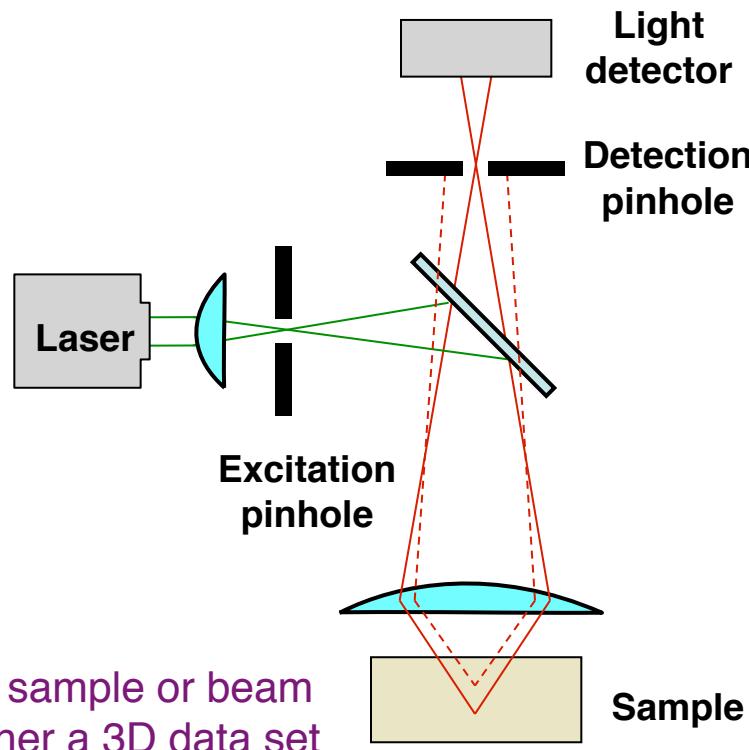


3D fluorescence microscopy

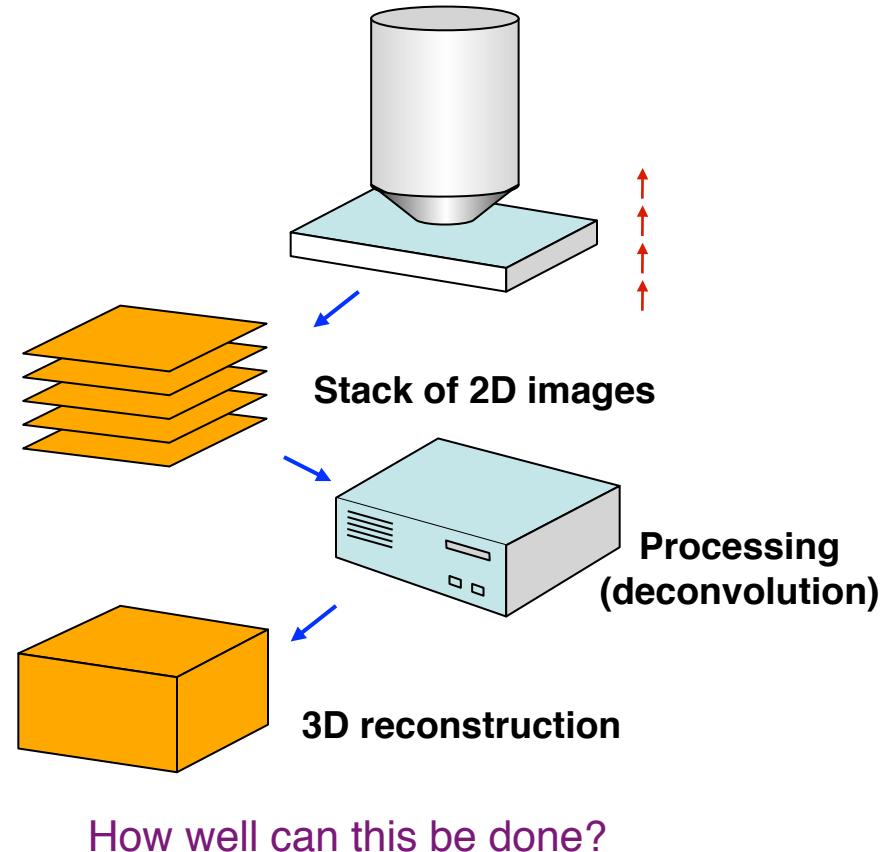
For 3D, acquire a “focal series” (stack) of images:
Take an image, refocus the sample, take another image, refocus, etc.

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

Approach 1:
Physically exclude the blur by
confocal microscopy



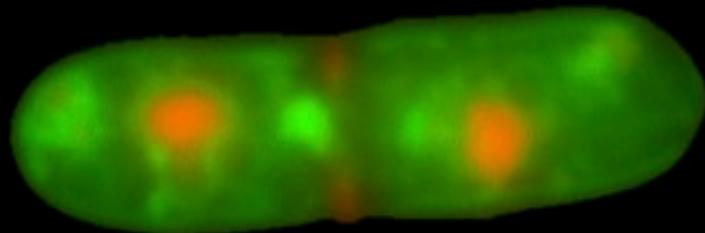
Approach 2:
Remove the blur computationally



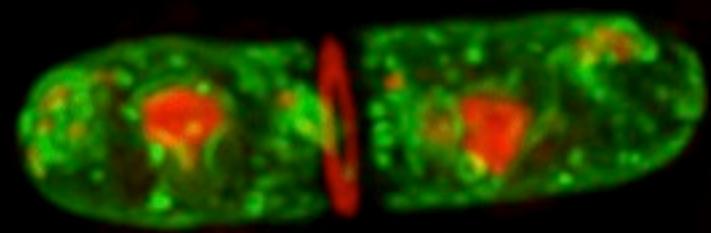
Deconvolution of 3D data

(Dividing fission yeast cell)

Raw data



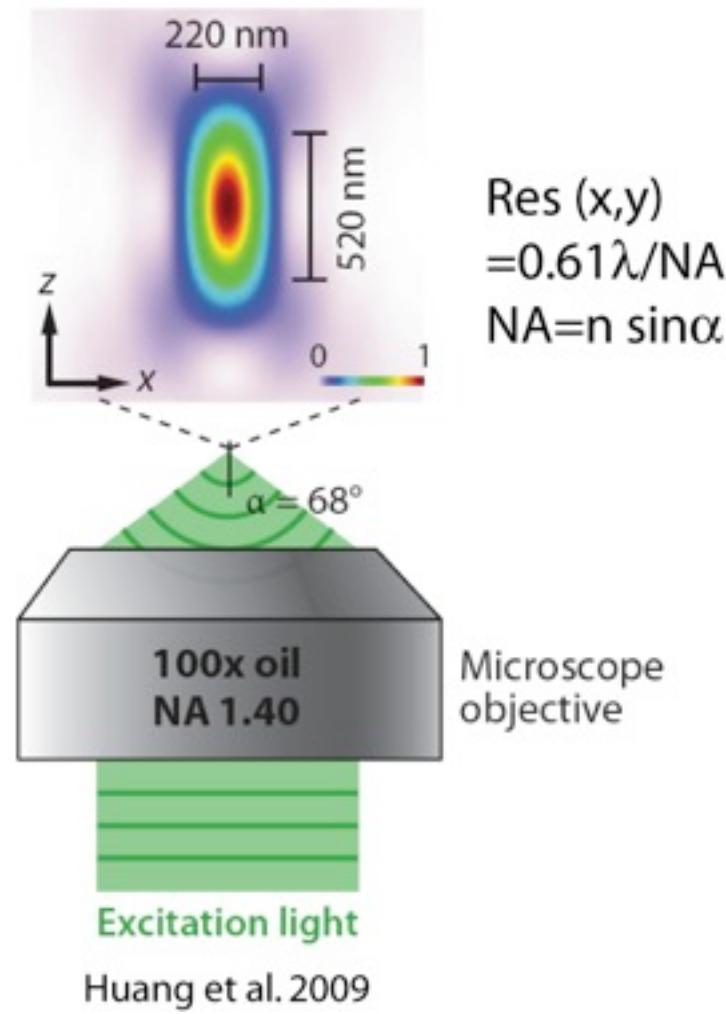
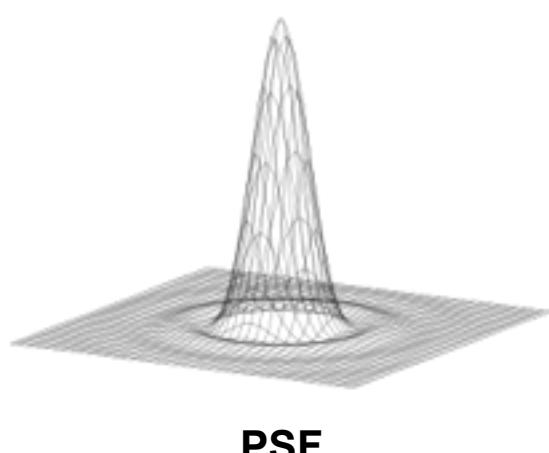
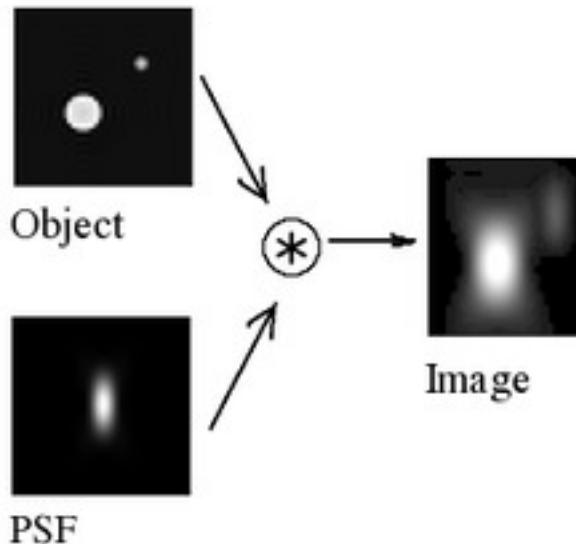
Deconvolved



4 μm

4 μm

Light microscopy imaging is defined by the Point Spread Function



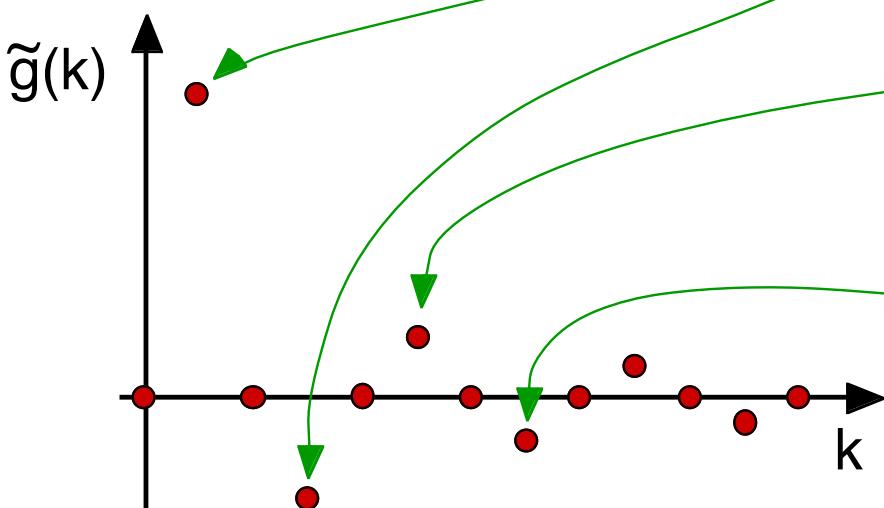
Huang et al. 2009

The Fourier Transform

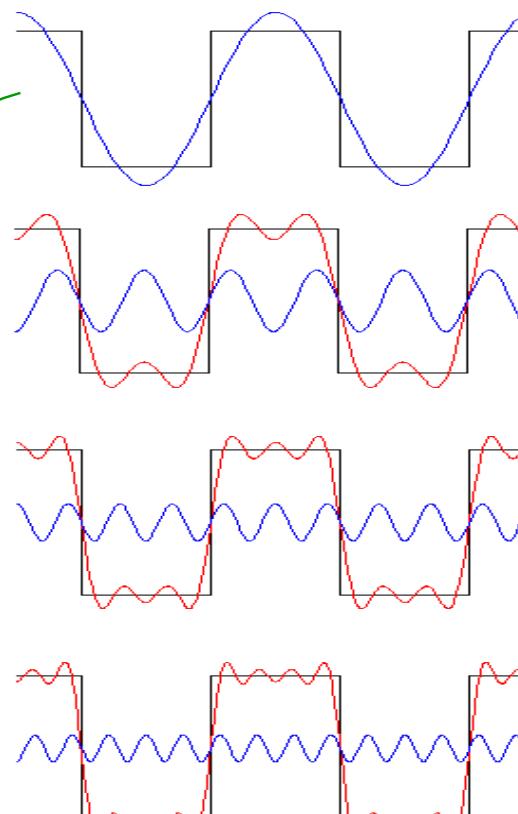
Any (nice) function $g(x)$ can be *equally well* described as a sum of waves.

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

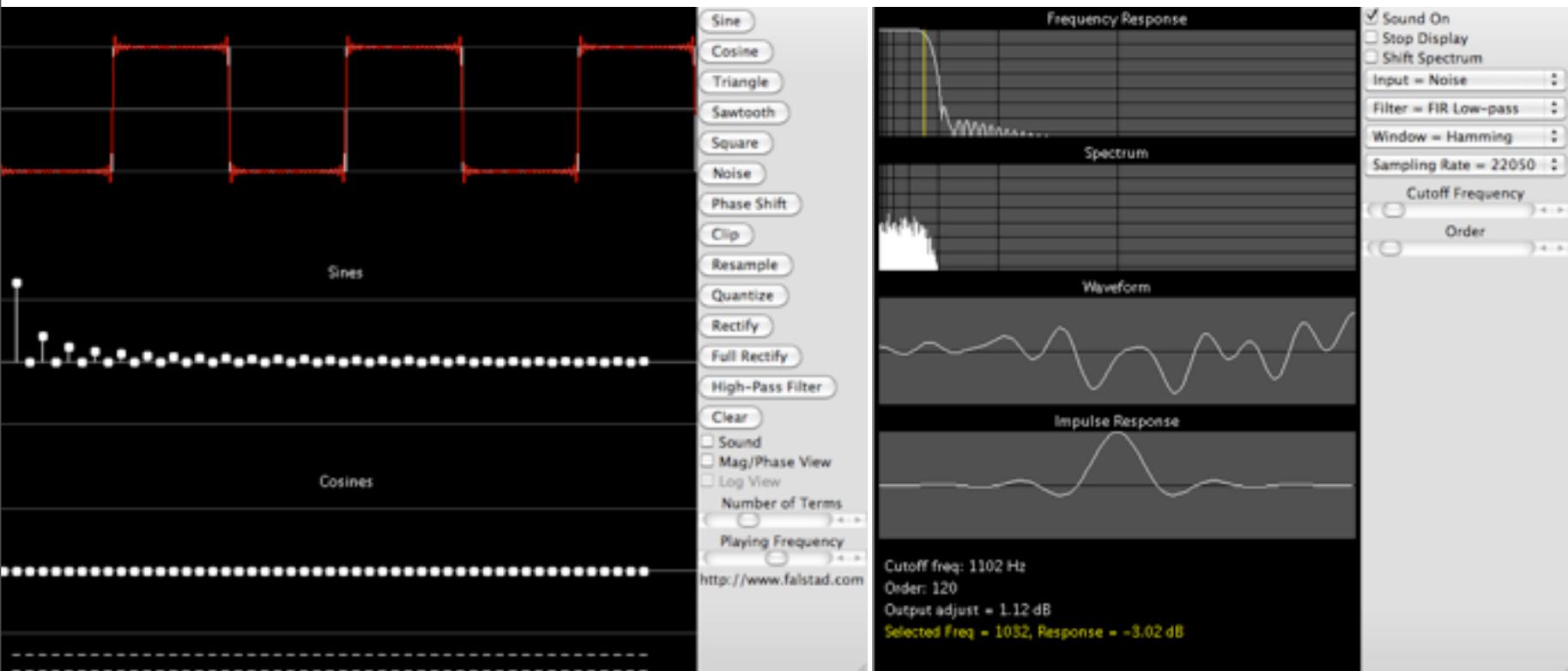


$$\tilde{g}(k) = \int g(x) e^{-2\pi i k x} dx$$



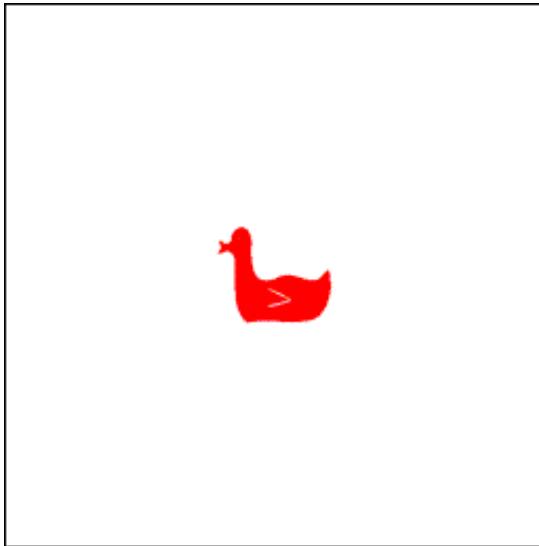
Fourier Transform and Digital Filter Applets

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

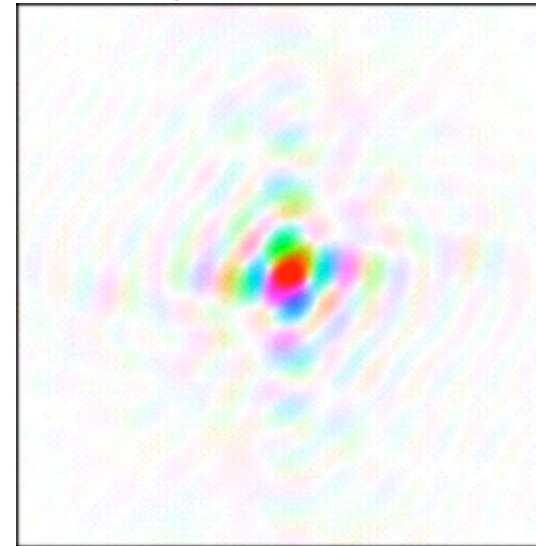


Low Resolution Duck

A Duck

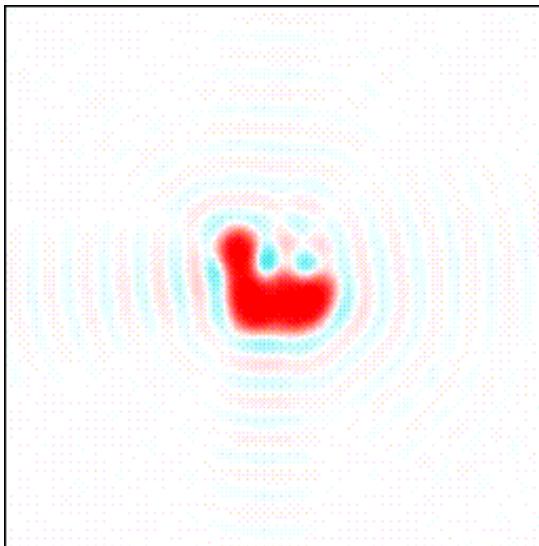


and its scattering function (Fourier Transform):



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

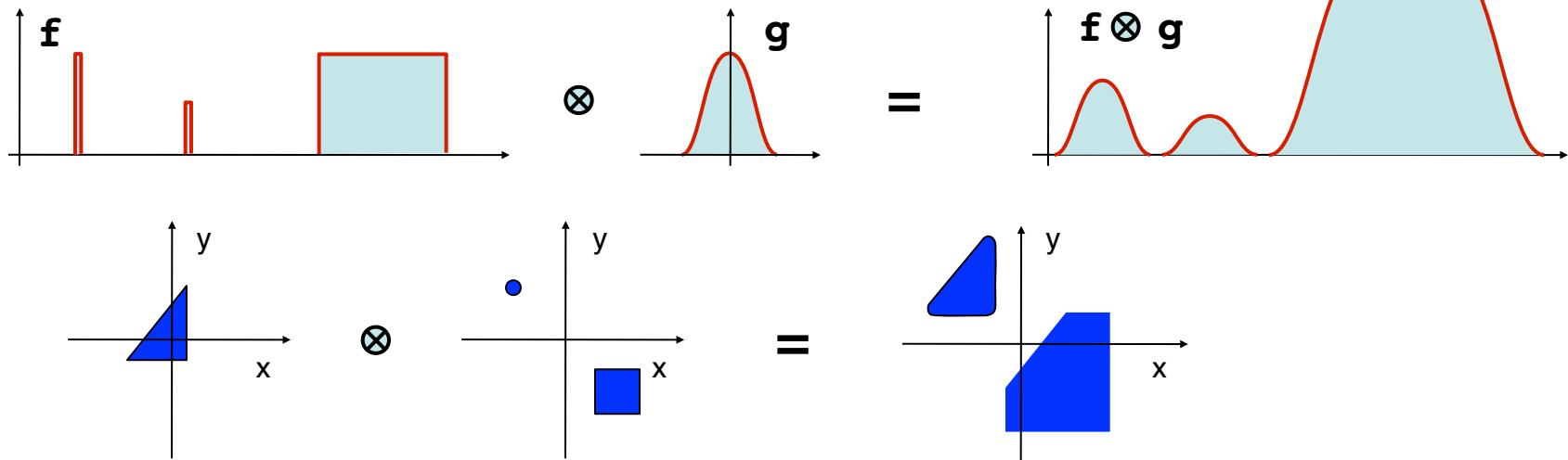
then $\tilde{h}(k) = \tilde{f}(k) \tilde{g}(k)$

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

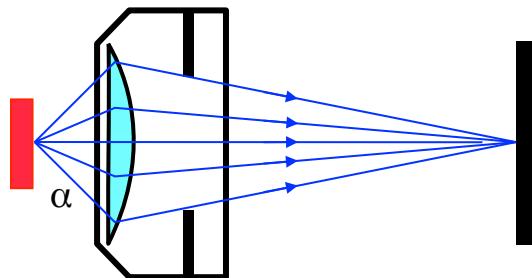
Symmetry: $g \otimes f = f \otimes g$

So what is a convolution, intuitively?

- “Blurring”
- “Drag and stamp”



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



α = maximum angle

$$\text{N.A.} = \eta \sin \alpha,$$

the highest spatial frequency is then $f_c = (2\eta \sin \alpha)/\lambda$
 $f_c = 0.178 \mu\text{m}$ for $\lambda = 500\text{nm}$, 1.4NA oil immersion lens

using Raleigh's criterion, the smallest separation
 between two points that can be resolved is $1/(1.22 \cdot f_c)$
 $d_{min} = 0.146 \mu\text{m}$

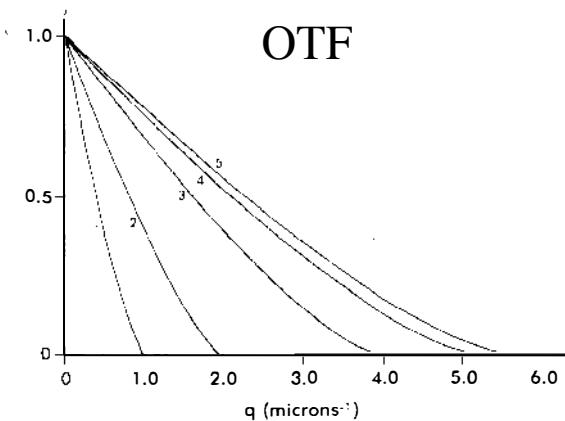


Figure 3 The in-focus contrast-transfer functions for several Zeiss lenses are compared. The curves are (1) 10 × 0.25 N.A., (2) 25 × 0.5 N.A., (3) 50 × 1.0 N.A. oil, (4) 100 × 1.25 N.A. oil, (5) 63 × 1.4 N.A. oil.

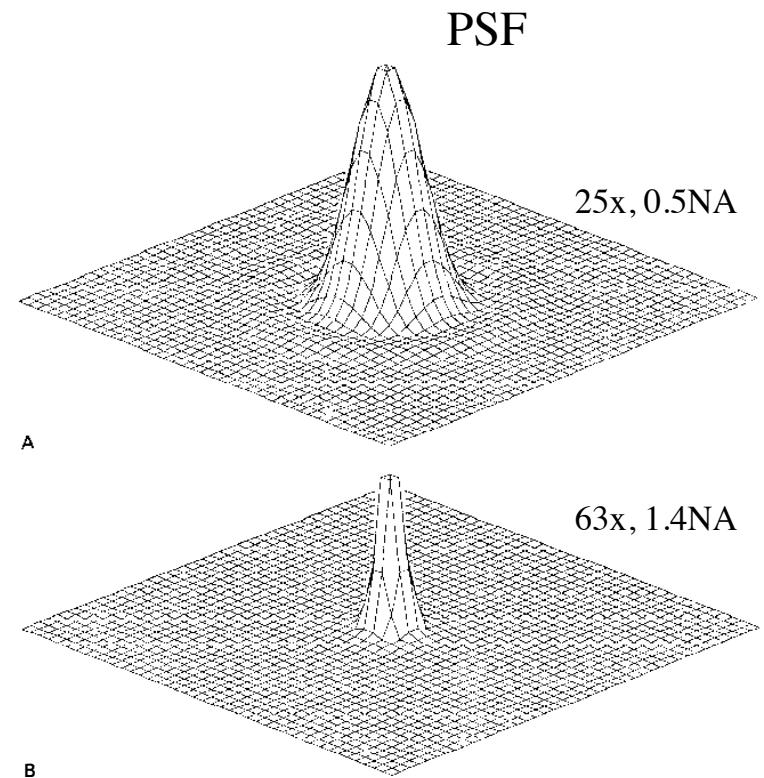
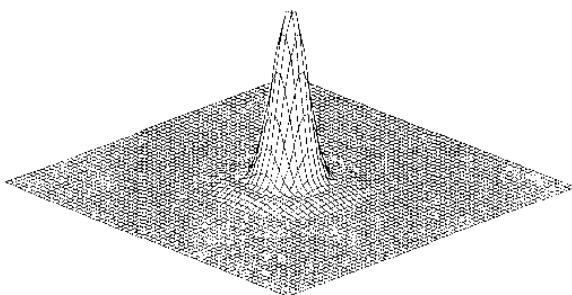
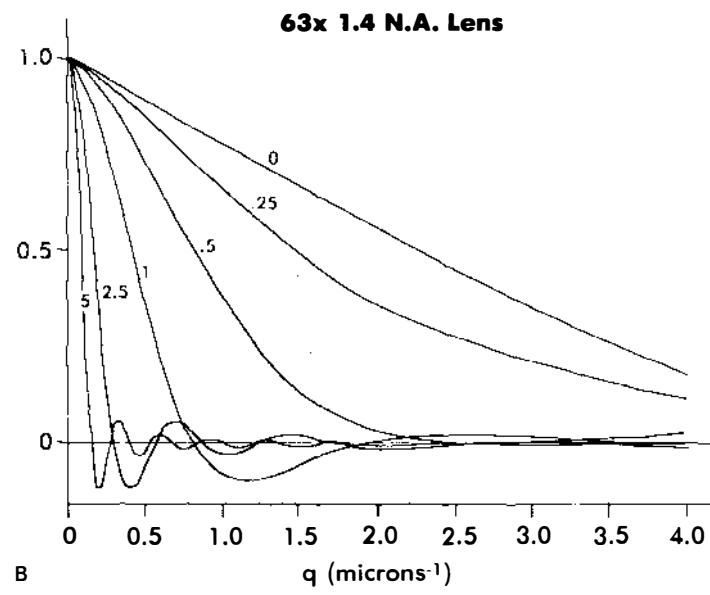
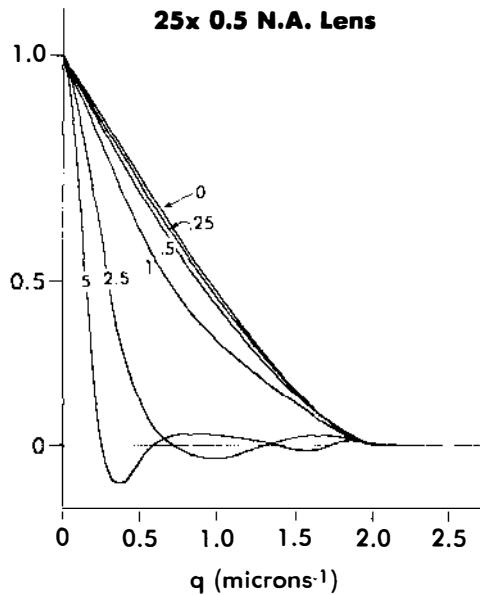
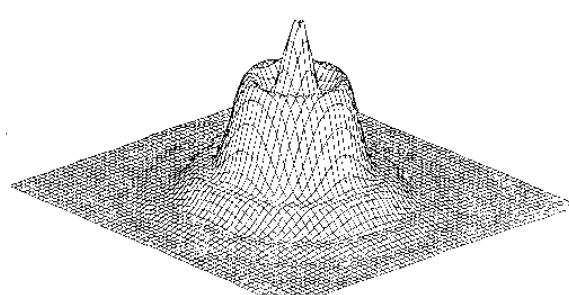


Figure 4 The point-spread functions calculated by Fourier transformation of the CTF's of Figure 3. (A) 25 × 0.5 N.A. lens (#2); (B) 63 × 1.4 N.A. lens (#5). Each grid square is $\lambda/6$ or 833 Å. Note how much sharper the 1.4 N.A. lens is than the 0.5 N.A. lens.

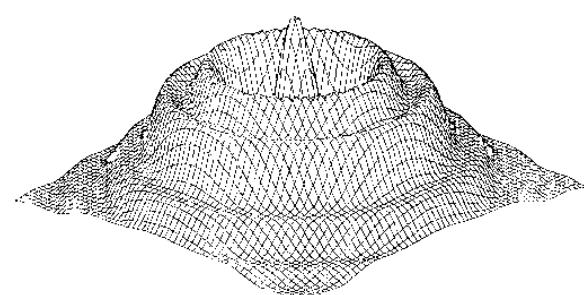
OTF, PSF of a defocused lens



25x lens 0 μm defocus



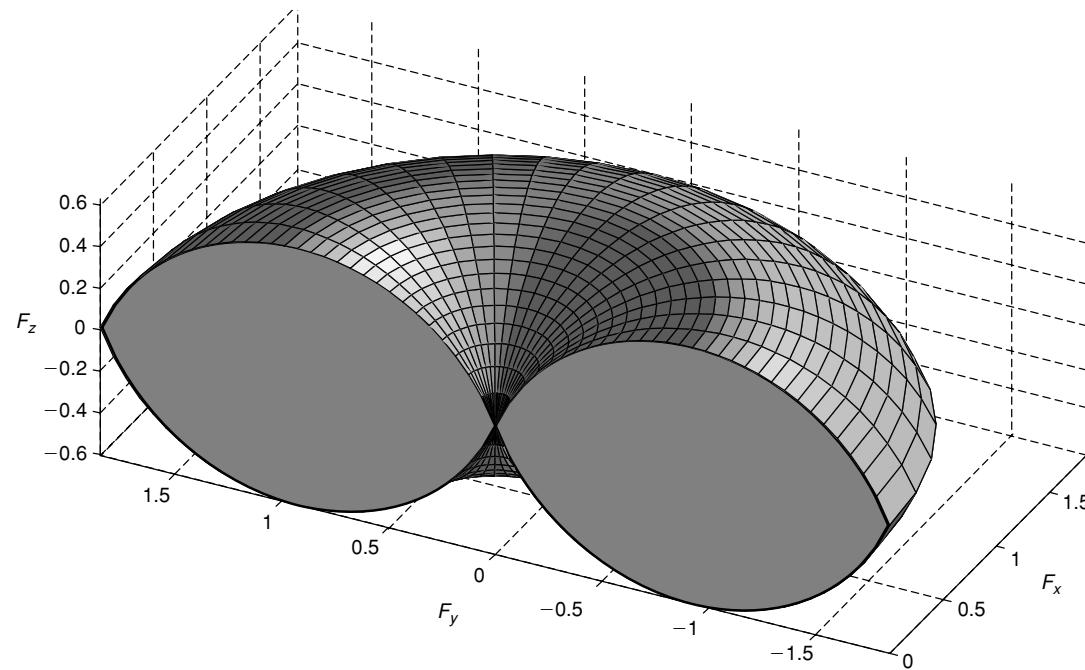
25x lens 2.5 μm defocus



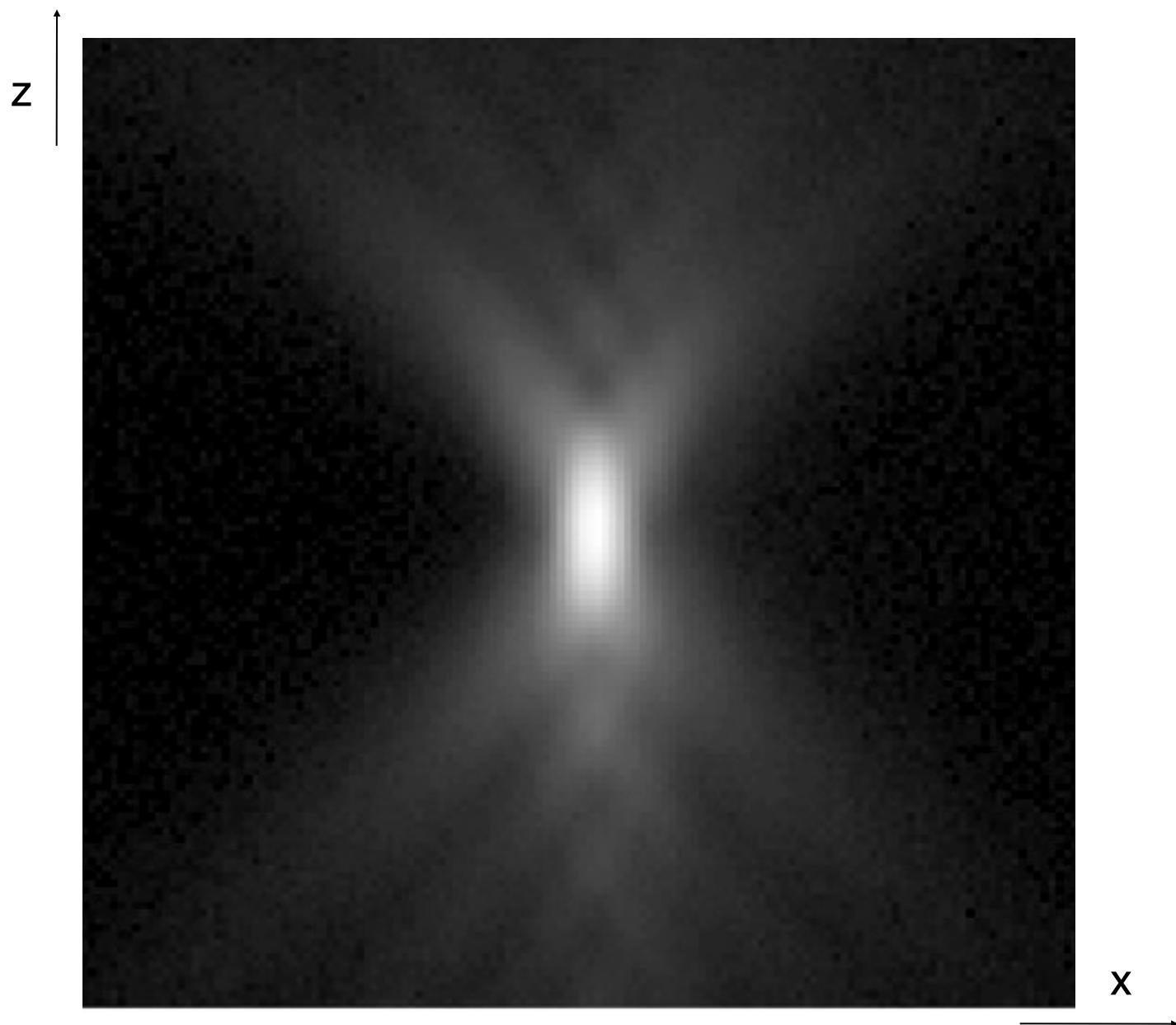
25x lens 5 μm defocus

3D OTF

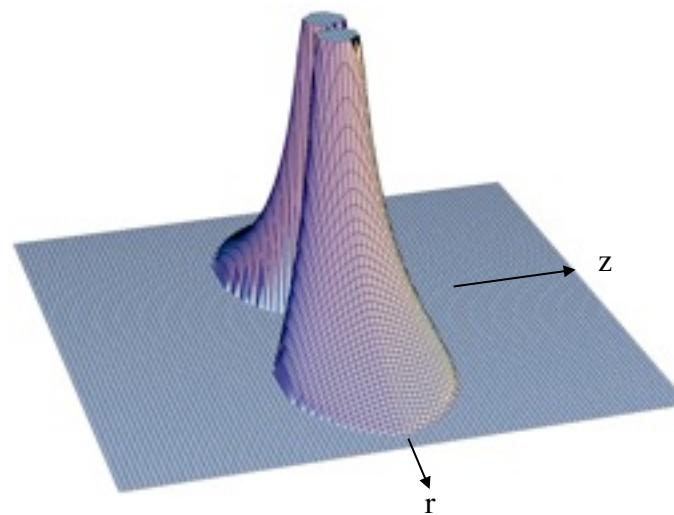
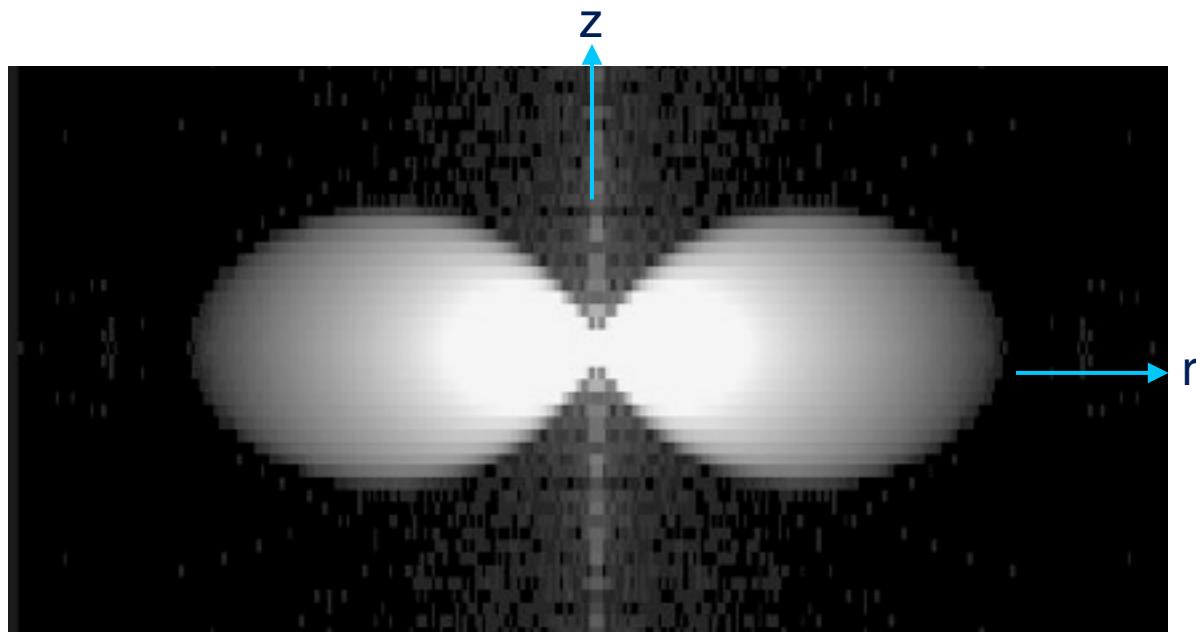
Combining different defocus OTFs from a stack of optical sections gives a donut shaped observable region (support) in Fourier Space



Experimentally measured PSF



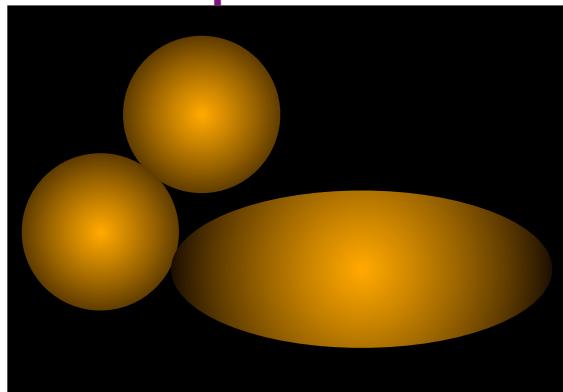
Experimentally measured OTF



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

PSF, OTF & deconvolution

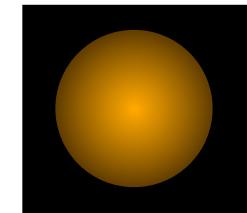
In real space:



Observed Image(r)



True Object(r)



Point Spread
Function, PSF(r)

In reciprocal space the convolution becomes a product:

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

This suggests:

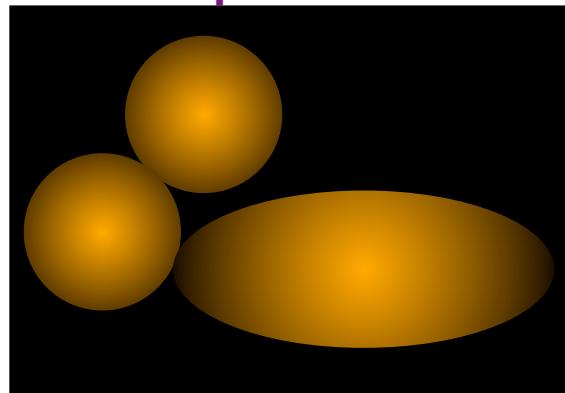
$$\text{Object} = \frac{\text{Image}}{\text{OTF}}$$

??? ("Deconvolution")

PSF is called the
"Optical Transfer Function",
OTF

PSF, OTF & deconvolution

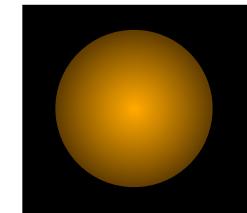
In real space:



Observed Image(r)



True Object(r)



Point Spread
Function, PSF(r)

In reciprocal space the convolution becomes a product:

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

This suggests:

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

PSF is called the
“Optical Transfer Function”,
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

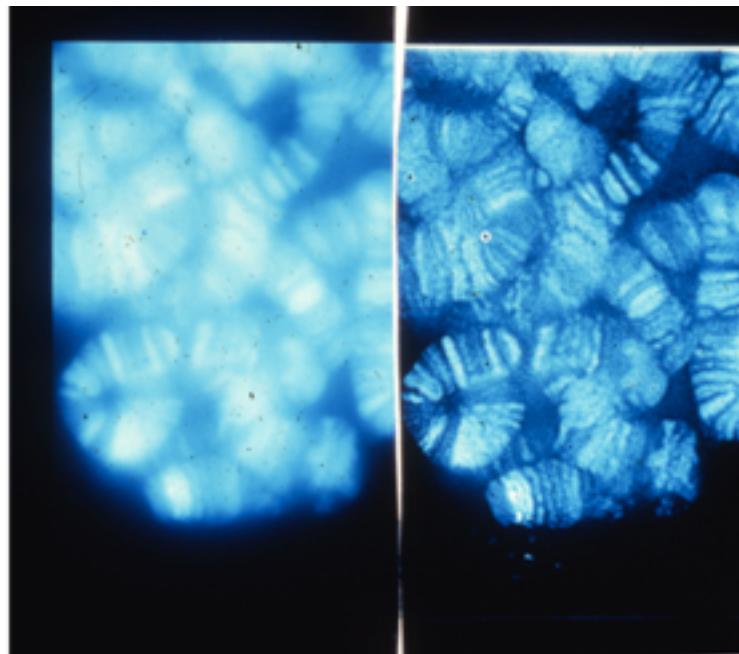
$$I_j \approx c_1 [O_j - c_2 (\text{PSF}_{\Delta z} \otimes O_{j+1} + \text{PSF}_{-\Delta z} \otimes O_{j-1})]$$

for speed do the convolutions as Fast Fourier Transforms (FFT), multiplication, FFT⁻¹

$$\text{PSF}_{\Delta z} \otimes O_{j+1} = \text{FFT}^{-1} [\text{FFT}(O_{j+1}) \cdot \text{OTF}_{\Delta z}]$$

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

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

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

or if OTF is complex:

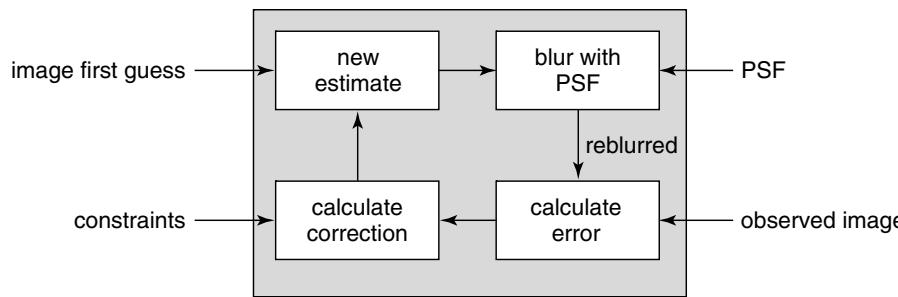
$$\frac{\text{Image} \cdot \text{OTF}^*}{\text{OTF} \cdot \text{OTF}^* + \gamma}$$

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 $I^0 = O$

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

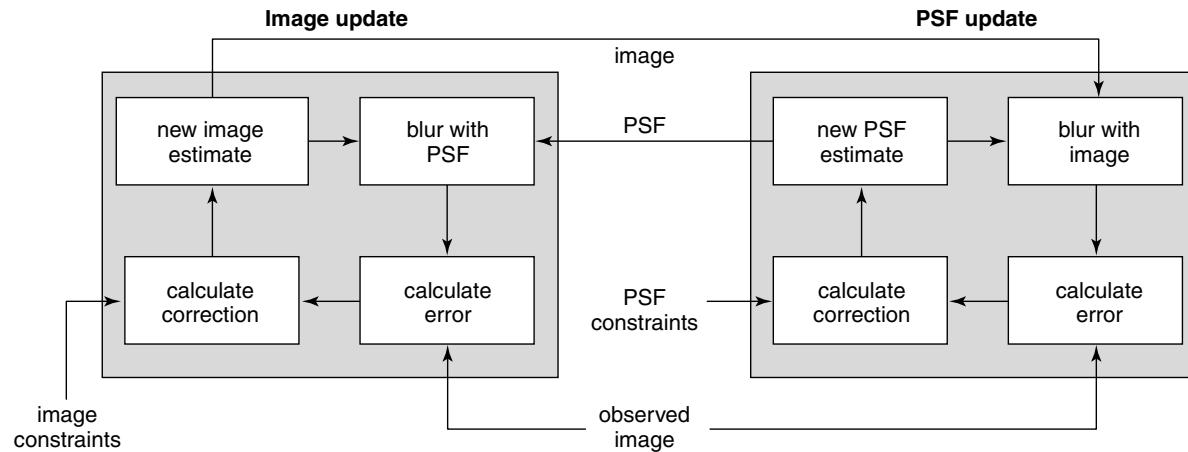
or use multiplicative update (Gold's method)

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

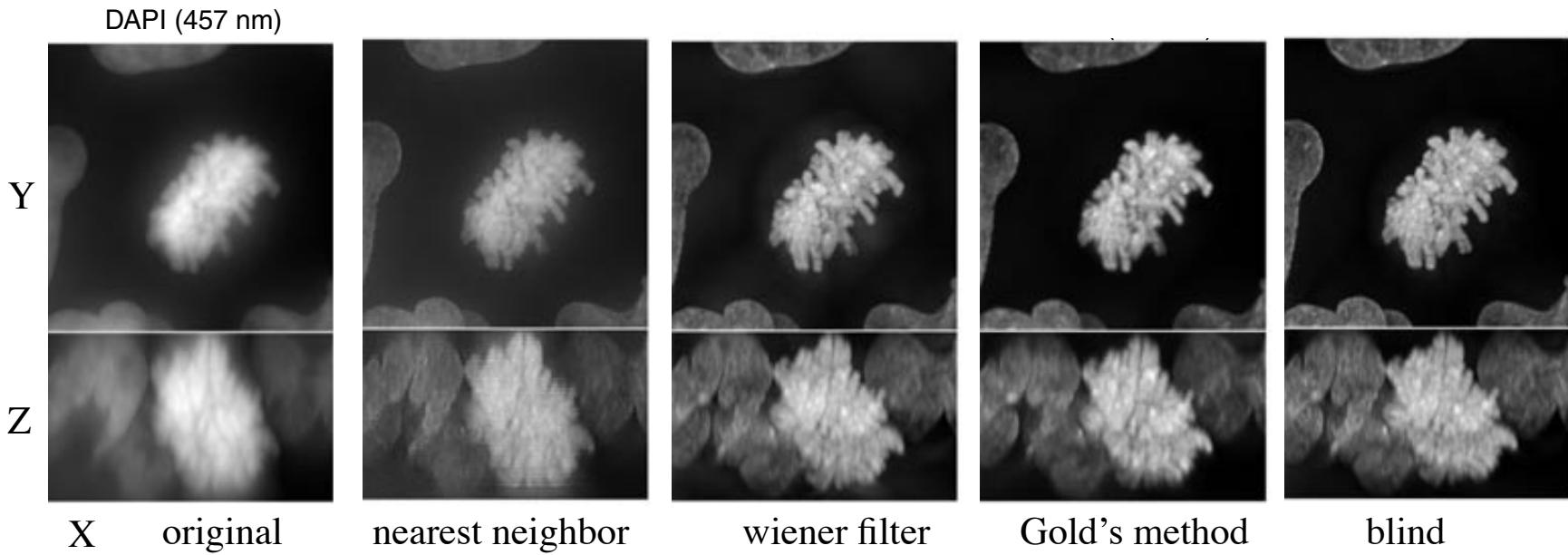
Deconvolution strategies

what if your PSF is not accurate?

blind deconvolution seeks to estimate both Image and PSF



Some comparisons: Hela cells, DAPI stained

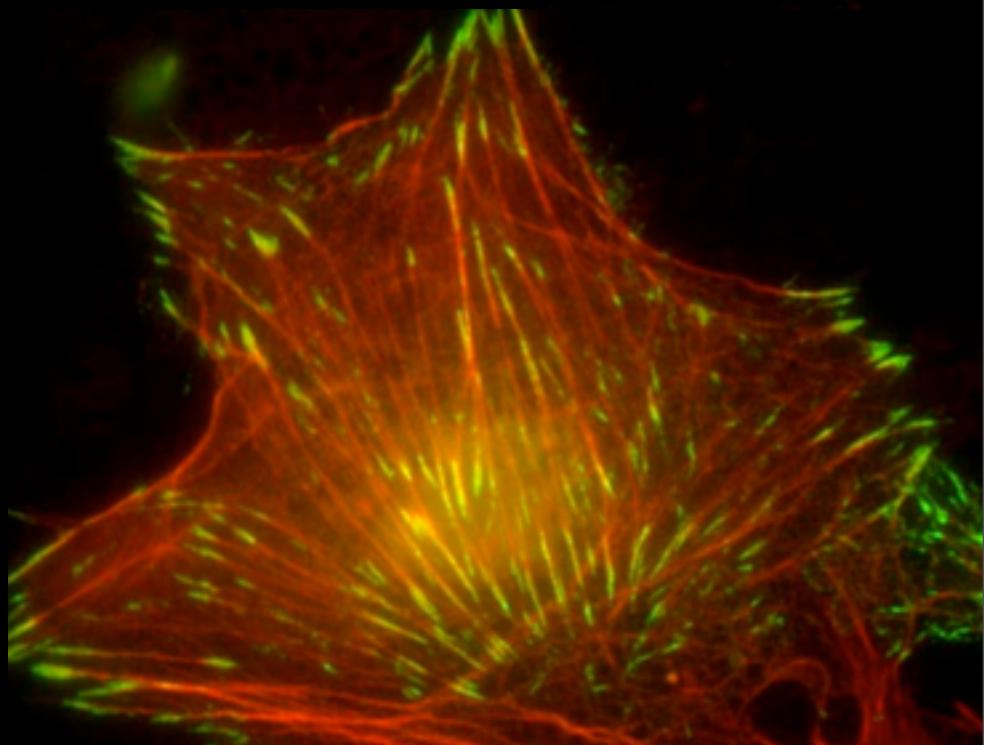
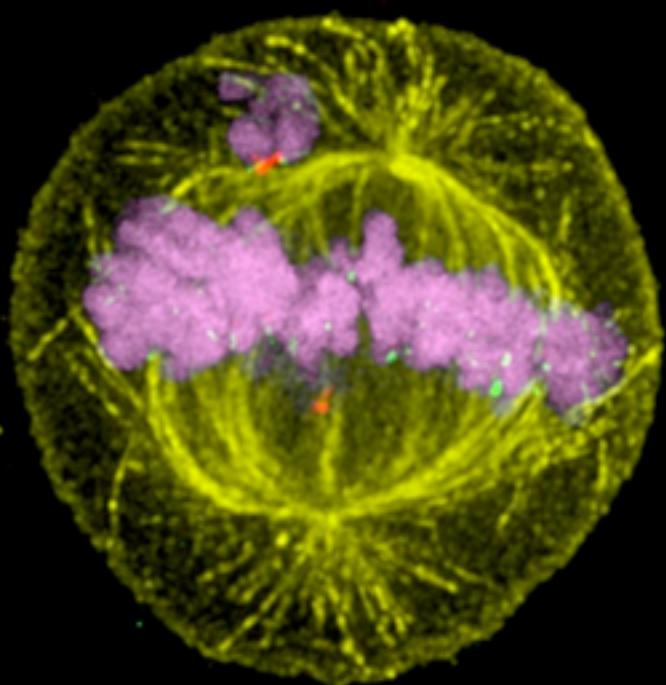
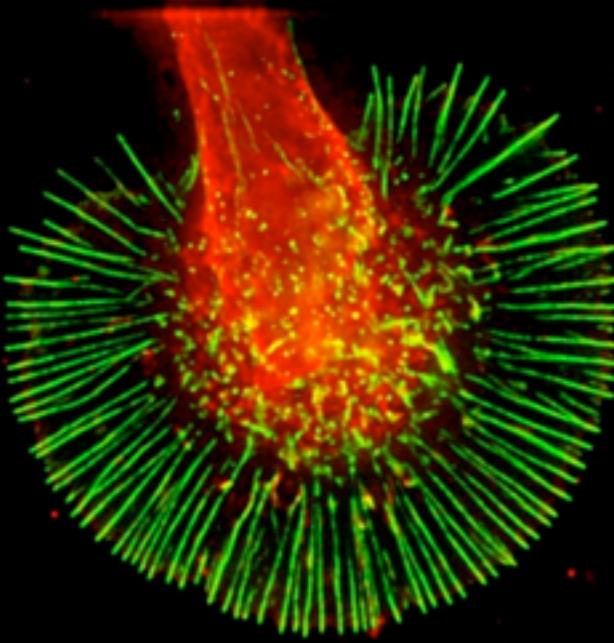
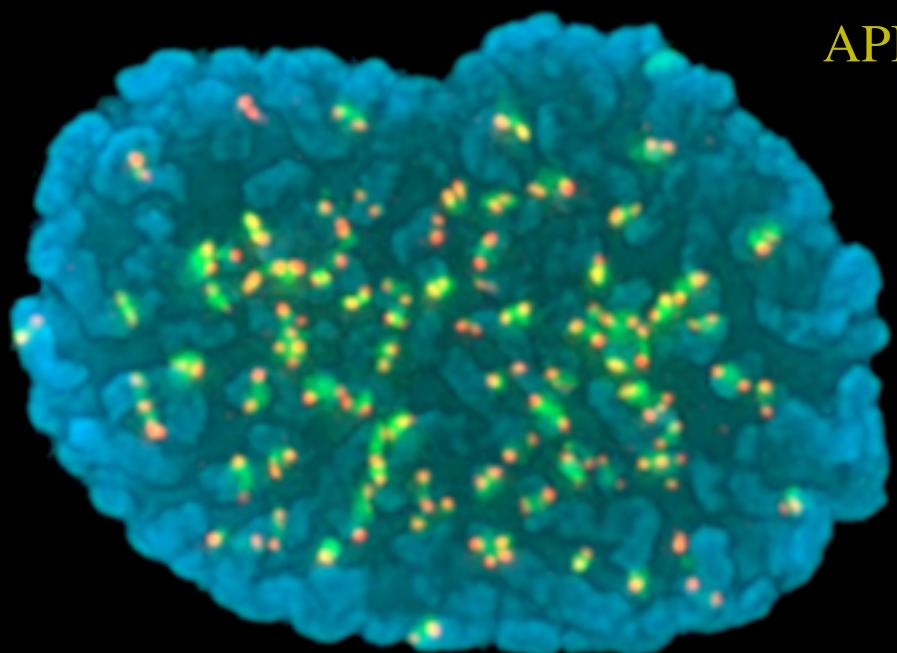


now many related variations

practical issues are signal to noise, accuracy of PSF

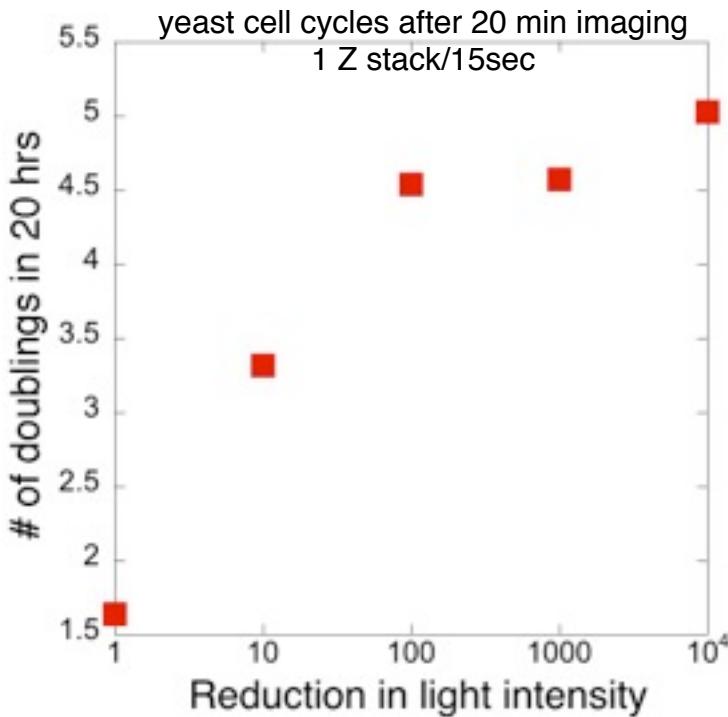
if sample is very thick then OSF can vary throughout sample
(minimize by matching index of refraction)

API web site

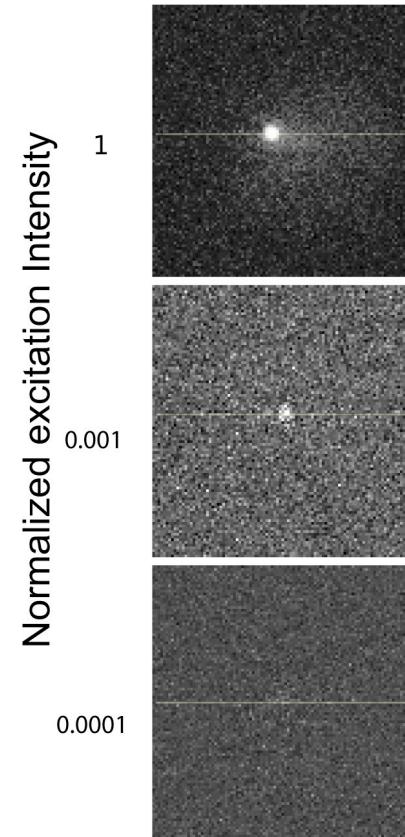


The in vivo imaging problem: phototoxicity

the challenge of live imaging:



maximum allowable photon flux that allows unperturbed viability is $4.8 \text{ nW}/\mu\text{m}^2$



“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

$$g_{opt}(r) = \arg \min_g \sum_r (h(r) * g(r) - f(r))^2 + \lambda R(g)$$



Regularization functional
defines how noise is handled

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

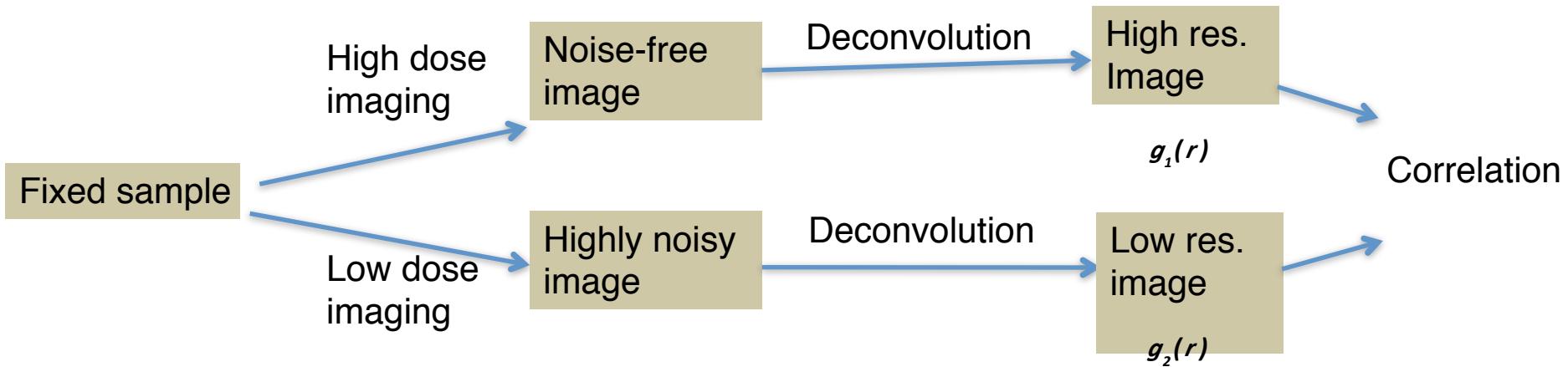
use entropy based optimization (ER-Decon)

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

α : Nonlinearity factor

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

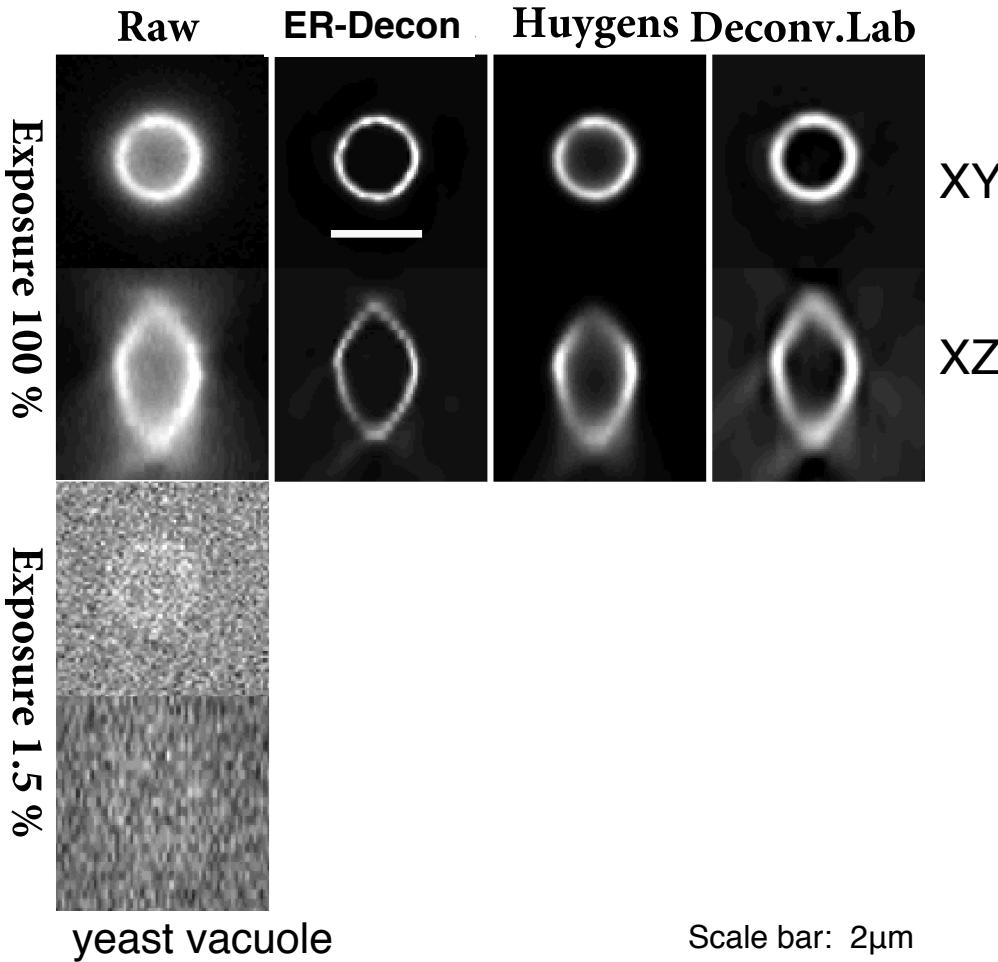
Validate by quantitative comparison to high dose image



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

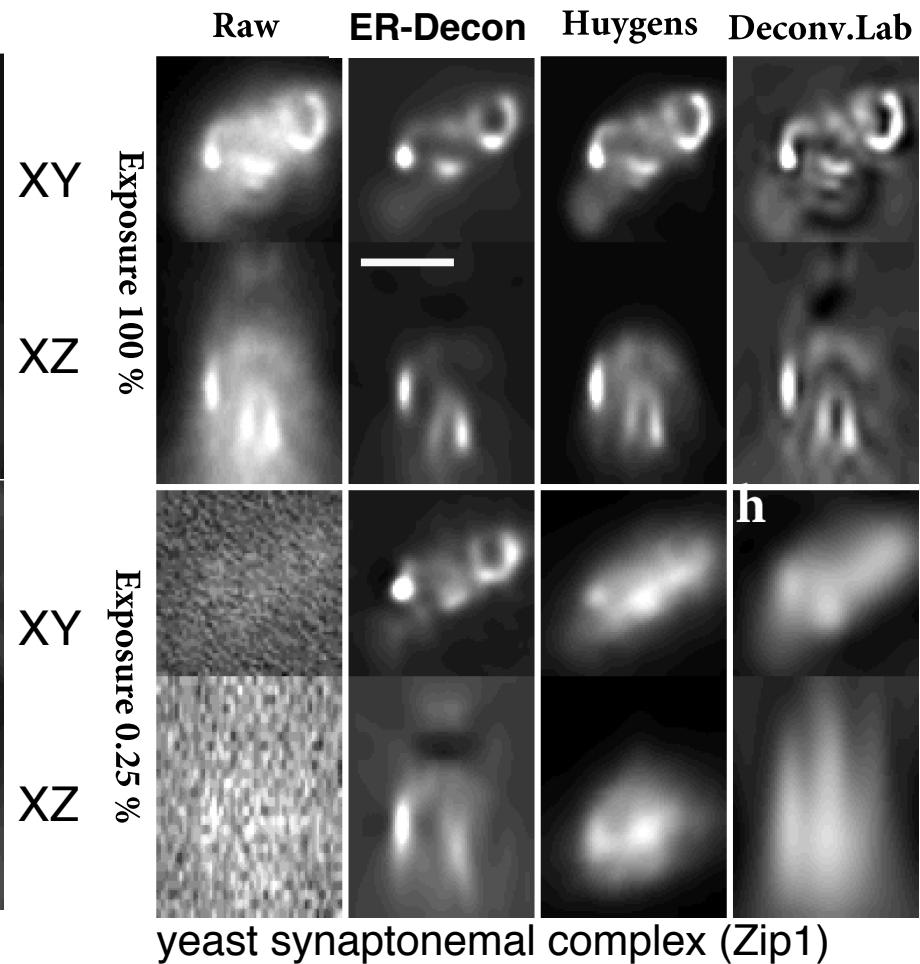
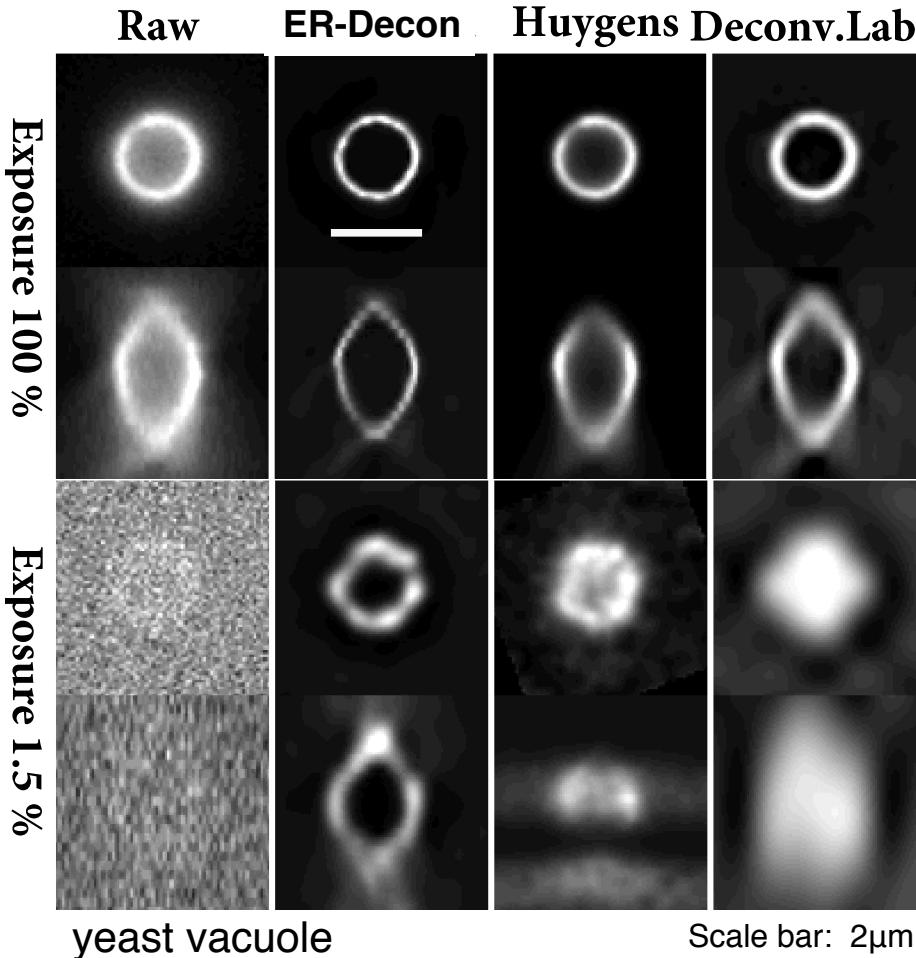
- (1) **Huygens** – commercially available method
(uses MLE approach)
- (2) **DeconvolutionLab (DeconLAB)** from Unser's group at EPFL -
best among free packages (uses wavelet approach)

Remarkable signal recovery simultaneous with deconvolution



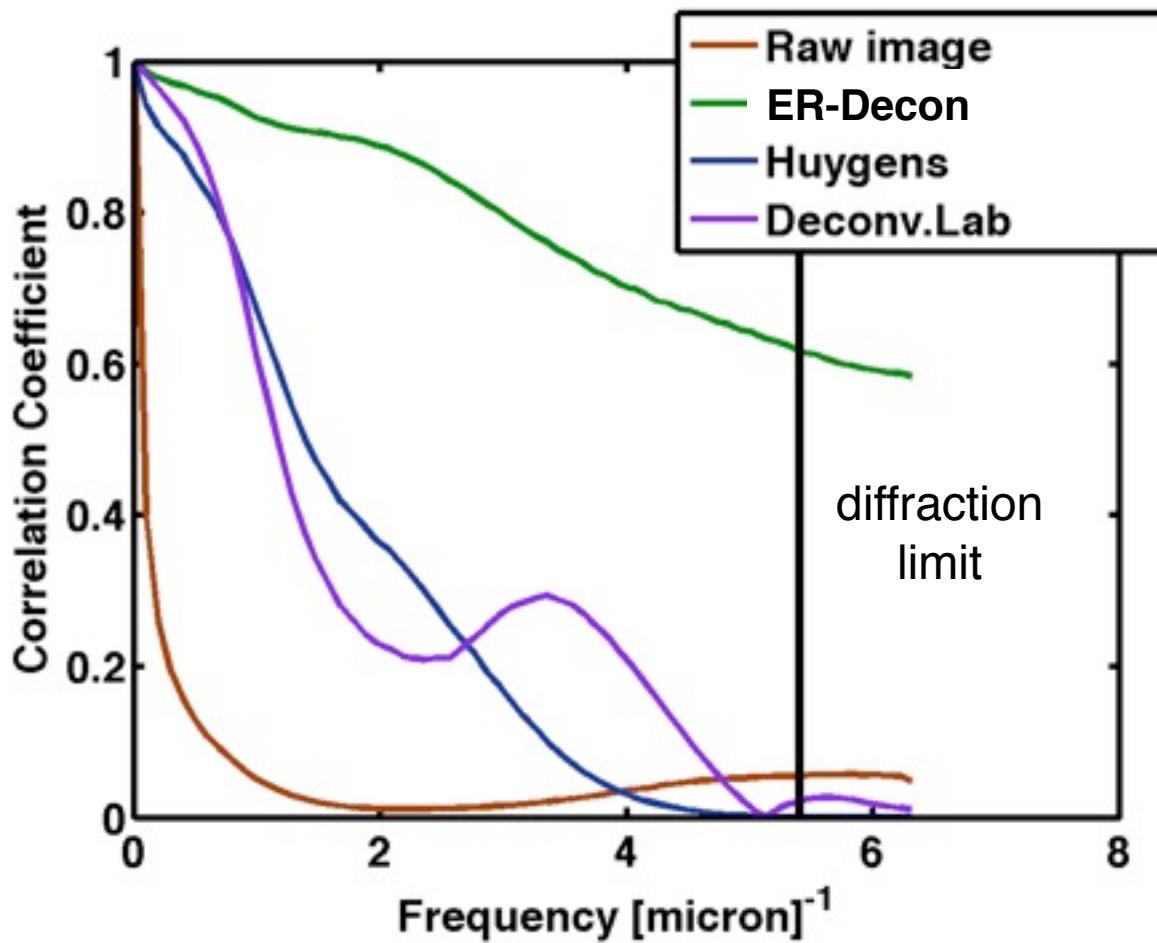
M. Arigovindan

Remarkable signal recovery simultaneous with deconvolution



M. Arigovindan

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



*enabling technology for *in vivo* imaging*

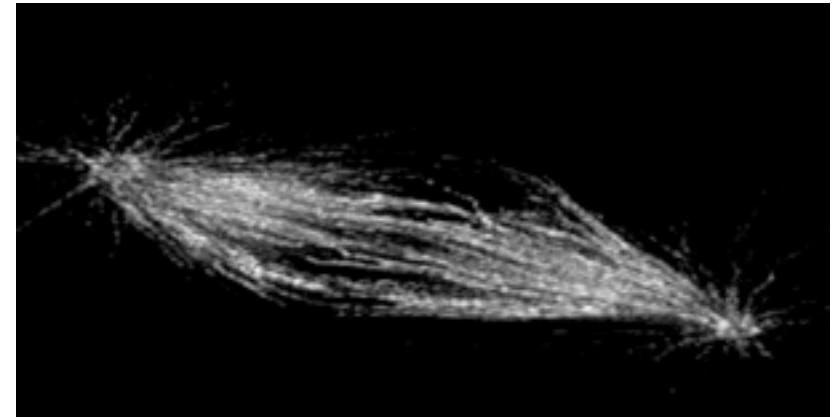
M. Arigovindan

ER-Decon deconvolution of SIM data

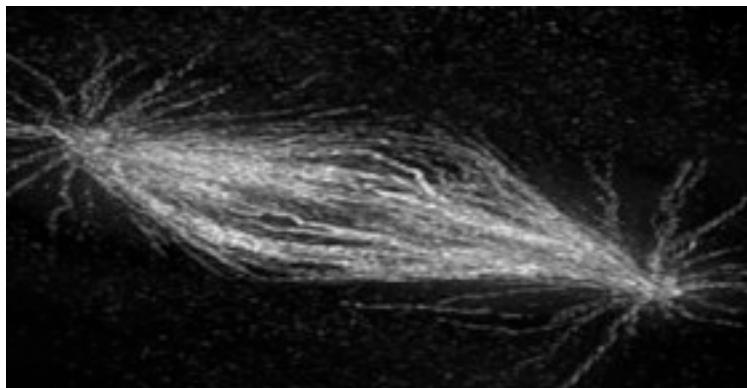
Widefield



SIM-Wiener filtering

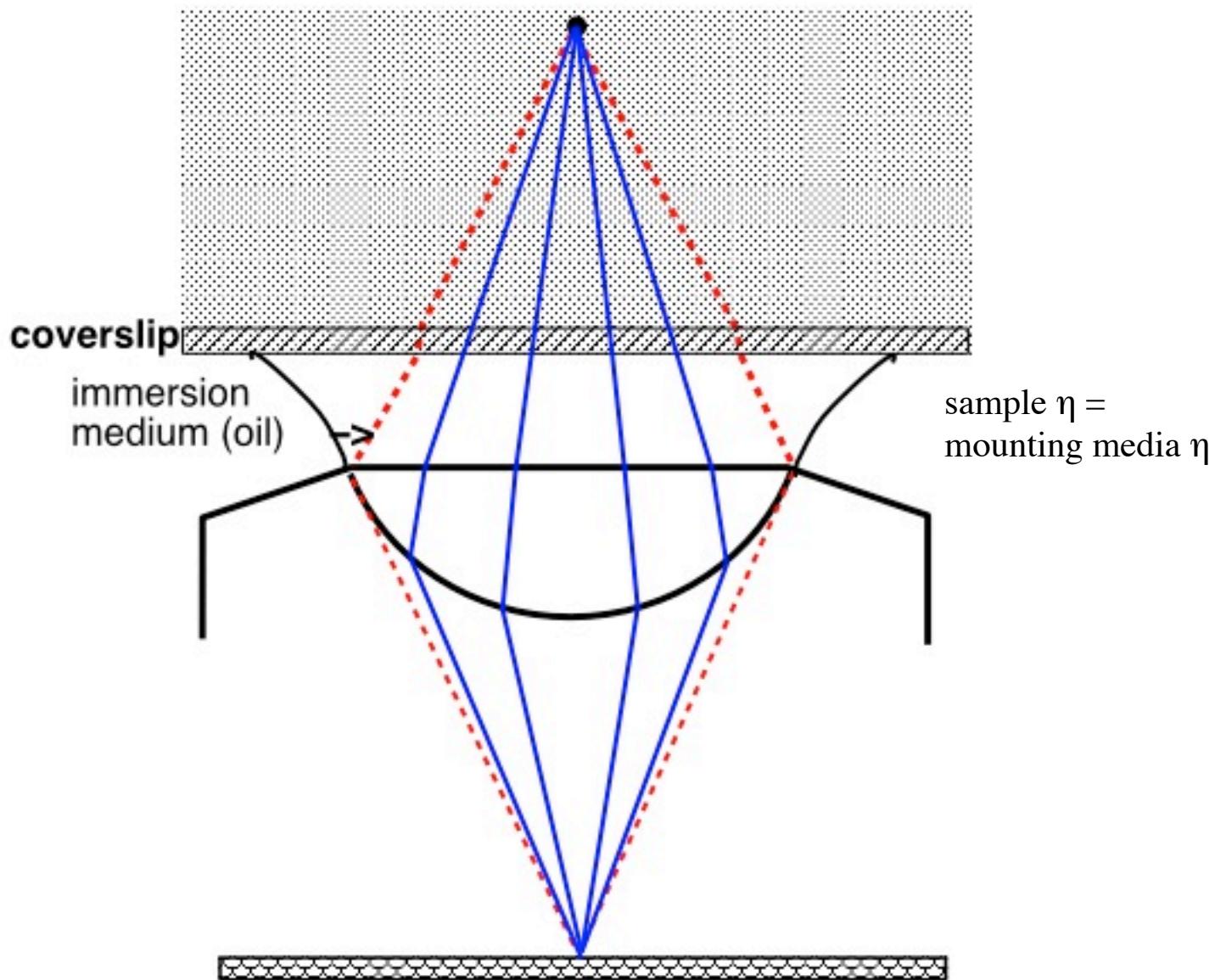


SIM + ER-Decon

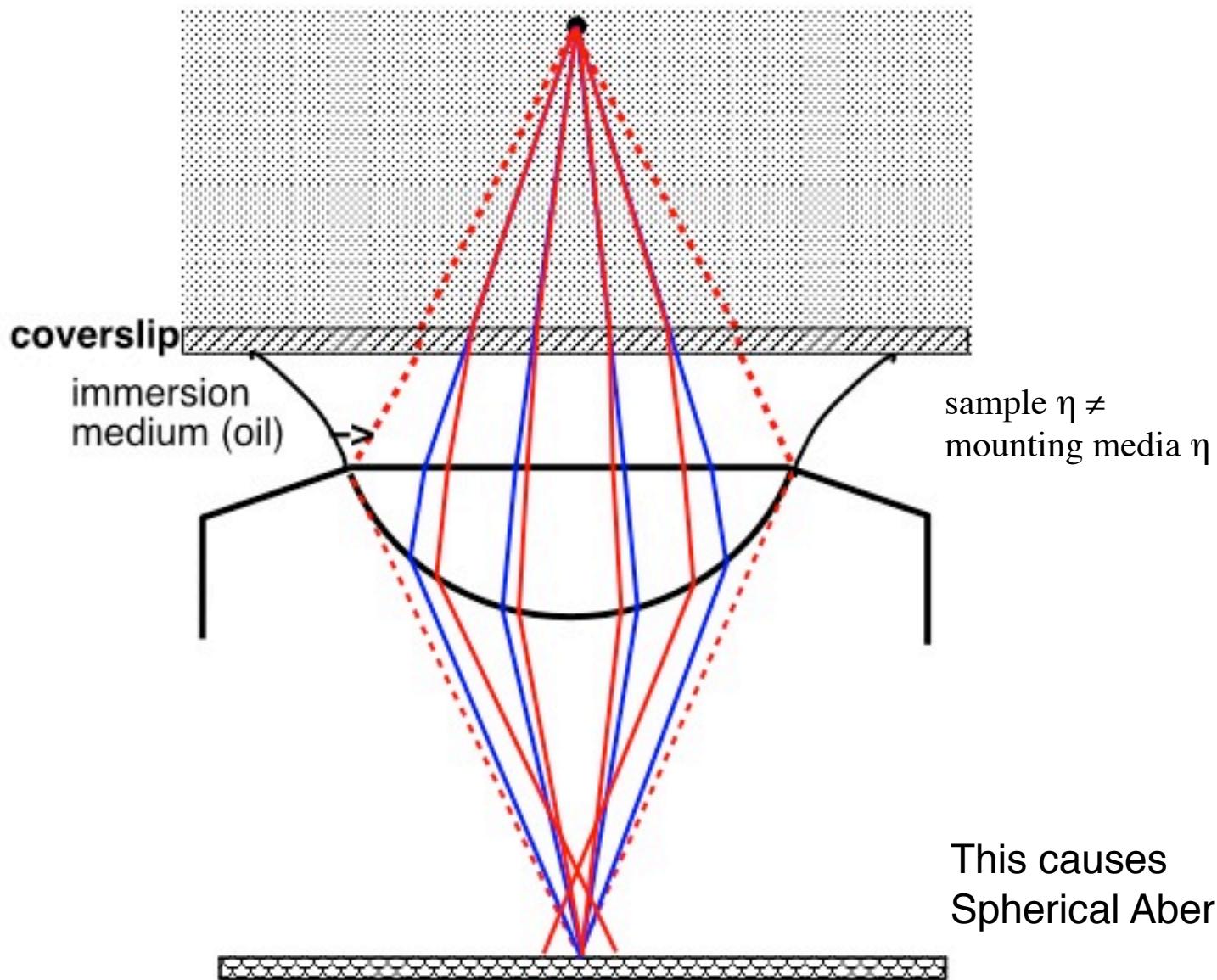


M. Arigovindan

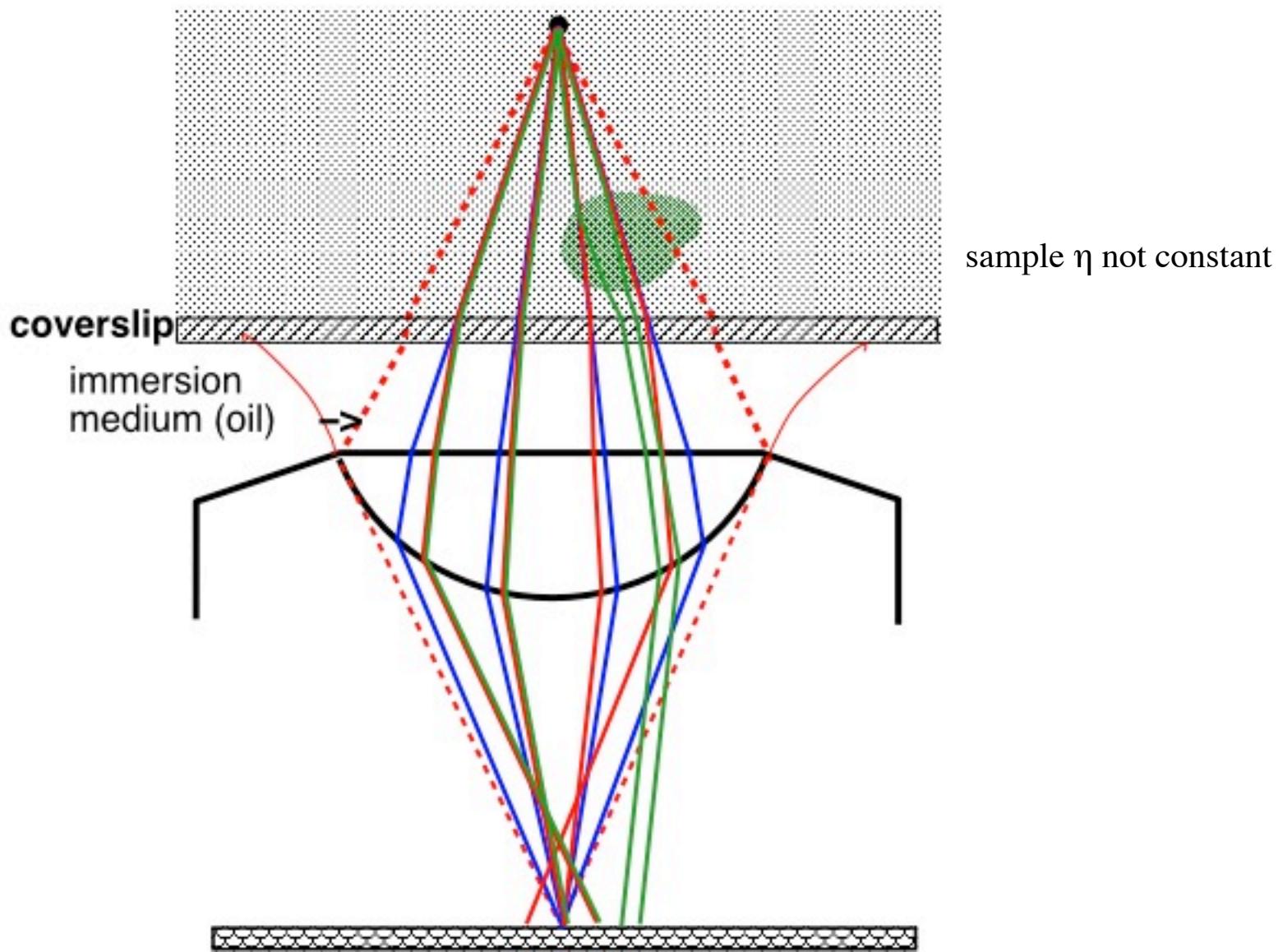
Properties of the Sample Affect Image Formation in Microscopy



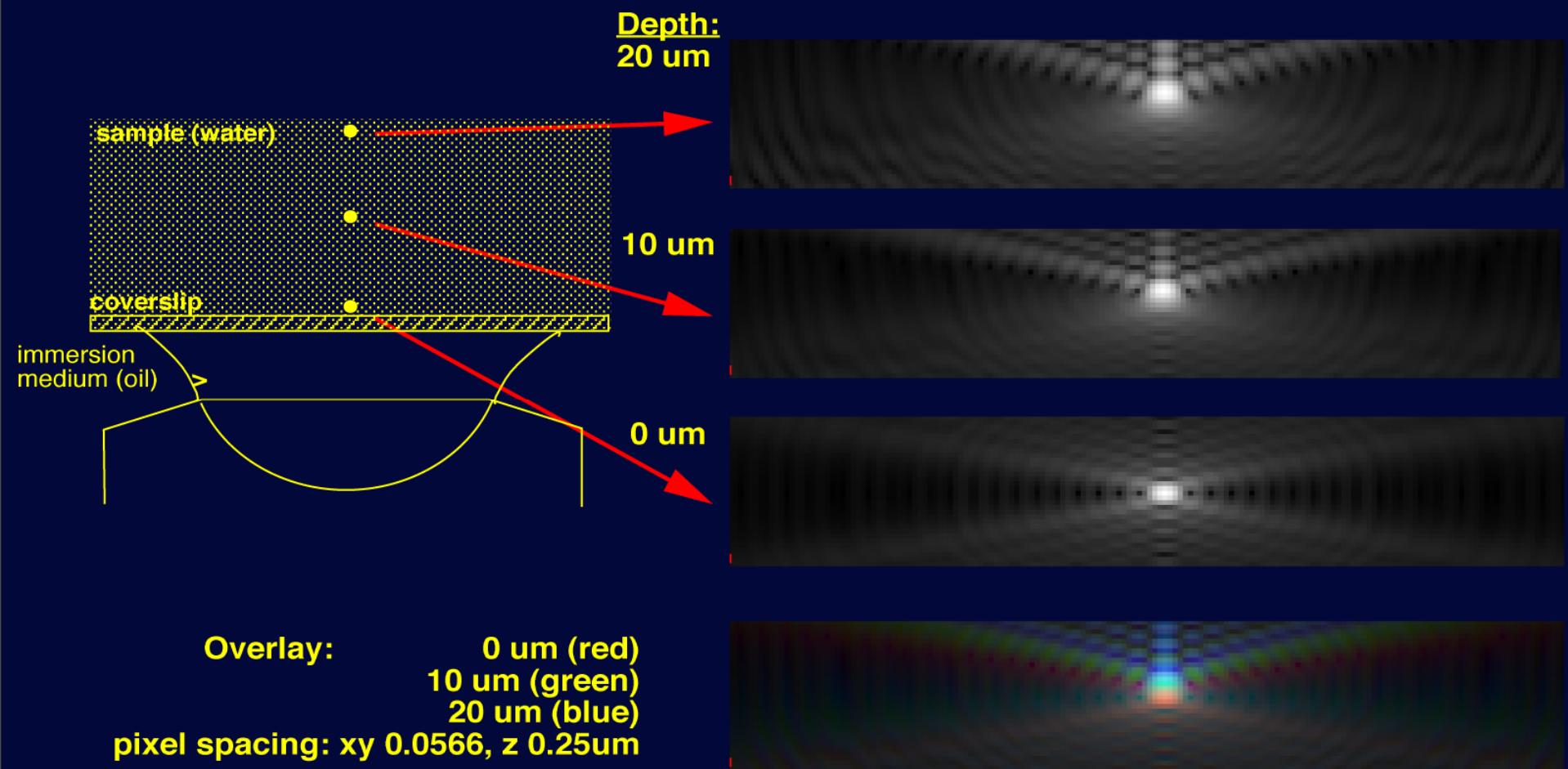
Properties of the Sample Affect Image Formation in Microscopy



Properties of the Sample Affect Image Formation in Microscopy



Depth-Dependent Effects in Water: Simulations of PSFs



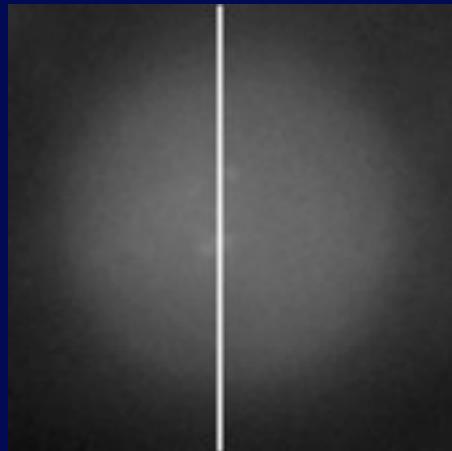
Hansel

Depth Dependent Deconvolution: Biological Data

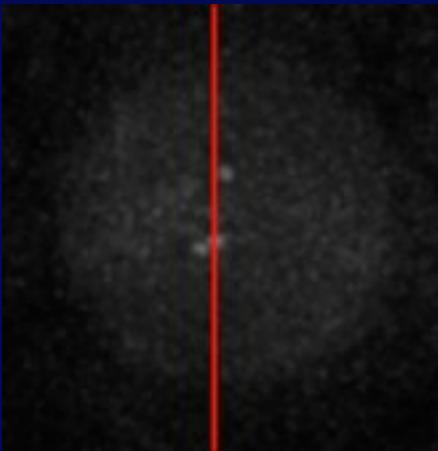
Line Profile Through 1 Spot in Nucleus

Section ~7 .6 μm into sample

Measured Data

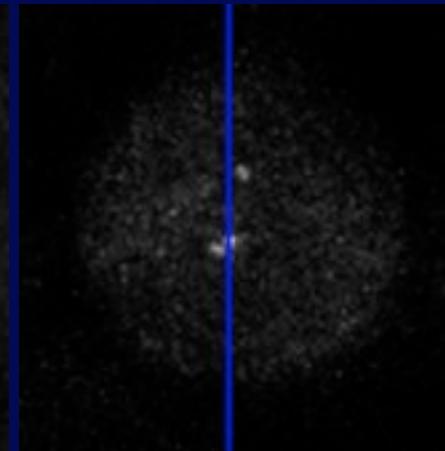


Spatially-Invariant



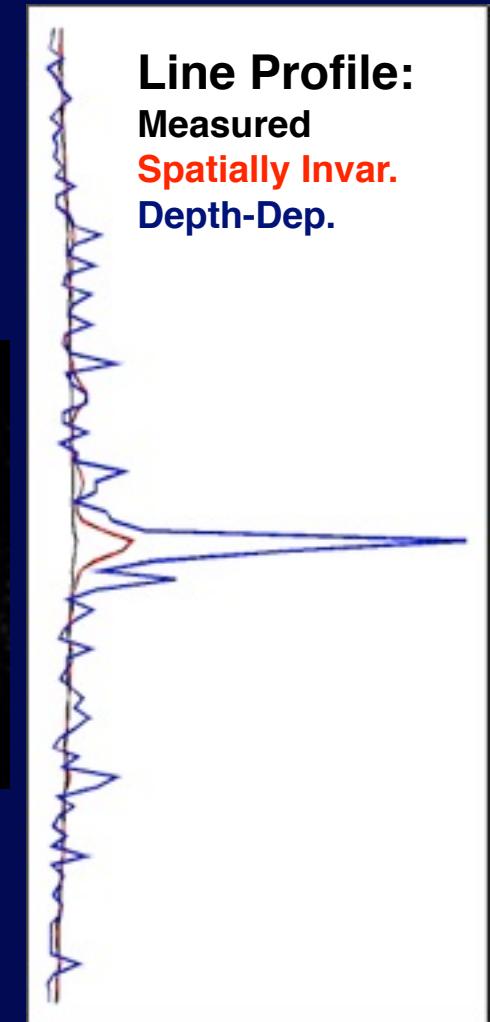
Post-Deconvolution

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)

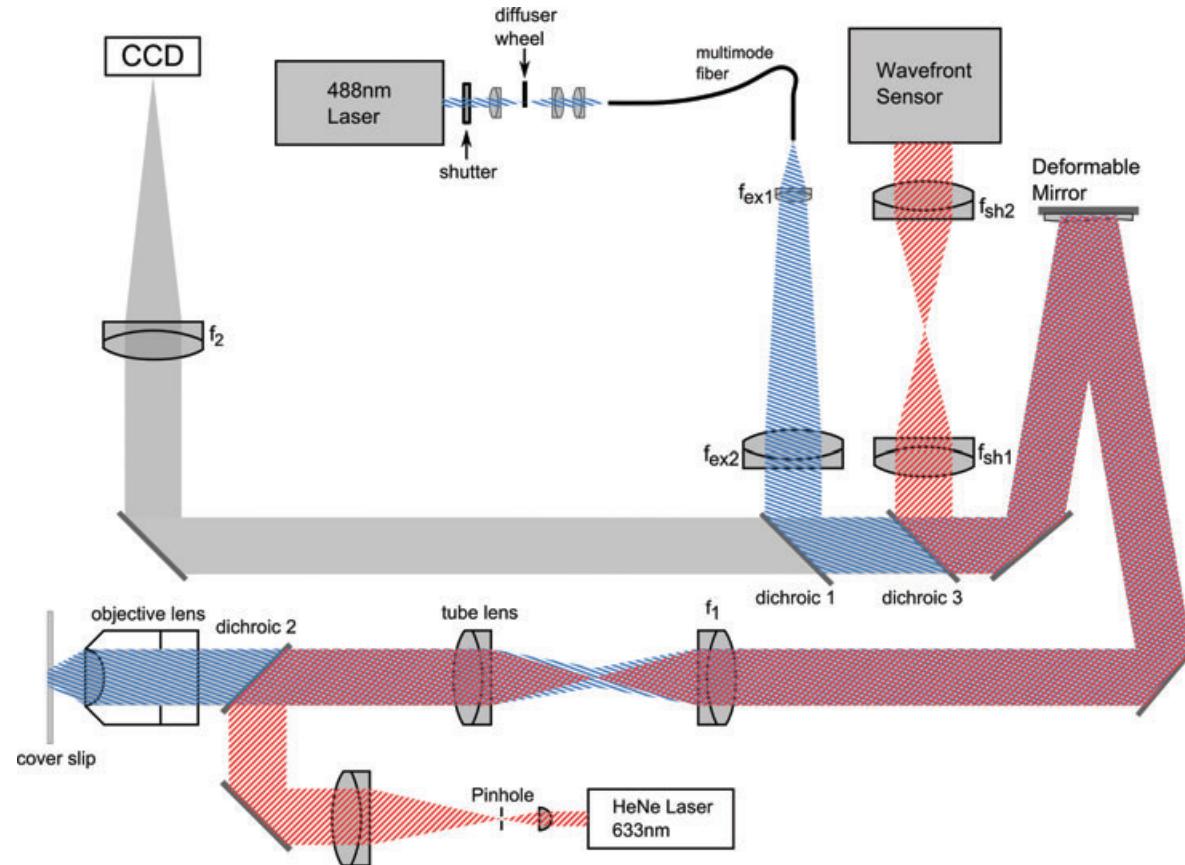
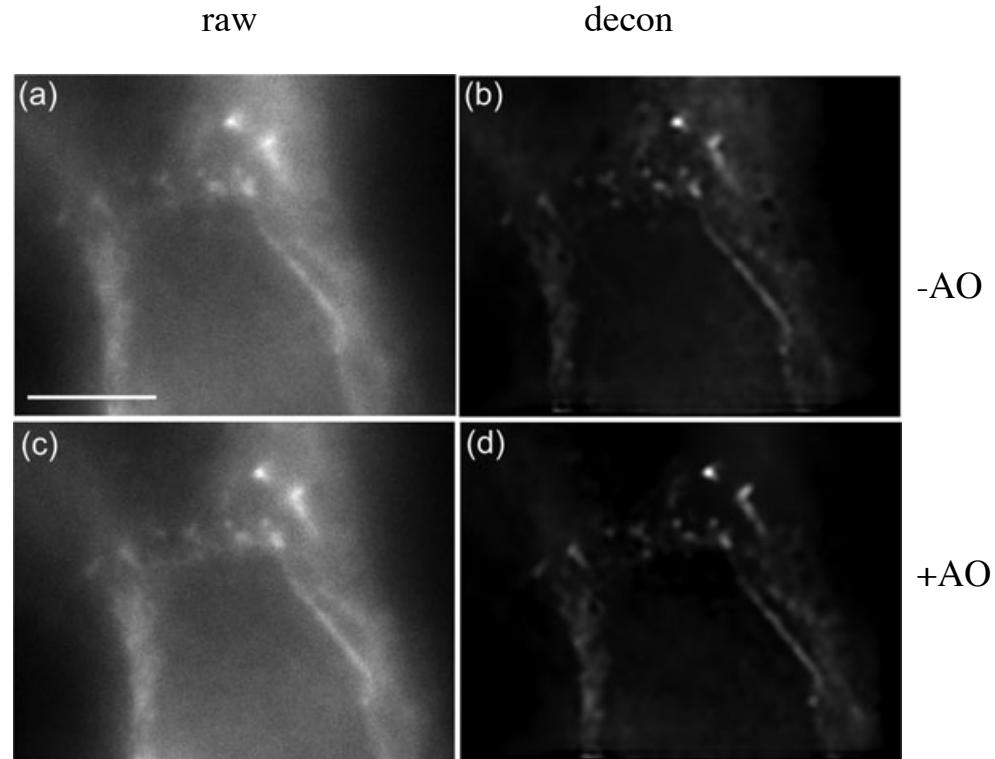
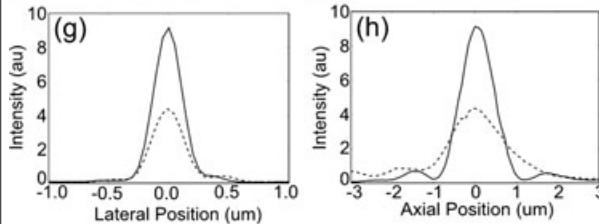
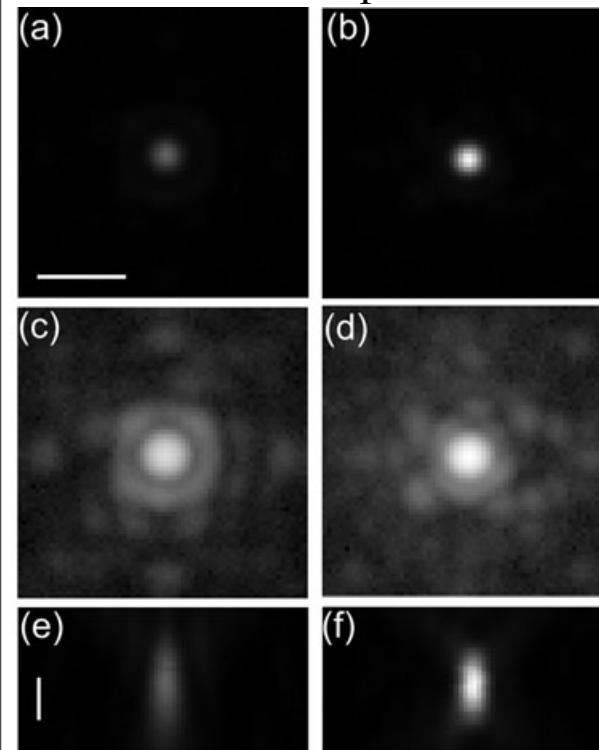


Fig. 2. Microscope layout. Grey represents the emission path. The blue striped beam is the excitation light and the red striped beam is the reference beam. See text for details.

Adaptive optics can correct for depth dependent effects

flat mirror mirror shaped to correct
 spherical aberration



Alexa 488-phalloidin stained mouse cells:
images at $4.4\mu\text{m}$ below cover slip

Structured illumination microscopy: the idea

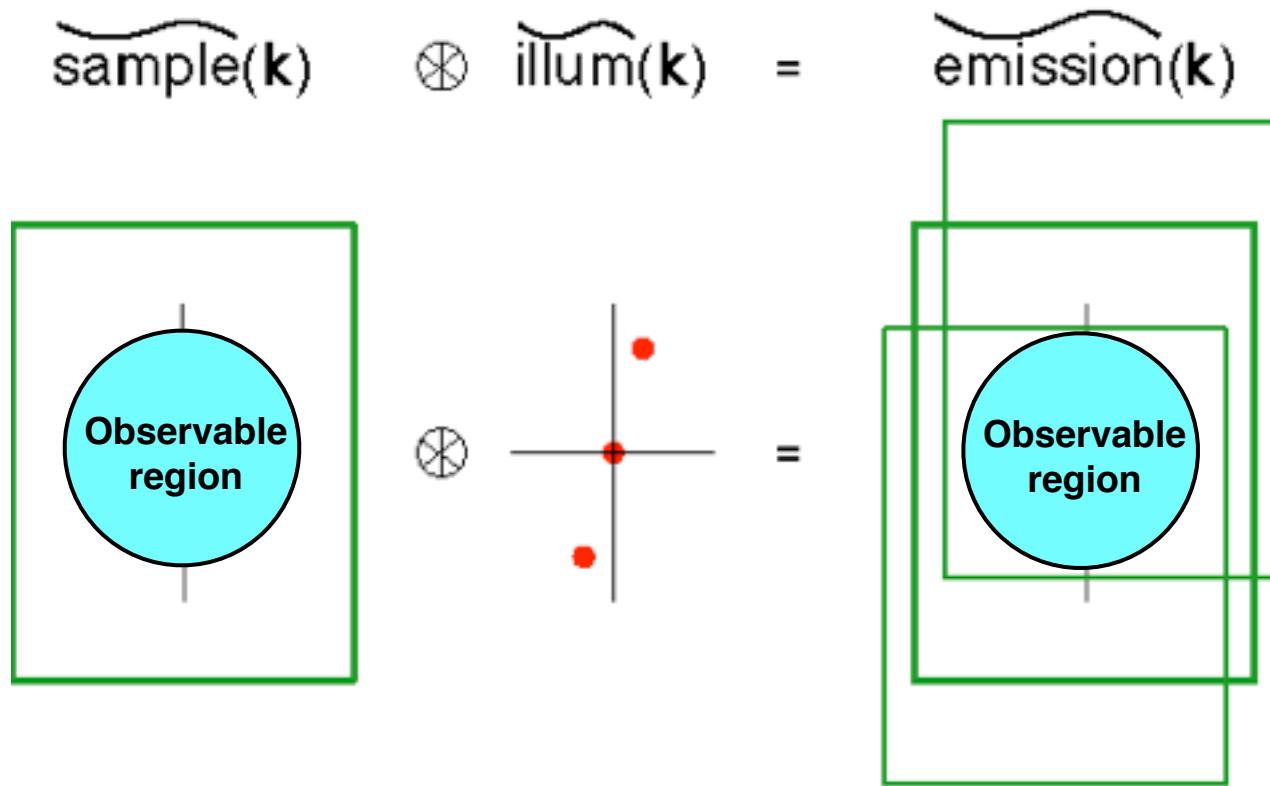
Two patterns
superposed
multiplicatively
give rise to
moiré fringes



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

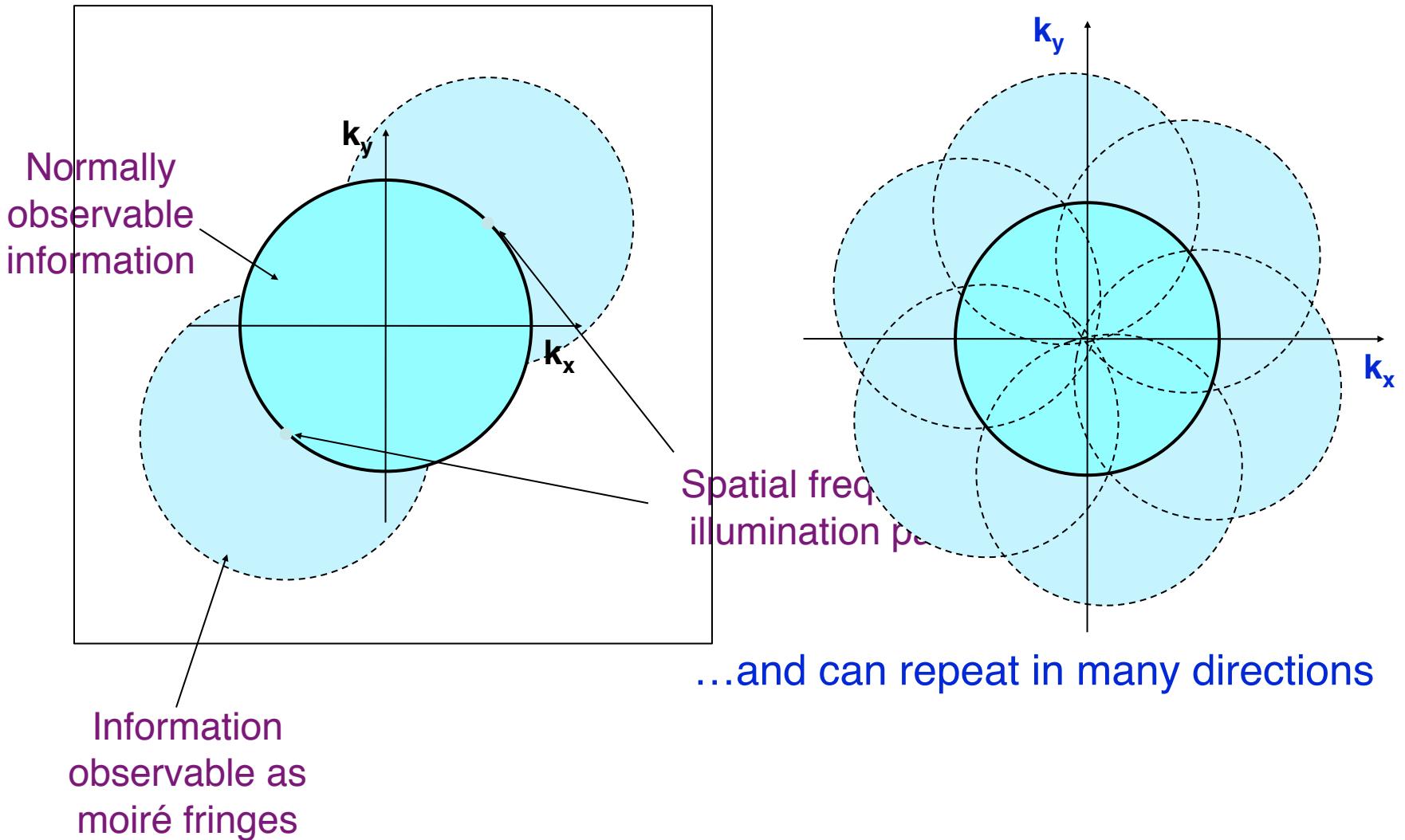
- *Illuminate the sample with a light pattern*
- *Observe moiré fringes between the pattern and the sample structure*
- *Deduce otherwise unresolvable information about the sample*

Illuminate sample with parallel stripes



*The emitted light contains 3 superimposed information components shifted by $0, \pm$ the inverse stripe spacing
Record 3 images ($0^\circ, 120^\circ, 240^\circ$ shifts) to sort out 3 components*

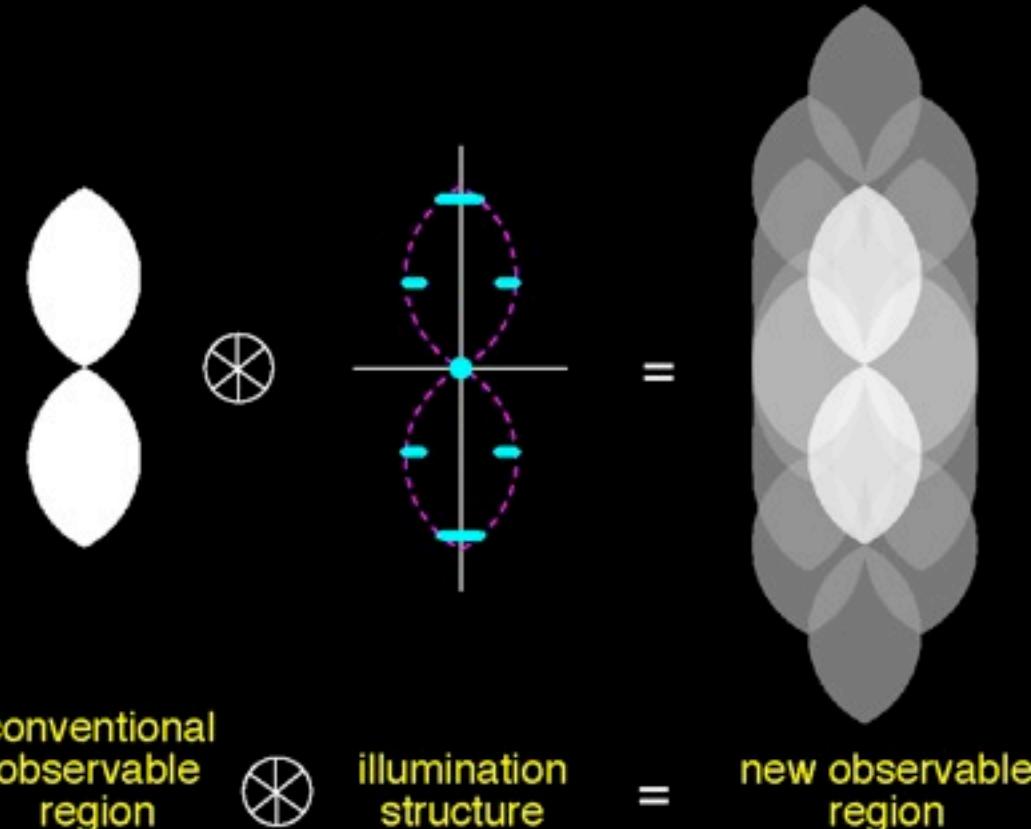
Resolution extension by Structured Illumination



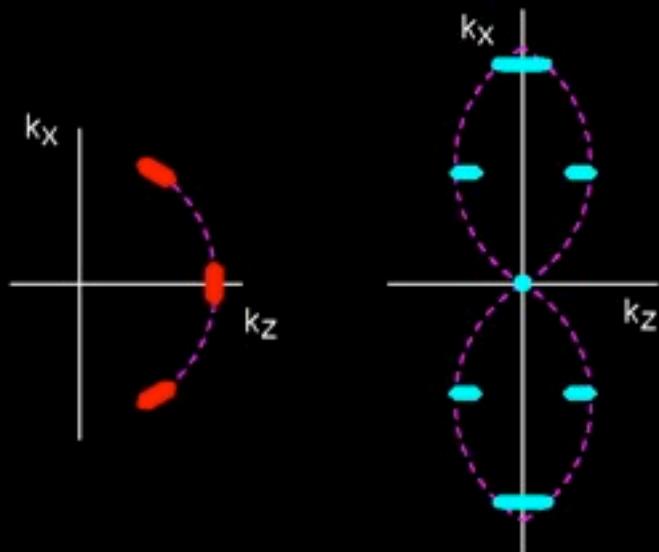
Q: What about 3D?

A: Use 3 beams

(admit diffraction orders 1, 0 and +1)



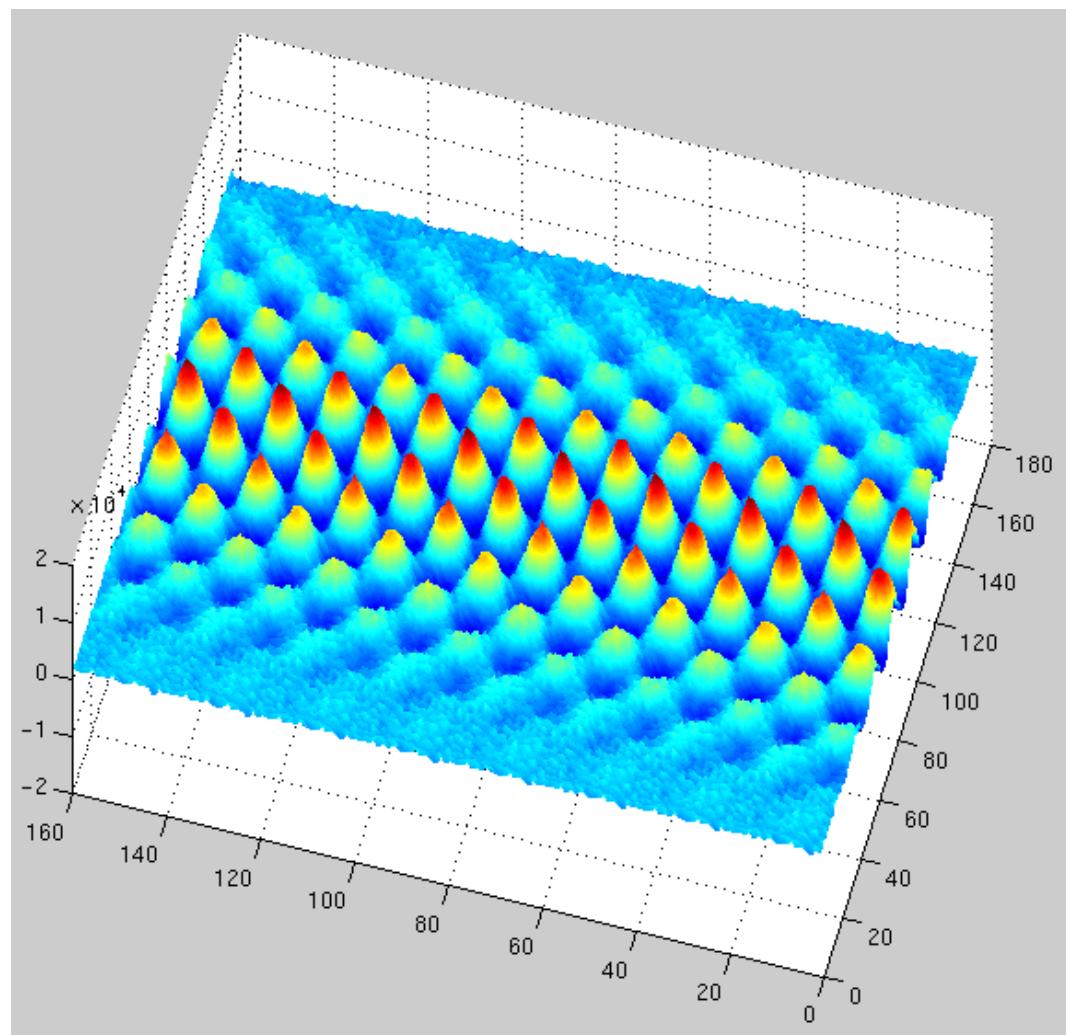
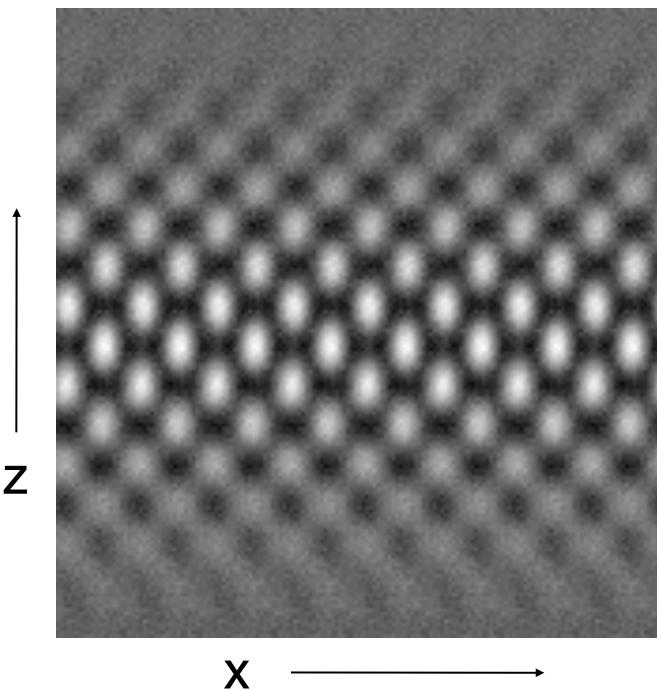
3 coherent beams \rightarrow 7 intensity components



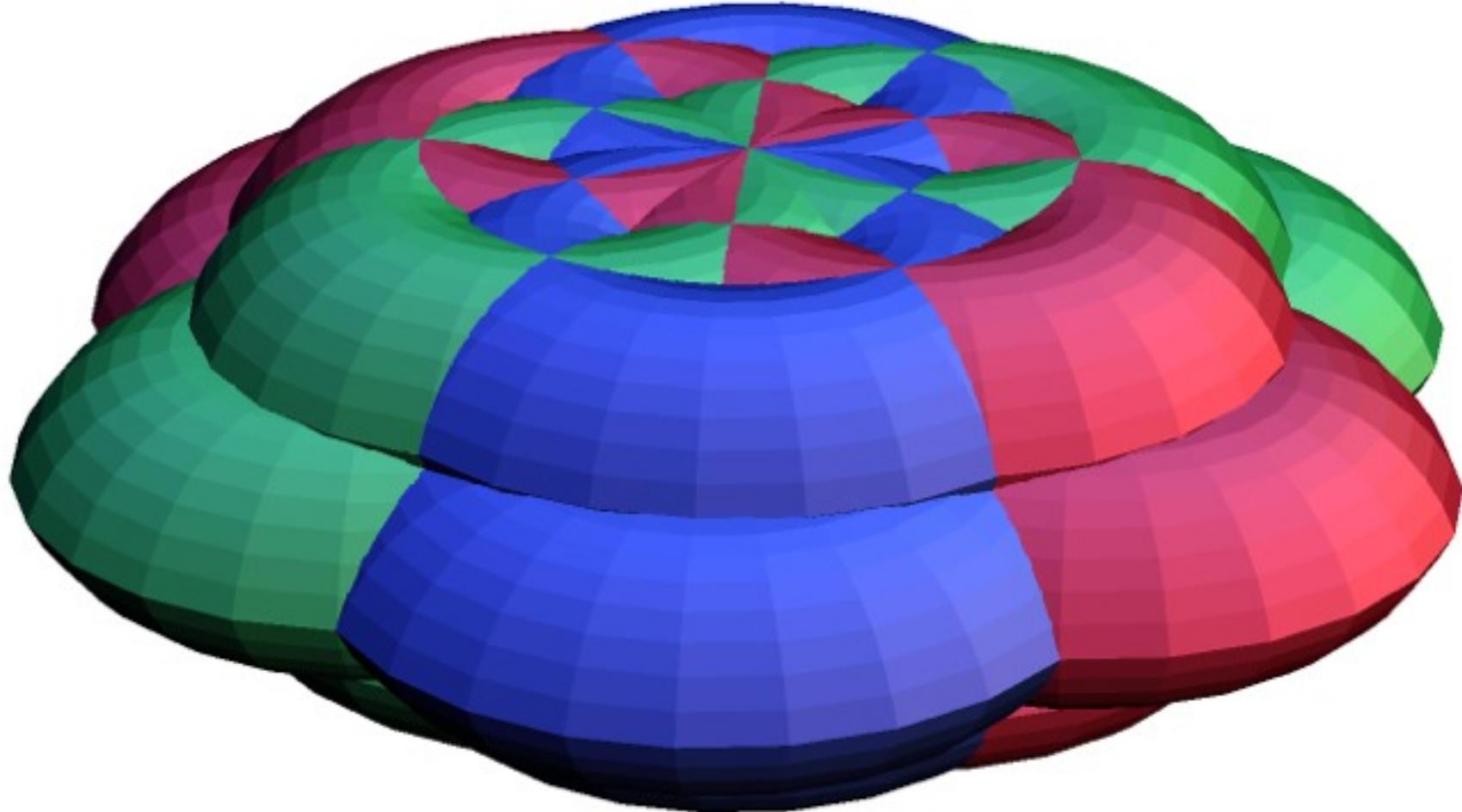
Covers full "confocal" OTF support without discarding light

Same procedure as for 2D,
except need 5 phases

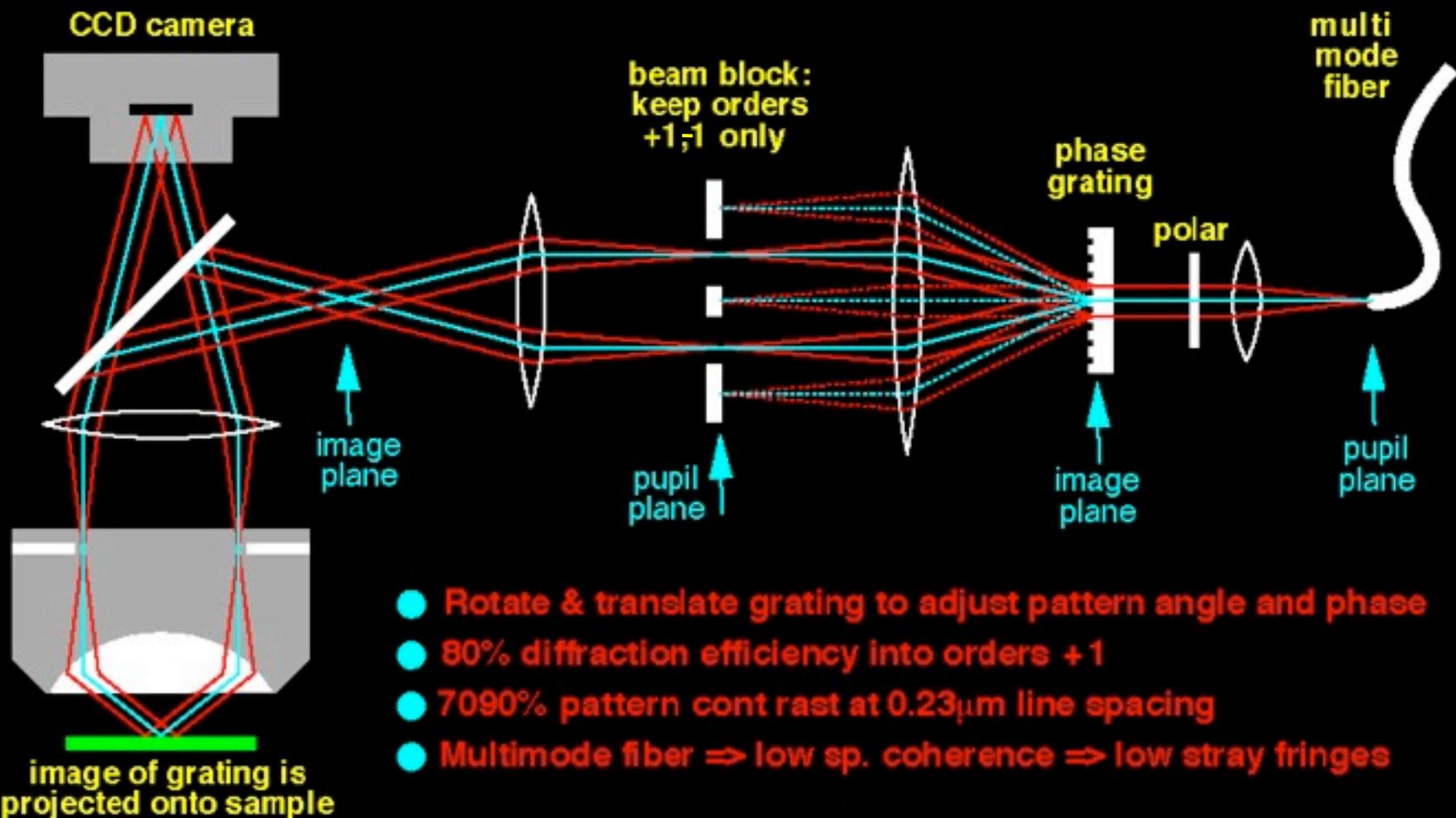
illumination intensity in real space



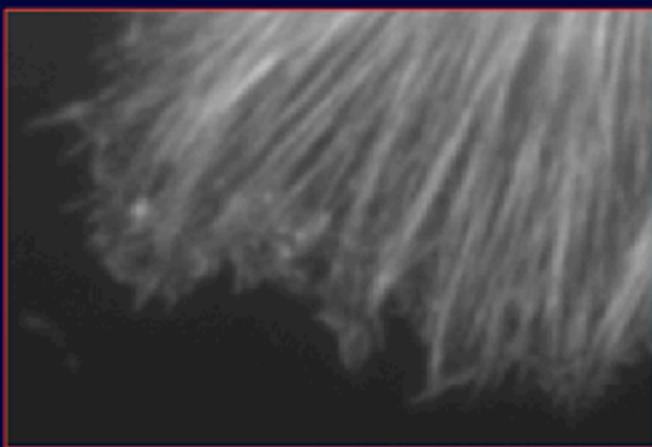
Observable Region Structured Illumination (3 orientations)



Structured illumination apparatus

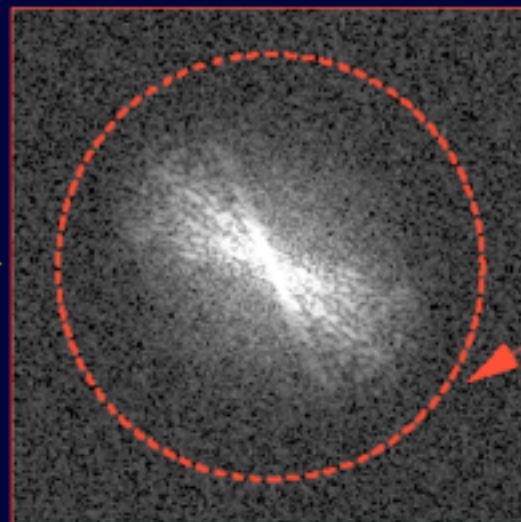


Real space images
(raw data)



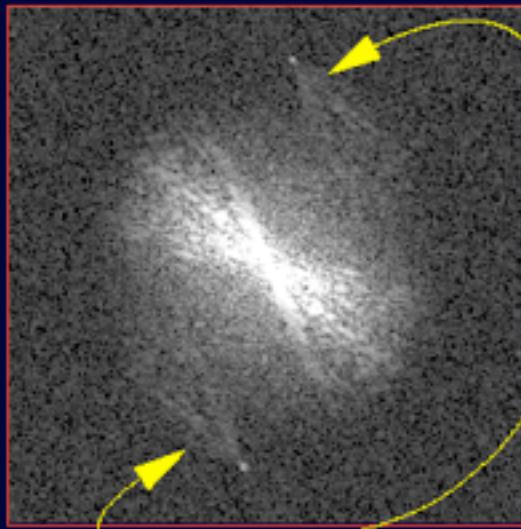
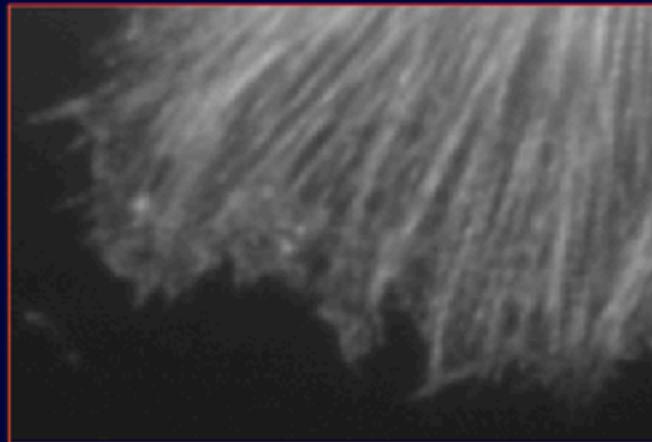
under
normal
illumination

Fourier transforms



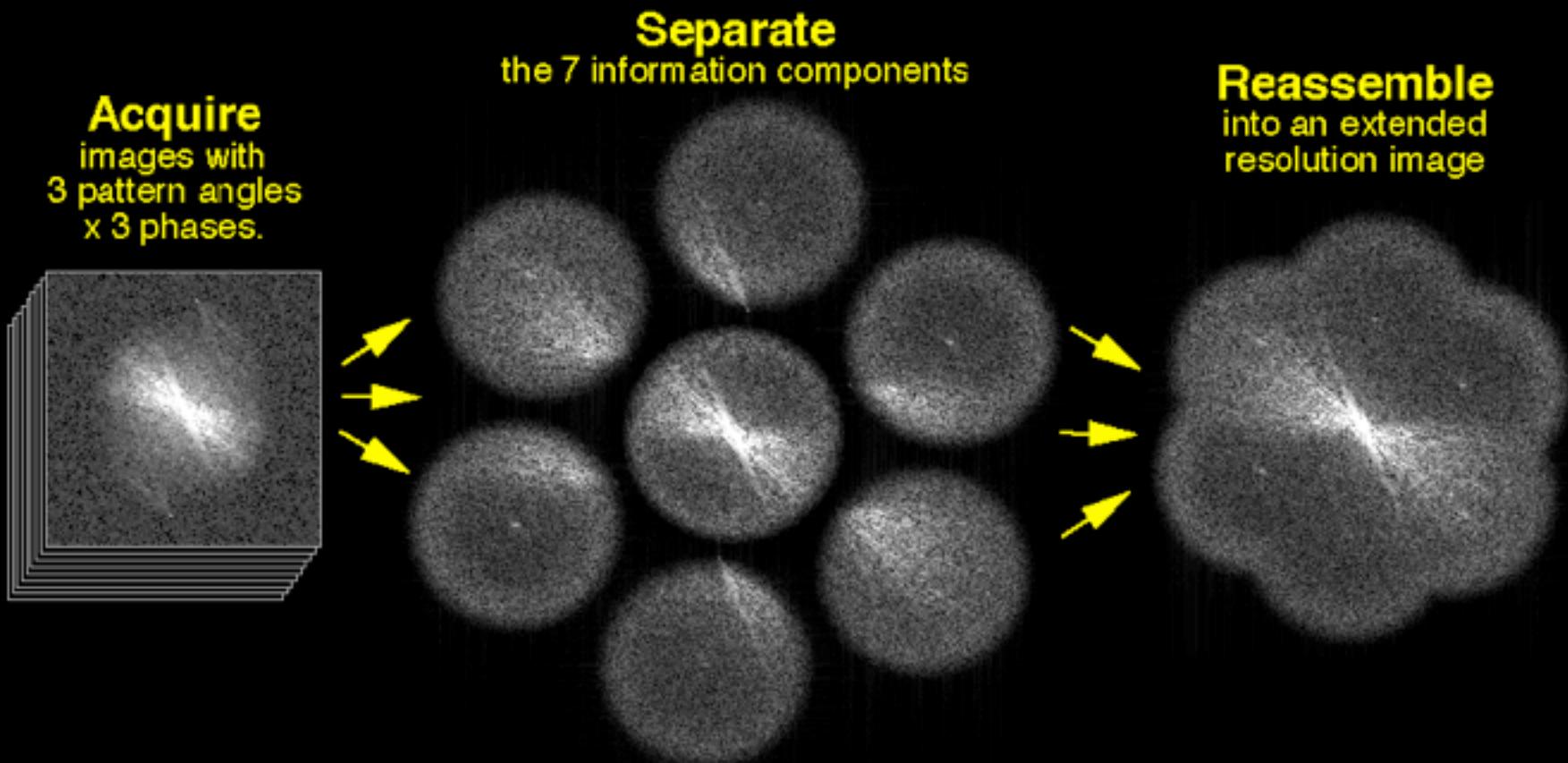
(theoretical
resolution
limit)

under
structured
illumination



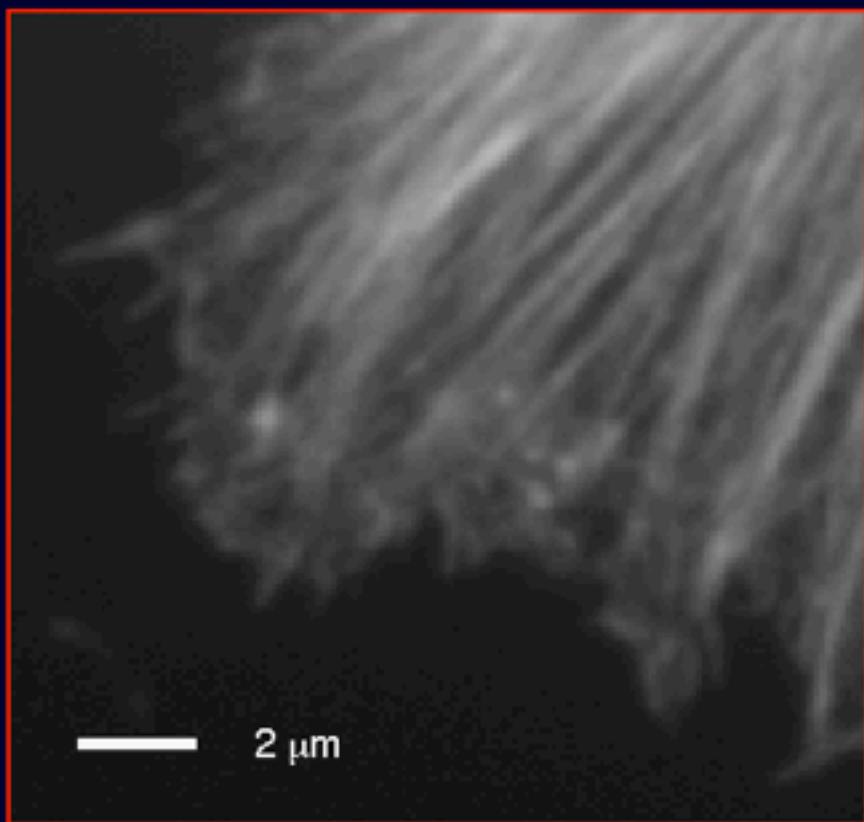
displaced
information
(i.e. moire
fringes)

Reconstruction in reciprocal space

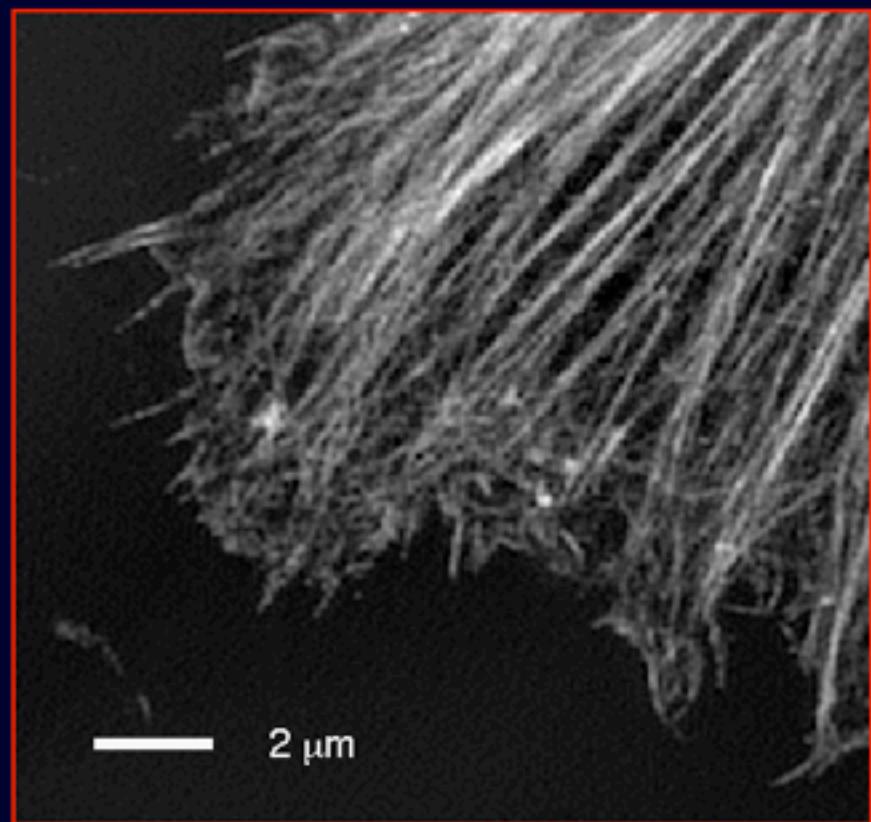


Resolution comparison Actin in a HeLa cell

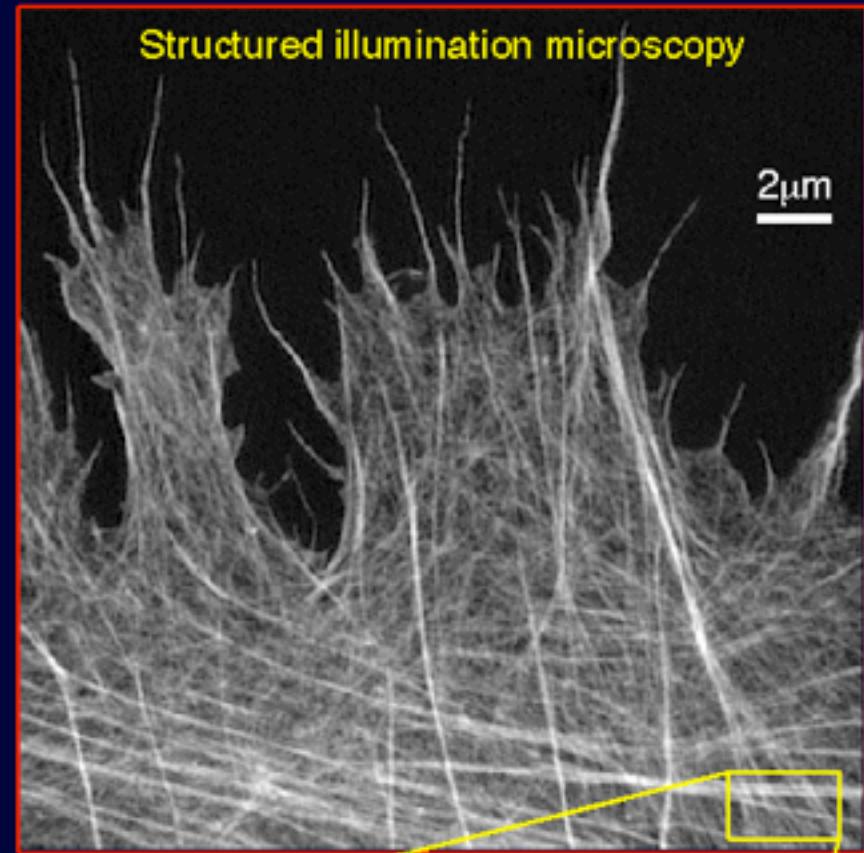
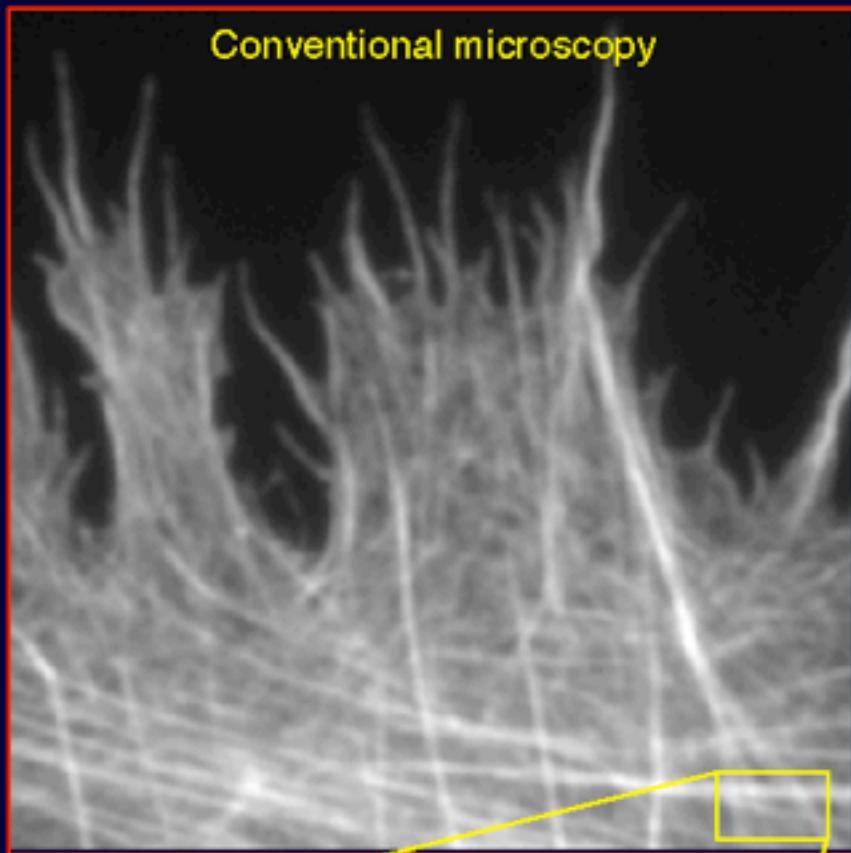
Conventional microscopy



Structured illumination
final reconstruction

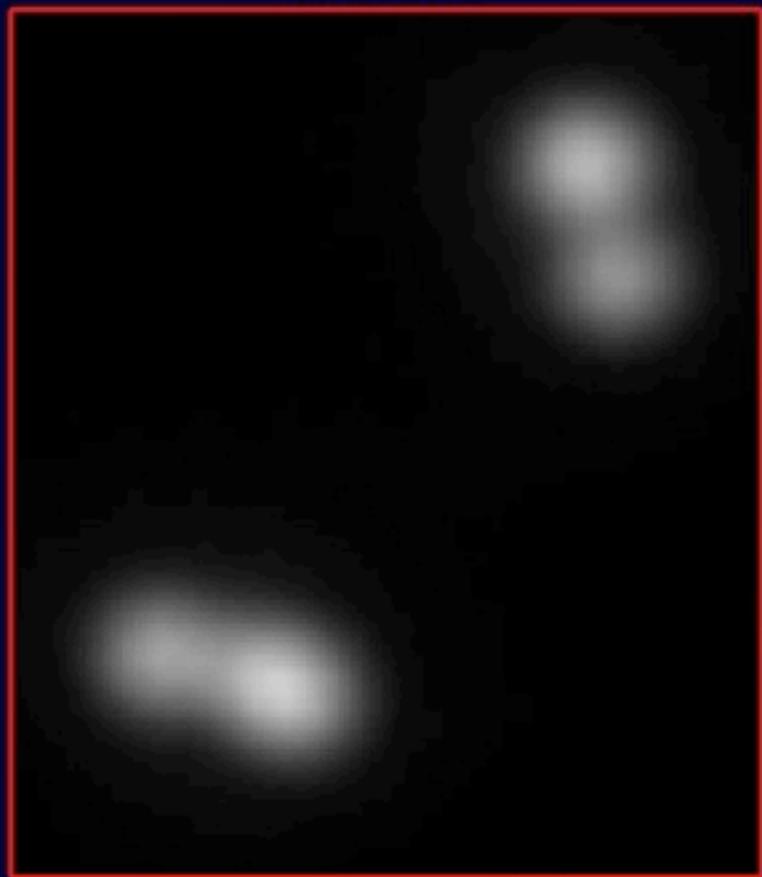


Actin in a HeLa cell

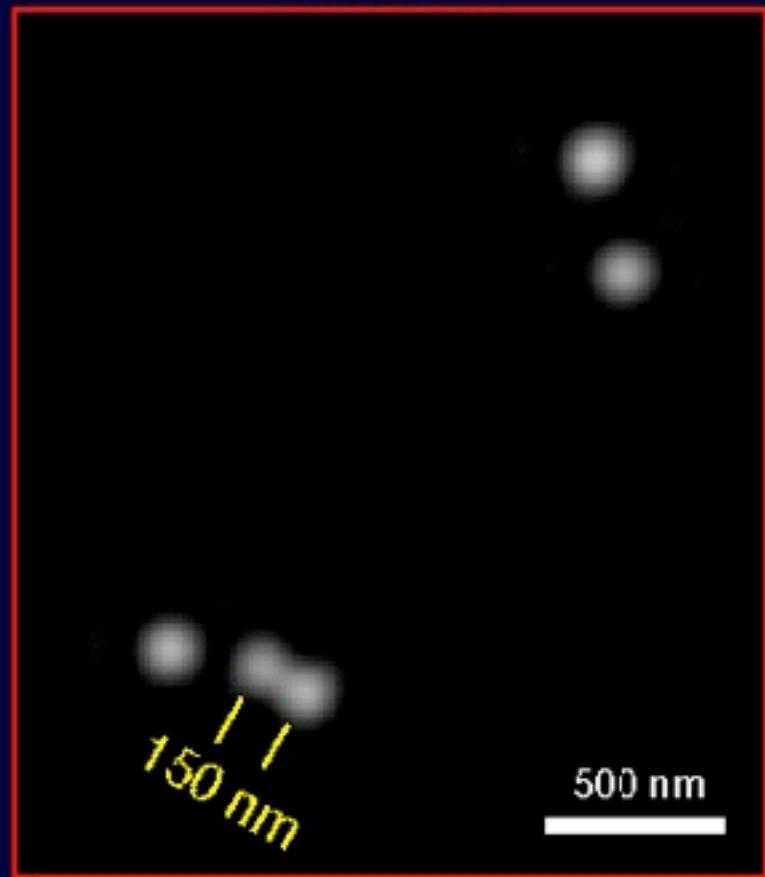


120 nm microspheres

Conventional
microscopy

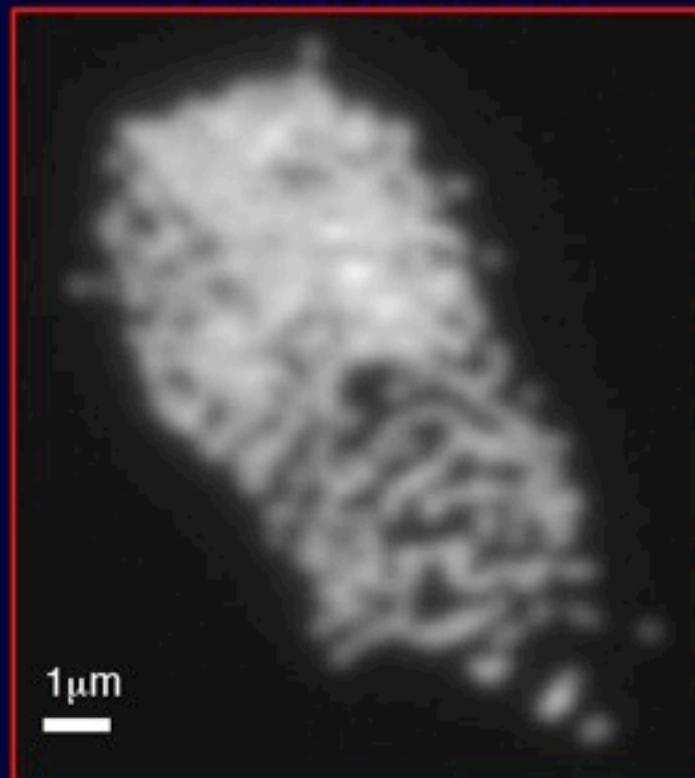


Structured illumination
microscopy

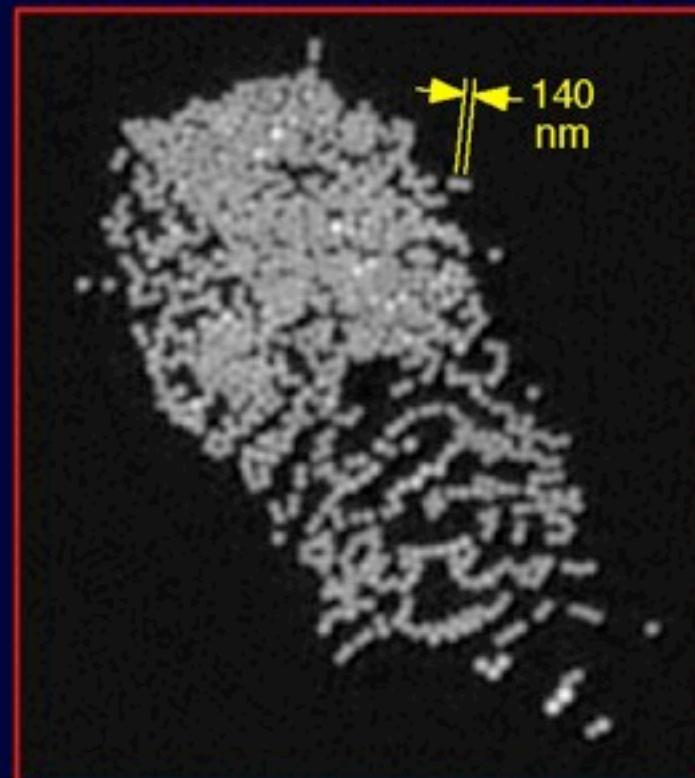


120 nm microspheres

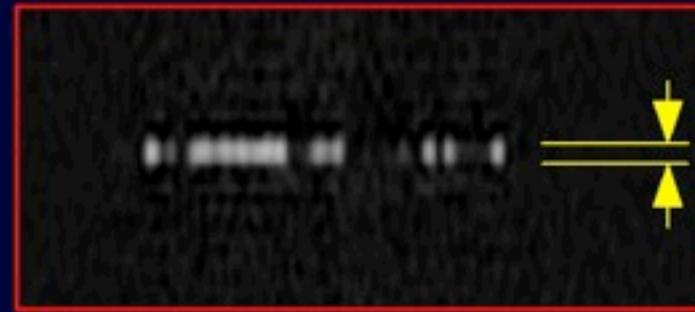
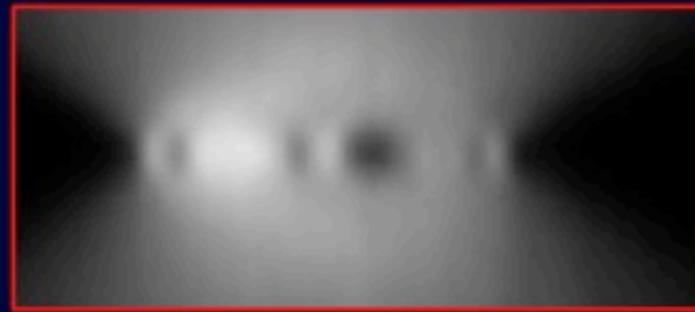
Conventional microscopy

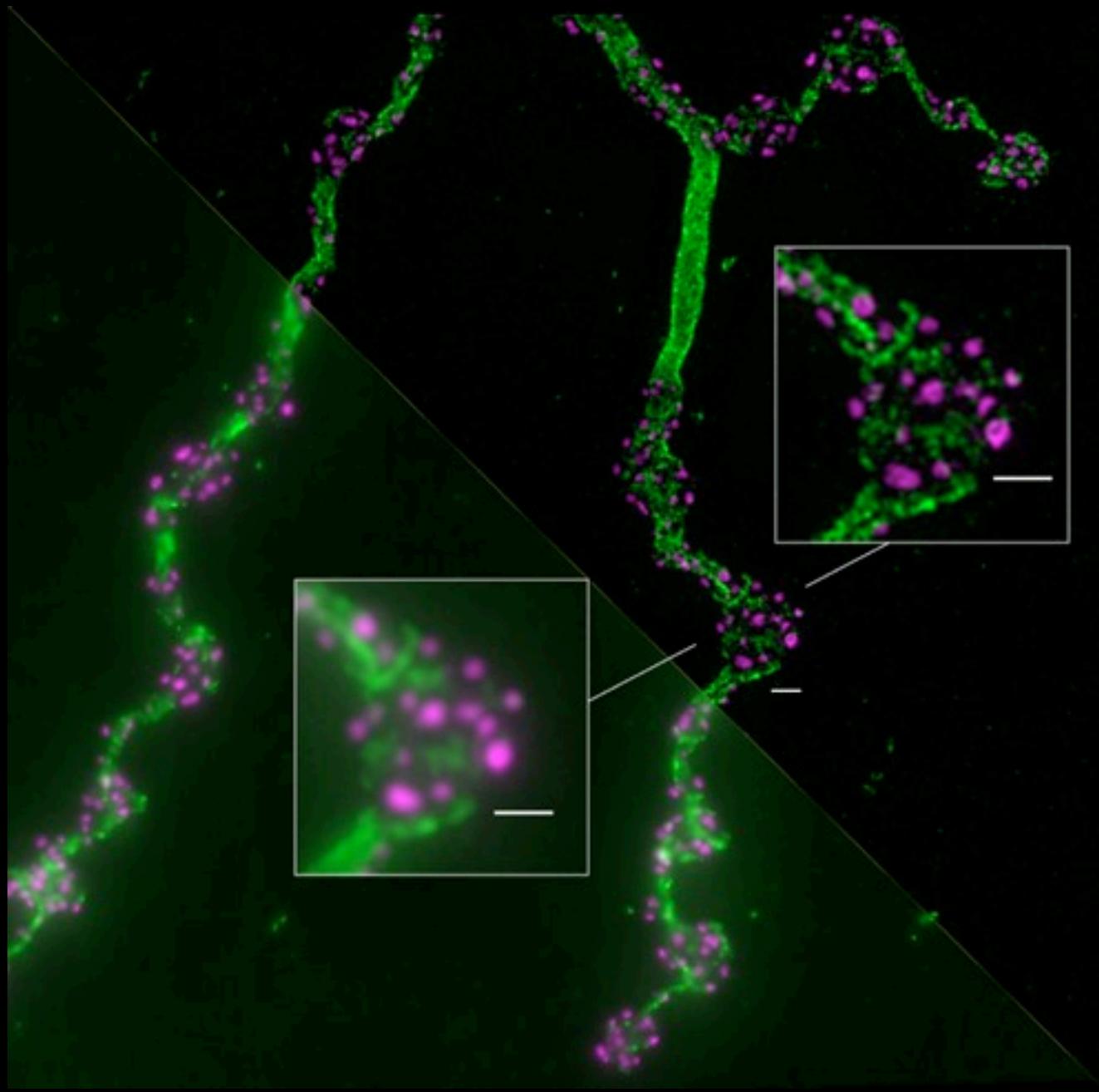


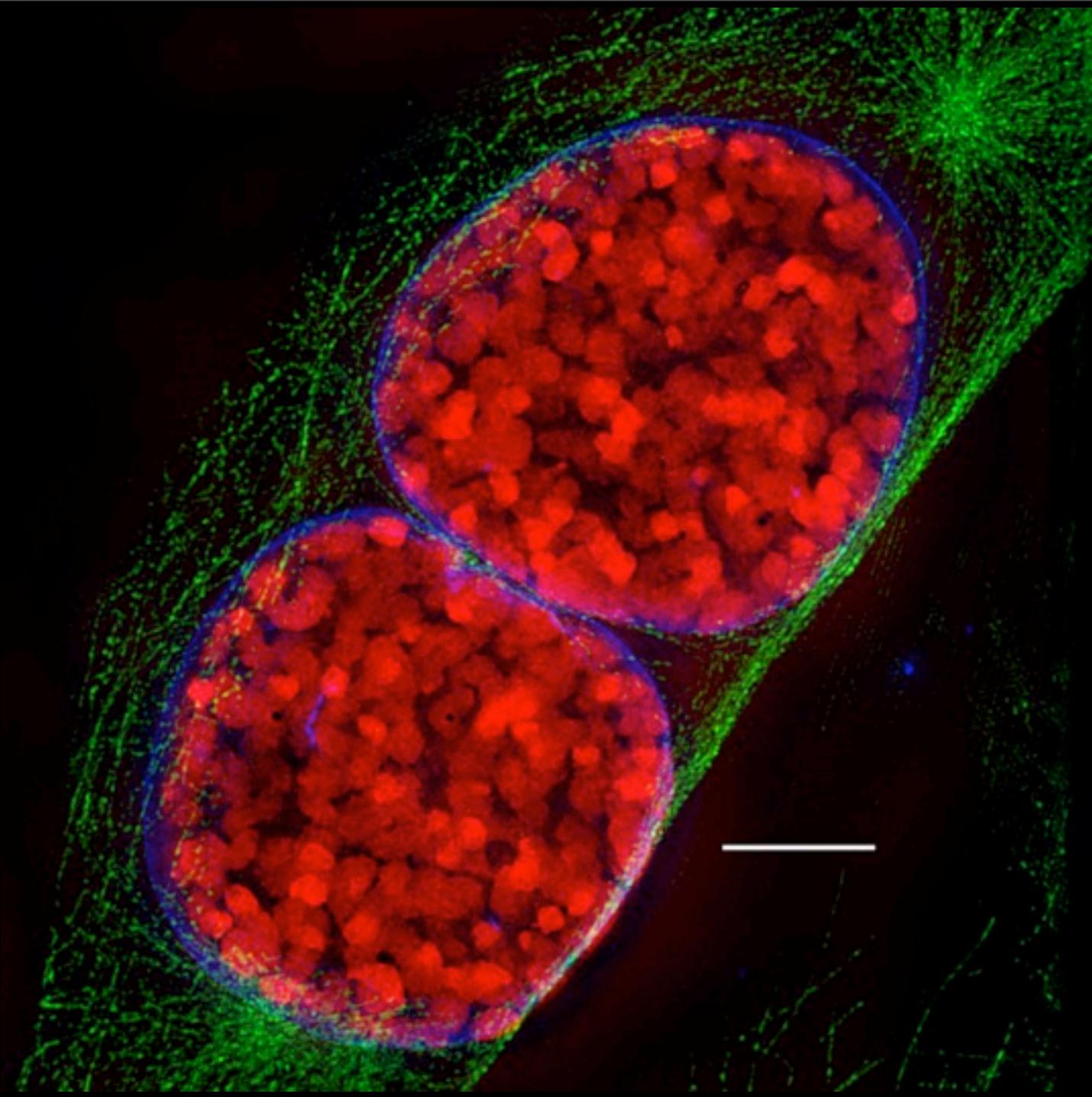
Structured illumination microscopy



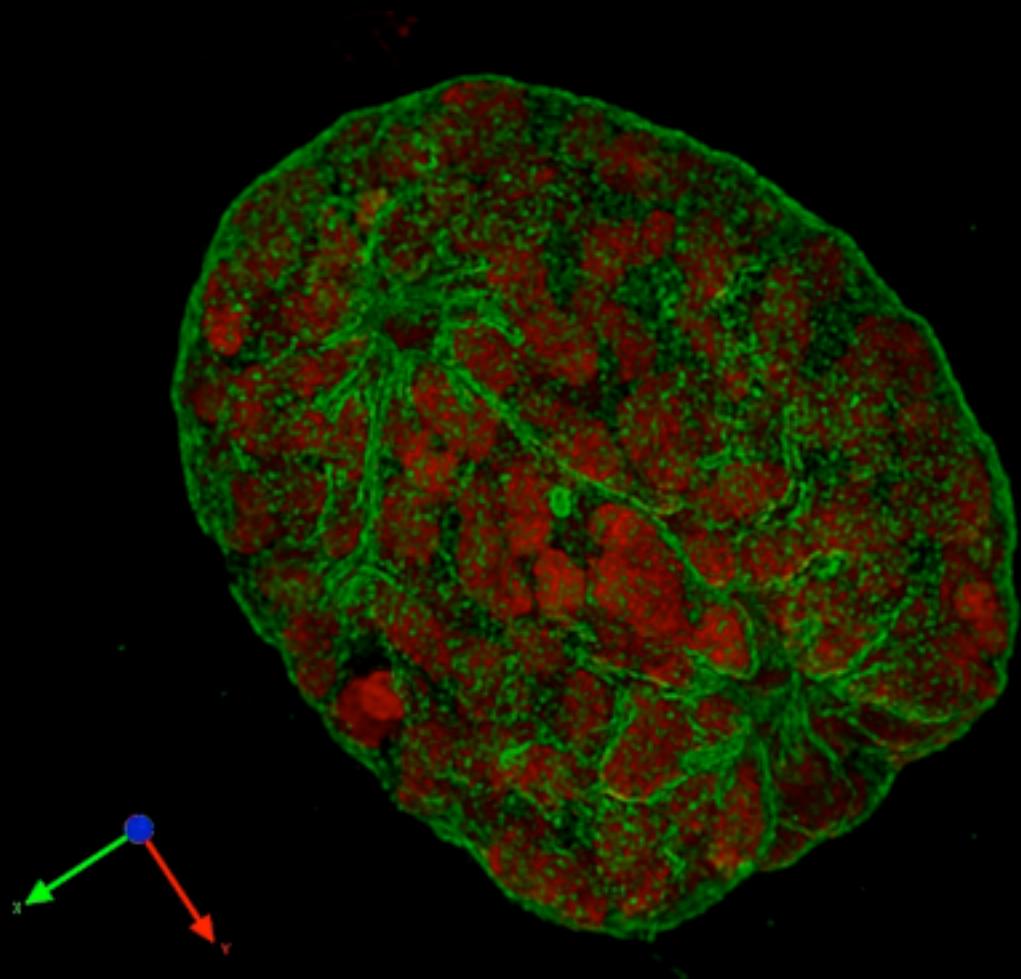
XZ



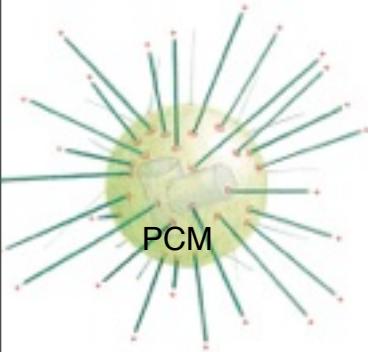




Thursday, May 9, 13

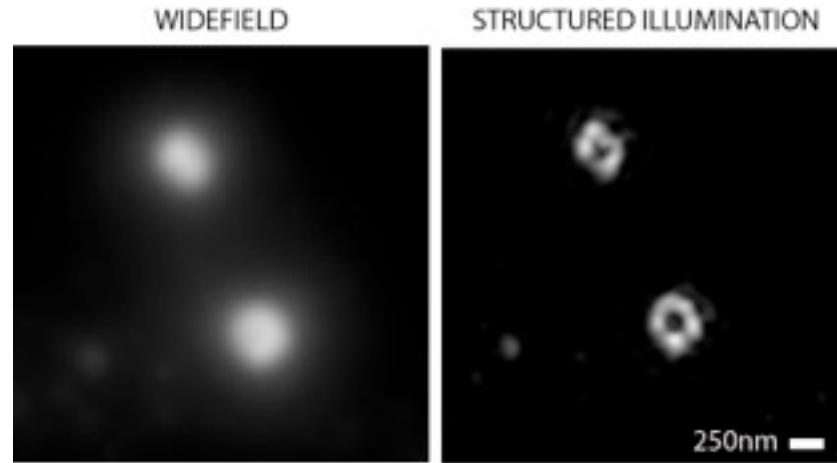


PCM long considered amorphous (decades of EM)



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

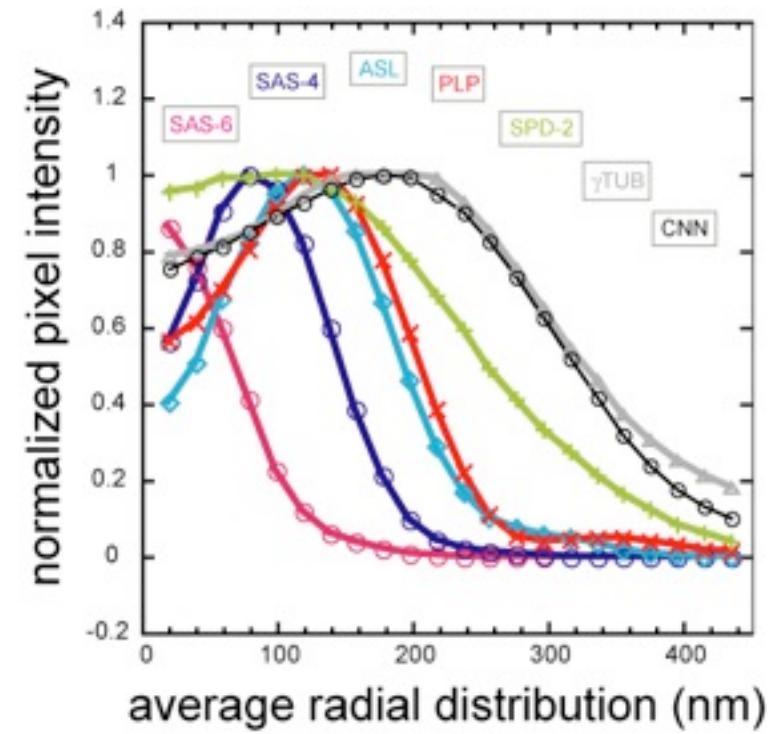
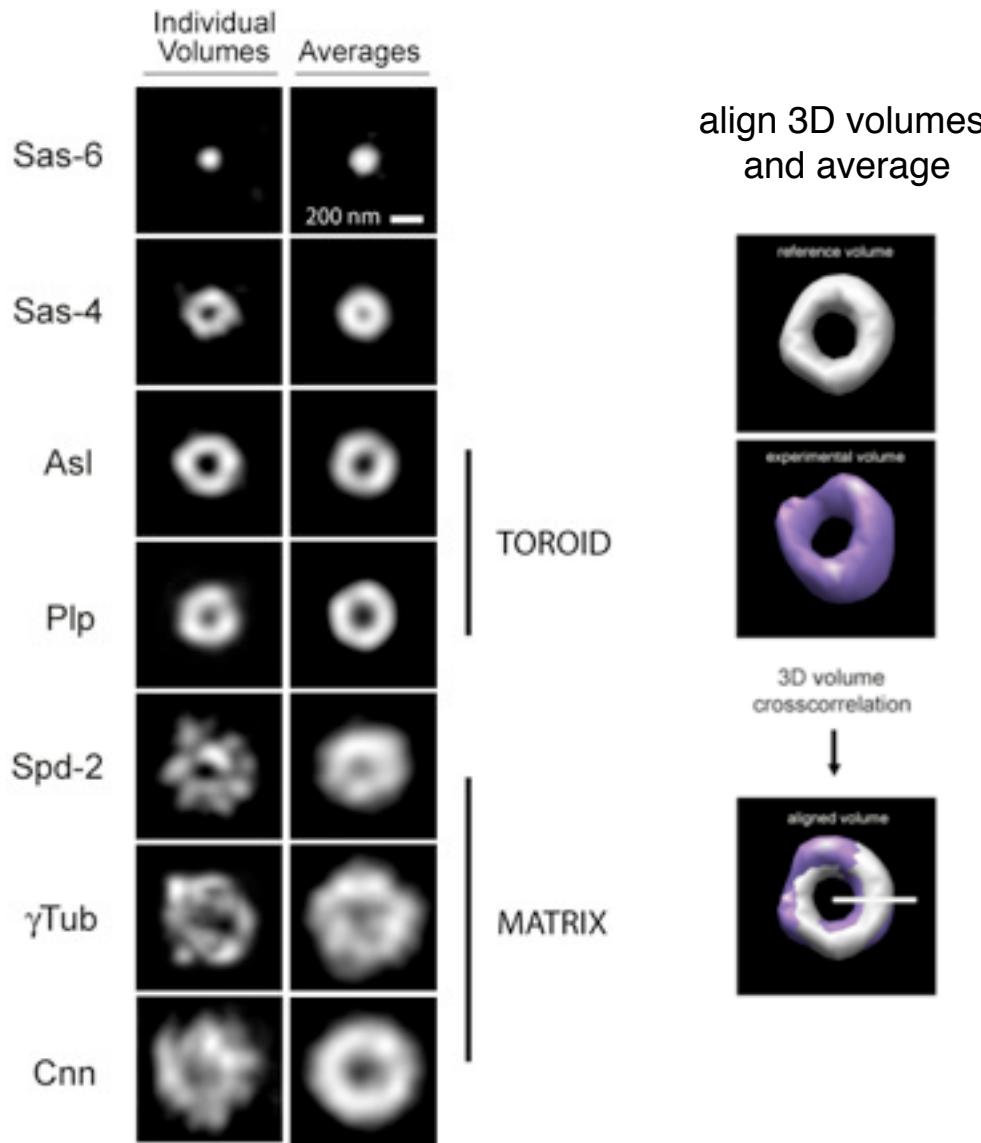
=> only tractable with sub-diffraction microscopy



combination of SIM/STORM provides maximal confidence/resolution

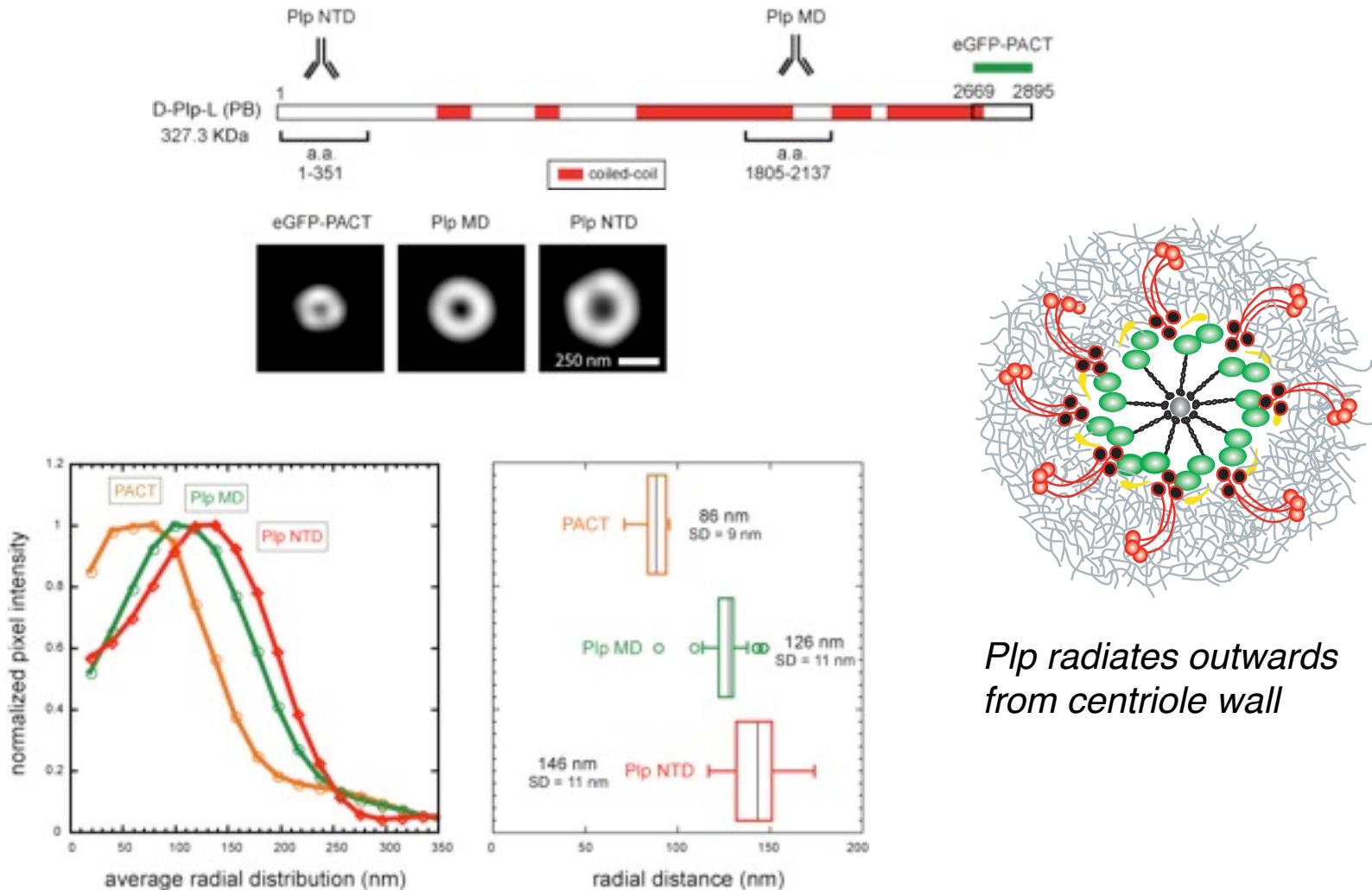
V. Mennella

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



V. Mennella, B. Keszthelyi

What is the molecular architecture of Plp?

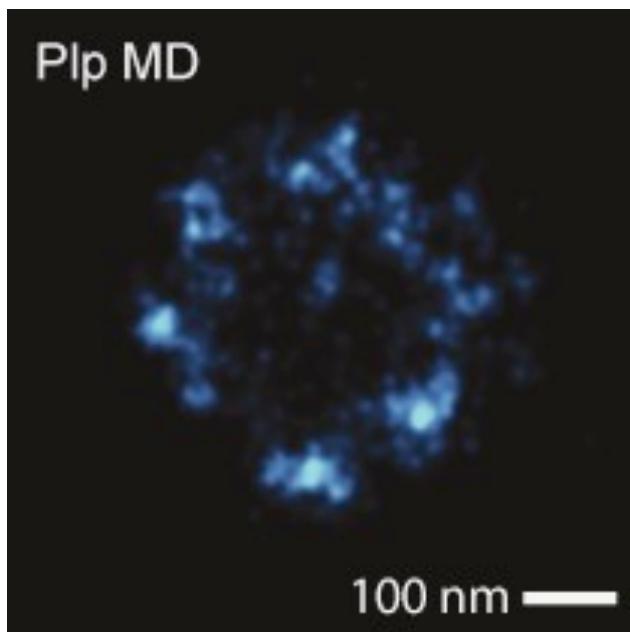


Plp radiates outwards from centriole wall

Molecular Structural information in cells!

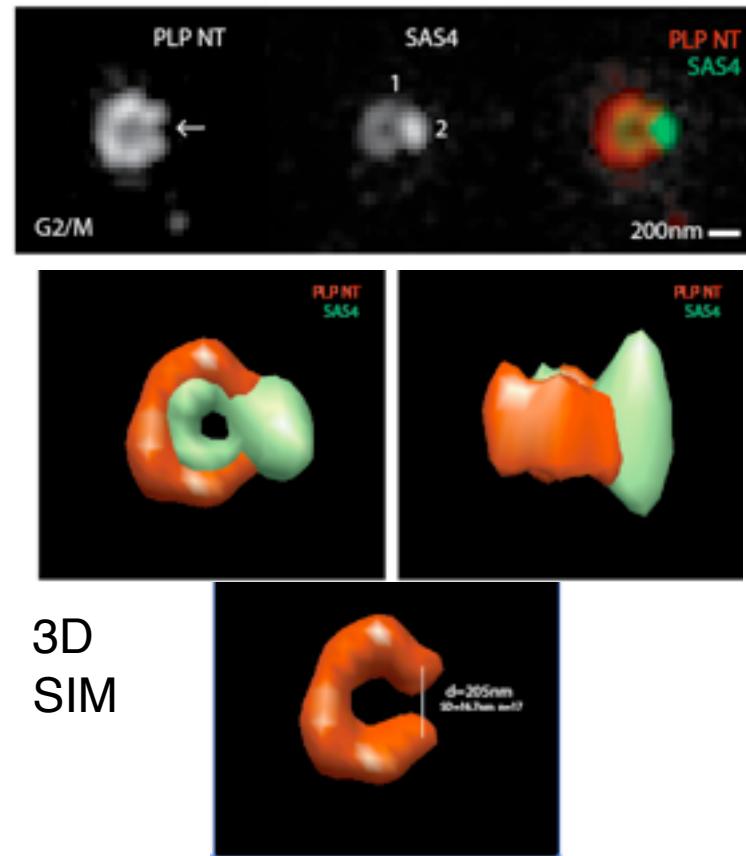
V. Mennella

PLP organized in symmetrical clusters that open for new procentriole



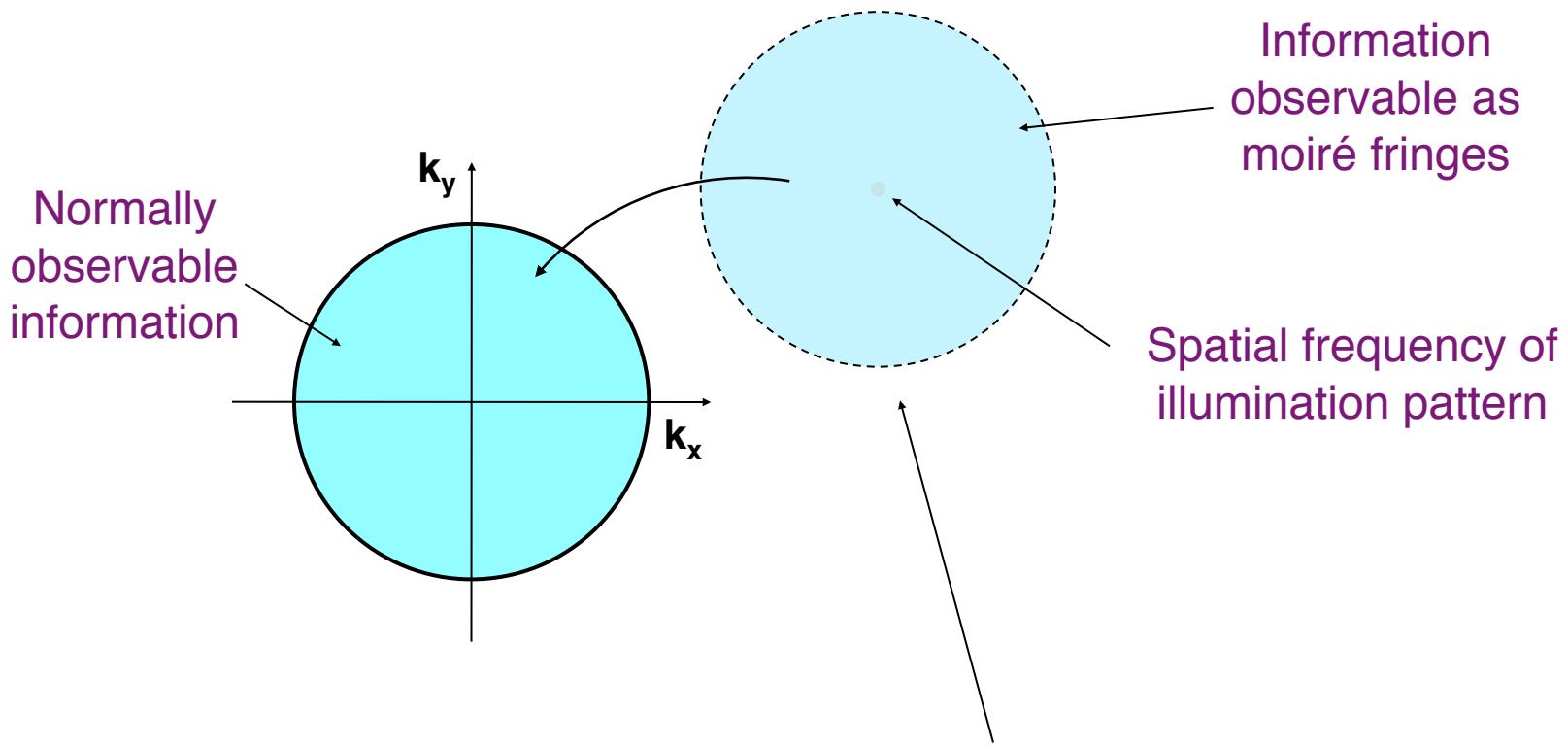
STORM ~ 9 fold symmetry

XYZ resolution:
32nm,37nm,59nm



V. Mennella, B. Huang

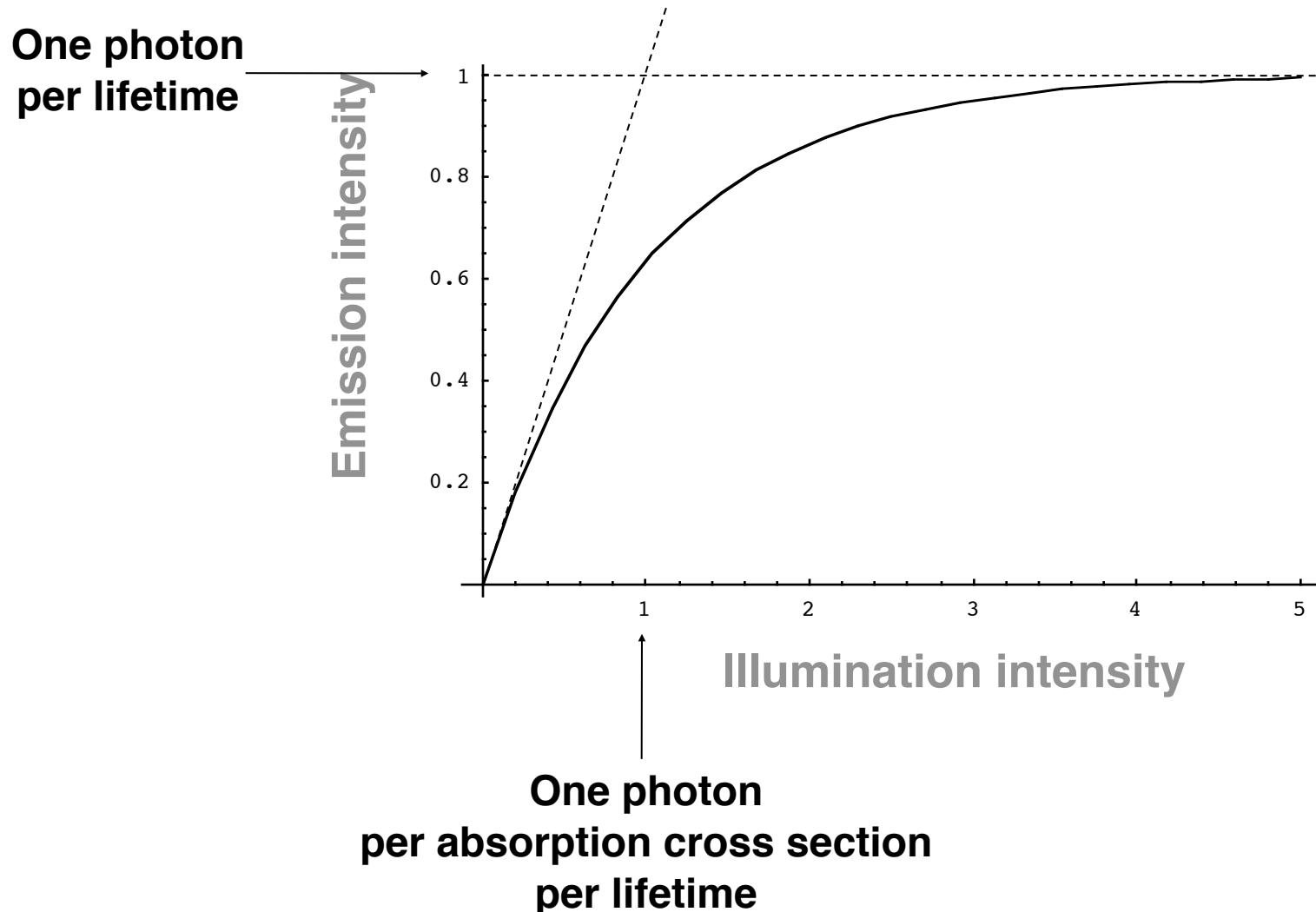
What are the limits of Structured Illumination?



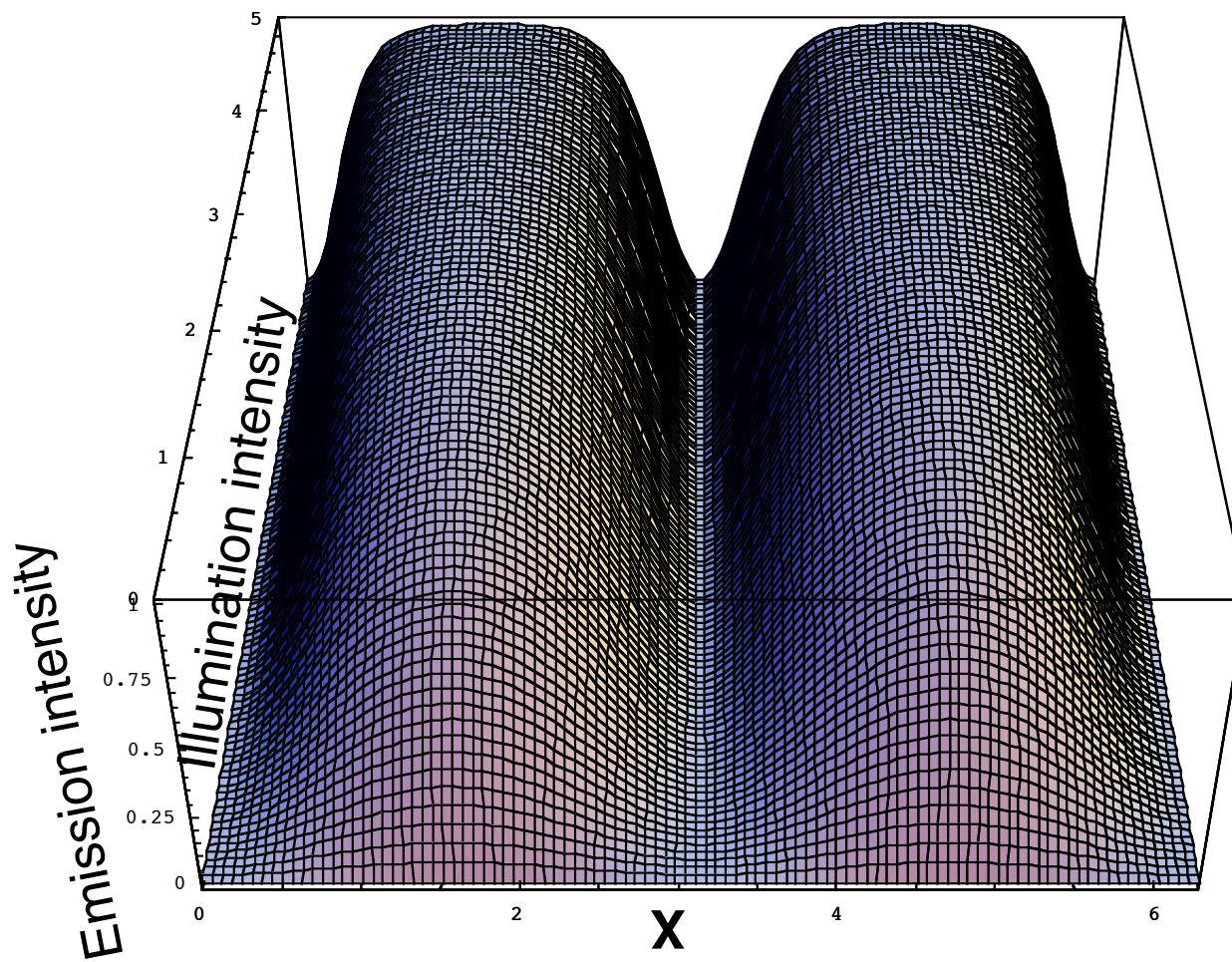
- Linear theory says this is impossible
- How about exploiting non-linear processes?

A simple source of nonlinearity: saturation

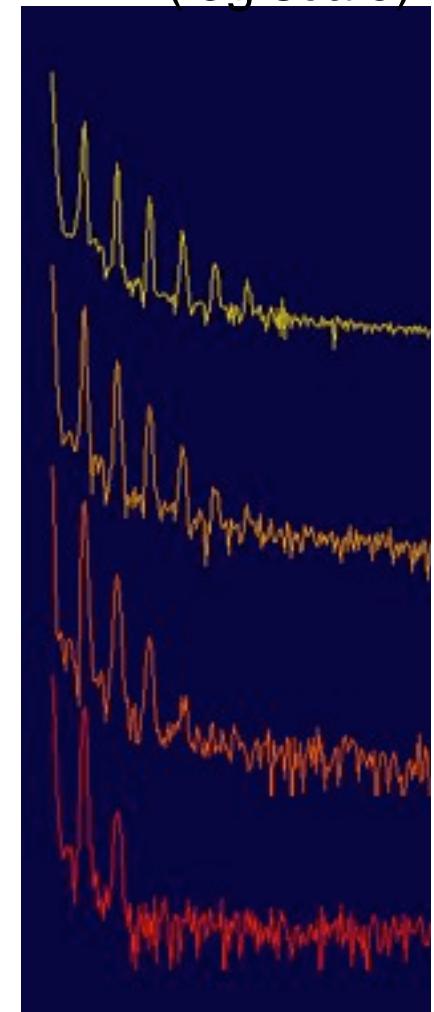
(R. Heintzmann Max-Planck Gottingen)



Saturated Structured Illumination



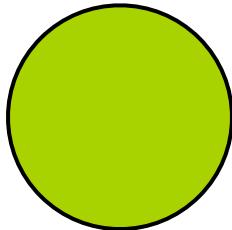
FT (log scale)



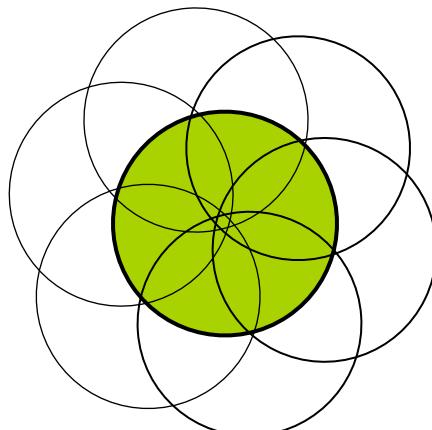
Resolution extension by nonlinear structured illumination

Effective observable regions

Conventional
microscopy



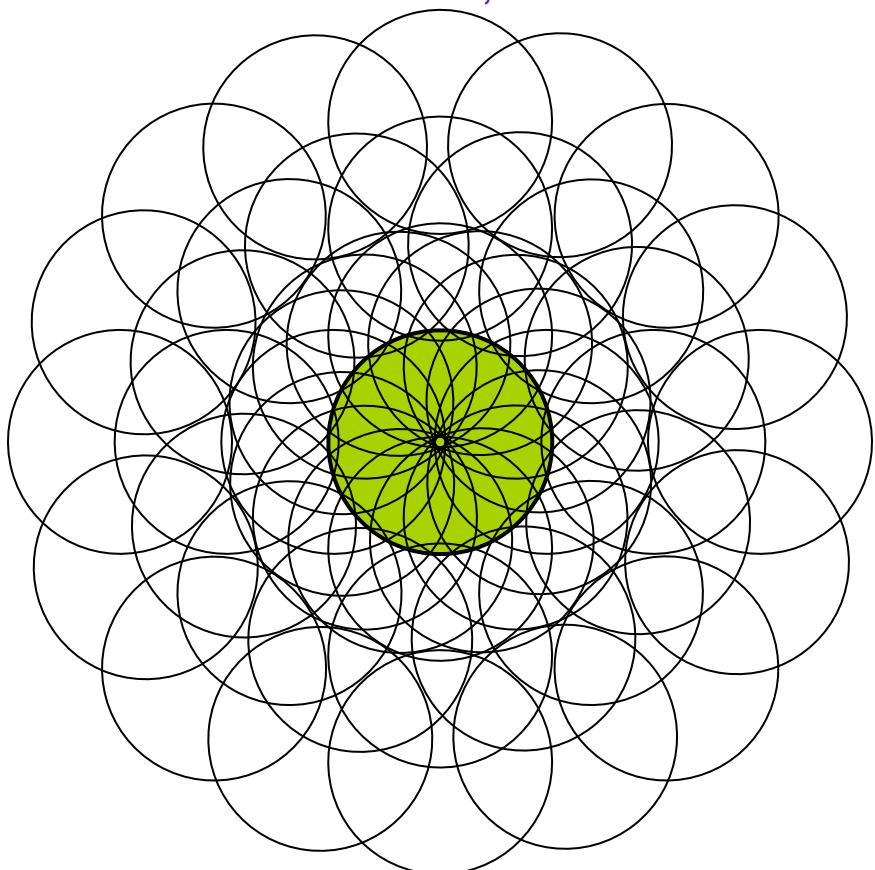
200 nm res.



100 nm res.

Linear
Structured
illumination
3 directions

Nonlinear structured illumination
2 new harmonics, 8 directions



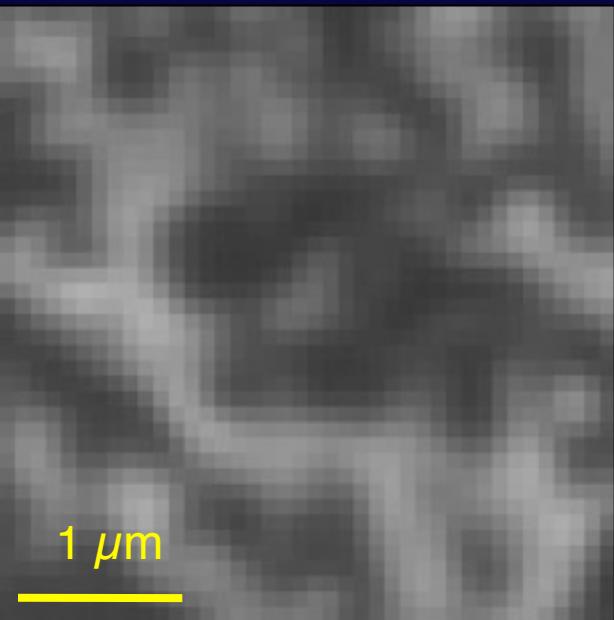
50 nm res.

Non-linear structured illumination

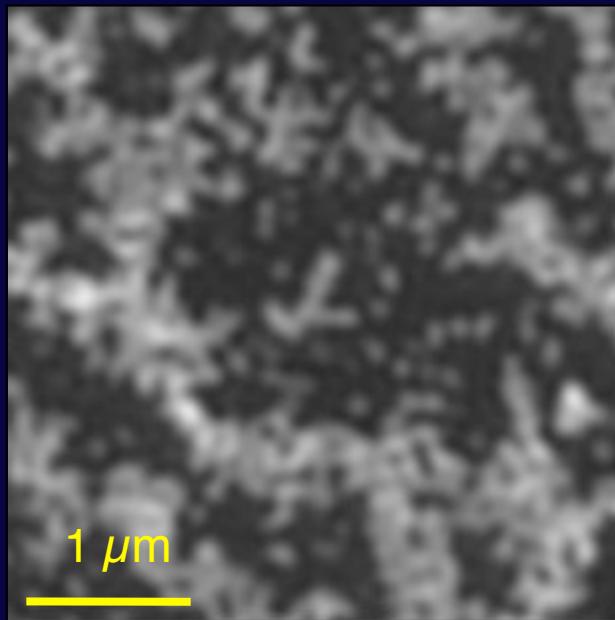
Conventional
microscopy

Linear
structured
illumination

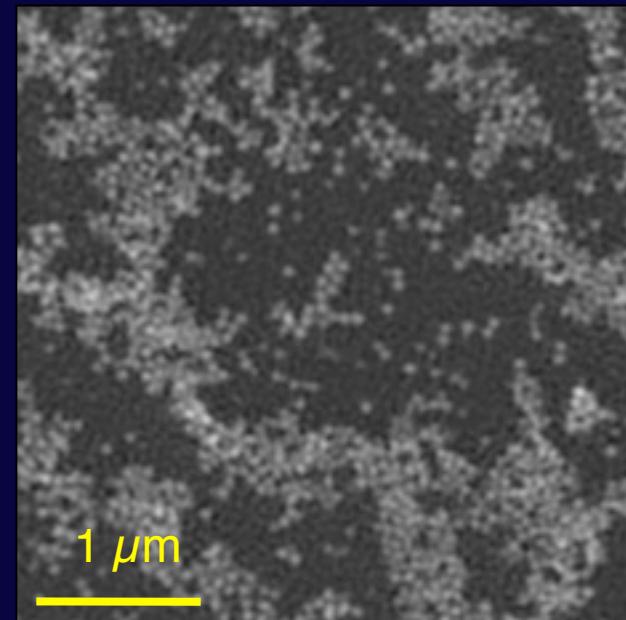
Saturated
structured
illumination



**~250 nm resolution
(diffraction limit)**



~120 nm resolution



46 nm resolution!

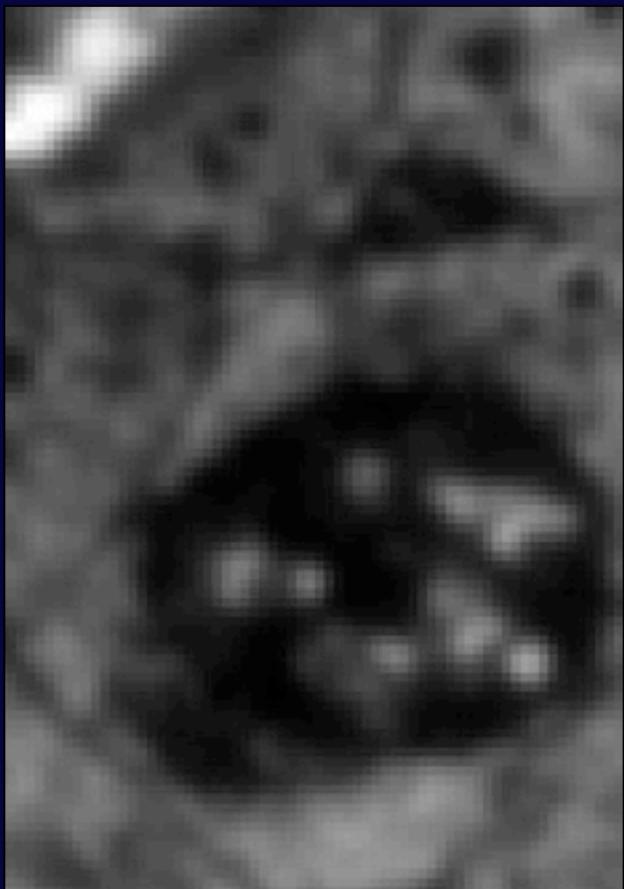
50 nm microspheres

Drosophila embryo section DNA/RNA stain

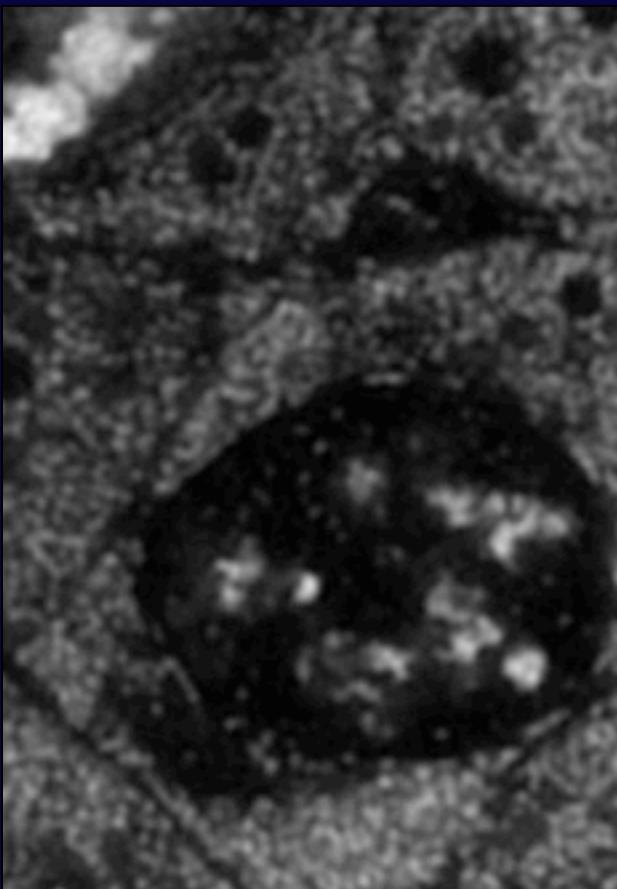
Conventional
microscopy

Linear
structured
illumination

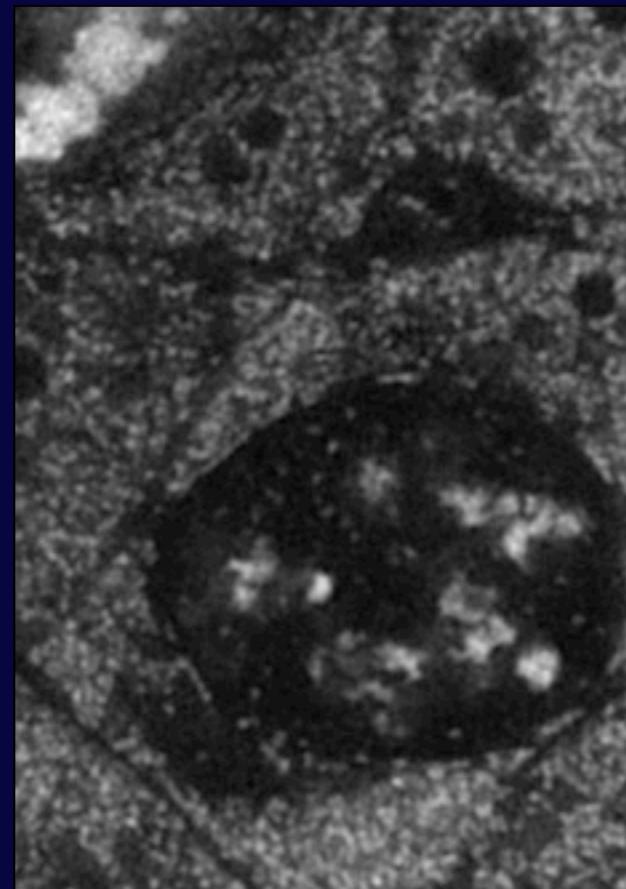
Saturated
structured
illumination
(1 new harmonic)



Min. FWHM \approx 280 nm



Min. FWHM \approx 110 nm



Min. FWHM \approx 80 nm

Going beyond the diffraction limit: more light collection angles

We know:

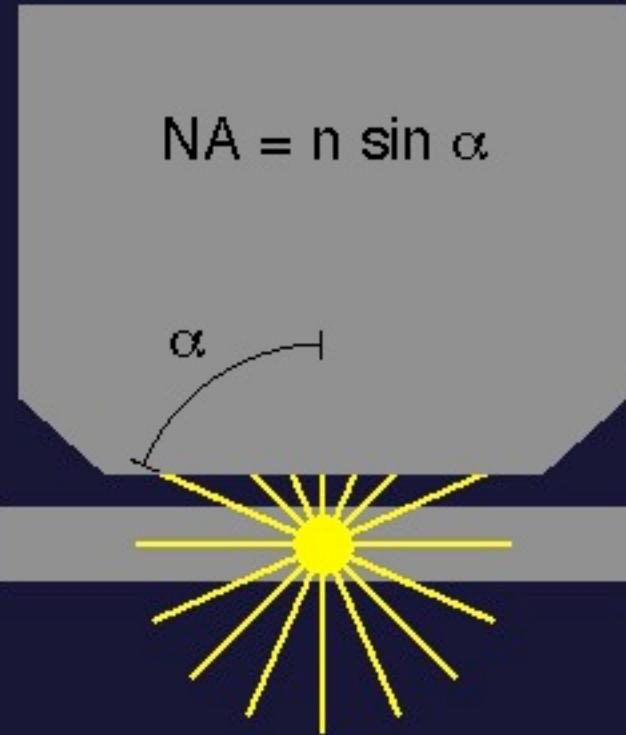
Higher NA



Gathering light over
larger set of angles

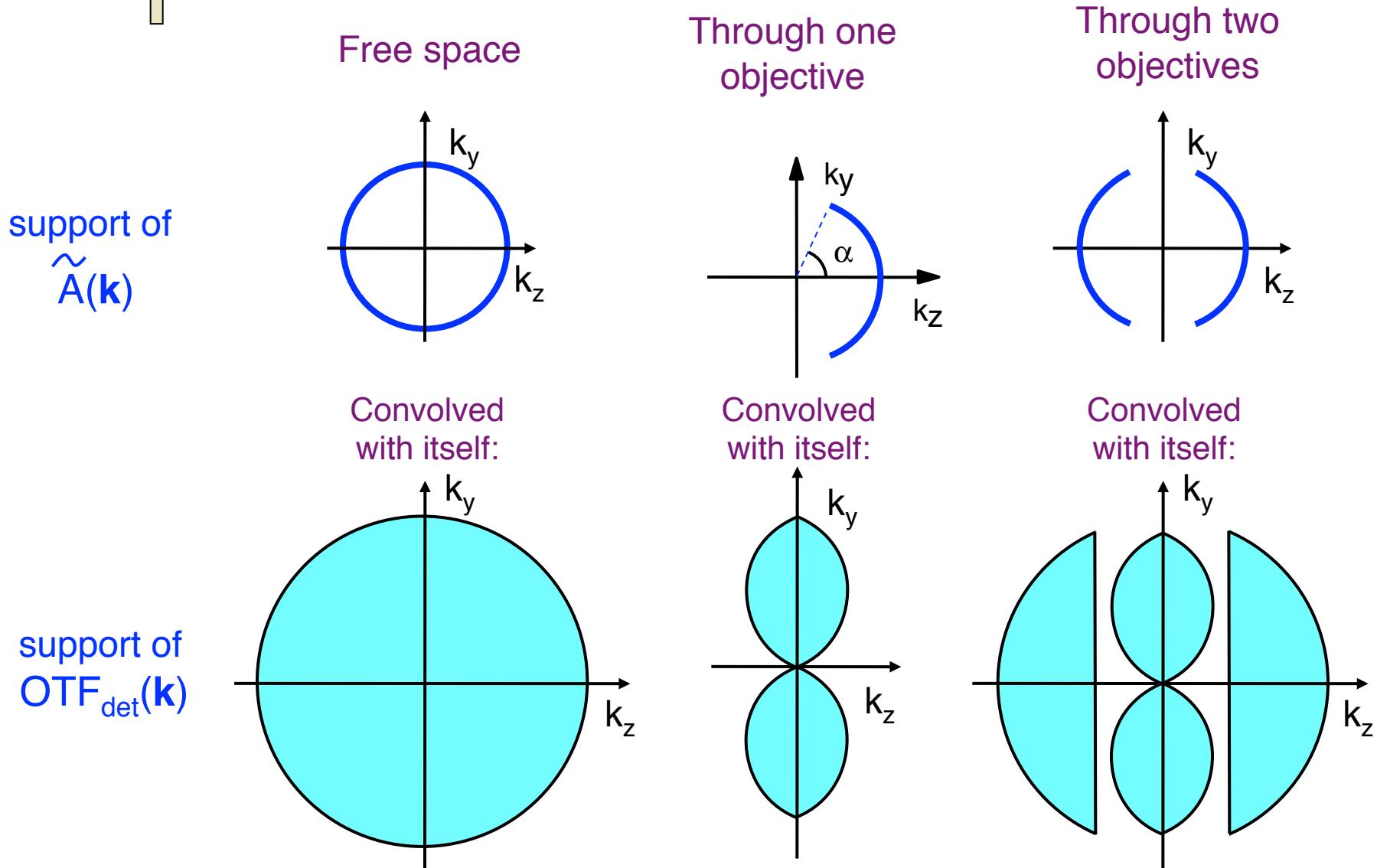
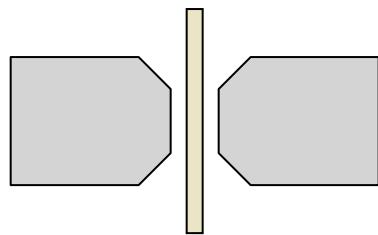


Higher resolution

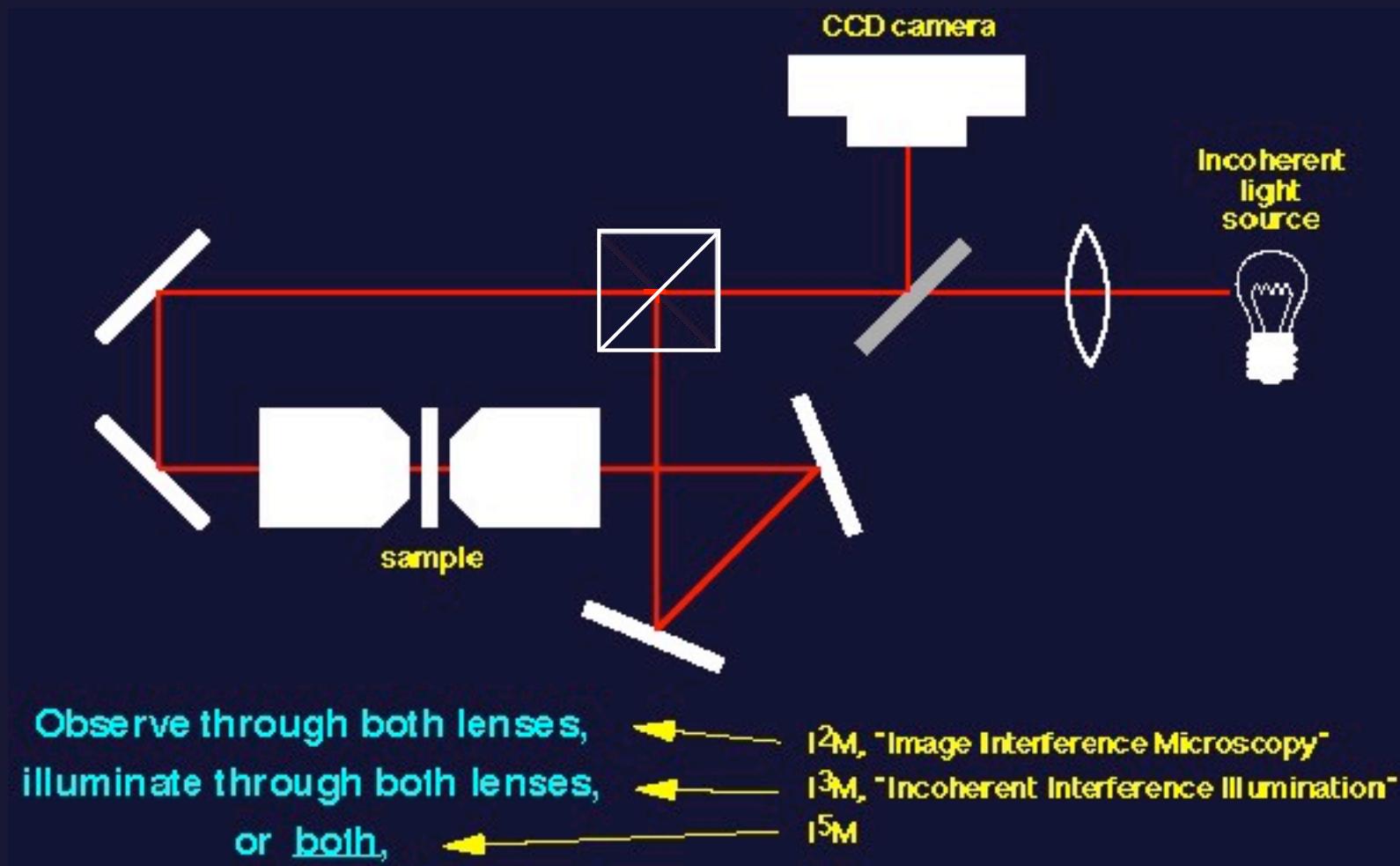


...So what about gathering the light
emitted toward the back side?

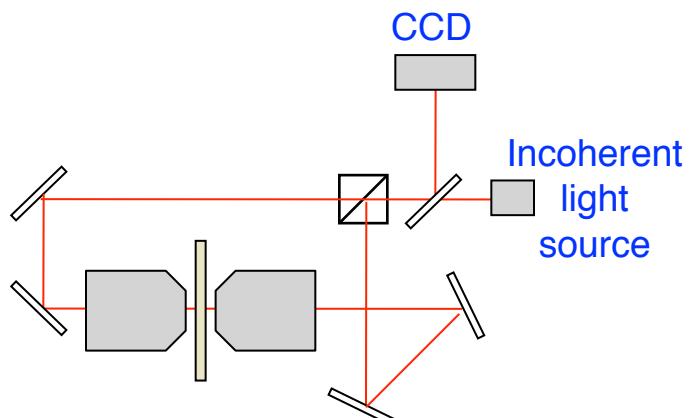
OTF when detecting through two lenses



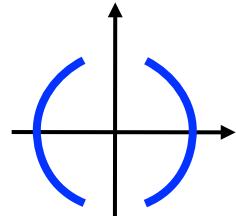
I5M concept



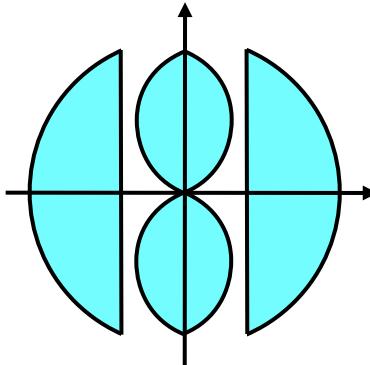
I₅M OTF



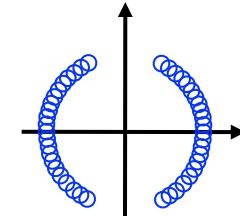
Detection angles



OTF_{det}



Illum. angles

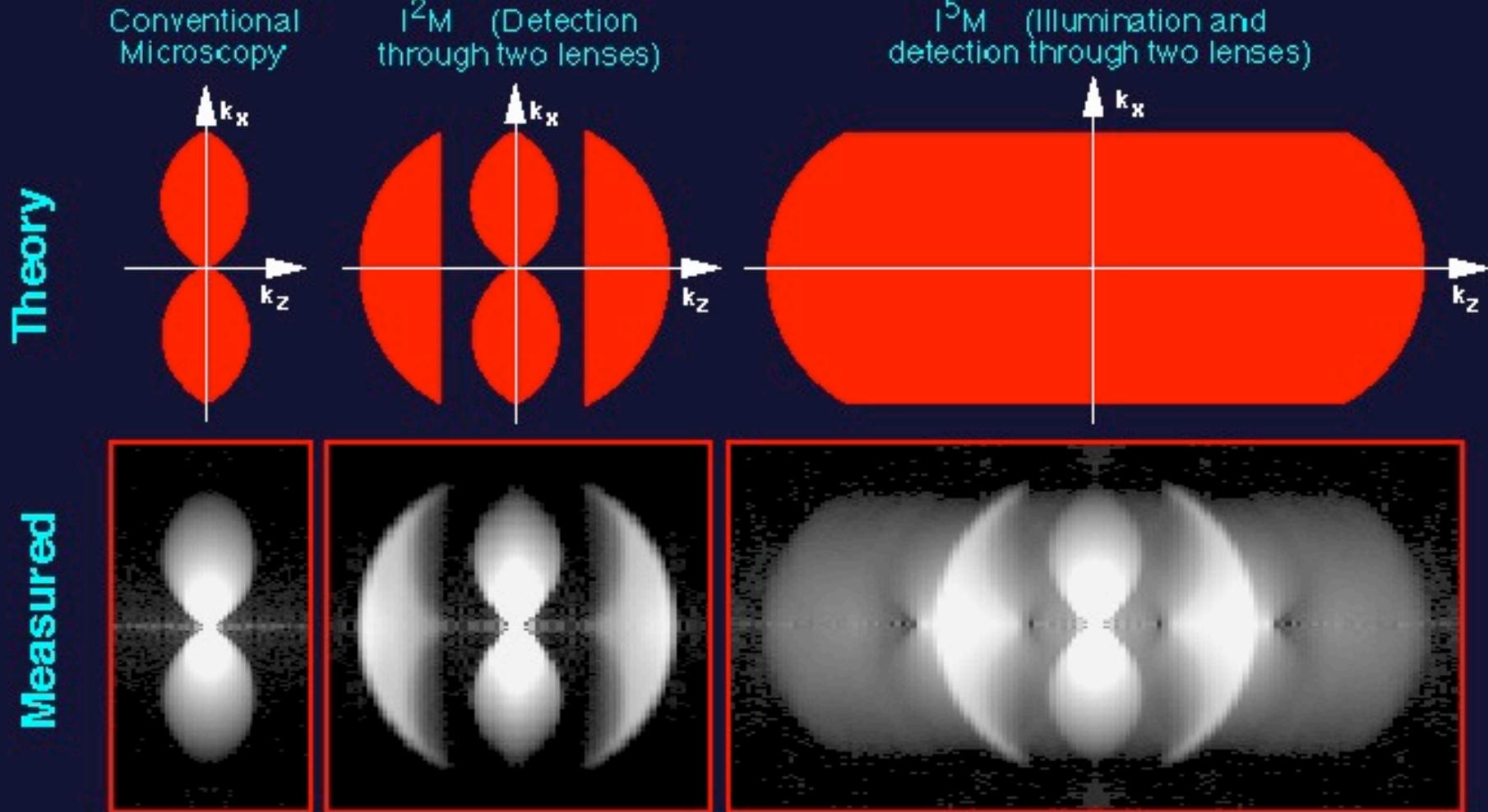


$\tilde{\text{illum}}$

$$\text{OTF}_{\text{eff}} = \text{OTF}_{\text{det}} \otimes \tilde{\text{illum}} = \text{OTF}_{\text{eff}}$$

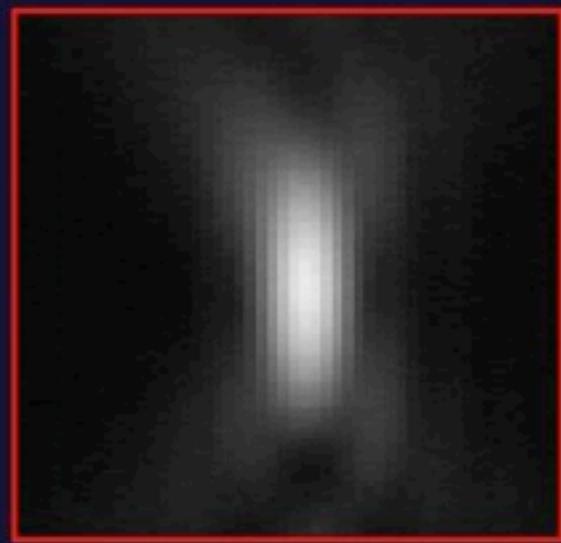
Diagram illustrating the calculation of the effective OTF (OTF_{eff}) as the product of the detection OTF (OTF_{det}) and the illumination function ($\tilde{\text{illum}}$). The resulting effective OTF is shown as a large cyan circle.

Observable Information [Optical Transfer Functions]

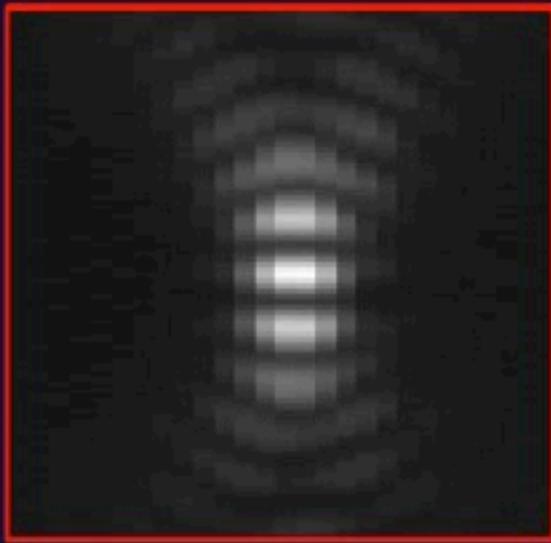


Deconvolution of bead data

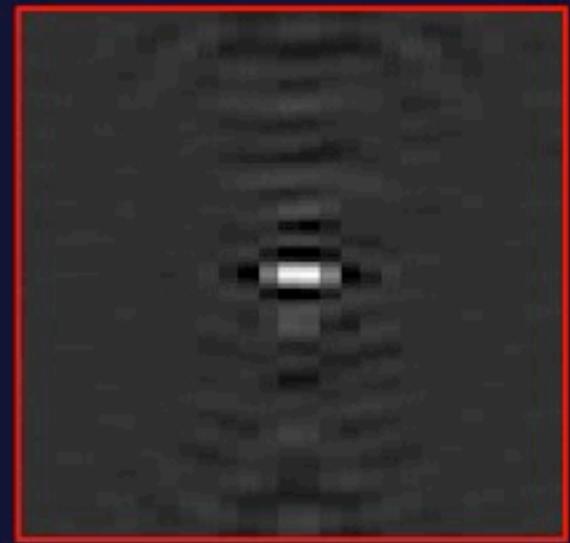
One lens



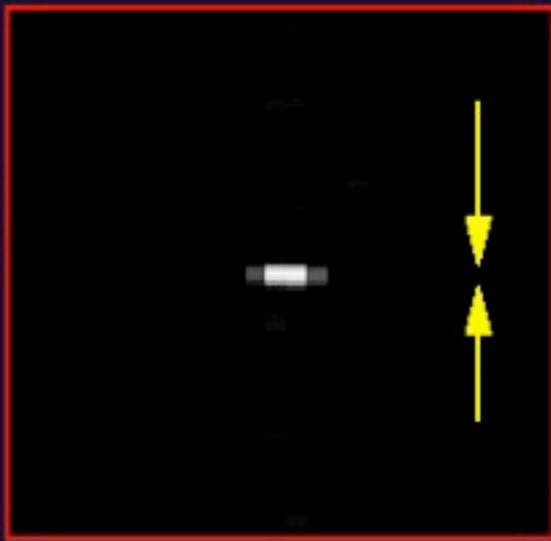
Two lens det. & illum.



After Wiener filtering



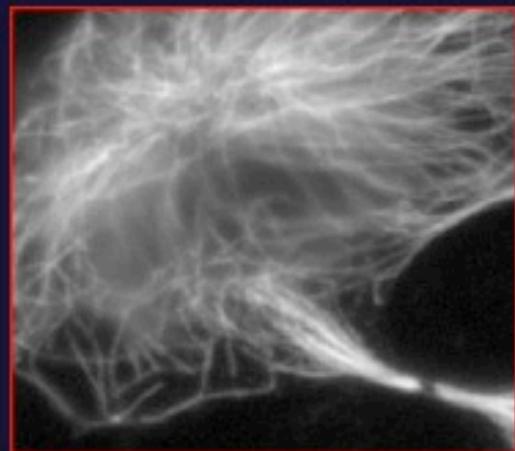
After iterative
deconvolution
using
positivity
constraint



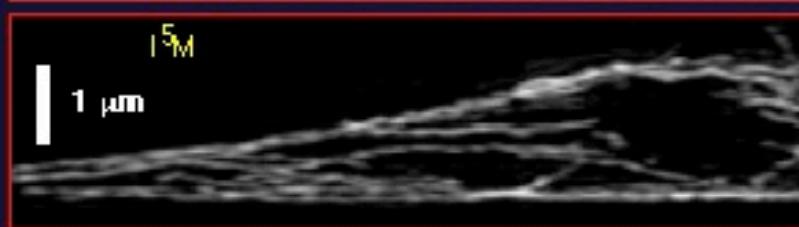
FWHM
~70nm

Microtubules in a recently divided PtK2 cell

XY
view
of area.

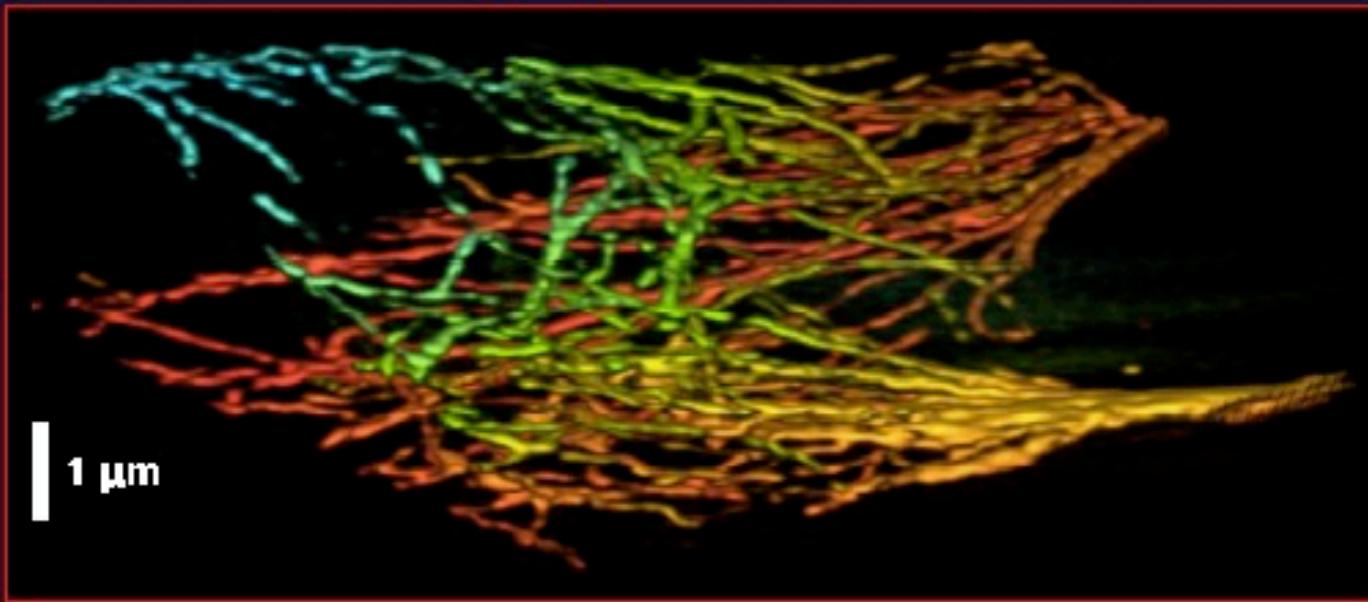


conventional microscopy
+ deconvolution

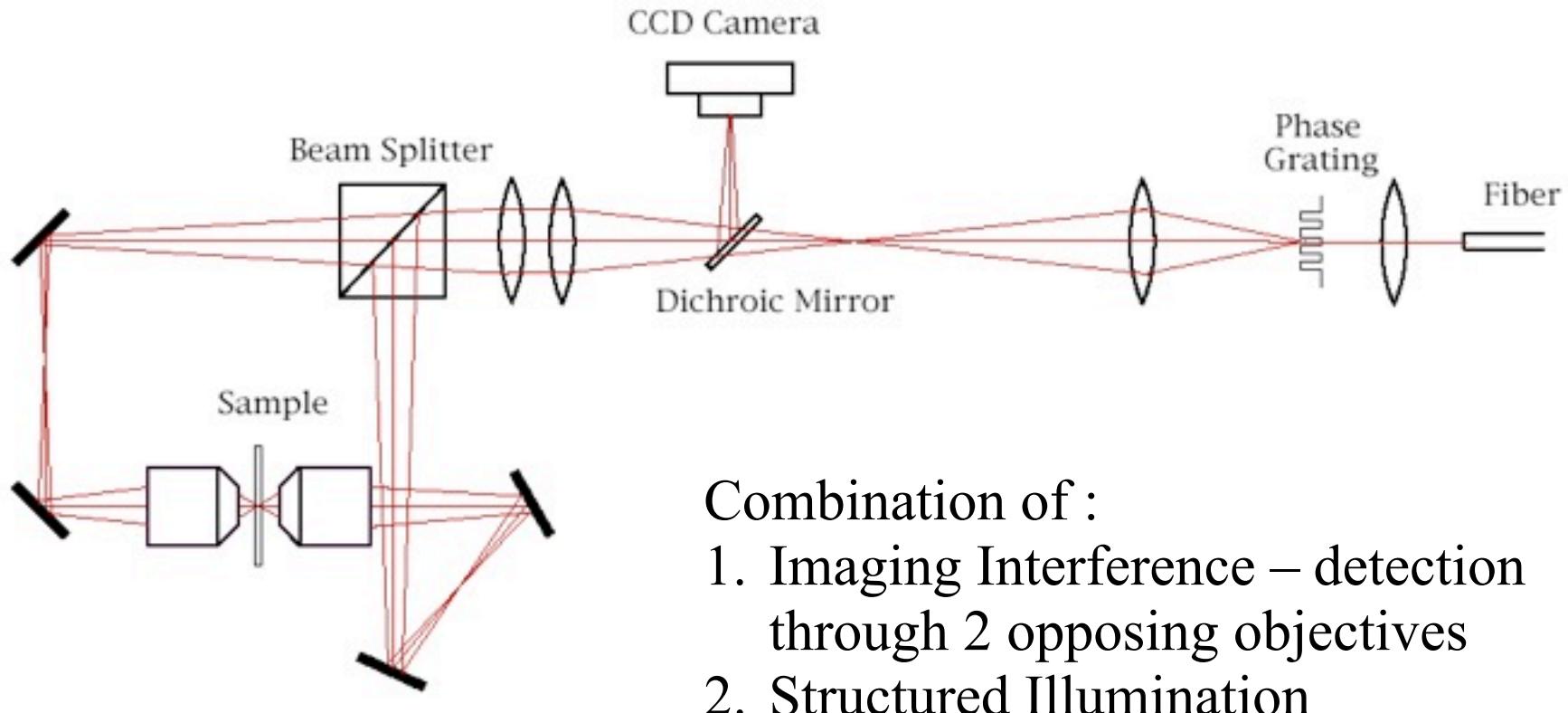


XZ
projections
1 μm slices

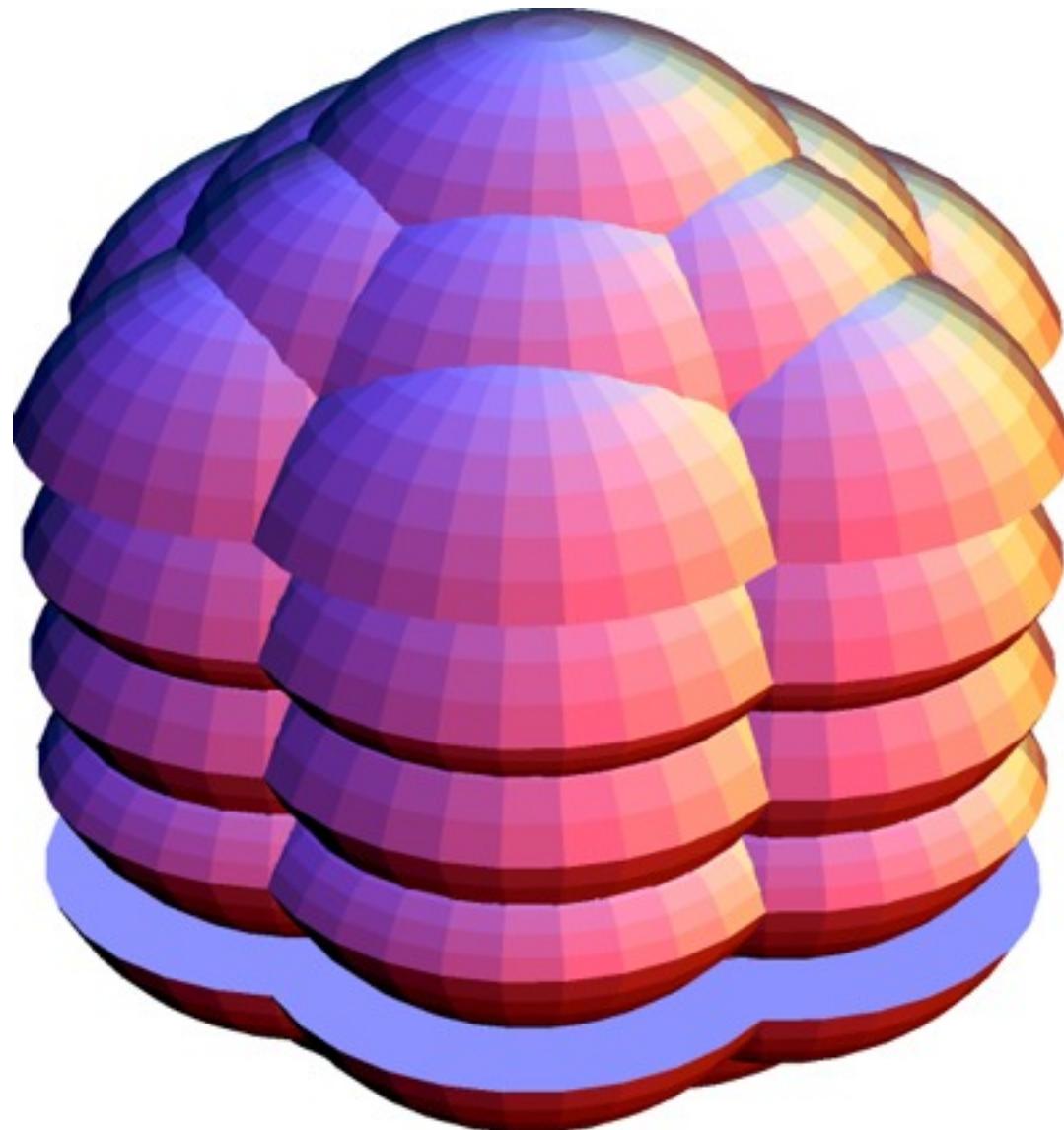
15
3D view



Combine methods: I⁵S use two lenses to collect more angles

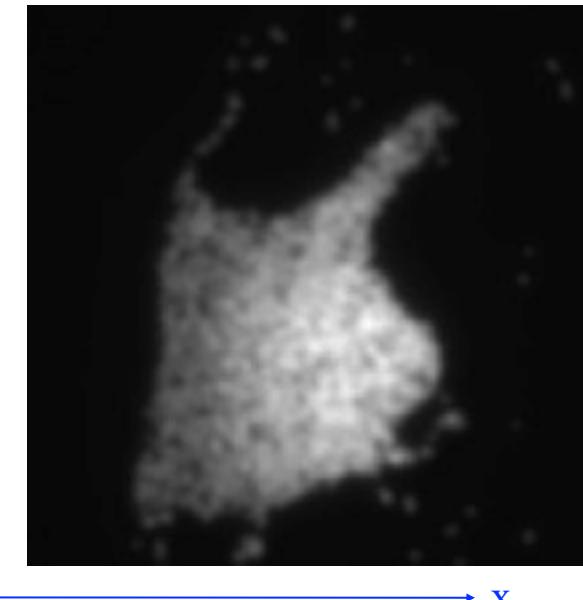


Observable Region through 2 opposing objectives
Structured Illumination (3 orientations) – I⁵S

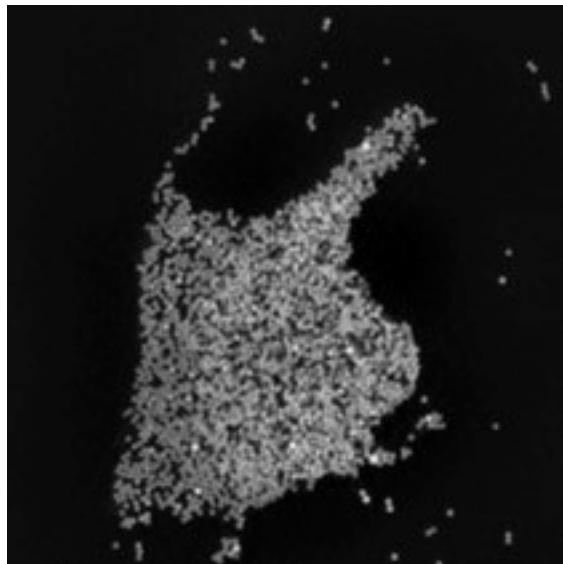


Comparison of Resolving Power

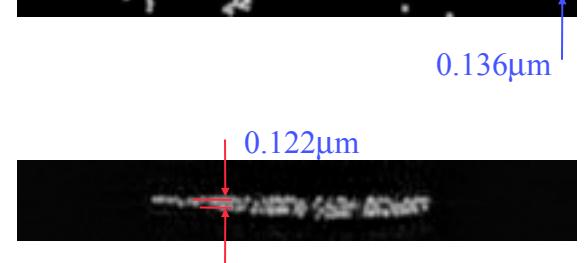
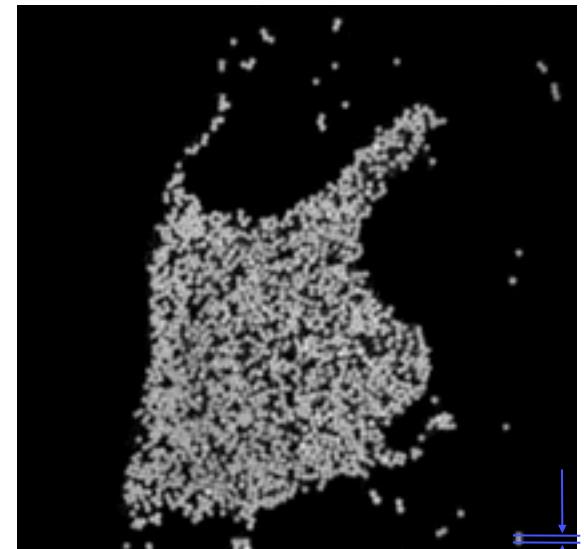
Conventional



Structured Illum.



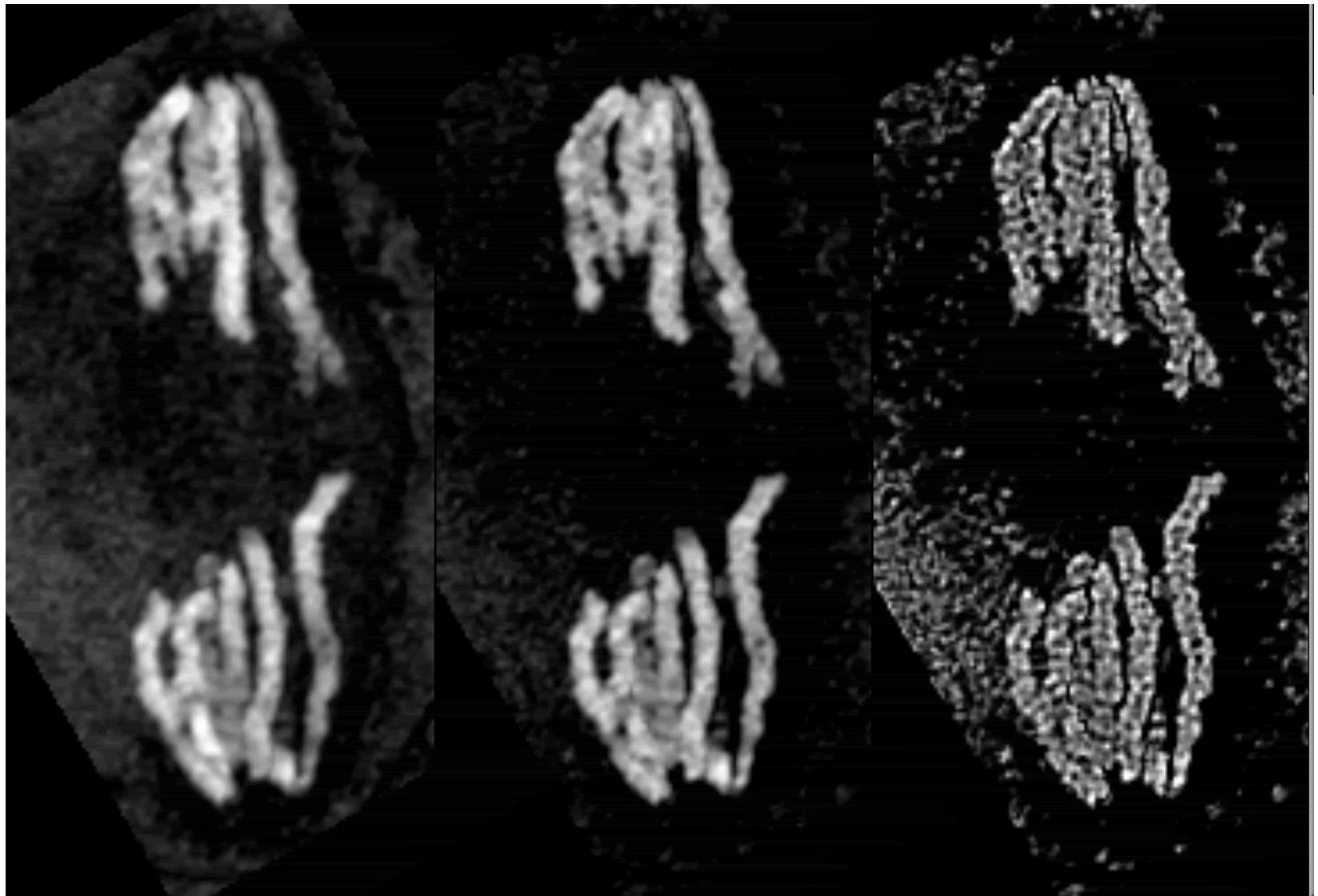
I⁵S



Sample: 0.12μm red-fluorescence microspheres

Lin Shao, Mats Gustafsson

Drosophila Anaphase Chromosomes (.2μm wide)



Lin Proc.

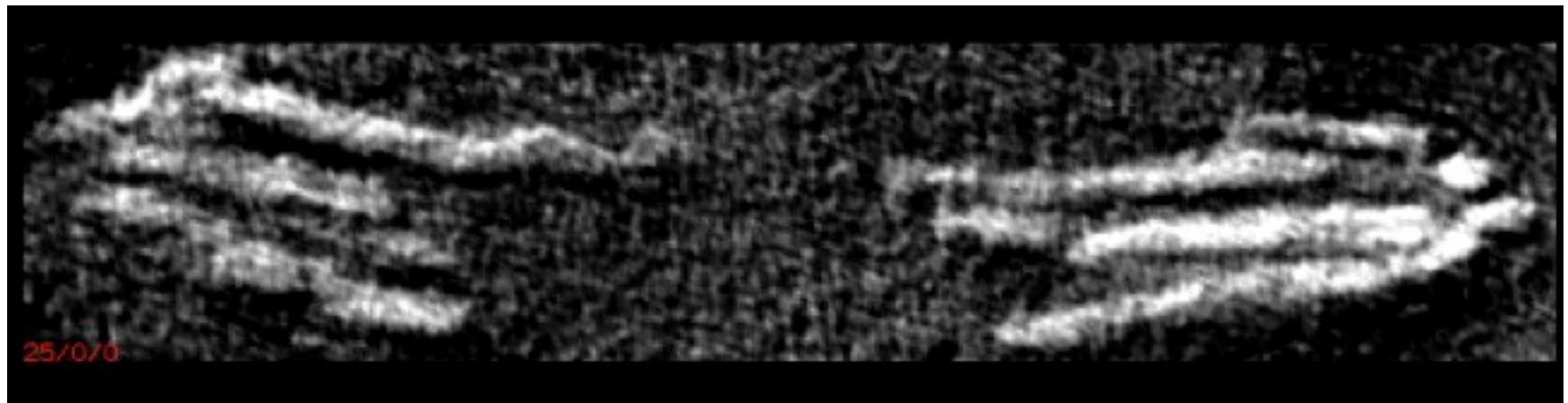
Decon

Decon + LCE

Lin Shao

Comparison between OM and EM-Tomo

EM



OM

