MICROSCOPIA OTTICA IN BIOLOGIA CELLULARE [675SM]

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Contrasting techniques with laboratory

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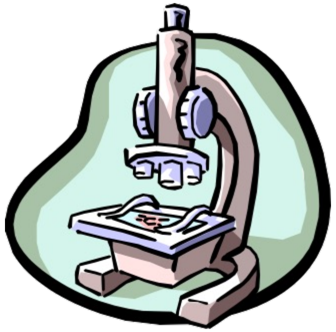
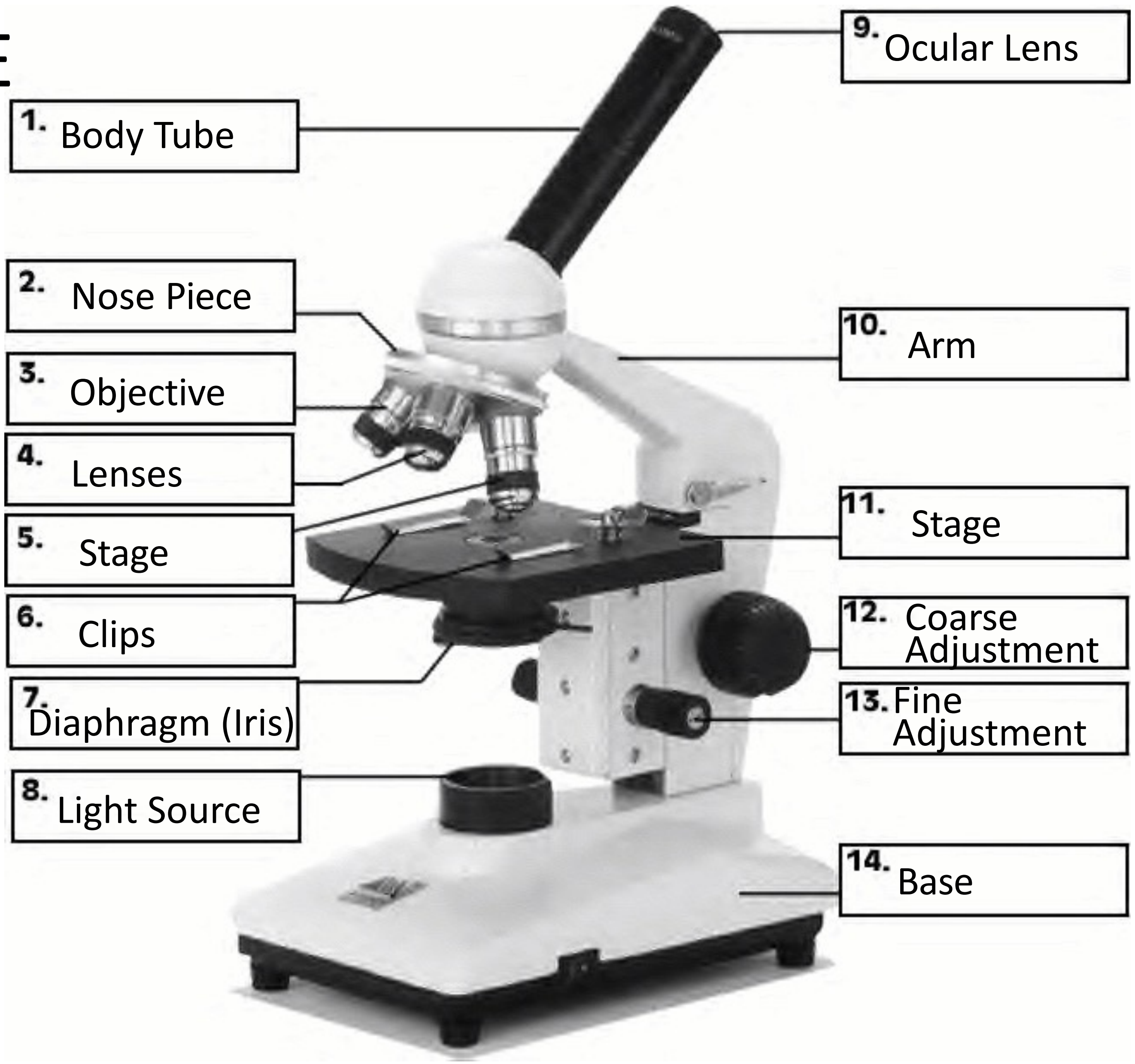
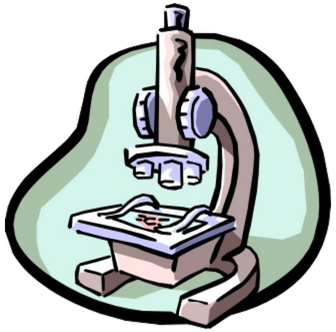
Last time….OPTICAL MICROSCOPY



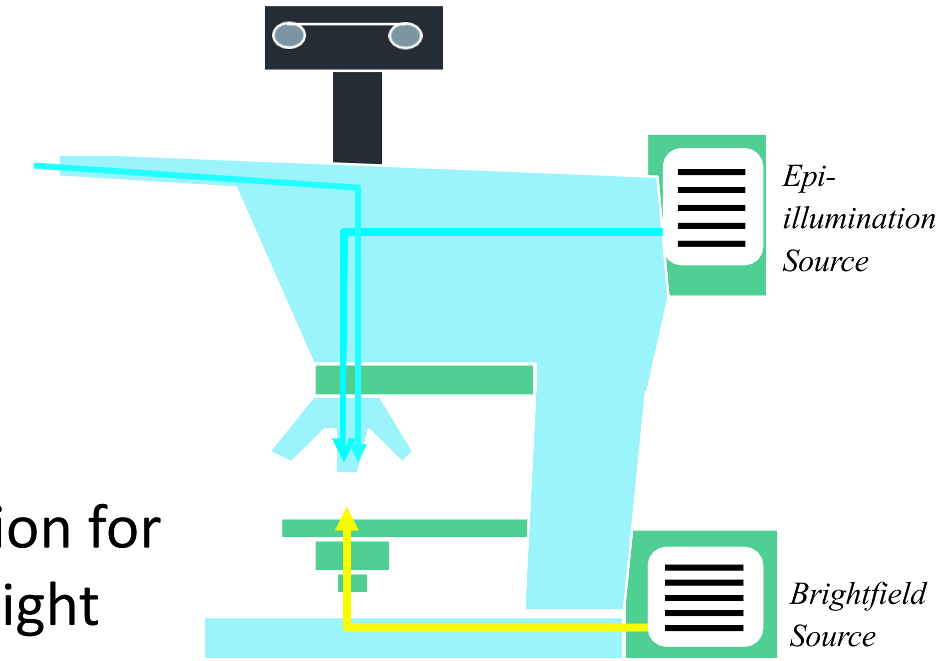
1. How a microscope works
2. Magnification vs resolution
3. Numerical aperture and working distance
4. Objectives
5. Point-spread function and Airy disk
6. Optical abberations

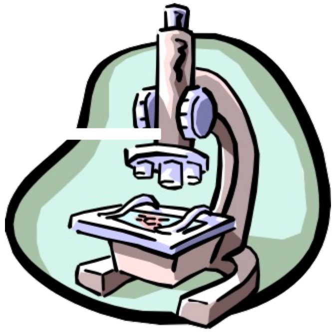
MICROSCOPE


1. Body Tube
2. Nose Piece
3. Objective
4. Lenses
5. Stage
6. Clips
7. Diaphragm (Iris)
8. Light Source
9. Ocular Lens
10. Arm
11. Stage
12. Coarse Adjustment
13. Fine Adjustment
14. Base



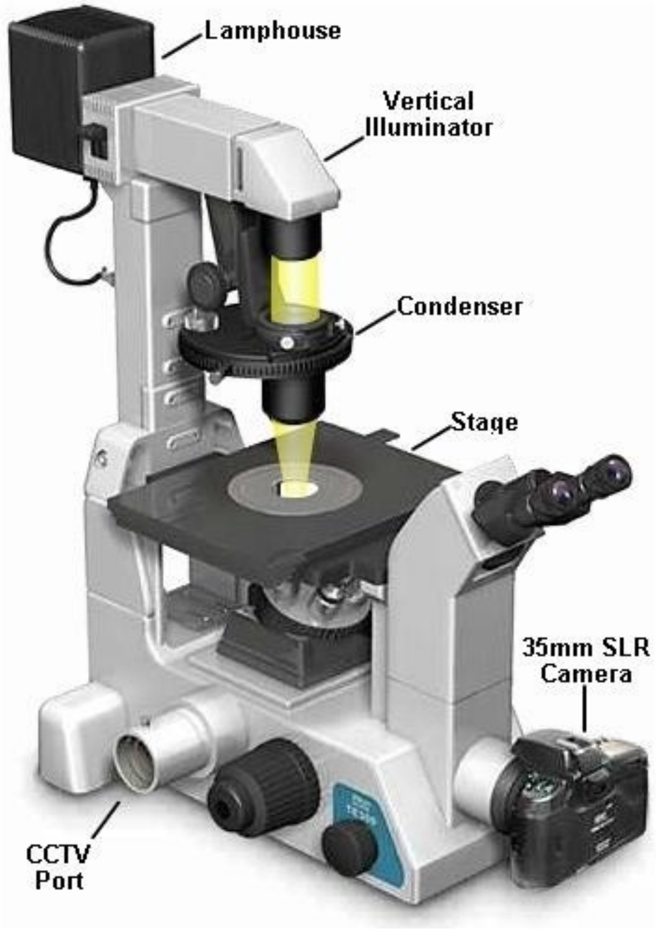
UPRIGHT MICROSCOPE

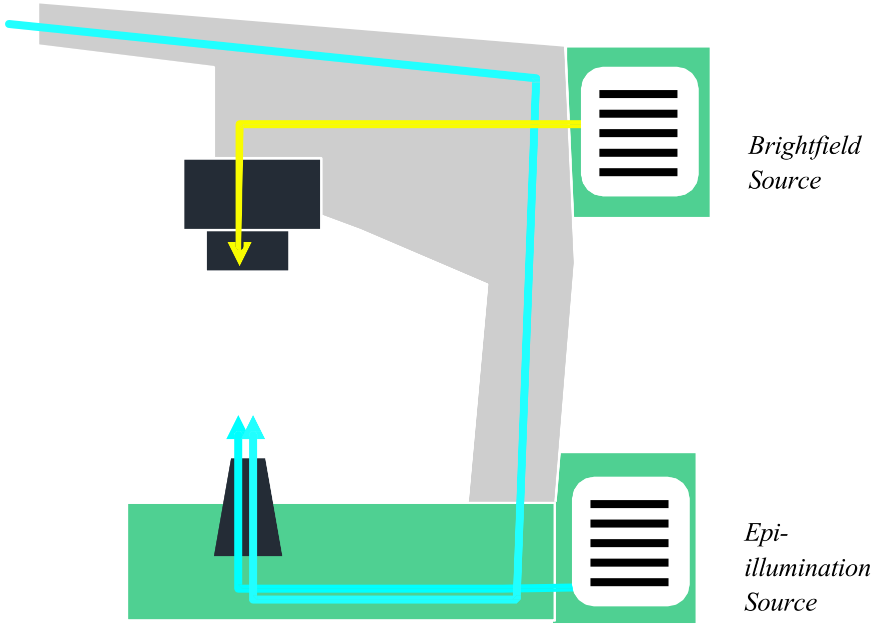


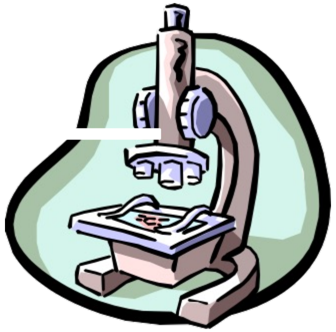


Transmitted and reflected light: transmission for bright field and reflection for flourescent light

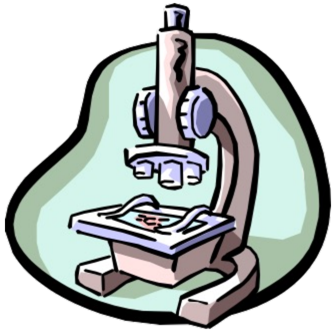
INVERTED MICROSCOPE

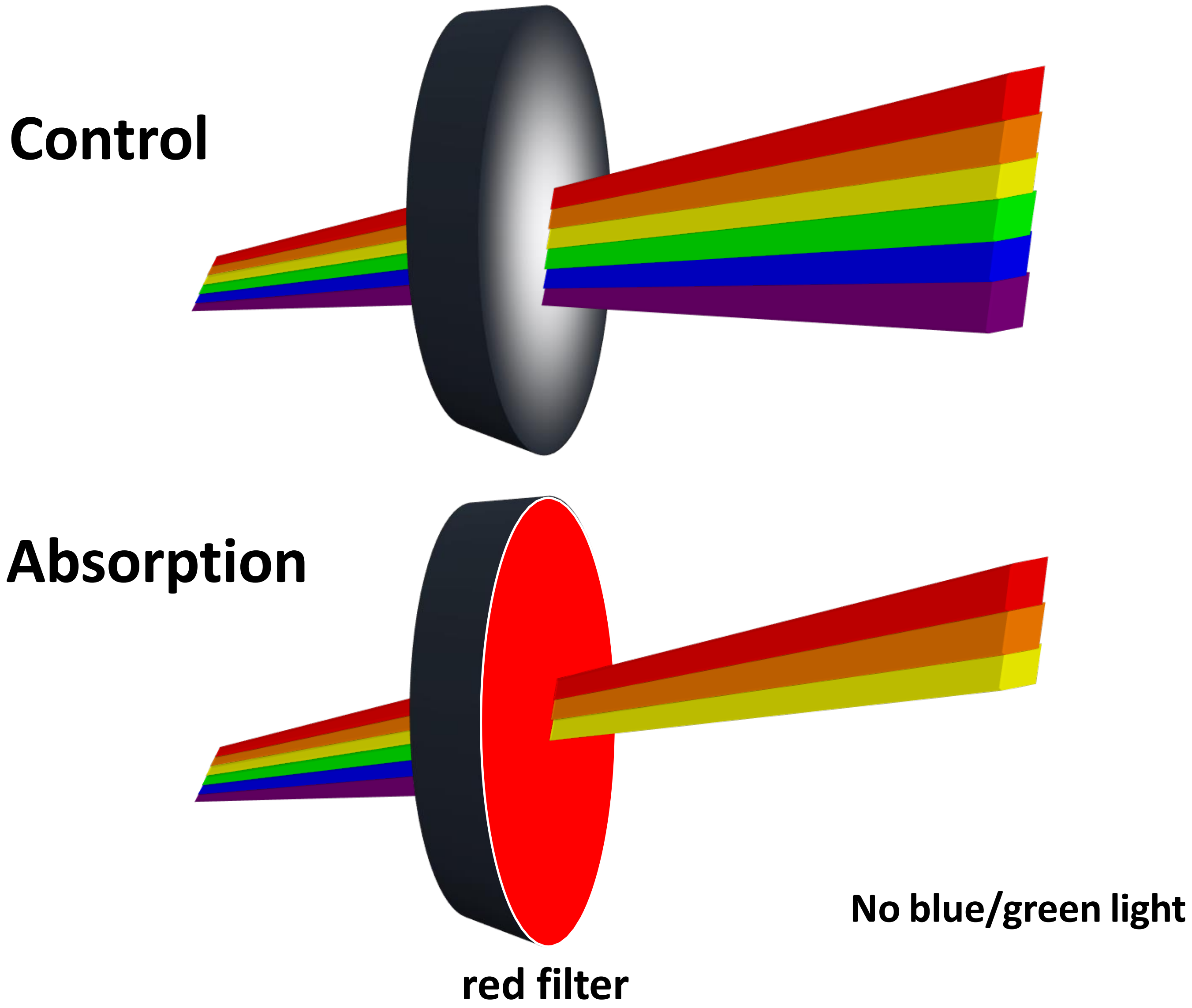




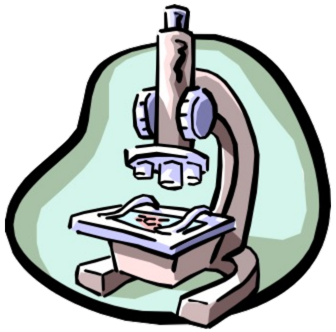


ABSORPTION

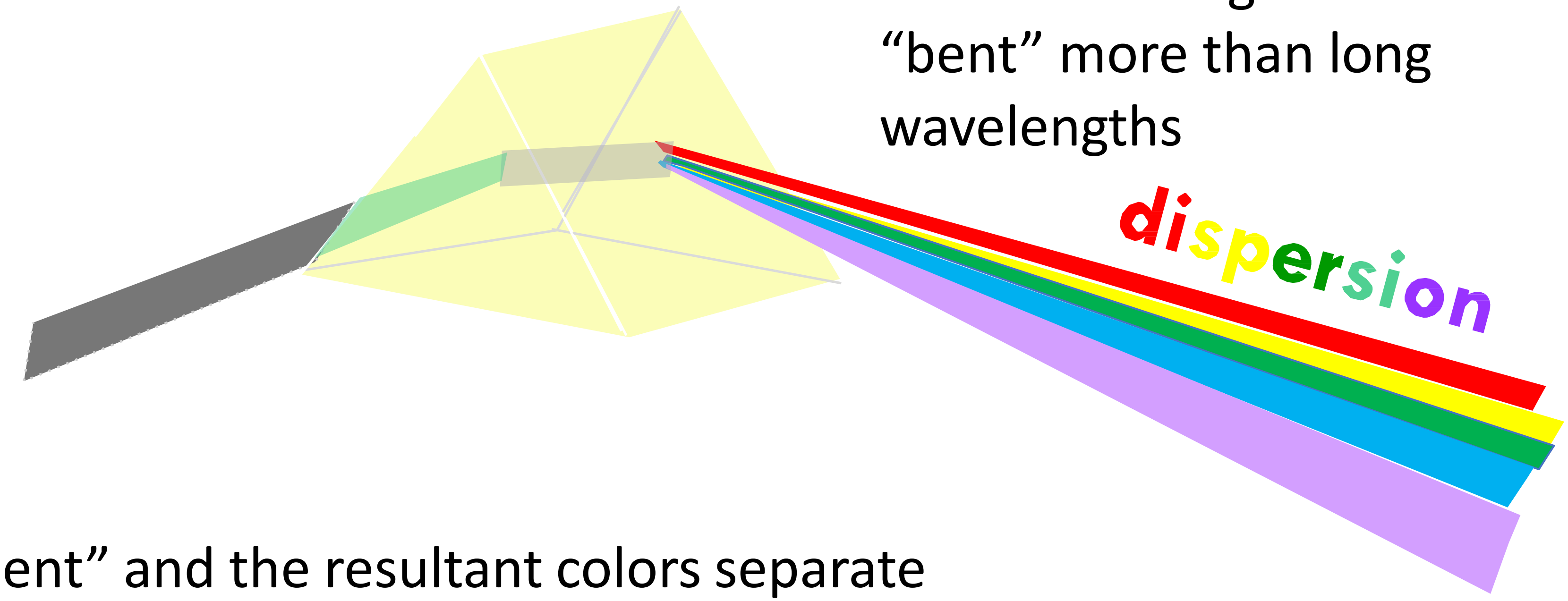




REFRACTION & DISPERSION



Short wavelengths are "bent" more than long wavelengths

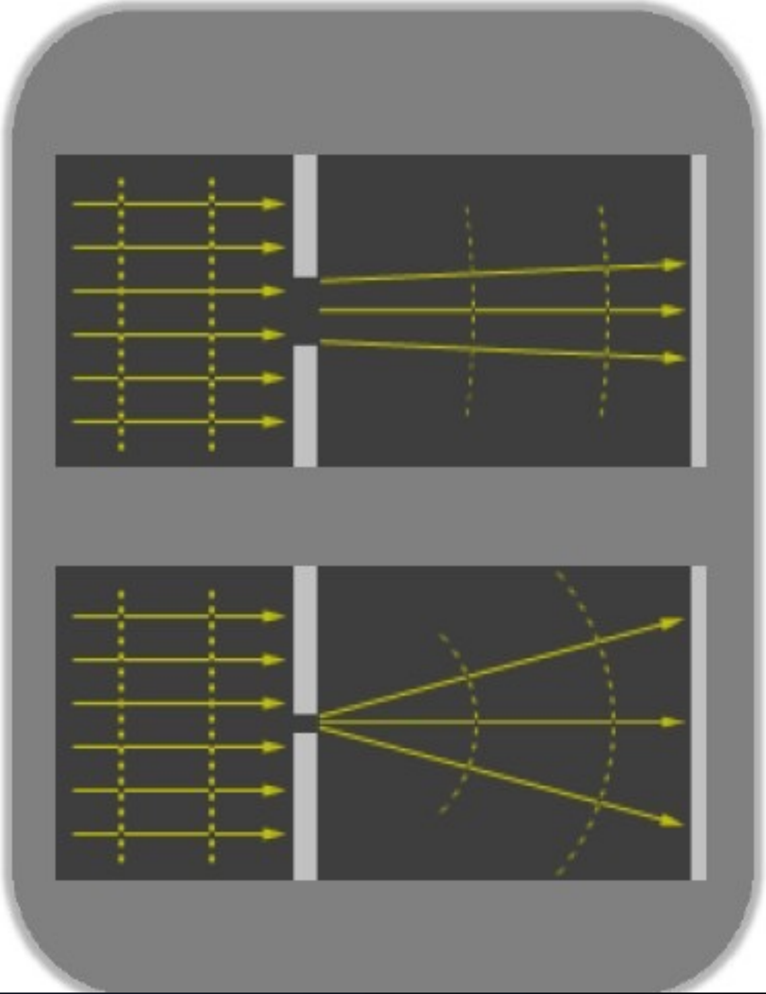


Light is "bent" and the resultant colors separate (dispersion). Red is least refracted, violet most refracted.

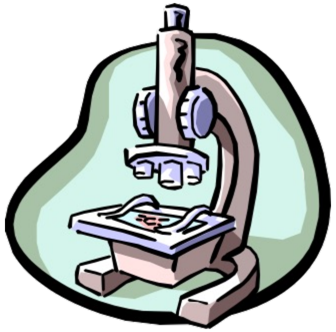
DIFFRACTION

Diffraction: Rays

- Parallel rays incident on an aperture, say rays from a point source at infinity, begin to diverge.
- The smaller the aperture, the larger the divergence.
- This can be explained if we consider light as a wave phenomenon

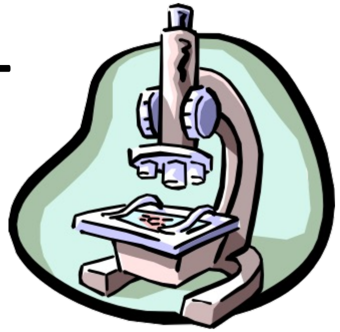


This lesson: CONTRASTING TECHNIQUES



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

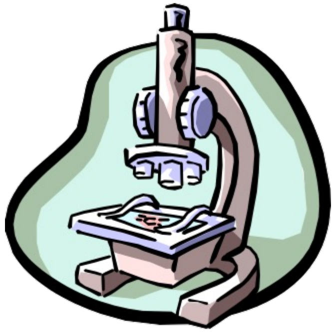
CONTRAST GENERATION FOR TRANSMITTED LIGHT



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

  >>> Phase shift / Polarization / Interference

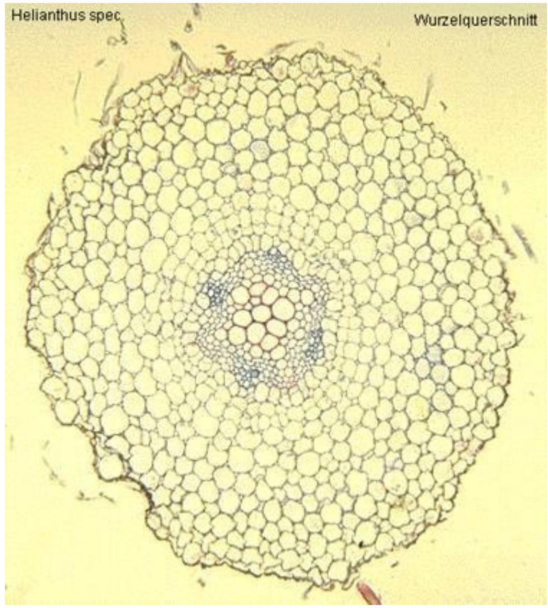
BRIGHTFIELD



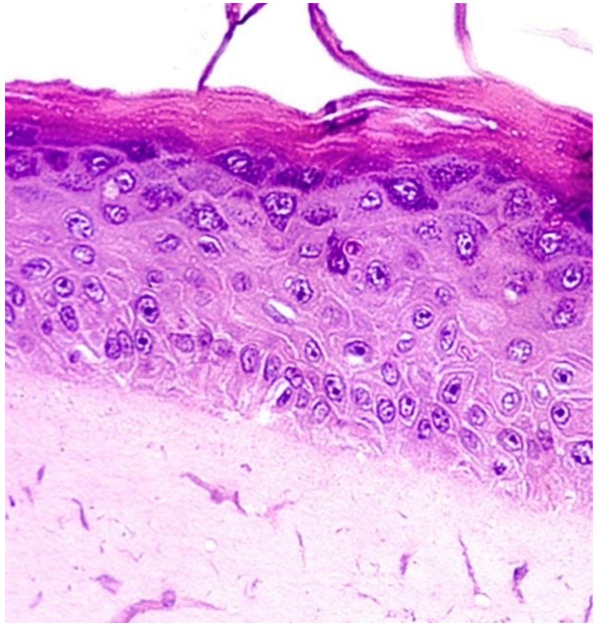
Principle:
Light is transmitted through the sample and absorbed by it.

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.

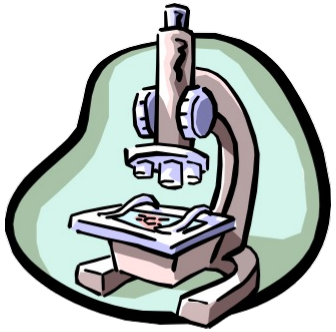


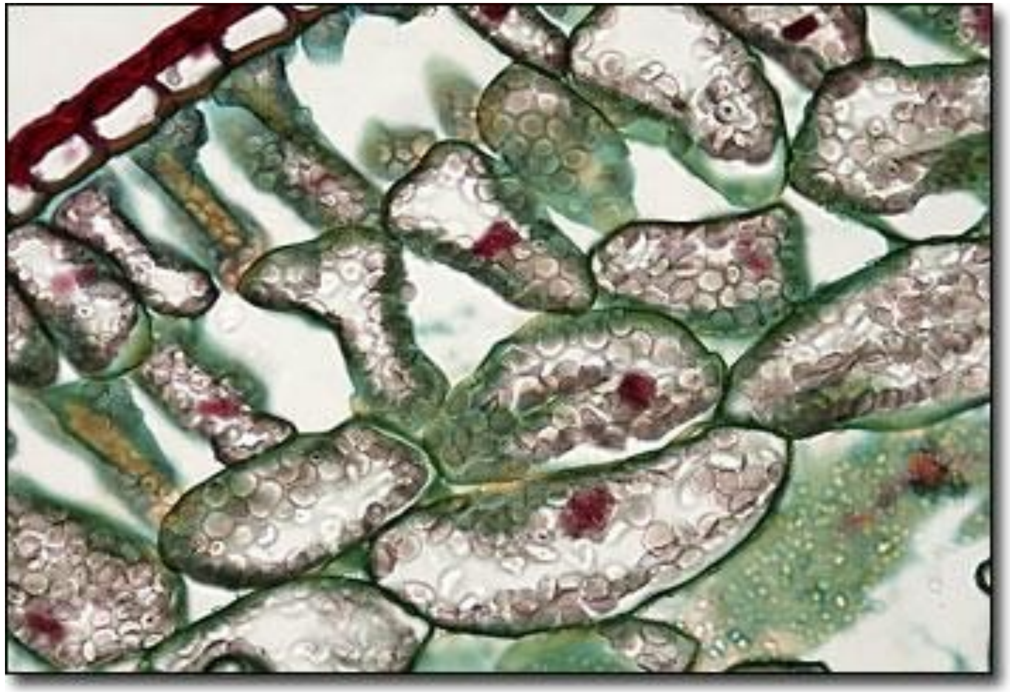
Cross section of sunflower root
(http: /www.zum.de/Faecher/Materialien/beck/12/bs12-5.htm)



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

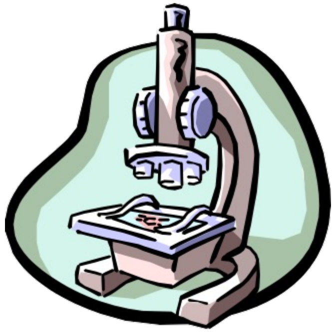
BRIGHTFIELD





- Bright Field is the most universal technique used in light microscope.
- Usually used in samples with colorimetric staining or good contrast; dark sample absorbing light on bright background
- Particularily useful on samples with intrinsic colour (e.g. chloroplasts)
- AI programs using mashine-learning techniques on stained samples to be able to identify cells/organelles etc.. without staining in Bright Field.

DARKFIELD

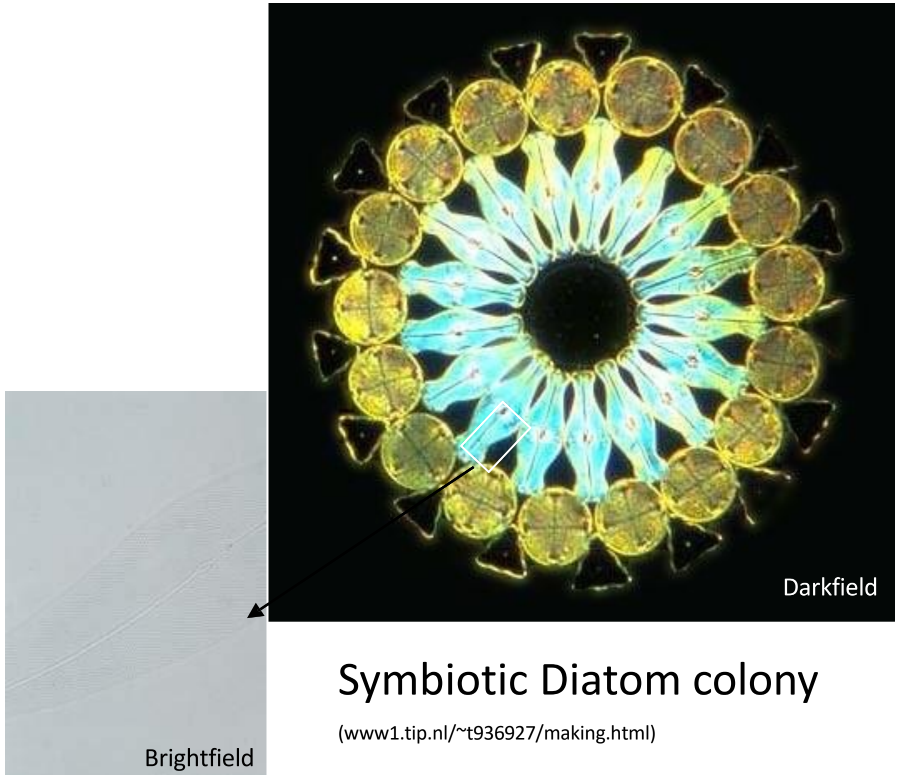


Principle:

The illuminating rays of light are directed through the sample from the side by putting a dark disk 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:

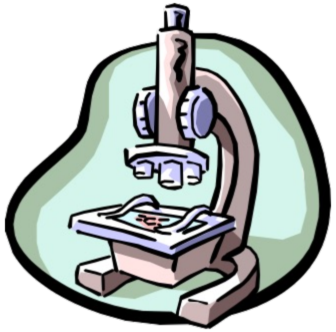
- Diatoms and other unstained or colourless specimens



Symbiotic Diatom colony

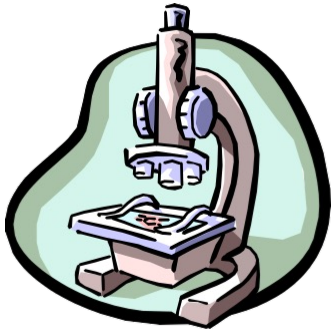
(www1.tip.nl/~t936927/making.html)

DARKFIELD

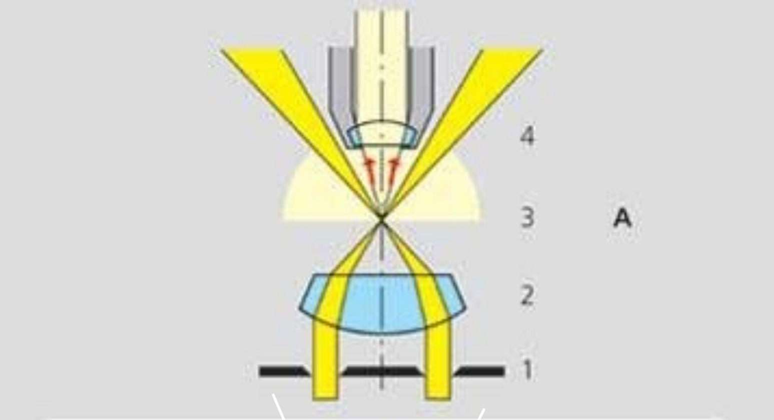


Fine structures can often not be seen in front of a bright background.

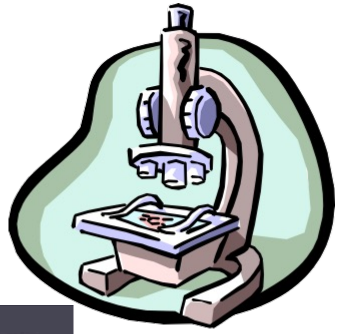
DARKFIELD

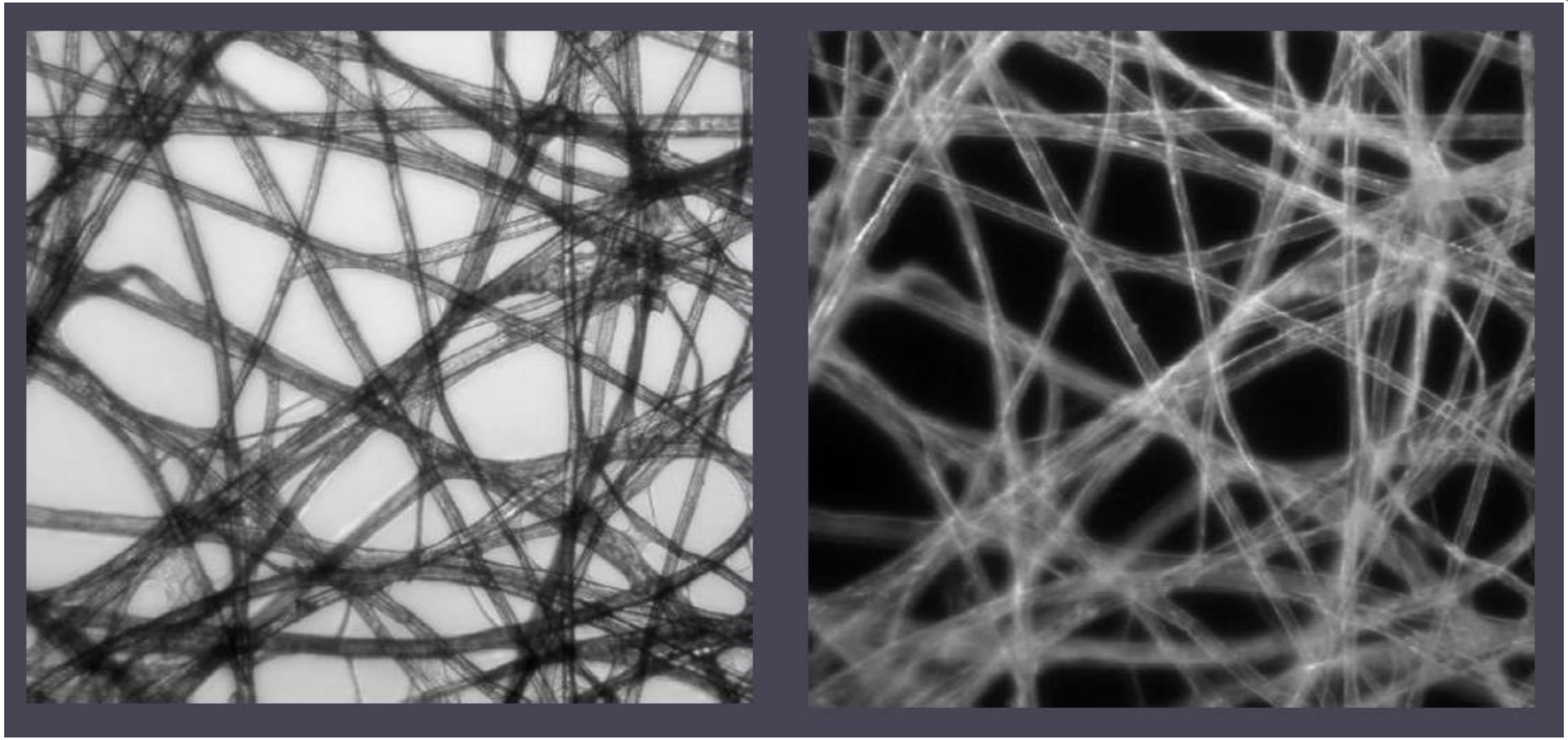


- Dark background against which objects are brilliantly illuminated.
- This is accomplished by a special condenser that transmits a hollow cone of light.
- Most of the light directed through the condenser does not enter the objective, the field is dark.
- However, some of the light rays will be scattered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

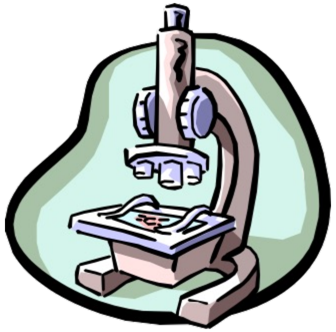


DARKFIELD



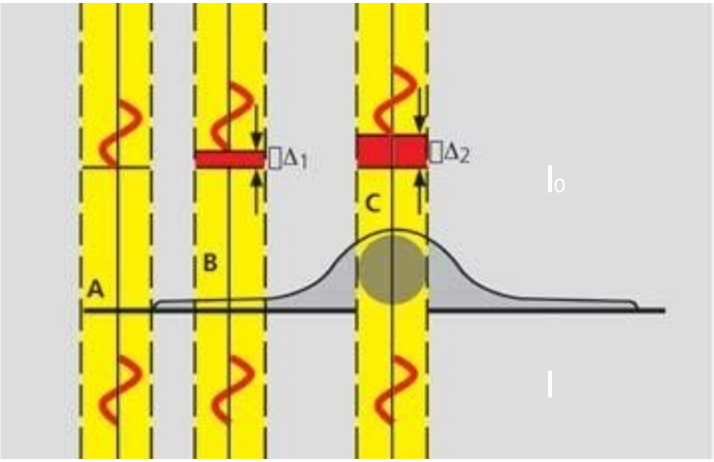


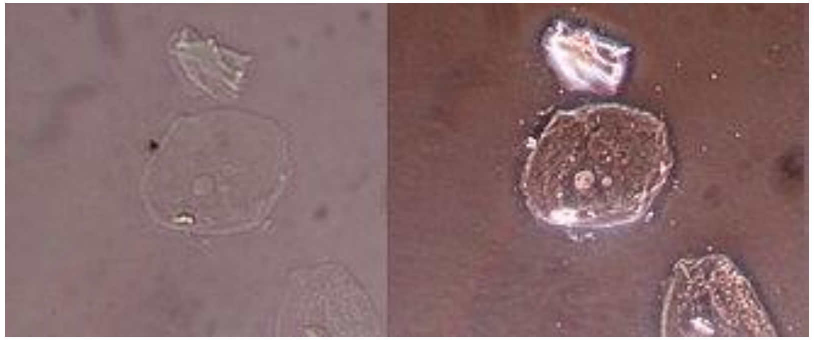
PHASE CONTRAST

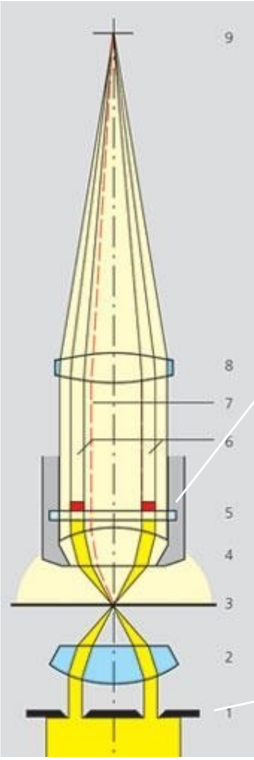


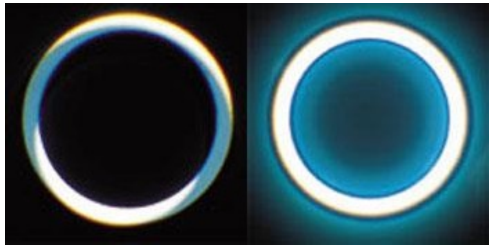
Principle:

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

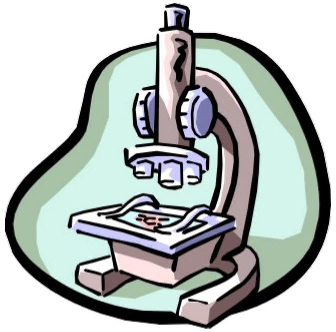






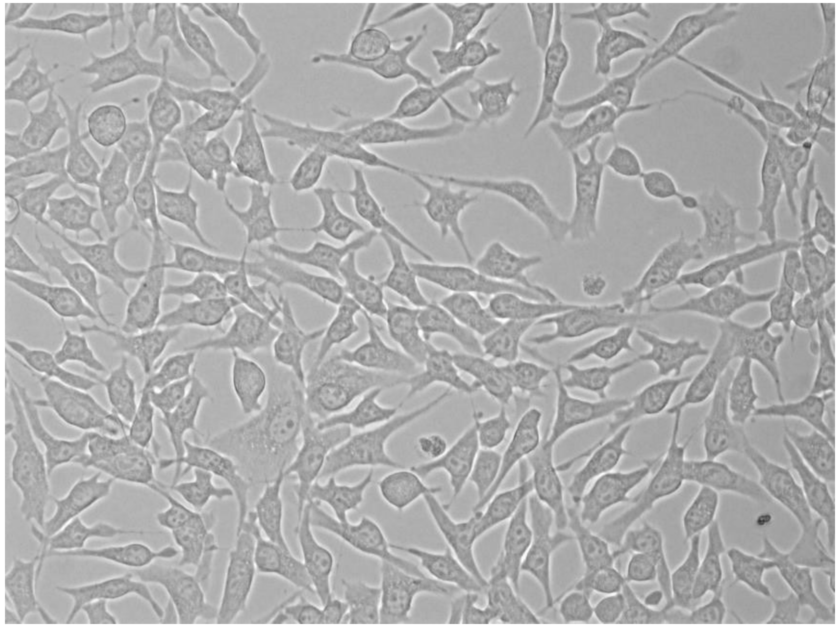


PHASE CONTRAST

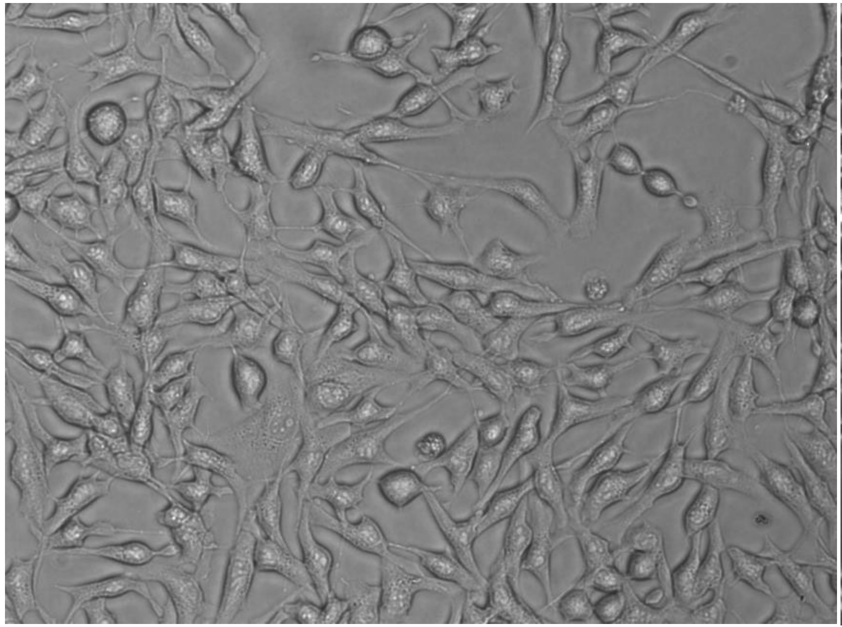


Phase contrast in practice

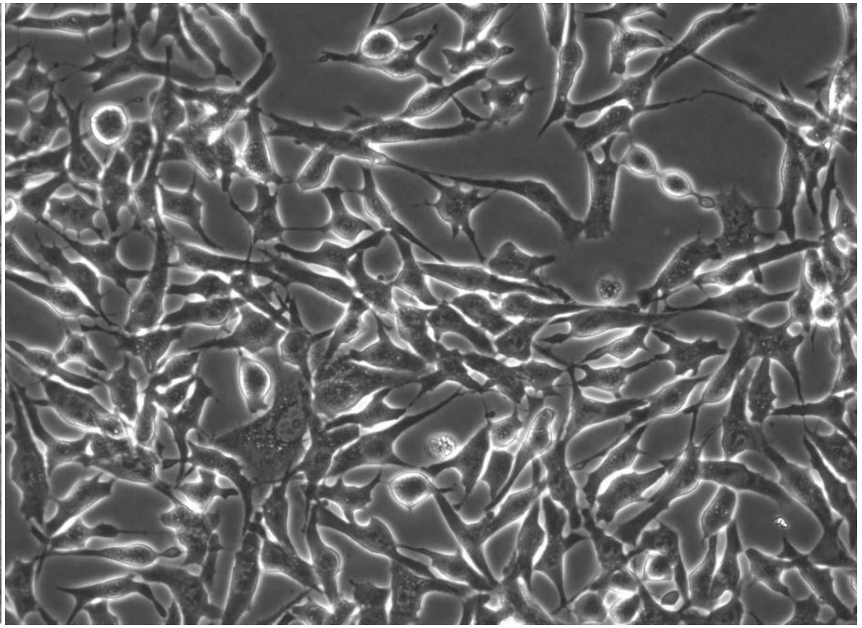
Application: Phase contrast is the most commonly used contrasting technique
All tissue culture microscopes and many time-lapse microscopes are set up for phase contrast.



brightfield

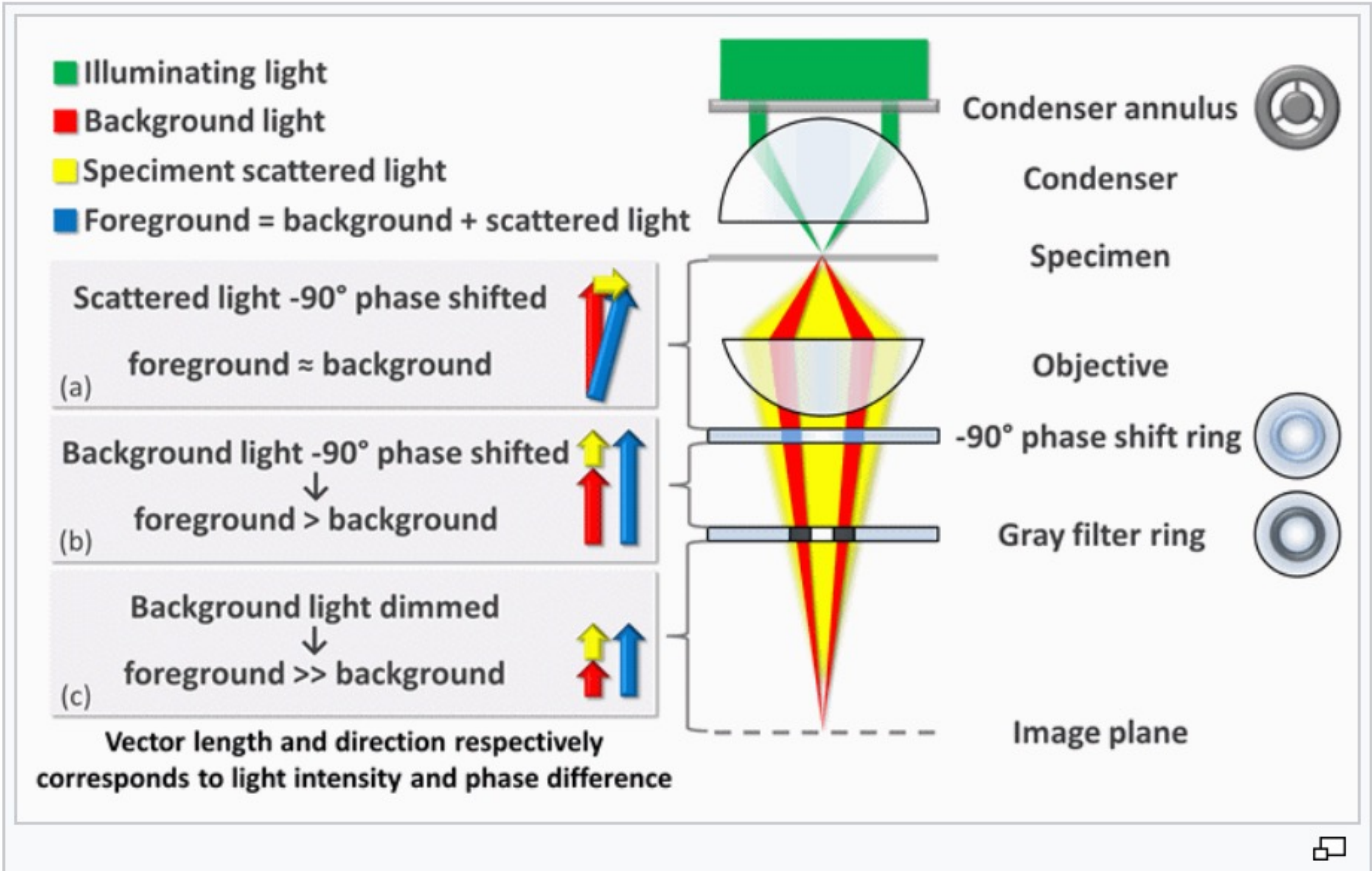


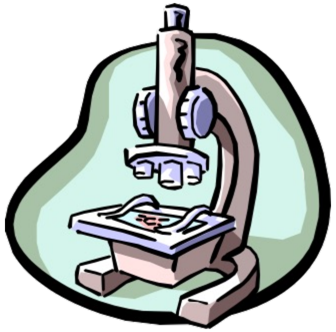
wrong phase stop



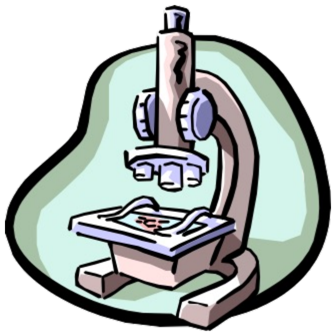
right phase stop

PHASE CONTRAST

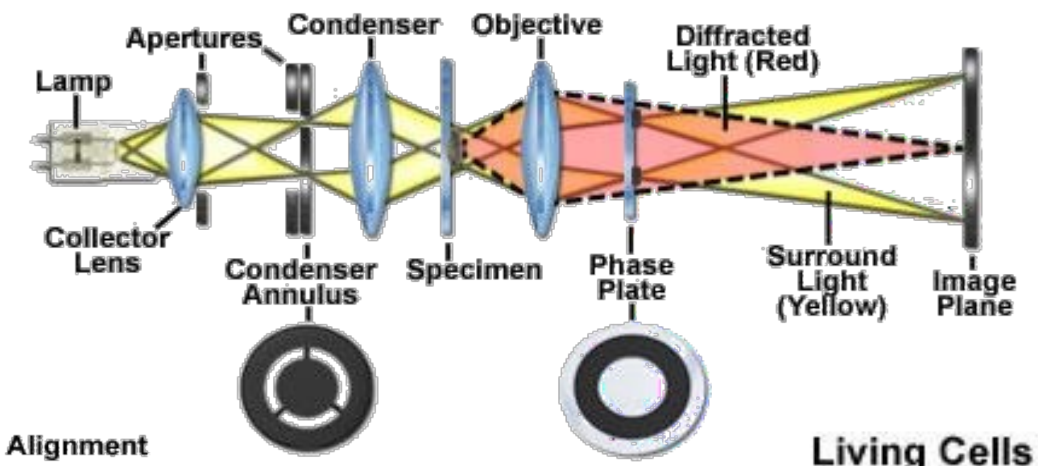




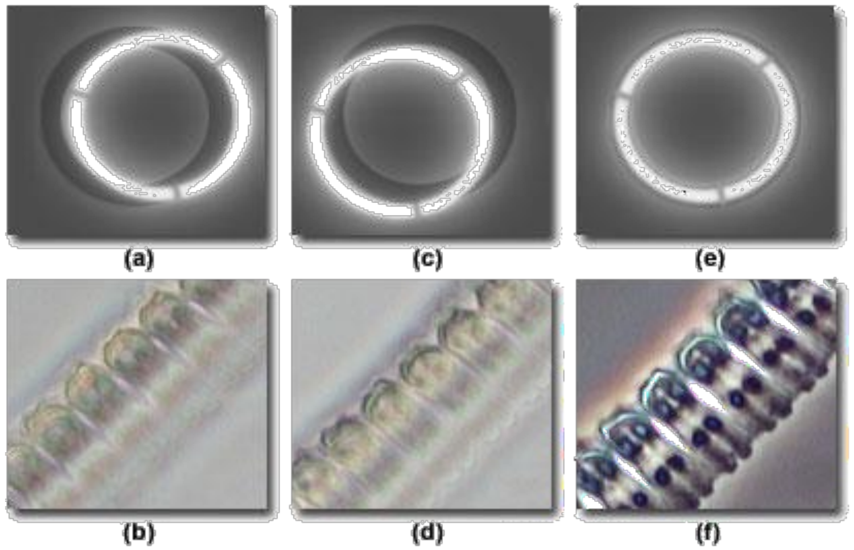
PHASE CONTRAST



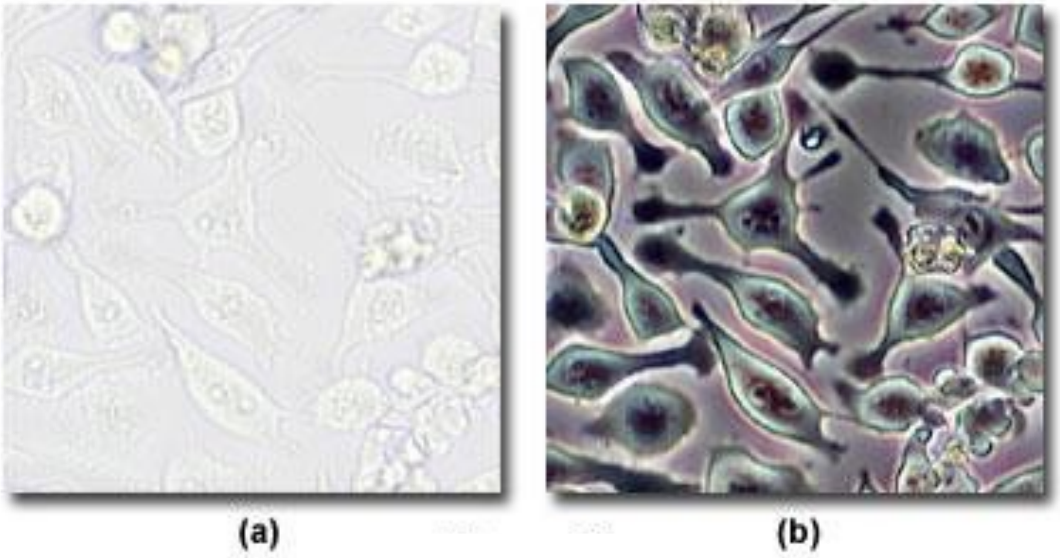
Phase Contrast Microscope Optical Train



Phase Contrast Optical System Alignment

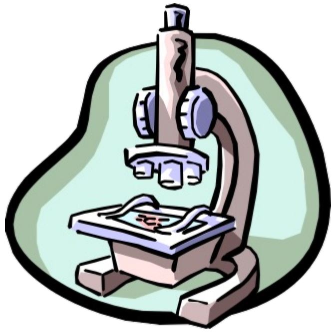


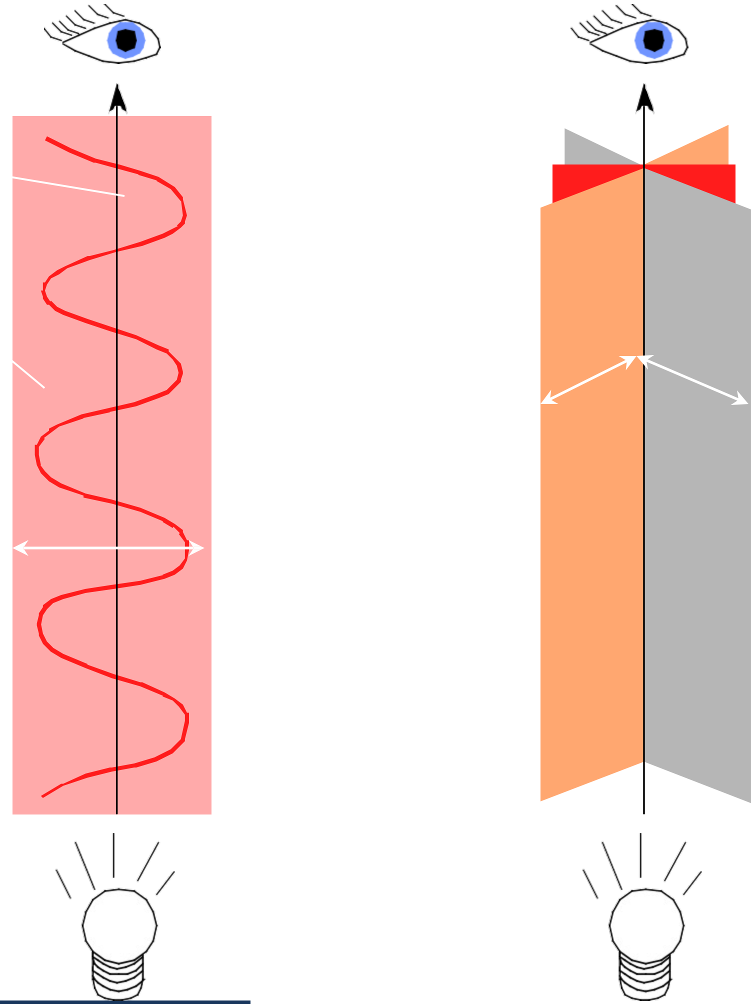
Living Cells in Brightfield and Phase Contrast



THEORY & APPL. LIGHT MICROSCOPY

POLARISATION

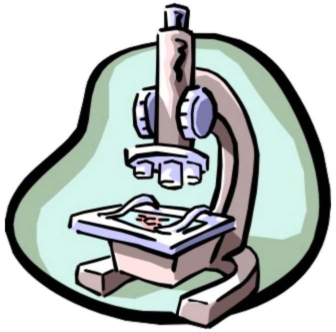


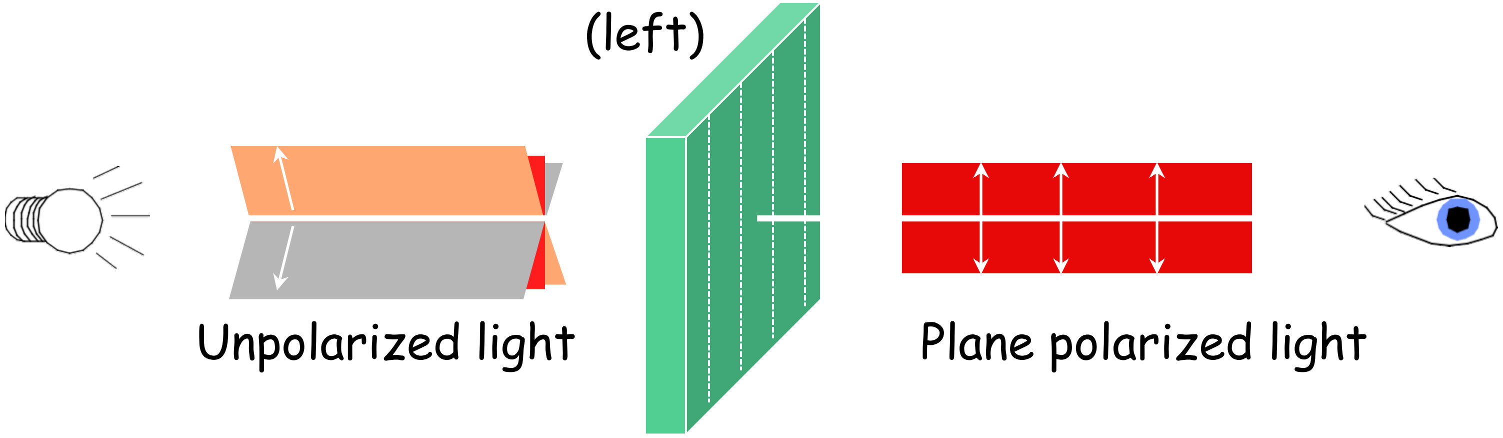


light vibrates in all planes that contain the light ray (i.e., all planes perpendicular to the propagation direction)

POLARISATION

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



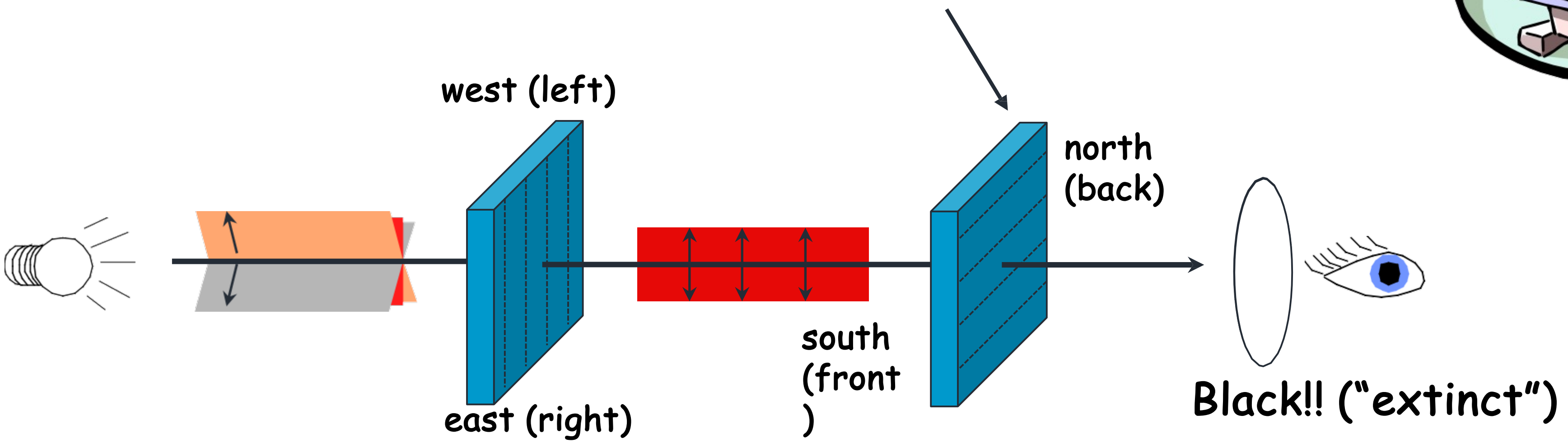


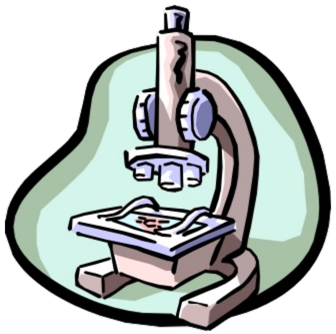
Only the component of light vibrating in E-W direction can pass through lower polarizer – **light intensity decreases**

Though polarized, still white light!

POLARISATION

2) Insert the **upper polarizer**



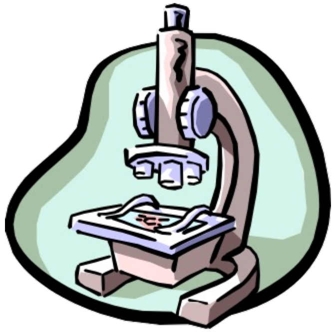


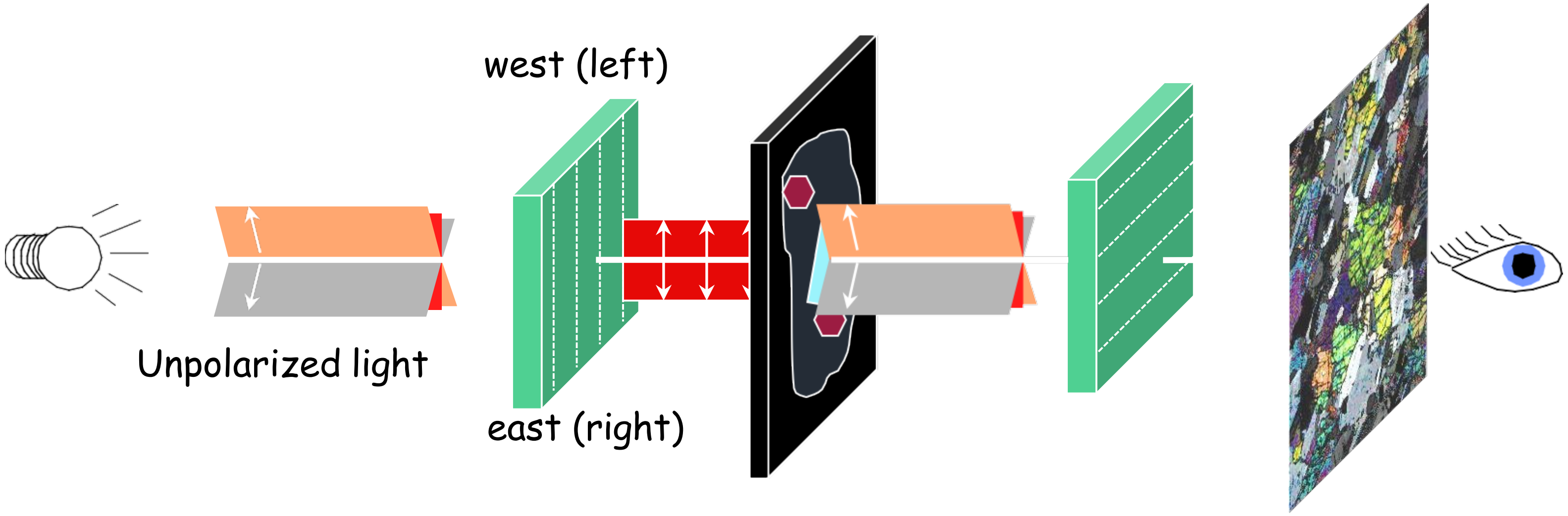
Now what happens?
What reaches your eye?

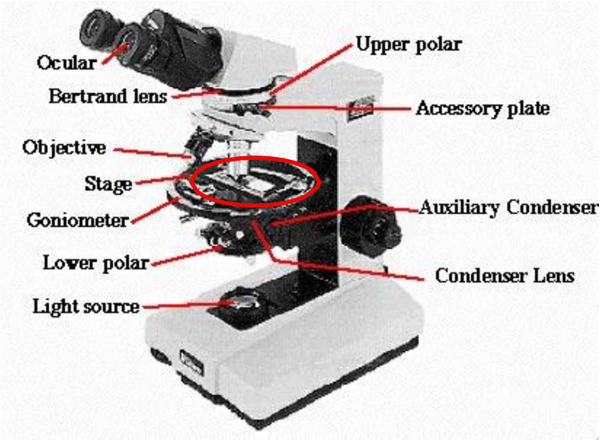
Why would anyone design a microscope that prevents light from reaching your eye???

POLARISATION

3) Now insert a **thin section**







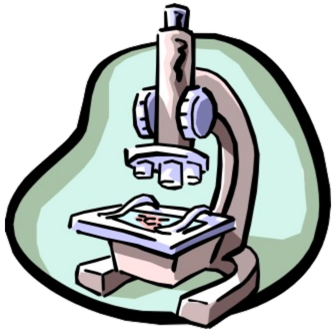
Light vibrating E-W

Light vibrating in many planes and with many wavelengths

Light and colors reach eye!

How does this work??

POLARISATION CONTRAST

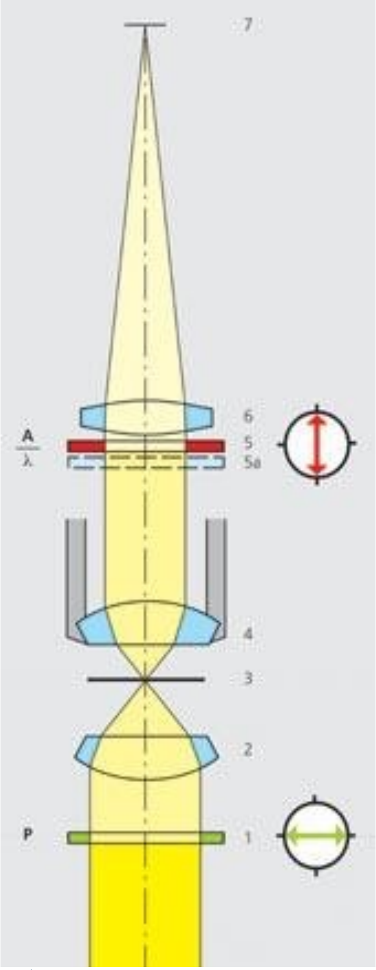


Principle:

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:

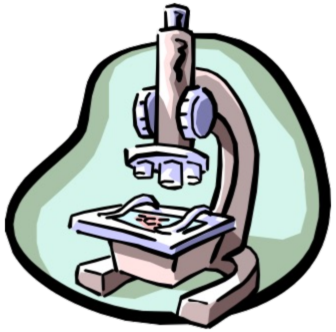
Polarization contrast is used to look at materials with birefringent properties, in which the refractive index depends on the vibration direction of the incident light, e.g. crystals or polymers.

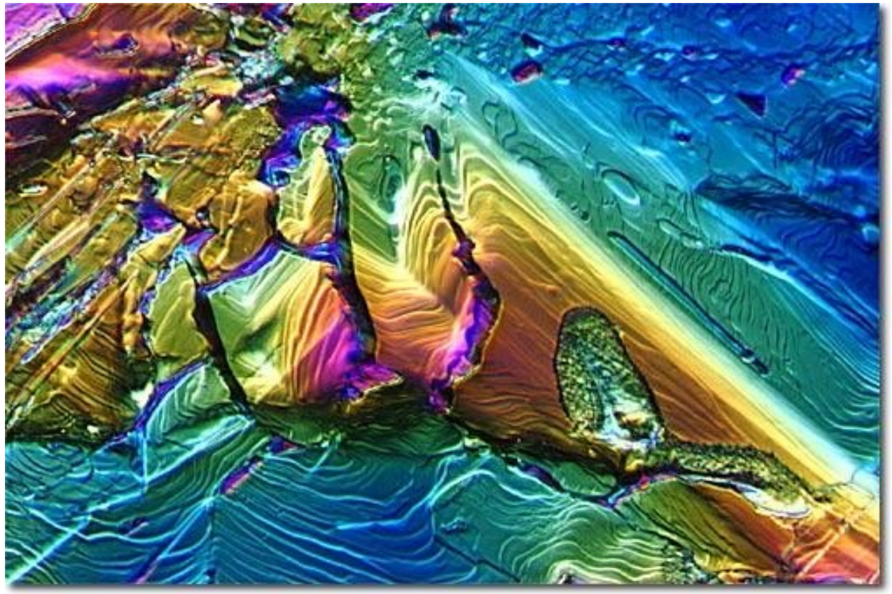


Brightfield

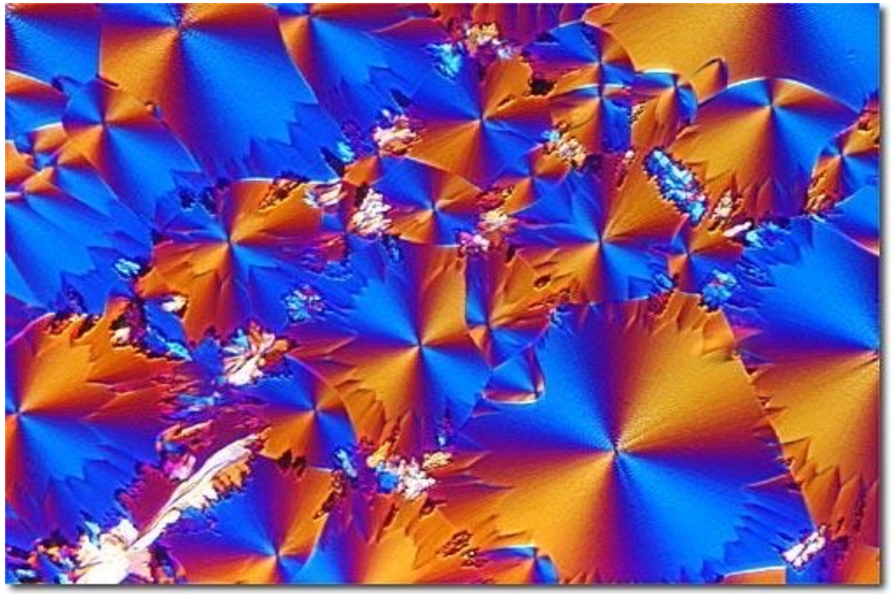
Polarization contrast

Polarization contrast with Lambda plate



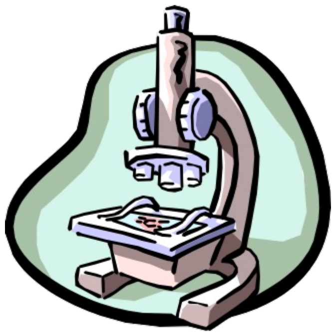


Glutaric Acid Crystallites



Dinosaur Bone

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

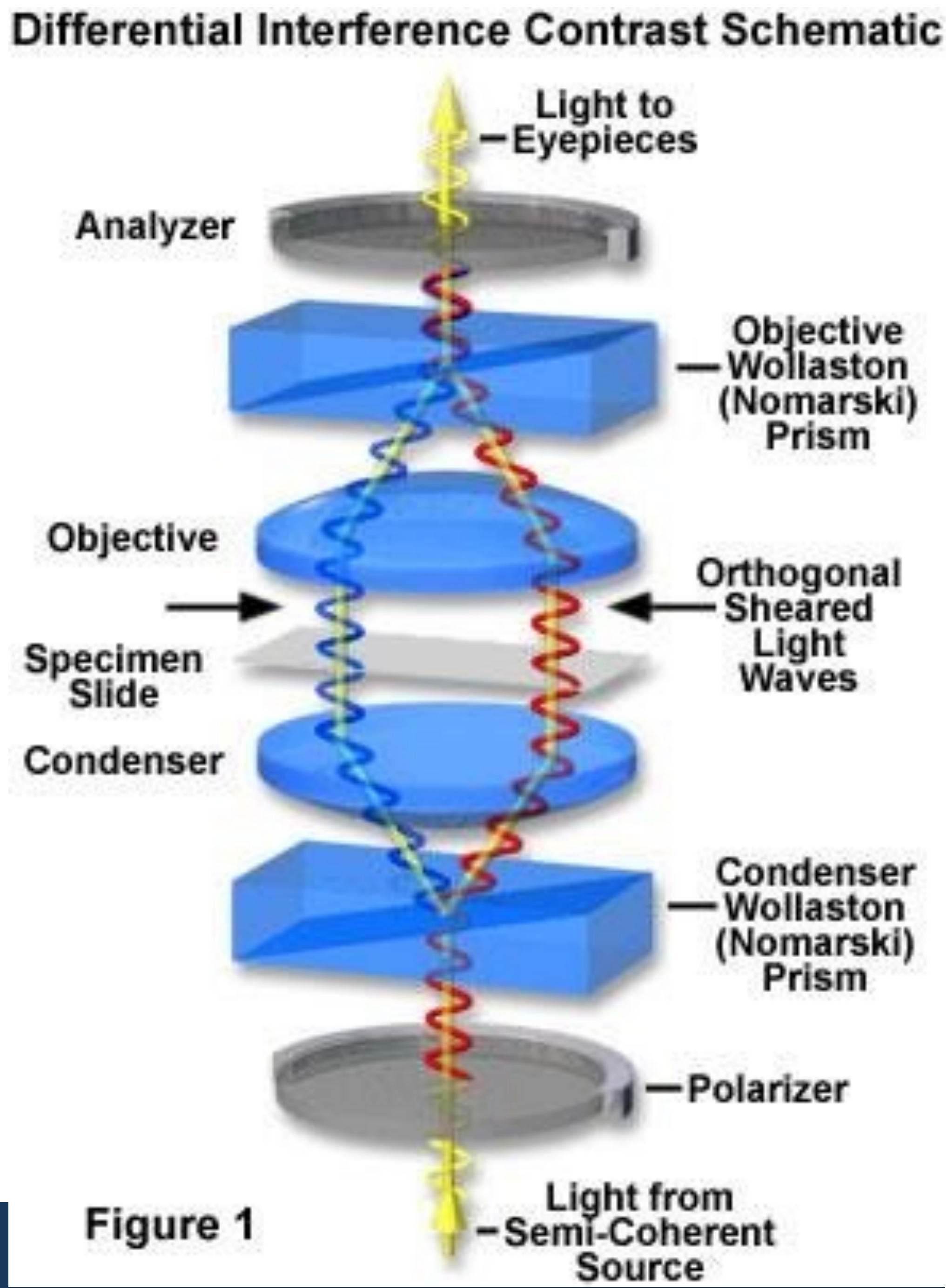
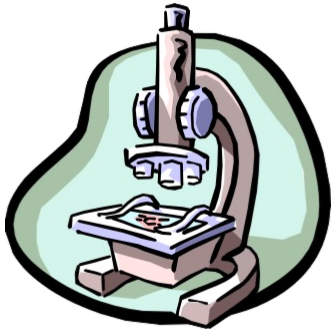
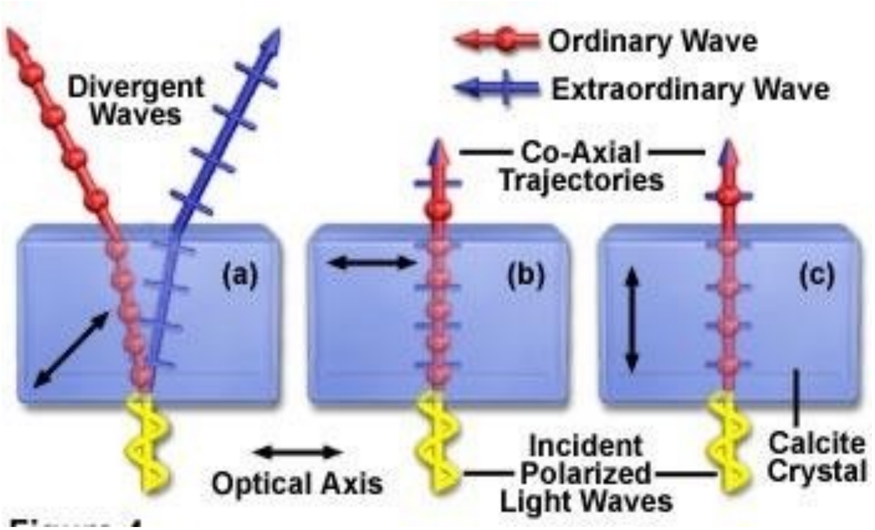

Differential Interference Contrast Schematic
Light to Eyepieces
Analyzer
Objective Wollaston (Nomarski) Prism
Objective
Orthogonal Sheared Light Waves
Specimen Slide
Condenser
Condenser Wollaston (Nomarski) Prism
Polarizer
Light from Semi-Coherent Source

Figure 1



BIREFRINGENCE



Bi-Refraction in Calcite Crystals

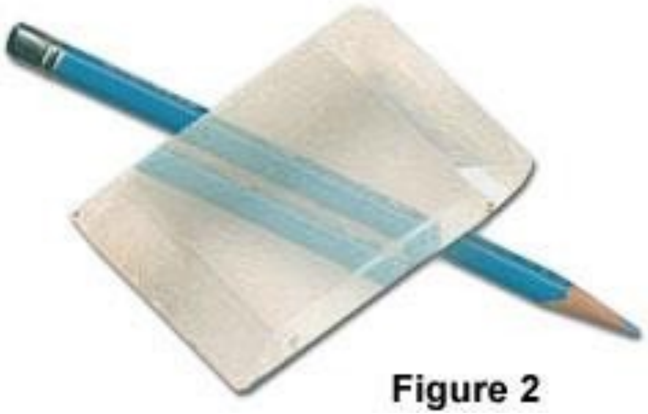
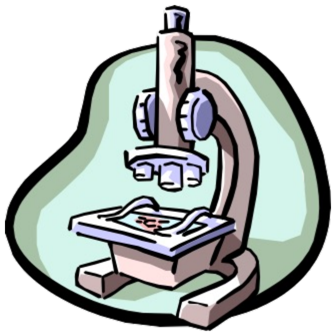


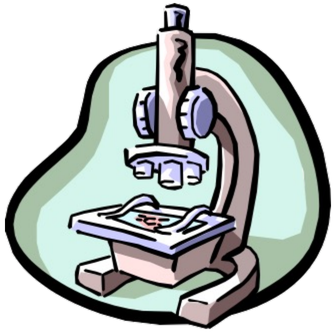
Figure 2

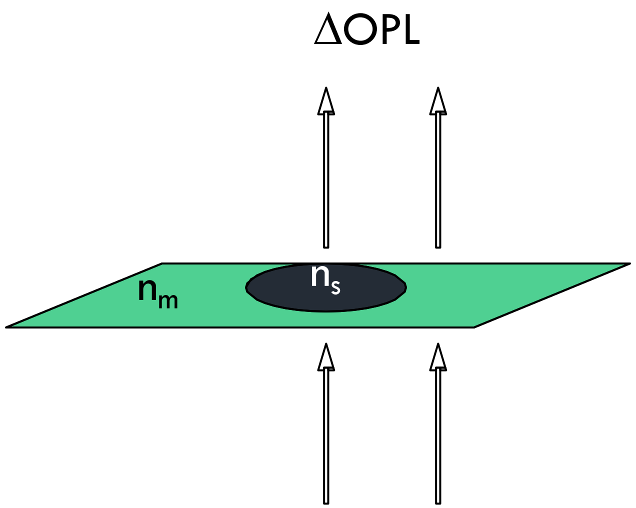


- Birefringent materials have different indices of refraction for light polarized parallel or perpendicular to the optical axis.

- Two beams with orthogonal polarization are produced if illumination is at an angle to optical axis

Differential Interference Contrast (DIC)

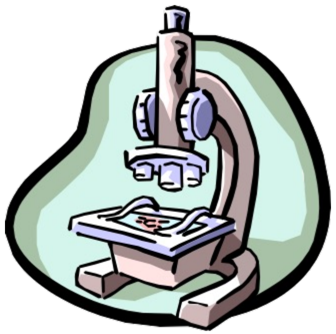


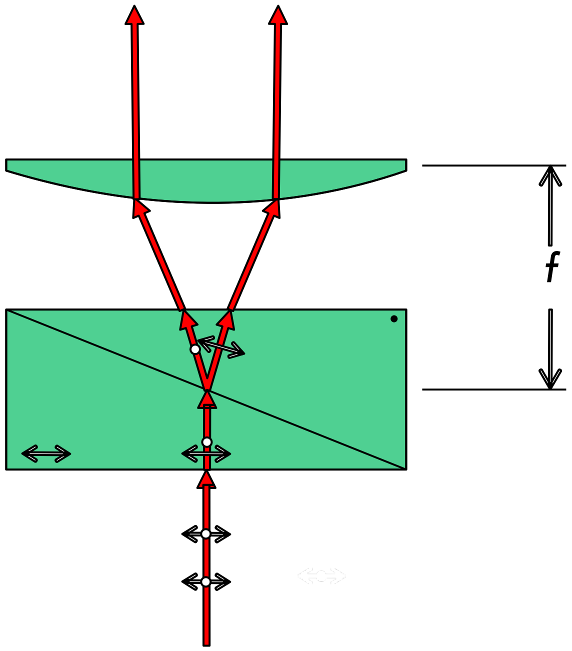


The idea:

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

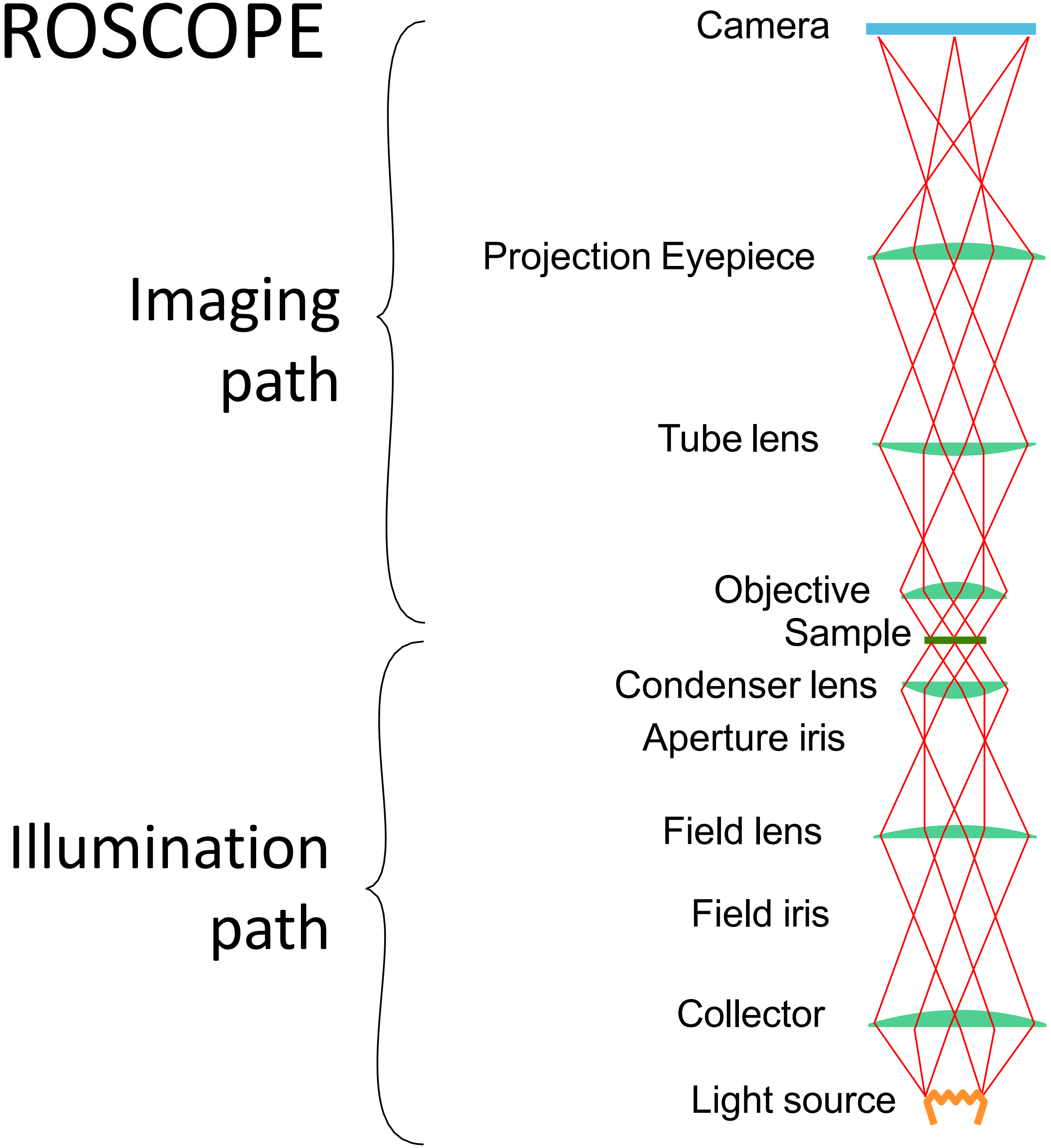
WOLLASTON / NOMARSKI PRISMS

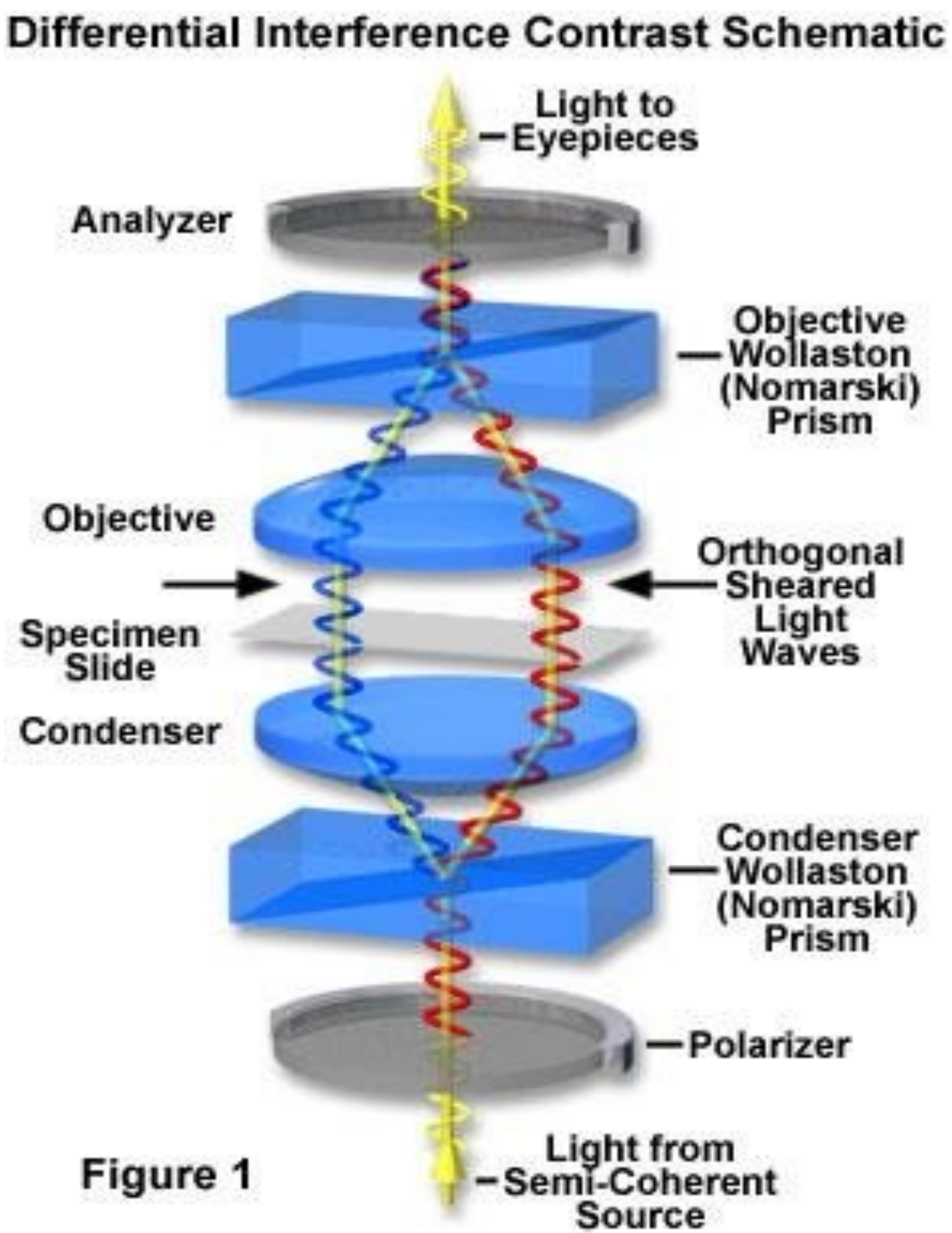




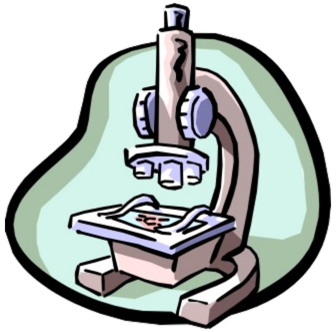
- Two pieces of cemented calcite/quartz
- Produce orthogonally polarized beams propagating at different angles
- Placed at a back focal plane, this produces the two beams that will probe the OPL difference of our sample

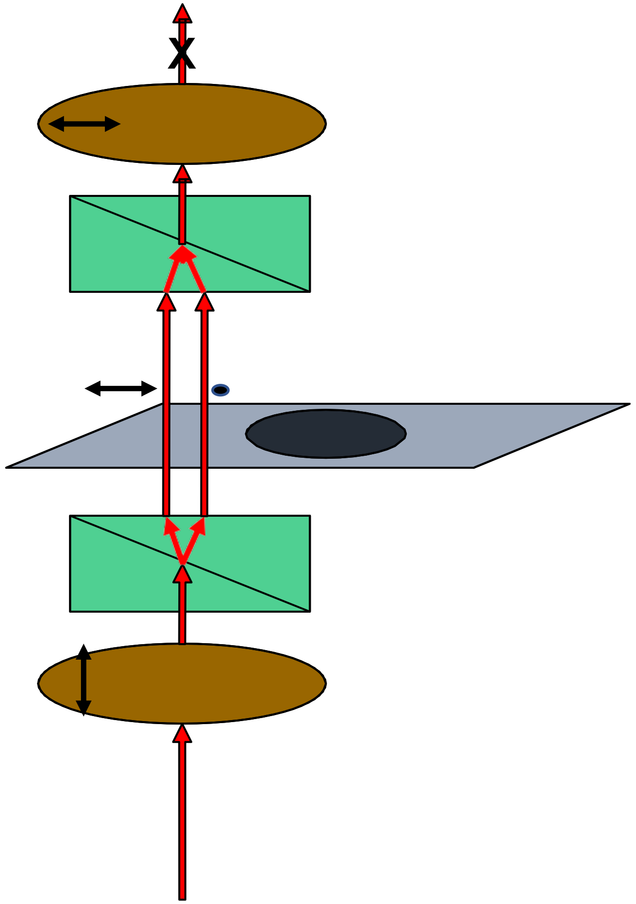
THE DIFFERENTIAL INTERFERENCE CONTRAST (DIC) MICROSCOPE





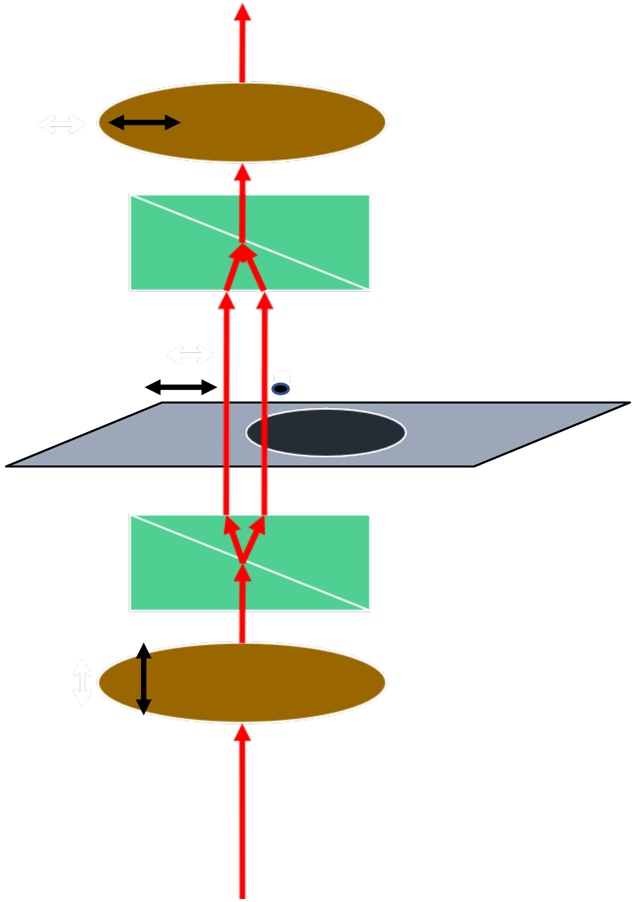
HOW DIC GENERATES CONTRAST

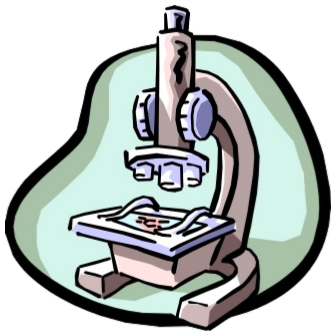




- Both beams see same OPL
- Emerge in phase
- Regenerate initial polarization
- No light makes it through analyzer

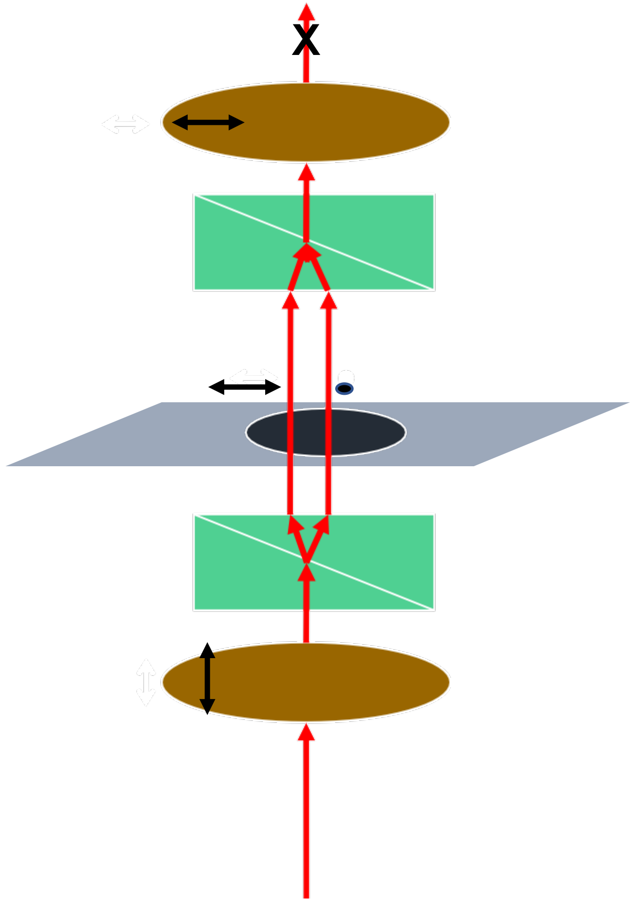
HOW DIC GENERATES CONTRAST



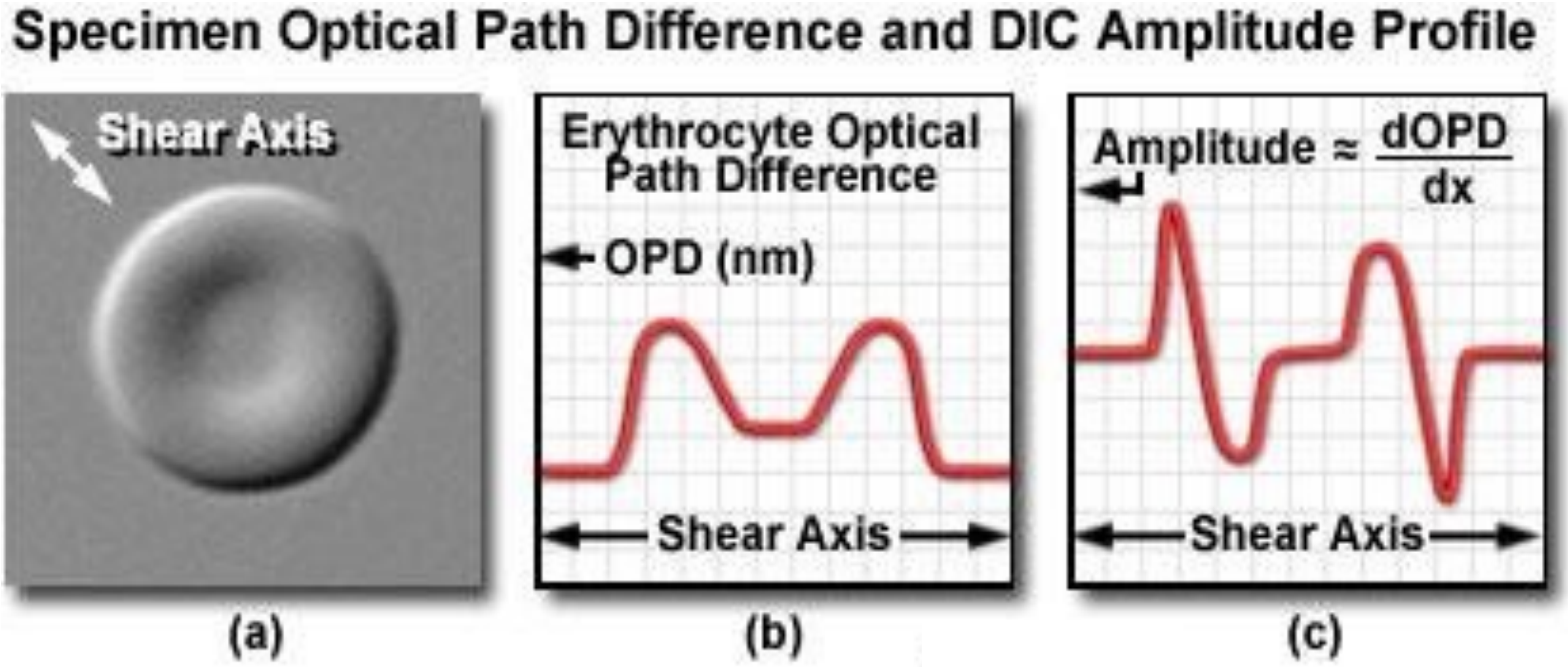


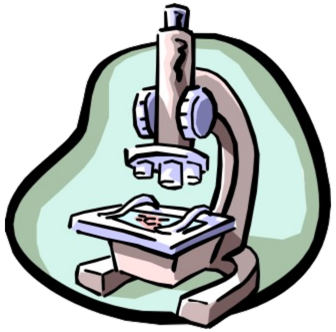
- Beams see different OPL
- Right beam is phase retarded
- Generate elliptical polarization
- Light makes it through analyzer

HOW DIC GENERATES CONTRAST



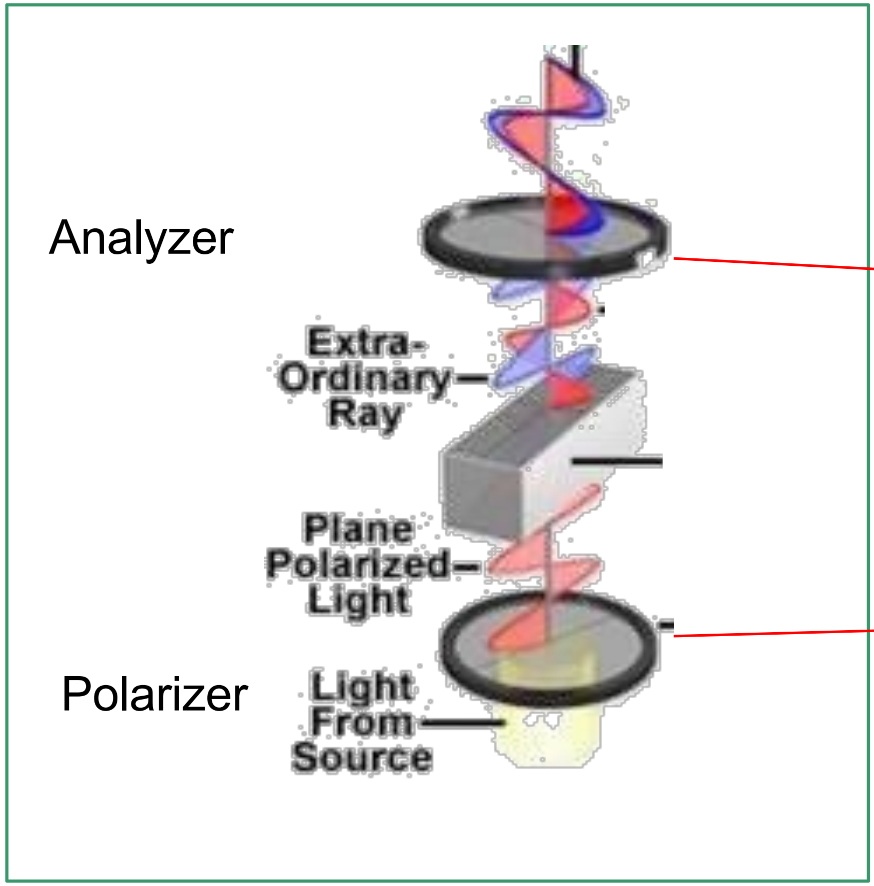
- Both beams see same OPL
- Emerge in phase
- Regenerate initial polarization
- No light makes it through analyzer

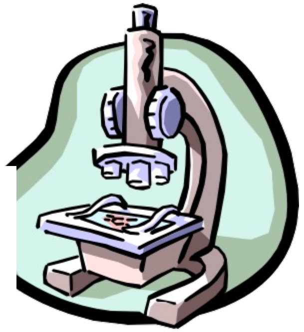

Specimen Optical Path Difference and DIC Amplitude Profile
Shear Axis
Erythrocyte Optical Path Difference
OPD (nm)
Shear Axis
Amplitude ≈ dOPD/dx
Shear Axis
(a)
(b)
(c)



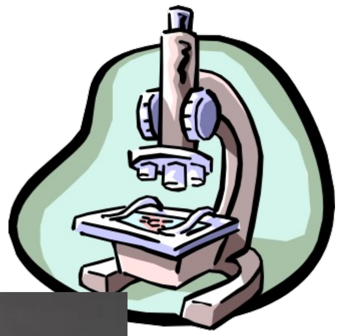
1. Contrast is directional
2. Contrast highlights edges
3. One end brighter, other is dimmer giving a pseudo – 3D image

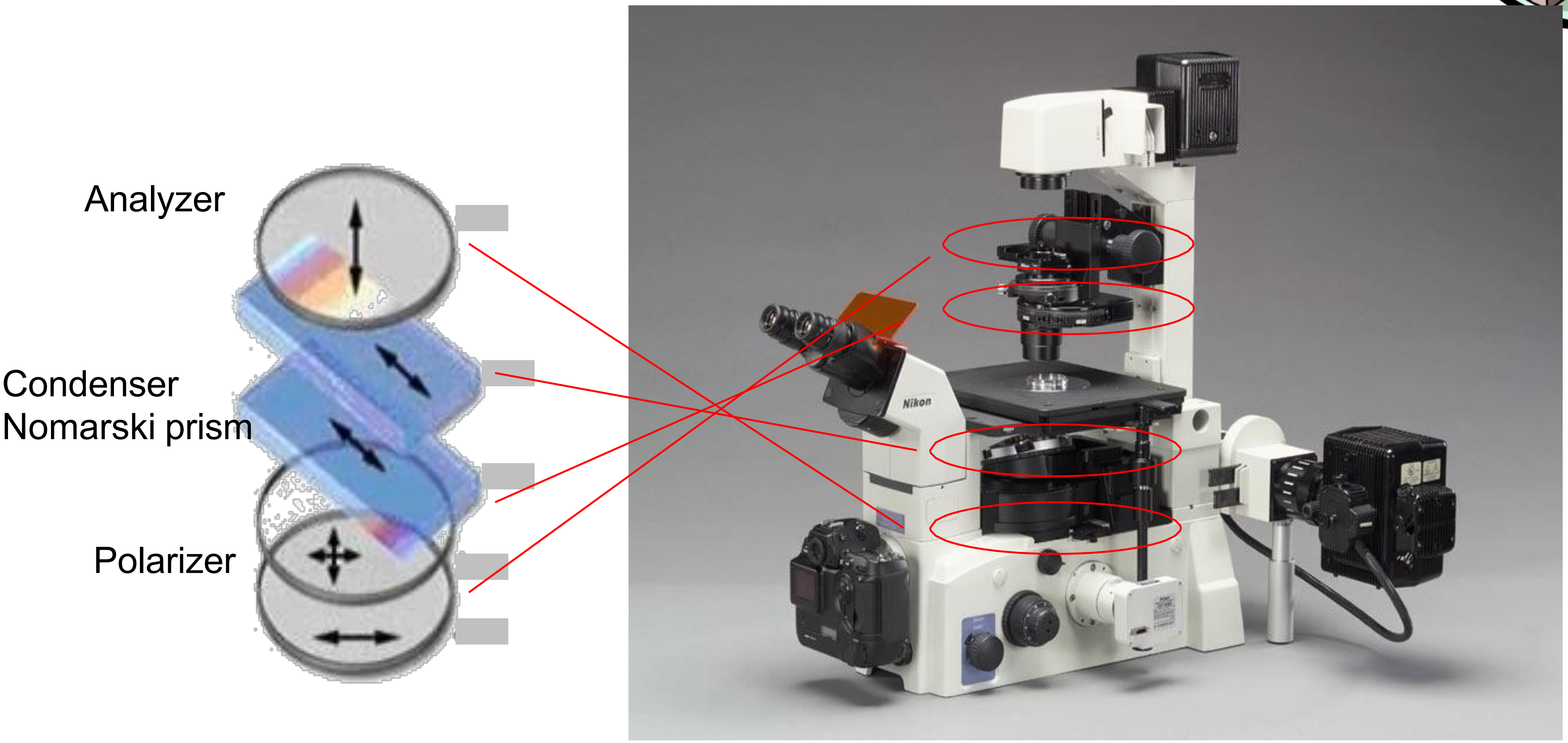
THE DIFFERENTIAL INTERFERENCE CONTRAST (DIC) MICROSCOPE



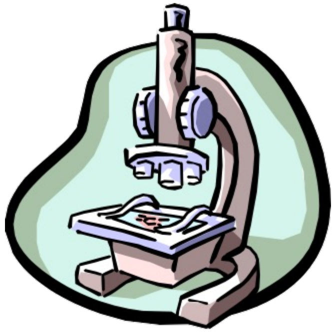


THE DIFFERENTIAL INTERFERENCE CONTRAST (DIC) MICROSCOPE



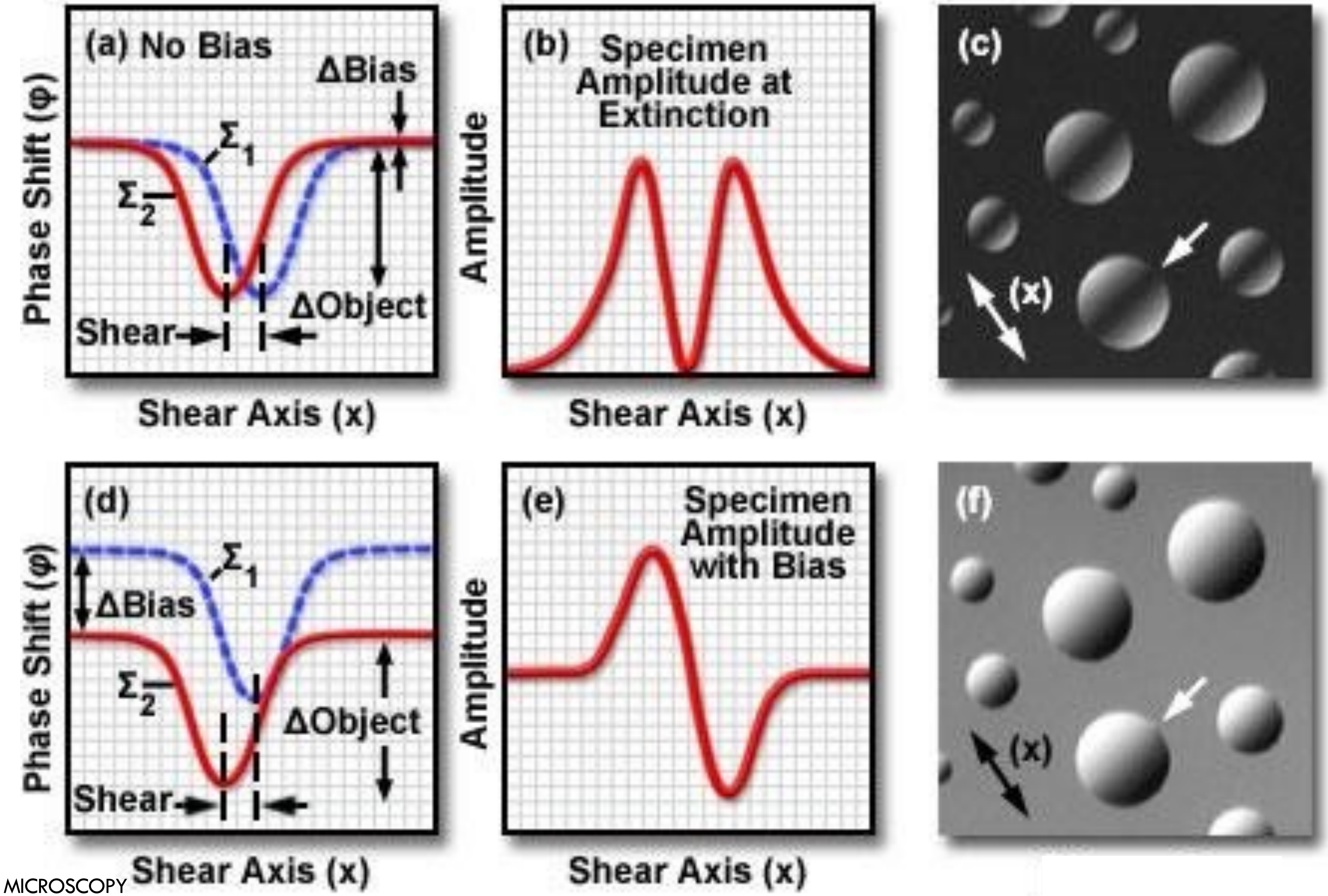


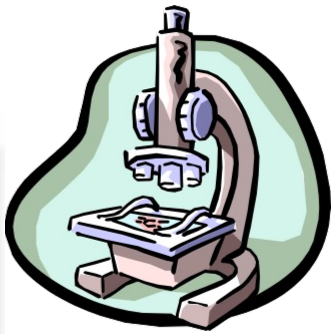
SHEAR IN IMAGE

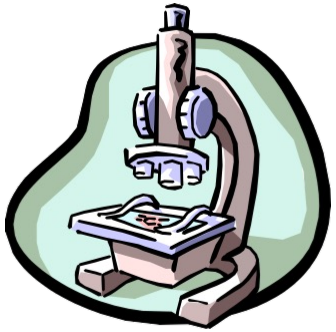


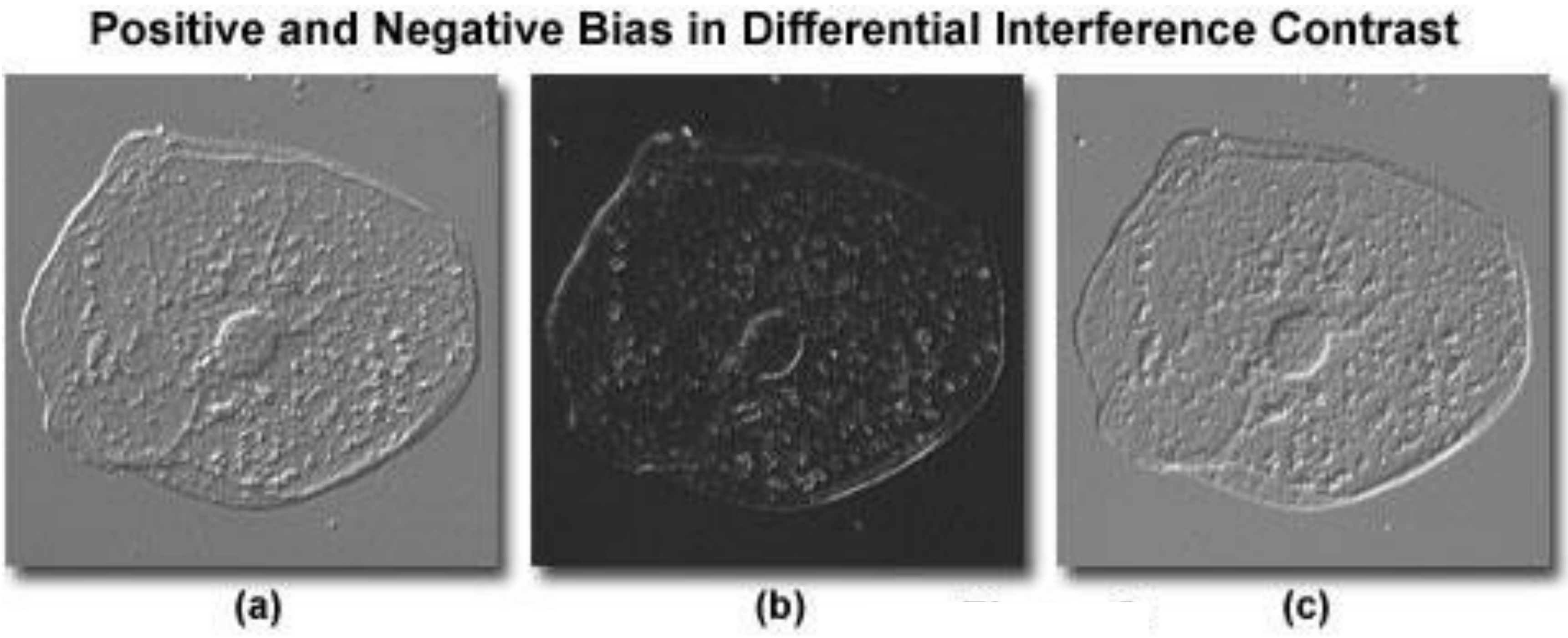
- Degree of shear is set by wollaston combination
- Bias of shear adjustable by shifting upper wollaston position to retard one beam more or less relative to other
- Cannot be used for quantitative measurements of dry mass
- But extremely useful for observing living cells

DIC Image Plane Wavefront Interference

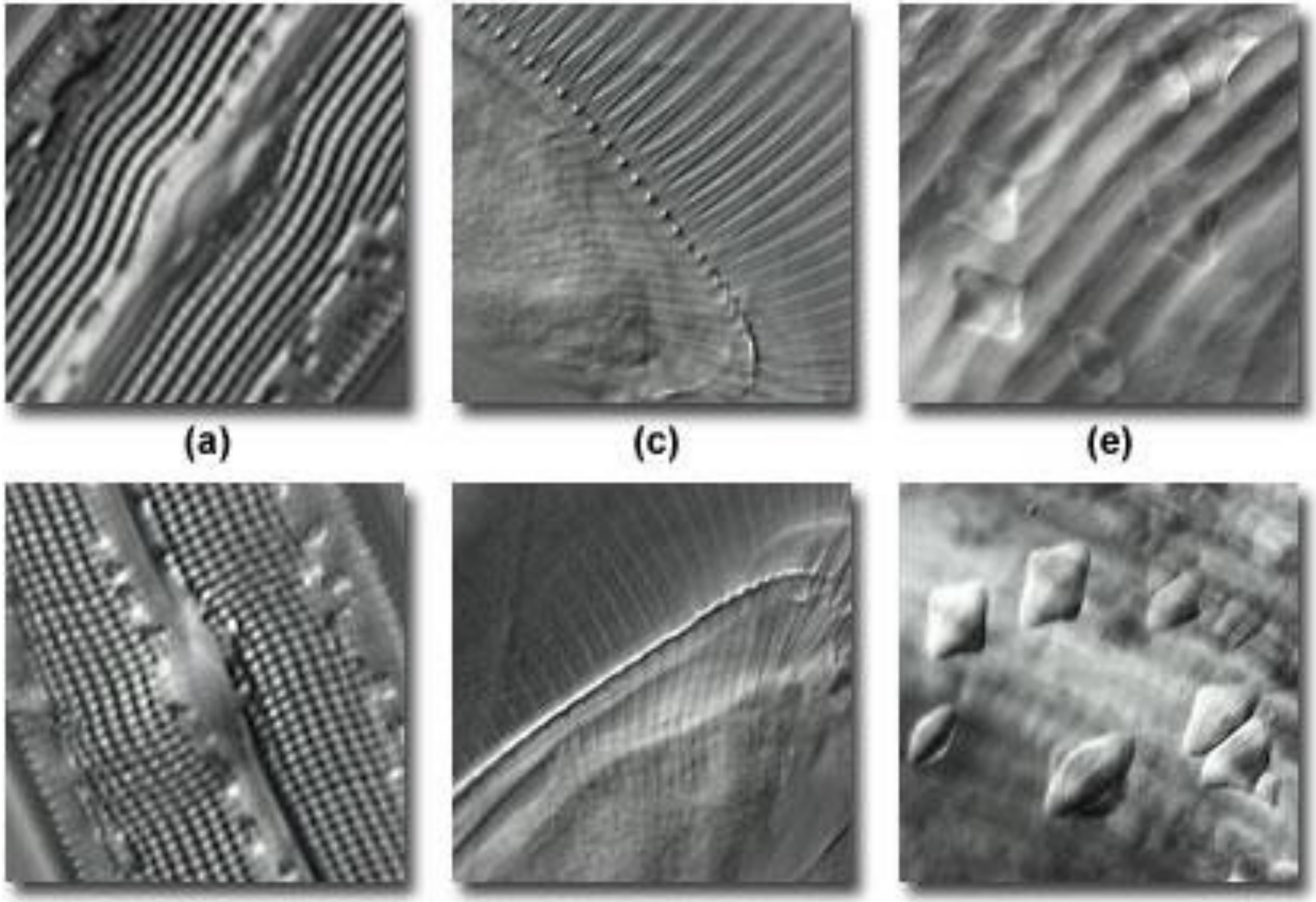






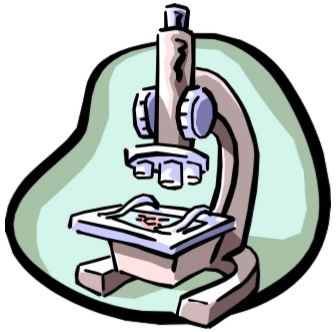

Positive and Negative Bias in Differential Interference Contrast
(a)
(b)
(c)

Effect of Specimen Orientation on DIC Images

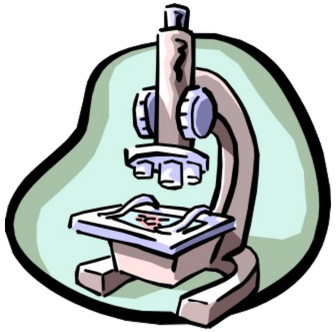


(a) (c) (e)

(b) (d) (f)

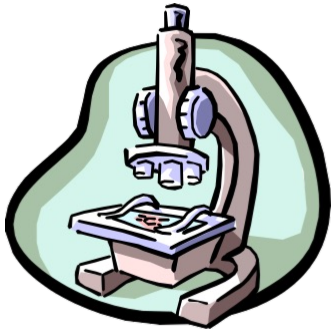


COMPARISON OF NOMARSKI AND PHASE CONTRAST OPTICS



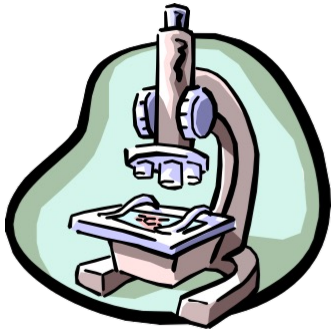
Phase Contrast	Nomarski
Cheaper	More expensive
Easier to set up	Fussy alignment
Uses less than full aperture of objective	Uses full aperture — closest to theoretical limit
Phase Halo — surrounds specimen and other changes	Shadow Effect — contrast greatest at shear direction maximum

COMPARISON OF NOMARSKI AND PHASE CONTRAST OPTICS



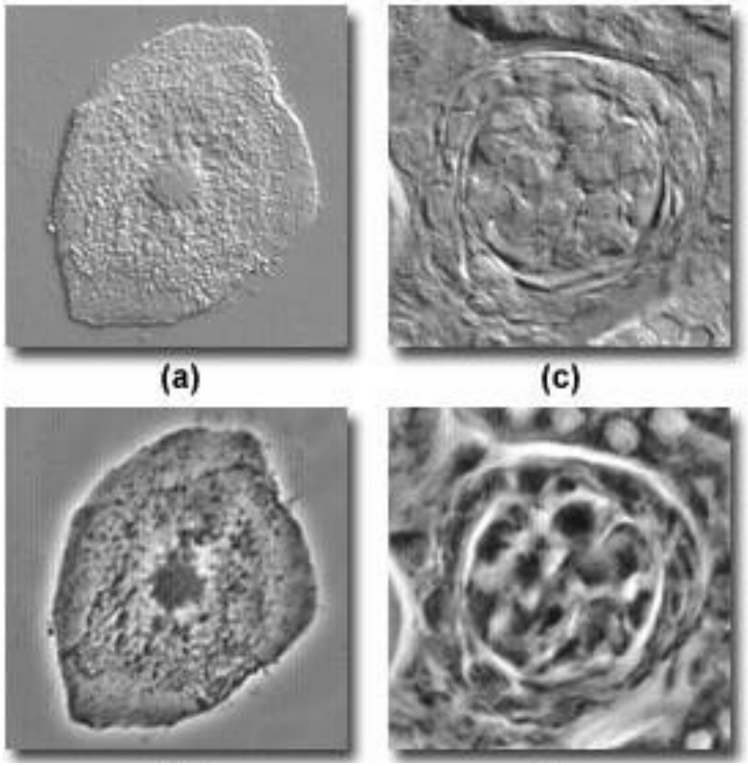
Phase Contrast	Nomarski
Insensitive to birefringence in specimen or slides	Optics disrupted by birefringence
Extremely large depth of field — sensitive to artifacts far out of plane of specimen	Extremely shallow depth of field — useful for optical sectioning of specimen
Doesn’t work well with stained specimens	Works well with stained specimens; optics can be adjusted to enhance contrast

DIC IS HIGHER RESOLUTION THAN PHASE CONTRAST

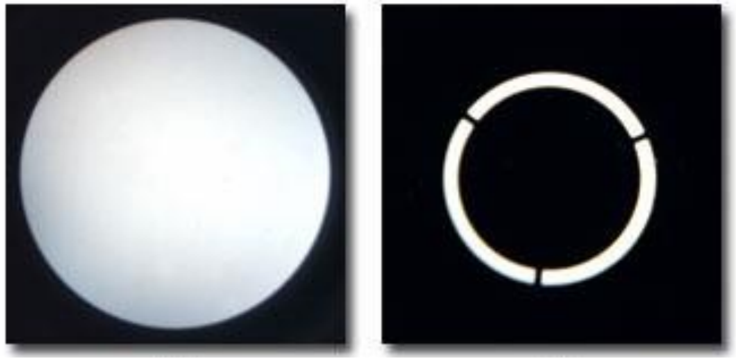


DIC

Phase



Microscope Apertures in DIC and Phase Contrast



Halos in Phase Contrast and DIC Microscopy

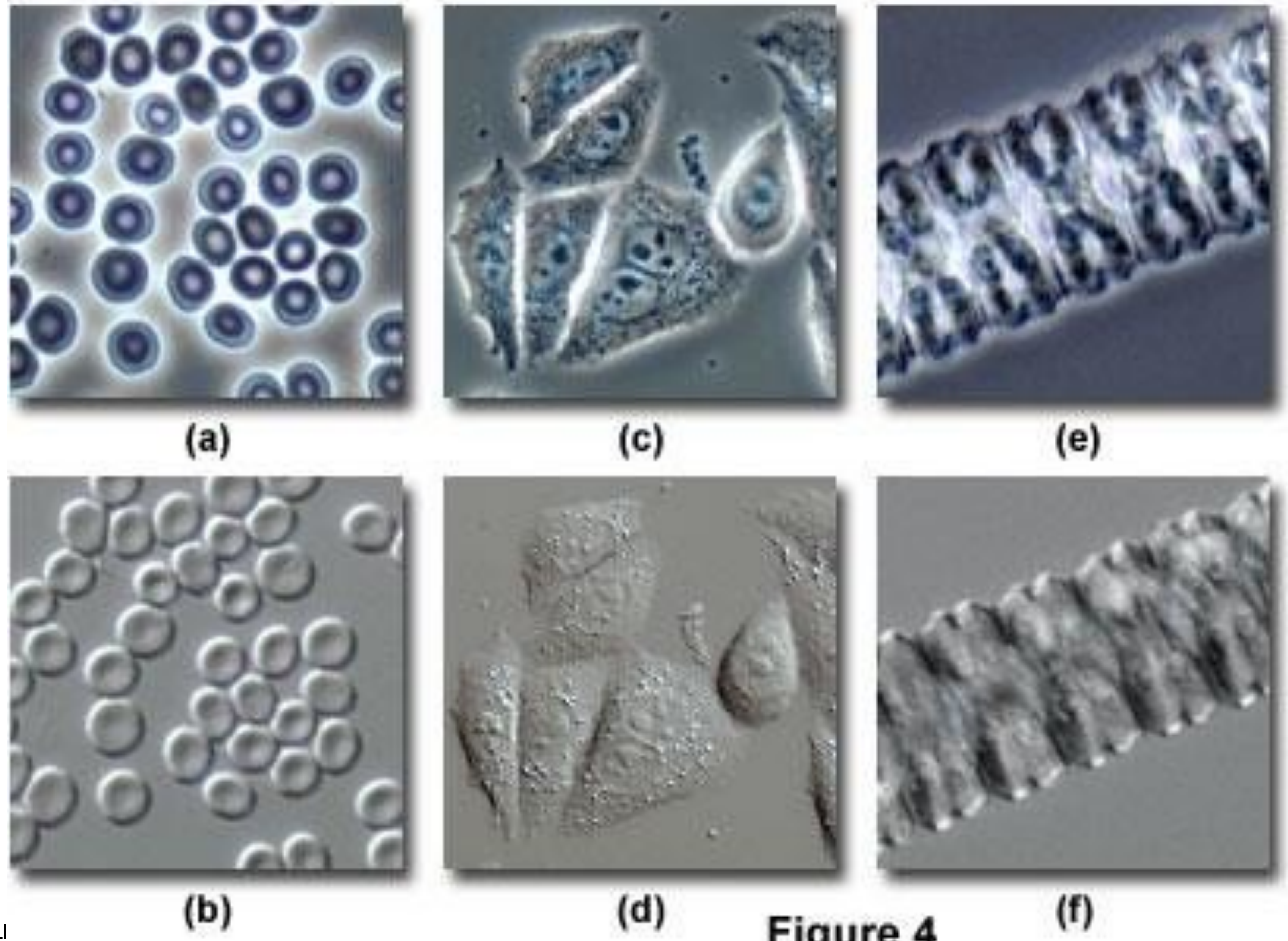
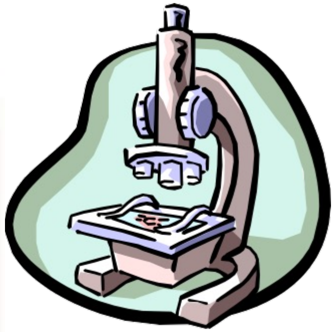
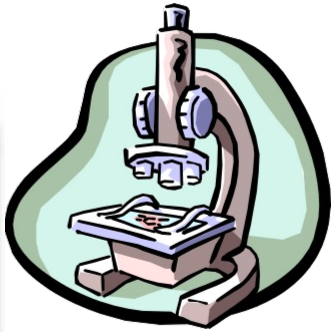


Figure 4



Transparent Specimens in Phase Contrast and DIC



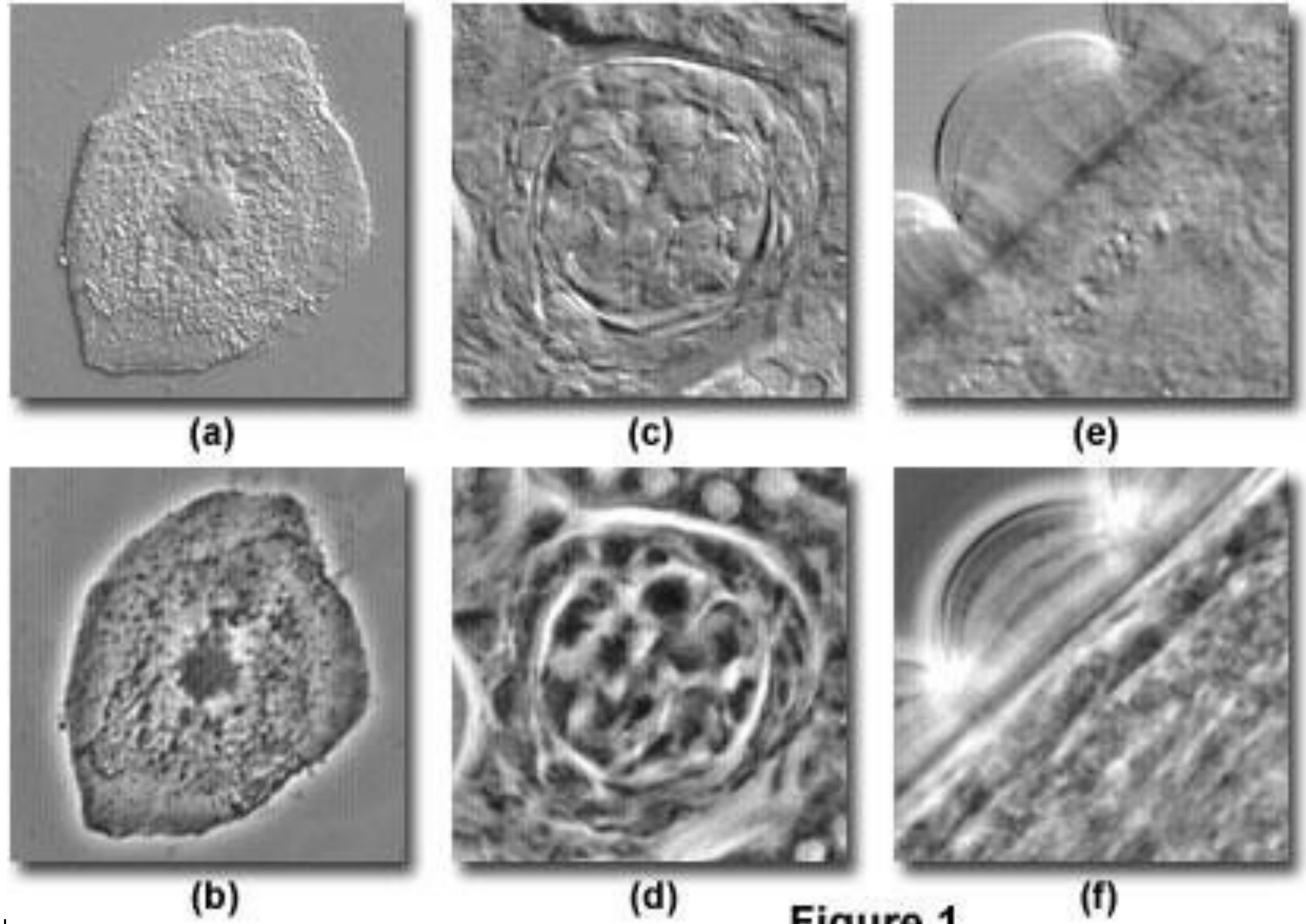


Figure 1

Birefringent Specimens in Phase Contrast and DIC

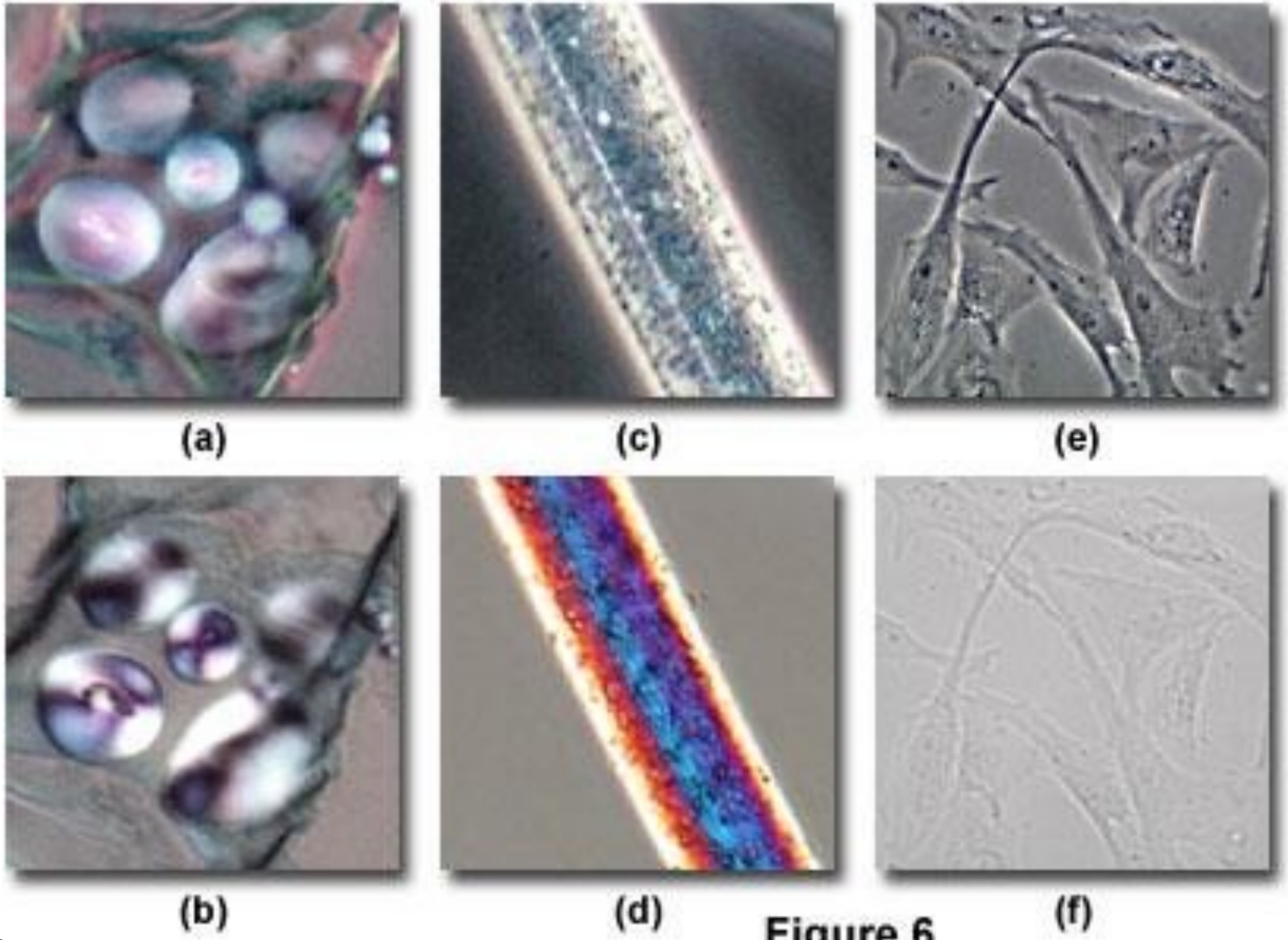
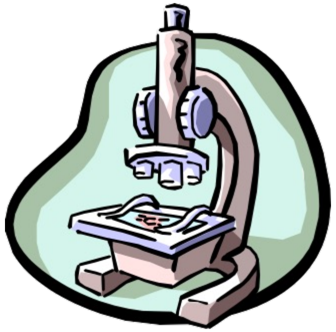
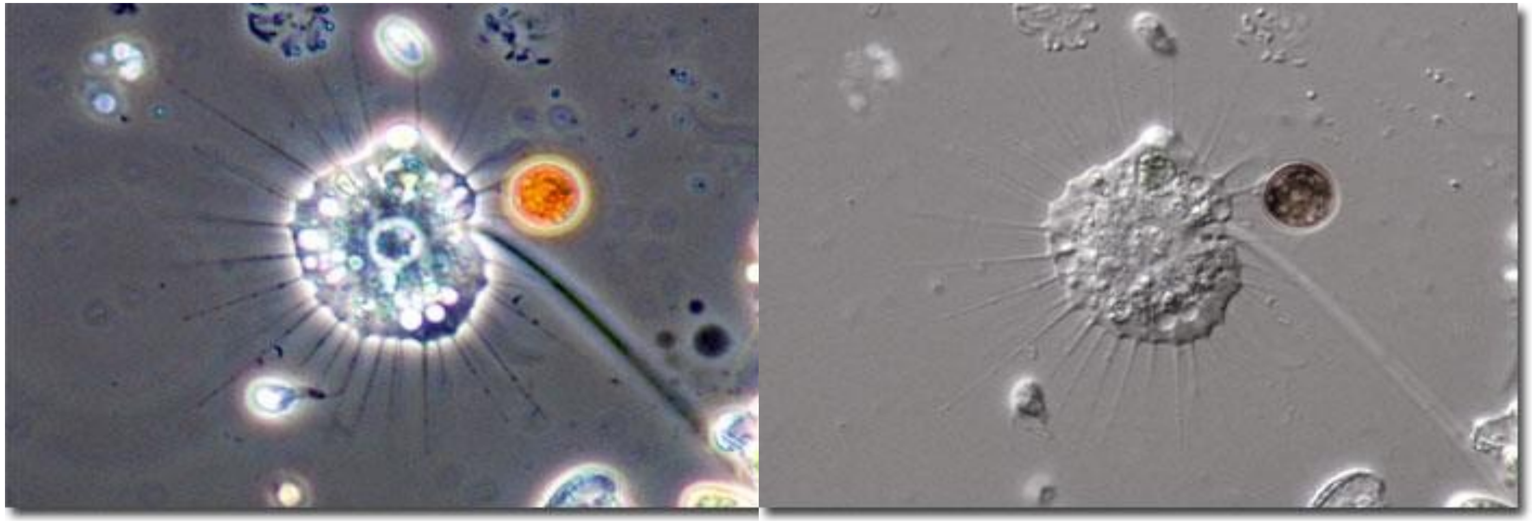


Figure 6

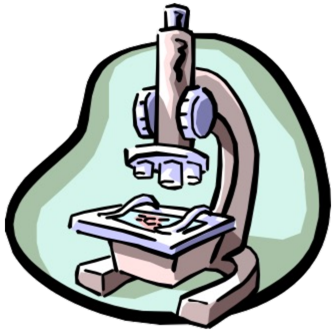


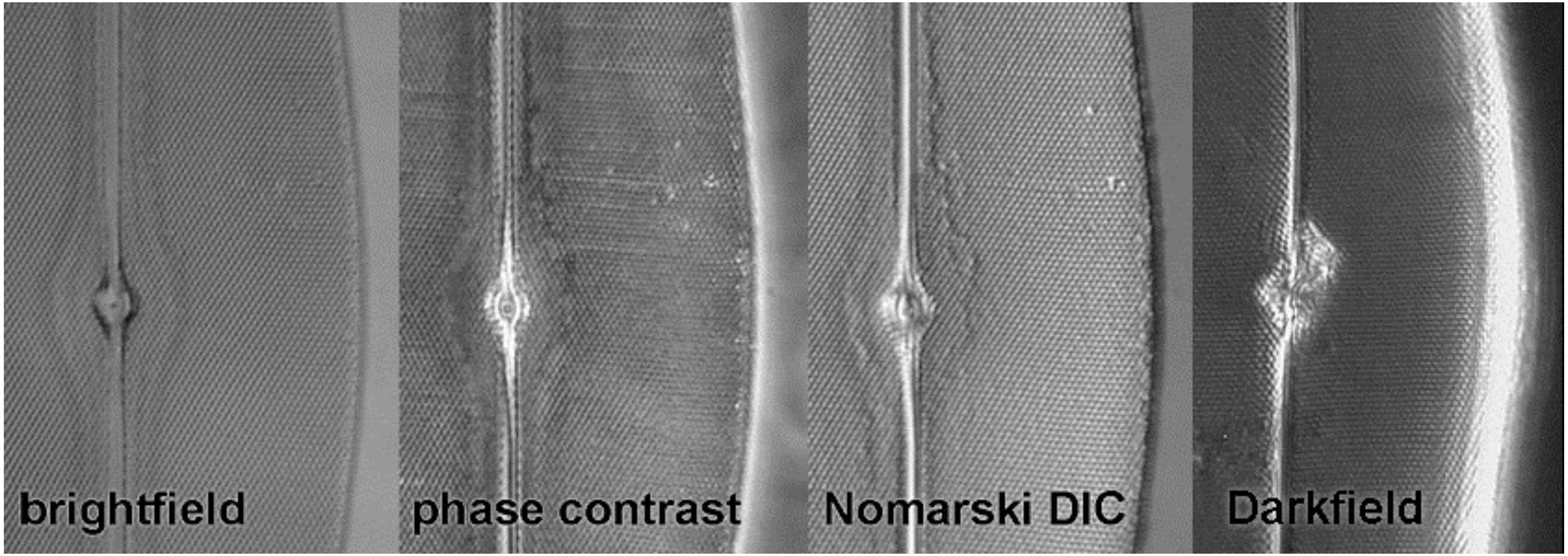
HeLa Cell Culture

Heliozoans
(*Actinophrys sol*)

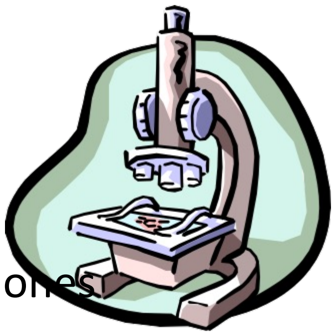


https://en.wikipedia.org/wiki/Polarized_light_microscopy#/media/File:Paper_Micrograph_Bright.png





CONTRASTING TECHNIQUES - A SUMMARY



- **Brightfield - absorption**

  Light transmitted through sample. Only useful for colored samples. Very little contrast in unstained ones.

- **Darkfield - scattering**

  Light directed from the side - only scattered light enters the microscope lenses -> sample appears as an illuminated object

- **Phase Contrast - phase interference**

  Incident light is out of phase with transmitted light. phases of the light are synchronized by an interference lens -> new image with greater contrast

- **Polarization Contrast – polarization**

  Polarized light for illumination. vibration direction of the polarized light is altered by a sample - light can pass through 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**