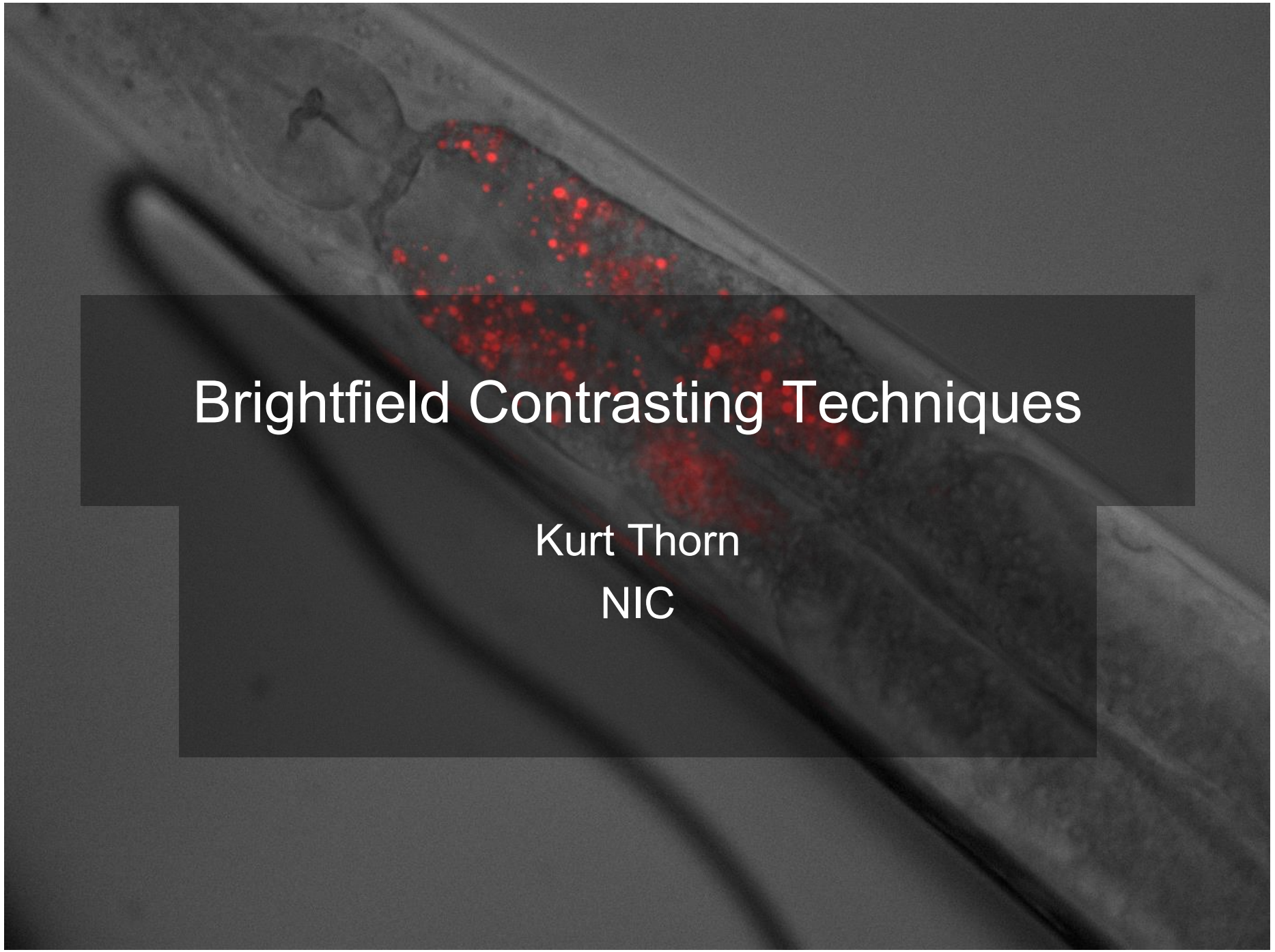
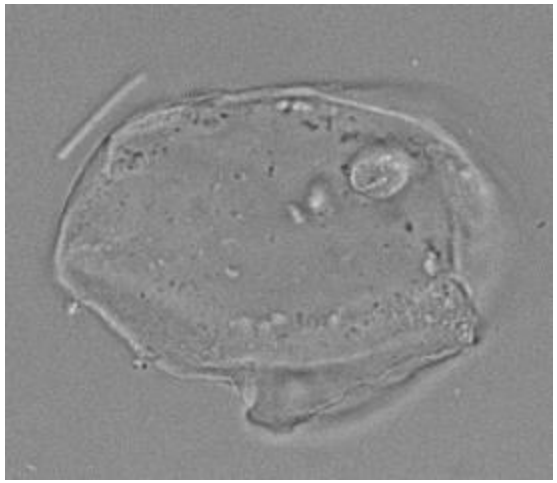


Brightfield Contrasting Techniques

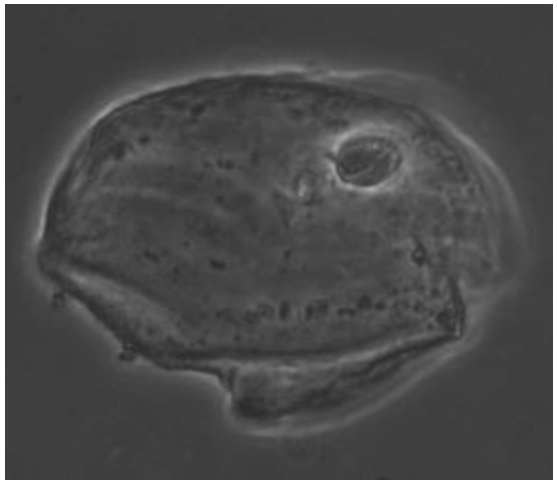
Kurt Thorn
NIC

Generating contrast in light microscopy

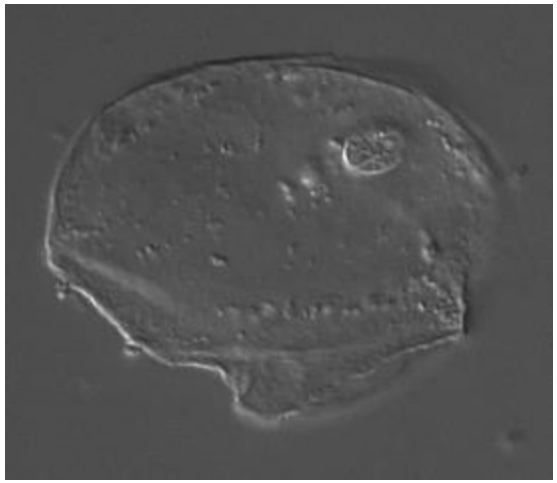
- Problem: Many biological specimens are thin and transparent and difficult to see.
- Solution:
  - Fluorescent staining
  - Brightfield contrasting techniques: DIC, Phase, others



Brightfield

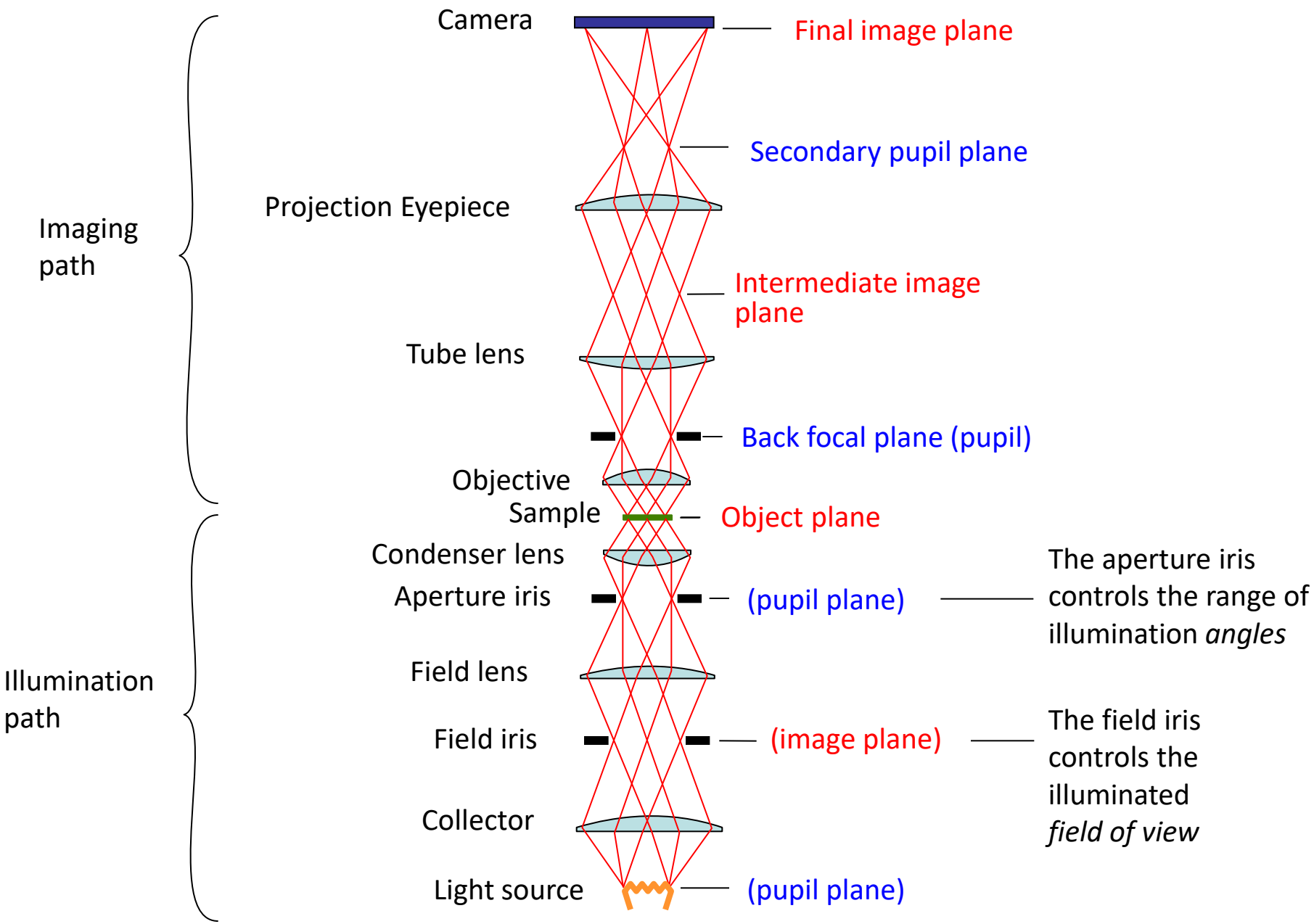


Phase Contrast



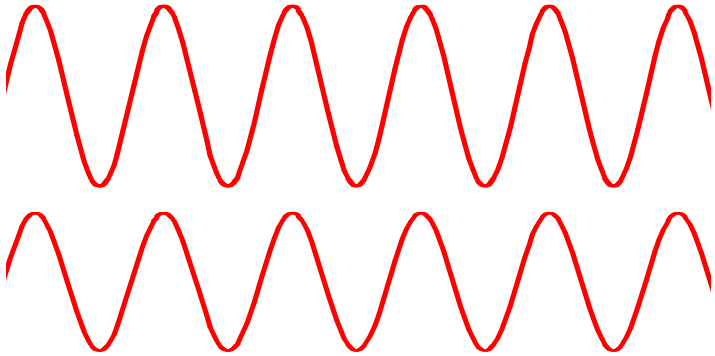
DIC

Review: The Trans-illumination Microscope

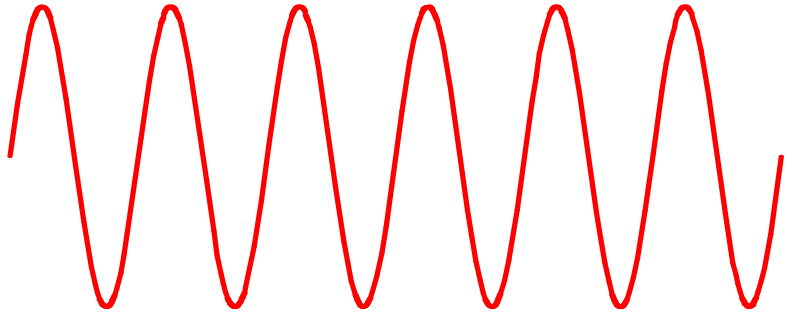


Reminder: Interference

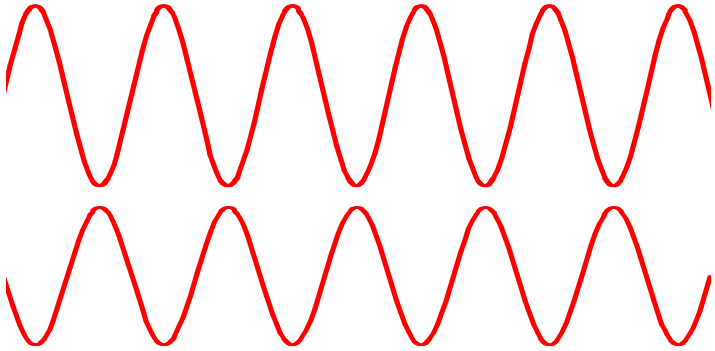
In phase



constructive interference

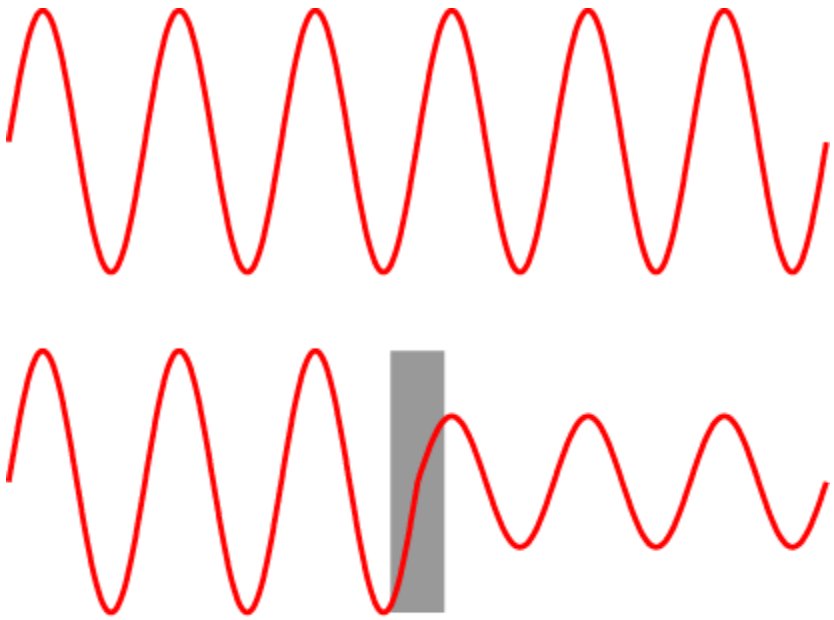


Opposite phase



destructive interference

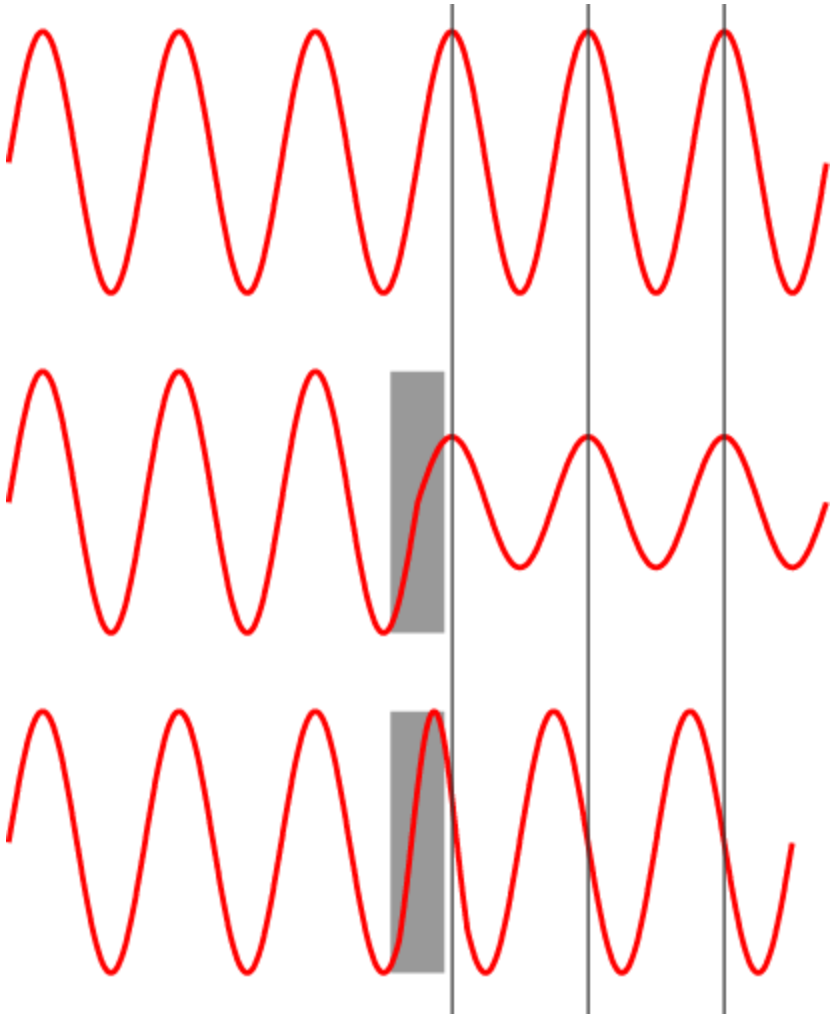
Amplitude and Phase Samples



Initial beam, unpurturbed

Amplitude sample

Amplitude and Phase Samples



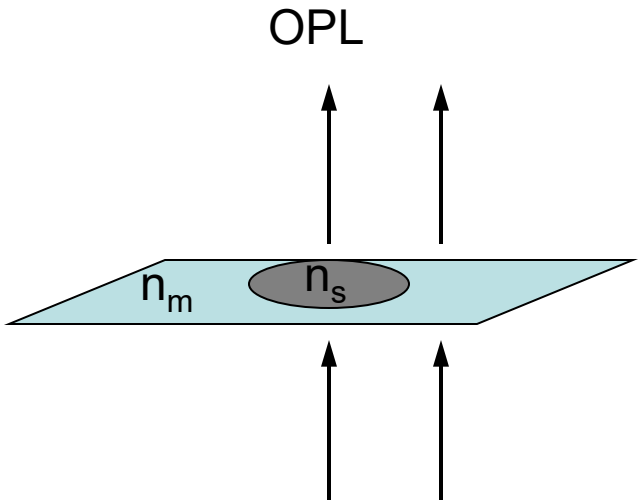
Initial beam, unpurturbed

Amplitude sample

Phase sample

Many biological samples are phase samples

Higher refractive indices slow down light



t = sample thickness , ~ 1 micron

n_s = sample refractive index , ~ 1.38

n_m = medium refractive index, ~ 1.33

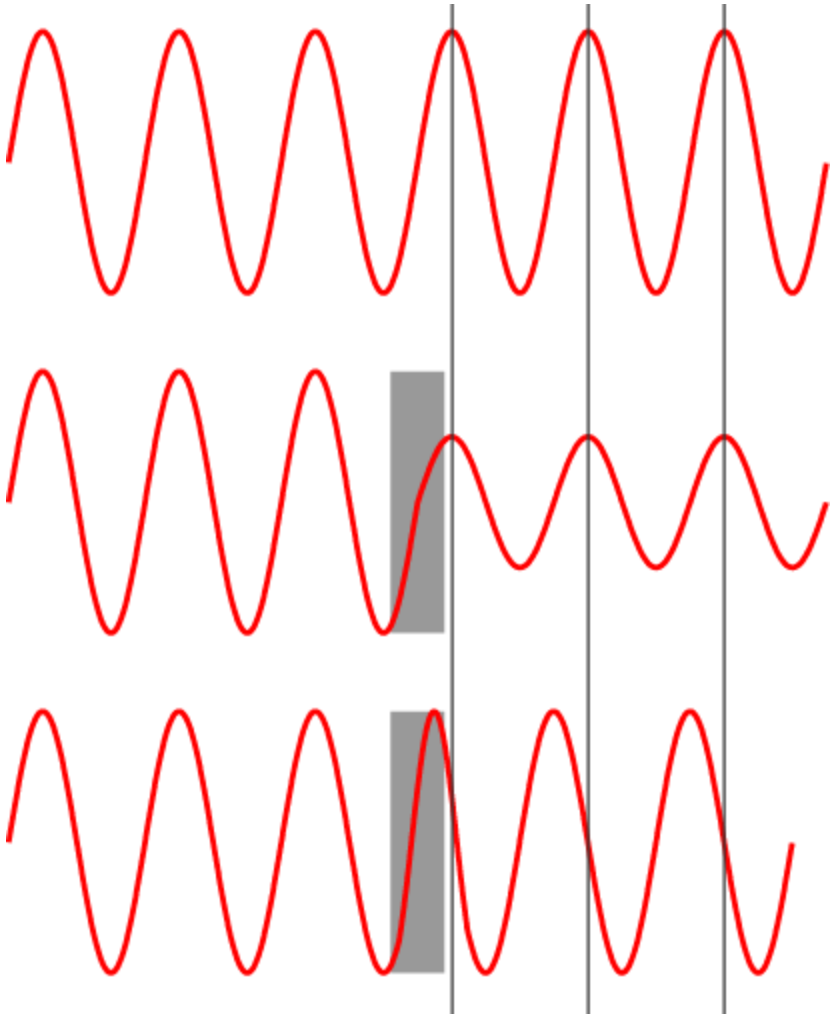
Optical path length (OPL) difference = t (n_s-n_m)

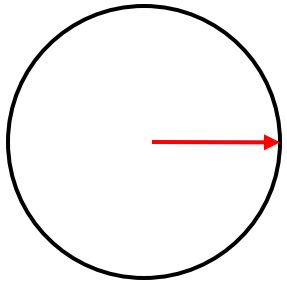
= 1 micron (1.38-1.33) = .05 microns = 50 nm,

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

Amplitude and Phase Samples

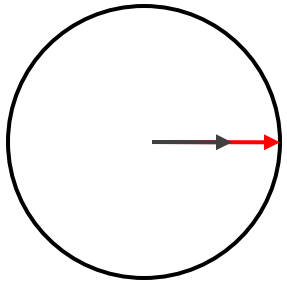
Represented as Vectors



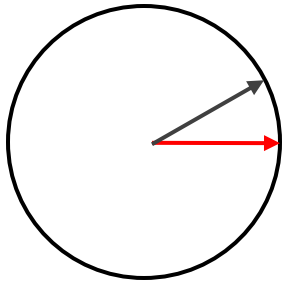


Vector length: amplitude

Rotation around circle: phase



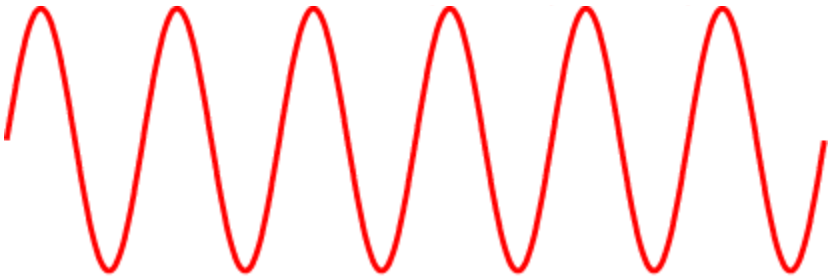
Only amplitude changes

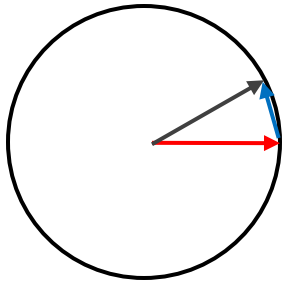


Only phase changes

How can we see a phase change?

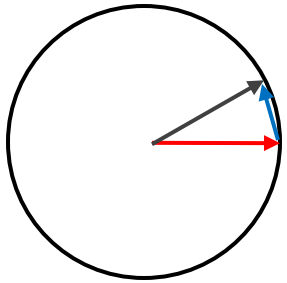
Problem: optical detectors are not phase sensitive; they only detect changes in amplitude





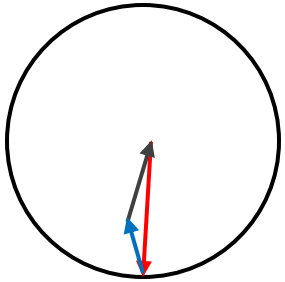
Decompose phase shift into an unpurturbed wave and a perturbed wave

Converting a phase change to an amplitude change



Unpurturbed wave (Surround wave)
Perturbed wave (Diffracted wave)
Resulting wave (Particle wave)

Imagine shifting phase of surround wave by 90˚

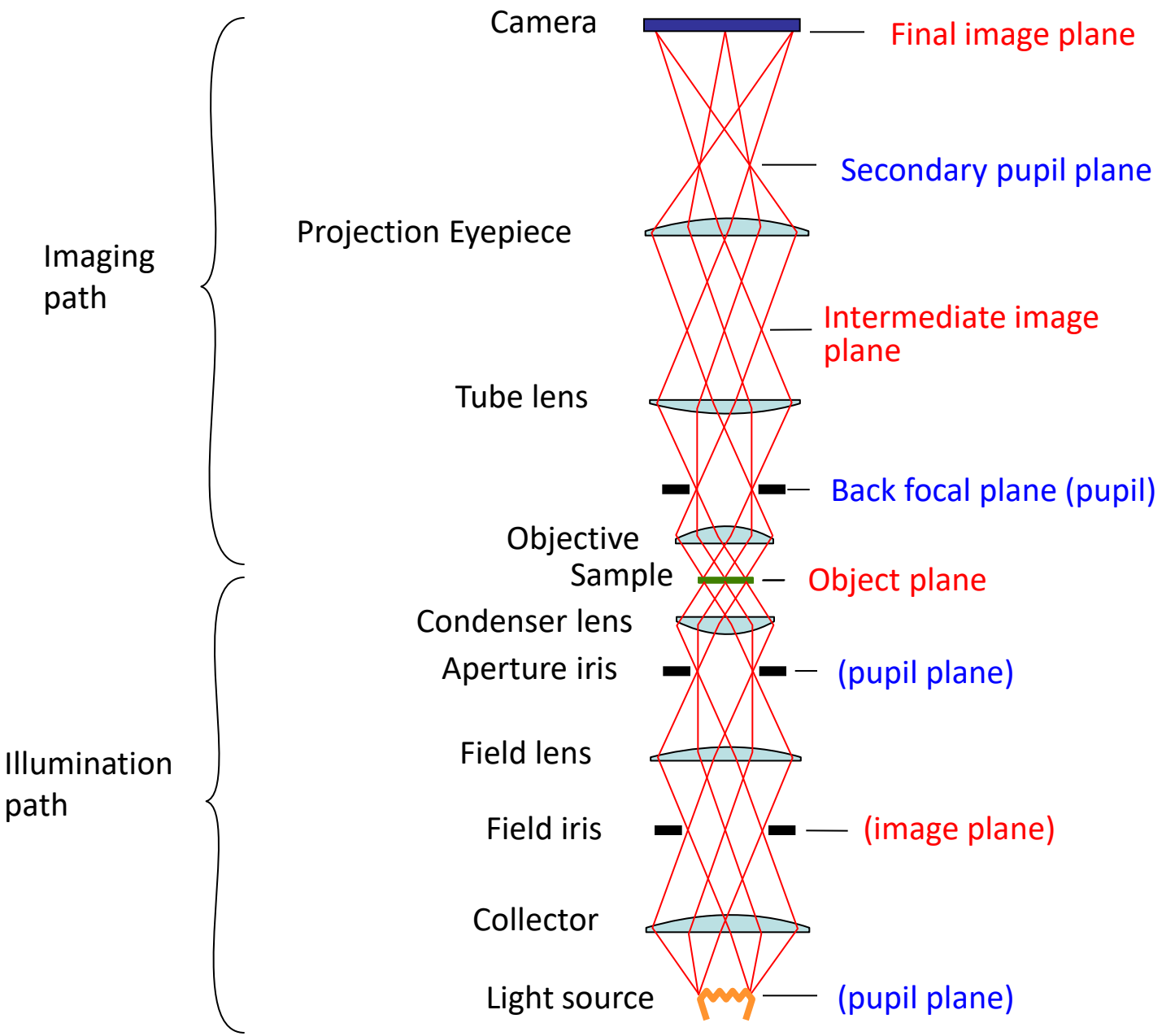


Resulting wave is now reduced in amplitude, and this amplitude change is detectable

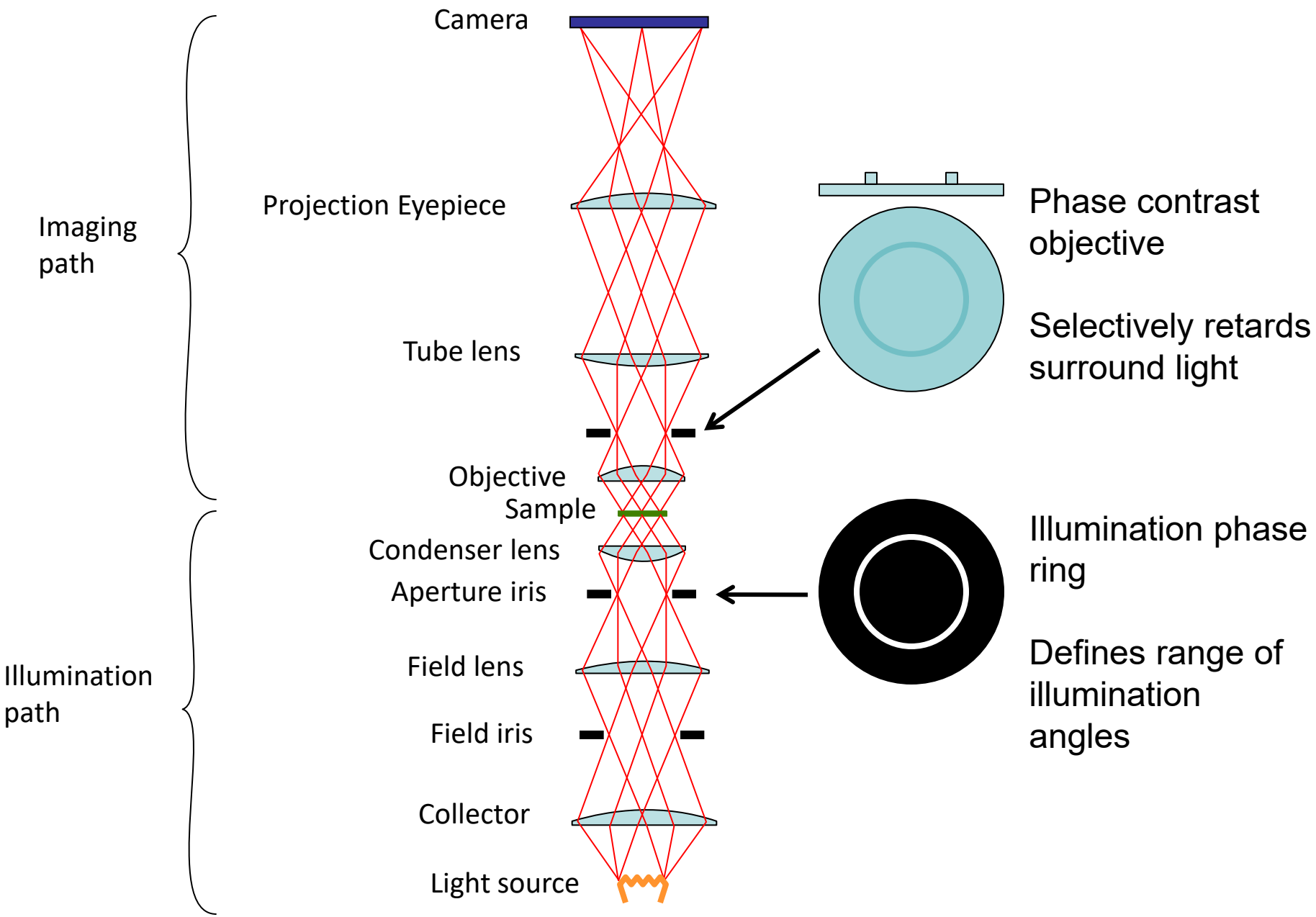
Phase contrast microscopy

- 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

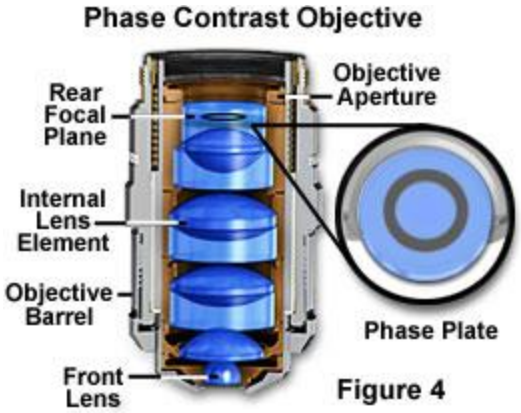


Figure 4

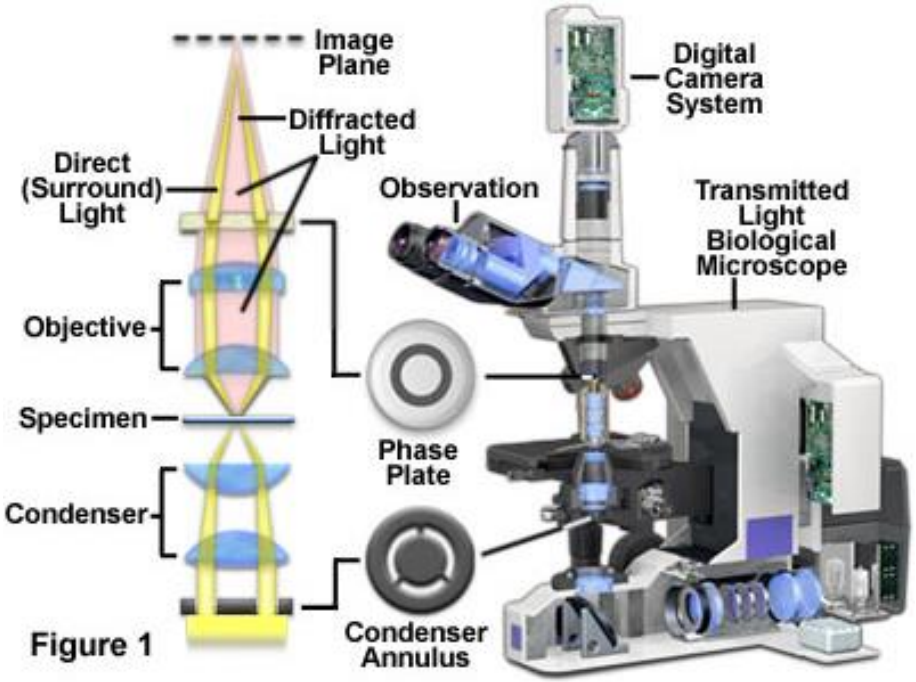
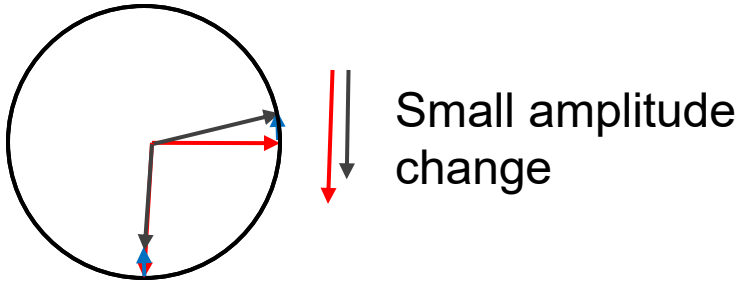
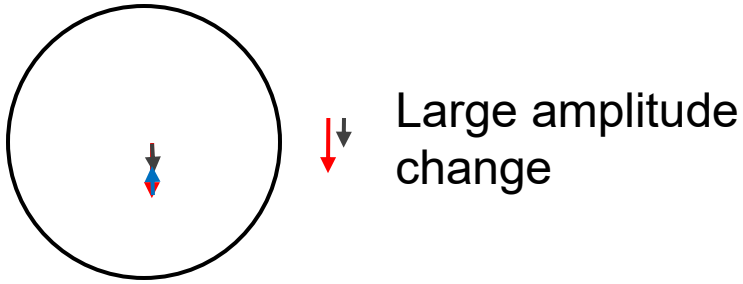


Figure 1

Phase Contrast – Further Refinements

- Typically, phase contrast ring in objective attenuates surround wave by ~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

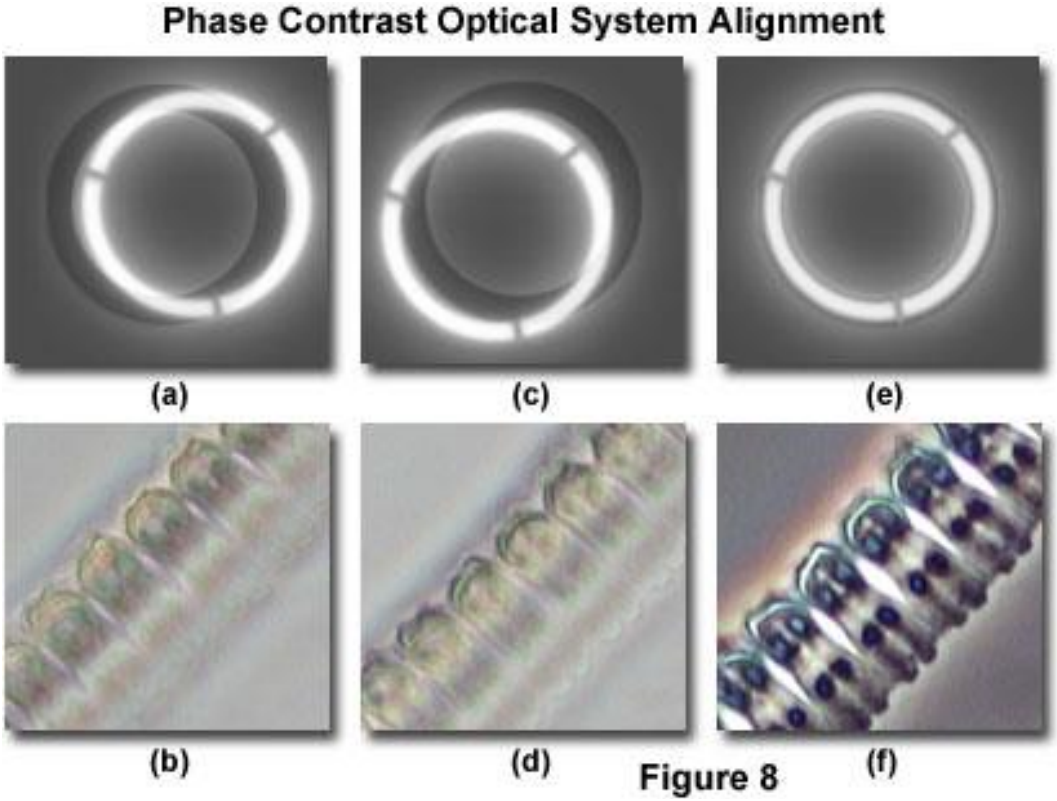
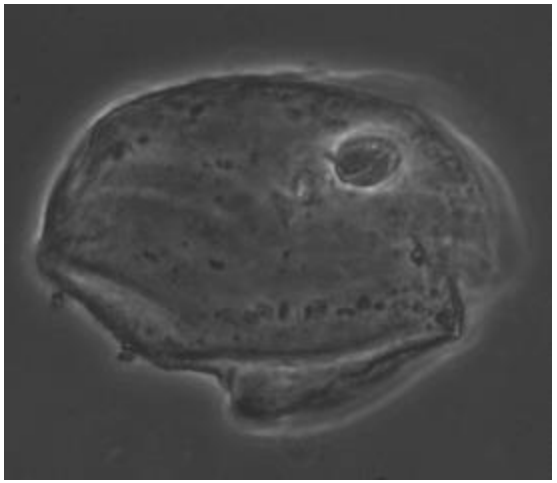


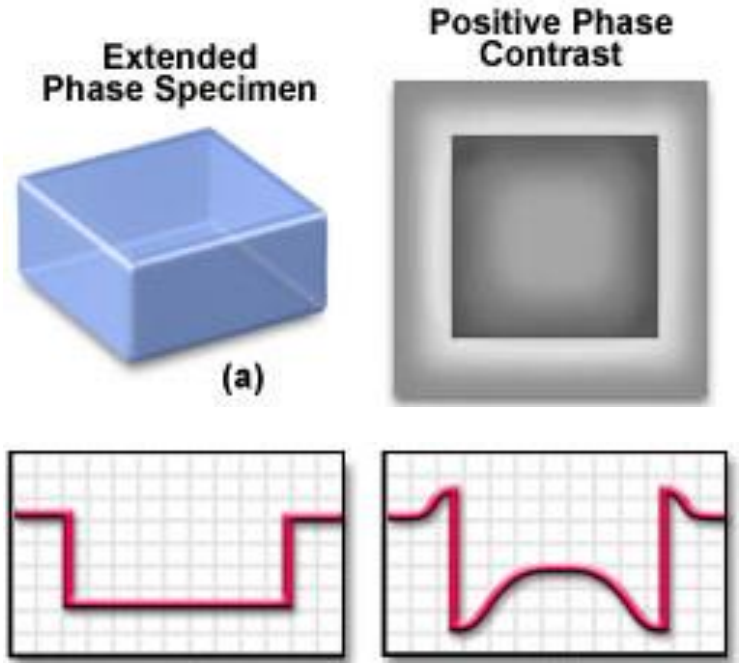
Figure 8

Back focal plane

Front focal plane

Limitations of Phase Contrast

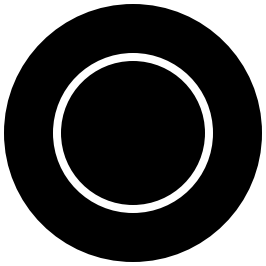




- Halos result from diffracted light that is intercepted by phase ring

- Shade off is caused by greater diffraction at edges of objects than their centers

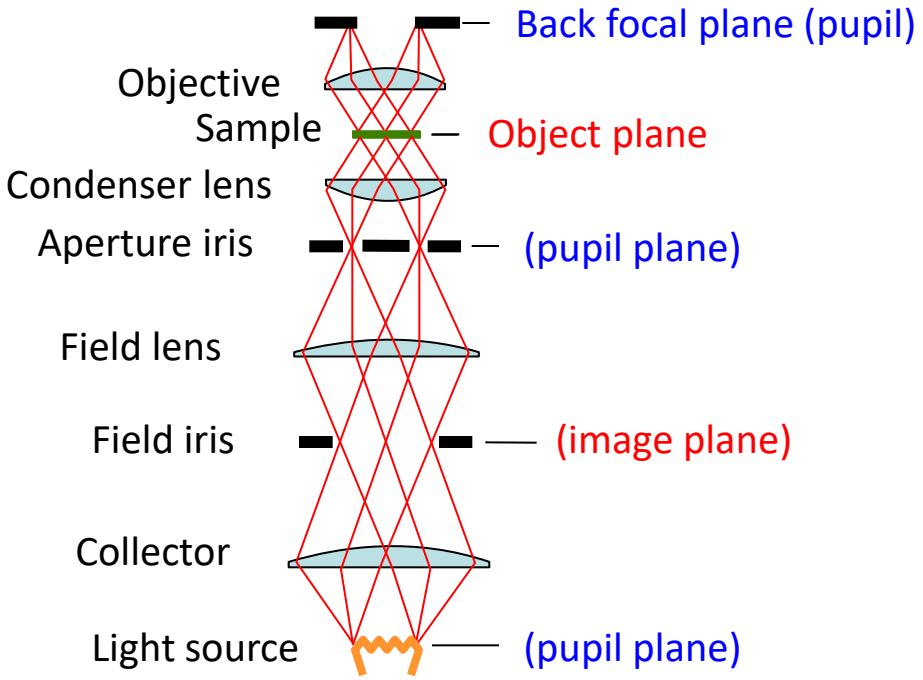
Limitations of Phase Contrast



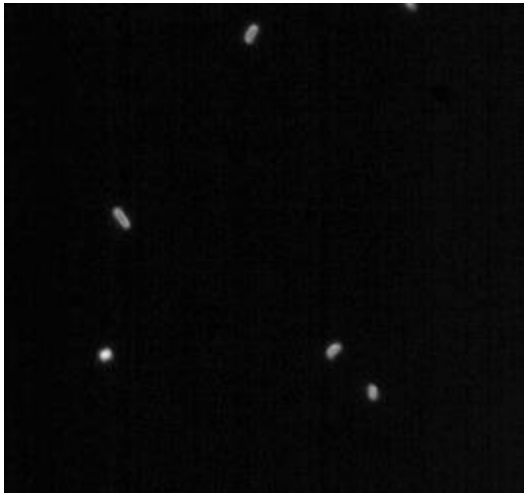
- Poor optical sectioning due to limited illumination aperture.
- For sufficiently thick samples, can get more than 360° phase shift, meaning thin and thick regions will be identical in contrast.

Darkfield Microscopy

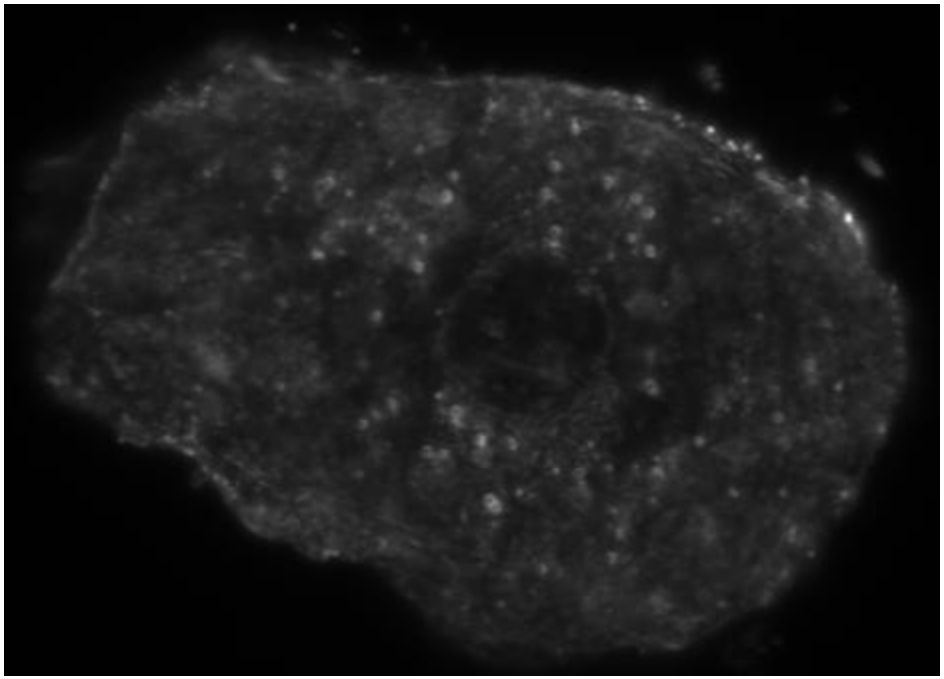
- Idea: throw away all non-diffracted light by illuminating with a condensor annulus at higher NA than the objective.
- No illumination light makes it through the objective, only diffracted light.



Darkfield Microscopy



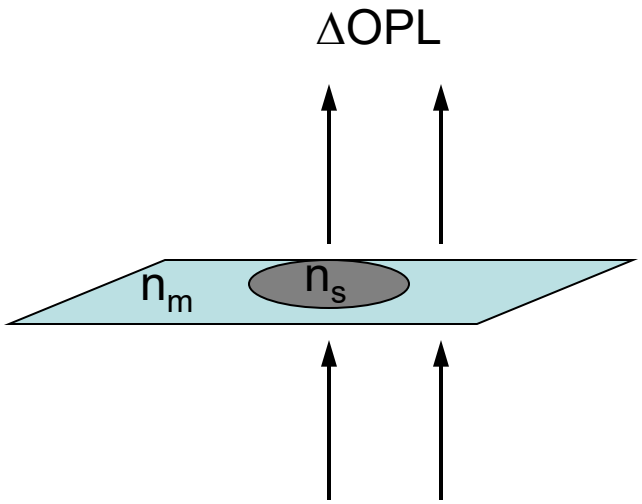
- Ideal for small particles, unstained microorganisms, etc.



- Can easily see subdiffraction particles (e.g. 40 nm gold)

- Not so good for large objects

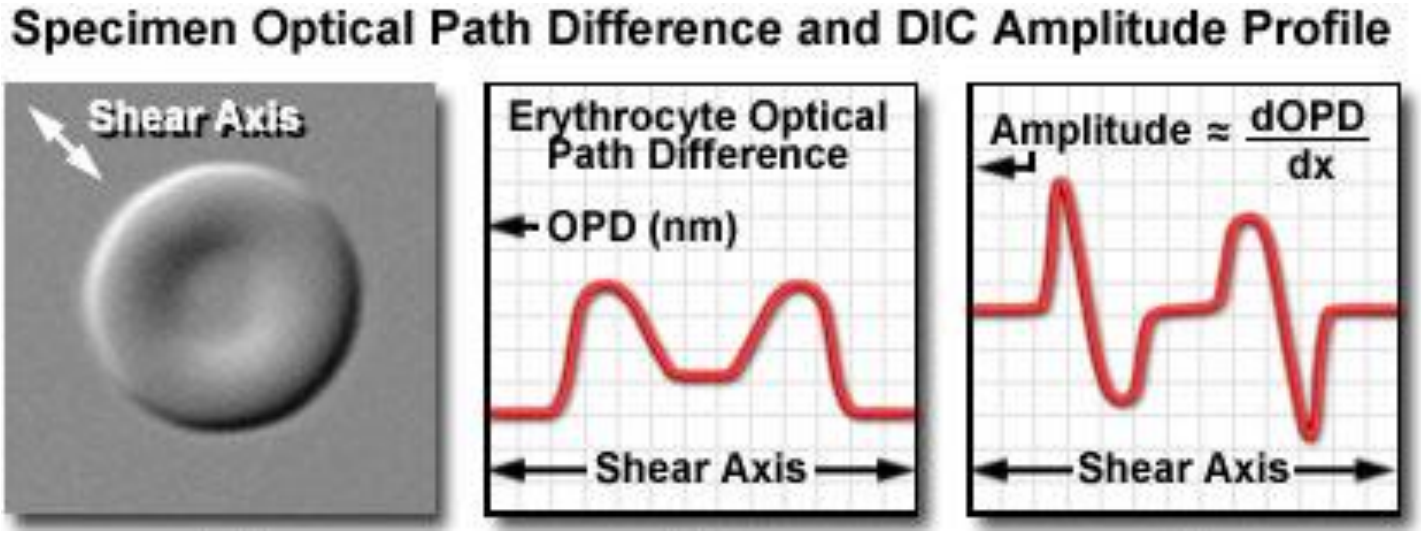
Differential Interference Contrast (DIC)



The idea:

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

What DIC accomplishes

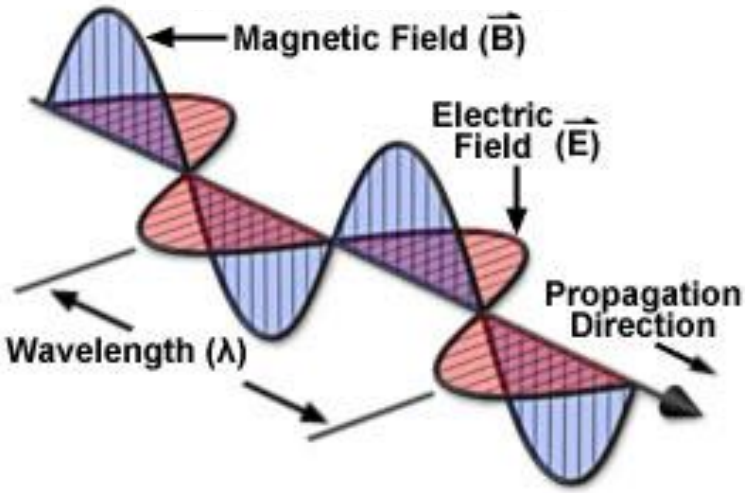


Converts relative differences in optical path length to differences in amplitude

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

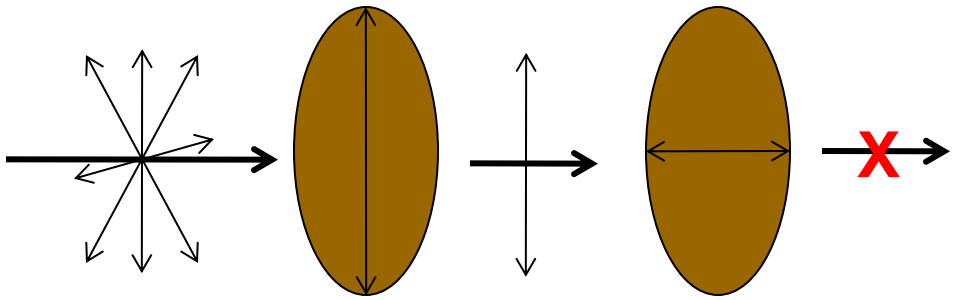
Polarization



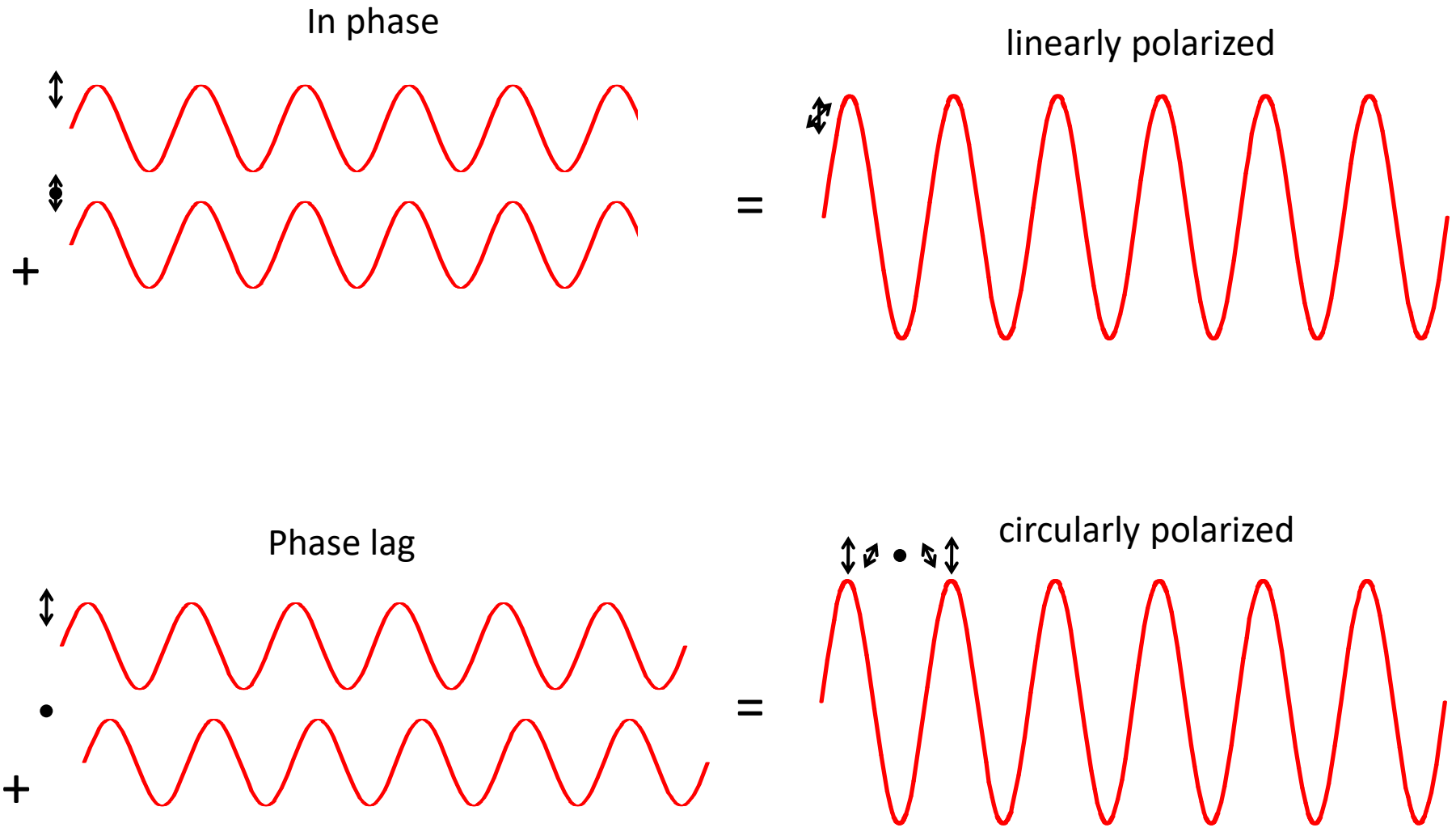
- Polarization: orientation of E-field.
- Most light sources produce unpolarized light – no preferred polarization angle

Polarizers

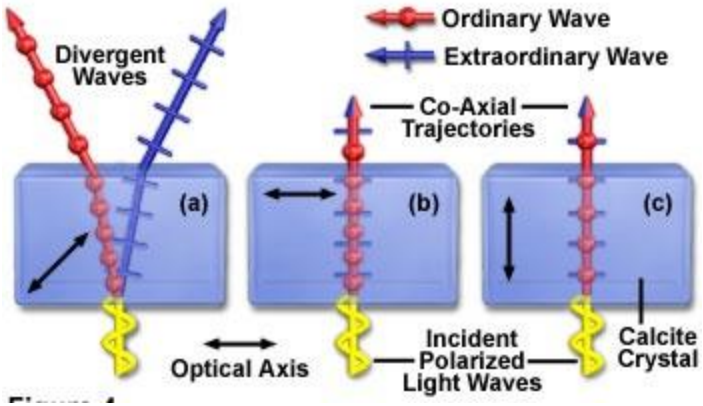
- Polarizers specifically transmit one polarization angle of light
- Crossed polarizers transmit no light



Interference and polarization



Birefringence



Bi-Refraction in Calcite Crystals

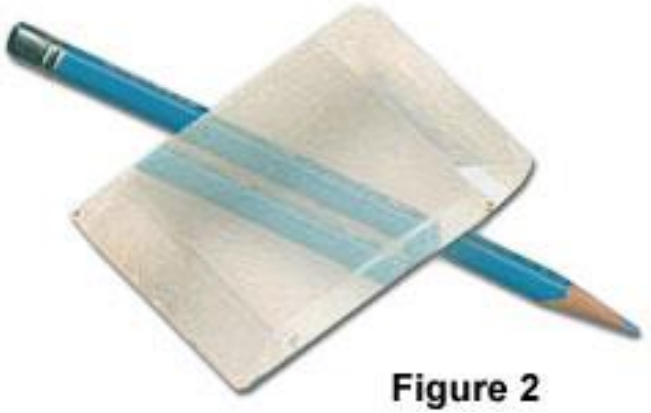
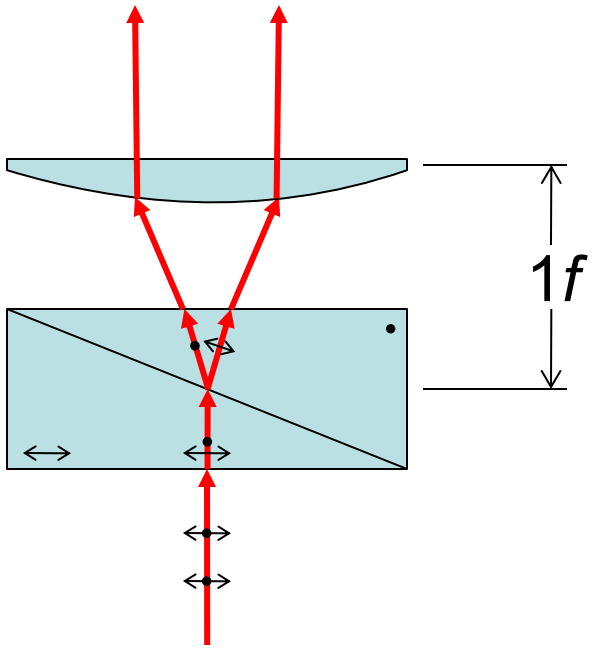


Figure 2

- 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

Imaging path

Illumination path

Camera

Projection Eyepiece

Tube lens

Objective

Sample

Condenser lens

Aperture iris

Field lens

Field iris

Collector

Light source

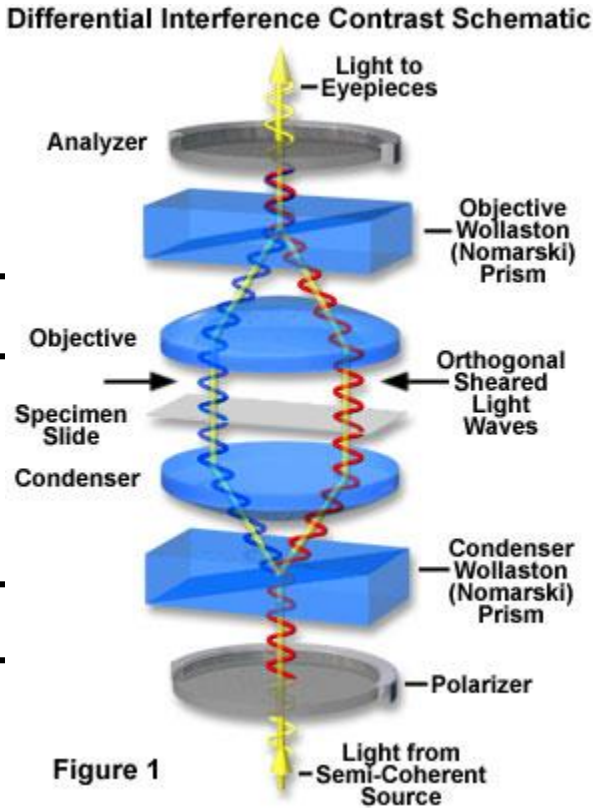
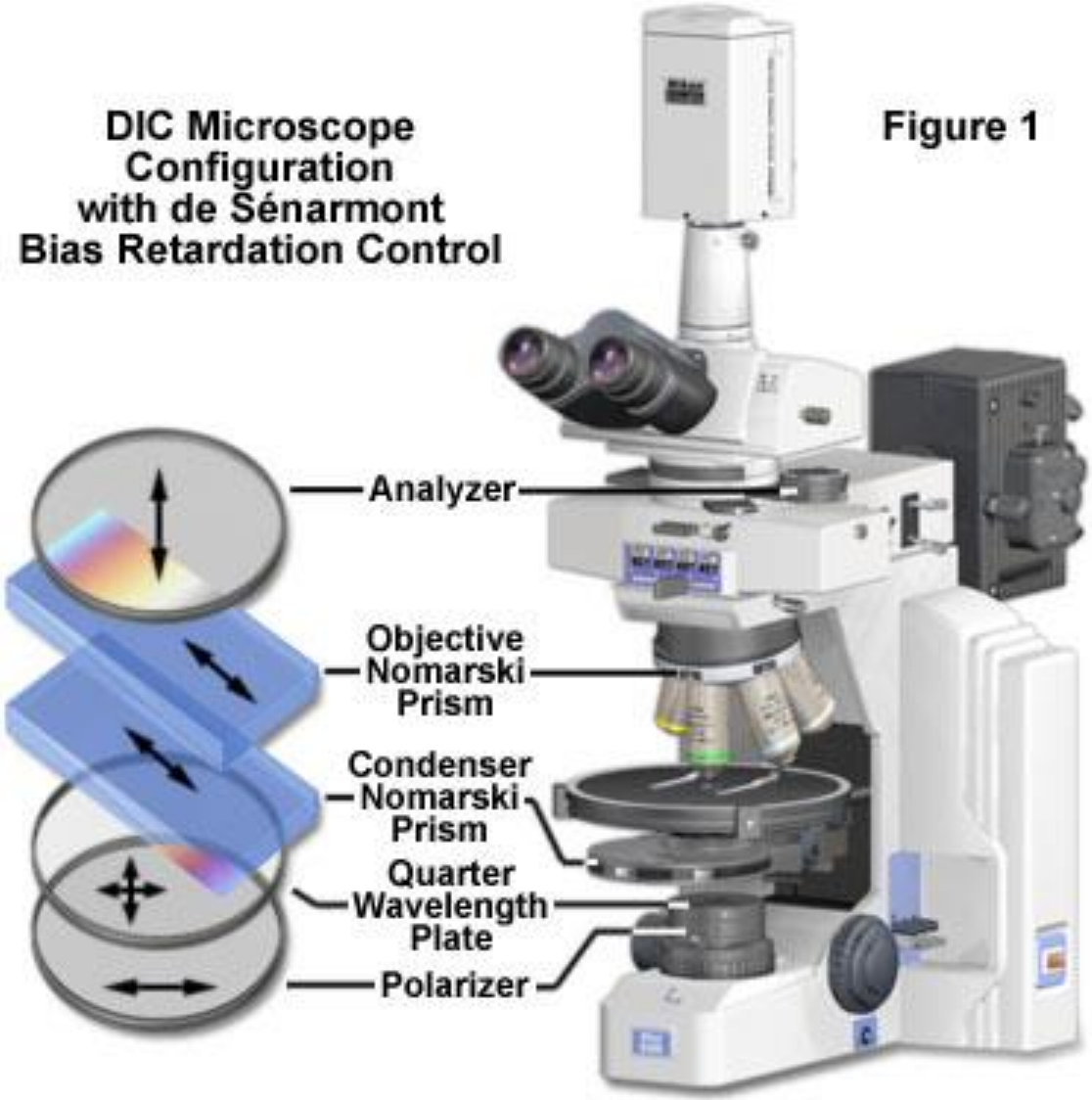


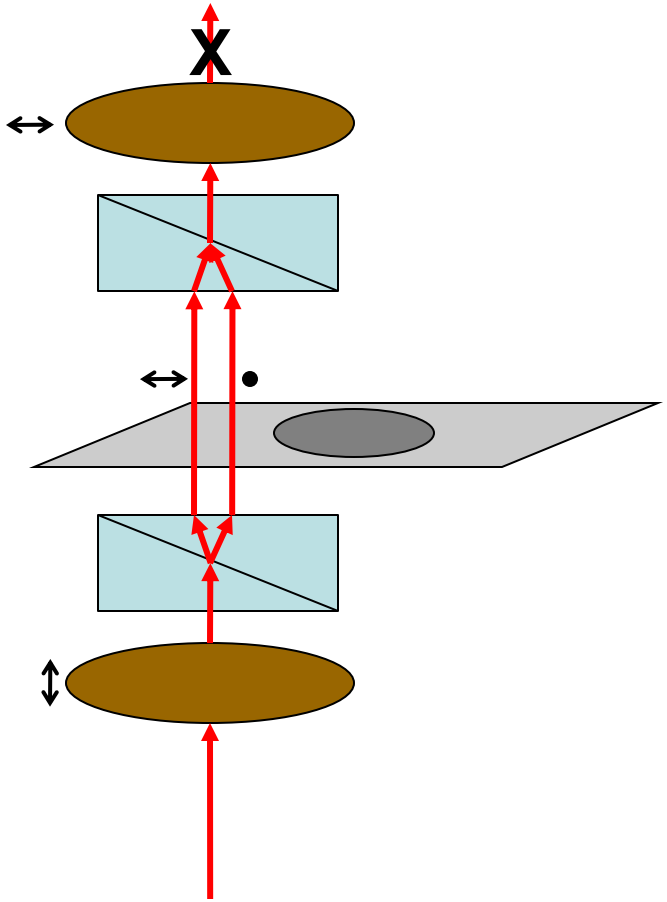
Figure 1

DIC Microscope Configuration with de Sénarmont Bias Retardation Control

Figure 1

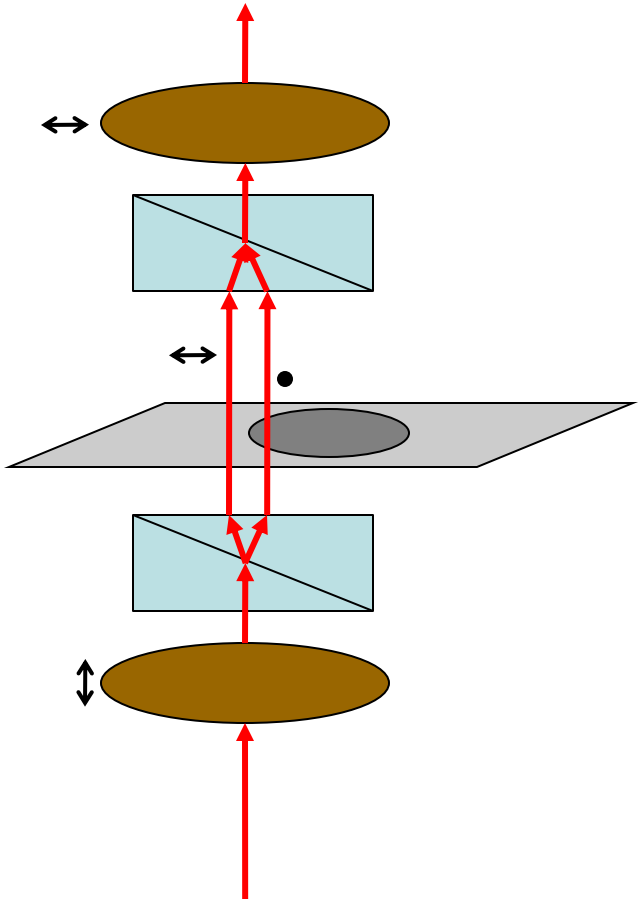


How DIC generates contrast



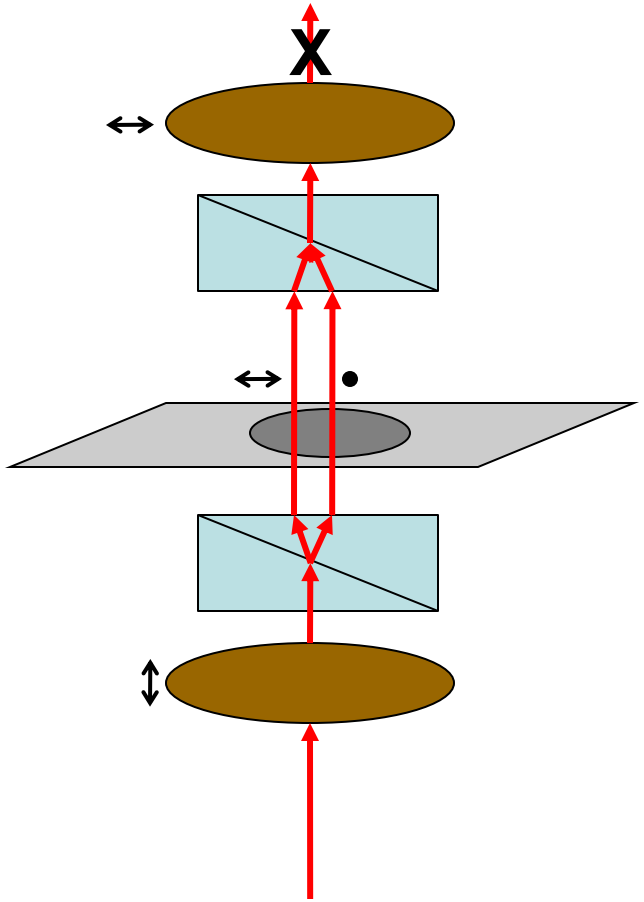
- Both beams see same OPL
- Emerge in phase
- Regenerate initial polarization
- No light makes it through analyzer

How DIC generates contrast



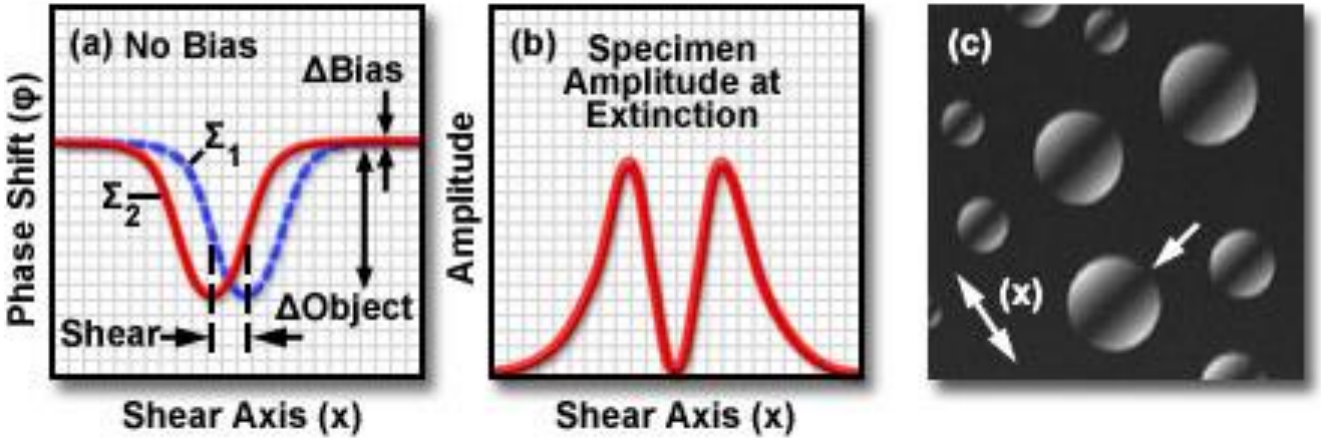
- Beams see different OPL
- Right beams is phase retarded
- Generate elliptical polarization
- Light makes it through analyzer

How DIC generates contrast

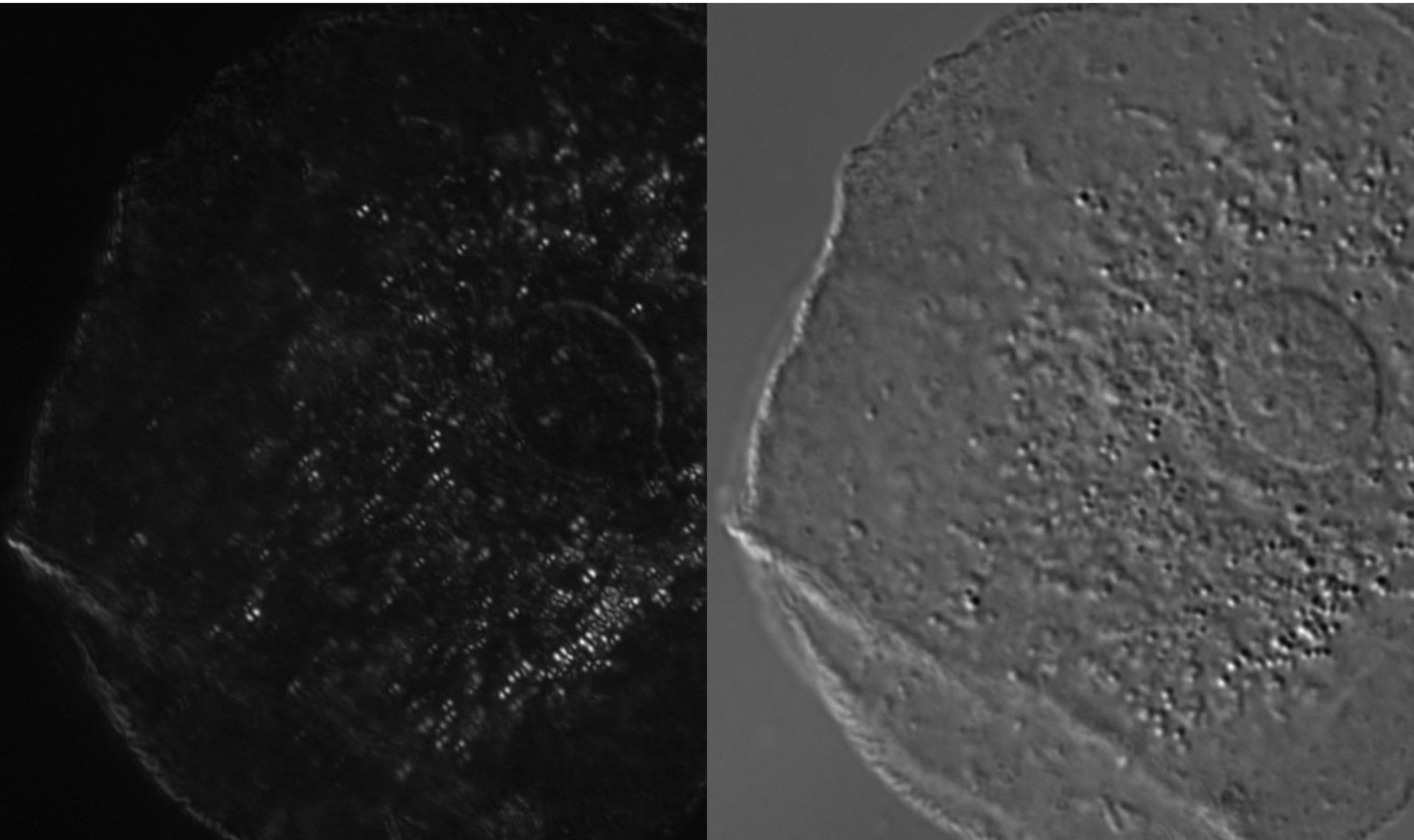


- Both beams see same OPL
- Emerge in phase
- Regenerate initial polarization
- No light makes it through analyzer

Role of Bias in DIC



Role of Bias in DIC



Bias adjusment in de Sénarmont DIC

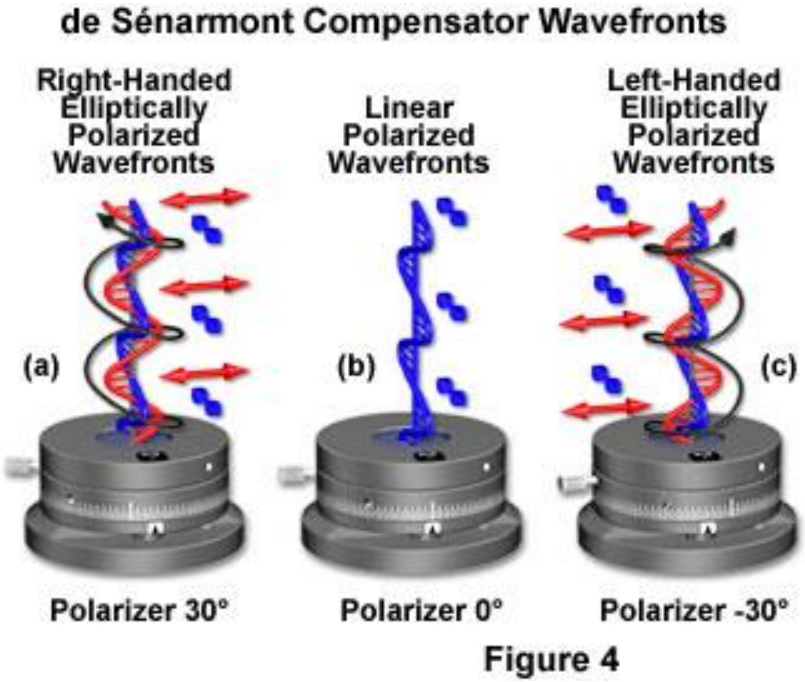


Figure 4

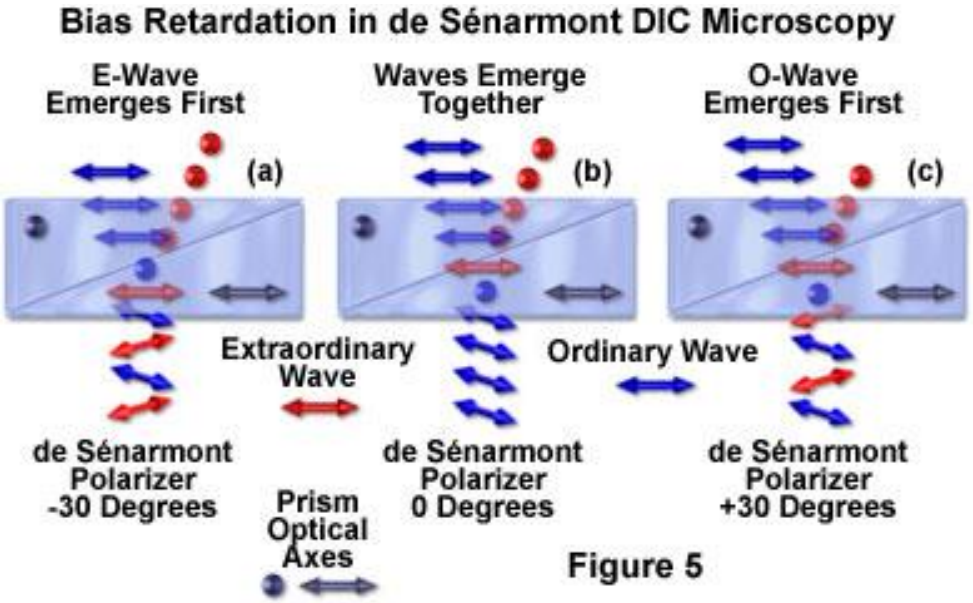
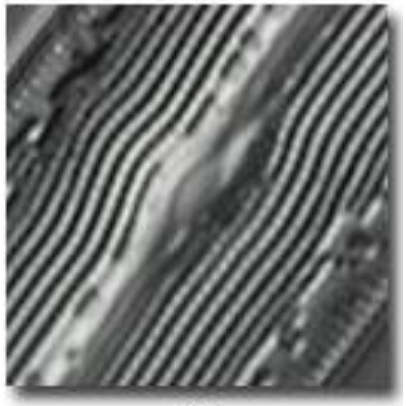


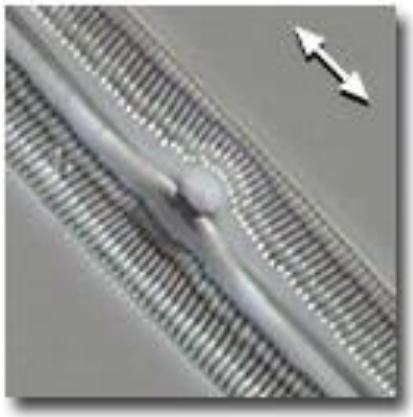
Figure 5

DIC is sensitive to specimen orientation



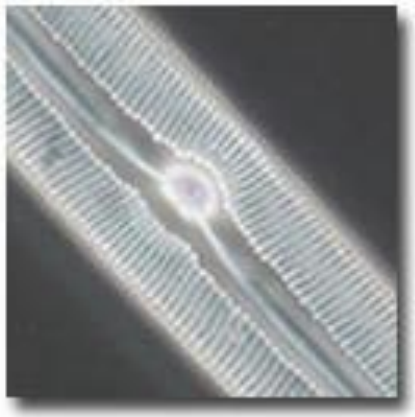
(a)

(b)



(a)

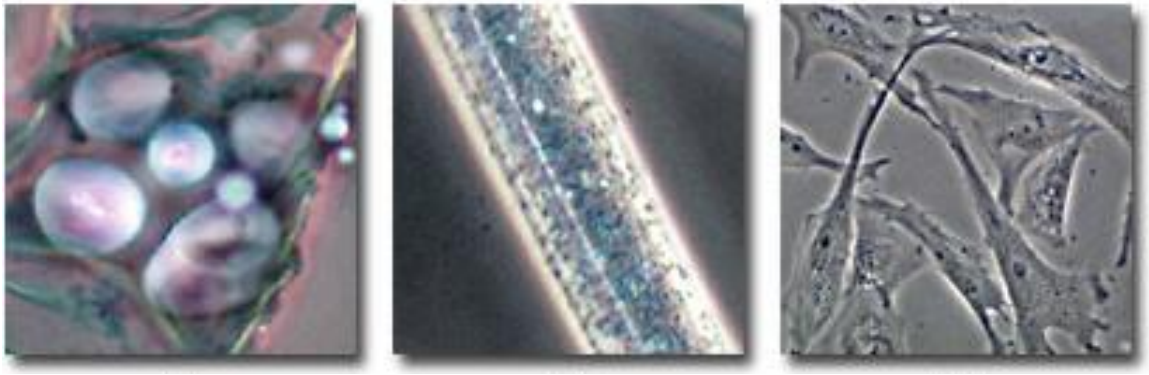
(b)



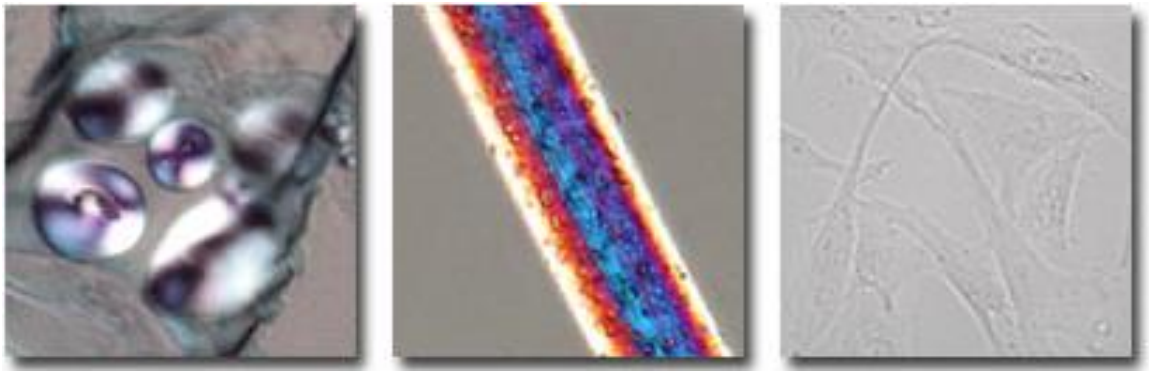
(c)

DIC doesn’t work on birefringent samples

Phase



DIC

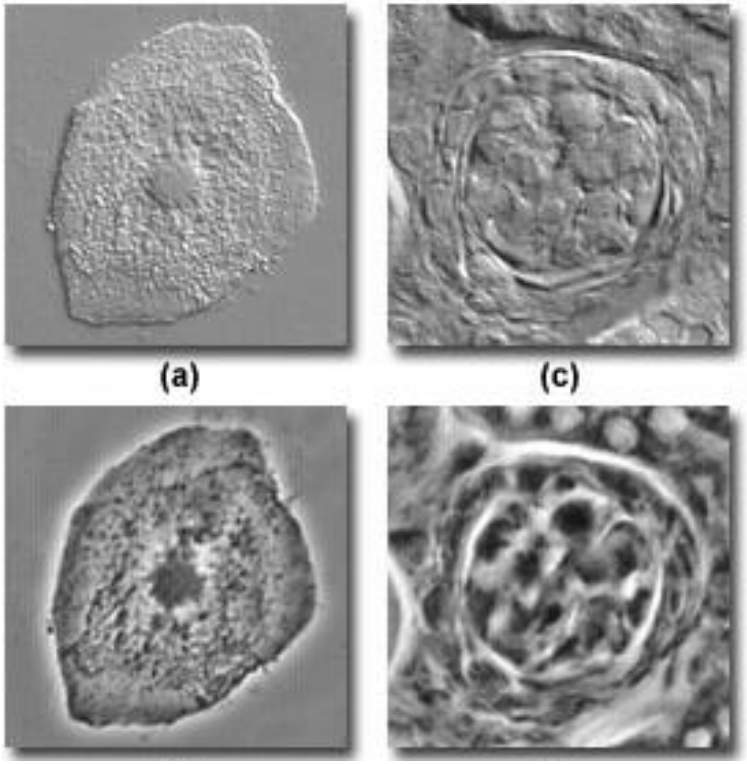


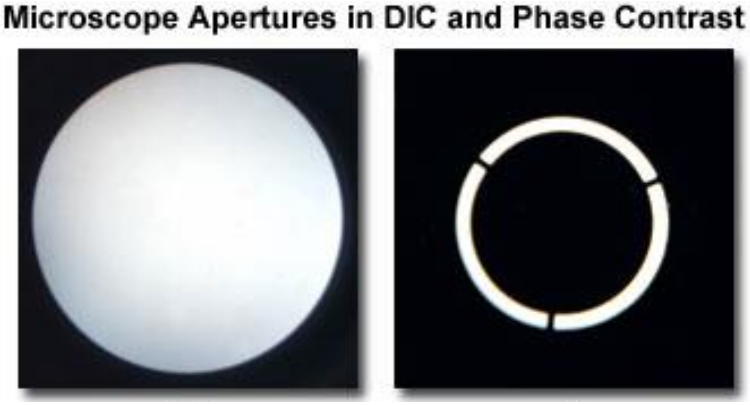
Can’t plate cells on or or cover cells with plastic.

DIC is higher resolution than phase contrast

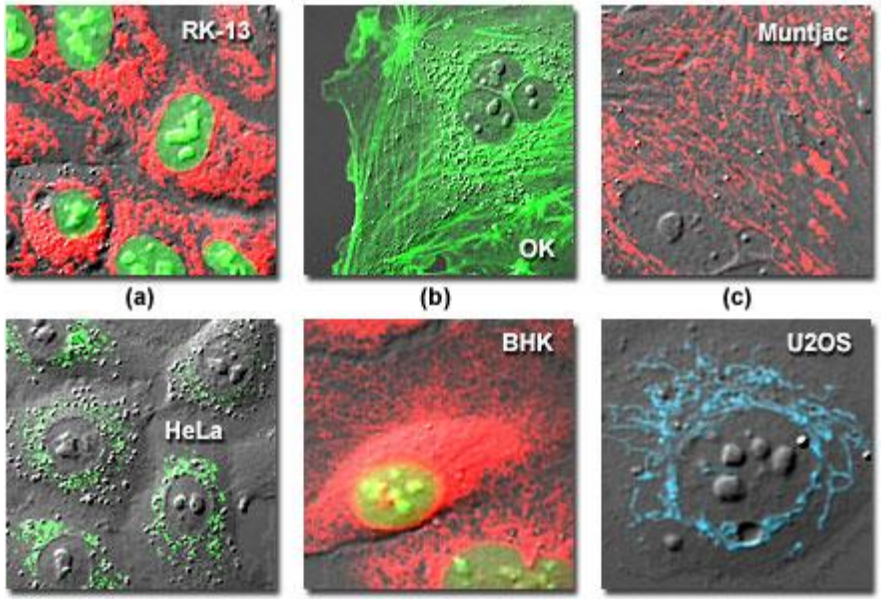
DIC

Phase





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

micro.magnet.fsu.edu

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

Hecht, “Optics”

Acknowledgements

Orion Weiner / Mats Gustafsson