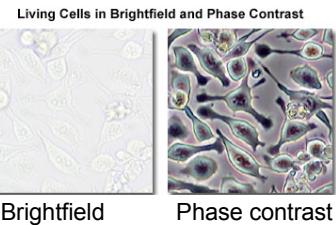


Generating contrast in light microscopy

Orion Weiner Tetrad Microscopy Bootcamp 2009



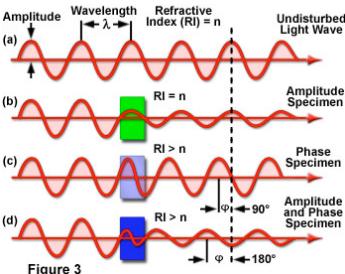
Problem-- many living unstained samples are thin and optically transparent

Hard to see by brightfield.

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

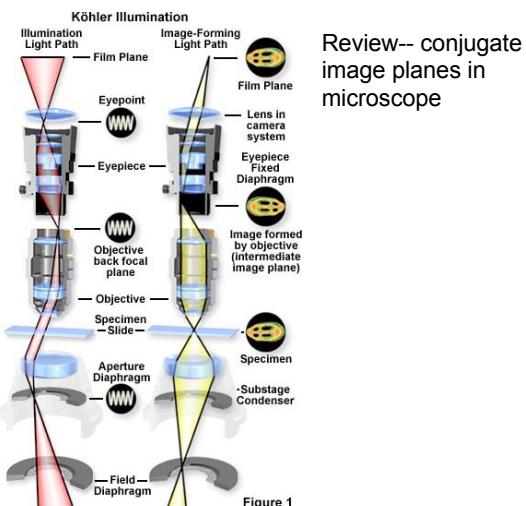
Absorption is not the only way samples interact with light. (polarization, phase shift)

Amplitude and Phase Specimens



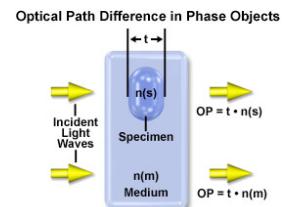
Your eyes are good at seeing differences in amplitude (intensity) and wavelength (color), but not phase or polarization

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



Review-- conjugate image planes in microscope

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

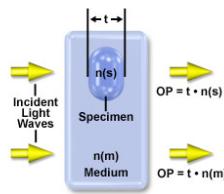
$n(m)$ = refractive index of medium. Cell medium 1.335

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

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

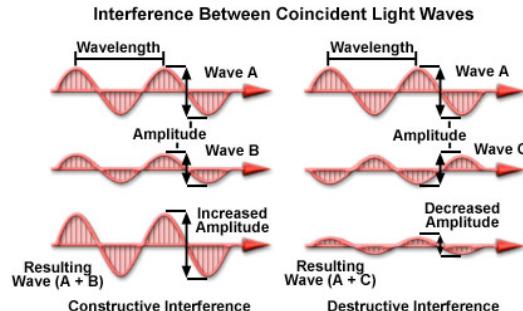
which is about **1/4 the wavelength of green light (488 nm)**

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

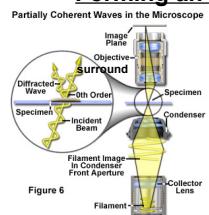
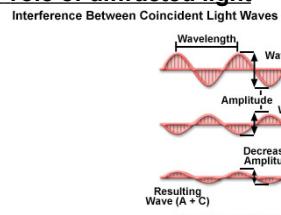
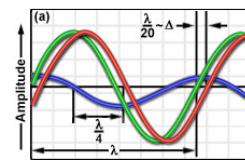


Figure 6
Partially Coherent Waves in the Microscope



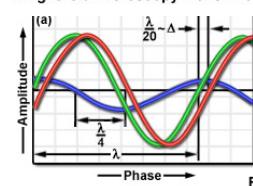
Interference Between Coincident Light Waves



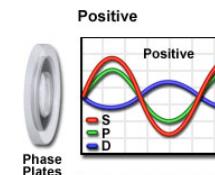
S= surround (undiffracted)
D= diffracted wave
P= particle wave (S+D)

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

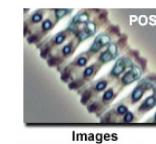
Brightfield Microscopy Wave Phase Relationships



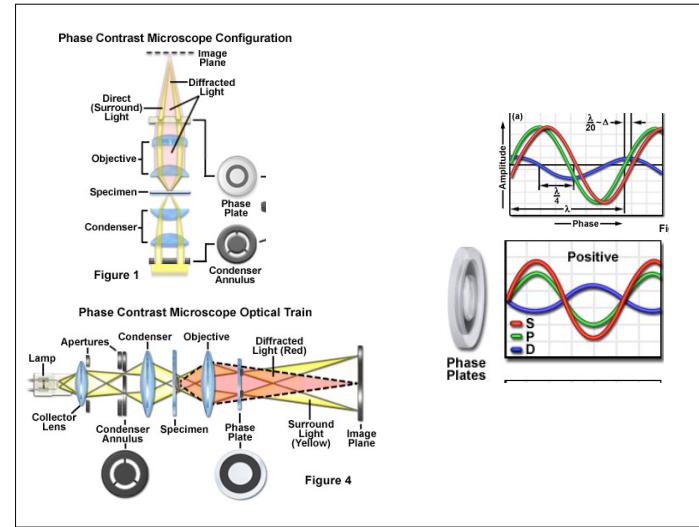
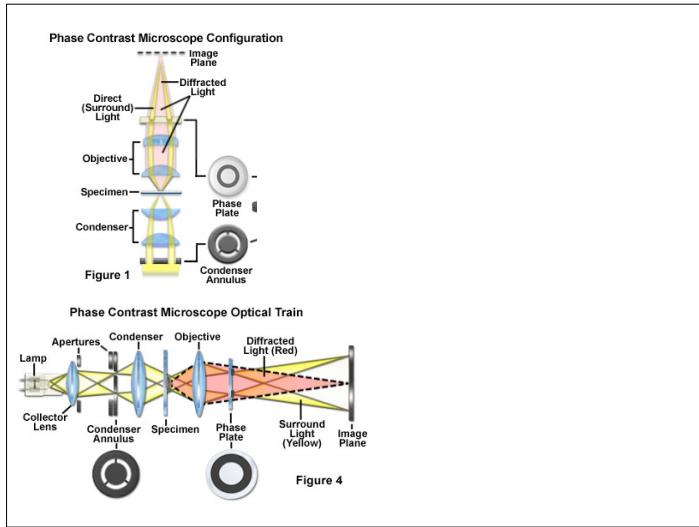
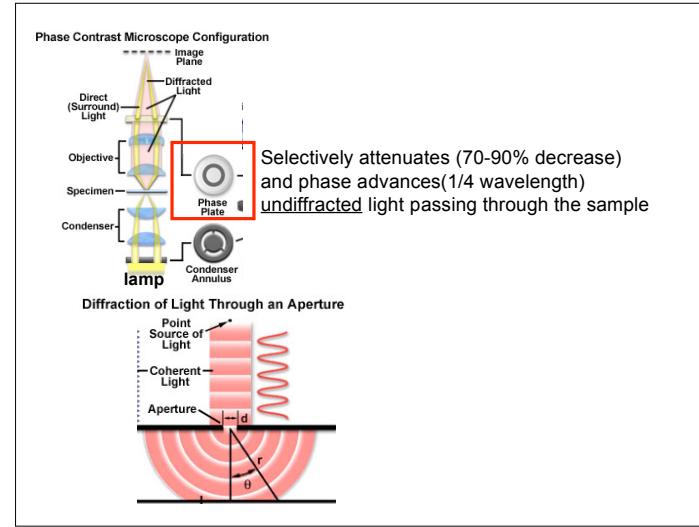
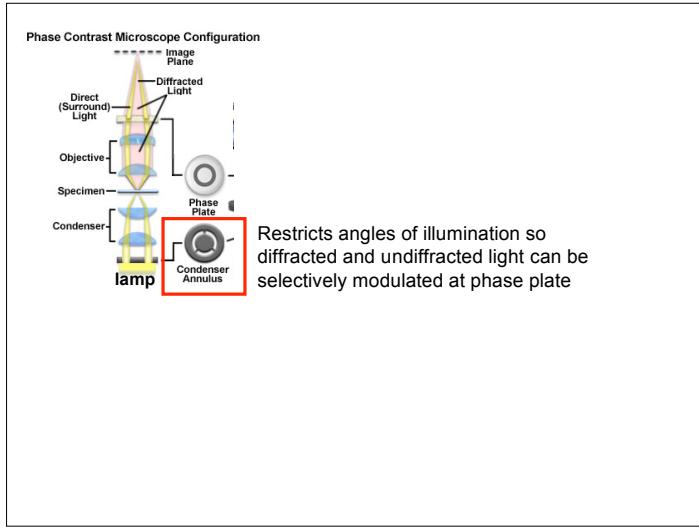
We would rather have D closer to S in amplitude and phase shift to be $\gamma/2$ (vs $\gamma/4$) for max interference and contrast



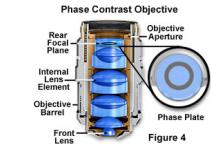
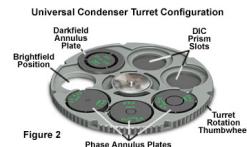
Positive Phase Contrast Systems



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



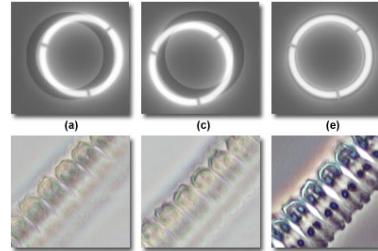
Where these elements live in the microscope



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

(separates surround and diffracted light)

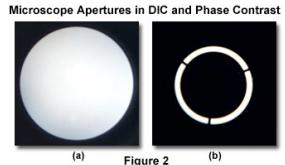
Phase Contrast Optical System Alignment



Back focal plane

Image plane

Limitations of Phase Contrast

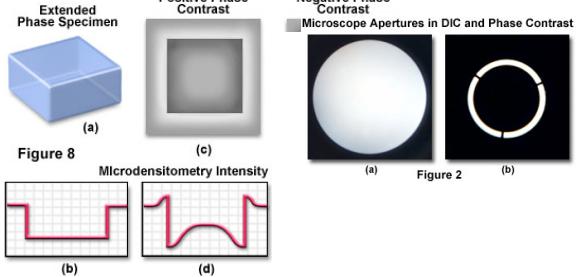


Poor for thick samples for two reasons

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)

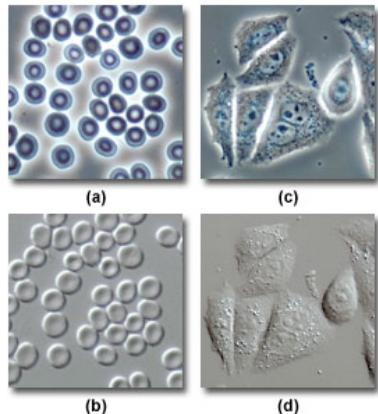
Limitations of Phase Contrast

Shade-Off in Positive and Negative Phase Contrast

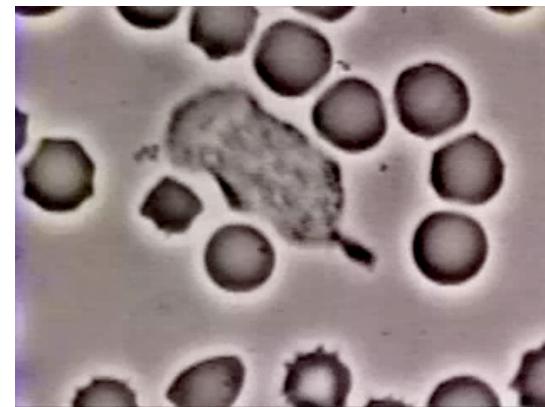


Halos -- some diffracted light (esp low spatial frequency and center of objects) also captured by phase plate, leading to localized contrast reversal. Can limit resolution.

Halos in Phase Contrast and DIC Microscopy

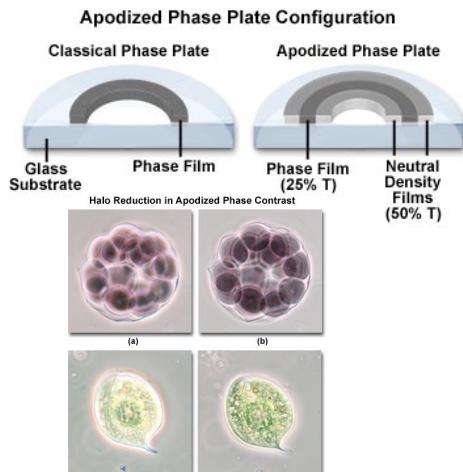


Example of Phase-- chemotaxing neutrophil

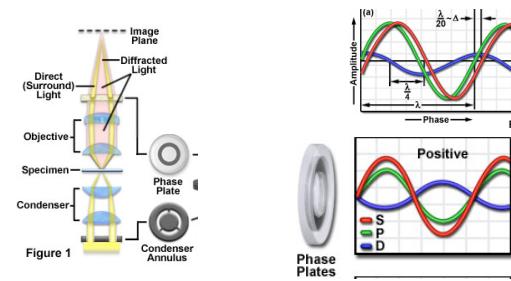


David Roger

Halos in phase contrast can be decreased by apodization



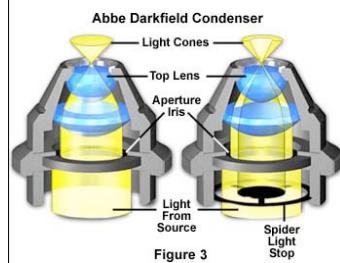
Review of Phase



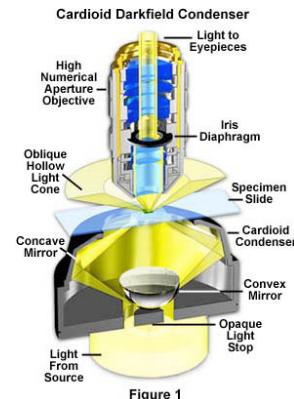
What if we were to increase contrast further by throwing away all non-diffracted light?

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



Radiolarian in Brightfield and Darkfield Illumination

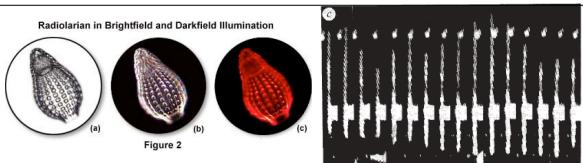
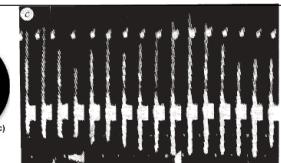


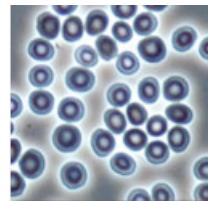
Figure 2



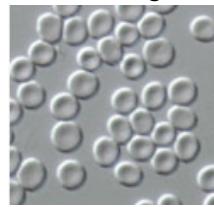
First direct visualization of microtubule dynamic instability

- 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

DIC: an alternative technique for enhancing contrast



Phase



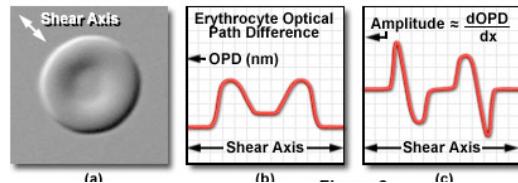
DIC (Differential Interference Contrast)

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

Specimen Optical Path Difference and DIC Amplitude Profile



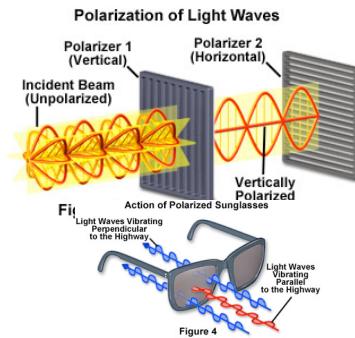
Converts relative differences in optical path length to differences in amplitude

Features of a DIC image



1. Contrast is directional
2. Contrast highlights edges
3. One end brighter, other is dimmer than background leading to pseudoshadowed, almost 3d image

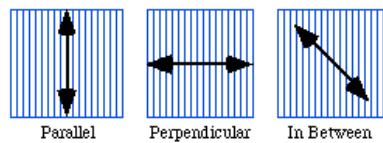
Review of light polarity, polarizers



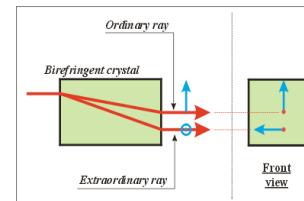
Birefringence



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



Bi-Refraction in Calcite Crystals

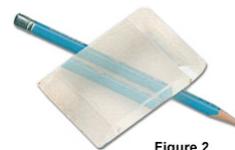
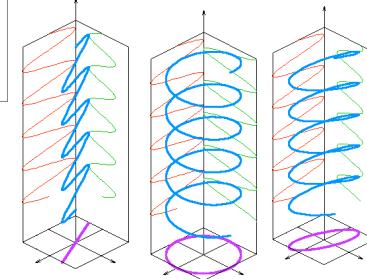
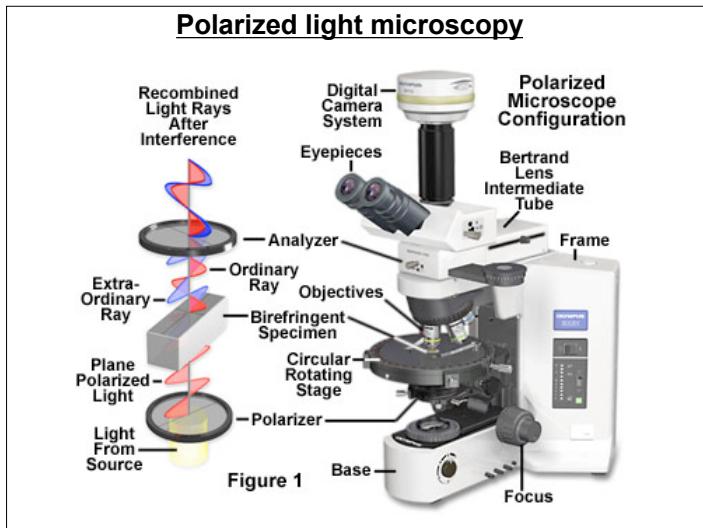


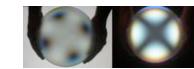
Figure 2





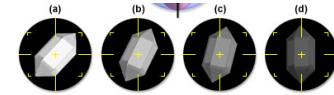
Polarized Light microscopy

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

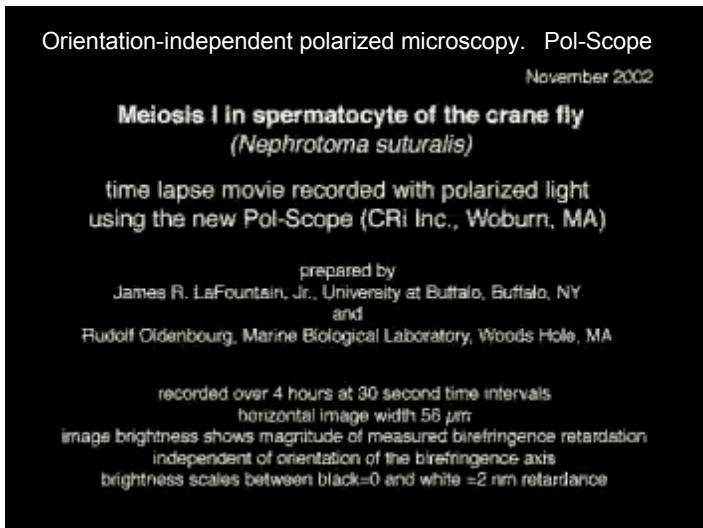


Requires strain-free optics

Depends on orientation, so rotating stage desirable

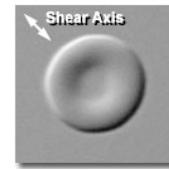


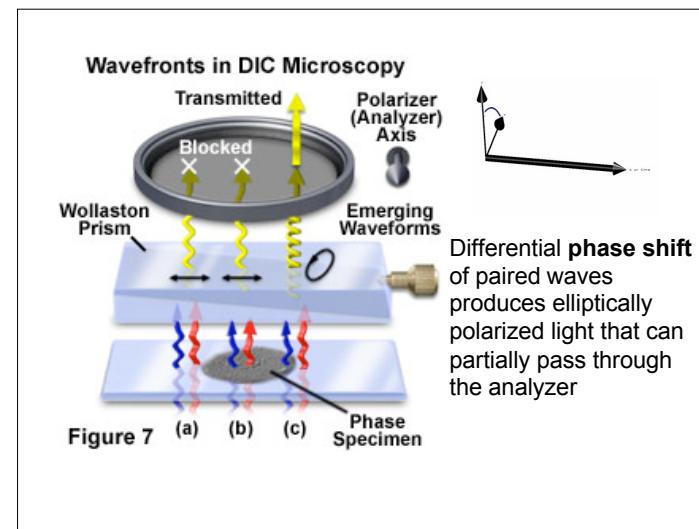
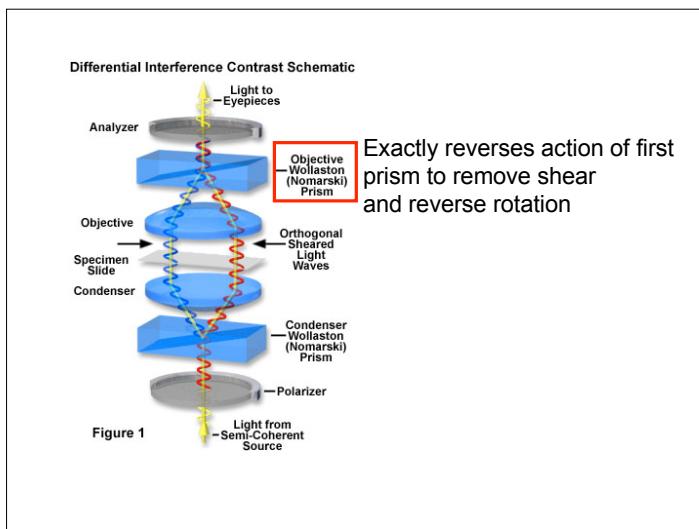
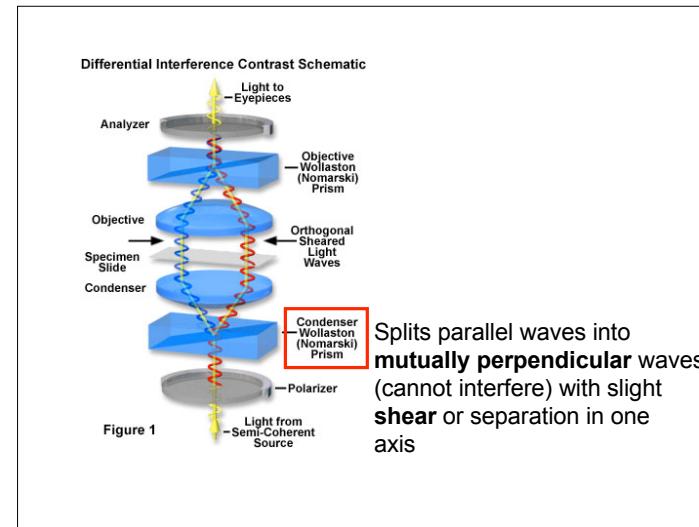
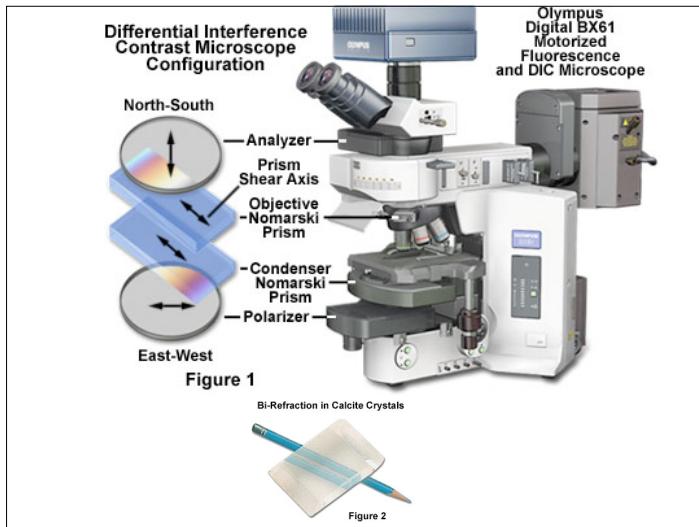
Compatible with fluorescence microscopy (good way to read out orientation of certain chromophores)



Can use modification of polarization microscope
for non-birefringent samples

-- DIC converts optical path difference into polarity changes





Role of Bias in DIC

DIC Image Plane Wavefront Interference

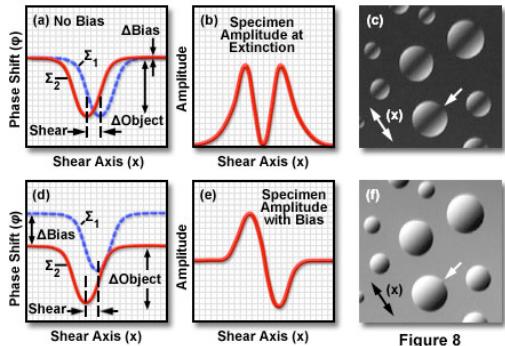


Figure 8

Ways to introduce bias in DIC

1. Translate Prisms relative to one another

Wollaston Prism Interference Fringes

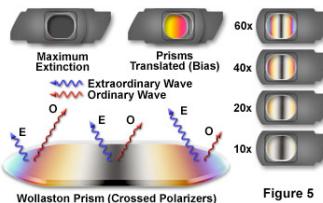


Figure 5

de Séamont Compensator Configuration

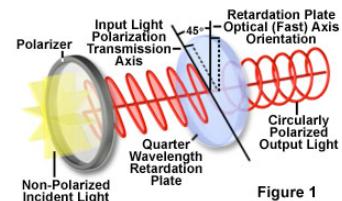


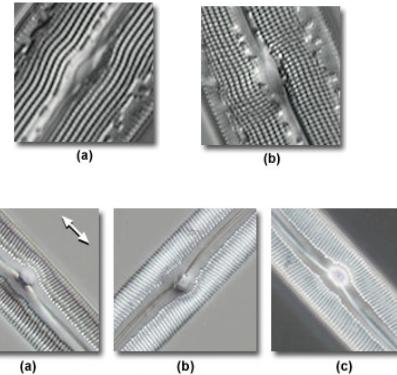
Figure 1

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

Example of DIC-- 3t3 movement



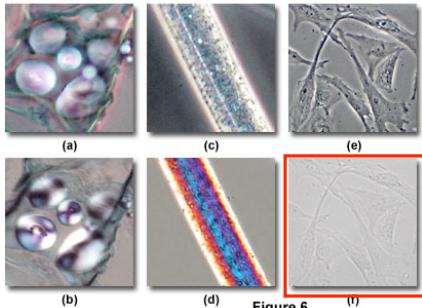
Because of directional contrast, DIC is sensitive to specimen orientation



DIC but not phase is orientation-dependent

Phase better than DIC for birefringent samples

Birefringent Specimens in Phase Contrast and DIC



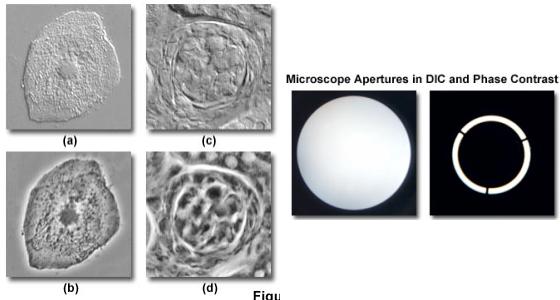
DIC not compatible with birefringent samples (can't plate cells on or cover cells with plastic).

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

Transparent Specimens in Phase Contrast and DIC



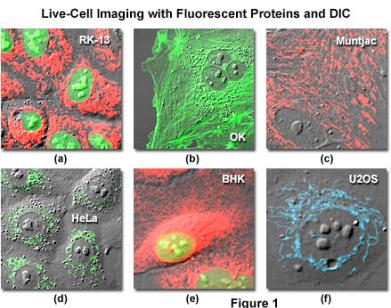
In lab you will examine effect of closing down condenser aperture on ability to do optical sectioning (zebrafish)

Example of DIC-- C. elegans development



DIC is good for thick samples

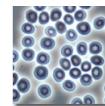
Phase Contrast and DIC often used in conjunction with fluorescence microscopy



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

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)



Thanks!

Phase microscopy

microscopyu.com

DIC microscopy

<http://micro.magnet.fsu.edu/primer/techniques/dic/dicintro.html>

Lab today

(Phase and Nomarski alignment handout)
Cheek cells, S2 cells, zebrafish