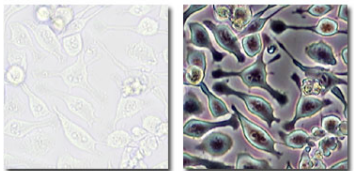
Generating contrast in light microscopy

Orion Weiner Principles & Practice of Light Microscopy 2013

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)

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

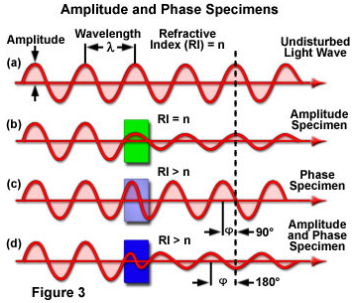


Figure 3

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

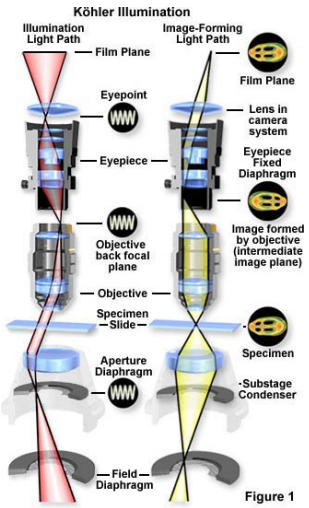
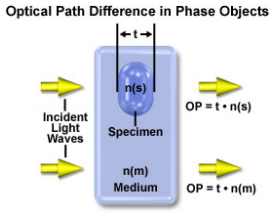


Figure 1

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

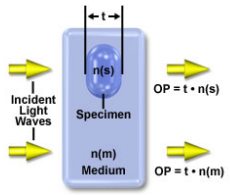
n(m) = refractive index of medium. Cell medium 1.335

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

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

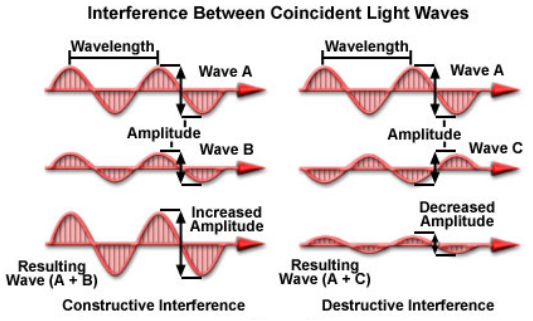
which is about 1/4 the wavelength of green light (488 nm)

What Phase Microscopy accomplishes

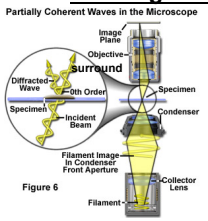


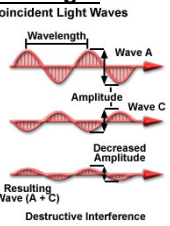
Converts differences in optical path length to differences in amplitude

Review-- interference of light waves with same wavelength

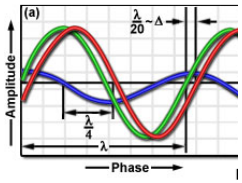


Forming an image-- role of diffracted light





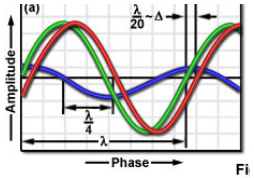
Brightfield Microscopy Wave Phase Relationships



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

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

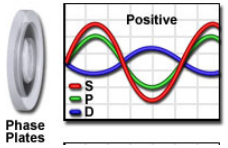
Brightfield Microscopy Wave Phase Relationships



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

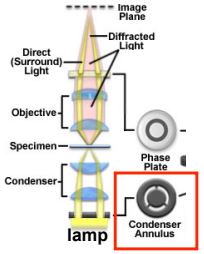
We would rather have D closer to S in amplitude
and phase shift to be γ/2 (vs γ/4) for max interference and contrast

Positive **Phase Contrast Systems**



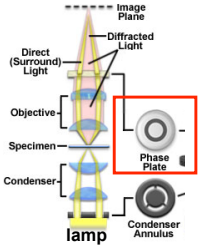
Need way of independently controlling amplitude and phase of S + D.

Phase Contrast Microscope Configuration



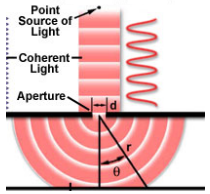
Restricts angles of illumination so diffracted and undiffracted light can be selectively modulated at phase plate

Phase Contrast Microscope Configuration



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

Diffraction of Light Through an Aperture



Phase Contrast Microscope Configuration

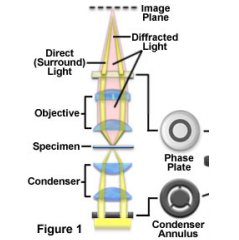


Figure 1

Phase Contrast Microscope Optical Train

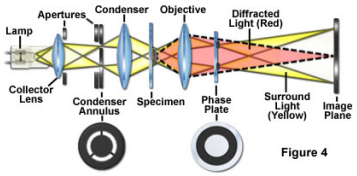


Figure 4

Phase Contrast Microscope Configuration

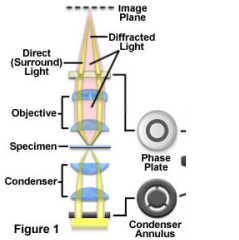


Figure 1

Phase Contrast Microscope Optical Train

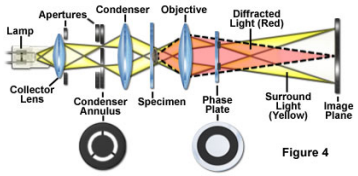
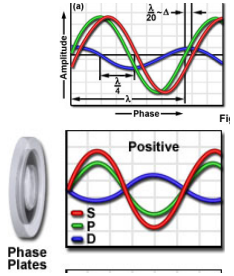
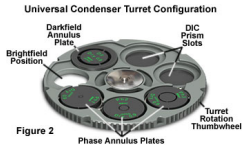
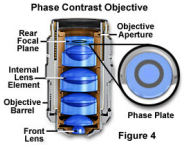


Figure 4



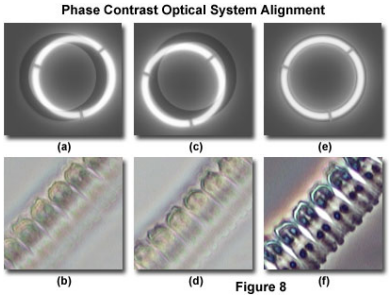
Where these elements live in the microscope





<u>Proper alignment of condenser annulus and phase plate are essential for phase microscopy</u>

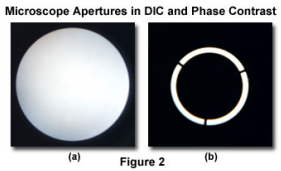
(separates surround and diffracted light)



Back focal plane

Image plane

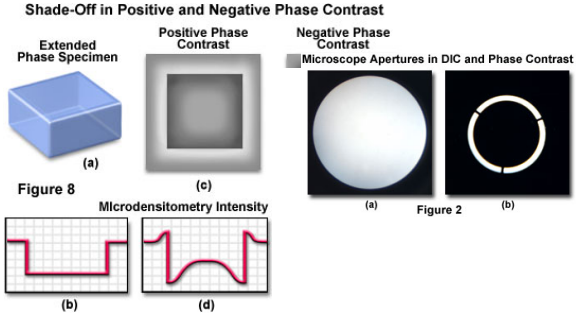
<u>Limitations of Phase Contrast</u>



Poor for thick samples for two reasons

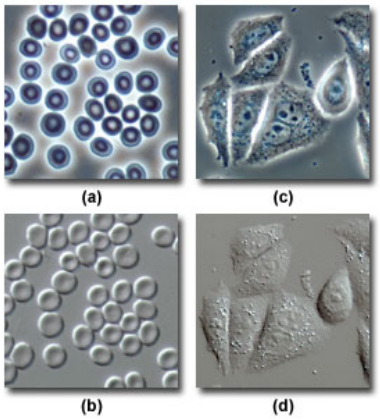
1. Poor lateral (z) resolution due to limited aperture
2. Sufficiently thick samples can shift light more than 1 wavelength (so thin and thick sections can have similar brightness for biological samples thicker than about 10 microns)

<u>Limitations of Phase Contrast</u>

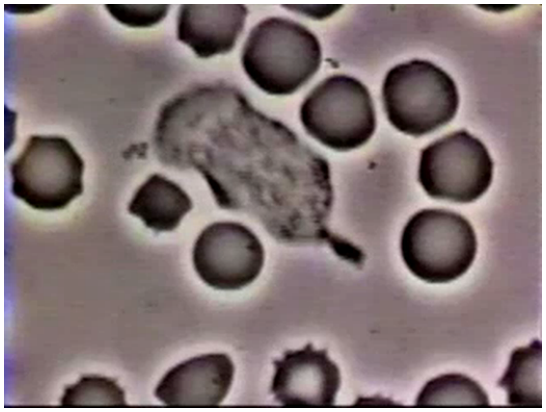


Halos -- some diffracted light (esp low spatial frequency and center of objects) also captured by phase plate, leading to localized contrast reversal. Can limit resolution.

Halos in Phase Contrast and DIC Microscopy

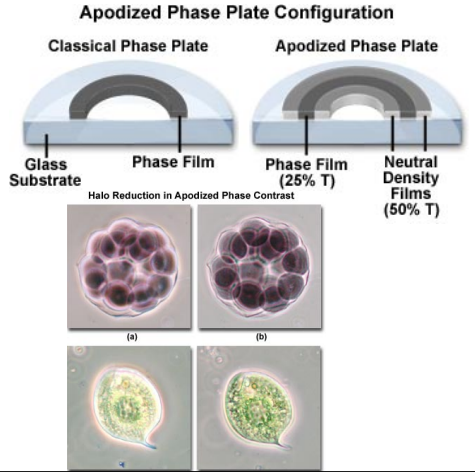


Example of Phase-- chemotaxing neutrophil

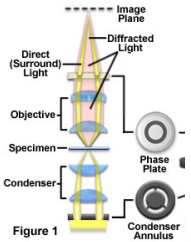


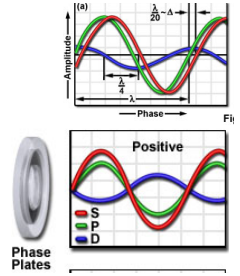
David Roger

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

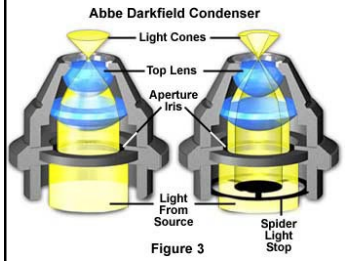


Figure 3

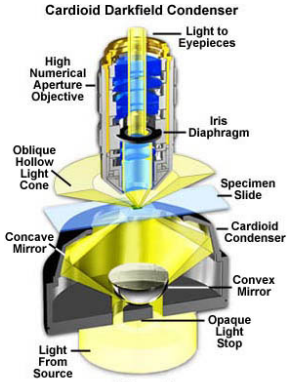


Figure 1

Similar to phase, projects cone of light onto specimen, but with higher NA than objective, so no surround light enters objective

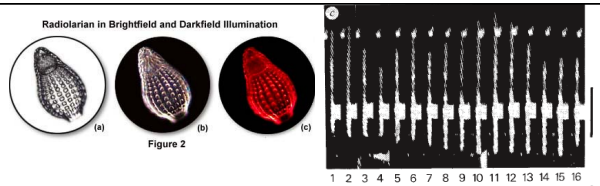


Figure 2

First direct visualization of microtubule dynamic instability

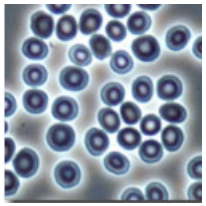
-Darkfield good for imaging unstained microorganisms,

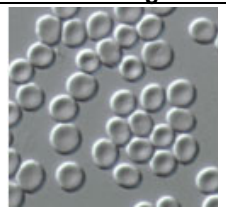
-even sub-resolution objects such as flagella (20nm diameter) visible with darkfield.

-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

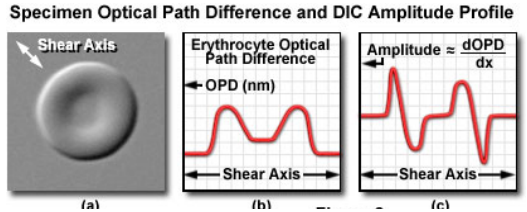

Phase


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

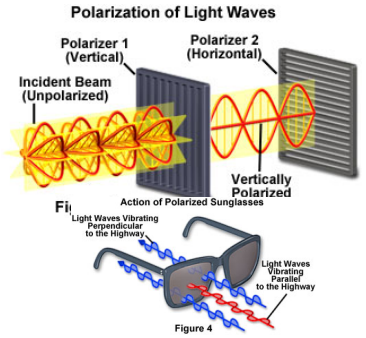


Converts relative differences in optical path length to differences in amplitude

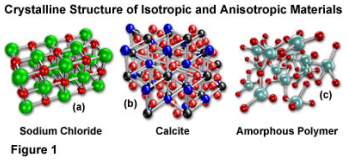
Features of a DIC image

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

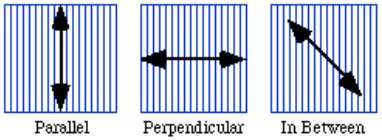
Review of light polarity, polarizers



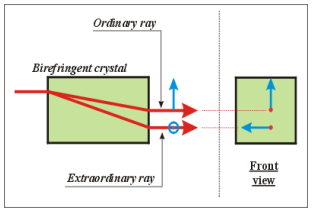
Birefringence



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

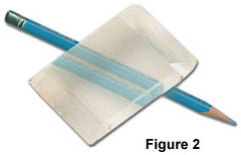
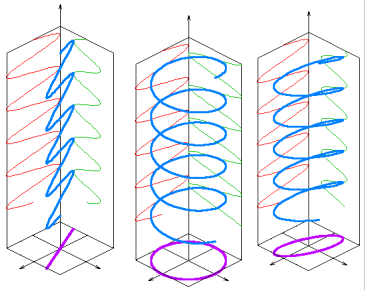


Figure 2



Polarized light microscopy

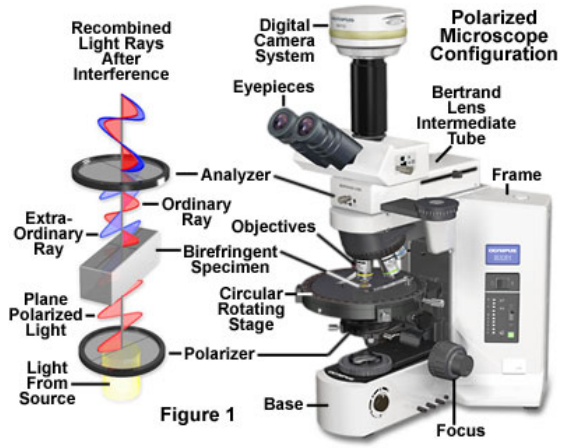
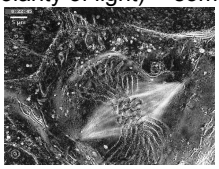
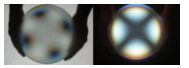


Figure 1

Polarized Light microscopy

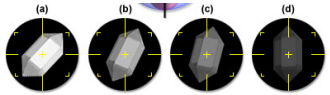
Only works with birefringent samples (those that alter polarity of light) -- some polymers such as microtubules





Requires strain-free optics

Depends on orientation, so rotating stage desirable



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

Orientation-independent polarized microscopy. Pol-Scope

November 2002

Meiosis I in spermatocyte of the crane fly
(Nephrotoma suturalis)

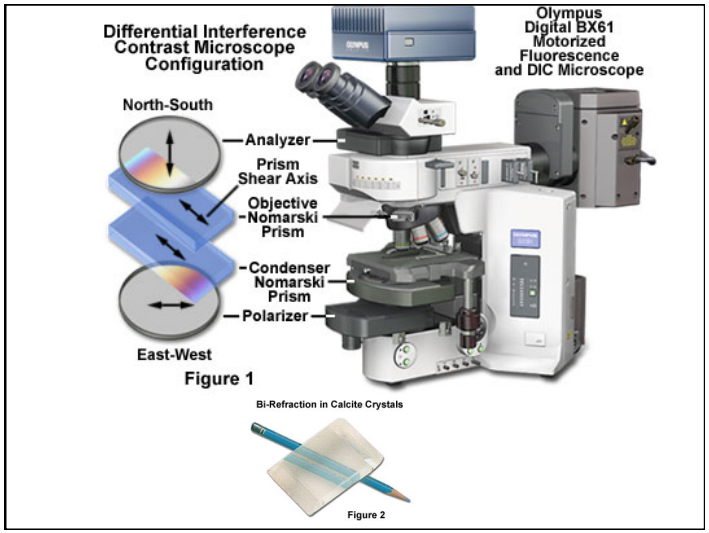
time lapse movie recorded with polarized light
using the new Pol-Scope (CRi Inc., Woburn, MA)

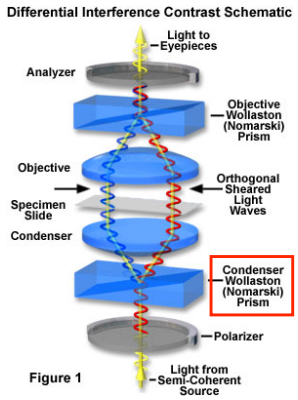
prepared by
James R. LaFountain, Jr., University at Buffalo, Buffalo, NY
and
Rudolf Oldenbourg, Marine Biological Laboratory, Woods Hole, MA

recorded over 4 hours at 30 second time intervals
horizontal image width 56 μm
image brightness shows magnitude of measured birefringence retardation
independent of orientation of the birefringence axis
brightness scales between black=0 and white =2 nm retardance

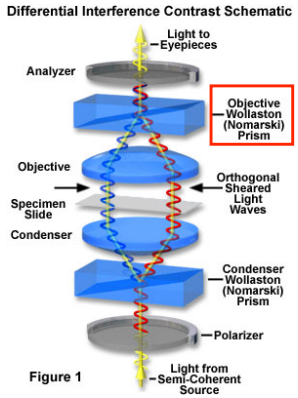
Can use modification of polarization microscope for non-birefringent samples

-- **DIC converts optical path difference into polarization changes (and then this into amplitude)**

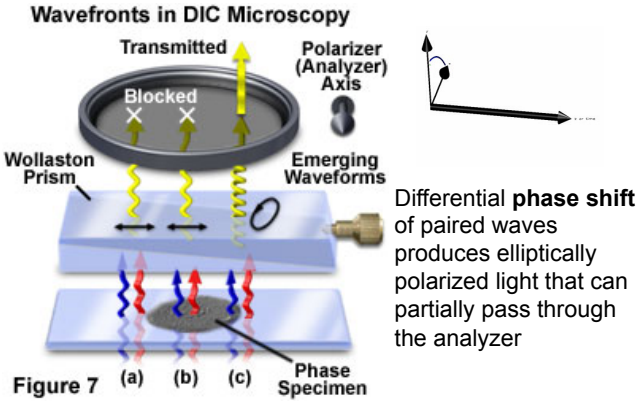




Splits parallel waves into **mutually perpendicular** waves (cannot interfere) with slight **shear** or separation in one axis



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

Role of Bias in DIC

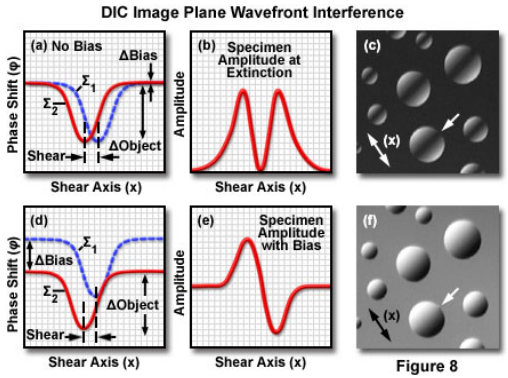


Figure 8

Ways to introduce bias in DIC

1. Translate Prisms relative to one another

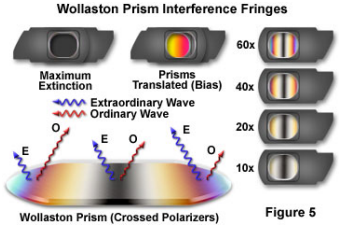


Figure 5

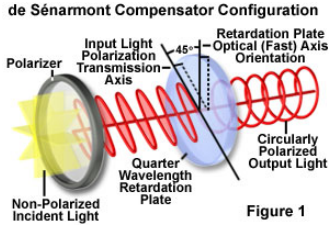
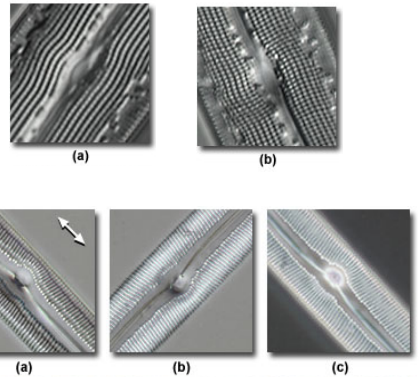


Figure 1

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

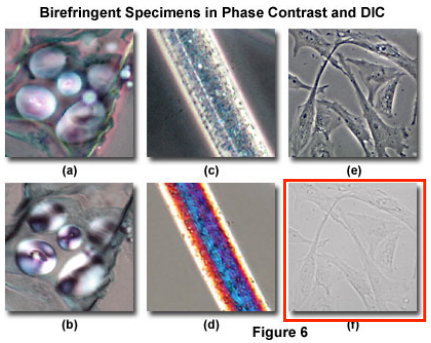
Example of DIC-- 3t3 movement

Because of directional contrast, DIC is sensitive to specimen orientation



DIC but not phase is orientation-dependent

Phase better than DIC for birefringent samples

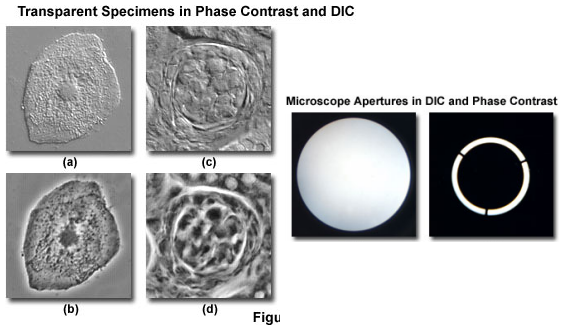


DIC not compatible with birefringent samples (can't plate cells on or or cover cells with plastic).

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

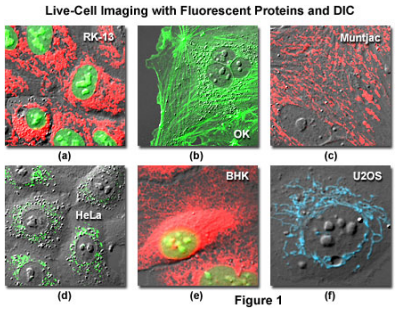


In lab you will examine effect of closing down condenser aperture on ability to do optical sectioning (zebrafish)

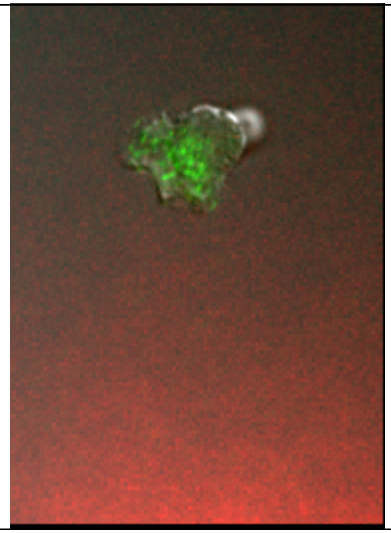
Example of DIC-- C. elegans development

DIC is good for thick samples

Phase Contrast and DIC often used in conjunction with fluorescence microscopy

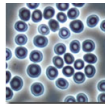


To provide cellular or organismal reference.
Phase and DIC are much more general (and less toxic) detection tools than fluorescence.

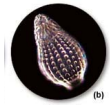


Review:

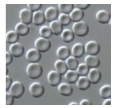
Phase-- converts optical path length into contrast



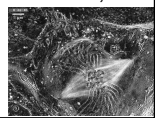
Darkfield-- images only diffracted light



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