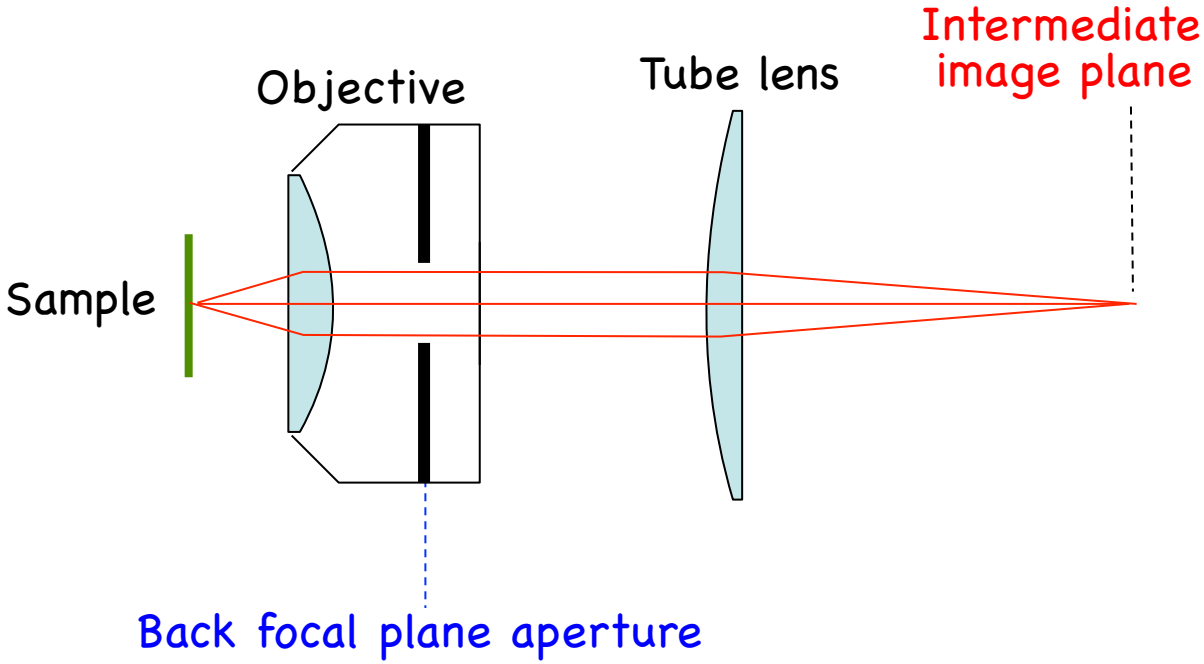
Principles & Practice of Light Microscopy 2

- Resolution
- Aberrations
- The Point Spread Function
- The Optical Transfer Function
- Spatial frequencies

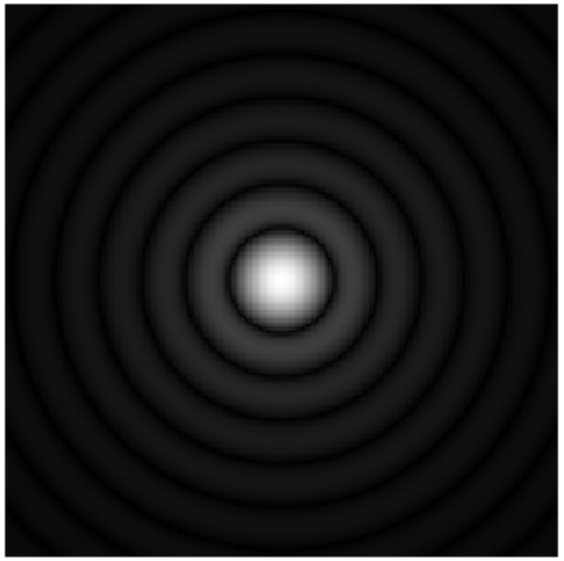
Edited by: Zvi Kam, Weizmann
For Advance Light Microscopy course

RESOLUTION

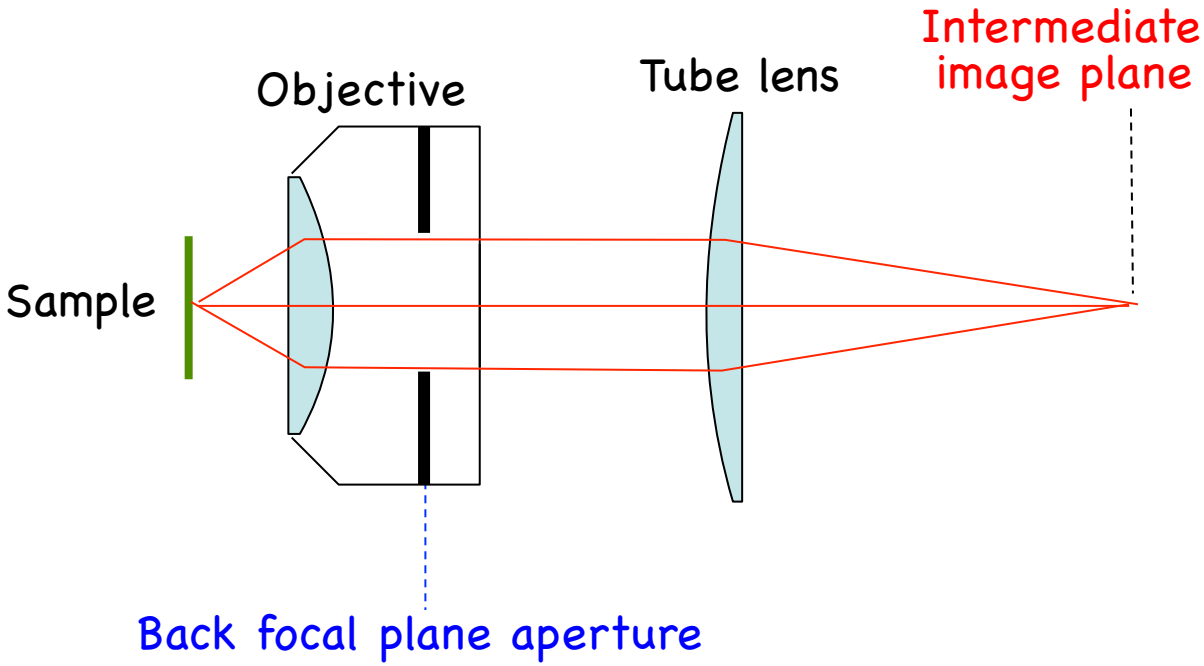
Aperture and Resolution



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



Aperture and Resolution



Diffraction spot
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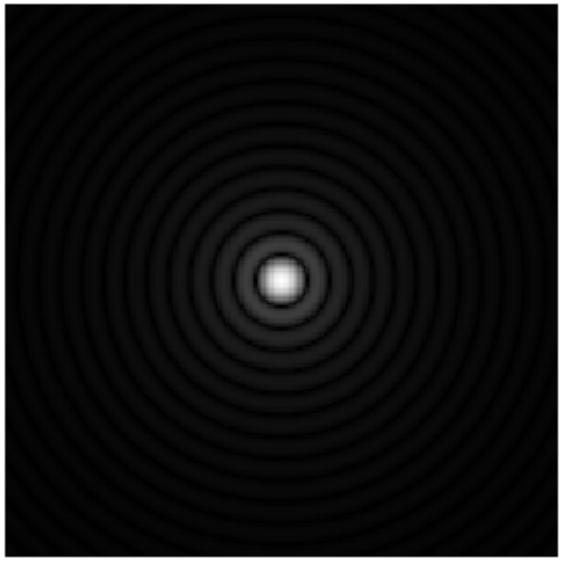
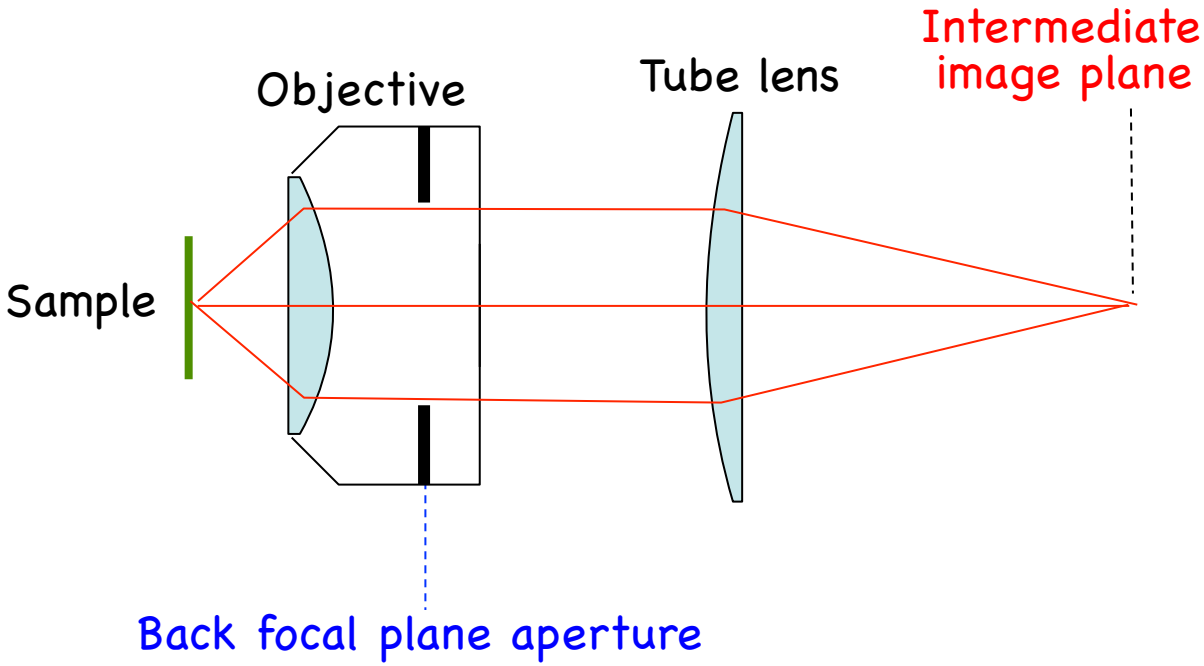
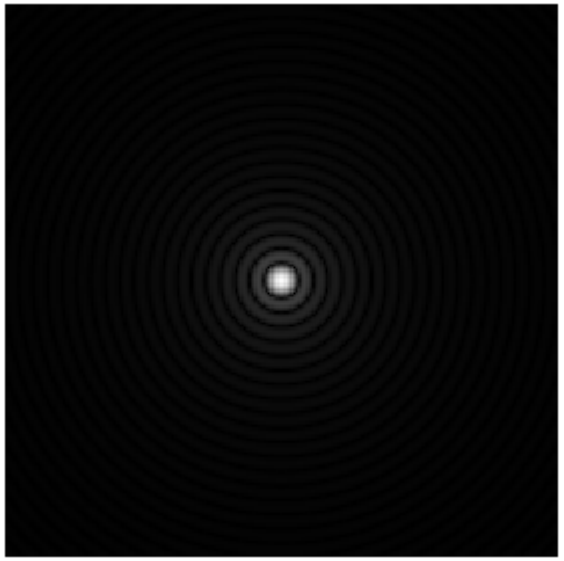


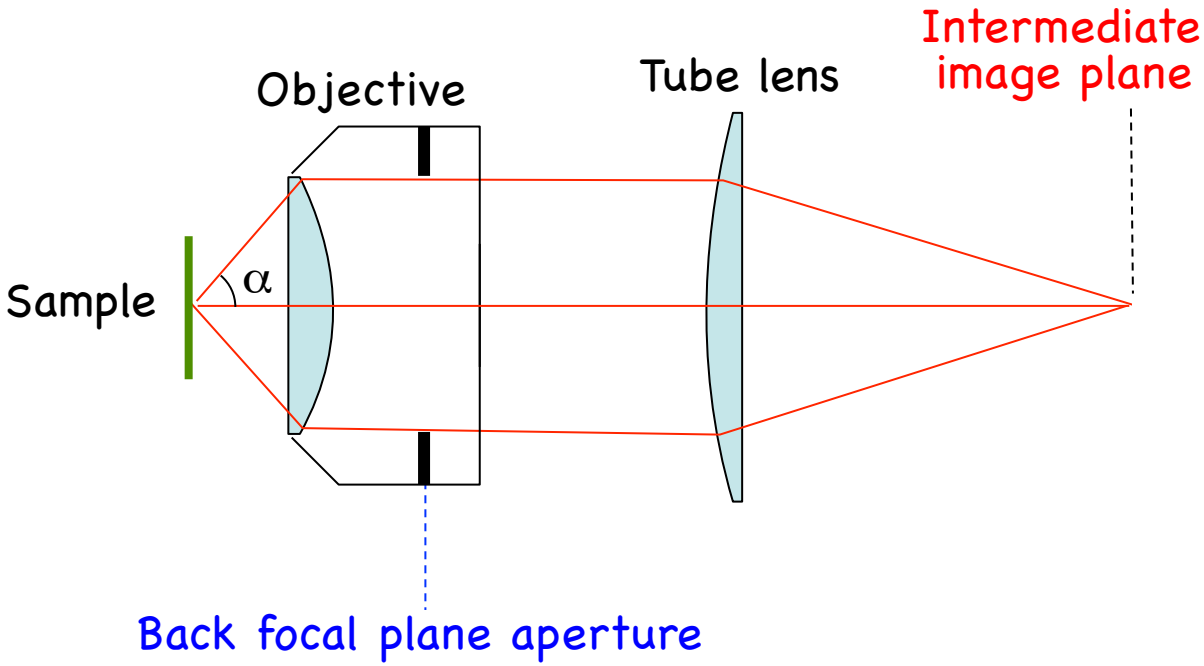
Aperture and Resolution



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



Aperture and Resolution



Diffraction spot
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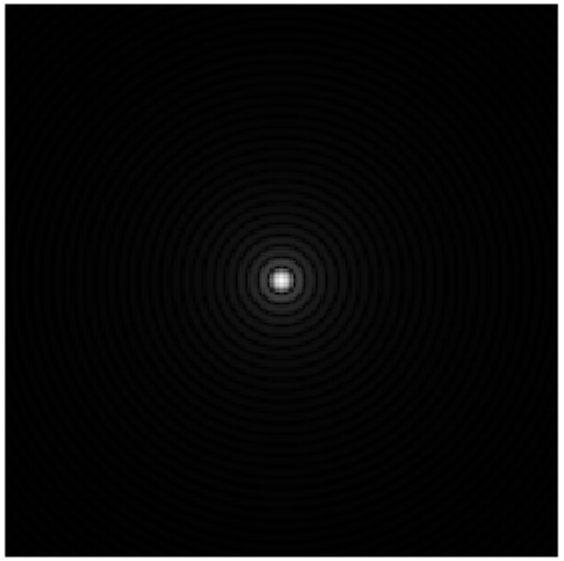


- Image resolution improves with Numerical Aperture (NA)

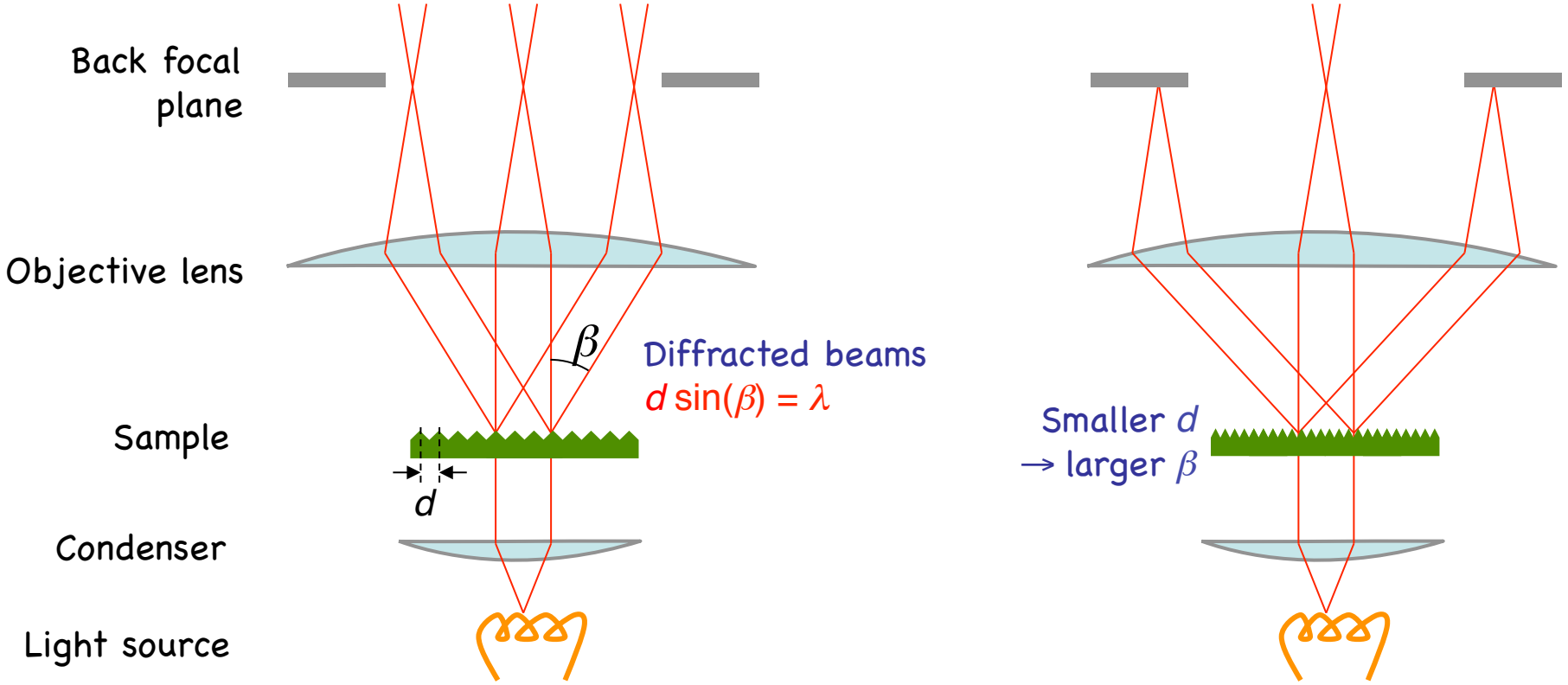
$$NA = n \sin(\alpha)$$

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

Resolution

Ernst Abbe's argument (1873)

Consider a striped sample ≈ a diffraction grating



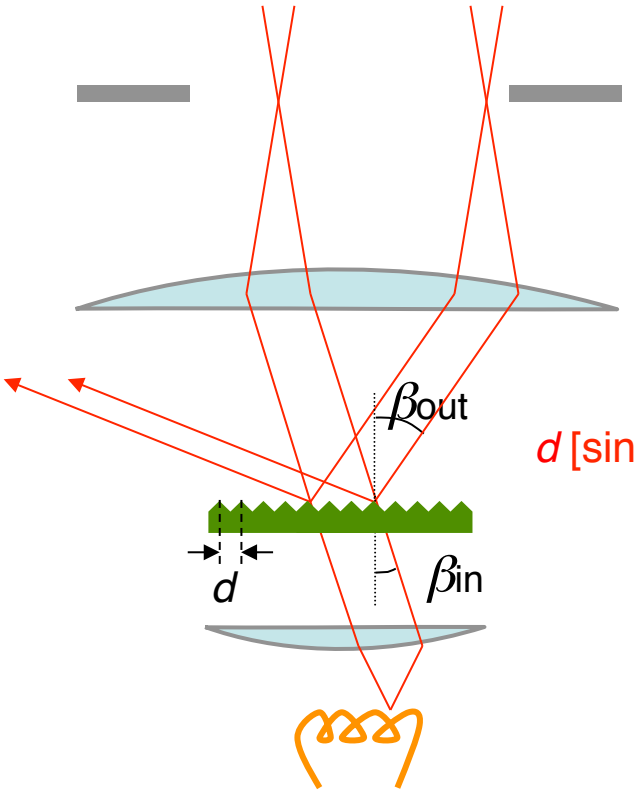
If $\beta > \alpha$, only one spot makes it through
⇒ no interference ⇒ no image formed

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 $\beta_{in} < \alpha$.

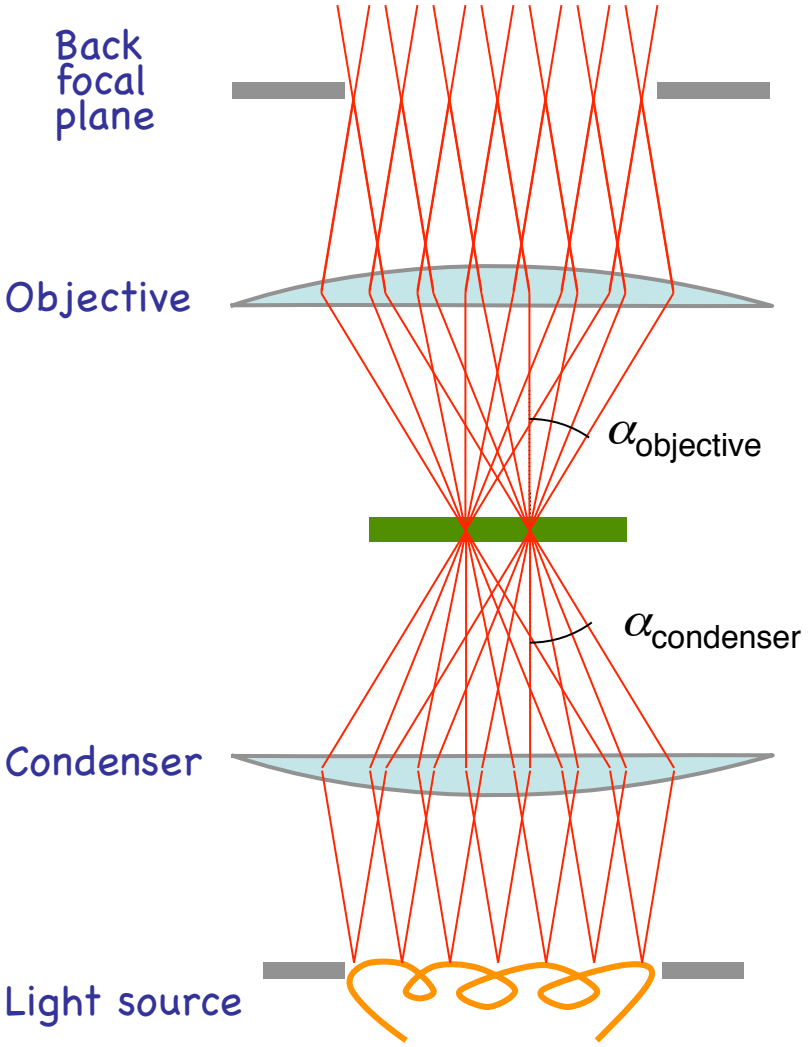
Resolution (smallest resolvable *d*)
with incoherent illumination (all possible illumination directions):

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

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

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

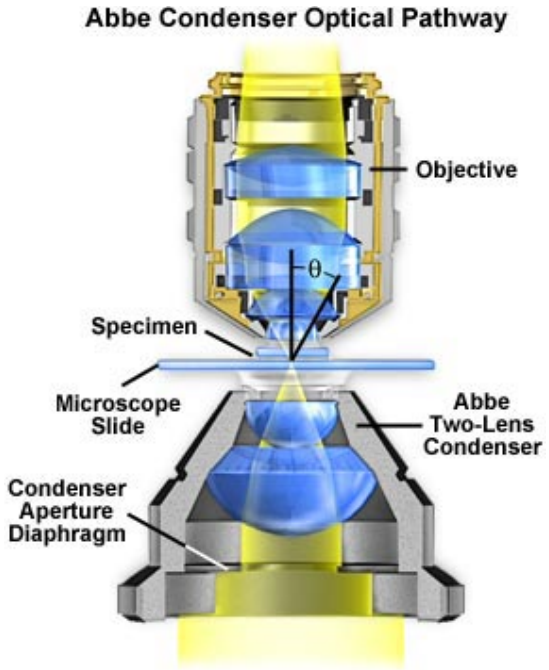
$\alpha_{condenser} \geq \alpha_{objective}$

$\Leftrightarrow$

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

$\Rightarrow$ 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:

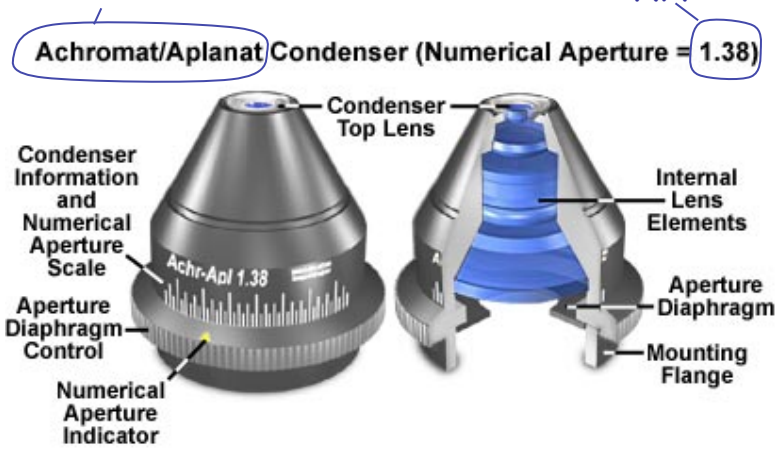
- Low mag objectives have large FOV,
- High mag objectives have large α

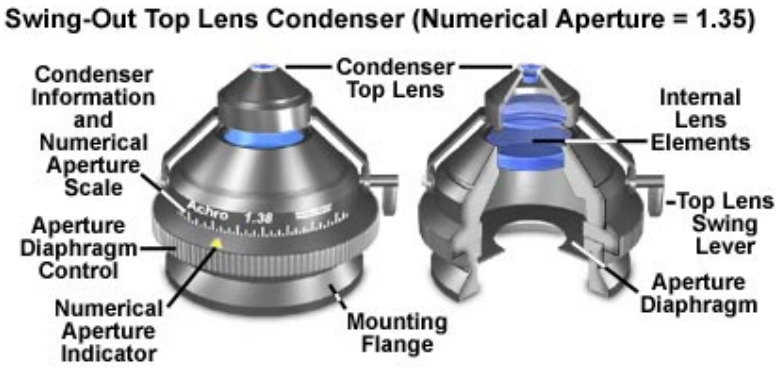
(With 2X and 100x objectives we need $(100/2)^2$ = 2500 times more light than any objective uses!)

Solutions:

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

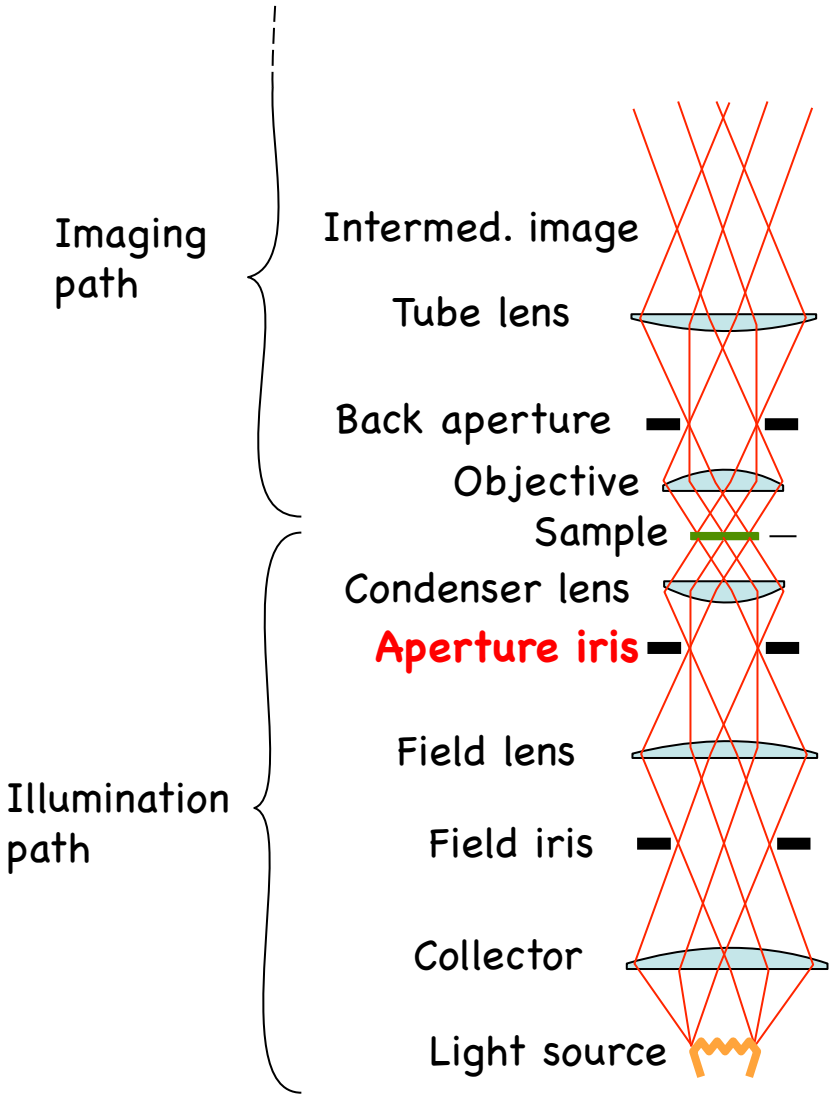
Grade of correction NA





Aperture, Resolution & Contrast

Can adjust the condenser NA with the **aperture iris**

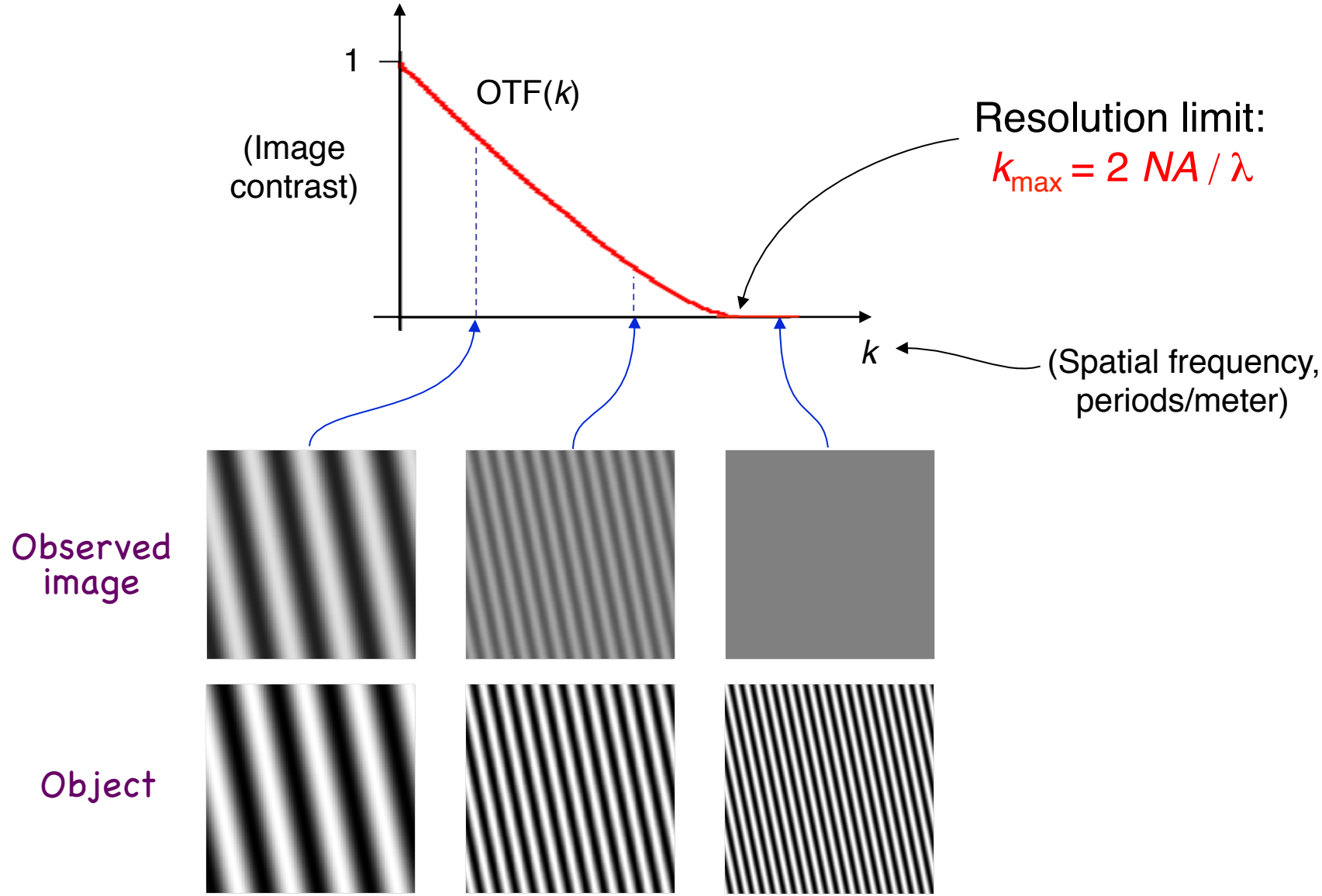


Q: Don't we always want it full open??

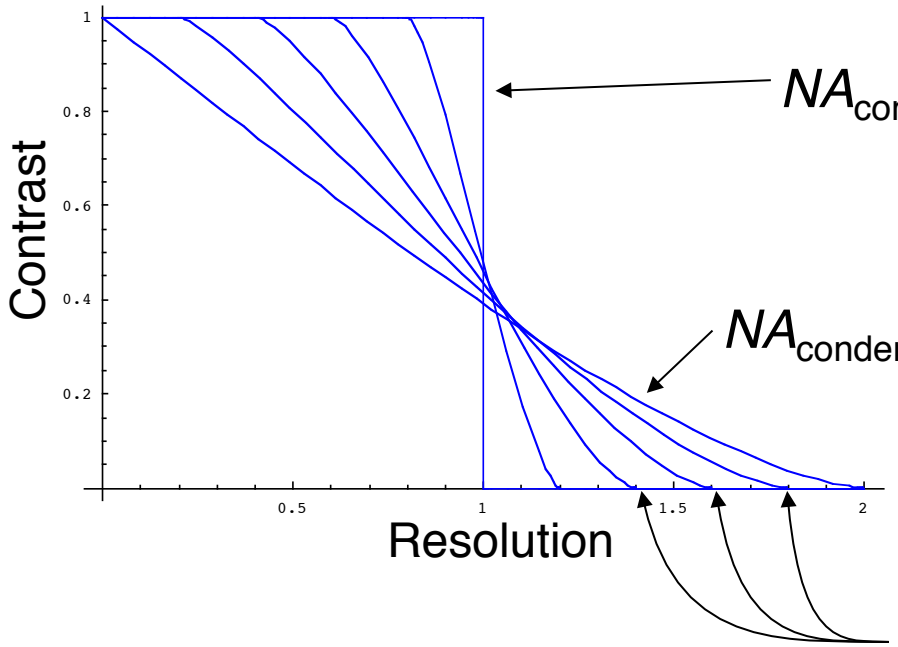
A: **No**

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

Spatial frequencies & the Optical Transfer Function (OTF)



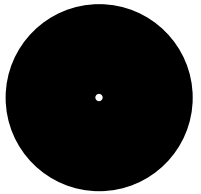
Resolution & Contrast vs. Illumination aperture



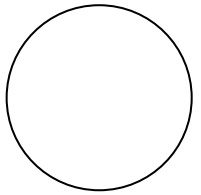
$NA_{condenser} \approx 0$

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

Pupil appearance



("Coherent illumination")



(= Full aperture, "incoherent illumination")

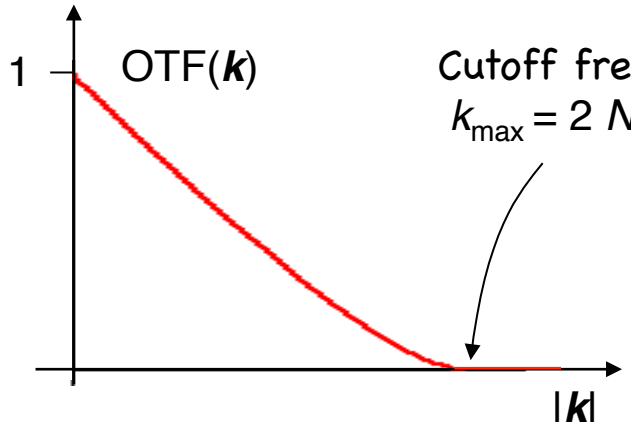
Resolution limit:

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

Increasing the illumination aperture
increases resolution
but decreases contrast

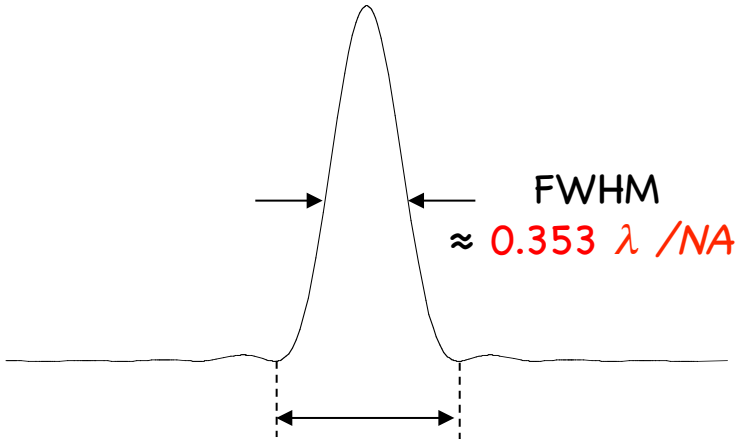
Definitions of Resolution

As the OTF cutoff frequency



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

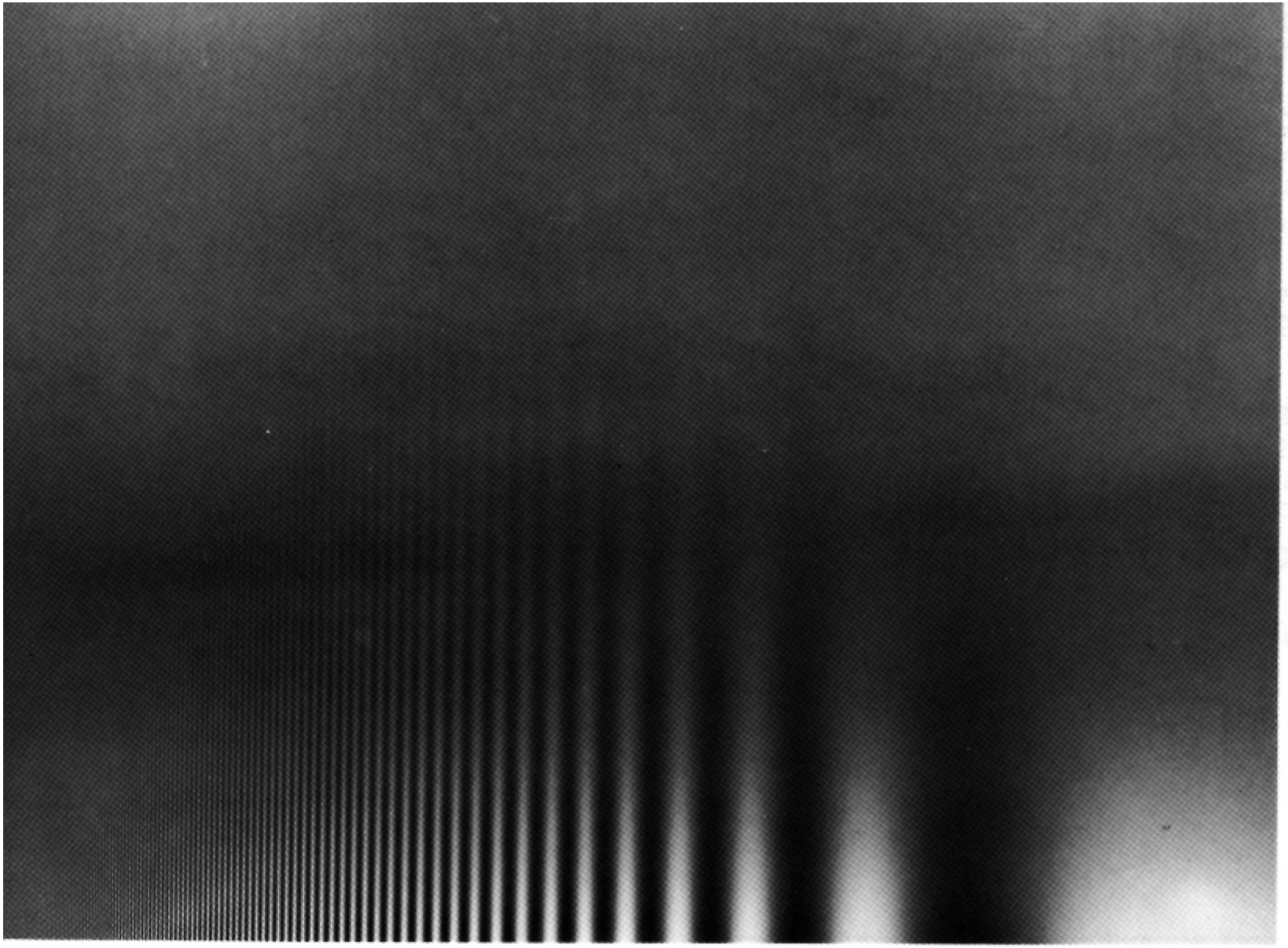
As the Full Width at Half Max (FWHM) of the PSF

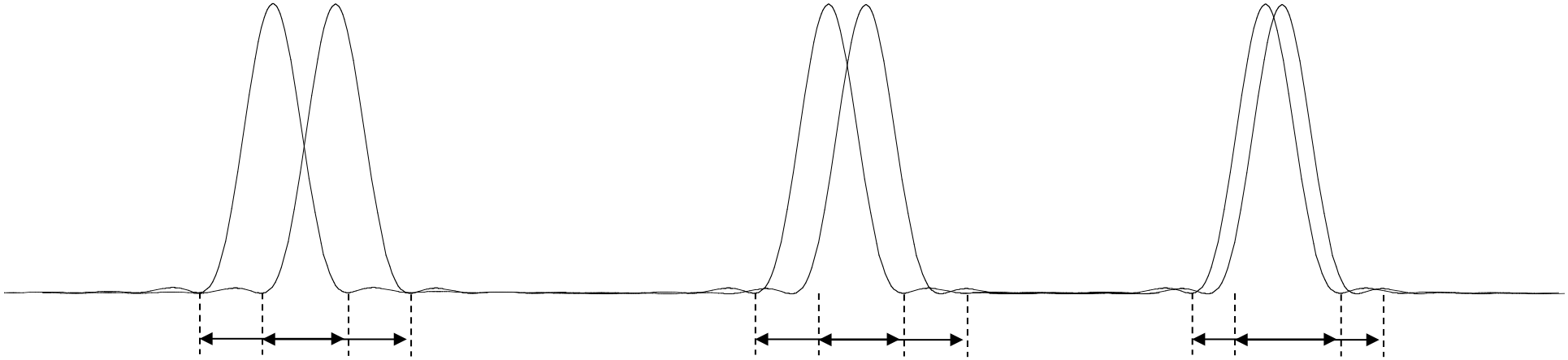


As the diameter of the Airy disk (first dark ring of the PSF)
= "Rayleigh criterion"

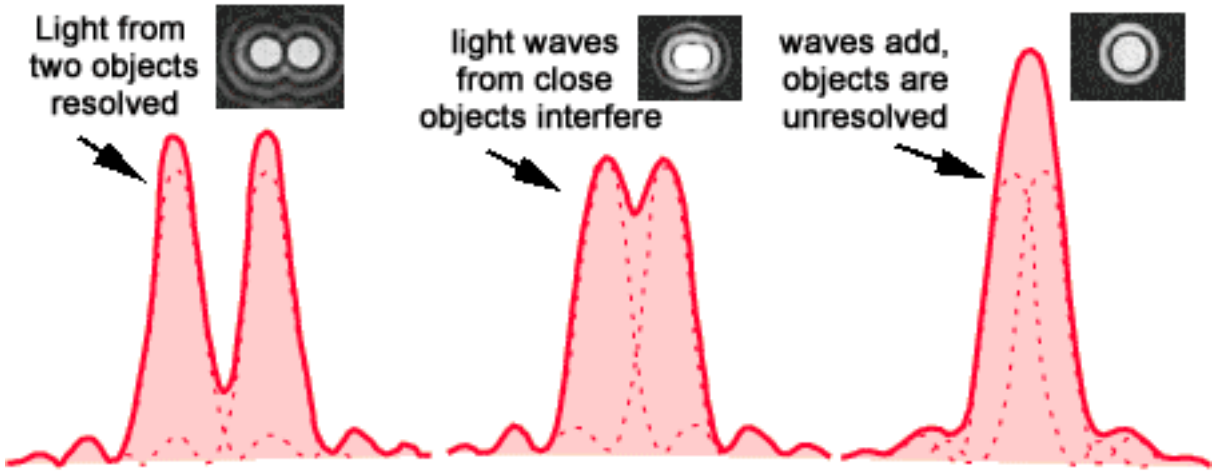
Airy disk diameter
≈ 0.61 λ /NA

Contrast-resolution link





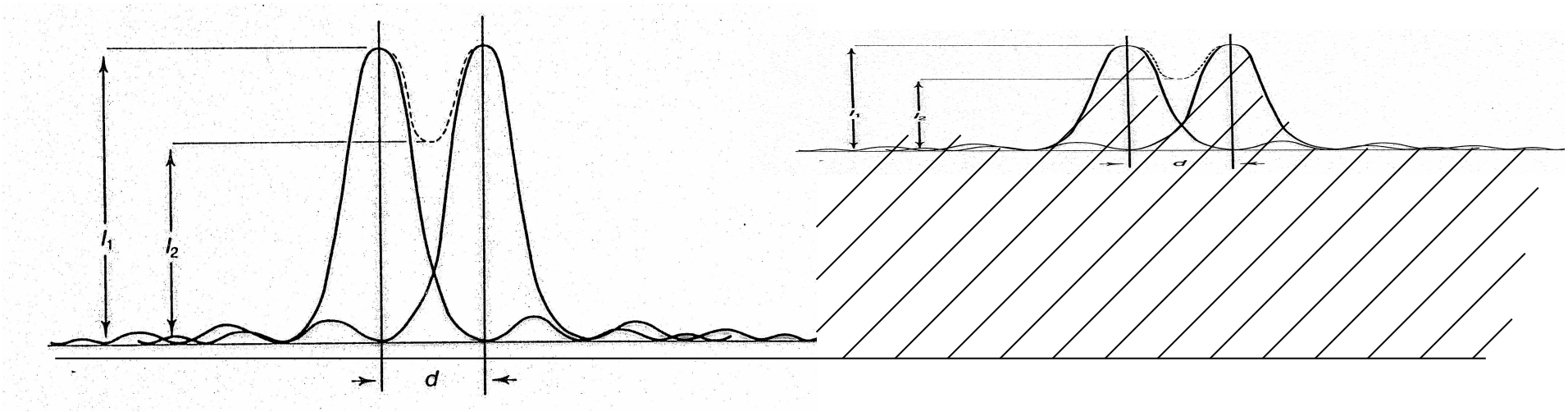
Rayliegh criterion: zero background



$I_2 / I_1 \sim 70\%$ $d=0.61\lambda / NA$ $NA=1.4$ $d=0.22\ \mu m$

What is the complex optics for?

The practice of microscopy,
or:
The fight for contrast.



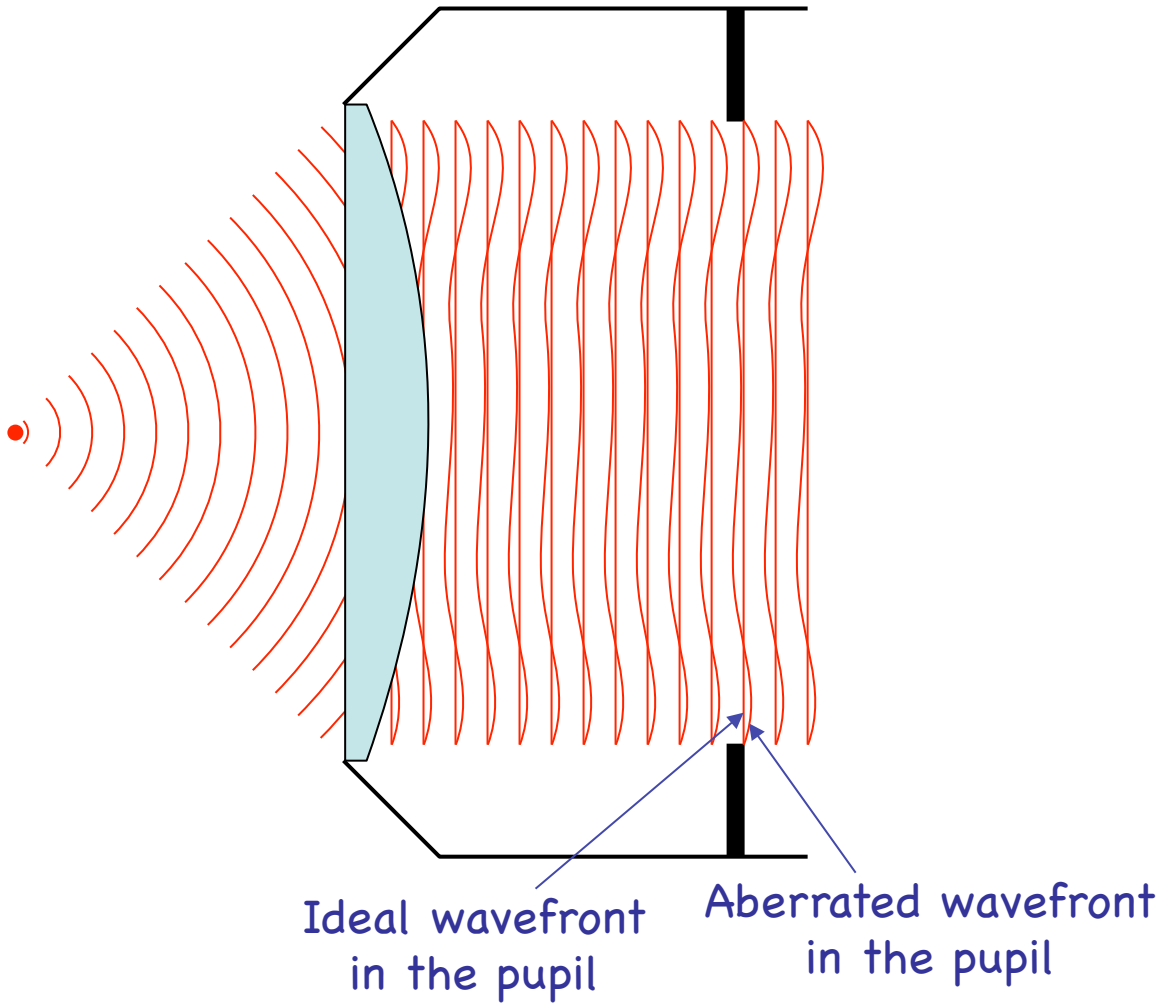
Aberrations

They are the enemy

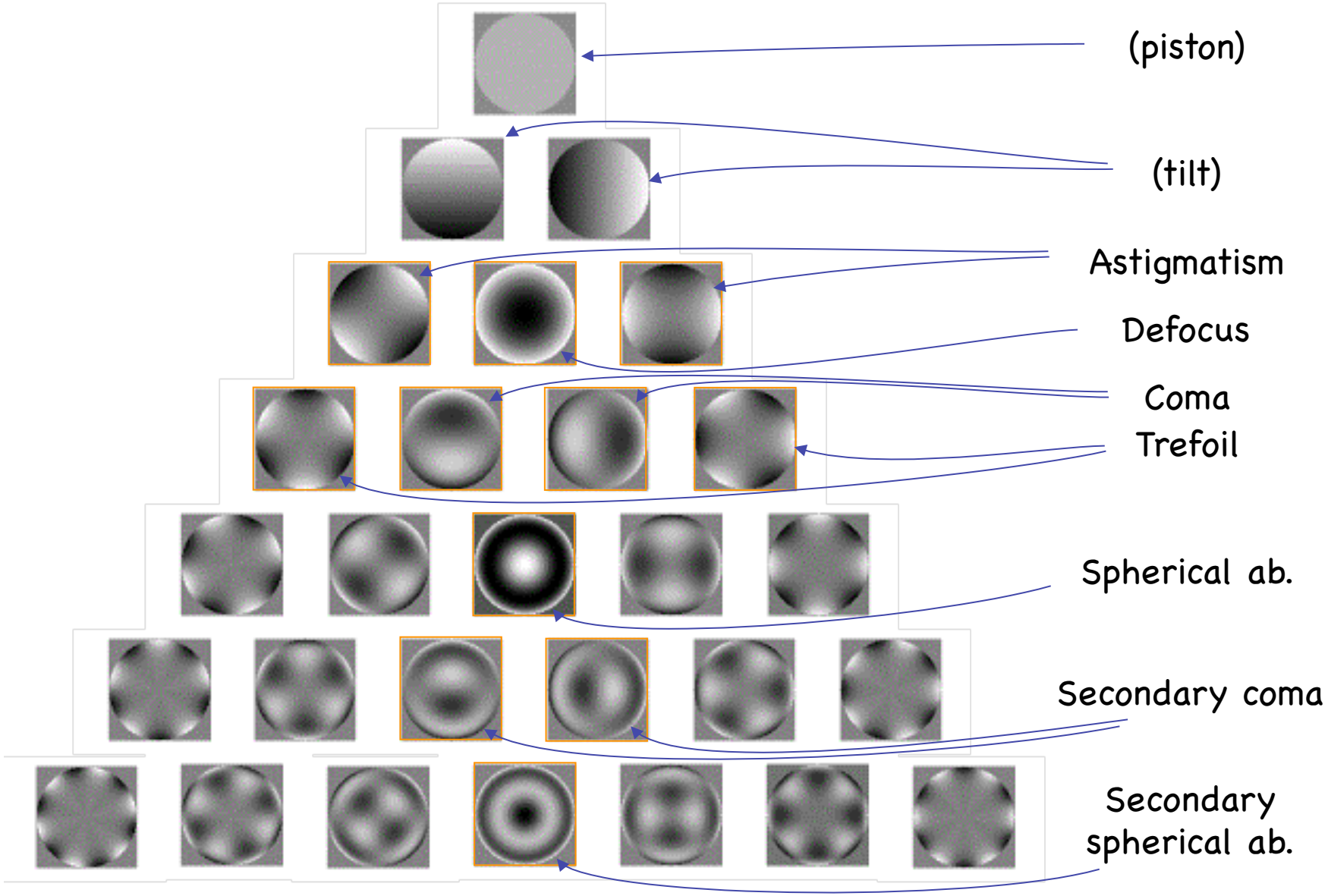
Aberrations: wave description

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

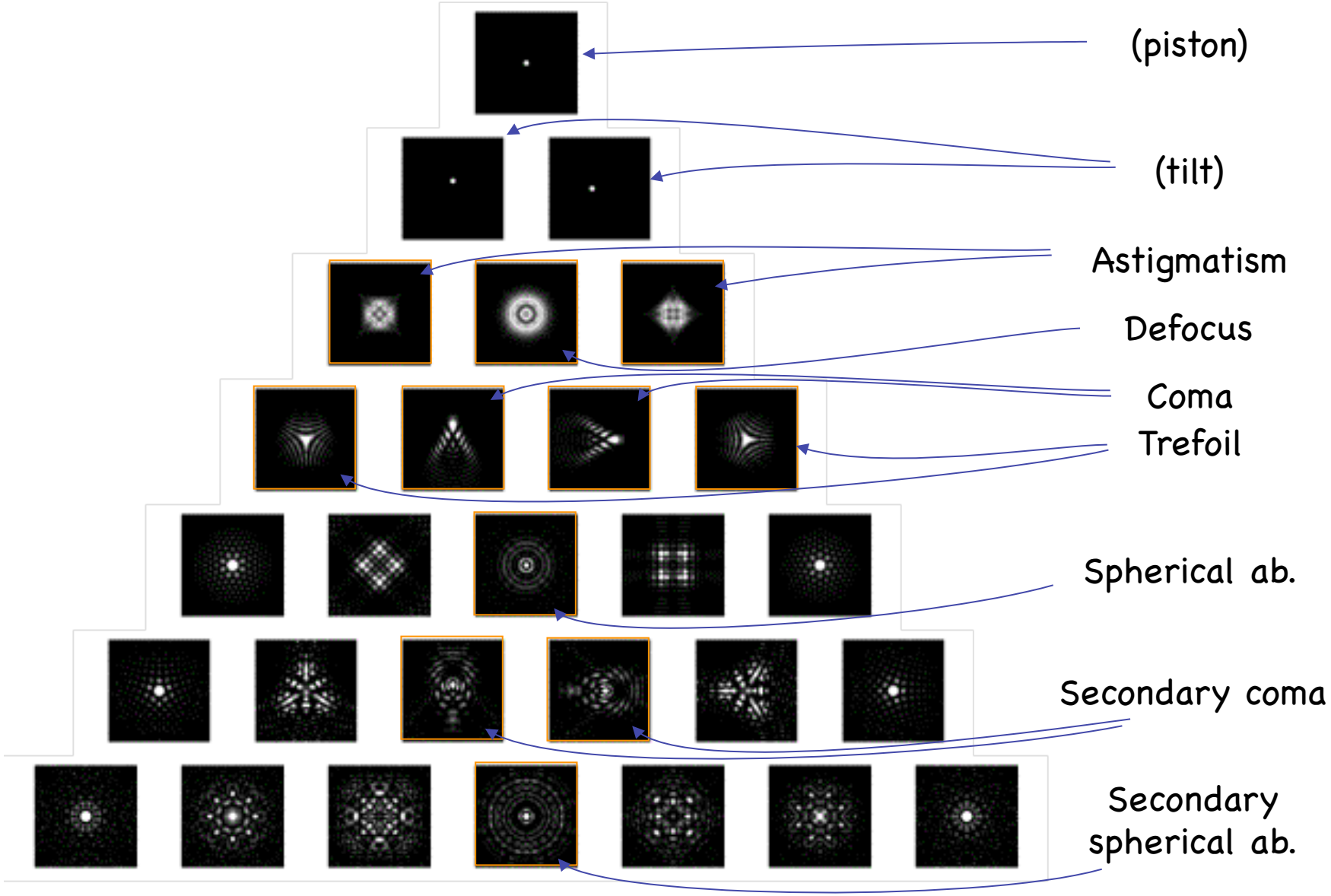
Wavefront Aberrations



Wavefront Aberrations



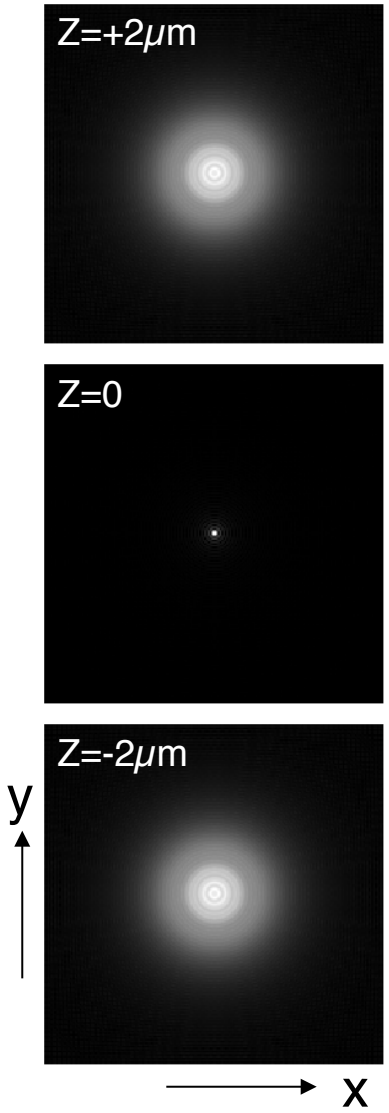
PSF Aberrations



The 3D Point Spread Function (PSF)

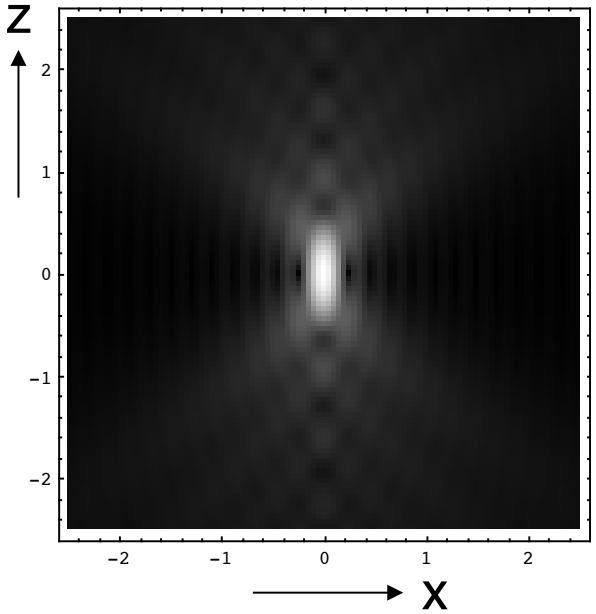
The image of a point object

2D PSF for different defocus

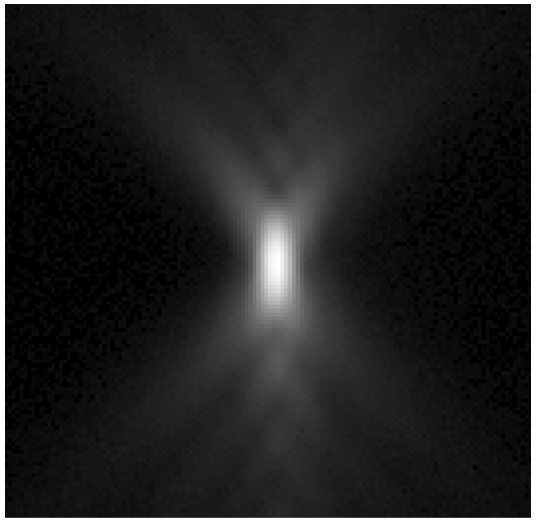


3D PSF

Calculated

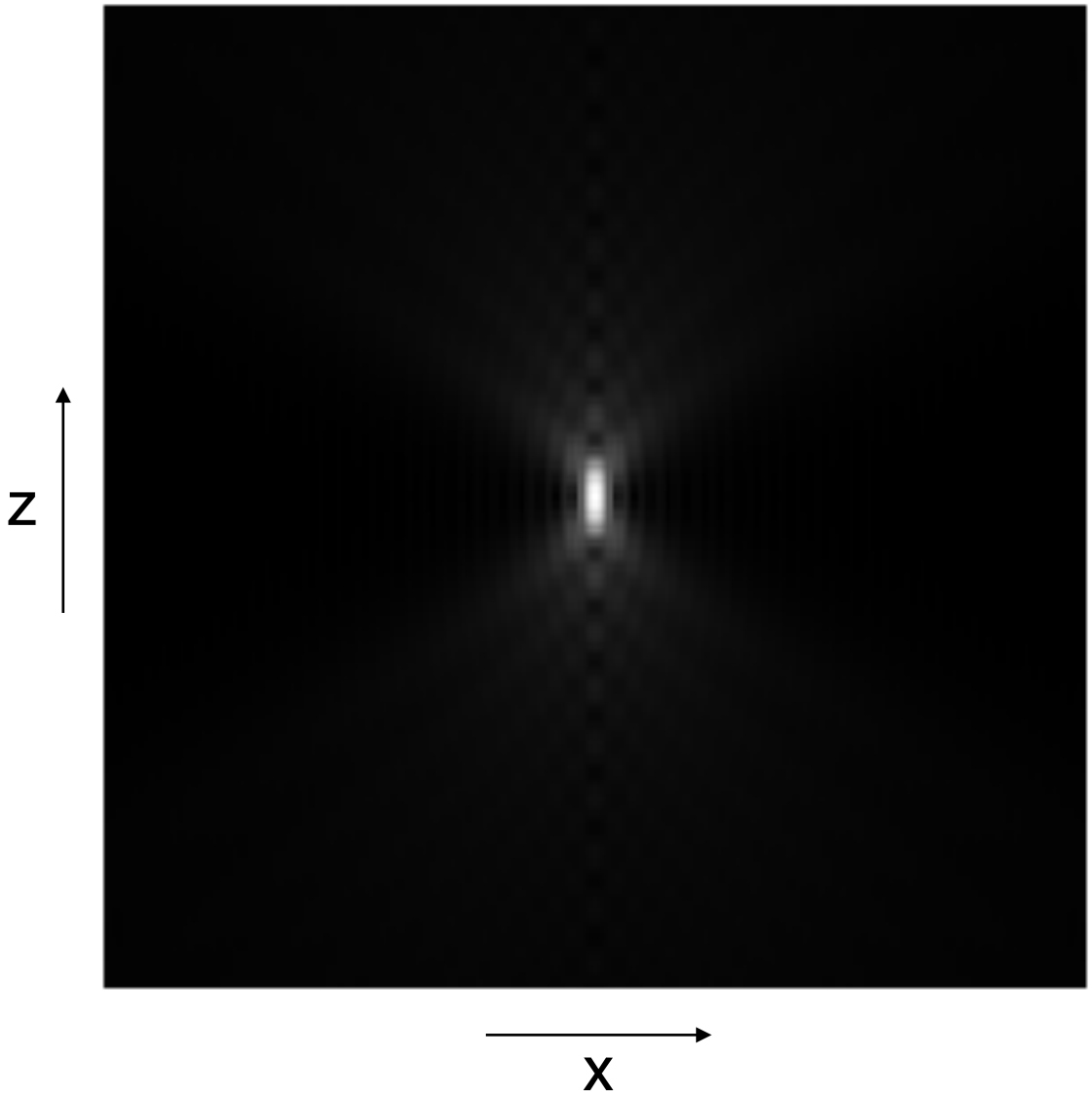


Measured



Spherical Aberration

Point spread function



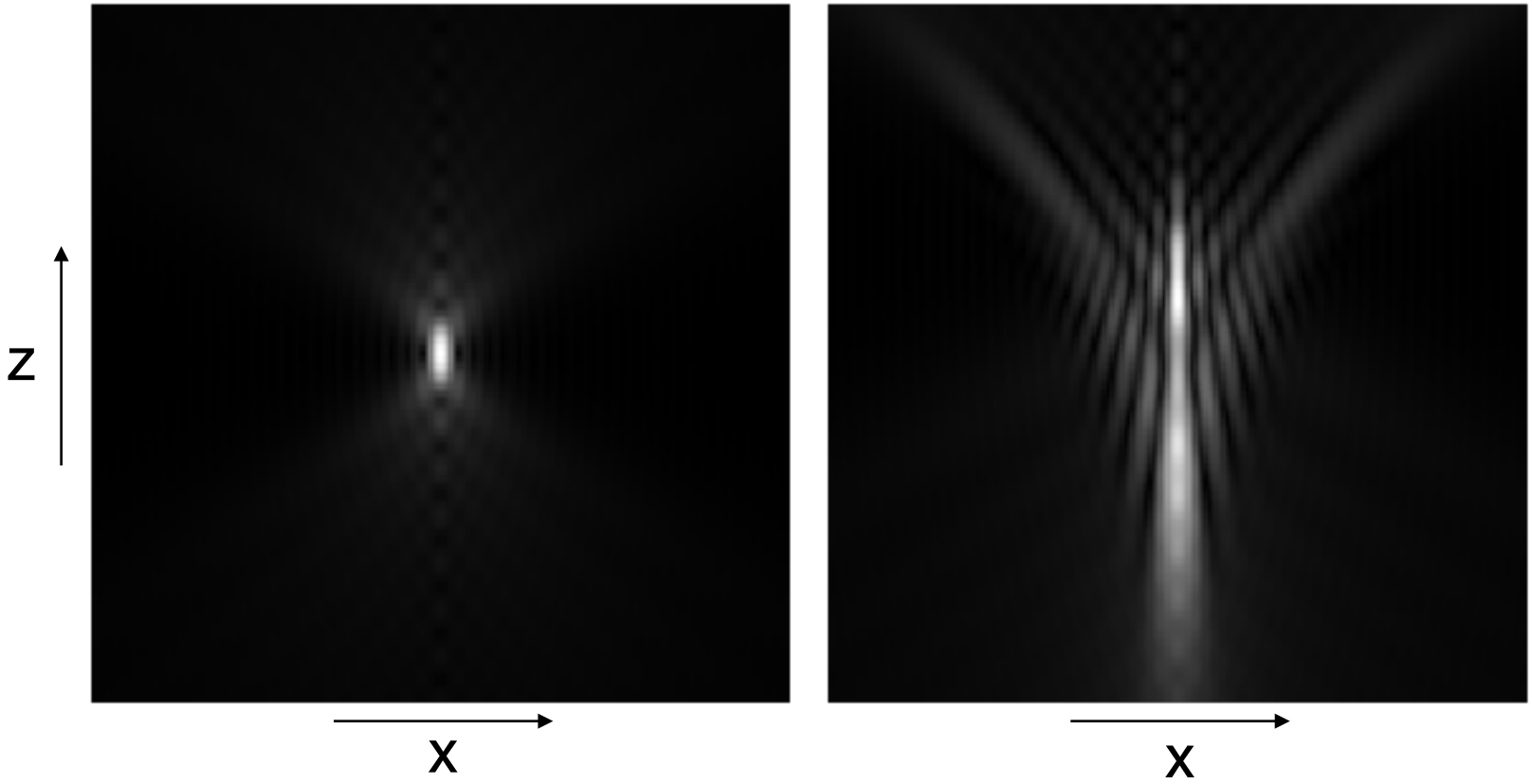
0-1 wave p-p
of primary
spherical ab.

Spherical Aberration

Point spread functions

Ideal

1 wave of spherical ab

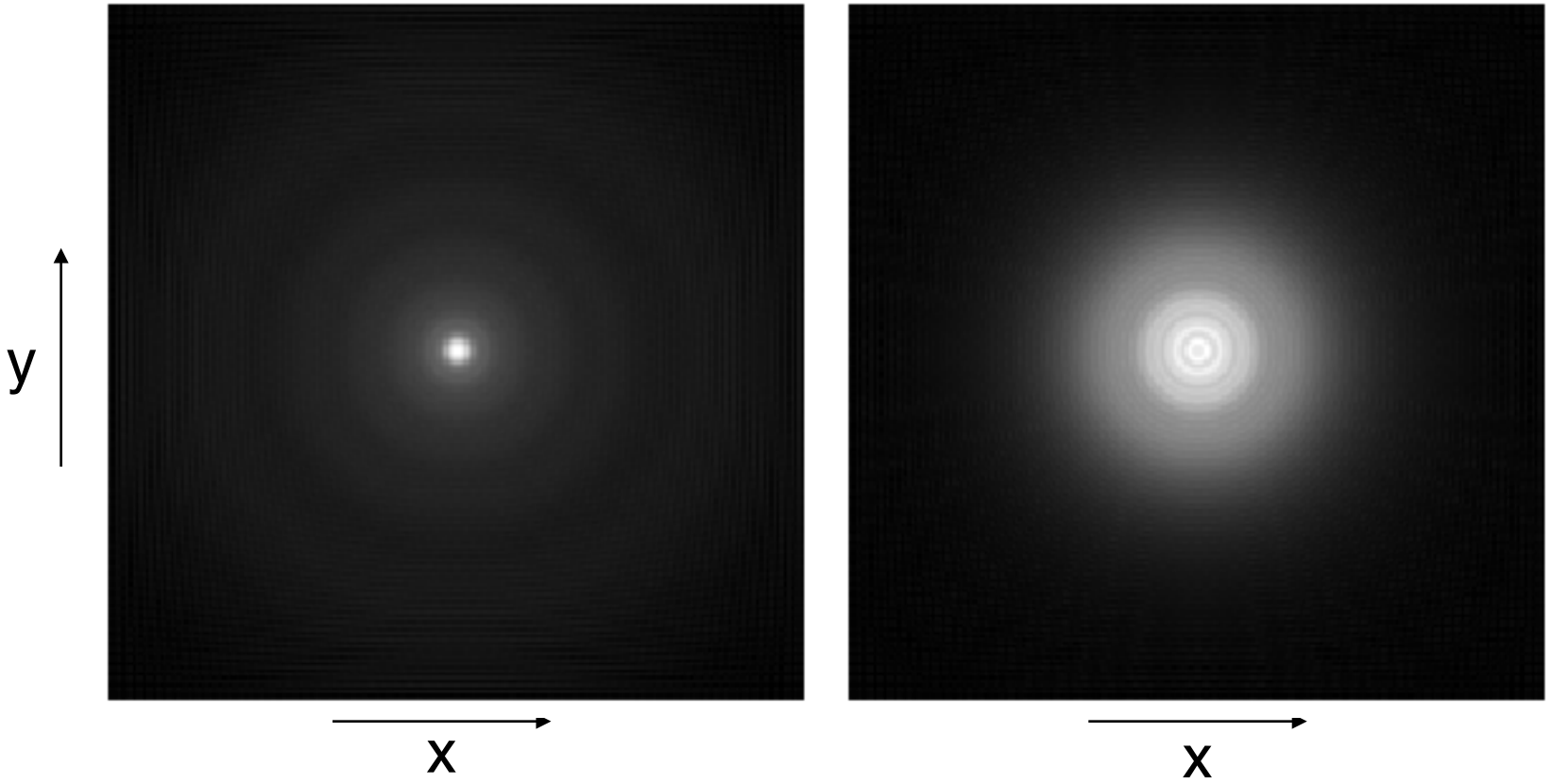


Spherical Aberration

Point spread functions

Ideal

1 wave of spherical ab



Sources of Spherical Aberration

Design compromises

Manufacturing tolerances

Immersion fluid index error

Temperature variation

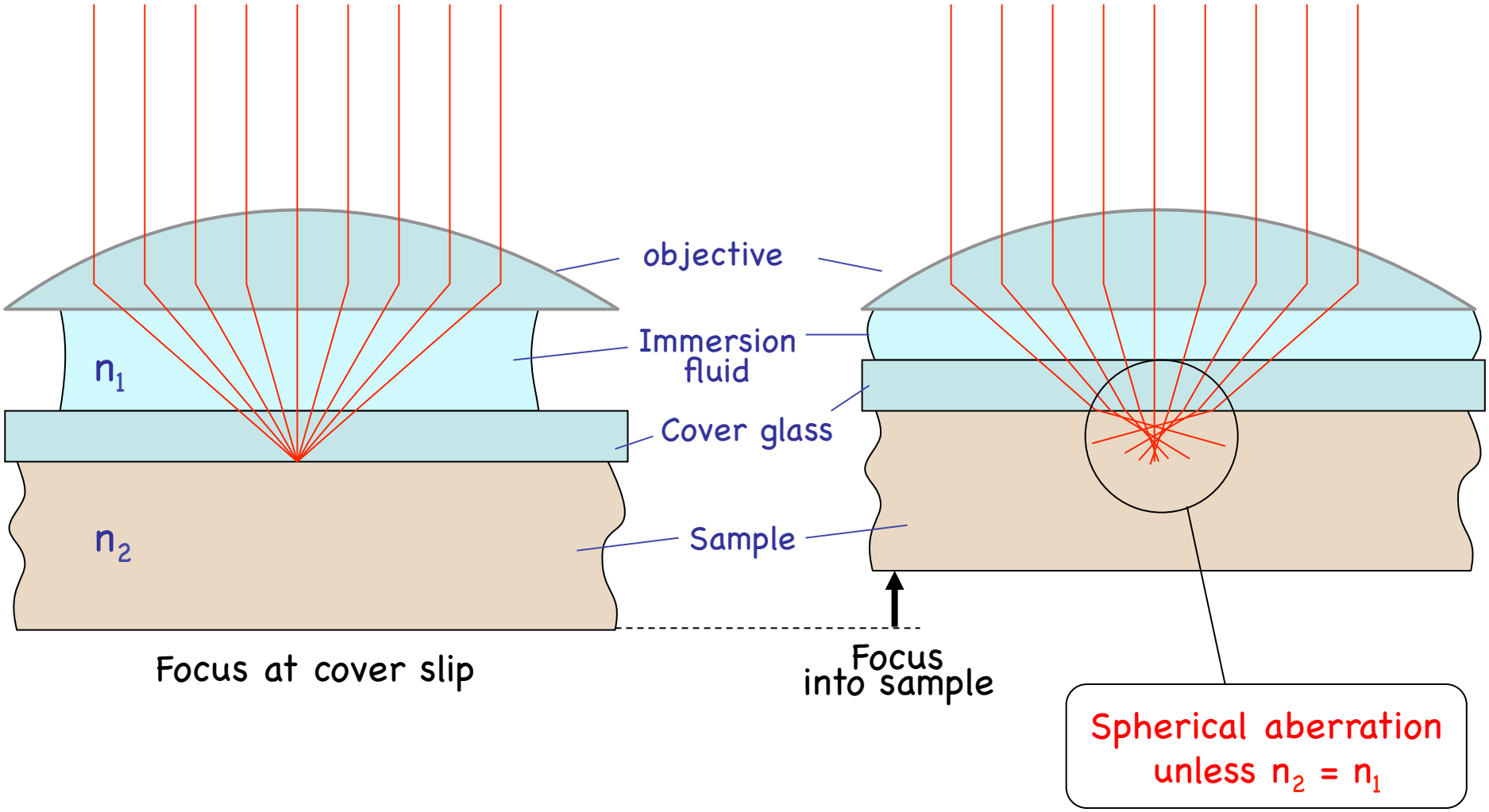
Cover slip thickness

(high-NA objectives except oil immersion)

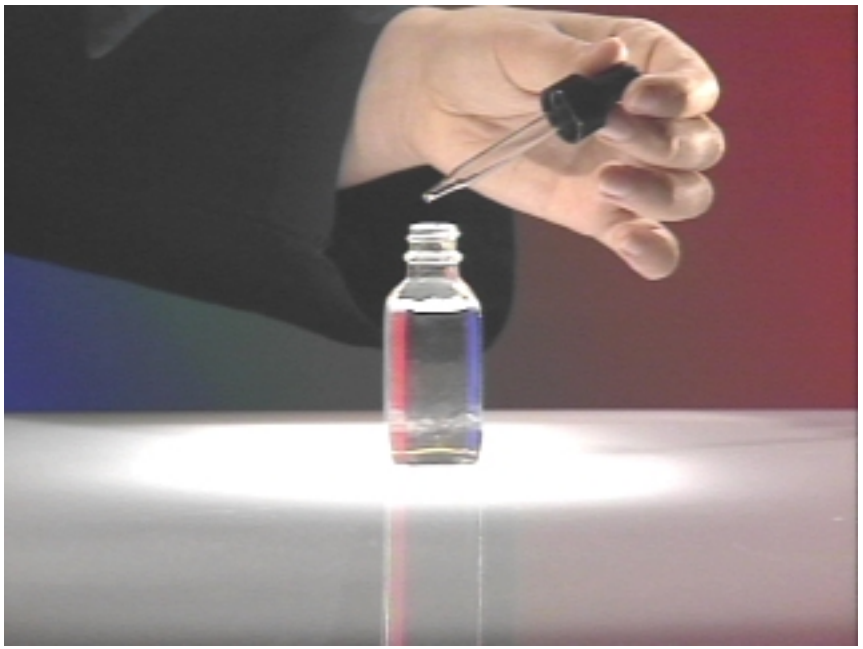
Correction collar setting

Sample refractive index mismatch

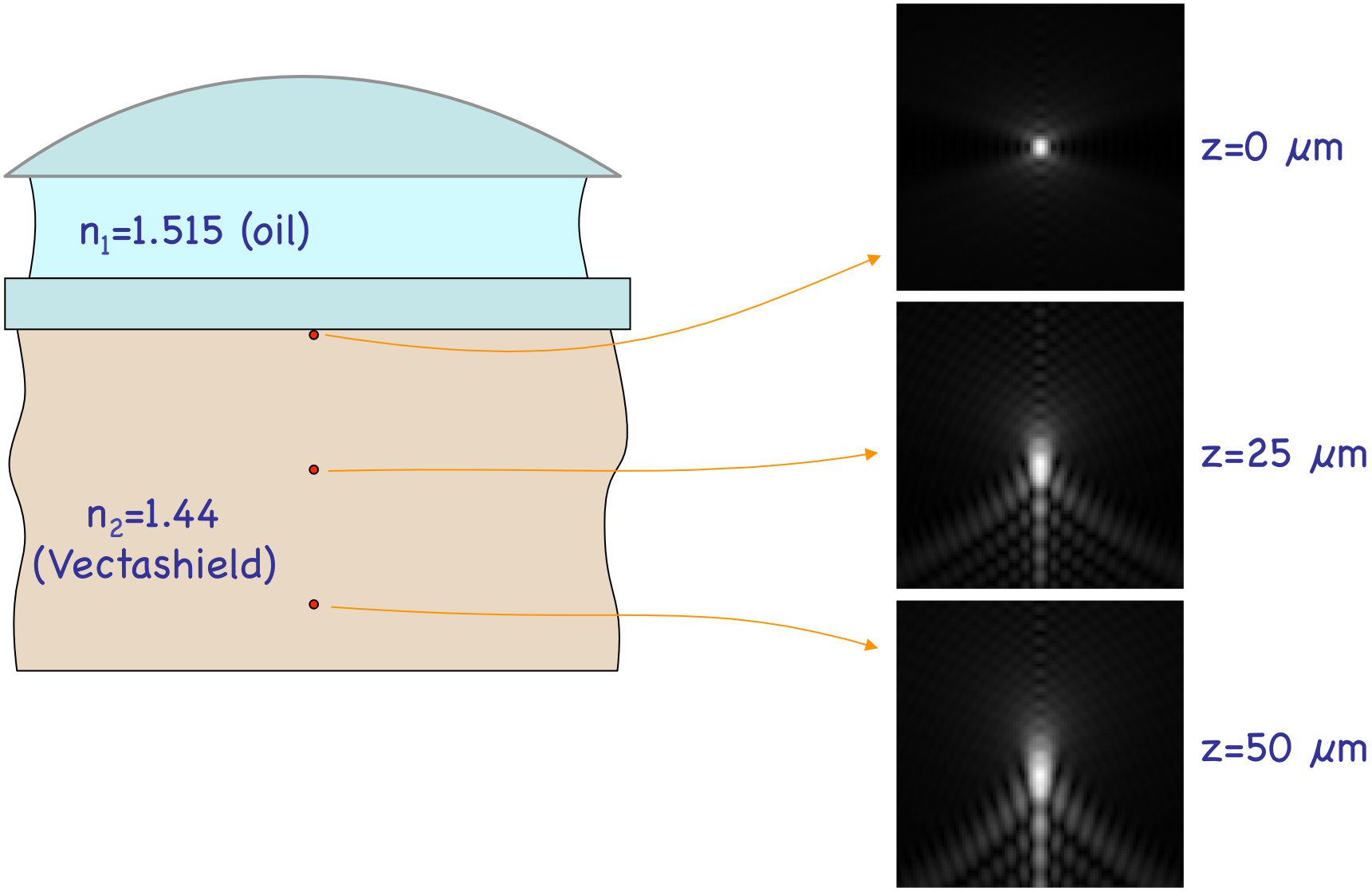
Index Mismatch
& Spherical Aberration



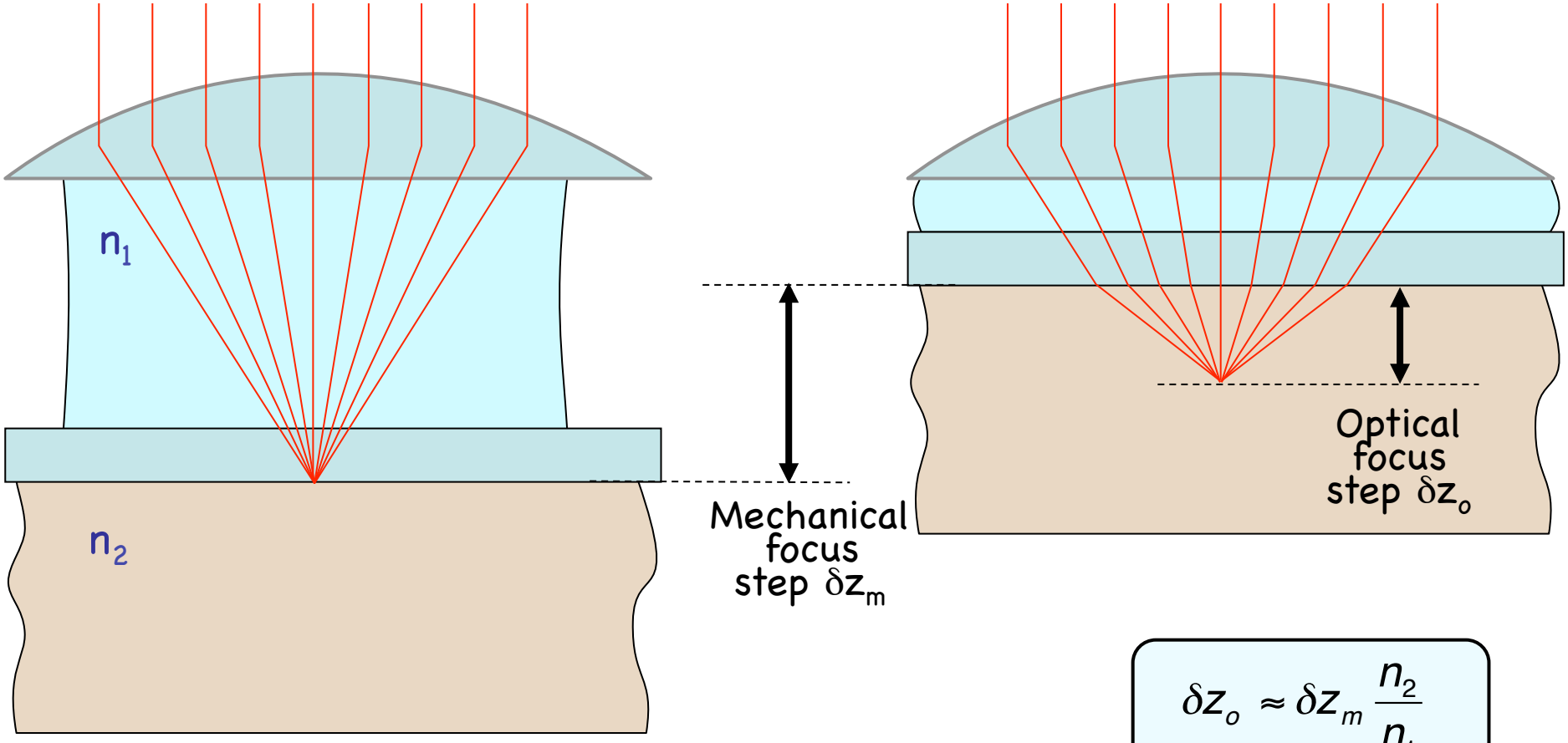
Index matching



Index Mismatch
& Spherical Aberration



Index Mismatch & Axial Scaling

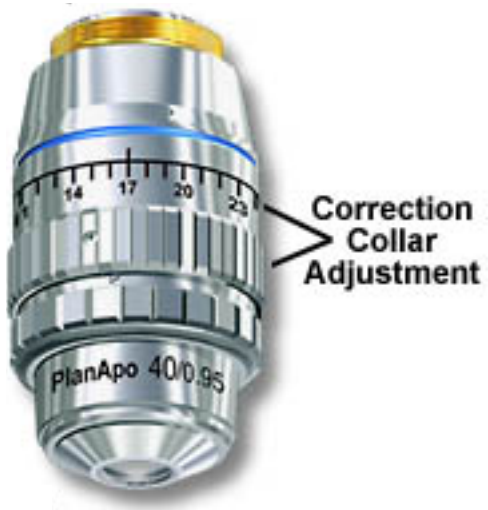


$$\delta z_o \approx \delta z_m \frac{n_2}{n_1}$$

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

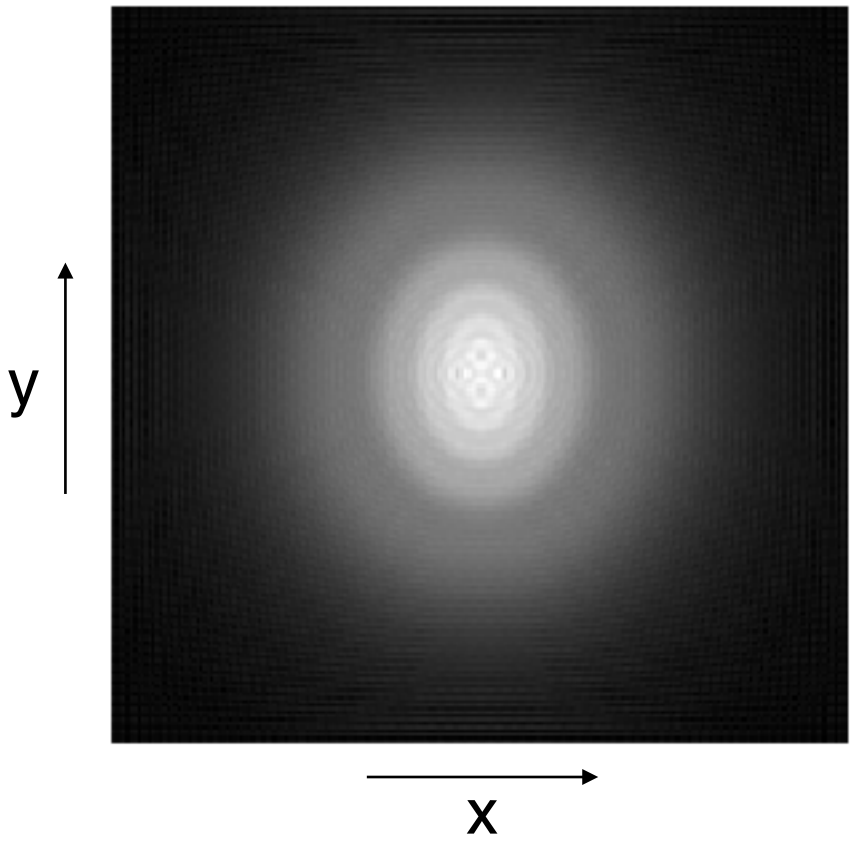
Getting Rid of Spherical Aberration

- Use correct and consistent cover slips
- Pick a room with stable temperature ≈ 20°C
- Use special immersion iof for 37°C live work
- Adjust correction collar
- If no collar, adjust immersion medium index
- **Use an objective that is matched to the mounting medium:**
  - For aqueous samples, use a water immersion objective
  - For fixed samples viewed with oil immersion objectives, ideally use a mounting medium with index≈1.515
  - For fixed samples in commercial media, ideally use a glycerol immersion objective



Astigmatism

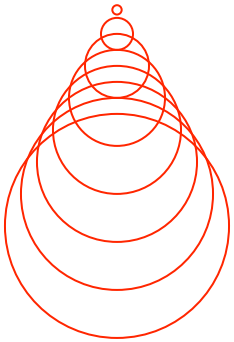
Point spread function

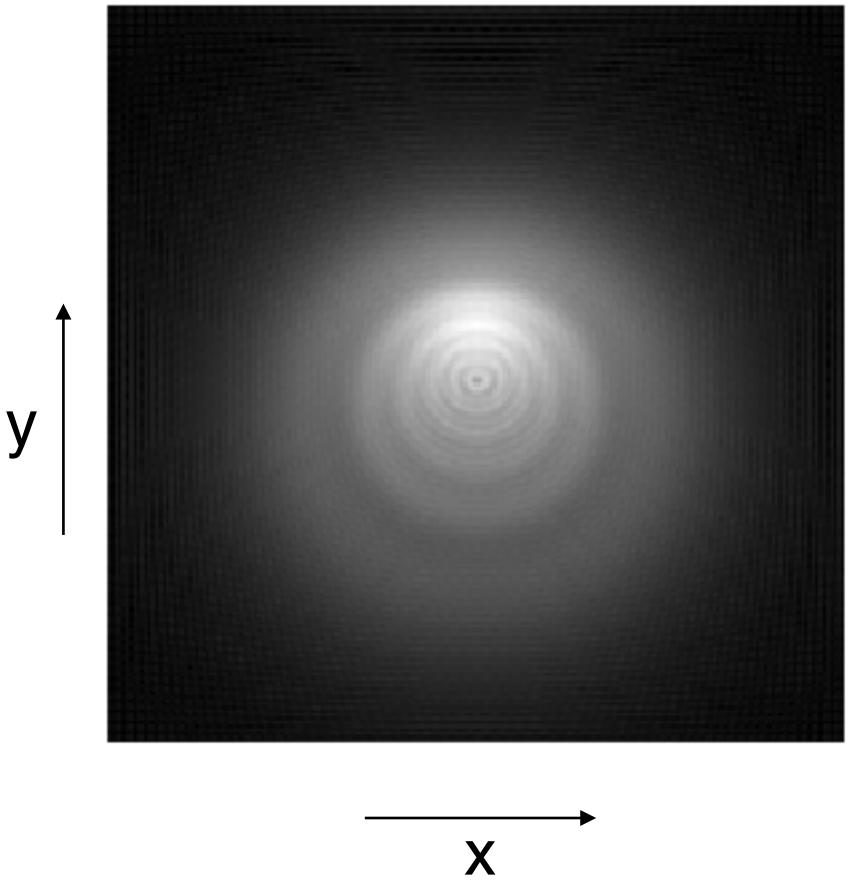


1 wave p-p of
x-y astigmatism

Coma

Point spread function



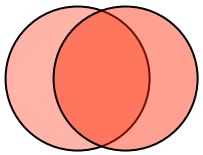


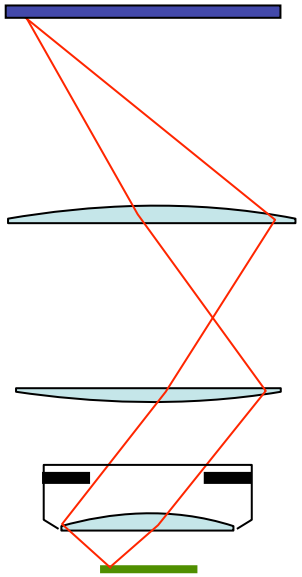
1 wave p-p of
y coma

Sources of Astigmatism & Coma

Off-axis (edges of field of view)

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

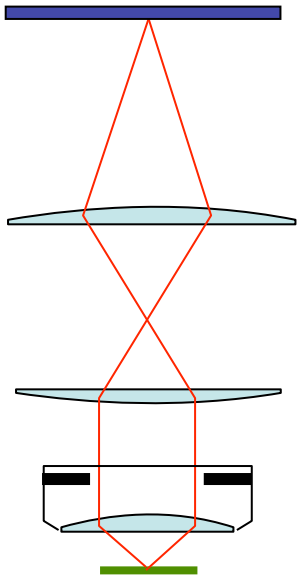




On-axis (center of field of view)

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

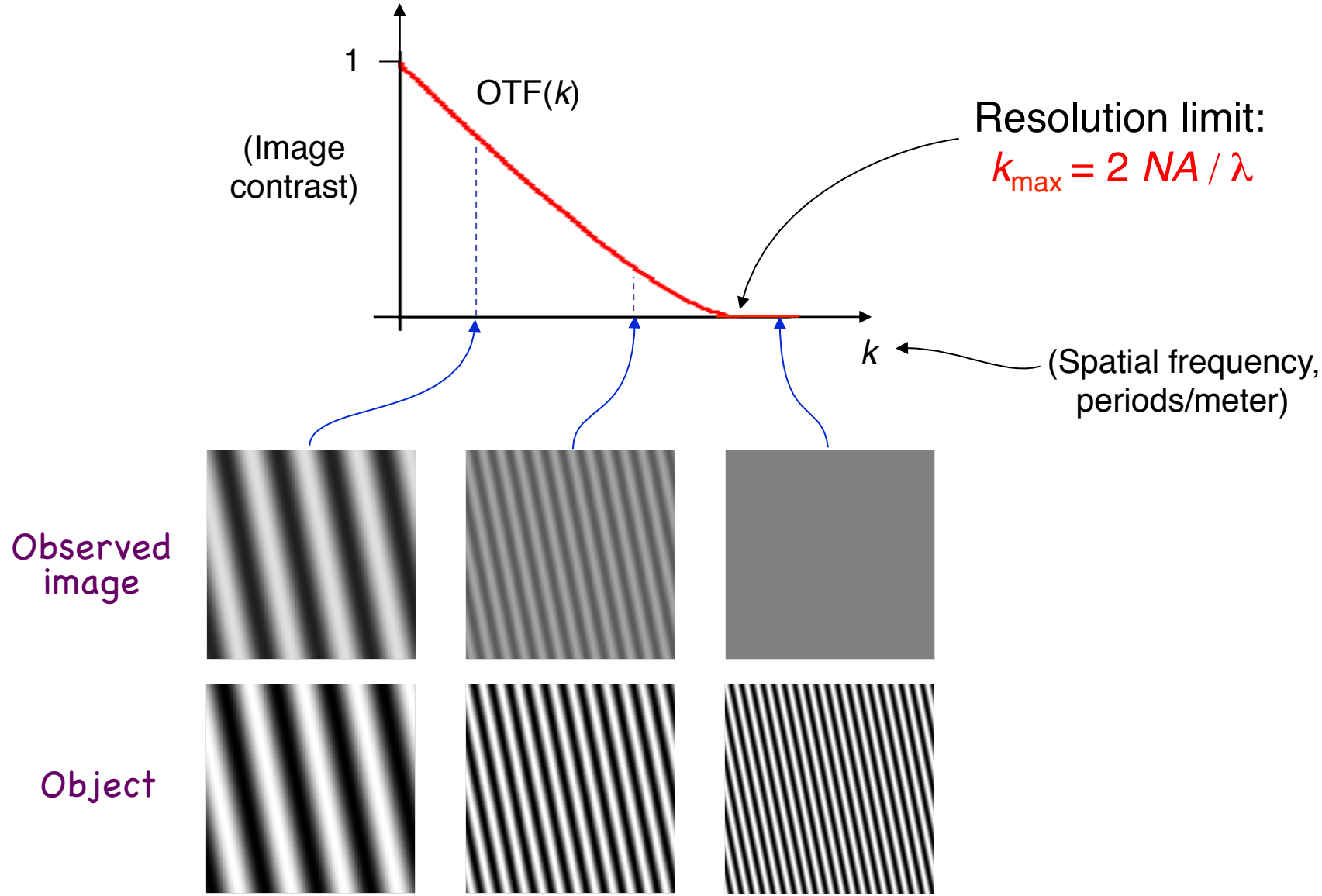
- manufacturing or assembly tolerances
- dirt or abuse
- Misalignment (tilt, off-axis shift of something)
- bad downstream components (mirrors, dichroics, filters...)
- **Air bubble** in the immersion fluid or sample
- **Tilted cover slip**
  (dry and water-immersion high-NA lenses)



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

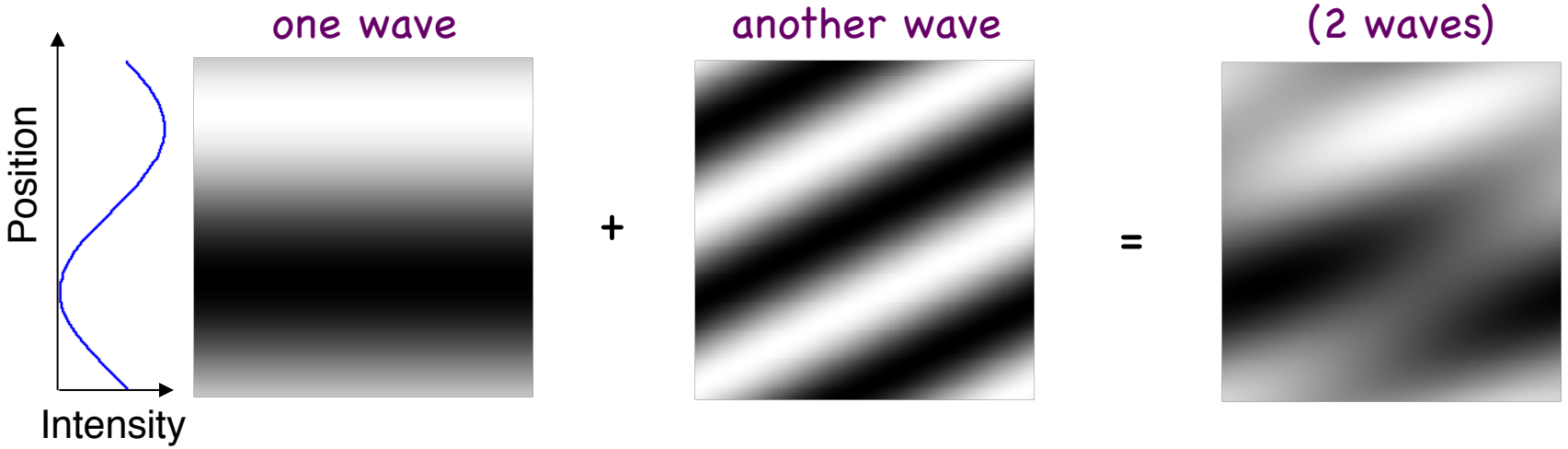
For the record only, not for this course
Attribute to Mats Gustafsson

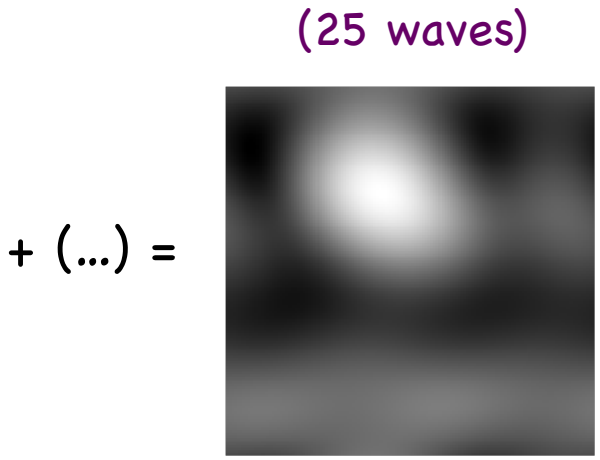
The response to pure waves is well-defined by the Optical Transfer Function (OTF)

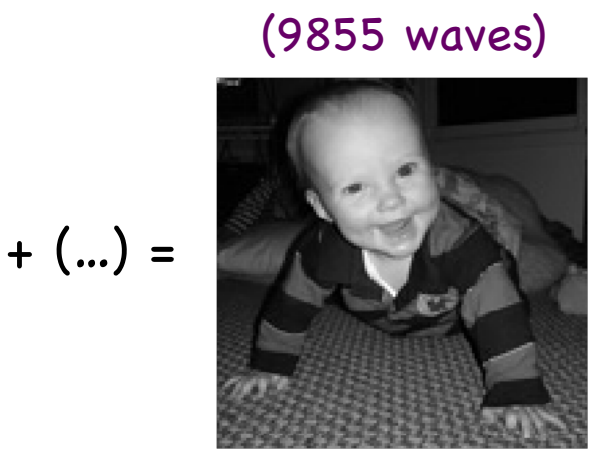


Think of Images as Sums of Waves

... or "spatial frequency components"







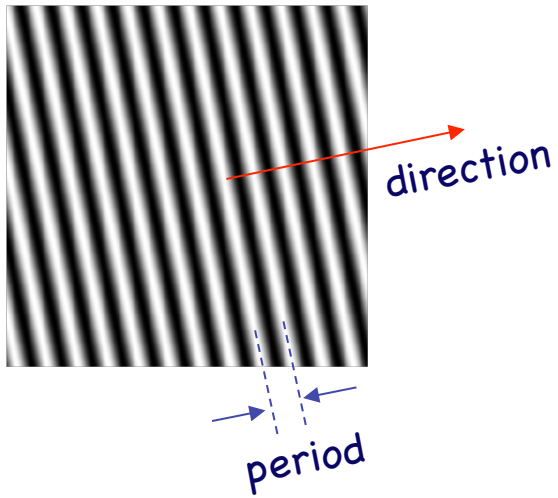
Frequency Space

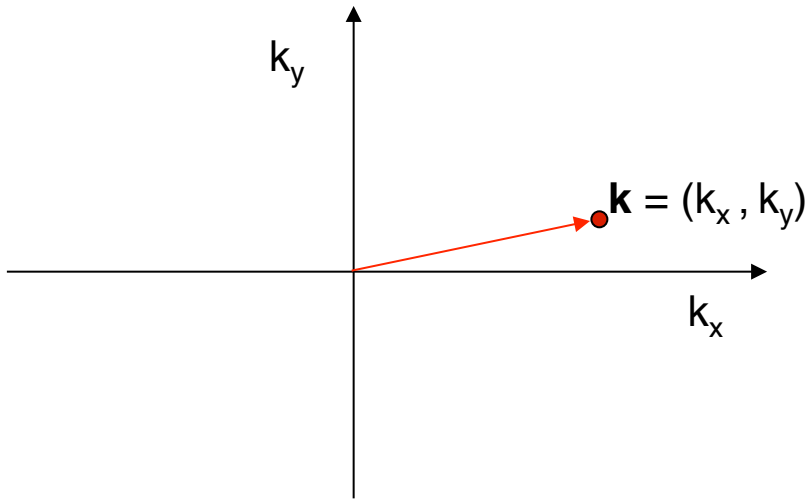
To ***describe*** a wave,
we need to specify its:

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

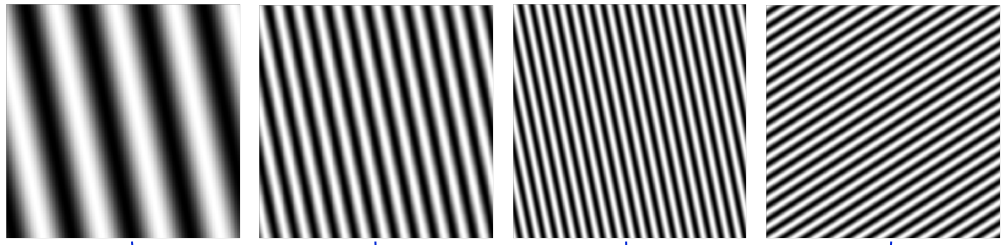
- Frequency (how many periods/meter?) → Distance from origin
- Direction → Direction from origin
- Amplitude (how strong is it?) → Magnitude of value
- Phase (where are the peaks & troughs?) → Phase of value

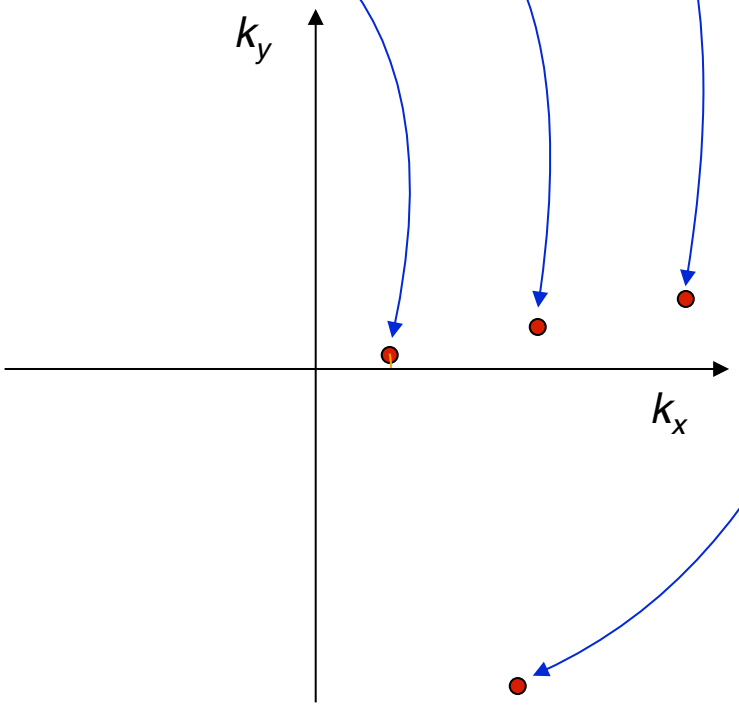
complex



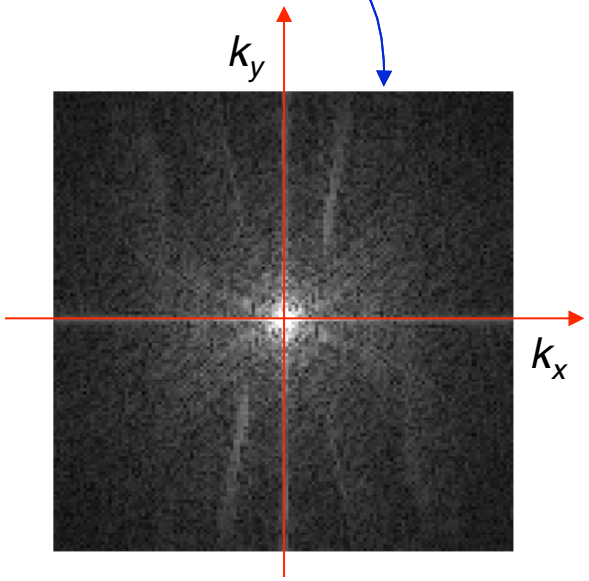


Frequency Space
and the *Fourier Transform*





Fourier Transform



Properties of the Fourier Transform

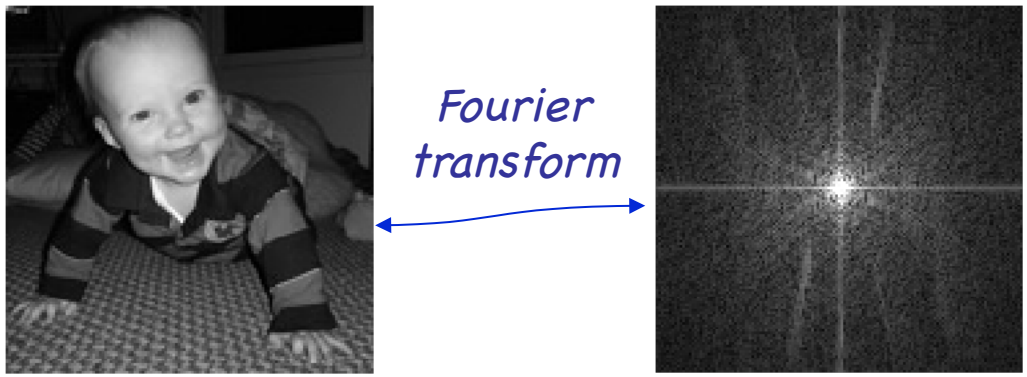
$$F(\mathbf{k}) = \int f(\mathbf{r}) e^{2\pi i \mathbf{k}\cdot\mathbf{r}} d\mathbf{r}$$

Completeness:

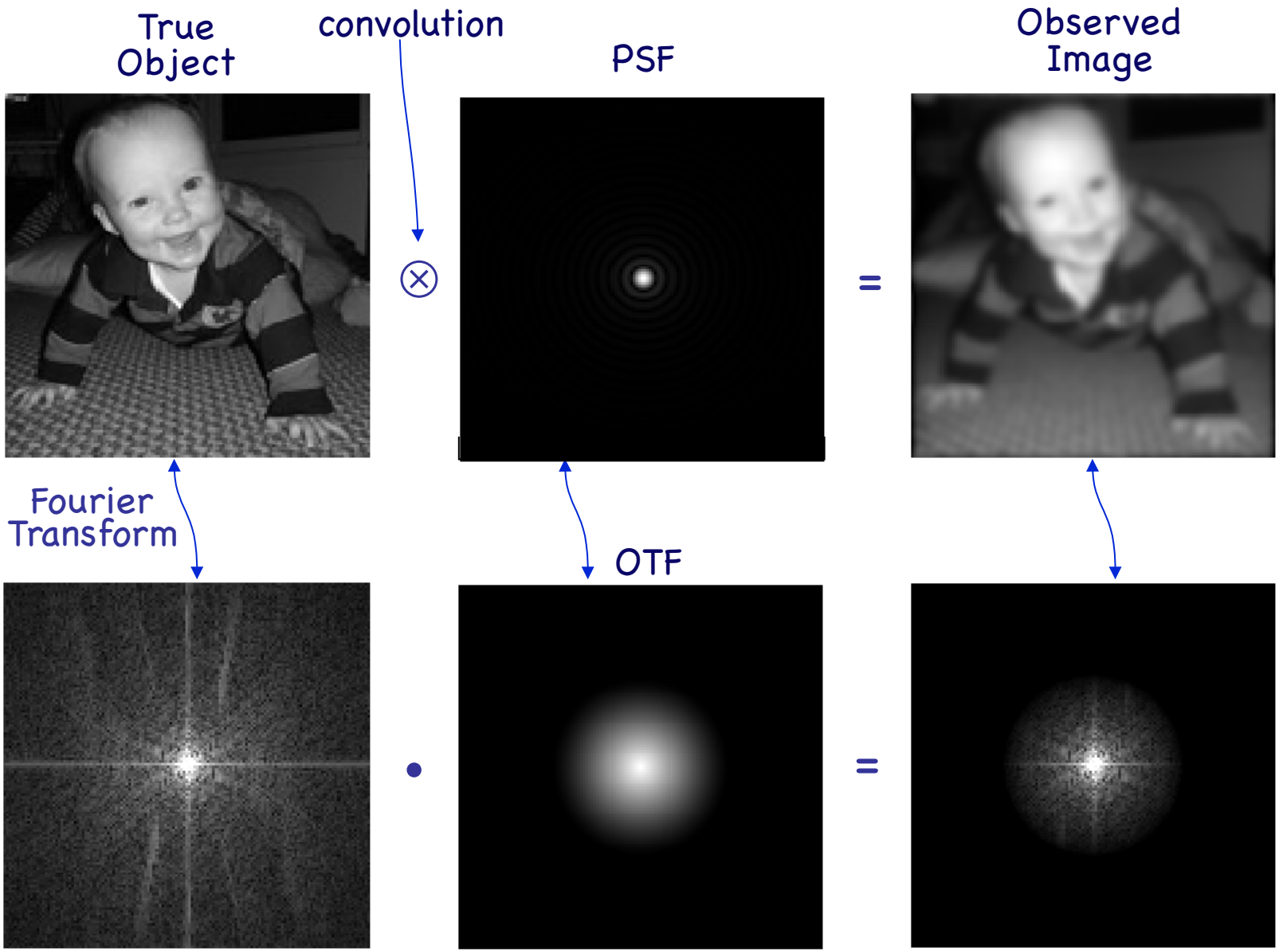
The Fourier Transform contains **all** the information of the original image

Symmetry:

The Fourier Transform of the Fourier Transform is the original image



The OTF and the PSF are Fourier transforms of each other



Convolutions

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

Why do we care?

- They are everywhere…
- The **convolution theorem**:

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

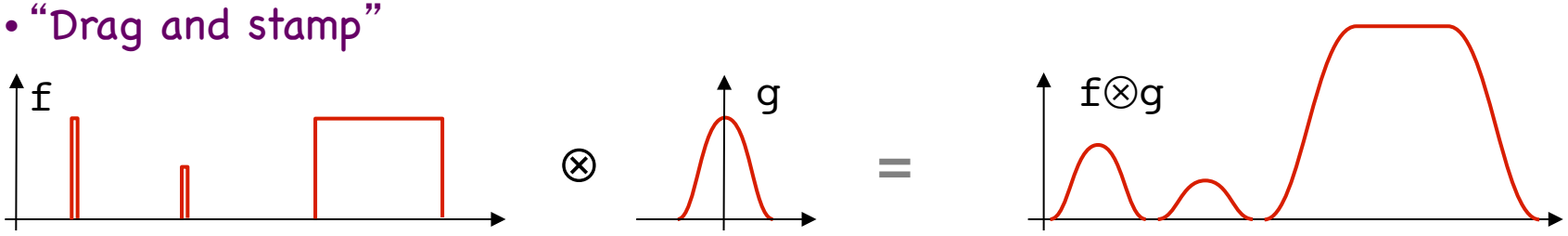
then $\tilde{h}(\mathbf{k}) = \tilde{f}(k)\, \tilde{g}(\mathbf{k})$

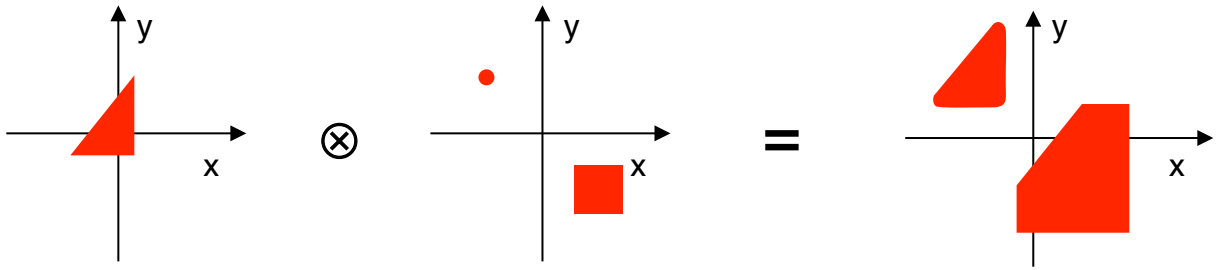
A convolution in real space becomes a product in frequency space & vice versa

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

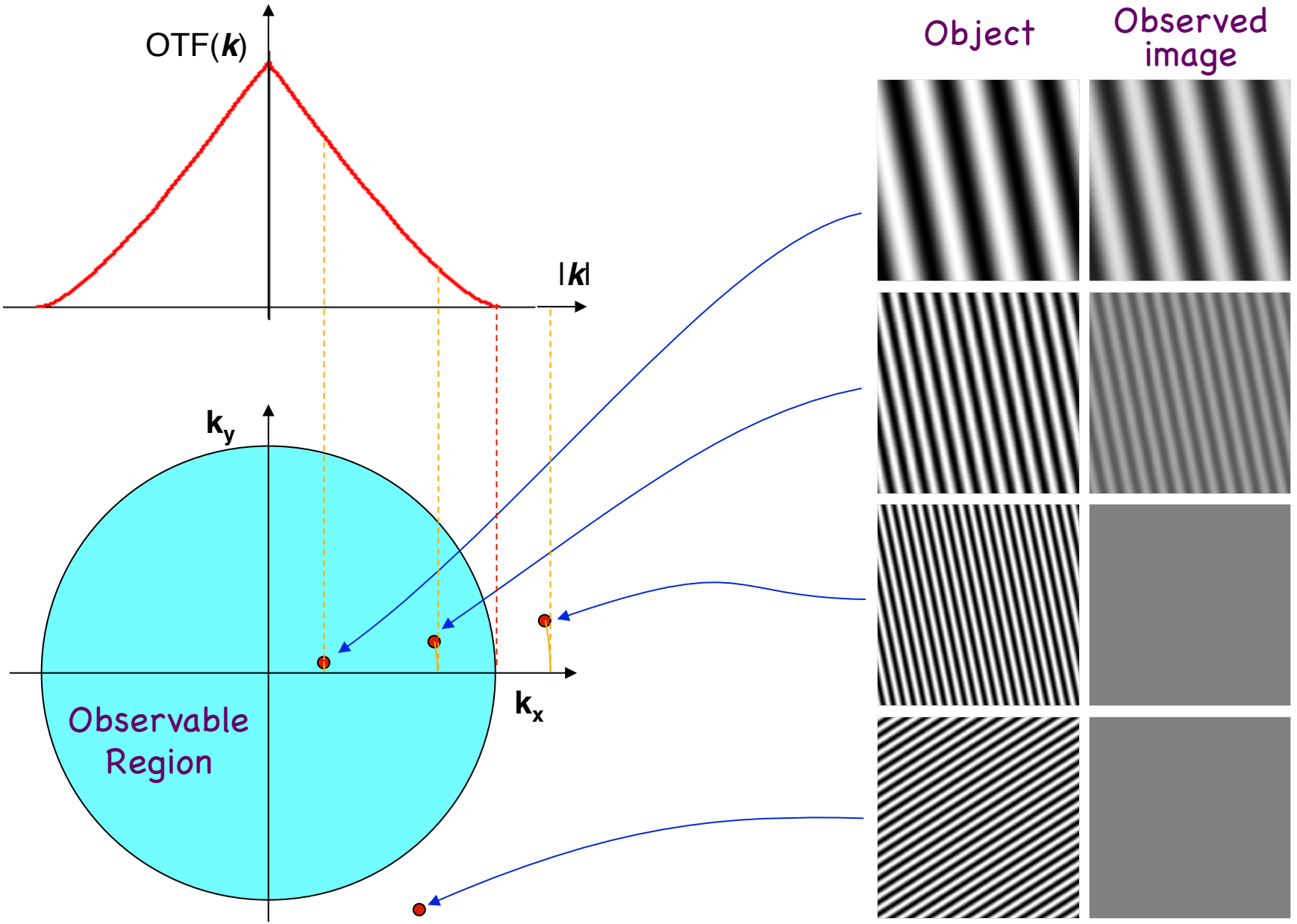
So what **is** a convolution, intuitively?

- "Blurring"
- "Drag and stamp"

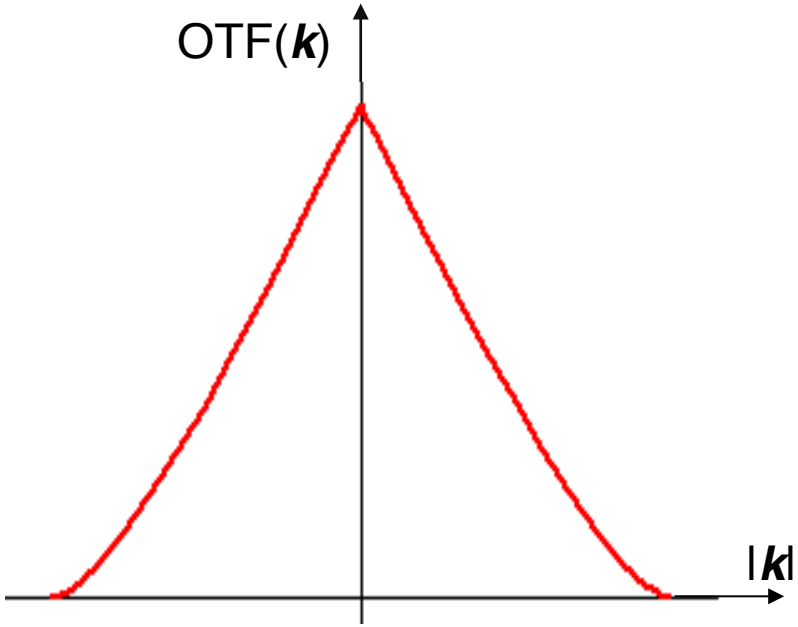




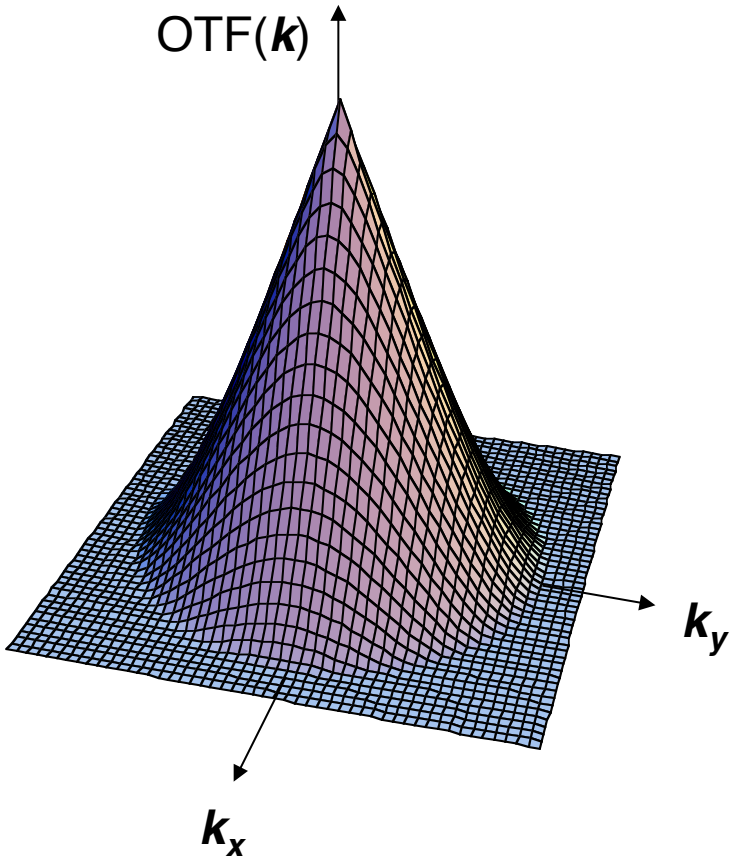
The Transfer Function Lives in Frequency Space



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

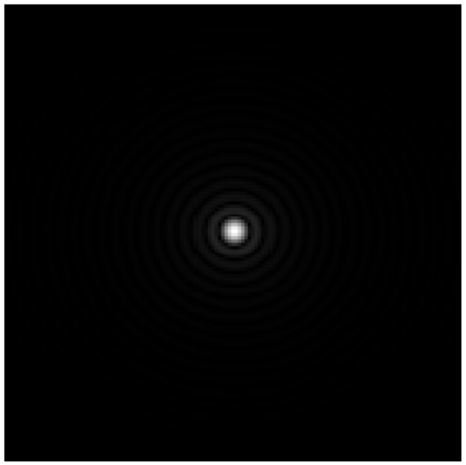


(Idealized calculations)



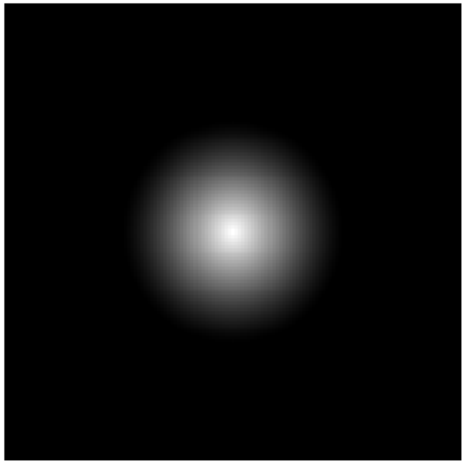
The 3D OTF

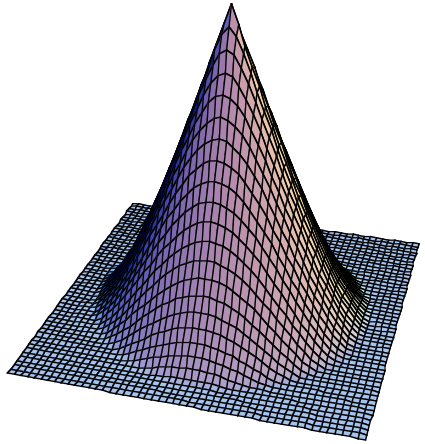
2D PSF



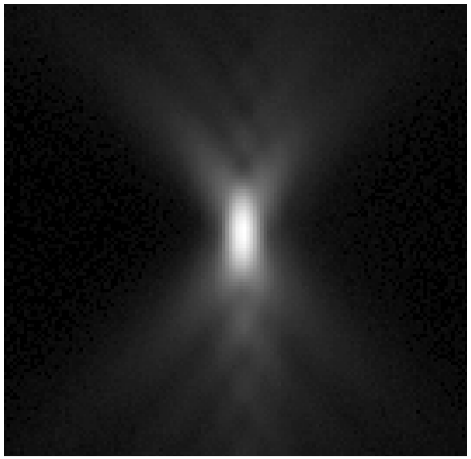
2D F.T.

2D OTF



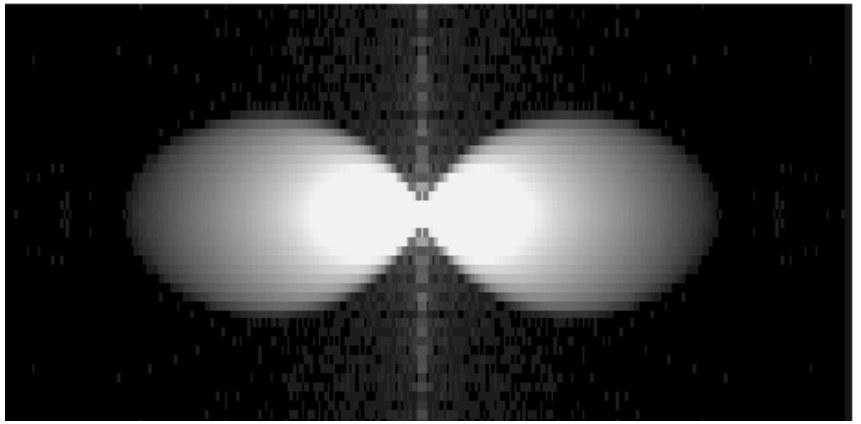


3D PSF

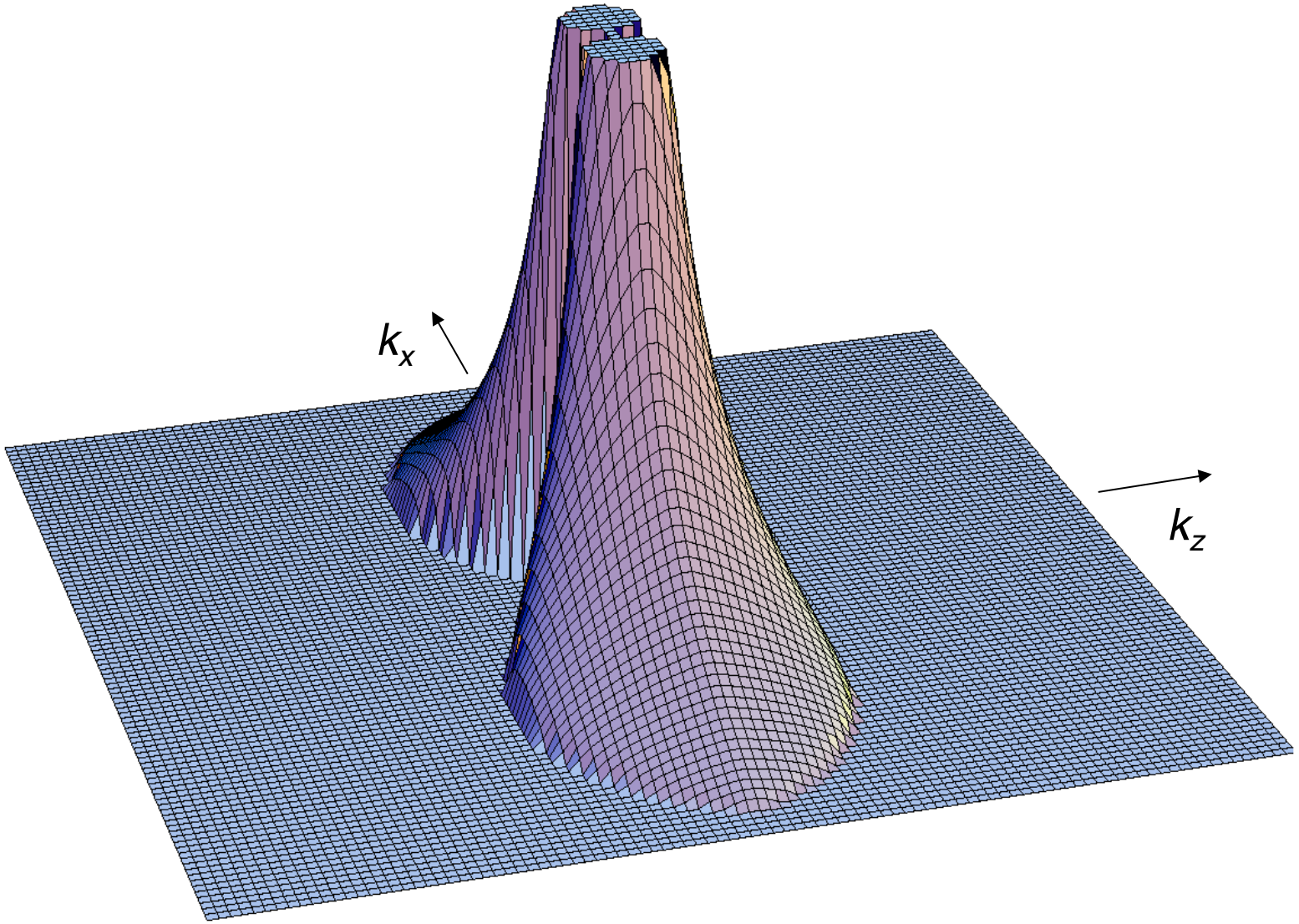


3D F.T.

3D OTF



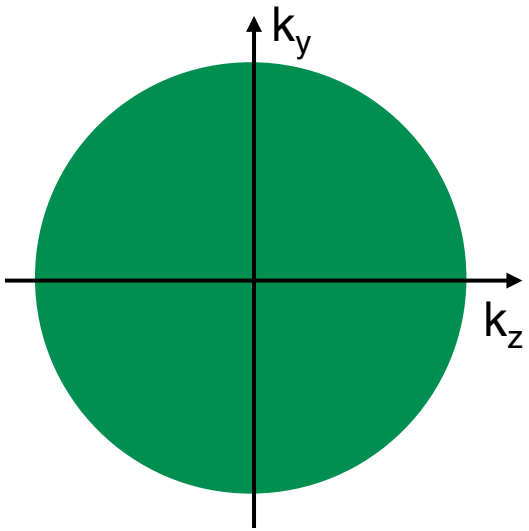
Values of the 3D OTF

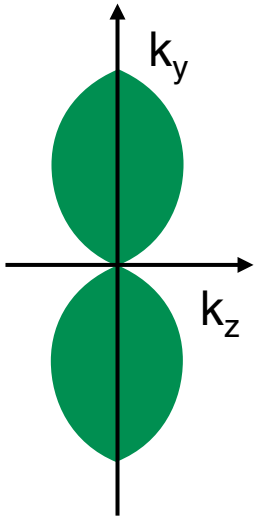


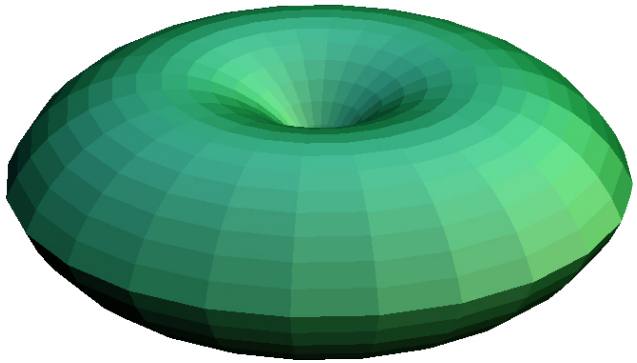
3D Observable Region

= OTF support

= Region where the OTF is non-zero







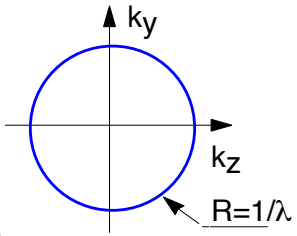
Why this weird region?

Monochromatic light

In free space

Monochromatic light has only one wavelength λ → only one $|\boldsymbol{k}| = 1 / \lambda$

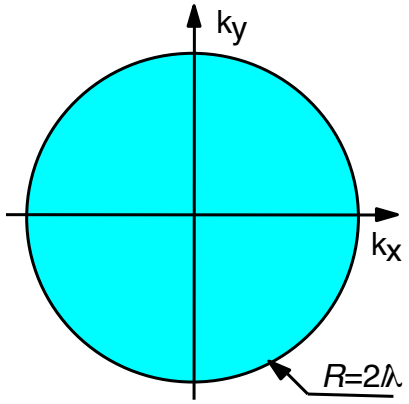
support of $\widetilde{A}(\boldsymbol{k})$ (amplitude)



→ The light amplitude $A(\mathbf{i})$ has Fourier components only on a shell of radius $1 / \lambda$

The intensity $I(\boldsymbol{r}) = |A(\boldsymbol{r})|^2$

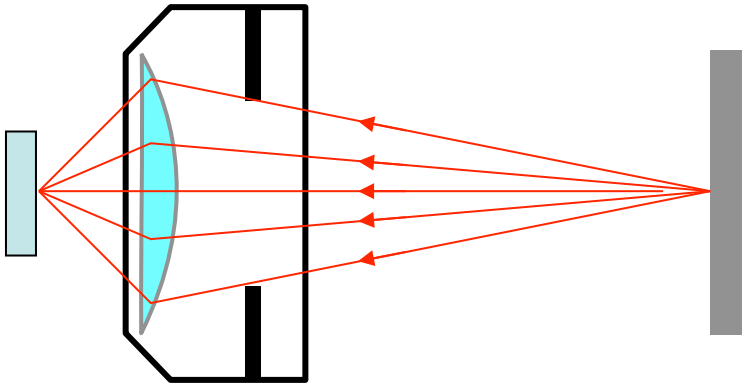
support of $\widetilde{I}(\boldsymbol{k})$ (intensity)



In reciprocal space, this product A^*A becomes a convolution $\widetilde{A} \otimes \widetilde{A}$ of the shell with itself, which is a sphere of twice the radius

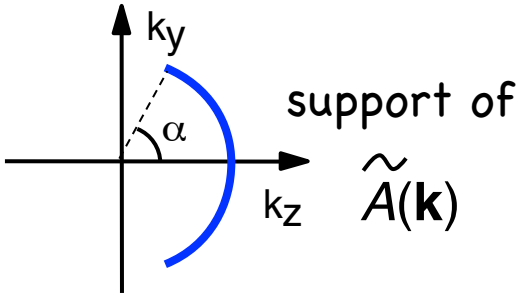
This sphere indicates all frequency components of the light intensity that can ever be non-zero

Consider light **from** a camera pixel **into** the sample.
What are the Fourier components of this light?



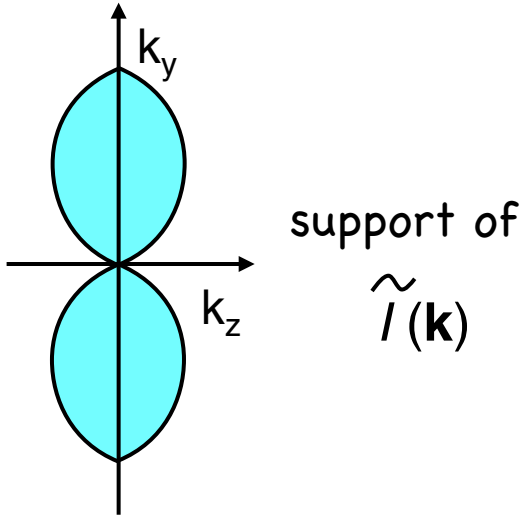
Light enters the sample only
from those directions
allowed by the aperture angle α

Only a bowl-shaped segment of the shell
makes it through the objective:

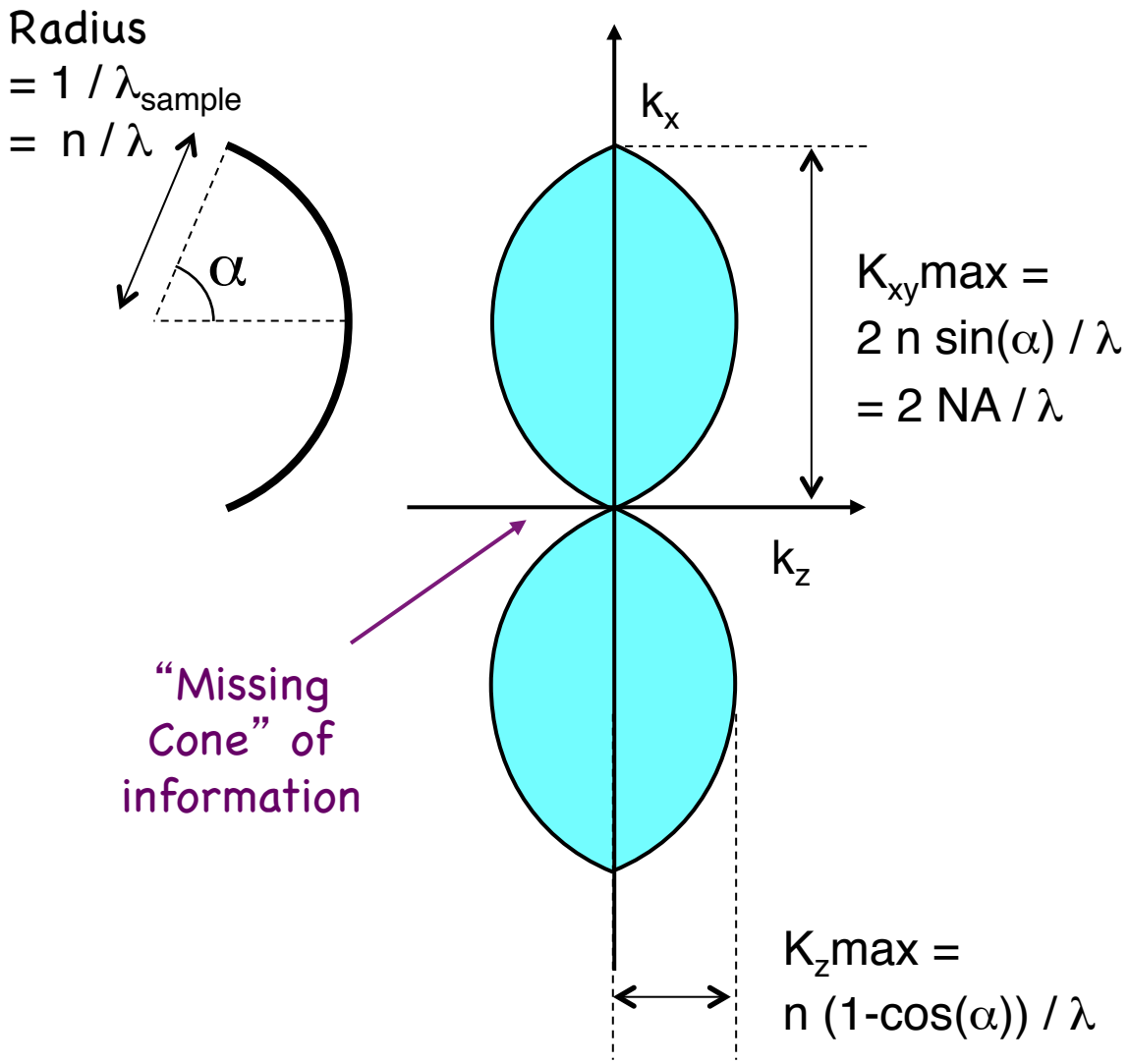


The intensity now becomes
a convolution of the **bowl** with itself,
which has a a donut-shaped
region of support.

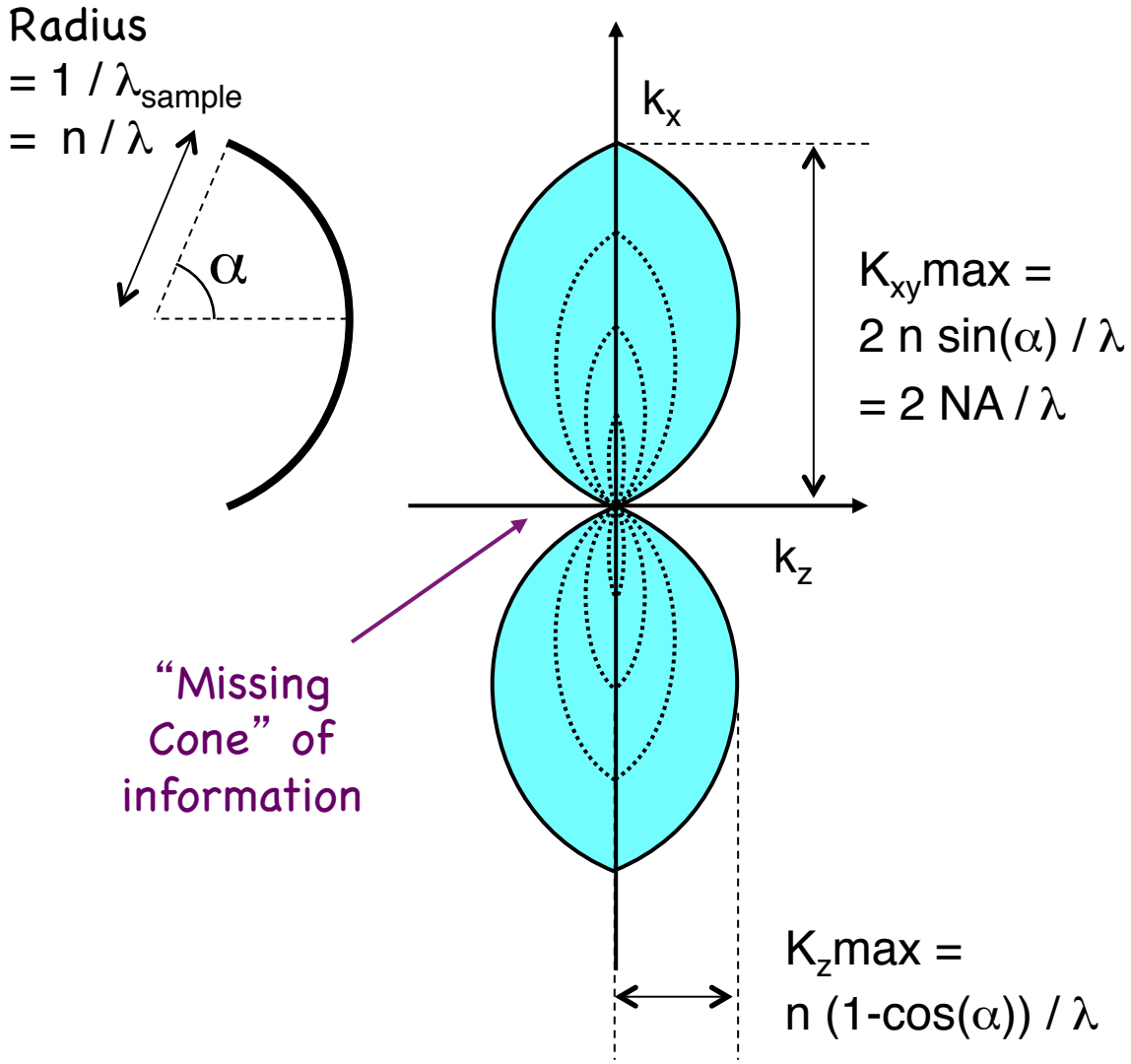
This is the **OTF support**:
the set of sample frequencies that can
be detected through the microscope



So what is the resolution?



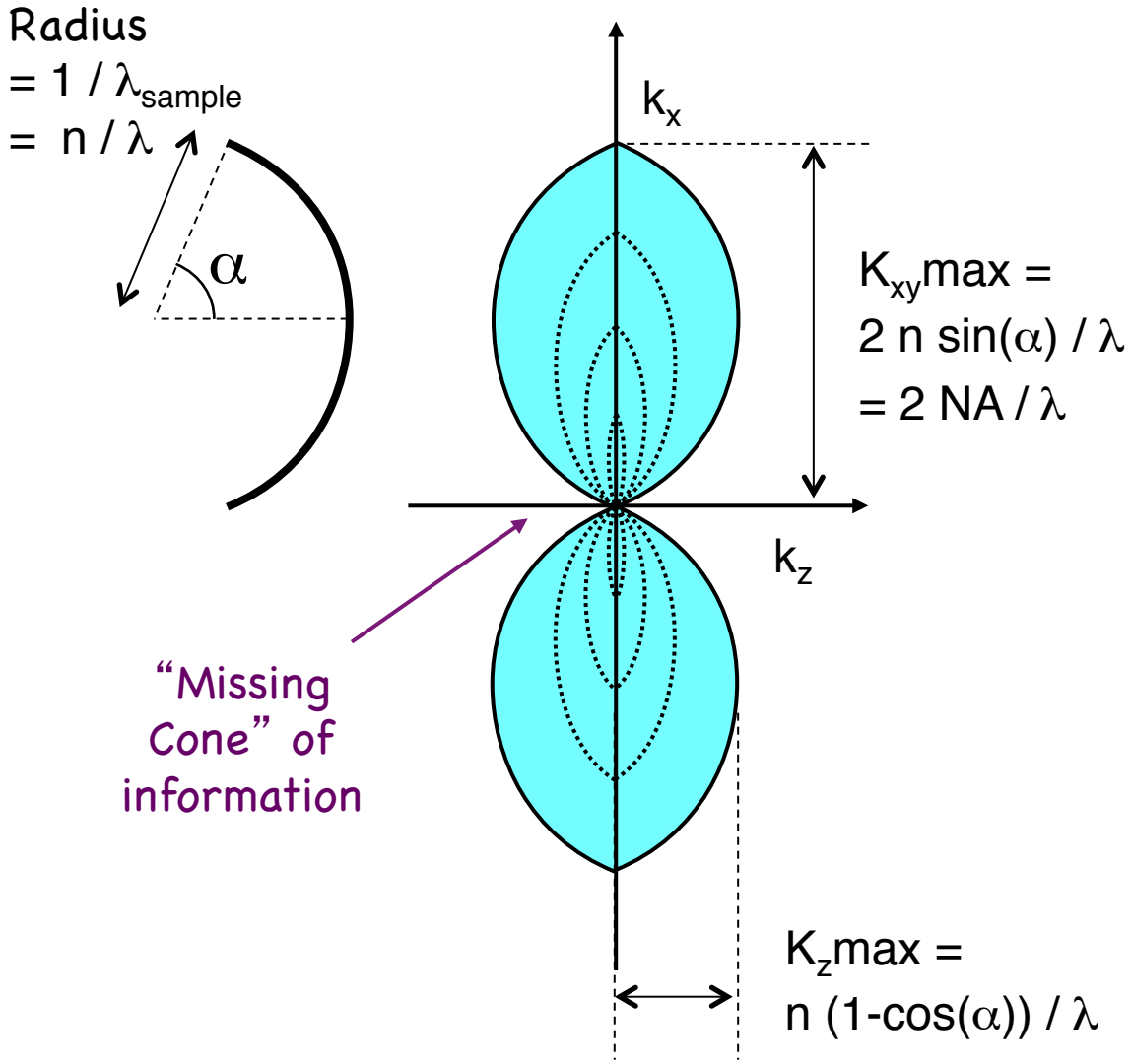
So what is the resolution?



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

NA = 1.4
n=1.515
→ $\alpha = 67.5°$
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