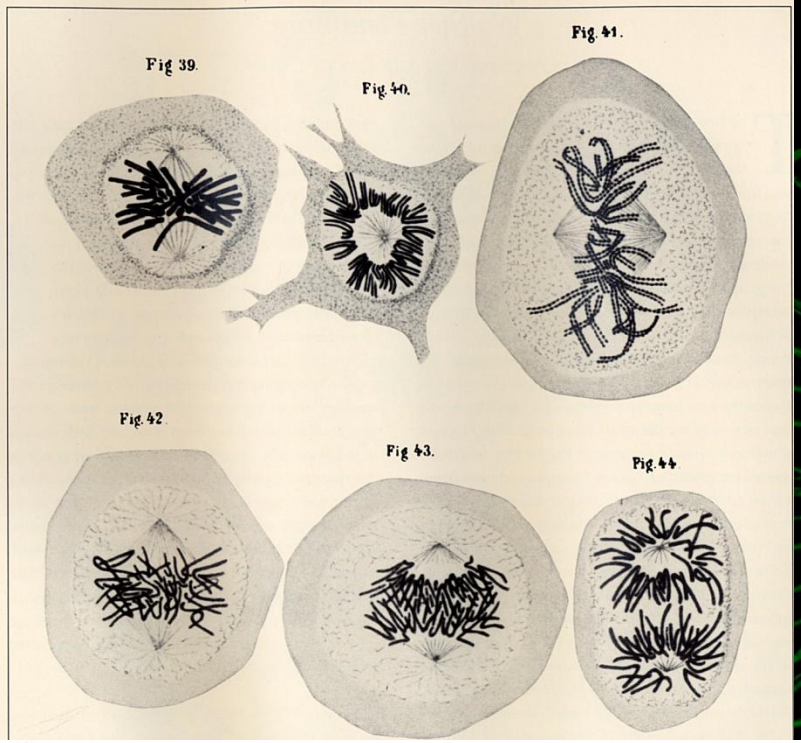
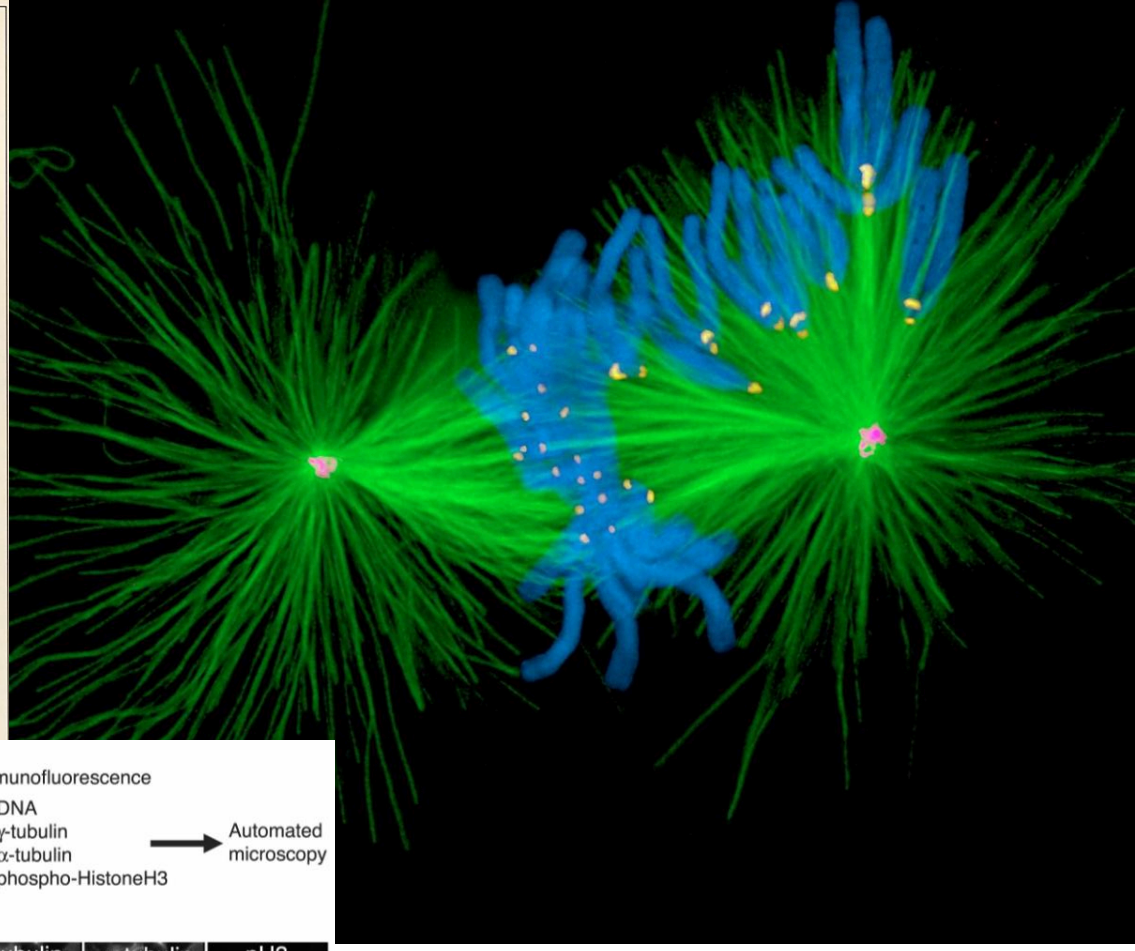
Introduction to Light Microscopy

(Image: T. Wittman, Scripps)

The Light Microscope

- Four centuries of history
- Vibrant current development
- One of the most widely used research tools





a

14000 dsRNA
(96-well plate x 146)

S2 cell
+ Cdc27 dsRNA

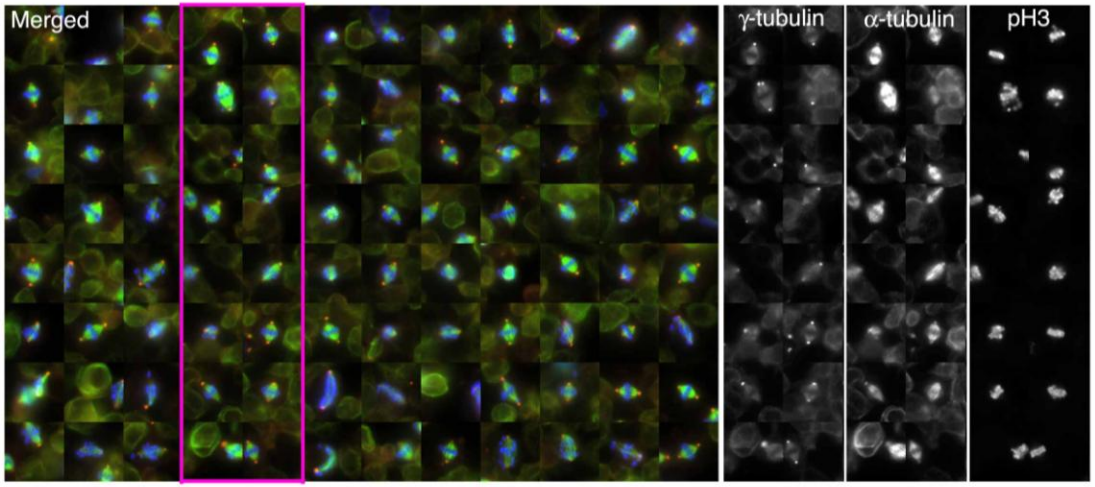
4 days

96-well, glass-bottom plate
(ConA-coated)

Immunofluorescence
DNA
γ-tubulin
α-tubulin
phospho-HistoneH3

Automated microscopy

b



Major Imaging Functions of the Microscope

- Magnify
- Resolve features
- Generate Contrast
- Capture and Display Images

An Upright Epifluorescence Microscope

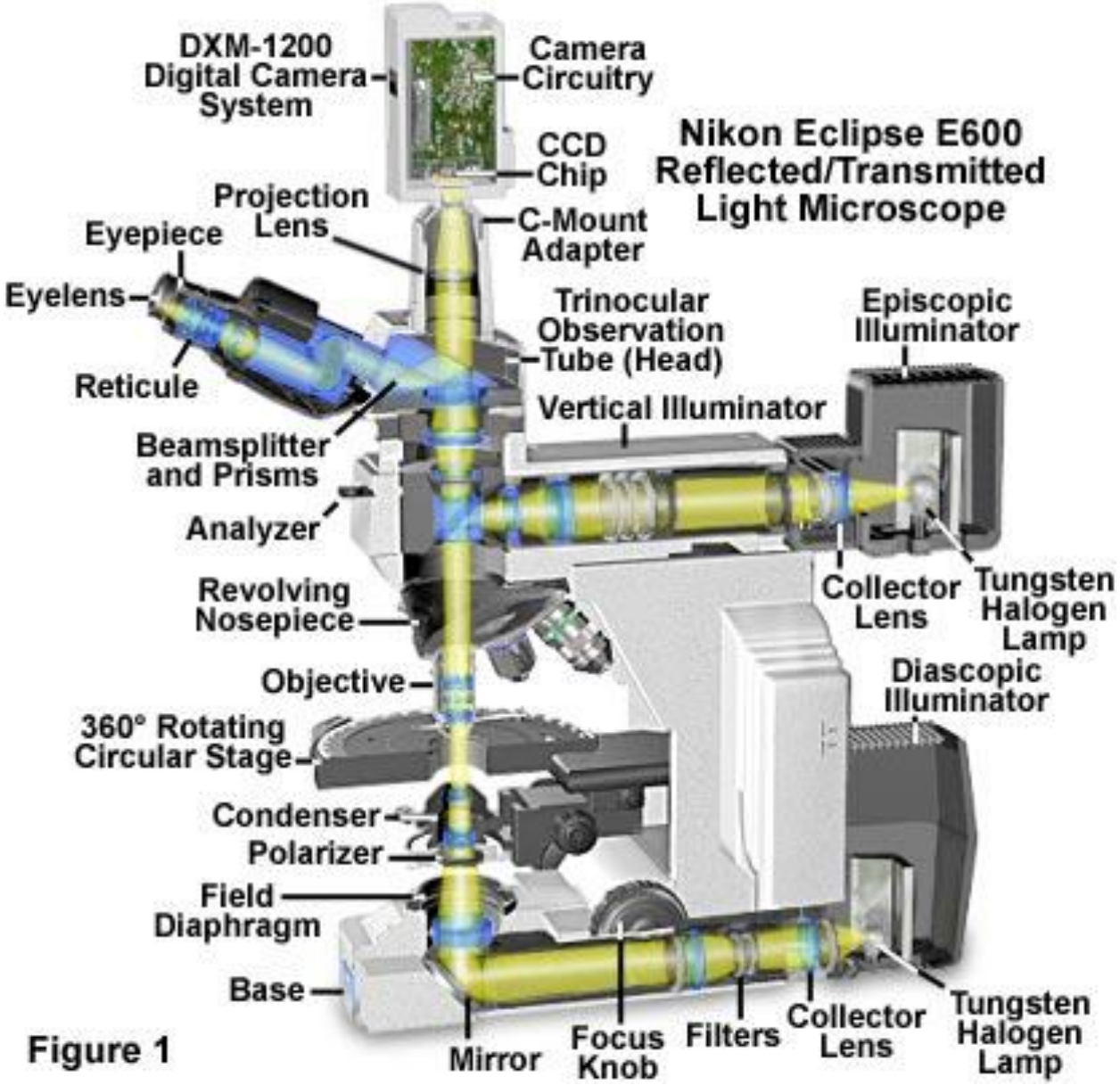
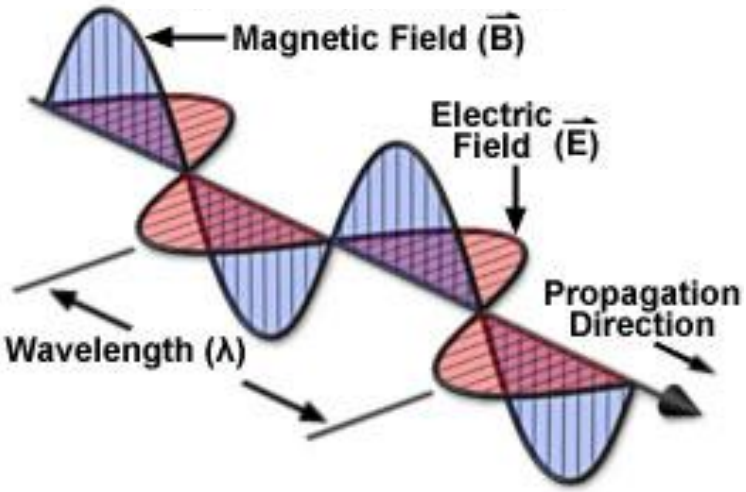


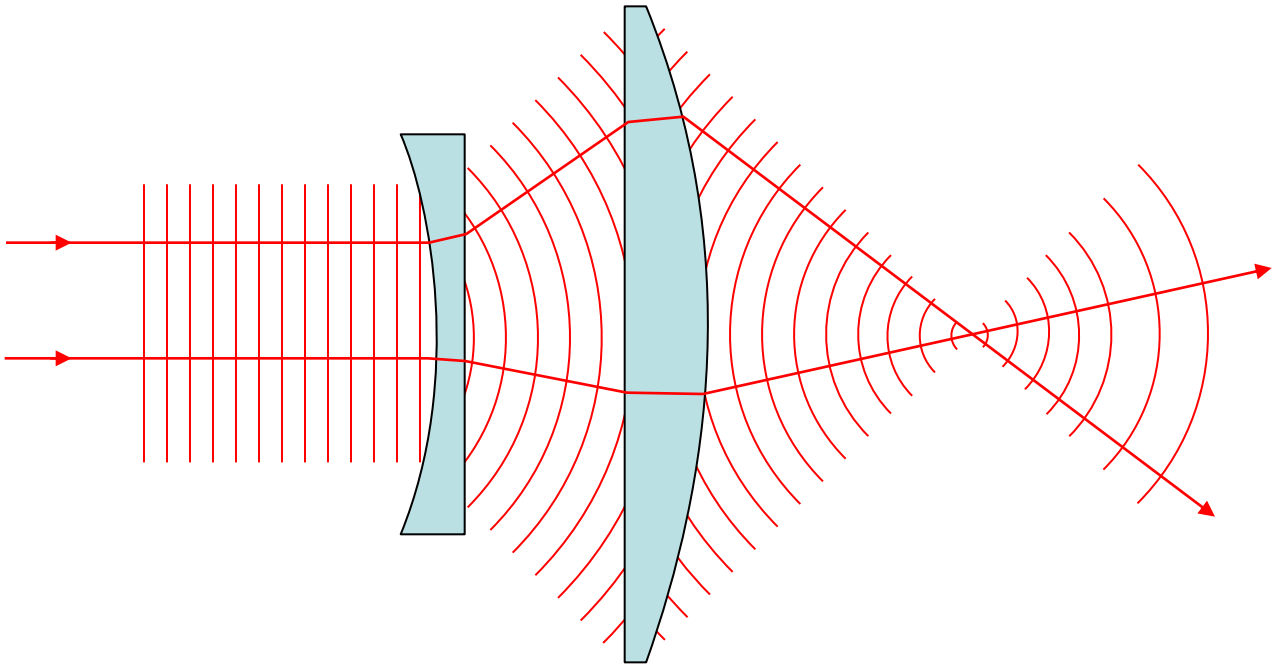
Figure 1

Waves vs. Photons vs. Rays

- Quantum wave-particle duality
- Rays: photon trajectories
- Rays: propagation direction of waves



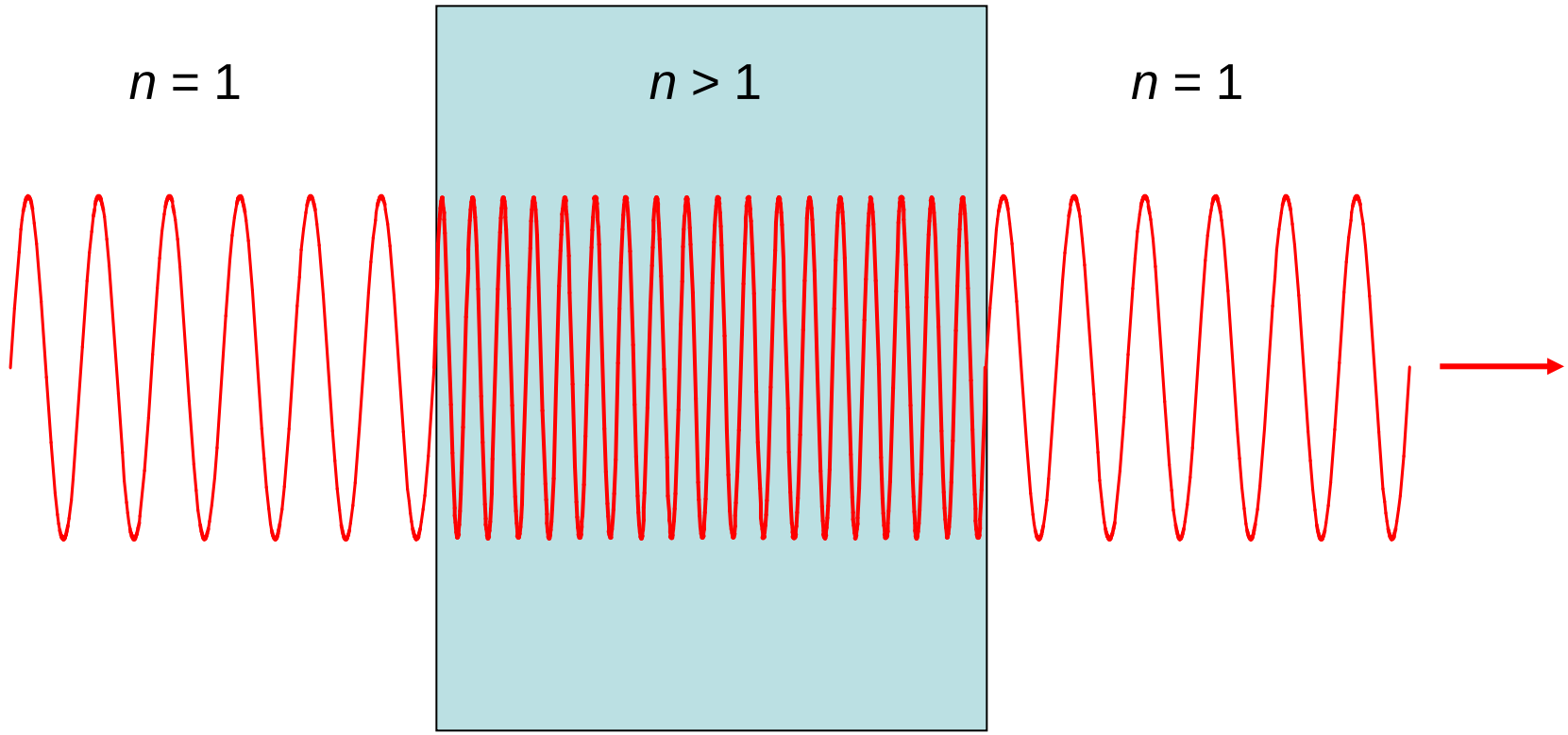
Rays are perpendicular to wavefronts



Light travels more slowly in matter

The speed ratio is the ***Index of Refraction, n***

$$v = c/n$$

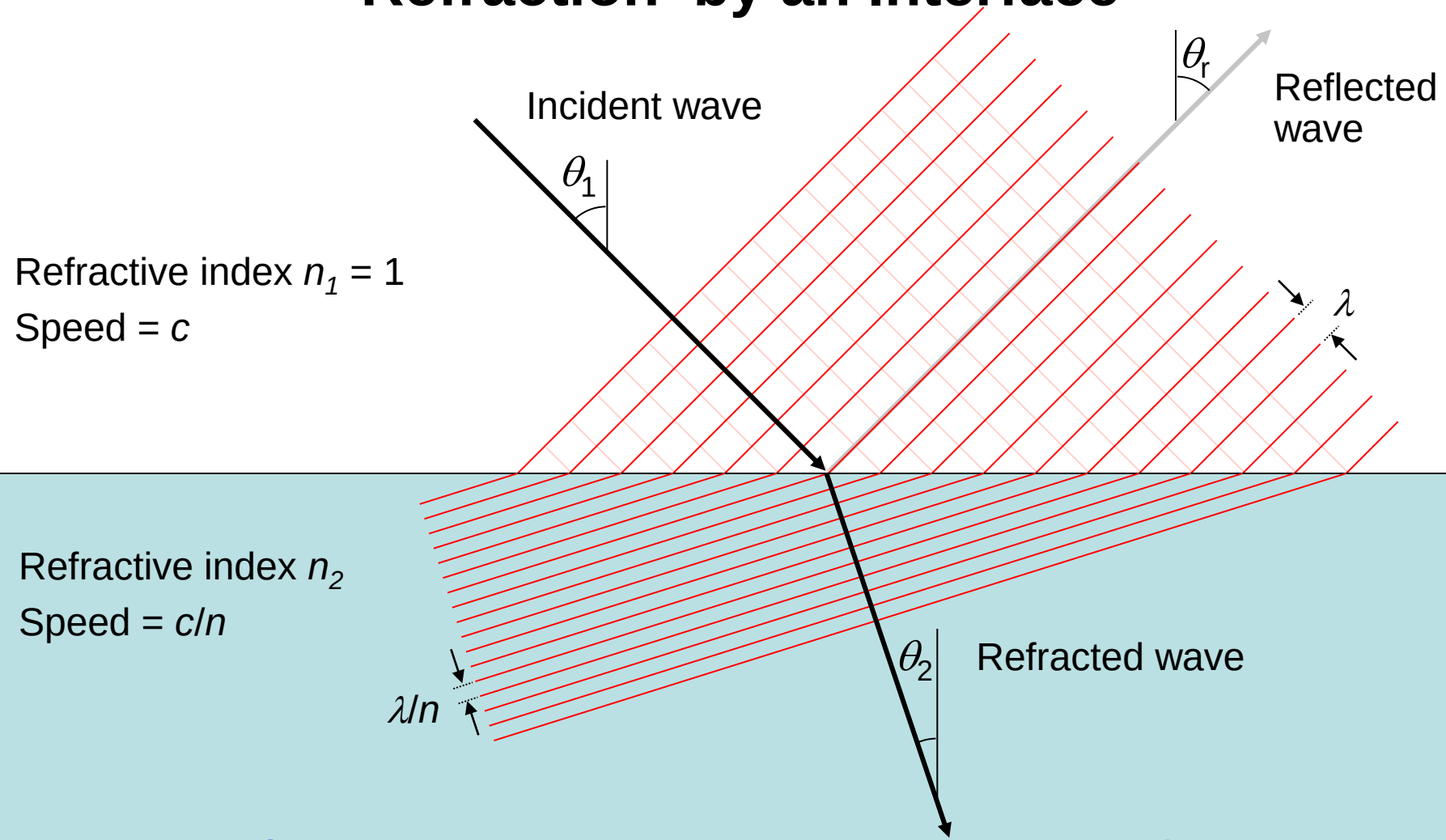


Refractive Index Examples

• Vacuum	1
• Air	1.0003
• Water	1.333
• Cytoplasm	1.35–1.38 ?
• Glycerol	1.475 (anhydrous)
• Immersion oil	1.515
• Fused silica	1.46
• Optical glasses	1.5–1.9
• Diamond	2.417

Depends on wavelength and temperature

Refraction by an Interface



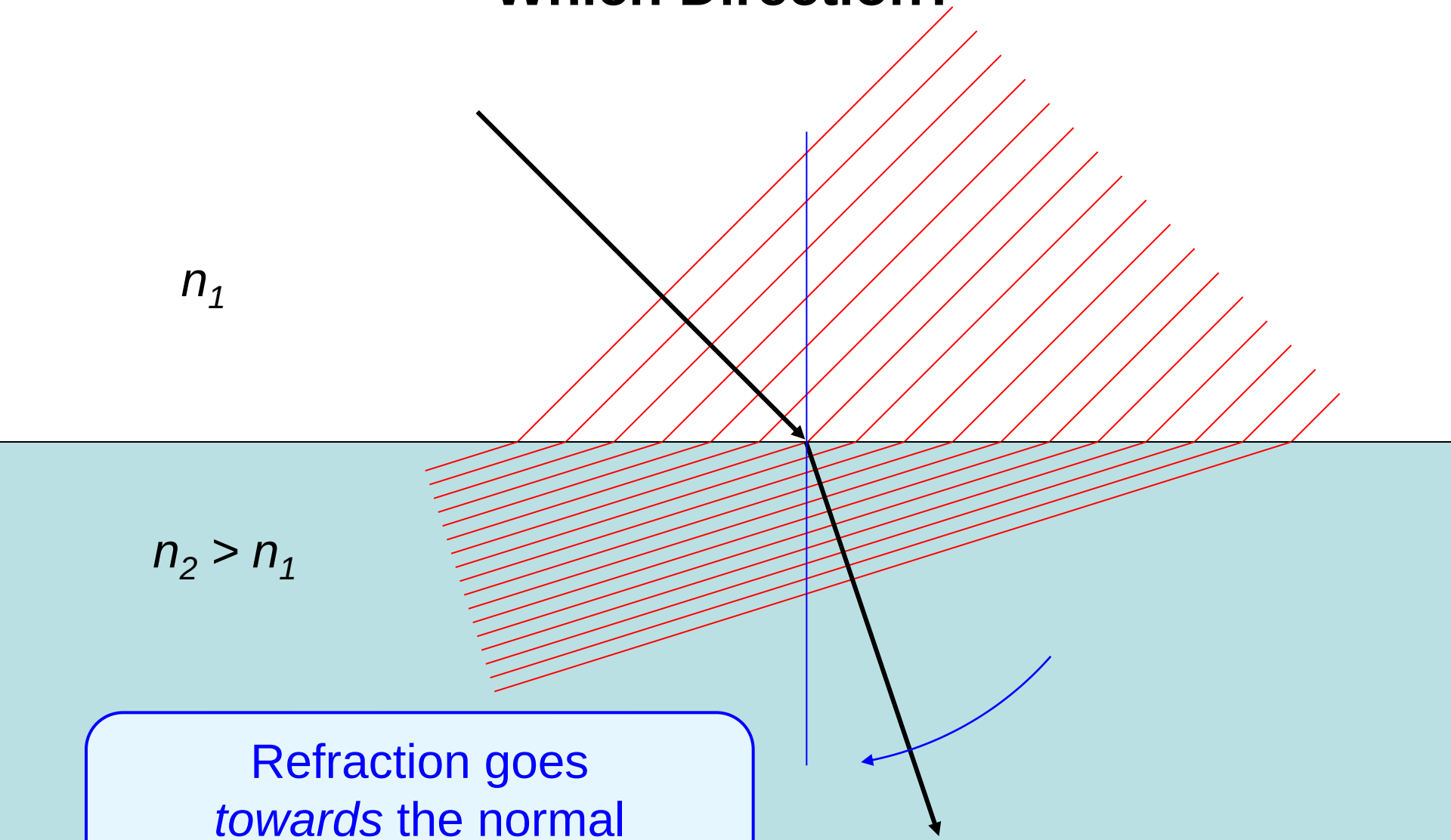
$\Rightarrow$ Snell's law:

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

Mirror law:

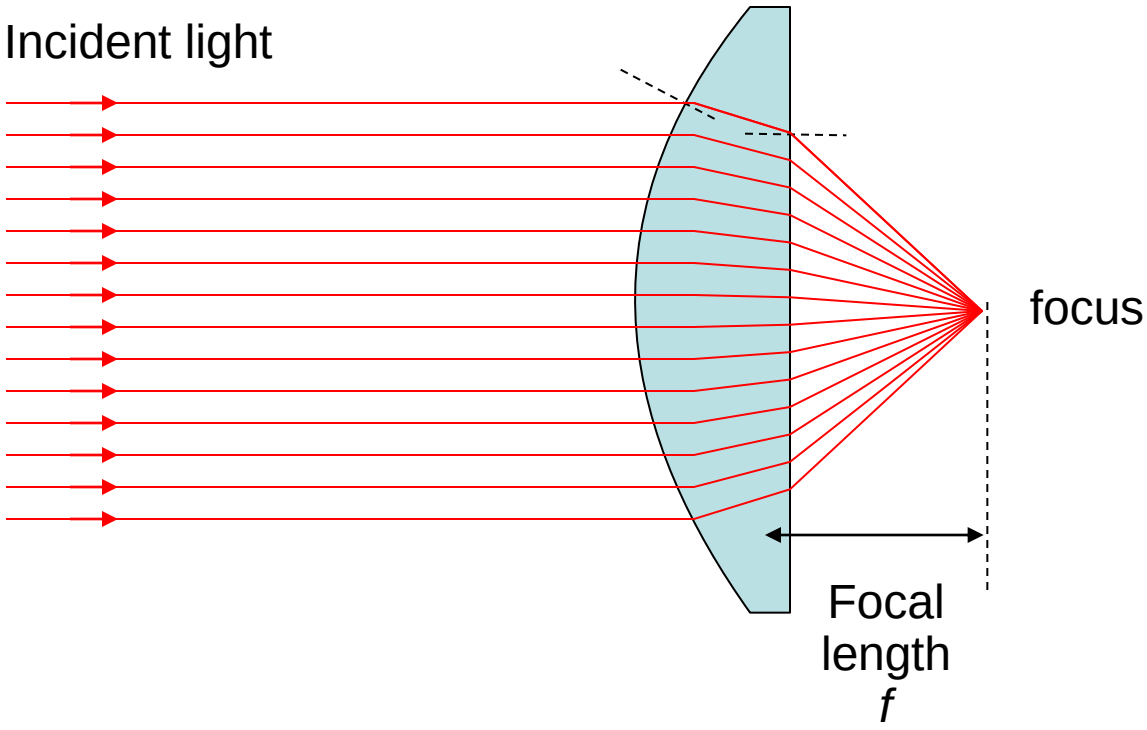
$$\theta_r = \theta_1$$

Which Direction?



Refraction goes
towards the normal
in the *higher*-index medium

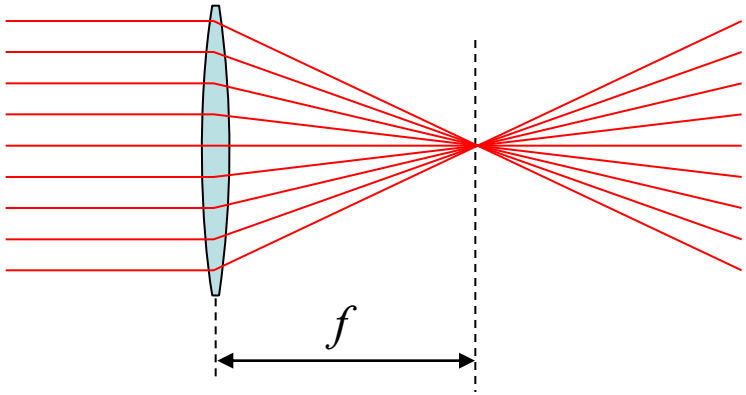
Lenses work by refraction



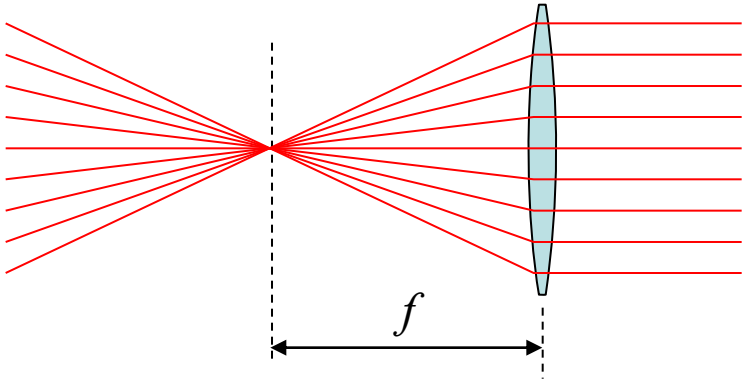
Ray Tracing Rules of Thumb

(for thin ideal lenses)

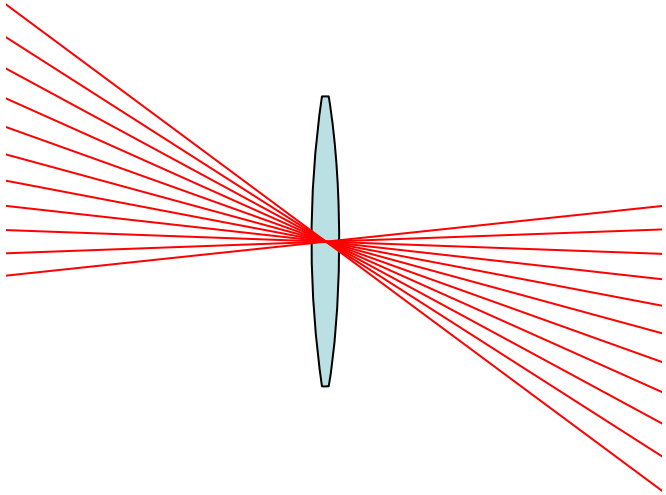
Parallel rays converge at the focal plane



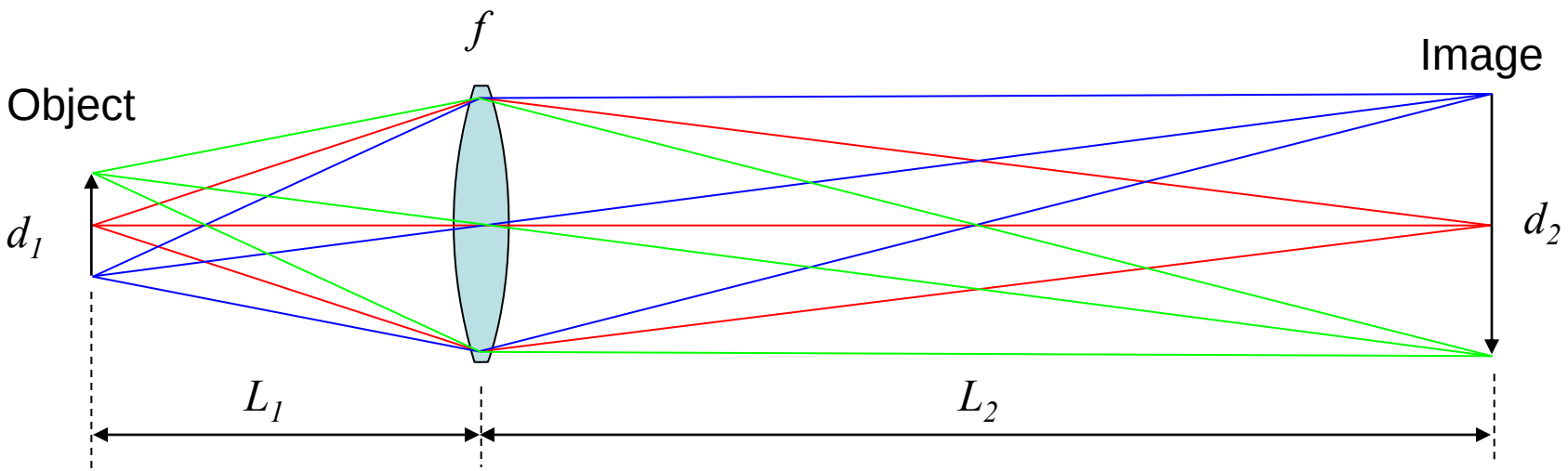
Rays that cross in the focal plane end up parallel



Rays through the lens center are unaffected



Imaging

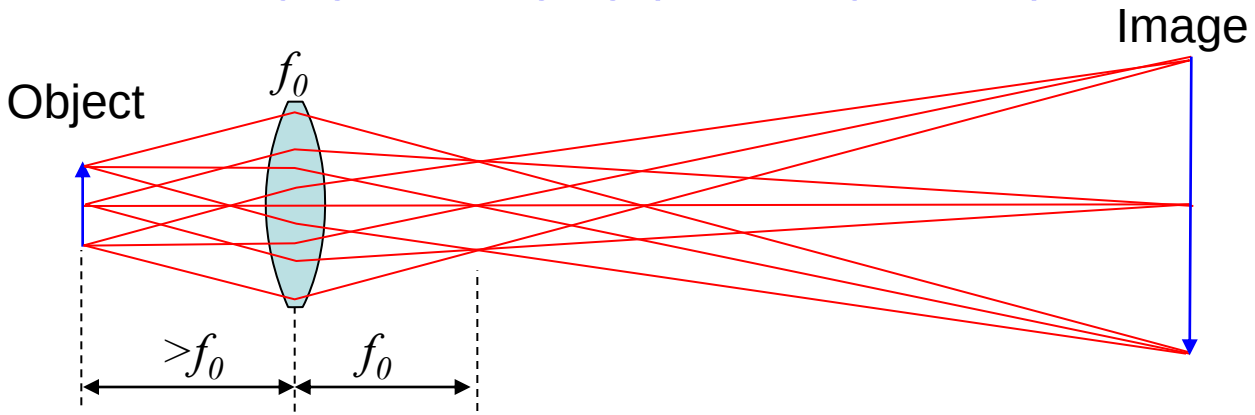


The lens law: $$\frac{1}{L_1}+\frac{1}{L_2}=\frac{1}{f}$$

Magnification: $$M=\frac{d_2}{d_1}=\frac{L_2}{L_1}$$

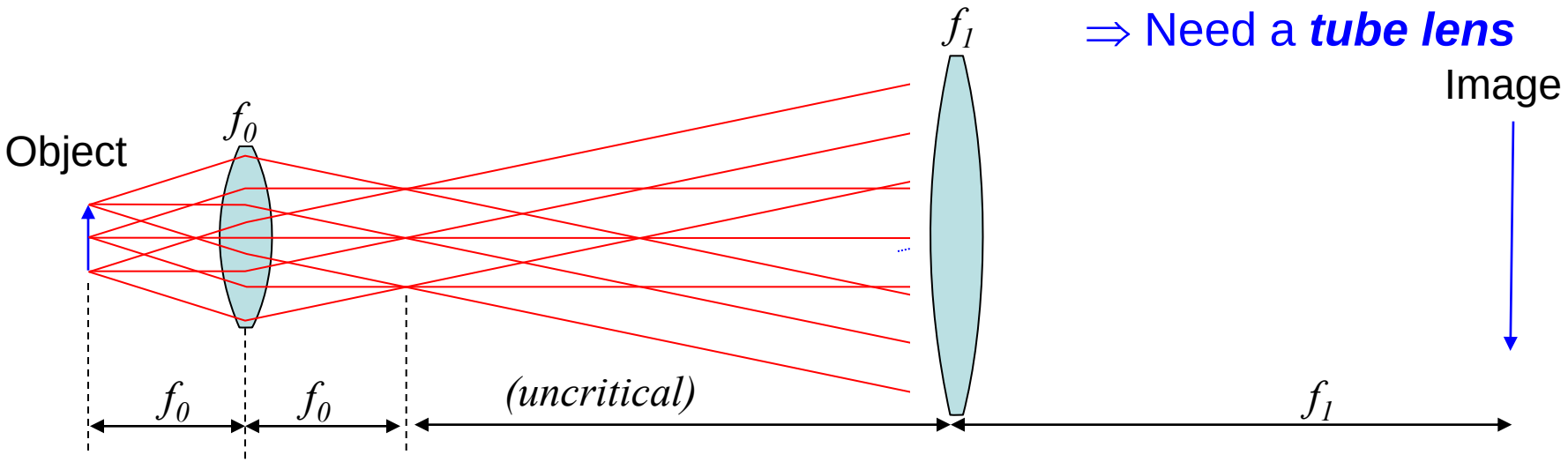
Finite vs. Infinite Conjugate Imaging

- Finite conjugate imaging (older objectives)



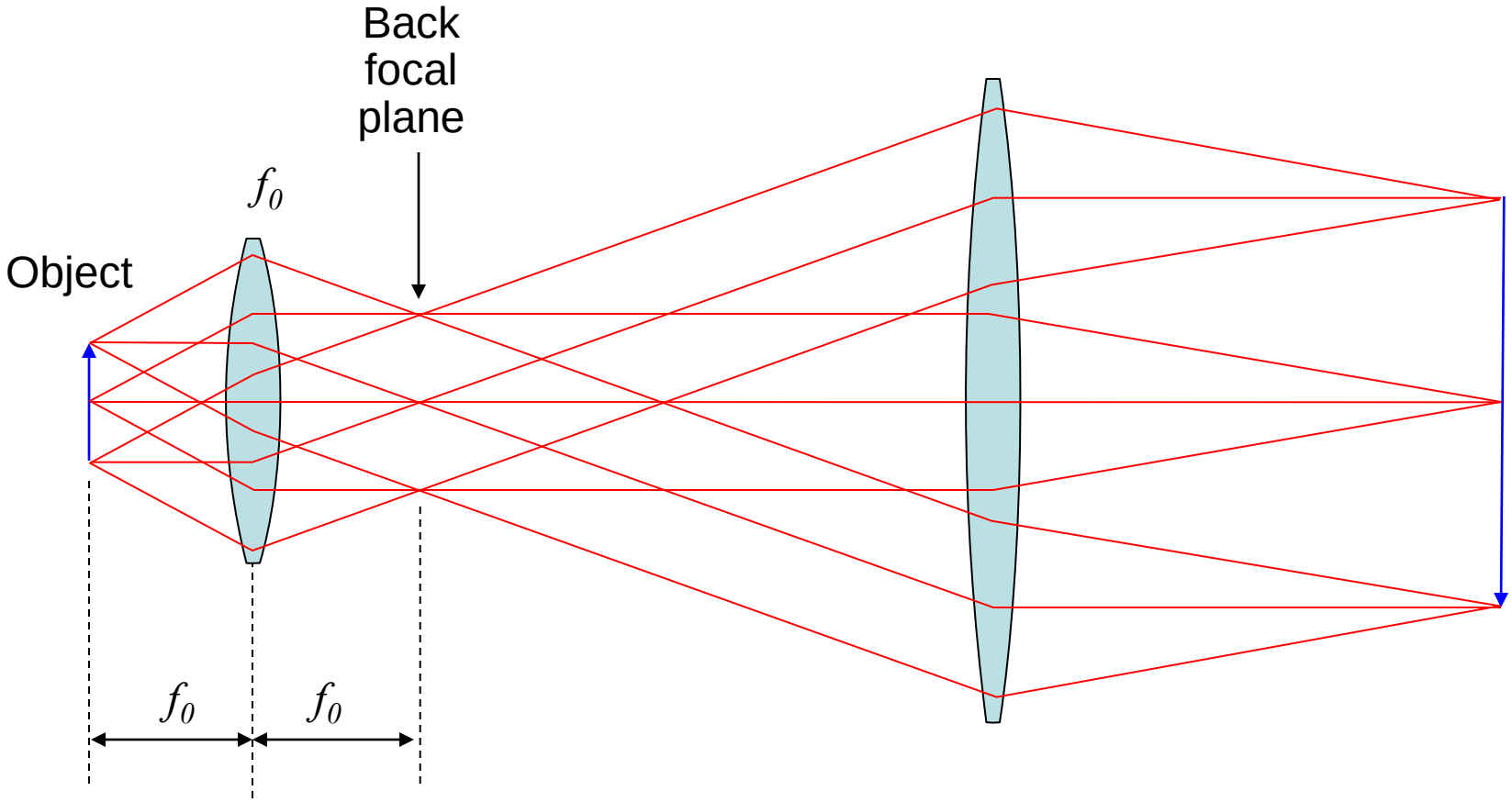
- Infinite conjugate imaging (modern objectives).

Image at infinity
$\Rightarrow$ Need a ***tube lens***



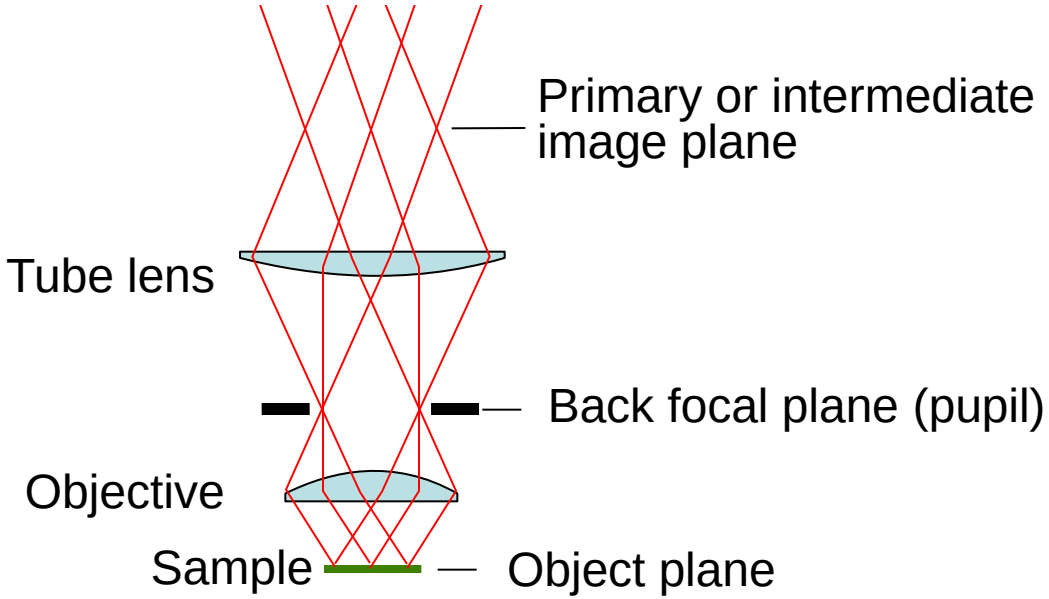
Magnification: $M = \frac{f_1}{f_o}$

Back focal plane

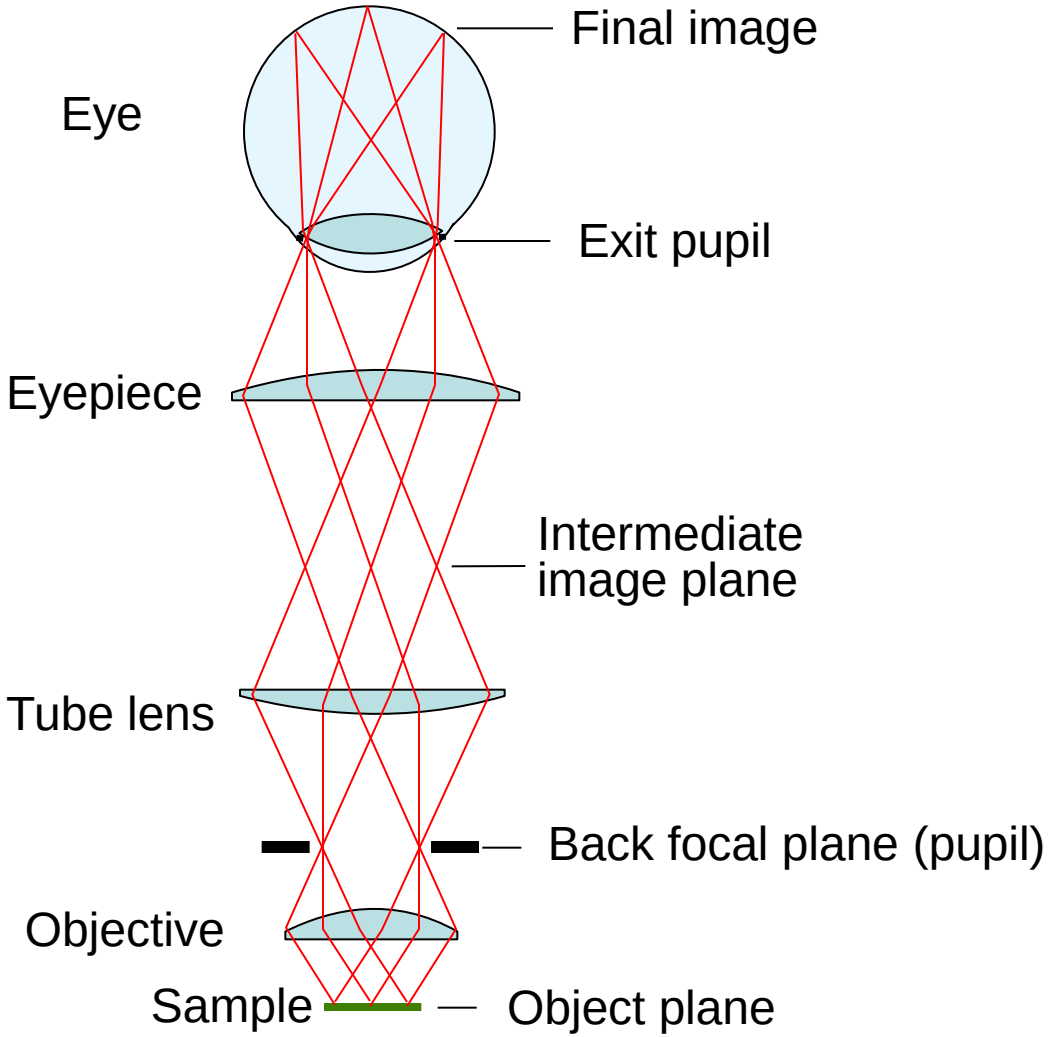


Rays that leave the object with the same angle meet in the objective's *back focal plane*

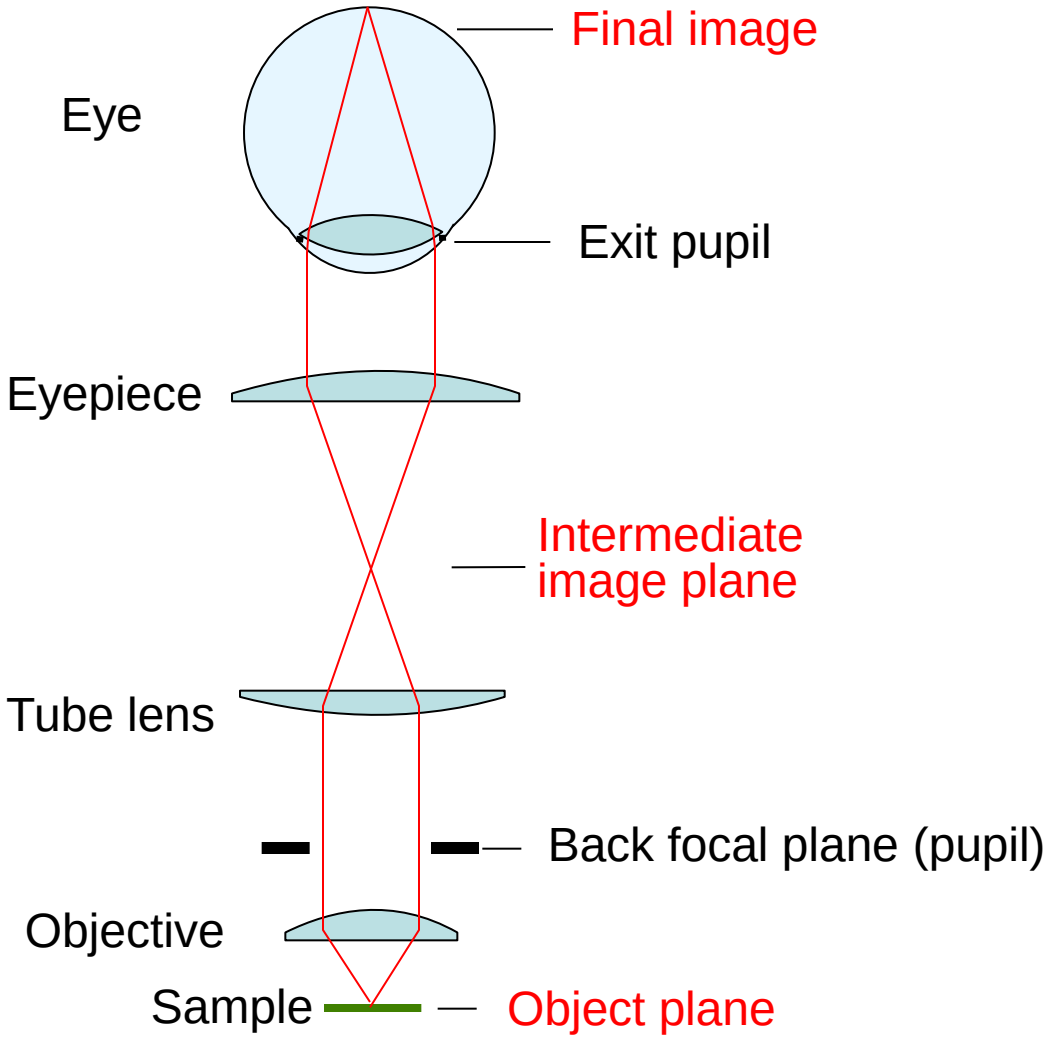
The Compound Microscope



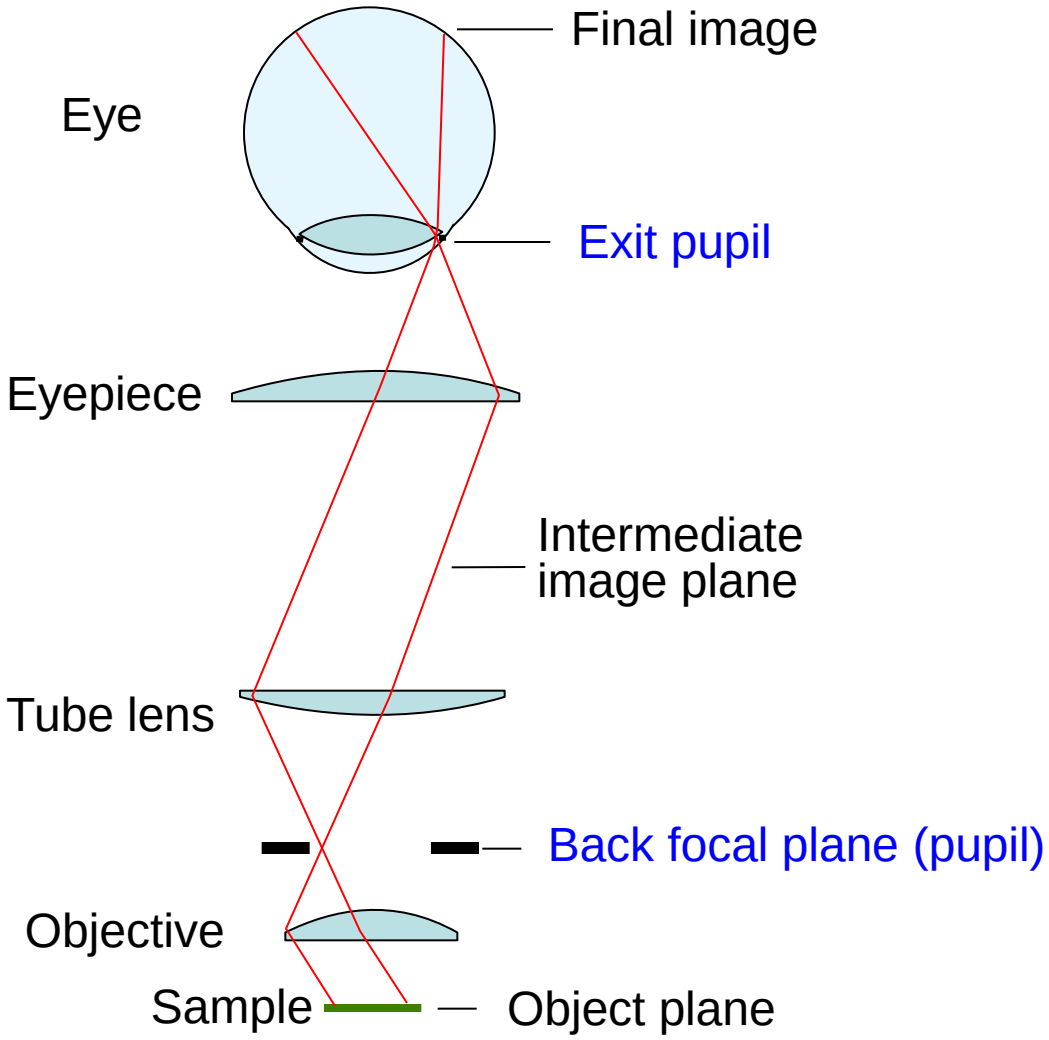
The Compound Microscope



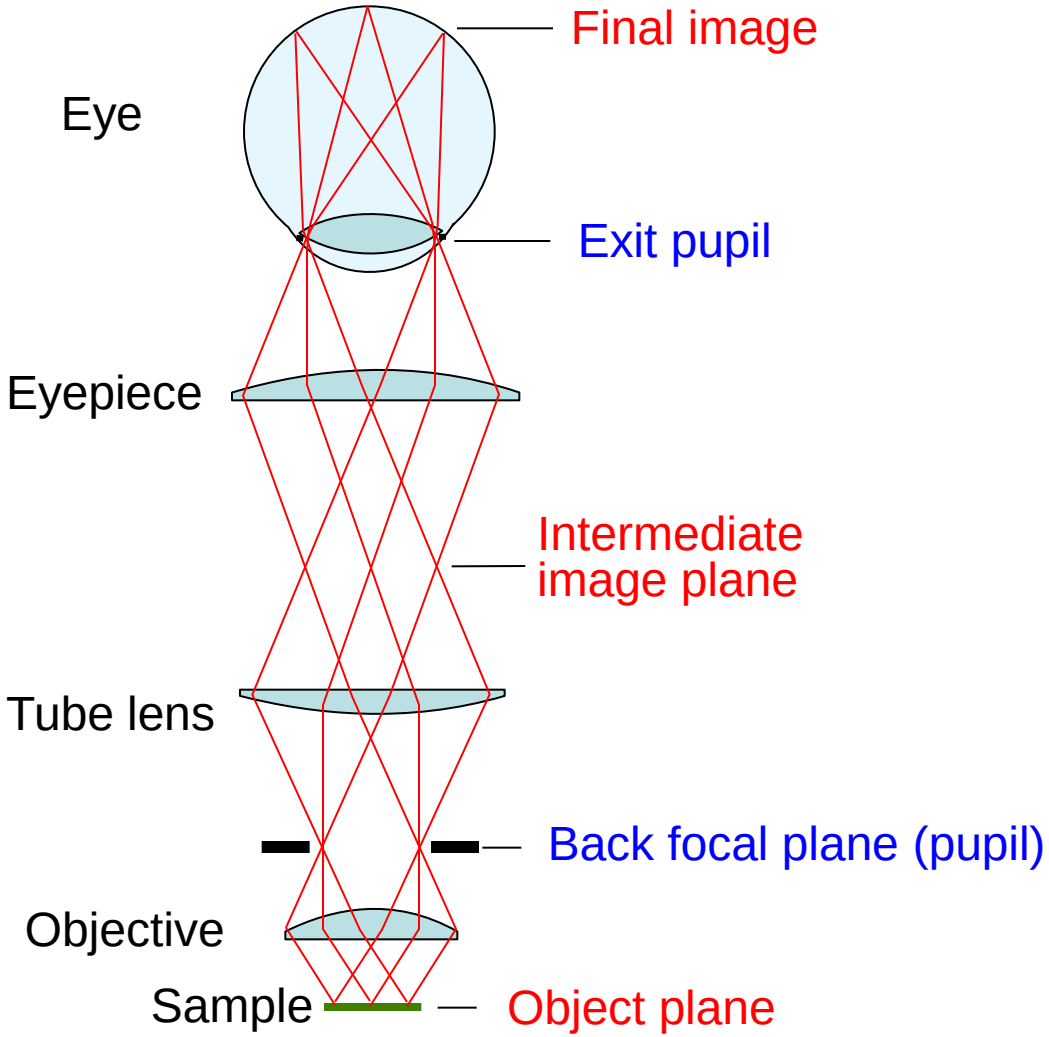
The Compound Microscope



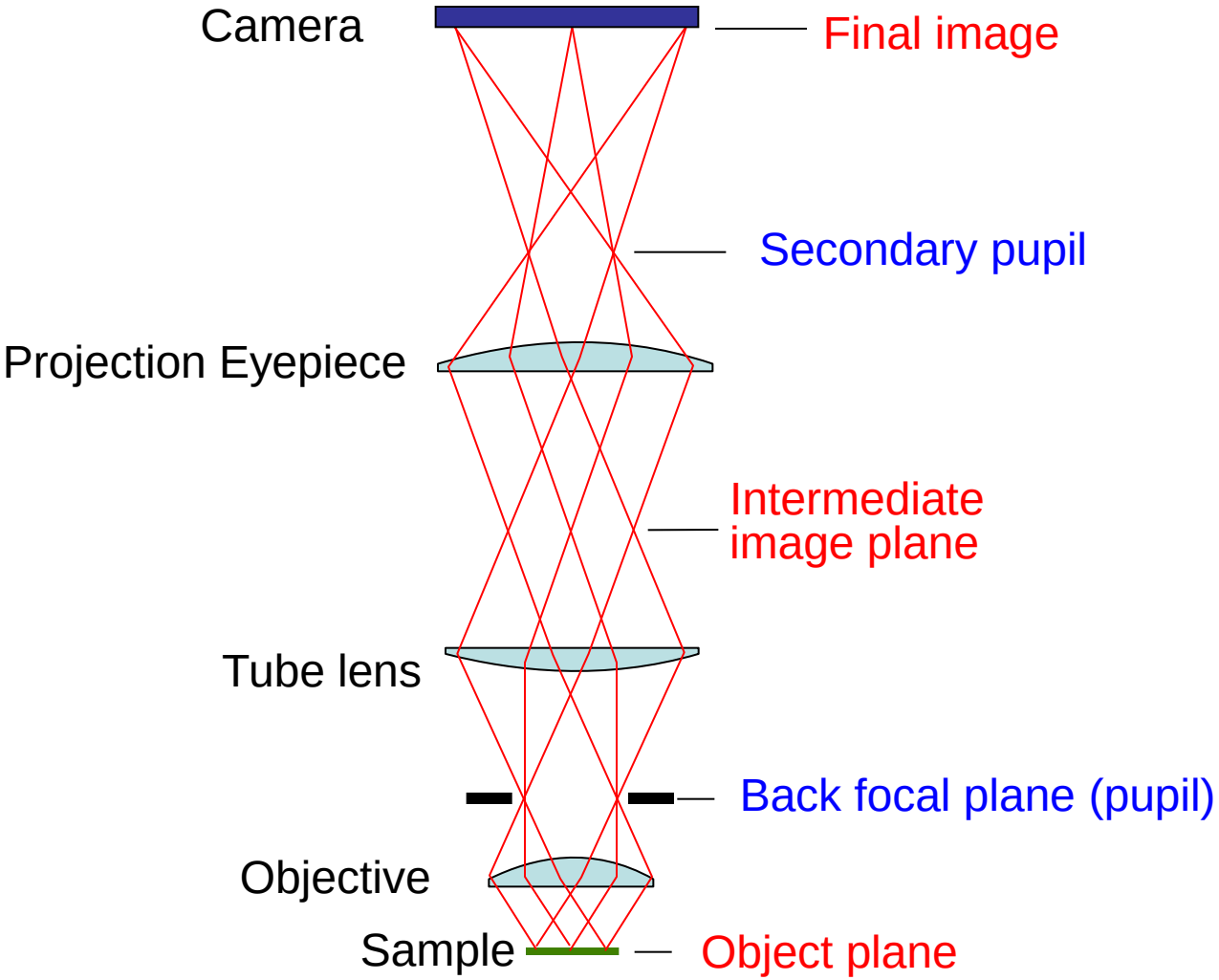
The Compound Microscope



The Compound Microscope



The Compound Microscope

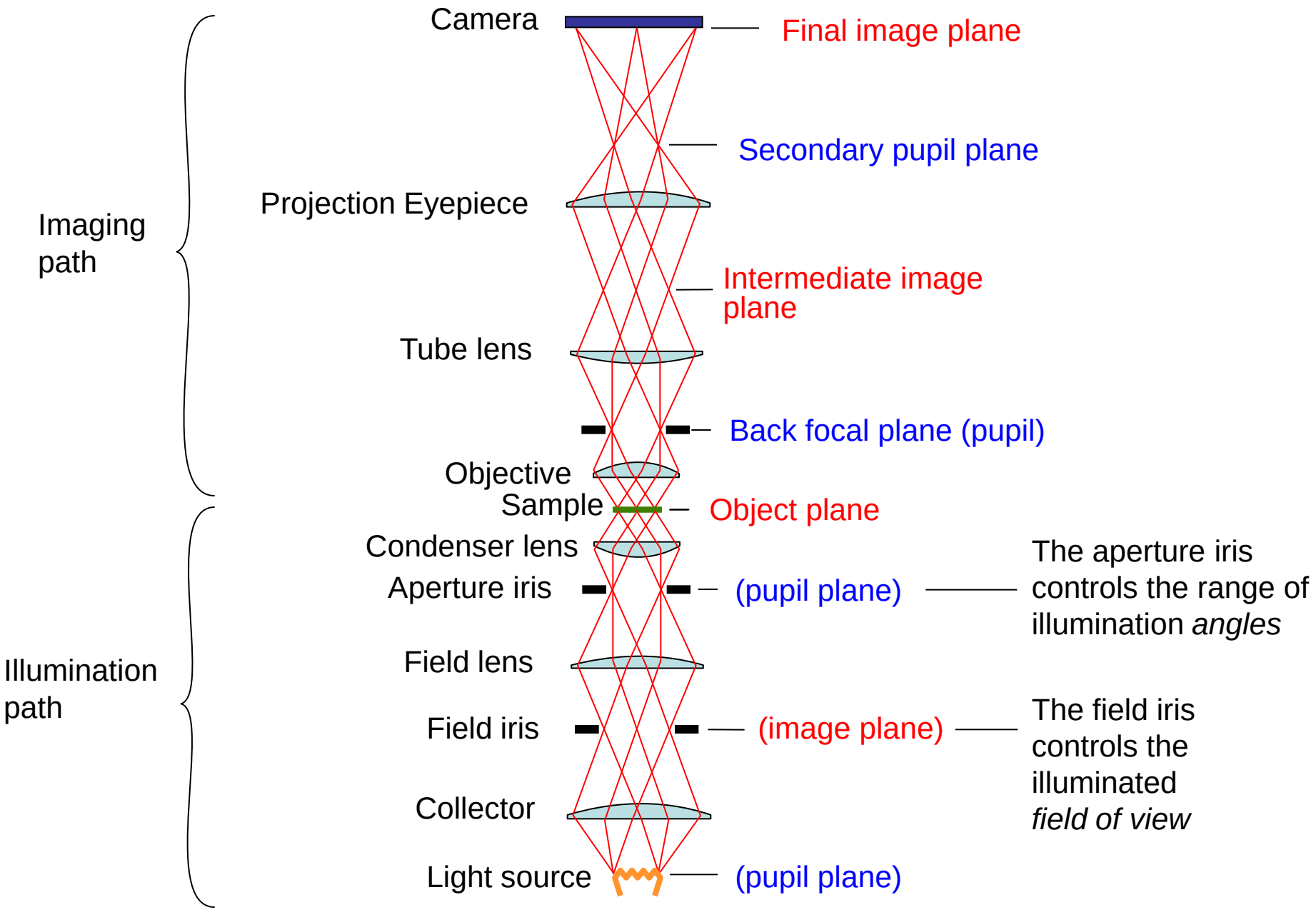


Eyepieces (Oculars)

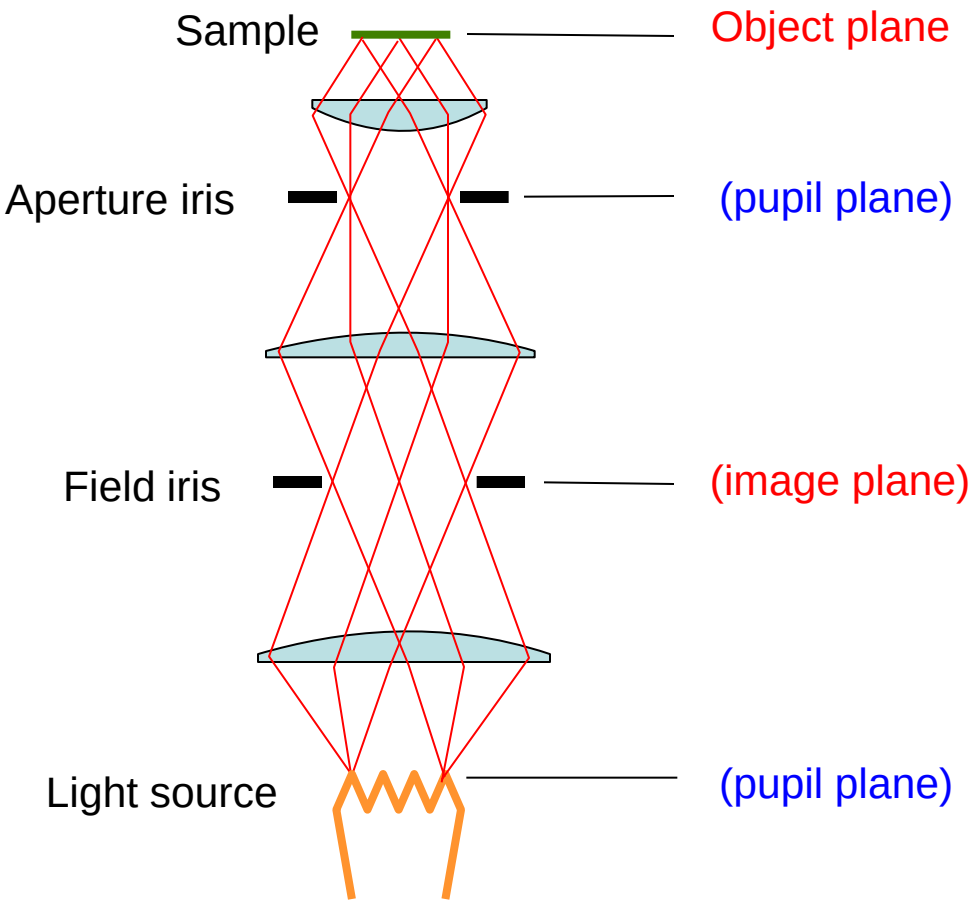
Features

- Magnification (10x typical)
- “High eye point” (exit pupil high enough to allow eyeglasses)
- Diopter adjust (at least *one* must have this)
- Reticle or fitting for one
- Eye cups

Trans-illumination Microscope

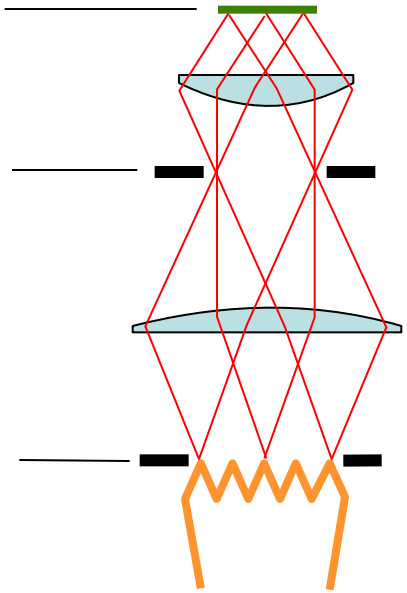


Köhler Illumination



- Each light source point produces a parallel beam of light at the sample
- Uniform light intensity at the sample even if the light source is “ugly” (e.g. a filament)

Critical Illumination



- The source is imaged onto the sample
- Usable only if the light source is perfectly uniform

Conjugate Planes in A Research Microscope

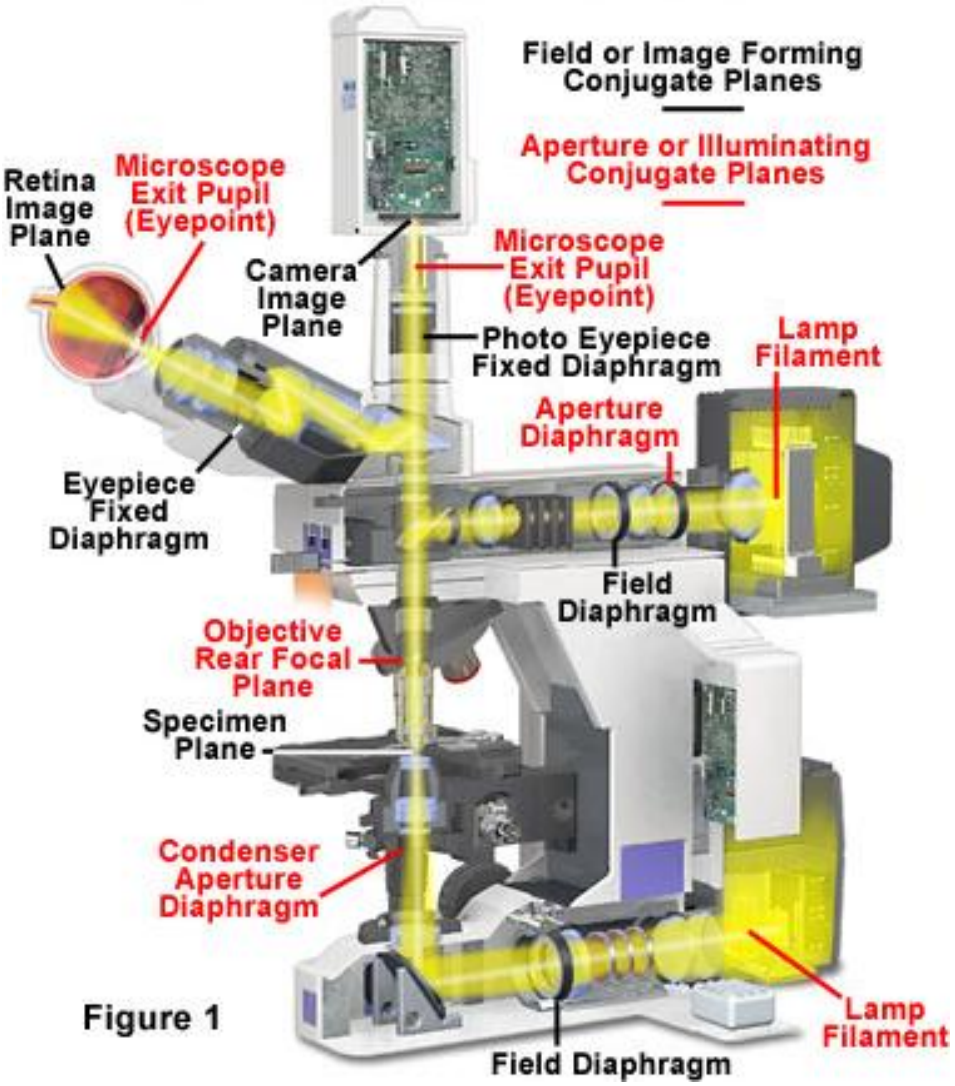


Figure 1

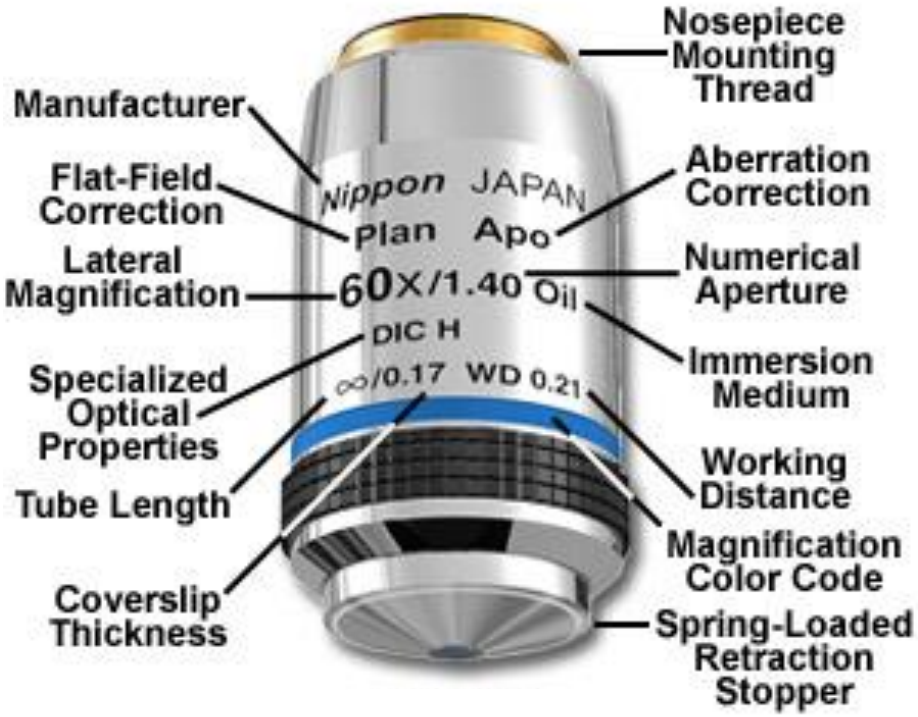
How view the pupil planes?

Two ways:

- “Eyepiece telescope”
- “Bertrand lens”

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

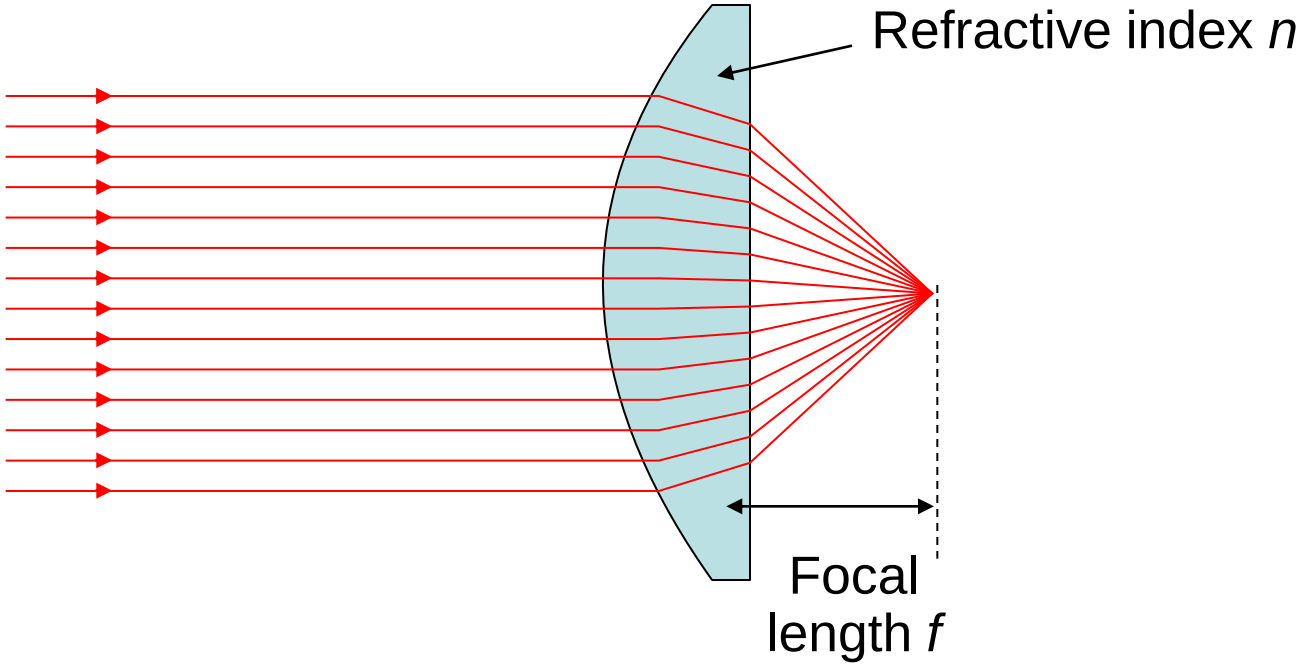
Some examples:

10x/0.3 WD = 15.2mm

20x/0.75 WD = 1.0mm

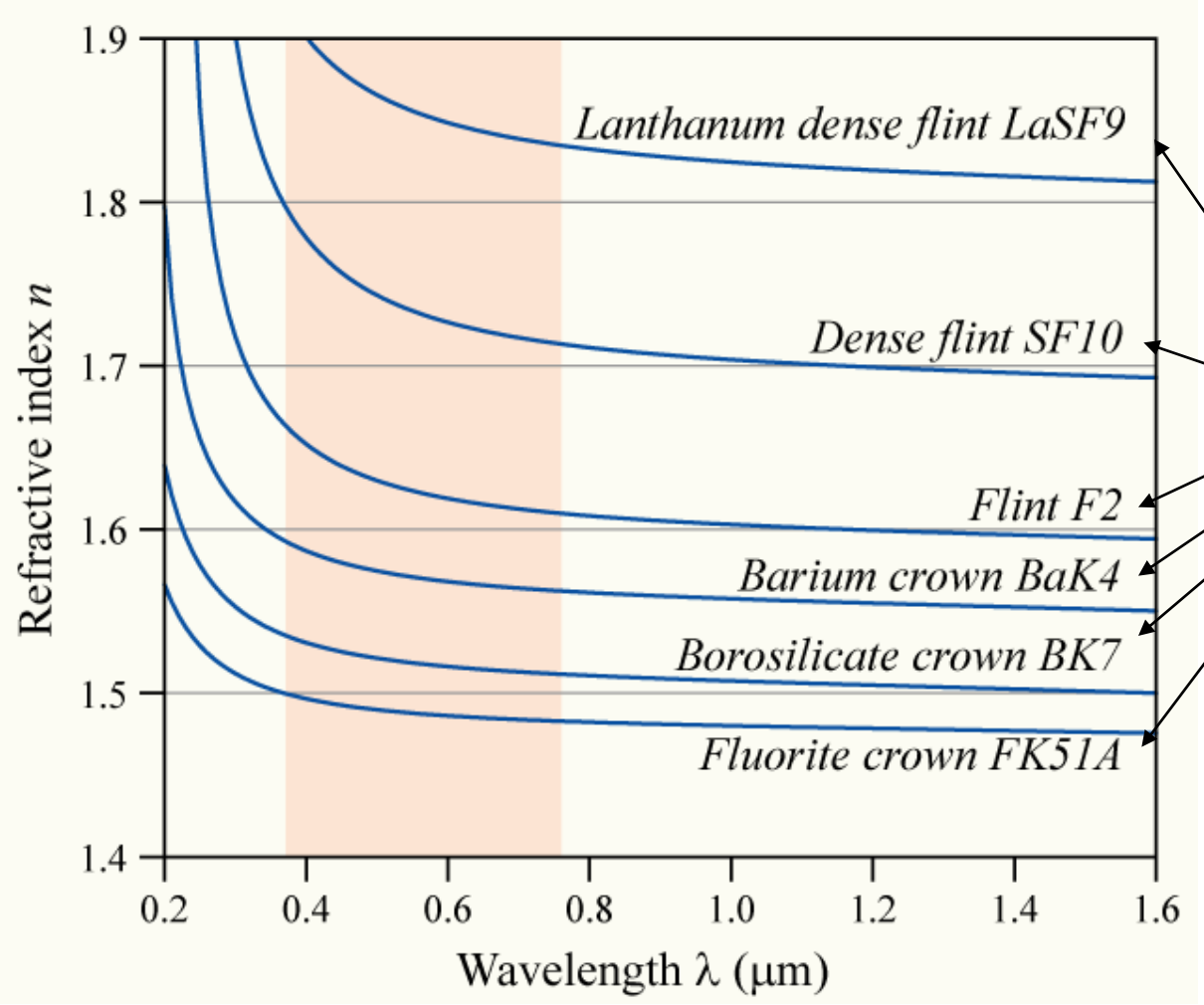
100x/1.4 WD = 0.13mm

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



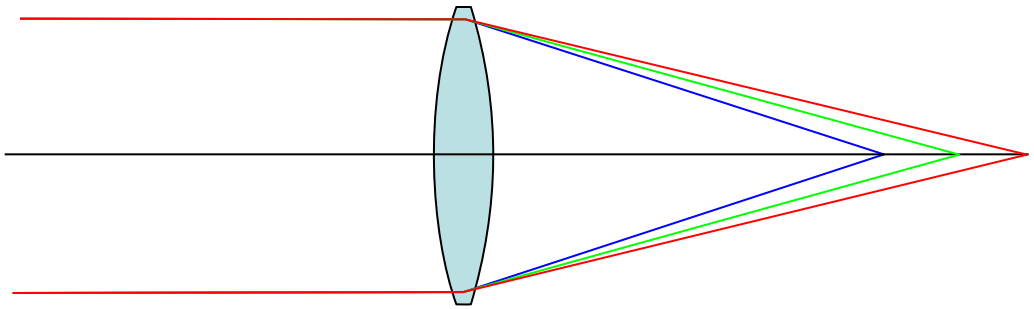
$f \propto 1/(n-1)$

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

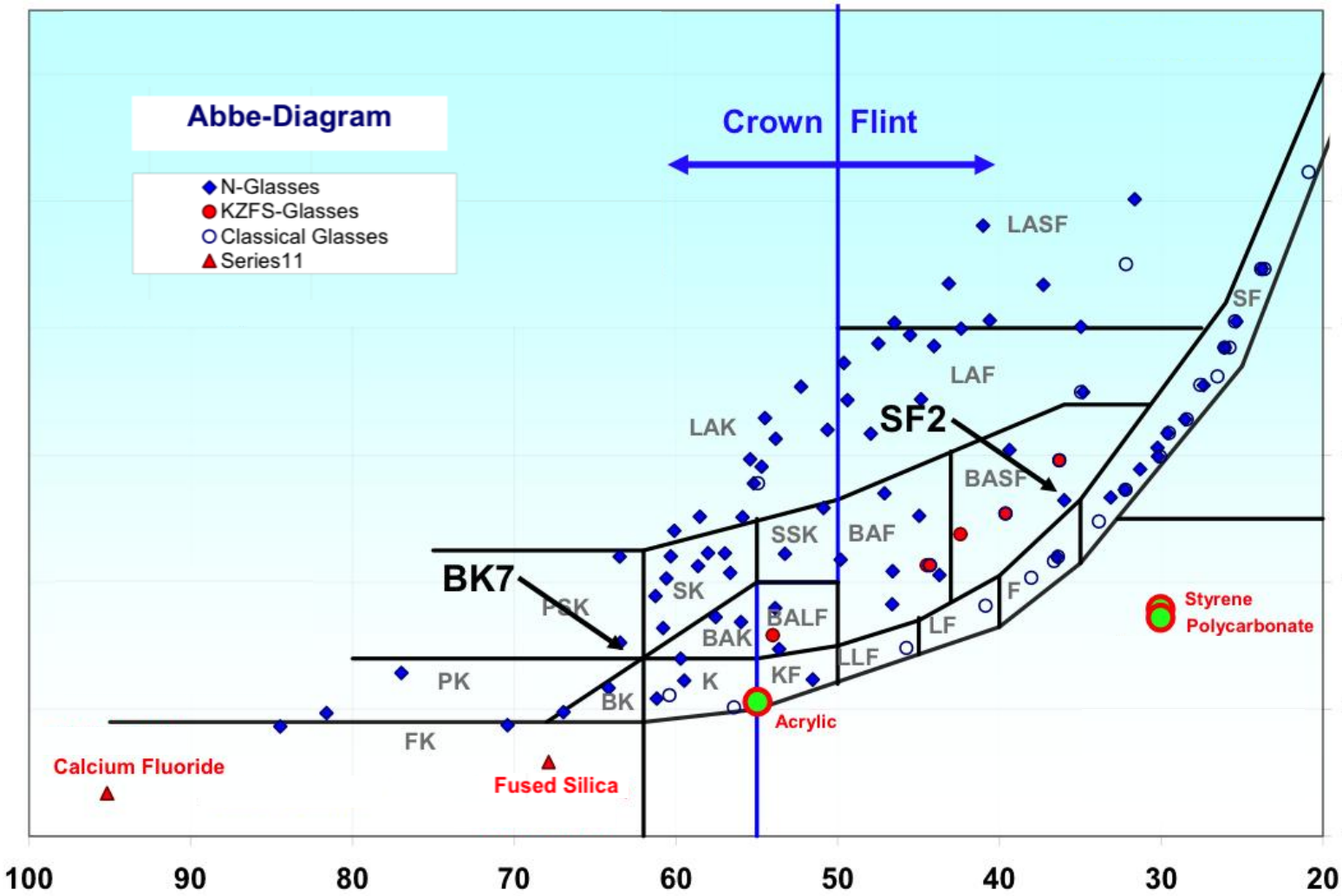


⇒ Chromatic aberration

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

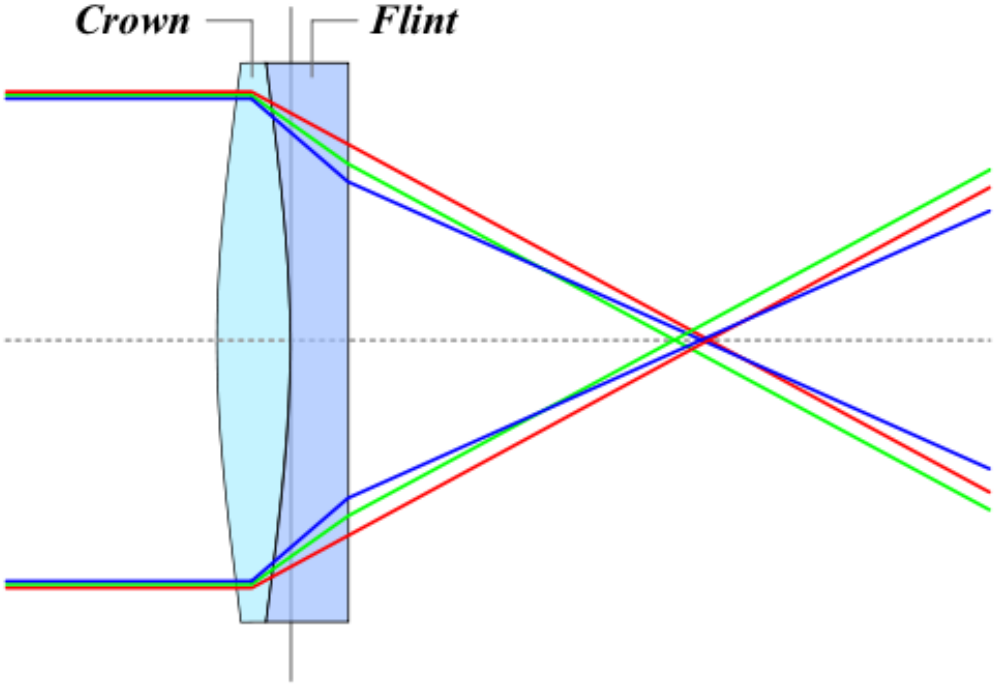


Dispersion vs. refractive index of different glass types

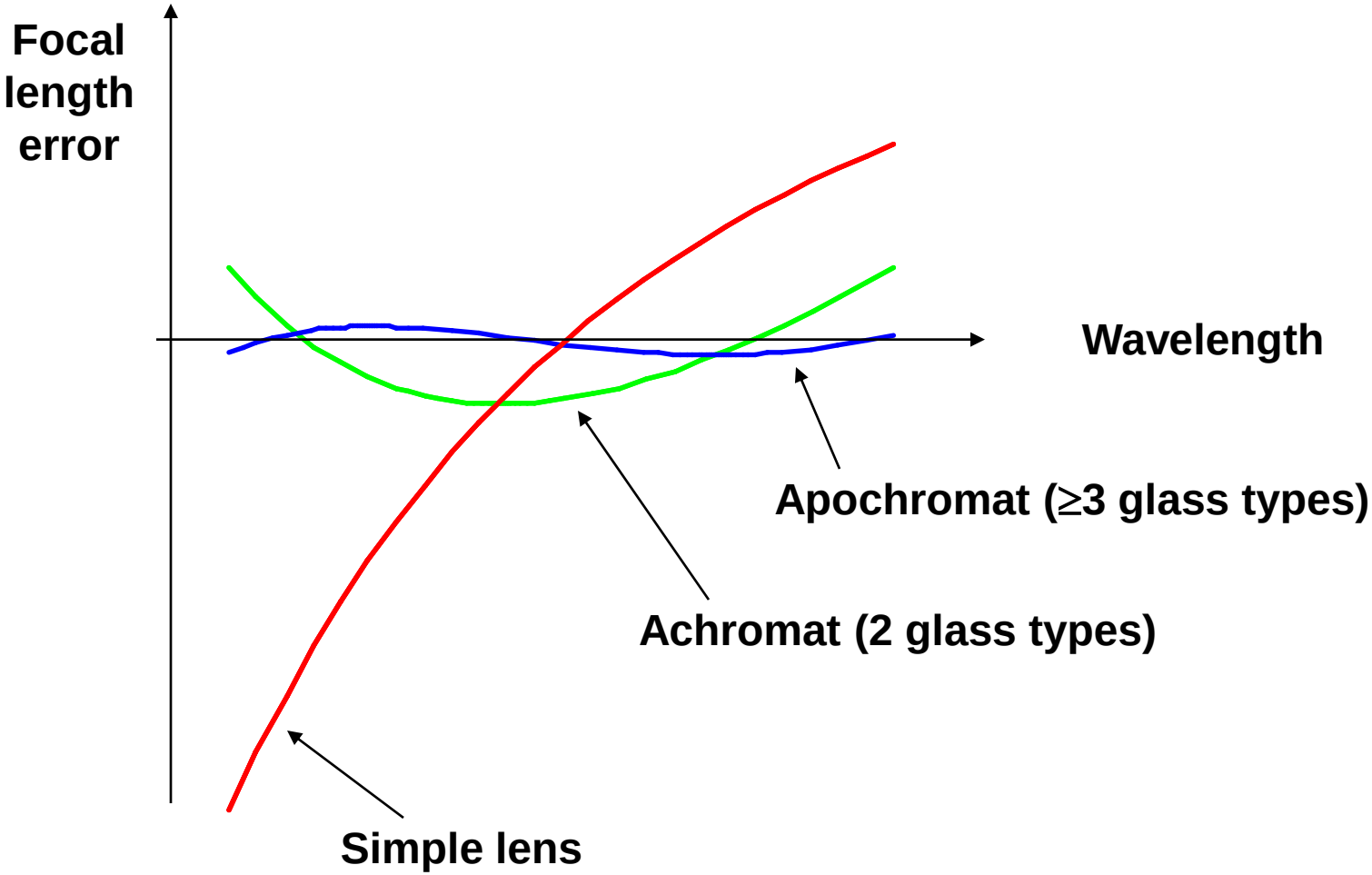


Achromatic Lenses

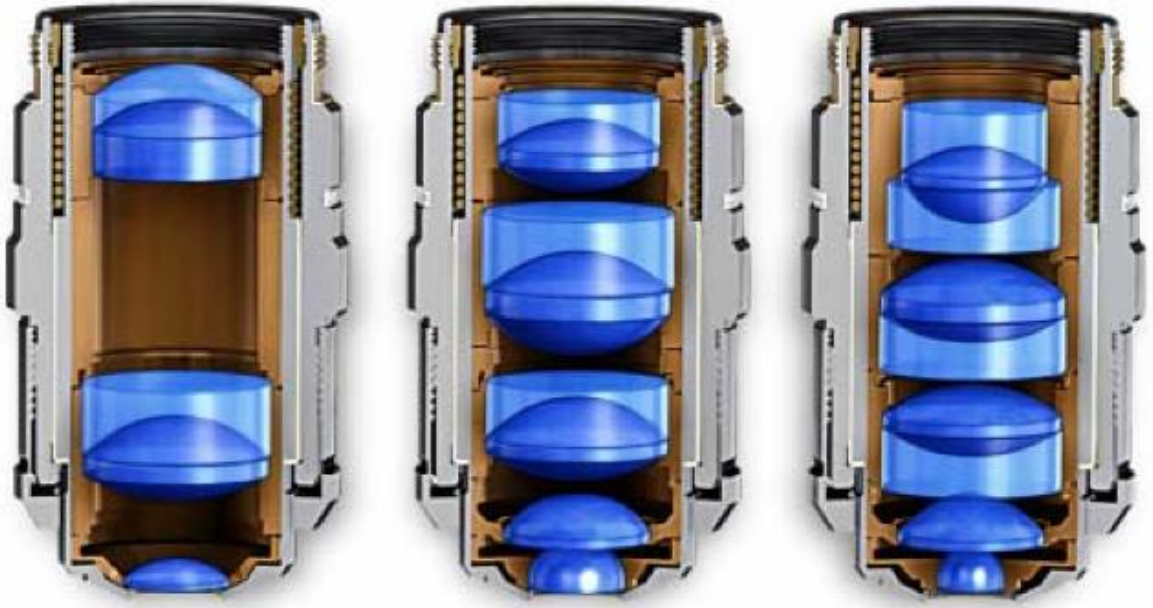
- Use a weak negative flint glass element to compensate the dispersion of a positive crown glass element



Achromats and Apochromats



Correction classes of objectives



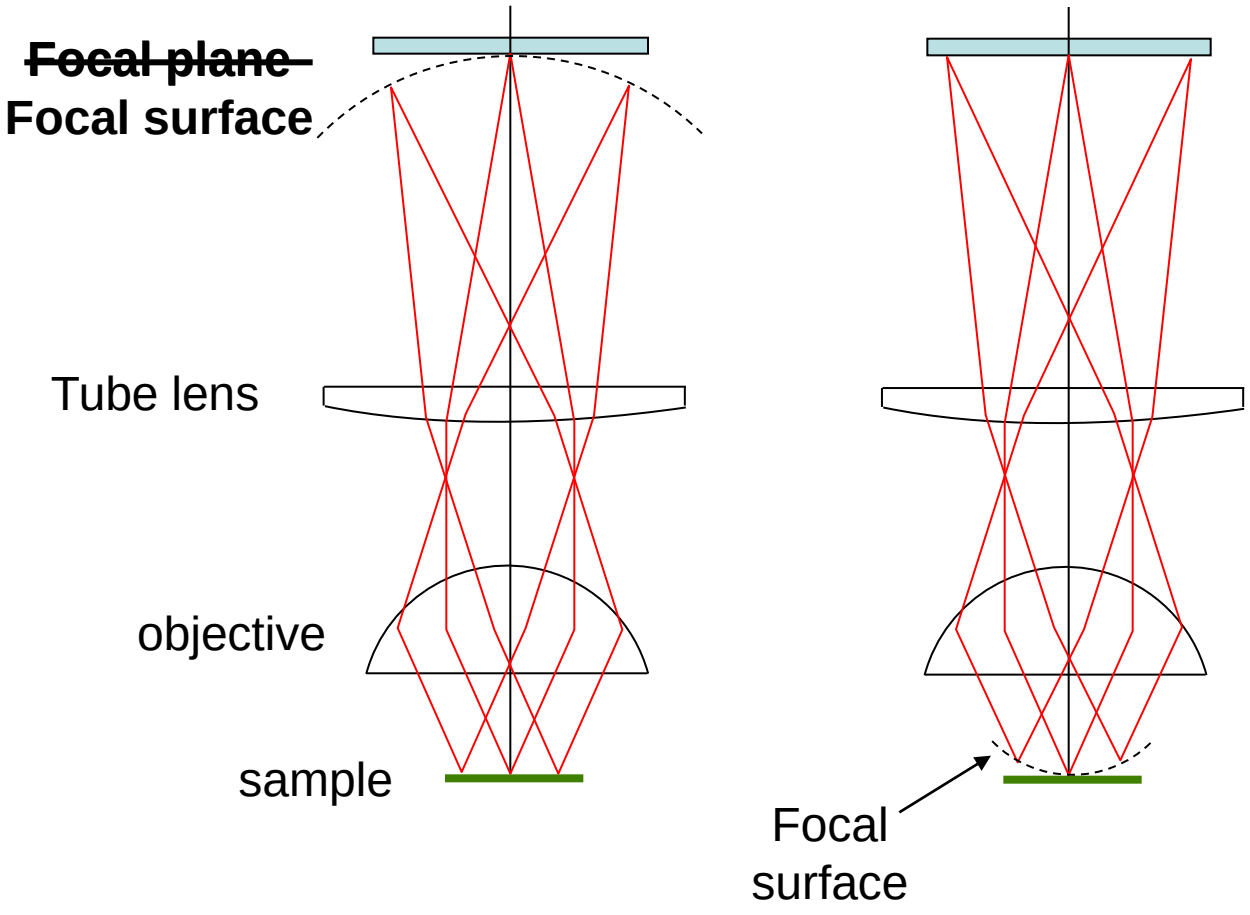
Achromat
(cheap)

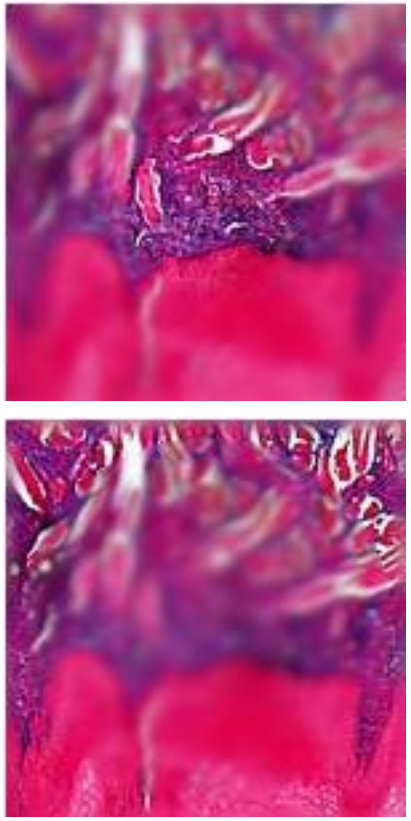
Fluor
"semi-apo"
(good correction, high UV transmission)

Apochromat
(best correction)

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

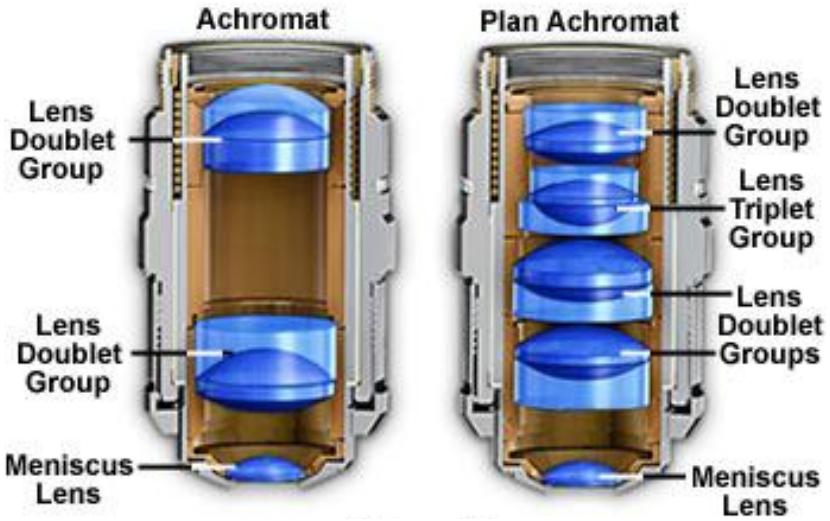
Curvature of Field





Plan objectives

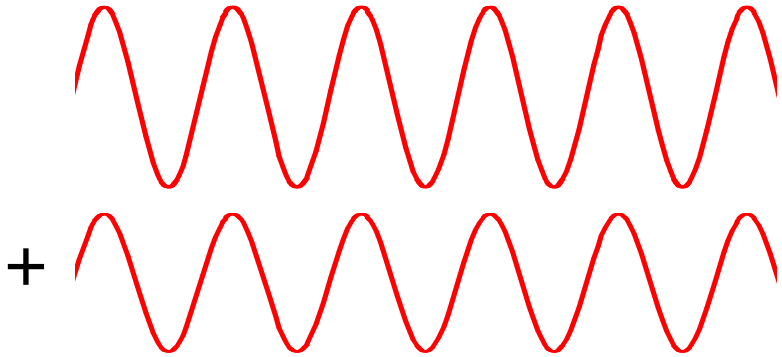
- Corrected for field curvature
- More complex design
- Needed for most photomicrography



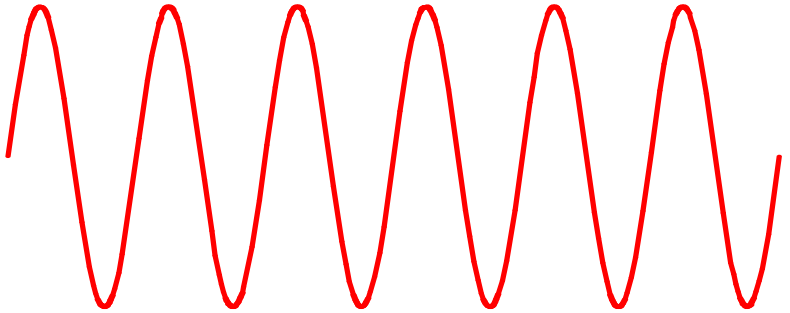
- **Plan-Apochromats** have the highest performance (and highest complexity and price)

Interference

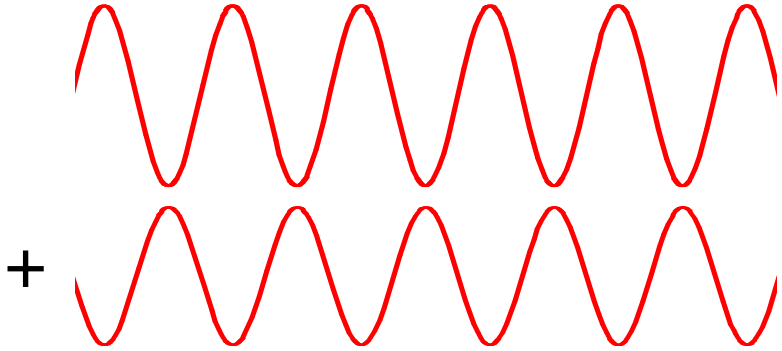
In phase



constructive interference

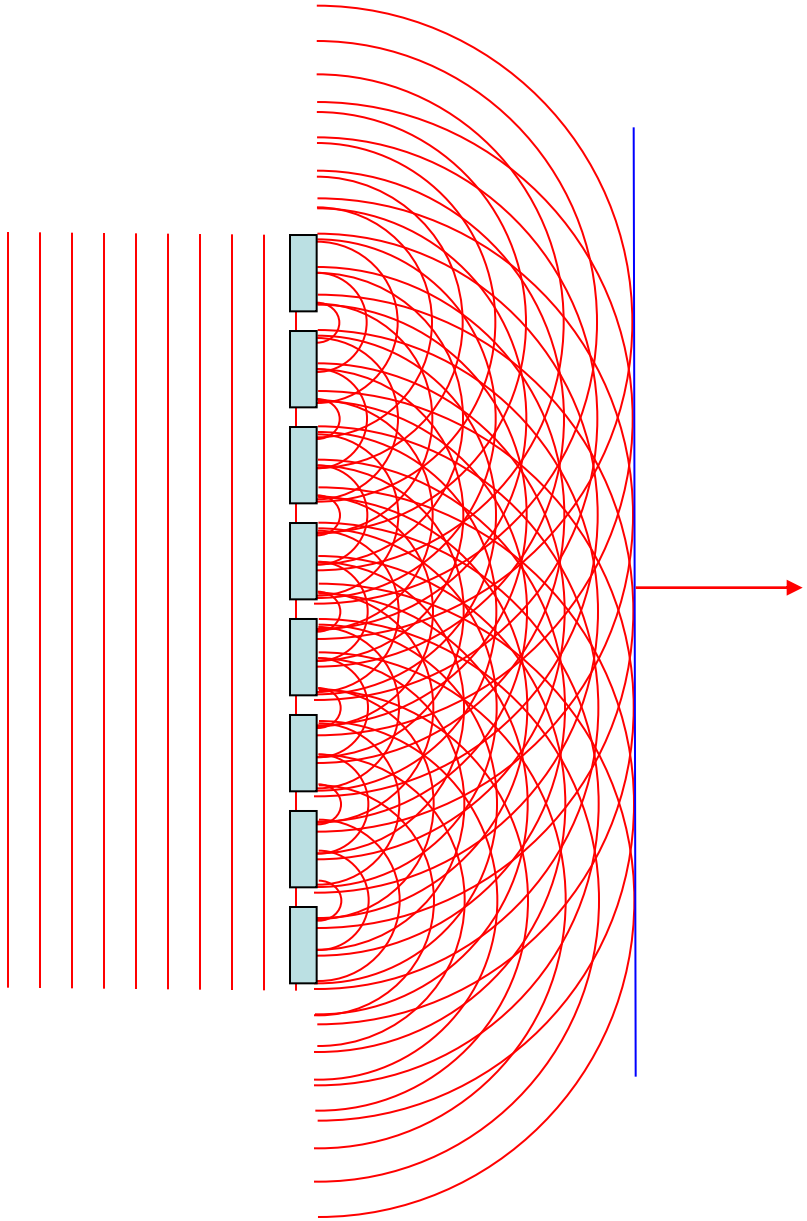


Opposite phase

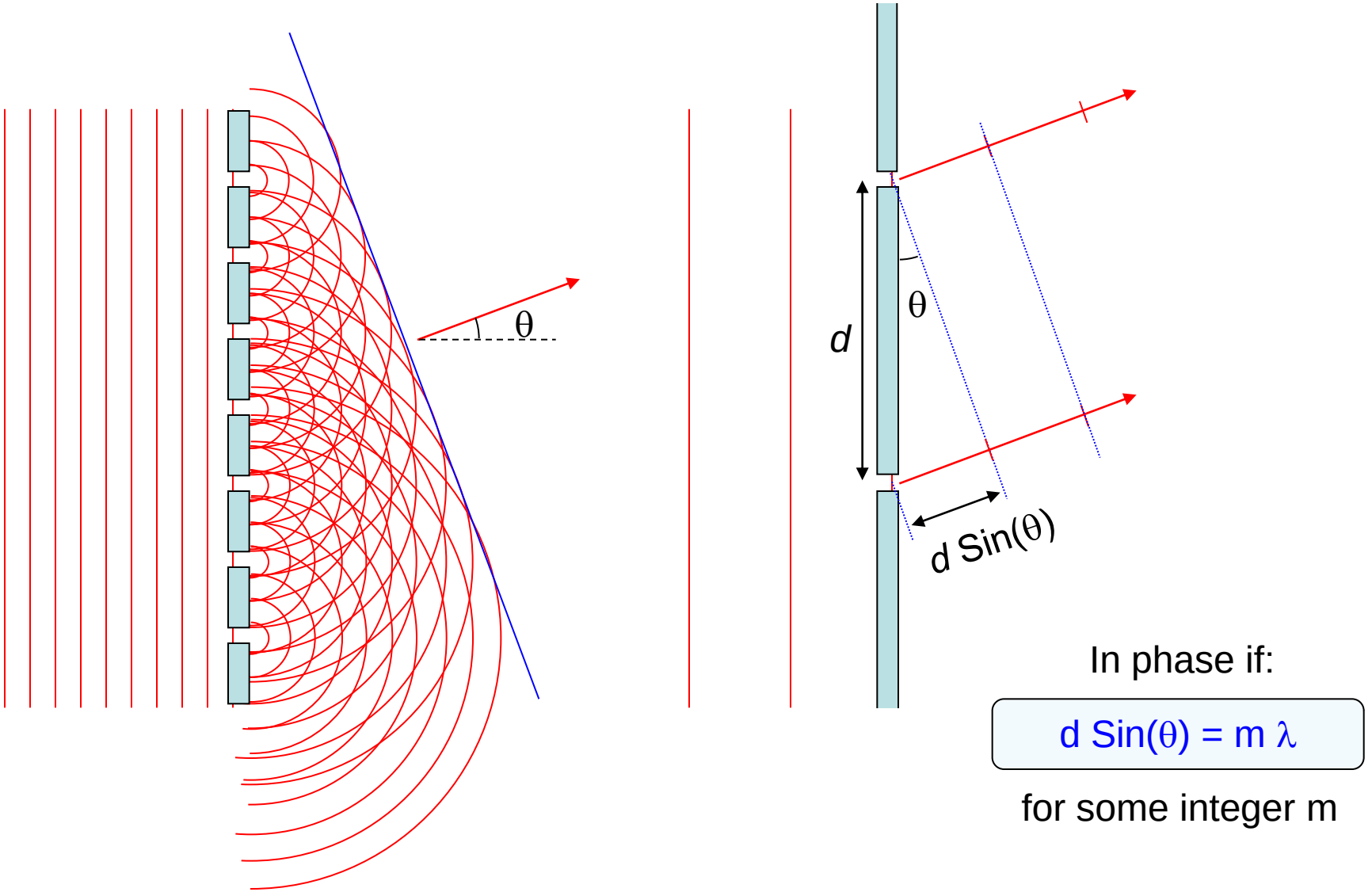


destructive interference

Diffraction by a periodic structure (grating)



Diffraction by a periodic structure (grating)

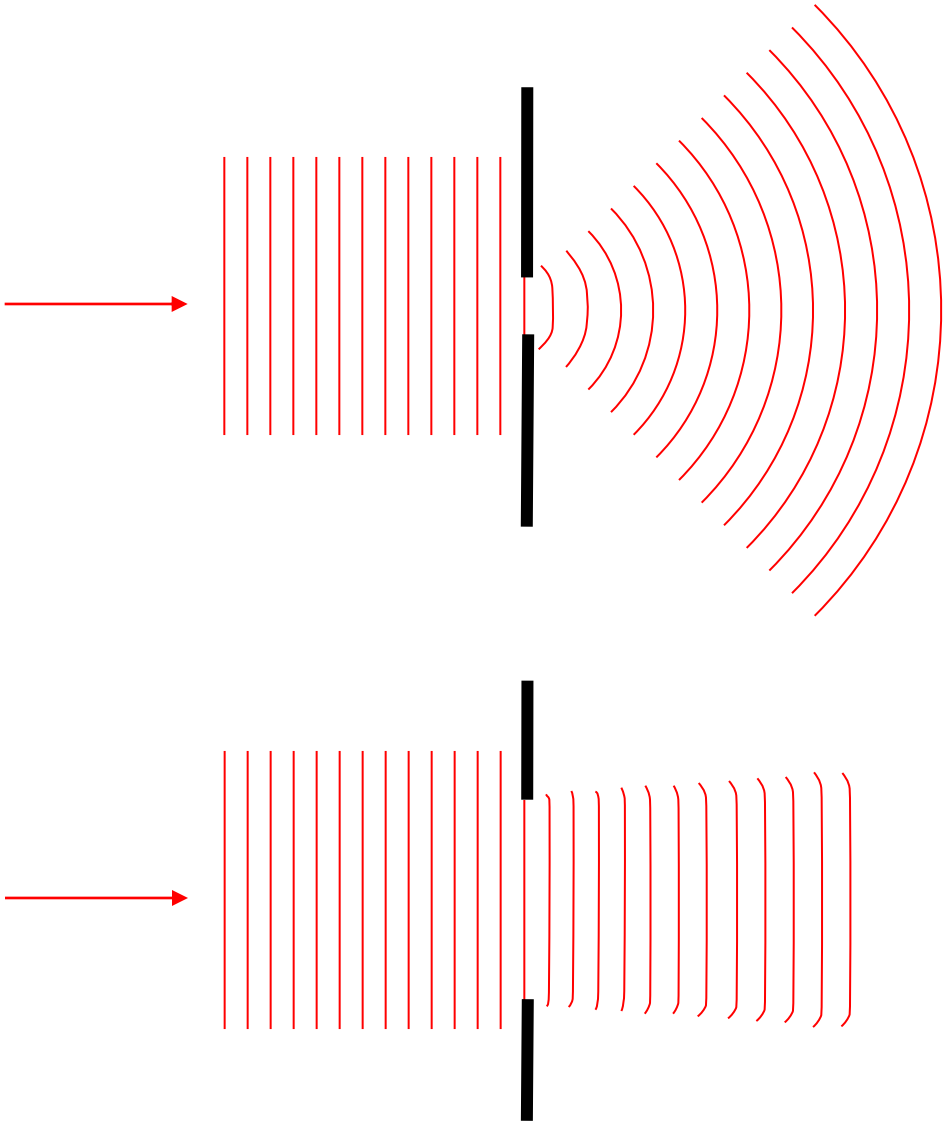


In phase if:

$$d \sin(\theta) = m \lambda$$

for some integer m

Diffraction by an aperture drawn as waves



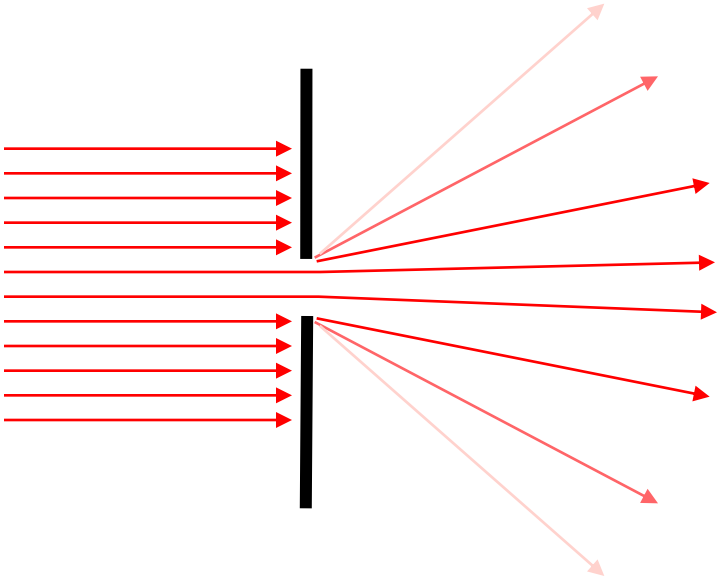
Light spreads to new angles

Larger aperture

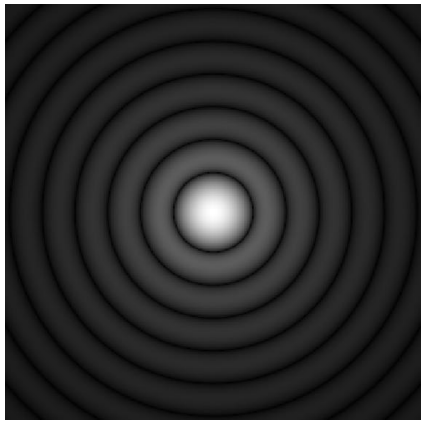
⇔

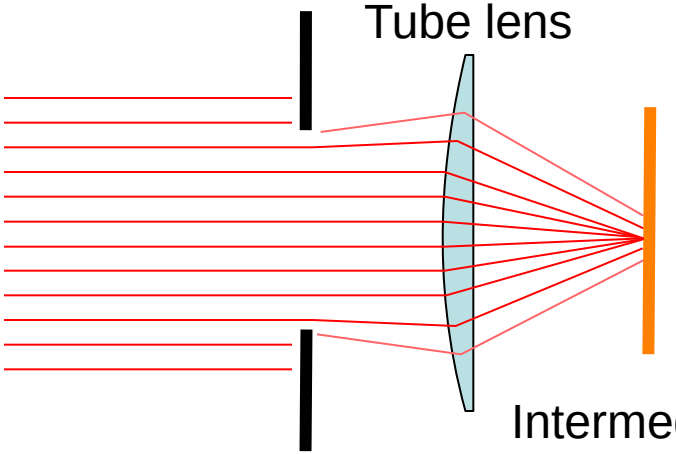
weaker diffraction

Diffraction by an aperture drawn as rays



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



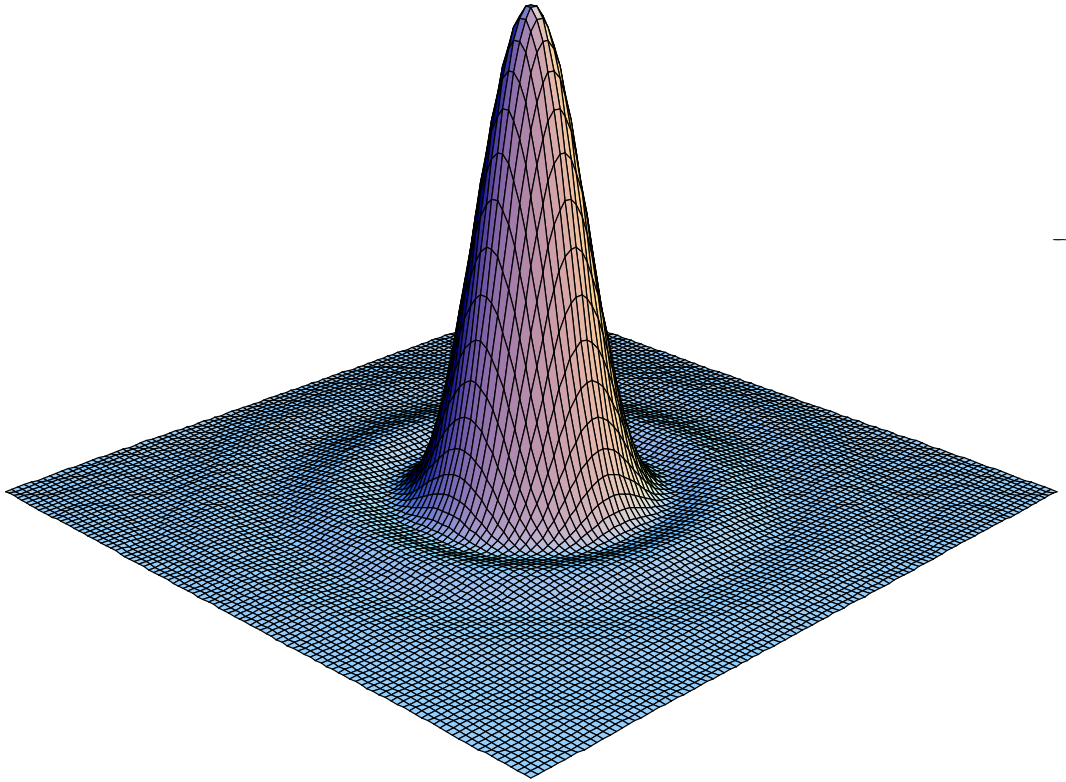


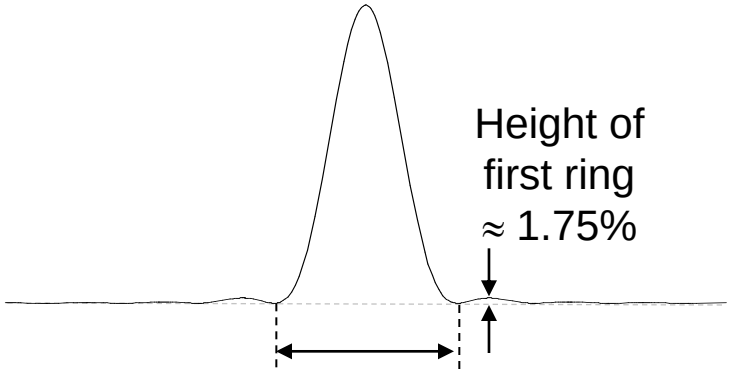
…or can be formed at a finite distance by a lens…

…as happens in a microscope

The Airy Pattern

= the far-field diffraction pattern from a round aperture

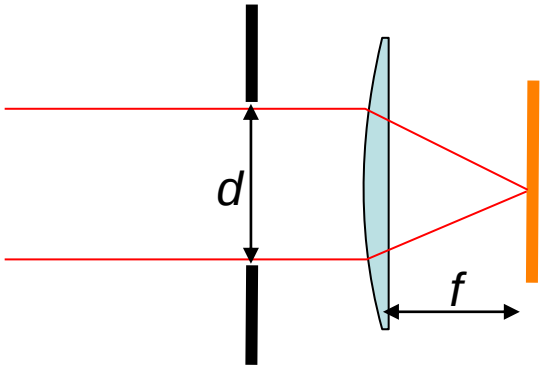




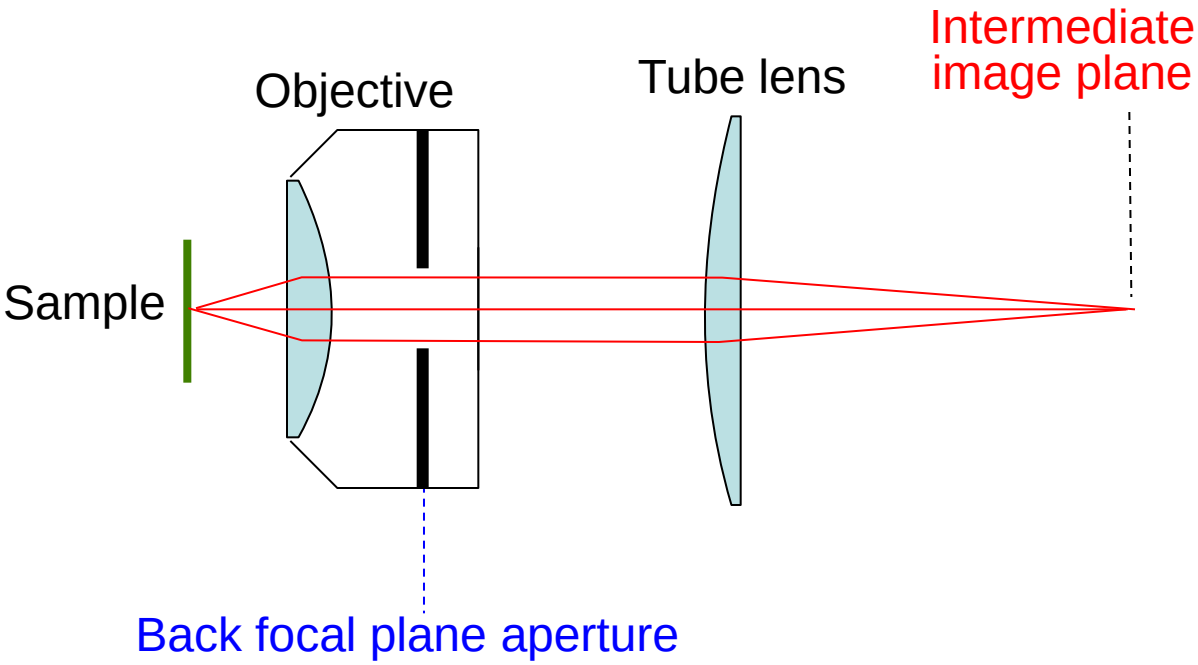
"Airy disk" diameter

$\boldsymbol{d = 2.44\ \lambda\ f/d}$

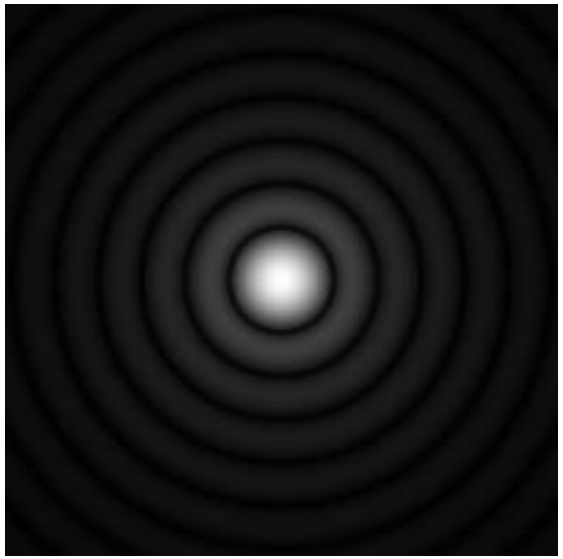
(for small angles d/f)



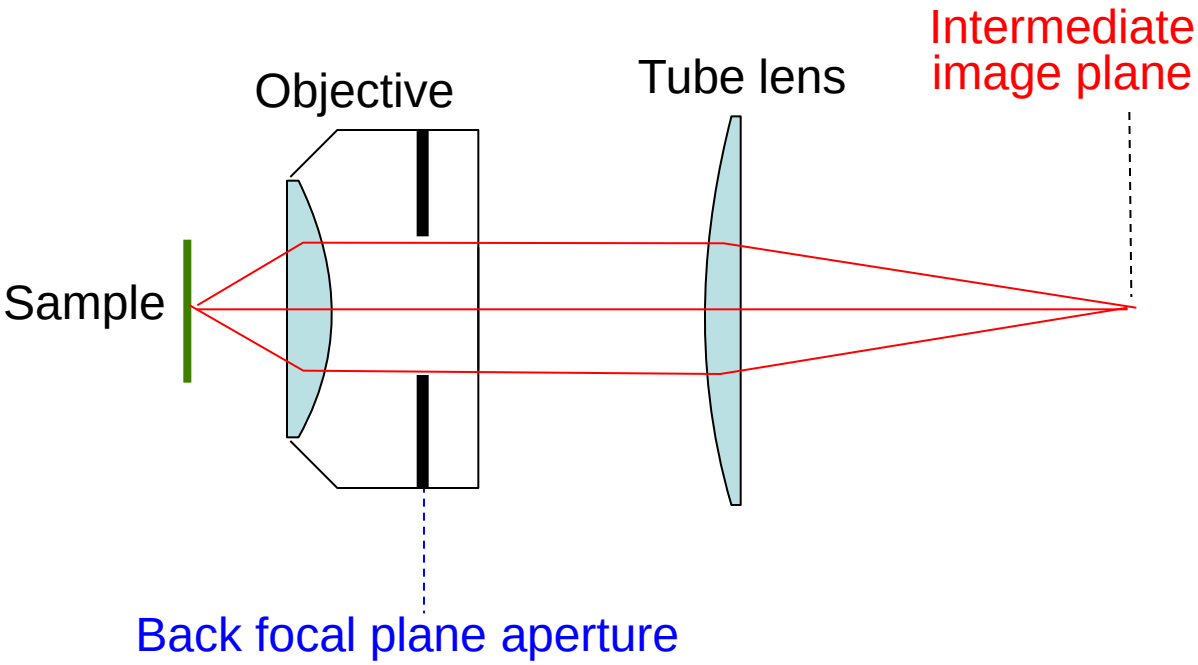
Aperture and Resolution



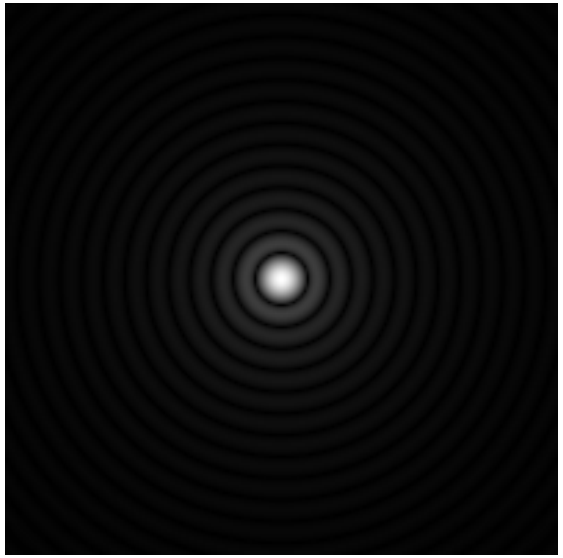
Diffraction spot
on image plane
= ***Point Spread Function***



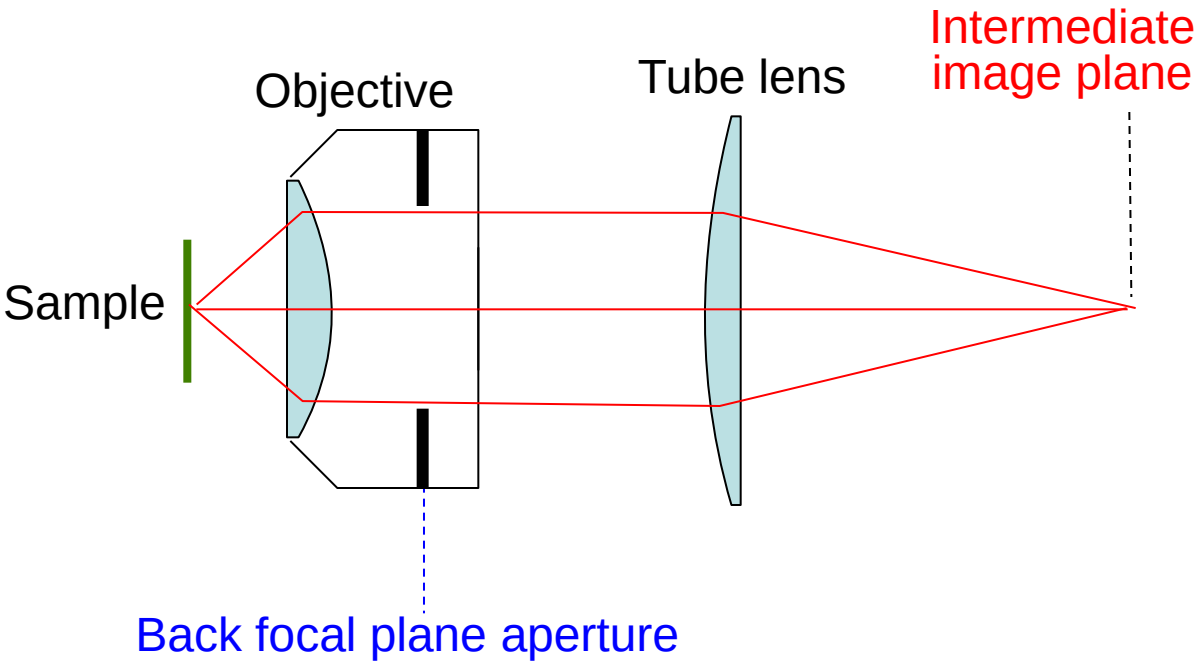
Aperture and Resolution



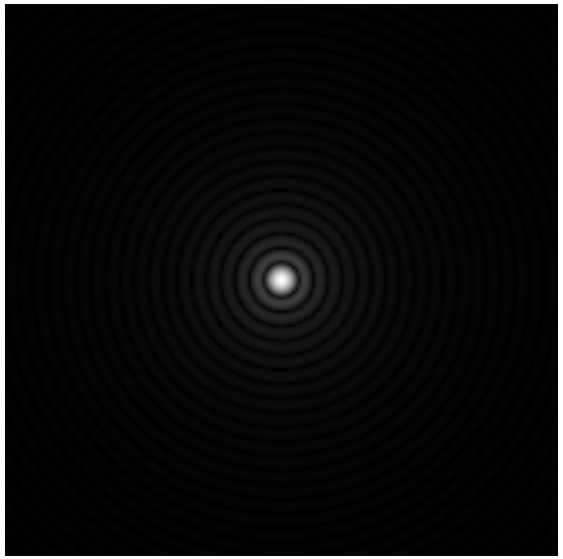
Diffraction spot
on image plane
= ***Point Spread Function***



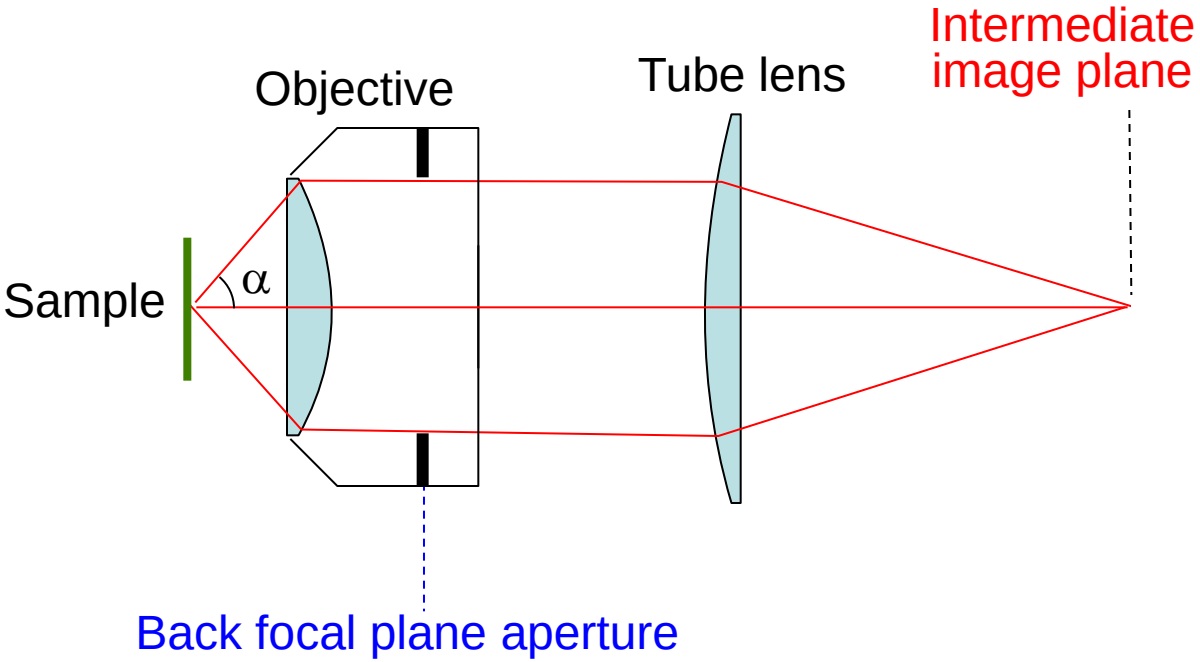
Aperture and Resolution



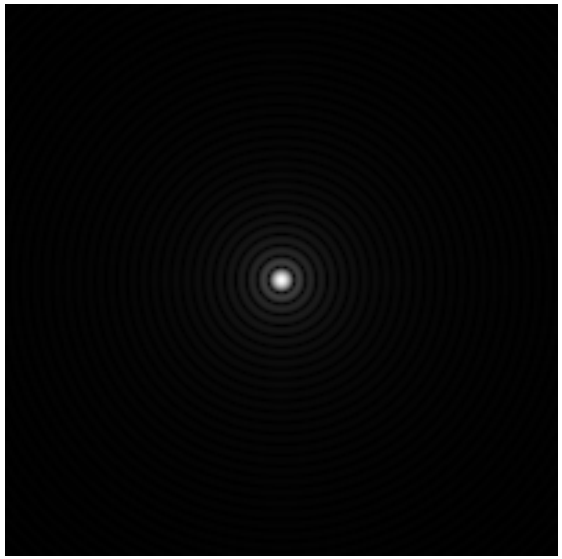
Diffraction spot
on image plane
= ***Point Spread Function***



Aperture and Resolution



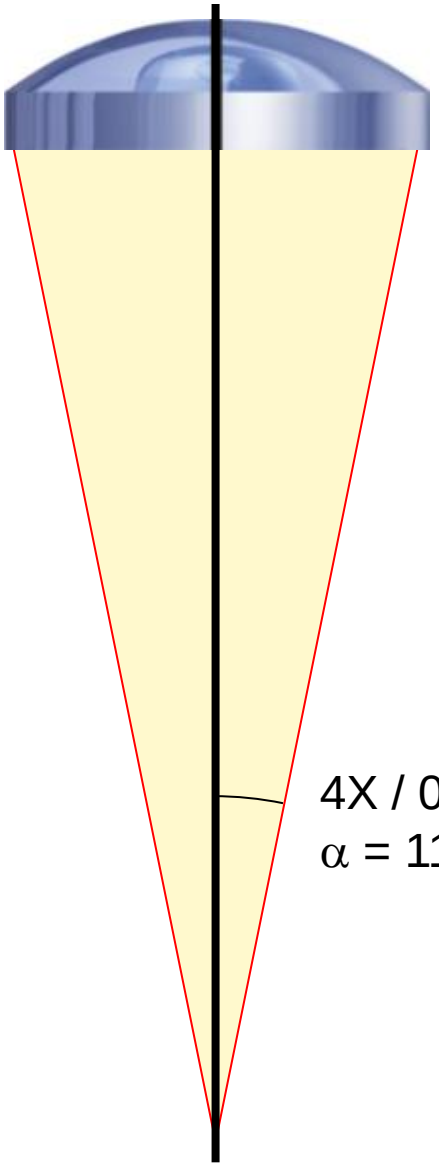
Diffraction spot on image plane (resolution)



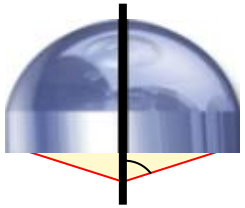
- Image resolution improves with ~~aperture size~~ Numerical Aperture (NA)

$NA = n \sin(\alpha)$ where: α = light gathering angle
n = refractive index of sample

Numerical Aperture

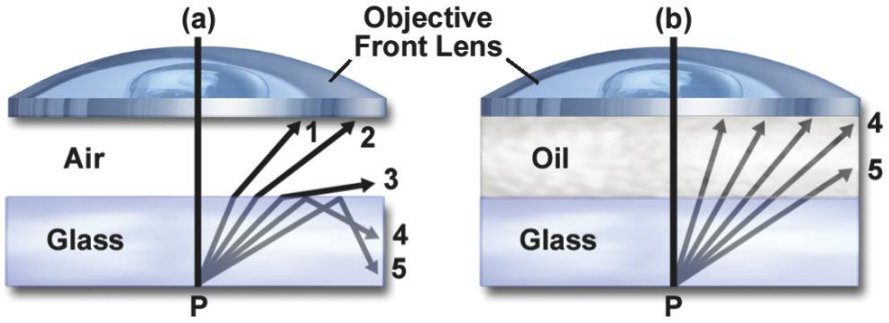


4X / 0.20 NA
α = 11.5°



100X / 0.95 NA
α = 71.8°

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)

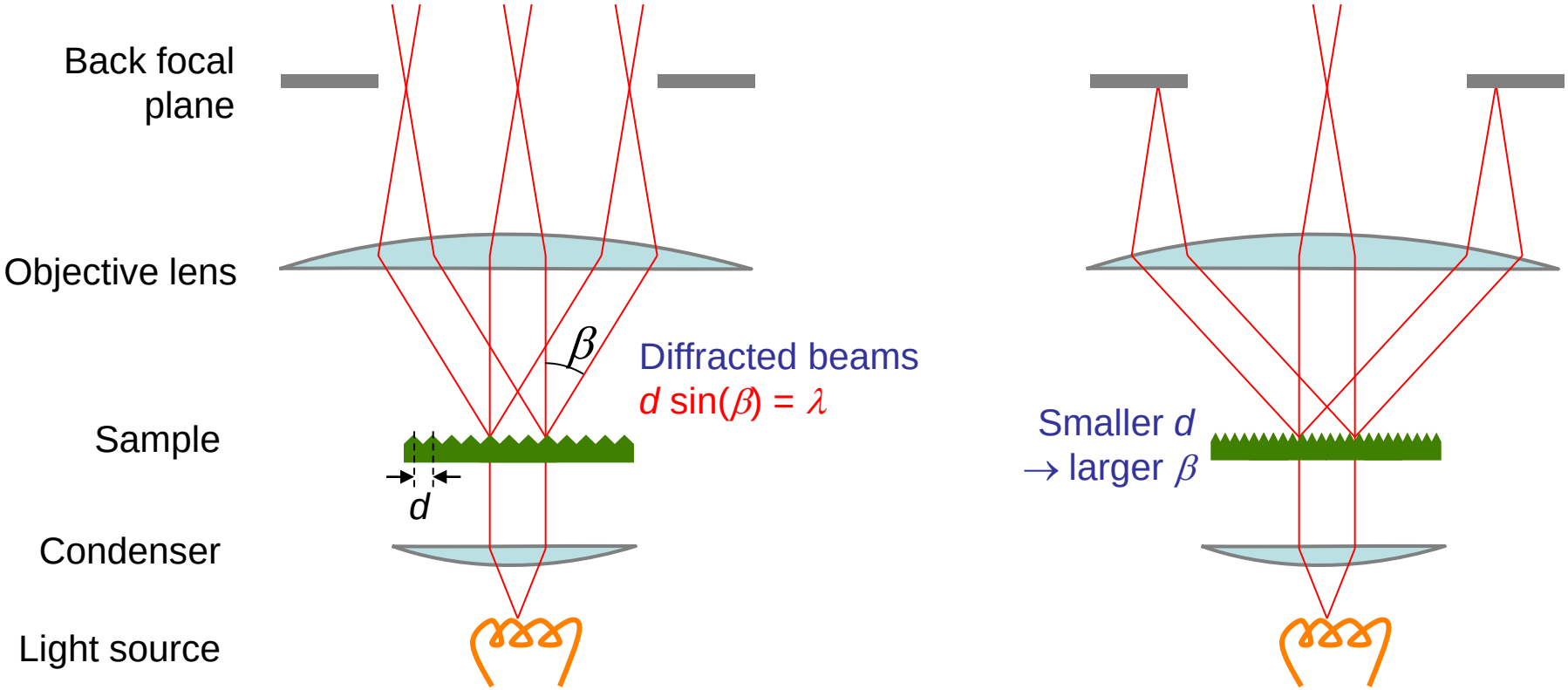
Glycerol immersion:
- n ≈ 1.45 (85%)
- max NA ≈ **1.35** (Leica)

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

Resolution

Ernst Abbe's argument (1873)

Consider a striped sample ≈ a diffraction grating



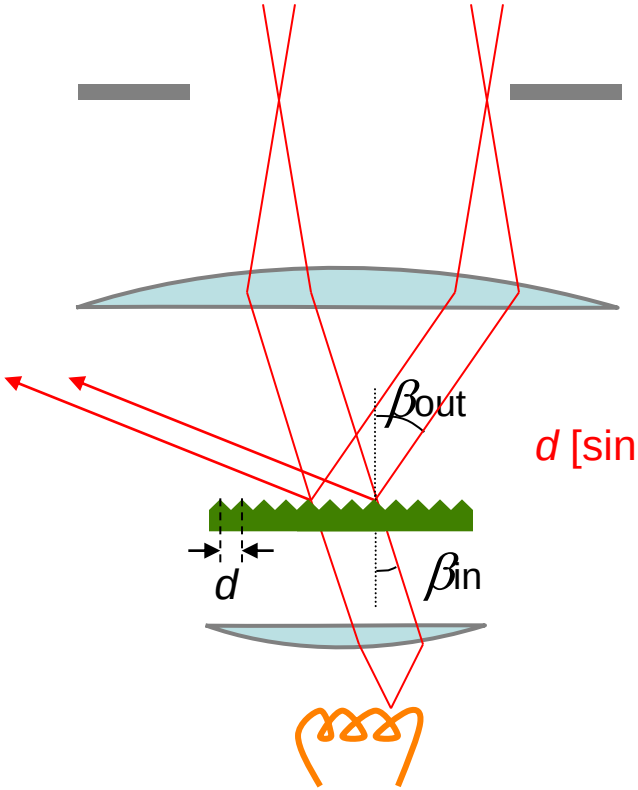
If $\beta > \alpha$, only one spot makes it through
$\Rightarrow$ no interference $\Rightarrow$ no image formed

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 $\beta_{in} < \alpha$.

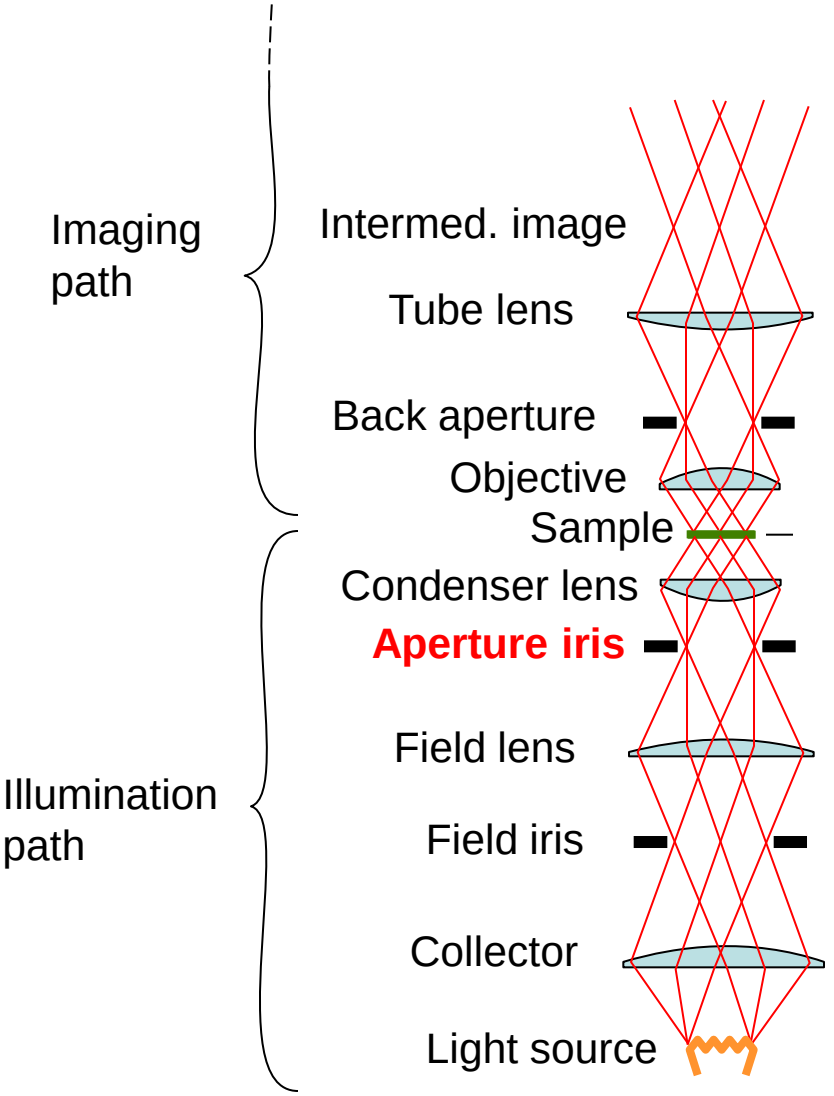
Resolution (smallest resolvable *d*)
with incoherent illumination (all possible illumination directions):

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

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

Aperture, Resolution & Contrast

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

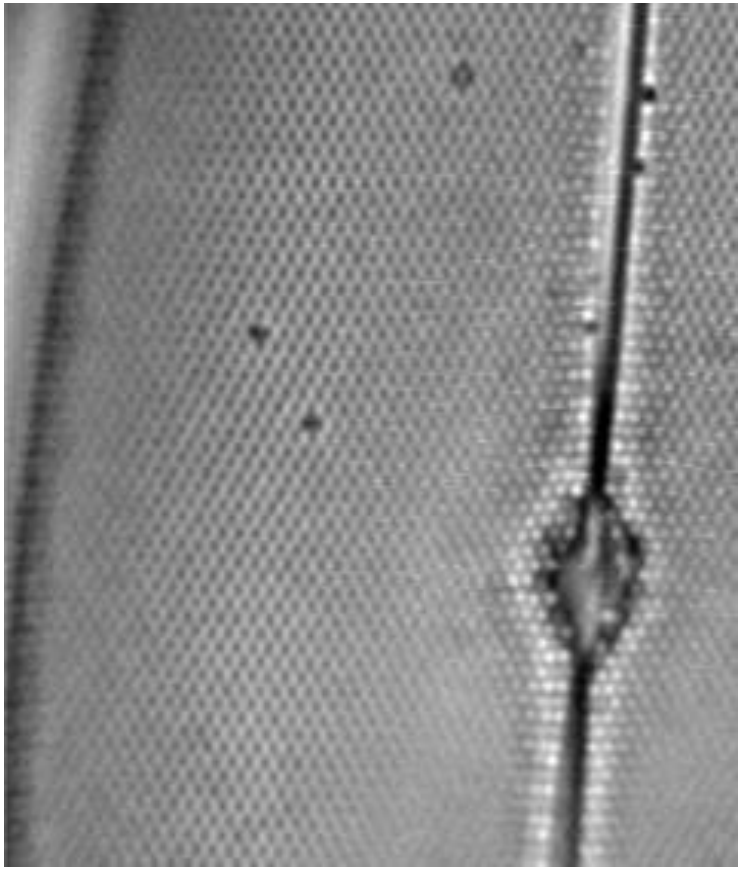


Q: Don't we always want it full open??

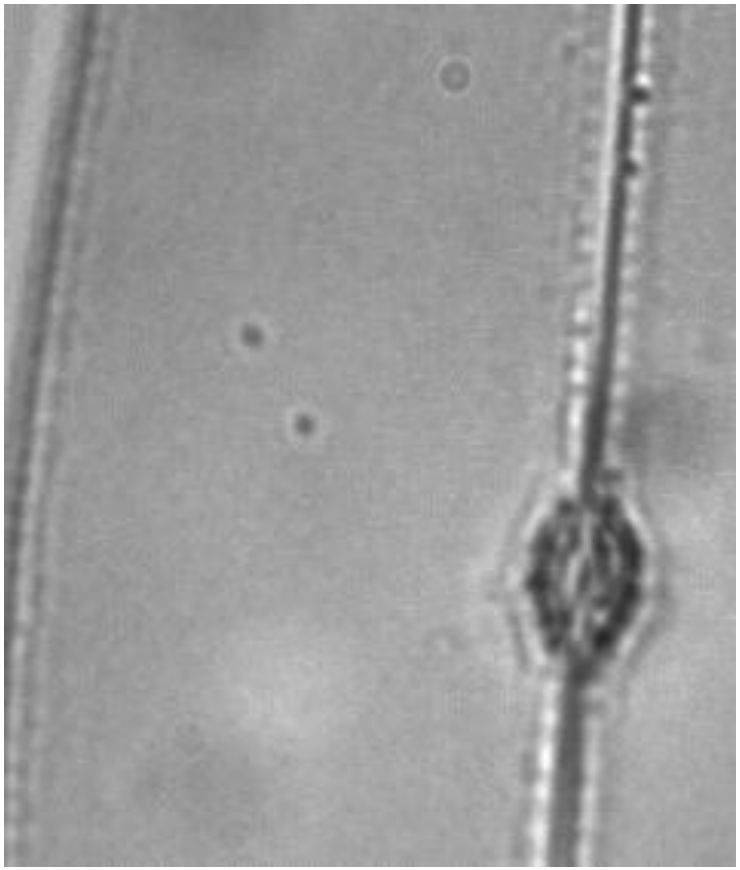
A: **No**

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

NA and Resolution



High NA Objective



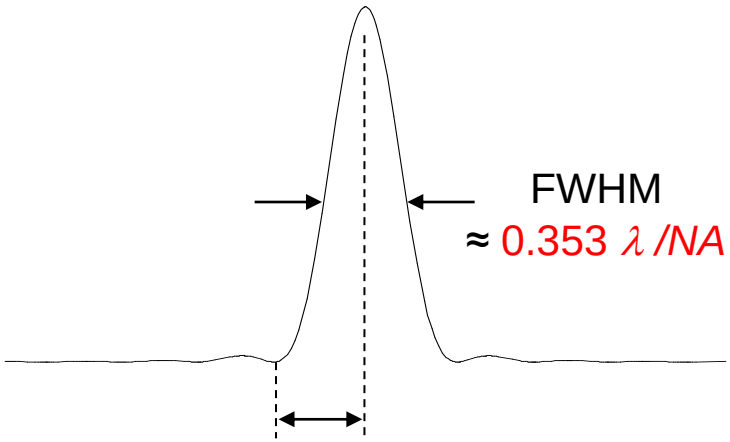
Low NA Objective

Alternate Definitions of Resolution

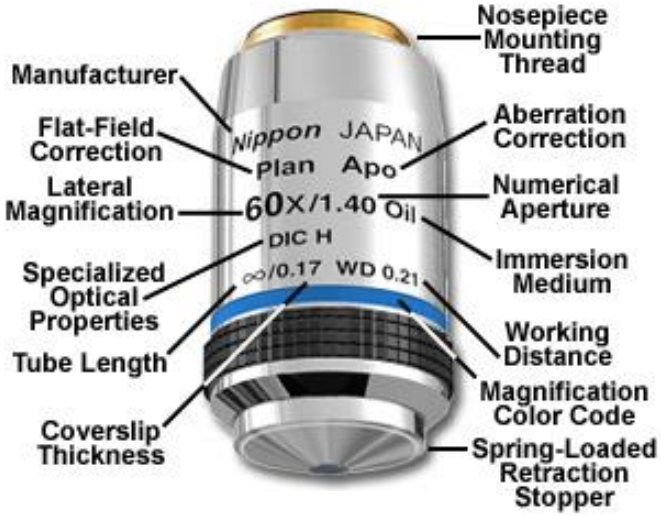
As the Full Width at Half Max (FWHM) of the PSF

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

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