Microscopy: Fundamental Principles and Practical Approaches

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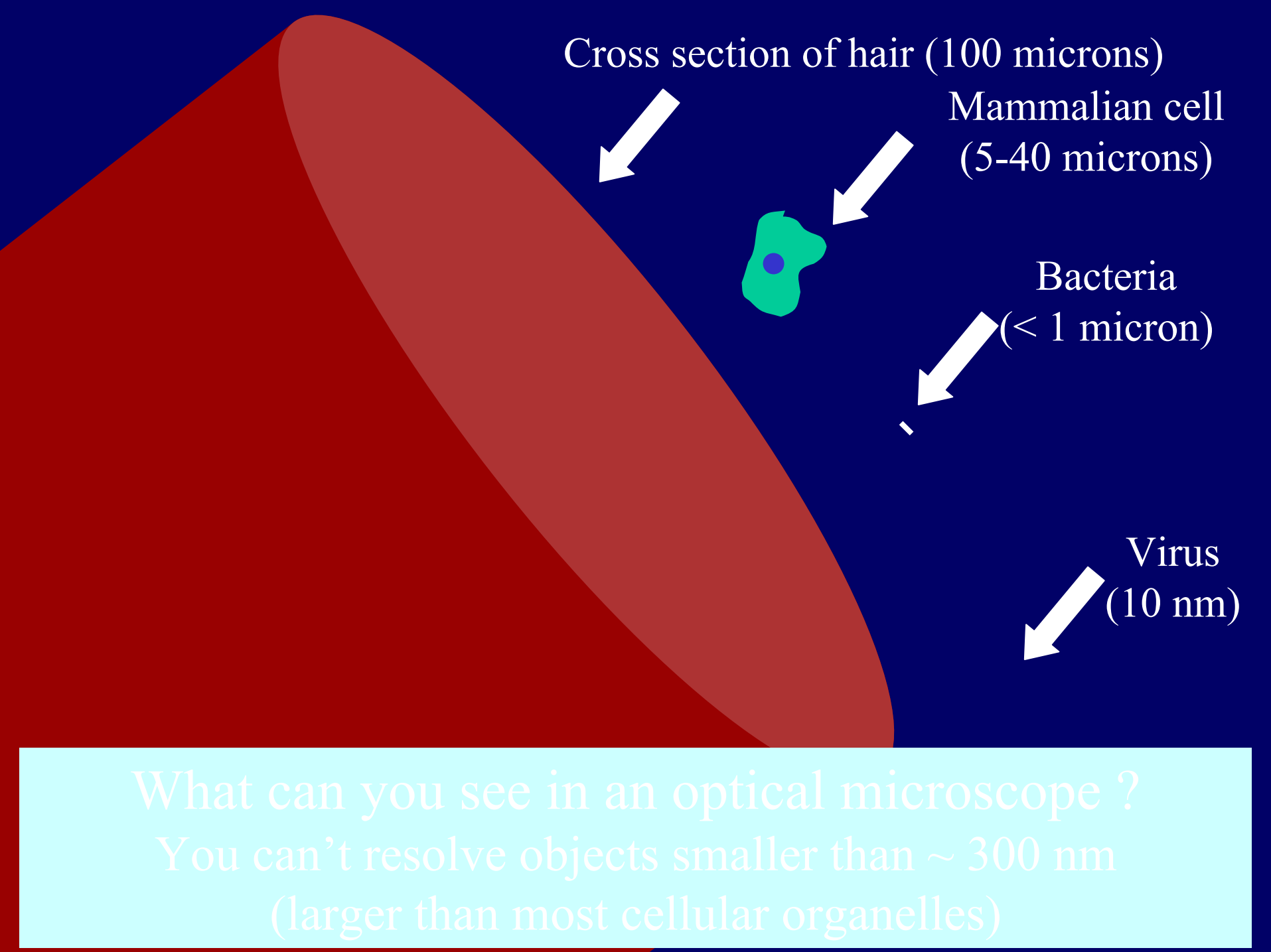
Online Resource:
http://micro.magnet.fsu.edu/primer/index.html

Book: Murphy, D.B. *Fundamentals of Light Microscopy and Electronic Imaging*. *Wiley-Liss 2001*

Overview

- Image Formation: Diffraction and Interference
- Limits to Resolution: Numerical Aperture and Immersion Objectives
- Light Path and Köhler Illumination
- Getting Contrast: Phase Contrast
- Getting Contrast: DIC
- Bothersome Aberrations



What can you see in an optical microscope ?

You can't resolve objects smaller than ~ 300 nm

(larger than most cellular organelles)

Lenses

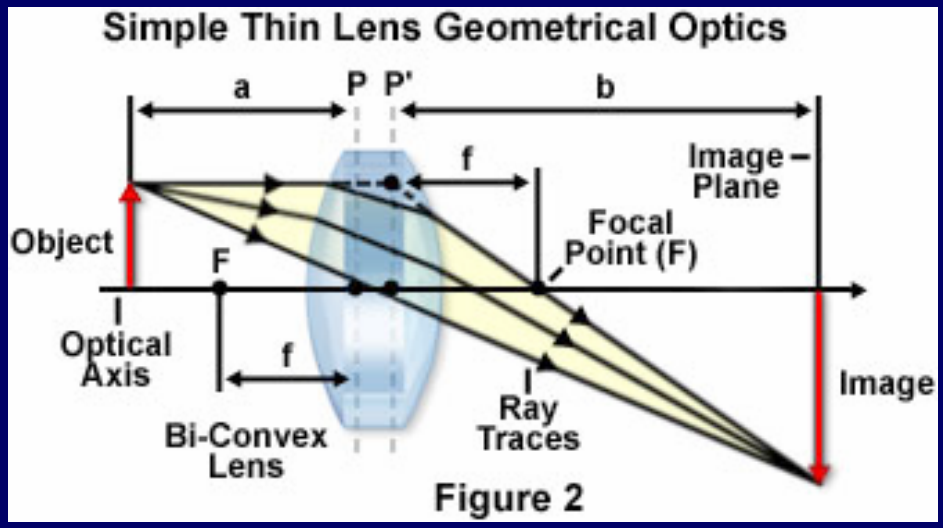


Figure 2

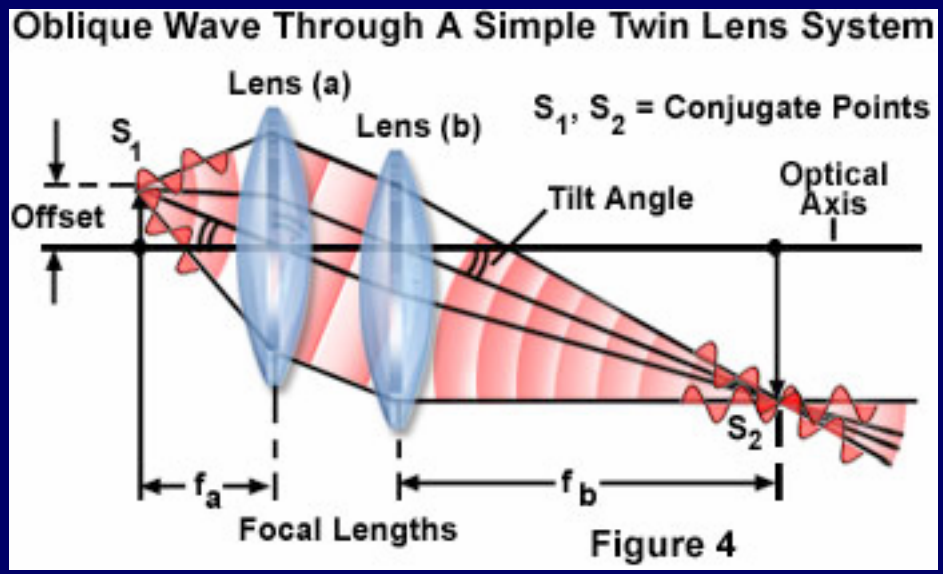
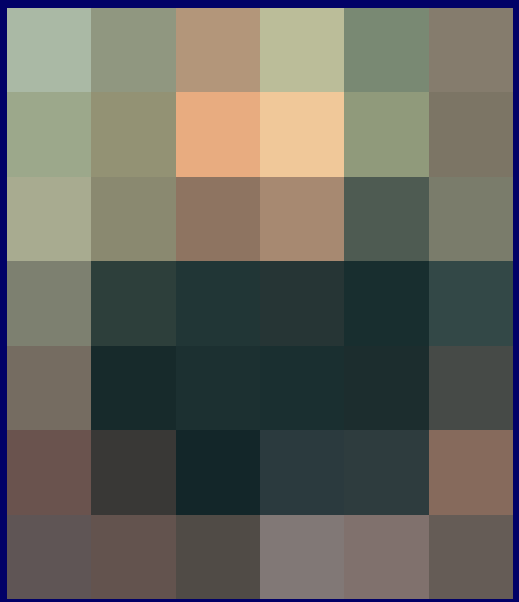


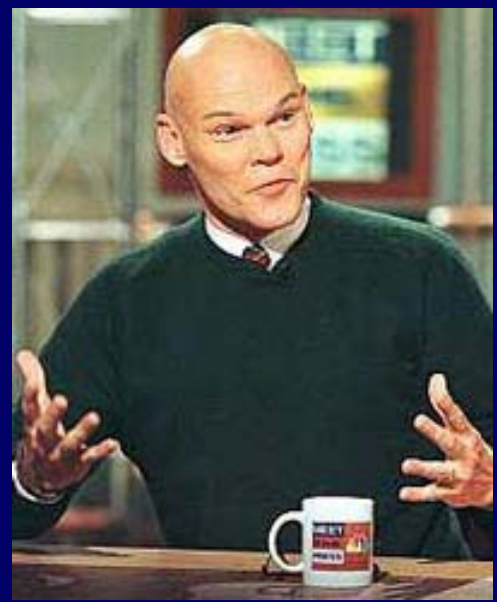
Figure 4

James Carville Says:

James Carville Says:

"It's the RESOLUTION, stupid!!!"





Diffraction

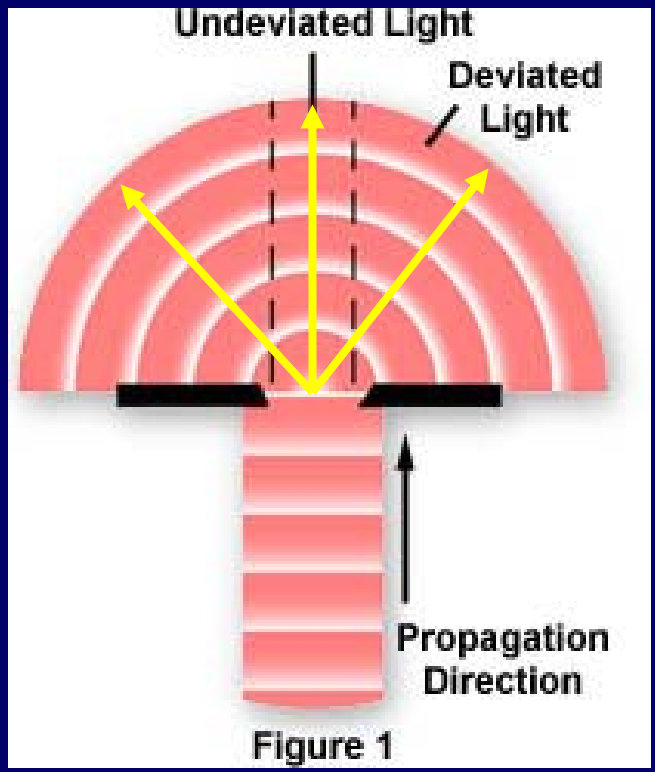
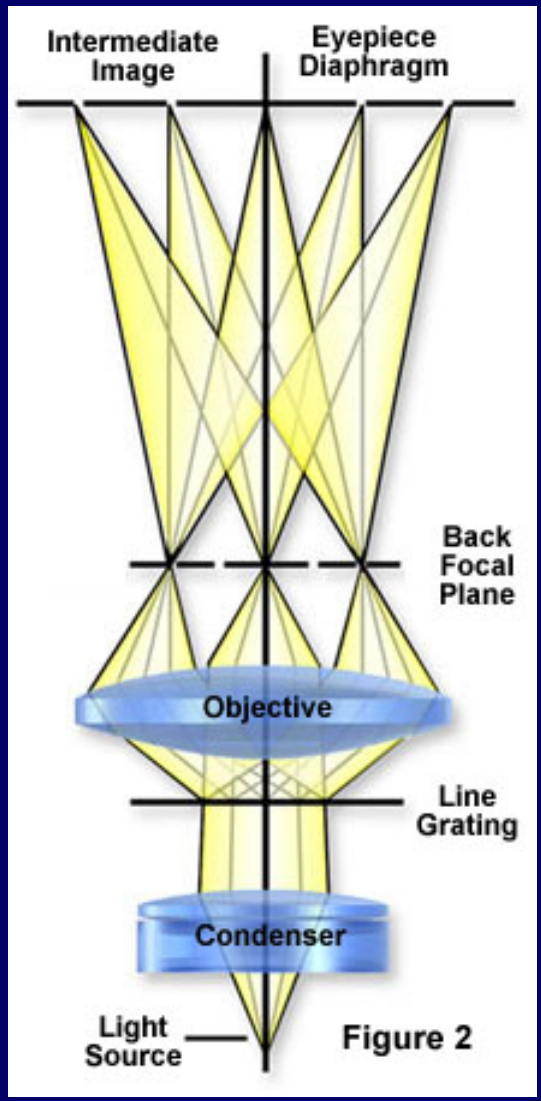


Figure 1

Diffraction, Interference and Image Formation



Diffraction and Spacing in the Specimen

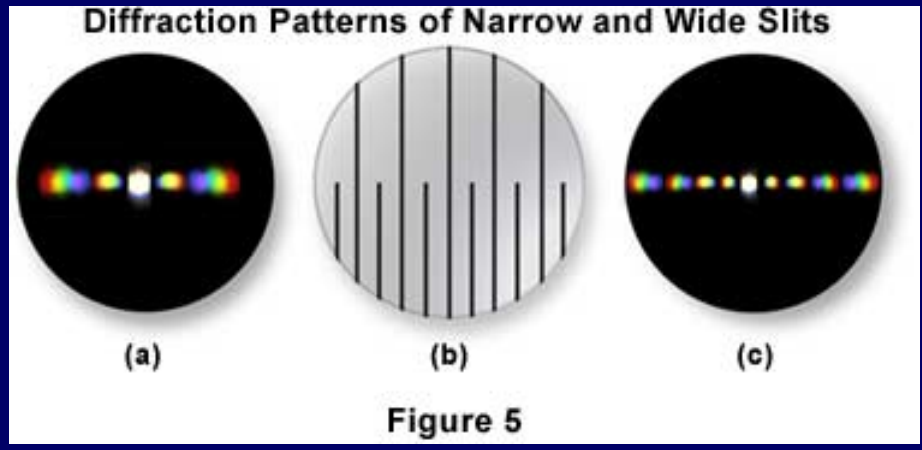


Figure 5

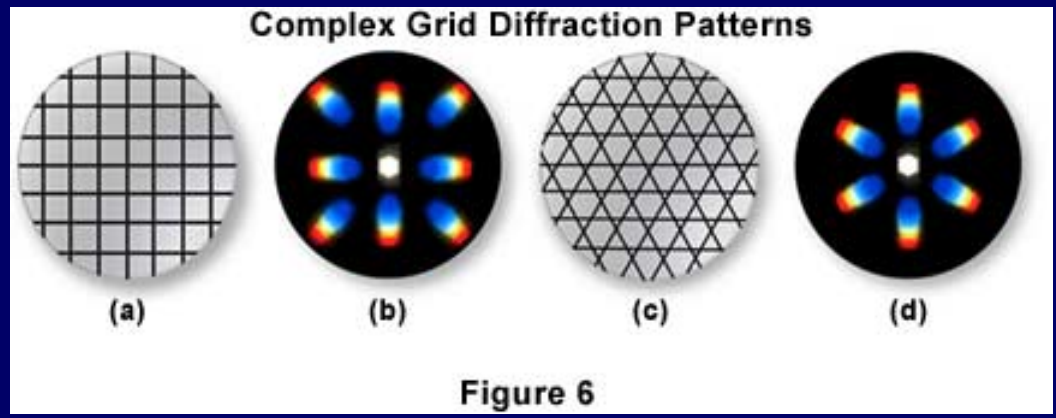
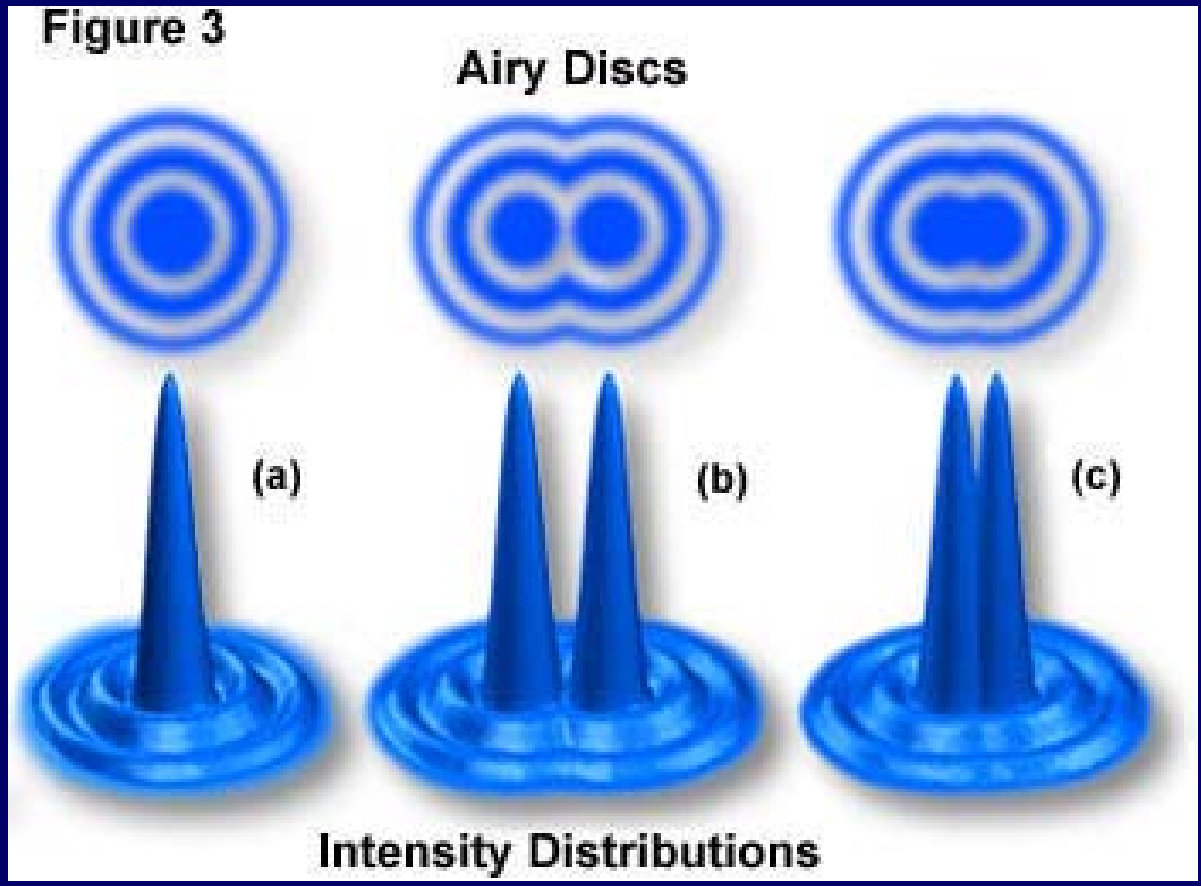


Figure 6

- Abbe: The details of a specimen will be resolved if the objective capture the 0^{th} and 1^{st} diffracted orders (or any two orders).

Resolution Between Two Objects



Different criteria specify different spacings between the images to achieve “resolution”

Resolution is Dictated by Numerical Aperture

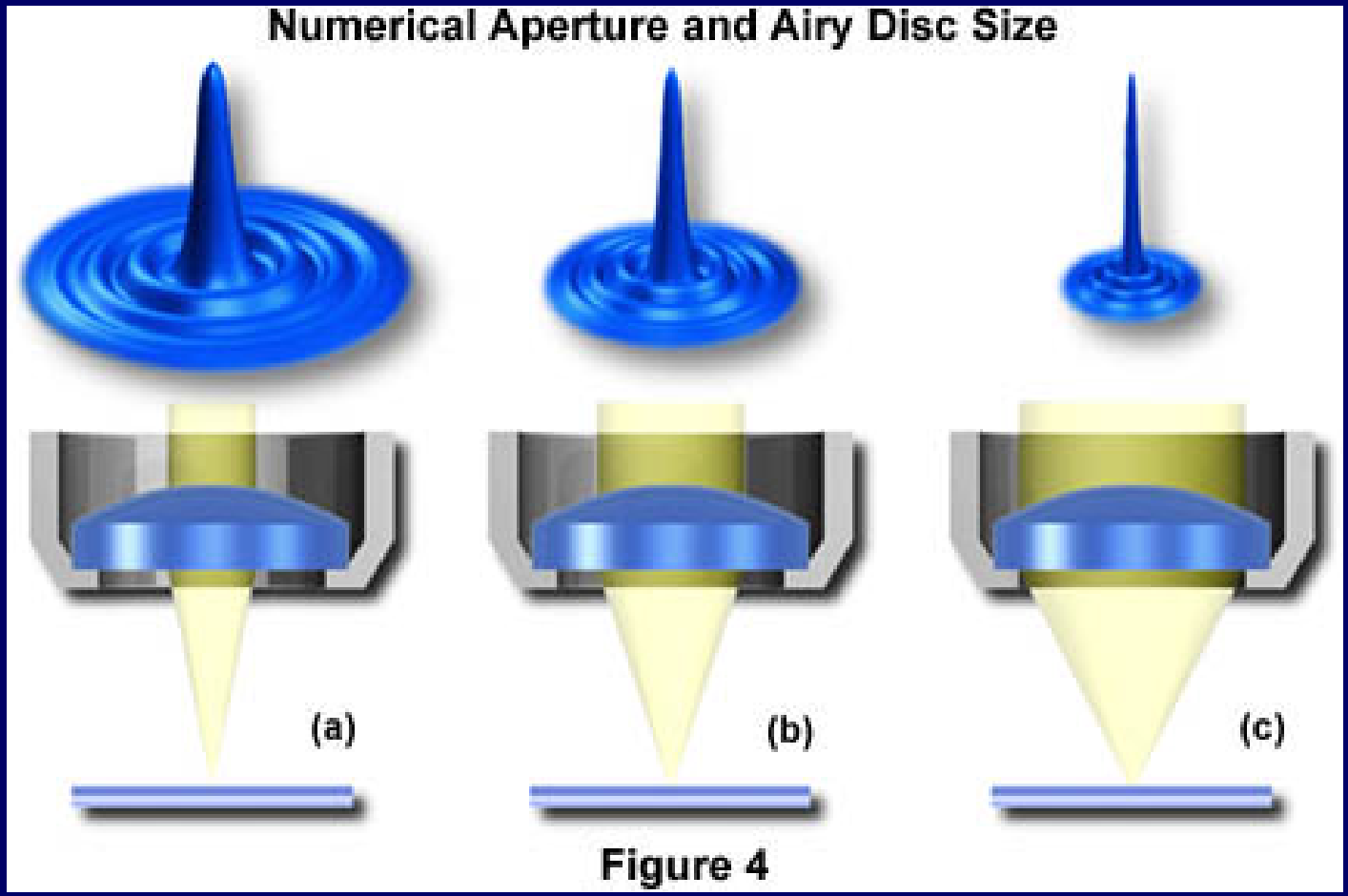
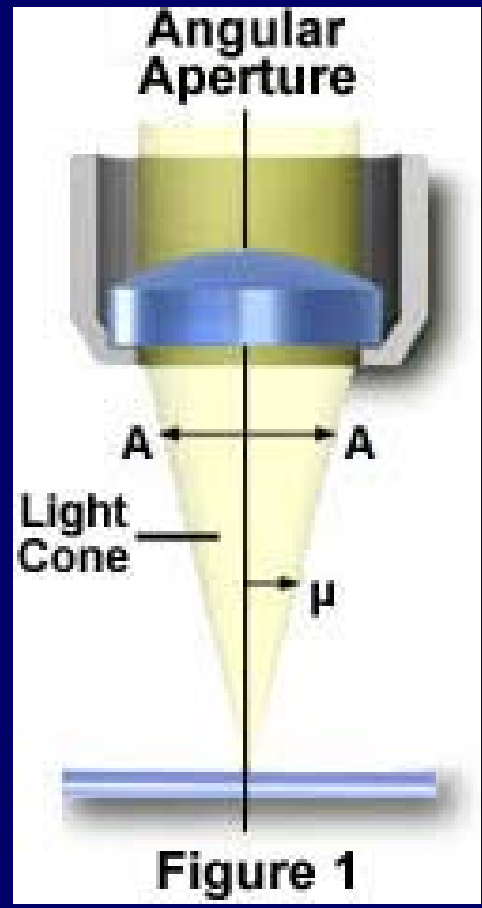


Figure 4

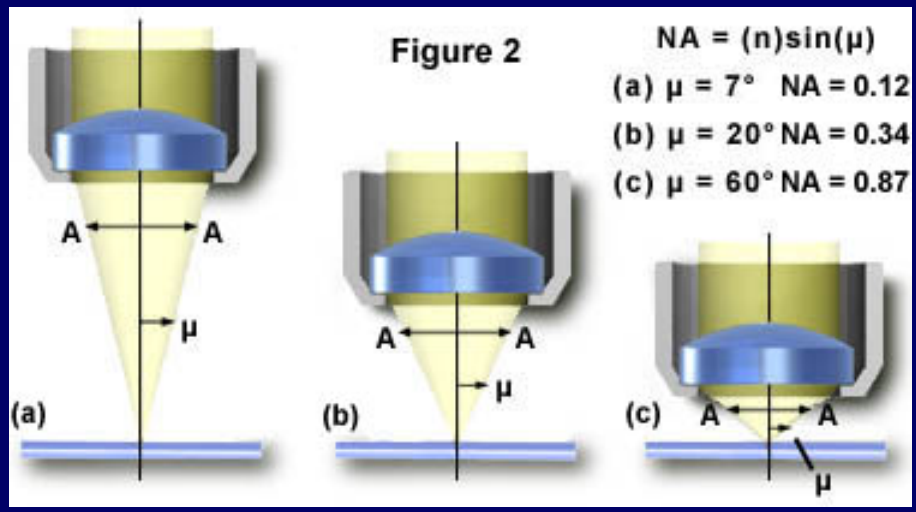
The smaller the NA, the bigger the focal spot,
And the less resolution obtained

Numerical Aperture

- A measure of the angle of the cone of illumination captured by the objective
- **NA=*n(sinθ)***
- ***θ is the angular aperture***
- ***n is the refractive index of the immersion medium***



N.A.



- In practice it is difficult to achieve N.A.s above 0.95 with dry objectives.
- The refractive index of the medium between the objective and the specimen is increased by using oils (n=1.51) or water (n=1.33)

Immersion Medium

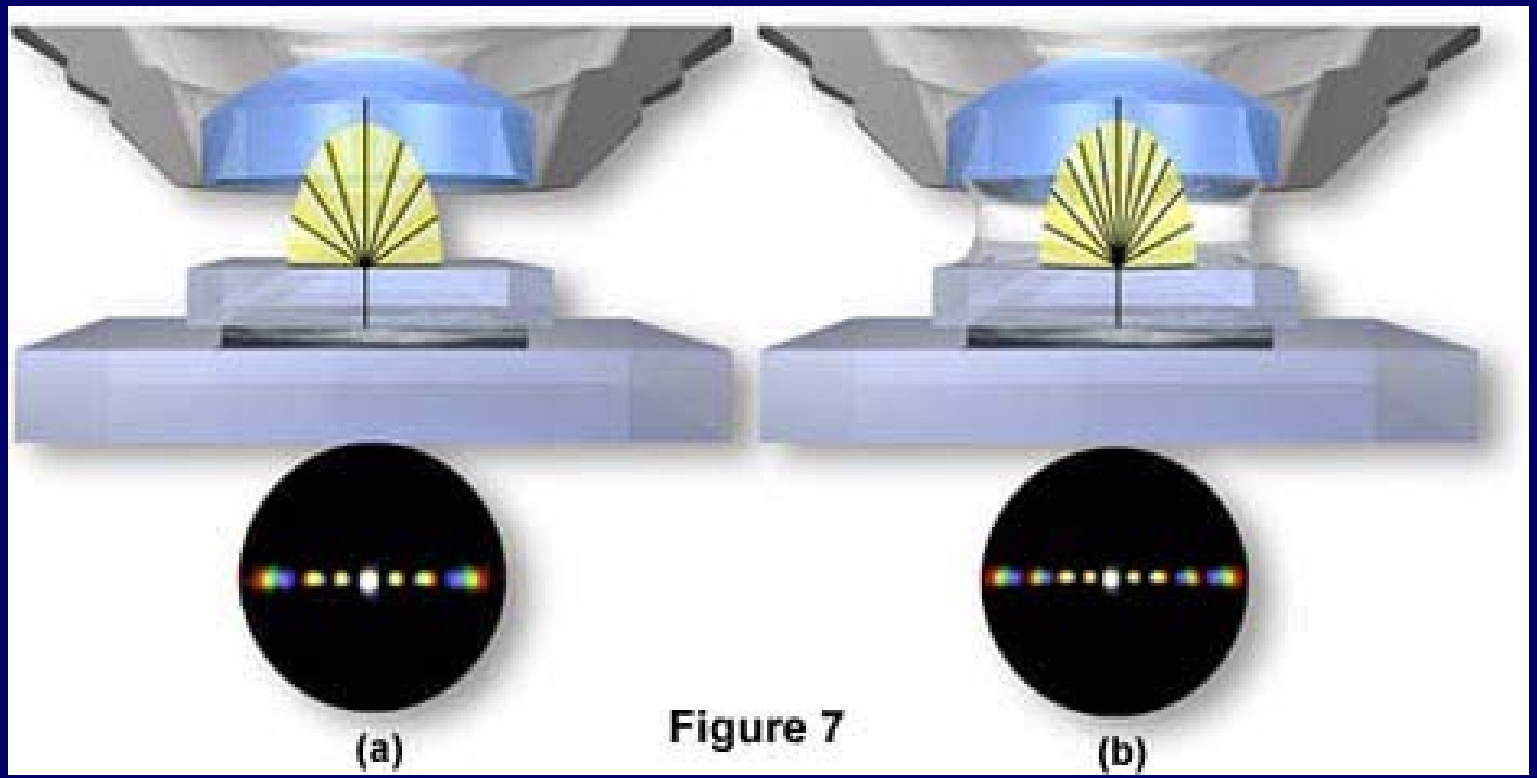
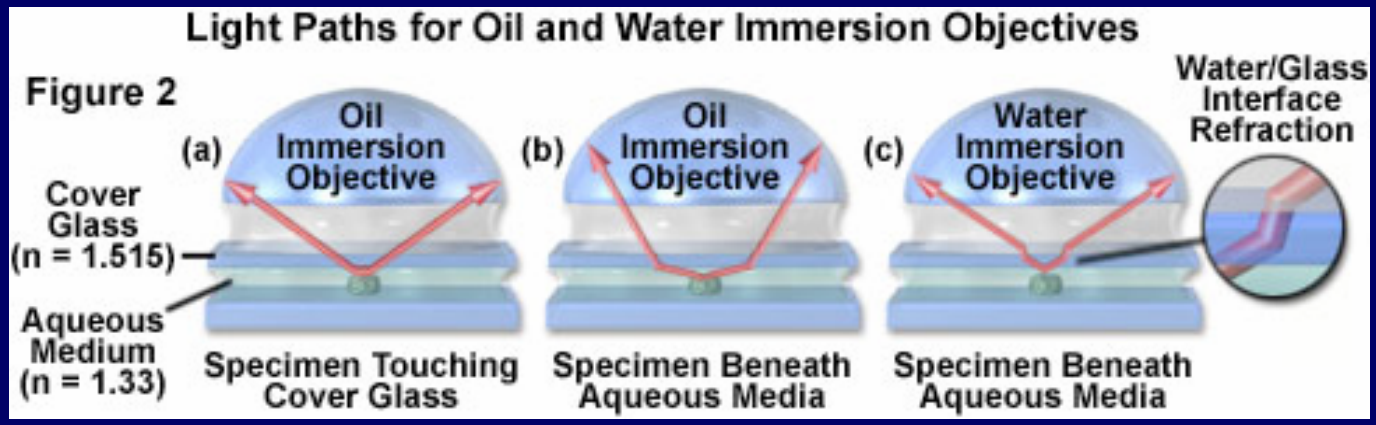
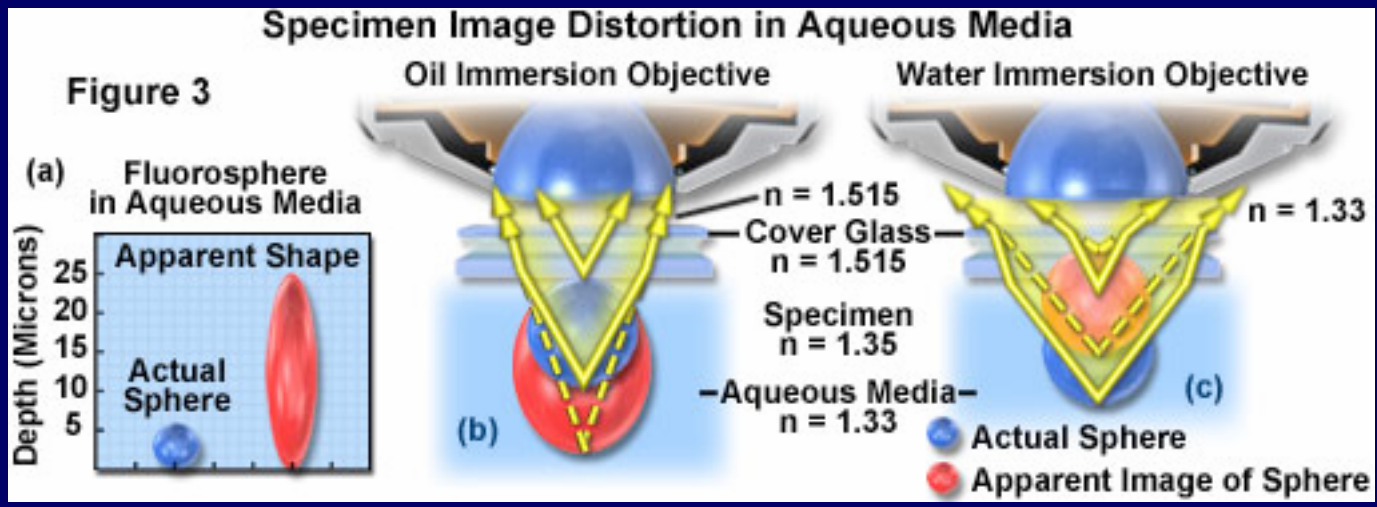


Figure 7





- Oil immersion objectives can have higher NA, and hence resolution
- Spherical aberrations at micron distances from the coverglass can be problematic

Criteria for Maximum Resolution

$R=\lambda/2NA$

$R=0.61\lambda/NA$ (Rayleigh Criterion)

$R=1.22\ \lambda/(NA(obj) + NA(cond))$

So at NA=0.95

360 nm	R=0.19 micrometers
450 nm	R=0.24
550 nm	R=0.29
700 nm	R=0.37

Resolution of light microscopy

Horizontal

$$1.22 \times \Lambda / (N.A._{objective} + N.A._{condenser})$$

e.g. 488 nm light, N.A. 1.4 = 213 nm

Vertical

$$2 \times \Lambda \times n / (N.A._{objective})^2$$

e.g. 488 nm light, oil, N.A. 1.4 = 754 nm

where:

Λ is the wavelength of light

N.A. is numerical aperture

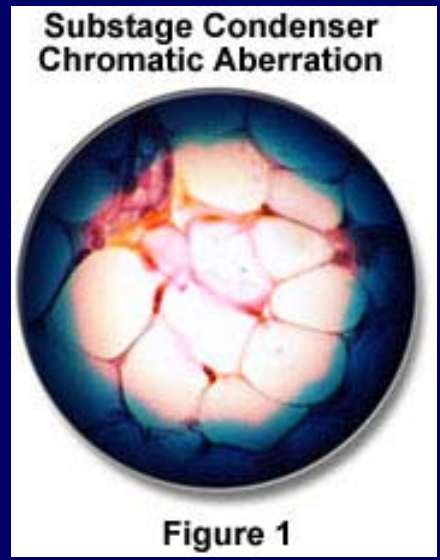
n is the refractive index of the sample medium

Markings on the Objective

Figure 1

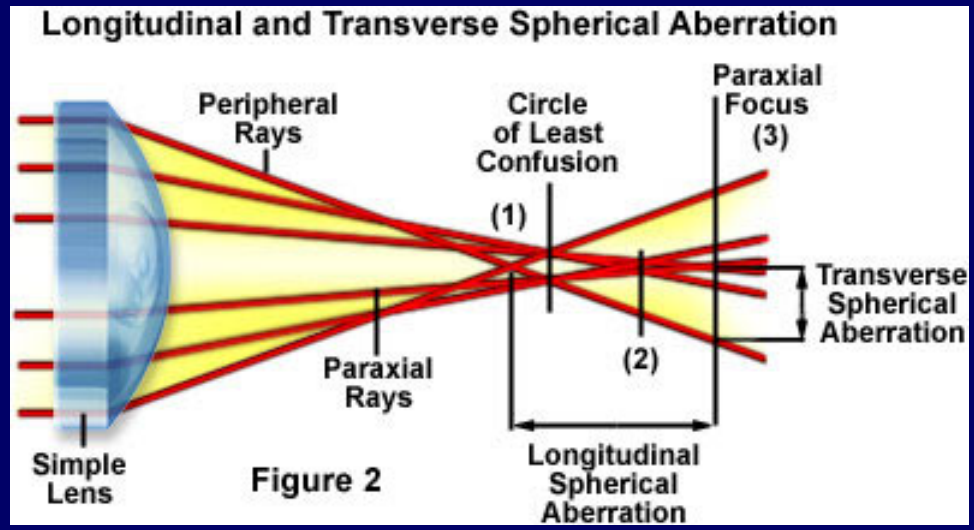
Aberrations/Corrections

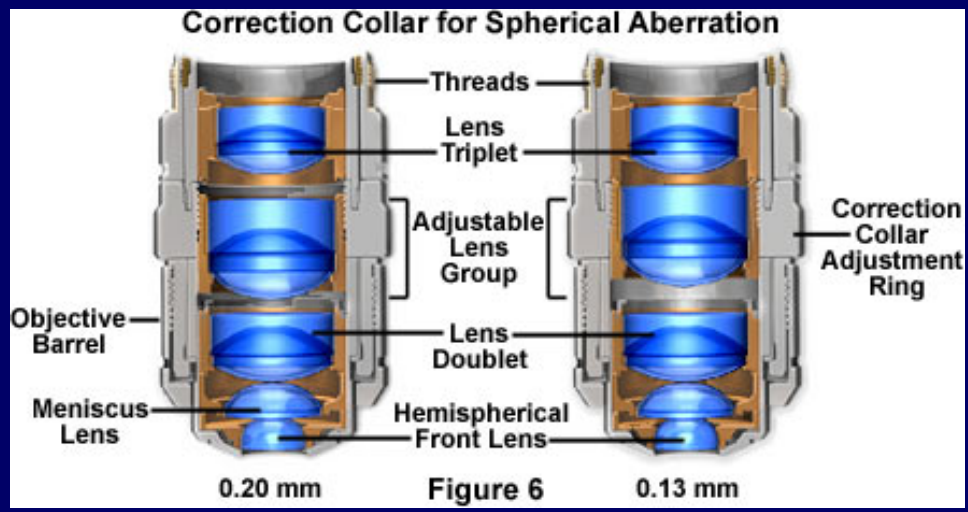
- Chromatic aberrations:
- Achro, Achromat, Apochromat (wider range of wavelengths), Fluor



Aberrations/Corrections

- Spherical Aberrations
- Light passing through the periphery of lens not brought to focus with light through center
- Lenses are well corrected for standard 17 mm cover glass, or have adjustment collar





Aberrations/ Corrections

- Flat Field Corrections
- Plan

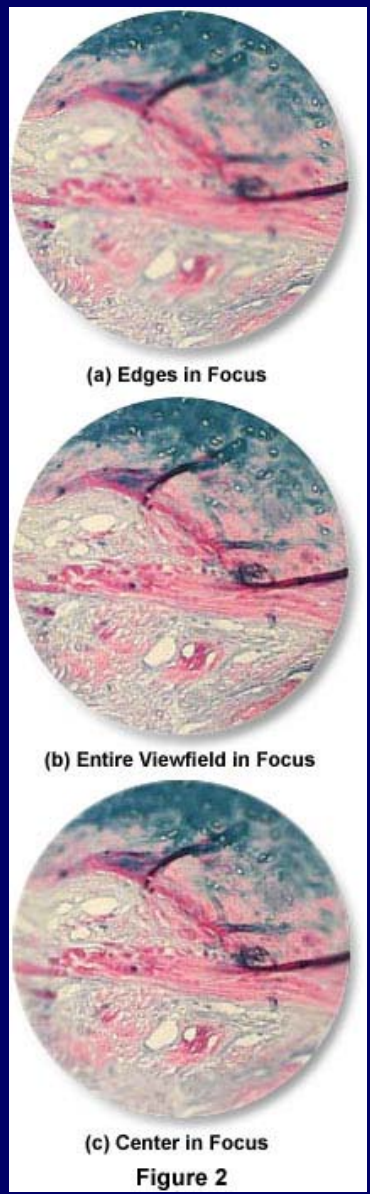
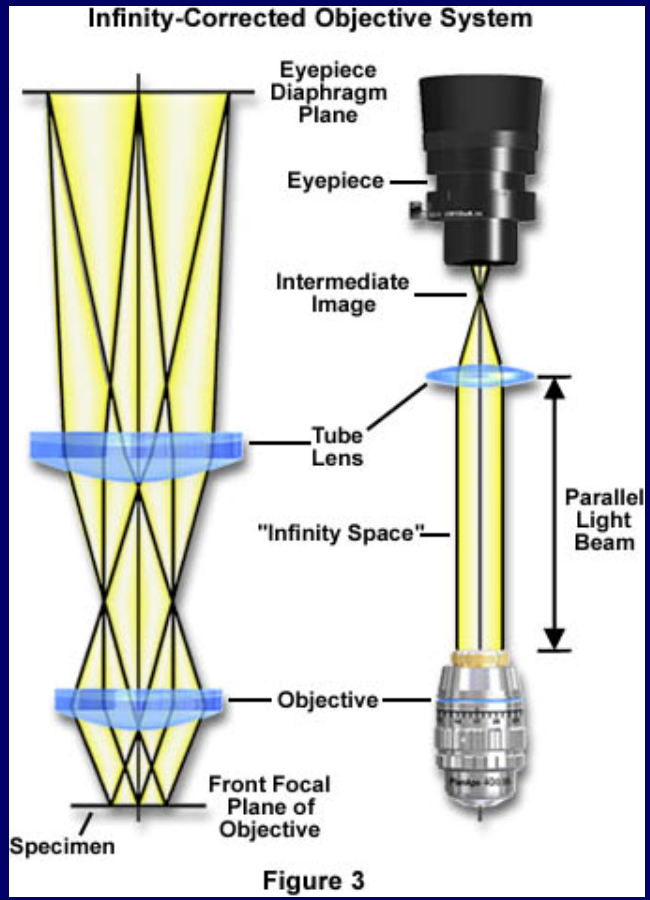


Figure 2

Mechanical Tube Length

160 mm fixed



Infinity

Köhler Illumination is Absolutely Required for Good Transmitted Light Contrast.

There are two sets of conjugate Optical planes in the microscope:

1. Aperture or Illumination Plane

2. Focus (Object), Image Plane

These two are Fourier transforms of each other -- This means that they are related in specific ways.

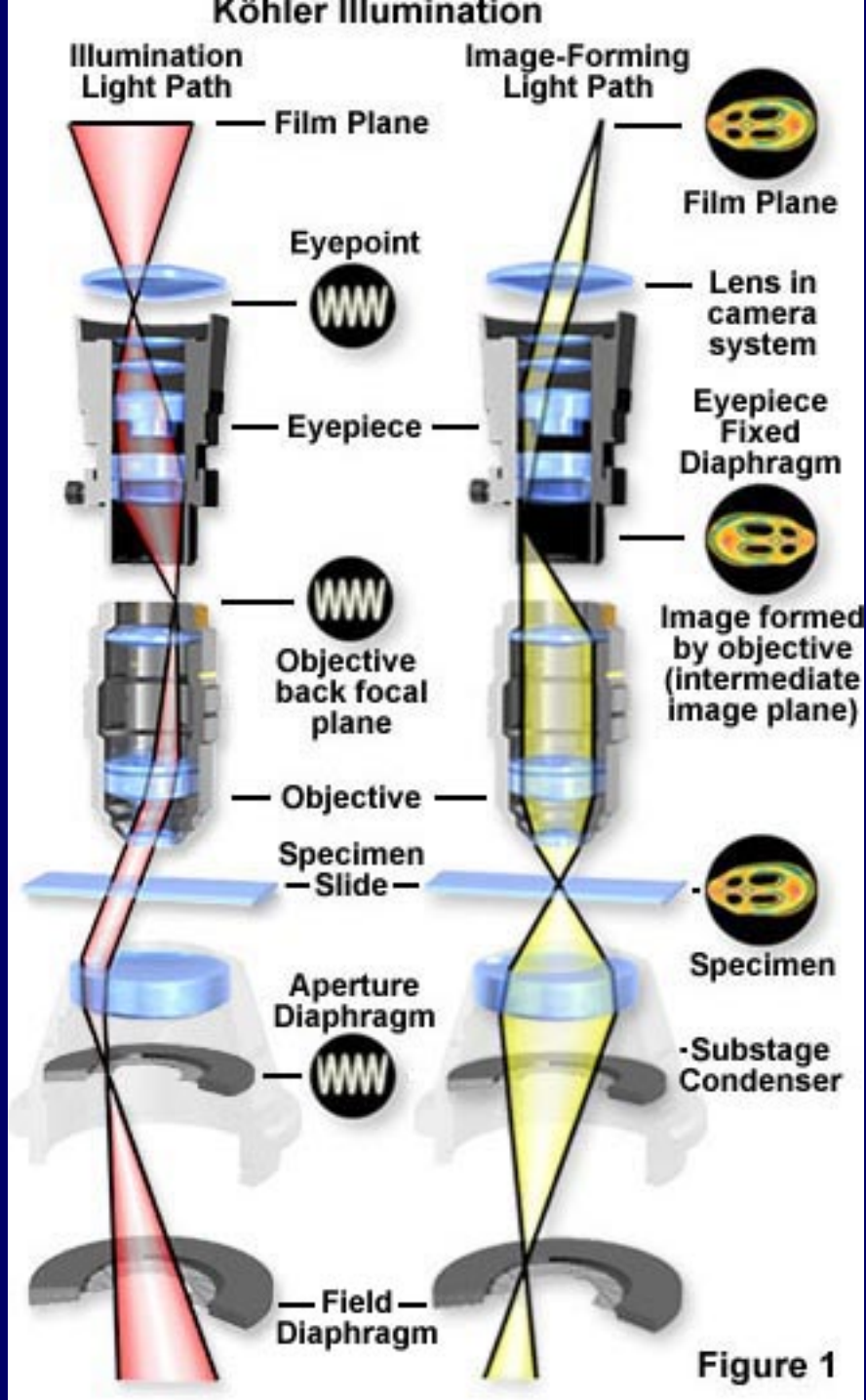


Figure 1

Proper Alignment of the Condensor

Focus and Center the Illumination

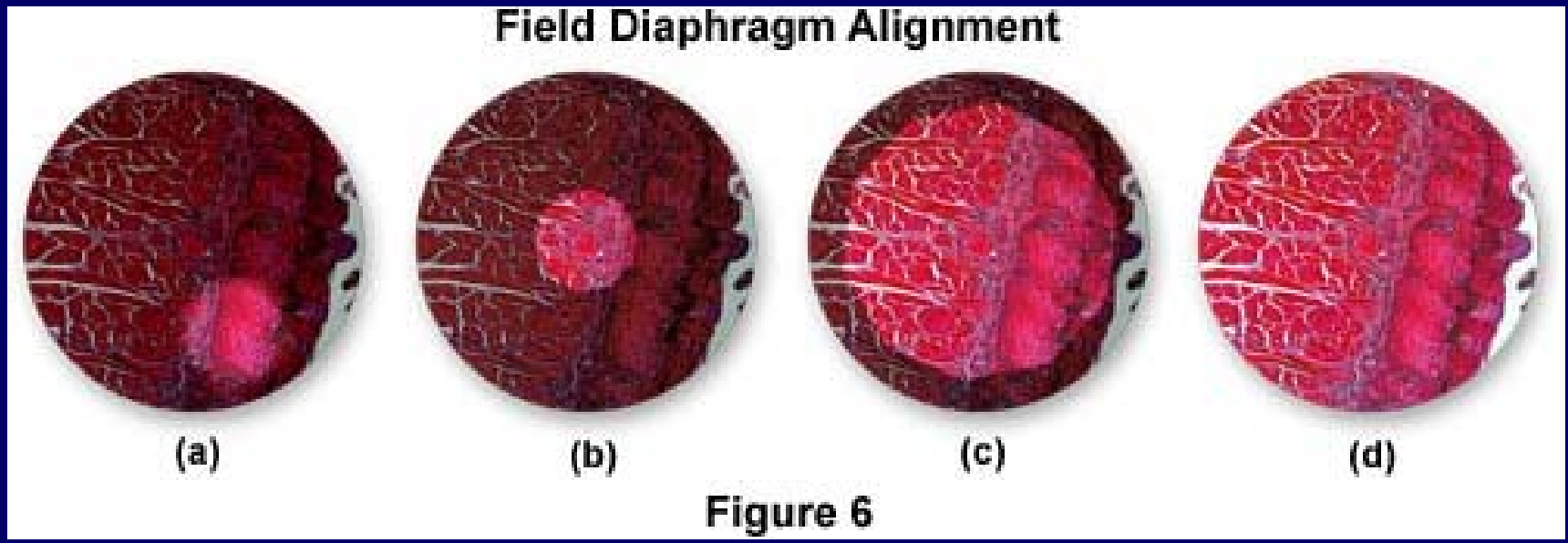
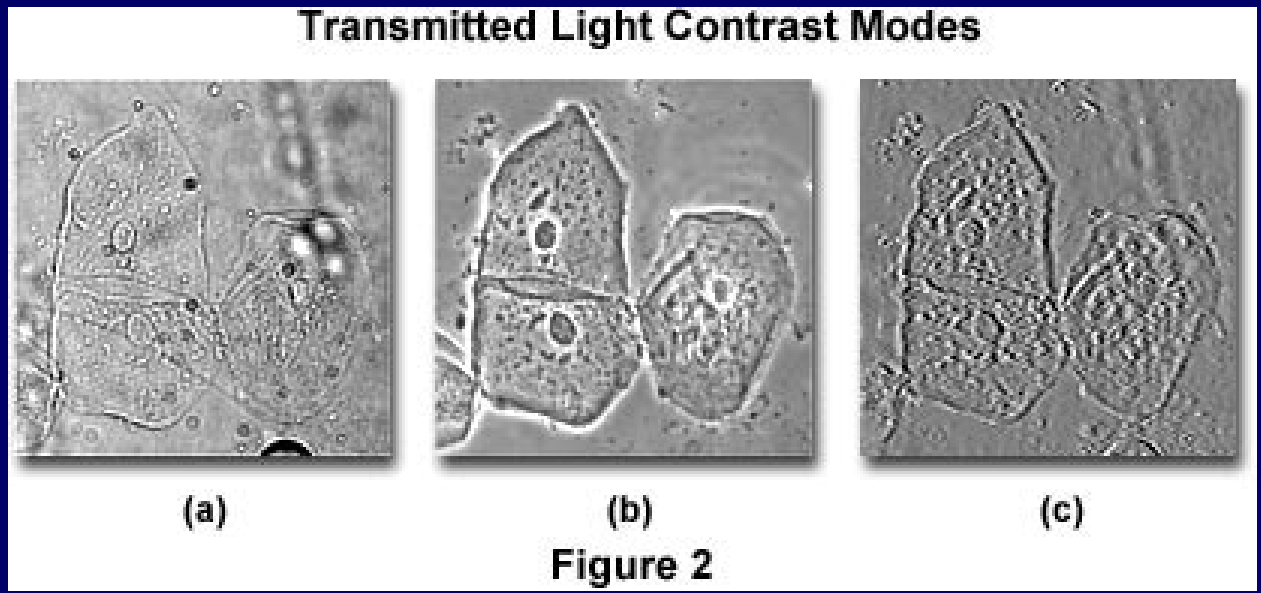


Figure 6

1. Close diaphragm
2. Focus diaphragm in image field
3. Center diaphragm in field
4. Open the diaphragm to fill the field

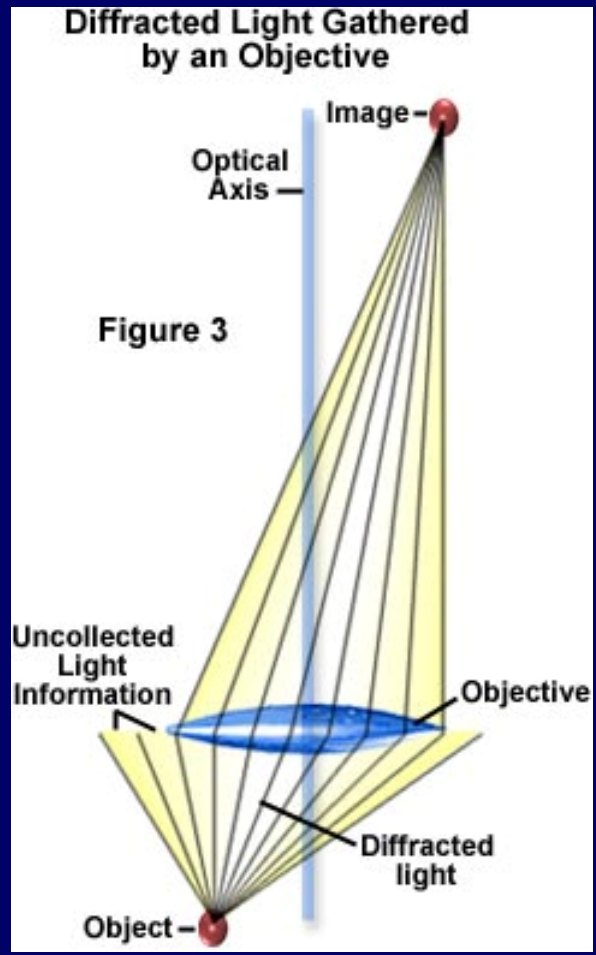
Contrast

- Unstained biological specimens usually have low contrast in bright field images
- Phase contrast and differential interference contrast use different optical tricks to introduce contrast based on changes in the refractive index across the specimen

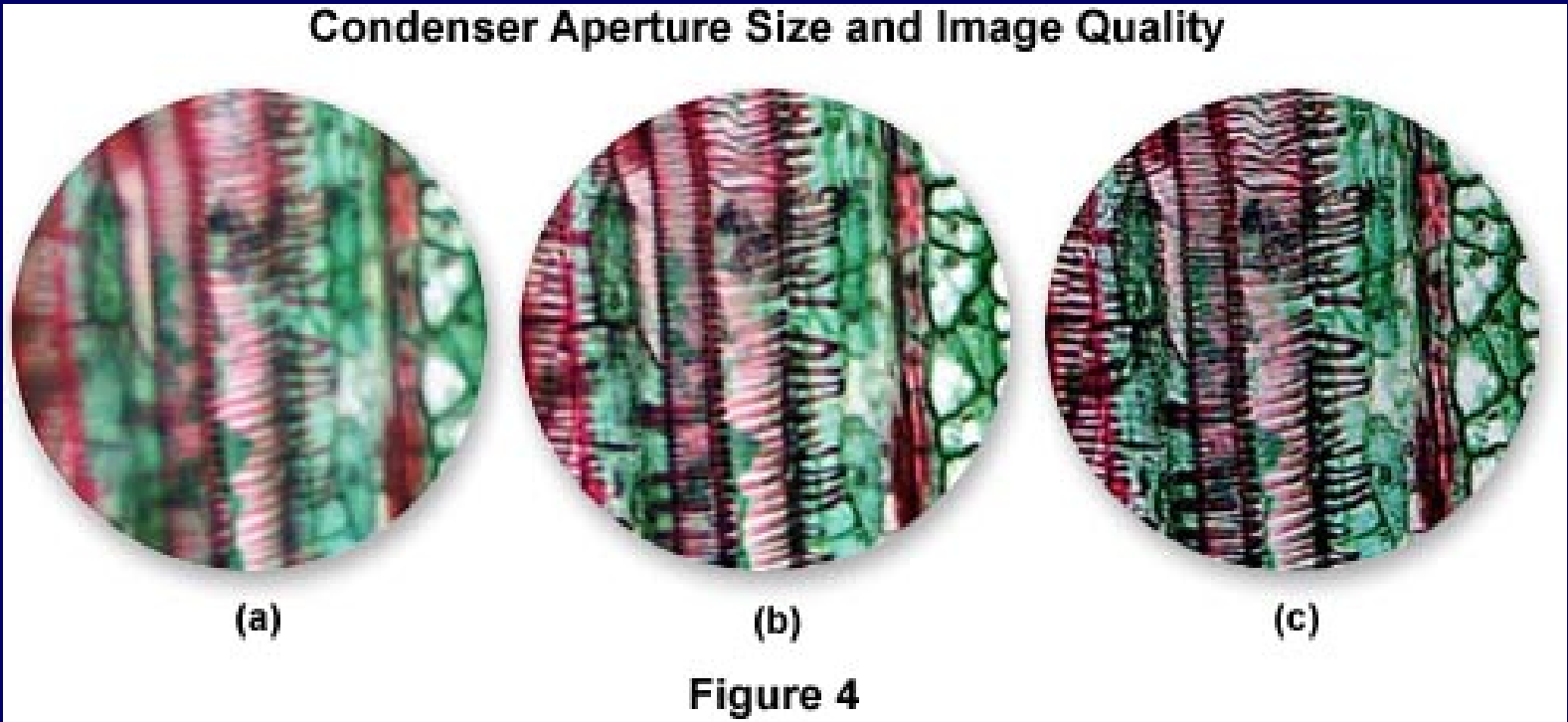


Biological Specimens as Phase Objects

- Visibility of light after interference is a function of coherence
- Can be maximized by decreasing the size of the conenser diaphragm, but at cost to resolution (decrease NA)



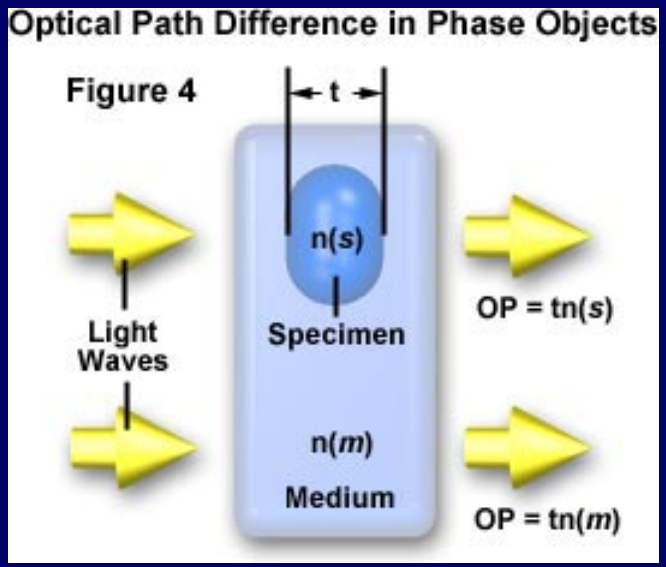
Contrast and Resolution Vary with Illumination



NA = 100% NA =75% NA = 25%

Note! For many contrast methods, including DIC, Hoffman and Fluorescence, resolution is given by the smallest NA in the system

Optical Path Difference

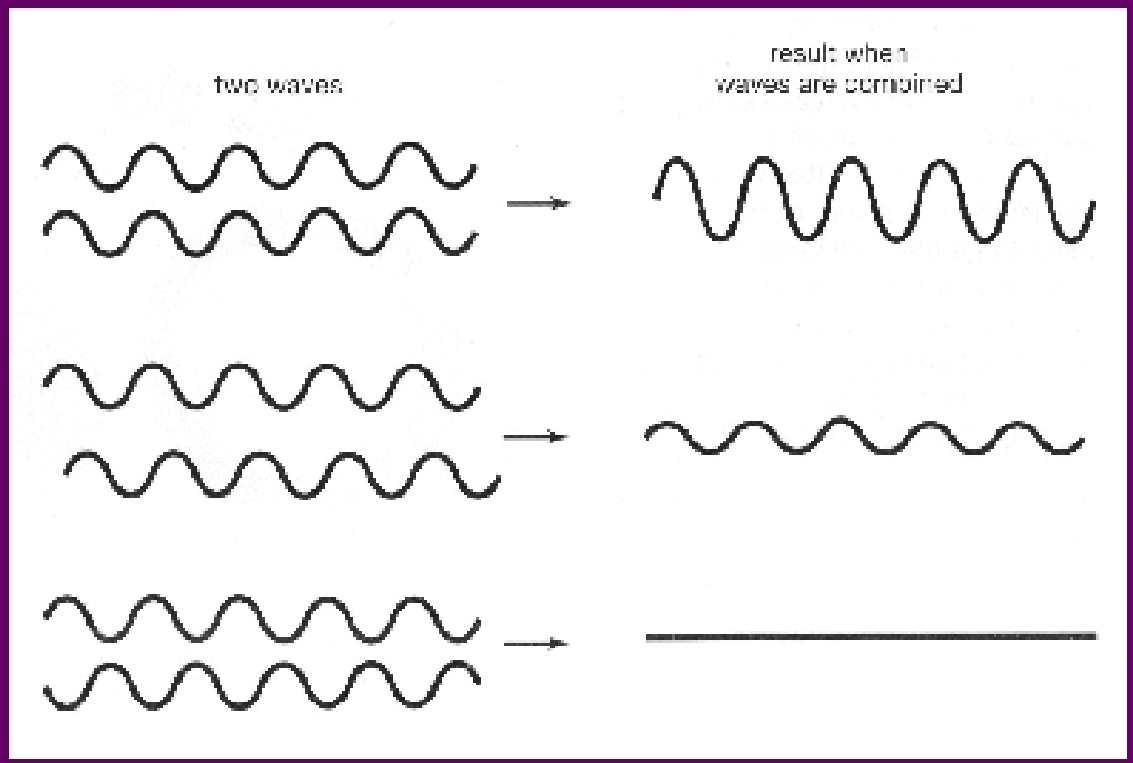


- OPD=t(n(s)-n(m))
- Phase Difference

$\delta = (2\pi/\lambda)(OPD)$

•Optical path differences in unstained specimens are small but give phase differences that are exploited in the phase contrast microscope.

Constructive and Destructive Interference



constructive → White

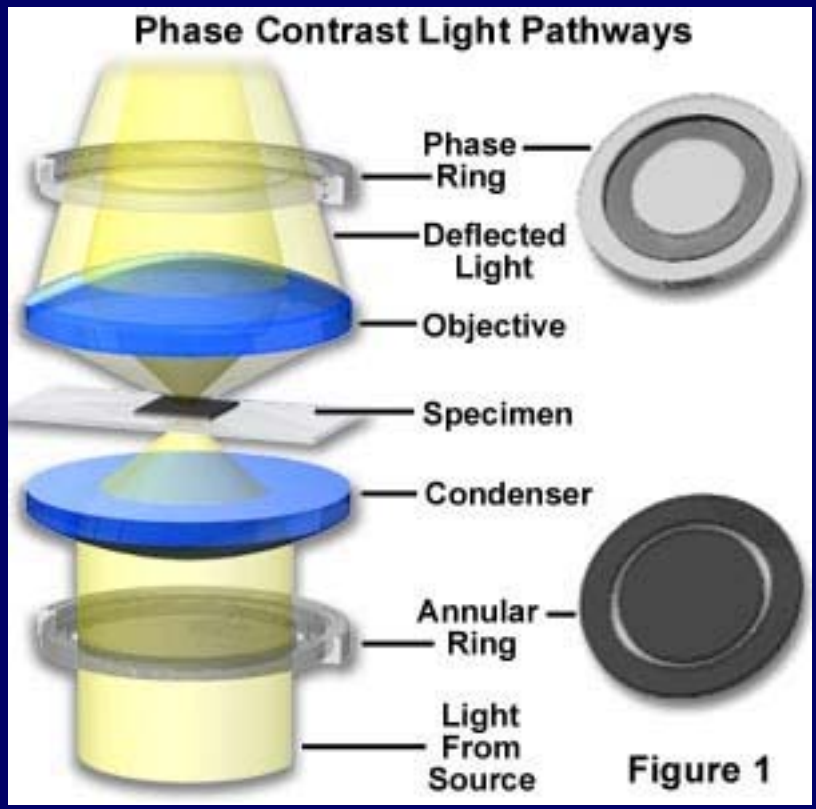
→ Gray

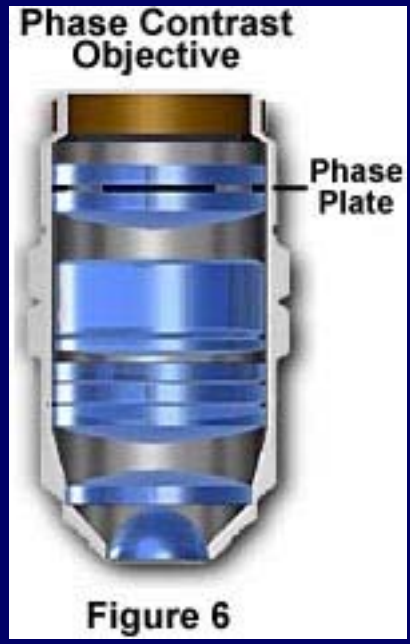
destructive → Black

Phase Contrast

- Unstained specimens that do not absorb light retard its phase by ~1/4 wavelength compared to undeviated light
- Direct zeroth order light passes through specimen undeviated, diffracted light lags behind by ~1/4 wavelength, but in interference this is not sufficient to observably reduce intensity
- Phase microscope speeds up direct light by ¼ wavelength, so that it ends up ½ wavelength out of phase with the diffracted light, giving destructive interference (black)

Phase Contrast (most common method)





Phase Contrast illuminates a ring, but in this case the ring is in the aperture plane. Unscattered light is "phase delayed" for maximum interference.

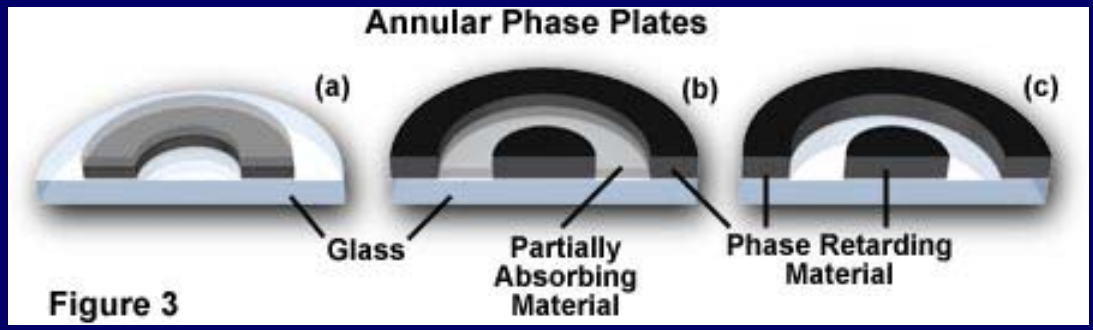


Figure 3

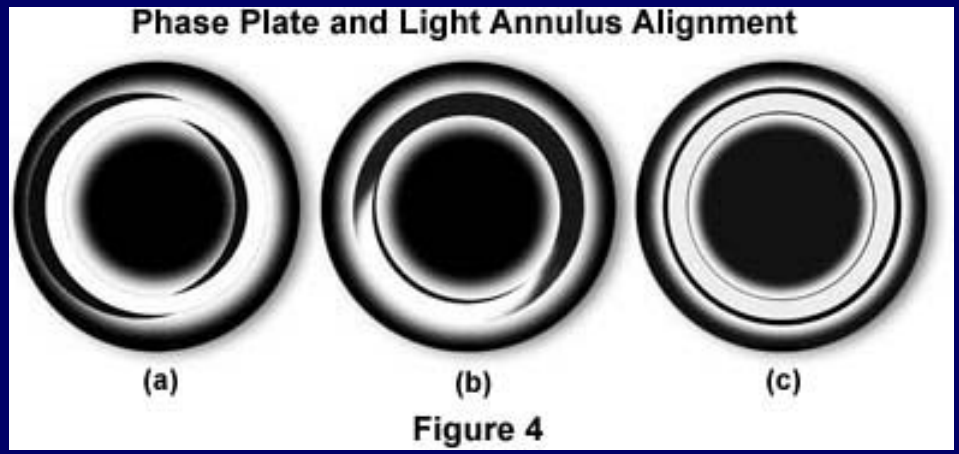
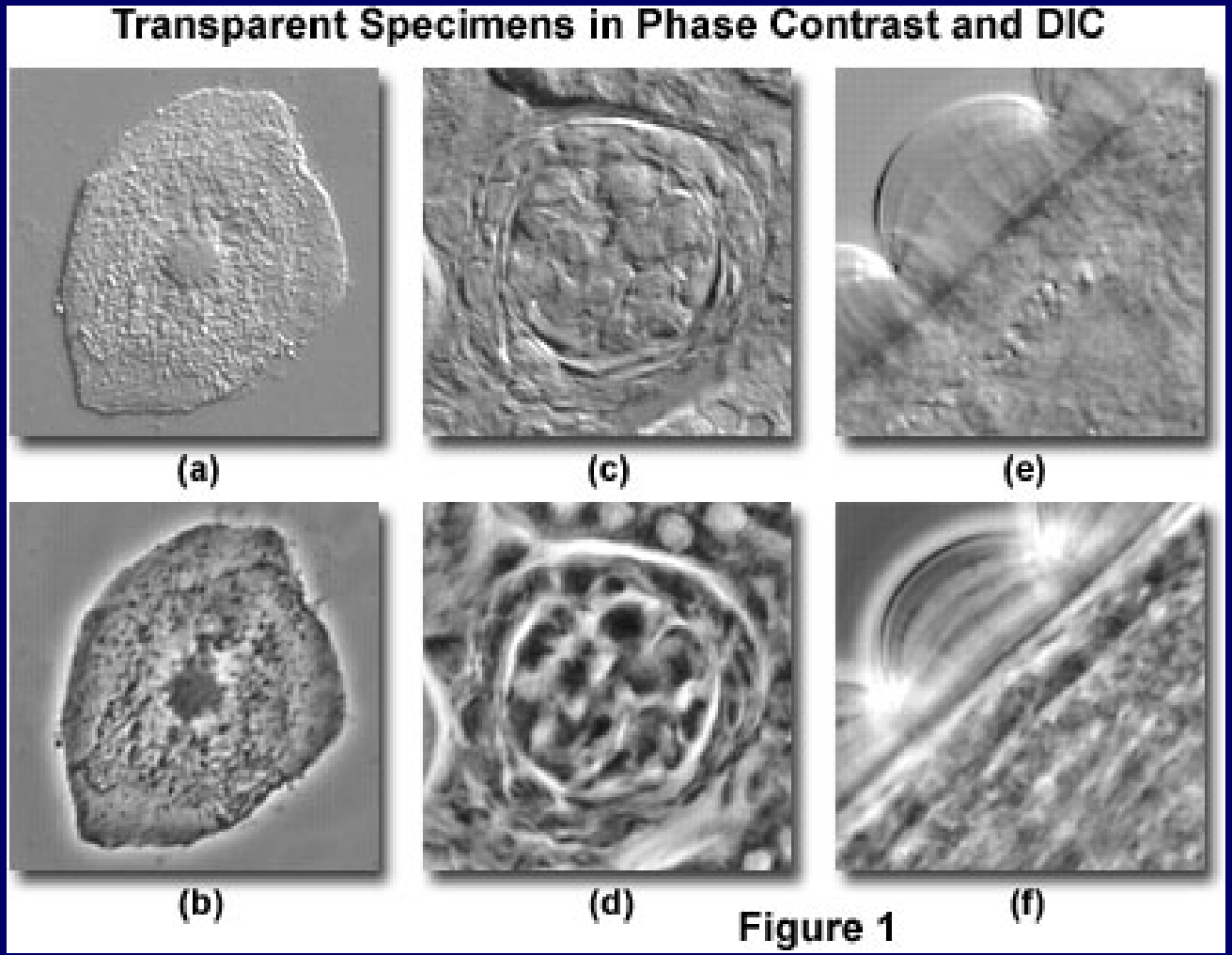


Figure 4

Limitations

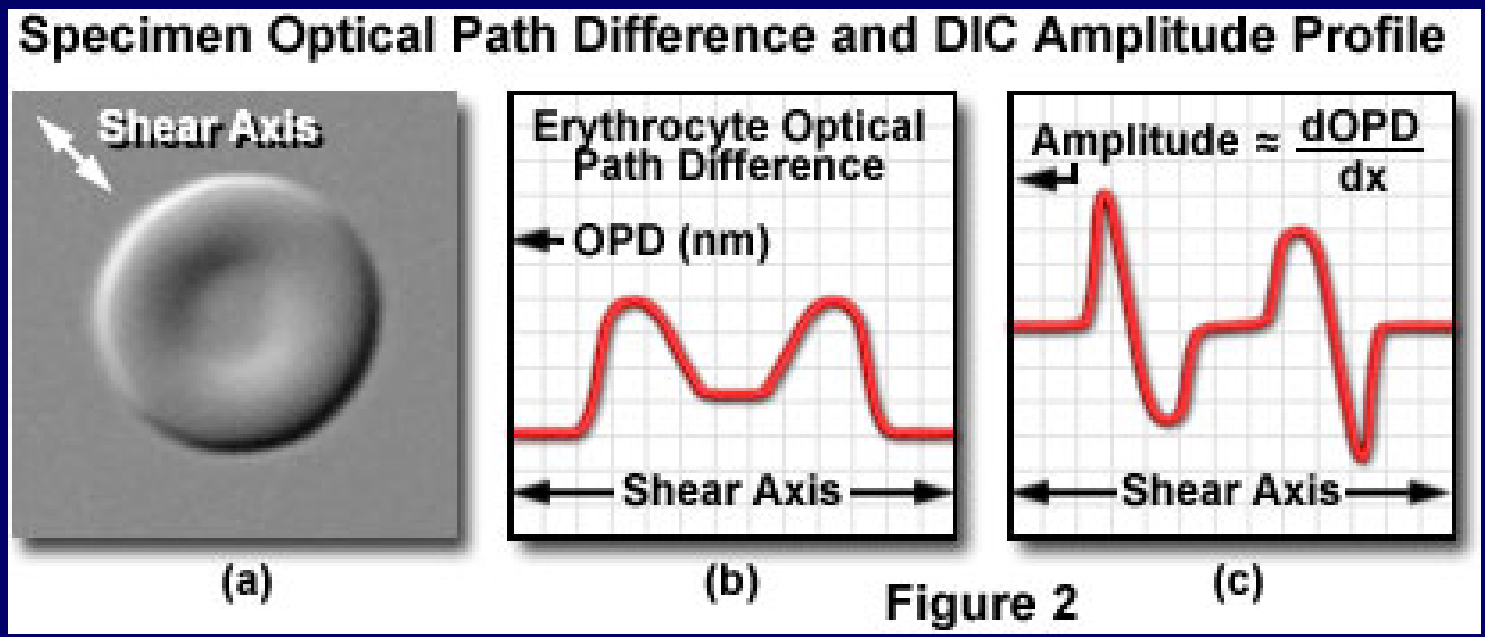
- Halos
- Phase annuli limit working NA, hence resolution
- Poor for thick specimens due to phase shifts from planes above and below focus

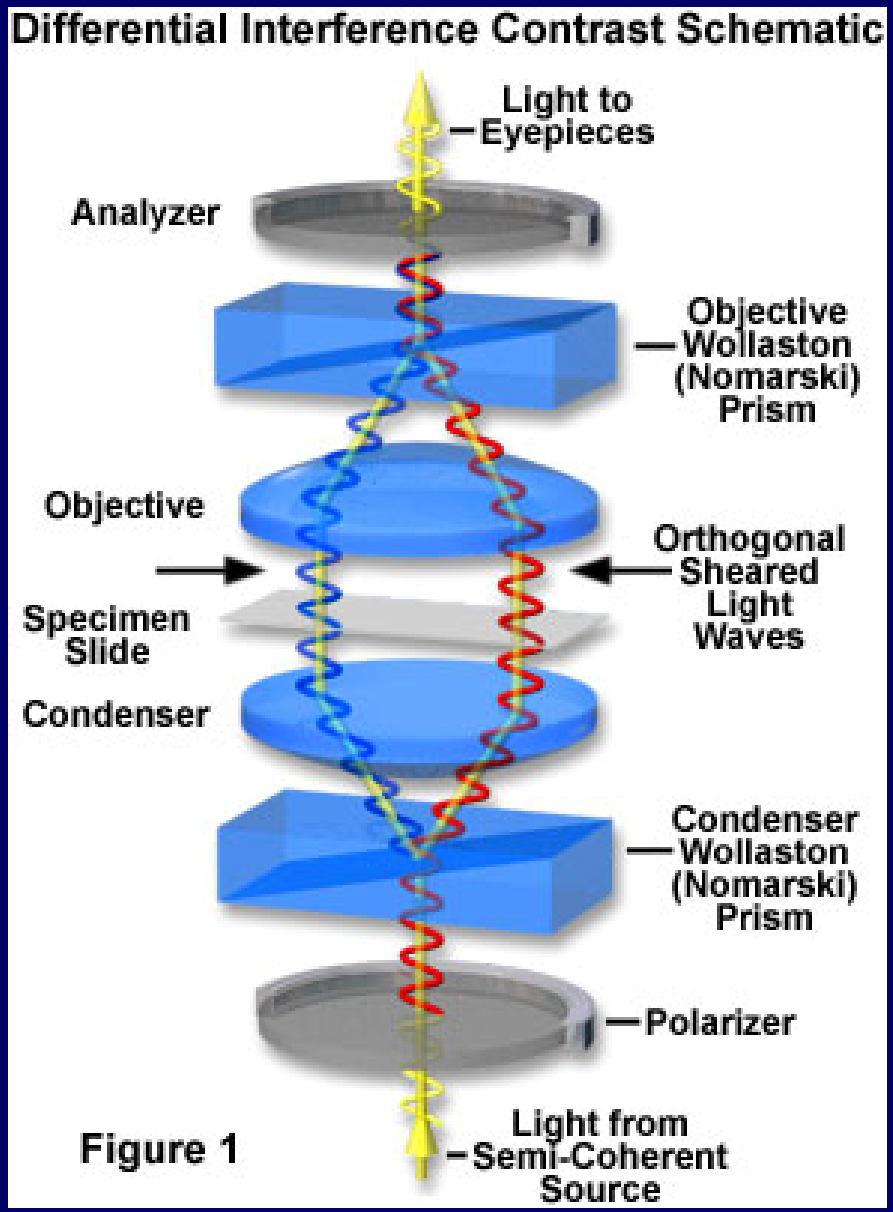
Phase and DIC

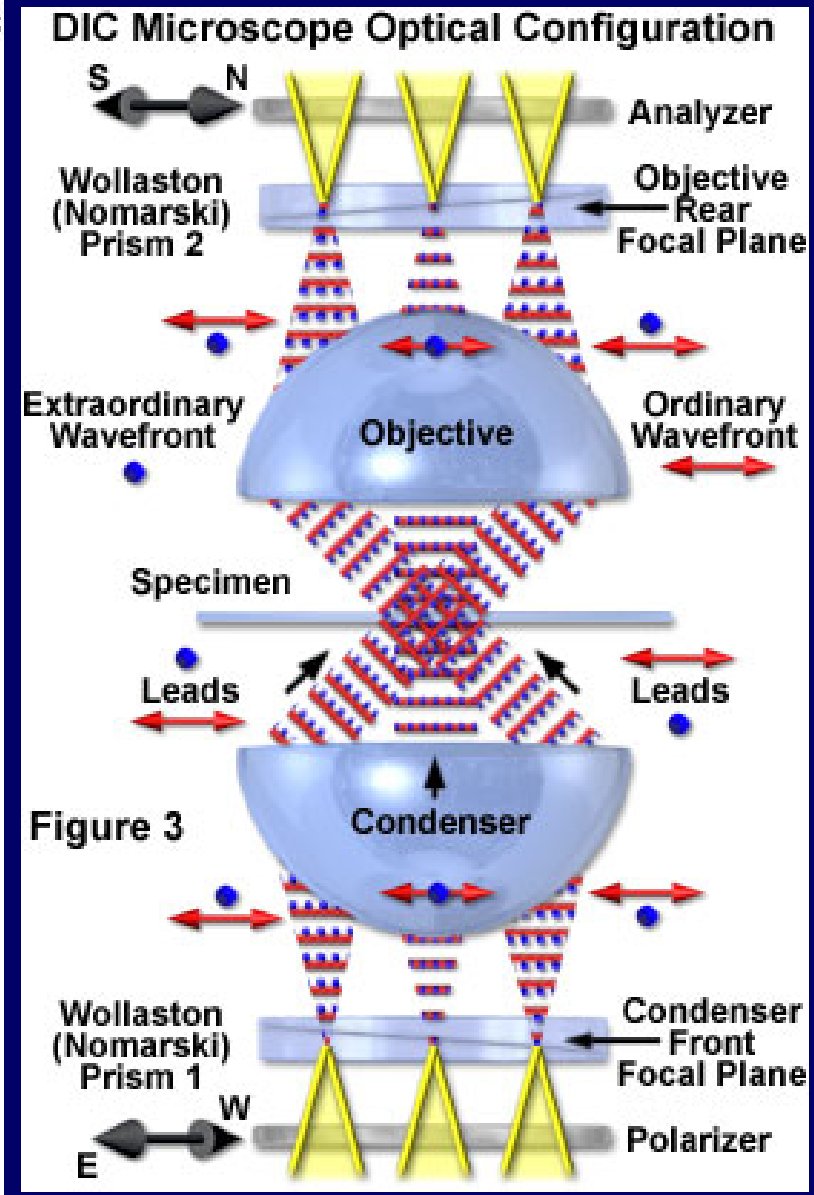


Phase and DIC

- Phase: intensity based on optical path variation- high OPD=dark, low OPD=light
- DIC: intensity variation based on magnitude of gradients in OPD. Sharp gradients give pseudo relief shading. Shallow gradients appear with similar intensity to background




Differential Interference Contrast Schematic
Light to Eyepieces
Analyzer
Objective Wollaston (Nomarski) Prism
Objective
Orthogonal Sheared Light Waves
Specimen Slide
Condenser
Condenser Wollaston (Nomarski) Prism
Polarizer
Light from Semi-Coherent Source
Figure 1


DIC Microscope Optical Configuration
S
N
Analyzer
Wollaston (Nomarski) Prism 2
Objective Rear Focal Plane
Extraordinary Wavefront
Objective
Ordinary Wavefront
Specimen
Leads
Leads
Condenser
Figure 3
Wollaston (Nomarski) Prism 1
Condenser Front Focal Plane
W
E
Polarizer

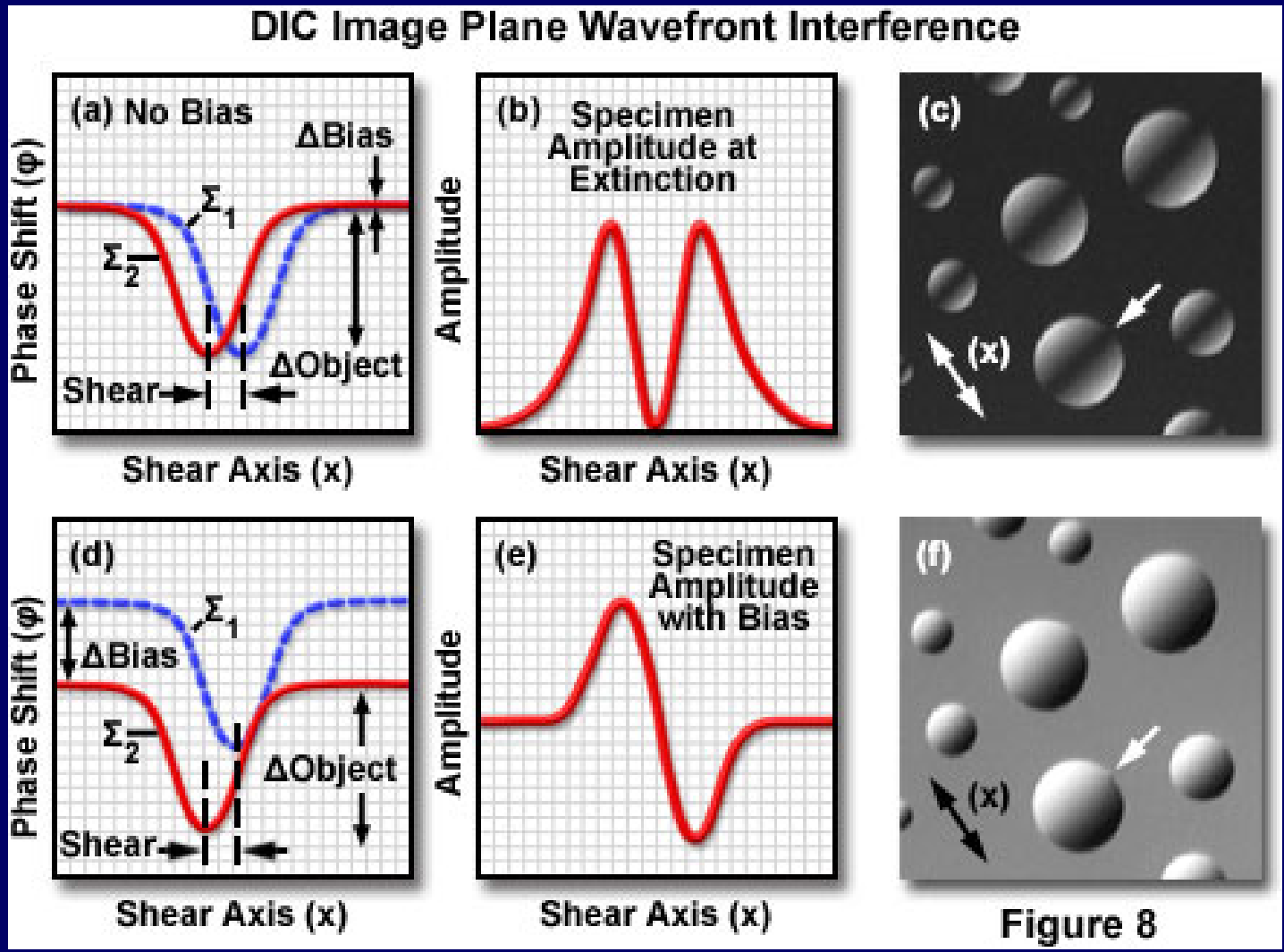
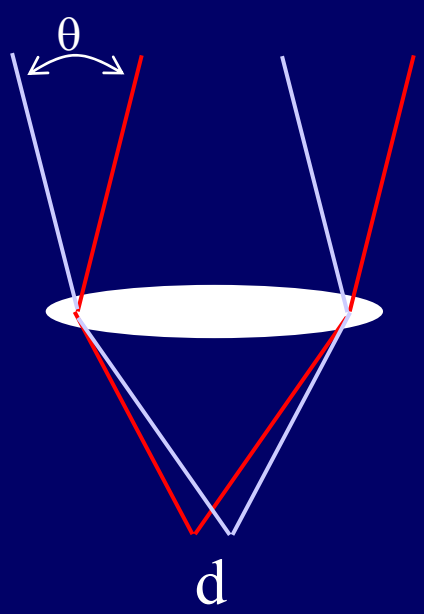

DIC Image Plane Wavefront Interference
(a) No Bias
ΔBias
Σ1
Σ2
ΔObject
Shear
Phase Shift (φ)
Shear Axis (x)
(b) Specimen Amplitude at Extinction
Amplitude
Shear Axis (x)
(c)
(x)
(d)
ΔBias
Σ1
Σ2
ΔObject
Shear
Phase Shift (φ)
Shear Axis (x)
(e) Specimen Amplitude with Bias
Amplitude
Shear Axis (x)
(f)
(x)

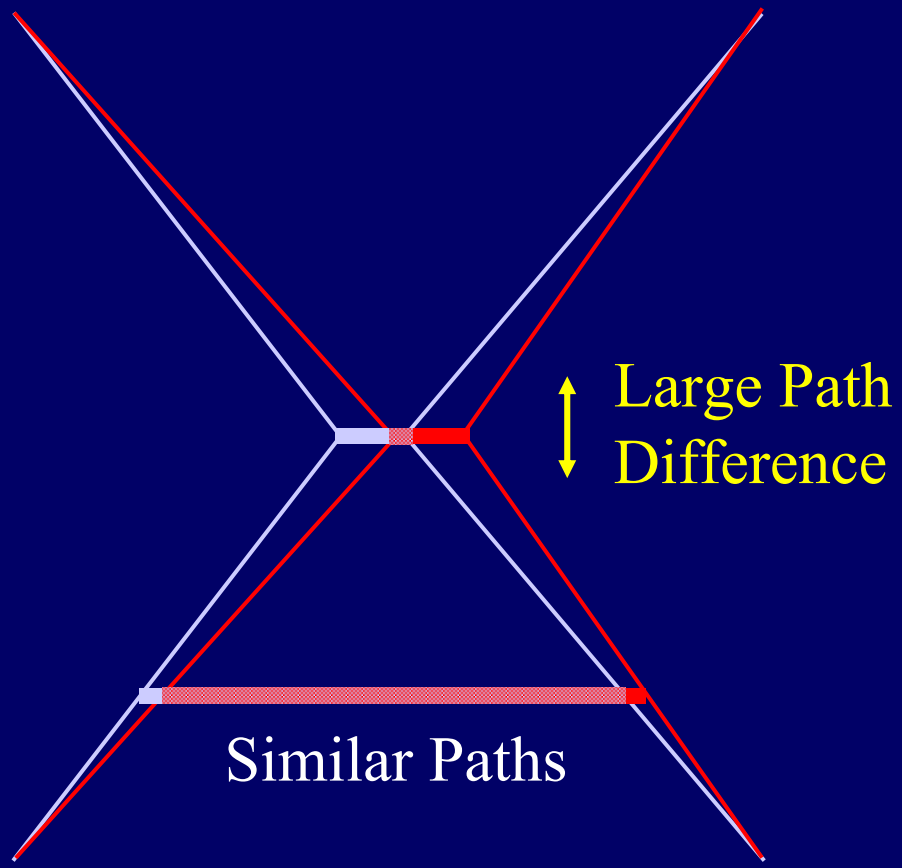
Figure 8

DIC Allows Optical Sectioning

Angles in back aperture correspond to positions in the object/image



Wollaston Prisms
Separate Two Polarizations
To Different Angles



Advantages and Disadvantages of DIC

- Capable of high resolution, no halos, optical sectioning is possible.
- Cannot image through tissue culture plastics, harder to set up, requires well-corrected objectives