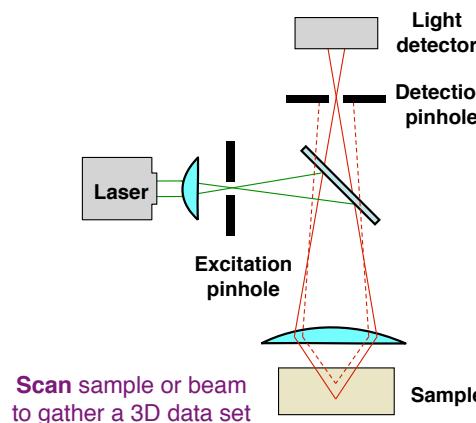


# 3D fluorescence microscopy

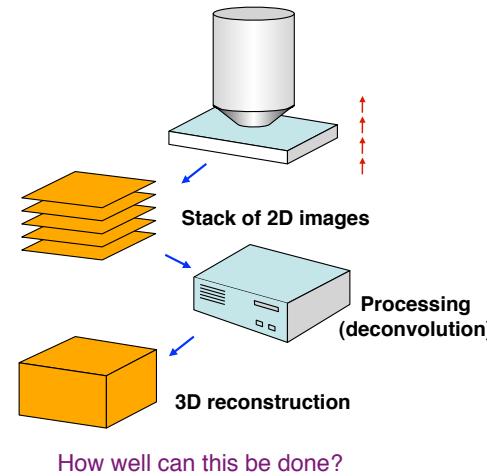
For 3D, acquire a “focal series” (stack) of images:  
Take an image, refocus the sample, take another image, refocus, etc.

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

**Approach 1:**  
**Physically exclude the blur by**  
**confocal microscopy**



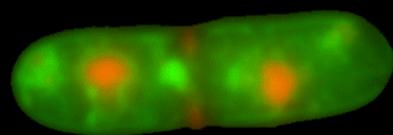
**Approach 2:**  
**Remove the blur computationally**



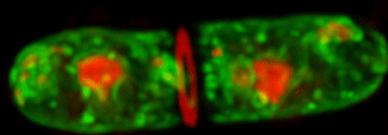
## Deconvolution of 3D data

(Dividing fission yeast cell)

Raw data



Deconvolved



4 μm

4 μm

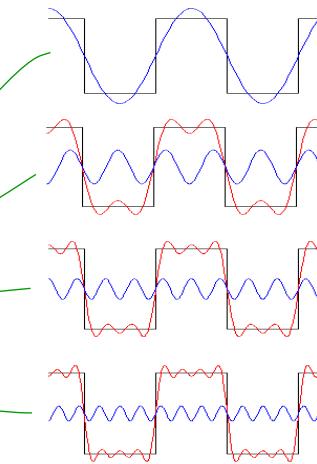
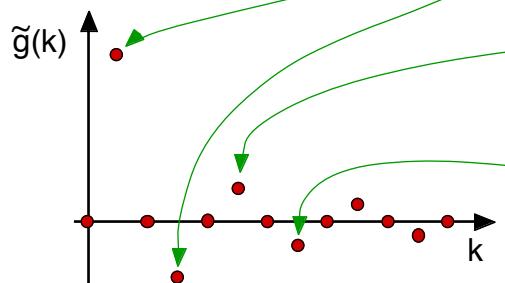
## The Fourier Transform

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

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

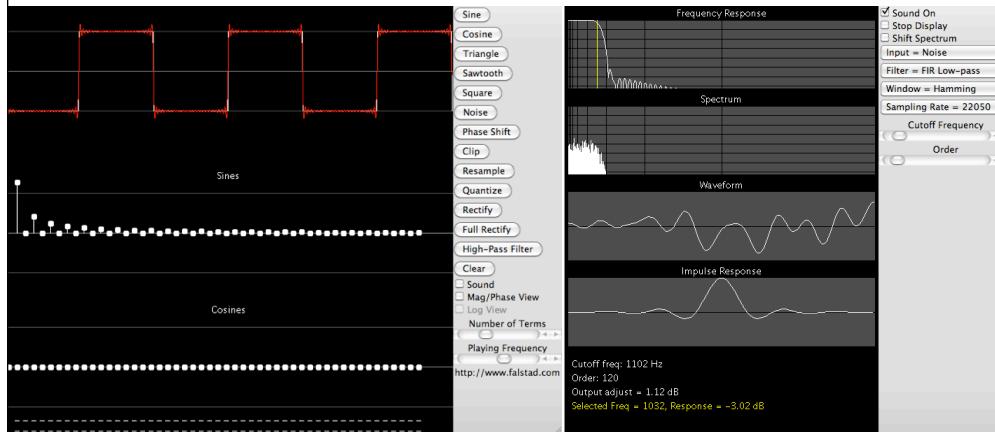
The “Fourier transform”  $\tilde{g}(k)$  specifies the amplitude  $A$  and the phase  $\phi$  for the component wave of wavelength  $L = 1/k$

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



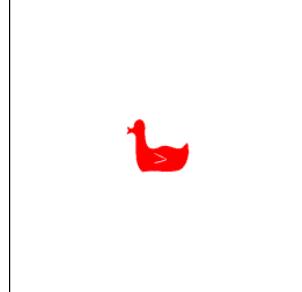
# Fourier Transform and Digital Filter Applets

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

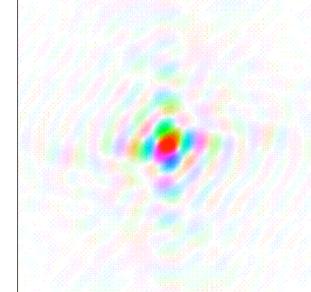


## Low Resolution Duck

A Duck

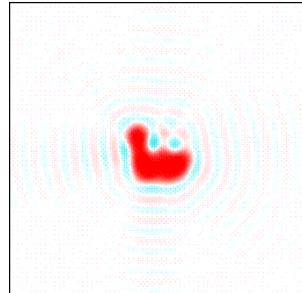


and its scattering function (Fourier Transform):

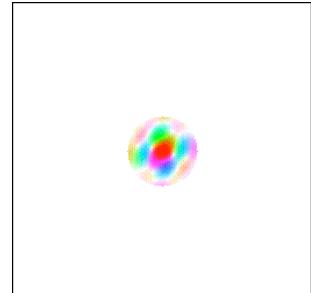


a blurred duck

<=



Using only low resolution data



## Convolutions

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

Why do we care?

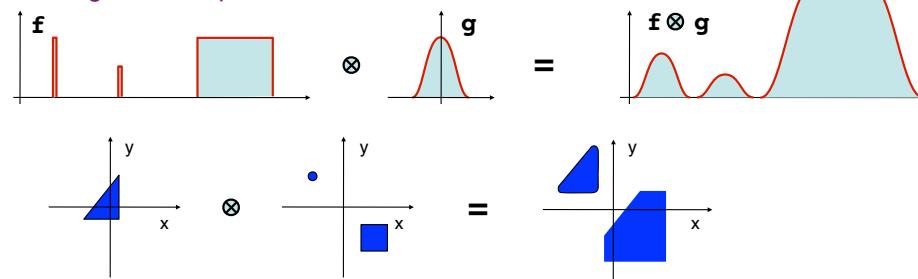
- They are everywhere...
- The convolution theorem:  
If  $h(r) = (f \otimes g)(r)$ ,  
then  $\tilde{h}(k) = \tilde{f}(k) \tilde{g}(k)$

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

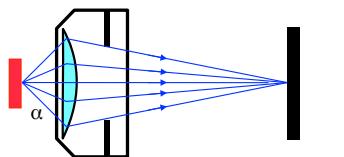
Symmetry:  $g \otimes f = f \otimes g$

So what is a convolution, intuitively?

- "Blurring"
- "Drag and stamp"



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



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

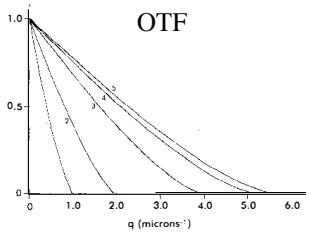


Figure 3 The in-focus contrast-transfer functions for several Zeiss lenses are compared. The curves are (1) 10  $\times$  0.25 N.A., (2) 25  $\times$  0.5 N.A., (3) 50  $\times$  1.0 N.A. oil, (4) 100  $\times$  1.25 N.A. oil, (5) 63  $\times$  1.4 N.A. oil.

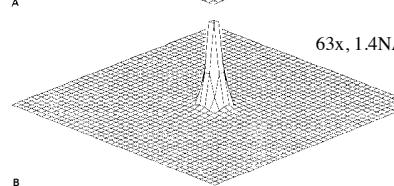
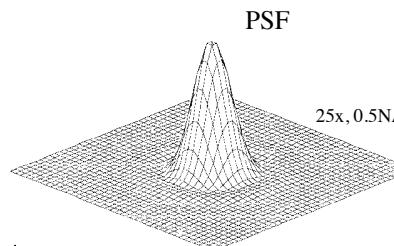
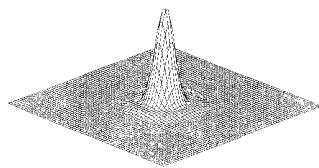
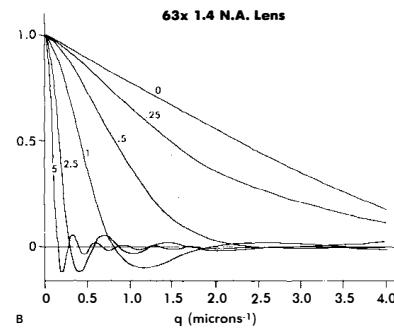
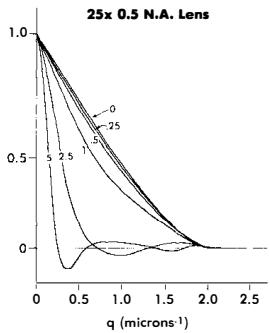
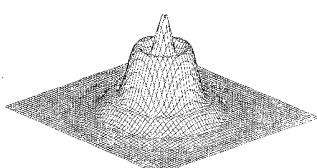


Figure 4 The point-spread functions calculated by Fourier transformation of the CTF's of Figure 3. (A) 25  $\times$  0.5 N.A. lens (#2); (B) 63  $\times$  1.4 N.A. lens (#5). Each grid square is  $\lambda/6$  or 833 Å. Note how much sharper the 1.4 N.A. lens is than the 0.5 N.A. lens.

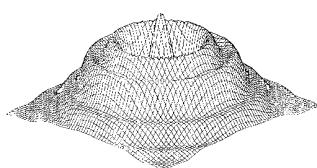
## OTF, PSF of a defocused lens



25x lens 0  $\mu\text{m}$  defocus

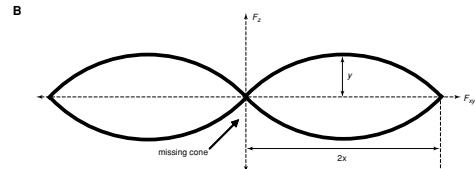
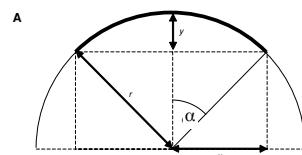


25x lens 2.5  $\mu\text{m}$  defocus

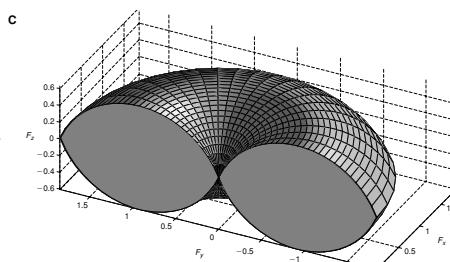


25x lens 5  $\mu\text{m}$  defocus

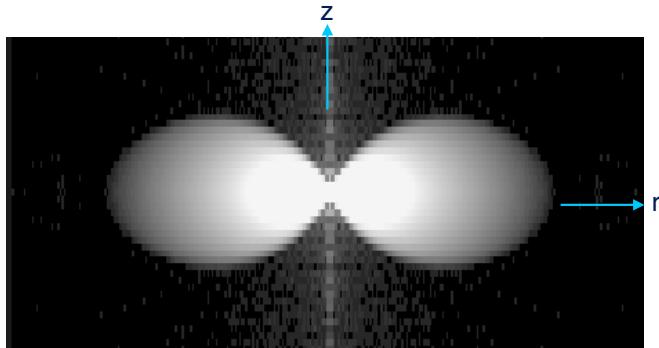
## 3D OTF



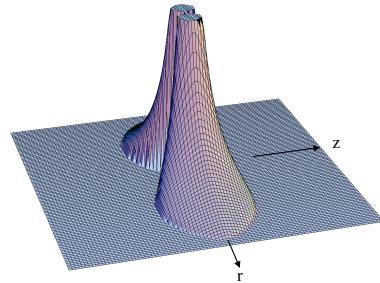
Only a bowl-shaped segment of the shell makes it through the objective:  
The intensity now becomes a convolution of the **bowl** with itself, which has  
a donut-shaped region of support.



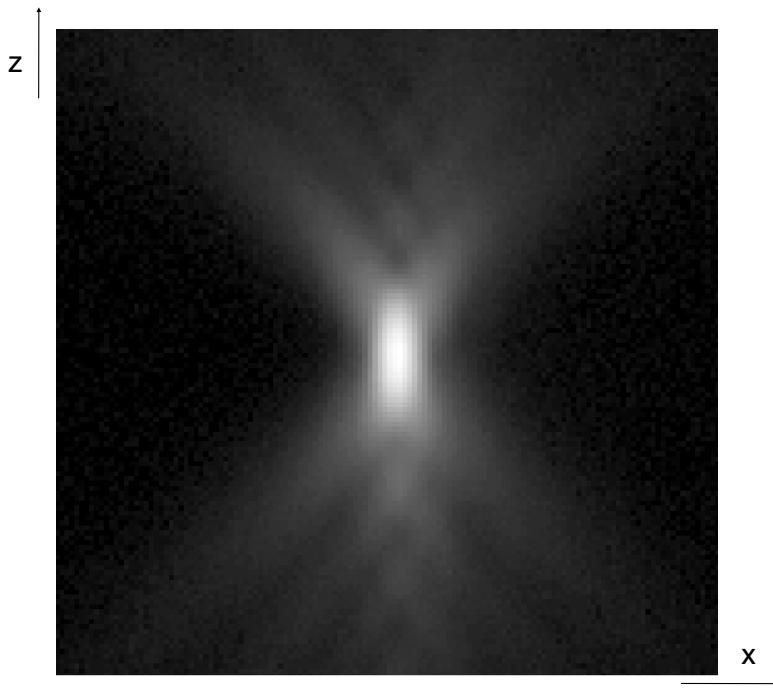
## Experimentally measured OTF



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

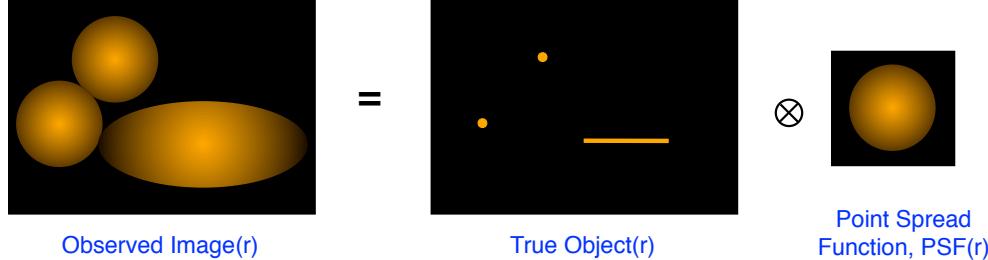


### Experimentally measured PSF



## PSF, OTF & deconvolution

In real space:



In reciprocal space the convolution becomes a product:

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

This suggests:

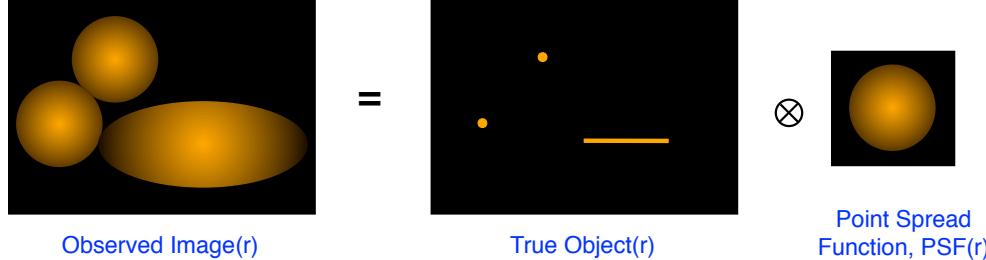
$$\text{Object} = \frac{\text{Image}}{\text{OTF}}$$

$\text{PSF}$  is called the  
“Optical Transfer Function”,  
OTF

??? (“Deconvolution”)

## PSF, OTF & deconvolution

In real space:



In reciprocal space the convolution becomes a product:

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

This suggests:

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

$\text{PSF}$  is called the  
“Optical Transfer Function”,  
OTF

??? (“Deconvolution”)

What's the catch??

A: We can't divide by  $\text{OTF}(k)$  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

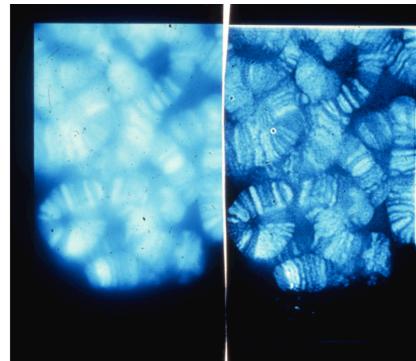
$$I_j \approx c_1 [ O_j - c_2 (PSF_{\Delta z} \otimes O_{j+1} + PSF_{-\Delta z} \otimes O_{j-1}) ]$$

for speed do the convolutions as Fast Fourier Transforms (FFT), multiplication, FFT<sup>-1</sup>

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

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

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

$\gamma$  is related to the signal to noise, sets maximum amplification

or if OTF is complex:

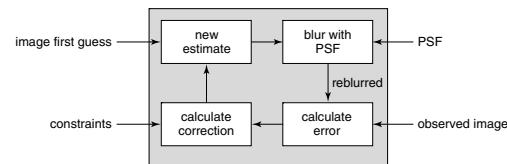
$$\frac{\text{Image} \cdot \text{OTF}^*}{\text{OTF} \cdot \text{OTF}^* + \gamma}$$

## Deconvolution strategies

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

family of iterative constrained methods

work by calculating convolution, followed by update



arithmetic update (vanCittert's method)

start with  $I^0 = O$

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

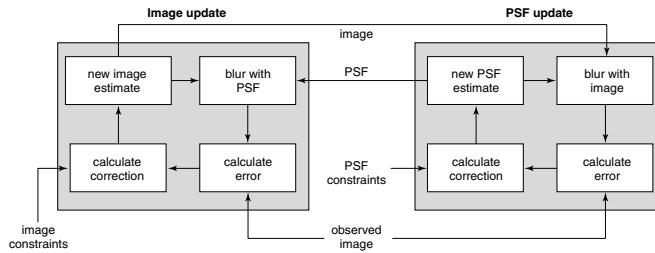
or use multiplicative update (Gold's method)

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

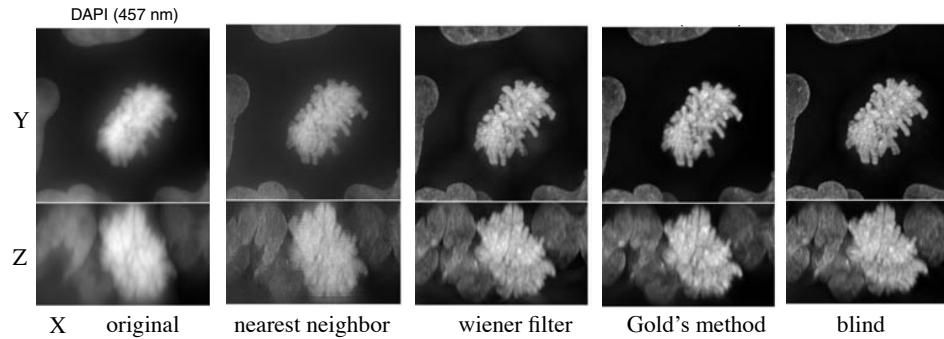
## Deconvolution strategies

what if your PSF is not accurate?

blind deconvolution seeks to estimate both Image and PSF



## Some comparisons: Hela cells, DAPI stained

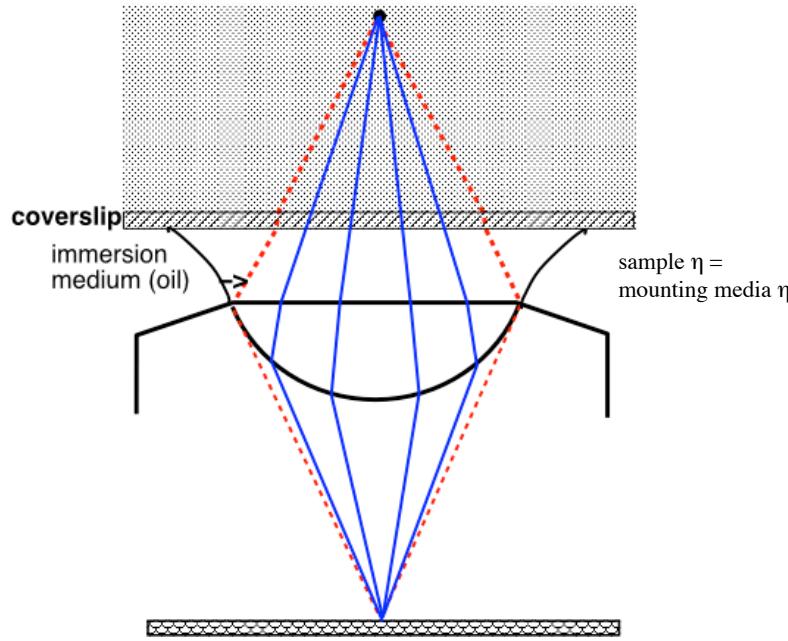


now many related variations

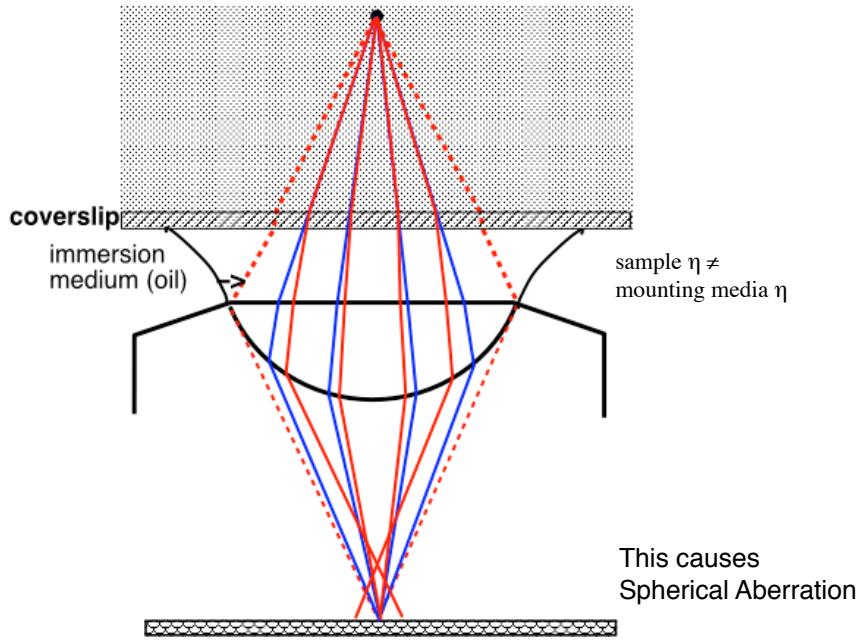
practical issues are signal to noise, accuracy of PSF

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

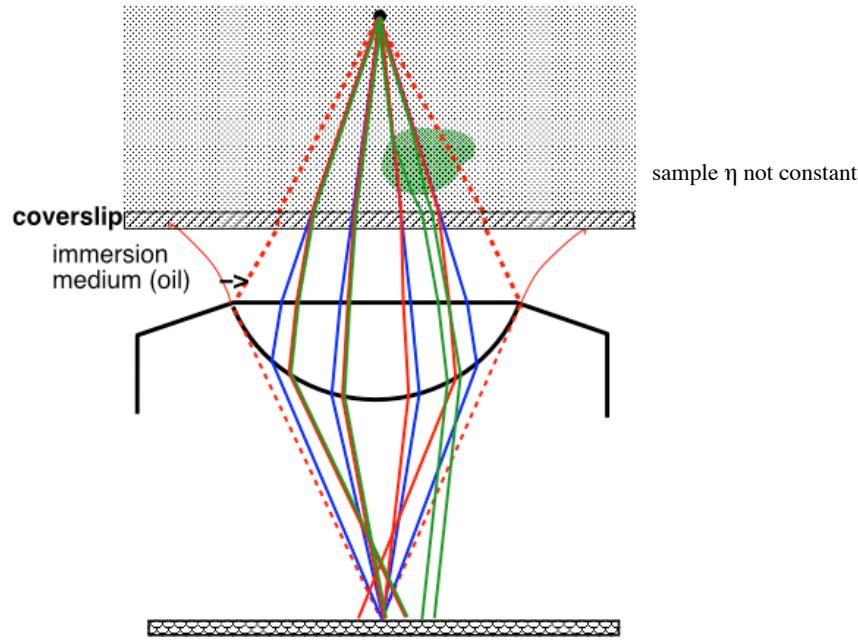
## Properties of the Sample Affect Image Formation in Microscopy



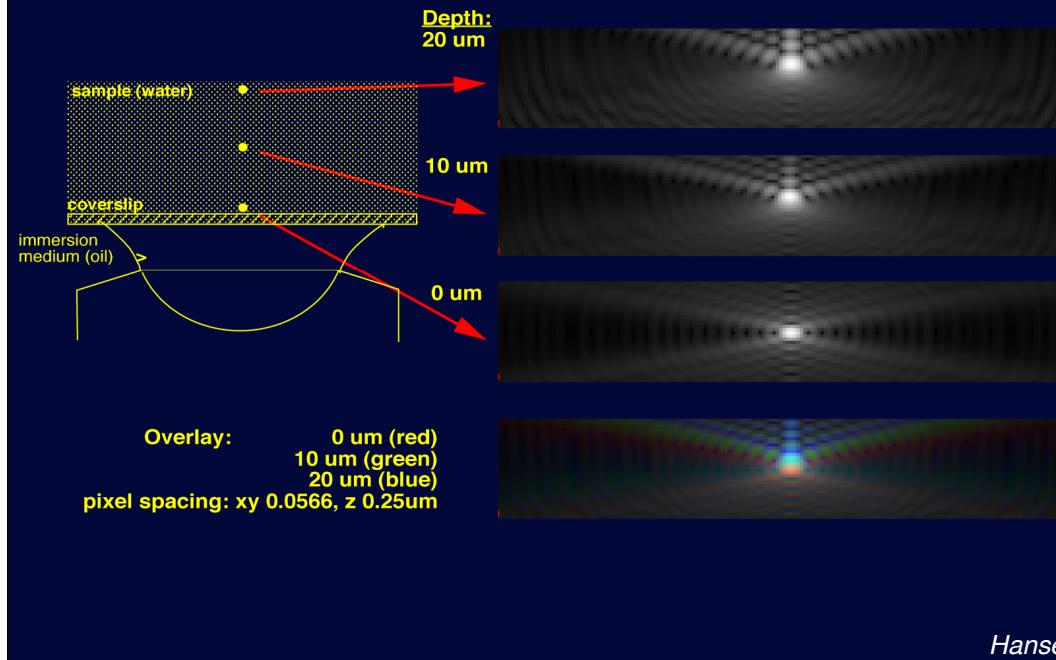
## Properties of the Sample Affect Image Formation in Microscopy



## Properties of the Sample Affect Image Formation in Microscopy

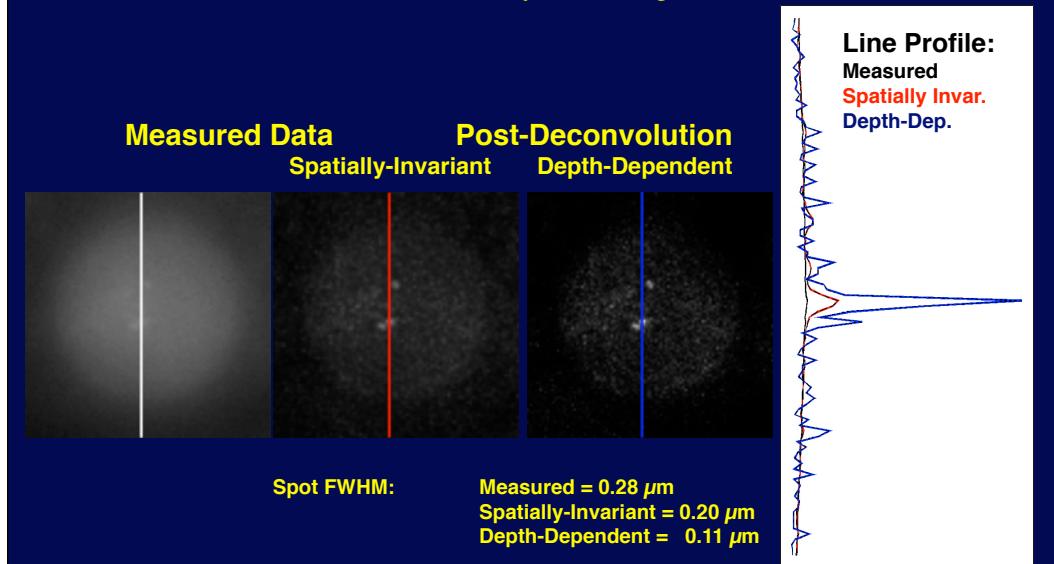


## Depth-Dependent Effects in Water: Simulations of PSFs



## Depth Dependent Deconvolution: Biological Data

Line Profile Through 1 Spot in Nucleus  
Section ~7 .6  $\mu\text{m}$  into sample



Hanser

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

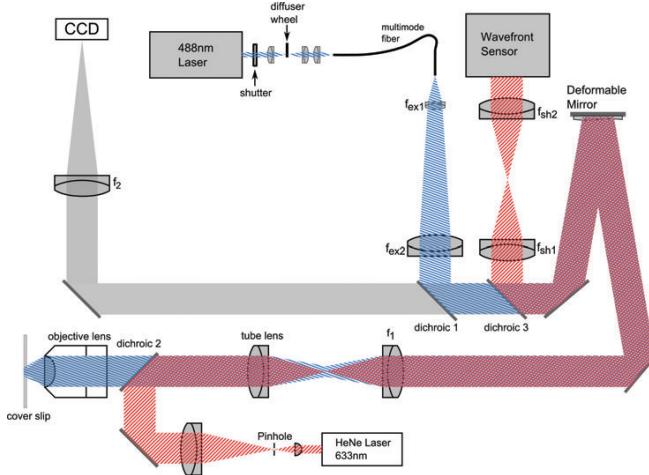
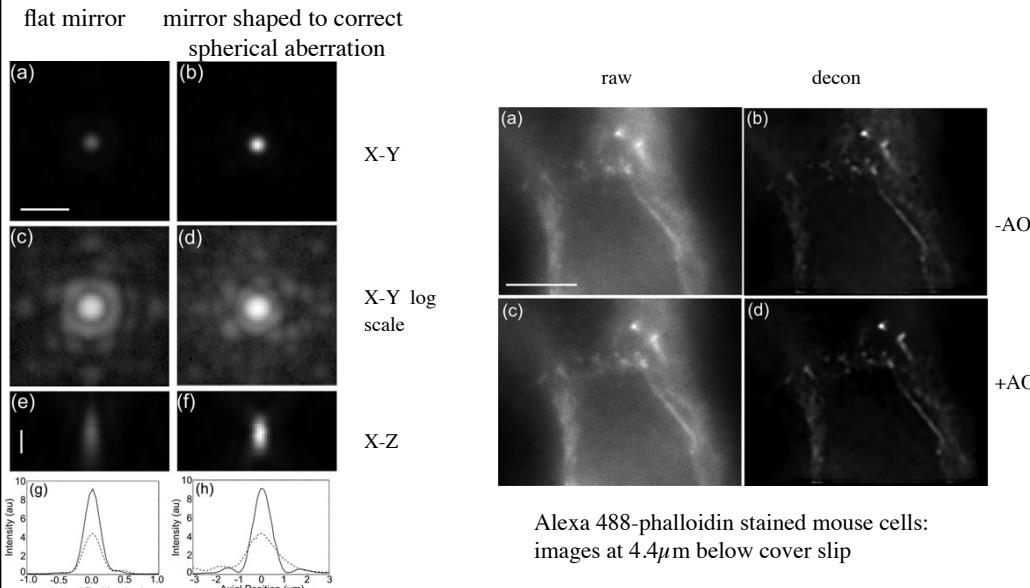


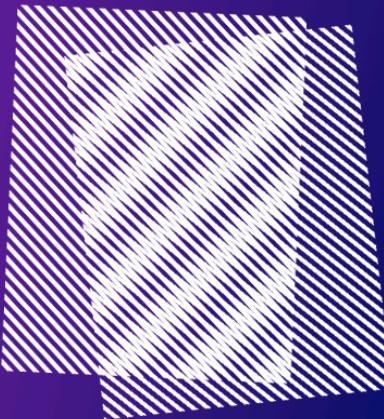
Fig. 2. Microscope layout. Grey represents the emission path. The blue striped beam is the excitation light and the red striped beam is the reference beam.  
See text for details.

## Adaptive optics can correct for depth dependent effects



## Structured illumination microscopy: the idea

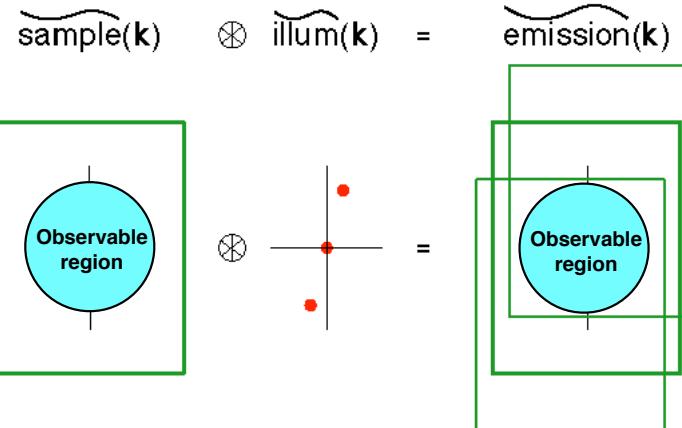
Two patterns  
superposed  
multiplicatively  
give rise to  
**moiré fringes**



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*

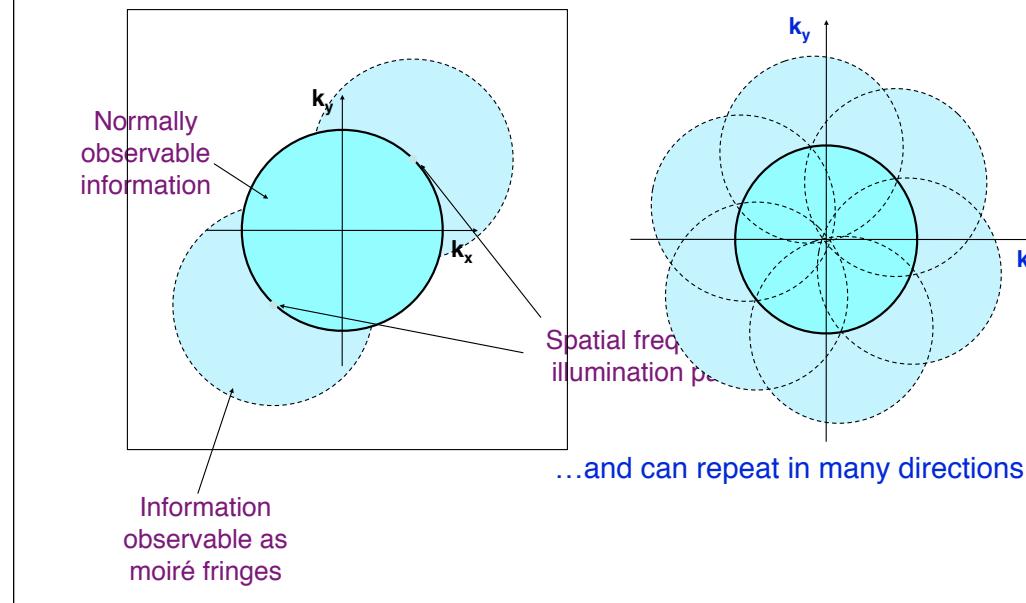
## *Illuminate sample with parallel stripes*



*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*

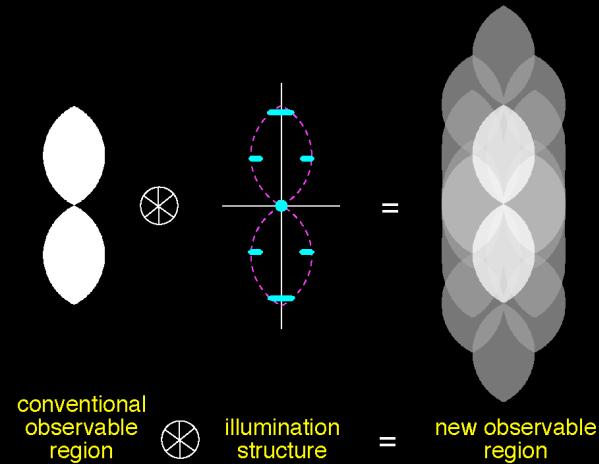
## *Resolution extension by Structured Illumination*



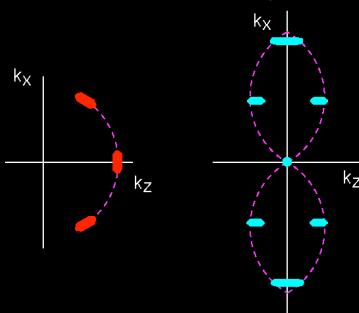
Q: What about 3D?

A: Use 3 beams

(admit diffraction orders 1, 0 and +1 )



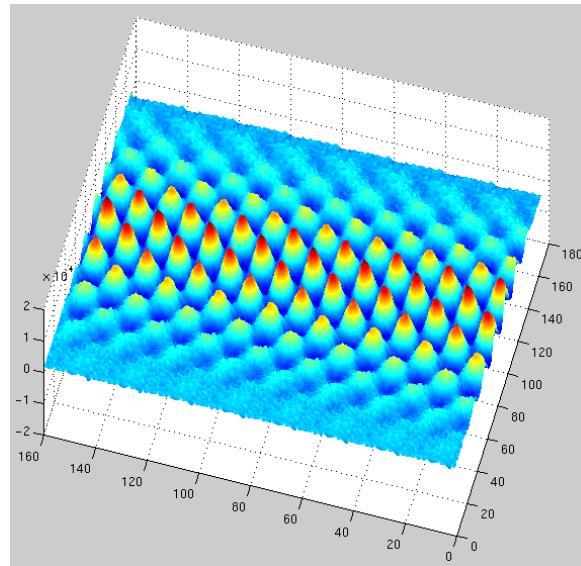
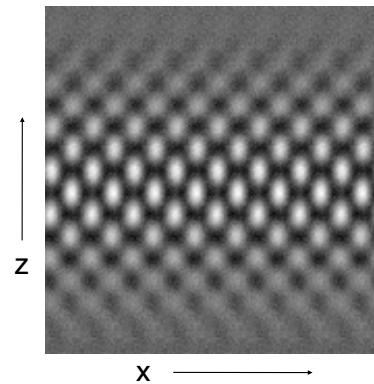
3 coherent  
beams → 7 intensity  
components



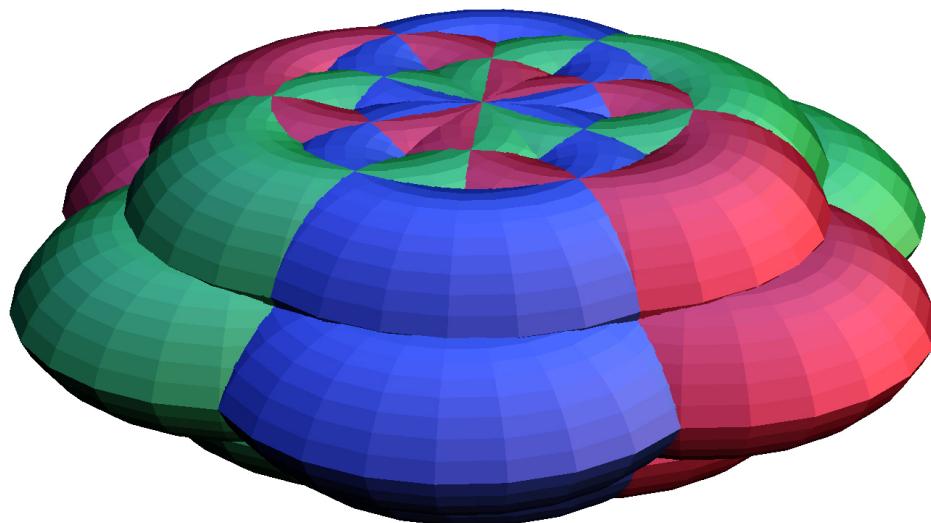
Covers full "confocal" OTF support  
without discarding light

Same procedure as for 2D,  
except need 5 phases

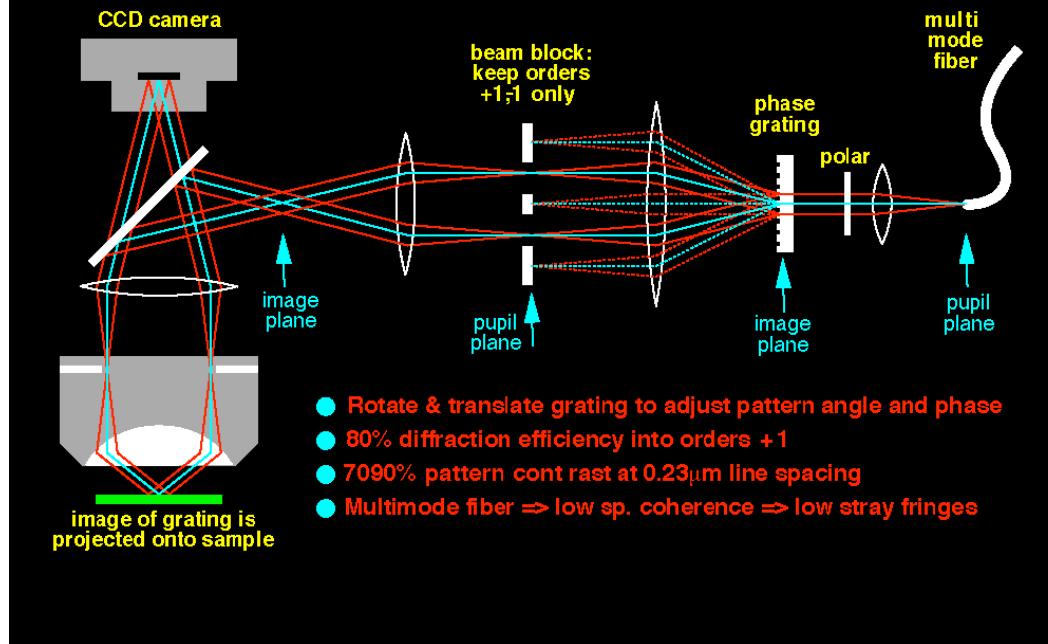
### illumination intensity in real space

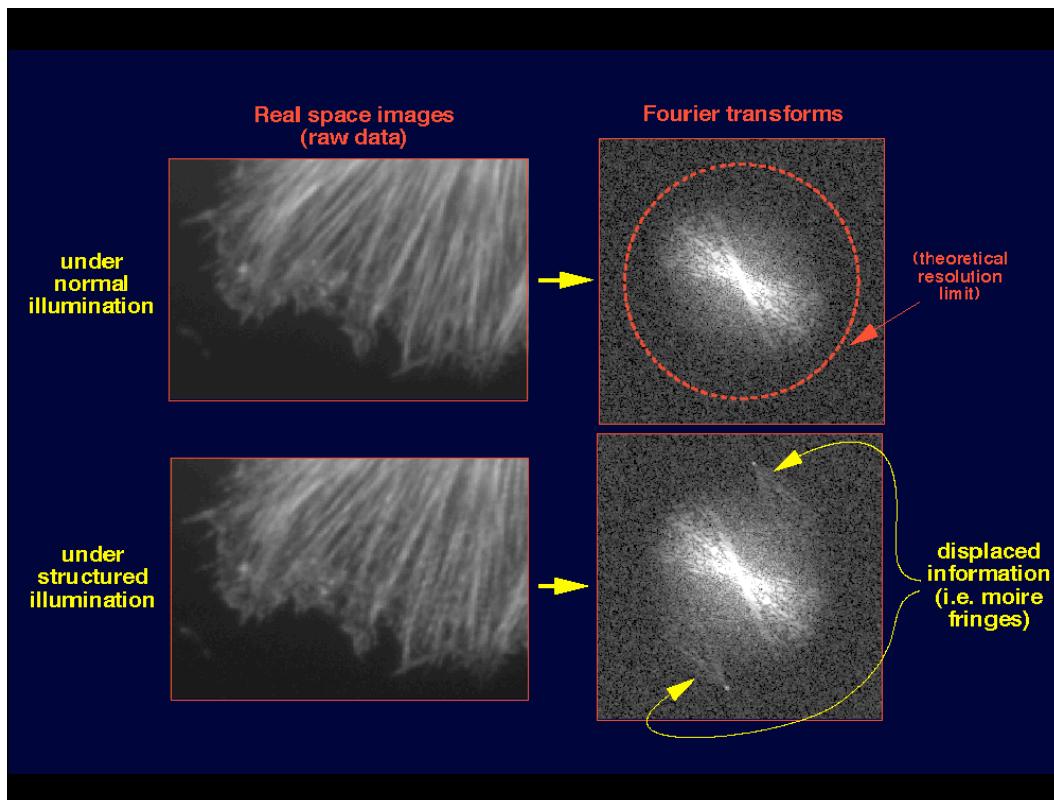


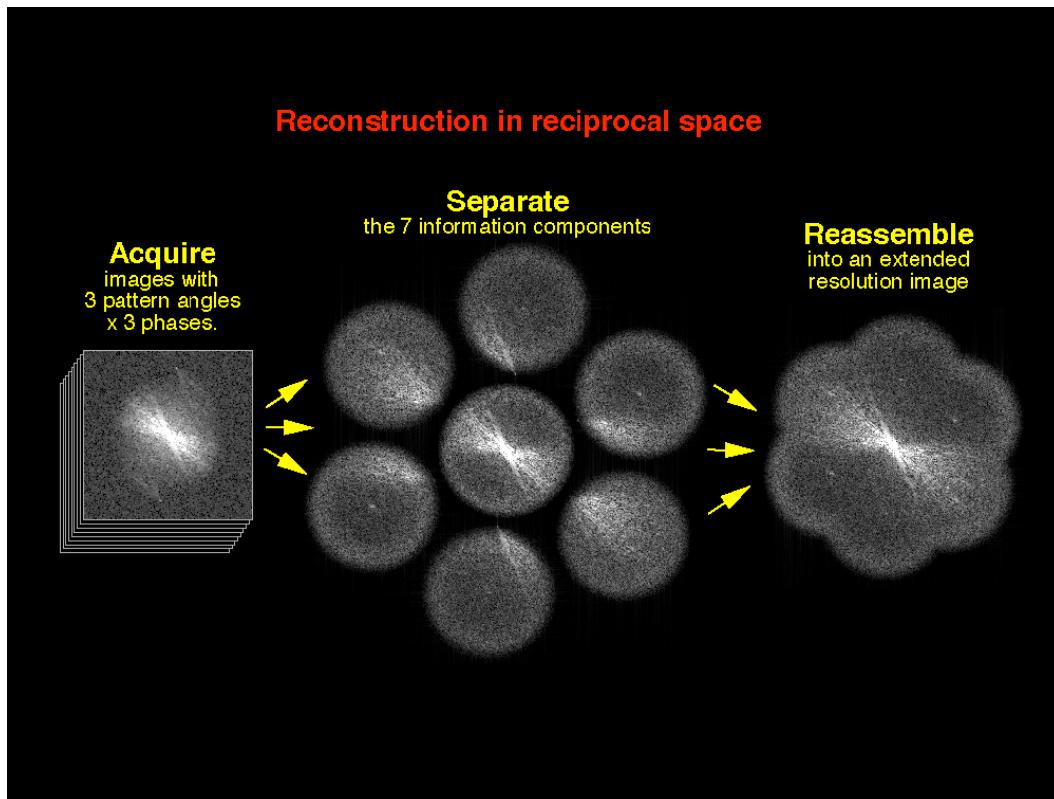
Observable Region  
Structured Illumination (3 orientations)



## Structured illumination apparatus

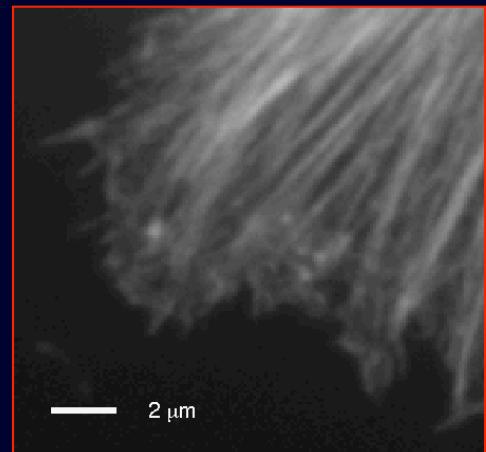




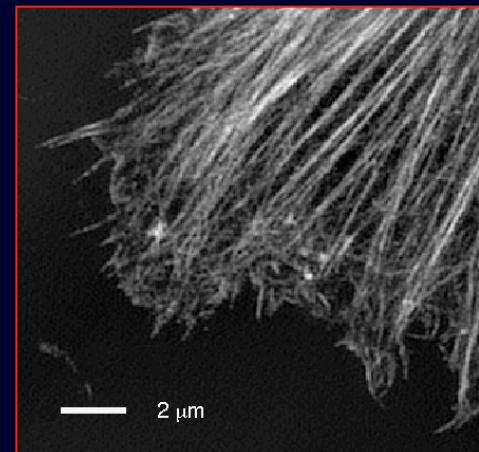


**Resolution comparison**  
Actin in a HeLa cell

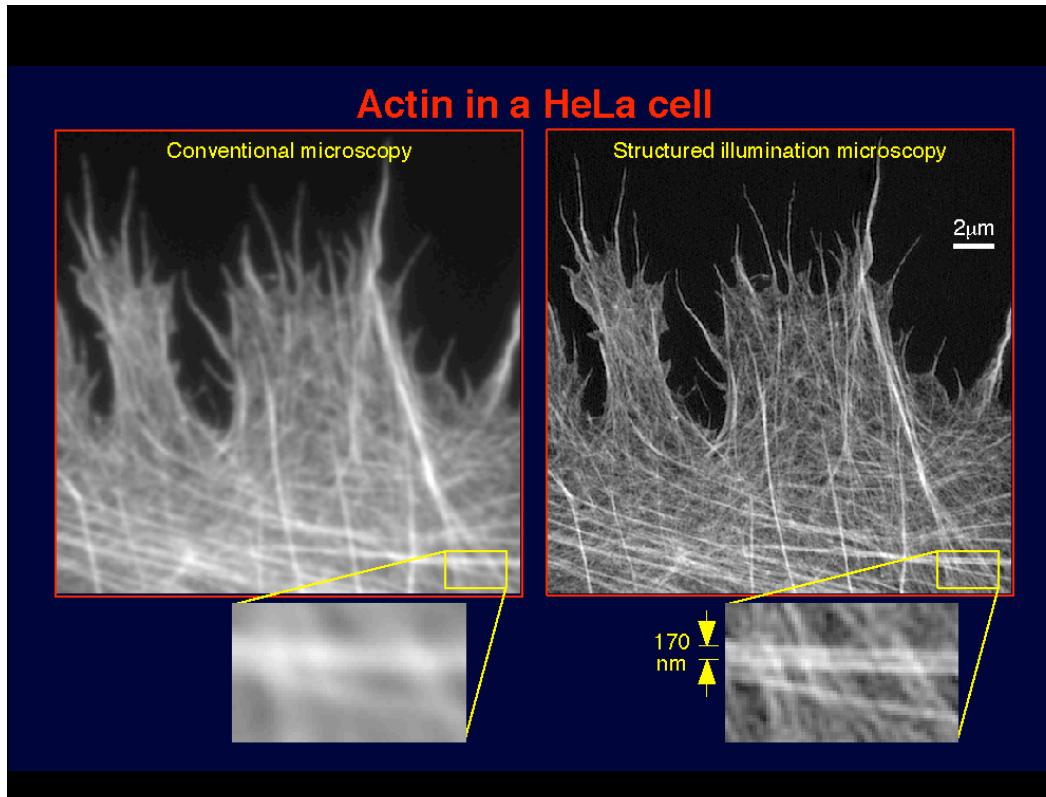
Conventional microscopy



Structured illumination  
final reconstruction

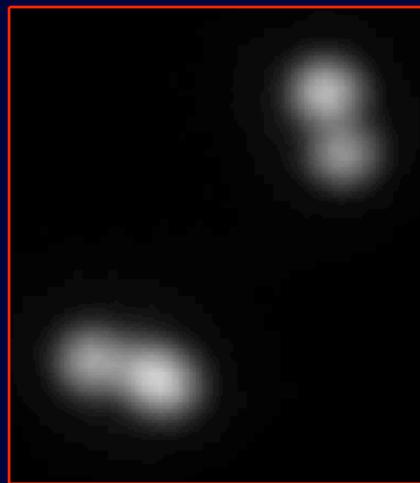


## Actin in a HeLa cell

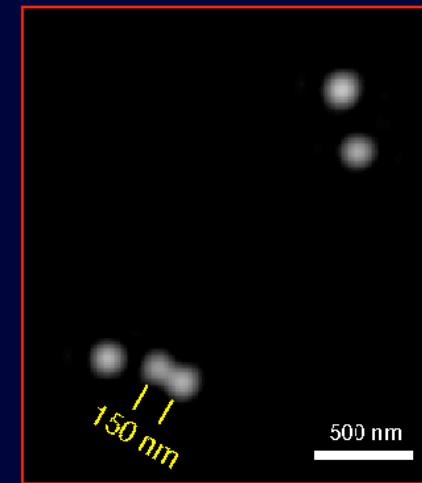


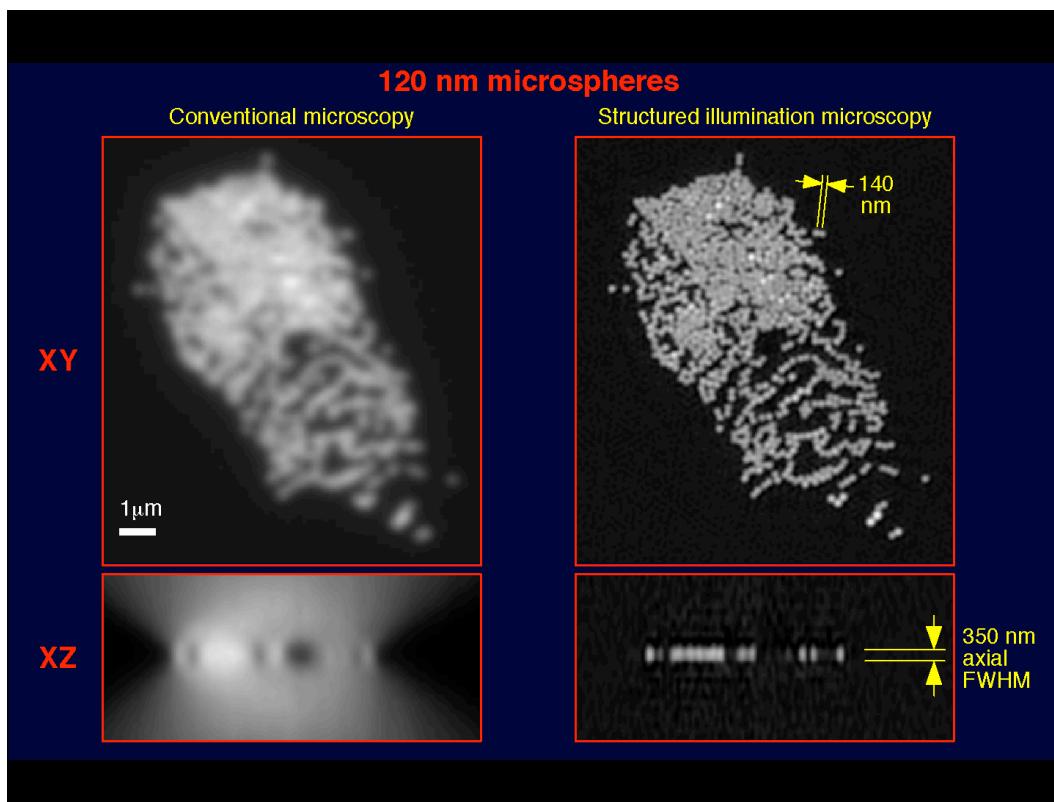
## 120 nm microspheres

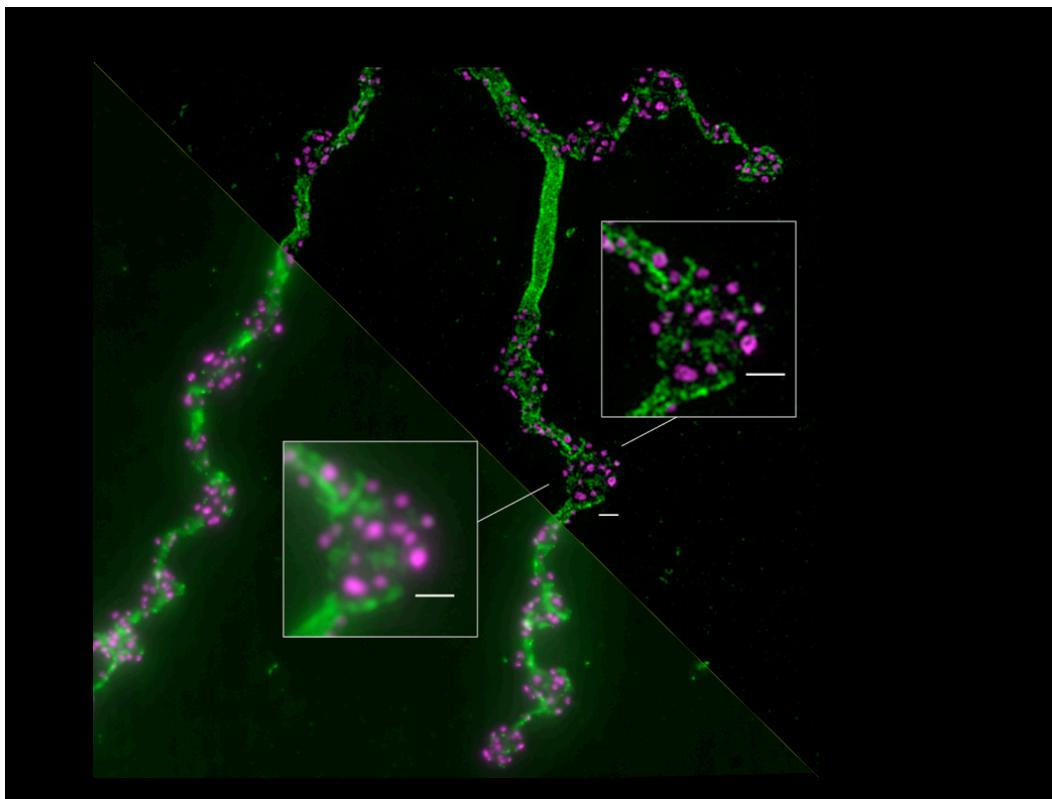
Conventional  
microscopy

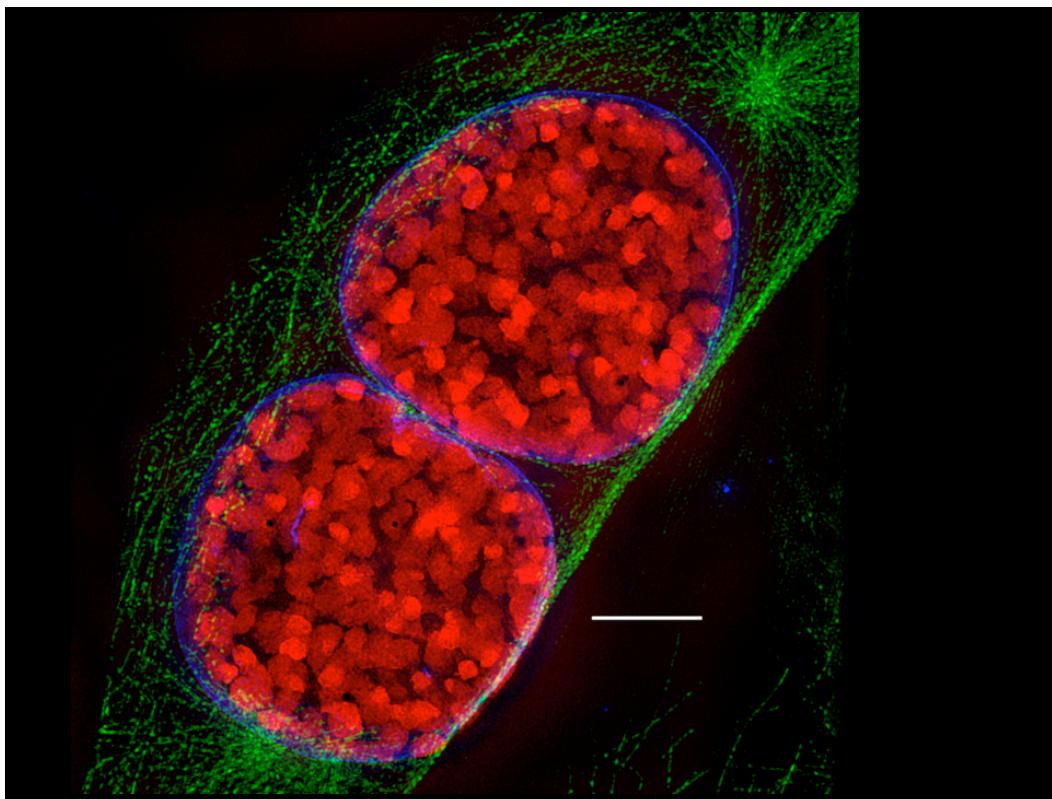


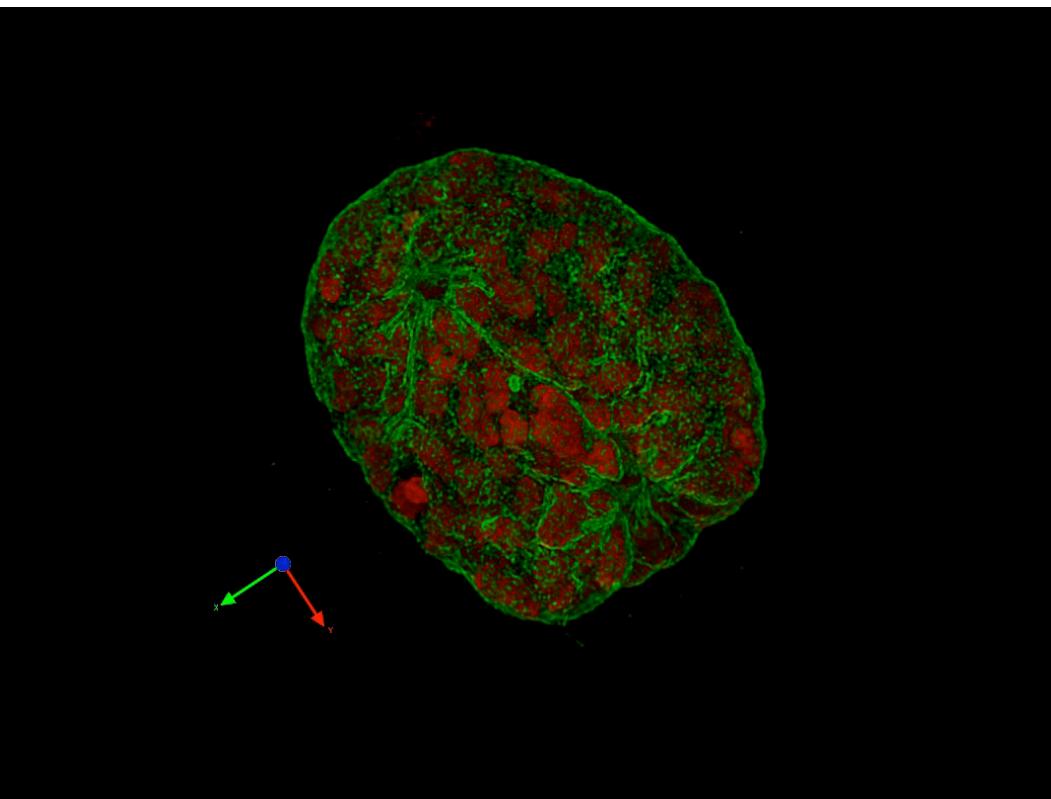
Structured illumination  
microscopy



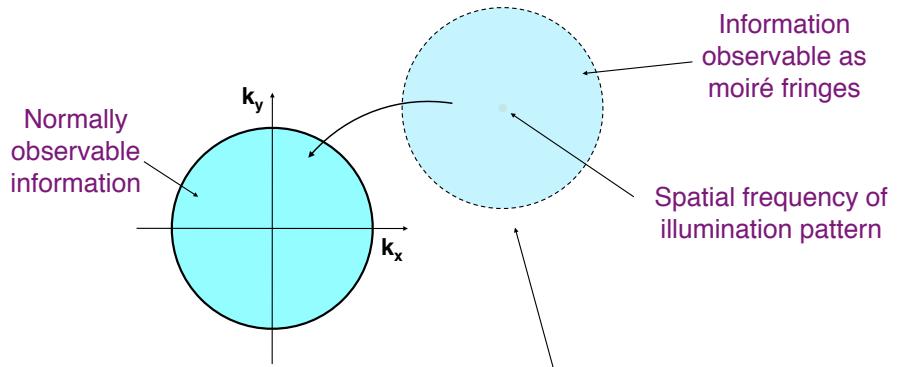








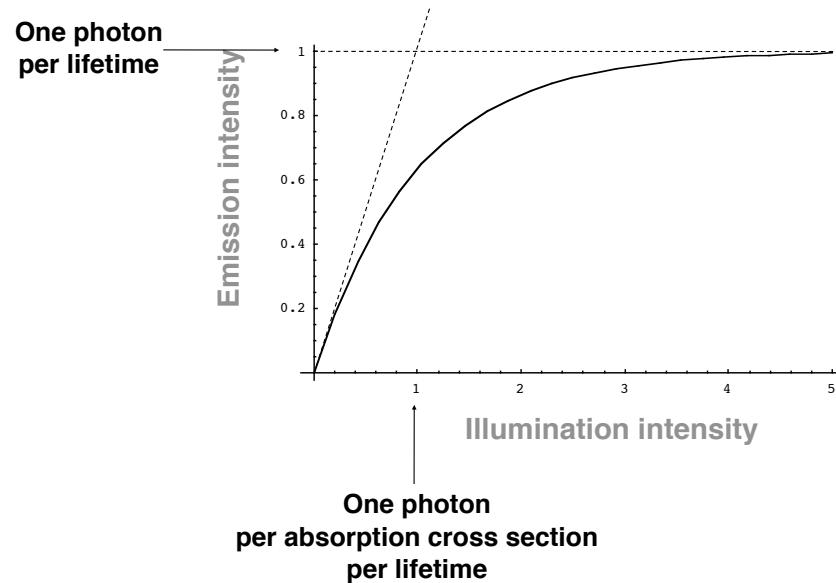
## *What are the limits of Structured Illumination?*



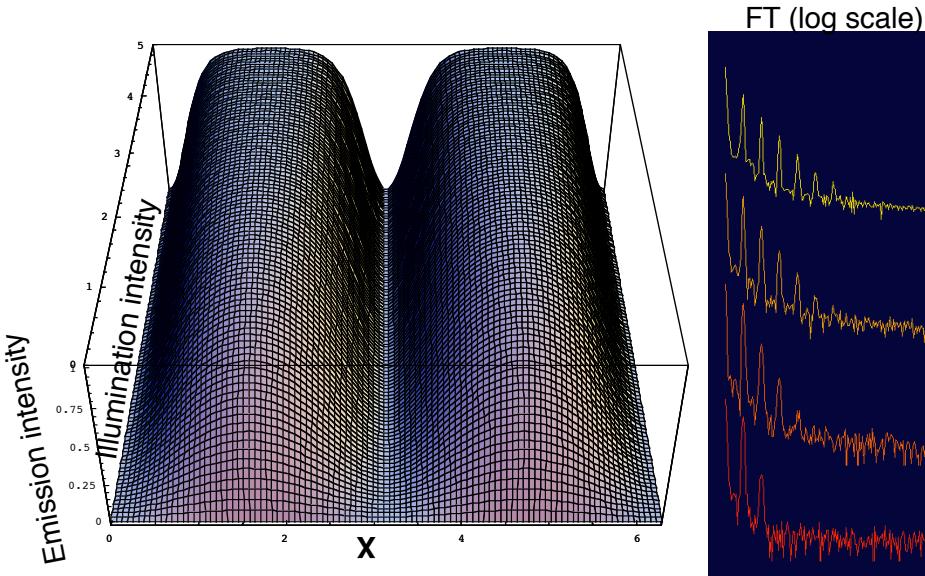
- Linear theory says this is impossible
- How about exploiting non-linear processes?

## *A simple source of nonlinearity: saturation*

(R. Heintzmann Max-Planck Gottingen)



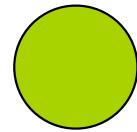
## *Saturated Structured Illumination*



## *Resolution extension by nonlinear structured illumination*

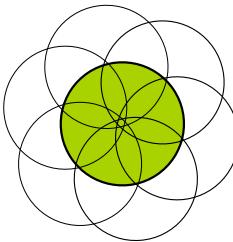
Effective observable regions

Conventional  
microscopy



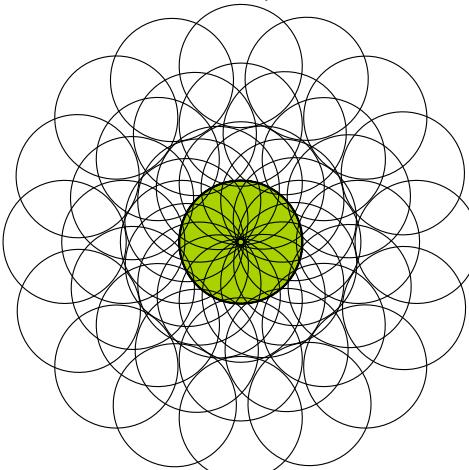
200 nm res.

Linear  
Structured  
illumination  
3 directions



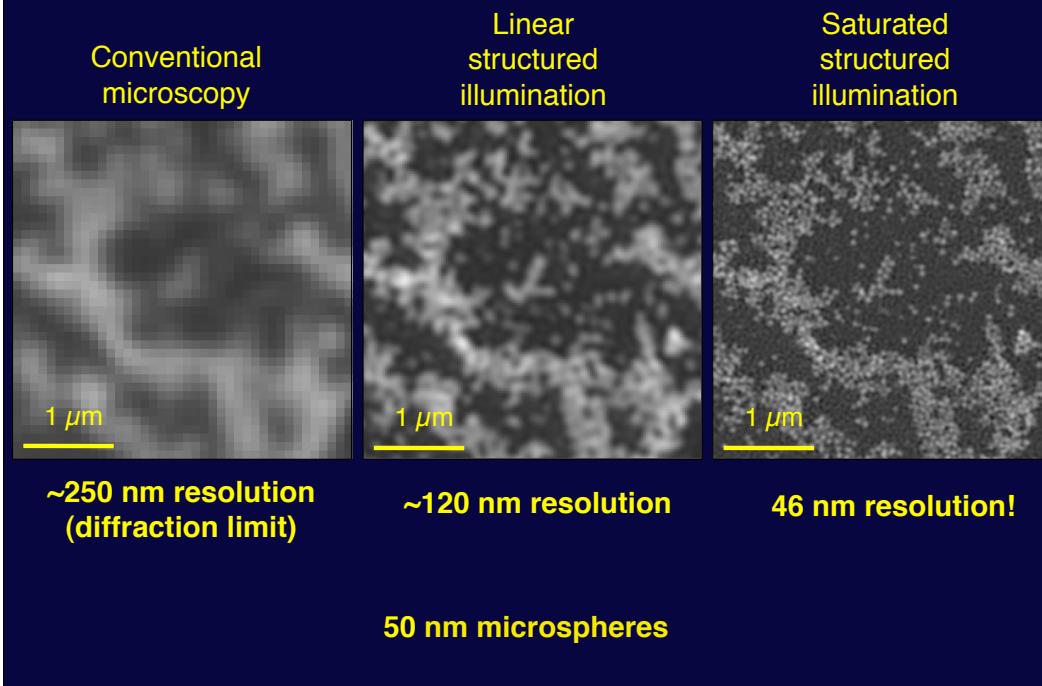
100 nm res.

Nonlinear structured illumination  
2 new harmonics, 8 directions

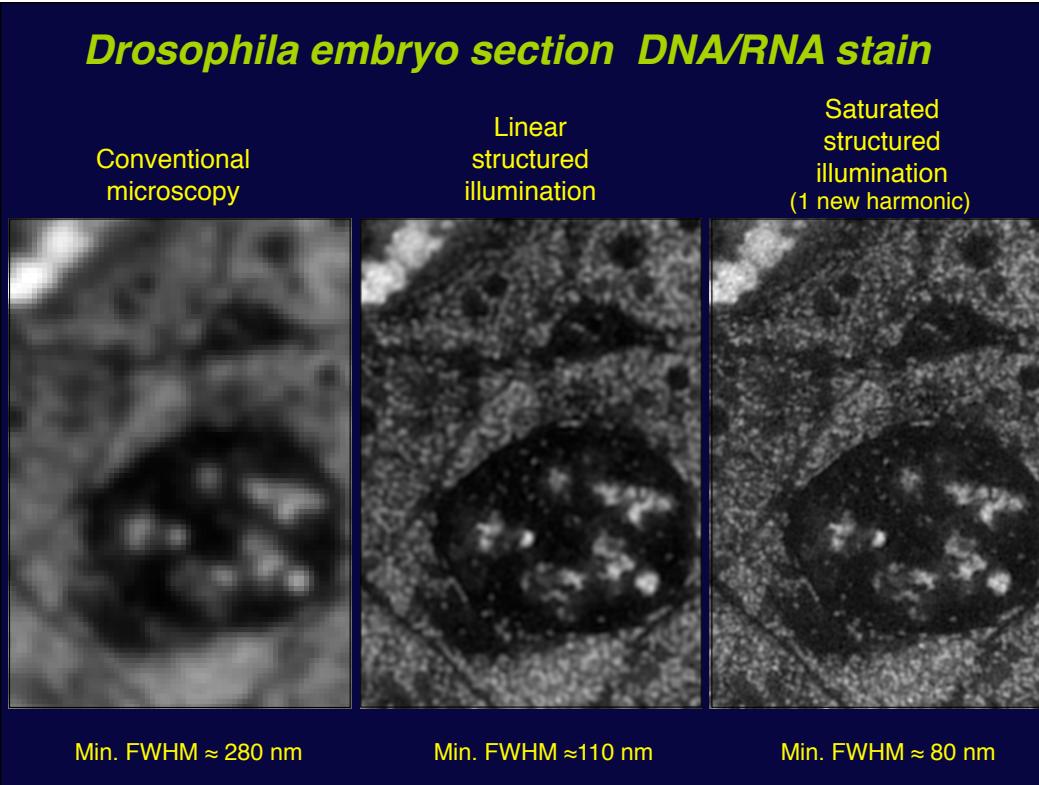


50 nm res.

## *Non-linear structured illumination*



### *Drosophila* embryo section DNA/RNA stain



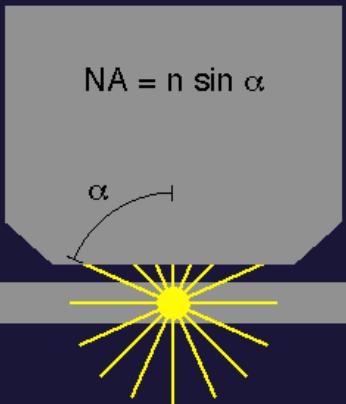
## Going beyond the diffraction limit: more light collection angles

We know:

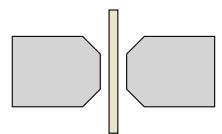
Higher NA  
→

Gathering light over  
larger set of angles  
→

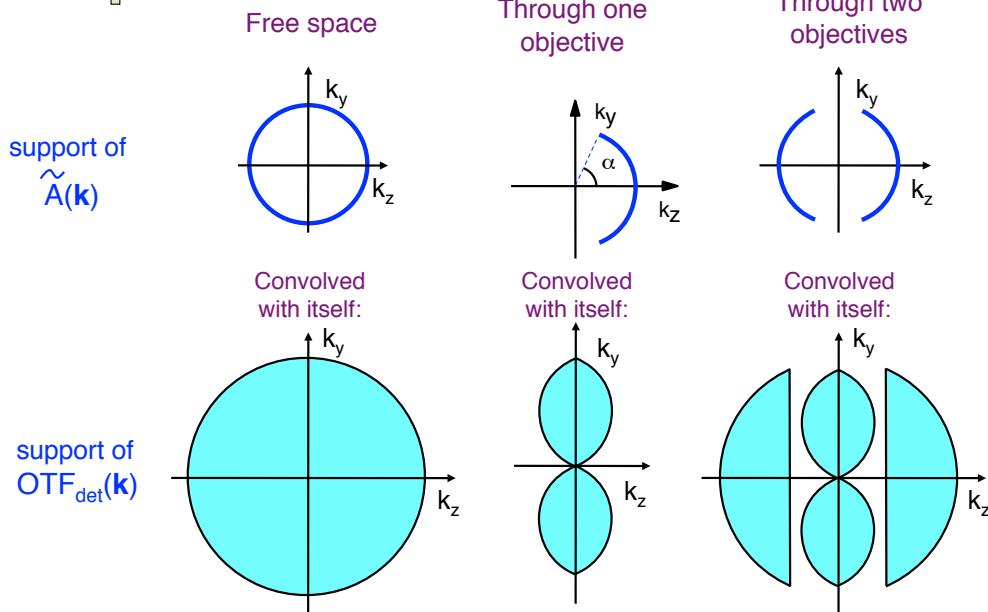
Higher resolution



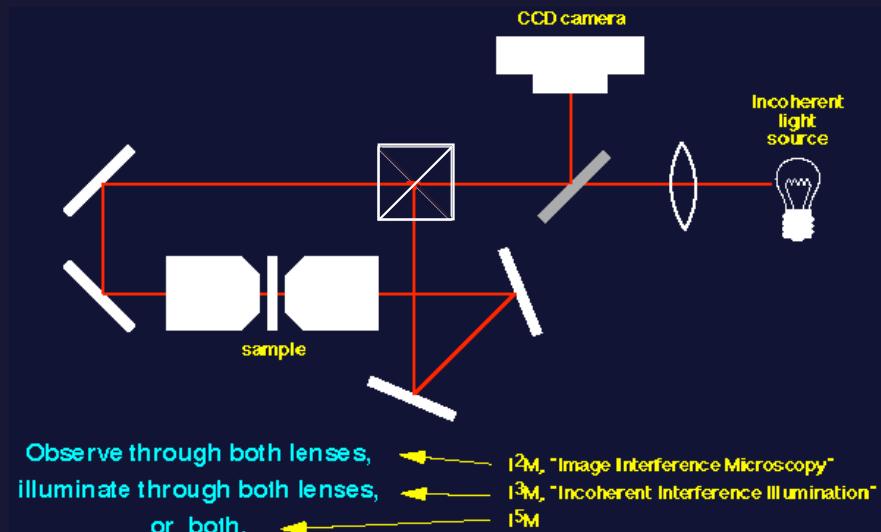
...So what about gathering the light  
emitted toward the back side?

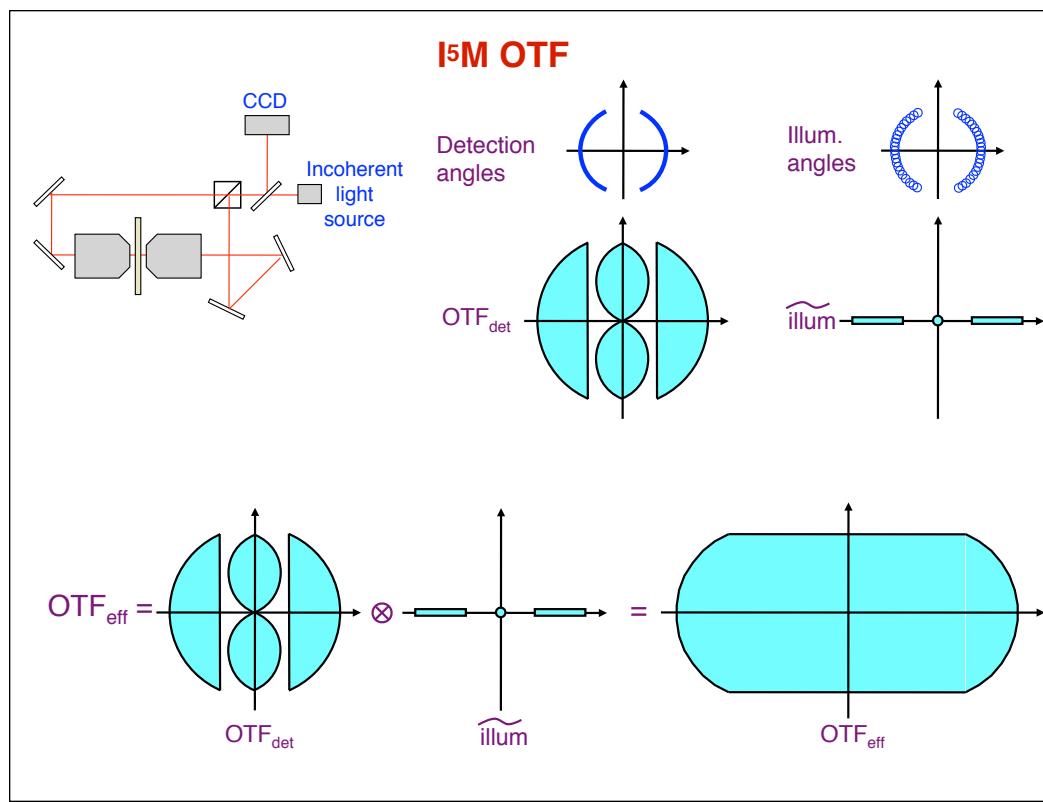


## OTF when detecting through two lenses

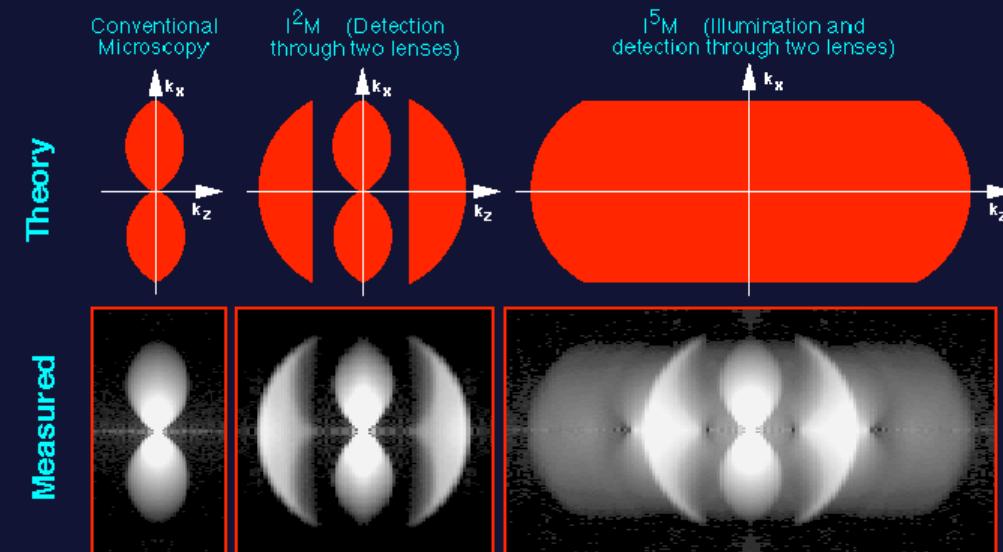


## I<sup>5</sup>M concept

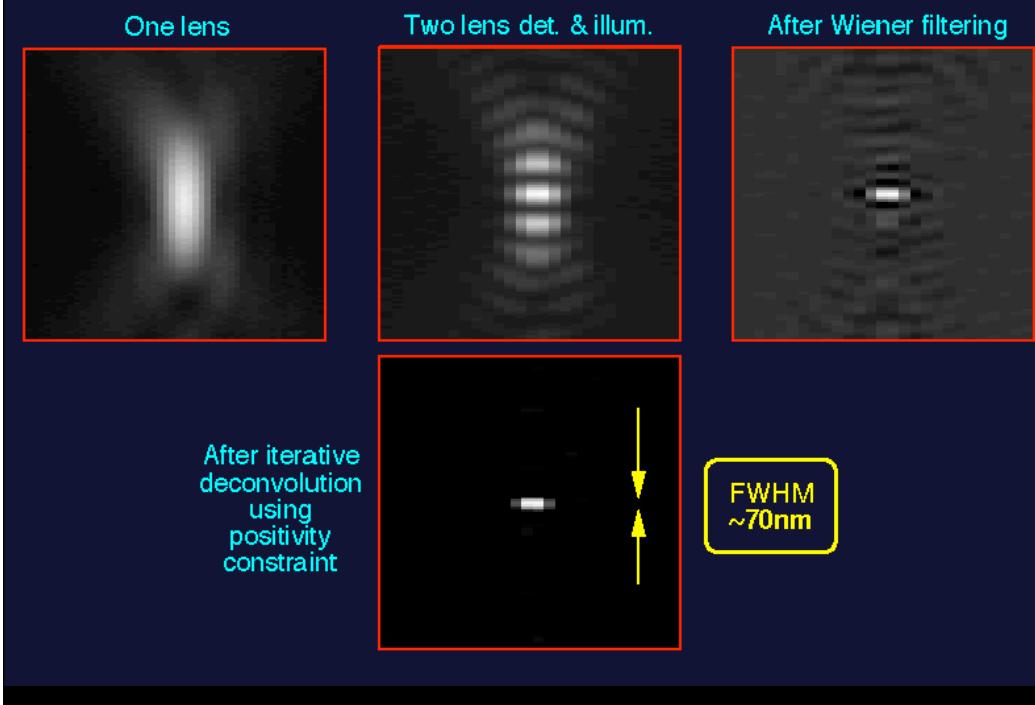




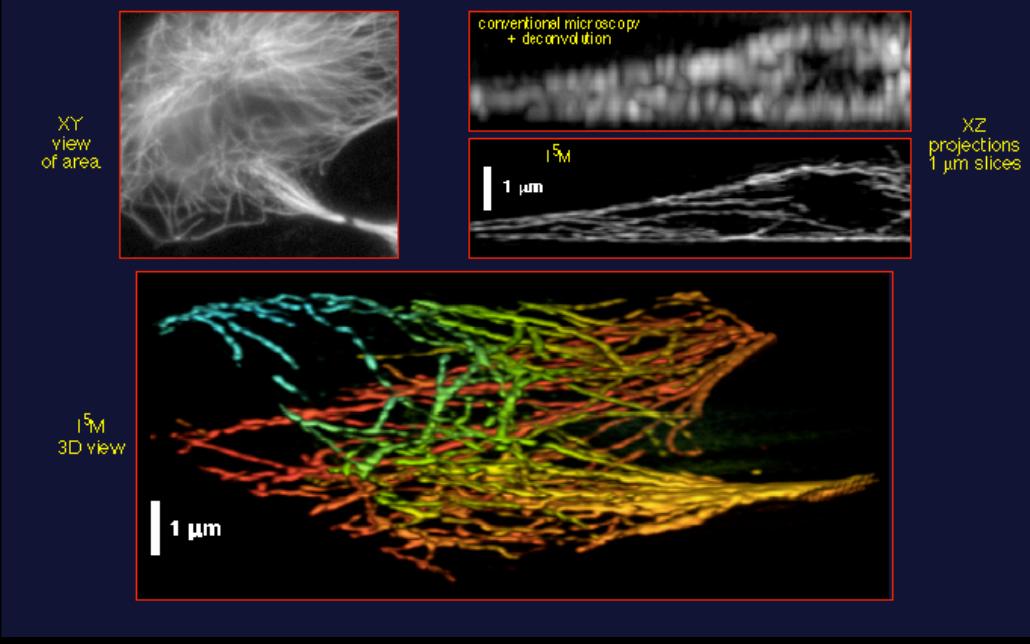
## Observable Information [ Optical Transfer Functions ]



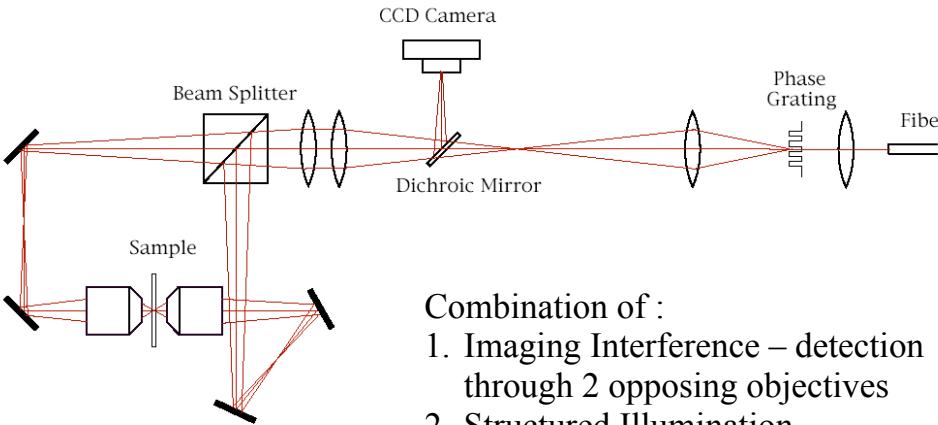
## Deconvolution of bead data



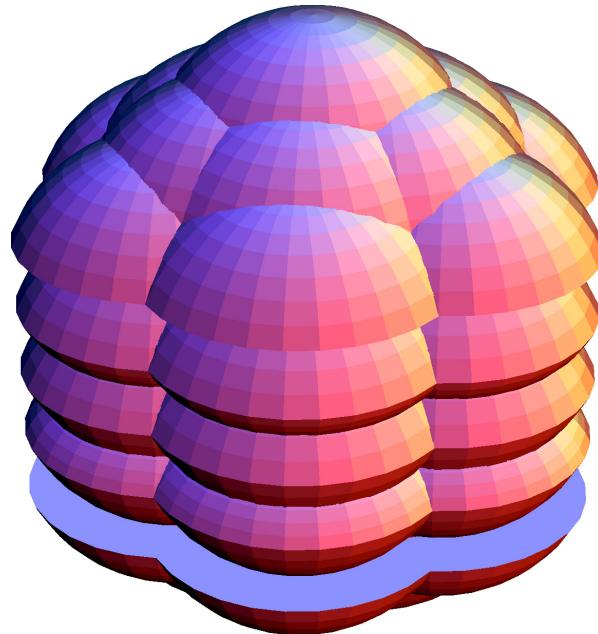
## Microtubules in a recently divided PtK2 cell



*Combine methods: I<sup>5</sup>S  
use two lenses to collect more angles*

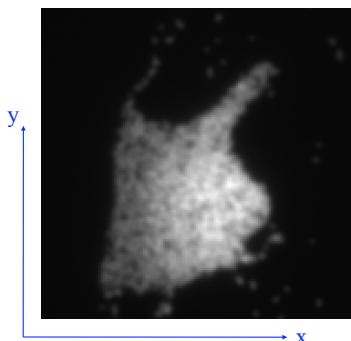


Observable Region through 2 opposing objectives  
Structured Illumination (3 orientations) – I<sup>5</sup>S

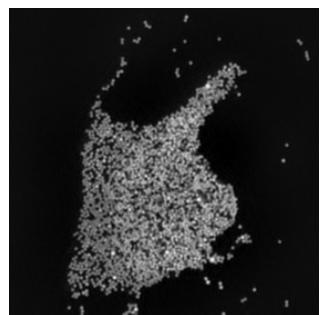


## *Comparison of Resolving Power*

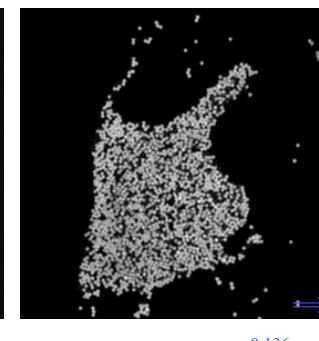
Conventional



Structured Illum.



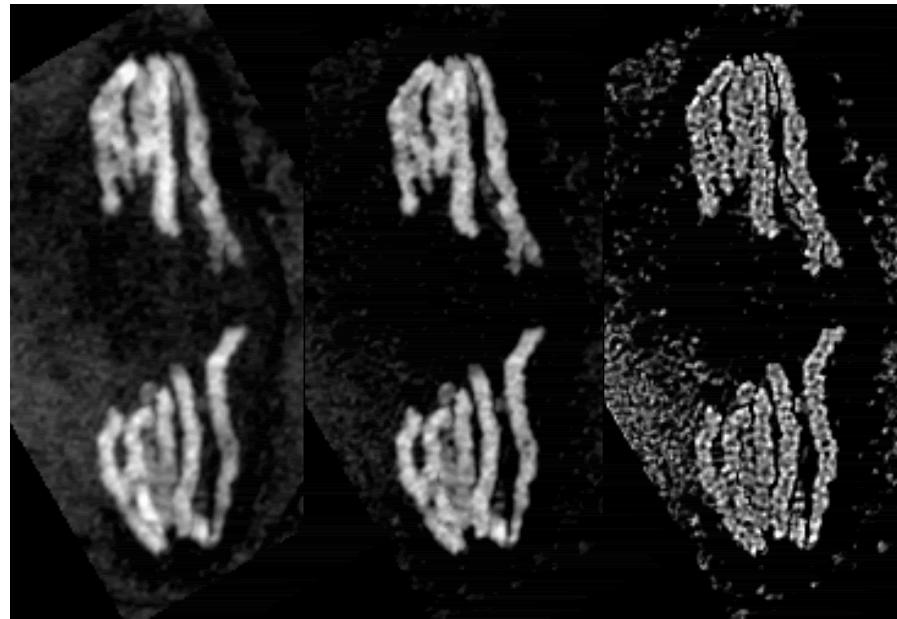
I<sup>5</sup>S



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*

