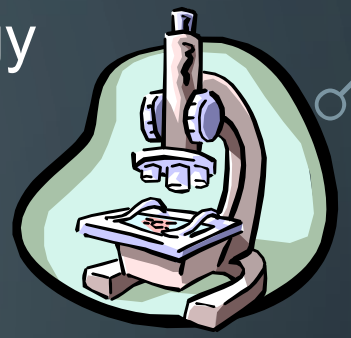


Light microscopy in Cellular Biology

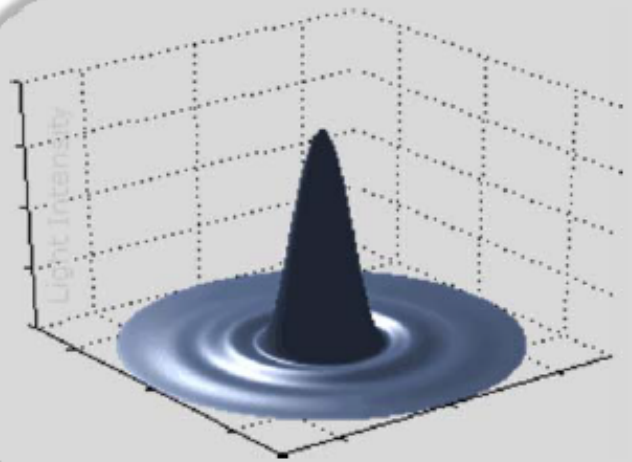
Gabriele Baj
gbaj@units.it



Diffraction and Point Spread Function

Diffraction is the explanation for why
an object point-source spreads out to form a finite image spot

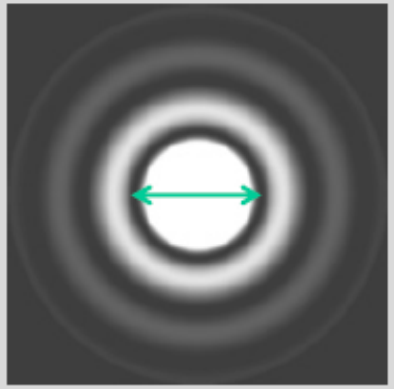
For an optical system with circular aperture
the finite image spot forms an Airy Disk (Point Spread Function)



$$PSF(x) = \frac{[2 \cdot J_1(u)]^2}{u^2}$$

$$with \quad u = \frac{\pi \cdot x}{\lambda \cdot f/\#}$$

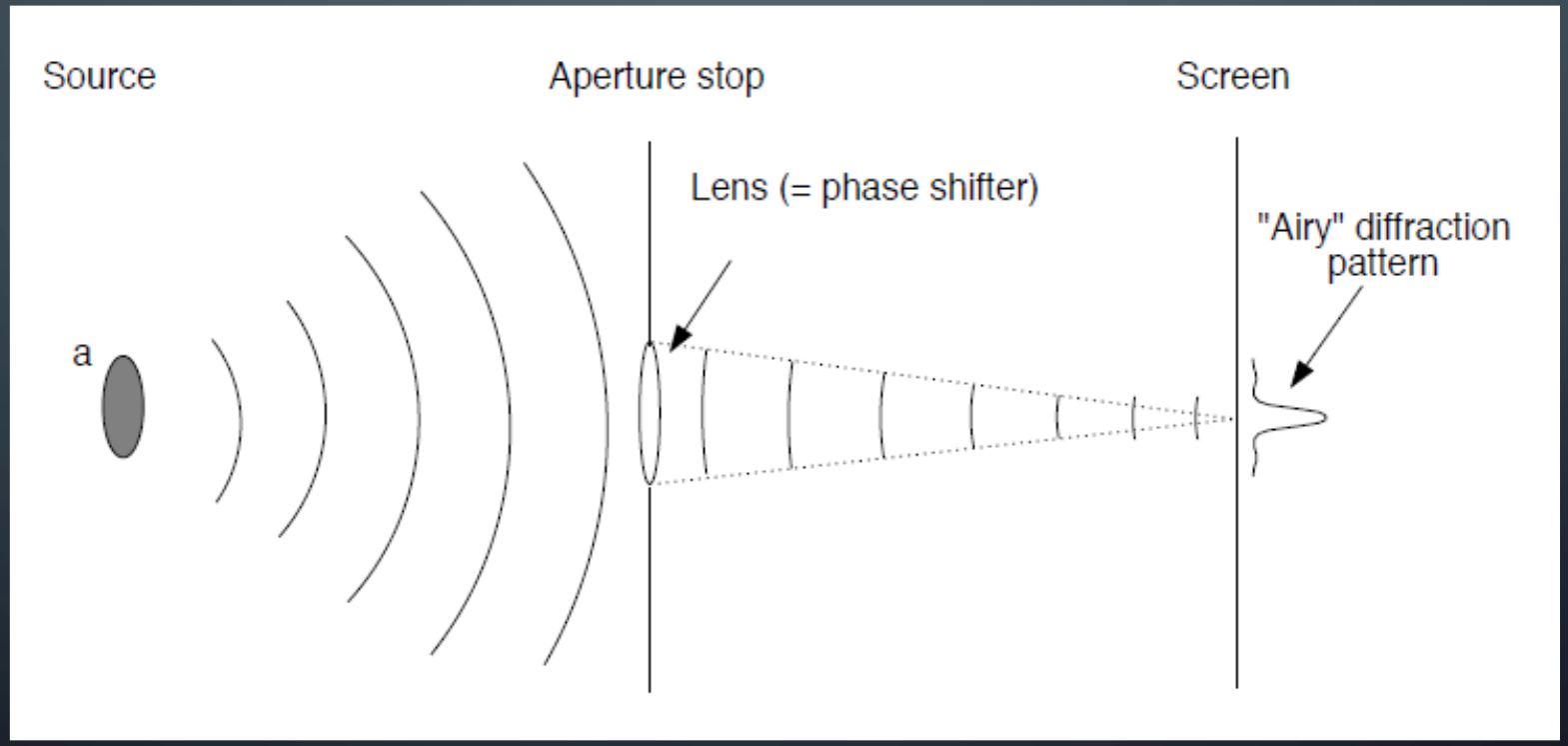
$$and \quad x_{cutoff} = 1.22 \cdot \lambda \cdot f/\#$$

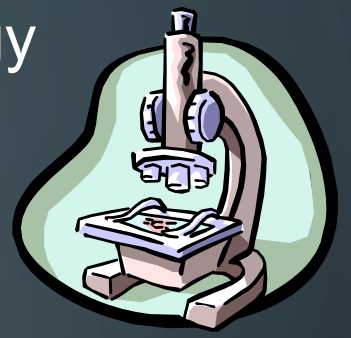


Airy disk diameter: 2.44 λ f/#



Diffraction and Point Spread Function





Diffraction and Point Spread Function

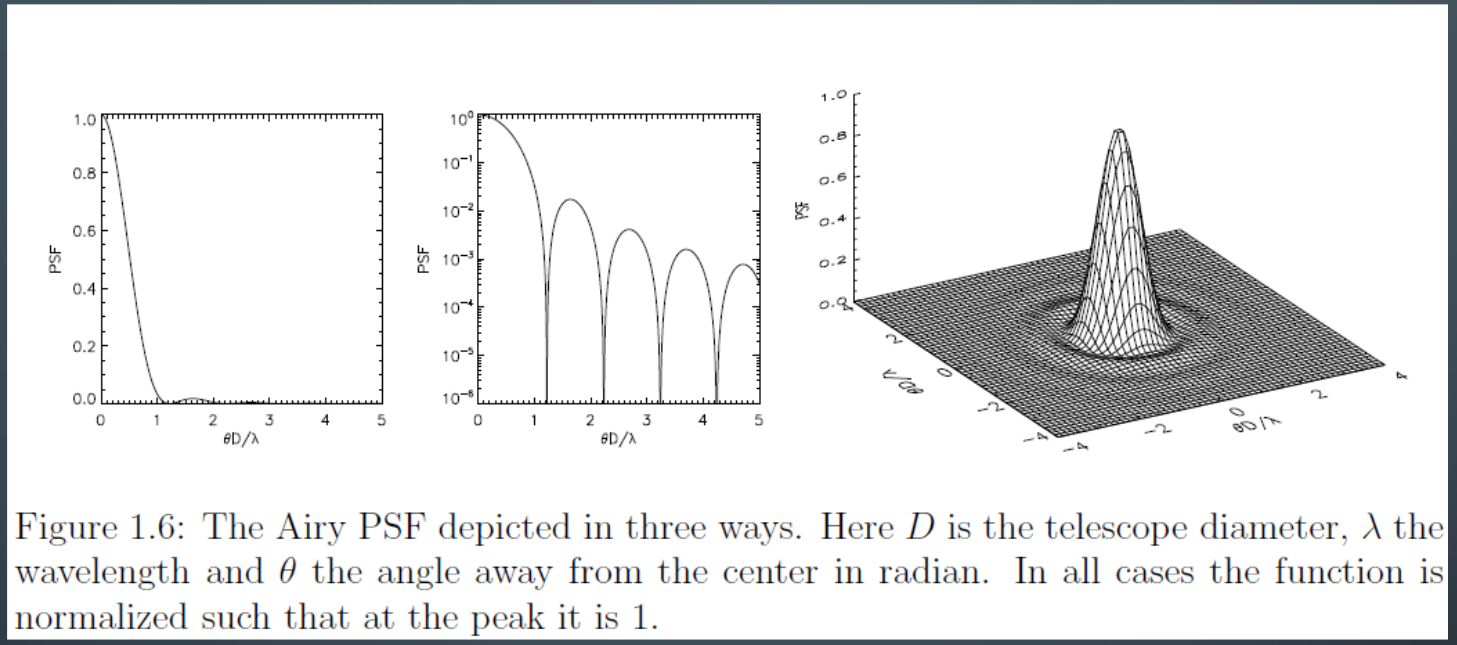
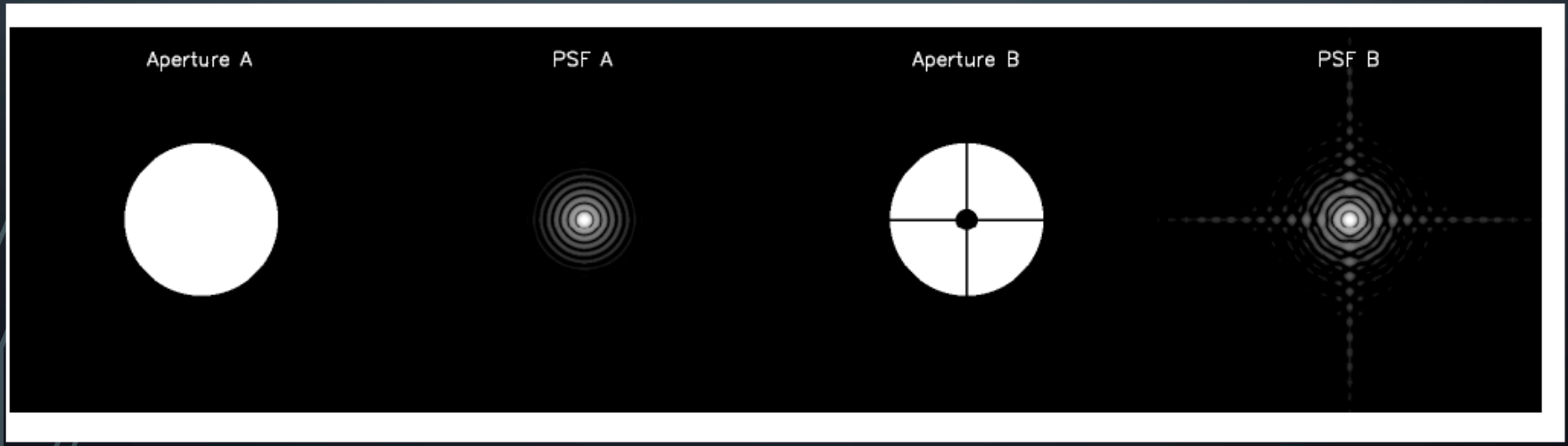
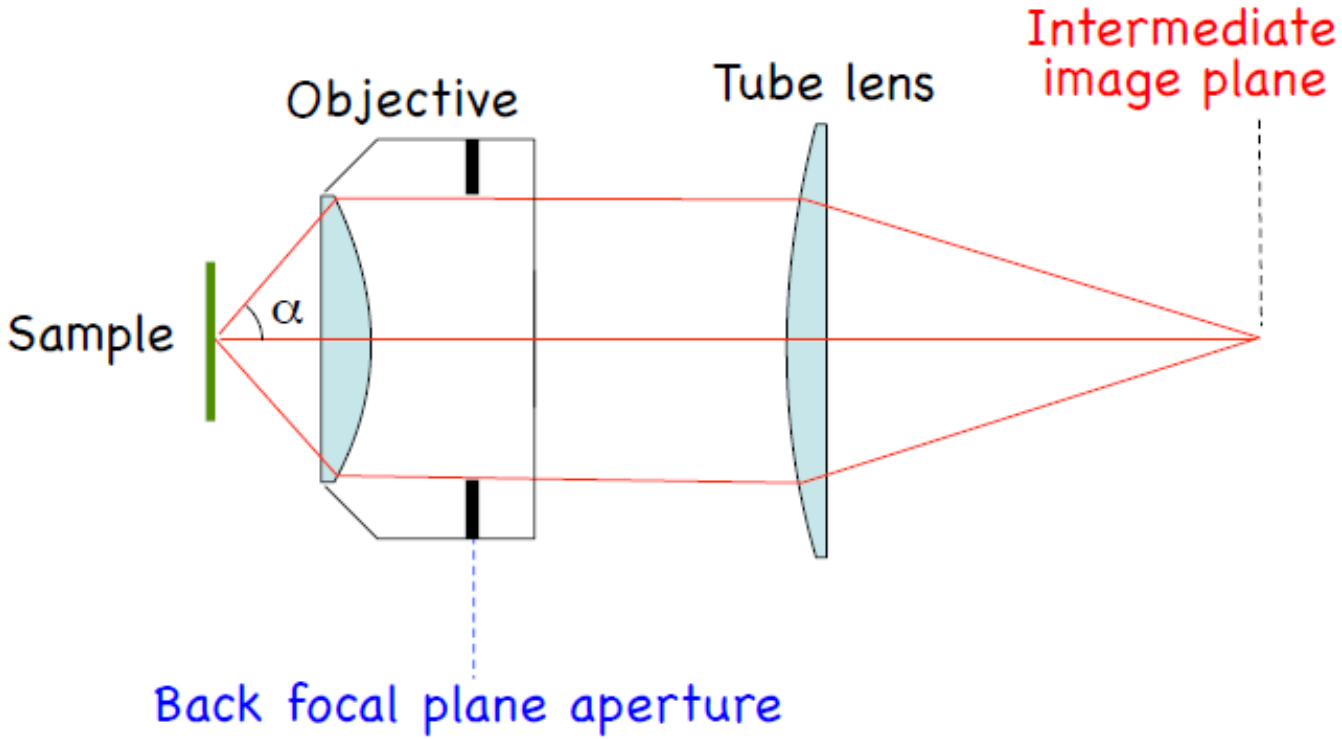


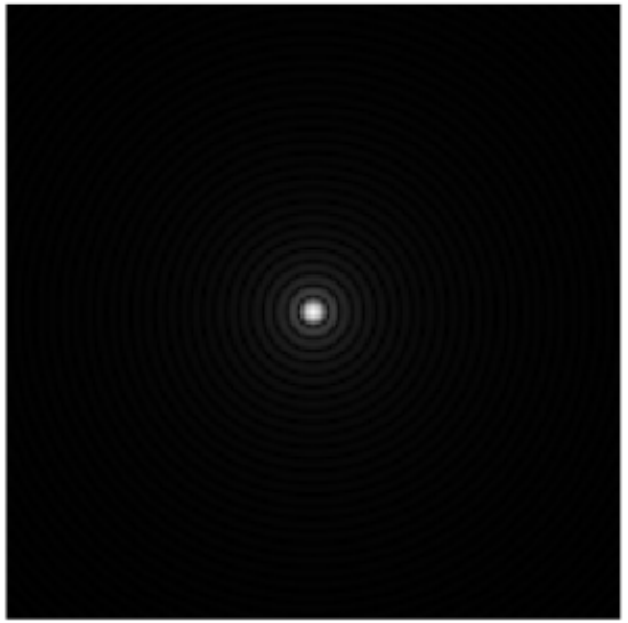
Figure 1.6: The Airy PSF depicted in three ways. Here D is the telescope diameter, λ the wavelength and θ the angle away from the center in radian. In all cases the function is normalized such that at the peak it is 1.

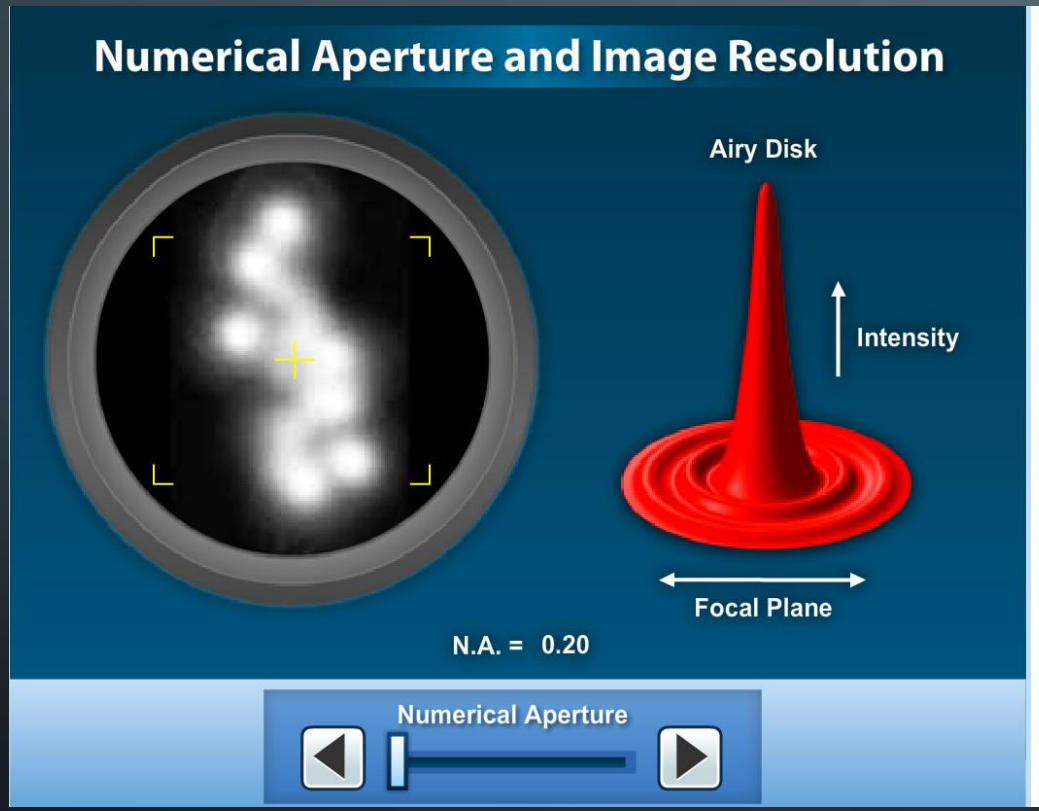


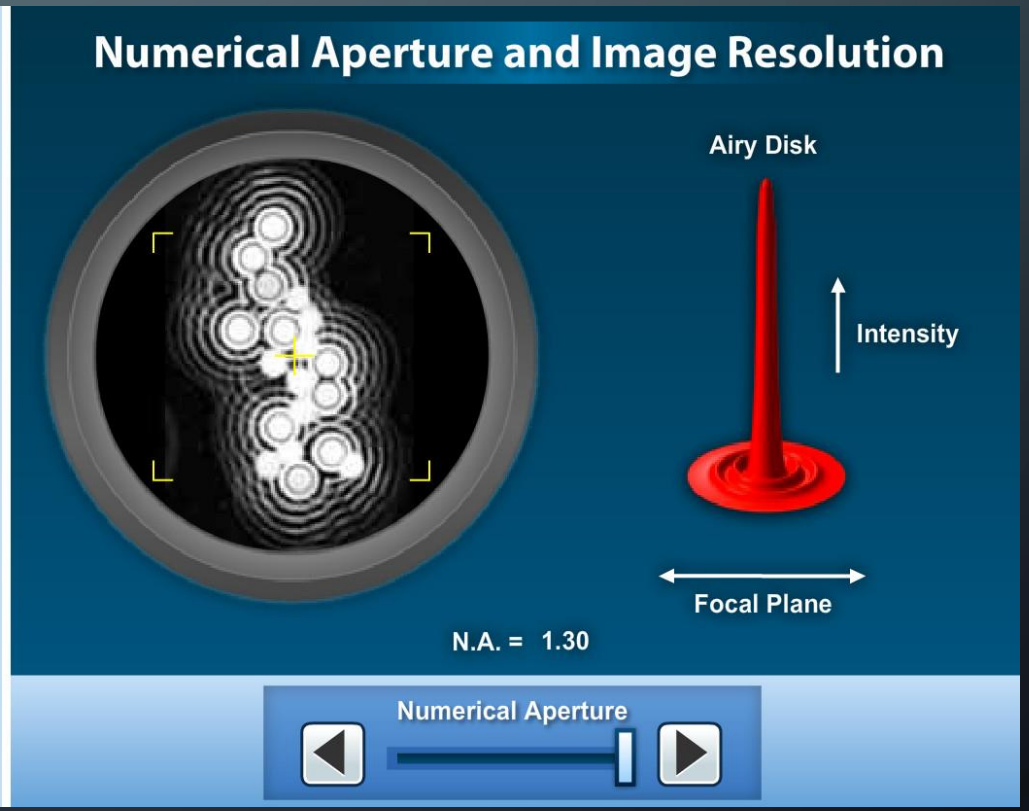
Aperture and Resolution

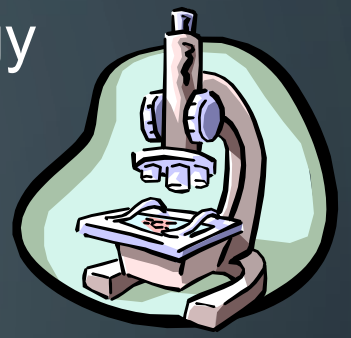


Diffraction spot
on image plane
= ***Point Spread Function***




Numerical Aperture and Image Resolution
Airy Disk
Intensity
Focal Plane
N.A. = 0.20
Numerical Aperture


Numerical Aperture and Image Resolution
Airy Disk
Intensity
Focal Plane
N.A. = 1.30
Numerical Aperture



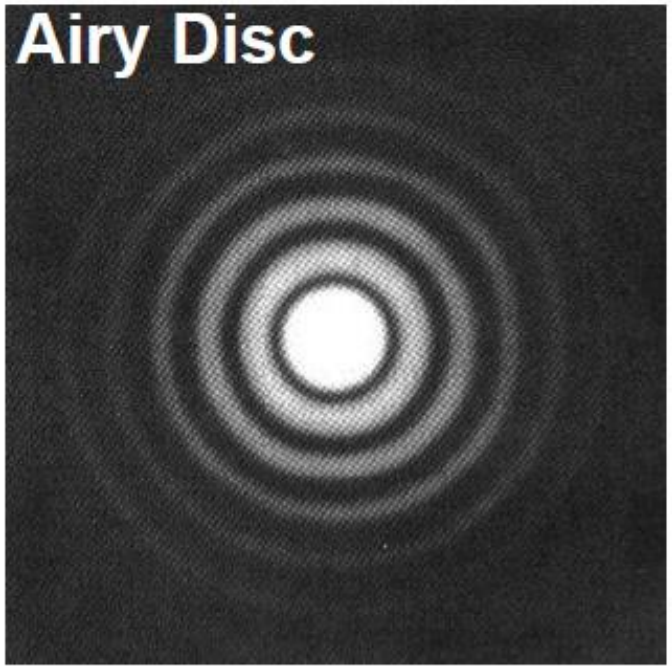
Diffraction and Point Spread Function

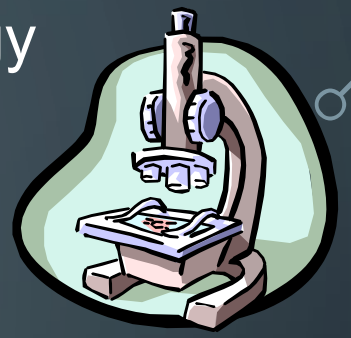
The Point Spread Function

The PSF for a perfect optical system is not a point, but is made up a core surrounded by concentric rings of diminishing intensity

It is called the Airy disc.

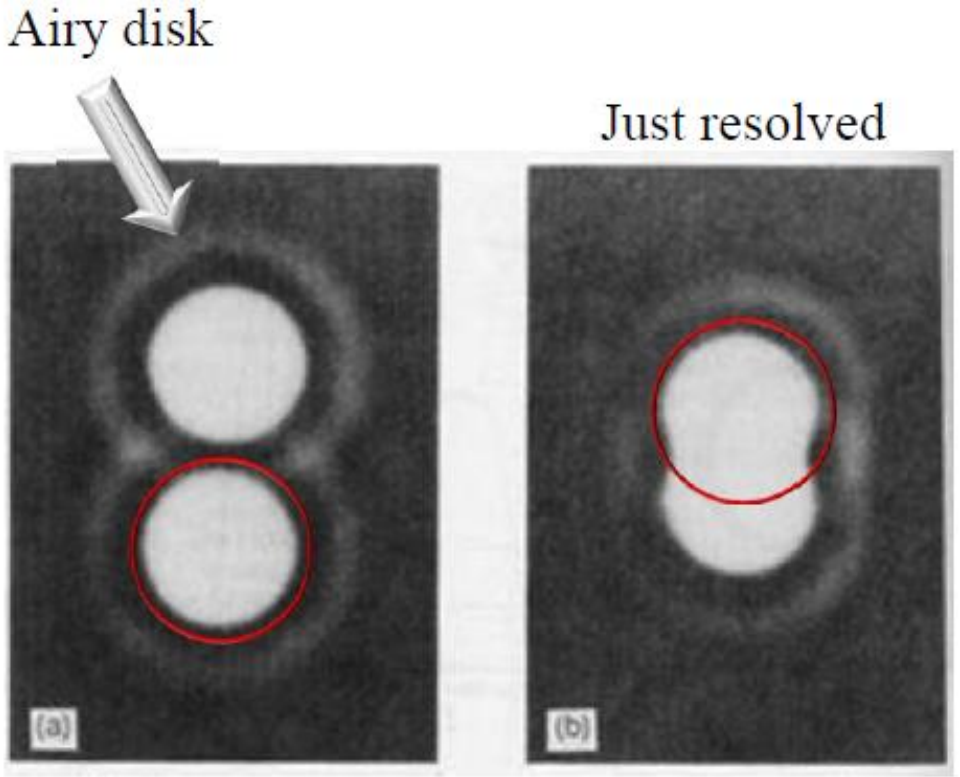
It is called the Airy disc.

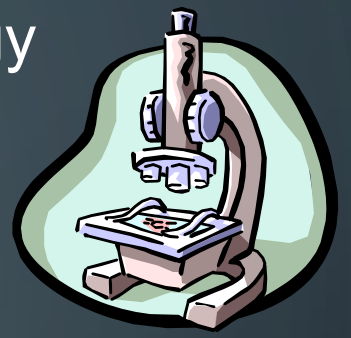




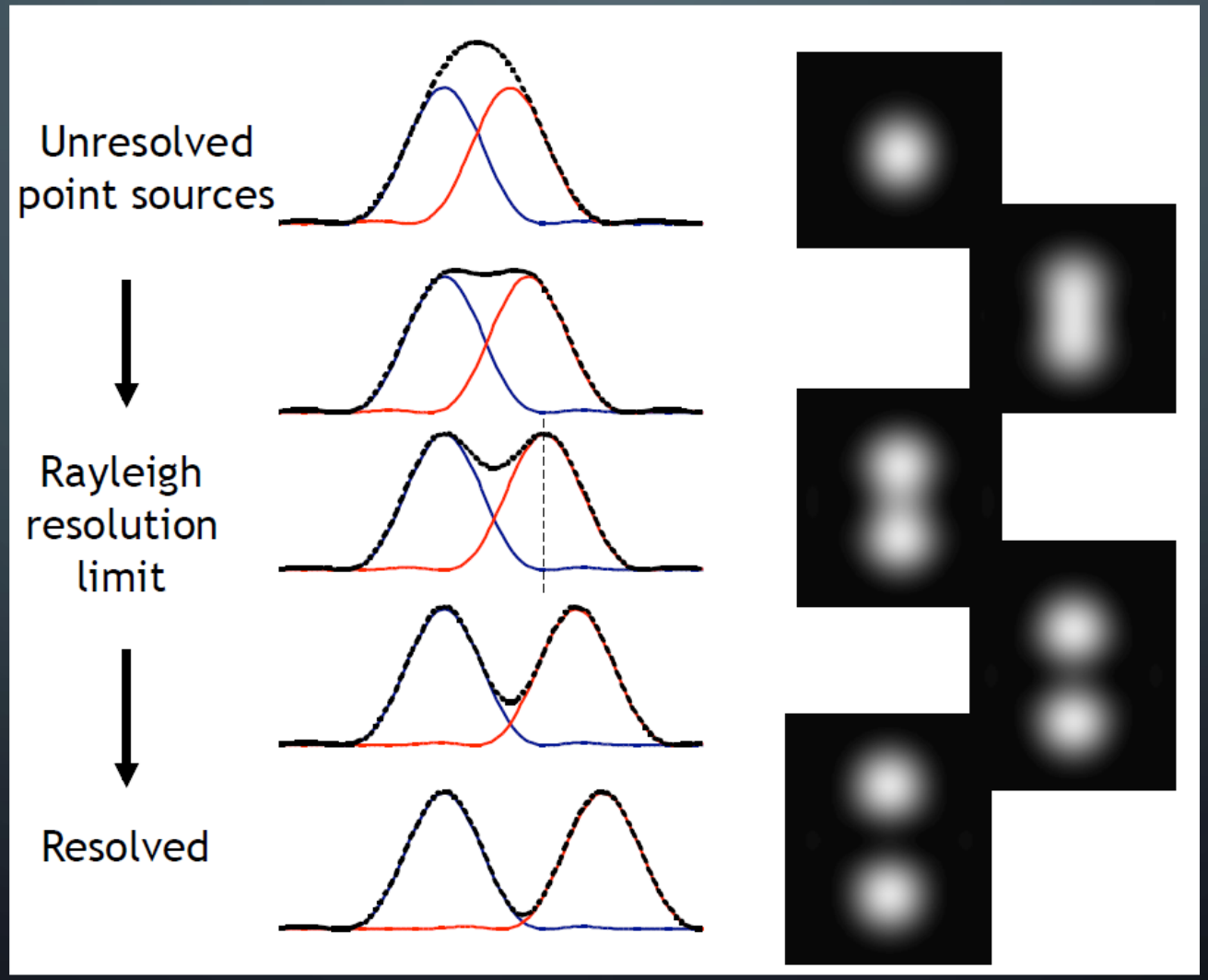
Angular resolution criterion for diffraction

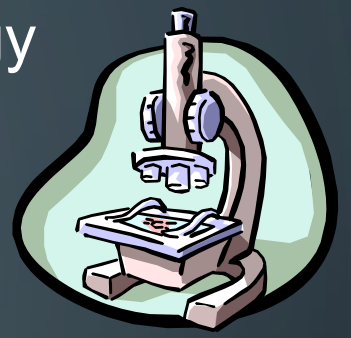
- **Rayleigh criterion** is a measure of spatial resolution
- Two point sources are "just resolved" when the diffraction maximum of one image coincides with the first minimum of the other





Resolution


Unresolved
point sources
Rayleigh
resolution
limit
Resolved



The Concept of Magnification

Magnification of the Microscope

- M Microscope = M Objective X M Eyepiece X M Intermediate Factor

M = Magnification

- Example: Objective = 60 x

  Eyepiece = 10 x

  Intermediate Factor = 1 x

  Overall M = 600 x





Objectives



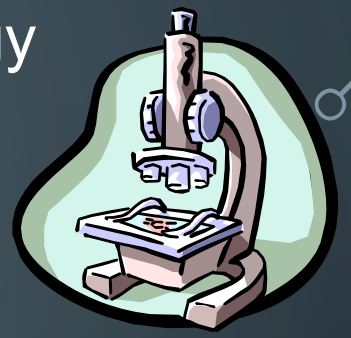
Figure 1



Objectives



Figure 1



Objectives & Co

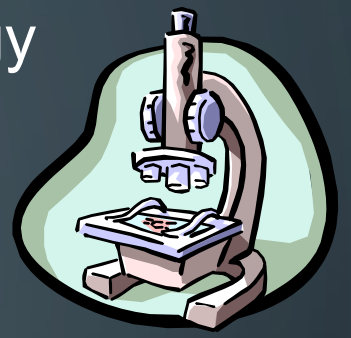
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http://www.microscopyu.com/tutorials/java/objectives/nuaperture/index.html

http://www.microscopyu.com/tutorials/java/objectives/immersion/index.html

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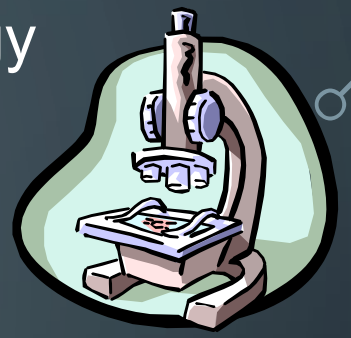


The characteristics of objectives



Figure 1





YOUR FRIEND - THE OBJECTIVE

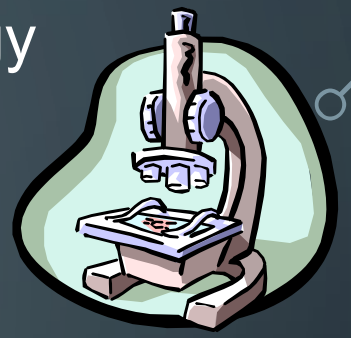
Objectives can be classified into transmitted light and reflected-light (Epi) versions.



Figure 1



Figure 1



OBJECTIVE TYPES

- CP-Achromat

  Good colour correction – exactly for two wavelengths. Field flatness in the image center, refocusing also covers the peripheral areas. For fields of view up to dia. 18 mm. Versions for phase contrast.

- Achroplan

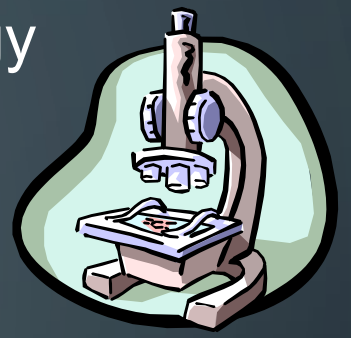
  Improved Achromat objectives with good image flatness for fields of view with dia. 20 or even 23 mm. Achroplan for transmitted light and Achroplan Ph for phase contrast.

- Plan-Neofluar

  Excellent colour correction for at least three wavelengths. Field flattening for the field of view with dia. 25 mm. Highly transmitting for UV excitation at 365 nm in fluorescence. All methods possible, special high-quality variants are available for Pol and DIC.

- Plan-Apochromat

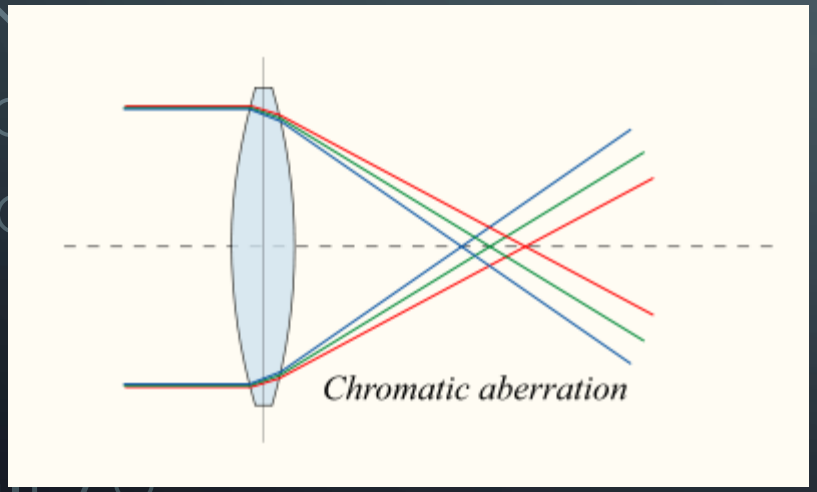
  Perfect colour rendition (correction for four wavelengths!). Flawless image flatness for fields of view with dia. 25 mm. Highest numerical apertures for a resolving power at the very limits of the physically possible.

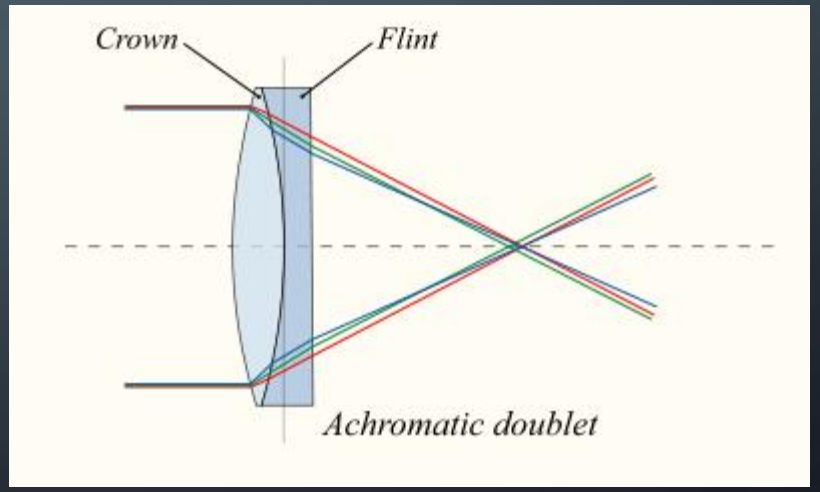


CHROMATIC ABERRATION

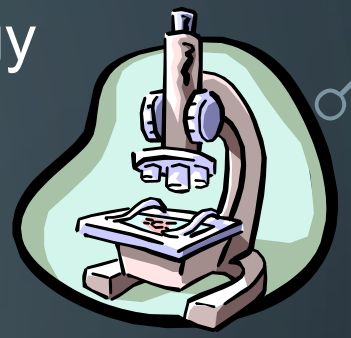


Chromatic aberration is caused by a lens having different refractive indexes for different wavelengths. Since the focal length of a lens is dependent on the refractive index, different wavelengths will be focused on different positions in the focal plane.
Chromatic aberration is seen as fringes of colour around the image.





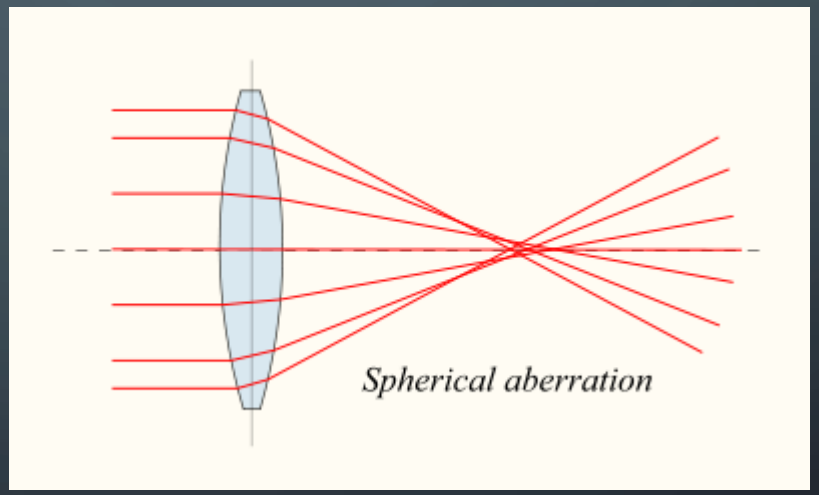
It can be minimised by using an achromatic doublet (= achromat) in which two materials with differing dispersion are bonded together to form a single lens.

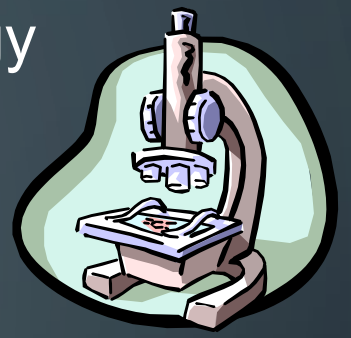




SPHERICAL ABERRATION

Spherical aberration causes beams parallel to but away from the lens axis to be focussed in a slightly different place than beams close to the axis. This manifests itself as a blurring of the image.





The characteristics of objectives



Figure 1





Numerical Aperture (N.A.)

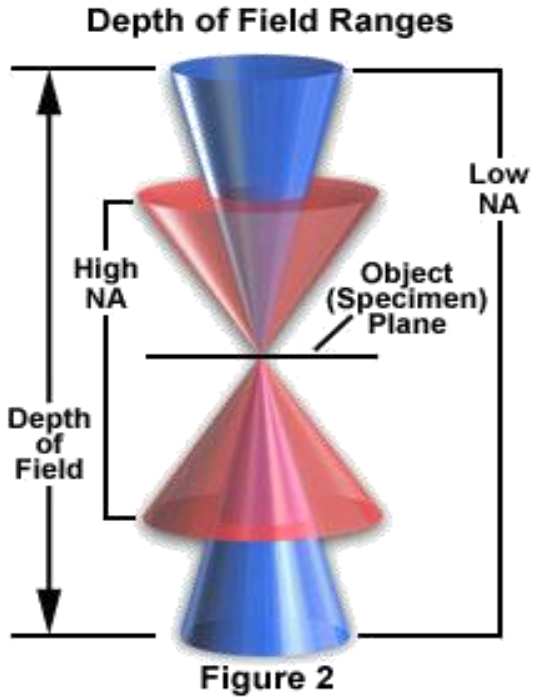


Figure 2

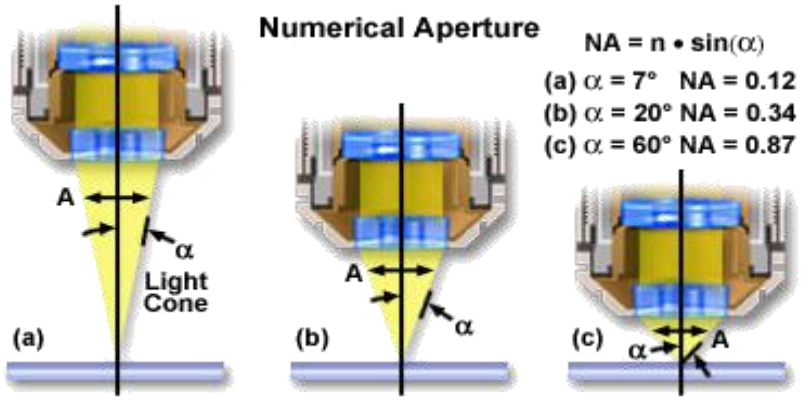


Figure 1

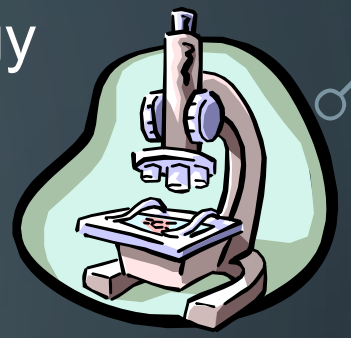
Numerical Aperture = N.A. = n · sin α

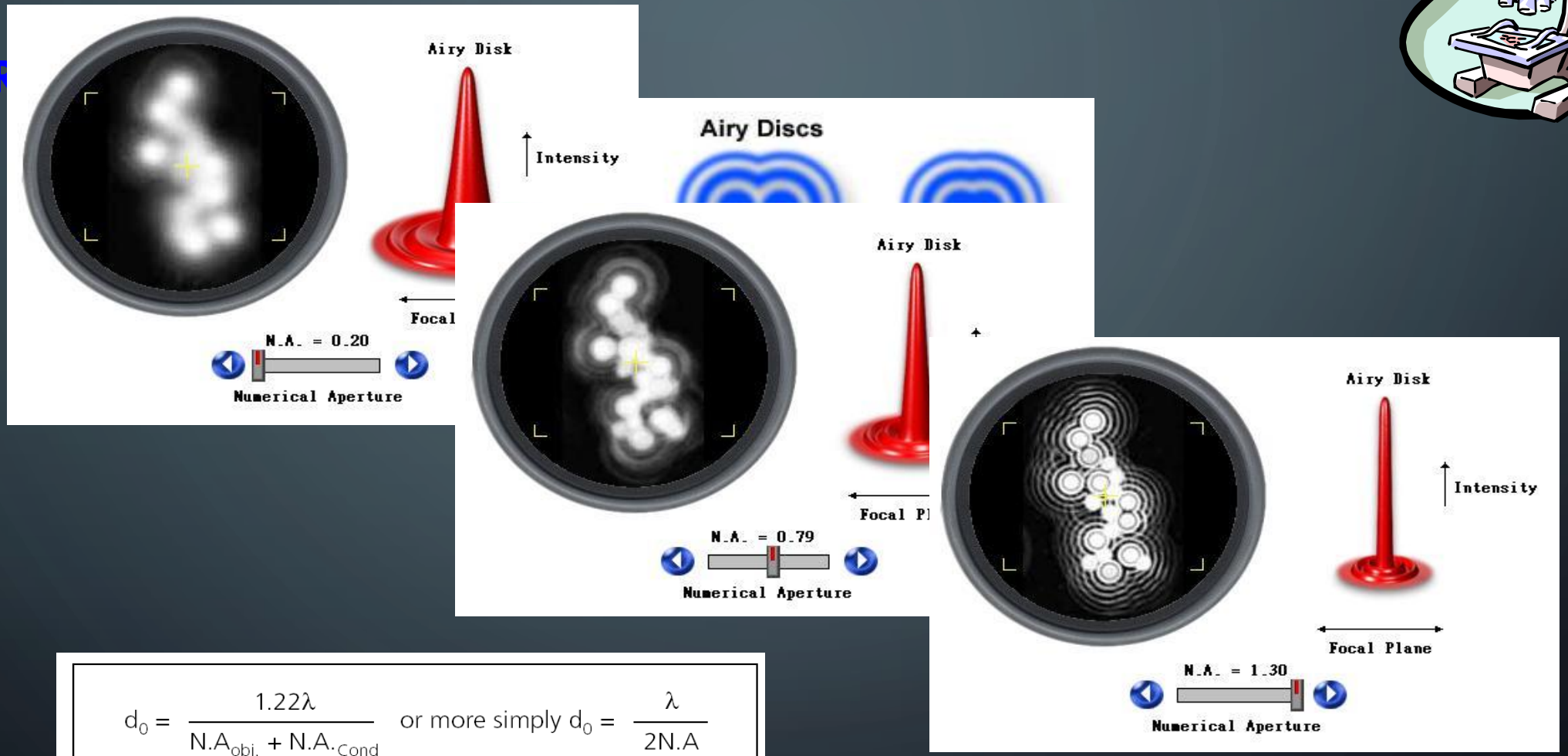
α is half the opening angle of the objective.

n is the refractive index of the immersion medium used between the objective and the object.

(n = 1 for air; n = 1.51 for oil or glass)

2022 - Light microscopy in Cellular Biology

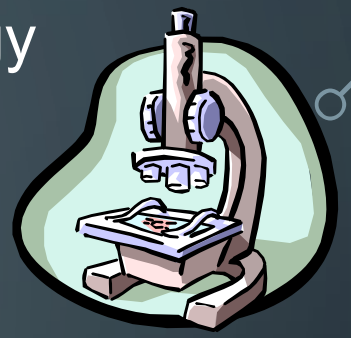




$$d_0 = \frac{1.22\lambda}{N.A_{obj.} + N.A._{Cond}} \quad \text{or more simply } d_0 = \frac{\lambda}{2N.A}$$

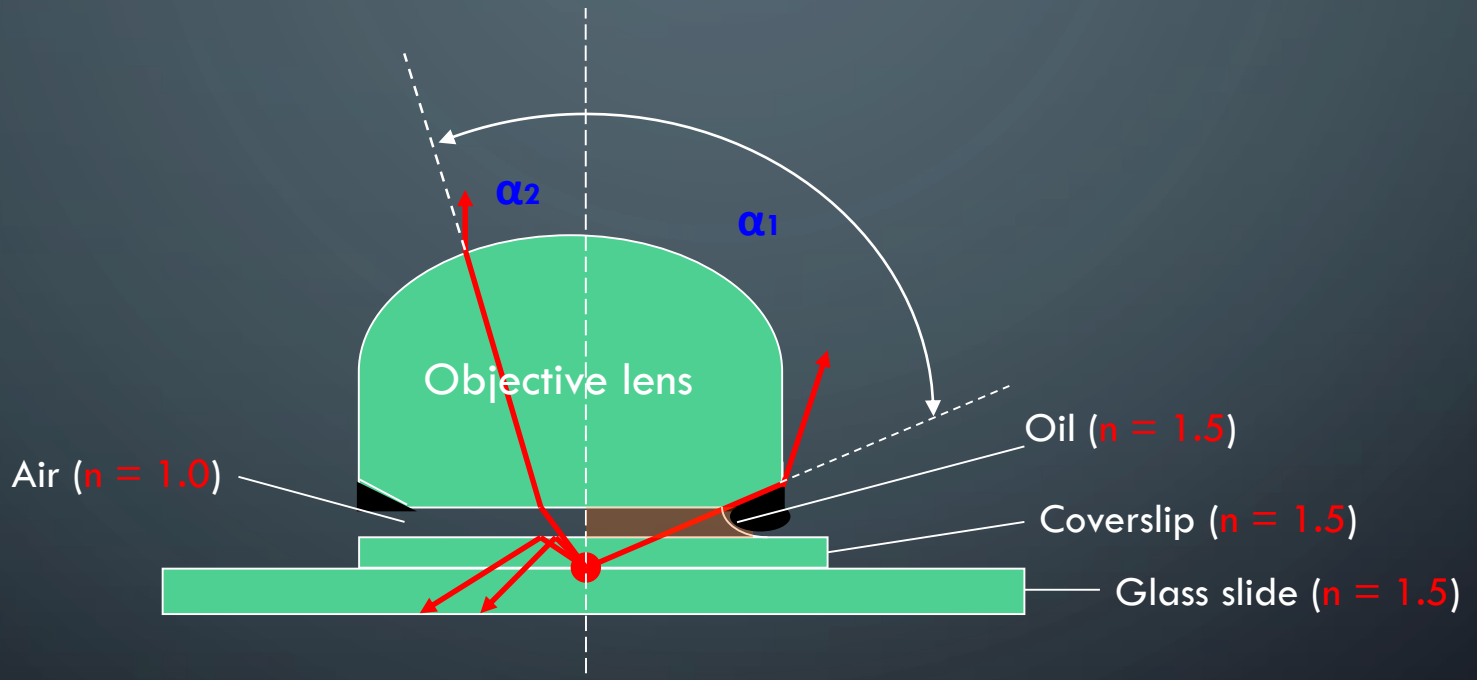
λ = wavelength of light, e.g. 550 nm (green)

- **Resolving power, the limit up to which two small objects are still seen separately.**



What is the numerical aperture?

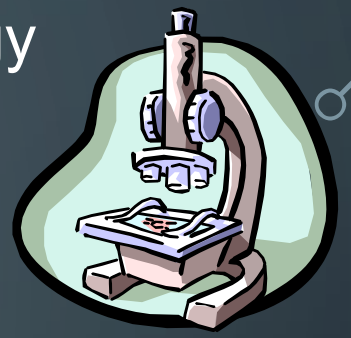
NA is an estimate of how much light from the sample is collected by the objective



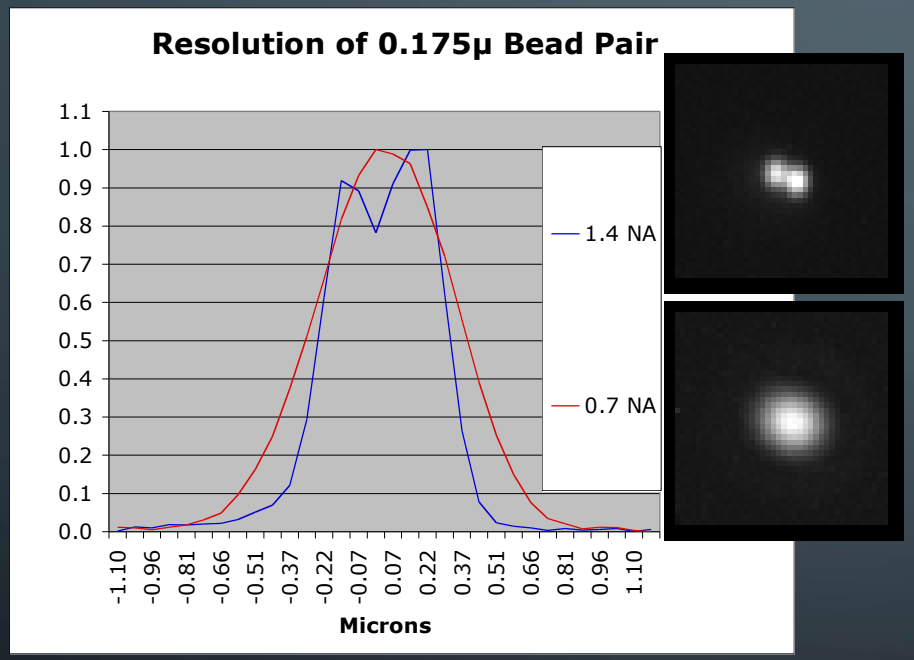
$$NA = n \sin \alpha$$

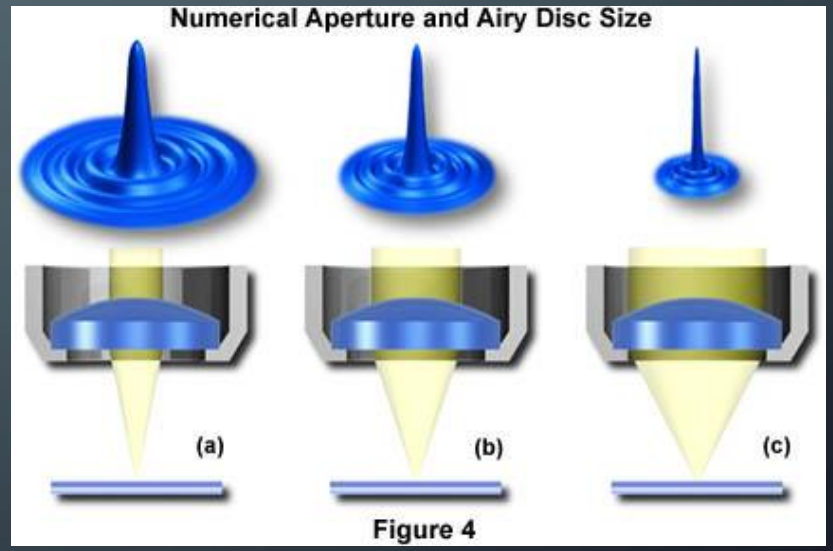
n = refractive index

α = angle of incident illumination



Numerical aperture, NOT magnification determines resolution!





Increasing NA

A lens with a larger NA will be able to visualize finer details and will also collect more light and give a brighter image than a lens with lower NA.

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



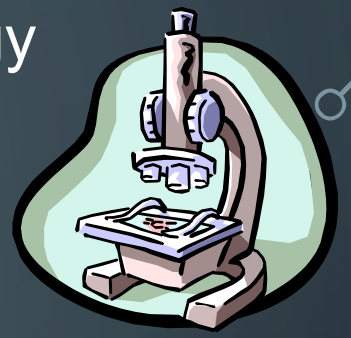
CONTRASTING TECHNIQUES

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

Contrast Generation for Transmitted Light

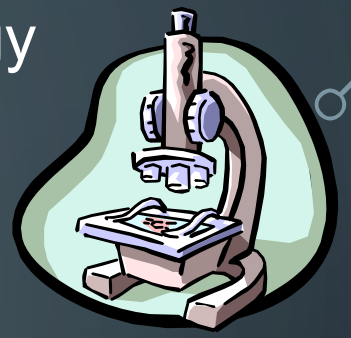
- Brightfield >>> absorbance
- Darkfield >>> diffraction
- Phase Contrast >>> Phase shift
- Differential Interference Contrast (DIC) Microscopy

>>> Phase shift / Polarization / Interference

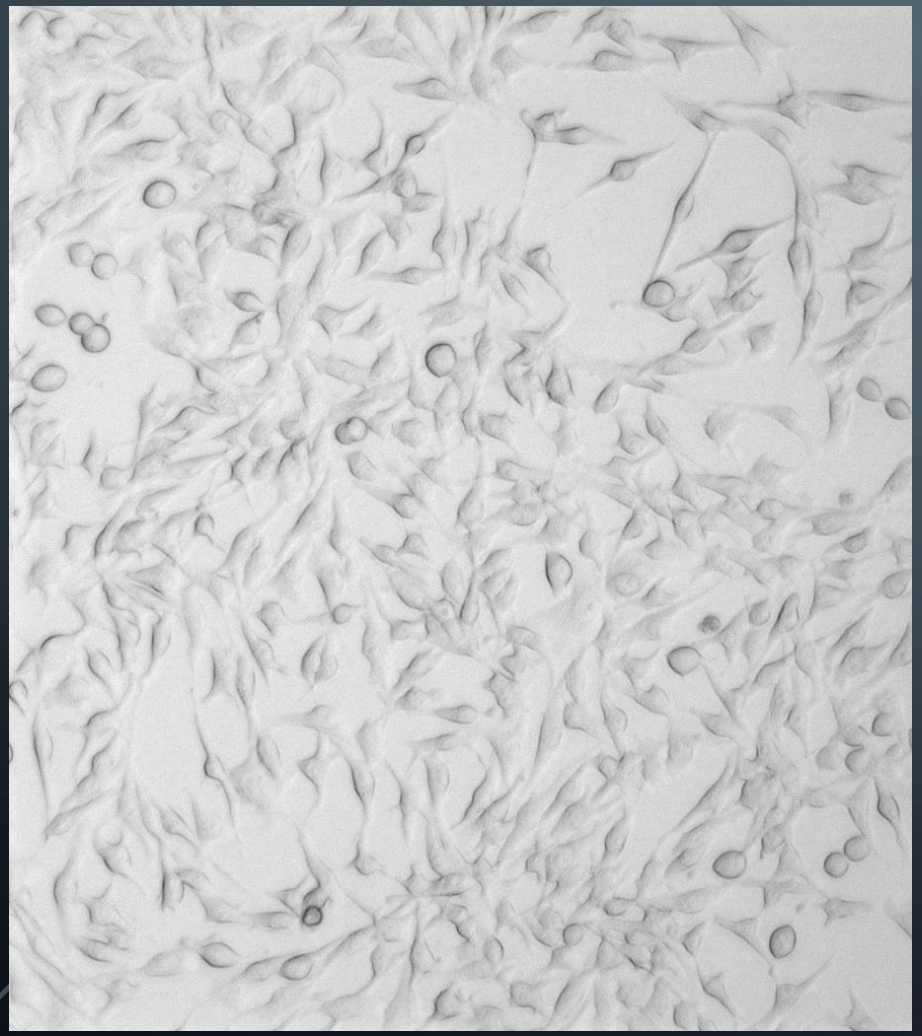


Contrast Generation for Transmitted Light

- Darkfield and Phase Contrast Microscopy
- Polarized Light and Polarization Microscopy
- Differential Interference Contrast (DIC) Microscopy



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



Which properties can be used?

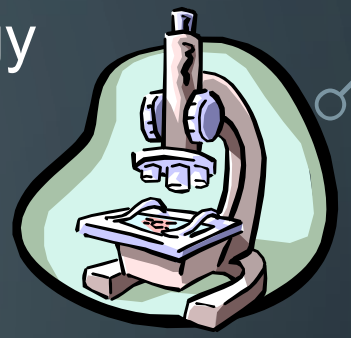
Absorption

Scattering

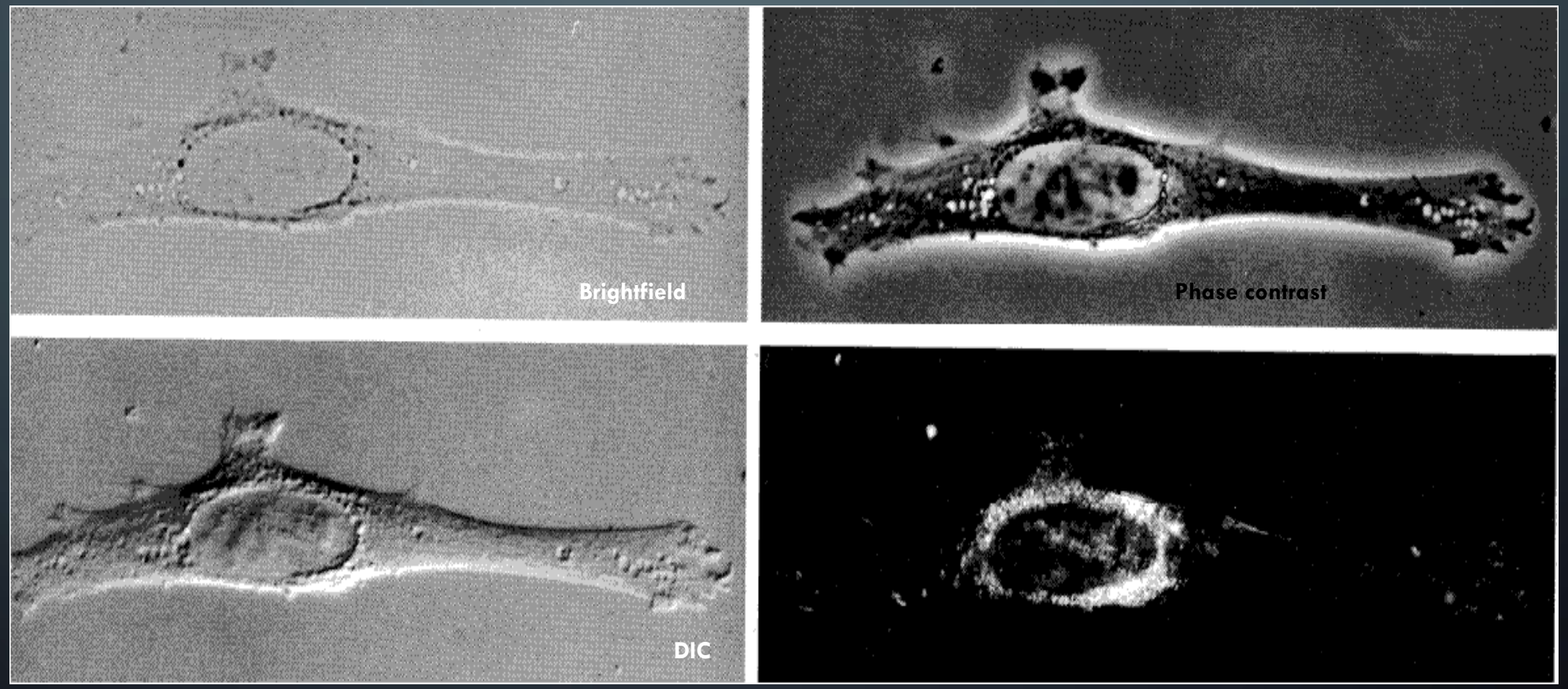
Refraction

Phase

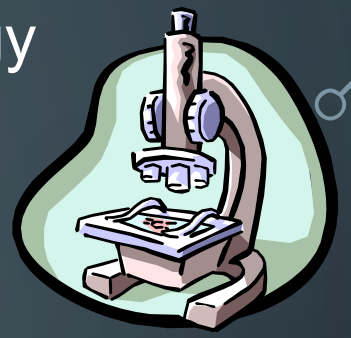
Polarization



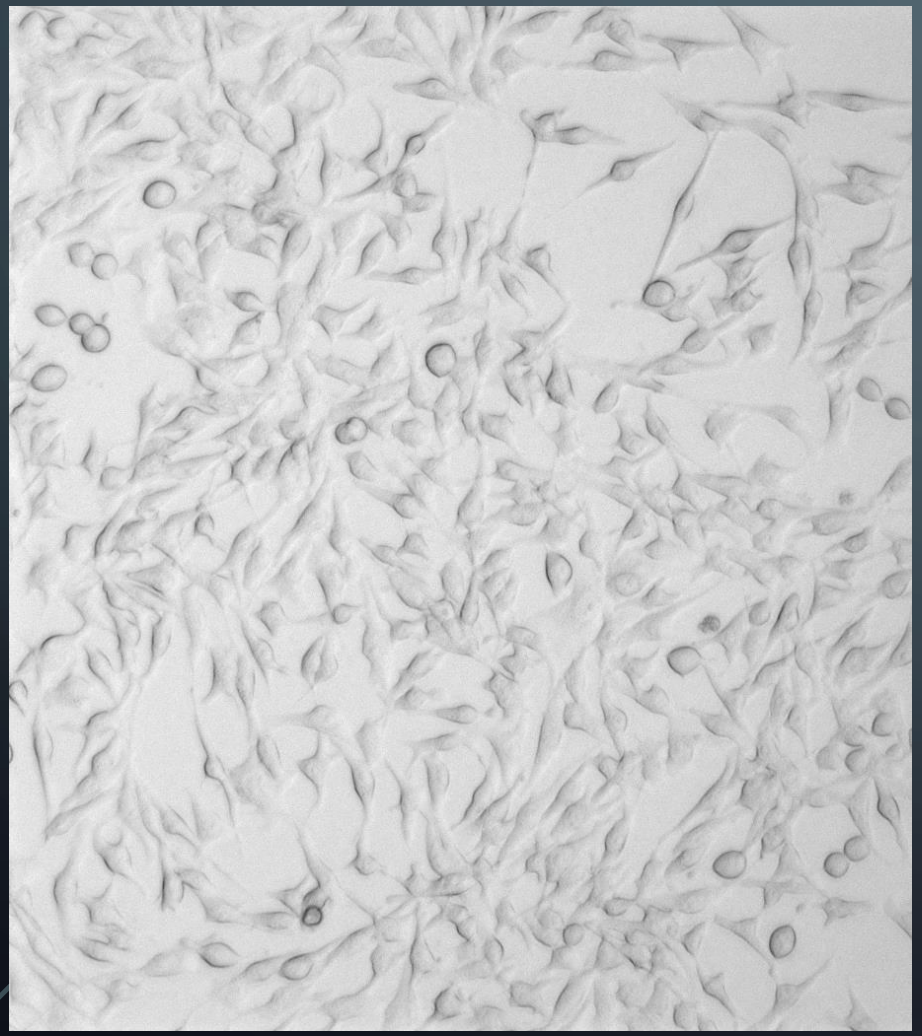
CONTRASTING TECHNIQUES



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



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



Which properties can be used?

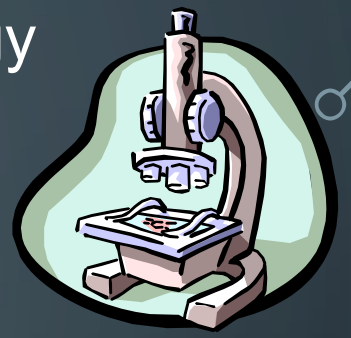
Absorption

Scattering

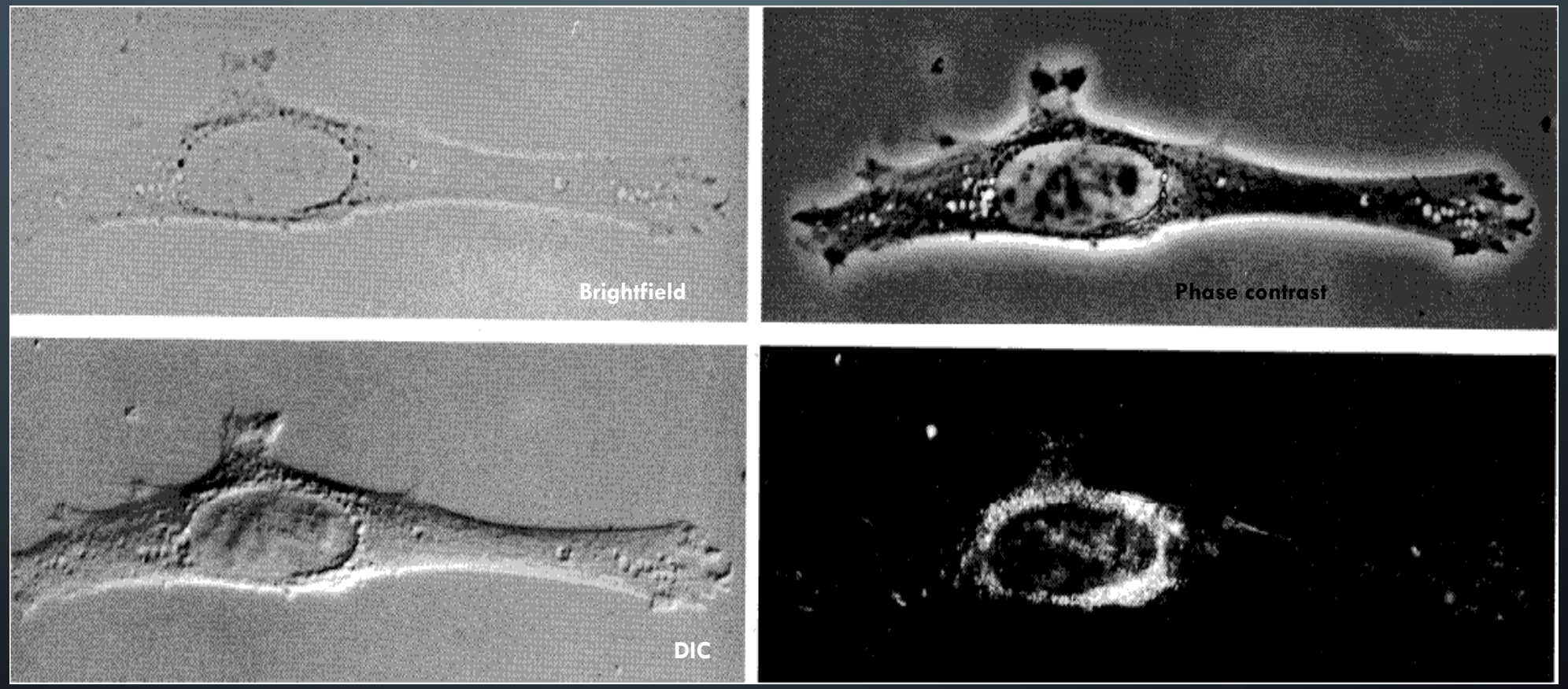
Refraction

Phase

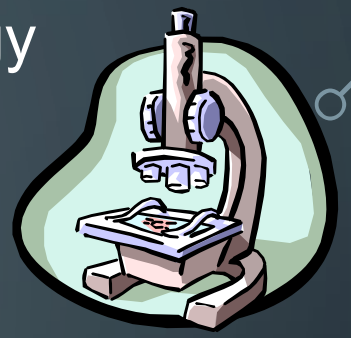
Polarization



CONTRASTING TECHNIQUES



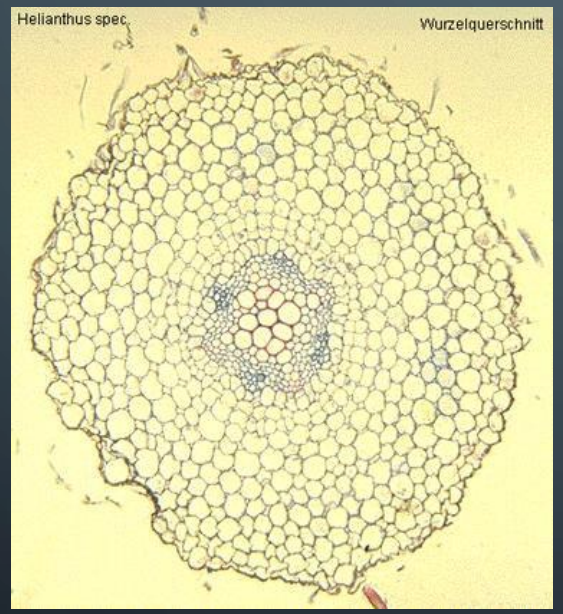
Taken from: http://fig.cox.miami.edu/~cmallery/150/Fallsyll.htm



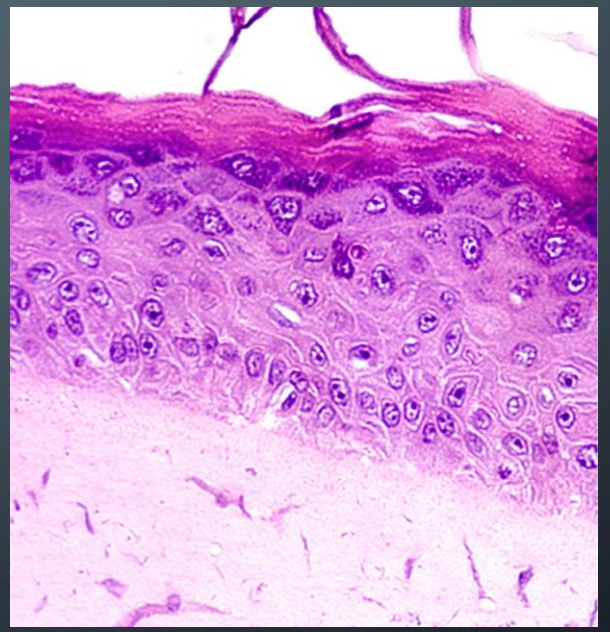
BRIGHTFIELD

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

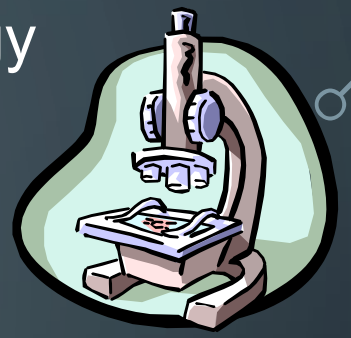
Application: Only useful for specimens that can be contrasted via dyes. Very little contrast in unstained specimens. With a bright background, the human eye requires local intensity fluctuations of at least 10 to 20% to be able to recognize objects.



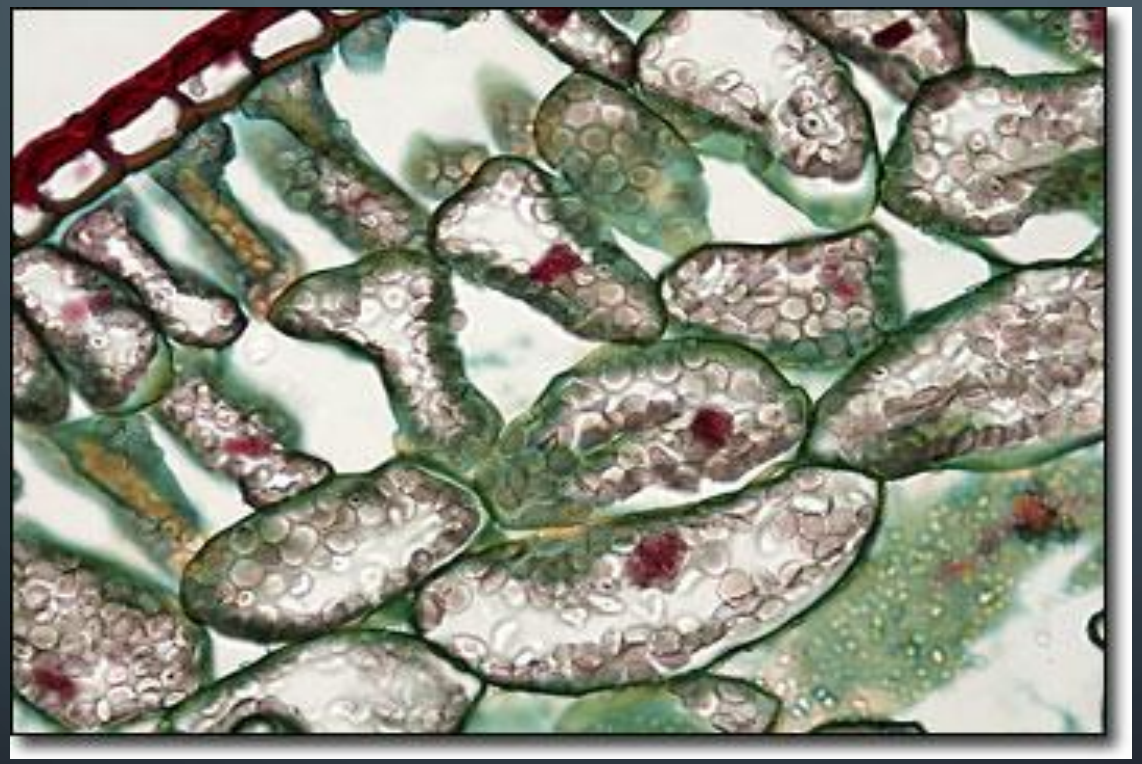
Cross section of sunflower root
(http://www.zum.de/Faecher/Materialien/beck/12/bs12-5.htm)



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



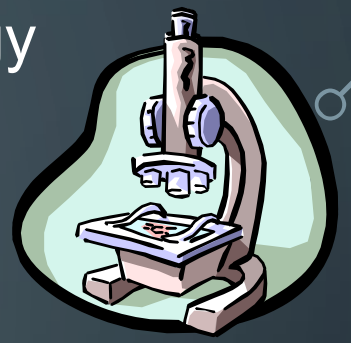
Bright Field



- 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

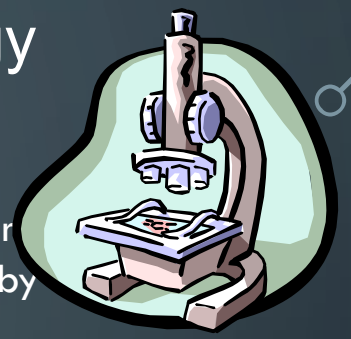




- Fine structures can often not be seen in front of a bright background.

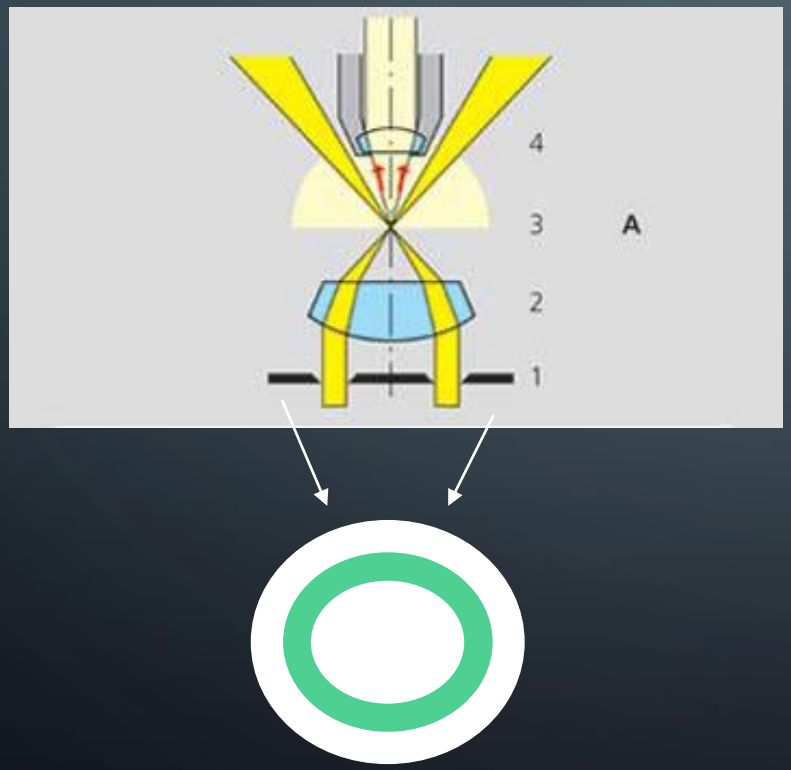
2022 - Light microscopy in Cellular Biology

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







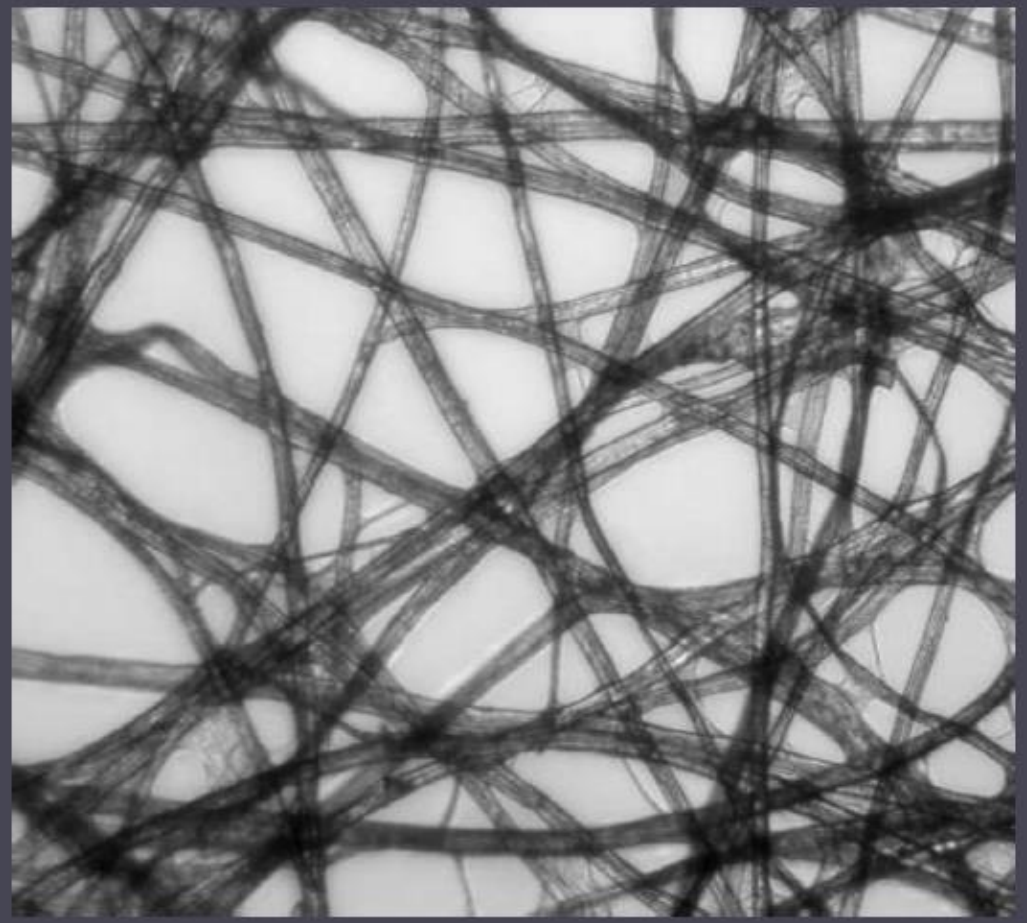
Symbiotic Diatom colony

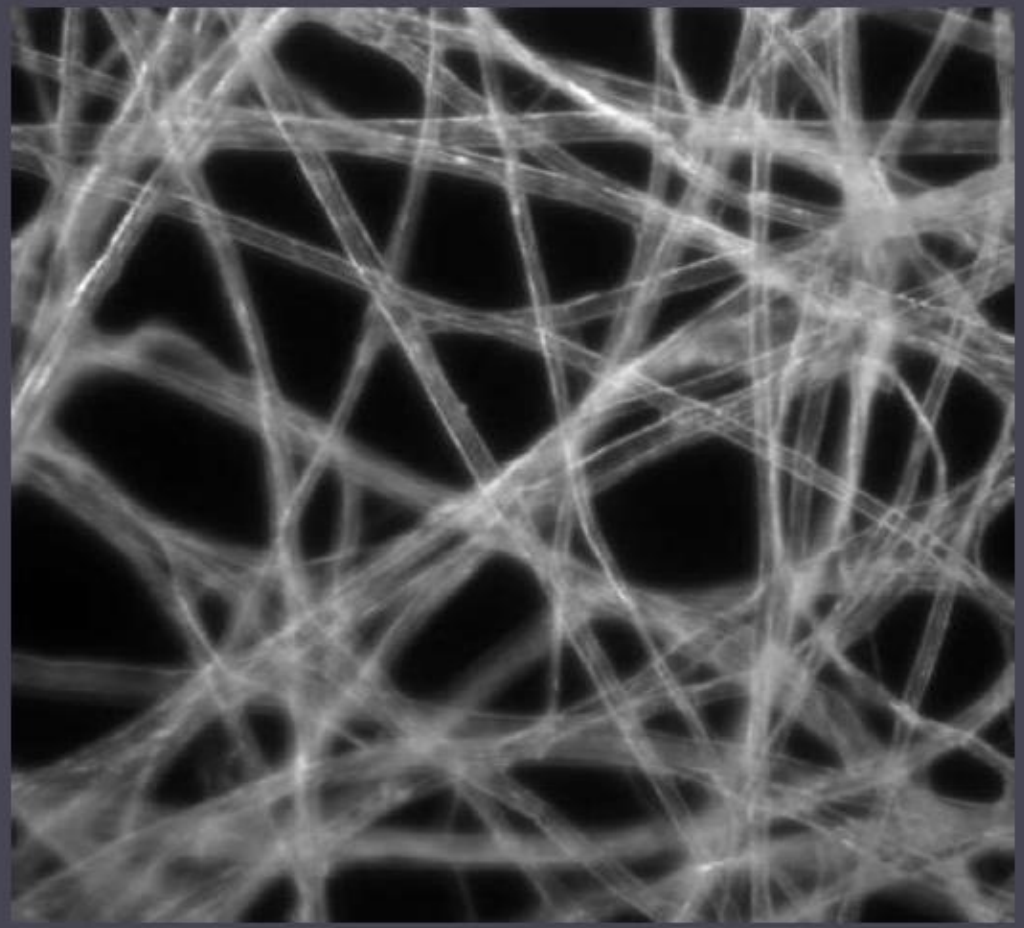
(www1.tip.nl/~t936927/making.html)

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

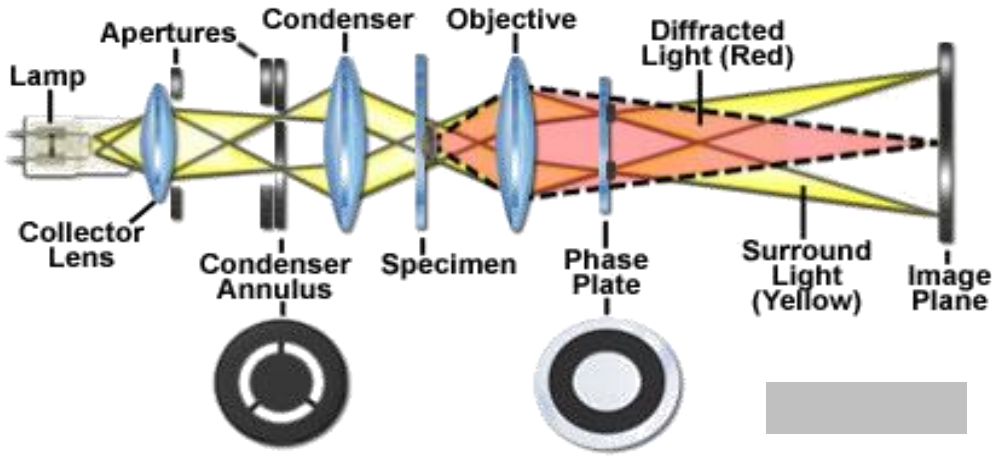
Dark-Field Microscopy

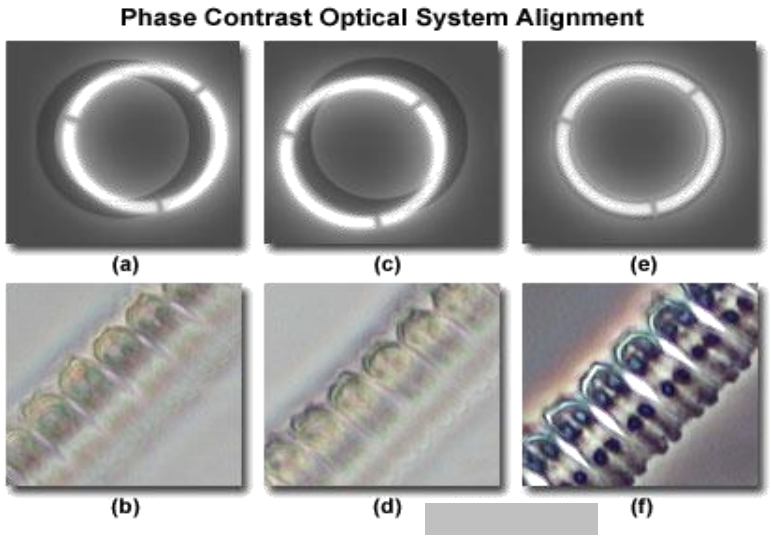
- Dark background against which objects are brilliantly illuminated.
- This is accomplished by equipping with a special condenser that transmits a hollow cone of light.
- Most of the light directed through the condenser does not enter the objective, the field is dark.
- However, some of the light rays 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

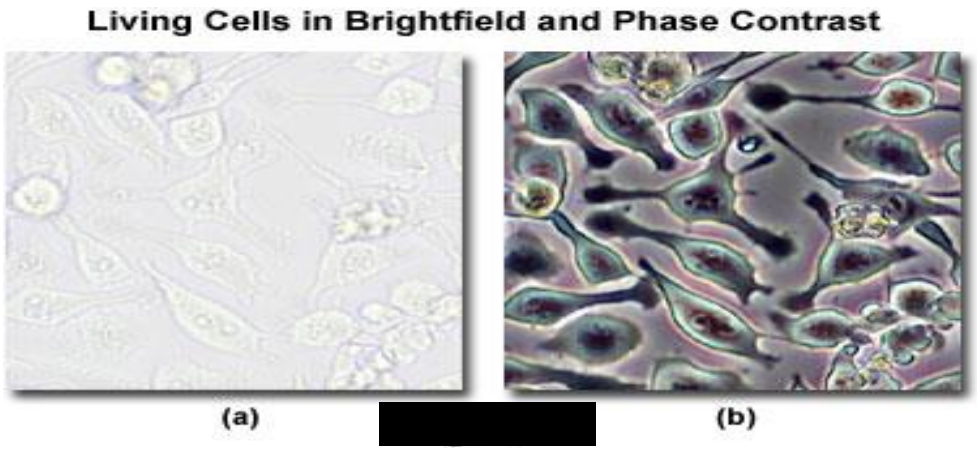




Phase Contrast

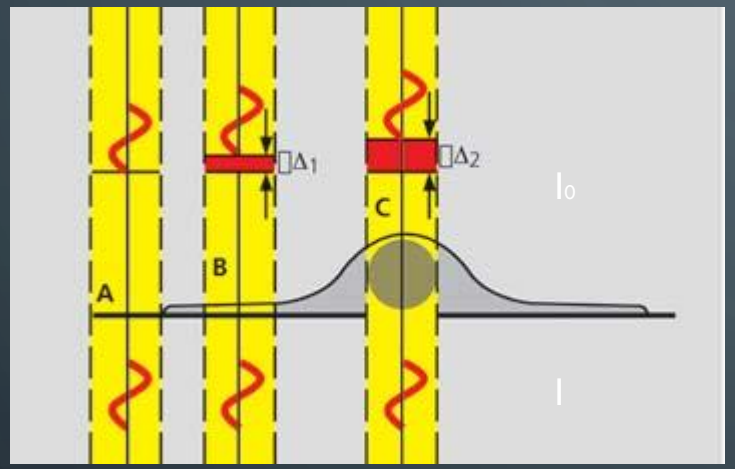


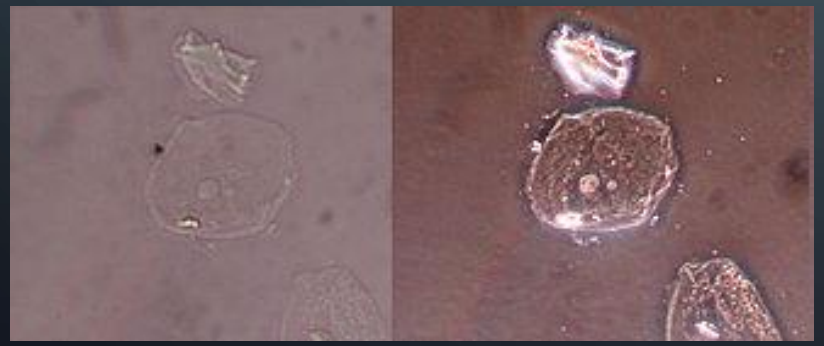


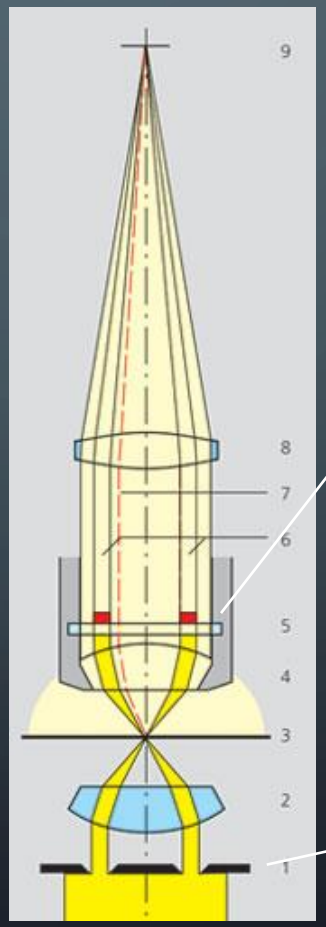


PHASE CONTRAST IN THEORY

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

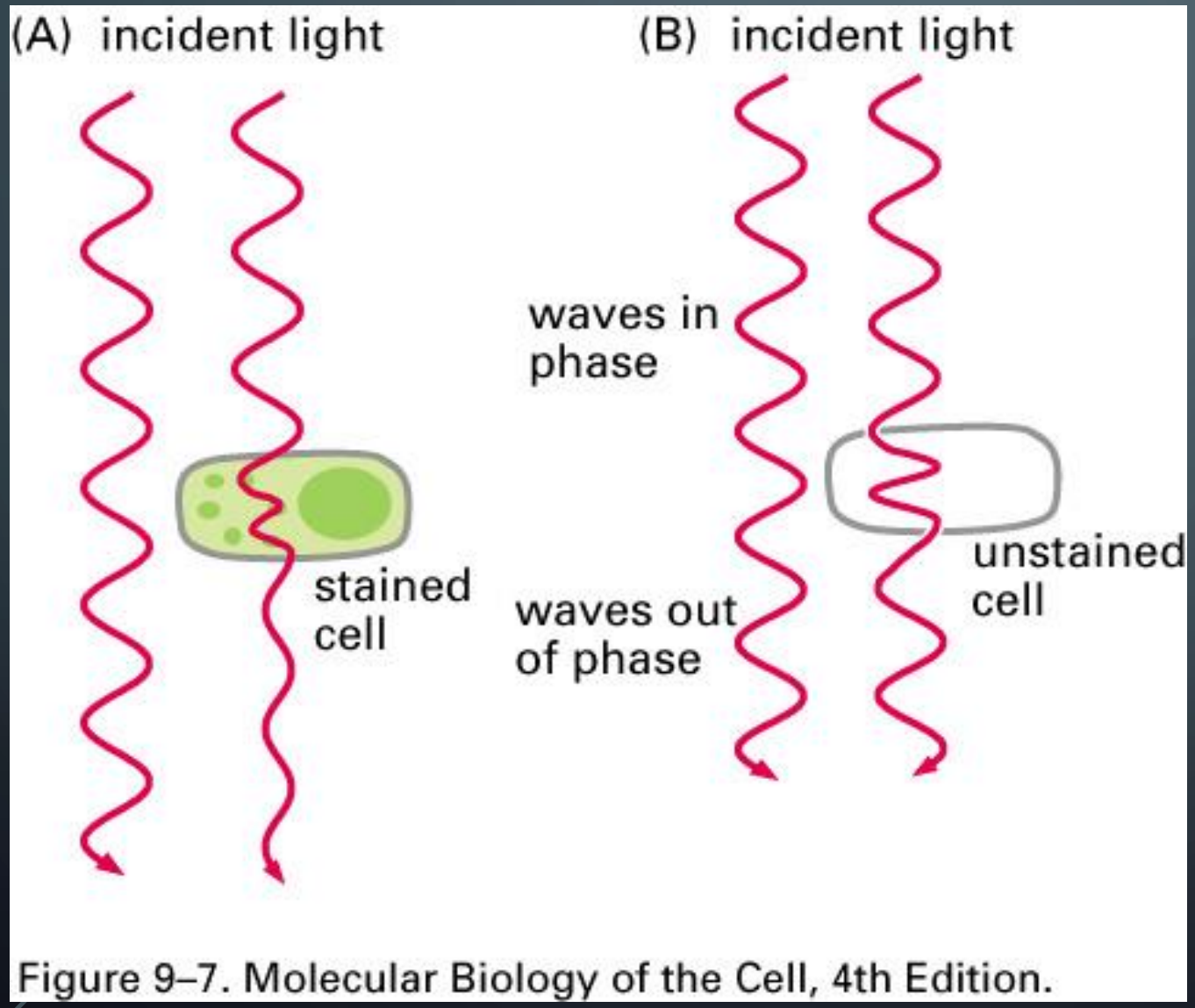
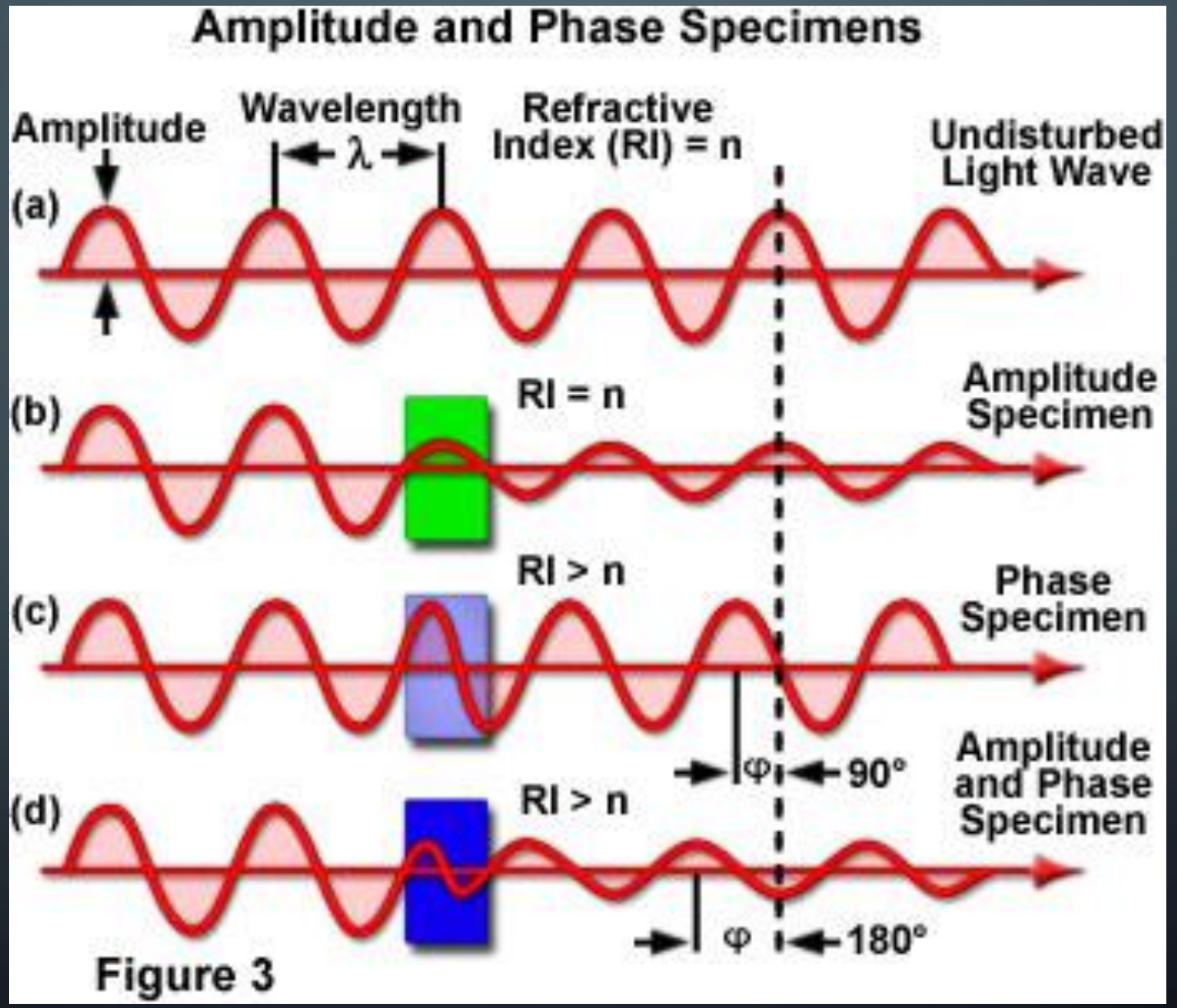


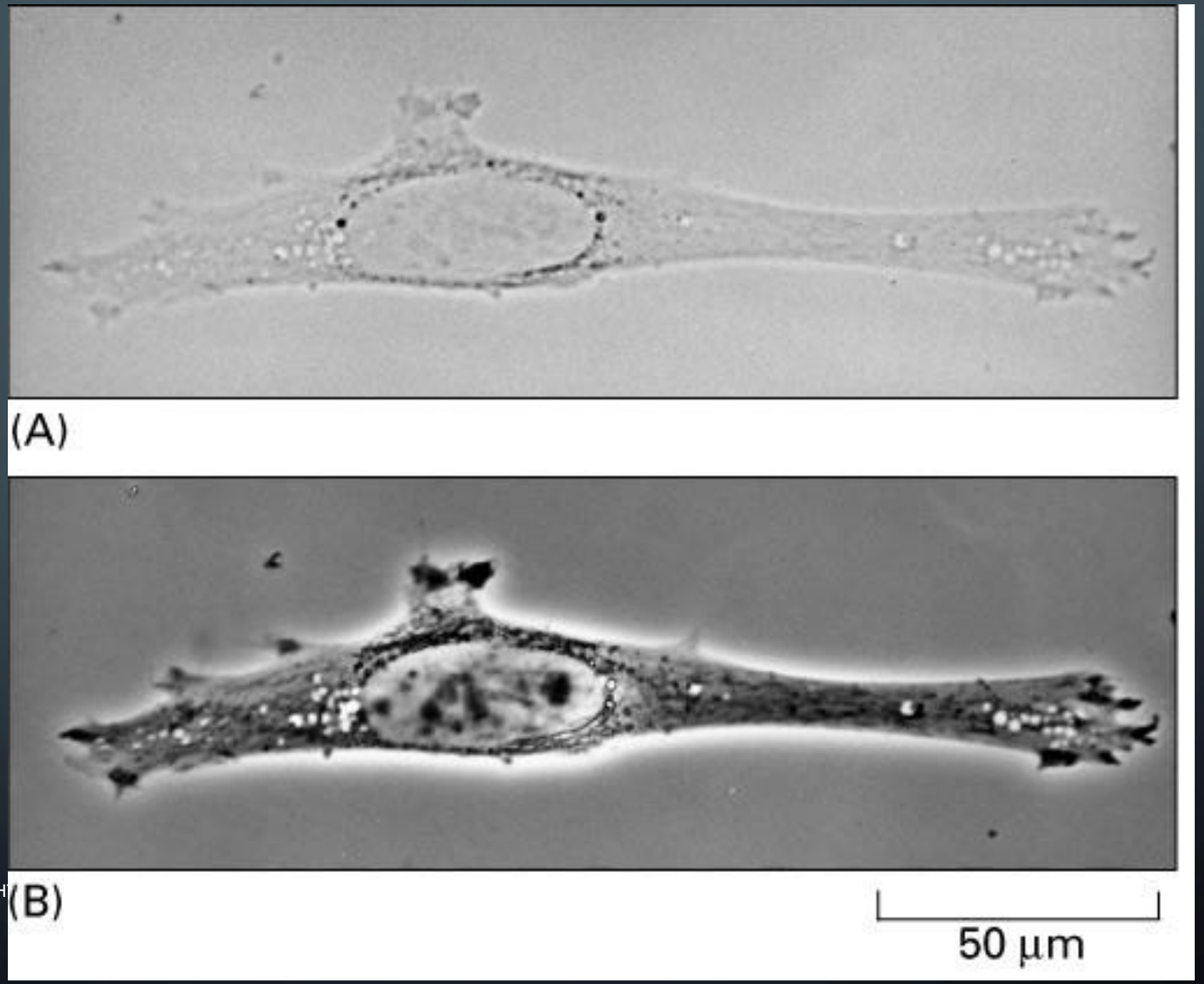
Figure 9–7. Molecular Biology of the Cell, 4th Edition.

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

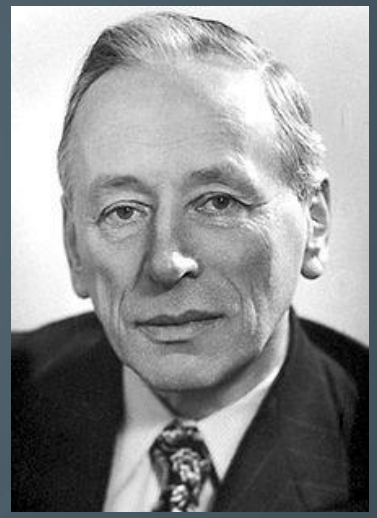

Amplitude and Phase Specimens
Amplitude
Wavelength
λ
Refractive
Index (RI) = n
Undisturbed
Light Wave
(a)
(b)
RI = n
Amplitude
Specimen
(c)
RI > n
Phase
Specimen
φ
90°
Amplitude
and Phase
Specimen
(d)
RI > n
φ
180°
Figure 3

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?


(A)
(B)
50 µm

FRITS ZERNIKE (1888–1966)



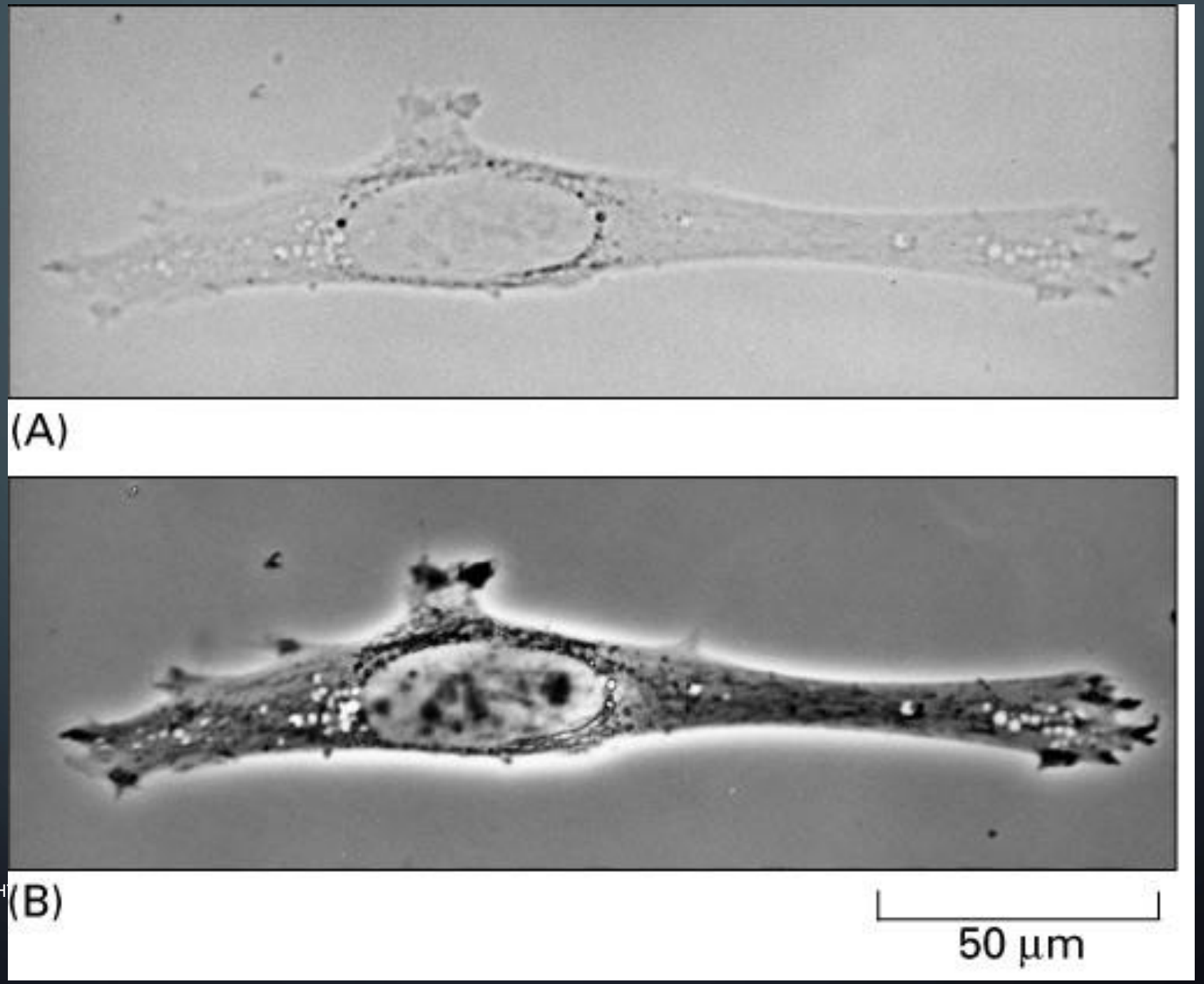
- Dutch physicist
- Developed vector notation for theory of light propagation through phase objects
- Invented phase contrast optics in 1930; not manufactured until 1941 by Zeiss

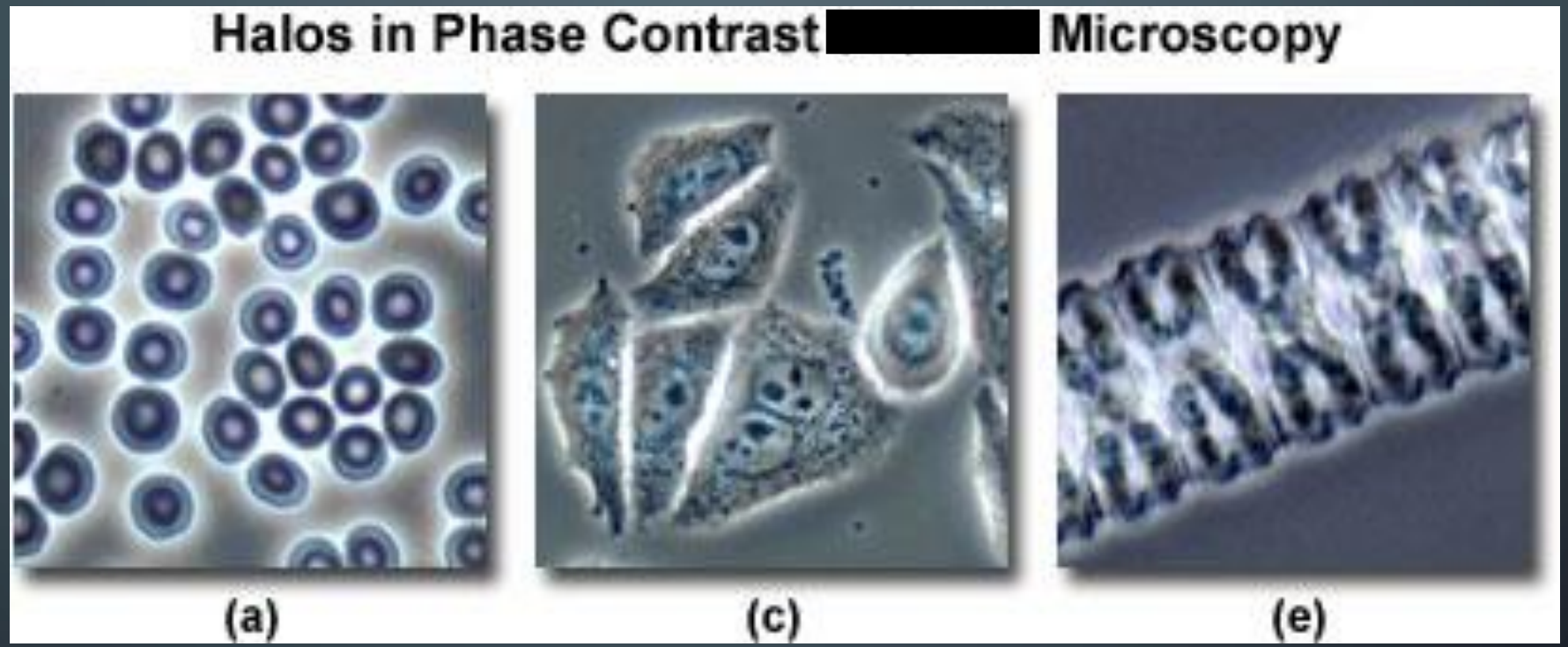
PHASE CONTRAST IMAGING

- 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)

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

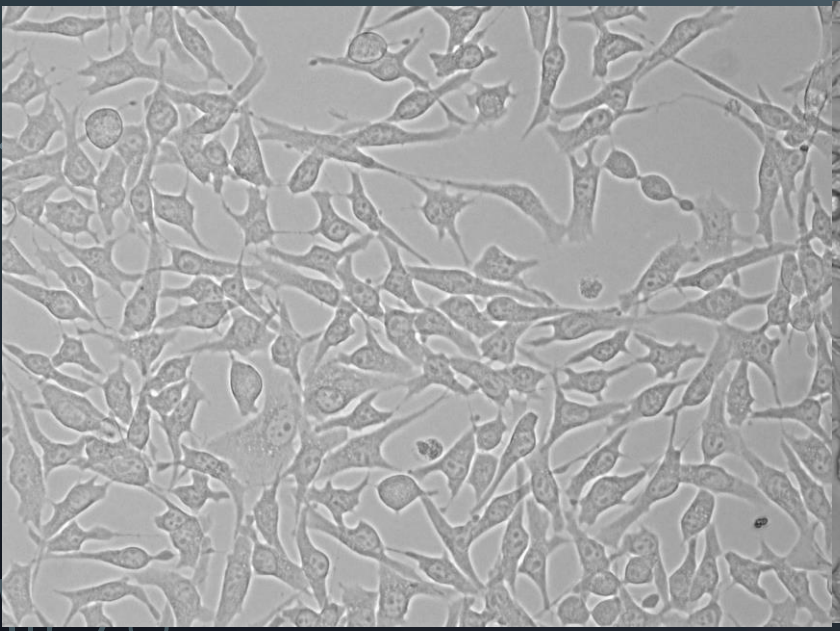

(A)
(B)
50 µm


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

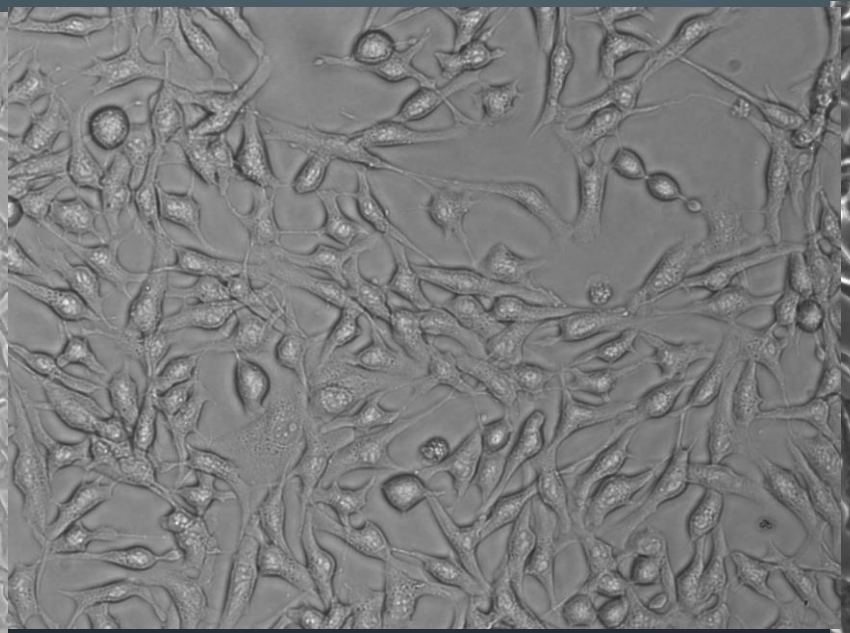


Phase contrast in practice

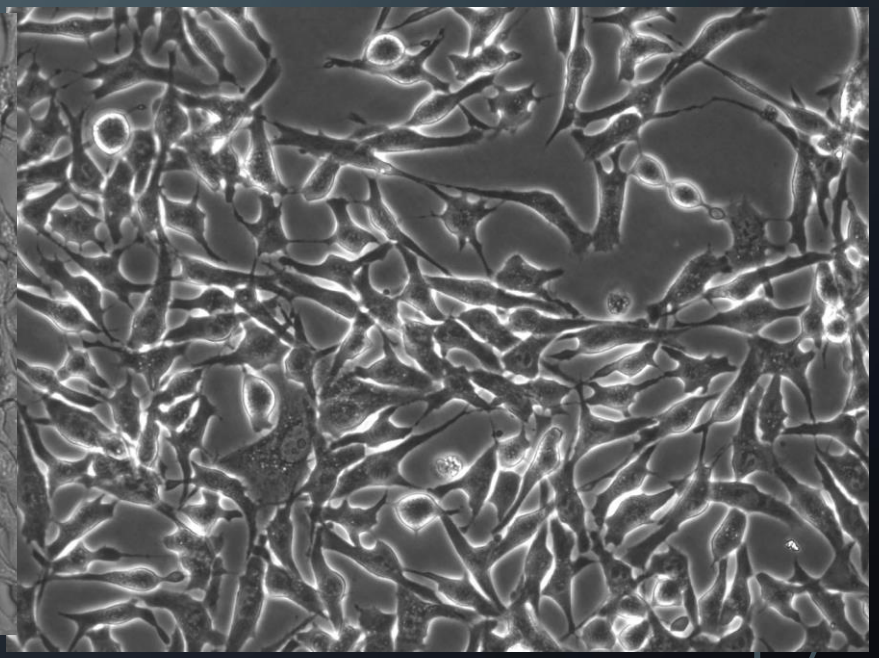
Application: Phase contrast is the most commonly used contrasting technique All tissue culture microscopes and the time-lapse microscopes are set up for phase.



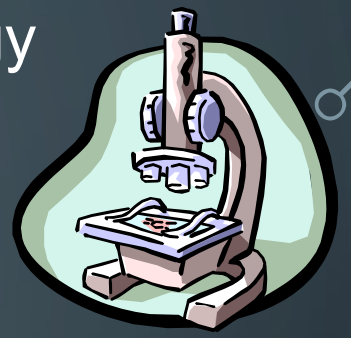
brightfield



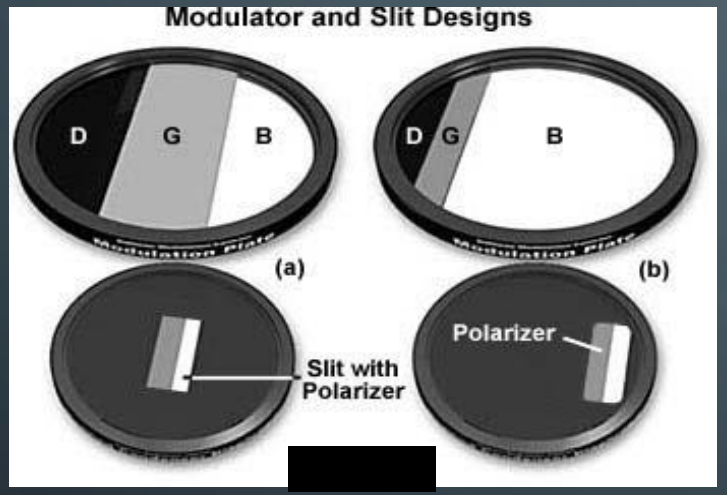
wrong phase stop

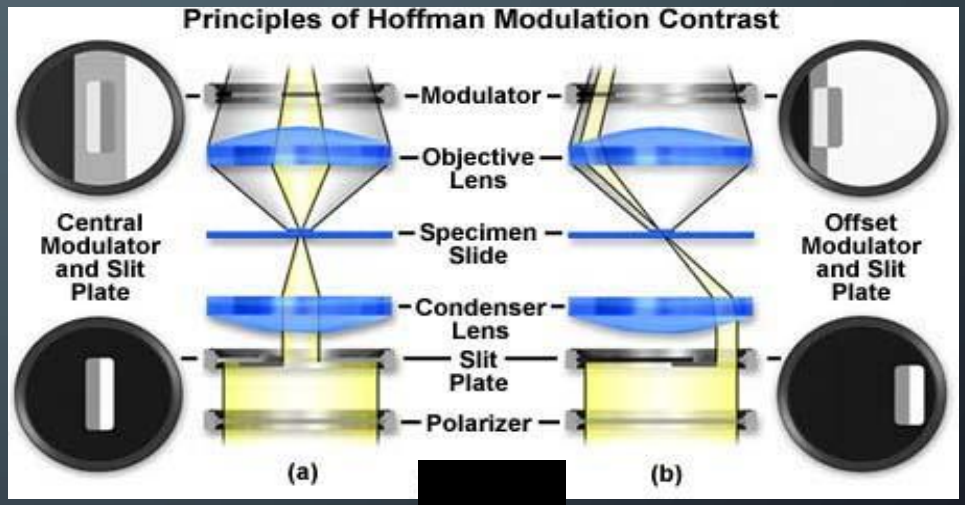


right phase stop

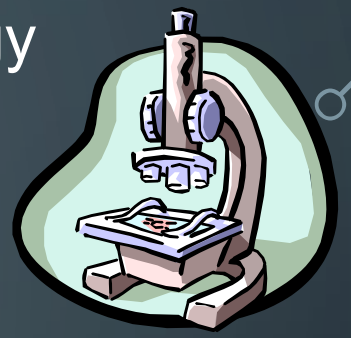


Hoffman Modulation Contrast



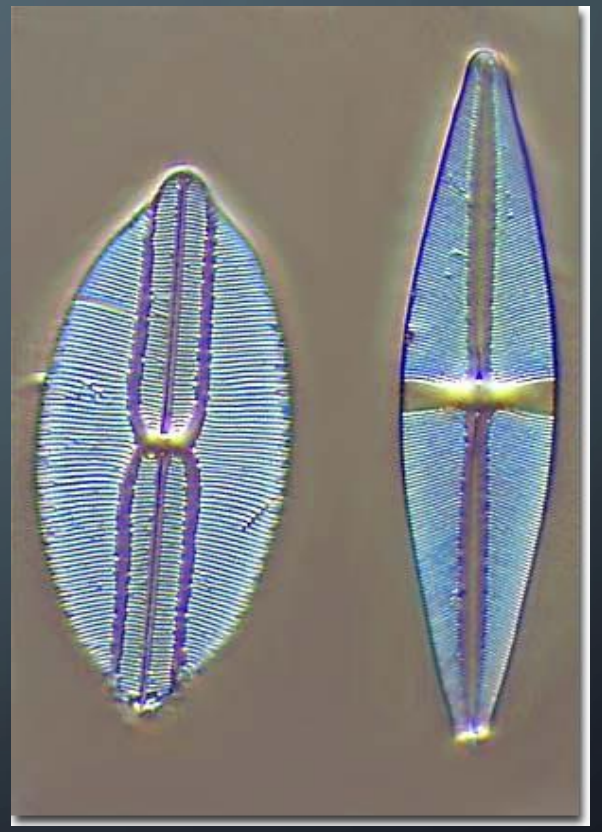


D : dark, 1% transmittance
G : gray, 15% transmittance
B : bright, 100% transmittance



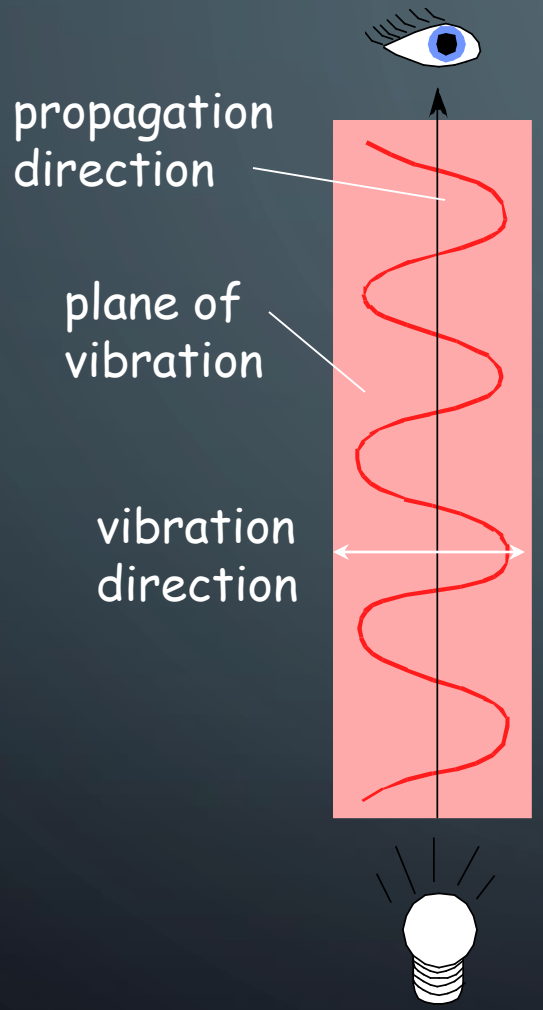
Hoffman Modulation Contrast

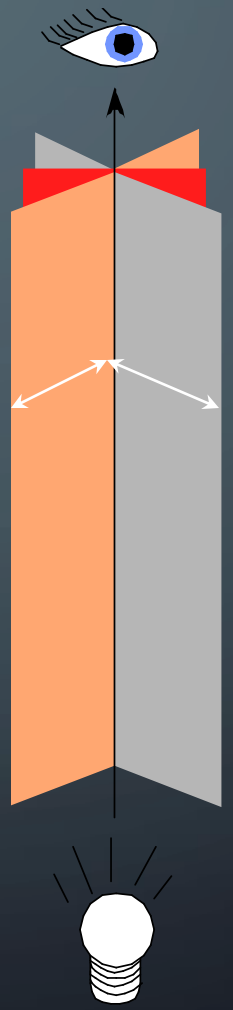
- Increase visibility and contrast in unstained and living material by detecting optical gradients (or slopes) and converting them into variations of light intensity.



Diatoms

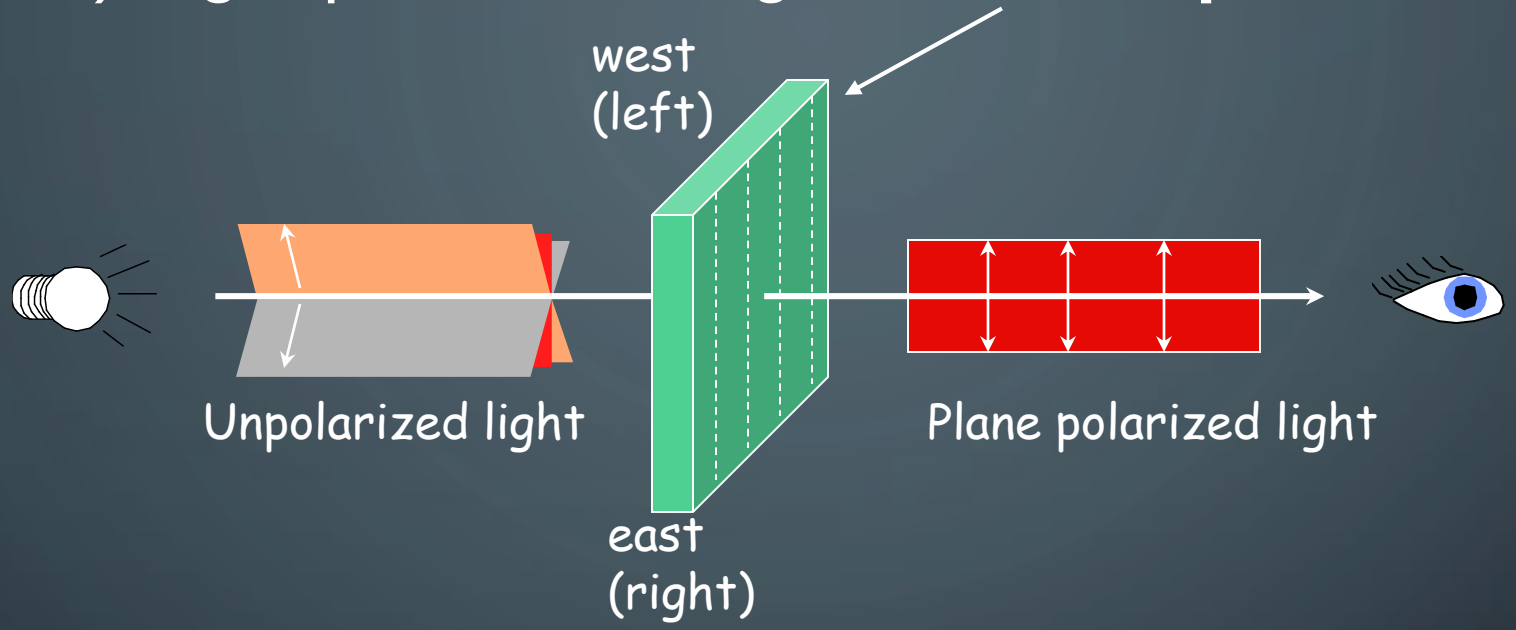
What happens as light moves through the scope?





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

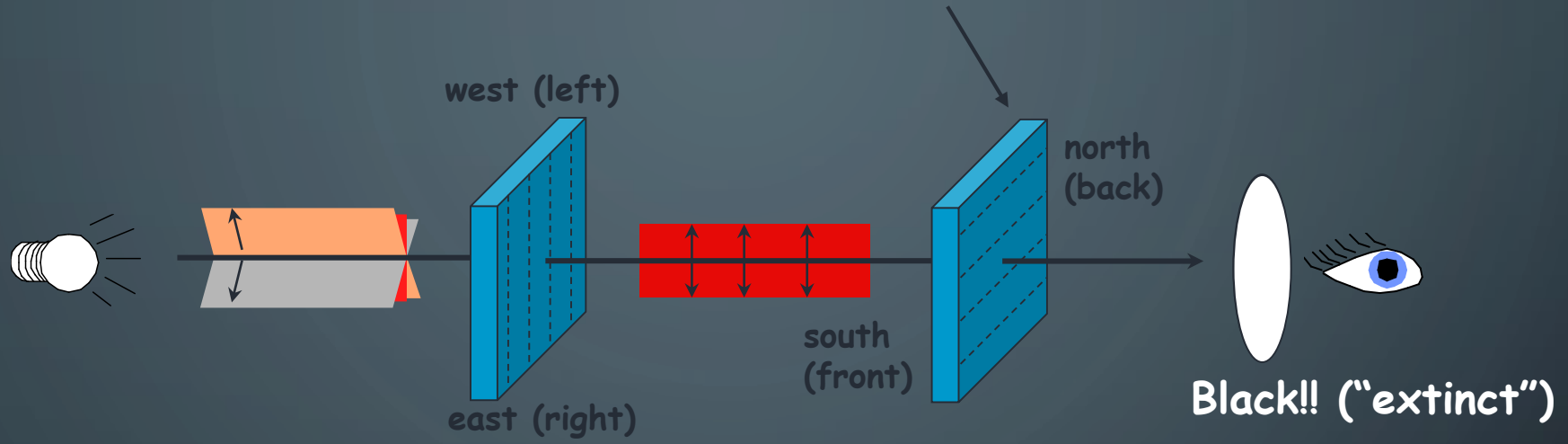
1) Light passes through the **lower polarizer**



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**

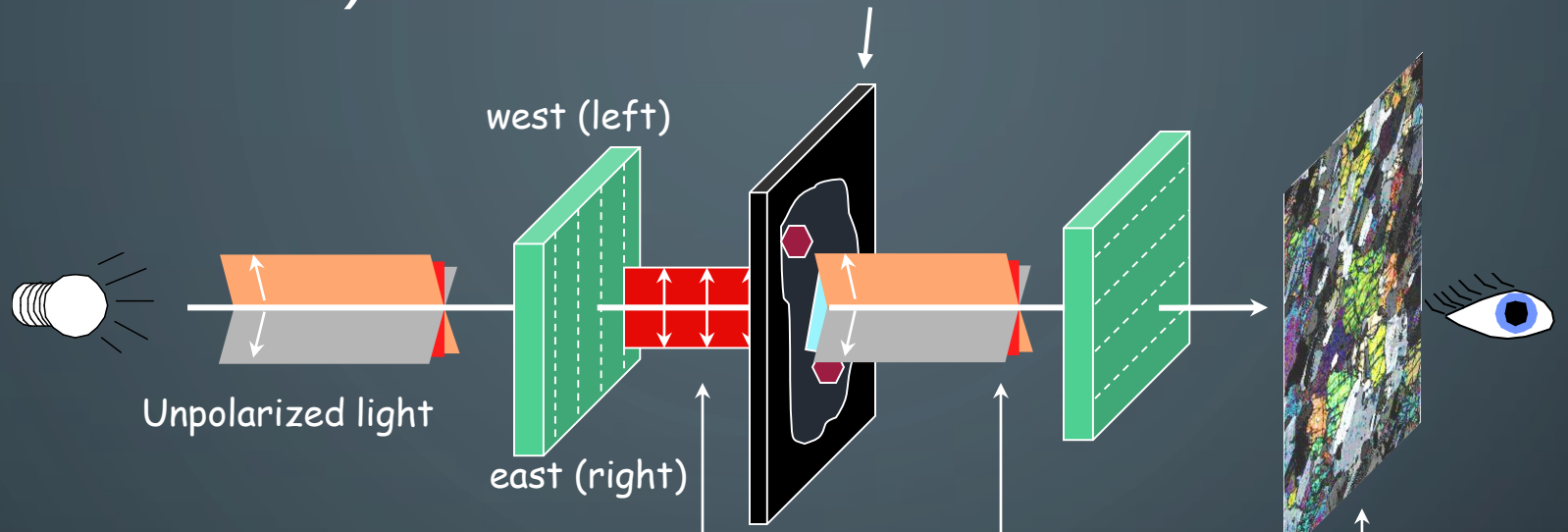


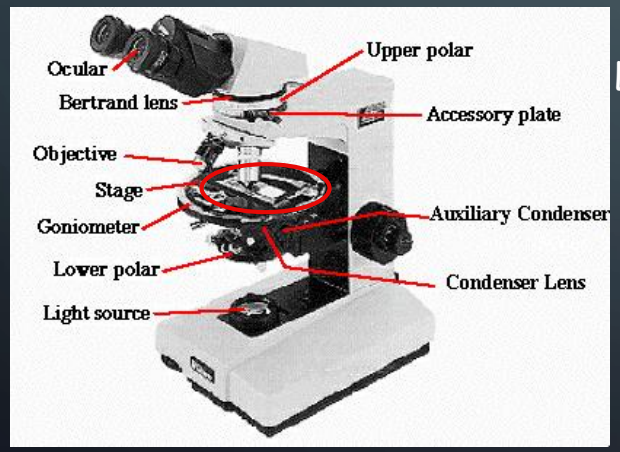
Now what happens?
What reaches your eye?

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

© Jane Selverstone, University of New Mexico, 2003

3) Now insert a **thin section**

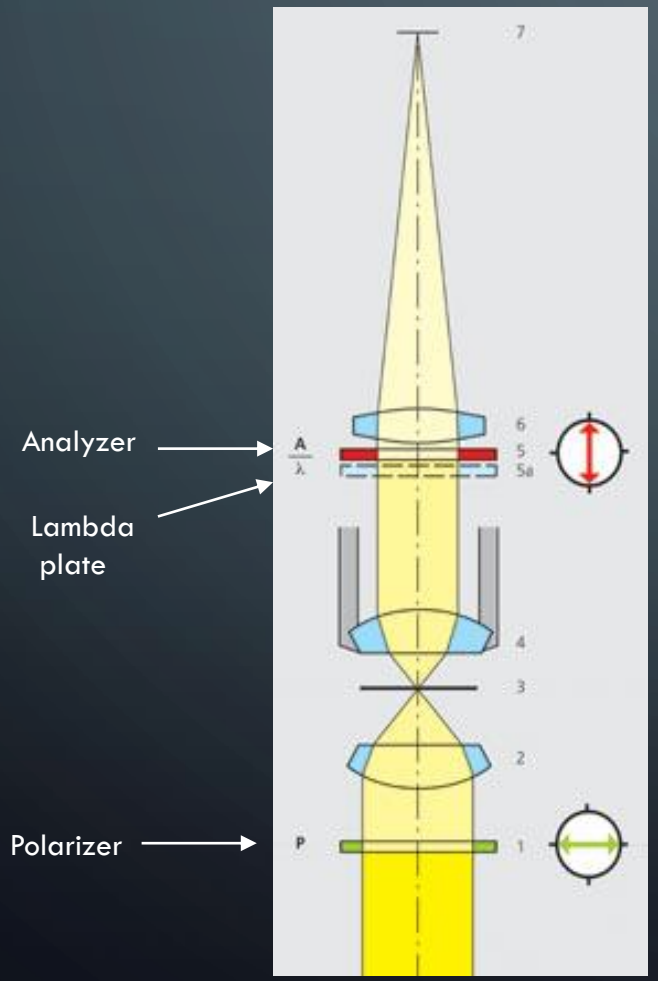




How does this work??

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



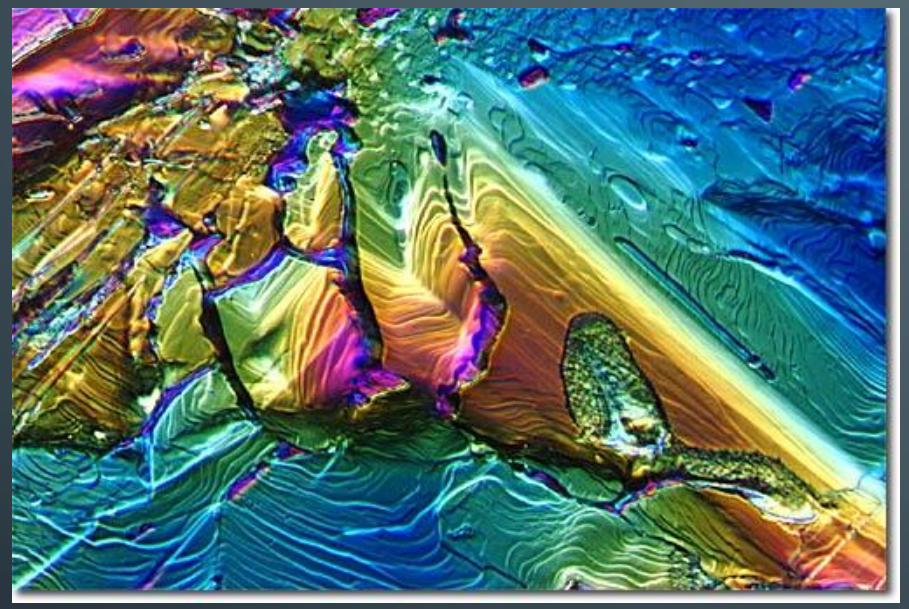
Polarization contrast



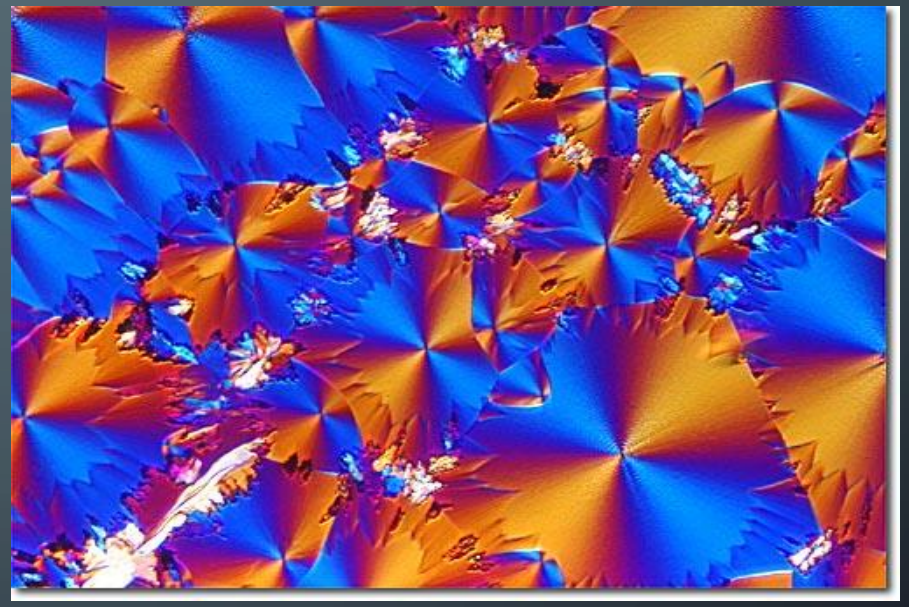
Polarization contrast with Lambda plate

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

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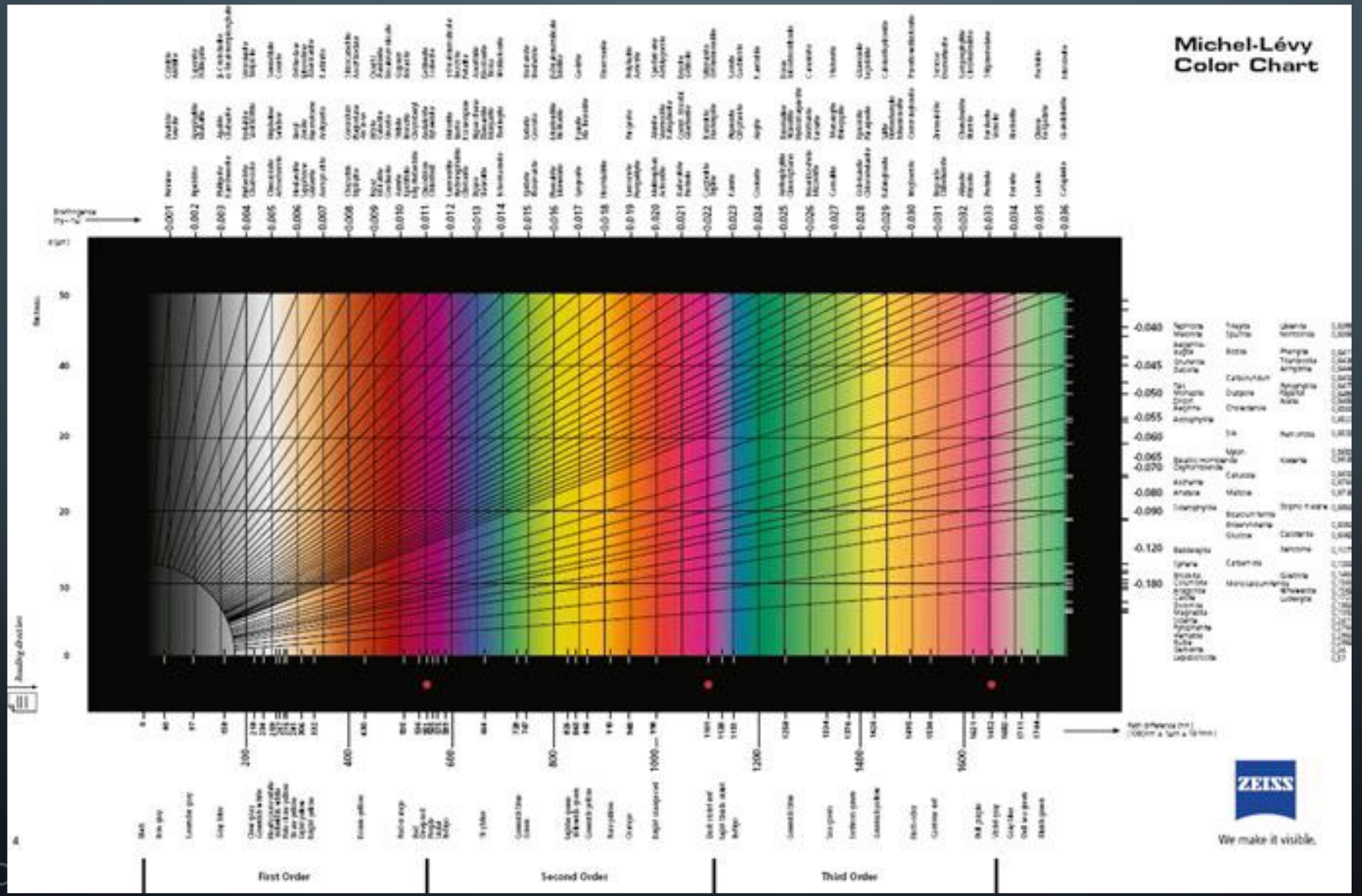
Glutaric Acid Crystallites



Dinosaur Bone

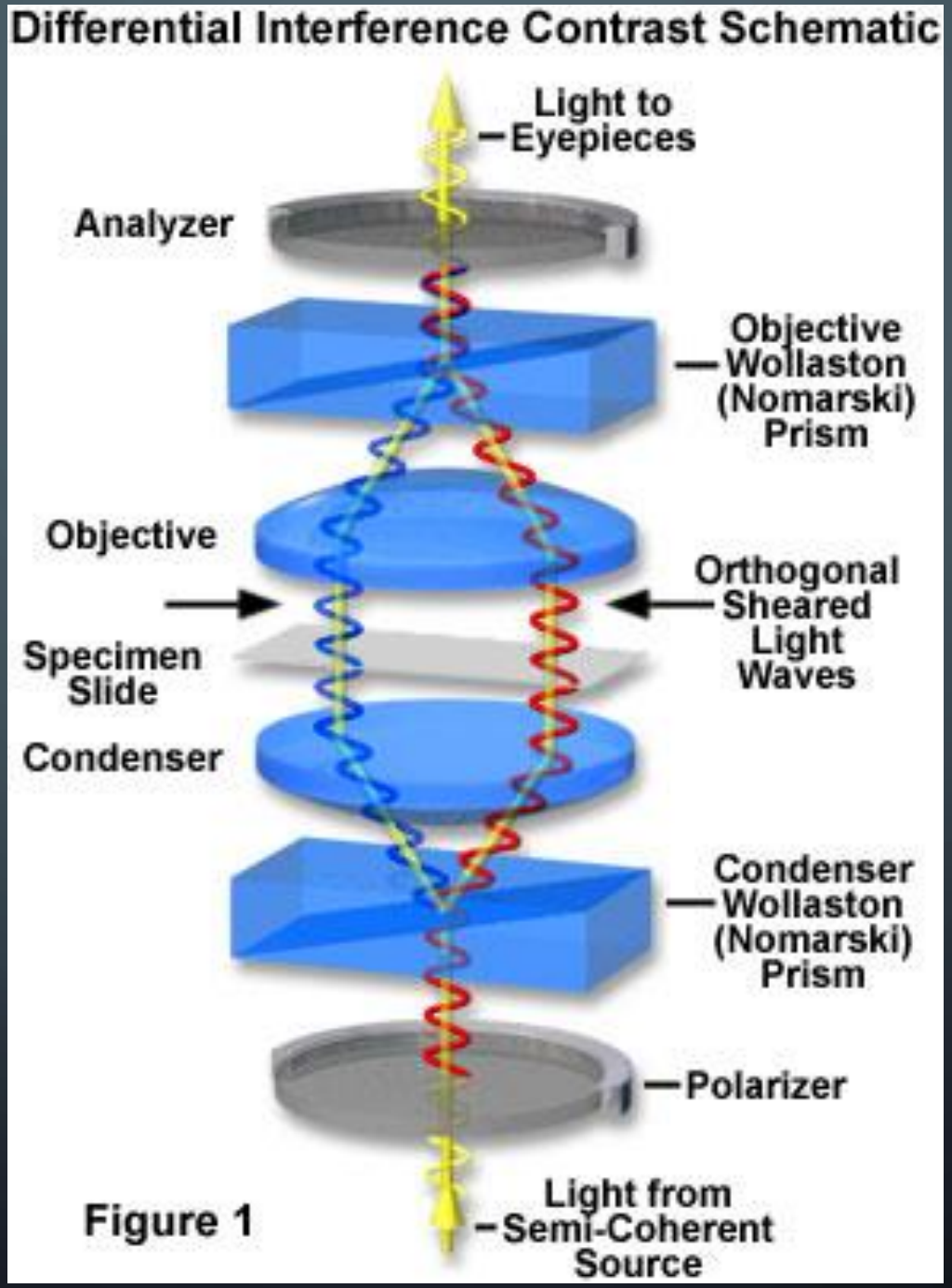
2022 - Light microscopy in Cellular Biology



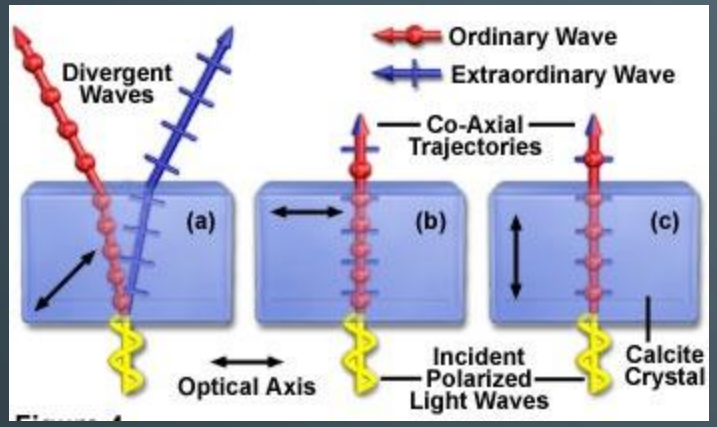


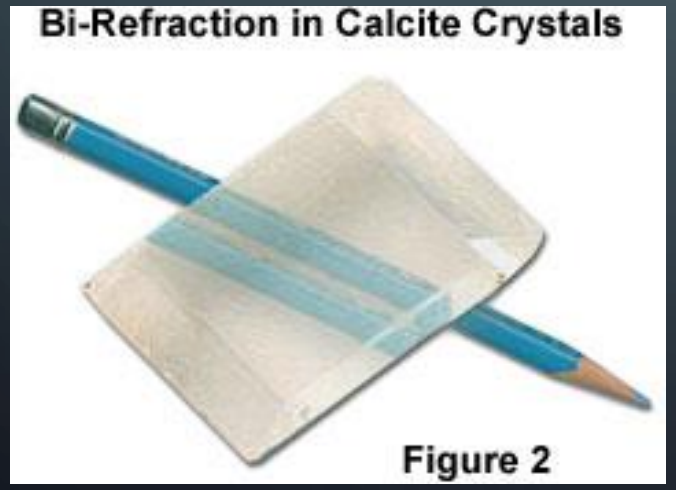
NOMARSKI IMAGE

- 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

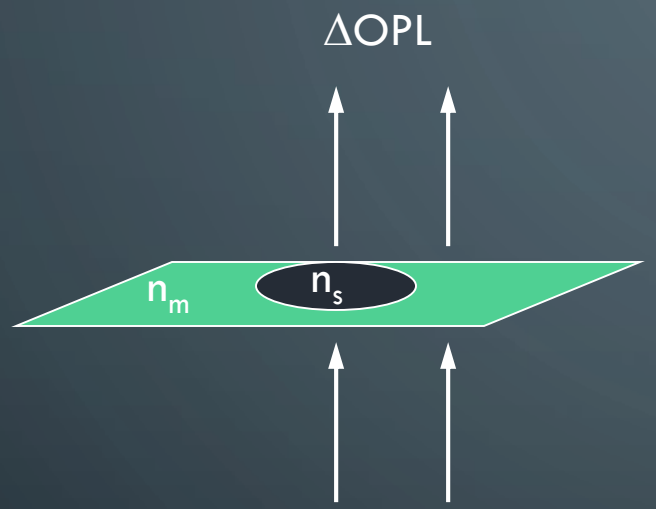
BIREFRINGENCE





- 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

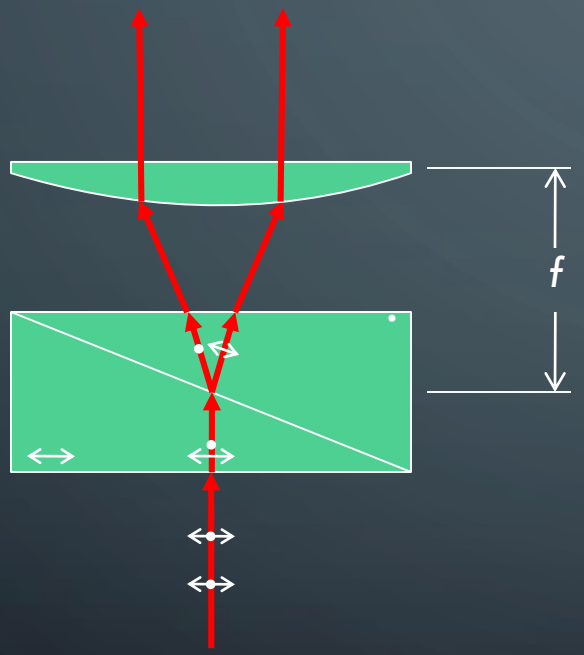
Differential Interference Contrast (DIC)



The idea:

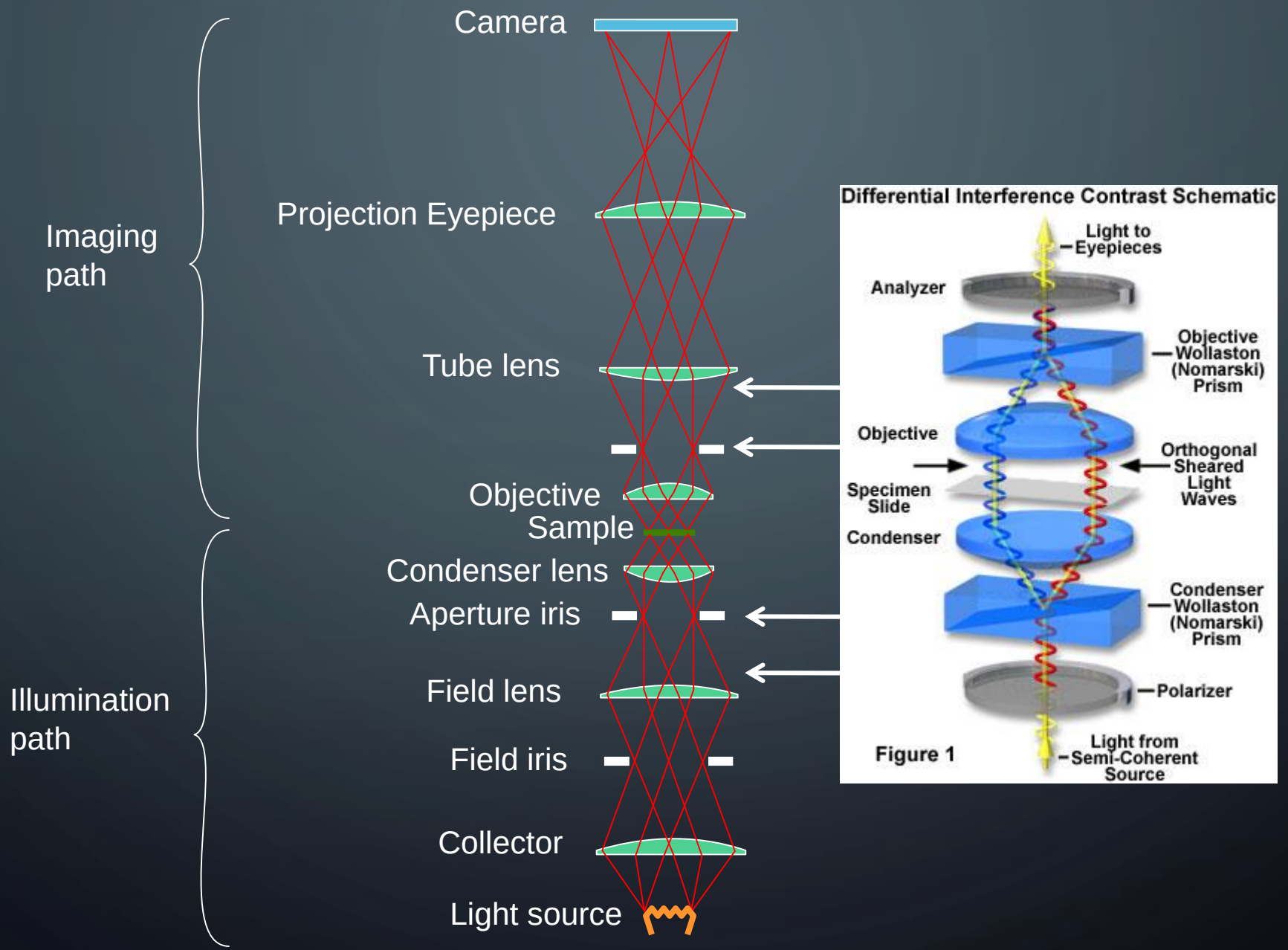
Use two beams and interference to measure the path length difference between adjacent points in the sample

WOLLASTON / NOMARSKI PRISMS

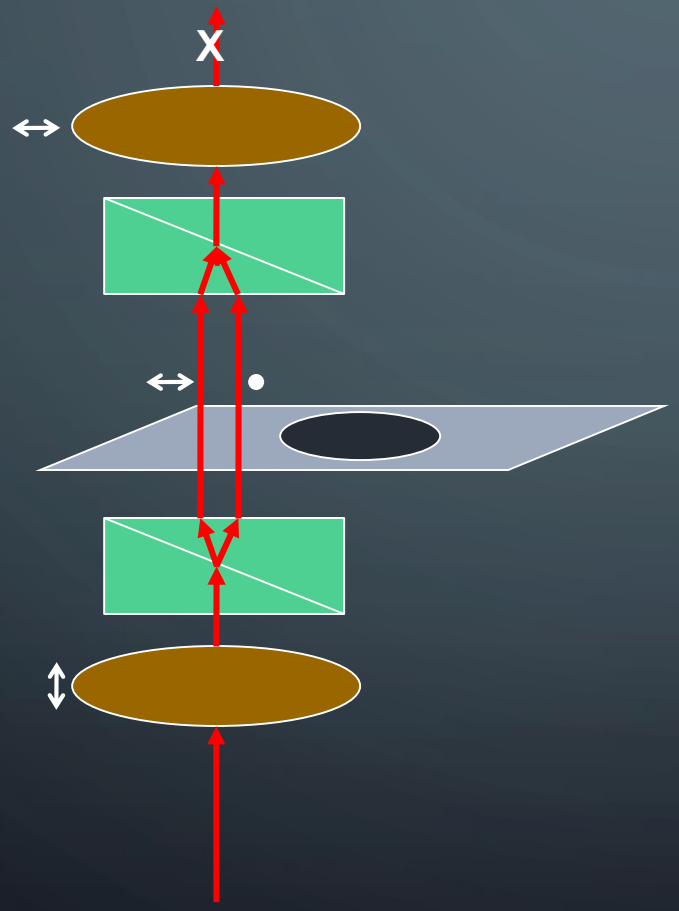


- Two pieces of cemented calcite / quartz
- Produce orthogonally polarized beams propagating at different angles
- Placed at a back focal plane, this produces the two beams that will probe the OPL difference of our sample

THE DIFFERENTIAL INTERFERENCE CONTRAST (DIC) MICROSCOPE

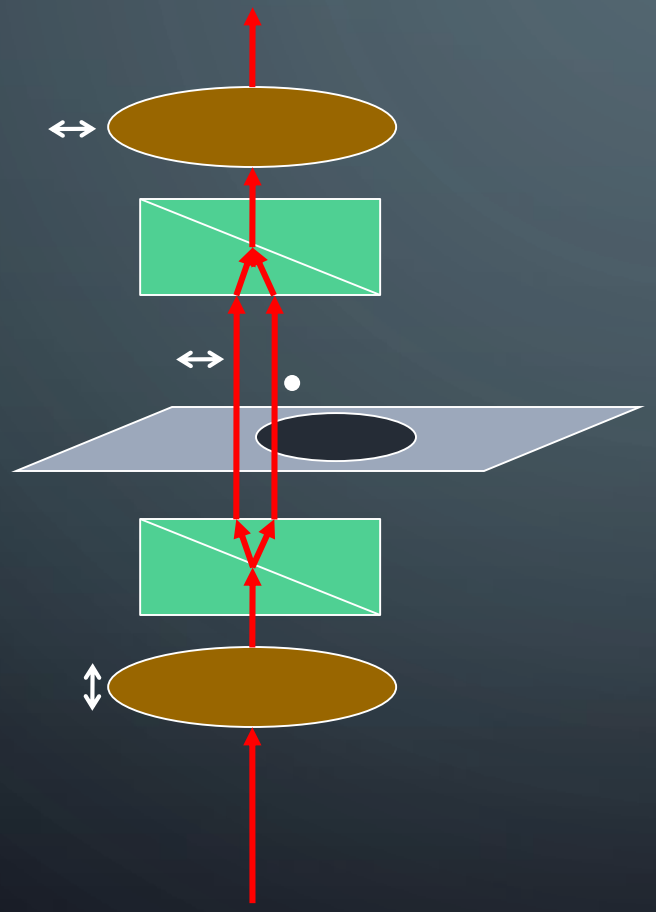


HOW DIC GENERATES CONTRAST



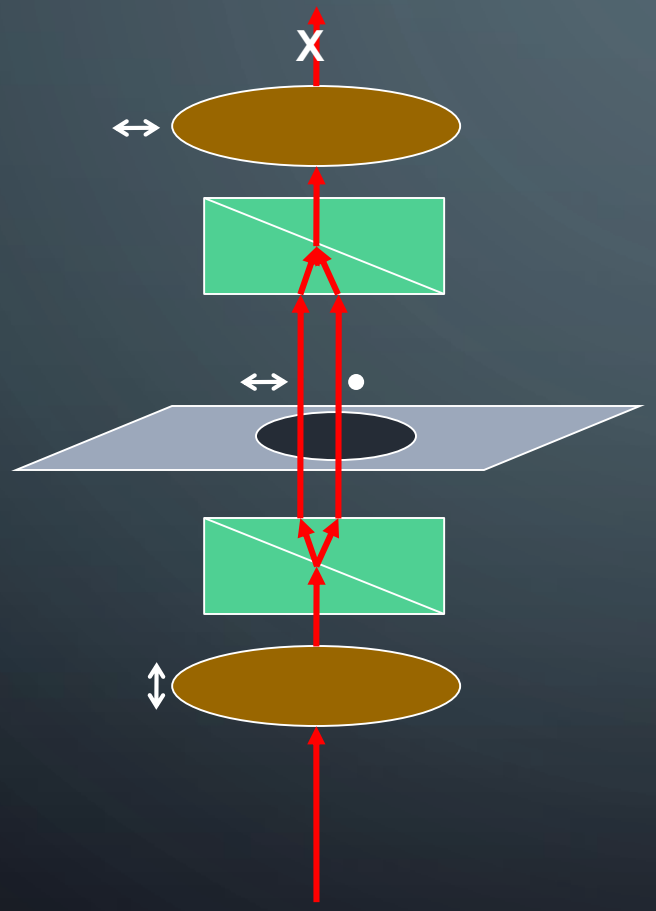
- Both beams see same OPL
- Emerge in phase
- Regenerate initial polarization
- No light makes it through analyzer

HOW DIC GENERATES CONTRAST

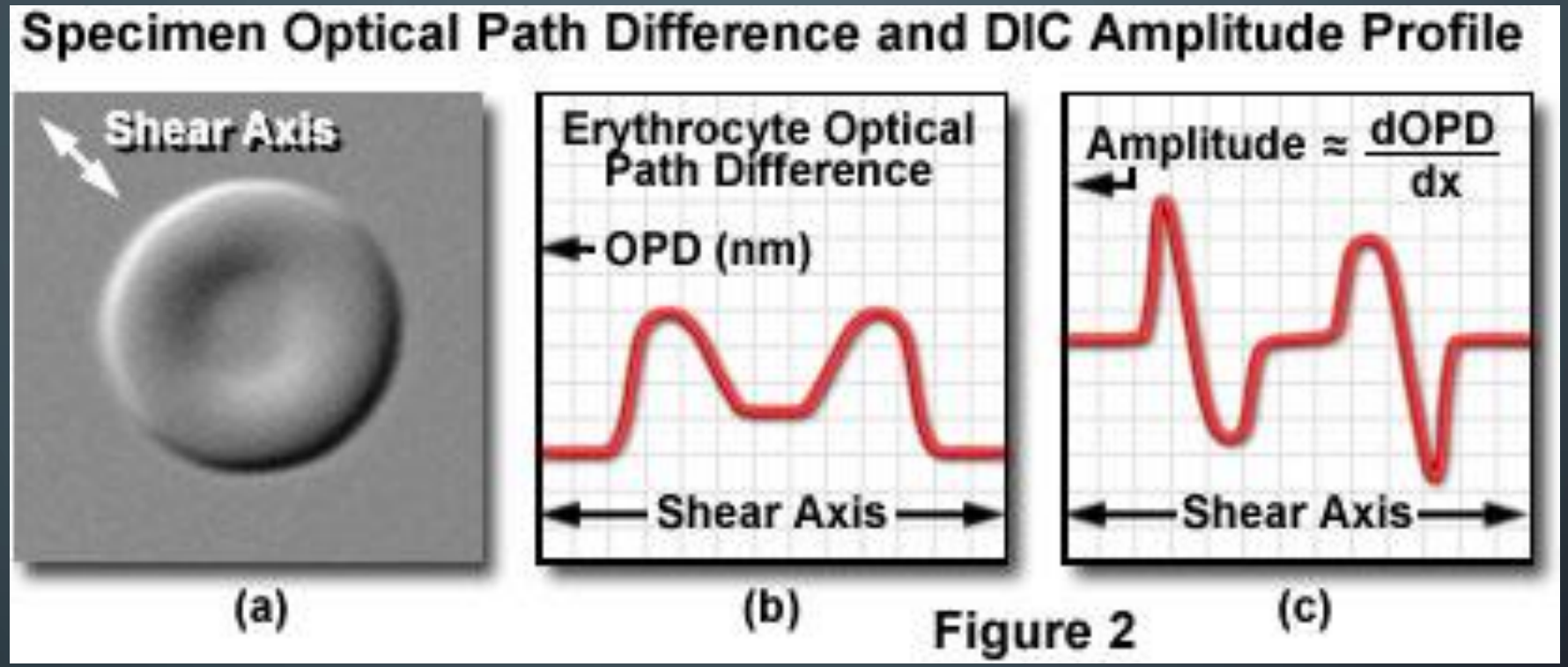


- Beams see different OPL
- Right beam is phase retarded
- Generate elliptical polarization
- Light makes it through analyzer

HOW DIC GENERATES CONTRAST



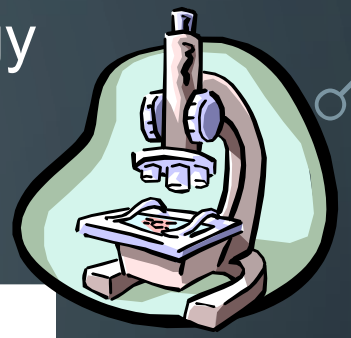
- Both beams see same OPL
- Emerge in phase
- Regenerate initial polarization
- No light makes it through analyzer


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

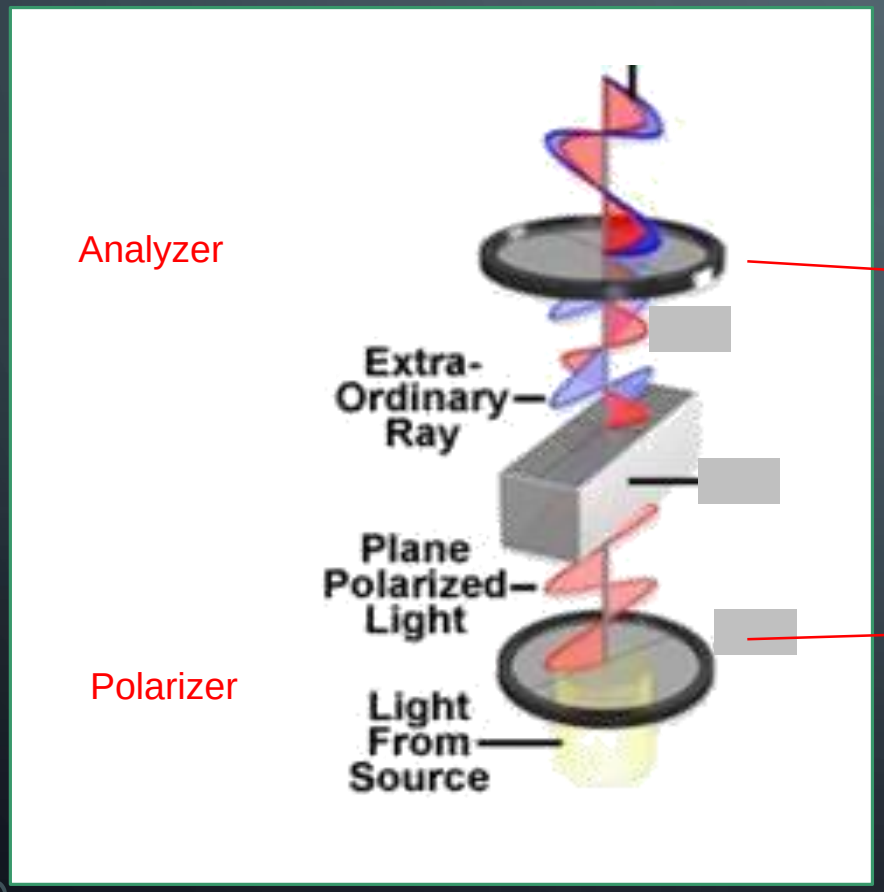
Features of a DIC image



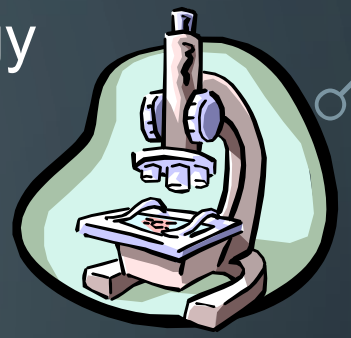
1. Contrast is directional
2. Contrast highlights edges
3. One end brighter, other is dimmer giving a pseudo – 3D image



Polarization





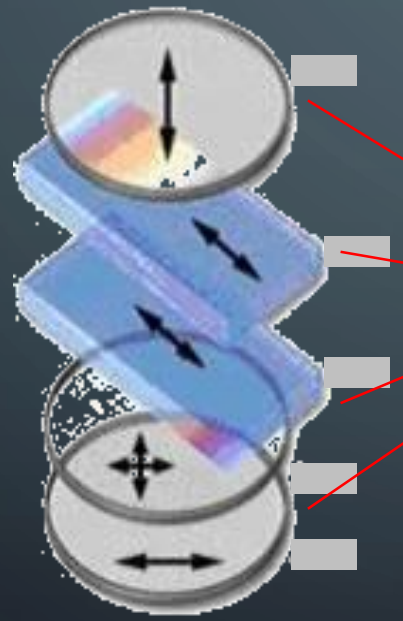


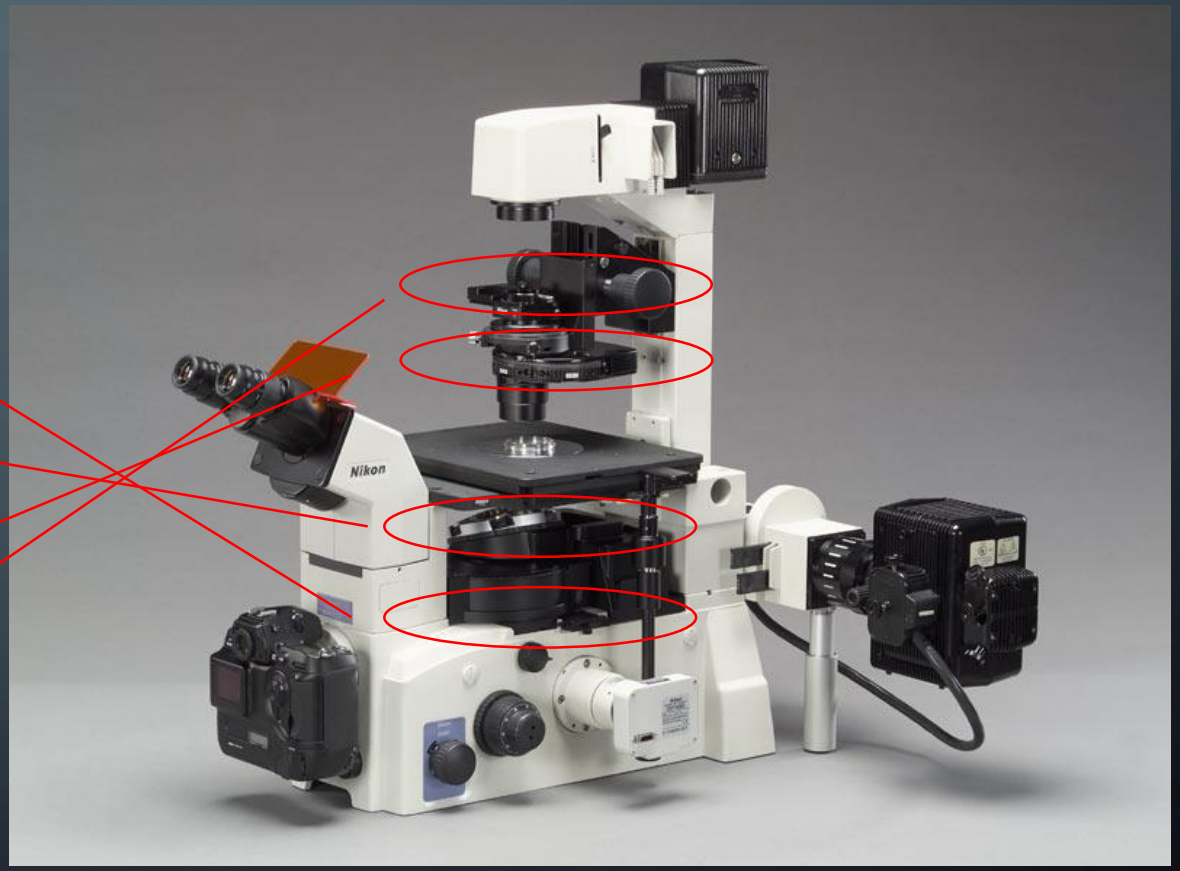
Differential interference contrast (DIC)

Analyzer

Condenser
Nomarski prism

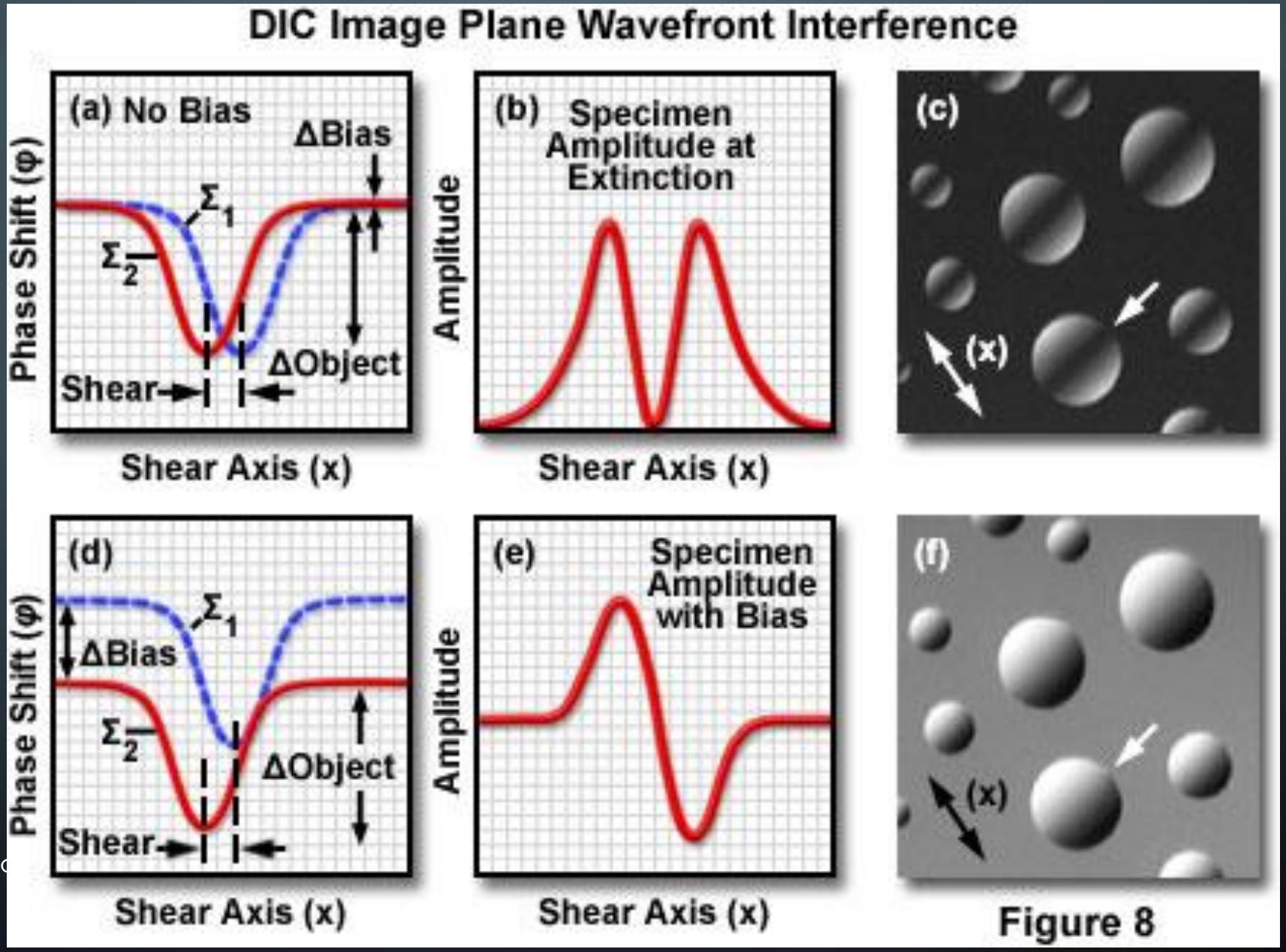
Polarizer





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

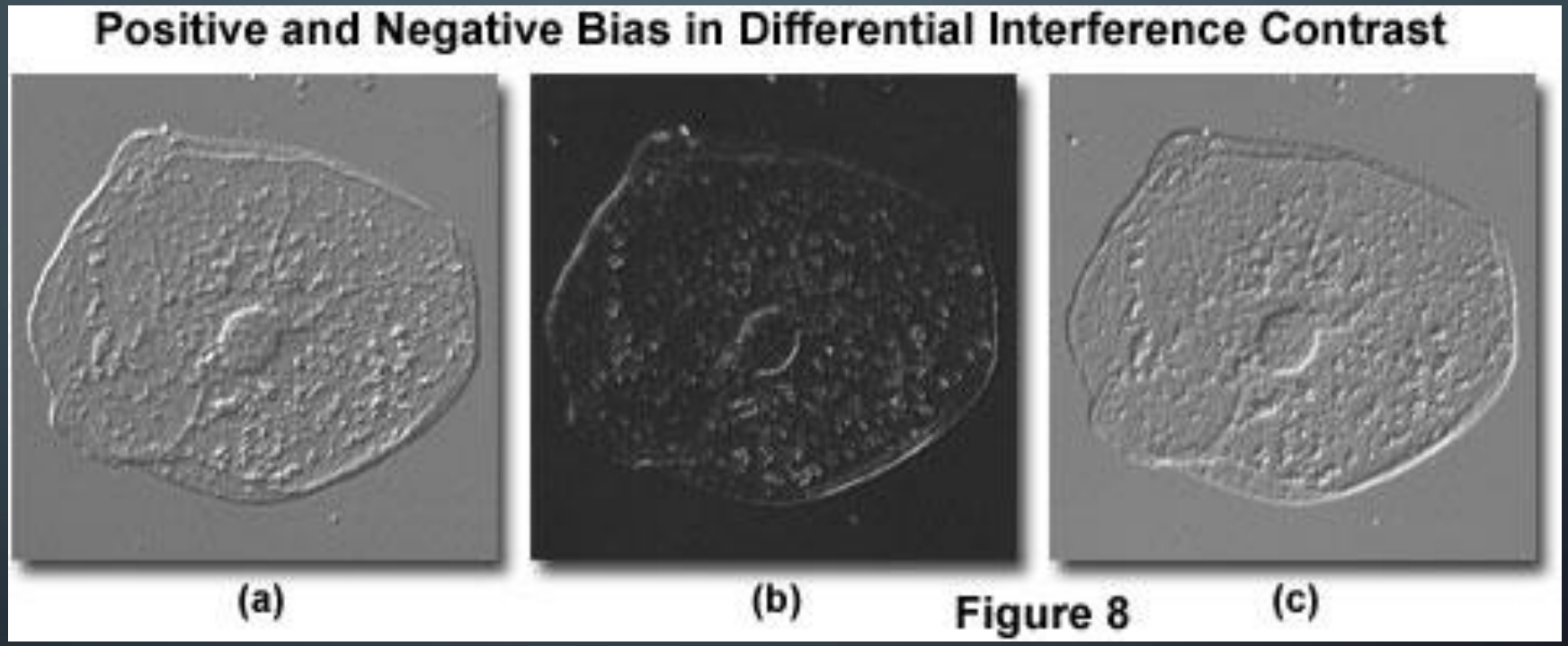
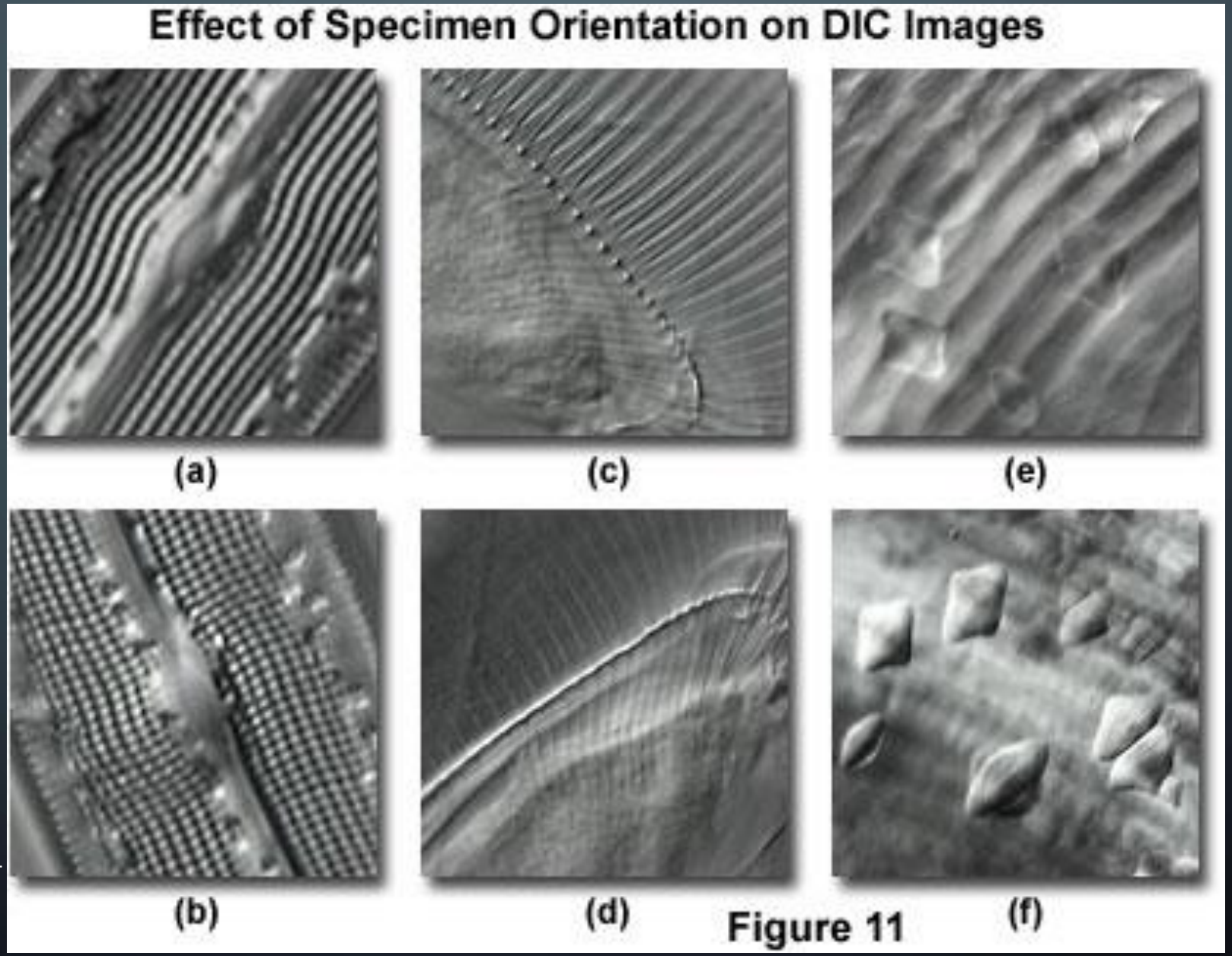

Positive and Negative Bias in Differential Interference Contrast
(a)
(b)
(c)

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

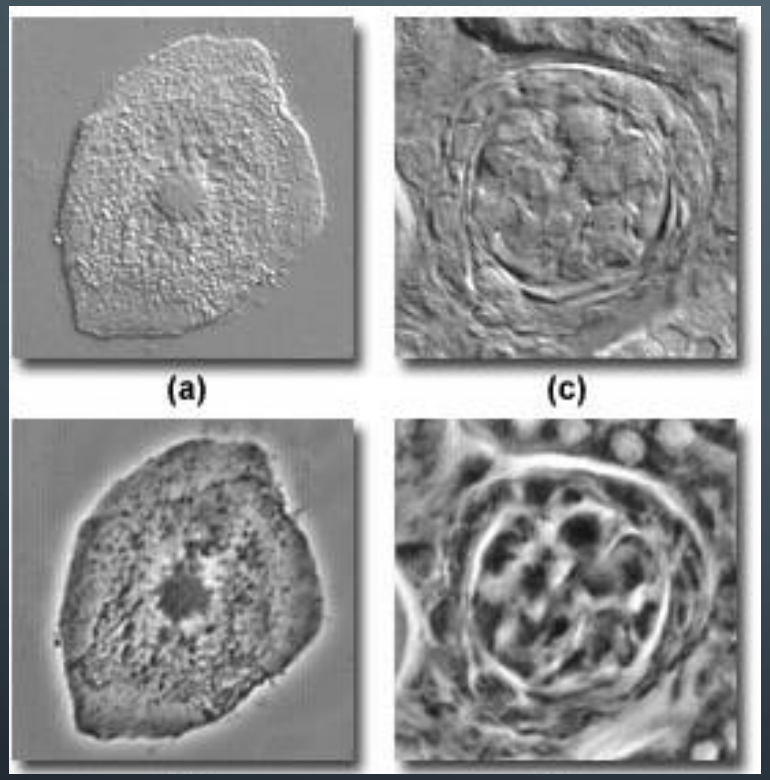
Extremely shallow depth of field — useful for optical sectioning of specimen

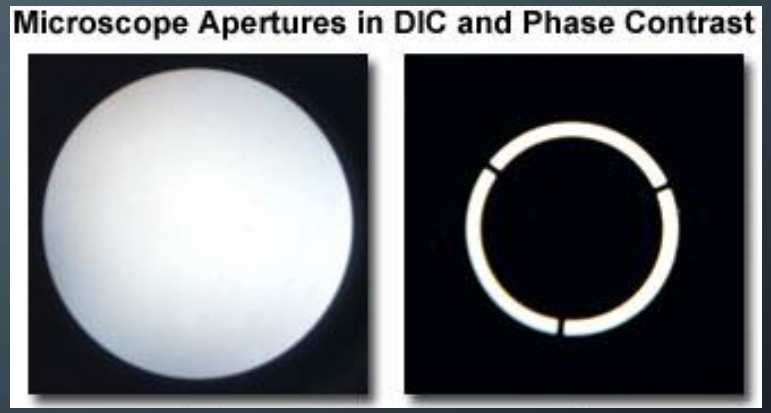
Works well with stained specimens; optics can be adjusted to enhance contrast

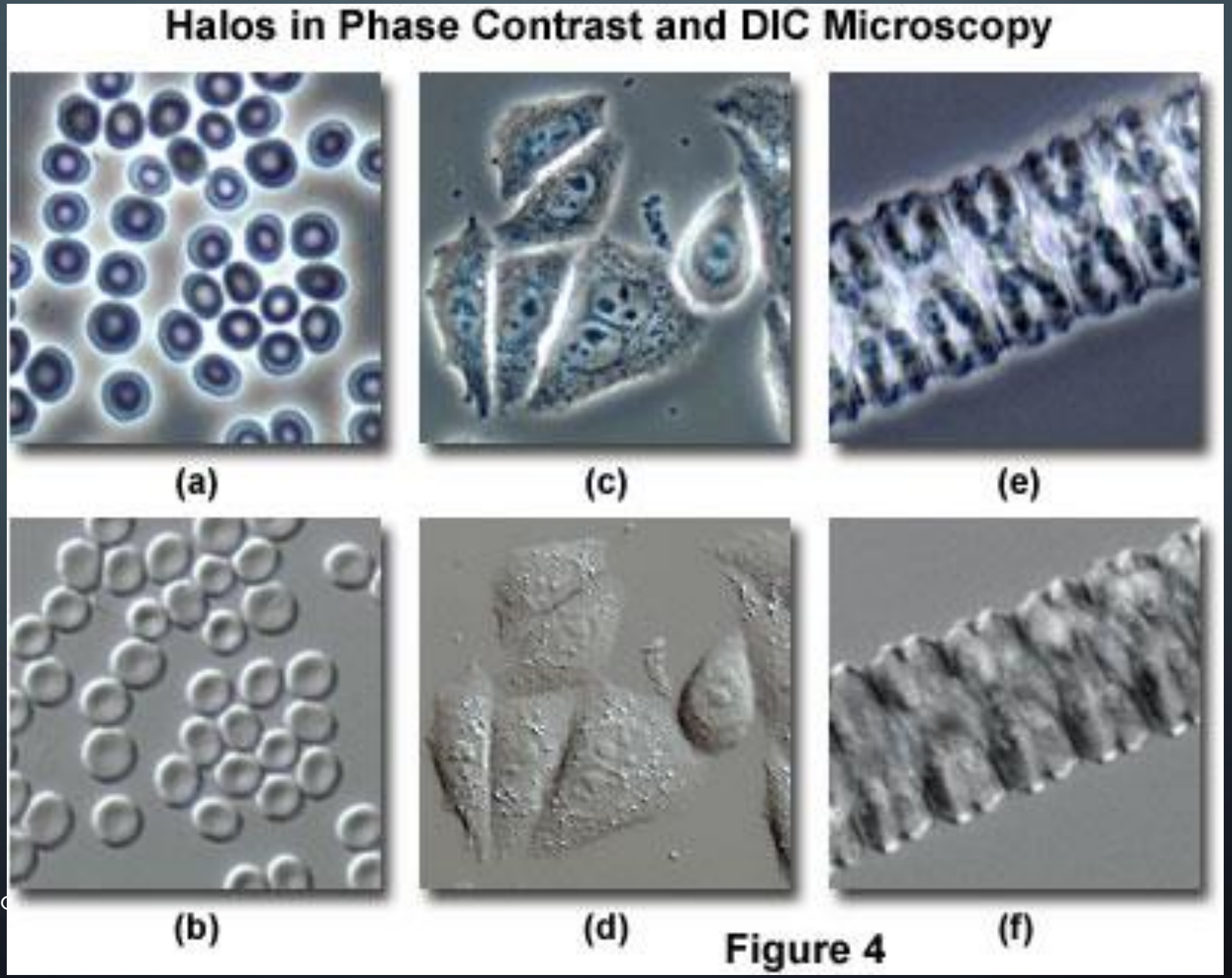
DIC IS HIGHER RESOLUTION THAN PHASE CONTRAST

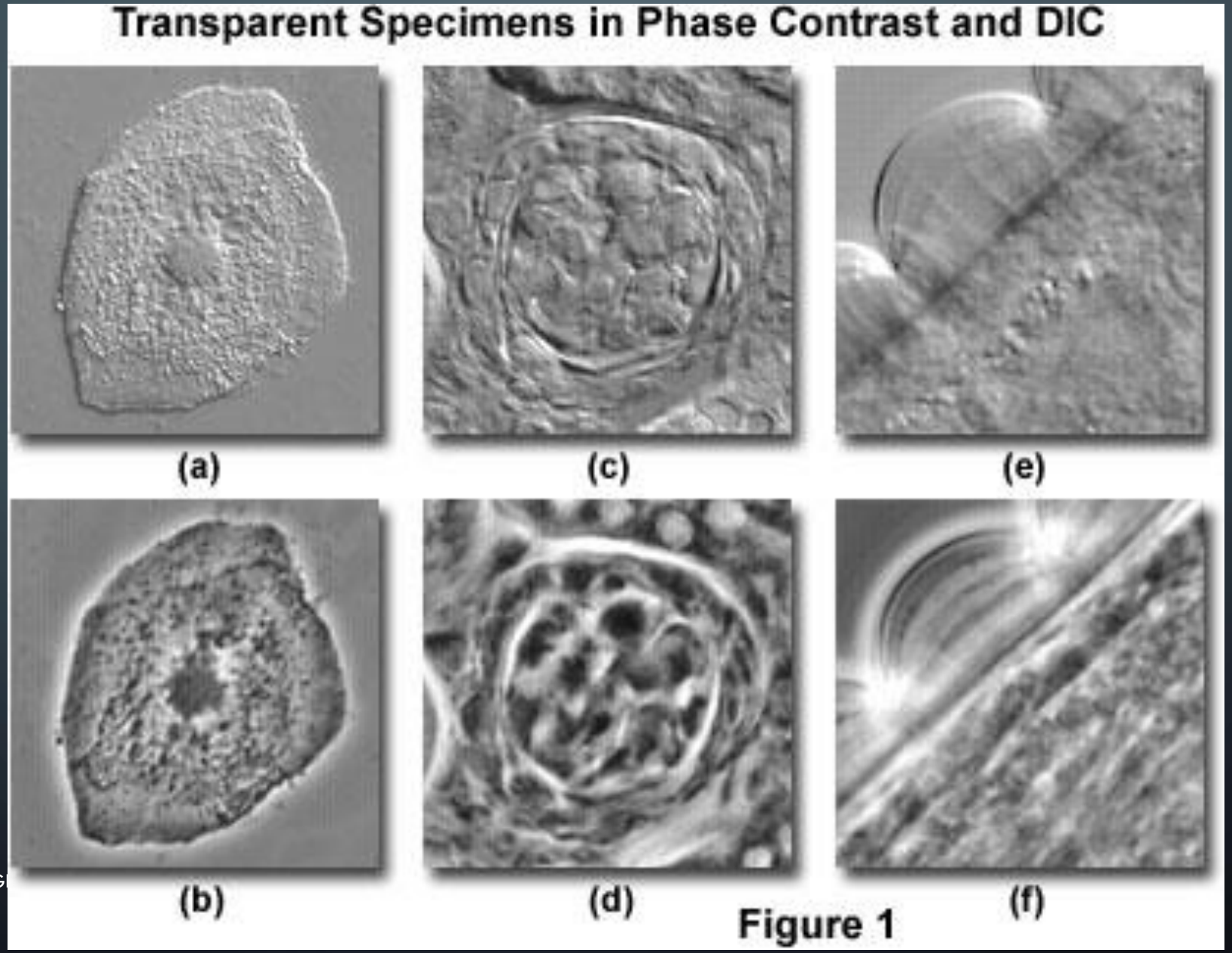
DIC

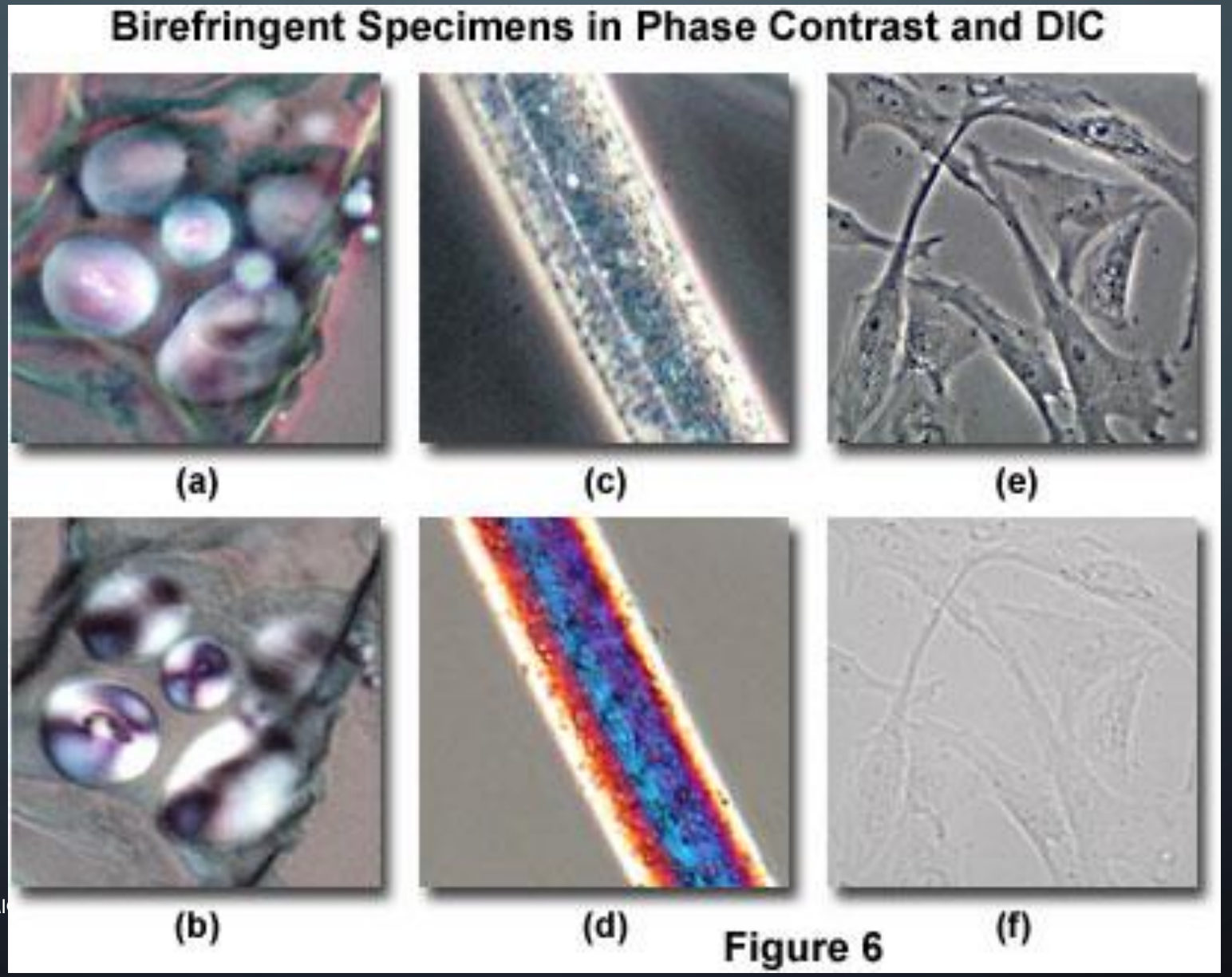
Phase





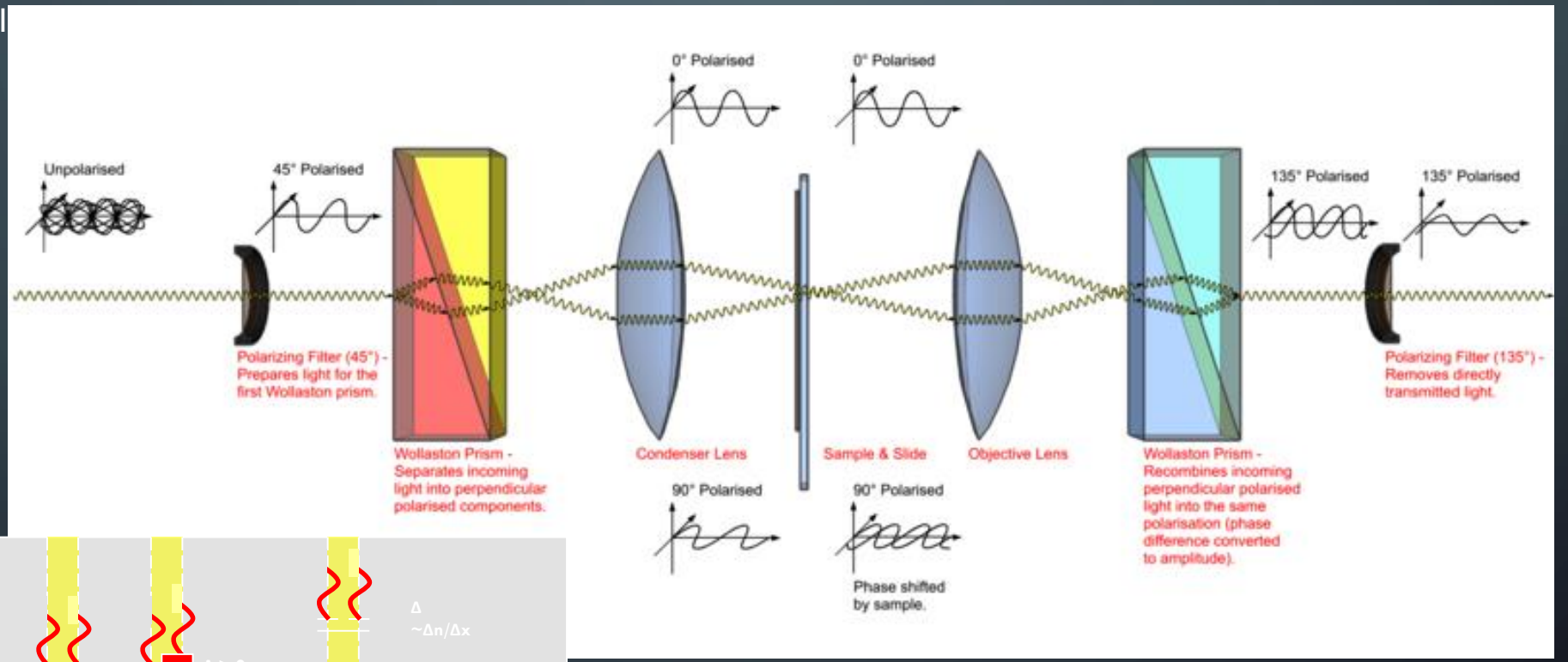

Halos in Phase Contrast and DIC Microscopy
(a)
(c)
(e)
(b)
(d)
(f)
Figure 4

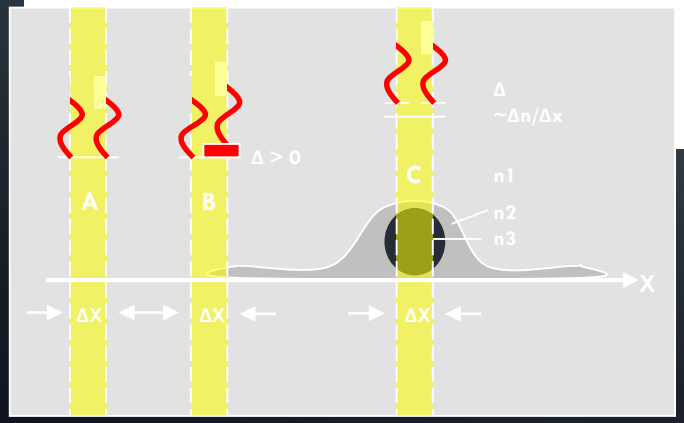

Transparent Specimens in Phase Contrast and DIC
(a)
(c)
(e)
(b)
(d)
(f)
Figure 1

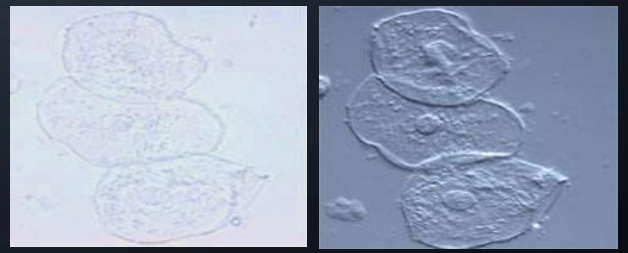

Birefringent Specimens in Phase Contrast and DIC
(a)
(c)
(e)
(b)
(d)
(f)
Figure 6

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

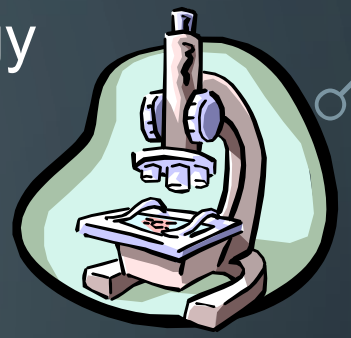






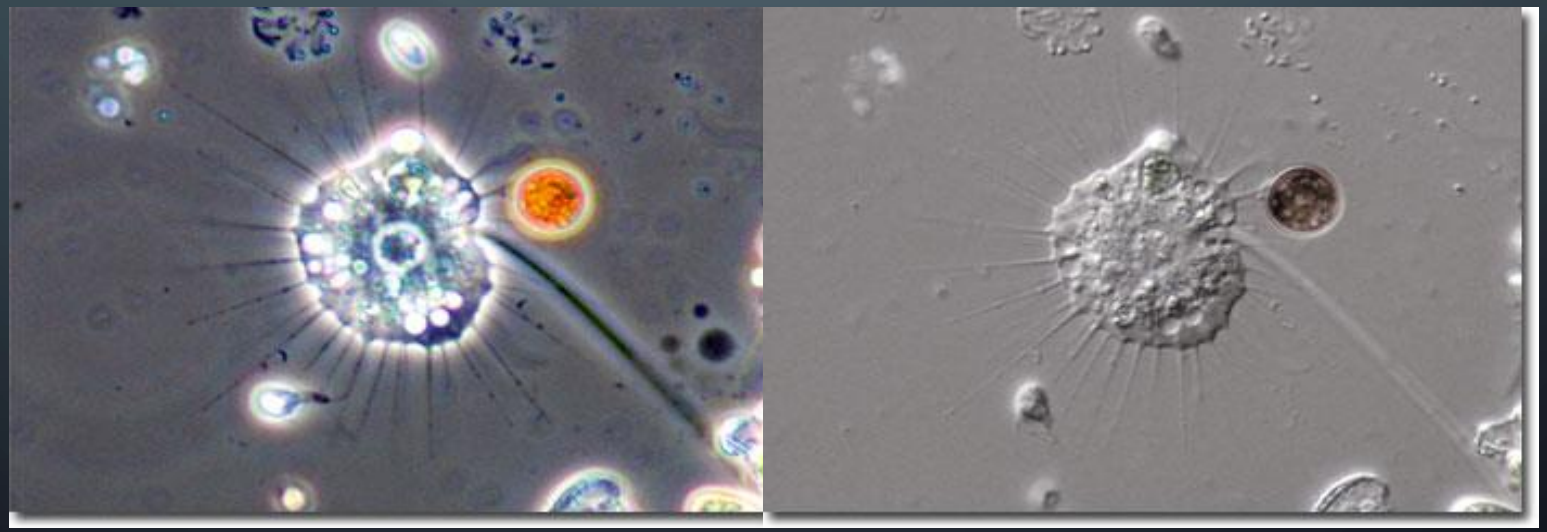
→ *most of our microscopes are set up for DIC*

2022 - Light microscopy in Cellular Biology



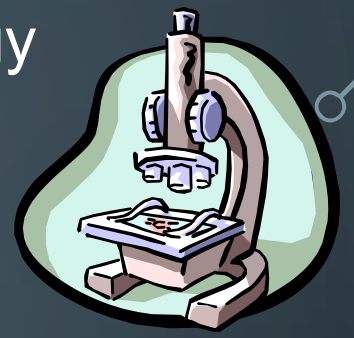
HeLa Cell Culture



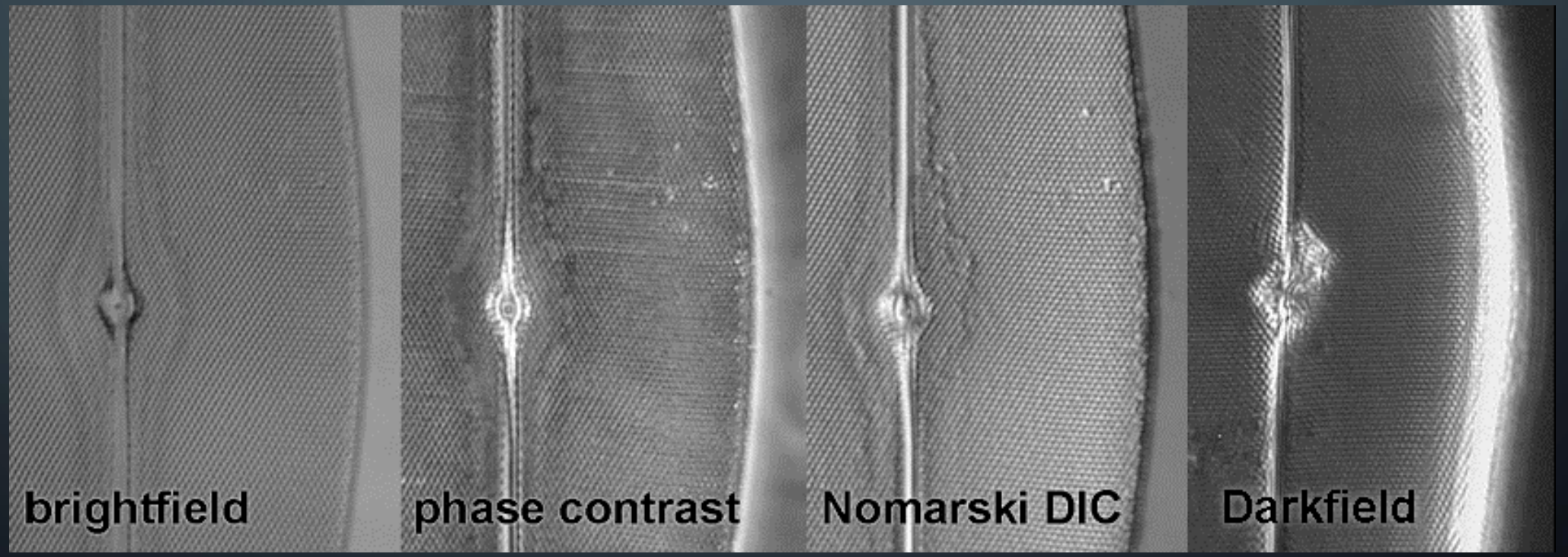


Heliozoans (*Actinophrys sol*)

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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

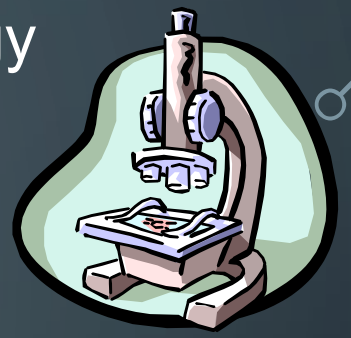
- Polarization Contrast -polarization

  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 (Fluorescent)



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.

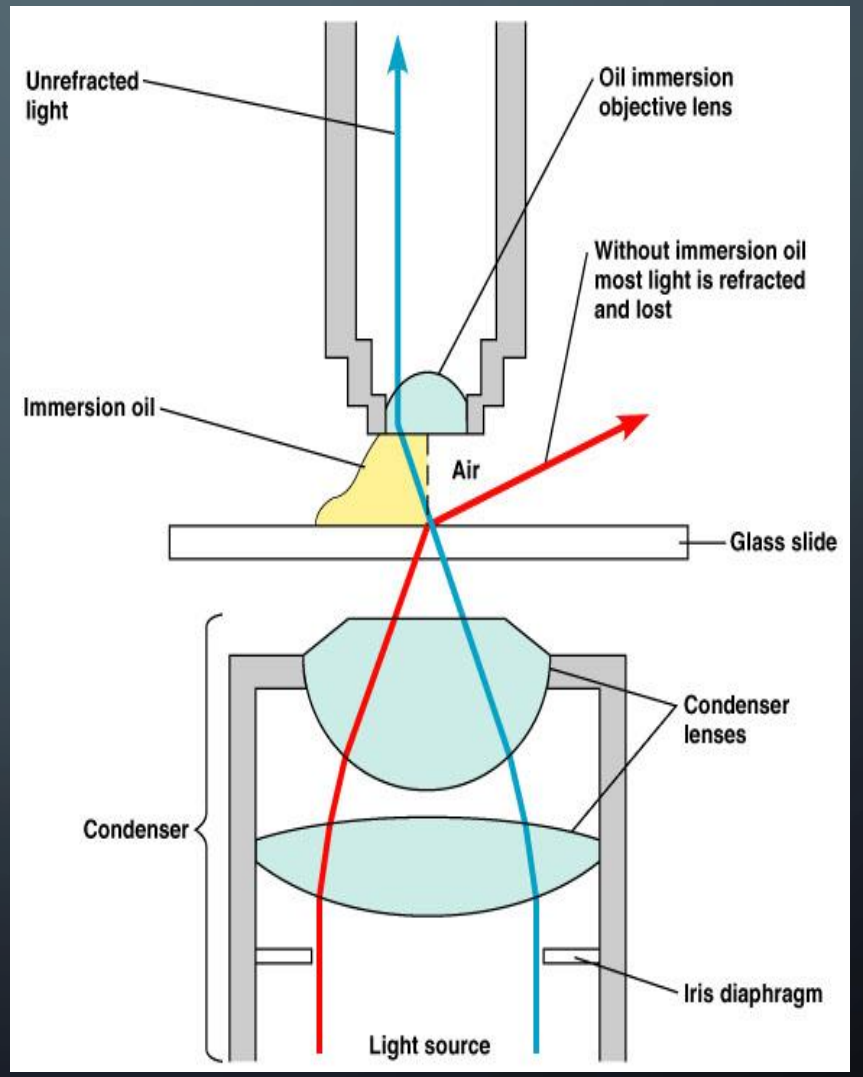
RI of Air- 1.0

RI of Water -1.3

RI of Glycerol- 1.47

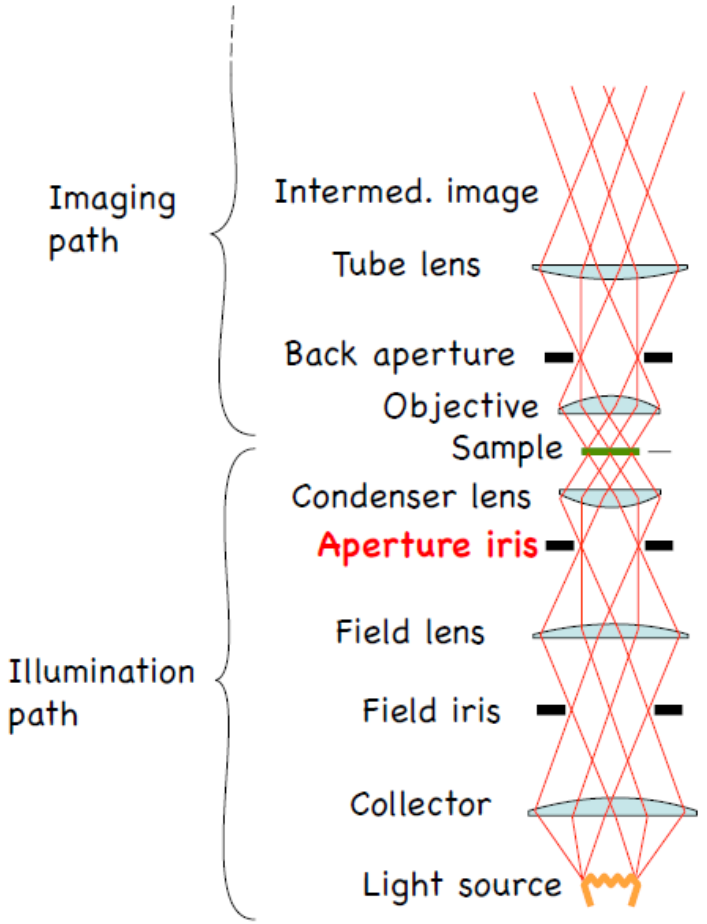
RI of Glass(avg)- 1.5

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