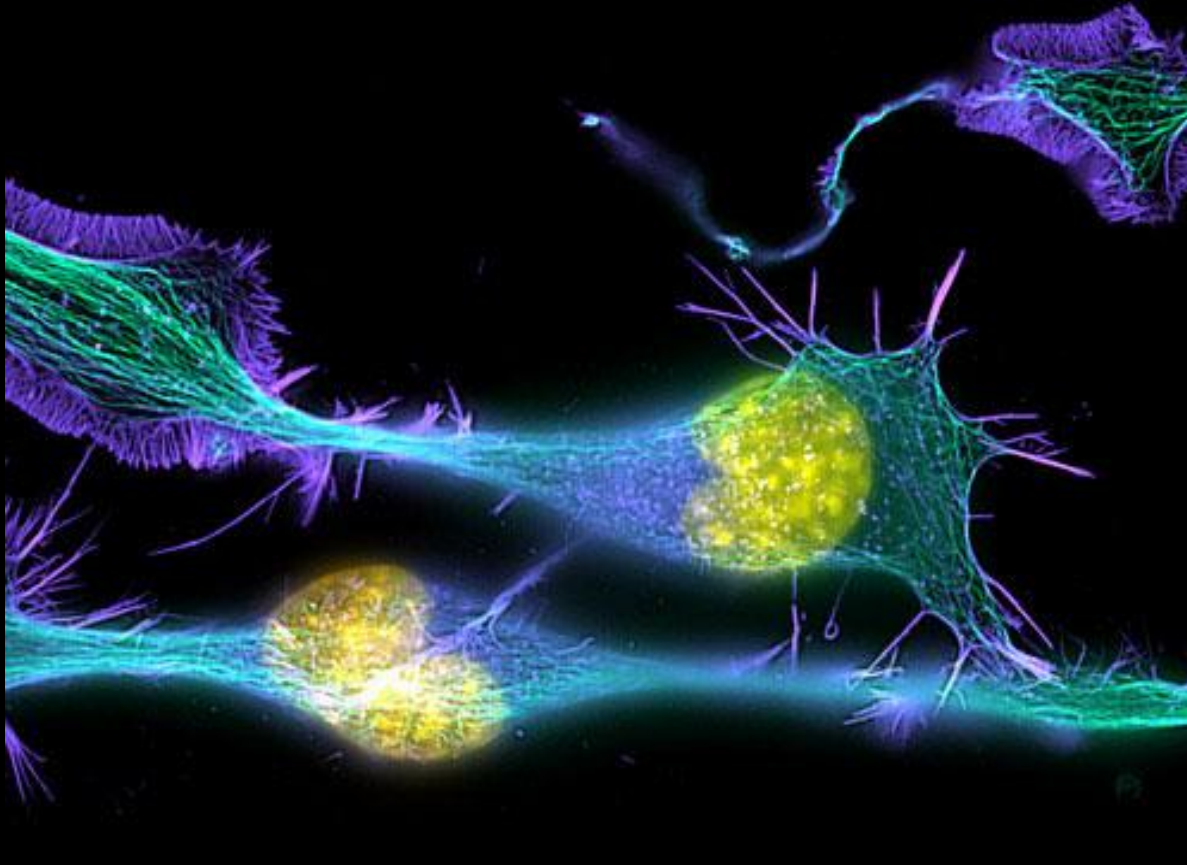
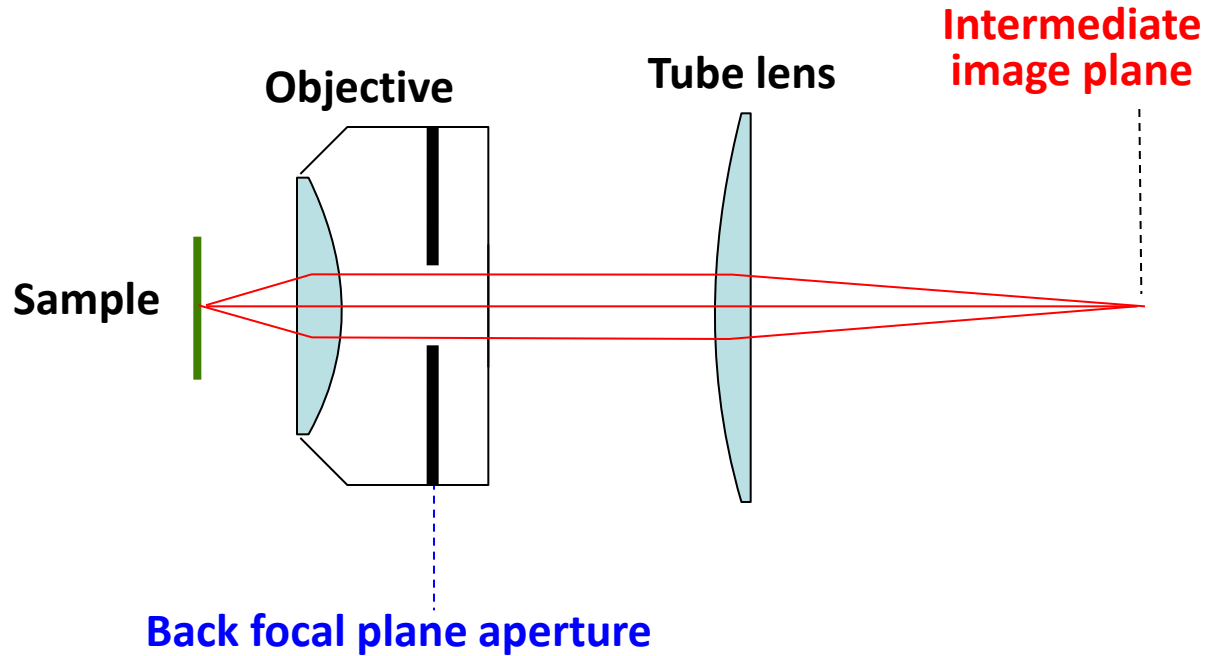


# Principles & Practice of Light Microscopy: 2

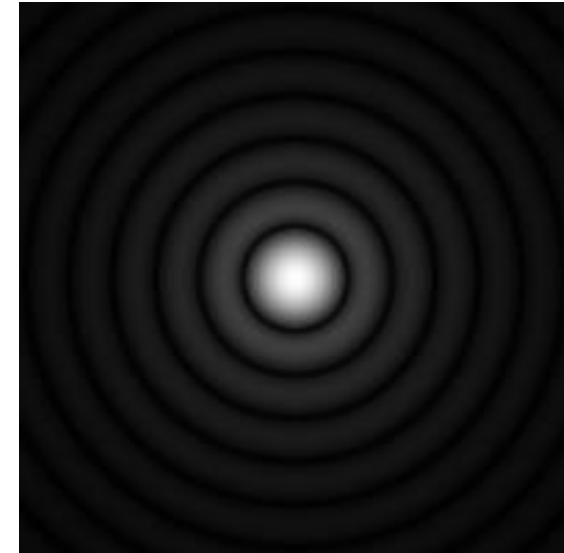


(Image: T. Wittman, Scripps)

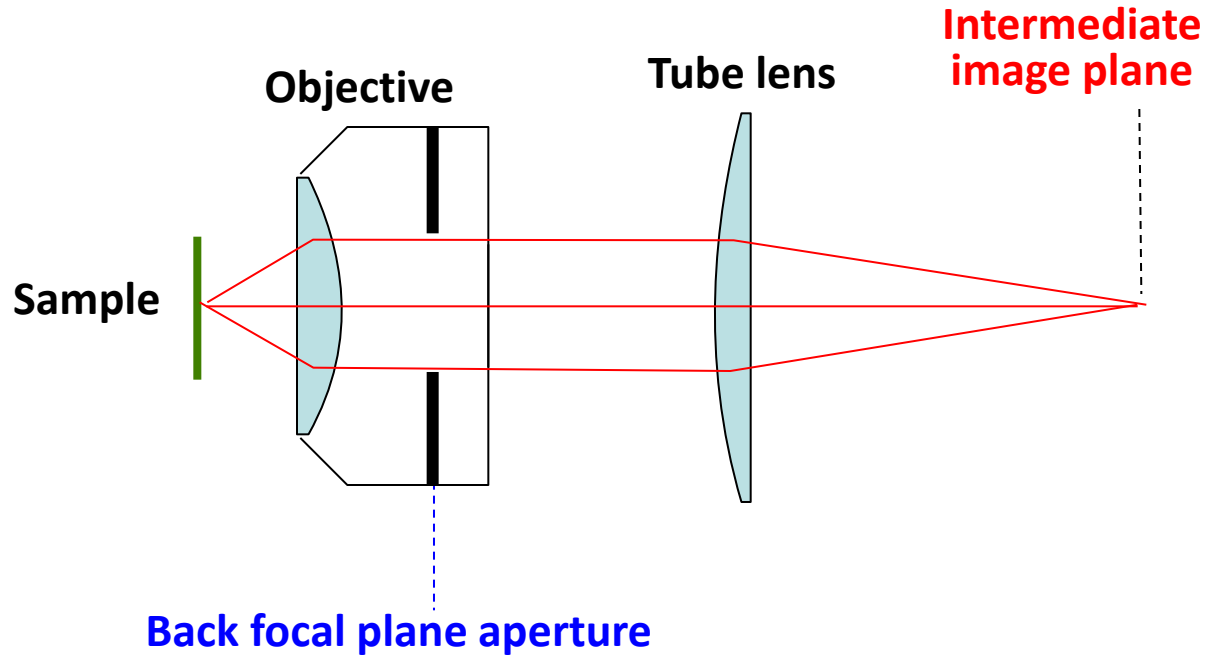
# Aperture and Resolution



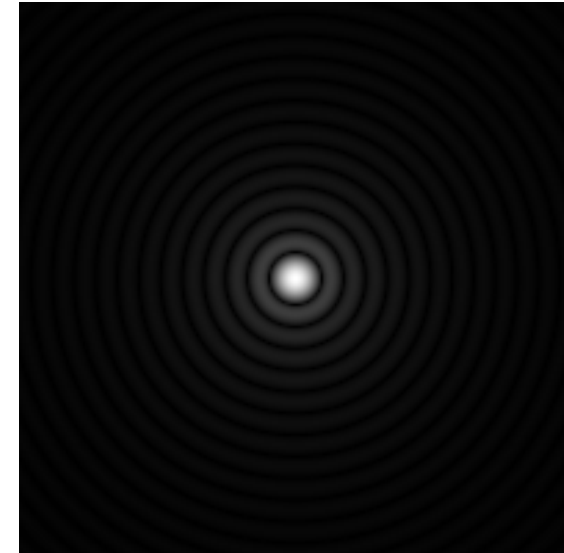
Diffraction spot  
on image plane  
= *Point Spread Function*



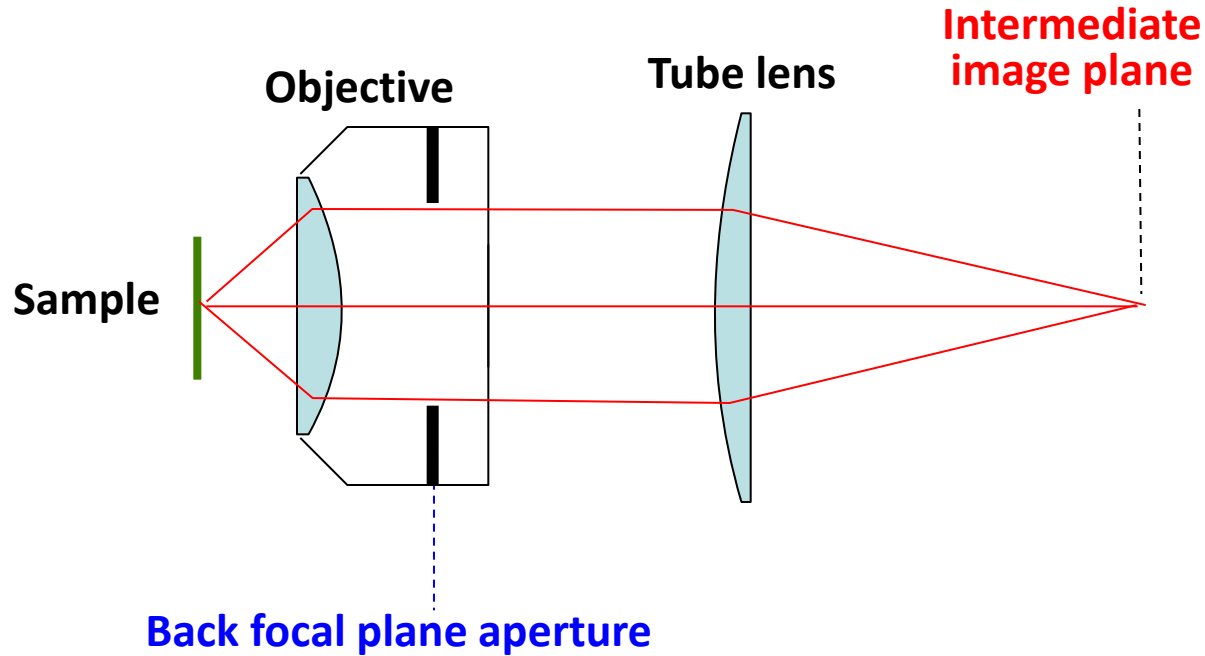
# Aperture and Resolution



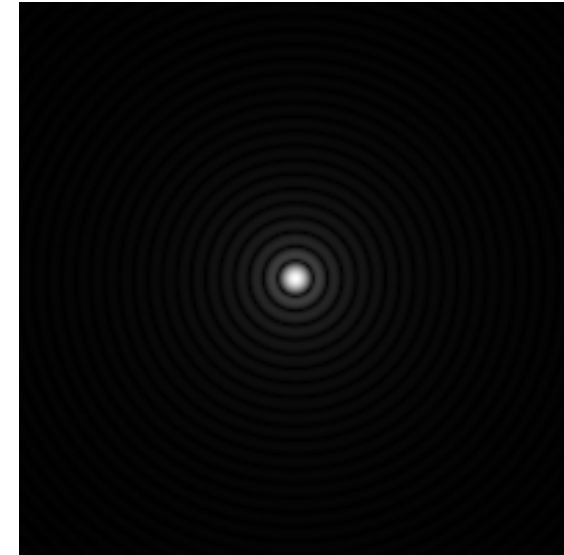
Diffraction spot  
on image plane  
= *Point Spread Function*



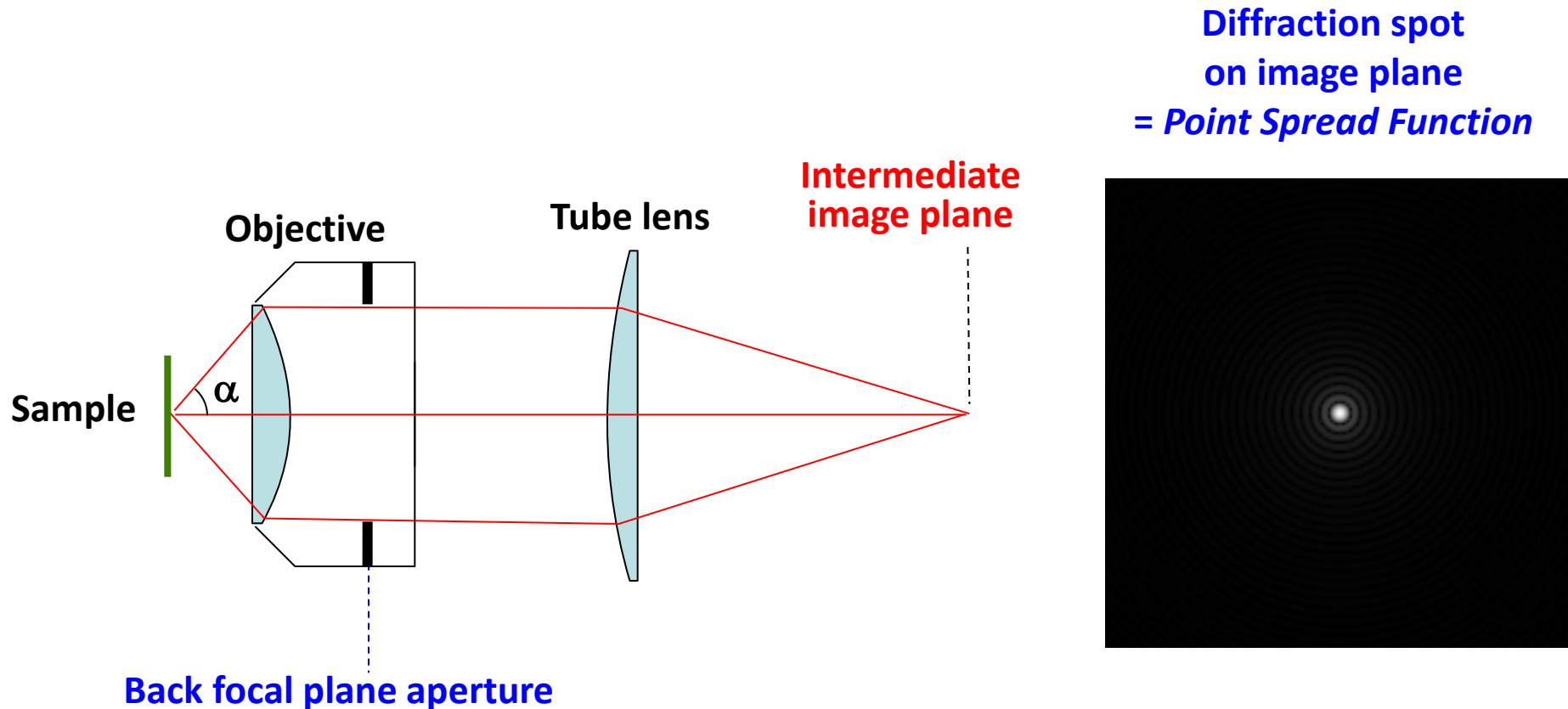
# Aperture and Resolution



Diffraction spot  
on image plane  
= *Point Spread Function*



# Aperture and Resolution



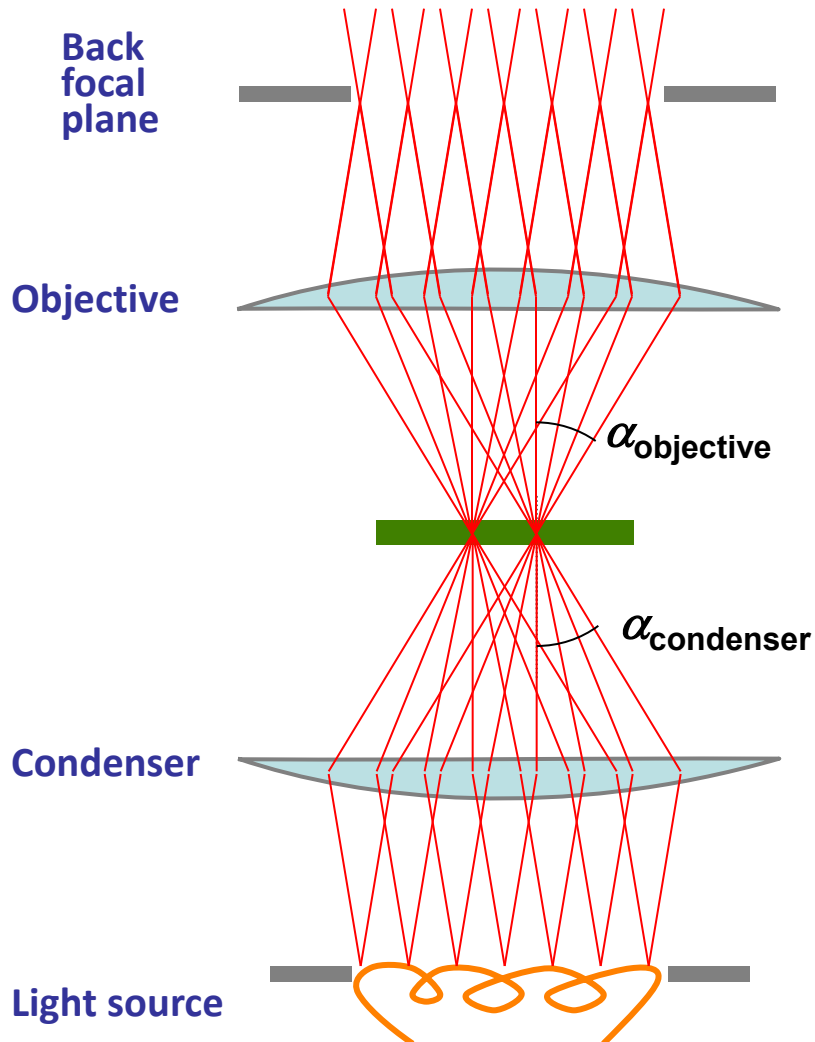
- Image resolution improves with Numerical Aperture (NA)

$$NA = n \sin(\alpha)$$

where:  $\alpha$  = light gathering angle  
 $n$  = refractive index of sample

# Filling the back focal plane

*In trans-illumination microscopy, to get maximum resolution, the illumination must “fill the back focal plane”*



For the highest resolution,  
we need to have

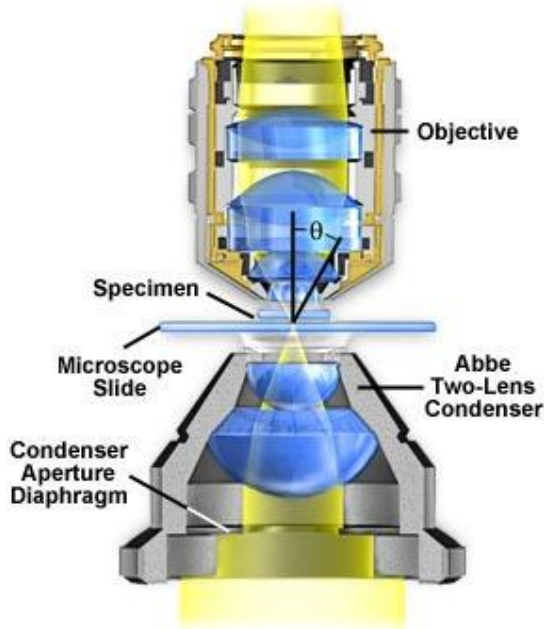
$$\alpha_{\text{condenser}} \geq \alpha_{\text{objective}}$$

$$NA_{\text{condenser}} \geq NA_{\text{objective}}$$

$\Rightarrow$  with oil immersion objectives,  
we need an *oil immersion condenser*!

# The Condenser

Abbe Condenser Optical Pathway



## Tasks:

- Illuminate at all angles  $< \alpha_{\text{objective}}$
- Concentrate light on the field of view for *all* objectives to be used

## Problem:

- Low mag objectives have large FOV,
- High mag objectives have large  $\alpha$   
(With 2X and 100x objectives we need  $(100/2)^2 = 2500$  times more light than any objective uses!)

## Solutions:

- Compromise
- Exchangable condensers, swing-out front lenses,...

## Grade of correction

NA

Achromat/Aplanat Condenser (Numerical Aperture = 1.38)

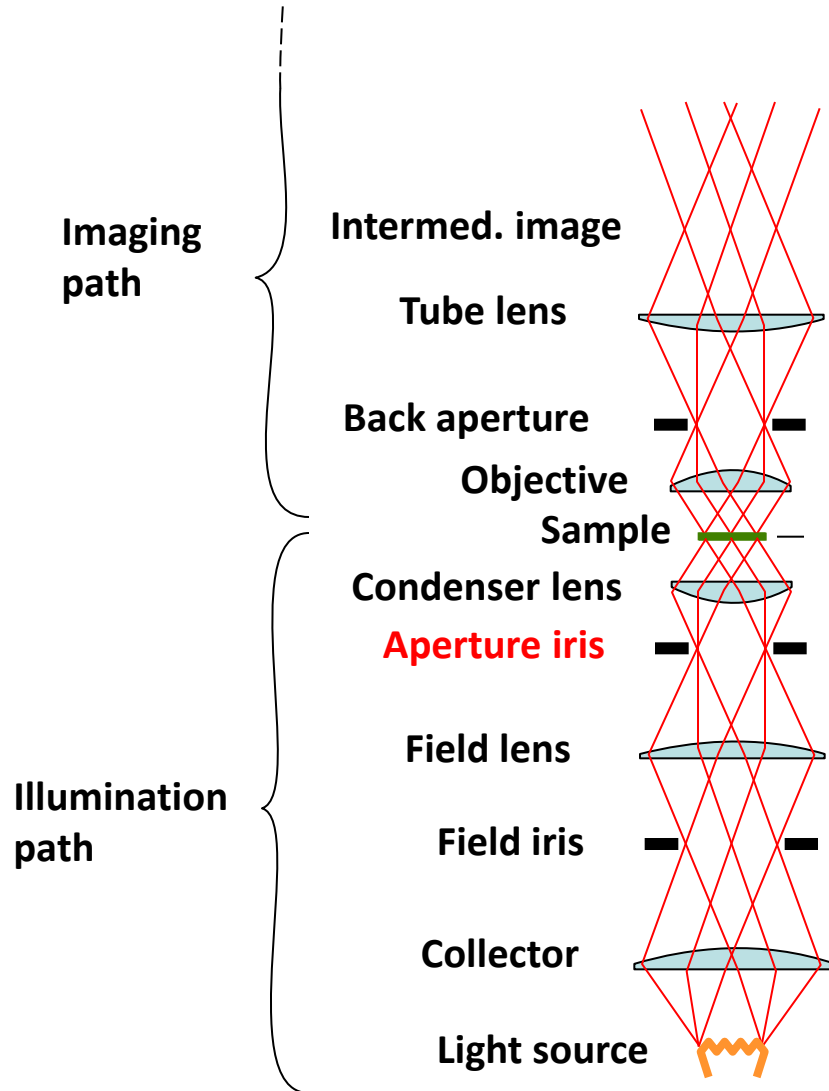


Swing-Out Top Lens Condenser (Numerical Aperture = 1.35)



# Aperture, Resolution & Contrast

Can adjust the condenser NA with the aperture iris



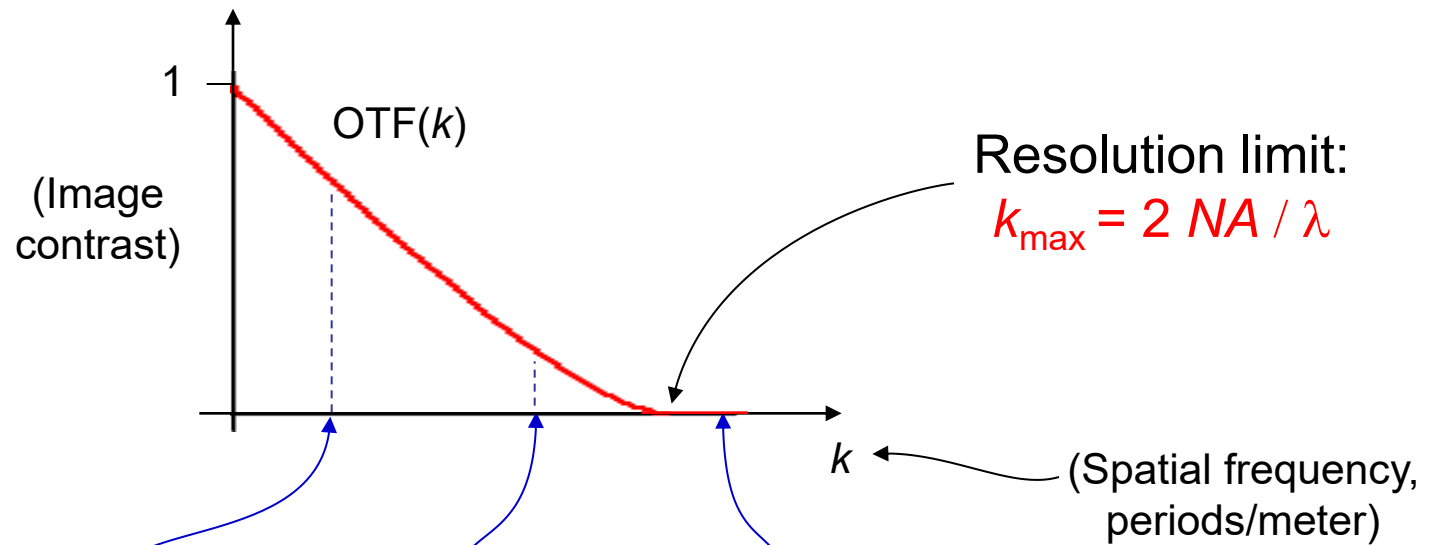
**Q: Don't we always want it full open??**

**A: No**

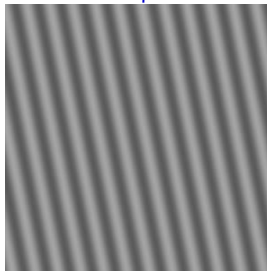
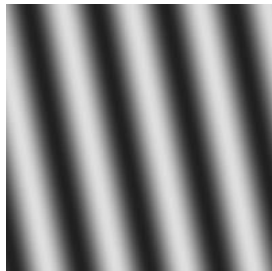
**Why? Tradeoff:  
resolution vs. contrast**



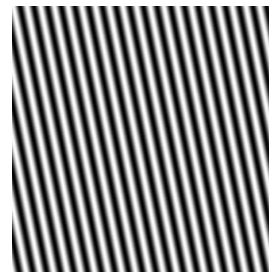
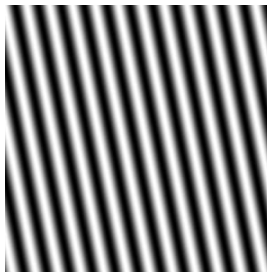
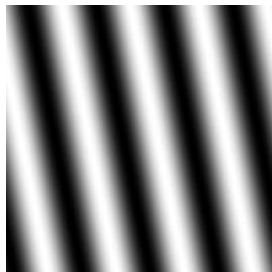
# Spatial frequencies & the Optical Transfer Function (OTF)



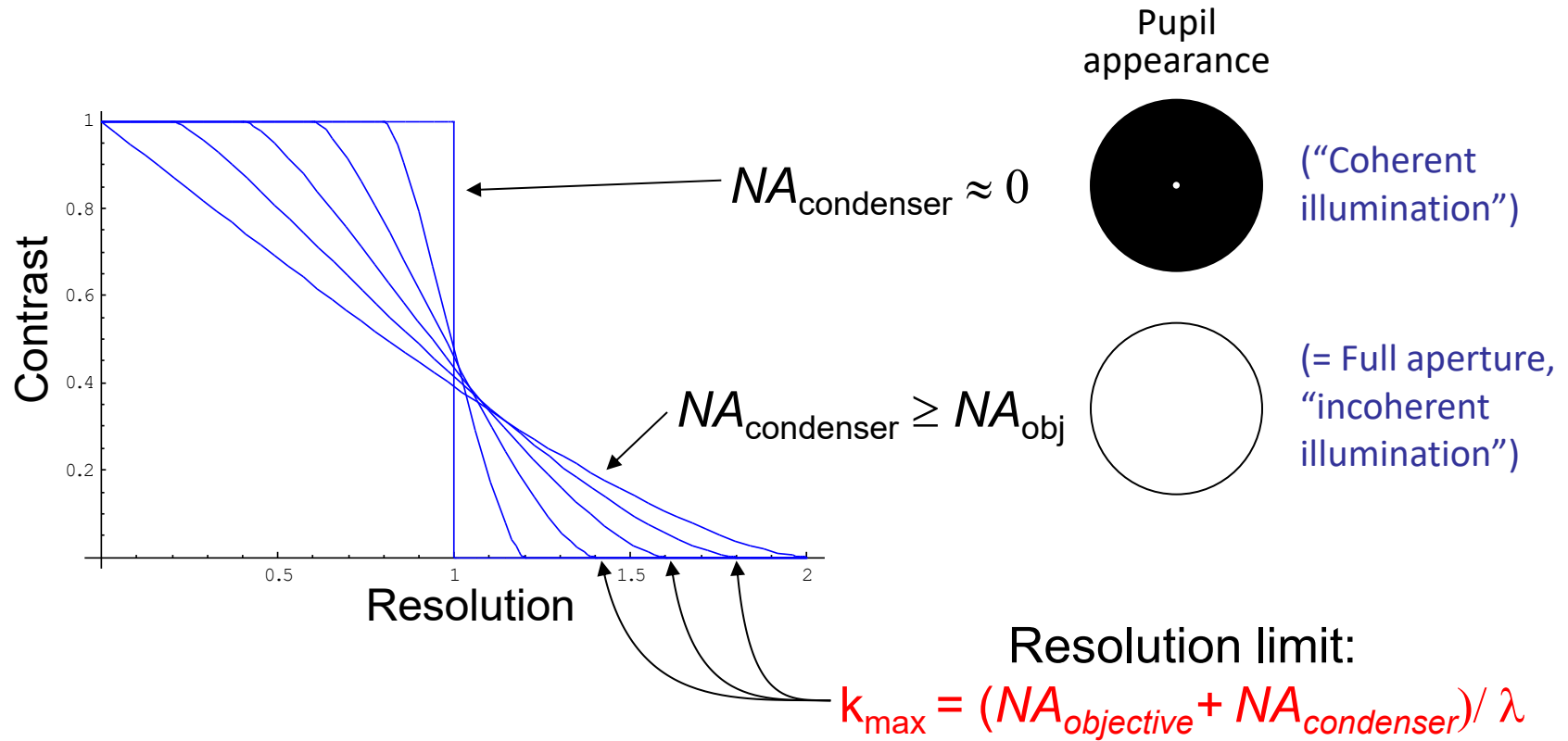
Observed image



Object



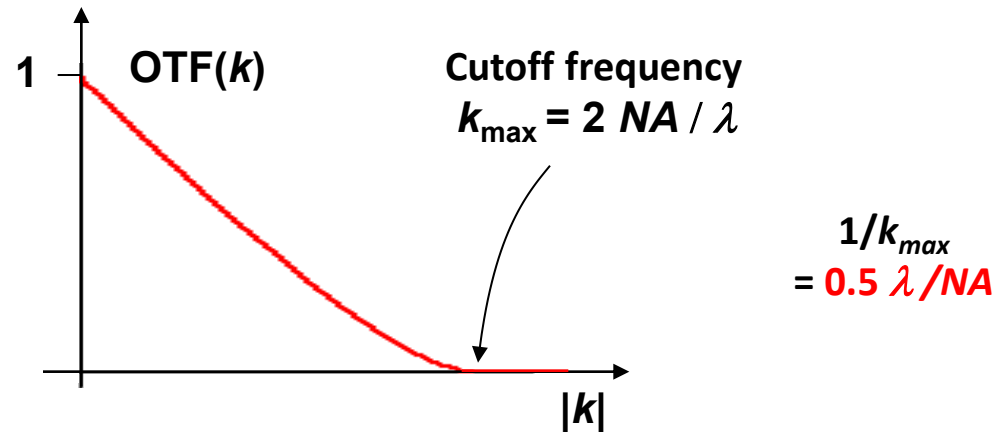
# Resolution & Contrast vs. Illumination aperture



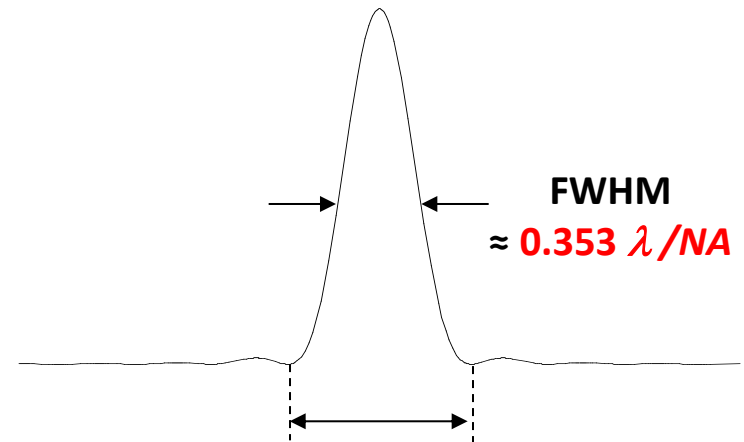
Increasing the illumination aperture  
increases resolution  
but decreases contrast

# Definitions of Resolution

As the OTF cutoff frequency



As the Full Width at Half Max (FWHM) of the PSF



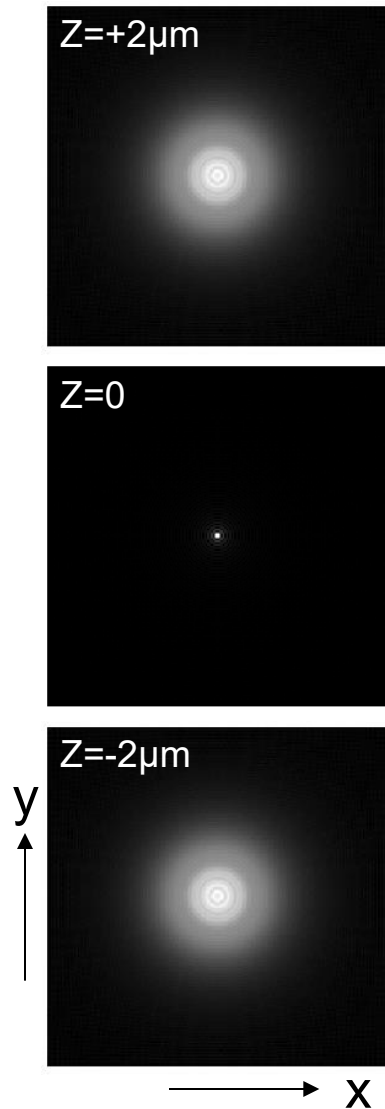
As the diameter of the Airy disk (first dark ring of the PSF)  
= "Rayleigh criterion"

Airy disk diameter  
 $\approx 0.61 \lambda / NA$

# The 3D Point Spread Function (PSF)

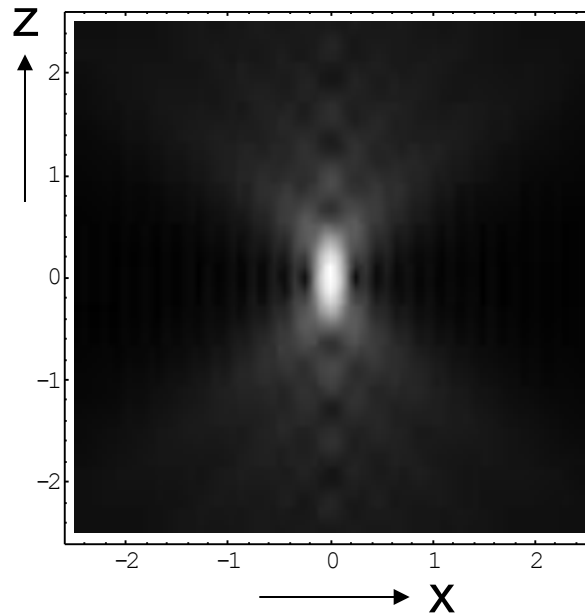
The image of a point object

2D PSF  
for different defocus

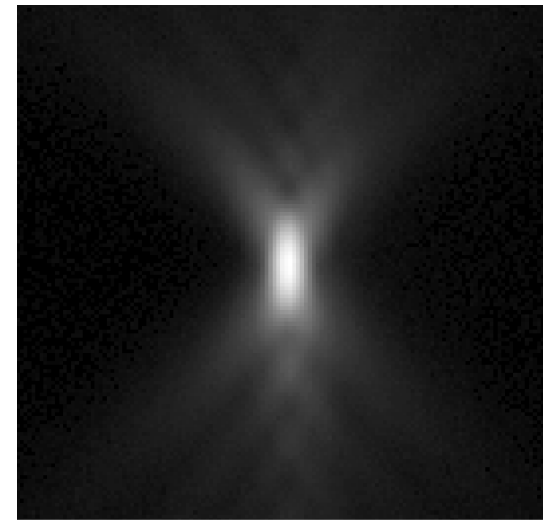


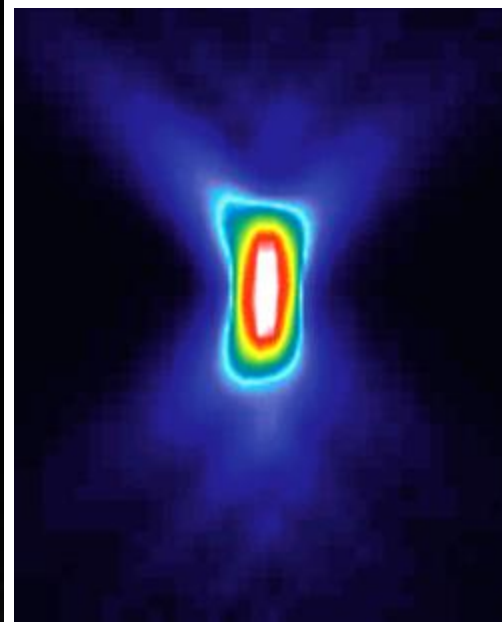
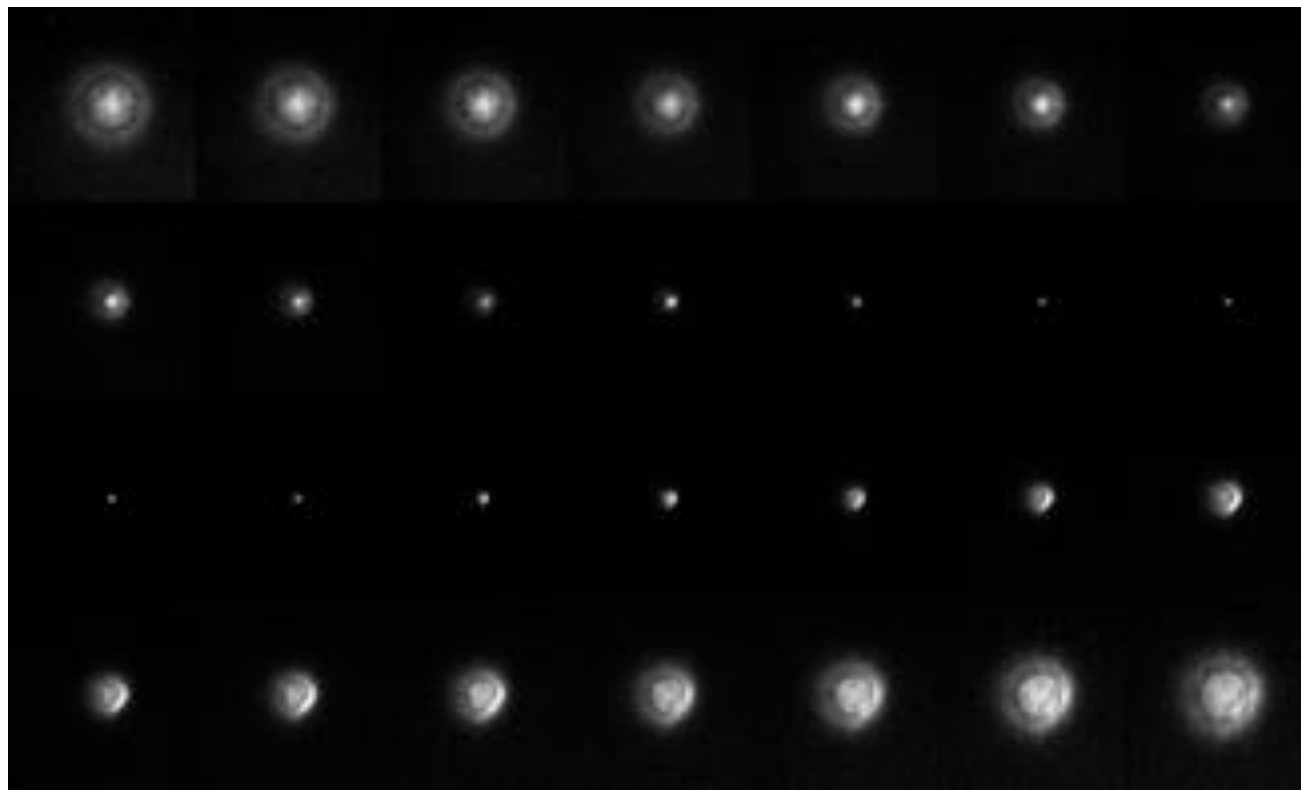
3D PSF

Calculated



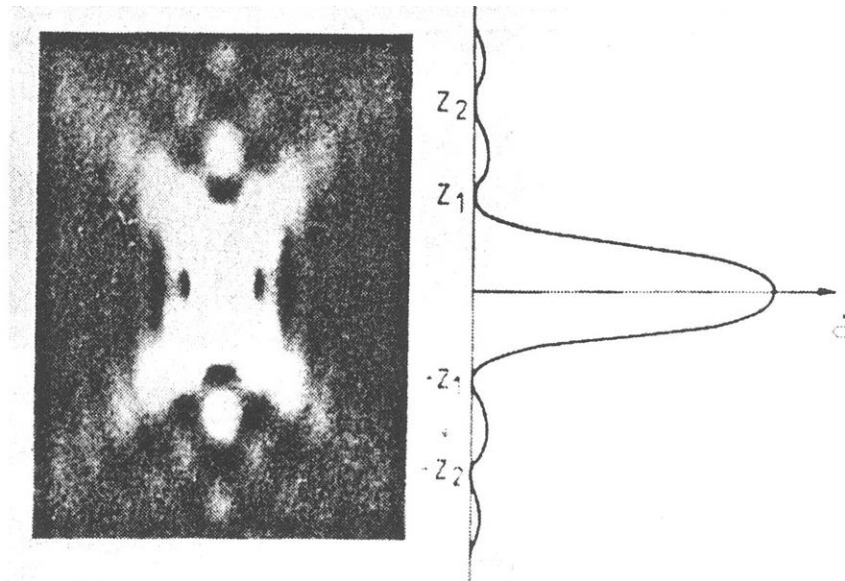
Measured





**X - Z**

# Z-resolution, a.k.a. depth of field, for widefield microscopy



Z-resolution:  
 $2\lambda n / NA^2$

$NA$	Resolution (nm; X-Y)	depth of field ( $\mu m$ )
0.3	1017	11.1
0.75	407	1.77
0.95	321	1.11
1.4	218	0.773

# Summary: Numerical Aperture and Resolution

- Numerical aperture, ***not magnification***, sets the smallest details you can resolve in an image
- Increasing NA also increases the amount of light collected by the lens, thereby increasing the brightness of the image – this scales as  $NA^2$

## Specifications for some common objectives

<b>Magnification</b>	<b>NA</b>	<b>Resolution (nm)</b>	<b>Depth of Field (nm)</b>	<b>Light gathering (arb. units)</b>
10	0.3	1017	16830	0.09
20	0.75	407	2690	0.56
40	0.95	321	1680	0.90
40	1.3	235	896	1.69
60	1.2	254	926	1.44
60	1.4	218	773	1.96
100	1.4	218	773	1.96



# Aberrations

They are the enemy

# Aberrations

- Chromatic aberrations

  - Longitudinal chr. Ab.

  - Lateral chr. Ab.

- Wavefront aberrations

  - Spherical aberration

  - Astigmatism

  - Coma

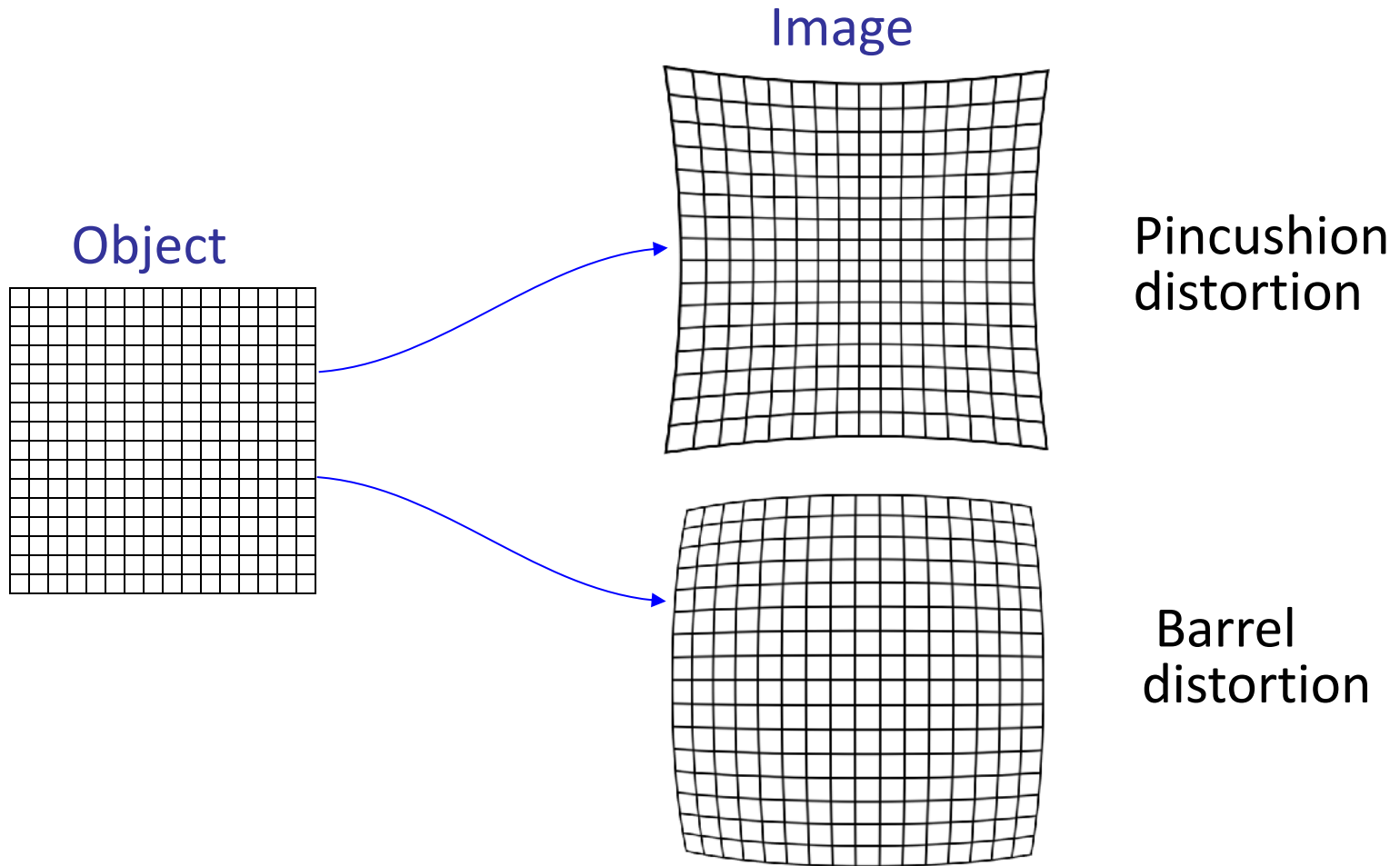
  - ...

- Curvature of field

- Distortion

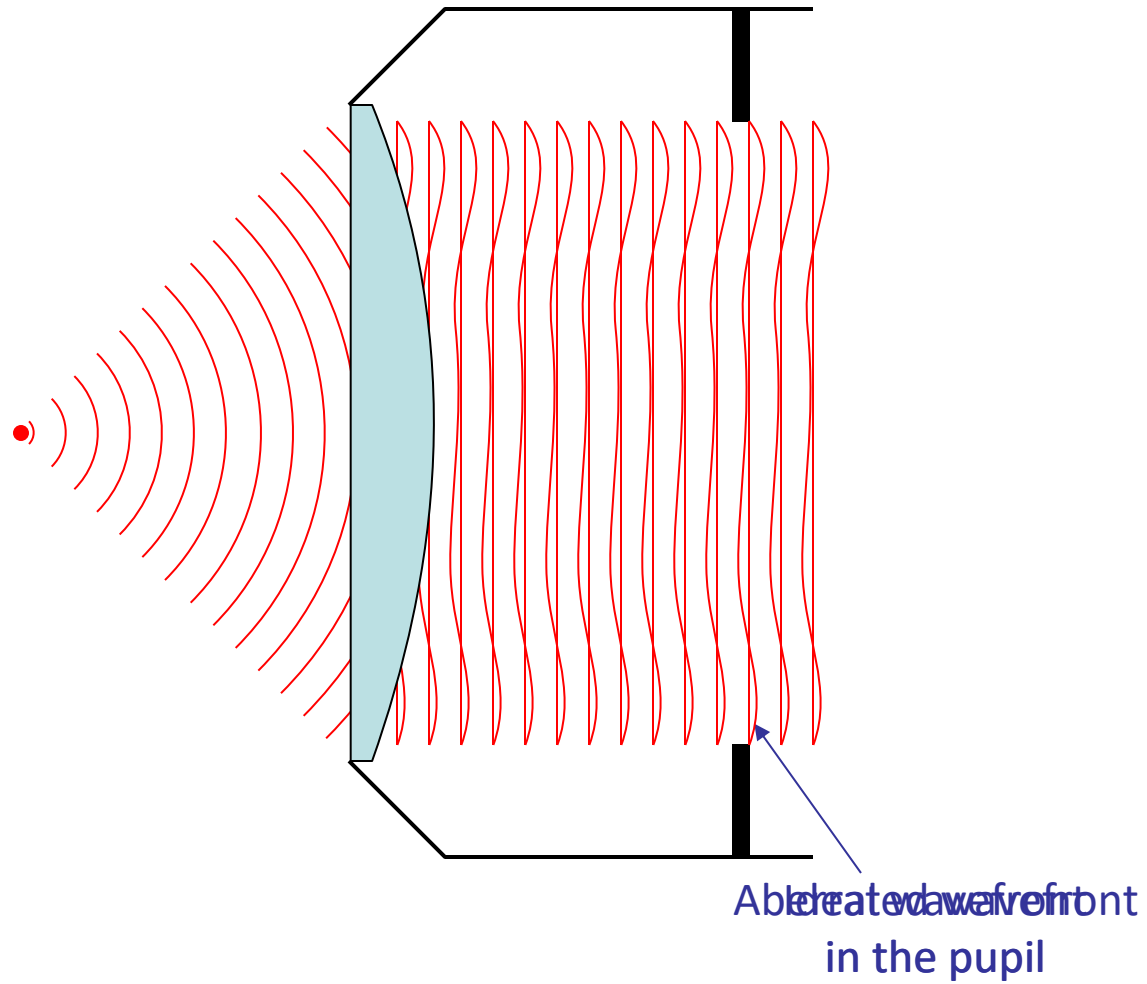
# Geometric Distortion

= Radially varying magnification

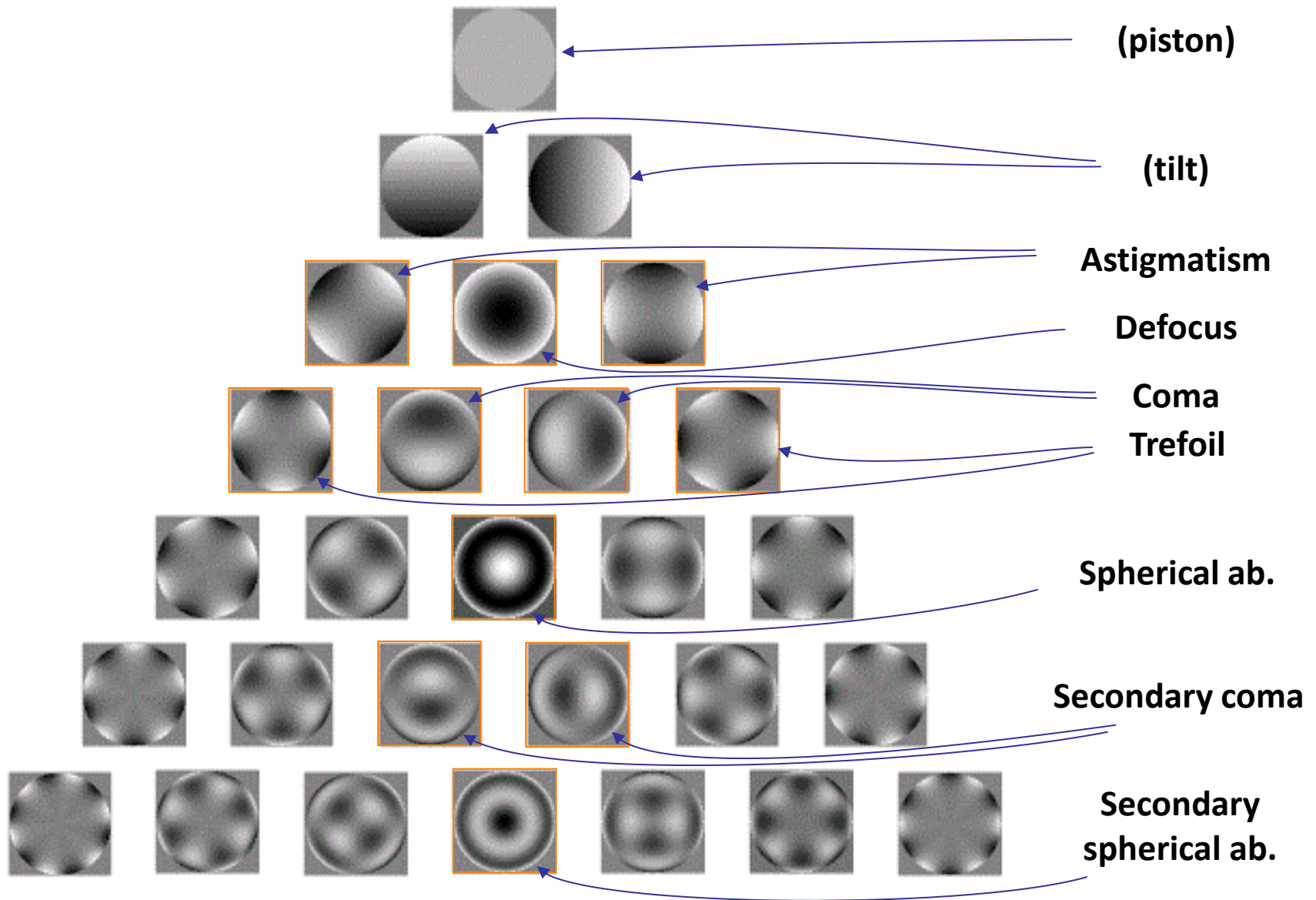


May be introduced by the projection eyepiece

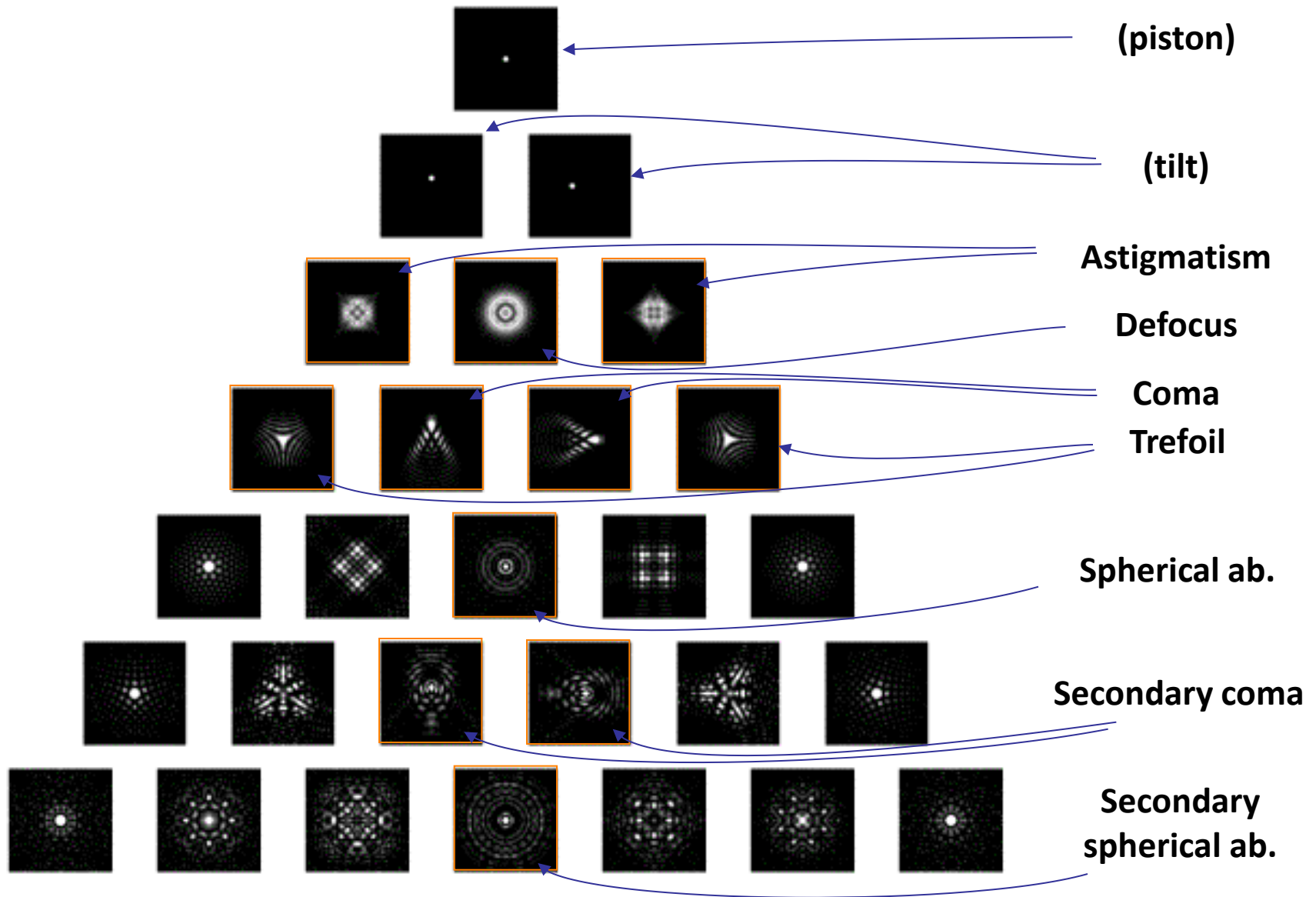
# Wavefront Aberrations



# Wavefront Aberrations

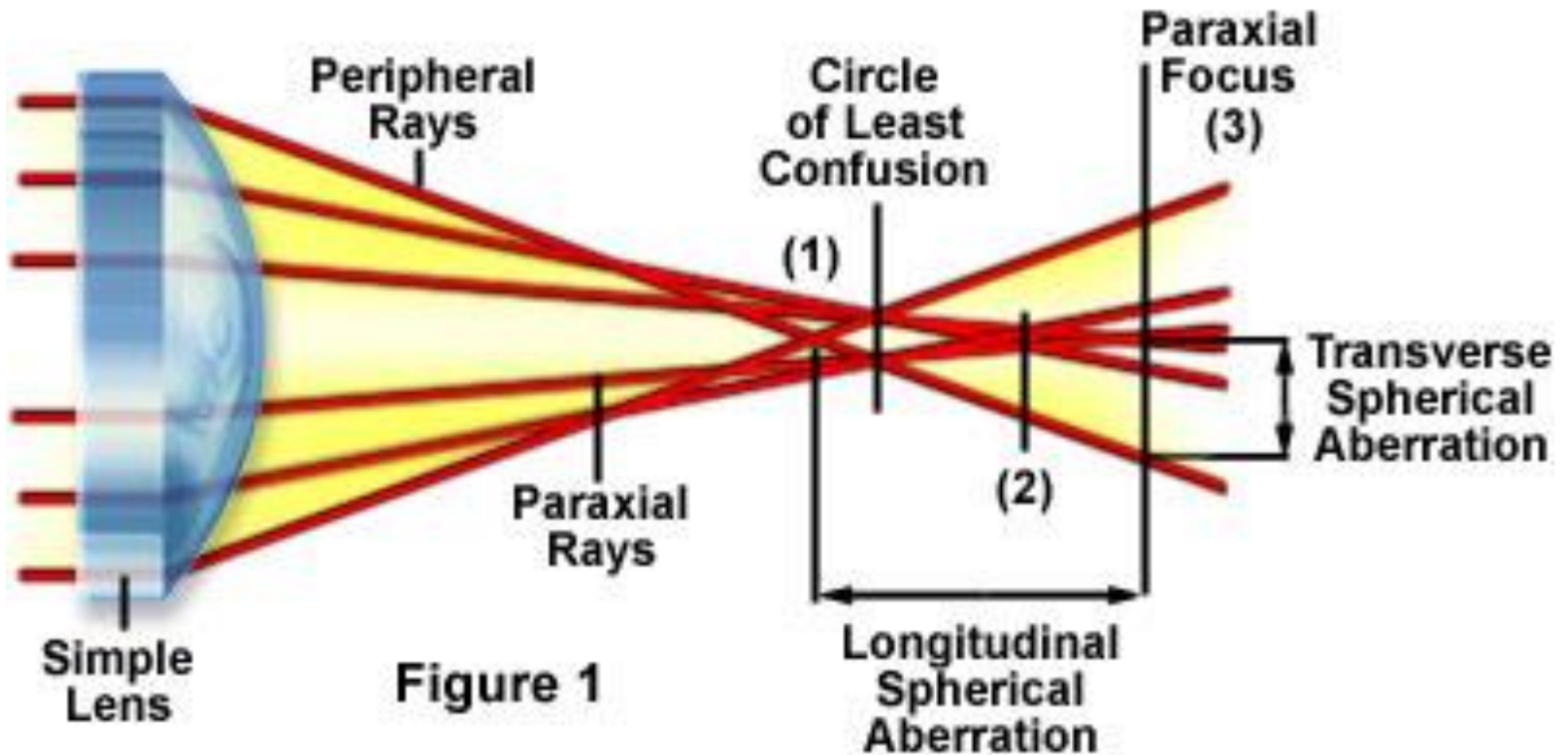


# PSF Aberrations



# Spherical Aberration

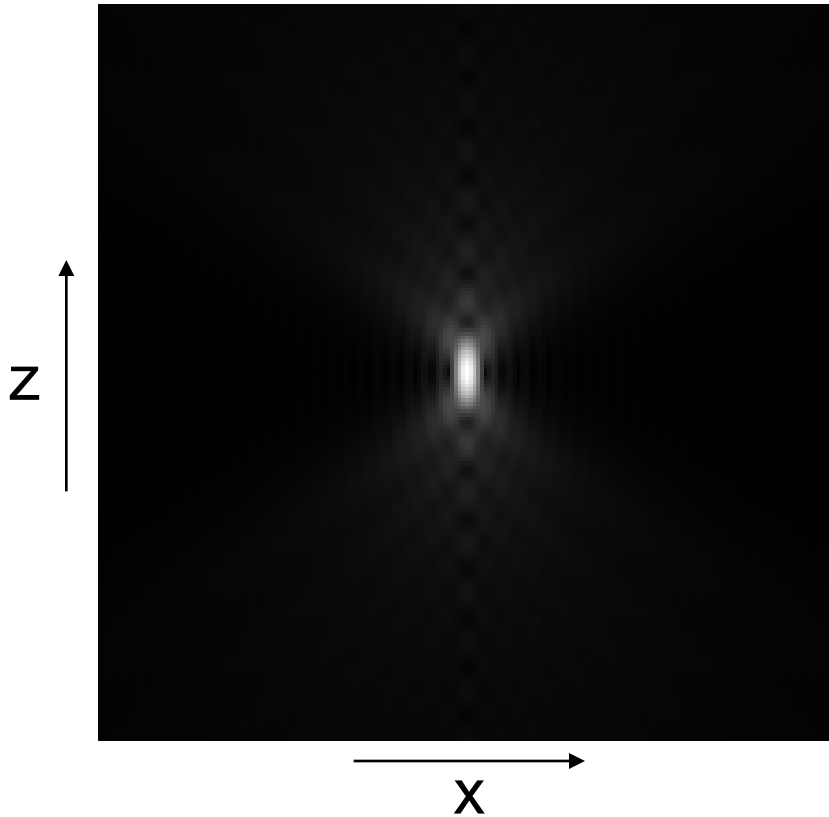
## Longitudinal and Transverse Spherical Aberration



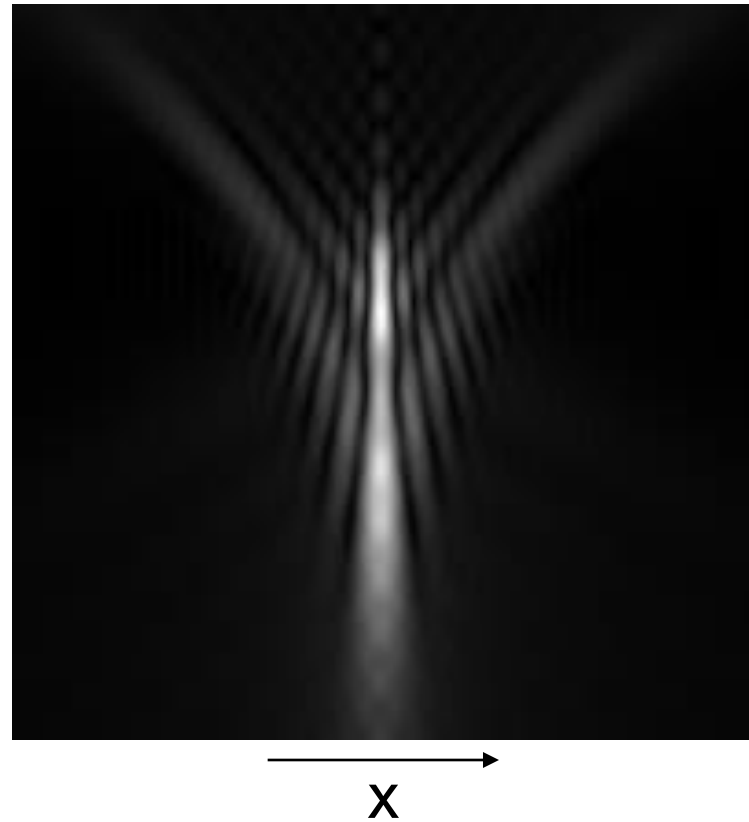
# Spherical Aberration

## Point spread functions

Ideal

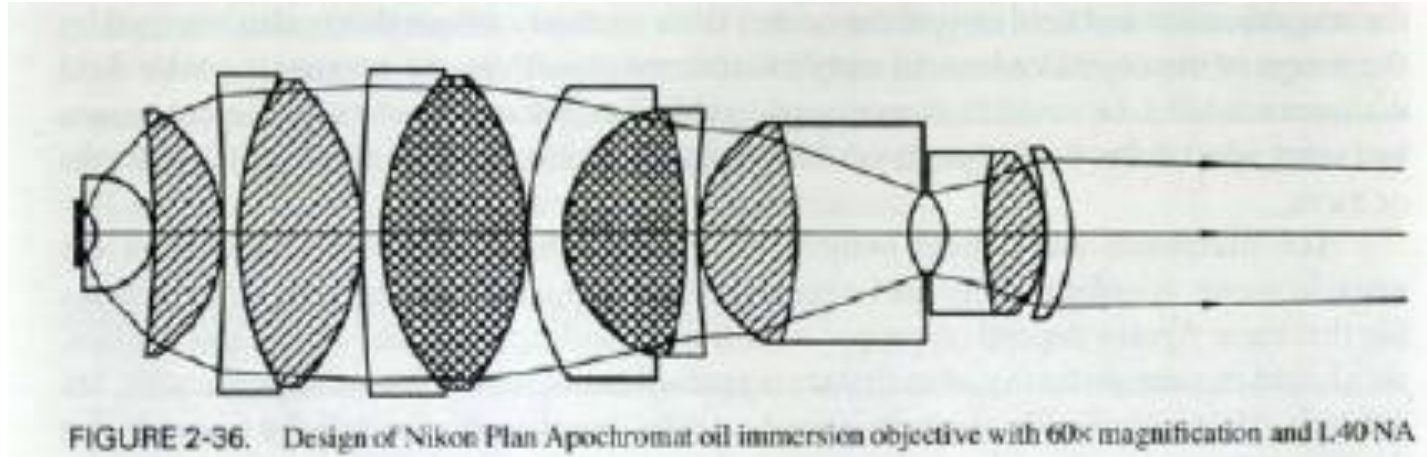


1 wave of spherical ab





# Causes of spherical aberration



- Modern objectives are complicated!
- The optical design requires specifying the optical path length between the sample and the back focal plane of the lens
- $OPL = l_1 n_1 + l_2 n_2 + \dots + l_n n_n$

# Sources of Spherical Aberration

Design compromises

Manufacturing tolerances

Immersion fluid index error

Temperature variation

Cover slip thickness

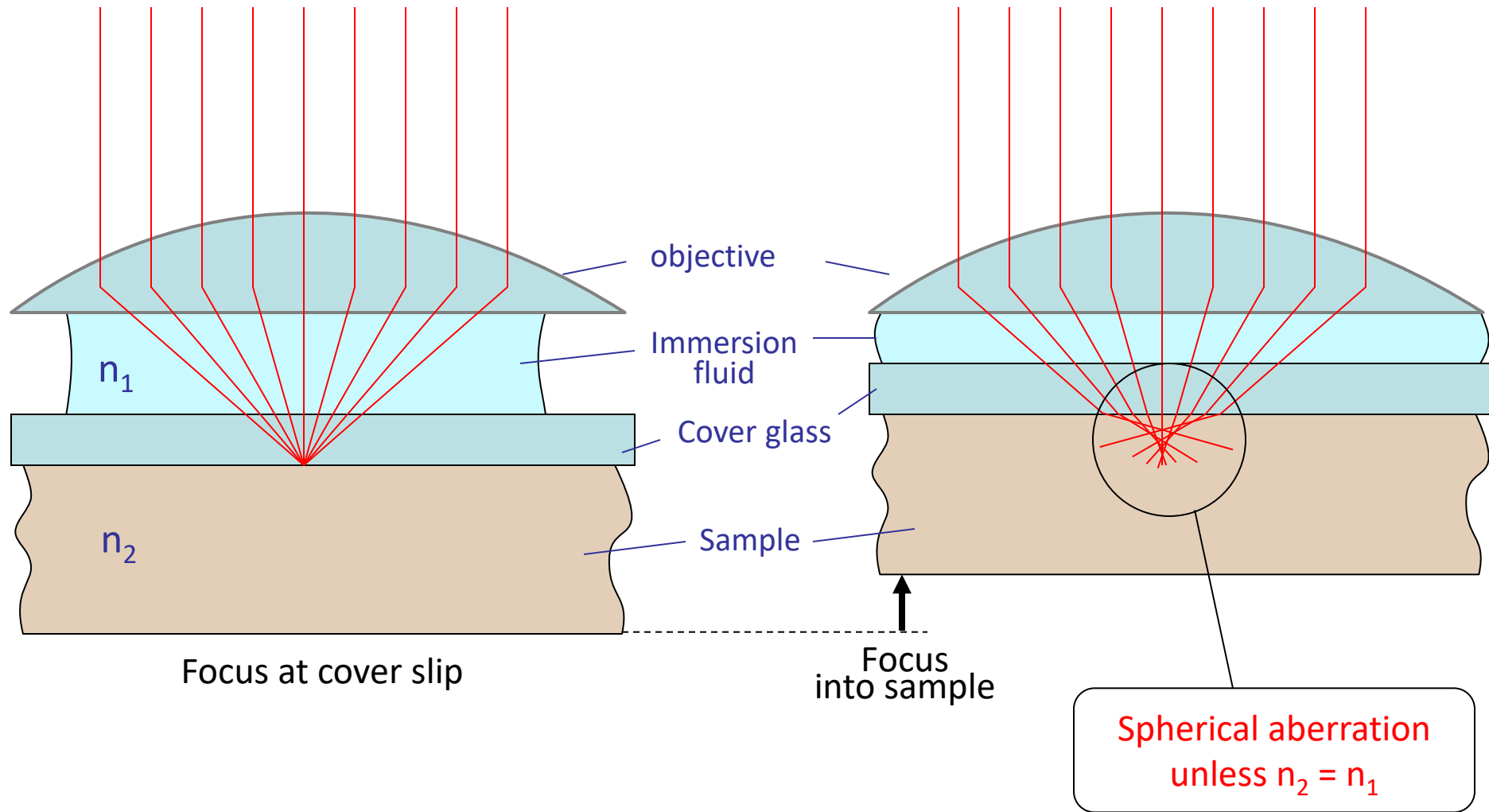
(high-NA objectives except oil immersion)

Correction collar setting

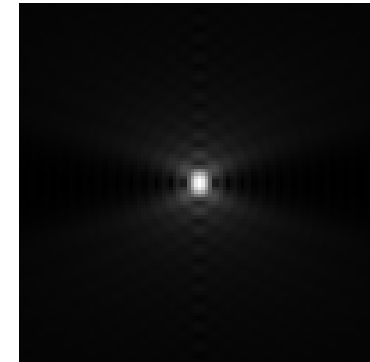
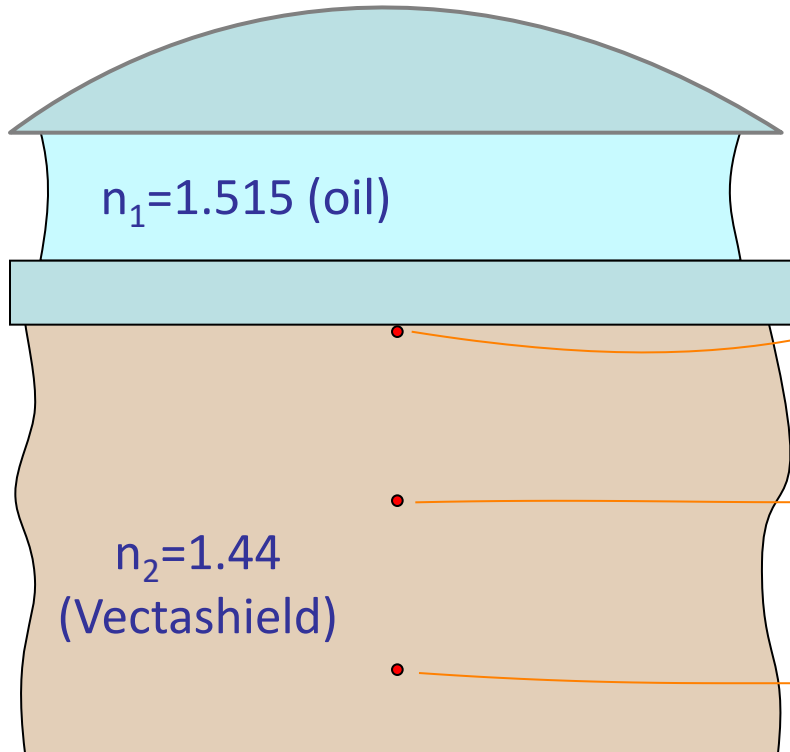
***Sample refractive index mismatch***



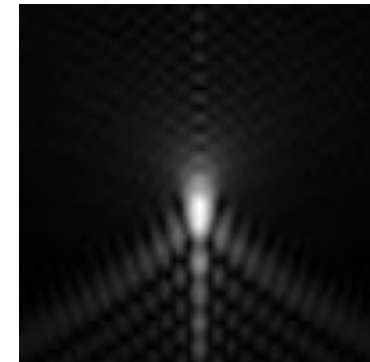
# Index Mismatch & Spherical Aberration



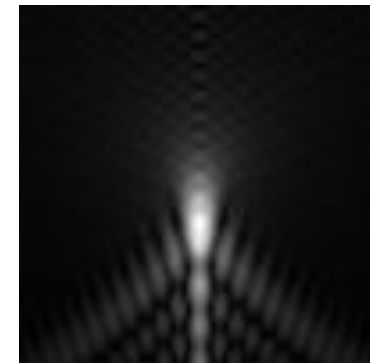
# Index Mismatch & Spherical Aberration



$z=0\ \mu\text{m}$



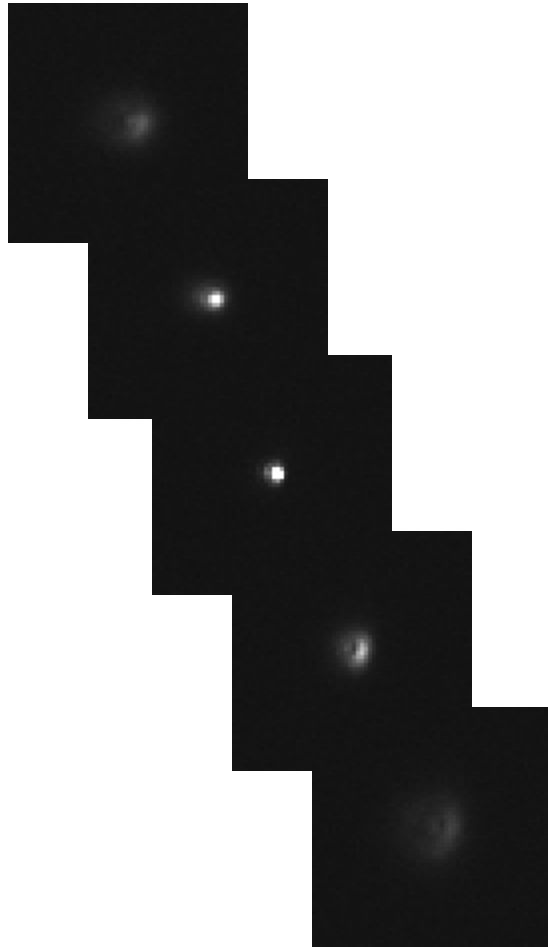
$z=25\ \mu\text{m}$



$z=50\ \mu\text{m}$

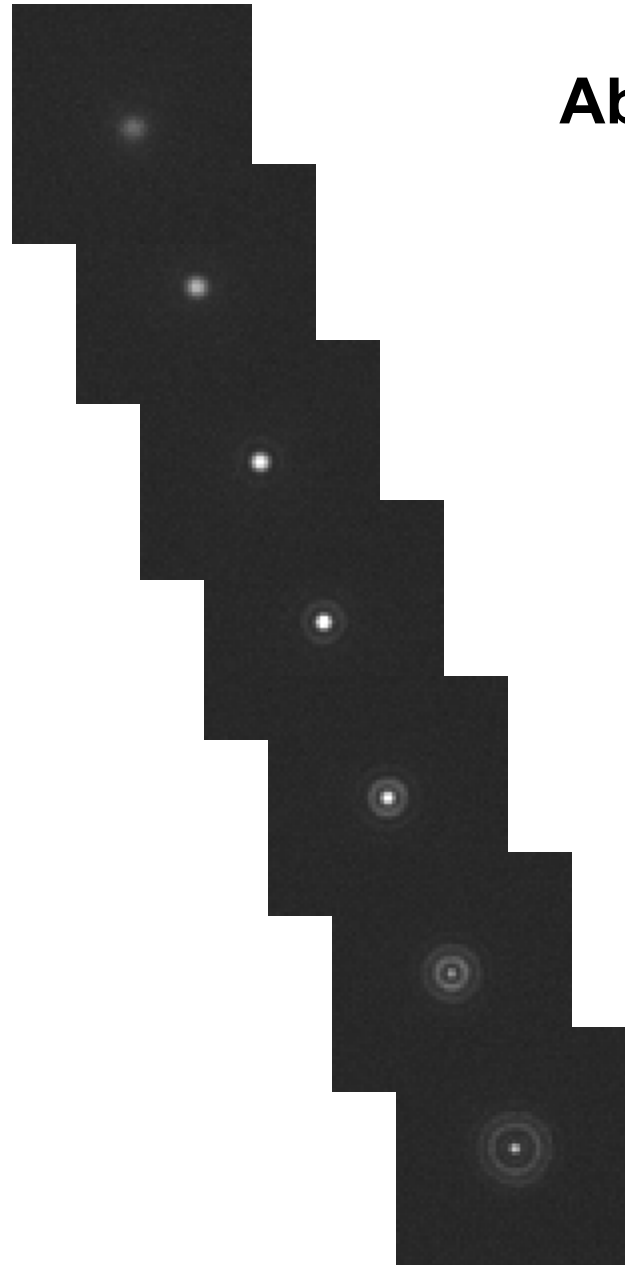
# How to recognize spherical aberration

**Unaberrated**



**0.5  $\mu\text{m}$  steps**

**Aberrated**

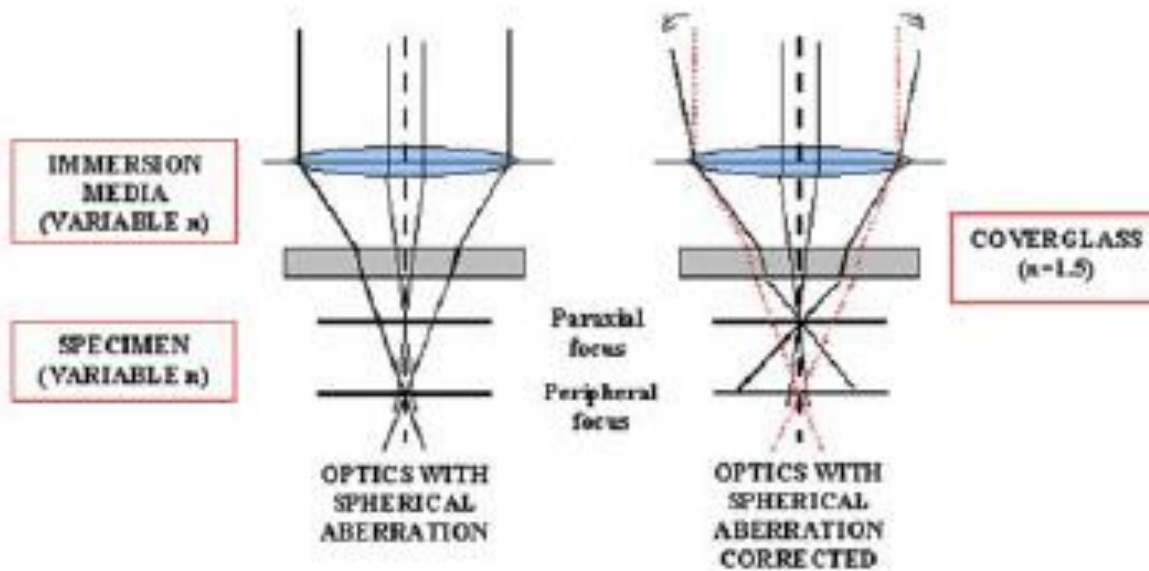


**1  $\mu\text{m}$  steps**

# What can you do about spherical aberration?

- Use 0.17 mm coverslips ( $\sim$  #1.5)
- Work close to the coverslip
- Match lenses to the refractive index of your samples, and vice versa
  - For aqueous samples, use water immersion / water dipping lenses
  - For fixed samples and oil immersion lenses, mount your sample in a medium with  $n = 1.515$
- Adjust objective correction collar when available
- Use lower NA lenses

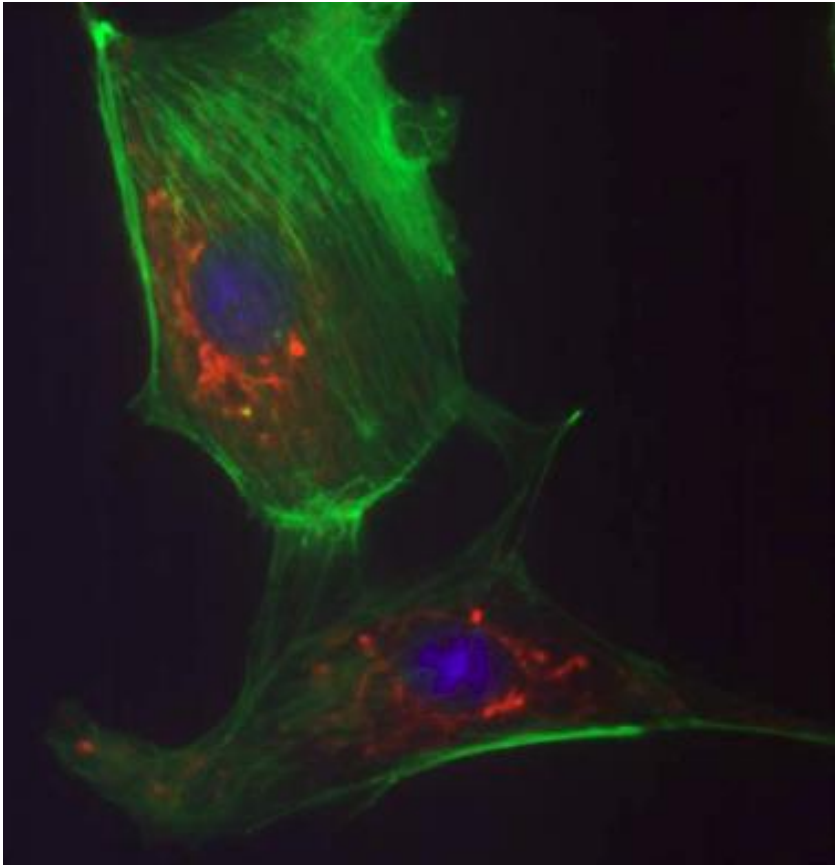
# Correction collars



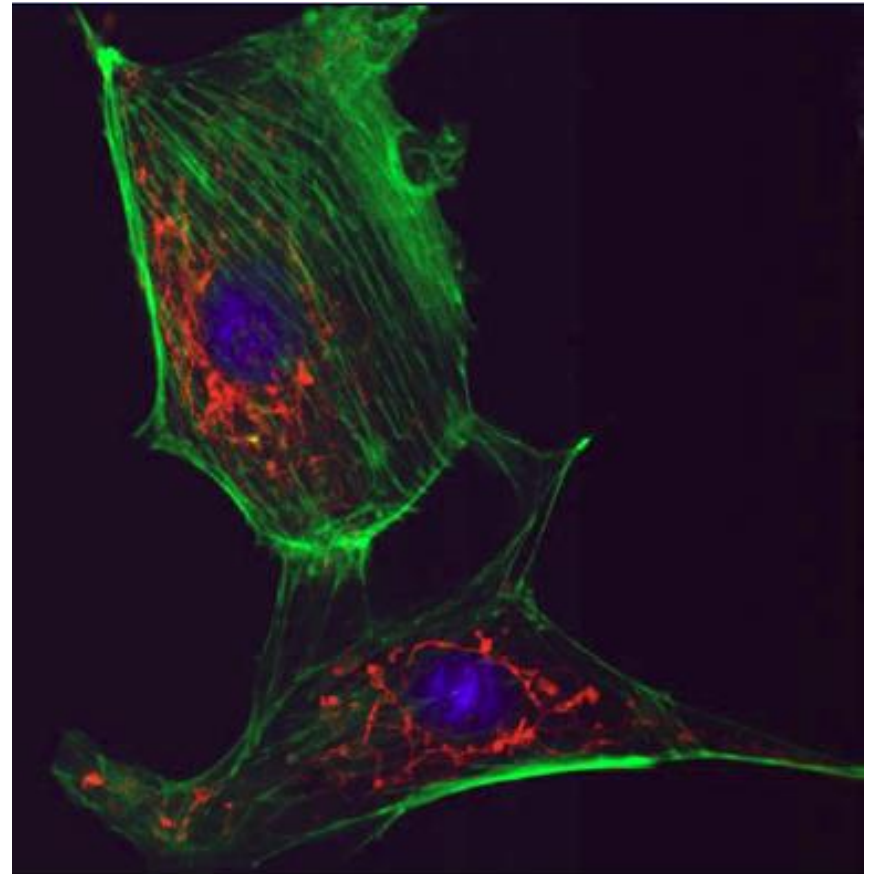
A correction collar can only eliminate spherical aberration at a single focal plane

# Example

Aberrated

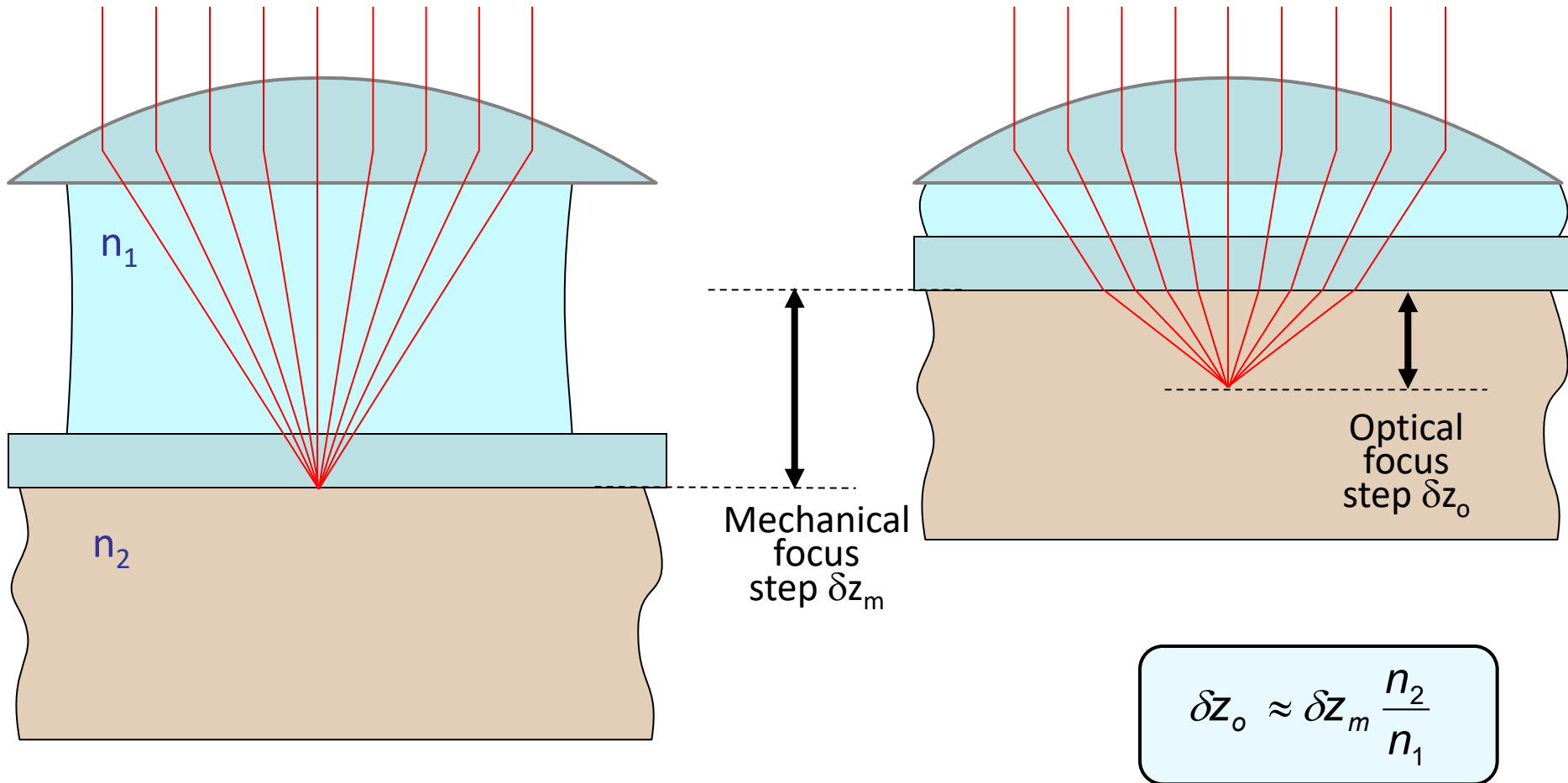


Unaberrated





# Index Mismatch & Axial Scaling

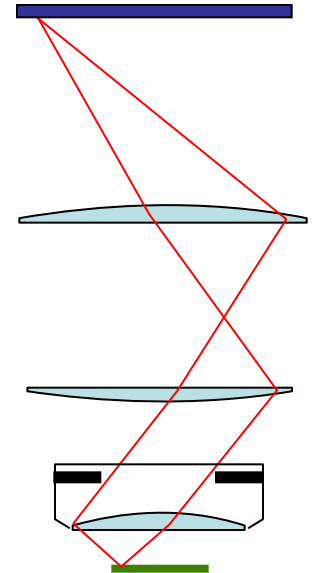


If there is index mismatch,  
your z pixel size is not what you think

# Sources of Astigmatism & Coma

## Off-axis (edges of field of view)

- All objectives have some
- Present in the design
- You get what you pay for

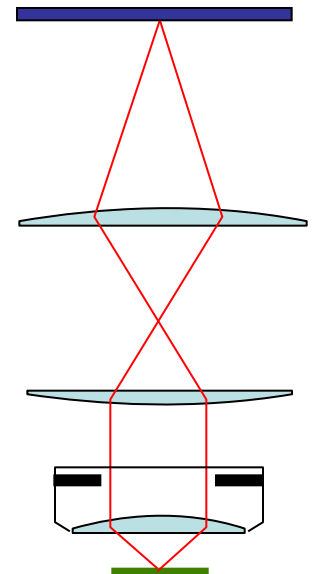


## On-axis (center of field of view)

Should be none, by symmetry.

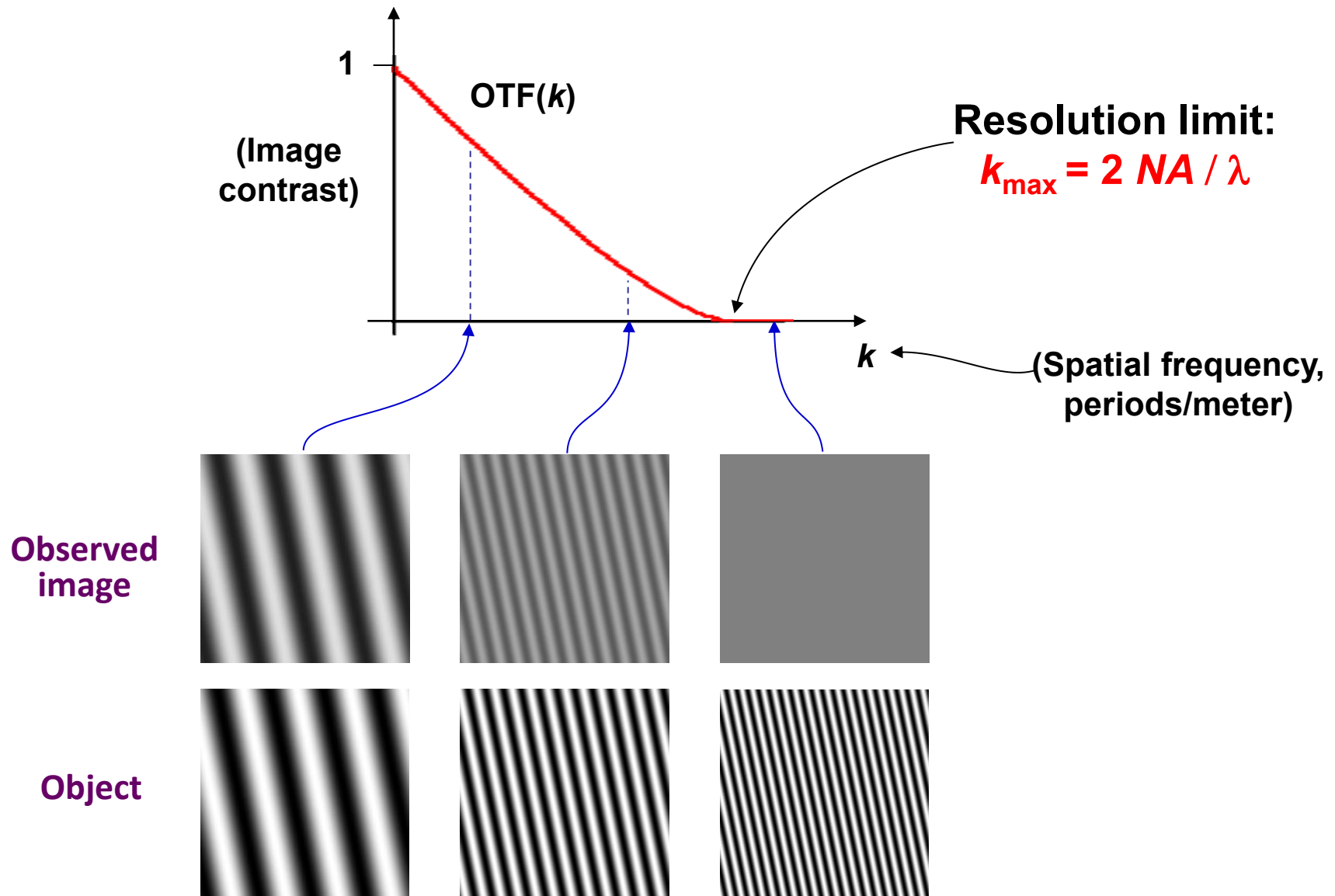
If they are there, they could be from:

- manufacturing or assembly tolerances
- dirt or abuse
- Misalignment (tilt, off-axis shift of something)
- **bad downstream components** (mirrors, dichroics, filters...)
- **Air bubble** in the immersion fluid or sample
- **Tilted cover slip**  
(dry and water-immersion high-NA lenses)



**More about  
Spatial frequencies & the  
Optical Transfer Function (OTF)**

# The response to pure waves is well-defined by the Optical Transfer Function (OTF)



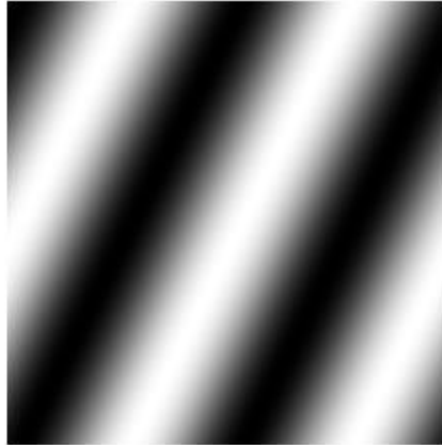
# Think of Images as Sums of Waves

... or “spatial frequency components”

one wave



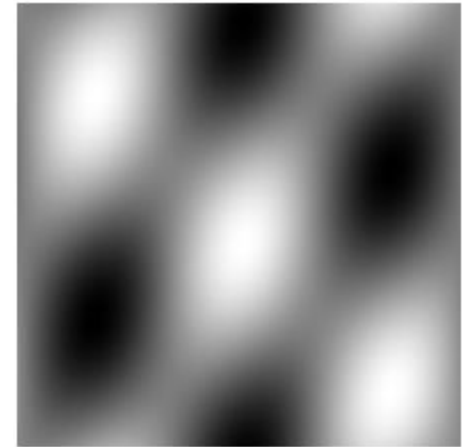
another wave



+

=

(2 waves)



(25 waves)



+ (...) =

(10000 waves)



+ (...) =

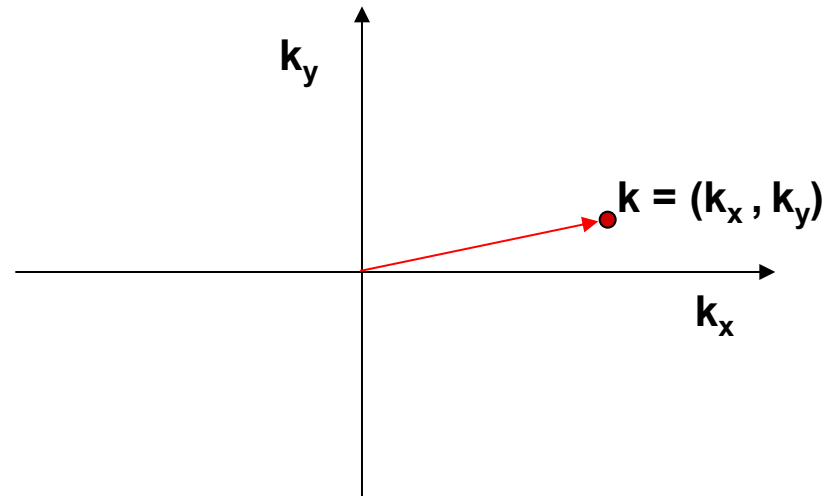
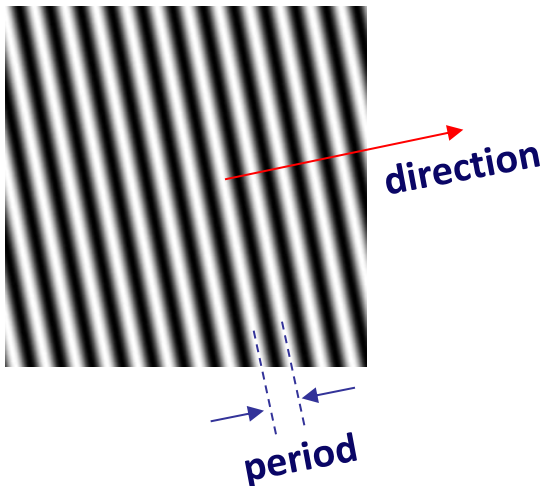
# Frequency Space

To *describe* a wave,  
we need to specify its:

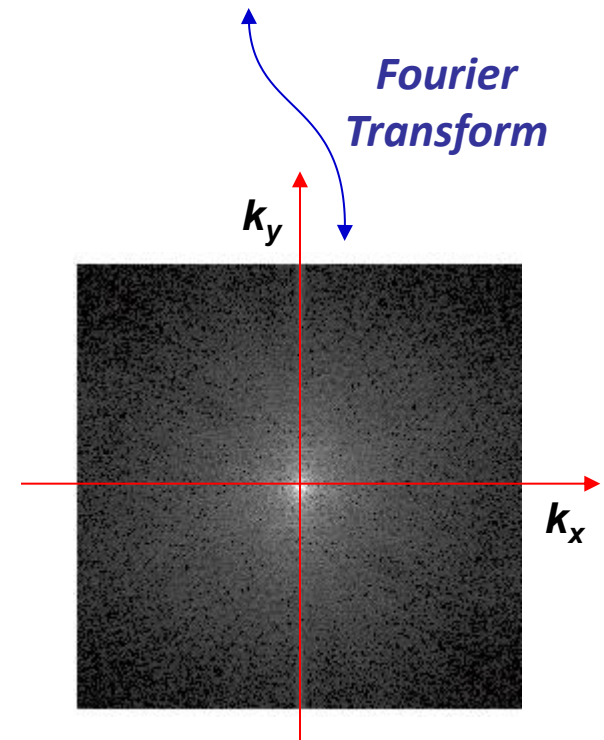
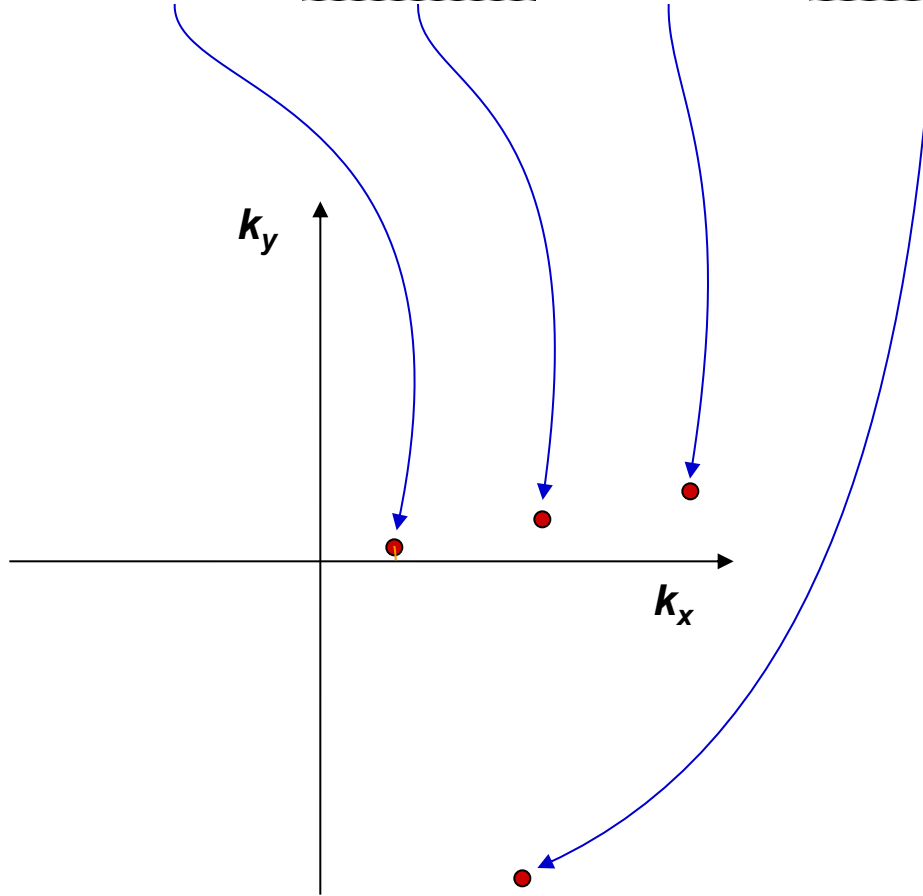
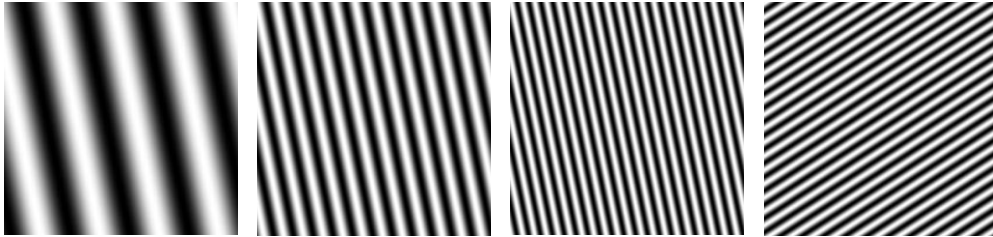
- **Frequency** (how many periods/meter?)
- **Direction**
- **Amplitude** (how strong is it?)
- **Phase** (where are the peaks & troughs?)

Can describe it by  
*a value at a point*

Distance from origin  
Direction from origin  
Magnitude of value  
Phase of value  
*complex*



# Frequency Space and the *Fourier Transform*



# Properties of the Fourier Transform

$$F(\mathbf{k}) = \int f(\mathbf{r}) e^{2\pi i \mathbf{k} \cdot \mathbf{r}} d\mathbf{r}$$

**Completeness:**

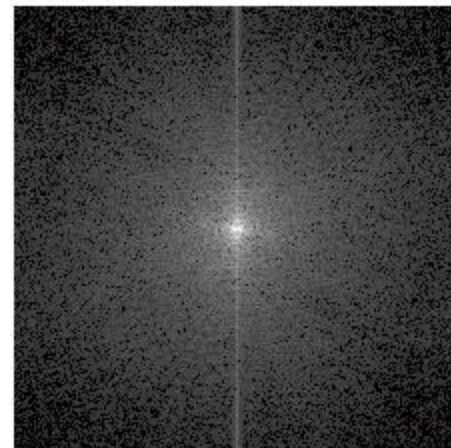
The Fourier Transform contains all the information of the original image

**Symmetry:**

The Fourier Transform of the Fourier Transform is the original image

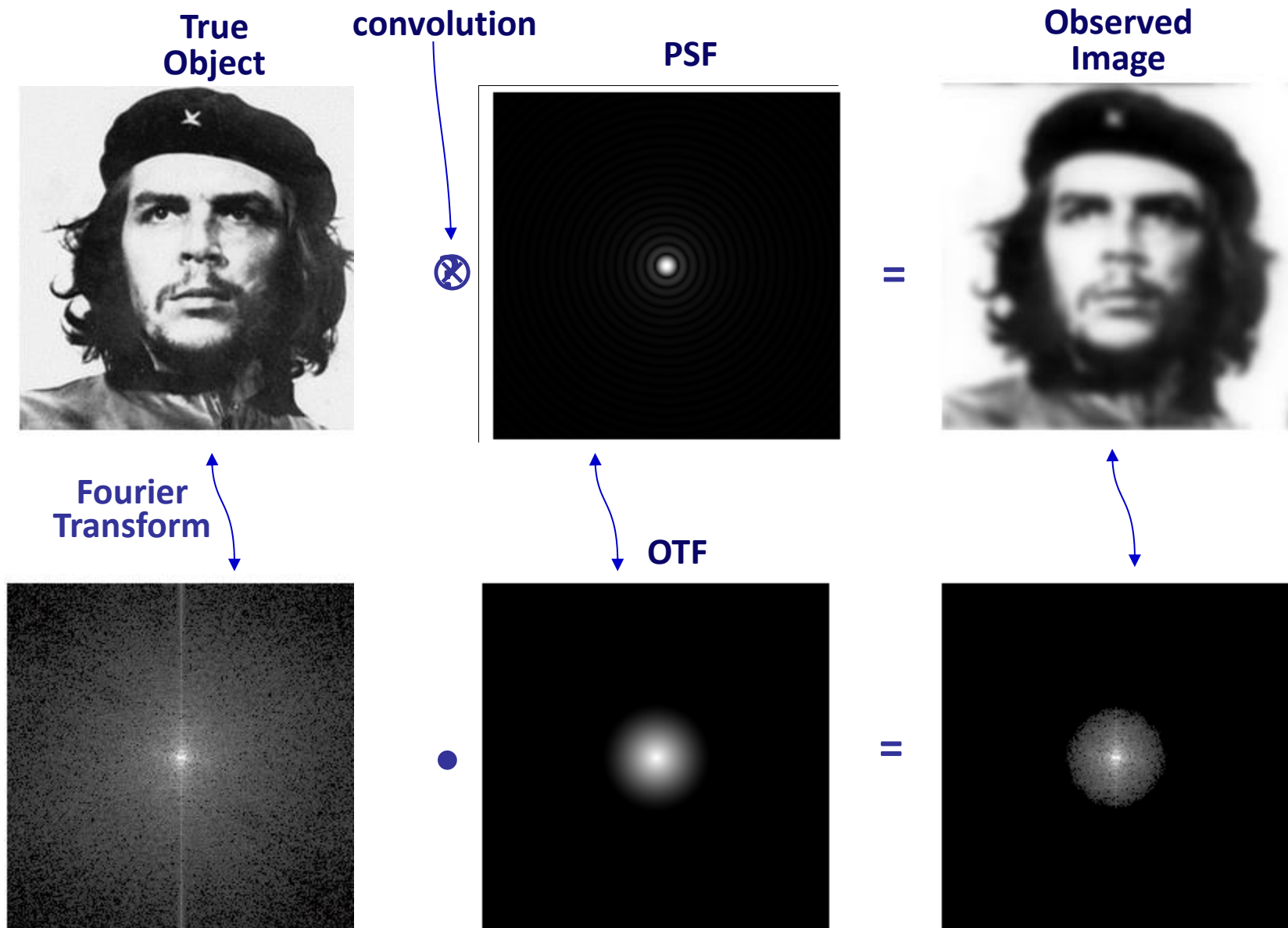


*Fourier  
transform*





# The OTF and Imaging



# Convolutions

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

Why do we care?

- They are everywhere...
- The convolution theorem:

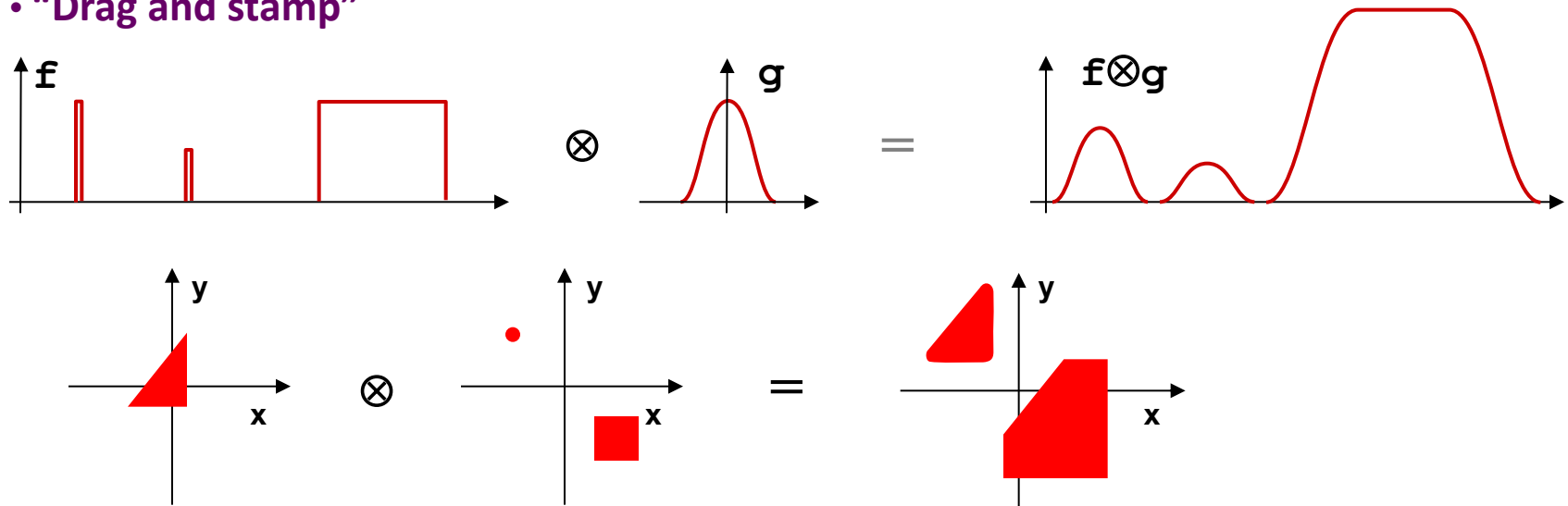
If  $h(r) = (f \otimes g)(r)$ ,  
 then  $\tilde{h}(k) = \tilde{f}(k) \tilde{g}(k)$

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

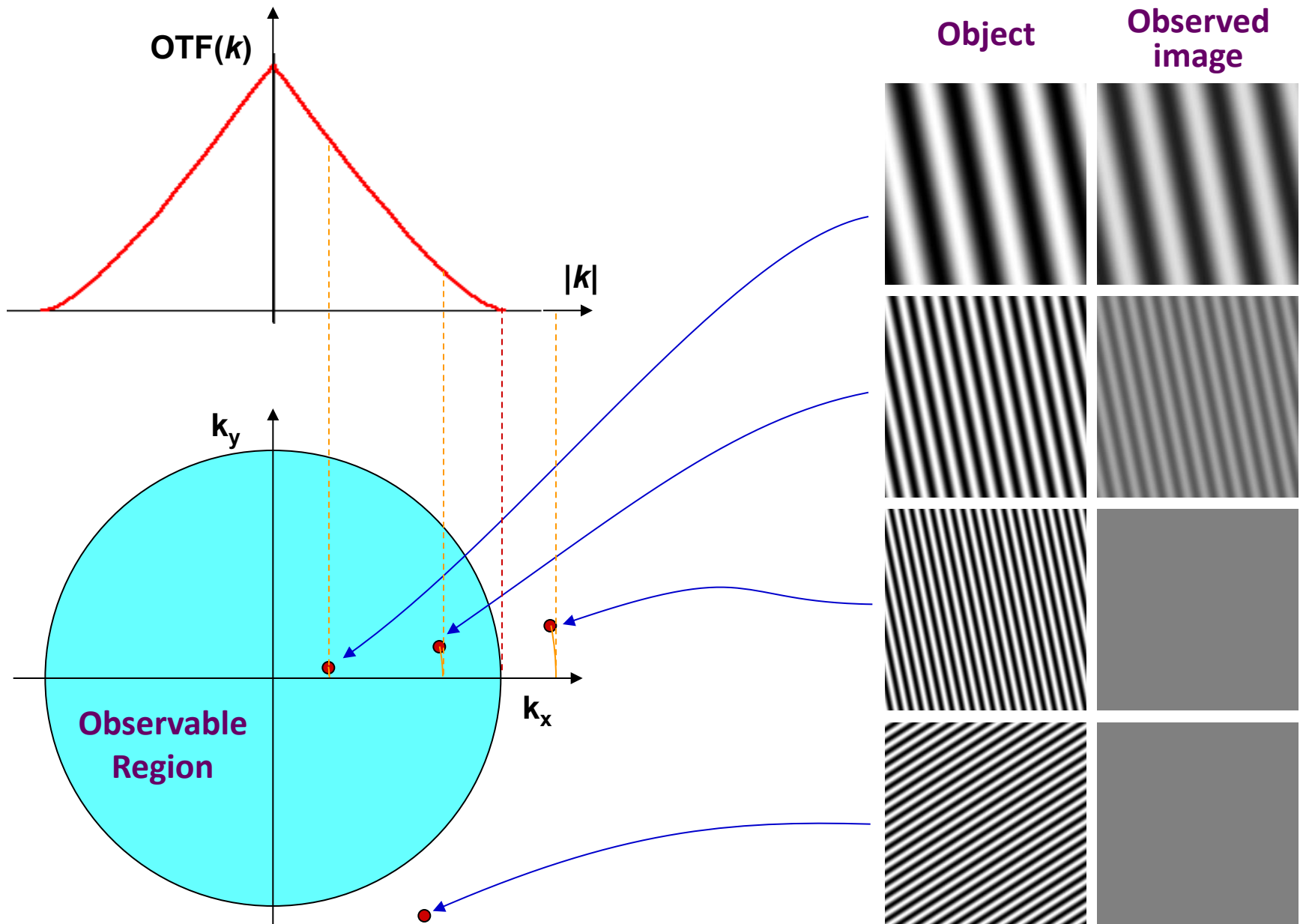
Symmetry:  $g \otimes f = f \otimes g$

So what is a convolution, intuitively?

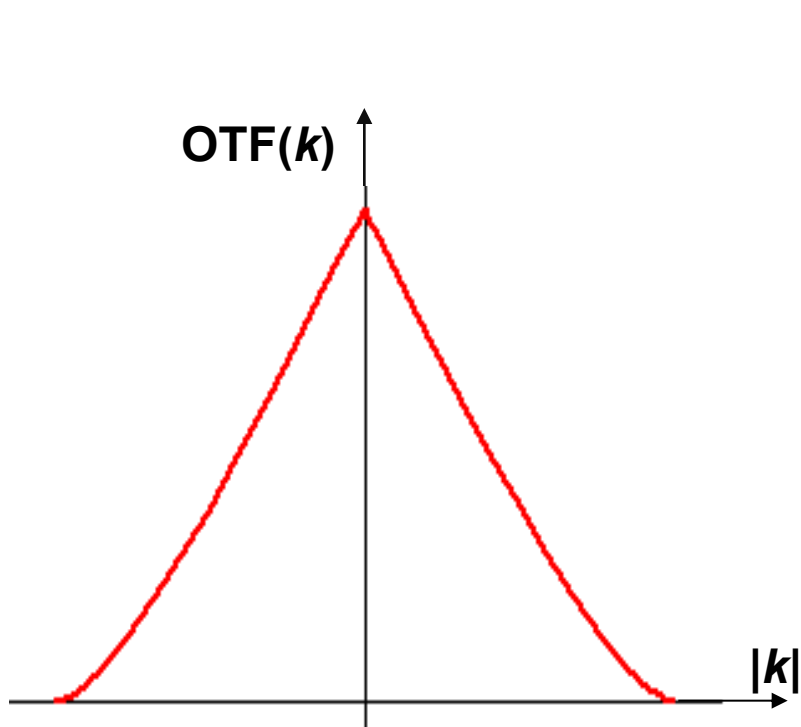
- “Blurring”
- “Drag and stamp”



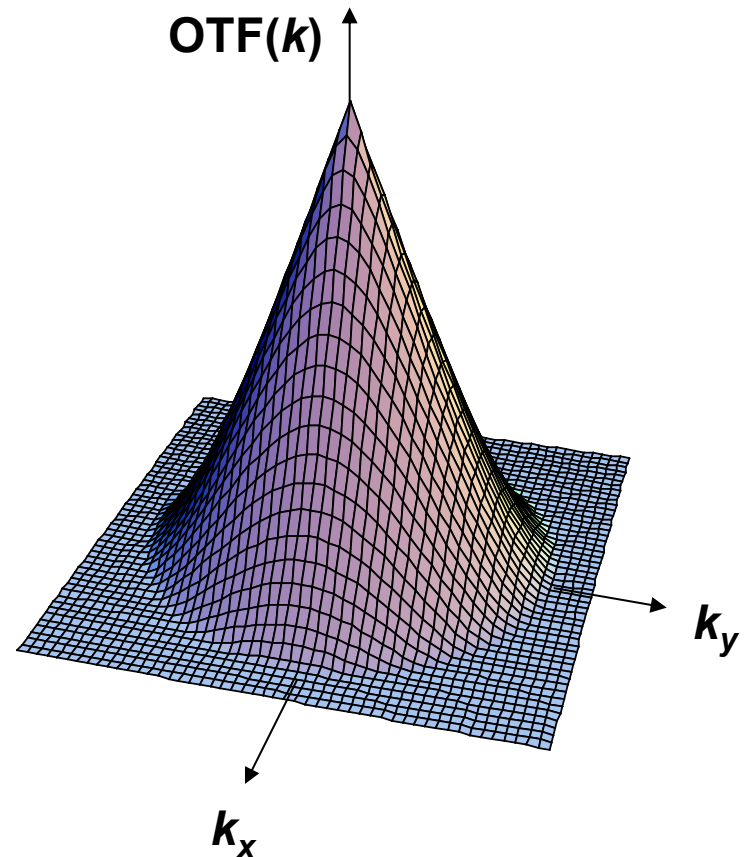
# The Transfer Function Lives in Frequency Space



# The 2D In-focus Optical Transfer Function (OTF)

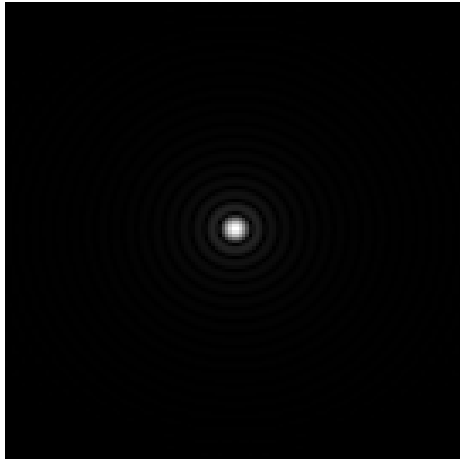


(Idealized  
calculations)

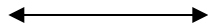


# The 3D OTF

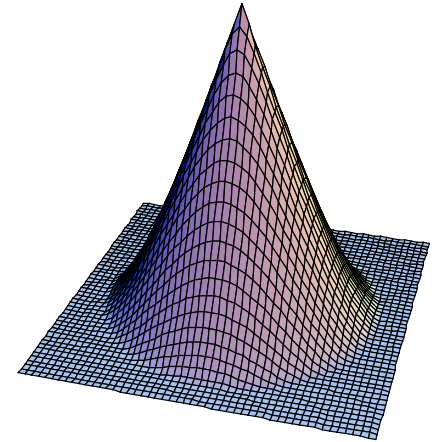
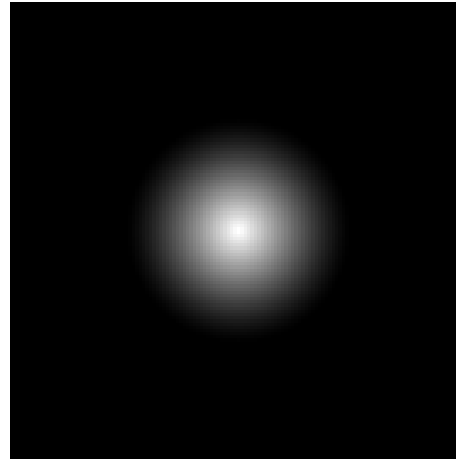
2D PSF



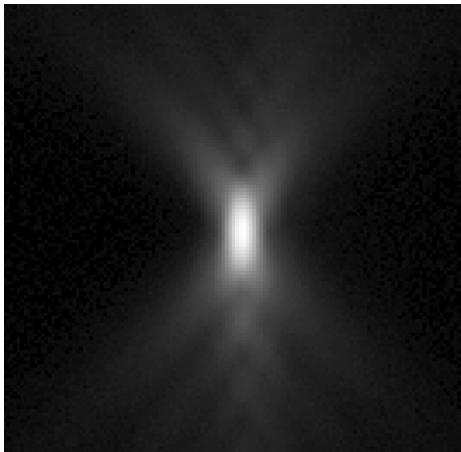
2D F.T.



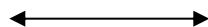
2D OTF



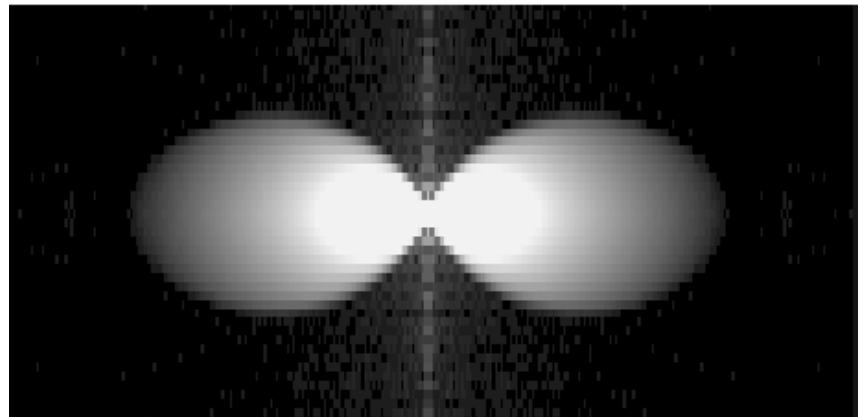
3D PSF



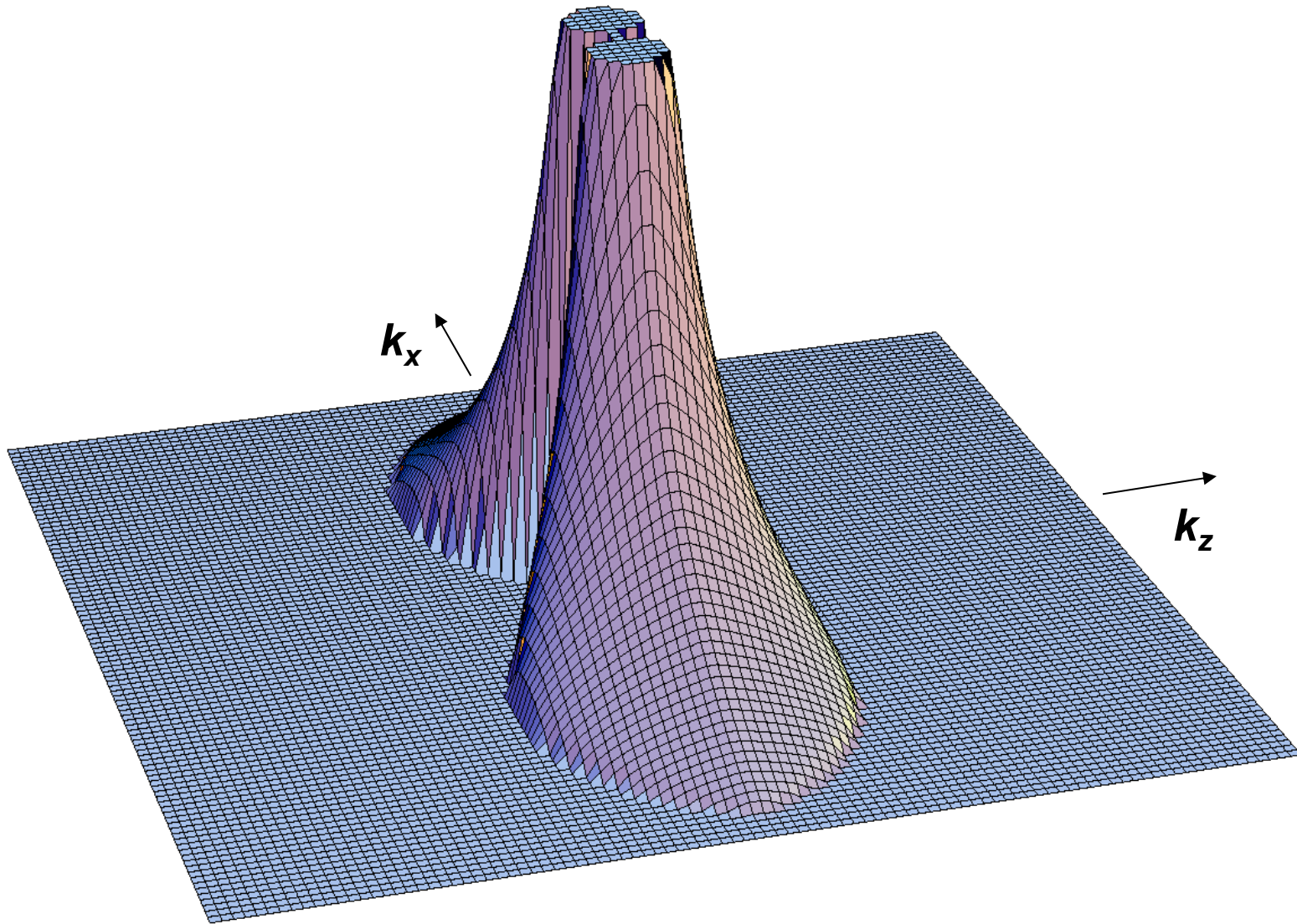
3D F.T.



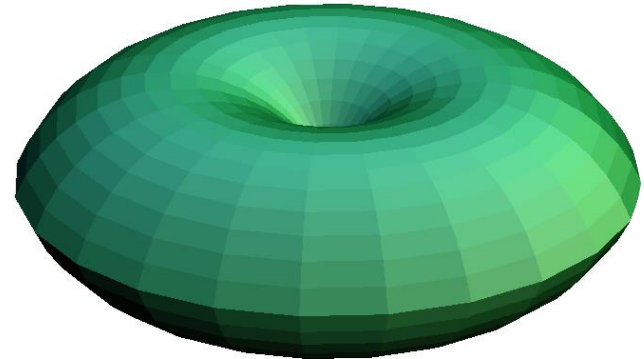
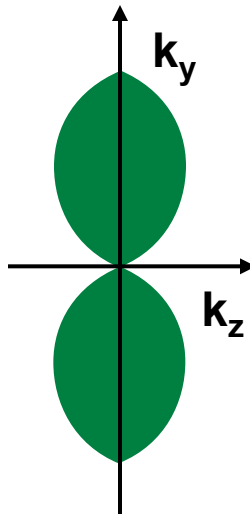
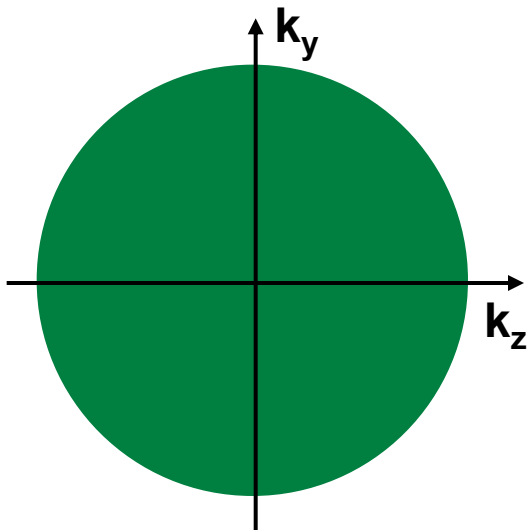
3D OTF



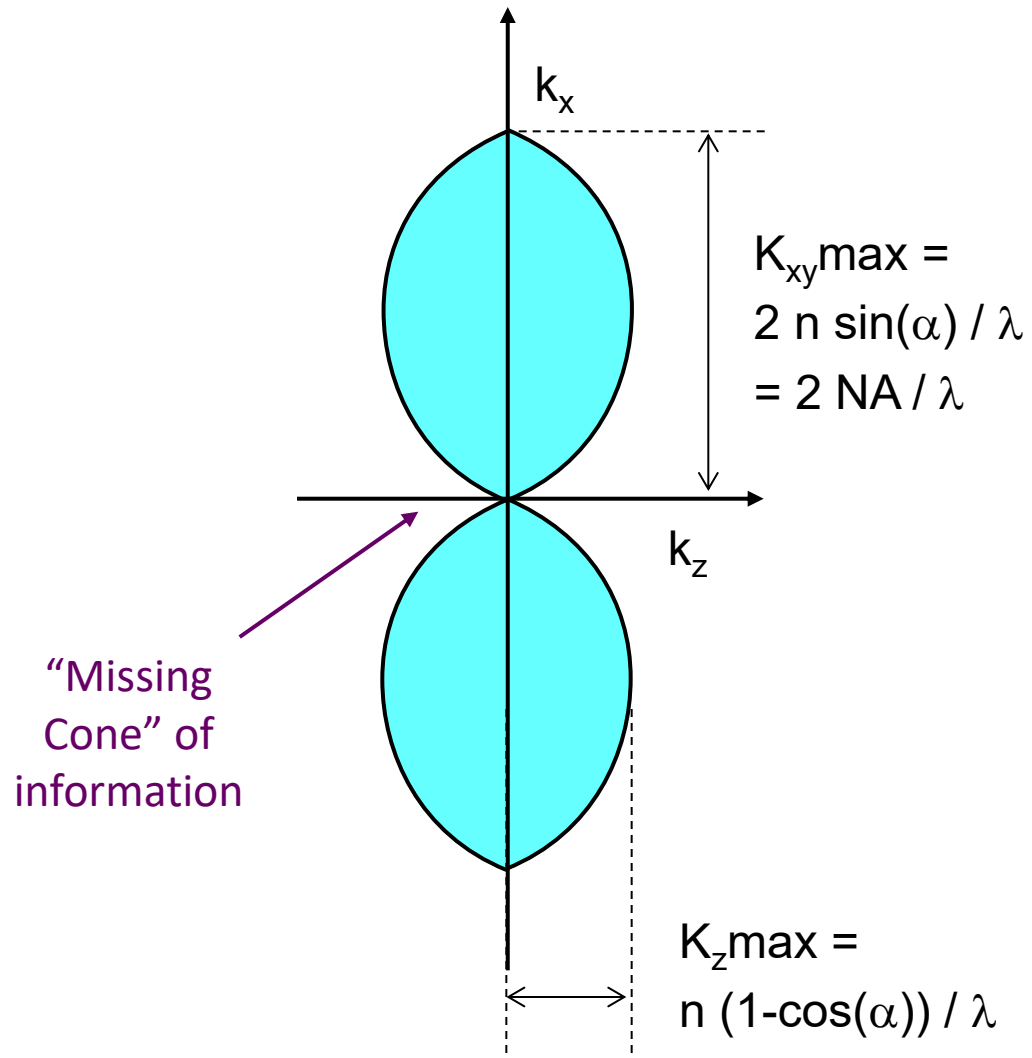
# Values of the 3D OTF



**3D Observable Region**  
= OTF support  
= Region where the OTF is non-zero

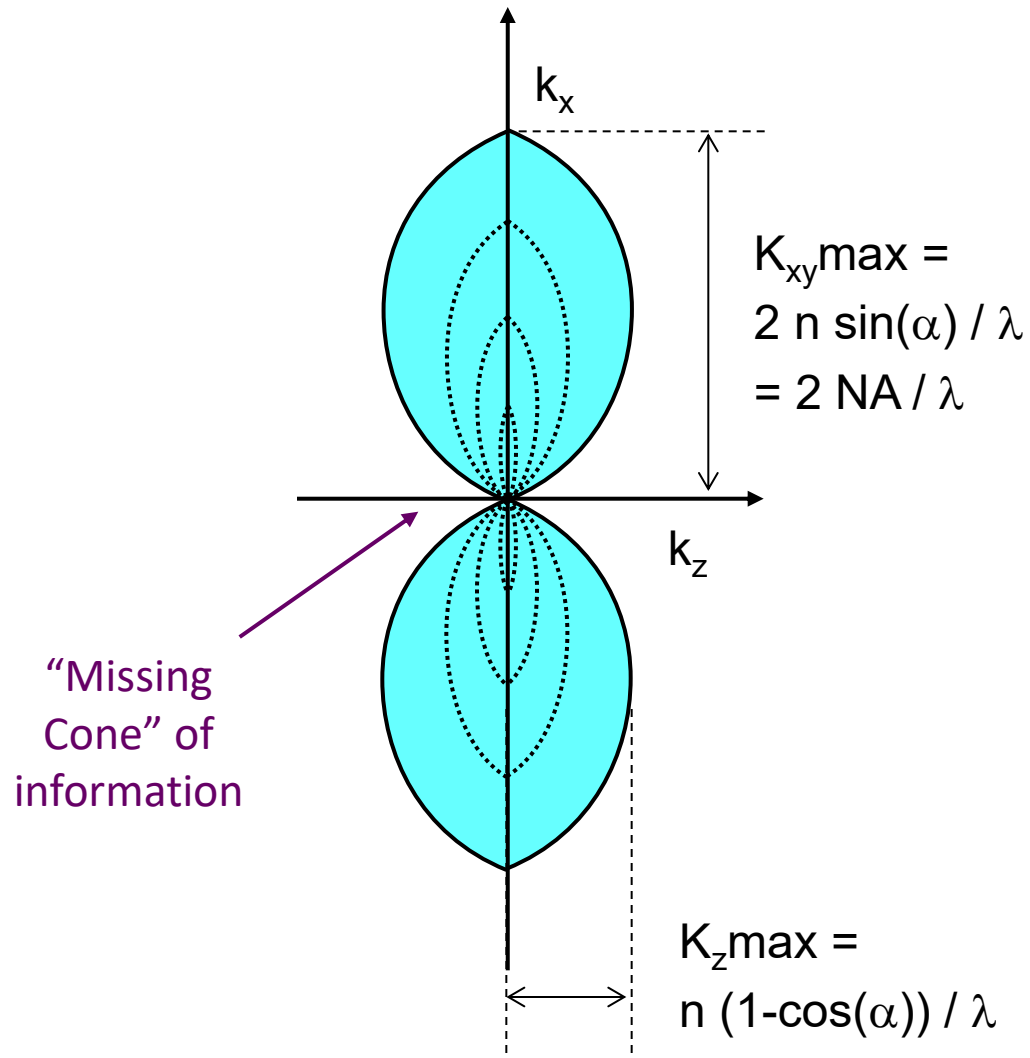


## So what is the resolution?





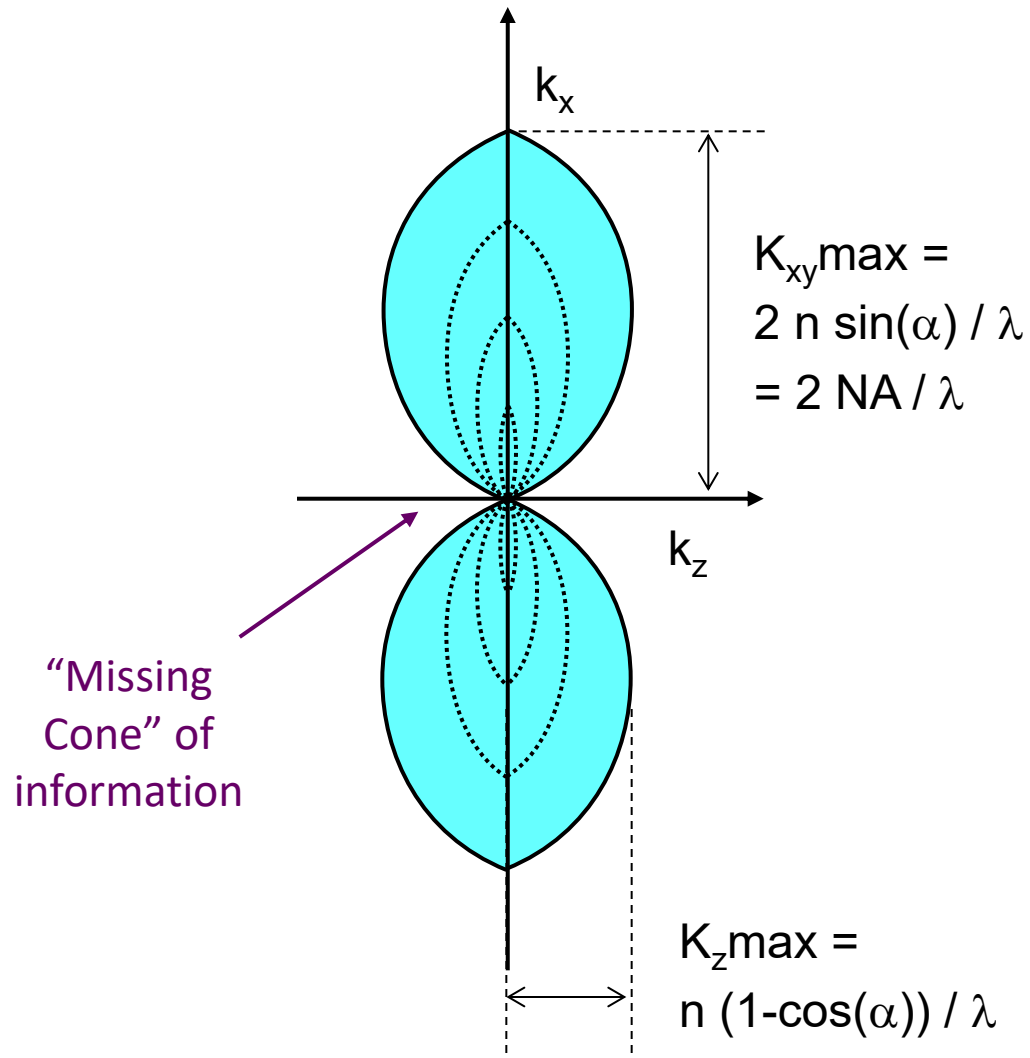
# So what is the resolution?



Lowering the NA  
Degrades the  
axial resolution  
faster than the  
lateral resolution

But low axial resolution  
= long *depth of field*  
This is *good*,  
if 2D is enough

## So what is the resolution?



Example:  
a high-end objective

$$\text{NA} = 1.4$$

$$n = 1.515$$

$$\rightarrow \alpha = 67.5^\circ$$

$$\lambda = 600 \text{ nm}$$



Lateral (XY) resolution:

$$1 / K_{xy} \text{max} = 0.21 \text{ } \mu\text{m}$$

Axial (Z) resolution:

$$1 / K_z \text{max} = 0.64 \text{ } \mu\text{m}$$

# Nomenclature

- Optical Transfer Function, OTF  
Complex value with amplitude and phase
- Contrast Transfer Function, CTF
- Modulation Transfer Function, MTF  
Same thing without the phase information

# Resources

Slides available at: <http://nic.ucsf.edu/edu.html>

<http://www.microscopyu.com>

<http://micro.magnet.fsu.edu>

Douglas B. Murphy “Fundamentals of Light Microscopy and Electronic Imaging”

James Pawley, Ed. “Handbook of Biological Confocal Microscopy, 3rd ed.”

# Acknowledgements

Steve Ross, Mats Gustafsson