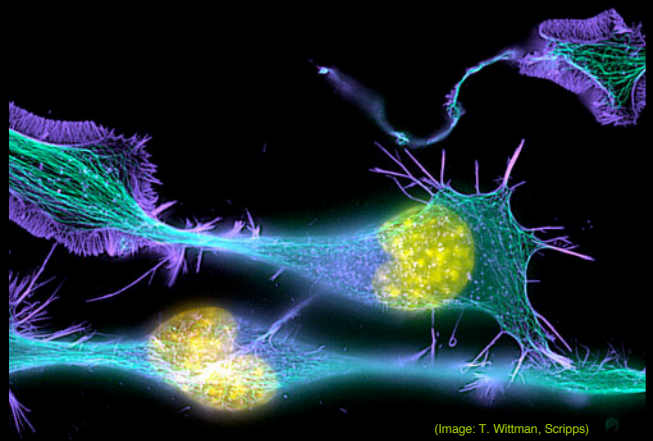
Principles & Practice of Light Microscopy



Principles and Practice of Light Microscopy

- Lectures Mondays 10–12 in BH212

  Mats Gustafsson: mats@msg.ucsf.edu, 514-4385

- Labs Wed ***or*** Th 4–6 in BH309, starting April 18 (pick one of the two lab groups)

  Orion Weiner: orion.weiner@ucsf.edu, 514-4508

  John Sedat: sedat@msg.ucsf.edu, 476-4156

  Kurt Thorn: kurt.thorn@ucsf.edu, 514-9709

- Optional reading materials:
  - Douglas Murphy, *Fundamentals of Light Microscopy and Digital Imaging* ($85 at Amazon - sorry!)
  - micro.magnet.fsu.edu
  - www.microscopyu.com

Lecture course (draft)

Apr 2: Light, refraction, diffraction, ray optics, lenses, images. The light microscope, numerical aperture, Köhler illumination.

Apr 9: *No lecture*

Apr 16: Resolution and contrast, aberrations, spatial frequencies and the Fourier transform, the point spread function, the optical transfer function.

Apr 23: Phase contrast, DIC, darkfield, polarization microscopy.

Apr 30: Fluorescence, probes, photobleaching, filters and dichroics, fluorescent proteins.

May 7 : TIRF, FRET, FRAP, FLIP, FLIM, photo-activation, image fluorescence correlation spectroscopy, optical tweezers, single molecule microscopy, live cell techniques.

May 14: Confocal, spinning disk, multi-photon, second/third harmonic generation, coherent anti-Stokes Raman microscopy (CARS)

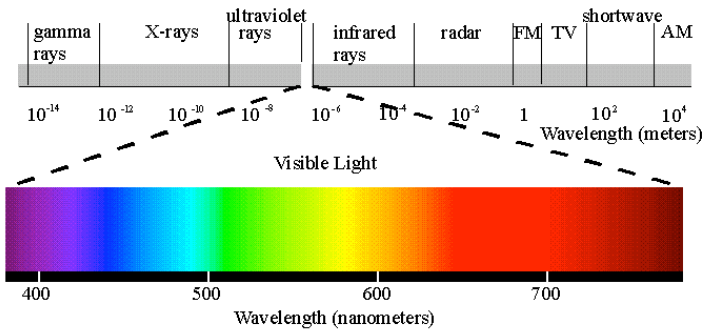
May 21: Detectors, light sources, noise. Image analysis and filtering: scaling, gamma, filtering, filtering artifacts, image arithmetic, ratioing, linear unmixing, segmentation.

May 28: Deconvolution, advanced techniques: 4Pi, structured illumination, SPIM, PALM/FPALM/STORM…

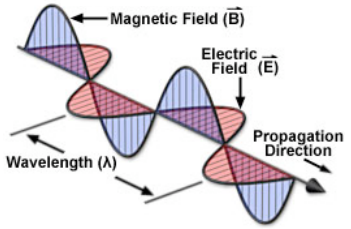
The Light Microscope

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

Electromagnetic Waves



Light as an Electromagnetic Wave



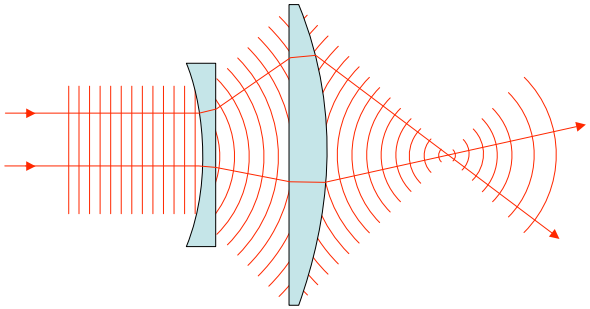
Most matter interacts mostly with the electric field
⇒ We will ignore the magnetic field

Polarization = direction of electric field

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

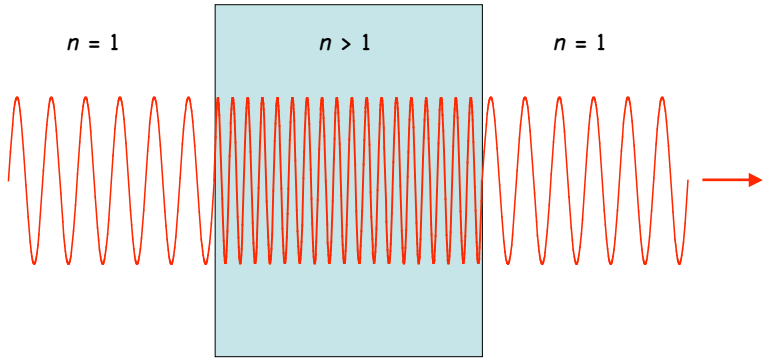
Rays are perpendicular to wavefronts



Light travels more slowly in matter

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

$v = c/n$

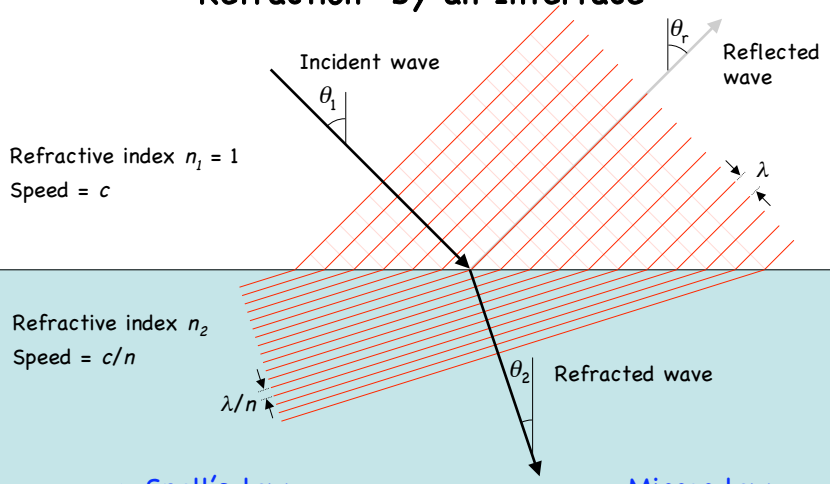


Refractive Index Examples

• 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



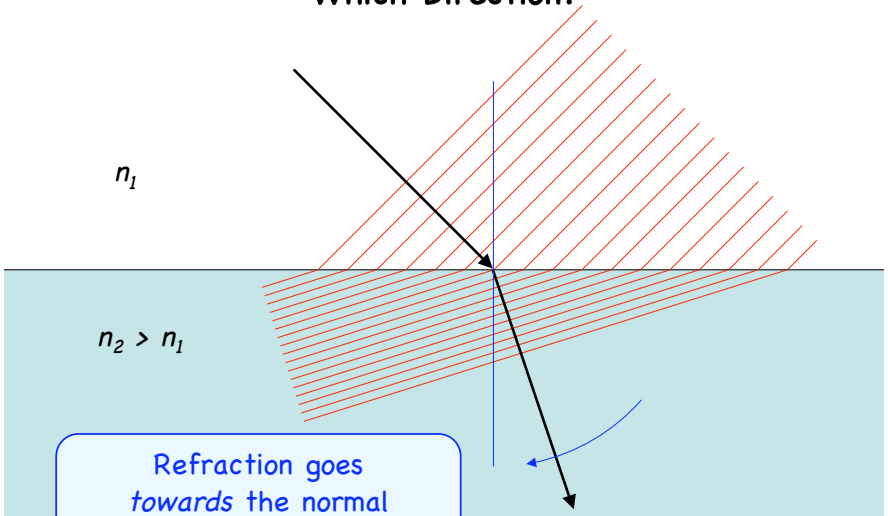
⇒ Snell's law:

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

Mirror law:

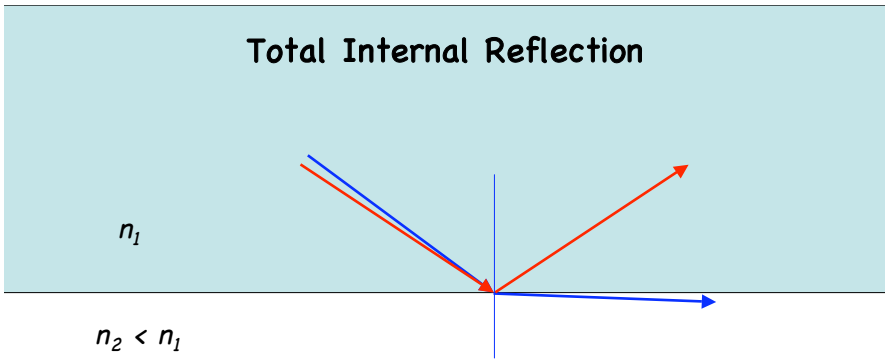
$\theta_r = \theta_1$

Which Direction?



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

Total Internal Reflection

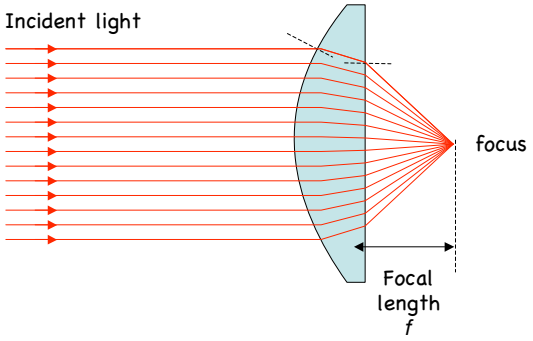


Snell's Law: $n_1 \sin(\theta_1) = n_2 \sin(\theta_2)$

If $n_1 \sin(\theta_1) > n_2$, then $\sin(\theta_2)$ would have to exceed 1.
Impossible ⇒ No light can be transmitted
⇒ All is reflected: *Total internal reflection*

Happens only when going *to* a *lower*-index medium

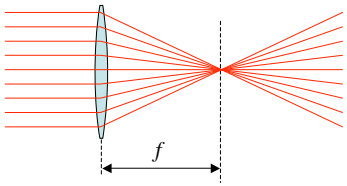
Lenses work by refraction



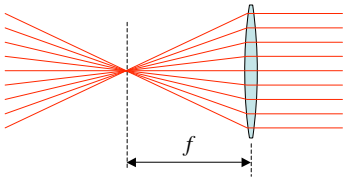
Ray Tracing Rules of Thumb

(for thin ideal lenses)

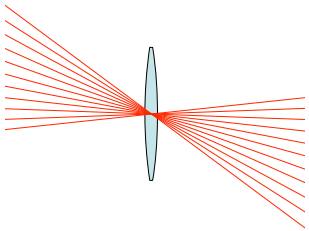
Parallel rays converge at the focal plane



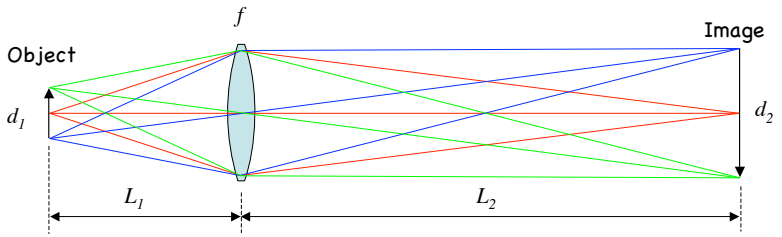
Rays that cross in the focal plane end up parallel



Rays through the lens center are unaffected



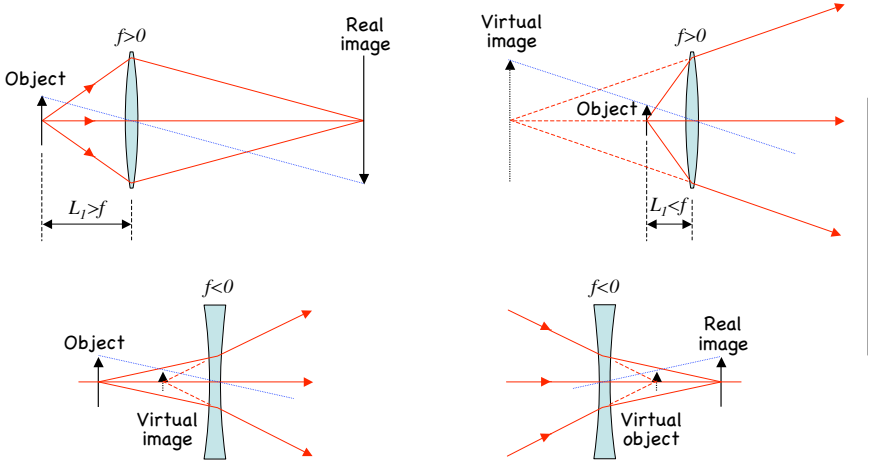
Imaging



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

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

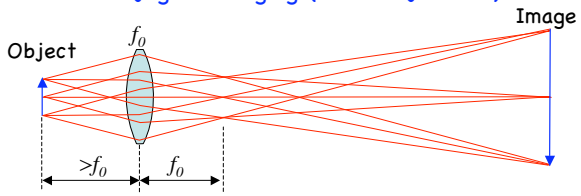
Real and virtual images



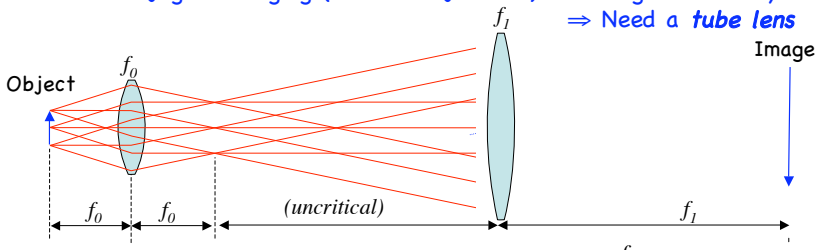
The same lens law applies: Negative lenses have negative f
Virtual objects or images have negative values of L_1 or L_2

Finite vs. Infinite Conjugate Imaging

- Finite conjugate imaging (older objectives)

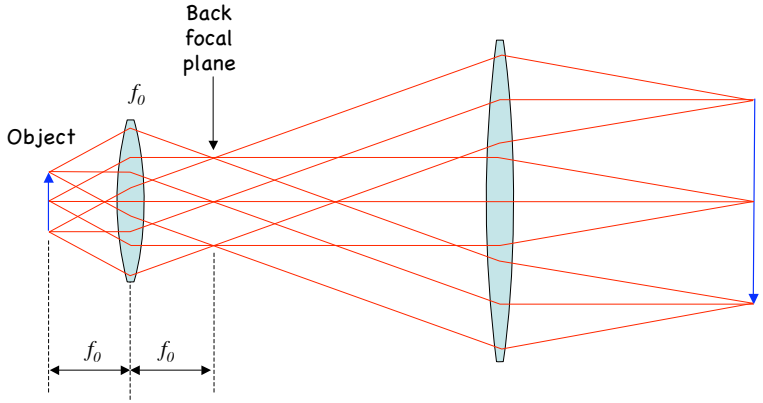


- Infinite conjugate imaging (modern objectives). Image at infinity ⇒ Need a ***tube lens***



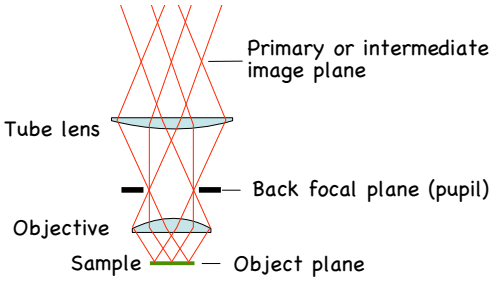
Magnification: $M = \frac{f_1}{f_o}$

Back focal plane

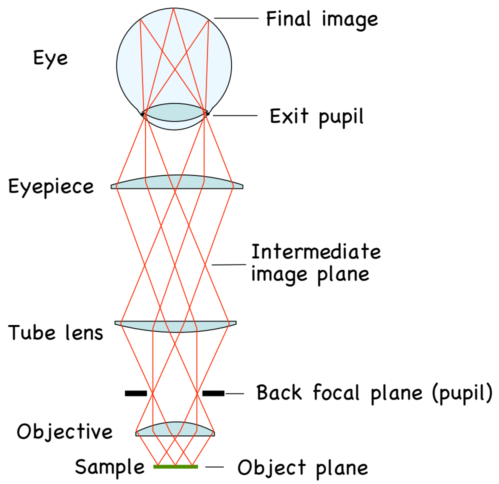


Rays that leave the object with the same angle meet in the objective's *back focal plane*

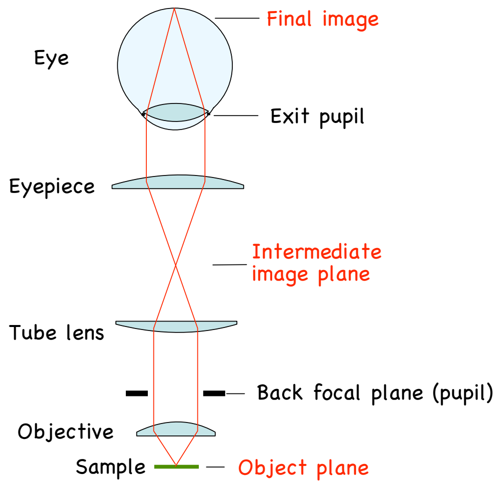
The Compound Microscope



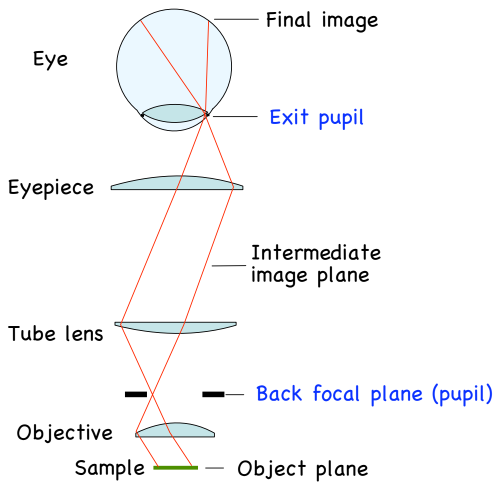
The Compound Microscope



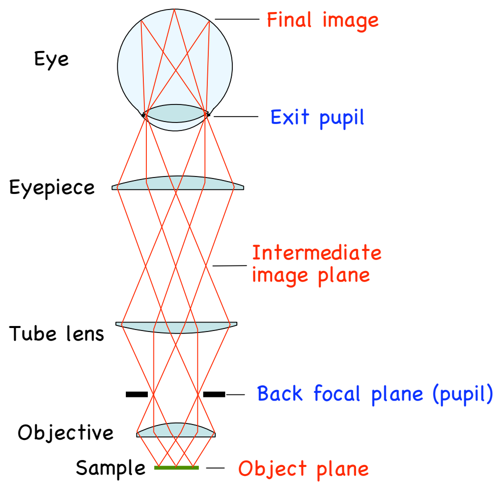
The Compound Microscope



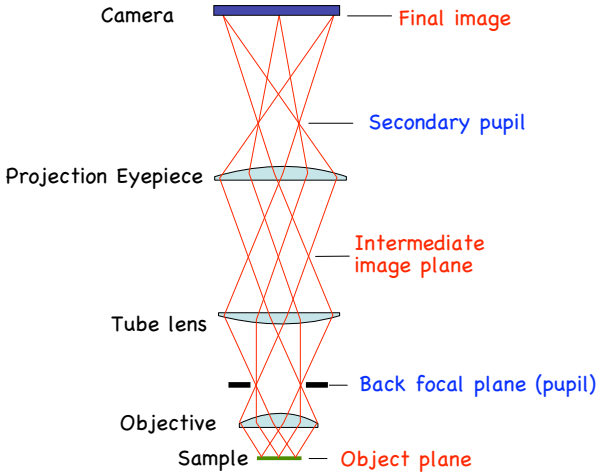
The Compound Microscope



The Compound Microscope



The Compound Microscope

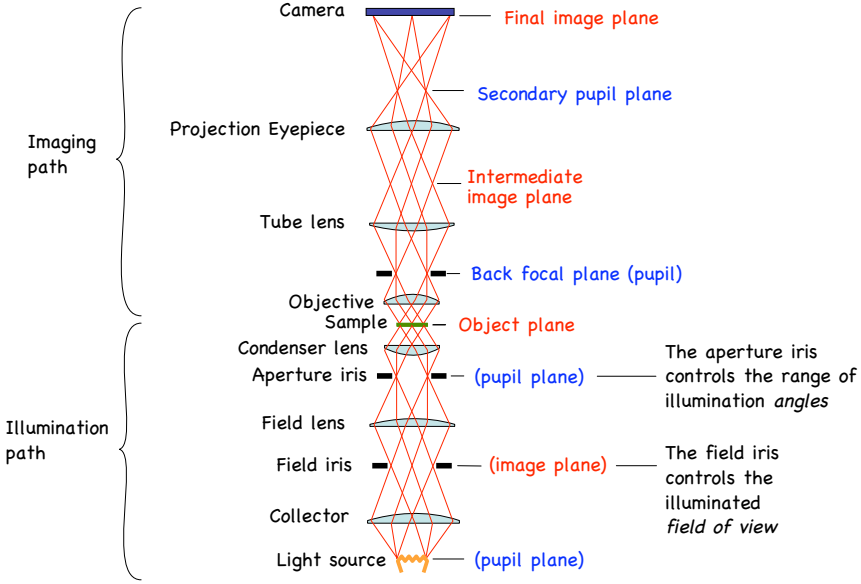


Eyepieces (Oculars)

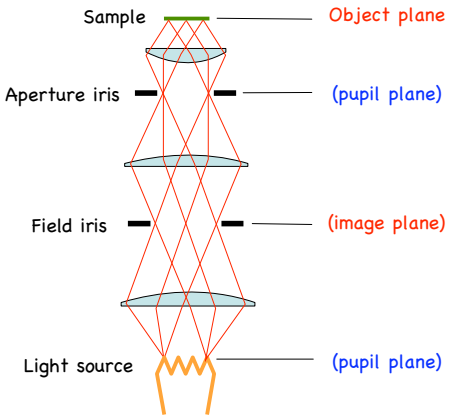
Features

- 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

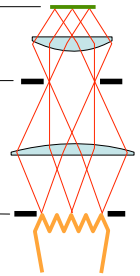


Köhler Illumination



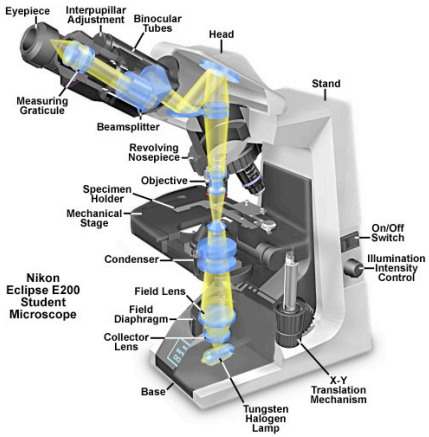
- Each light source point produces a parallel beam of light at the sample
- Uniform light intensity at the sample even if the light source is "ugly" (e.g. a filament)

Critical Illumination

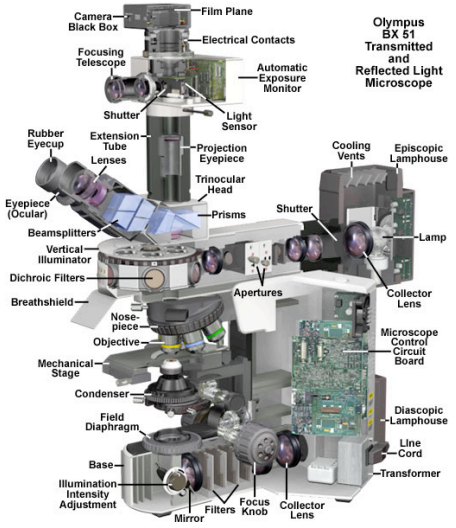


- The source is imaged onto the sample
- Usable only if the light source is perfectly uniform

A Simple Microscope



A Research Microscope



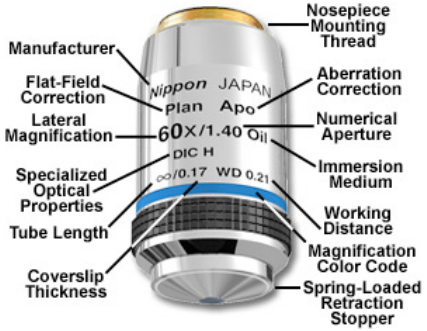
How view the pupil planes?

Two ways:

- "Eyepiece telescope"
- "Bertrand lens"

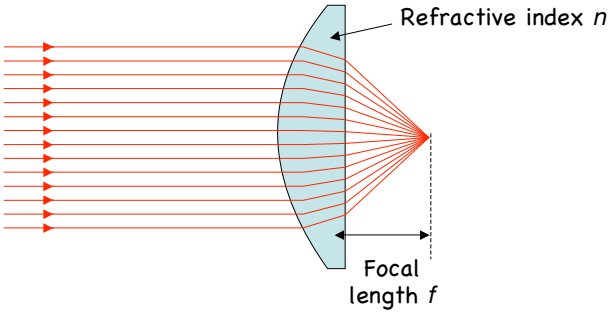
By far the most important part:

the Objective Lens



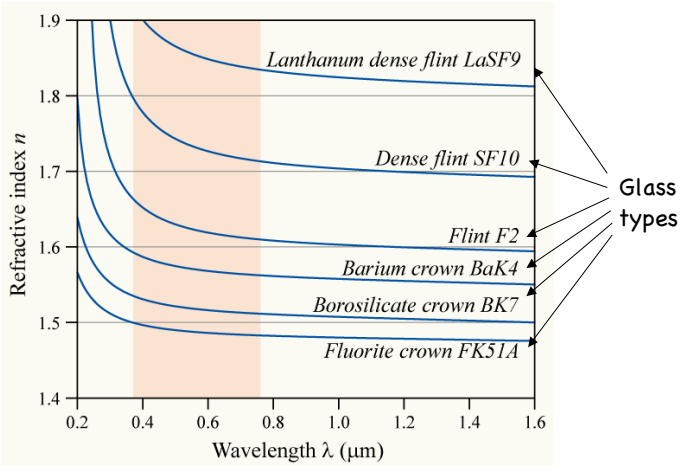
Each major manufacturer sells 20-30 different ***categories*** of objectives.
What are the important distinctions?

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



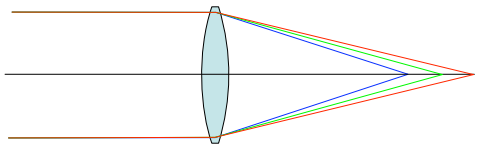
$f \propto 1/(n-1)$

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

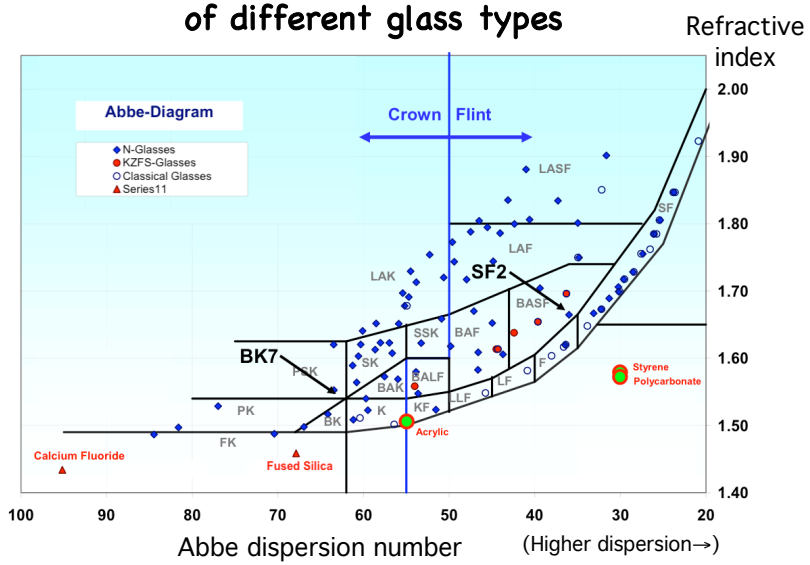


⇒ Chromatic aberration

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

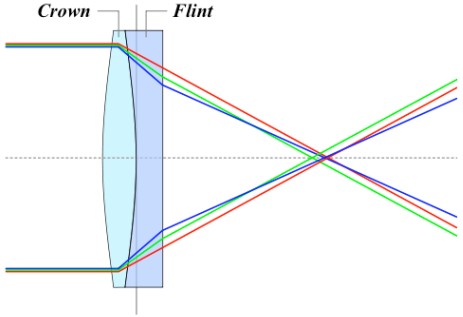


Dispersion vs. refractive index of different glass types

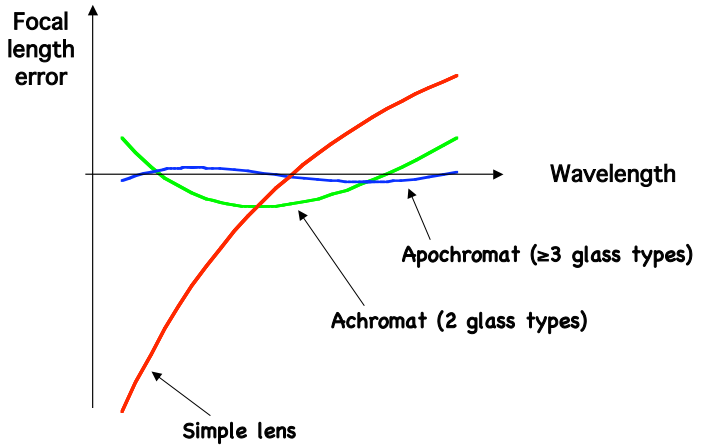


Achromatic Lenses

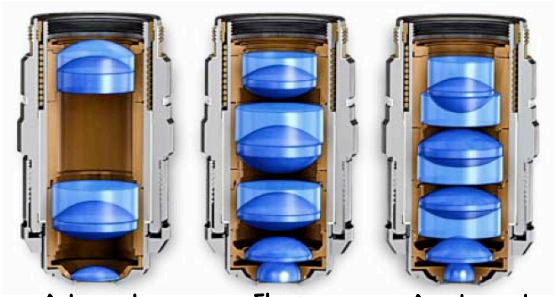
- Use a weak negative flint glass element to compensate the dispersion of a positive crown glass element



Achromats and Apochromats



Correction classes of objectives



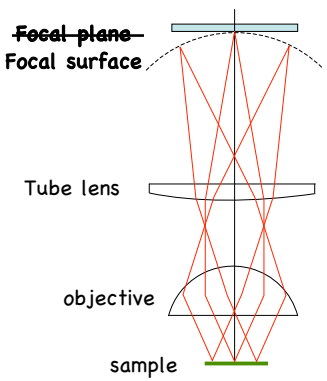
Achromat (cheap)

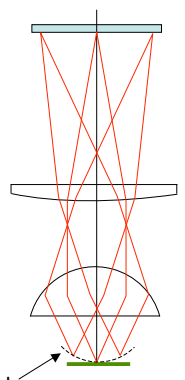
Fluor "semi-apo" (good correction, high UV transmission)

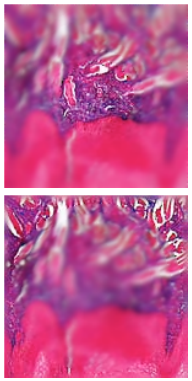
Apochromat (best correction)

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

Curvature of Field

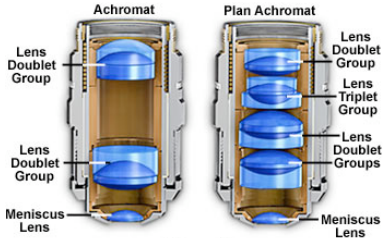






Plan objectives

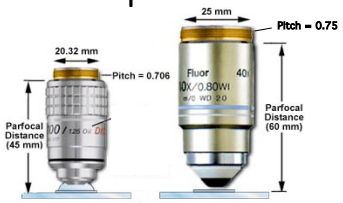
- Corrected for field curvature
- More complex design
- Needed for most photomicrography



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

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)

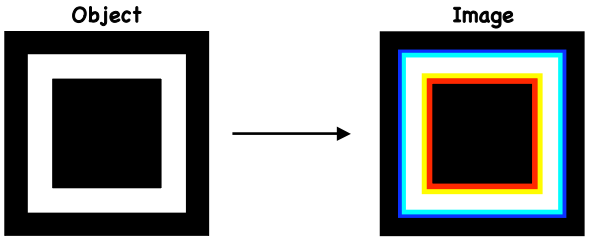
LCA correction:	
In objective	In tube lens
Nikon	Leica
Olympus	Zeiss

⇒ mixing brands can produce severe LCA

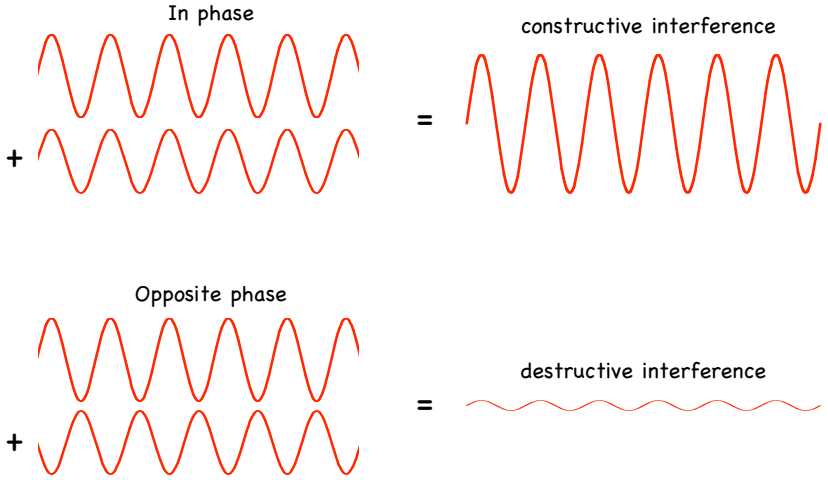
Lateral chromatic aberration

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

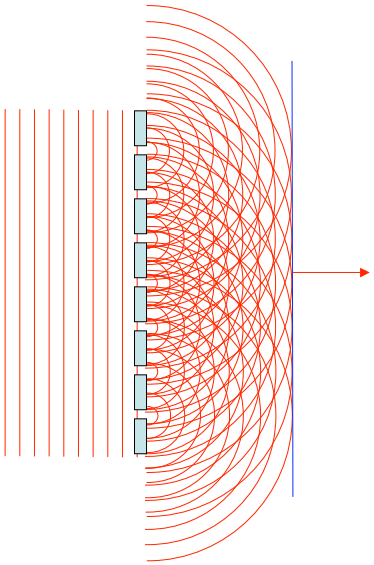
= Different magnification for different colors



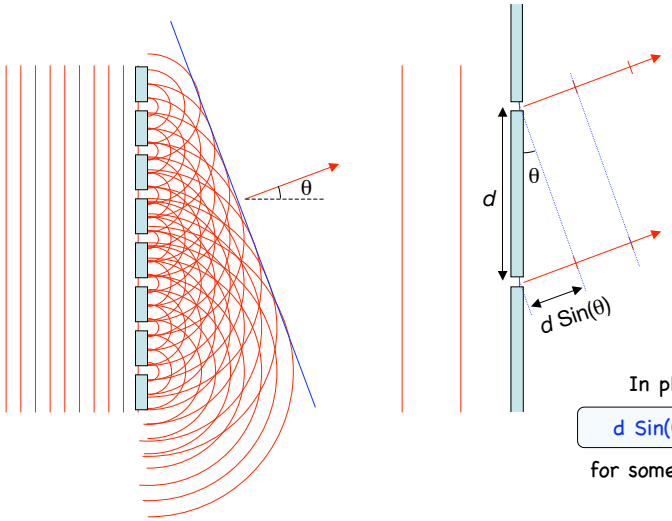
Interference



Diffraction by a periodic structure (grating)



Diffraction by a periodic structure (grating)

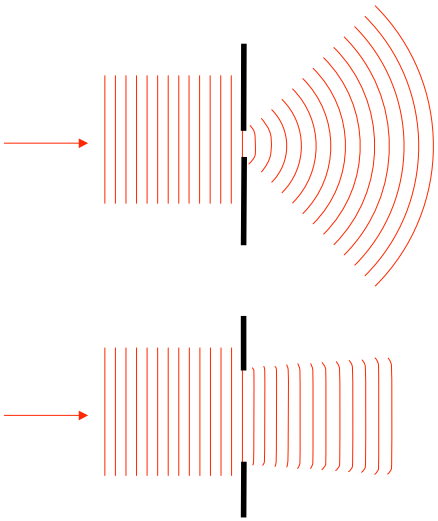


In phase if:

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

for some integer m

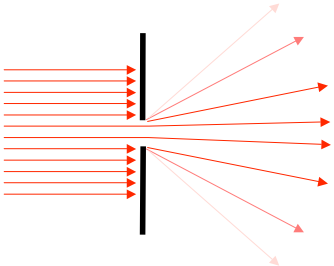
Diffraction by an aperture drawn as waves



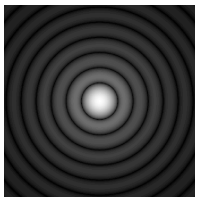
Light spreads to new angles

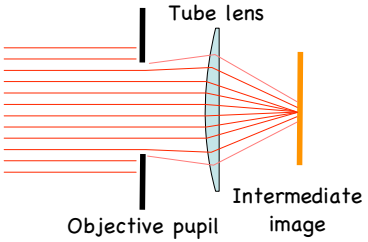
Larger aperture
⇔
weaker diffraction

Diffraction by an aperture drawn as rays



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



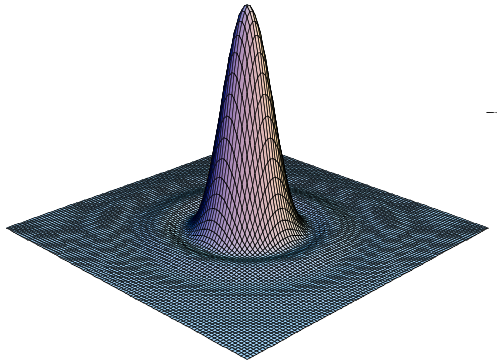


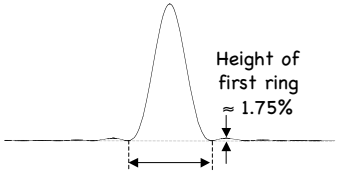
…or can be formed at a finite distance by a lens…

…as happens in a microscope

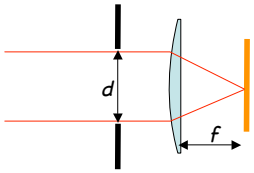
The Airy Pattern

= the far-field diffraction pattern from a round aperture

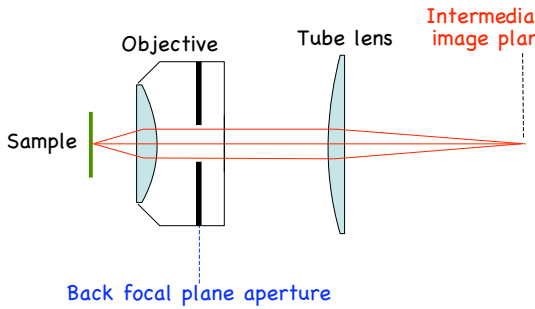




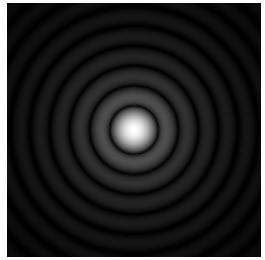
"Airy disk" diameter
$d = 2.44\ \lambda\ f/d$
(for small angles d/f)



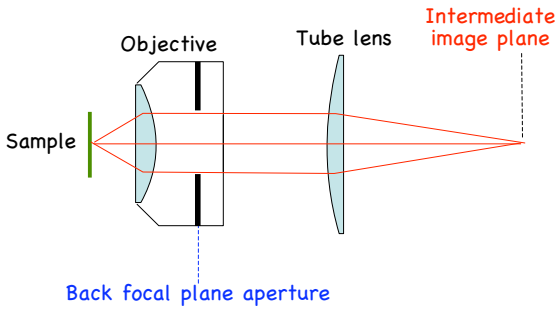
Aperture and Resolution



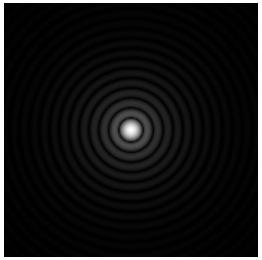
Diffraction spot
on image plane
(resolution)



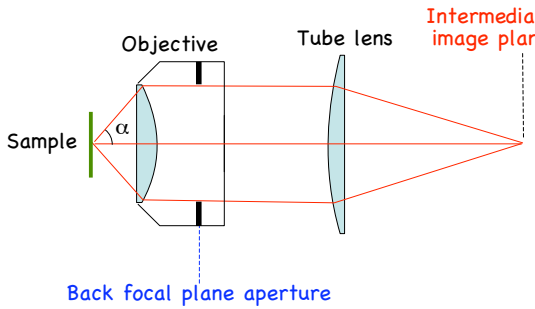
Aperture and Resolution



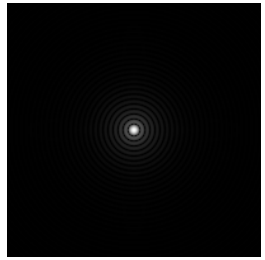
Diffraction spot
on image plane
(resolution)



Aperture and Resolution



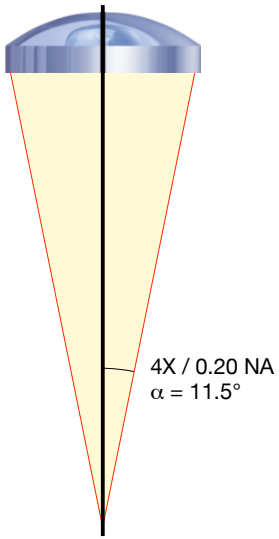
Diffraction spot
on image plane
(resolution)

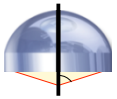


- Image resolution improves with ~~aperture size~~ Numerical Aperture (NA)

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

Numerical Aperture



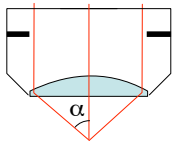


Numerical Aperture

Compare:

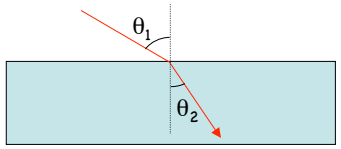
Numerical Aperture:

$NA = n \sin(\alpha)$



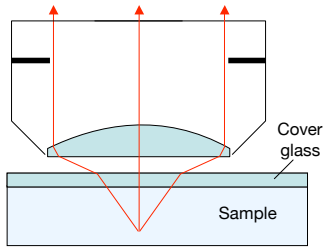
Snell's law:

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



- 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

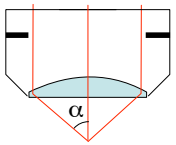


Numerical Aperture

Compare:

Numerical Aperture:

$NA = n \sin(\alpha)$



Snell's law:

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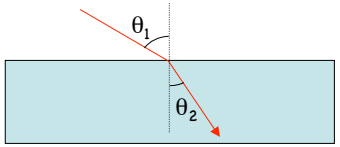
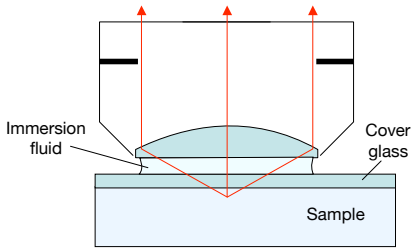


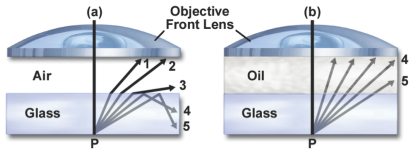
- n sin(θ) doesn't change at horizontal interfaces
- sin(anything) ≤ 1

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

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



Immersion Objectives

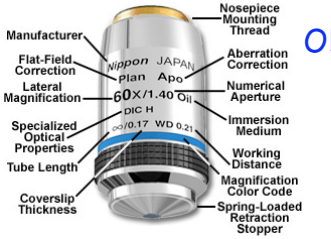


NA can approach the index of the immersion fluid

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

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 for Polarized Light	Strain-Free, Low Birefringence,
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)