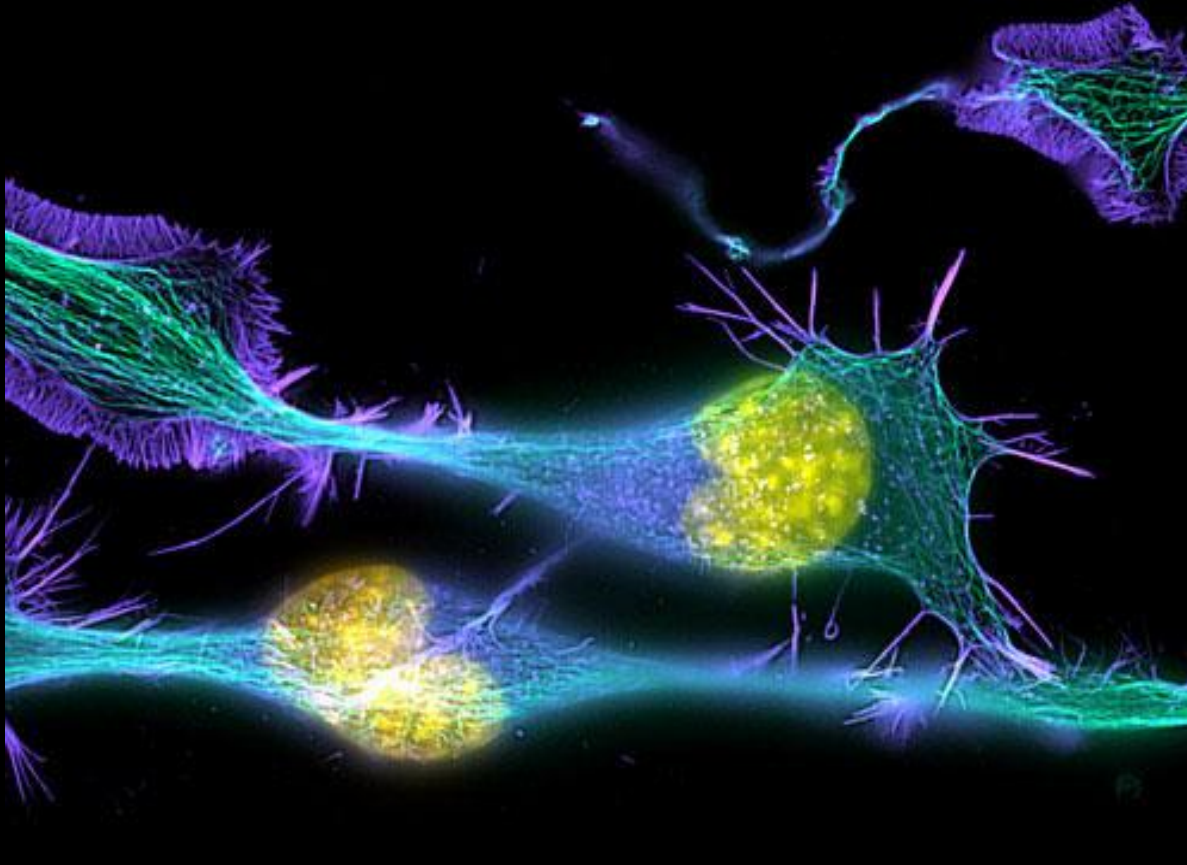
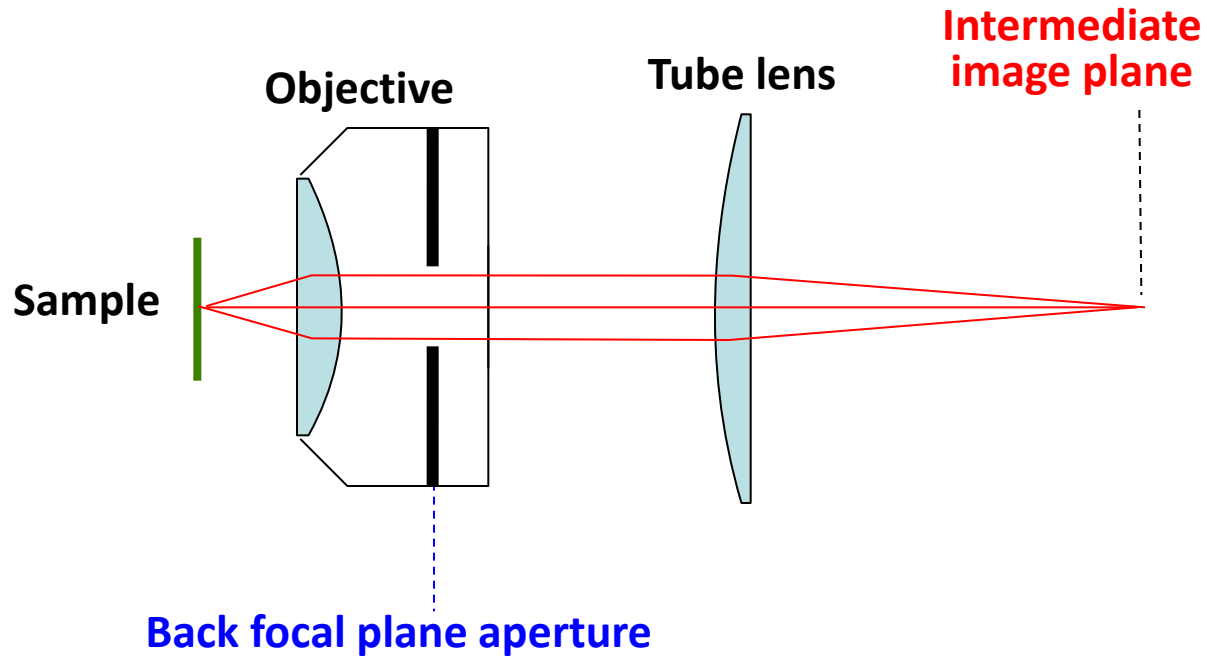


Principles & Practice of Light Microscopy: 2

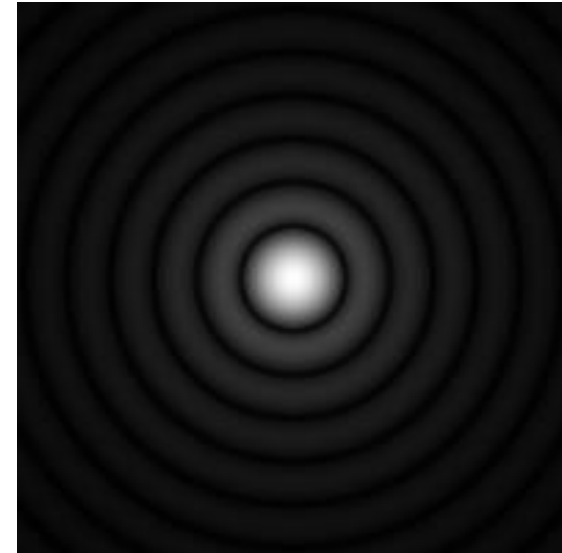


(Image: T. Wittman, Scripps)

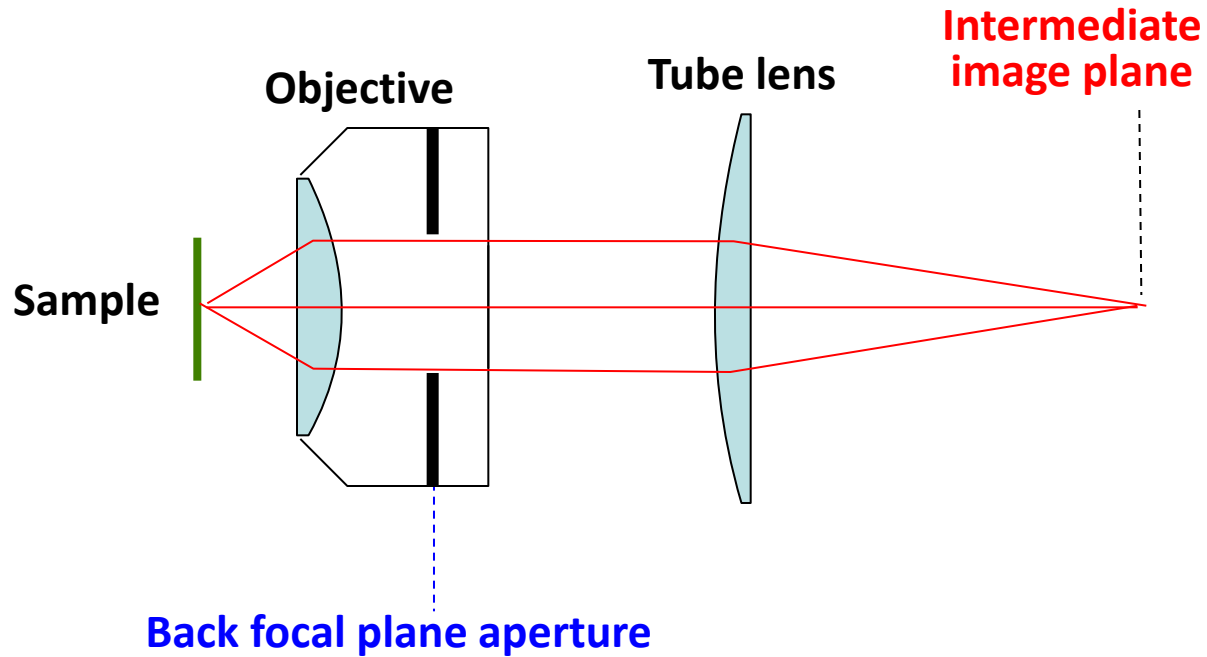
Aperture and Resolution



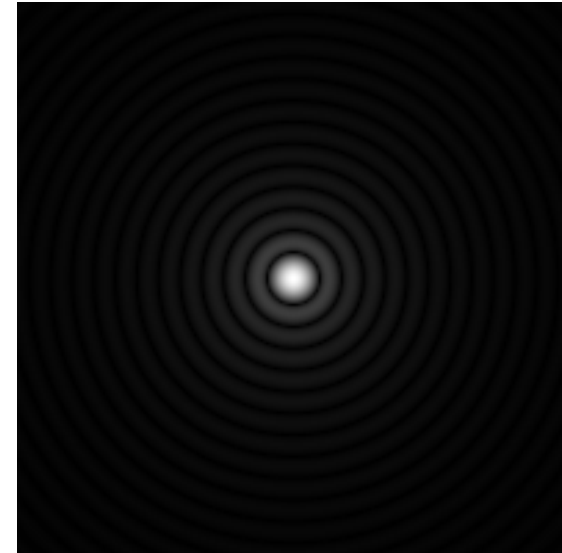
Diffraction spot
on image plane
= *Point Spread Function*



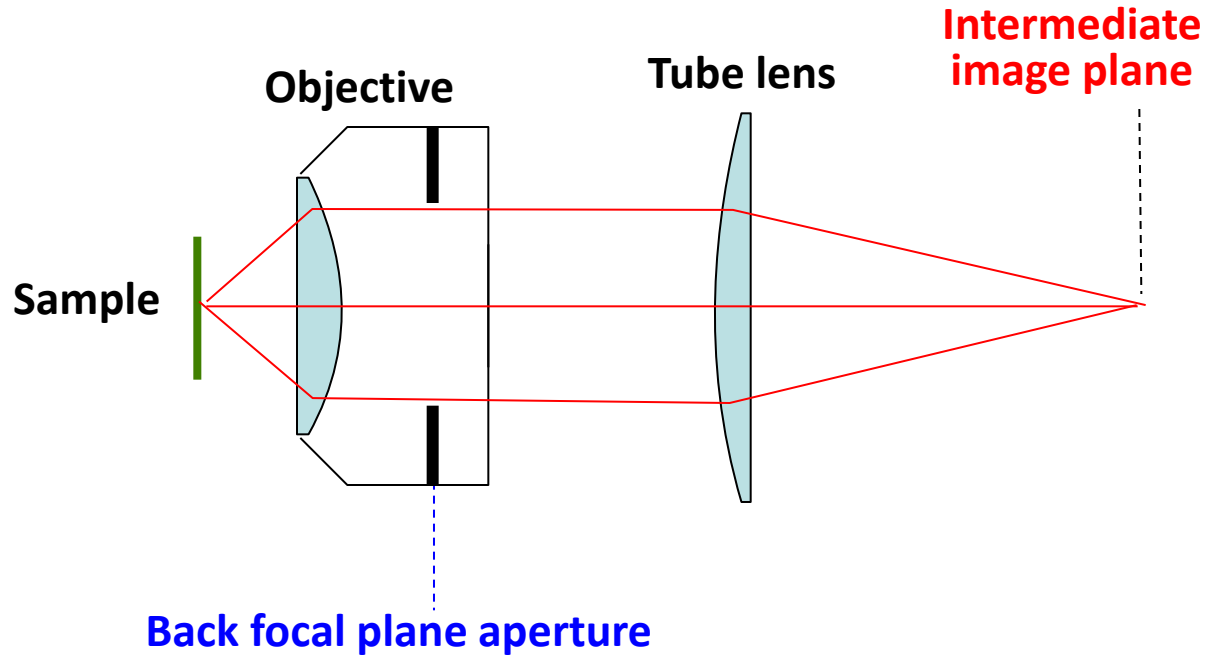
Aperture and Resolution



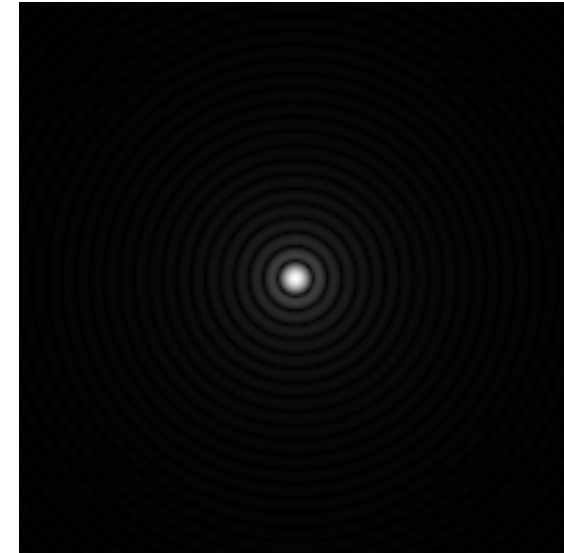
**Diffraction spot
on image plane
= *Point Spread Function***



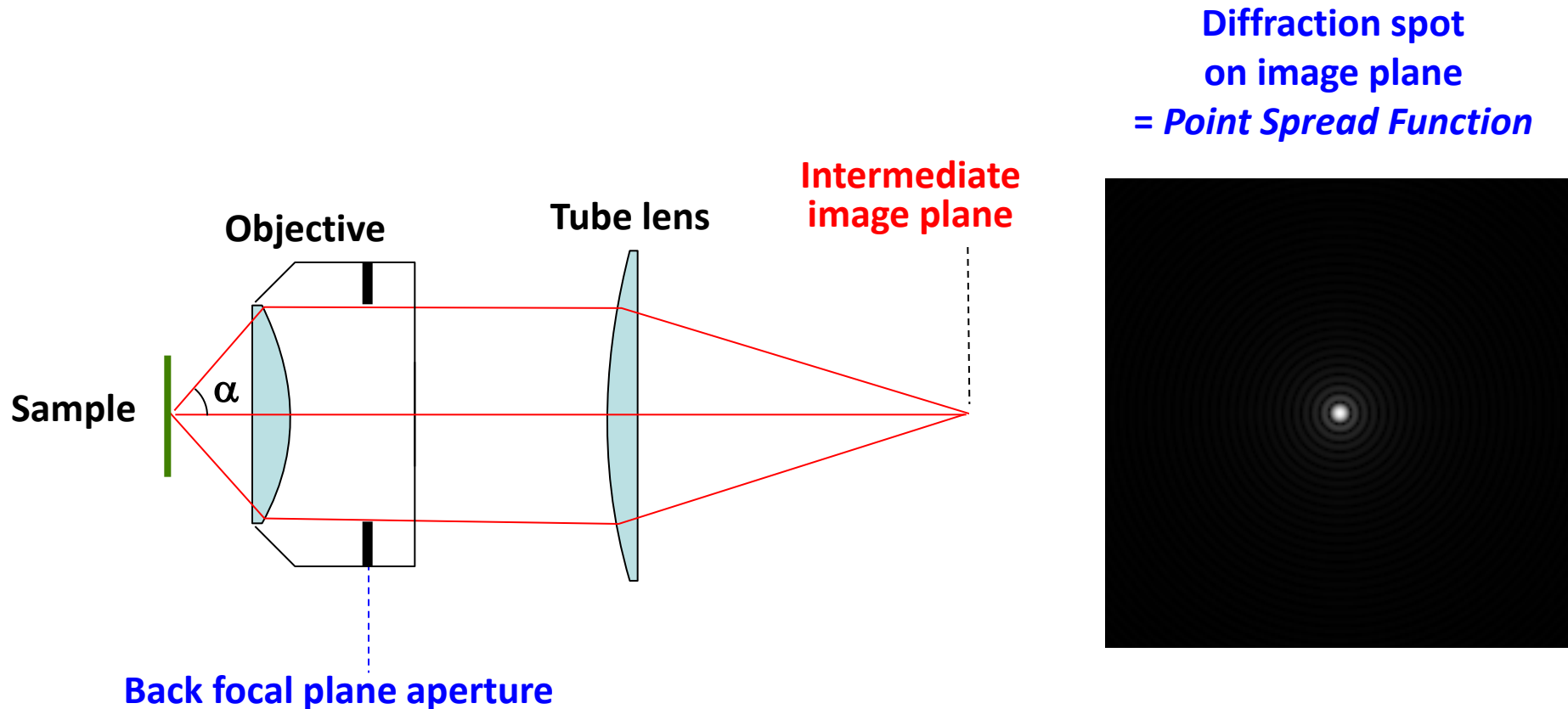
Aperture and Resolution



Diffraction spot
on image plane
= *Point Spread Function*



Aperture and Resolution



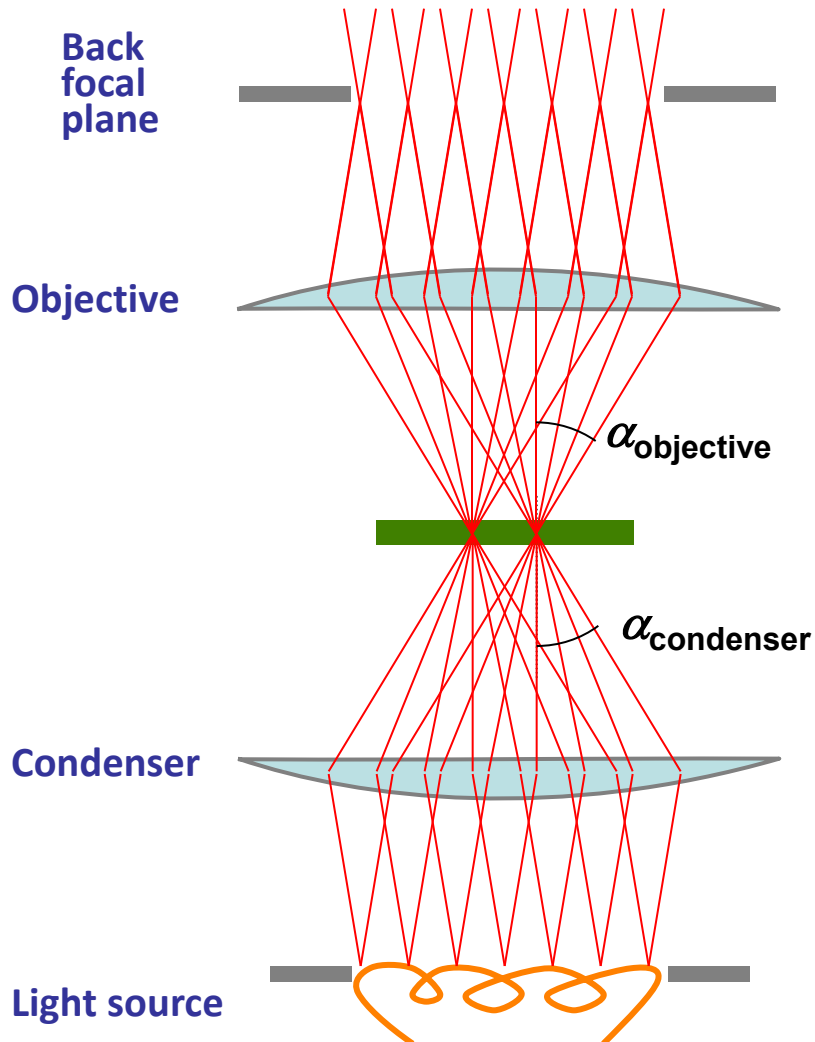
- Image resolution improves with Numerical Aperture (NA)

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

where: α = 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

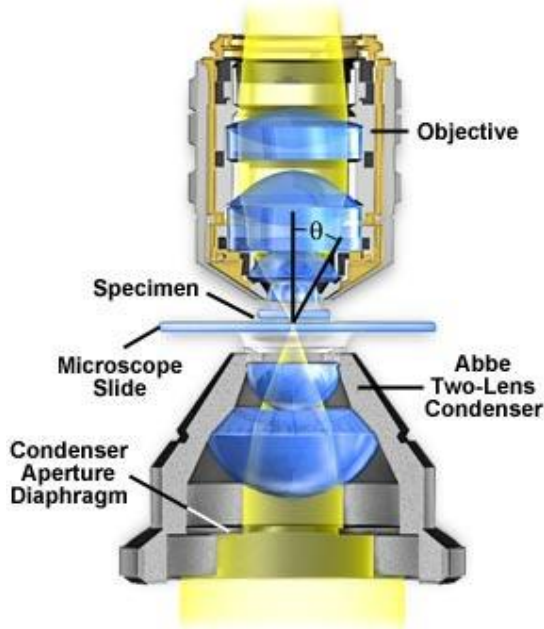
$$\alpha_{\text{condenser}} \geq \alpha_{\text{objective}}$$

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

\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 α
(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)

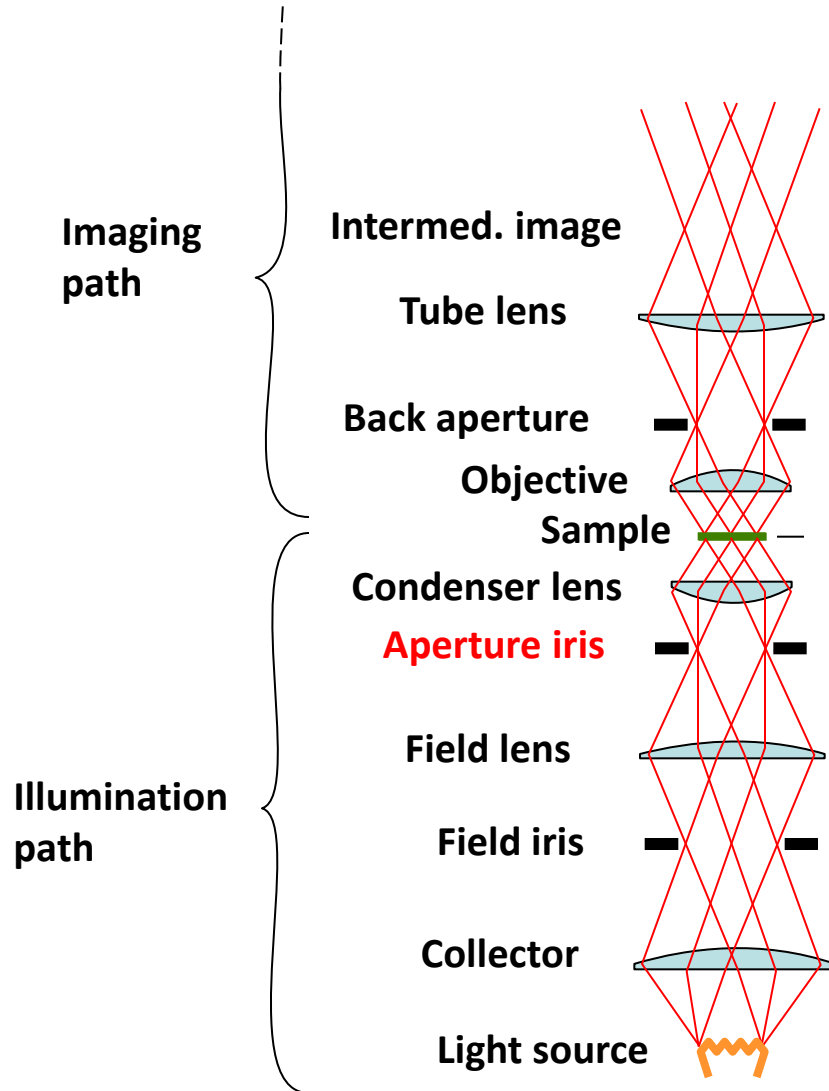


Swing-Out Top Lens Condenser (Numerical Aperture = 1.35)



Aperture, Resolution & Contrast

Can adjust the condenser NA with the aperture iris

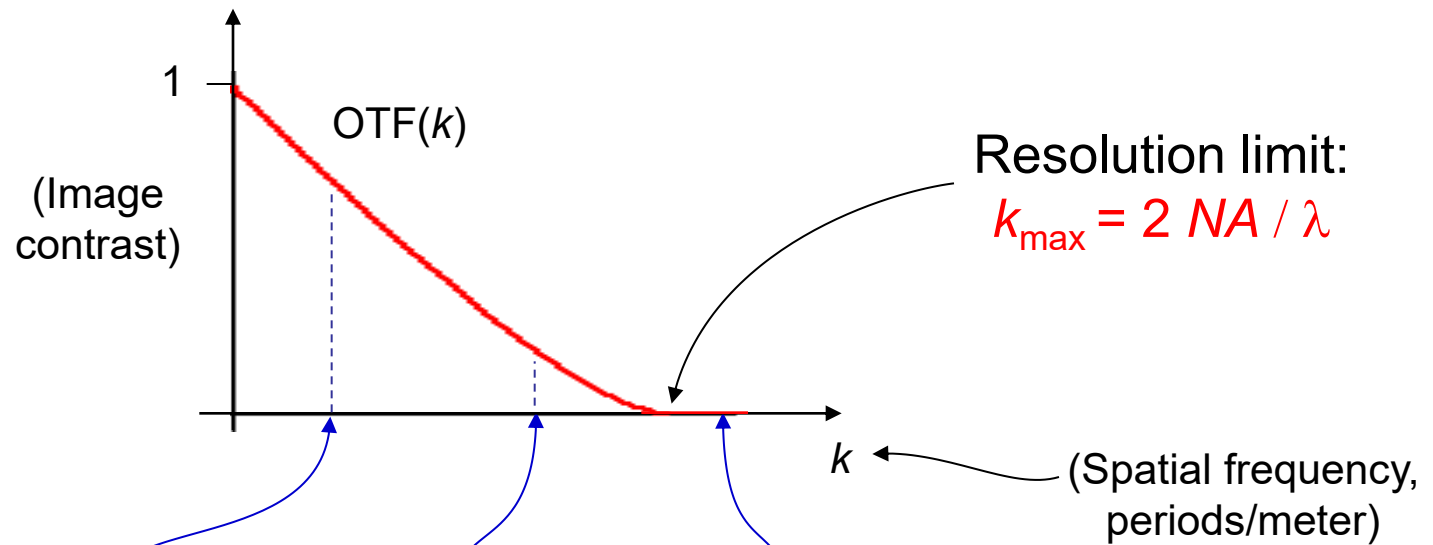


Q: Don't we always want it full open??

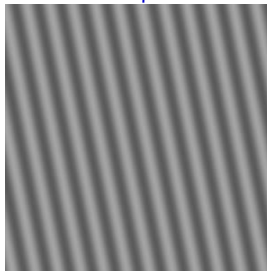
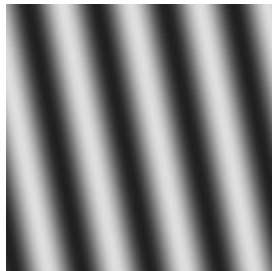
A: No

Why? Tradeoff:
resolution vs. contrast

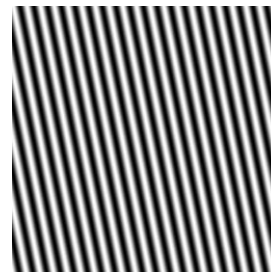
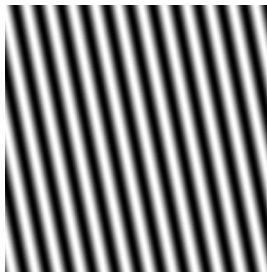
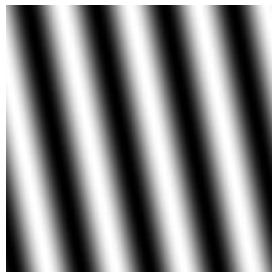
Spatial frequencies & the Optical Transfer Function (OTF)



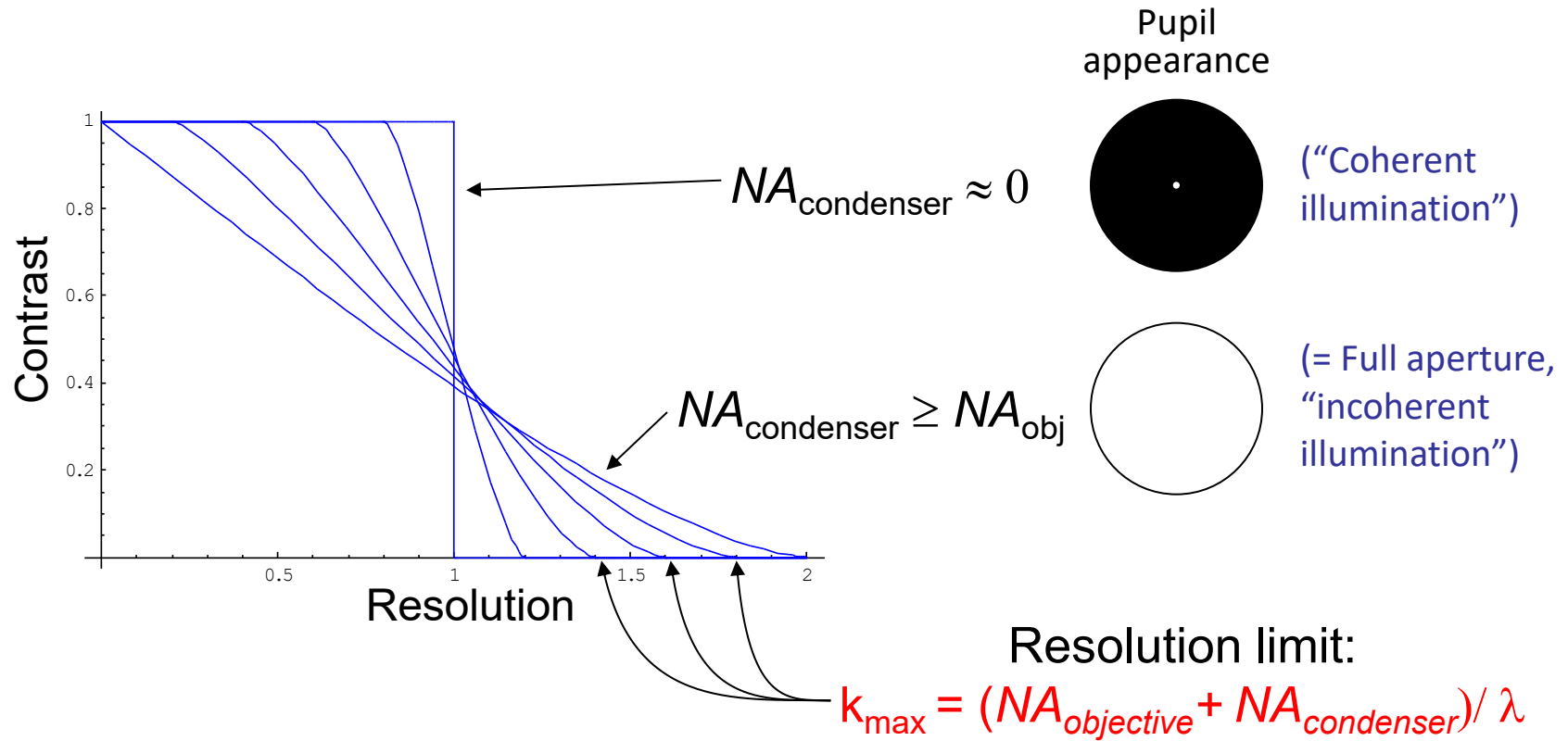
Observed image



Object



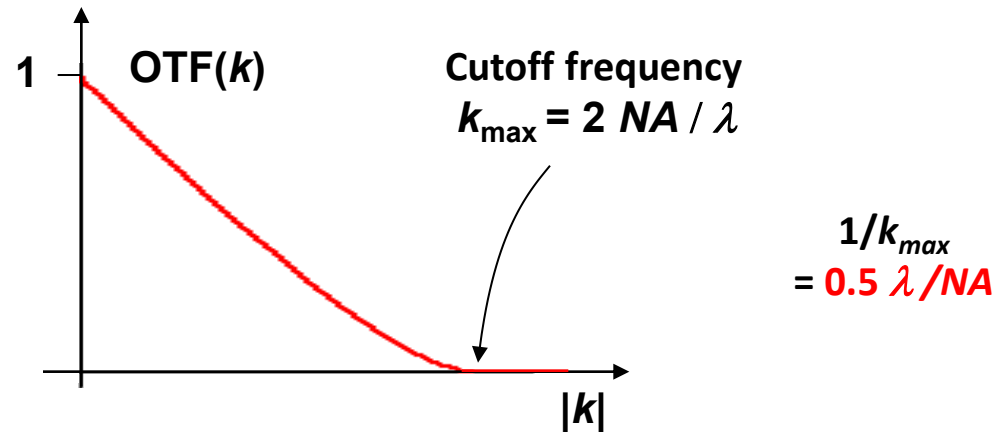
Resolution & Contrast vs. Illumination aperture



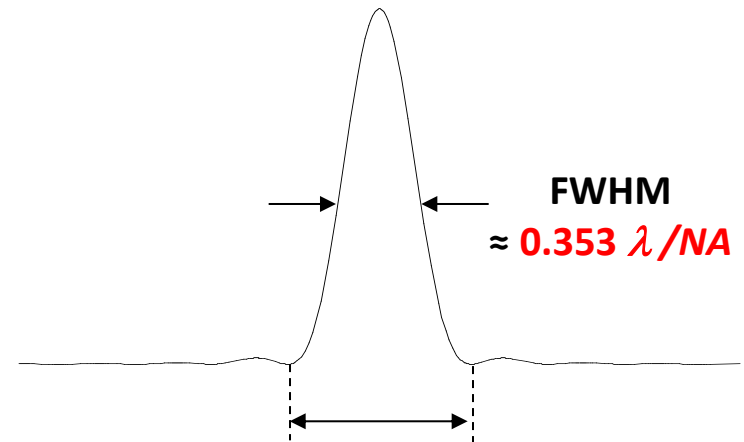
Increasing the illumination aperture
increases resolution
but decreases contrast

Definitions of Resolution

As the OTF cutoff frequency



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



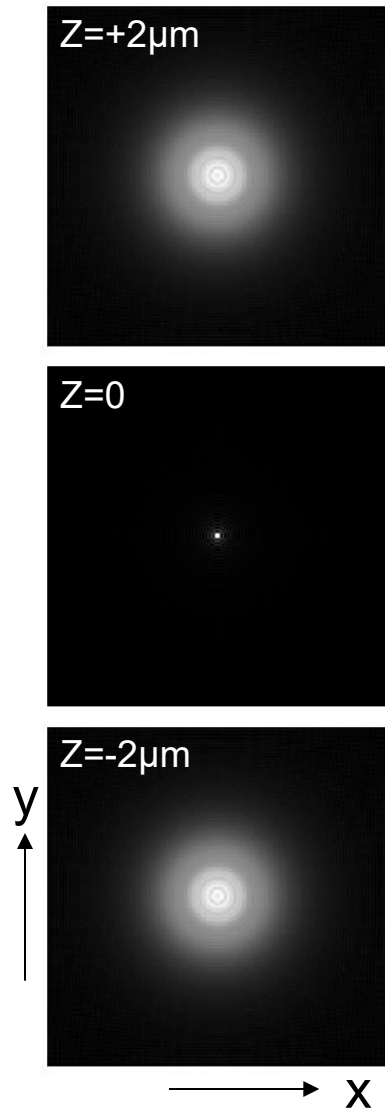
As the diameter of the Airy disk (first dark ring of the PSF)
= "Rayleigh criterion"

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

The 3D Point Spread Function (PSF)

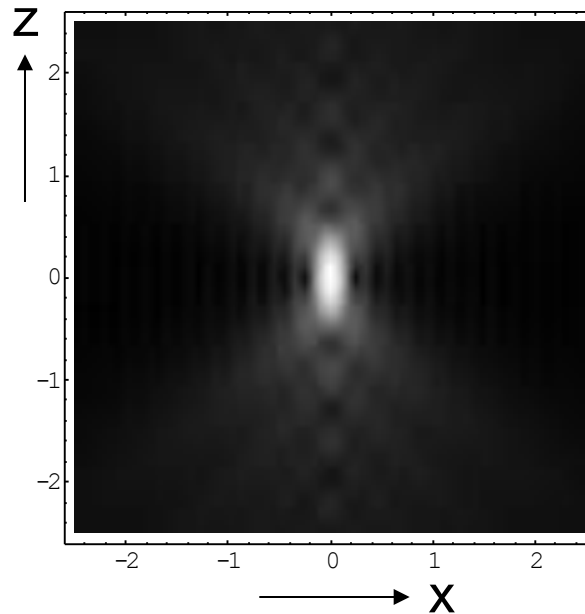
The image of a point object

2D PSF
for different defocus

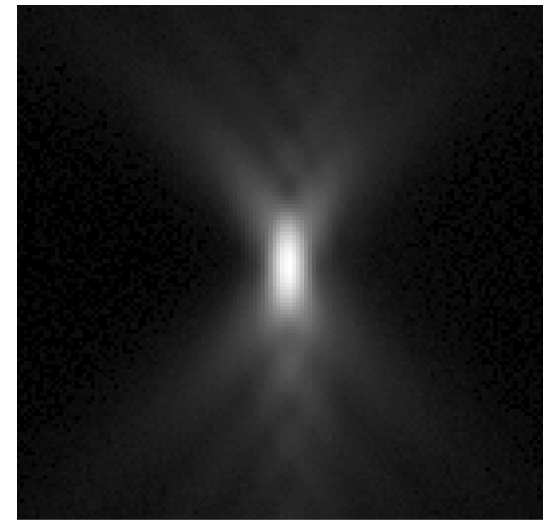


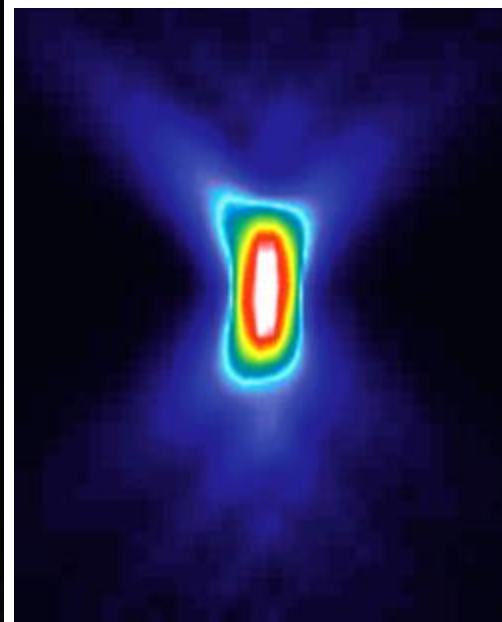
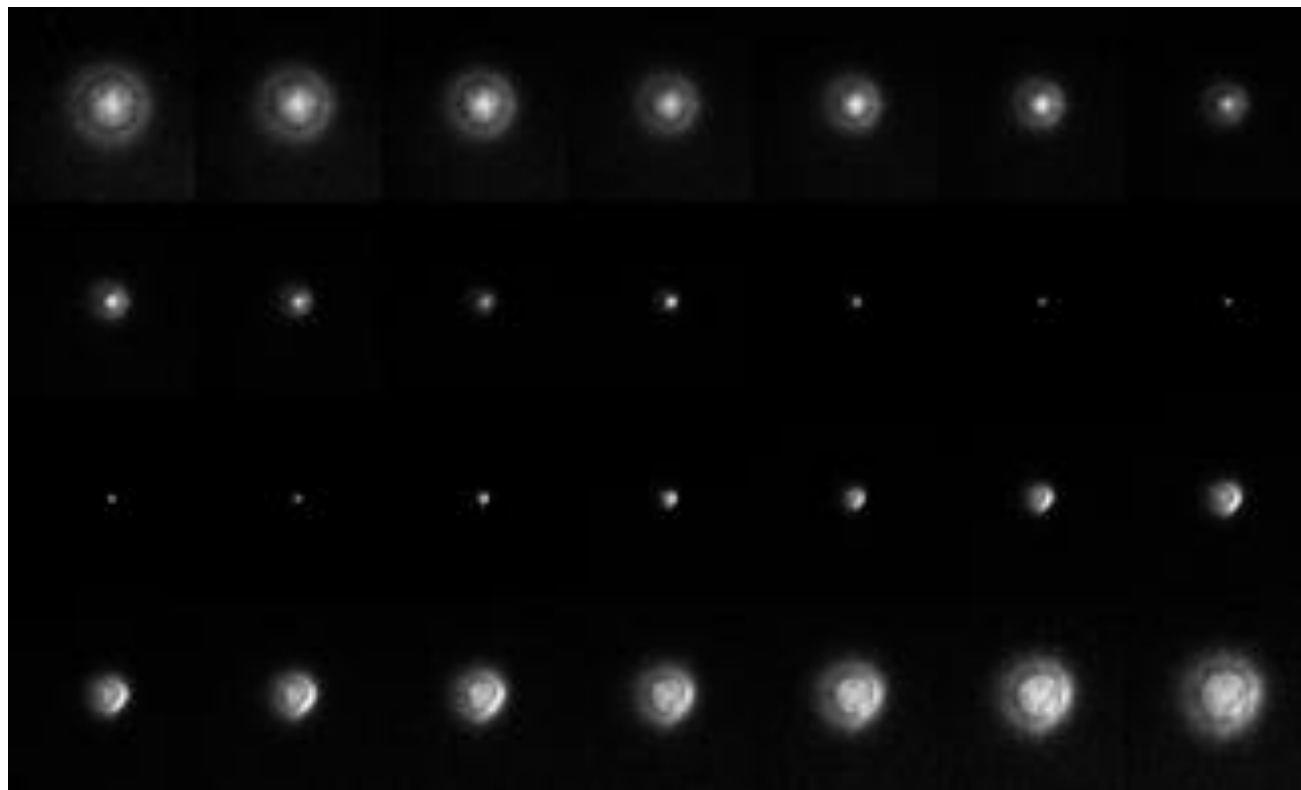
3D PSF

Calculated



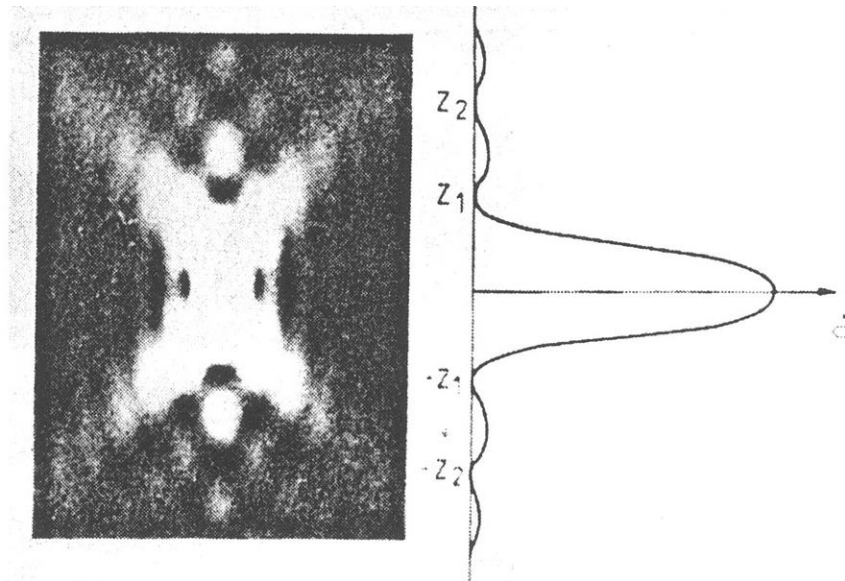
Measured





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 (μm)
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

Magnification	NA	Resolution (nm)	Depth of Field (nm)	Light gathering (arb. units)
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

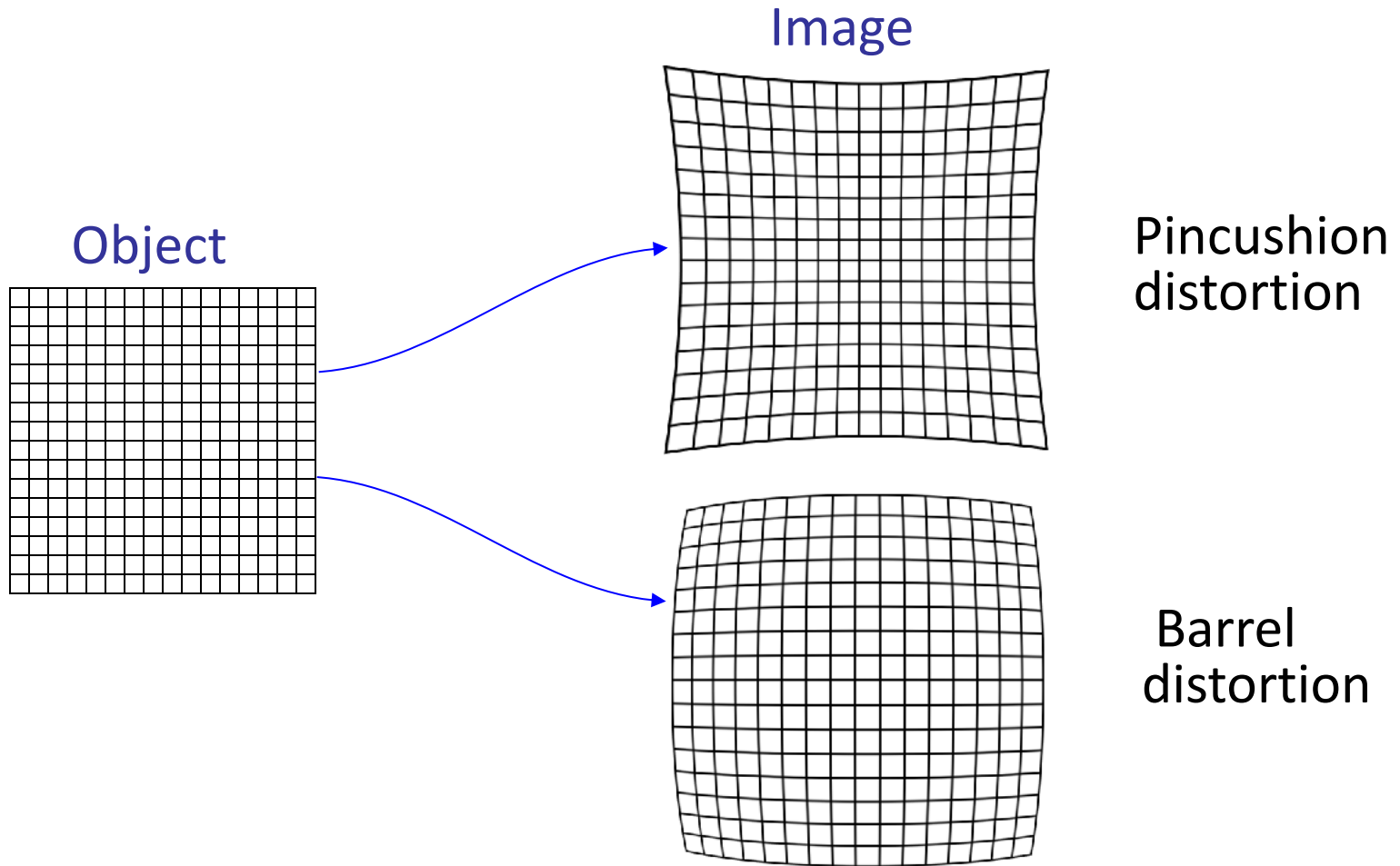
 - ...

- Curvature of field

- Distortion

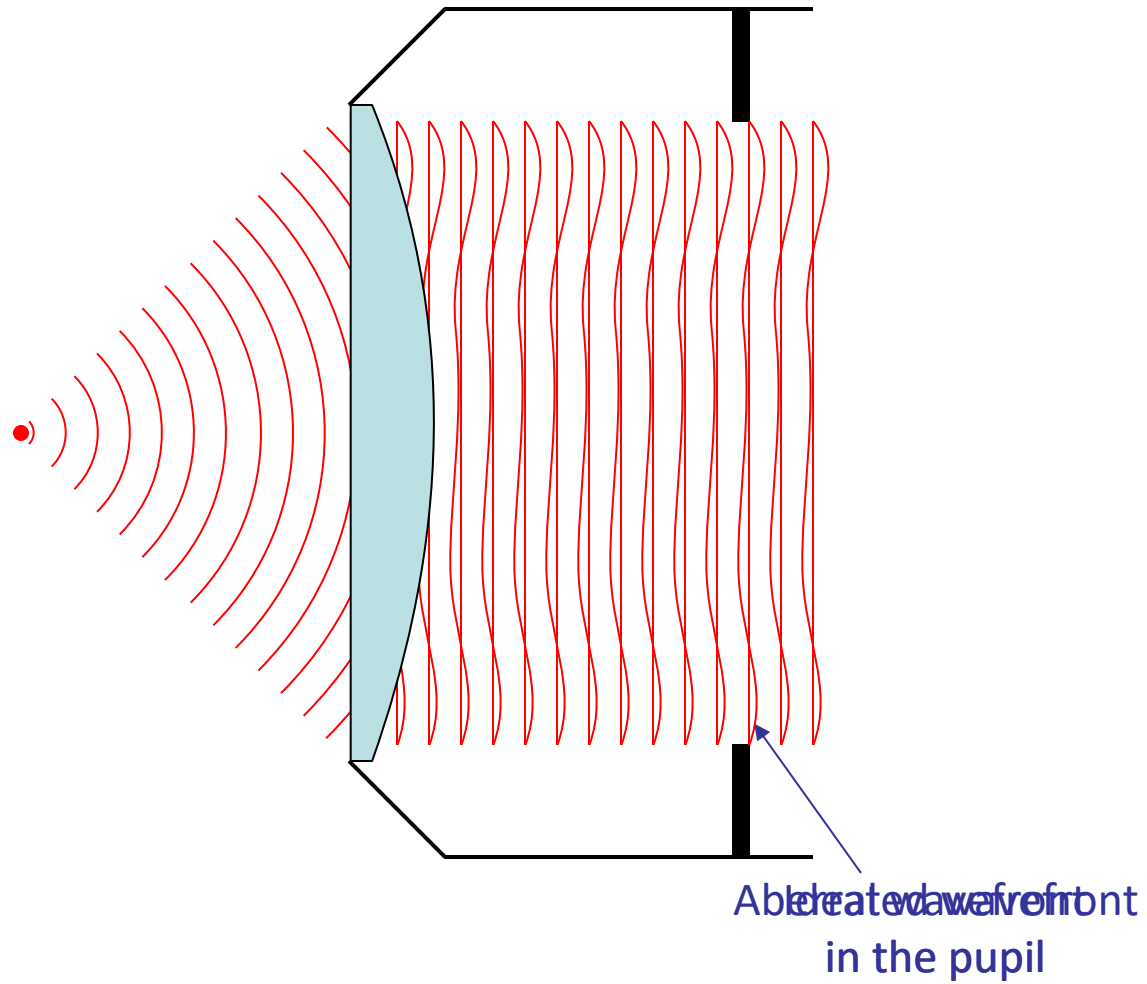
Geometric Distortion

= Radially varying magnification

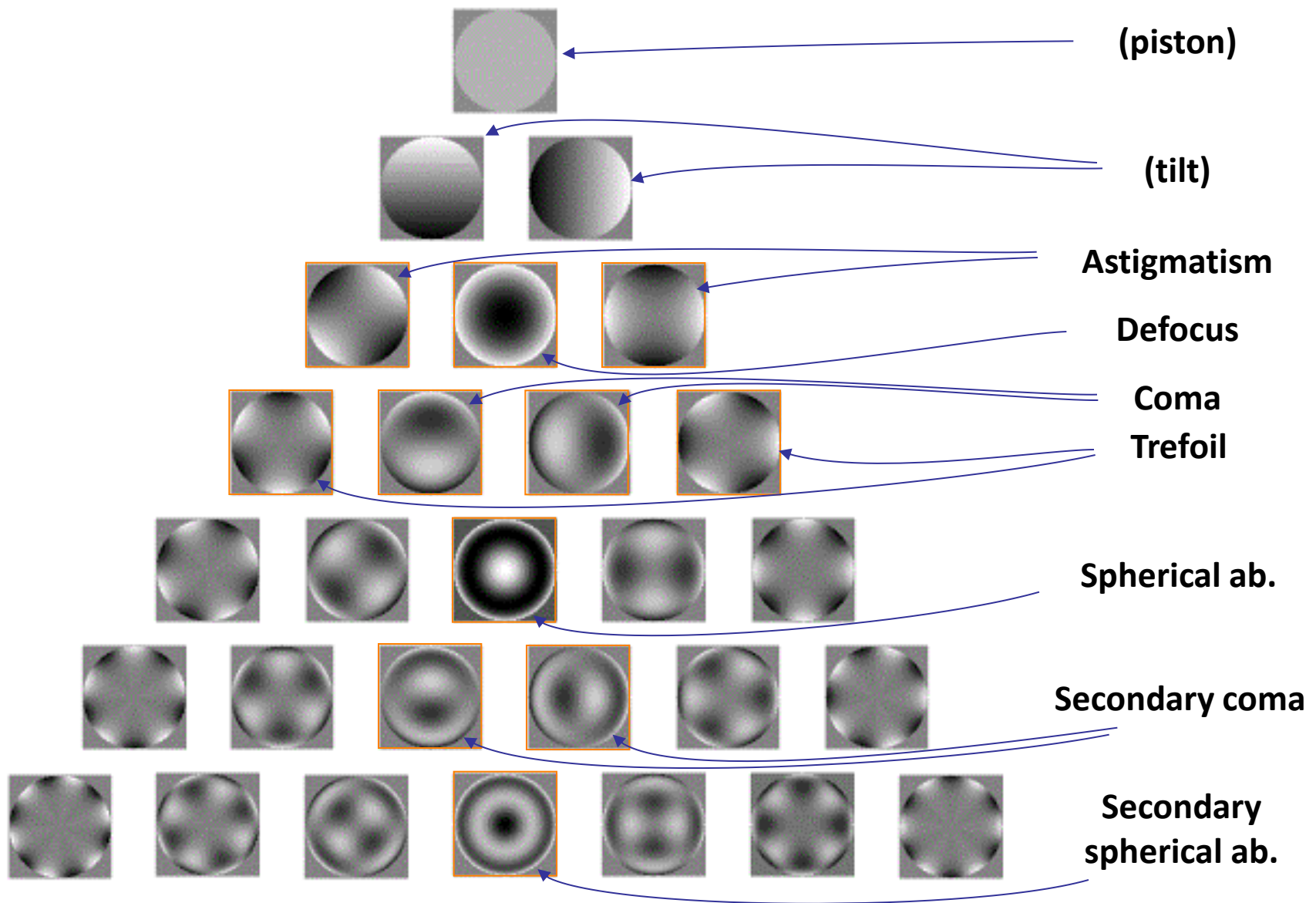


May be introduced by the projection eyepiece

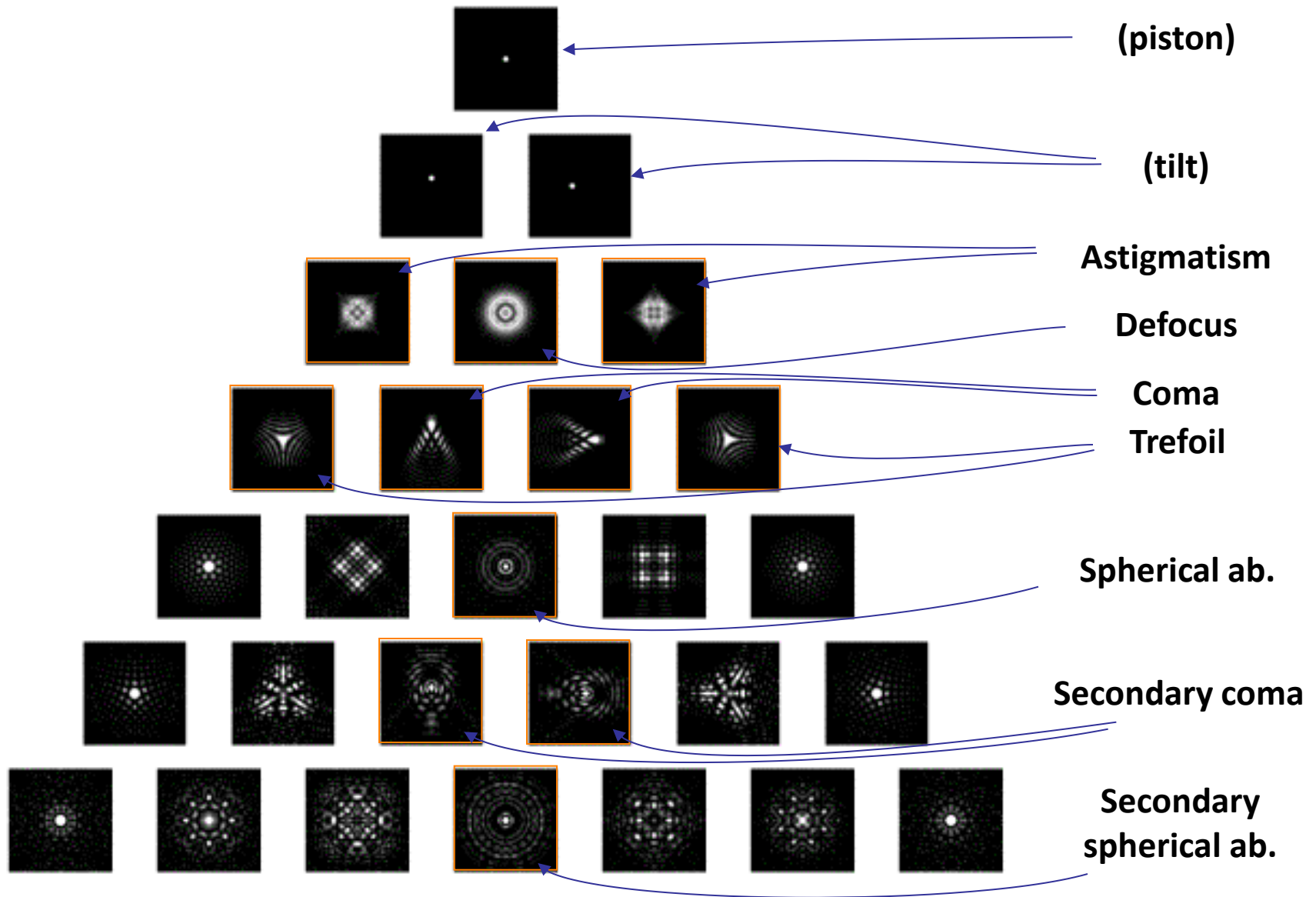
Wavefront Aberrations



Wavefront Aberrations

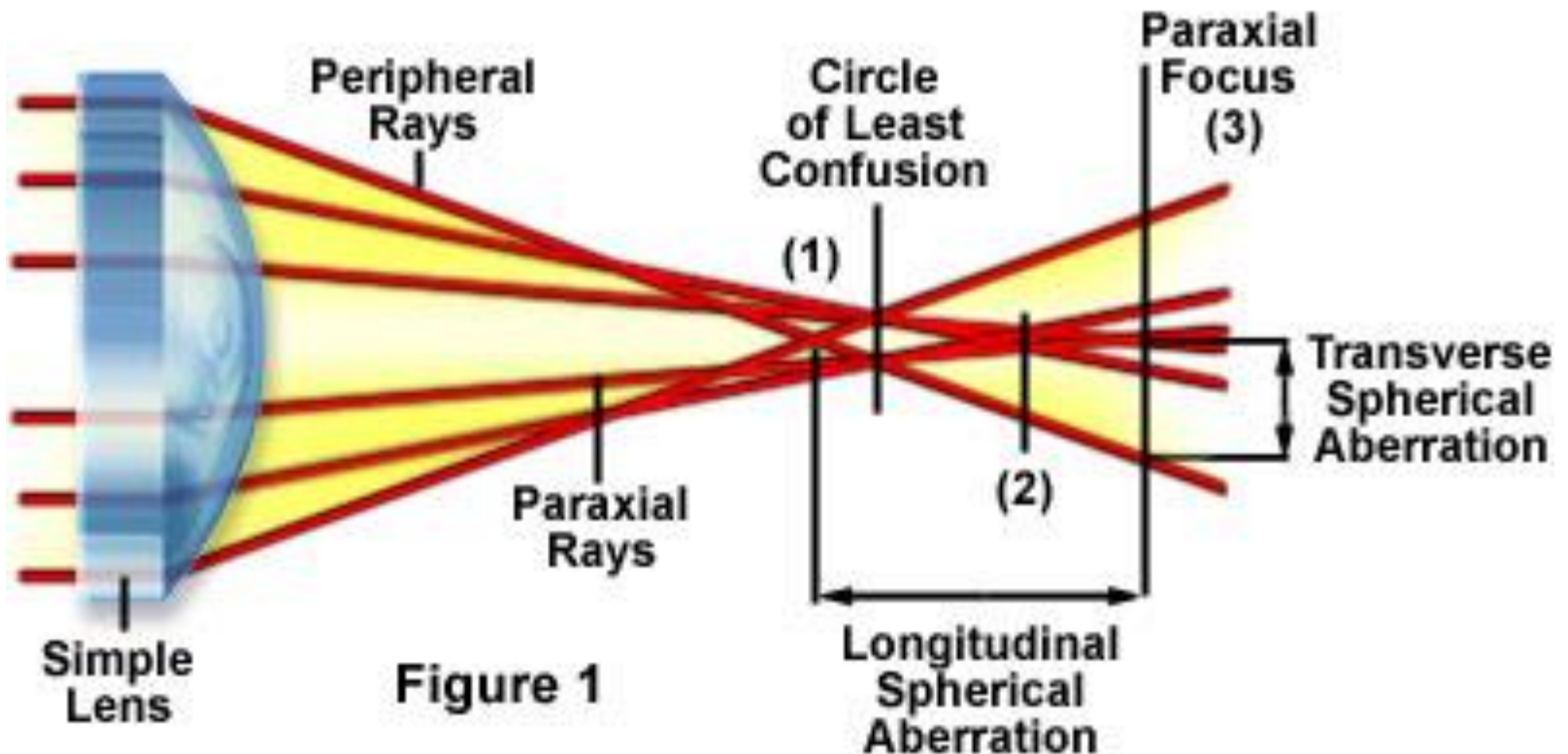


PSF Aberrations



Spherical Aberration

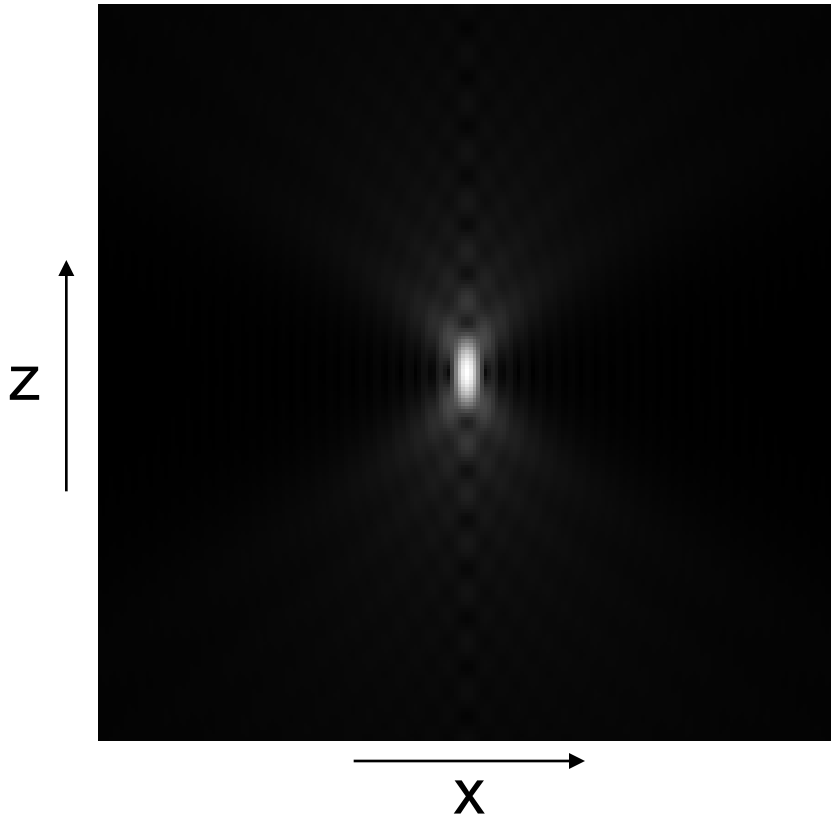
Longitudinal and Transverse Spherical Aberration



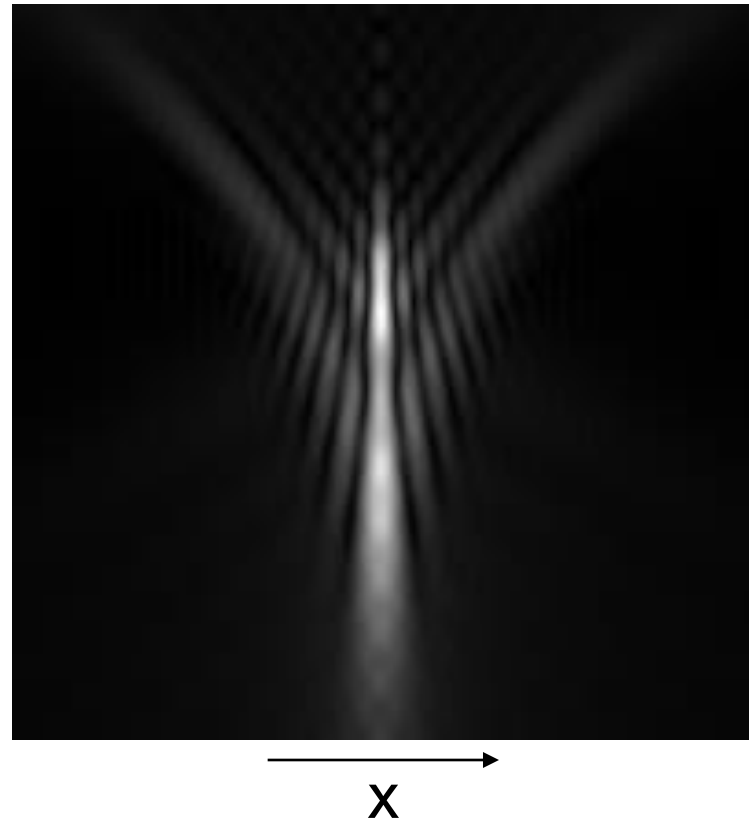
Spherical Aberration

Point spread functions

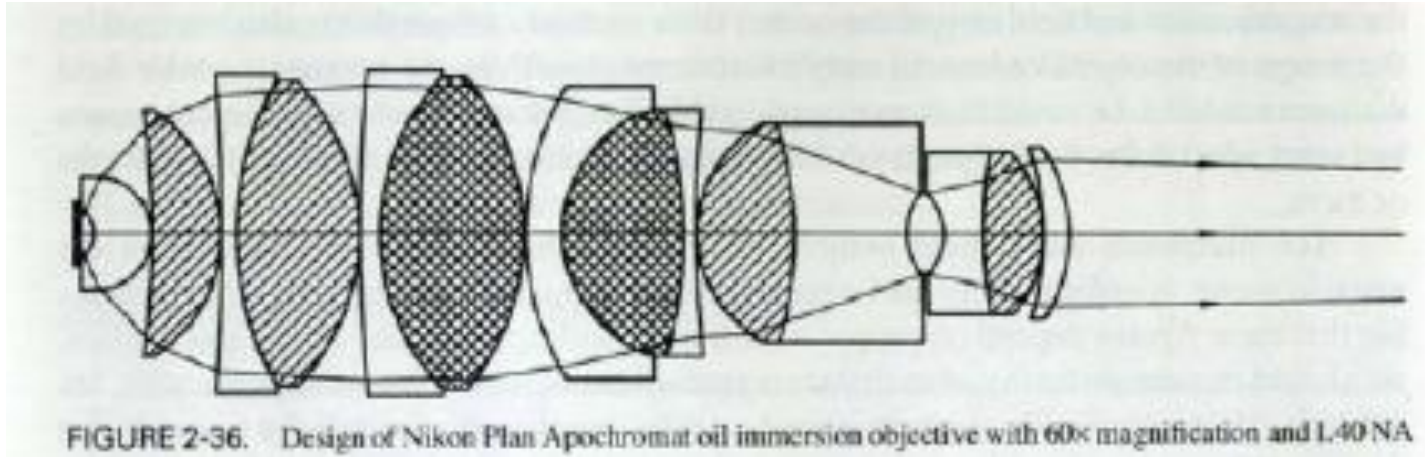
Ideal



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

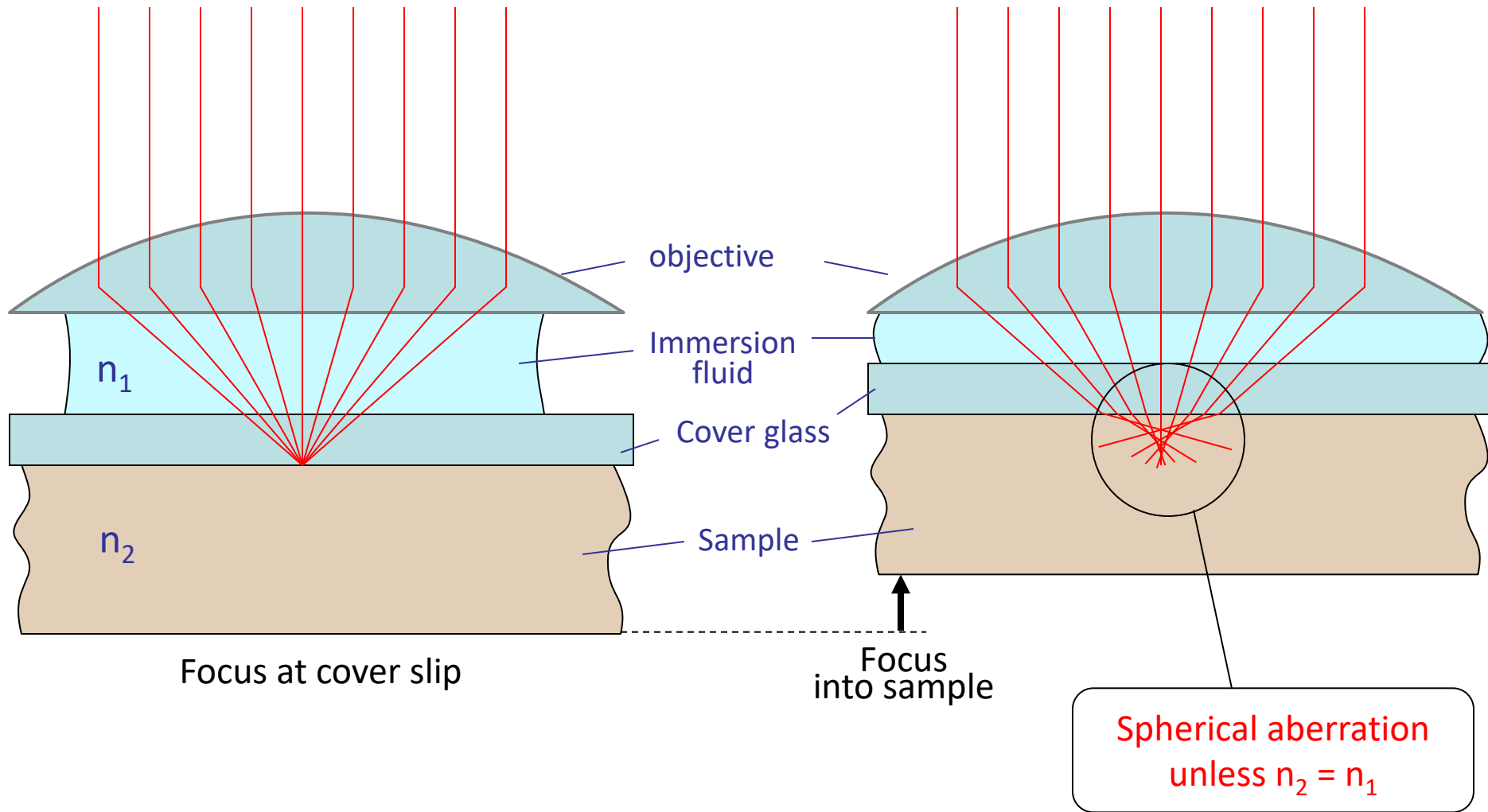
(high-NA objectives except oil immersion)

Correction collar setting

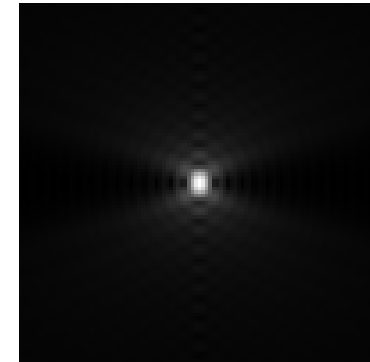
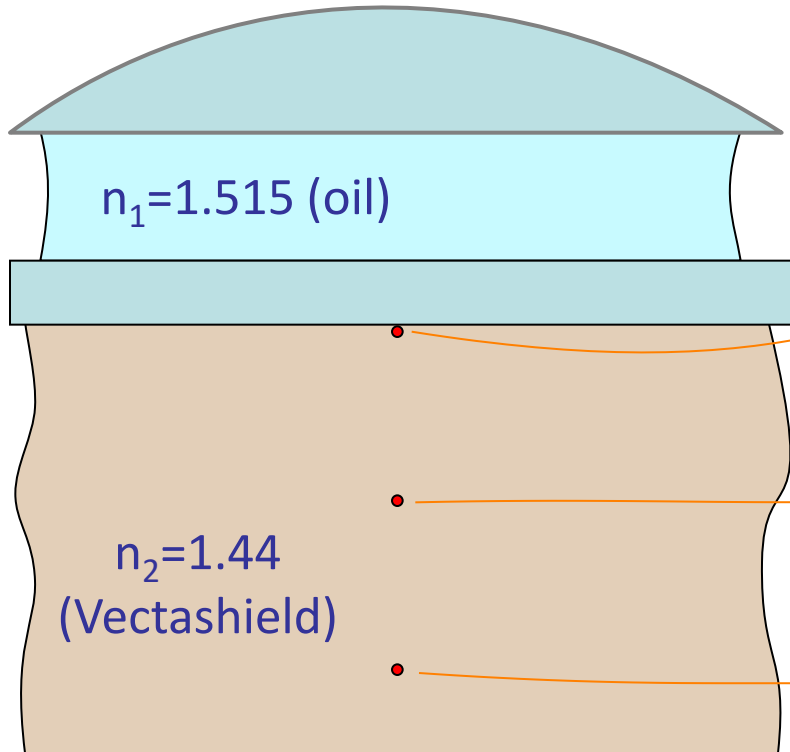
Sample refractive index mismatch



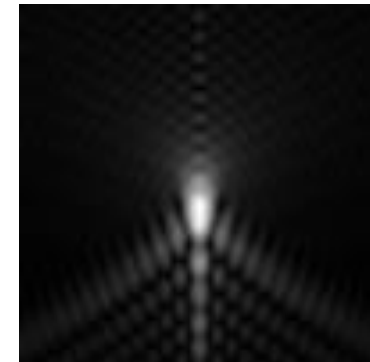
Index Mismatch & Spherical Aberration



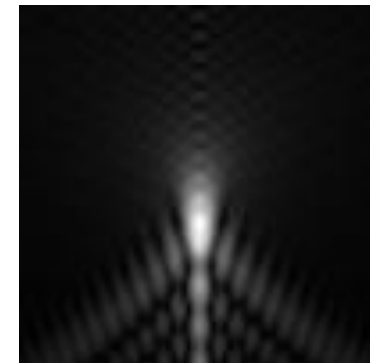
Index Mismatch & Spherical Aberration



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



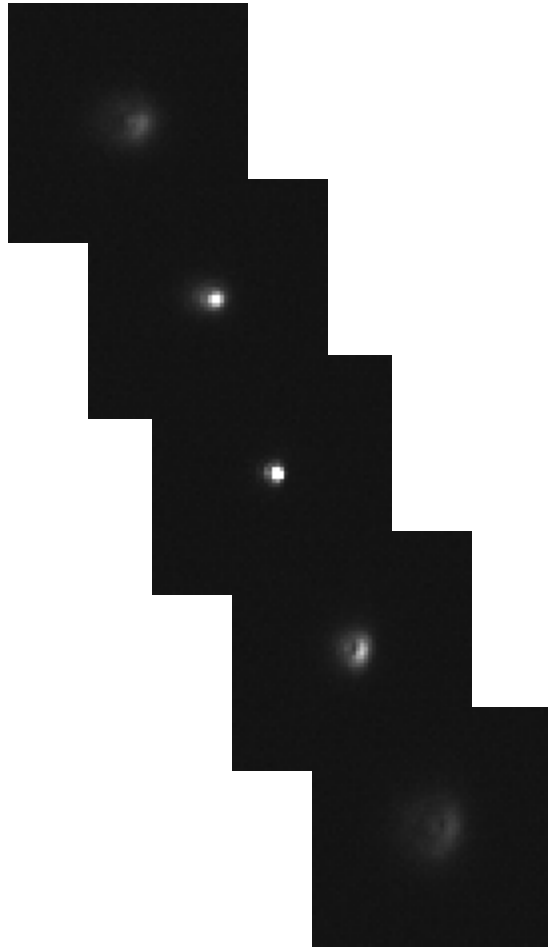
$z=25\ \mu\text{m}$



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

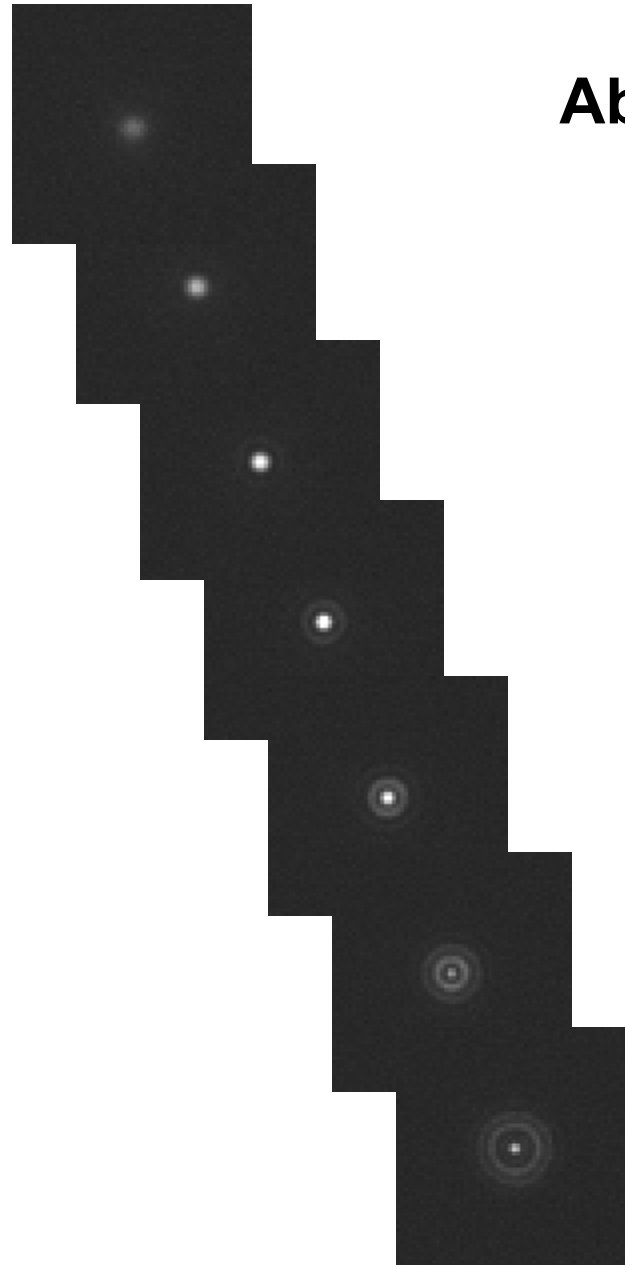
How to recognize spherical aberration

Unaberrated



0.5 μm steps

Aberrated

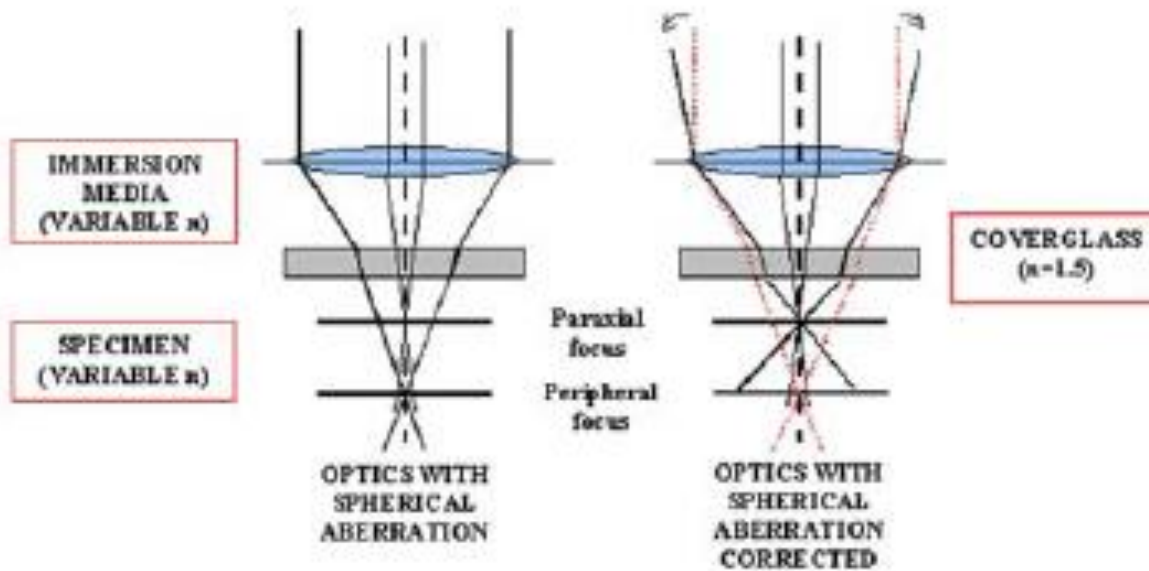


1 μm steps

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

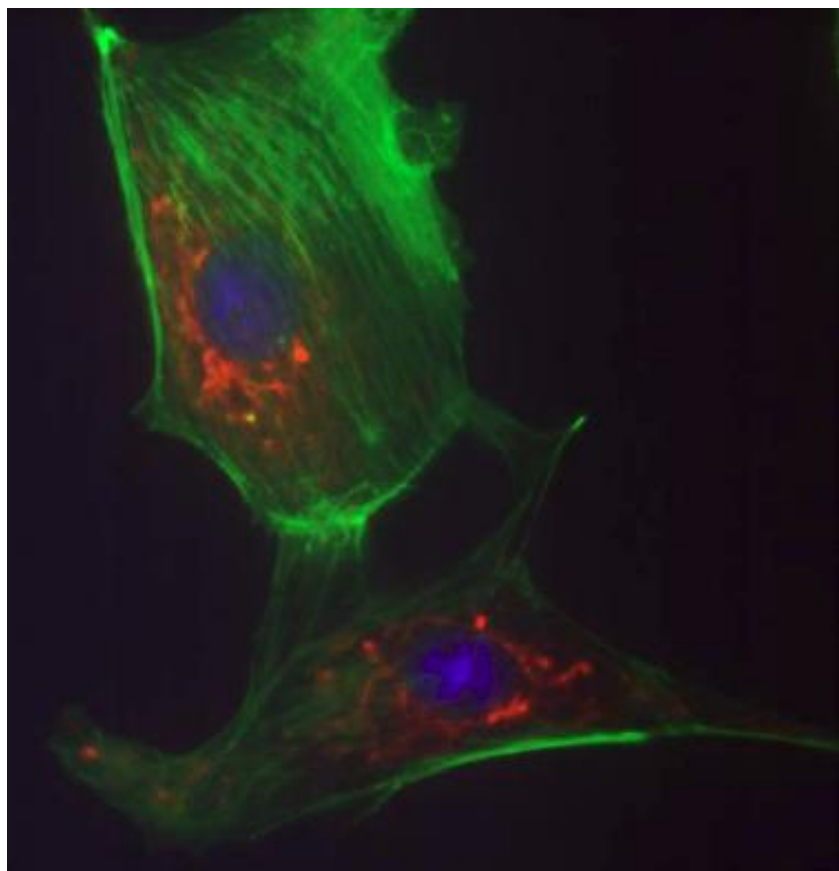
Correction collars



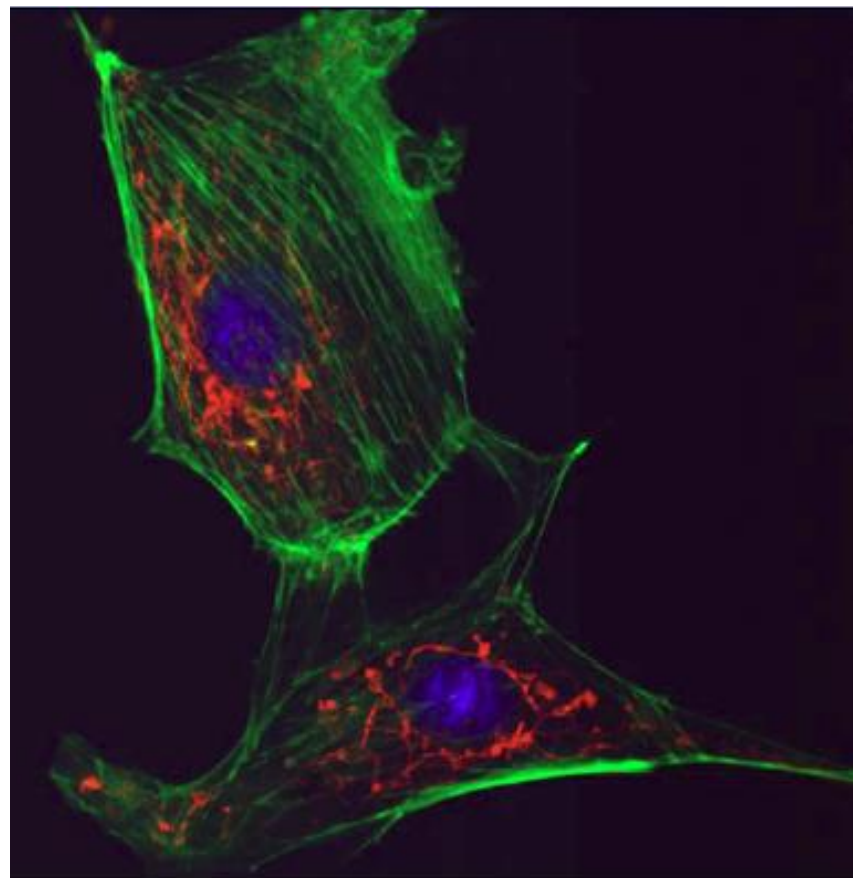
A correction collar can only eliminate spherical aberration at a single focal plane

Example

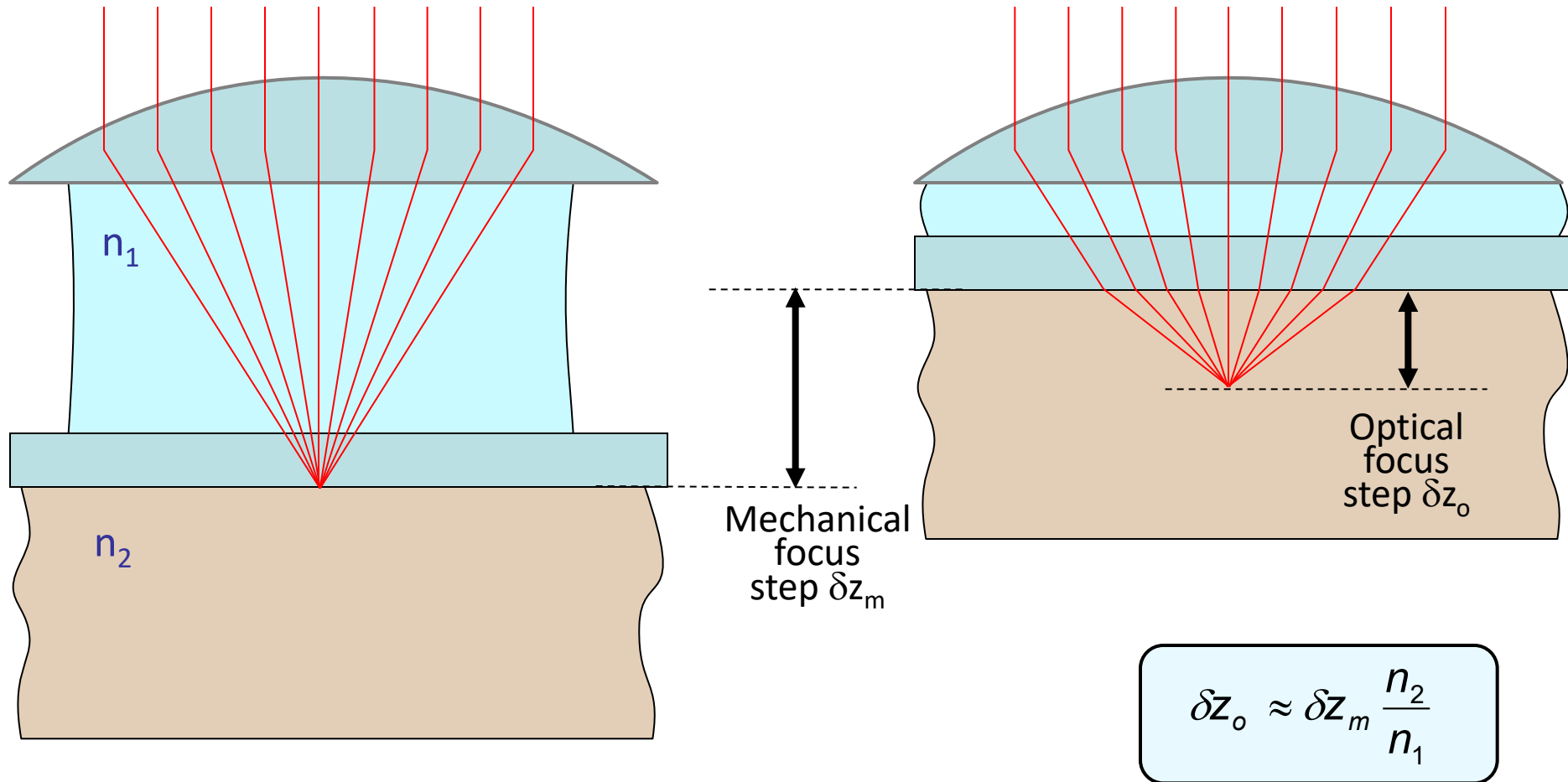
Aberrated



Unaberrated



Index Mismatch & Axial Scaling

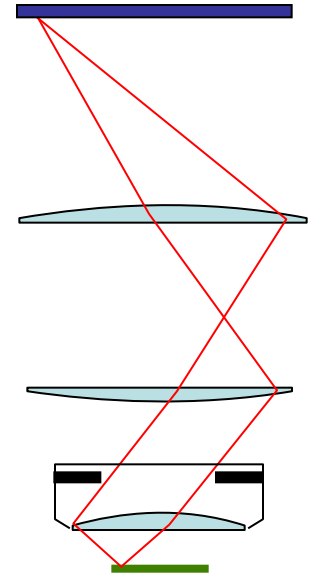


If there is index mismatch,
your z pixel size is not what you think

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

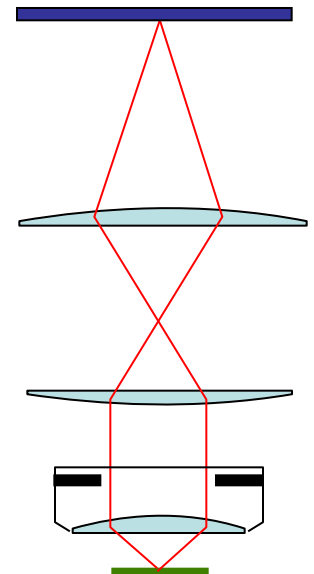


On-axis (center of field of view)

Should be none, by symmetry.

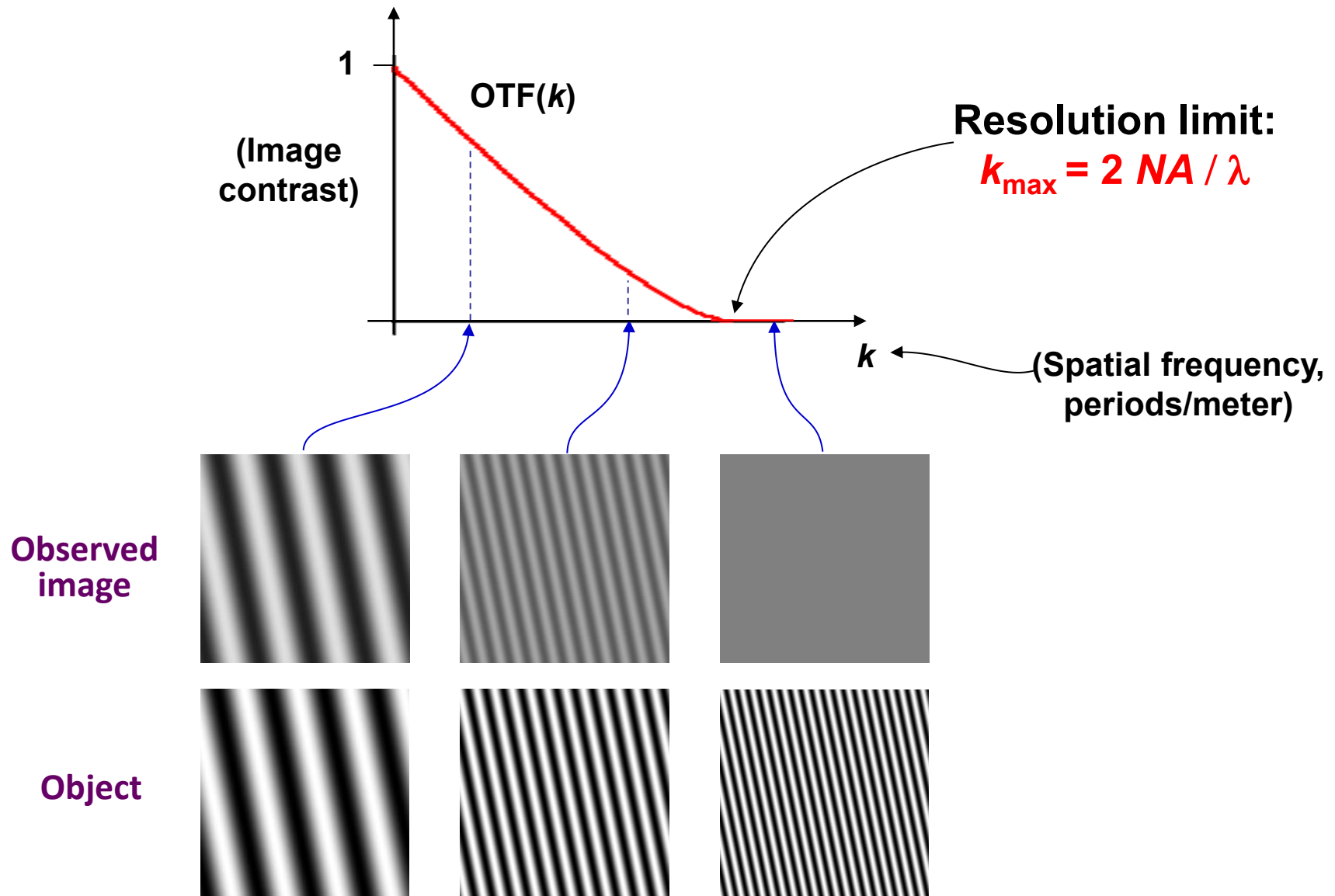
If they are there, they could be from:

- 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)



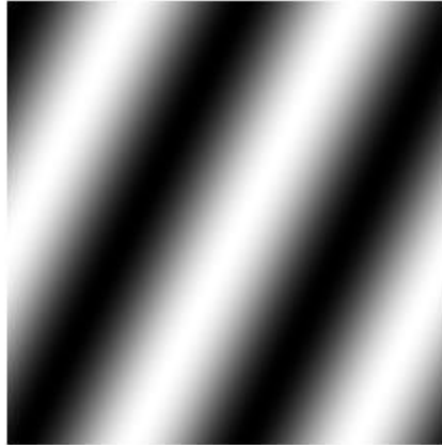
Think of Images as Sums of Waves

... or “spatial frequency components”

one wave



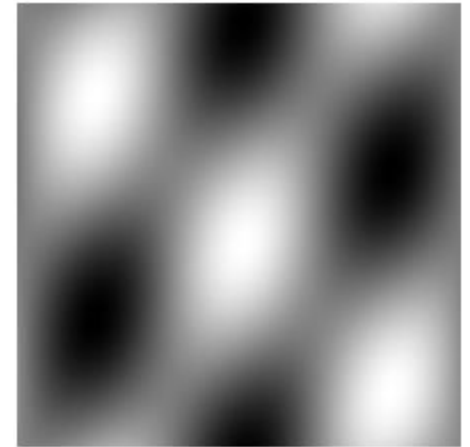
another wave



+

=

(2 waves)



(25 waves)



+ (...) =

(10000 waves)



+ (...) =

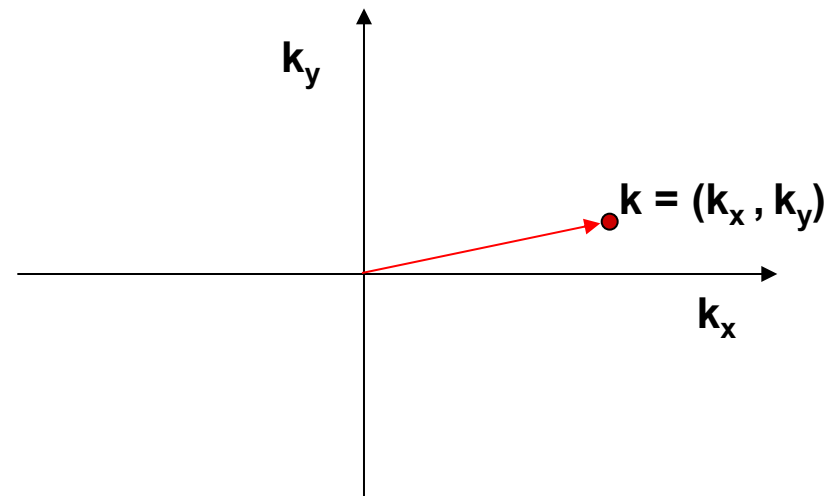
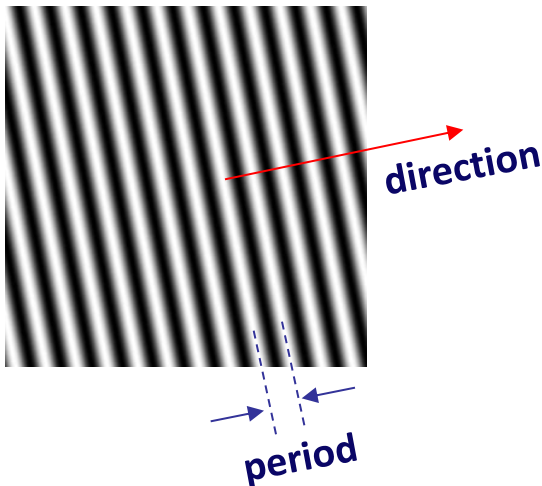
Frequency Space

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

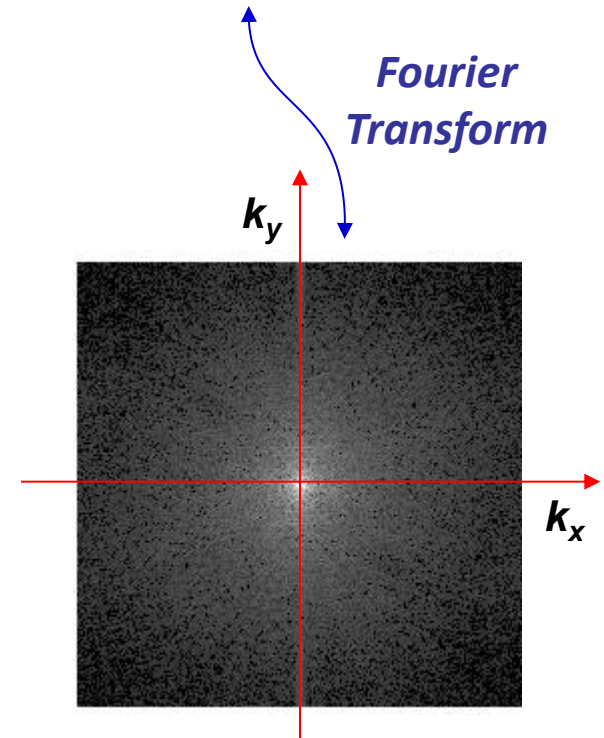
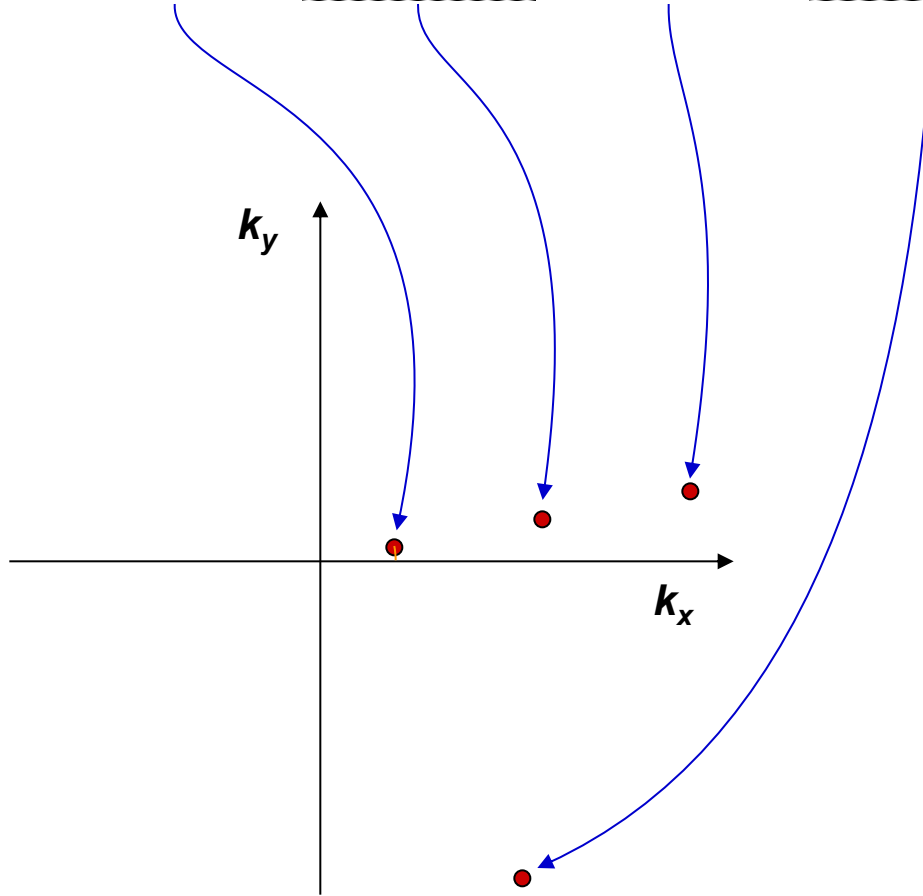
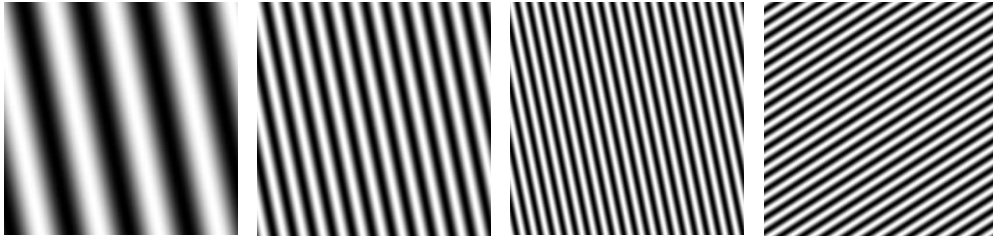
- **Frequency** (how many periods/meter?)
- **Direction**
- **Amplitude** (how strong is it?)
- **Phase** (where are the peaks & troughs?)

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:

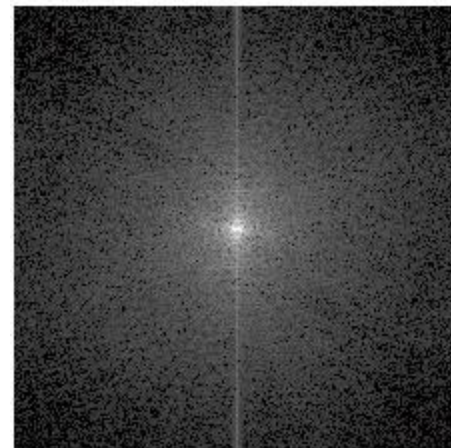
The Fourier Transform contains all the information of the original image

Symmetry:

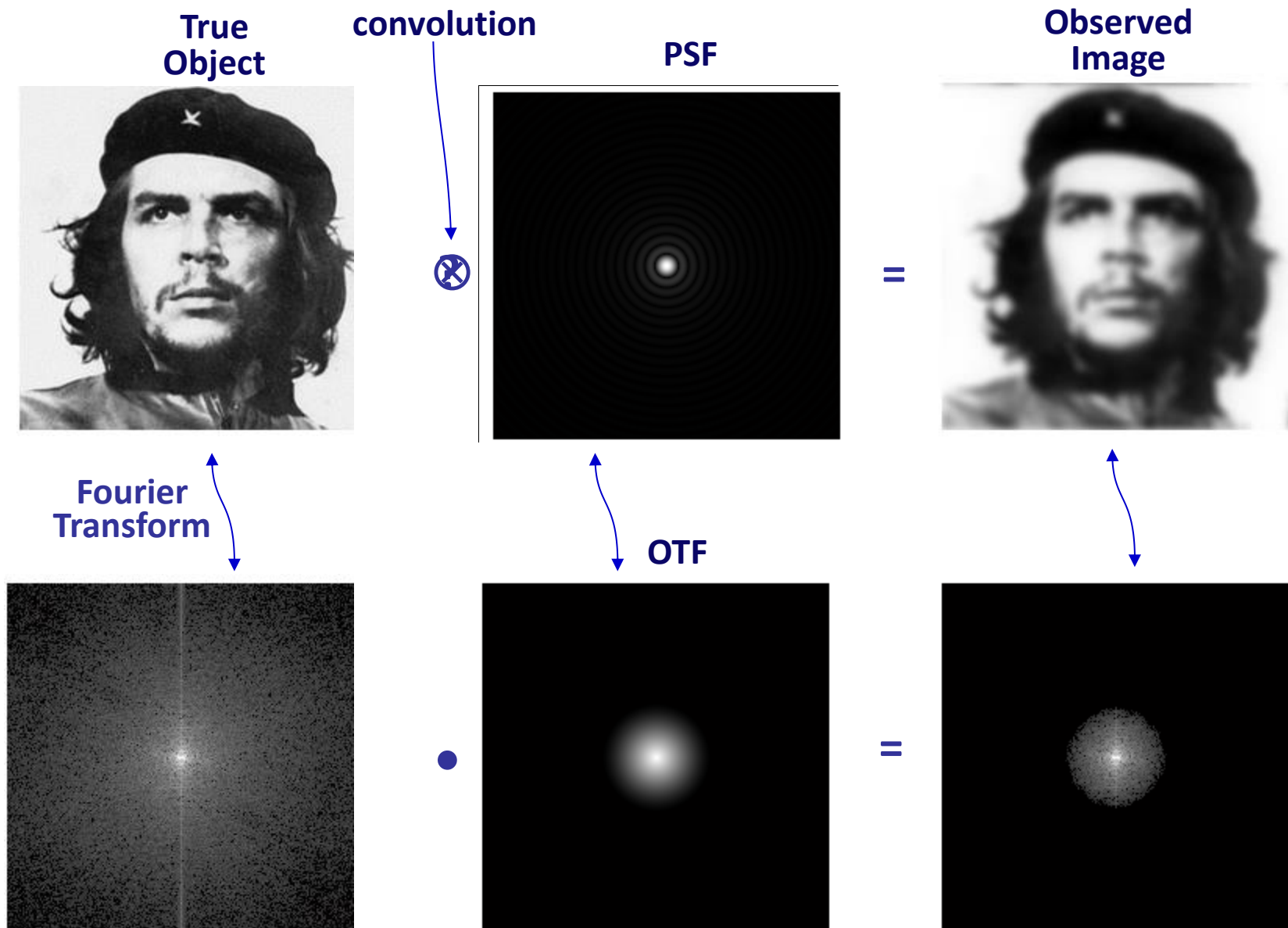
The Fourier Transform of the Fourier Transform is the original image



*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:

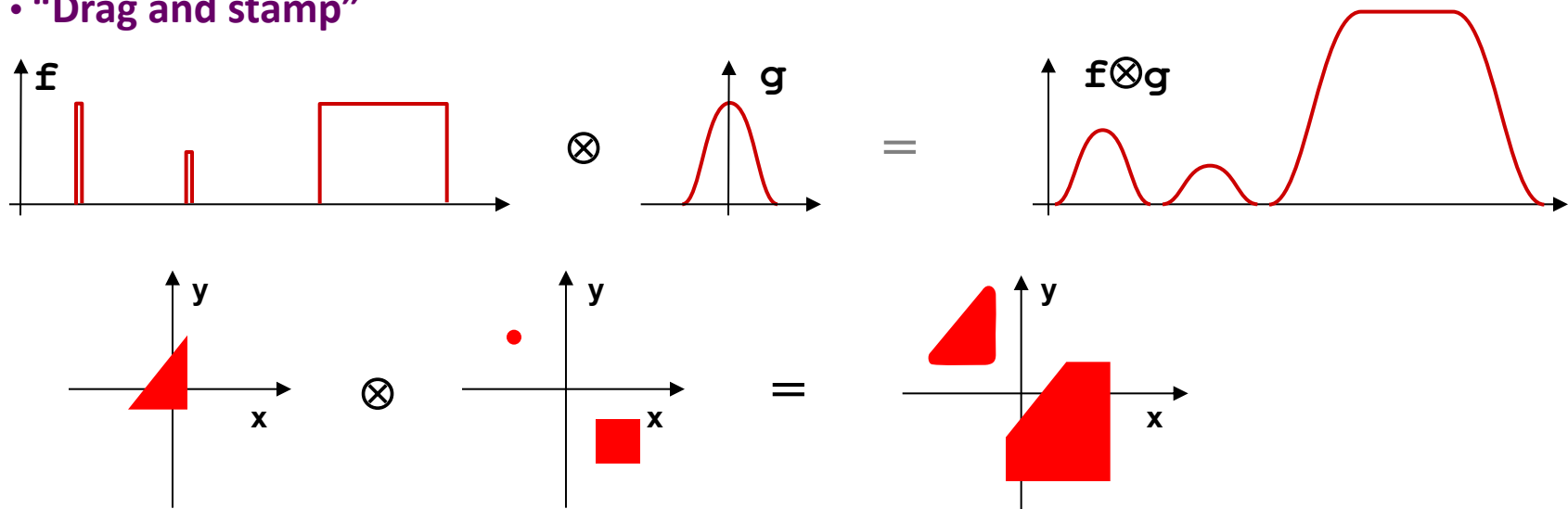
If $h(r) = (f \otimes g)(r)$,
 then $\tilde{h}(k) = \tilde{f}(k) \tilde{g}(k)$

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

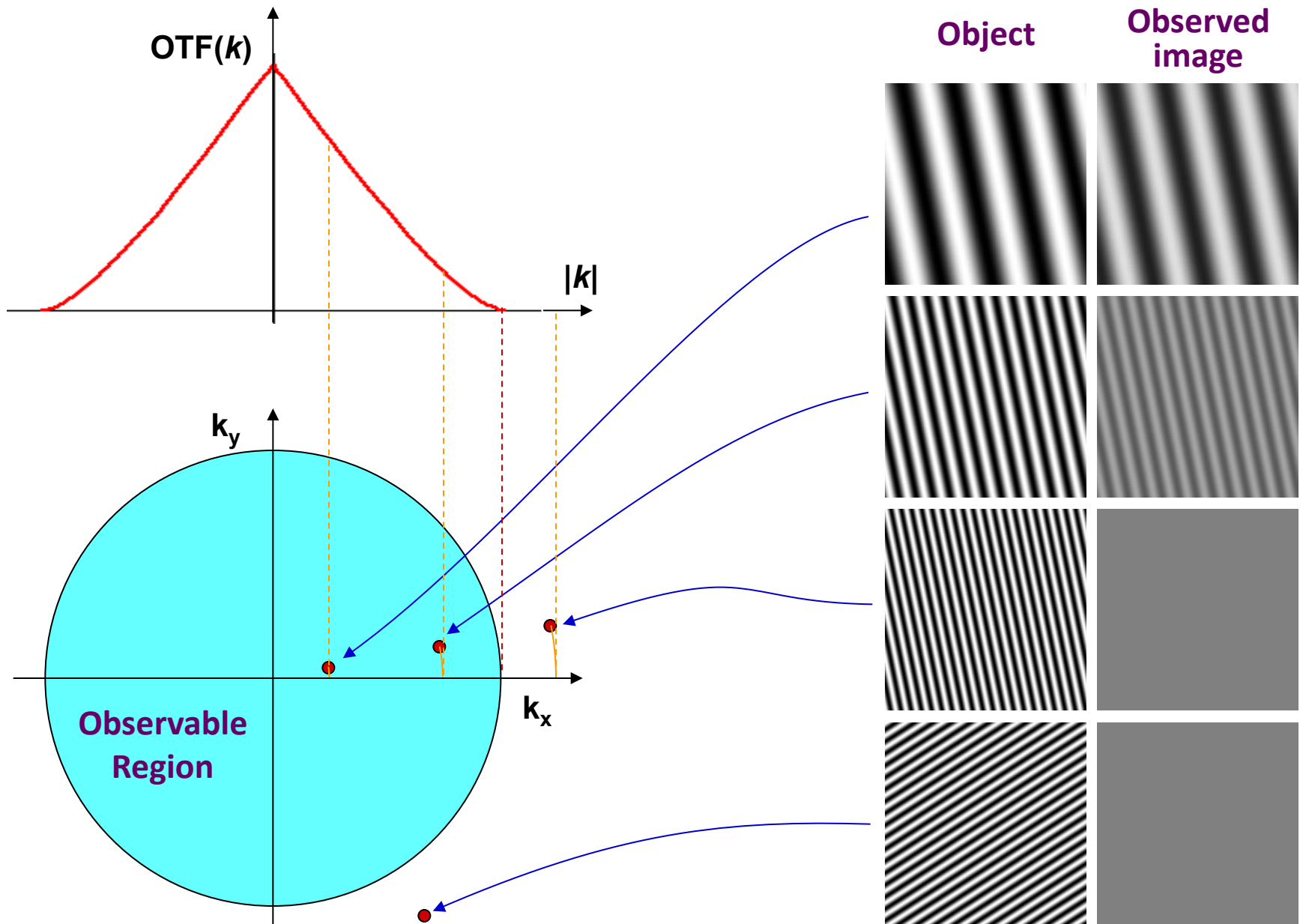
Symmetry: $g \otimes f = f \otimes g$

So what is a convolution, intuitively?

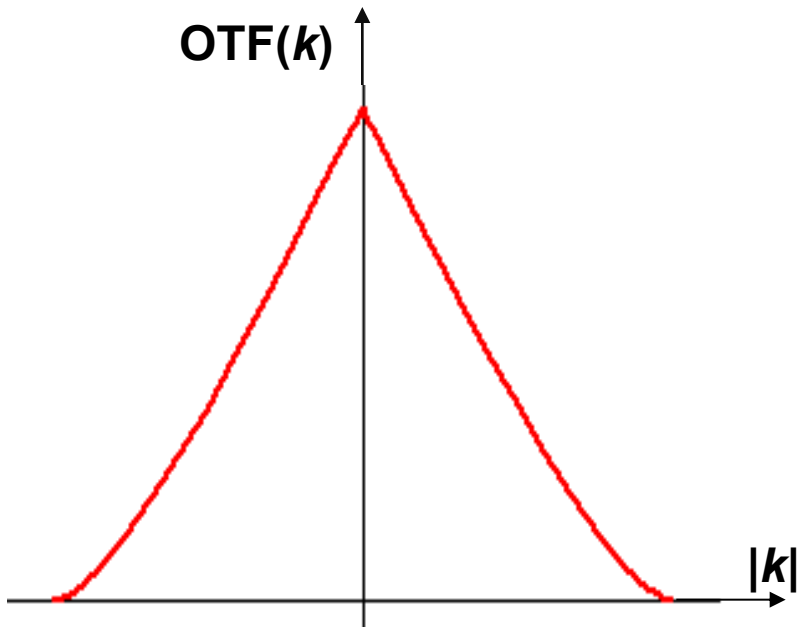
- “Blurring”
- “Drag and stamp”



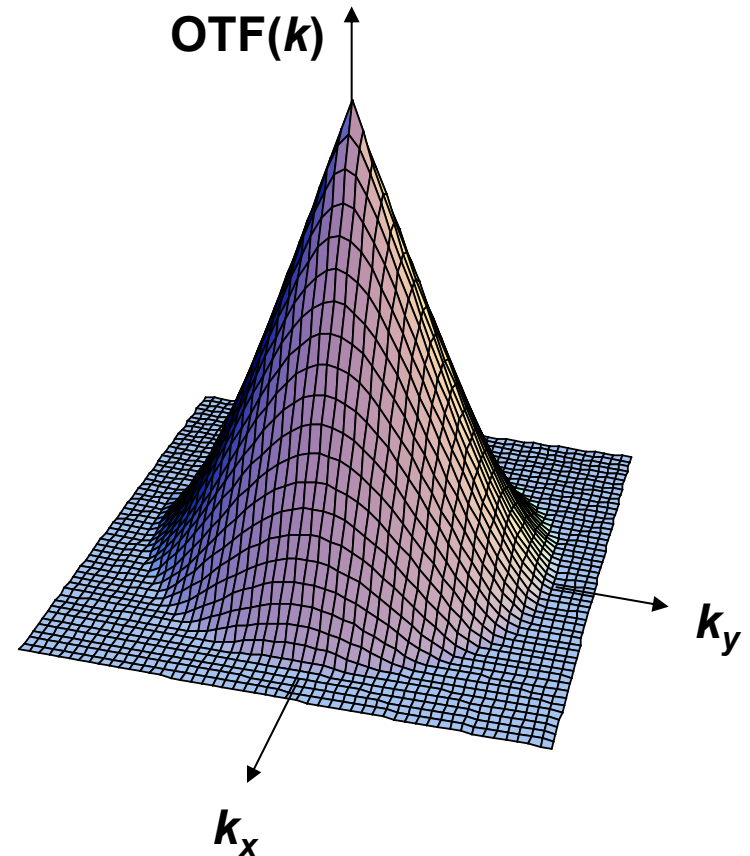
The Transfer Function Lives in Frequency Space



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

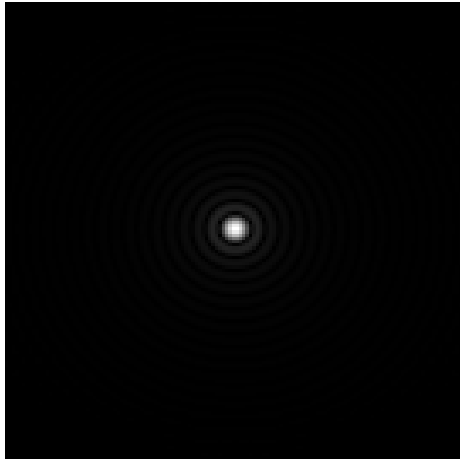


(Idealized
calculations)

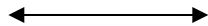


The 3D OTF

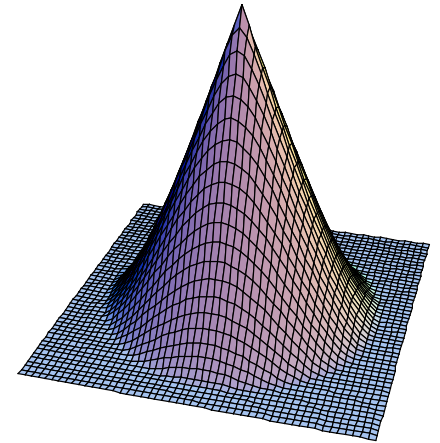
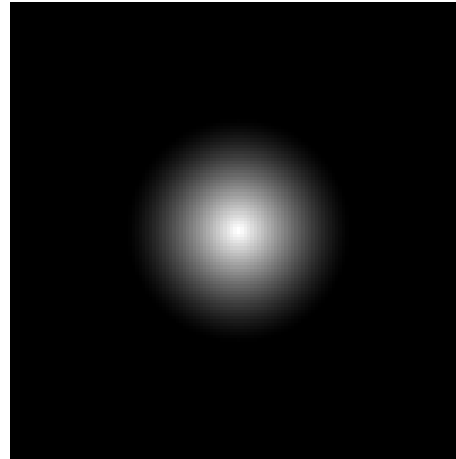
2D PSF



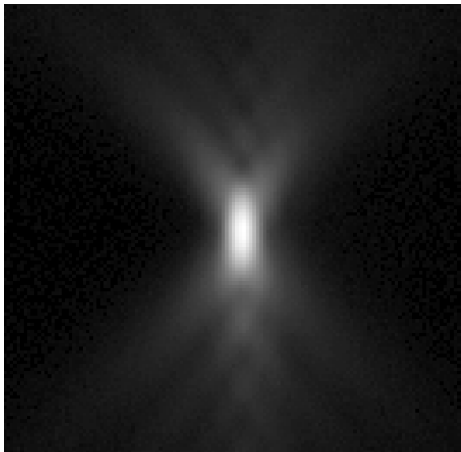
2D F.T.



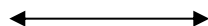
2D OTF



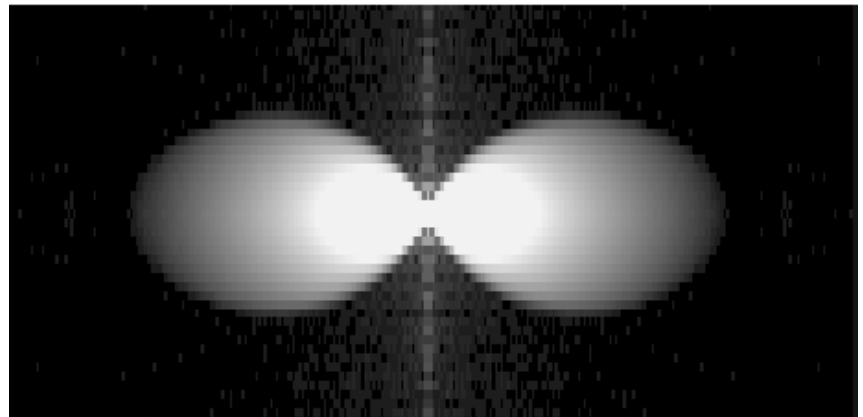
3D PSF



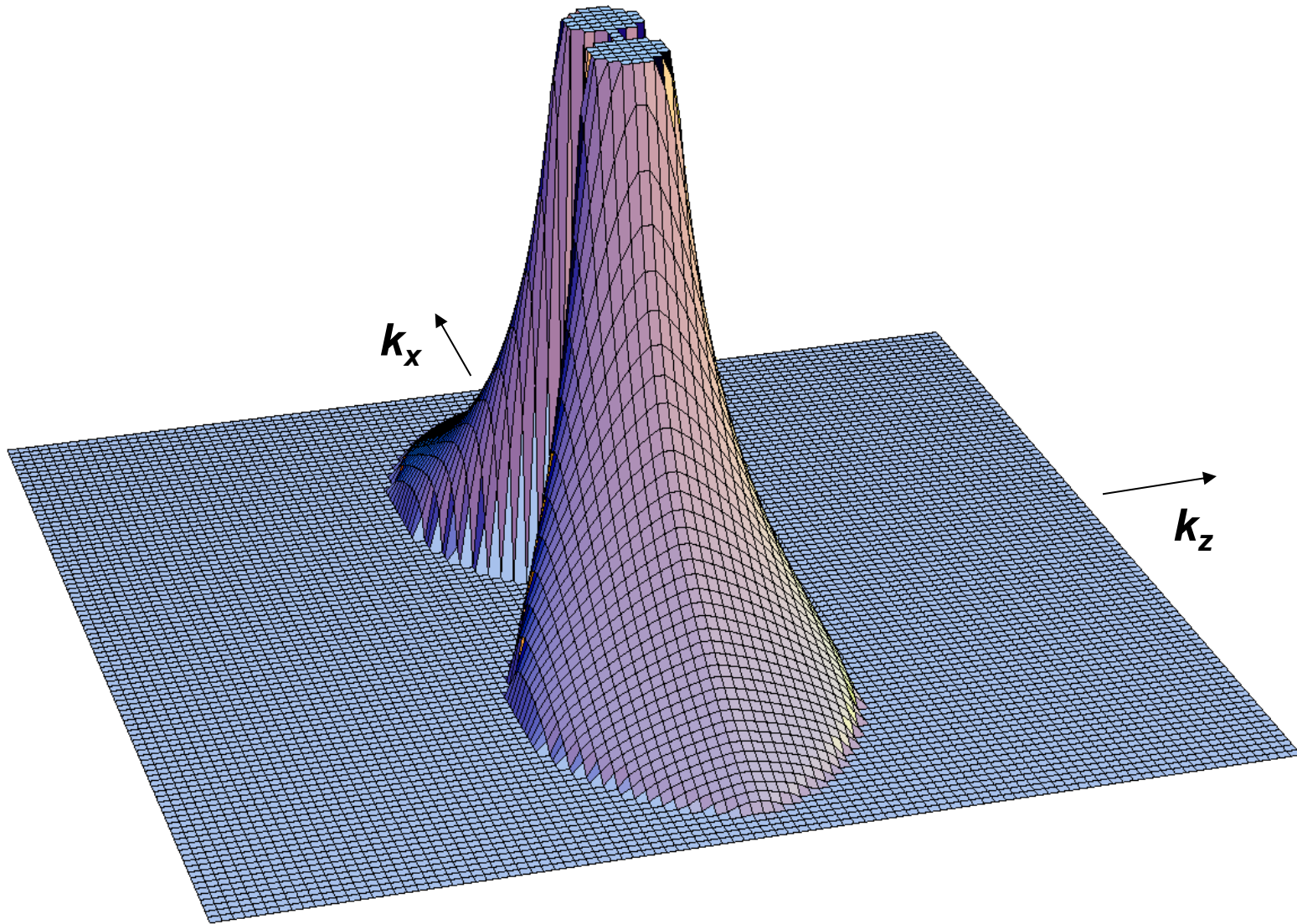
3D F.T.



3D OTF



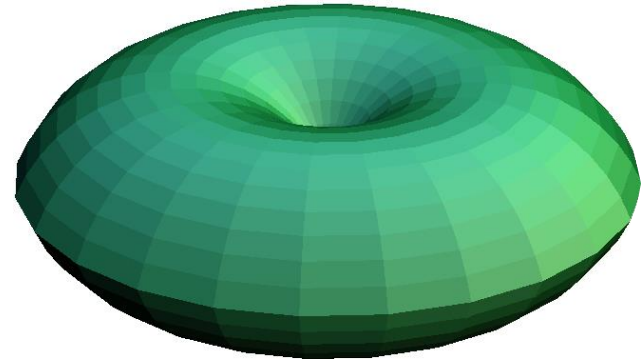
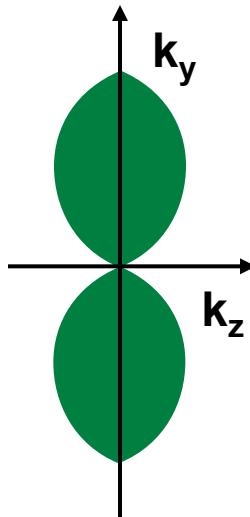
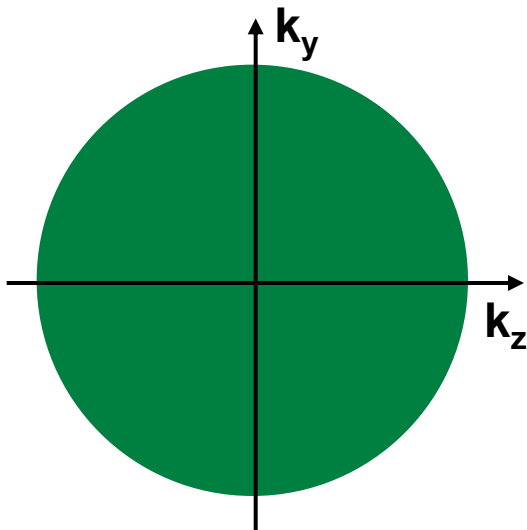
Values of the 3D OTF



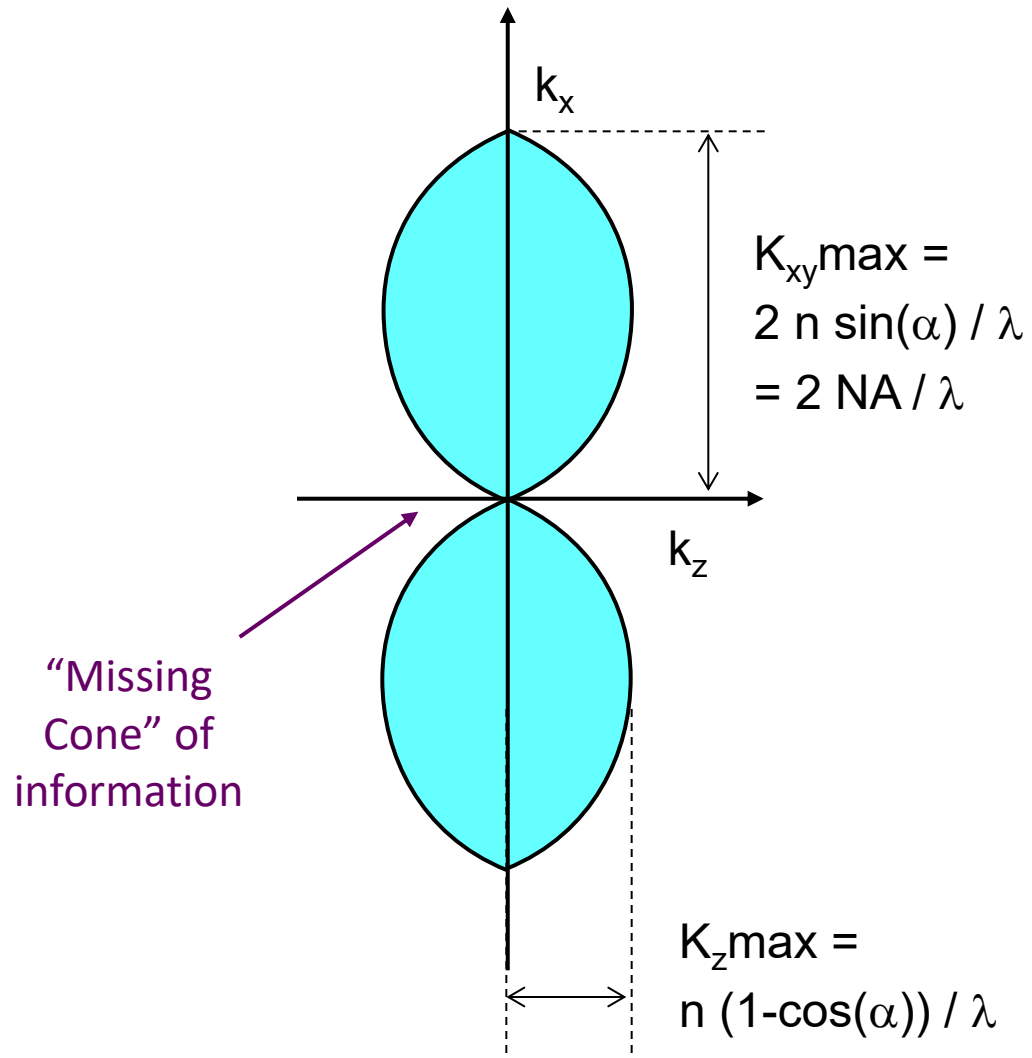
3D Observable Region

= OTF support

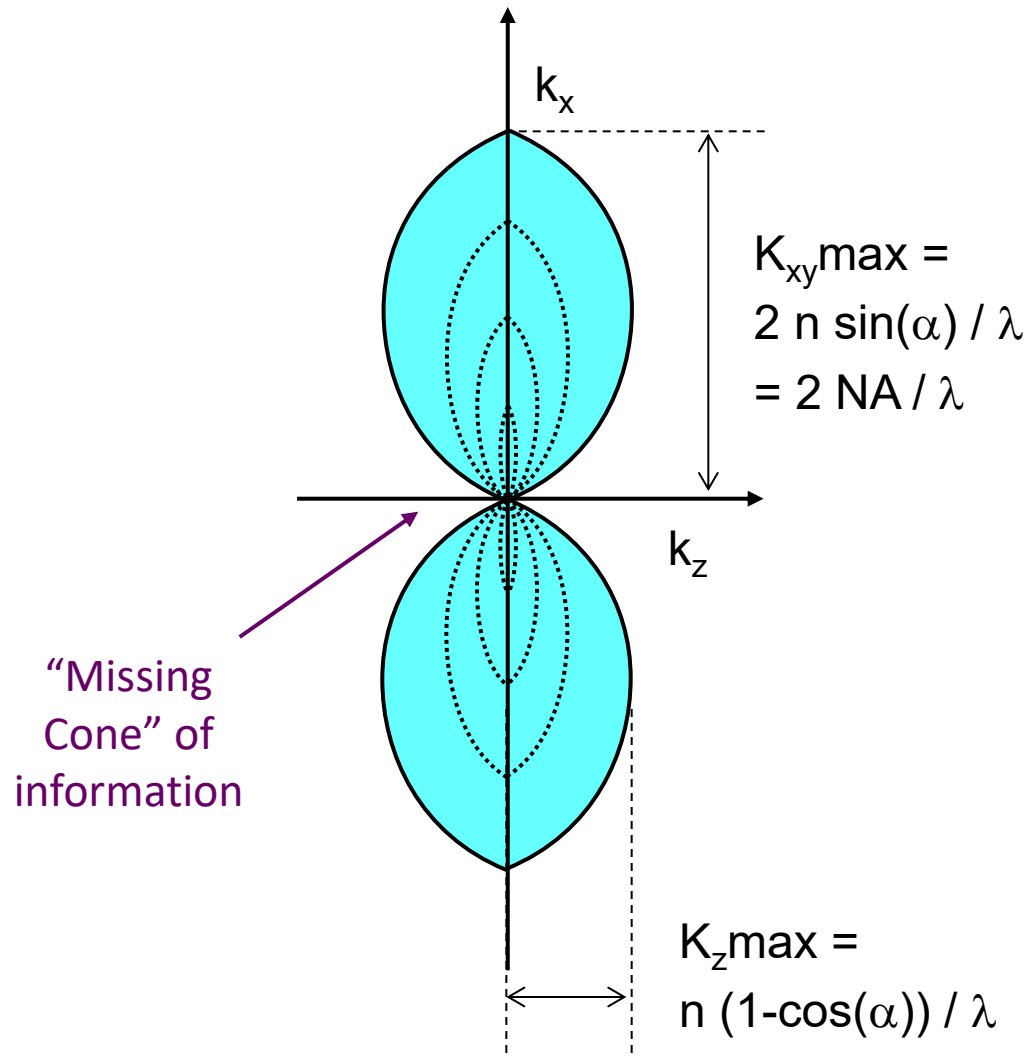
= Region where the OTF is non-zero



So what is the resolution?



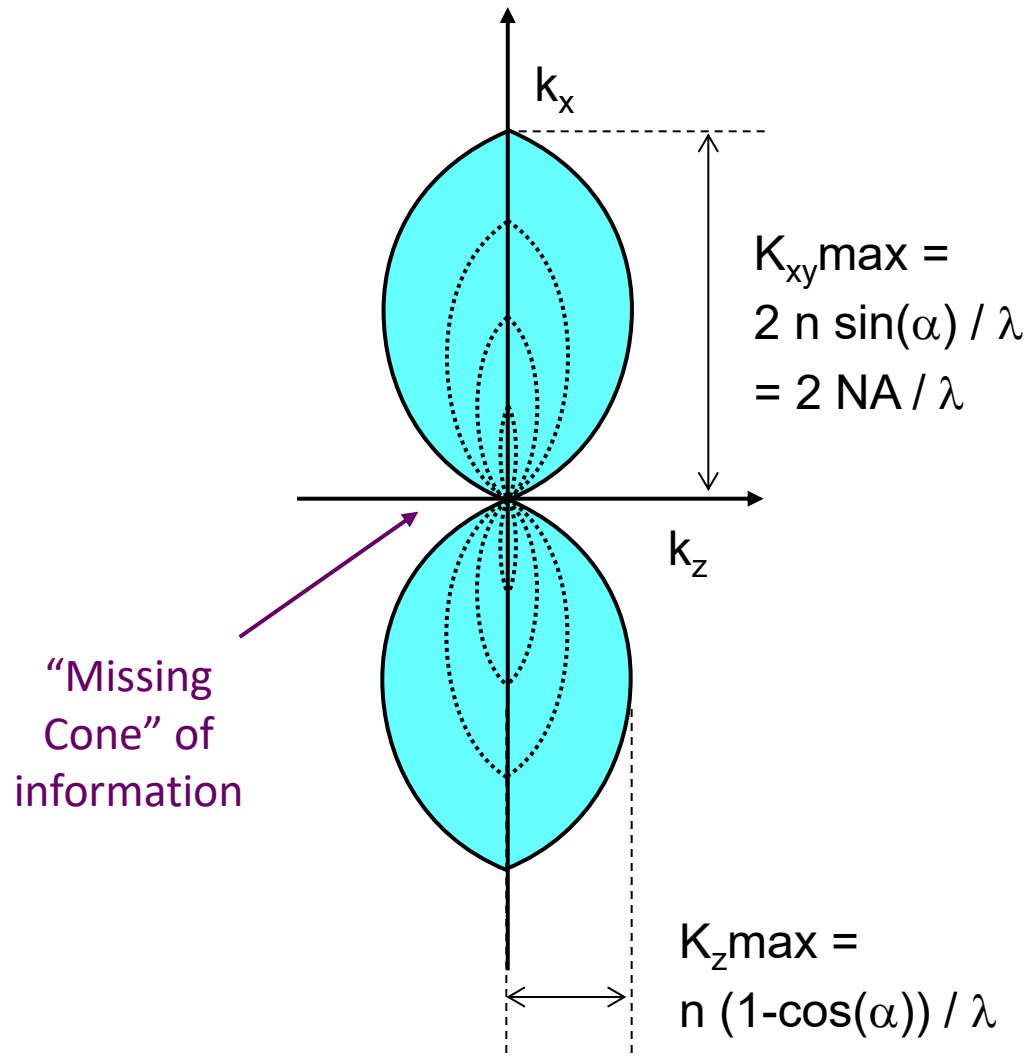
So what is the resolution?



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