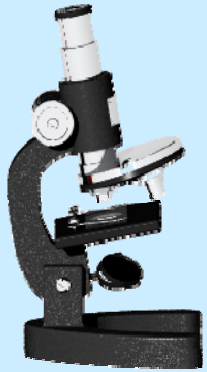
2006 Oct 30, Nov 6, 13, 20 & 27

An Introduction to the Theory and Practice of Light Microscopy

$E = h\nu$



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The Five Talk Plan

- Oct 30. A brief history of microscopy, theory of operation, key parts of a typical microscope for transmitted light, Kohler illumination, the condenser, objectives, Nomarski, phase contrast, resolution

- Nov 06. Fluorescence: Why use it, fluorescence principals, contrast, resolution, filters, dichroic filter cubes, immuno staining, fluorescent proteins, dyes.

- Nov 13. Detectors, sampling & digital images: Solid state digital cameras, Photomultipliers, noise, image acquisition, Nyquist criterion/resolution, pixel depth, digital image types/color/compression

- Nov 20. Confocal Microscopy: Theory, sensitivity, pinhole, filters, 3-D projection/volume renders

- Nov 27. Advanced Confocal: Live cell imaging, co-localization, blead through/cross talk, FRAP, fluorescence recovery after photobleaching, deconvolution

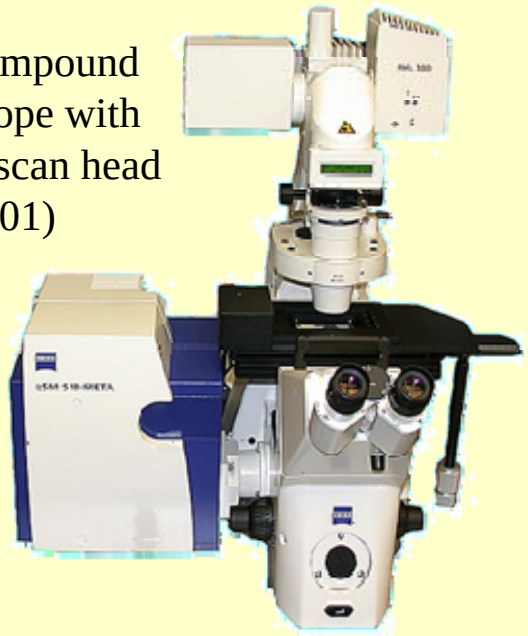
History and Evolution

- 4 Centuries of light microscopy!
- Improvements: optics – cameras – lasers – filters - dyes – computing – molecular biology - etc.
- Imaging transmitted, DIC, phase contrast, fluorescence. Live cell techniques – dyes targeting organelles, time lapse, spectral detection, FRAP, FRET
- Resolution improving techniques

The First Compound Microscope (circa 1595)

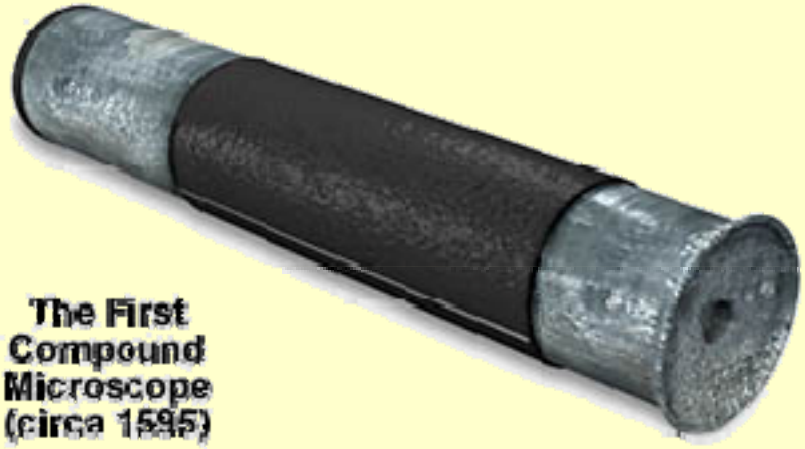
~400 years

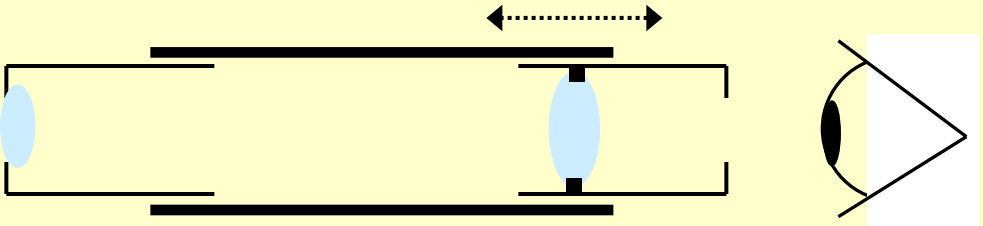
Zeiss compound microscope with confocal scan head (2001)



Janssen

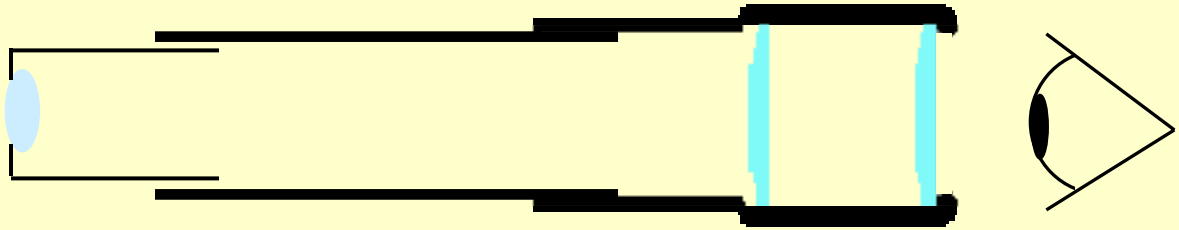
Zacharias Janssen was a Dutch spectacle-maker credited with inventing the first compound microscope in ~1590. Magnifications ~3x to ~9x.





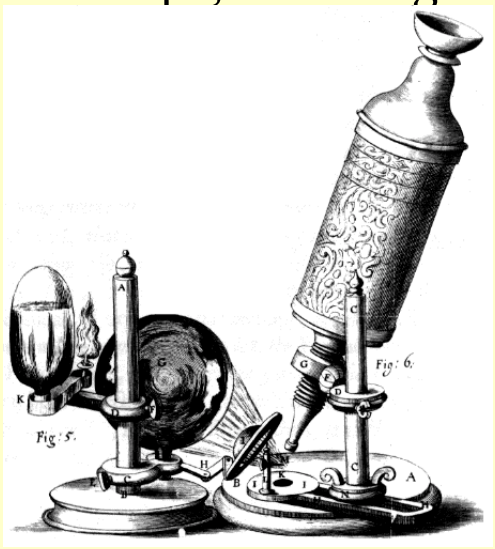
Huygens

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 micrometer. Resolution ~5

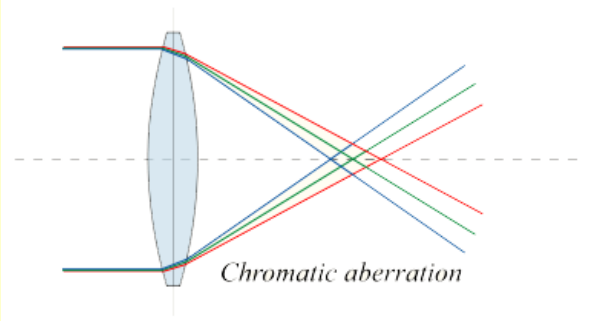


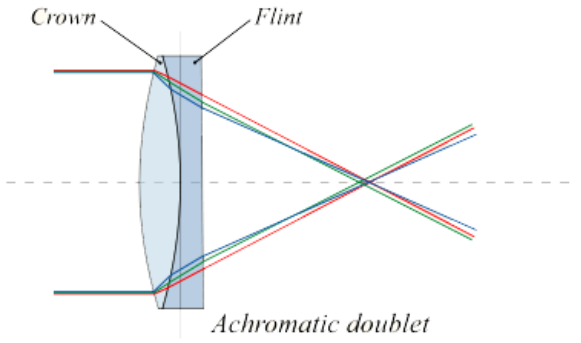
Leeuwenhoek

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!

E = hν

Chester Moore Hall & John Dollond

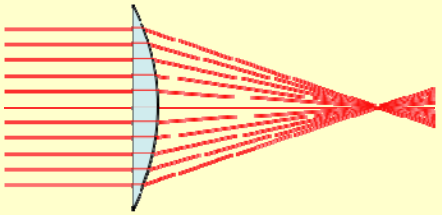




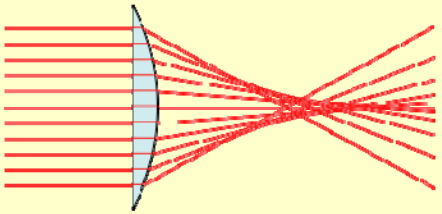
• Achromatic refracting lens was invented in 1733 by an English barrister named Chester Moore Hall
• Patented by John Dollond.

Lister

Tight focus



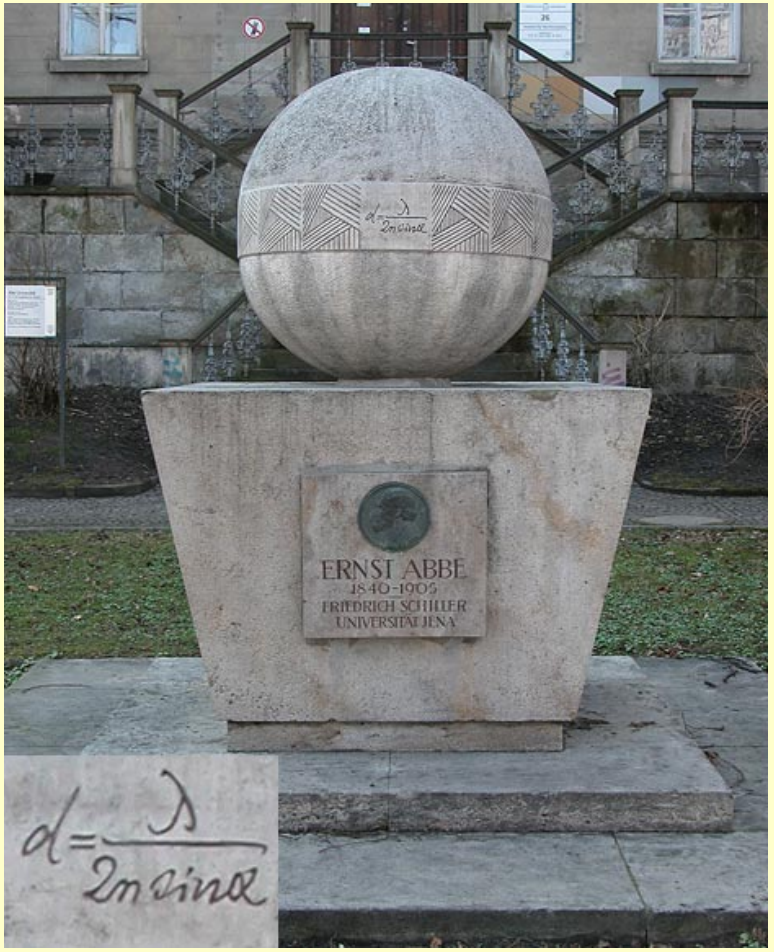
Spherical aberration



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

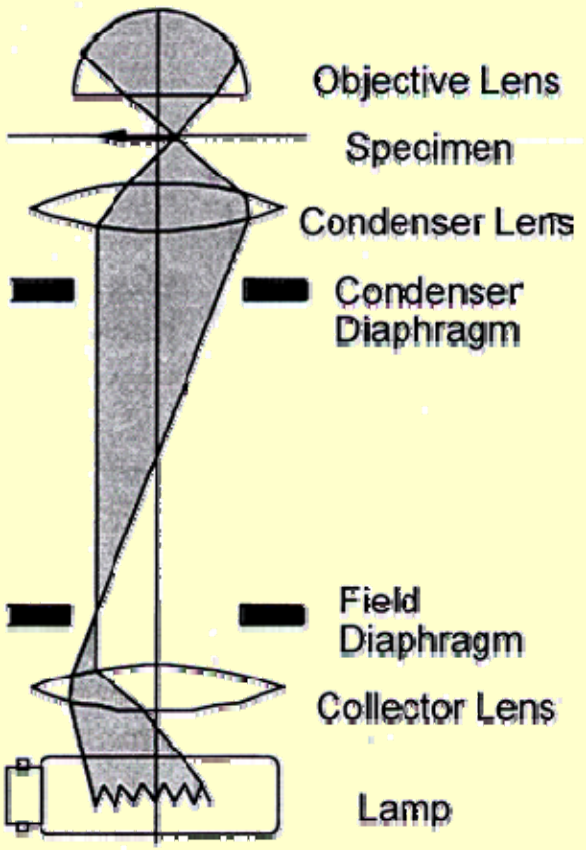
Ernst Abbe (1840-1905) applied mathematical principles to the design of lenses, which dramatically facilitated the manufacturing high quality optical instruments by the Carl Zeiss corporation. Abbe's realization that the performance of a microscope was limited by the diffraction of light was not well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



The Abbe limit formula $d = \frac{\lambda}{2n\sin\alpha}$

Köhler

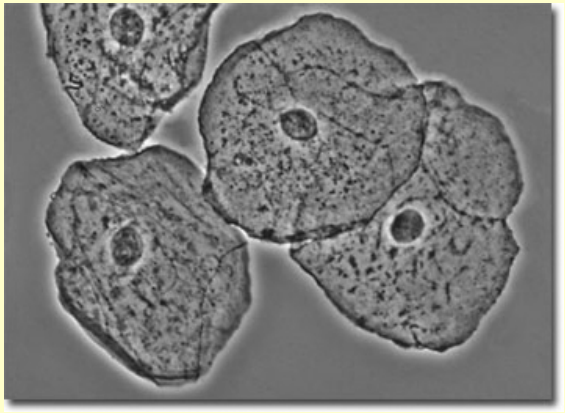
In 1893 August Köhler (1866-1948) invented a method of providing optimum illumination of a microscope specimen while working at the Zeiss Corporation. Improved resolution and evenness of light illumination made photomicrography possible.



E = hν

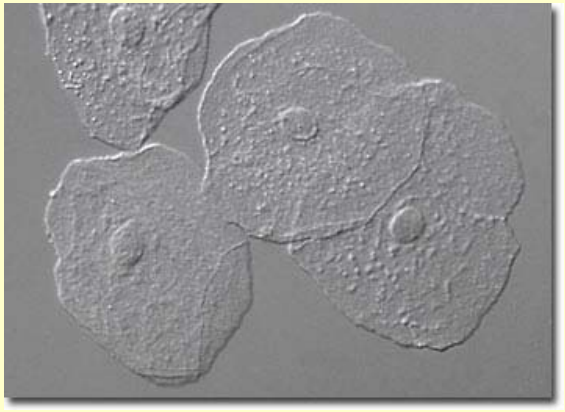
Zernike

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



Nomarski

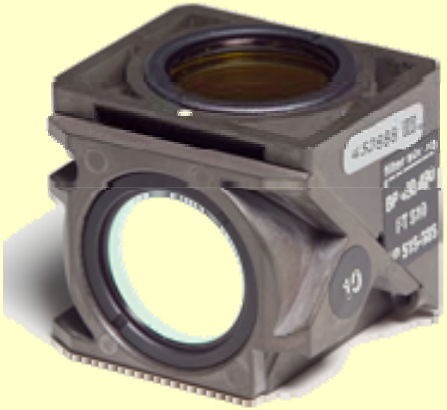
Georges Nomarski (1919-1997) developed the differential interference contrast (DIC) microscopy technique, which bares his name.



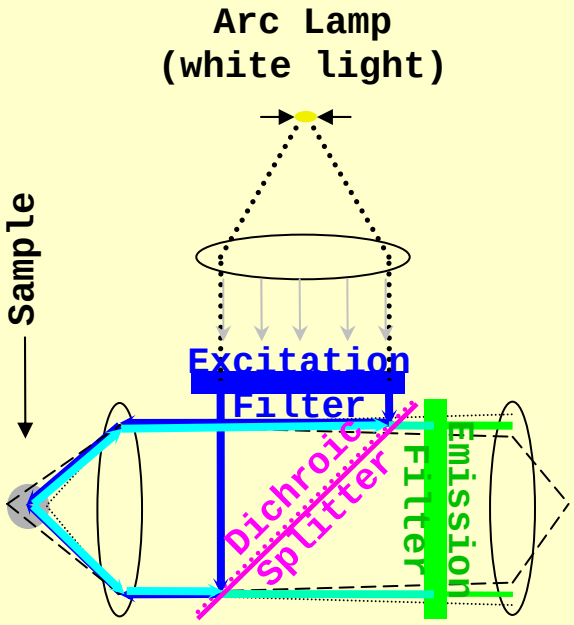
Ploem

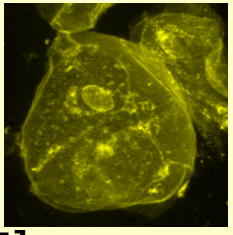
Johas Ploem invented the epi-illumination cube used in fluorescence microscopy.

Dichroic filter cube



Johan Sebastiaan Ploem
(1927-Present)

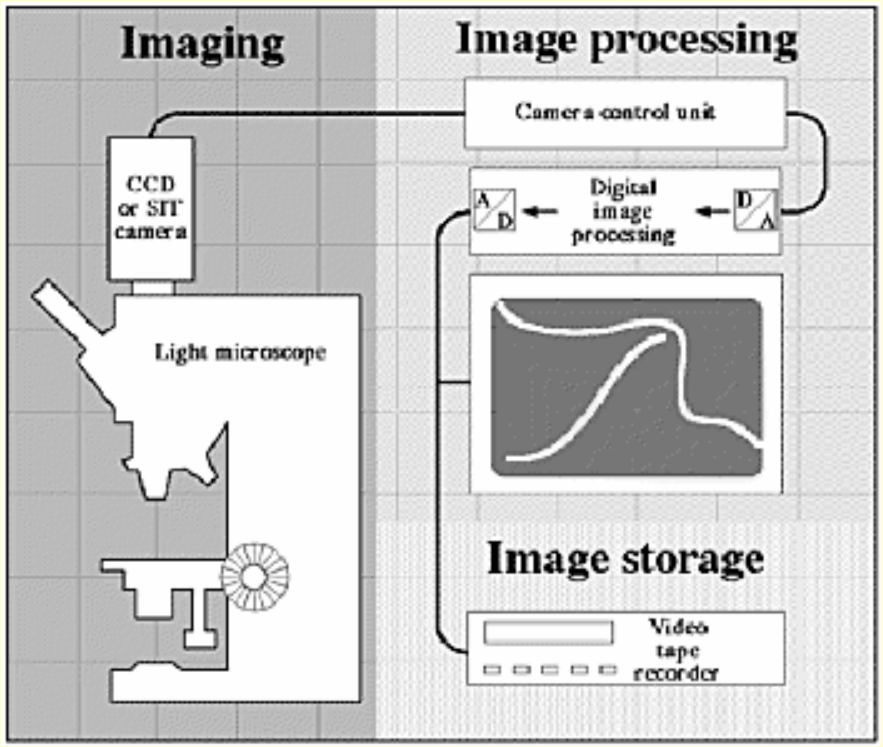




Fluorescence
image

Inoue & D. Allen & N. Allen

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



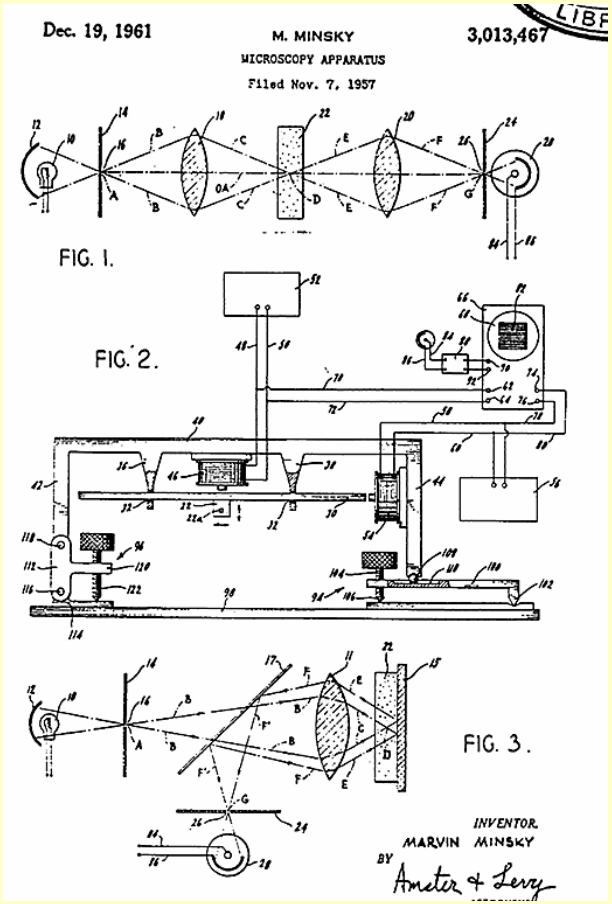
Shinya Inoue

Nina Allen

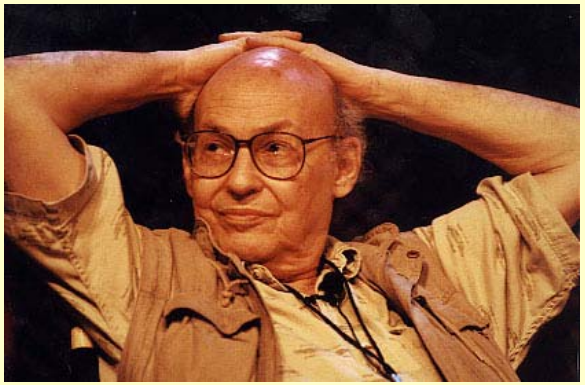
E = hν

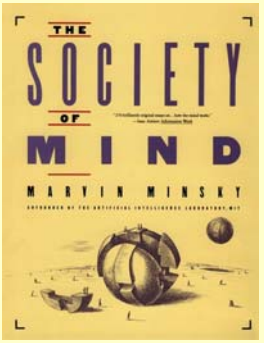
Marvin Minsky

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



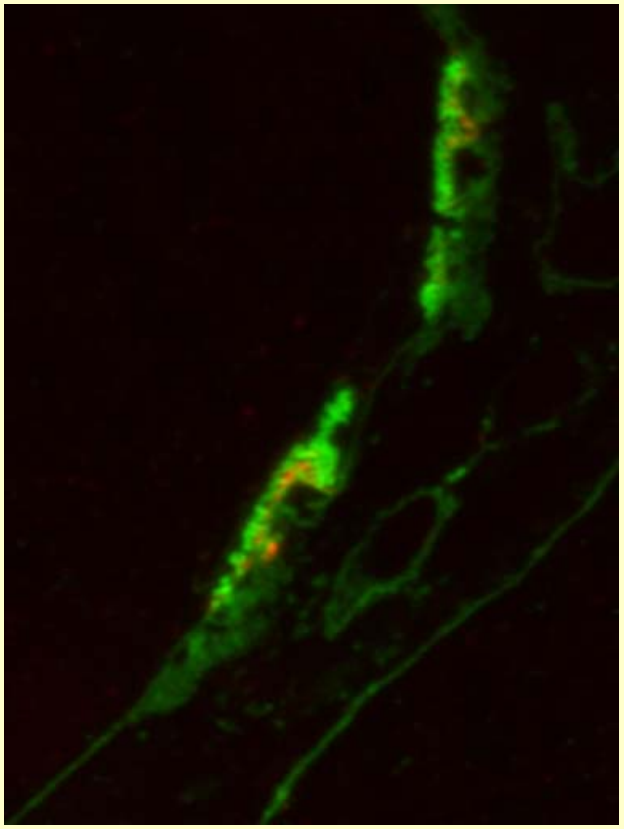
Practical Confocal microscope systems became available in the late 1980s. Yields improved contrast, resolution and optical sectioning.



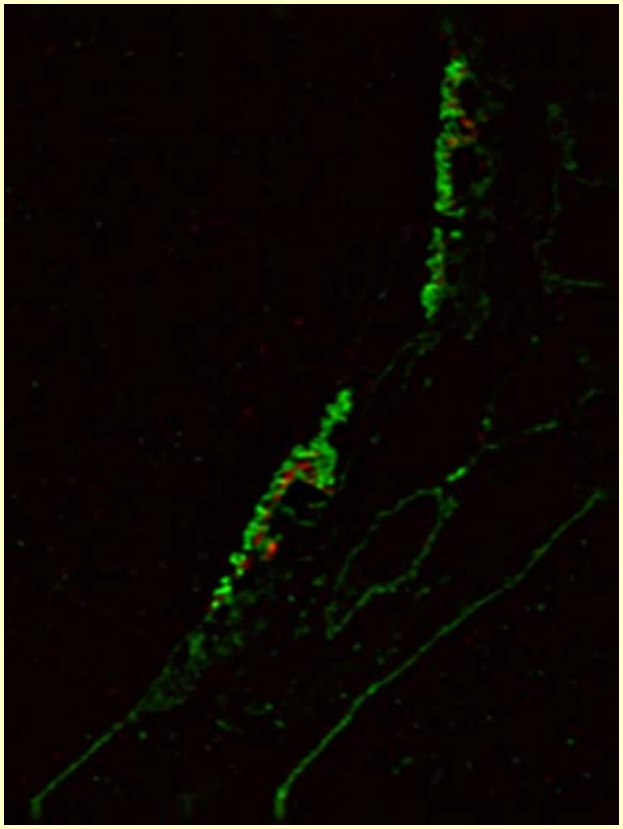


Sedat, Amos & Agrad

1980s Digital deconvolution microscopy removes haze.



Mathematical
Transformation
+ time



+ a powerful computer
with extensive storage

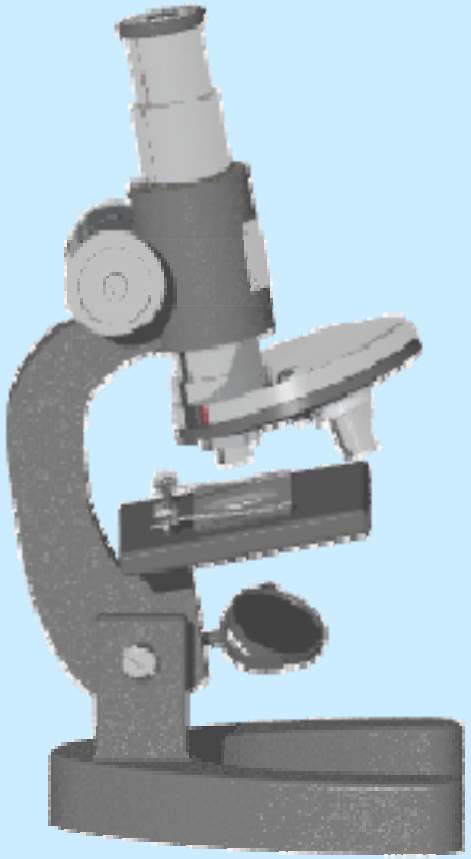
History Summary - Recent Evolution

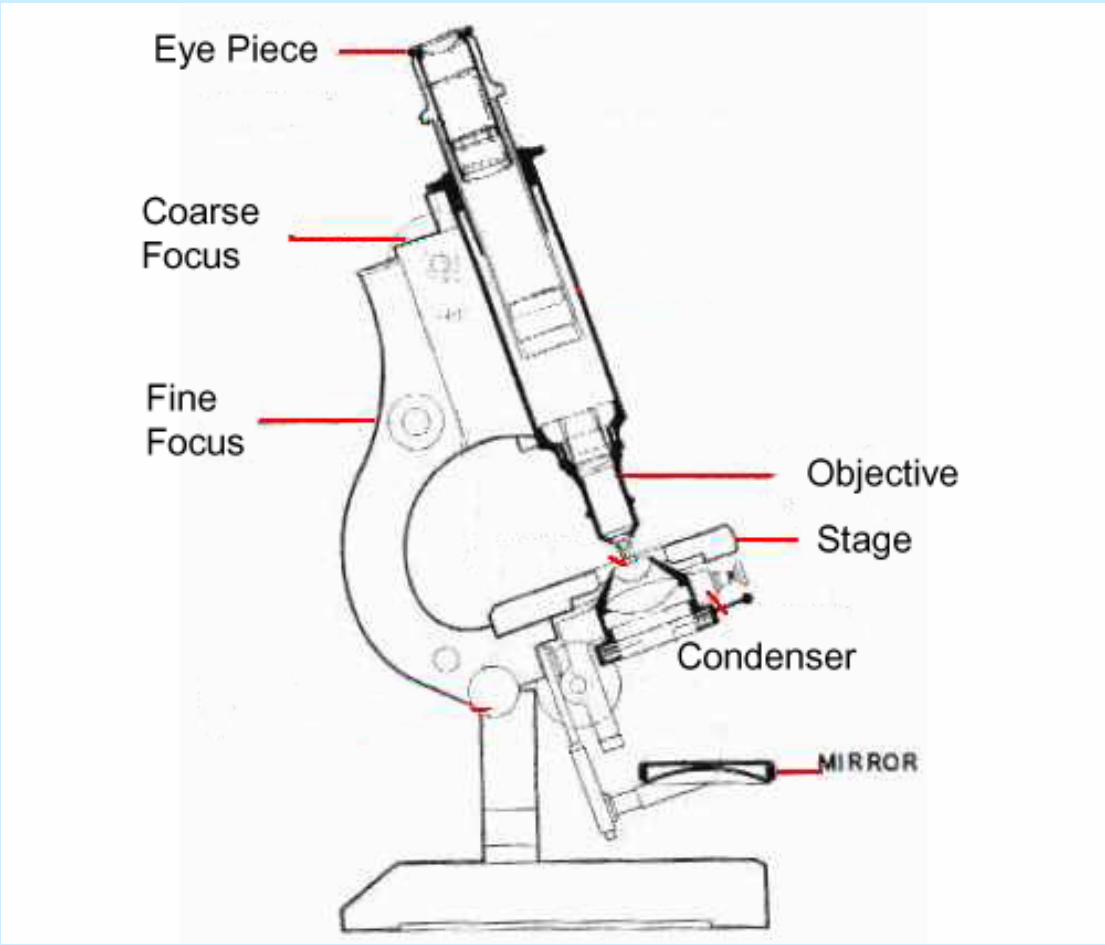
Dyes – fixed, vital, indicators
Immunostaining /Antibodies
Molecular Biology
Illumination
Lasers
Electronics
Cameras – CCDs, Intensifiers, high speed
Optics – ATOF, ATOM, fibers
Computers
Algorithms & software
Techniques – Time lapse, FRAP, FRET, FLIM
Control systems – focus, x-y movement, shutters
Live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

Key Parts of a typical microscope

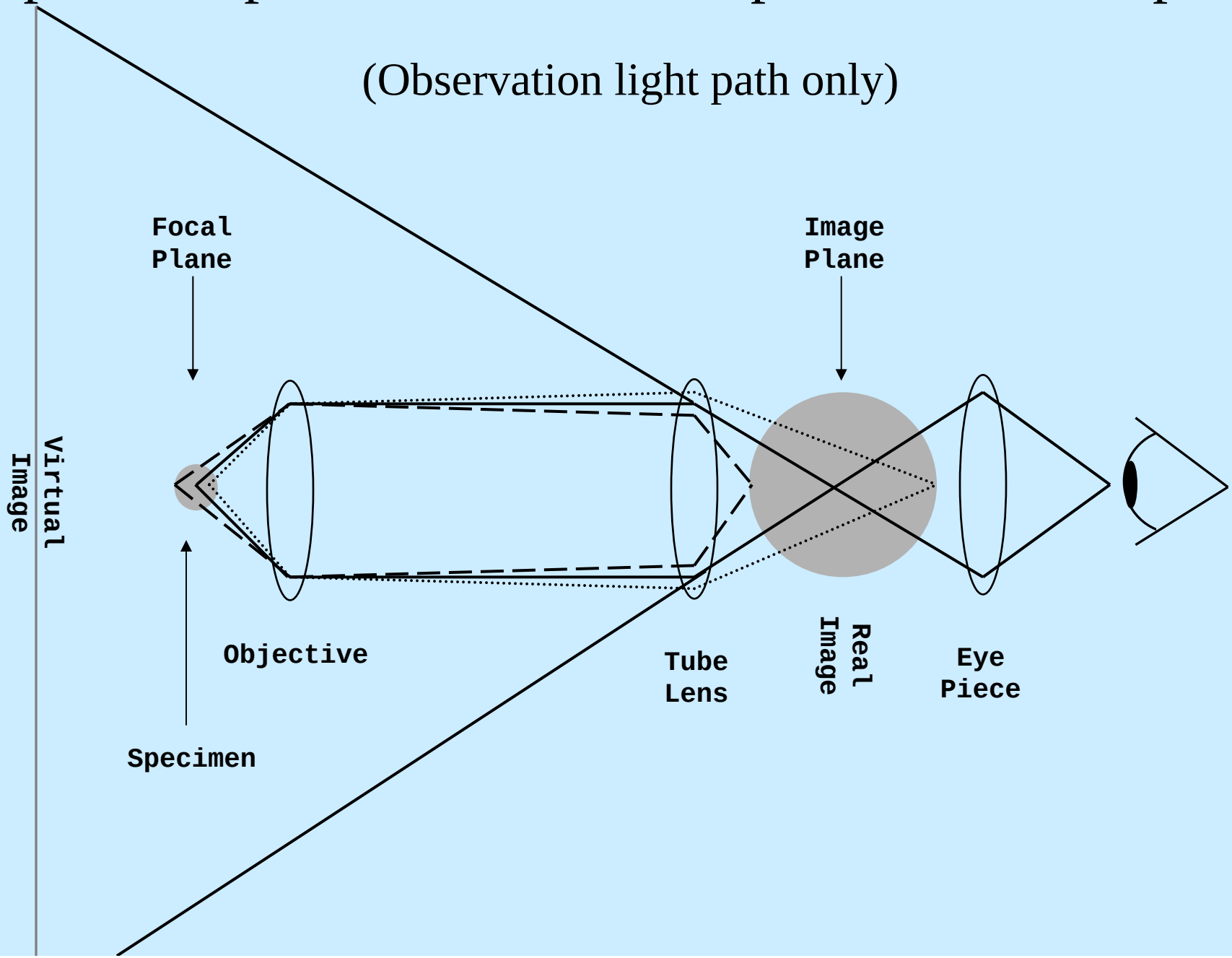




Note that the lamp is missing.

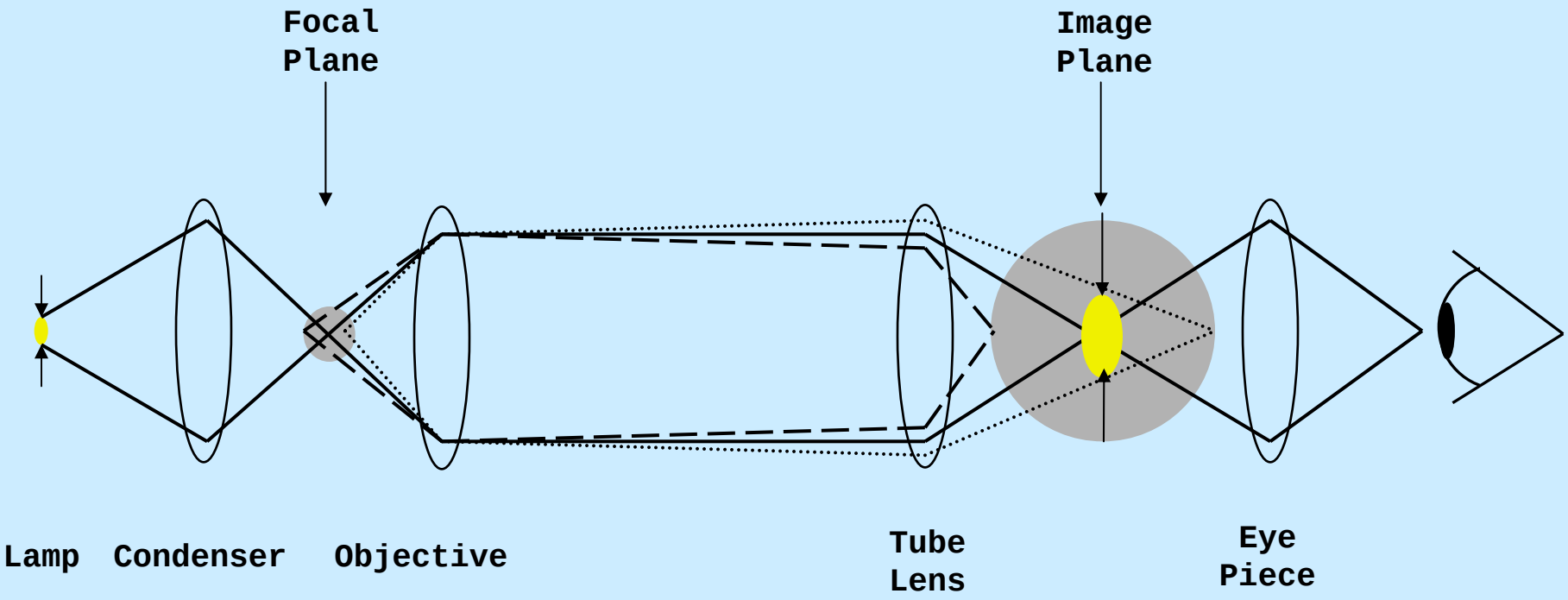
Simplified Optical Path of a Compound Microscope

(Observation light path only)



Simplified Optical Path

Illumination and observation light paths



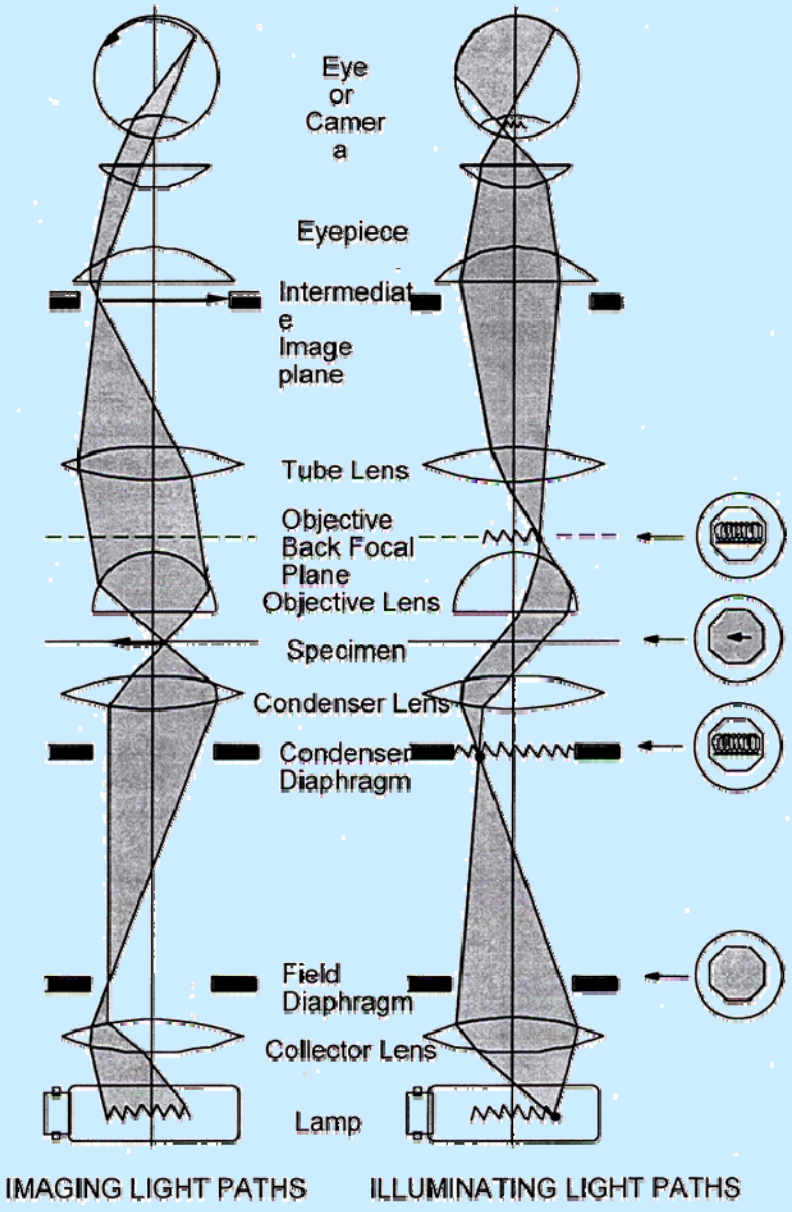
Problem: See image of lamp filament

Kohler Illumination

Condenser has double apertures

Condenser aperture changes the cone angle of light at the specimen

Field diaphragm changes diameter of light at the specimen

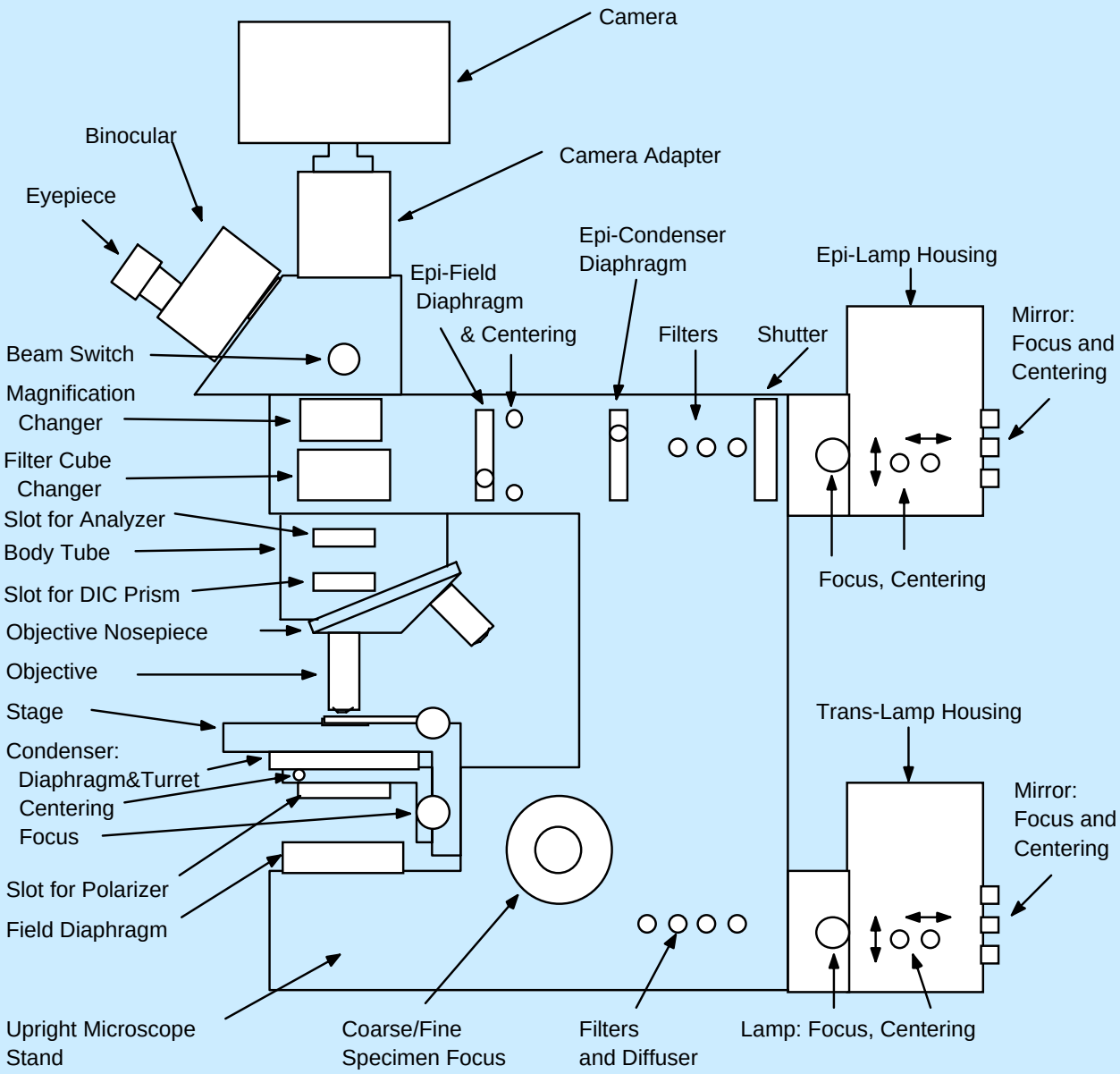


The Condenser

- Is your friend
  - When trying to find your 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)

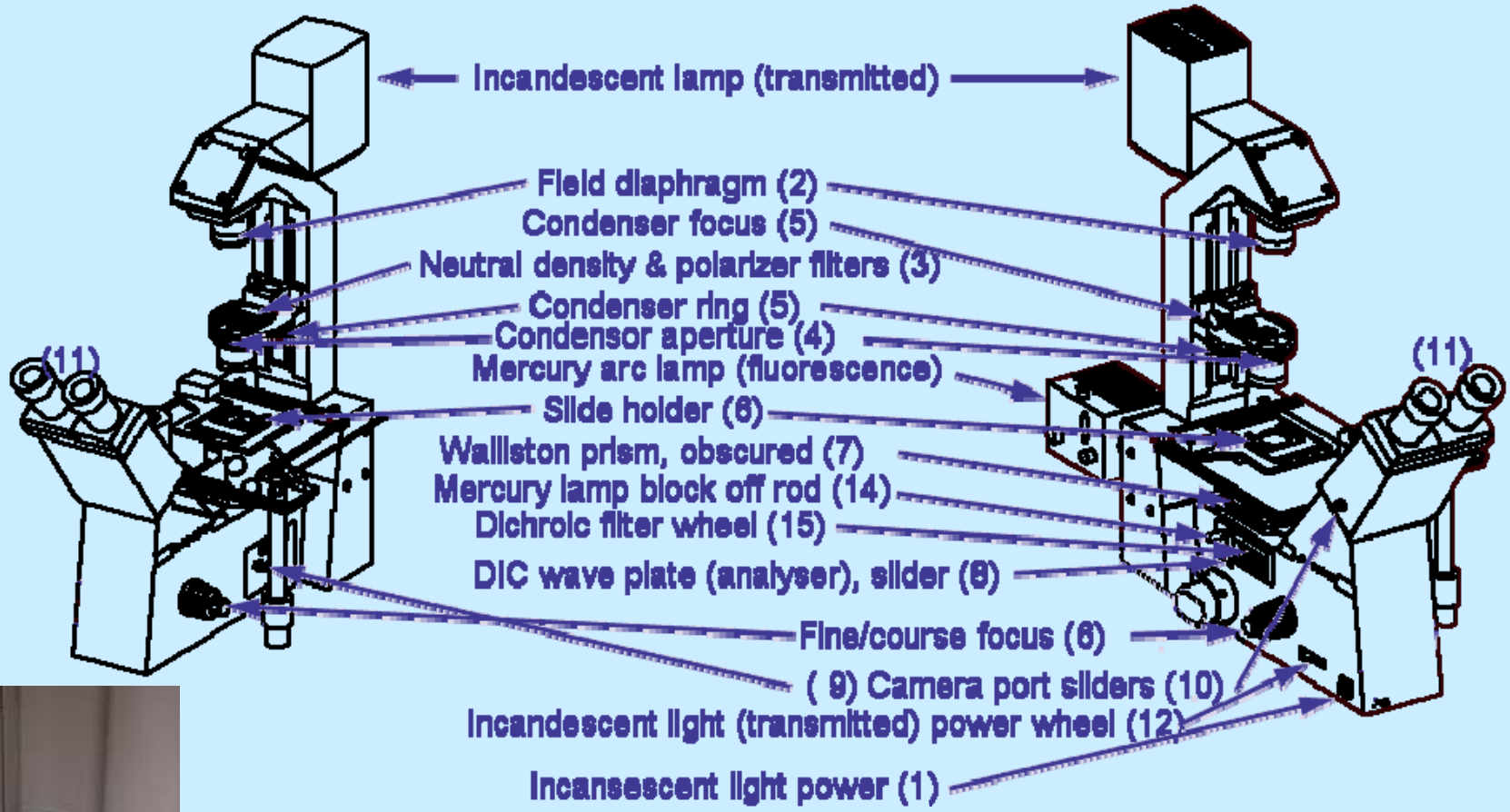
Parts of a typical microscope

MICROSCOPE COMPONENTS



From E. Salmon

Parts of a typical microscope



Objectives

- Magnification
- Numerical Aperture
- Immersion – oil – glycerol – water – air
- Tube length system (infinity)
- Cover slip (0.17 mm or 0 or variable)
- WD = working distance
  Corrections – cover slip – iris
- Field and abberation
- Plan Apo – Fluor – Acromat

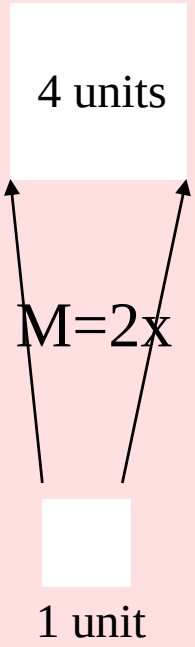
40x NeoPlan 0.4 NA cover
slip thickness adjustment correction

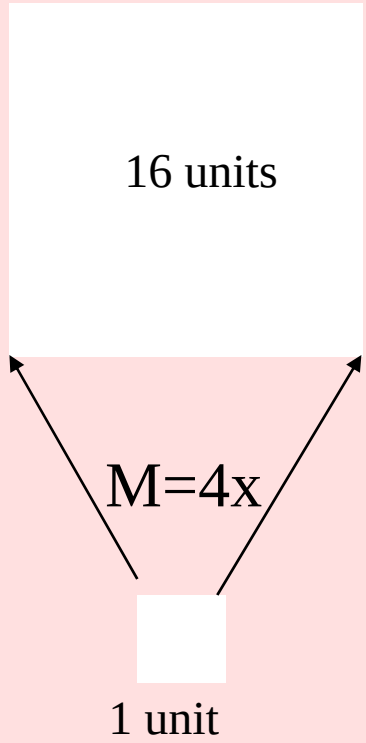
Figure 1

Magnification

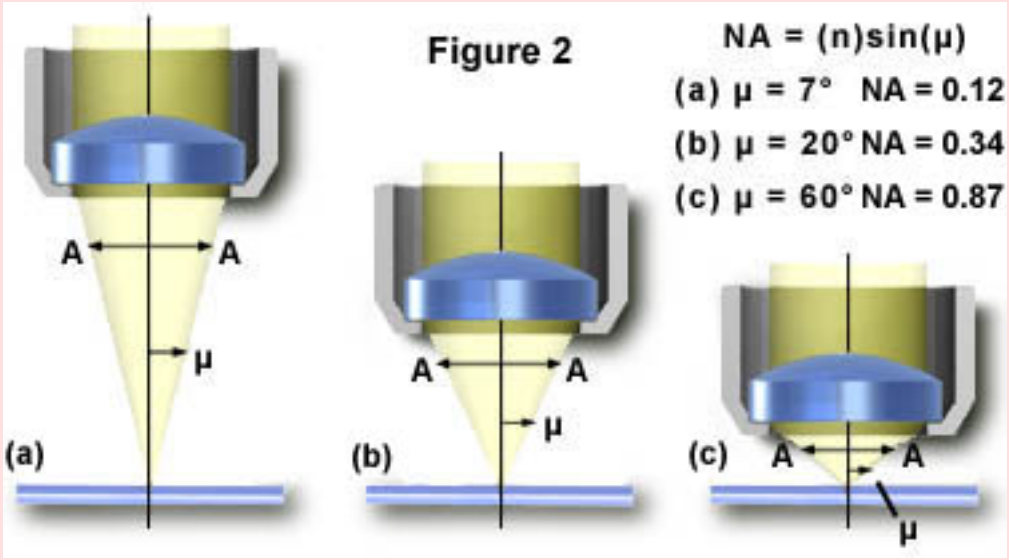
- Note more magnification (M) gives less light intensity at detector. (due to inverse square law & also often more absorbing lens elements)

- Brightness proportional to $1 / M^2$





Numerical Aperture (NA)



Numerical aperture is loosely related to resolving power.

High NA leads to smaller working distance.

u = half angle of cone of illumination

n = refractive index of medium

- Brightness proportional to NA^2

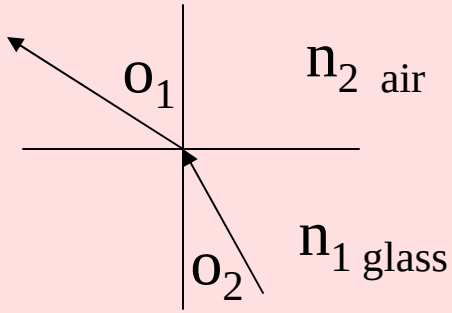
Immersion

Refractive Indexes:

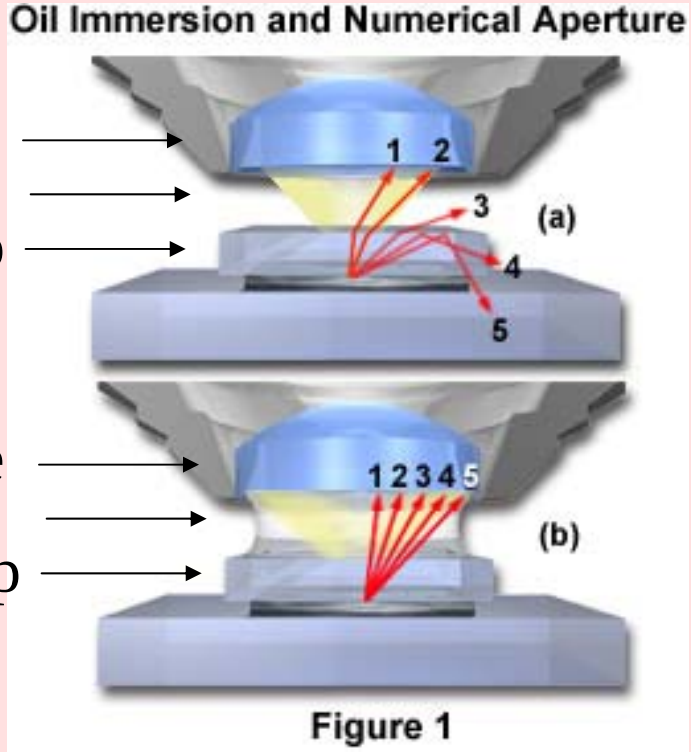
- Dry (air) 1.00
- Water 1.33
- Glycerol 1.47
- Immersion oil, glass 1.52

Snell's law of refraction

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



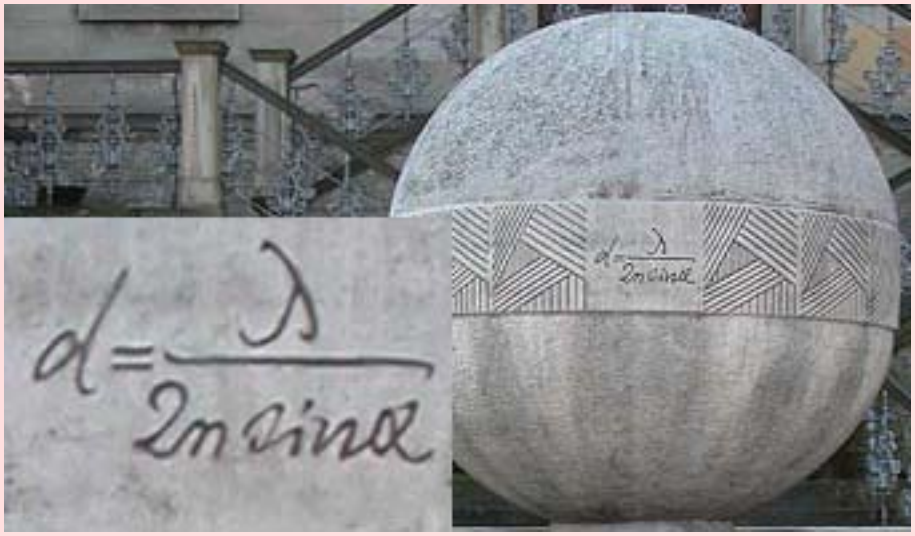
(n = refractive index)

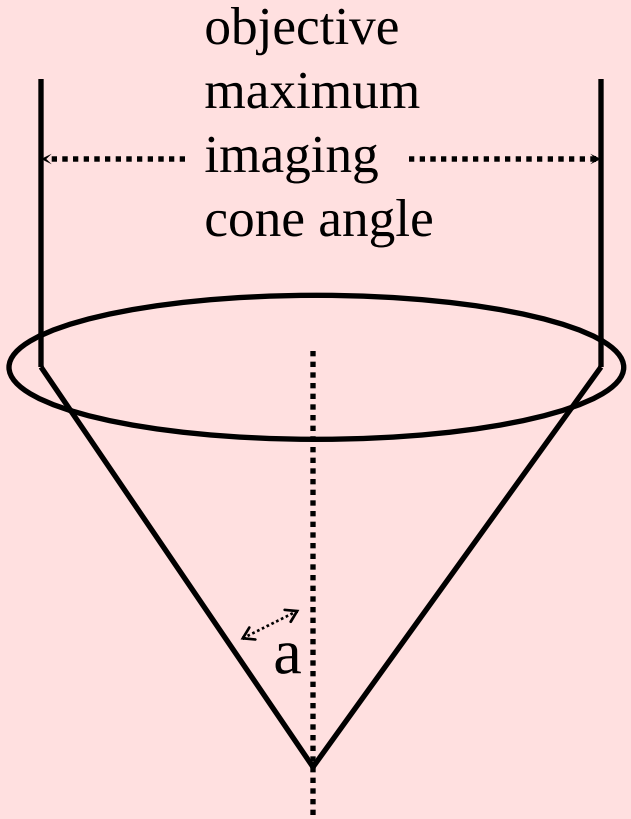


Numerical Aperture (NA) - resolution

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

Wide Field d = λ / 2 NA
= 0.22 μm





Tube length

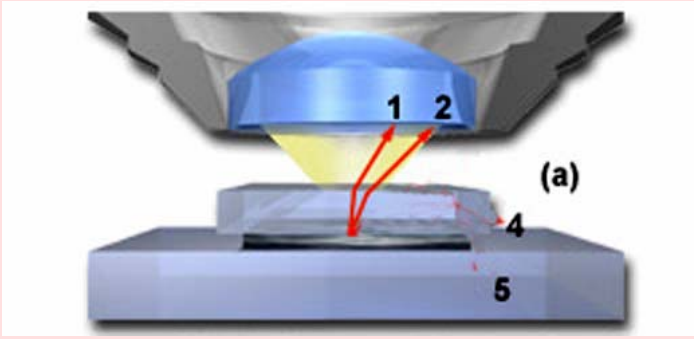
- Modern scopes are “infinity” corrected
- Older scopes may be 160 mm

Figure 1

Cover Slips

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

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



Typical Objectives

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 **

Care of Objectives

Gentle, gentle, gentle, gentle!

When rotating objective turret. Slowly!

When changing slides. Focus away. Remove slide slowly!

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

Cleaning:

Should be infrequent with dry lenses.

Lens tissue or surgical cotton only! Never Kim Wipes, Kleenex, Q-Tips, etc.

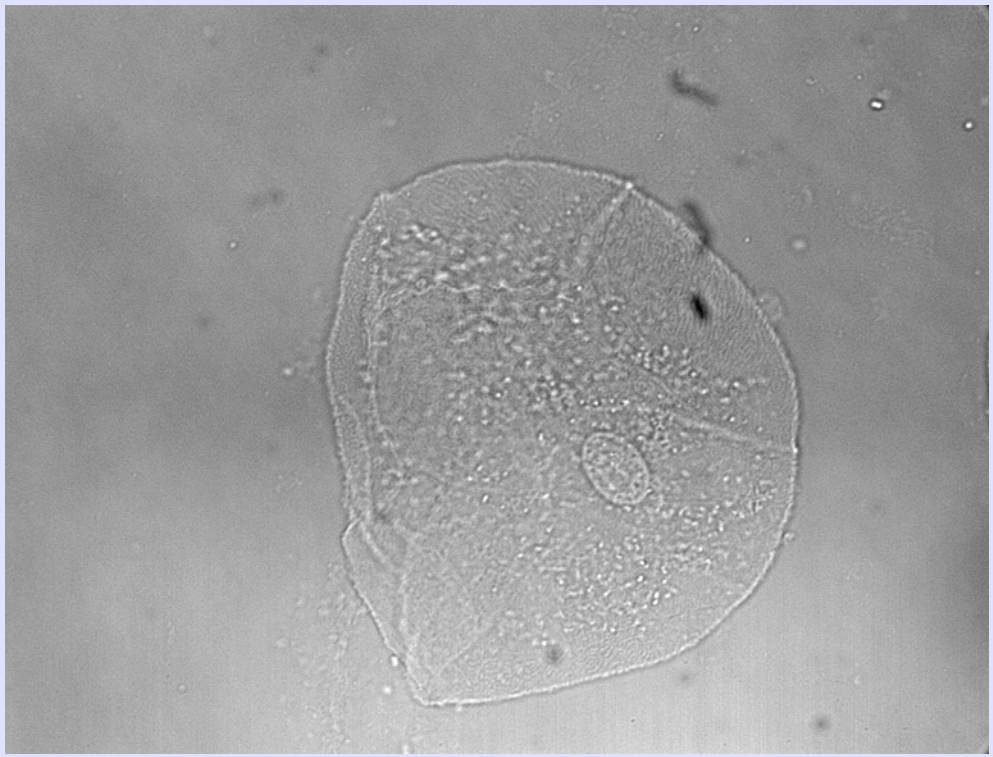
With immersion lenses, blot front lens gently. Do not wipe or rub.

Dry or immersion lenses of residue or oil use coated lens cleaner (pH ~6.0 to 7.0, non-ionic detergent, short chain alcohol)

Common Modes of Imaging

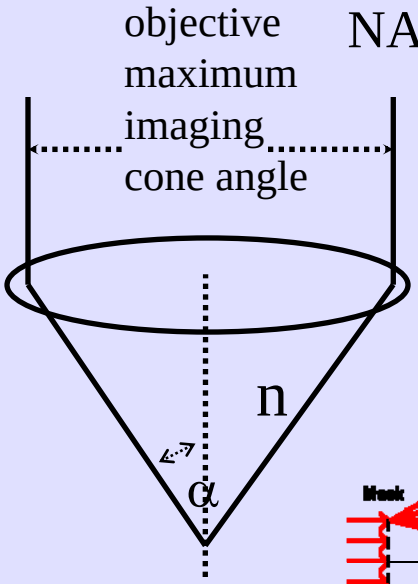
- Transmitted
  - Wide field (standard transmitted)
  - Phase contrast
  - Nomarski (DIC)
  - Polarization (material science)
- Epi-illumination (including confocal)
  - Fluorescence
  - Reflection (material science)

Transmitted light – (wide field)

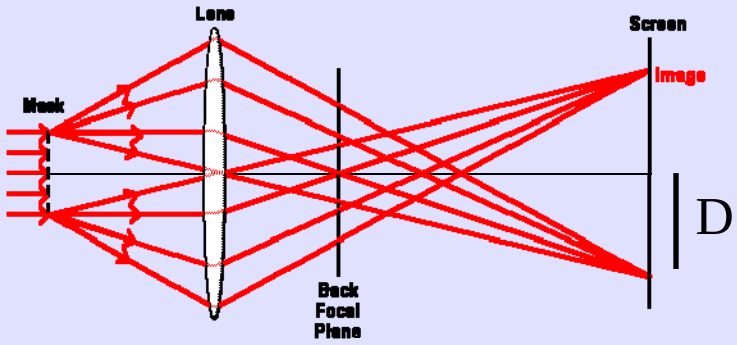


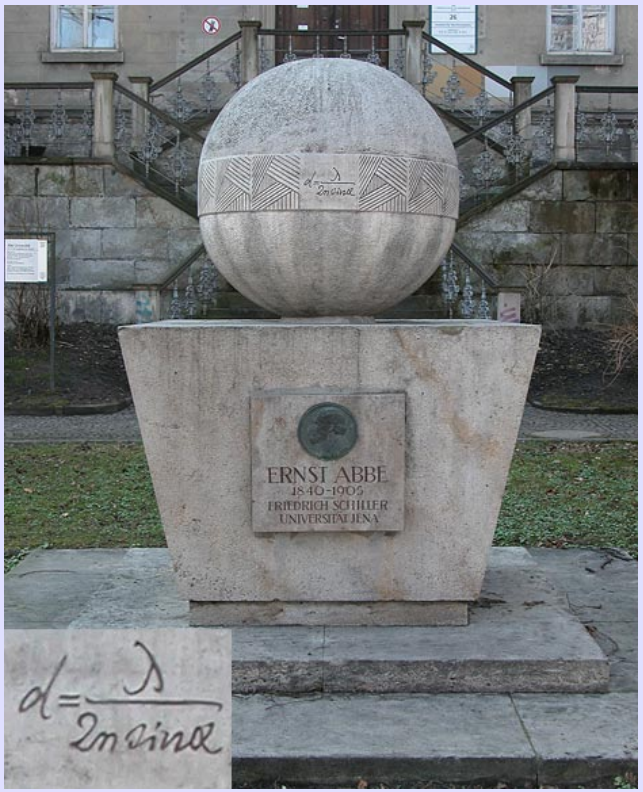
Live Buccal Epithelial cell (unstained)

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

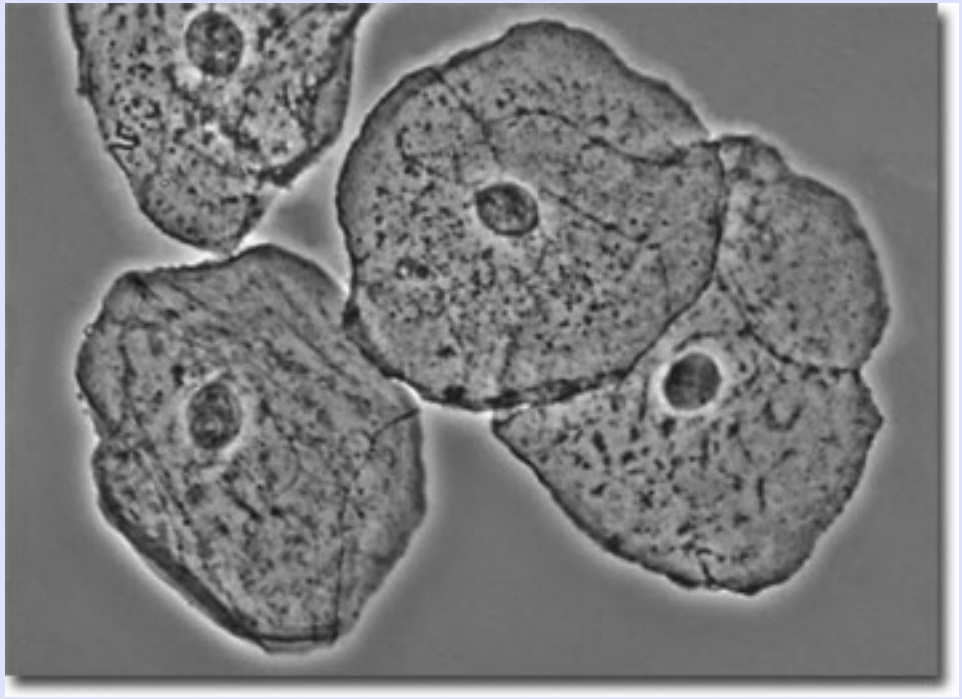
Interference of these rays in the image plane

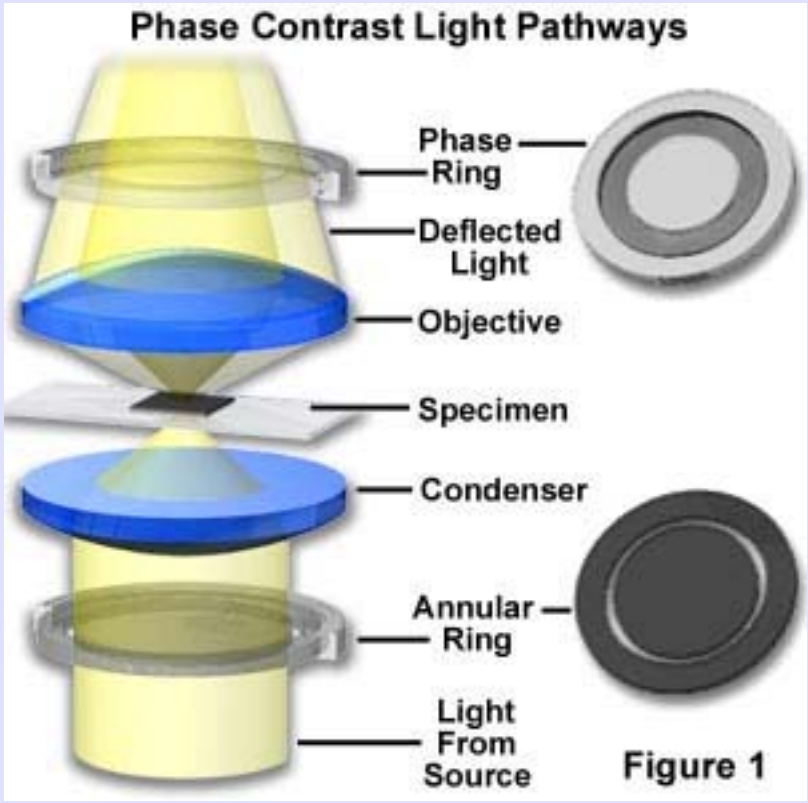
$D = \lambda / 2n \sin(\alpha)$

n = refractive index of medium

λ = wavelength of light

Phase Contrast

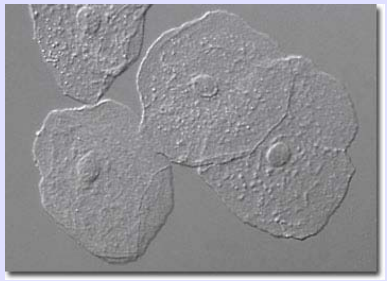




Phase contrast

Easy to set up.
Works through plastic
Loose resolution

Normarski (Differential Interference Contrast, DIC)



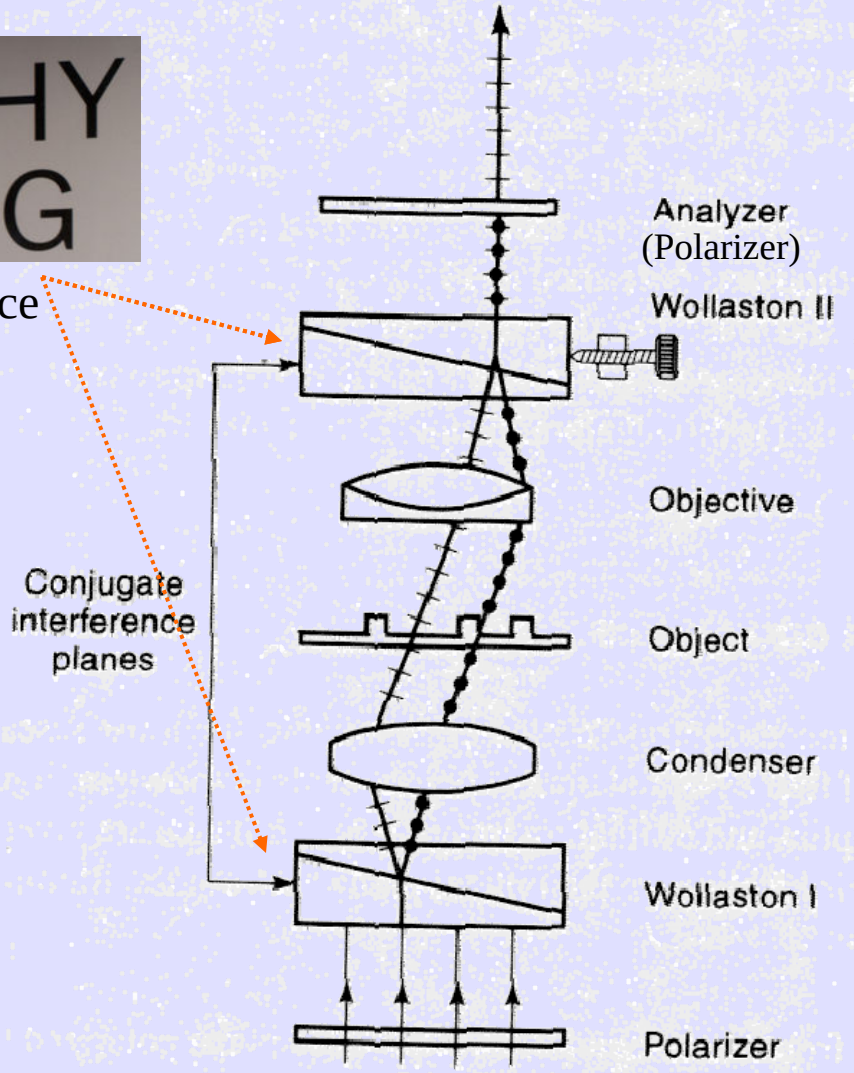
Birefringence

Setup, setup, setup!

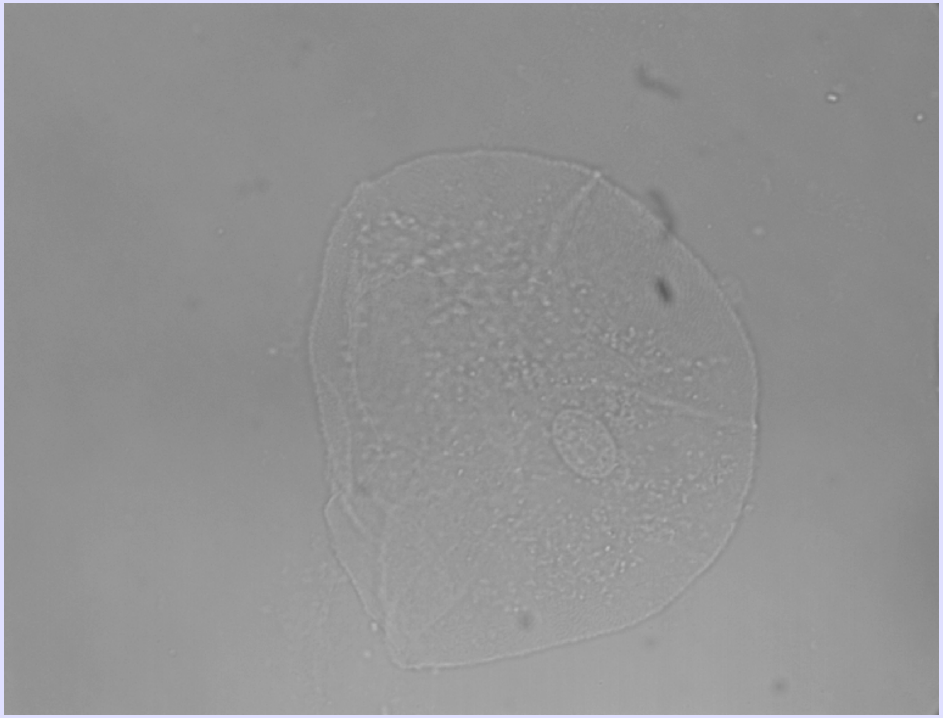
1. Kohler illumination
2. Cross polarizers
3. Push in Wollaston prisms
4. Adjust shear (Wollaston II)

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

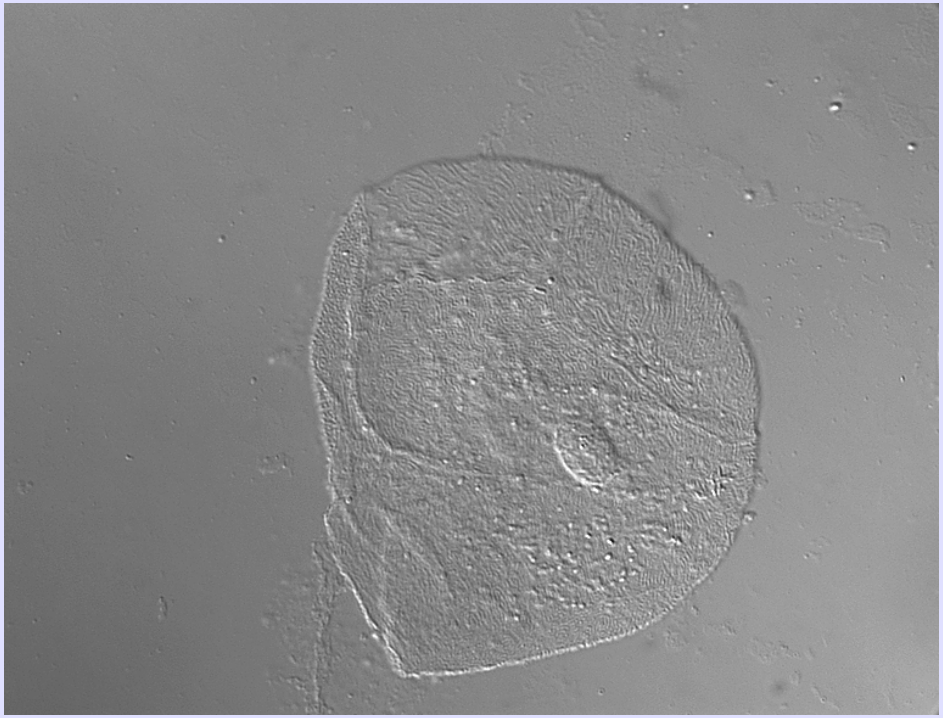
But easy to do once practiced.



Nomarski enhances local gradients of refractive differences



Wide field



Nomarski (DIC)

Standard White Light Microscope

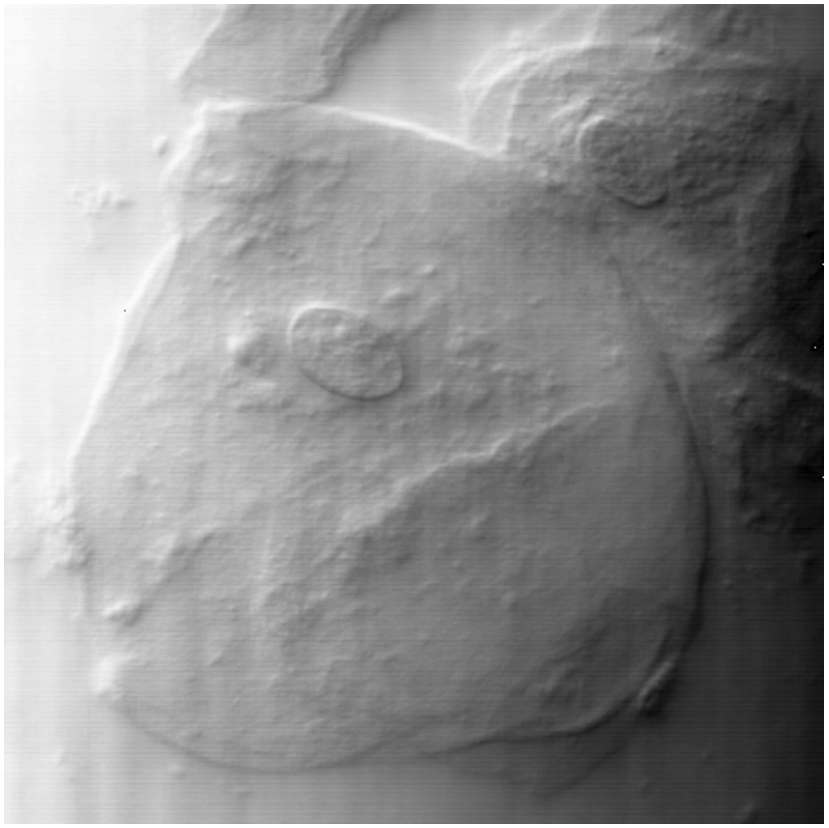
Transmitted light microscopy
- Sample needs to be mostly transparent
- Dyes give contrast/color

Reflected light microscopy
- Sample needs to be reflective
- See surface

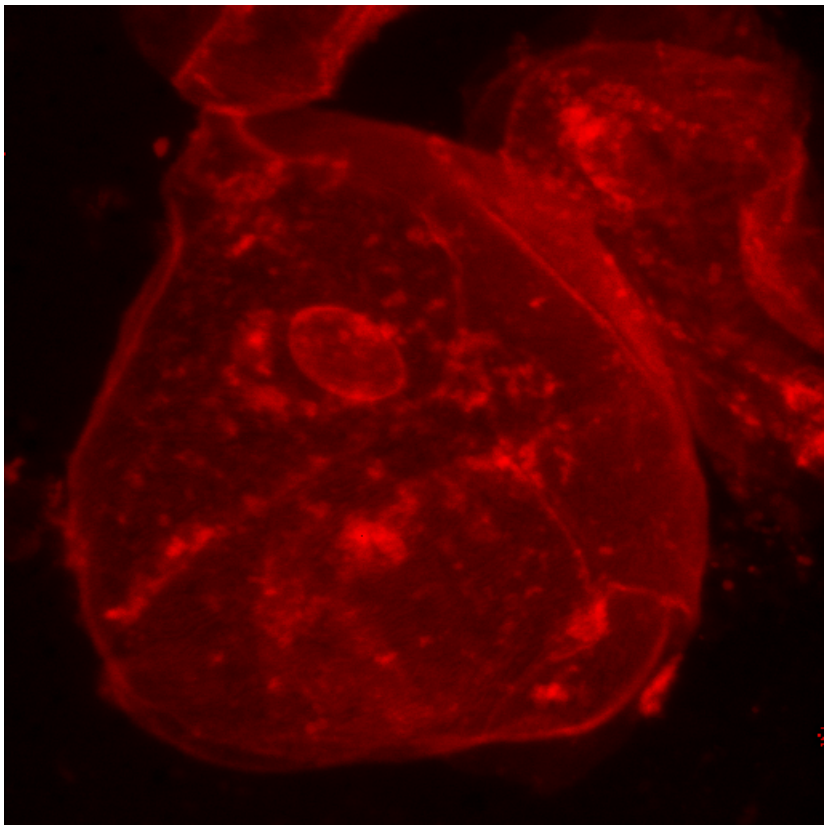
Exciting light adds background – reduces contrast

Fluorescence – Live Buccal Epithelial cells

More on November 6!

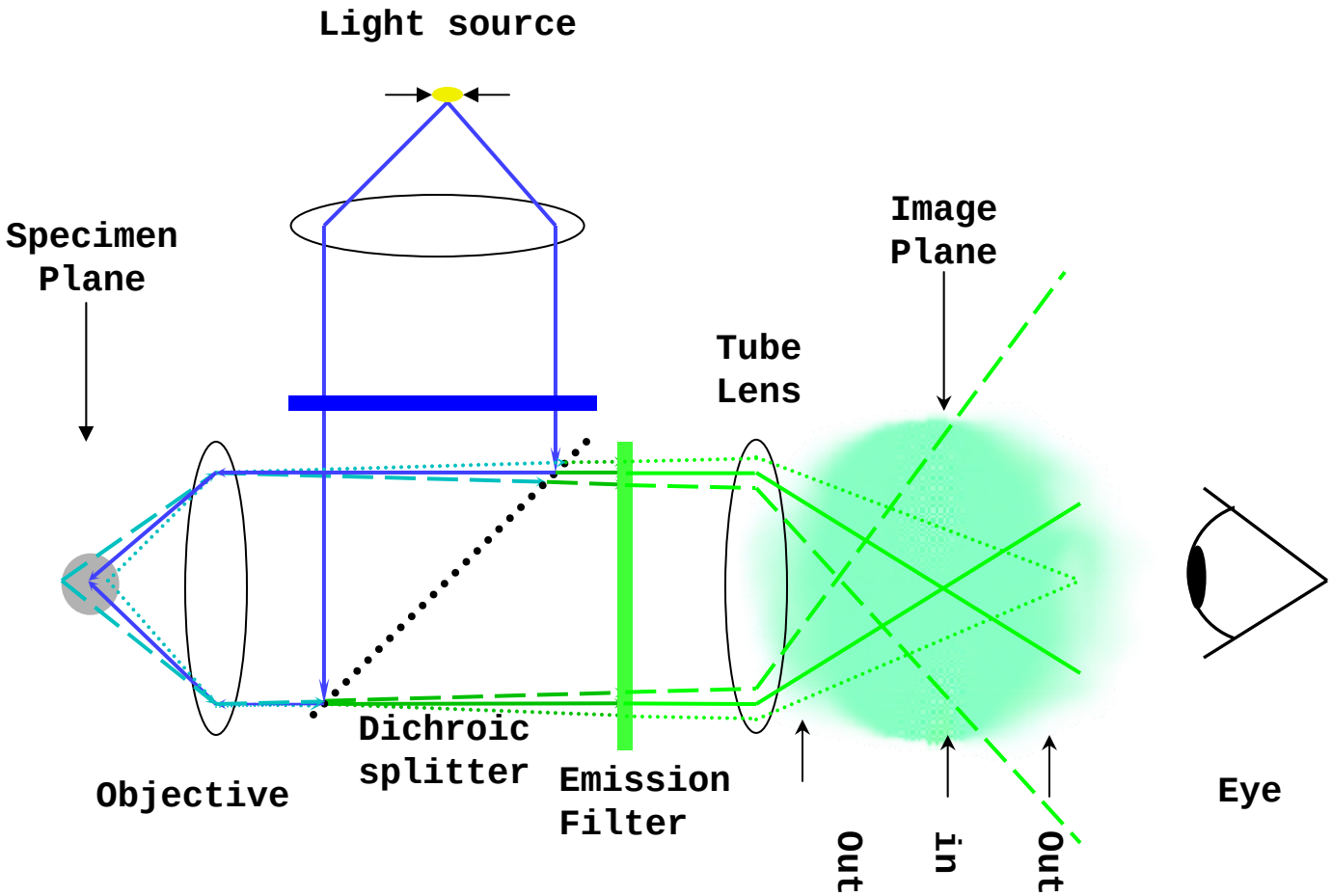


Transmitted



Fluorescence
FM 1-43 membrane dye

Limitation of wide field



A solution is confocal imaging: November 20!

Barely scratched the surface of light microscopy.

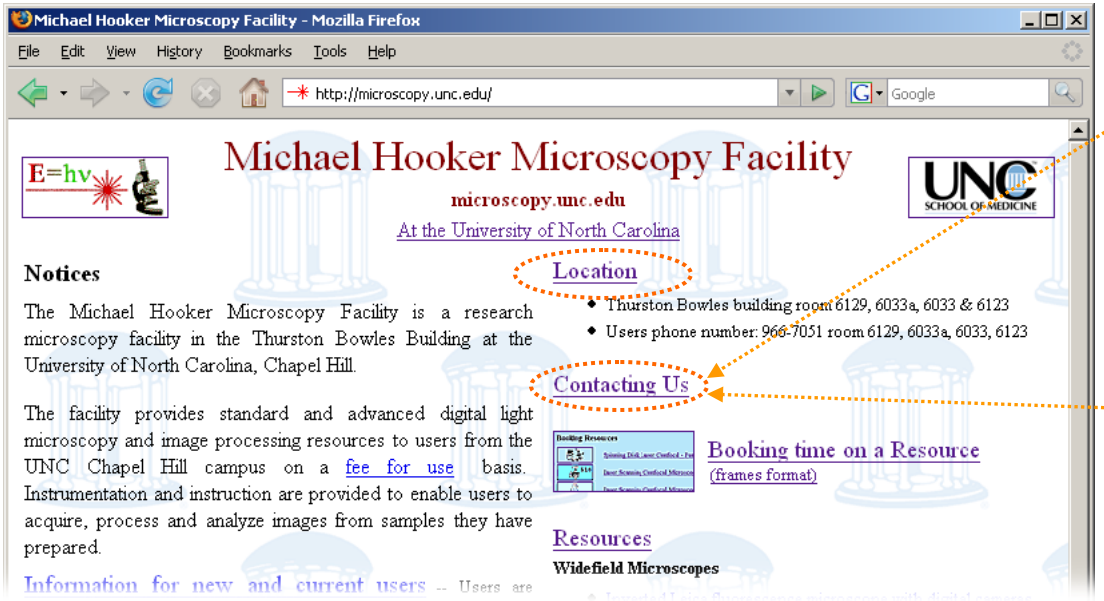
References

- Fundamentals of Light Microscopy and Electronic Imaging, D. Murphy
- Handbook of Biological Confocal Microscopy, 2nd (1995) or 3rd (2006) ed., James Pawley
- Microscopy from the Very Beginning, 2nd ed., Carl Zeiss Microscopy

Hydroethidrine Mosquito larva from a pond in Durham

Michael Hooker Microscopy Facility

http://microscopy.unc.edu



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Kohler Illumination