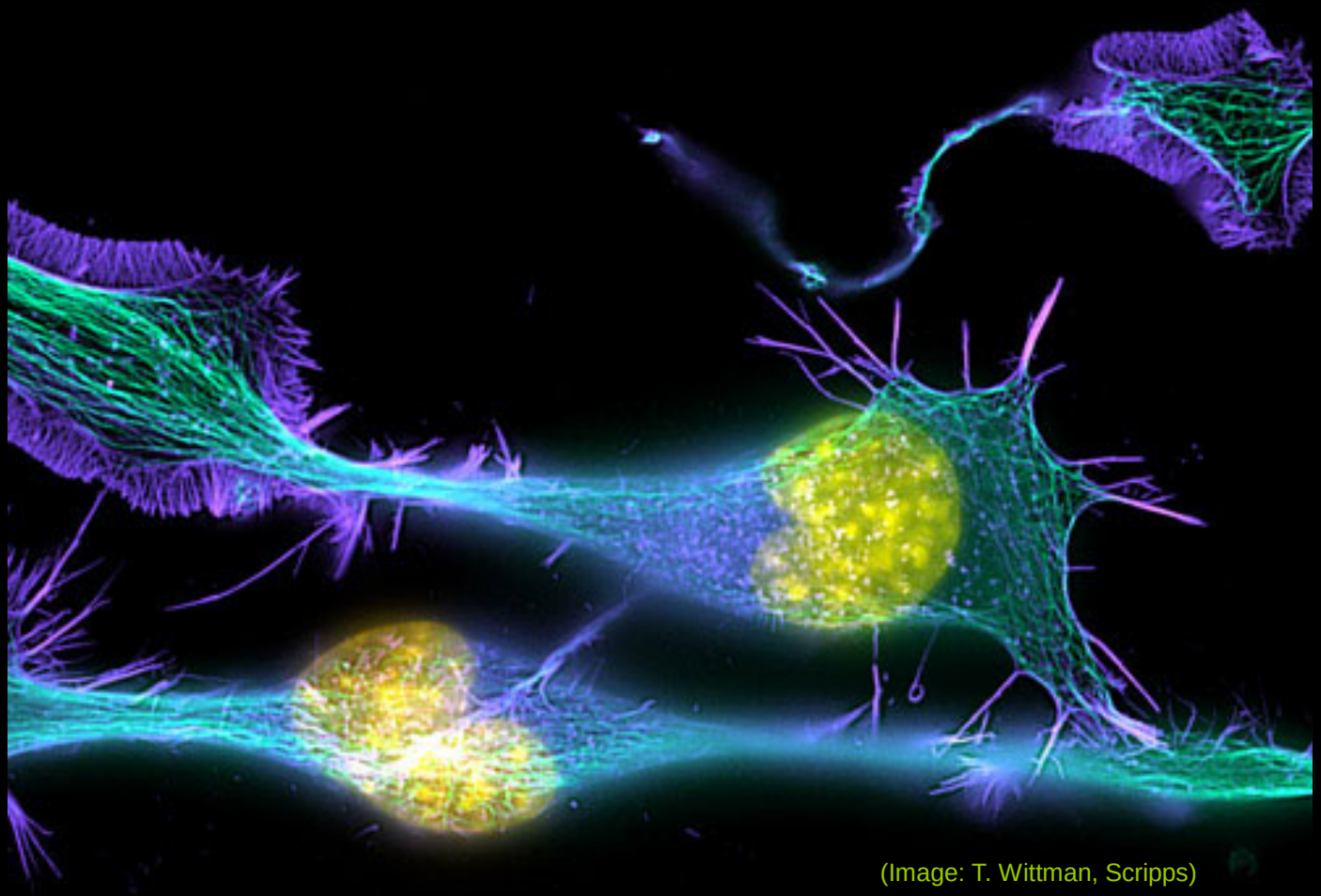
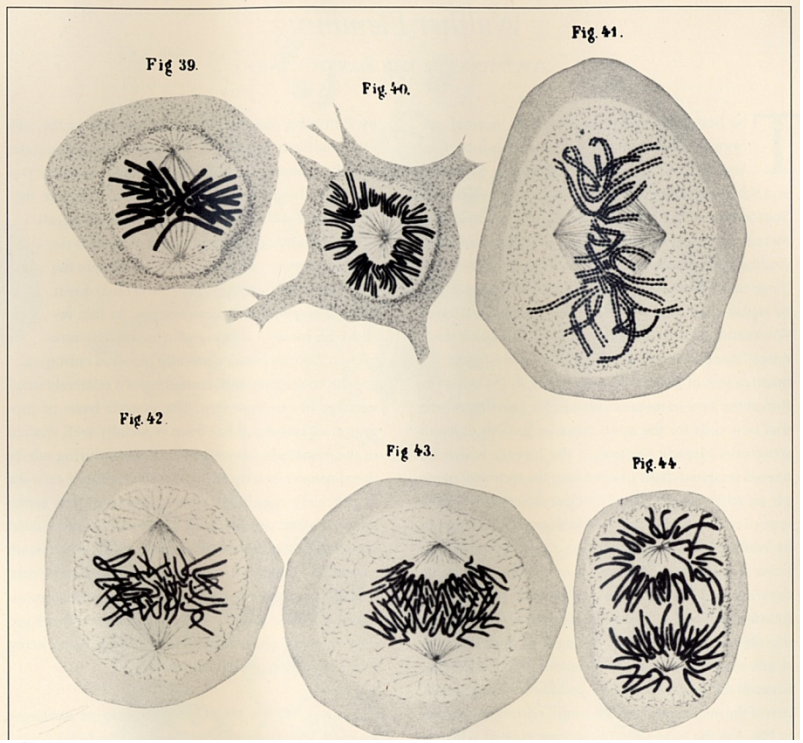
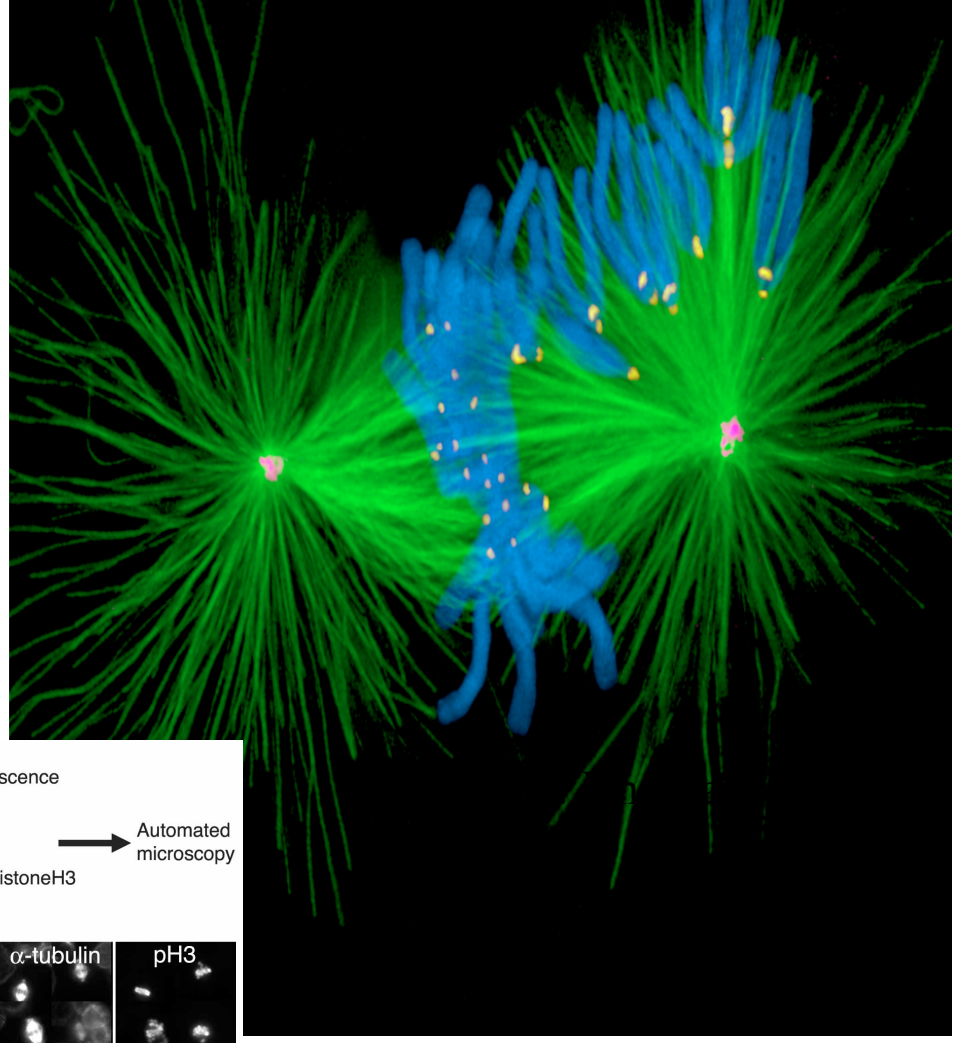


(Image: T. Wittman, Scripps)

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

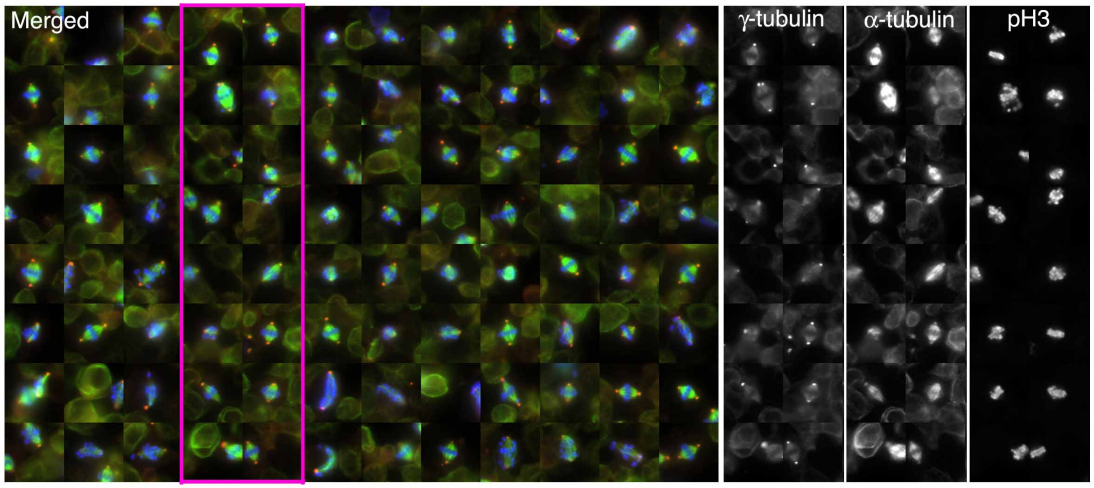
96-well, glass-bottom plate
(ConA-coated)

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

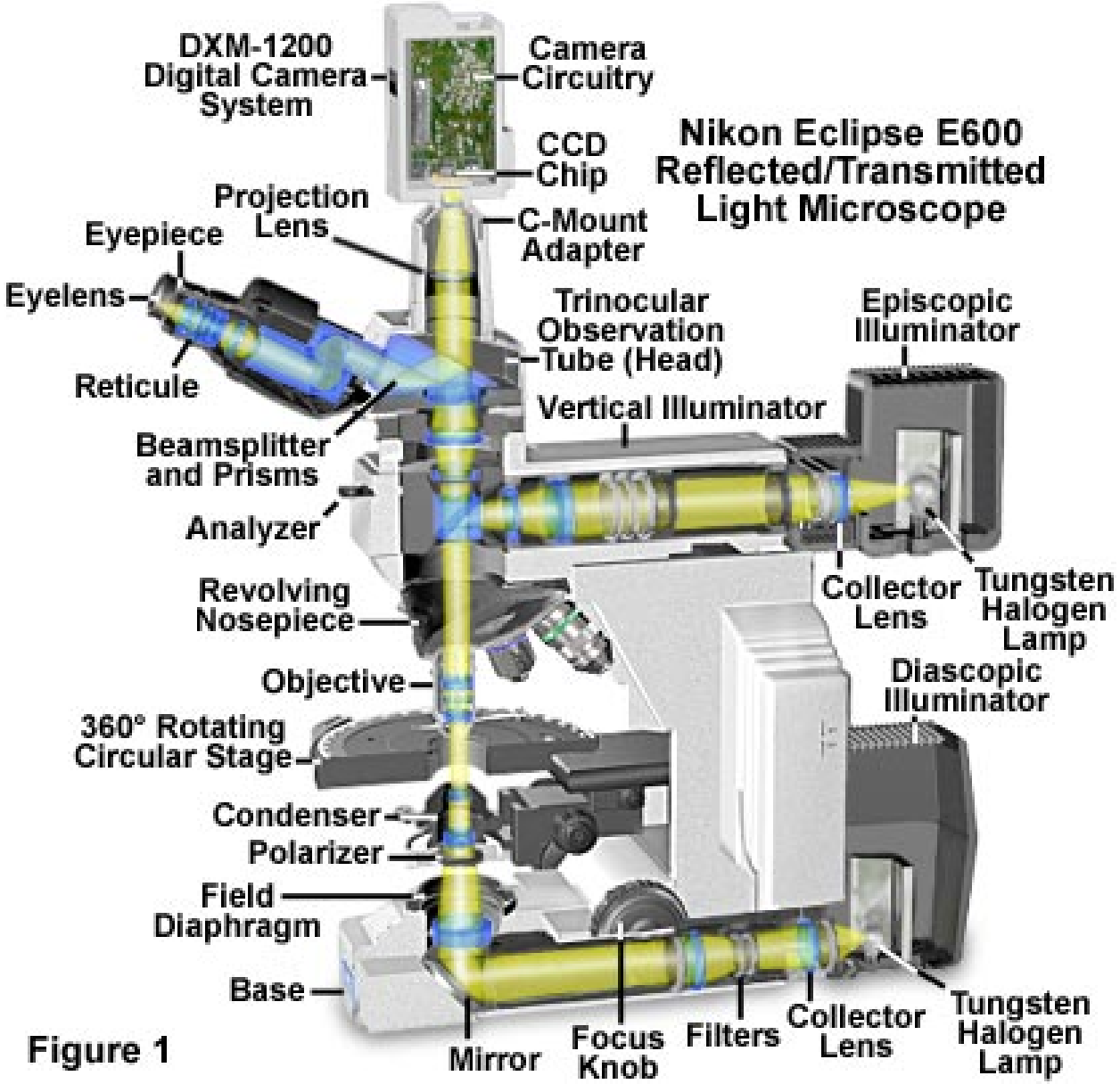
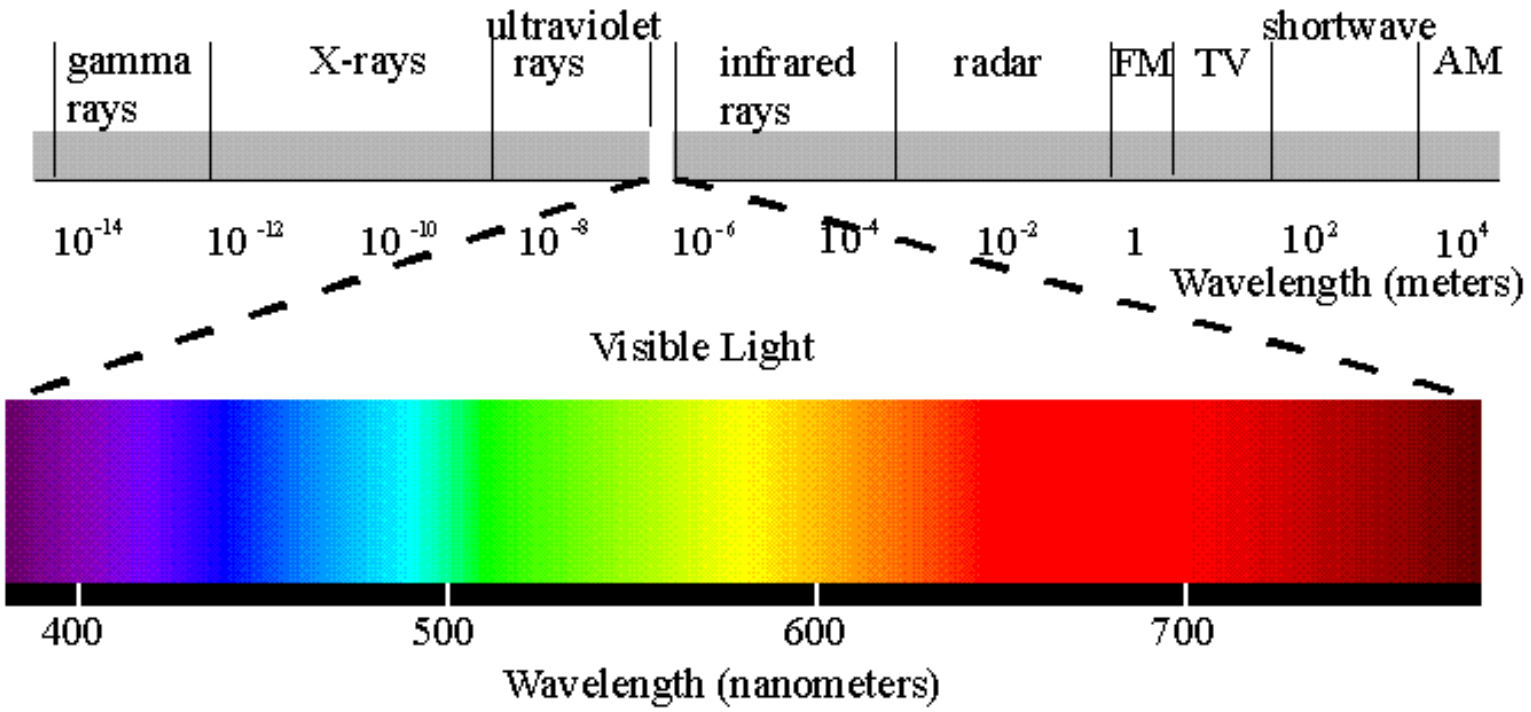


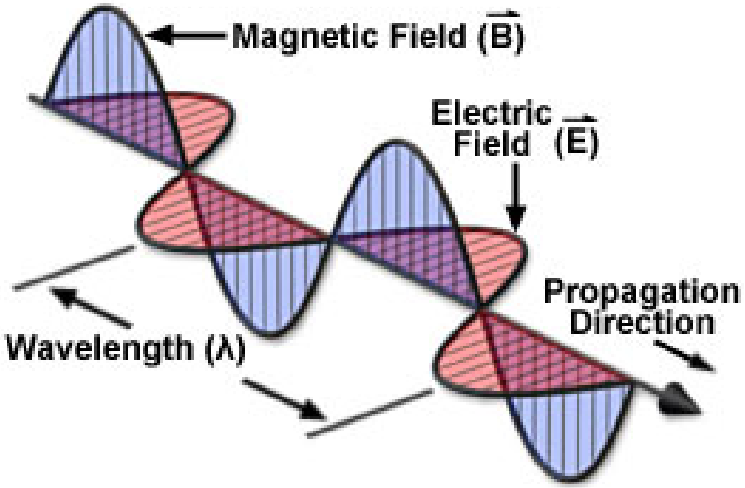
Figure 1

Electromagnetic Waves

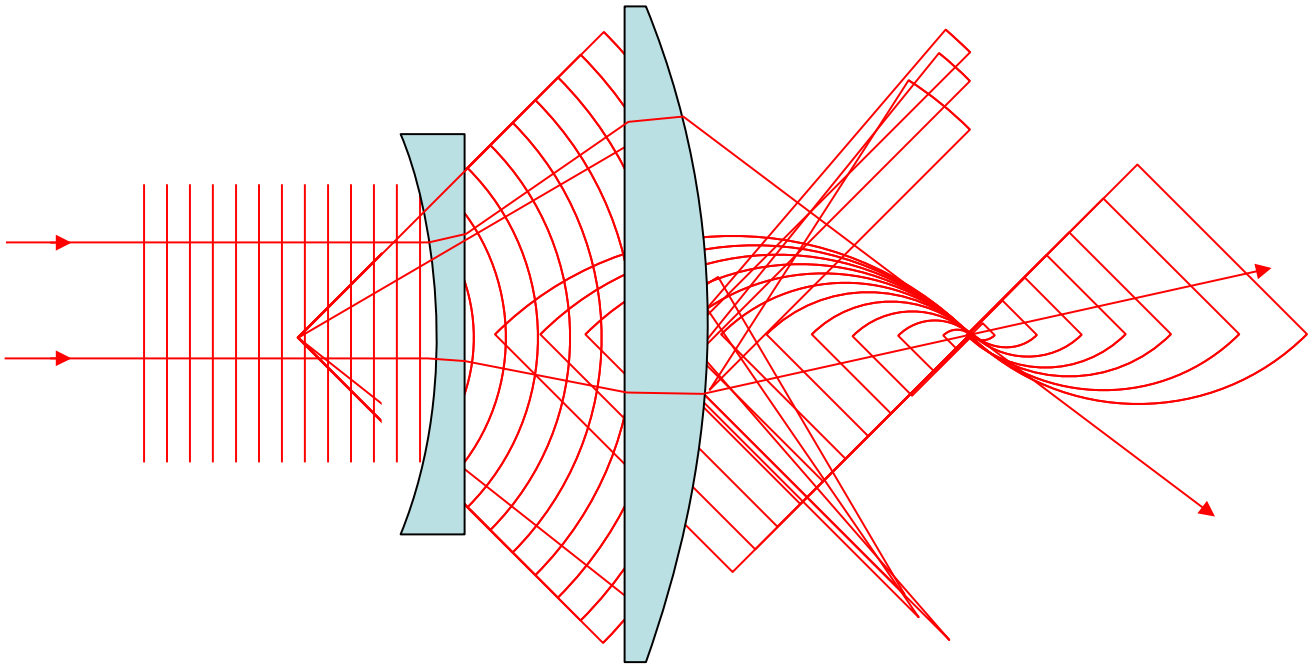


Waves vs. Photons vs. Rays

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



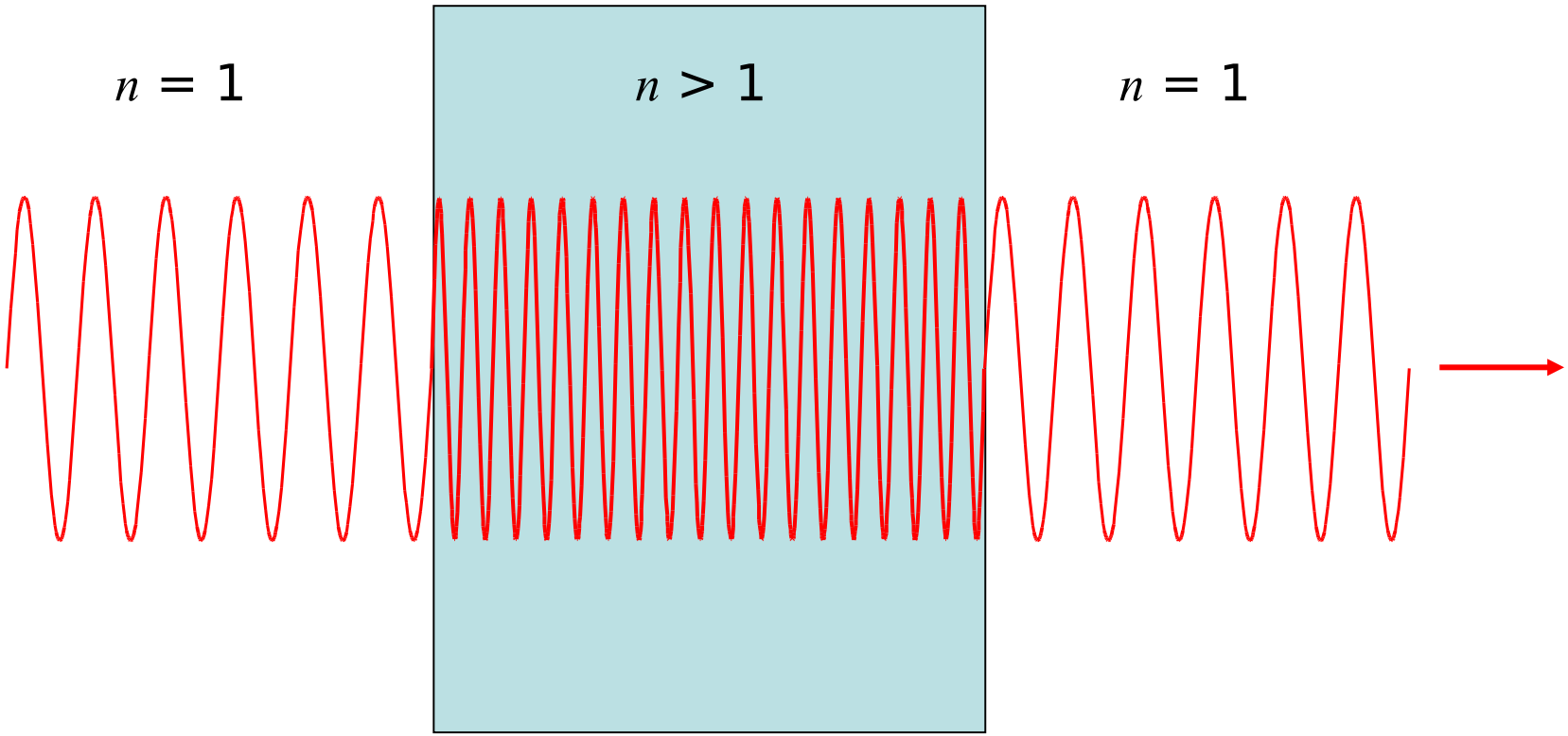
Rays are perpendicular to wavefronts



Light travels more slowly in matter

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

$$v = c/n$$

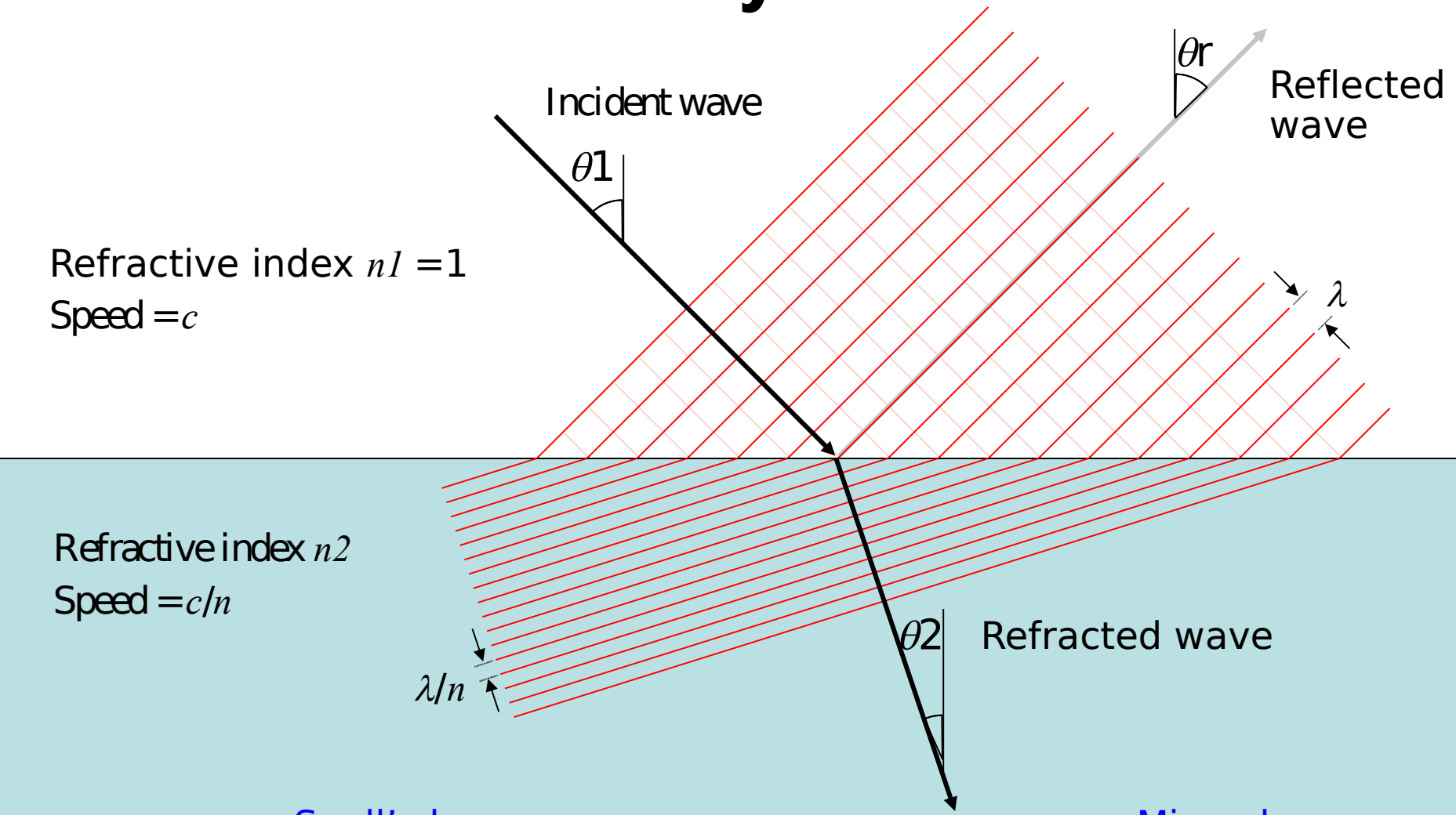


Refractive Index Examples

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



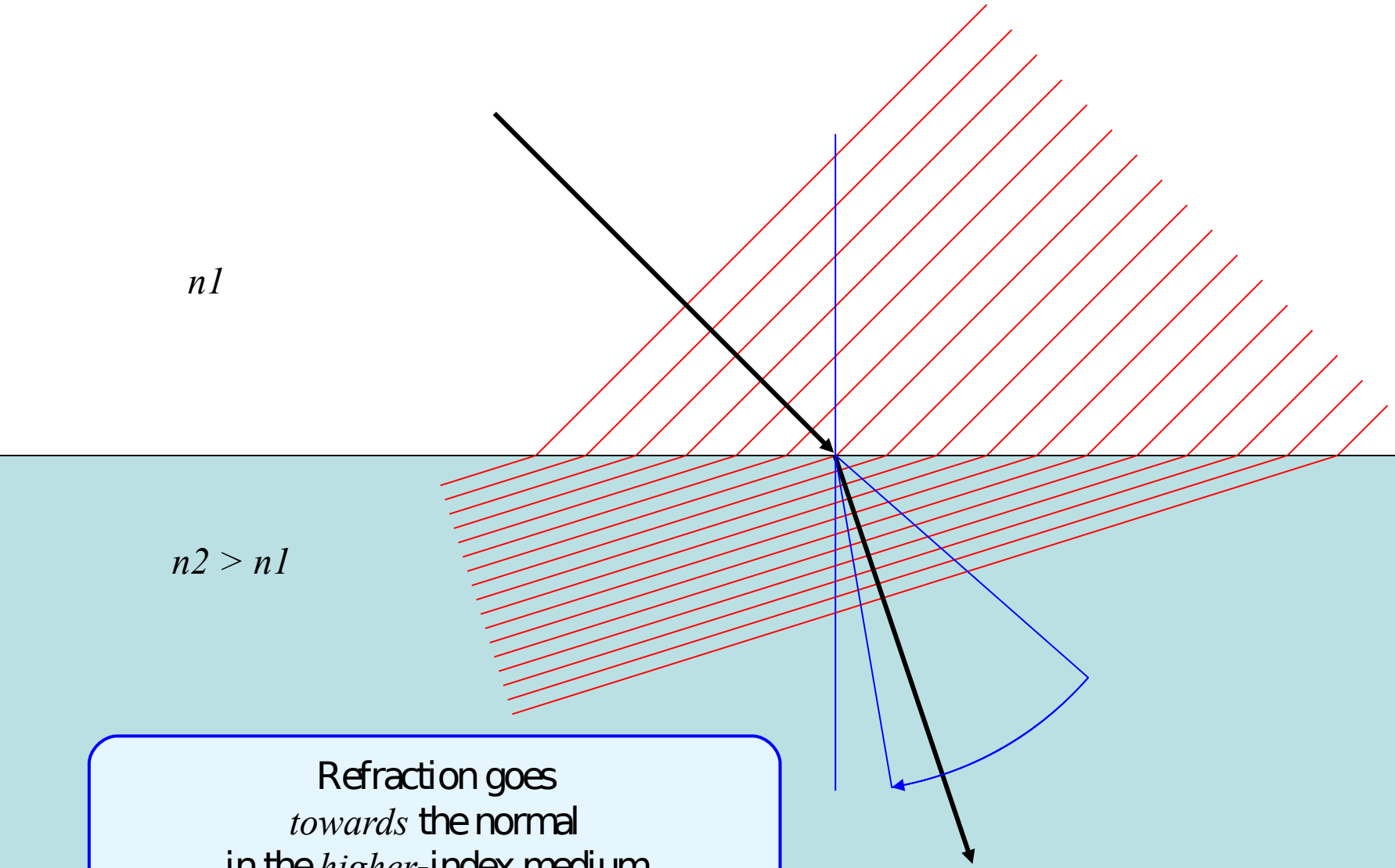
⇒ Snell's law:

$$n1 \sin(\theta 1) = n2 \sin(\theta 2)$$

Mirror law:

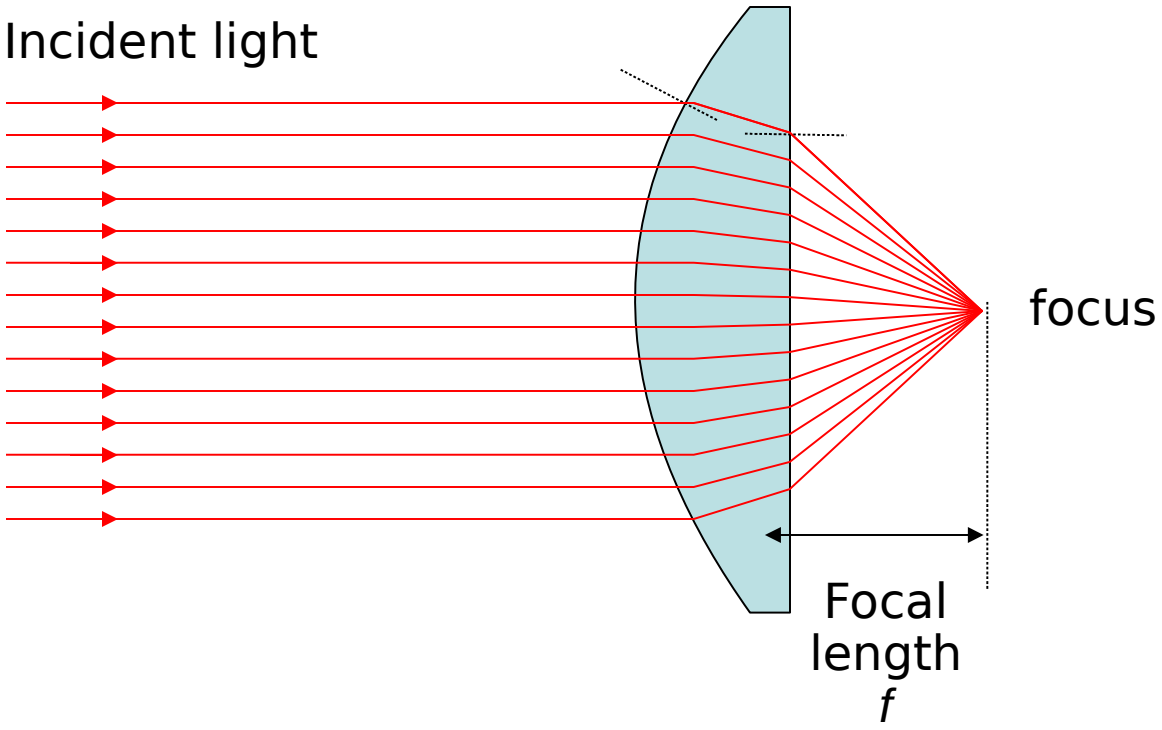
$$\theta r = \theta 1$$

Which Direction?



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

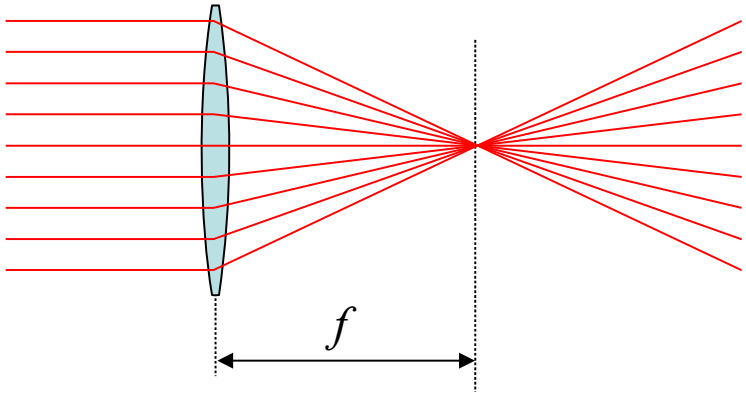
Lenses work by refraction



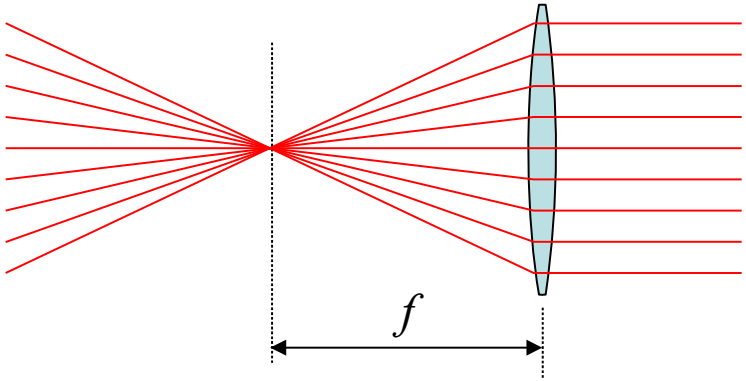
Ray Tracing Rules of Thumb

(for thin ideal lenses)

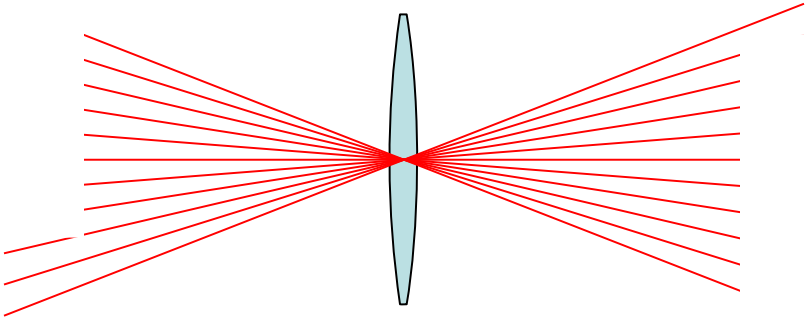
Parallel rays converge at the focal plane



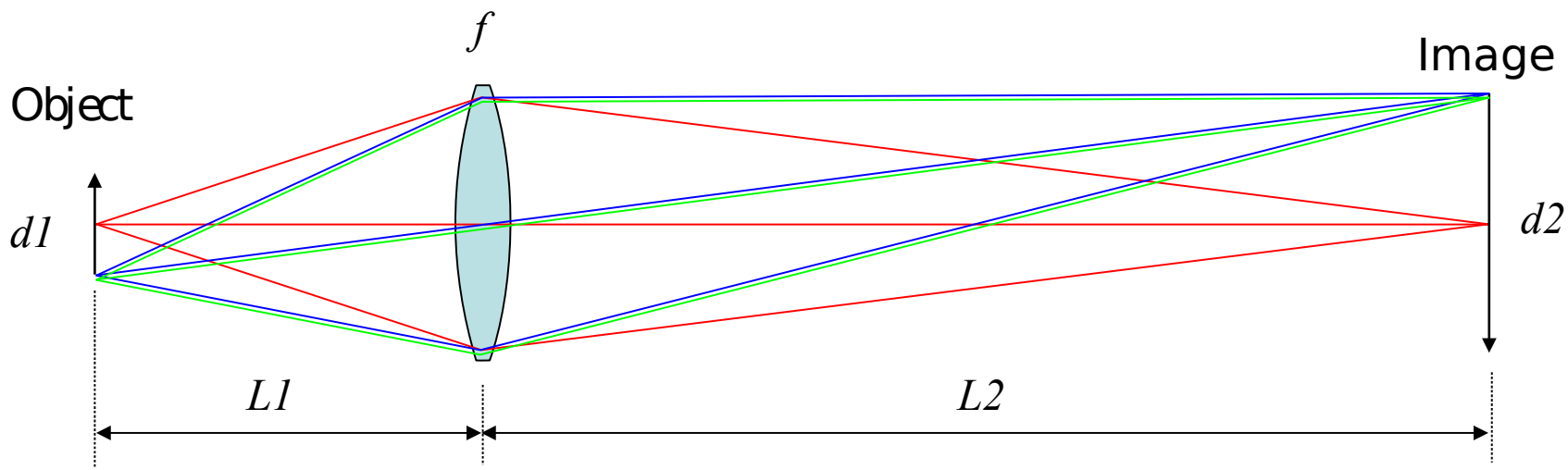
Rays that cross in the focal plane end up parallel



Rays through the lens center are unaffected



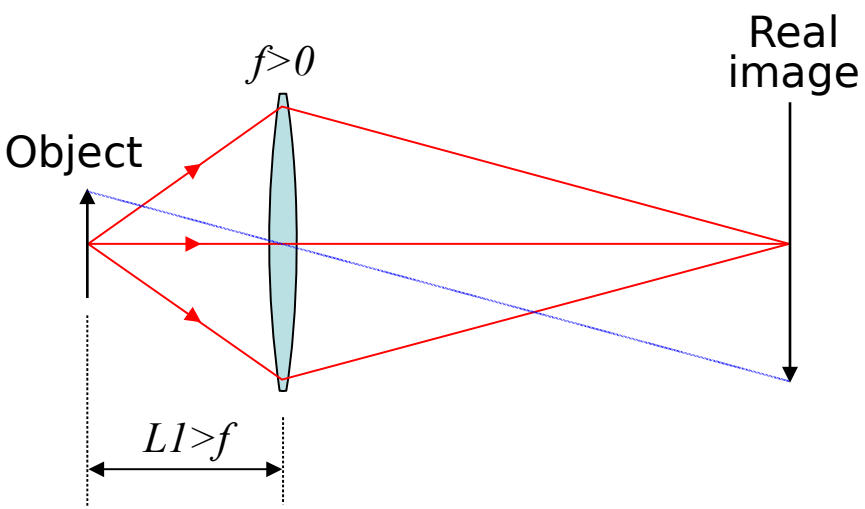
Imaging

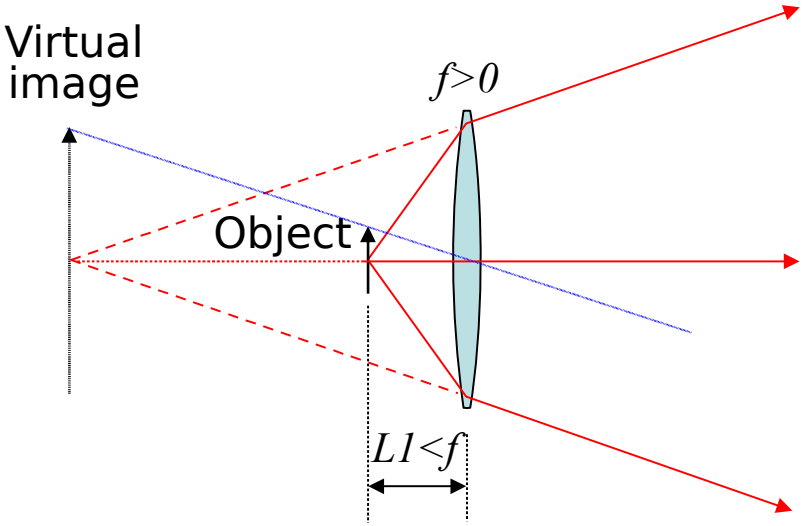


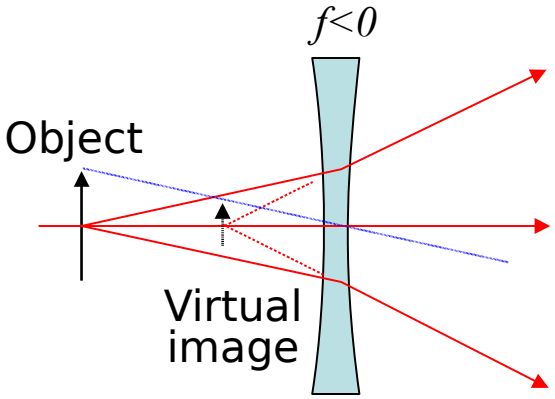
The lens law: $\frac{1}{L_1} + \frac{1}{L_2} = \frac{1}{f}$

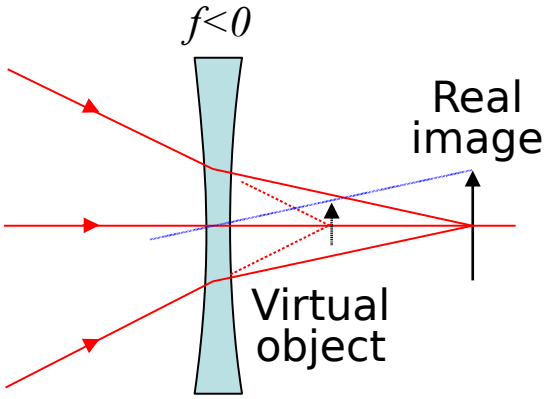
Magnification: $M = \frac{d_2}{d_1} = \frac{L_2}{L_1}$

Real and virtual images





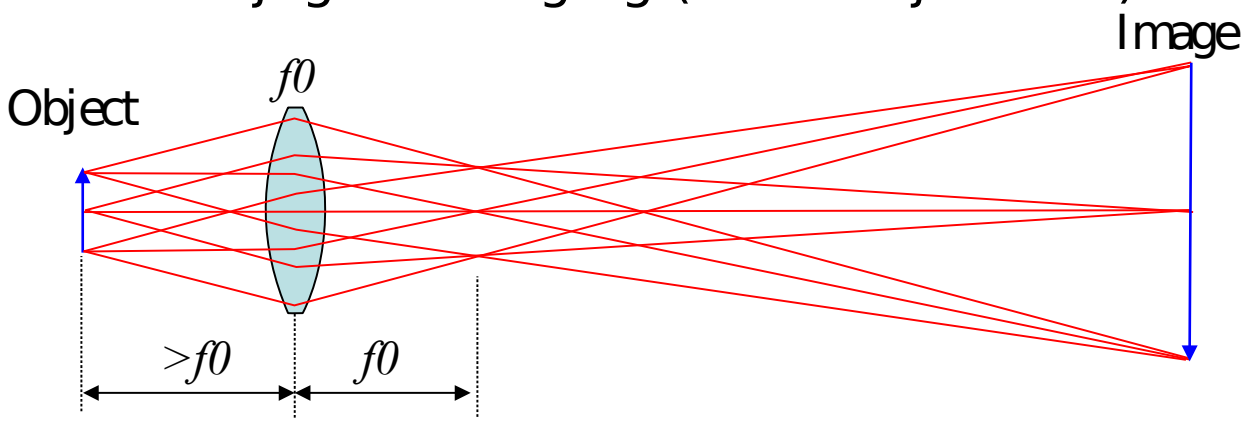




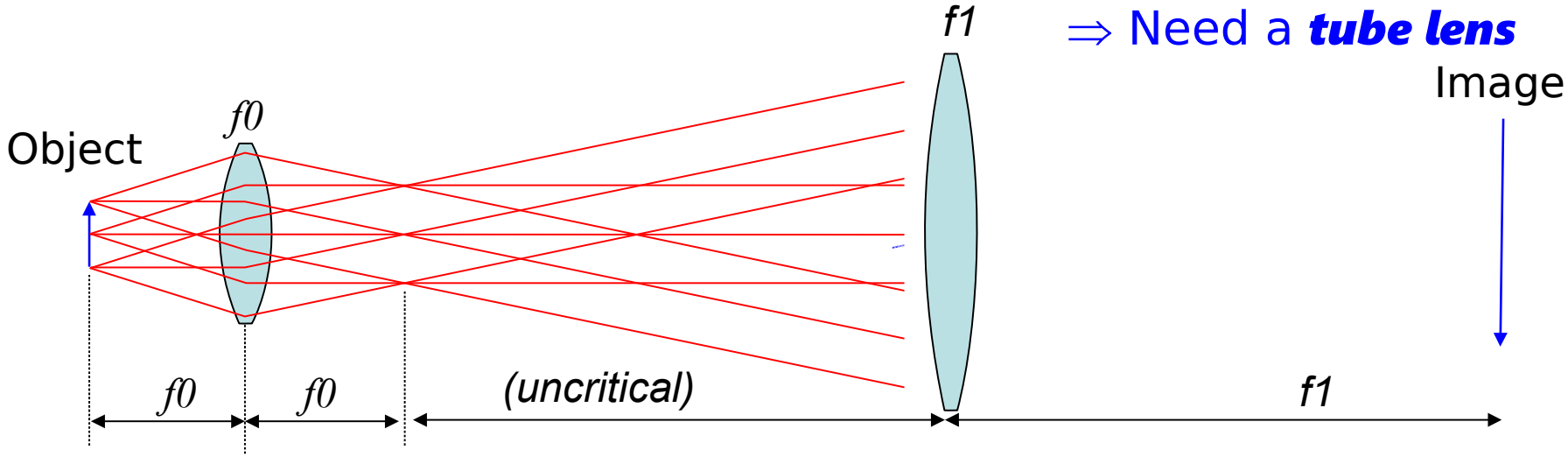
The same lens law applies: Negative lenses have negative f
Virtual objects or images have negative values of $L1$ or $L2$

Finite vs. Infinite Conjugate Imaging

- Finite conjugate imaging (older objectives)

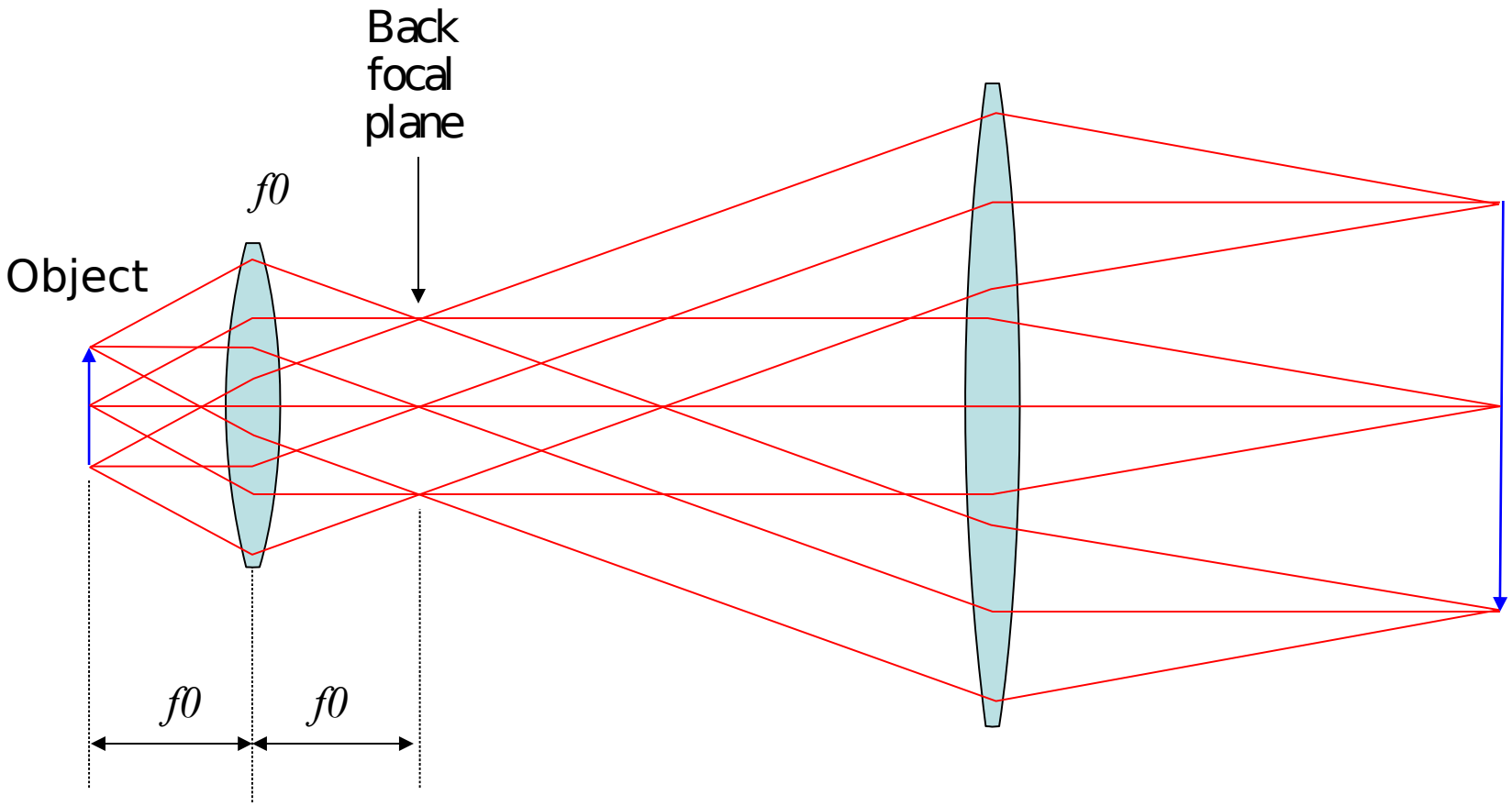


- Infinite conjugate imaging (modern objectives). Image at infinity



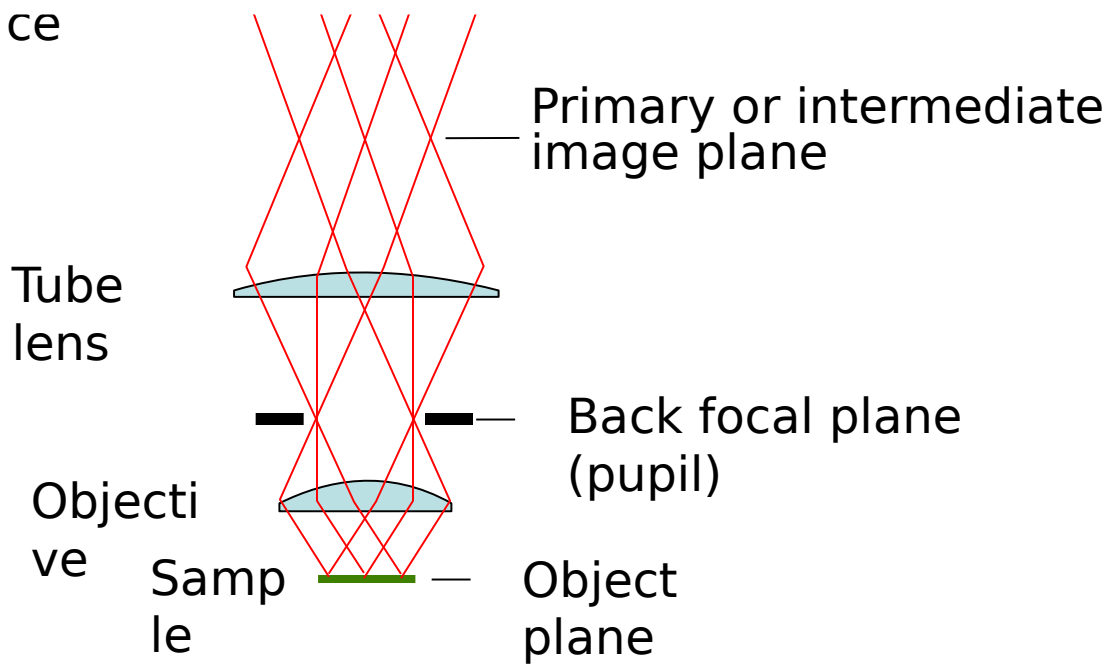
Magnification: $M = \frac{f_1}{f_0}$

Back focal plane

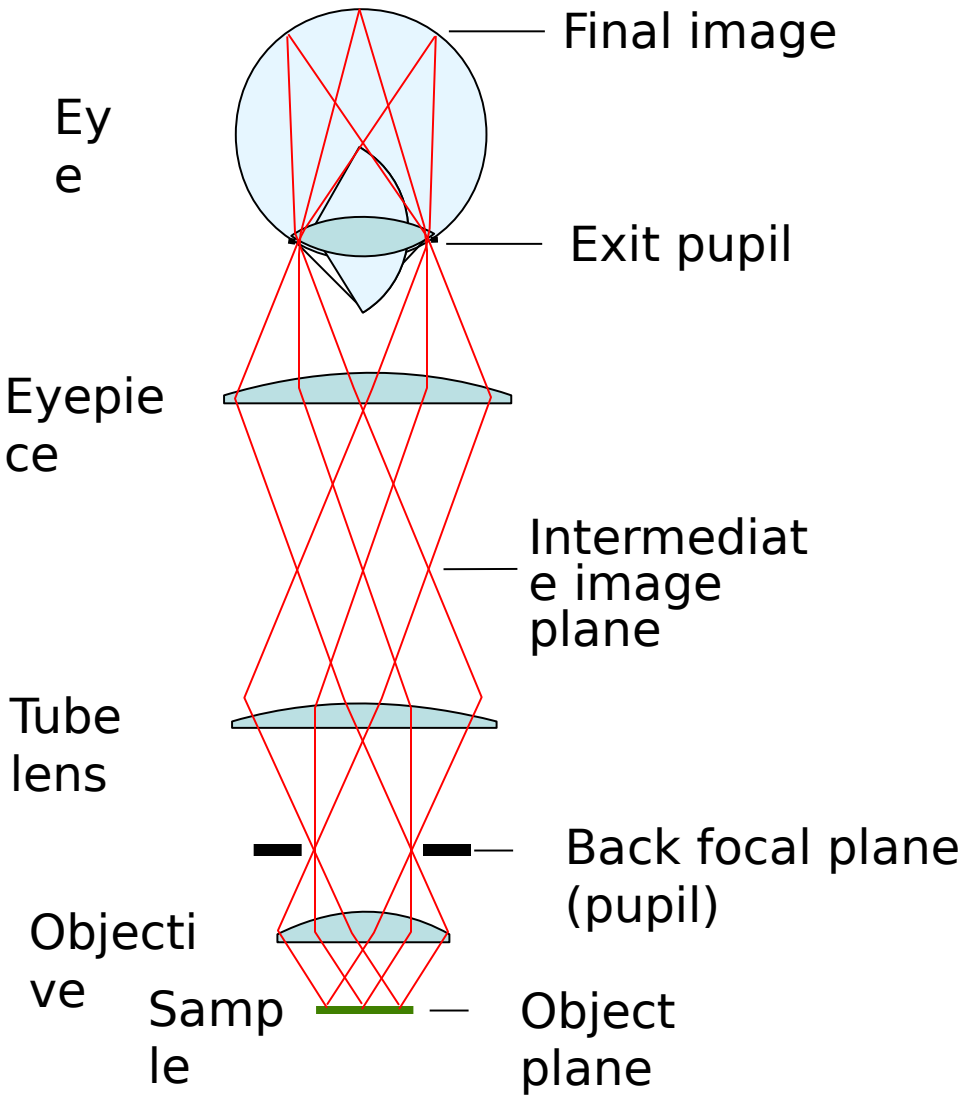


Rays that leave the object with the same angle
meet in the objective's *back focal plane*

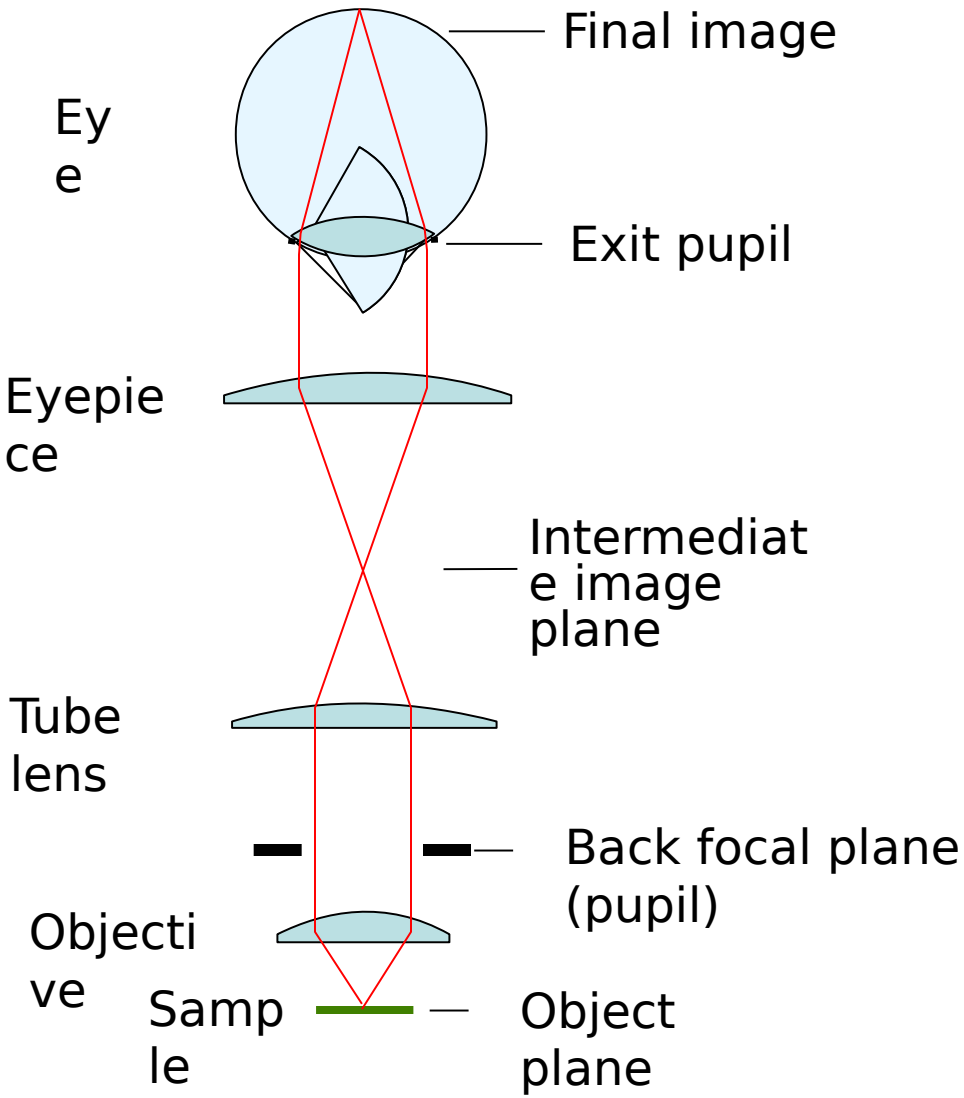
The Compound Microscope



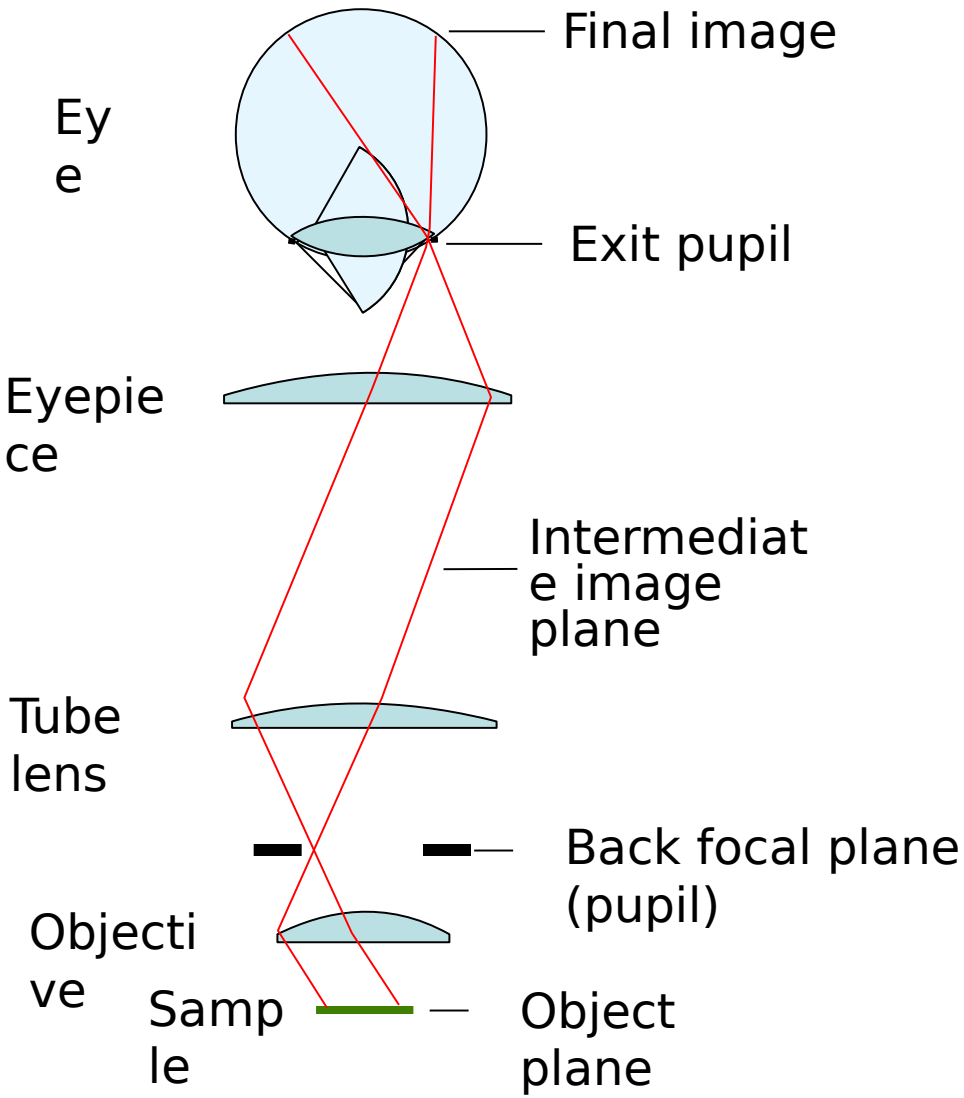
The Compound Microscope



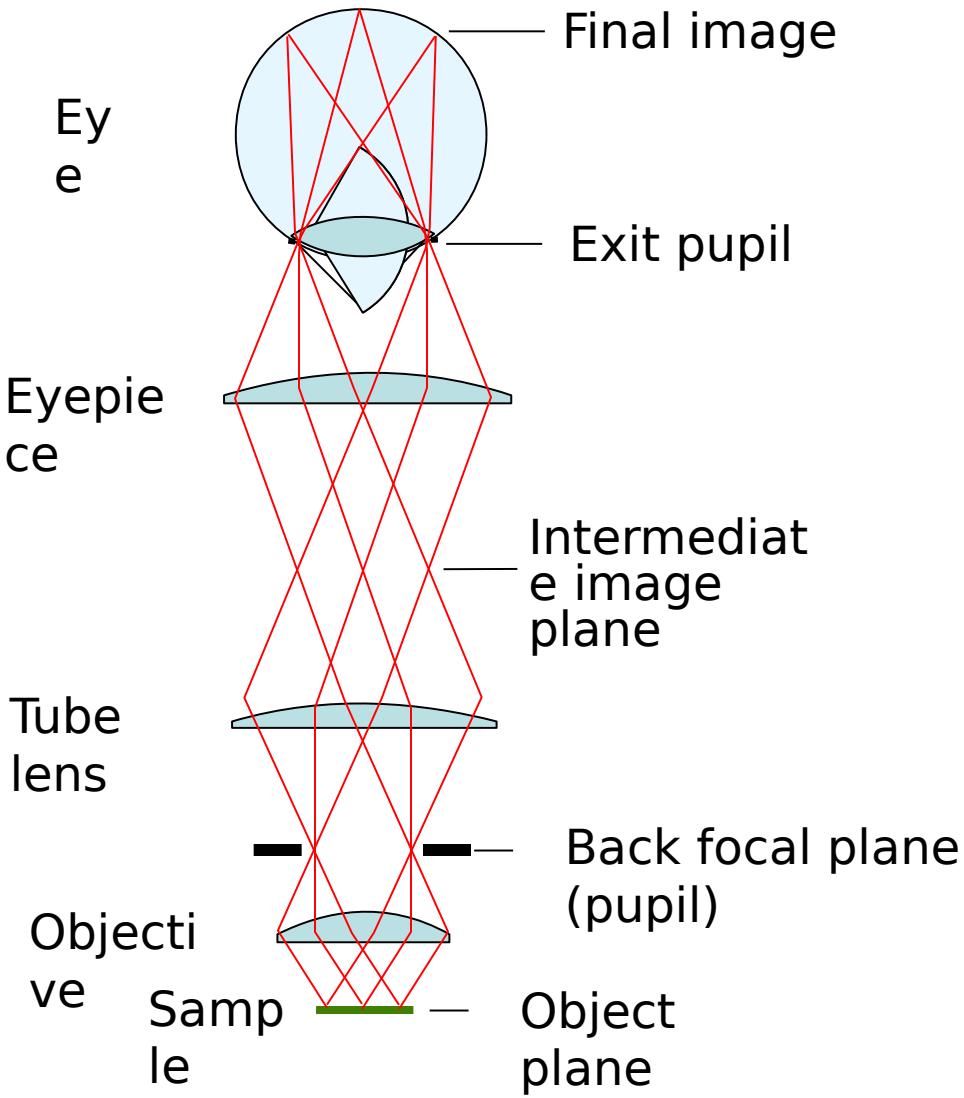
The Compound Microscope



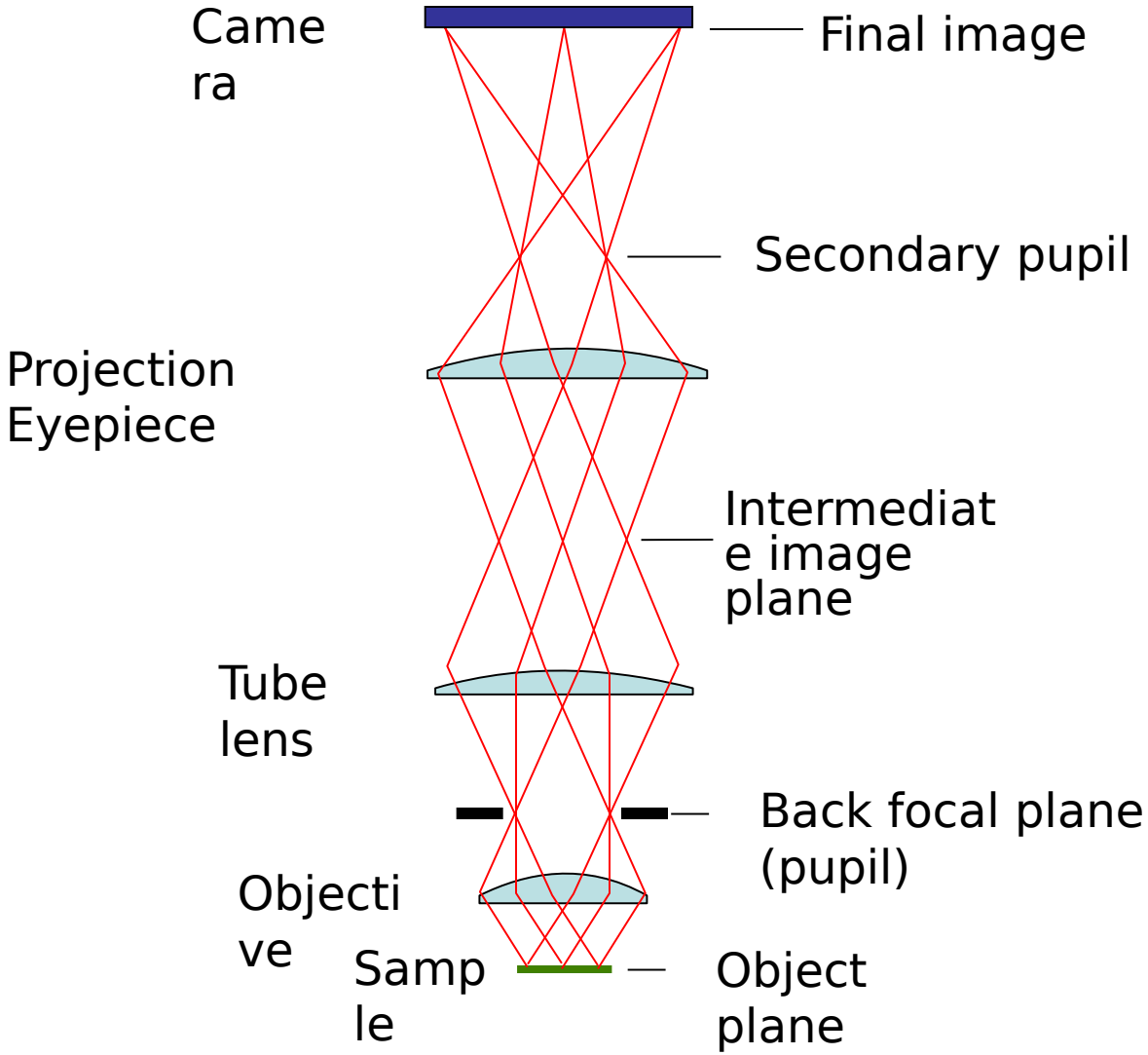
The Compound Microscope



The Compound Microscope



The Compound Microscope

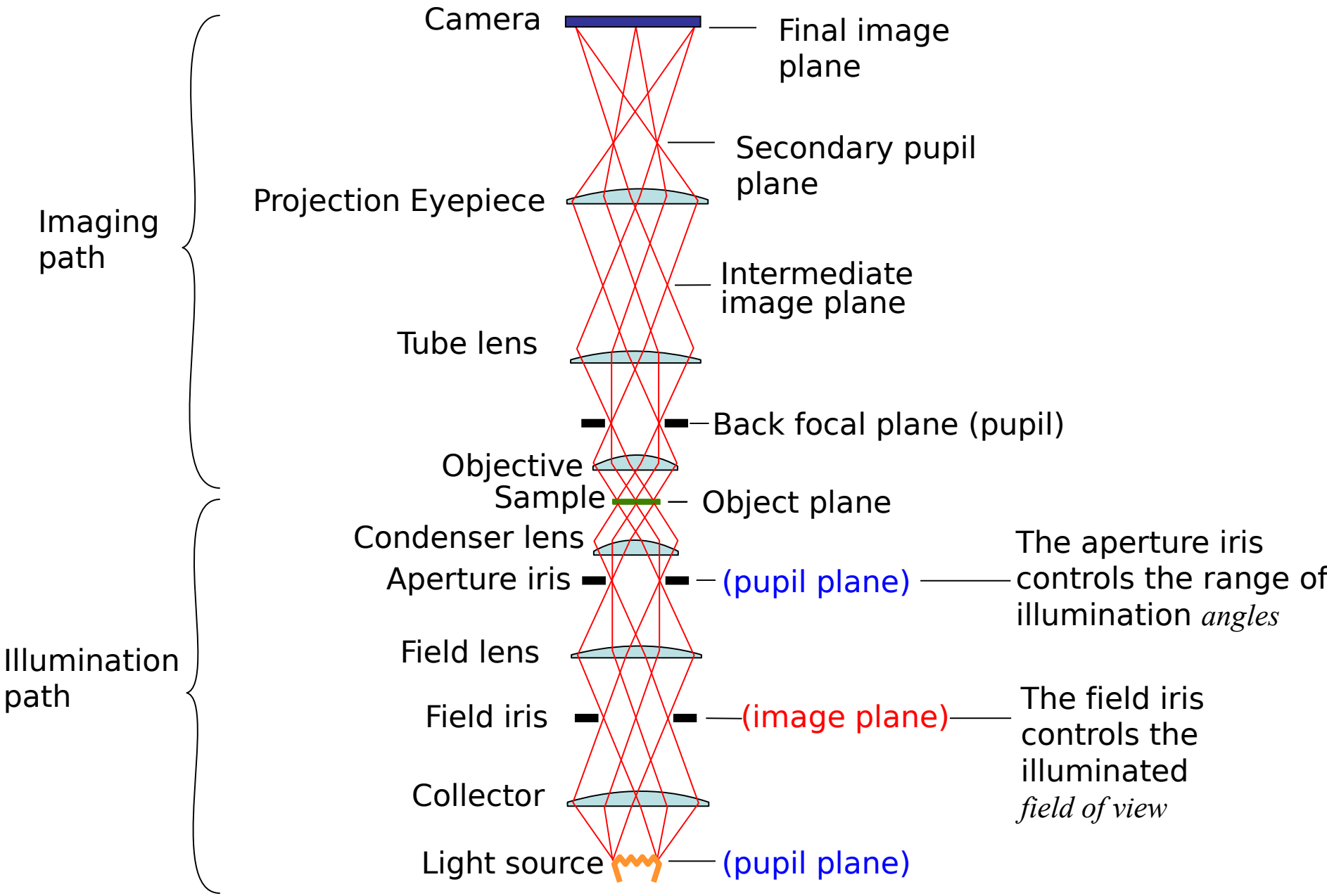


Eyepieces (Oculars)

Features

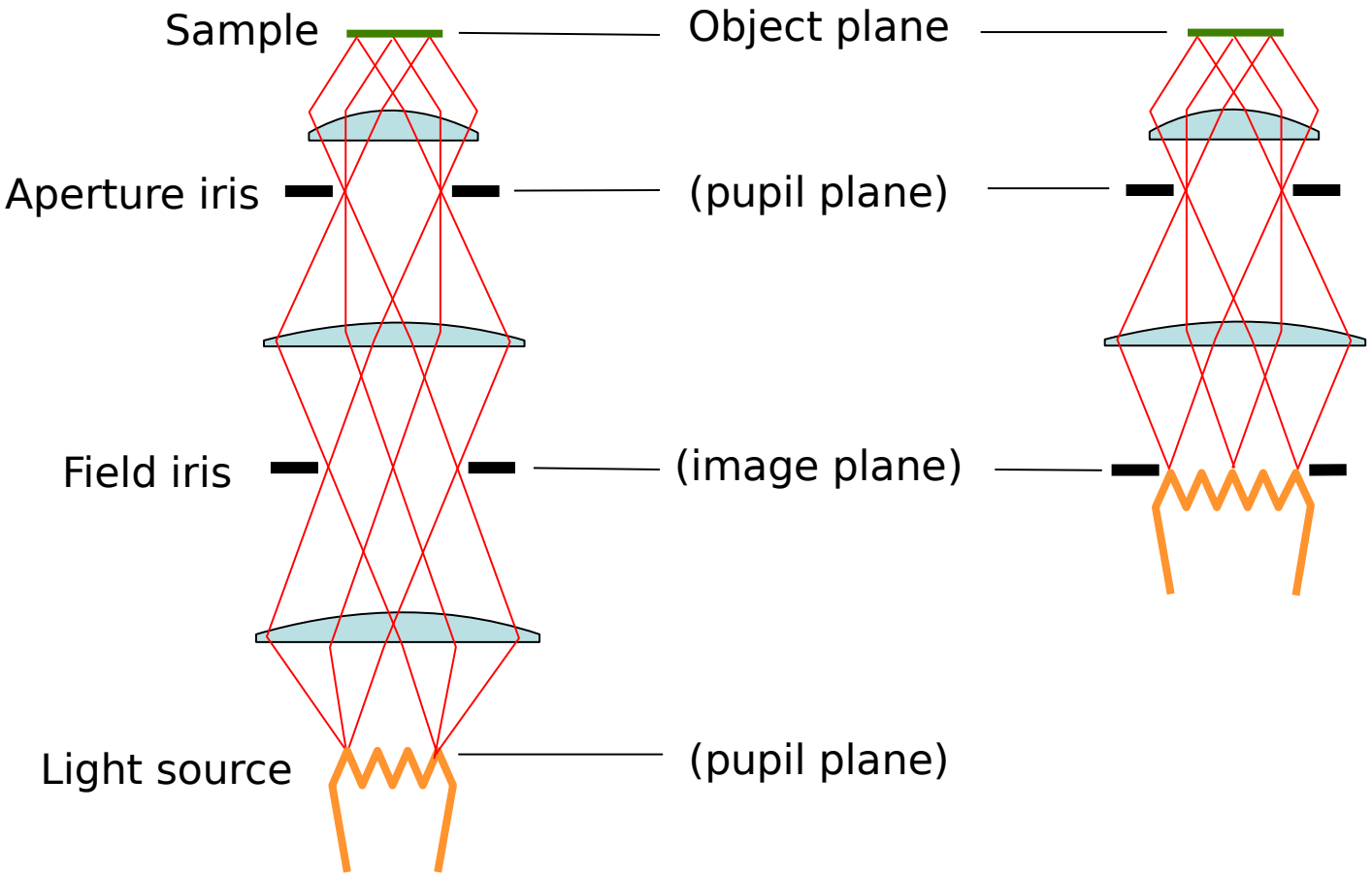
Magnification (10x typical)
"High eye point" (exit pupil high enough to allow eyeglasses)
Diopter adjust (at least *one* must have this)
Reticle or fitting for one
Eye cups

Trans-illumination Microscope



Köhler Illumination

Critical 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)

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

Conjugate Planes in A Research Microscope

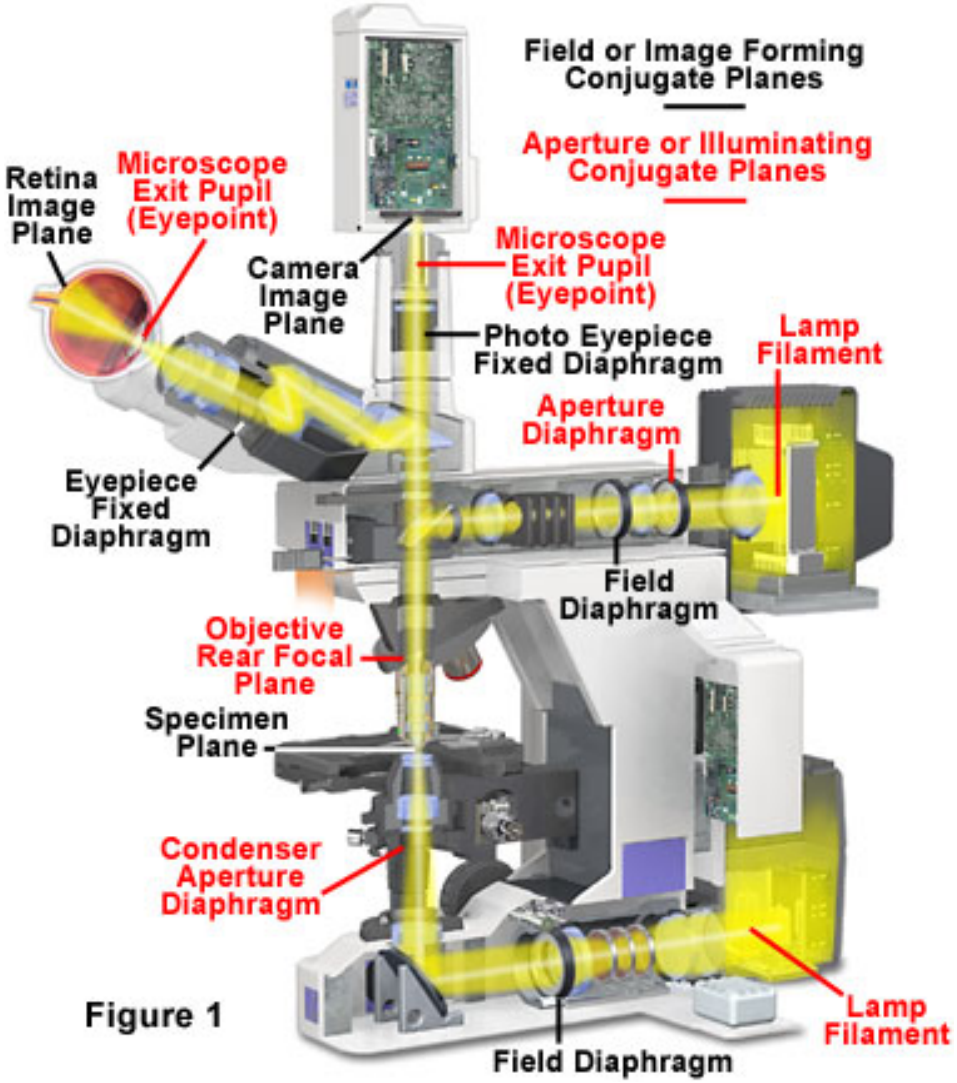


Figure 1

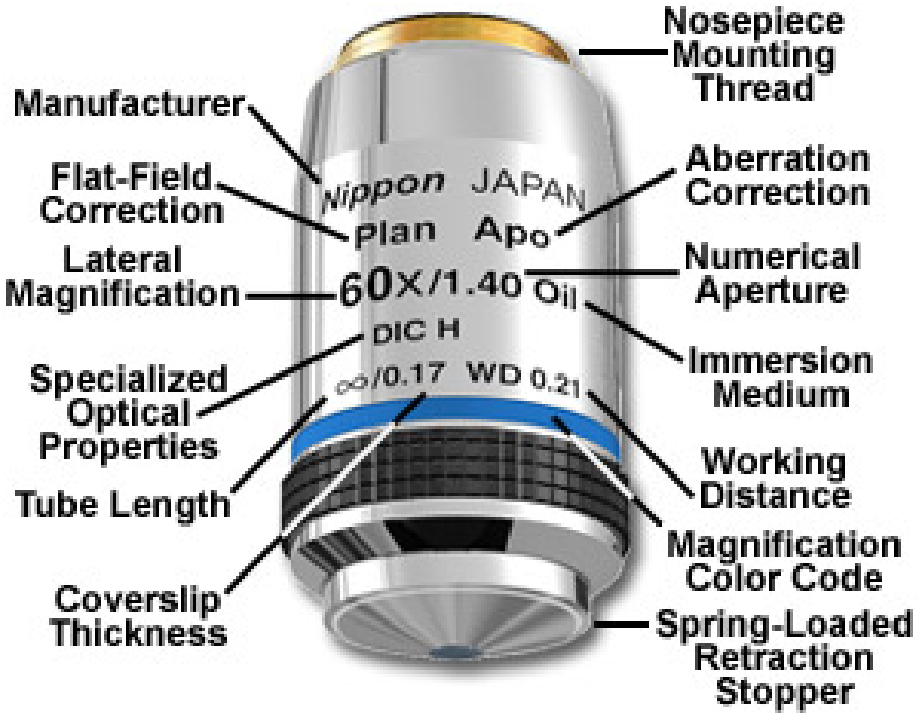
How view the pupil planes?

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

Figure 1

In general, high NA lenses have short working distances

However, extra-long working distance objectives do exist

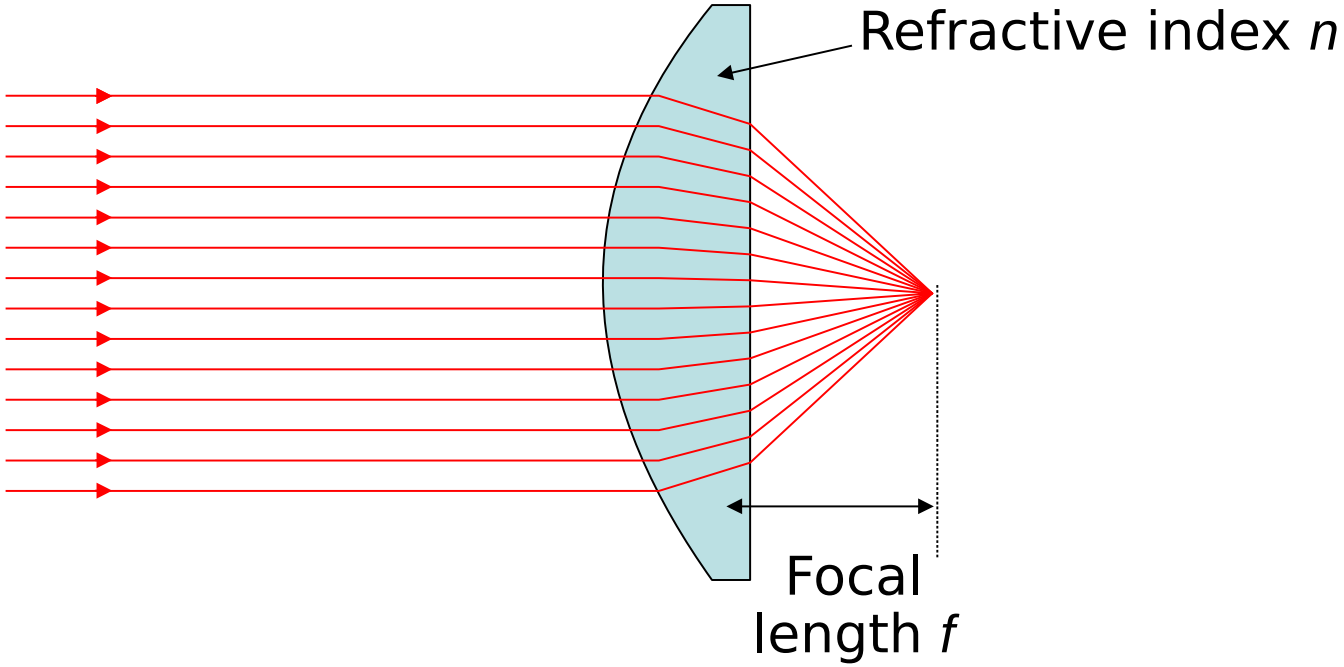
Some examples:

10x/0.3 WD = 15.2mm

20x/0.75 WD = 1.0mm

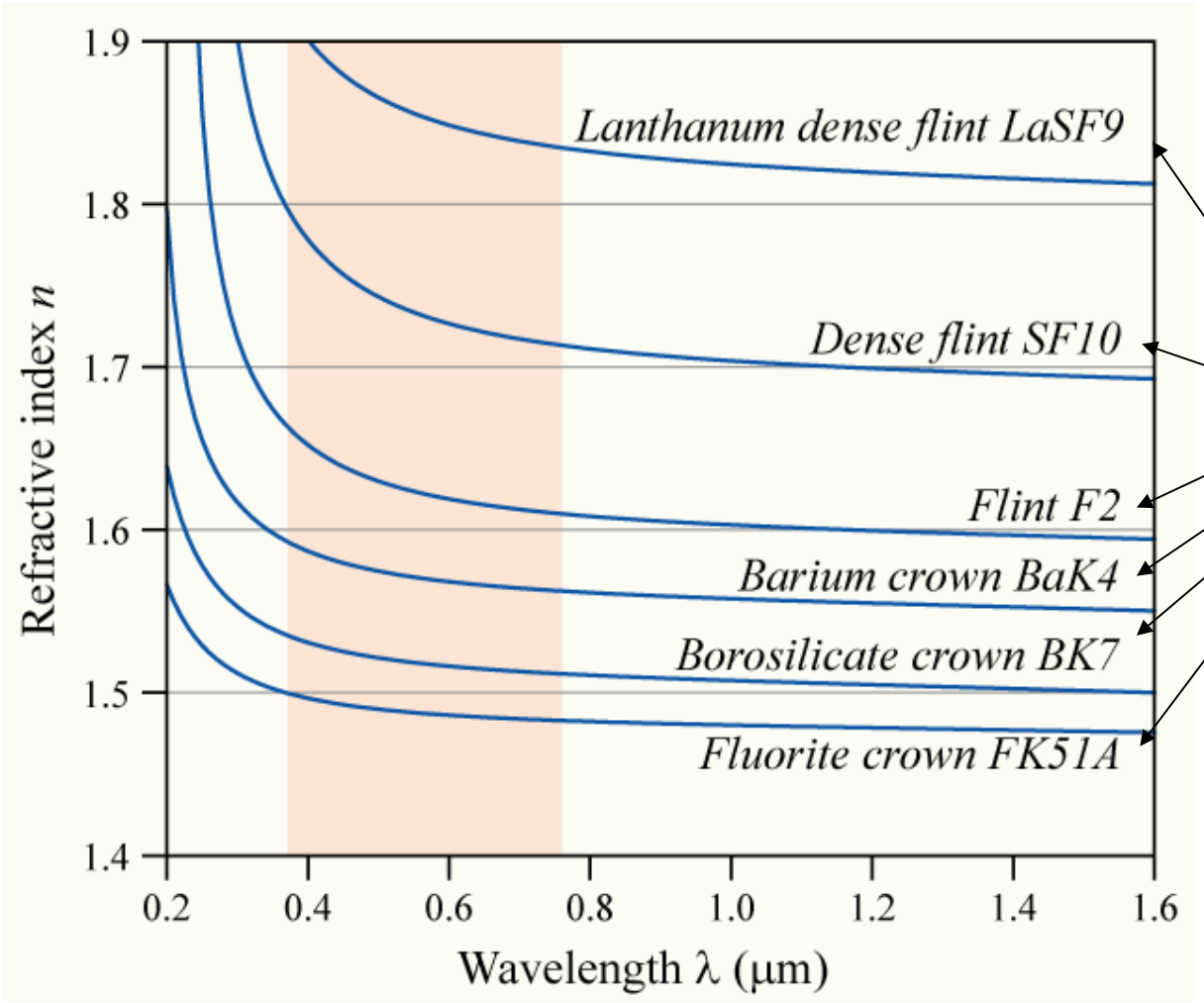
100x/1.4 WD = 0.13mm

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



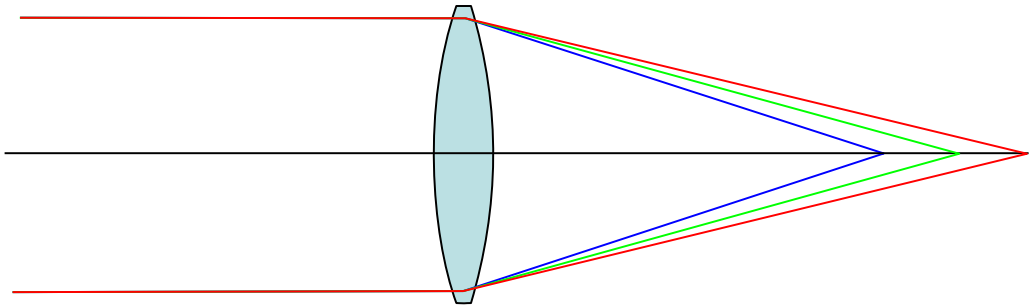
$f \propto 1/(n-1)$

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

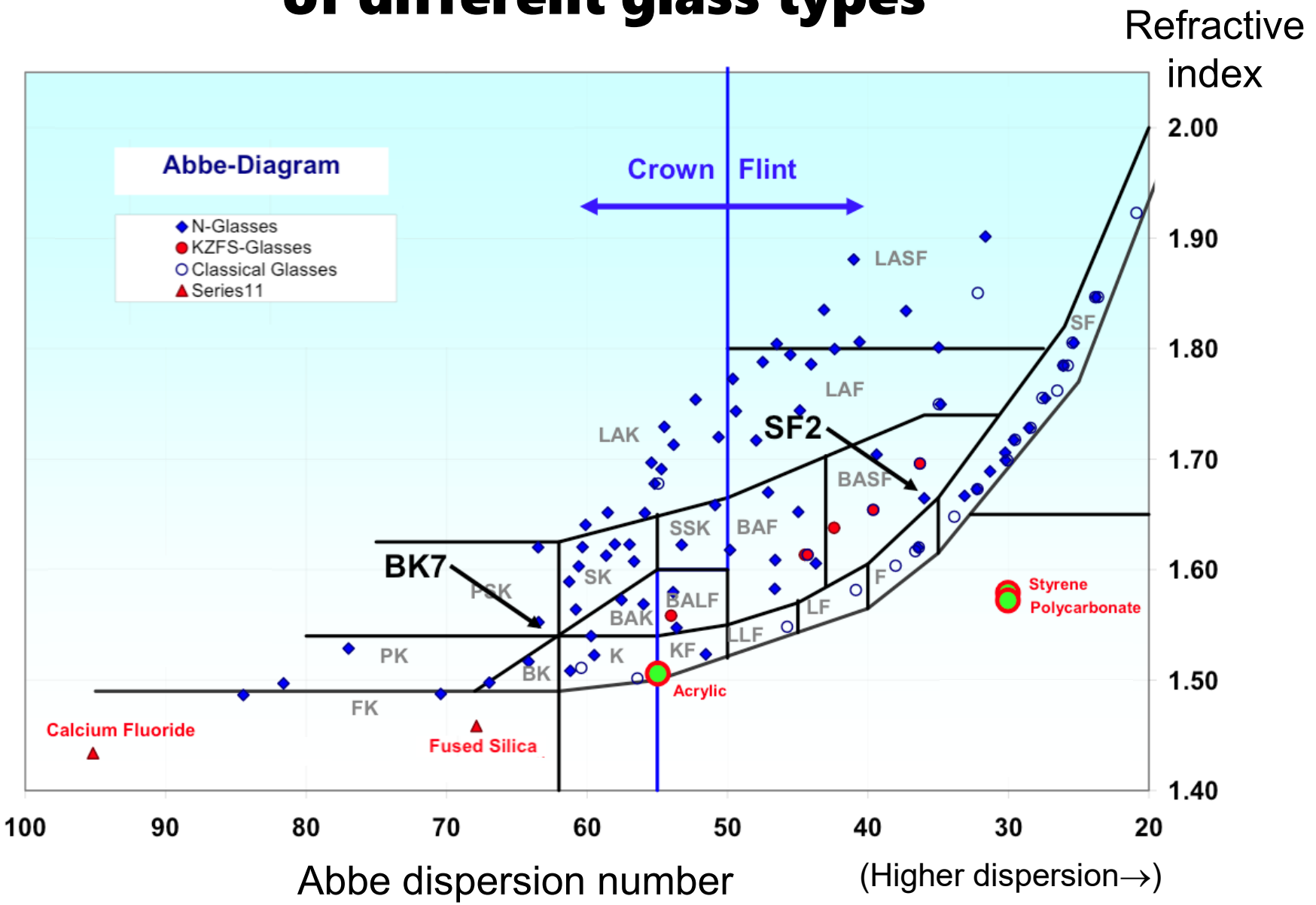


⇒ Chromatic aberration

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

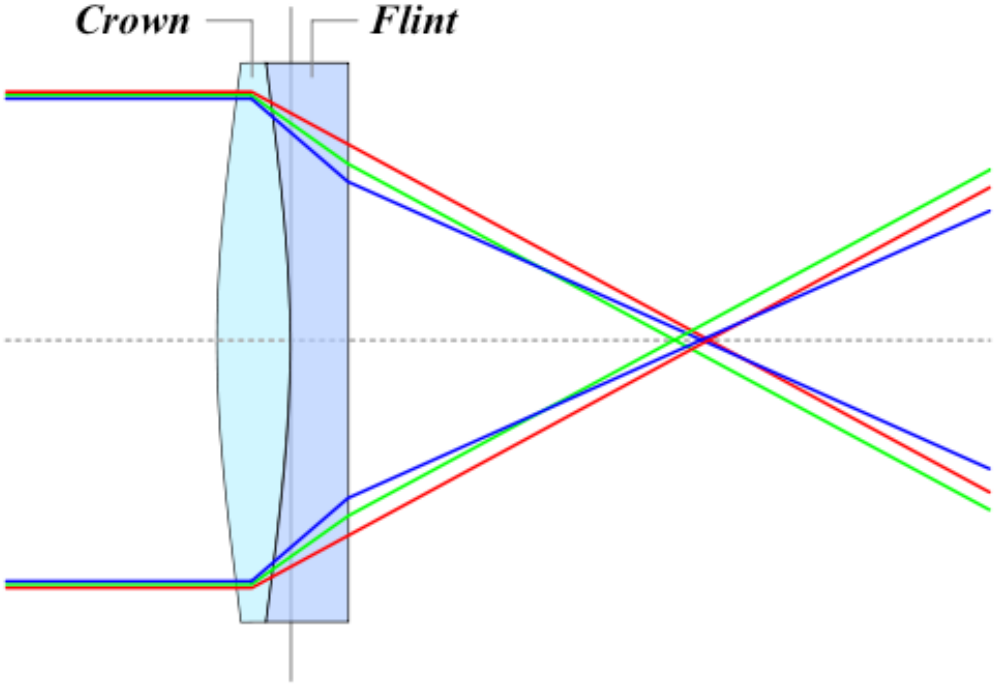


Dispersion vs. refractive index of different glass types

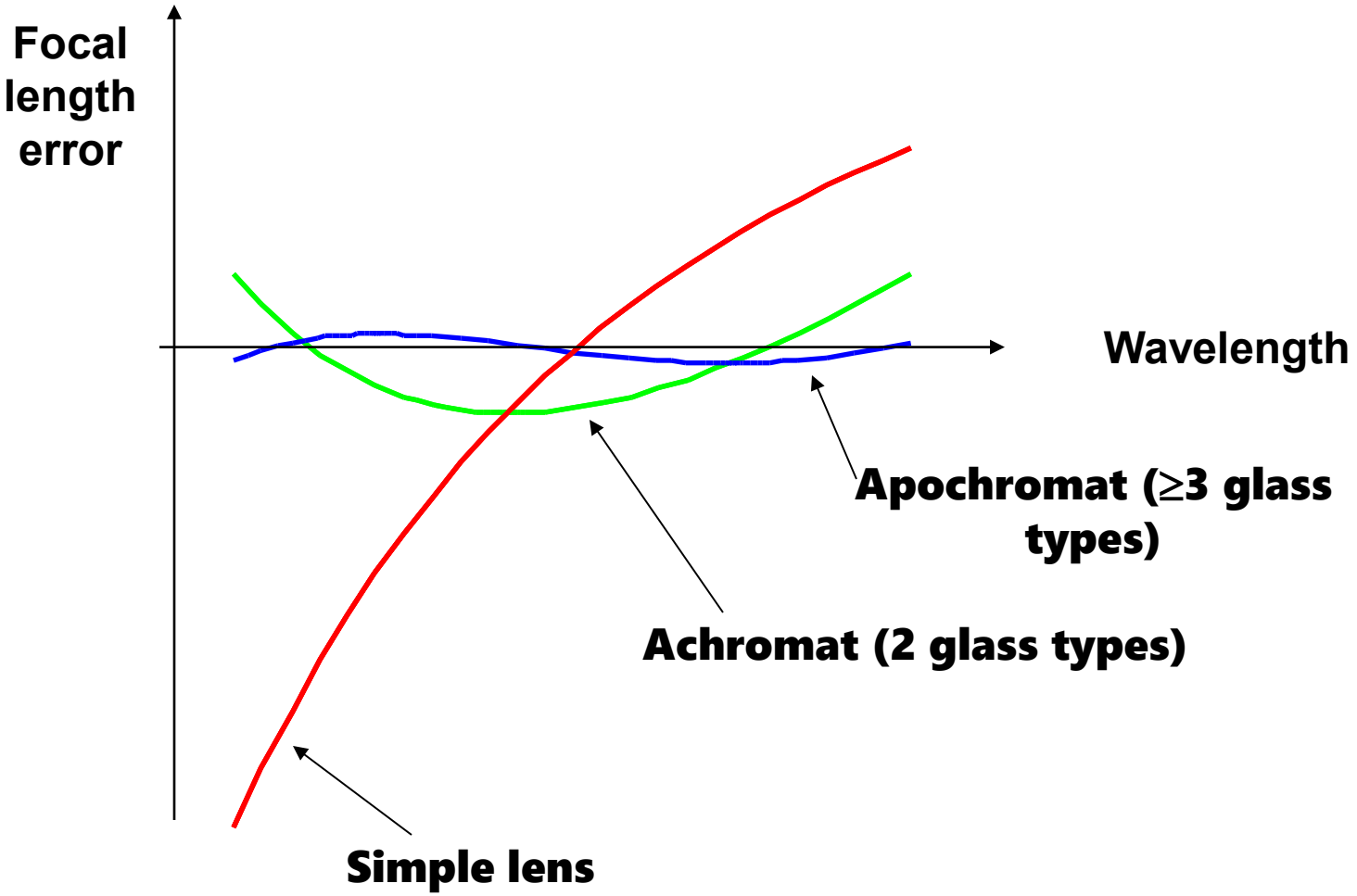


Achromatic Lenses

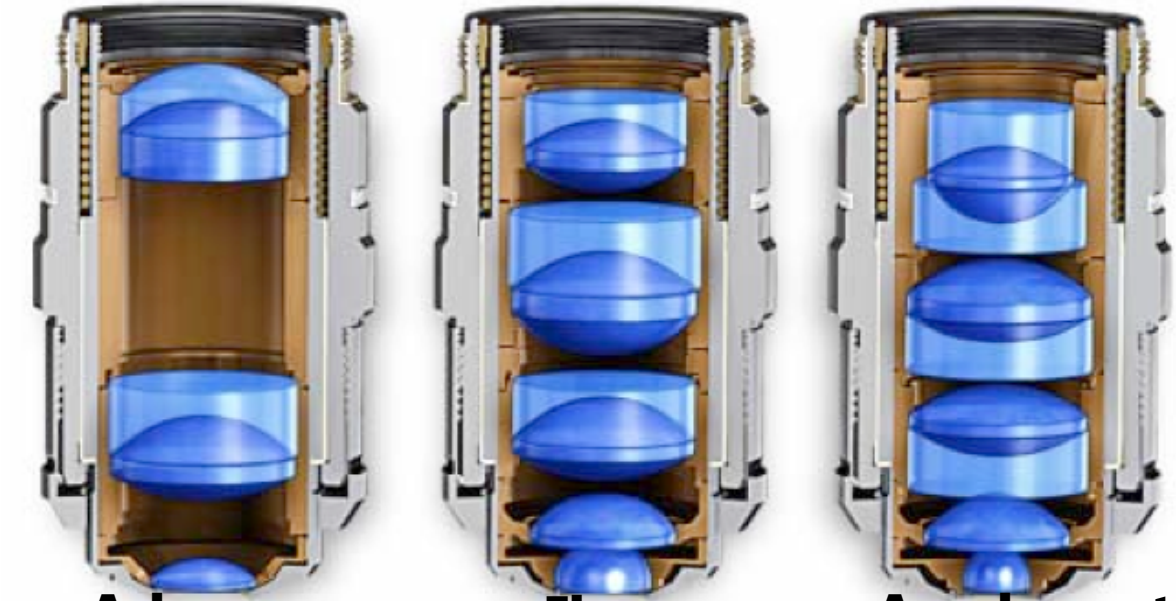
- Use a weak negative flint glass element to compensate the dispersion of a positive crown glass element



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 ⟶

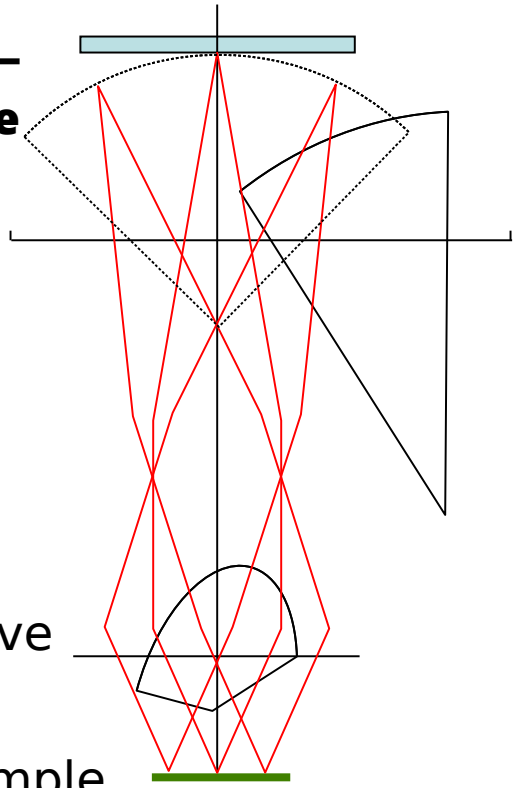
Curvature of Field

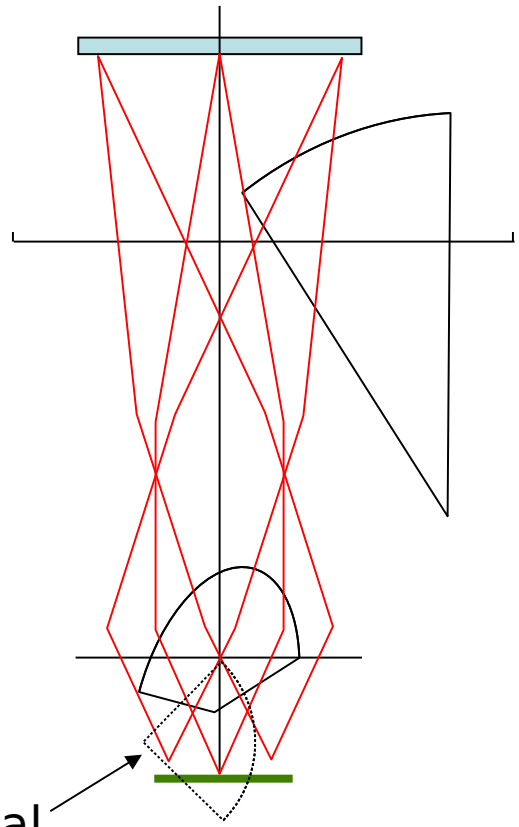
~~Focal plane~~
Focal surface

Tube lens

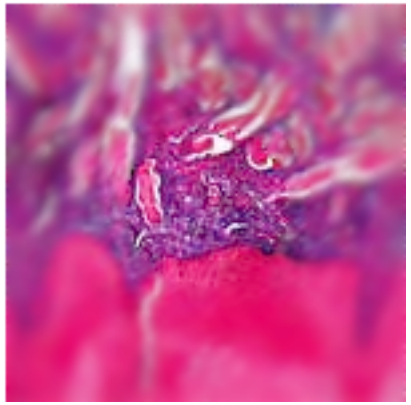
objective

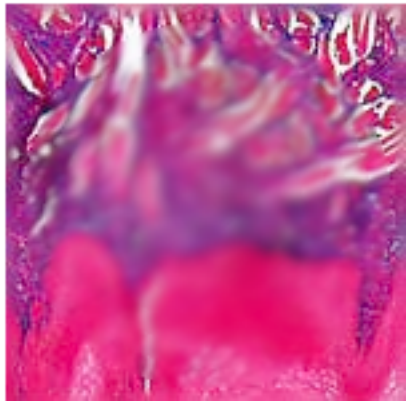
sample





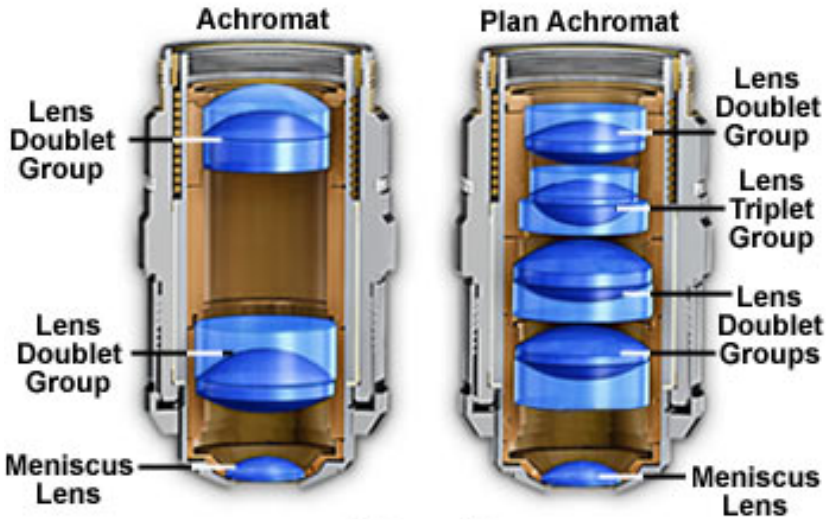
Focal
surface





Plan objectives

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



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

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)

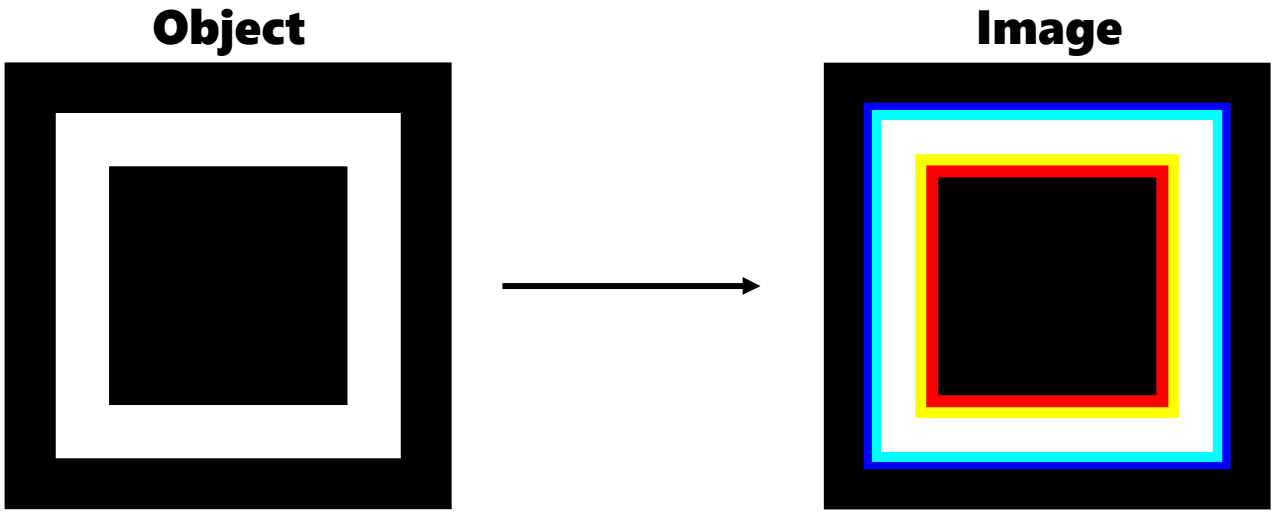
⇒ mixing brands can produce severe LCA

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

Lateral chromatic aberration

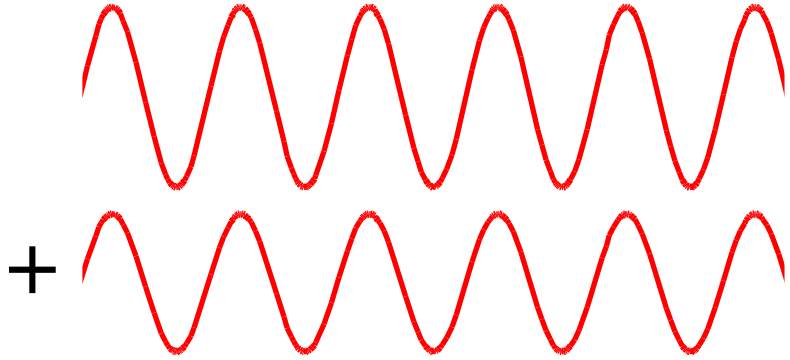
(= LCA, lateral color, chromatic difference of magnification)

= Different magnification for different colors

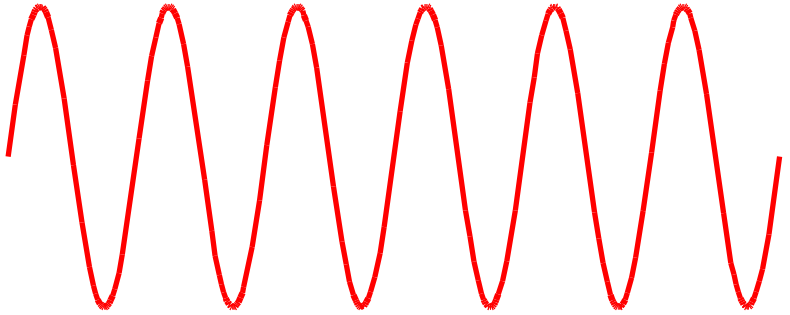


Interference

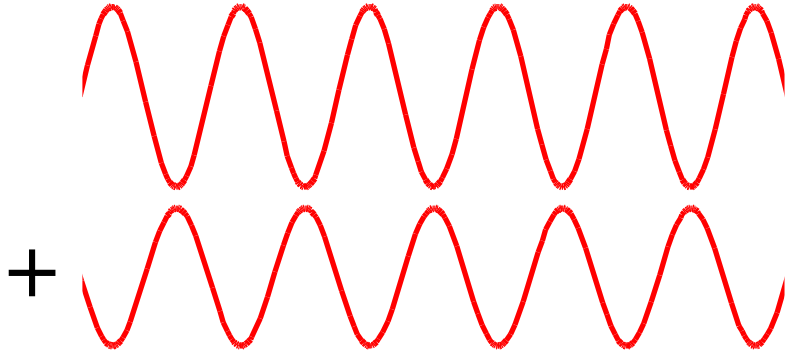
In phase



constructive interference

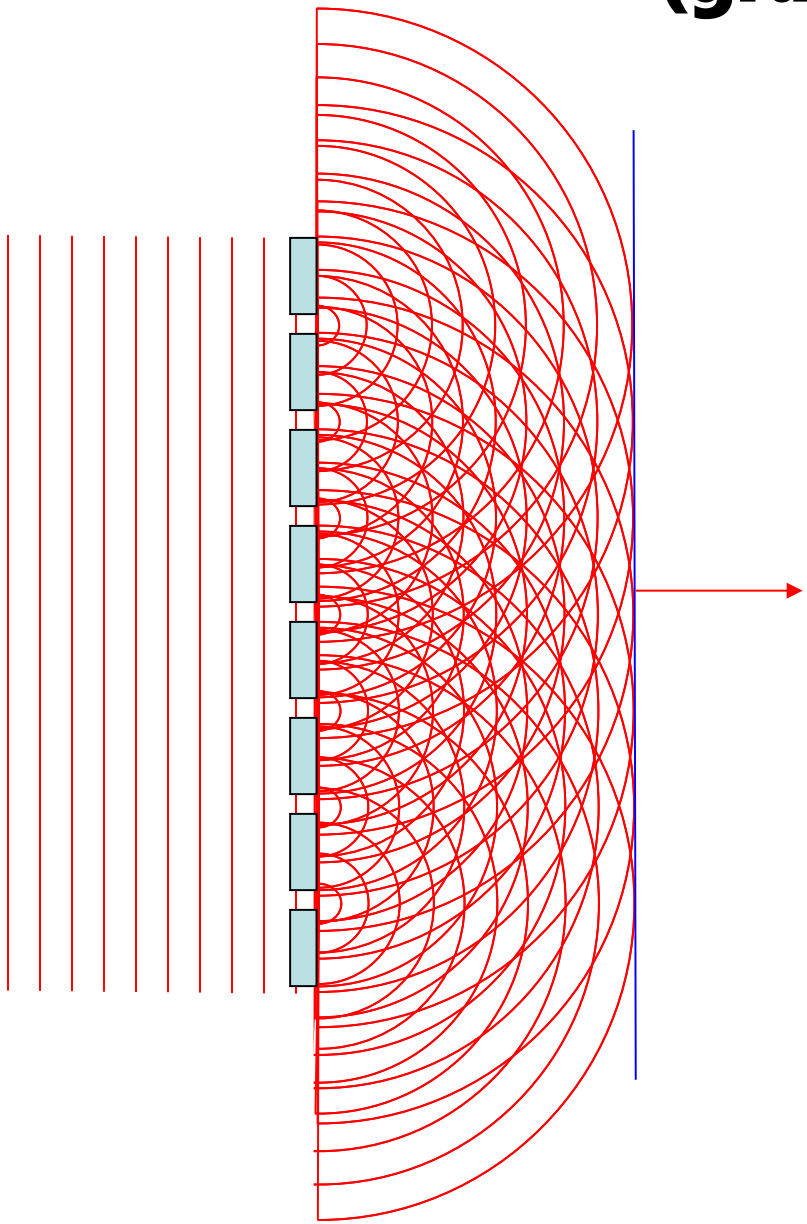


Opposite phase

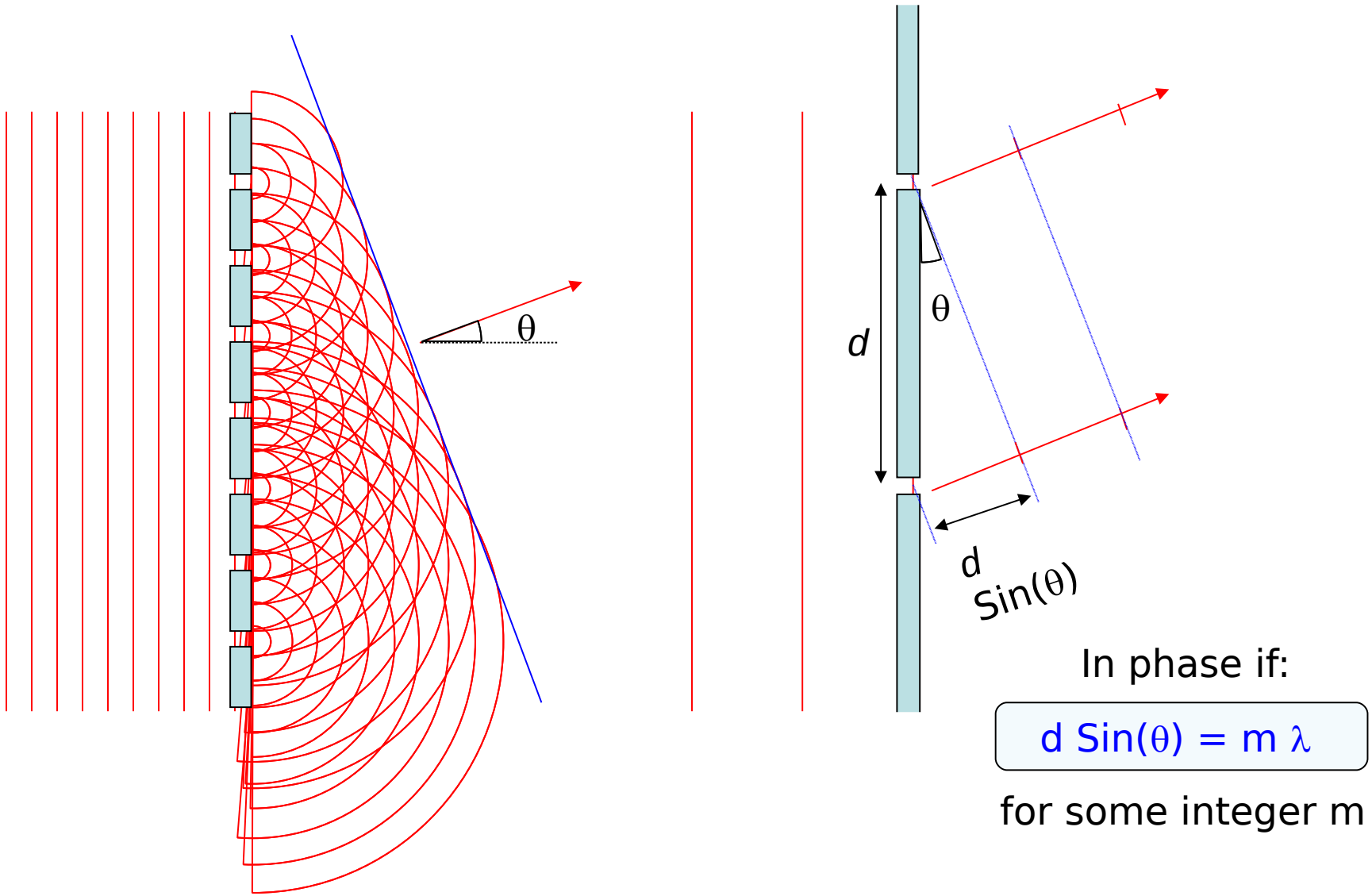


destructive interference

Diffraction by a periodic structure (grating)



Diffraction by a periodic structure (grating)



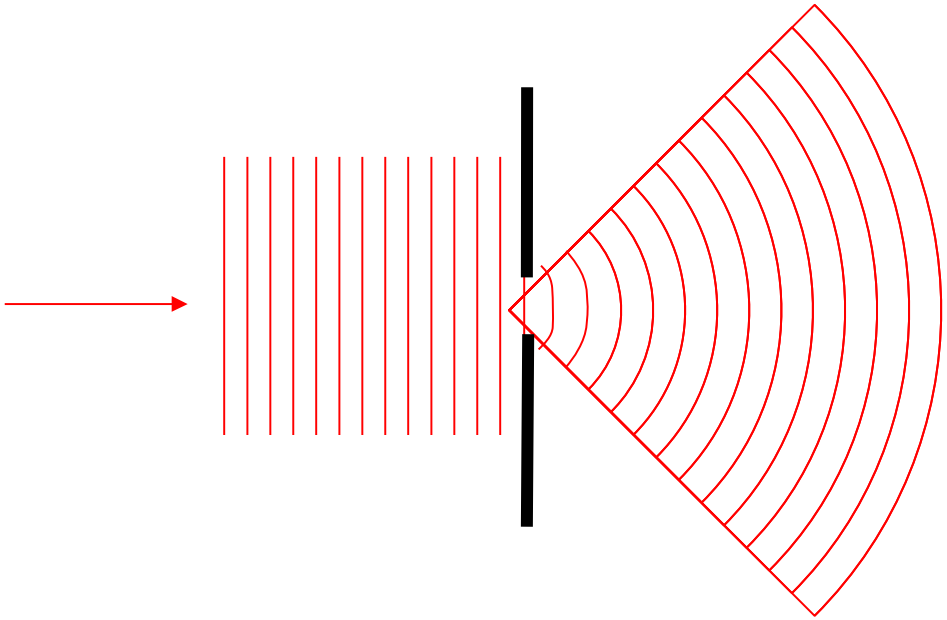
In phase if:

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

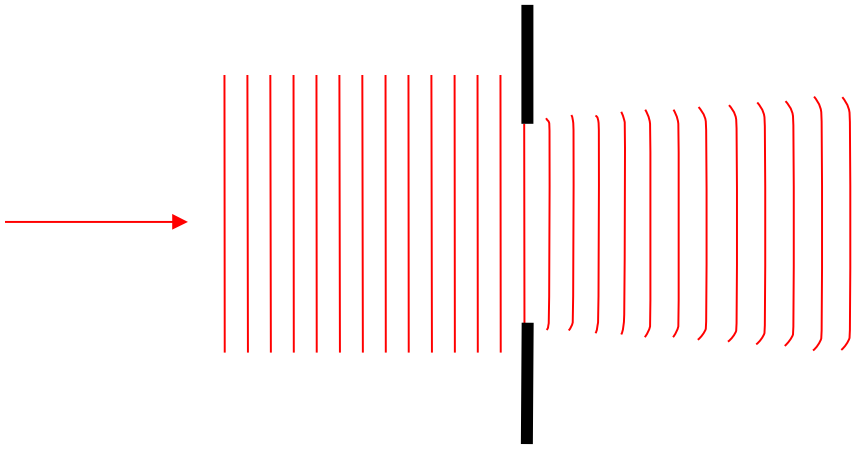
for some integer m

Diffraction by an aperture

drawn as waves



Light spreads to new angles



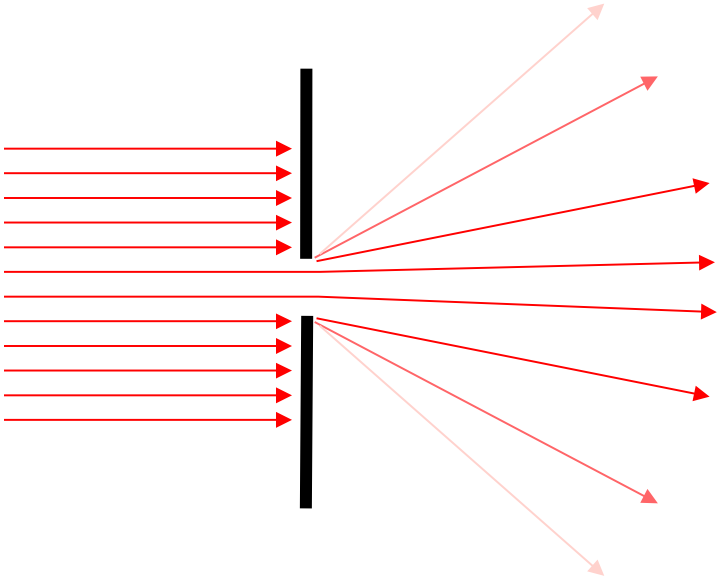
Larger aperture

⇔

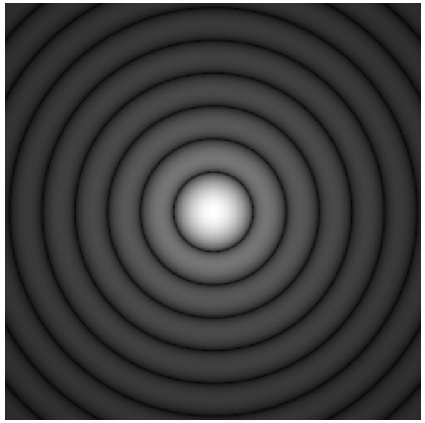
weaker diffraction

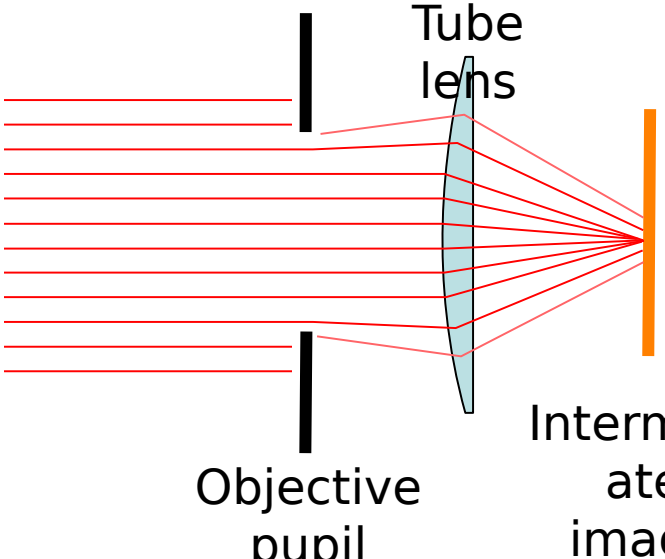
Diffraction by an aperture

drawn as rays



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



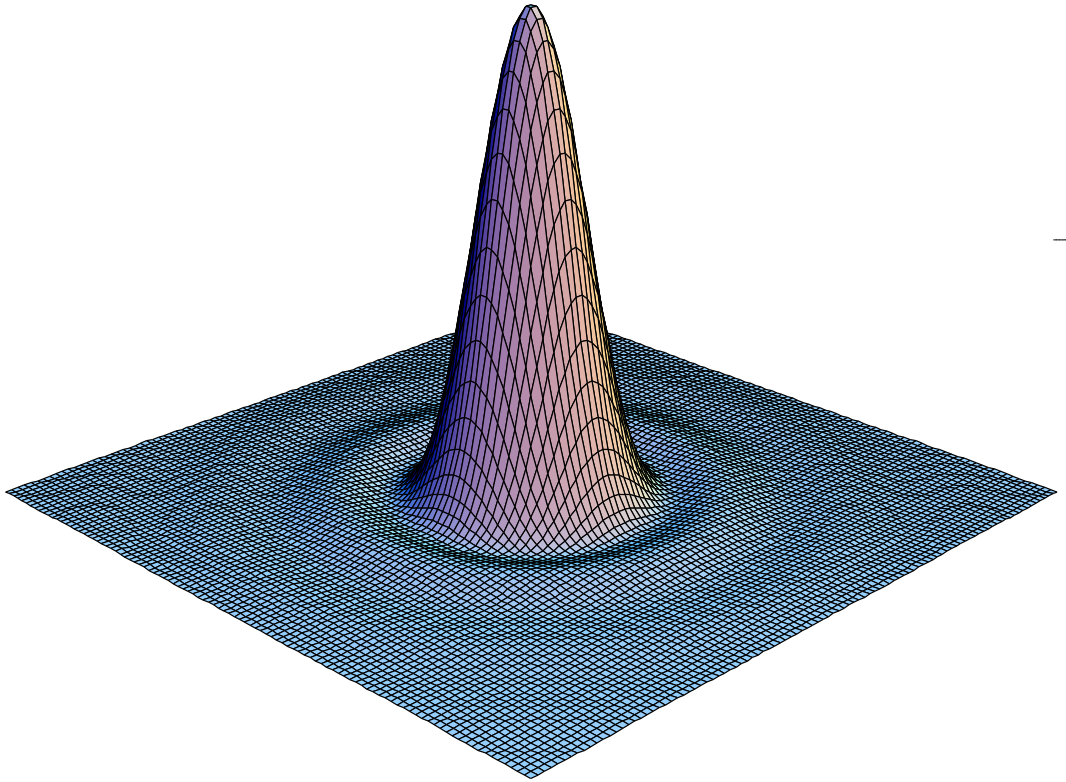


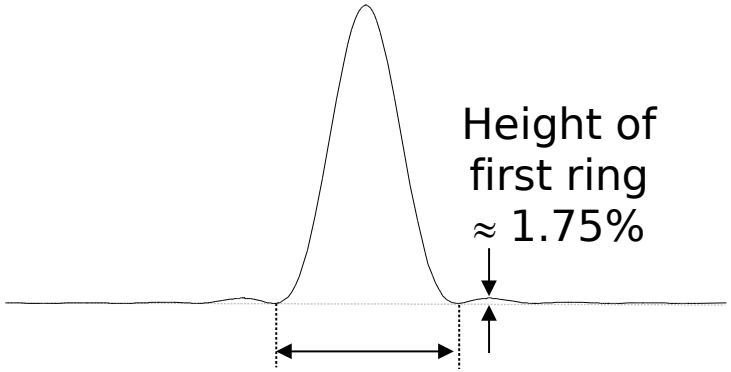
…or can be formed at a finite distance by a lens…

…as happens in a microscope

The Airy Pattern

= the far-field diffraction pattern from a round aperture

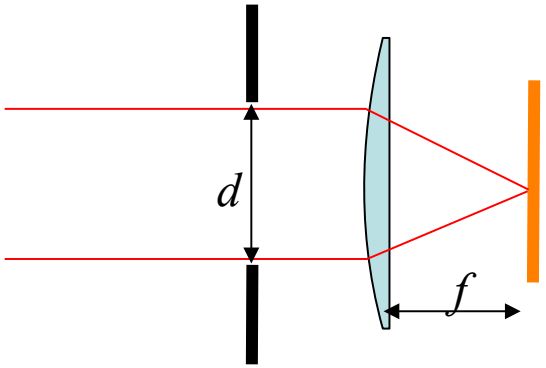




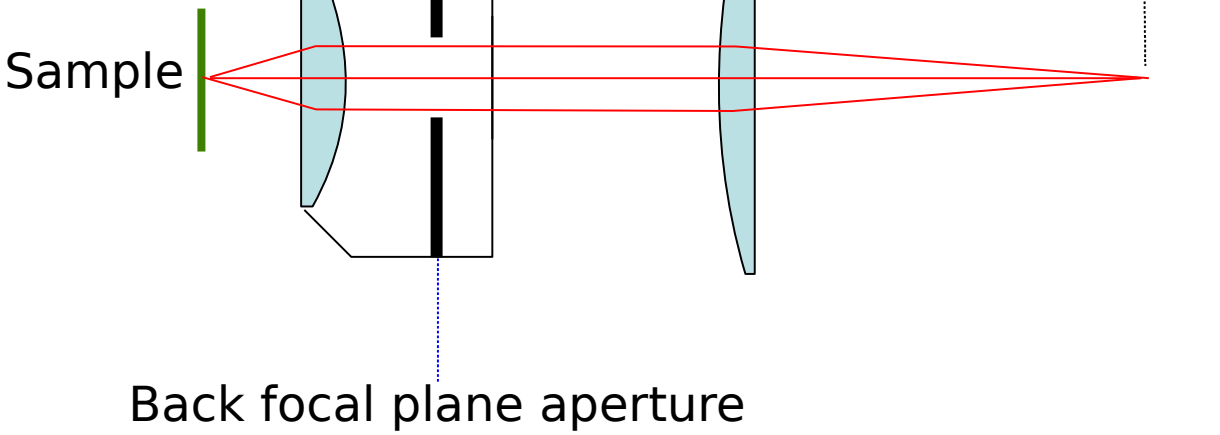
"Airy disk" diameter

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

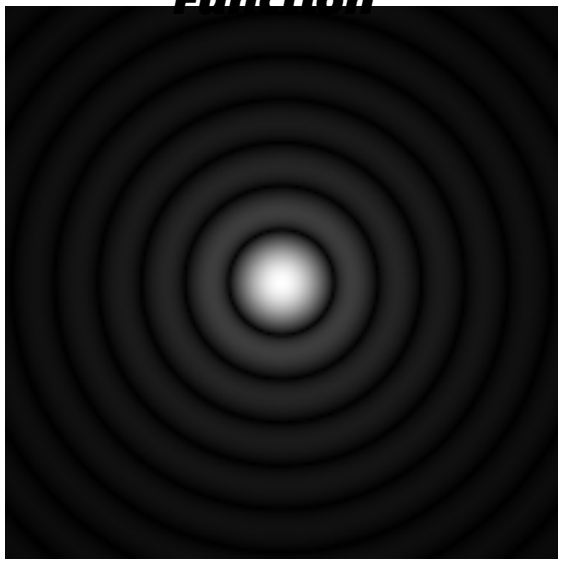
(for small angles d/f)



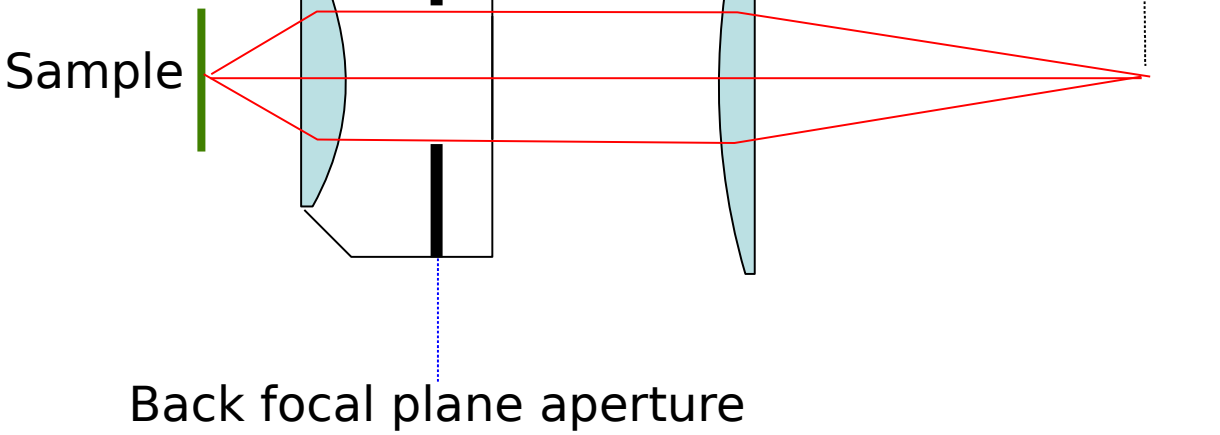
Aperture and Resolution



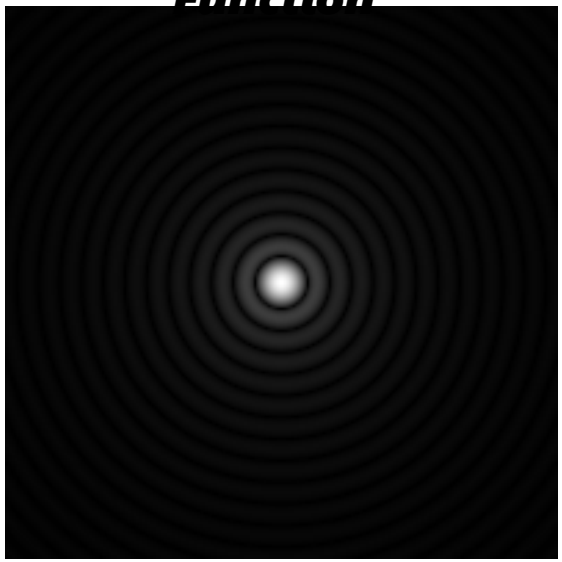
Diffraction spot
on image plane
= ***Point Spread***
Function



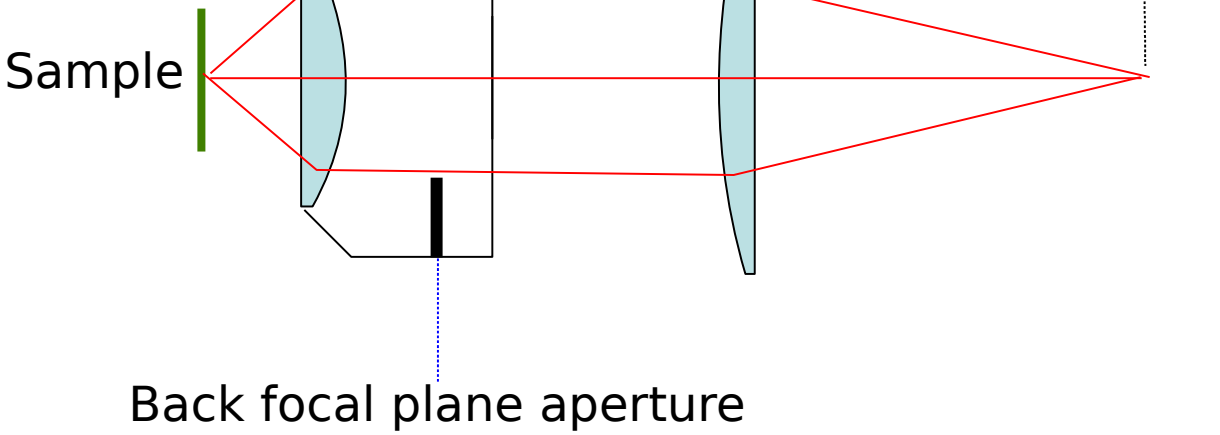
Aperture and Resolution



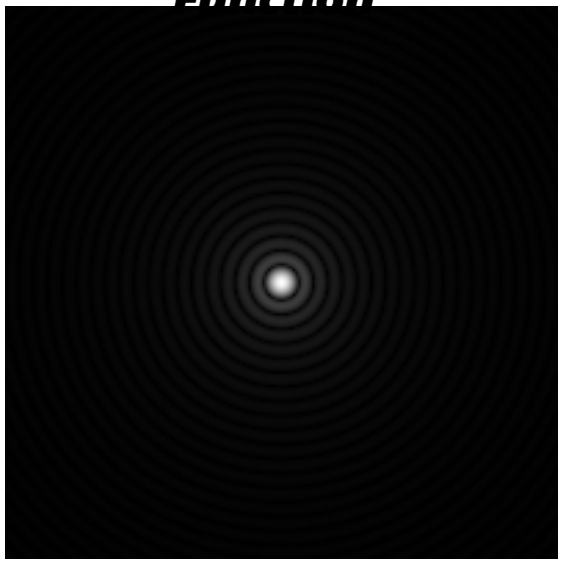
Diffraction spot
on image plane
= ***Point Spread***
Function



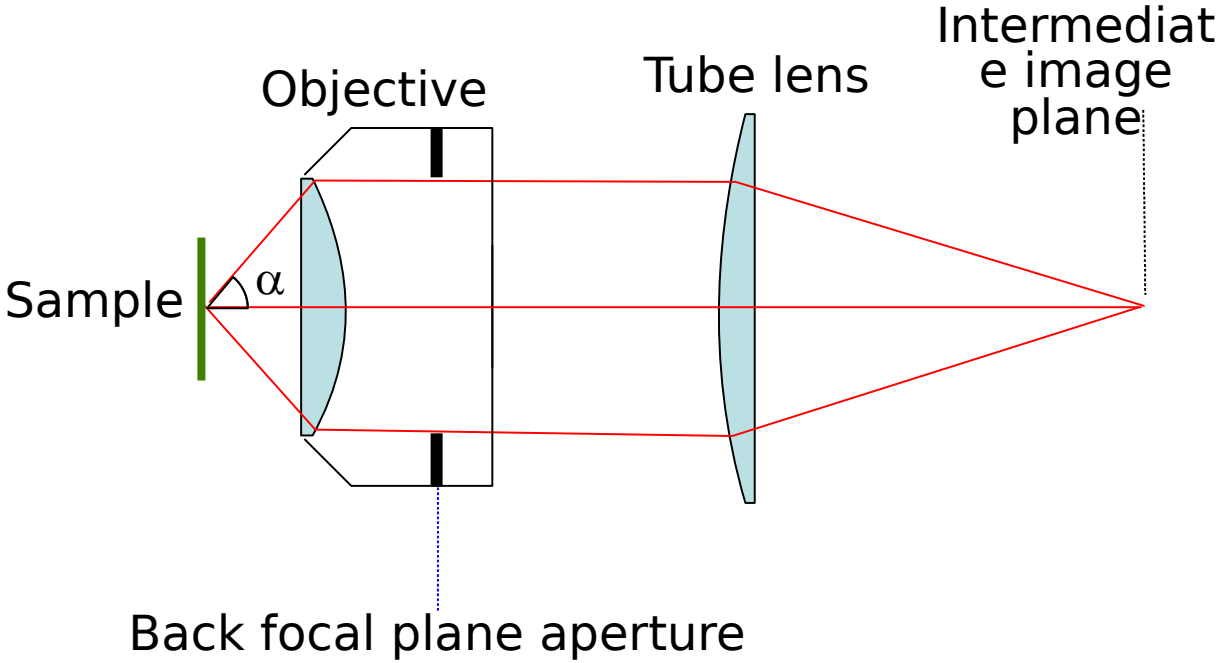
Aperture and Resolution



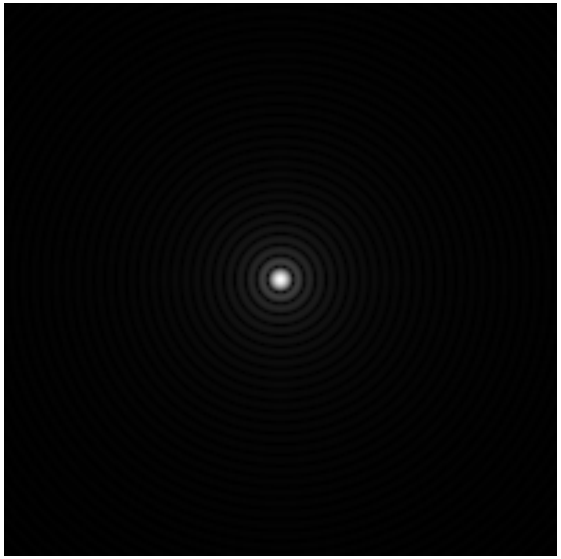
Diffraction spot
on image plane
= ***Point Spread***
Function



Aperture and Resolution



Diffraction spot
on image plane
(resolution)

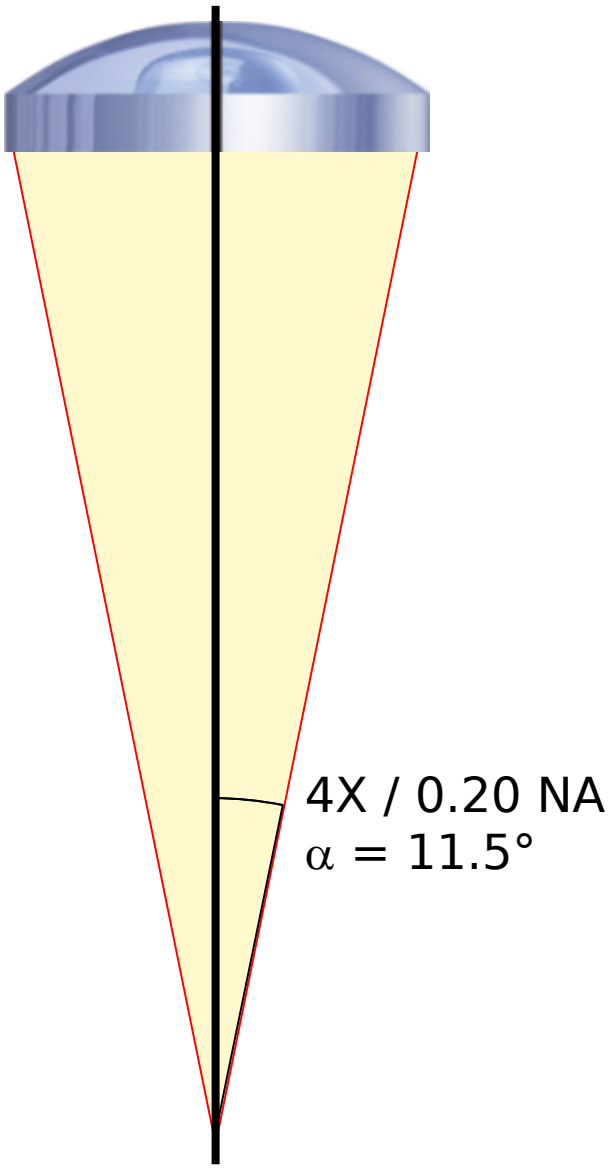


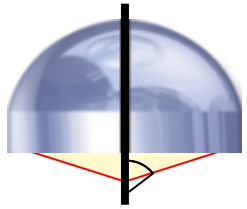
- Image resolution improves with ~~aperture size~~ Numerical Aperture (NA)

$NA = n \sin(\alpha)$

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

Numerical Aperture



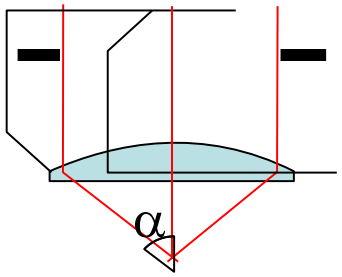


100X / 0.95 NA
α = 71.8°

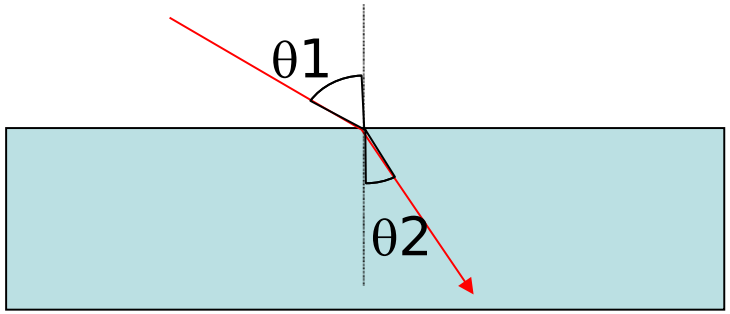
Numerical Aperture

Compare:

Numerical Aperture:
NA = n sin(α)

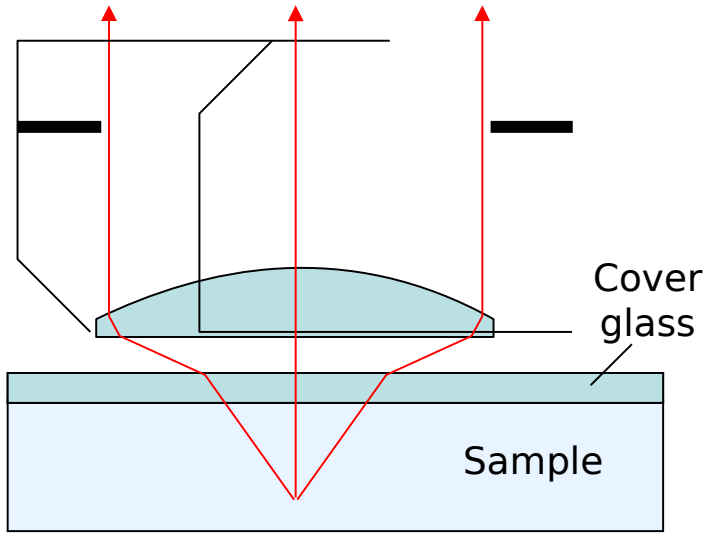


Snell's law:
n1 sin(θ1) = n2 sin(θ2)



- n sin(θ) doesn't change at horizontal interfaces
- sin(anything) ≤ 1

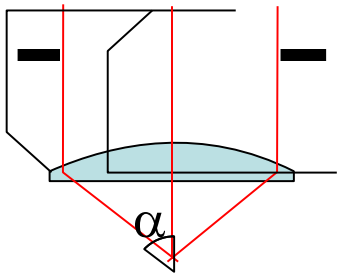
⇒ NA cannot exceed the *lowest* n between the sample and the objective lens



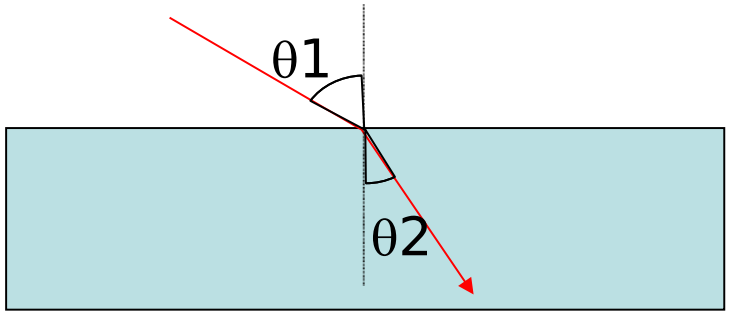
Numerical Aperture

Compare:

Numerical Aperture:
NA = n sin(α)



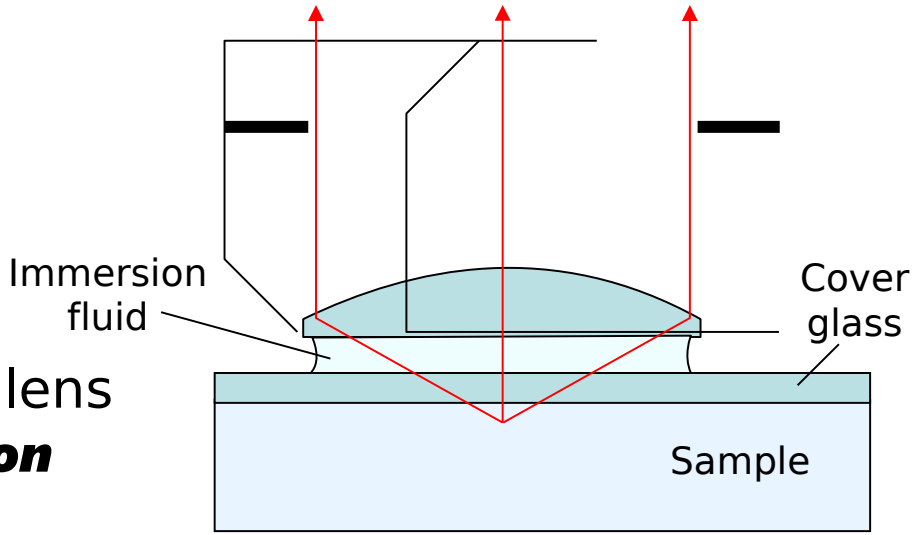
Snell's law:
n1 sin(θ1) = n2 sin(θ2)



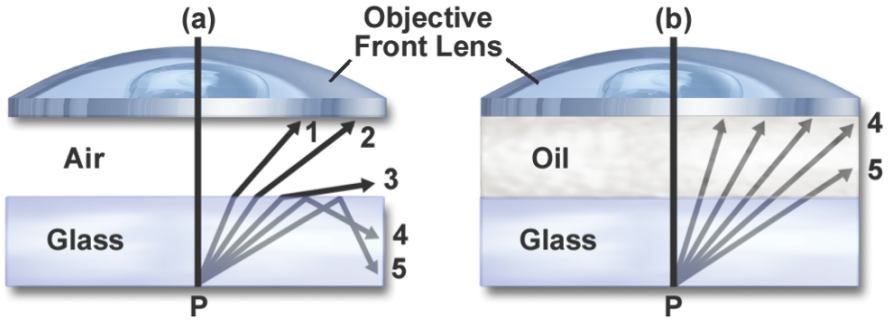
- n sin(θ) doesn't change at horizontal interfaces
- sin(anything) ≤ 1

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

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



Immersion Objectives



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%)

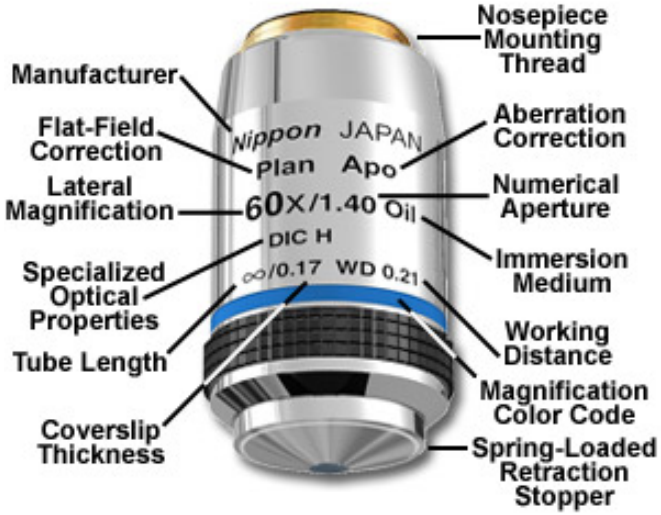
max NA ≈ **1.35** (Leica)

Water immersion:

n ≈ 1.33

max NA ≈ **1.2**

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