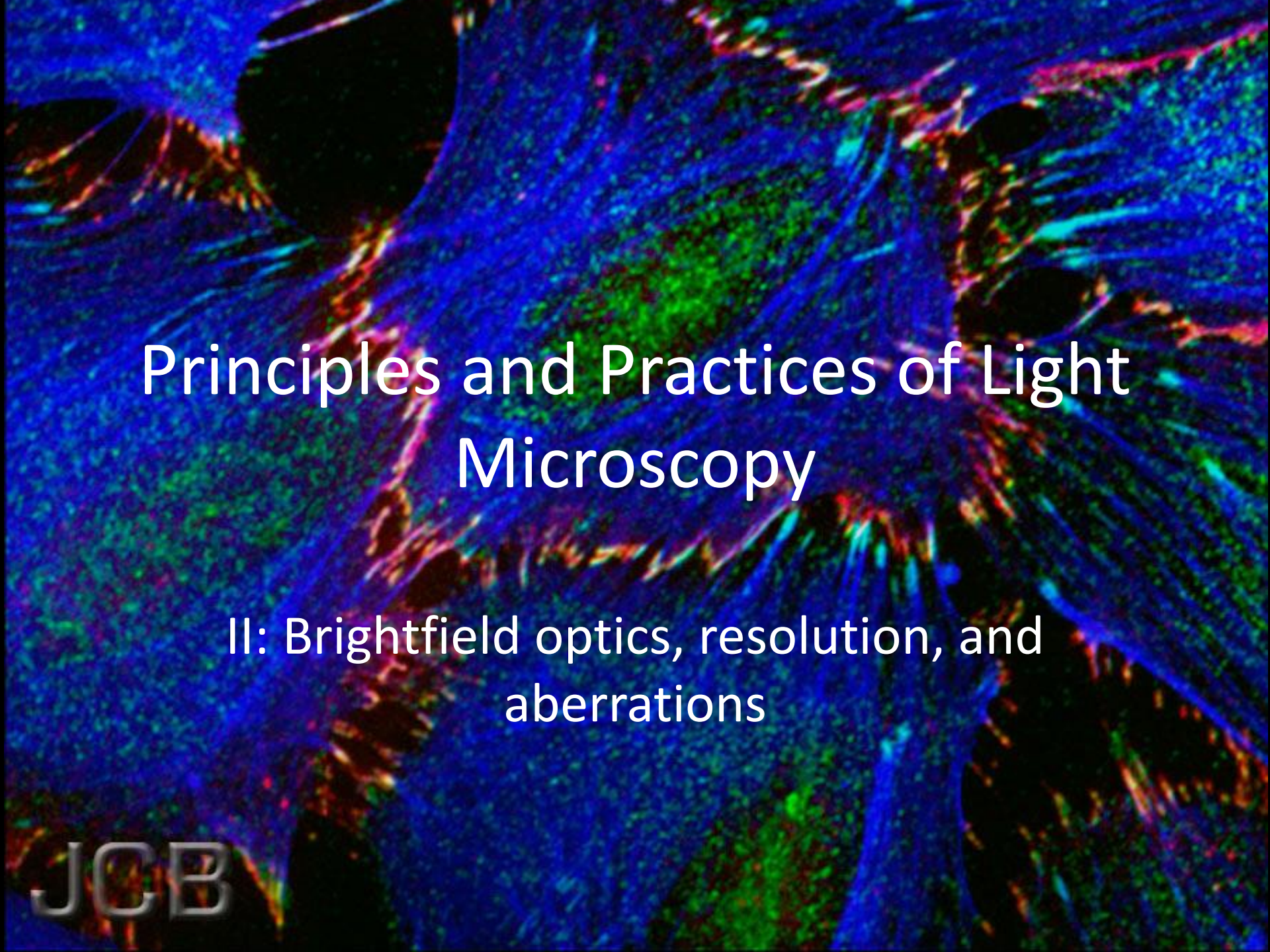
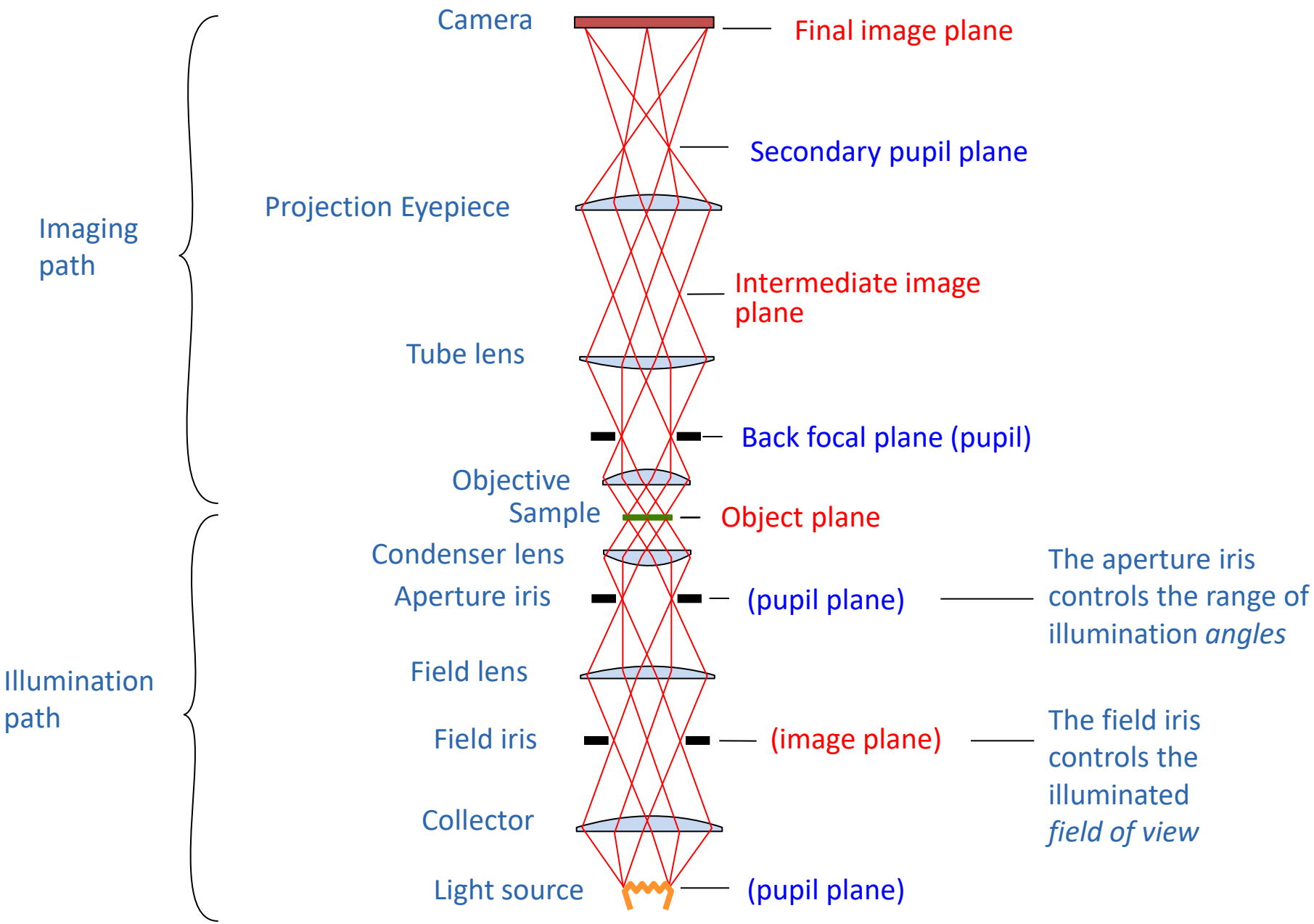


Principles and Practices of Light Microscopy

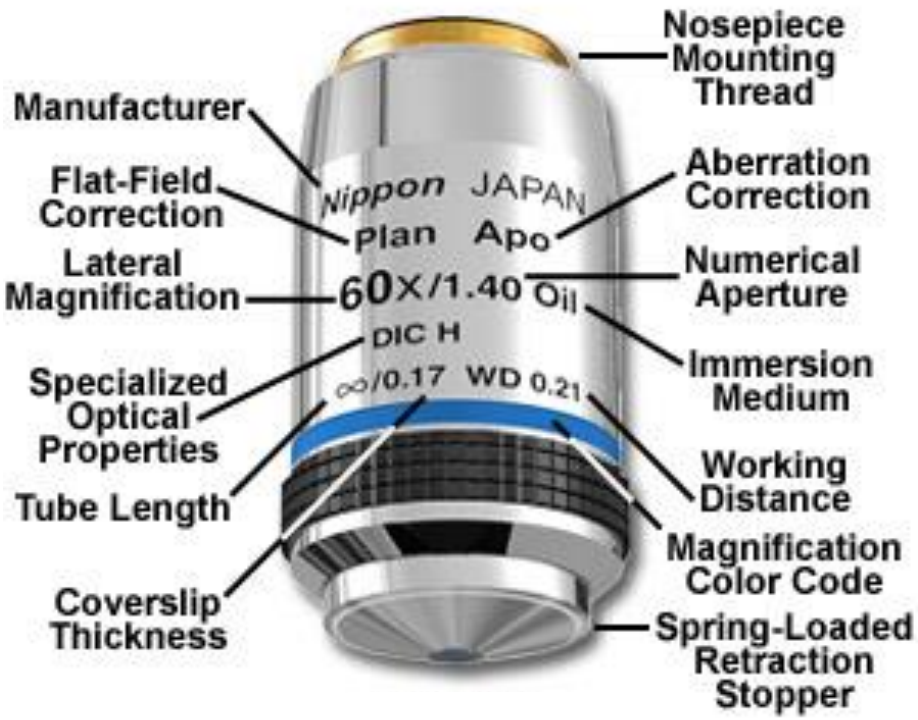
II: Brightfield optics, resolution, and aberrations

Trans-illumination Microscope



By far the most important part:

the Objective Lens



Each major manufacturer sells 20-30 different ***categories*** of objectives.
What are the important distinctions?

Working Distance

In general, high NA lenses have short working distances

However, extra-long working distance objectives do exist

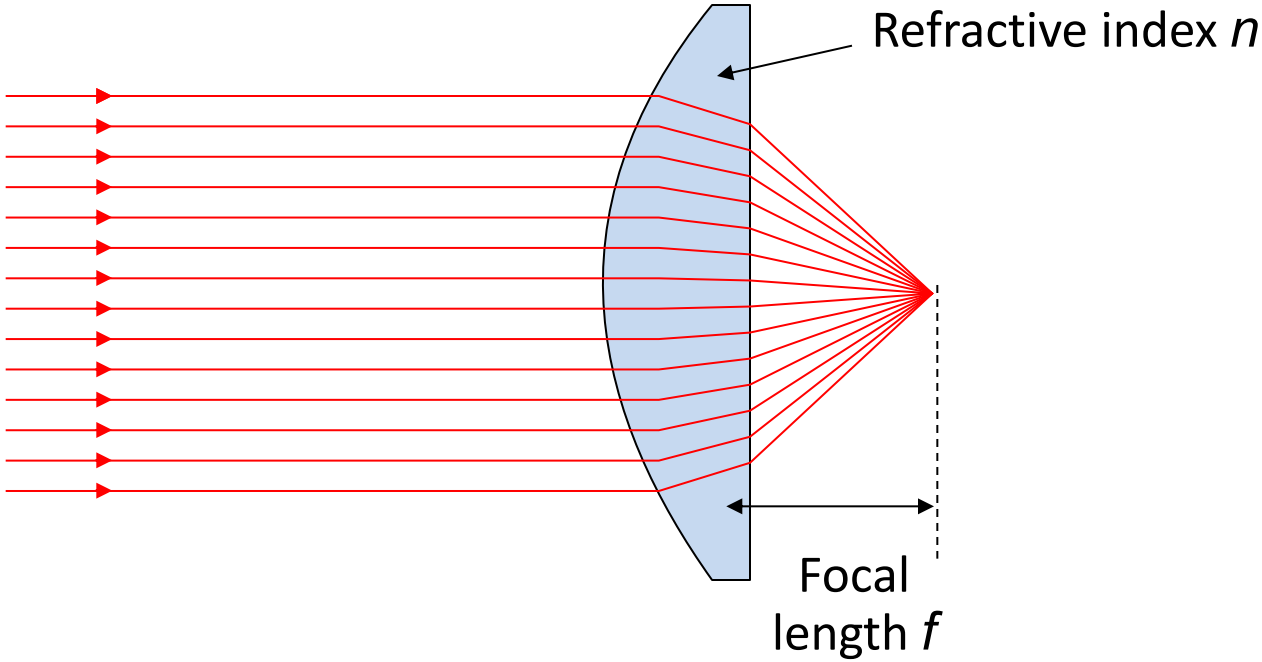
Some examples:

10x/0.3 WD = 15.2mm

20x/0.75 WD = 1.0mm

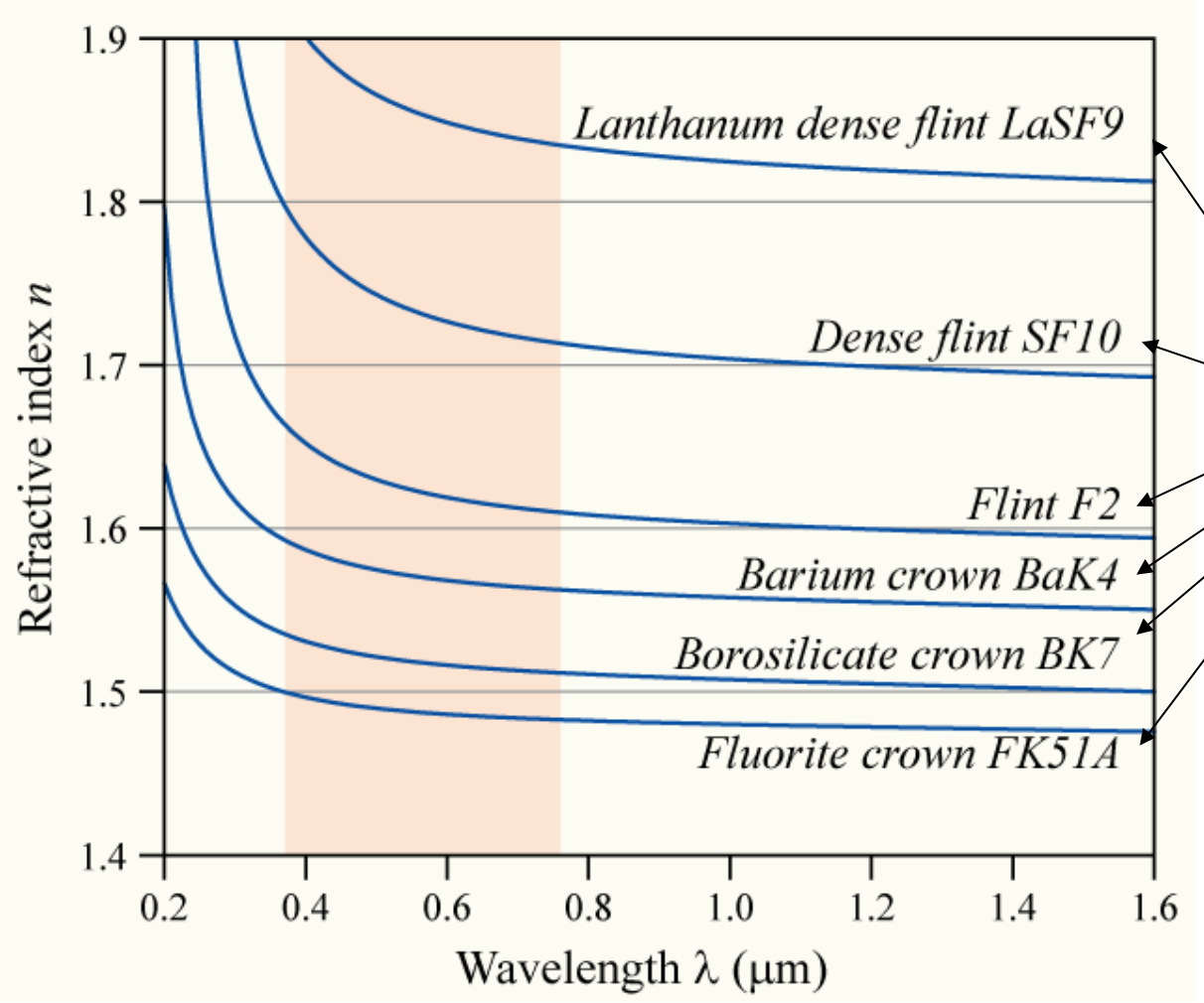
100x/1.4 WD = 0.13mm

The focal length of a lens depends on the refractive index…



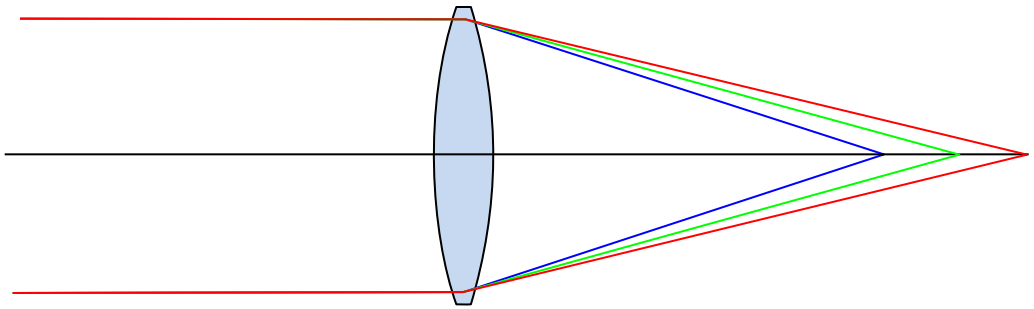
$f \propto 1/(n-1)$

… and the refractive index depends on the wavelength (“dispersion”)

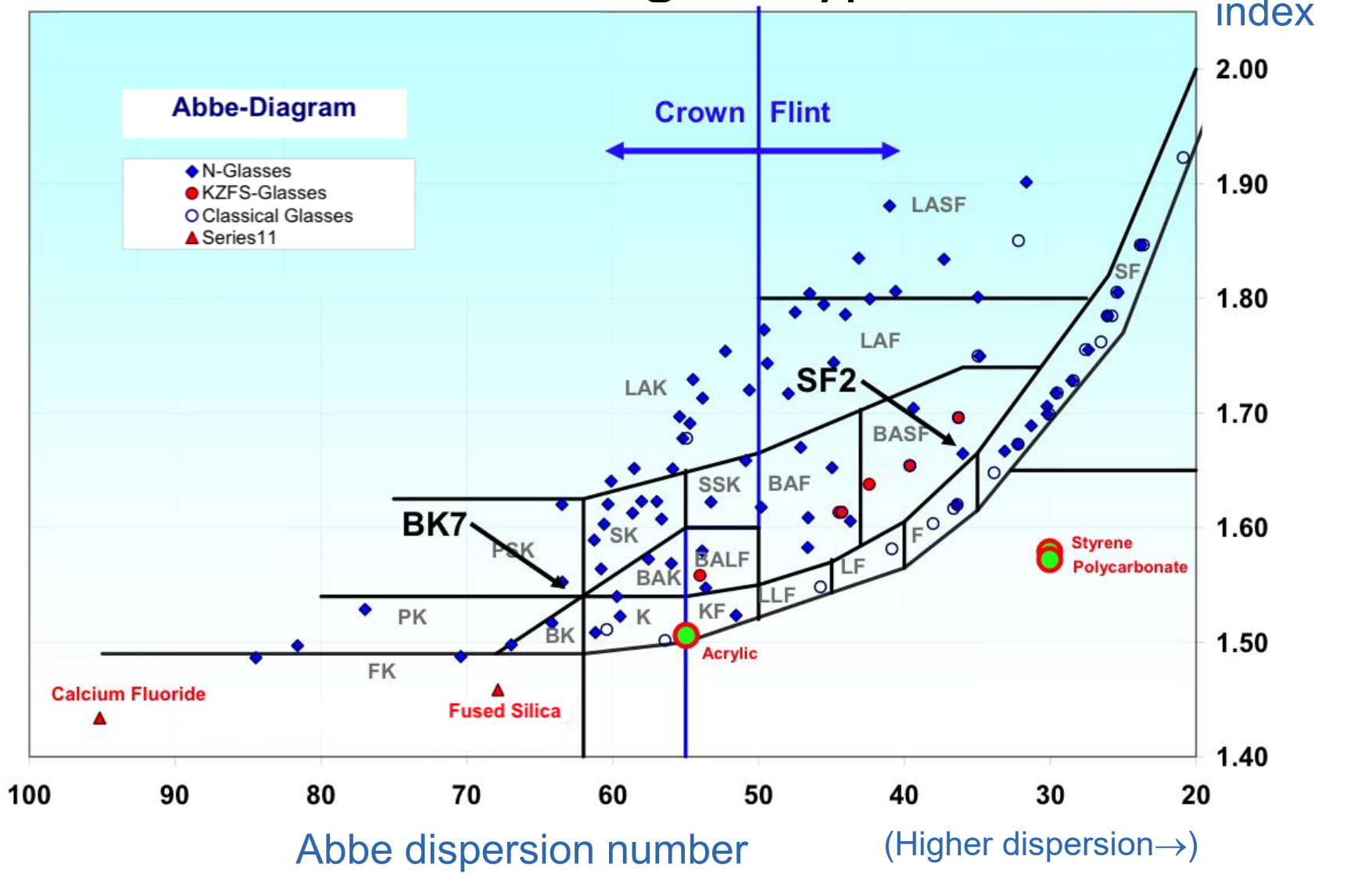


$\Rightarrow$ Chromatic aberration

- Different colors get focused to different planes
- Not good…

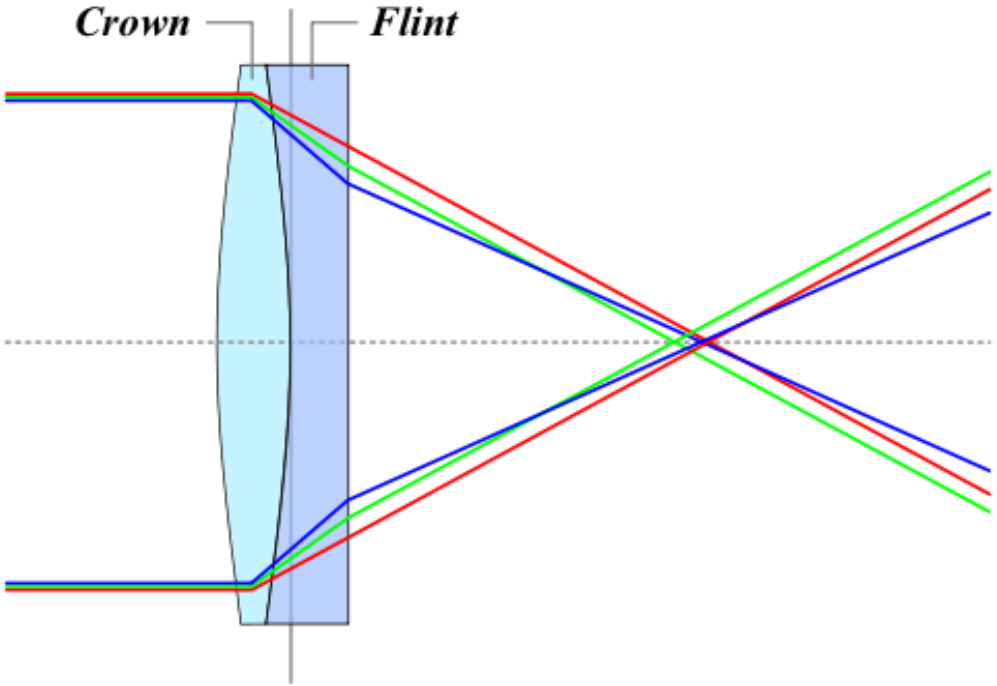


Dispersion vs. refractive index of different glass types

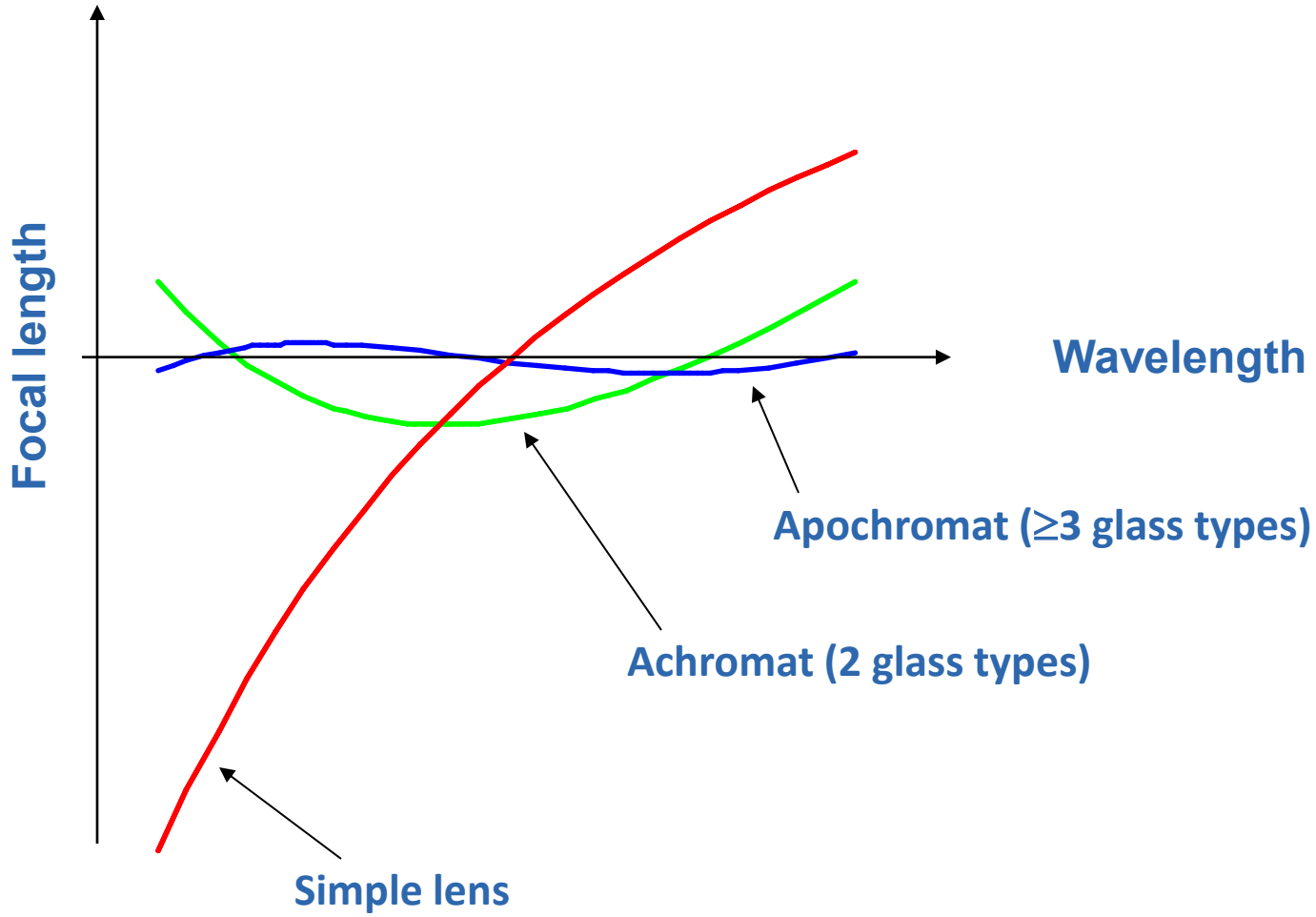


Achromatic Lenses

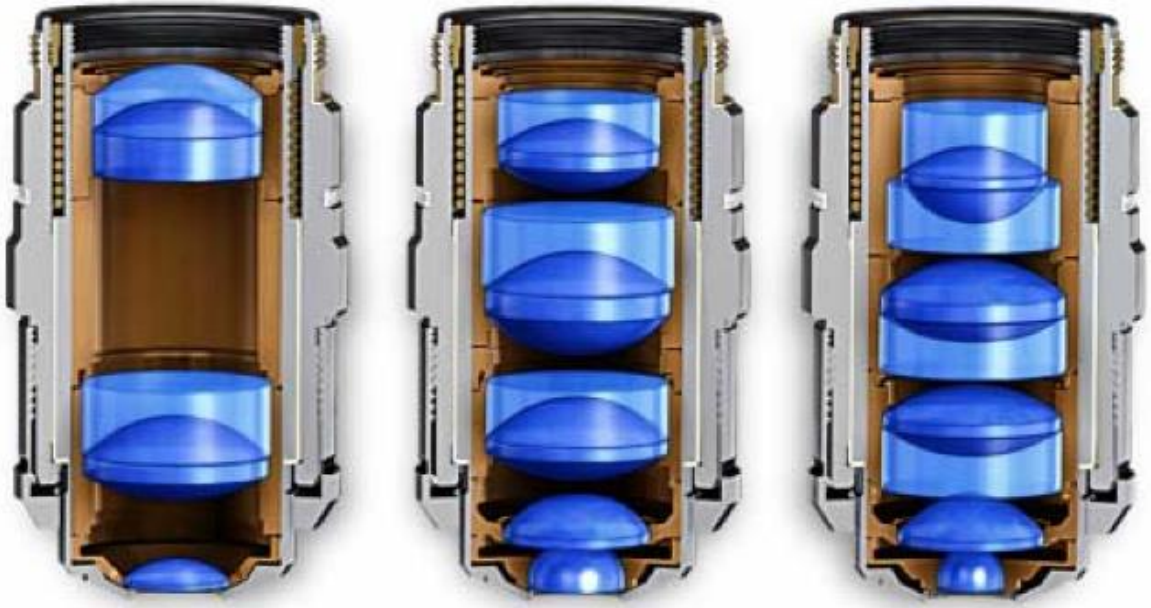
- Use a weak negative flint glass element to compensate the dispersion of a positive crown glass element



Achromats and Apochromats



Correction classes of objectives



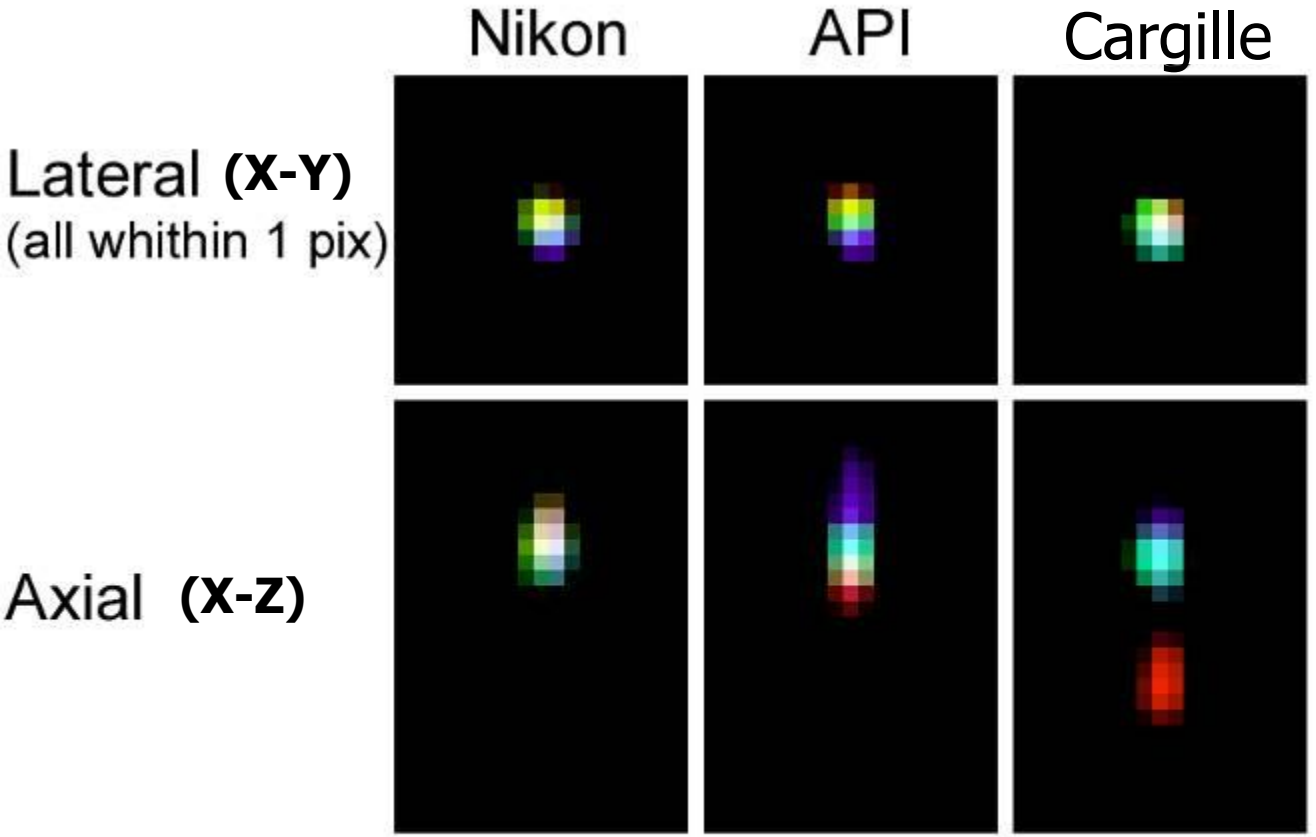
Achromat
(cheap)

Fluor
"semi-apo"
(good correction, high UV transmission)

Apochromat
(best correction)

Correction for other (i.e. monochromatic) aberrations also improves in the same order ⟶

Using the wrong immersion oil can induce axial chromatic aberration



Steve Ross

Putting one brand of objectives onto another brand of microscope?

Usually a bad idea:

- May not even fit

- May get different magnification than is printed on the objective

Tube lens focal length	
Nikon	200
Leica	200
Olympus	180
Zeiss	165

- Incompatible ways of correcting lateral chromatic aberration (LCA)

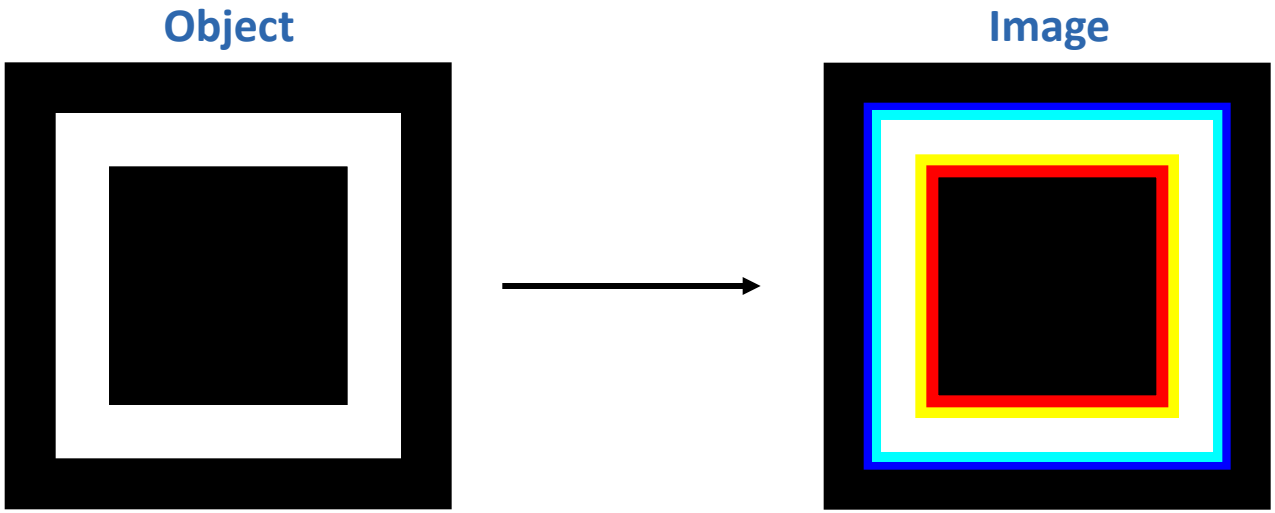
⇒ mixing brands can produce severe LCA

LCA correction:	
In objective	In tube lens
Nikon	Leica
Olympus	Zeiss

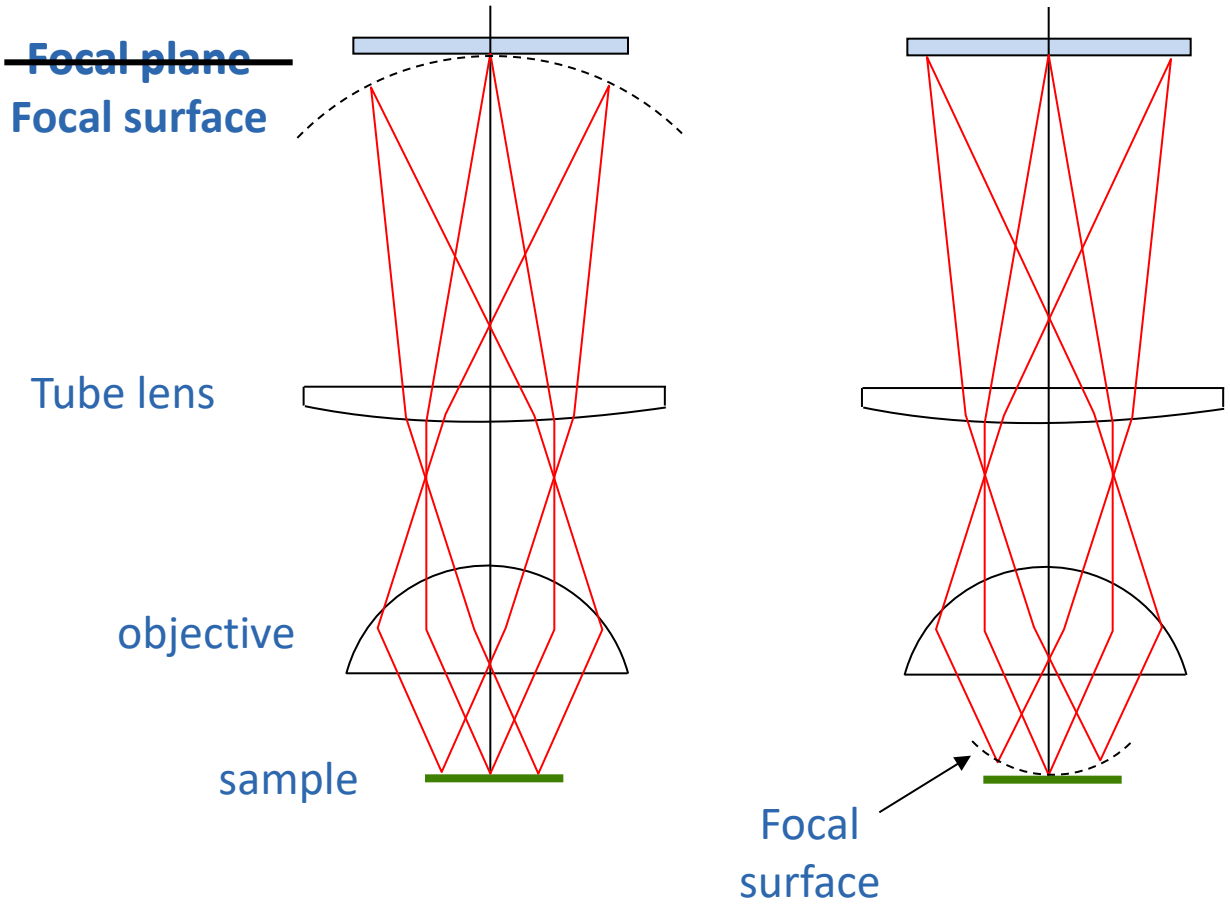
Lateral chromatic aberration

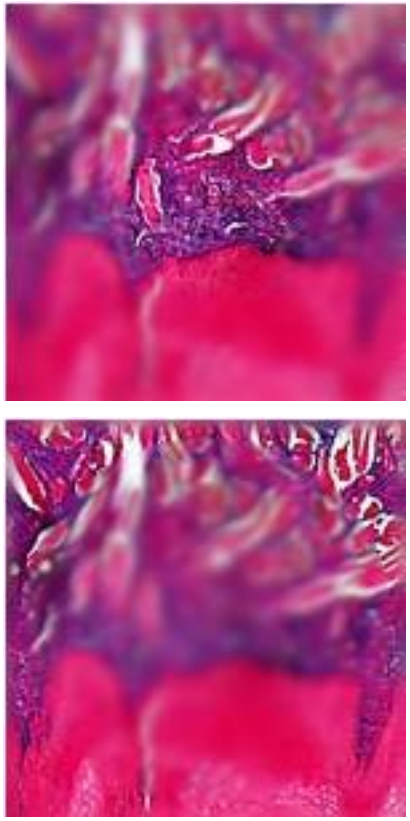
(= LCA, lateral color, chromatic difference of magnification)

= Different magnification for different colors



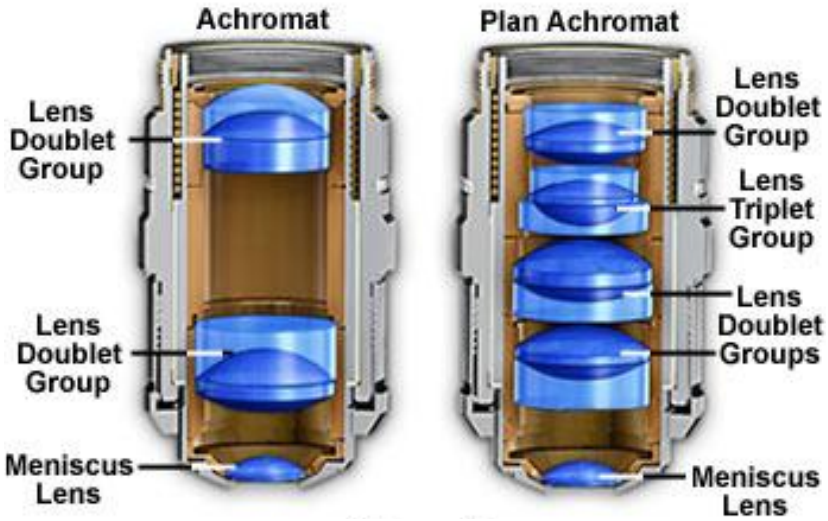
Curvature of Field





Plan objectives

- Corrected for field curvature
- More complex design
- Needed for most photomicrography



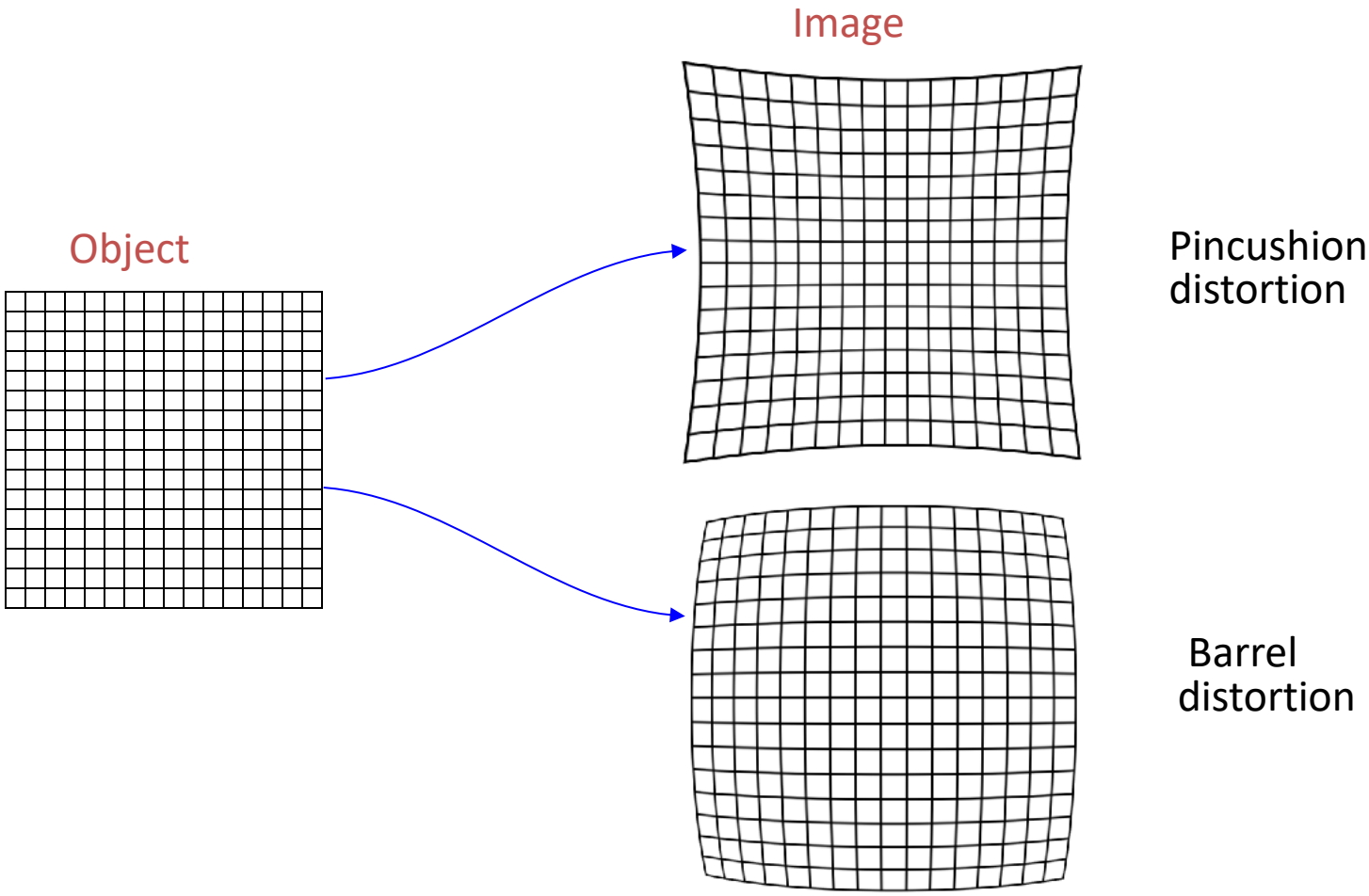
- **Plan-Apochromats** have the highest performance (and highest complexity and price)

Aberrations

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

Geometric Distortion

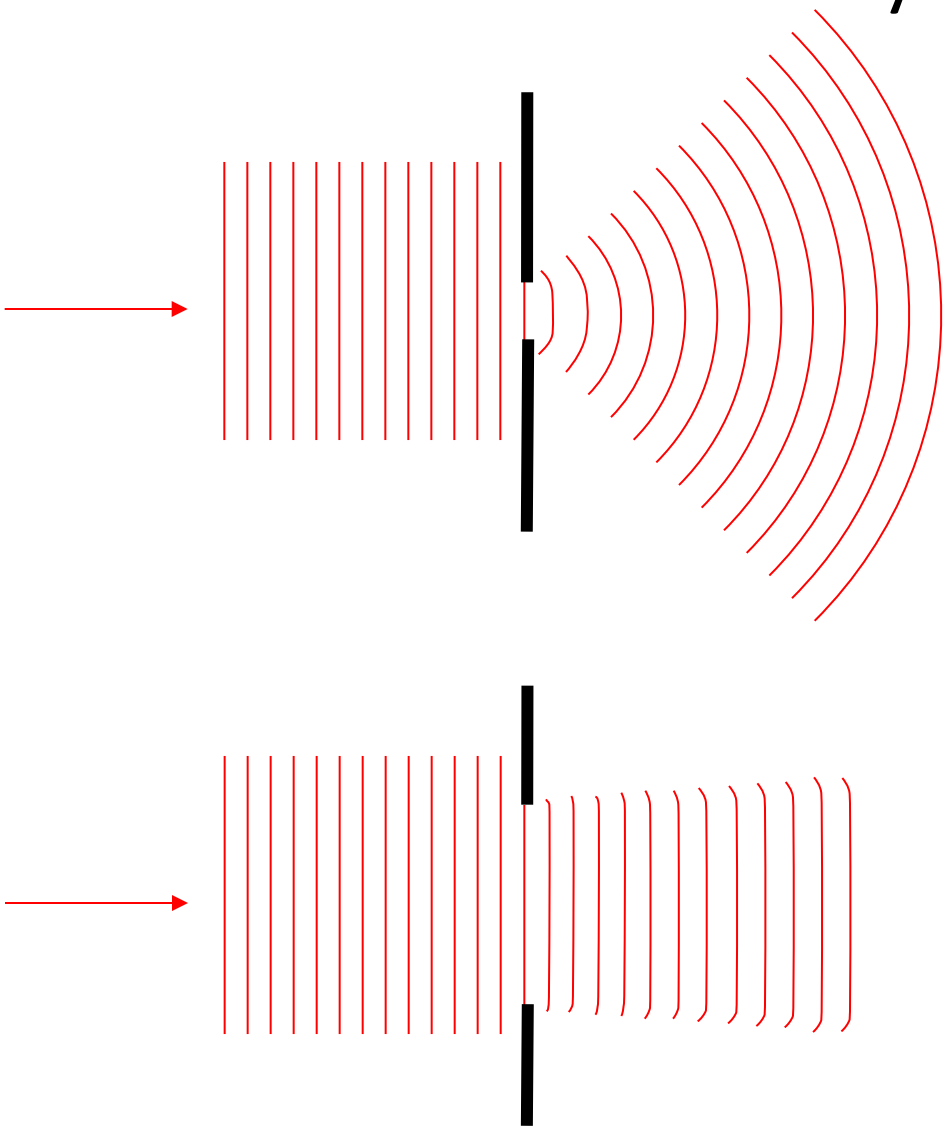
= Radially varying magnification



May be introduced by the projection eyepiece

Diffraction by an aperture

drawn as waves



Light spreads to new angles

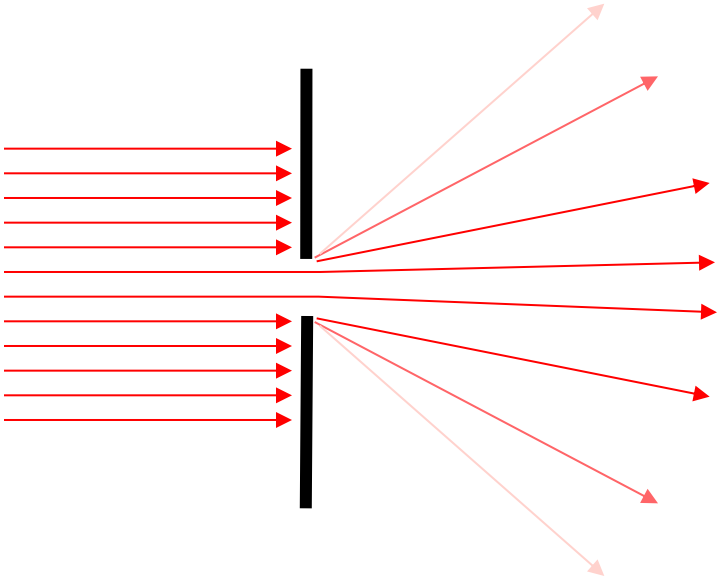
Larger aperture

⇔

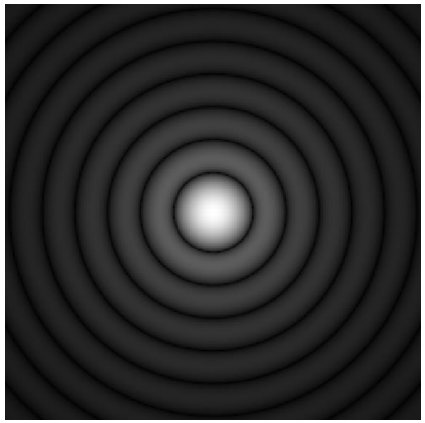
weaker diffraction

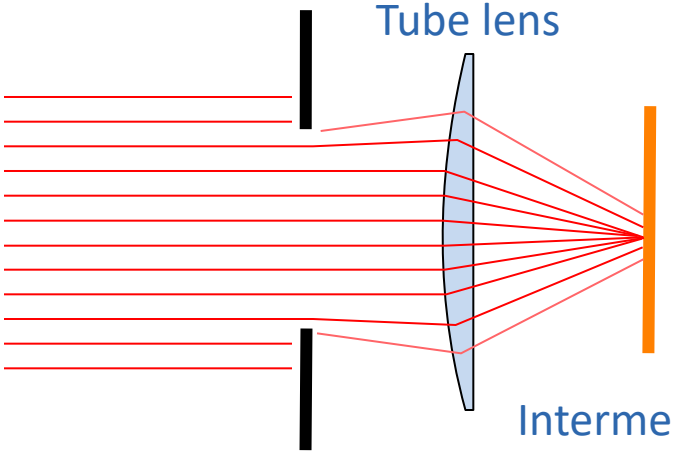
Diffraction by an aperture

drawn as rays



The pure, “far-field” diffraction pattern is formed at ∞ distance…



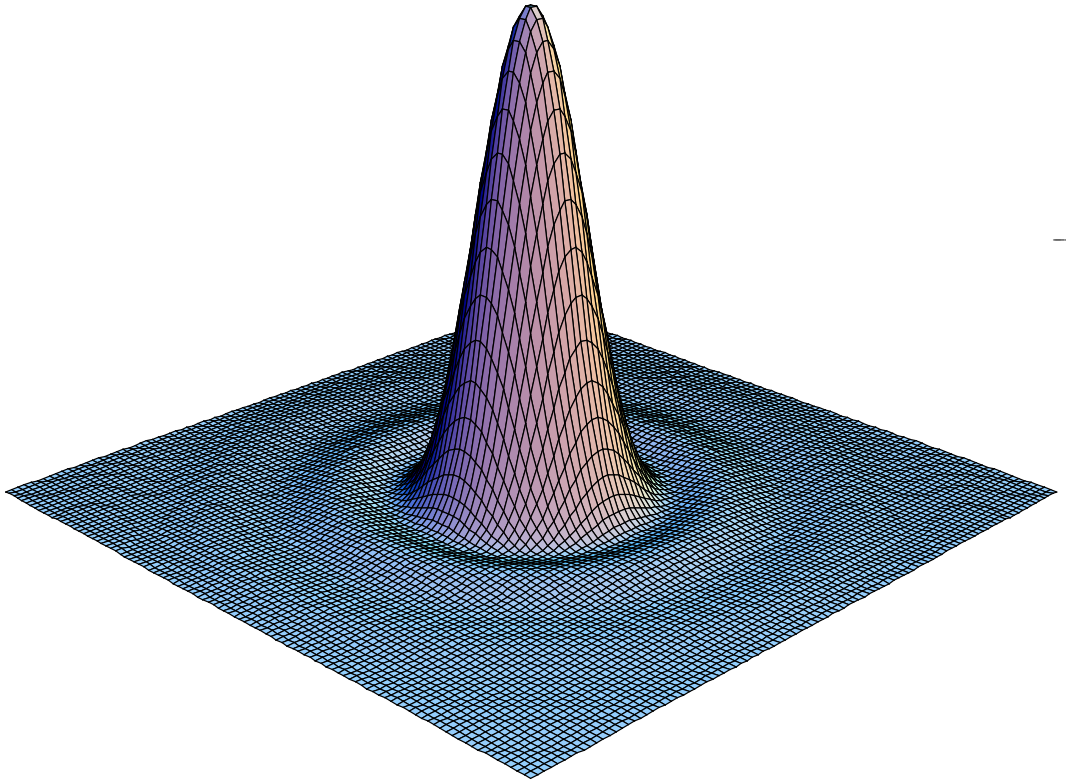


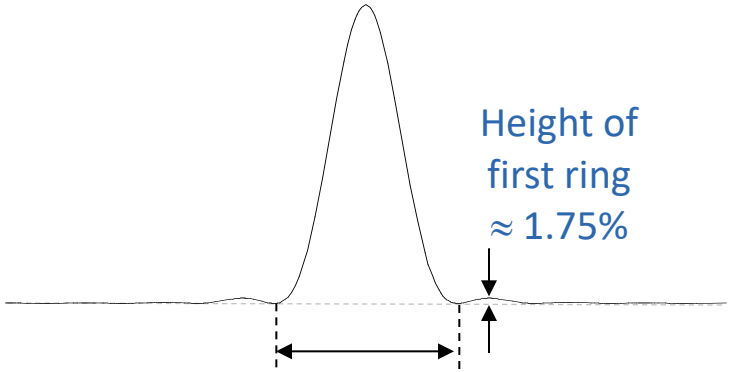
…or can be formed at a finite distance by a lens…

…as happens in a microscope

The Airy Pattern

= the far-field diffraction pattern from a round aperture

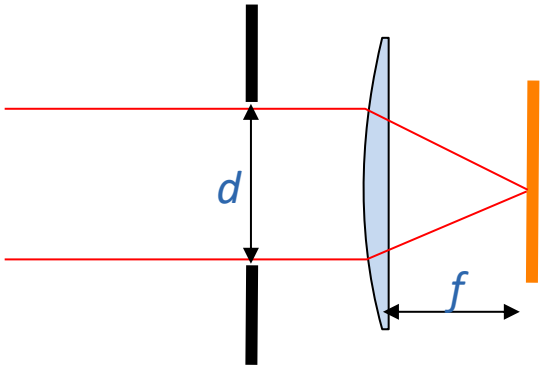




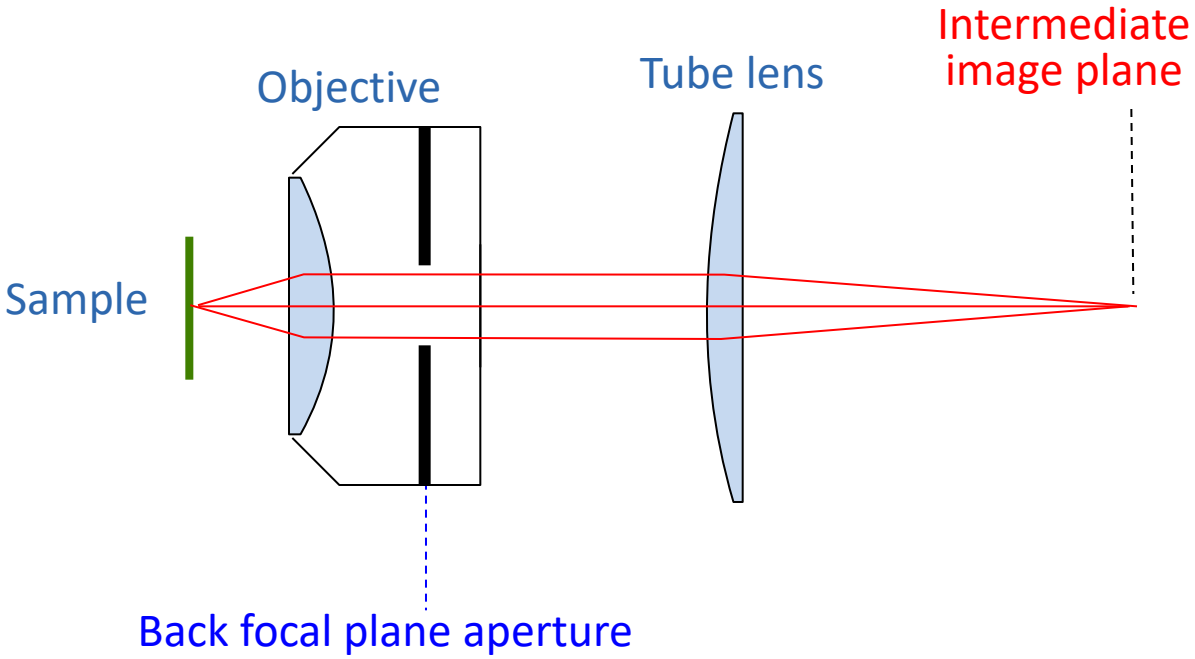
"Airy disk" diameter

$d = 2.44\,\lambda f/d$

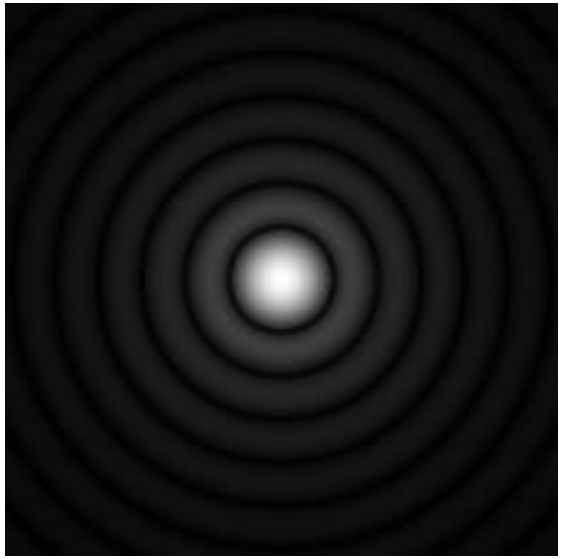
(for small angles d/f)



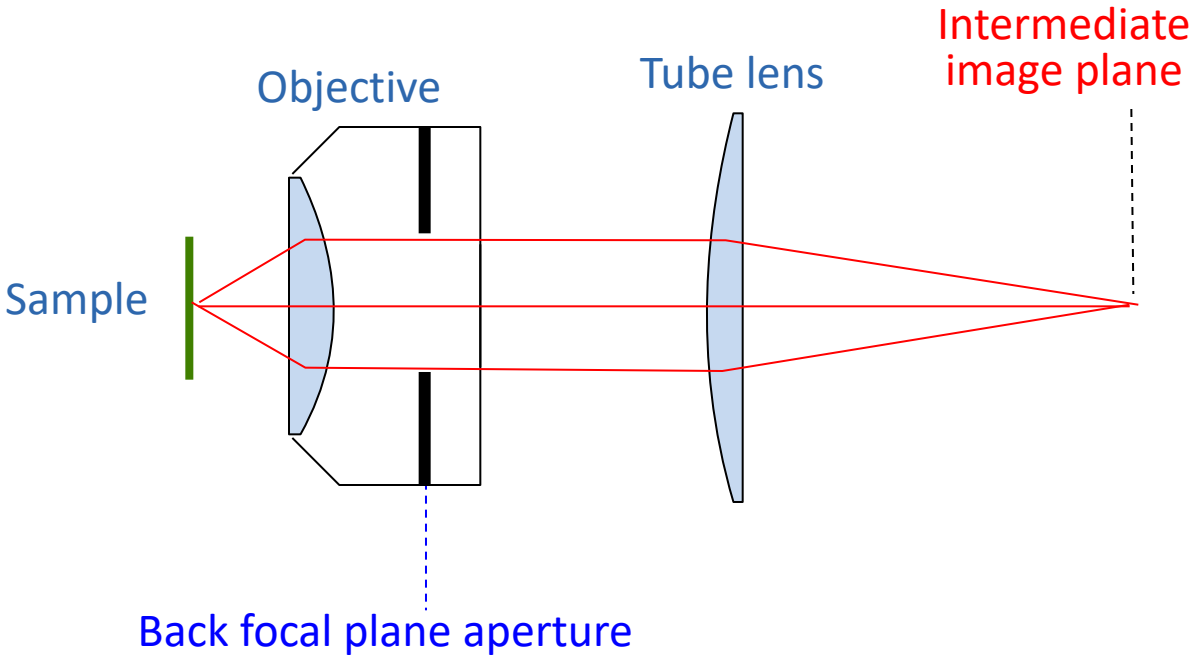
Aperture and Resolution



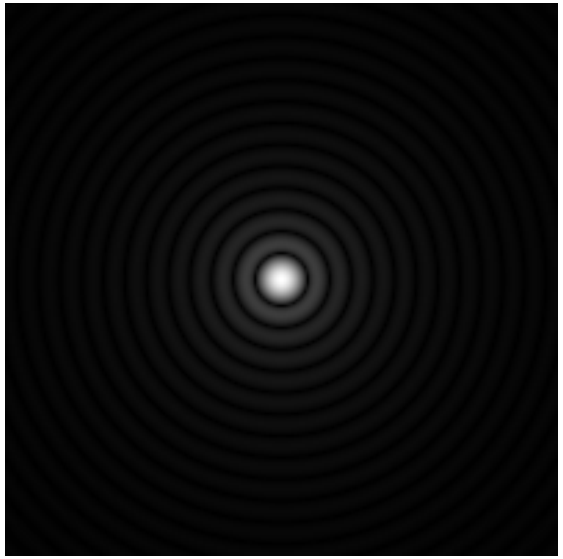
Diffraction spot
on image plane
= ***Point Spread Function***



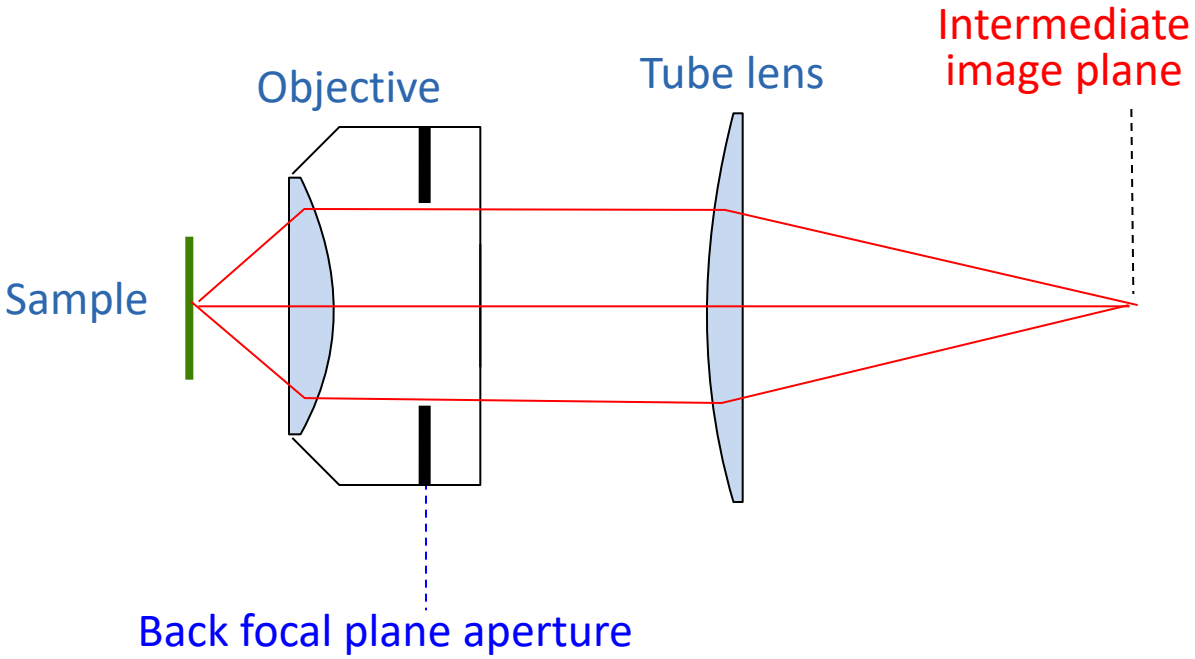
Aperture and Resolution



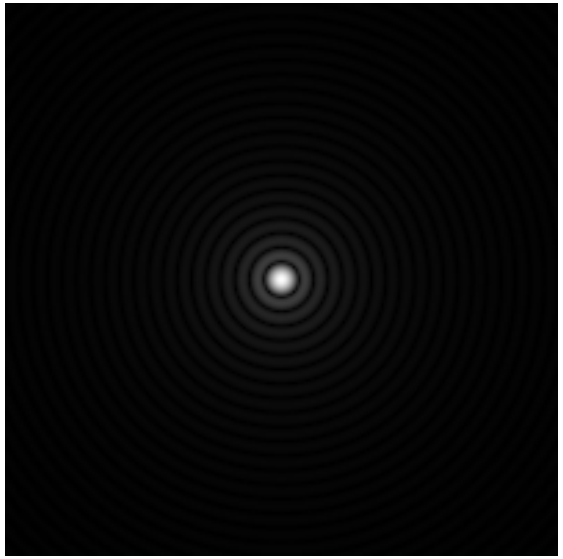
Diffraction spot
on image plane
= ***Point Spread Function***



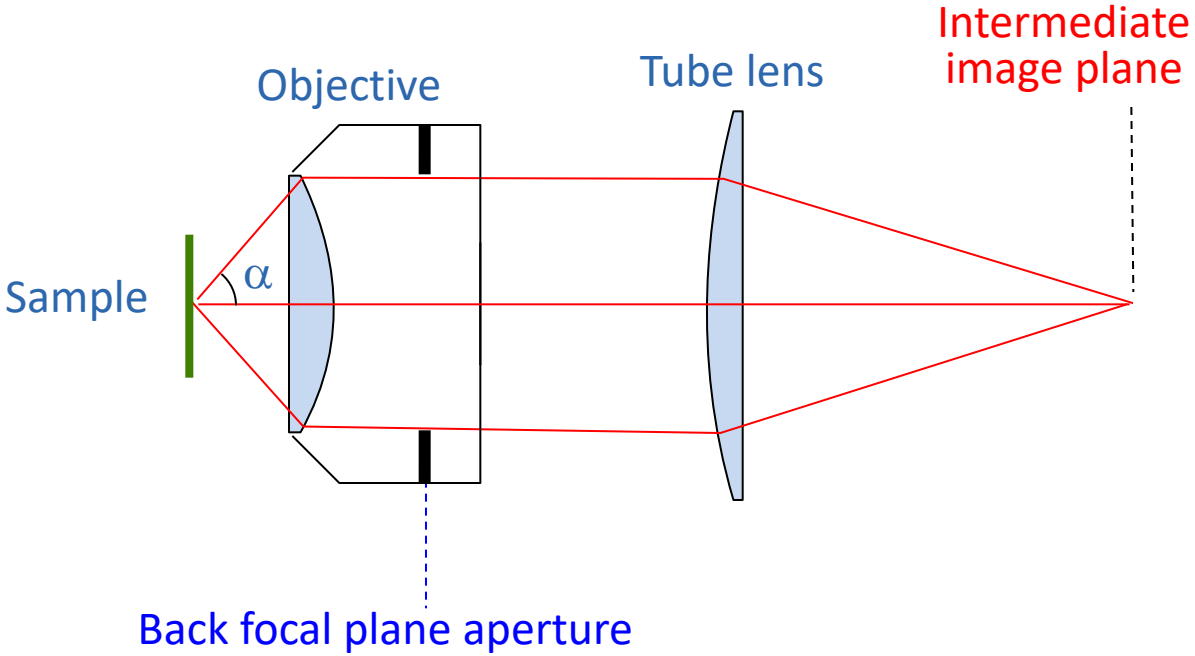
Aperture and Resolution



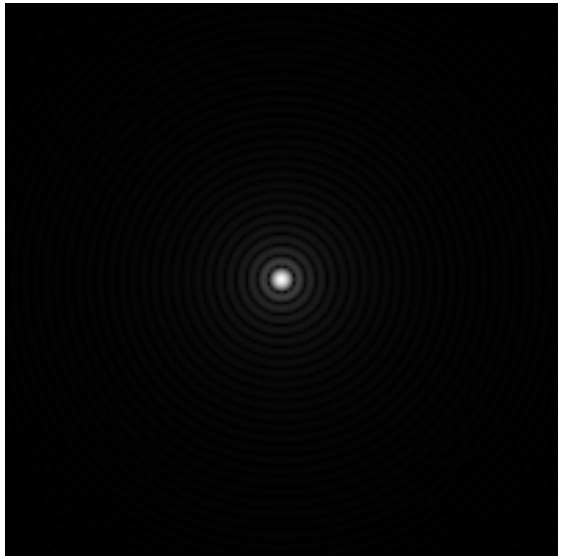
Diffraction spot
on image plane
= ***Point Spread Function***



Aperture and Resolution



Diffraction spot on image plane (resolution)



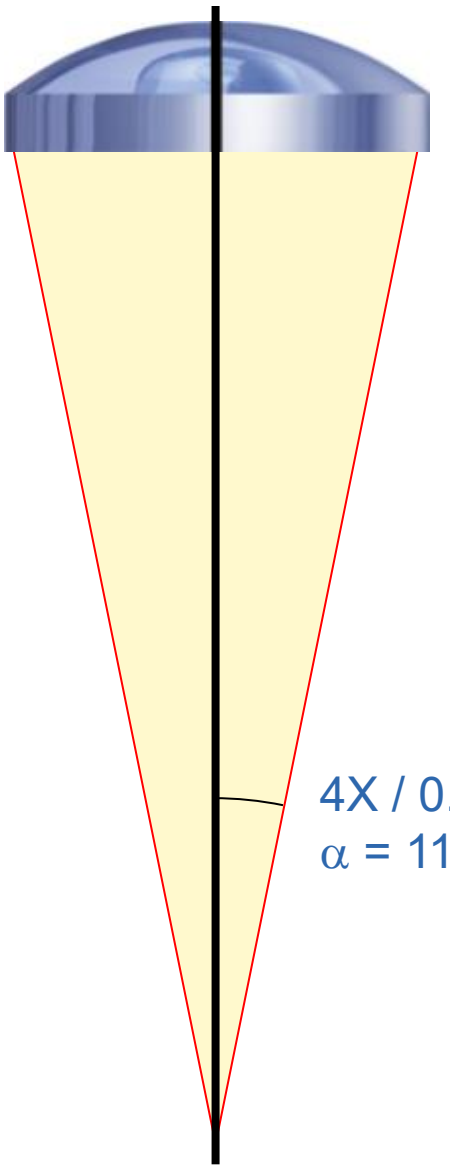
- Image resolution improves with ~~aperture size~~ Numerical Aperture (NA)

$NA = n \sin(\alpha)$

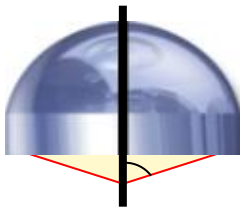
where:

α = light gathering angle
n = refractive index of sample

Numerical Aperture



4X / 0.20 NA
α = 11.5°



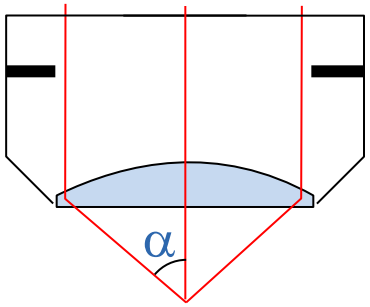
100X / 0.95 NA
α = 71.8°

Numerical Aperture

Compare:

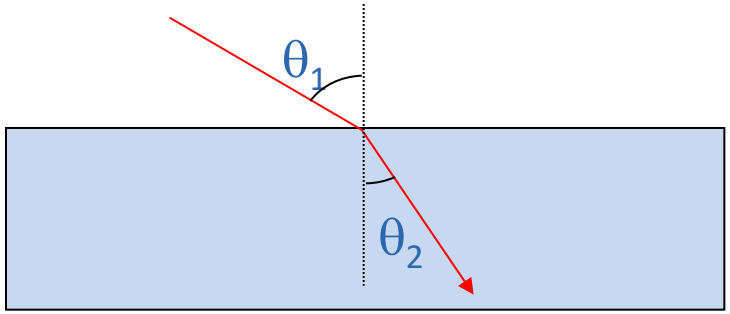
Numerical Aperture:

$NA = n \sin(\alpha)$



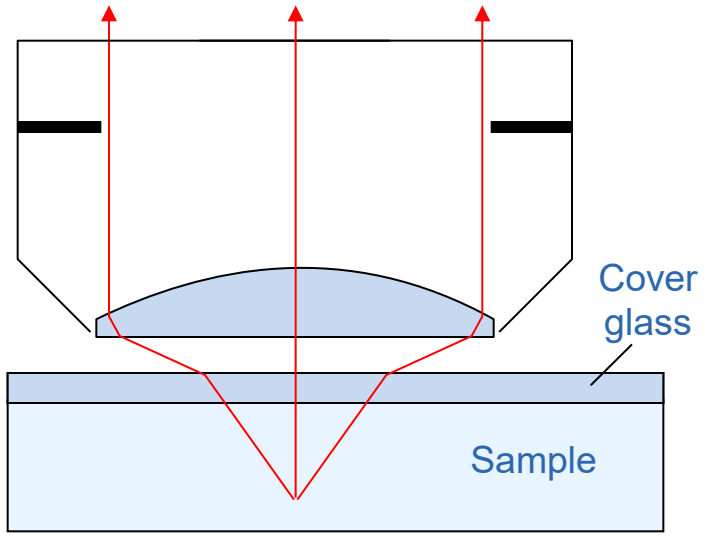
Snell's law:

$n_1 \sin(\theta_1) = n_2 \sin(\theta_2)$



- $n \sin(\theta)$ doesn't change at horizontal interfaces
- $\sin(\text{anything}) \leq 1$

$\Rightarrow$ NA cannot exceed the *lowest* n between the sample and the objective lens

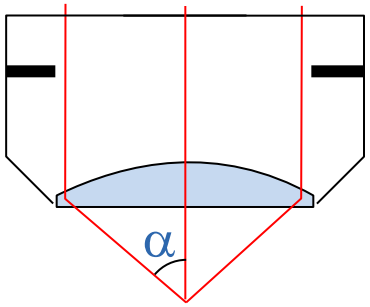


Numerical Aperture

Compare:

Numerical Aperture:

$NA = n \sin(\alpha)$



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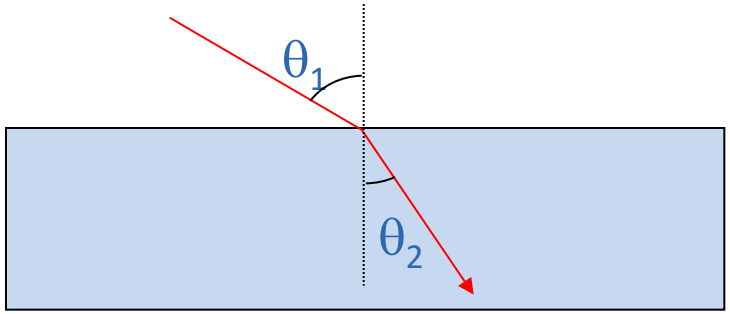
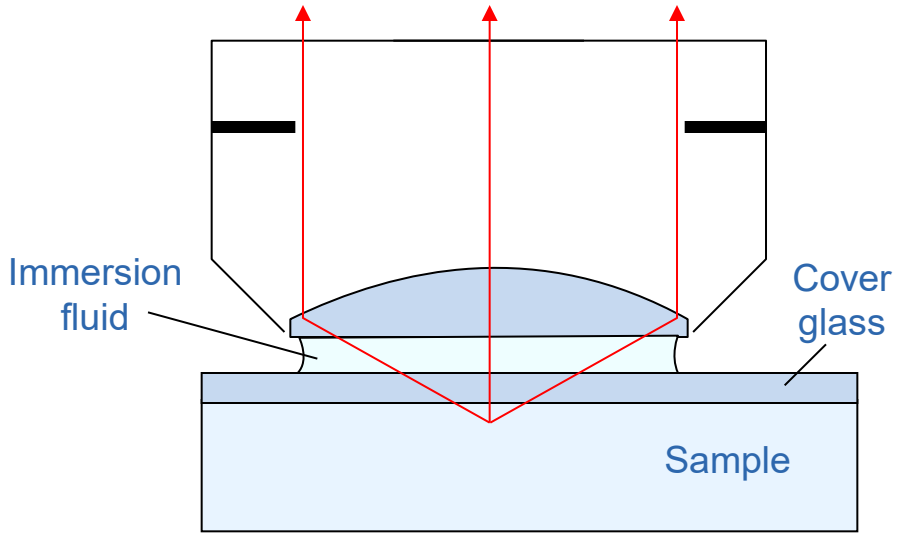


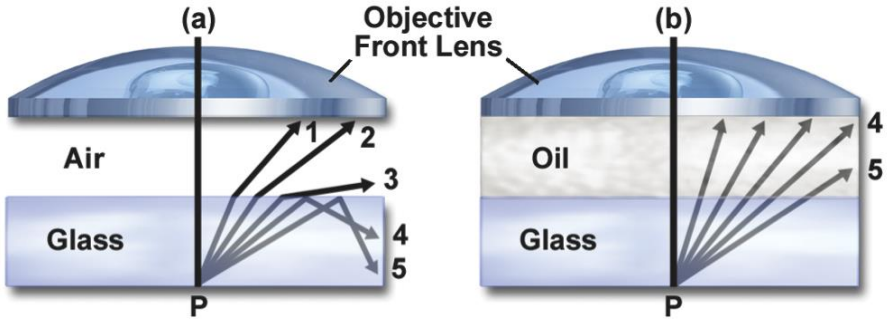
- $n \sin(\theta)$ doesn't change at horizontal interfaces
- $\sin(\text{anything}) \leq 1$

$\Rightarrow$ NA cannot exceed the *lowest* n between the sample and the objective lens

$\Rightarrow$ NA >1 requires ***fluid immersion***



Immersion Objectives



⇒ NA cannot exceed
the *lowest* n between the
sample and the objective lens

⇒ NA >1 requires ***fluid immersion***

NA can approach
the index of the
immersion fluid

Oil immersion:
- n ≈ 1.515
- max NA ≈ **1.4** (1.45–1.49 for TIRF)

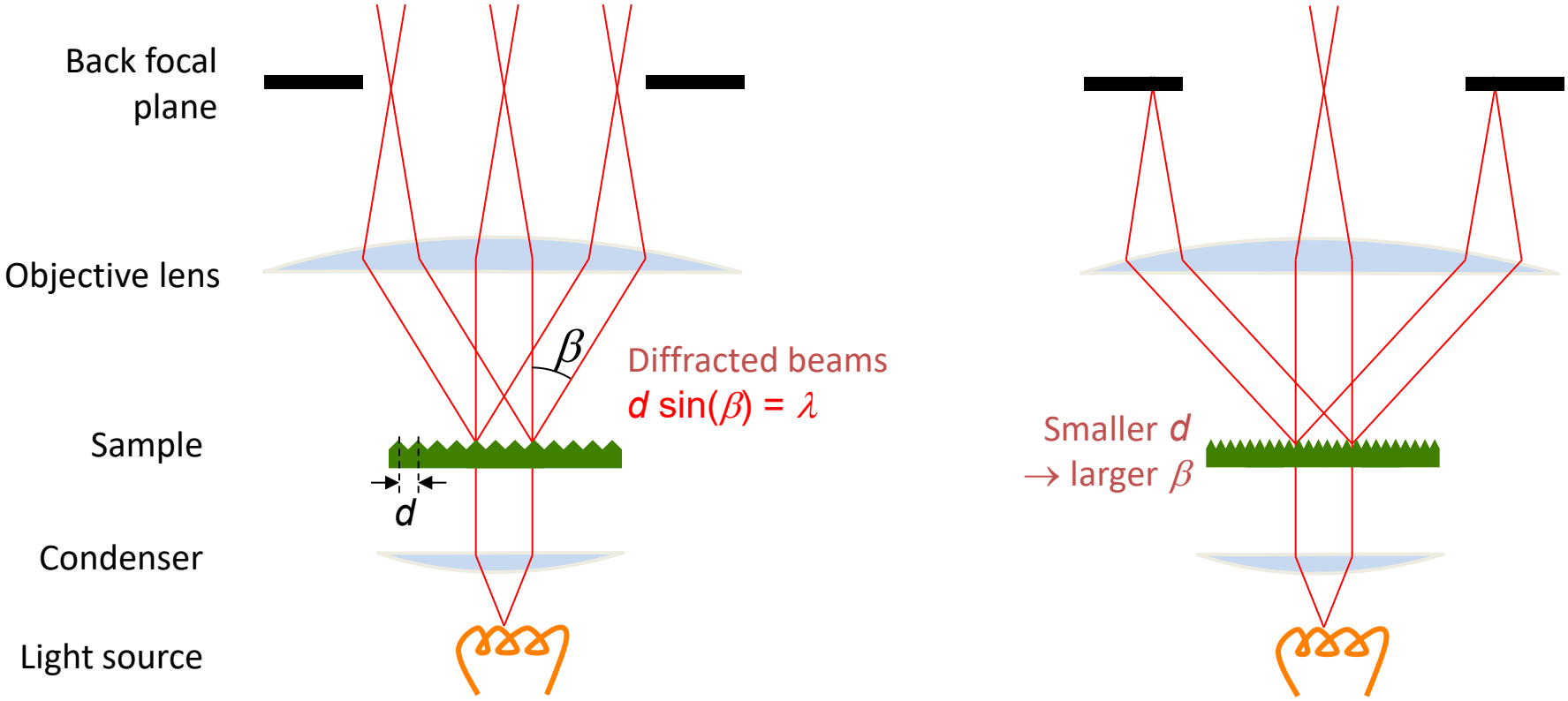
Glycerol immersion:
- n ≈ 1.45 (85%)
- max NA ≈ **1.35** (Leica)

Water immersion:
- n ≈ 1.33
- max NA ≈ **1.2**

Resolution

Ernst Abbe's argument (1873)

Consider a striped sample ≈ a diffraction grating



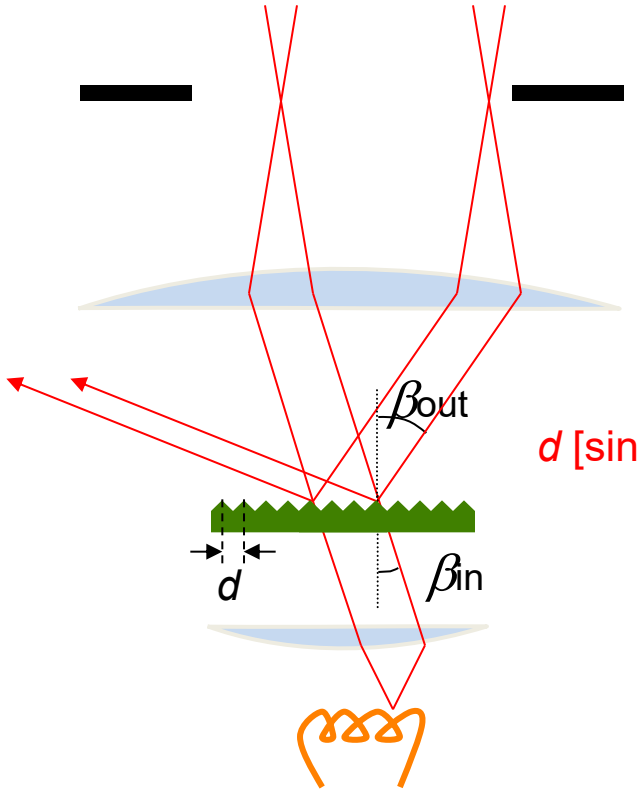
If $\beta > \alpha$, only one spot makes it through
$\Rightarrow$ no interference $\Rightarrow$ no image formed

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 $\beta_{in} < \alpha$.

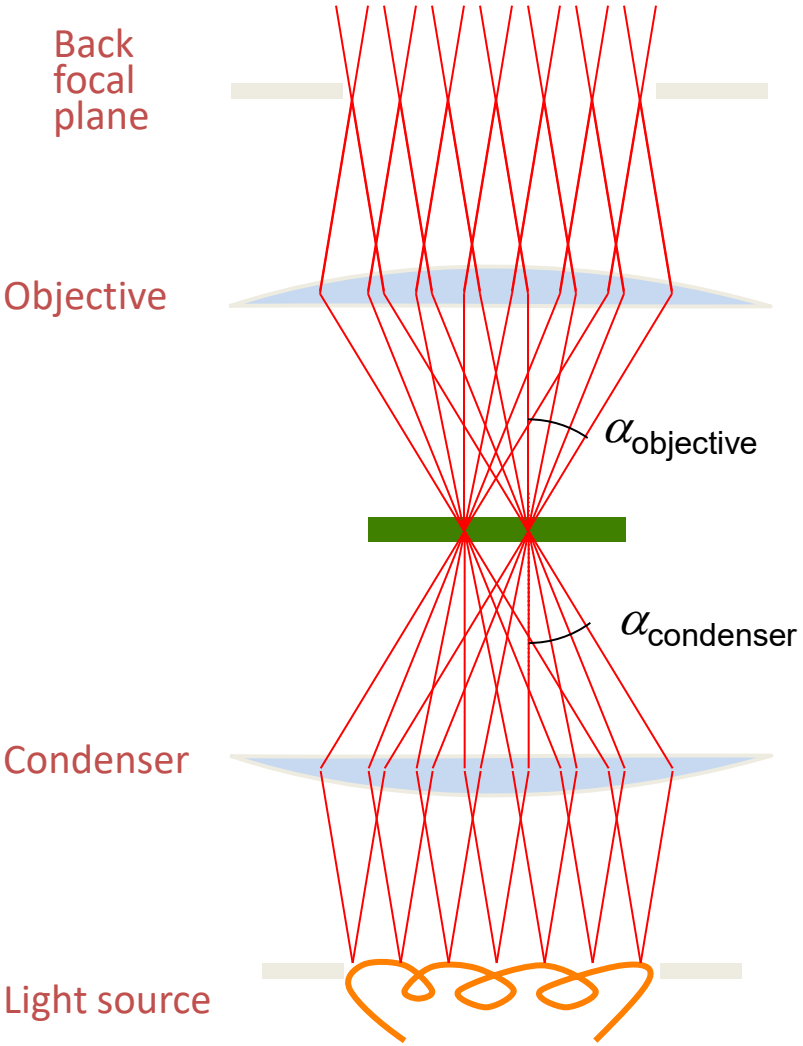
Resolution (smallest resolvable d)
with incoherent illumination (all possible illumination directions):

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

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

Filling the back focal plane

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



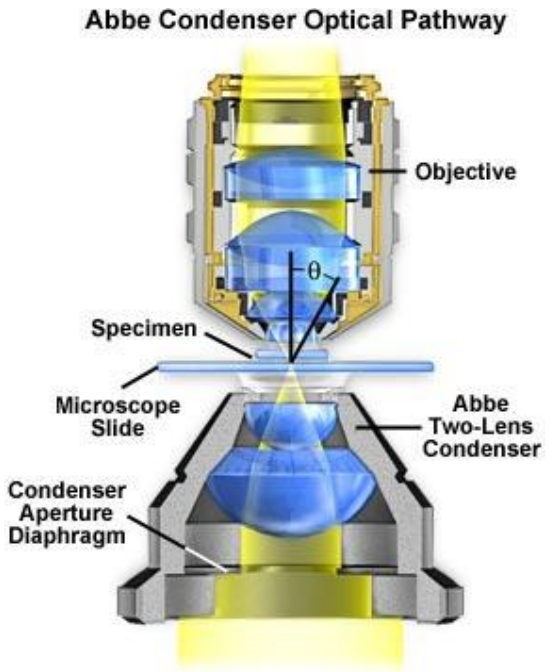
For the highest resolution, we need to have

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

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

$\Rightarrow$ 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

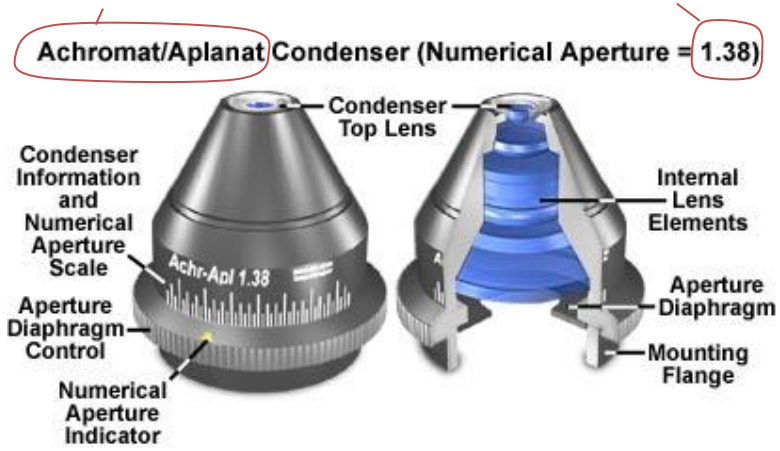
(With 2X and 100x objectives we need $(100/2)^2 = 2500$ times more light than any objective uses!)

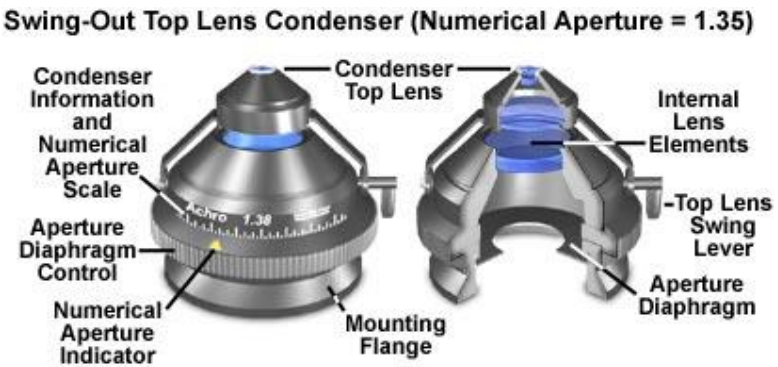
Solutions:

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

Grade of correction

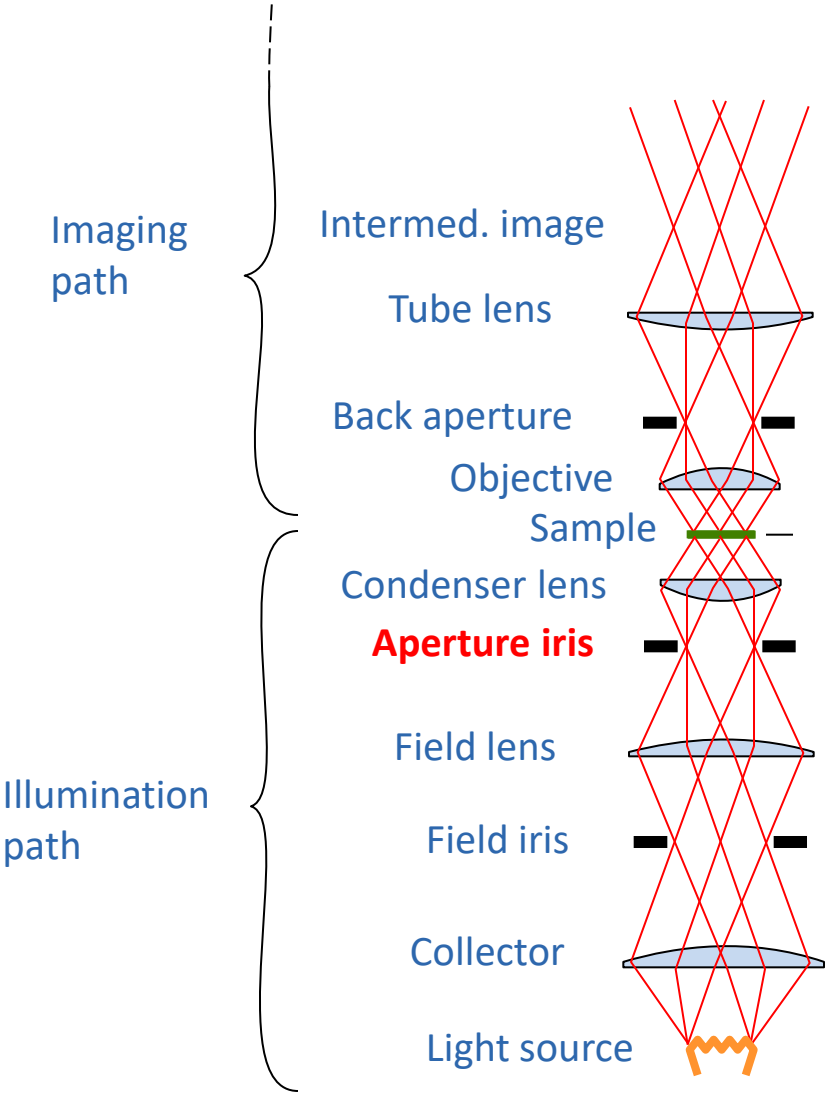
NA





Aperture, Resolution & Contrast

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

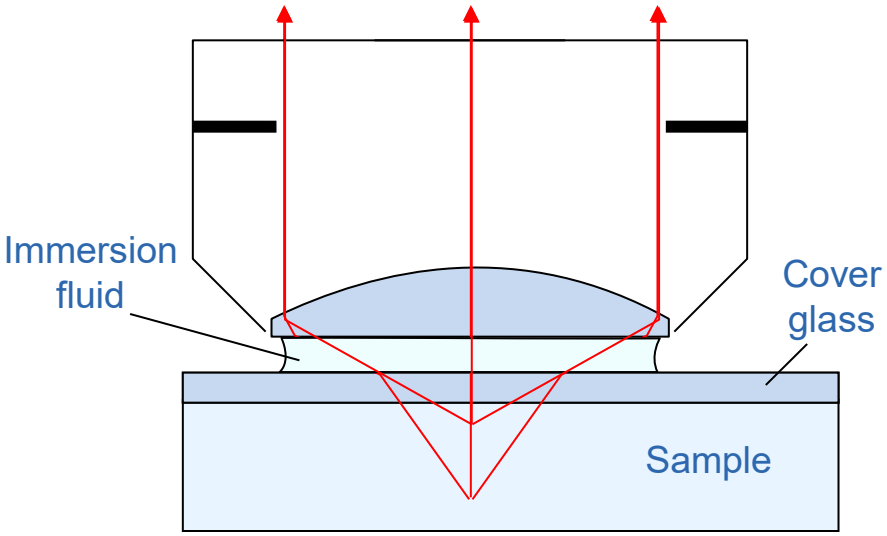


Q: Don't we always want it full open??

A: **No**

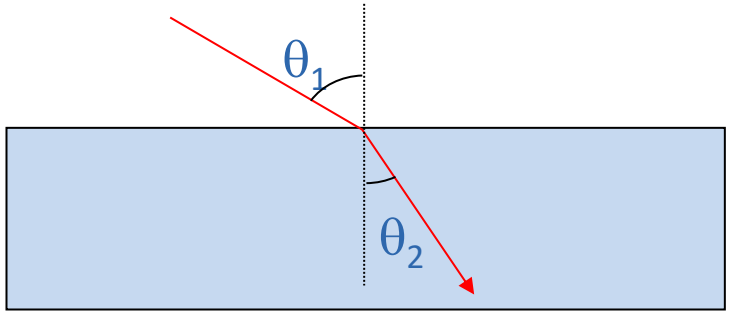
Why? Tradeoff: resolution vs. **contrast**

Numerical Aperture and Resolution



Snell's law:

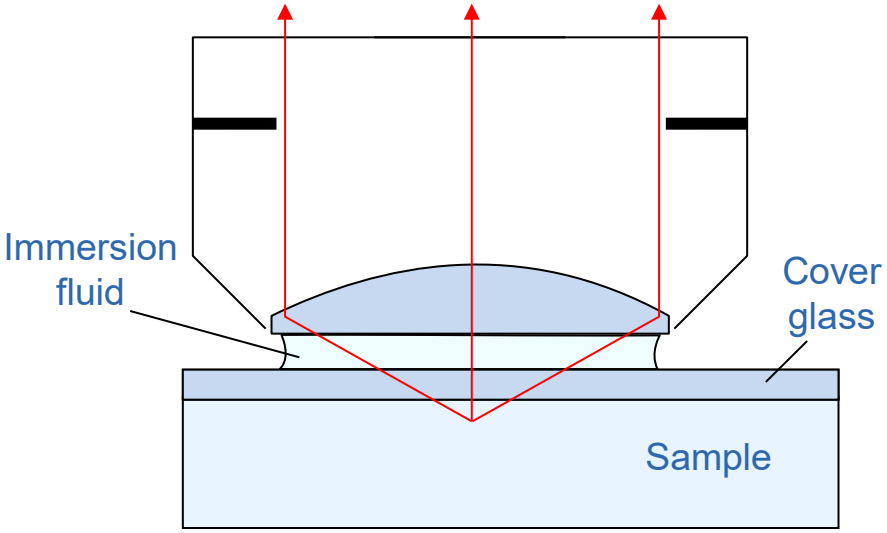
$n_1 \sin(\theta_1) = n_2 \sin(\theta_2)$



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

$$d_{min} = \frac{\lambda}{NA} = \frac{\lambda}{n \sin(\theta)}$$

Numerical Aperture and Resolution



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

$$d_{min} = \frac{\lambda}{NA} = \frac{\lambda}{n \sin(\theta)}$$

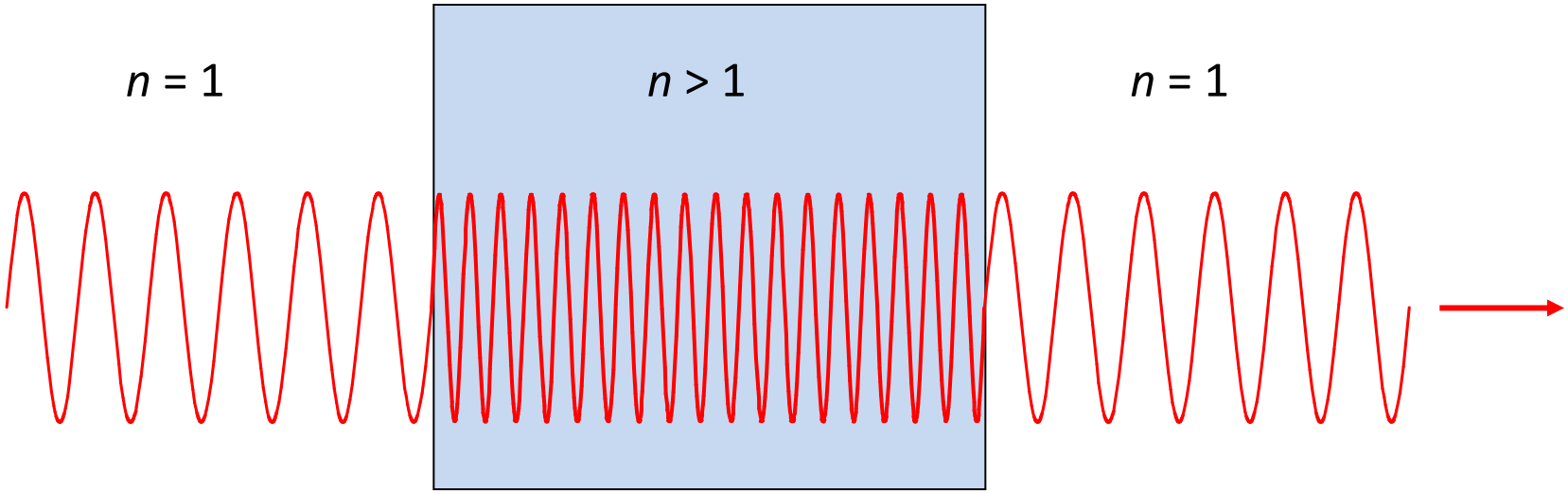
Sample, coverglass, immersion fluid, and top lens of objective all have same refractive index.

What happens if we change that refractive index?

Resolution improves with RI! Why?

Light travels more slowly in matter

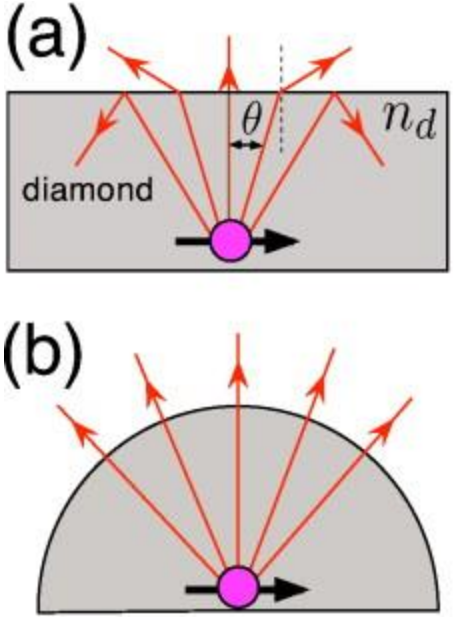
And the wavelength shortens



Recall Abbe's experiment:

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

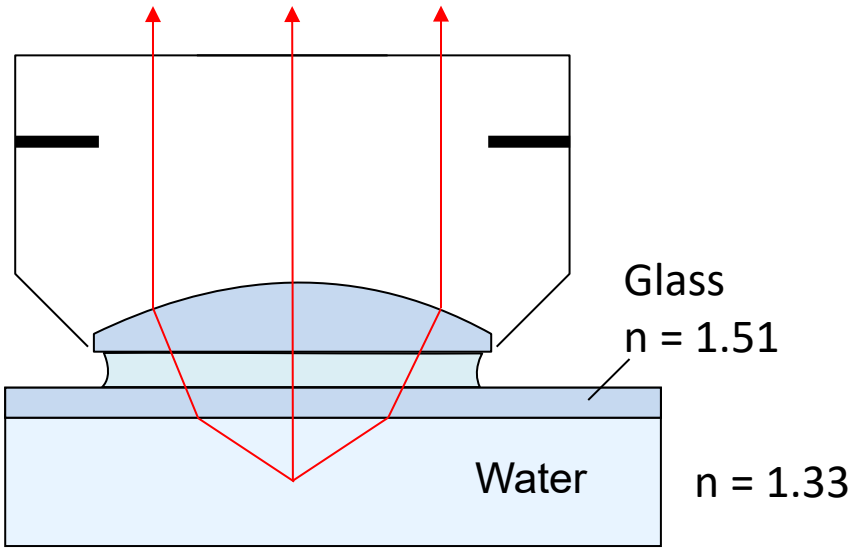
Modifying RI to change resolution



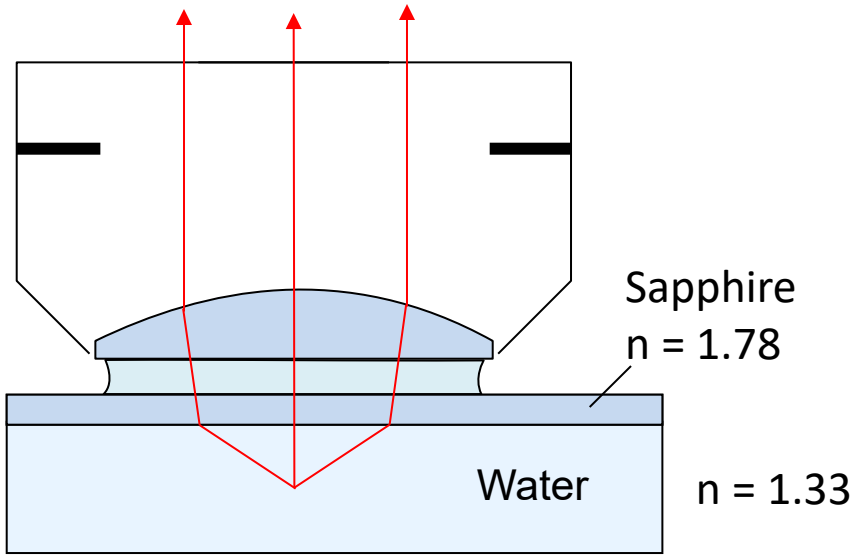
Olympus 1.65 NA TIRF lens:
Uses sapphire coverslips (n=1.76), diiodomethane (n=1.74)

Diamond solid immersion lens
Appl. Phys. Lett. 97, 241902 (2010)

Why not use NA 1.65 lenses?



NA 1.4 oil immersion objective



NA 1.65 oil immersion objective

What's the largest angle we can collect?

$$n_1 \sin\theta_1 = n_2 \sin\theta_2$$

$$1.33 \sin 90 = n_2 \sin\theta_2$$

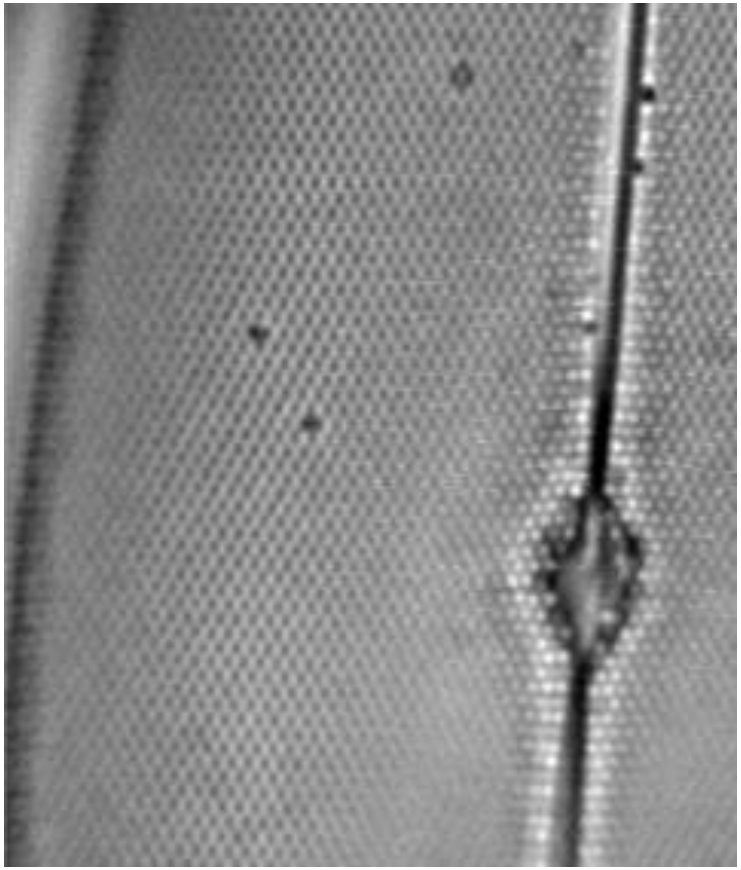
$$1.33/n_2 = \sin\theta_2$$

Effective NA is limited by the sample

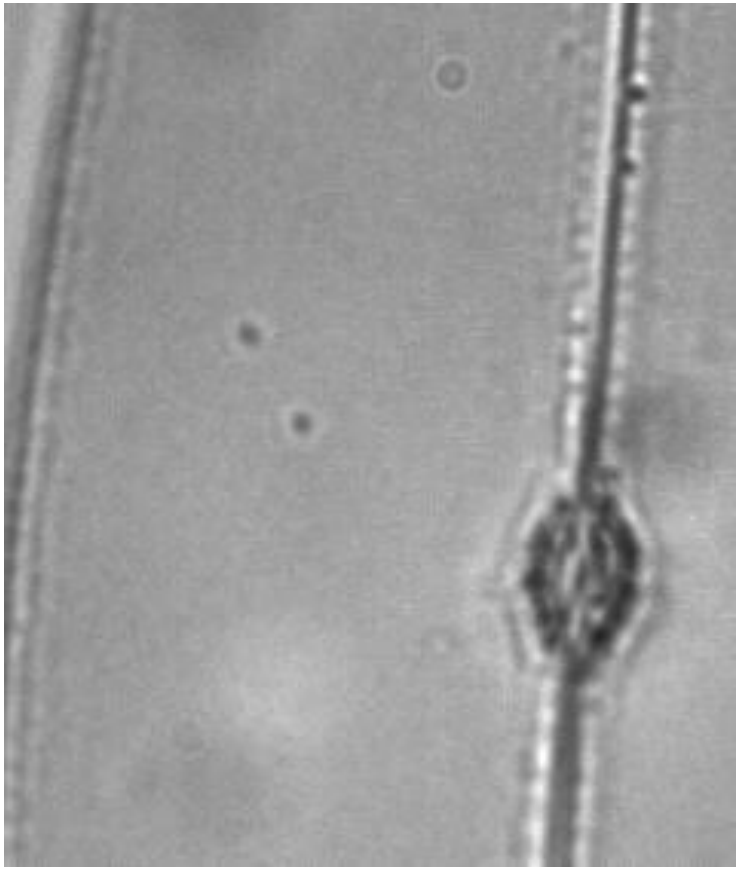
- To use a high NA objective, need a high RI mounting medium

Water	1.33
Glycerol	1.45
Vectashield	1.44
Prolong Gold	1.39 – 1.46
2,2-thiodiethanol	1.52
Methyl Salicylate	1.53
Benzyl benzoate	1.57

NA and Resolution



High NA Objective



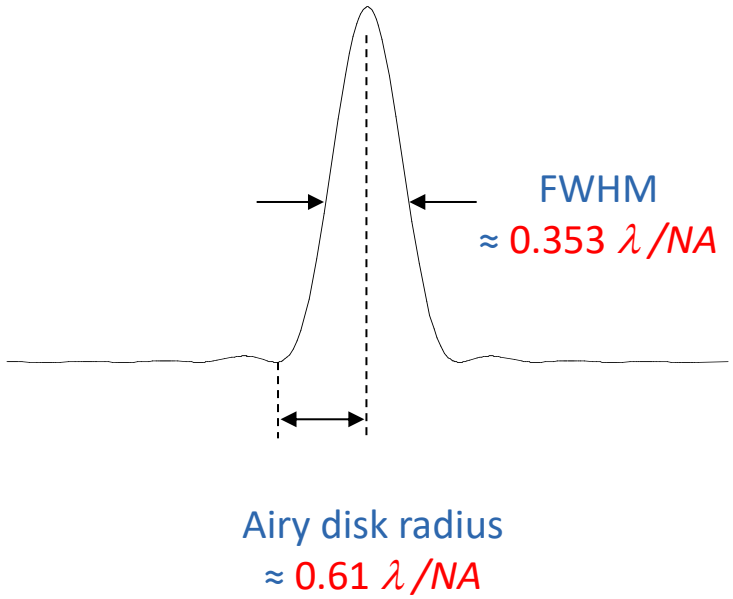
Low NA Objective

Alternate Definitions of Resolution

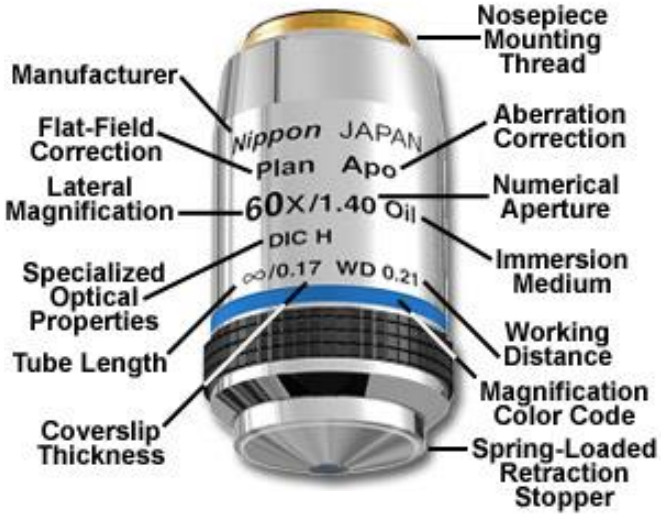
As the Full Width at Half Max (FWHM) of the PSF

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

(Probably most common definition)



Objective Types



Basic properties

- Magnification
- Numerical Aperture (NA)
- Infinite or finite conjugate
- Cover slip thickness if any
- Immersion fluid if any

Correction class

- Achromat
- Fluor
- Apochromat

Field flatness

- Plan or not

Phase rings for phase contrast

- Positive or negative
- Diameter of ring (number)

Special Properties

- Strain free for Polarization or DIC

Features

- Correction collar for spherical aberration
- Iris
- Spring-loaded front end
- Lockable front end

Further reading

www.microscopyu.com

micro.magnet.fsu.edu

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

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

Acknowledgements

Ron Vale / Mats Gustafsson / Steve Ross