

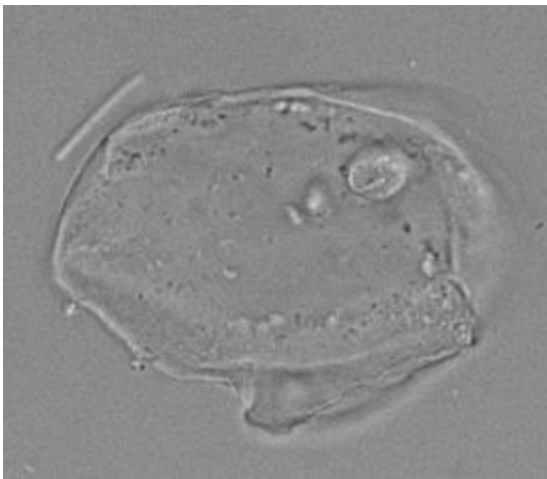
A grayscale micrograph of a biological specimen, possibly a cell or tissue section, with numerous bright red fluorescent spots concentrated in the center. The background is dark and textured. A semi-transparent dark gray rectangle is overlaid on the image, containing the title and author information.

# Brightfield Contrasting Techniques

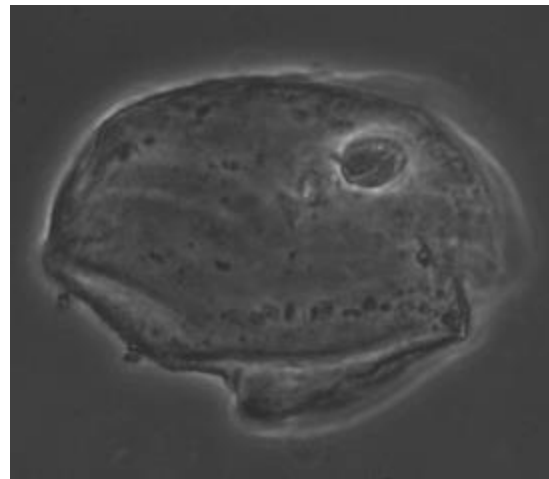
Kurt Thorn  
NIC

# Generating contrast in light microscopy

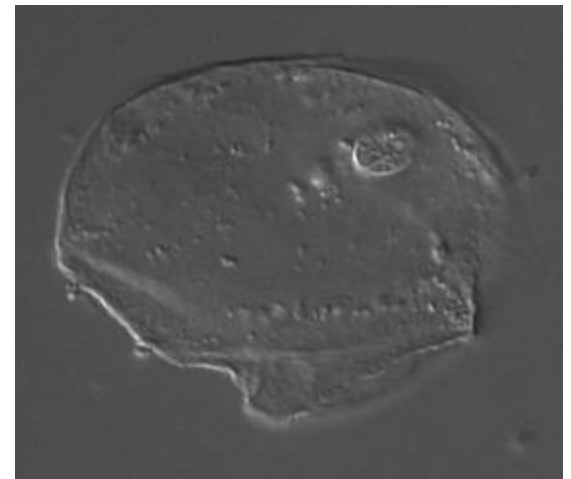
- Problem: Many biological specimens are thin and transparent and difficult to see.
- Solution:
  - Fluorescent staining
  - Brightfield contrasting techniques: DIC, Phase, others



Brightfield

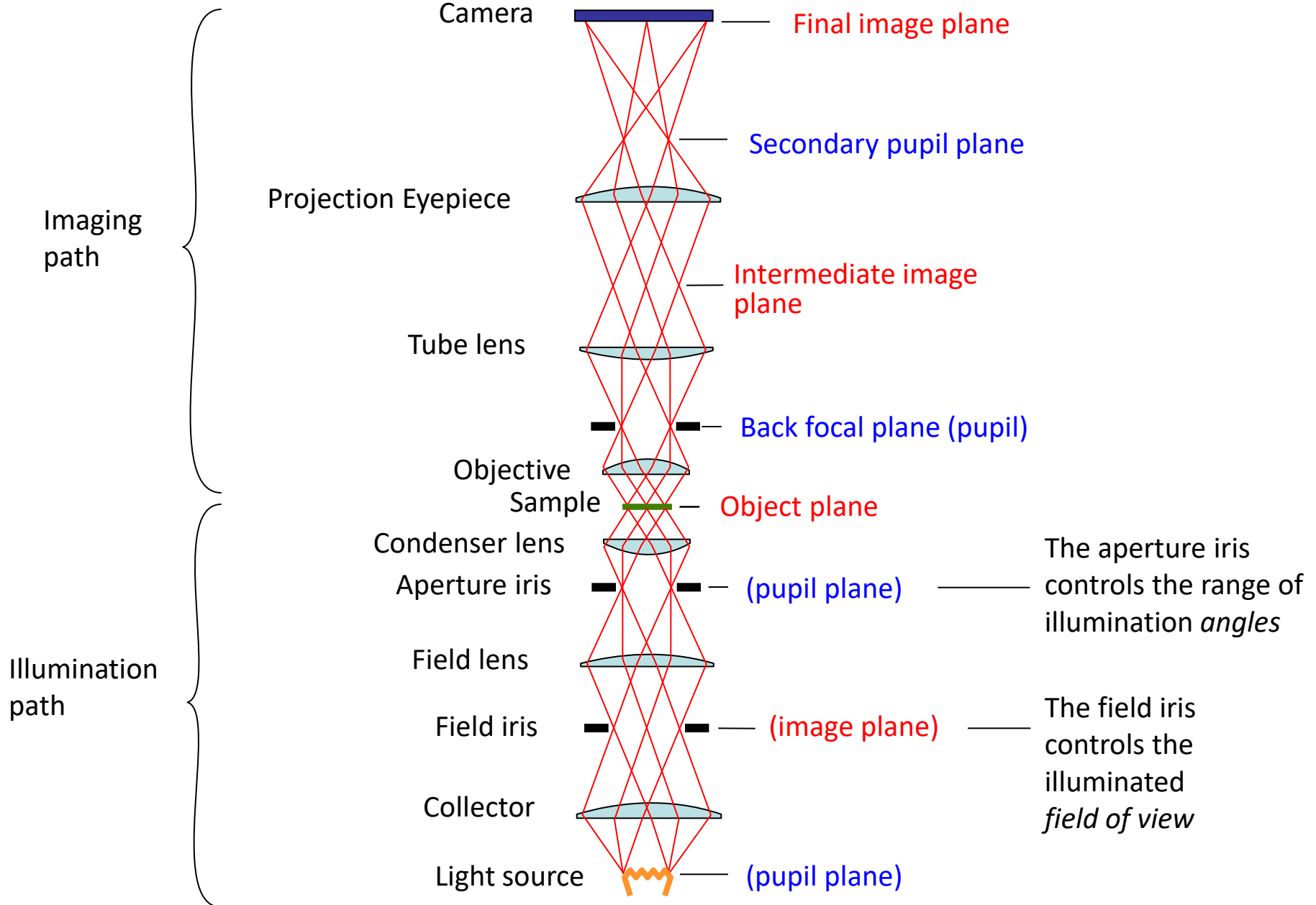


Phase Contrast



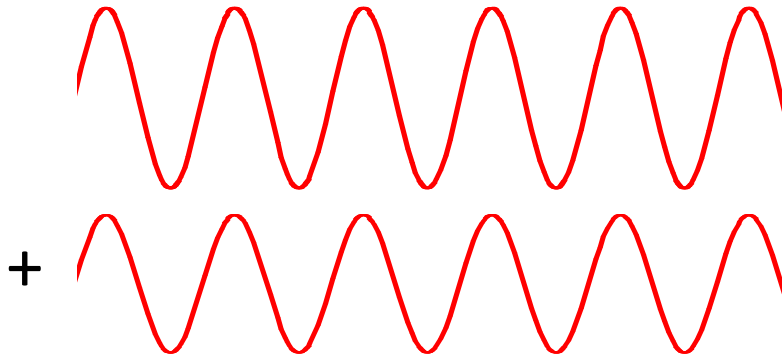
DIC

# Review: The Trans-illumination Microscope

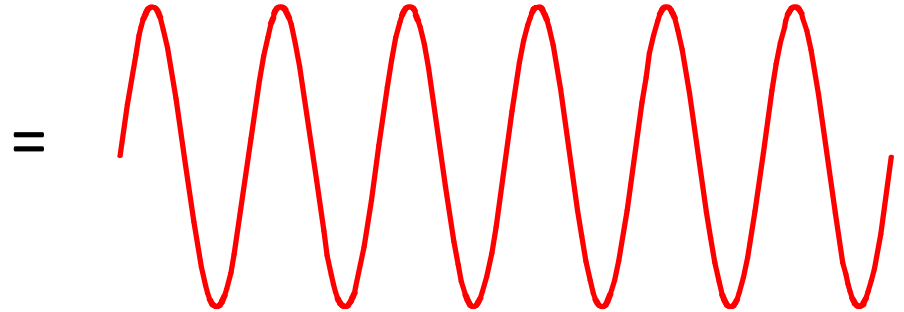


# Reminder: Interference

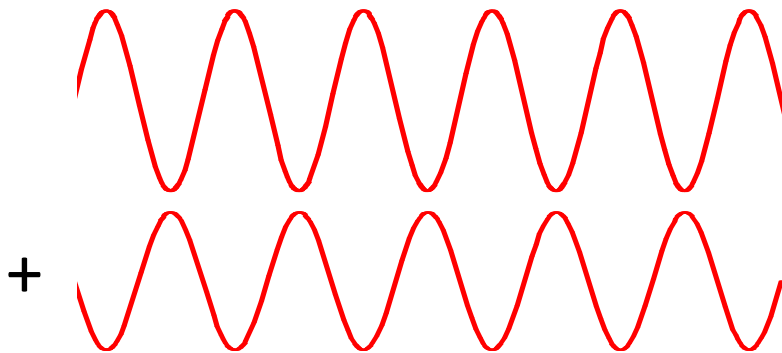
In phase



constructive interference



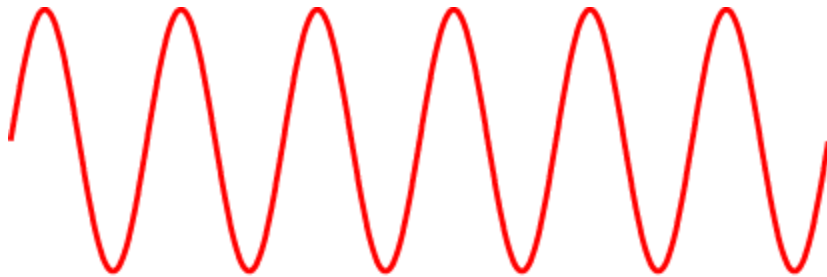
Opposite phase



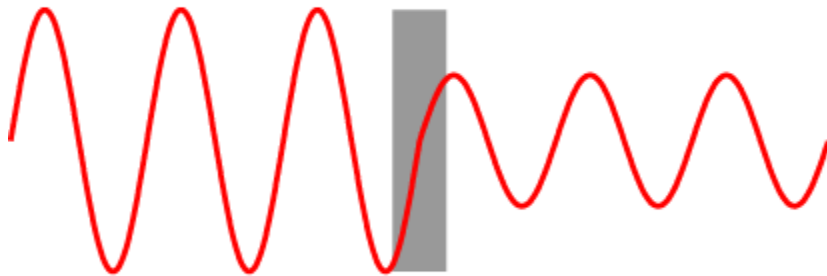
destructive interference



# Amplitude and Phase Samples

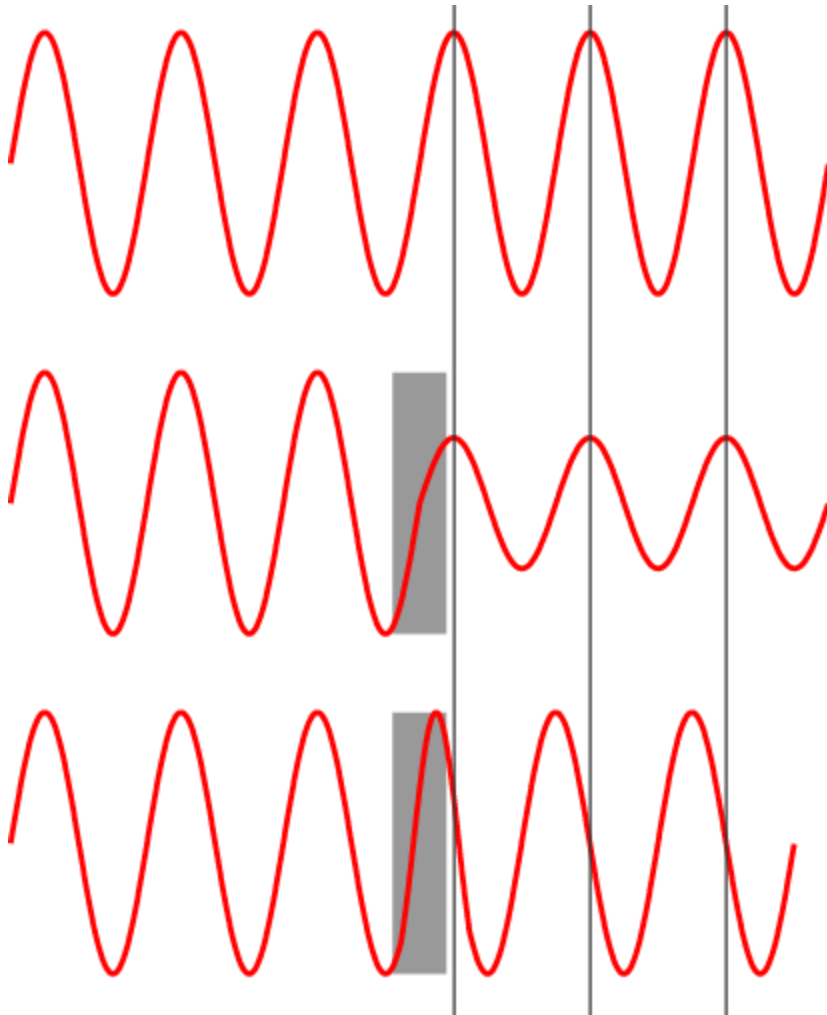


Initial beam, unperturbed



Amplitude sample

# Amplitude and Phase Samples



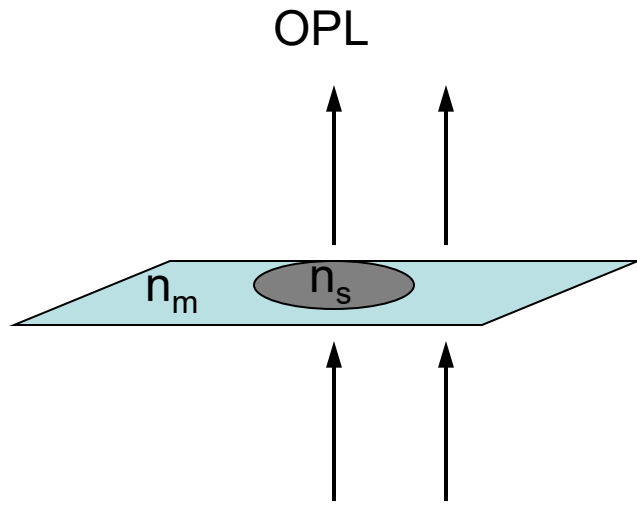
Initial beam, unperturbed

Amplitude sample

Phase sample

# Many biological samples are phase samples

Higher refractive indices slow down light



$t$  = sample thickness ,  $\sim 1$  micron

$n_s$  = sample refractive index ,  $\sim 1.38$

$n_m$  = medium refractive index,  $\sim 1.33$

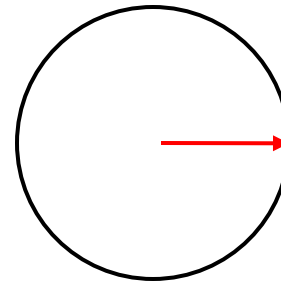
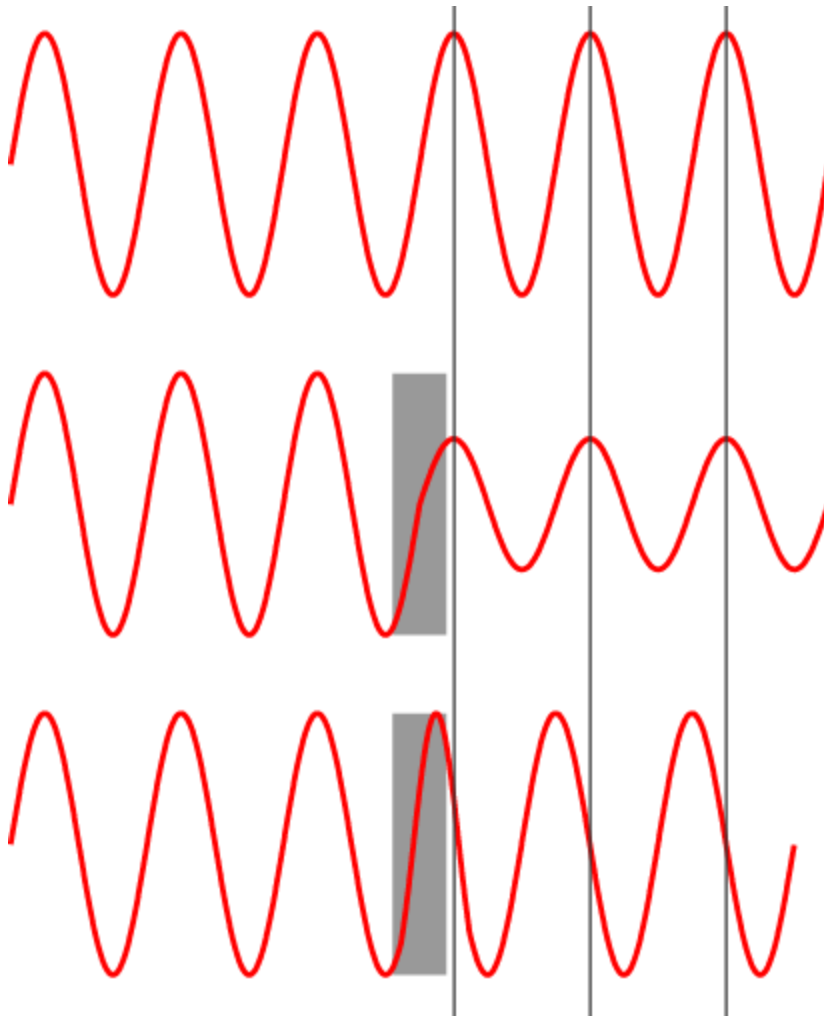
Optical path length (OPL) difference =  $t (n_s - n_m)$

=  $1 \text{ micron} (1.38 - 1.33) = .05 \text{ microns} = 50 \text{ nm}$ ,

which is about  $1/10$  the wavelength of green light (488 nm)

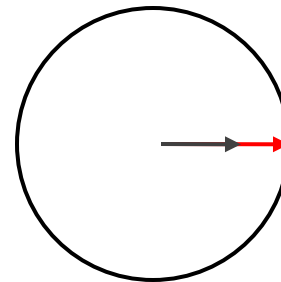
# Amplitude and Phase Samples

Represented as Vectors

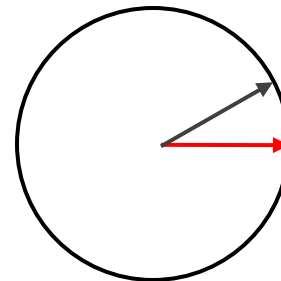


Vector length:  
amplitude

Rotation around  
circle: phase



Only amplitude  
changes

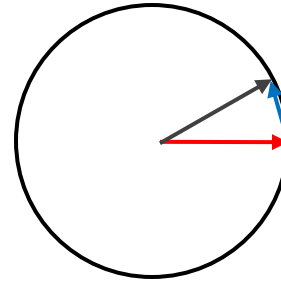
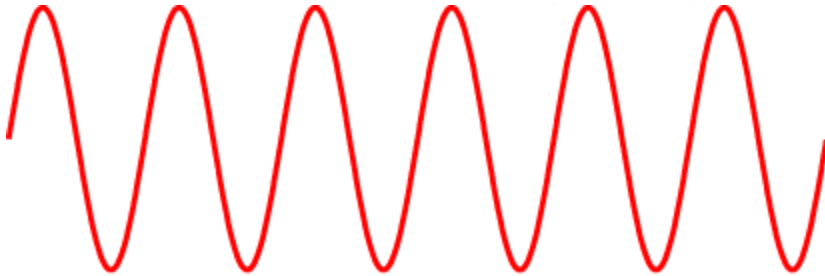


Only phase  
changes



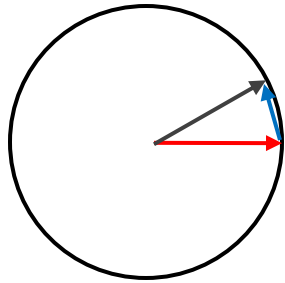
# How can we see a phase change?

Problem: optical detectors are not phase sensitive; they only detect changes in amplitude



Decompose phase shift into an **unperturbed wave** and a **perturbed wave**

# Converting a phase change to an amplitude change

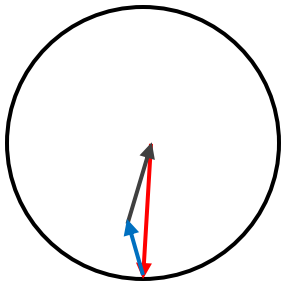


Unperturbed wave (Surround wave)

Perturbed wave (Diffracted wave)

Resulting wave (Particle wave)

Imagine shifting phase of surround wave by  $90^\circ$

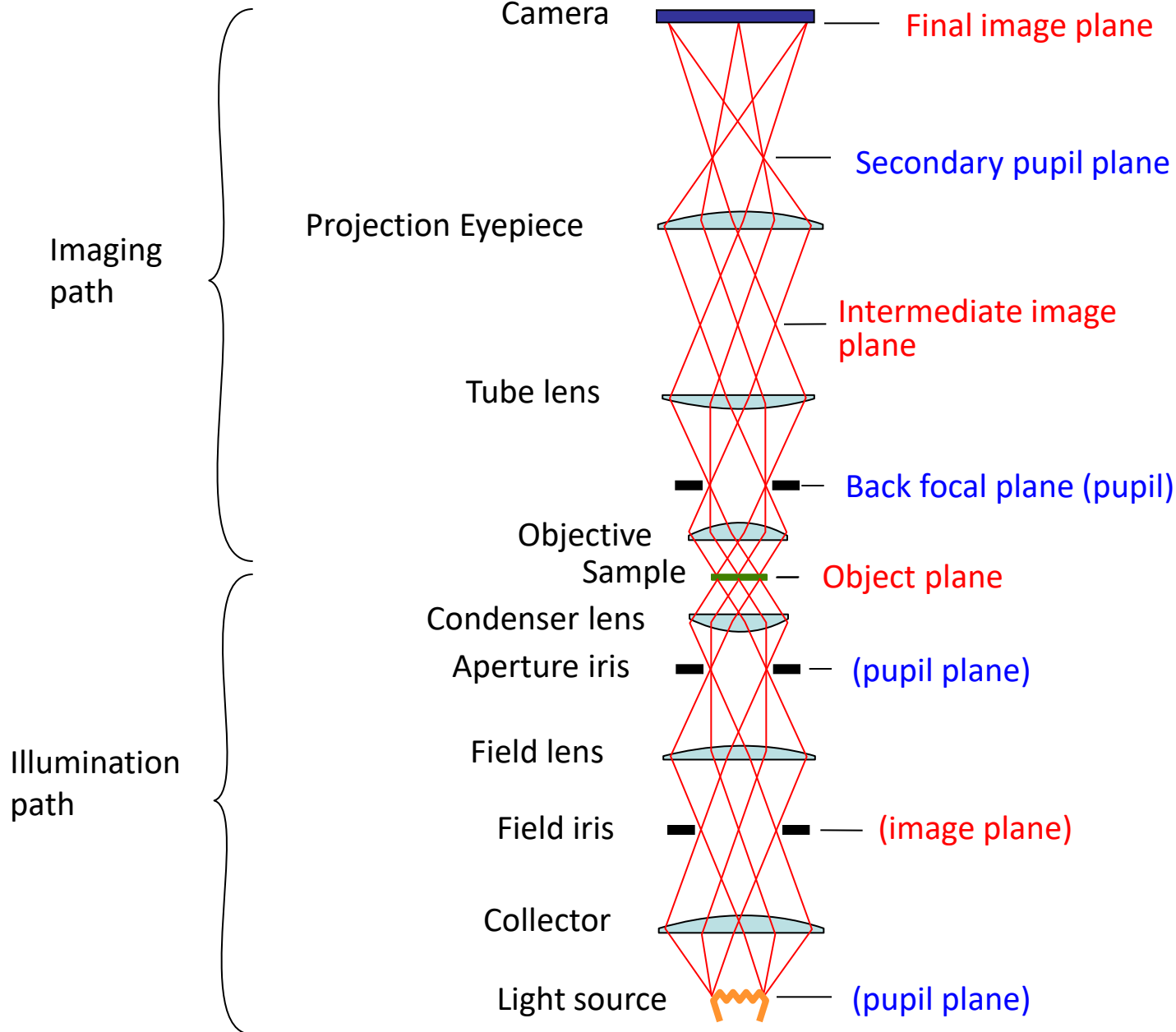


Resulting wave is now reduced in amplitude,  
and this amplitude change is detectable

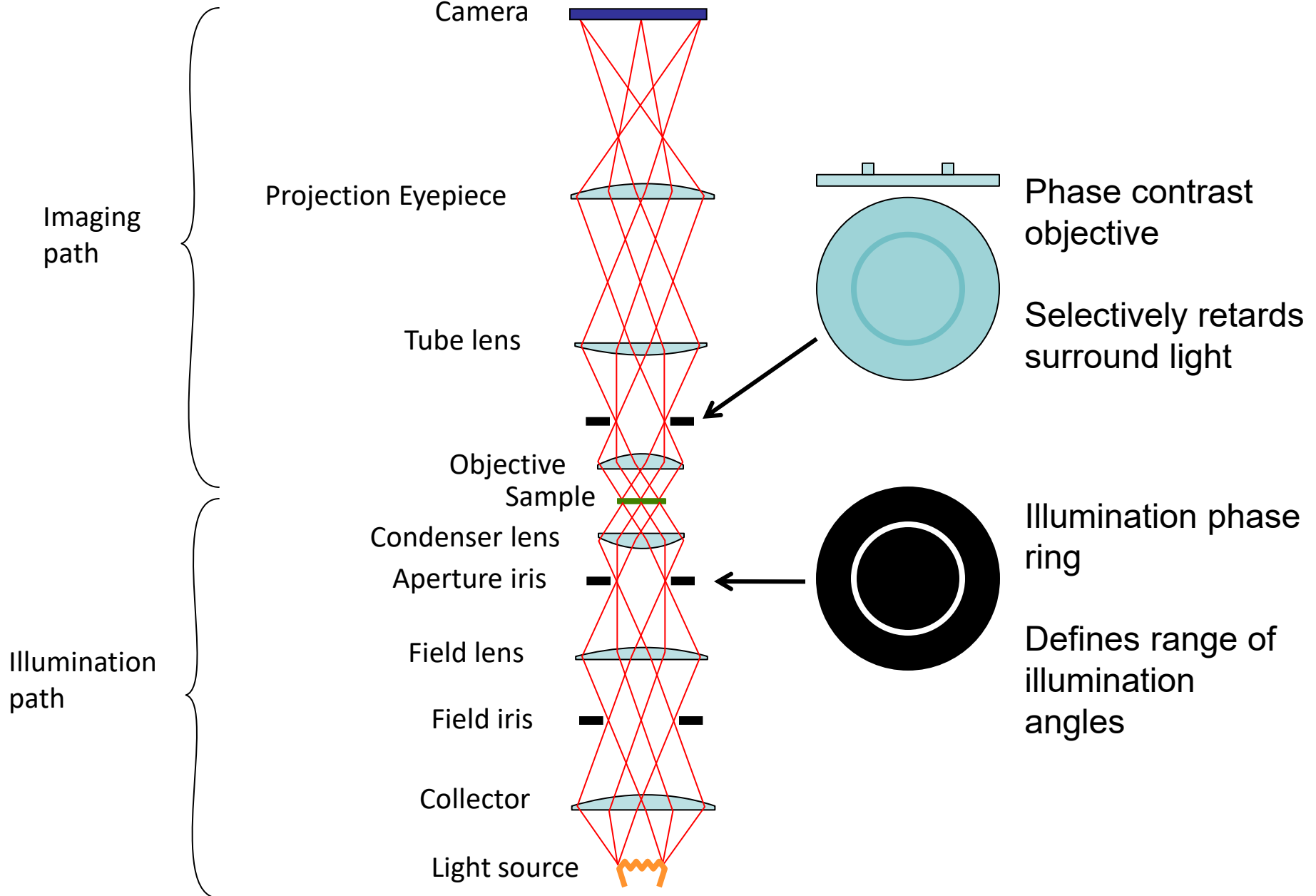
# Phase contrast microscopy

- Need to selectively shift the phase of the surround wave. How to do this?
- The sample will scatter light in all directions, so if we illuminate with a small range of angles we can specifically alter the phase at those angles

# The phase contrast microscope

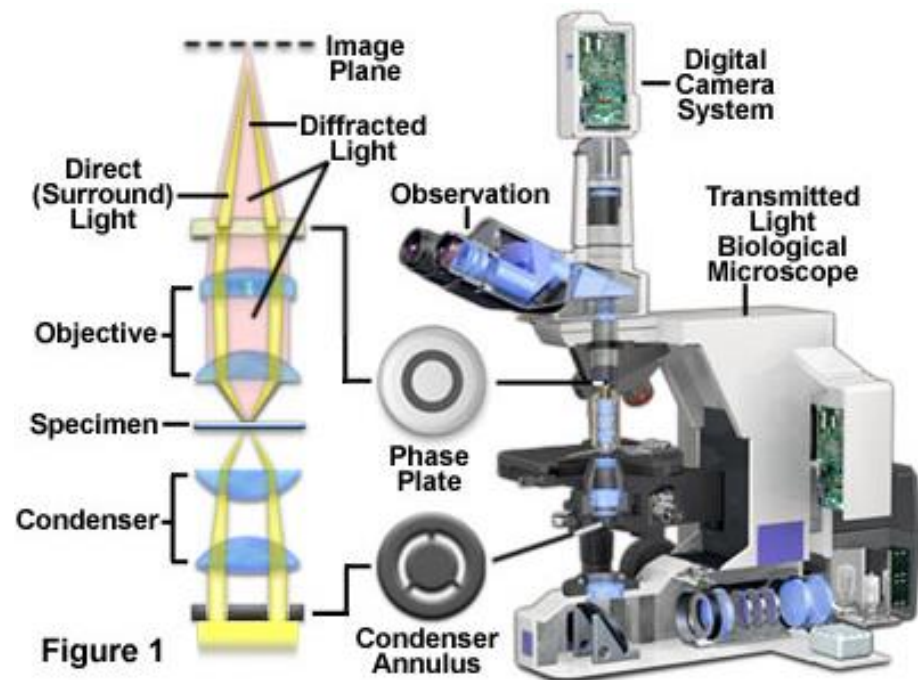
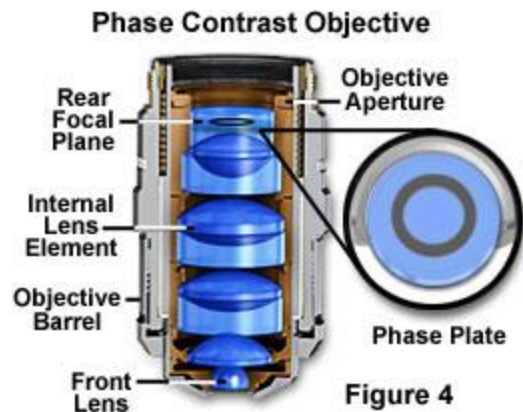


# The phase contrast microscope



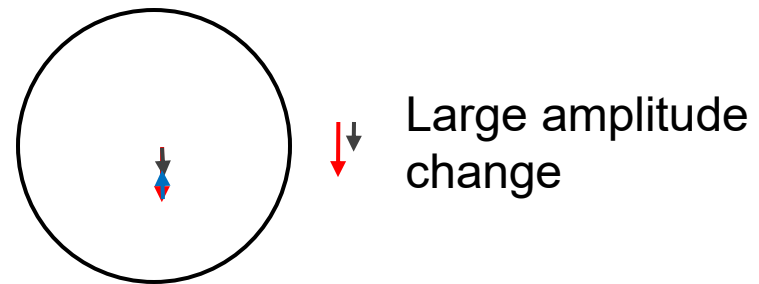
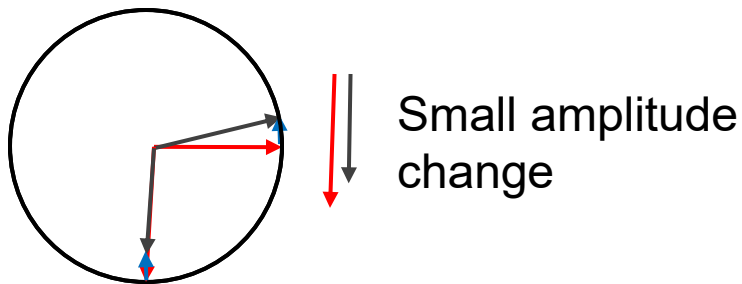
# Phase Contrast in the Microscope

Phase ring is typically built into objective



# Phase Contrast – Further Refinements

- Typically, phase contrast ring in objective attenuates surround wave by  $\sim 75\%$
- Enhances visibility of small phase differences



# Phase Contrast – Further Refinements



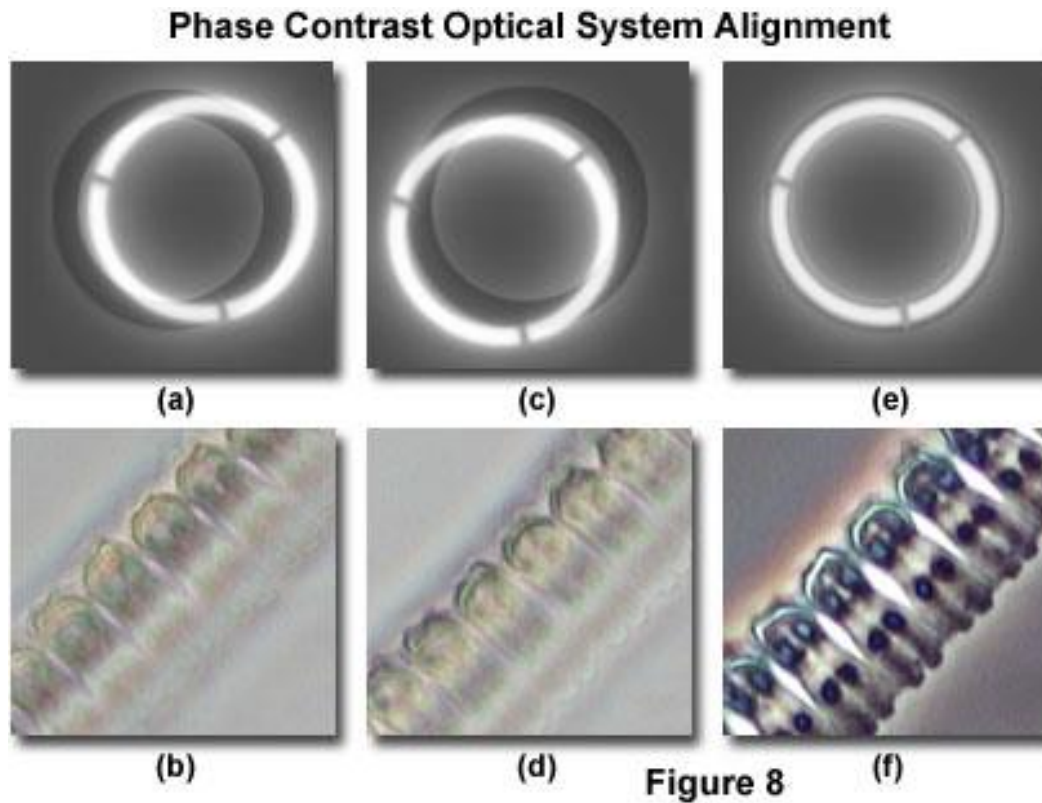
- Negative phase contrast
  - Retards surround wave, so objects which advance phase (low refractive index) are brighter



- Positive phase contrast
  - Advances surround wave, so objects which retard phase (high refractive index) are brighter



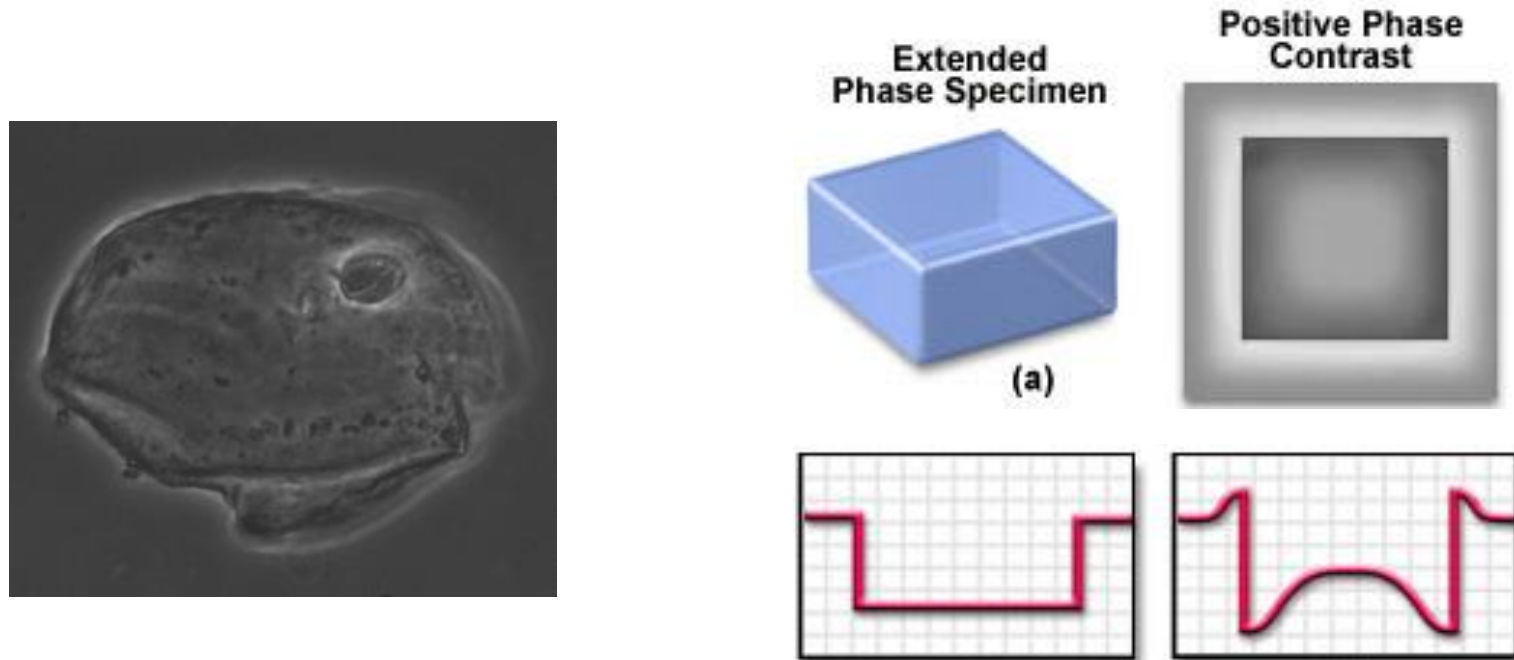
# Alignment of phase rings



Back focal plane

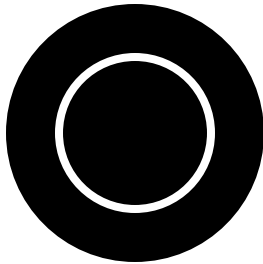
Front focal plane

# Limitations of Phase Contrast



- Halos result from diffracted light that is intercepted by phase ring
- Shade off is caused by greater diffraction at edges of objects than their centers

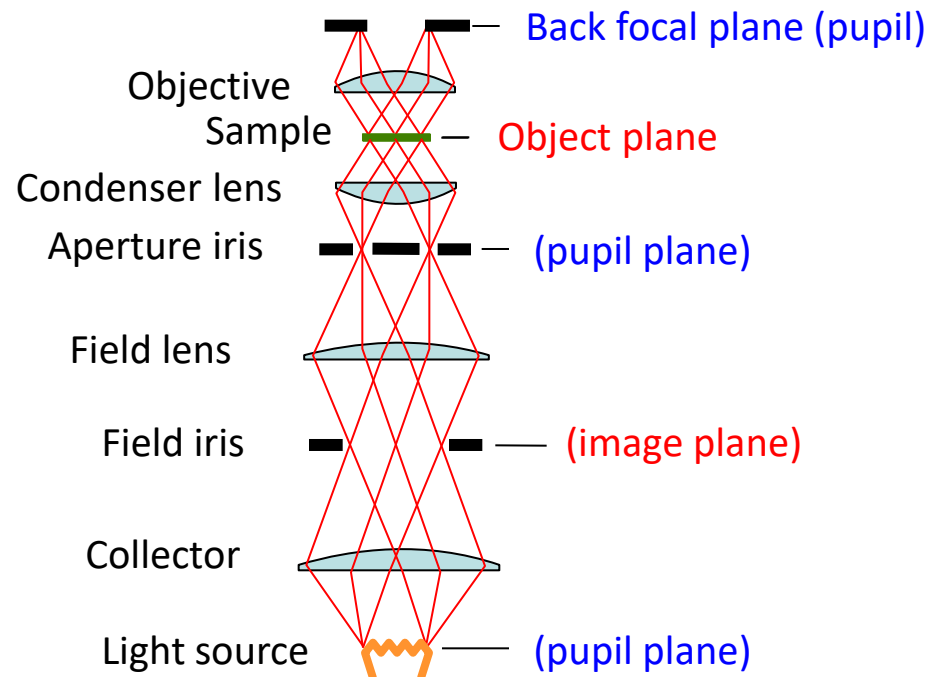
# Limitations of Phase Contrast



- Poor optical sectioning due to limited illumination aperture.
- For sufficiently thick samples, can get more than  $360^\circ$  phase shift, meaning thin and thick regions will be identical in contrast.

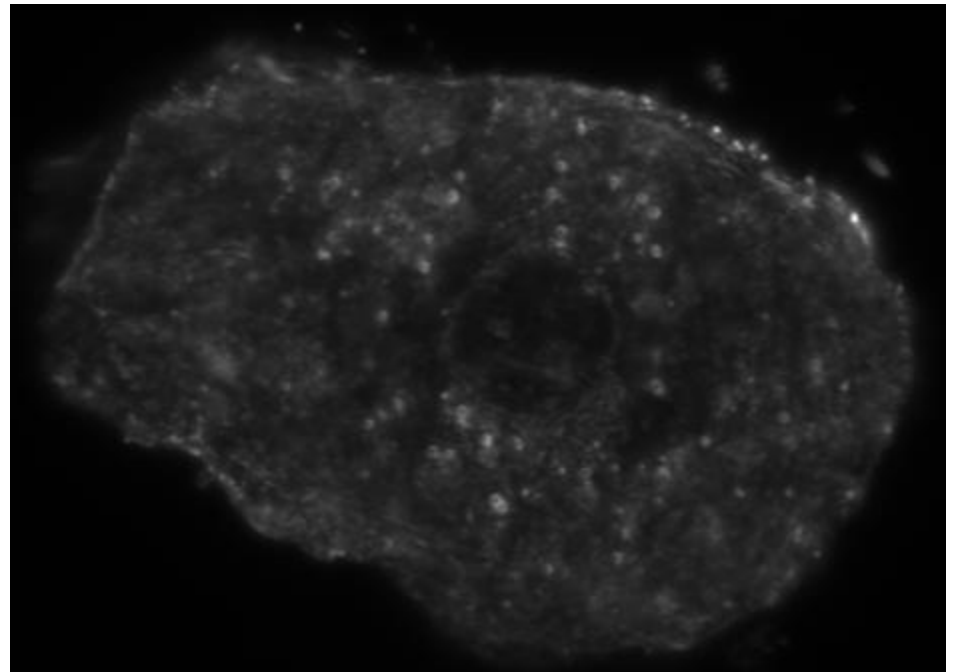
# Darkfield Microscopy

- Idea: throw away all non-diffracted light by illuminating with a condenser annulus at higher NA than the objective.
- No illumination light makes it through the objective, only diffracted light.

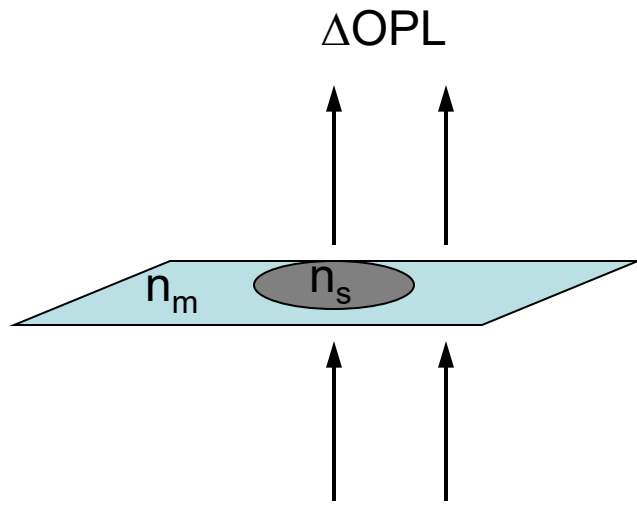


# Darkfield Microscopy

- Ideal for small particles, unstained microorganisms, etc.
- Can easily see subdiffraction particles (e.g. 40 nm gold)
- Not so good for large objects



# Differential Interference Contrast (DIC)

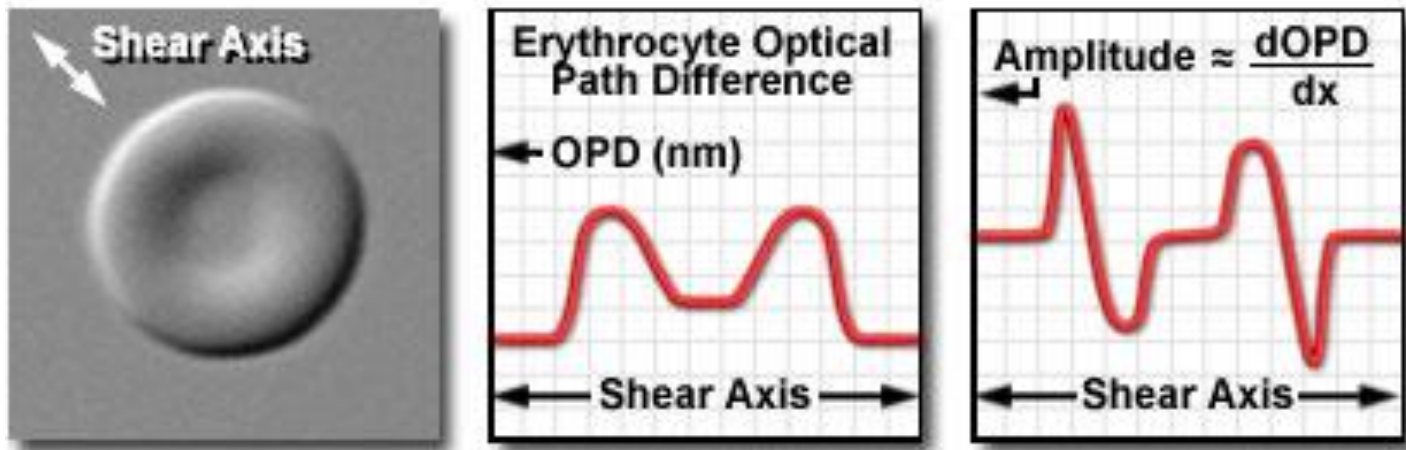


The idea:

Use two beams and interferometry to measure the path length difference between adjacent points in the sample

# What DIC accomplishes

**Specimen Optical Path Difference and DIC Amplitude Profile**



Converts relative differences in optical path length to differences in amplitude

# Features of a DIC image

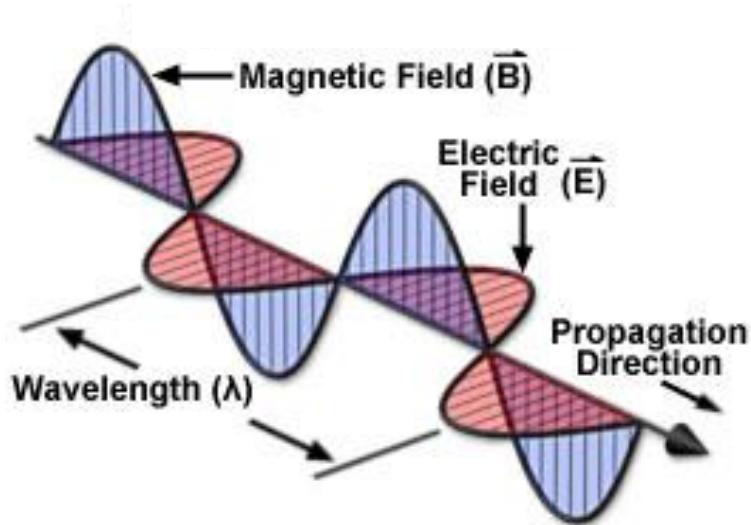


1. Contrast is directional
2. Contrast highlights edges
3. One end brighter, other is dimmer giving a pseudo – 3D image



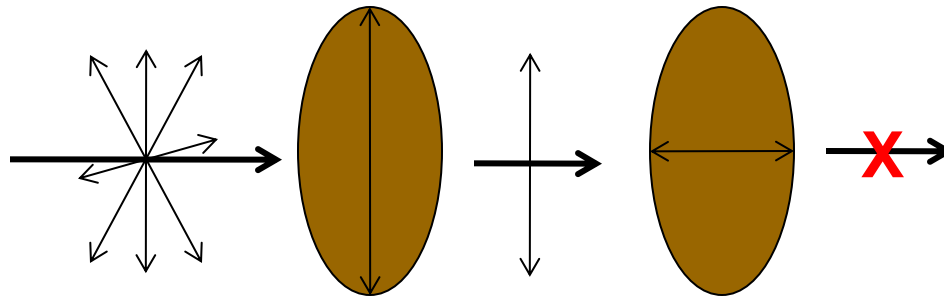
# Polarization

- Polarization: orientation of E-field.
- Most light sources produce unpolarized light – no preferred polarization angle



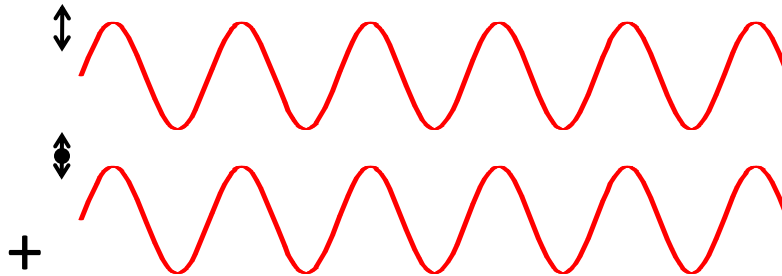
# Polarizers

- Polarizers specifically transmit one polarization angle of light
- Crossed polarizers transmit no light

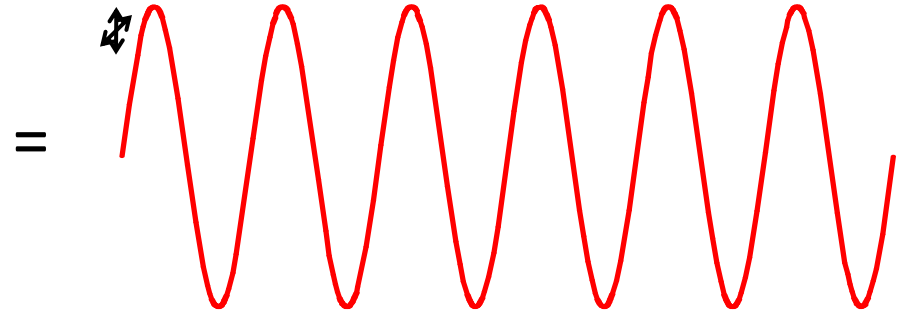


# Interference and polarization

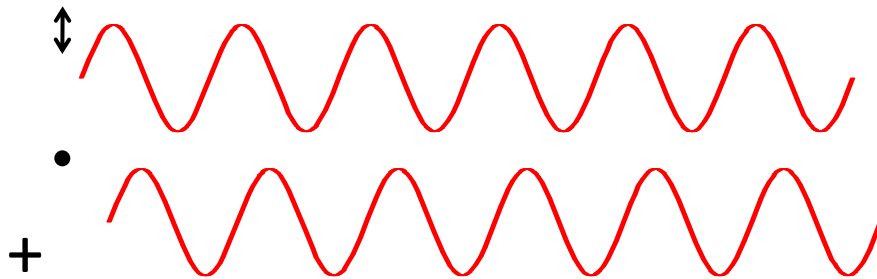
In phase



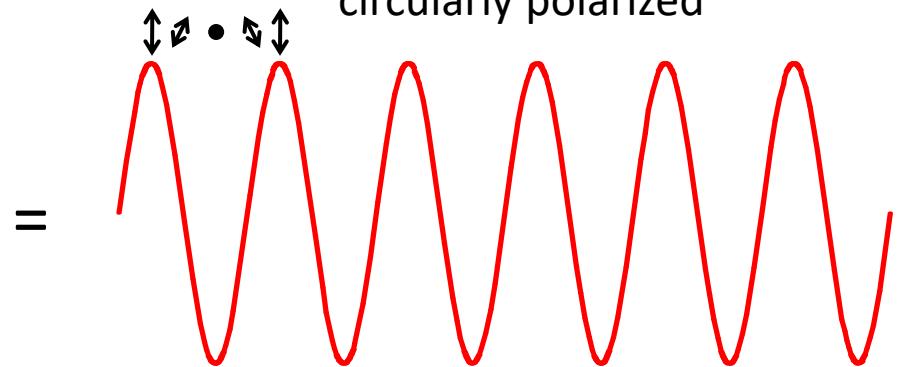
linearly polarized



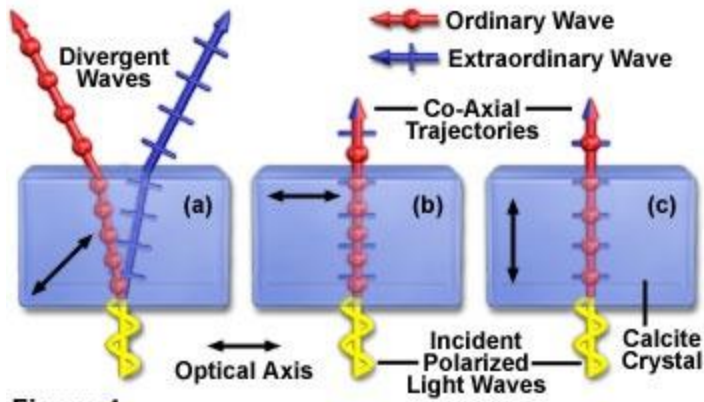
Phase lag



circularly polarized



# Birefringence



Bi-Refraction in Calcite Crystals

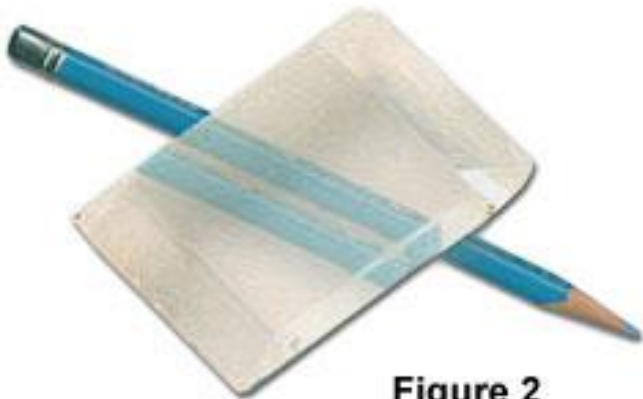
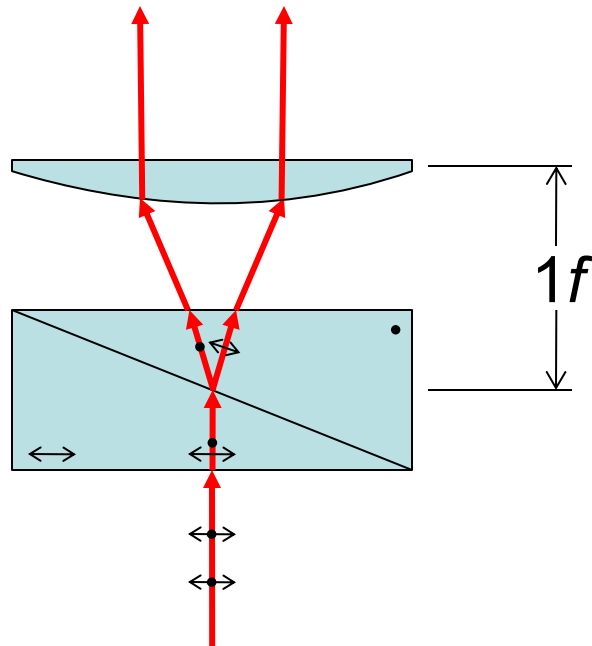


Figure 2

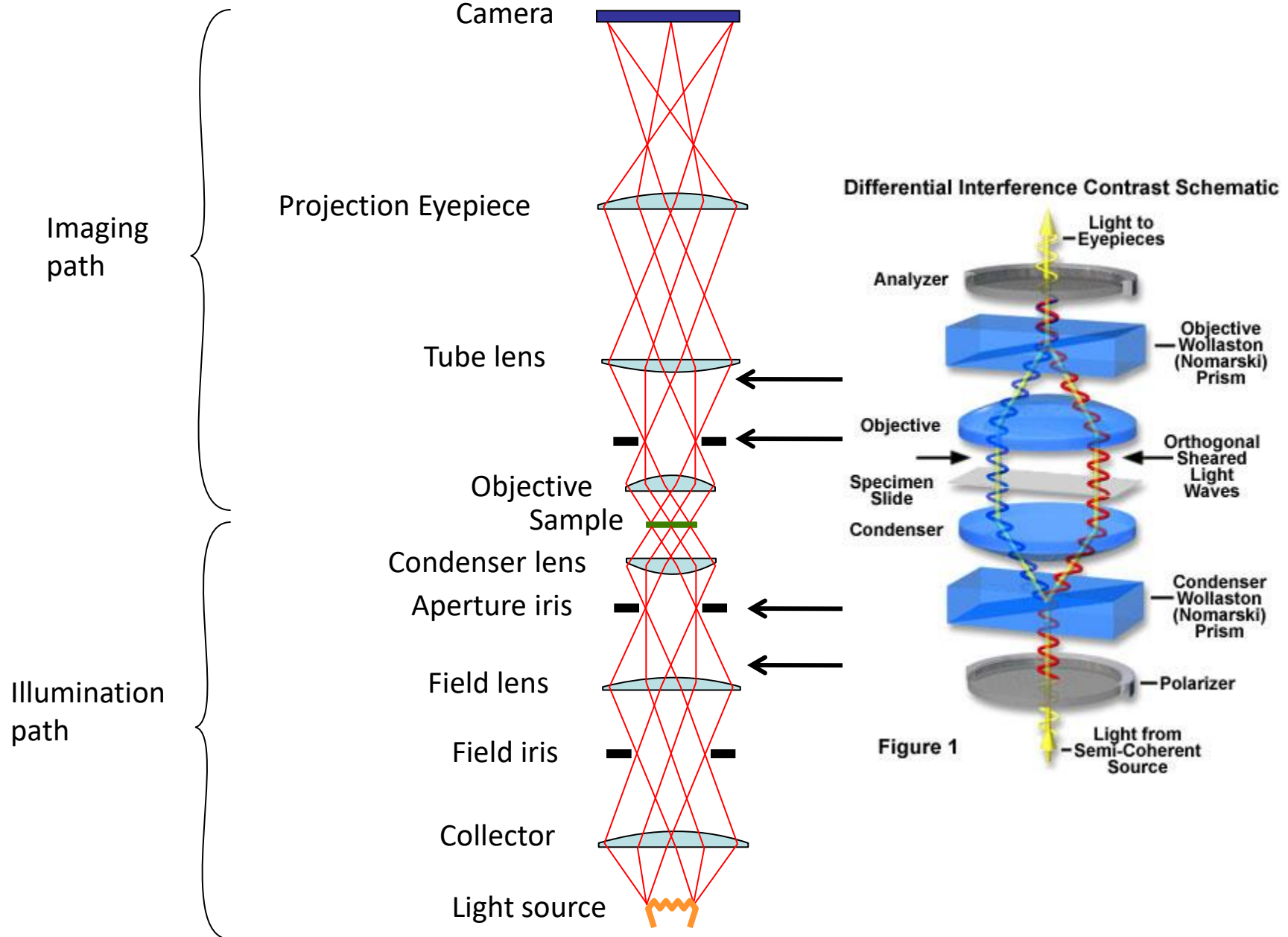
- Birefringent materials have different indices of refraction for light polarized parallel or perpendicular to the optical axis.
- For unpolarized light two beams with orthogonal polarization are produced illuminated not along optical axis

# Wollaston / Nomarski Prisms



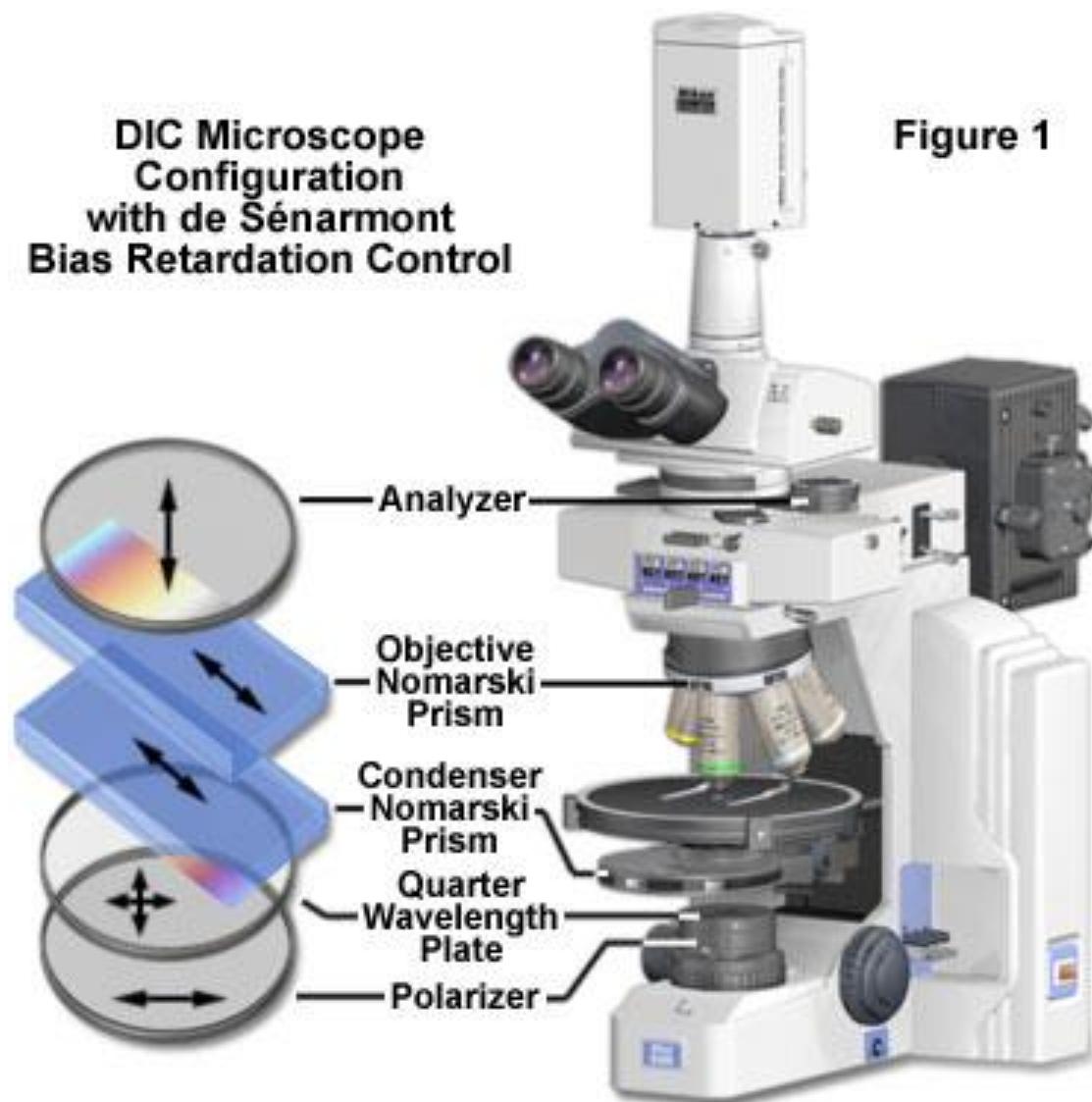
- Two pieces of cemented calcite / quartz
- Produce orthogonally polarized beams propagating at different angles

# The differential interference contrast (DIC) microscope

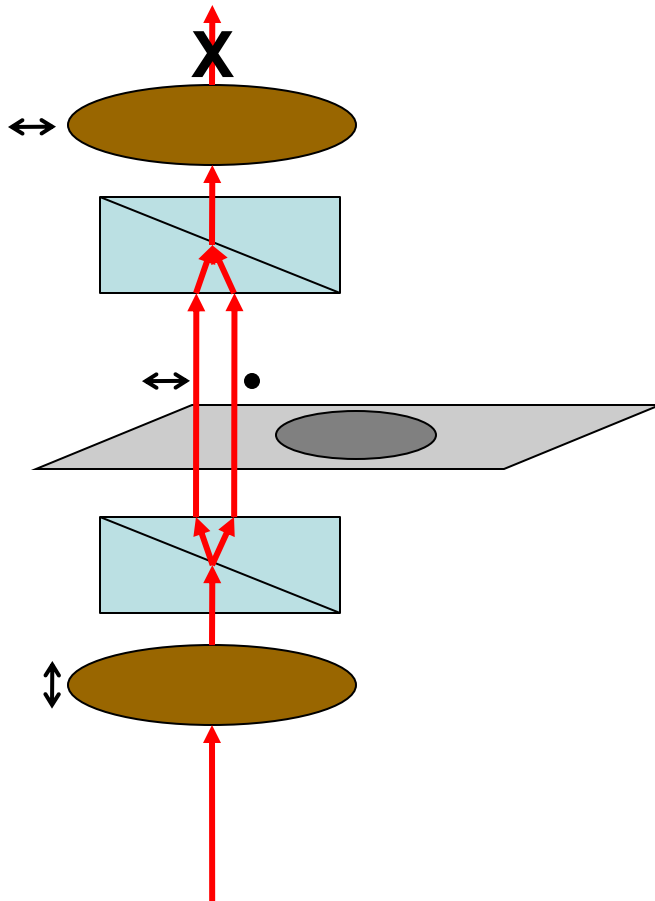


**DIC Microscope  
Configuration  
with de Sénarmont  
Bias Retardation Control**

**Figure 1**



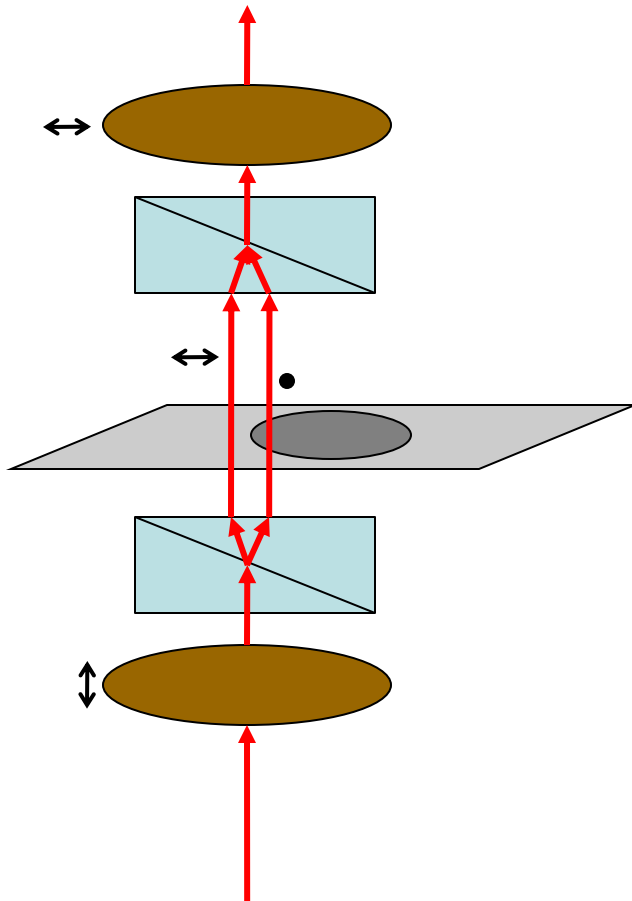
# How DIC generates contrast



- Both beams see same OPL
- Emerge in phase
- Regenerate initial polarization
- No light makes it through analyzer

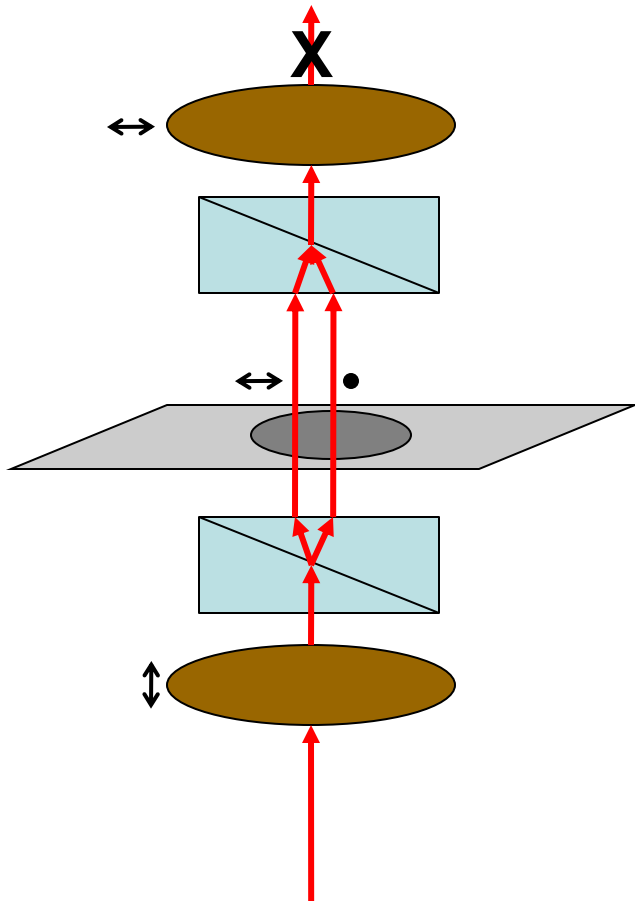


# How DIC generates contrast



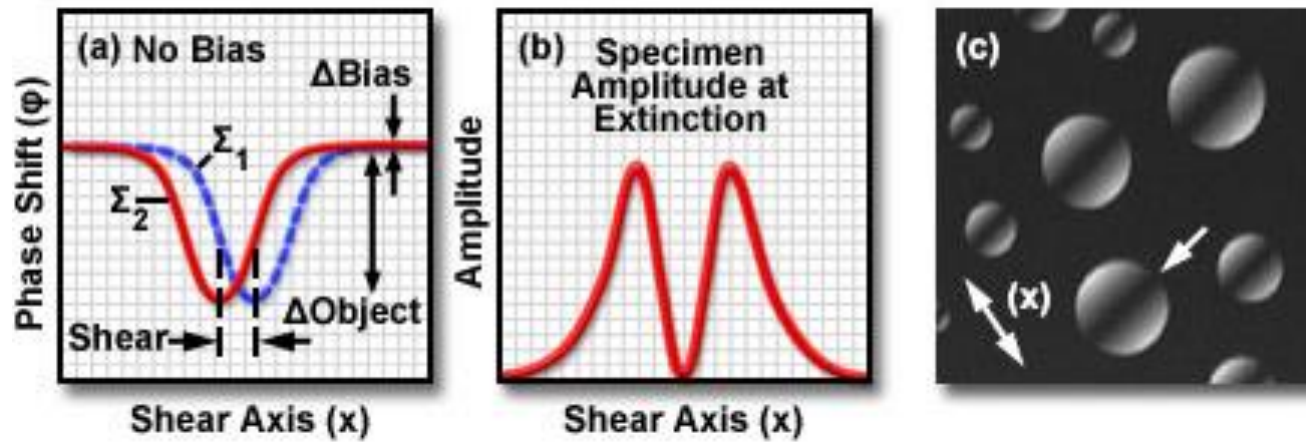
- Beams see different OPL
- Right beams is phase retarded
- Generate elliptical polarization
- Light makes it through analyzer

# How DIC generates contrast

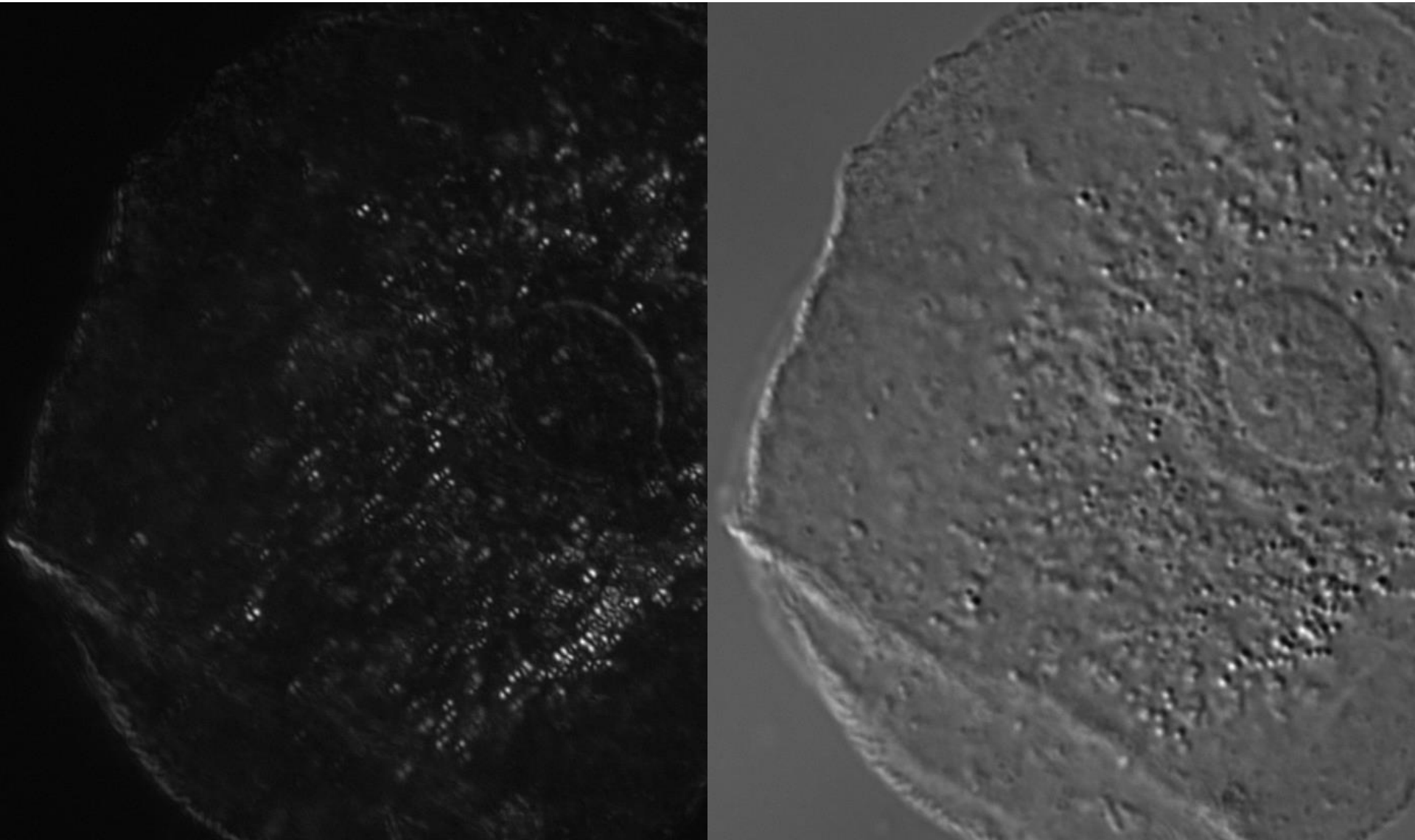


- Both beams see same OPL
- Emerge in phase
- Regenerate initial polarization
- No light makes it through analyzer

# Role of Bias in DIC



# Role of Bias in DIC



# Bias adjustment in de Sénarmont DIC

## de Sénarmont Compensator Wavefronts

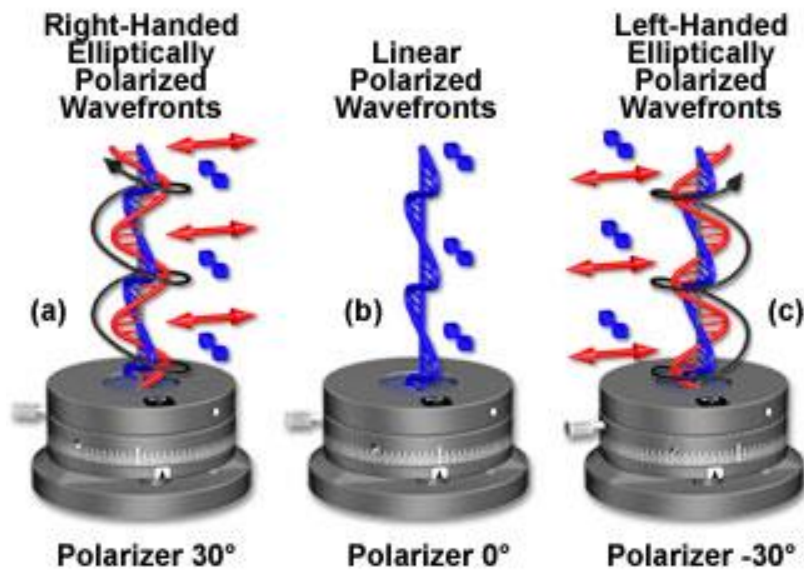


Figure 4

## Bias Retardation in de Sénarmont DIC Microscopy

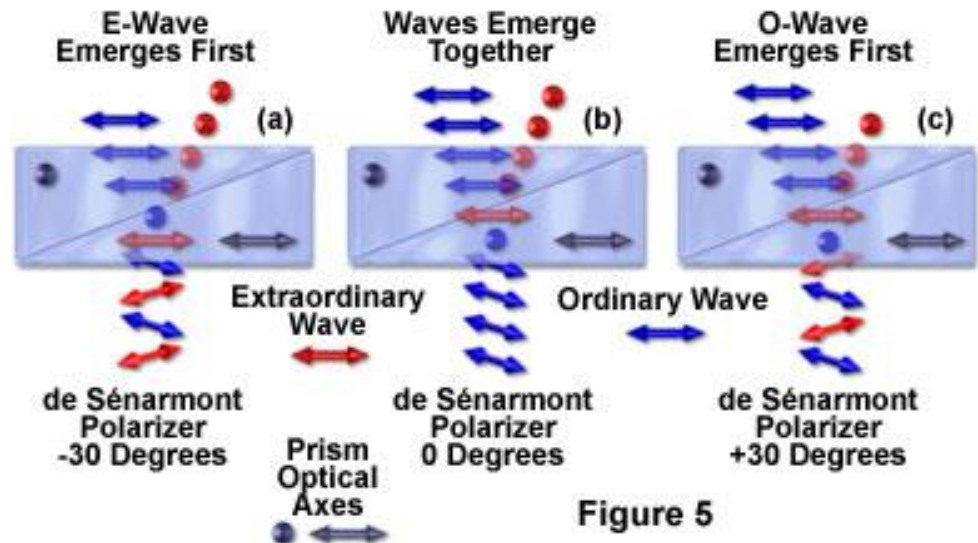
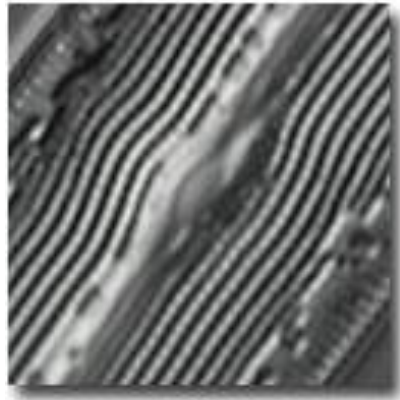
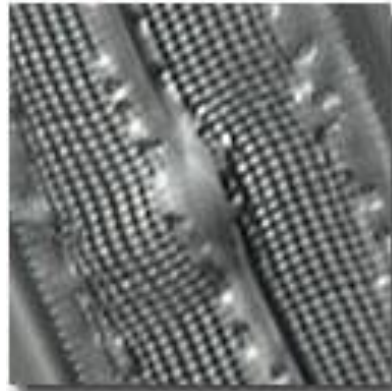


Figure 5

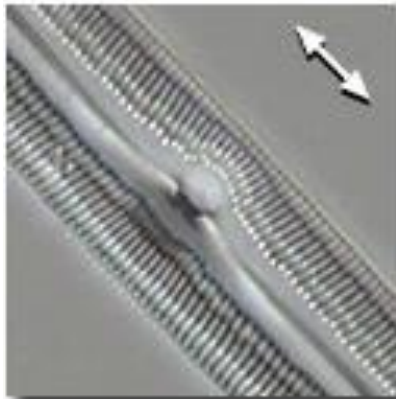
# DIC is sensitive to specimen orientation



(a)



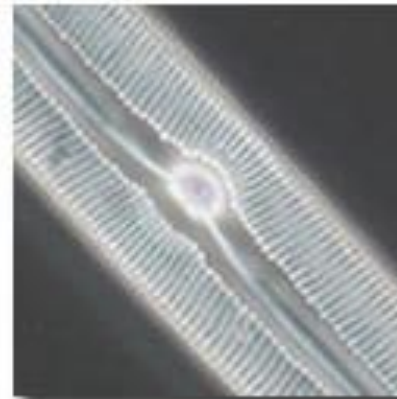
(b)



(a)



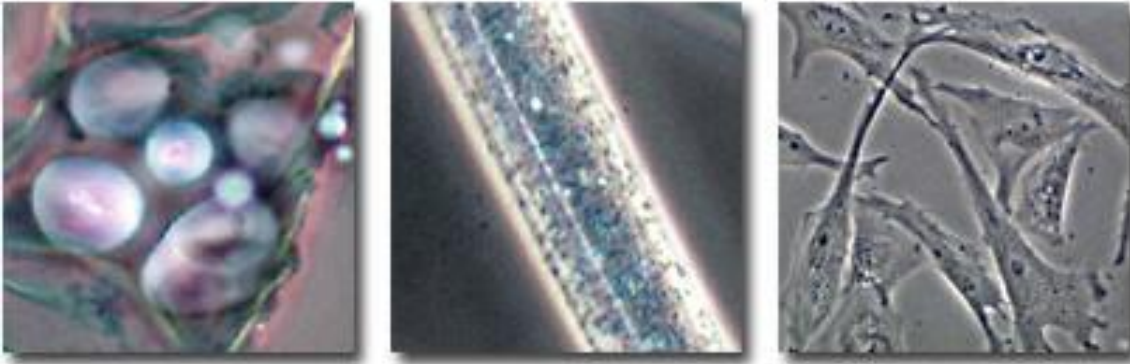
(b)



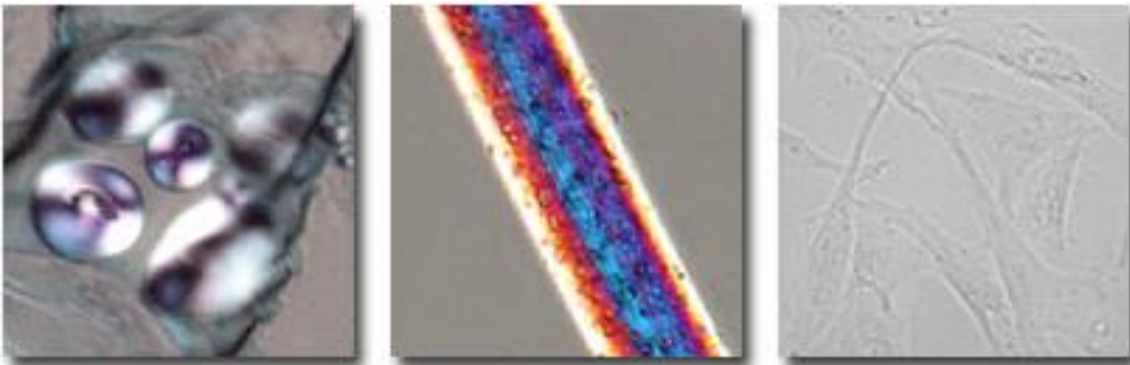
(c)

# DIC doesn't work on birefringent samples

Phase



DIC

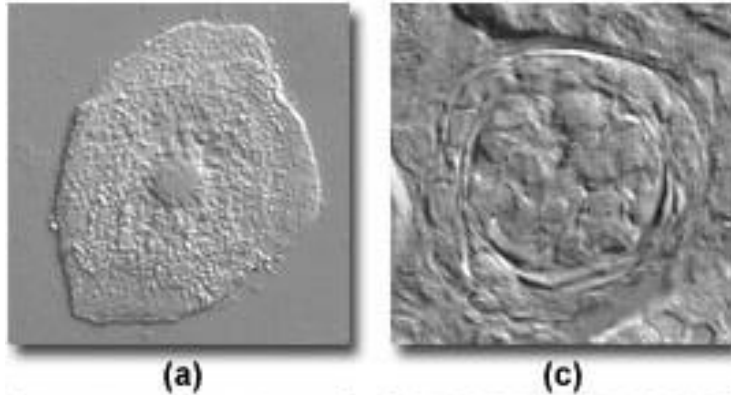


Can't plate cells on or  
cover cells with  
plastic.



DIC is higher resolution than phase contrast

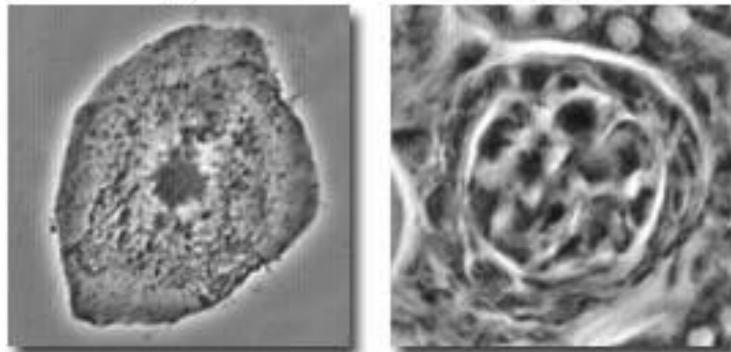
DIC



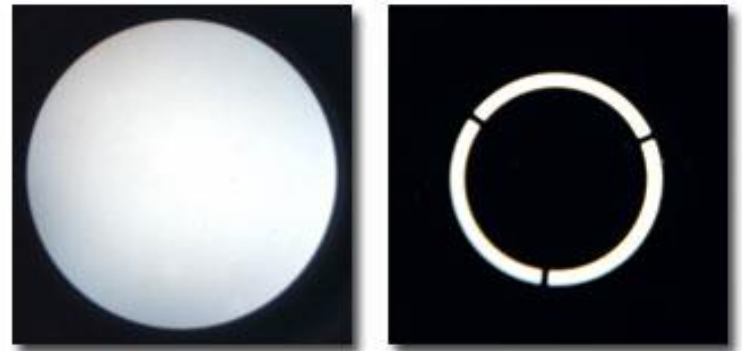
(a)

(c)

Phase

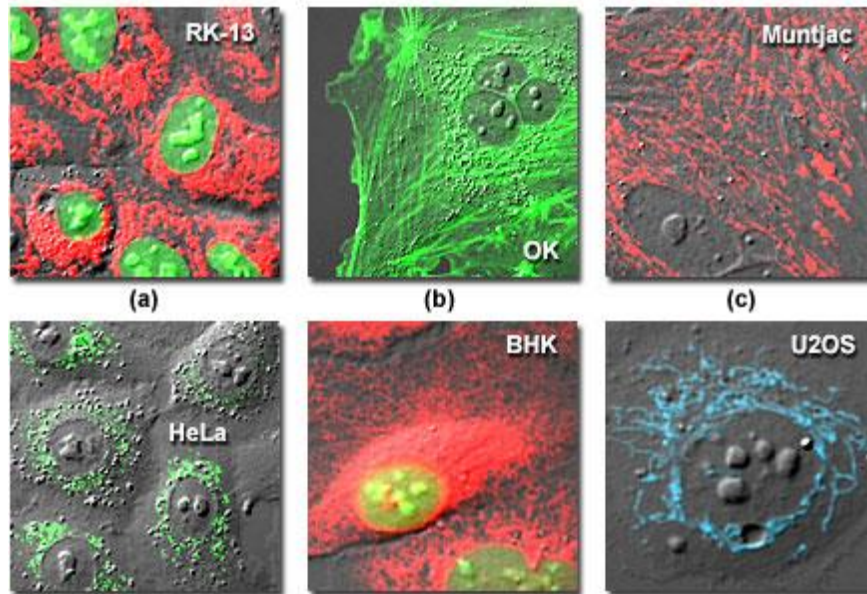


Microscope Apertures in DIC and Phase Contrast





# Combining Phase / DIC with fluorescence



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

Phase and DIC do degrade fluorescence performance slightly

## Further reading

[www.microscopyu.com](http://www.microscopyu.com)

[micro.magnet.fsu.edu](http://micro.magnet.fsu.edu)

Douglas B. Murphy, “Fundamentals of Light Microscopy and Electronic Imaging”

Hecht, “Optics”

## Acknowledgements

Orion Weiner / Mats Gustafsson