# Principles & Practice of Light Microscopy: 2
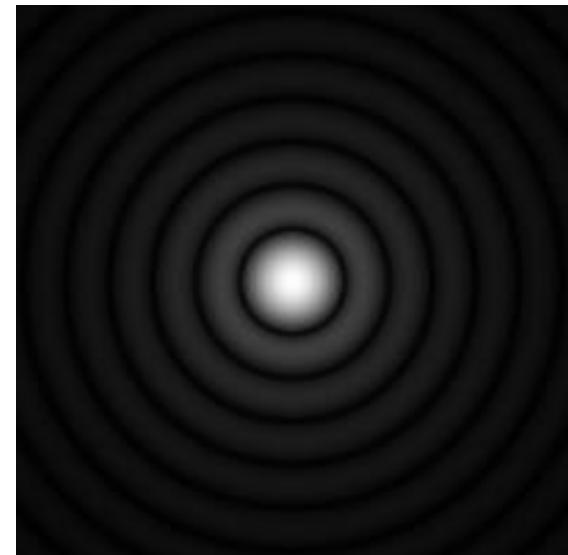

(Image: T. Wittman, Scripps)
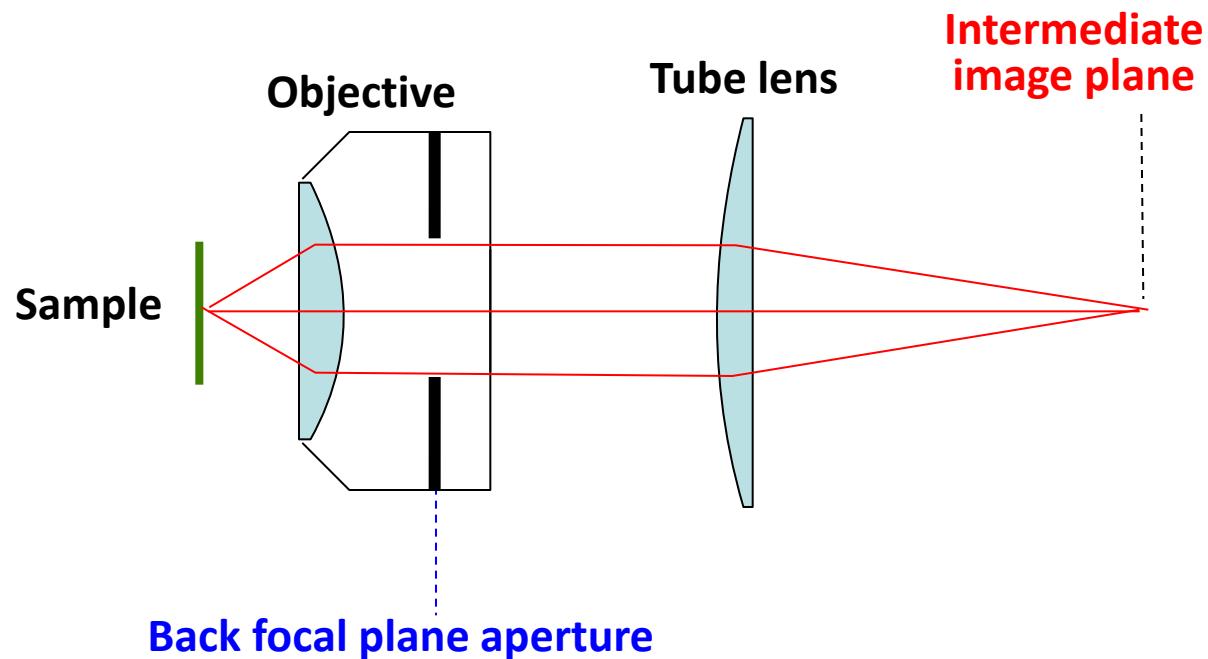
Aperture and Resolution
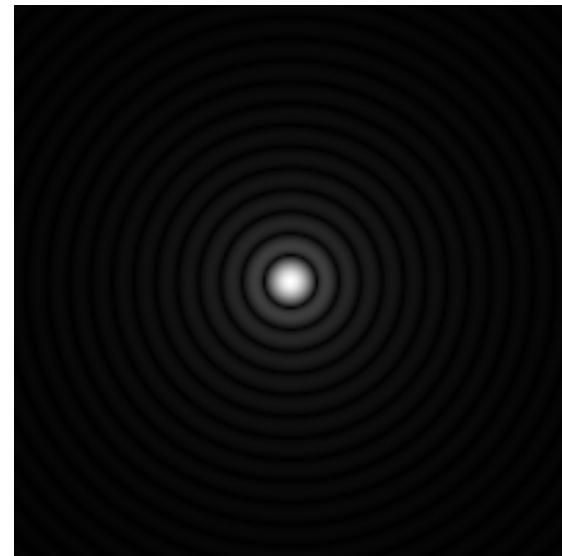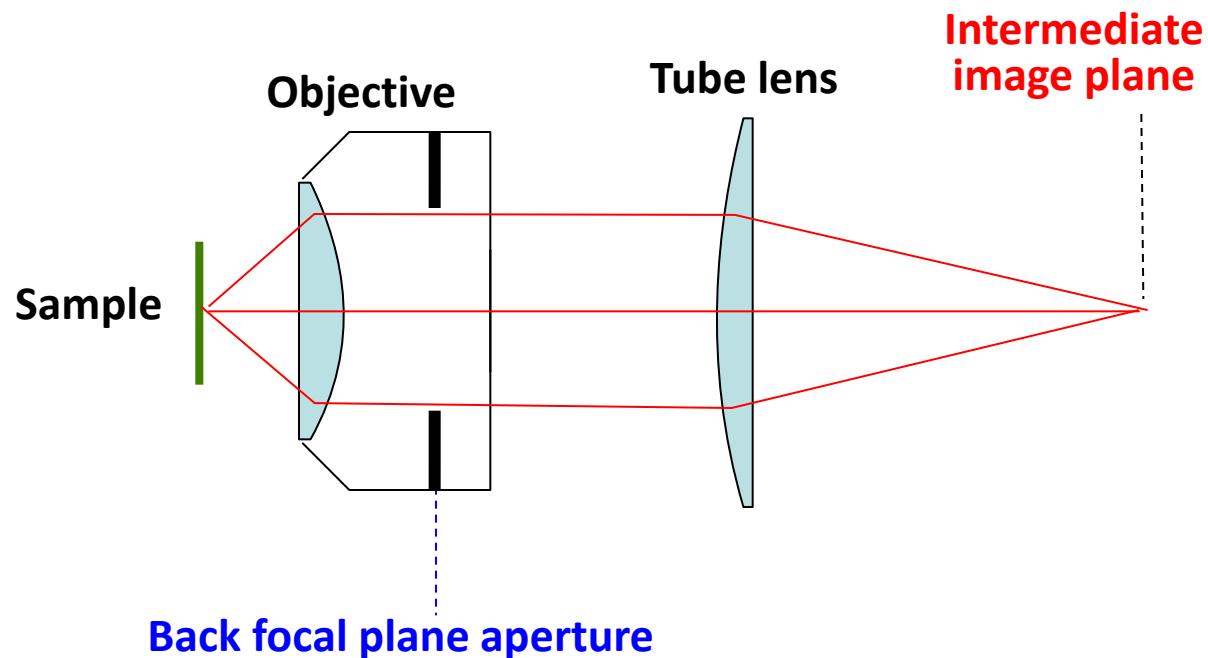
Objective

Tube lens

Intermediate image plane

Sample

Back focal plane aperture

Diffraction spot on image plane = *Point Spread Function*

Aperture and Resolution

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

Objective

Tube lens

**Intermediate
image plane**

Sample

Back focal plane aperture

Aperture and Resolution

Objective

Tube lens

Intermediate image plane

Sample

Back focal plane aperture

Diffraction spot on image plane = *Point Spread Function*

Aperture and Resolution

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

Objective **Tube lens** **Intermediate image plane**

Sample α

**Back focal plane aperture**
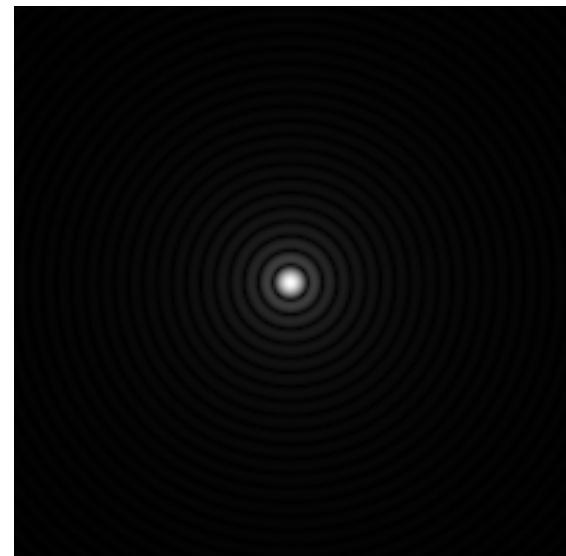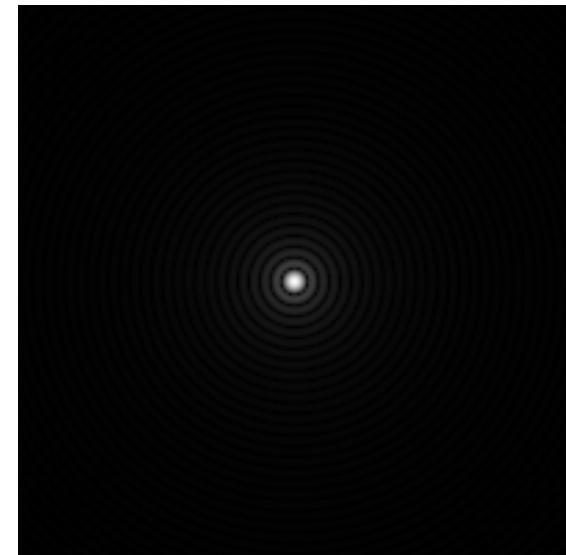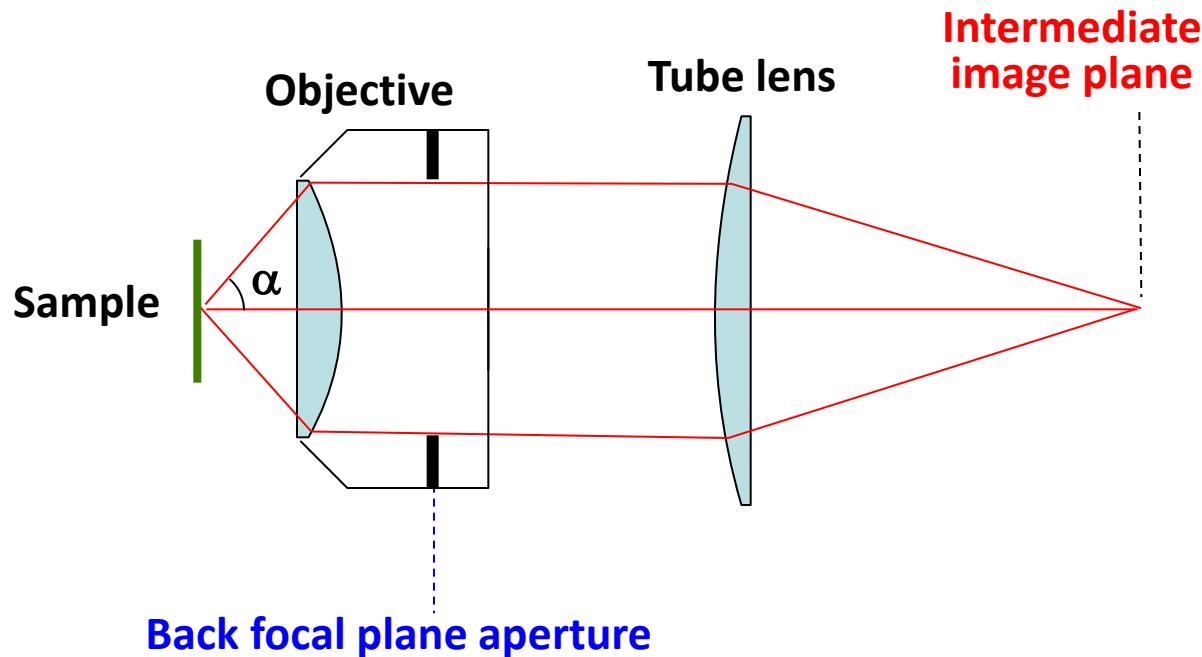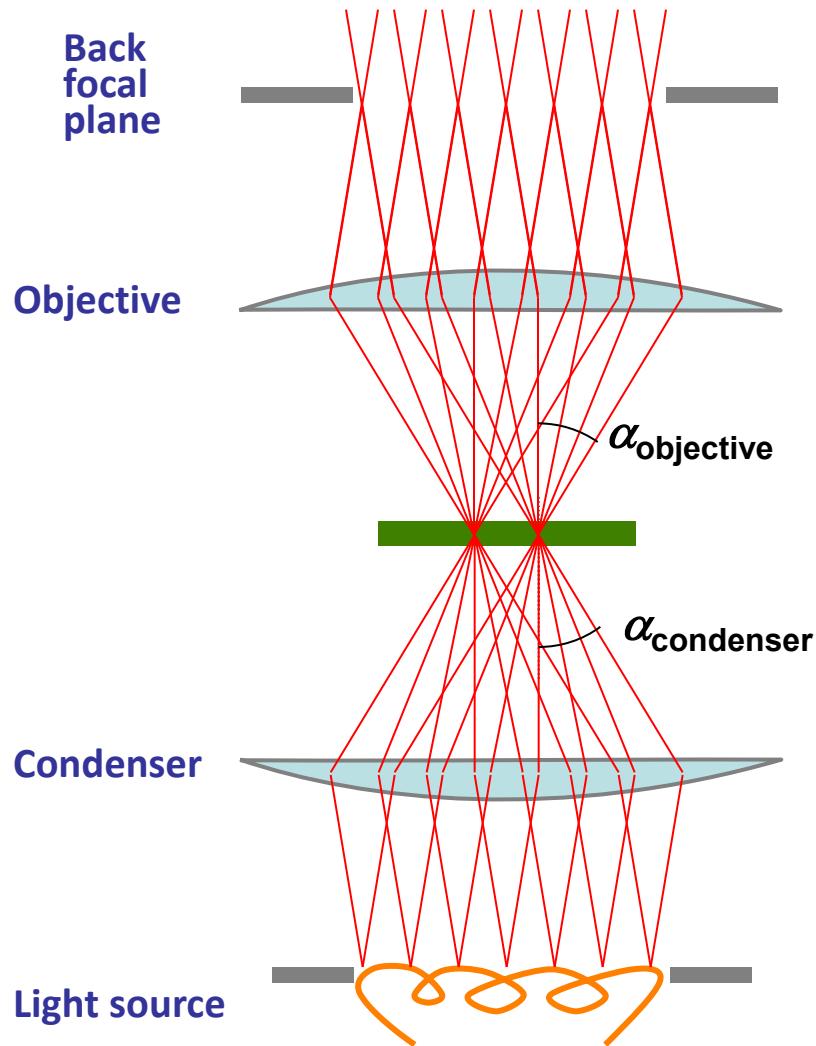
- **Image resolution improves with Numerical Aperture (NA)**

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

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"

Back focal plane

Objective

$\alpha_{\text{objective}}$

$\alpha_{\text{condenser}}$

Condenser

**Light source**

For the highest resolution,
we need to have
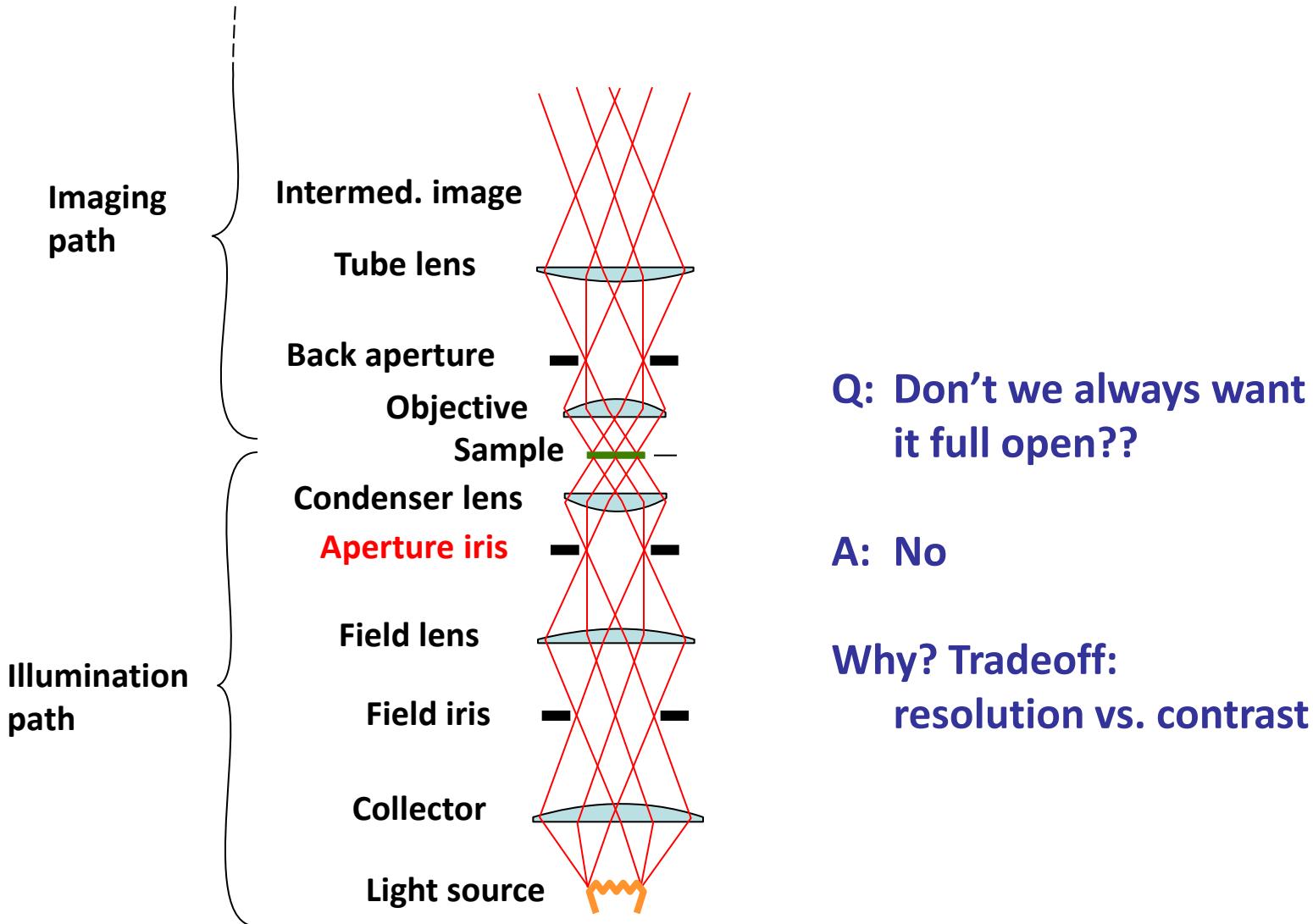
$\alpha_{\text{condenser}} \geq \alpha_{\text{objective}}$

$NA_{\text{condenser}} \geq NA_{\text{objective}}$
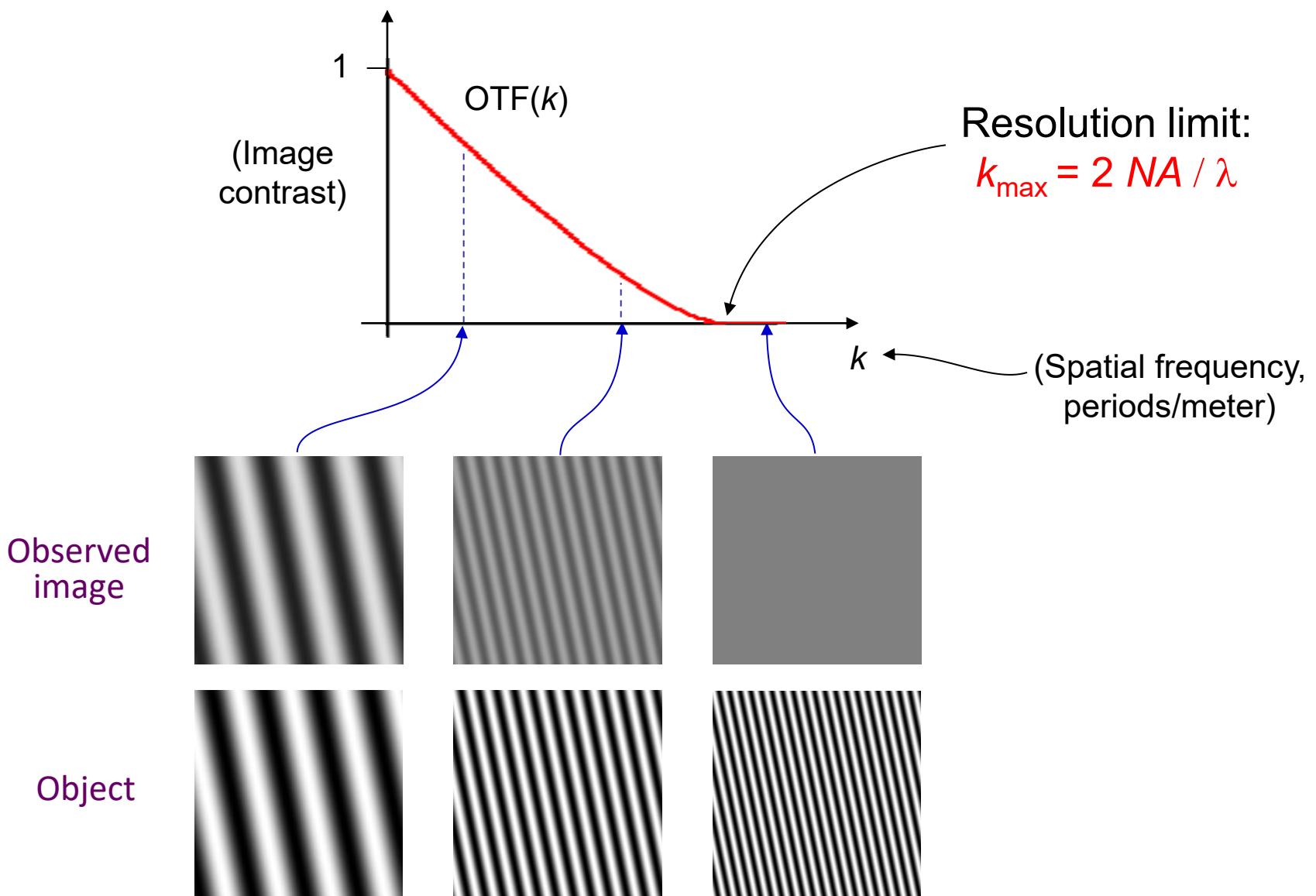
$\Rightarrow$ with oil immersion objectives,
we need an *oil immersion condenser!*

Aperture, Resolution & Contrast
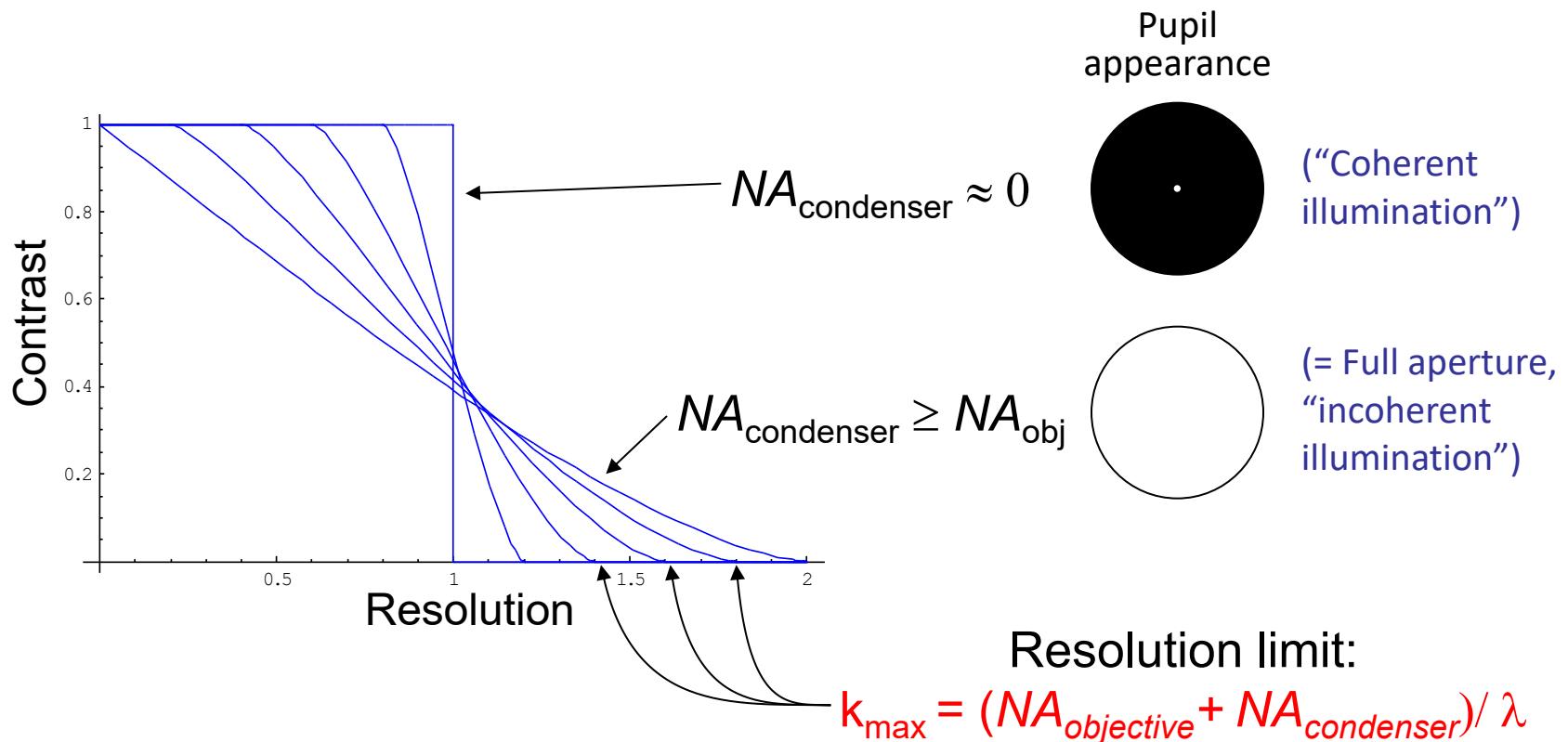
Can adjust the condenser NA with the aperture iris

Imaging path

Illumination path

Intermed. image

Tube lens

Back aperture

Objective

Sample

Condenser lens

Aperture iris

Field lens

Field iris

Collector

Light source

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

A: No

Why? Tradeoff:
  resolution vs. contrast

# Spatial frequencies & the Optical Transfer Function (OTF)

Resolution limit:
$k_{max} = 2\ NA\ /\ \lambda$

OTF(k)

(Image contrast)

k (Spatial frequency, periods/meter)

Observed image

Object
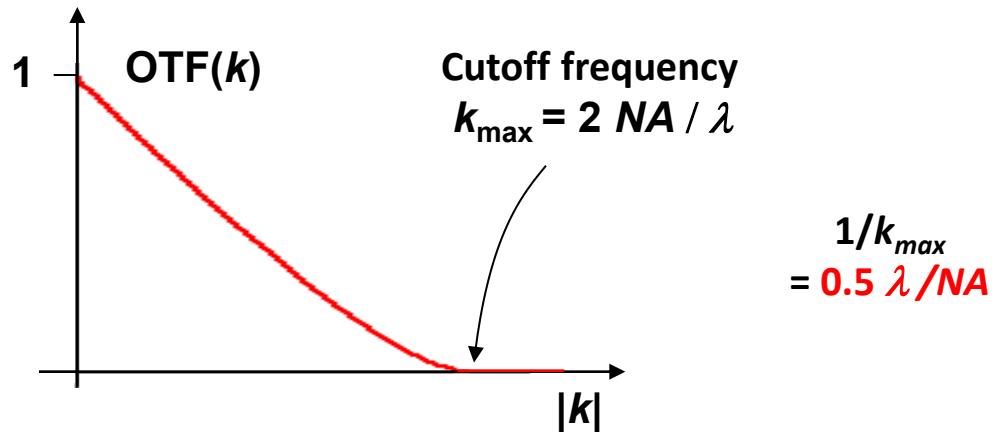
Resolution & Contrast vs. Illumination aperture

$NA_{\text{condenser}} \approx 0$

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

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
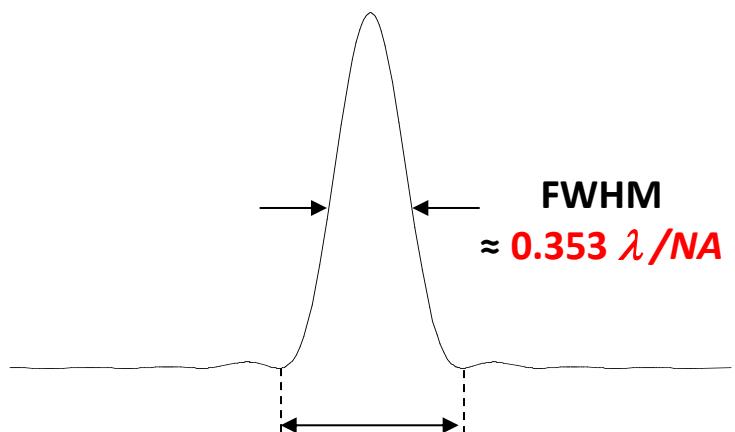
Definitions of Resolution

As the OTF cutoff frequency

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

OTF(k)

1

Cutoff frequency
$$k_{max} = 2\,NA/\lambda$$

$|k|$

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

FWHM
$$\approx 0.353\,\lambda/NA$$

As the diameter of the Airy disk
(first dark ring of the PSF)
= "Rayleigh criterion"
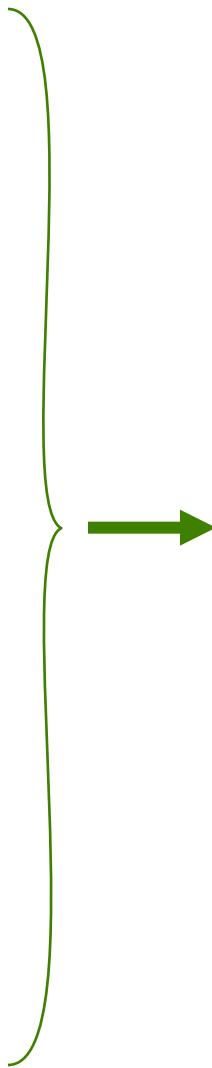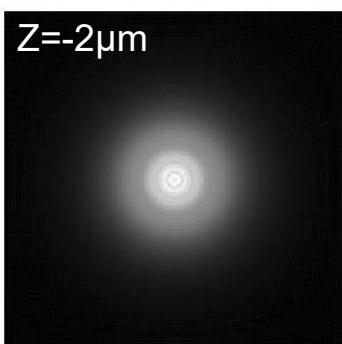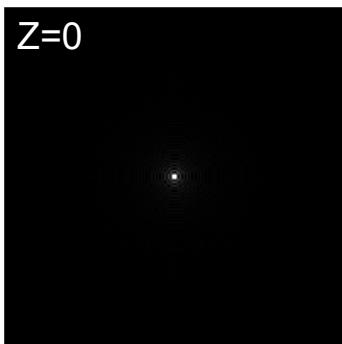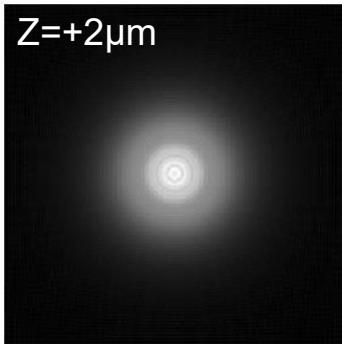
Airy disk diameter
$$\approx 0.61\,\lambda/NA$$

The 3D Point Spread Function (PSF)
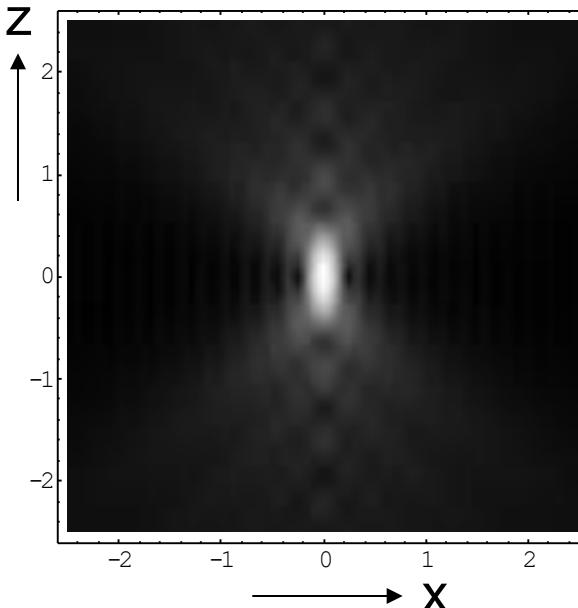
## The image of a point object

**2D PSF
for different defocus**

Z=+2μm

Z=0

Z=-2μm

y

x
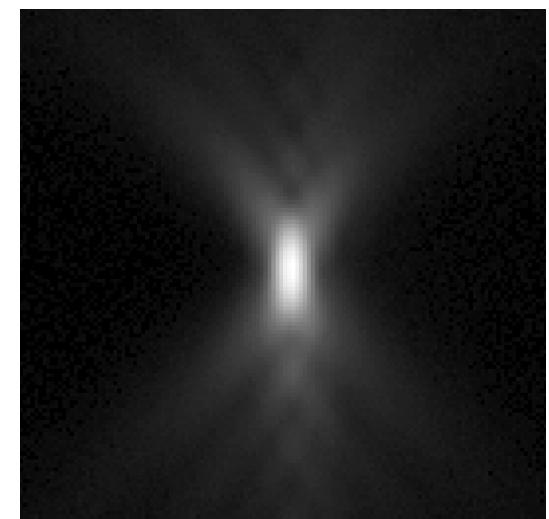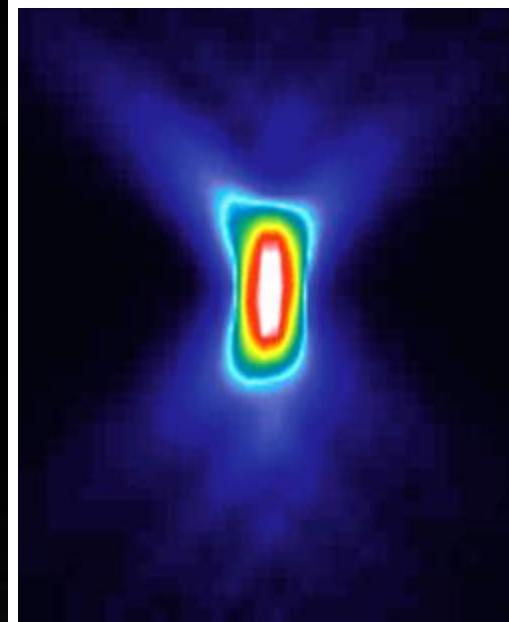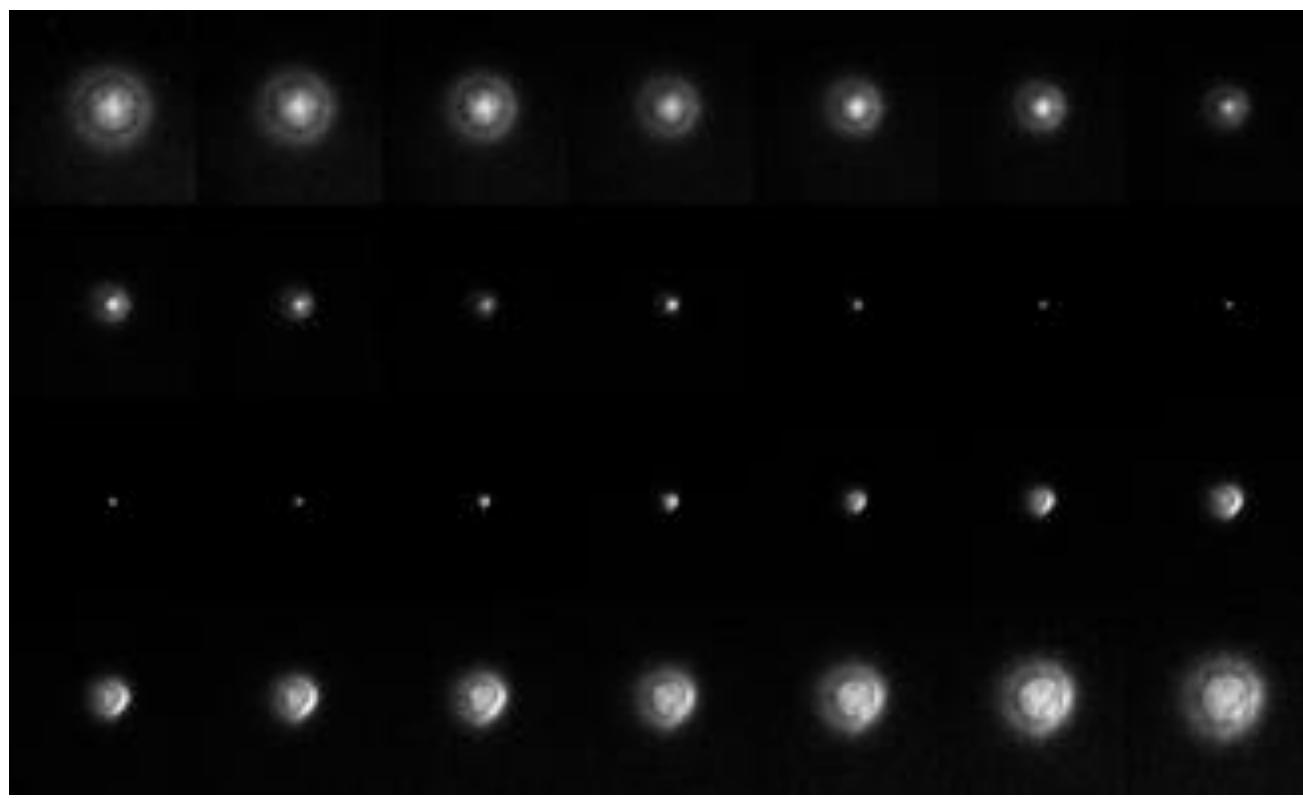
3D PSF

Calculated

Measured

Z

2

1

0

−1

−2

−2   −1   0   1   2

x
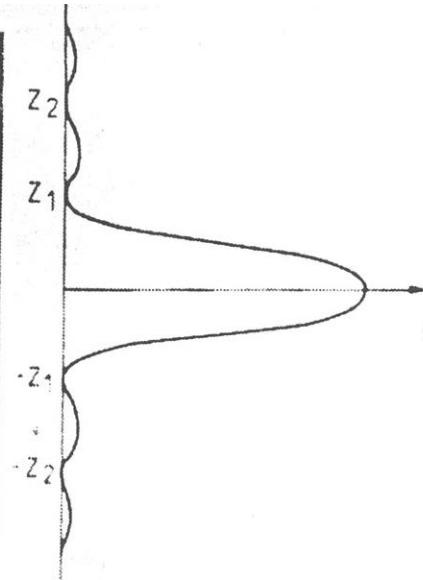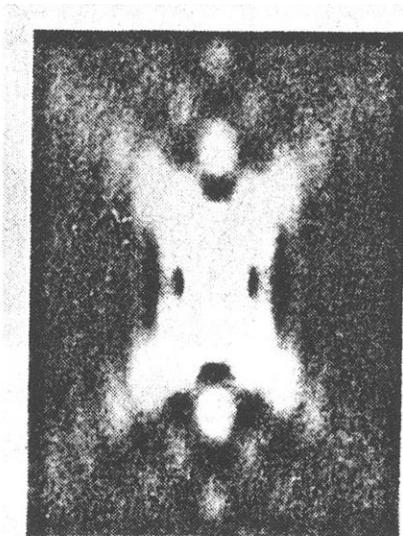
X - Z

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
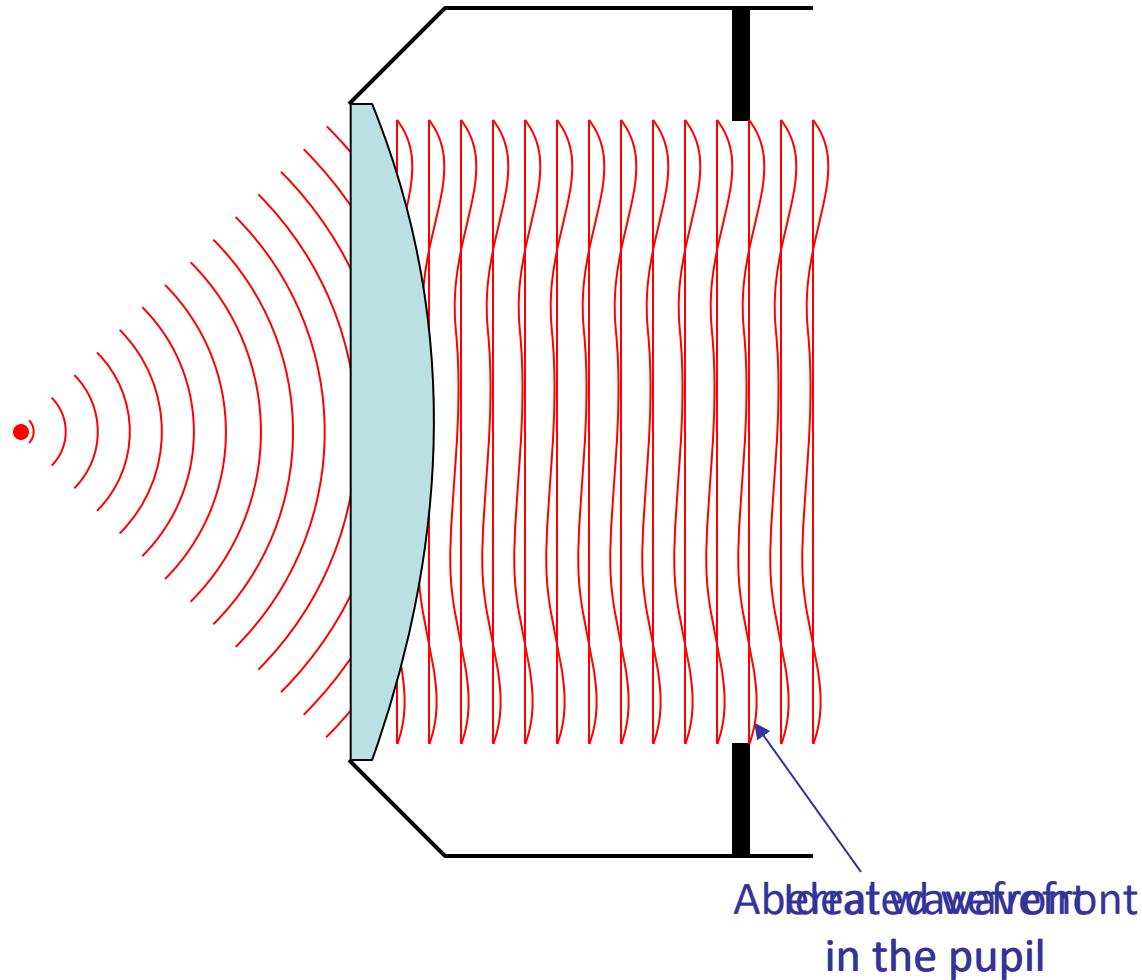
Aberrations

They are the enemy
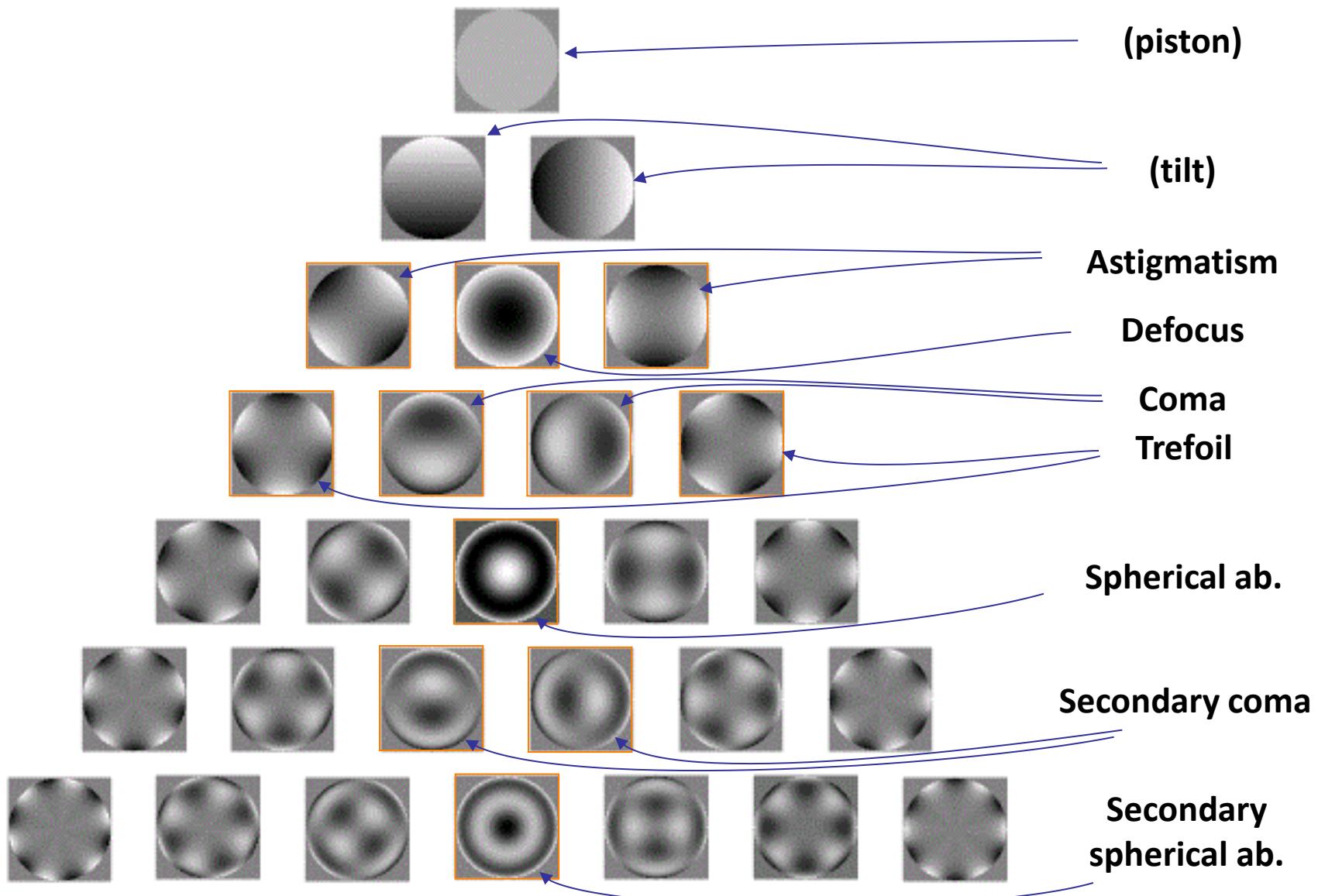
Aberrations

- Chromatic aberrations
  Longitudinal chr. Ab.
  Lateral chr. Ab.

- Wavefront aberrations
 Spherical aberration
 Astigmatism
  Coma
  …

- Curvature of field

- Distortion

Wavefront Aberrations

Aberrated wavefront
in the pupil

Wavefront Aberrations

(piston)

(tilt)

Astigmatism

Defocus

Coma

Coma

Trefoil

Spherical ab.

Secondary coma

Secondary spherical ab.

PSF Aberrations

(piston)

(tilt)

Astigmatism

Defocus

Coma

Coma
Trefoil

Spherical ab.

Secondary coma

Secondary
spherical ab.

Spherical Aberration

Longitudinal and Transverse Spherical Aberration
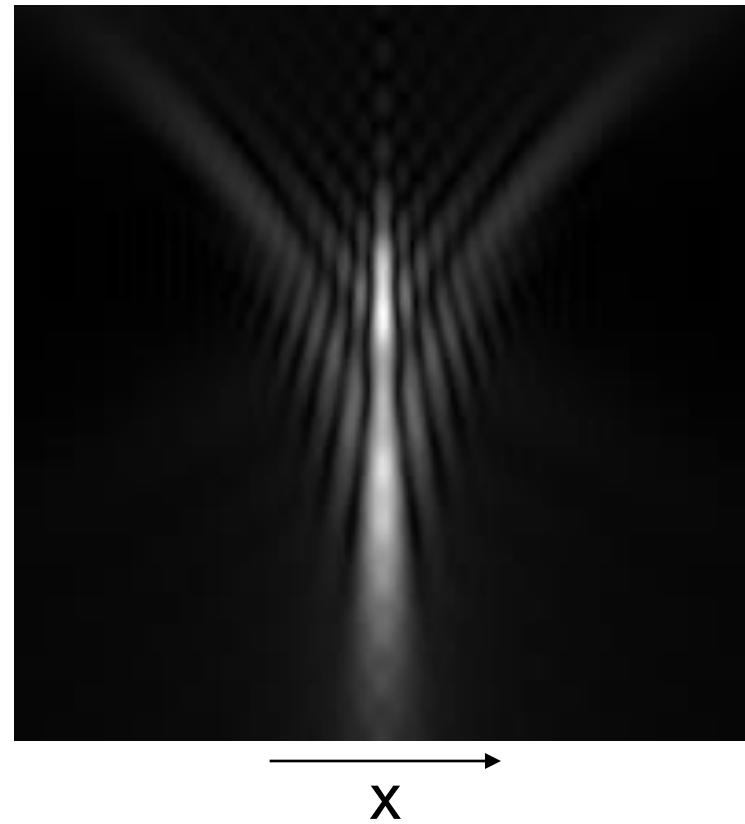
Figure 1

Spherical Aberration

Point spread functions

Ideal

1 wave of spherical ab

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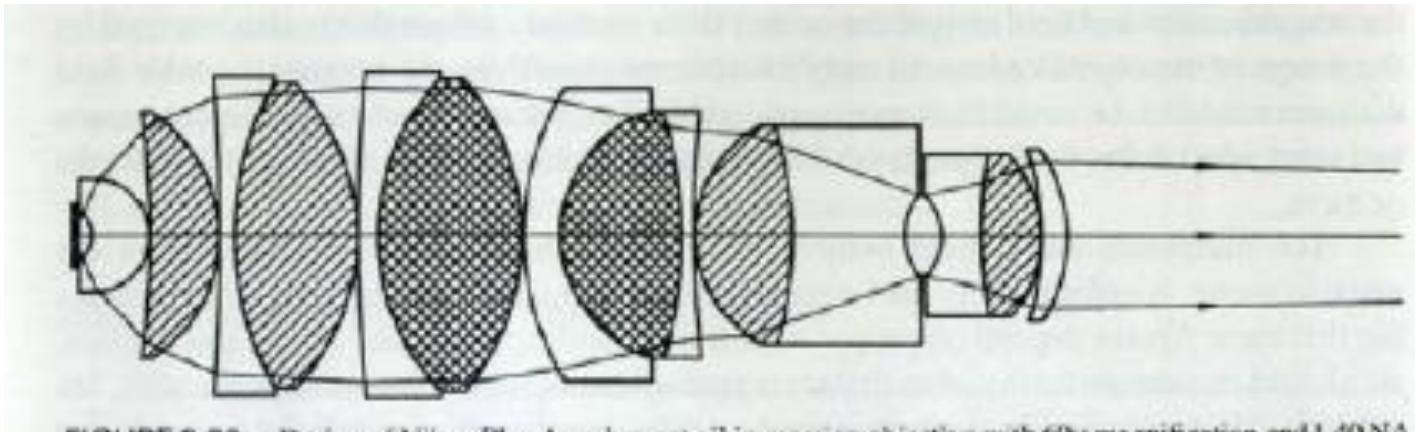
FIGURE 2-36.   Design of Nikon Plan Apochromat oil immersion objective with 60× magnification and 1.40 NA

- 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
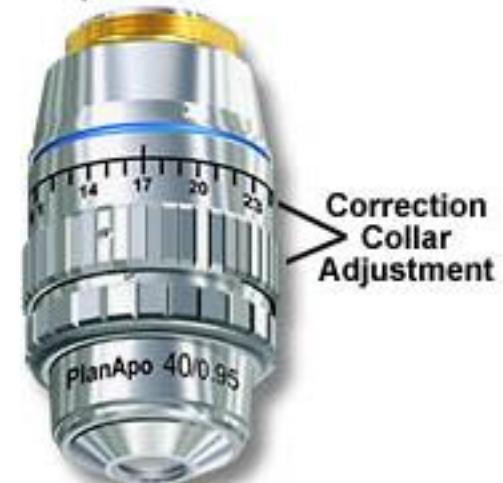
Temperature variation

Cover slip thickness
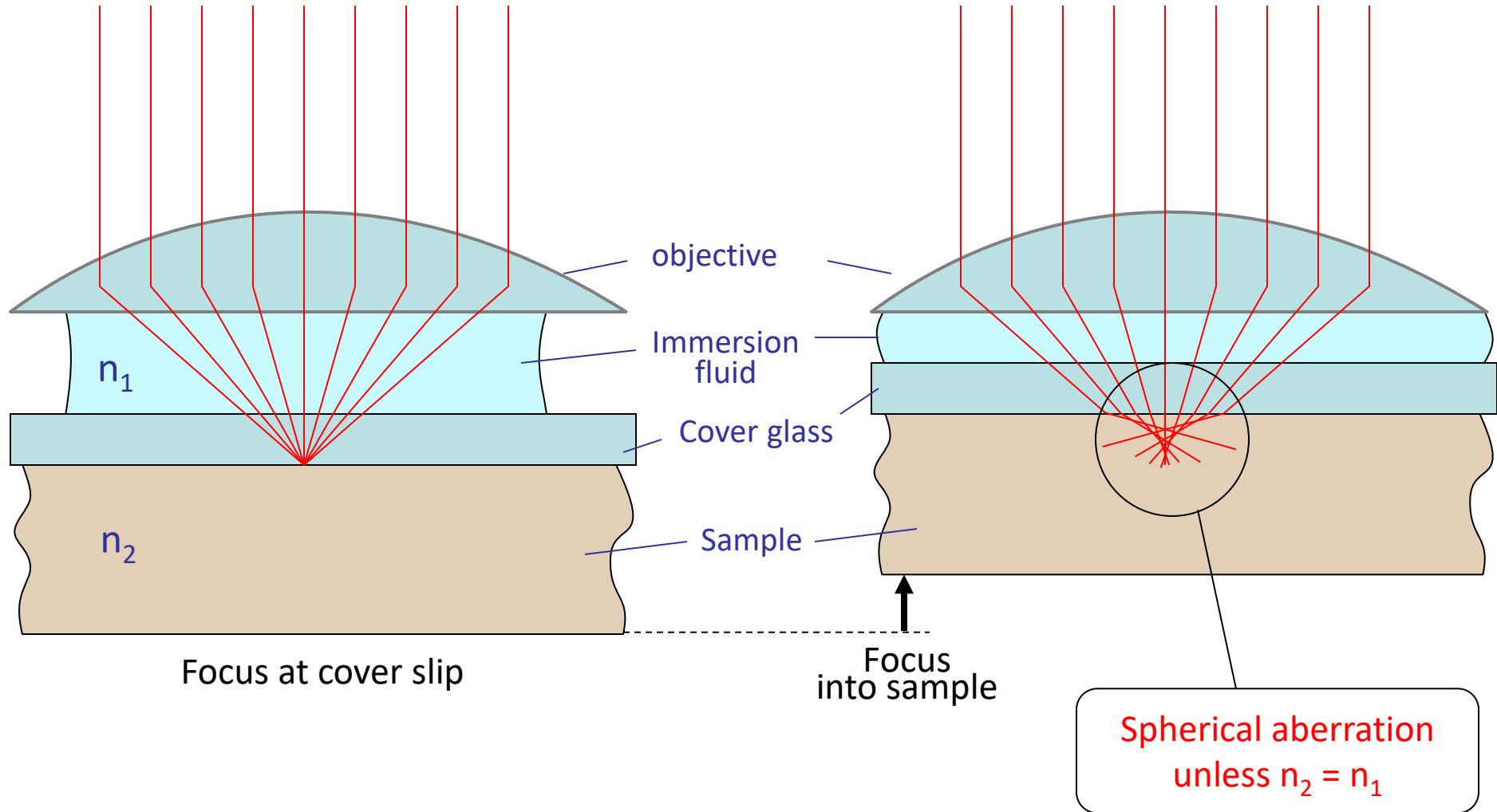
    (high-NA objectives except oil immersion)

Correction collar setting
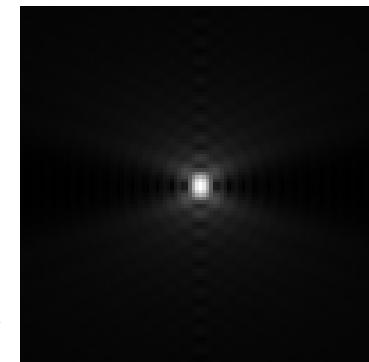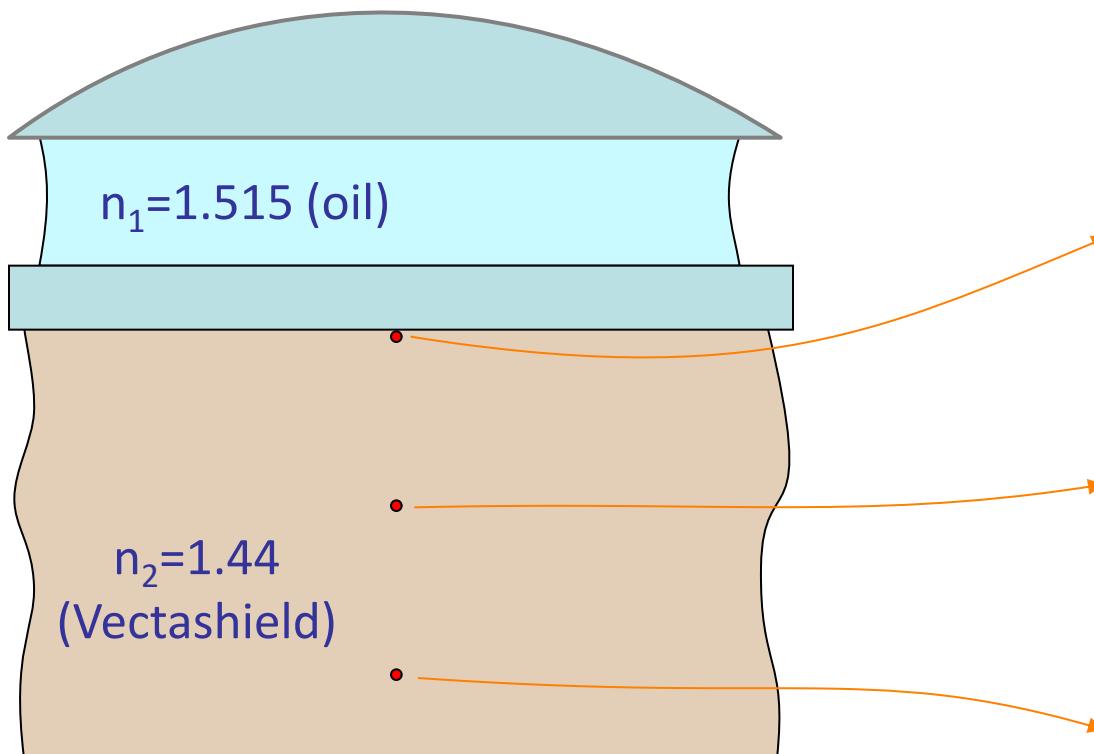
Sample refractive index mismatch
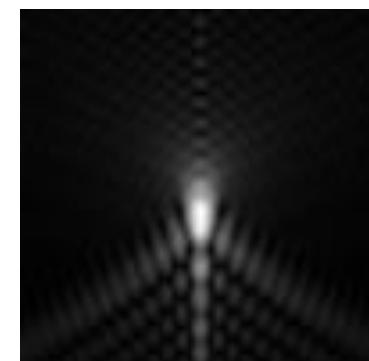

Correction Collar Adjustment

Index Mismatch
& Spherical Aberration

objective

Immersion fluid

n_1

Cover glass

n_2

Sample

Focus at cover slip

Focus into sample

Spherical aberration unless $n_2 = n_1$

Index Mismatch
& Spherical Aberration

$n_1=1.515$ (oil)

$n_2=1.44$
(Vectashield)

z=0 µm

z=25 µm
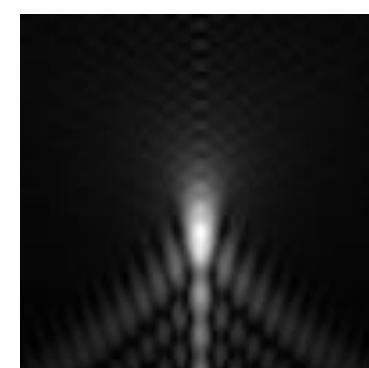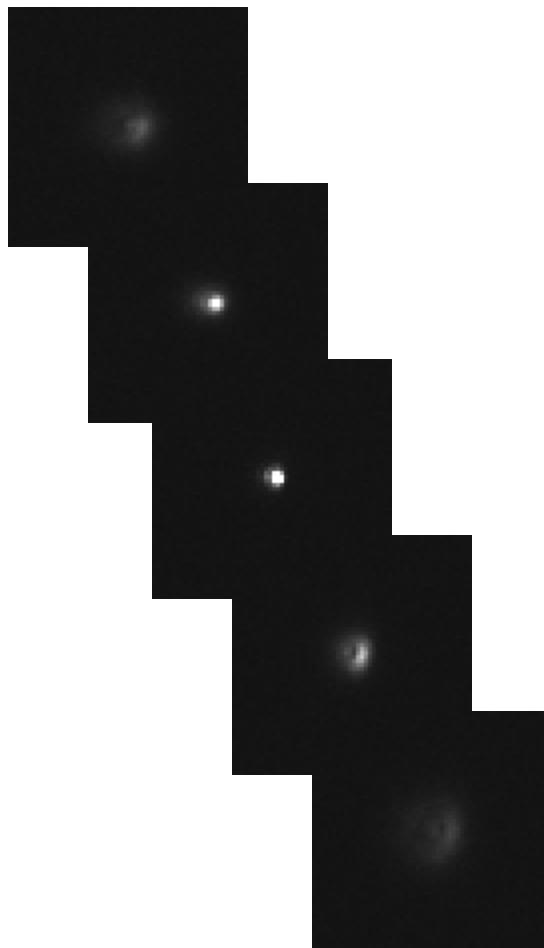
z=50 µm
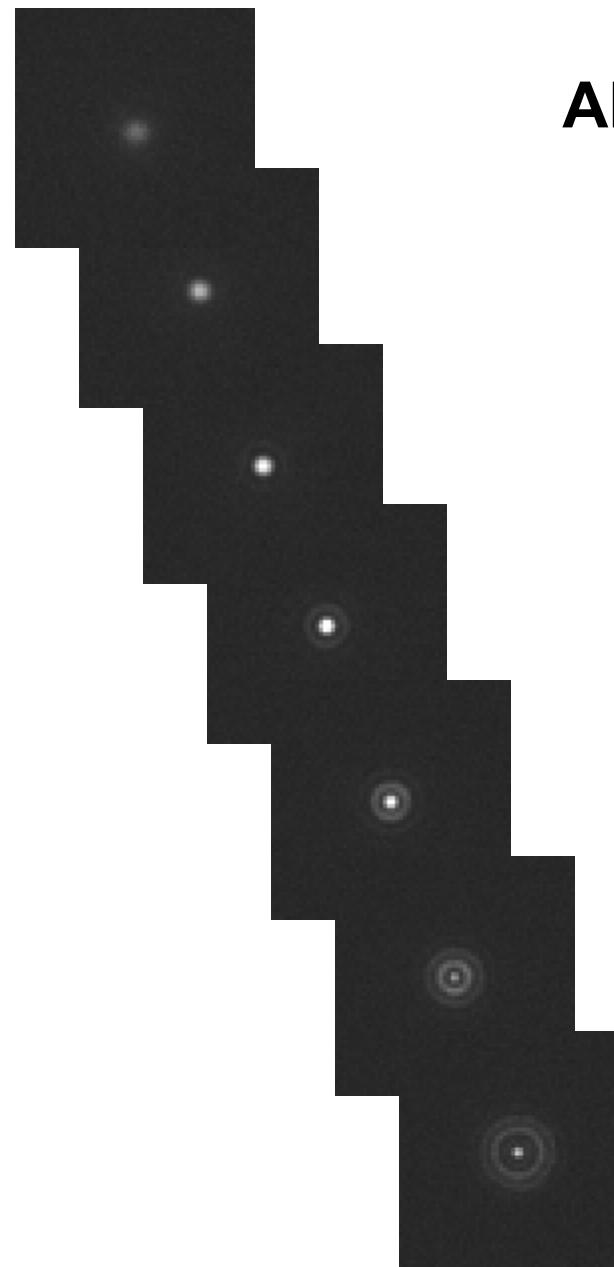
How to recognize spherical aberration

Unaberrated

Aberrated

0.5 μm steps
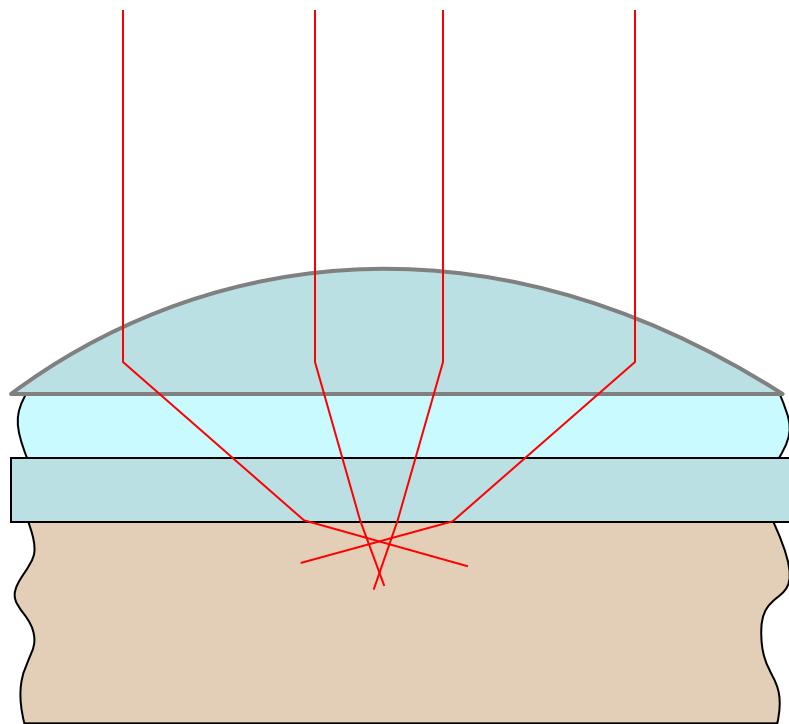
1 μm steps
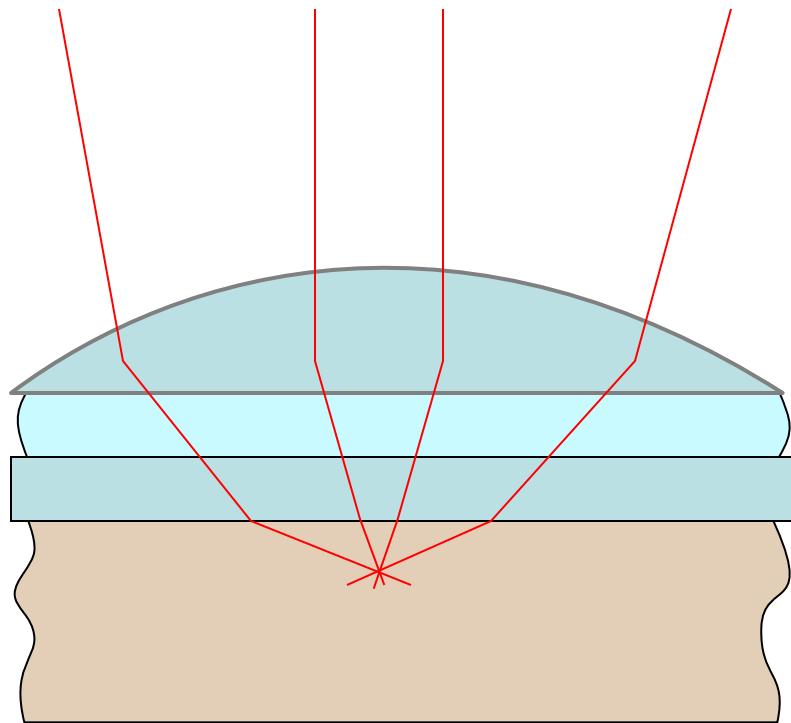
What can you do about spherical aberration?

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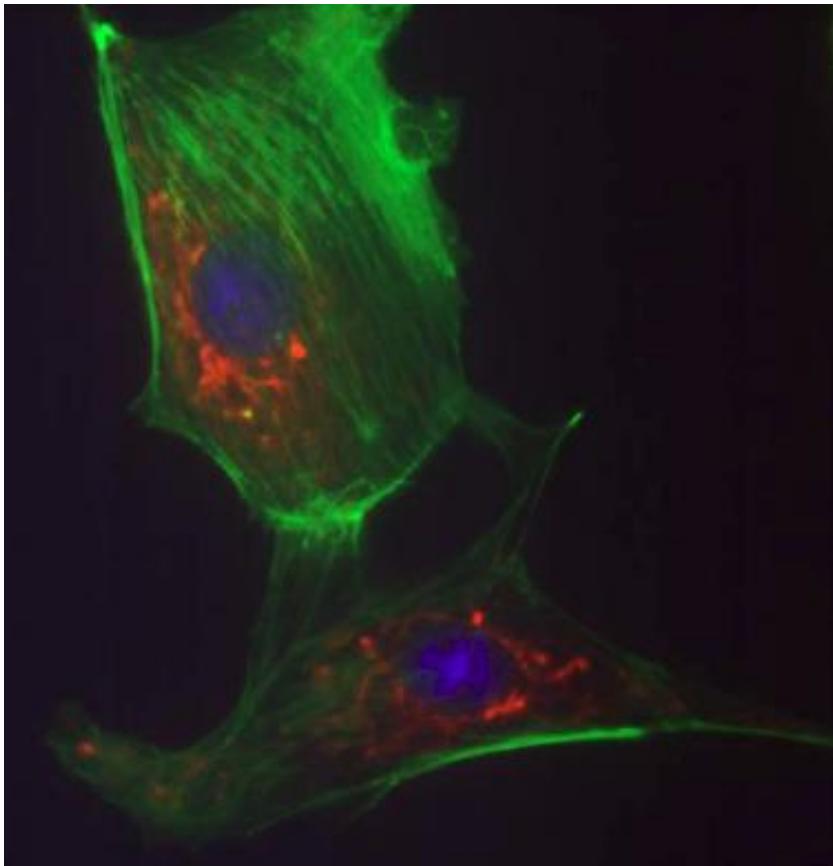
Correction collars
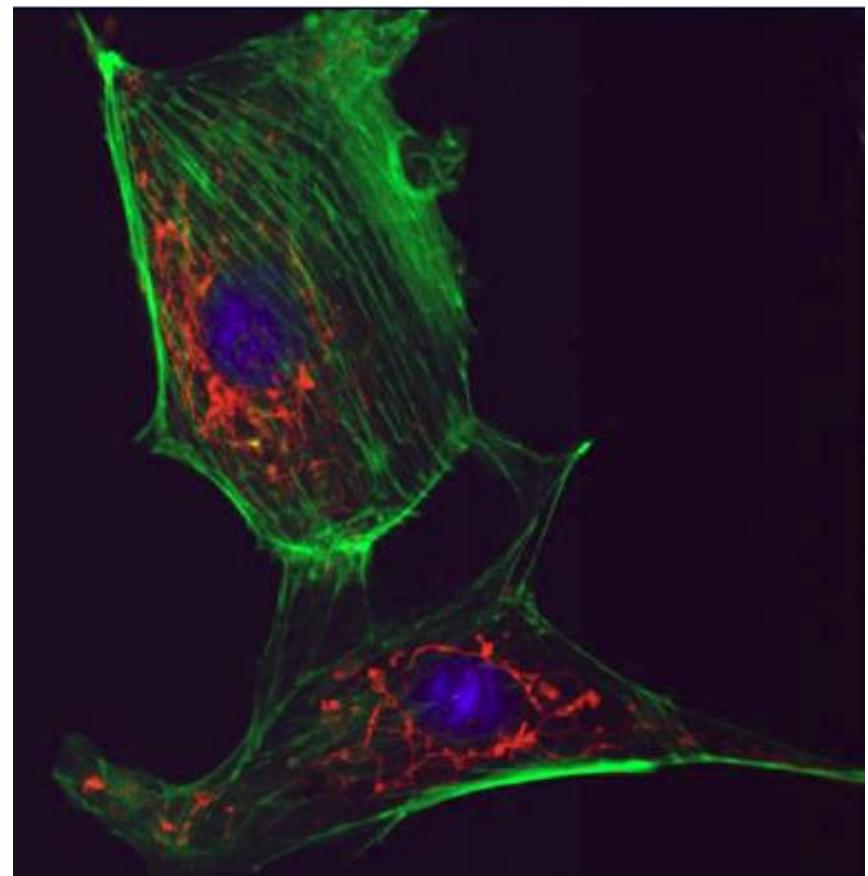
A correction collar can only eliminate spherical aberration at a single focal plane

Example
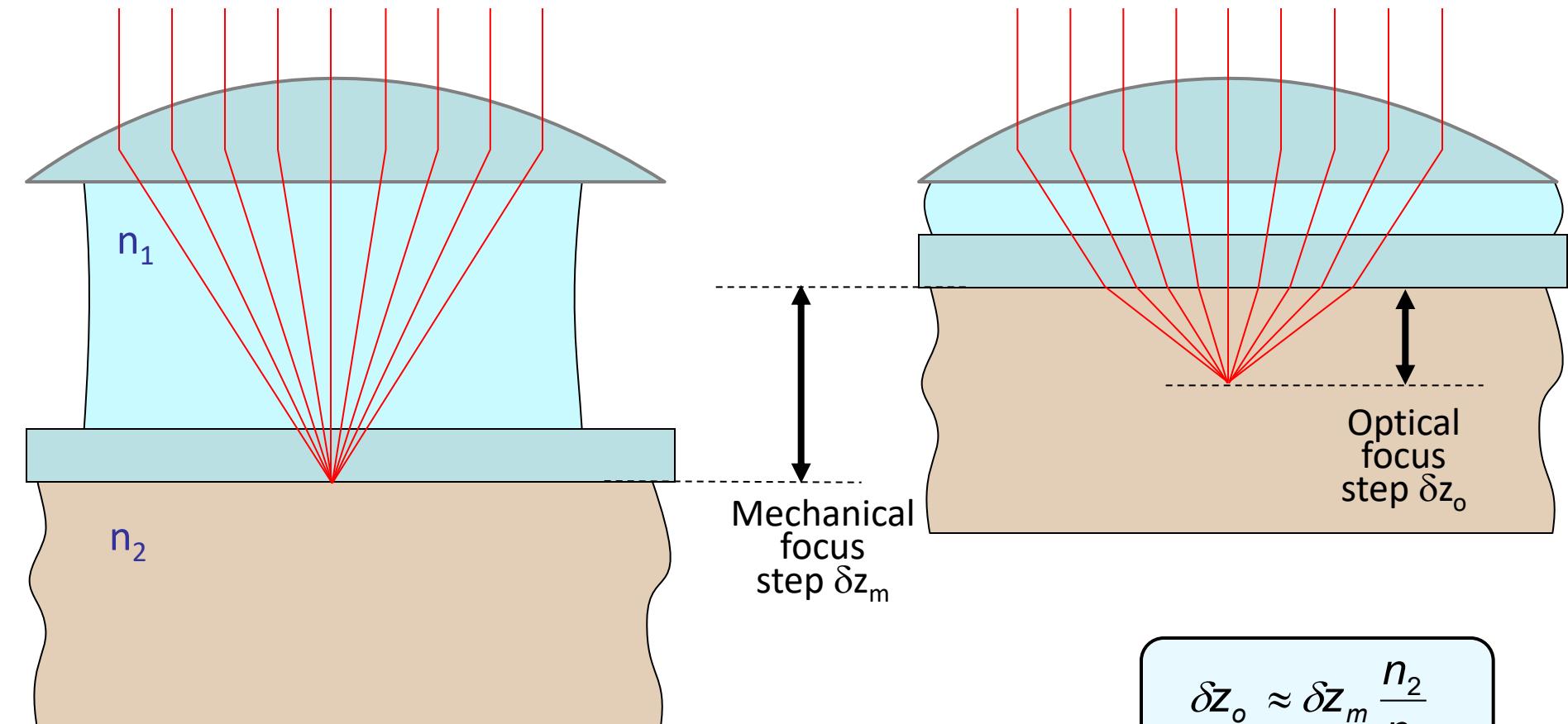
Aberrated

Unaberrated

Index Mismatch & Axial Scaling

n_1

n_2

Mechanical
focus
step δz_m

Optical
focus
step δz_o
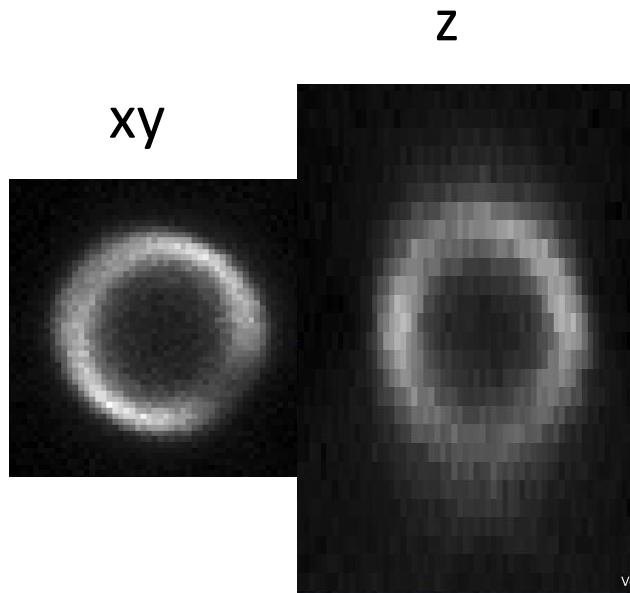
$$\delta z_o \approx \delta z_m \frac{n_2}{n_1}$$
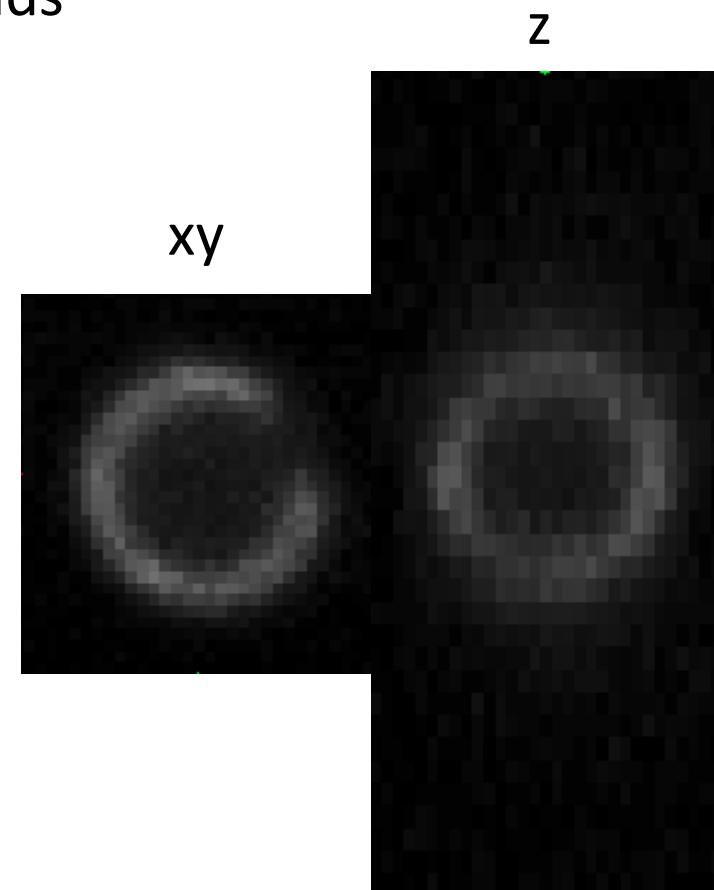
If there is index mismatch,
your z pixel size is not what you think

Index Mismatch & Axial Scaling

## 2 μm beads

xy

z

n = 1.32

xy

z

n = 1.52

Mark Chan
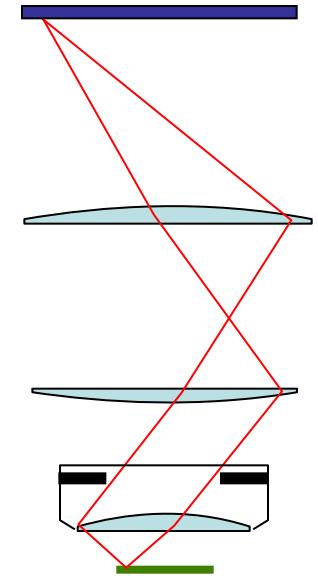
Sources of Astigmatism & Coma

Off-axis   (edges of field of view)

- All objectives have some
- Present in the design
- You get what you pay for
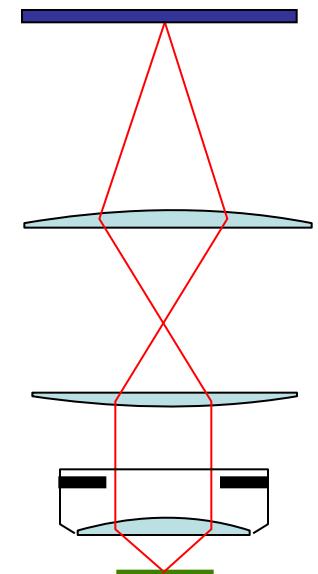
## On-axis   (center of field of view)

Should be none, by symmetry.
If they are there, they could be from:
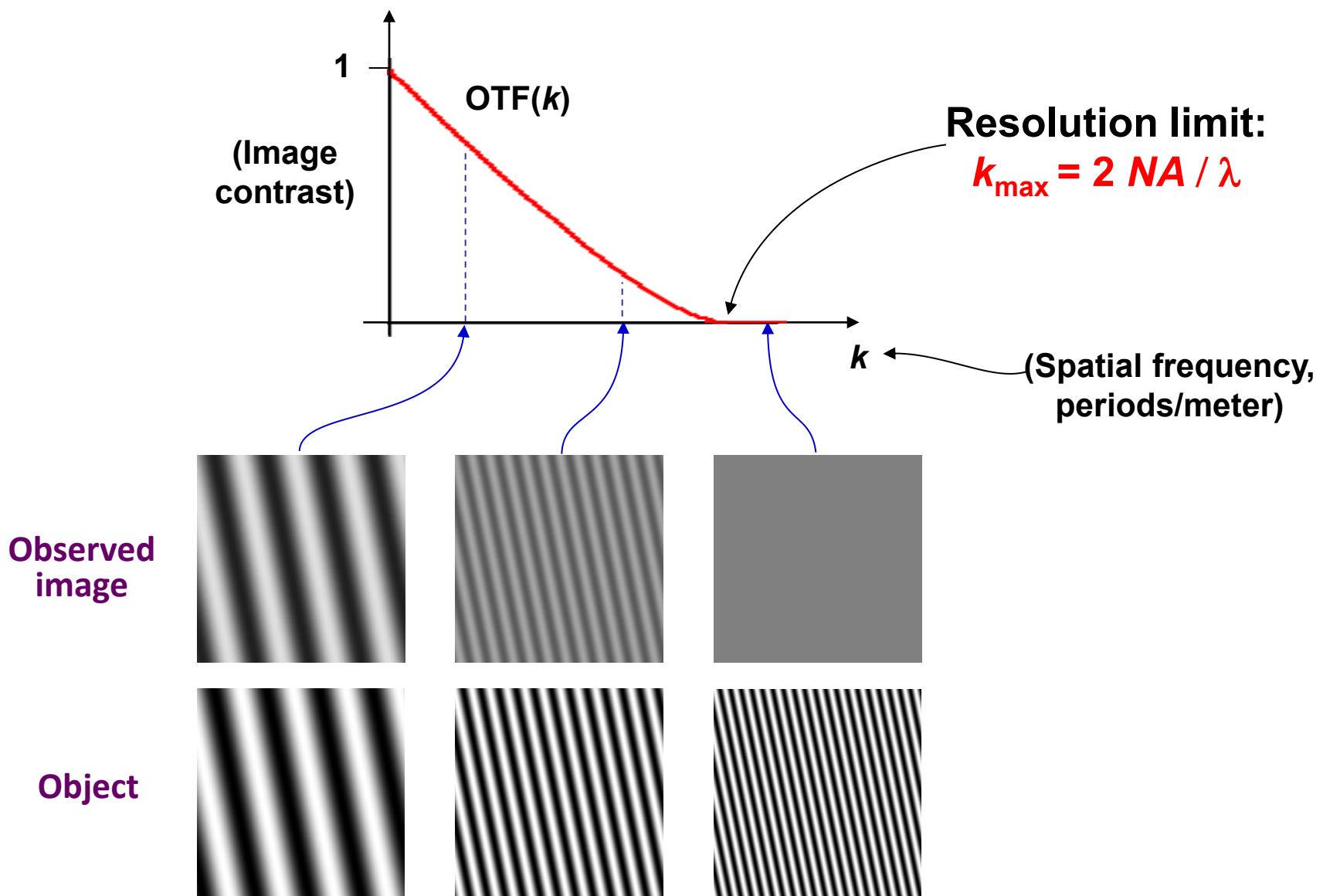
- manufacturing or assembly tolerances
- dirt or abuse
- Misalignment (tilt, off-axis shift of something)
- **bad downstream components** (mirrors, dichroics, filters…)
- **Air bubble** in the immersion fluid or sample
- **Tilted cover slip**
    (dry and water-immersion high-NA lenses)

More about
Spatial frequencies & the
Optical Transfer Function (OTF)
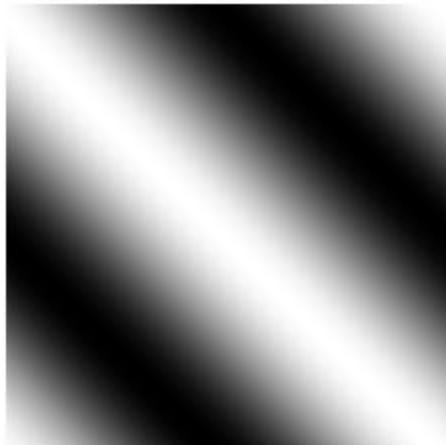
# The response to pure waves is well-defined by the Optical Transfer Function (OTF)

1

OTF(k)

(Image contrast)

Resolution limit:
k_{max} = 2 NA / λ

k

(Spatial frequency, periods/meter)

Observed image

Object

Think of Images as Sums of Waves
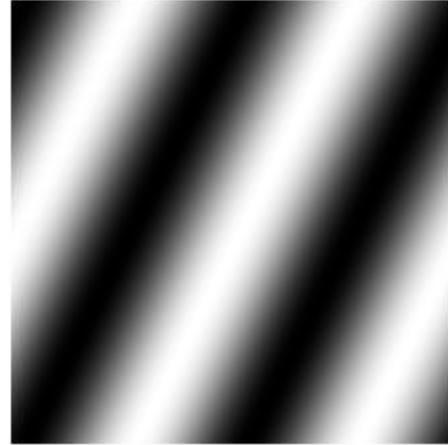
... or "spatial frequency components"
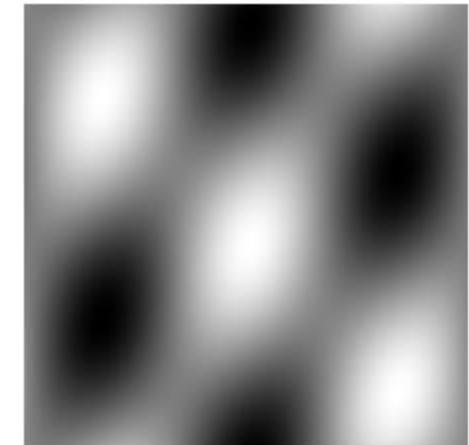
one wave   **another wave**   **(2 waves)**
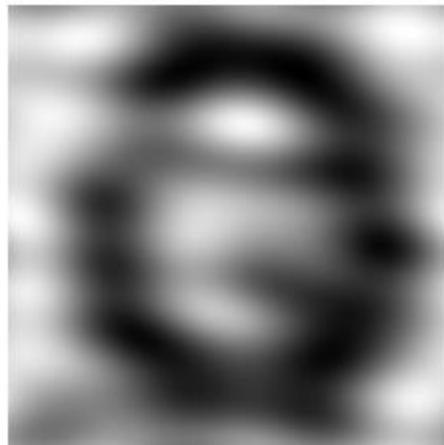
$+$ $=$
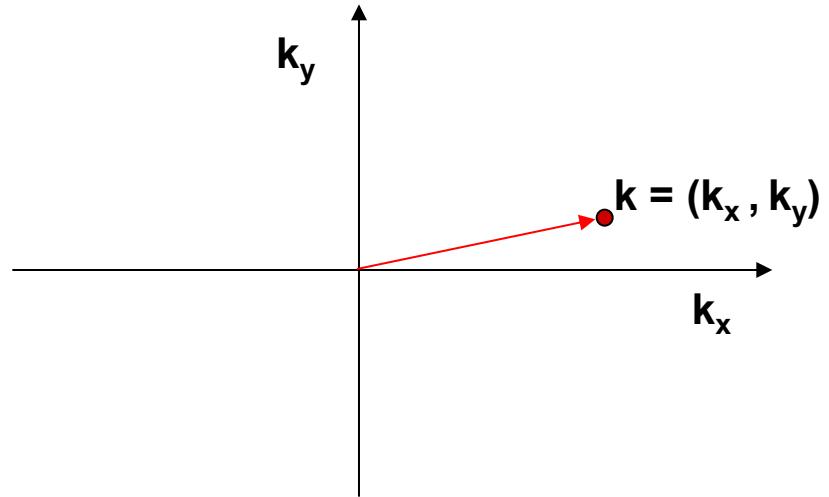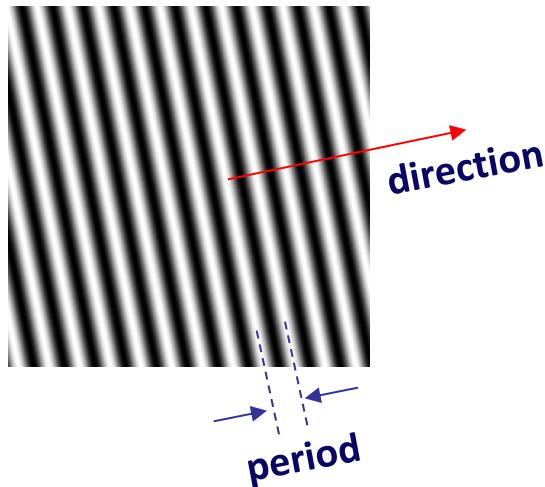
(25 waves)   **(10000 waves)**

$+ (...) =$ $+ (...) =$
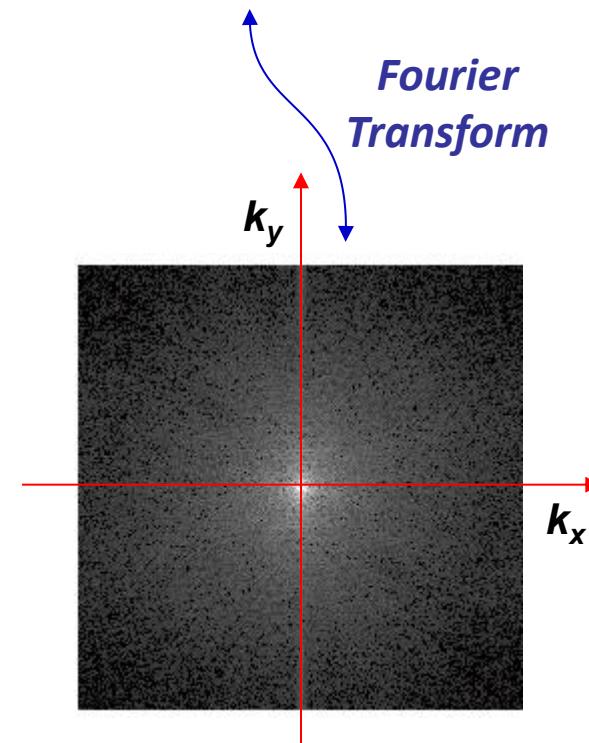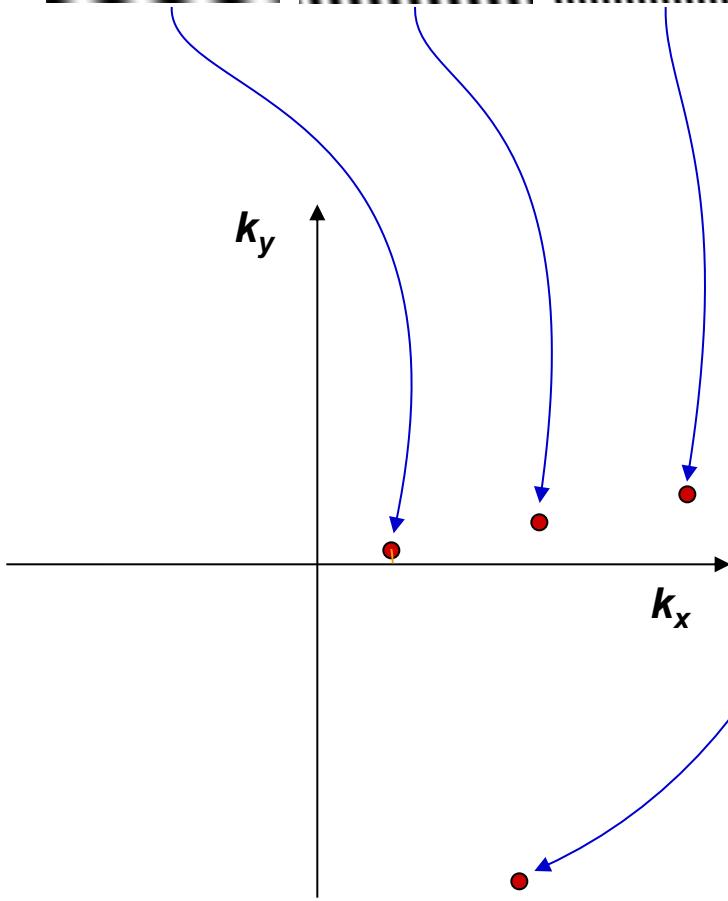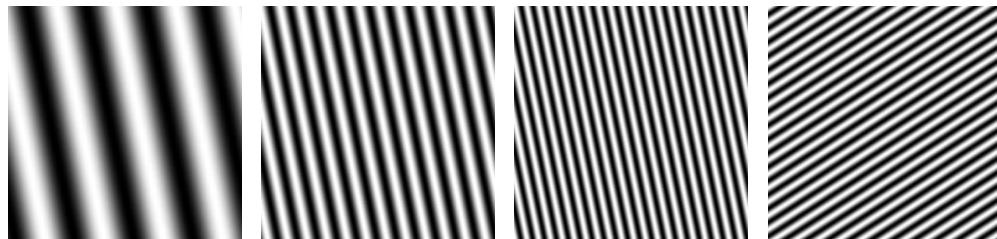
# Frequency Space

**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

direction

period

k_y

$k = (k_x , k_y)$

k_x

Frequency Space
and the *Fourier Transform*

k_y

k_x

Fourier Transform

k_y

k_x

Properties of the Fourier Transform

$$F(\mathbf{k}) = \int f(\mathbf{r})e^{2\pi i \mathbf{k} \bullet \mathbf{r}} d\mathbf{r}$$
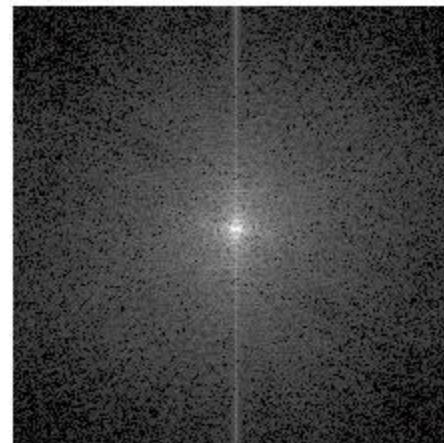
Completeness:
**The Fourier Transform contains all the information of the original image**
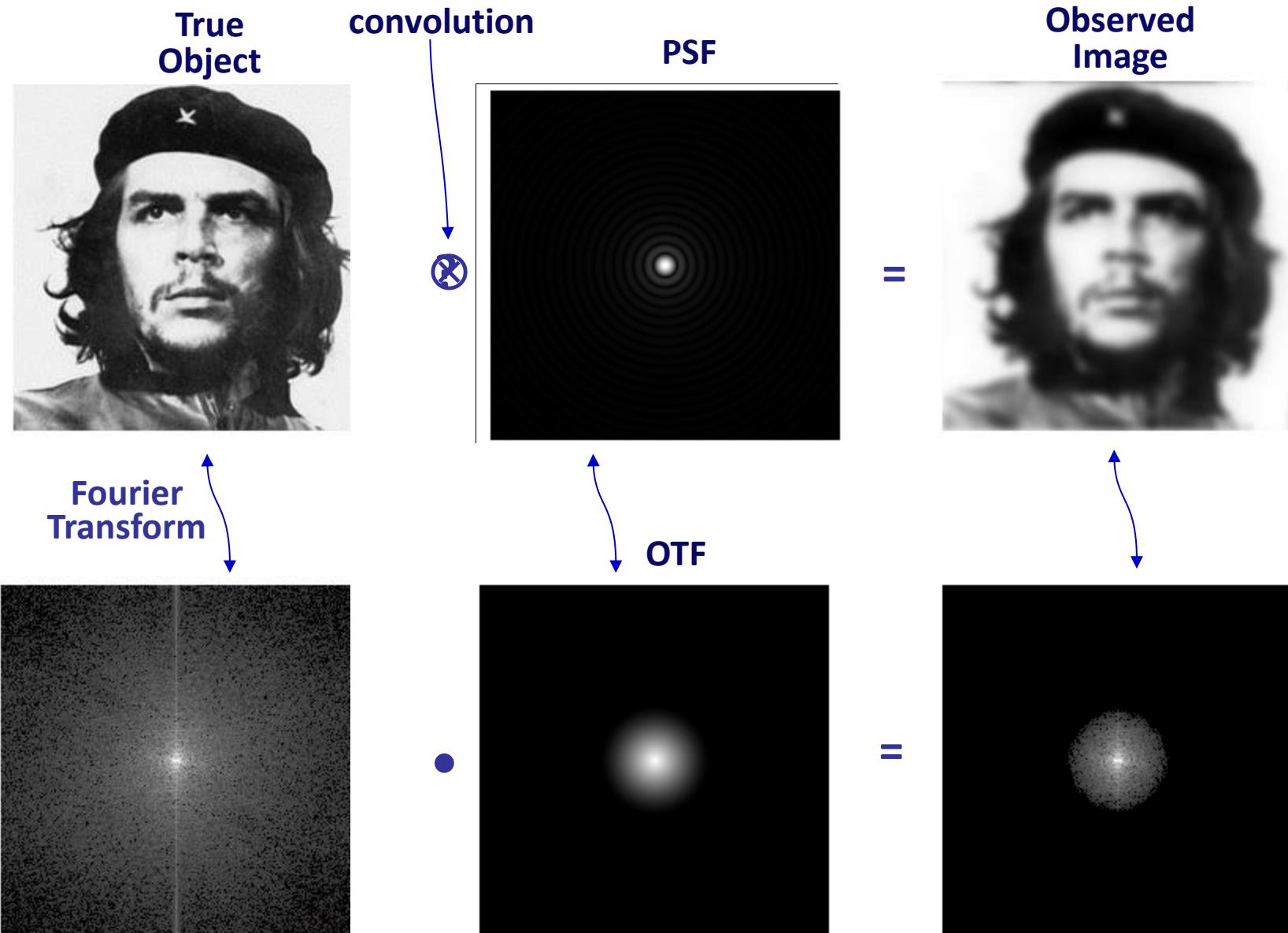
Symmetry:
The Fourier Transform of the Fourier Transform is the original image

Fourier transform

The OTF and Imaging

True Object

convolution

PSF

Observed Image

$\otimes$

=

Fourier Transform

OTF

$\bullet$

=

Convolutions

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

Why do we care?
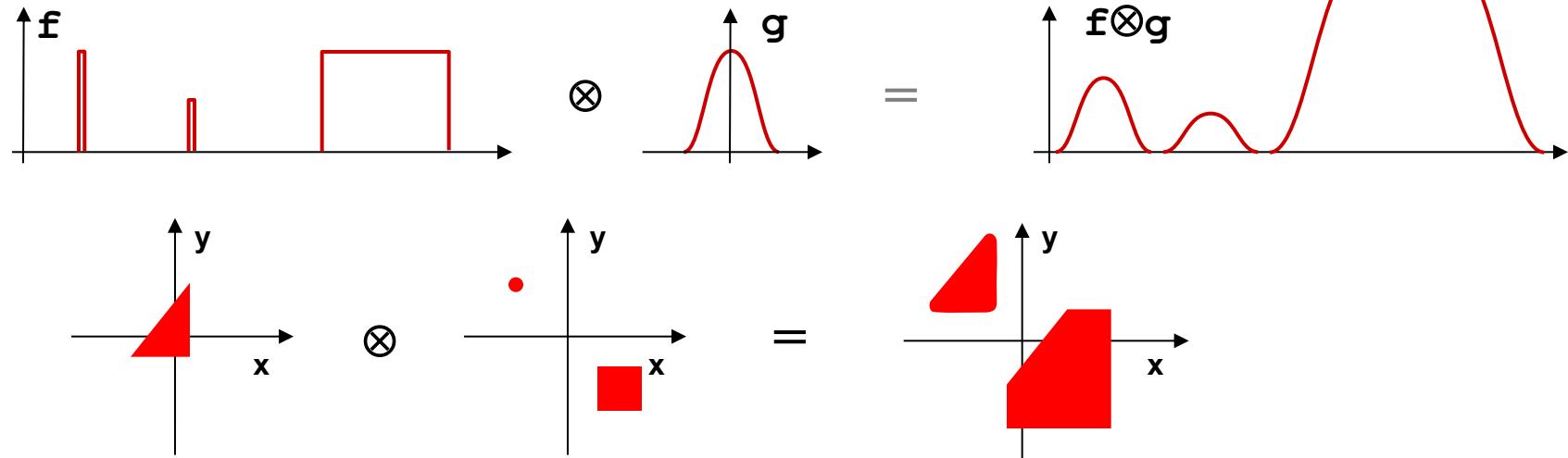
- **They are everywhere…**
- **The <u>convolution theorem</u>:**

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

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

**A convolution in real space becomes a product in frequency space & vice versa**
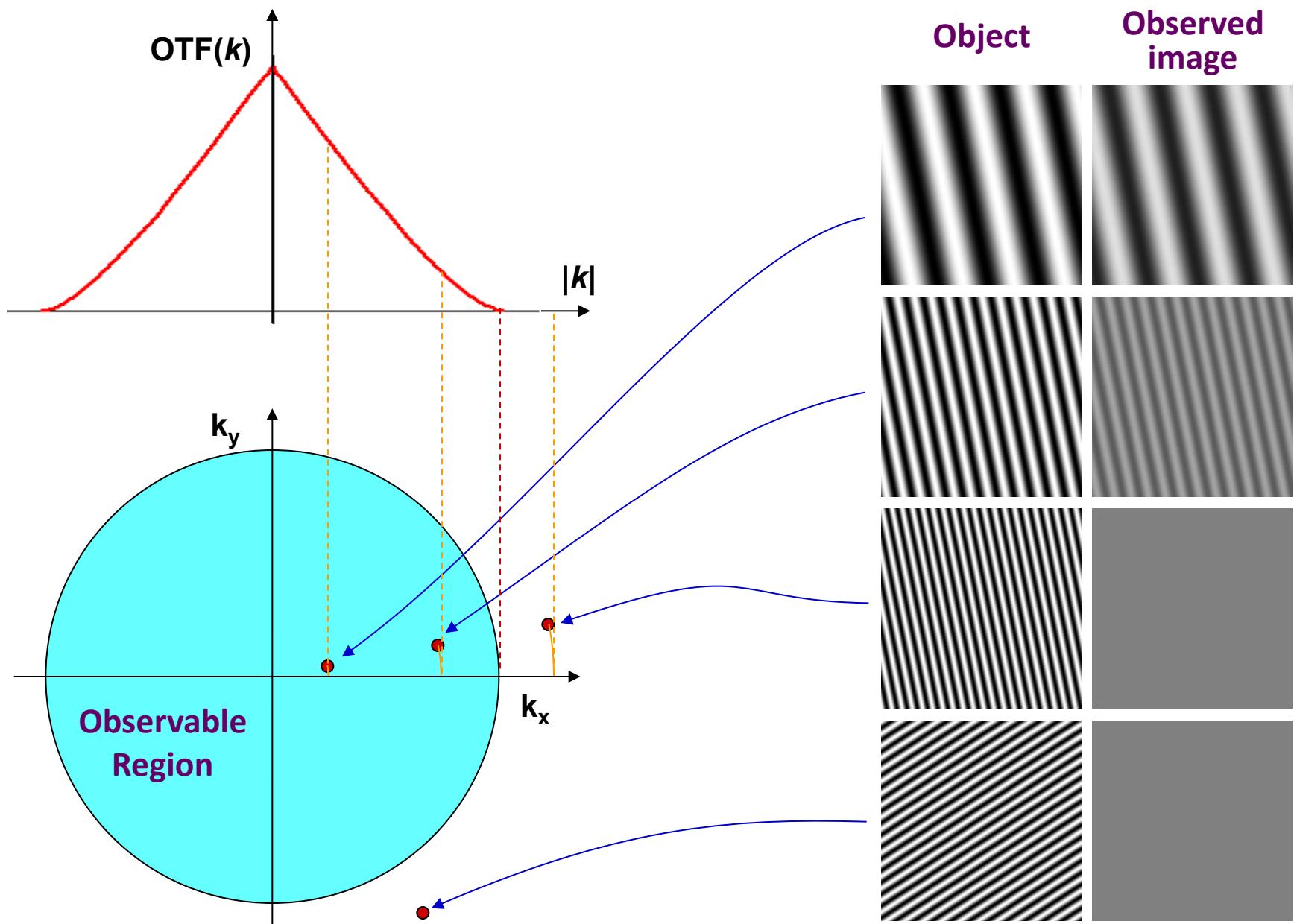
Symmetry: $g \otimes f = f \otimes g$

**So what <u>is</u> a convolution, intuitively?**

- **"Blurring"**
- **"Drag and stamp"**
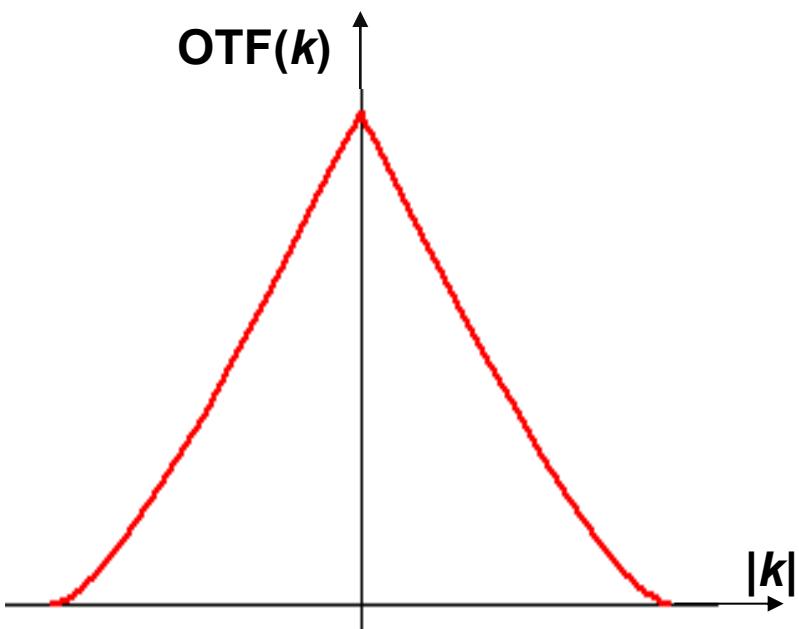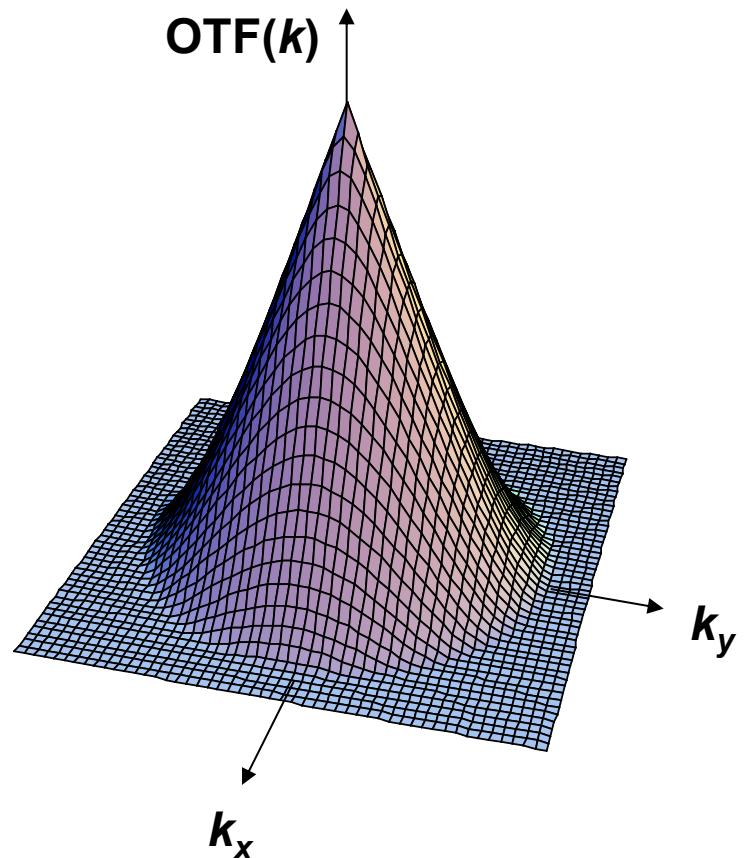
The Transfer Function Lives in Frequency Space

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

OTF(k)

$|k|$

OTF(k)

k_y

k_x
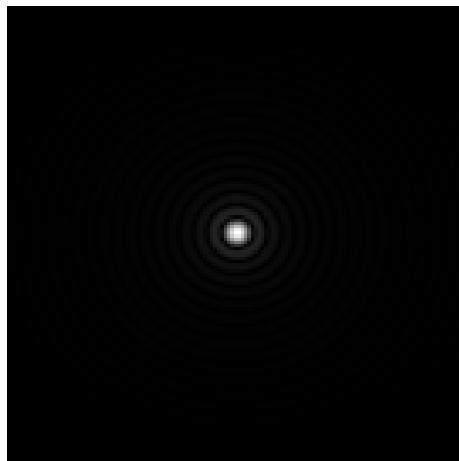
(Idealized calculations)
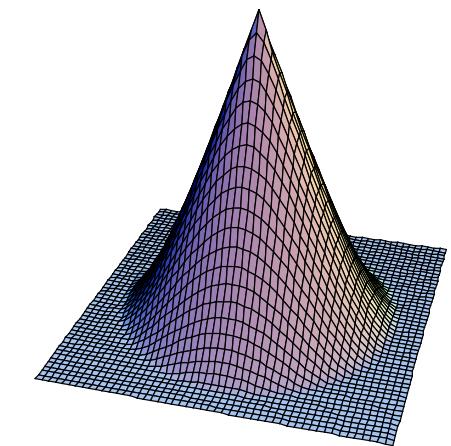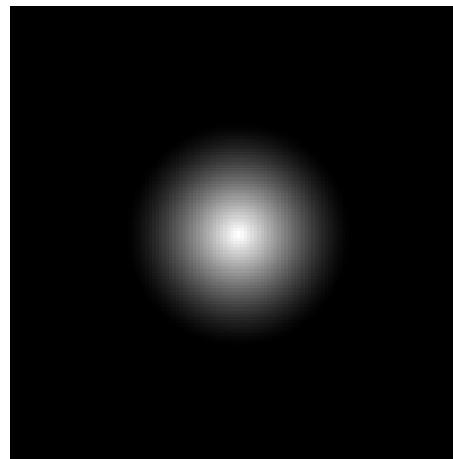
The 3D OTF

2D PSF
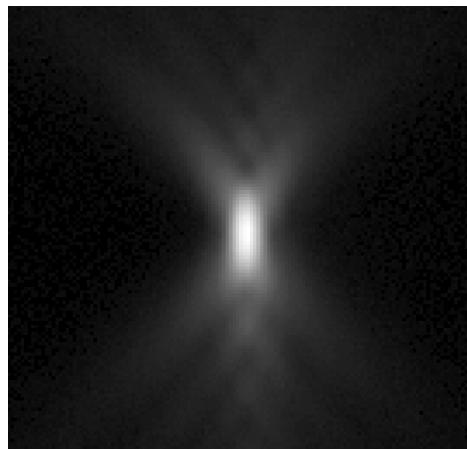
2D OTF

2D F.T.
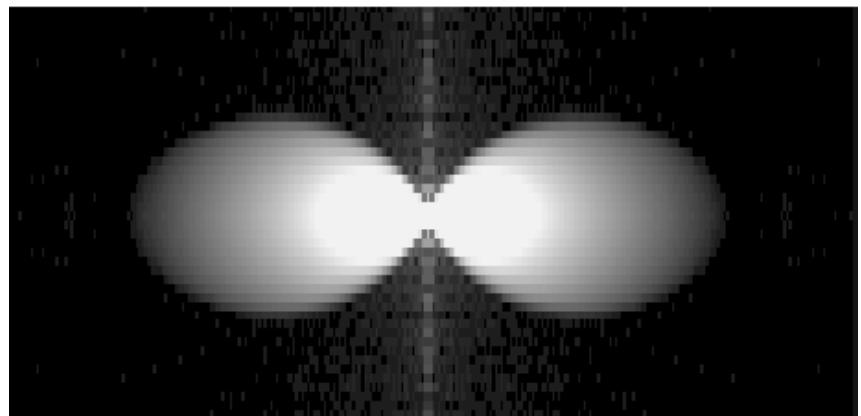
3D PSF
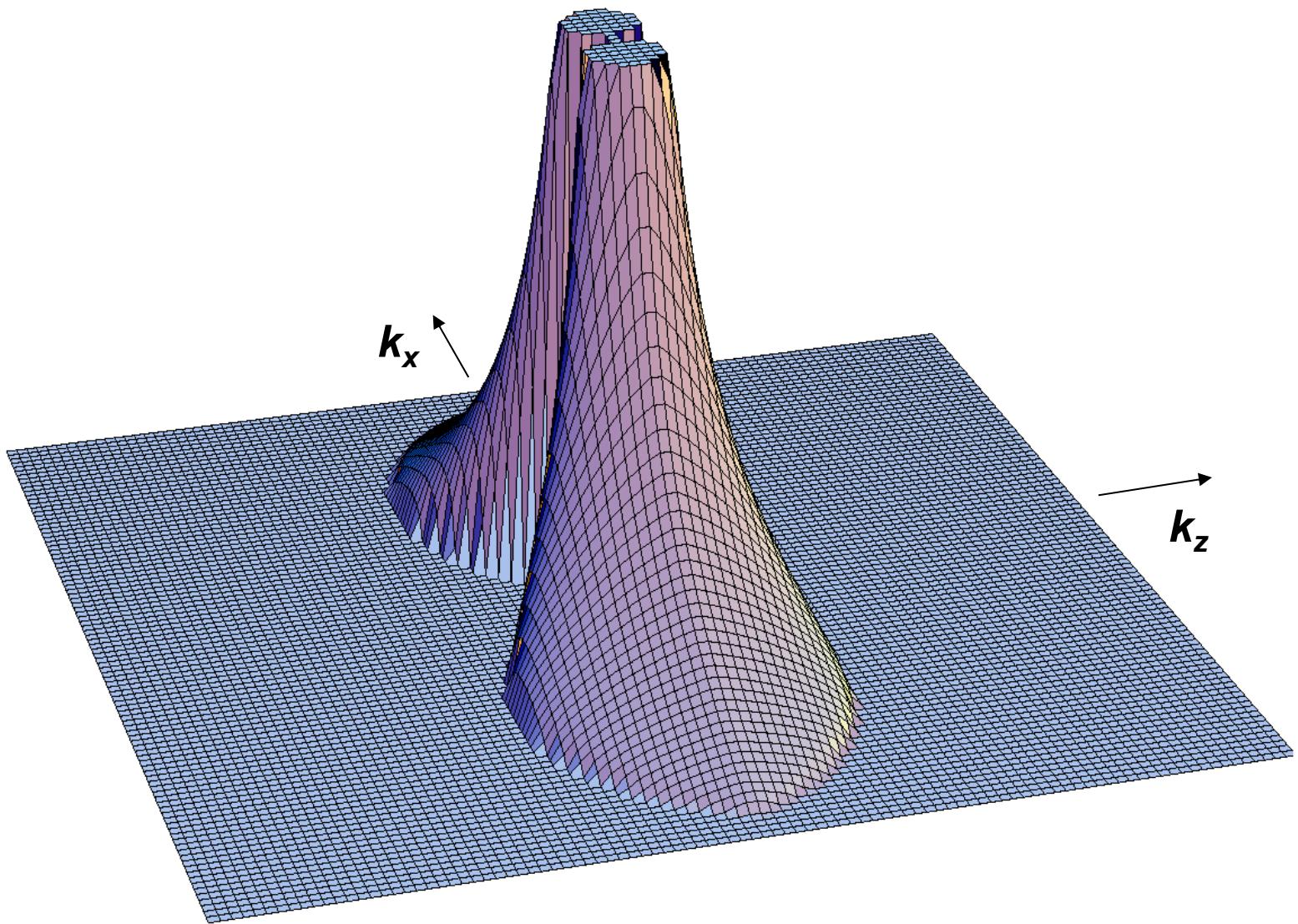
3D OTF

3D F.T.
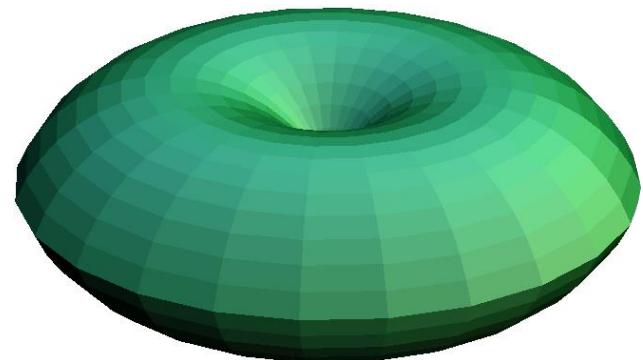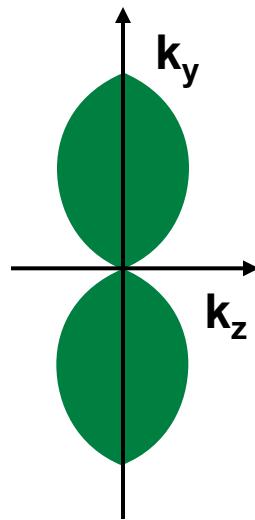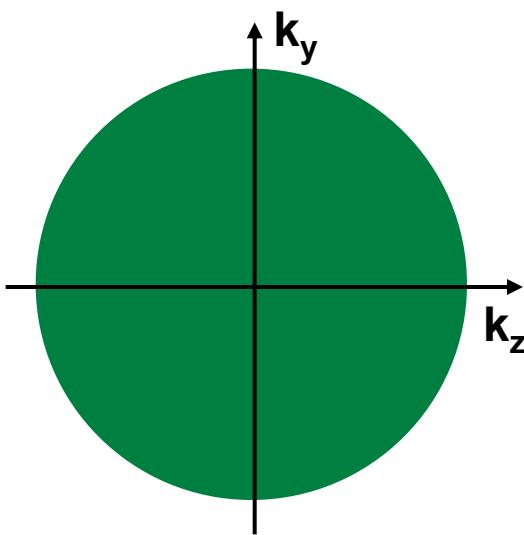
Values of the 3D OTF
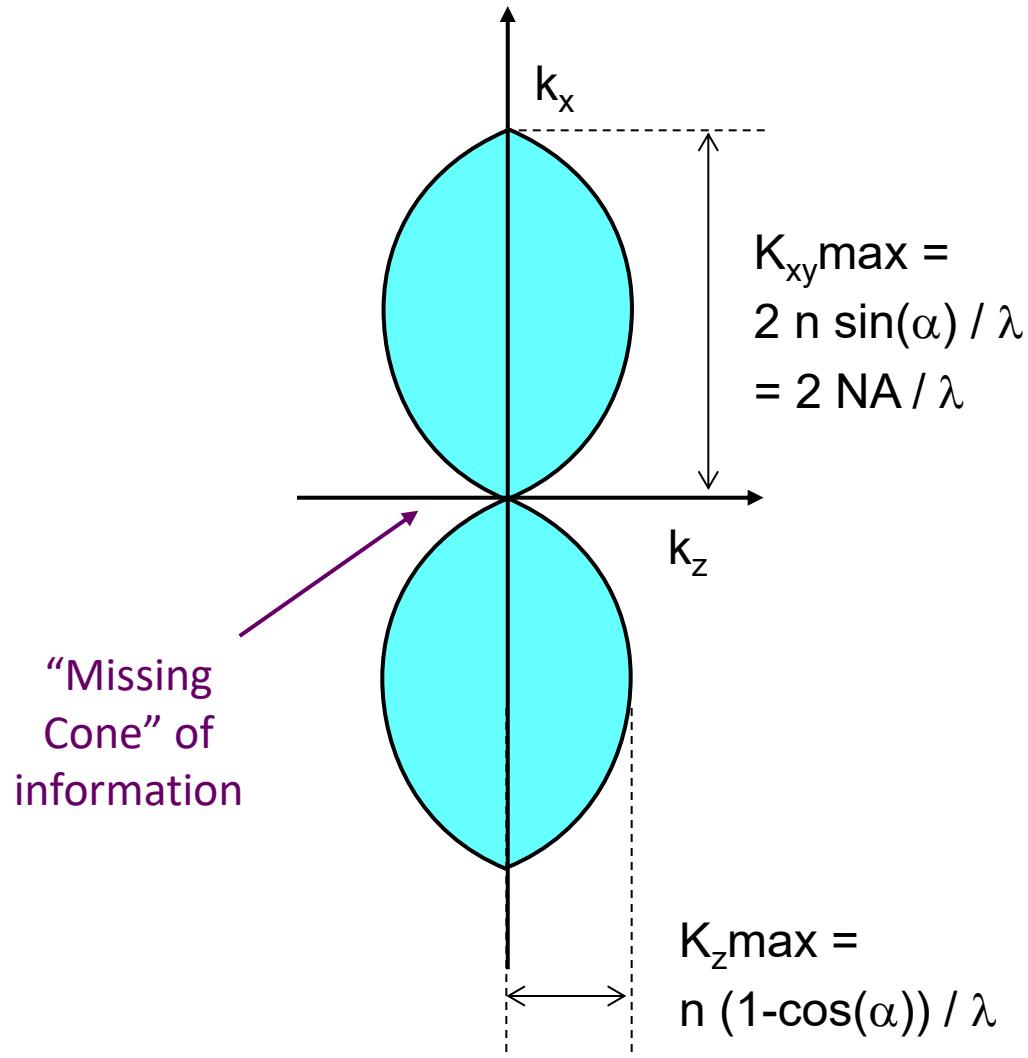
3D Observable Region

= OTF support
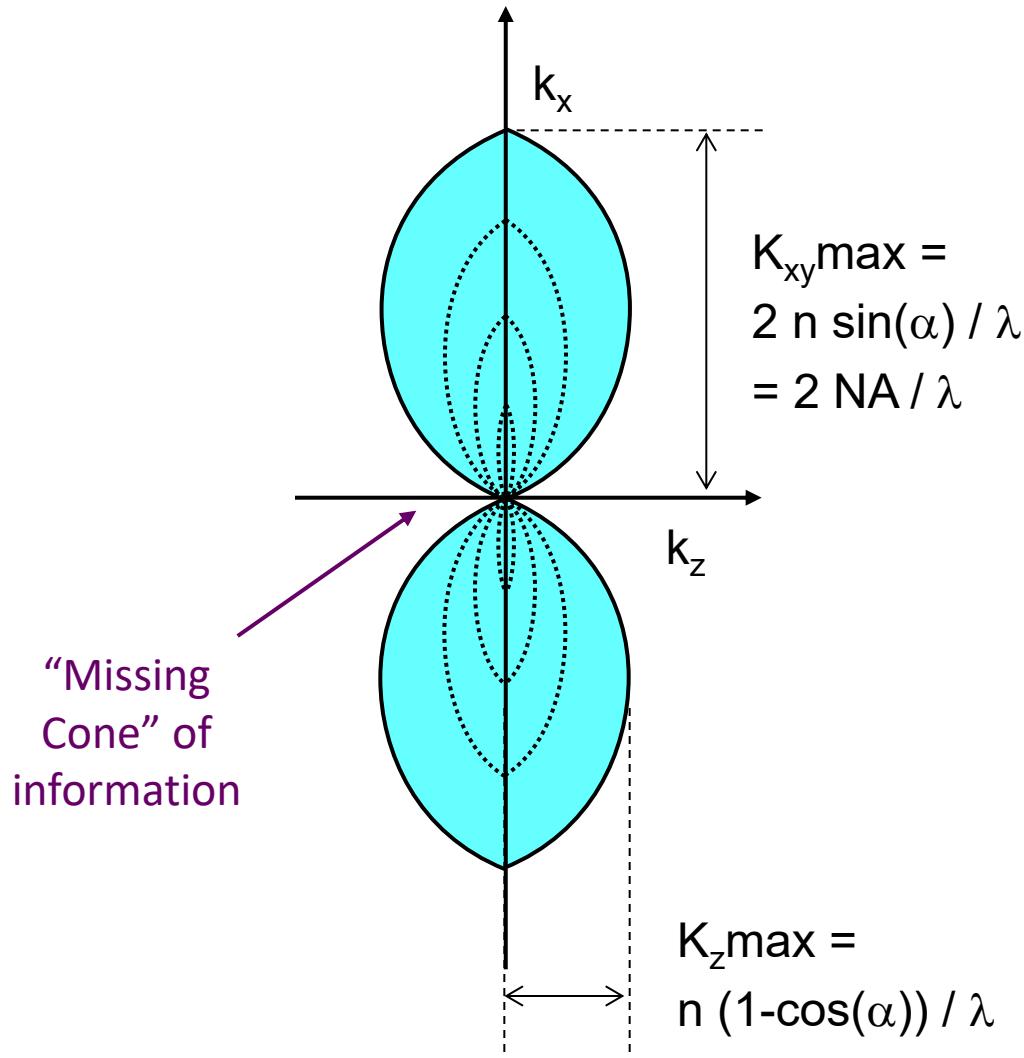
= Region where the OTF is non-zero

So what is the resolution?

k_x

k_z

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

"Missing Cone" of information

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

So what is the resolution?

$K_{xy}max =$

$2\,n\,\sin(\alpha)\,/\,\lambda$

$= 2\,NA\,/\,\lambda$

k_x

k_z

"Missing Cone" of information

$K_z max =$

$n\,(1-\cos(\alpha))\,/\,\lambda$
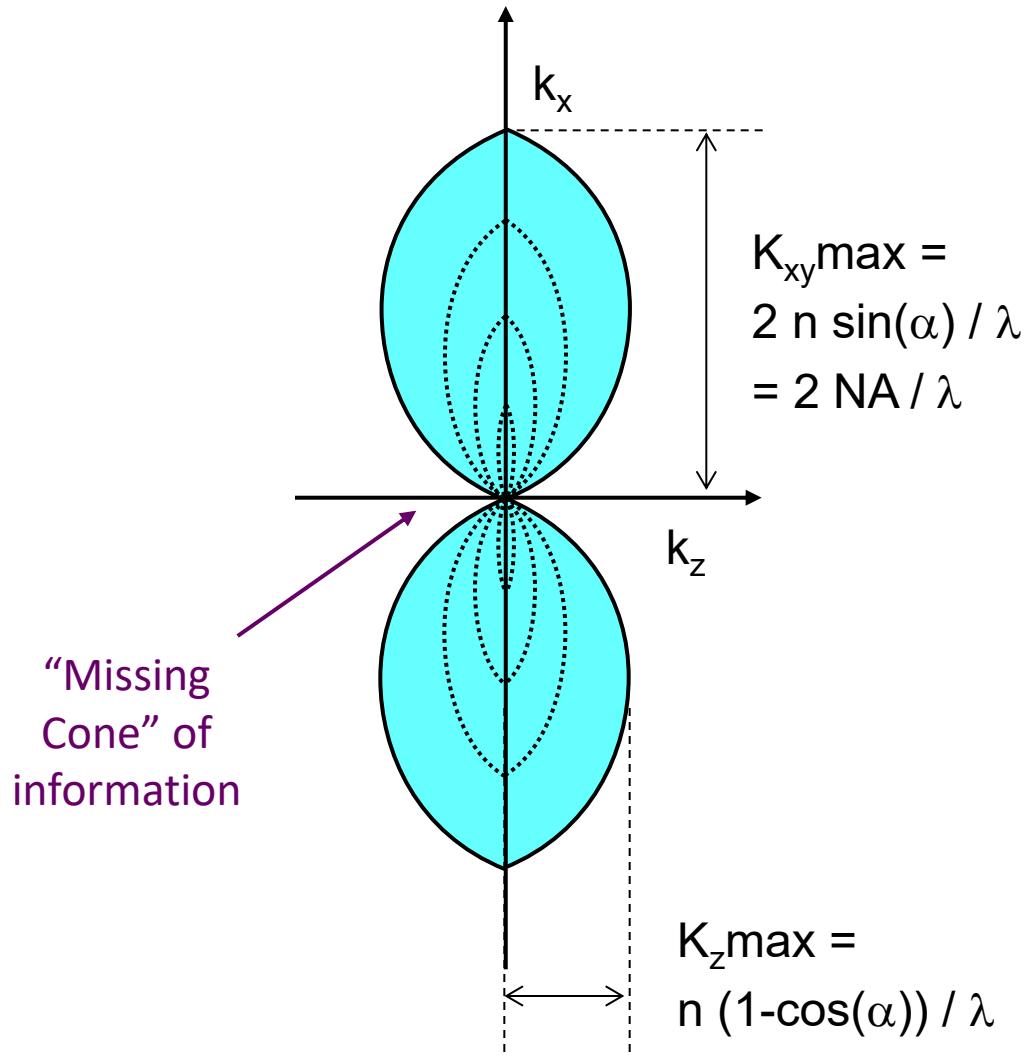
Lowering the NA Degrades the axial resolution faster than the lateral resolution

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

So what is the resolution?

k_x

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

k_z

"Missing Cone" of information

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

Example:
a high-end objective

$NA = 1.4$
$n = 1.515$
→ $\alpha = 67.5°$
$\lambda = 600$ 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