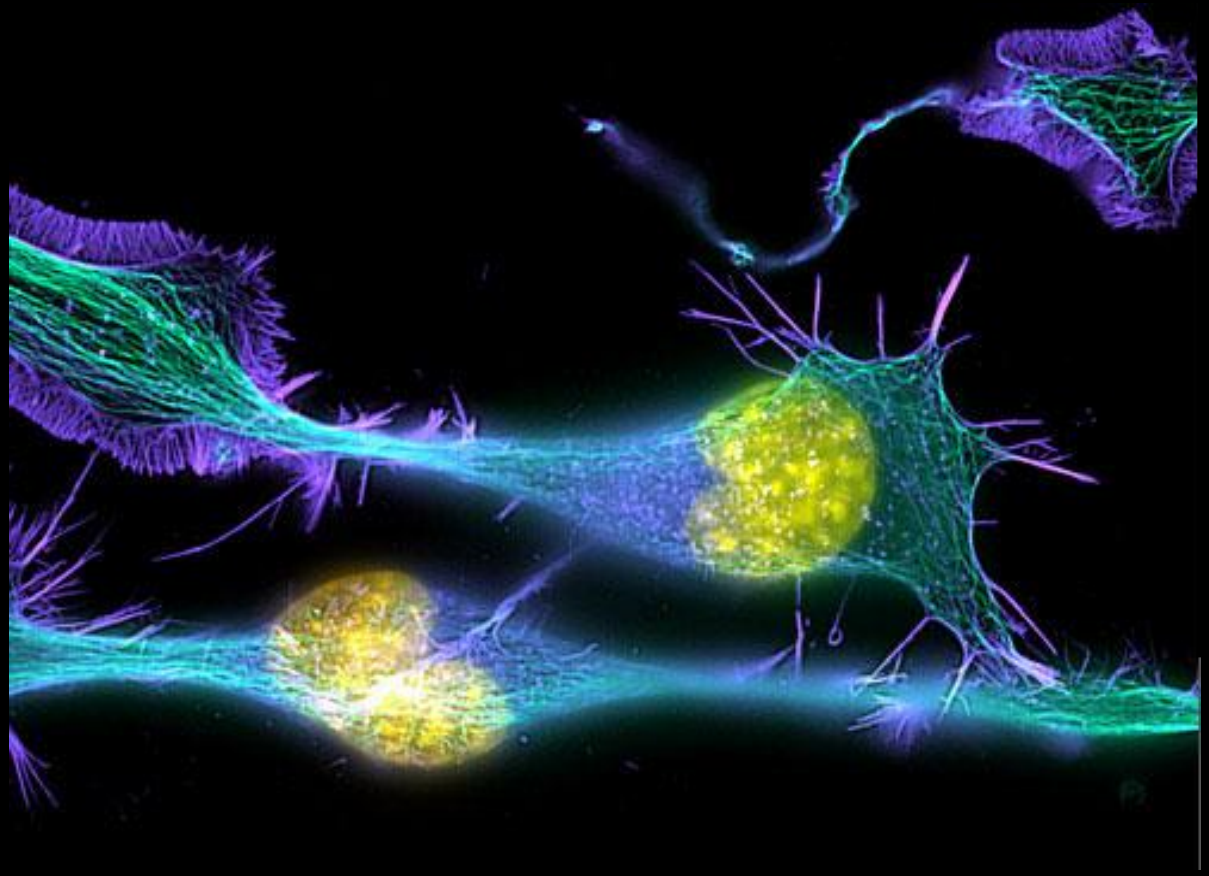
Principles & Practice of Light Microscopy: 2



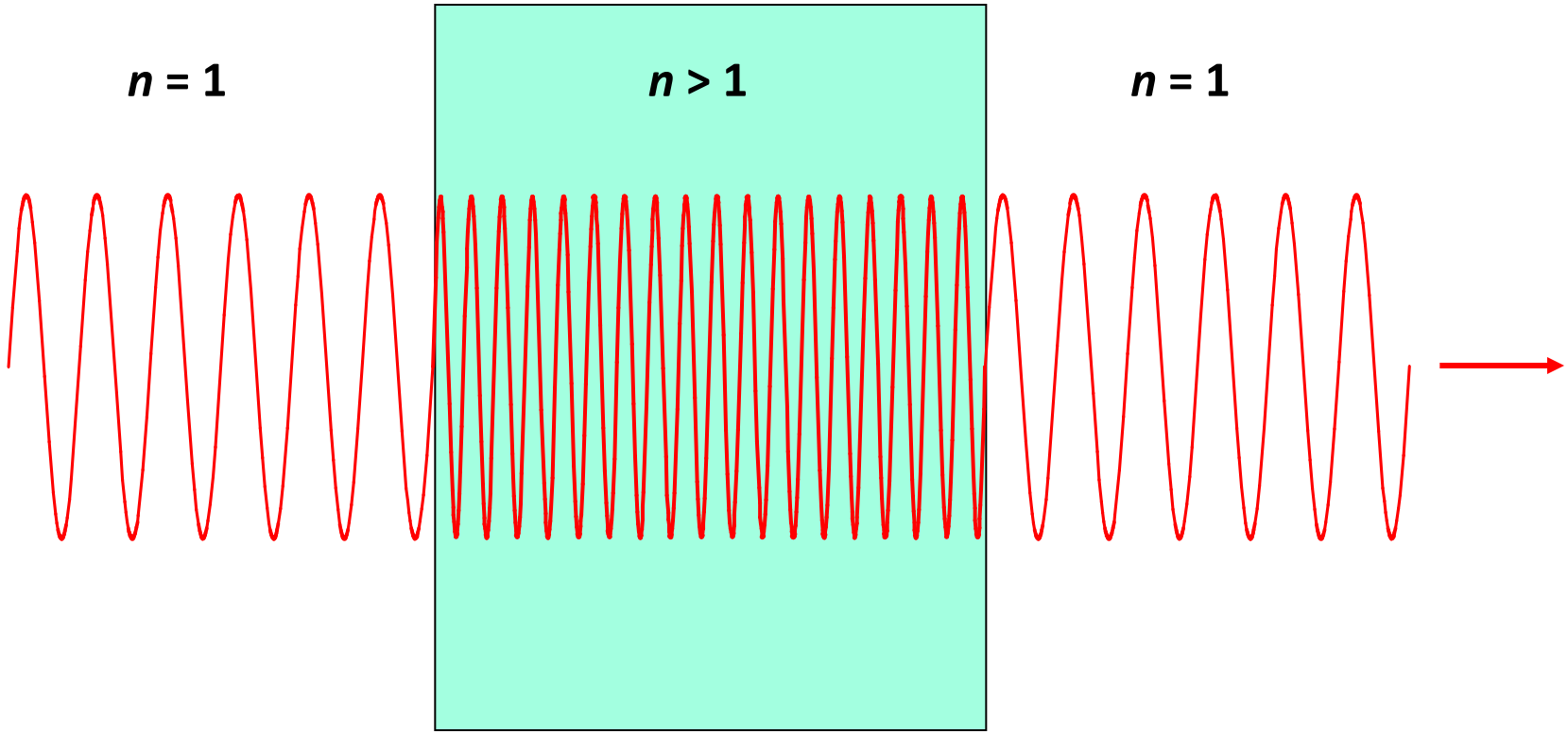
(Image: T. Wittman, Scripps)

Light travels more slowly in matter

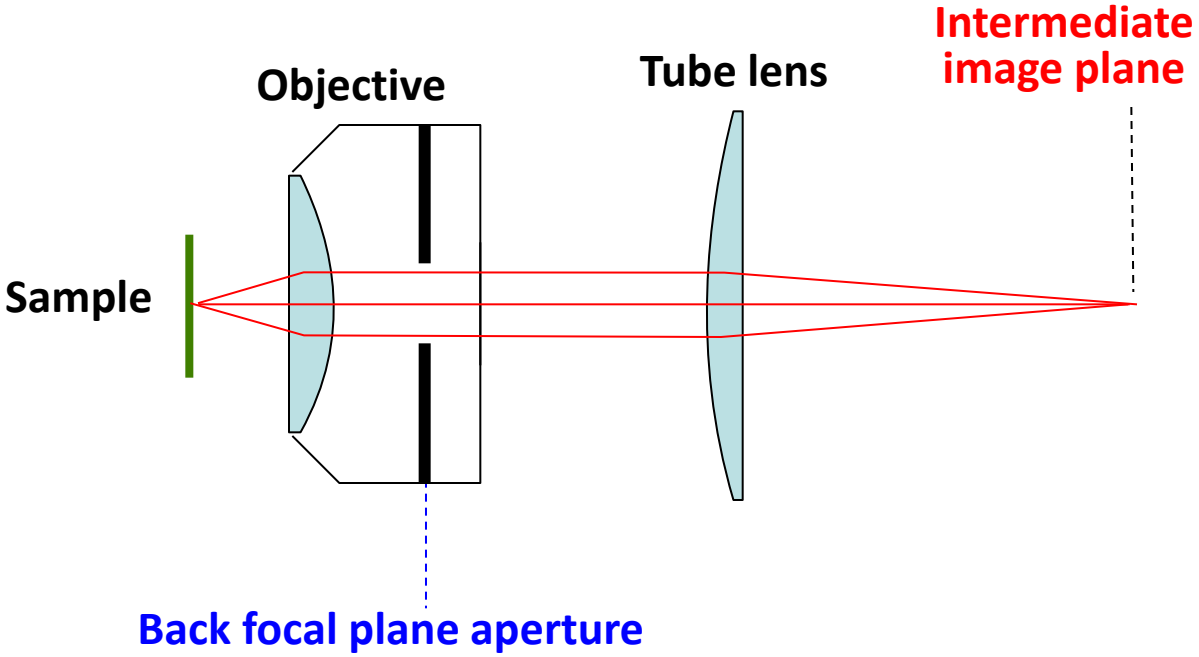
Velocity slower
Wavelength slower

Frequency unchanged
Energy unchanged

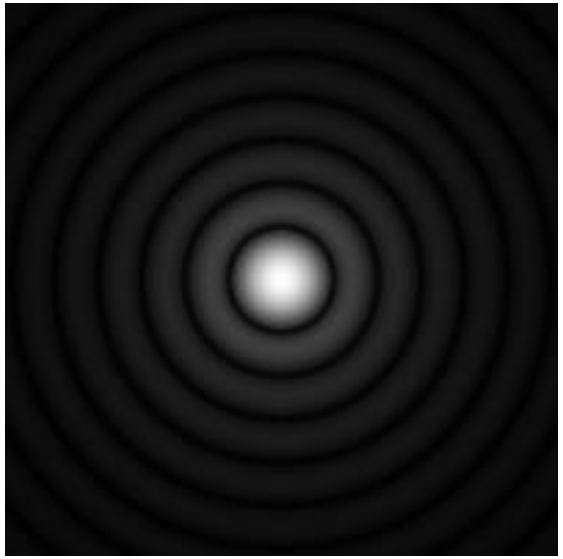
$E = h\nu$
$\nu = c/\lambda$



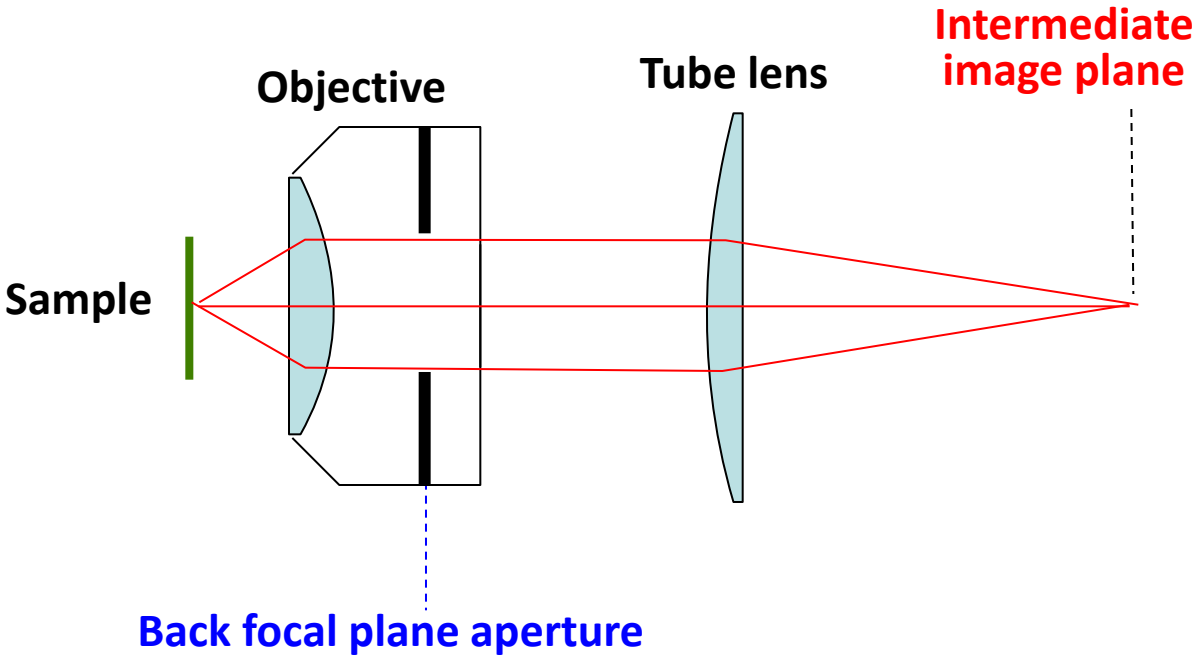
Aperture and Resolution



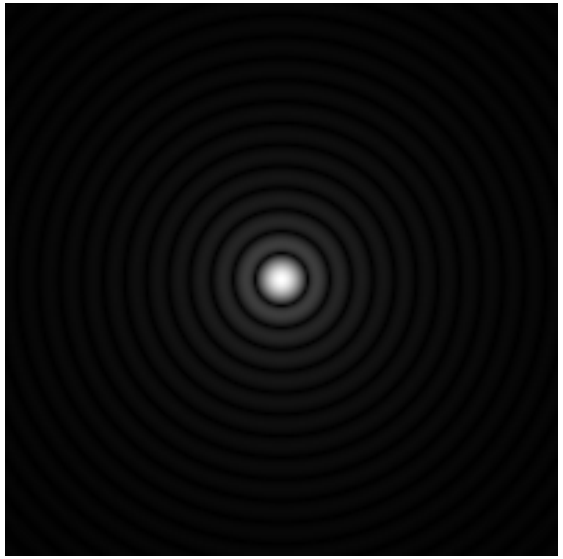
Diffraction spot on image plane = *Point Spread Function*



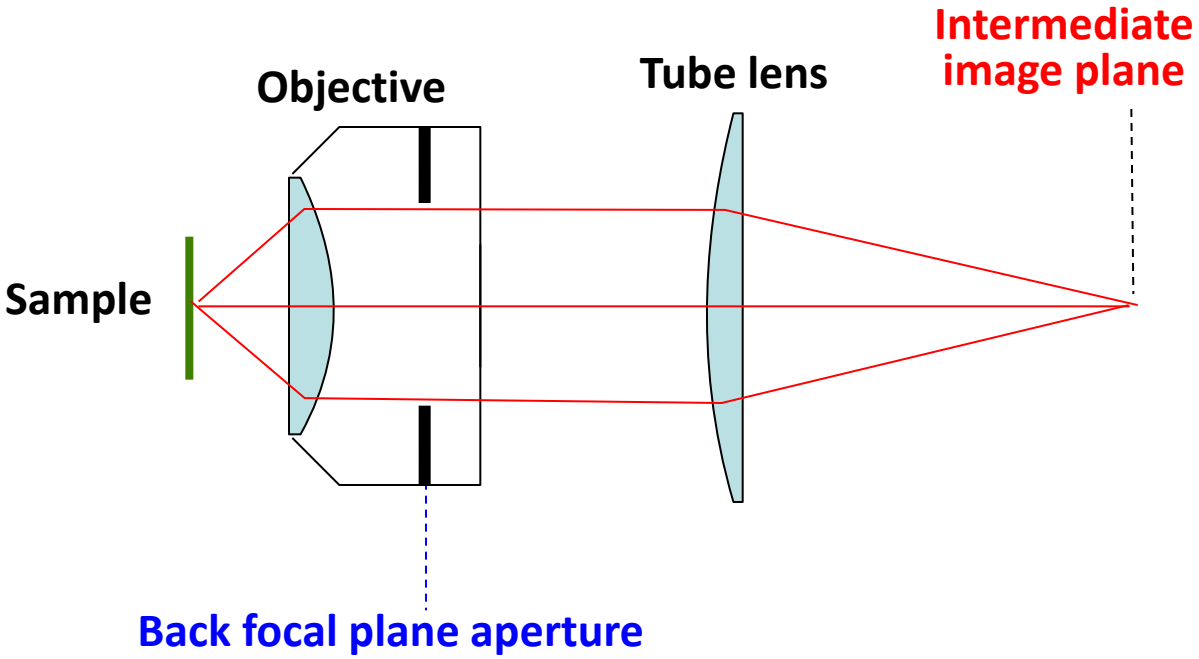
Aperture and Resolution



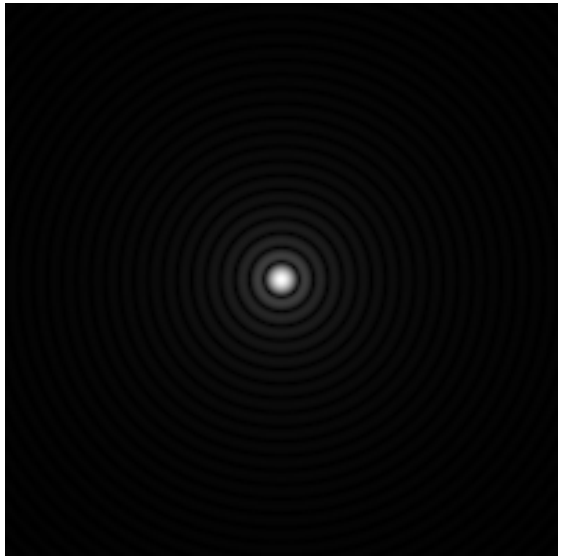
Diffraction spot on image plane = *Point Spread Function*



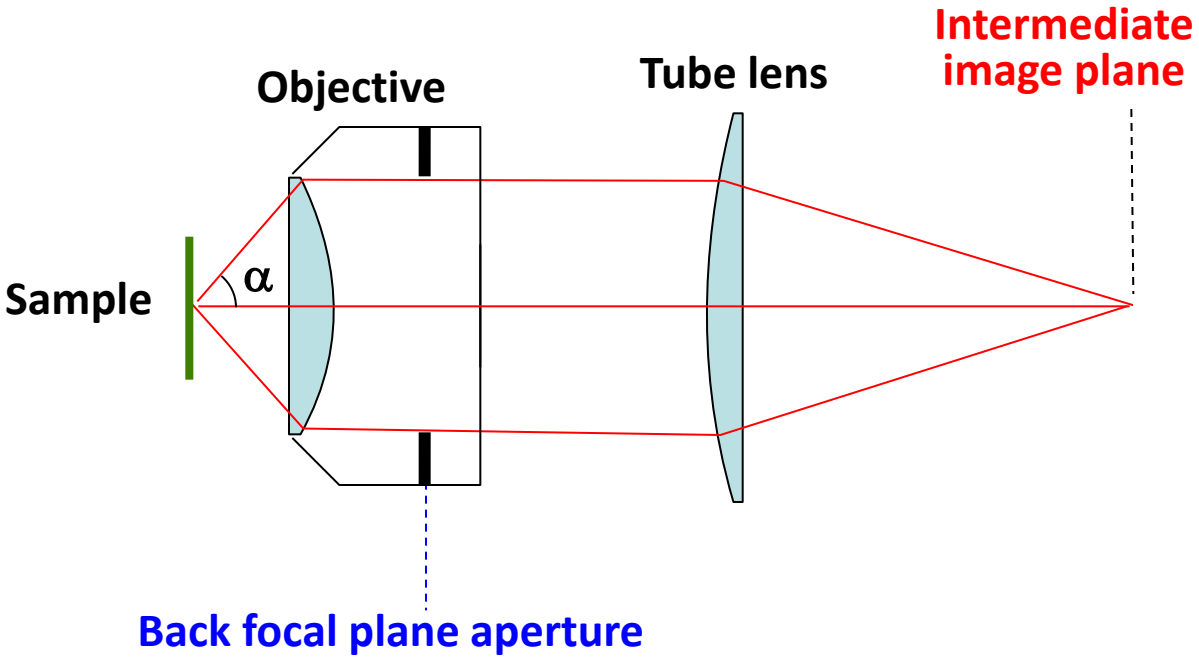
Aperture and Resolution



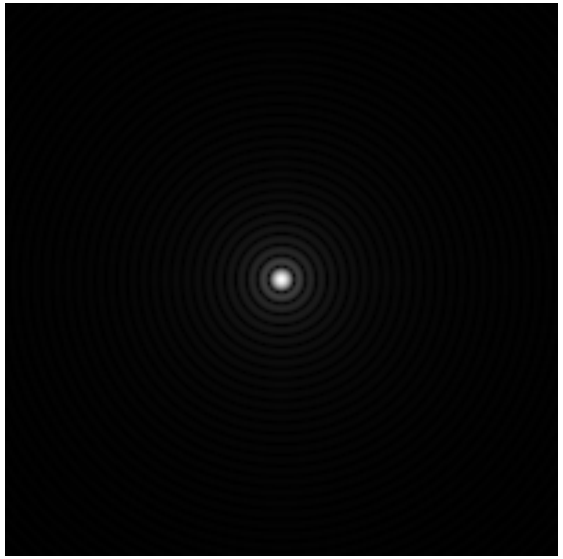
Diffraction spot on image plane = *Point Spread Function*



Aperture and Resolution



Diffraction spot on image plane = *Point Spread Function*



- **Image resolution improves with Numerical Aperture (NA)**

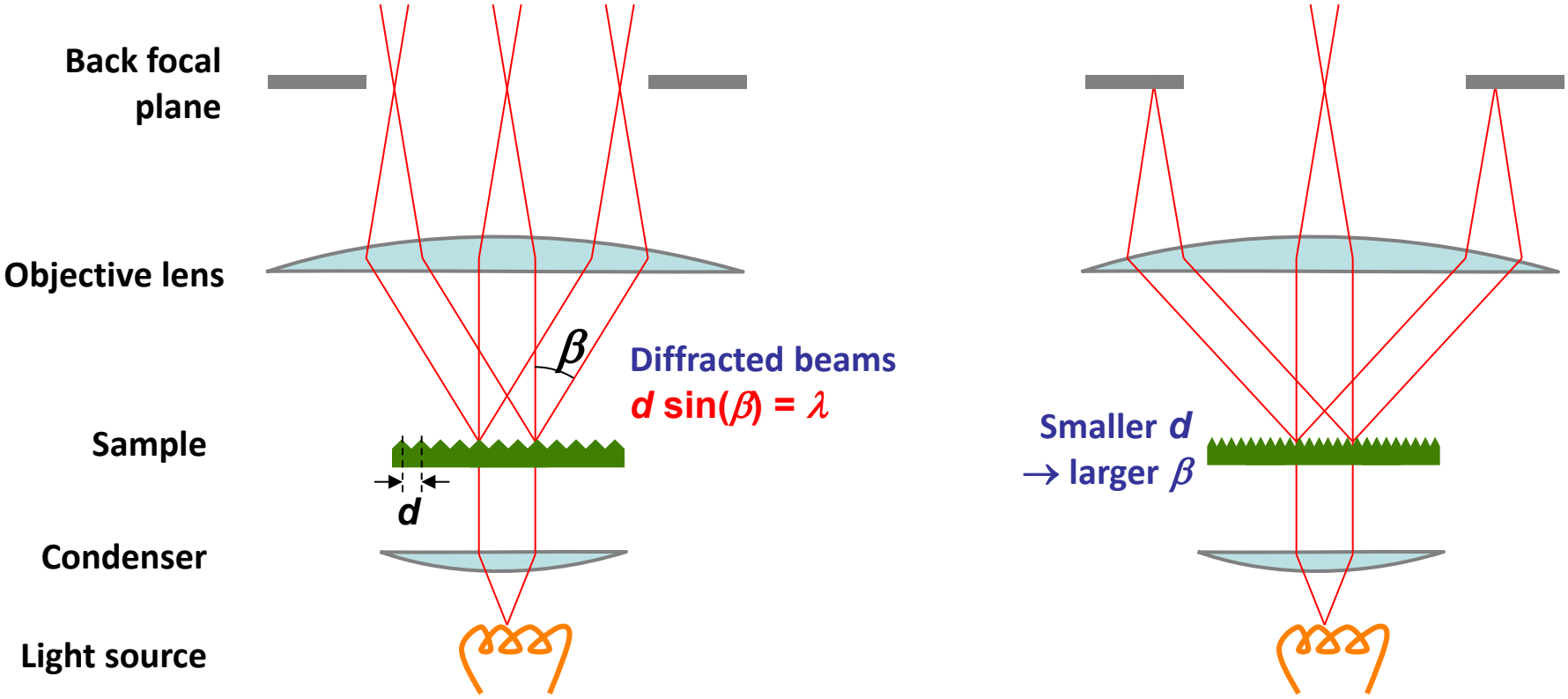
$$NA = n \sin(\alpha)$$

where: α = light gathering angle
n = refractive index of sample

Resolution

Ernst Abbe's argument (1873)

Consider a striped sample ≈ a diffraction grating



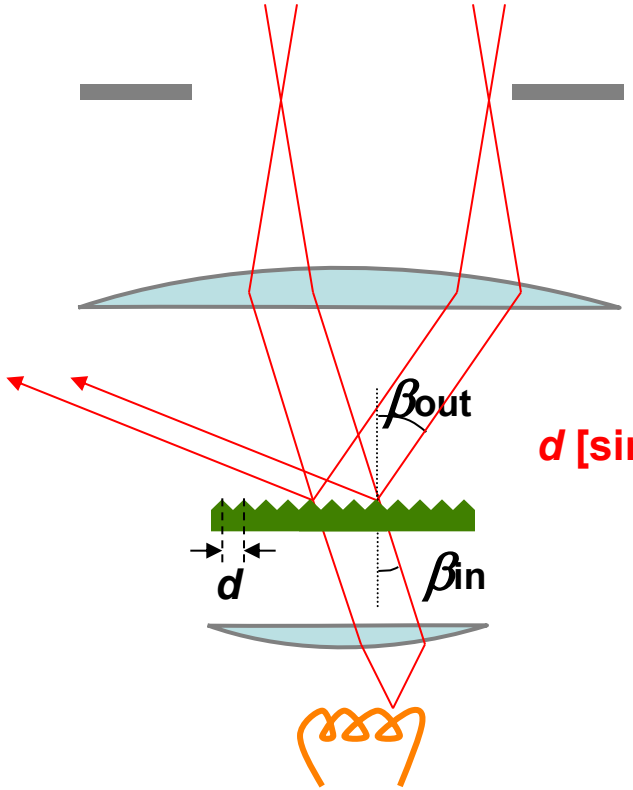
If $\beta > \alpha$, only one spot makes it through
$\Rightarrow$ no interference $\Rightarrow$ no image formed

Resolution (smallest resolvable d):

$$d_{min} = \lambda_{sample}/\sin(\alpha) = \lambda/n\sin(\alpha) = \lambda/NA$$

(Abbe's argument, continued)

Now consider oblique illumination (an off-axis source point):



One spot hopelessly lost, but two spots get through → interference → image formed!

$$d\,[\sin(\beta_{in}) + \sin(\beta_{out})] = \lambda$$

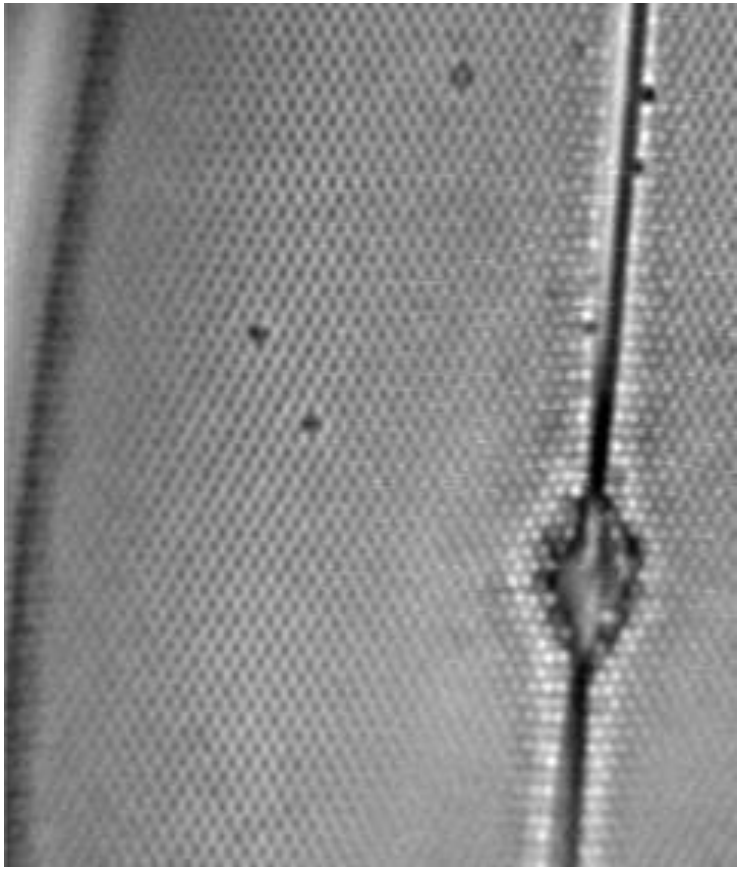
Two spots get through if $\beta_{out} < \alpha$ **and** $\beta_{in} < \alpha$.

Resolution (smallest resolvable *d*) with incoherent illumination (all possible illumination directions):

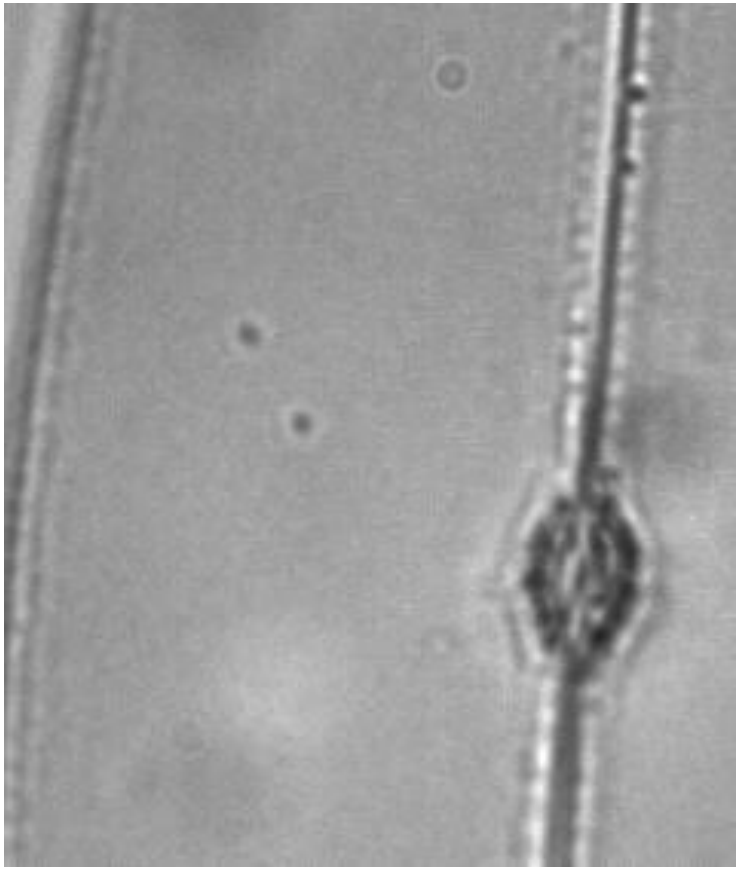
$$d_{min} = \lambda/(NA_{obj} + NA_{condenser})$$

→ $\lambda/2\ NA$ **if** $NA_{condenser} \geq NA_{obj}$ **("Filling the back focal plane")**

NA and Resolution



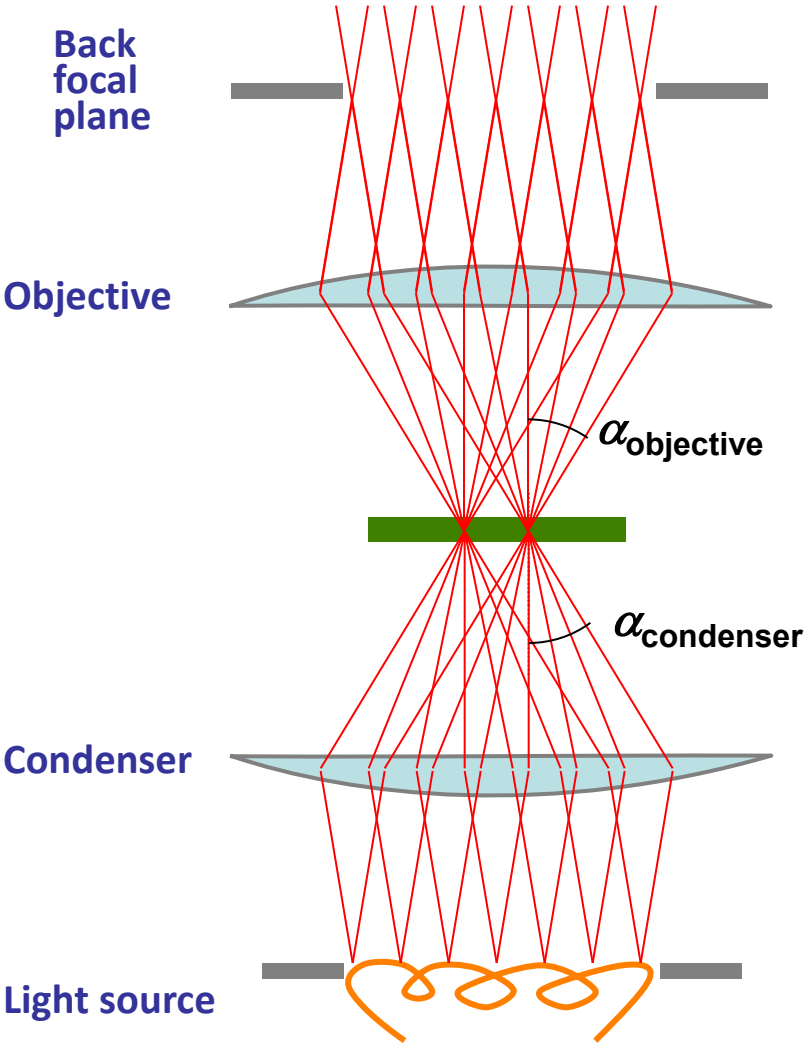
High NA Objective



Low NA Objective

Filling the back focal plane

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



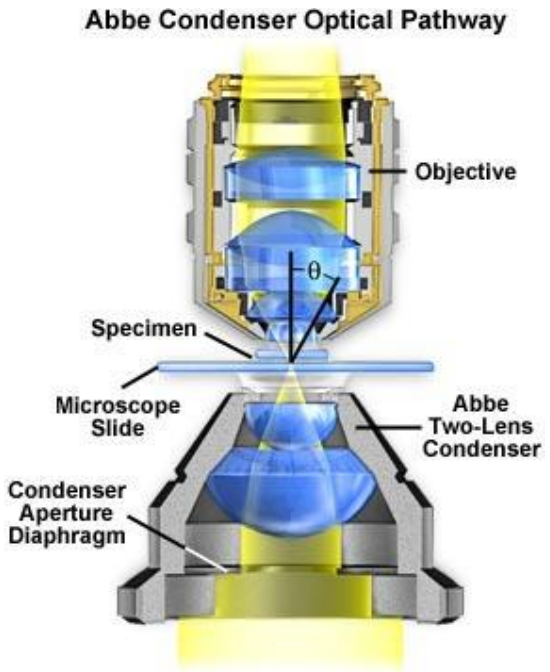
For the highest resolution, we need to have

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

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

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

The Condenser



Tasks:

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

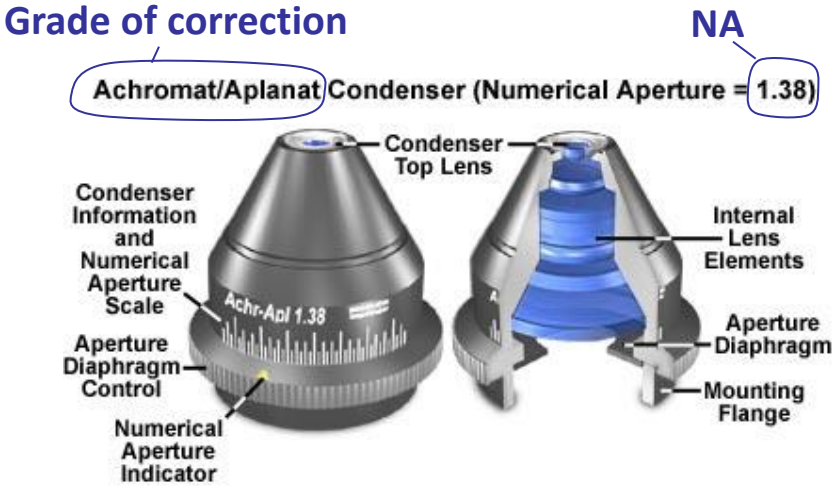
Problem:

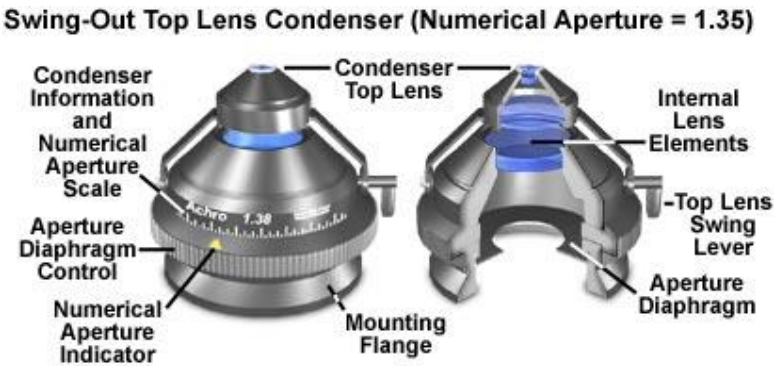
- Low mag objectives have large FOV,
- High mag objectives have large α

(With 2X and 100x objectives we need $(100/2)^2 = 2500$ times more light than any objective uses!)

Solutions:

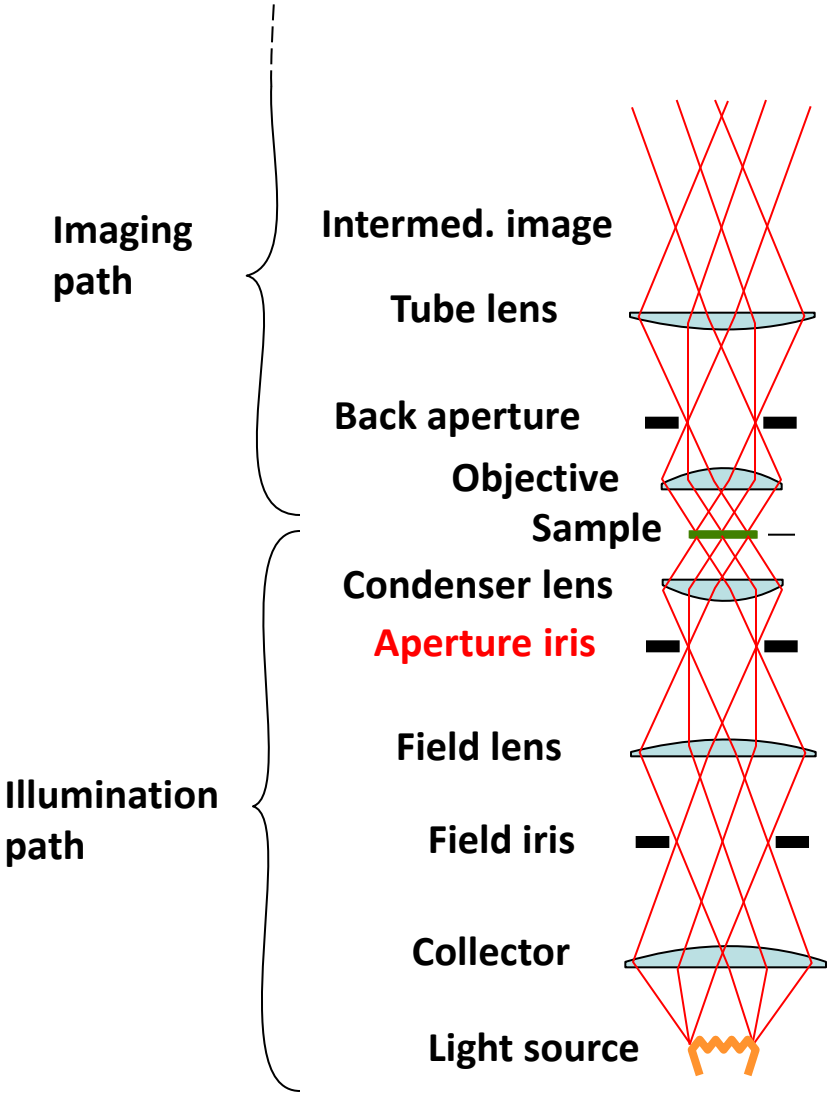
- Compromise
- Exchangable condensers, swing-out front lenses,…





Aperture, Resolution & Contrast

Can adjust the condenser NA with the aperture iris

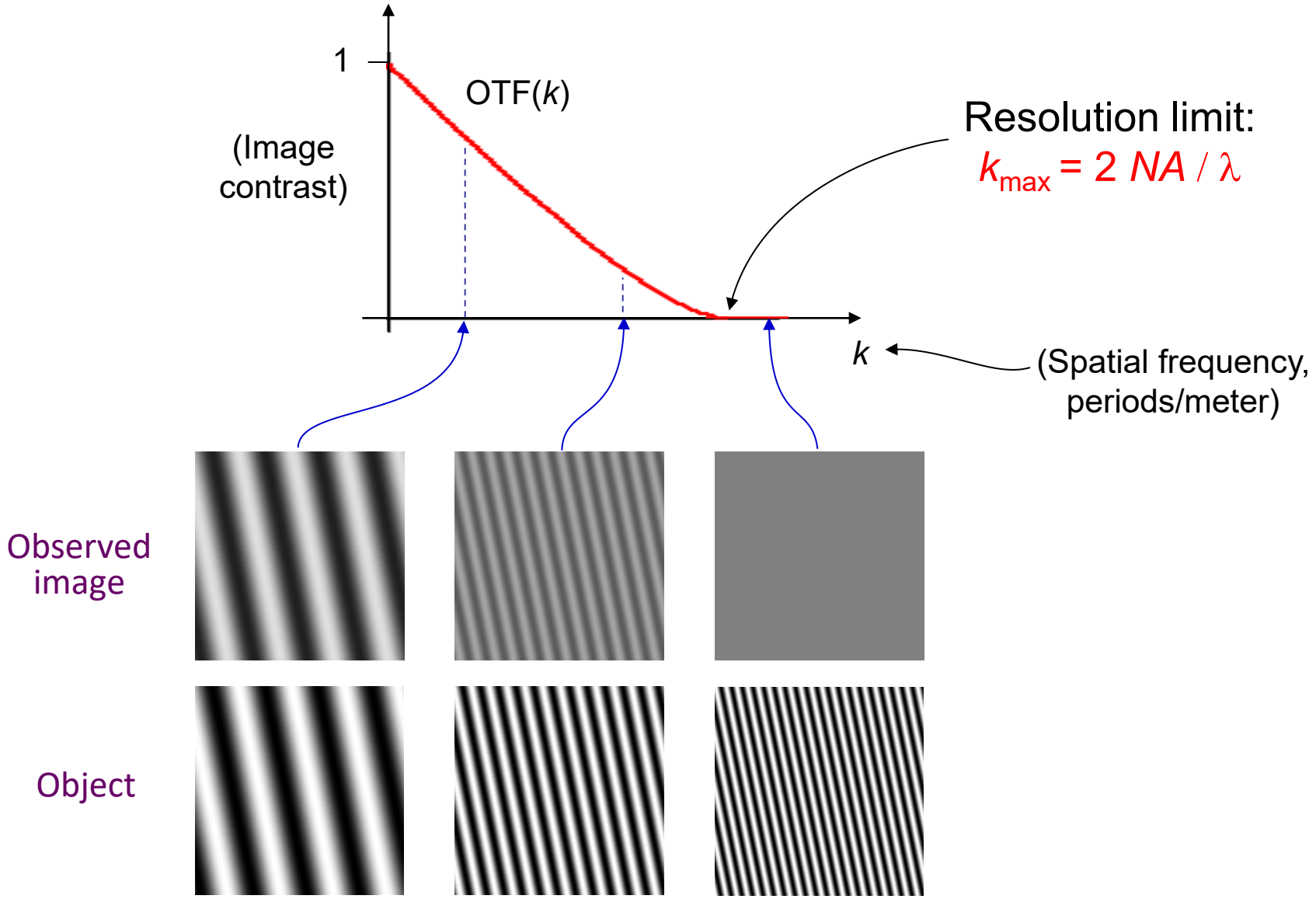


Q: Don't we always want it full open??

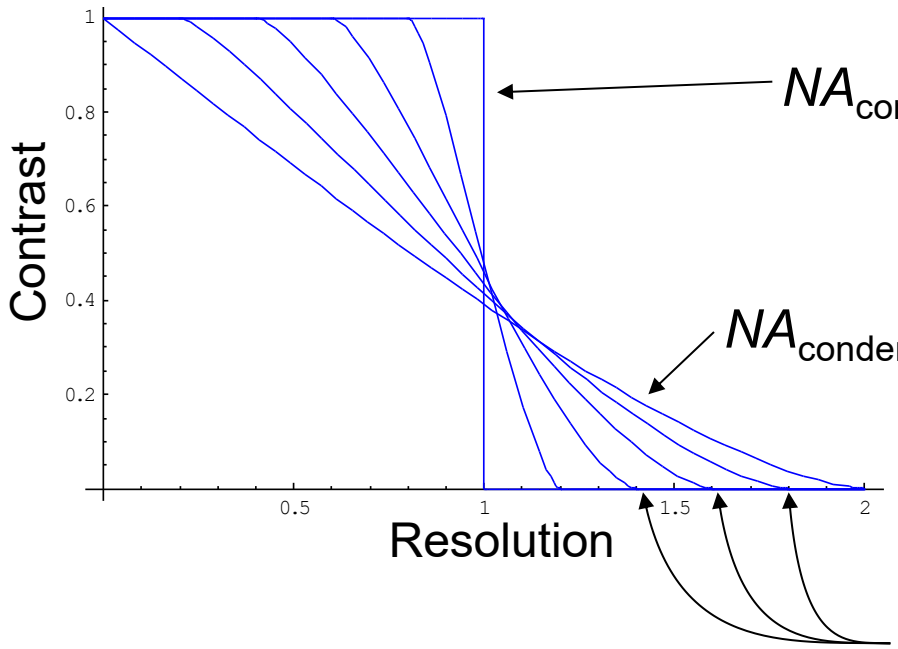
A: No

Why? Tradeoff: resolution vs. contrast

Spatial frequencies & the Optical Transfer Function (OTF)



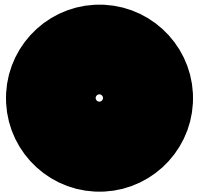
Resolution & Contrast vs. Illumination aperture



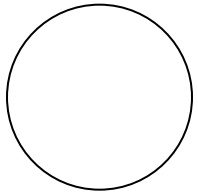
$NA_{condenser} \approx 0$

$NA_{condenser} \geq NA_{obj}$

Pupil appearance



("Coherent illumination")



(= Full aperture, "incoherent illumination")

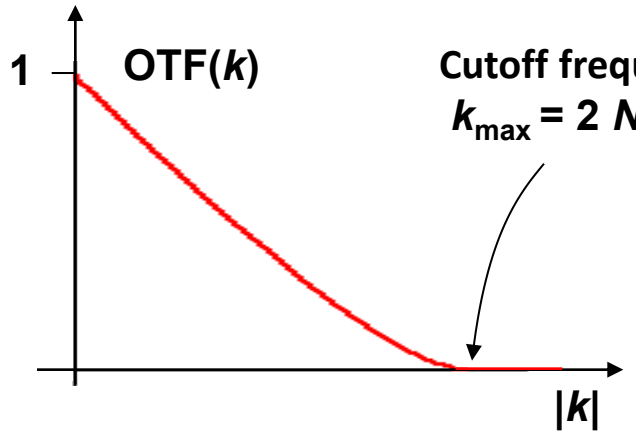
Resolution limit:

$k_{max} = (NA_{objective} + NA_{condenser}) / \lambda$

Increasing the illumination aperture
increases resolution
but decreases contrast

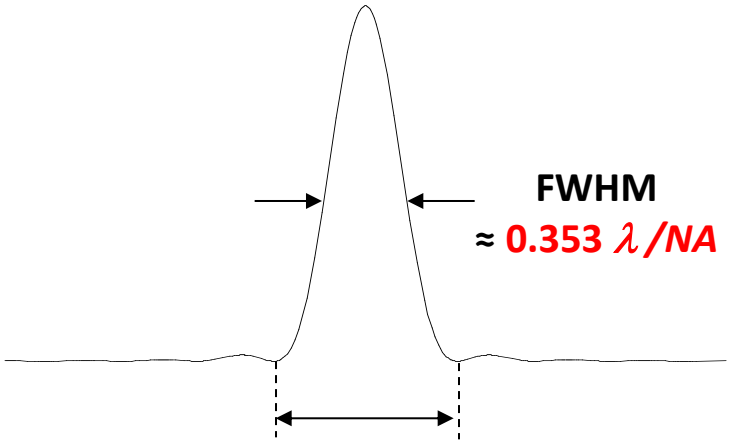
Definitions of Resolution

As the OTF cutoff frequency



$1/k_{max}$
$= 0.5\ \lambda/NA$

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



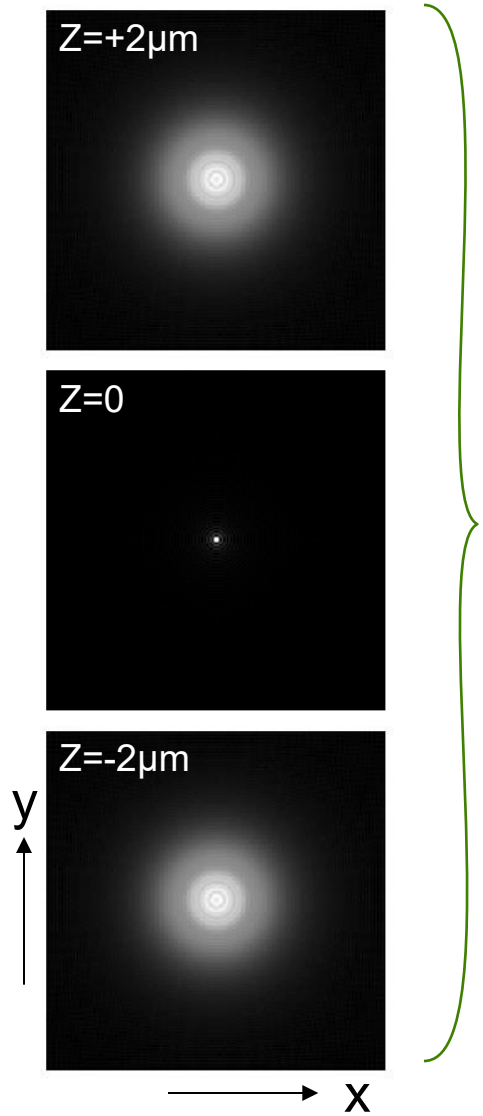
As the diameter of the Airy disk (first dark ring of the PSF)
= “Rayleigh criterion”

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

The 3D Point Spread Function (PSF)

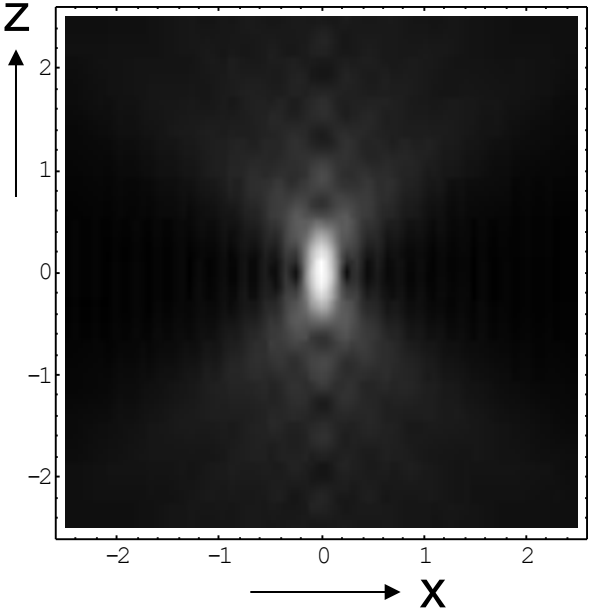
The image of a point object

2D PSF for different defocus

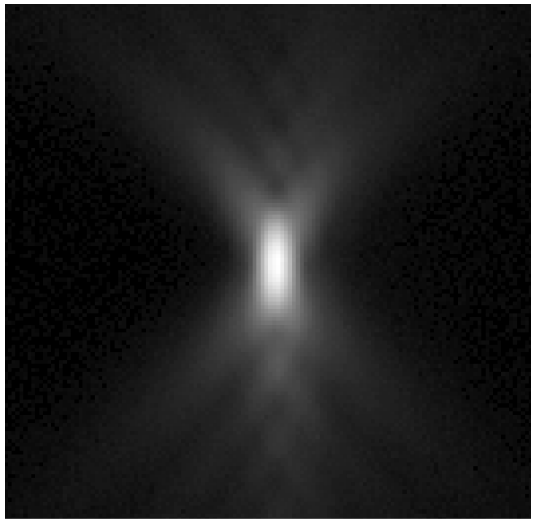


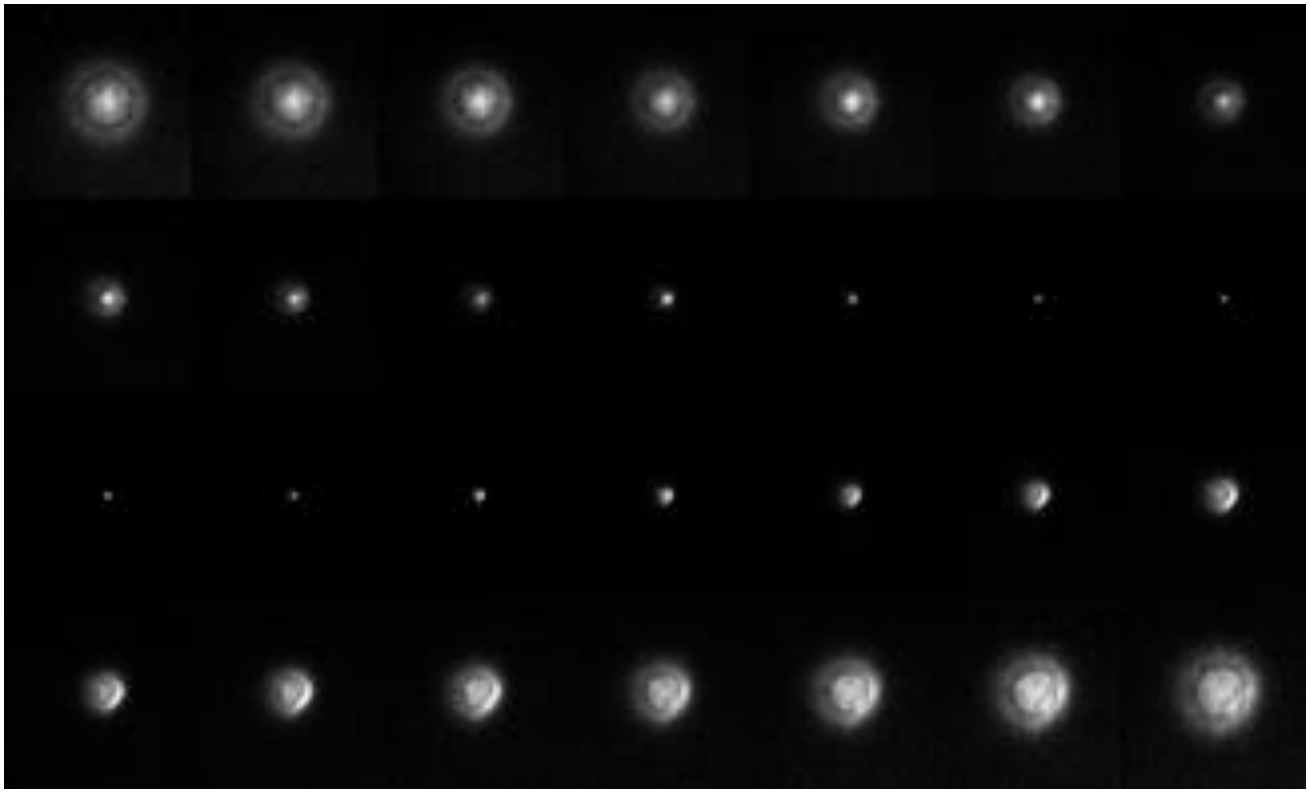
3D PSF

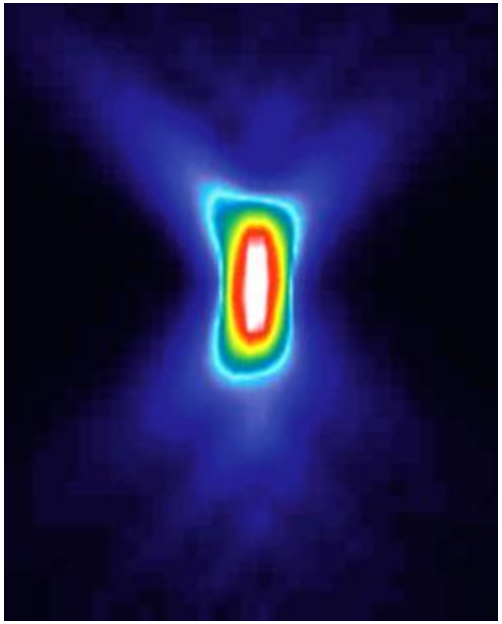
Calculated



Measured

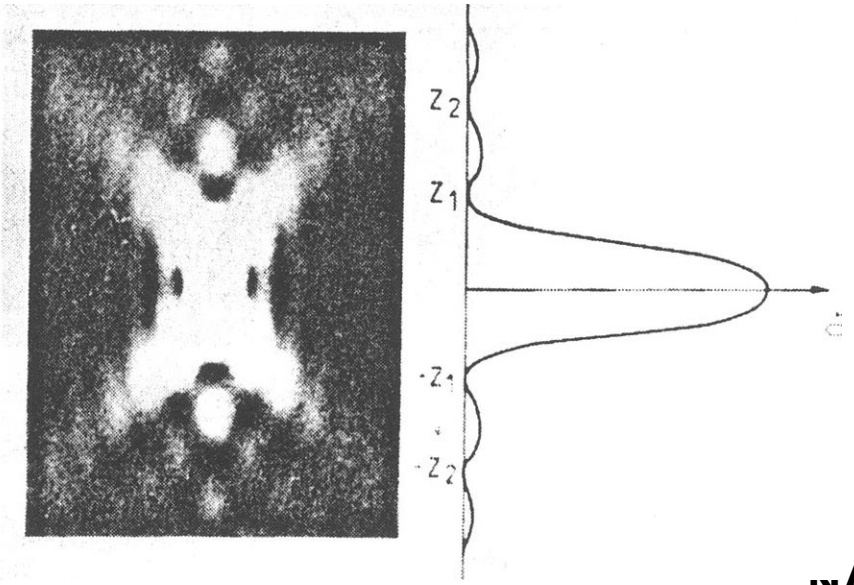






x - z

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

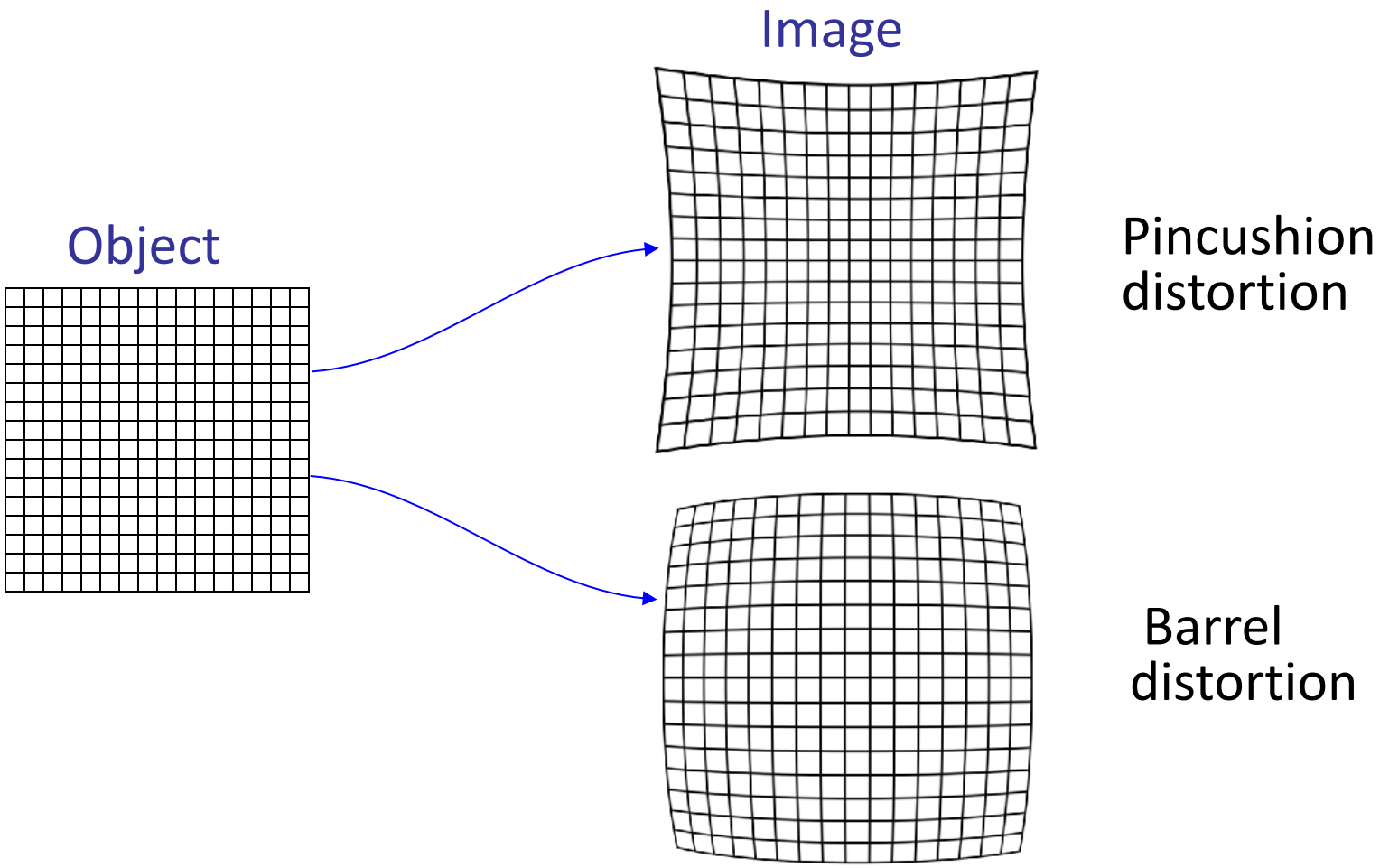
They are the enemy

Aberrations

- Chromatic aberrations
  - Longitudinal chr. Ab.
  - Lateral chr. Ab.
- Wavefront aberrations
  - Spherical aberration
  - Astigmatism
  - Coma
  - …
- Curvature of field
- Distortion

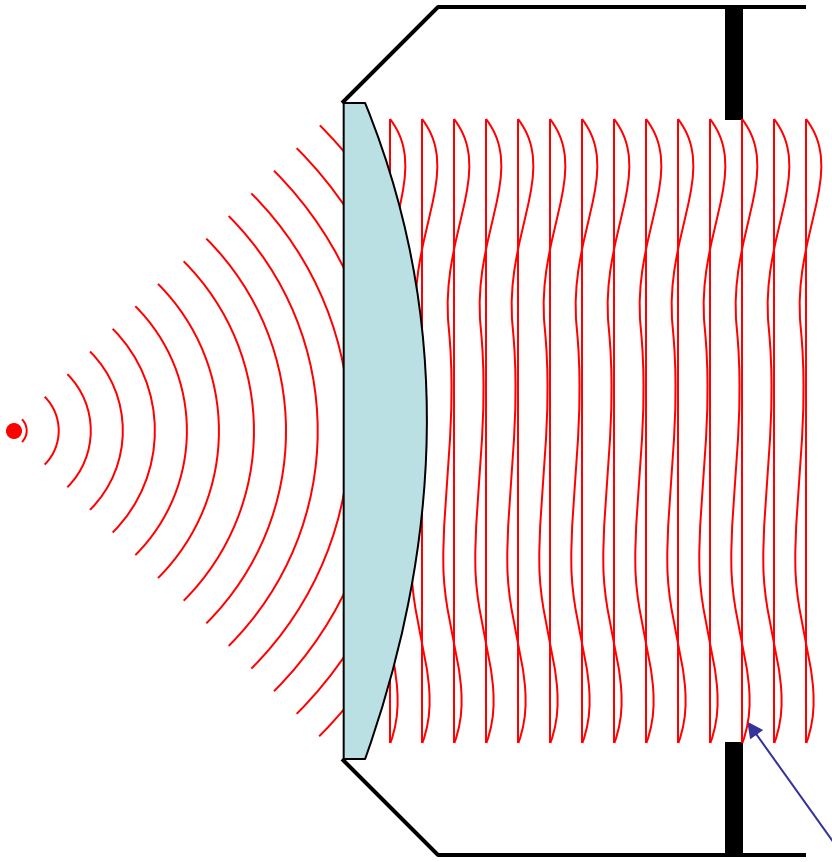
Geometric Distortion

= Radially varying magnification

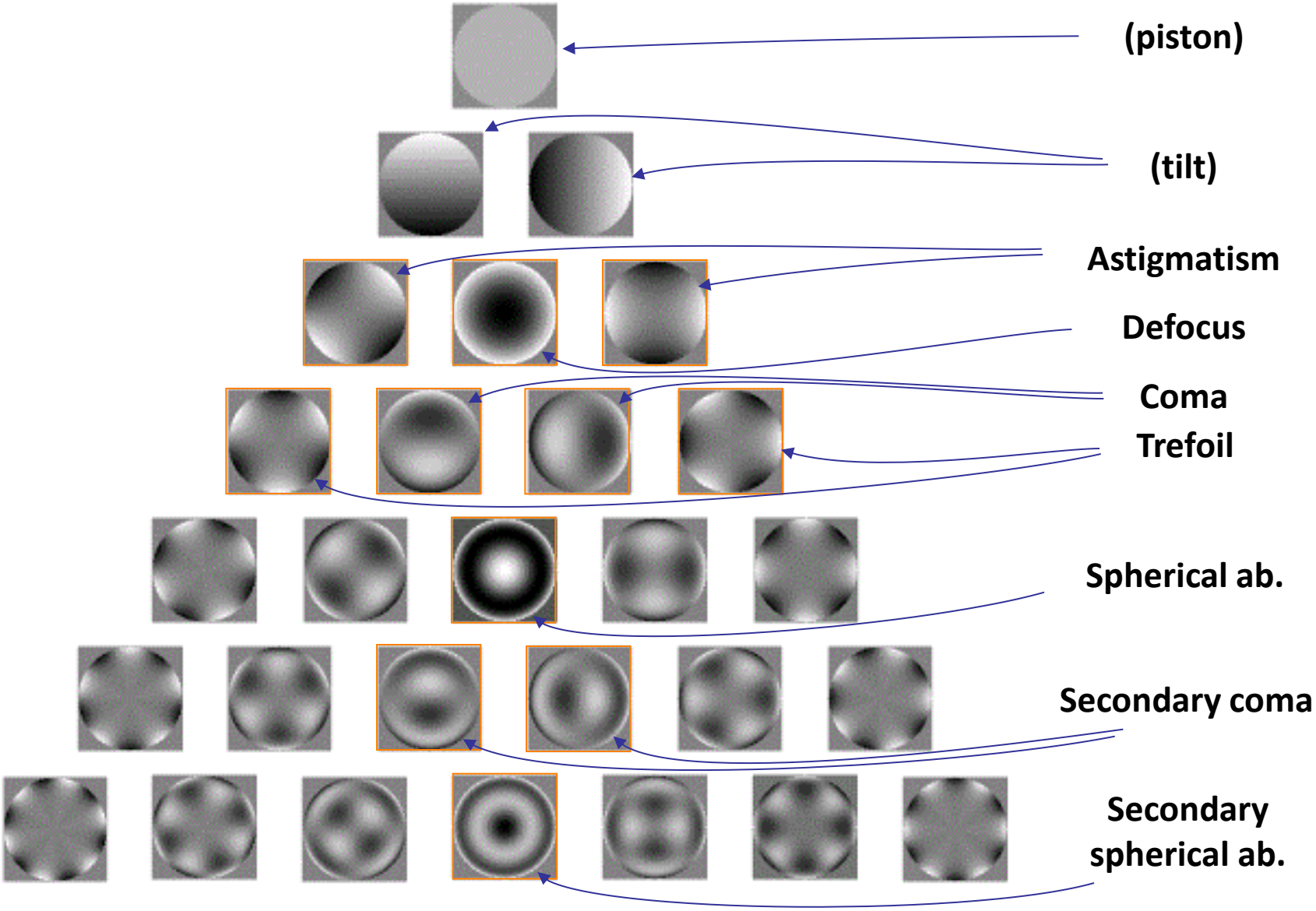


May be introduced by the projection eyepiece

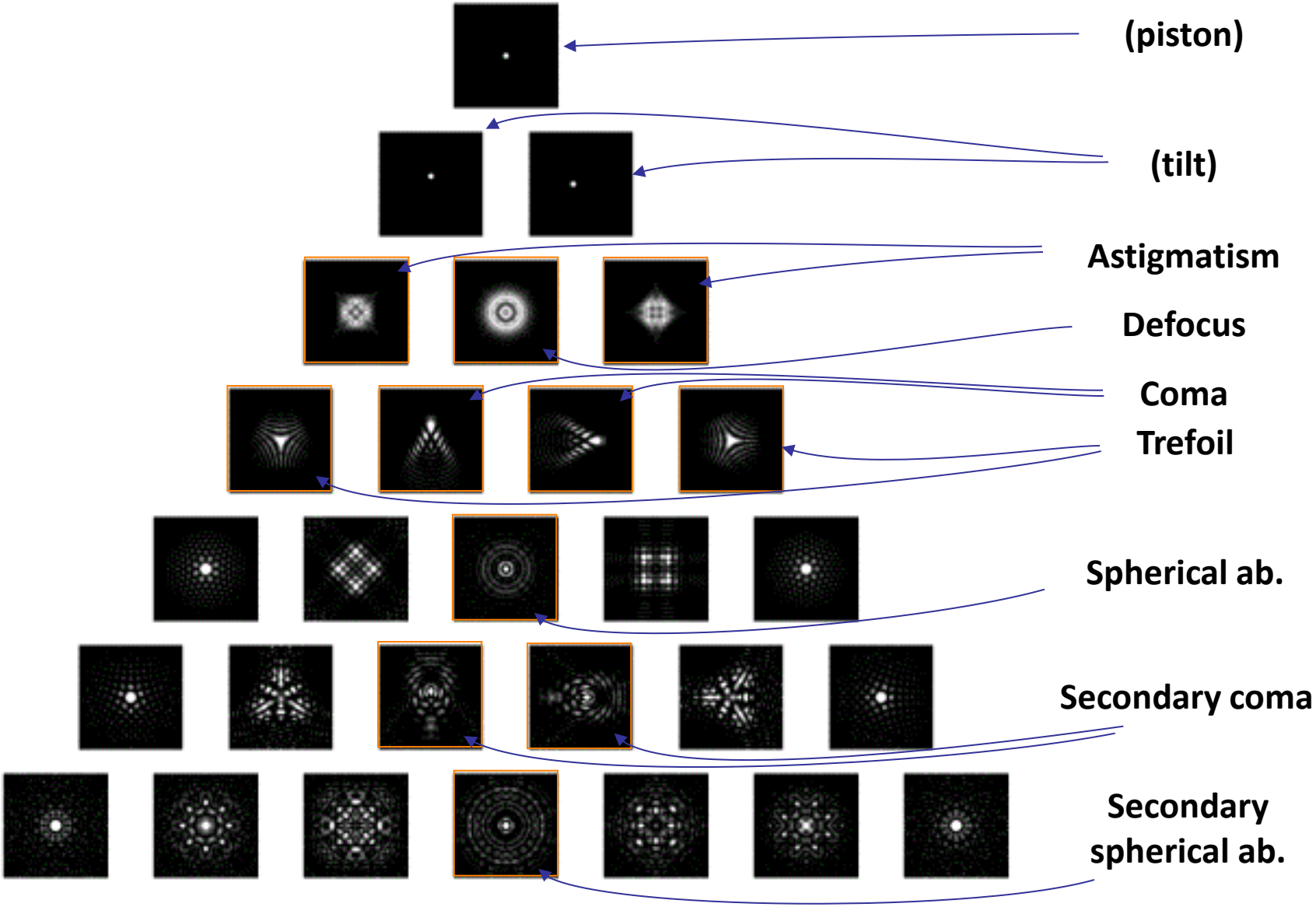
Wavefront Aberrations



Wavefront Aberrations



PSF Aberrations



Spherical Aberration

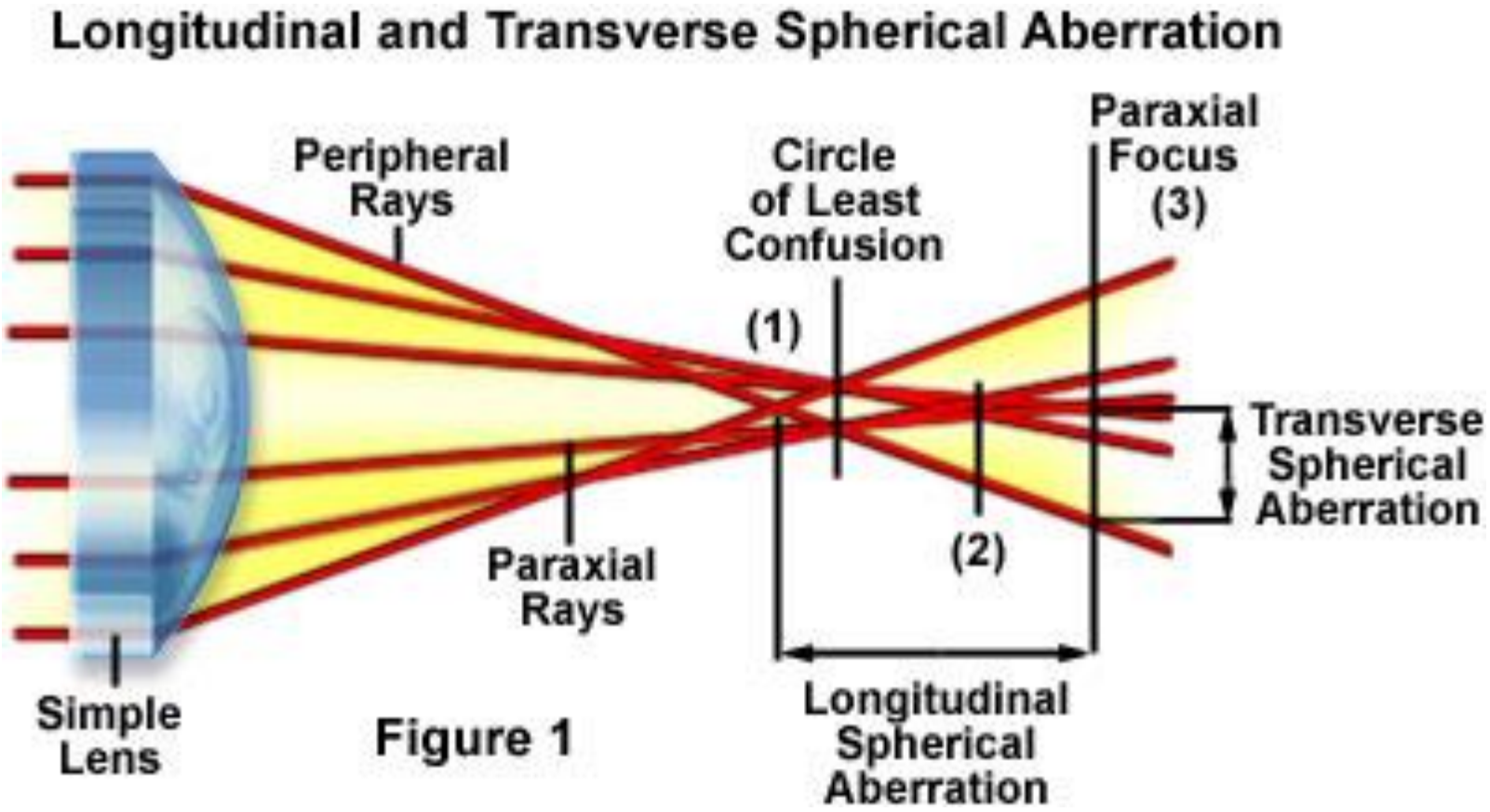


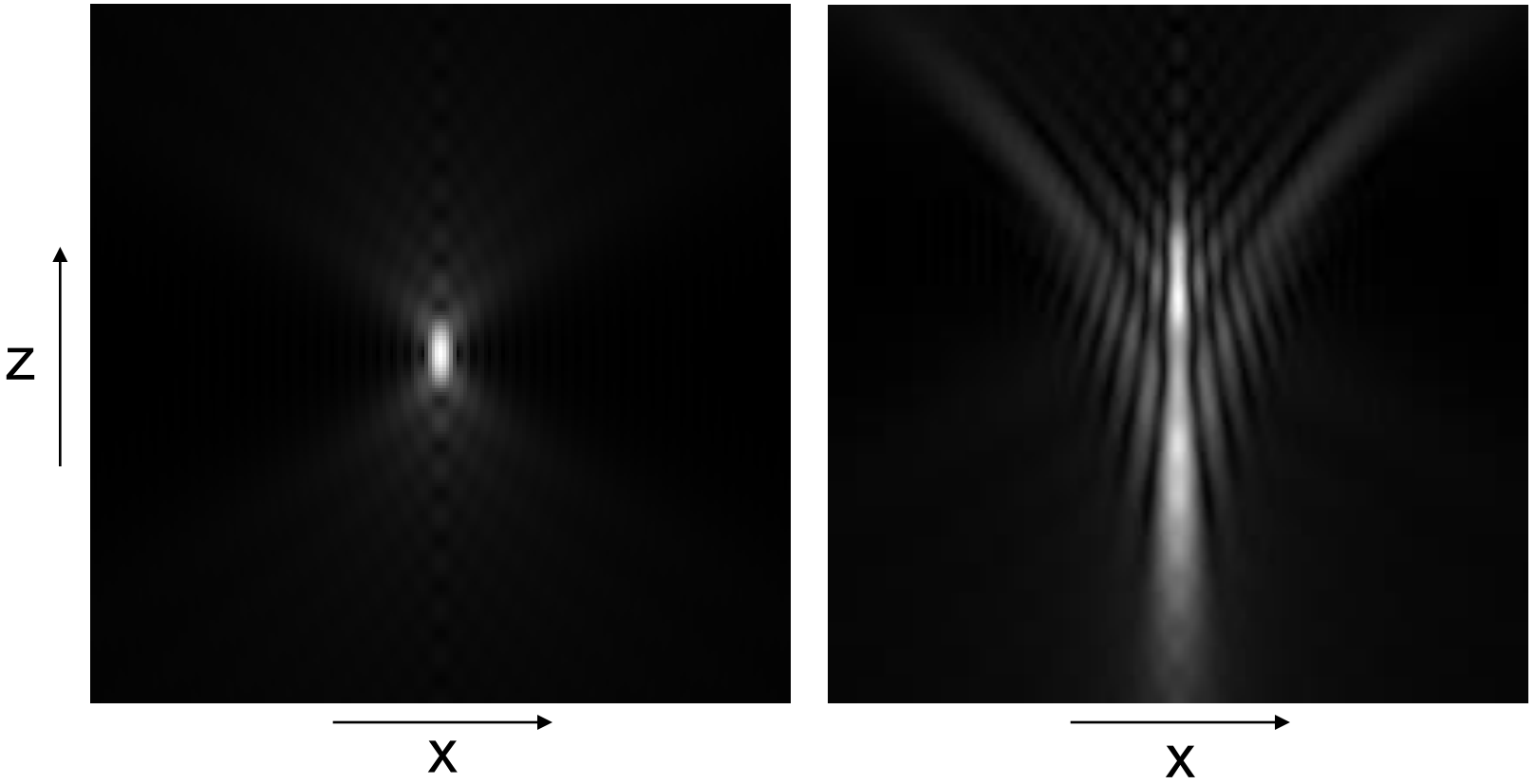
Figure 1

Spherical Aberration

Point spread functions

Ideal

1 wave of spherical ab



Causes of spherical aberration

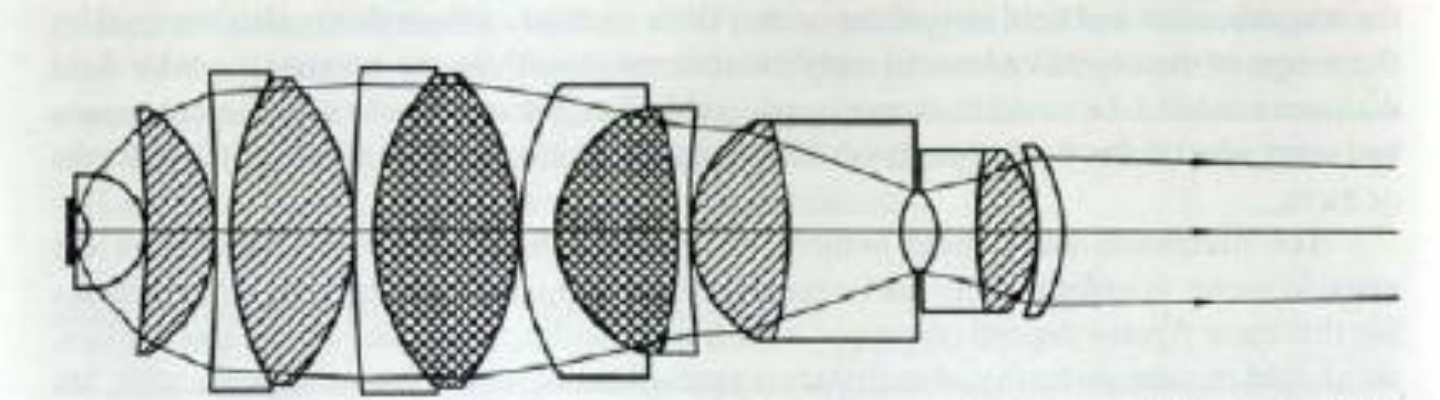


FIGURE 2-36. Design of Nikon Plan Apochromat oil immersion objective with 60× magnification and 1.40 NA

- 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 = $l_1n_1 + l_2n_2 + \ldots + l_nn_n$

Sources of Spherical Aberration

Design compromises

Manufacturing tolerances

Immersion fluid index error

Temperature variation

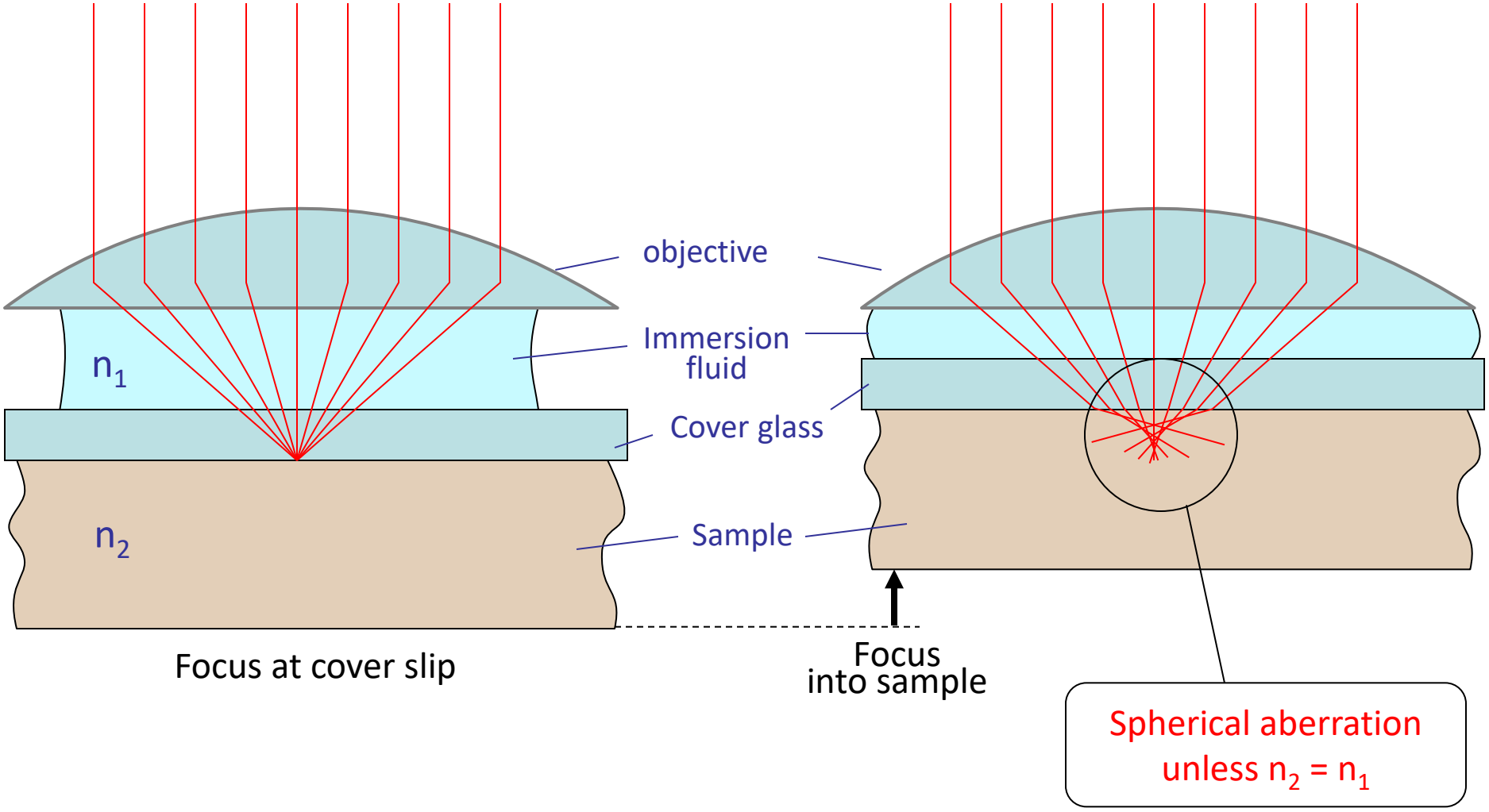
Cover slip thickness

(high-NA objectives except oil immersion)

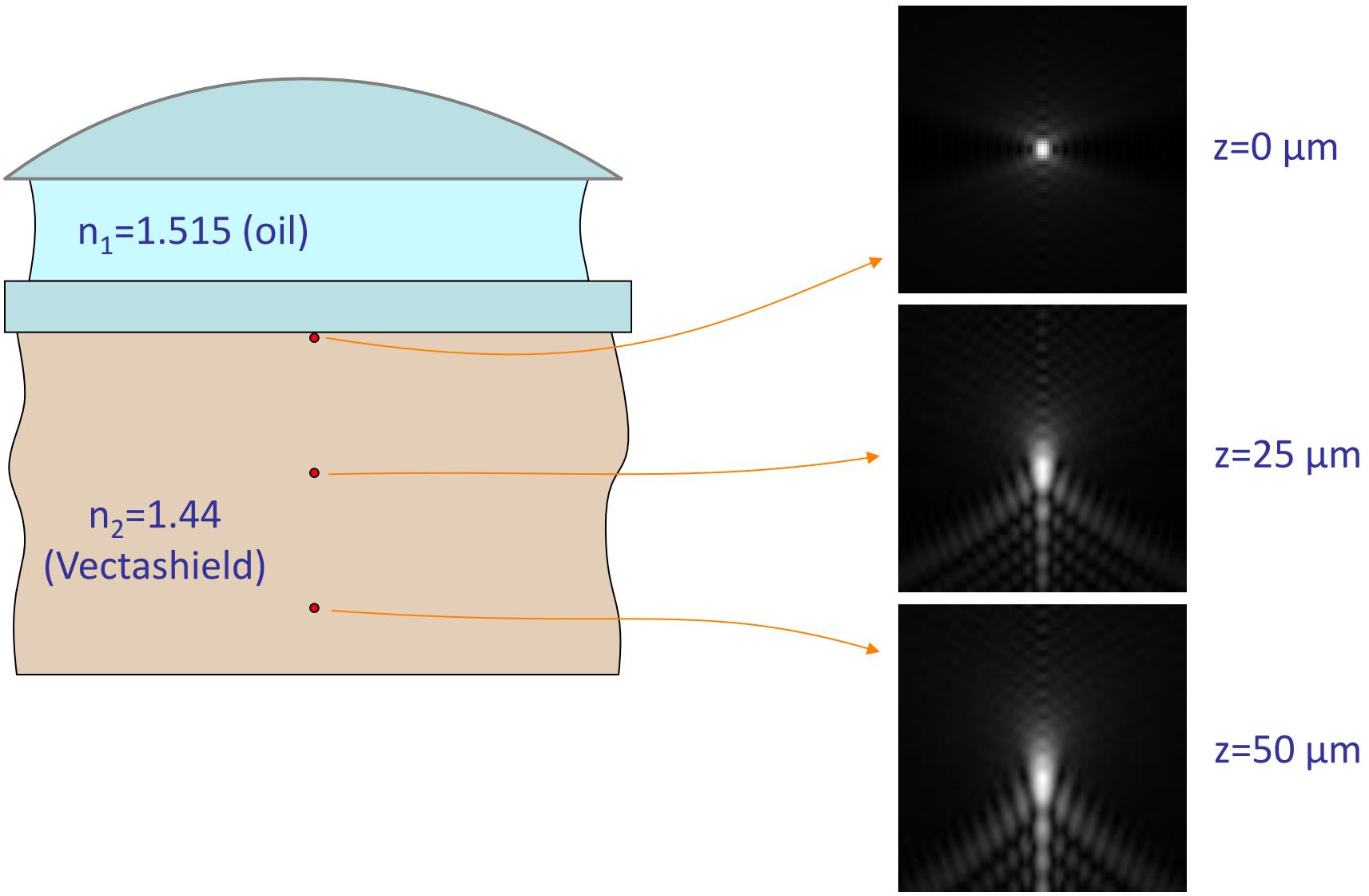
Correction collar setting

Sample refractive index mismatch

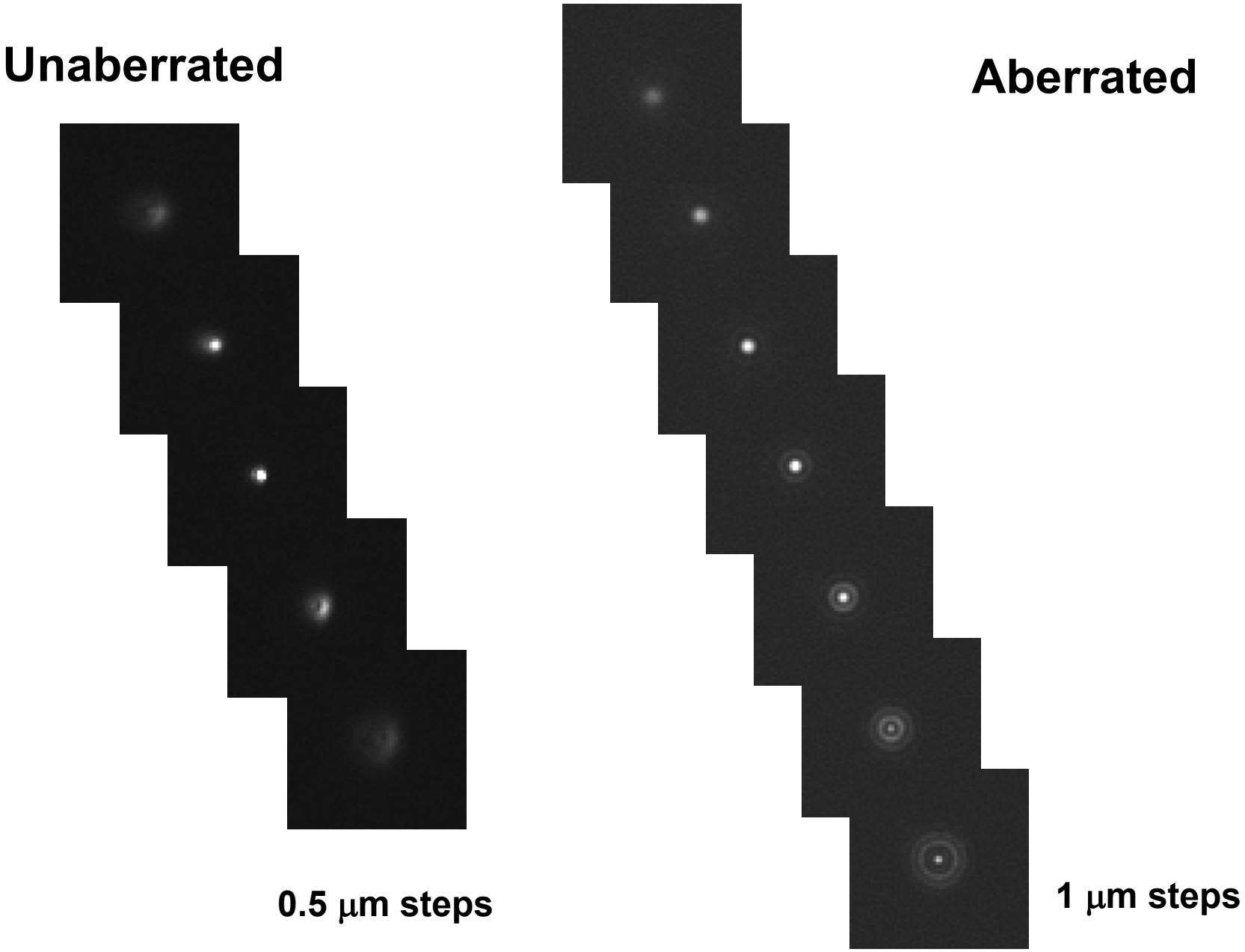
Index Mismatch
& Spherical Aberration



Index Mismatch
& Spherical Aberration



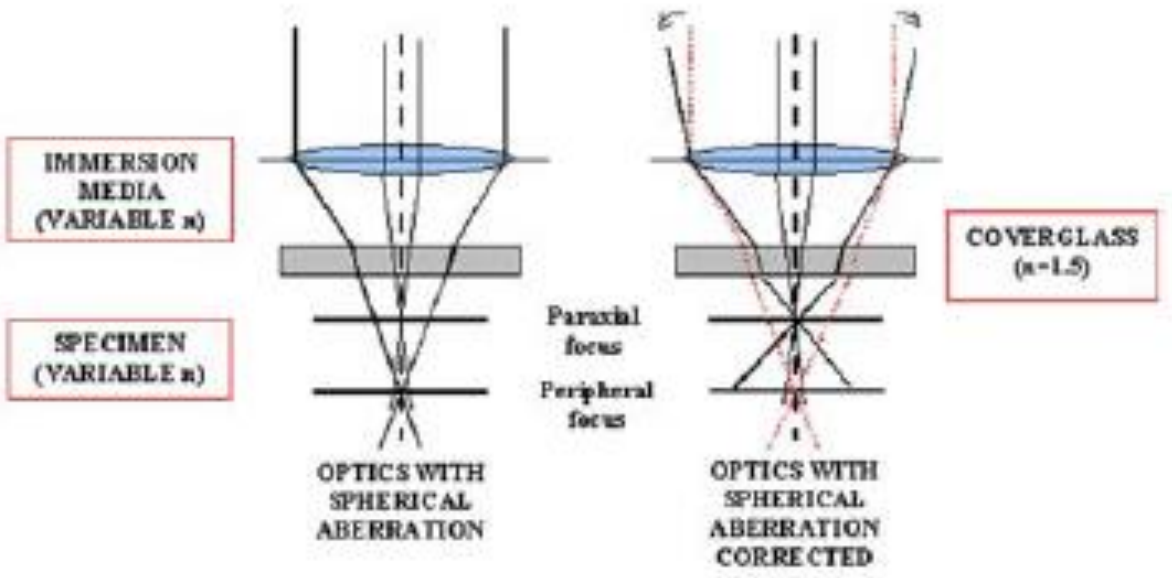
How to recognize spherical aberration



What can you do about spherical aberration?

- Use 0.17 mm coverslips (~ #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

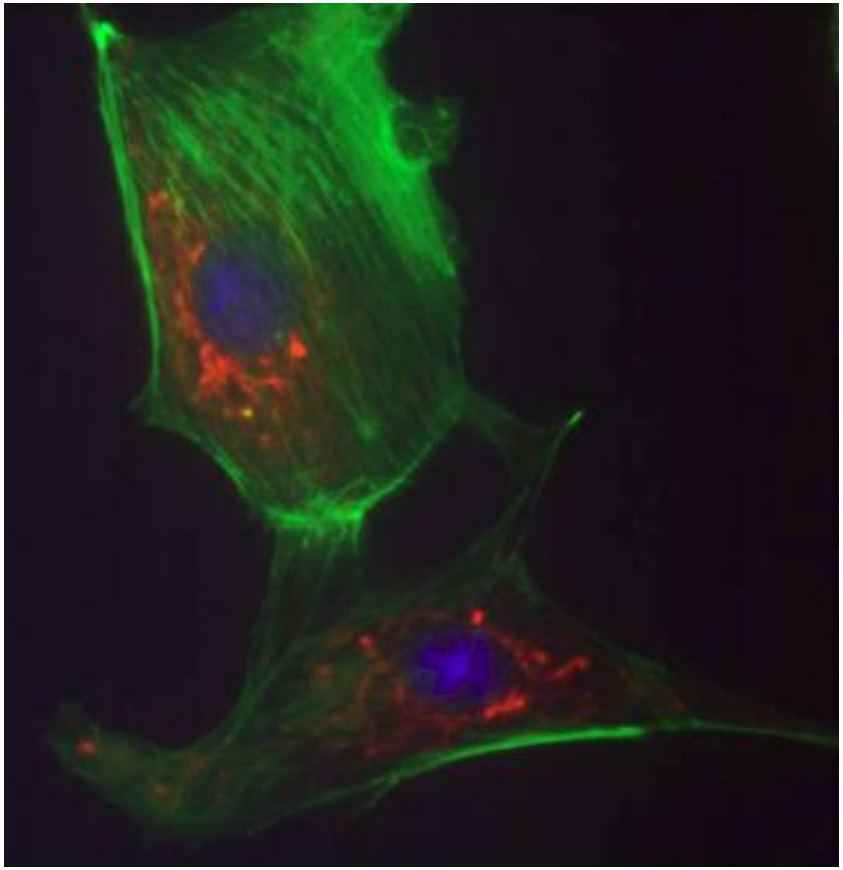
Correction collars



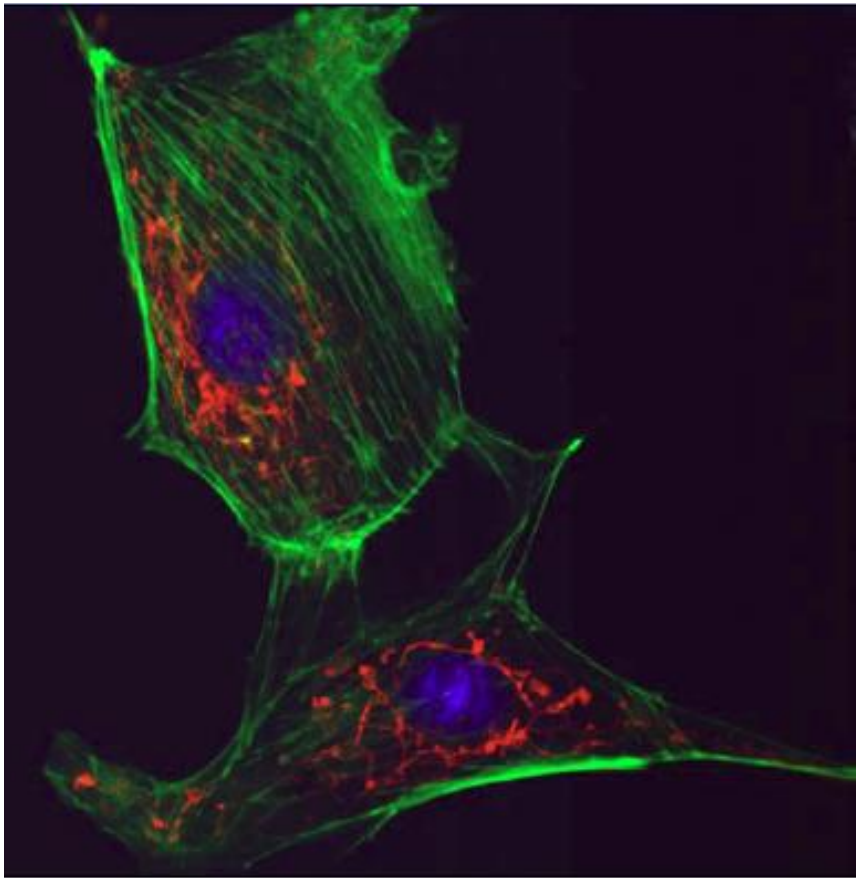
A correction collar can only eliminate spherical aberration at a single focal plane

Example

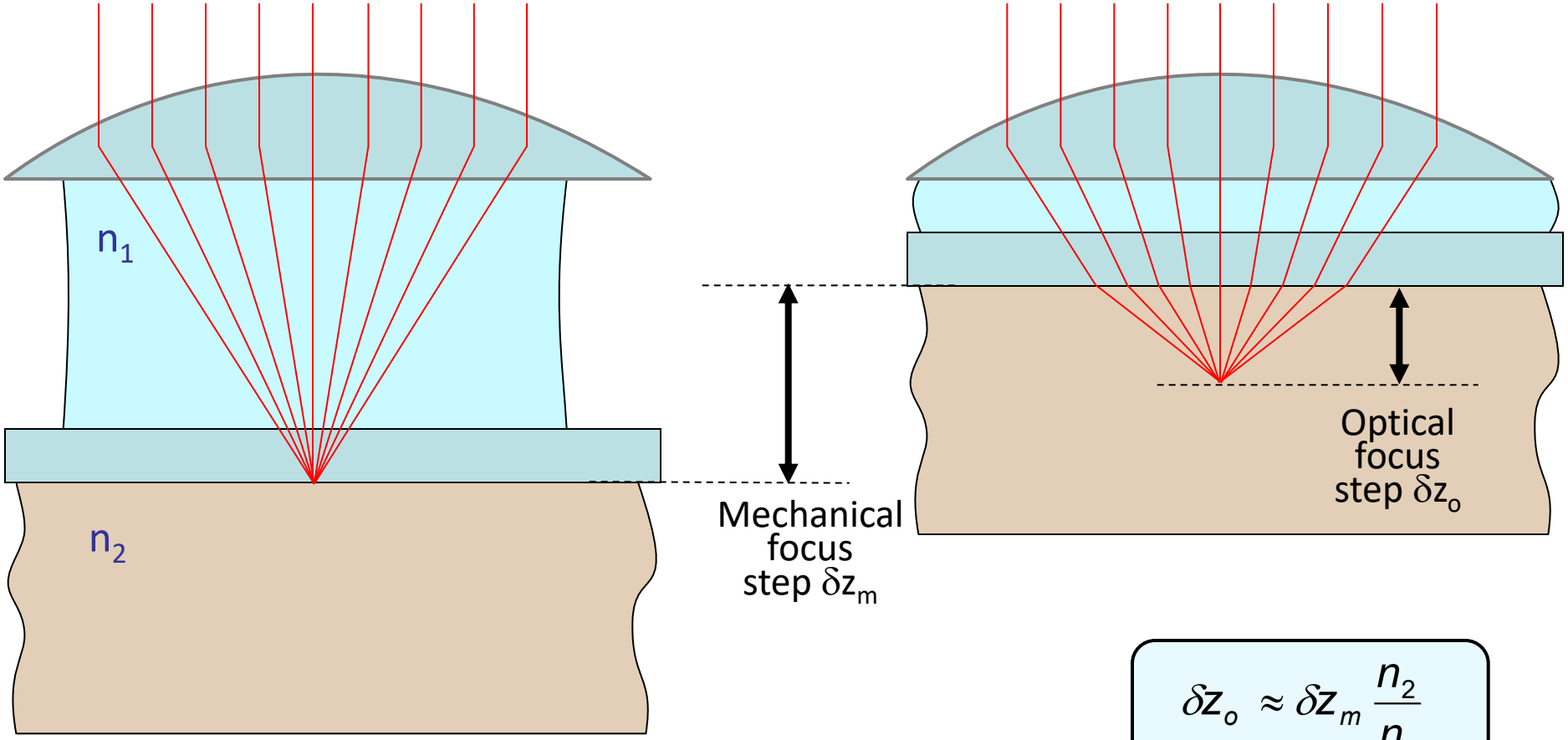
Aberrated



Unaberrated



Index Mismatch & Axial Scaling



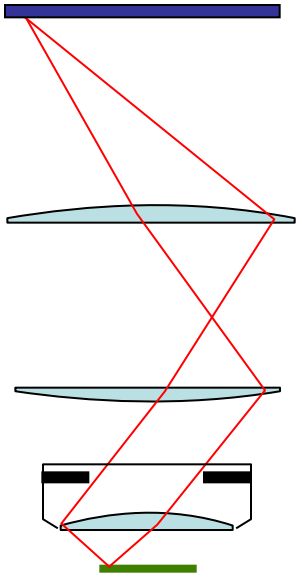
$$\delta z_o \approx \delta z_m \frac{n_2}{n_1}$$

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)

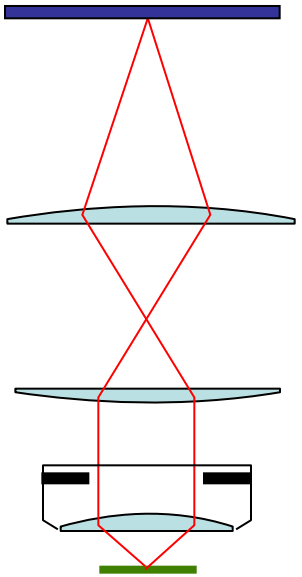
- All objectives have some
- Present in the design
- You get what you pay for



On-axis (center of field of view)

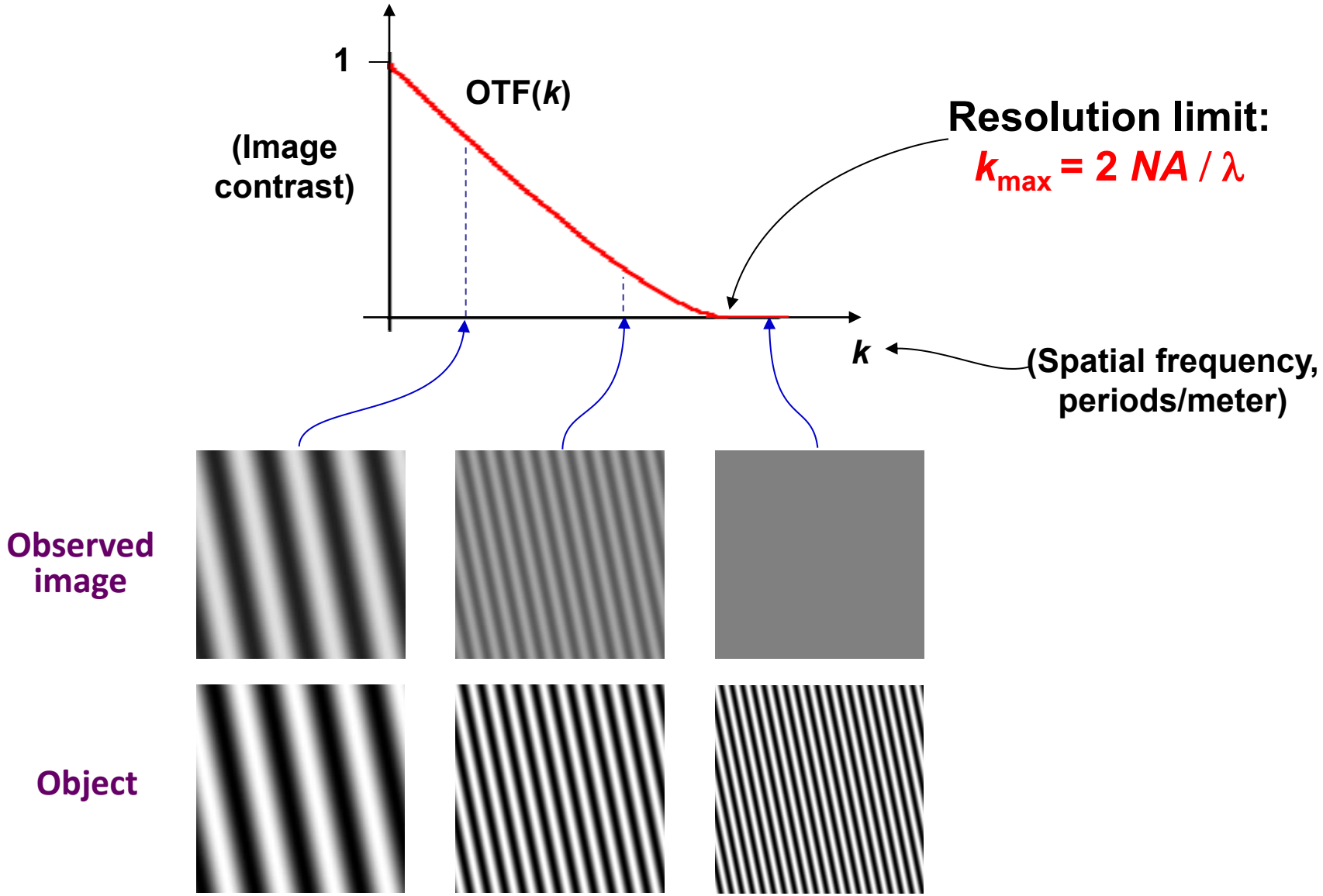
Should be none, by symmetry.
If they are there, they could be from:

- 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)



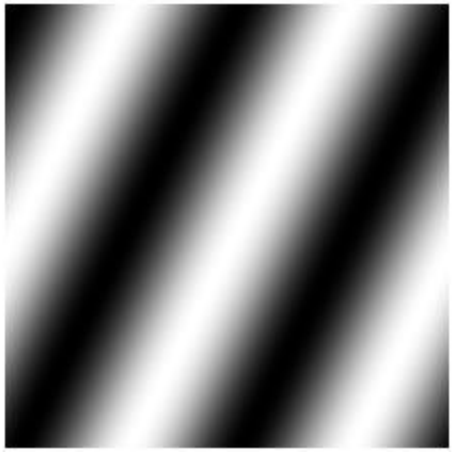
Think of Images as Sums of Waves

… or "spatial frequency components"

one wave

+

another wave



=

(2 waves)

+ (…) =

(25 waves)

+ (…) =

(10000 waves)

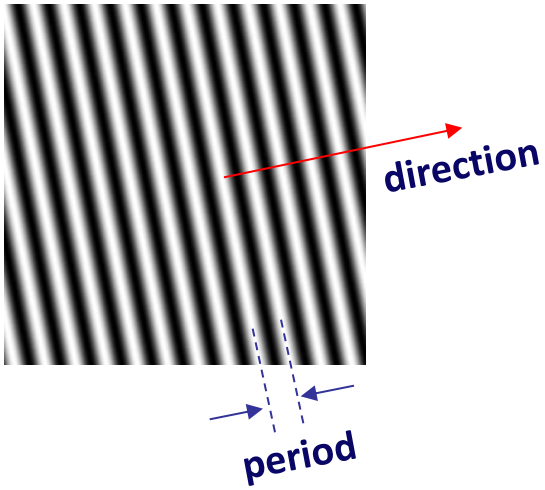
Frequency Space

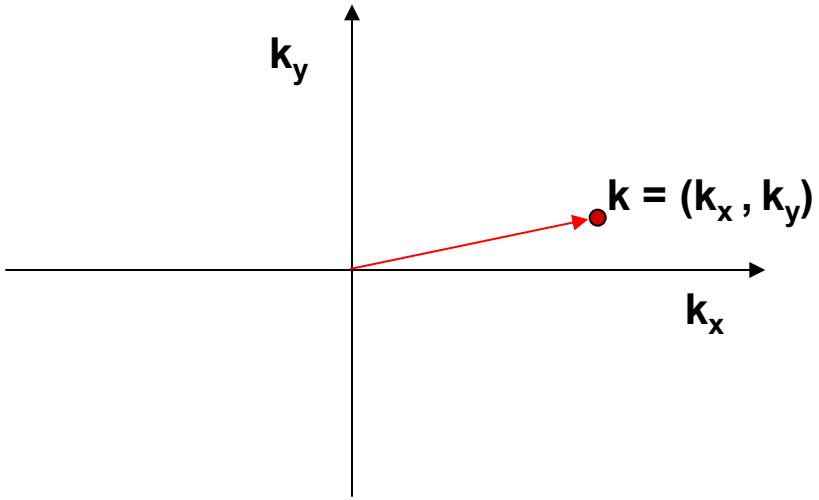
To *describe* a wave, we need to specify its:

Can describe it by a *value* at a *point*

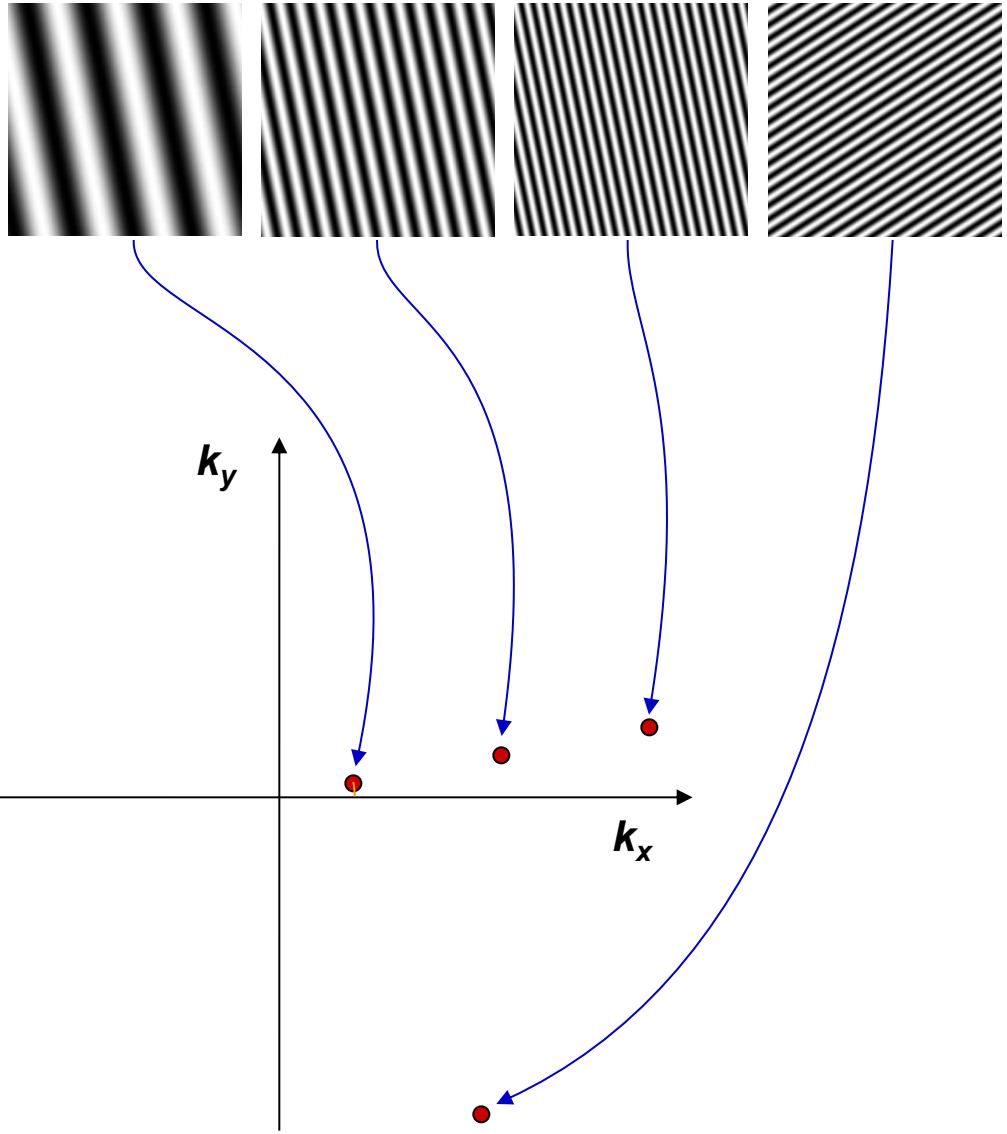
- **Frequency** (how many periods/meter?) → **Distance from origin**
- **Direction** → **Direction from origin**
- **Amplitude** (how strong is it?) → **Magnitude of value**
- **Phase** (where are the peaks & troughs?) → **Phase of value**

complex

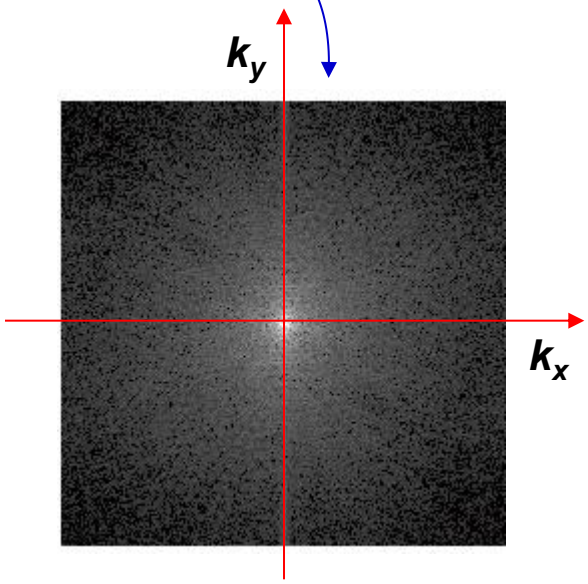




Frequency Space
and the *Fourier Transform*



Fourier Transform



Properties of the Fourier Transform

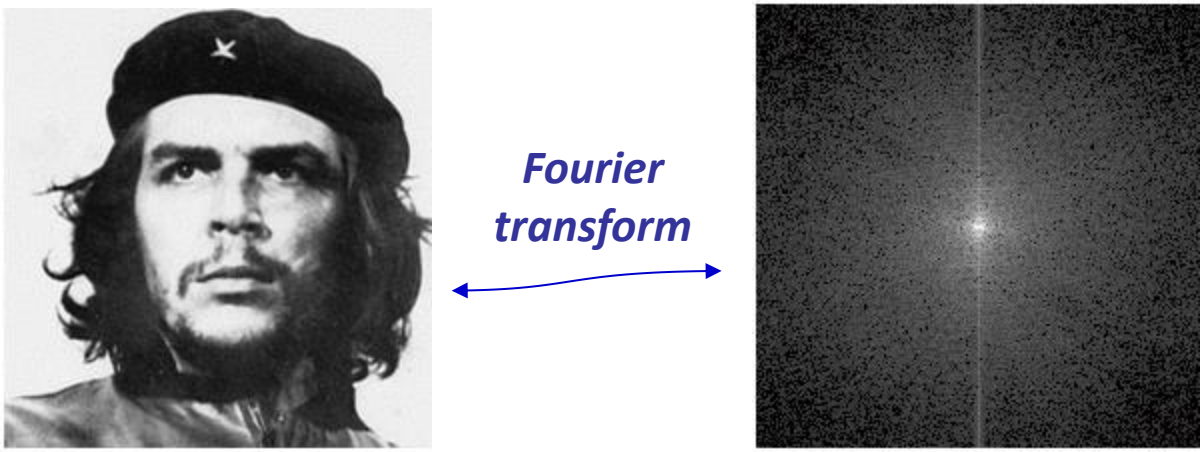
$$F(\mathbf{k}) = \int f(\mathbf{r}) e^{2\pi i \mathbf{k}\bullet\mathbf{r}} d\mathbf{r}$$

Completeness:

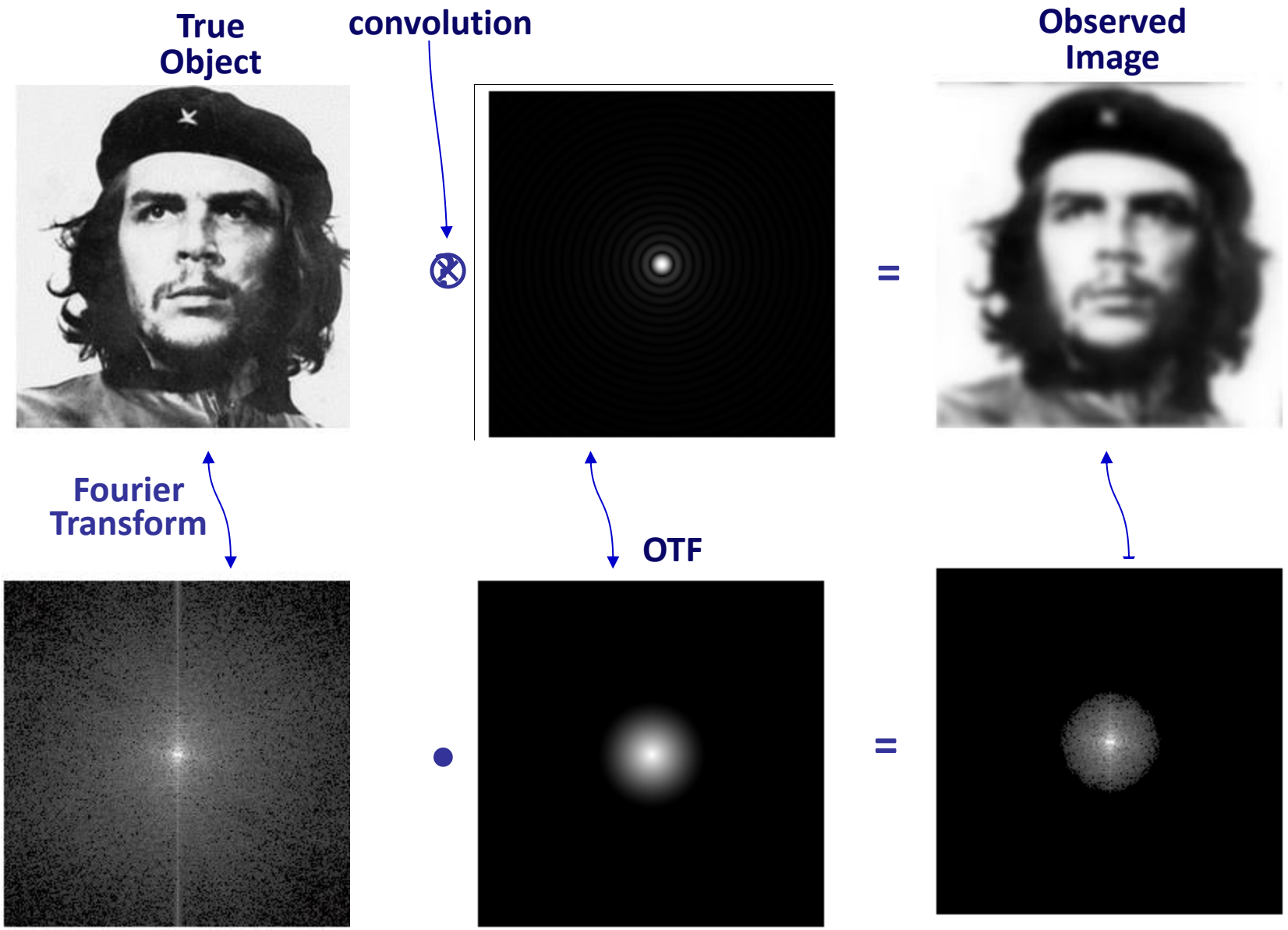
The Fourier Transform contains all the information of the original image

Symmetry:

The Fourier Transform of the Fourier Transform is the original image



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

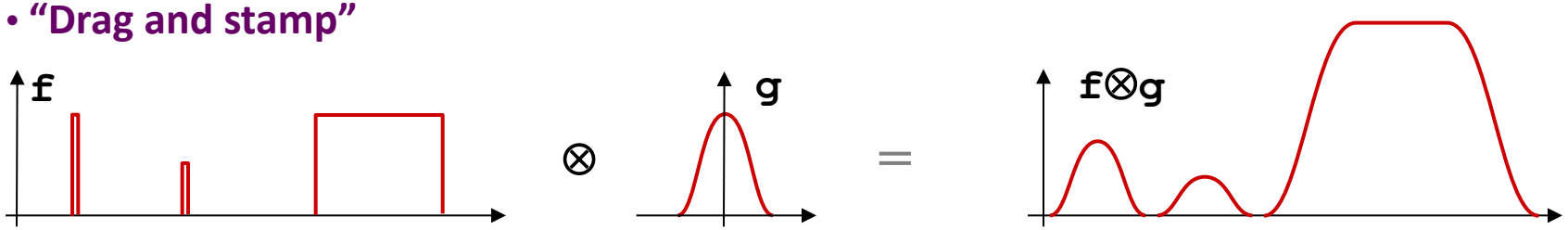
then $\tilde{h}(k) = \tilde{f}(k)\, \tilde{g}(k)$

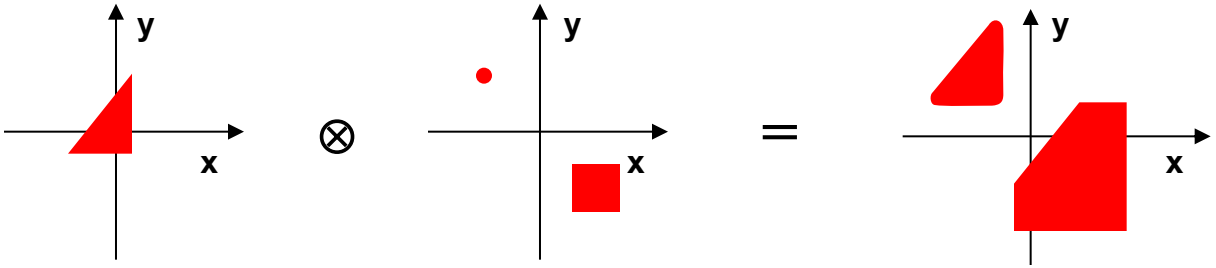
A convolution in real space becomes a product in frequency space & vice versa

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

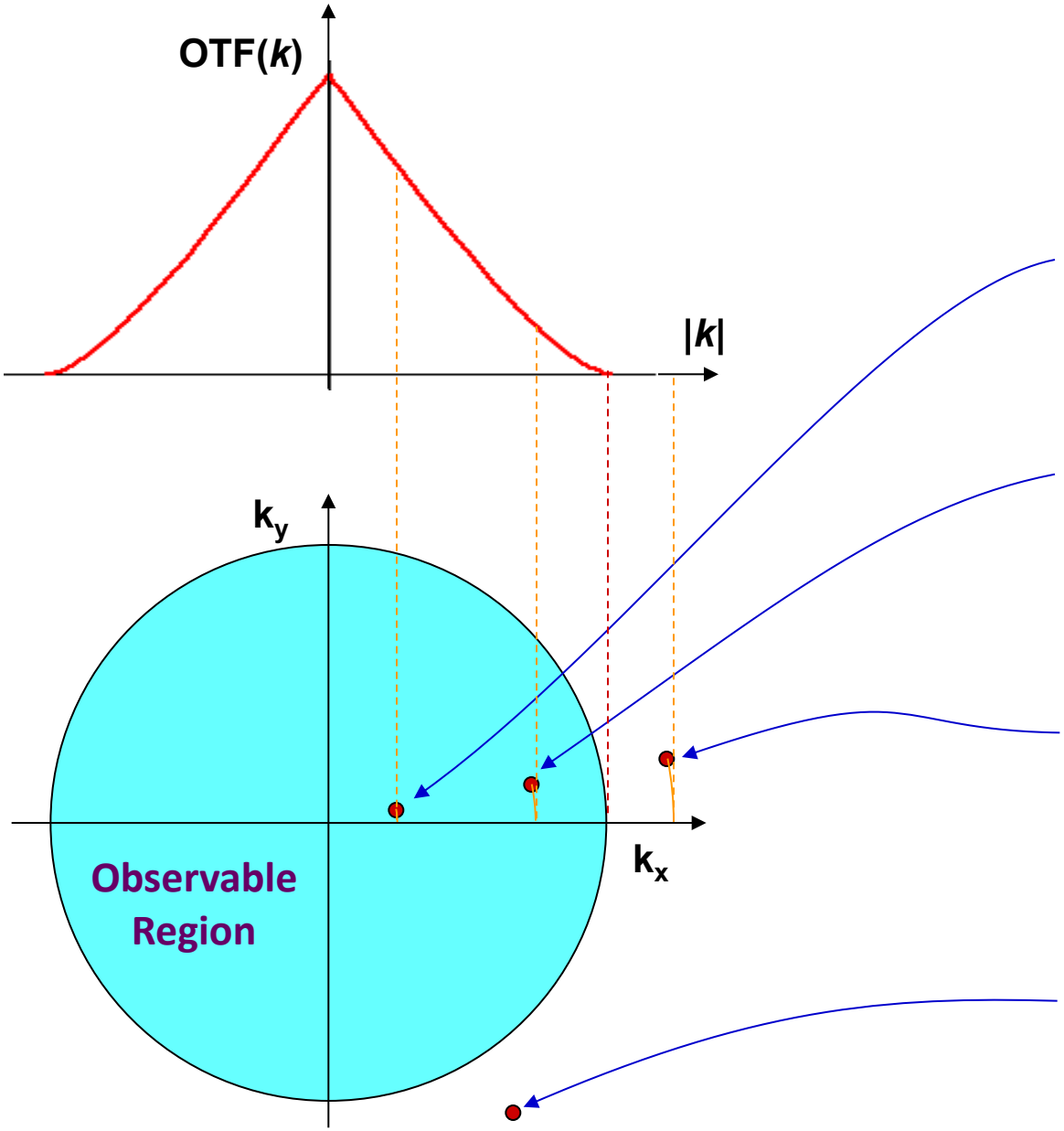
So what is a convolution, intuitively?

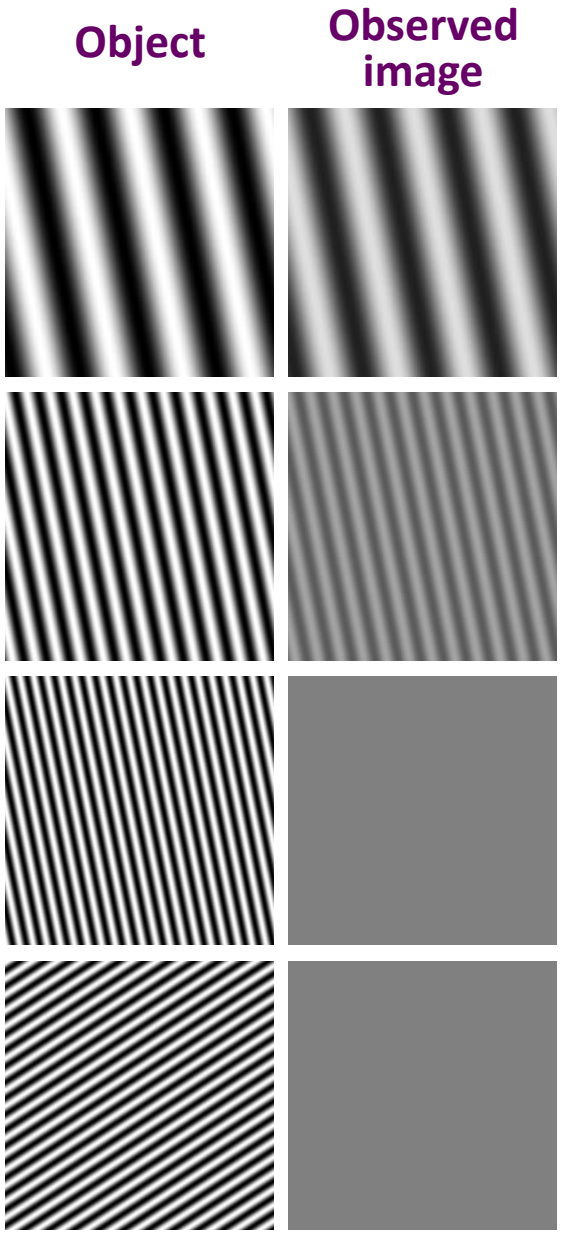
- "Blurring"
- "Drag and stamp"



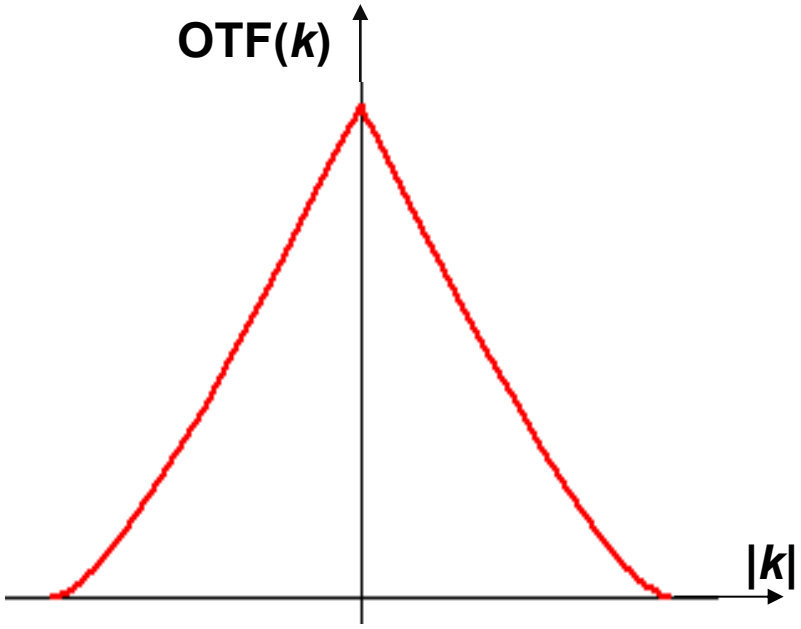


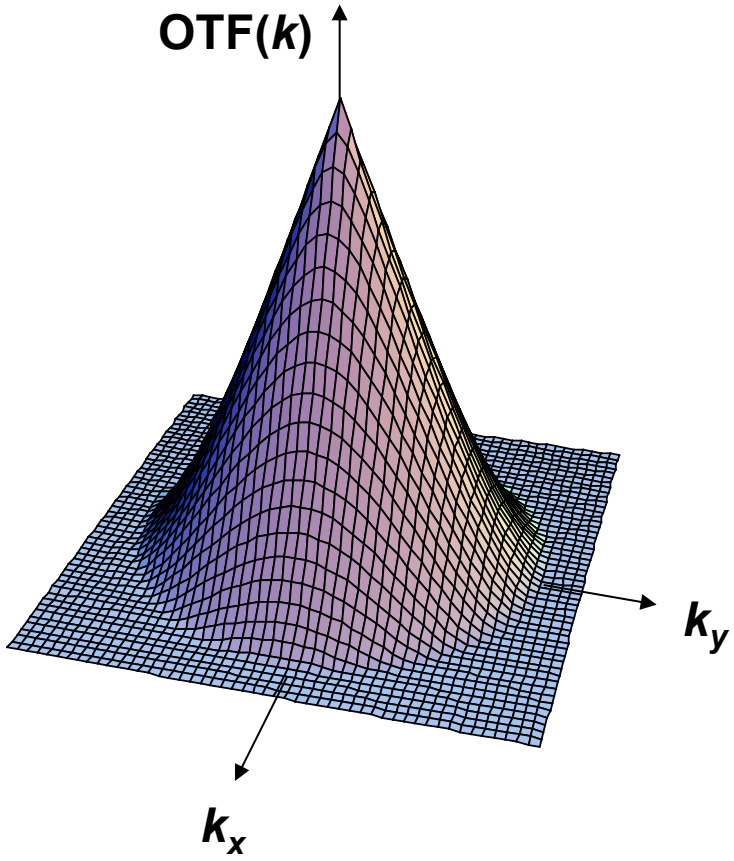
The Transfer Function Lives in Frequency Space





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

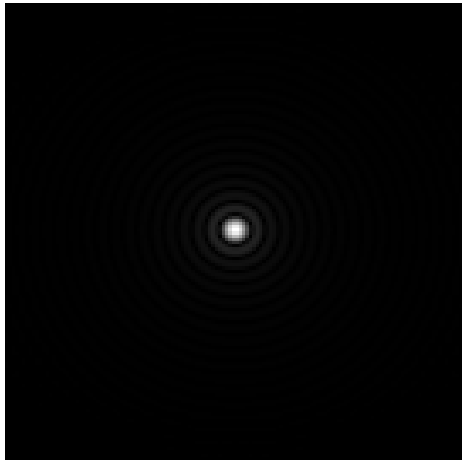




(Idealized calculations)

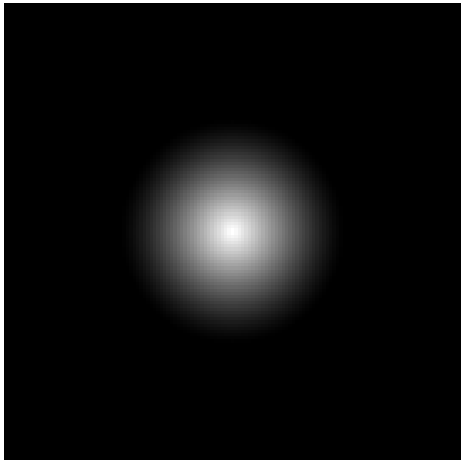
The 3D OTF

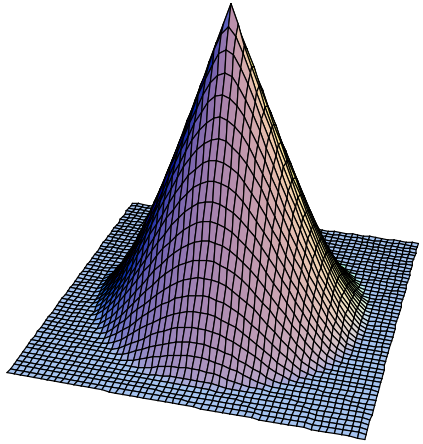
2D PSF



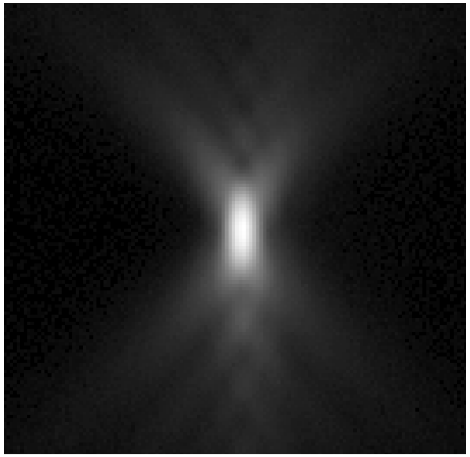
2D F.T.

2D OTF



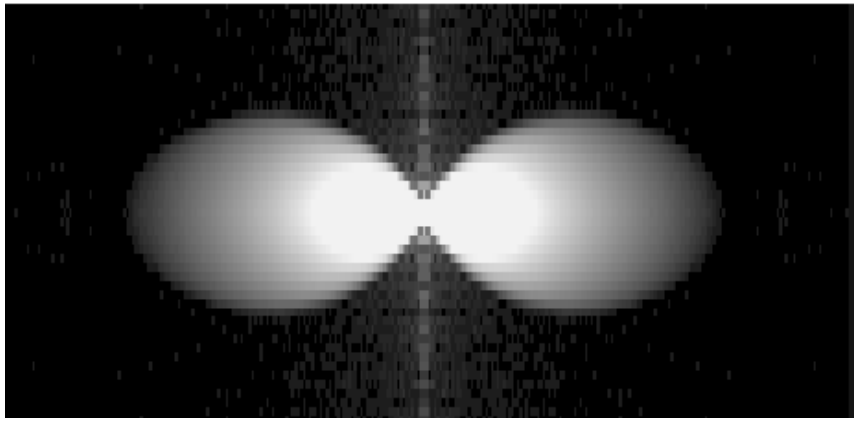


3D PSF

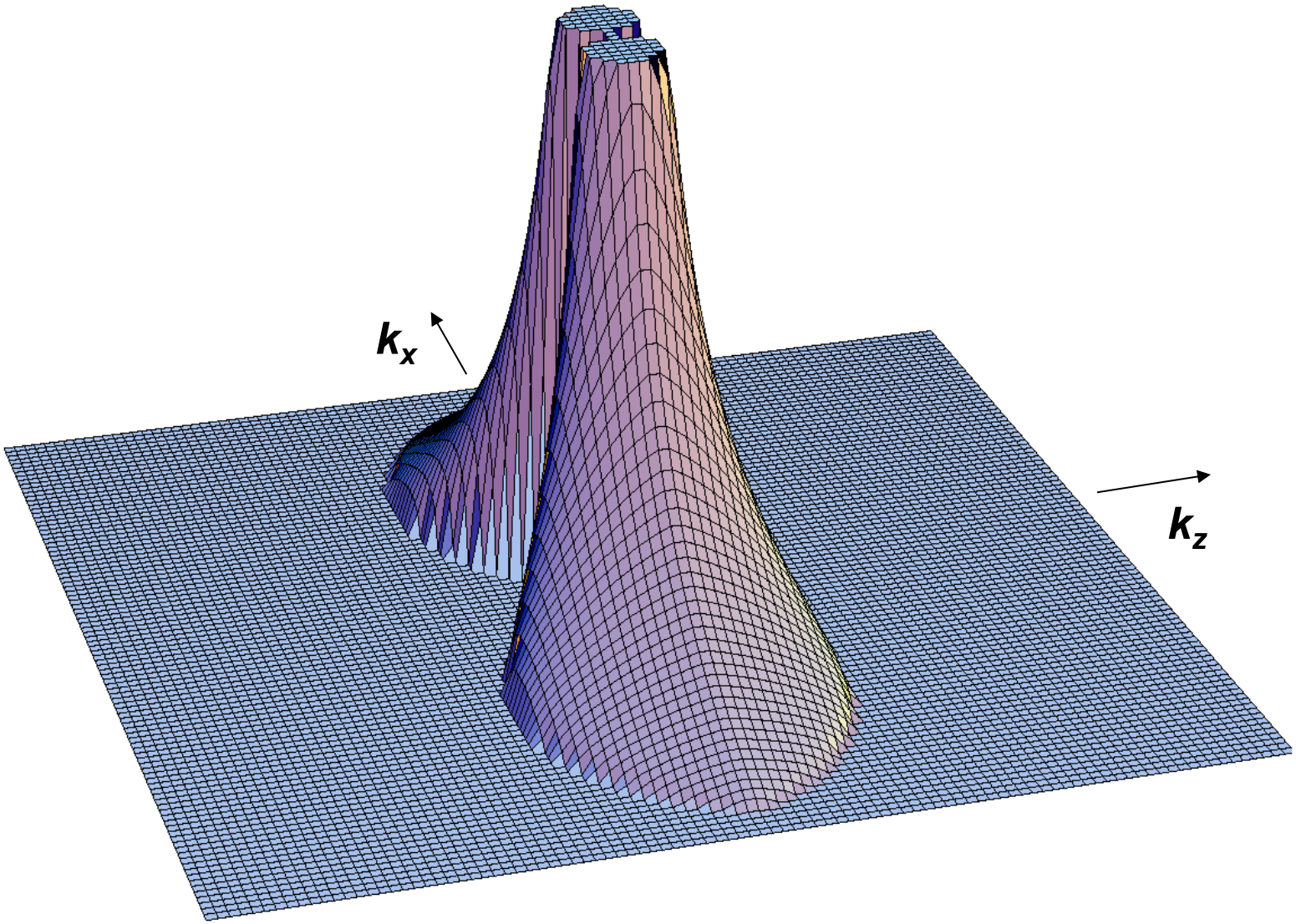


3D F.T.

3D OTF



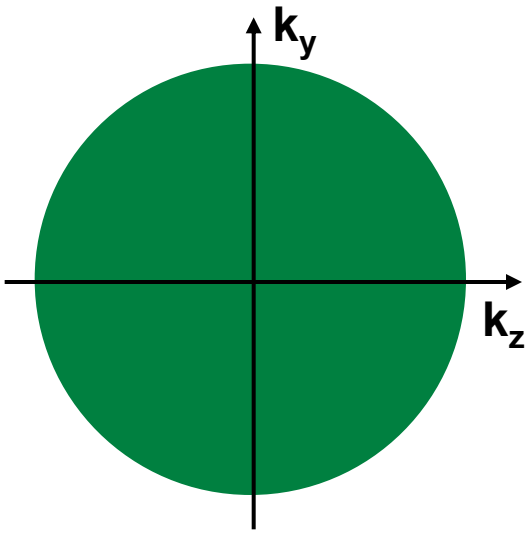
Values of the 3D OTF

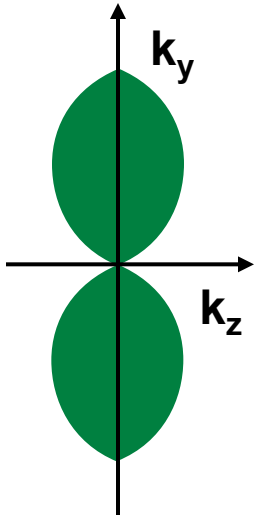


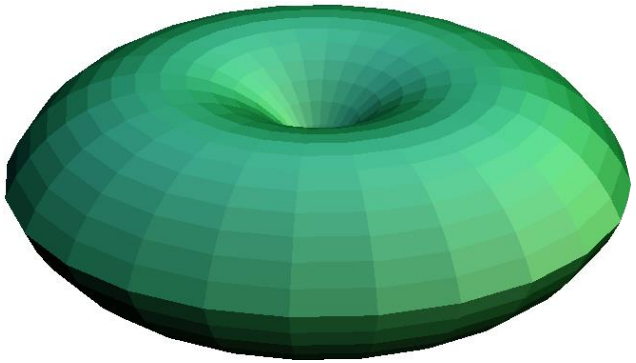
3D Observable Region

= OTF support

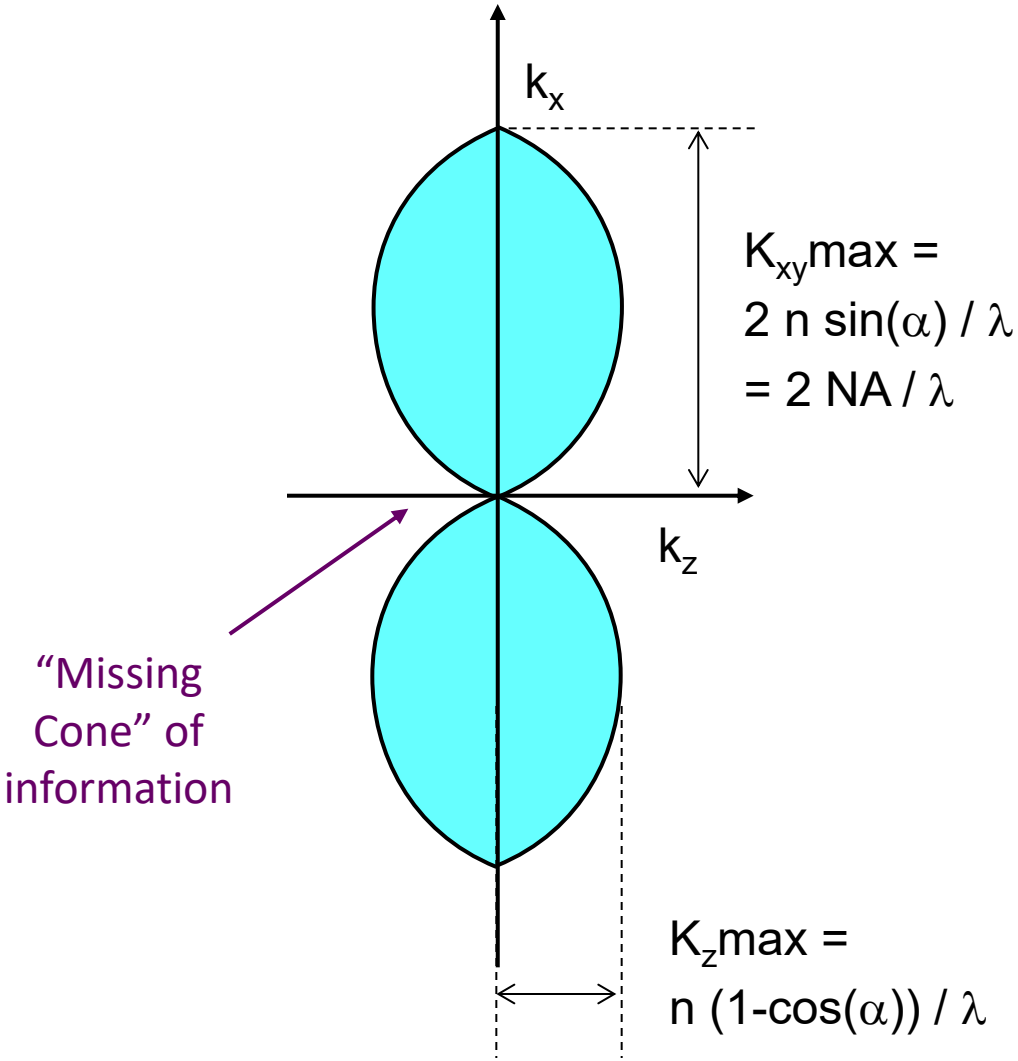
= Region where the OTF is non-zero



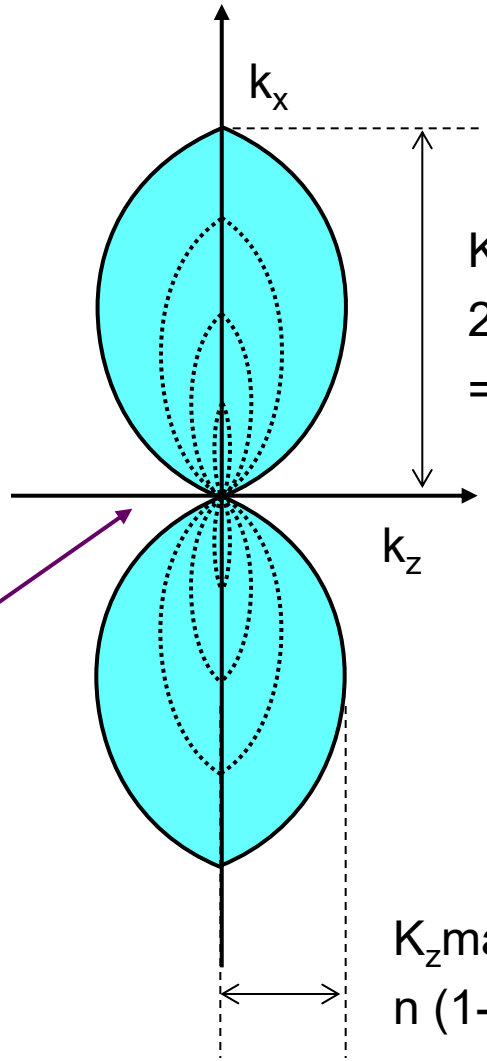




So what is the resolution?



So what is the resolution?



$K_{xy}max =$
$2\ n\ \sin(\alpha) / \lambda$
$= 2\ NA / \lambda$

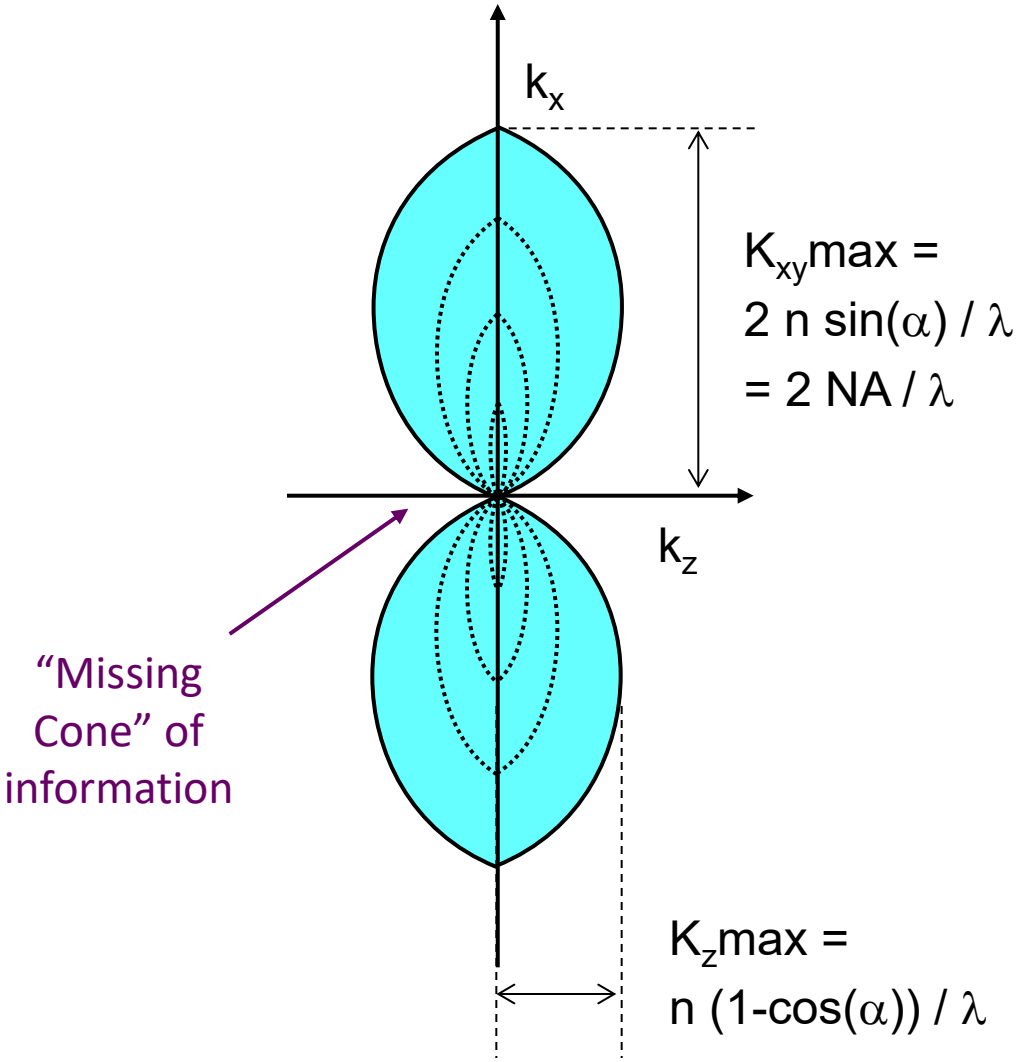
"Missing Cone" of information

$K_z max =$
$n\ (1-\cos(\alpha)) / \lambda$

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

NA = 1.4
n=1.515
→ α = 67.5°
λ = 600 nm

→

Lateral (XY) resolution:
$1/ K_{xy}max$ = 0.21 µm

Axial (Z) resolution:
$1/ K_z max$ = 0.64 µm

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