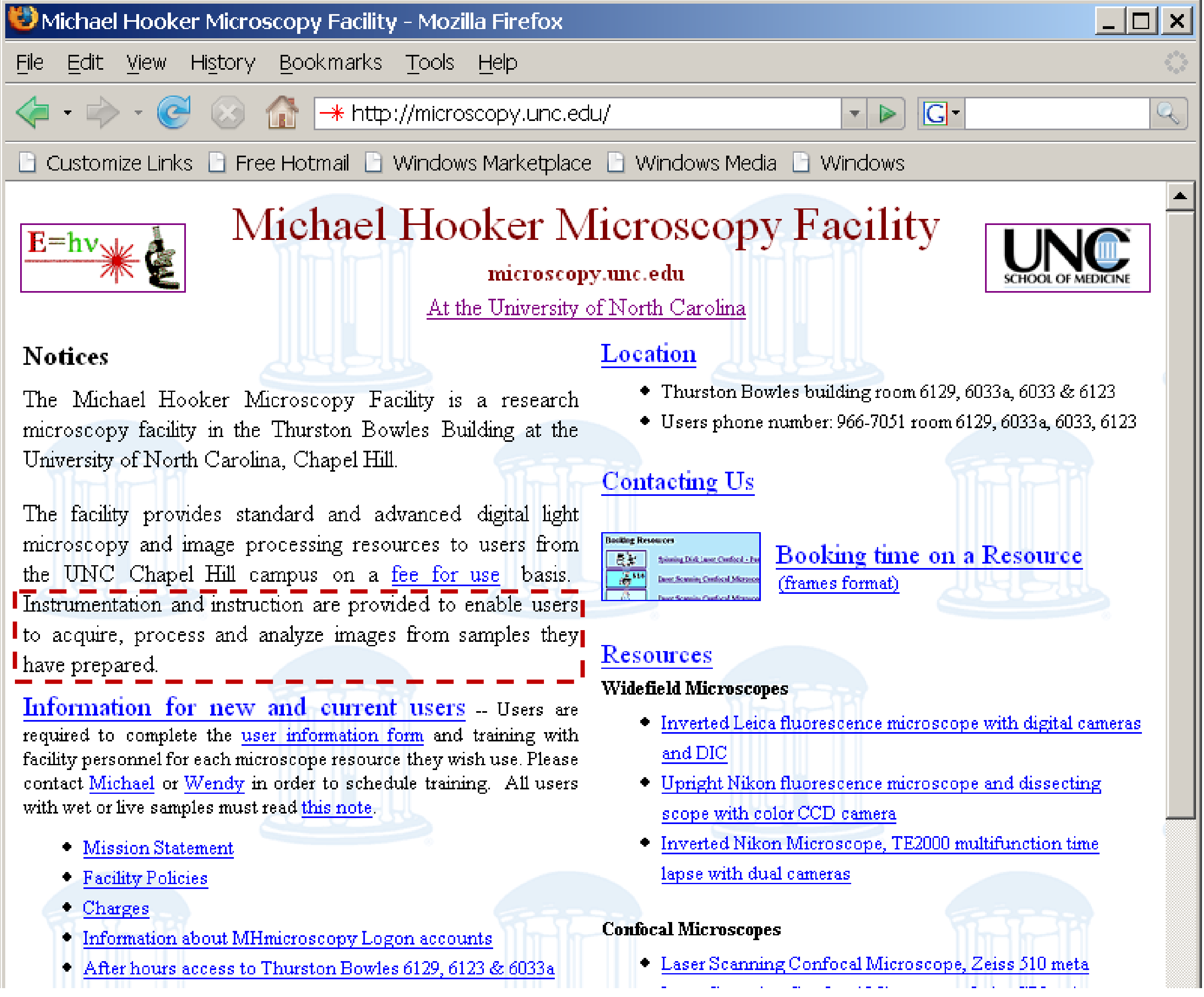
An Introductory Guide to Light Microscopy

16 Apr to 14 May 2007

Michael Chua
microscopy@unc.edu
843-3268
6007 Thurston Bowles

Wendy Salmon
wendy_salmon@med.unc.edu
966-7051
6129 Thurston Bowles


Michael Hooker Microscopy Facility - Mozilla Firefox
File Edit View History Bookmarks Tools Help
http://microscopy.unc.edu/
Customize Links
Free Hotmail
Windows Marketplace
Windows Media
Windows
E=hv
Michael Hooker Microscopy Facility
microscopy.unc.edu
At the University of North Carolina
UNC SCHOOL OF MEDICINE
Notices
The Michael Hooker Microscopy Facility is a research microscopy facility in the Thurston Bowles Building at the University of North Carolina, Chapel Hill.
The facility provides standard and advanced digital light microscopy and image processing resources to users from the UNC Chapel Hill campus on a fee for use basis. Instrumentation and instruction are provided to enable users to acquire, process and analyze images from samples they have prepared.
Information for new and current users -- Users are required to complete the user information form and training with facility personnel for each microscope resource they wish use. Please contact Michael or Wendy in order to schedule training. All users with wet or live samples must read this note.
Mission Statement
Facility Policies
Charges
Information about MHmicroscopy Logon accounts
After hours access to Thurston Bowles 6129, 6123 & 6033a
Location
Thurston Bowles building room 6129, 6033a, 6033 & 6123
Users phone number: 966-7051 room 6129, 6033a, 6033, 6123
Contacting Us
Booking Resources
Booking time on a Resource
(frames format)
Resources
Widefield Microscopes
Inverted Leica fluorescence microscope with digital cameras and DIC
Upright Nikon fluorescence microscope and dissecting scope with color CCD camera
Inverted Nikon Microscope, TE2000 multifunction time lapse with dual cameras
Confocal Microscopes
Laser Scanning Confocal Microscope, Zeiss 510 meta

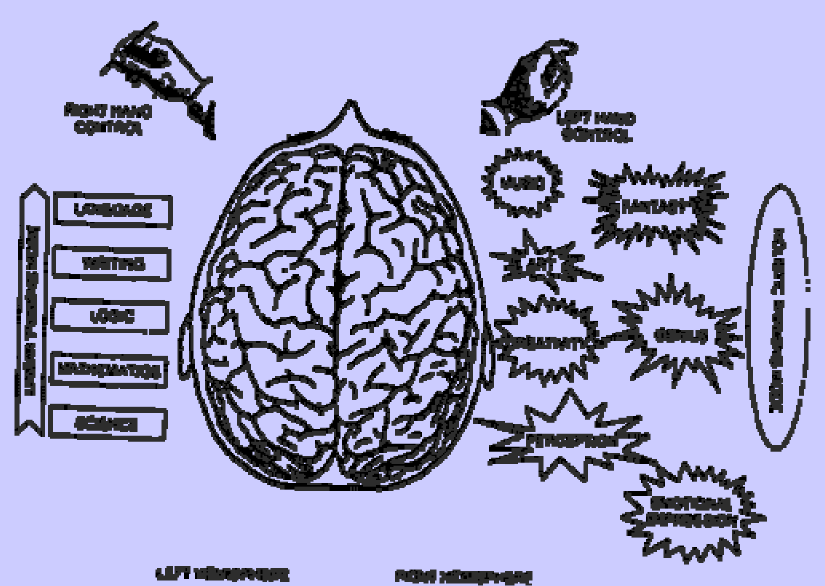
An Introductory Guide to Light Microscopy - Five Talk Plan

- Apr 16. A brief perspective of microscopy, theory of operation, key parts of a typical microscope for transmitted light, Kohler illumination, the condenser, objectives, Nomarski, phase contrast, resolution
- Apr 23. Fluorescence: Why use it, fluorescence principals, contrast, resolution, filters, dichroic filter cubes, immuno staining, fluorescent proteins, dyes.
- Apr 30. Detectors, sampling & digital images: Solid state digital cameras, Photomultipliers, noise, image acquisition, Nyquist criterion/resolution, pixel depth, digital image types/color/compression
- May 07. Confocal Microscopy: Theory, sensitivity, pinhole, filters, 3-D projection/volume renders
- May 14. Advanced Fluorescence/Confocal: Live cell imaging, co-localization, bleed through/cross talk, FRAP, fluorescence recovery after photobleaching, deconvolution

E = hν

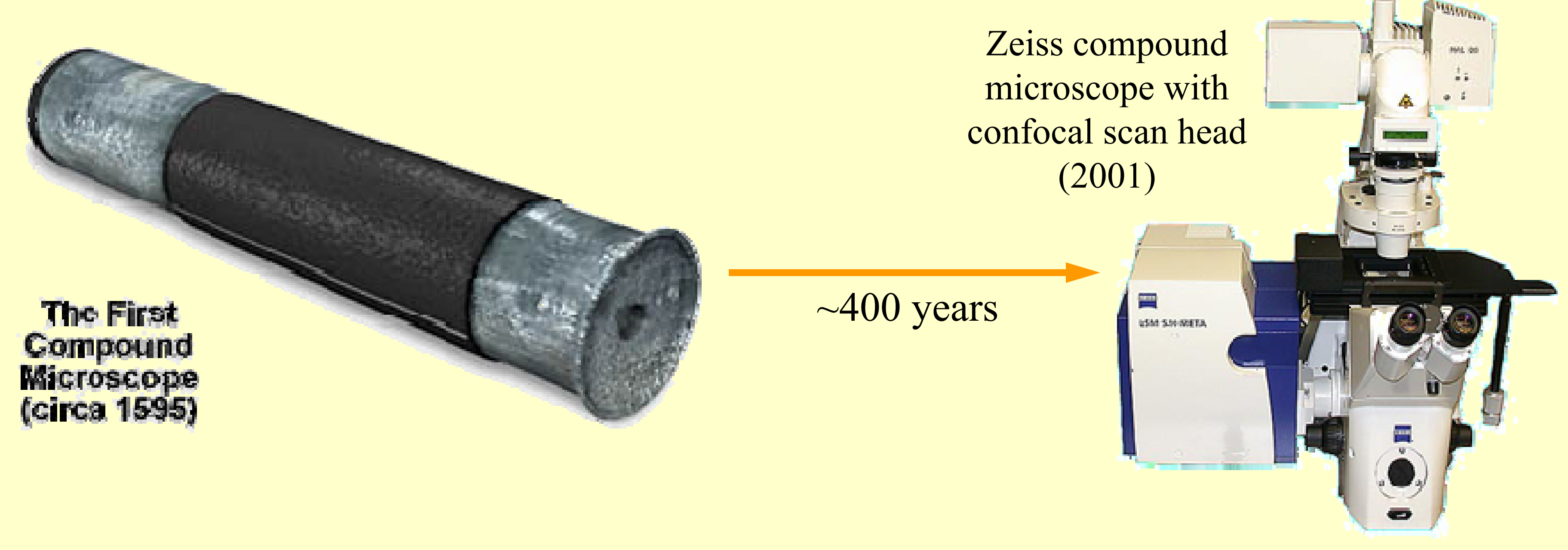
Today's Plan

- Brief perspective / evolution of imaging
- Basic Optics
- Parts of a microscope & Kohler Illumination
- Objectives
- Modes of Imaging common in Biomedical Research



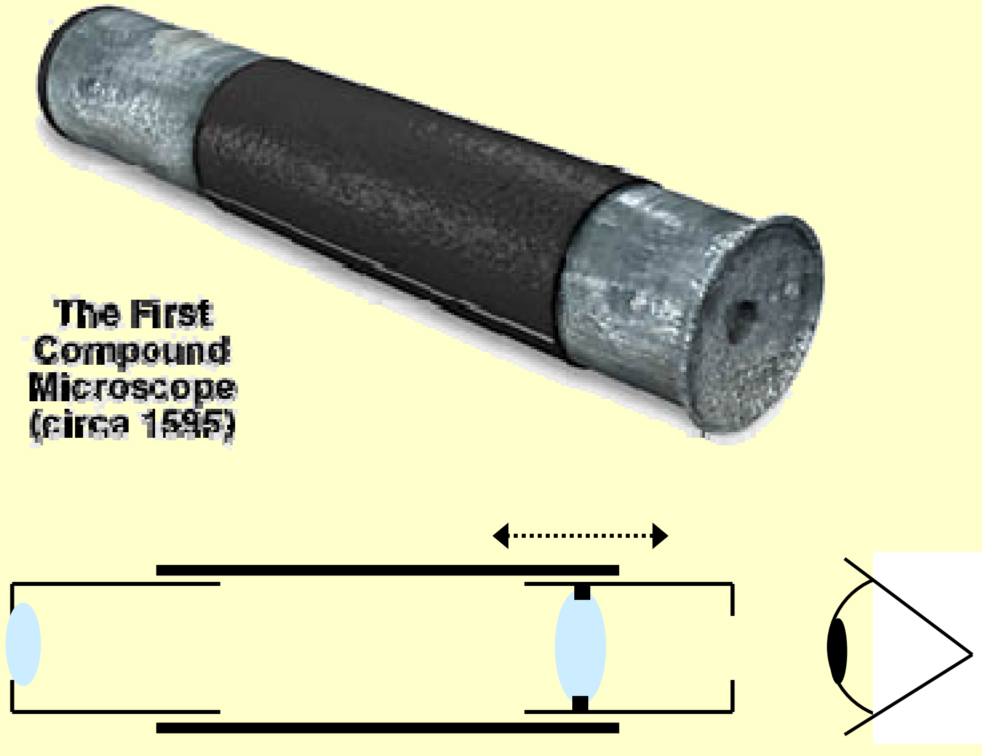
History and Evolution

- 4 Centuries of light microscopy!



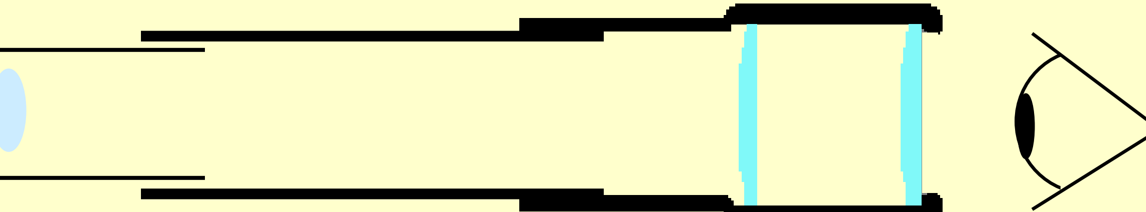
Janssen

Zacharias Janssen was a Dutch spectacle-maker credited with inventing the first compound microscope in ~1590. Magnifications ~3x to ~9x.



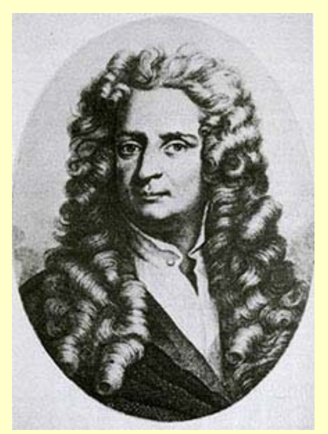
Huygens

Christiaan Huygens (1629–1695) was a Dutch mathematician, astronomer and physicist developed an improved two lens eye piece. Optical errors in two half curved lenses tend to cancel out. Can get more magnification.



Hooke

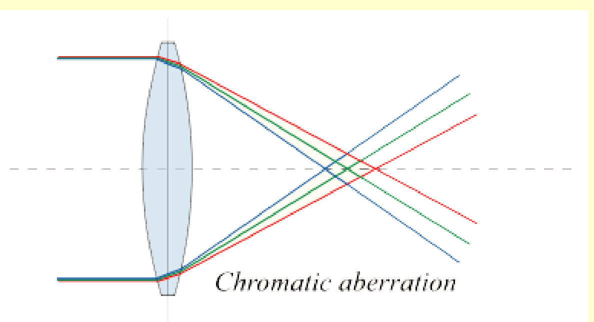
Robert Hooke improved the design of the new compound microscope, including a light source ~1655. Developed the micrometer. Resolution ~5 um.

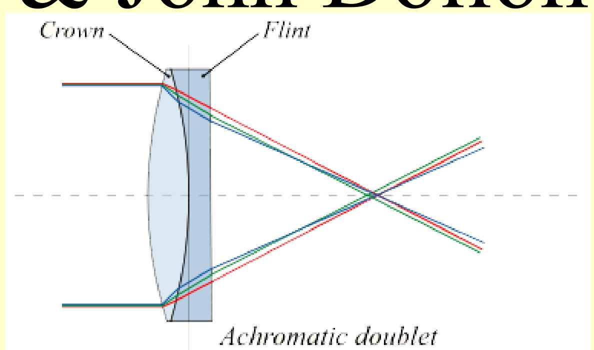


Leeuwenhoek

Anton van Leeuwenhoek simple microscope (~1675) used a single lens which yielded high magnifications (~70x to ~300x) and excellent resolution (~1 um). He reported seeing many kinds of microorganisms including bacteria!

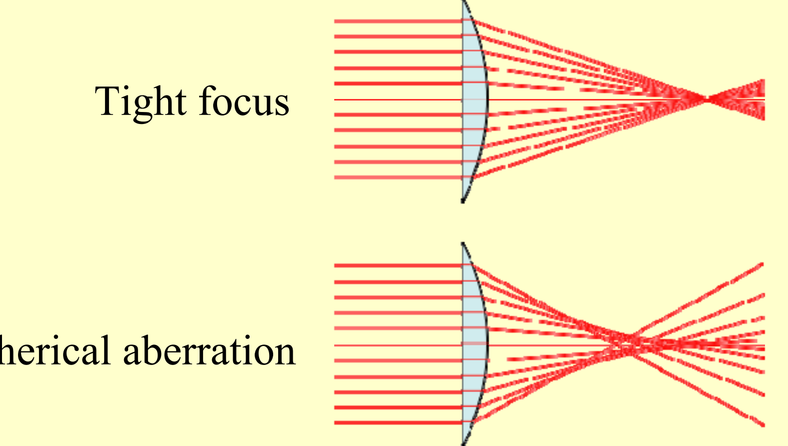
Chester Moore Hall & John Dollond





- Achromatic refracting lens was invented in 1733 by an English barrister named Chester Moore Hall
- Patented by John Dollond.

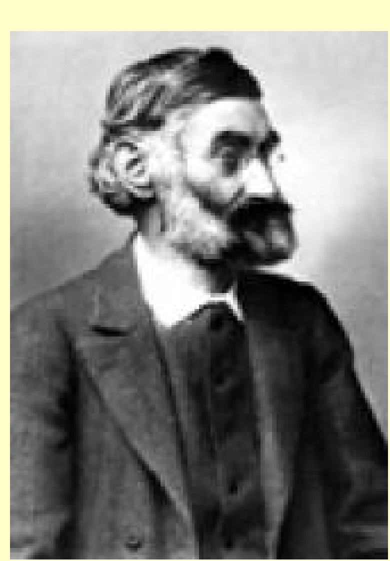
Lister

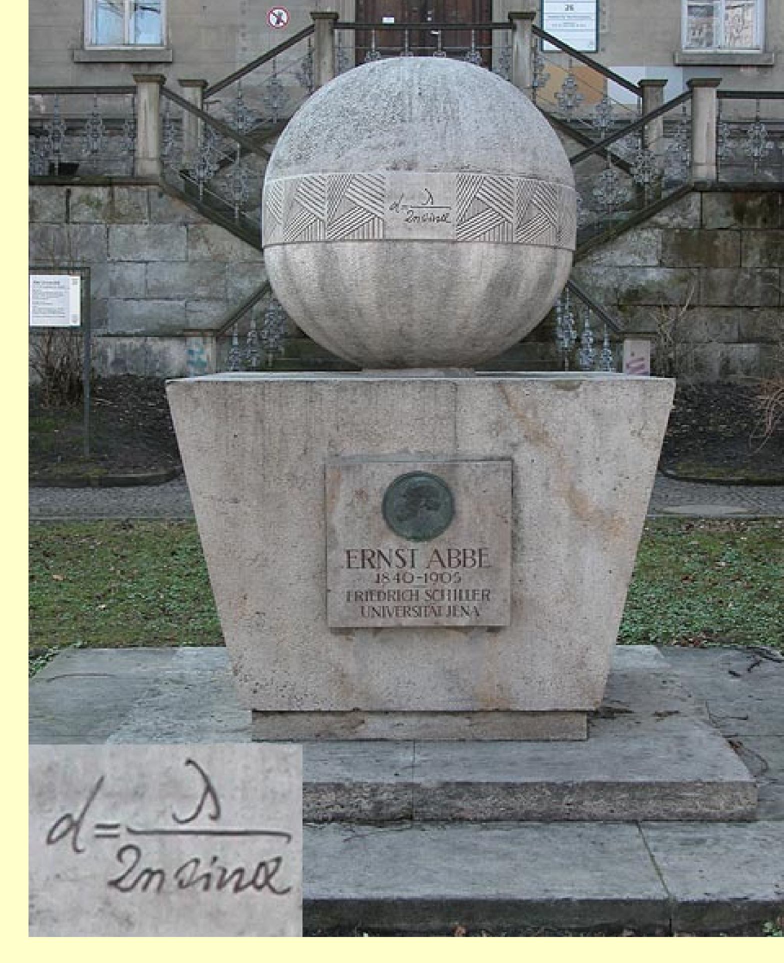


Joseph Jackson Lister (1786-1869) design and construct superior complex lenses by combining lenses of crown and flint glasses of different dispersion, but separated in order to both correct chromatic aberration and minimize spherical aberration.

Abbe

Ernst Abbe (1840-1905) applied mathematical principles to the design of lenses, which dramatically facilitated the manufacturing high quality optical instruments by the Carl Zeiss corporation. Abbe's realization that the performance of a microscope was limited by the diffraction of light was not well accepted for decades.

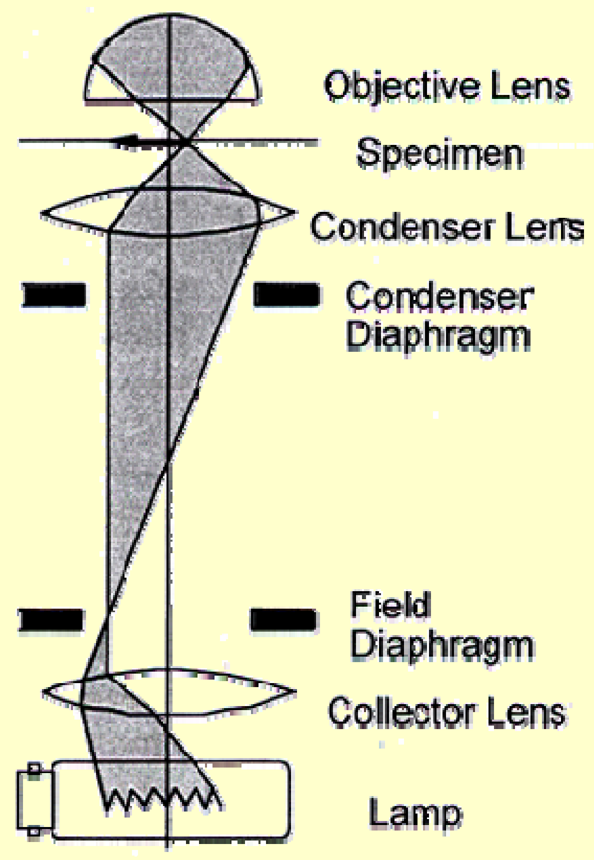


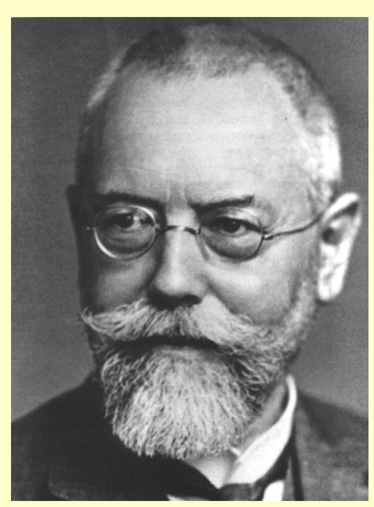


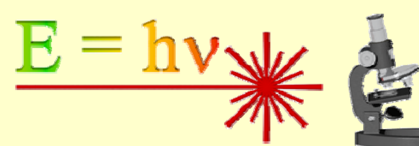
The Abbe limit formula

Köhler

In 1893 August Köhler (1866-1948) invented a method of providing optimum illumination of a microscope specimen while working at the Zeiss Corporation. Improved resolution and evenness of light illumination made photomicrography possible.

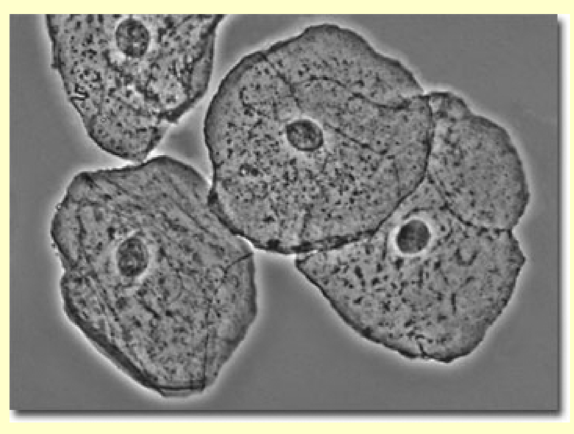






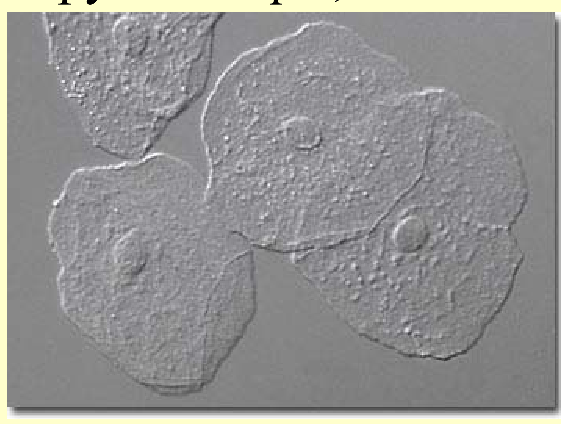
Zernike

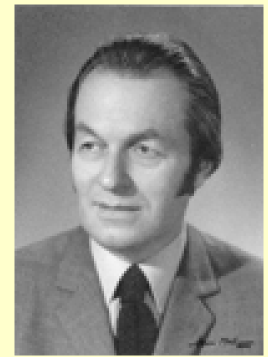
Frederik Zernike (1888–1966) invented phase contrast microscopy in 1933, a way to see unstained cells. Nobel prize 1953.



Nomarski

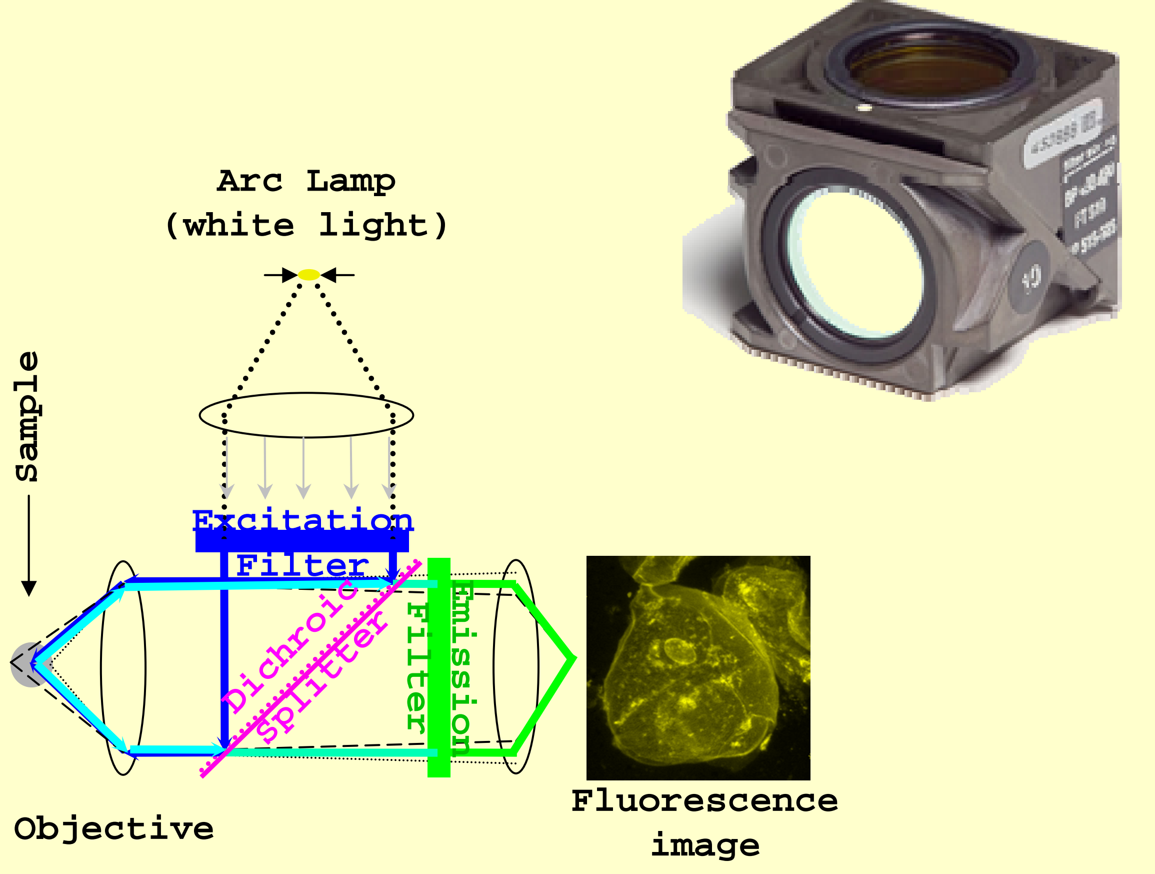
Georges Nomarski (1919-1997) developed the differential interference contrast (DIC) microscopy technique, which bares his name.





Ploem

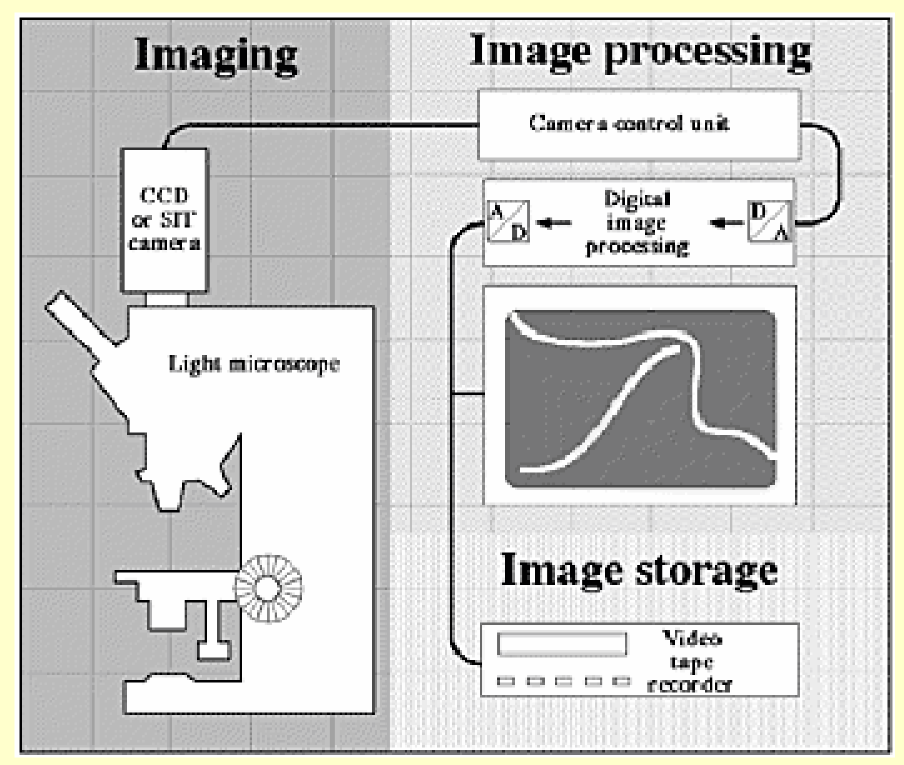
Johas Ploem invented the epi-illumination cube used in fluorescence microscopy.



Johan Sebastiaan Ploem
(1927-Present)

Inoue & D. Allen & N. Allen

Video enhanced microscopy – use electronic camera and computer generated contrast enhancement.

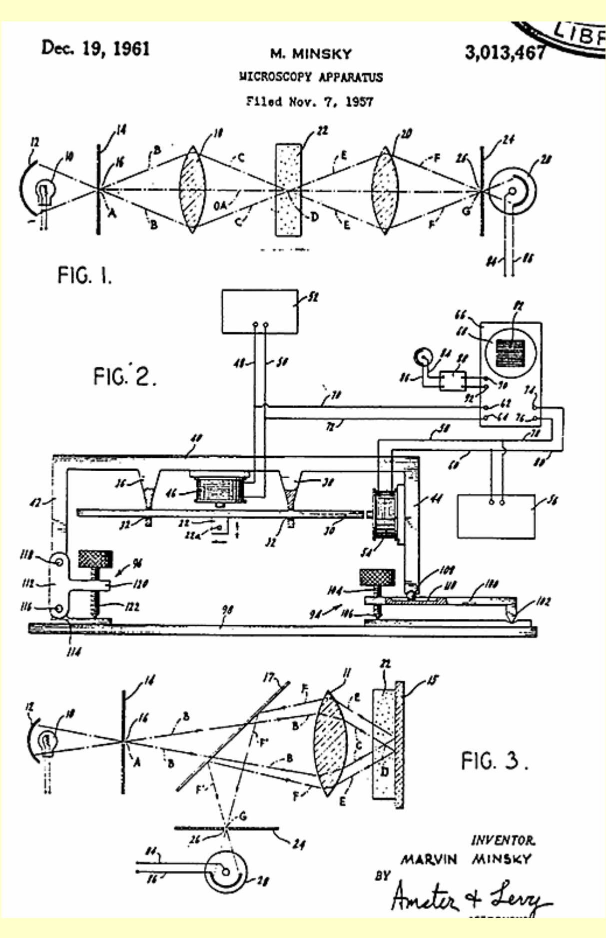


Shinya Inoue

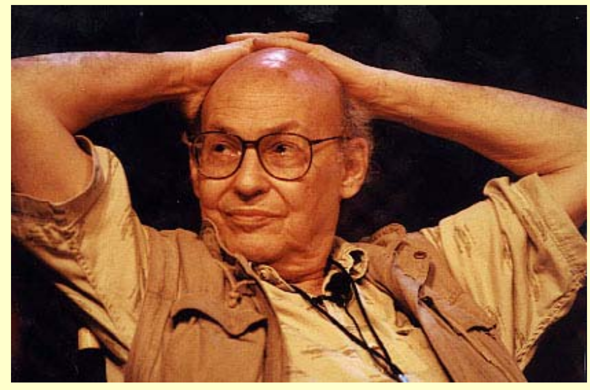
Bob & Nina Allen

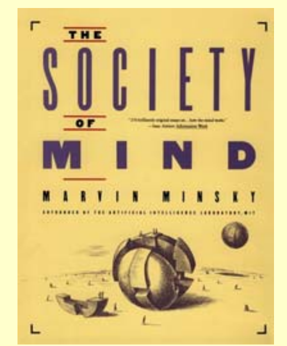
Marvin Minsky

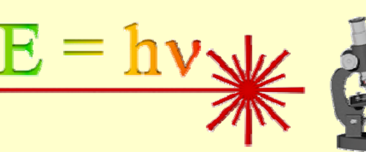
1957 Patented the Confocal Scanning Microscope: U.S.Patent 3013467



Practical Confocal microscope systems became available in the late 1980s. Yields improved contrast, resolution and optical sectioning.

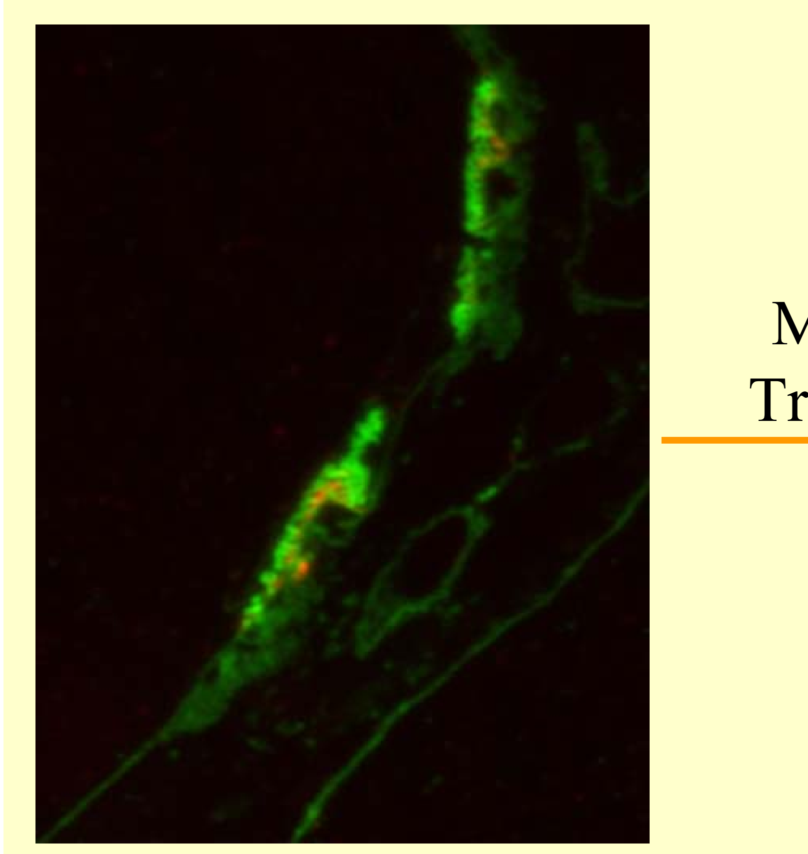




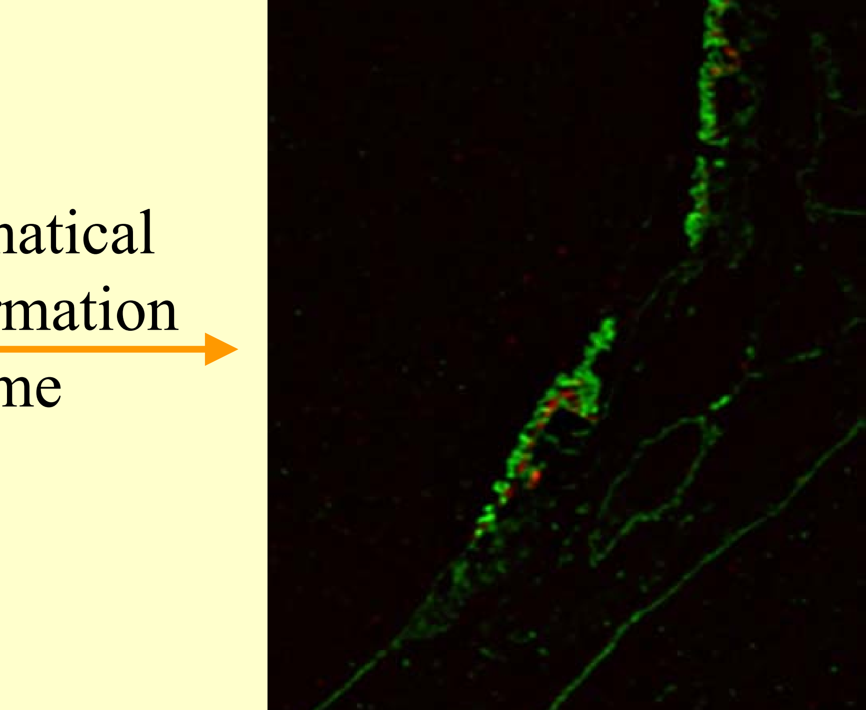


Sedat, Amos & Agard

1980s Digital deconvolution microscopy removes haze.



Mathematical Transformation + time



+ a powerful computer with extensive storage

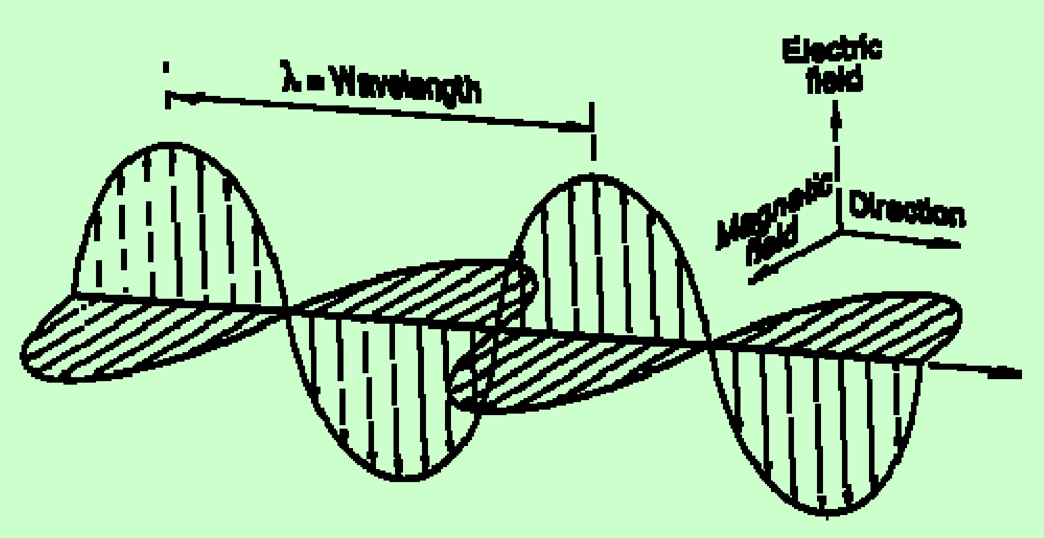
History - Recent Evolution+

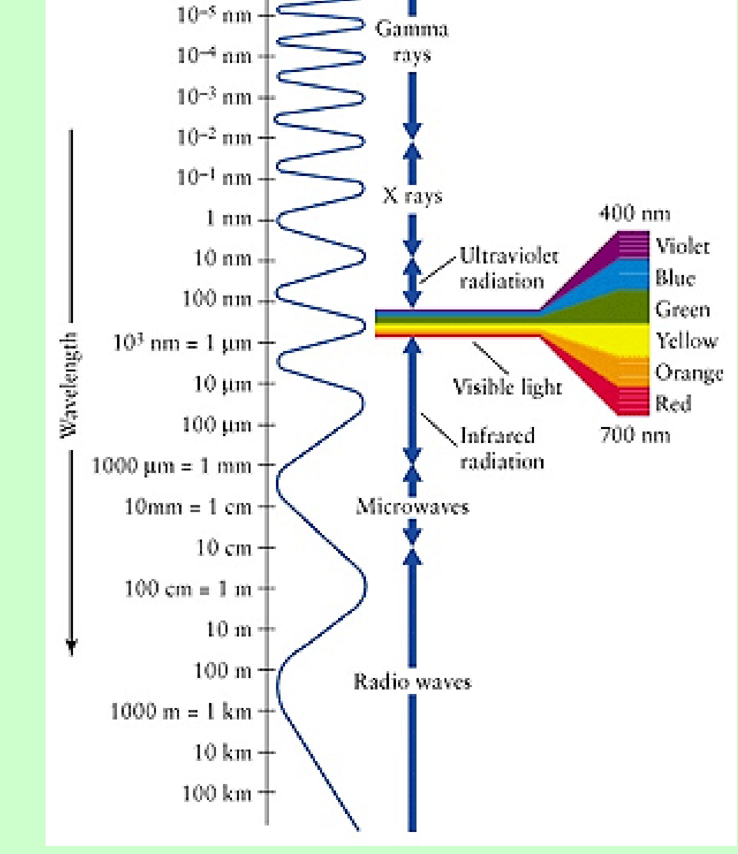
Dyes – fixed, vital, indicators
Immunostaining /Antibodies
Molecular Biology
Illumination
Lasers
Electronics
Cameras – CCDs, Intensifiers, high speed
Optics – AOTF, AOM, guide fibers
Computers
Algorithms & software
Techniques – Time lapse, FRAP, FRET, FLIM
Control systems – focus, x-y movement, shutters
Live cell environmental control

Better resolution
More sensitivity
Lower noise
Faster detection
Greater specificity
Easier analysis
Bigger storage
New capabilities

Increased complexity
Increased cost
More raw data

Optics – Light is an Electromagnetic Wave





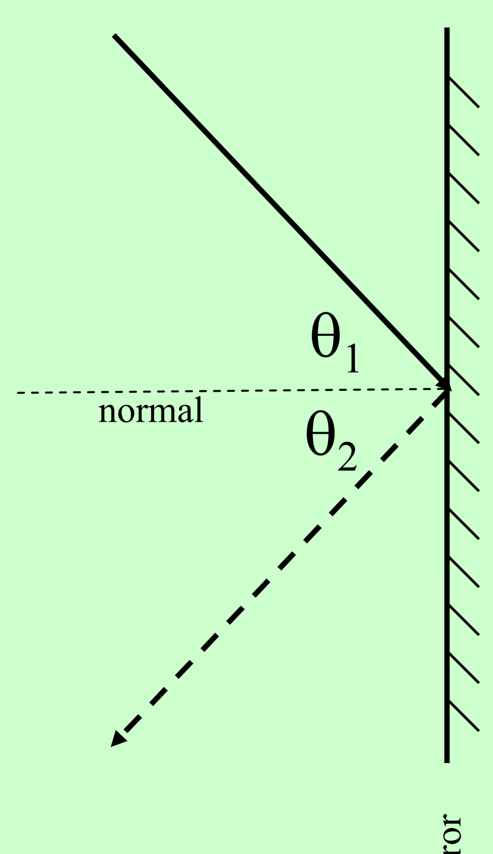
- **Wavelength** (can see)
- **Intensity** (can see)
- **Phase** (can not see)
- **Polarization** (can not see)

$$\mathbf{c = \nu\lambda}$$

ν = frequency λ = wavelength c = $3\text{x}10^8$ m/s velocity in a vacuum

e.g. Green: 550 nm, $54\text{x}10^{12}$ Hz (c.f. cell phone 1.9 GHz, 16 cm)

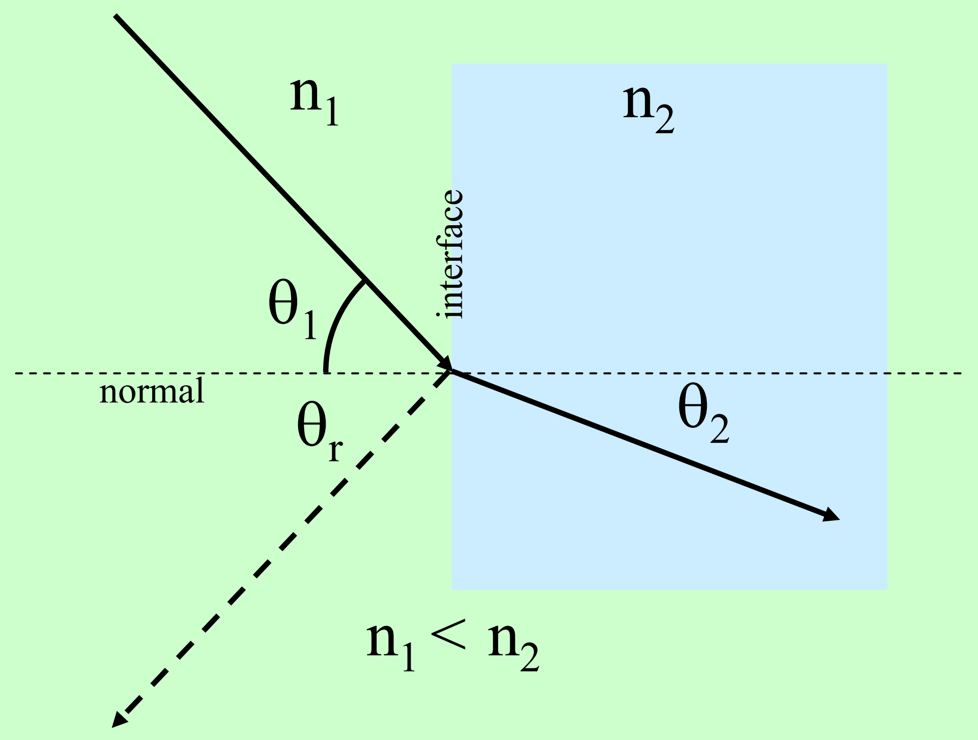
Optics - Reflection

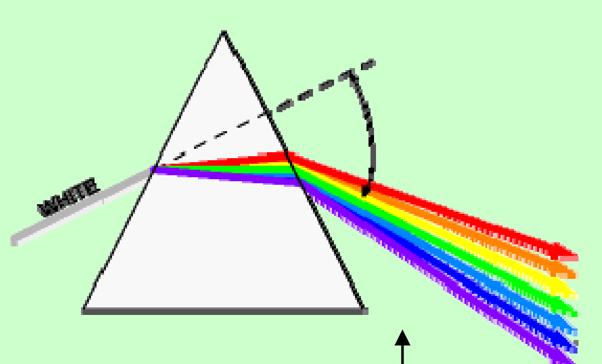


- Angle of reflection equals angle of incidence, $\theta_1 = \theta_2$
- No change in frequency or wavelength
- At larger angles, polarization reflection favors polarization *parallel* to the mirror plane, since light is wave phenomenon

- Can be due to EM wave interaction with:
  1. Dielectric and conductor, e.g. silver coating on back of glass,
  2. Plasmon – bouncing of EM waves off sea of vibrating electrons on metal surface,
  3. Interface at materials of different refractive index. E.g. Sunset light reflected off surface of the ocean

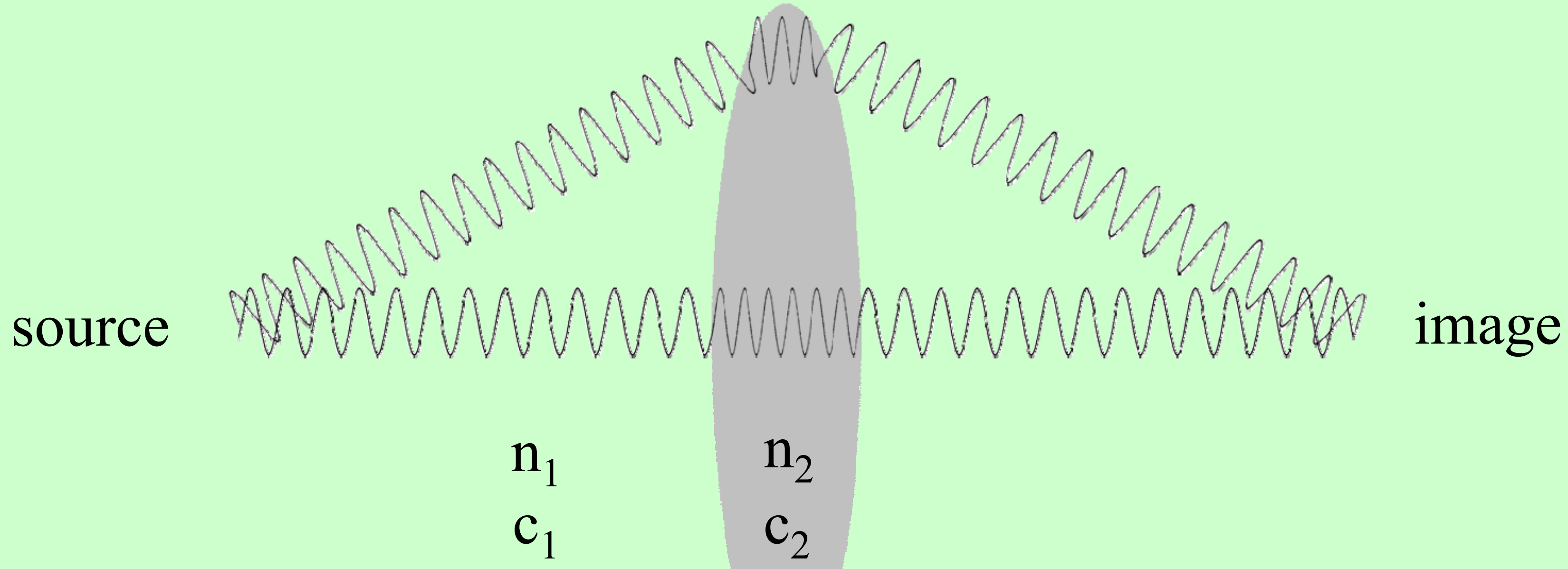
Optics – Refraction 1





- Light bent due to change in phase velocity in different media
- No change in wavelength
- Also get reflected component with larger difference in refractive indices
- Snell's law: $n_1 \sin(\theta_1) = n_2 \sin(\theta_2)$
- Wavelength dependent – more refraction with shorter wavelength
- Refractive index: $n = c / \text{velocity}_{\text{medium}}$, $(c = \text{velocity}_{\text{vacuum}})$

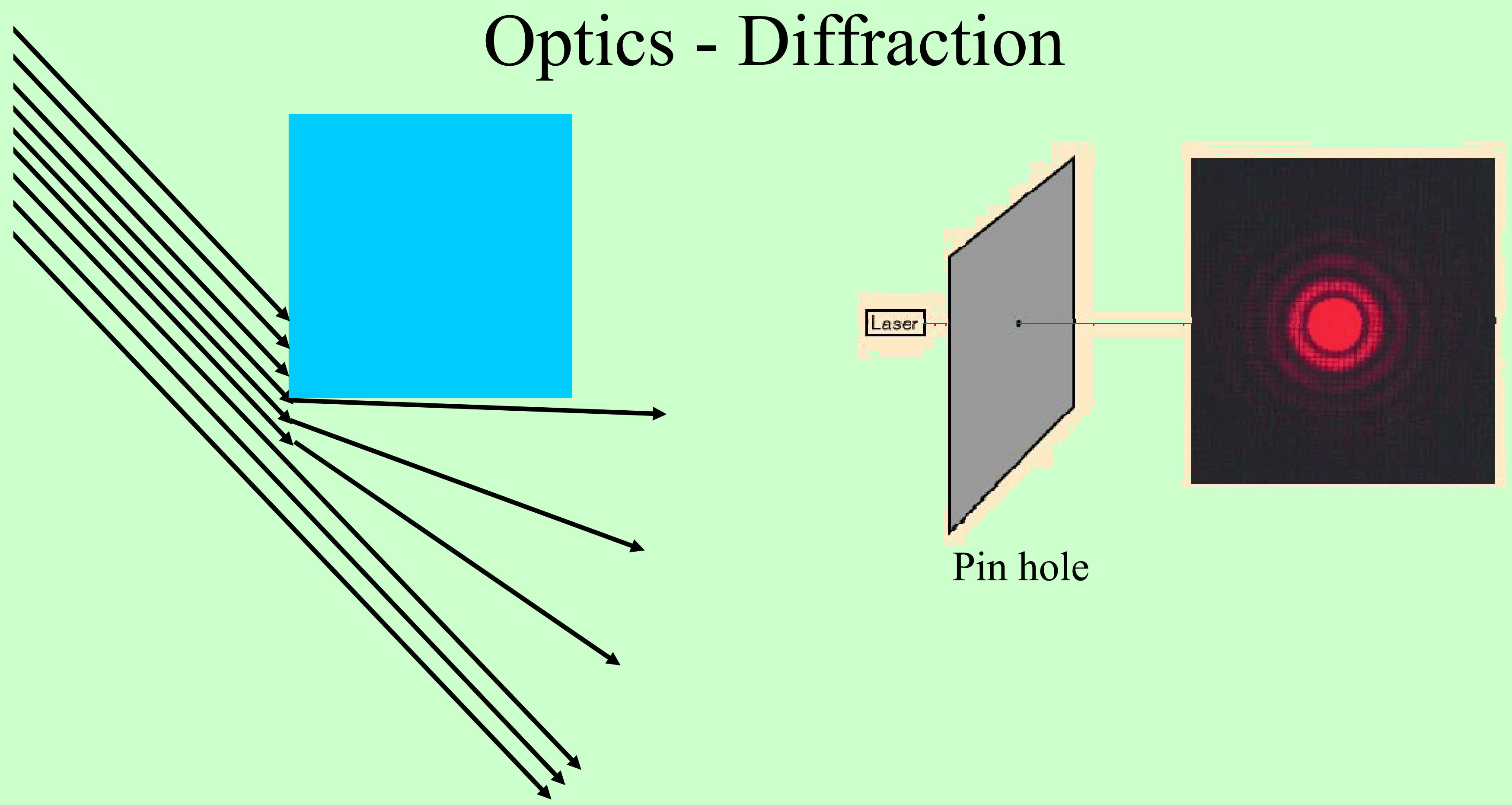
Optics – Refraction 2 Lens



Medium refractive index $n_1 < n_2$
Velocity in medium $c_2 < c_1$
Light is coherent at local region of source
Straight-through wave is retarded longer in thicker part of lens, where velocity is lower
In path shown above, light arrives in phase giving constructive interference

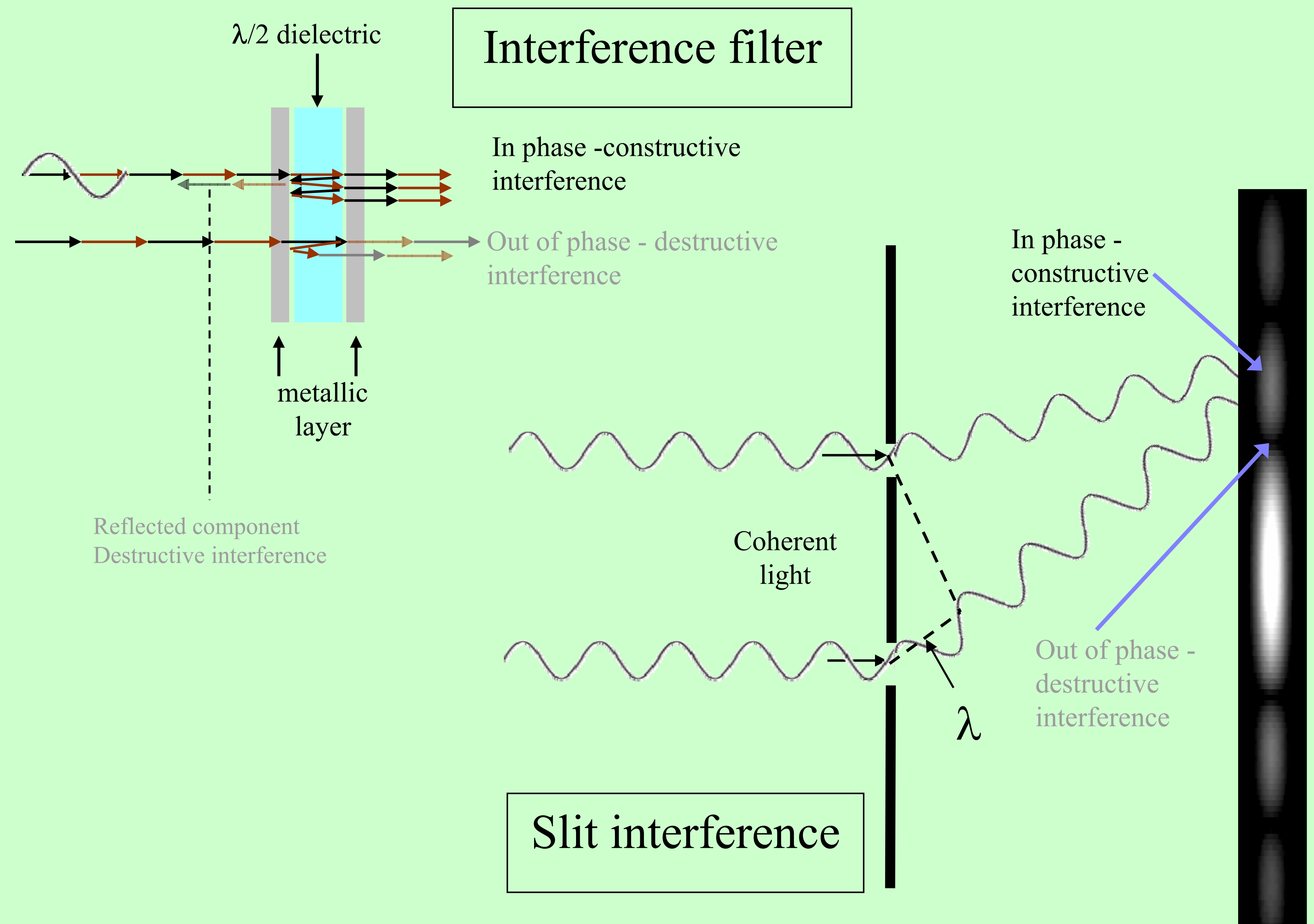
When light arrives out of phase, there is destructive interference

Optics - Diffraction

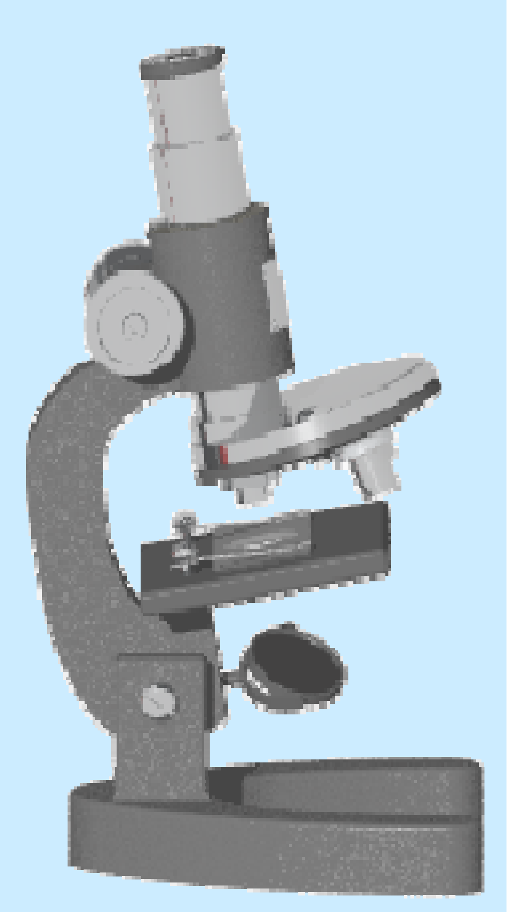


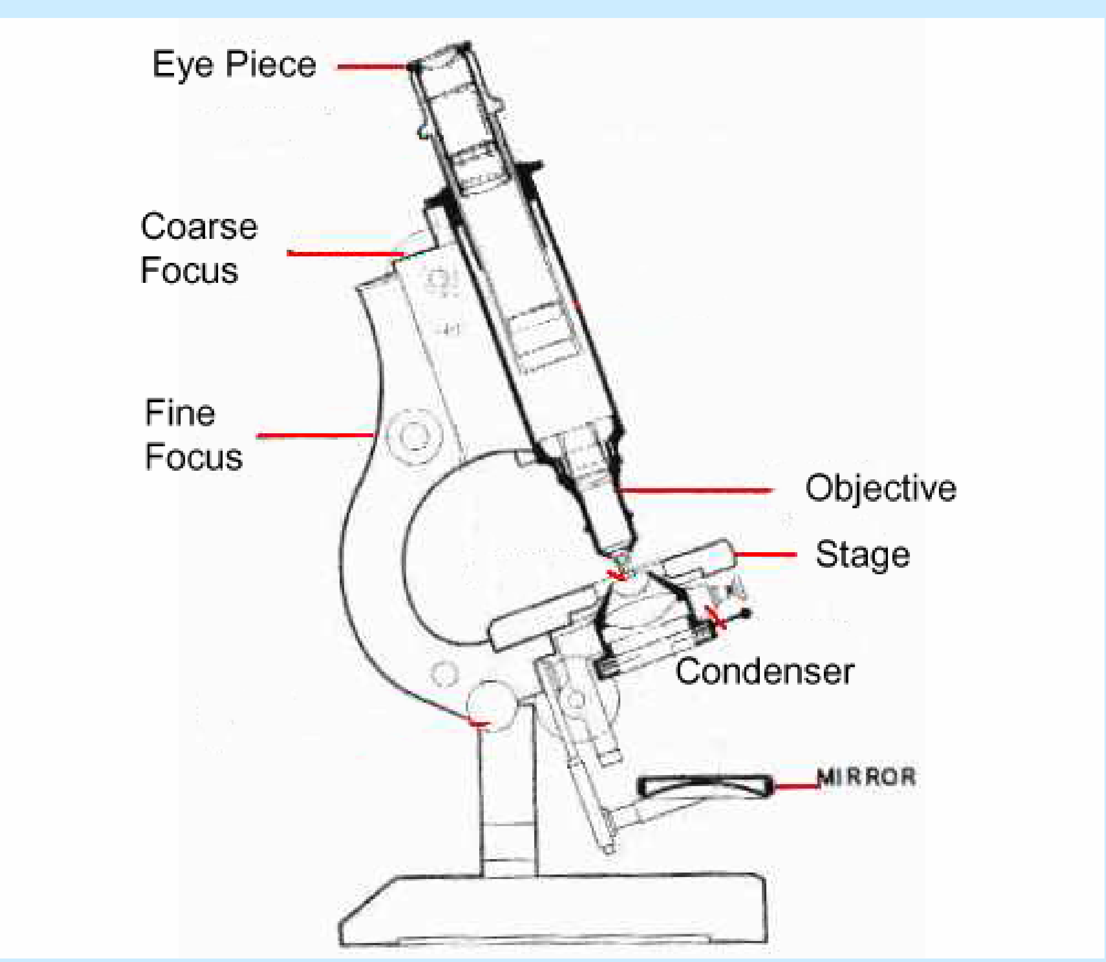
- Interaction of EM waves with small objects and edges
  - Interference effects
  - Summation/cancellation of in phase and out of phase waves
- No change in frequency or wavelength
- Will see later that diffraction limits optical resolution

Optics – Interference examples



Key Parts of a typical microscope

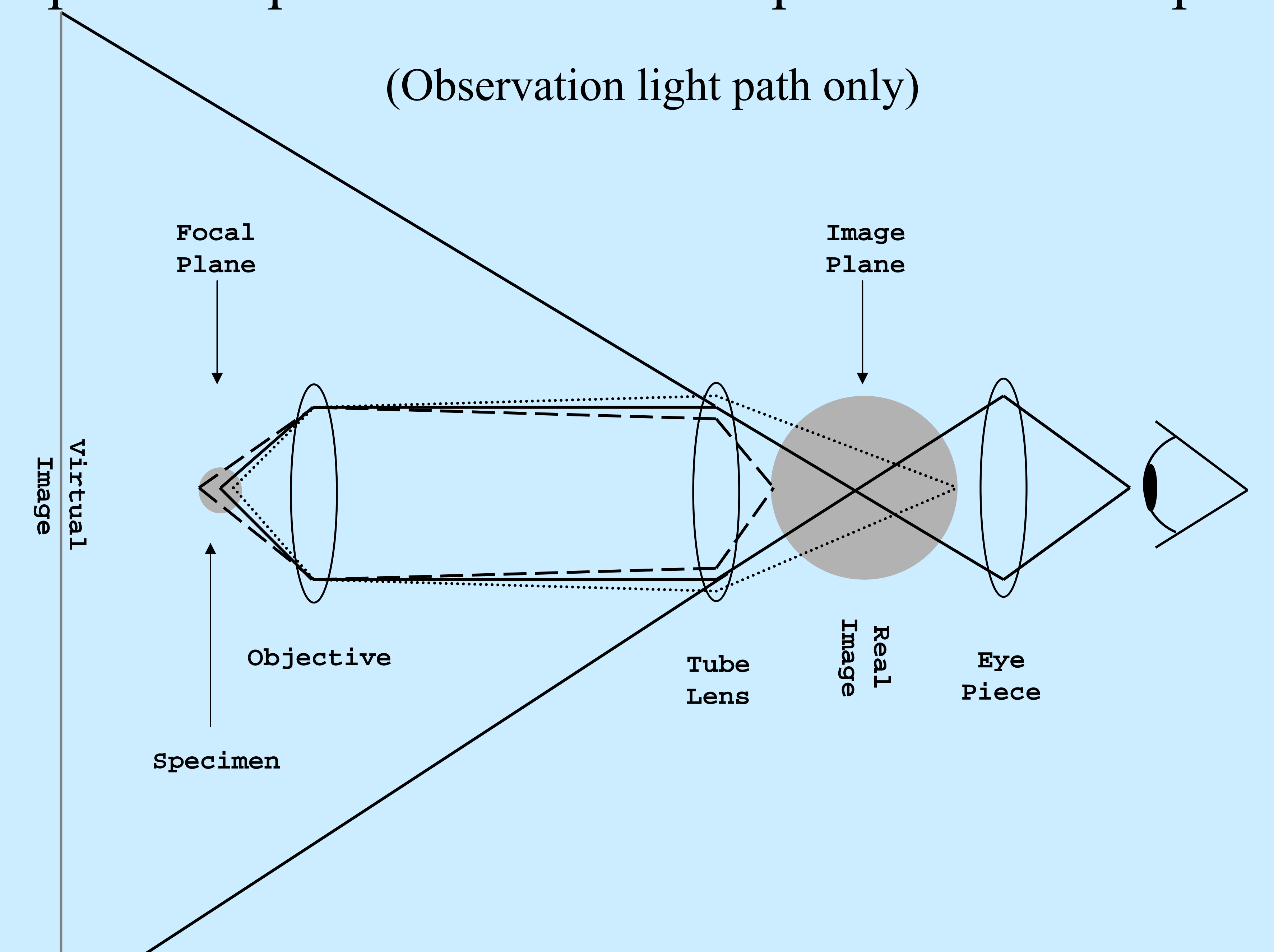




Note that the lamp is missing.

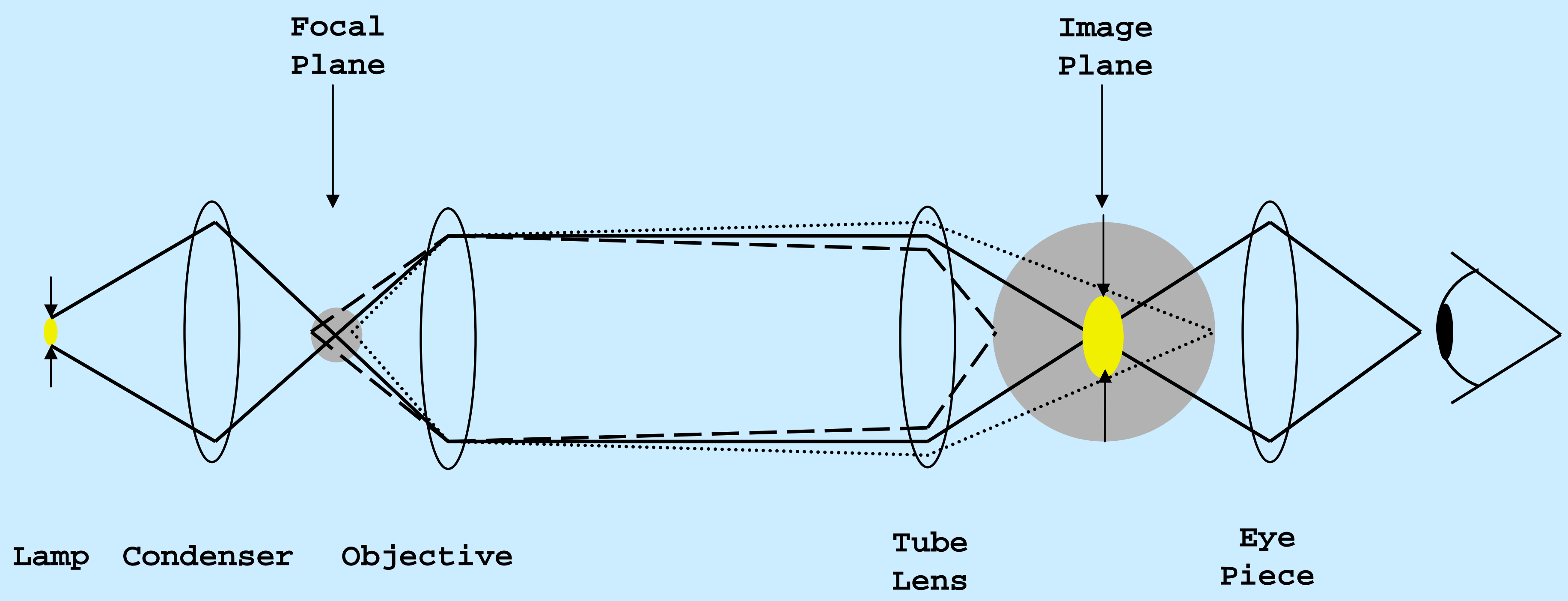
Simplified Optical Path of a Compound Microscope

(Observation light path only)



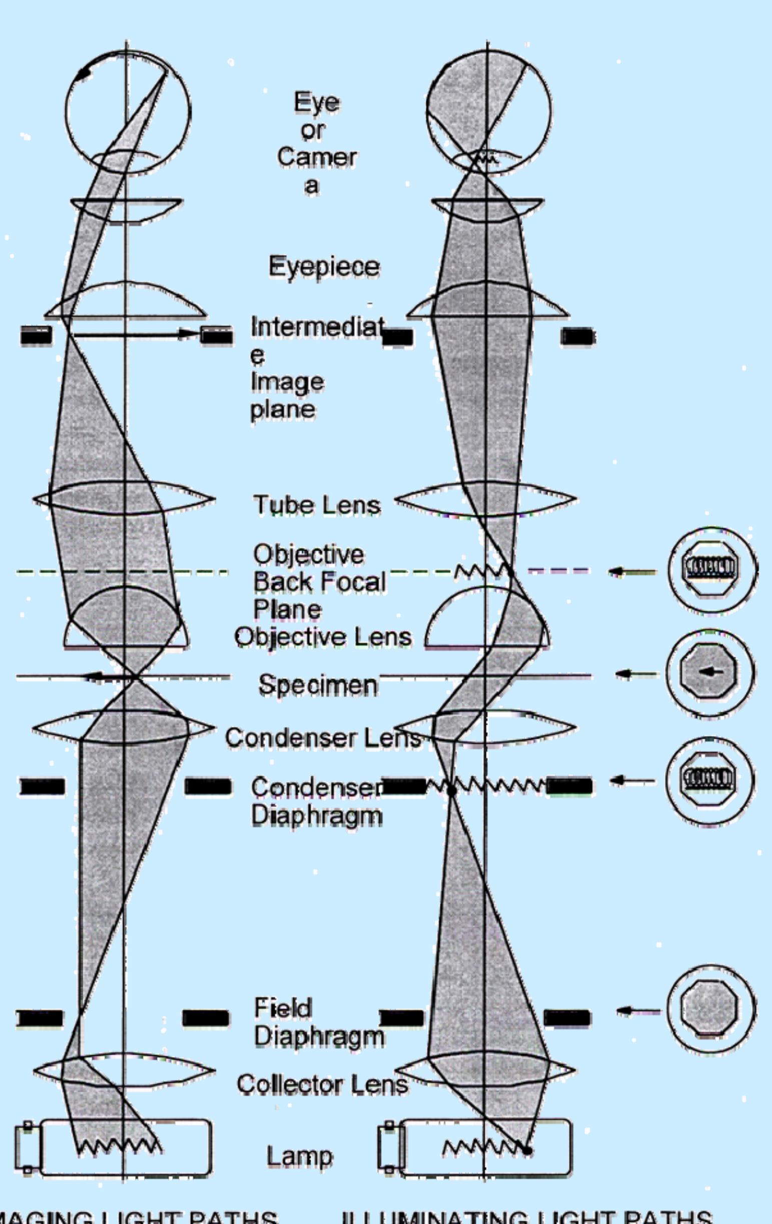
Simplified Optical Path

Illumination and observation light paths



Problem: See image of lamp filament

Kohler Illumination

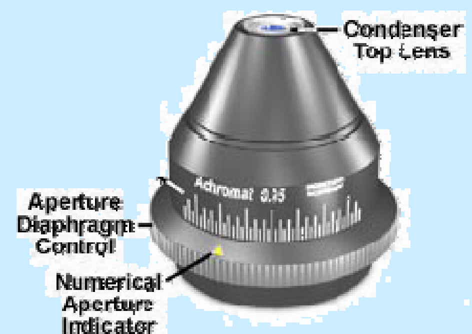


<u>Condenser has double apertures</u>

1. Condenser aperture changes the cone angle of light at the specimen

2. Field diaphragm changes diameter of light at the specimen

The Condenser



- …is your friend
  - When trying to find your sample
    - Minimum aperture gives:
      - Thick depth of focus (easy to find sample since greater better chance of being in focus)
      - High contrast (can see edges of colorless cells, and also see dust & scratches which is great for finding the sample)
      - Less light (but have more than enough anyway)
      - Poorer resolution (who cares when just locating sample)
  - When taking images
    - Maximum aperture provides:
      - Thinner depth of focus (less overlying material seen)
      - Lower contrast (more even background, no dust and scratches)
      - More light (good for dark samples)
      - Best resolution (camera will notice the difference but eye will not)

Parts of a typical microscope

MICROSCOPE COMPONENTS

Camera

Camera Adapter

Binocular

Eyepiece

Epi-Field
Diaphragm
& Centering

Epi-Condenser
Diaphragm

Filters

Shutter

Epi-Lamp Housing

Mirror:
Focus and
Centering

Beam Switch

Magnification
Changer

Filter Cube
Changer

Slot for Analyzer

Body Tube

Slot for DIC Prism

Objective Nosepiece

Objective

Stage

Condenser:
Diaphragm&Turret
Centering
Focus

Slot for Polarizer

Field Diaphragm

Focus, Centering

Trans-Lamp Housing

Mirror:
Focus and
Centering

Upright Microscope
Stand

Coarse/Fine
Specimen Focus

Filters
and Diffuser

Lamp: Focus, Centering

From E.D. Salmon

Parts of a typical microscope

Incandescent lamp (transmitted)
Field diaphragm (2)
Condenser focus (5)
Neutral density & polarizer filters (3)
Condenser ring (5)
Condensor aperture (4)
(11)
Mercury arc lamp (fluorescence)
Slide holder (6)
Wallisten prism, obscured (7)
Mercury lamp block off rod (14)
Dichroic filter wheel (15)
DIC wave plate (analyser), slider (8)
Fine/course focus (6)
(9) Camera port sliders (10)
Incandescent light (transmitted) power wheel (12)
Incansescent light power (1)
(11)

Objectives

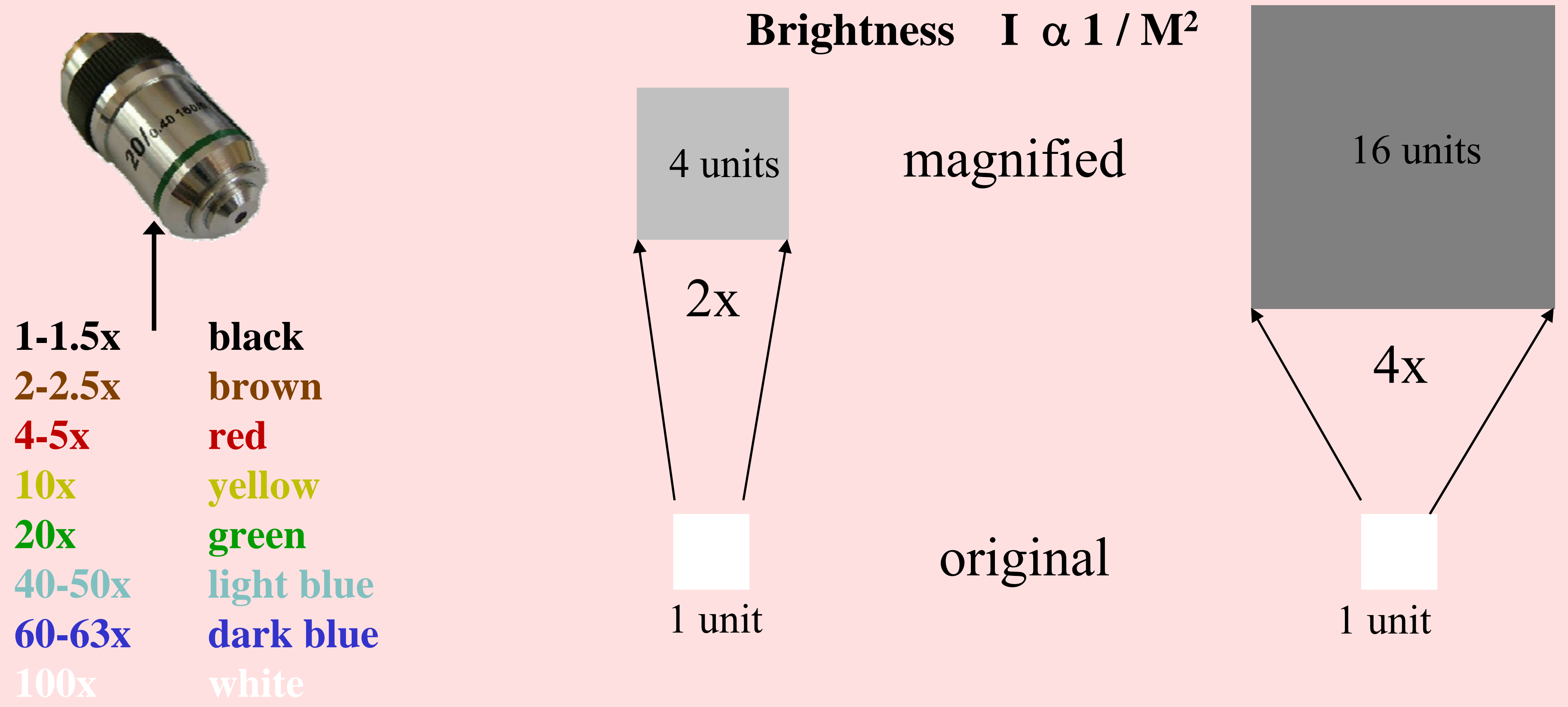
The objective is the 1ST imaging element
Determines the performance of the optical system

- Magnification
- Numerical Aperture (NA)
- Immersion: oil – glycerol – water – air
- Tube length system (infinity)
- Cover slip (0.17 mm or 0 or variable)
- WD = working distance
- Corr = Correction Collar
  - cover slip – iris – immersion medium
- Field curvature and aberration correction:
  - Plan Apo – Fluor – Acromat …

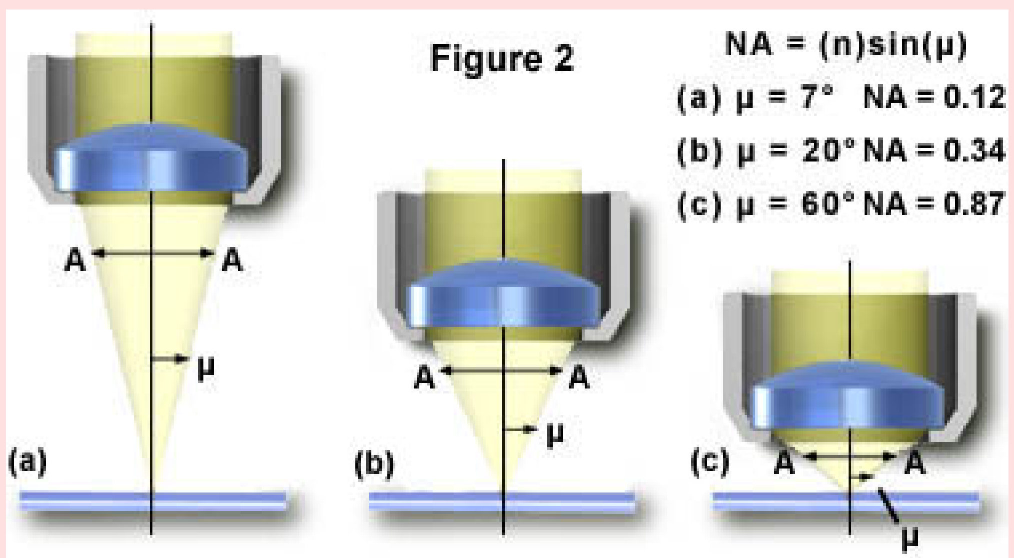
40x NeoPlan 0.4 NA cover slip thickness adjustment correction

Objectives - Magnification

- Note: more magnification (M) gives less light intensity at detector
  - Due to inverse square law
  - Often, lens elements that are also more absorbing
- Brightness proportional to $\mathbf{1 / M^2}$
- Color coded band shows magnification (roughly)



Objectives - Numerical Aperture (NA)



From Molecular Expressions

$$NA = n \cdot \sin(a)$$

a = half angle of cone of illumination

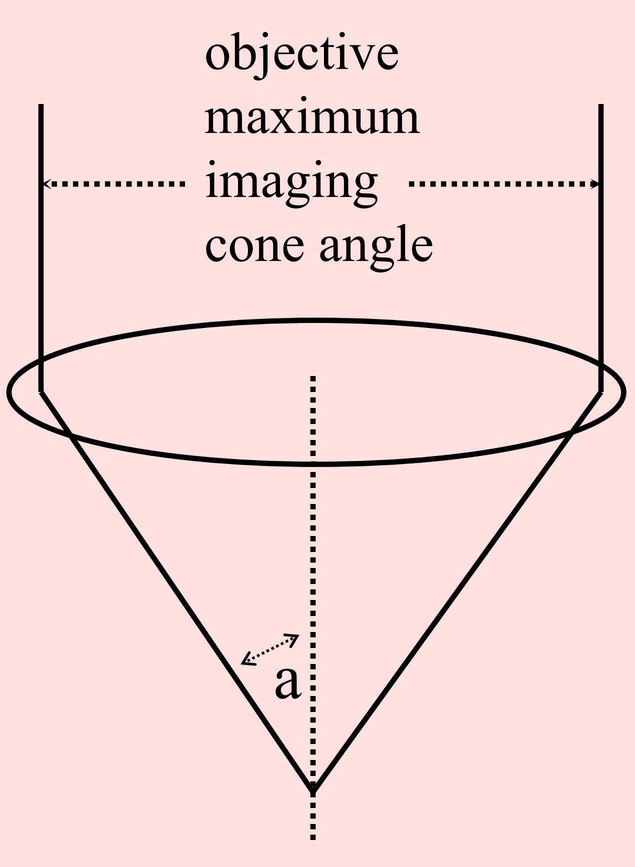
n = refractive index of medium

- Numerical aperture is related to light gathering and resolving power.
- High NA leads to smaller working distance.
- Transmission brightness is proportional to NA^2

Numerical Aperture (NA) - resolution

$NA = n \cdot \sin(a)$, a = half cone angle
n = refractive index of medium
λ = wavelength

Wide Field (d=distance) $d = \lambda / (2\ NA)$ $= 0.22\ \mu m$



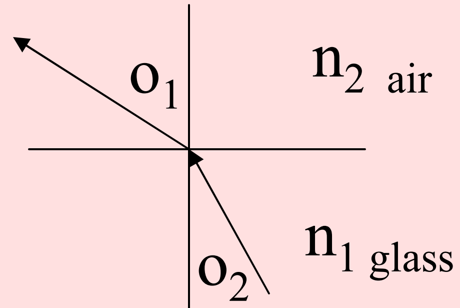
Objectives - Immersion

Refractive Indexes:

- Dry (air) = 1.0003
- Water = 1.33
- Glycerol = 1.47
- Immersion oil = 1.52
- Glass = 1.52

Snell's law of refraction

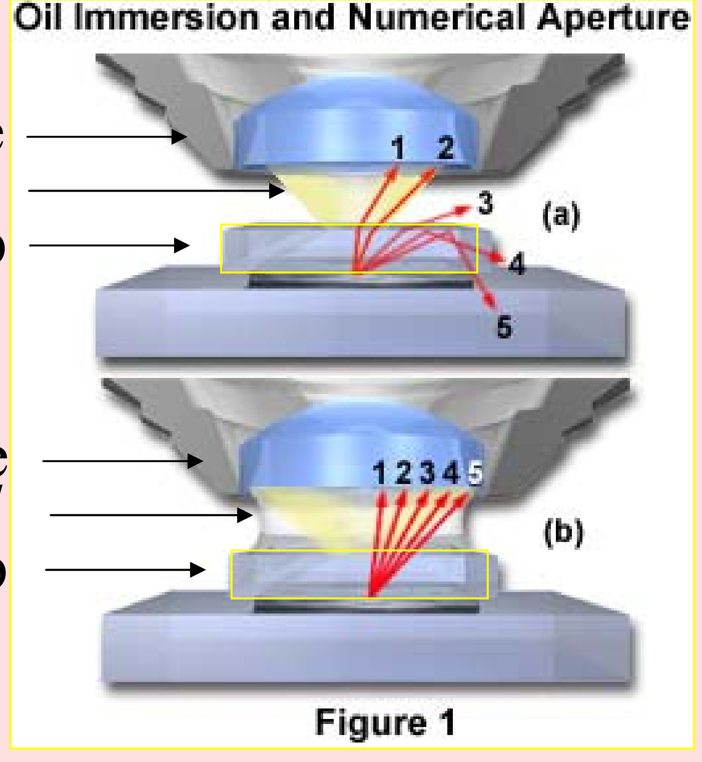
$$n_1 \cdot \sin(o_1) = n_2 \cdot \sin(o_2)$$



(n = refractive index)

objective
air
cover slip

objective
oil
cover slip

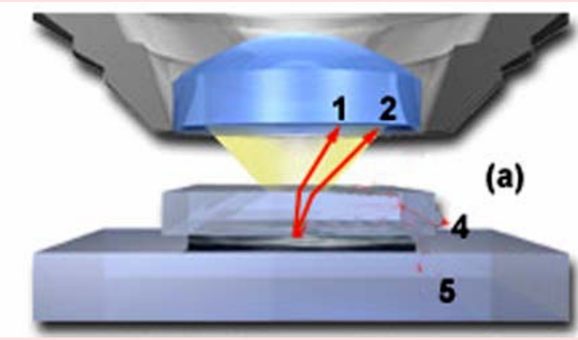


From Molecular Expressions

Objectives - Cover Slips

- For non-oil immersion objectives, the coverslip is really a lens element.
- Use (number) #1.5 for many objectives
- With higher magnification, non-immersion lenses with a cover slip correction collar, it is very important to adjust it appropriately

This objective's correction collar lists cover slip thickness in mm. Set to 0.17 for #1.5 cover slips, and/or use maximum contrast method if your sample has structures with good contrast for empirical adjustment while viewing.



From Molecular Expressions

Objectives - Tube length

- Modern scopes are "infinity" corrected
  - Allows focus by moving objective without change of magnification
  - Allows filters, cubes and other components between the objective and tube lens without causing distortion
- Older scopes may be 160 mm
- Need to match with microscope

Objectives – Corrections

- Coverslip – 0 to 2 mm
  - Note: culture dishes are 1 mm thick
- Immersion medium – oil, glycerol, water
- Aperture – Max. NA - reduced NA
  - Used for matching darkfield condenser NA or reducing glare; but loose resolution and brightness

Objectives – Special Properties

- DIC (Nomarki)
- Phase Contrast

- WD = Working distance

Objectives *						
Mag.	NA	type	WD	corrections	cover slip	Immersion
10x	0.3		3.6 mm		#1.5	air
16x	0.5	PL Fluotar	150 um		#1.5	oil/glycerol/water
20x	0.7	PL Apo	590 um		#1.5	air
40x	0.85	PL Apo	240 um	corr	0.14-0.18	water
40x	1.25 to 0.75	Apochromat	240 um	aperture	#1.5	oil
63x	1.4 to 0.6	PlanApo	90 um	aperture	#1.5	oil
63x	1.2	Apo	220 um	corr	0.14-0.18	water
L40x	0.8	HCX Apo	3 mm	U-V-I	none	water **
L63x	0.9	HCX Apo	2 mm	U-V-I	none	water **

Objective Type	Spherical Aberration	Chromatic Aberration	Field Curvature correction
Achromat	1 Color	2 Colors	No
Plan Achromat	1 Color	2 Colors	Yes
Fluorite	2-3 Colors	2-3 Colors	No
Plan Fluorite	3-4 Colors	2-4 Colors	Yes
Plan Apochromat	3-4 Colors	4-5 Colors	Yes

Care of Objectives

Gentle, gentle, gentle, gentle!

When rotating objective turret: Focus away 1st. Slowly!

When changing slides: Focus away. Remove slide slowly!

When unscrewing objectives: Two hands! Don't let drop. Store immediately in objective holder tube. Don't leave on table.

Cleaning:

- Should be infrequent with dry lenses.
- Lens tissue or surgical cotton only! Never Kim Wipes, Kleenex, Q-Tips, etc.
- With immersion lenses, blot front lens gently. Do not wipe or rub.
- Dry or immersion lenses clean off residue or oil use coated lens cleaner (pH ~6.0 to 7.0, non-ionic detergent, short chain alcohol)

Objective & Condenser NA – Resolution

$NA_{obj} = n_o \cdot \sin(a)$, a = half cone angle

n_o = refractive index of objective side medium

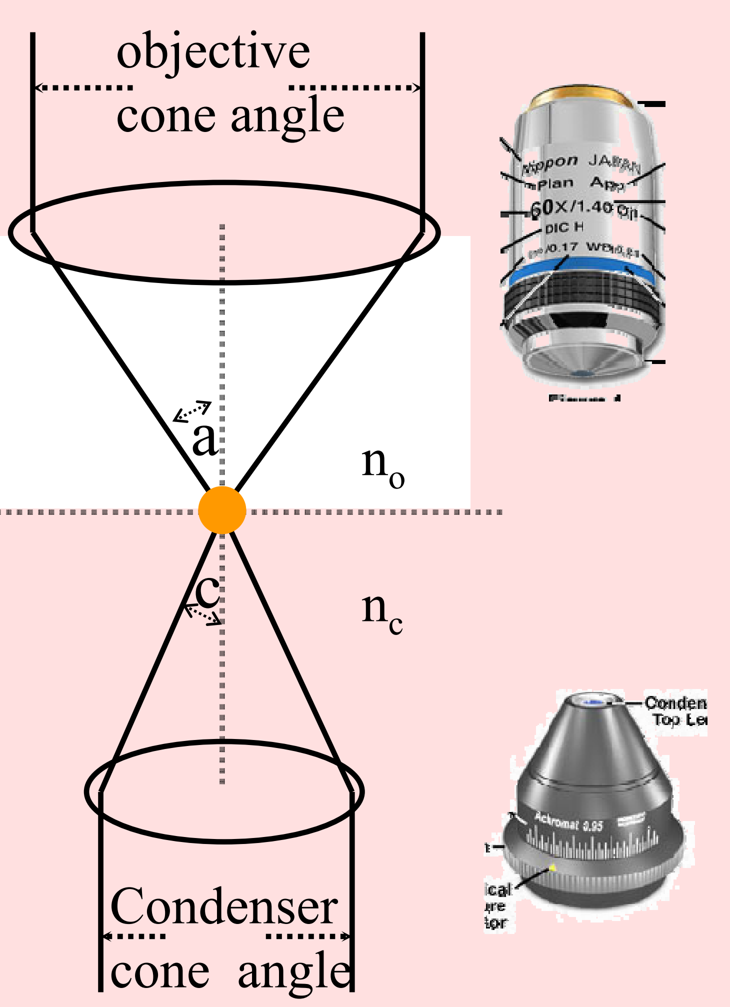
λ = wavelength

$NA_{cond} = n_c \cdot \sin(c)$ n_c = refractive index of condenser imm. medium

Approx. resolution $d = \lambda / 2\, NA$

Resolution with condenser

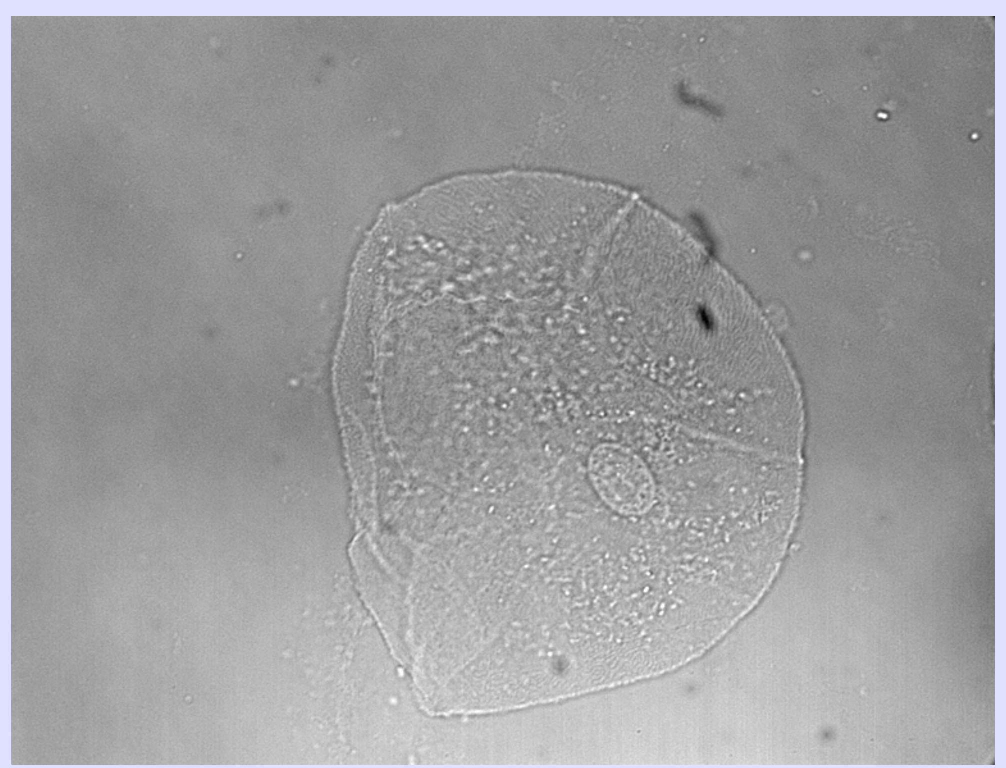
$$\mathbf{d = \lambda / (NA_{obj} + NA_{cond})}$$



Common Modes of Imaging

- Transmitted
  - Wide field (standard transmitted)
  - Phase contrast
  - Nomarski (DIC)
  - Polarization (material science)
- Epi-illumination (including confocal)
  - Fluorescence
  - Reflection (material science)

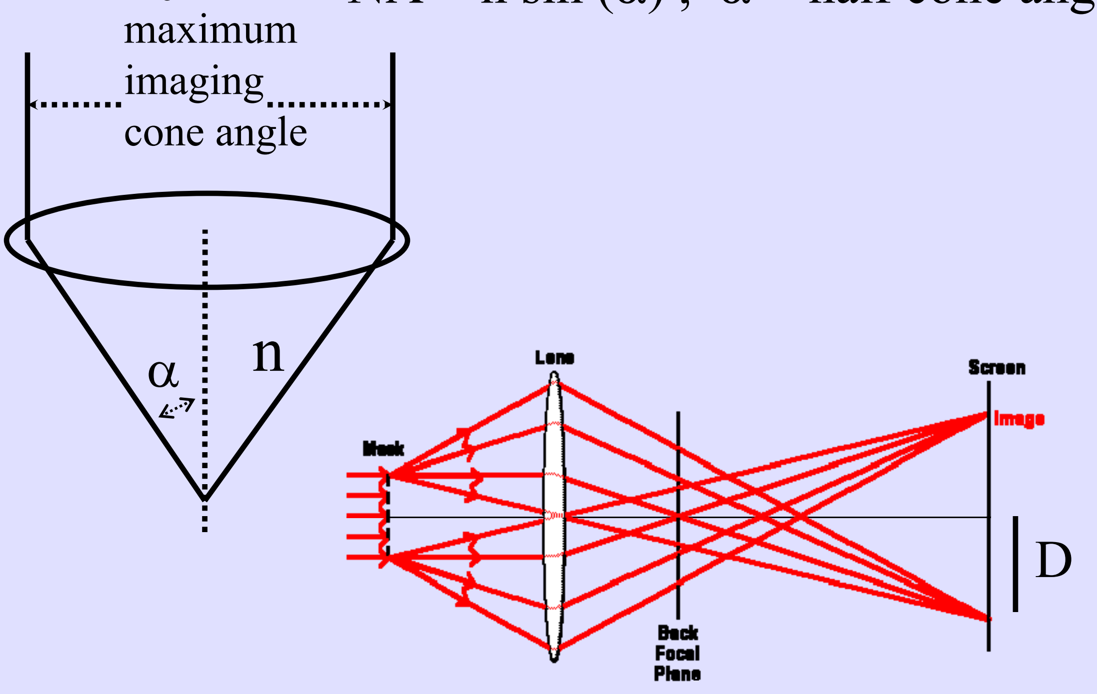
Transmitted light – (wide field)



Live Buccal Epithelial cell (unstained)

Wave Nature of Image Formation

NA = n·sin (α) , α = half cone angle



Ernst Abbe (1872) Image formation:-

Collection of diffracted rays around sample by objective

Interference of these rays in the image plane

$d = \lambda / (2n \sin(\alpha))$

n = refractive index of medium

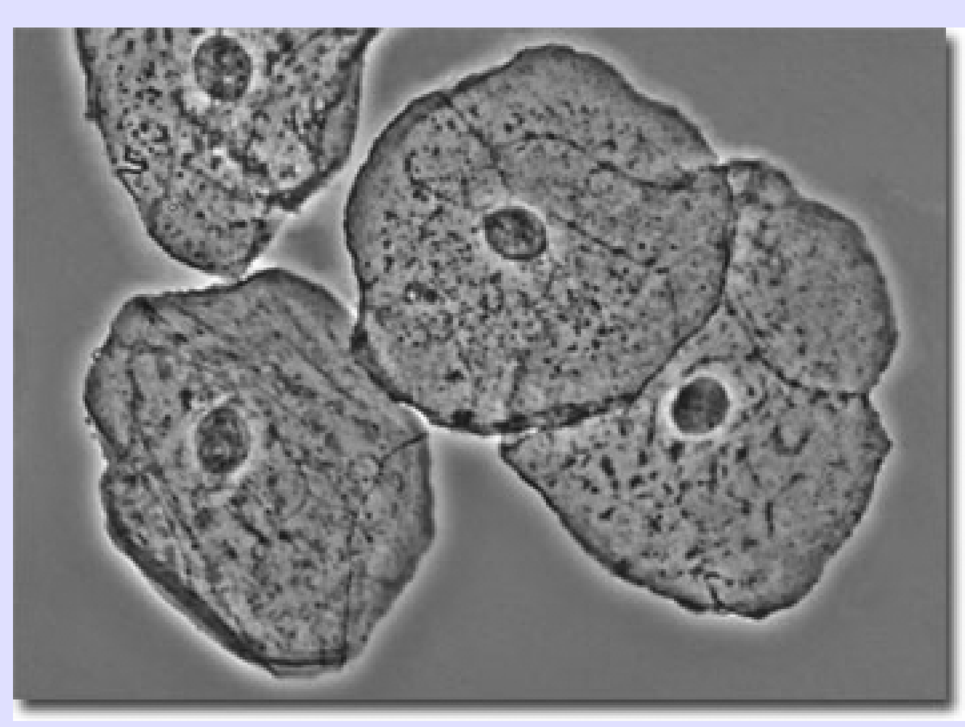
λ = wavelength of light

Both slits open

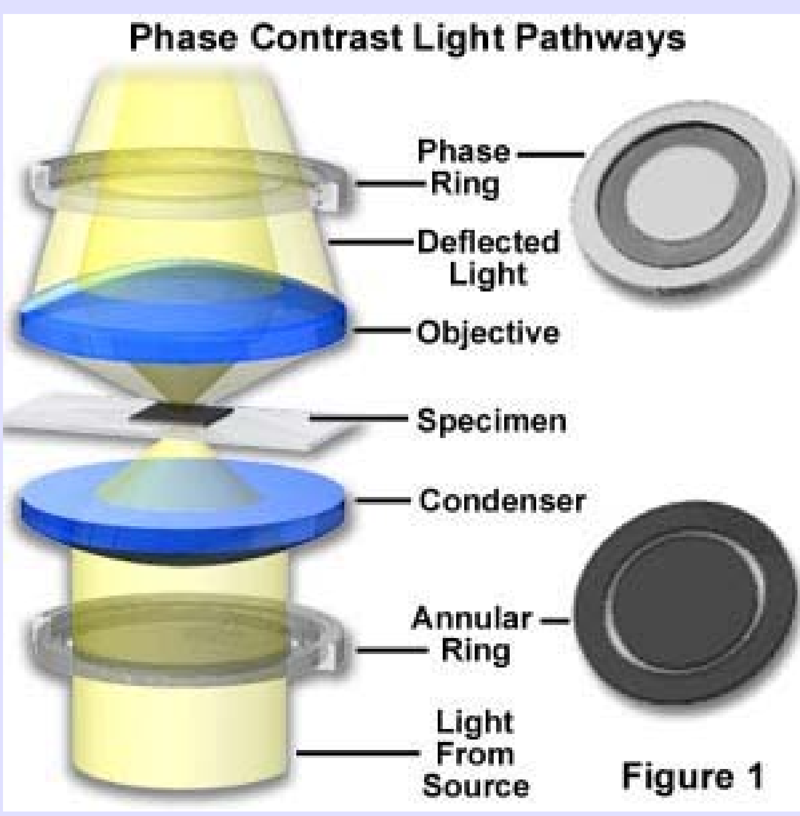
wave theory

particle theory

Phase Contrast



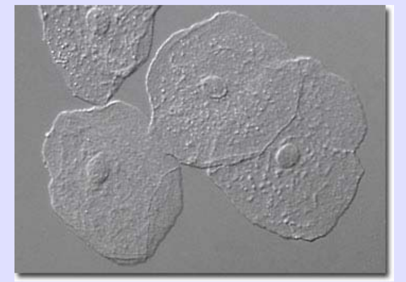
Phase contrast



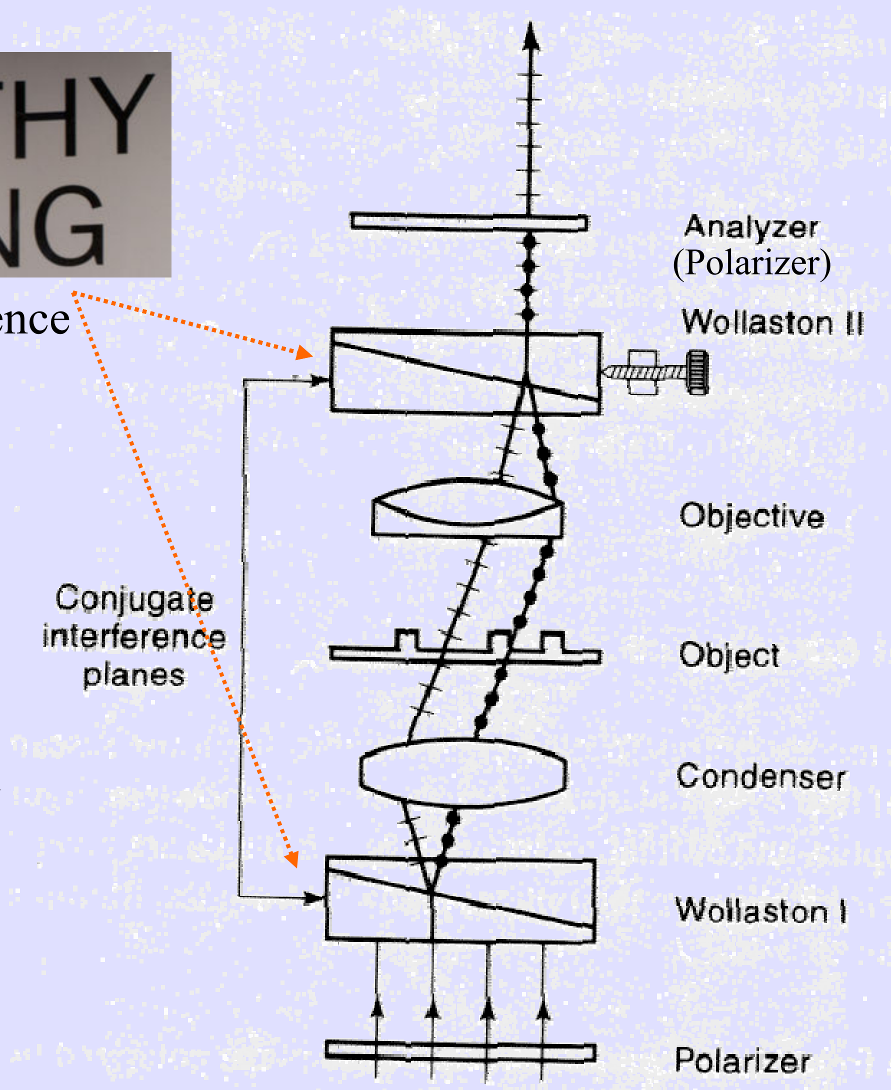
From Molecular Expressions

Easy to set up.
Works through plastic
Loose resolution

Normarski (Differential Interference Contrast, DIC)



Birefringence



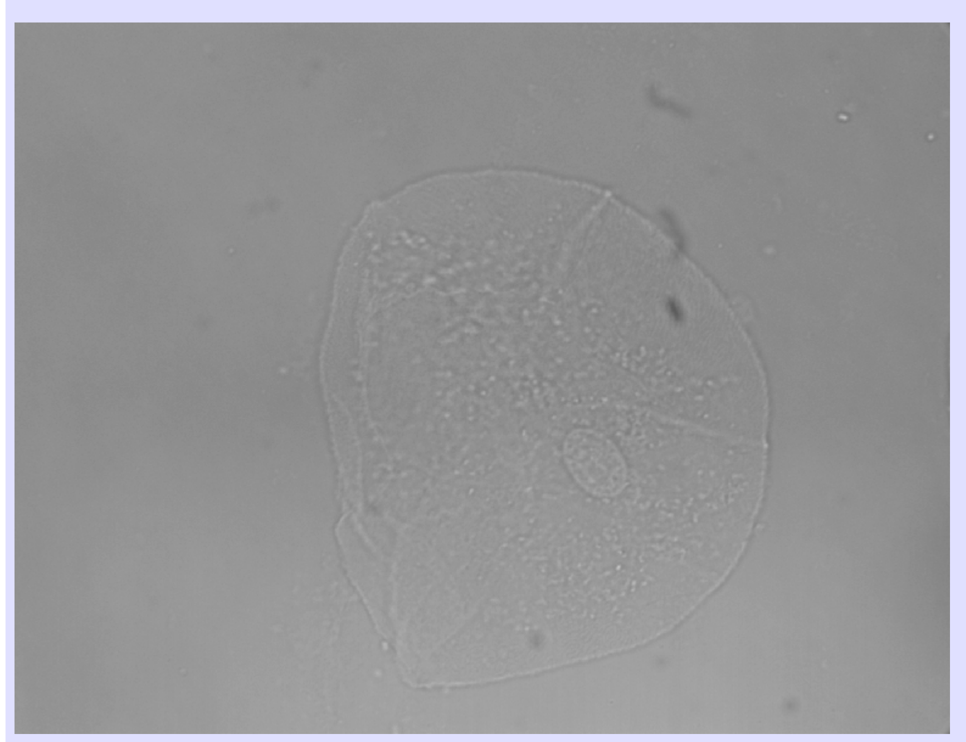
Setup, setup, setup!

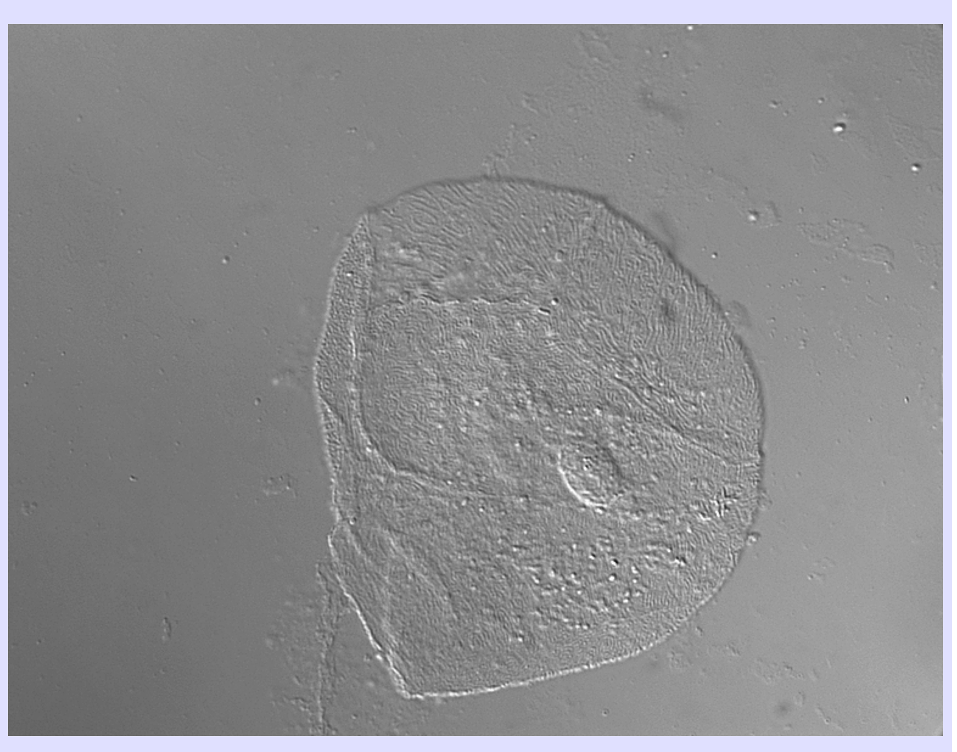
1. Kohler illumination
2. Cross polarizers
3. Push in Wollaston prisms
4. Adjust shear (Wollaston II)

No birefringent material in light path, e.g. plastic, collagen

But easy to do once practiced.

Nomarski enhances local gradients of refractive differences





Wide field

Nomarski (DIC)

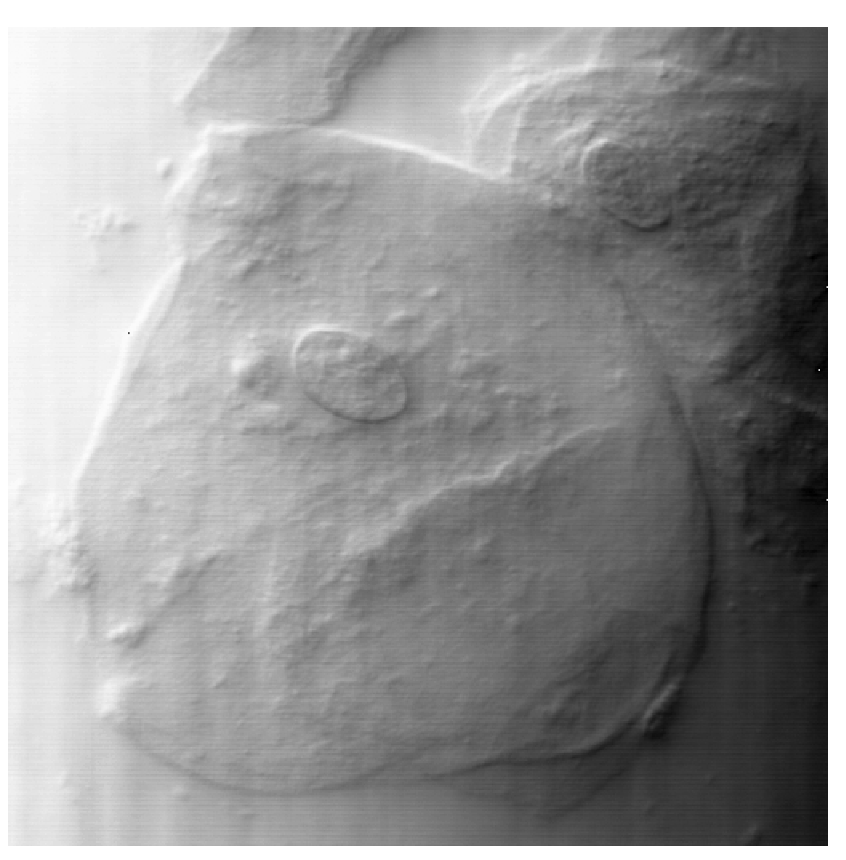
Standard Light Microscope

- Transmitted light microscopy
  - Sample needs to be mostly transparent
  - Dyes give contrast/color

- Exciting light adds background – reduces contrast

Fluorescence – Live Buccal Epithelial cells

More next Monday



Transmitted

Fluorescence
FM 1-43 membrane dye

Barely scratched the surface of light microscopy

- Some References
  - Fundamentals of Light Microscopy and Electronic Imaging, D. Murphy (2001)
  - Keller, E. H. 1995. Objective lenses for confocal microscopy. In Handbook of Biological Confocal Microscopy. pp. 111-126. [Ed. Pawley, J. H.] Plenum press, New York
  - Microscopy from the Very Beginning, 2nd ed., Carl Zeiss Microscopy
  - Salmon, E. D. and J. C. Canman. 1998. Proper Alignment and Adjustment of the Light Microscope. Current Protocols in Cell Biology 4.1.1-4.1.26, John Wiley and Sons, N.Y.

An Introductory Guide to Light Microscopy - Five Talk Plan

- Apr 16. A brief perspective of microscopy, theory of operation, key parts of a typical microscope for transmitted light, Kohler illumination, the condenser, objectives, Nomarski, phase contrast, resolution

- Apr 23. Fluorescence: Why use it, fluorescence principals, contrast, resolution, filters, dichroic filter cubes, immuno staining, fluorescent proteins, dyes.

- Apr 30. Detectors, sampling & digital images: Solid state digital cameras, Photomultipliers, noise, image acquisition, Nyquist criterion/resolution, pixel depth, digital image types/color/compression

- May 07. Confocal Microscopy: Theory, sensitivity, pinhole, filters, 3-D projection/volume renders

- May 14. Advanced Fluorescence/Confocal: Live cell imaging, co-localization, bleed through/cross talk, FRAP, fluorescence recovery after photobleaching, deconvolution

$E = h\nu$