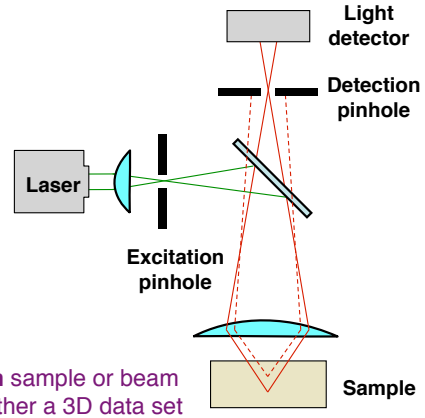
3D fluorescence microscopy

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

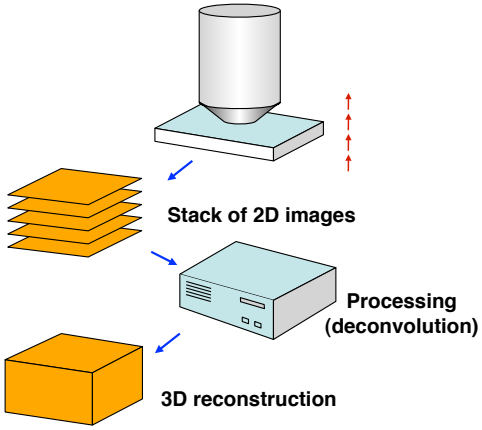
Problem: Each image contains out-of-focus blur from other focal planes

Approach 1:
Physically exclude the blur by confocal microscopy

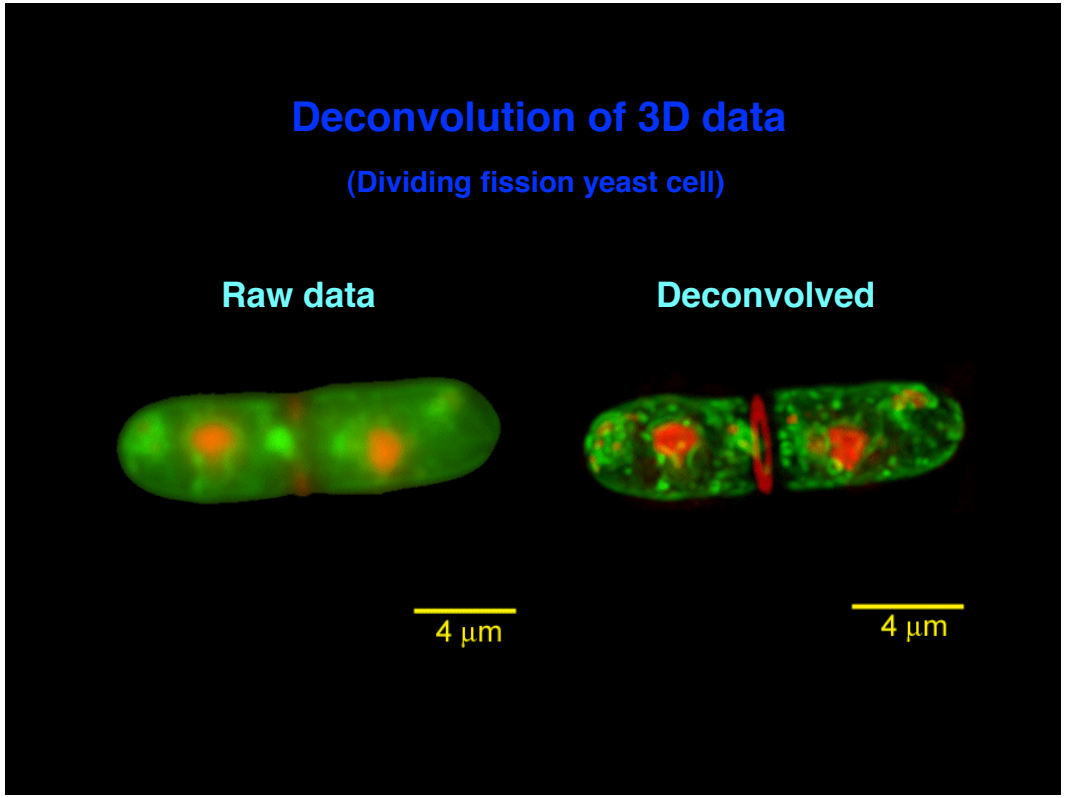


Scan sample or beam to gather a 3D data set

Approach 2:
Remove the blur computationally



How well can this be done?


Deconvolution of 3D data
(Dividing fission yeast cell)
Raw data
Deconvolved
4 μm
4 μm

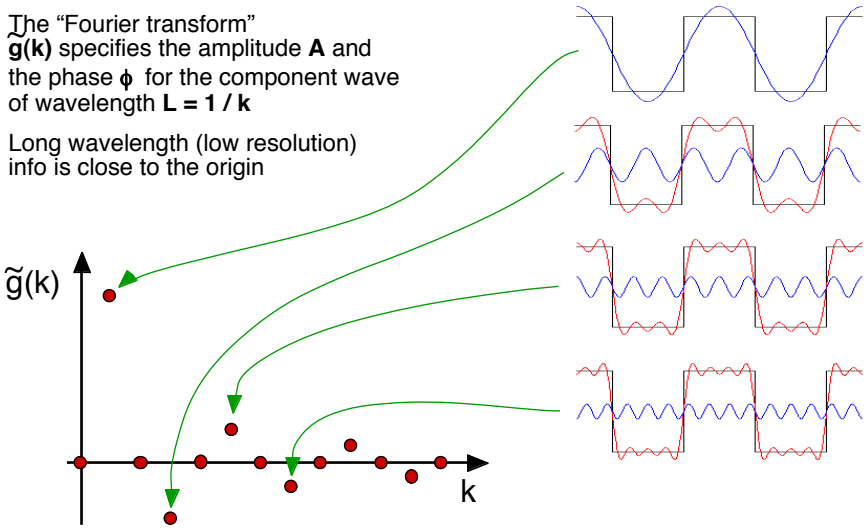
The Fourier Transform

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

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

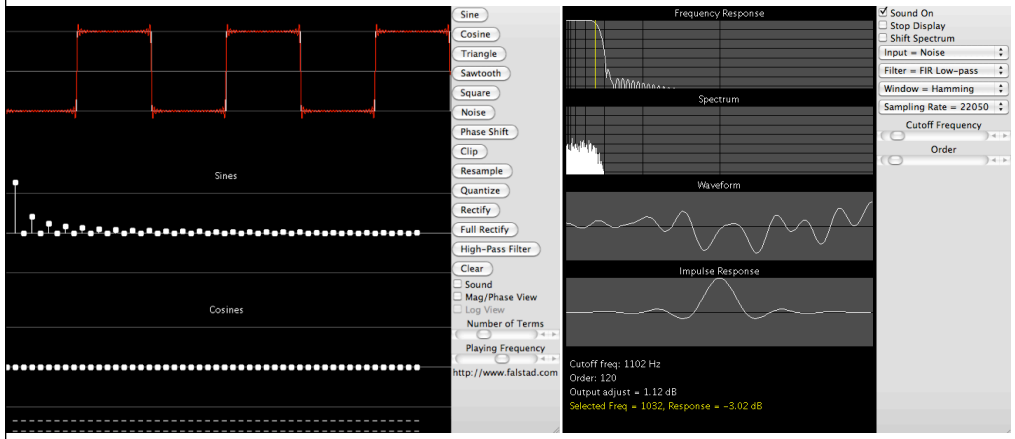
The "Fourier transform" $\tilde{g}(k)$ specifies the amplitude **A** and the phase ϕ for the component wave of wavelength **L = 1 / k**

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



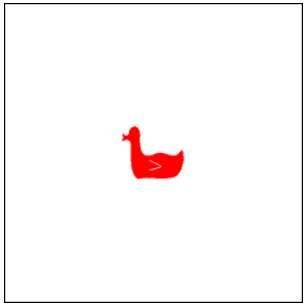
Fourier Transform and Digital Filter Applets

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

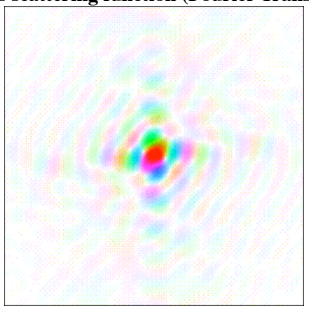


Low Resolution Duck

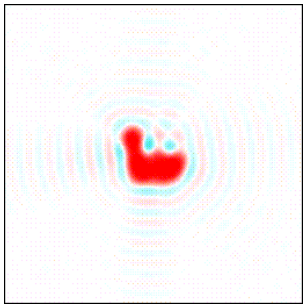
A Duck



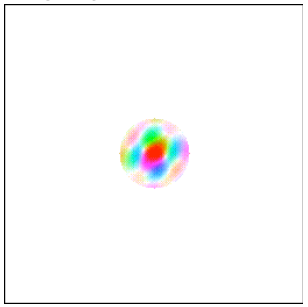
and its scattering function (Fourier Transform):



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

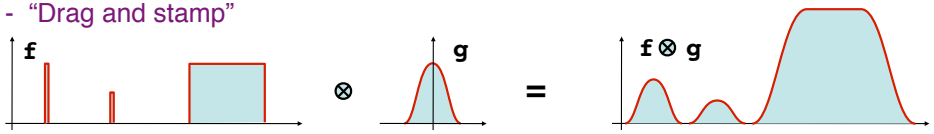
  then $\tilde{h}(k) = \tilde{f}(k)\, \tilde{g}(k)$

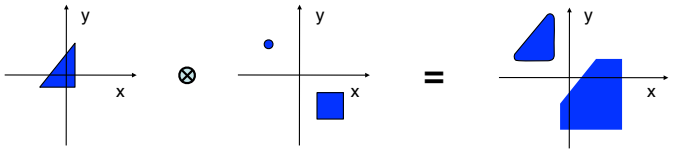
A convolution in real space becomes
a product in reciprocal space & vice versa

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

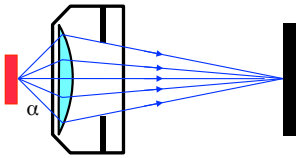
So what **is** a convolution, intuitively?

- "Blurring"
- "Drag and stamp"





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



α = maximum angle
N.A. $= \eta \sin \alpha$,

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 $1/(1.22 \bullet f_c)$
$d_{min} = 0.146\ \mu$m

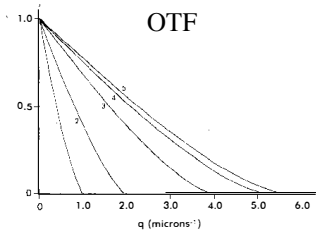


Figure 3 **The in-focus contrast-transfer functions for several Zeiss lenses are compared. The curves are (*1*) 10 × 0.25 N.A., (*2*) 25 × 0.5 N.A., (*3*) 50 × 1.0 N.A. oil, (*4*) 100 × 1.25 N.A. oil, (*5*) 63 × 1.4 N.A. oil.**

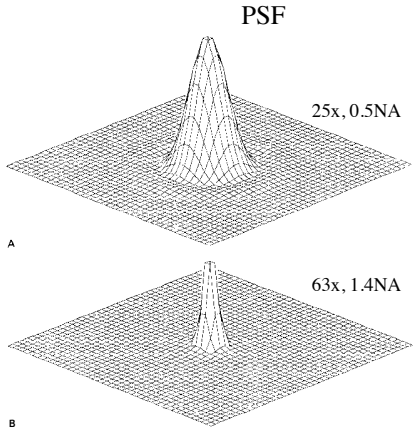
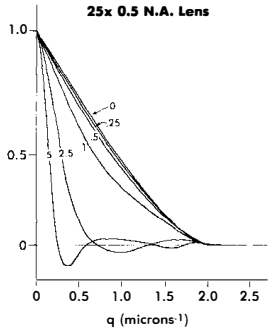
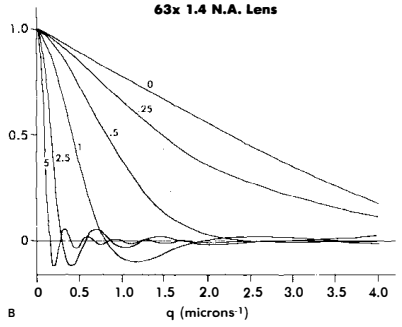
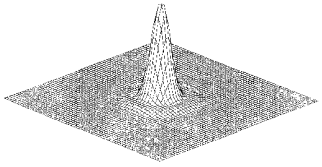


Figure 4 **The point-spread functions calculated by Fourier transformation of the CTF's of Figure 3. (*A*) 25 × 0.5 N.A. lens (# 2); (*B*) 63 × 1.4 N.A. lens (# 5). Each grid square is λ/6 or 833 Å. Note how much sharper the 1.4 N.A. lens is than the 0.5 N.A. lens.**

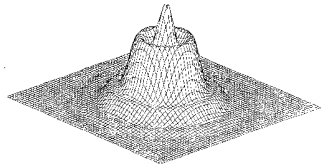
OTF, PSF of a defocused lens



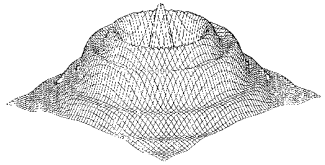




25x lens 0 μm defocus

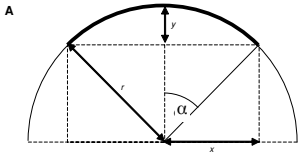


25x lens 2.5 μm defocus

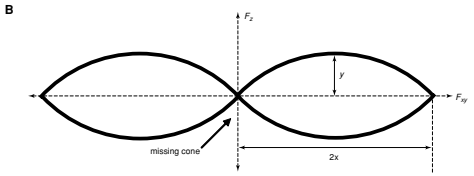


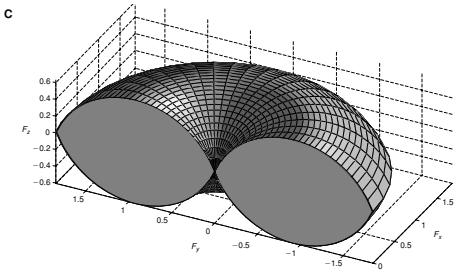
25x lens 5 μm defocus

3D OTF

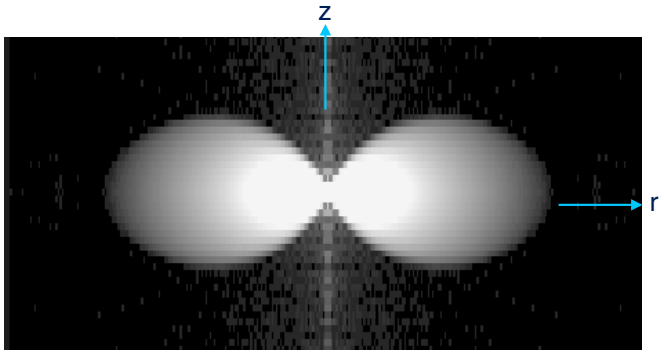


Only a bowl-shaped segment of the shell makes it through the objective: The intensity now becomes a convolution of the **bowl** with itself, which has a a donut-shaped region of support.

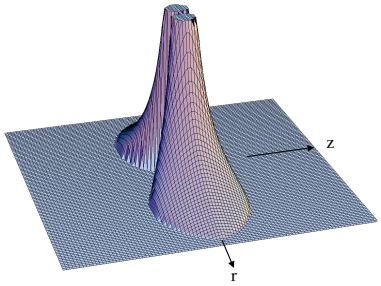




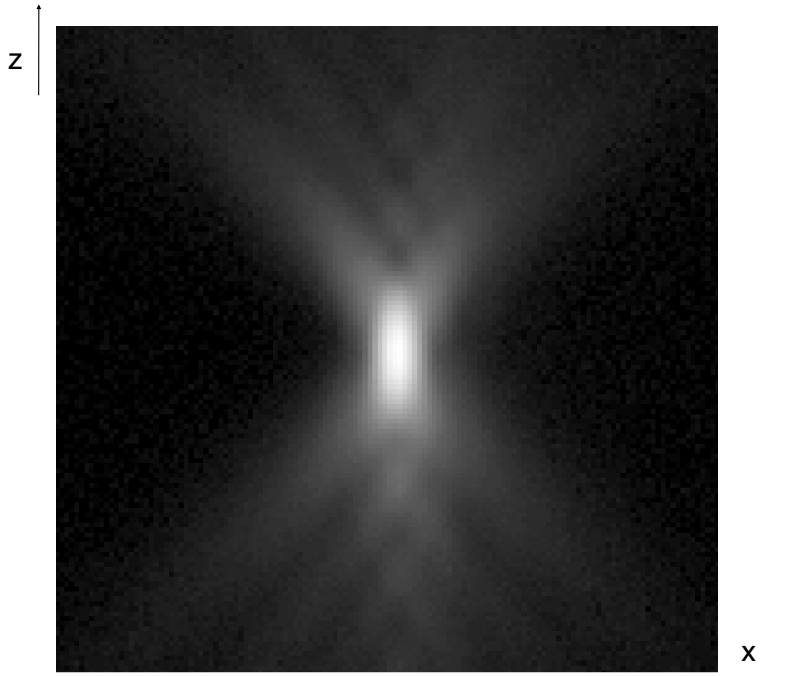
Experimentally measured OTF



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

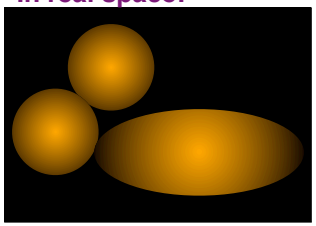


Experimentally measured PSF



PSF, OTF & deconvolution

In real space:

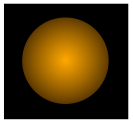


Observed Image(r)

=

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

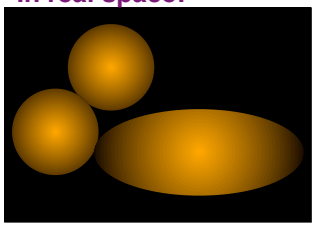
This suggests:

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

??? ("Deconvolution")

PSF, OTF & deconvolution

In real space:

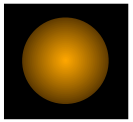


Observed Image(r)

=

True Object(r)

⊗



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This suggests:

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??? ("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

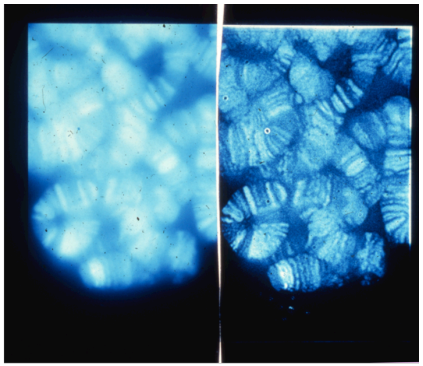
$$I_j \approx c_1[\, O_j - c_2(PSF_{\Delta z} \otimes O_{j+1} + PSF_{-\Delta z} \otimes O_{j-1}) \,]$$

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

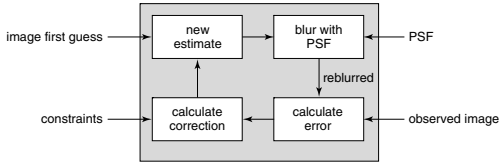
or if OTF is complex:

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

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 $I^{k+1} = 0$

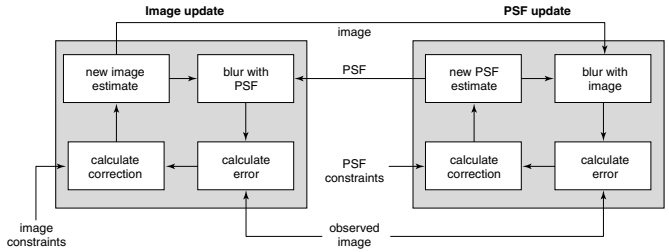
or use multiplicative update (Gold's method)

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

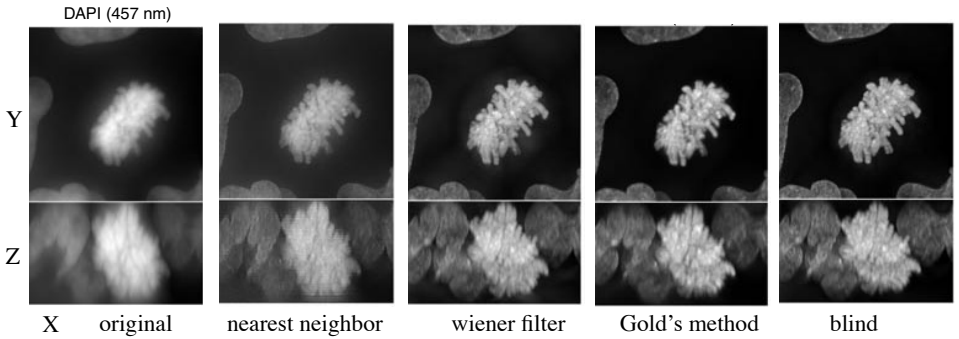
Deconvolution strategies

what if your PSF is not accurate?

blind deconvolution seeks to estimate both Image and PSF



Some comparisons: Hela cells, DAPI stained

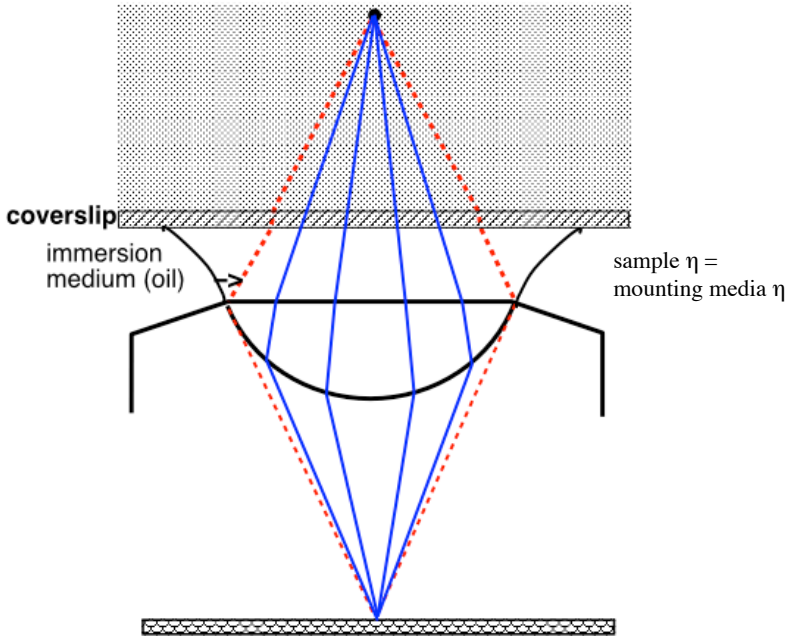


now many related variations

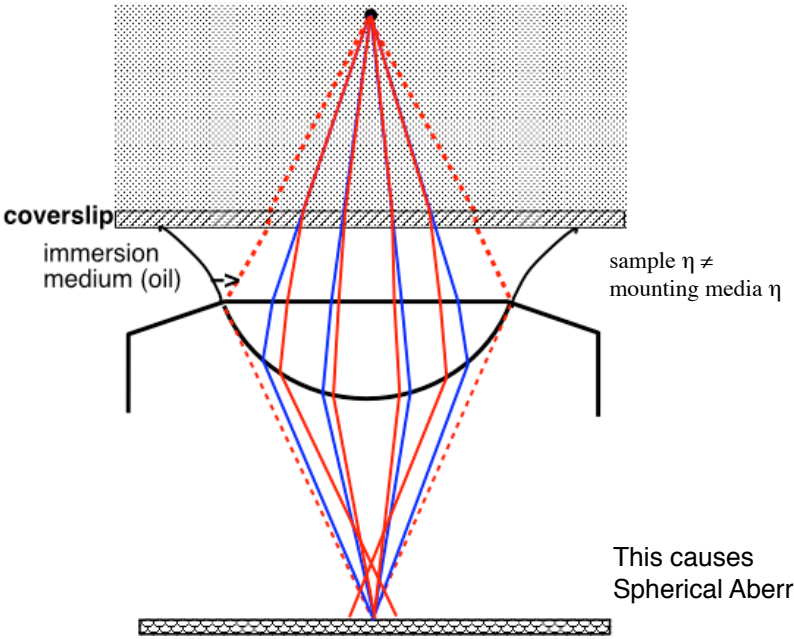
practical issues are signal to noise, accuracy of PSF

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

Properties of the Sample Affect Image Formation in Microscopy

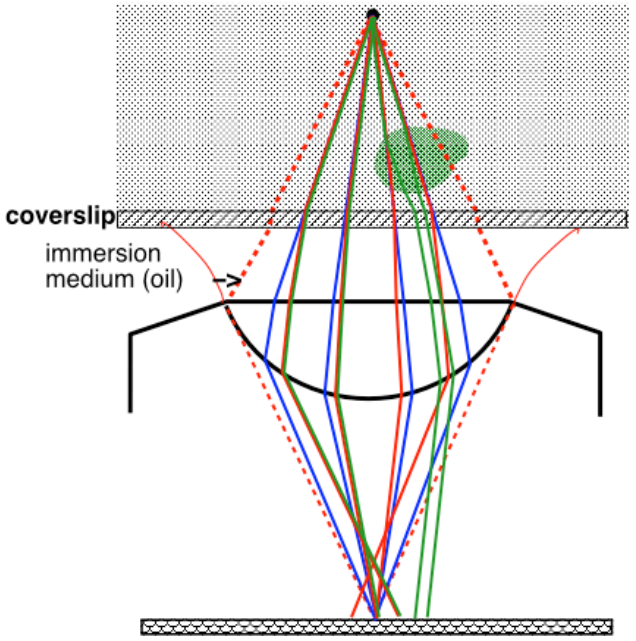


Properties of the Sample Affect Image Formation in Microscopy



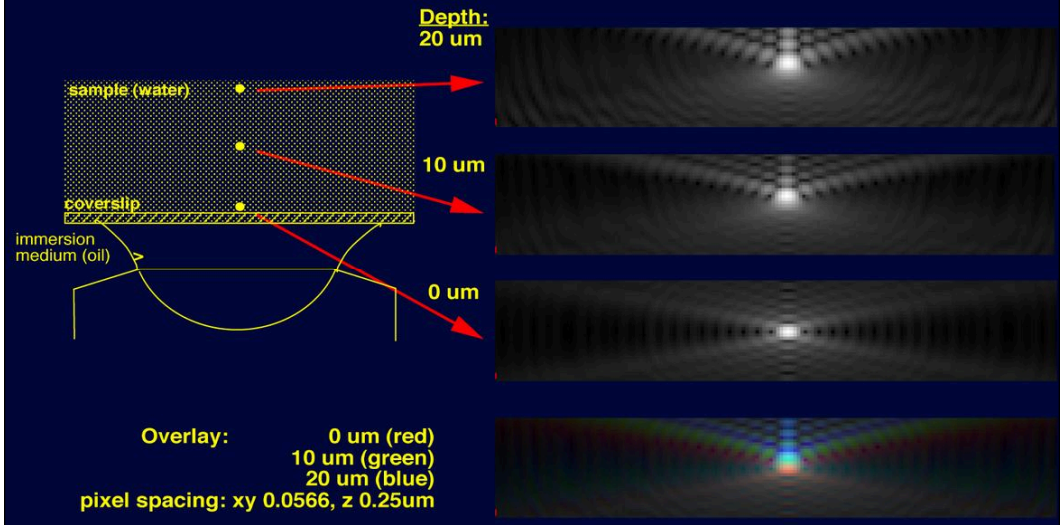
This causes
Spherical Aberration

Properties of the Sample Affect Image Formation in Microscopy



sample η not constant

Depth-Dependent Effects in Water: Simulations of PSFs



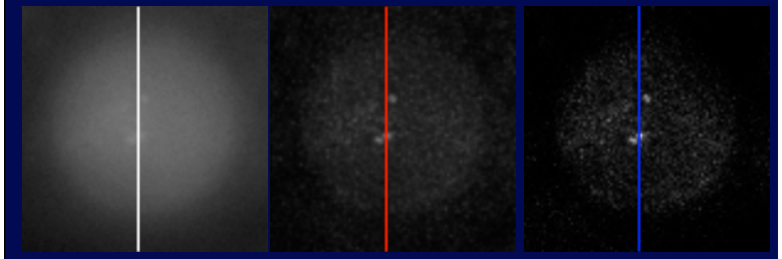
Overlay: 0 um (red)
10 um (green)
20 um (blue)
pixel spacing: xy 0.0566, z 0.25um

Hanse

Depth Dependent Deconvolution: Biological Data

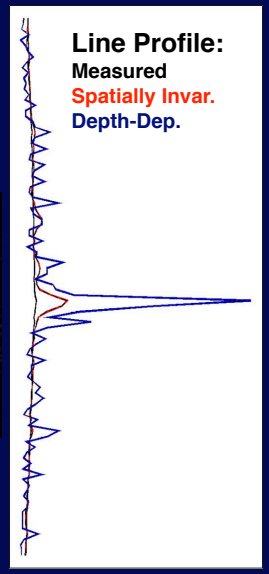
Line Profile Through 1 Spot in Nucleus
Section ~7 .6 μm into sample

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)

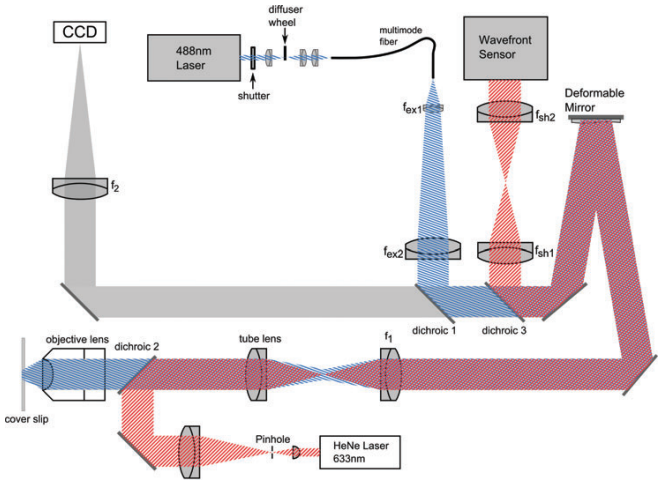
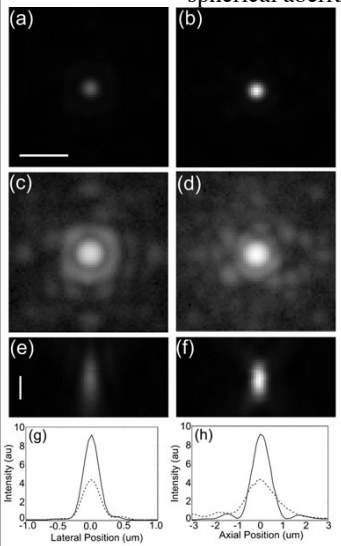


Fig. 2. Microscope layout. Grey represents the emission path. The blue striped beam is the excitation light and the red striped beam is the reference beam. See text for details.

Adaptive optics can correct for depth dependent effects

flat mirror

mirror shaped to correct spherical aberration



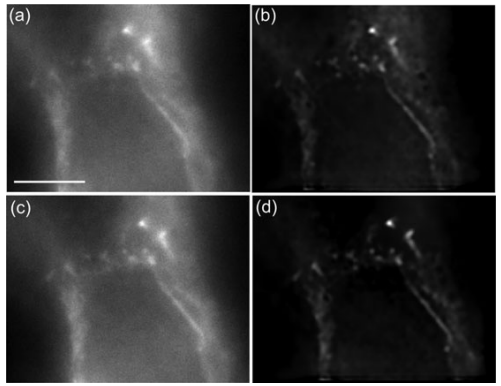
X-Y

X-Y log scale

X-Z

raw

decon



-AO

+AO

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

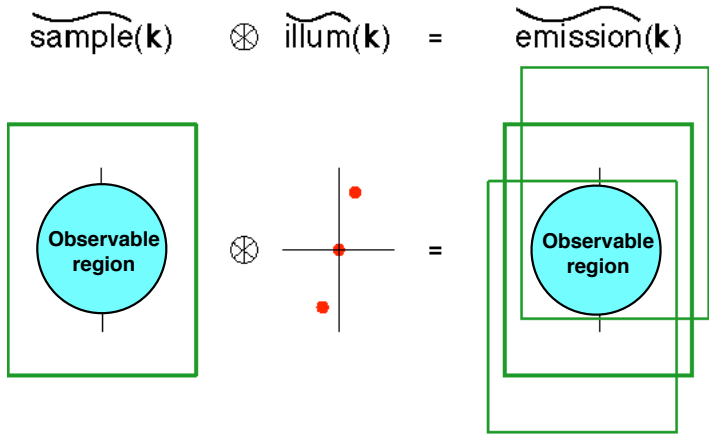
Structured illumination microscopy: the idea

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*

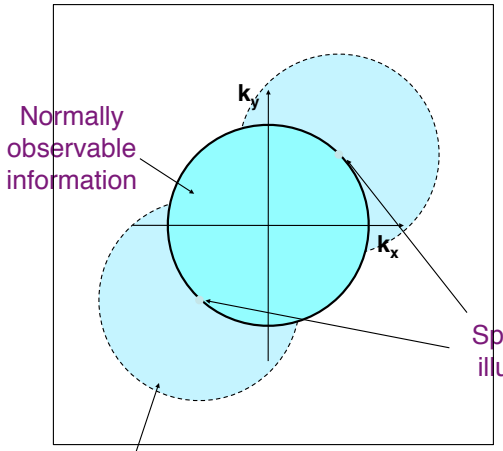
Illuminate sample with parallel stripes

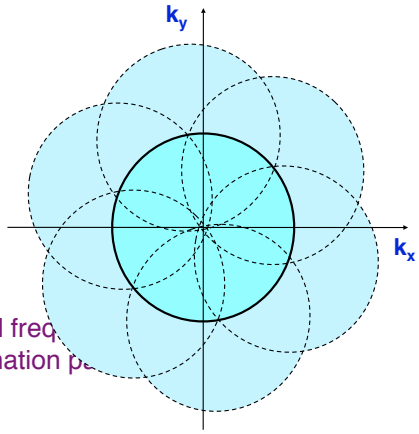


The emitted light contains 3 superimposed information components shifted by 0, ± the inverse stripe spacing

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

Resolution extension by Structured Illumination



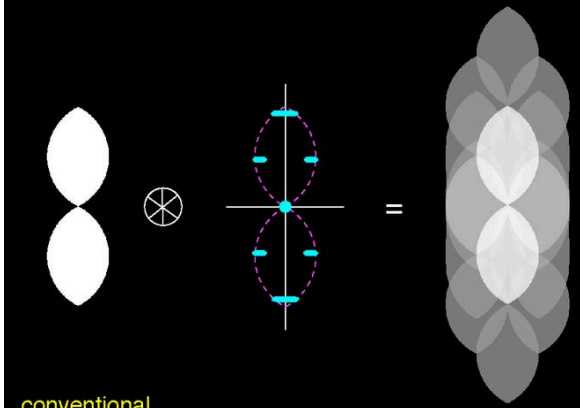


…and can repeat in many directions

Q: What about 3D?

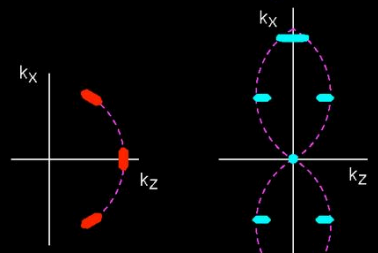
A: Use 3 beams

(admit diffraction orders 1, 0 and +1)



conventional observable region ⊛ illumination structure = new observable region

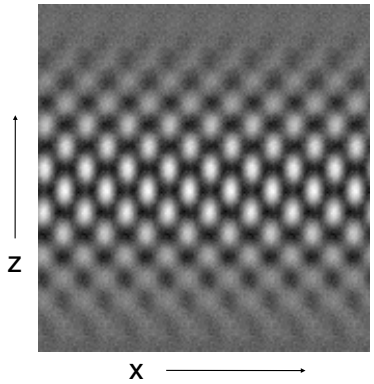
3 coherent beams → 7 intensity components

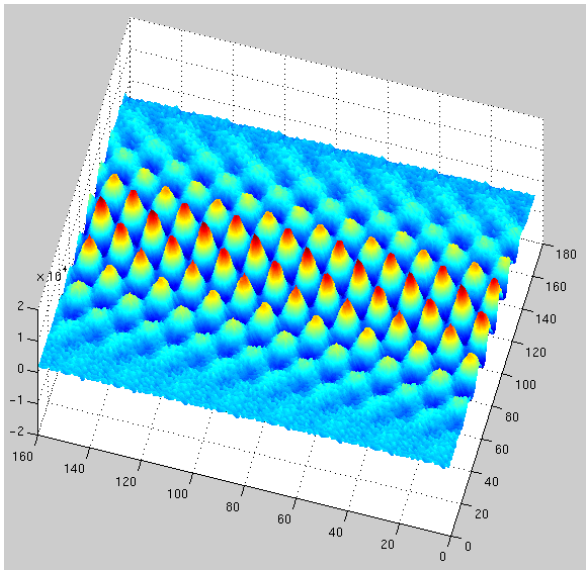


Covers full "confocal" OTF support without discarding light

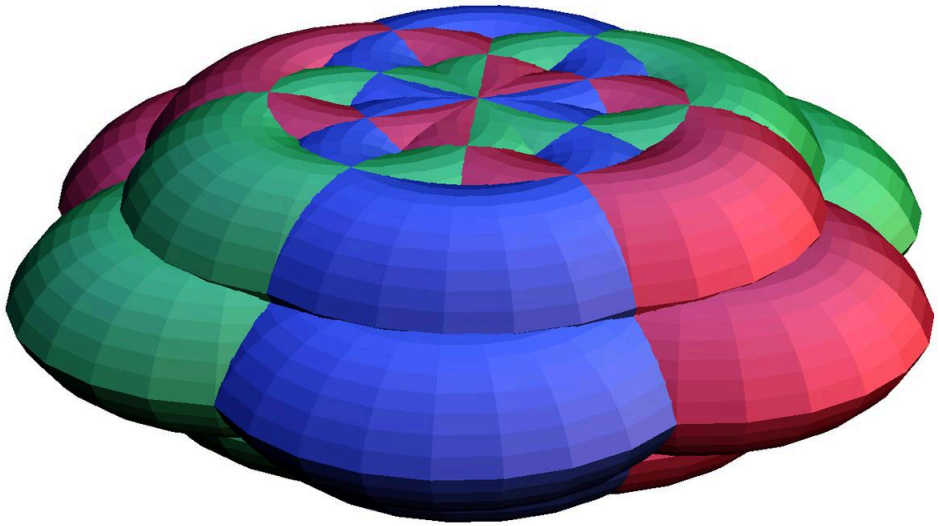
Same procedure as for 2D, except need 5 phases

illumination intensity in real space

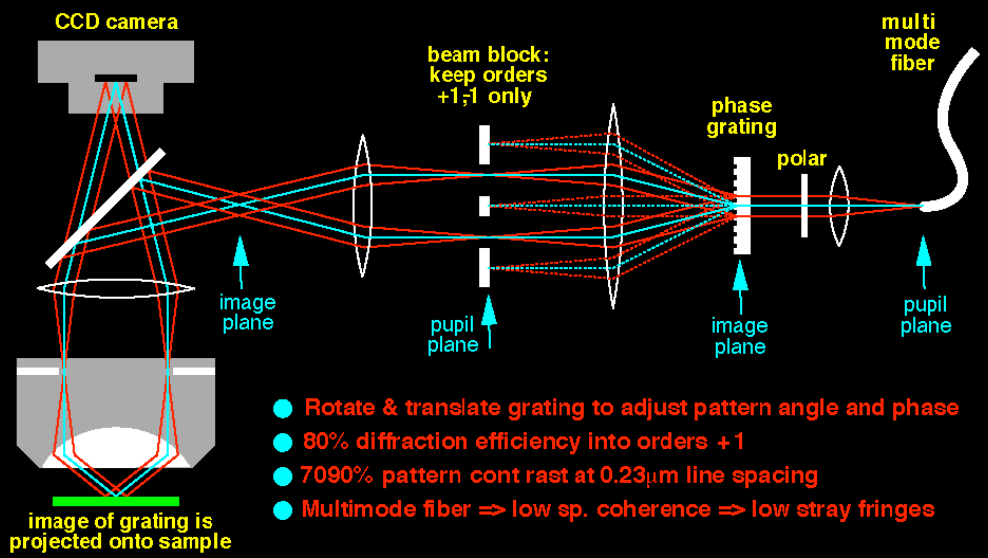




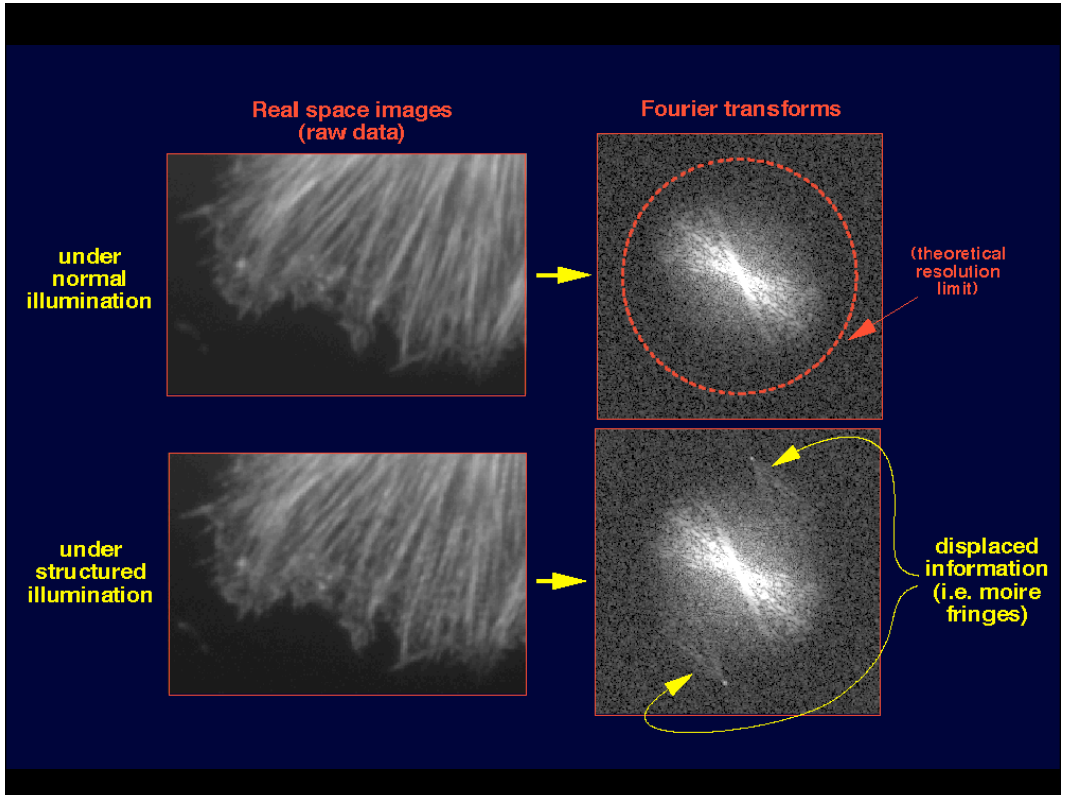
Observable Region
Structured Illumination (3 orientations)

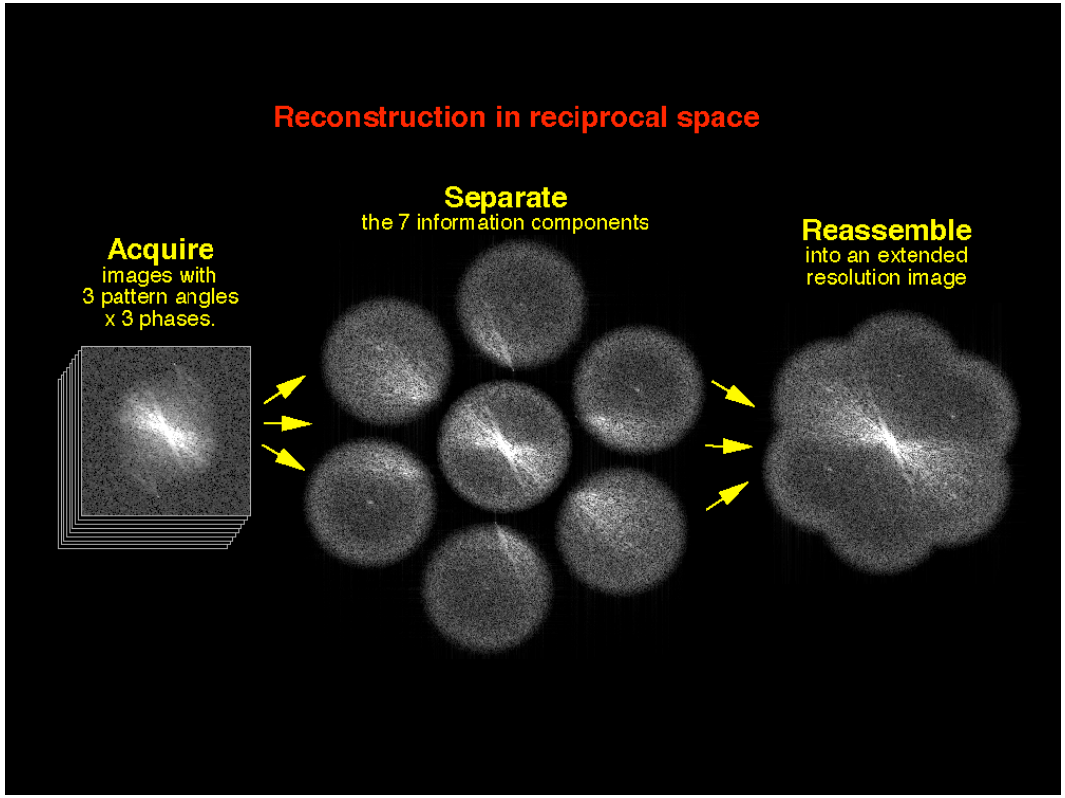


Structured illumination apparatus



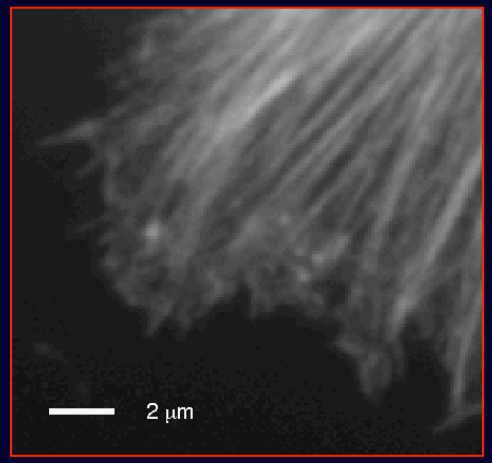
- Rotate & translate grating to adjust pattern angle and phase
- 80% diffraction efficiency into orders +1
- 7090% pattern cont rast at 0.23μm line spacing
- Multimode fiber => low sp. coherence => low stray fringes


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

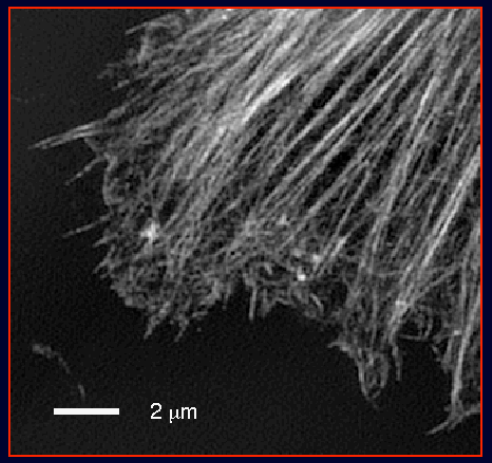

Reconstruction in reciprocal space
Acquire
images with
3 pattern angles
x 3 phases.
Separate
the 7 information components
Reassemble
into an extended
resolution image

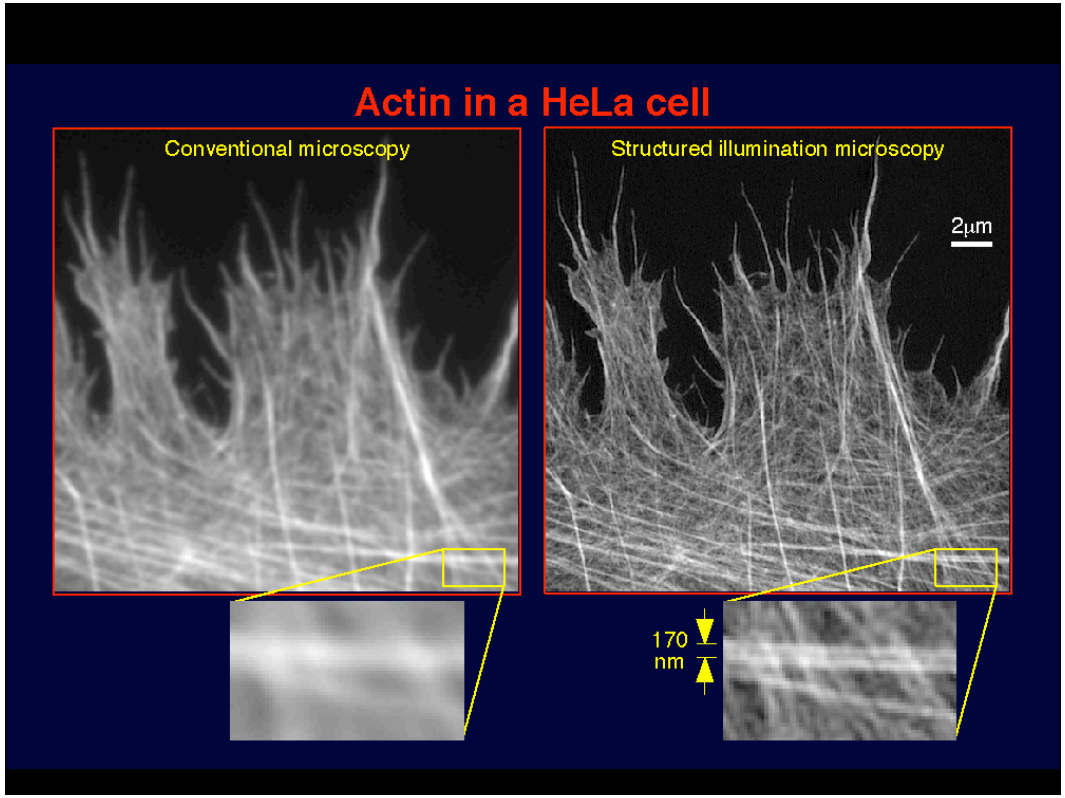
Resolution comparison
Actin in a HeLa cell

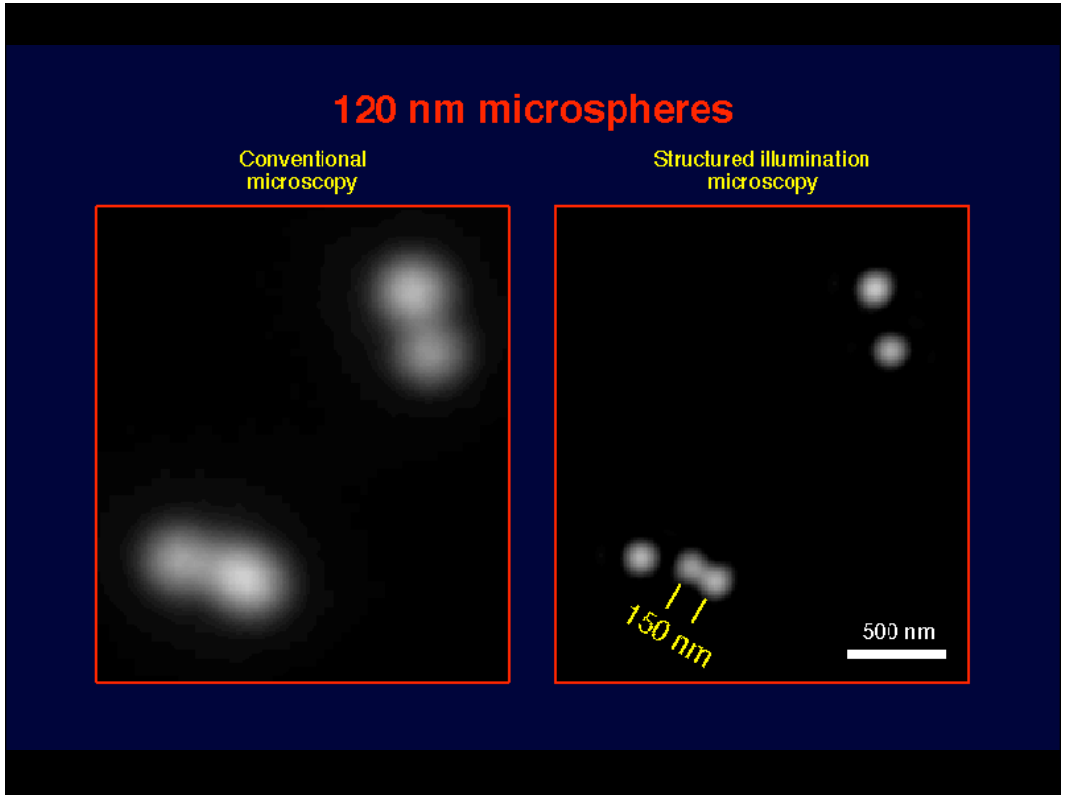
Conventional microscopy

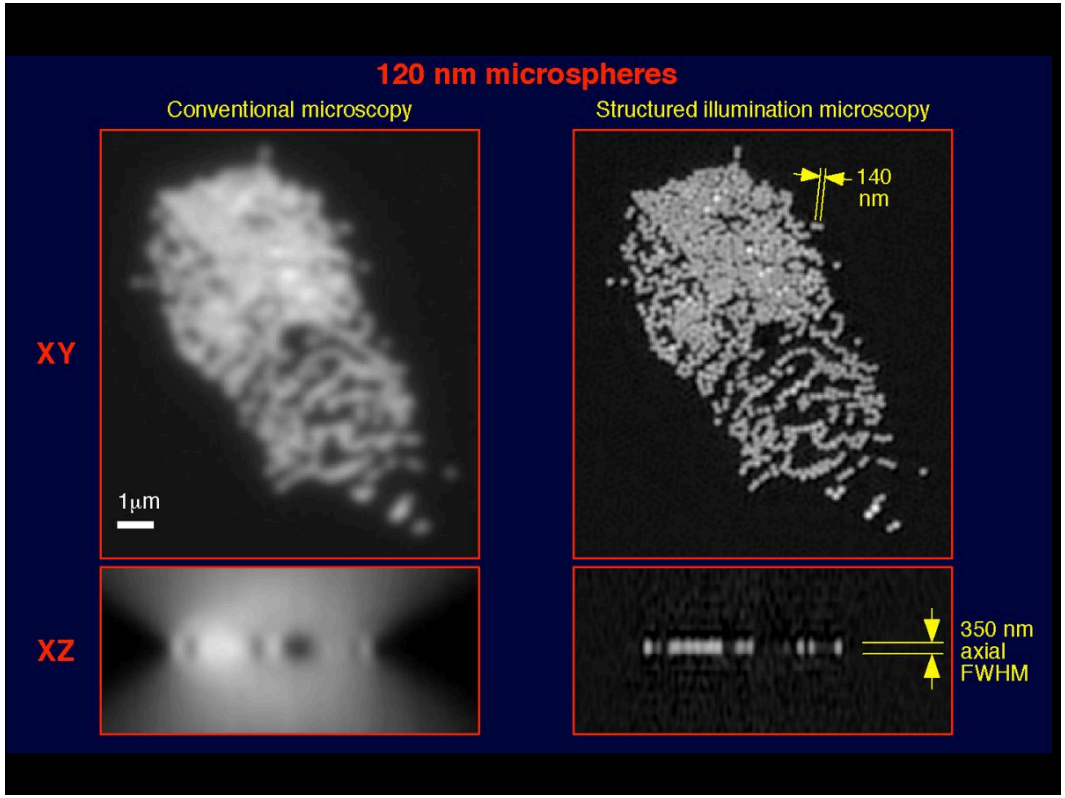


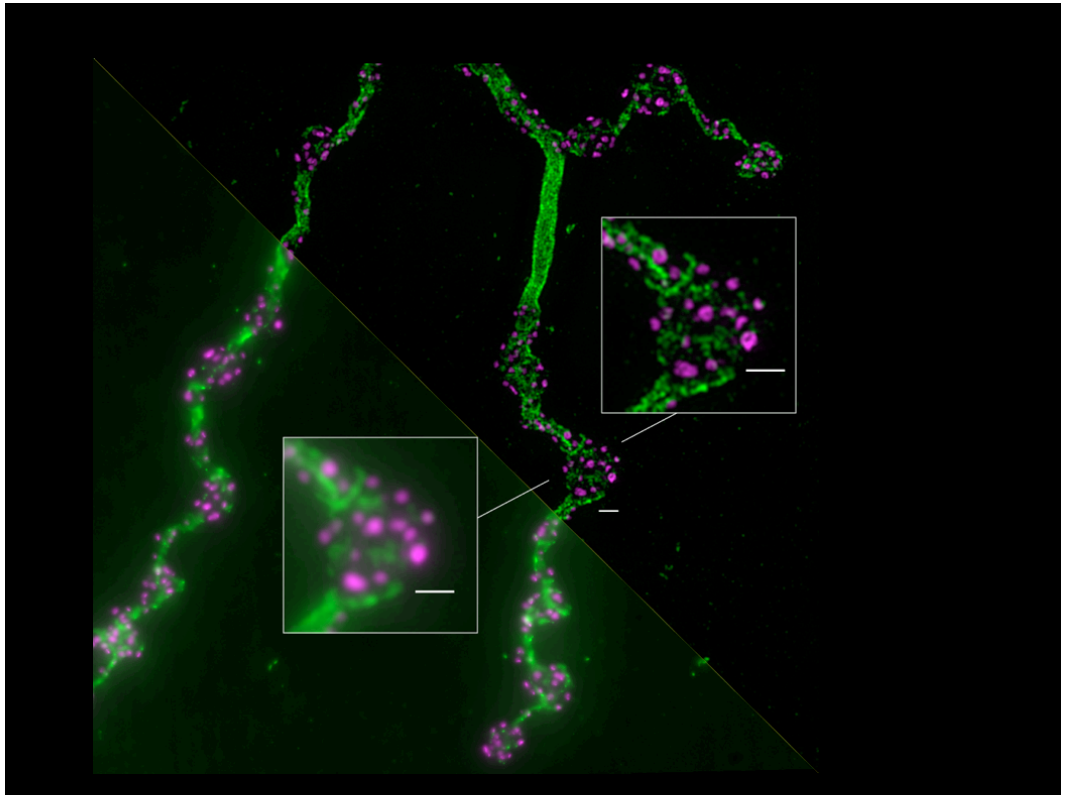
Structured illumination
final reconstruction

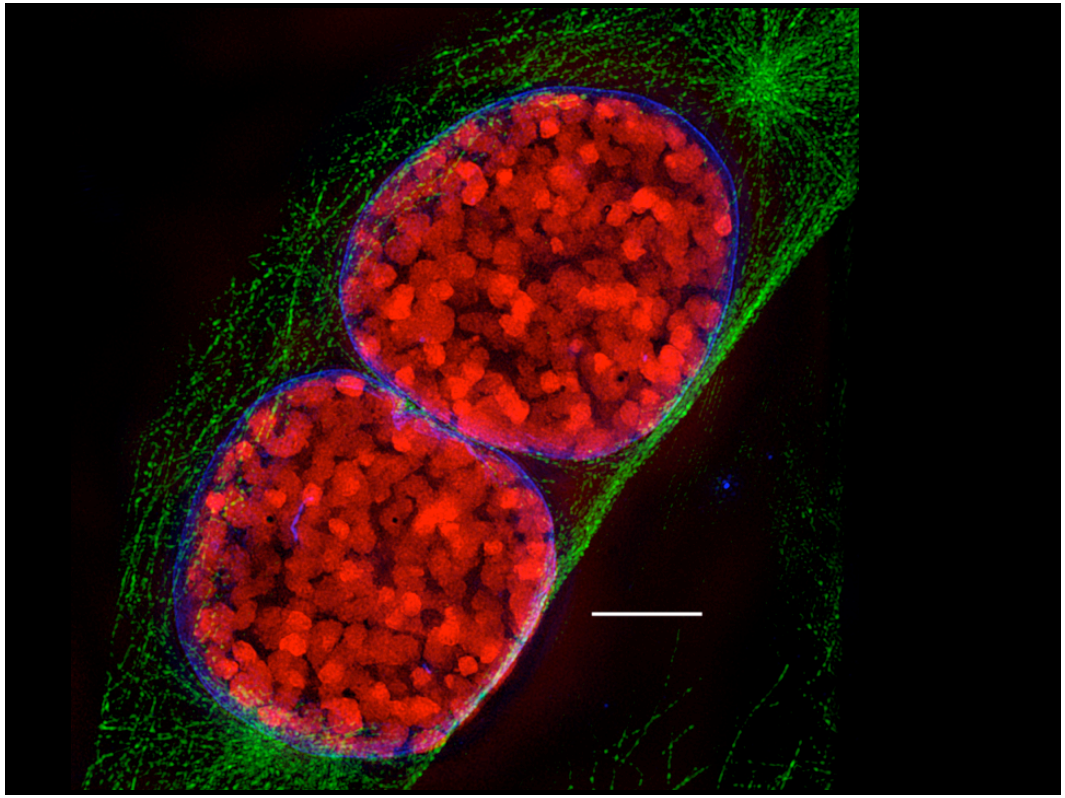


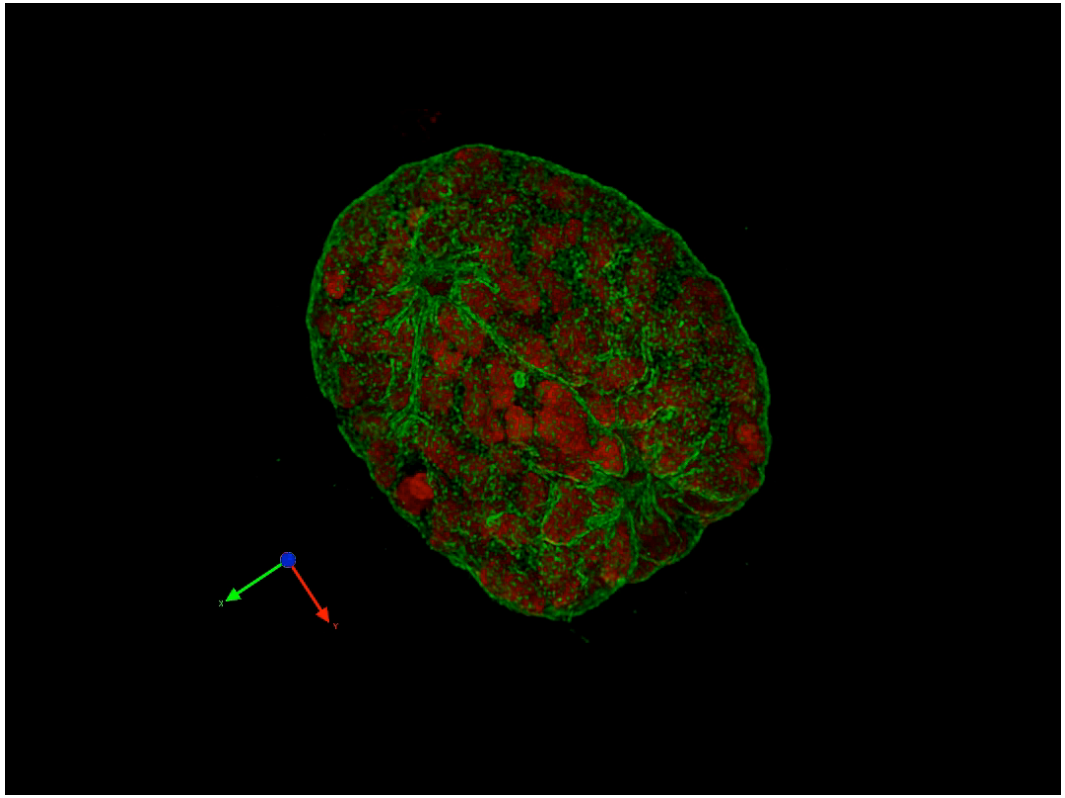

Actin in a HeLa cell
Conventional microscopy
Structured illumination microscopy
2μm
170
nm


120 nm microspheres
Conventional microscopy
Structured illumination microscopy
150 nm
500 nm

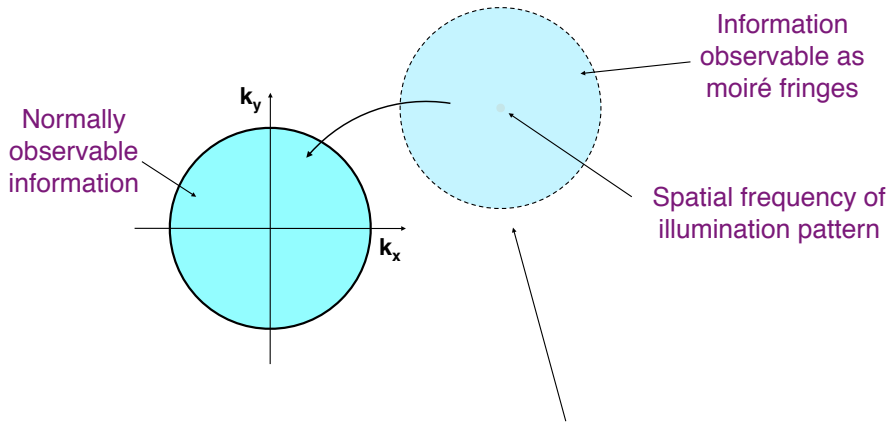

120 nm microspheres
Conventional microscopy
Structured illumination microscopy
XY
XZ
1μm
140 nm
350 nm axial FWHM






X
Y

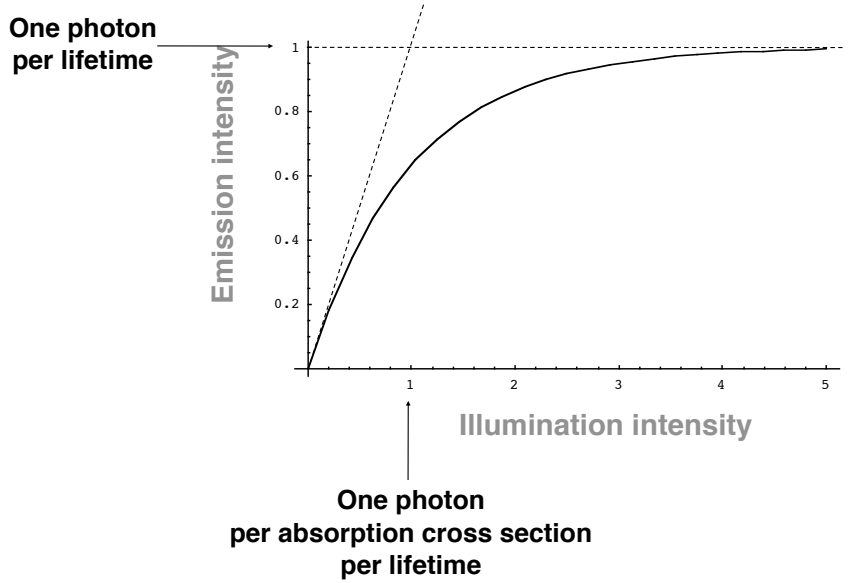
What are the limits of Structured Illumination?



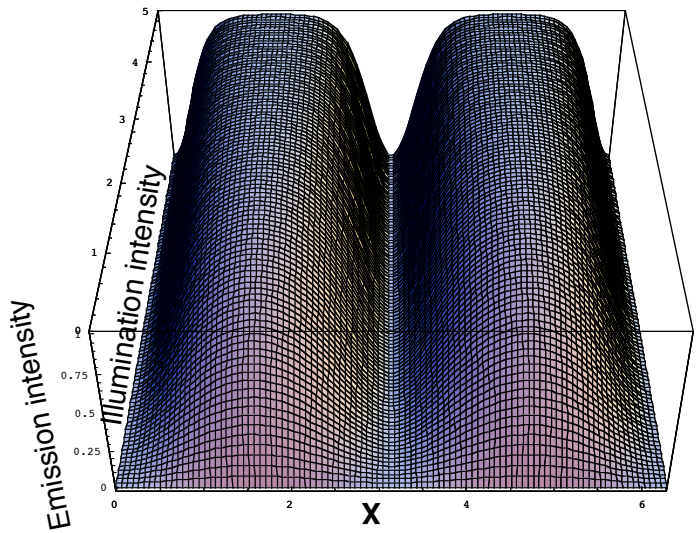
- Linear theory says this is impossible
- How about exploiting non-linear processes?

A simple source of nonlinearity: saturation

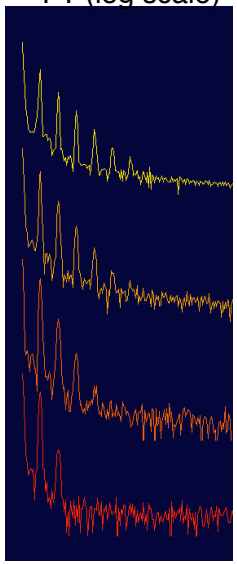
(R. Heintzmann Max-Planck Gottingen)



Saturated Structured Illumination

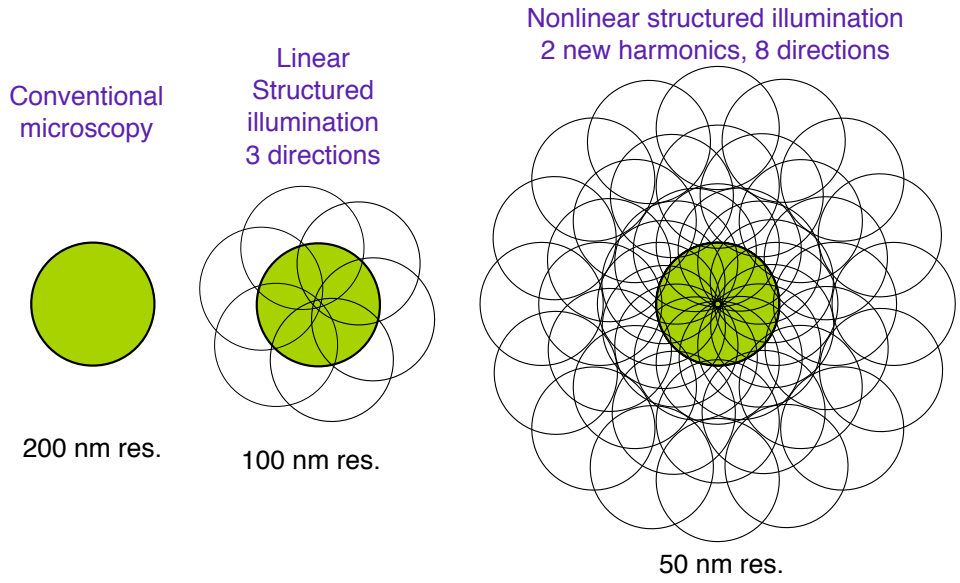


FT (log scale)

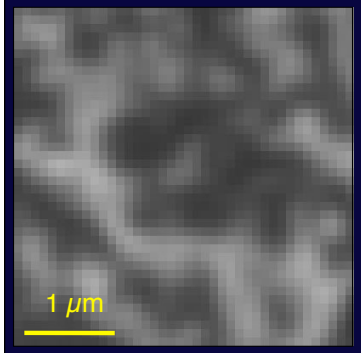
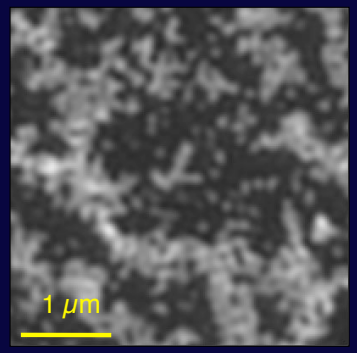
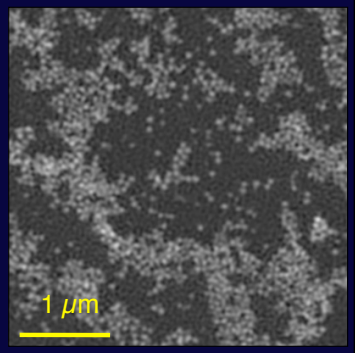


Resolution extension by nonlinear structured illumination

Effective observable regions

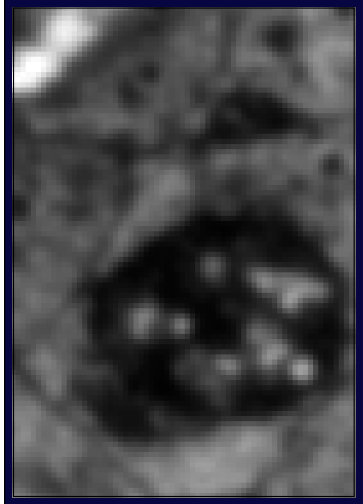
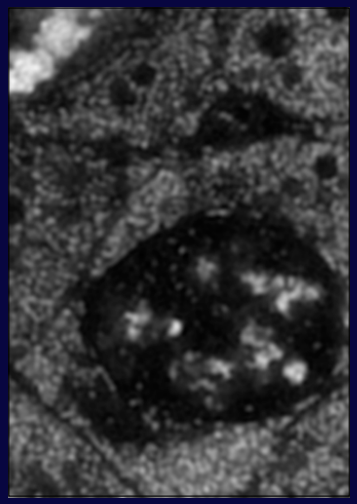
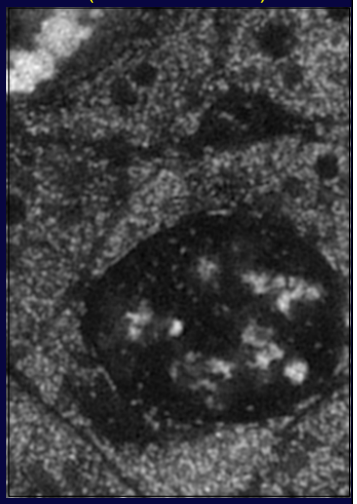


Non-linear structured illumination

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 ≈ 280 nm	Min. FWHM ≈110 nm	Min. FWHM ≈ 80 nm

Going beyond the diffraction limit: more light collection angles

We know:

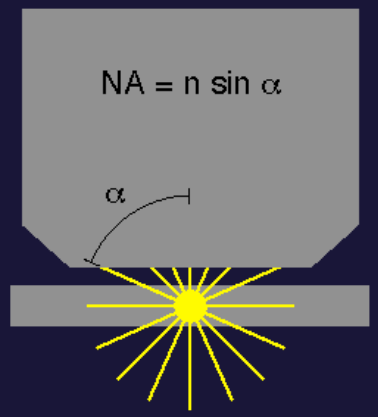
Higher NA

→

Gathering light over larger set of angles

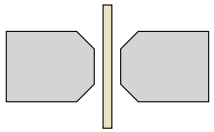
→

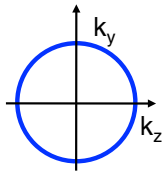
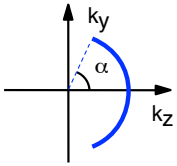
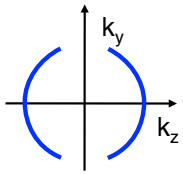
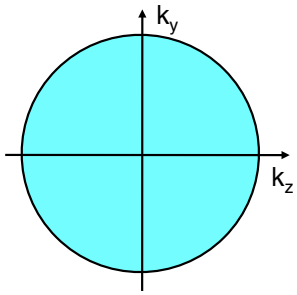
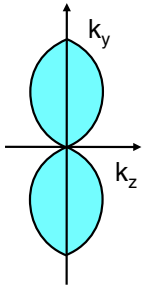
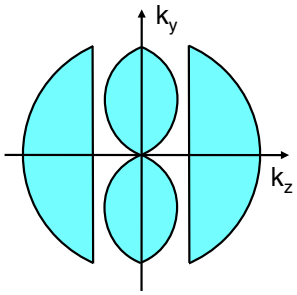
Higher resolution



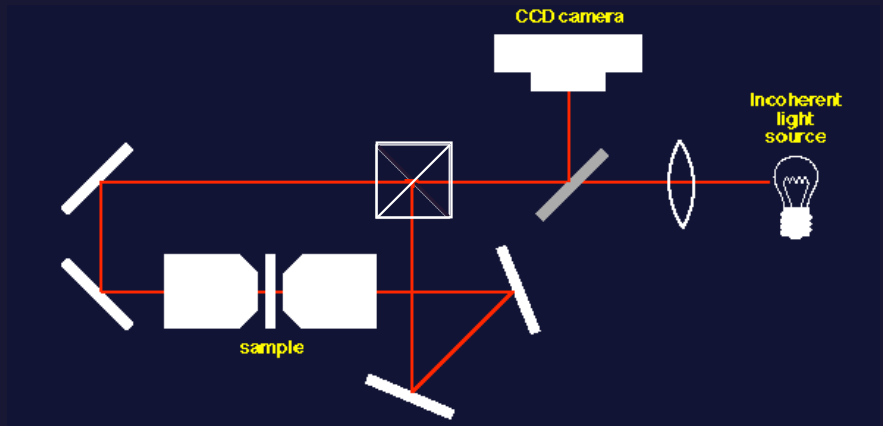
…So what about gathering the light emitted toward the back side?

OTF when detecting through two lenses



	Free space	Through one objective	Through two objectives
support of $\widetilde{A}(\mathbf{k})$			
Convolved with itself:			
support of $OTF_{det}(\mathbf{k})$			

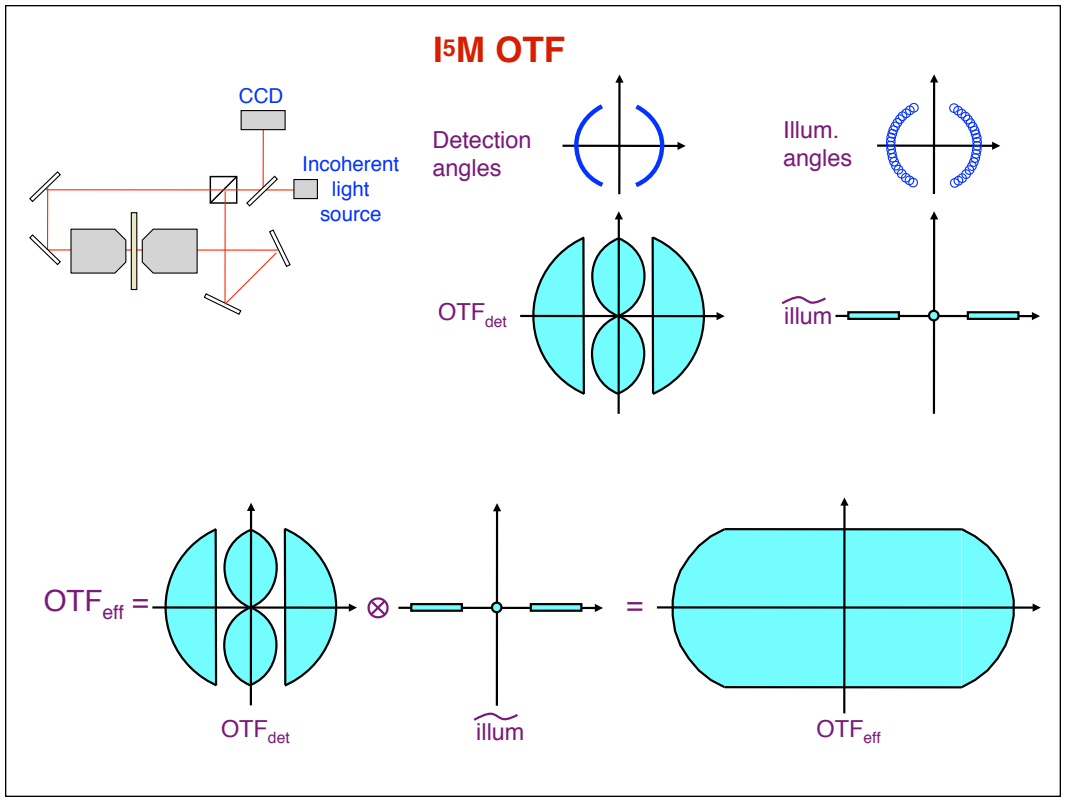
I5M concept

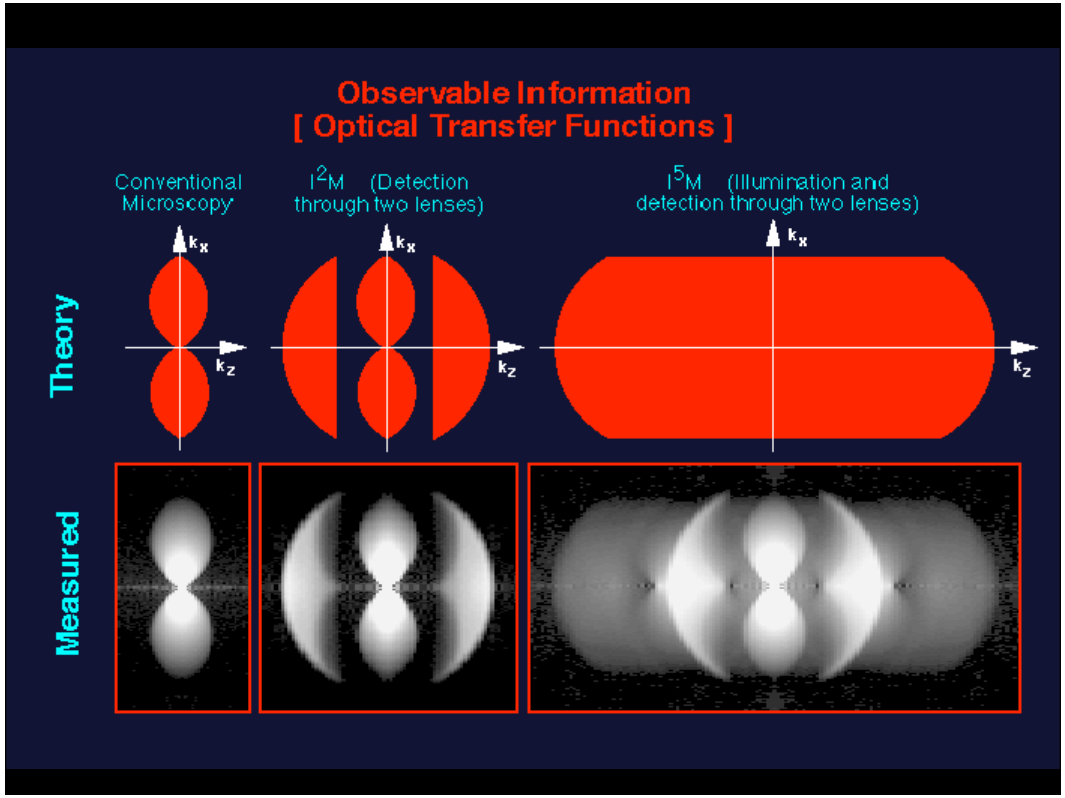


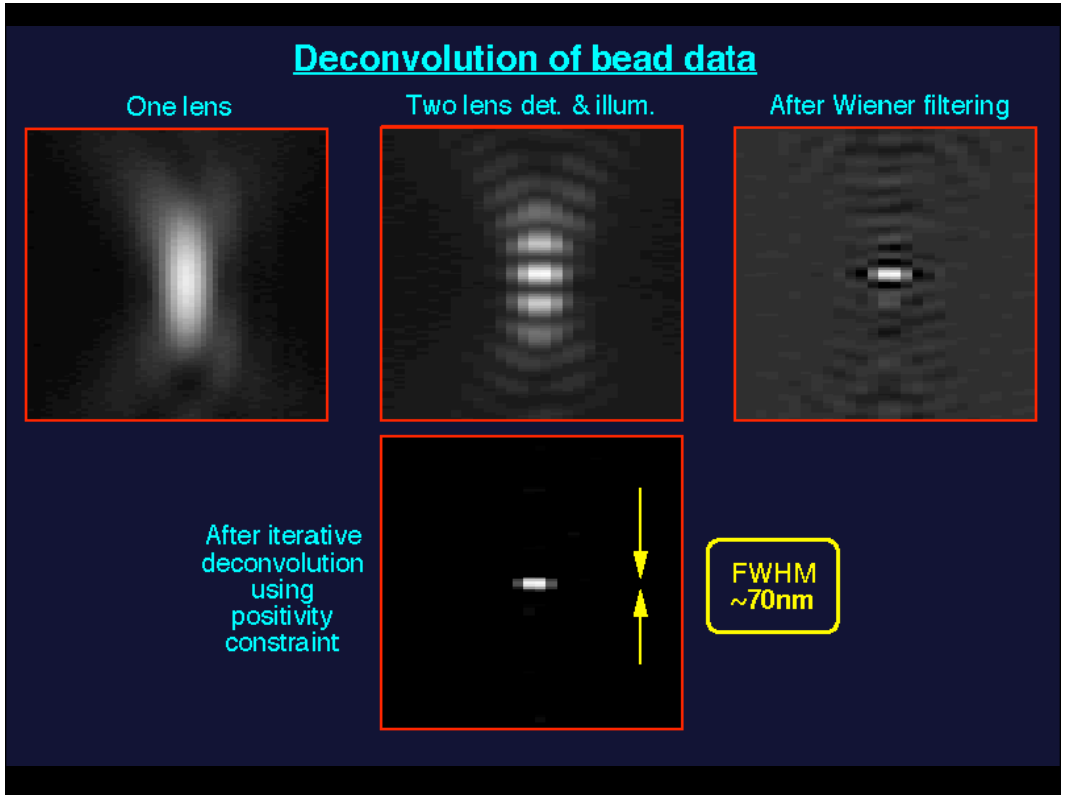
Observe through both lenses, ← I2M, "Image Interference Microscopy"

illuminate through both lenses, ← I3M, "Incoherent Interference Illumination"

or both, ← I5M

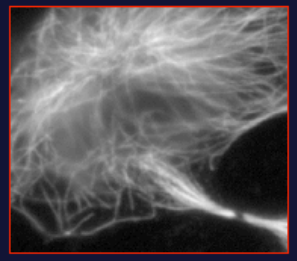

I5M OTF
CCD
Incoherent
light
source
Detection
angles
Illum.
angles
OTFdet
illum
OTFeff =
OTFdet
illum
=
OTFeff

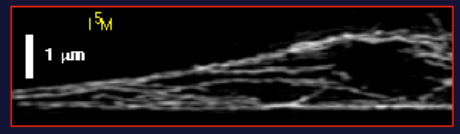

Observable Information
[Optical Transfer Functions]
Conventional Microscopy
I2M (Detection through two lenses)
I5M (Illumination and detection through two lenses)
Theory
Measured
kx
kz


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

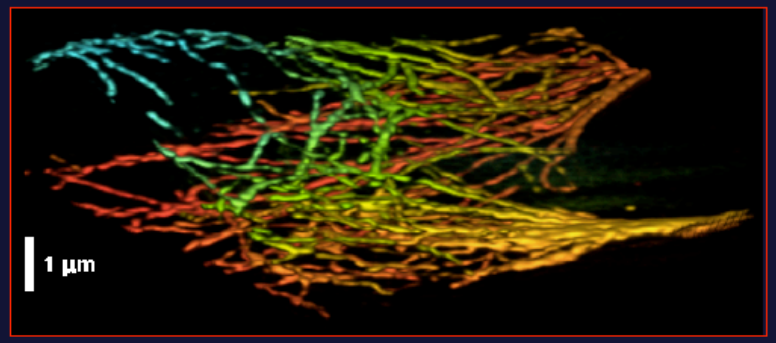
XY view of area



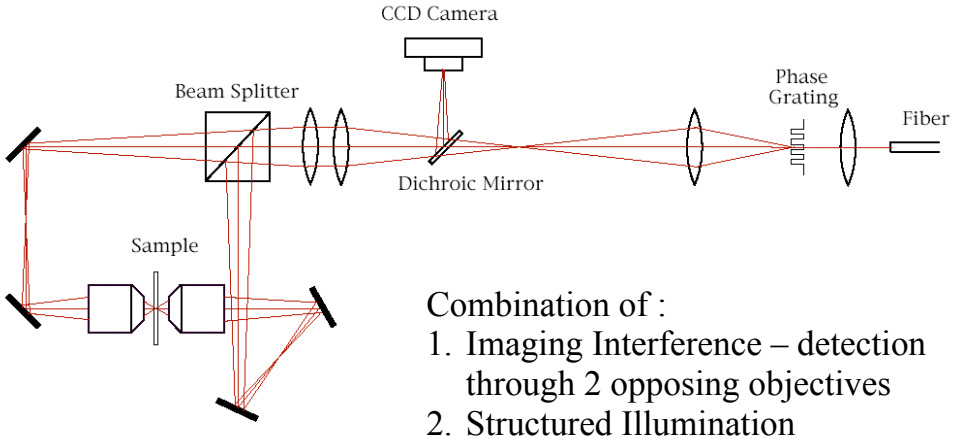


XZ projections 1 µm slices

I5M 3D view



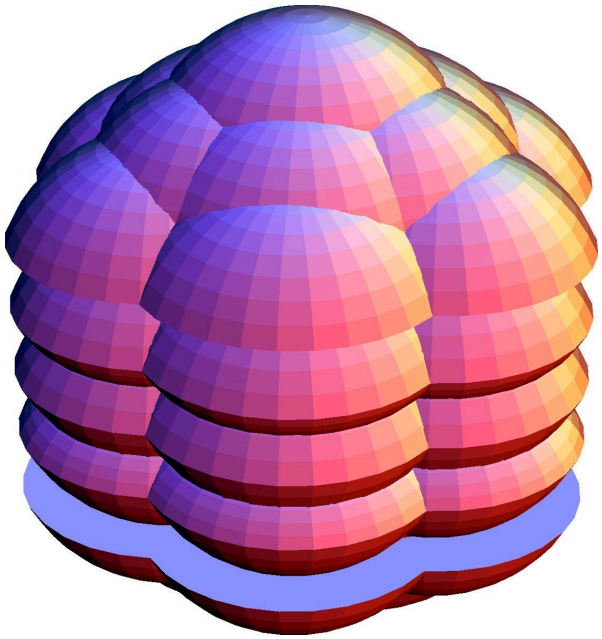
Combine methods: I^{5}S
use two lenses to collect more angles



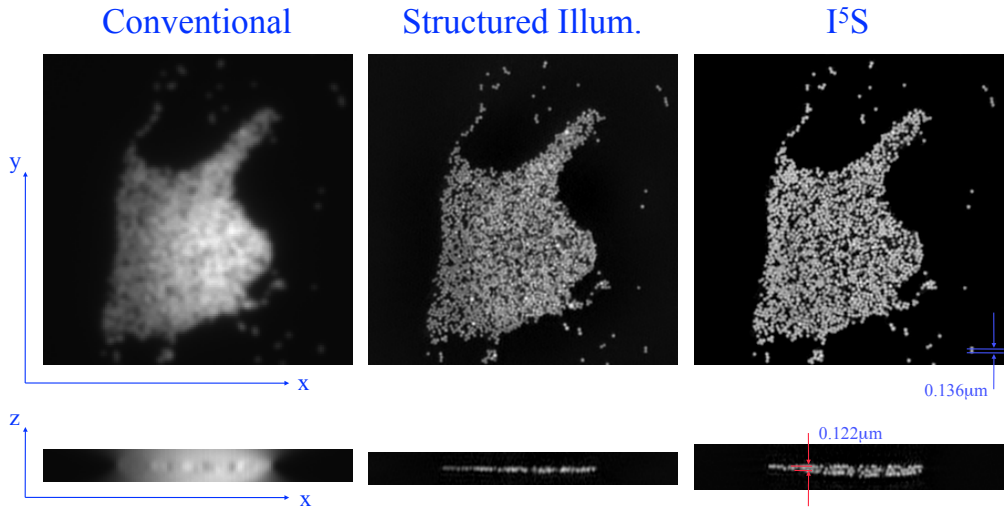
Combination of :

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

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



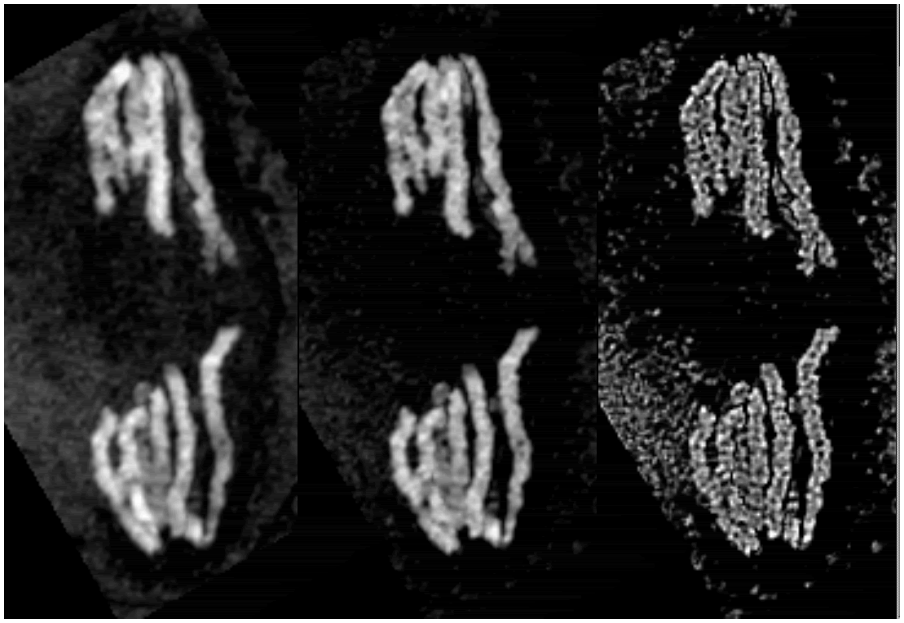
Comparison of Resolving Power



Sample: 0.12μm red-fluorescence microshperes

Lin Shao, Mats Gustafsson

Drosophila Anaphase Chromosomes (.2μm wide)

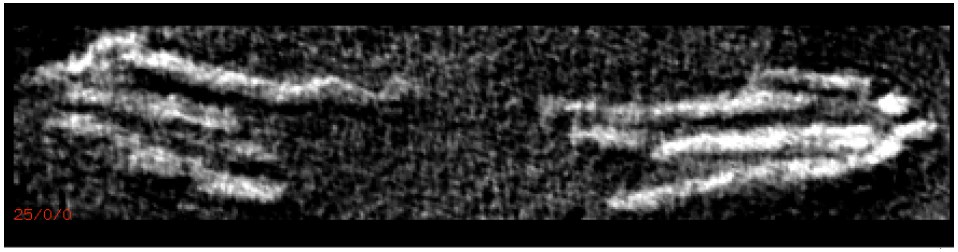


Lin Proc. **Decon** **Decon + LCE**

Lin Shao

Comparison between OM and EM-Tomo

EM



OM