Principles & Practice of Light Microscopy 3

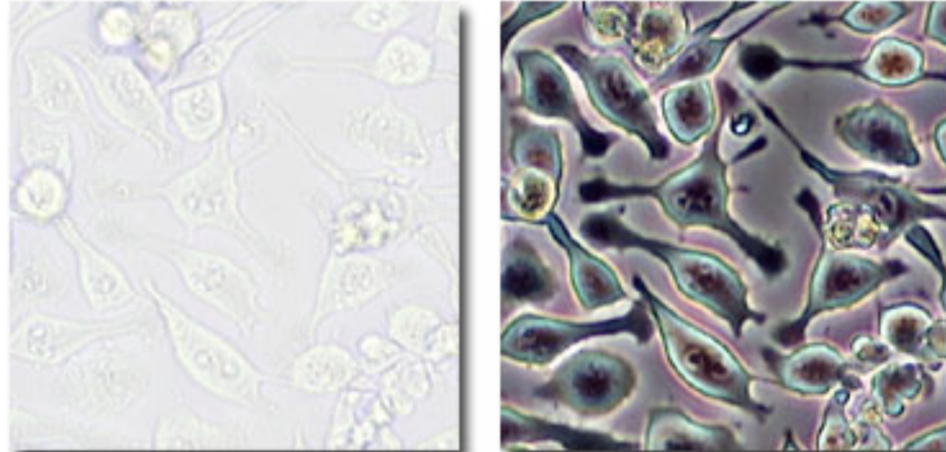
- CONTRAST

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

Enhancing contrast
In
Transmitted light microscopy

Generating contrast in light microscopy

Living Cells in Brightfield and Phase Contrast



Brightfield Phase contrast

Problem-- many living unstained samples are thin and optically transparent

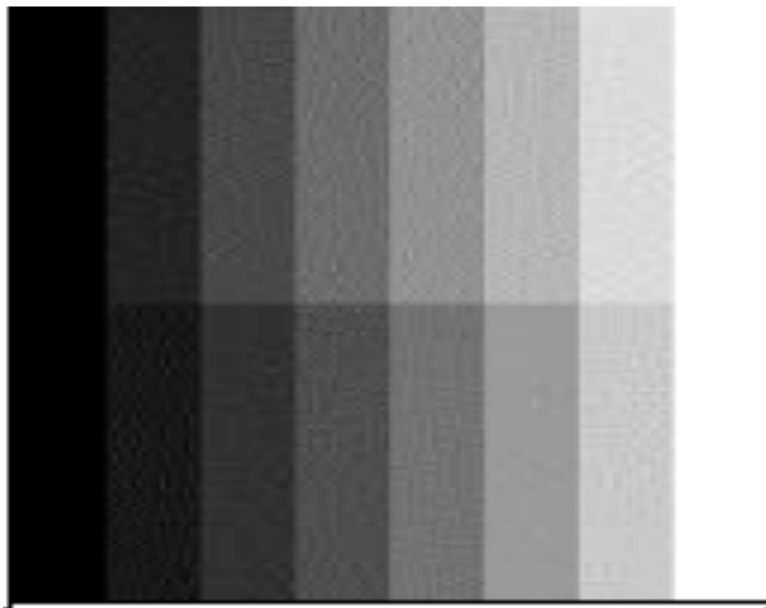
Hard to see by brightfield.

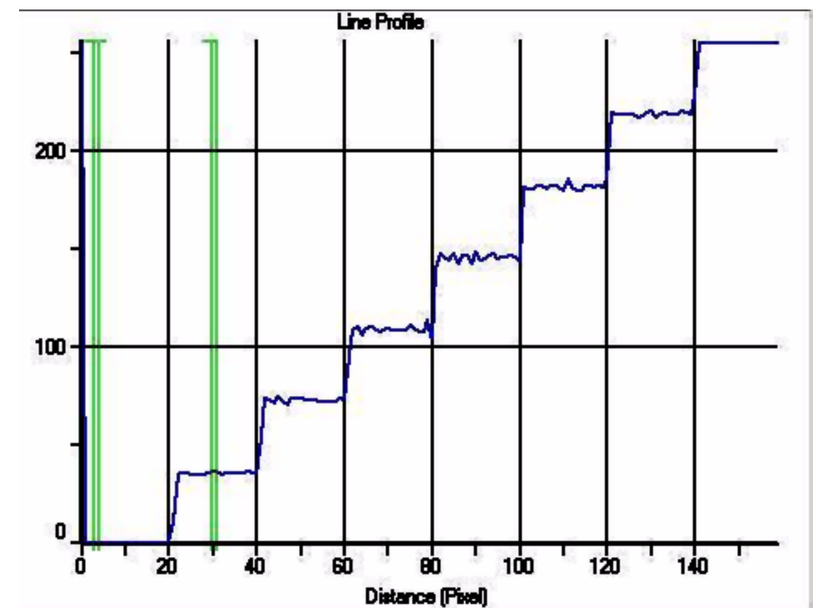
Solution-- transmitted light-based techniques for improving contrast (Phase, Darkfield, Polarization, DIC)

CONTRAST

Contrast describes our ability to distinguish two objects (or an object and its background) based on their relative intensities.

$$(I_1 - I_2)/(I_1 + I_2)$$





GENERATION OF CONTRAST

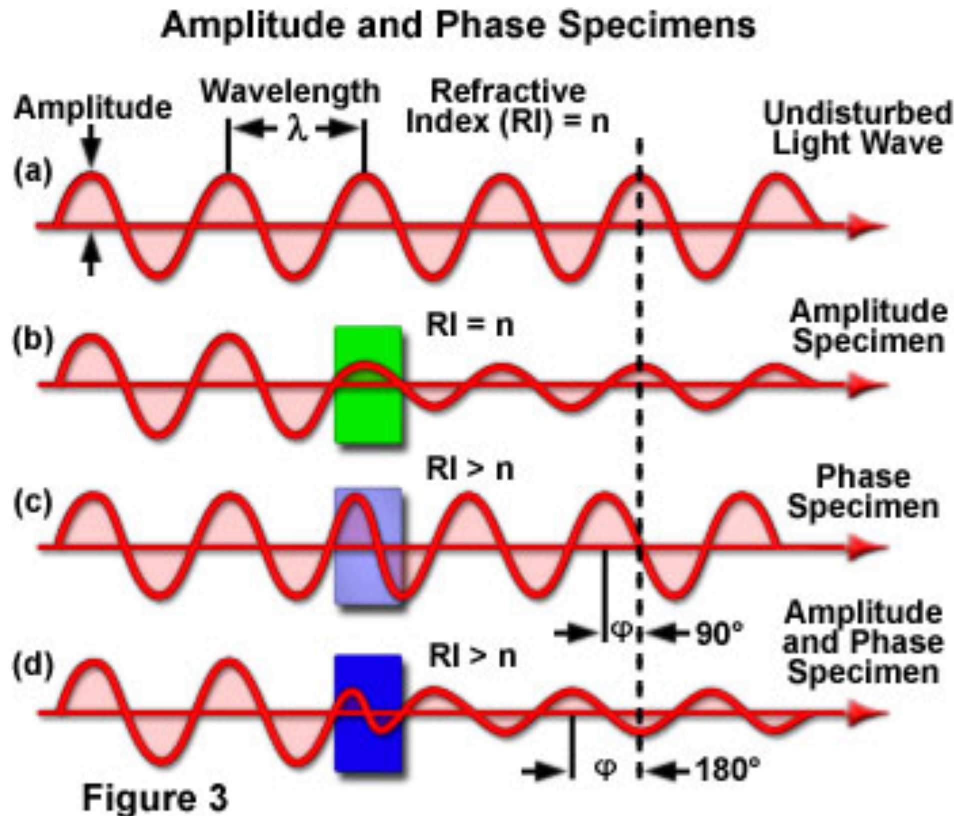
IMPORTANT CONCEPT

Microscopists look at thin objects which do not absorb much light. Therefore, they must find ways to increase constrast.

- Stains
- Darkfield
- Rheinberg illumination
- Phase contrast microscopy
- DIC (Nomarski)

Absorption is not the only way samples interact with light.

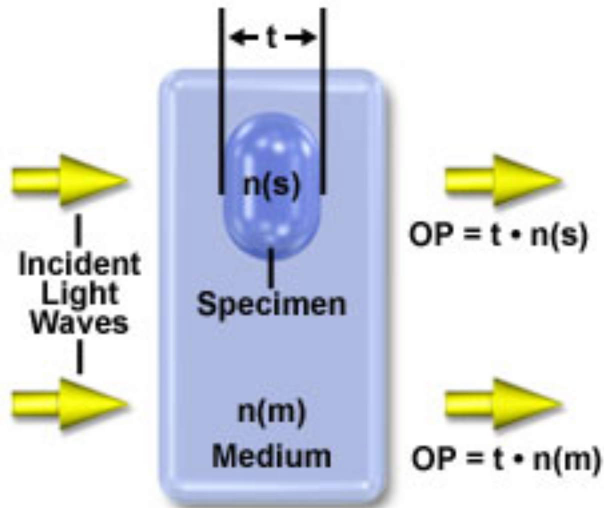
(polarization, phase shift)



Your eyes are good at seeing differences in amplitude (intensity) and wavelength (color), but not phase or polarization

Phase and DIC microscopy convert differences in phase to differences in amplitude

Samples of different refractive index change optical path length



t = sample thickness. Typical cell in monolayer = 5 microns

n(s) = refractive index of sample. Most cells 1.36

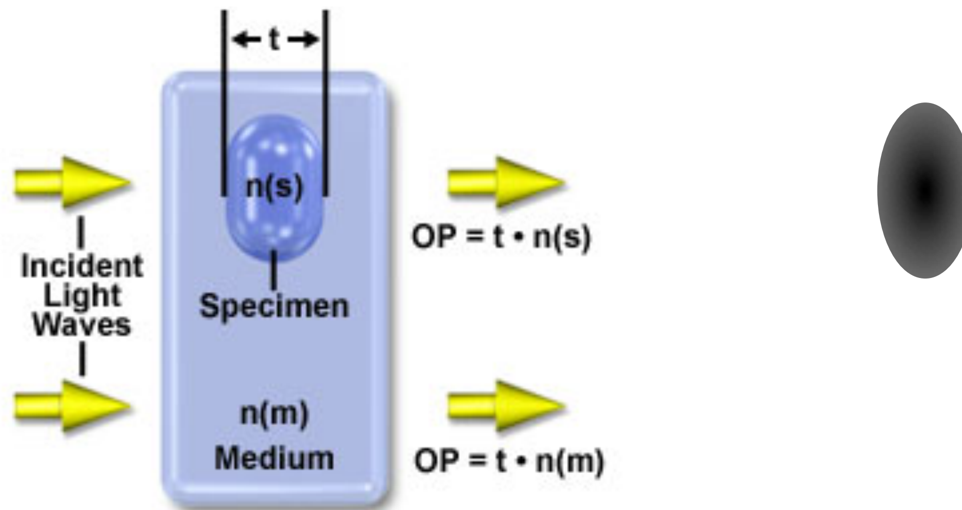
n(m) = refractive index of medium. Cell medium 1.335

Optical path difference = D = t $(n_s - n_m)$

= 5microns (1.36-1.335) = .125 microns= 125 nm,

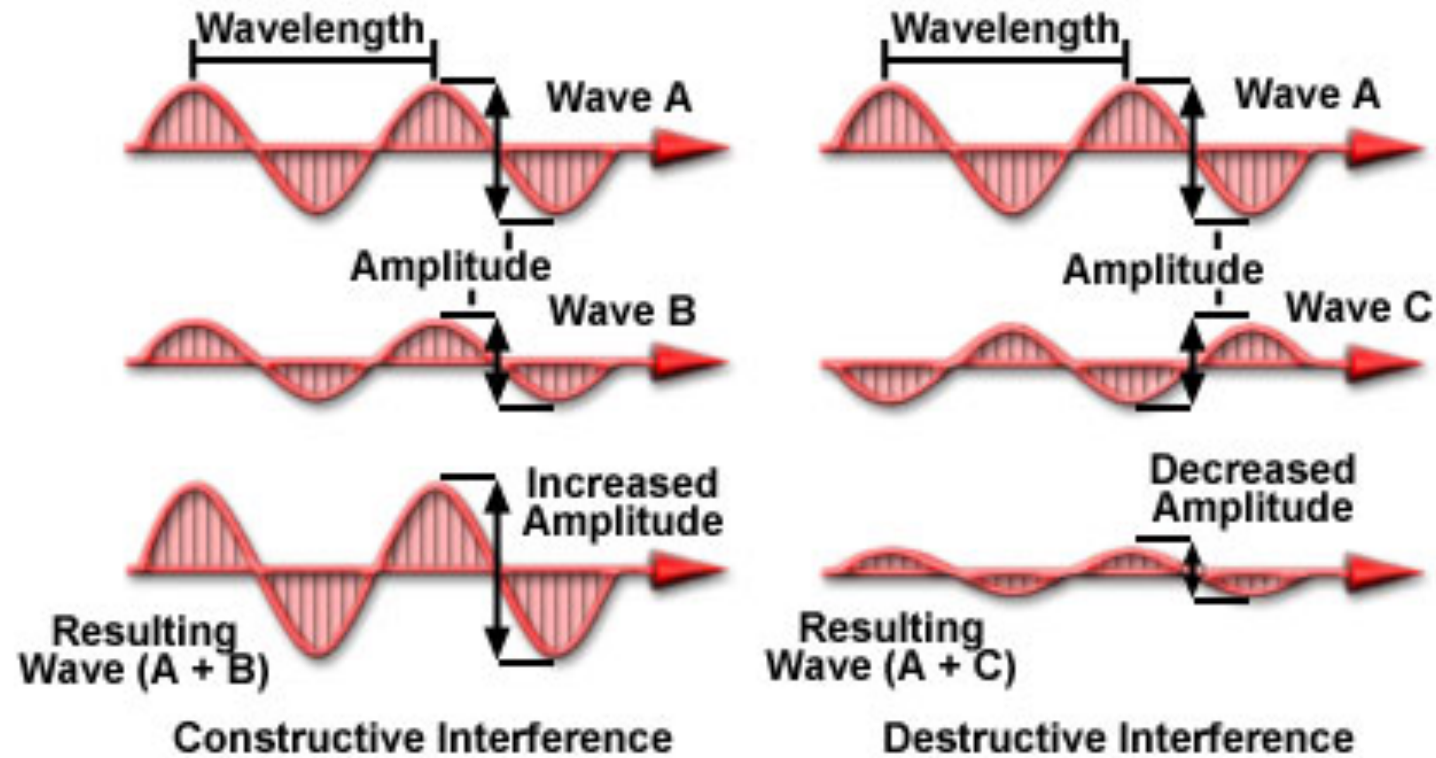
which is about 1/4 the wavelength of green light (488 nm)

What Phase Microscopy accomplishes

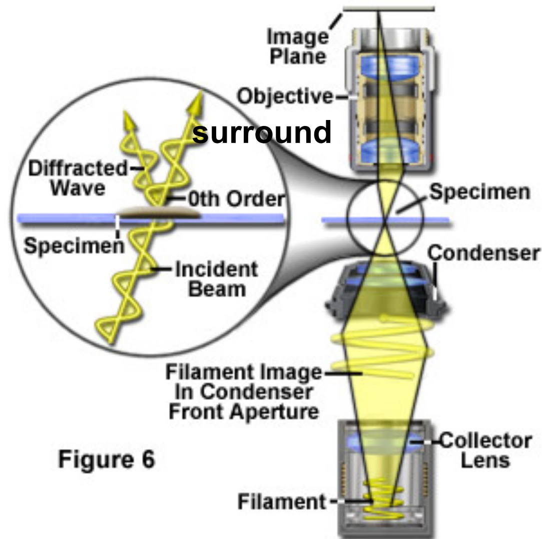


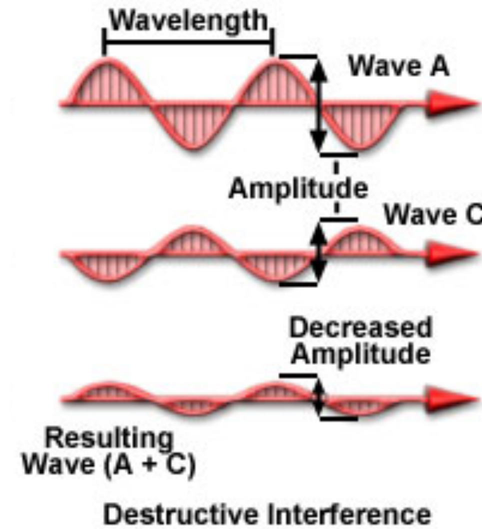
Converts differences in optical path length to differences in amplitude

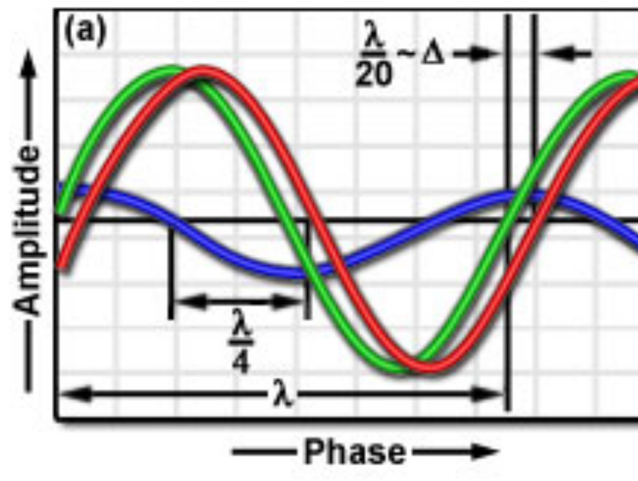
Review-- interference of light waves with same wavelength



Forming an image-- role of diffracted light

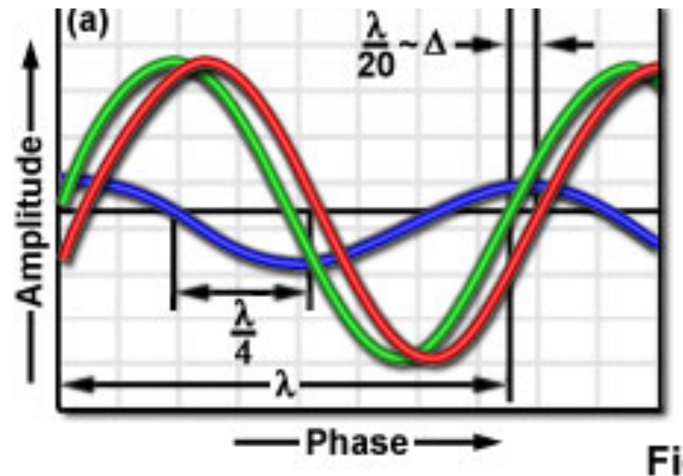






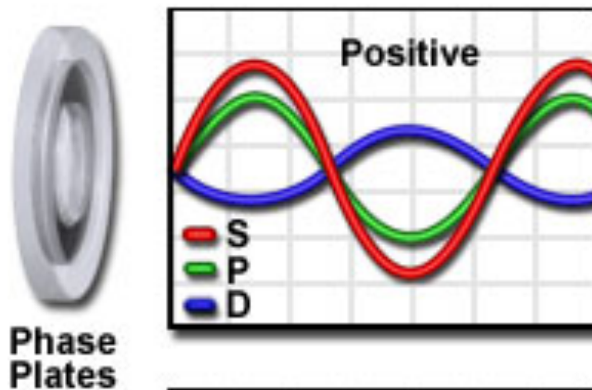
S= surround (undiffracted)
D= diffracted wave
P= particle wave (S+D)

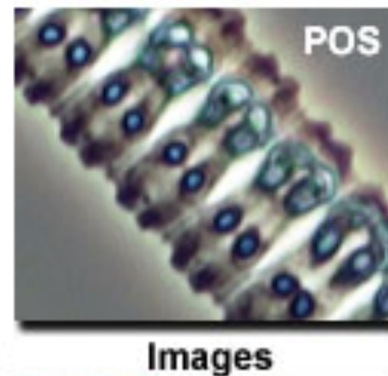
Because amplitude of surround and particle waves are almost identical, sample lacks contrast.



S= surround (undiffracted)
D= diffracted wave
P= particle wave (S+D)

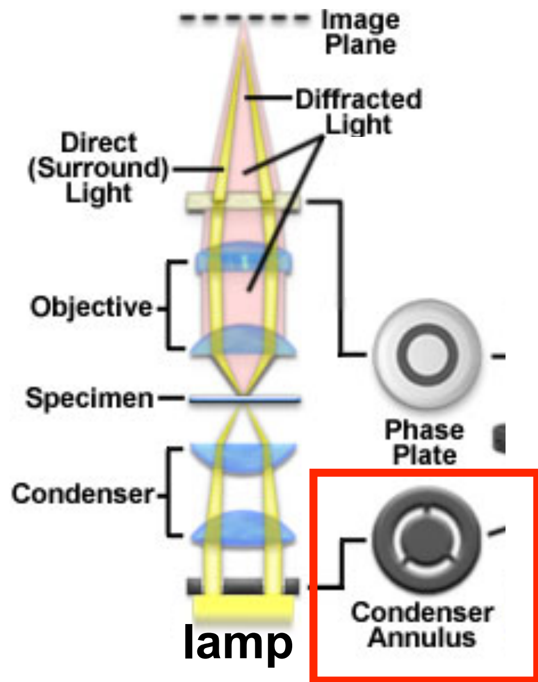
We would rather have D closer to S in amplitude
and phase shift to be γ/2 (vs γ/4) for max interference and contrast





Need way of independently controlling amplitude and phase of S + D.

Phase Contrast Microscope Configuration



Restricts angles of illumination so diffracted and undiffracted light can be selectively modulated at phase plate

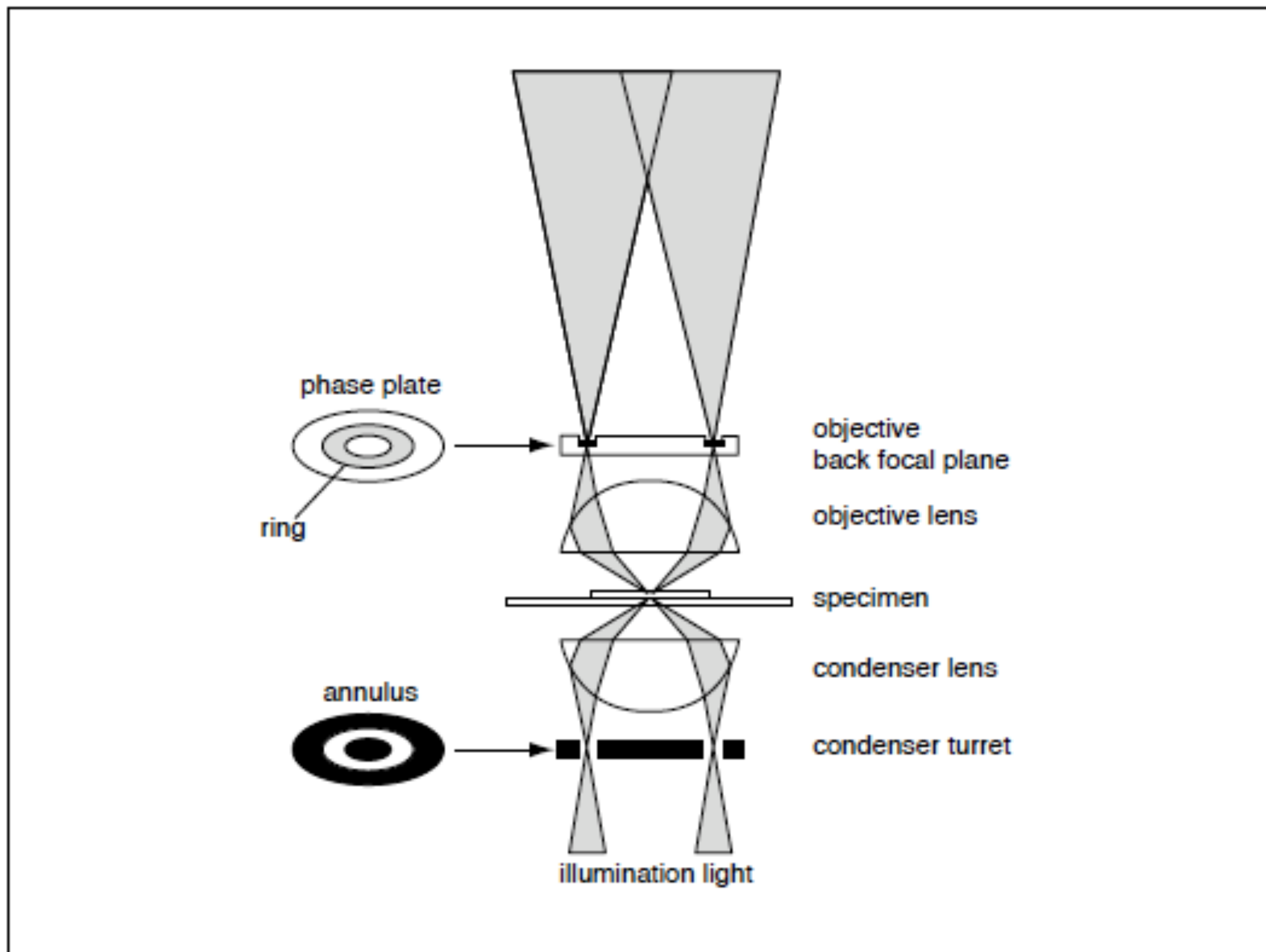
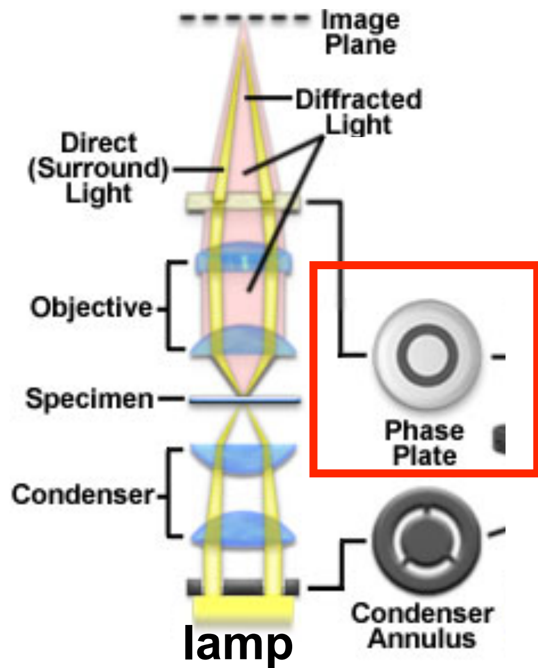
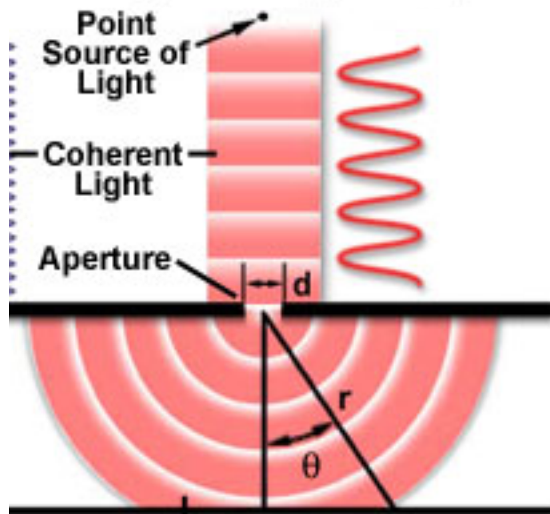


Figure 4.1.5 Illumination light path through the condenser annulus and objective phase ring in a microscope aligned for phase-contrast microscopy.



Selectively attenuates (70-90% decrease) and phase advances(1/4 wavelength) undiffracted light passing through the sample



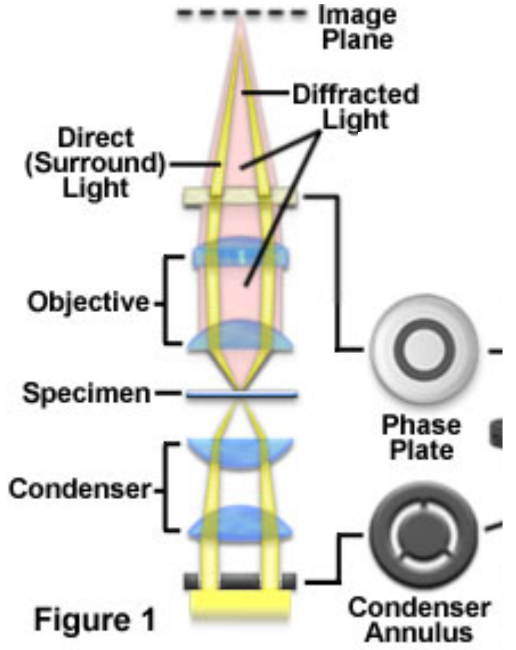


Figure 1

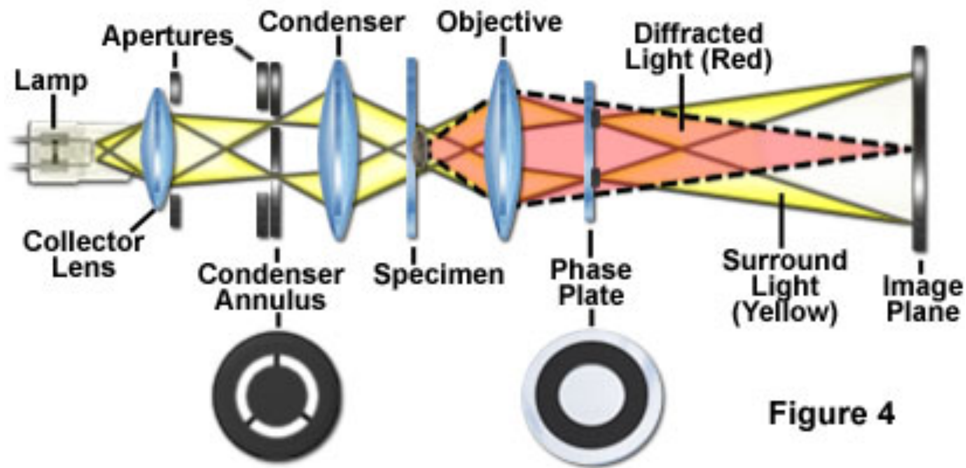
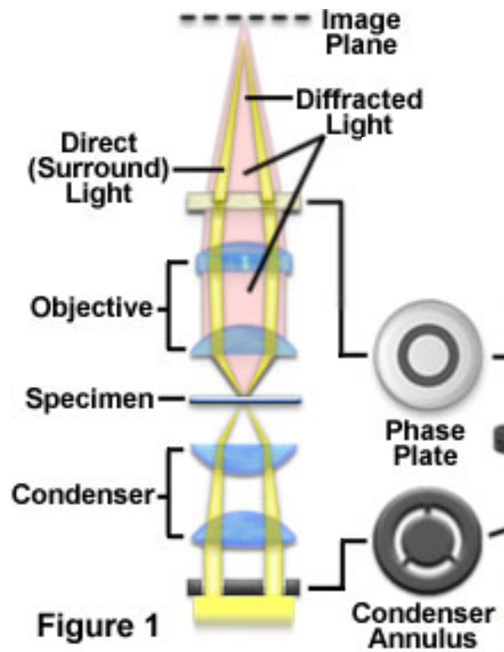
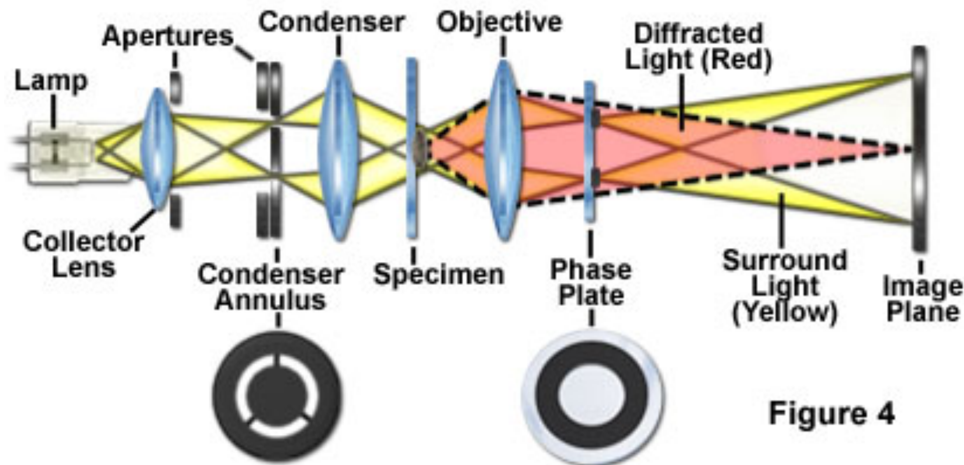


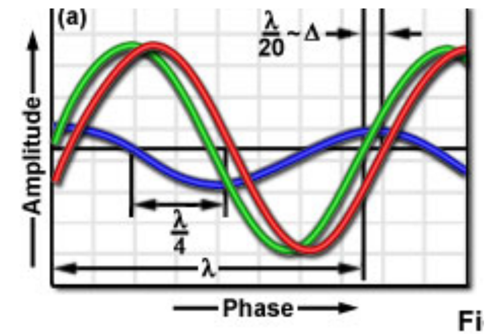
Figure 4



Phase Contrast Microscope Configuration
Image Plane
Diffracted Light
Direct (Surround) Light
Objective
Specimen
Condenser
Phase Plate
Condenser Annulus
Figure 1

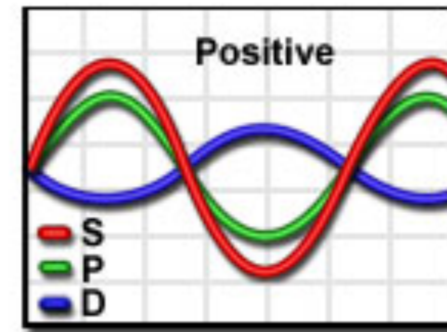


Phase Contrast Microscope Optical Train
Lamp
Apertures
Condenser
Objective
Diffracted Light (Red)
Collector Lens
Condenser Annulus
Specimen
Phase Plate
Surround Light (Yellow)
Image Plane
Figure 4



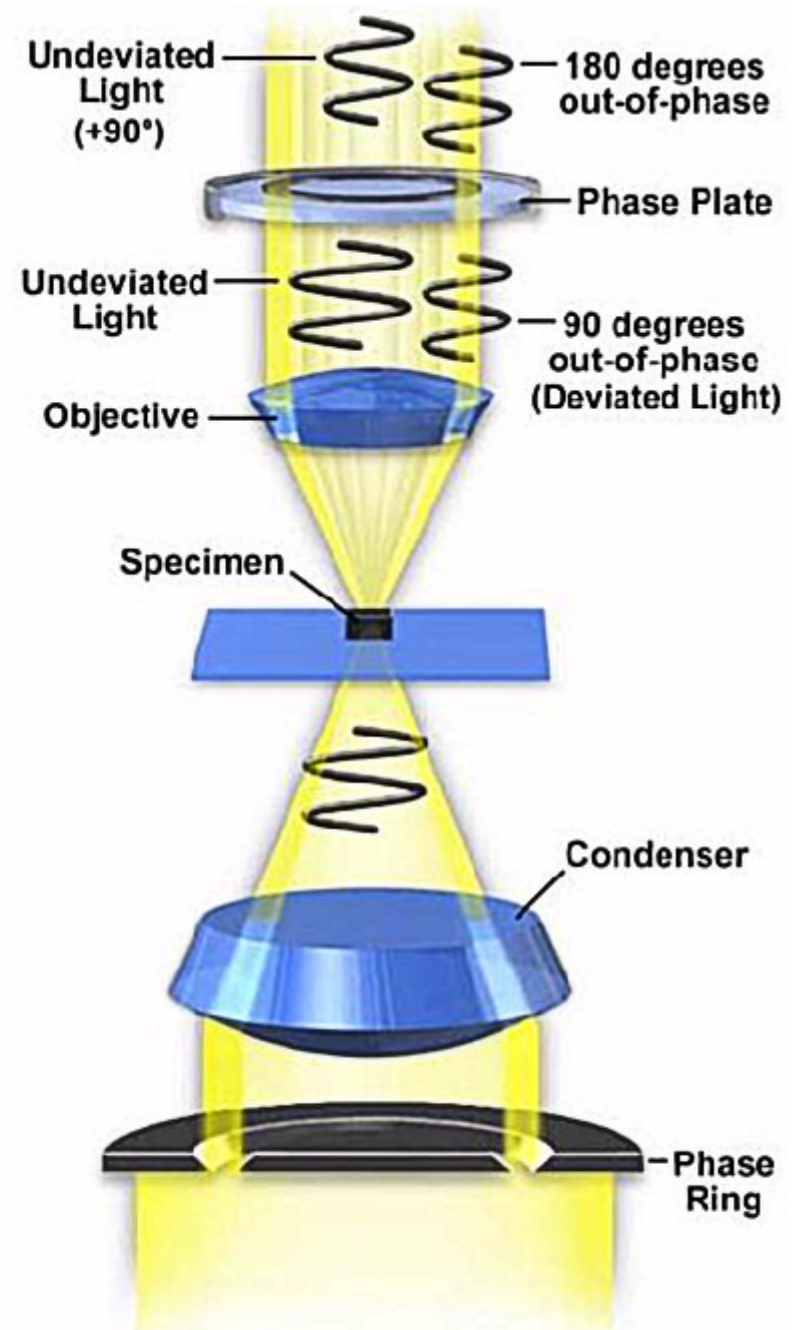
(a)
Amplitude
Phase
λ/20 ~ Δ
λ/4
λ
Fi

Phase Plates



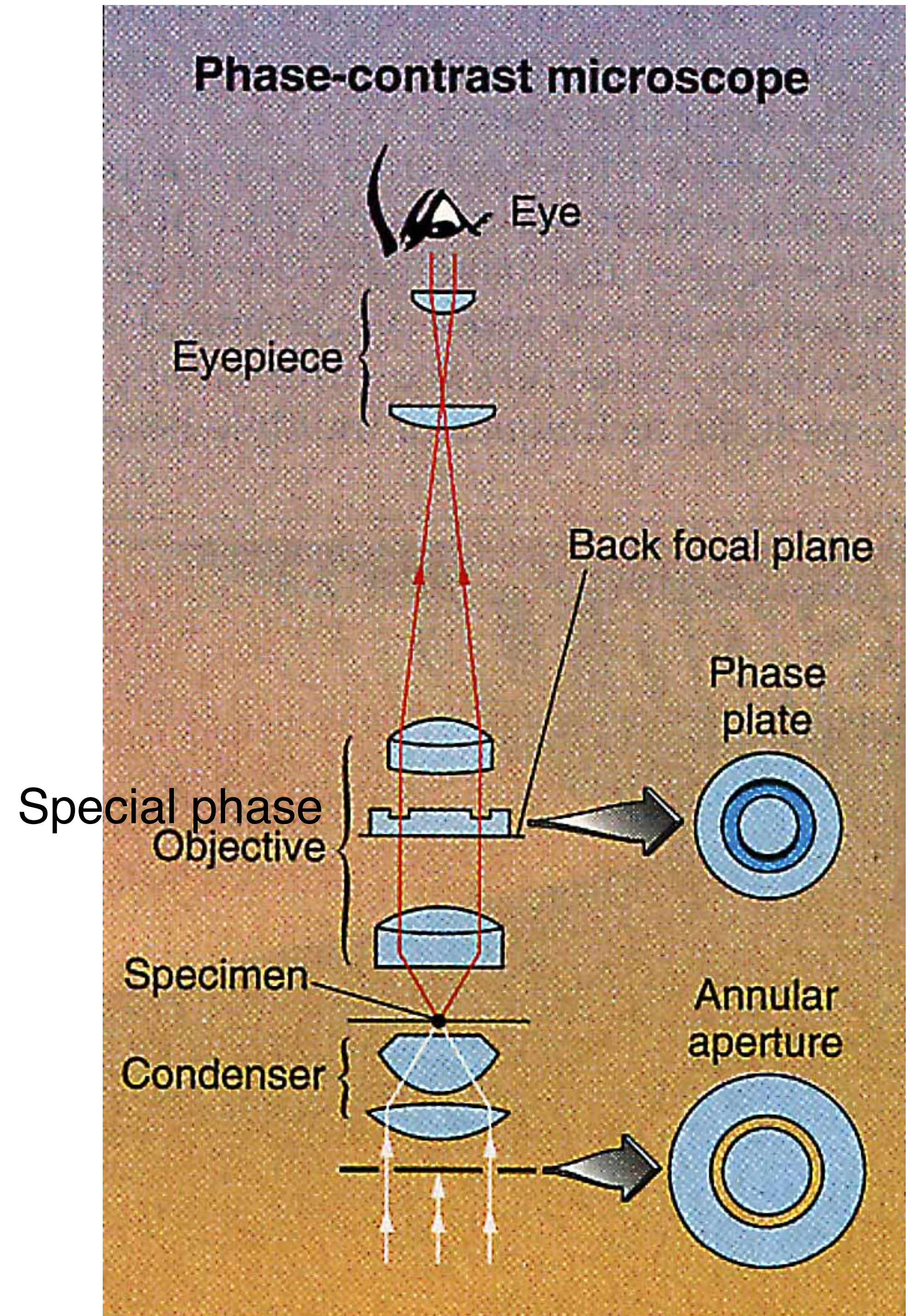
Positive
S
P
D

PHASE CONTRAST MICROSCOPY



Phase contrast:

Interference between
1. directly transmitted rays attenuated and phase-shifted in in the phase plate
and
2. rays diffracted (scattered) by refractive index variations in the sample



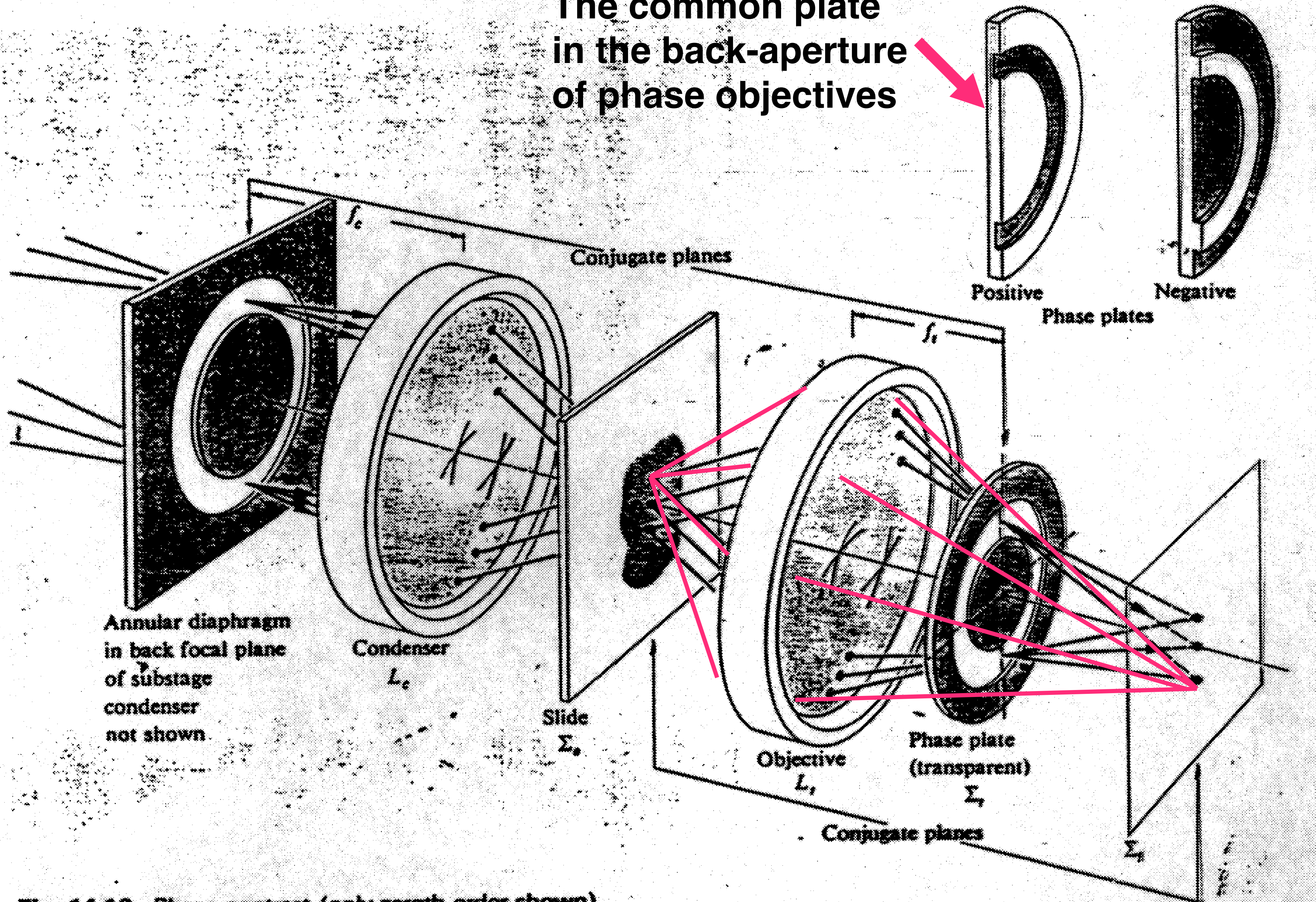
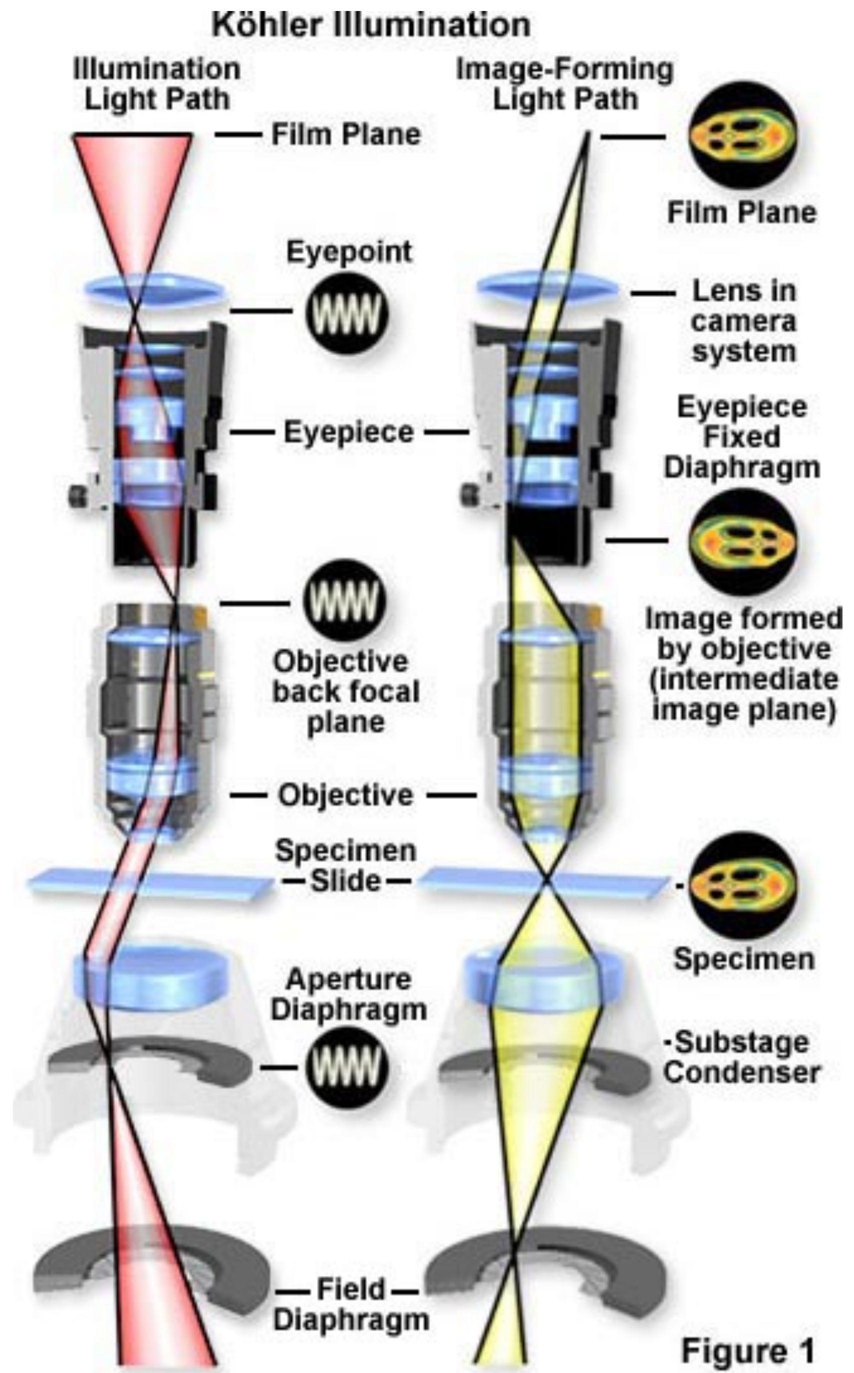


Fig. 14.19 Phase contrast (only zeroth order shown).



Review-- conjugate image planes in microscope

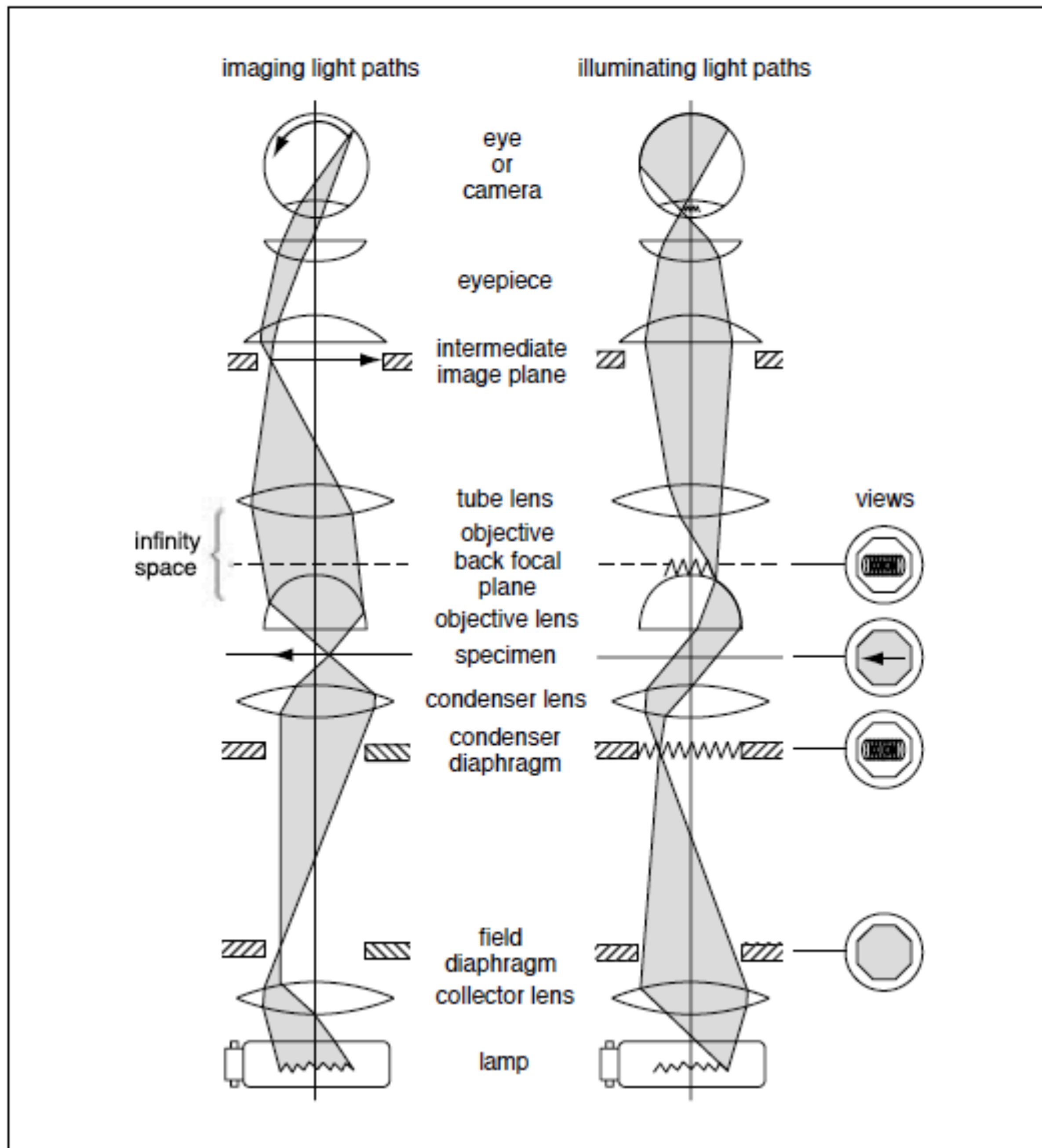


Figure 21.1.2 The imaging and illuminating light paths for a bright-field microscope aligned for transmitted light Köhler illumination. Modified from Keller (1998).

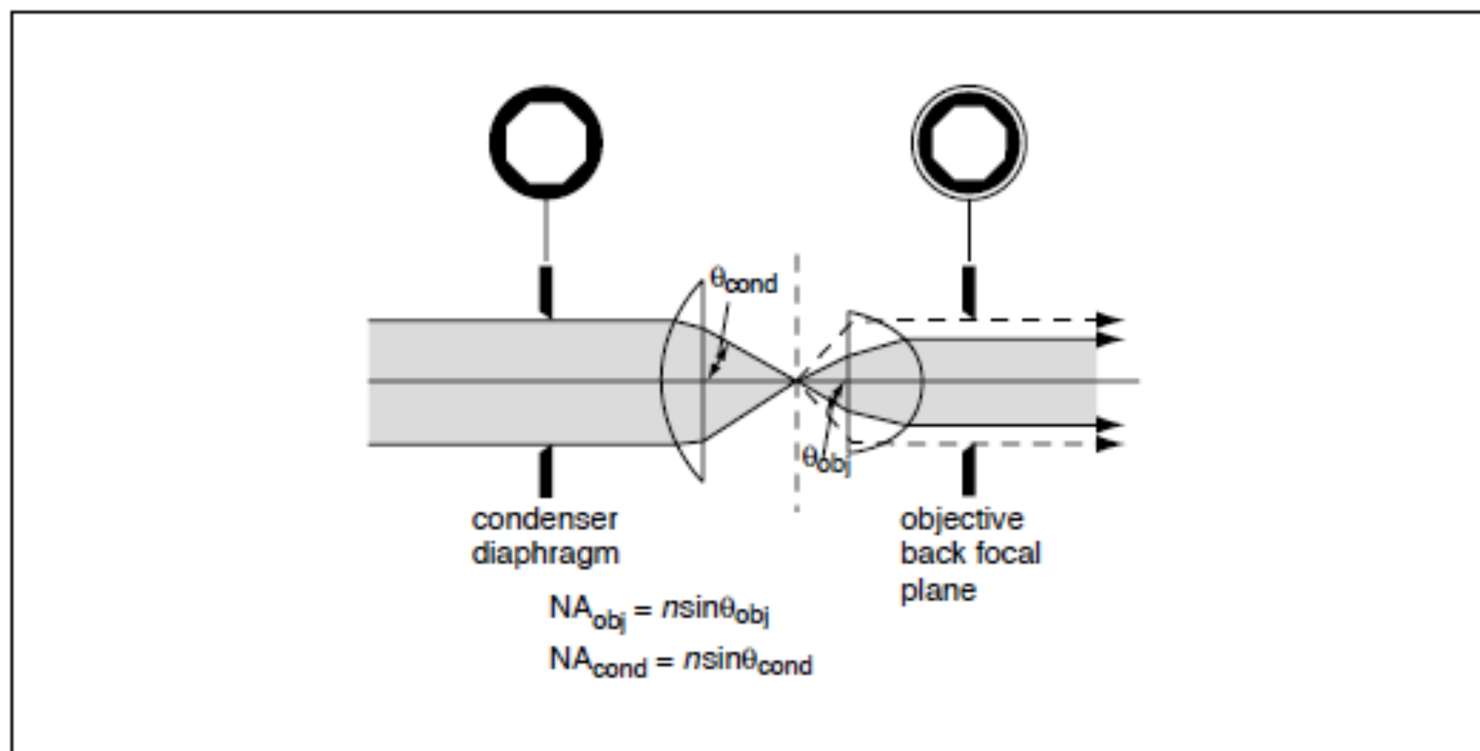


Figure 21.1.3 Numerical aperture (NA) of objective light collection and condenser illumination. The objective numerical aperture (NA_{obj}) depends on the angle of the cone of light from the specimen, which is accepted by the objective aperture while the numerical aperture of condenser illumination (NA_{cond}) is controlled by the condenser diaphragm and limited by the maximum NA of the condenser when the condenser diaphragm is wide open.

PHASE CONTRAST ALLIGNMENT

Phase Plate and Light Annulus Alignment

(a)

(b)

(c)

Proper alignment of condenser annulus and phase plate are essential for phase microscopy

(separates surround and diffracted light)

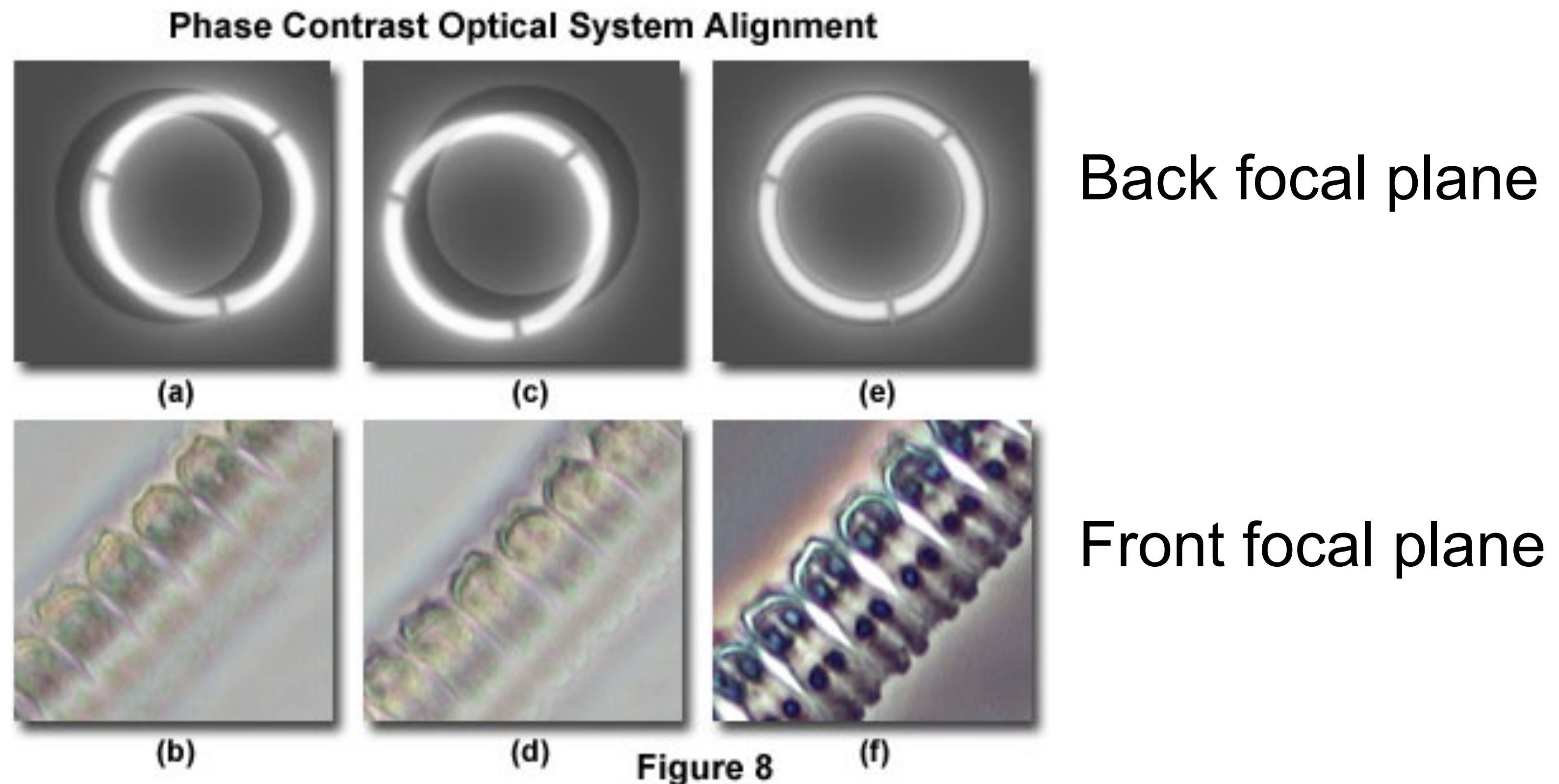


Figure 8

Limitations of Phase Contrast

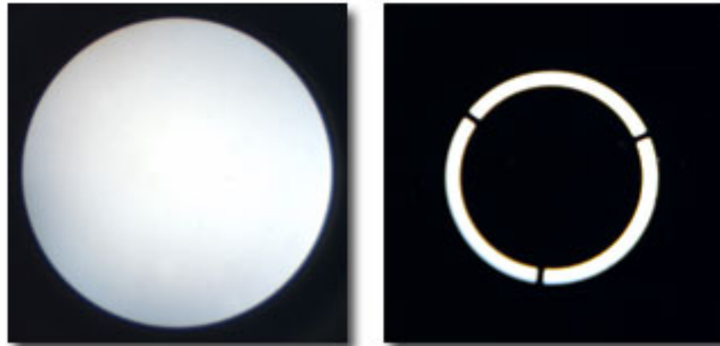
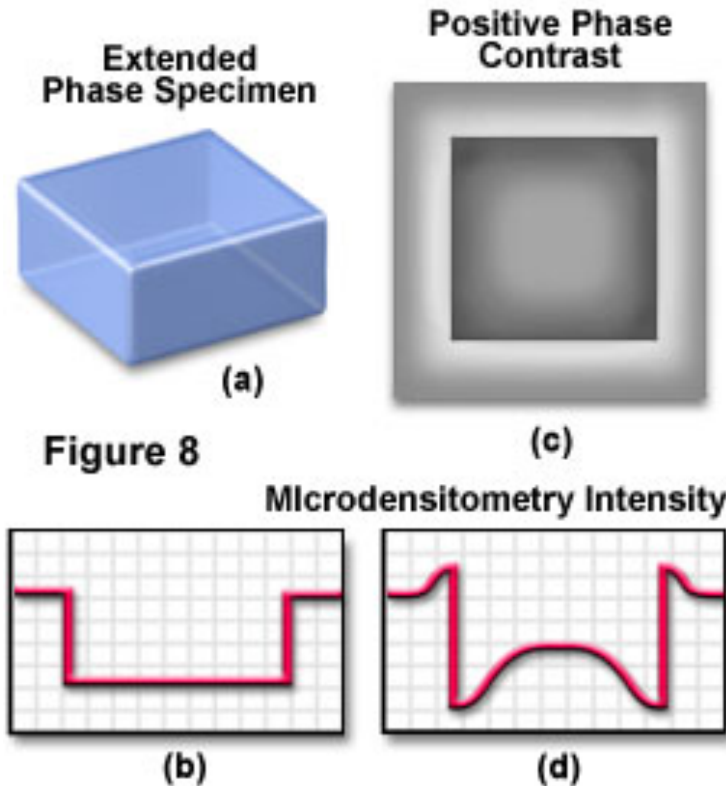


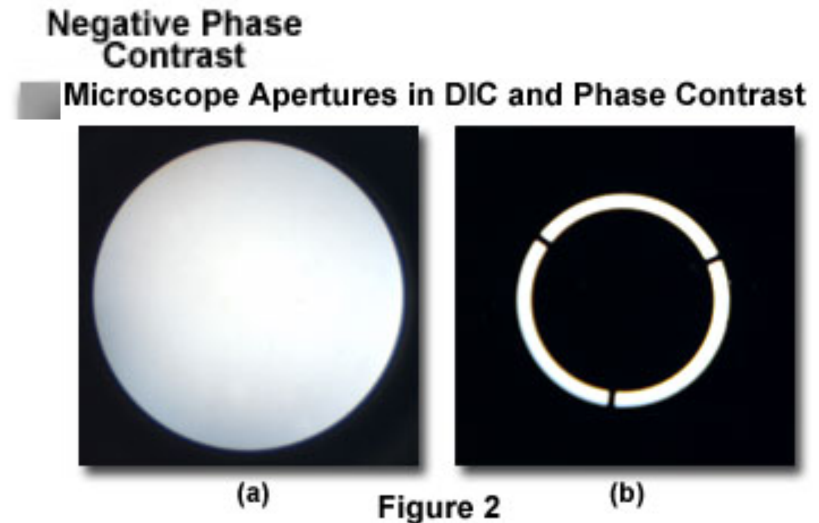
Figure 2

Poor for thick samples for two reasons

1. Poor lateral (z) resolution due to limited aperture
2. Sufficiently thick samples can shift light more than 1 wavelength (so thin and thick sections can have similar brightness for biological samples thicker than about 10 microns)

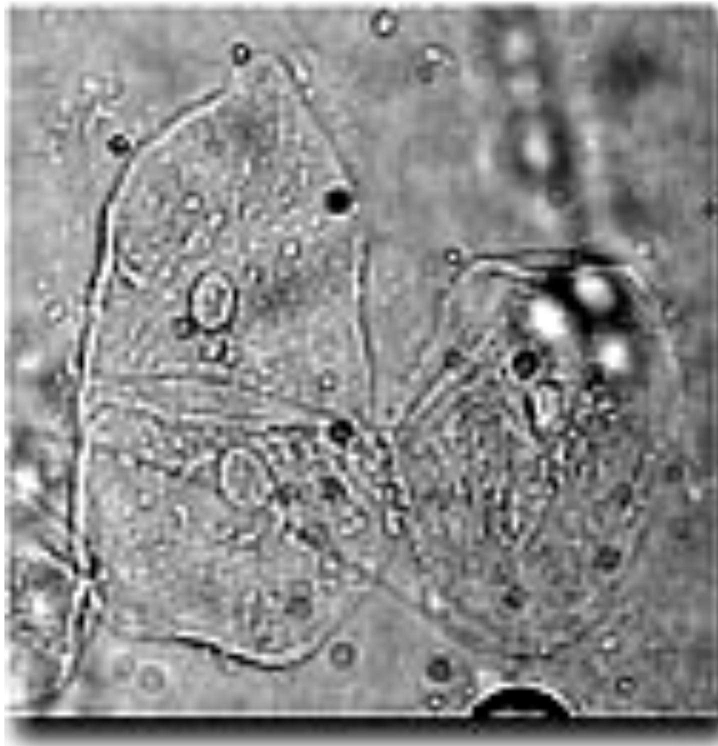
Limitations of Phase Contrast





Halos -- some diffracted light (esp low spatial frequency and center of objects) also captured by phase plate, leading to localized contrast reversal. Can limit resolution.

PHASE CONTRAST IMAGE



APODIZED PHASE CONTRAST

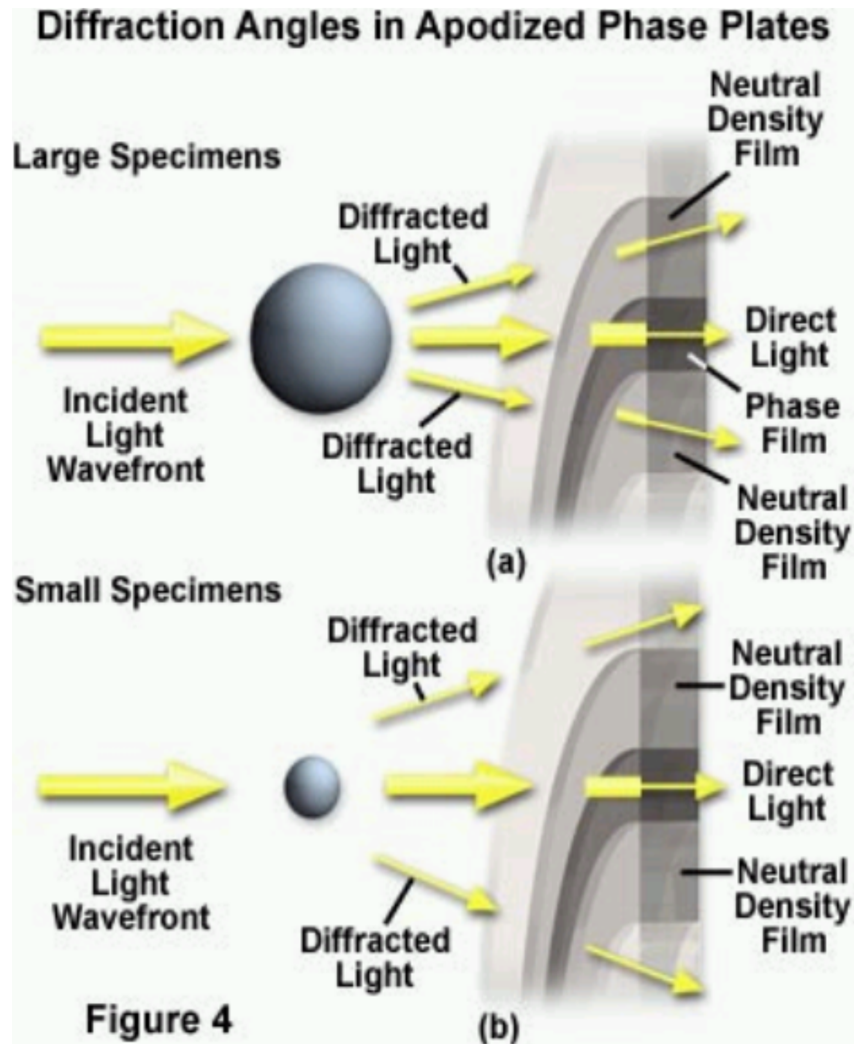


Figure 4

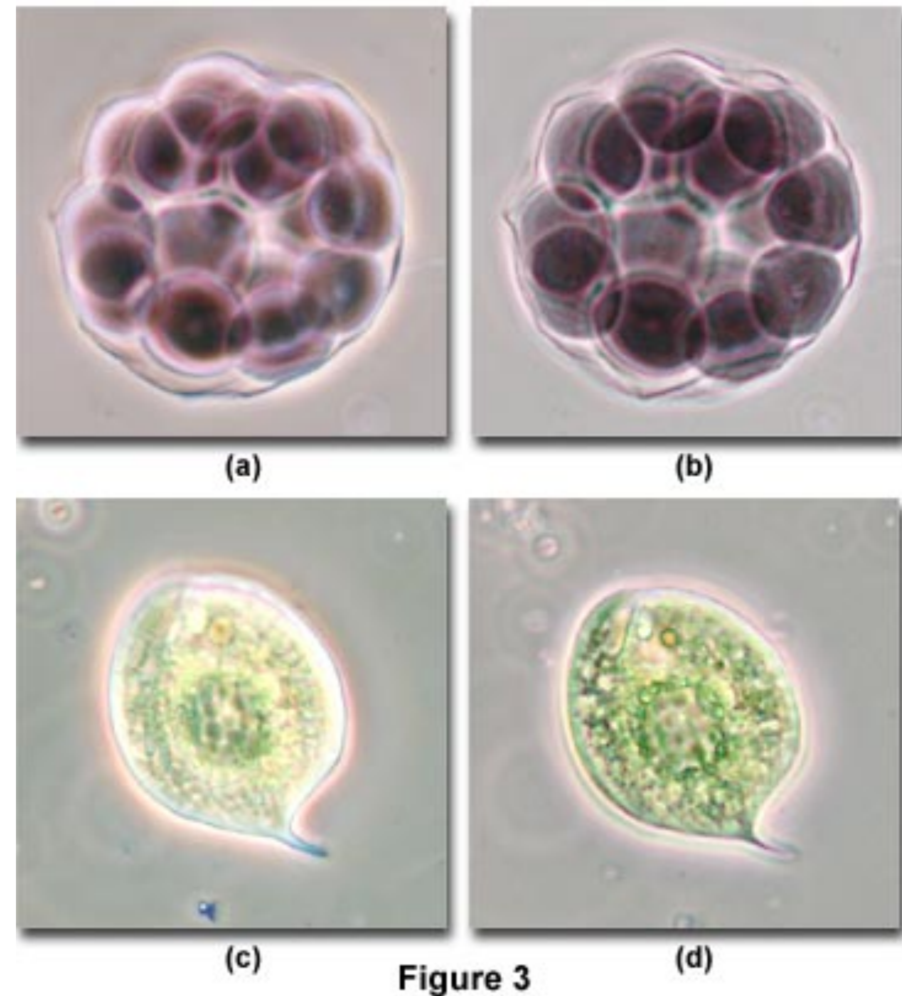
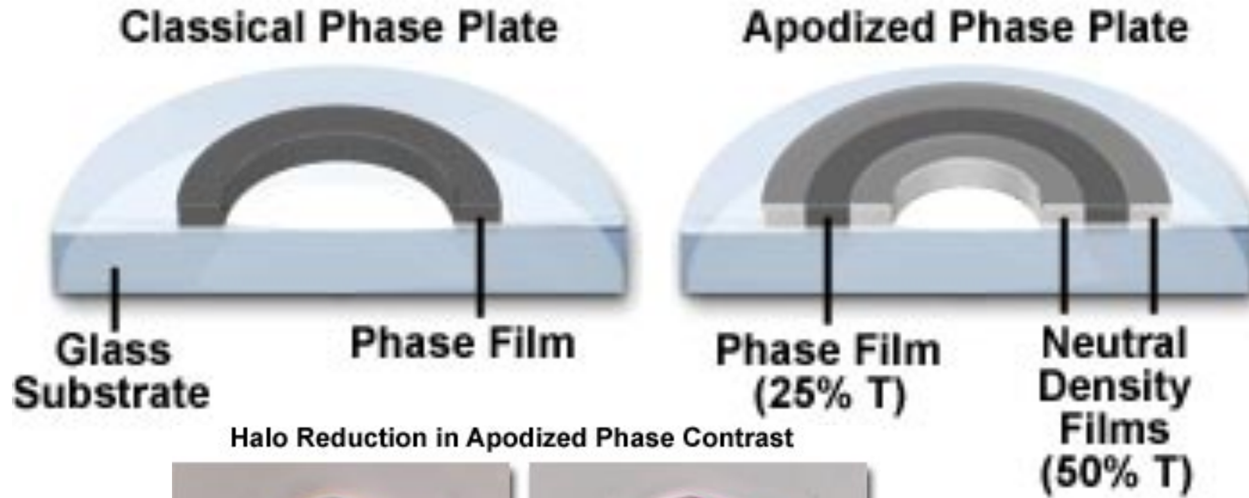
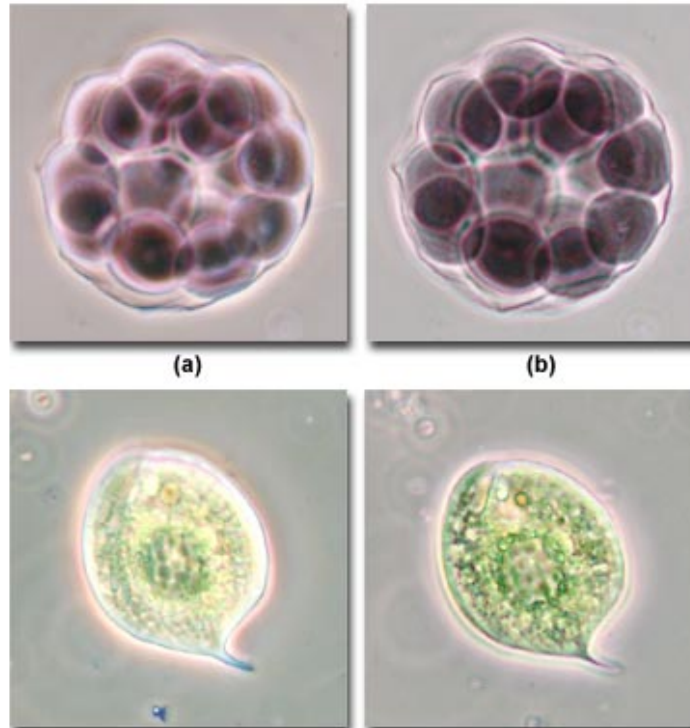


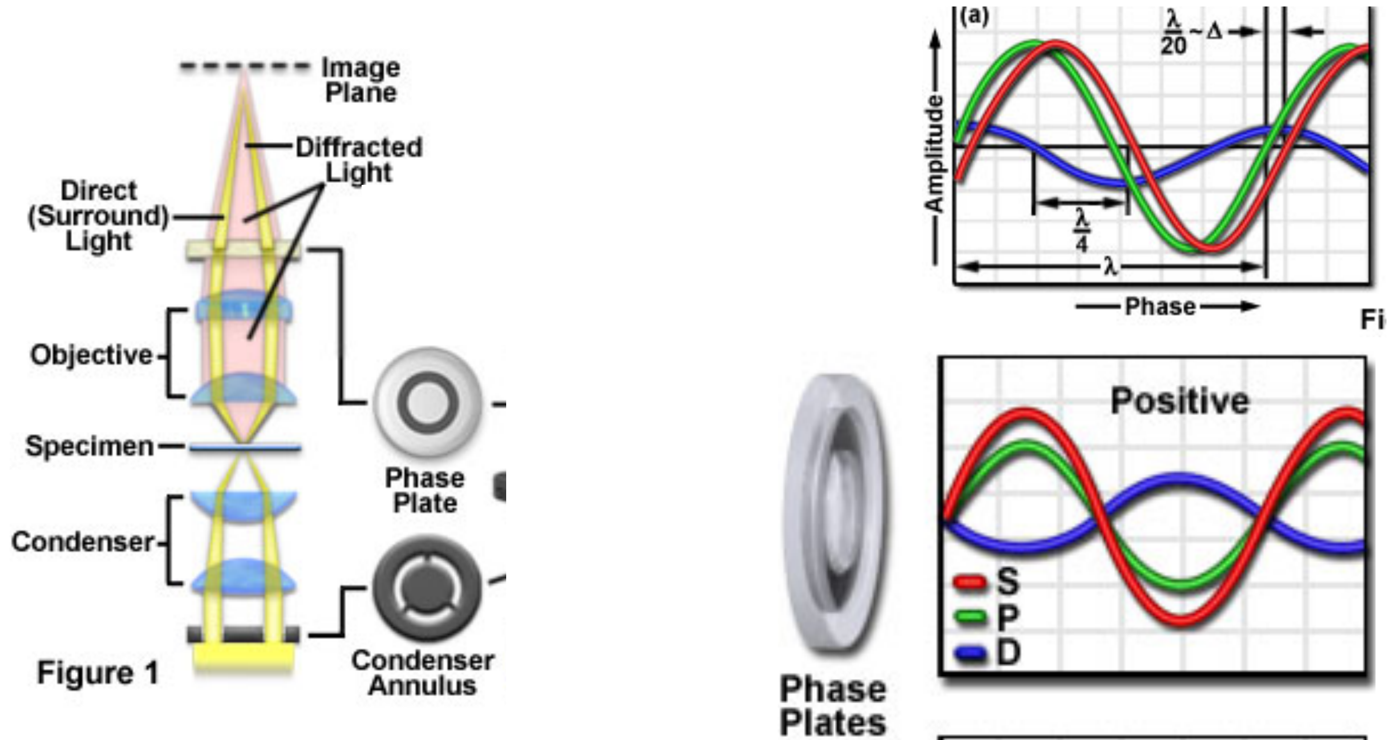
Figure 3

Halos in phase contrast can be decreased by apodization





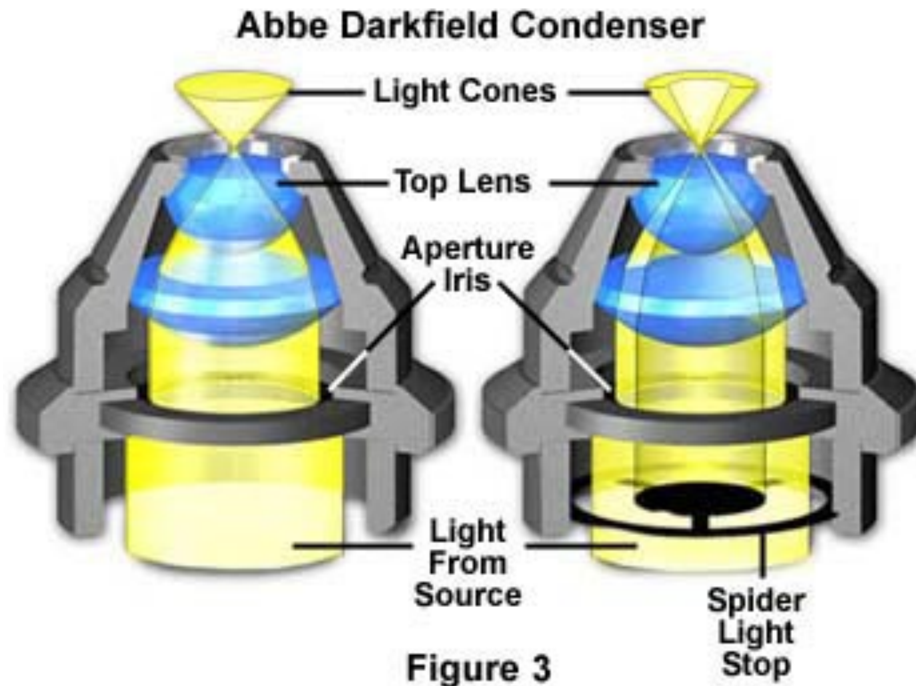
Review of Phase

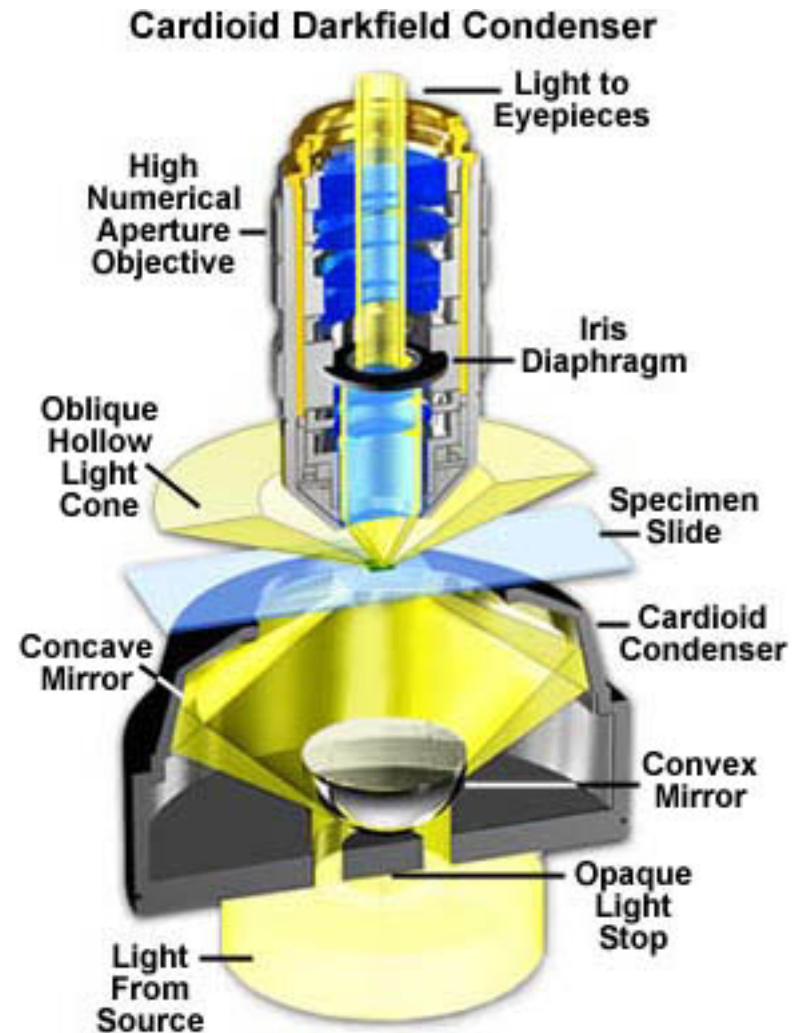


What if we were to increase contrast further by throwing away all non-diffracted light?

Darkfield microscopy

Darkfield images only diffracted light





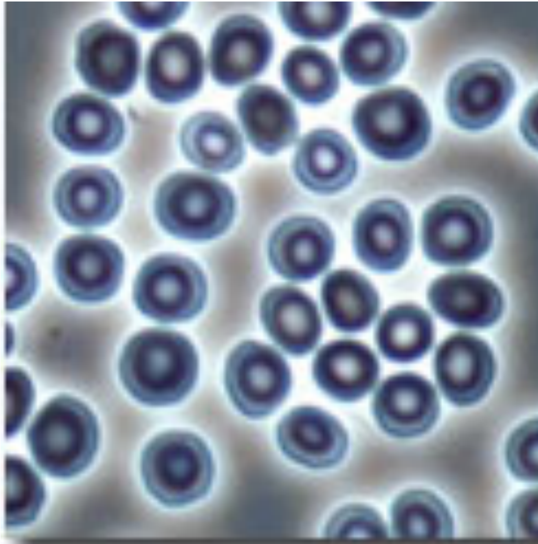
Similar to phase, projects

Cone of light onto specimen, but

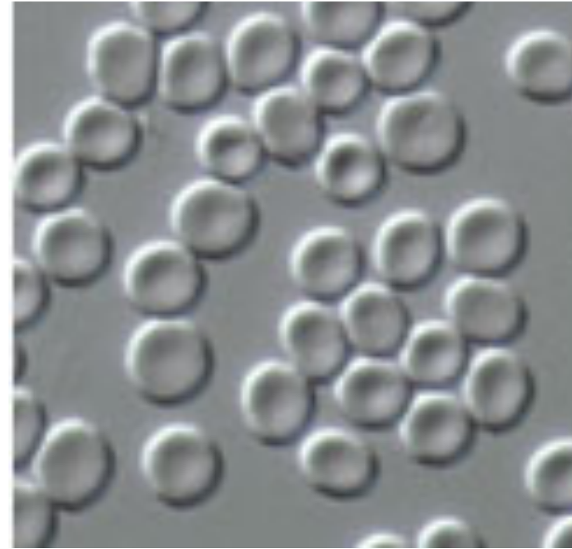
With higher NA than objective, so no

surround light enters objective

DIC: an alternative technique for enhancing contrast



Phase

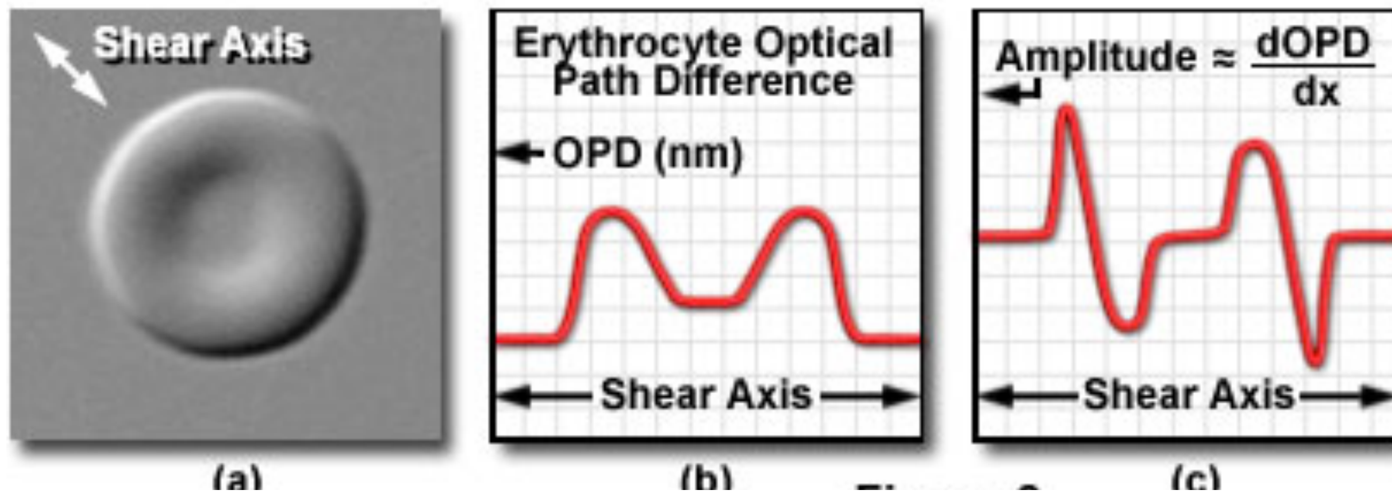


DIC (Differential Interference Contrast)

Also relies on phase shifts, but uses **differences in** optical path differences (vs absolute optical path for phase contrast)

Uses light polarization, dual beam interferometry

What DIC accomplishes



Converts relative differences in optical path length to differences in amplitude

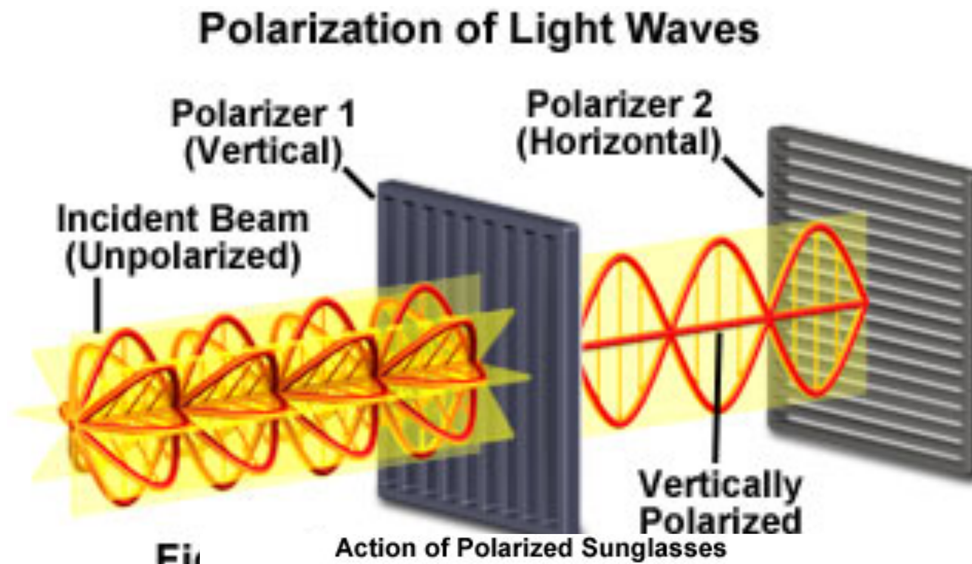
Features of a DIC image

1. Contrast is directional

2. Contrast highlights edges

3. One end brighter, other is dimmer than background leading to pseudoshadowed, almost 3d image

Review of light polarity, polarizers



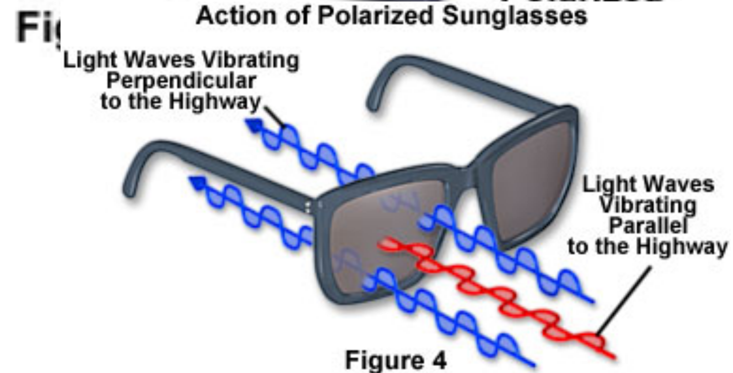


Figure 4

Birefringence

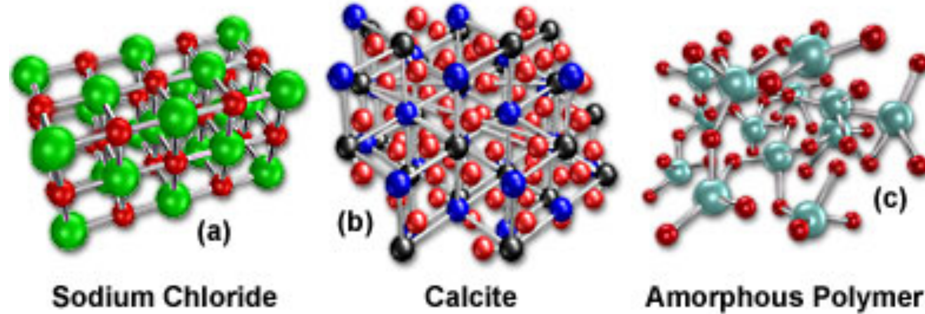
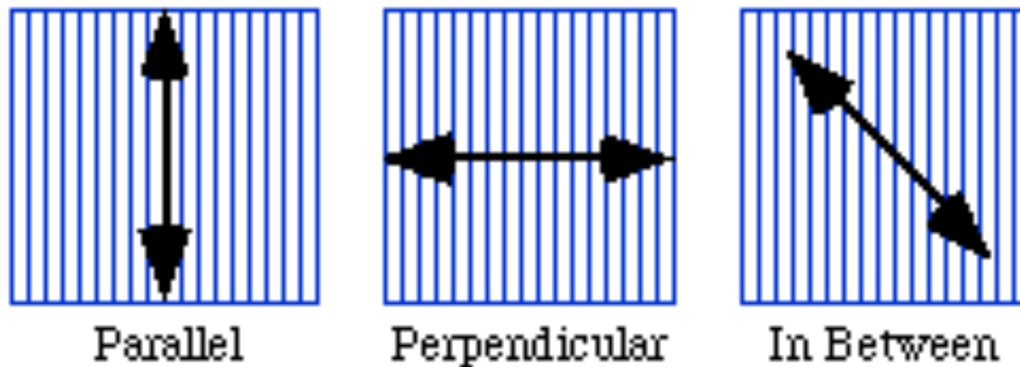
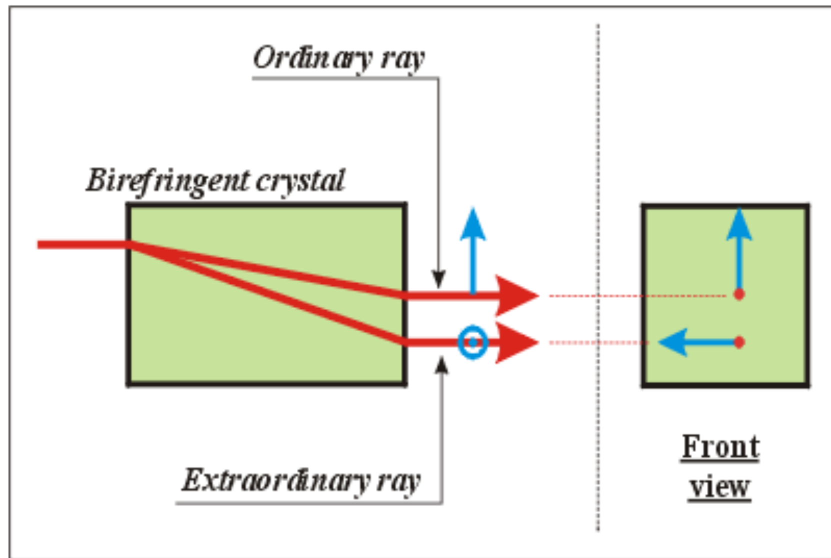


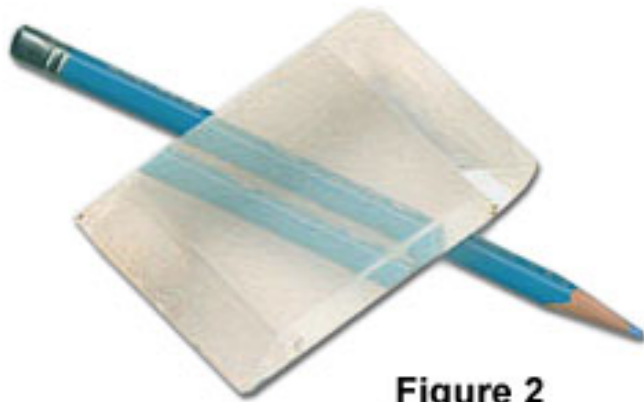
Figure 1

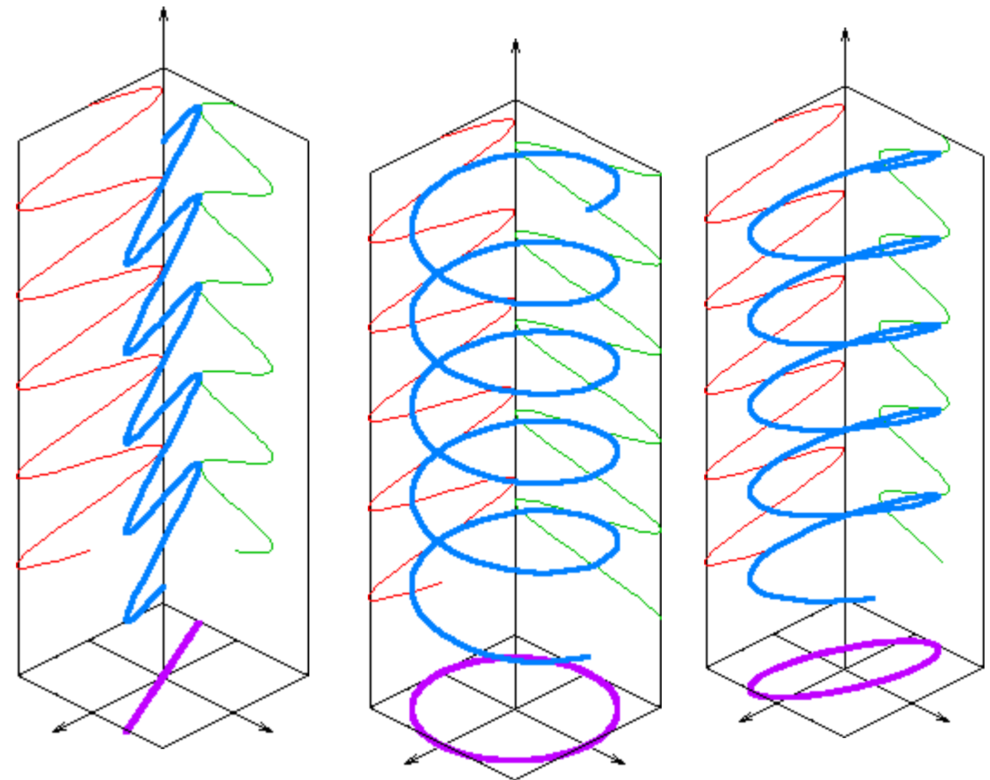
Birefringent materials have two indices of refraction (light travels through at different velocities depending on orientation) and can change polarization state of light.



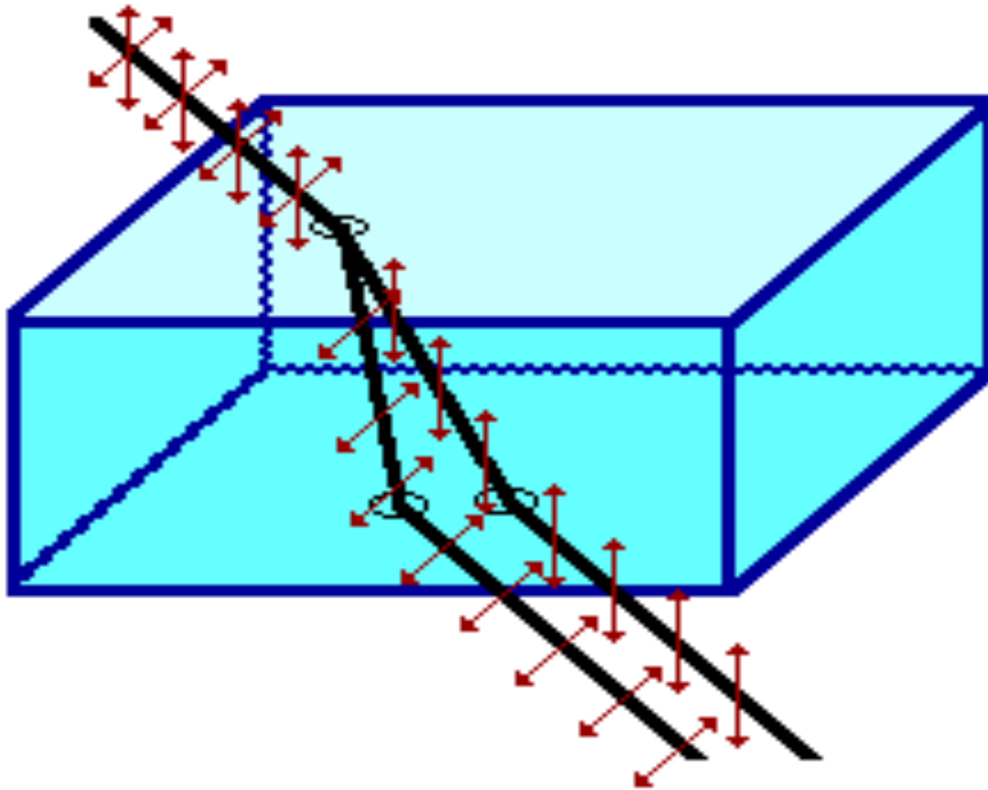
Consequences of birefringence on light polarity







BIREFRINGENCE



IMPORTANT CONCEPT

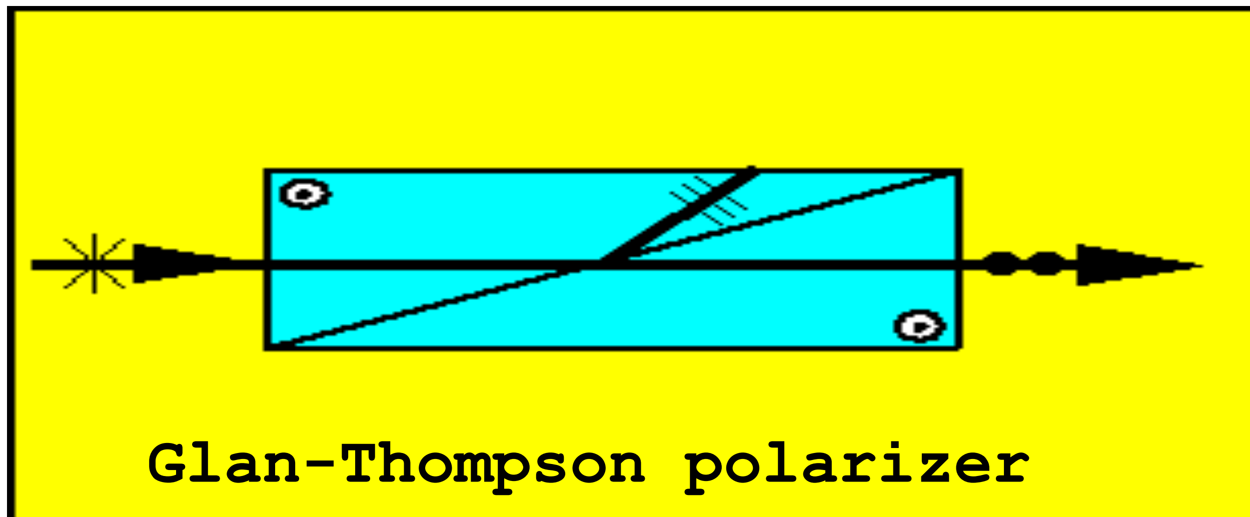
Index of Refraction (n)
may depend on Polarizatior

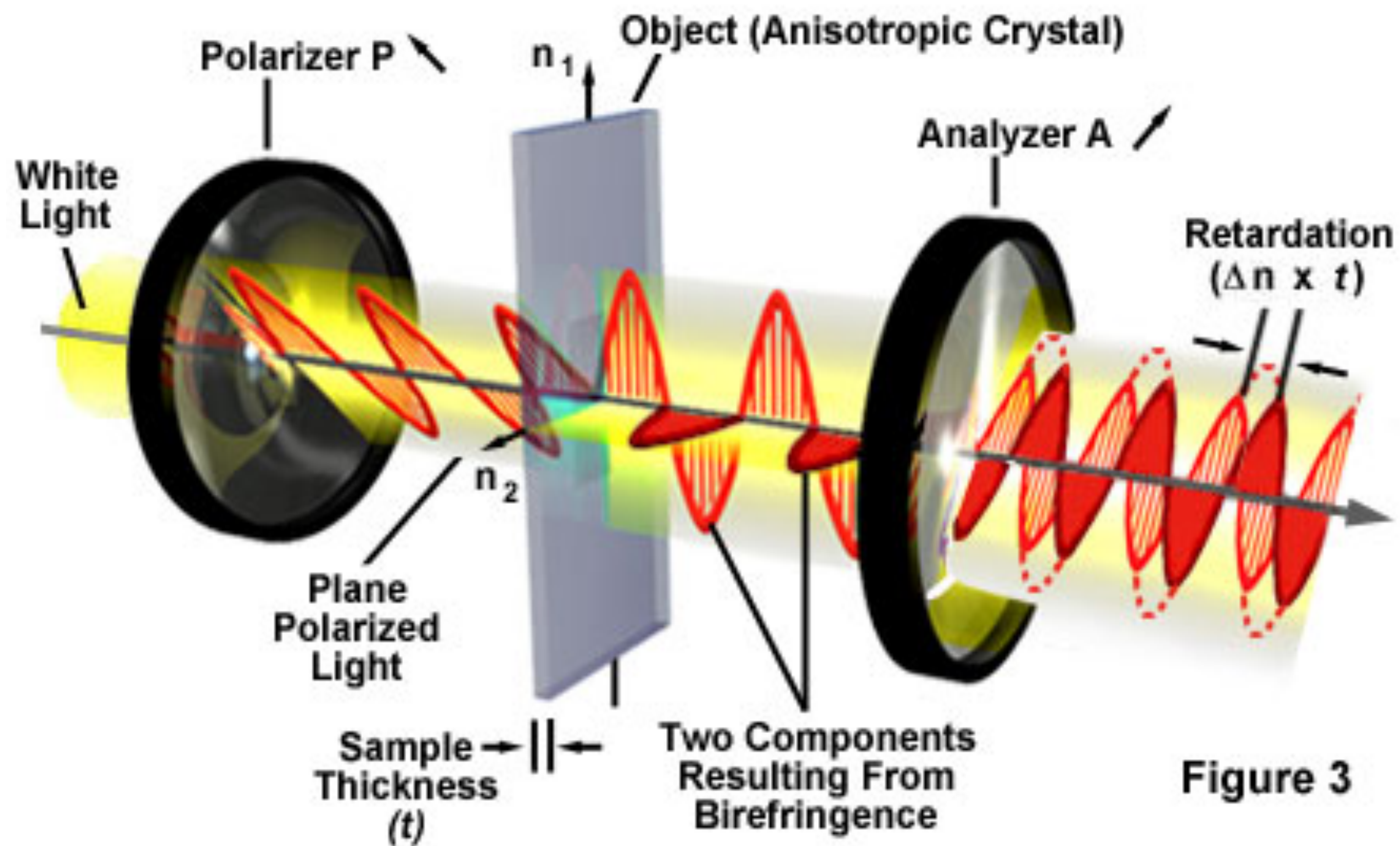
POLARIZERS

Relationship Between Long-Chain Molecule Orientation and the Orientation of the Polarization Axis

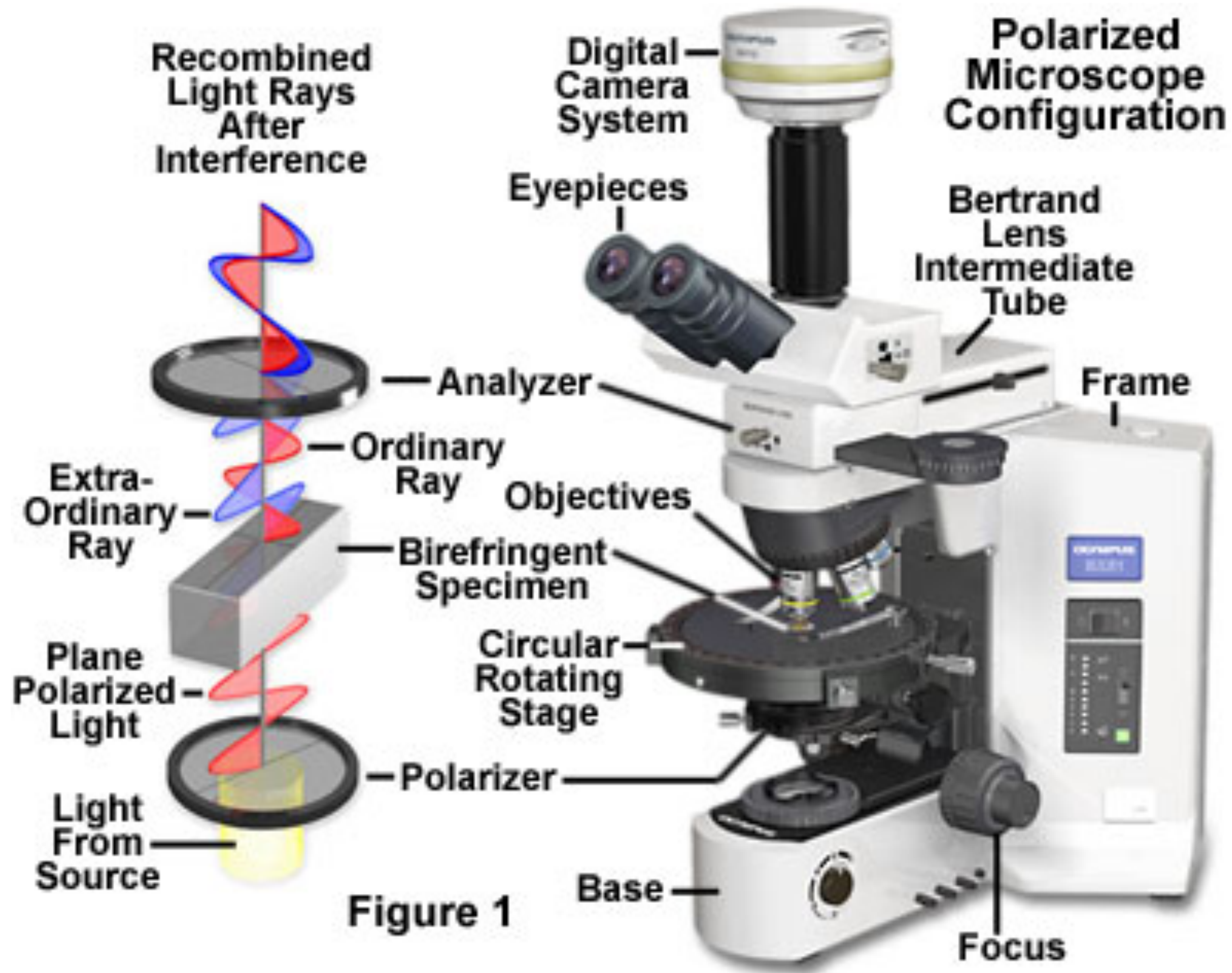
IMPORTANT CONCEPT

Absorption may depend on Polarization



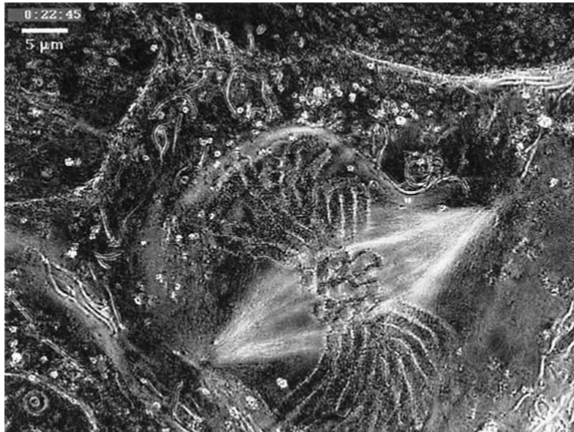

Birefringent Crystals Between Crossed Polarizers
Polarizer P
Object (Anisotropic Crystal)
n 1
Analyzer A
White Light
Retardation (Δn x t)
n 2
Plane Polarized Light
Sample Thickness (t)
Two Components Resulting From Birefringence
Figure 3

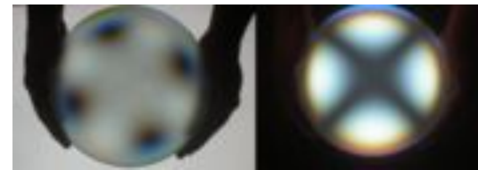
Polarized light microscopy



Polarized Light microscopy

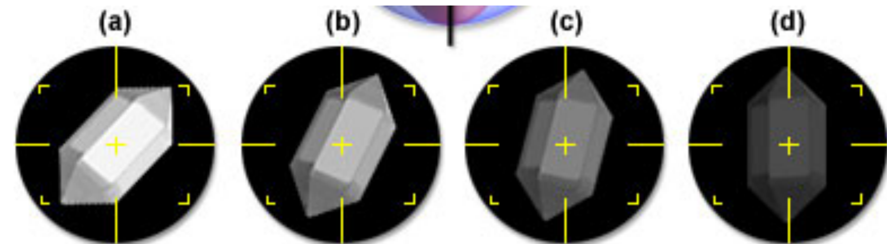
Only works with birefringent samples (those that alter polarity of light) -- some polymers such as microtubules





Requires strain-free optics

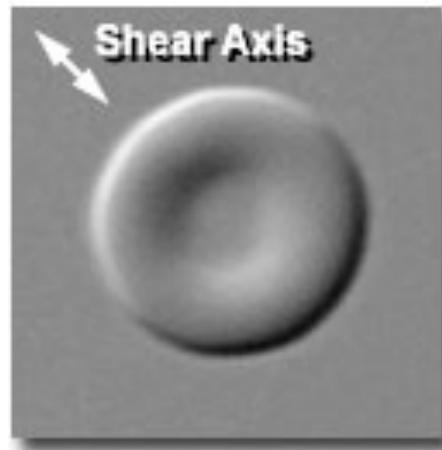
Depends on orientation, so rotating stage desirable

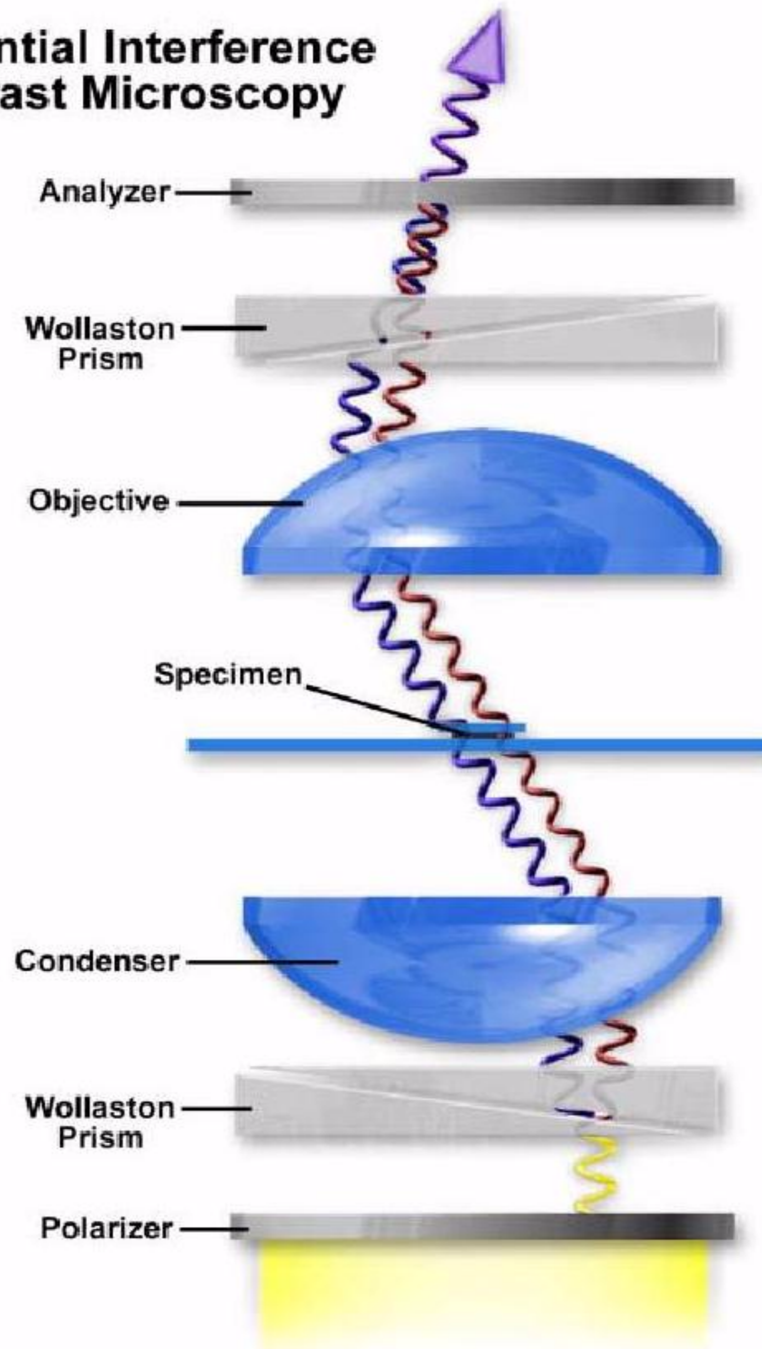


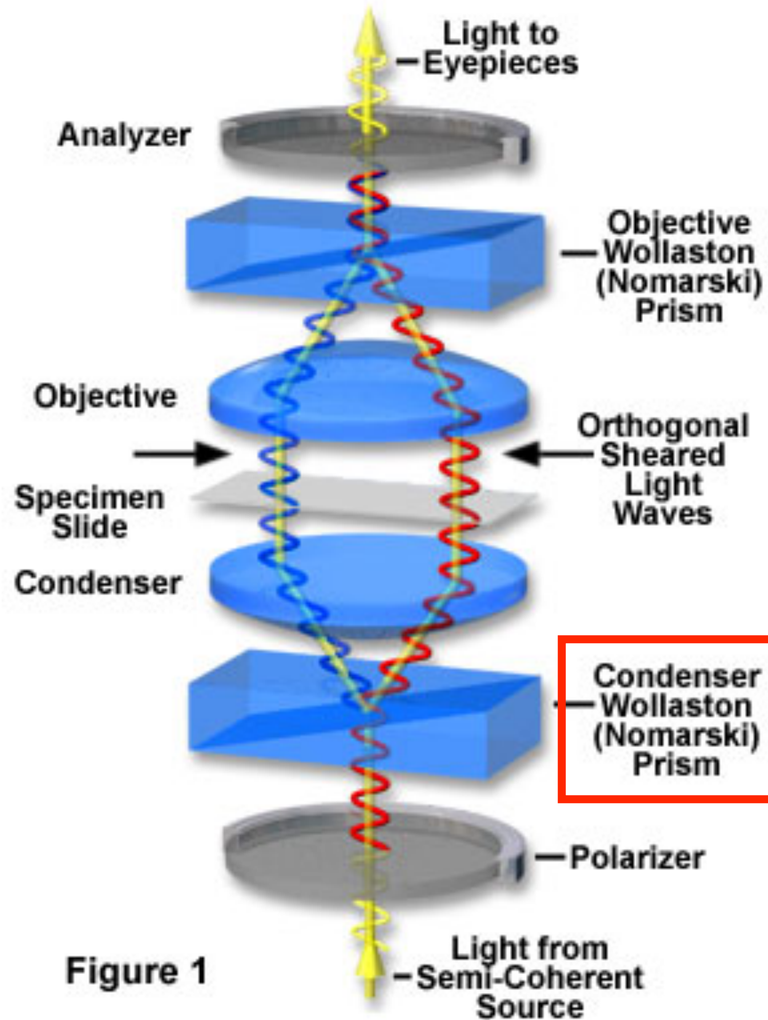
Compatible with fluorescence microscopy (good way to read out orientation of certain chromophores)

Can use modification of polarization microscope
for non-birefringent samples

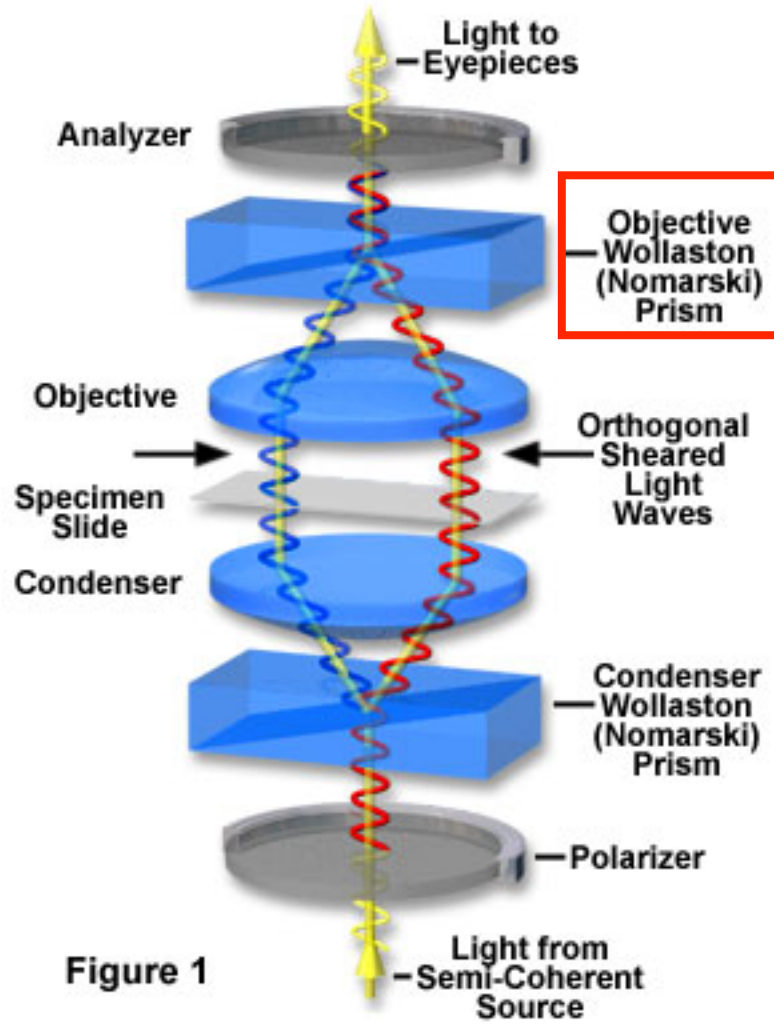
-- **DIC converts optical path difference into polarity changes**



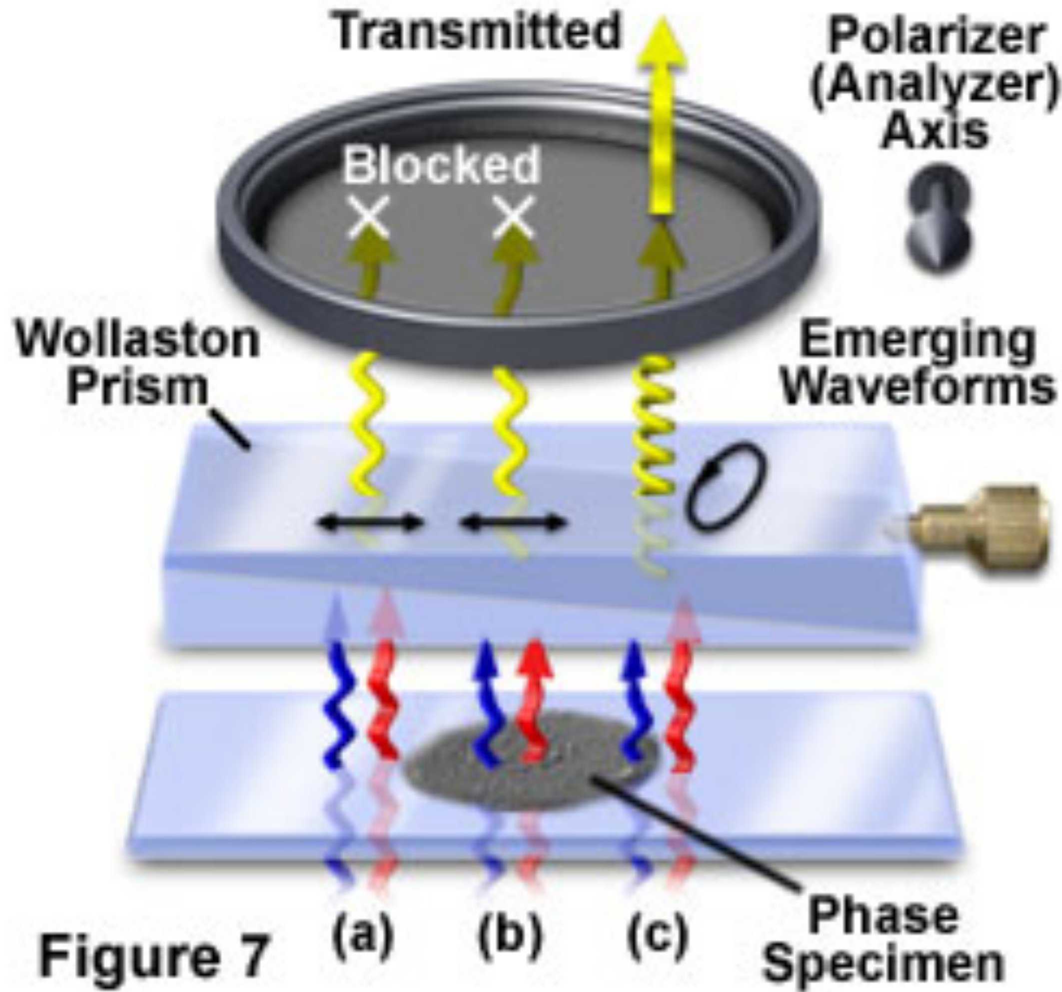

Differential Interference Contrast Microscopy
Analyzer
Wollaston Prism
Objective
Specimen
Condenser
Wollaston Prism
Polarizer



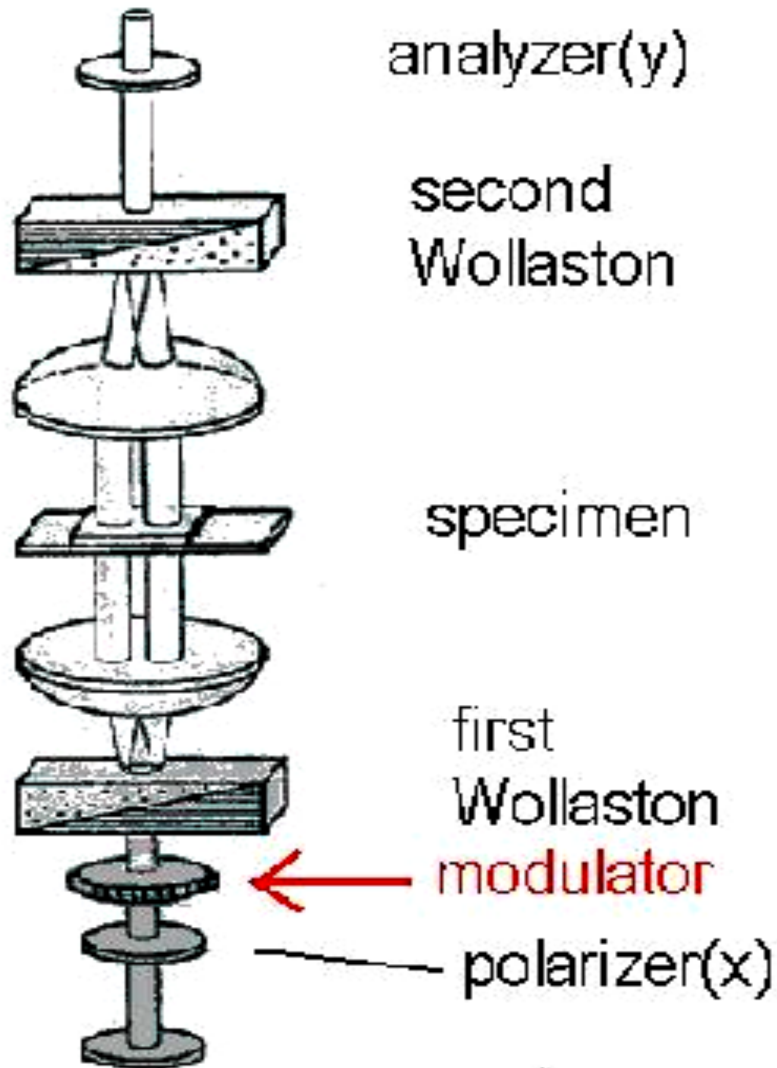
Splits parallel waves into **mutually perpendicular** waves (cannot interfere) with slight **shear** or separation in one axis



Exactly reverses action of first prism to remove shear and reverse rotation



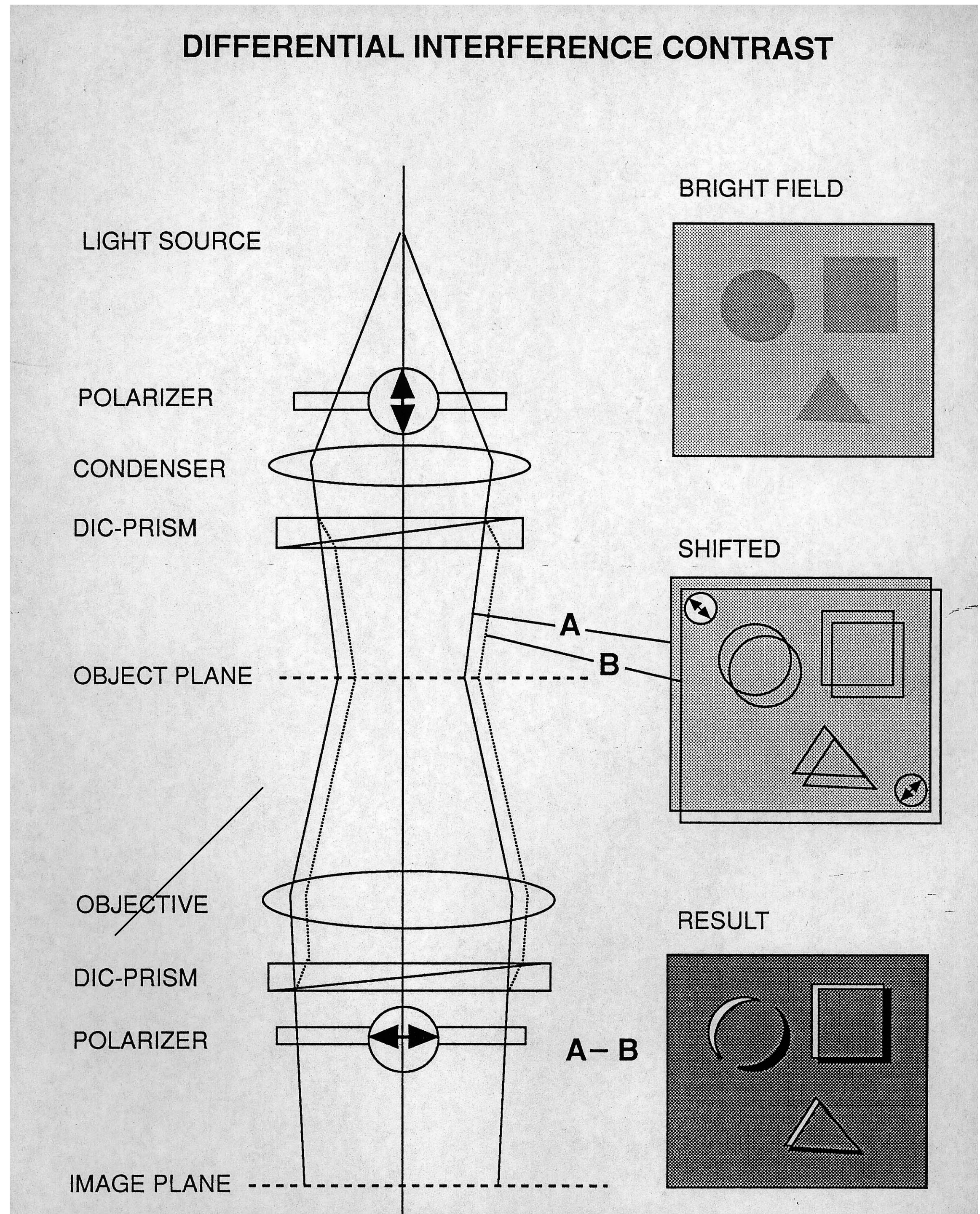
Differential **phase shift** of paired waves produces elliptically polarized light that can partially pass through the analyzer



Holzwarth, Webb, Kubinski, and Allen,
J. Microscopy, p*249-254* (1997)

Nomarski's Differential Interference Contrast (DIC):

Interference between righ and left handed circularly polarized and slightly SHIFTED in space images.



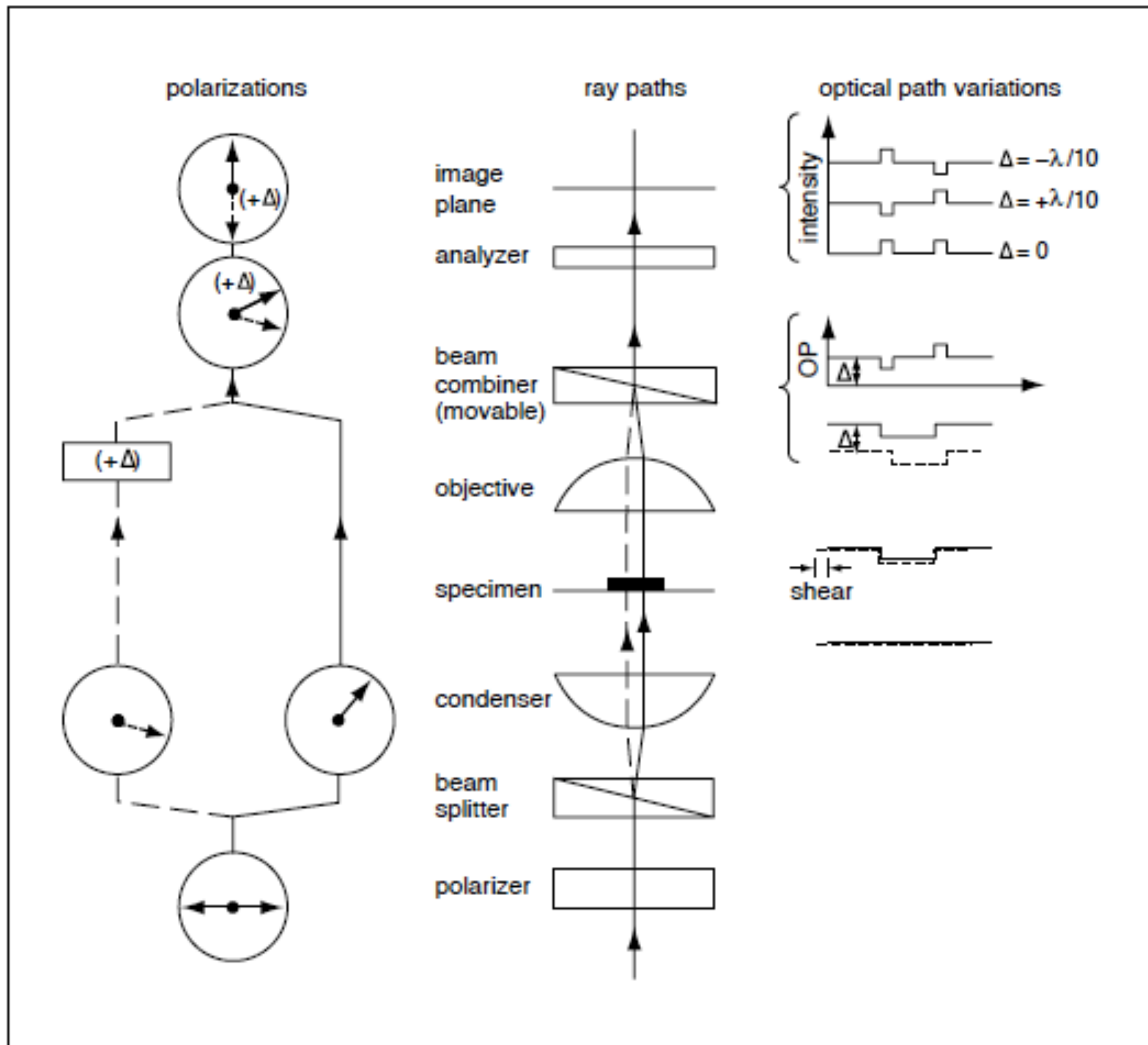
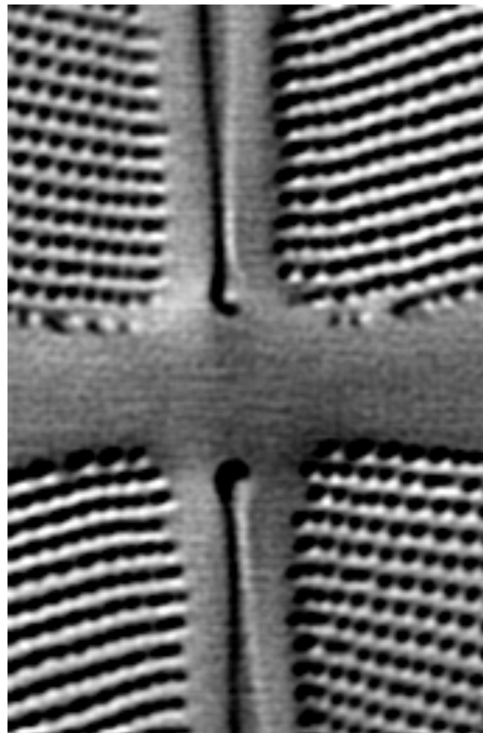
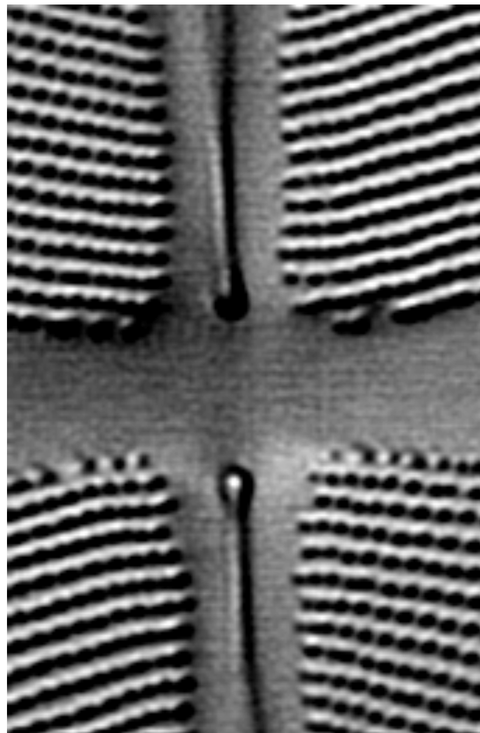


Figure 4.1.7 The optical system for DIC microscopy. From Salmon and Tran (1998), reprinted with permission from Academic Press.

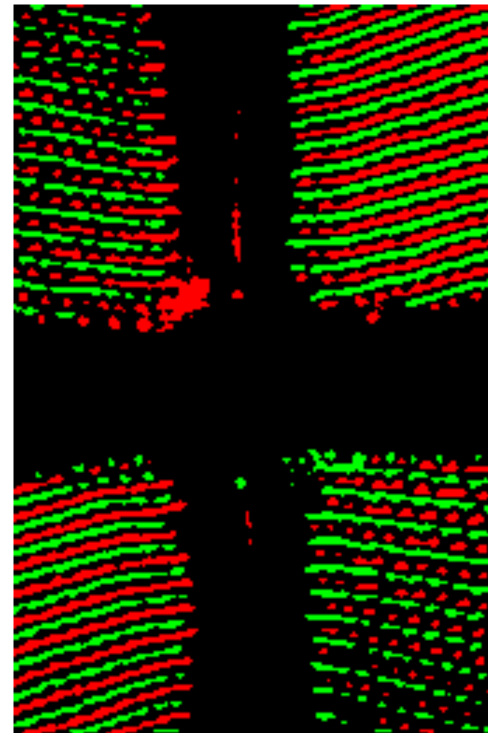
DIC IMAGES



X-pol



Y-pol



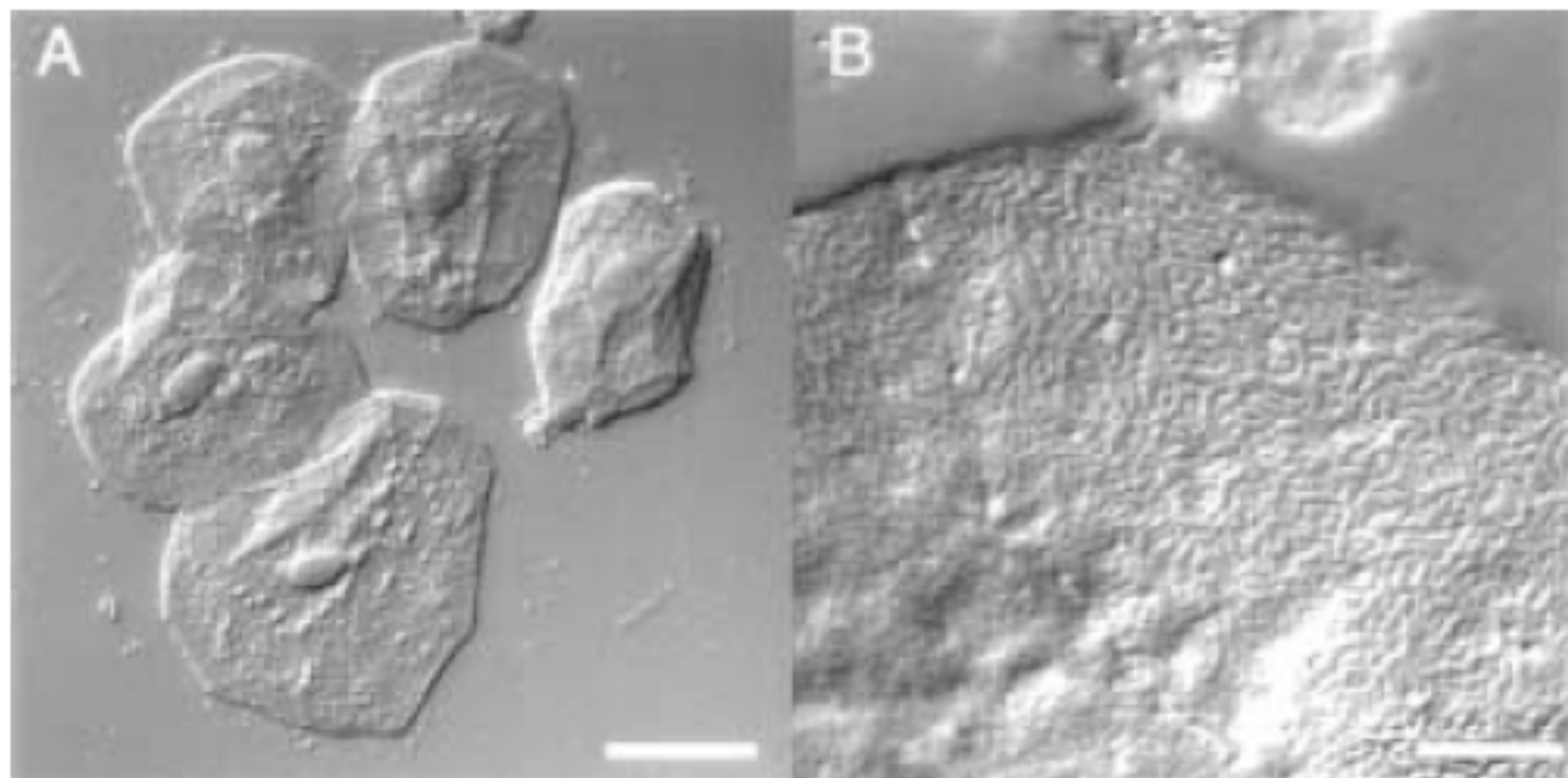
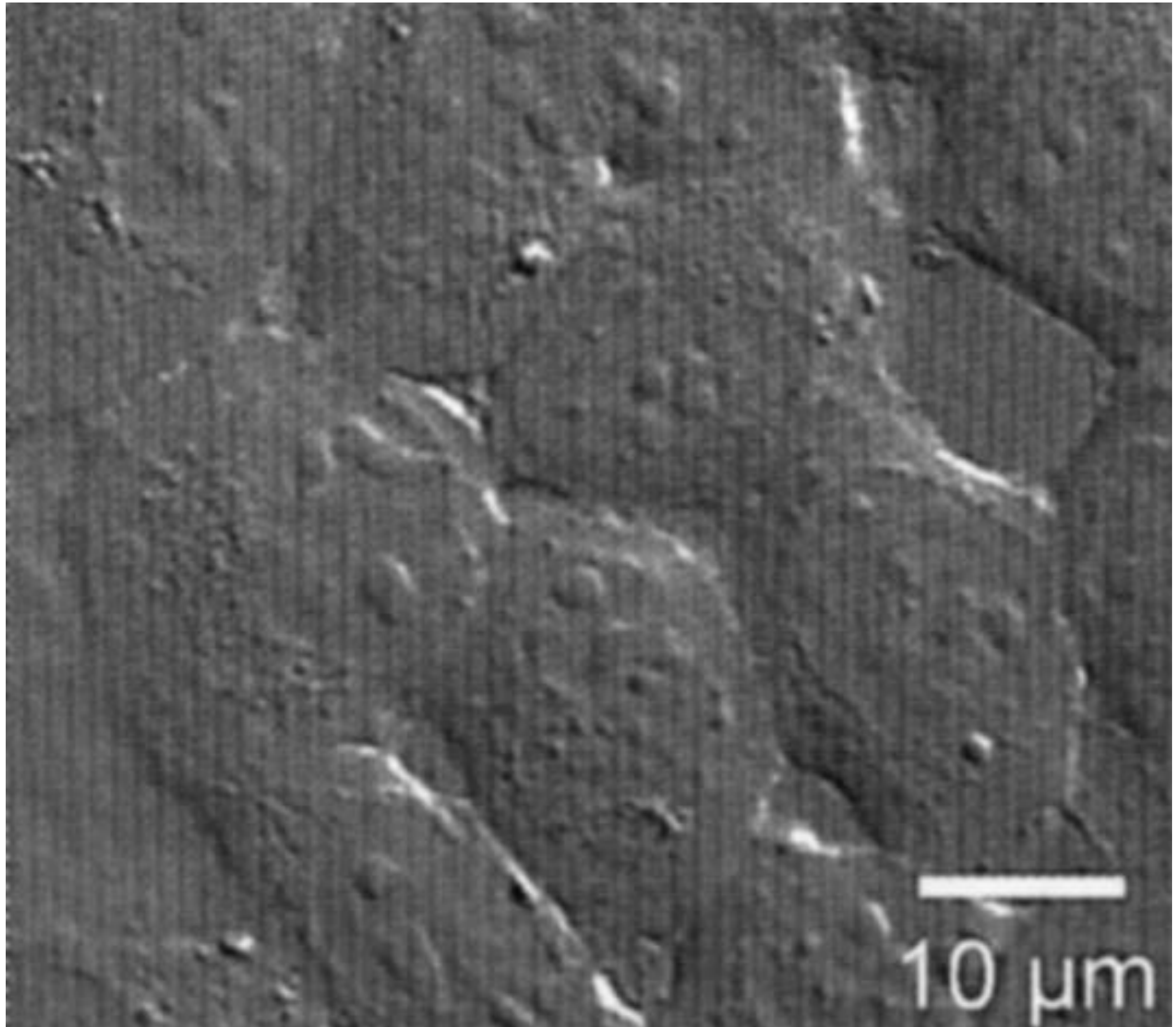
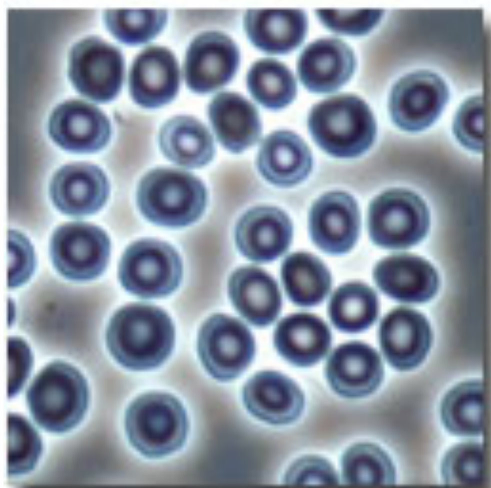


Figure 4.1.6 DIC images of a human cheek cell test specimen. (**A**) Low magnification of cheek cell preparation with a 20× objective. Bar = 20 μm. (**B**) High-resolution image of the surface of the cell at the top of (A) using a 60×/(NA = 1.4) Plan Apochromat objective and matching condenser ilumination. The ridges on the cell surface are often diffraction limited in width. Bar = 5 μm. From Salmon and Tran (1998), reprinted with permission from Academic Press.

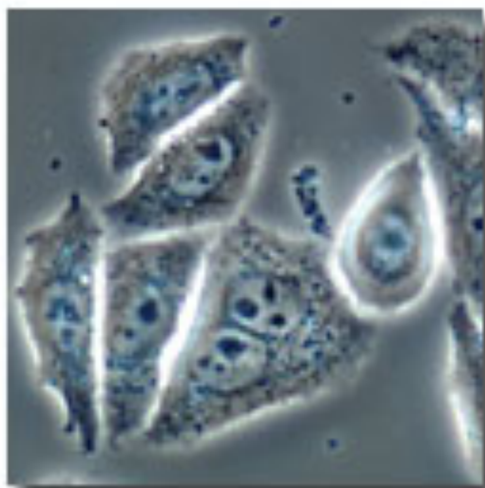
DIC image of cells



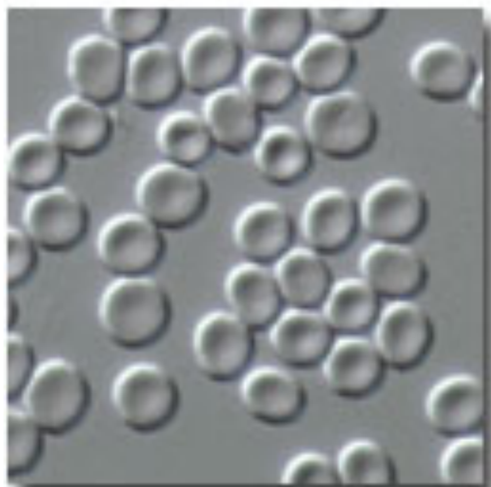
Halos in Phase Contrast and DIC Microscopy



(a)



(c)



(b)

(d)

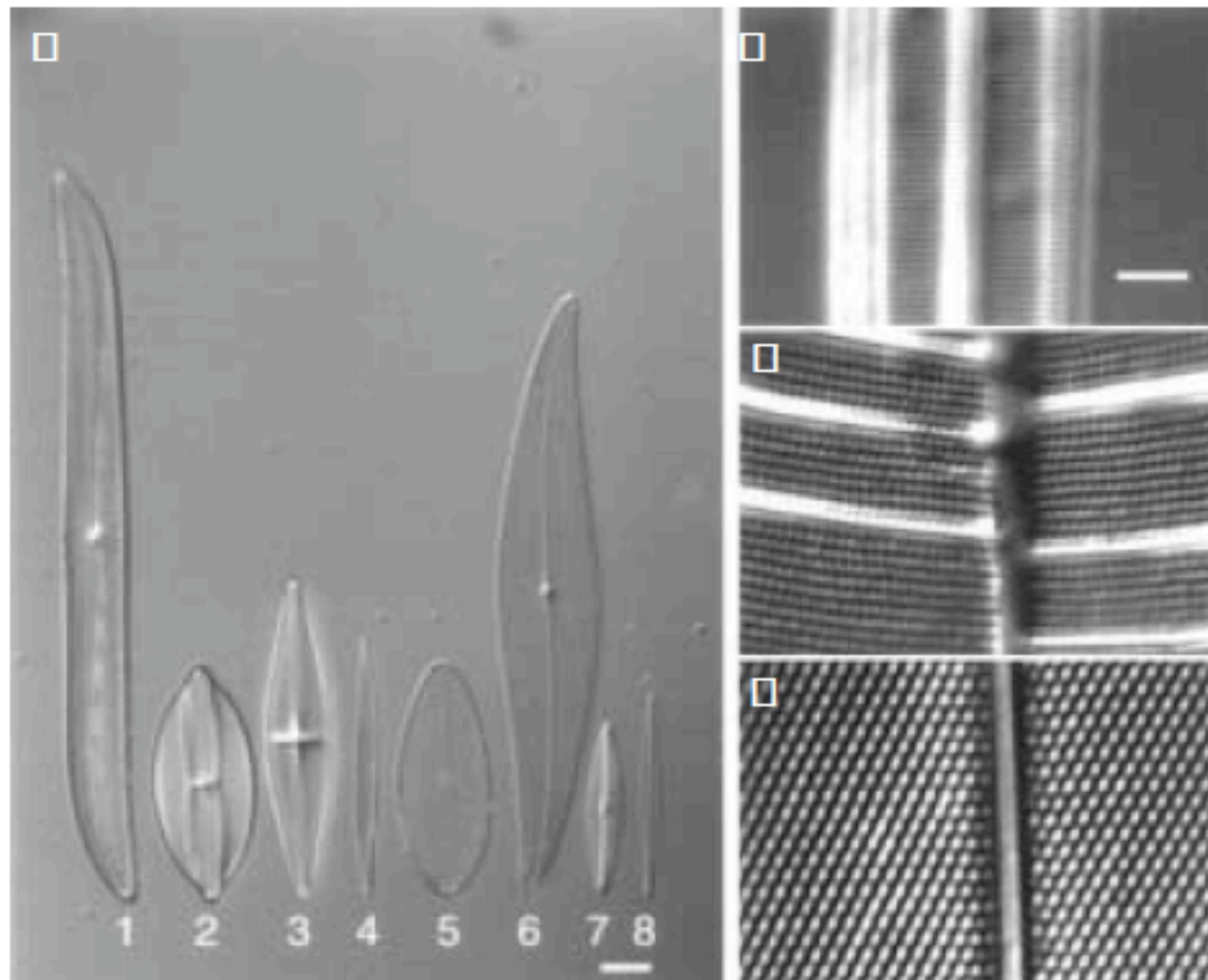


Figure 4.1.8 (**A**) The diatom test plate. The rows of pores are spaced in the silica shell by ~0.25 μm in *Amphipleura pellucida* (panel **B**; also number 8 on panel A), 0.41 μm in *Surrella gemma* (panel **C**; also number 5 on panel A), and 0.62 μm in *Pleurasigma angulatum* (panel **D**; also number 6 on panel A). Bar: panel A, 10 μm; panels B, C, and D, 2.5 μm. From Salmon and Tran (1998), reprinted with permission from Academic Press.

Differential Interference Contrast Microscope Configuration

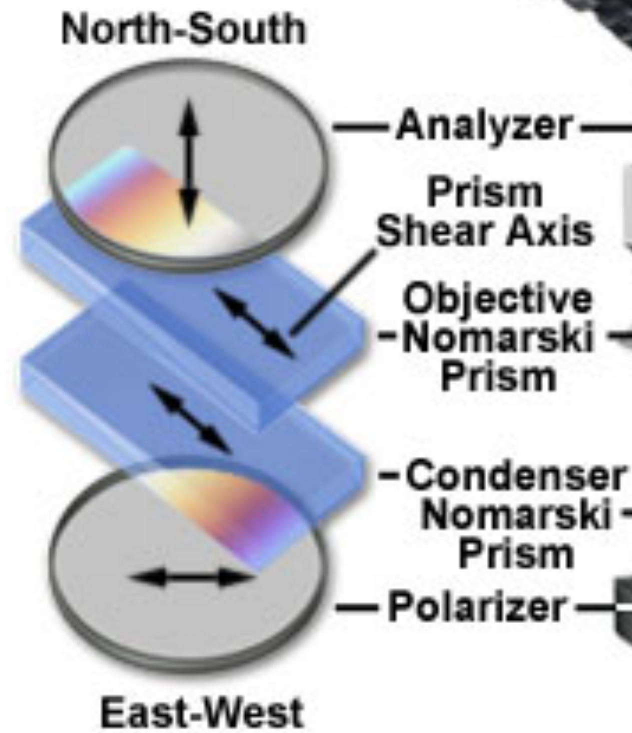


Figure 1

Bi-Refraction in Calcite Crystals

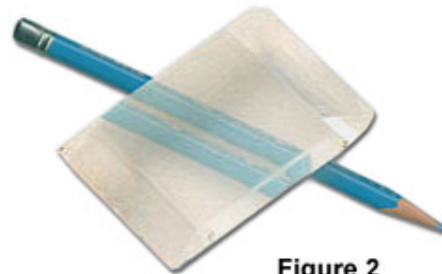


Figure 2

Role of Bias in DIC

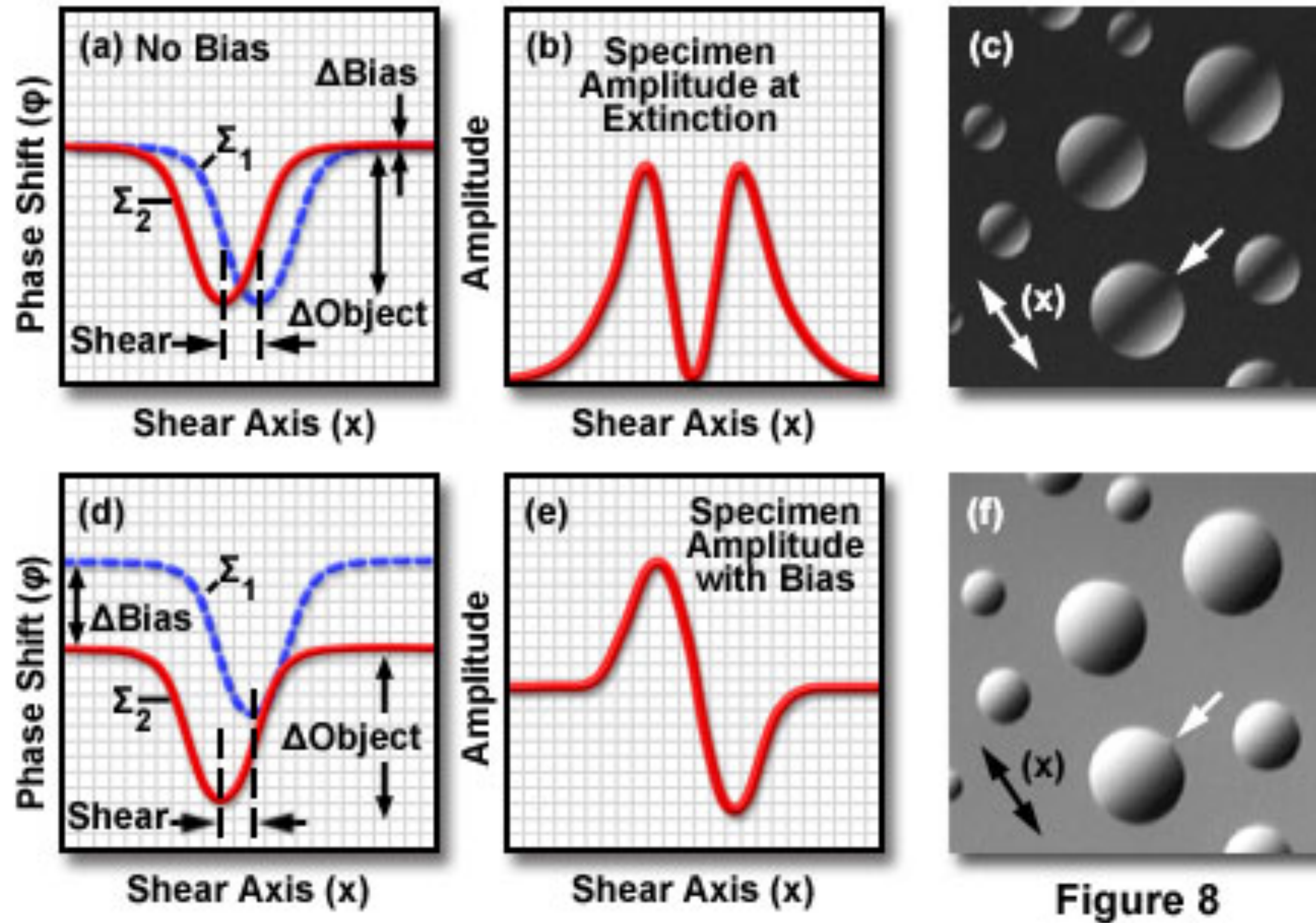
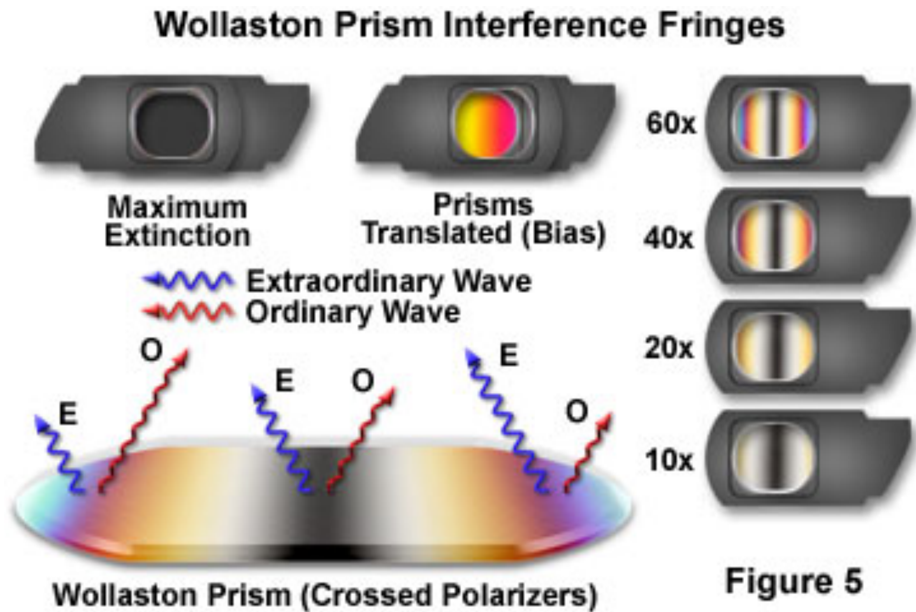
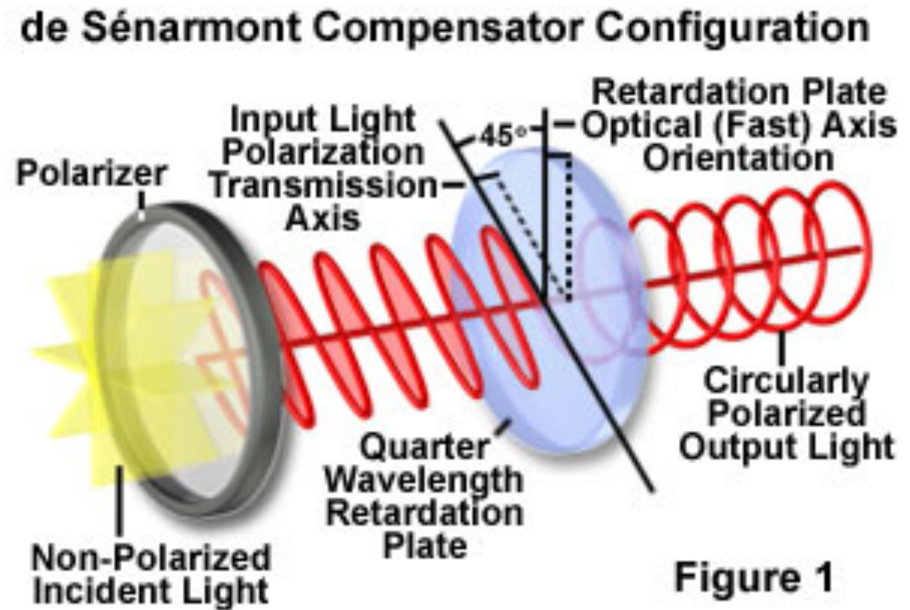


Figure 8

Ways to introduce bias in DIC

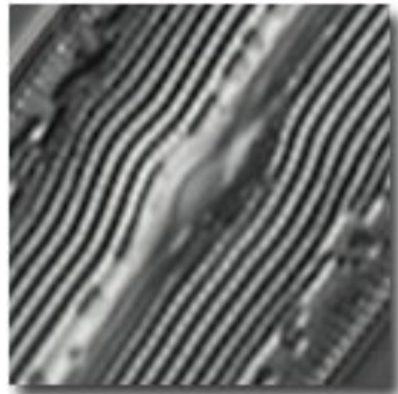
1. Translate Prisms relative to one another

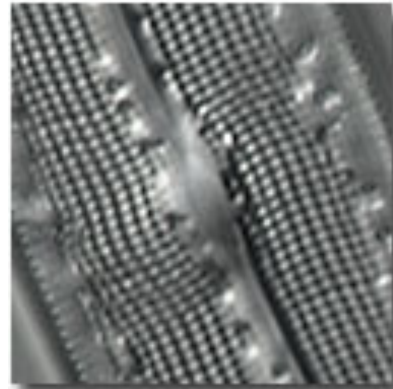


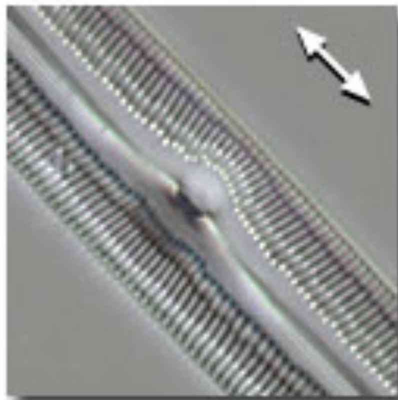


2. Rotate polarizer (in conjuction with wave retardation plate)

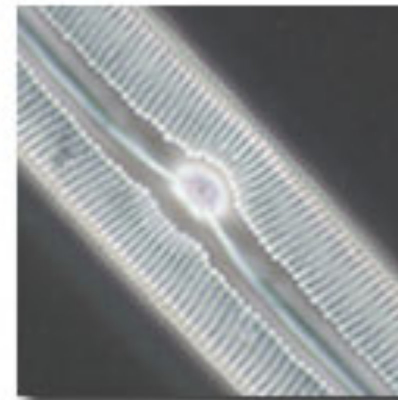
Because of directional contrast, DIC is sensitive to specimen orientation


(a)


(b)


(a)

(b)


(c)

DIC but not phase is orientation-dependent

Phase better than DIC for birefringent samples

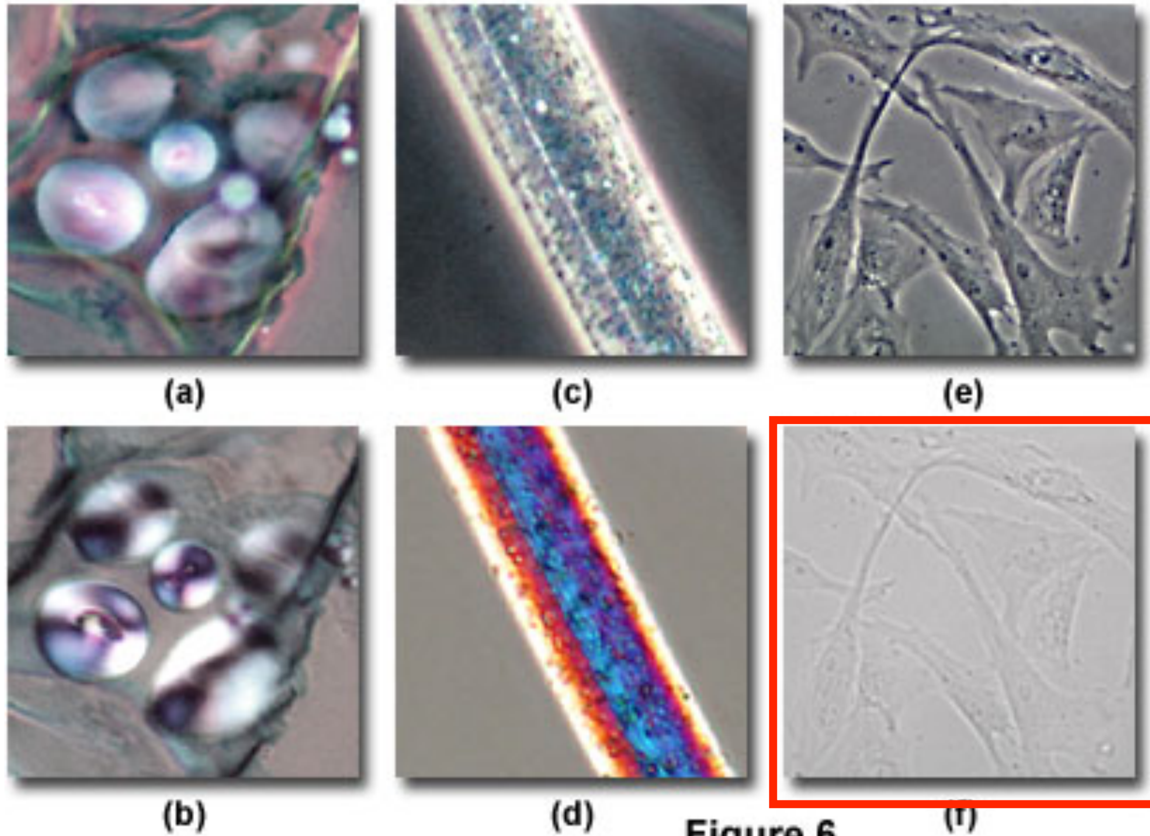
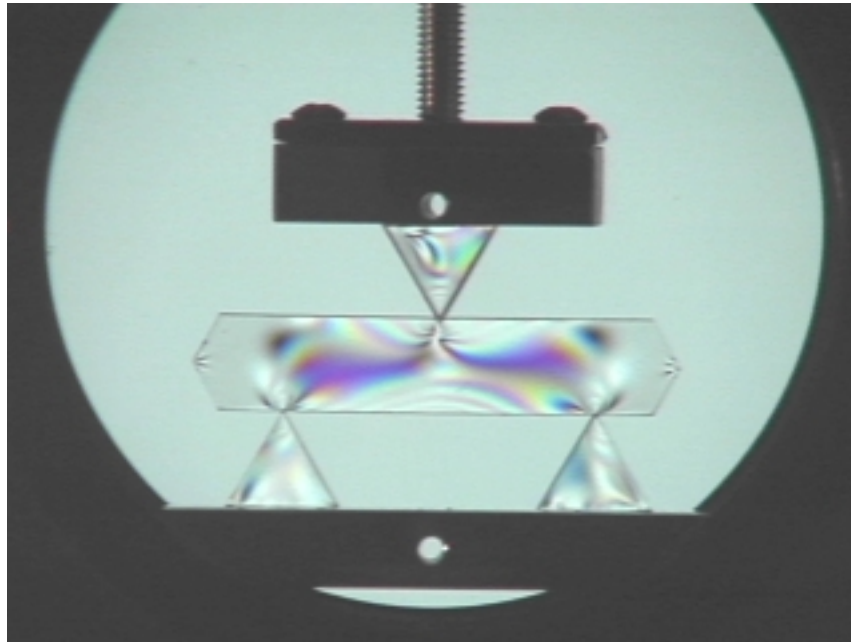


Figure 6

DIC not compatible with birefringent samples (can’t plate cells on or or cover cells with plastic).

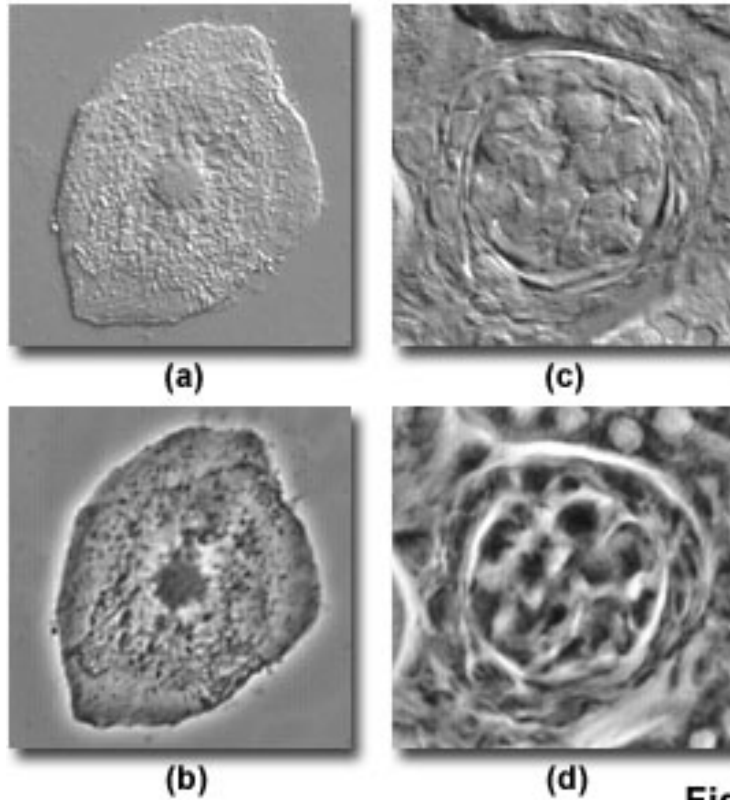
DIC is not working well in plastic dishes

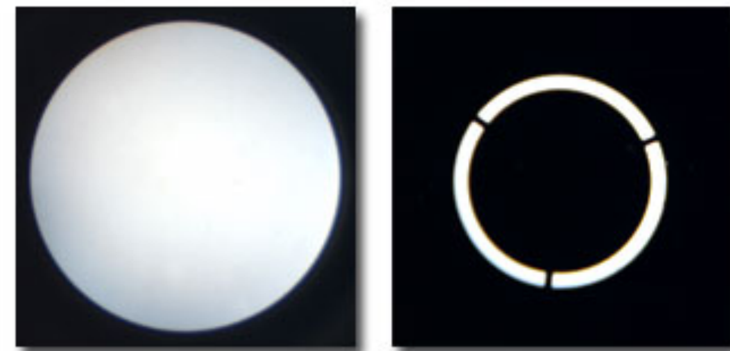


Comparison of Phase Contrast and DIC

	DIC	Phase Contrast
Sensitive to sample orientation	yes	no
Thick samples/optical sectioning	good	poor
Birefringent samples	poor	good

DIC gives superior lateral and axial resolution





examine effect of closing down condenser aperture on ability to do optical sectioning (C. elegans)

Phase Contrast and DIC often used in conjunction with fluorescence microscopy

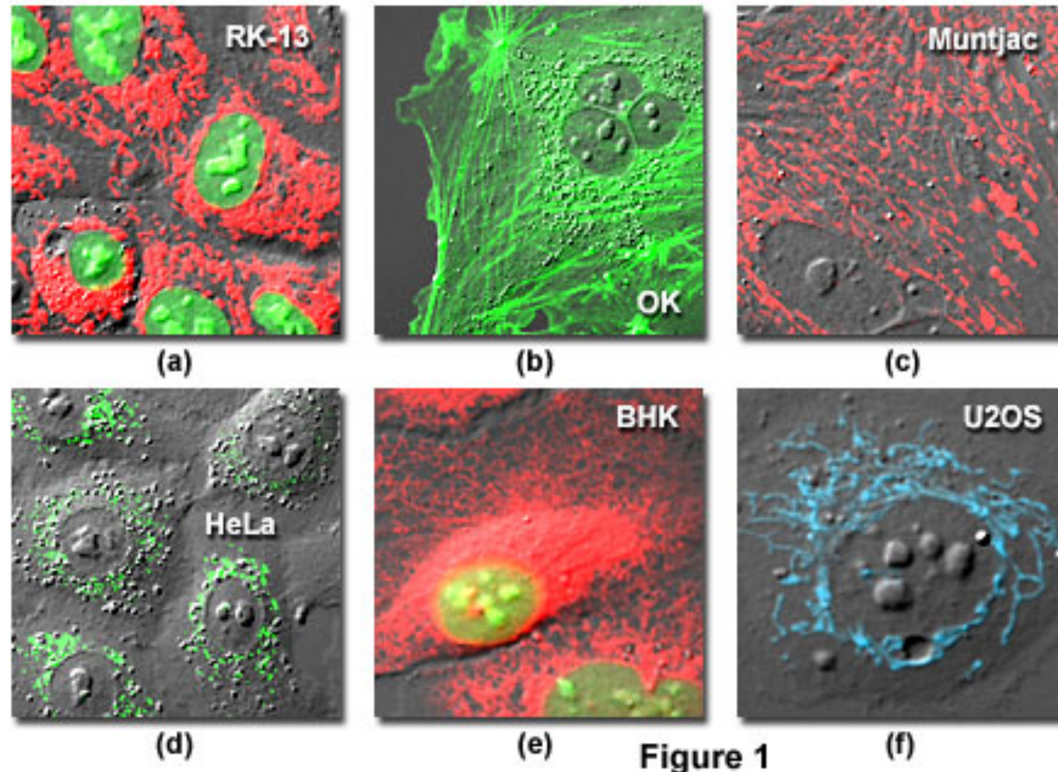
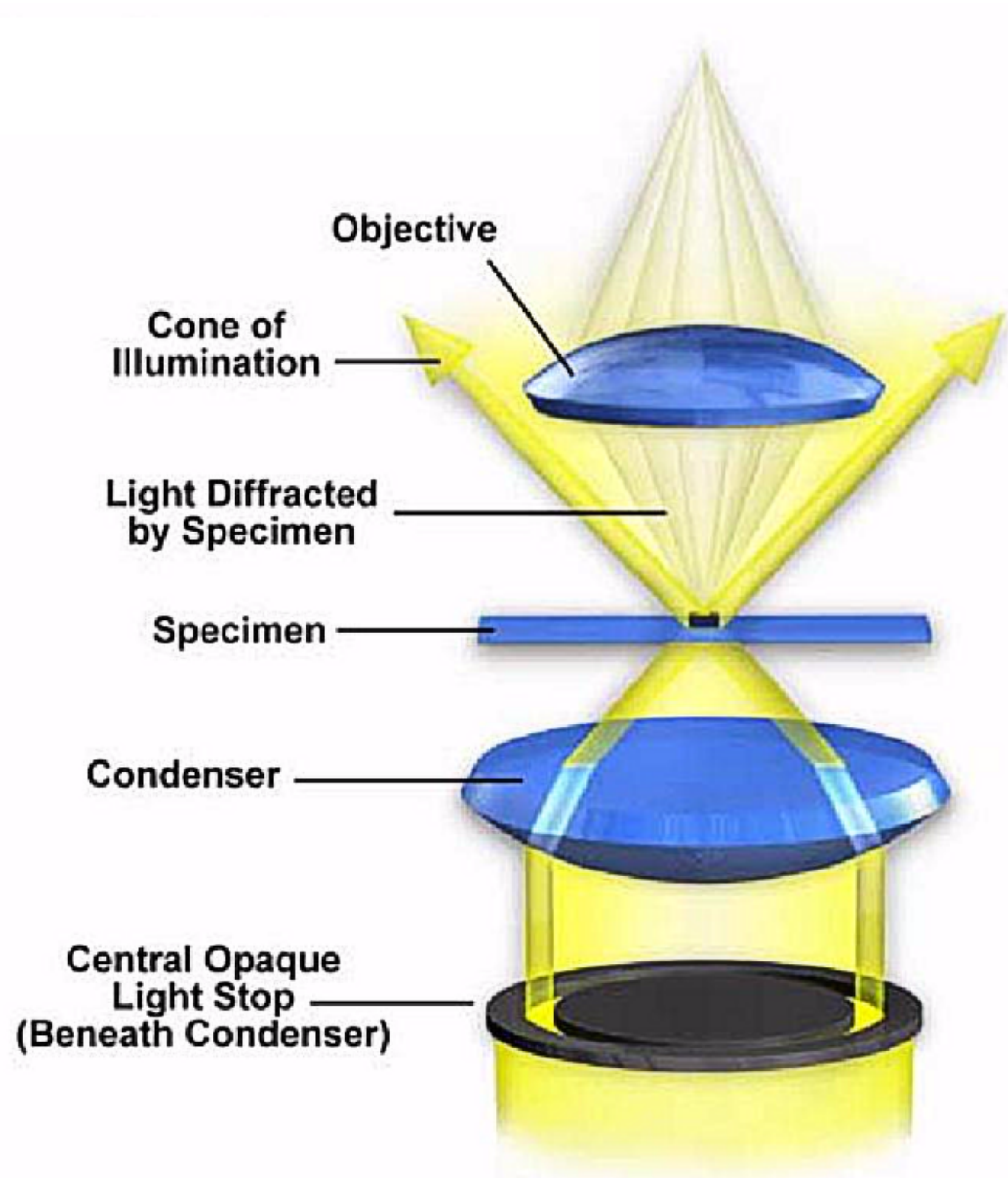


Figure 1

To provide cellular or organismal reference.
Phase and DIC are much more general (and less toxic detection tools than fluorescence.

DARKFIELD IMAGING



DARKFIELD IMAGES

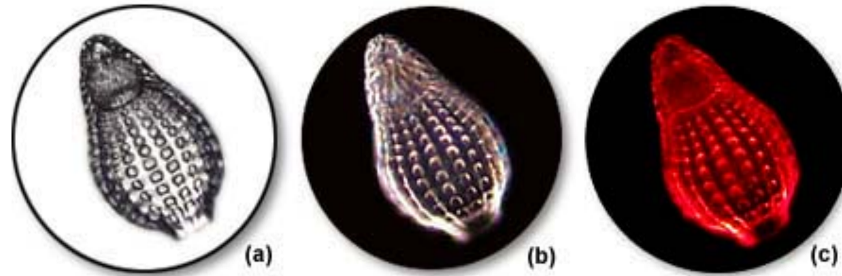
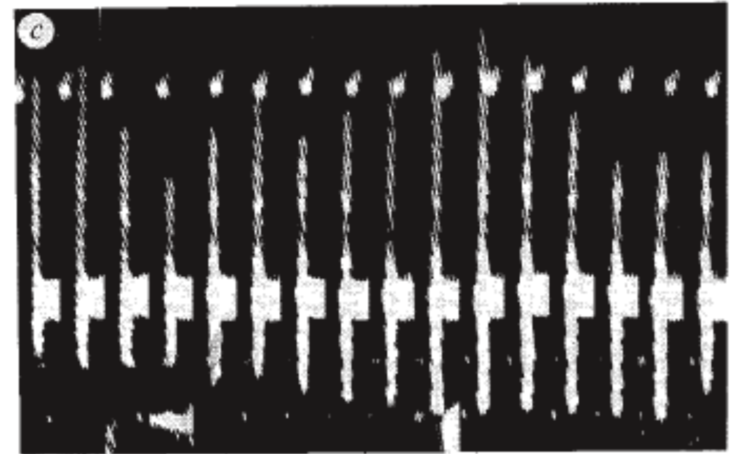


Figure 2



First direct visualization of microtubule dynamic instability

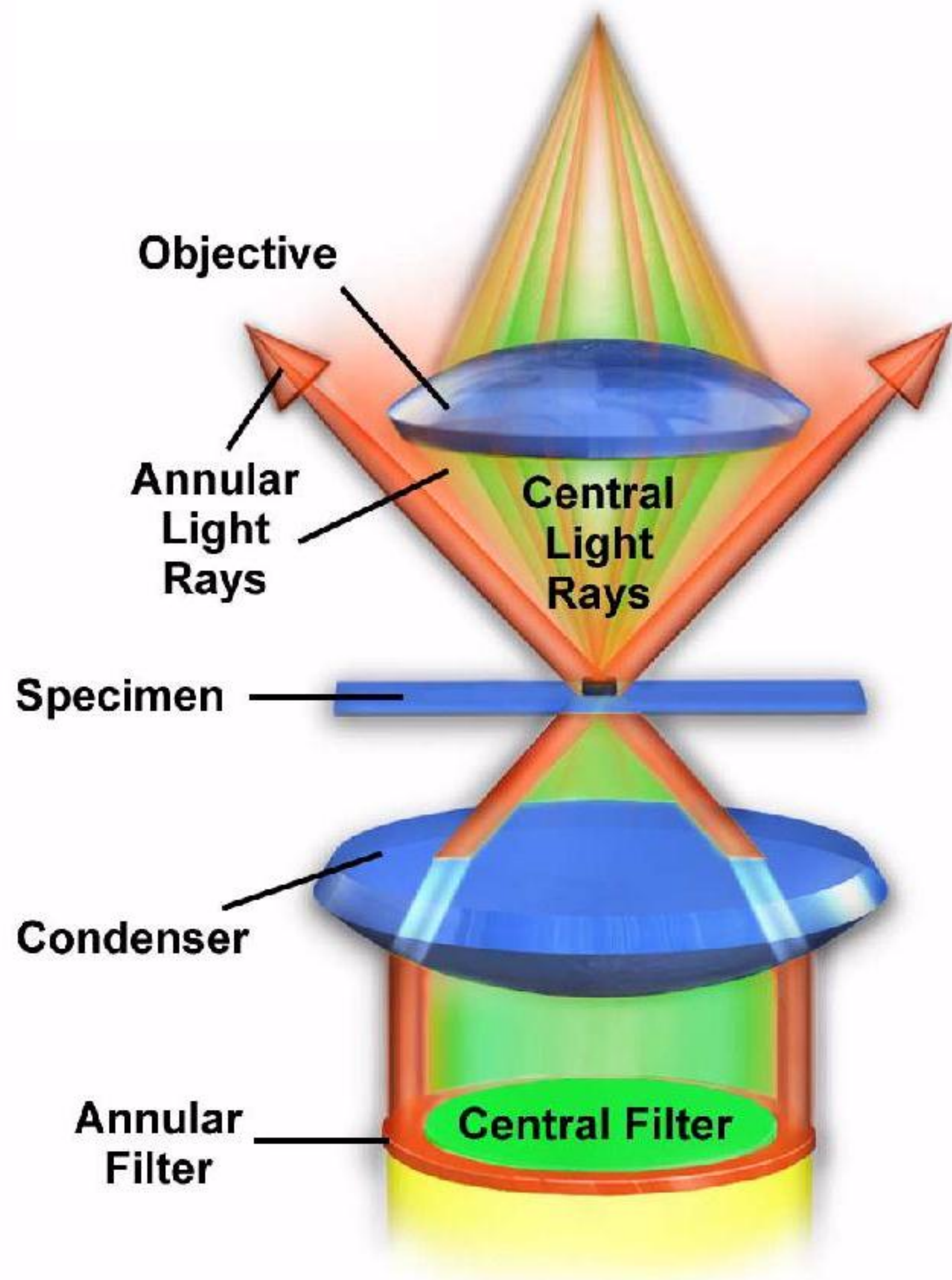
-Darkfield good for imaging unstained microorganisms,

-even sub-resolution objects such as flagella (20nm diameter) visible with darkfield.

-not good for internal structure

-Dust on sample, optics, bubbles in oil are not tolerated with this technique

RHEINBERG ILLUMINATION



RHEINBERG IMAGE

(a) Brightfield

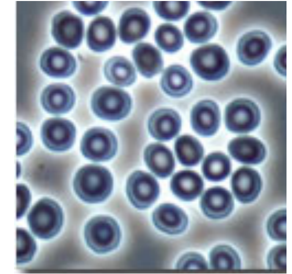
(b) Darkfield

(c) Rheinberg

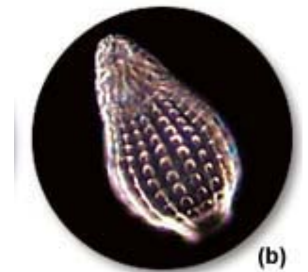
Figure 5

Review:

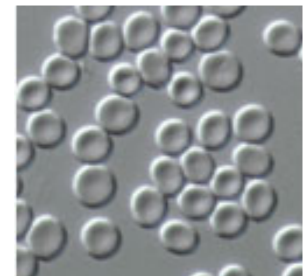
Phase-- converts optical path length into contrast



Darkfield-- images only diffracted light

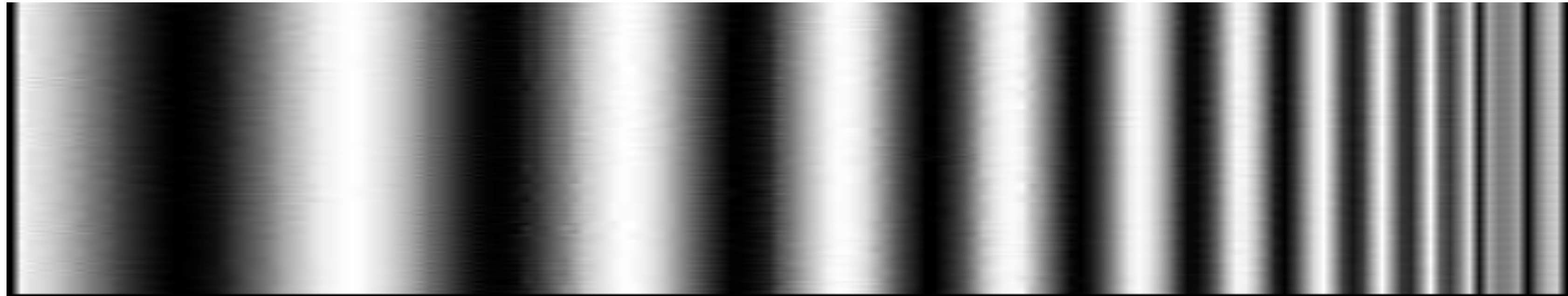


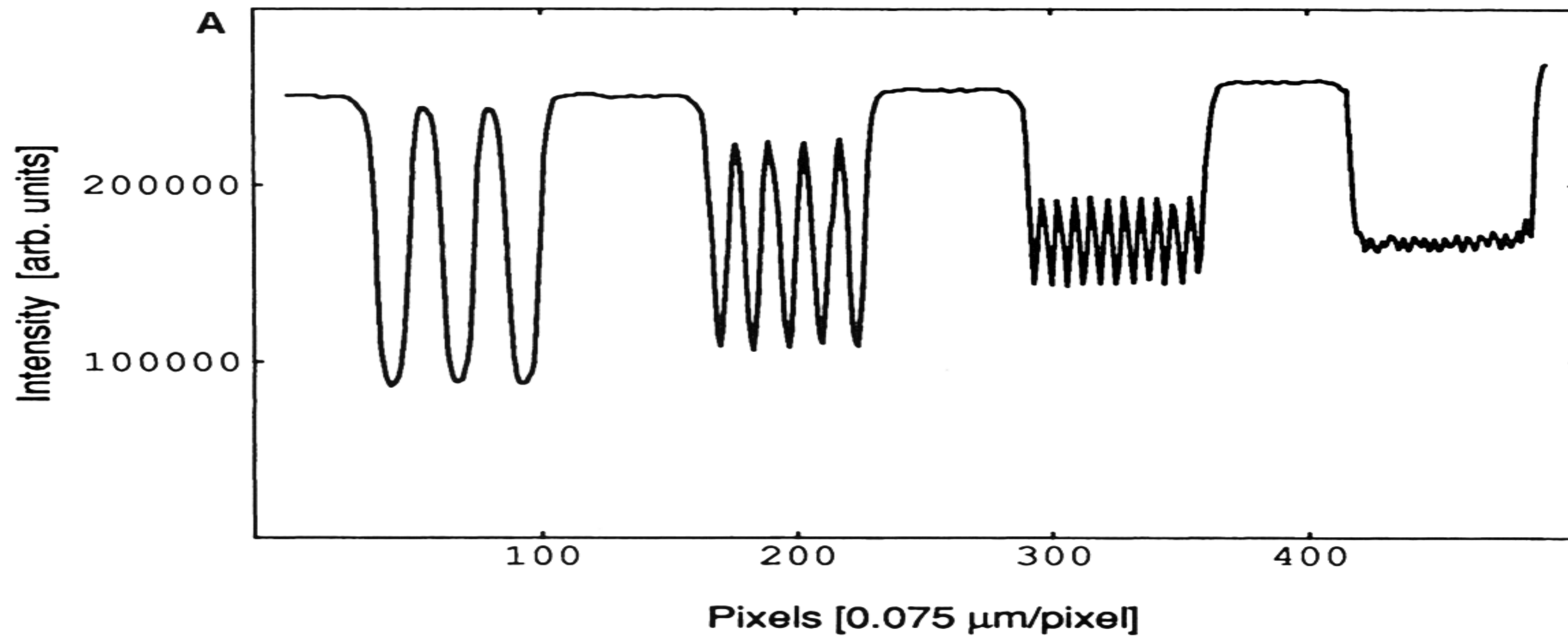
DIC-- contrasts region of sample with
local differences in optical path length



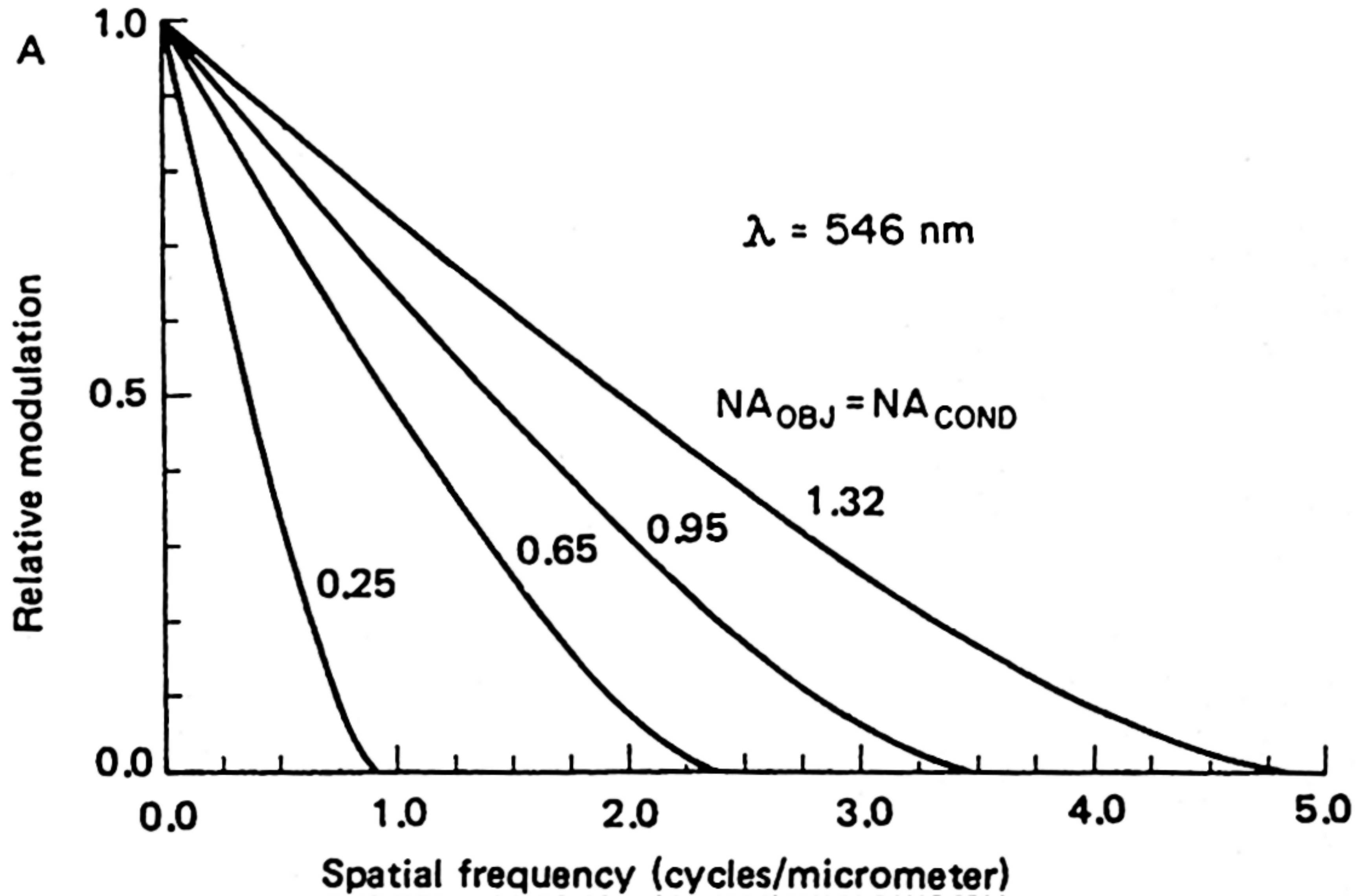
Polarization-- converts polarity information into contrast,
only works with birefringent samples
(polymers, some crystals)

CONTRAST and MTF

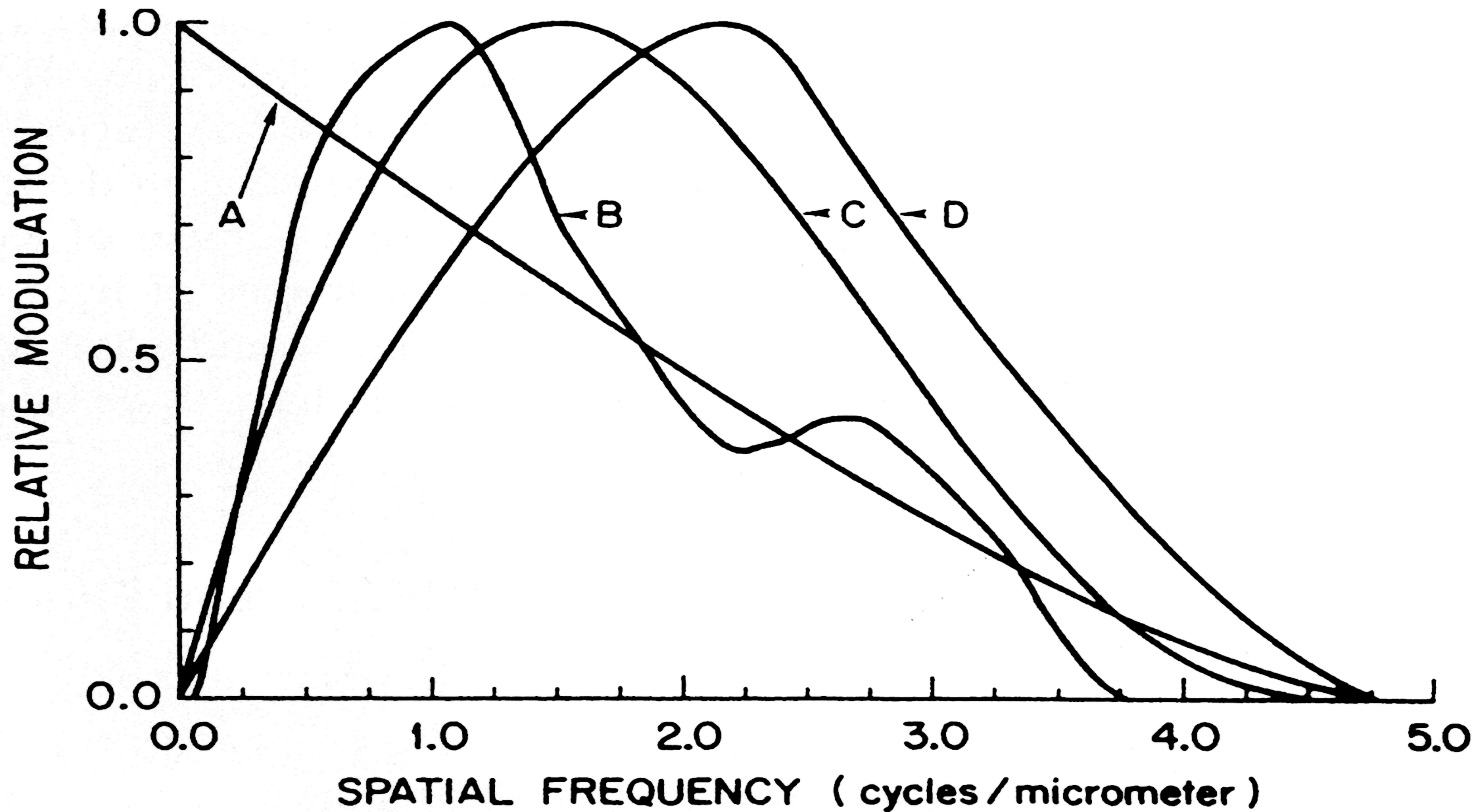




MTF



MTF WITH CONTRAST GENERATION



OTHER CONTRAST GENERATION METHODS

- Polarization
- Hoffman modulation/Oblique Illumination
- Interference
- Fluorescence
- QPM (DIC/phase contrast using three BF images).

TAKEHOME MESSAGES

- Numerical aperture determines resolution
- Empty magnification is bad
- **Contrast generation often lowers resolution,**
  but it is usually worth it
- Keep dirt off of the image planes
- Use ND filters to adjust illumination intensity

Phase microscopy
MicroscopyU
microscopyu.com

DIC microscopy
http://micro.magnet.fsu.edu/primer/techniques/dic/dicintro.html

Ted Salmon
(Phase and Nomarski alignment handout)