Phase contrast and DIC

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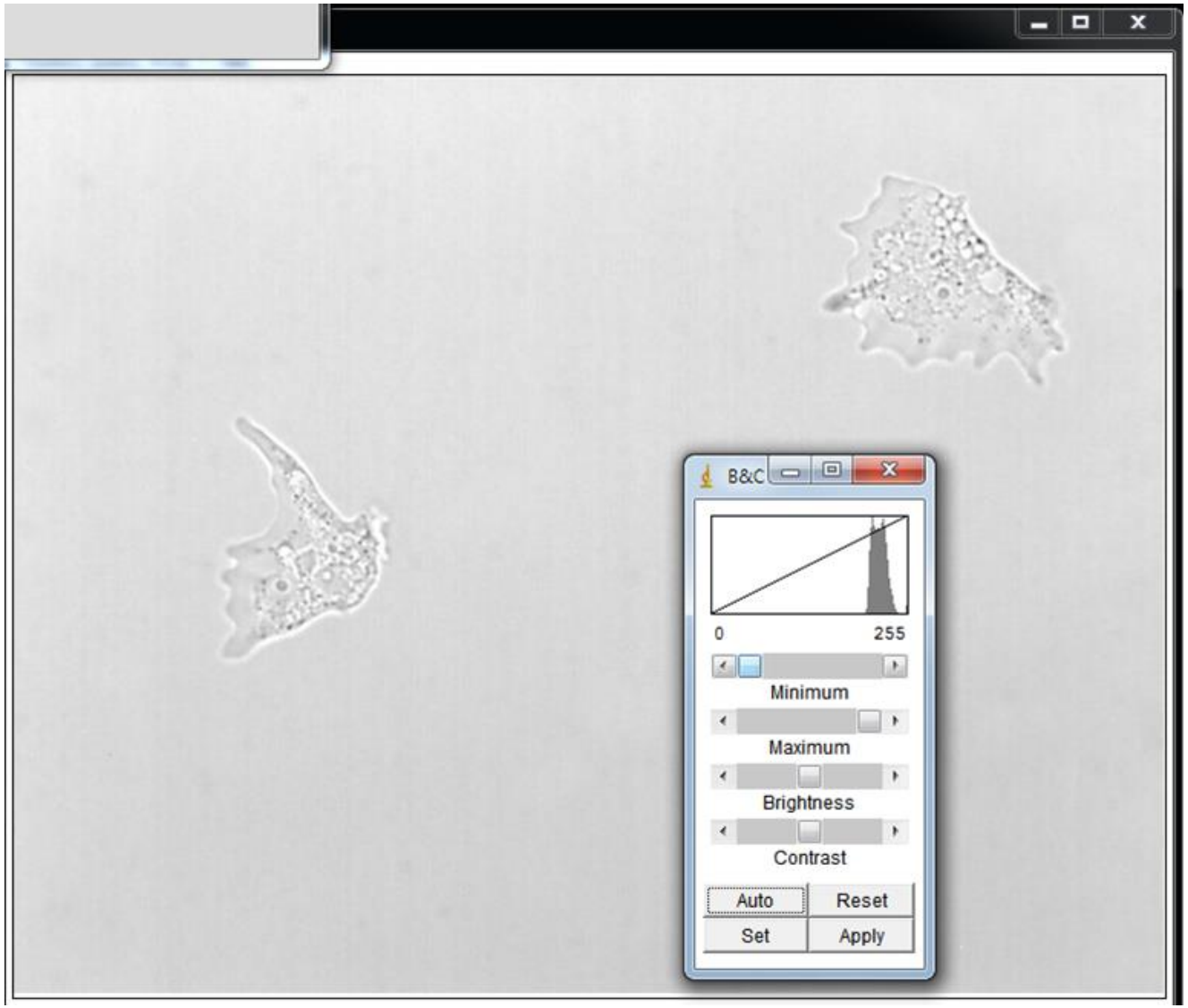
2013 March 25

Gerd A. Guenther
2011 Honorable Mention
Nikon Small World photomicrograhy competition
Image of a freshwater ciliate.

Enhancing contrast: *ex post facto*

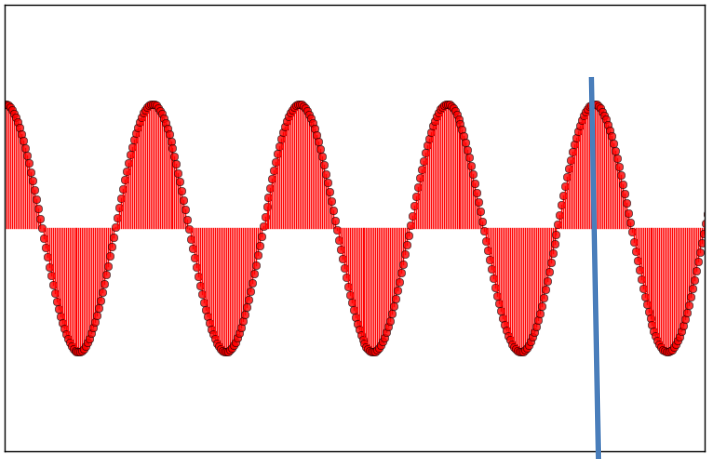


Why cells have poor contrast?

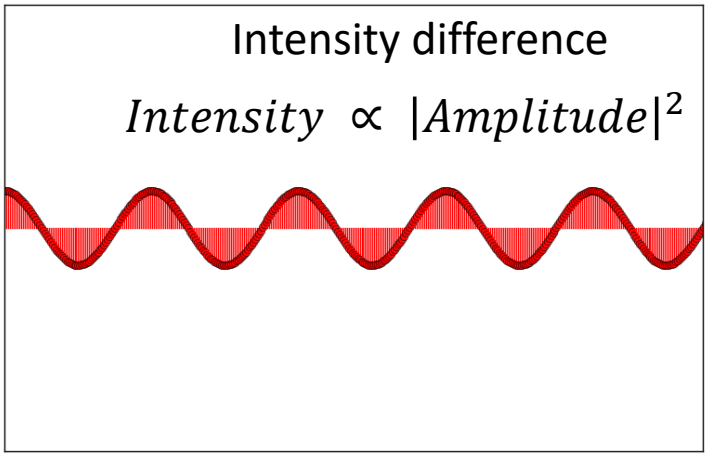
- Cameras and eyes are sensitive to the *intensity* of light
  - $Intensity \propto |Amplitude|^2$
- Amplitude of light wave is altered if object absorbs some light
- Amplitude not altered for transparent objects that absorb little (like cells)

Properties of light

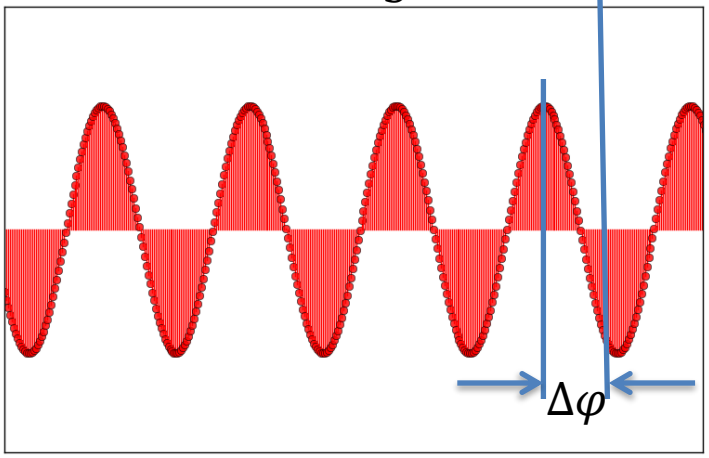
$y = \sin x$



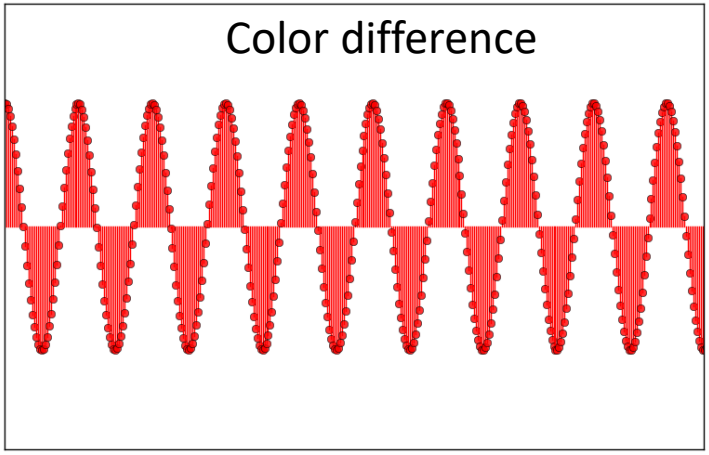
$y = 0.3 \sin x$



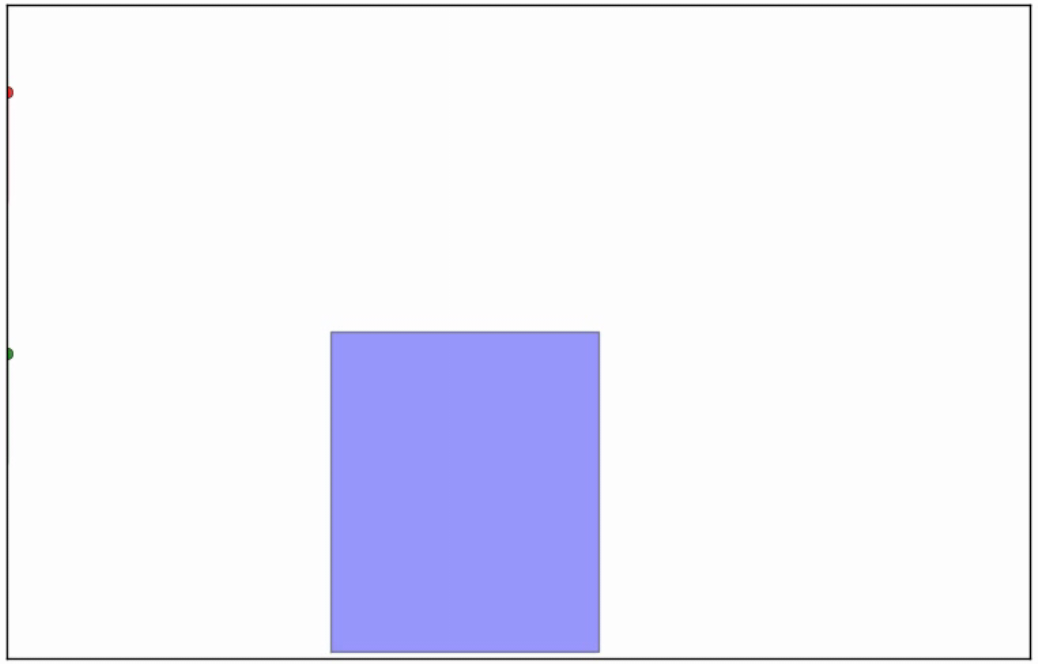
$y = \sin\left(x + {}^{2\pi}/_{3}\right)$



$y = \sin 2x$



Phase Objects

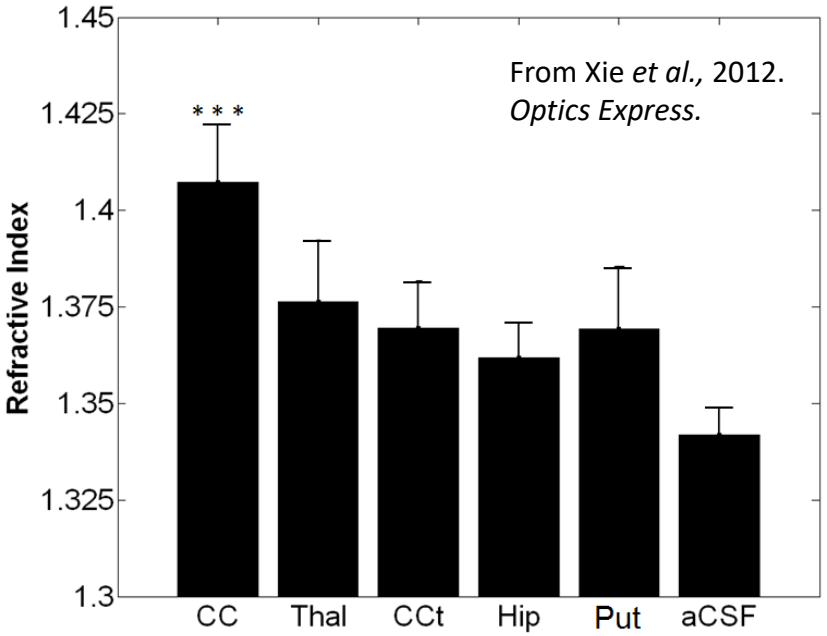


- Phase object introduce phase delay of light depending on:
  - ✓ Index of refraction
  - ✓ Thickness

- $n =$ refractive index
- $n = {}^{v_{vacuum}}/_{v_{media}}$
- $n = {}^{\lambda_{vacuum}}/_{\lambda_{media}}$

- Optical path length (OPL):
  - $OPL = n \times t$

Refractive indices of biological samples



Various regions of the brain

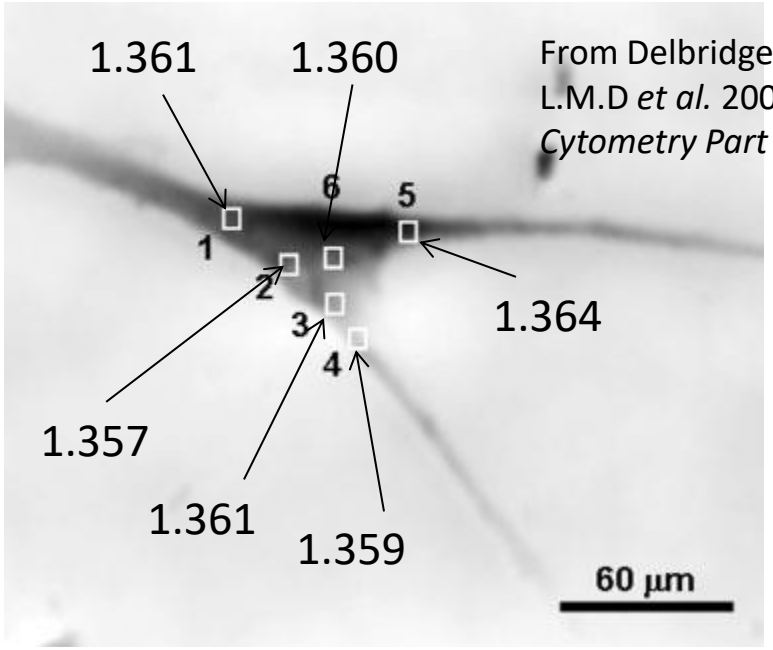
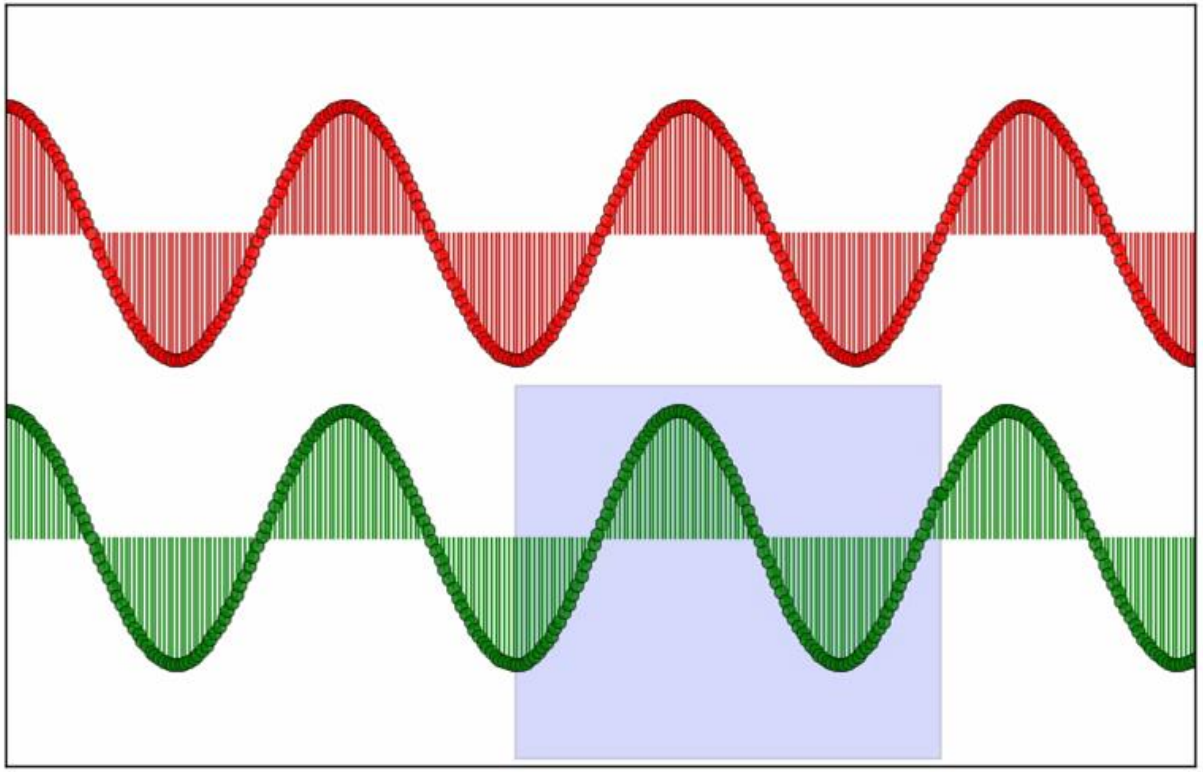


Image of smooth muscle cell

$$\Delta OPL = (n_{cell} - n_{media}) \times t \qquad \Delta\varphi = \frac{2\pi}{\lambda}(n_{cell} - n_{media}) \times t$$

$$\Delta OPL = (1.360 - 1.335) \times 5\ \mu m = 0.125\ \mu m$$

$$\Delta\varphi = \frac{2\pi}{0.5\ \mu m}(1.360 - 1.335) \times 5\ \mu m = 1.57\ \text{rad} = 90°$$



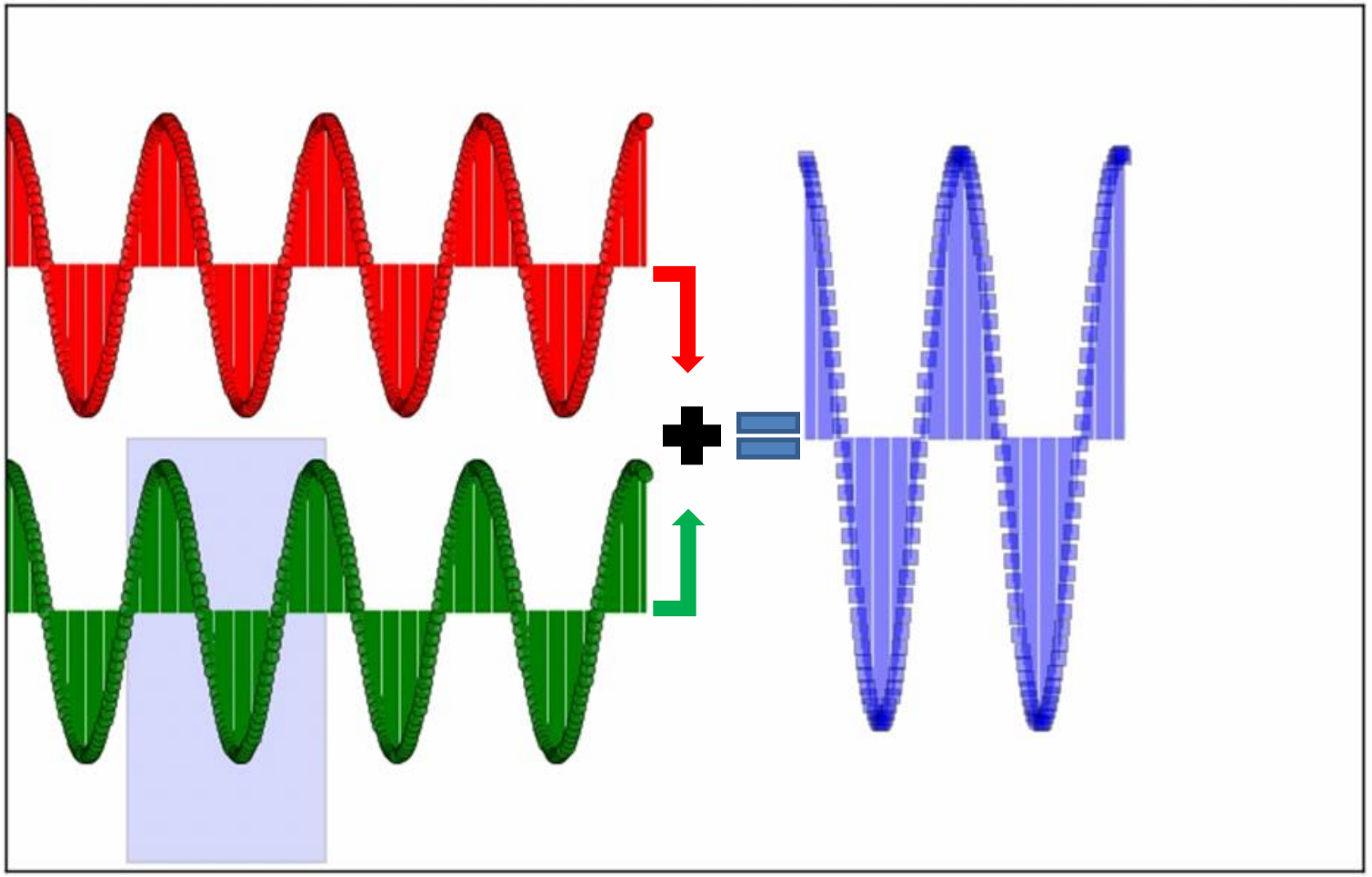
Phase difference between two waves depends on the optical path difference (OPD).

Optical path difference = difference in refractive index × distance

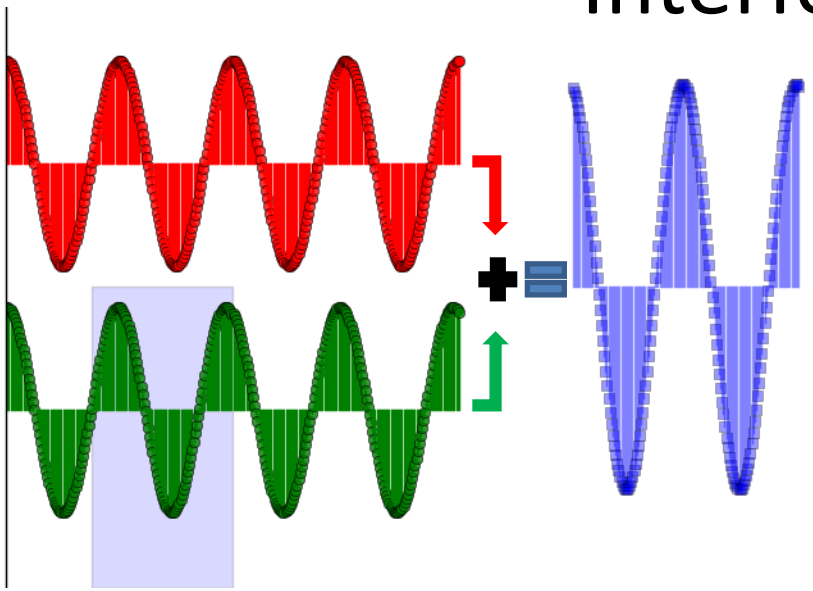
How to observe phase changes?

Must convert difference in phase to amplitude.

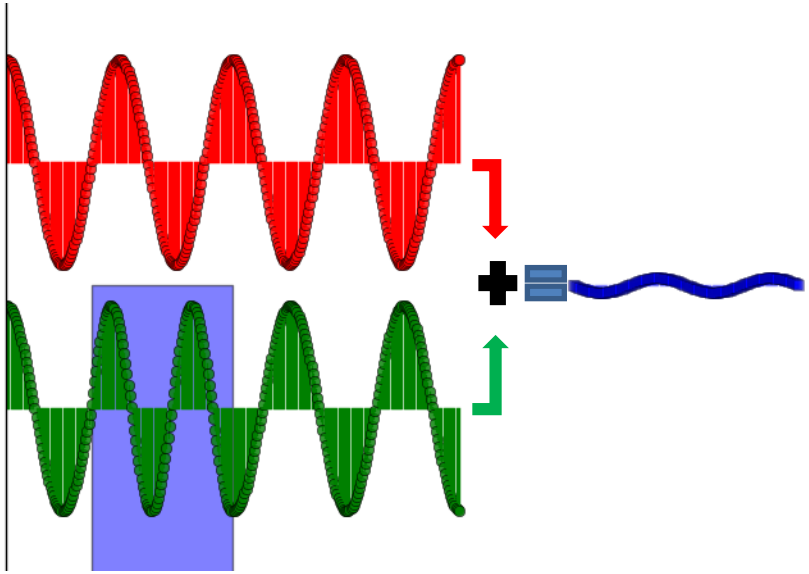
Solution: sum shifted wave with some reference



Interference



Waves that are *in phase* add constructively.



Waves that are 180° or λ/2 out of phase add destructively.

Phase contrast microscopy

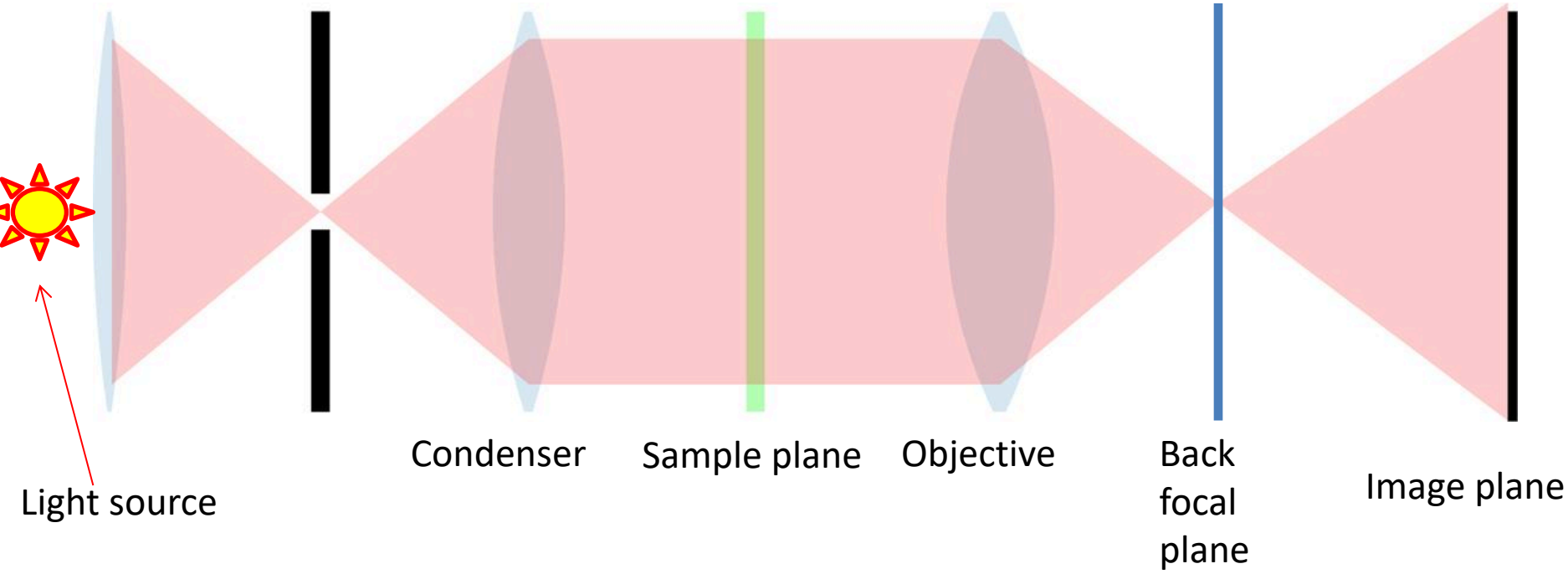
Frits Zernike

- Devised phase contrast microscopy in 1930s. Received Nobel Prize 1953.
- "…it is common knowledge that in all interference phenomena differences of phase are all-important. Why then had phases never been considered before … in the microscope?"
- Phases difficult to see
  - Must convert *phase* differences to *intensity* differences
- Phases difficult to define
  - Only *relative* phase matters

Light path for bright-field microscopy

No Sample

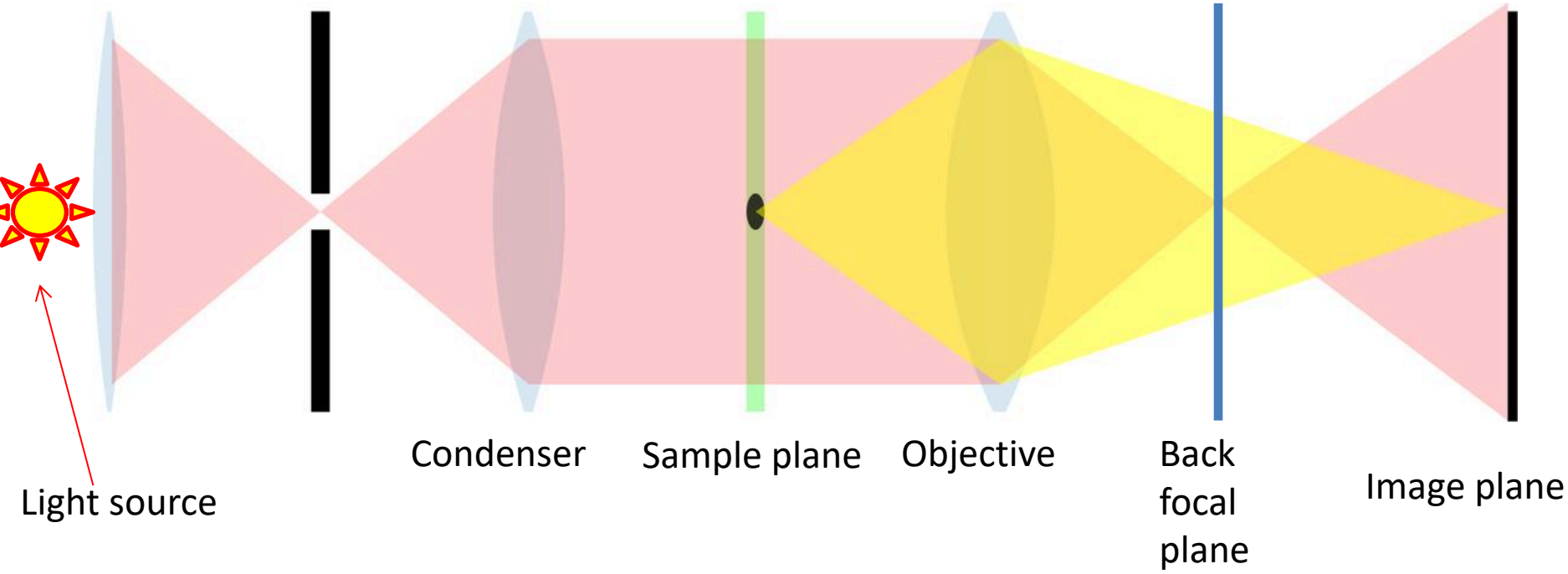
- Uniform illumination at the sample plane
- At objective's back focal plane, light occupies small spot
- Uniform intensity at the image plane



Light path for bright-field microscopy

With Sample

- Objective captures light from sample
- Light from point in sample focused to point at the image plane
- Light from sample occupies larger area in back focal plane



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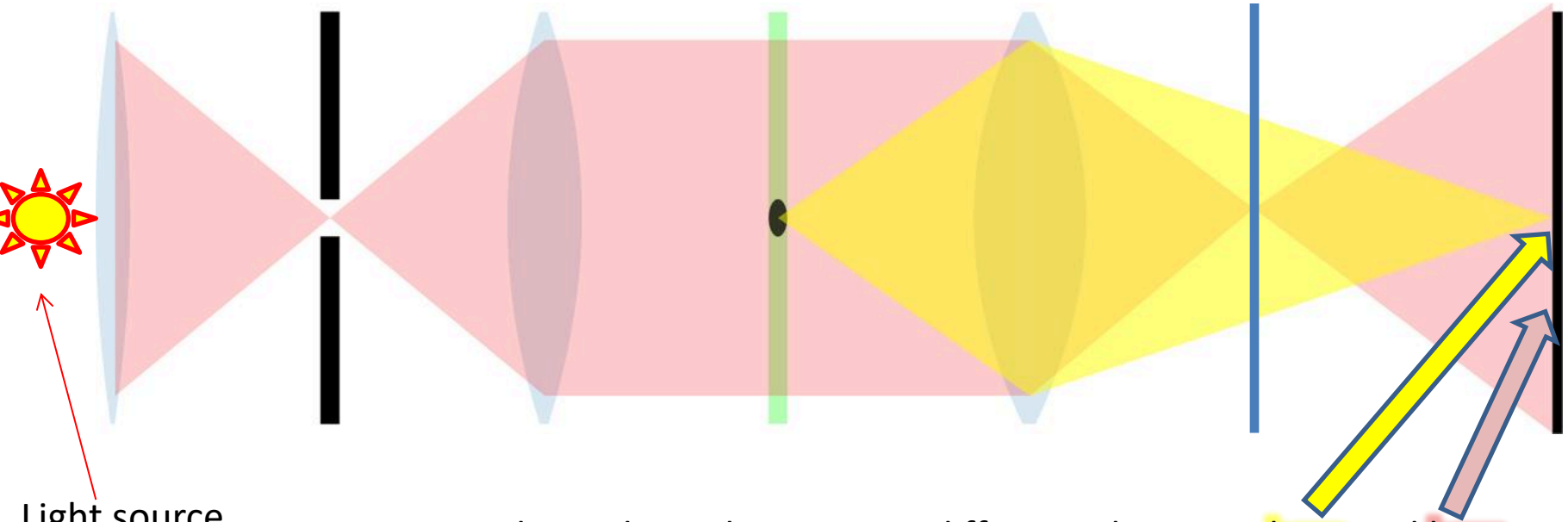
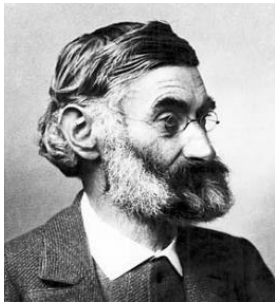
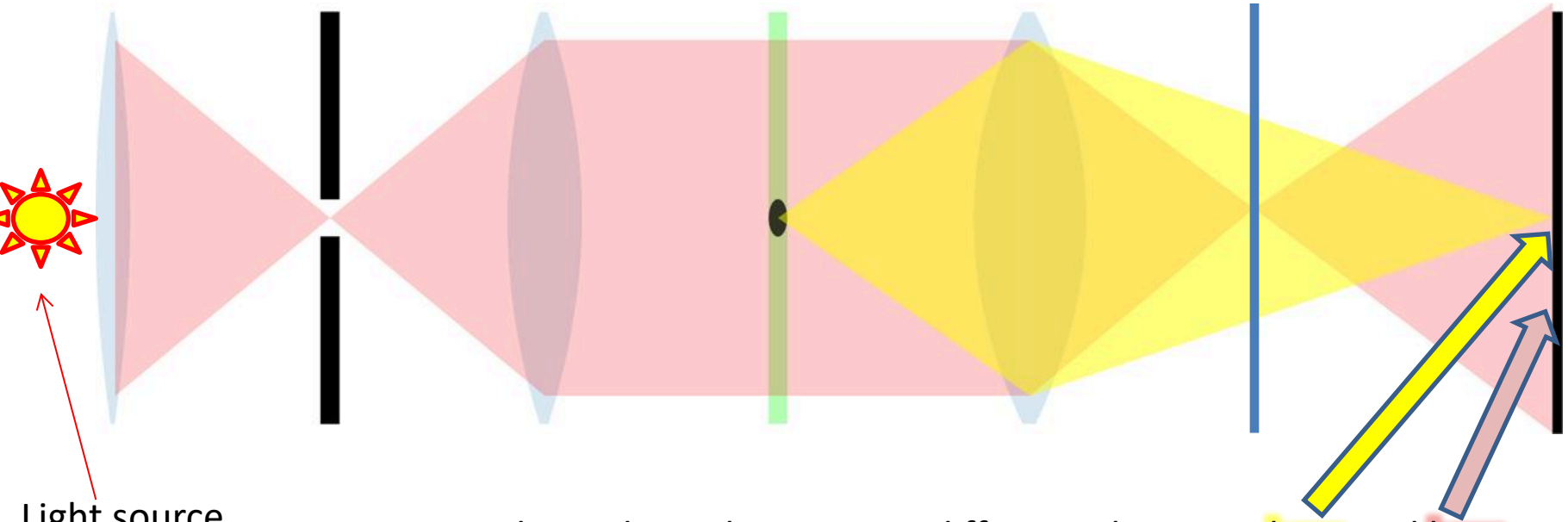


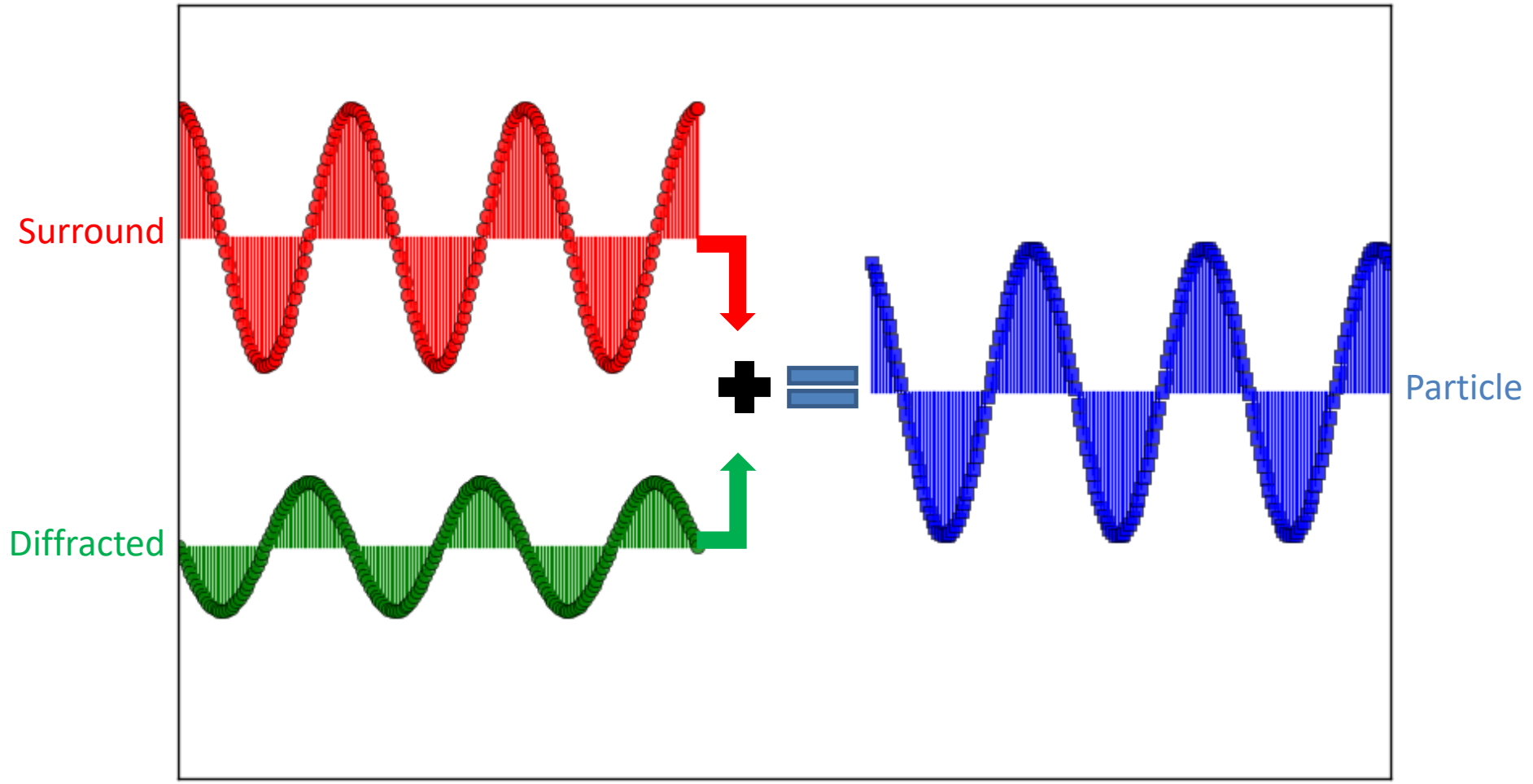
The microscope image is the interference effect of a diffraction phenomenon.
-Ernst Abbe



- Interference at image plane between
  surround or undiffracted light and
  diffracted light.
- Contrast depends on intensity of surround light and intensity of surround + diffracted (= particle) light.
- For phase objects: diffracted light is 90° out of phase with surround light



Superposition of two waves, 90° phase difference



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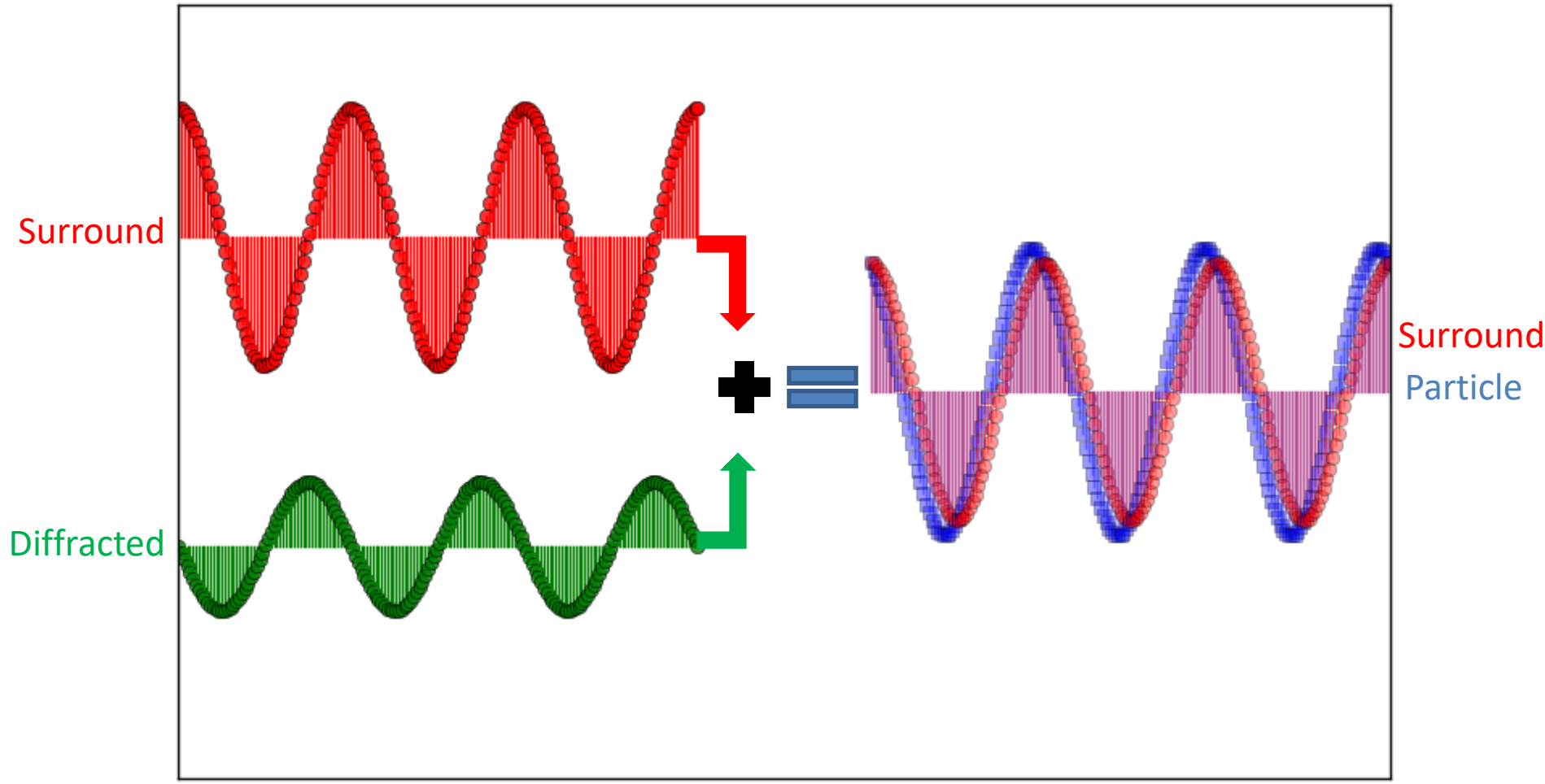
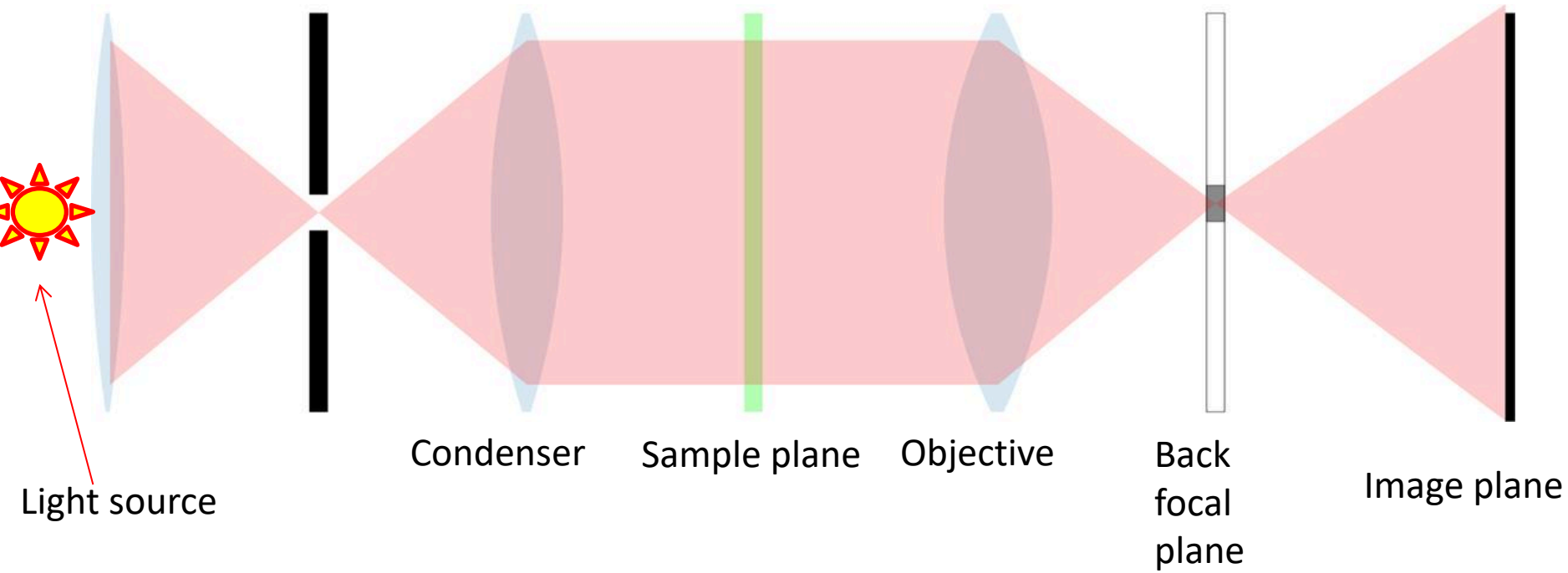


Phase contrast microscopy

The microscope image is the interference effect of a diffraction phenomenon.

-Ernst Abbe

To achieve larger amplitude difference between surround and particle wave: adjust phase difference between surround and diffracted light.



Phase contrast microscopy

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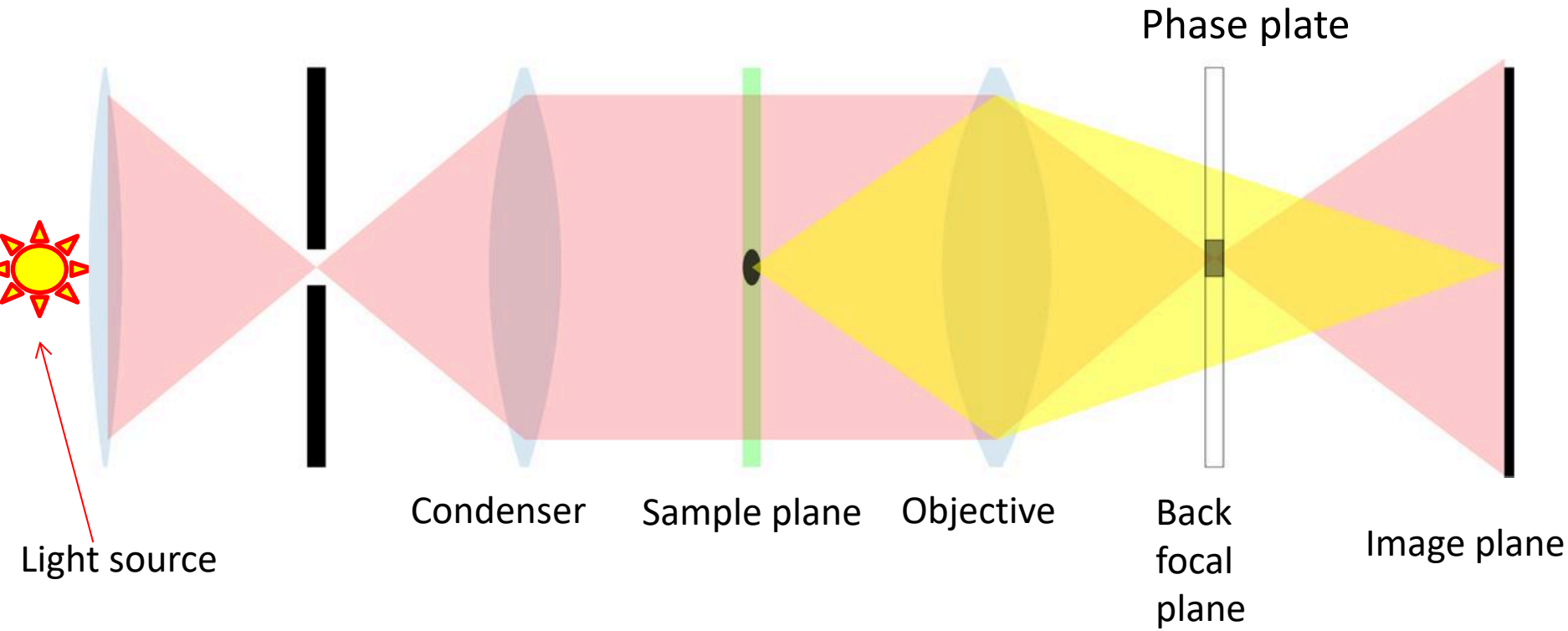
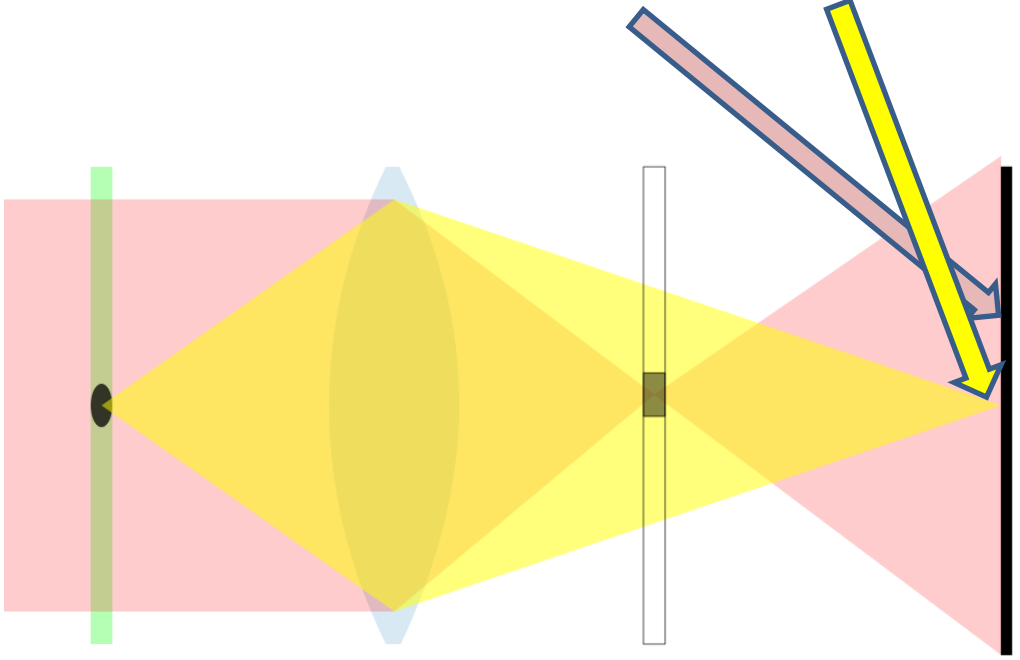
Phase plate shifts phase of surround light
but has little effect on diffracted light.



Phase contrast microscopy

… with equations

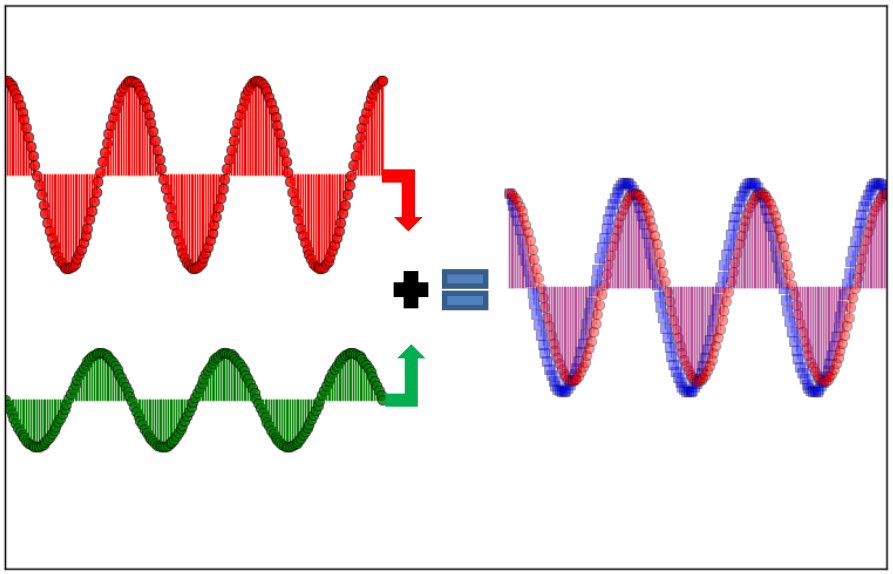
Field at the image plane: $U(x,y) = U_0 + U_1(x,y)$



Add $^{\pi}/_{2}$ shift to surround: $I(x,y) = a^2 + 2a\varphi(x,y)$

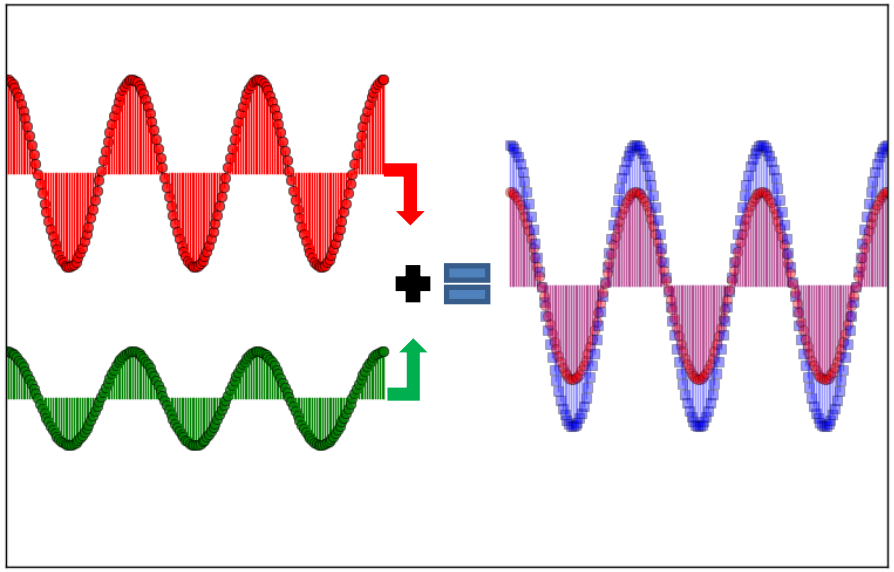
Add $^{-\pi}/_{2}$ shift to surround: $I(x,y) = a^2 - 2a\varphi(x,y)$

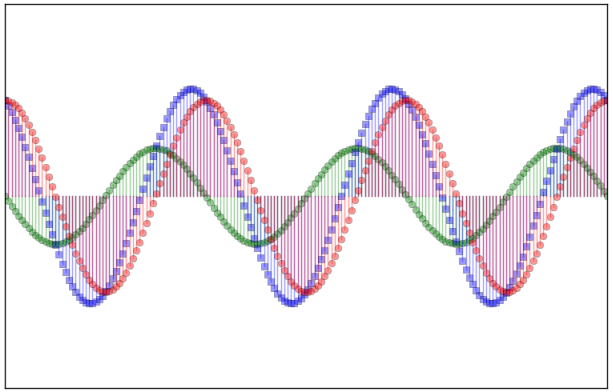
Surround wave 90° out of phase with diffracted wave.



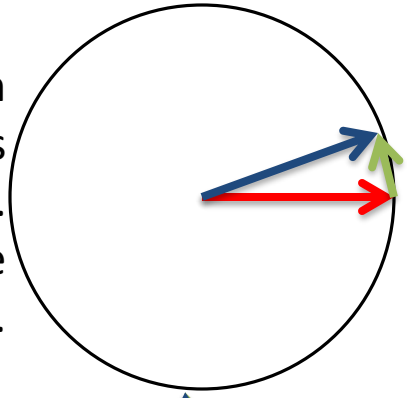
Surround wave
Particle wave

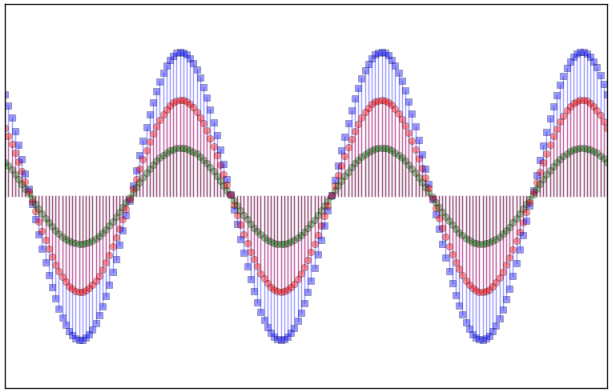
Surround wave in phase with diffracted wave.



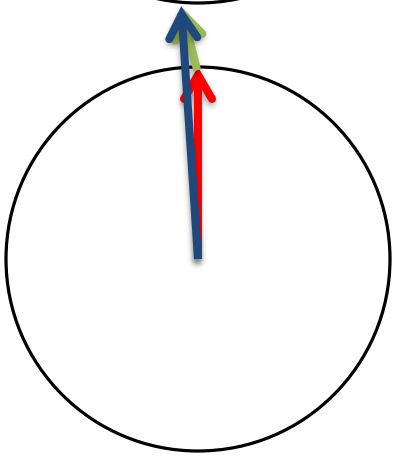


Vector's length gives its amplitude. Vector's angle gives its phase.

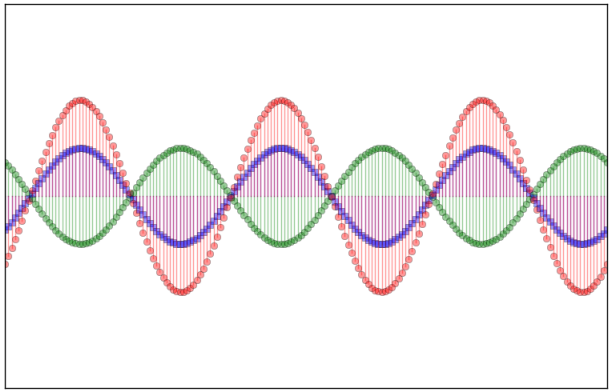




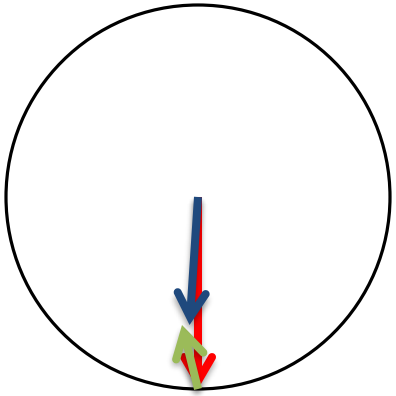
Negative Phase Contrast



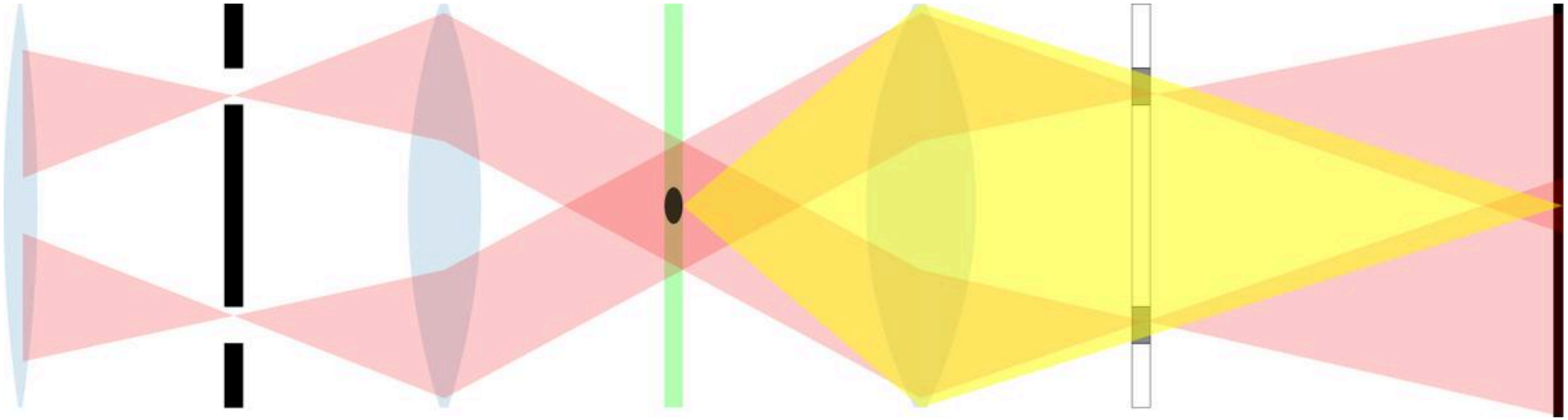
Surround wave is rotated by 90° but diffracted wave is untouched.



Positive Phase Contrast

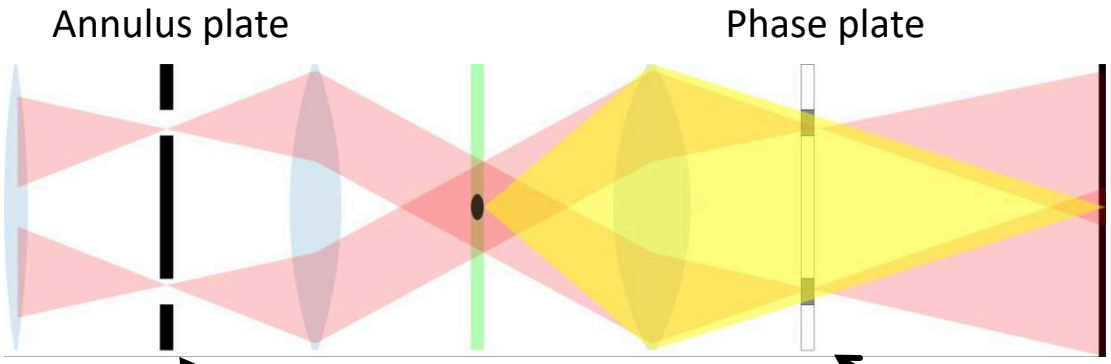


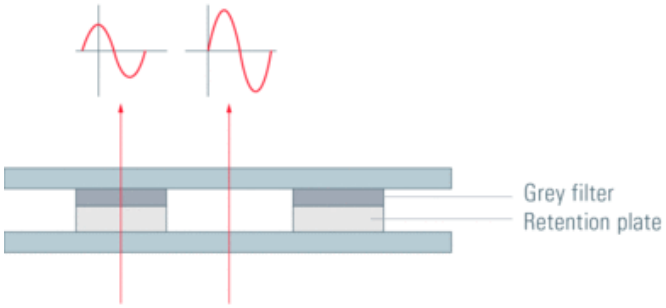
Condenser annulus and phase ring

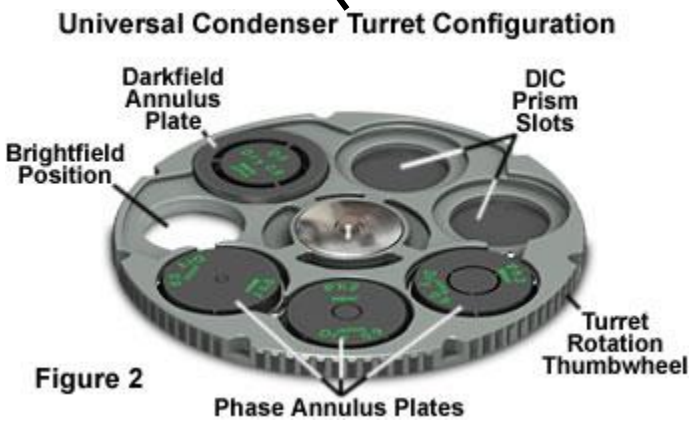


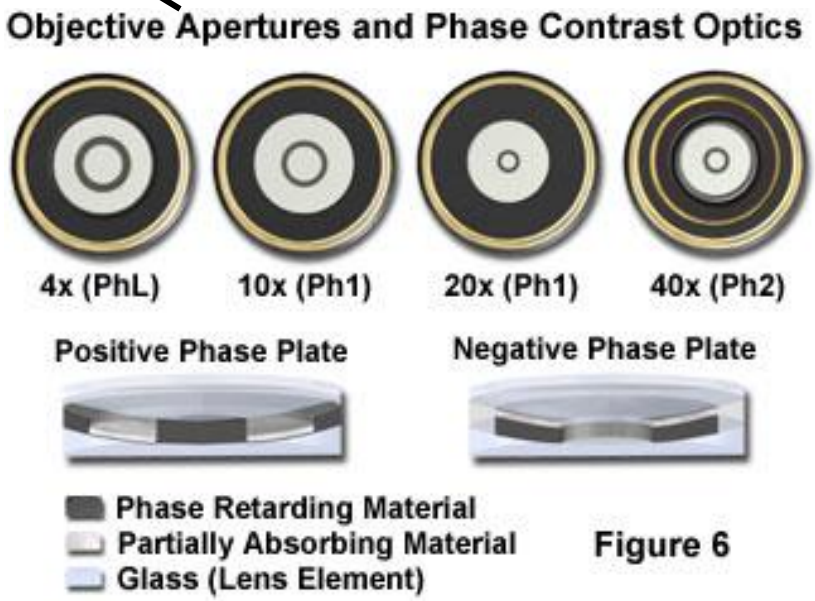
- Annulus allows more of the condenser aperture to be used

Phase Contrast Plates





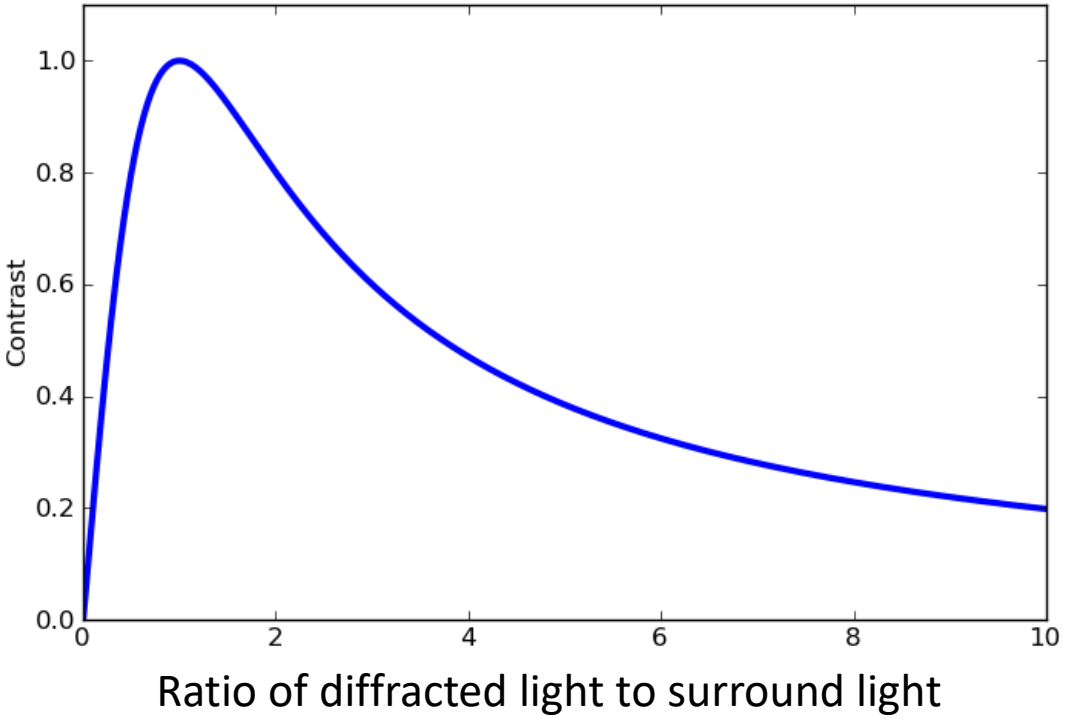




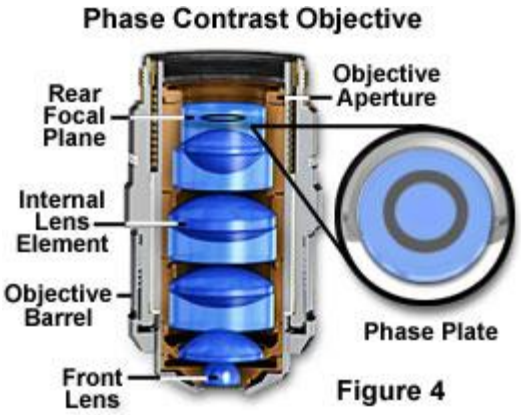
How to get the best contrast?

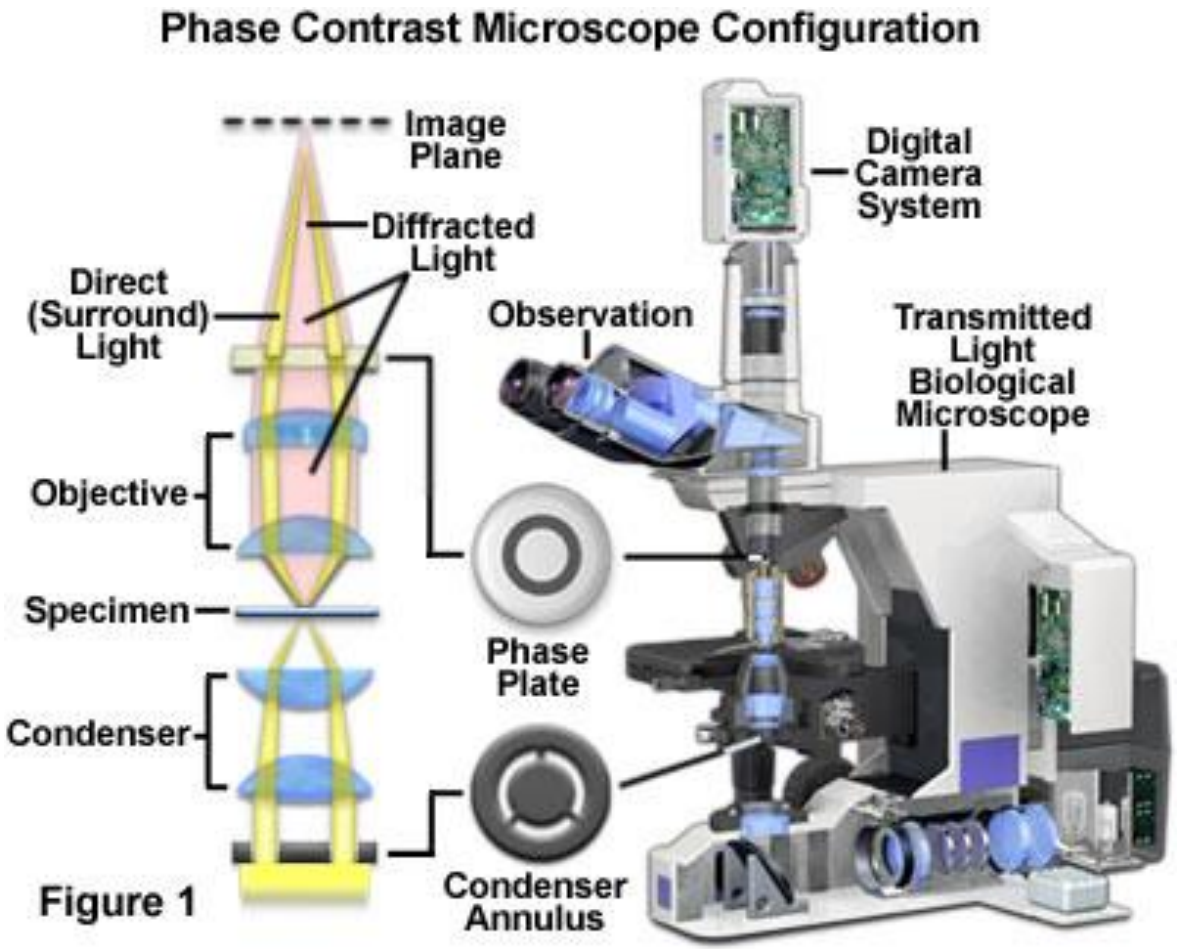
$$\gamma = \frac{I_{max} - I_{min}}{I_{max} + I_{min}}$$

In addition to introducing a phase shift, phase plate should attenuate the surround wave. Typically, surround wave is reduced by ~ 75%.



Phase plate is usually a part of the objective.

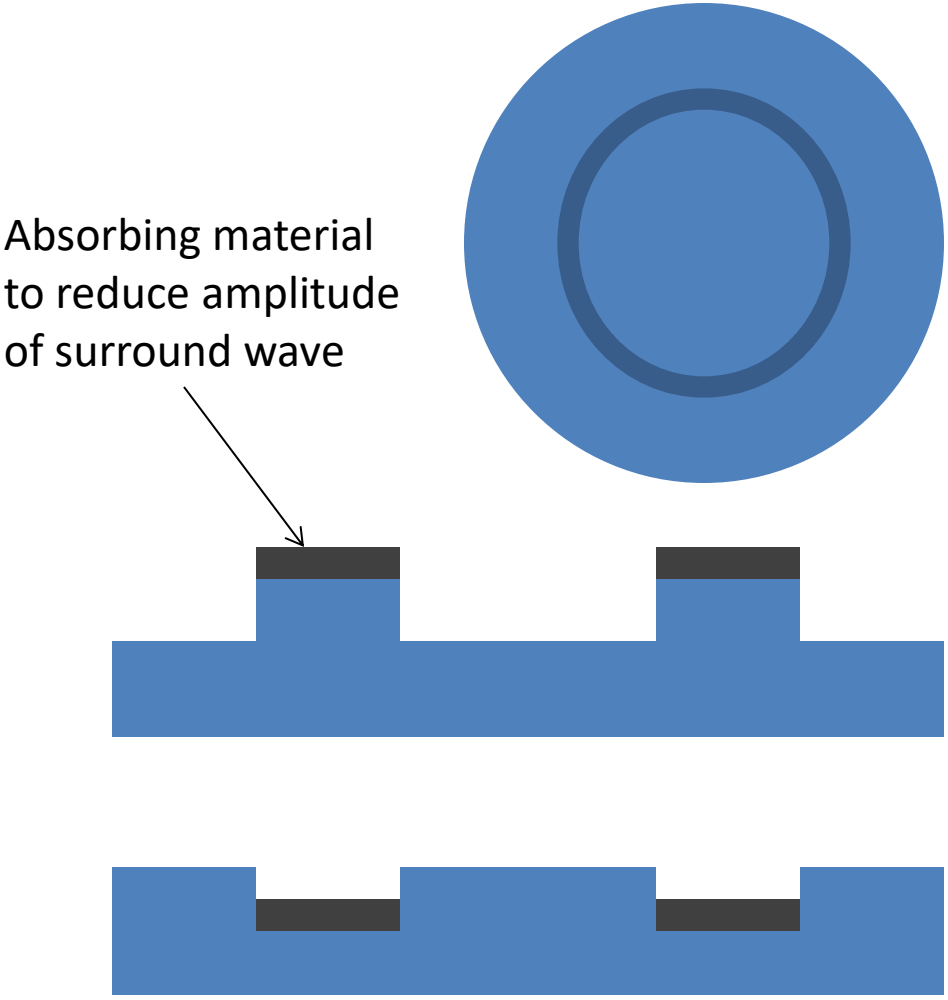




Condenser annulus and phase plate must be properly aligned for optimal imaging.

Alignment done by observing the phase plate and annulus together in the back focal plane.

Negative and Positive Phase Contrast Plates

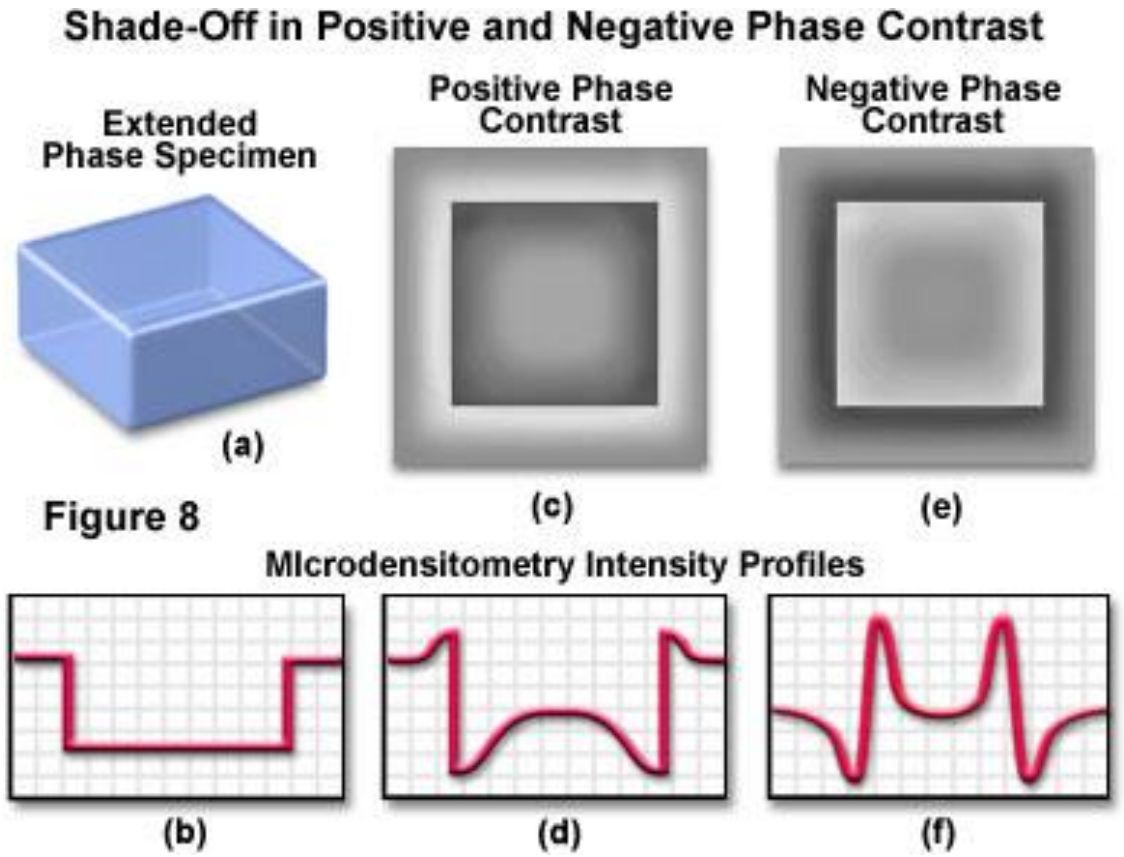


<u>Negative phase contrast</u>
Surround wave travels through more material and is therefore *retarded* in phase. Materials with *larger* OPL appear *darker*.

<u>Positive phase contrast</u>
Surround wave travels through less material and is therefore *advanced* in phase. Materials with *larger* OPL appear *brighter*.

Phase contrast is best for thin samples

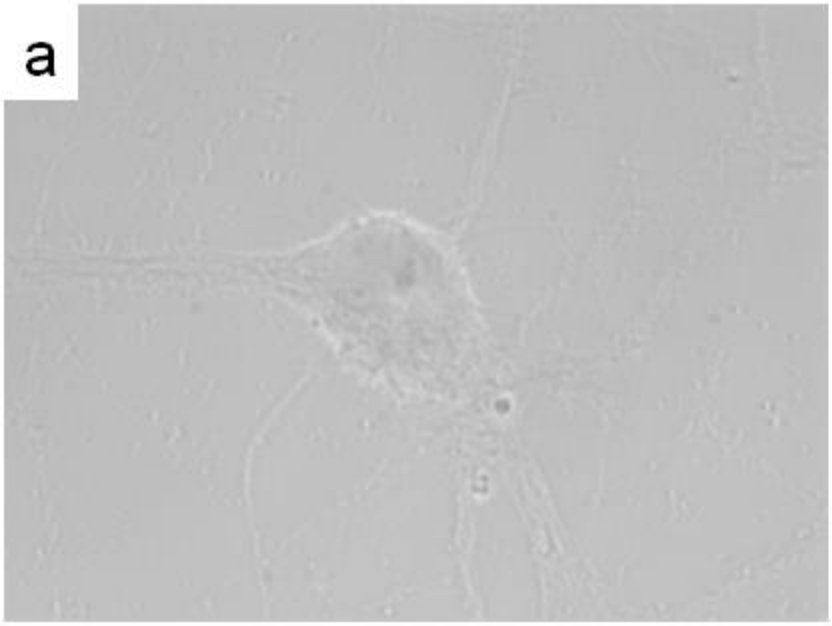
Imperfections of phase contrast technique: halo effects and shade-off



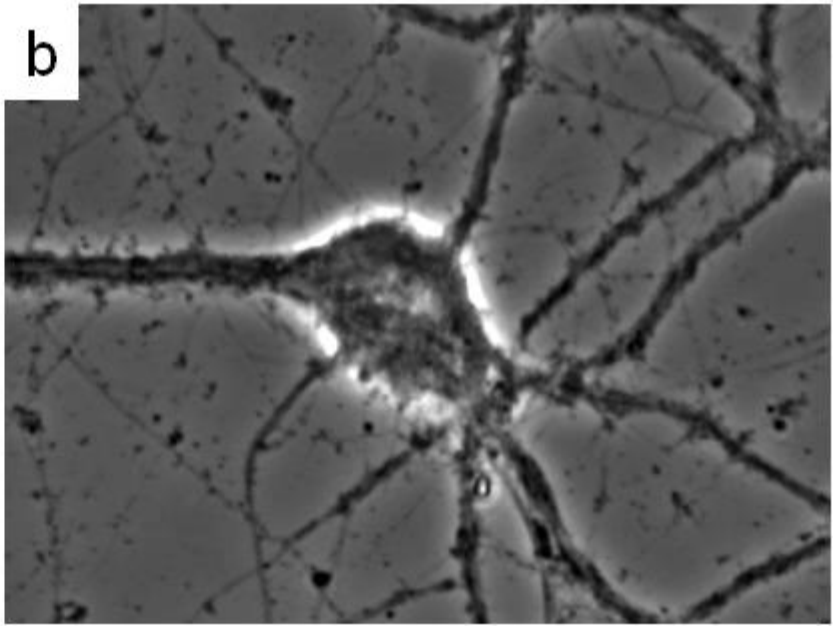
Some of the diffracted light enters phase ring and results in halo effect.

Because of larger diffraction from edges, extended objects have “shade-off” in the interiors.

Example of phase contrast image



Bright field image of neuron.



Phase contrast image.

Notice the halo effect around the body of the neuron.

Summary of Phase Contrast

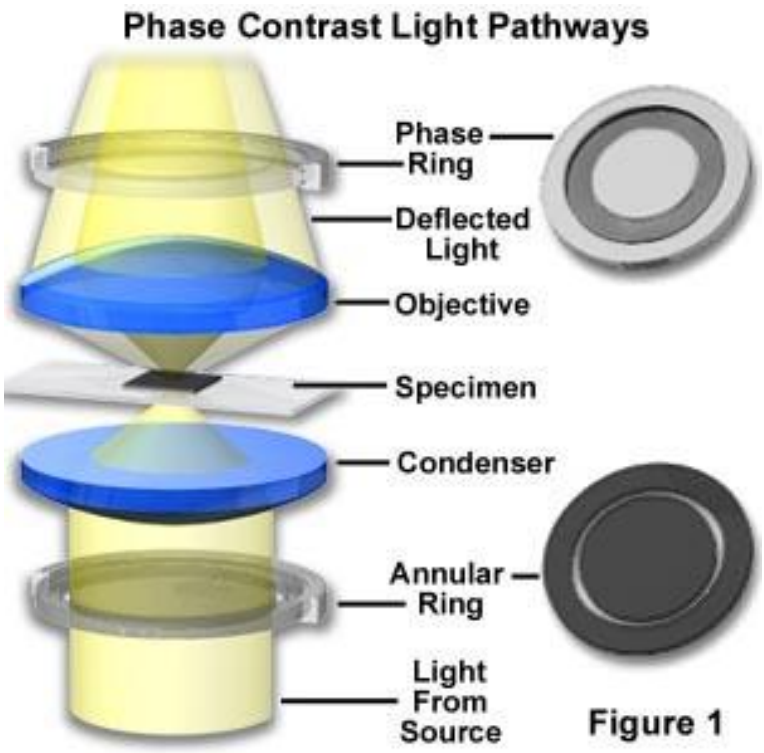
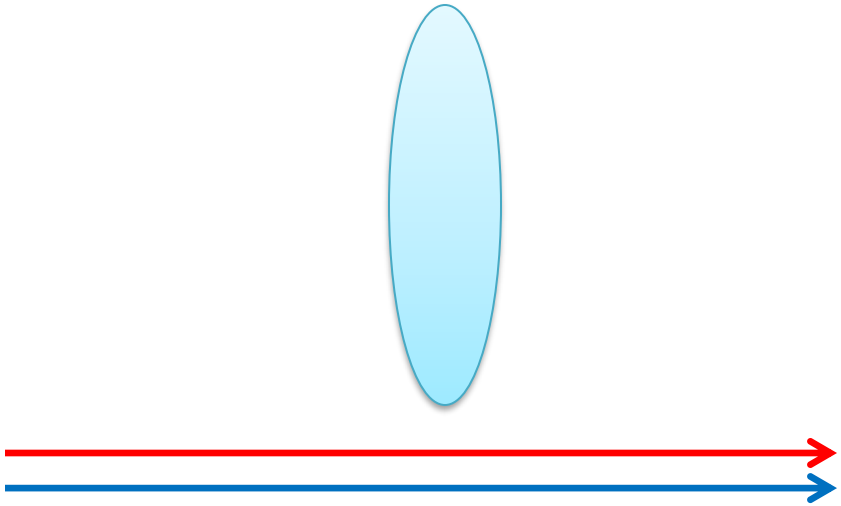


Figure 1

- Image = interference of diffracted and surround light
- With annulus, surround and diffracted light are separate in back focal plane
- $\pm \pi/2$ phase shift of surround light gives image that depends on phase of objects
- Attenuated surround light generates better contrast

Differential Interference Contrast

- Like phase contrast uses interference to convert phase difference to intensity difference
- Interferes pairs of neighboring waves that travel close together through sample



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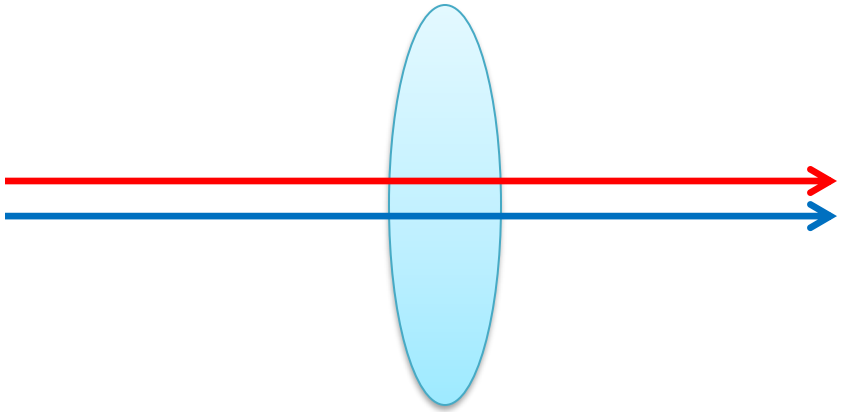

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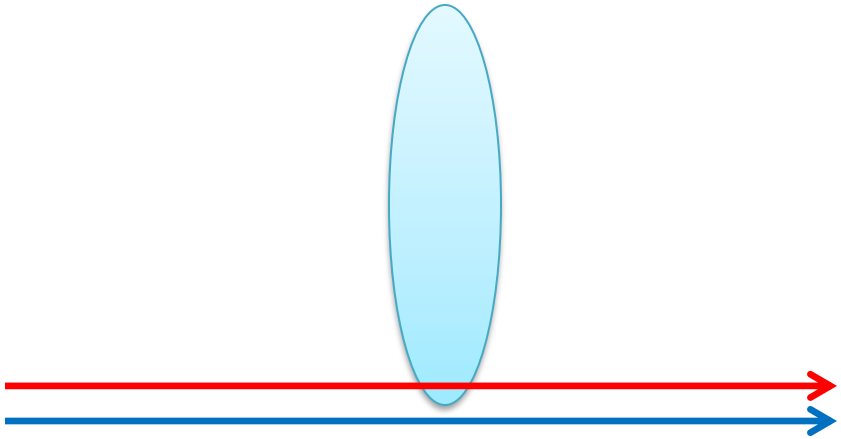

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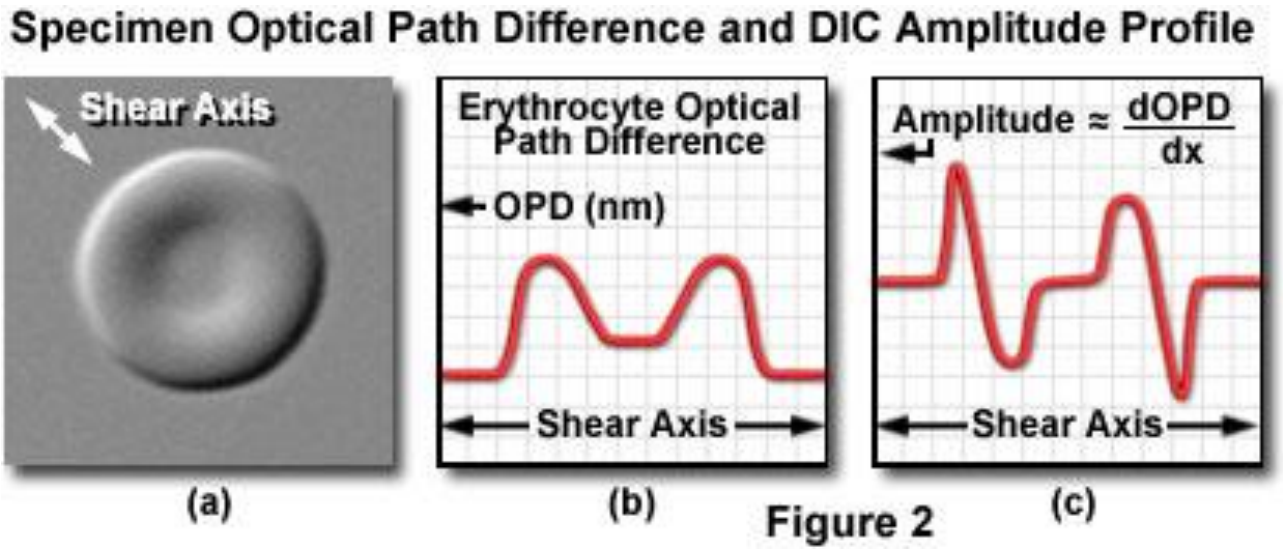
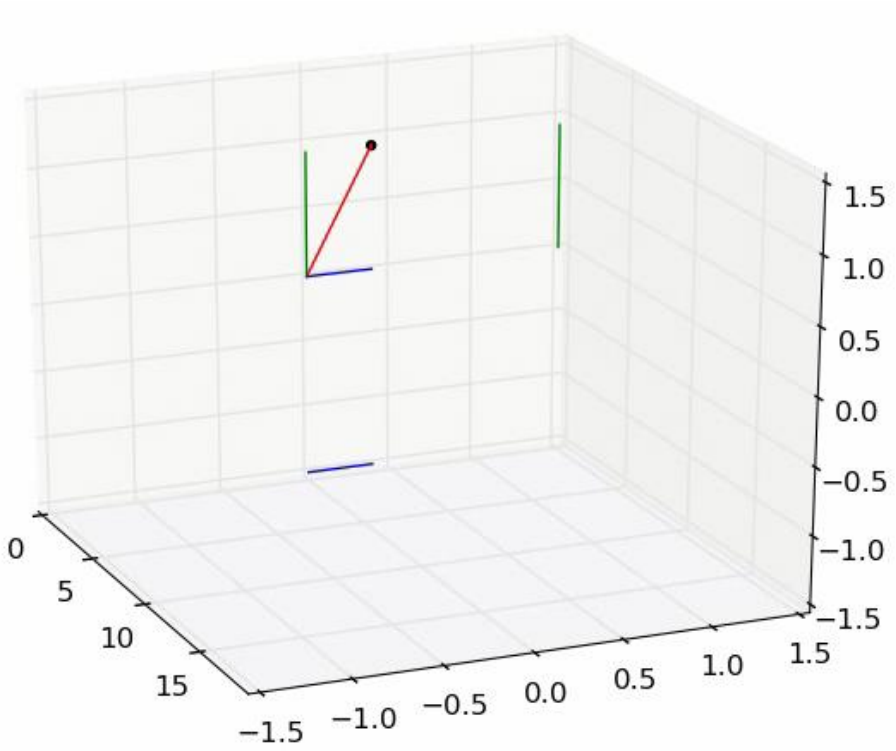


Image of a red blood cell.

OPD = refractive index difference × thickness

- Sensitive to phase gradients
- Contrast best along the direction of shear
- Objects appear shaded or in pseudo 3D relief
- Necessary optics: polarizers and prism beam splitters
- Uses full condenser and objective apertures

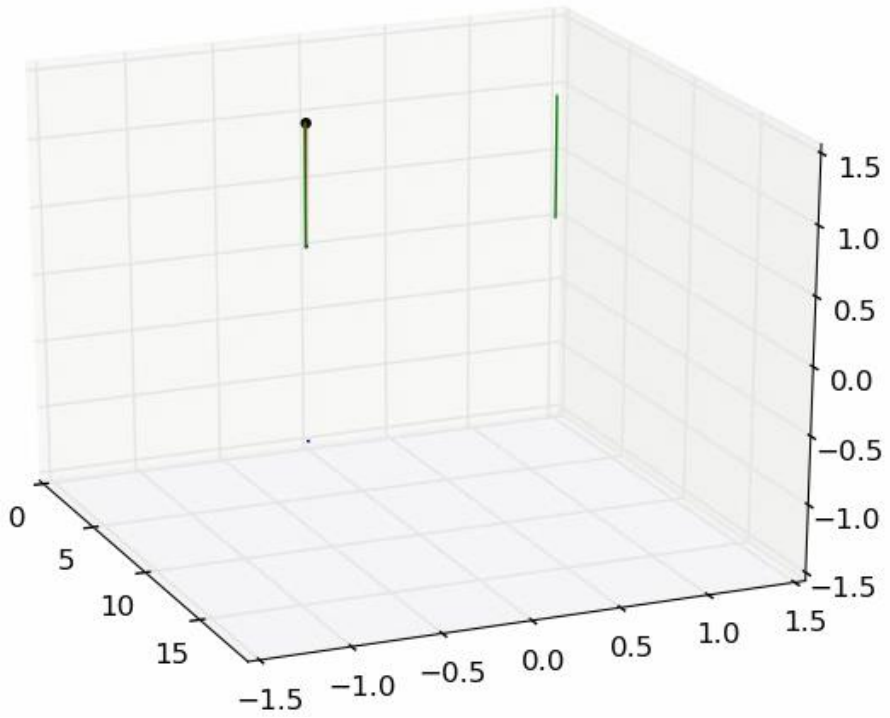
Polarization



- Polarization of light is perpendicular to direction of propagation.
- Light can be linearly polarized if field is oriented in a single direction.
- Light can be elliptically polarized if the field direction rotates as the wave propagates

$$\vec{E} = E_x\hat{x} + E_y\hat{y}$$

Polarization



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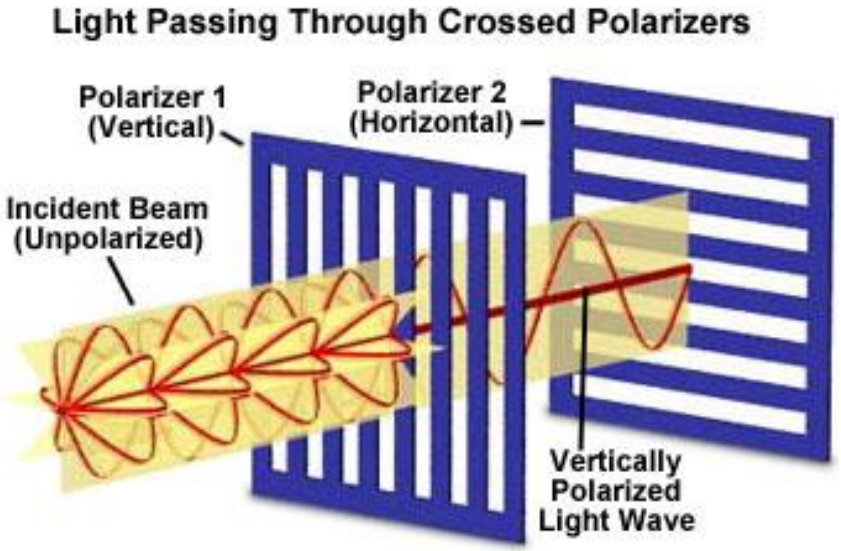
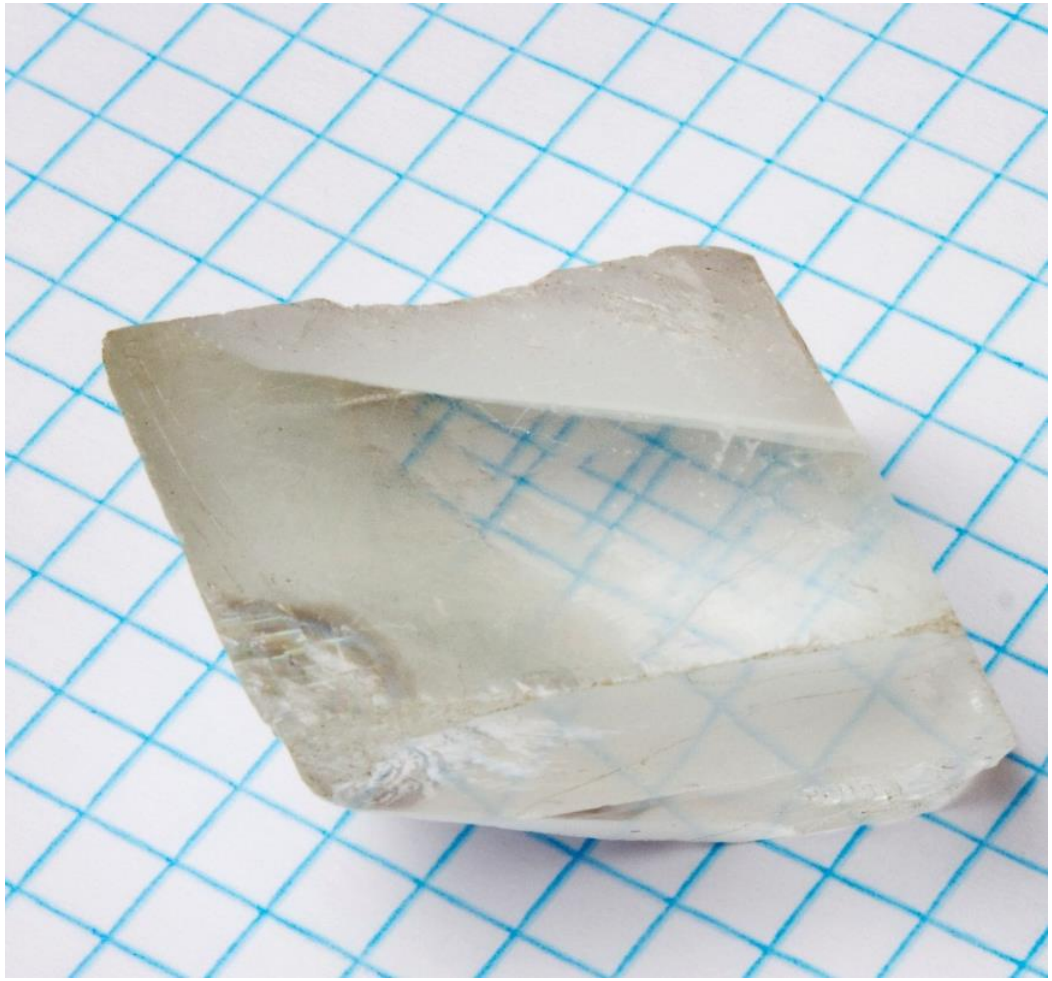
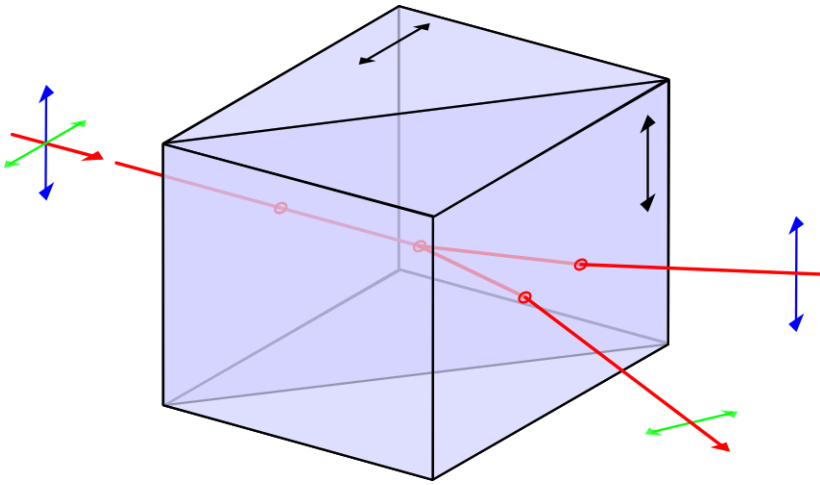


Figure 1

Birefringence



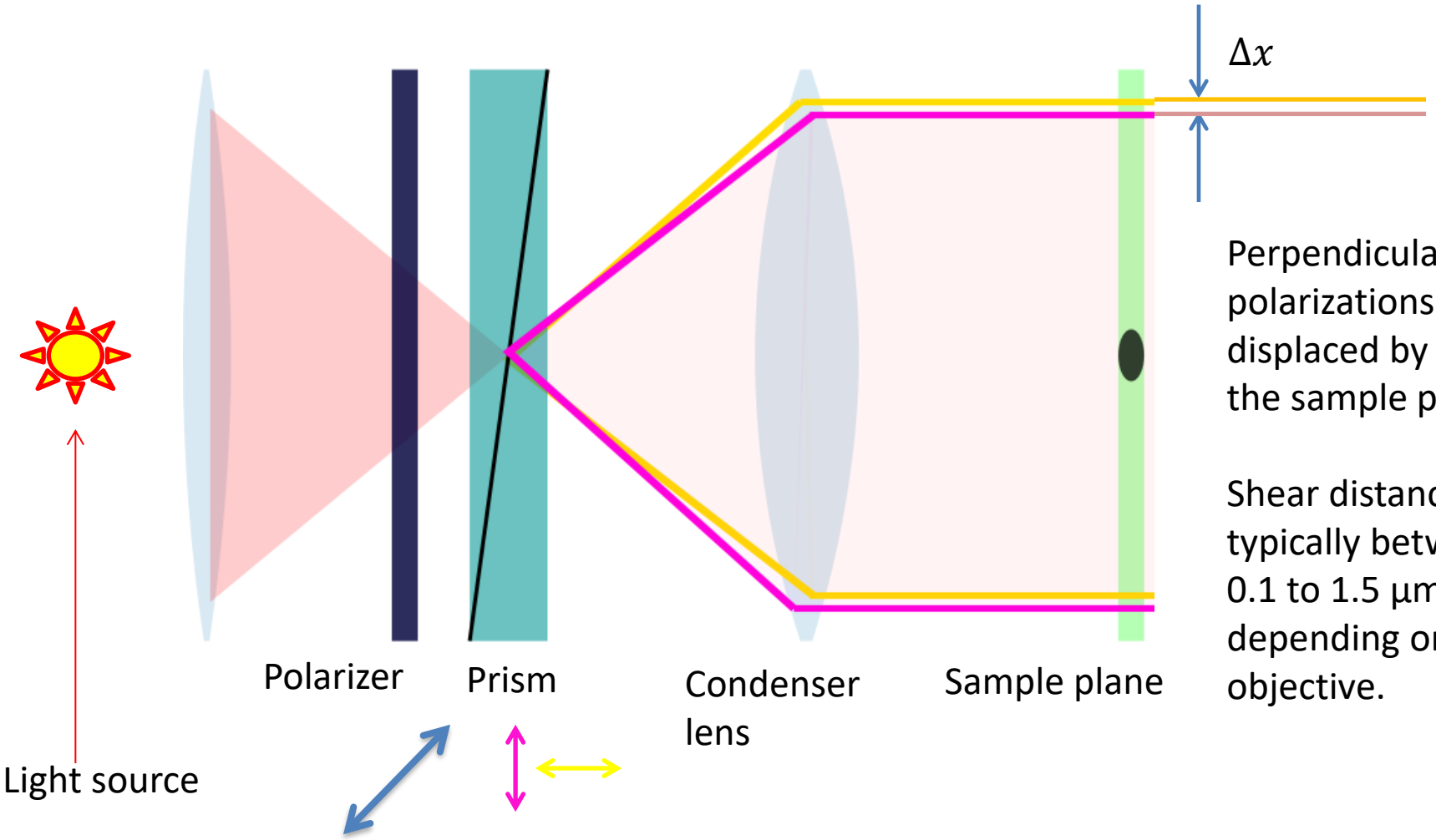
Wollaston Prism



Birefringent prism splits light into two orthogonally polarized beams.

The two beams transverse the sample and are recombined by another prism.

DIC: Illumination path



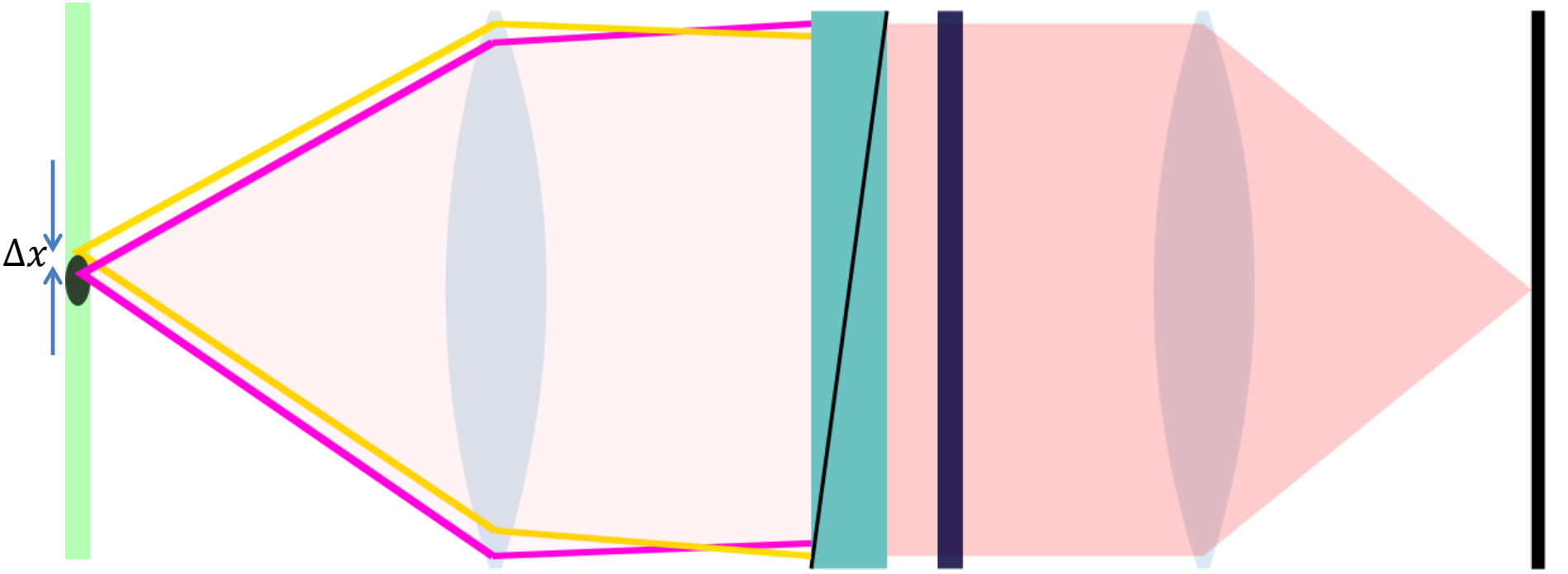
Perpendicular polarizations are displaced by Δx in the sample plane.

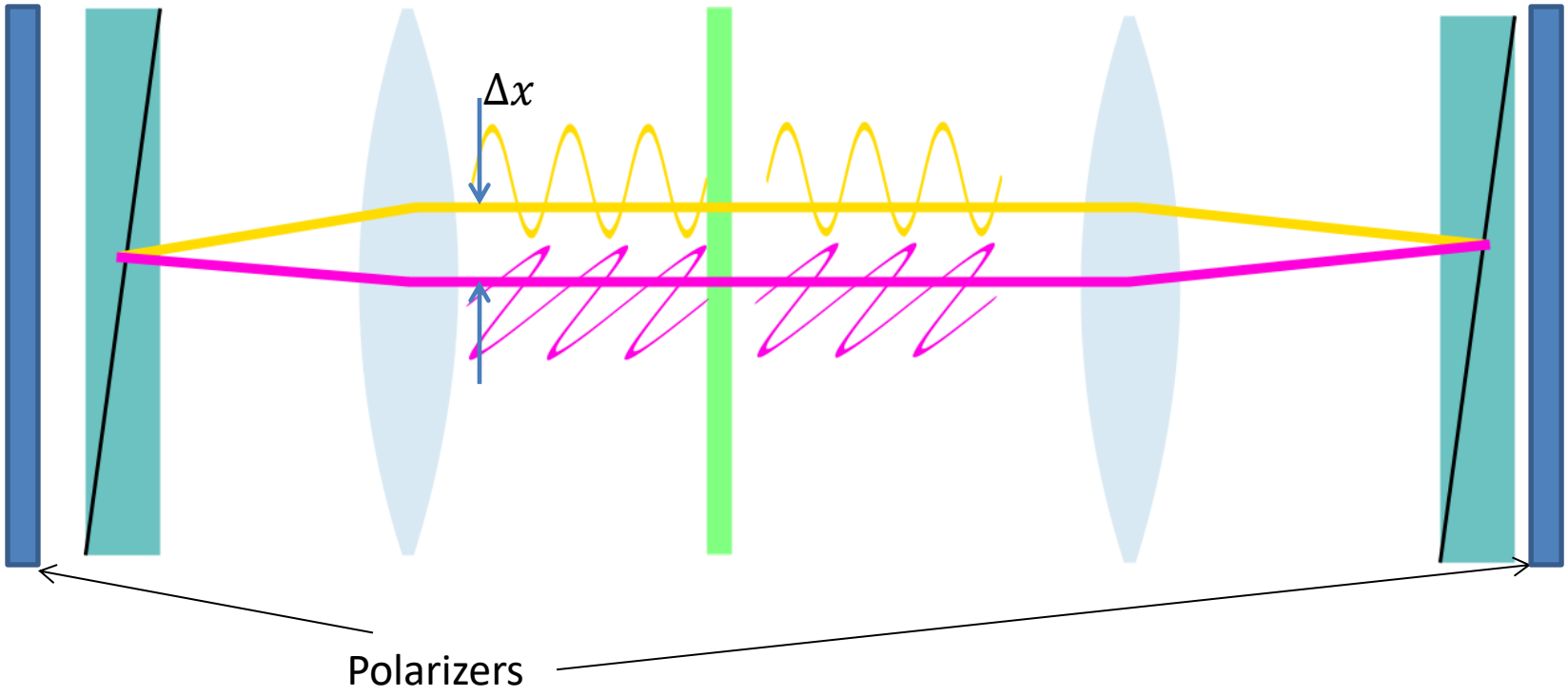
Shear distance typically between 0.1 to 1.5 µm depending on objective.

DIC: Detection path

Perpendicular polarizations are brought back together.

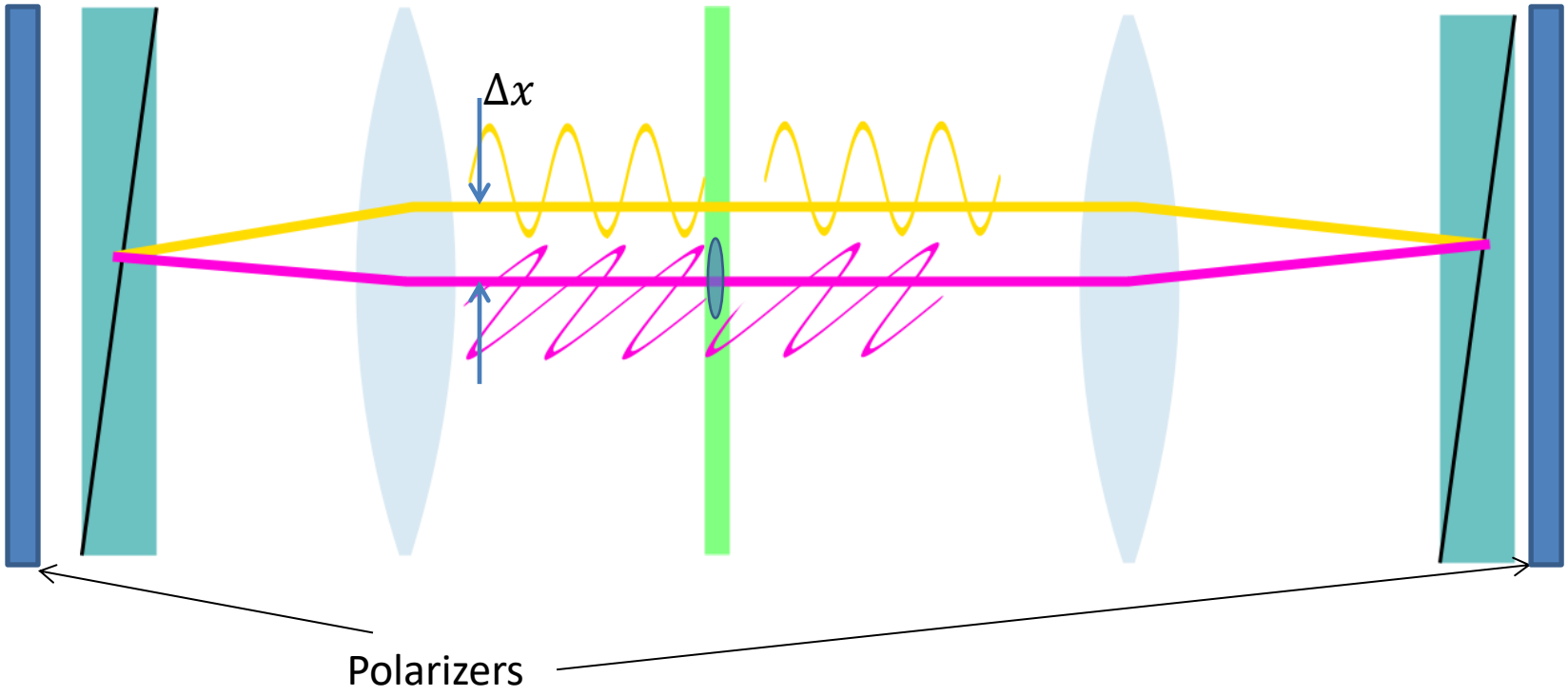
Unless there exists a phase difference between two polarizations, polarizer blocks light (extinction).





With polarizers crossed (i.e. perpendicular to each other):

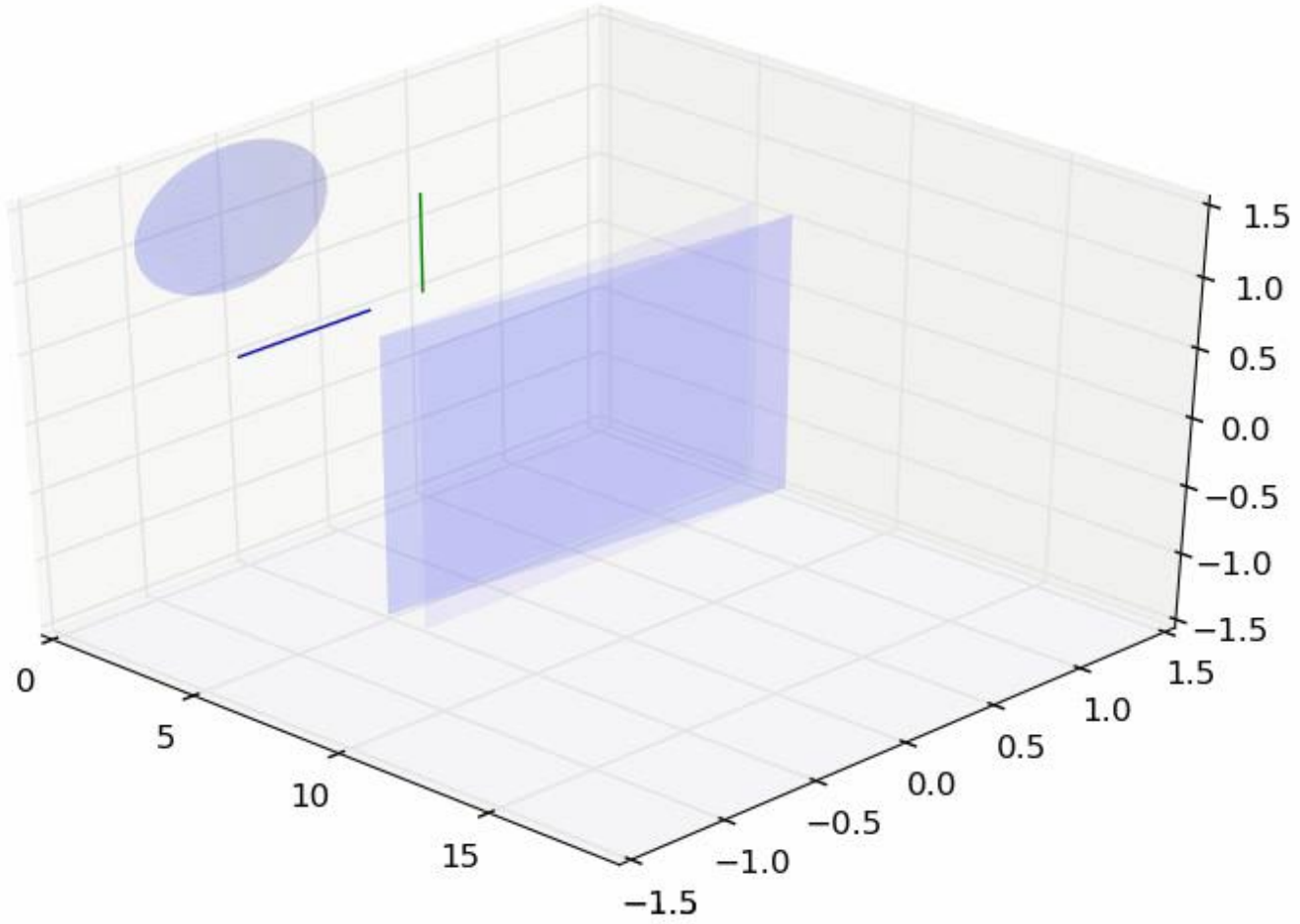
- Light is blocked if no phase difference between two displaced light paths
- With phase difference, light is transmitted to a degree depending on the phase difference



With polarizers crossed (i.e. perpendicular to each other):

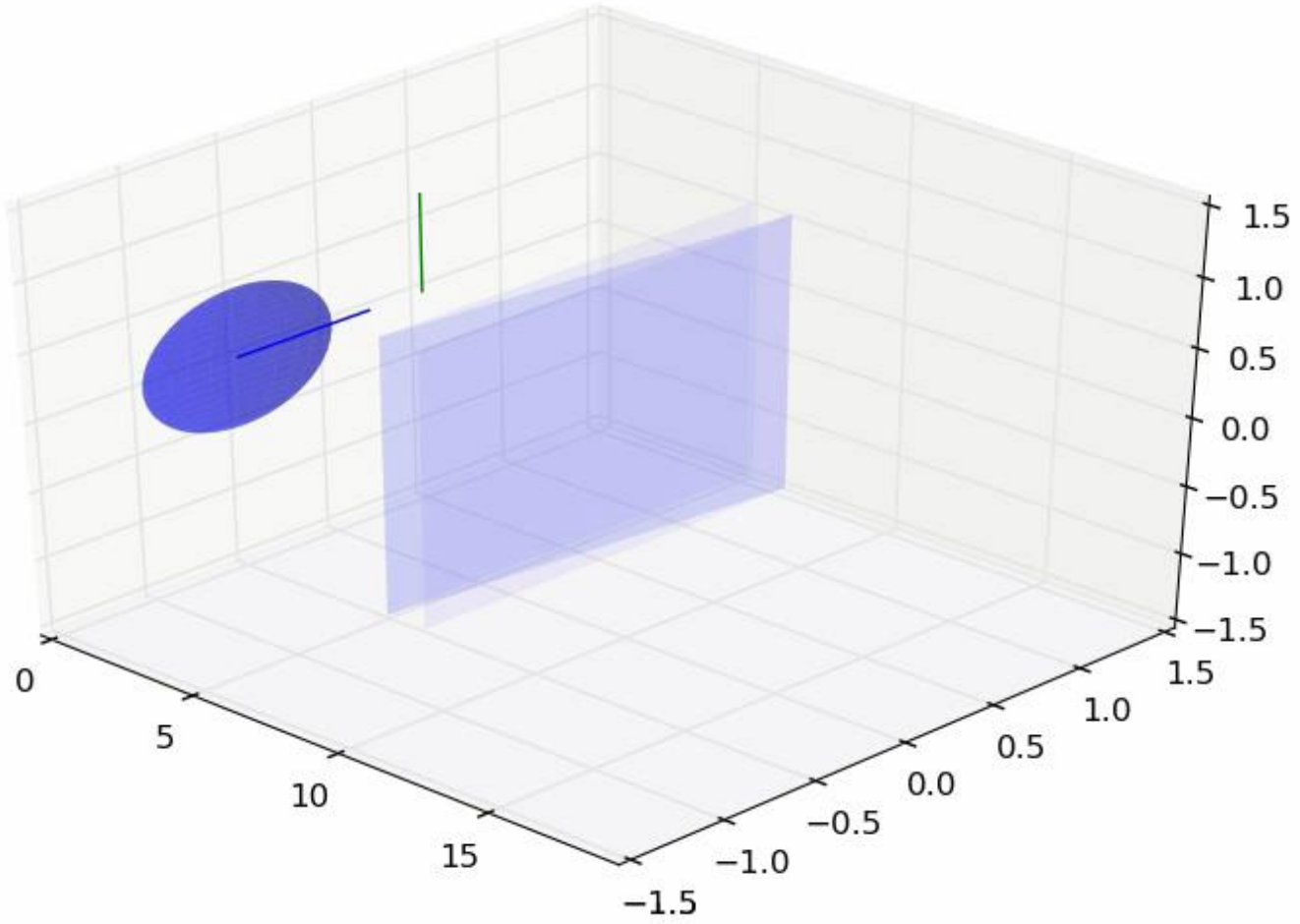
- Light is blocked if no phase difference between two displaced light paths
- With phase difference, light is transmitted to a degree depending on the phase difference

No phase difference between two sheared polarizations:
recombined beam is linearly polarized.



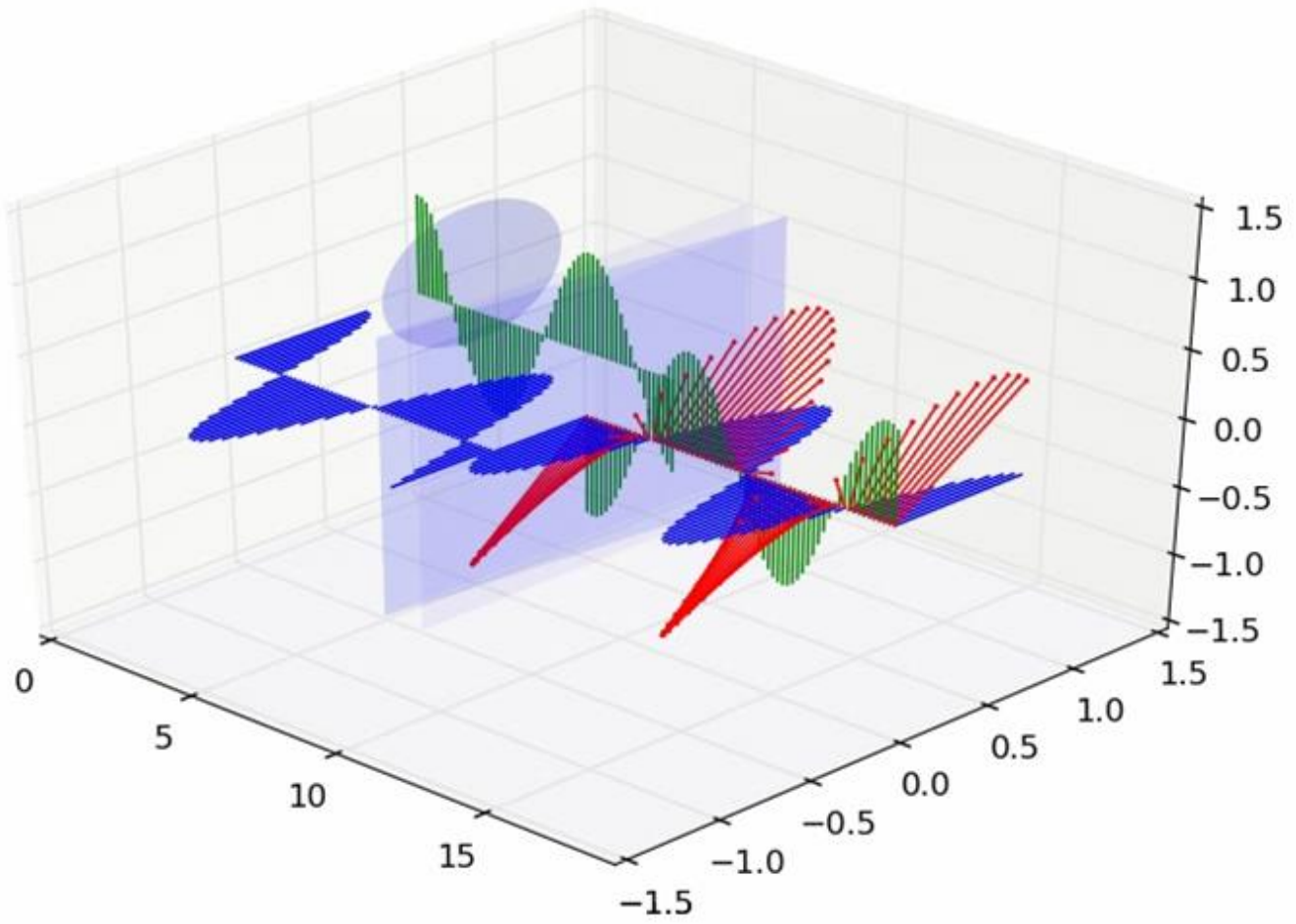
$$\vec{E} = E_x\hat{x} + E_y\hat{y}$$

With a phase difference between two sheared polarizations:
recombined beam is elliptically polarized.

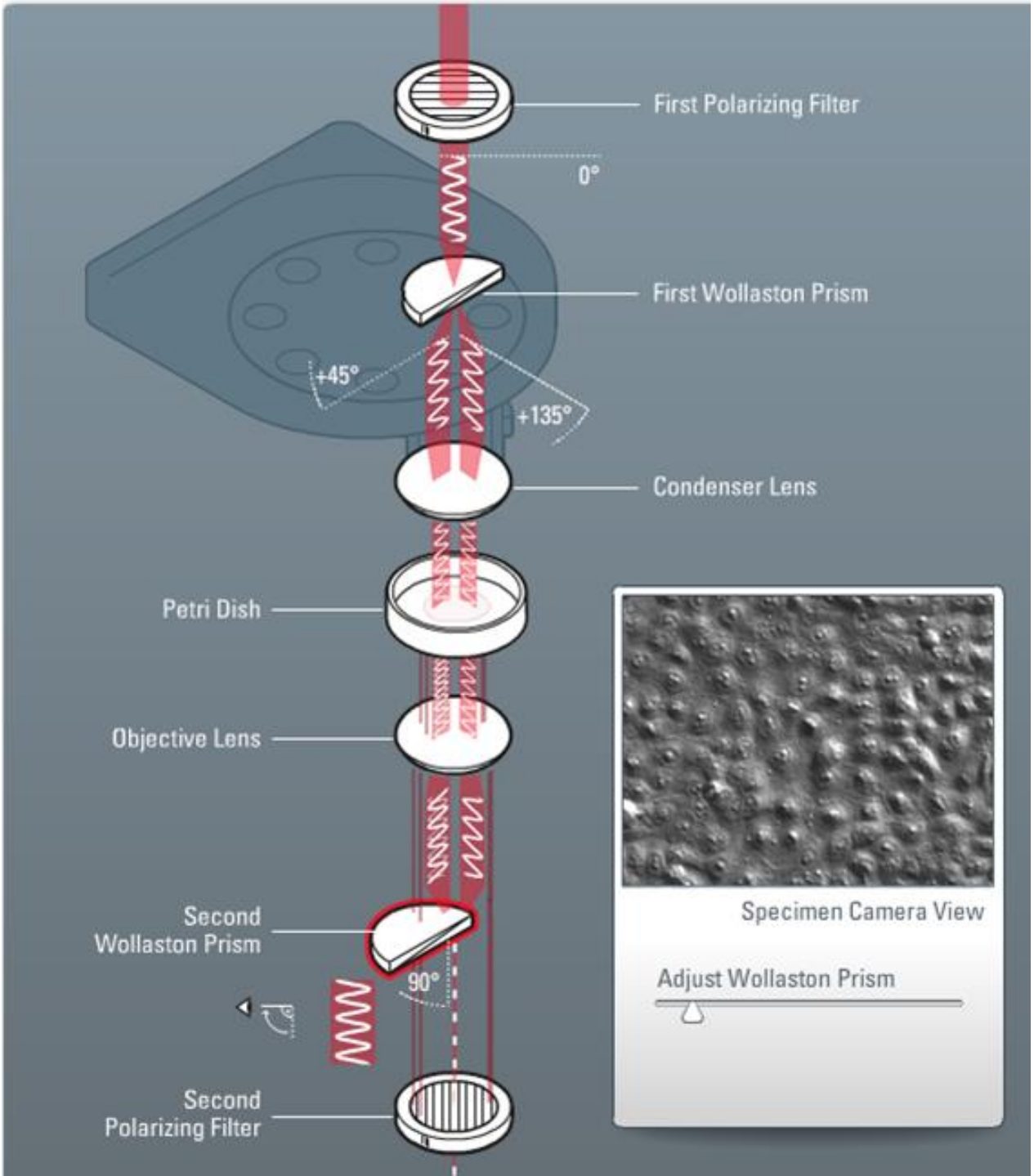


$$\vec{E} = E_x\hat{x} + E_y\hat{y}$$

Ellipticity of combined beam a function of the optical path difference.



$$\vec{E} = E_x\hat{x} + E_y\hat{y}$$



Contrast of image depends on phase difference of the separated polarizations.

Phase differences occur due to:

1. Optical path length difference in sample
2. Alignment of the two prisms and/or polarizer to introduce a “bias”.

Control of bias phase

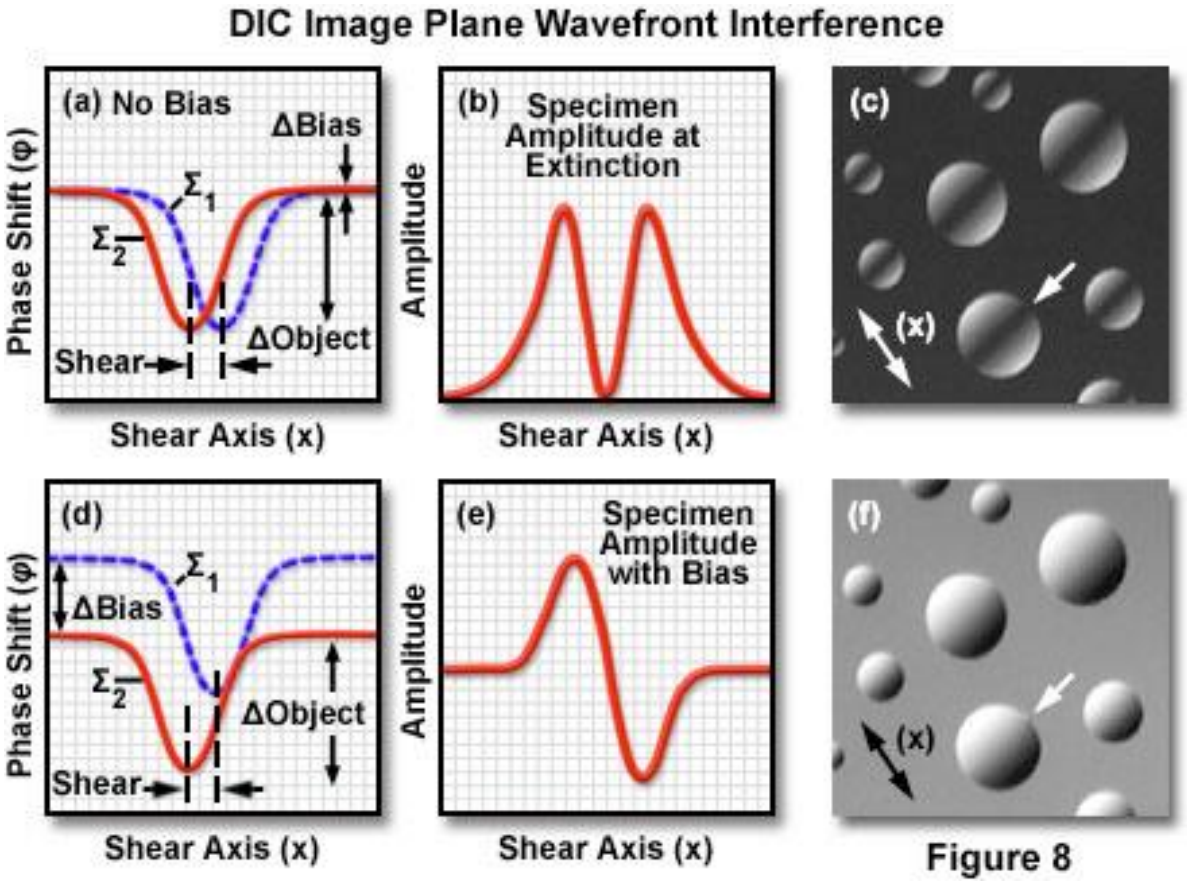
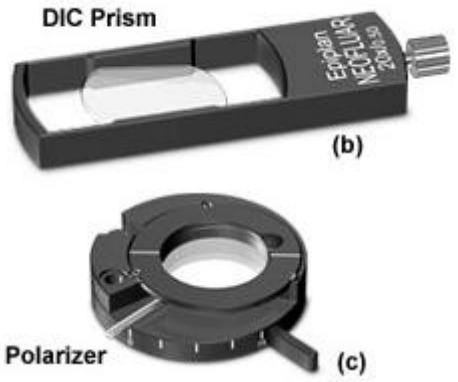
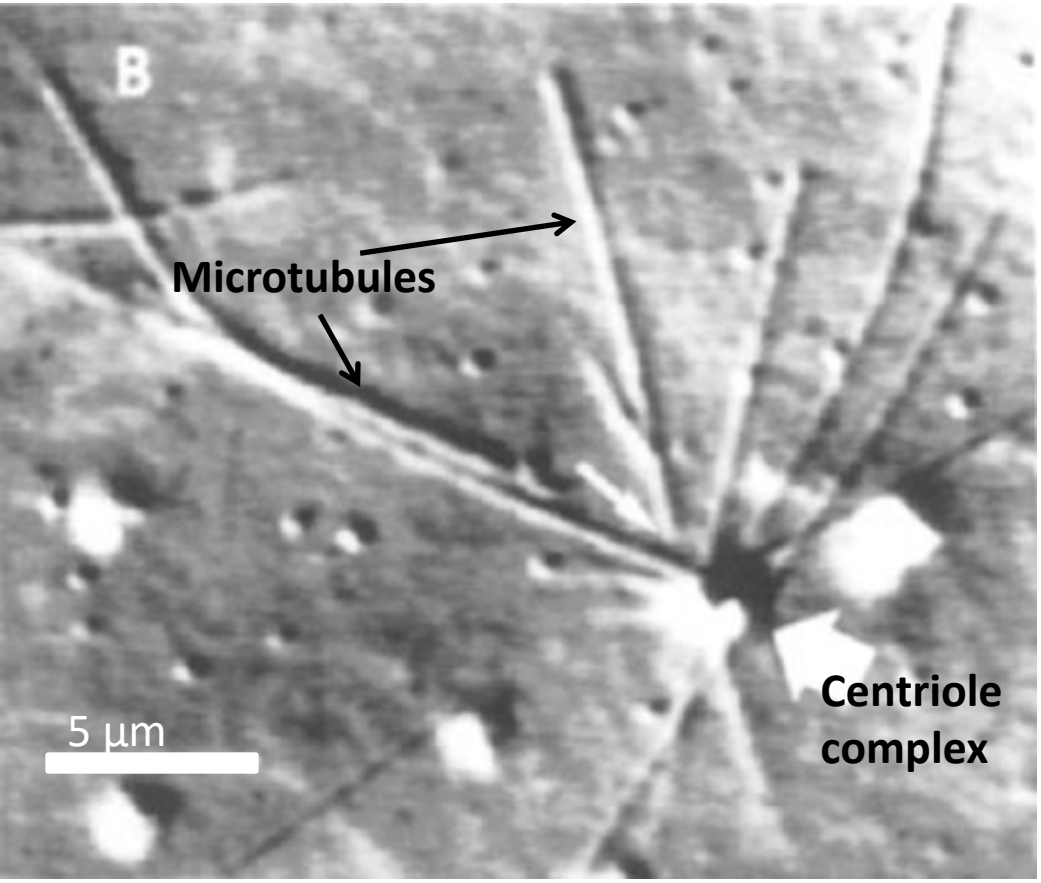


Figure 8

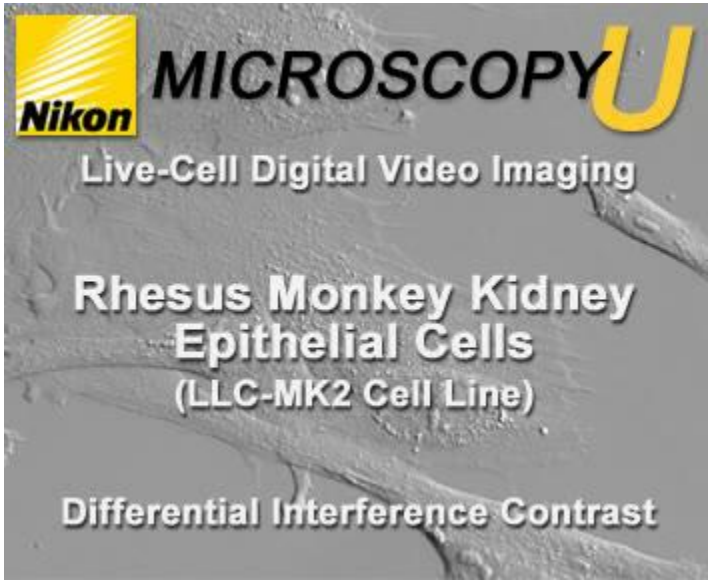
- Contrast controlled through "bias retardation" or "compensation"
- With no bias, no background light gets through
- Bias is introduced through sliding one of the prisms or, with a de Senarmont compensator, rotating a polarizer



DIC images



Gliksman *et al.* 1992. *J. Cell Biol*. 119: 1271-1276.



Can imaging be quantitative about phase differences?

$$I_{DIC}(x,y) \sim \Delta x \frac{\partial \varphi(x,y)}{\partial x}$$

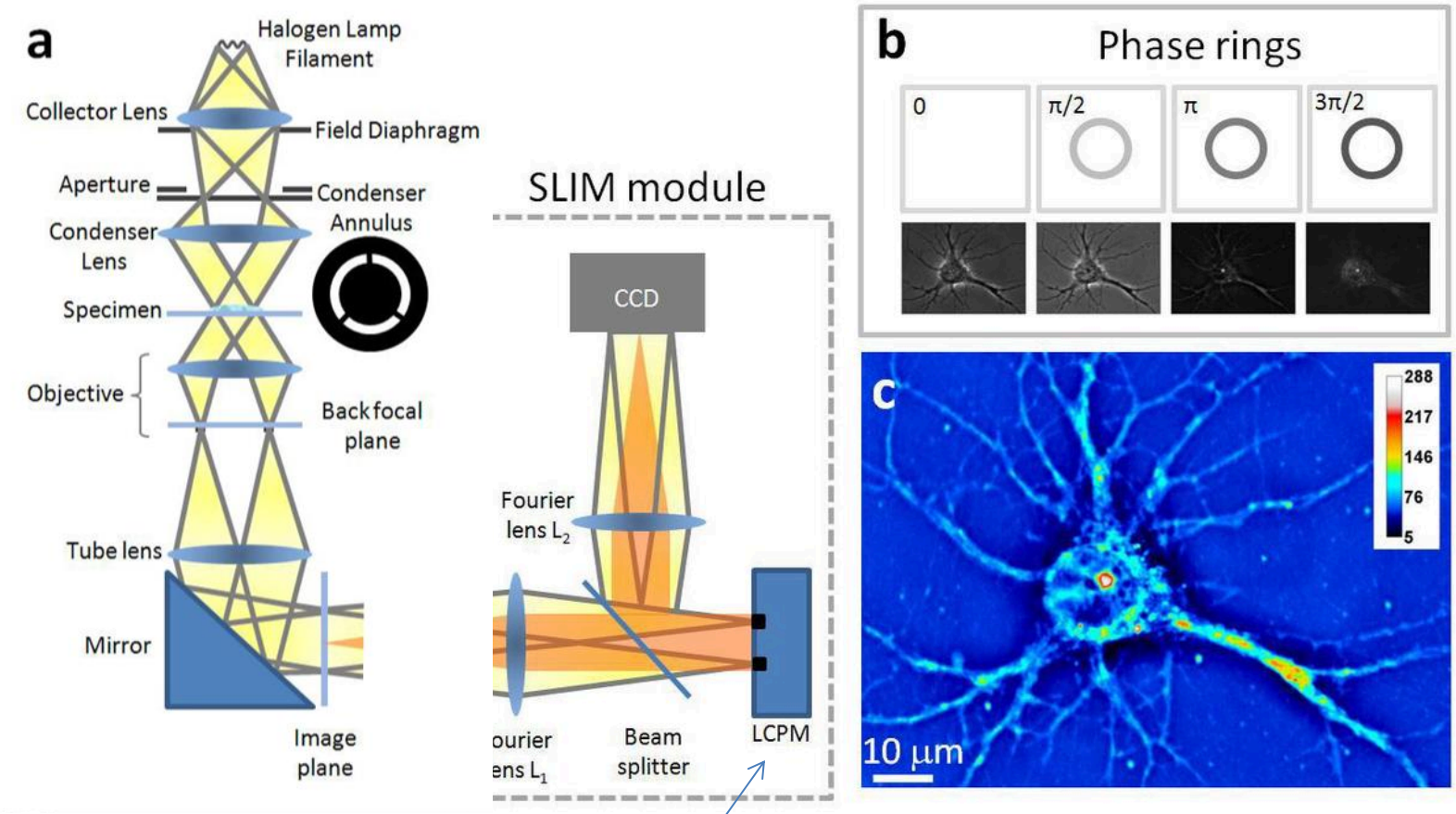
$$I_{PC}(x,y) \sim \varphi(x,y)$$

But these relations hold only for pure phase objects or where the phase of the object is not large.

Many approaches to *quantitative* phase microscopy:

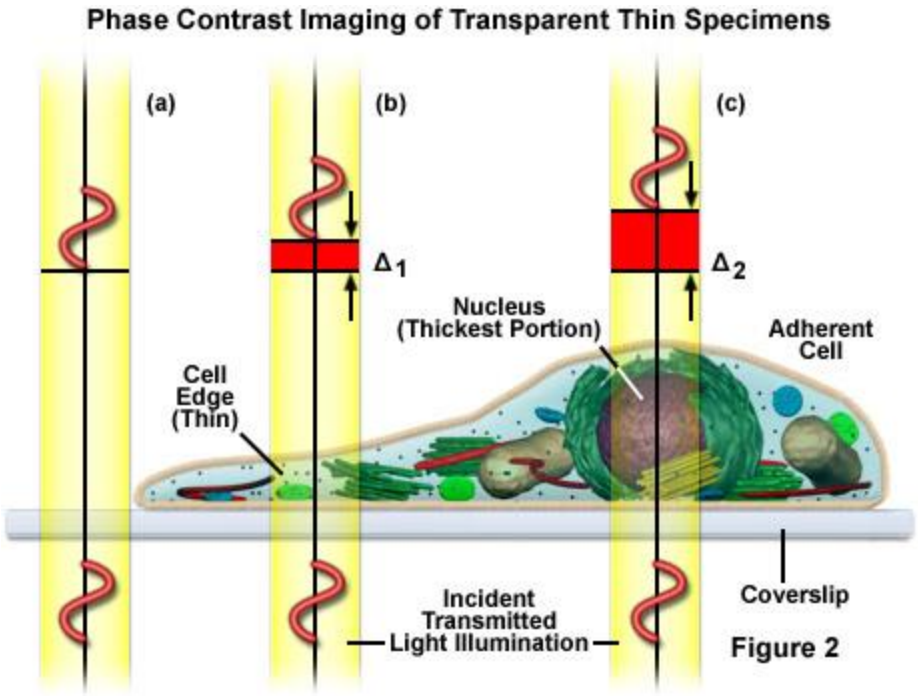
- Fast Fourier phase microscopy
- Phase-dispersion microscopy
- Spiral phase contrast microscopy
- Optical coherence microscopy
- Digital holographic microscopy
- Etc.

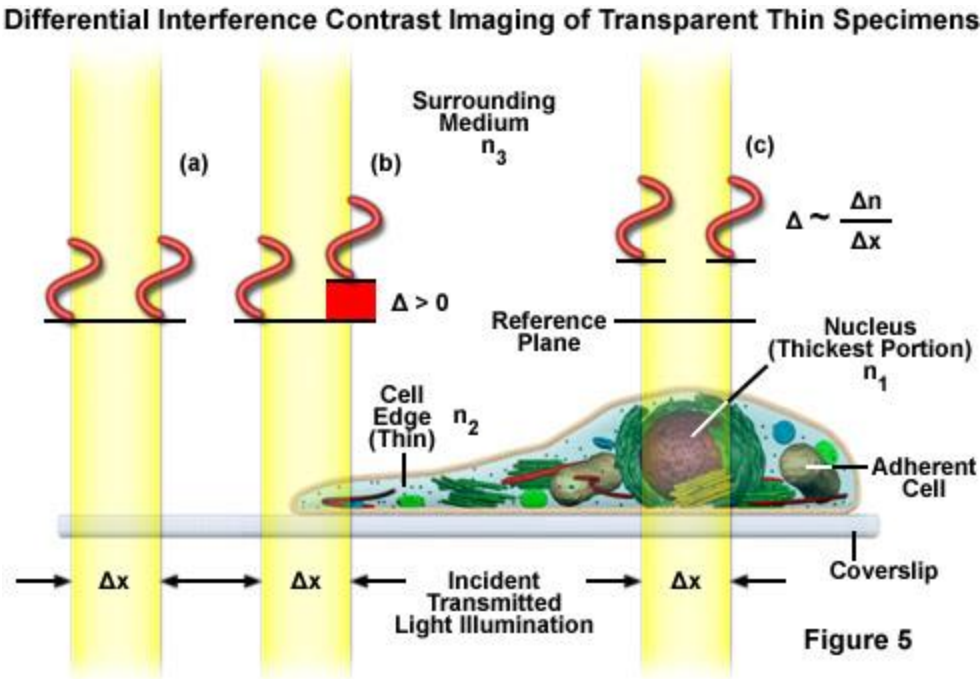
Spatial light interference microscopy



Controllable phase delay of surround light

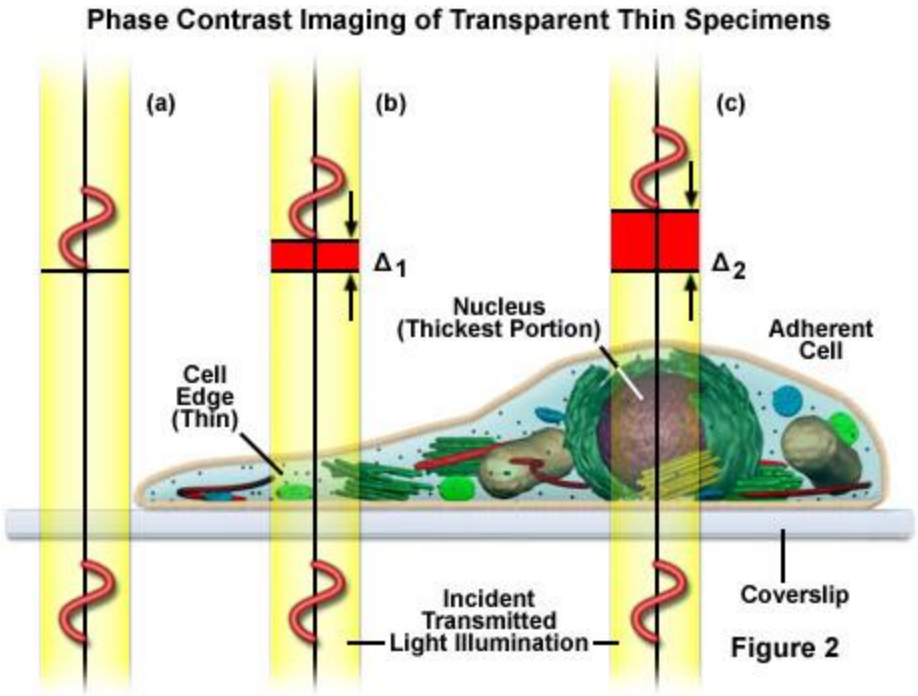
Recap

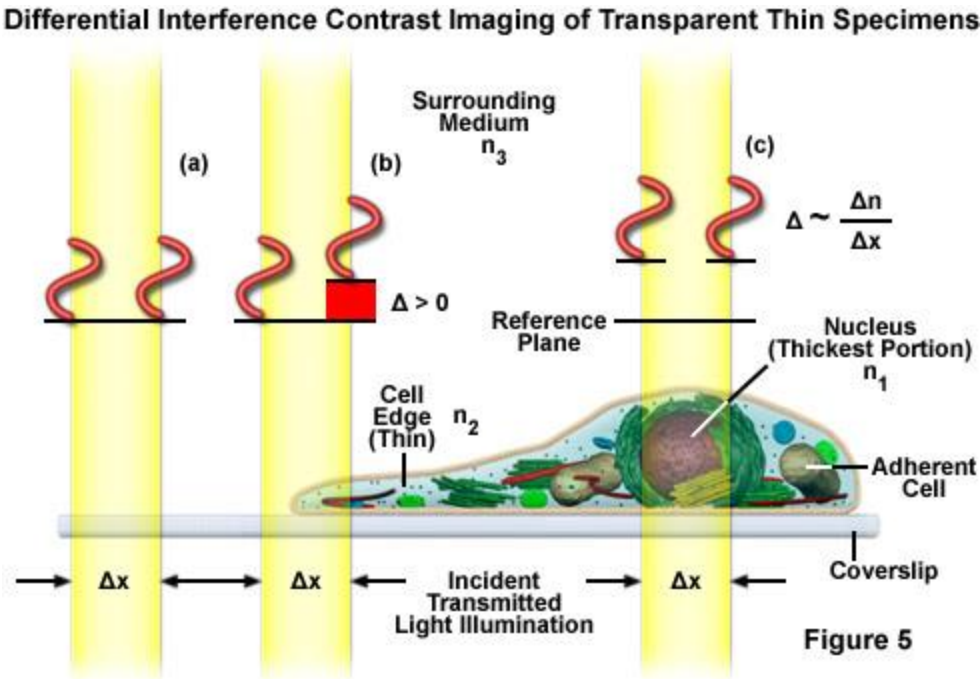




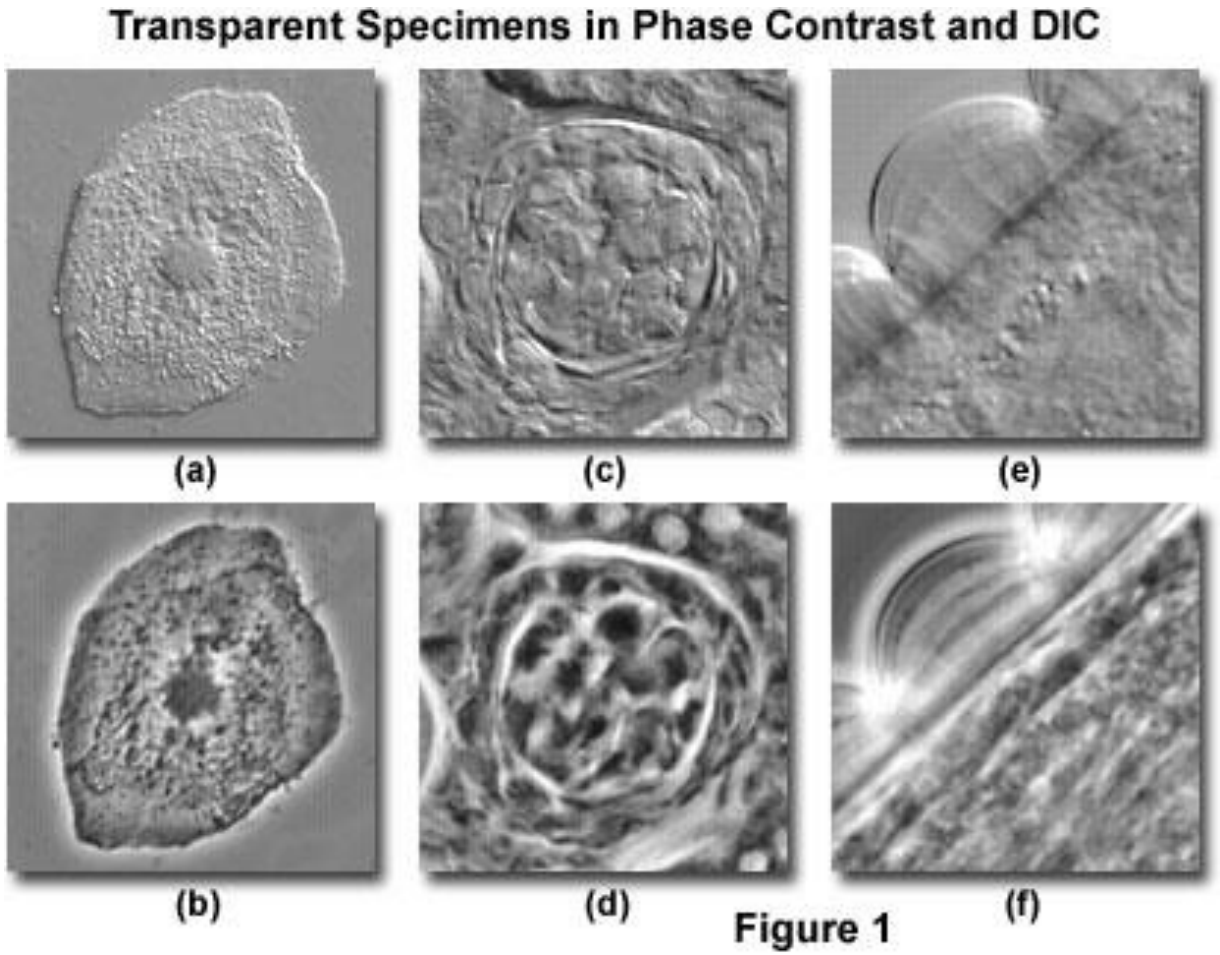
http://zeiss-campus.magnet.fsu.edu/articles/basics/contrast.html

Recap





http://zeiss-campus.magnet.fsu.edu/articles/basics/contrast.html



Cheek cell

Kidney tissue

Perisarc of hydrozoan

When to use phase contrast versus DIC?

	Phase Contrast	DIC
Imaging thick samples	Poor	Good
Imaging with birefringent materials	Good	Poor
Sensitive to sample orientation	No	Yes
Imaging of large phase shifts	Poor	Good

Additional Resources

- Websites:
  - http://www.microscopyu.com/
  - http://www.olympusmicro.com/
  - http://www.leica-microsystems.com/science-lab/
- Labs researching quantitative phase imaging:
  - Prof. Colin Sheppard http://www.bioeng.nus.edu.sg/optbioimaging/colin/research.asp
  - Prof. Gabriel Popescu http://light.ece.illinois.edu/
  - Prof. George Barbastathis http://3doptics.mit.edu/website/home
- Books:
  - *Advanced Light Microscopy* by M. Pluta
  - *Introduction to Optical Microscopy* by J. Mertz
  - *Quantitative Phase Imaging of Cells and Tissues* by G. Popescu