Principles & Practice of Light Microscopy 1

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

Principles and Practice of Light Microscopy

- Reading material
- Lectures
- Lab projects and report (oral & written)
- Journal Club (advanced topics)

Reading materials

Douglas Murphy, *Fundamentals of Light Microscopy and Digital Imaging*

M.W. Davidson1 & M. Abramowitz OPTICAL MICROSCOPY

www.microscopy.fsu.edu/primer/index.html

Giorgio Carboni, Fun Science Gallery

funsci.com/fun3_en/lens/lens.htm

VIDEO MICROSCOPY – 2nd Ed. **S. Inoue and K.R. Spring** **Plenum Press, NY 1997**

Web sites

micro.magnet.fsu.edu [Davidson & Abramowitz]

www.microscopy.fsu.edu

www.microscopyu.com [NIKON]

probes.invitrogen.com/resources/spectraviewe

http://microscope.fsu.edu/primer/anatomy/numaperture.html

http://micro.magnet.fsu.edu/primer/java/infinityoptics/magnification/index.html

www.cyto.purdue.edu/flowcyt/educate/pptslide.htm [CONFOCAL]

http://www.chroma.com/handbook.html [CHROMA – FILTERS]

Lectures

L-1: Properties of light: ray optics, reflection, refraction. Optical image formation. Microscope anatomy: Objective, Ocular, Upright/Inverted. Illumination. Geometrical-to-wave optics.

L-2: Resolution.

L-3: Contrast: Phase, DIC, darkfield, polarization.

L-4: Fluorescence: principles, probes, filters, sources, detectors, the biology. .

L-5: Special techniques: TIRF, FRET, FRAP, photo-activation, FLIP, FLIM, FCS, , single molecule microscopy, optical tweezers, X-RAY MICROSCOPY, AFM.

L-6: Scanning Confocal, spinning disk, multi-photon, second/third harmonic generation, coherent anti-Stokes Raman microscopy (CARS).

If time left and there is interest:

L-7: Advanced techniques: Deconvolution, 4Pi, SI, SPIM, PALM/FPALM, STORM STED.

L-8: Quantitative Analysis of Microscope Images

- Journal Club

Life-time imaging

Molecular motors (Block, Vale) nanopositioning

Tweezers

Z super-resolution by PSF correlation (Ben Simon)

Structured illumination

PALM [single, dual color, 2D, 3D] (Betzig et al.)

TIRF. Single-molecules imaging

STED (Hell et al.)

SPIM (Stelzer et al.)

Correlative Microscopy [EM+Light]

The Light Microscope

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

Landmarks in the History of Microscopy

Interdisciplinary step-by-step progress in science

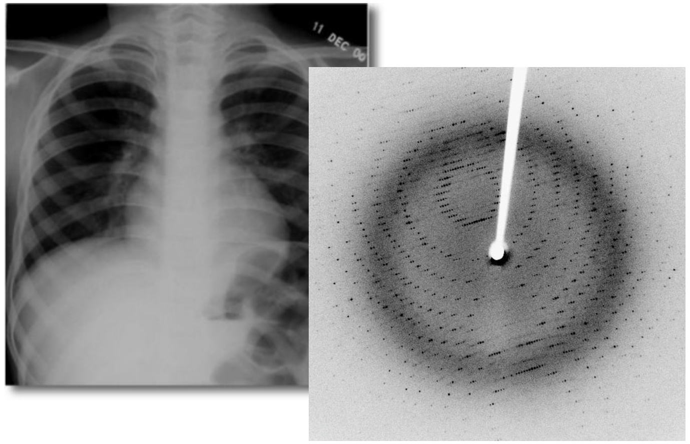
1900BC Egyptians use for cosmetics flat and spherical mirrors
Phoenicians Spherical glasses filled with water magnify
Greece Tales, 600BC, leave "cells" through morning due droplets
Alexandria school: +/-200C, Euclid, Hero and Ptolemy optics book
Middle age Arab scholars: Ibn al-Haytham (Alhazen) physical nature of light
1590 Zacharias Janssesn, Holland, builds two-lens microscope
1611 Kepler builds telescopes, suggests microscopes
1655* Hooke microscope - cork "cells"
1674* Leeuwenhoek use 1.5mm glass sphere magnifiers - protozoa
1683* Leeuwenhoek sees bacteria
1733 Chester Hall use doublets to correct chromatic aberration
1830 Airy, diffraction rings in star images
1833* Brown, nucleus in orchids
1838* Schleiden & Schwann cell theory
1876 Abbe's theory of diffraction in light microscopy
1879* Flemming, mitotic chromosomes
1881* Cajal use stains to see tissue anatomy
1882* Koch, microbiology (Cholera, Tubercolosis)
1886 Zeiss and Abbe design and build a diffraction limited microscope

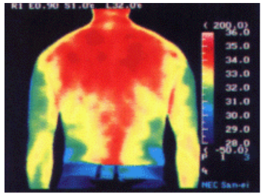
1898	Golgi use silver nitrate staining to see “his” apparatus
1924	Lacassagne use Marie Curie’s radium in Autoradiography
1924	de Brogli, electron’s wave character
1930	Lebedeff, interference microscope
1931*	Ruska, transmission EM. Commercialized: 1939 (Siemens)
1932*	Zernike, phase contrast microscope-> Cells in culture.
1941*	Coone, fluorescence microscopy
1945*	Porter, cells fixed in Osmium. Palade: organelles. Huxley: muscles
1952	Nomarski, Differential Interference Contrast (DIC)
1968	Gabor, lasers
1975	Ploem “pack”: excitation emission and dichroic filters
1977-80	Sheppard, Brakenhoff & Koester, scanning confocals
50’s	TV technology develops
70’s	Digital image processing
1981	Allen & Inoue, Video-enhanced microscopy
1983	Sedat & Agard 3D microscopy using “wide field” + deconvolution
1985	Boyde, Kino Nipkow-disk tandem confocal (spinning disk)
80’th	Scanning laser confocals
90’s	Near field, Tunneling and Atomic force microscopy. Below λ
80’s	pSec pulsed lasers
1997*	Webb, two photon confocal
2000-	Break the Abbe reolution limits: PALM, STED, SI

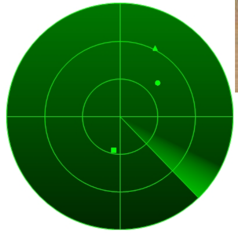
אין מוקדם ומאוחר בתורה...

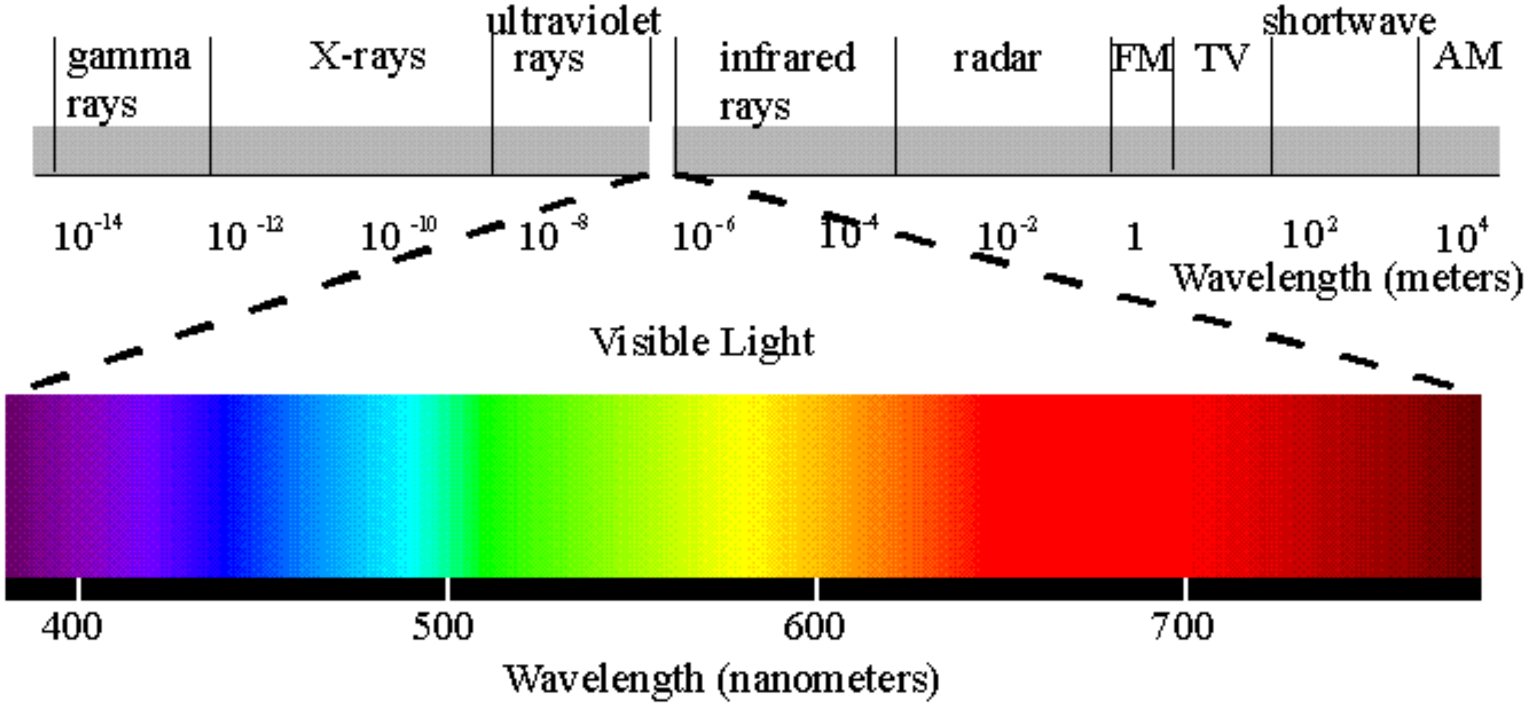
Some Q are asked before time,
But all can be answered at the end

Electromagnetic Waves









Q: why not use radar for microscopic imaging?

TYPES OF MICROSCOPES

* **Light (UV, visible, IR)**
* Raman
* Electron (SEM, TEM)
* X-Ray
* Near-field scanning microscopy
  - scanning tunneling
  - atomic force
  - near field optical

Light Microscopes

- Research Microscopes (cells, embryos, tissue sections)
- Tissue Culture Microscopes
- Stereoscopic Dissection Microscopes
- MacroScopes
- FiberScopes

Glossary of Microscope Modalities

SDM	Spinning Disk Microscopy	E. Boyde
3Decon	3D Deconvolution Microscopy	J.Sedat & D.Agard
LSCM	Laser Scanning Confocal Microscopy	Brakenhof
TIRF	Total Internal Reflection Fluorescence	D. Axelrod
SLEM	Selection Light and Electron Microscopy	
CLEM	Correlative Light and Electron Microscopy	
FCS	Fluorescence Correlation Spectroscopy	
FCCS	Fluorescence Cross-Correlation Spectroscopy	
RICS	Raster Scanning Correlation Spectroscopy	
FRAP	Fluorescence Recovery After Photobleaching	E. Elson
LSFM	Light Sheet Fluorescence Microscopy	
SPIM	Selective Plane Illumination Microscopy	E. Stelzer
DSLM	*** Microscopy	
FRET	Fluorescence (Forster) Resonance Energy Transfer	
FLIM	Fluorescence Life-Time Imaging	T. Jovin
BRET	Bio-Illumination Resonance Energy Transfer	
FUEL	Fluorescence by Unbound Excitation from Luminescence	
2P (2PE,3P)	Two-Photon / Multi-Photon Excitation Microscopy	W.W.Web
SPT	Single Particle Tracking	S. Block, R. Vale
SI (SIM)	Structured Illumination Microscopy	M. Gustafsson
PALM	Photo-activated Localization Microscopy	E. Betzig
STED	Stimulated Emission Depletion	S. Hell
STORM	Stochastic Optical Reconstruction Microscopy	S. Hell
RSFP	Reversible Switchable Fluorescence Proteins	

OVERVIEW

- Properties of light
- Optical image formation
- Microscope anatomy

Waves vs. Photons vs. Rays

- Quantum wave-particle duality
- EM field ≈ collective wave function for the photons
- Light intensity ∝ photon flux ∝ | field $|^2$
- Rays: photon trajectories
- Rays: propagation direction of waves

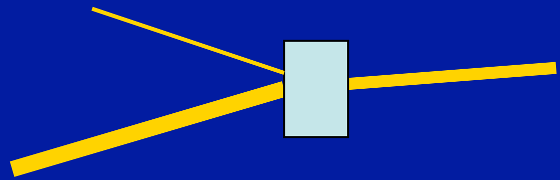
Modes of light interaction with matter

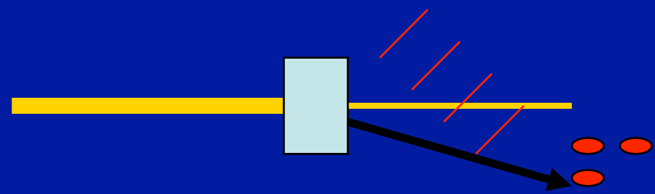
Rays	Waves	Particle Nature
Reflection	Interference	Absorption
Refraction	Diffraction	Scattering
	Polarization	Fluorescence

Interaction of Light with Matter

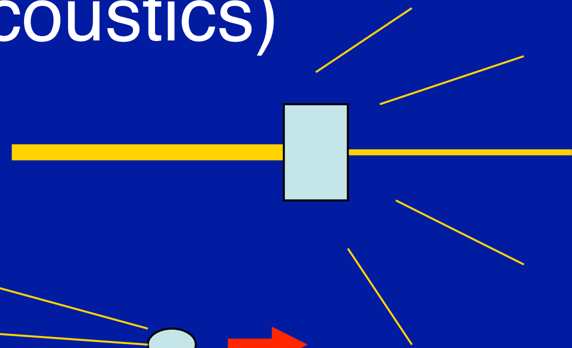
every mode has applications in microscopy.

Reflection
Refraction
Absorption

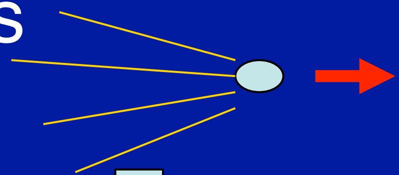




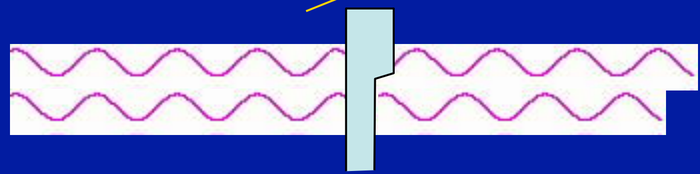
- light-to-chemistry (photobleaching/photoactivation)
- light-to-heat (absorption, photoacoustics)
- light-to-light: e.g. fluorescence

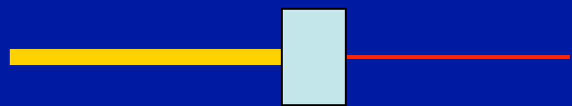


Scatter, diffraction
Radiation pressure: optical tweezers



Phase Shift - interference
Color Shift - fluorescence





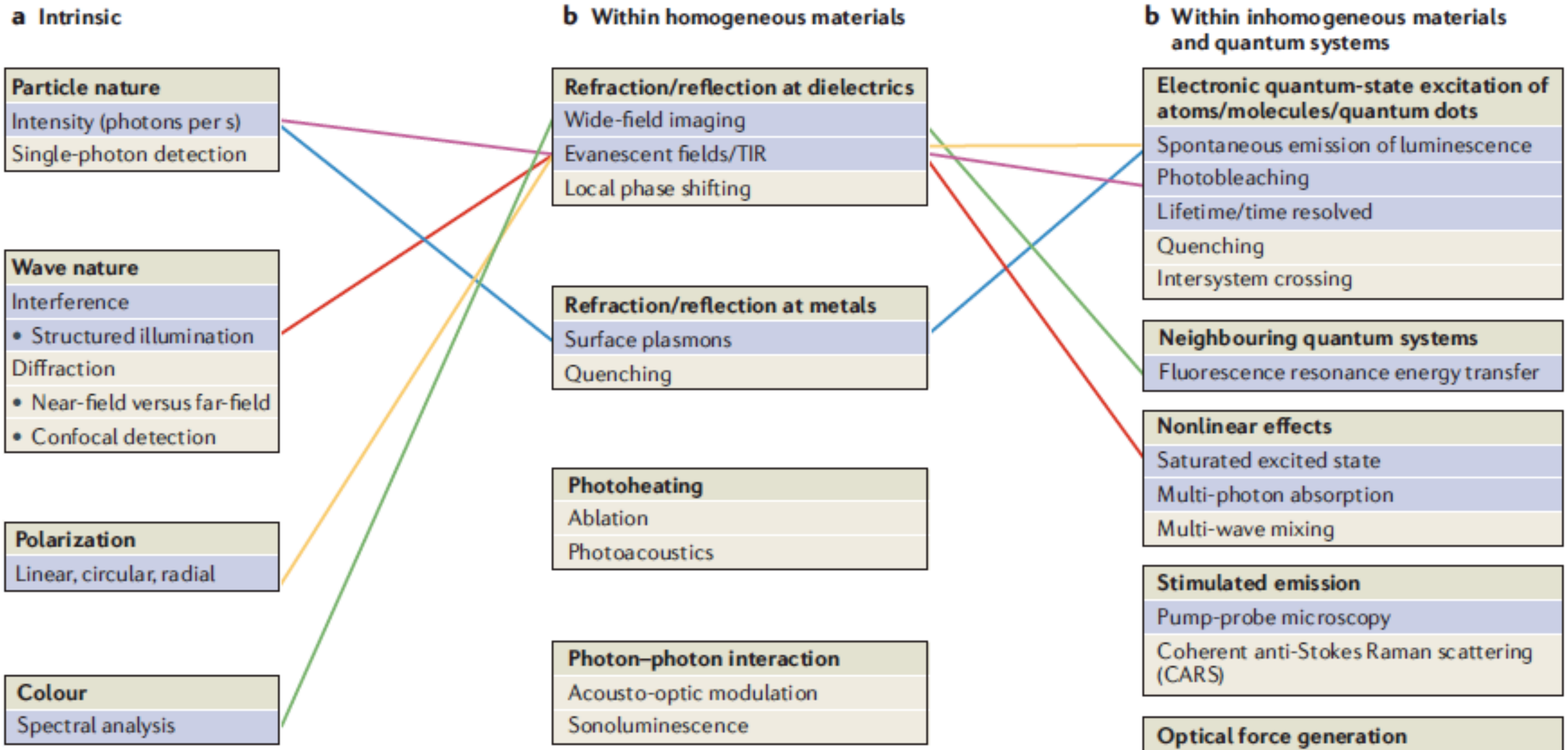
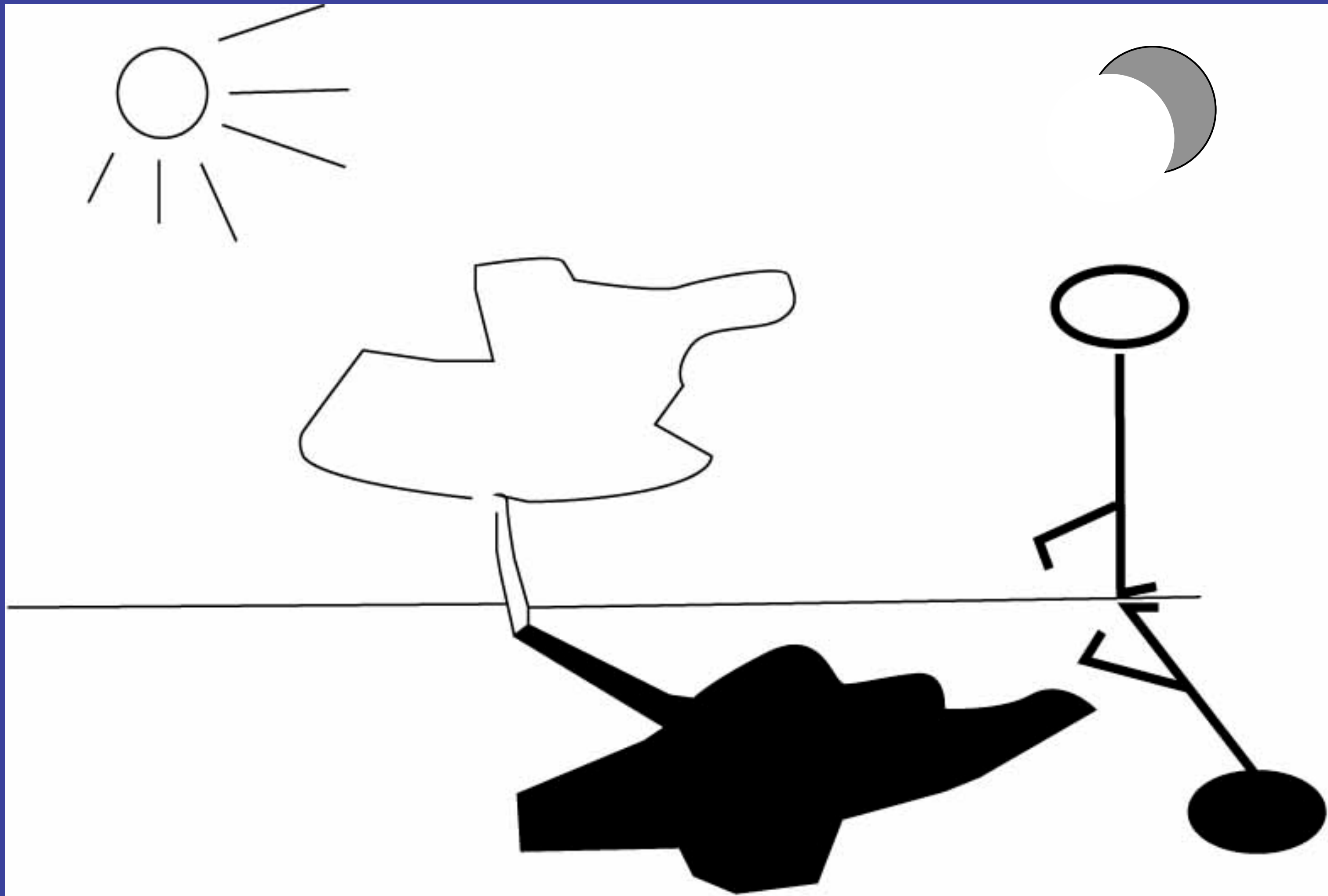


Figure 1 | **Combinations of fundamental properties of photons.** The properties of photons can be classed in three categories: (**a**) intrinsic properties of photons 'in vacuum' (that is, disregarding the material through which they travel); (**b**) properties observed in interactions with homogeneous materials (that is, materials with spatial variations that are larger than the wavelength of light); and (**c**) properties observed in interactions with material containing small inhomogeneities (that is, spatial variations at the size scale of the wavelength of light or smaller). The photon properties that are discussed in most detail in the main text are depicted in light blue. The properties that are used by the five combinations that are discussed in most detail in the main text are connected by coloured lines: nonlinear structured illumination total internal reflection fluorescence microscopy (TIRFM; red); surface-plasmon microscopy (SPM; blue); polarized TIRFM (yellow); TIR–FRAP (total internal reflection–fluorescence recovery after photobleaching) imaging microscopy (purple); and polarized fluorescence resonance energy transfer (pFRET) microscopy (green).

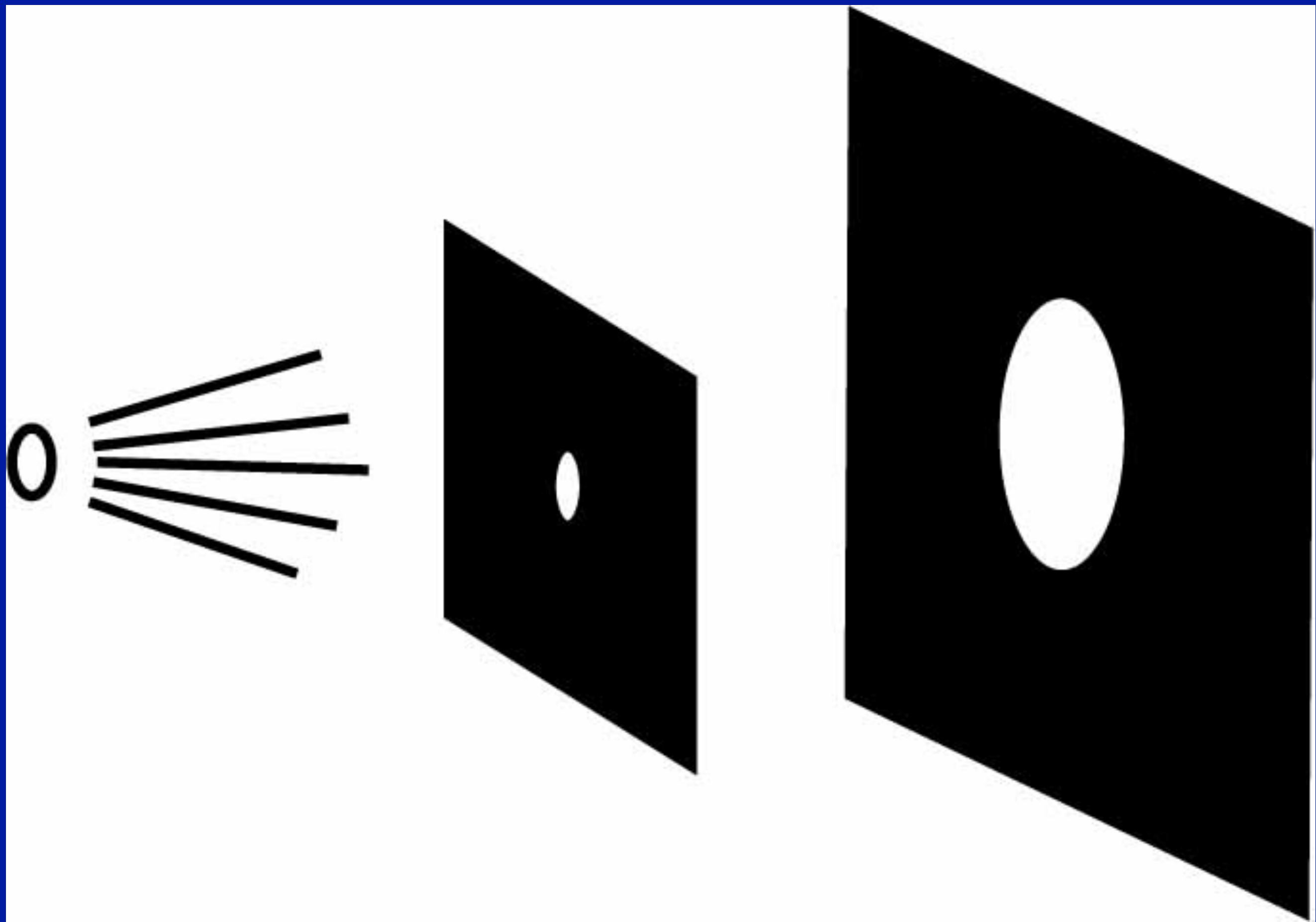
D. Axelrod

GEOMETRICAL OPTICS



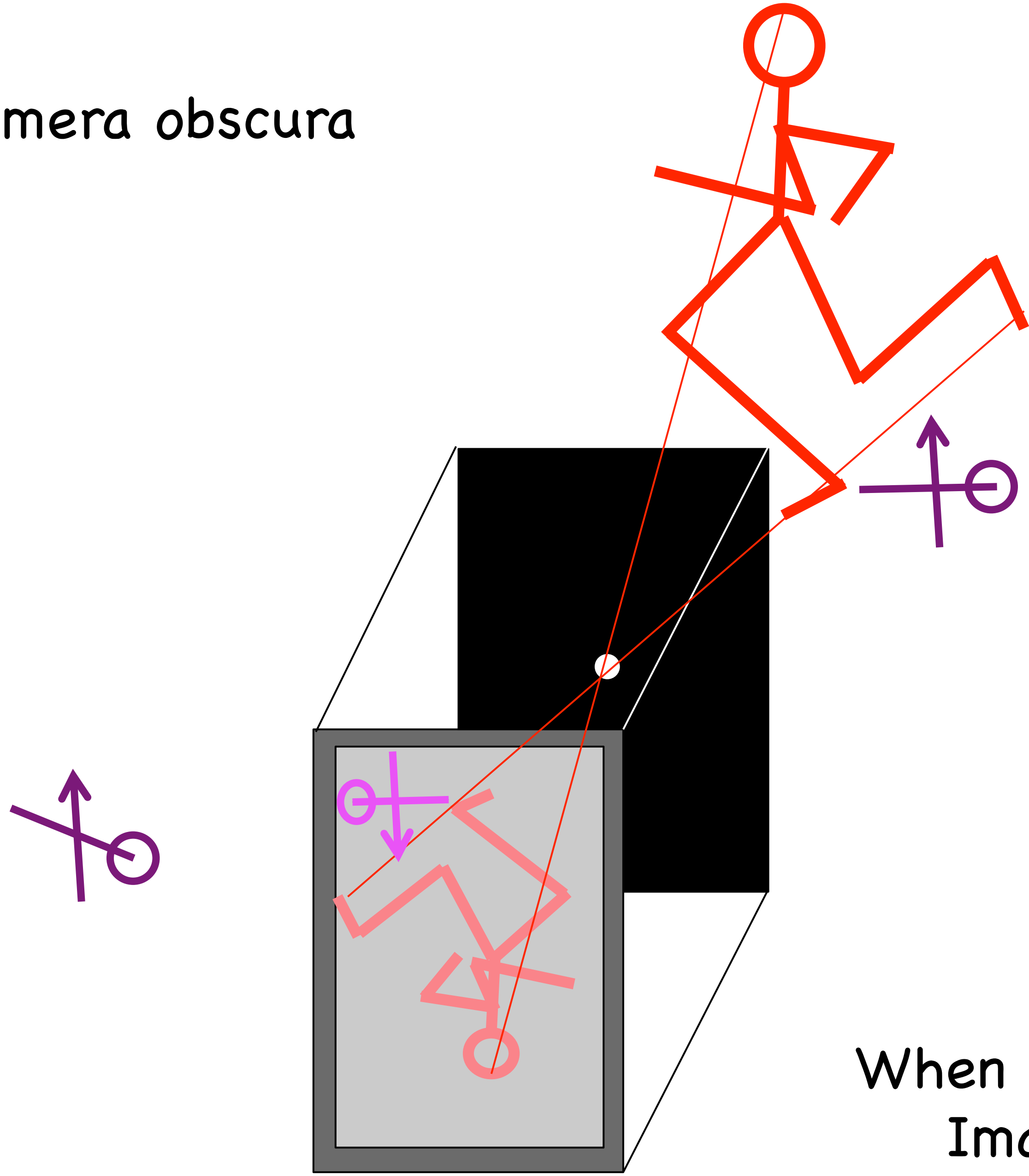
Rays go in straight lines: Shadows. Eclipse.

GEOMETRICAL OPTICS



Rays go in straight lines: round hole image is round
(always true?)
Q. How sharp is the image?

Camera obscura



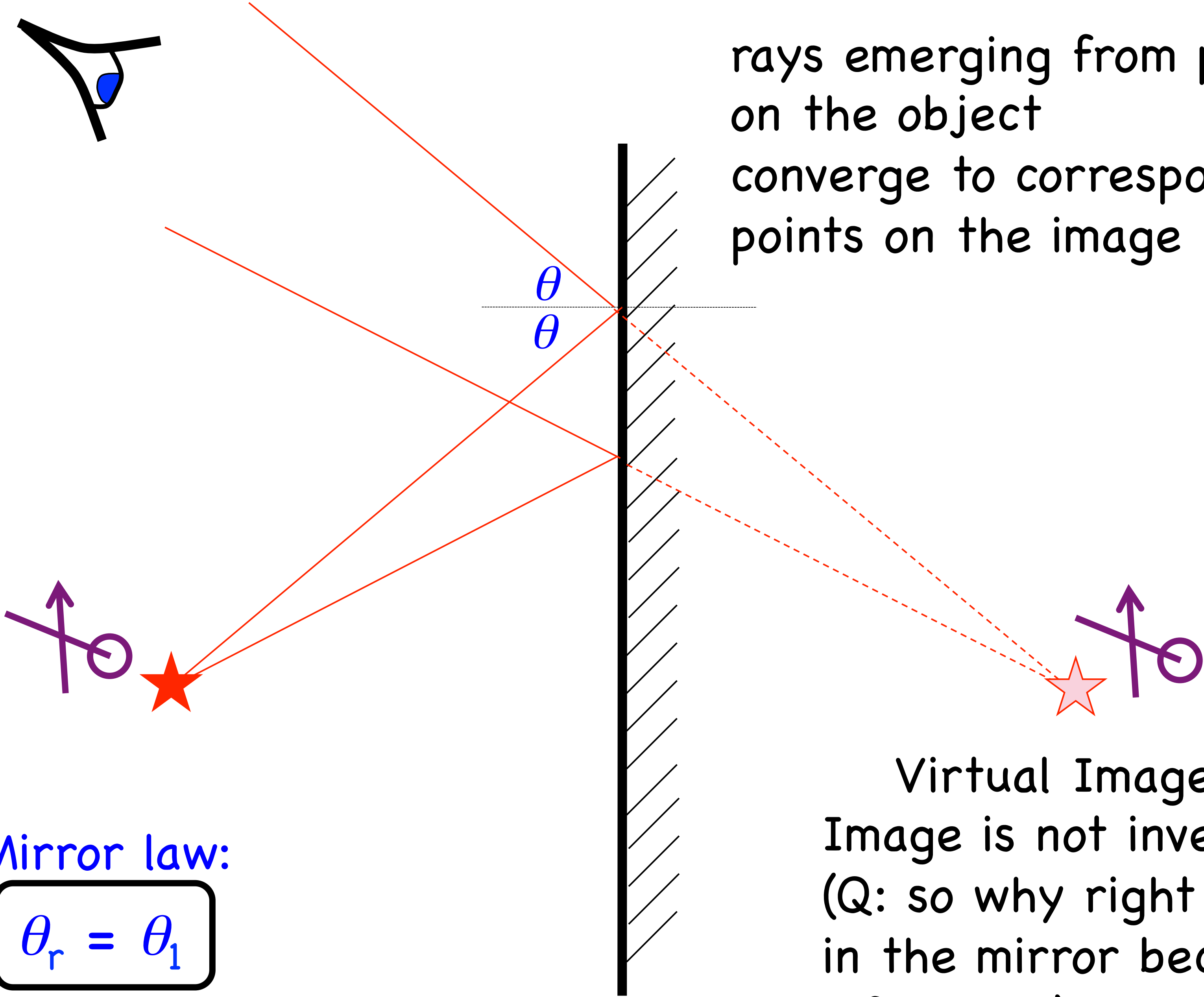
When pinhole is smaller:
Image is sharper
Image is dimmer

Image is inverted

Rays travel in straight lines

Unless…

Reflection in a flat mirror

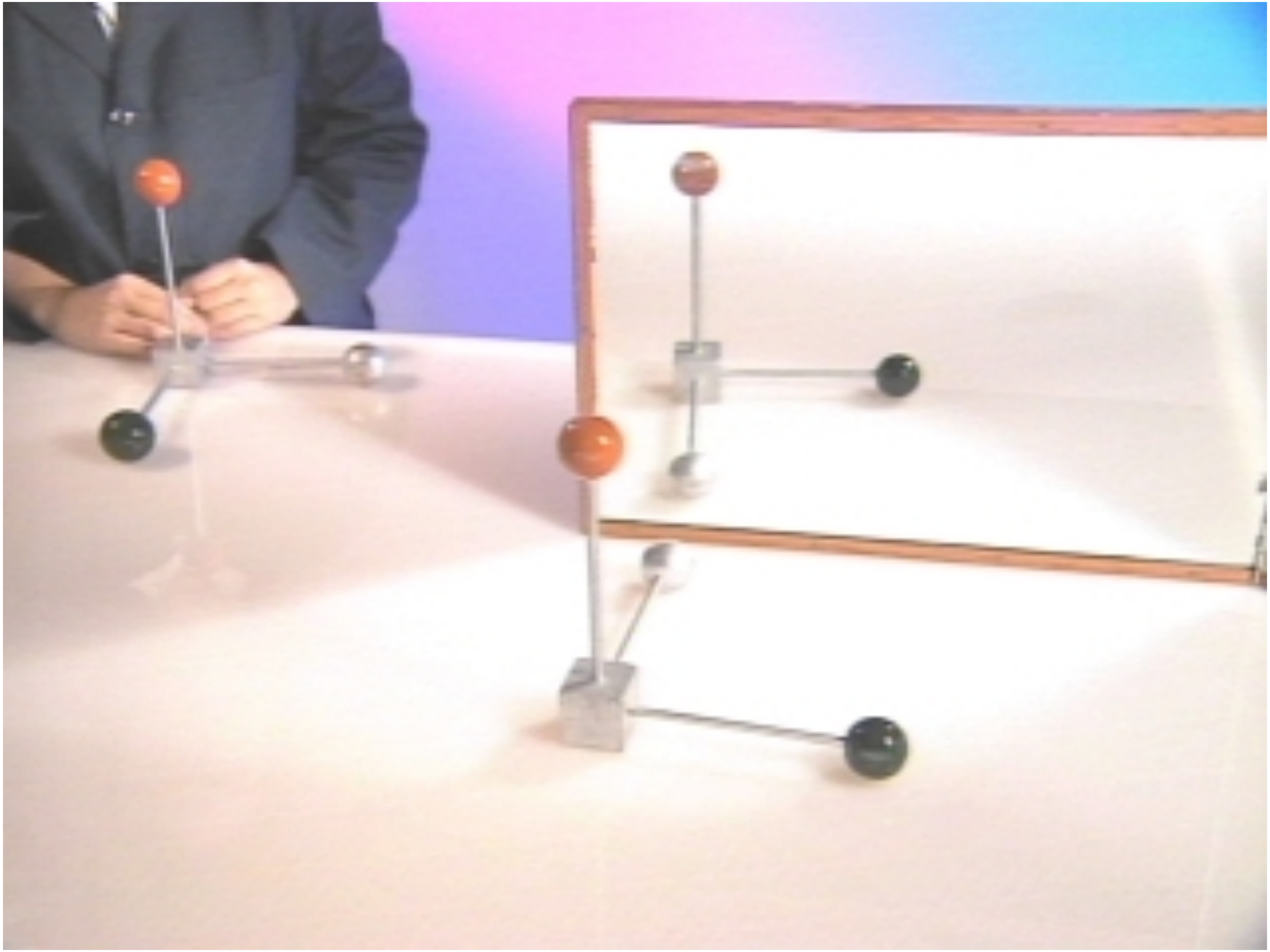


rays emerging from points on the object converge to corresponding points on the image

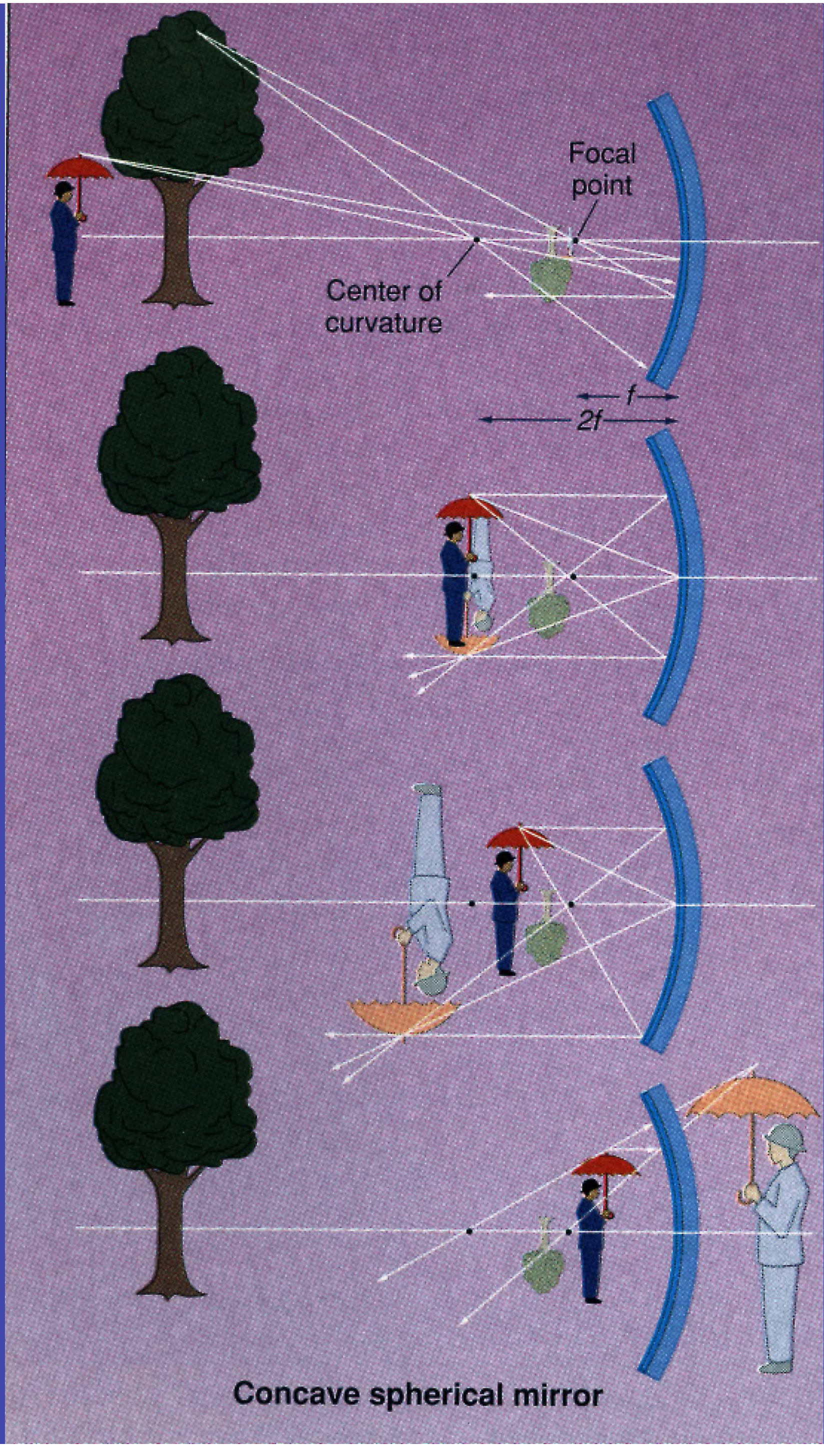
Virtual Image
Image is not inverted
(Q: so why right hand in the mirror becomes left hand?)

Mirror law:

$$\boxed{\theta_r = \theta_1}$$



REFLECTION
In a concave mirror



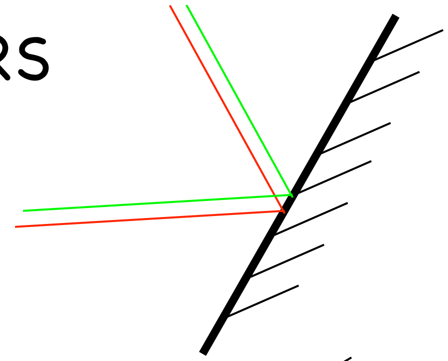
Something happens on the way through the Focus:

The image ran to infinity, and returned from the other side.

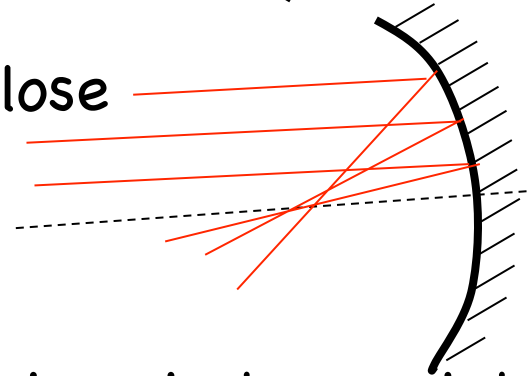
The image: where rays meet.

[Shaving mirror]

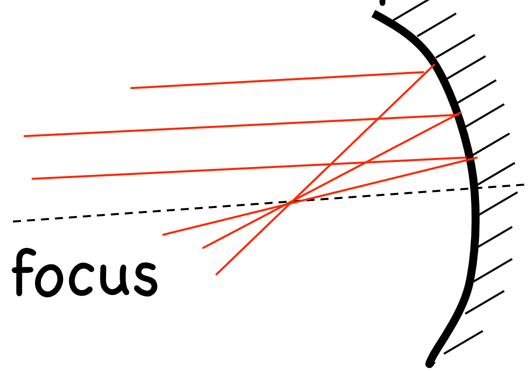
SOME INTERESTING FACTS ABOUT MIRRORS



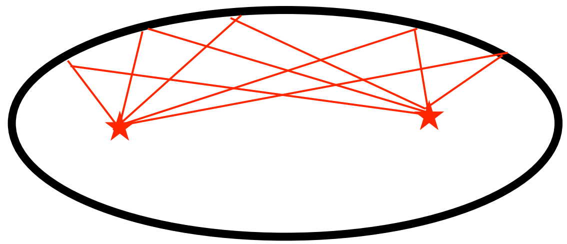
Mirrors have no chromatic aberrations
(all colors reflect at the same angle)



Spherical mirrors have sharp images for rays close to the axis, but rays at large angles "miss" the focus a bit



Parabolic mirrors project all rays parallel to their axis to a point (Telescopes use such mirrors) but cannot focus that well off axis



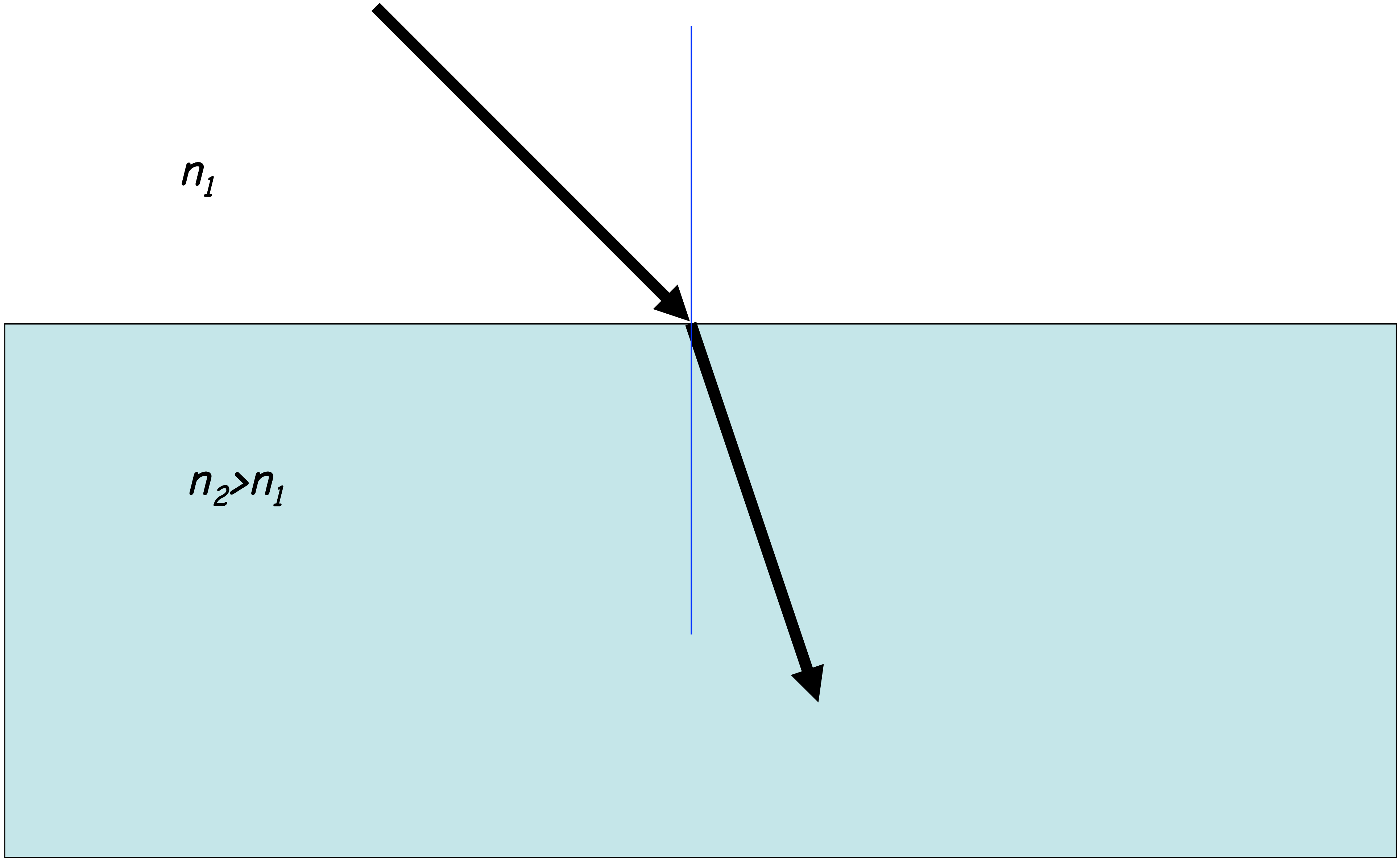
Elliptical mirrors image the first to the second focus

Q: Why not used (much) in microscopes?

Rays are reflected from mirrors

And create real and virtual images

Refraction

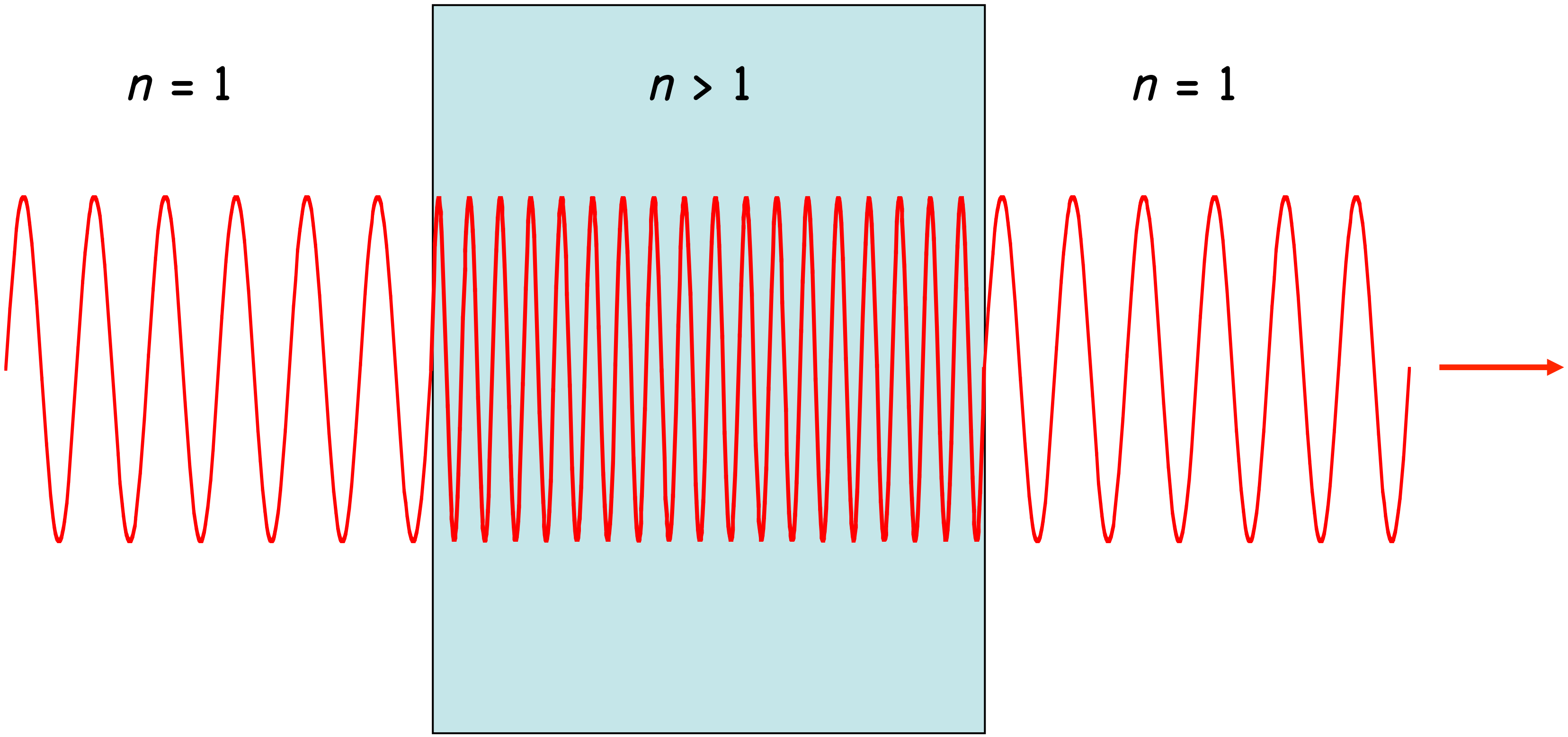


REFRACTION

Light travels more slowly in matter

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

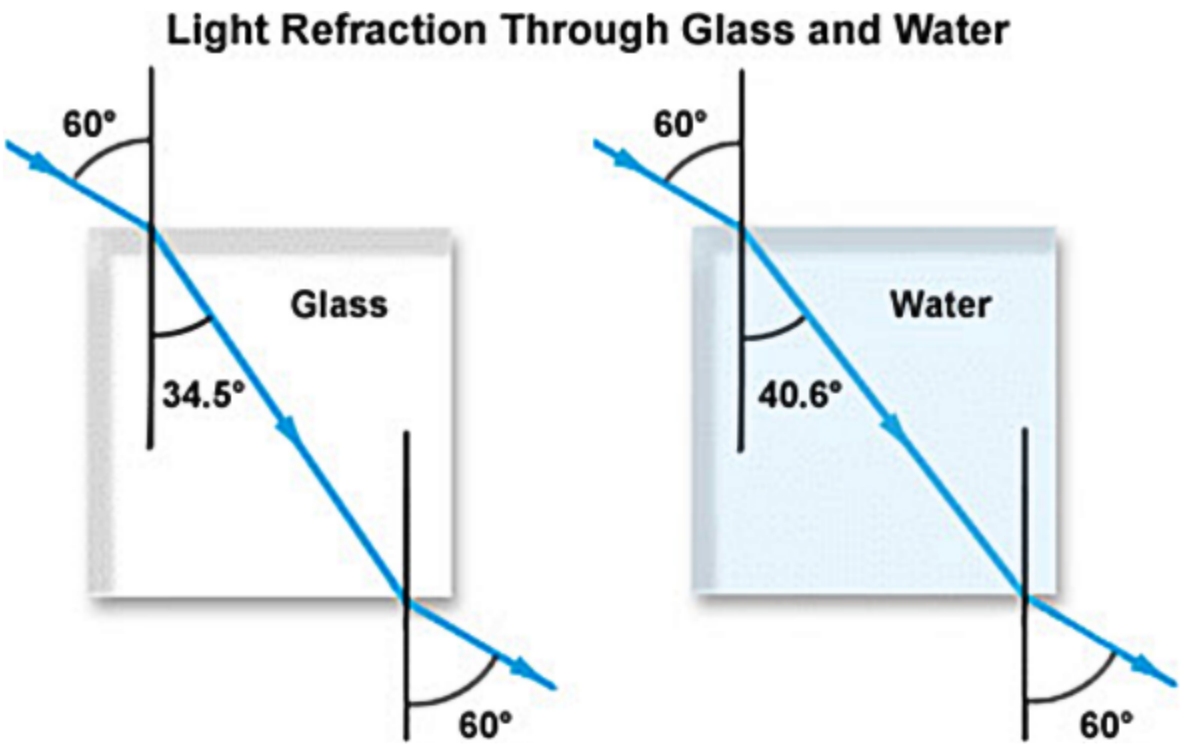
$$\boxed{v = c/n}$$



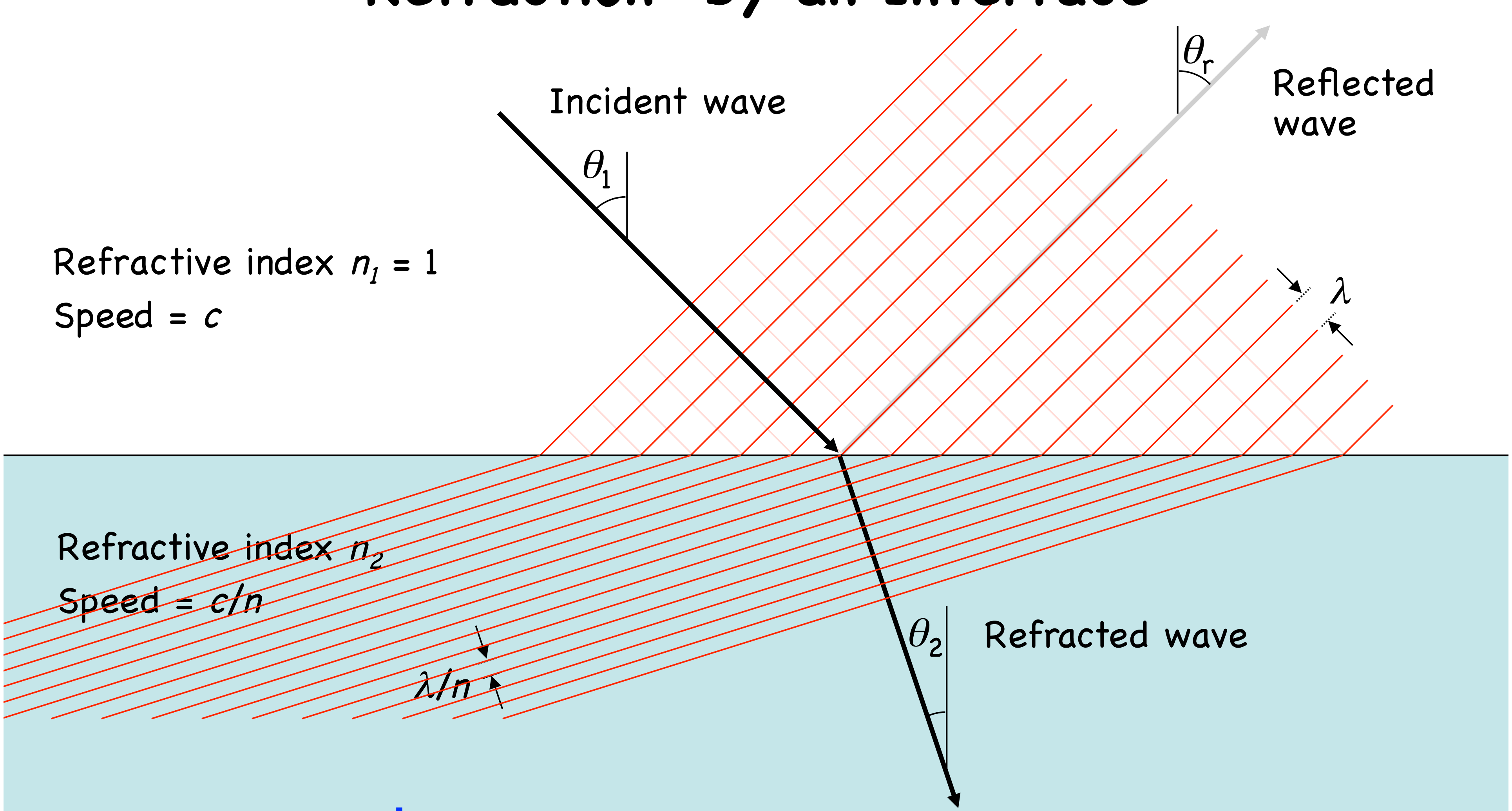
Refractive Index Examples

• Vacuum	1
• Air	1.0003
• Water	1.333
• Cytoplasm	~ 1.43
• Nucleus	~ 1.39
• 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


Light Refraction Through Glass and Water
60°
Glass
34.5°
60°
60°
Water
40.6°
60°

Refraction by an Interface



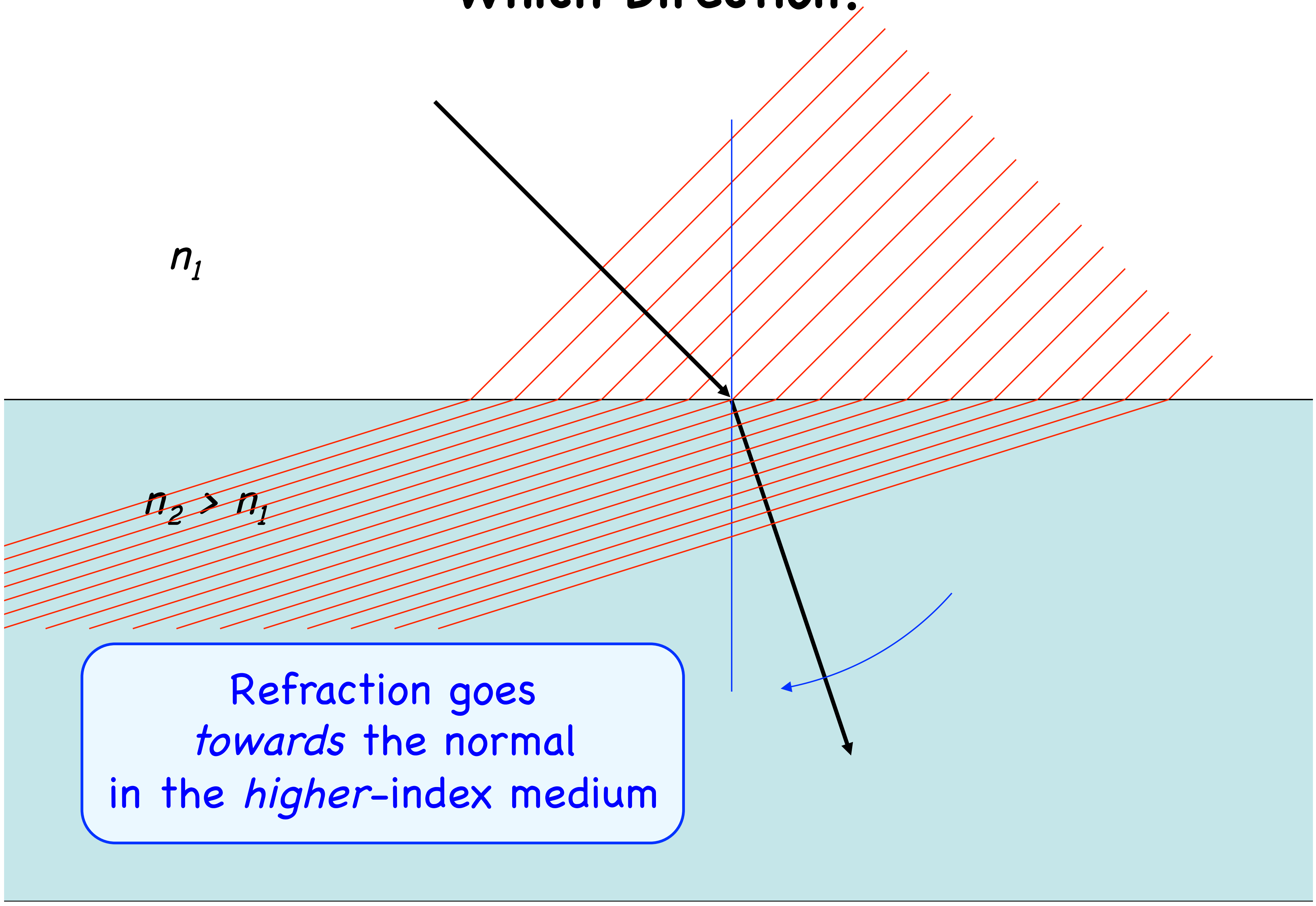
$\Rightarrow$ Snell's law:

$$\boxed{n_1 \operatorname{Sin}(\theta_1) = n_2 \operatorname{Sin}(\theta_2)}$$

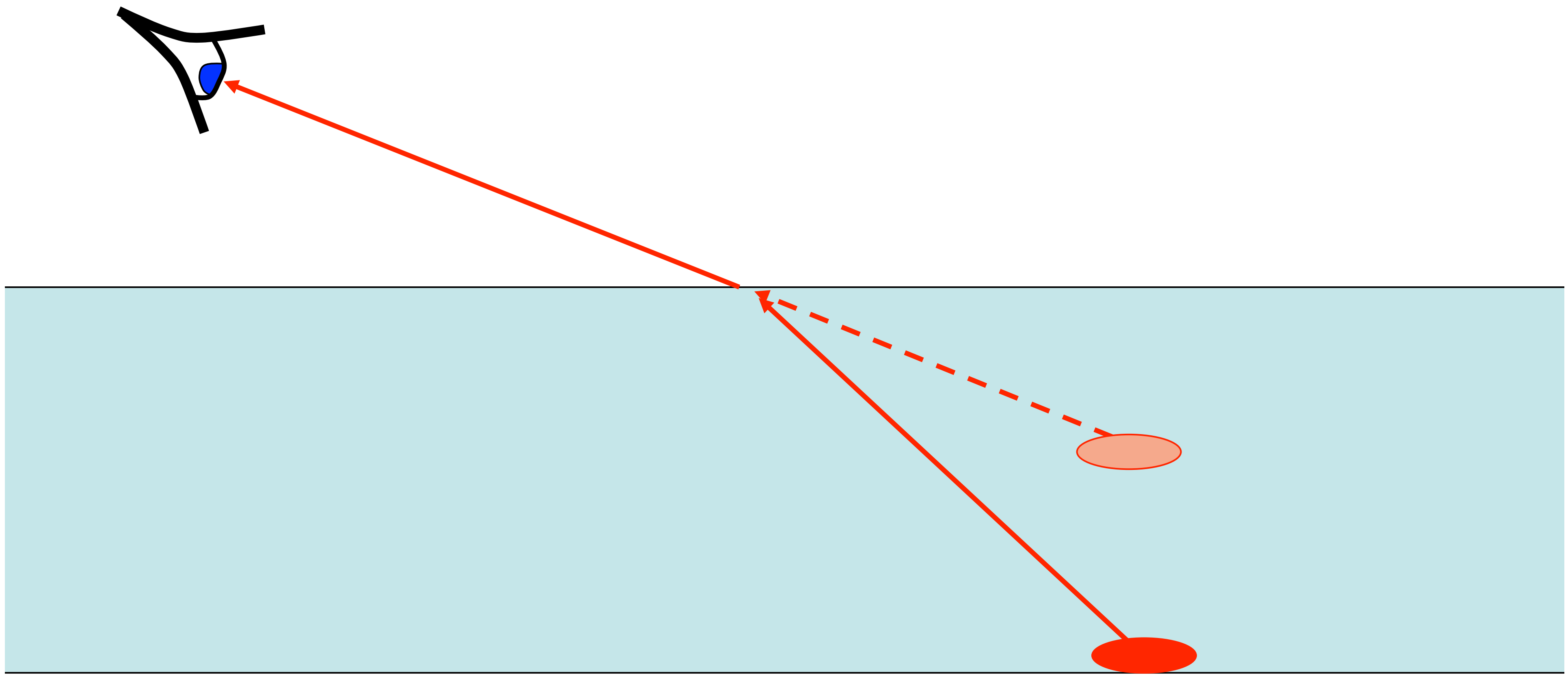
Mirror law:

$$\boxed{\theta_r = \theta_1}$$

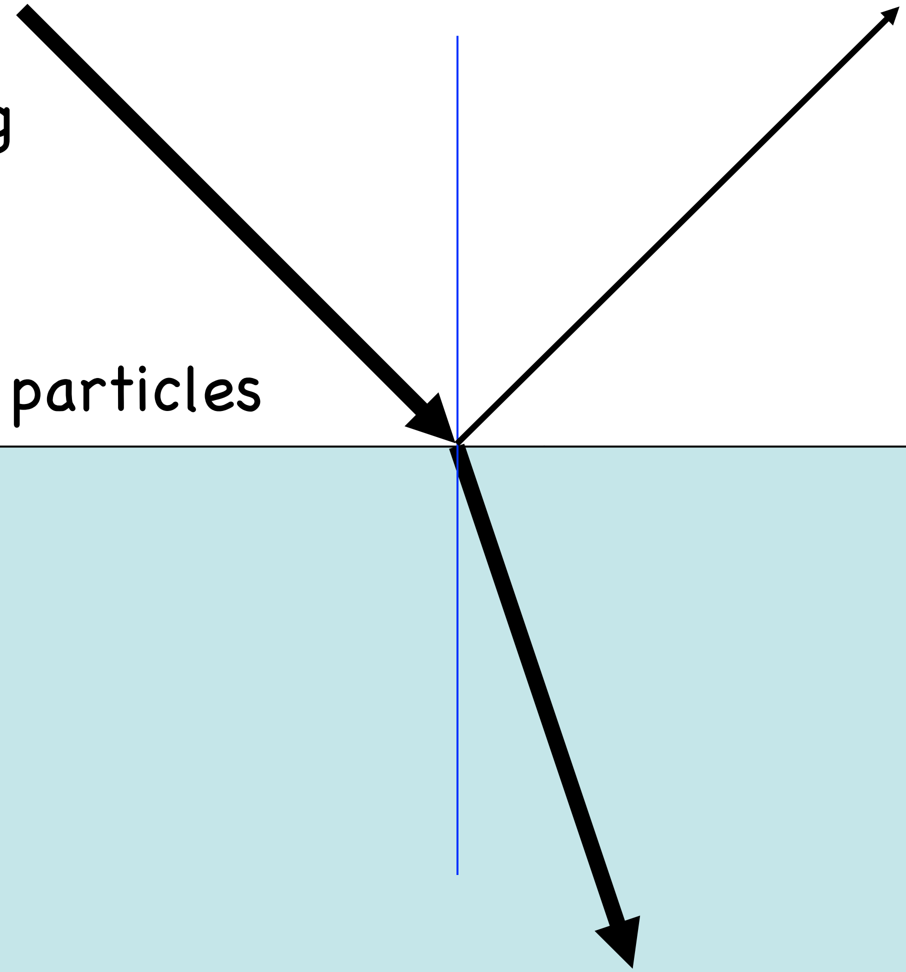
Which Direction?



Coin looks higher in the fountain than it really is



Refraction and reflection always together

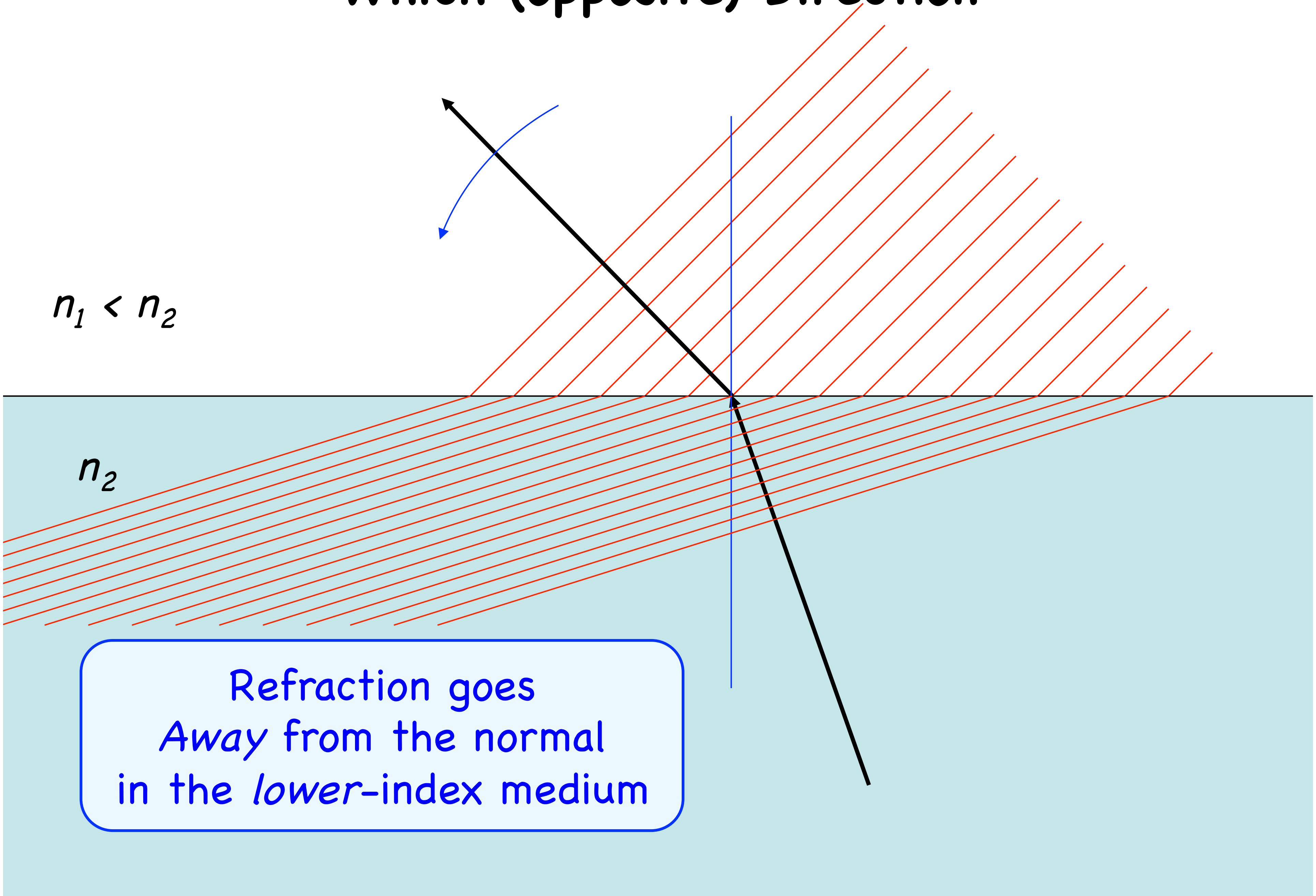


- antireflective coating
- Interference filters

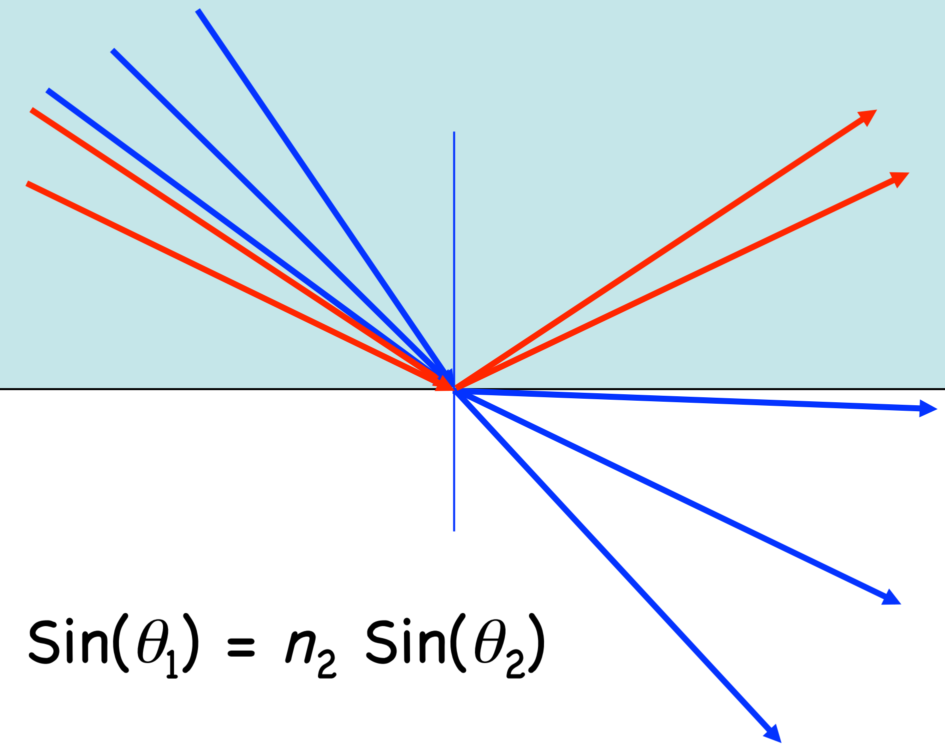
- Scattering by small particles

Q: If a typical high quality objective has 11 lenses, how much of the light will be transmitted through it?

Which (opposite) Direction



Total Internal Reflection



Snell's Law: $n_1 \, \text{Sin}(\theta_1) = n_2 \, \text{Sin}(\theta_2)$

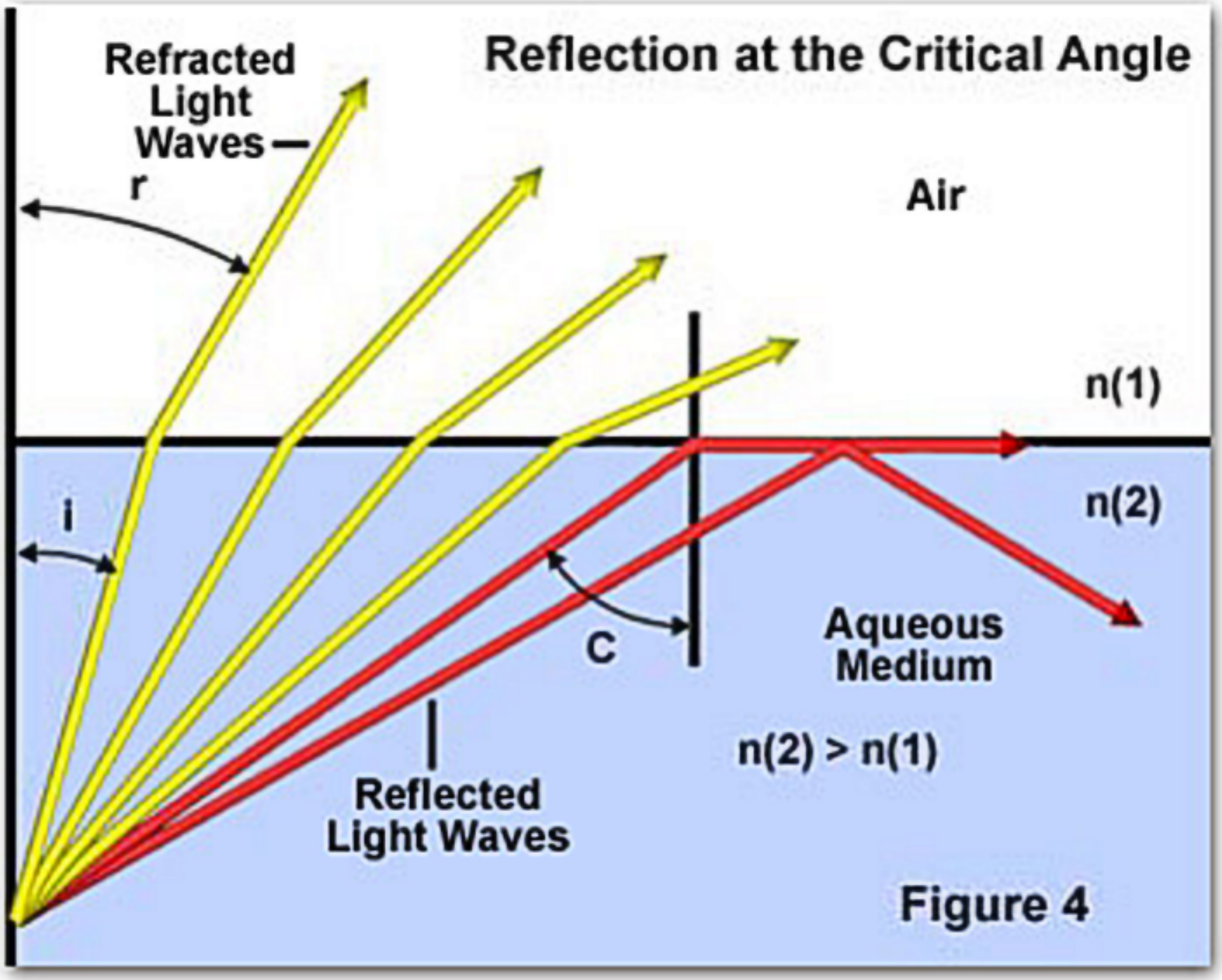
Beyond $n_2\text{Sin}(\theta_2) = n_1$, then $\text{Sin}(\theta_1)$ would have to exceed 1.

Impossible $\Rightarrow$ No light can be transmitted

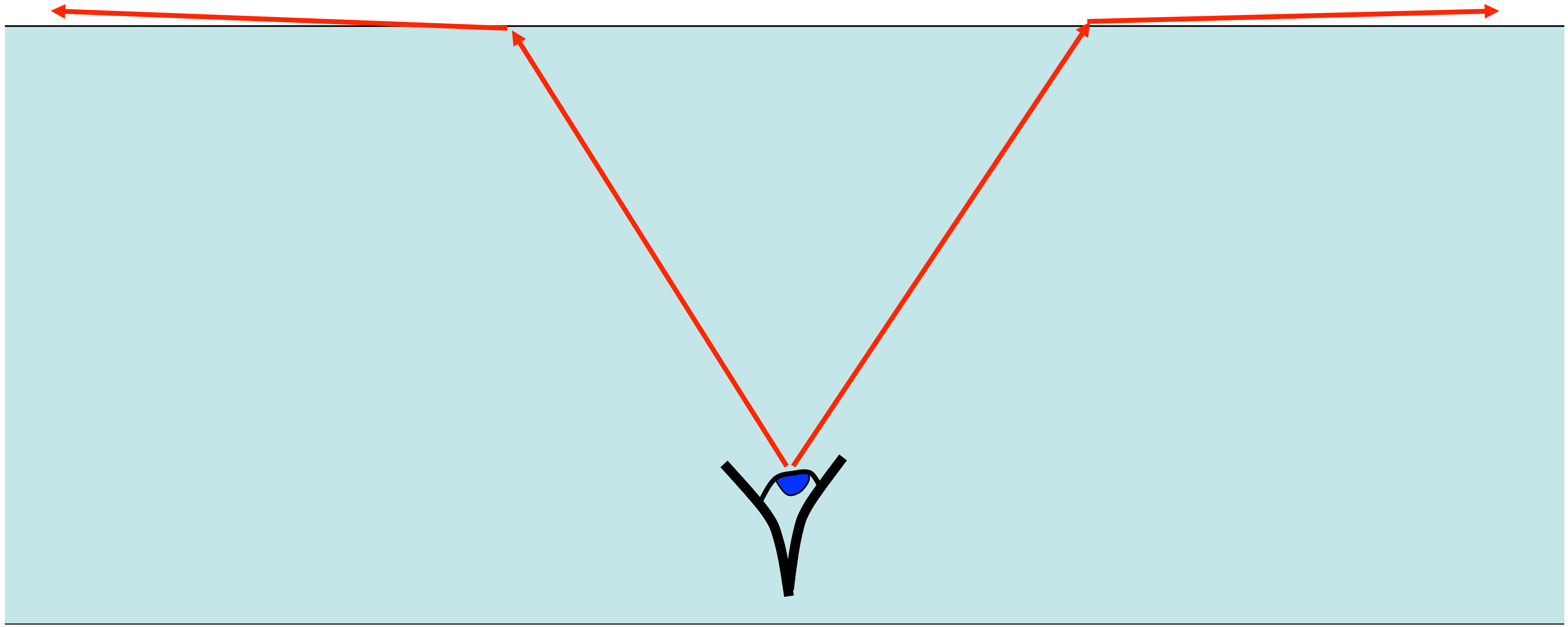
$\Rightarrow$ All is reflected: *Total internal reflection*

Happens only going *from high to lower* index medium

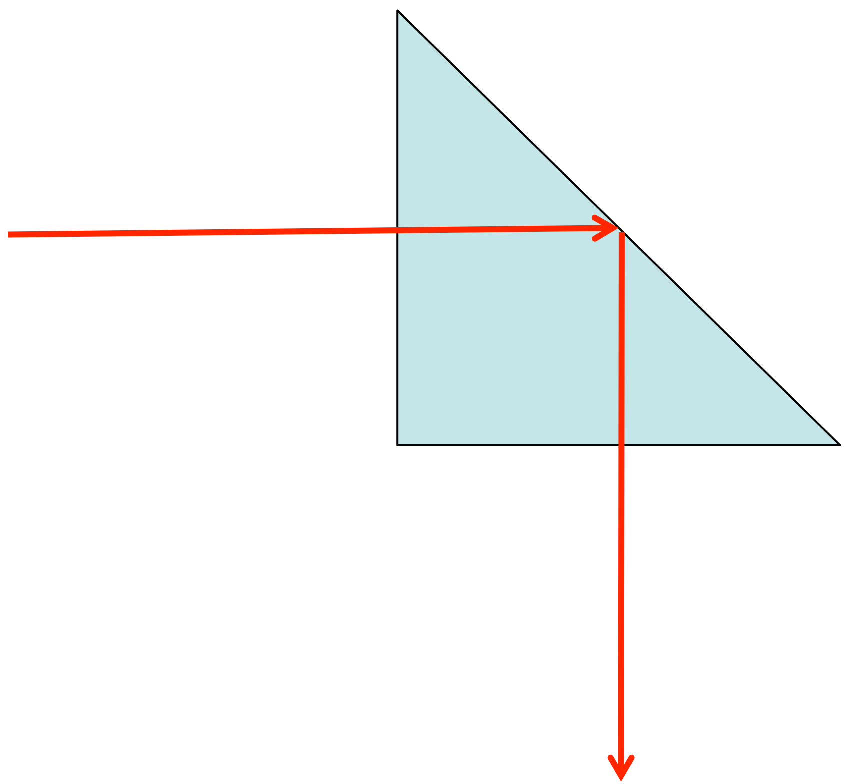
TOTAL INTERNAL REFLECTION



Horizon contracts to a cone looking up from under the water
(National Geographics underwater movies...)



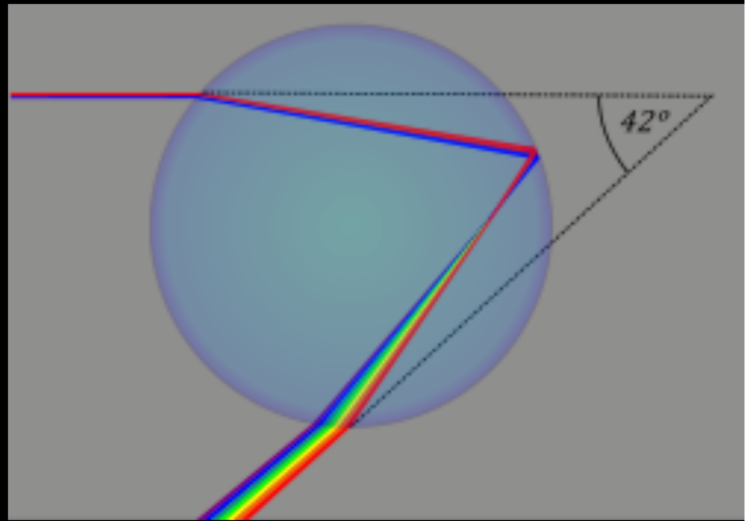
Prisms replace mirrors using total internal reflection



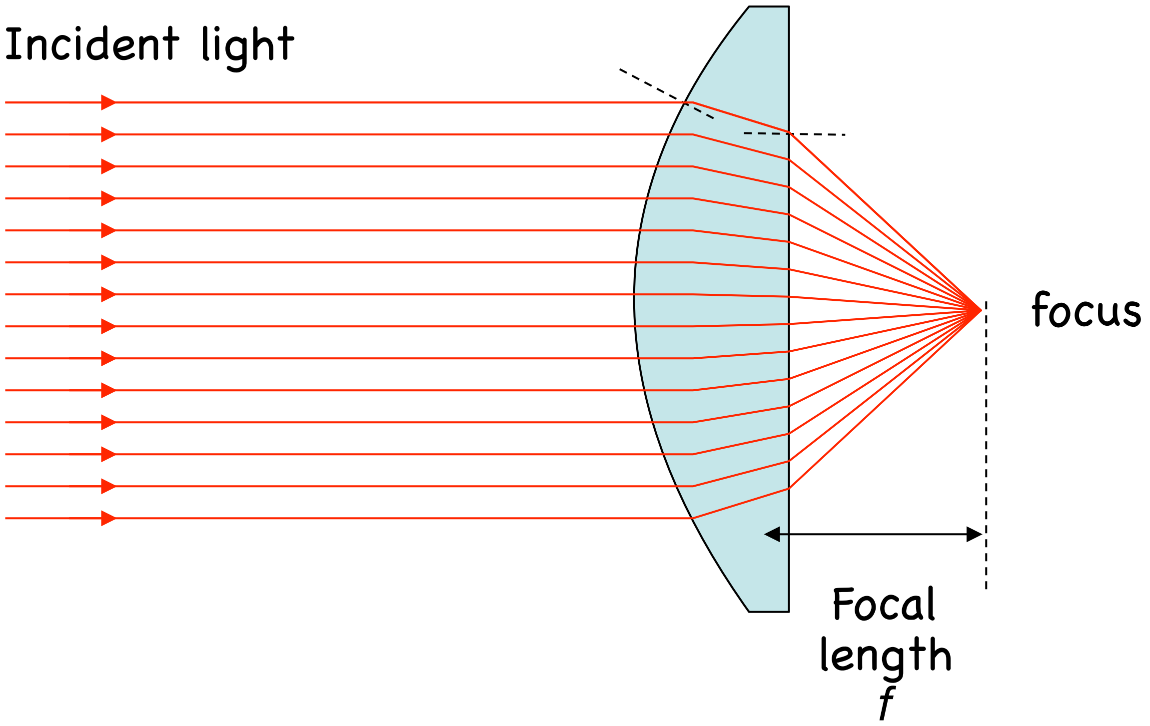
Reflection + refraction in rain drops -> rainbow

Q: why colors?

Why secondary rainbow has inverted color order?



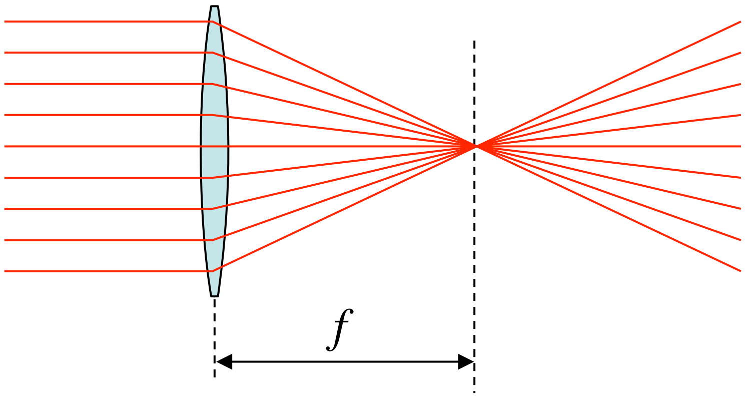
Lenses work by refraction



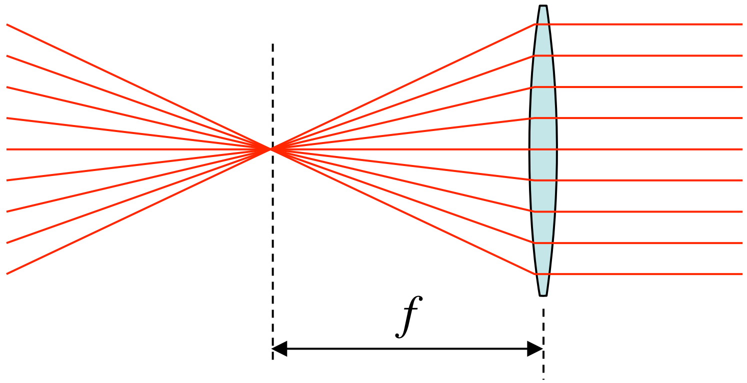
Ray Tracing 3 Rules of Thumb

(for thin ideal lenses and small ray angles: $\alpha \sim \sin\alpha \sim \tan\alpha$)

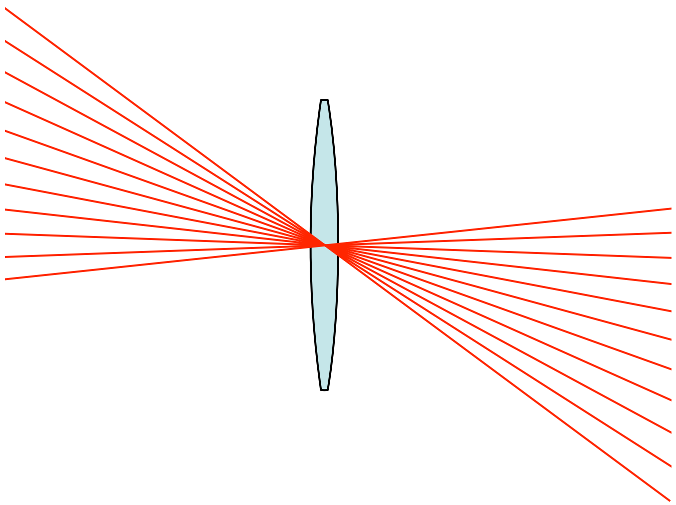
Parallel rays converge
at the focal plane



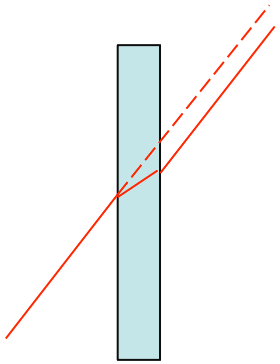
Rays that cross in the focal plane
end up parallel



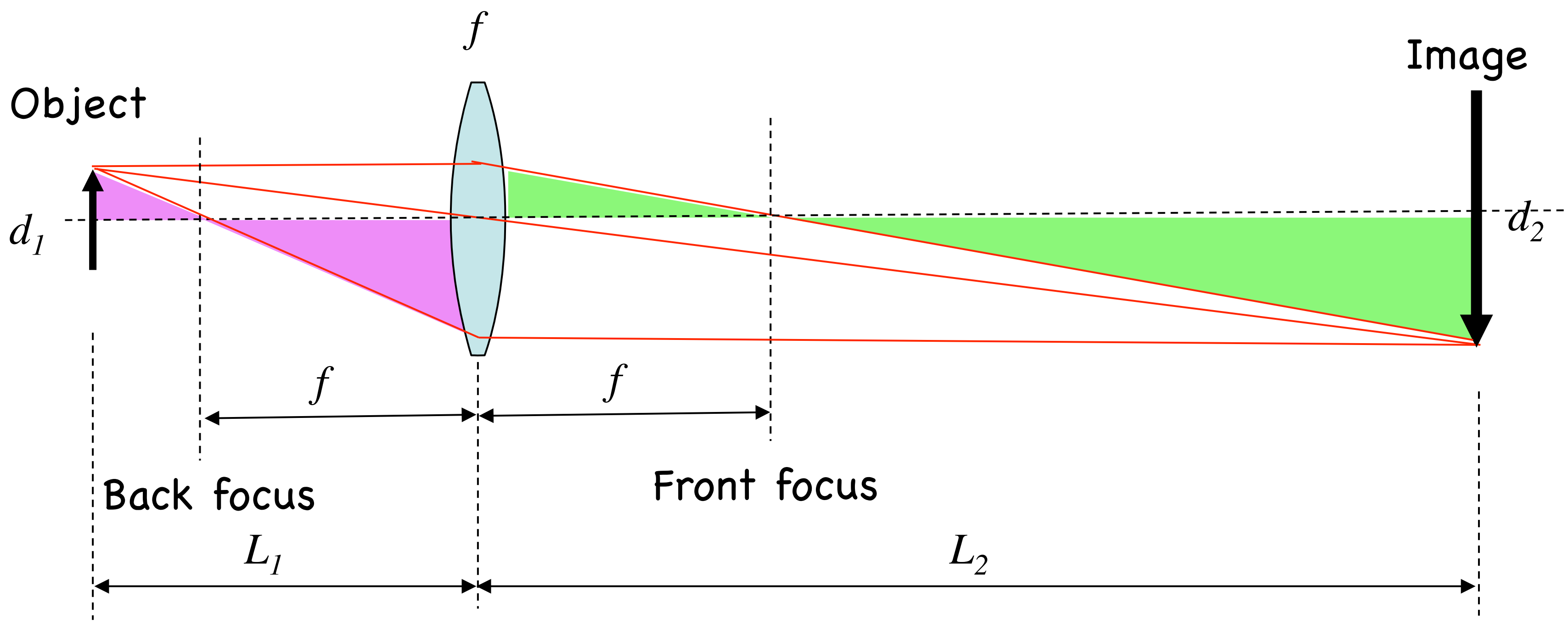
Rays through the lens center are unaffected



If thin
can neglect shift



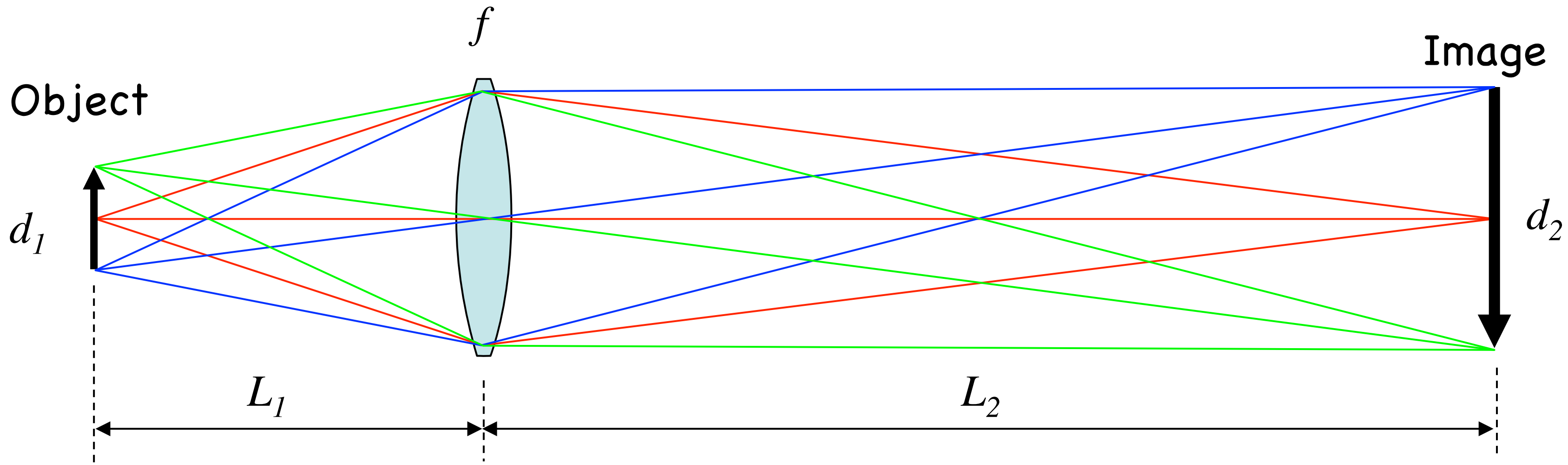
Building the image using 3 special rays



$$d_2/(L_2\text{-}f)=d_1/f$$

$$d_1/(L_1\text{-}f)=d_2/f$$

Image formation

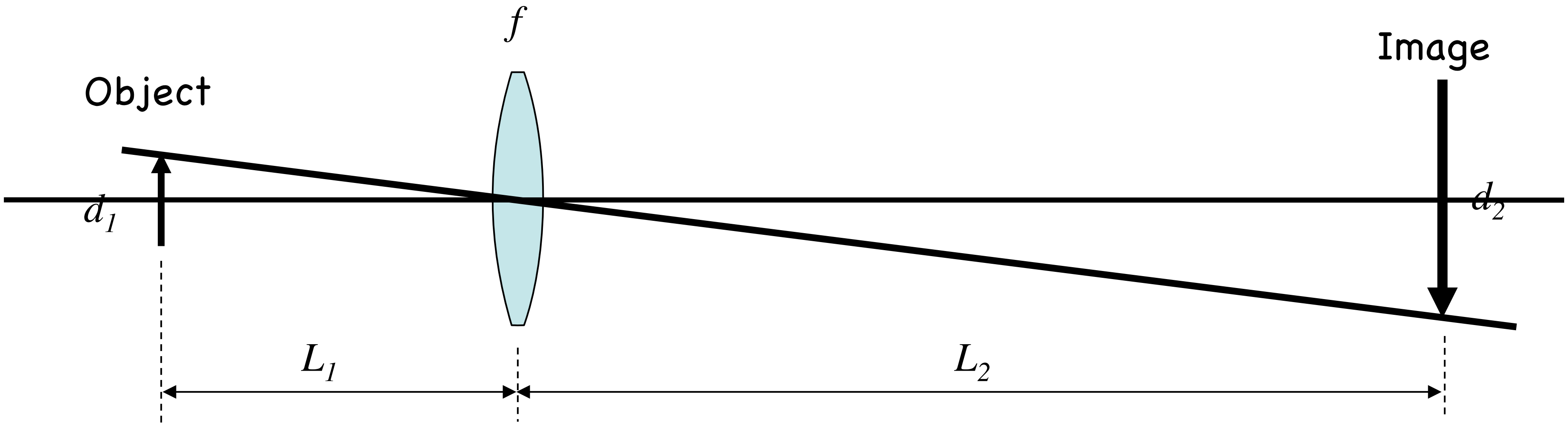


The lens law: $$\frac{1}{L_1}+\frac{1}{L_2}=\frac{1}{f}$$

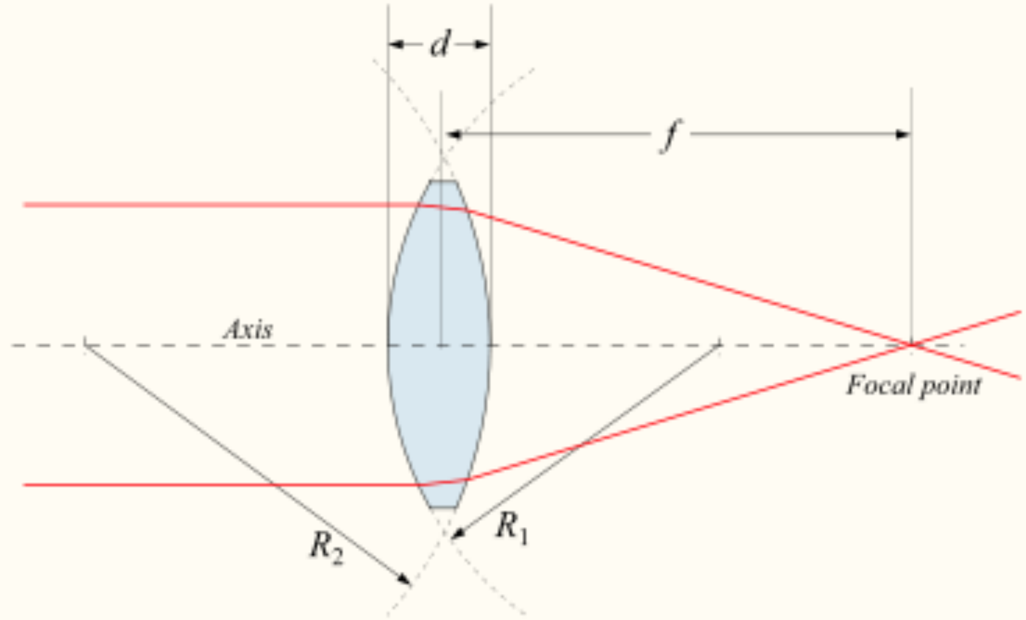
$$(L_1-f)*(L_2-f)=f^2$$

Neuton form

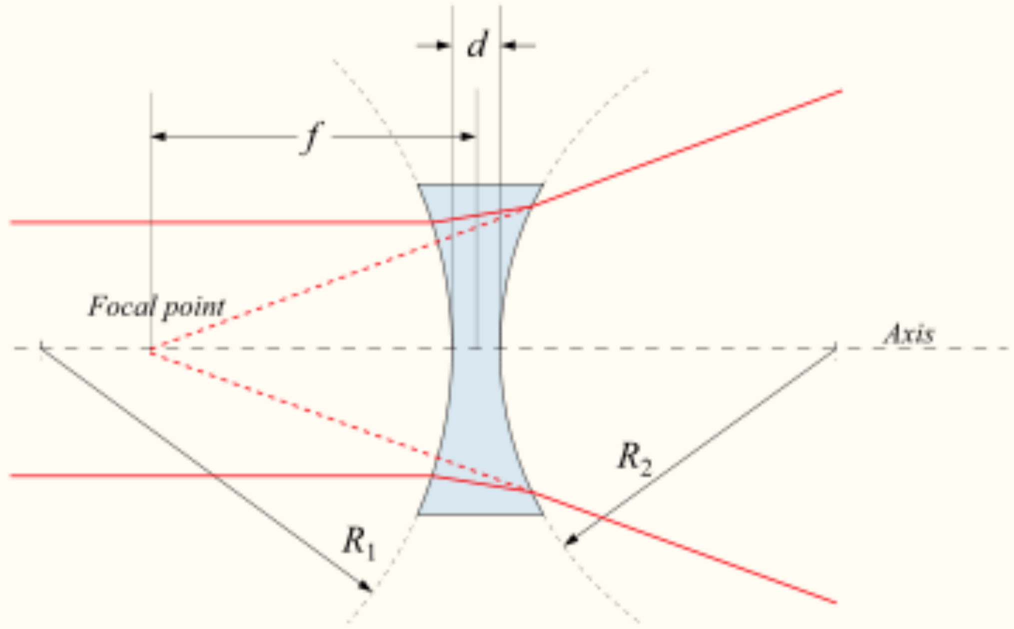
Imaging



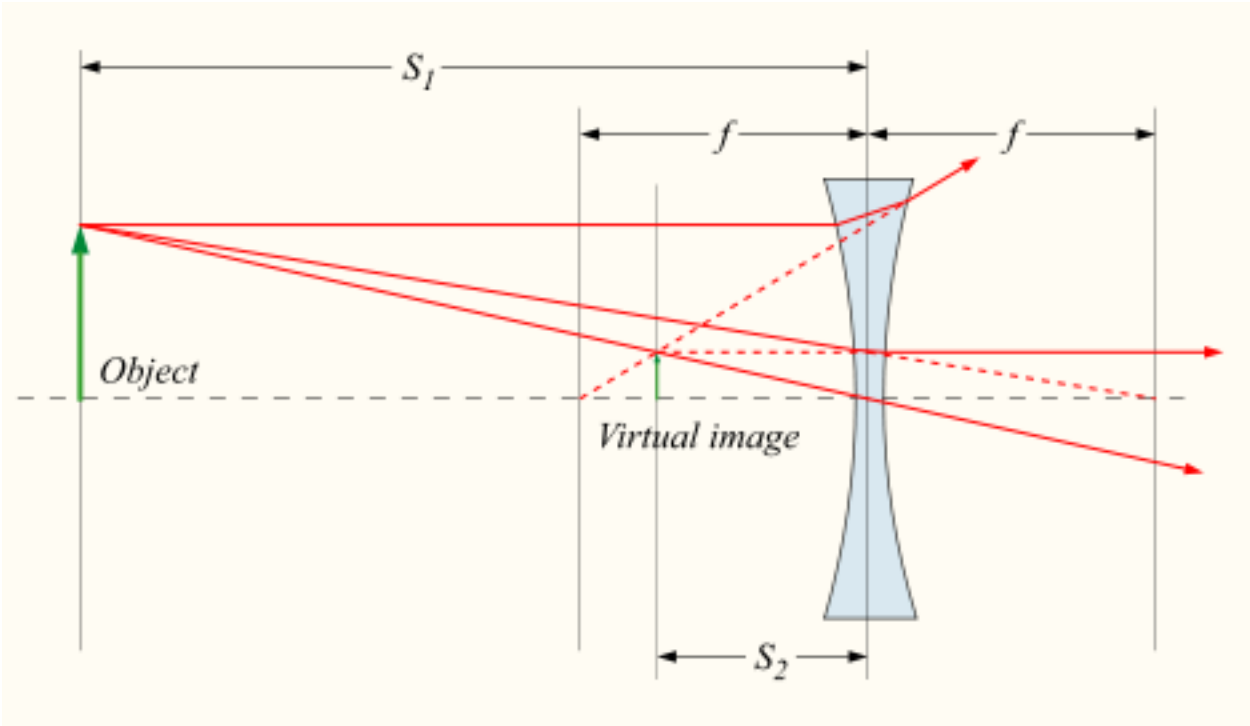
Magnification: $$M = \frac{d_2}{d_1} = \frac{L_2}{L_1}$$



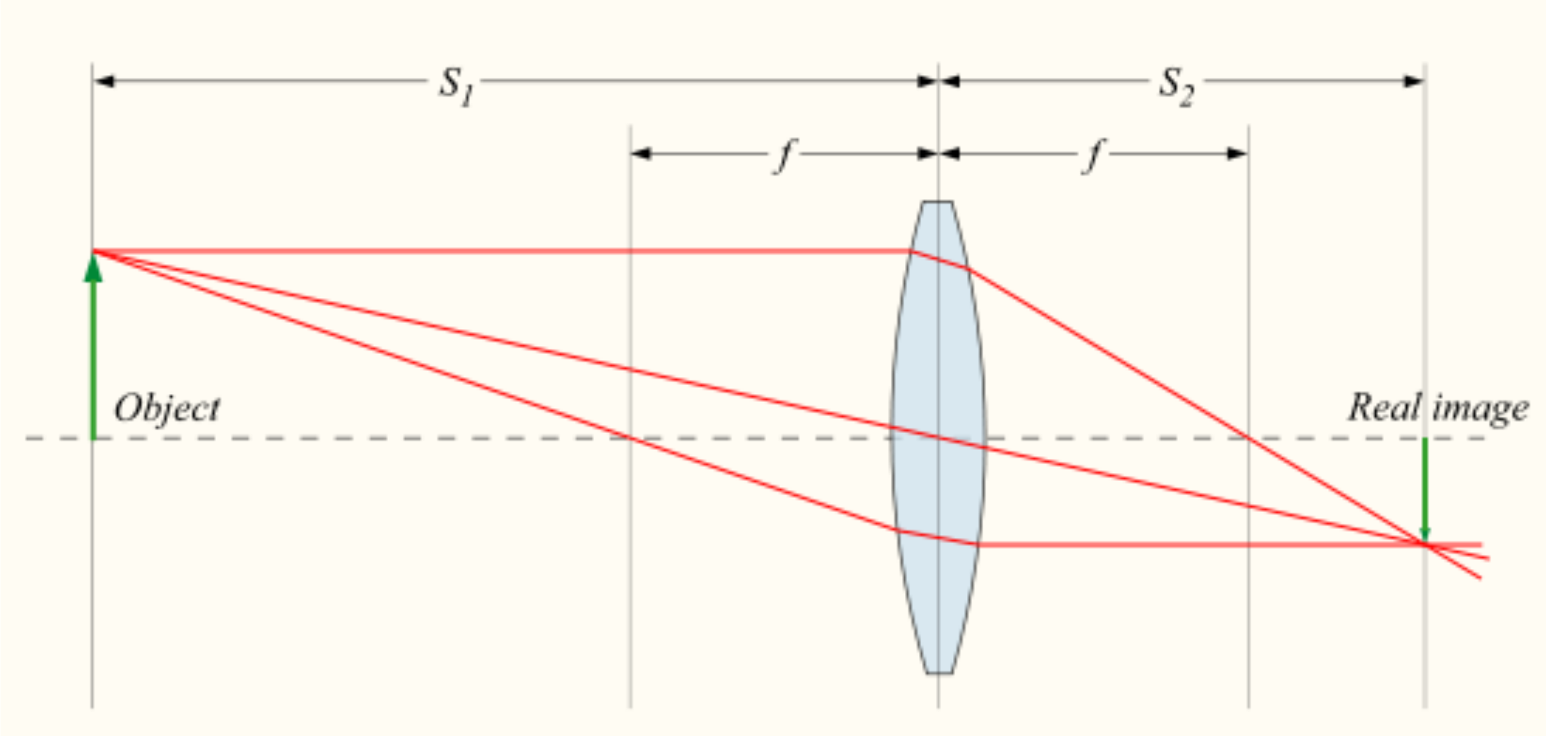
Positive (converging) lens

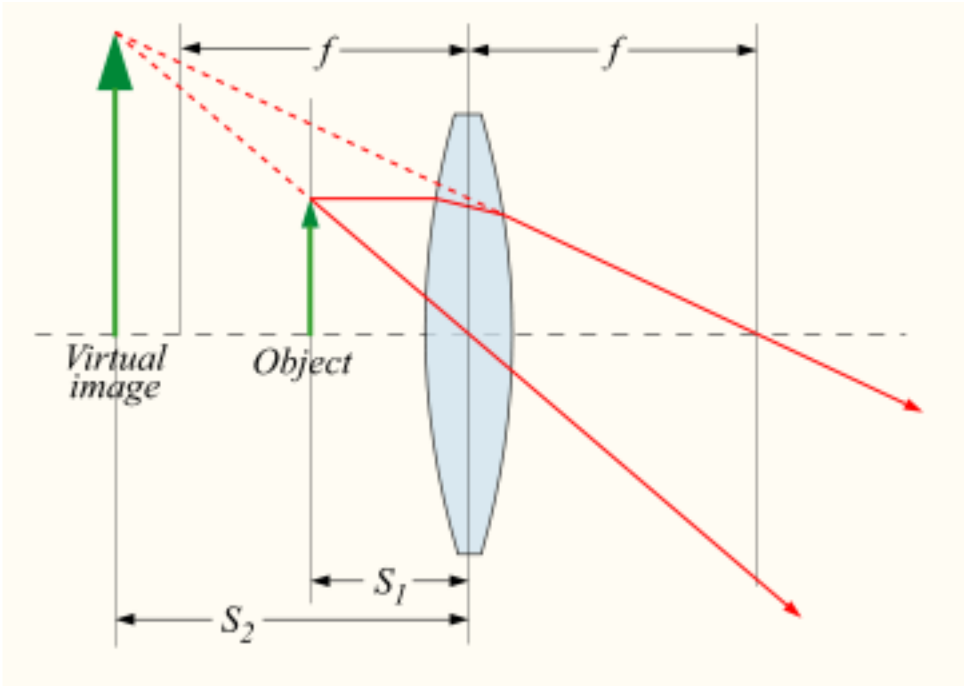


Negative (diverging) lens

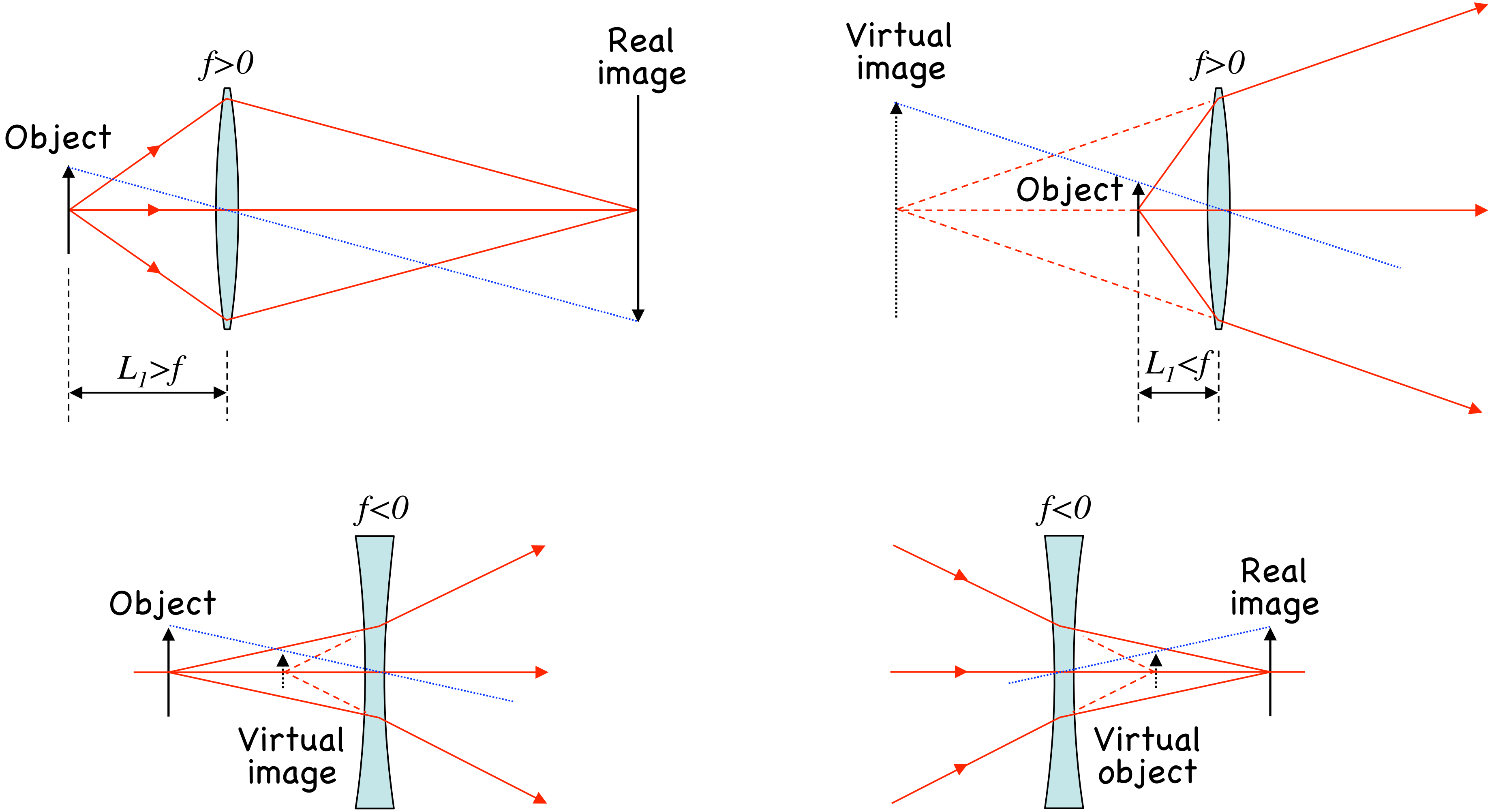


Used in microscope eyepiece


S_1
S_2
f
f
Object
Real image


f
f
Virtual image
Object
S_1
S_2

Real and virtual images



The same lens law applies: Negative lenses have negative f
Real images are inverted, Virtual images are upside up.
Virtual objects or images have negative values of L_1 or L_2

Image "escapes" to infinity

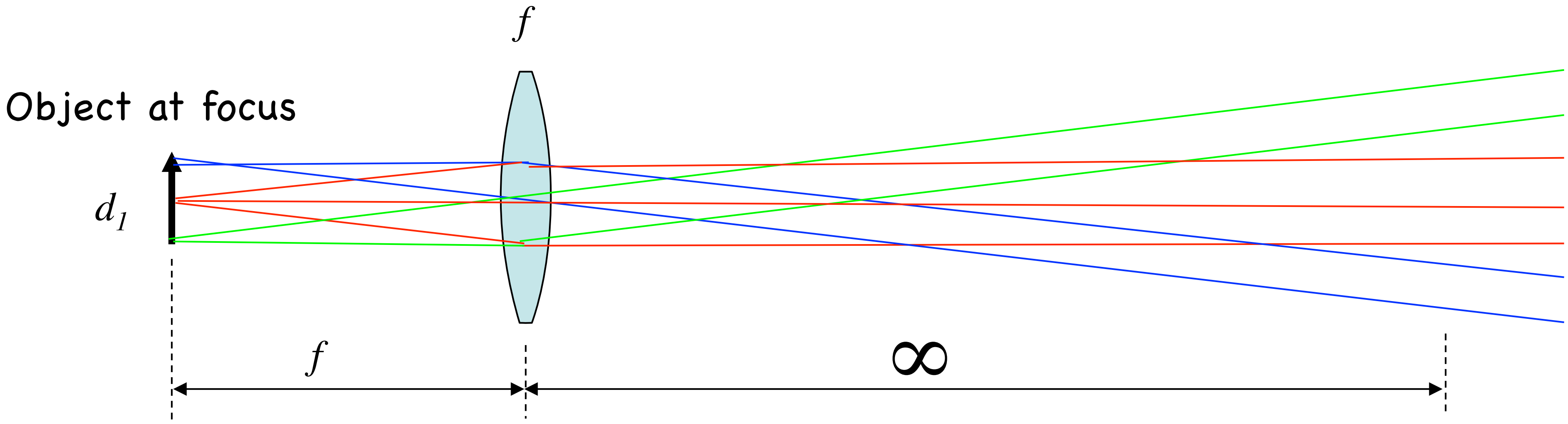
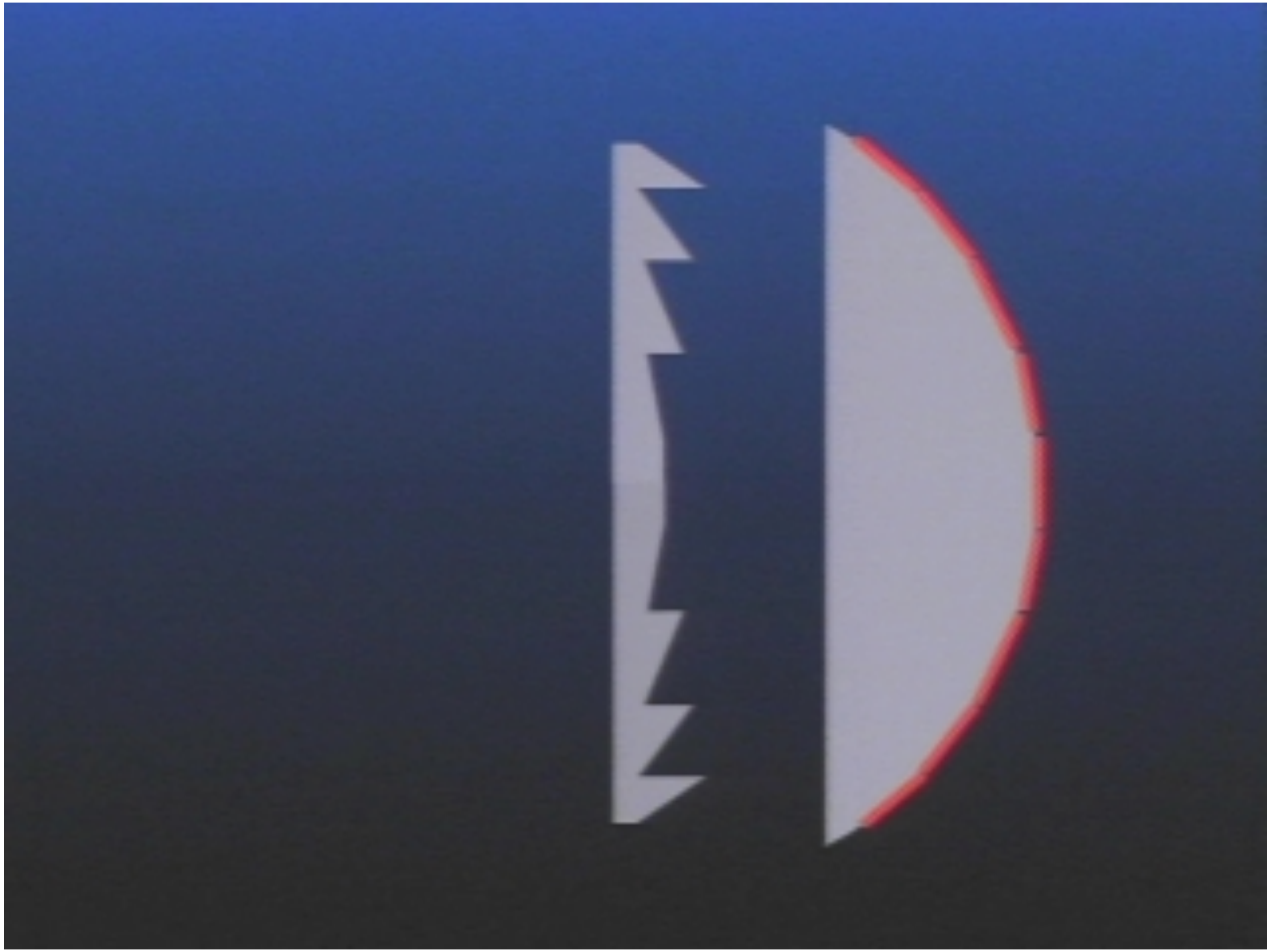


Image size also infinite

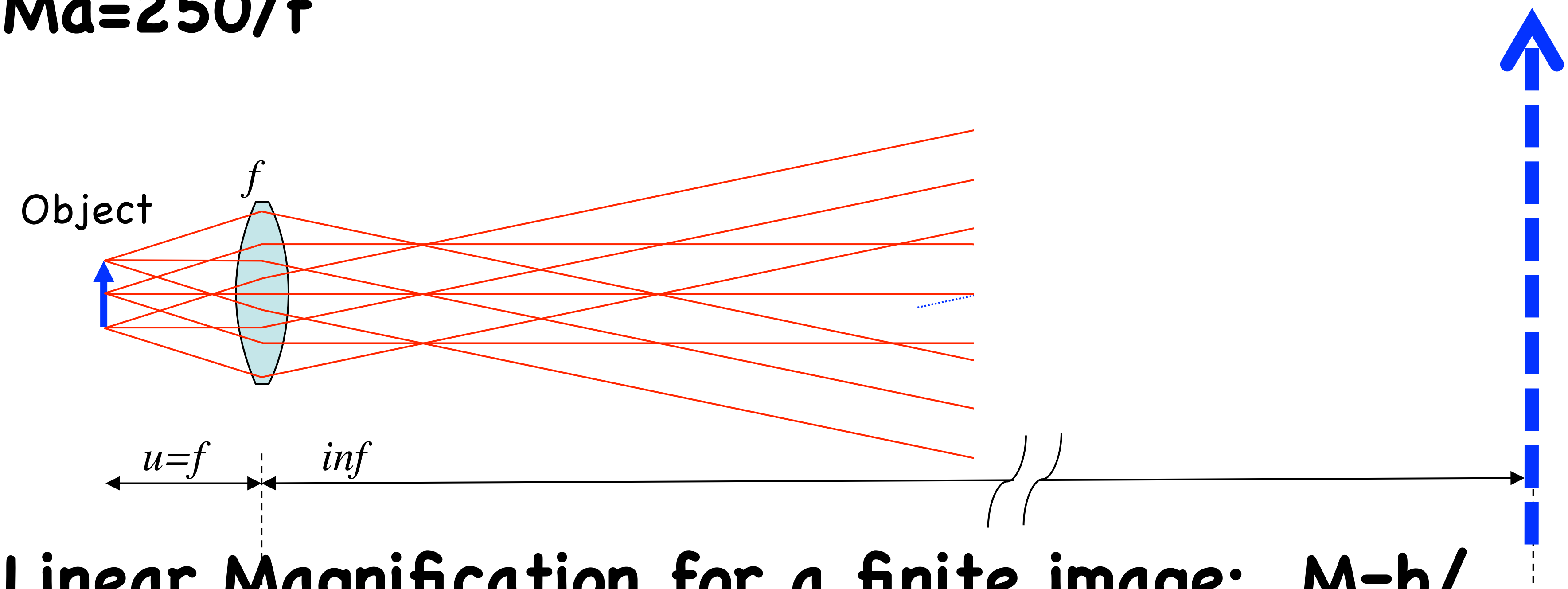
Angular size is defined
(e.g. stars)

Fresnel Lens

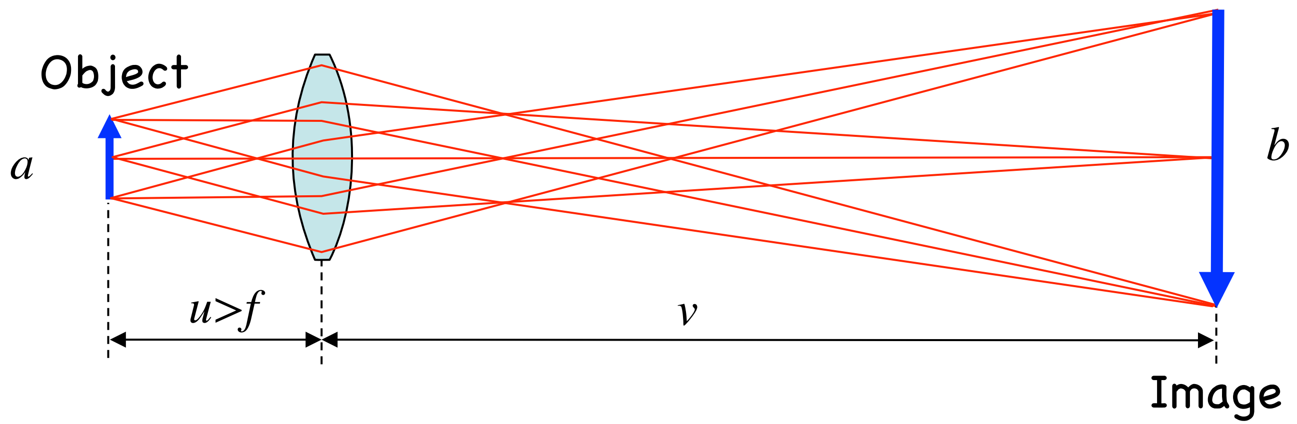


Magnification

Angular Magnification for image at infinity: Ma=250/f

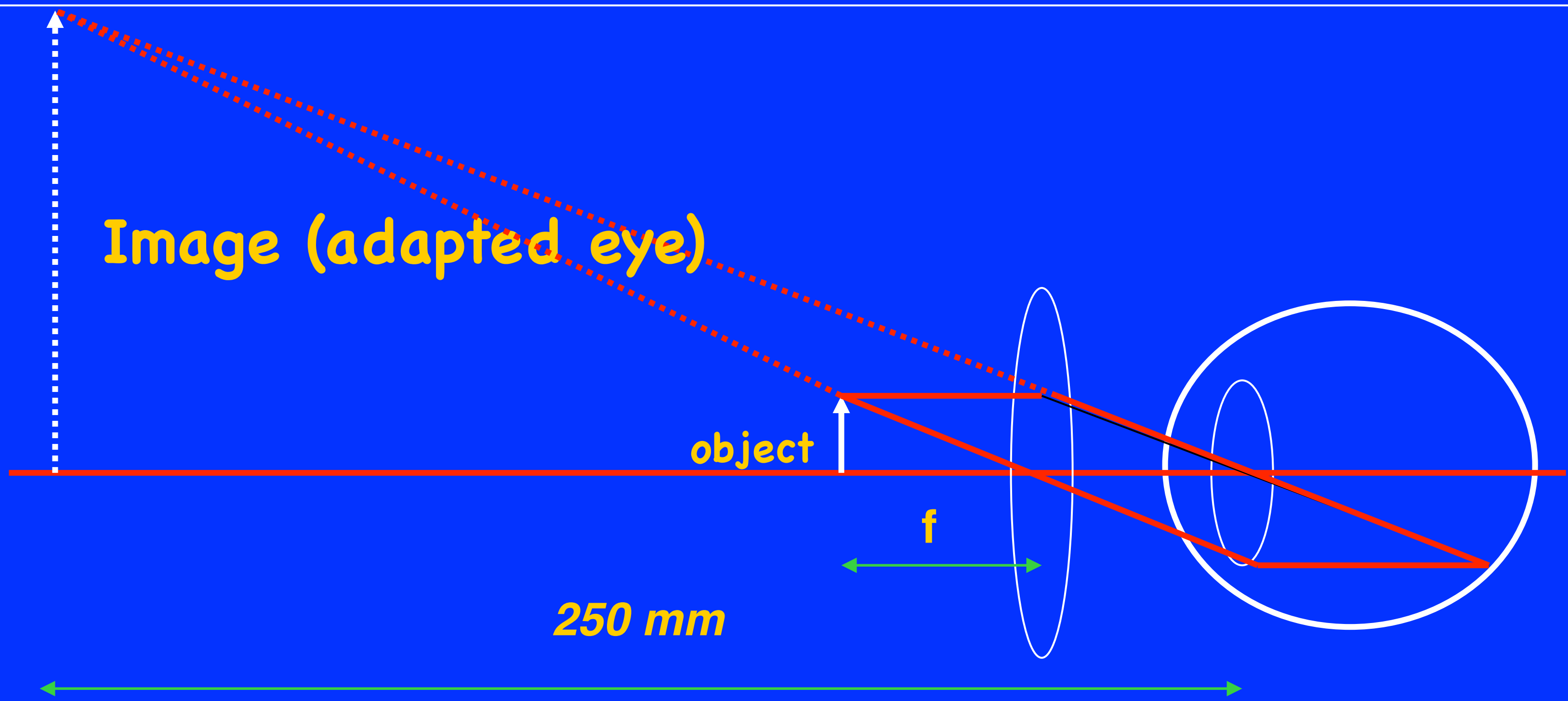


Linear Magnification for a finite image: M=b/a=v/u



To achieve
High mag
We use short f

VISUAL MAGNIFICATION



Single lens (simple) microscope *magnification:*

M= image size (angle) / object size (angle)

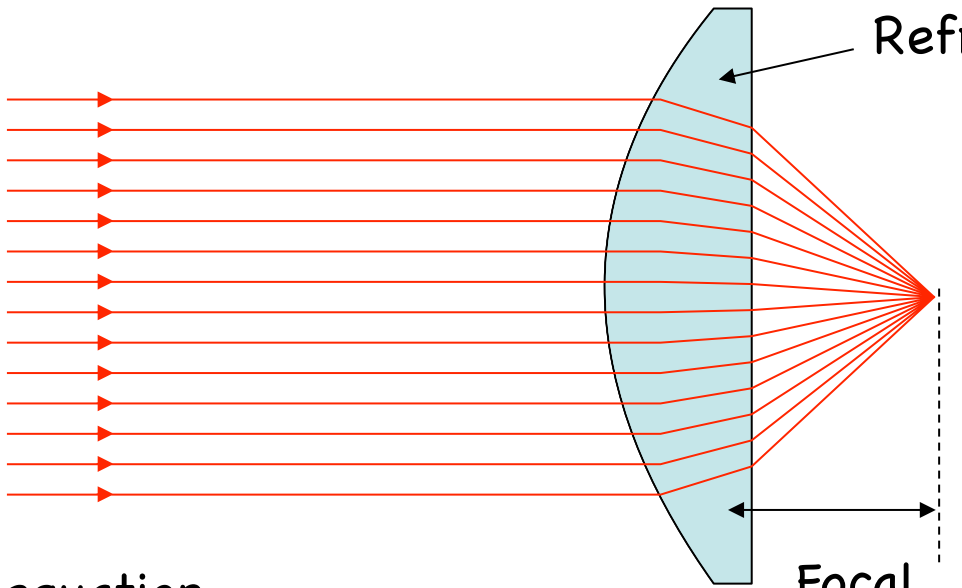
For eye adapted to see at 250mm: M=250/f+1 (why?)

for relaxed eye (see to infinity): M=250/f

for a typical magnifier f=50-20mm; M= 5-12.

Q: Why not magnify more? (Leeuwenhook did better!)

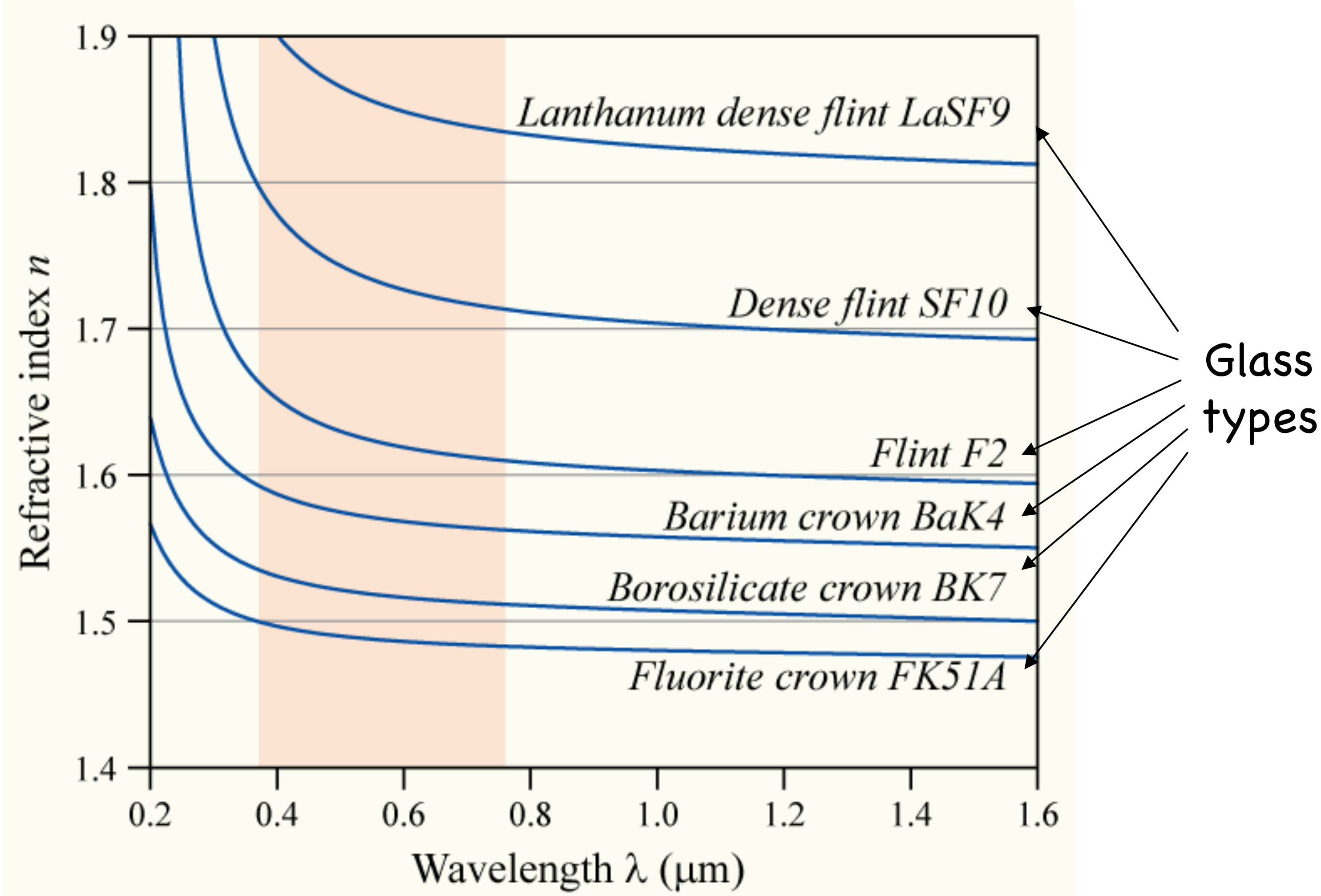
The focal length of a lens depends on the refractive index…



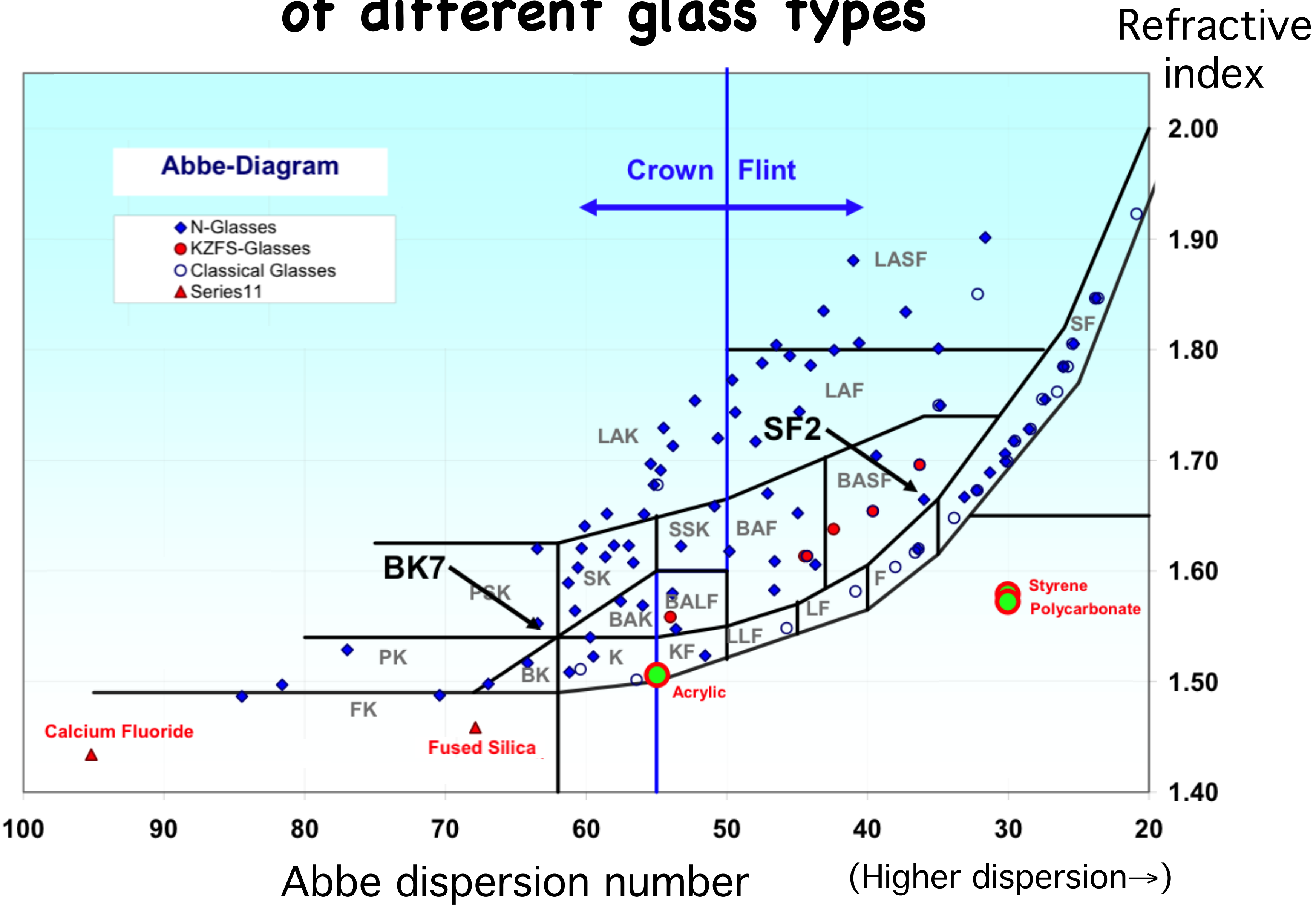
Lensmaker's equation
$1/f=(n-1)[1/r_1-1/r_2+(n-1)d/(nr_1r_2)]$

$$\boxed{f \propto r/(n-1) \sim 2\text{mm}}$$

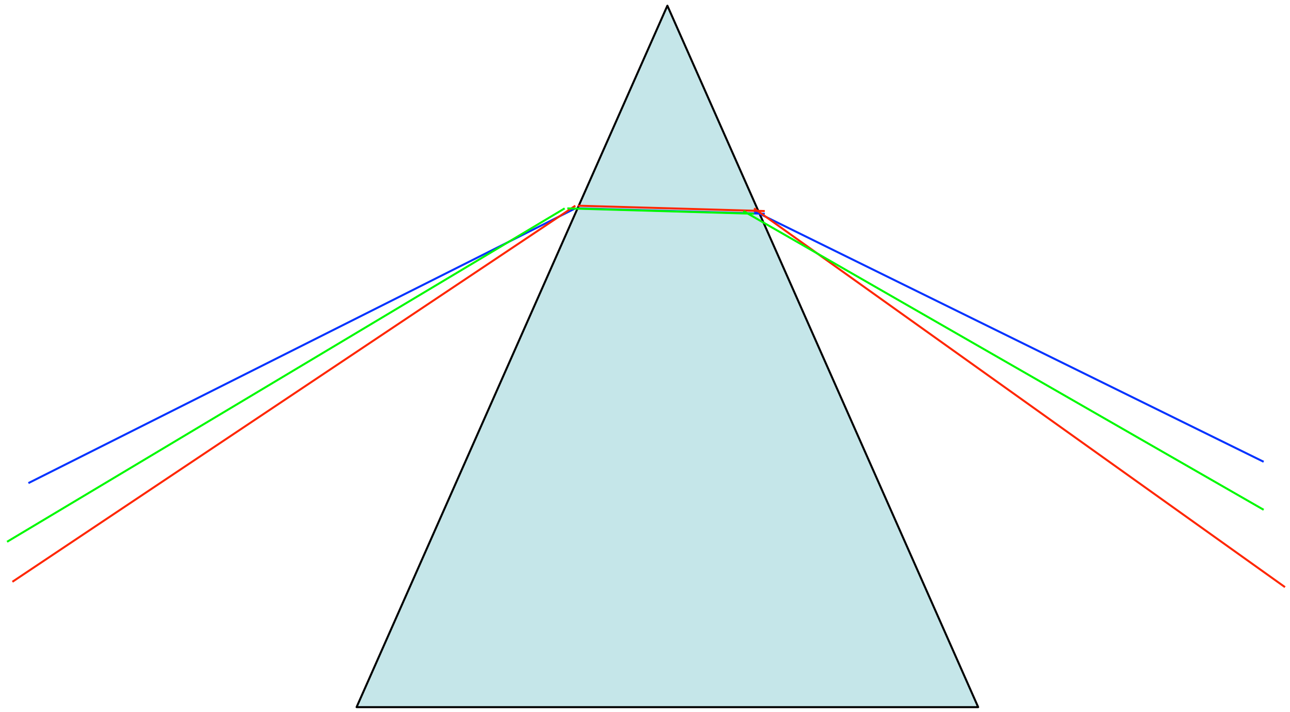
… and the refractive index depends on the wavelength ("dispersion")



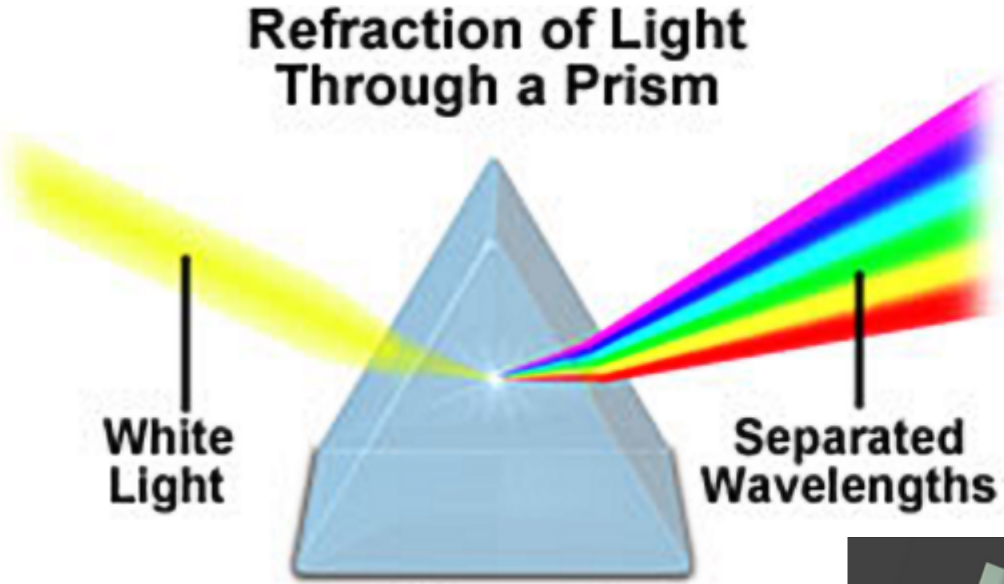
Dispersion vs. refractive index of different glass types

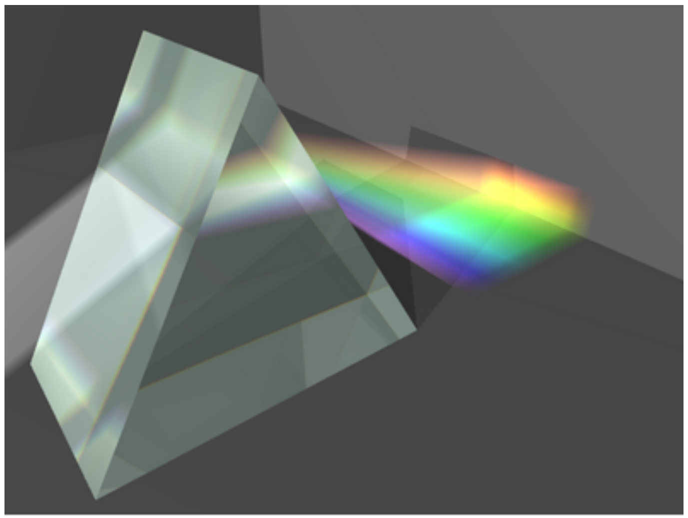


Refractive index depends on color



Index of refraction is usually a function of wavelength



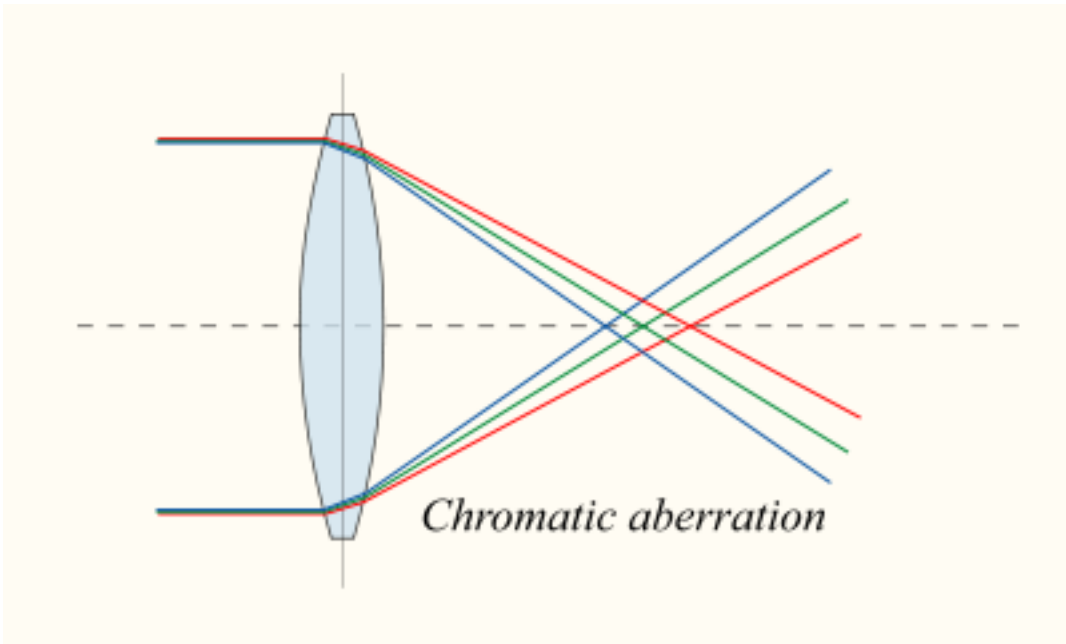


Q: what is the error in the image above?

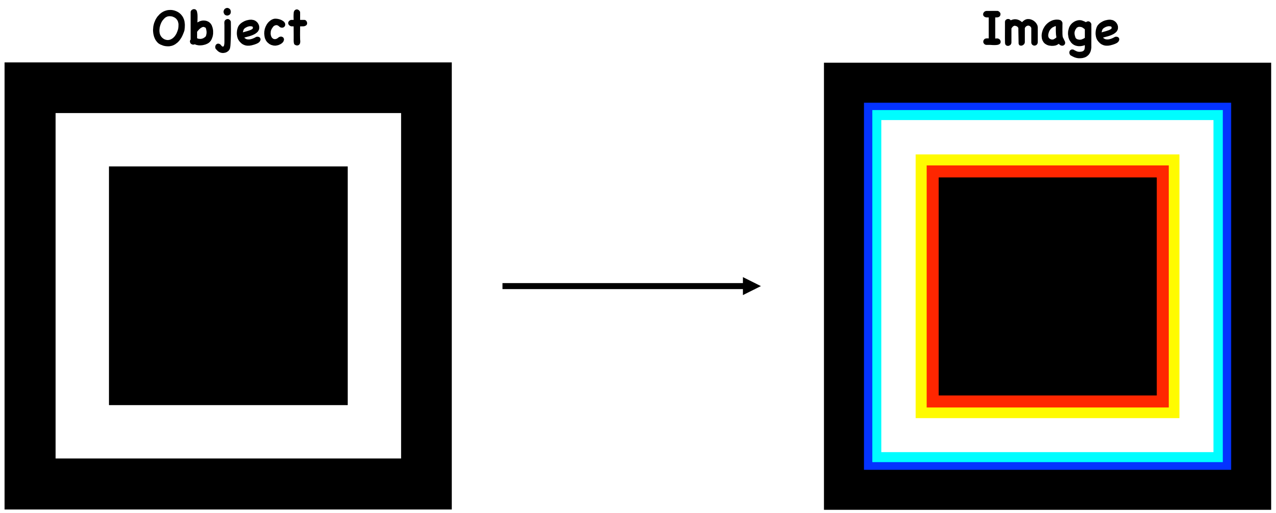
⇒ Chromatic aberration

Axial chromatic aberration

(difference in focus)

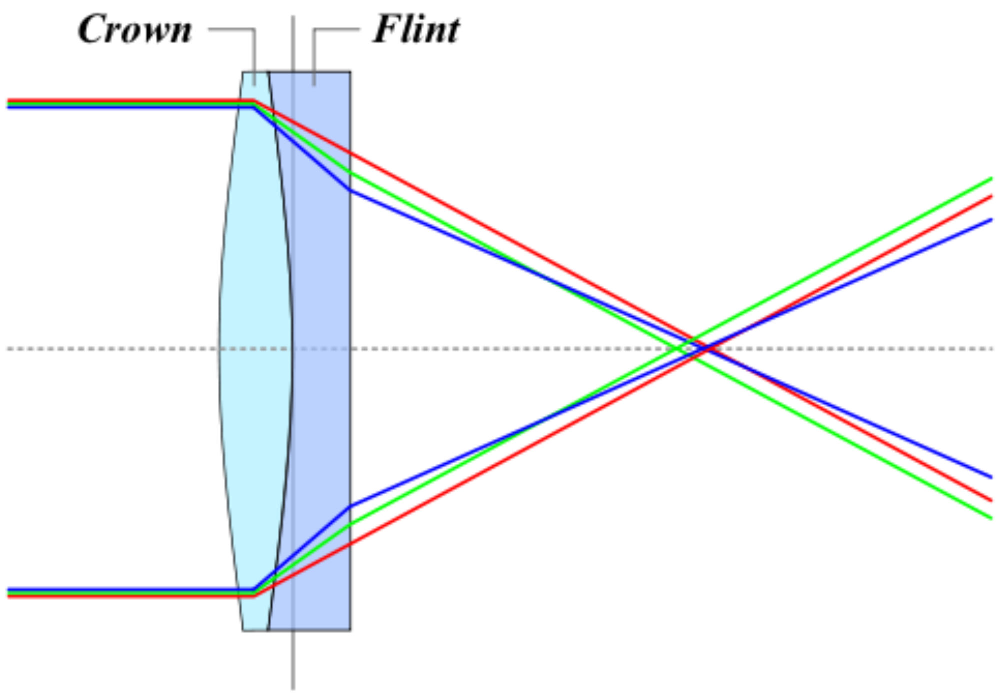


Lateral chromatic aberration
(difference in magnification)

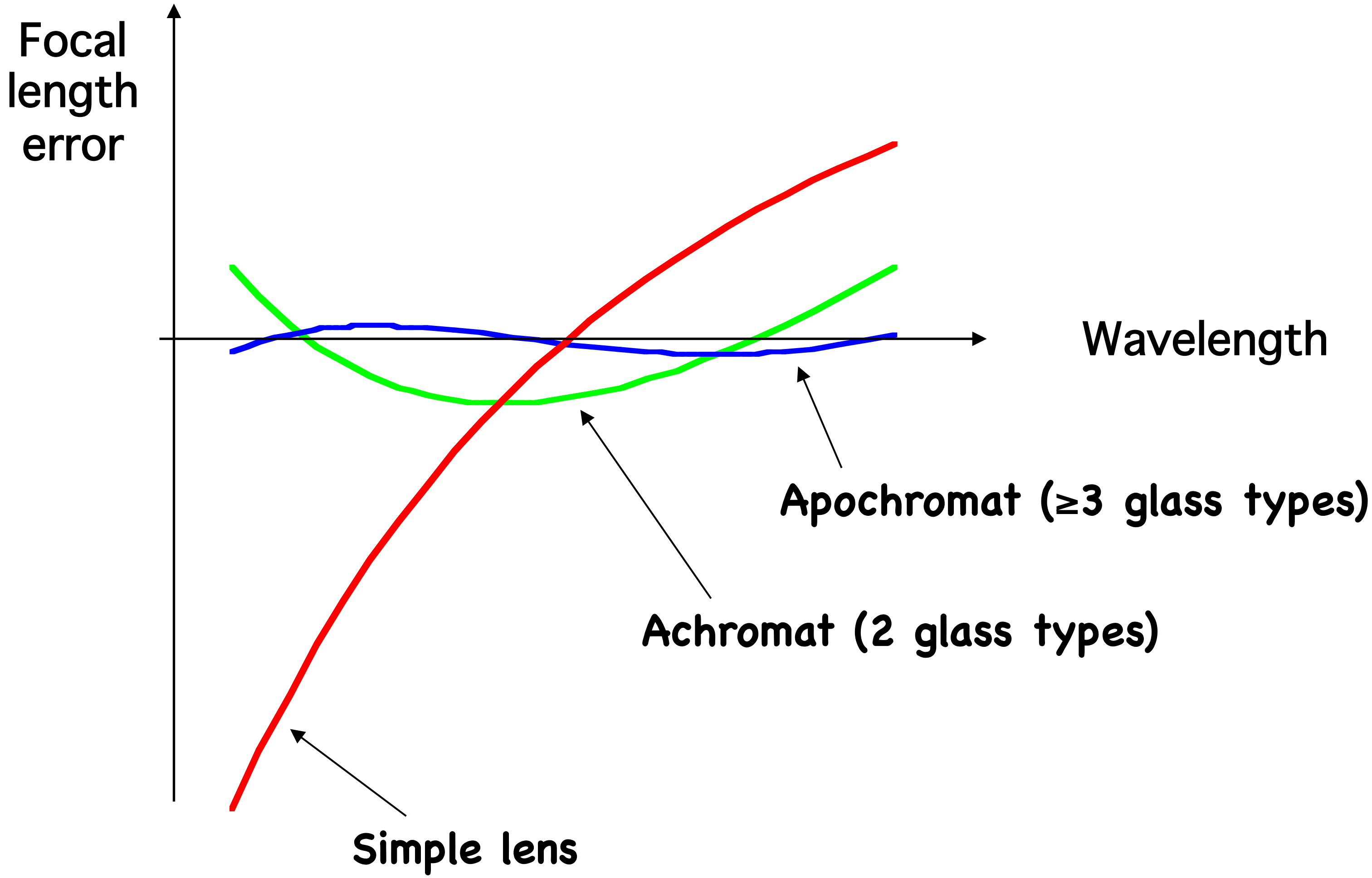


Achromatic Lenses

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

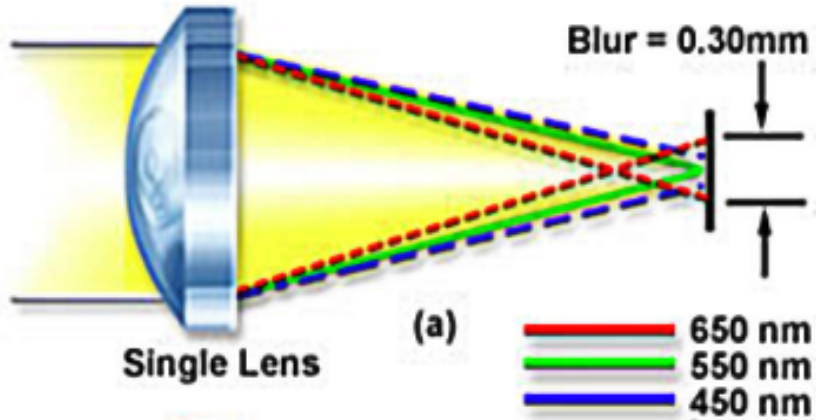


Achromats and Apochromats

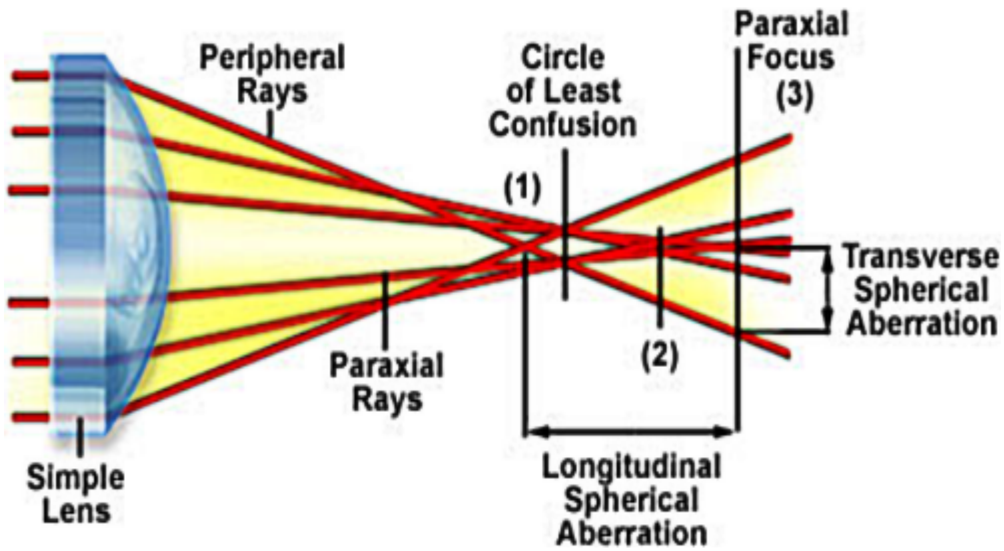


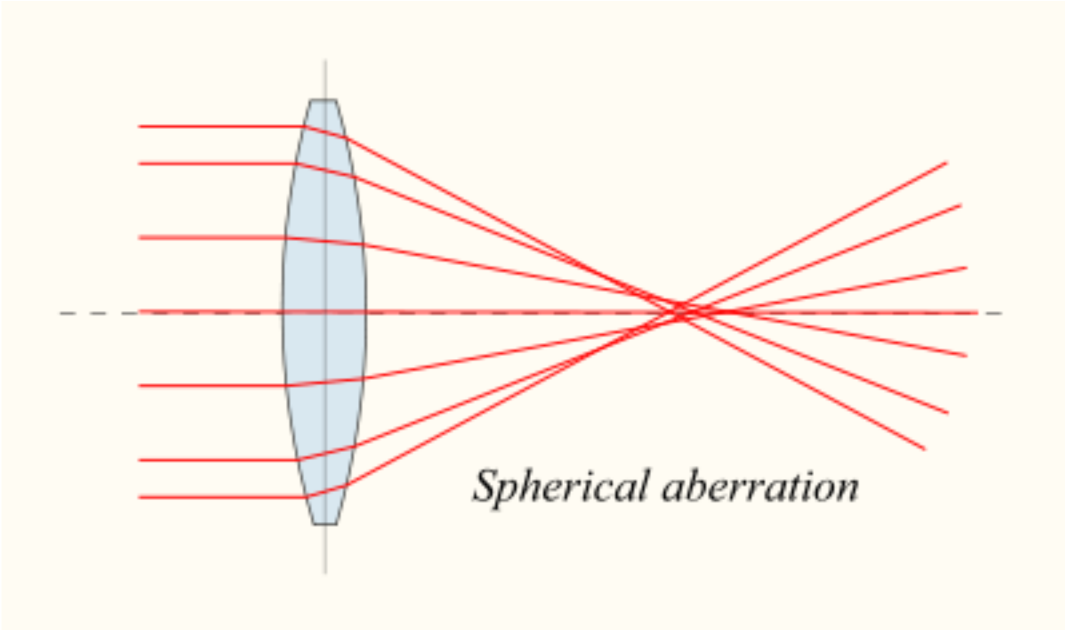
ABERRATIONS

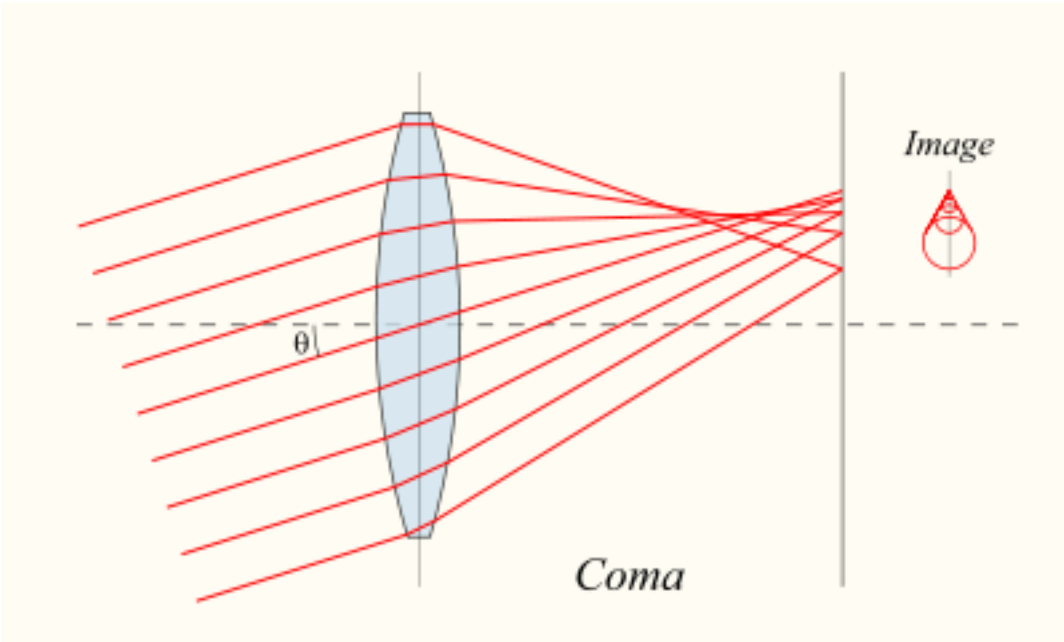
- CHROMATIC ABERRATION
  - due to $n(\lambda)$



- SPHERICAL ABERRATION
  - paraxial approx.







Images off axis, or misalignment of several lenses

ANATOMY OF A MICROSCOPE

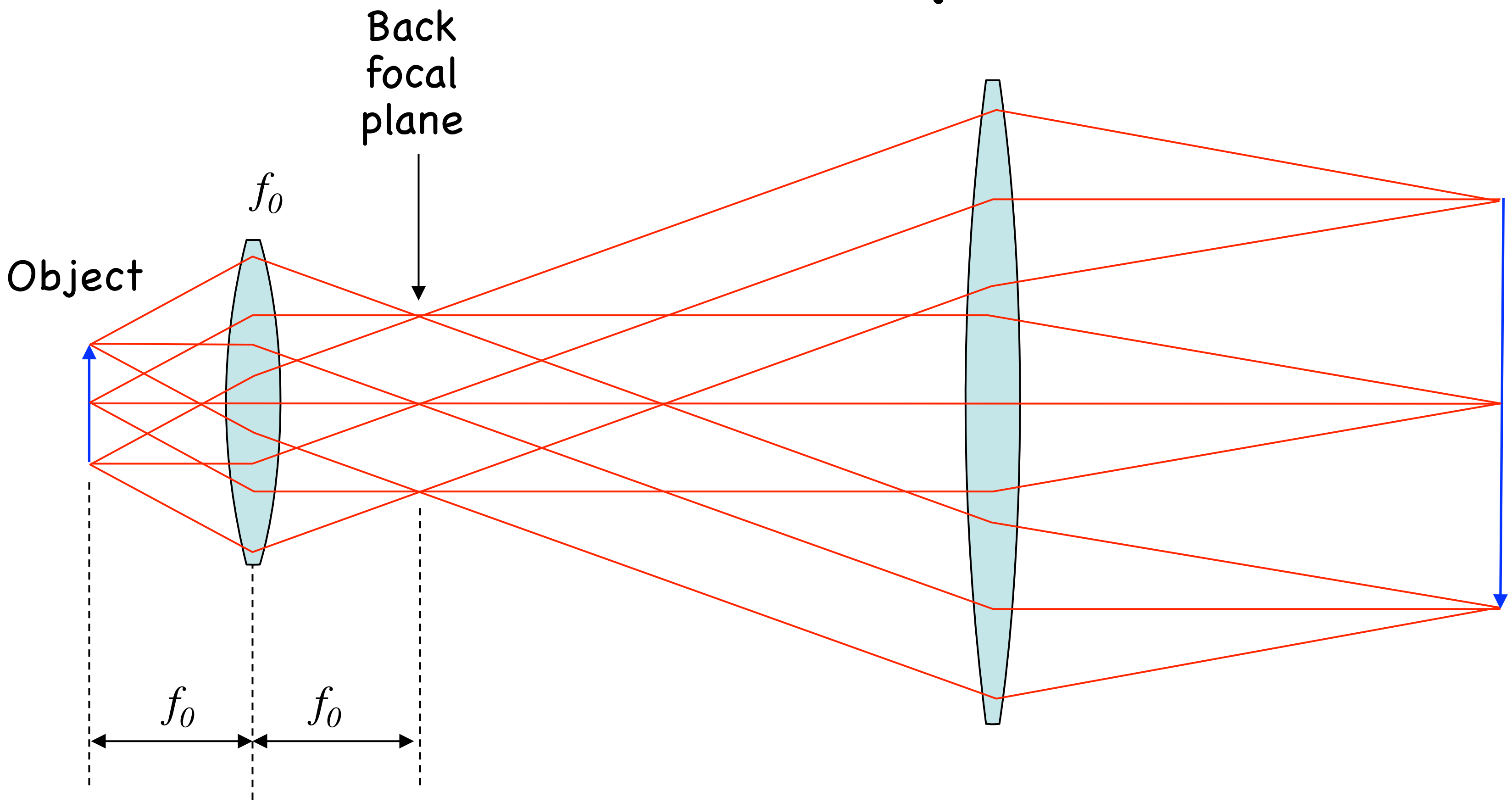
- Objectives
- Oculars
- Upright or Inverted
- Illumination

Magnifying glass (Leeuwenhoek)

Compound Microscope (Hooke)

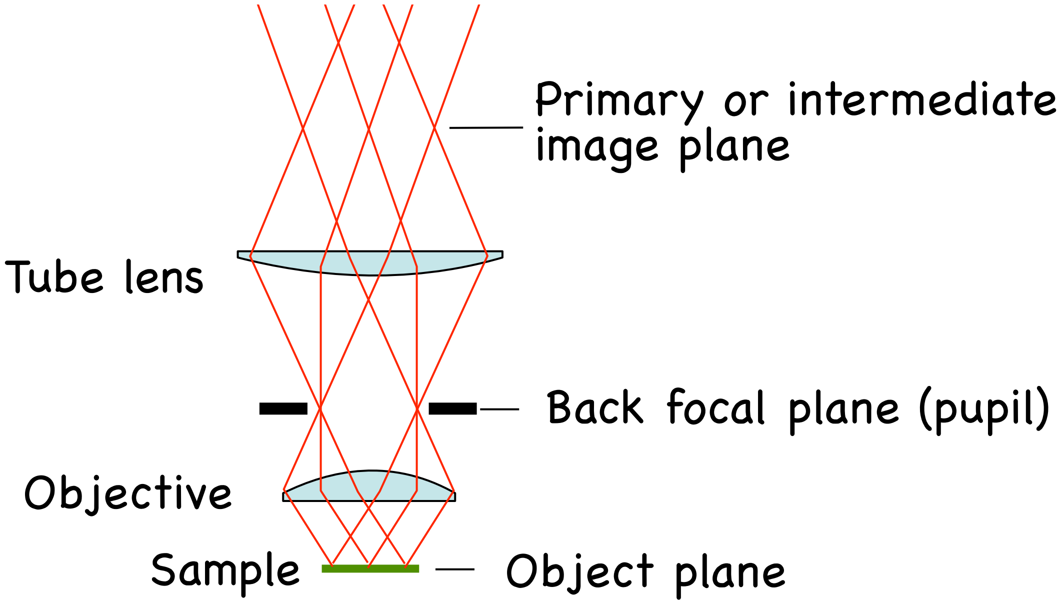
Q: why do we use compound microscopes

Back focal plane

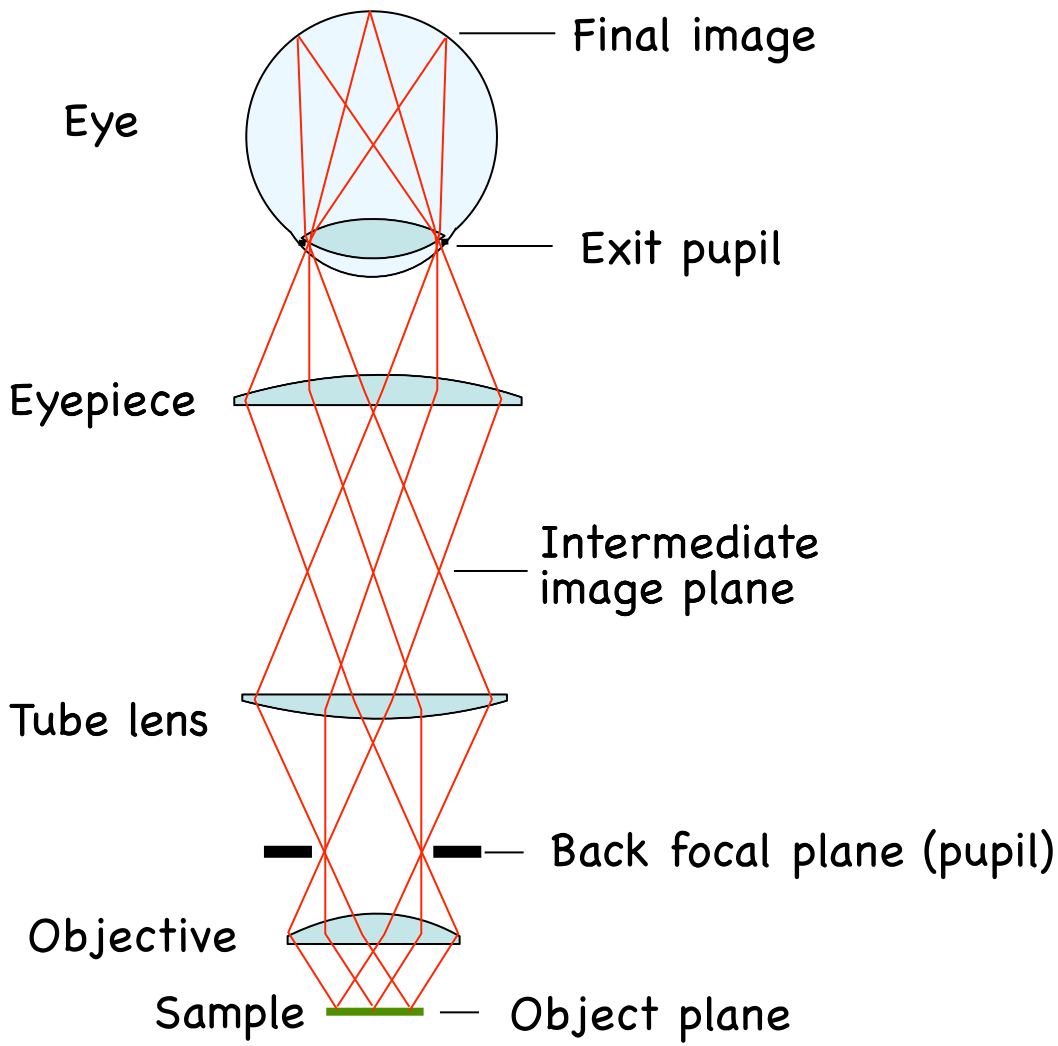


Rays that leave the object with the same angle
meet in the objective's *back focal plane*
Ray from every point in the object fill the back aperture

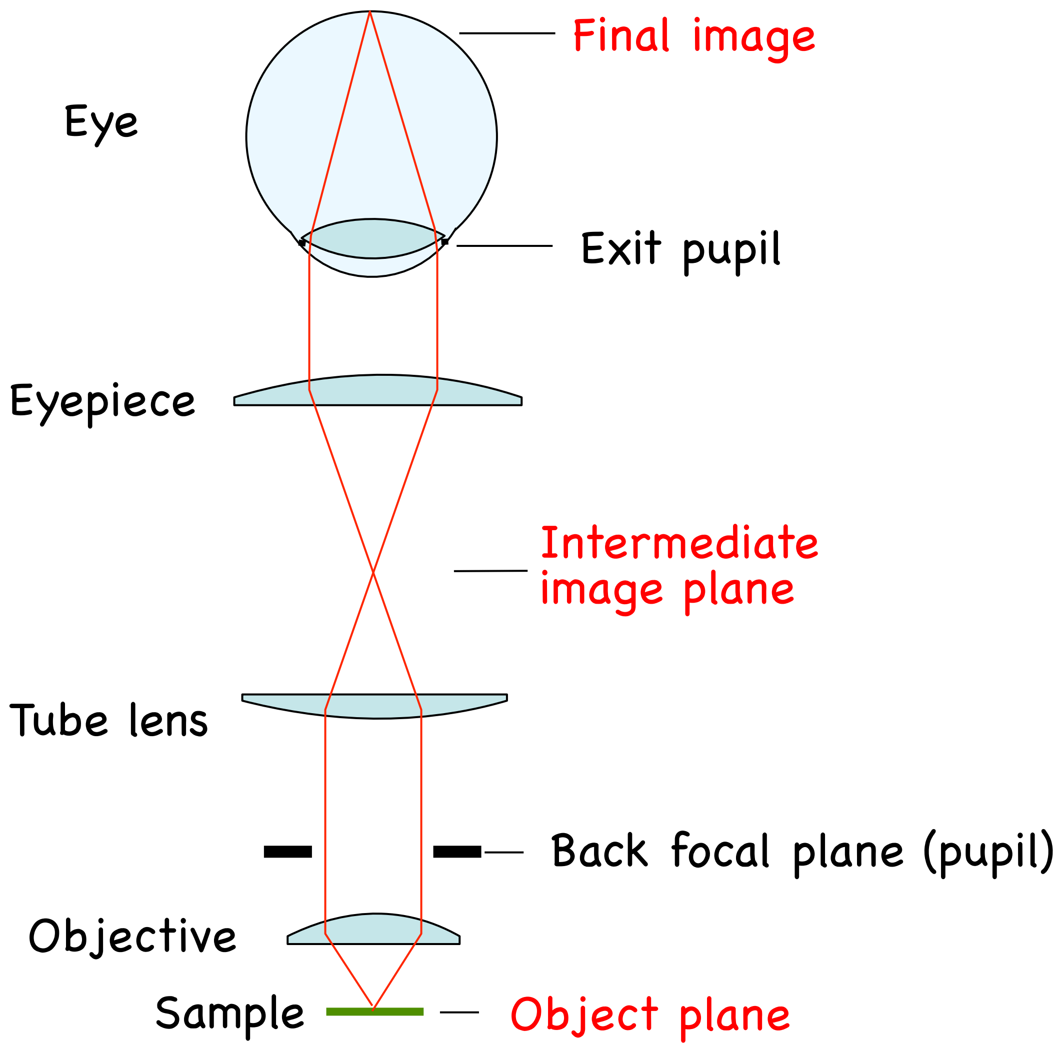
The Compound Microscope



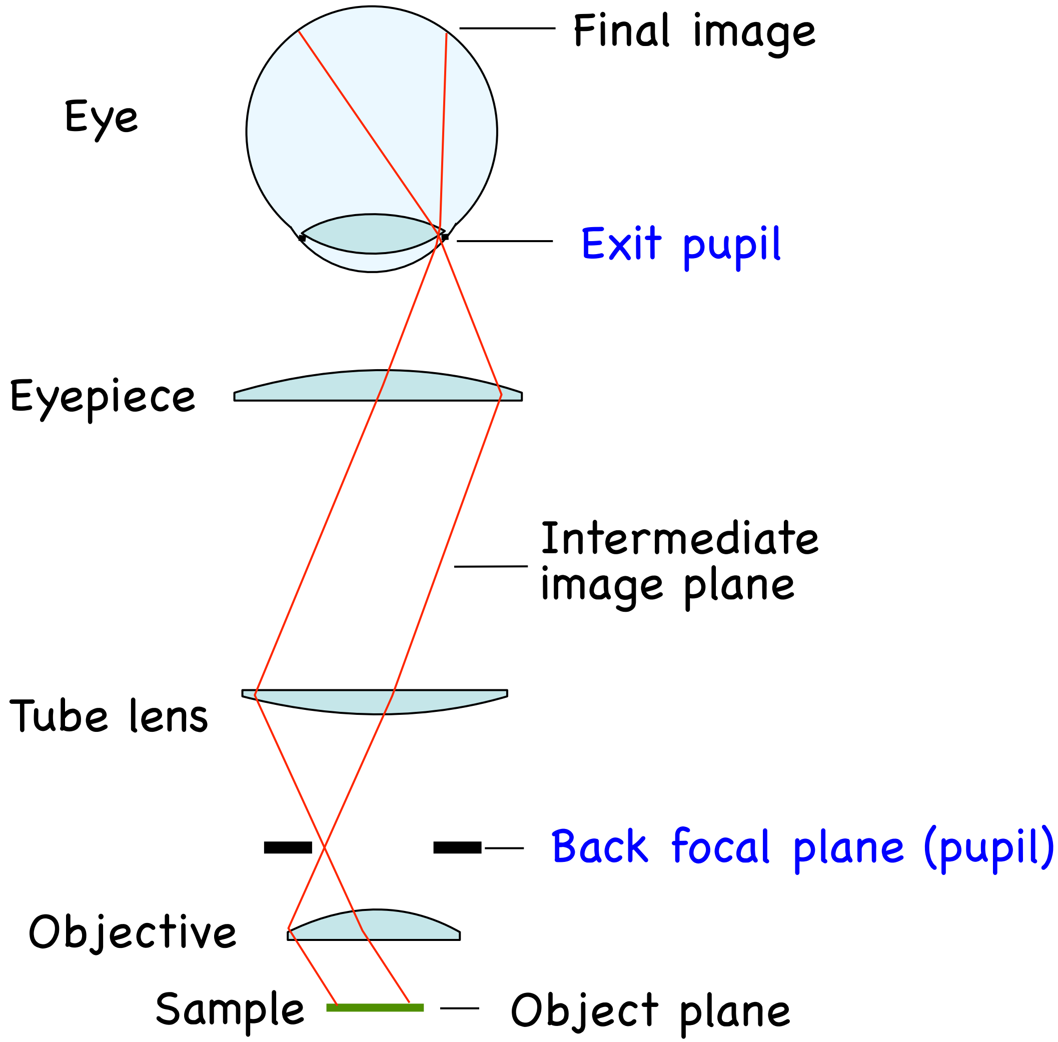
The Compound Microscope



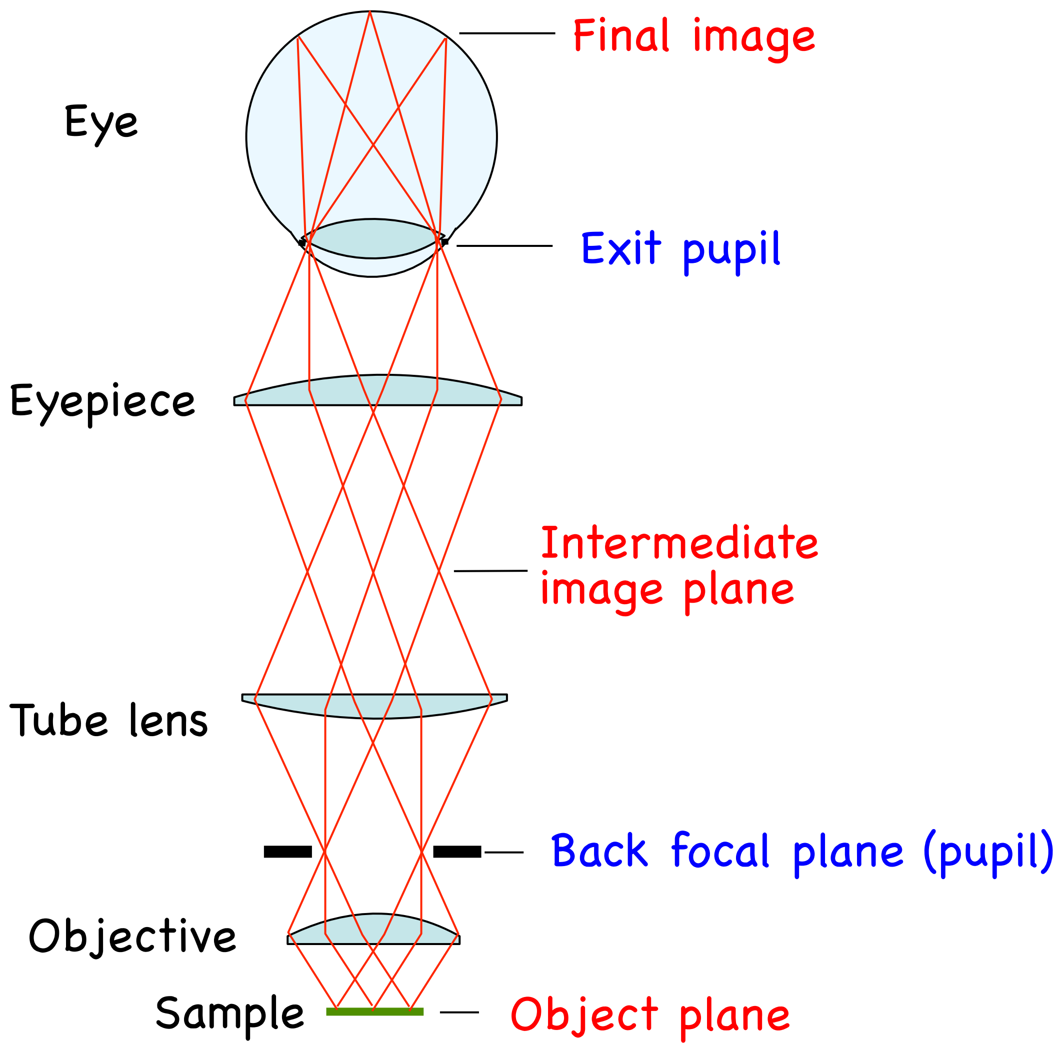
The Compound Microscope



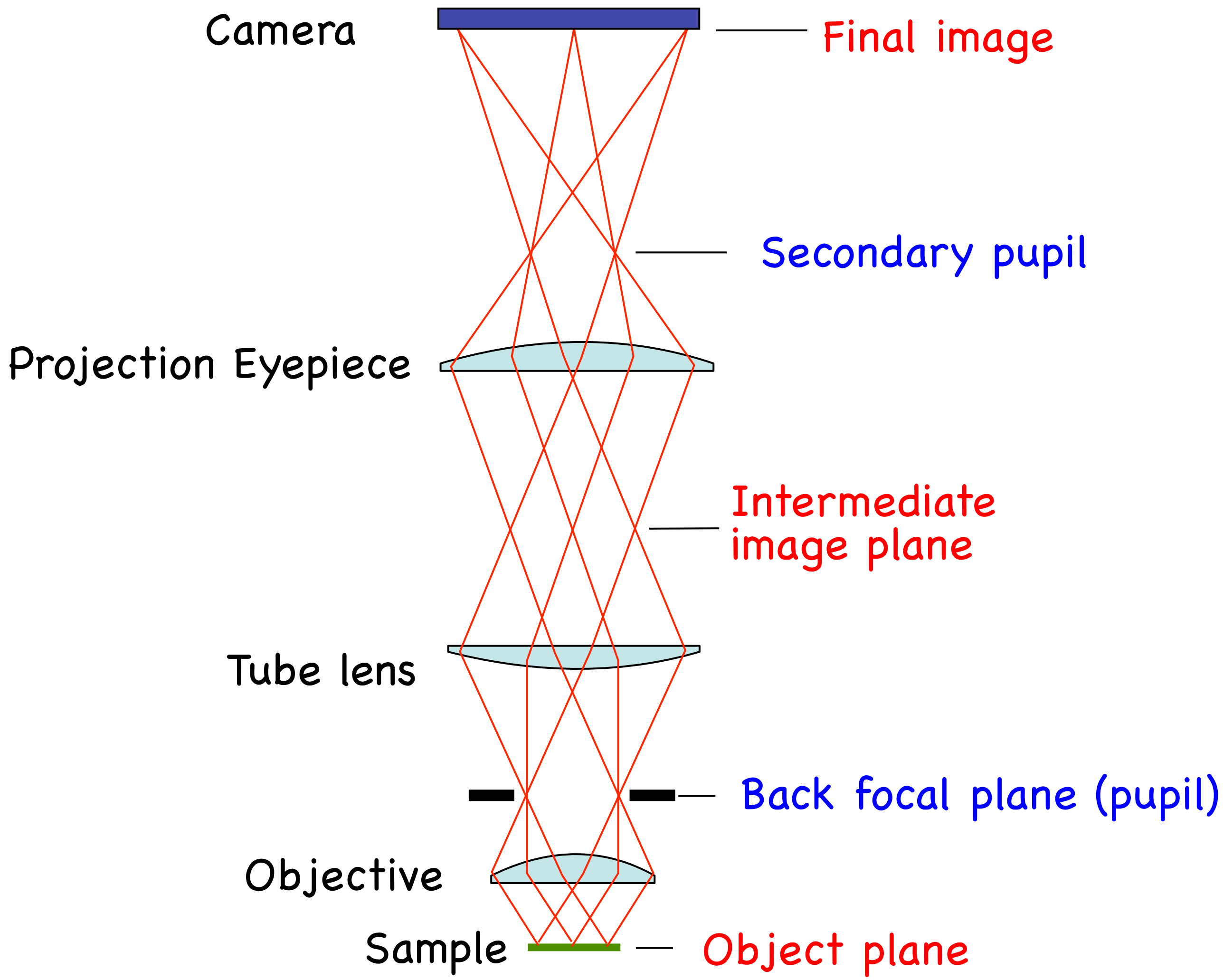
The Compound Microscope



The Compound Microscope

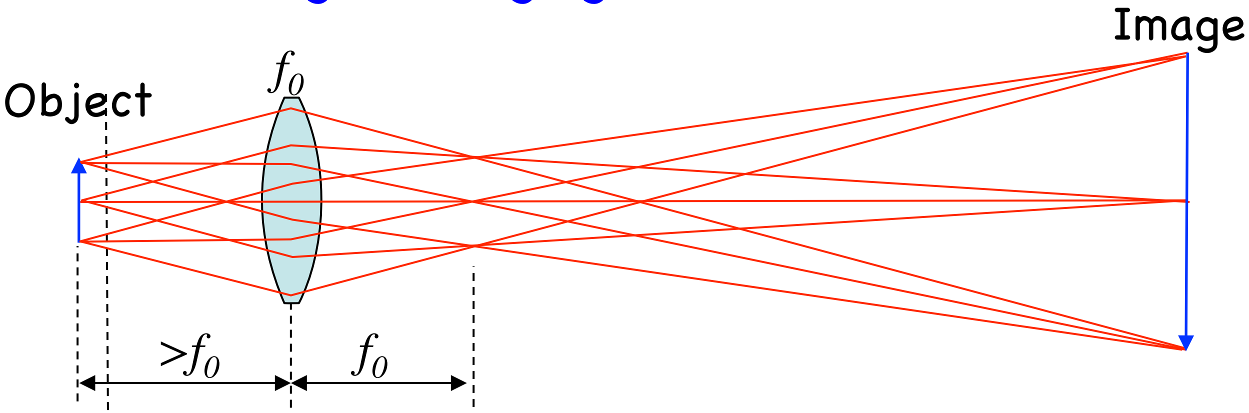


The Compound Microscope



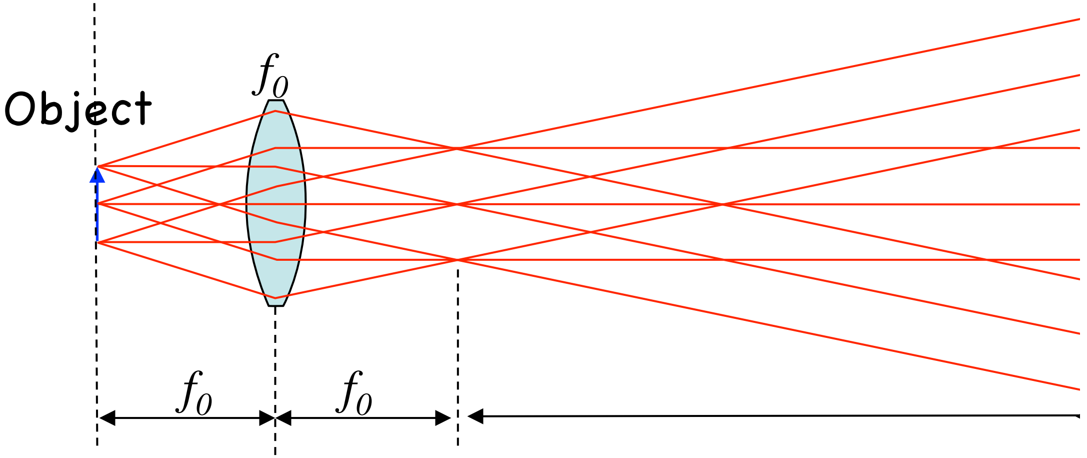
Finite vs. Infinite Conjugate Imaging

- Finite conjugate imaging (older objectives). Need relay lenses to add optics.



- Infinite conjugate imaging (modern objectives).

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



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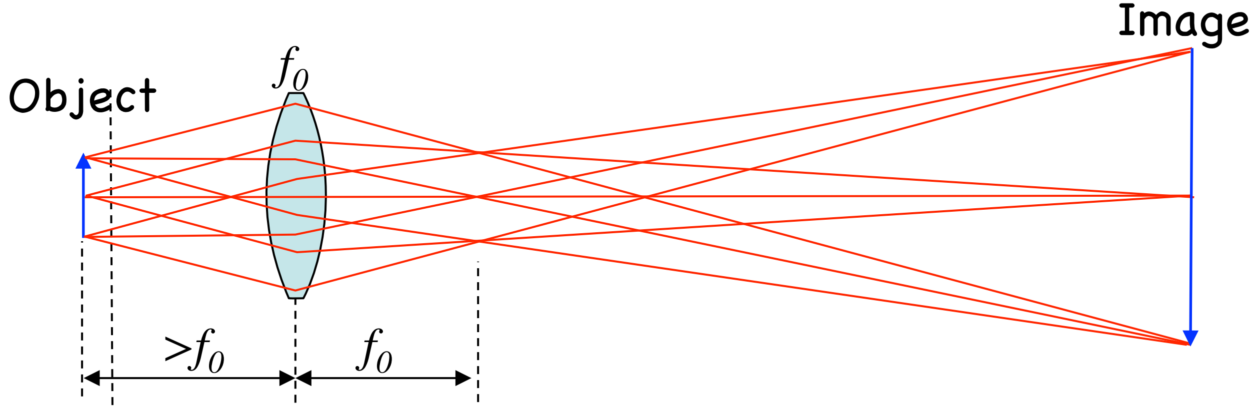

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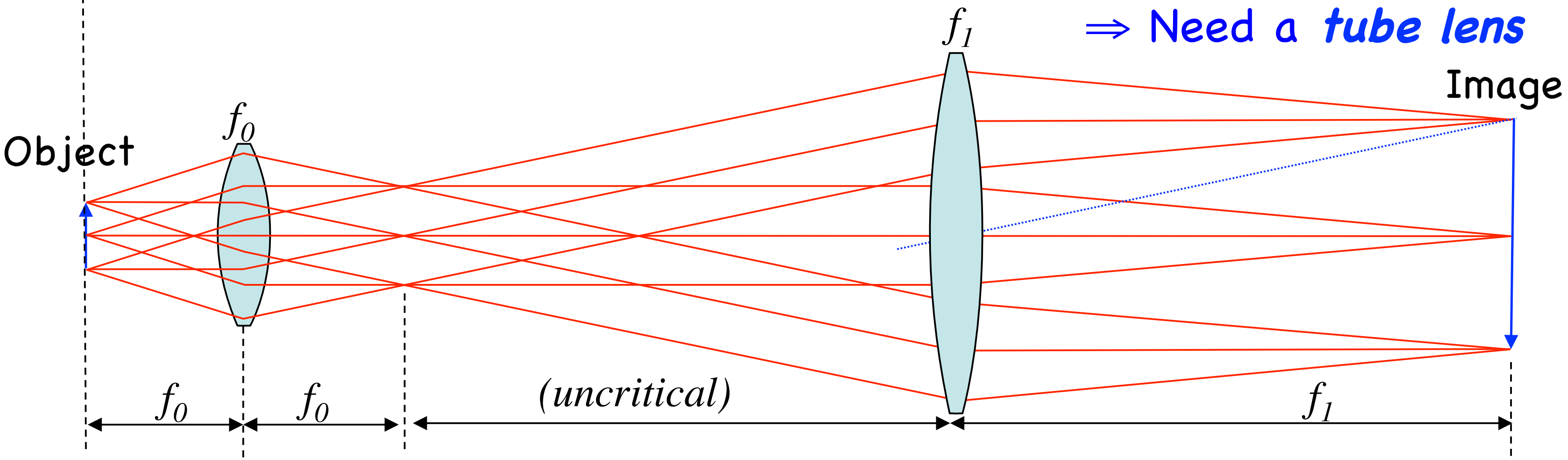
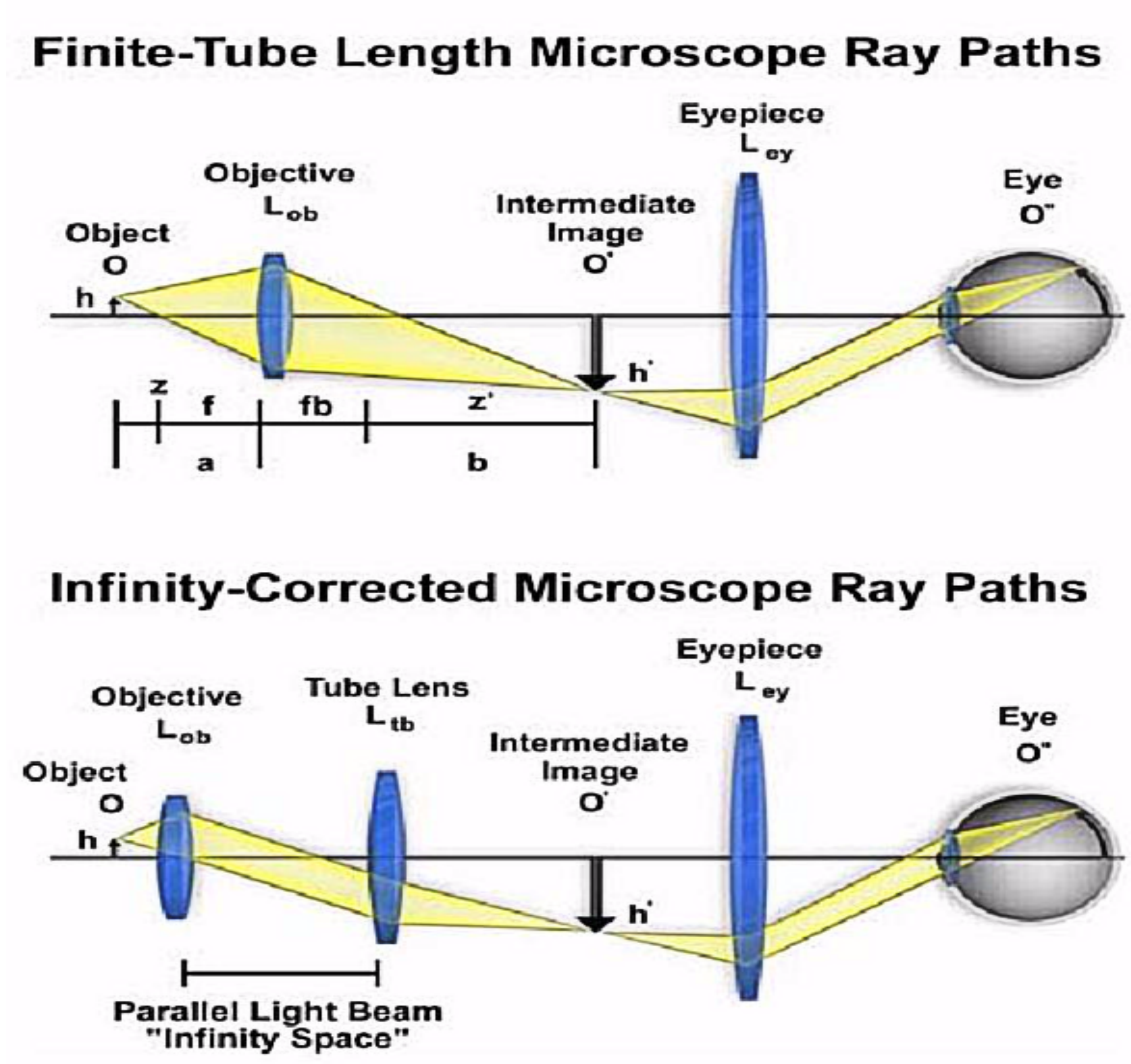
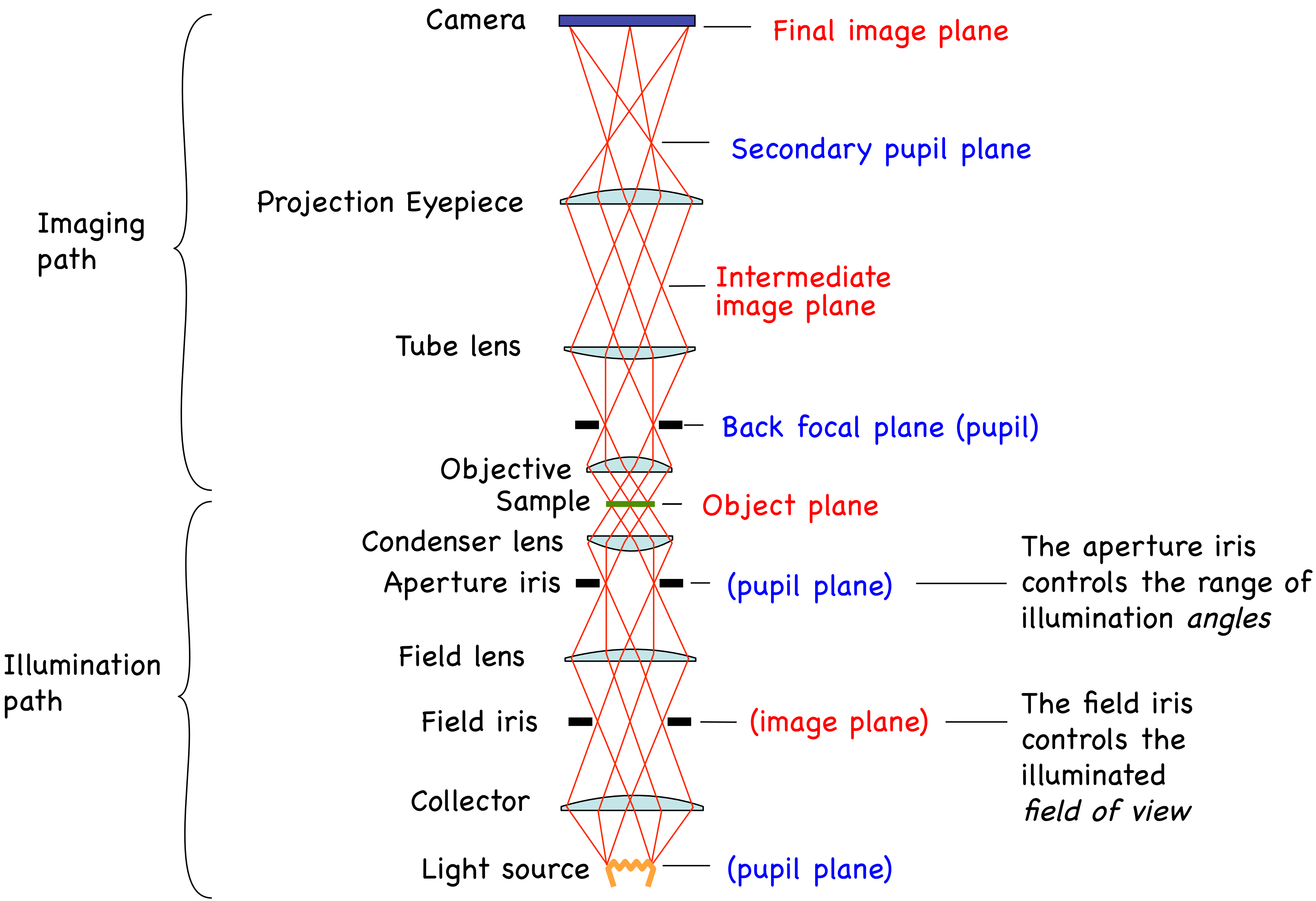


Magnification: $M = \dfrac{f_1}{f_o}$

INFINITY OPTICS

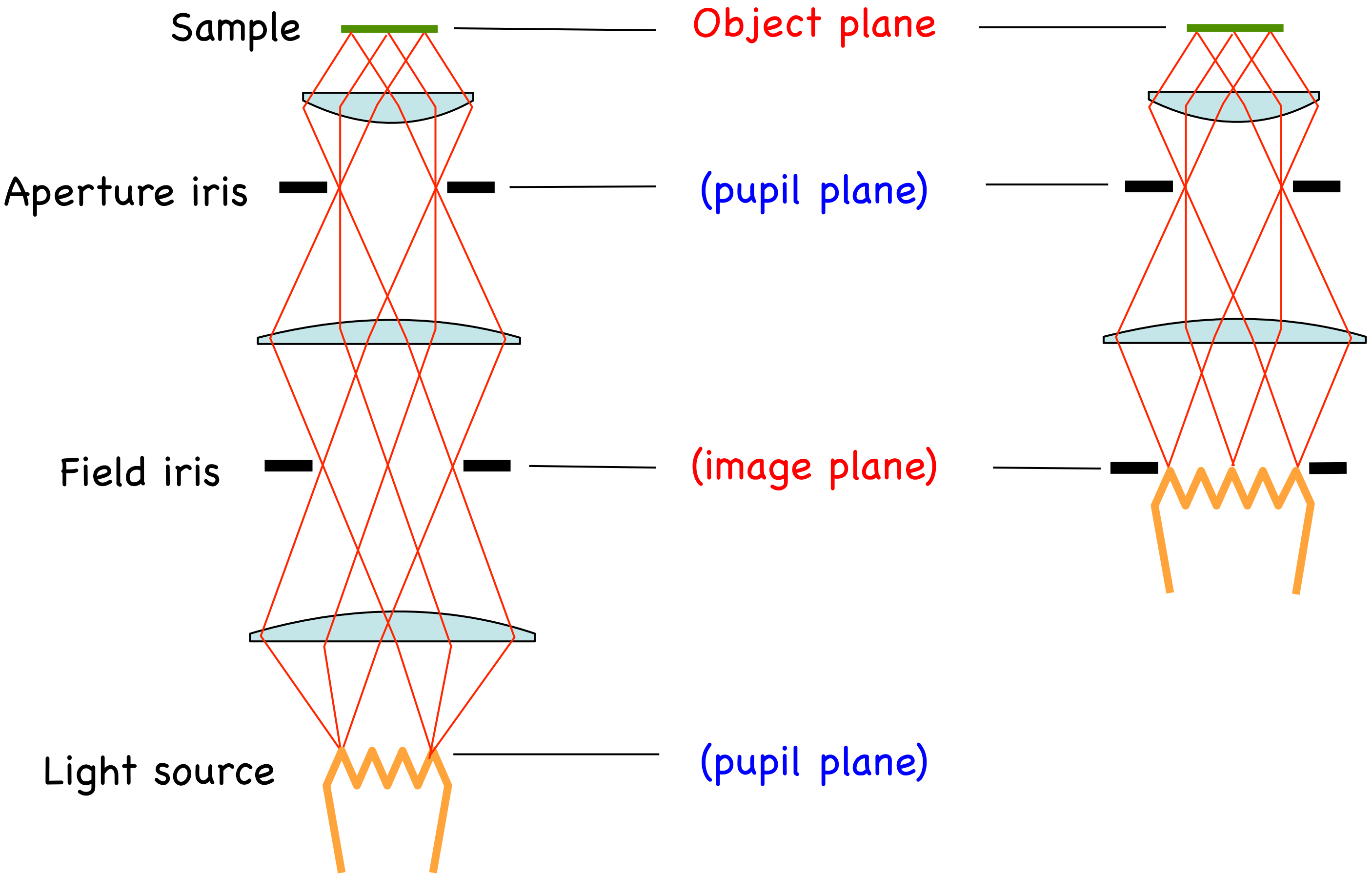


Trans-illumination Microscope



Köhler Illumination

Critical Illumination

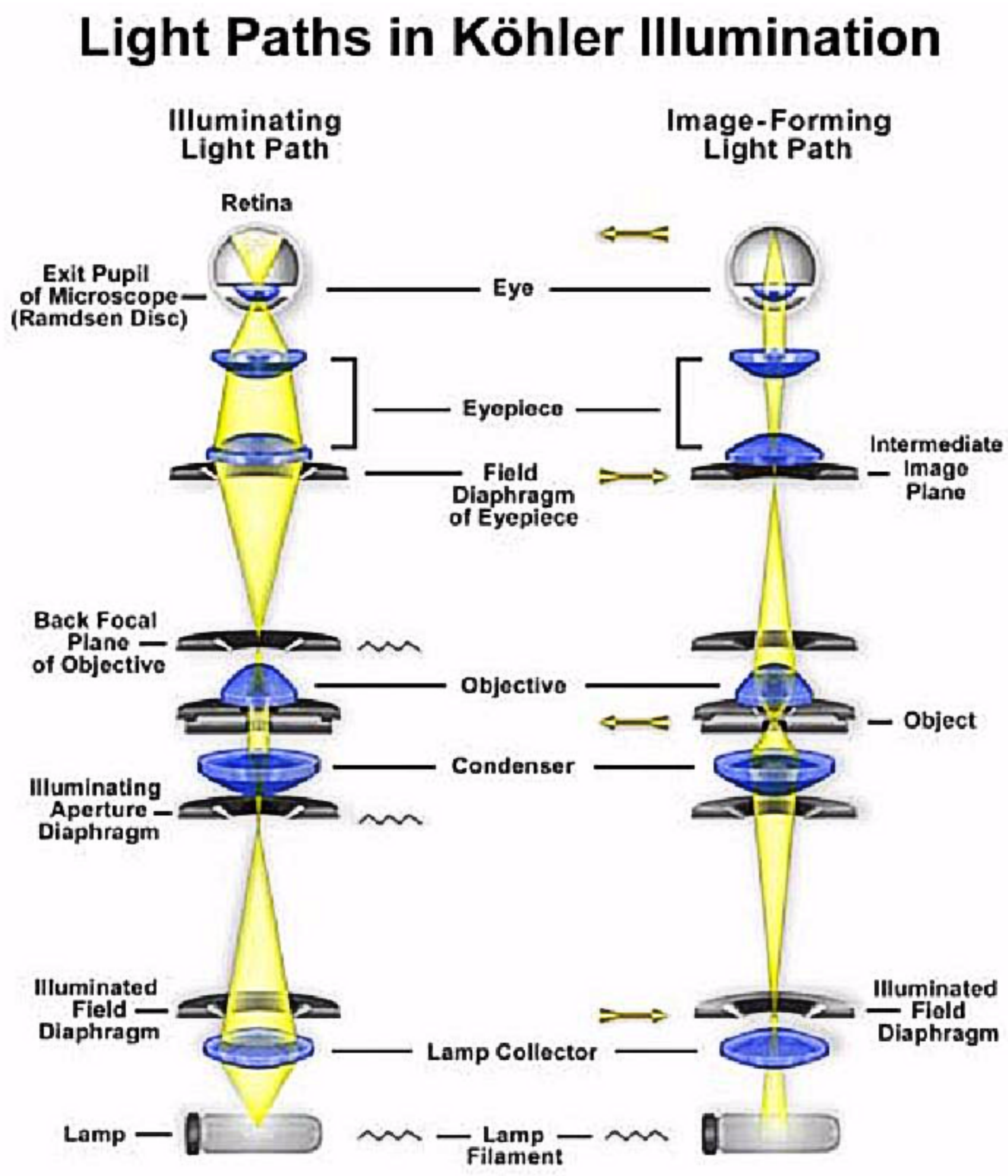


- Each light source point produces a parallel beam of light at the sample
- Uniform light intensity at the sample even if the light source is “ugly” (e.g. a filament)

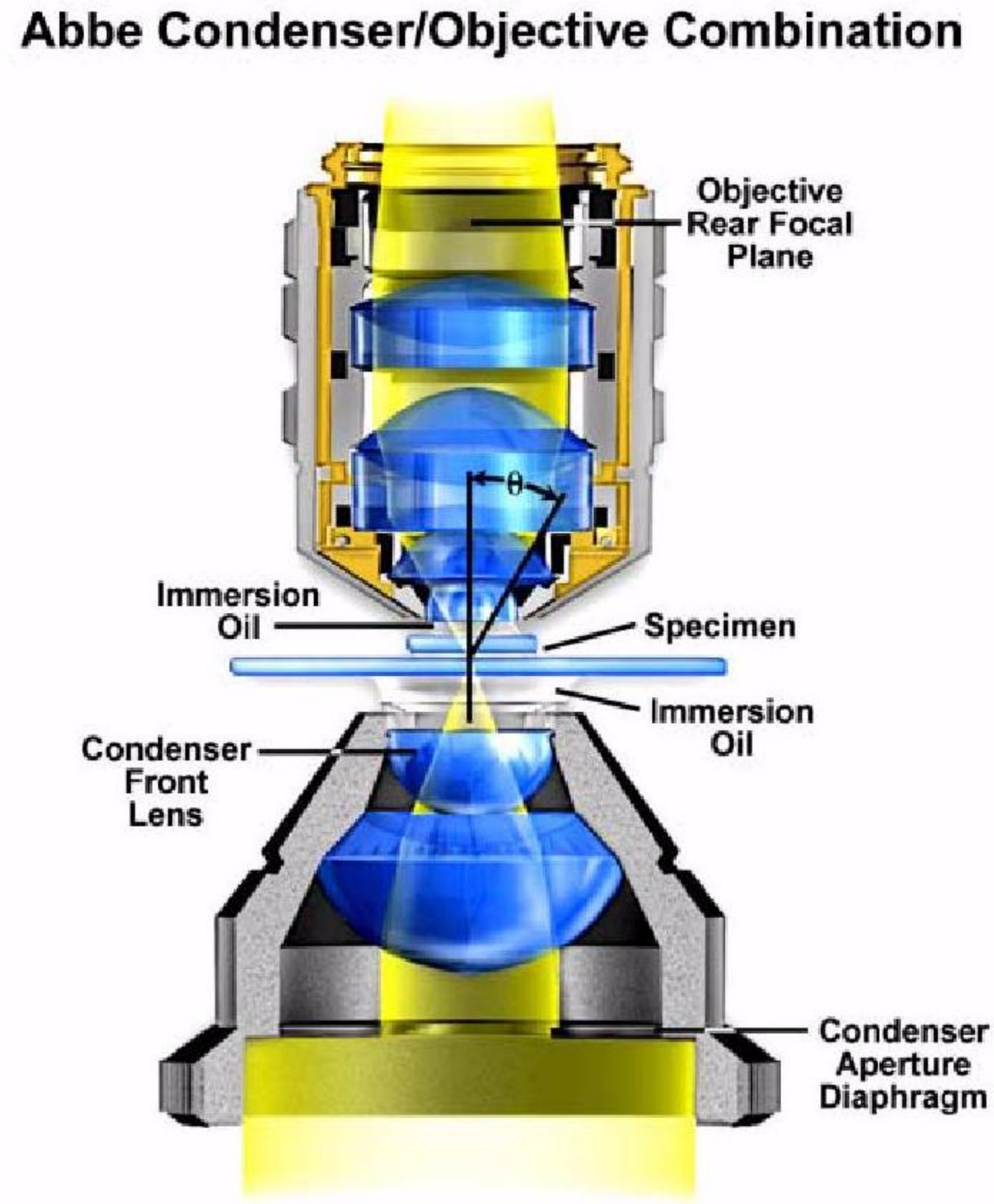
- The source is imaged onto the sample
- Usable only if the light source is perfectly uniform

ILLUMINATION

- Critical
  or
- Kohler



CONDENSER



Rayleigh Criterion:

resolution = $1.22\lambda/(NA_{cond} + NA_{obj})$

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 fitting for one
- Eye cups

Human eye resolves 1-2 minutes of arc.
Maximum useful magnification is about 500-1000 x NA.
Mag>1000NA: EMPTY MAGNIFICATION

Tube Lens

Matching camera pixel to linear magnification

Camera resolves 2-3 pixels length [Niquist sampling]

For camera:1000x1000 pixel 6μm in size
Objective: x100/1.4 resolution=0.2μm
Linear Mag=6x3/0.2=90
Camera Field of View=6/90=67μm

For camera:500x500 pixel 16μm in size
Objective: x100/1.4 resolution=0.2μm
Linear Mag=16x3/0.2=240
With 100x the actual image resolution~0.4μm

Q: What system would acquire better image resolution:
100X/0.95 with 16μm CCD pixel, or 50X/.95 with 8μm

How view the pupil planes?

Two ways:

- “Eyepiece telescope”
- “Bertrand lens”

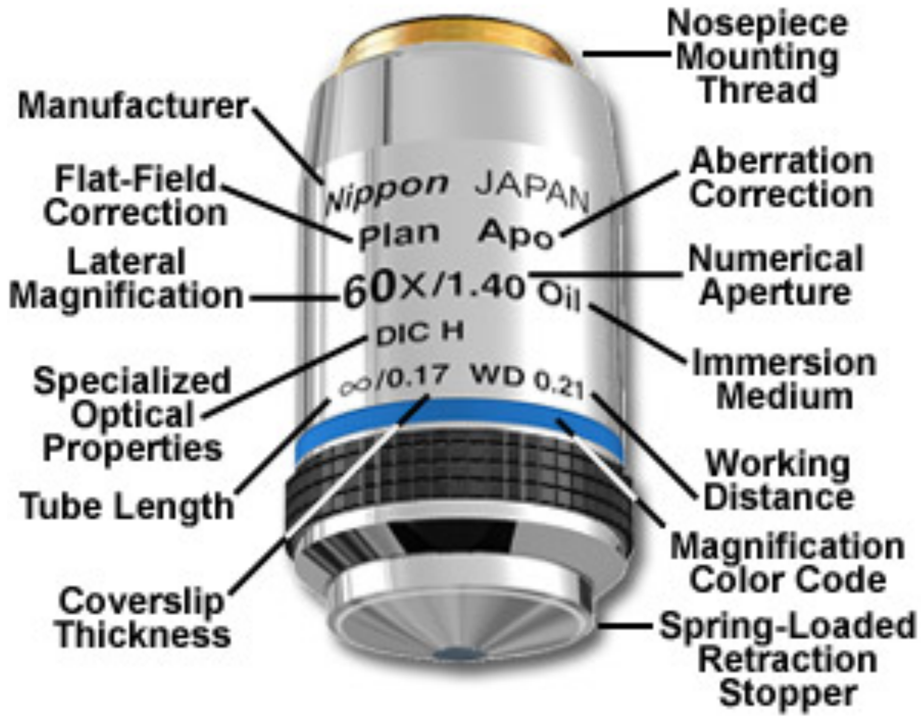
Why view the pupil planes?

Align illumination:

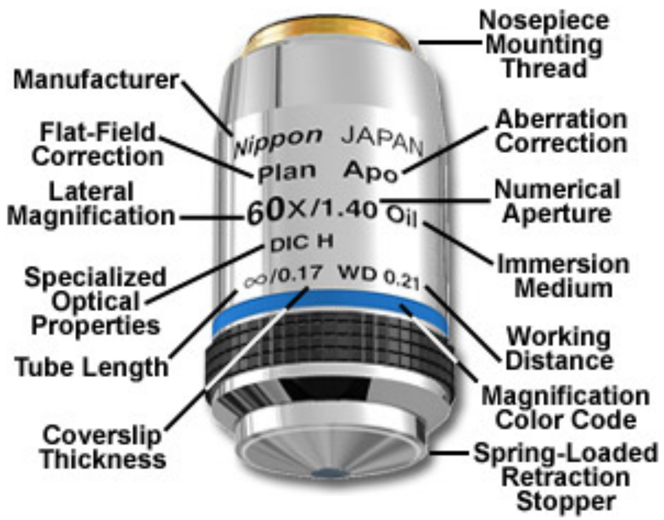
- Koehler
- Phase rings
- Nomarski

By far the most important part:

the Objective Lens



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



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

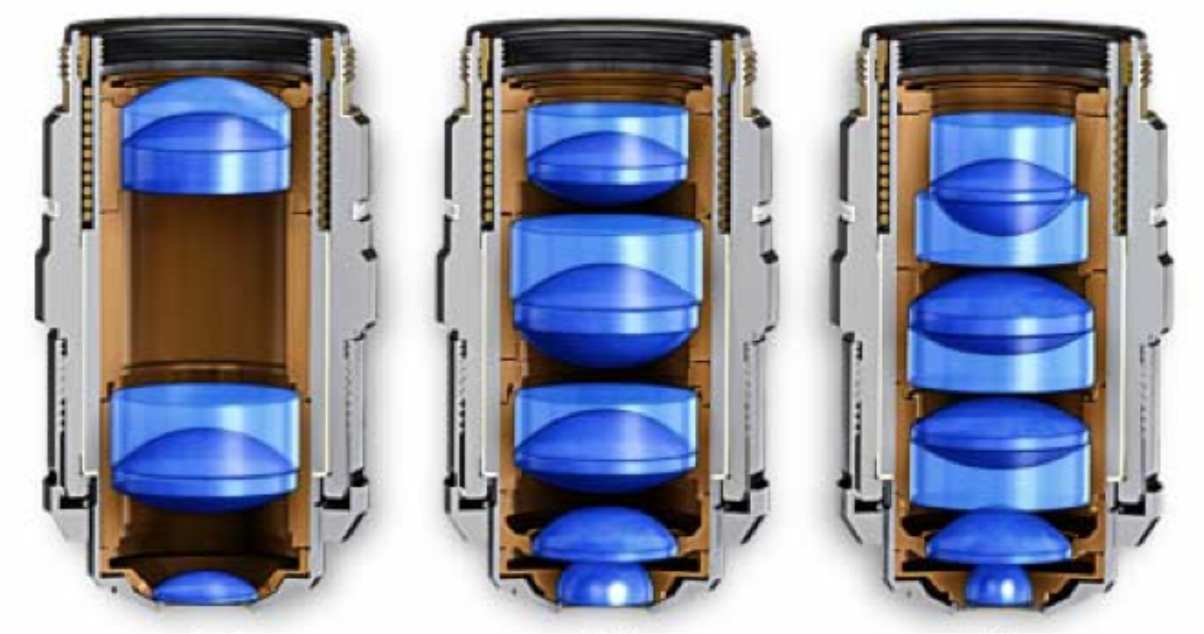
Field flatness
- Plan or not

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

Correction classes of objectives



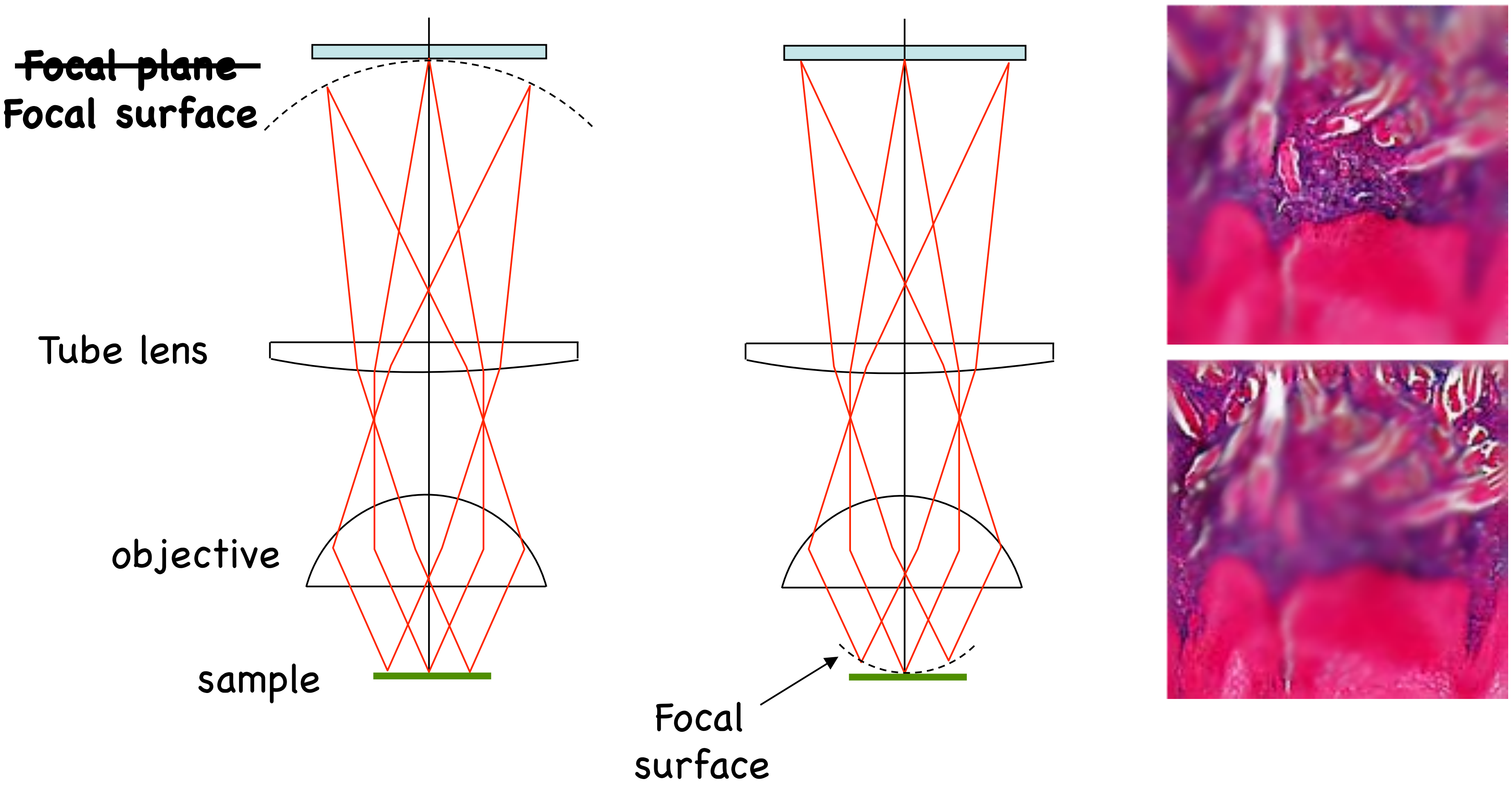
Achromat
(cheap)

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

Apochromat
(best correction)

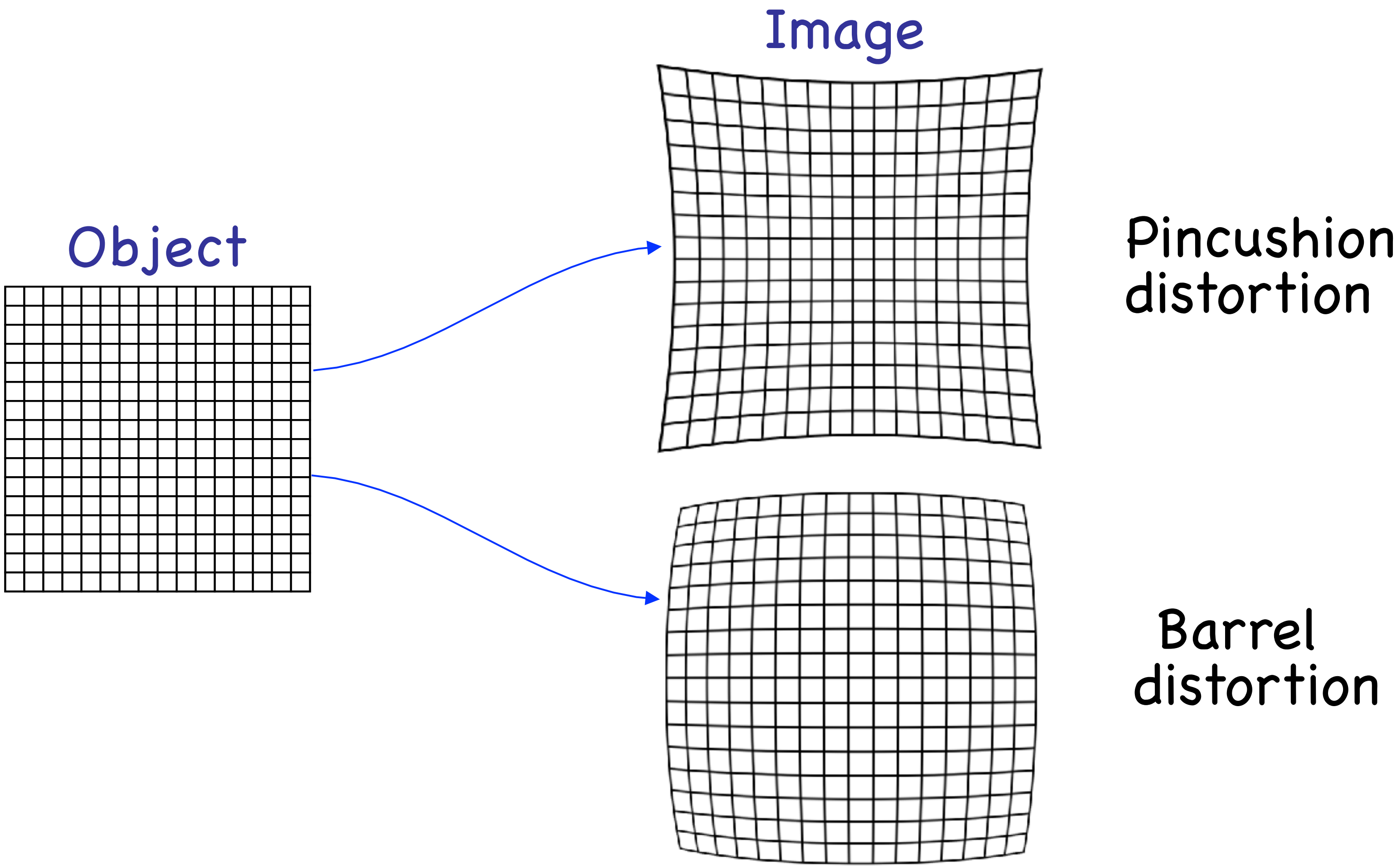
Correction for other (i.e. monochromatic) aberrations also improves in the same order

Curvature of Field



Geometric Distortion

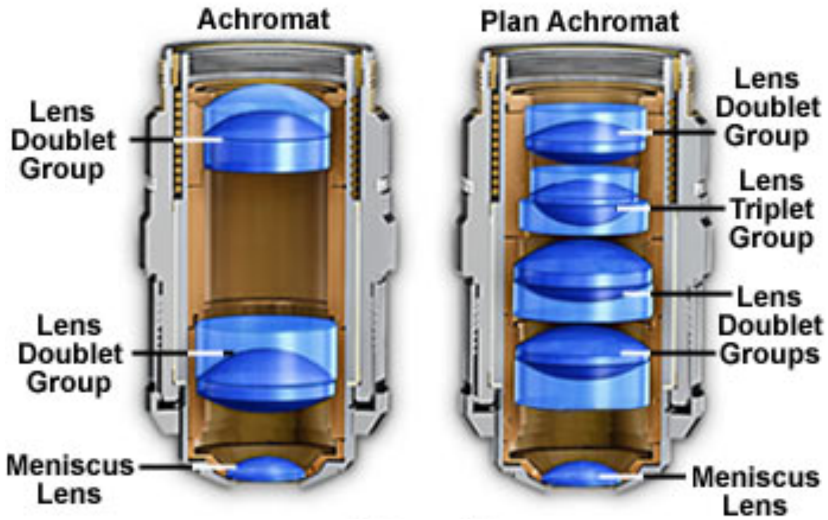
= Radially varying magnification



May be introduced by the projection eyepiece

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)

$\Rightarrow$ mixing brands can produce severe LCA

LCA correction:

In objective	In tube lens
Nikon	Leica
Olympus	Zeiss

Objective Designations

Abbreviation	Type
Achro, Achromat	Achromatic aberration correction
Fluor, Fl, Fluar, Neofluar, Fluotar	Fluorite aberration correction
Apo	Apochromatic aberration correction
Plan, Pl, Achroplan, Plano	Flat Field optical correction
EF, Acroplan	Extended Field (field of view less than Plan)
N, NPL	Normal field of view plan
Plan Apo	Apochromatic and Flat Field correction
UPLAN	Olympus Universal Plan (Brightfield, Darkfield, DIC, and Polarized Light)
LU	Nikon Luminous Universal (Brightfield, Darkfield, DIC, and Polarized Light)
L, LL, LD, LWD	Long Working Distance
ELWD	Extra-Long Working Distance
SLWD	Super-Long Working Distance
ULWD	Ultra-Long Working Distance
Corr, W/Corr, CR	Correction Collar
I, Iris, W/Iris	Adjustable numerical aperture (with iris diaphragm)
Oil, Oel	Oil Immersion
Water, WI, Wasser	Water Immersion
HI	Homogeneous Immersion
Gly	Glycerin Immersion
DIC, NIC	Differential or Nomarski Interference Contrast
CF, CFI	Chrome-Free, Chrome-Free Infinity-Corrected (Nikon)
ICS	Infinity Color-Corrected System (Zeiss)
RMS	Royal Microscopical Society objective thread size
M25, M32	Metric 25-mm objective thread;
Metric 32-mm objective thread	
Phase, PHACO, PC	Phase Contrast
Ph 1, 2, 3, etc.	Phase Condenser Annulus 1, 2, 3, etc.
DL, DLL, DM, BM	Phase Contrast: Dark Low, Dark Low Low, Dark medium, Bright Medium
PL, PLL	Phase Contrast: Positive Low, Positive Low Low
PM, PH	Phase Contrast: Positive Medium, Positive High Contrast (Regions with higher refractive index appear darker.)
NL, NM, NH	Phase Contrast: Negative Low, Negative Medium, Negative High Contrast (Regions with higher refractive index appear lighter.)
P, Po, Pol, SF	Strain-Free, Low Birefringence,
for Polarized Light	
U, UV, Universal	UV transmitting (down to approximately 340 nm) for UV-excited epifluorescence
UIS	Universal Infinity System (Olympus)
M	Metallographic (no coverslip)
NC, NCG	No Coverslip
EPI	Oblique or Epi illumination
TL	Transmitted Light
BBD, HD, B/D	Bright or Dark Field (Hell, Dunkel)
D	Darkfield
H	For use with a heating stage
U, UT	For use with a universal stage
DI, MI, TI	Interferometry, Noncontact, Multiple Beam (Tolanski)

Choosing Objectives

- Brightfield, phase, fluorescence, DIC ?
- Resolution and field of view
- Working distance
- Cover slip thickness
- Wavelength range
- Immersion medium
- Budget

From geometrical optics

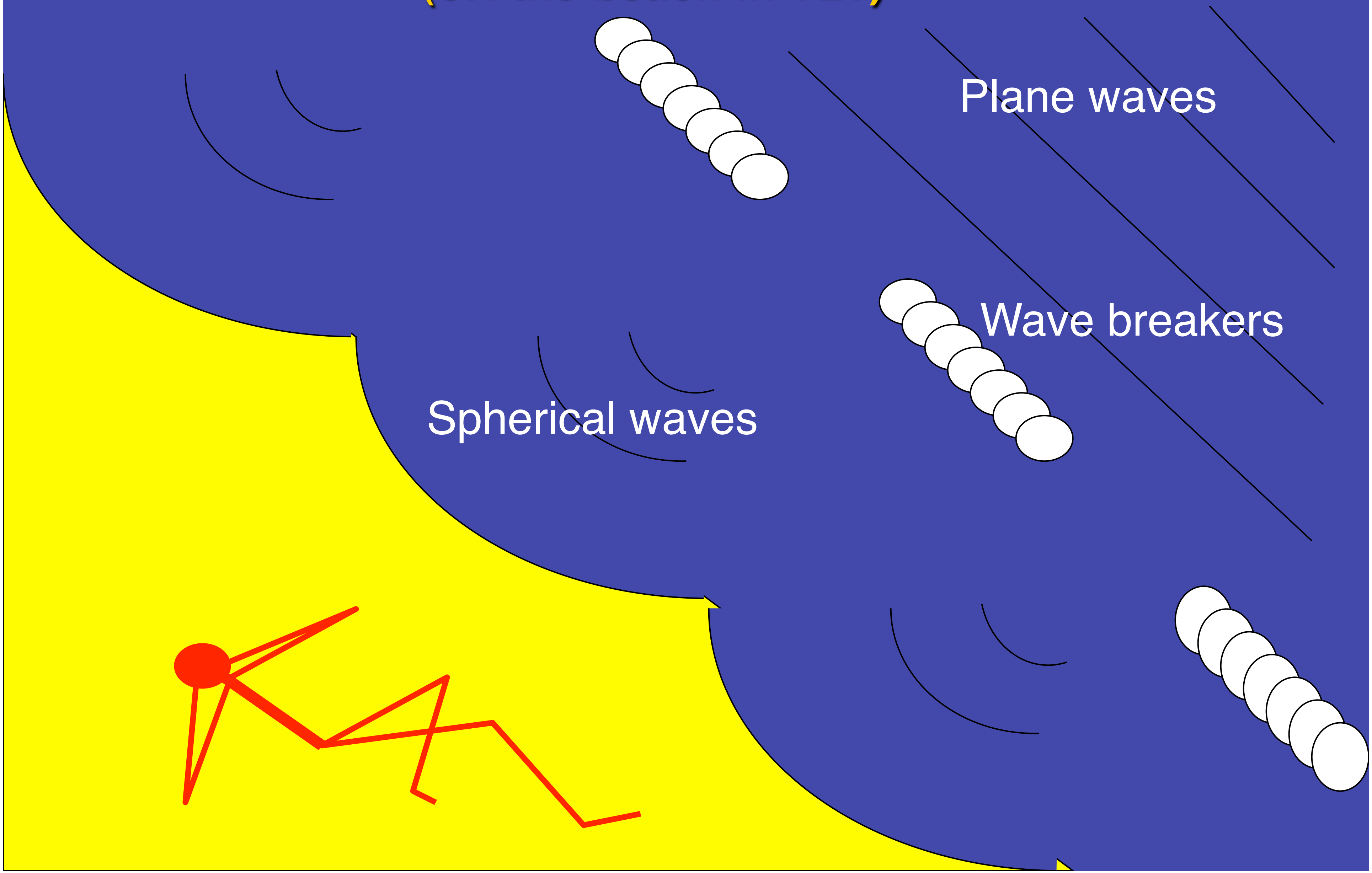
To

Wave optics

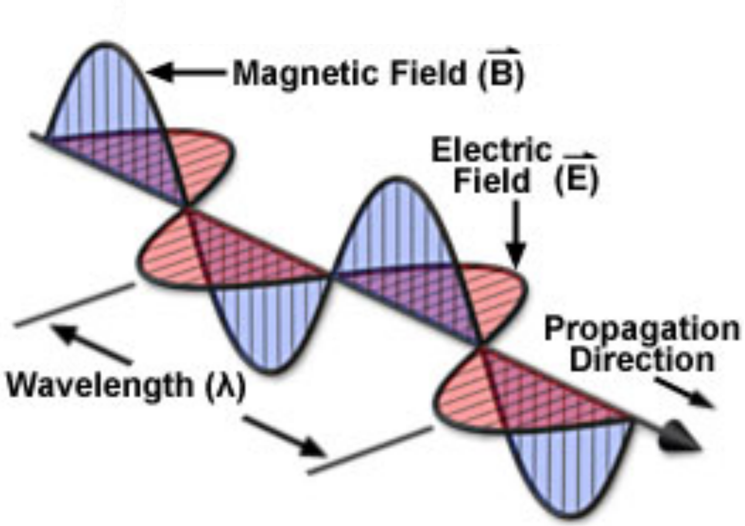
Or

Why we cannot correct optics to infinite sharpness

The Wave Nature of Light
(or: the beach in TLV)



Light as an Electromagnetic Wave



Refractive Index: n [~1-1.5]

Speed of Light: c [3 10^{10}cm/sec]

Wavelength: $\lambda = c/n/\nu$ [~0.5mm]

Wave Vector: $k = \omega n/c$

Frequency: $\nu = \omega/2\pi$ [6 10^{14}Hz]

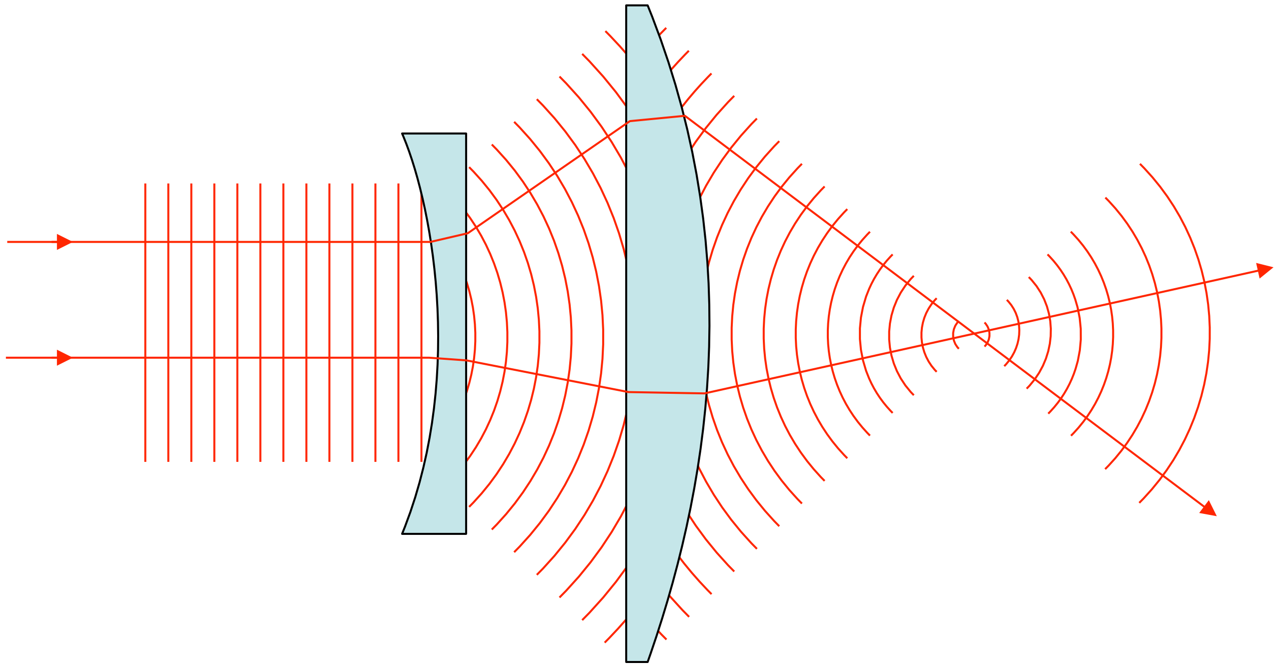
Plane wave Space-Time Equation:

$A \exp[kz\text{-}\omega t]$

Most matter interacts mostly with the electric field ⇒ Ignore the magnetic field

Polarization = direction of electric field

Rays are perpendicular to wavefronts

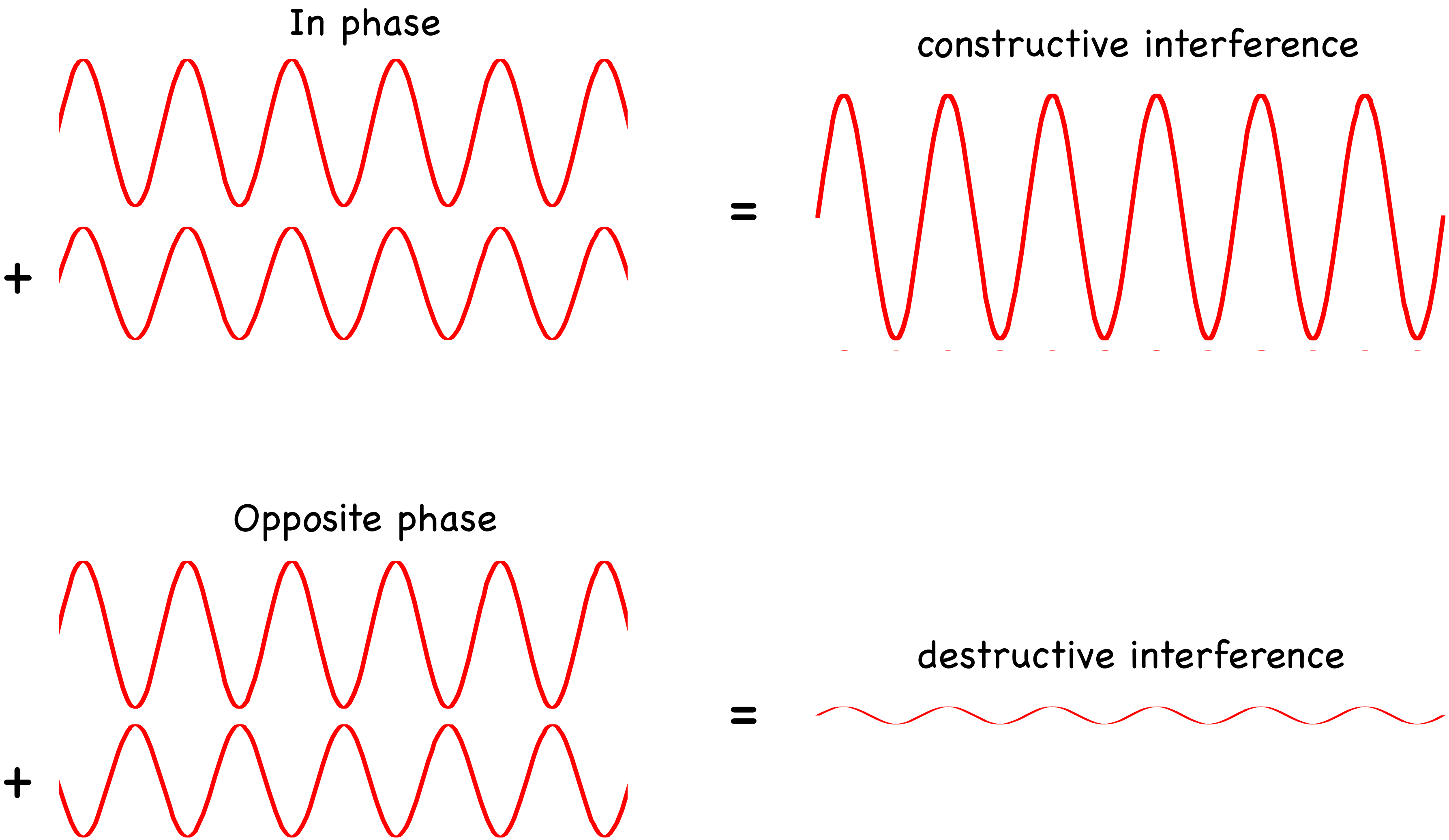


Space-time COHERENCE

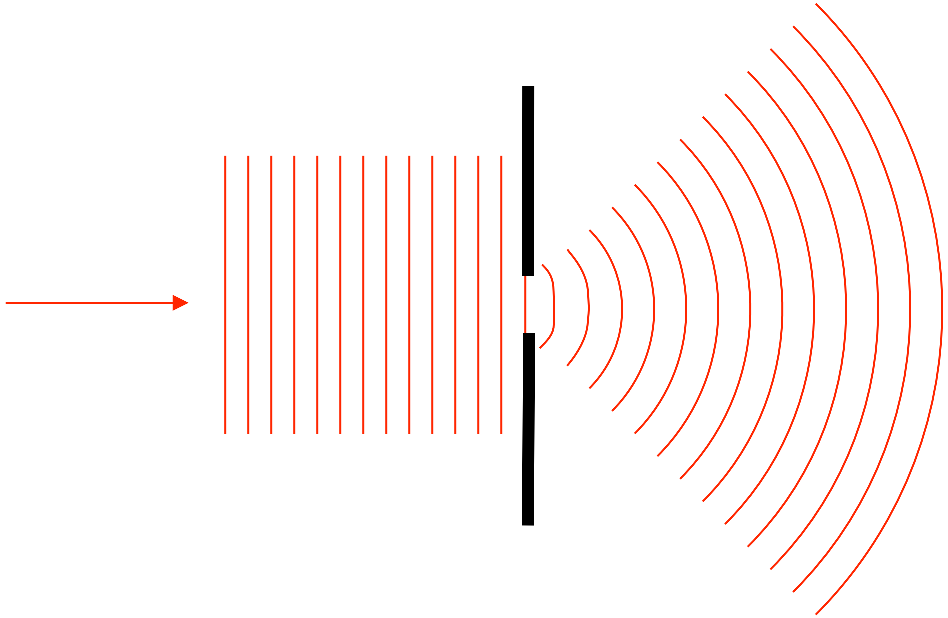
coherent light

incoherent light

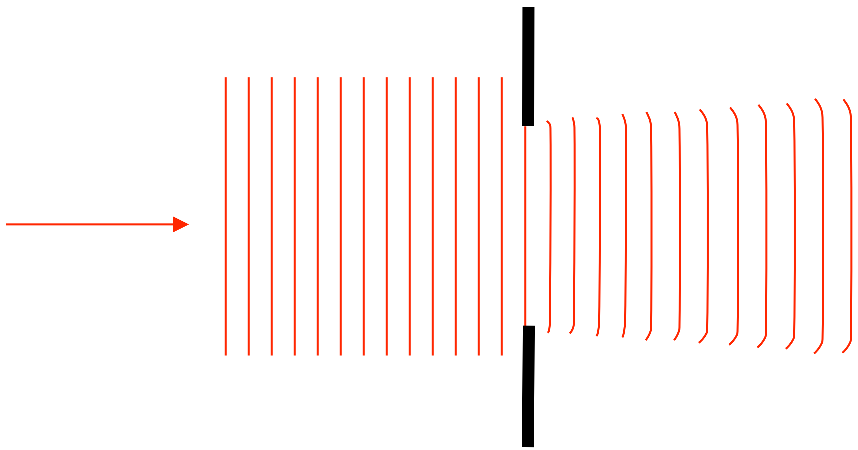
Interference



Diffraction by an aperture drawn as waves



Light spreads to new angles

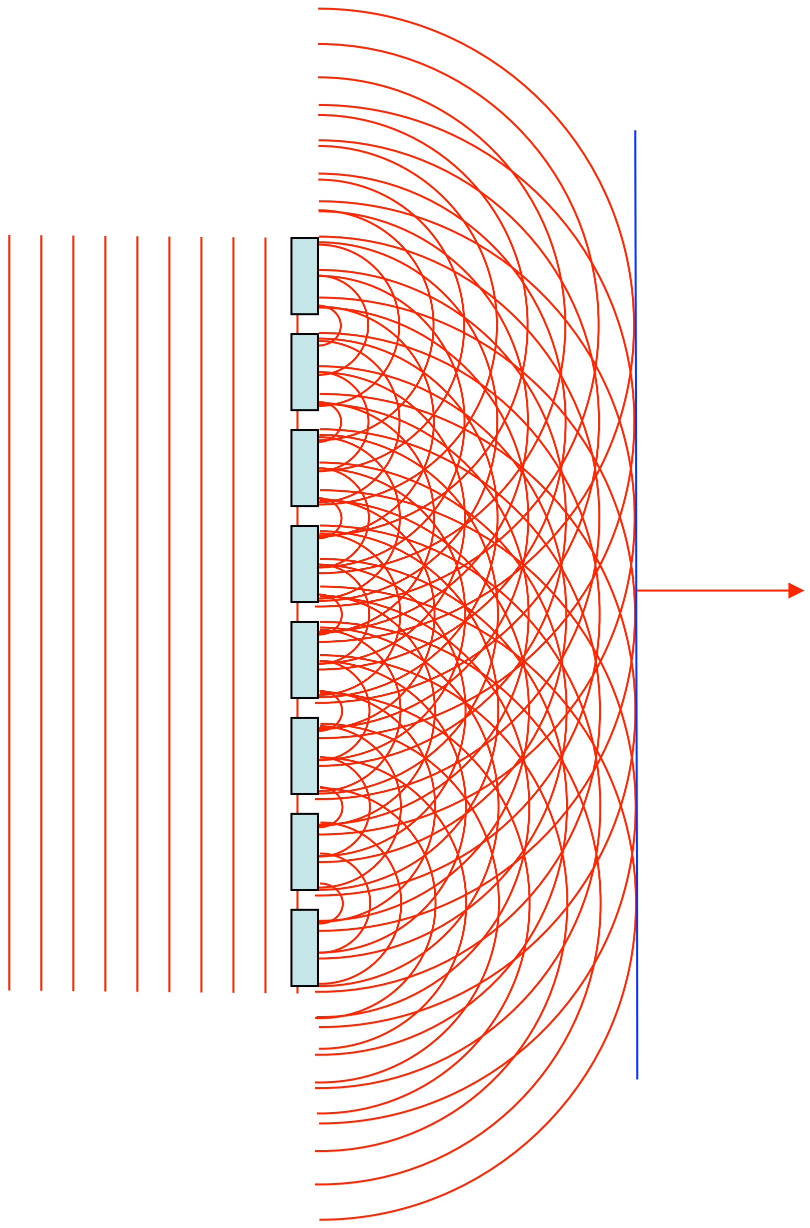


Larger aperture

⇔

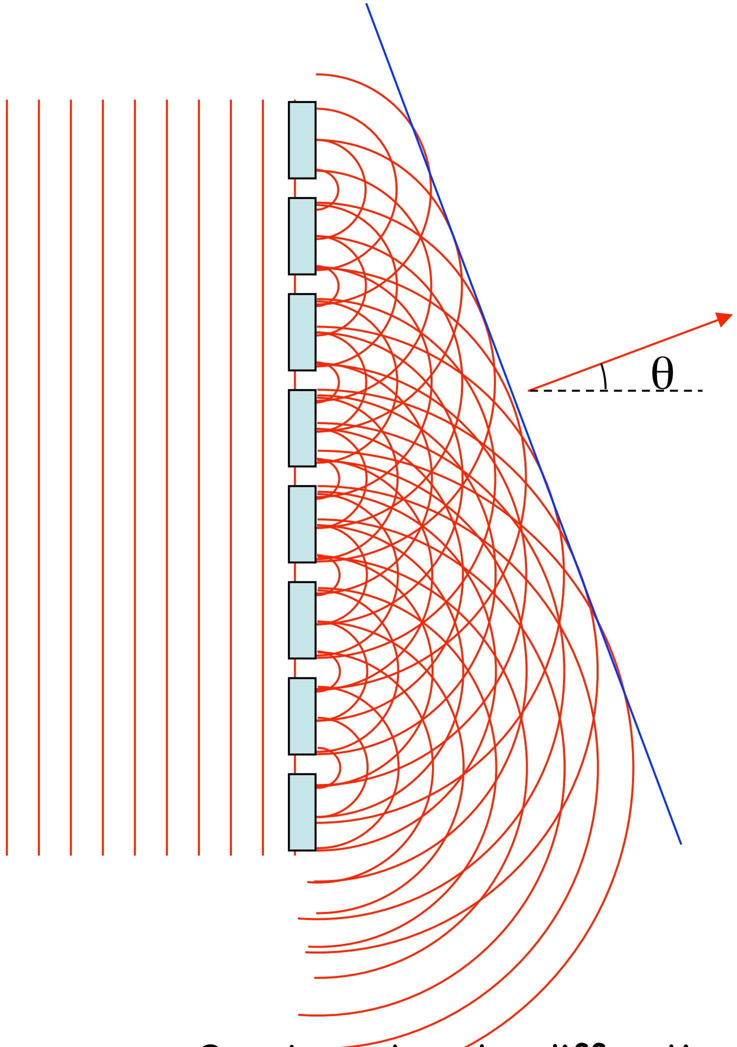
weaker diffraction

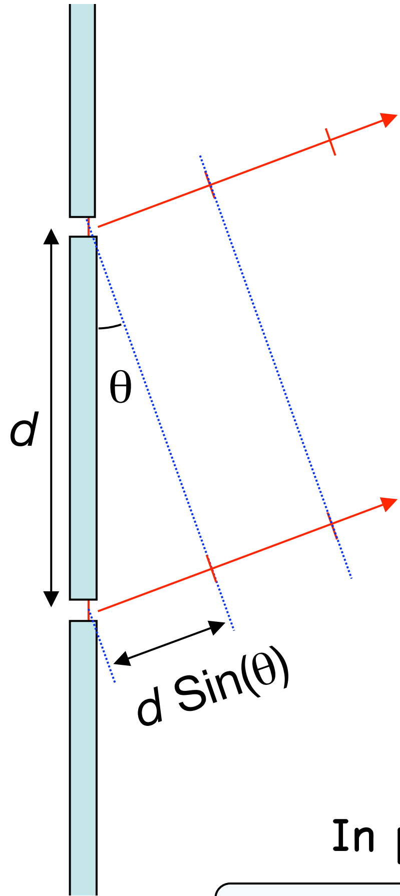
Diffraction by a periodic structure (grating)



Huygens-Fresnel principle

Diffraction by a periodic structure (grating)





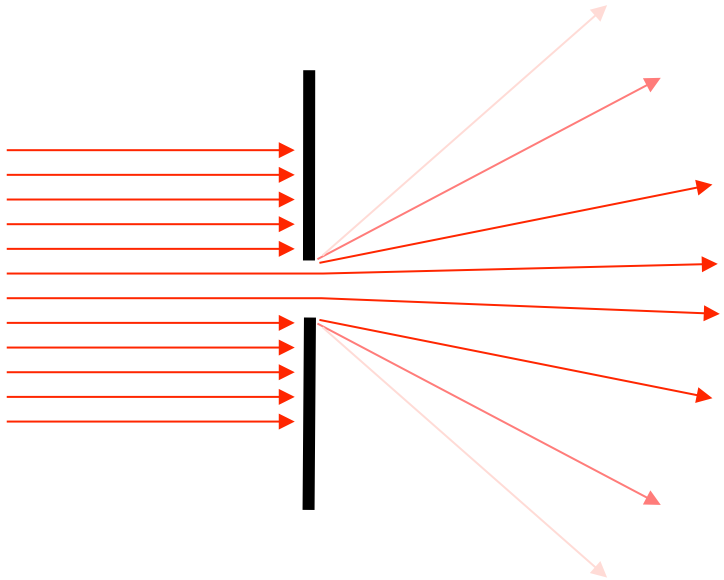
In phase if:

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

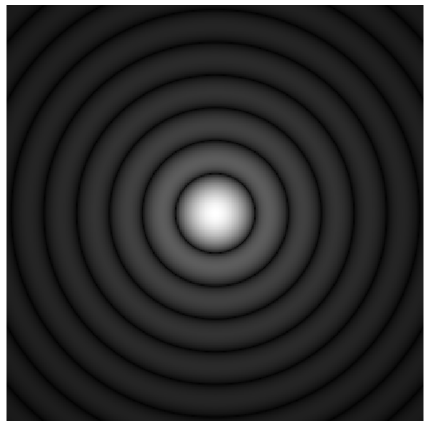
for some integer m

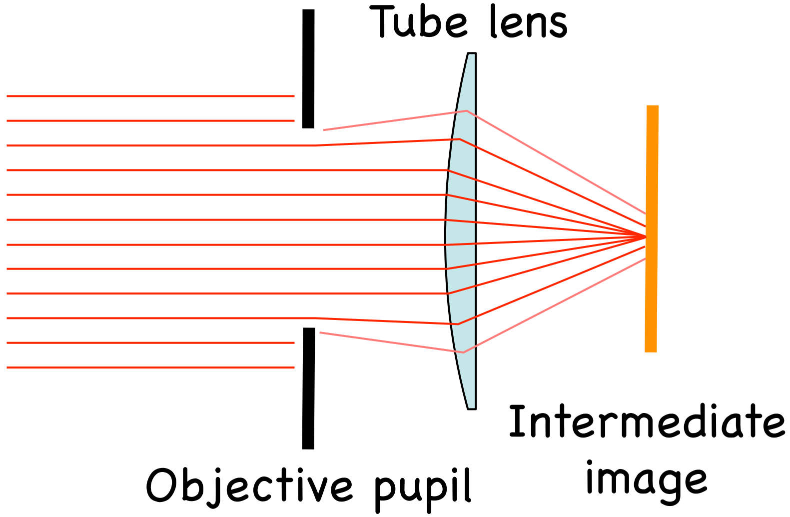
Q: why colors by diffraction from a CD

Diffraction by an aperture drawn as rays



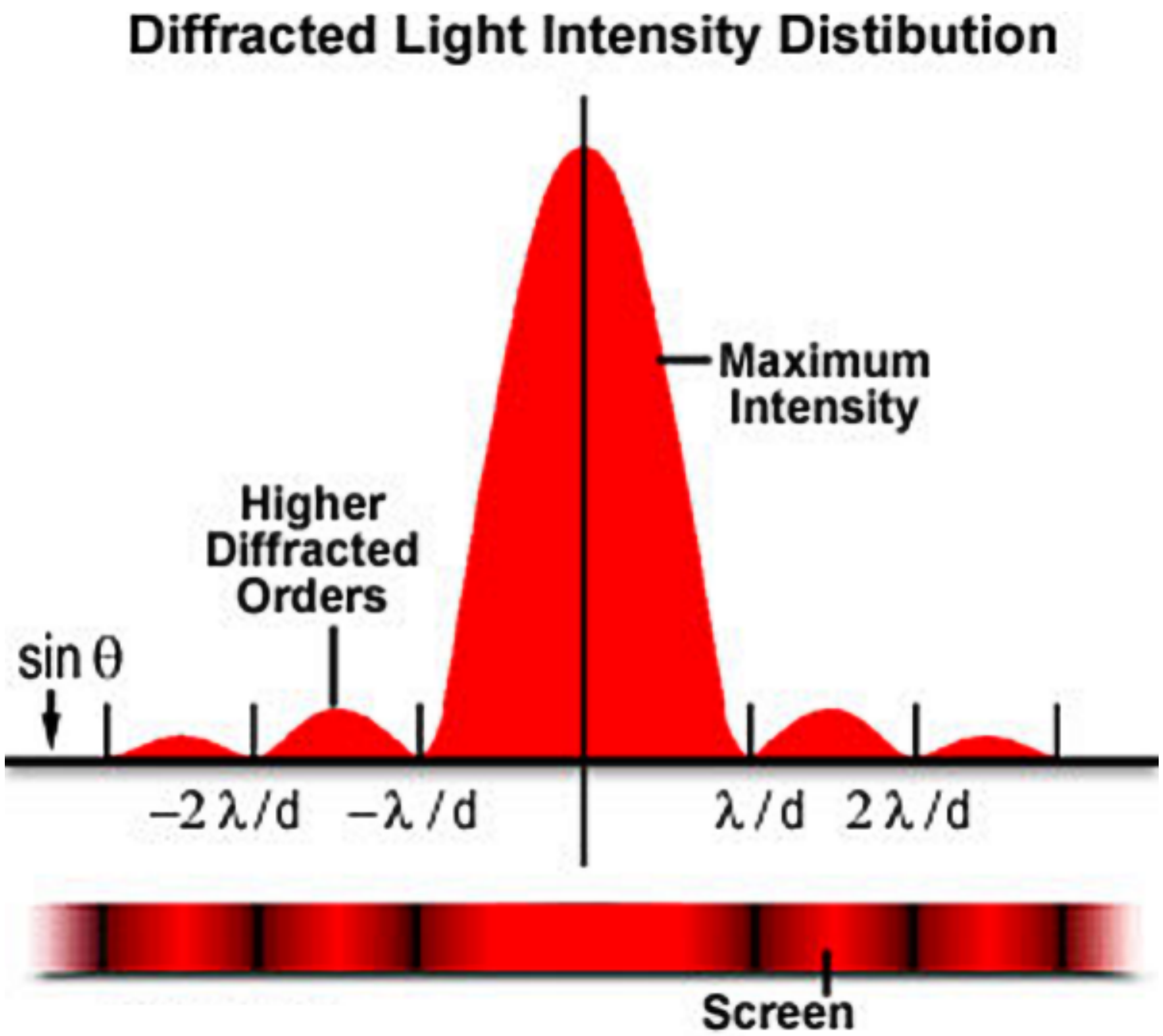
The pure, "far-field" diffraction pattern is formed at ∞ distance...





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

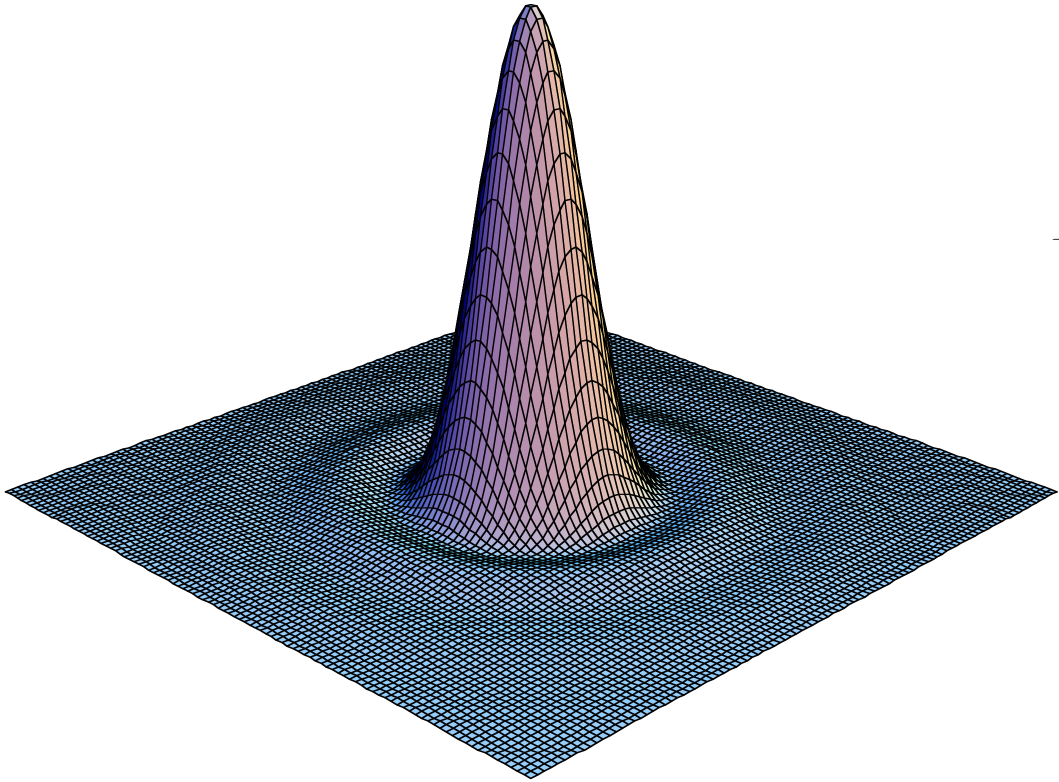
...as happens in a microscope

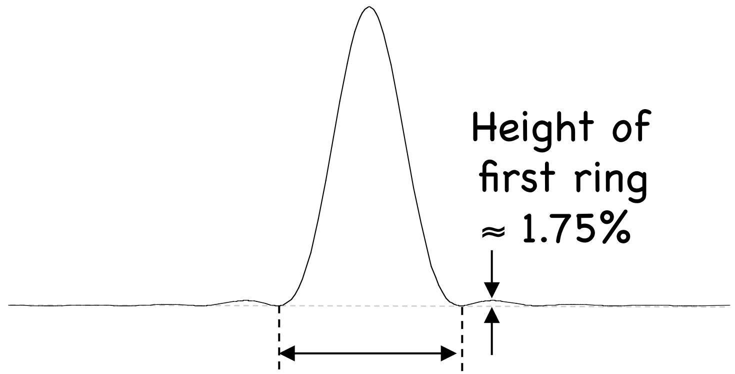


Slit: Sinc function [sin(θ)/θ]   Hole: Bessel function j(θ)

The Airy Pattern

= the far-field diffraction pattern from a round aperture

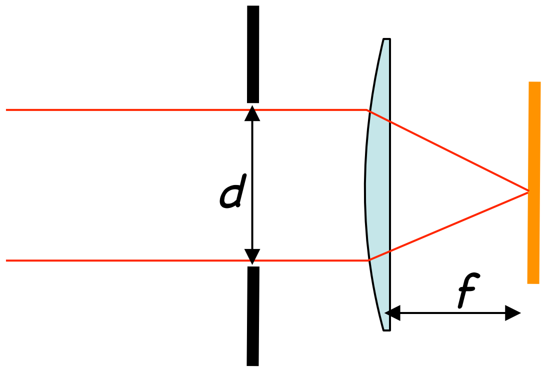




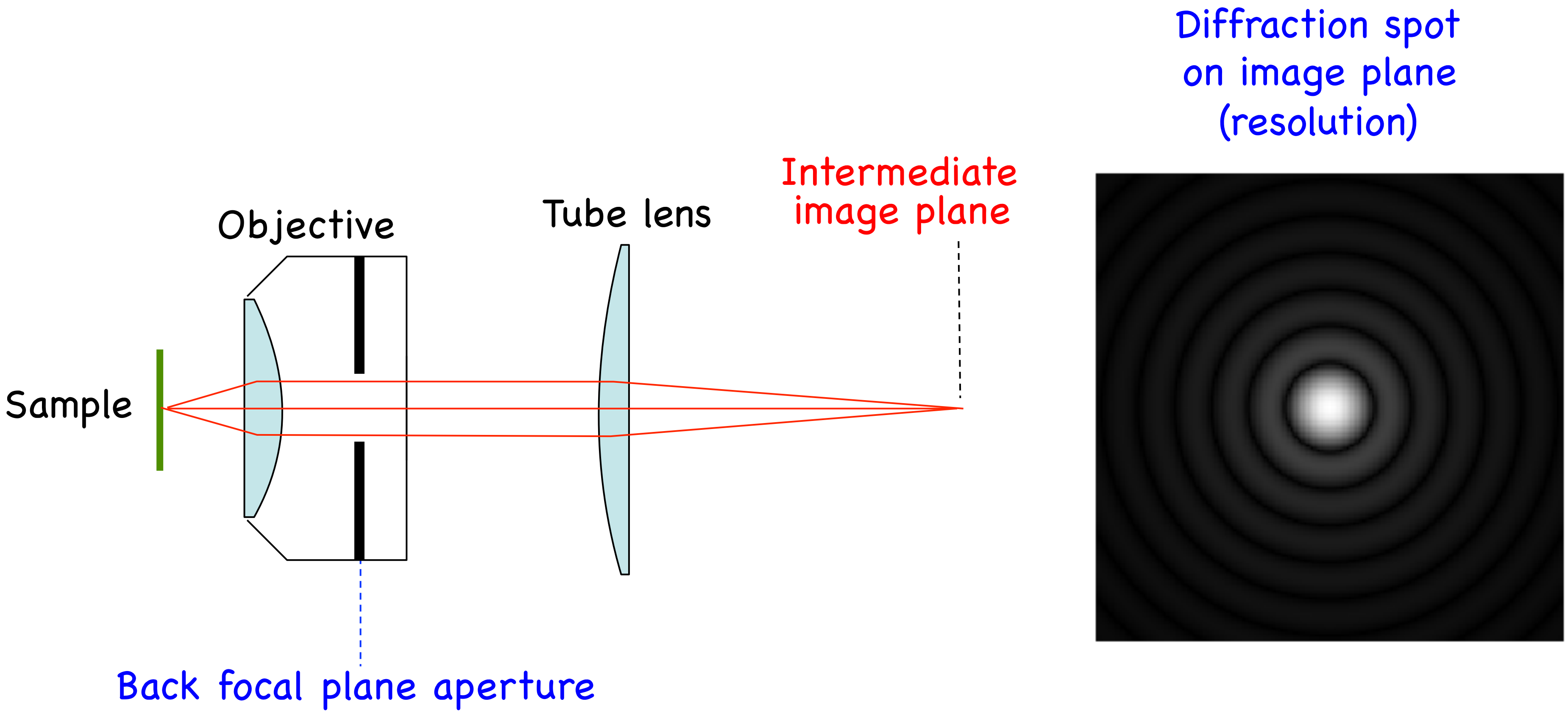
"Airy disk" diameter

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

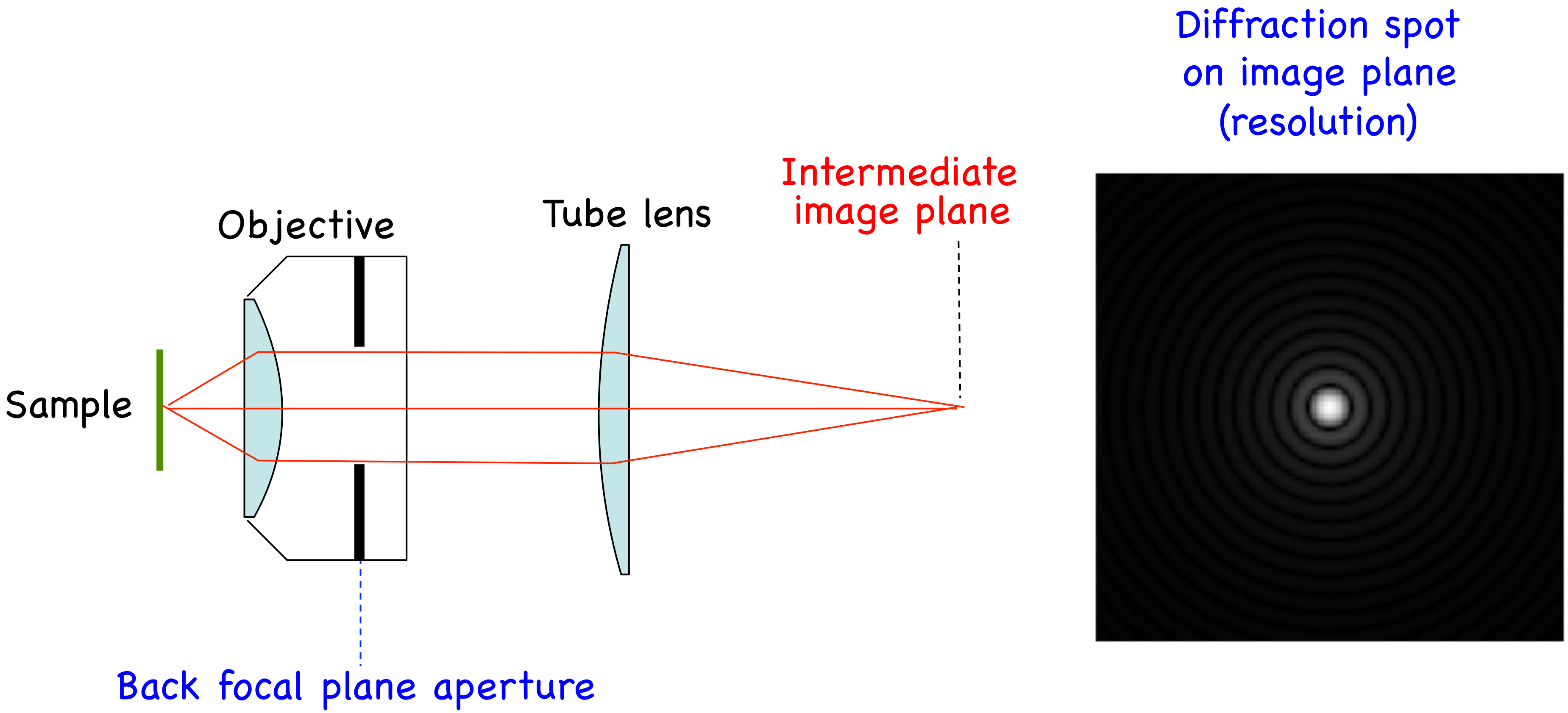
(for small angles d/f)



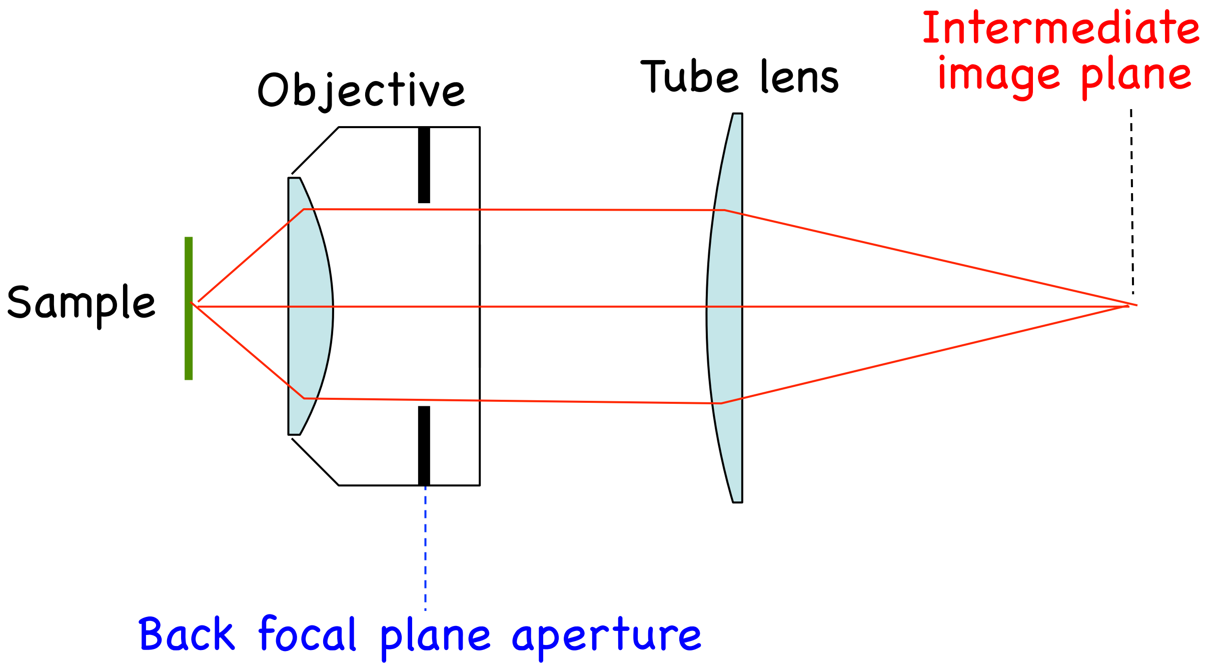
Aperture and Resolution



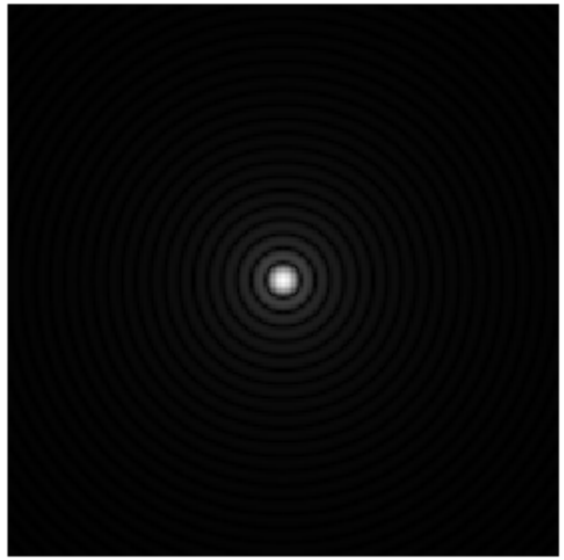
Aperture and Resolution



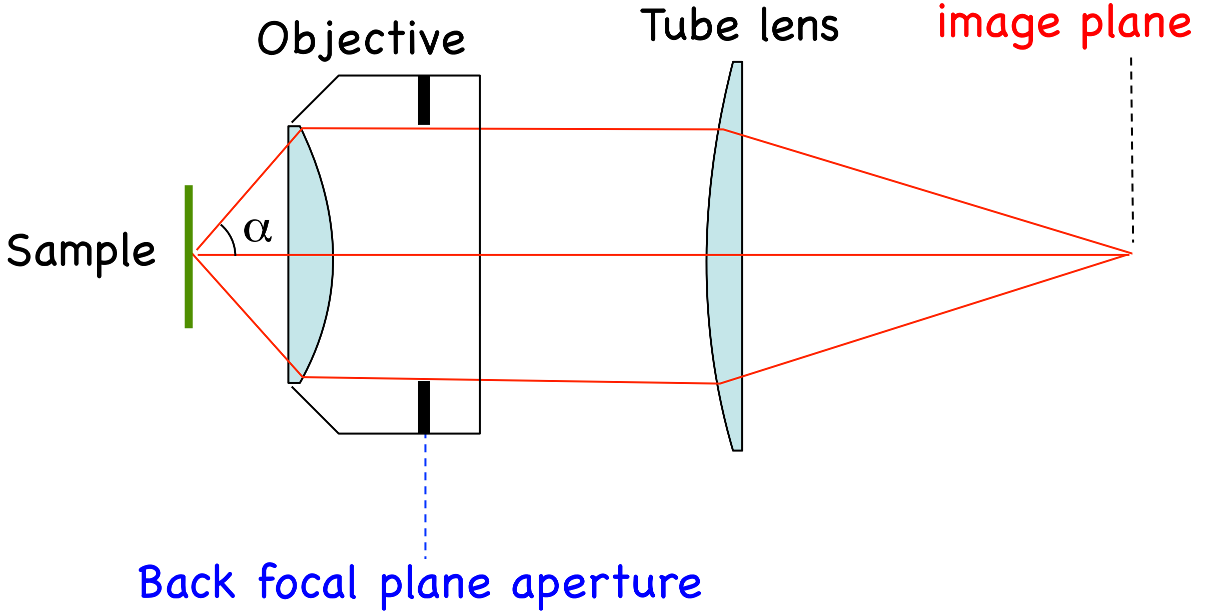
Aperture and Resolution

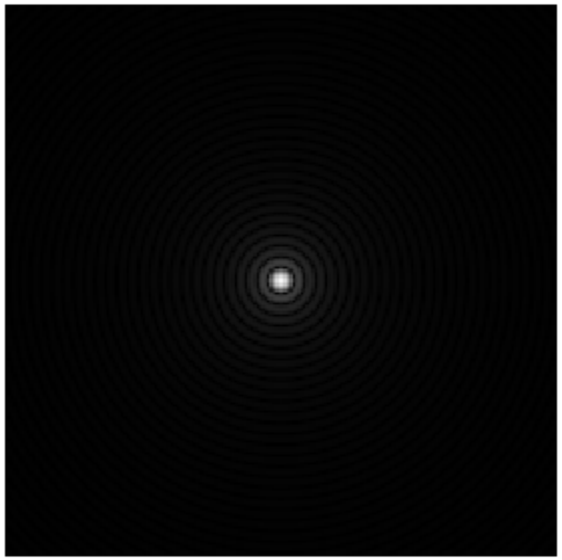


Diffraction spot on image plane (resolution)



Aperture and Resolution



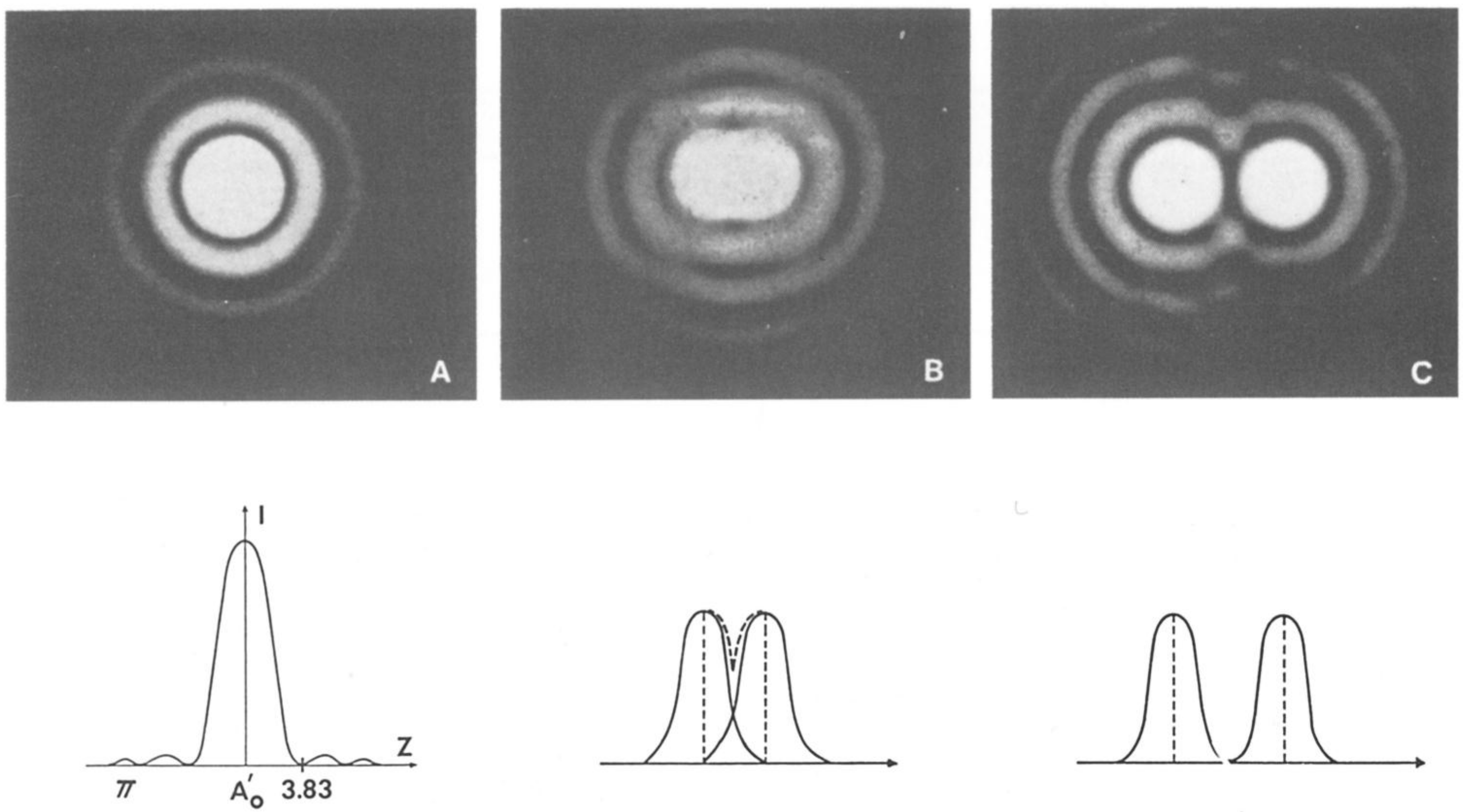


- Image resolution improves with ~~aperture size~~ Numerical Aperture (NA)

$$\boxed{NA = n \sin(\alpha)}$$

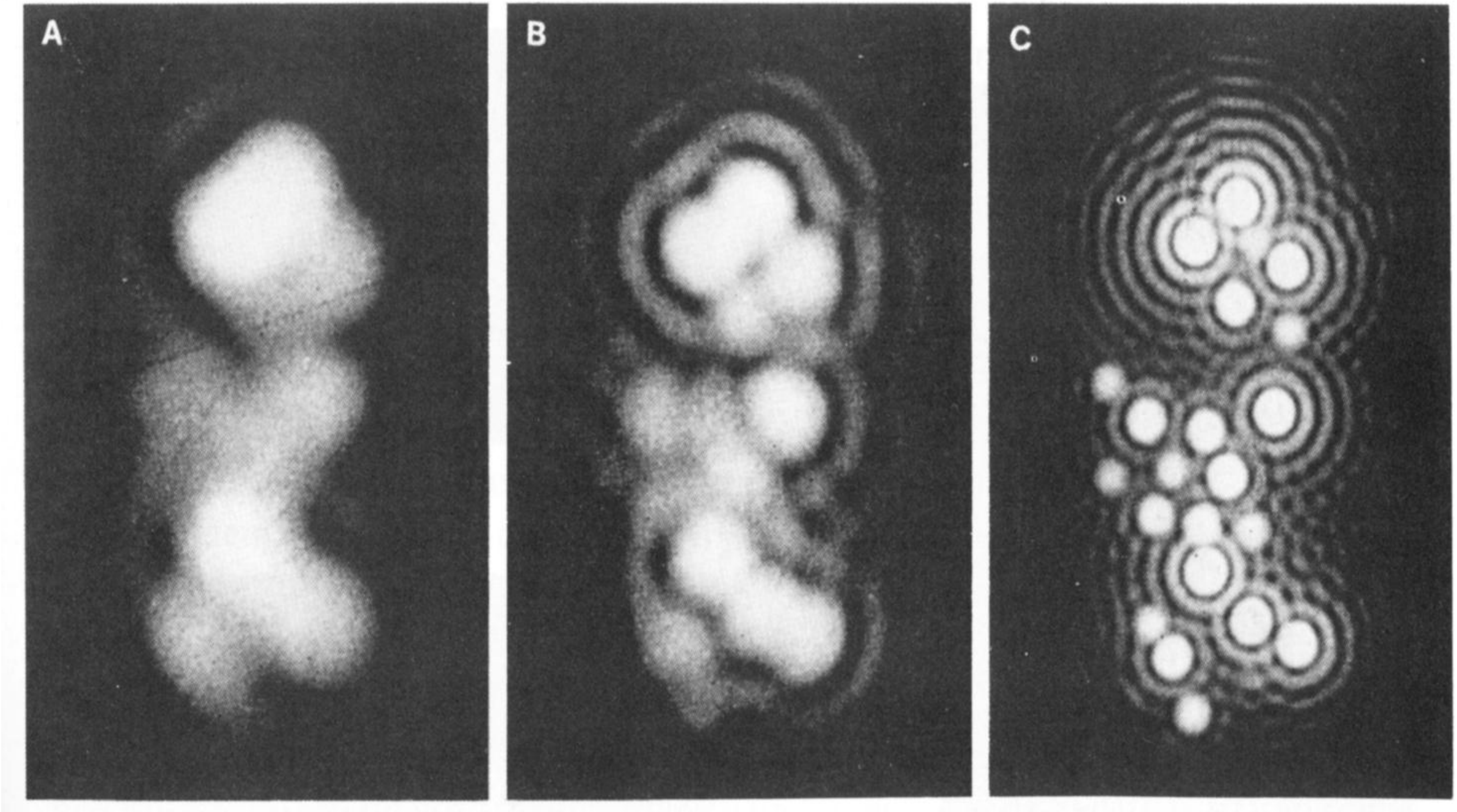
where:
α = light gathering angle
n = refractive index of sample

DIFFRACTION LIMITED IMAGING

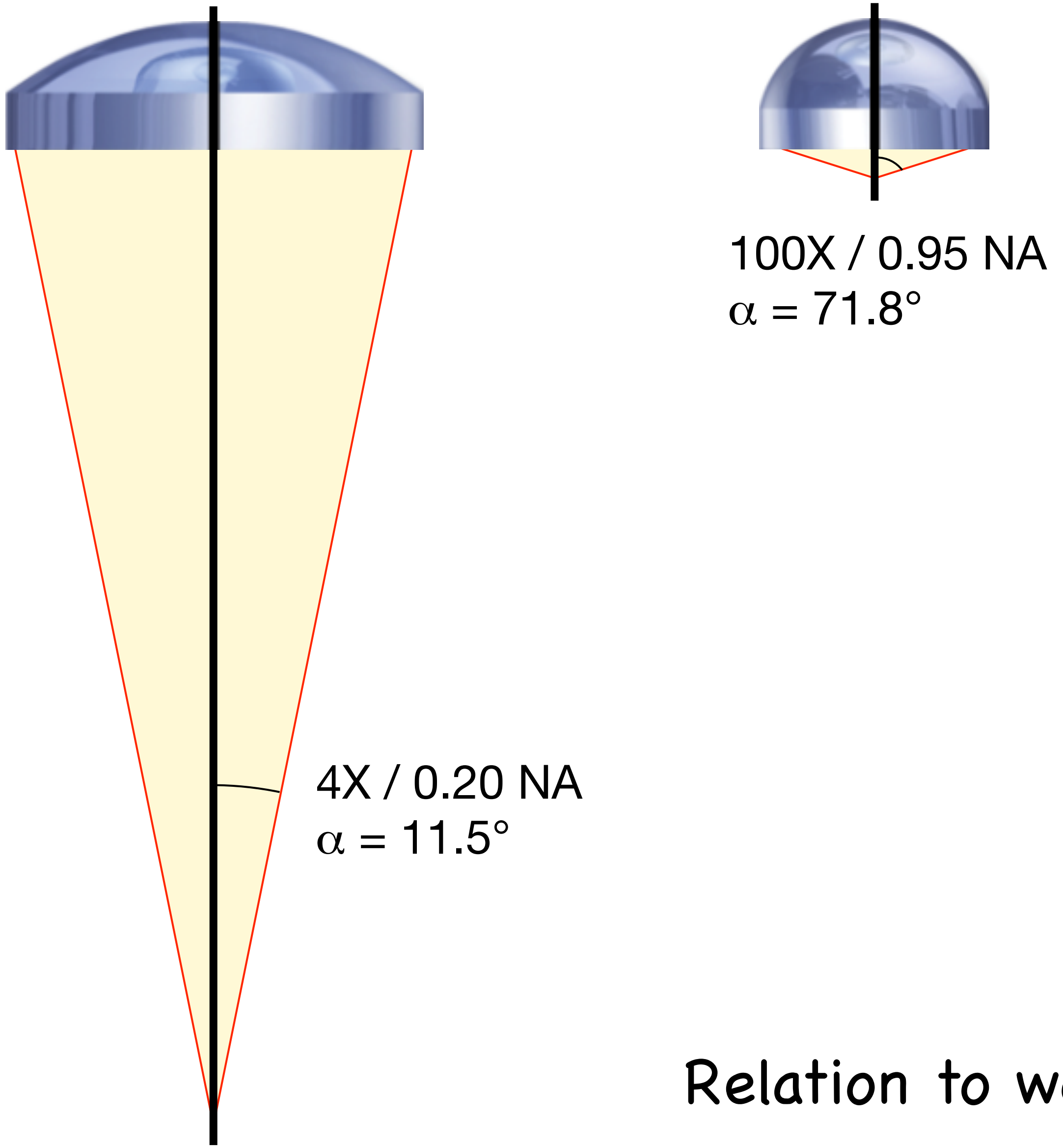


Rayleigh Criterion: resolution = 0.61λ/NA

EFFECT OF NA ON RESOLUTION



Numerical Aperture



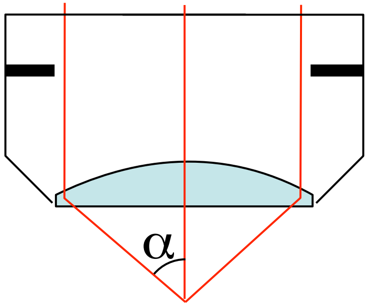
Relation to working distance

Numerical Aperture

Compare:

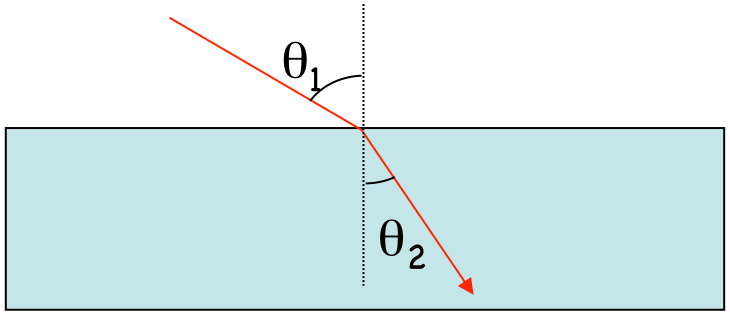
Numerical Aperture:

$NA = n \sin(\alpha)$



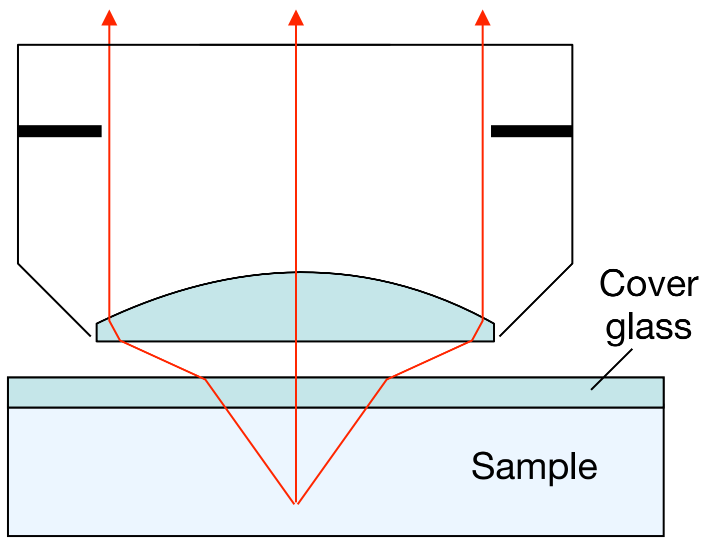
Snell's law:

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



- $n \sin(\theta)$ 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

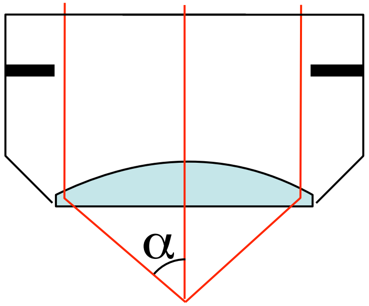


Numerical Aperture

Compare:

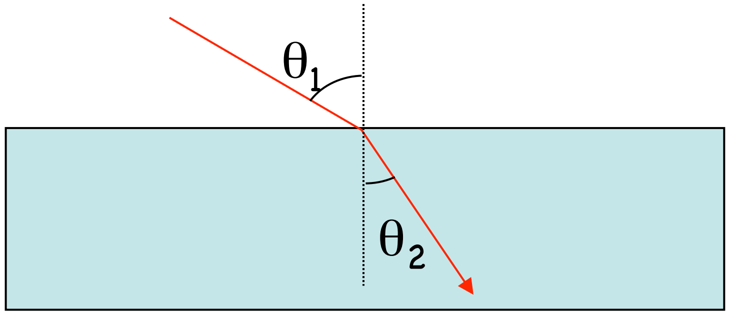
Numerical Aperture:

$NA = n \sin(\alpha)$



Snell's law:

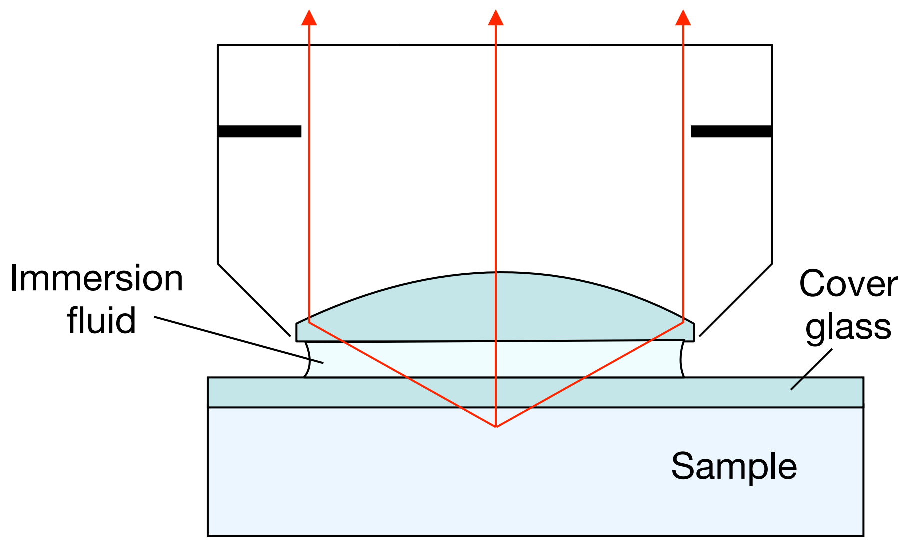
$n_1 \sin(\theta_1) = n_2 \sin(\theta_2)$



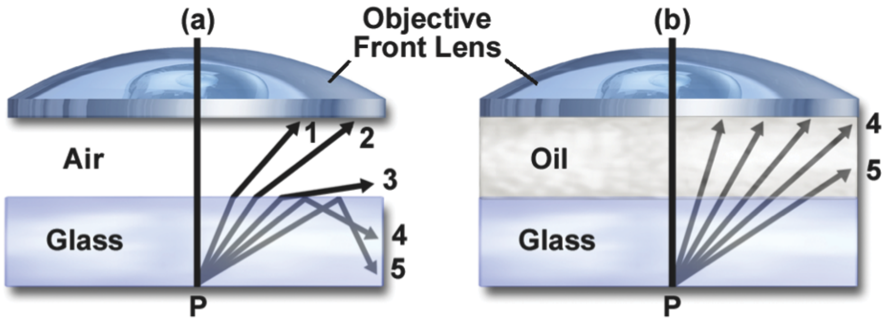
- $n \sin(\theta)$ doesn't change at horizontal interfaces
- $\sin(\text{anything}) \leq 1$

⇒ 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**

Effect of Objective Magnification and Numerical Aperture on Image Brightness

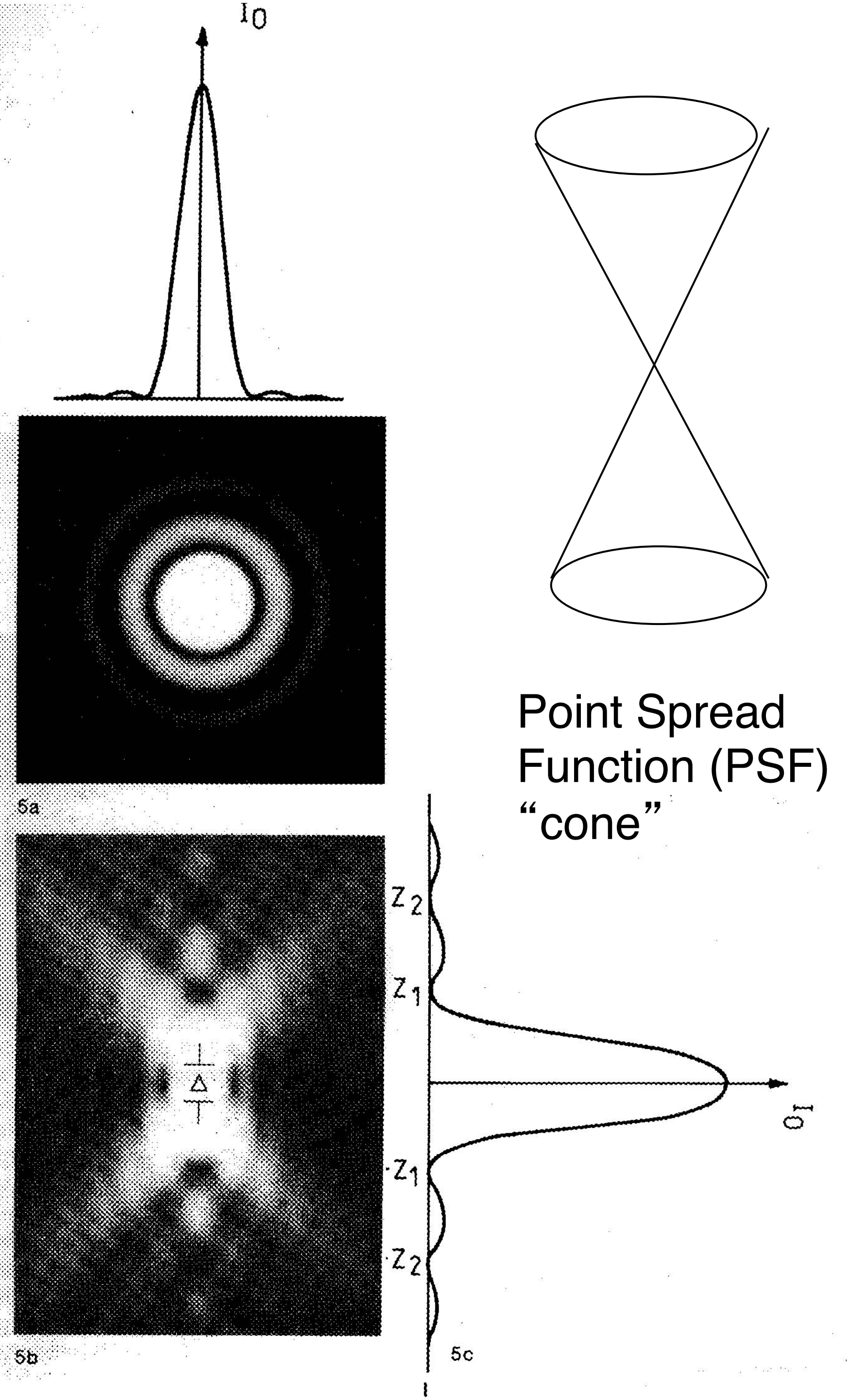
$$F_{(trans)} = 10^4 \cdot NA^2 / M^2$$

$$F_{(epi)} = 10^4 \cdot \left(NA^2 / M\right)^2$$

Magnification	Numerical Aperture	F(trans)	F(epi)
10x	0.25	6.25	0.39
10x	0.45	20.2	4.10
20x	0.50	6.25	1.56
20x	0.75	14.0	7.90
40x	0.65	2.64	1.11
40x (oil)	1.30	11.0	18.0
60x	0.85	2.01	1.45
60x (oil)	1.40	5.4	10.6
100x (oil)	1.40	1.96	3.84
100x (oil)	1.45	2.10	4.42

From: http://www.microscopyu.com/articles/formulas/formulasimagebrightness.html

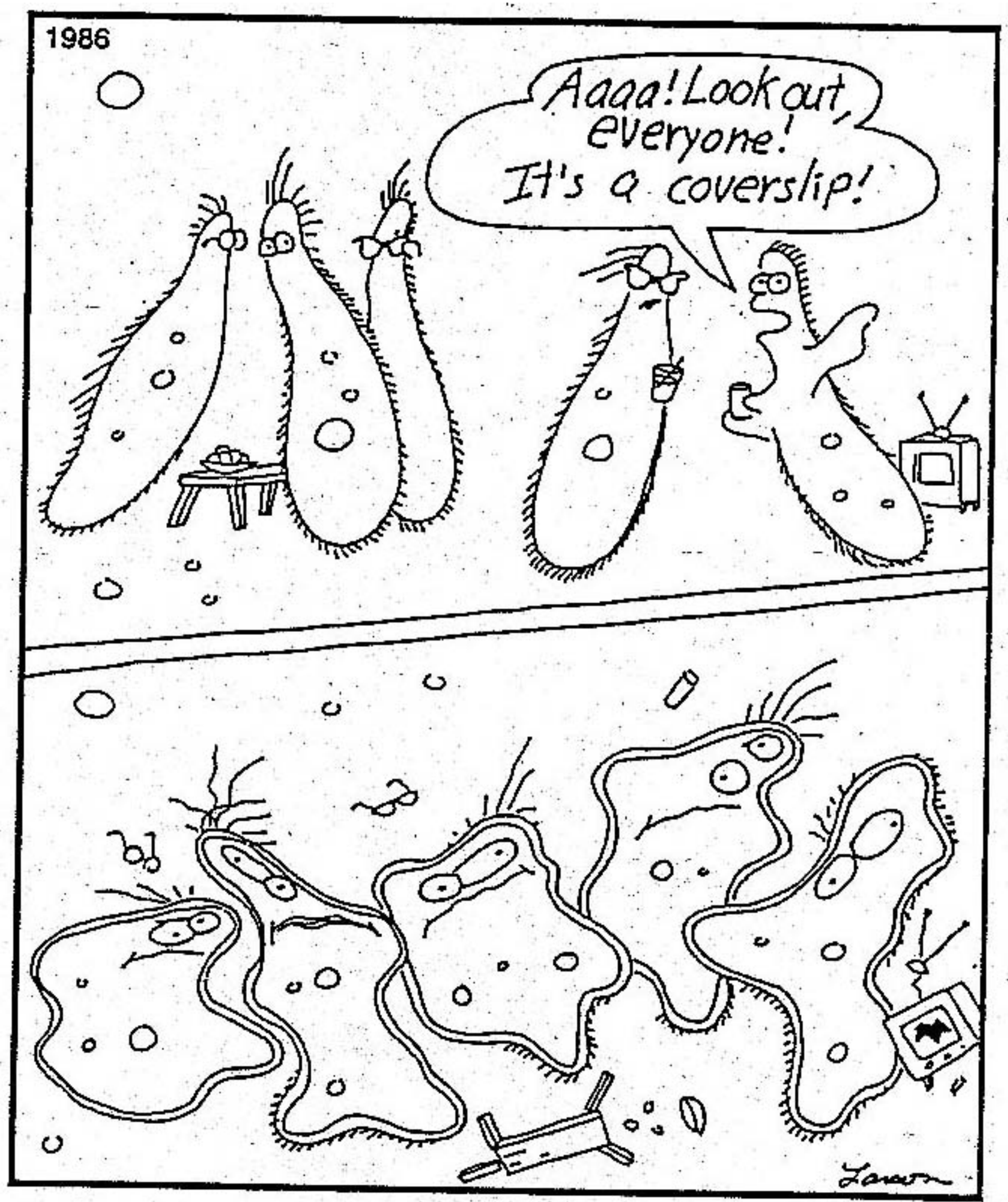
The 3D diffraction image of a point source in the Microscope (3D PSF): Lateral and axial resolution limmited by λ



From Larson diary

Three-Dimensional Imaging.

Live cell Imaging require special sample preparation and mounting.



Life on a microscope slide

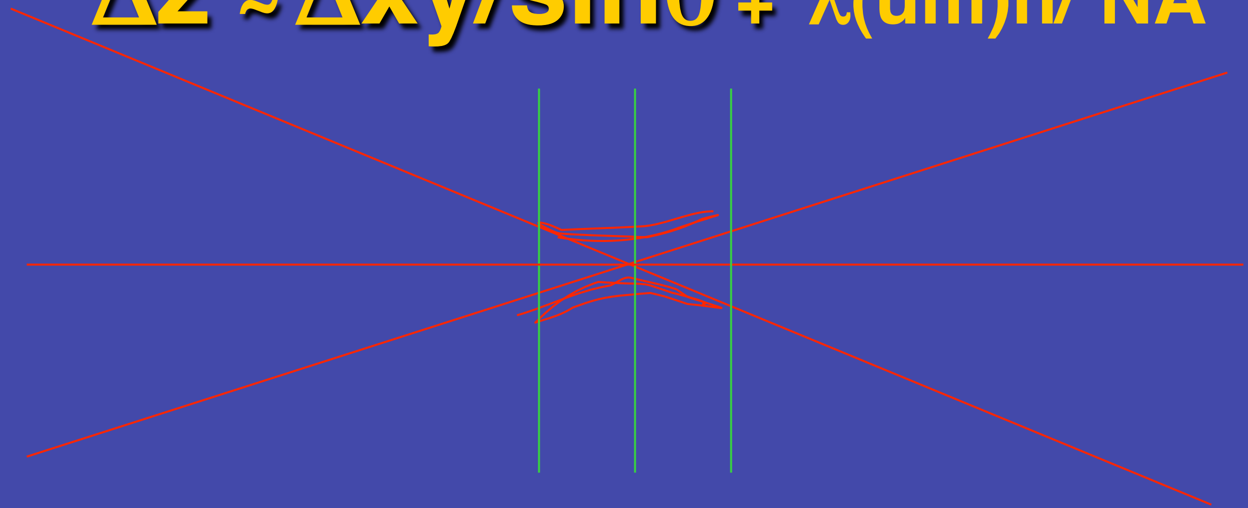
DEPTH OF FOCUS, Δz

3D resolution.

$$NA = n * \sin\theta$$

$$\Delta xy = 1.22\lambda / 2NA$$

$$\Delta z \sim \Delta xy / \sin\theta + \lambda(um) n / NA^2$$

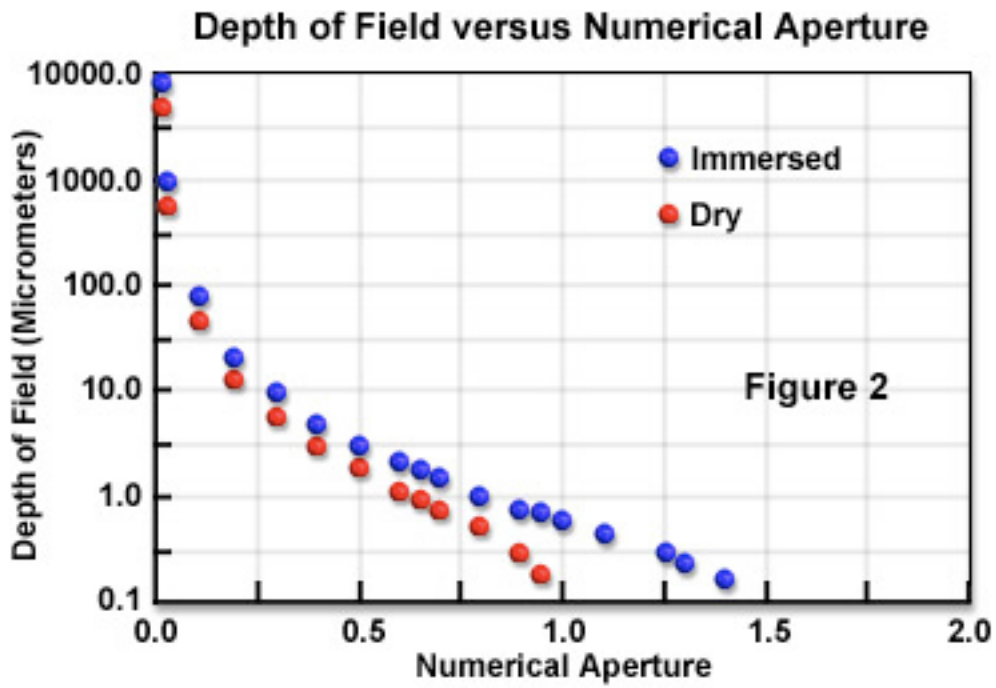


Axial resolution, Δz, contributions by geometrical + wave optics

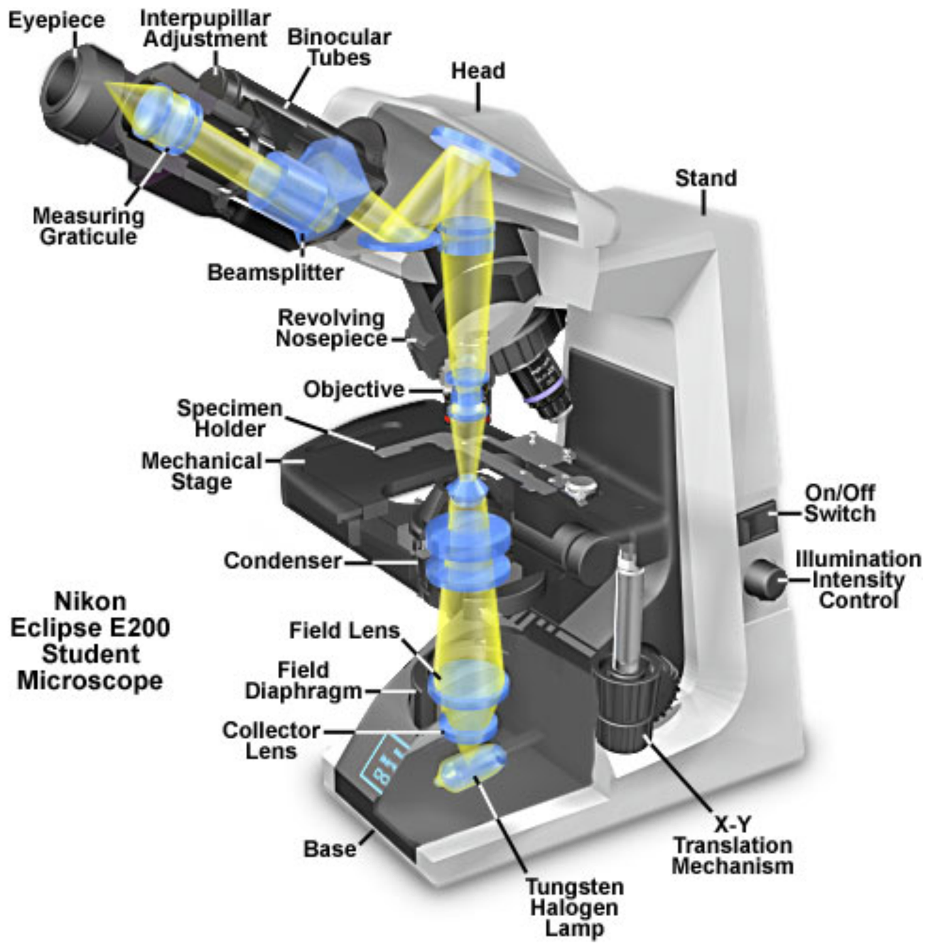
Objective	Lateral Resolution $0.61\lambda/NA$ µm	Axial Resolution $n/NA[\lambda/NA+d/M]$
X4/0.1	3.05	56.
X10/.25	1.02	8.5
X20/.4	0.61	5.8
X20/.7	0.44	1.0
X40/.65	0.51	1.2
X40/.95	0.34	0.7
X60/.95	0.34	0.7
X100/.95	0.34	0.7
X100/1.4	0.22	0.6

* $\lambda=0.5\mu m$
* $n=1.34$
* $d=0.1\mu m$

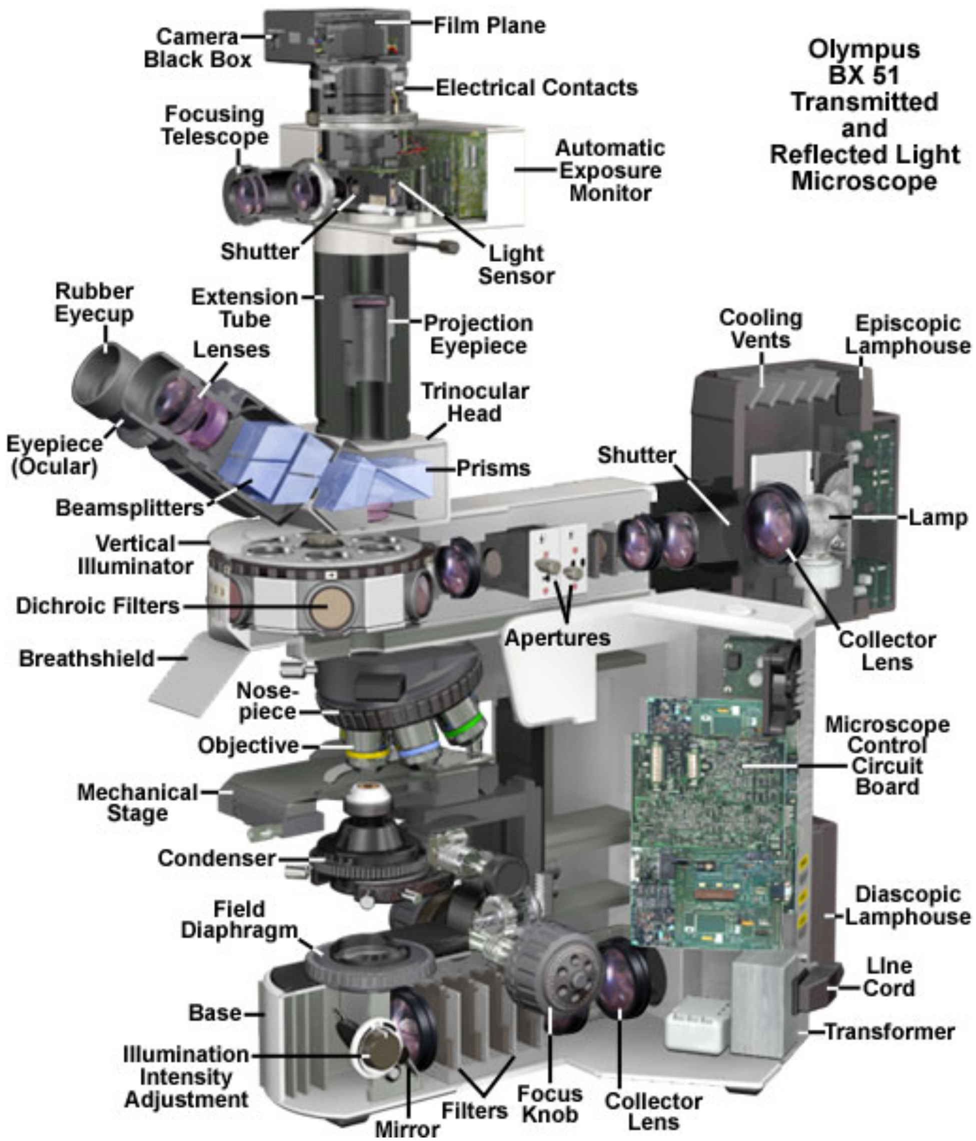
Resolution in µm



A Simple Microscope



A Research Microscope



INVERTED MICROSCOPE