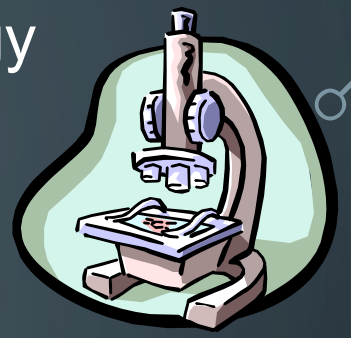


Light microscopy in Cellular Biology

Gabriele Baj
gbaj@units.it

Resolving power

- The ability to distinguish two adjacent points as distinct & separate is known as resolving power.
- Mere increase in size without the ability to distinguish structural details is not beneficial.
- In other words, the largest magnification produce by a microscope may not be the most useful because the image obtained may be unclear or fuzzy.
- The more lines or dots per unit area that can be seen separately, the greater is the resolving power.
- It is a function of the wavelengths of lights used & the numerical aperture of the lens system.

Numerical Aperture

- In optics, the numerical aperture (NA) of an optical system is a dimensionless number that characterizes the range of angles over which the system can accept or emit light.
- The sine value of half-aperture angle multiplied by the refractive index *n* of the medium gives the numerical aperture (NA)

- Thus,

$$NA = n \sin \theta$$

Magnification

- Magnification beyond the resolving power is of no value since the larger image will be less distinct in detail & fuzzy in appearance.
- The situation is analogous to the of a movie screen: If we move closer to the screen the image is larger but is also less sharp than when viewed from distance.
- Most laboratory microscopes are equipped with three objectives, each capable of a different degree of magnification.
- The total magnification of the system is determined by magnification of the objective by that of eyepiece.

The limit of Resolution

- The limit of Resolution is the smallest distance by which two objects can be separated & still be distinguished as two separate objects.
- The greatest resolution in optical microscopy can be obtained with the shortest wavelength of visible light & an objective with maximum NA.
- The relationship between NA & limit of resolution can be expressed as follows:

$$d = \lambda / 2NA$$

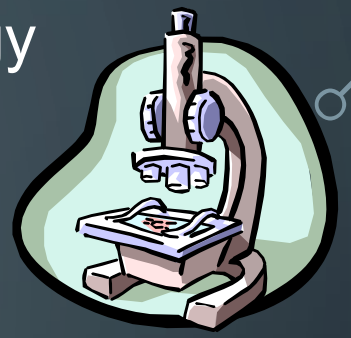
Here, d= Resolution

λ= Wavelength of light

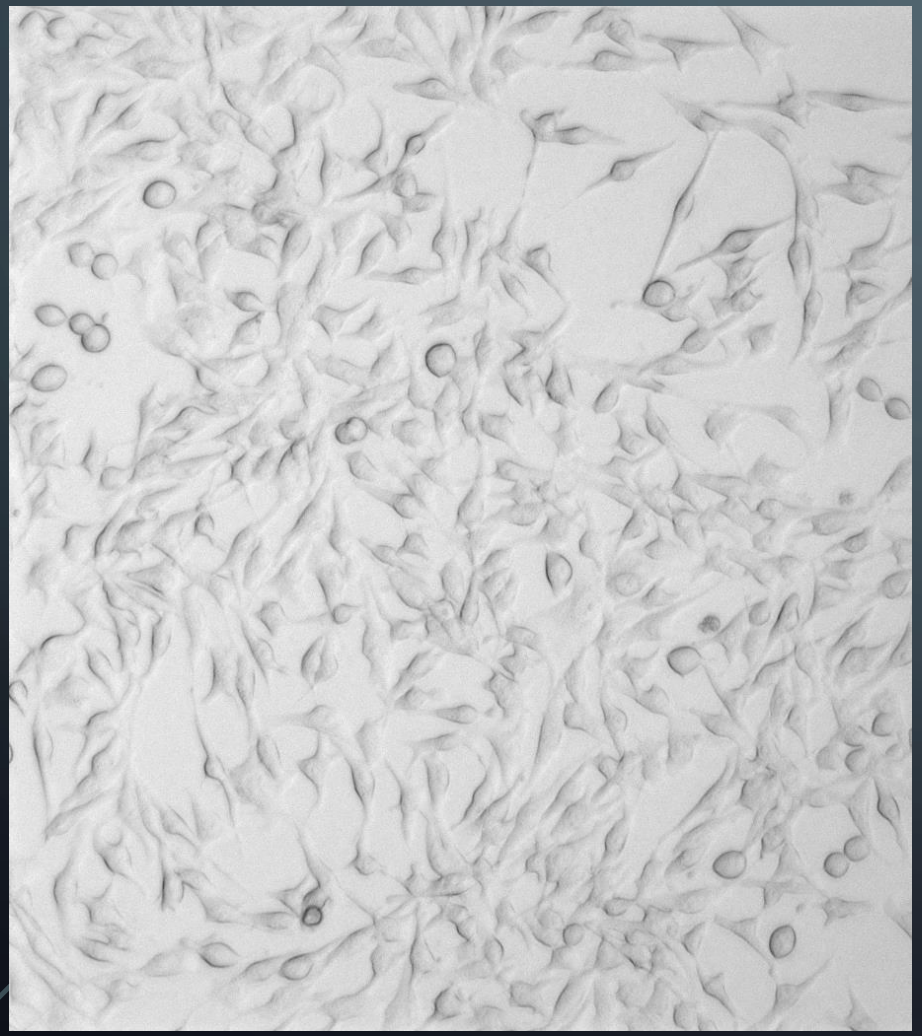
Contrast Generation for Transmitted Light

- Brightfield >>> absorbance
- Darkfield >>> diffraction
- Phase Contrast >>> Phase shift
- Differential Interference Contrast (DIC) Microscopy

>>> Phase shift / Polarization / Interference



HOW CAN WE USE THE PROPERTIES OF LIGHT TO CREATE CONTRAST?



Which properties can be used?

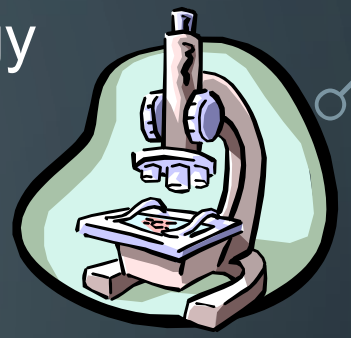
Absorption

Scattering

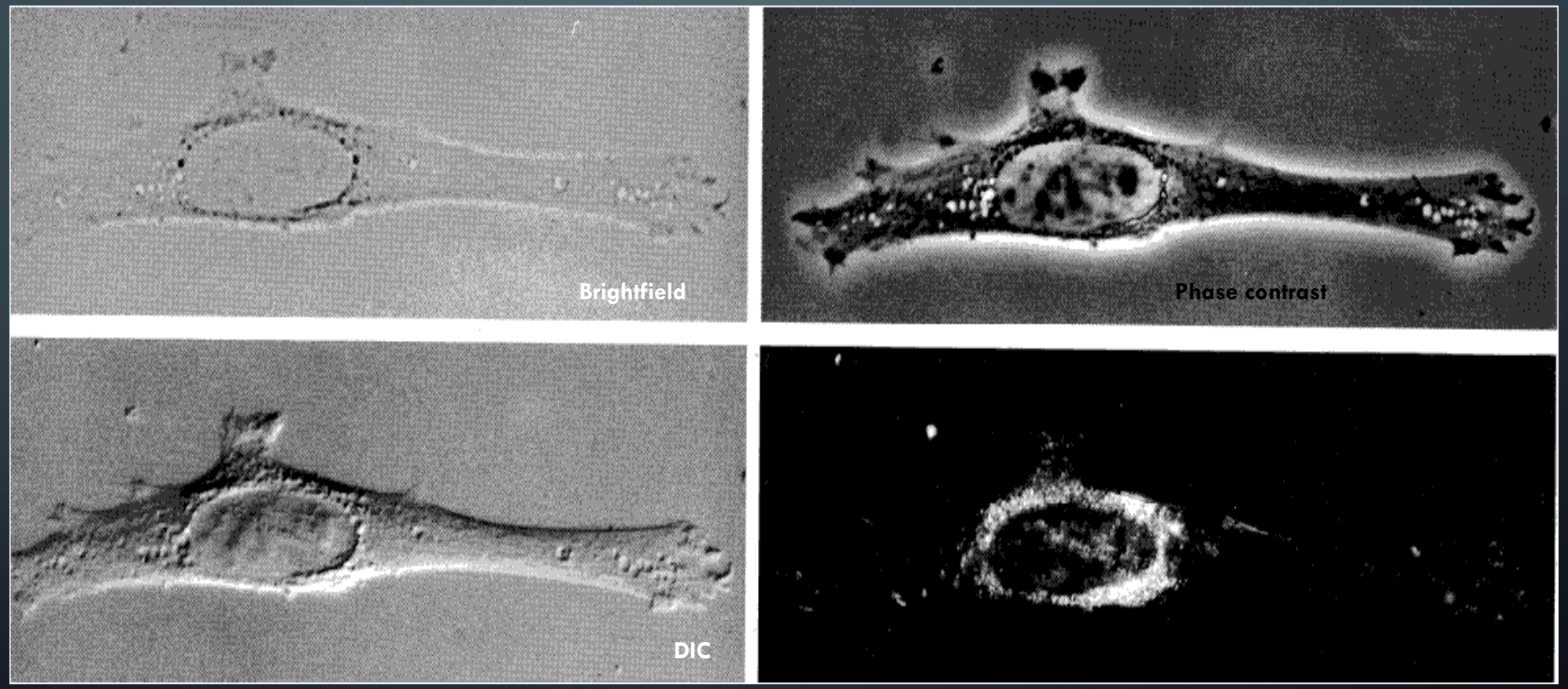
Refraction

Phase

Polarization



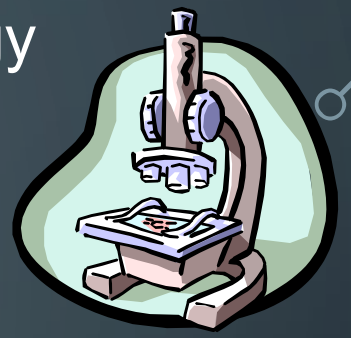
CONTRASTING TECHNIQUES



Taken from: http://fig.cox.miami.edu/~cmallery/150/Fallsyll.htm

CONTRASTING TECHNIQUES

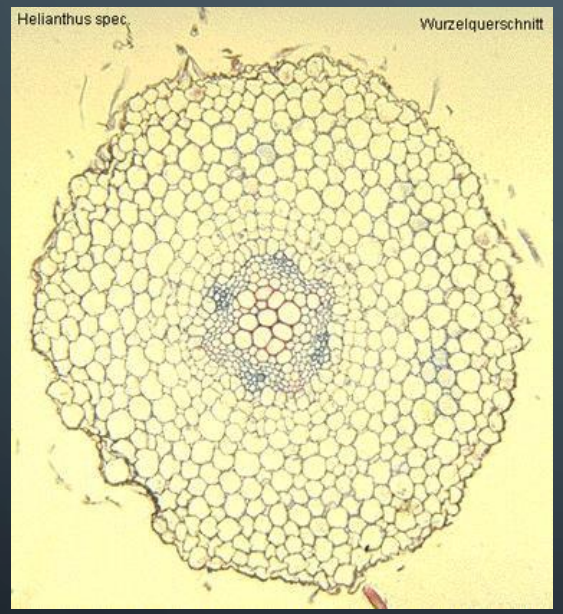
- Brightfield
- Darkfield
- Phase Contrast
- Polarization Contrast
- Differential Interference Contrast (DIC)



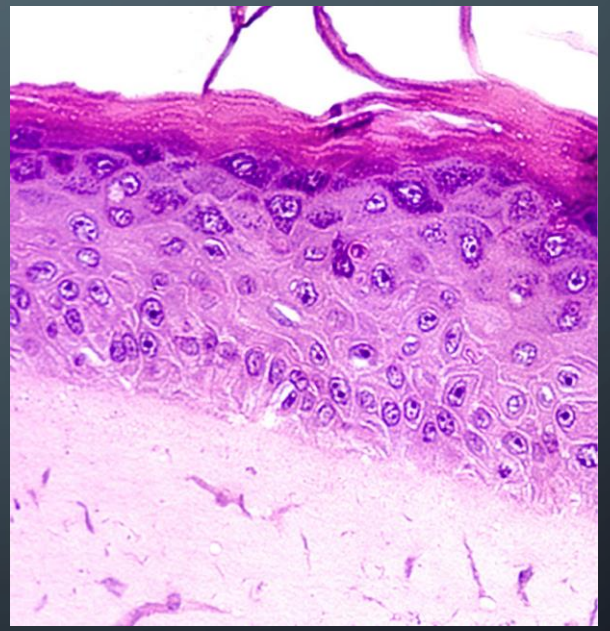
BRIGHTFIELD

Principle: Light is transmitted through the sample and absorbed by it.

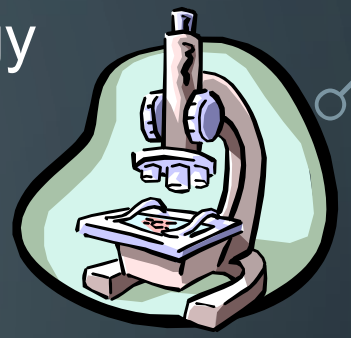
Application: Only useful for specimens that can be contrasted via dyes. Very little contrast in unstained specimens. With a bright background, the human eye requires local intensity fluctuations of at least 10 to 20% to be able to recognize objects.



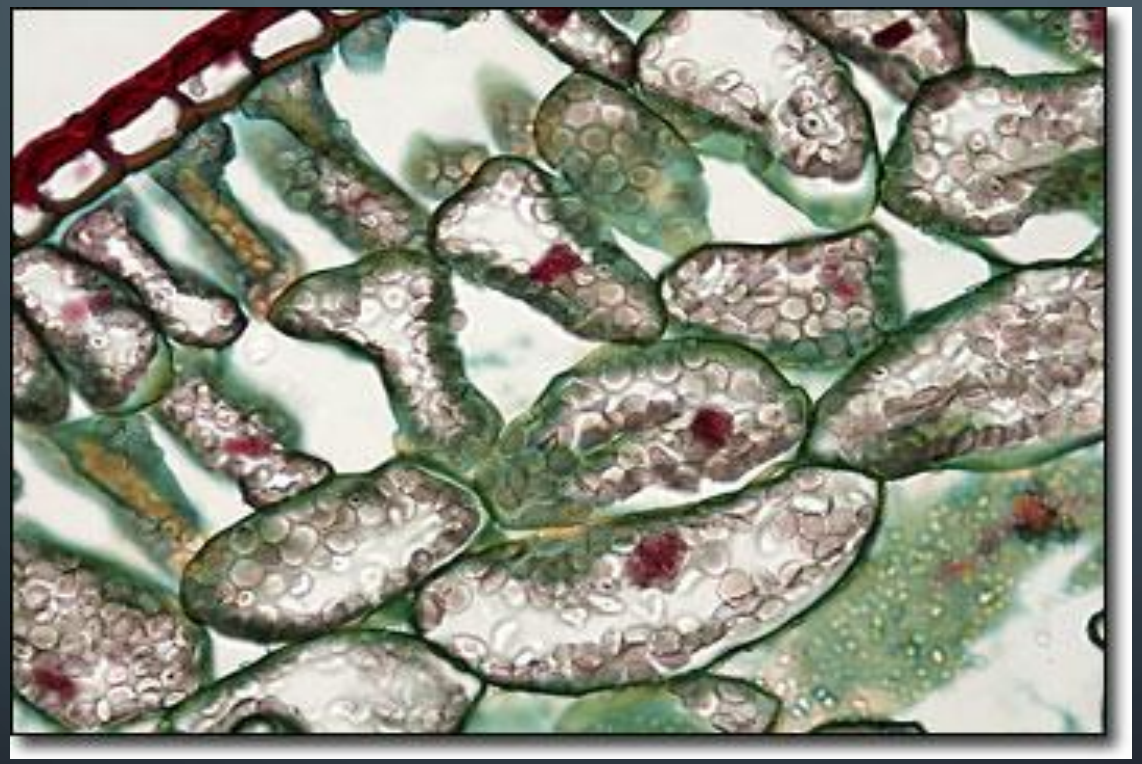
Cross section of sunflower root
(http://www.zum.de/Faecher/Materialien/beck/12/bs12-5.htm)



Piece of artificially grown skin
(www.igb.fhg.de/.../dt/PI_BioTechnica2001.dt.html)



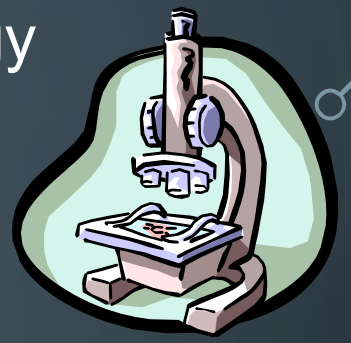
Bright Field



- Bright Field is the most universal technique used in light microscope.
- Usually used in samples with colorimetric staining or good contrast.

Bright-Field Microscopy

- Dark sample on a bright background.
- The Microscopic field is brightly lighted & the samples appear dark because they absorb some of the light.
- Usually, cells do not absorb much light, but staining them with a dye greatly increases their light absorbing capacity.
- Bright field illumination is useful for samples which have an intrinsic colour, for example chloroplasts in plant cells.
- Some of the light is absorbed by stains, pigmentation, or dense areas of the sample and this contrast allows us to see the specimen.



Dark Field

- Fine structures can often not be seen in front of a bright background.

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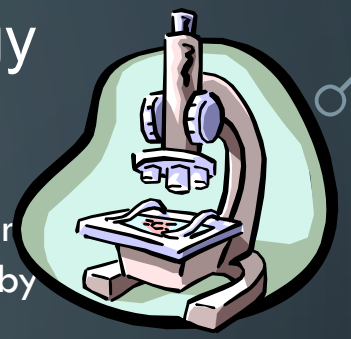
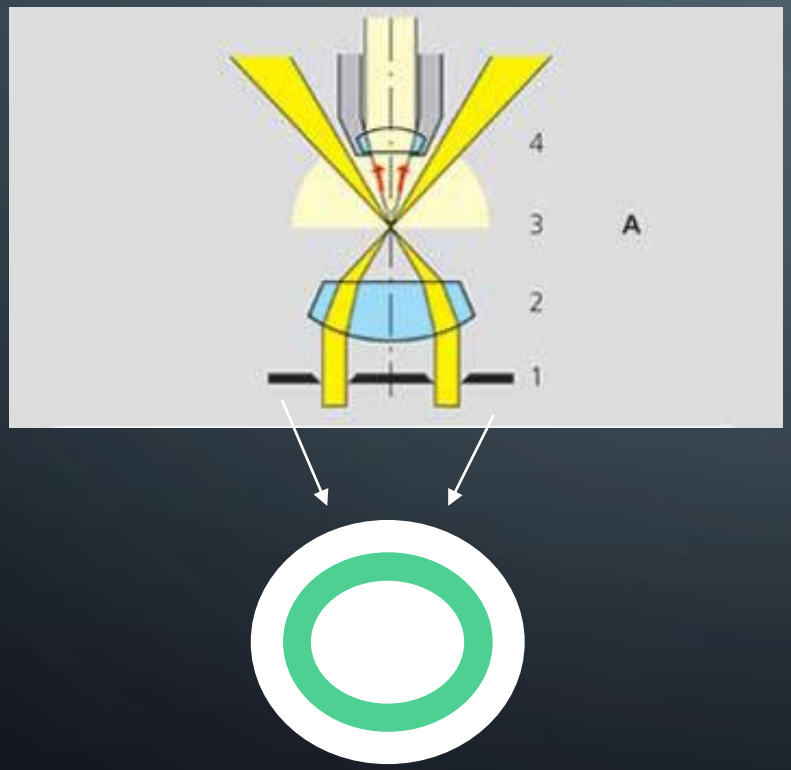
DARKFIELD



Principle: The illuminating rays of light are directed through the sample from the side by putting a dar into the condenser that hinders the main light beam to enter the objective. Only light that is scattered by structures in the sample enters the objective.

Application: People use it a lot to look at Diatoms and other unstained/colourless specimens



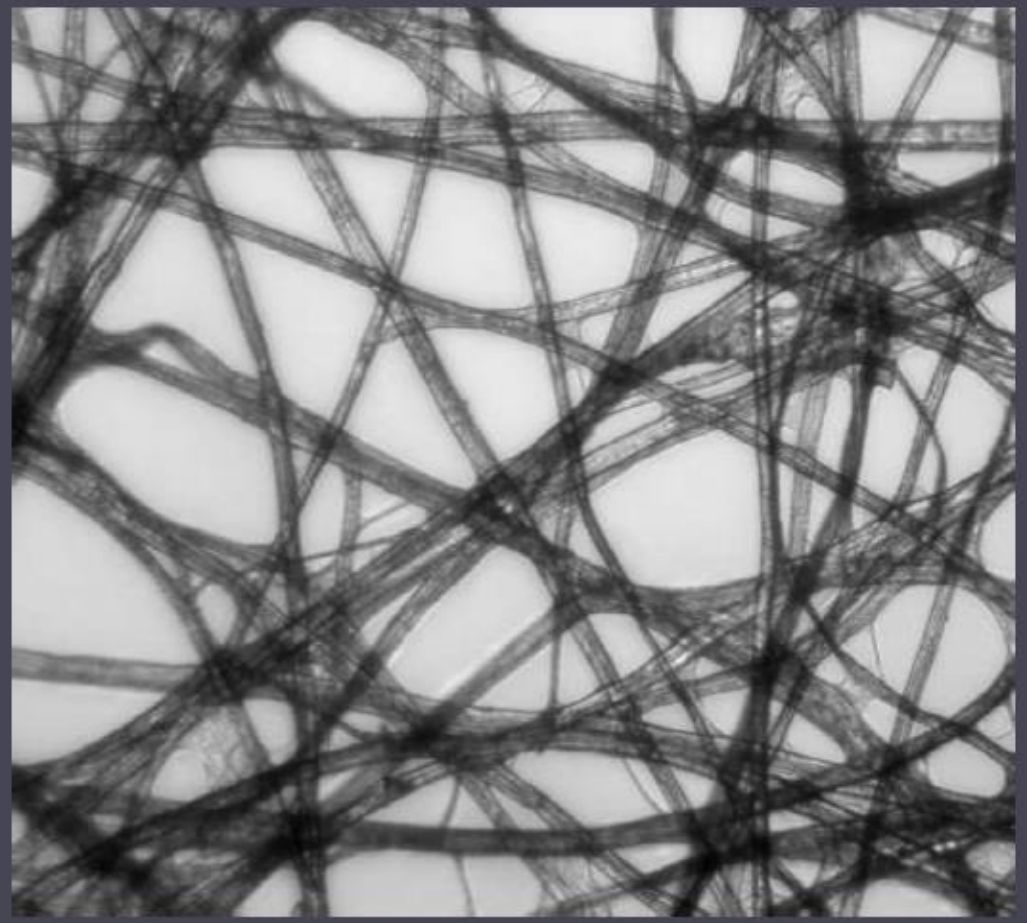
Symbiotic Diatom colony

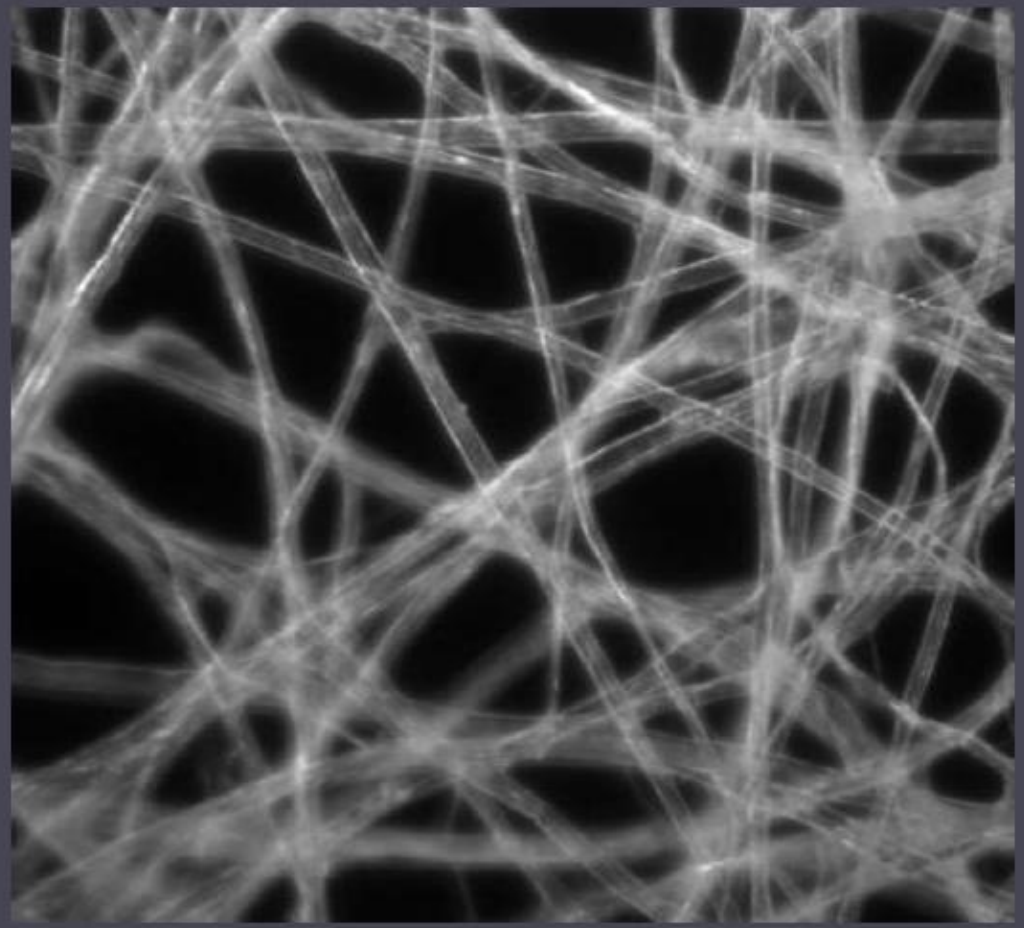
(www1.tip.nl/~t936927/making.html)

→ *we do not have microscopes set up for darkfield*

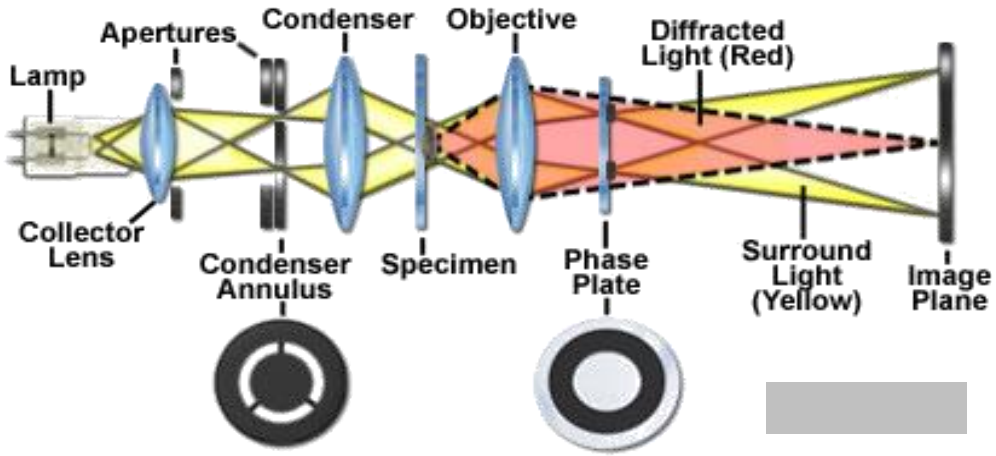
Dark-Field Microscopy

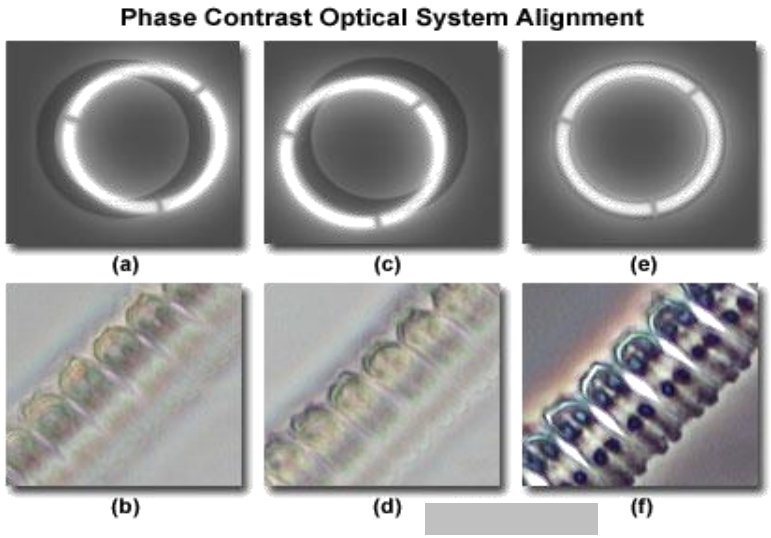
- Dark background against which objects are brilliantly illuminated.
- This is accomplished by equipping with a special condenser that transmits a hollow cone of light.
- Most of the light directed through the condenser does not enter the objective, the field is dark.
- However, some of the light rays will be scattered if the medium contains objects.
- The diffracted light will enter the objective & reach the eye, thus the object will appear bright in an dark background.
- Best for observing pale objects, unstained cells

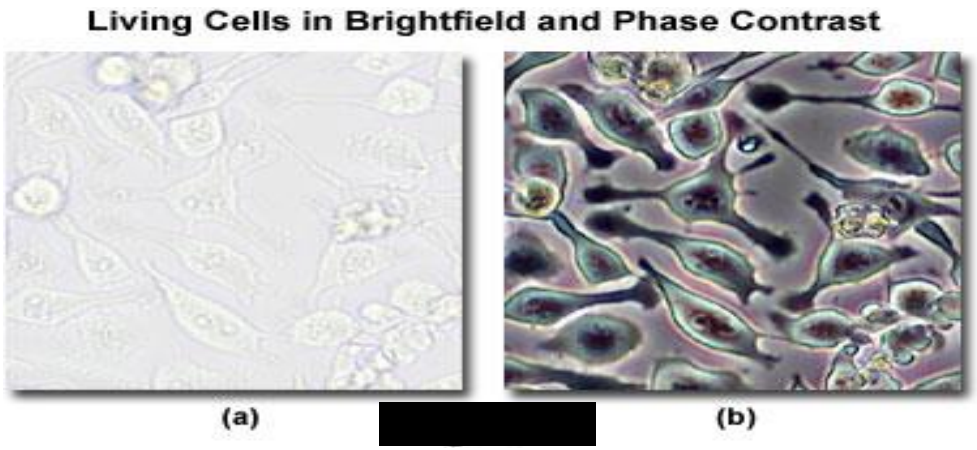




Phase Contrast

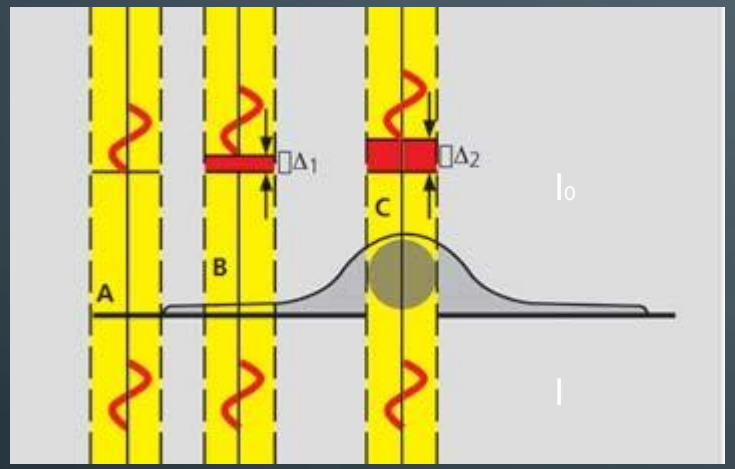


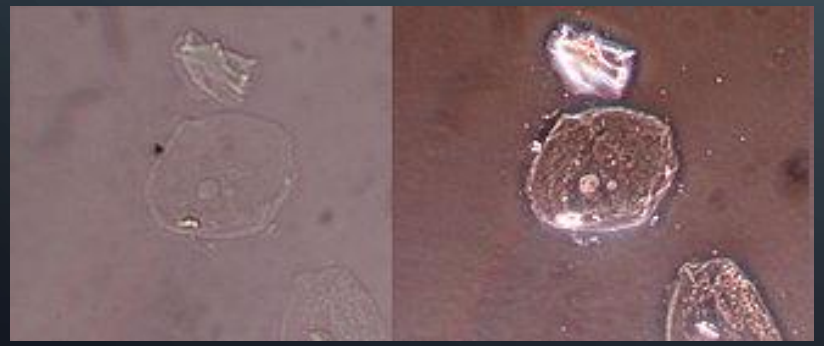


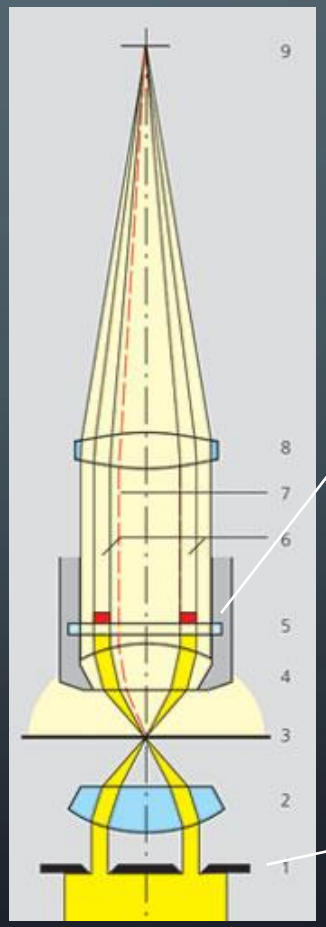


PHASE CONTRAST IN THEORY

Principle: Incident light [Io] is out of phase with transmitted light [I] as it was slowed down while passing through different parts of the sample and when the phases of the light are synchronized by an interference lens, a new image with greater contrast is seen.







Phase ring

not aligned aligned

Phase stops

PHASE CONTRAST IN THEORY

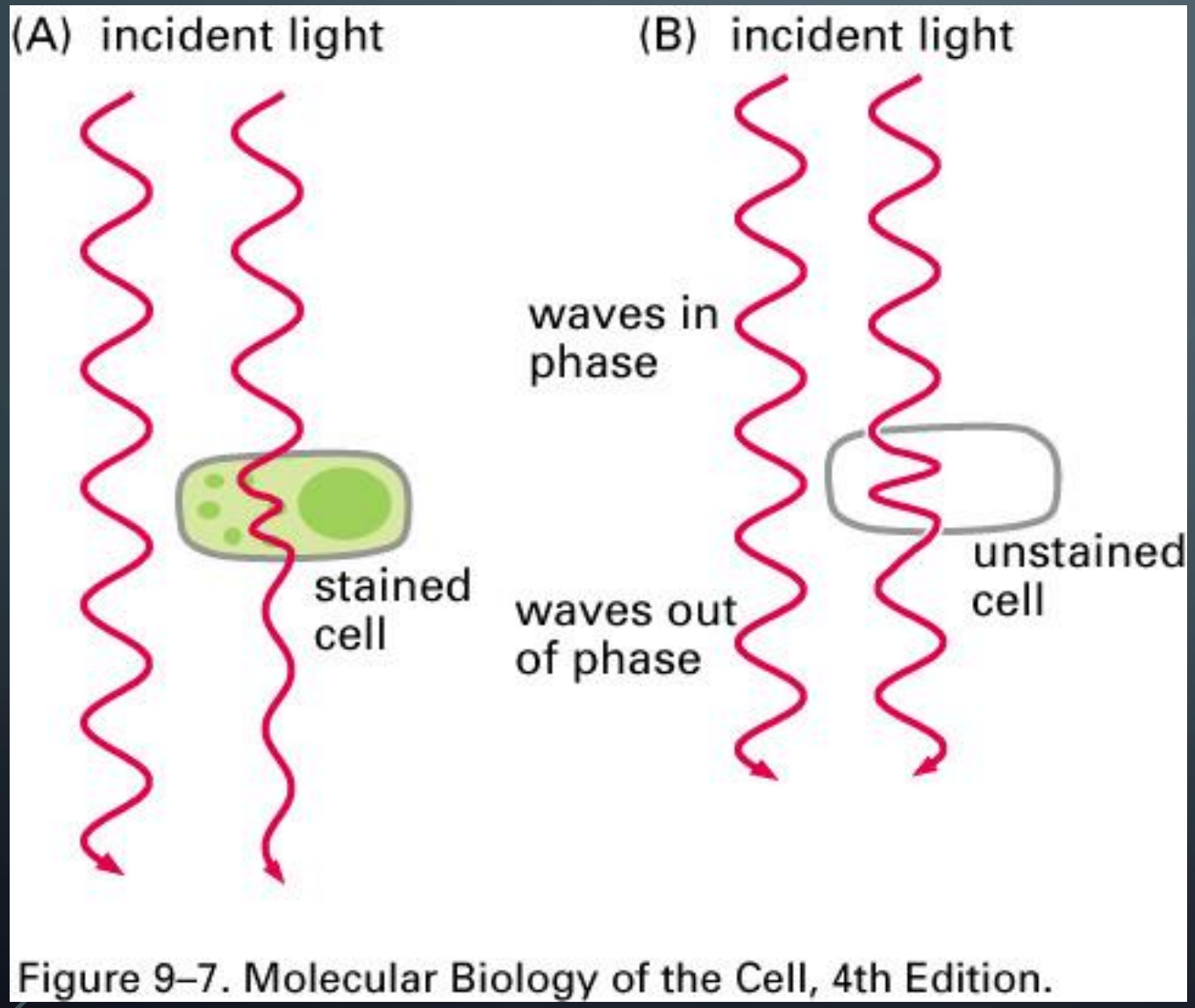


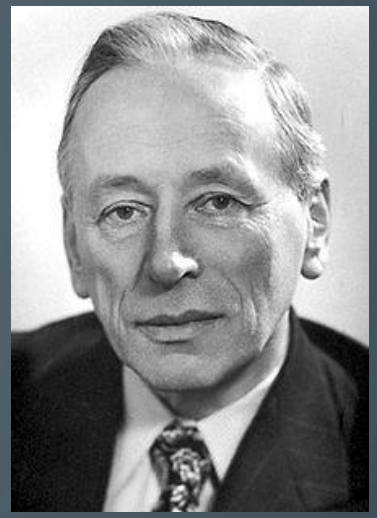
Figure 9–7. Molecular Biology of the Cell, 4th Edition.

- Do not absorb light
- Difference in index of refraction between specimen and background

PHASE DIFFERENCES

- Our eyes cannot see this
- Eyes set for amplitude differences, so cell is essentially transparent
- **But —** information **is** present in light beams from specimen and in image
- How do we see this?

FRITS ZERNIKE (1888–1966)

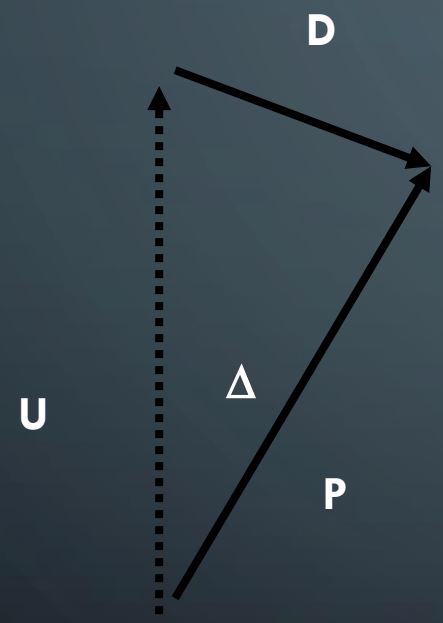


- Dutch physicist
- Developed vector notation for theory of light propagation through phase objects
- Invented phase contrast optics in 1930; not manufactured until 1941 by Zeiss

PHASE CONTRAST IMAGING

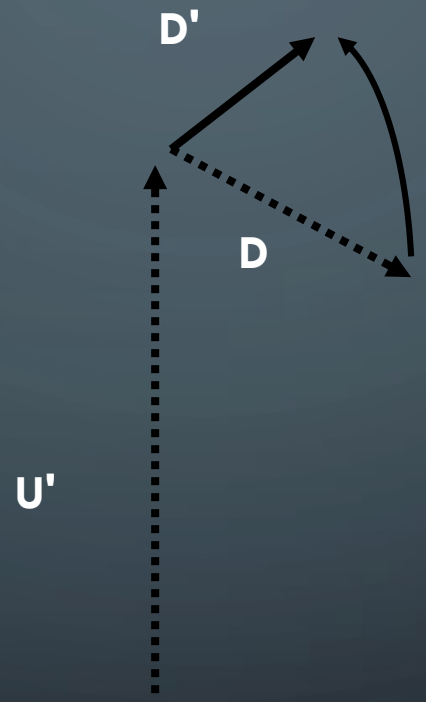
- Basic principle:
  - Shift phases (Δs) and/or amplitudes of U and D differentially
  - This can produce a change in amplitude of P (length of vector)

In specimen

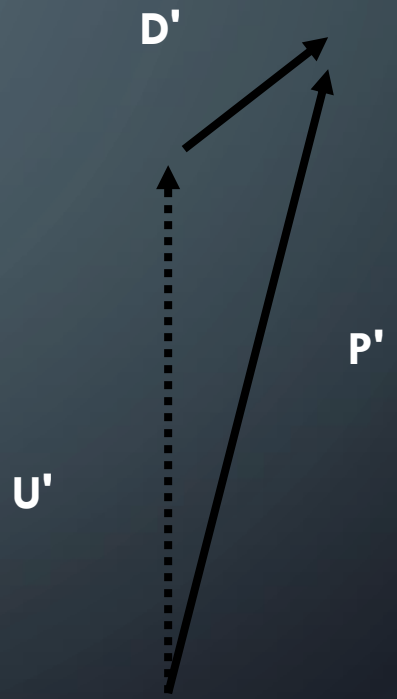


$U = P$

In microscope



At image plane

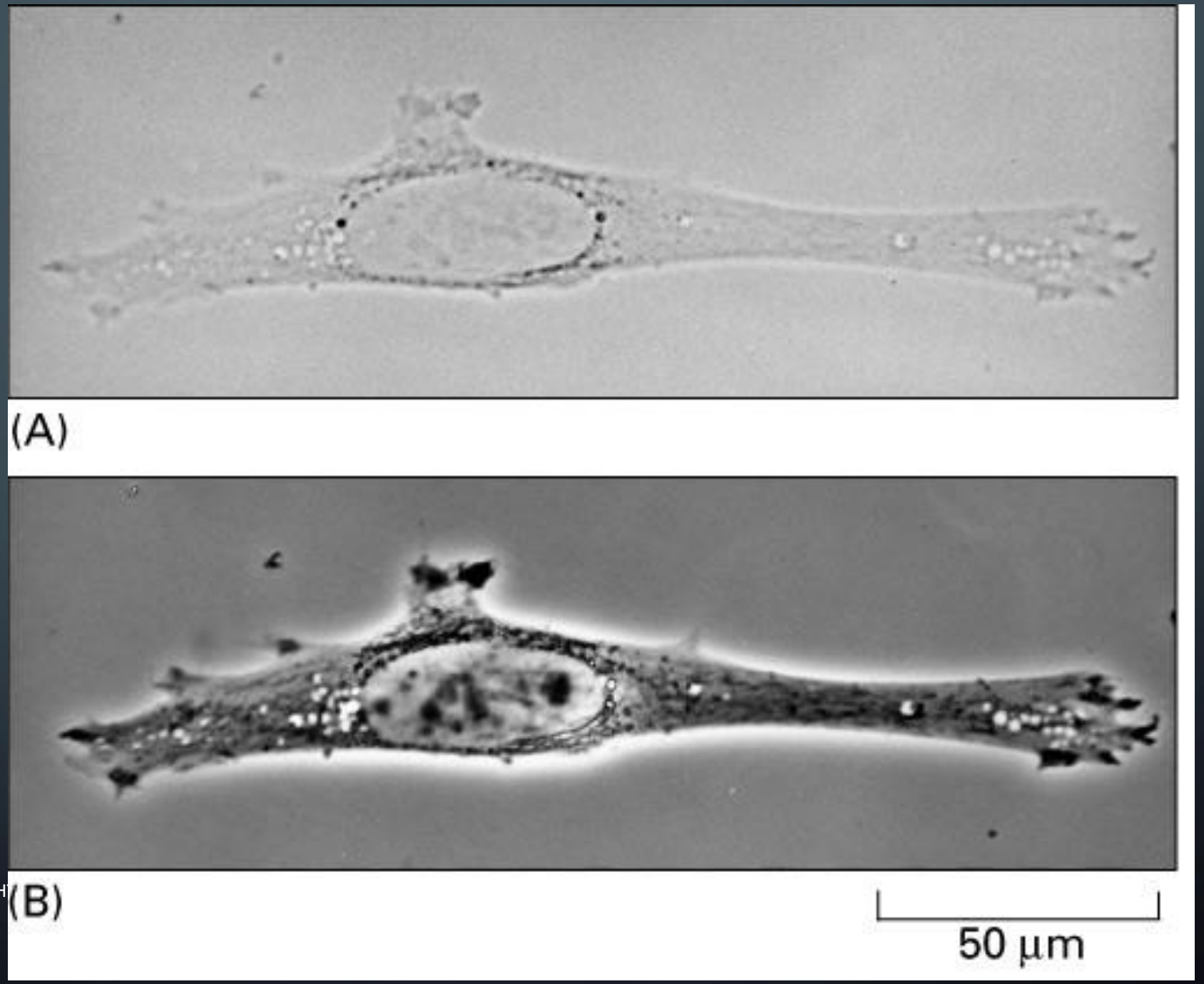


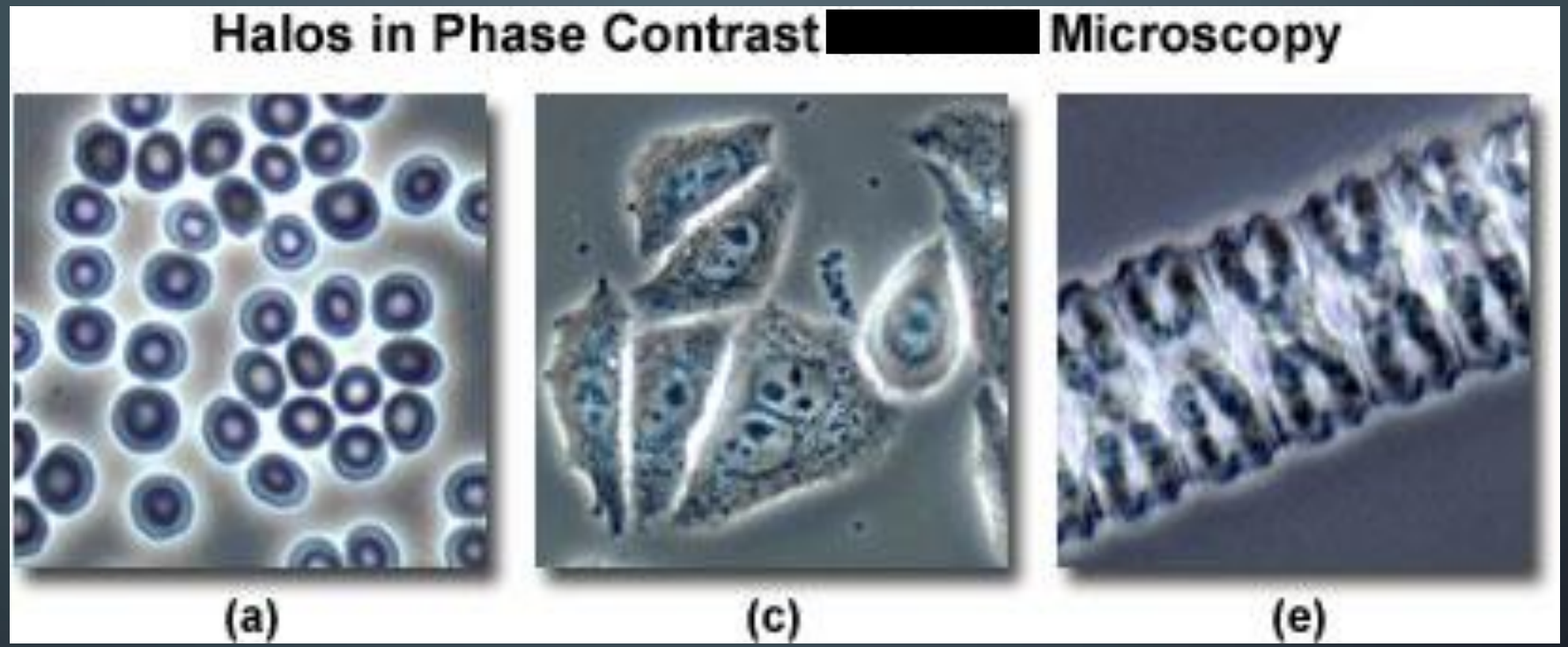
$U' \neq P'$

Amplitude!

PHASE CONTRAST OPTICS

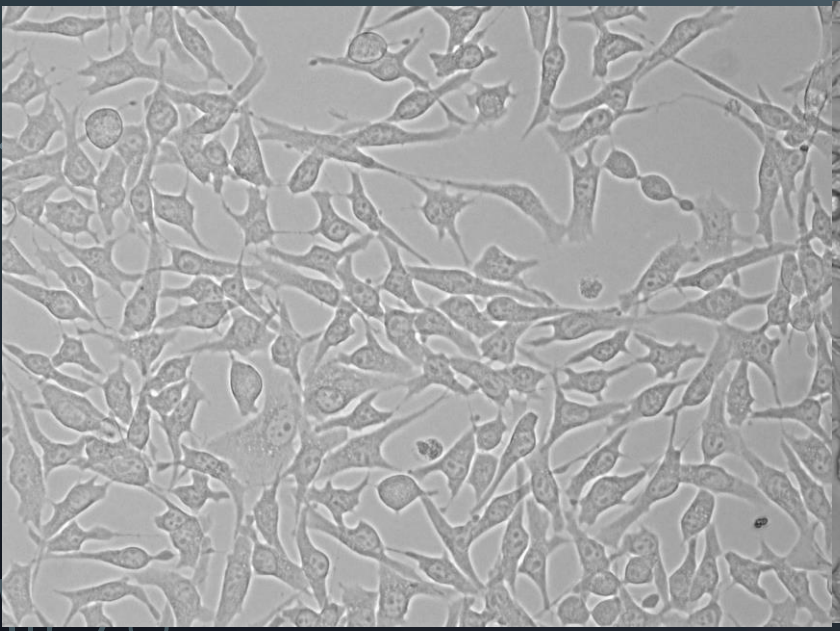
- Physically separates U and D light and subjects one or the other to phase shift and/or amplitude shift
- In theory, any shift of U and D are possible
- In practice, a shift of $\pm 90^\circ$ ($\lambda/4$) is appropriate for most biological specimens


(A)
(B)
50 µm

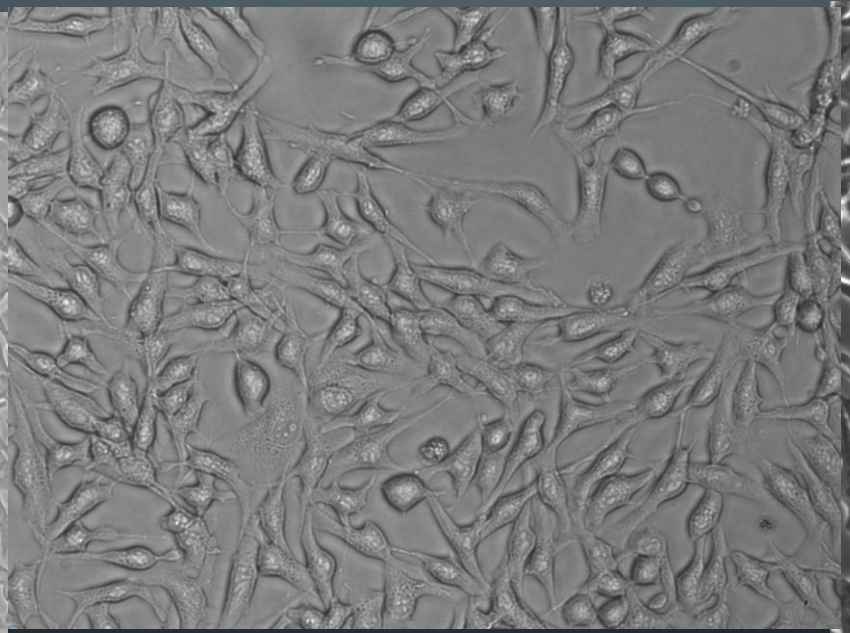

Halos in Phase Contrast [redacted] Microscopy
(a)
(c)
(e)

Phase contrast in practice

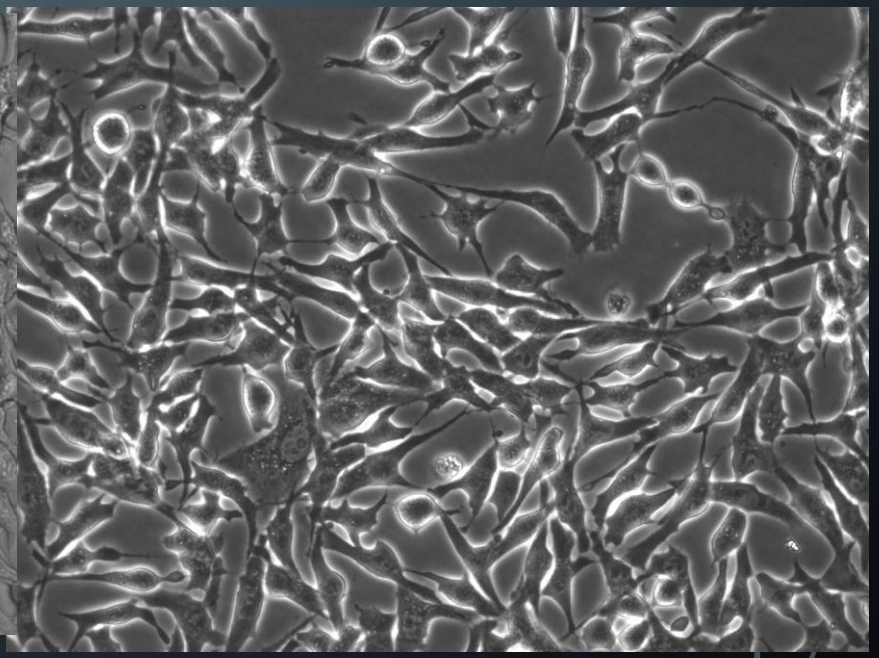
Application: Phase contrast is the most commonly used contrasting technique All tissue culture microscopes and the time-lapse microscopes are set up for phase.



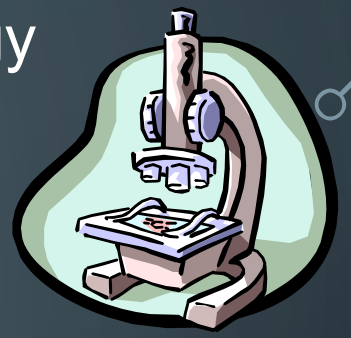
brightfield



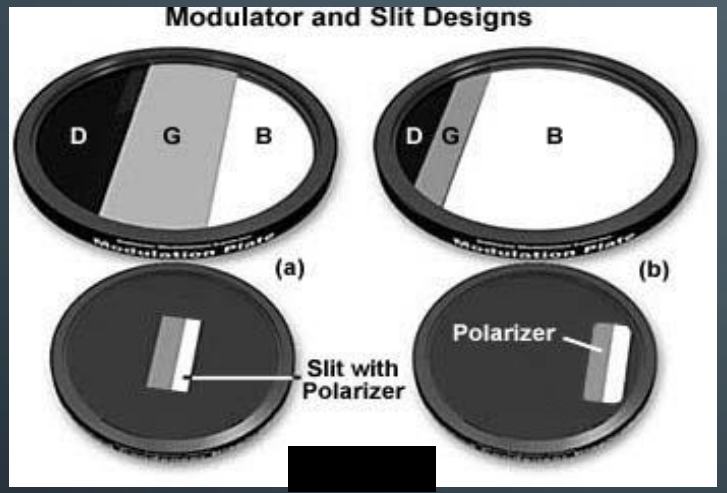
wrong phase stop

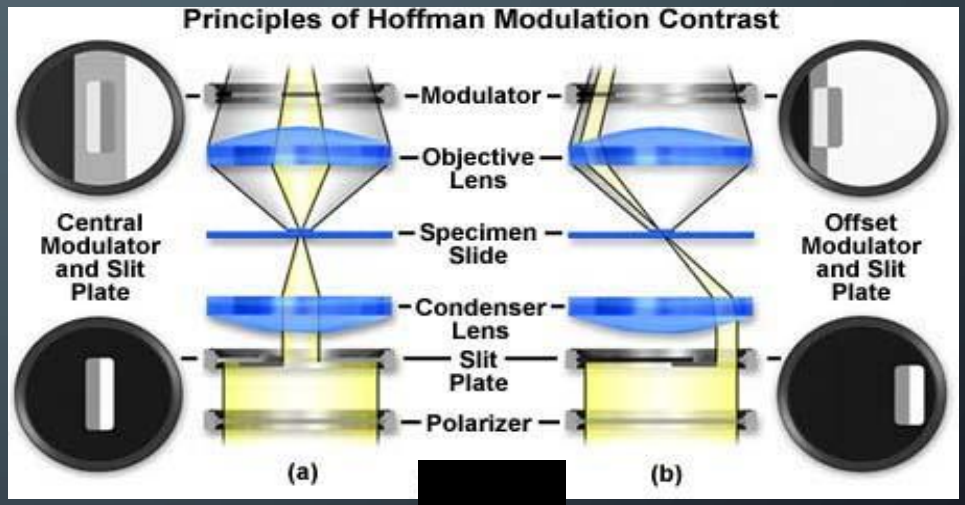


right phase stop

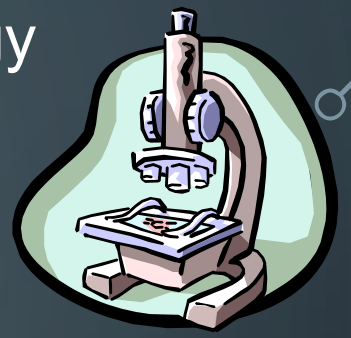


Hoffman Modulation Contrast



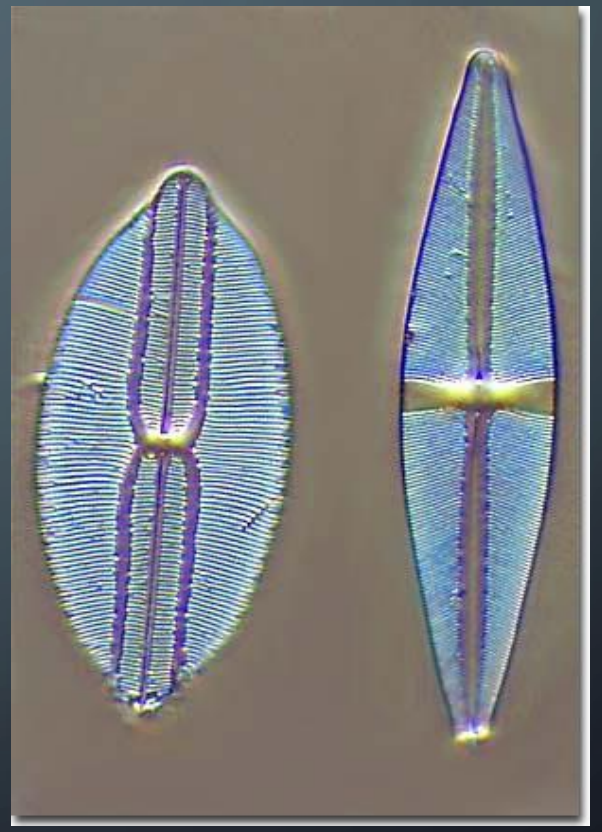


D : dark, 1% transmittance
G : gray, 15% transmittance
B : bright, 100% transmittance



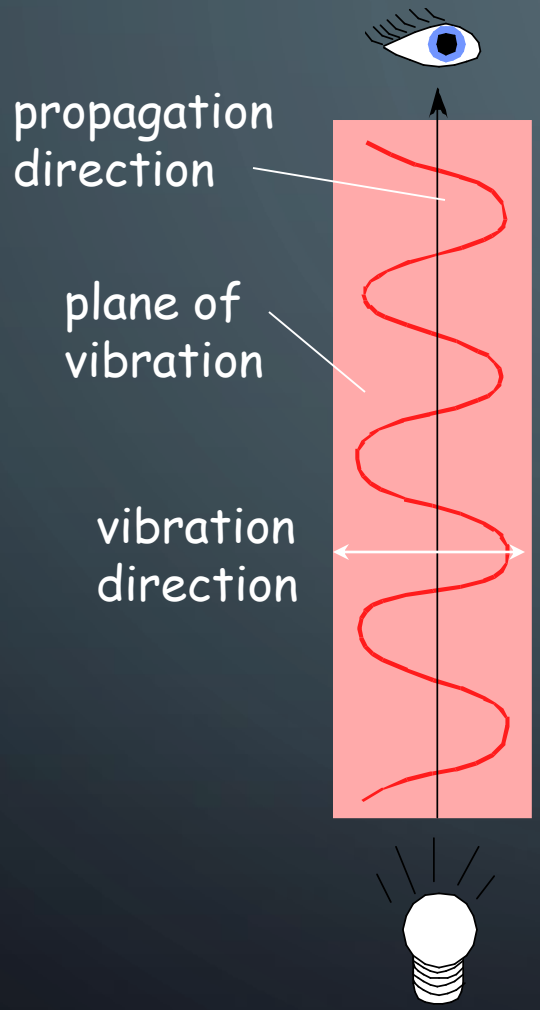
Hoffman Modulation Contrast

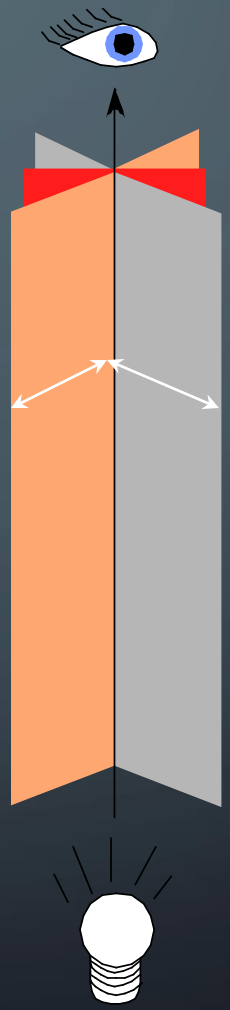
- Increase visibility and contrast in unstained and living material by detecting optical gradients (or slopes) and converting them into variations of light intensity.



Diatoms

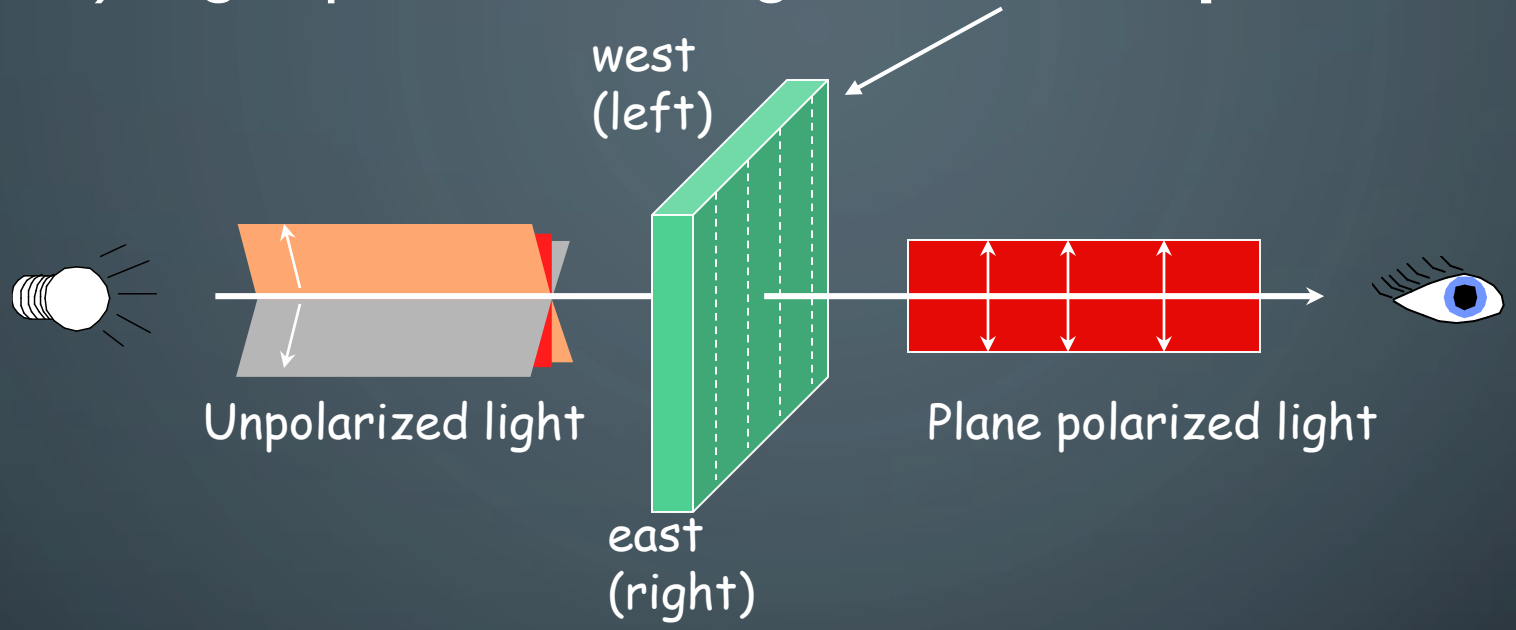
What happens as light moves through the scope?





light vibrates in all planes that contain the light ray (i.e., all planes perpendicular to the propagation direction

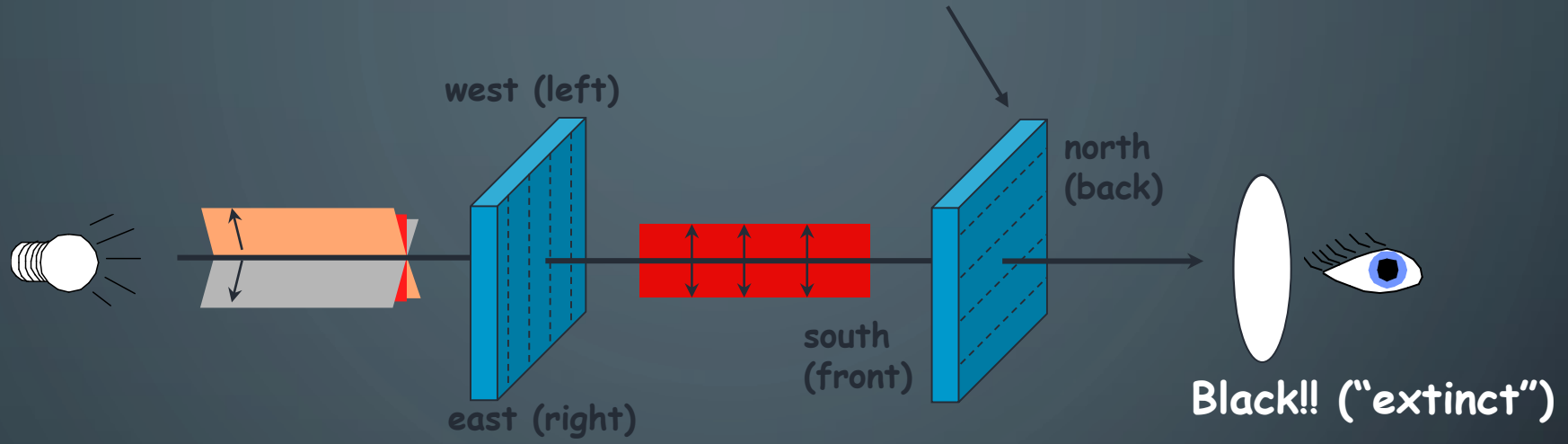
1) Light passes through the **lower polarizer**



Only the component of light vibrating in E-W direction can pass through lower polarizer – **light intensity decreases**

Though polarized, still white light!

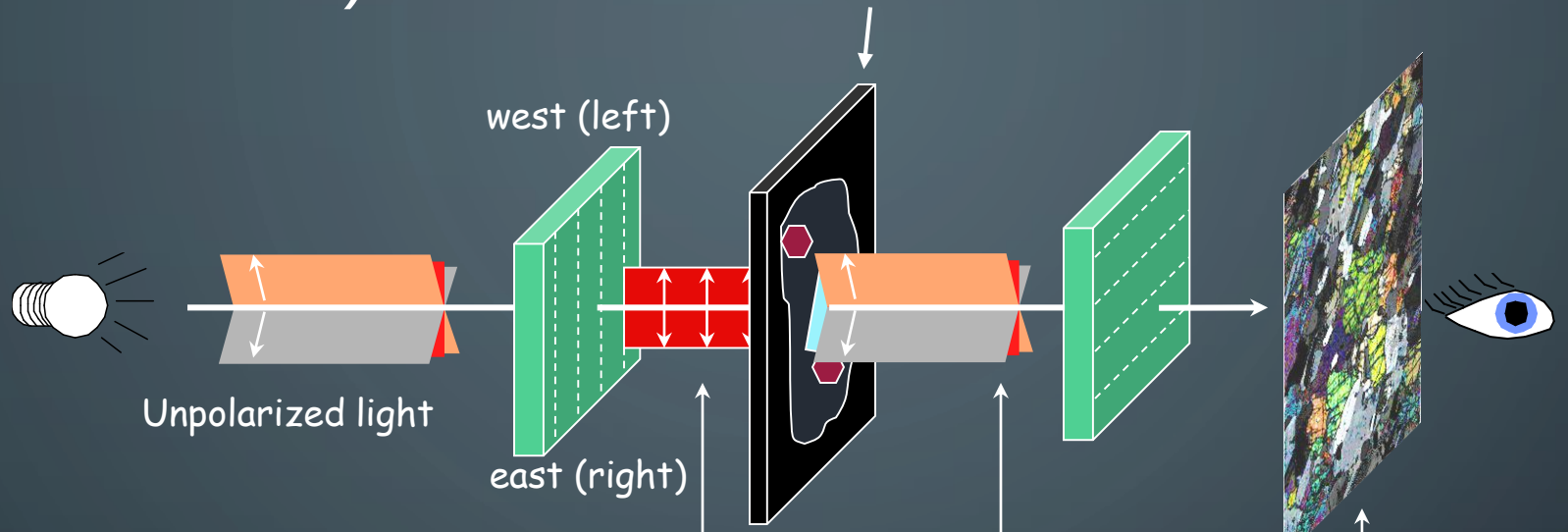
2) Insert the **upper polarizer**

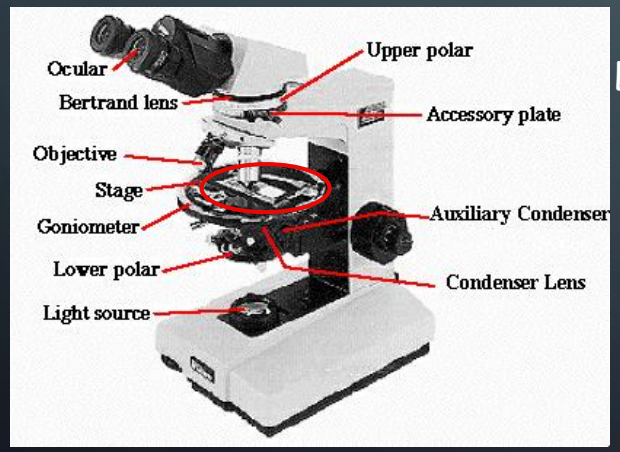


Now what happens?
What reaches your eye?

Why would anyone design a microscope that prevents light from reaching your eye???

3) Now insert a **thin section**

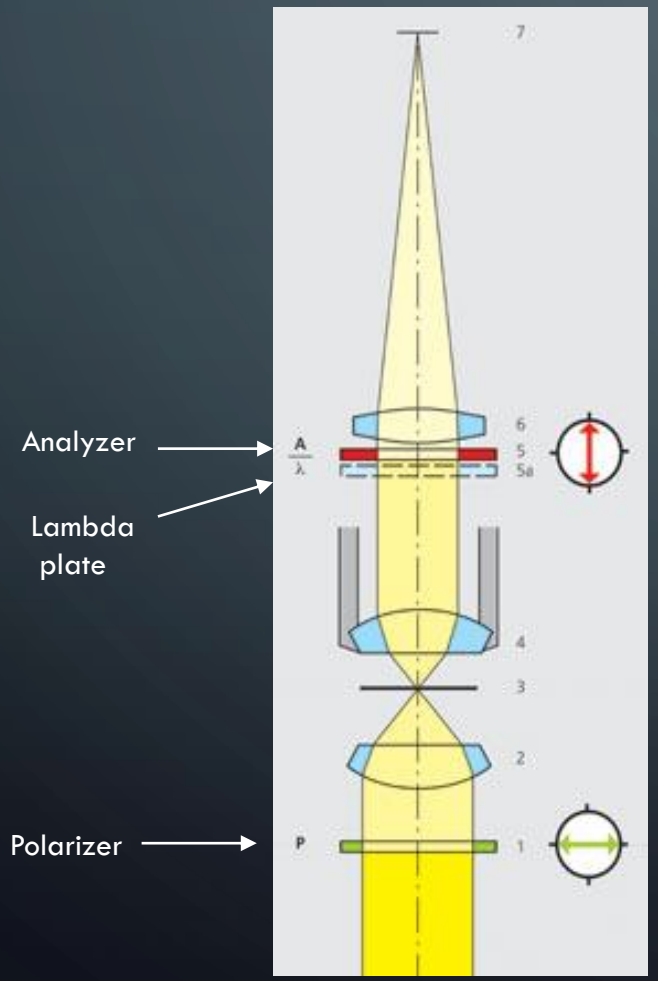




How does this work??

POLARIZATION CONTRAST

Principle: Polarized light is used for illumination. Only when the vibration direction of the polarized light is altered by a sample placed into the light path, light can pass through the analyzer. The sample appears light against a black background. A lambda plate can be used to convert this contrast into colours.



Application: Polarization contrast is used to look at materials with birefringent properties, in which the refractive index depends on the vibration direction of the incident light, e.g. crystals or polymers.

Brightfield

Polarization contrast

Polarization contrast with Lambda plate

→ *we do not have microscopes set up for polarization contrast*

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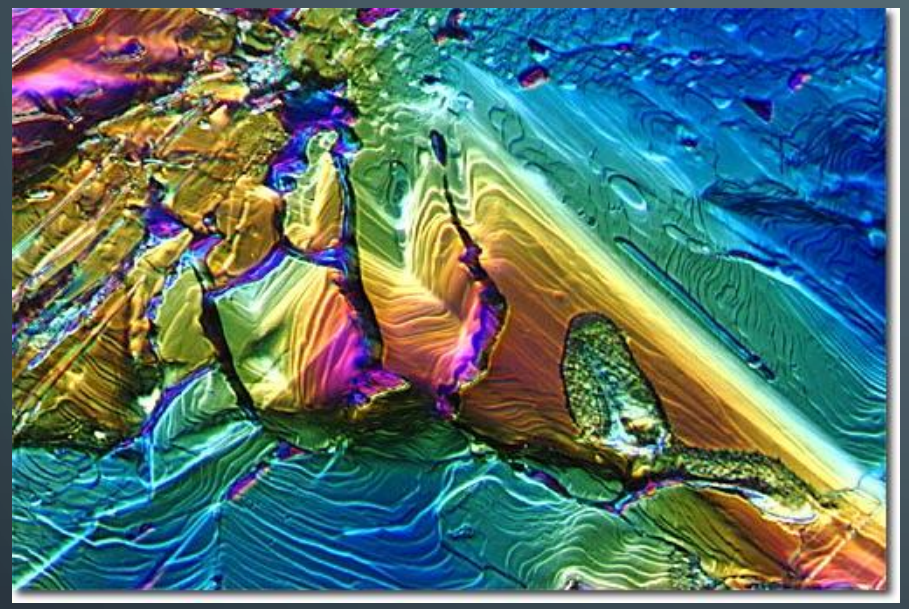
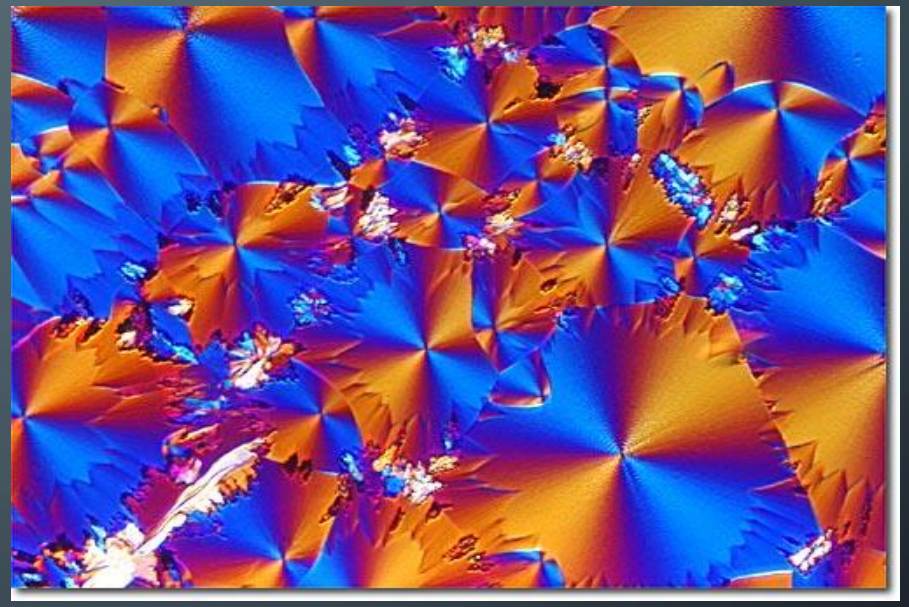
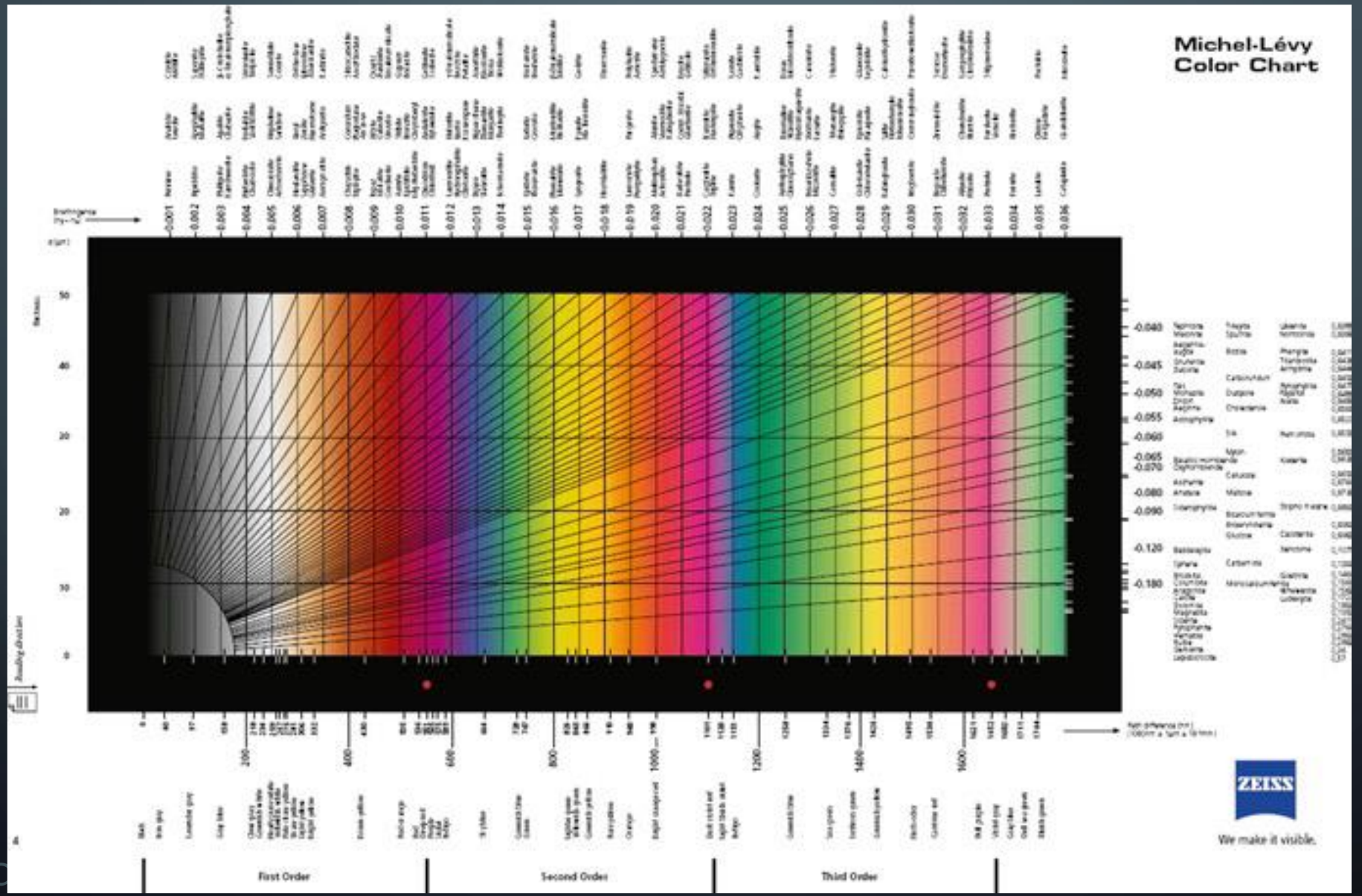


Glutaric Acid Crystallites



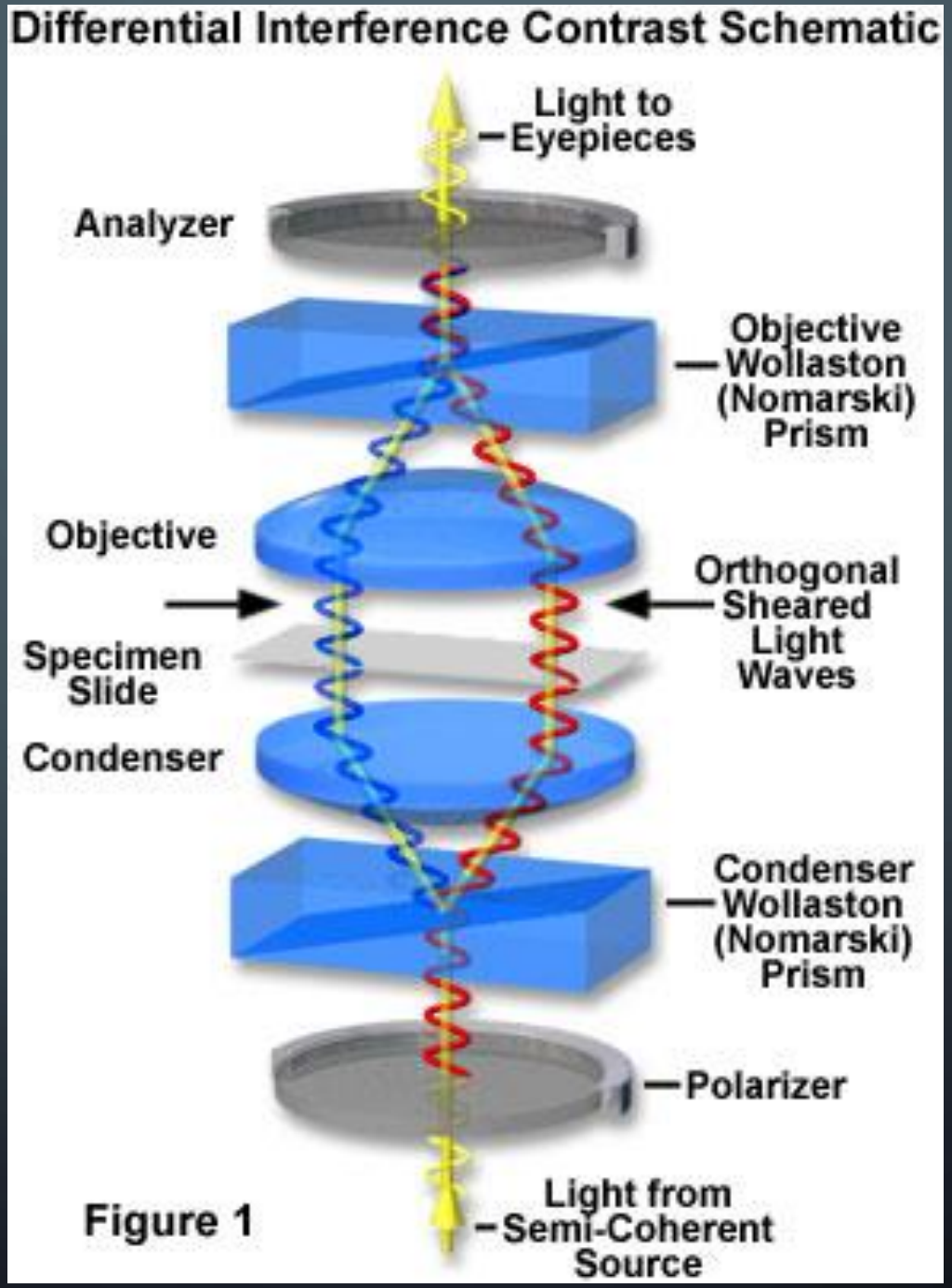
Dinosaur Bone

2020 - Light microscopy in Cellular Biology

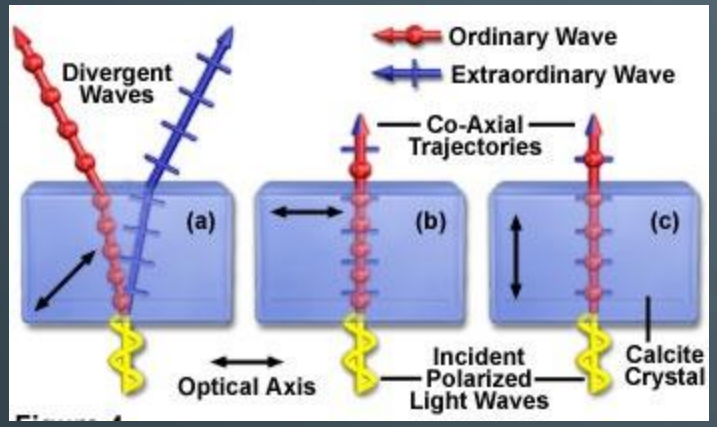


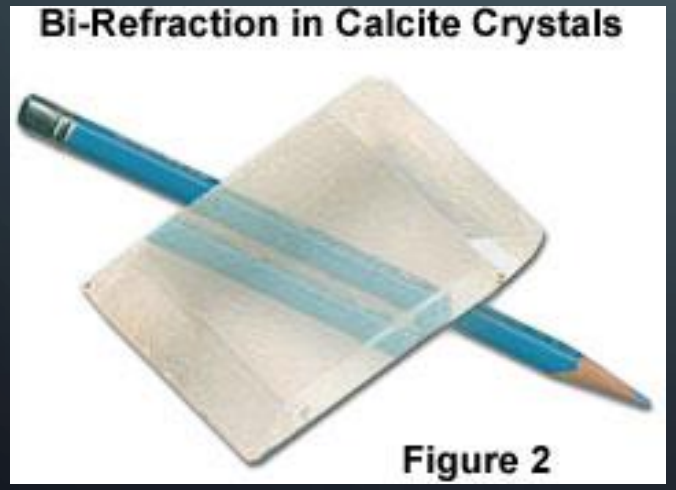
NOMARSKI IMAGE

- Result is extinction (shadow) on one side of specimen and reinforcement (bright) on the other
- Shear of image
- False relief 3D image
- Consider wavefront diagrams


Differential Interference Contrast Schematic
Light to Eyepieces
Analyzer
Objective Wollaston (Nomarski) Prism
Objective
Orthogonal Sheared Light Waves
Specimen Slide
Condenser
Condenser Wollaston (Nomarski) Prism
Polarizer
Light from Semi-Coherent Source
Figure 1

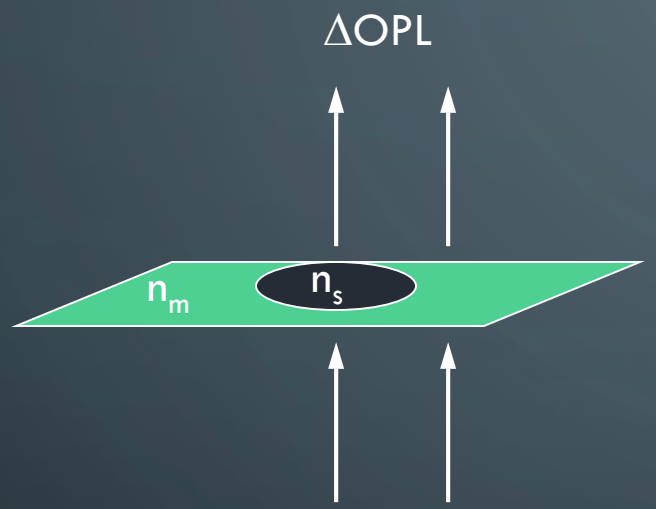
BIREFRINGENCE





- Birefringent materials have different indices of refraction for light polarized parallel or perpendicular to the optical axis.
- Two beams with orthogonal polarization are produced if illumination is at an angle to optical axis

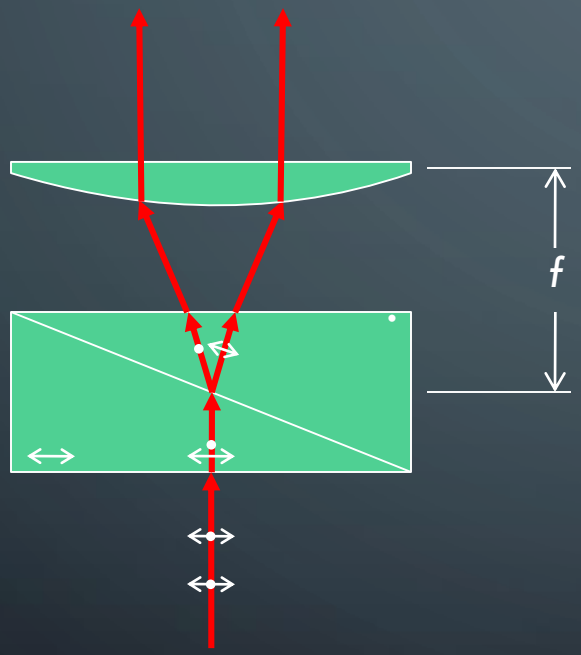
Differential Interference Contrast (DIC)



The idea:

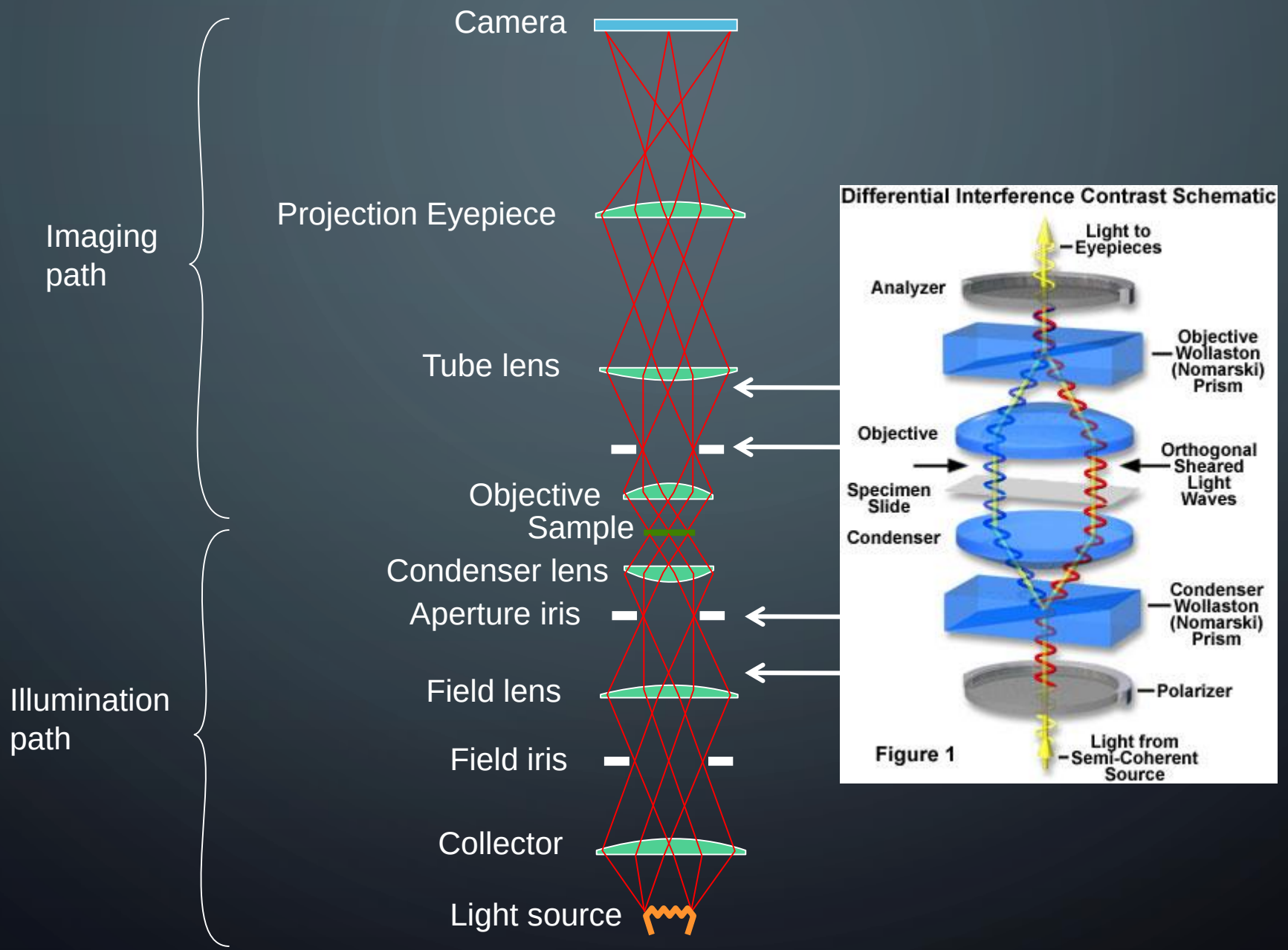
Use two beams and interference to measure the path length difference between adjacent points in the sample

WOLLASTON / NOMARSKI PRISMS

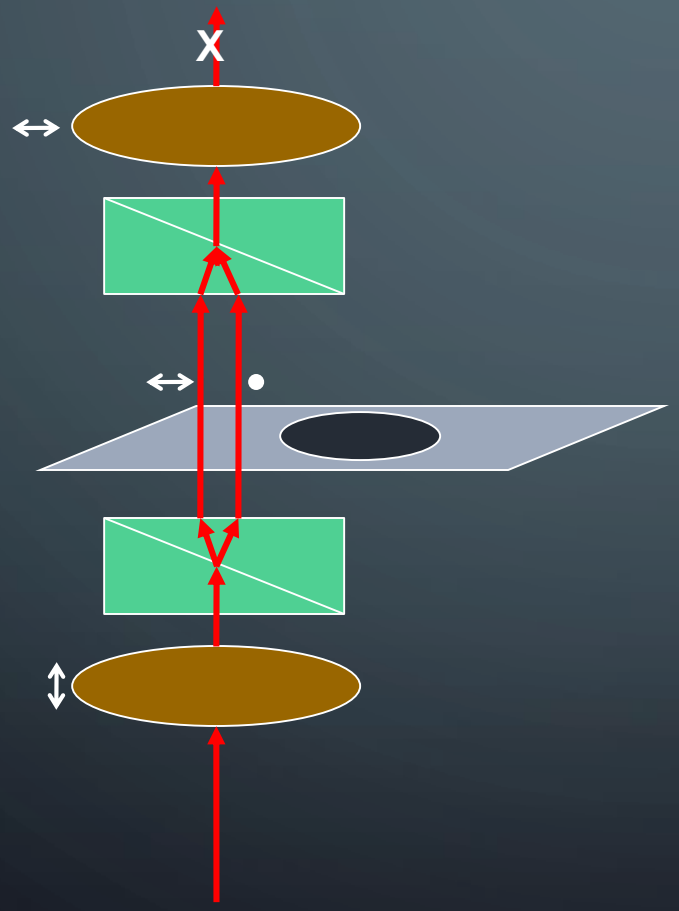


- Two pieces of cemented calcite / quartz
- Produce orthogonally polarized beams propagating at different angles
- Placed at a back focal plane, this produces the two beams that will probe the OPL difference of our sample

THE DIFFERENTIAL INTERFERENCE CONTRAST (DIC) MICROSCOPE

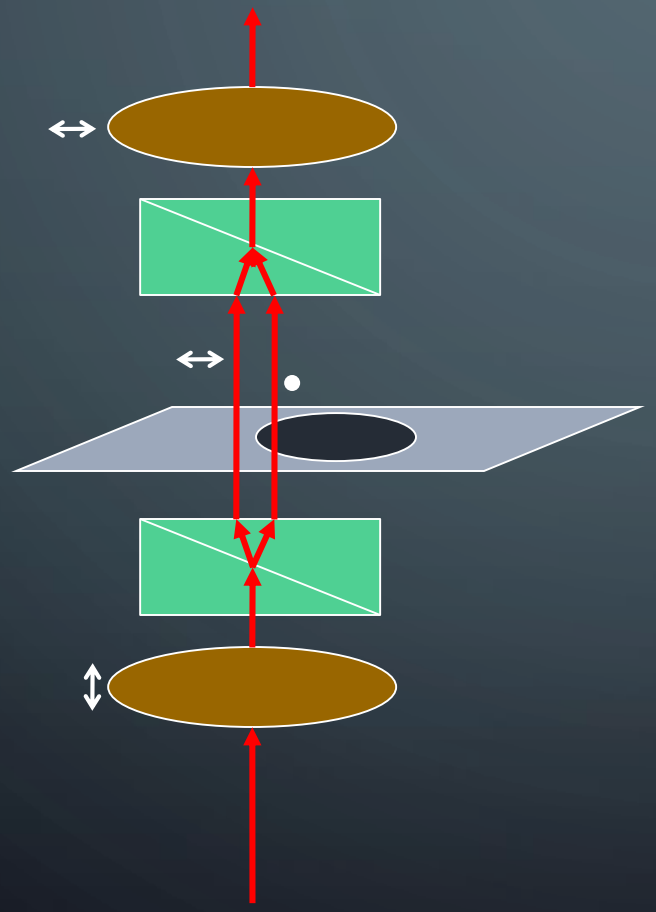


HOW DIC GENERATES CONTRAST



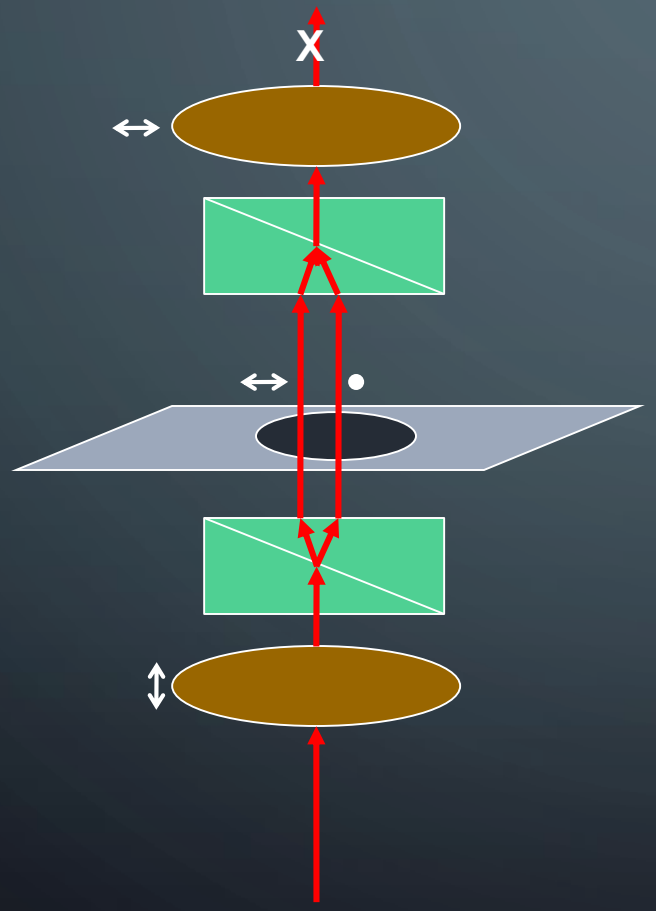
- Both beams see same OPL
- Emerge in phase
- Regenerate initial polarization
- No light makes it through analyzer

HOW DIC GENERATES CONTRAST

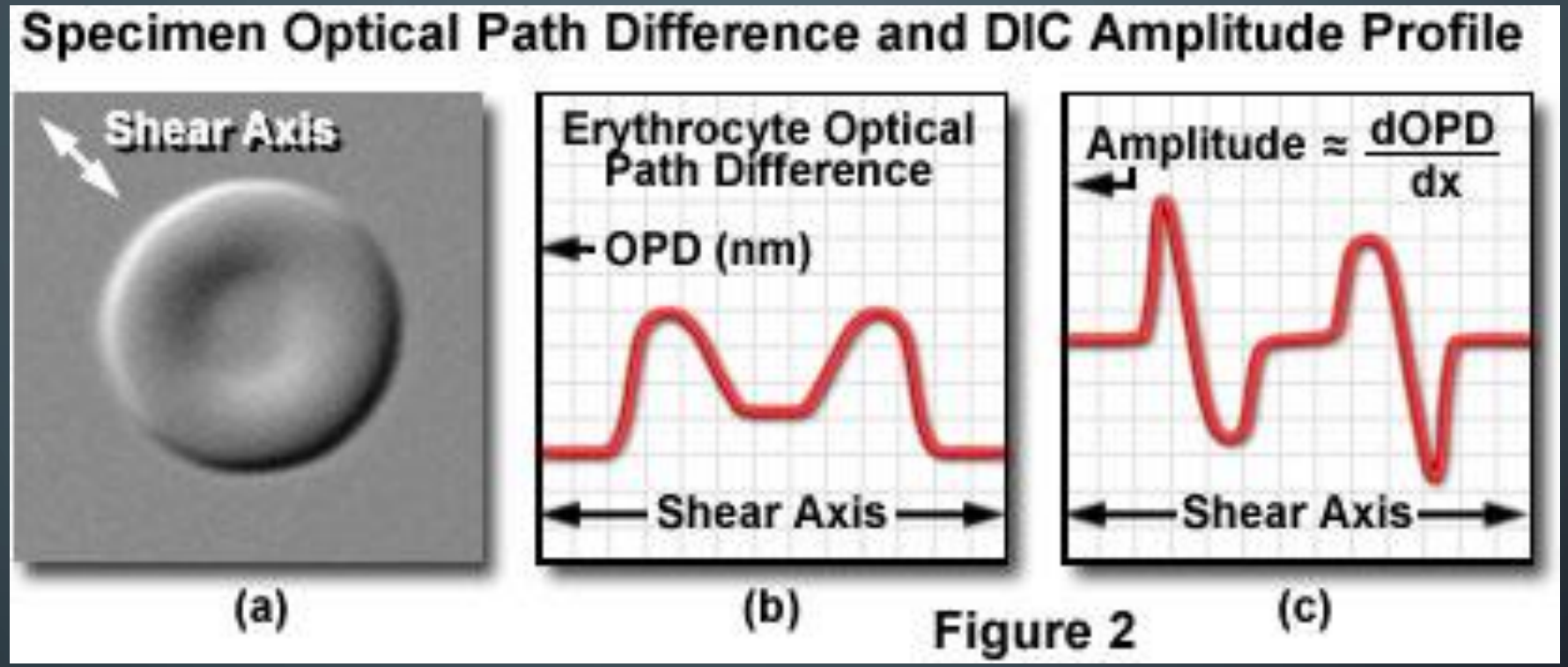


- Beams see different OPL
- Right beam is phase retarded
- Generate elliptical polarization
- Light makes it through analyzer

HOW DIC GENERATES CONTRAST

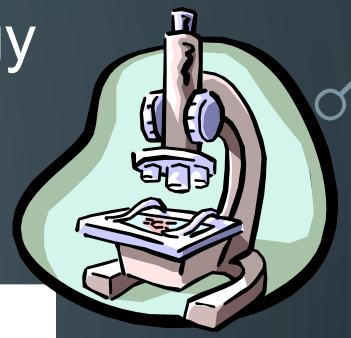


- Both beams see same OPL
- Emerge in phase
- Regenerate initial polarization
- No light makes it through analyzer

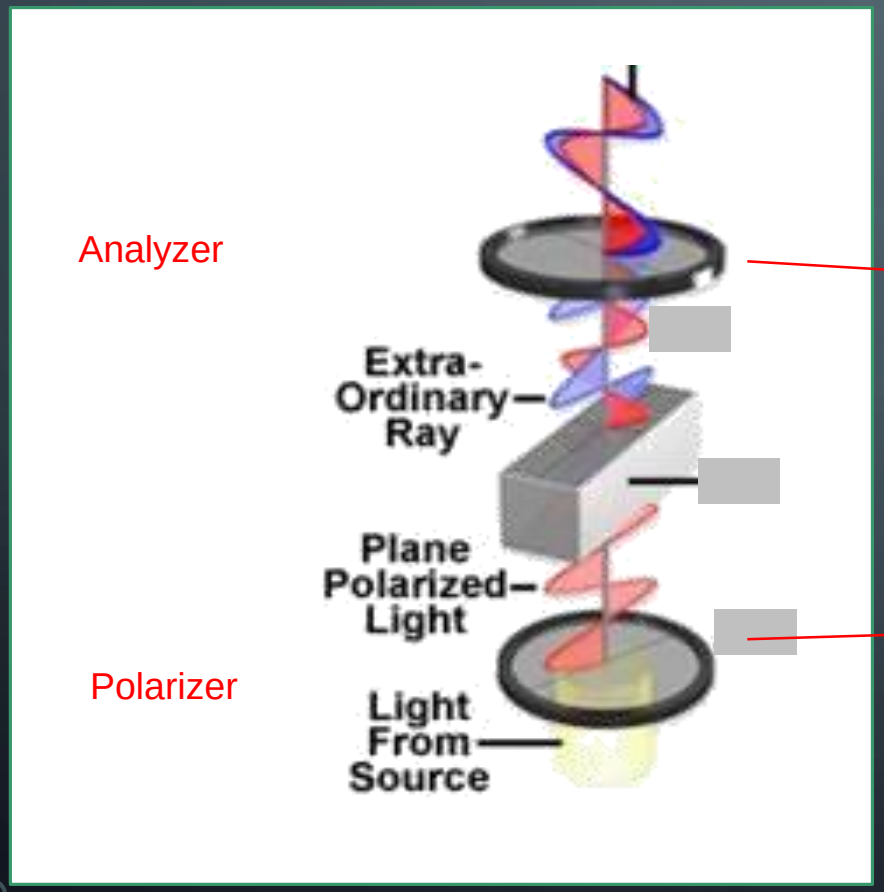

Specimen Optical Path Difference and DIC Amplitude Profile
Shear Axis
Erythrocyte Optical Path Difference
OPD (nm)
Shear Axis
Amplitude ≈ dOPD/dx
Shear Axis
(a)
(b)
(c)
Figure 2

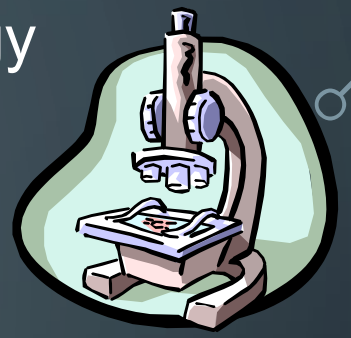
Features of a DIC image

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



Polarization



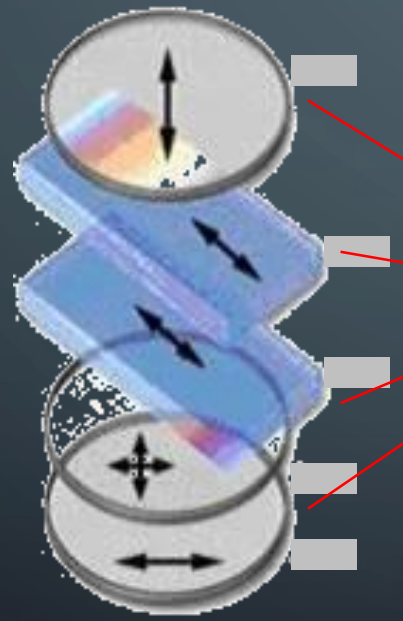


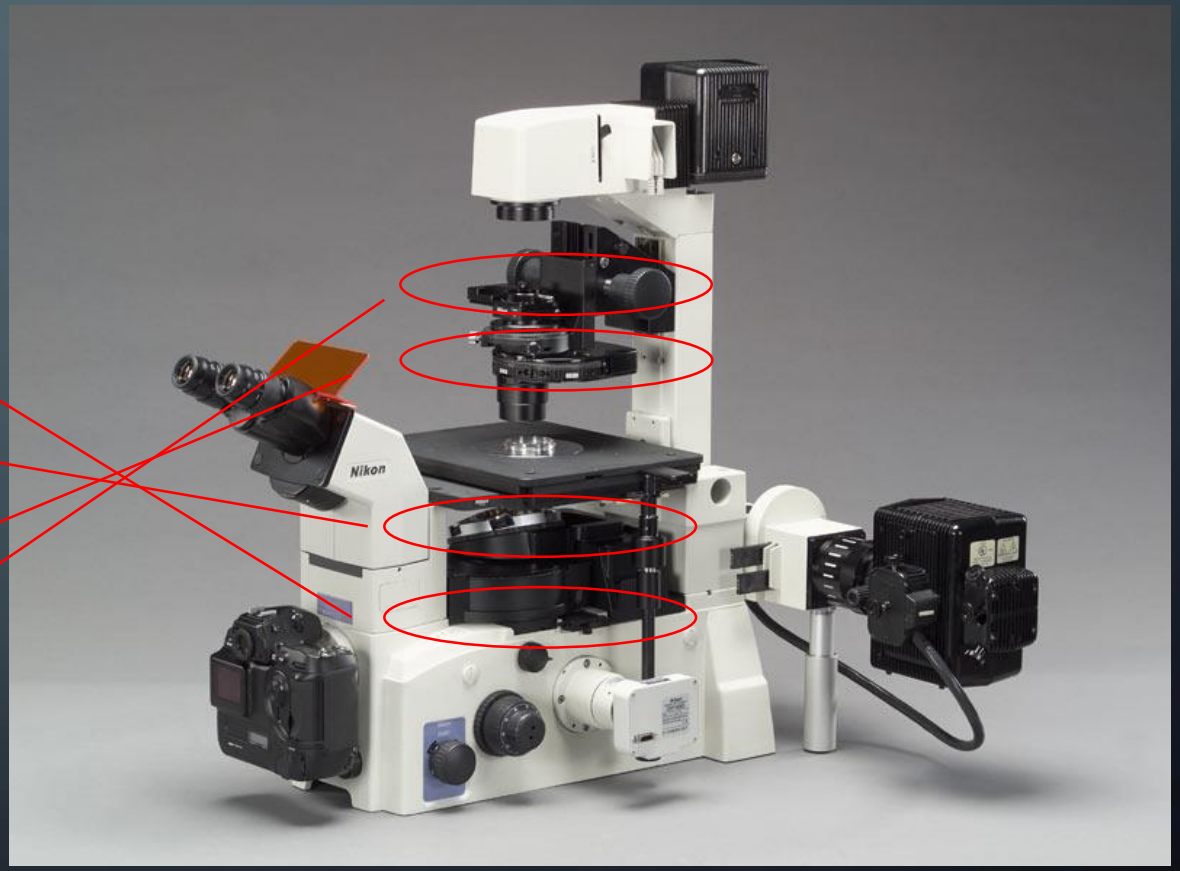
Differential interference contrast (DIC)

Analyzer

Condenser
Nomarski prism

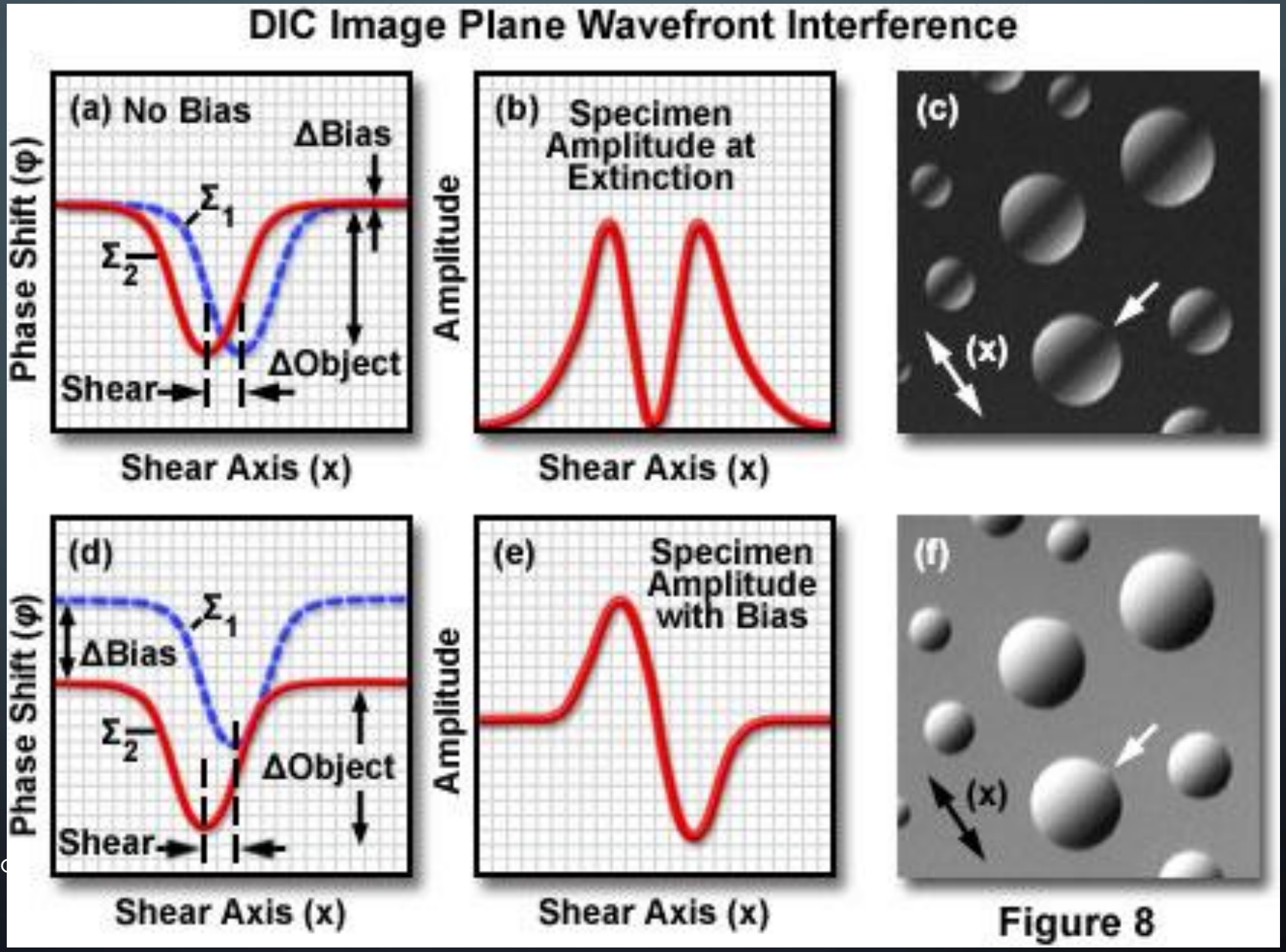
Polarizer





SHEAR IN IMAGE

- Degree of shear is set by wollaston combination
- Bias of shear adjustable by shifting upper wollaston position to retard one beam more or less relative to other
- Cannot be used for quantitative measurements of dry mass
- But extremely useful for observing living cells


DIC Image Plane Wavefront Interference
(a) No Bias
ΔBias
Σ1
Σ2
ΔObject
Shear
Phase Shift (φ)
Shear Axis (x)
(b) Specimen Amplitude at Extinction
Amplitude
Shear Axis (x)
(c)
(x)
(d)
ΔBias
Σ1
Σ2
ΔObject
Shear
Phase Shift (φ)
Shear Axis (x)
(e) Specimen Amplitude with Bias
Amplitude
Shear Axis (x)
(f)
(x)
Figure 8

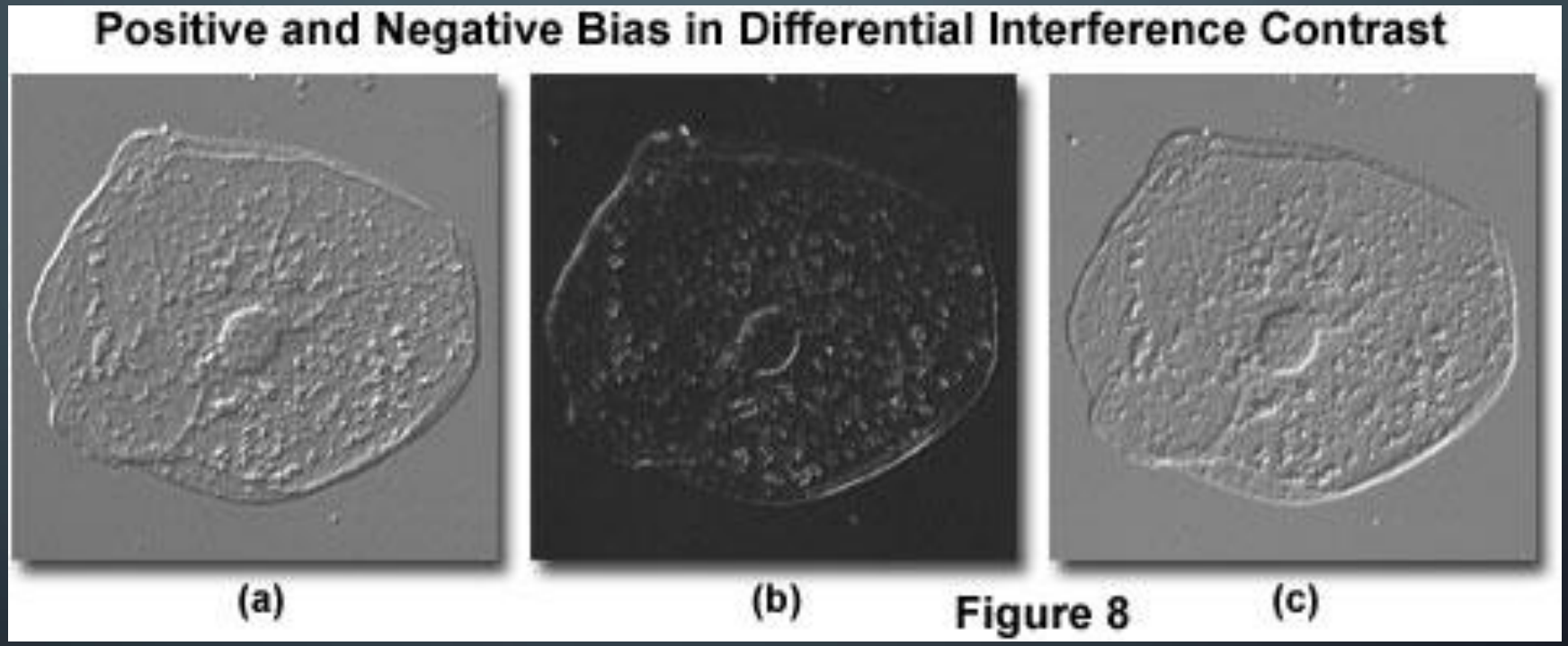
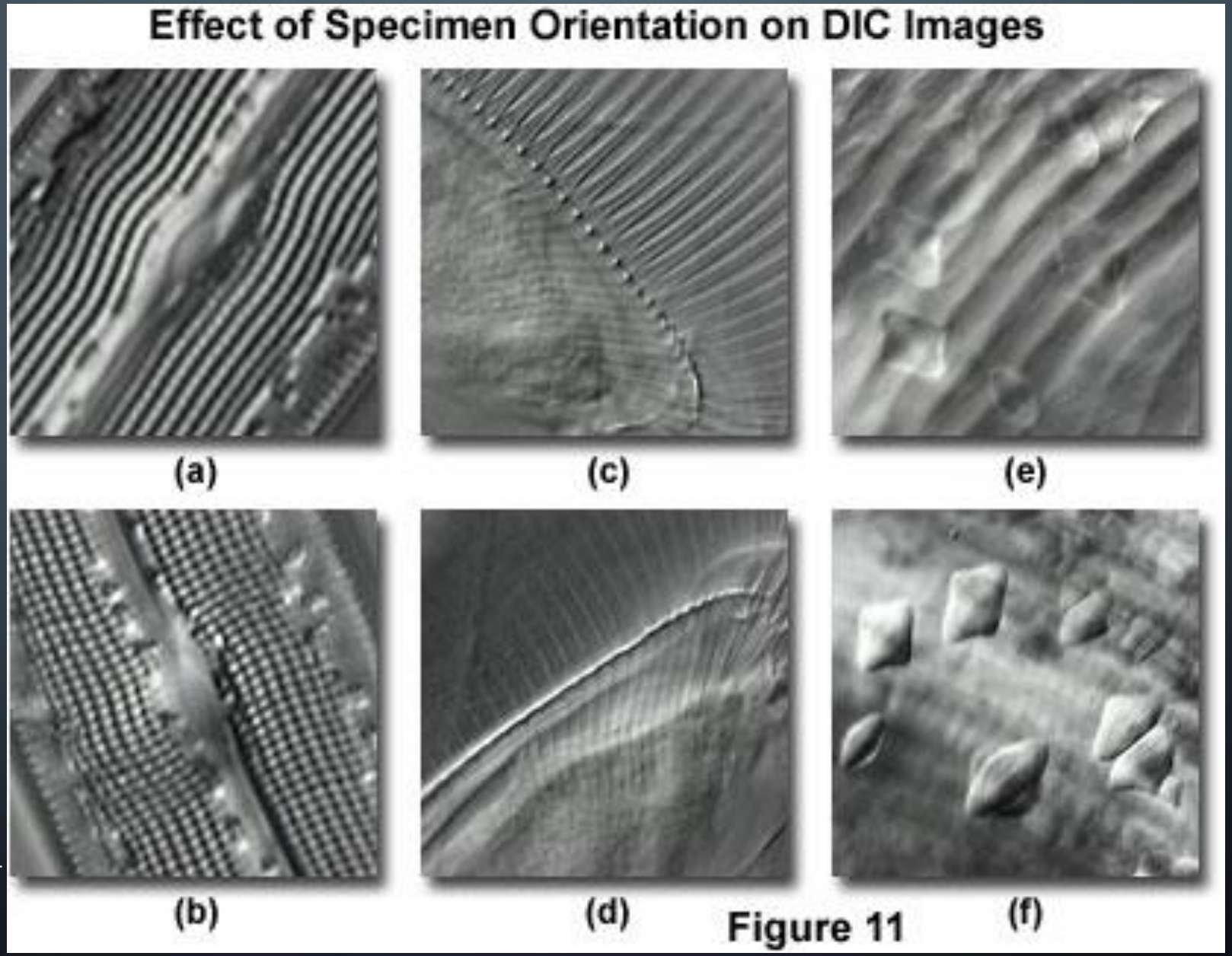

Positive and Negative Bias in Differential Interference Contrast
(a)
(b)
(c)

Figure 8


Effect of Specimen Orientation on DIC Images
(a)
(c)
(e)
(b)
(d)
(f)
Figure 11

COMPARISON OF NOMARSKI AND PHASE CONTRAST OPTICS

Phase Contrast	Nomarski
Cheaper	More expensive
Easier to set up	Fussy alignment
Uses less than full aperture of objective	Uses full aperture — closet to theoretical limit
Phase Halo — surrounds specimen and other changes in i.r.	Shadow Effect — contrast greatest at shear direction maximum

Phase Contrast

Insensitive to birefringence in specimen or slides

Extremely large depth of field — sensitive to artifacts far out of plane of specimen

Doesn't work well with stained specimens

Nomarski

Optics disrupted by birefriengence

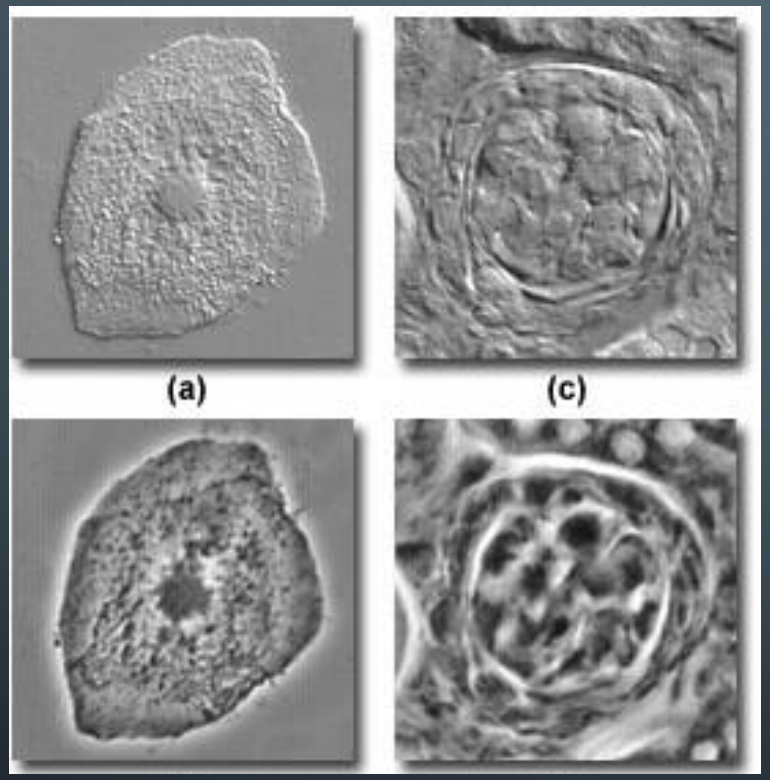
Extremely shallow depth of field — useful for optical sectioning of specimen

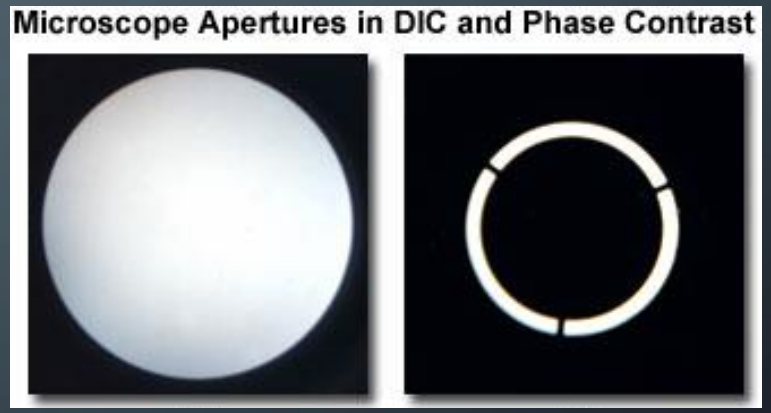
Works well with stained specimens; optics can be adjusted to enhance contrast

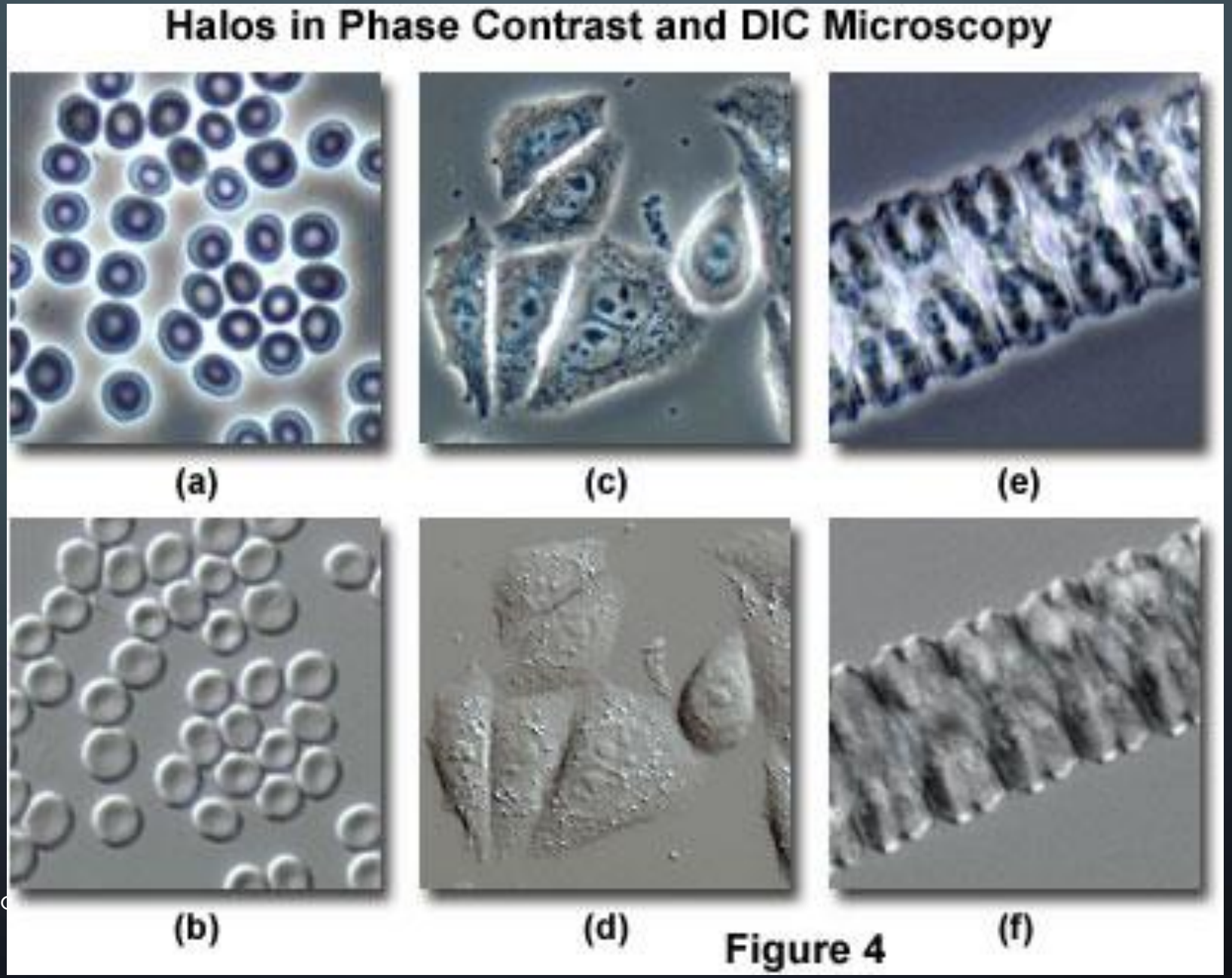
DIC IS HIGHER RESOLUTION THAN PHASE CONTRAST

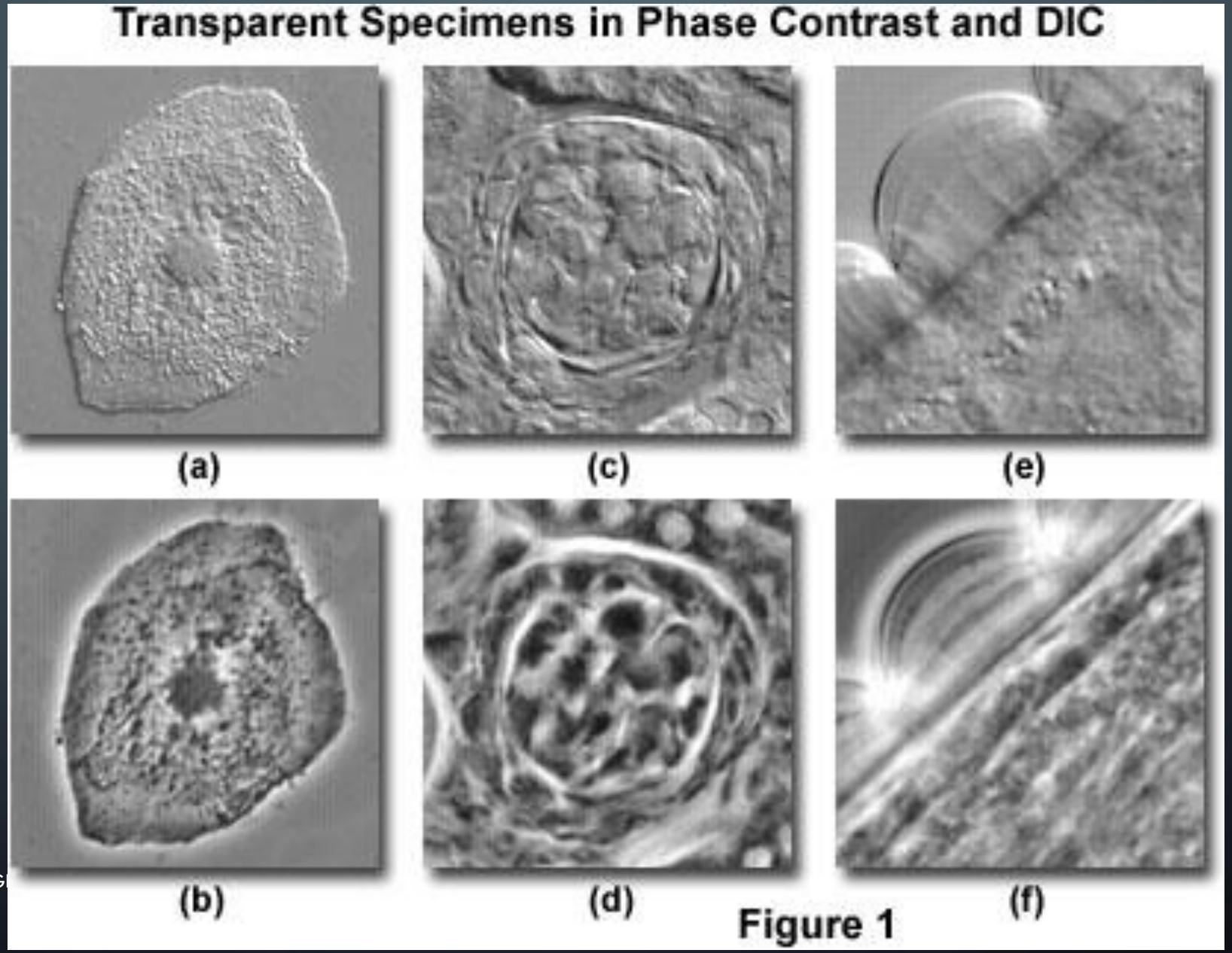
DIC

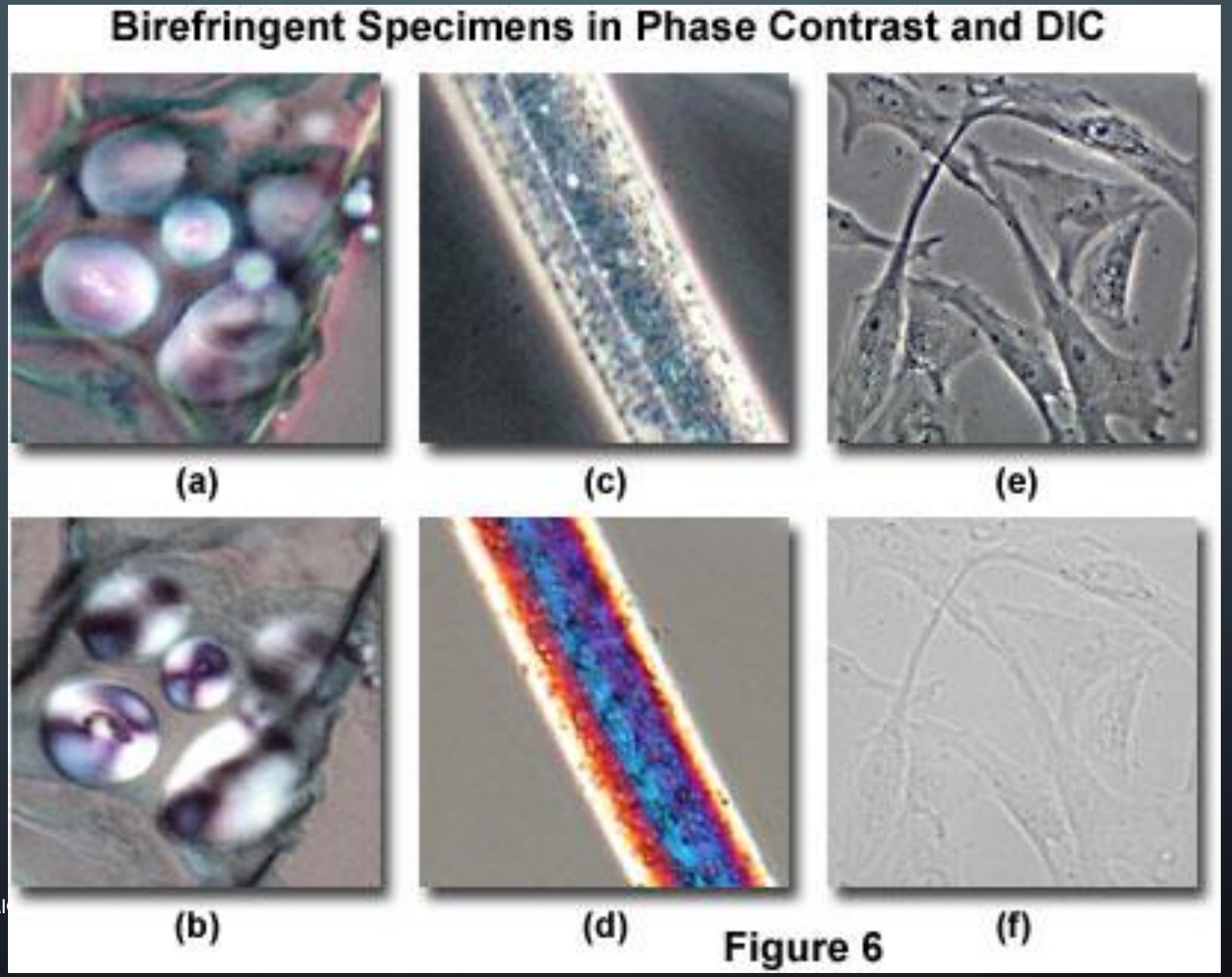
Phase





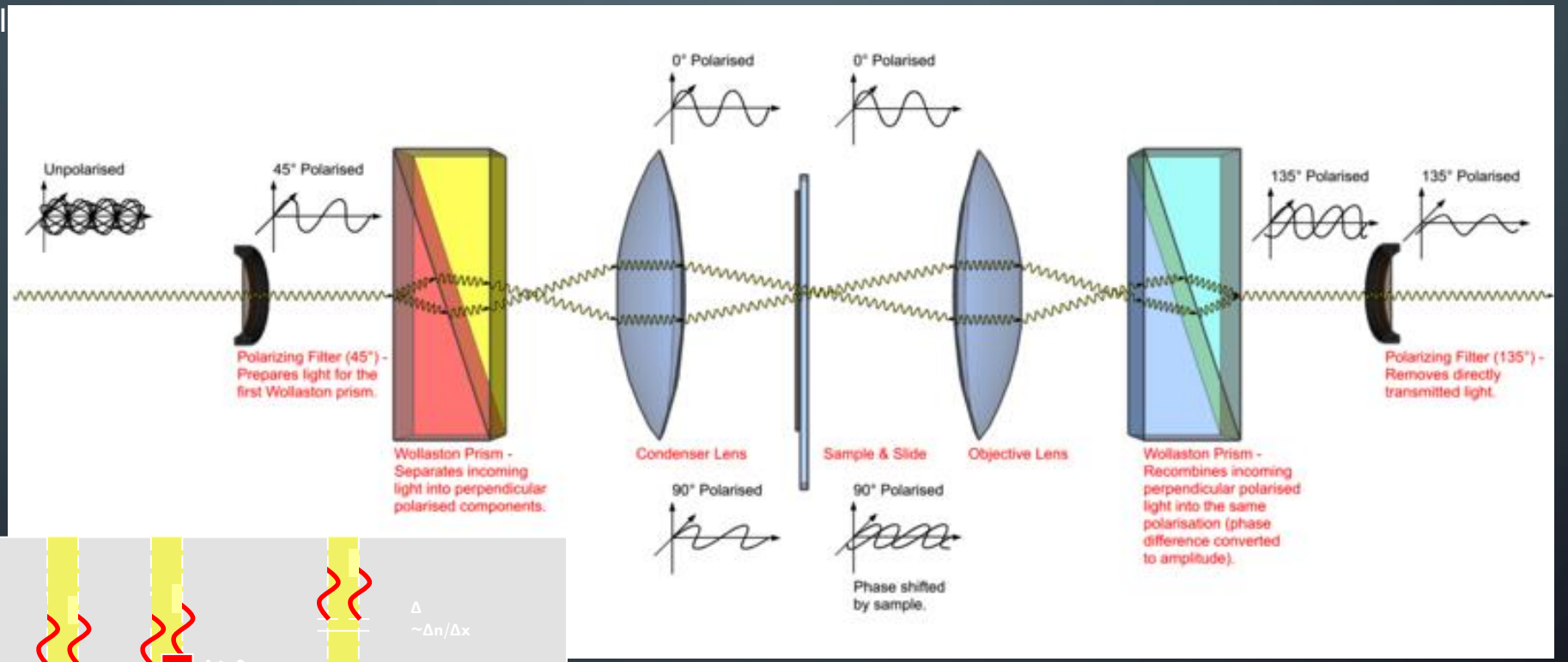

Halos in Phase Contrast and DIC Microscopy
(a)
(c)
(e)
(b)
(d)
(f)
Figure 4

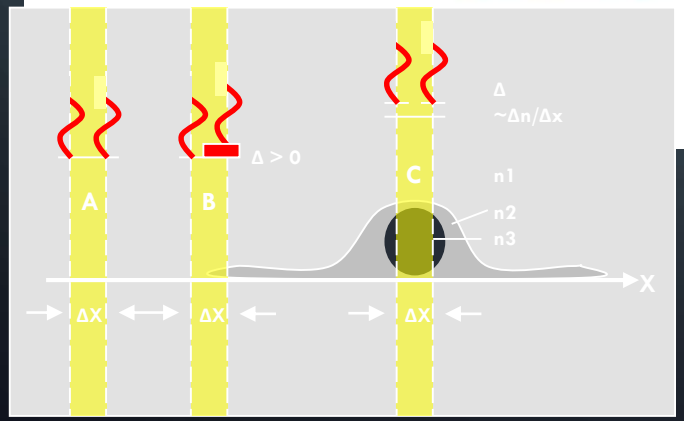

Transparent Specimens in Phase Contrast and DIC
(a)
(c)
(e)
(b)
(d)
(f)
Figure 1

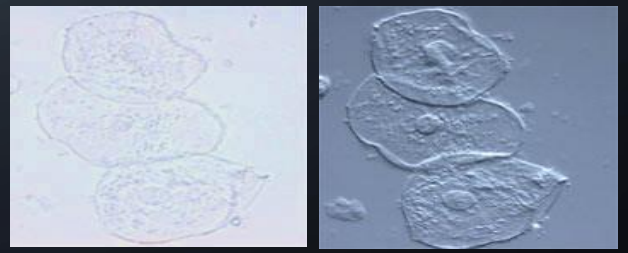

Birefringent Specimens in Phase Contrast and DIC
(a)
(c)
(e)
(b)
(d)
(f)
Figure 6

D(IFFERENTIAL) I(NTERFERENCE) C(ONTRAST)

Principle: Also known as Nomarski microscopy. Uses polarized light for illumination. Synchronizing of the different phases of incident and transmitted light is done by a set of prisms and filters introduced into the l

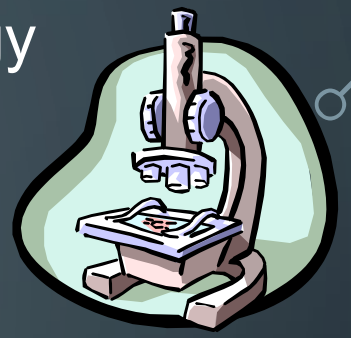




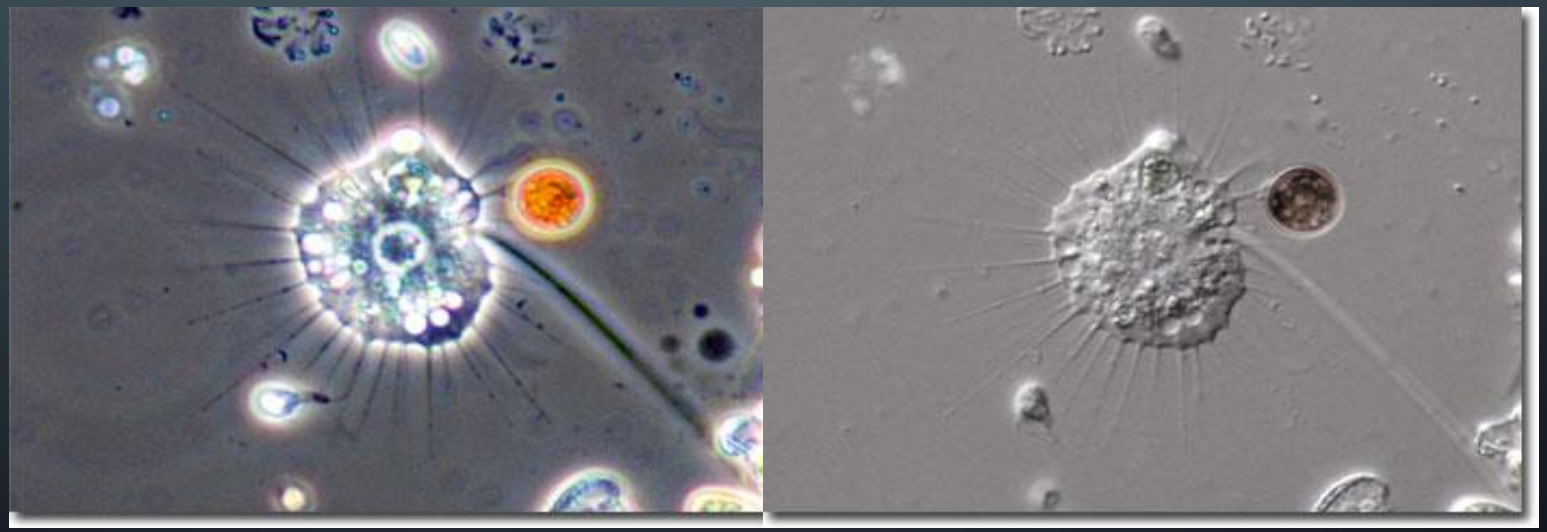


→ *most of our microscopes are set up for DIC*

2020 - Light microscopy in Cellular Biology



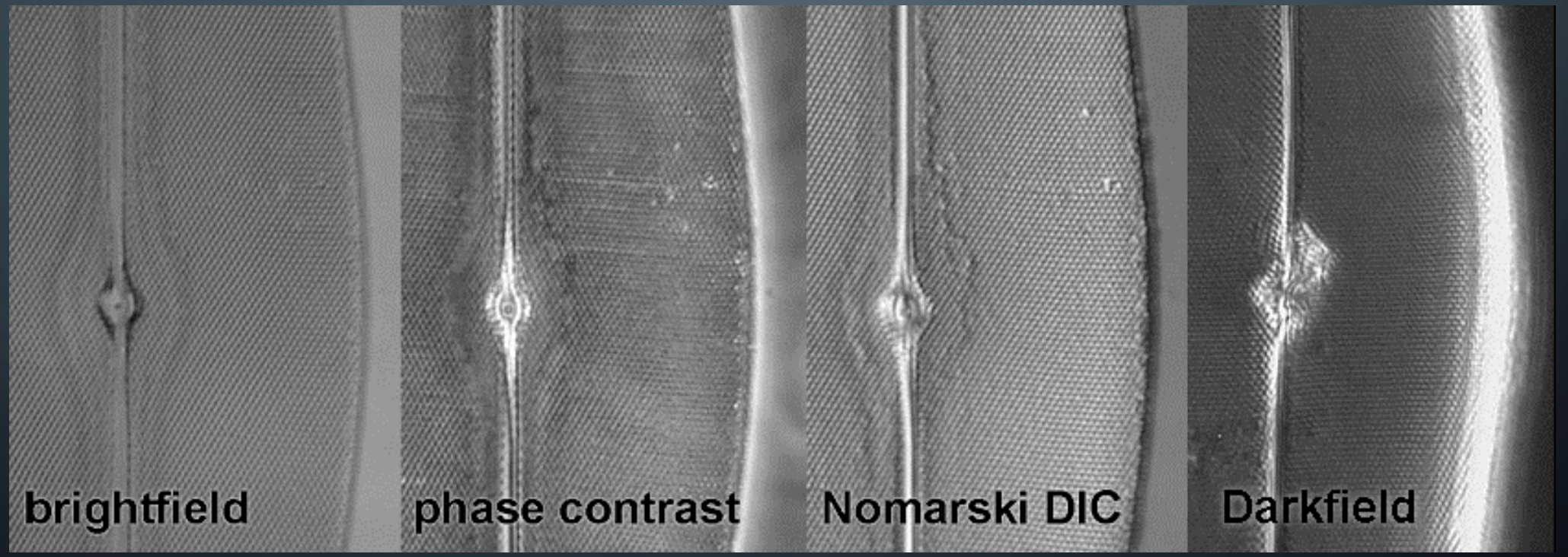
HeLa Cell Culture



Heliozoans (*Actinophrys sol*)

2020 - Light microscopy in Cellular Biology

https://en.wikipedia.org/wiki/Polarized_light_microscopy#/media/File:Paper_Micrograph_Bright.png



CONTRASTING TECHNIQUES - A SUMMARY

- **Brightfield -absorption**

  Light is transmitted through the sample. Only useful for specimens that can be contrasted via dyes. Very little contrast in unstained specimens.

- **Darkfield -scattering**

  The illuminating rays of light are directed from the side so that only scattered light enters the microscope lenses, consequently the cell appears as an illuminated object against the view.

- **Phase Contrast- phase interference**

  Incident light [Io] is out of phase with transmitted light [I] and when the phases of the light are synchronized by an interference lens, a new image with greater contrast is seen

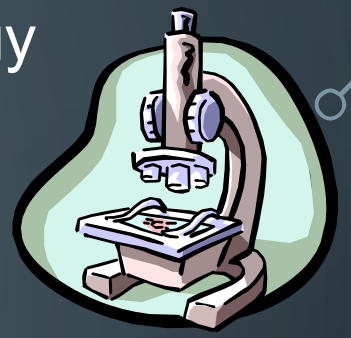
- **Polarization Contrast -polarization**

  Uses polarized light for illumination. Only when the vibration direction of the polarized light is altered by a sample placed into the light path, light can pass through the analyzer. The sample appears light against a black background.

- **Differential Interference Contrast (DIC) – polarization + phase interference**

  Also known as Nomarski microscopy. Synchronizing of the different phases of incident and transmitted light is done by a set of special condenser lens mounted below the stage of a microscope

- **Fluorescence Contrast (Fluorescein)**



REFRACTIVE INDEX

•Refractive index is the light-bending ability of a medium.

• The light may bend in air so much that it misses the small high-magnification lens.

• Immersion oil is used to keep light from bending.

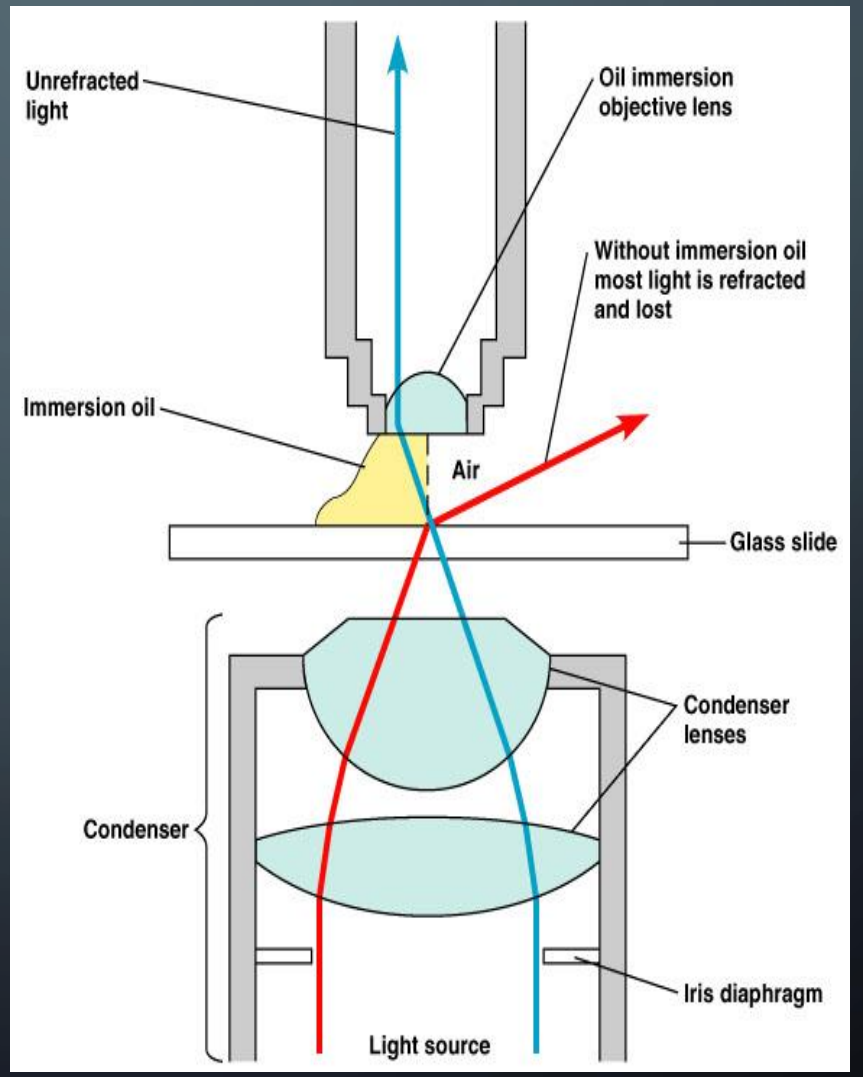
RI of Air- 1.0

RI of Water -1.3

RI of Glycerol- 1.47

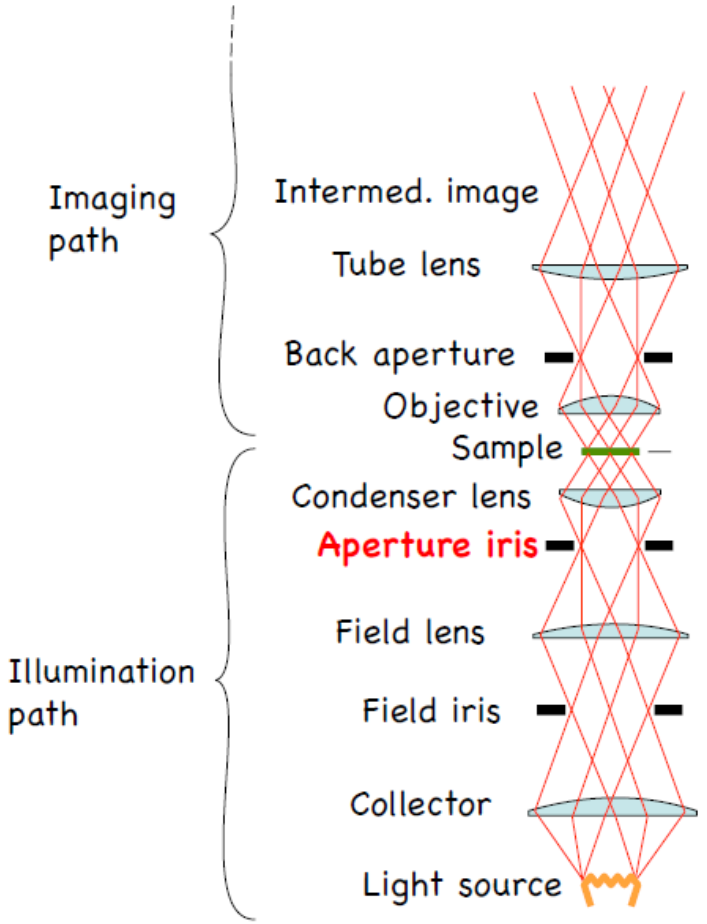
RI of Glass(avg)- 1.5

RI of Oil- 1.52



Aperture, Resolution & Contrast

Can adjust the condenser NA with the **aperture iris**



Q: Don't we always want it full open??

A: **No**

Why? Tradeoff: resolution vs. **contrast**

Increasing the illumination aperture increases resolution but decreases contrast