Transfer Functions]



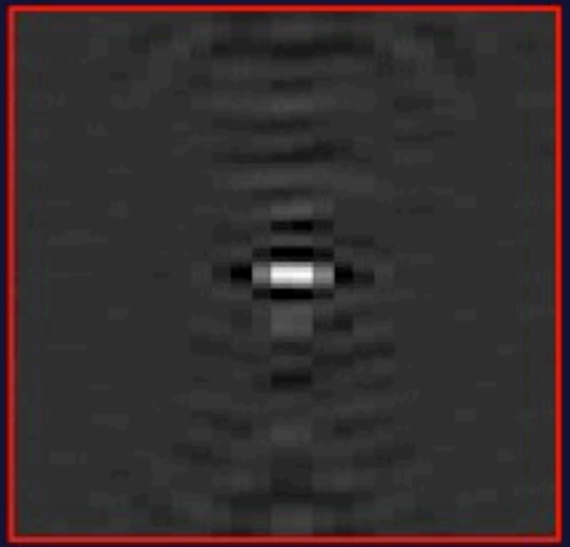
Deconvolution of bead data

One lens

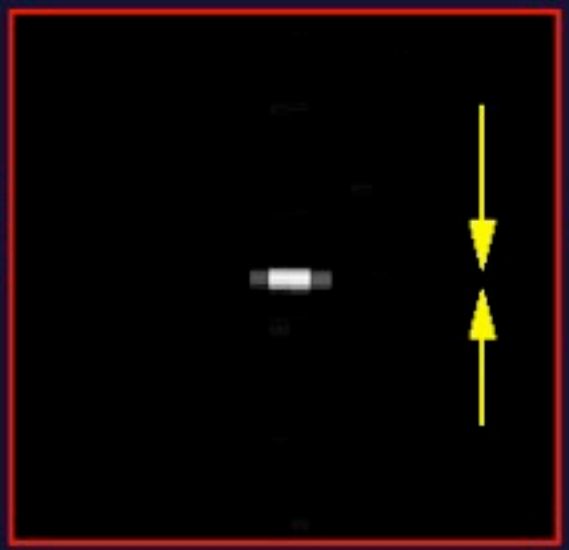


Two lens det. & illum.

After Wiener filtering



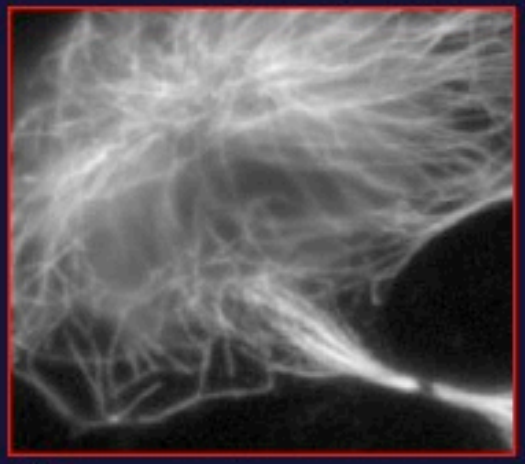
After iterative deconvolution using positivity constraint

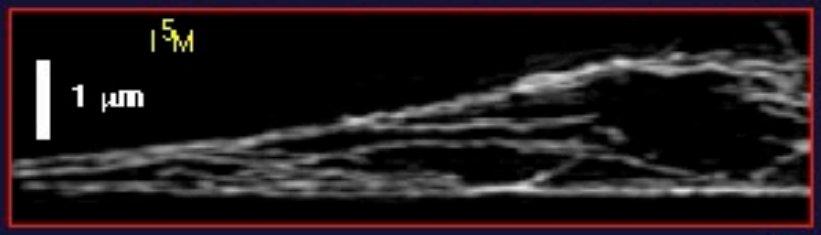


FWHM ~70nm

Microtubules in a recently divided PtK2 cell

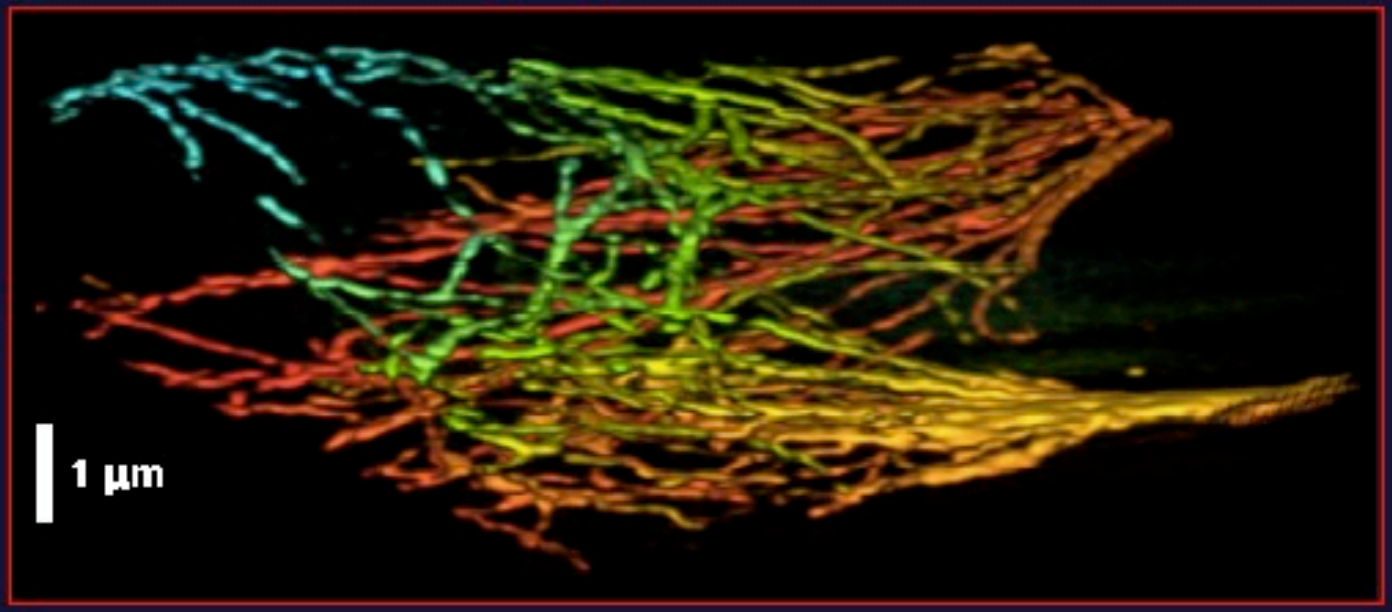
XY view of area



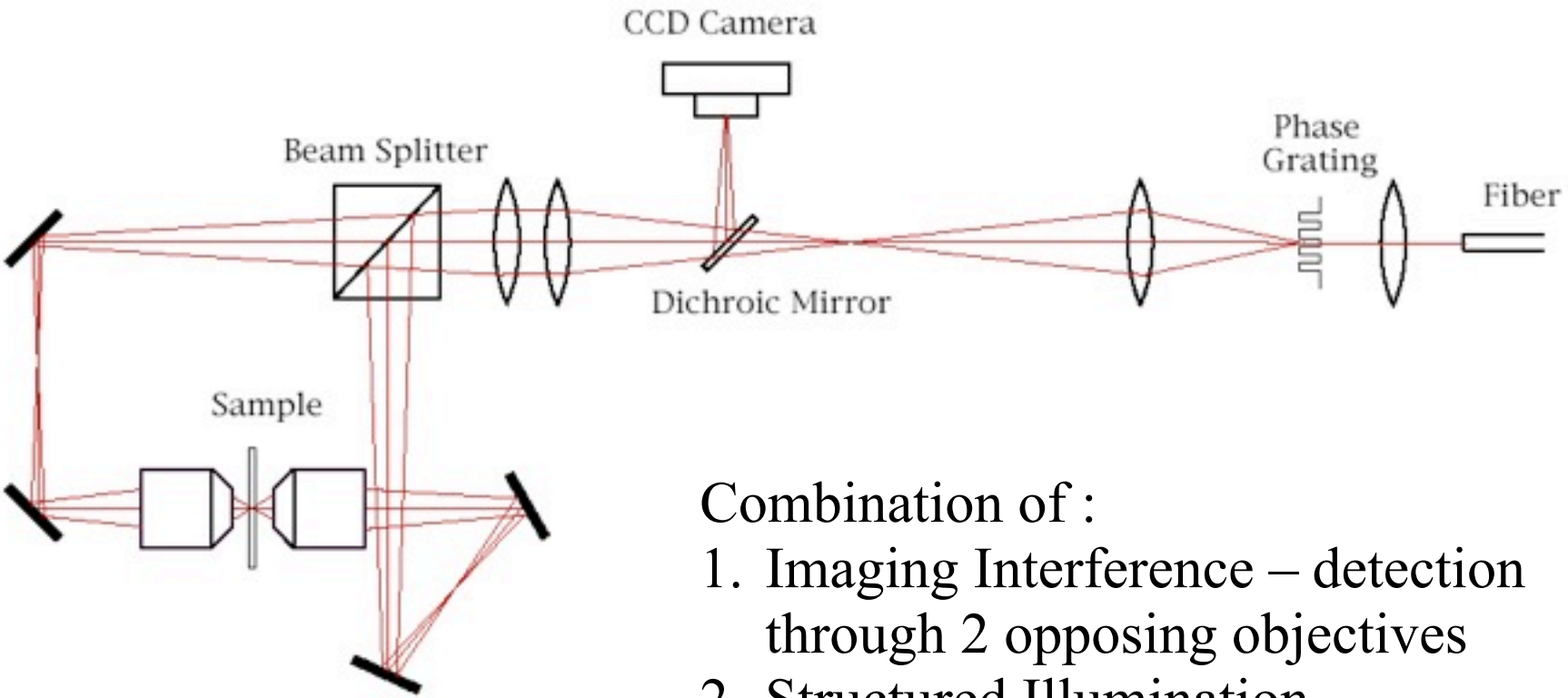


XZ projections 1 µm slices

I5M 3D view



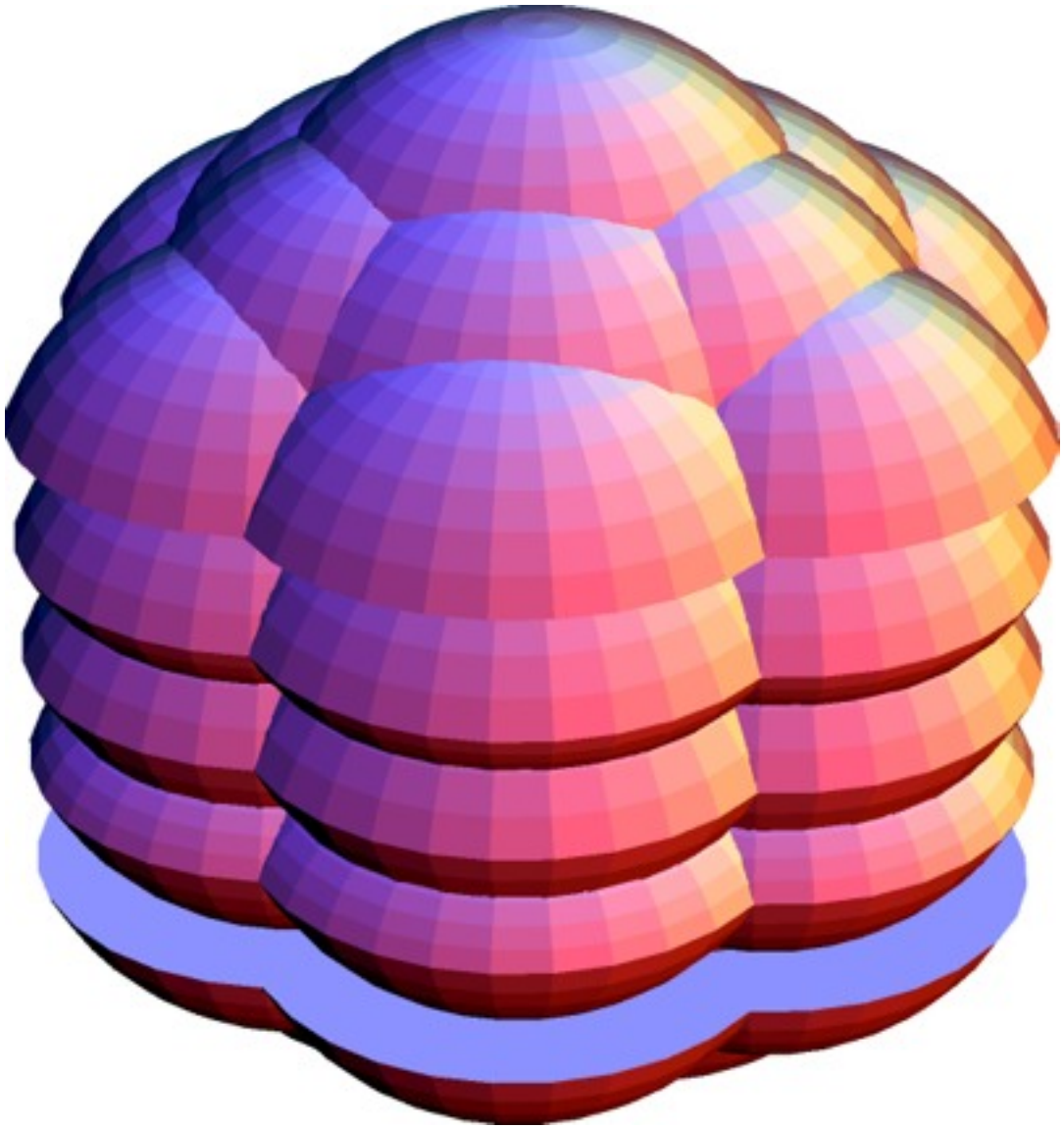
Combine methods: I^5S
use two lenses to collect more angles



Combination of :

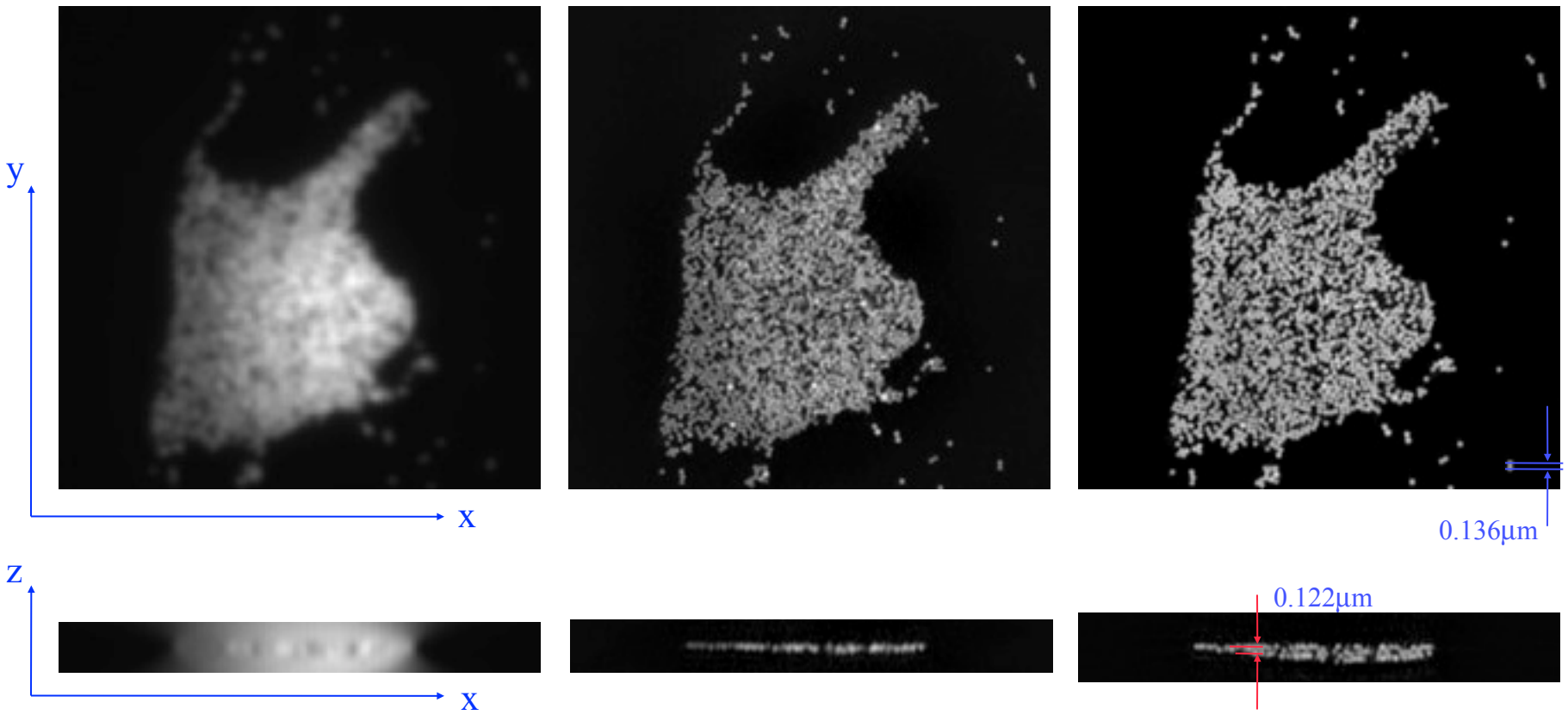
1. Imaging Interference – detection through 2 opposing objectives
2. Structured Illumination

Observable Region through 2 opposing objectives
Structured Illumination (3 orientations) – I^5S



Comparison of Resolving Power

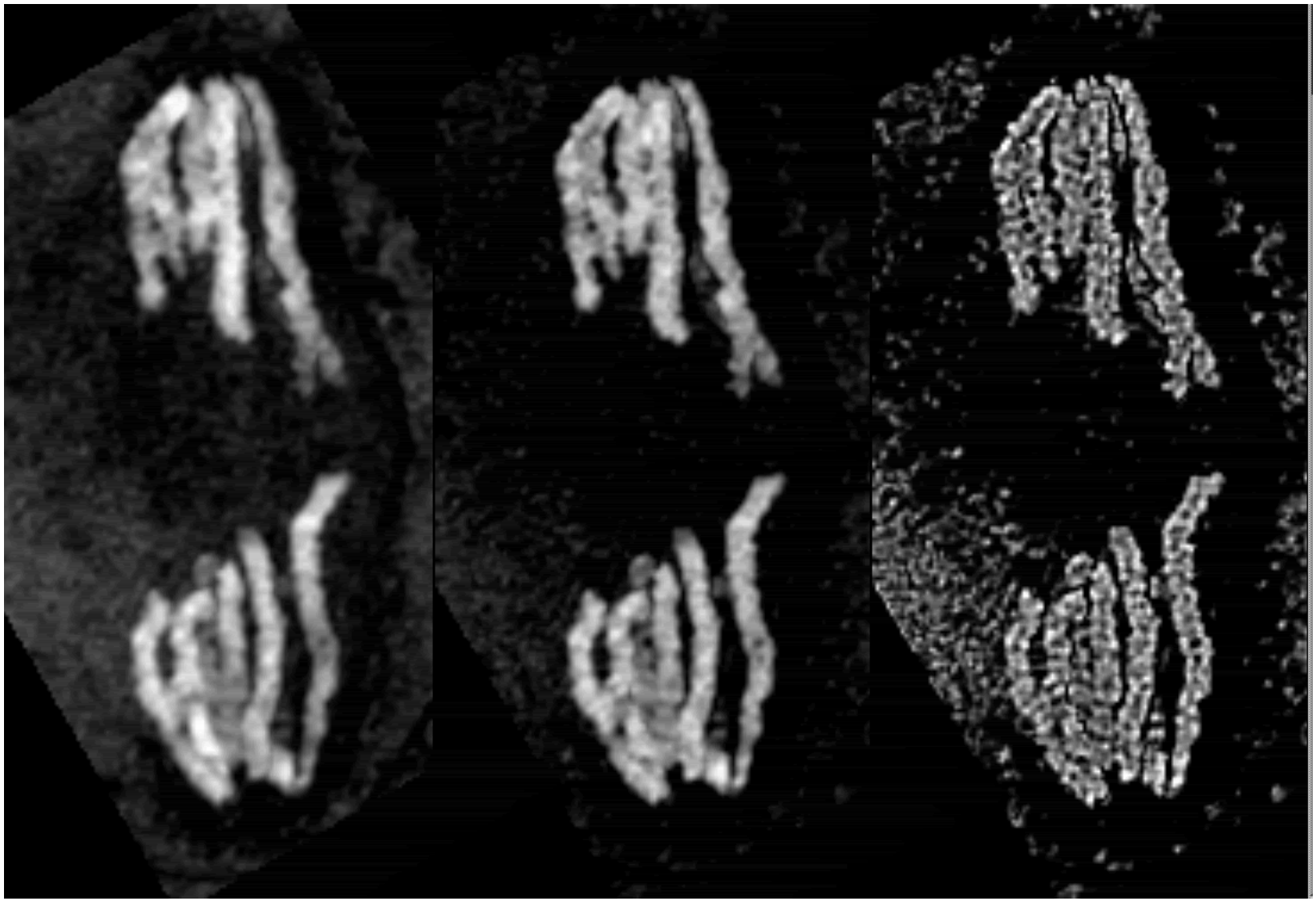
Conventional | Structured Illum. | I^5S



Sample: 0.12μm red-fluorescence microshperes

Lin Shao, Mats Gustafsson

Drosophila Anaphase Chromosomes (.2μm wide)

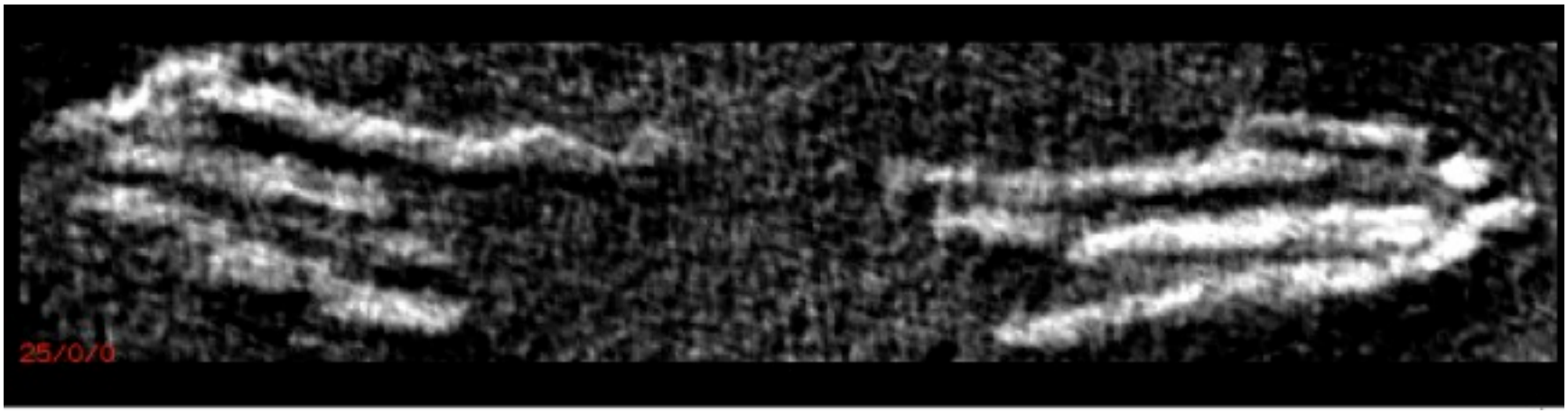


Lin Proc. **Decon** **Decon + LCE**

Lin Shao

Comparison between OM and EM-Tomo

EM



OM