Light microscopy in Cellular Biology

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gbaj@units.it

2021 - Light microscopy in Cellular Biology

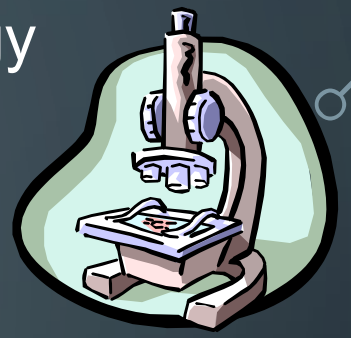


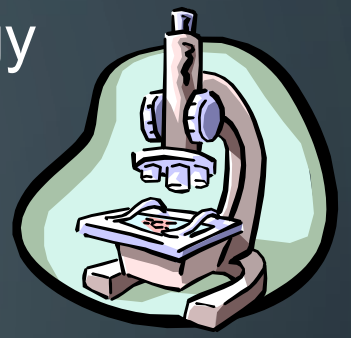
Image Formation

Lenses and Image Formation

Microscope Imaging and Koehler Illumination

Diffraction and Point Spread Function

Objectives and Eyepieces



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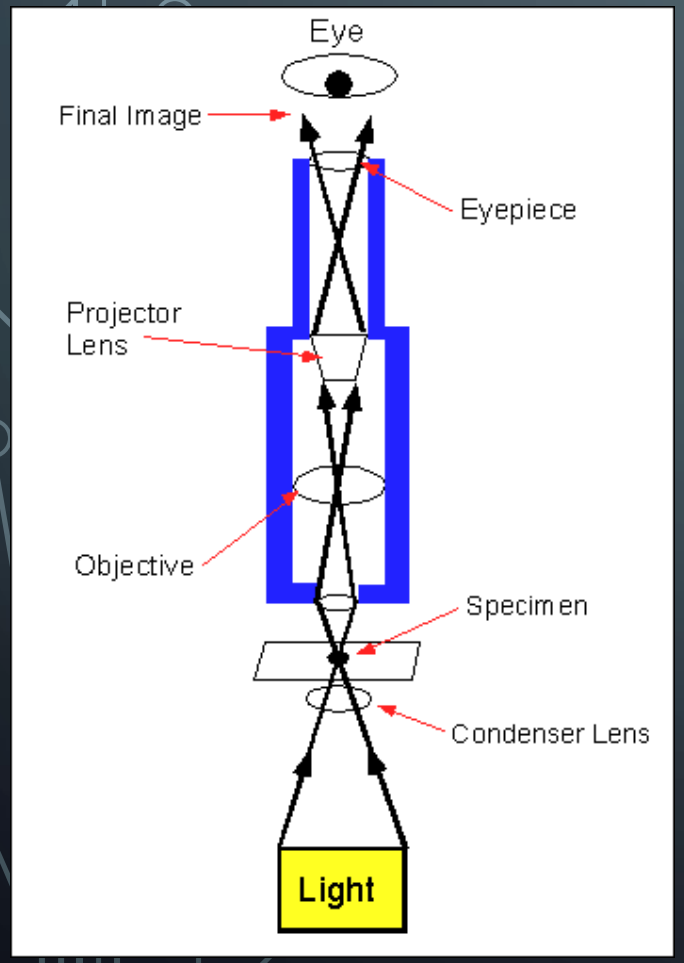
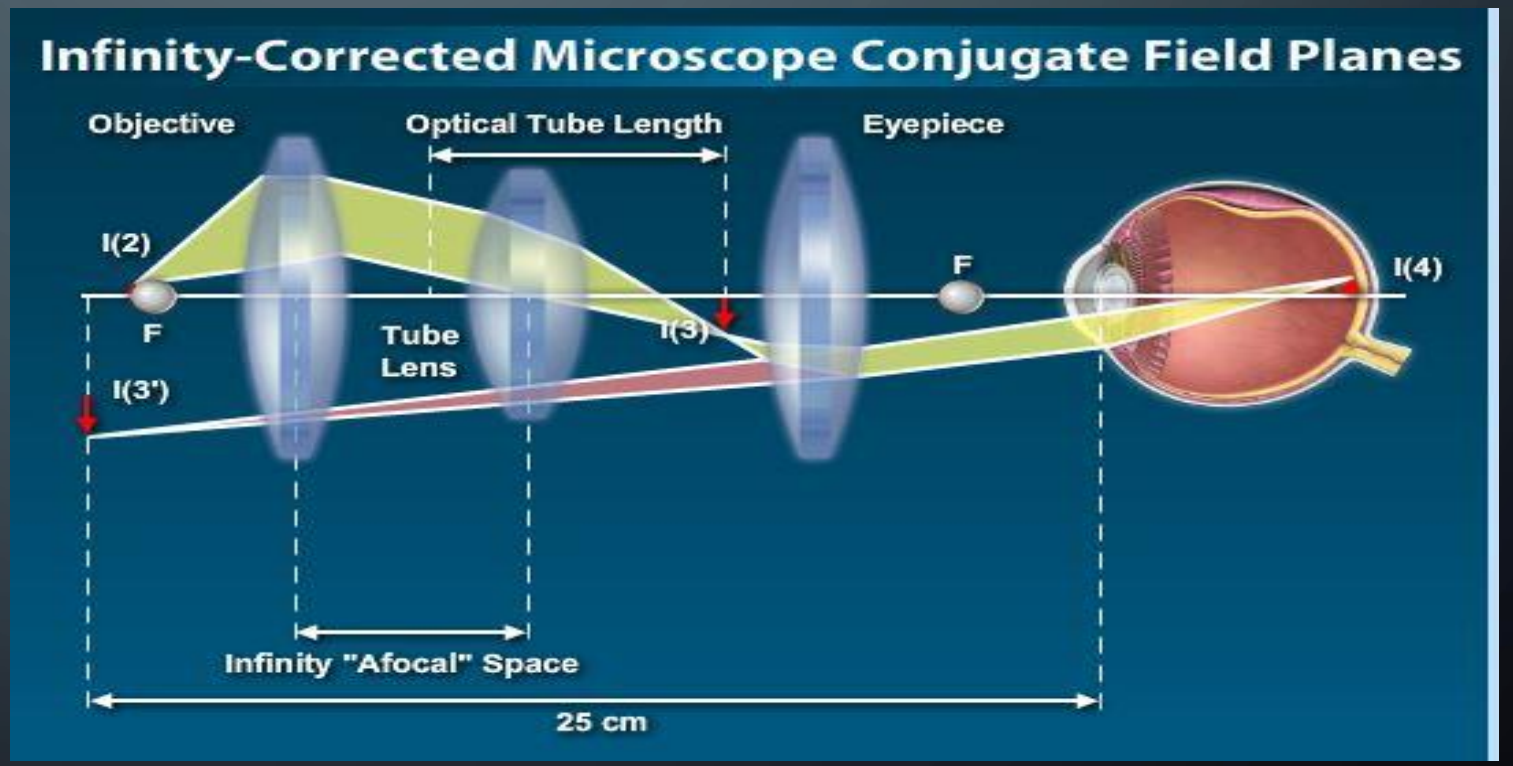




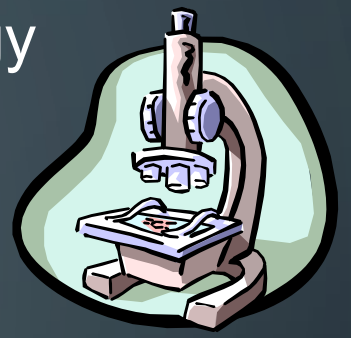
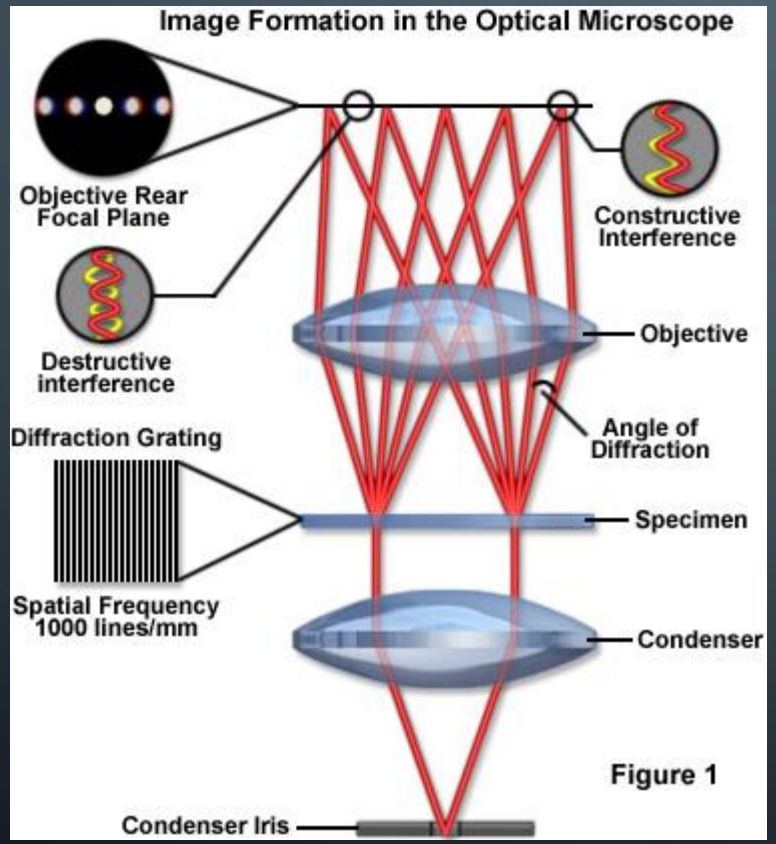
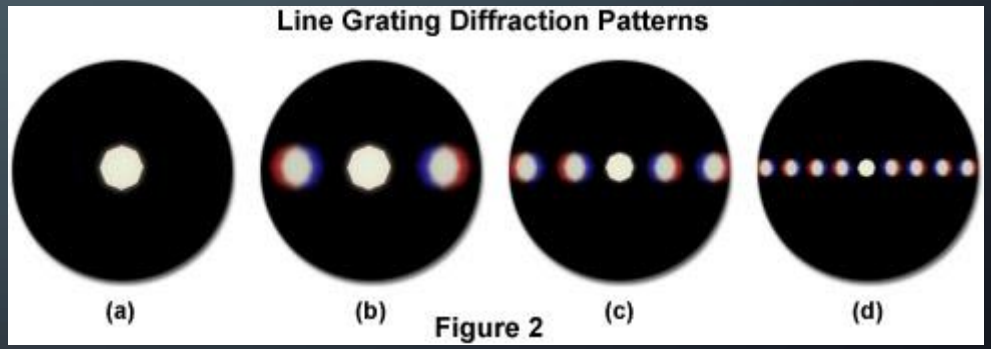
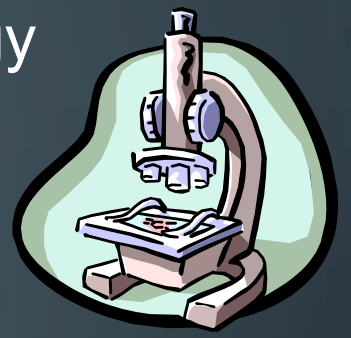


Image Formation







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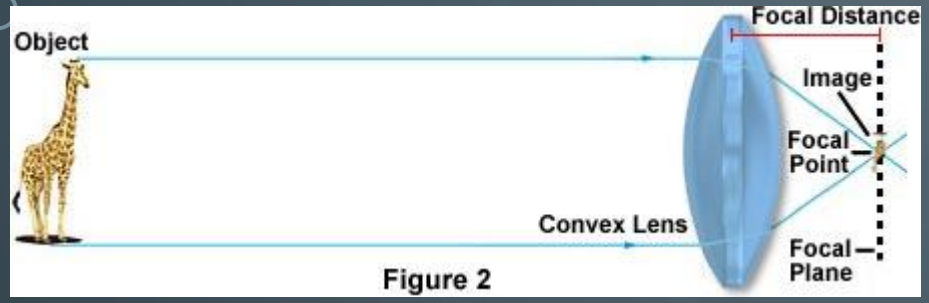


Figure 2

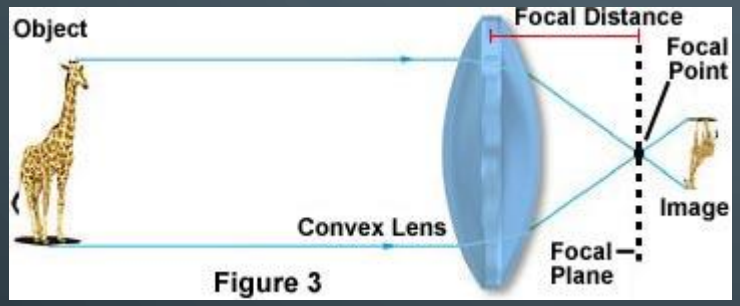


Figure 3

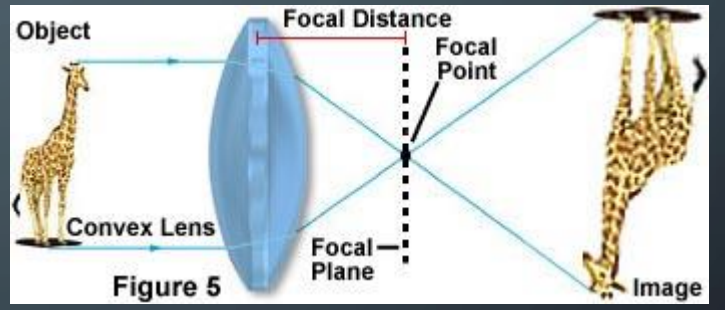


Figure 5

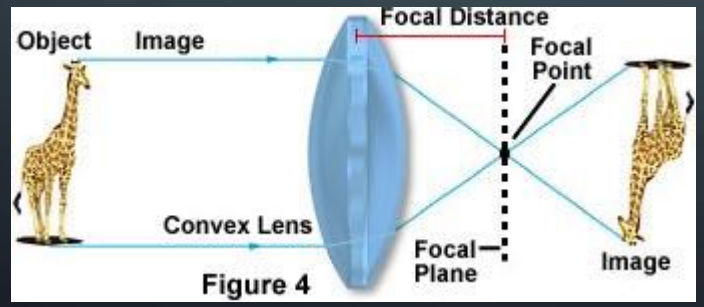
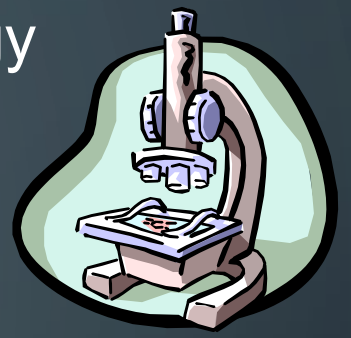
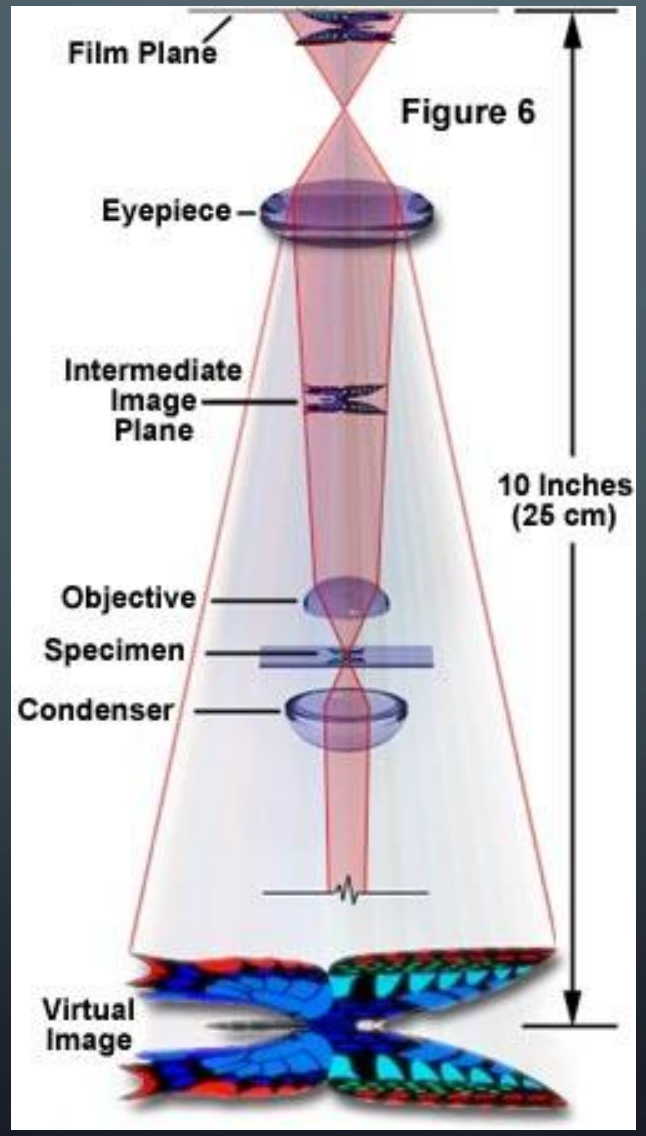


Figure 4



Lenses and Image Formation



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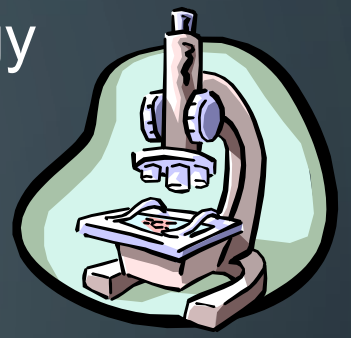
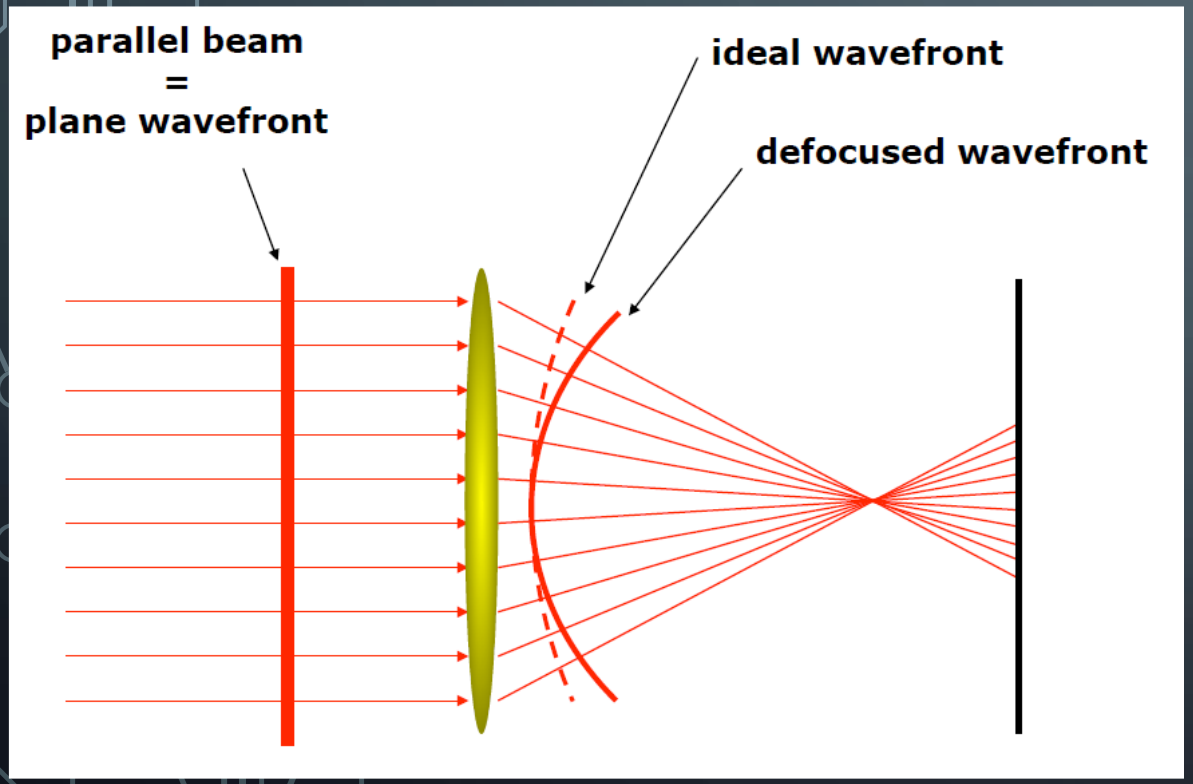
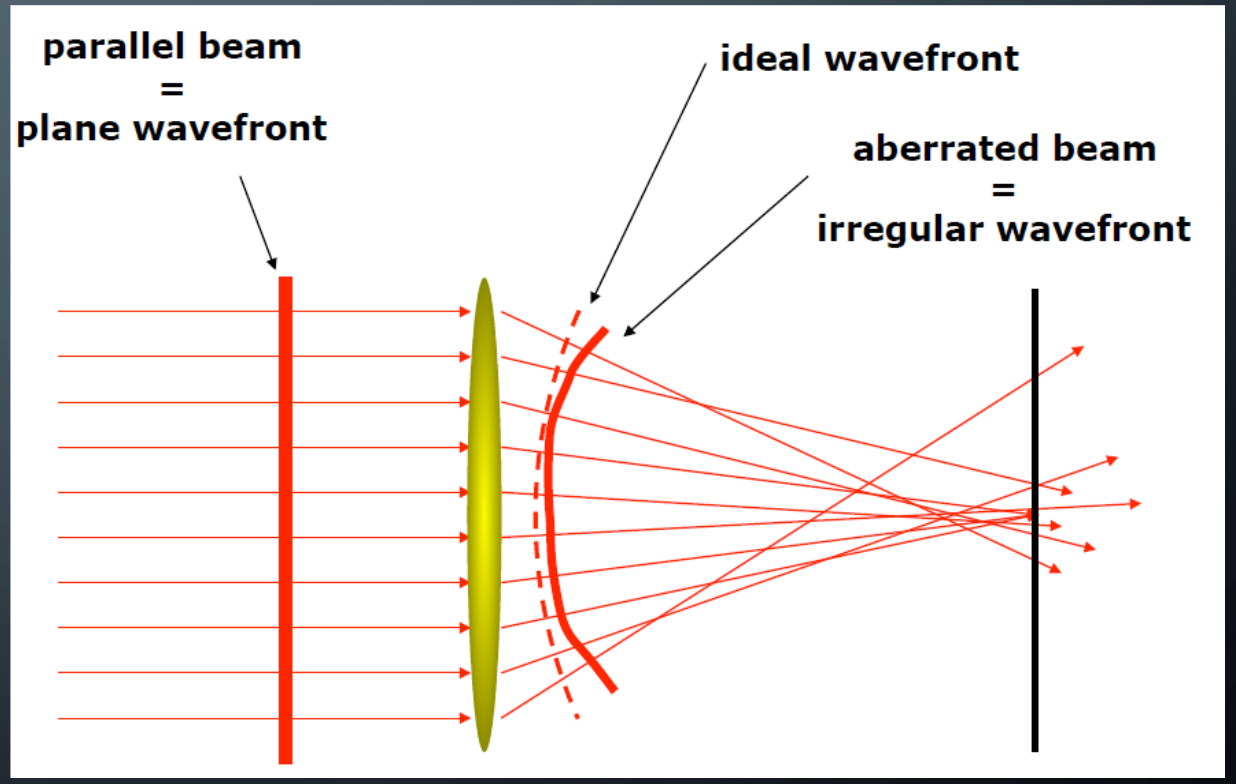
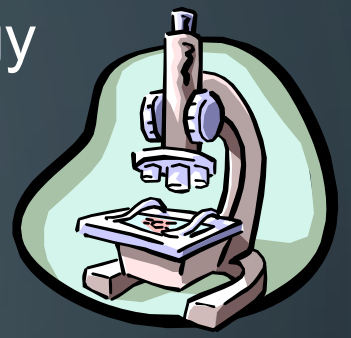


OUT of focus



Aberrations





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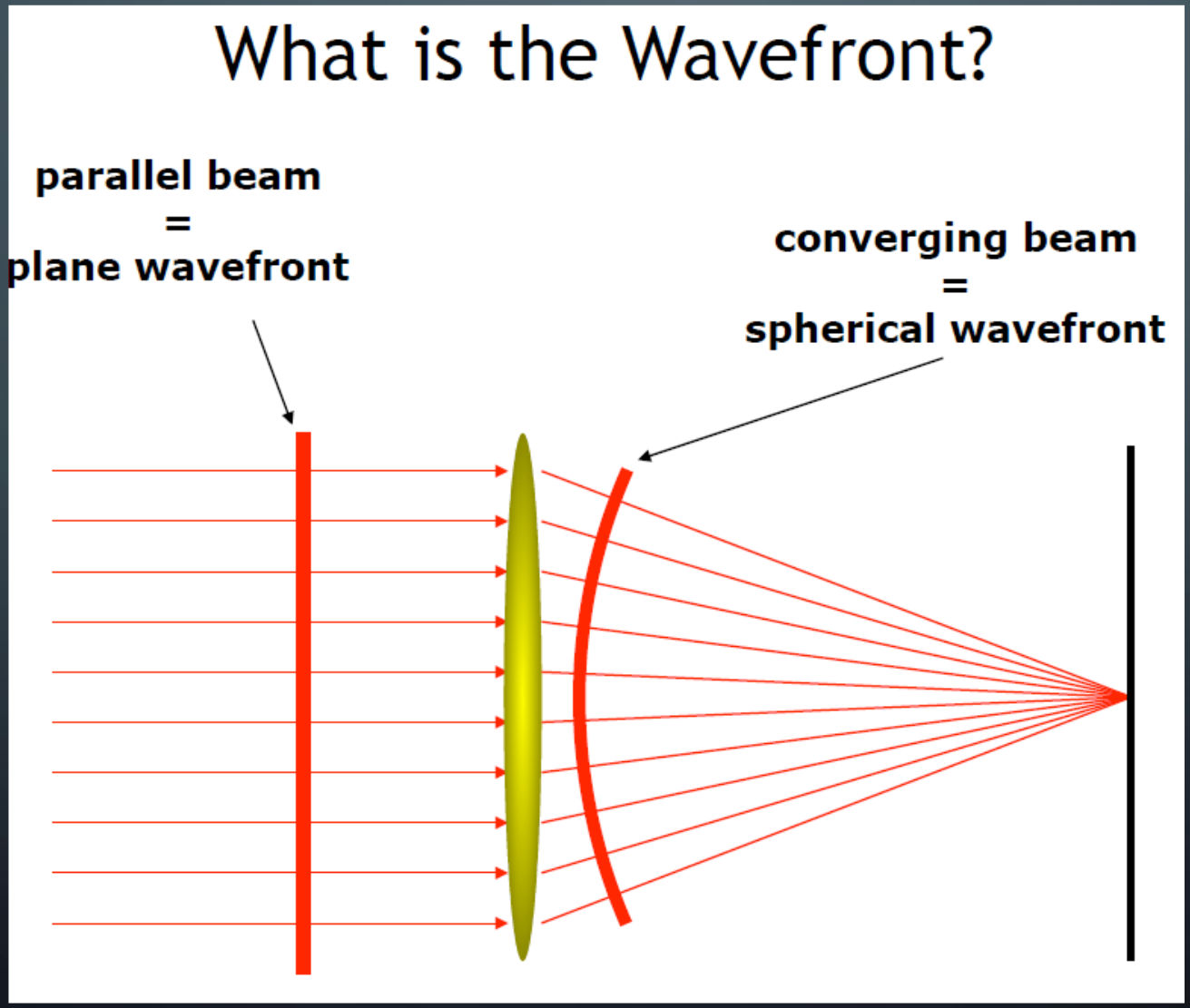
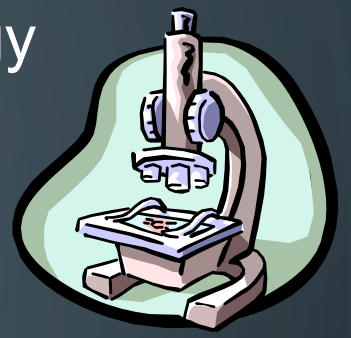
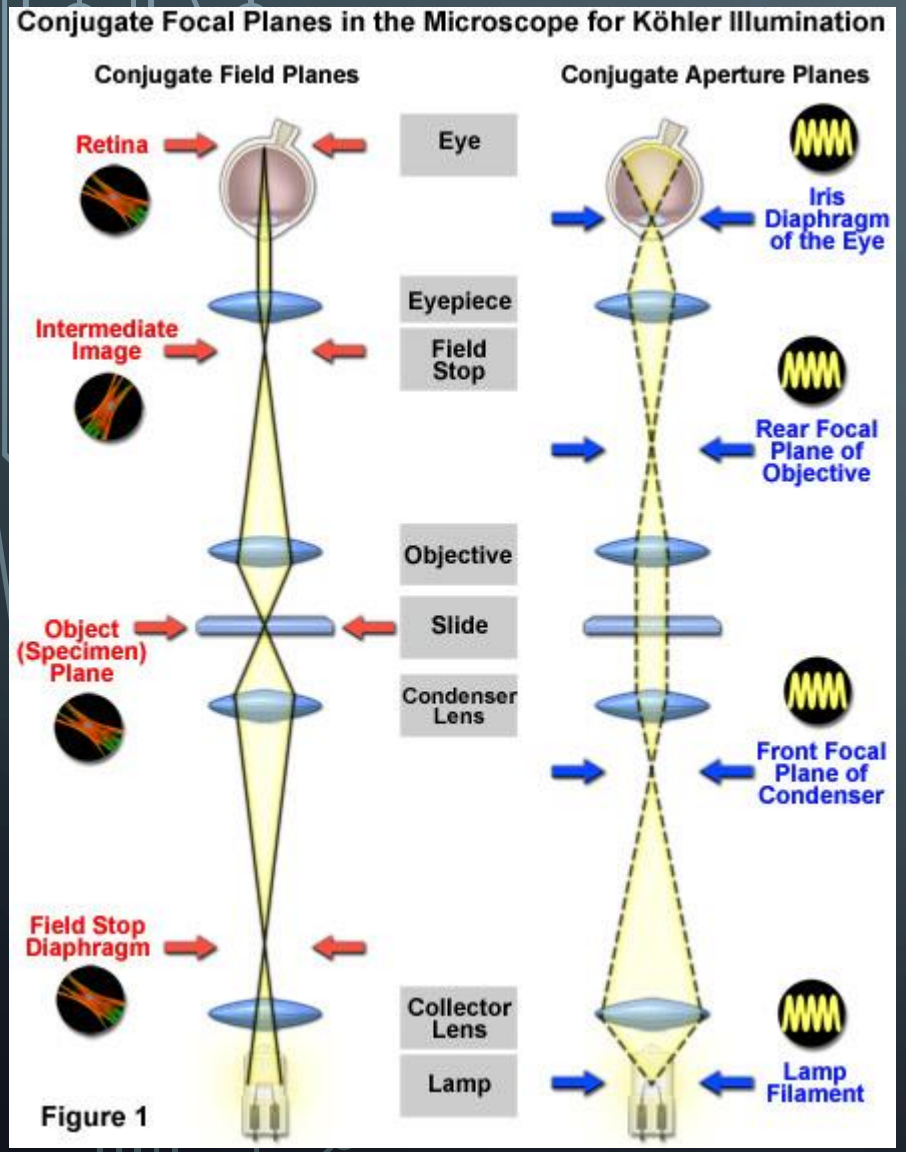




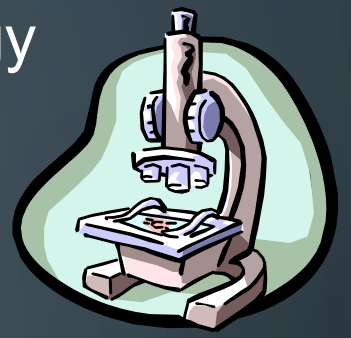
Microscope Imaging

http://zeiss-campus.magnet.fsu.edu/tutorials/index.html

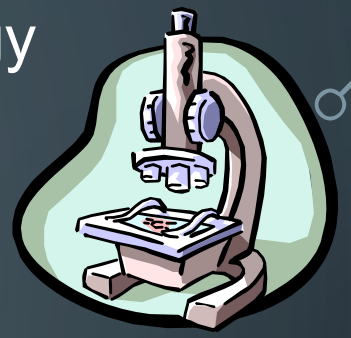
http://zeiss-campus.magnet.fsu.edu/tutorials/basics/transmittedlightopticalpathway/indexflash.html



Koehler Illumination



http://zeiss-campus.magnet.fsu.edu/tutorials/basics/microscopealignment/indexflash.html

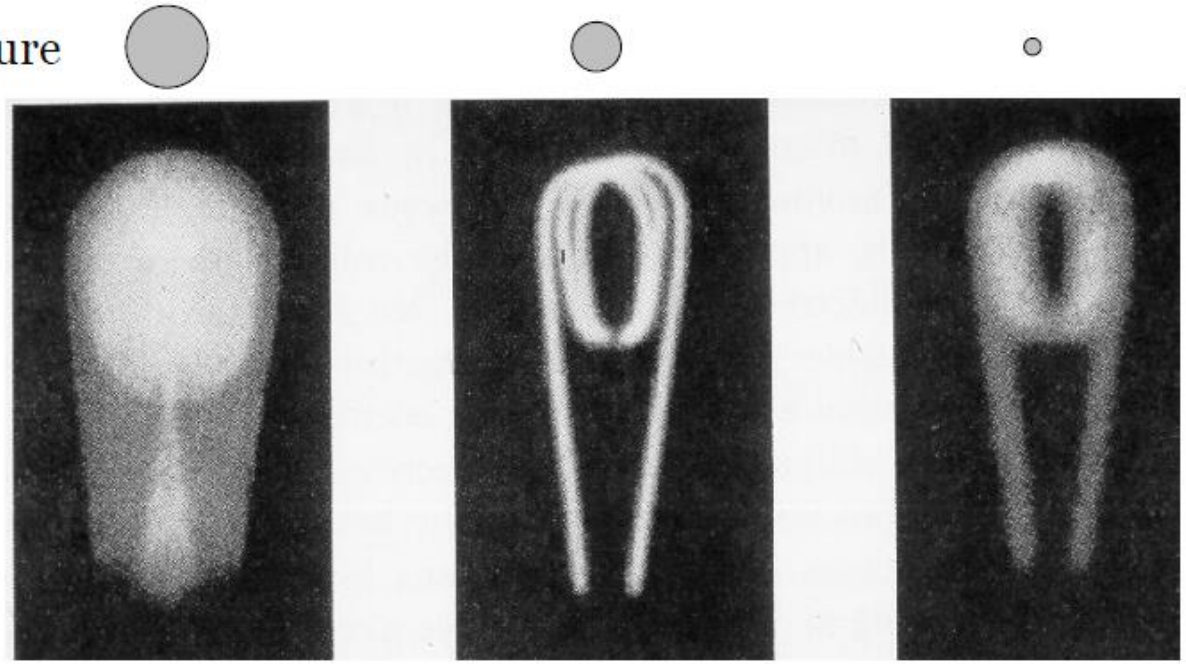


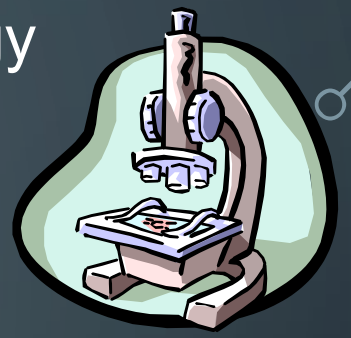
Diffraction

Diffraction limits image sharpness as the pinhole becomes small

(From Jenkins and White)

Aperture

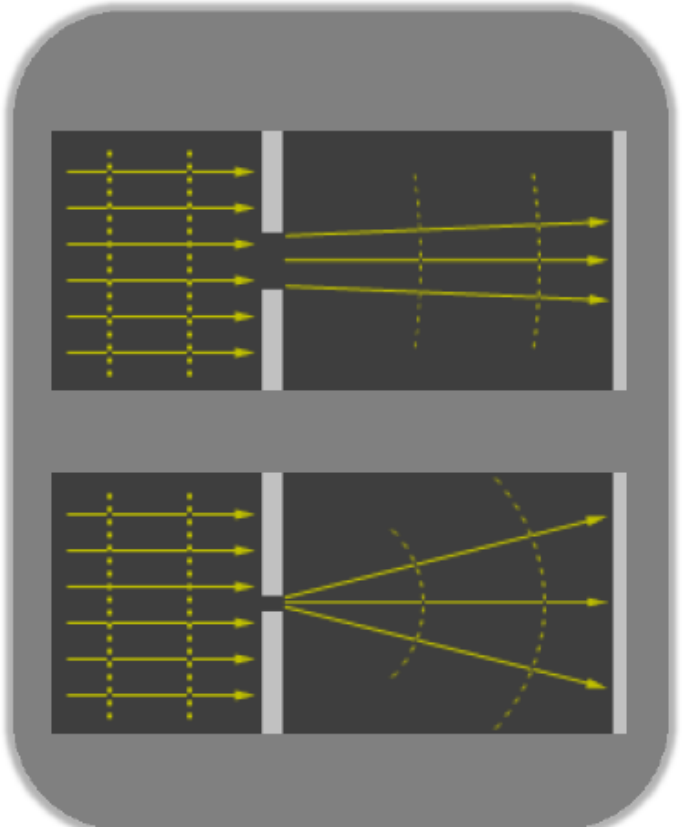




Diffraction

Diffraction: Rays

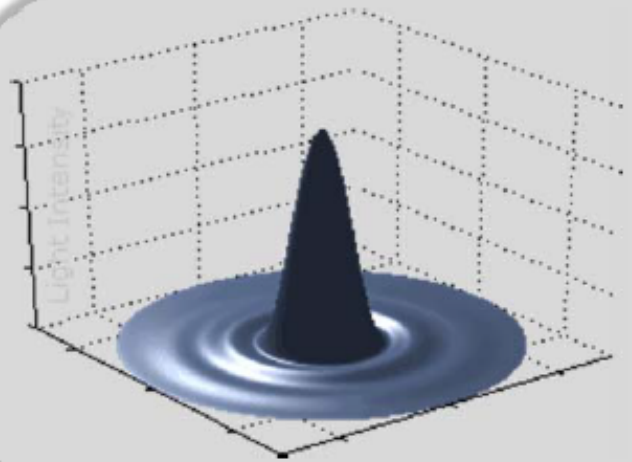
- Parallel rays incident on an aperture, say rays from a point source at infinity, begin to diverge.
- The smaller the aperture, the larger the divergence.
- This can be explained if we consider light as a wave phenomenon



Diffraction and Point Spread Function

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

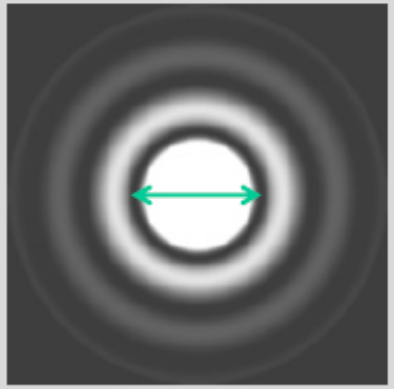
For an optical system with circular aperture
the finite image spot forms an Airy Disk (Point Spread Function)



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

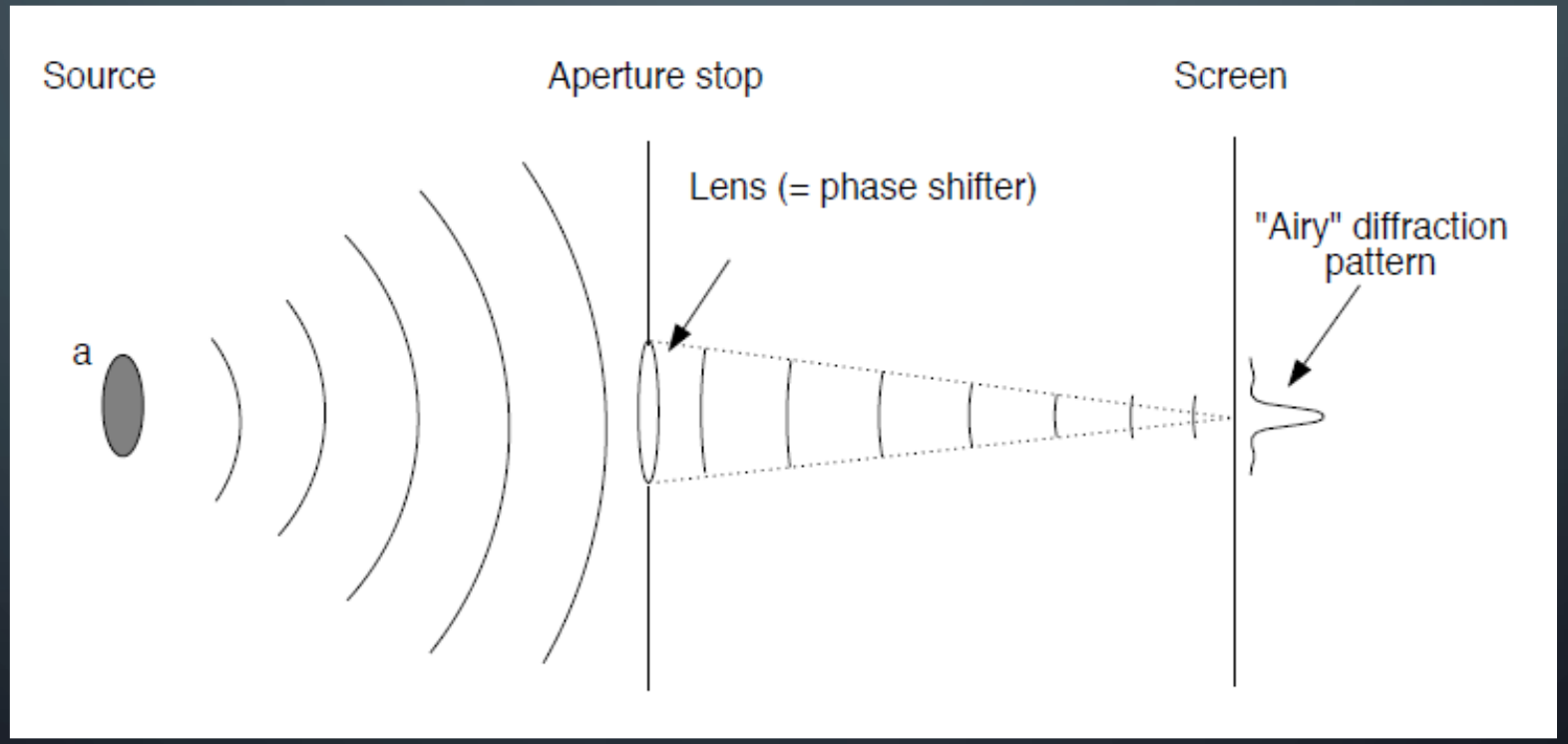
$$with \quad u = \frac{\pi \cdot x}{\lambda \cdot f/\#}$$

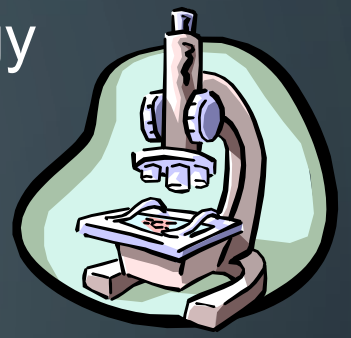
$$and \quad x_{cutoff} = 1.22 \cdot \lambda \cdot f/\#$$



Airy disk diameter: 2.44 λ f/#

Diffraction and Point Spread Function





Diffraction and Point Spread Function

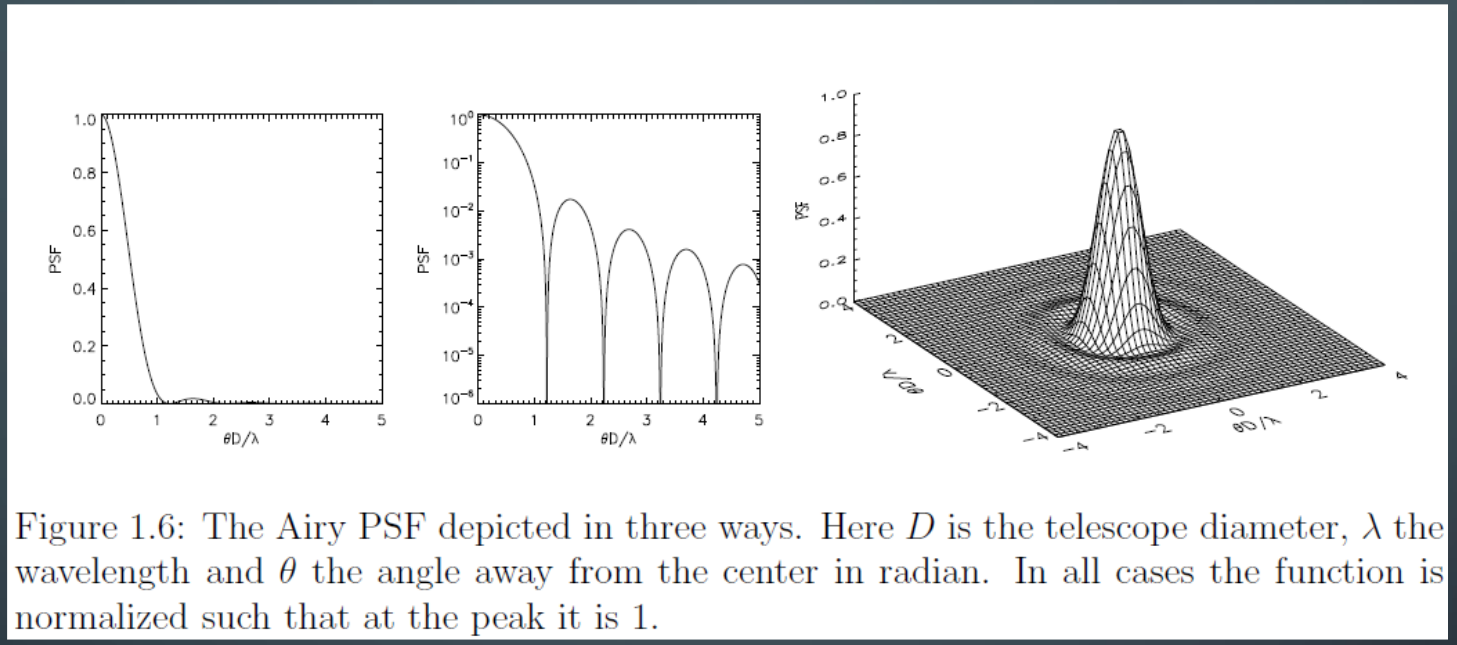
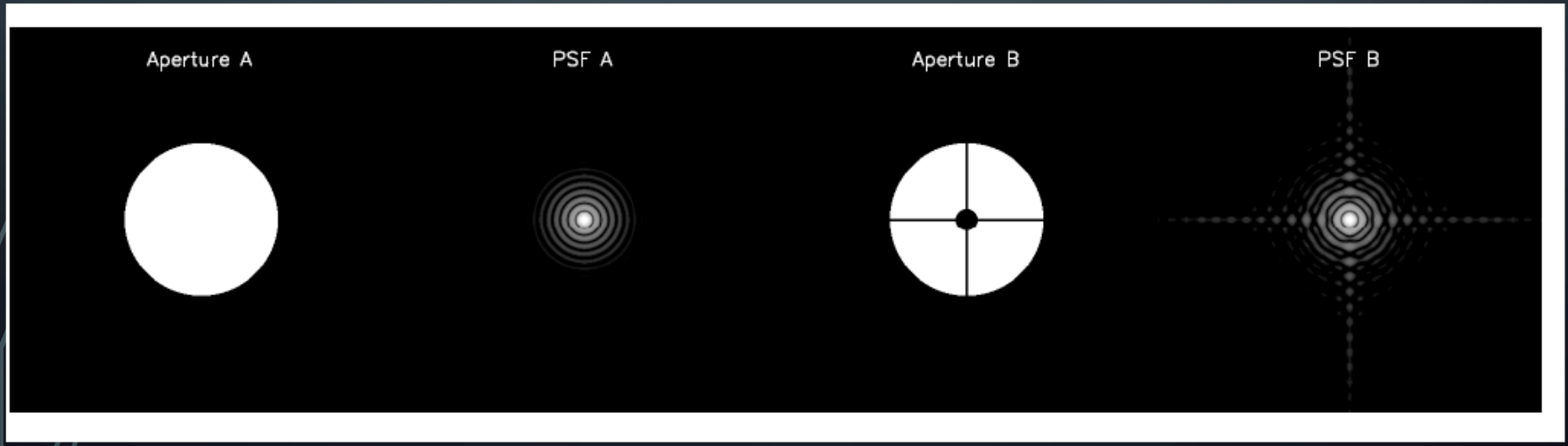
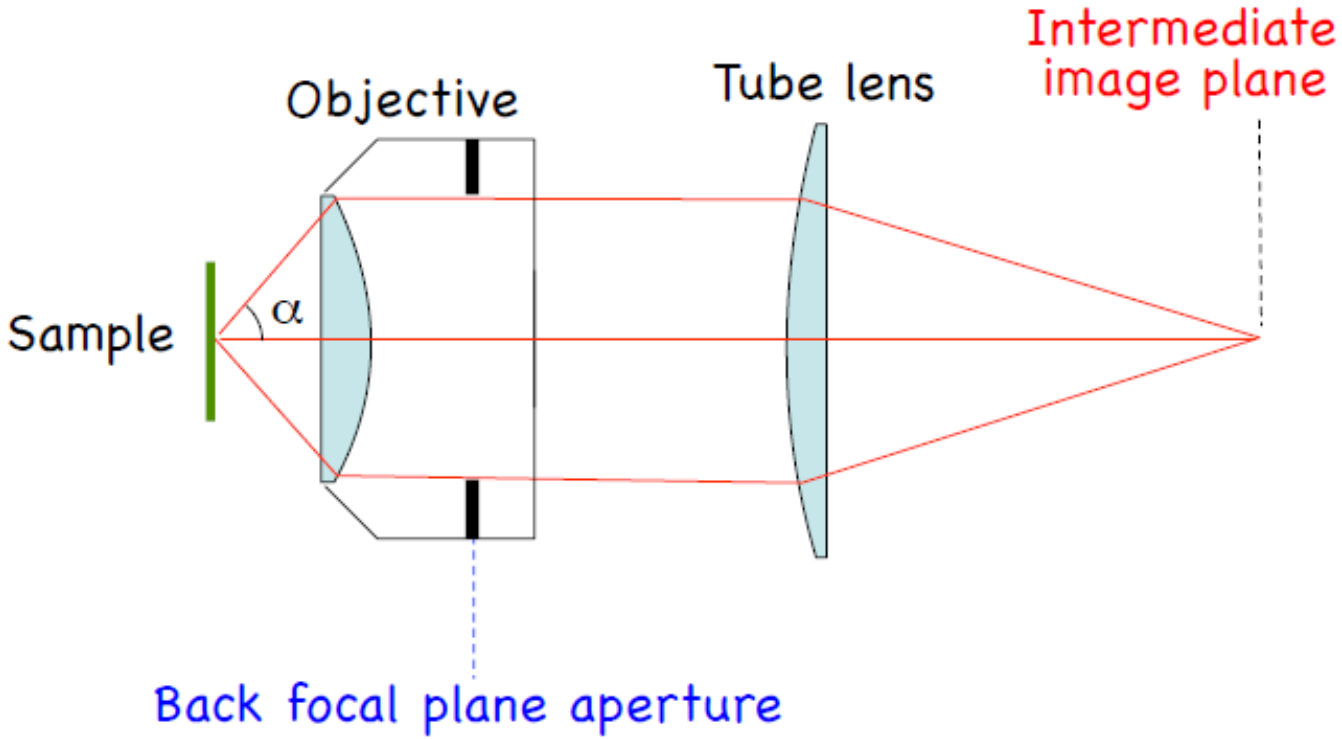


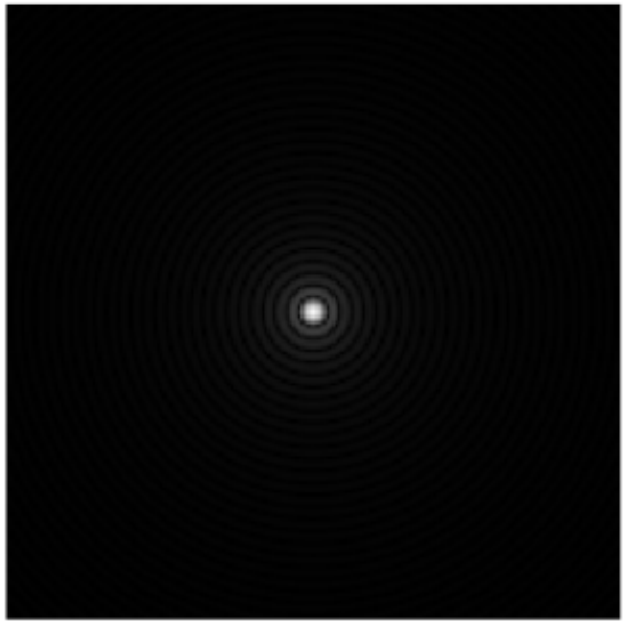
Figure 1.6: The Airy PSF depicted in three ways. Here D is the telescope diameter, λ the wavelength and θ the angle away from the center in radian. In all cases the function is normalized such that at the peak it is 1.

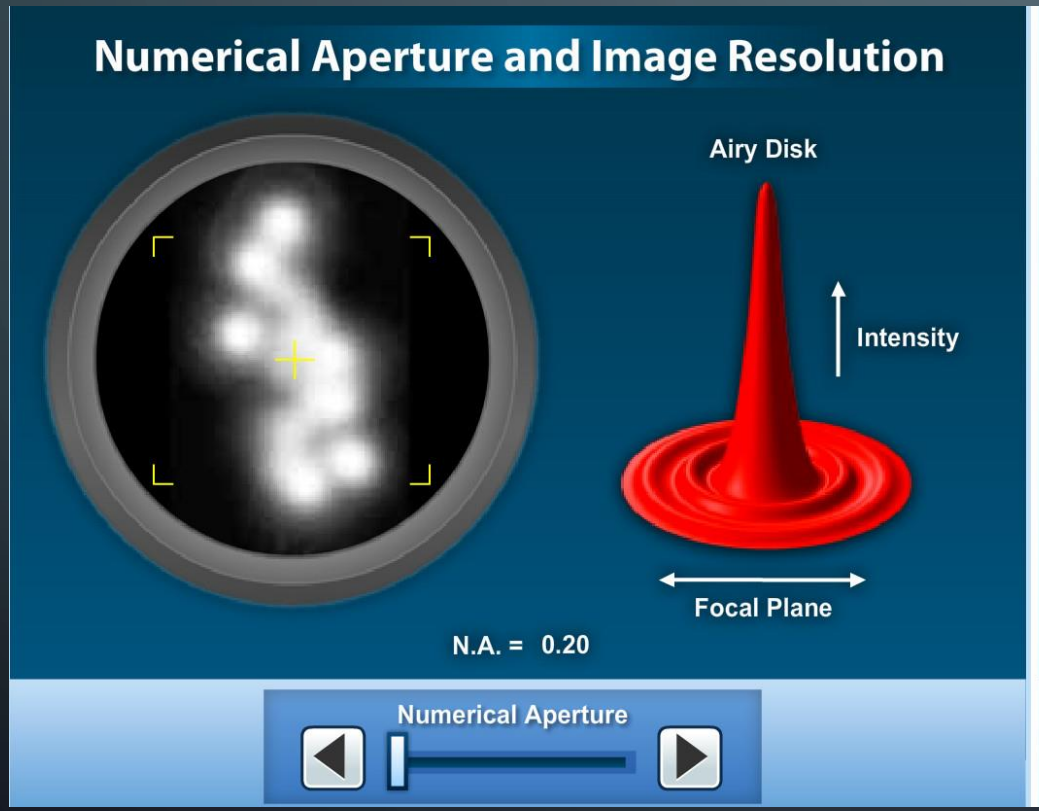


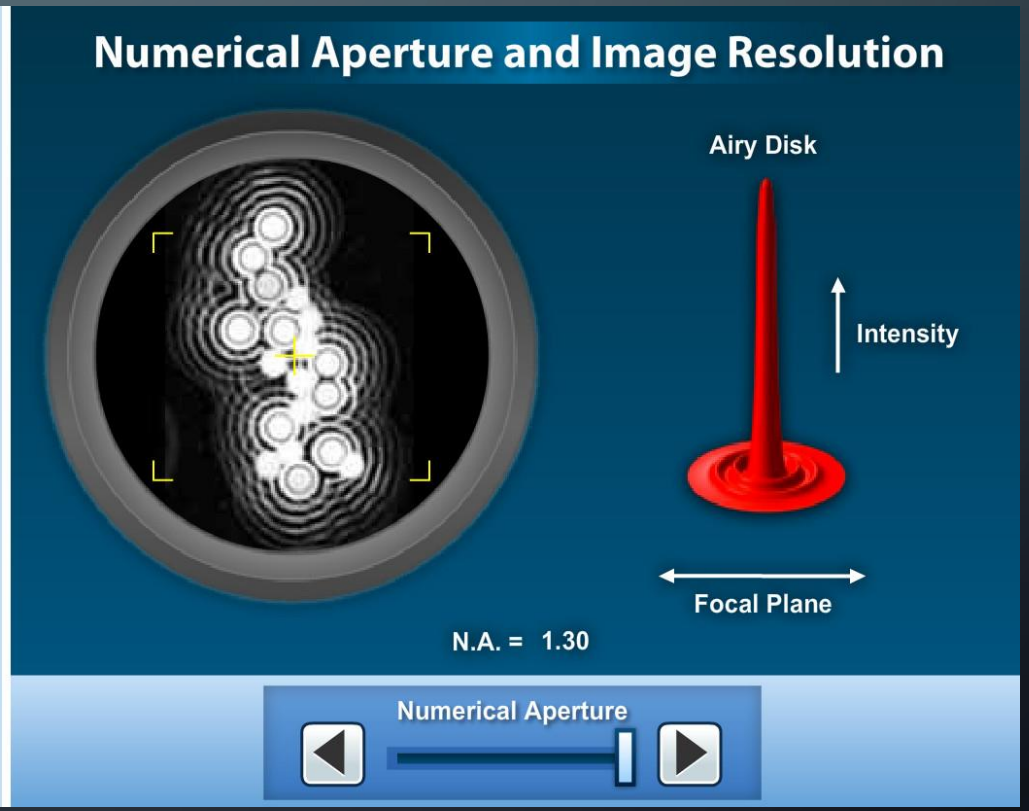
Aperture and Resolution

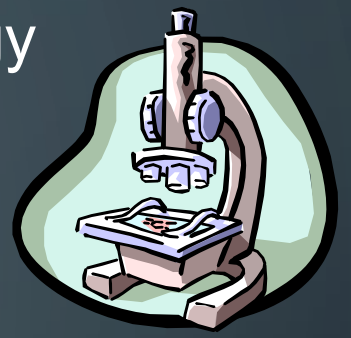


Diffraction spot
on image plane
= ***Point Spread Function***




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



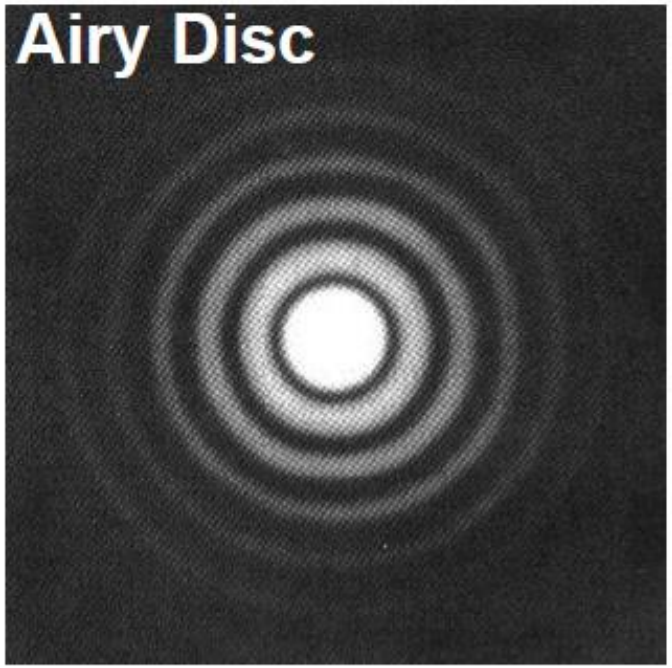
Diffraction and Point Spread Function

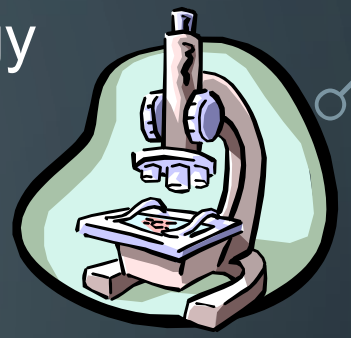
The Point Spread Function

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.

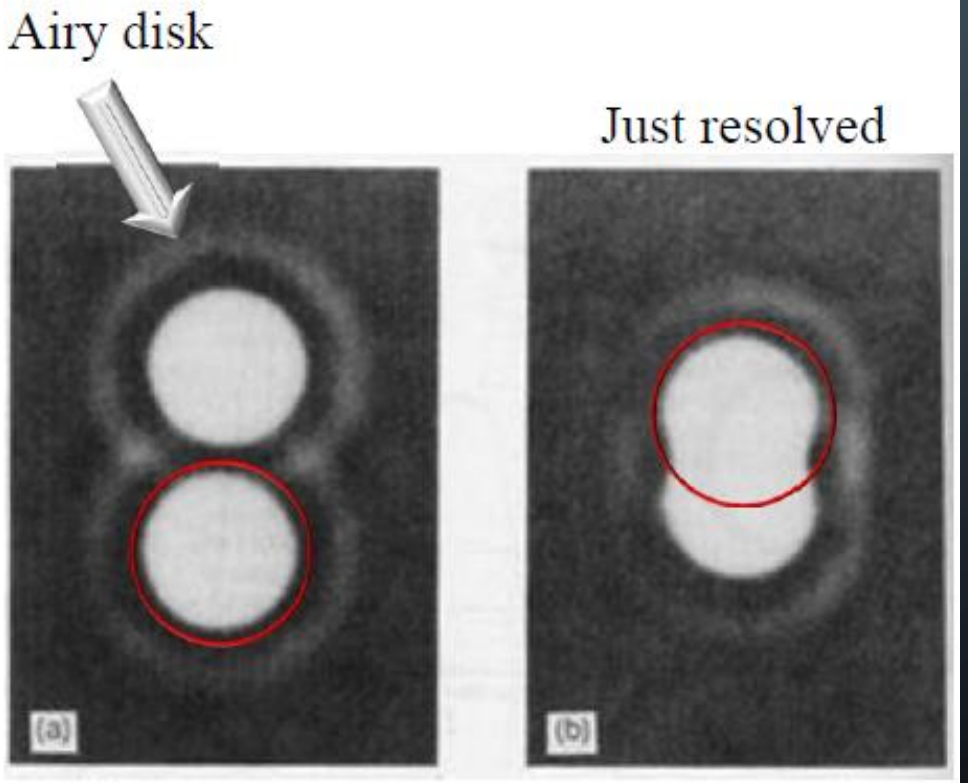
It is called the Airy disc.

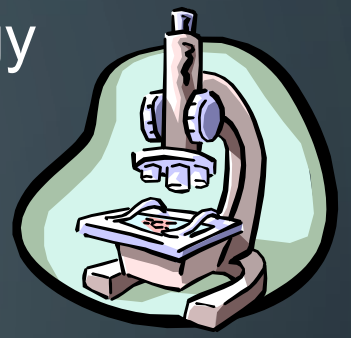




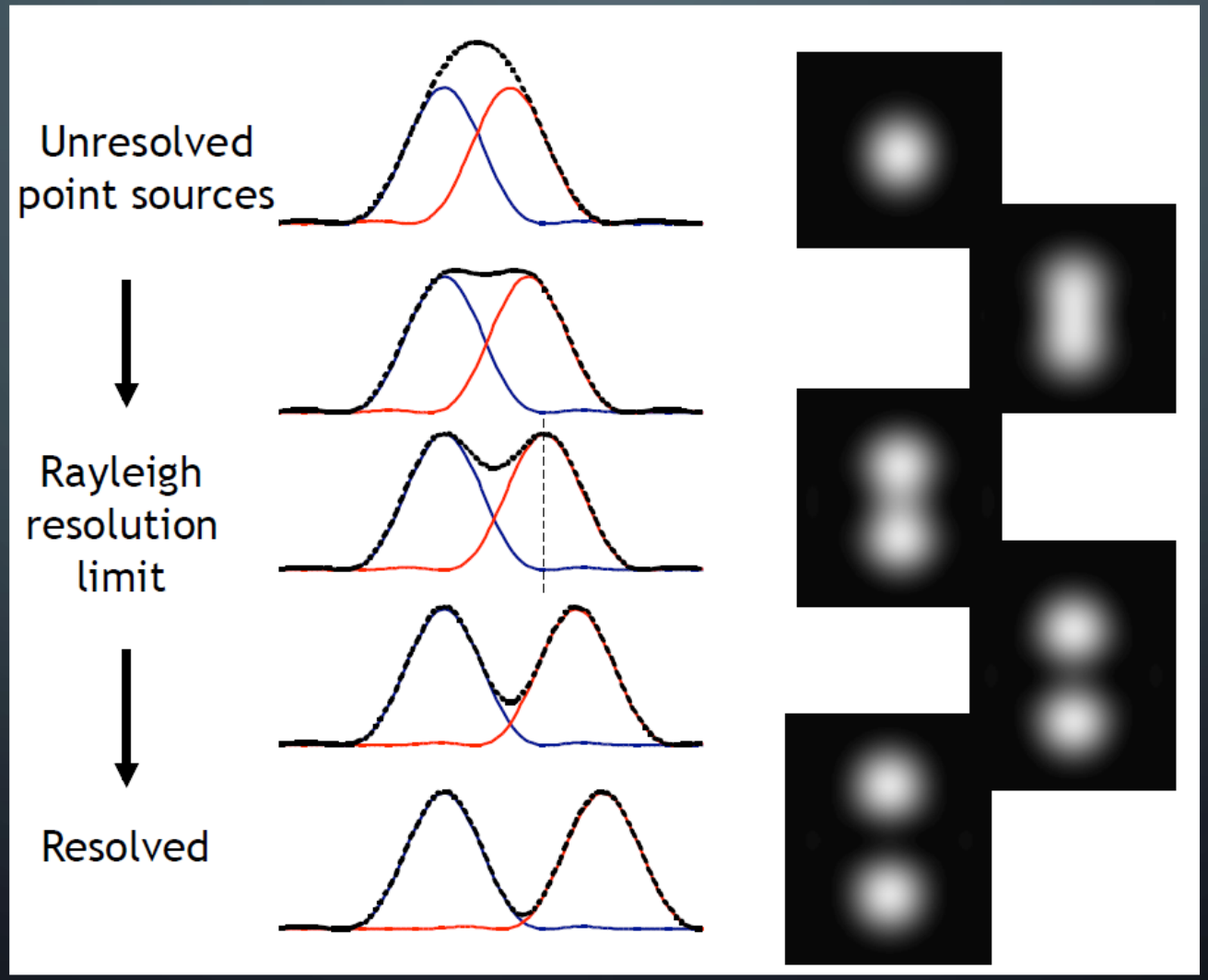
Angular resolution criterion for diffraction

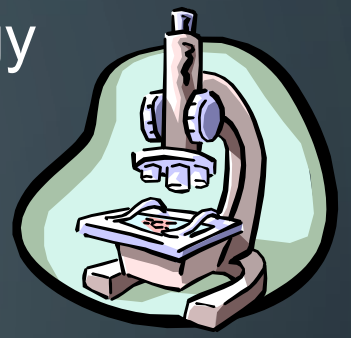
- **Rayleigh criterion** is a measure of spatial resolution
- Two point sources are "just resolved" when the diffraction maximum of one image coincides with the first minimum of the other





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

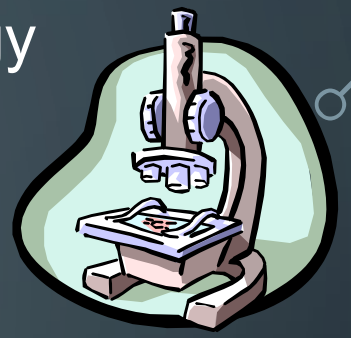
  Overall M = 600 x

Objectives

Figure 1

Objectives

Figure 1



Objectives & Co

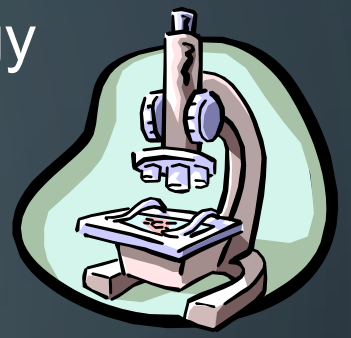
http://zeiss-campus.magnet.fsu.edu/tutorials/basics/transmittedlightopticalpathway/indexflash.html

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

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

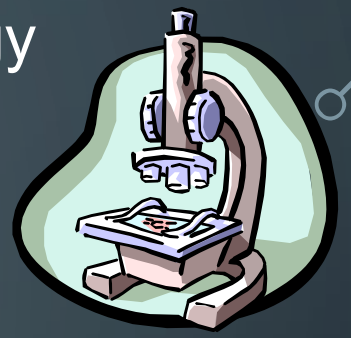
http://www.microscopyu.com/tutorials/java/aberrations/slipcorrection/index.html

http://www.microscopyu.com/articles/optics/index.html



The characteristics of objectives

Figure 1



YOUR FRIEND - THE OBJECTIVE

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

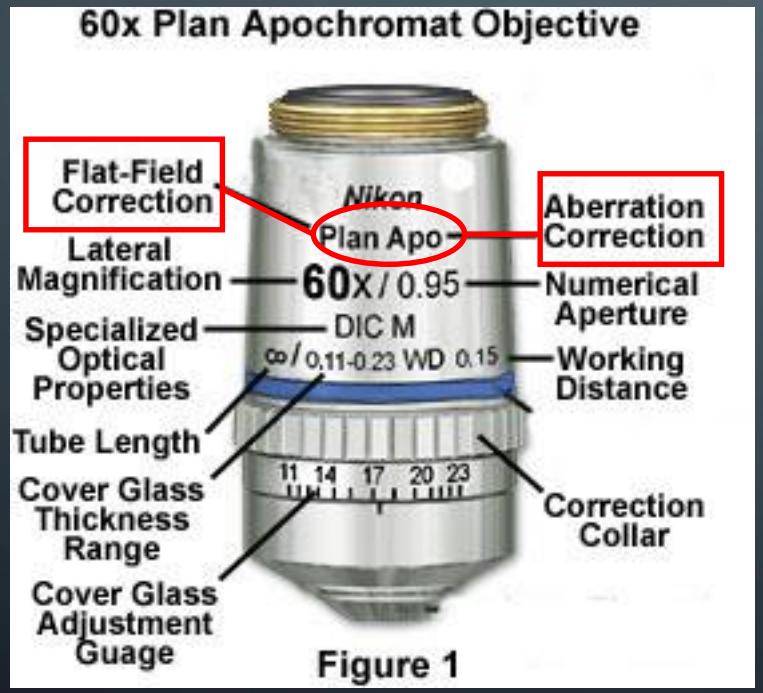


Figure 1

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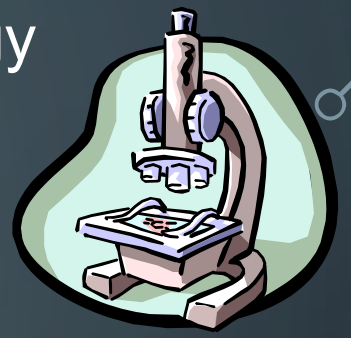


OBJECTIVE TYPES

- 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

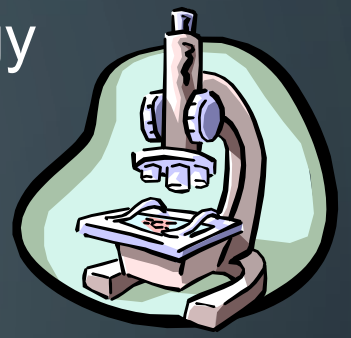
  Improved Achromat objectives with good image flatness for fields of view with dia. 20 or even 23 mm. Achroplan for transmitted light and Achroplan Ph for phase contrast.

- 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.

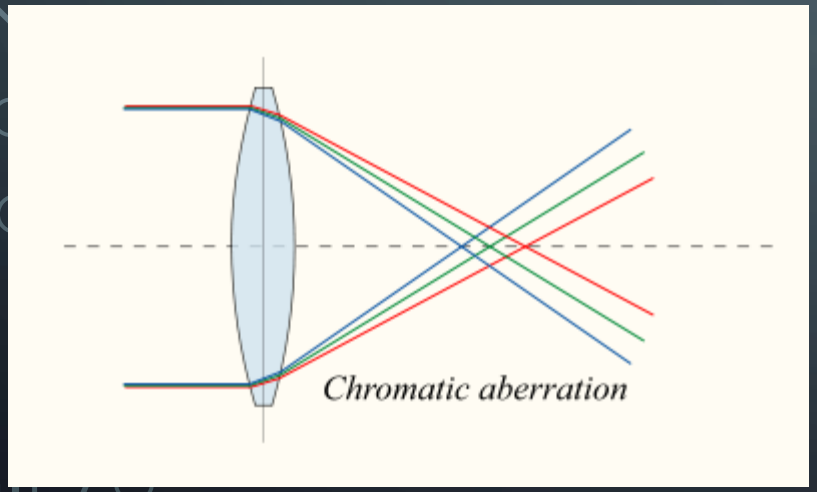
- Plan-Apochromat

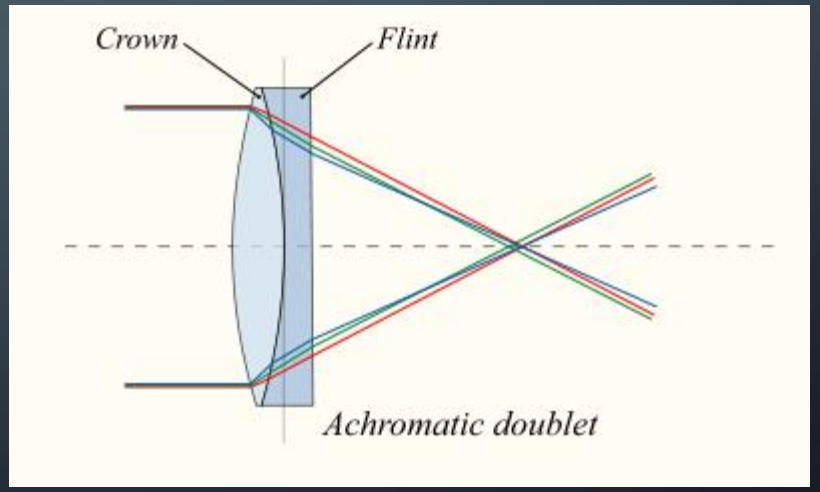
  Perfect colour rendition (correction for four wavelengths!). Flawless image flatness for fields of view with dia. 25 mm. Highest numerical apertures for a resolving power at the very limits of the physically possible.



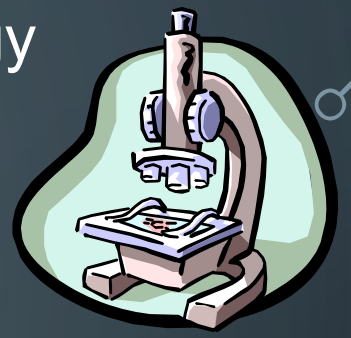
CHROMATIC ABERRATION

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.



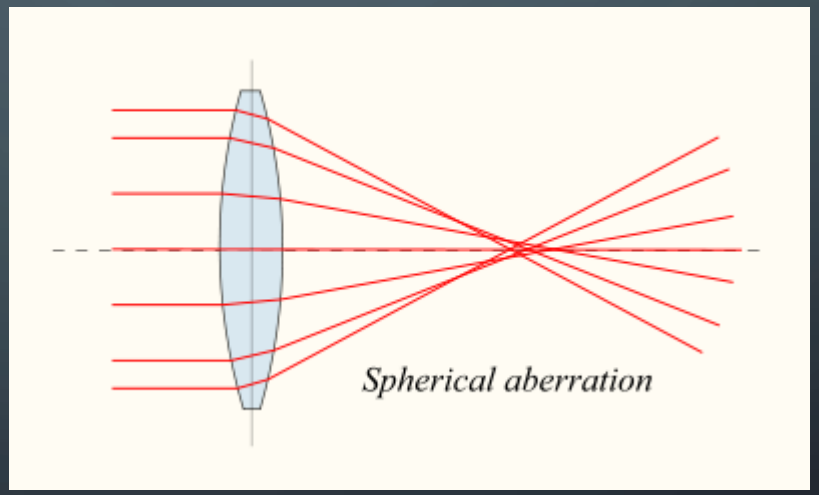


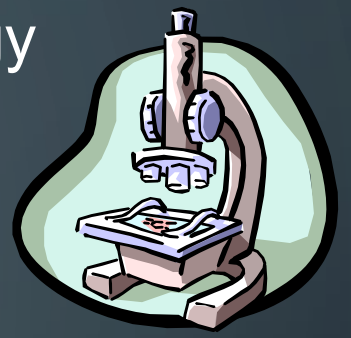
It can be minimised by using an achromatic doublet (= achromat) in which two materials with differing dispersion are bonded together to form a single lens.



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.)

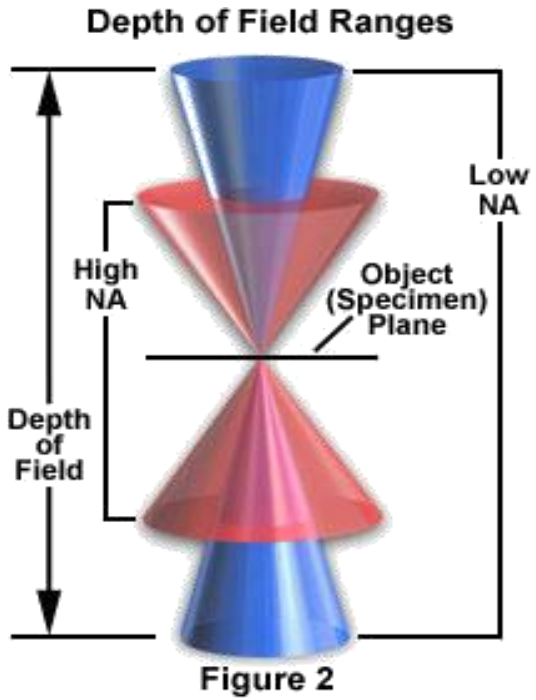


Figure 2

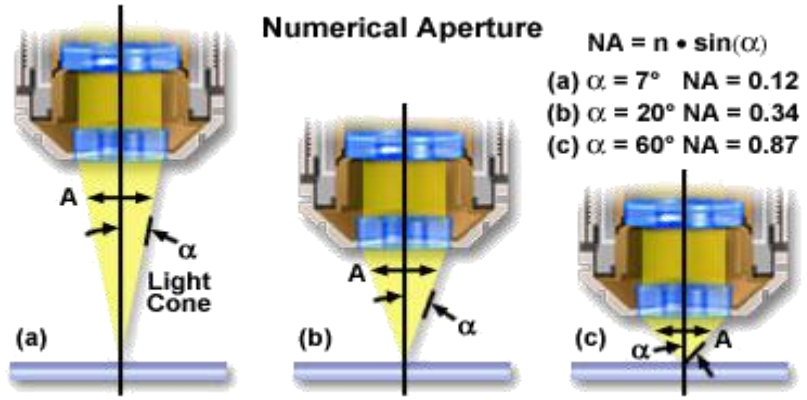


Figure 1

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)

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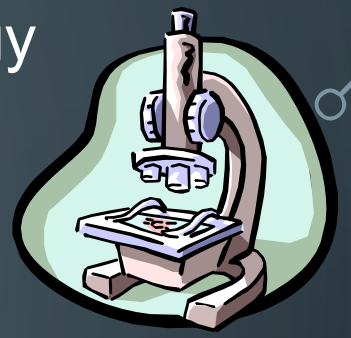
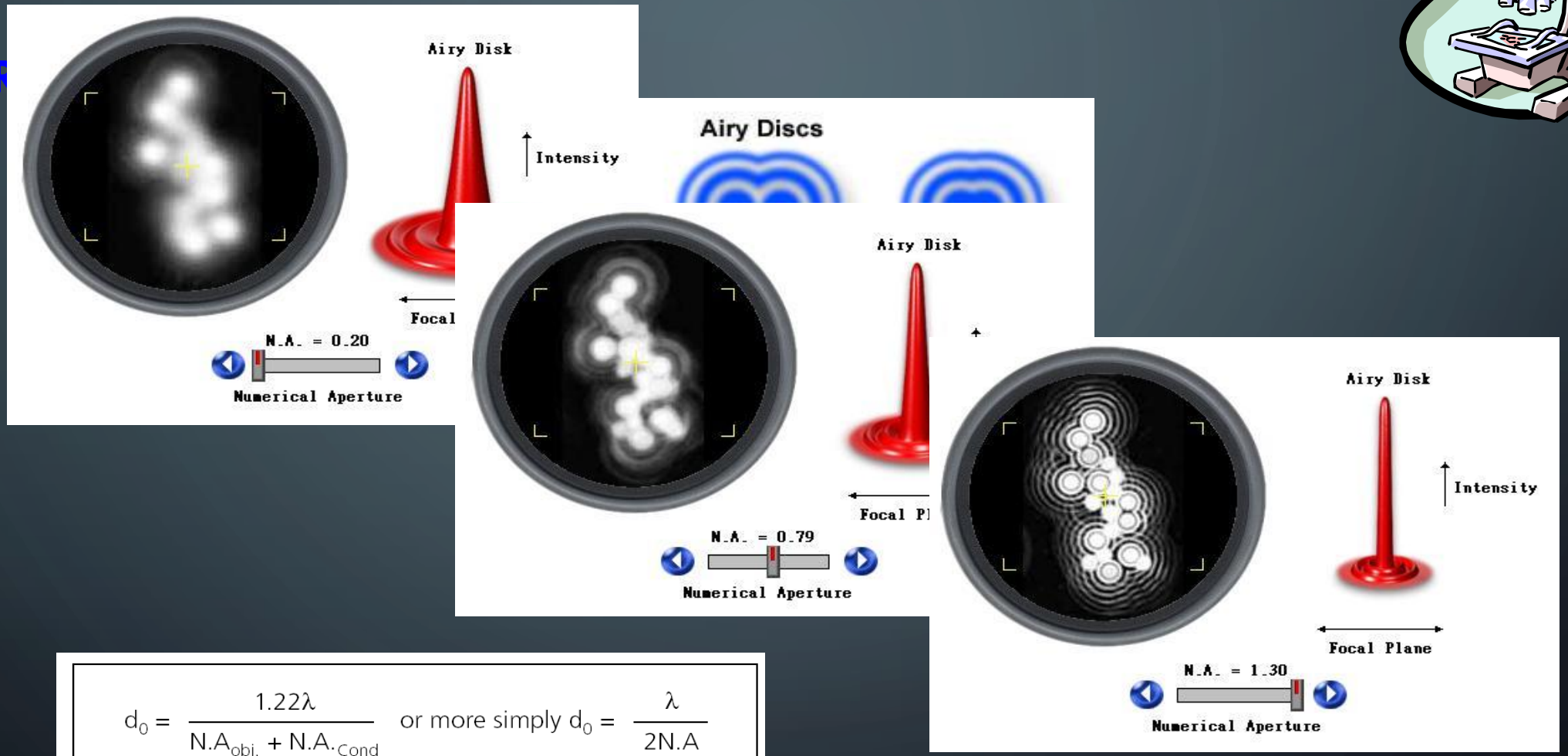
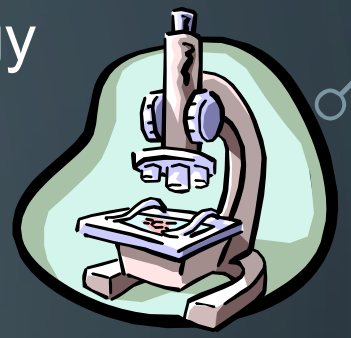




$$d_0 = \frac{1.22\lambda}{N.A_{obj.} + N.A._{Cond}} \quad \text{or more simply } d_0 = \frac{\lambda}{2N.A}$$

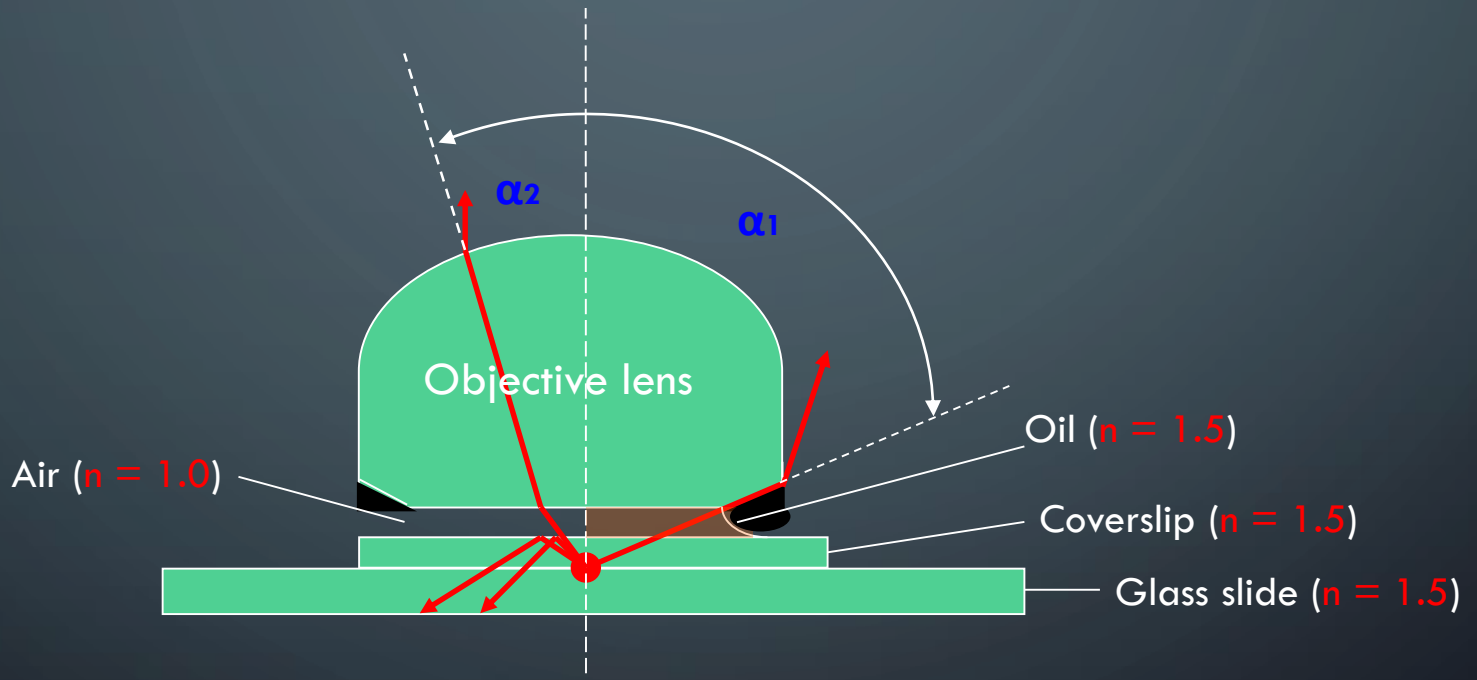
λ = wavelength of light, e.g. 550 nm (green)

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



What is the numerical aperture?

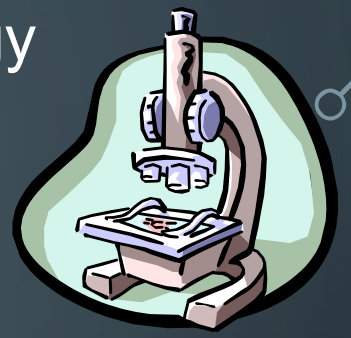
NA is an estimate of how much light from the sample is collected by the objective



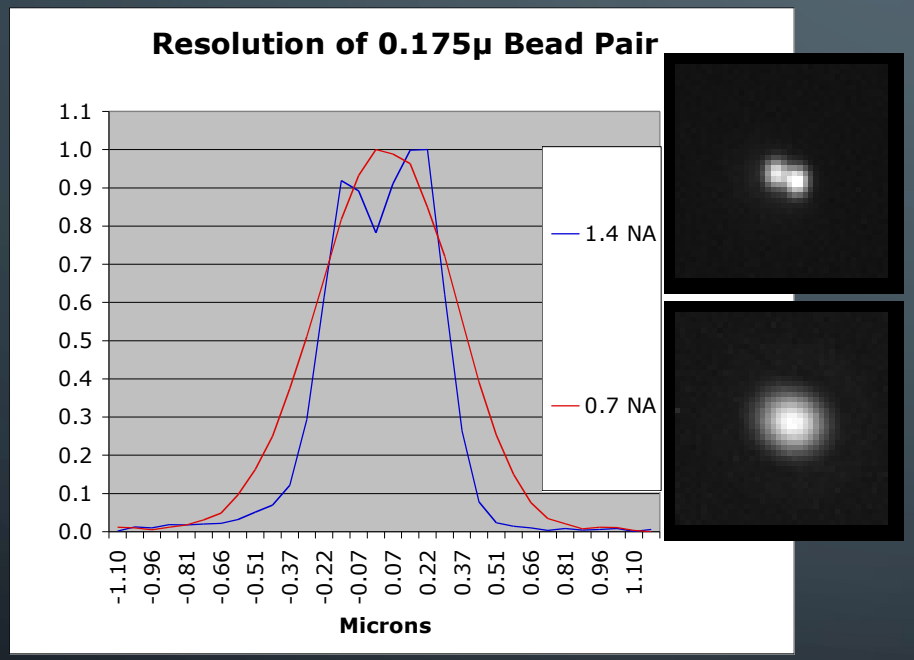
$$NA = n \sin \alpha$$

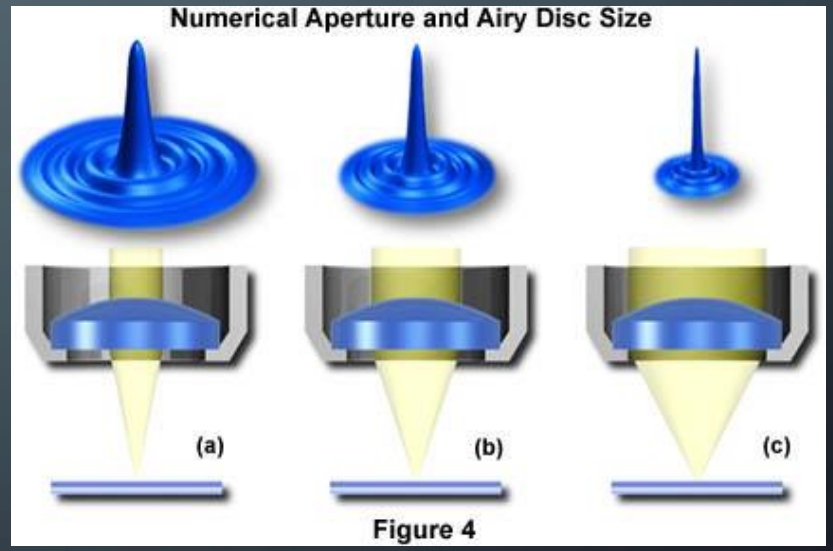
n = refractive index

α = angle of incident illumination



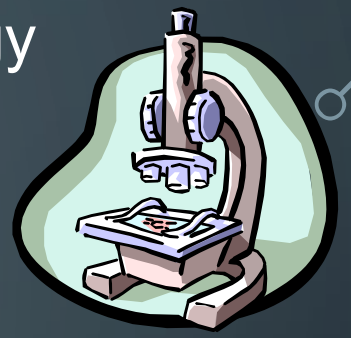
Numerical aperture, NOT magnification determines resolution!





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.



Contrast Generation for Transmitted Light

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

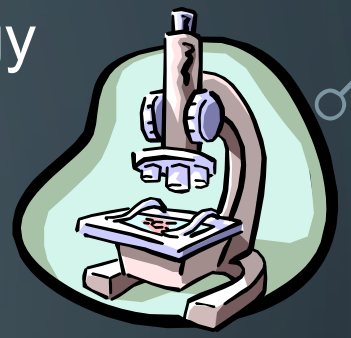
Contrast Generation for Transmitted Light

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

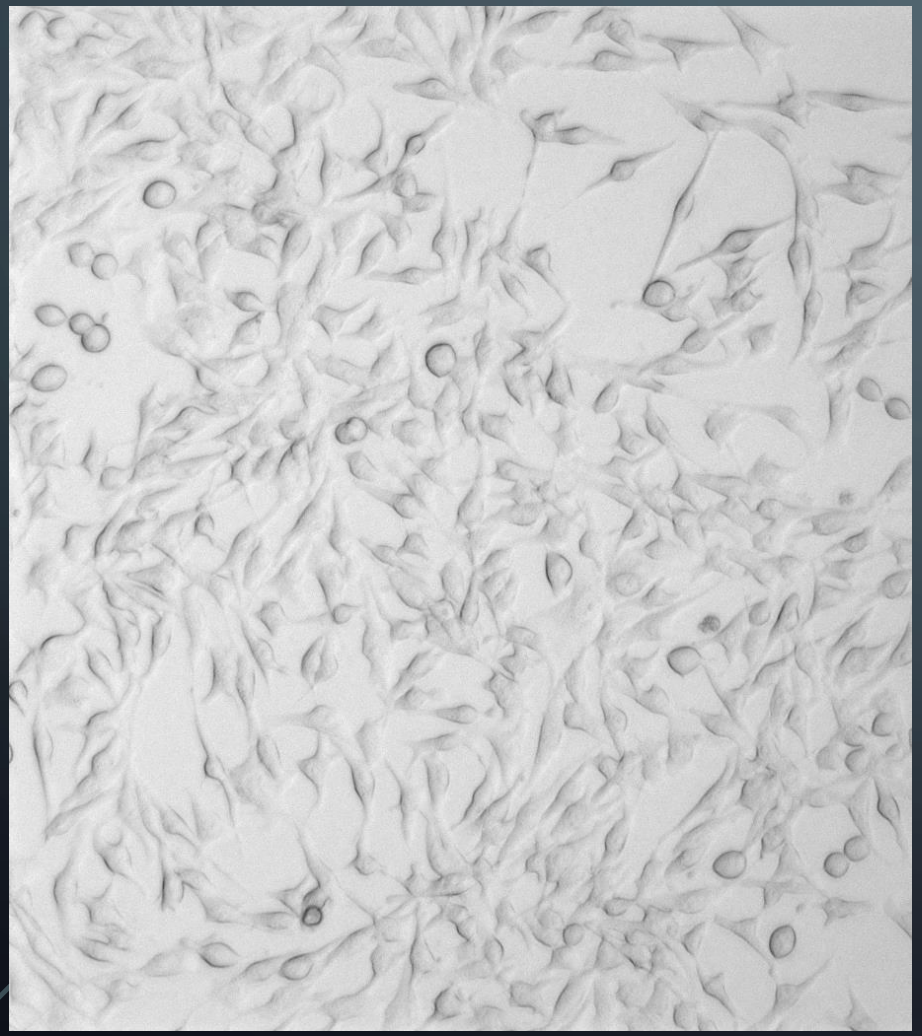
>>> Phase shift / Polarization / Interference

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.



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



Which properties can be used?

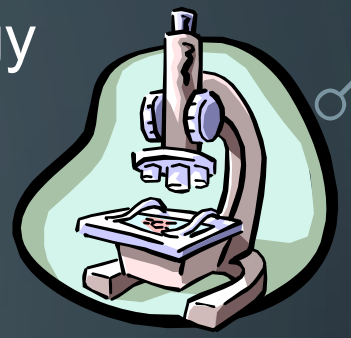
Absorption

Scattering

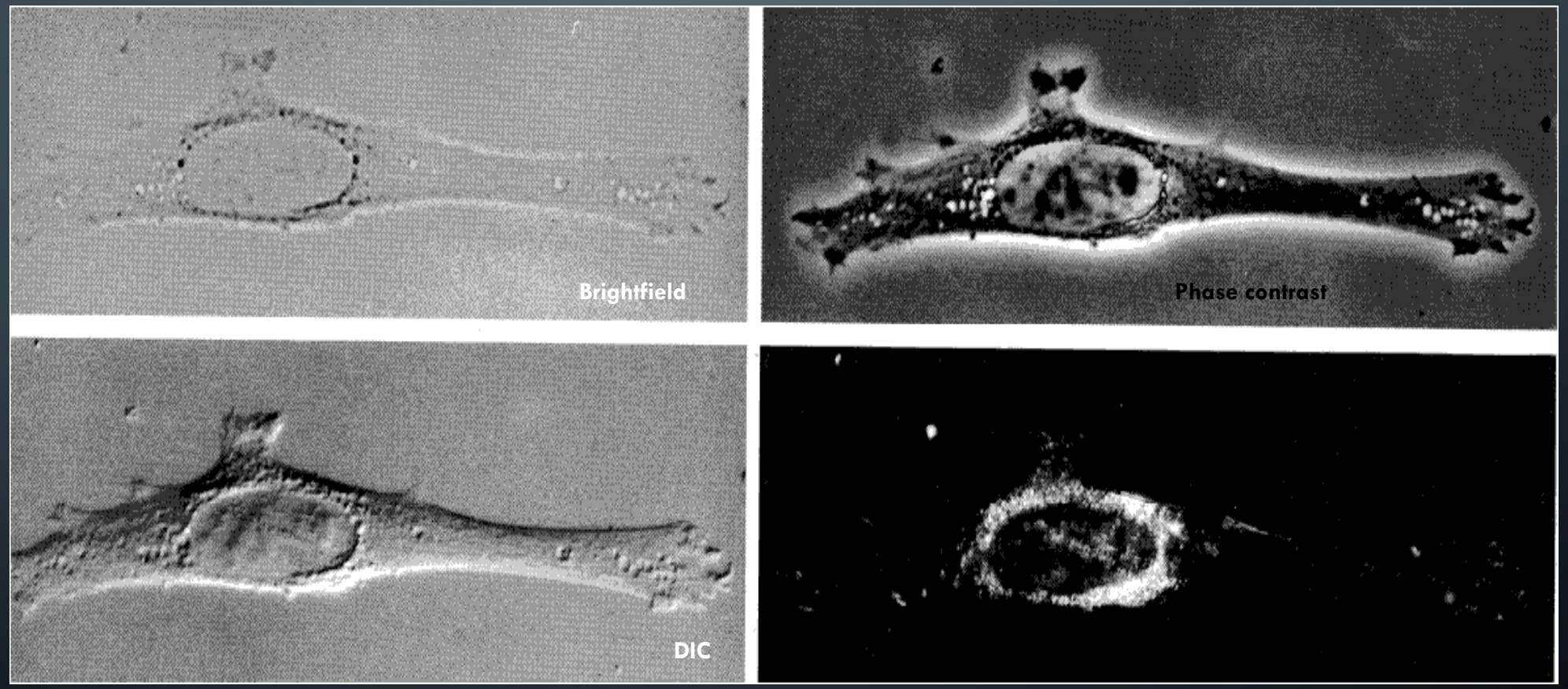
Refraction

Phase

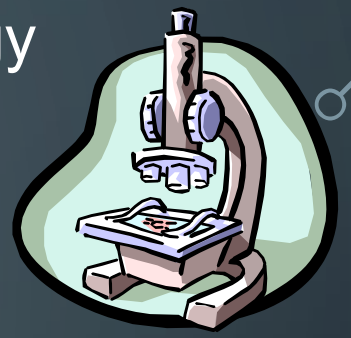
Polarization



CONTRASTING TECHNIQUES

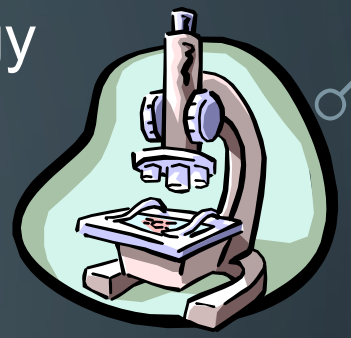


Taken from: http://fig.cox.miami.edu/~cmallery/150/Fallsyll.htm



CONTRASTING TECHNIQUES

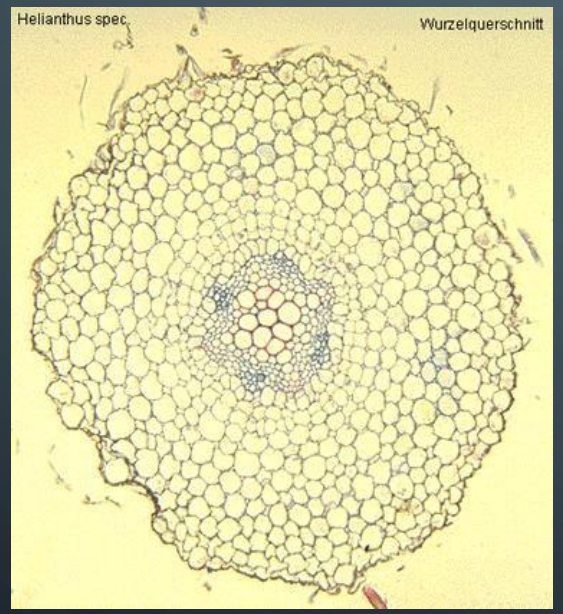
- Brightfield
- Darkfield
- Phase Contrast
- Polarization Contrast
- Differential Interference Contrast (DIC)



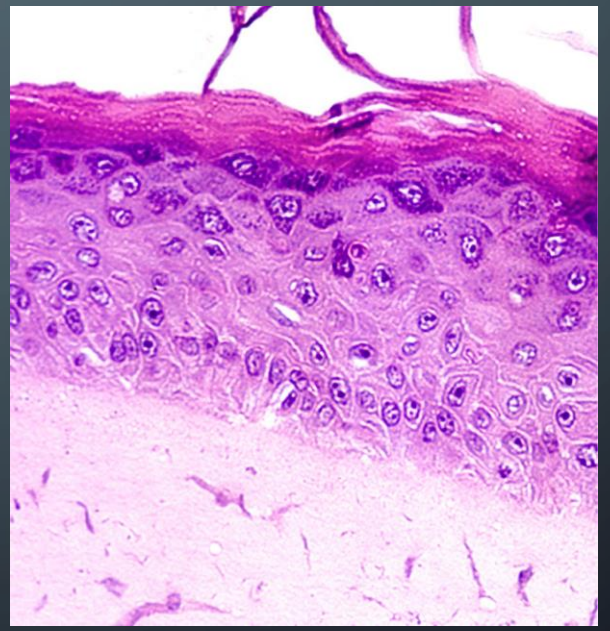
BRIGHTFIELD

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

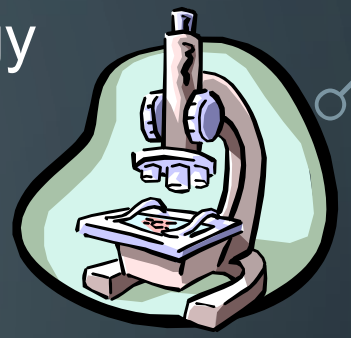
Application: Only useful for specimens that can be contrasted via dyes. Very little contrast in unstained specimens. With a bright background, the human eye requires local intensity fluctuations of at least 10 to 20% to be able to recognize objects.



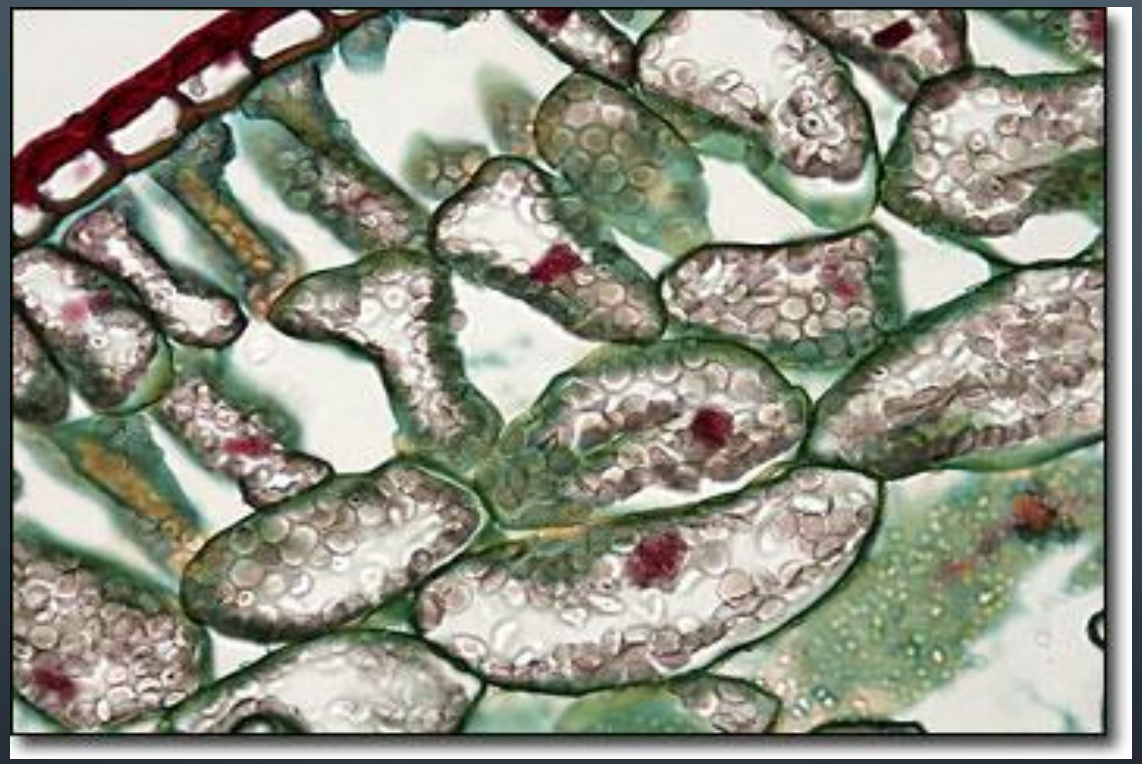
Cross section of sunflower root
(http://www.zum.de/Faecher/Materialien/beck/12/bs12-5.htm)



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



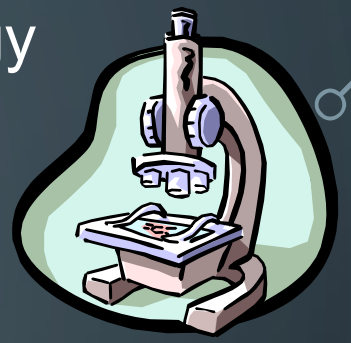
Bright Field



- 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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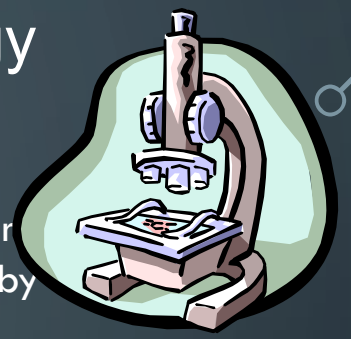
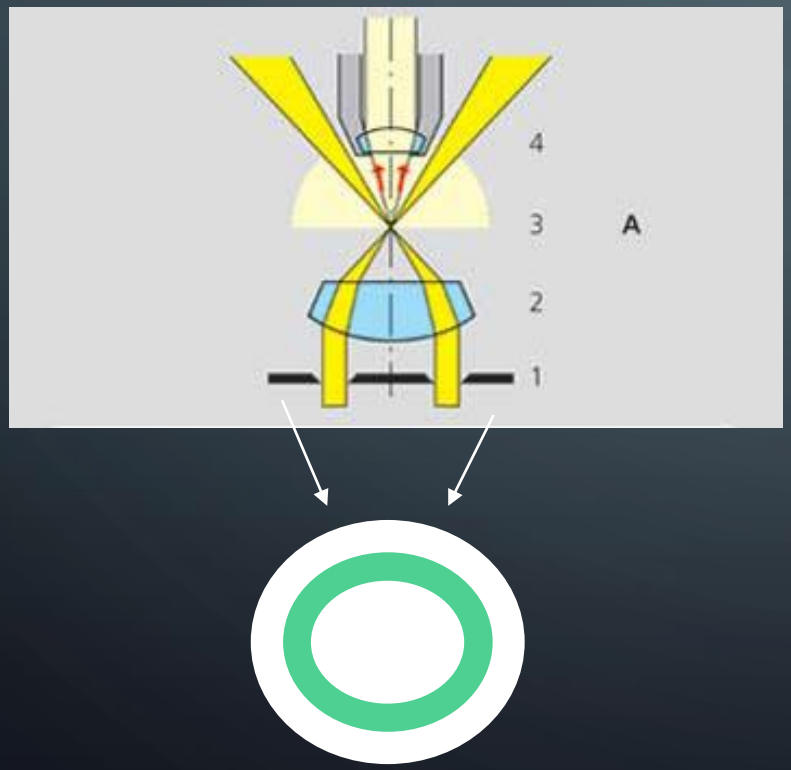
DARKFIELD



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



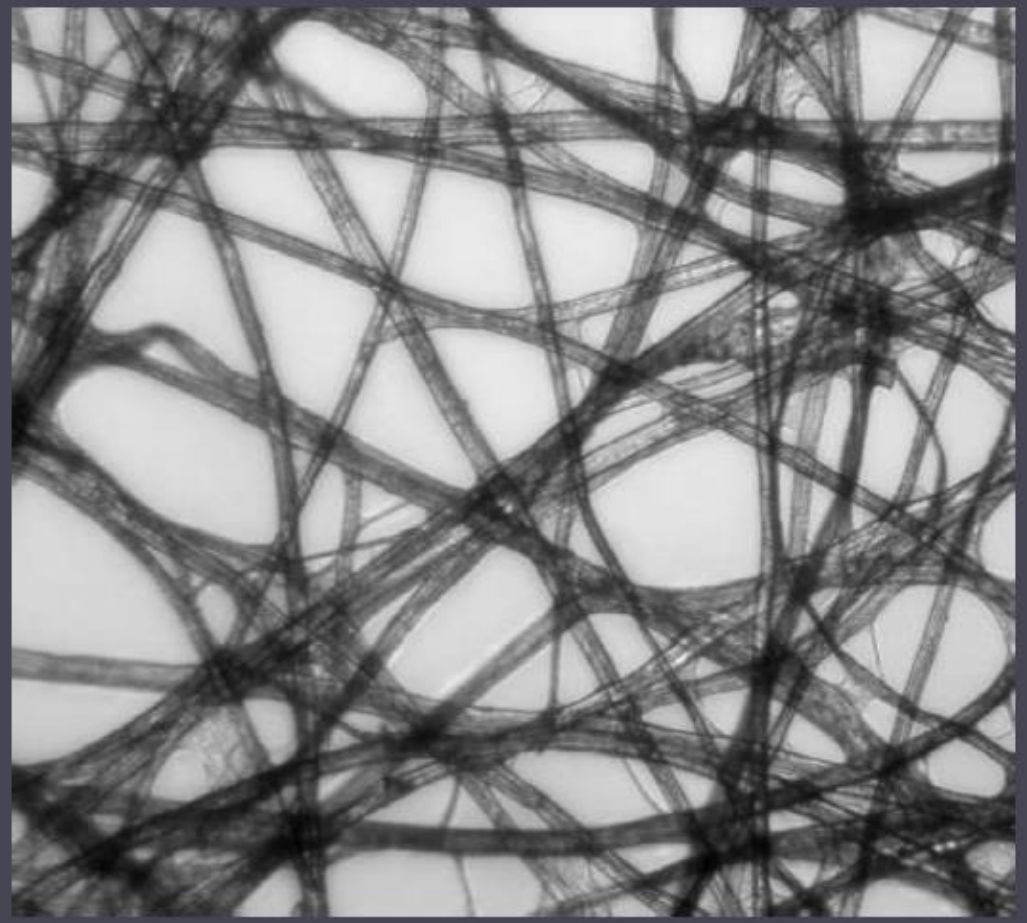
Symbiotic Diatom colony

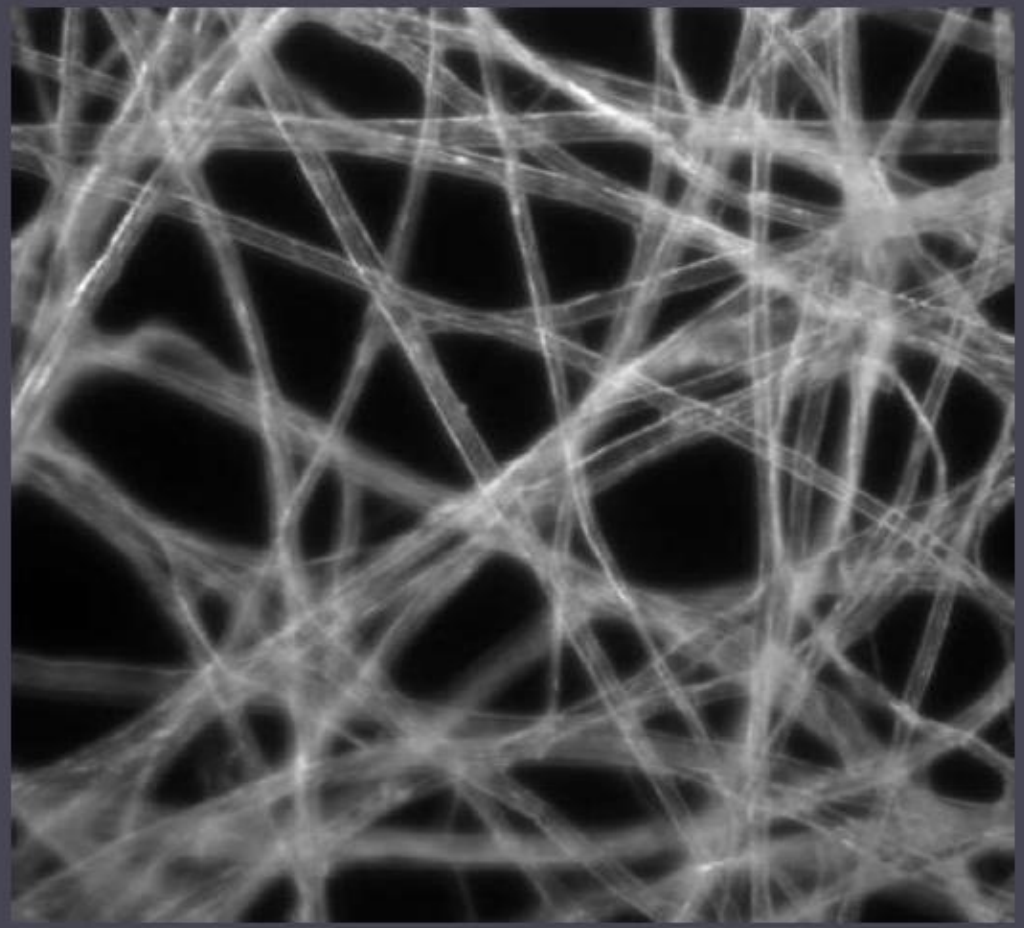
(www1.tip.nl/~t936927/making.html)

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

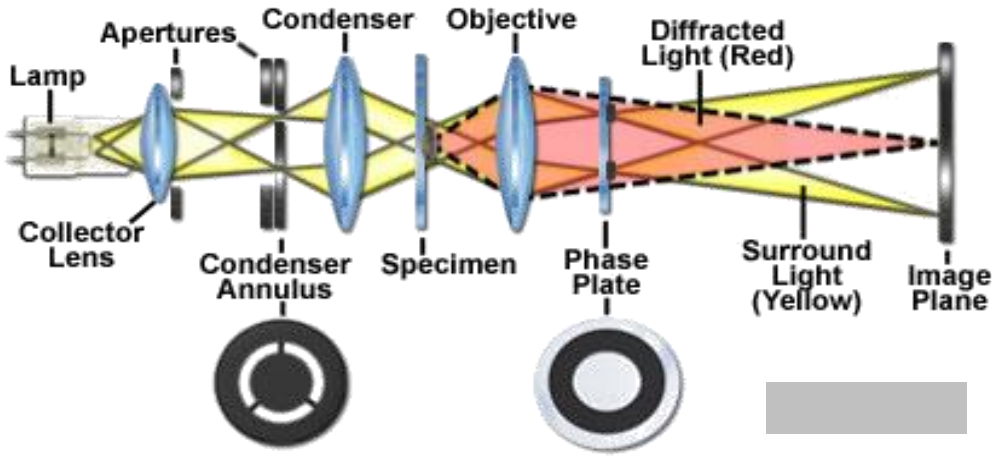
Dark-Field Microscopy

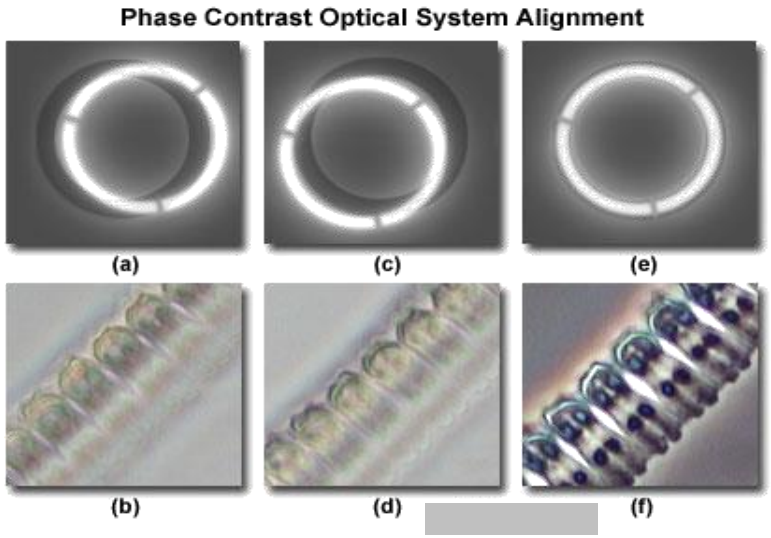
- Dark background against which objects are brilliantly illuminated.
- This is accomplished by equipping with a special condenser that transmits a hollow cone of light.
- Most of the light directed through the condenser does not enter the objective, the field is dark.
- However, some of the light rays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

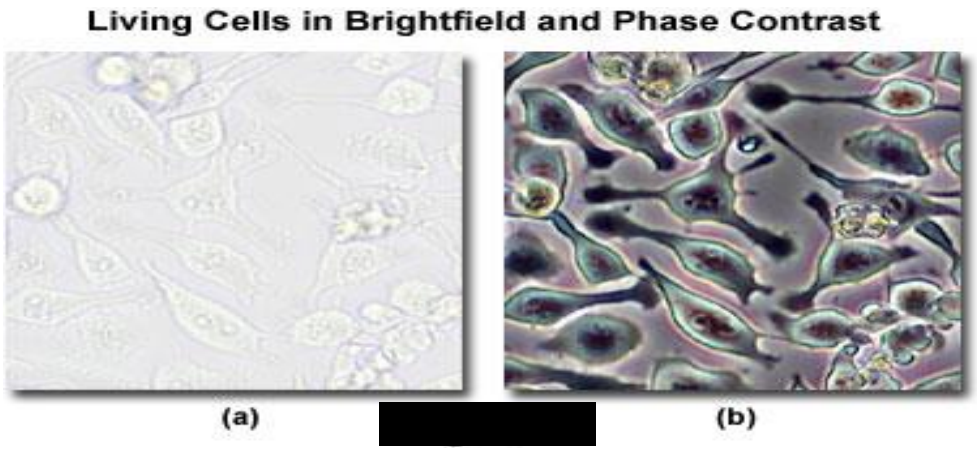




Phase Contrast

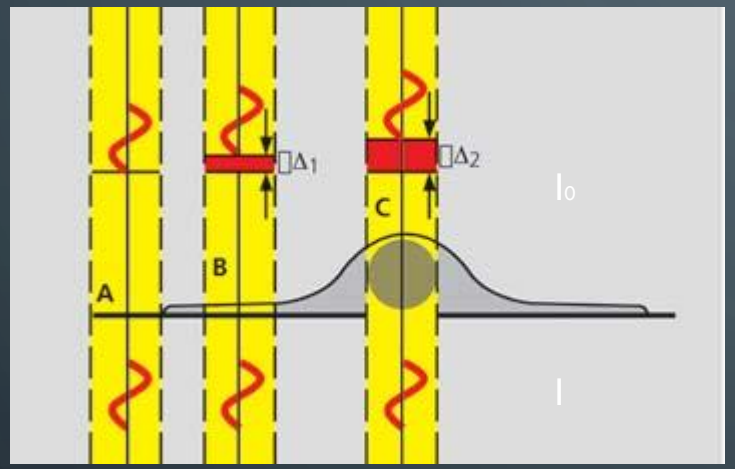


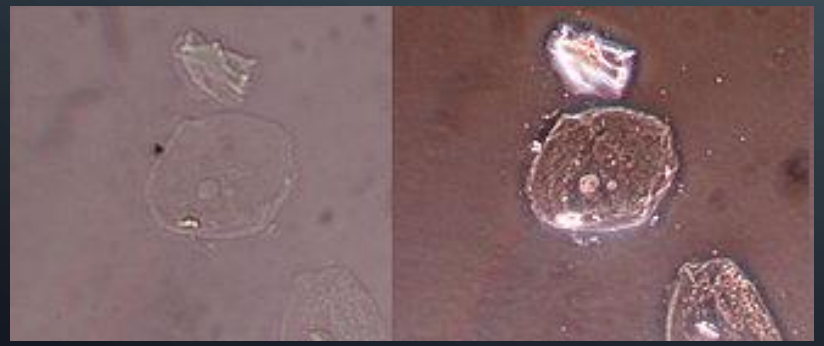


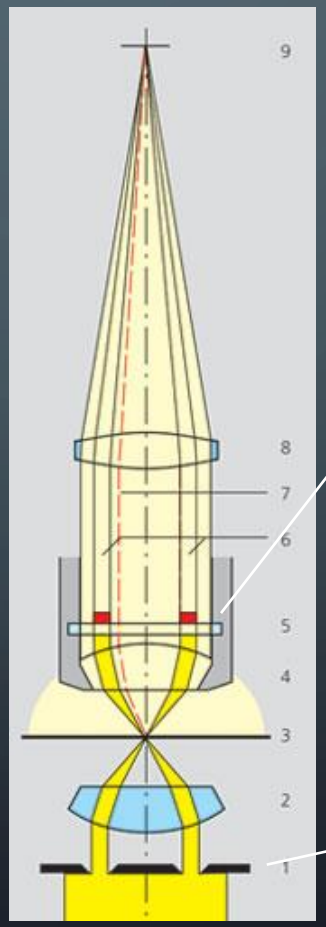


PHASE CONTRAST IN THEORY

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

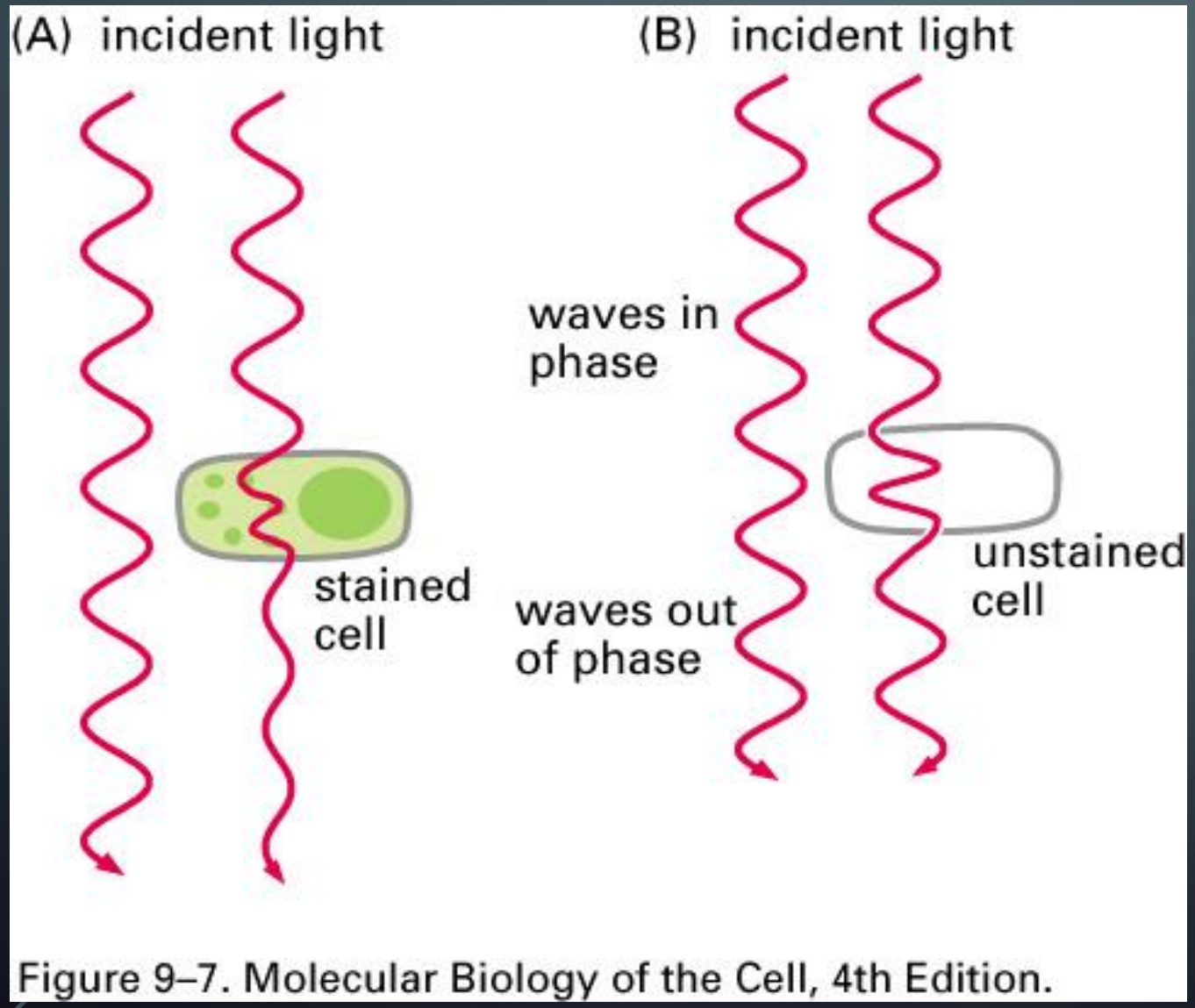
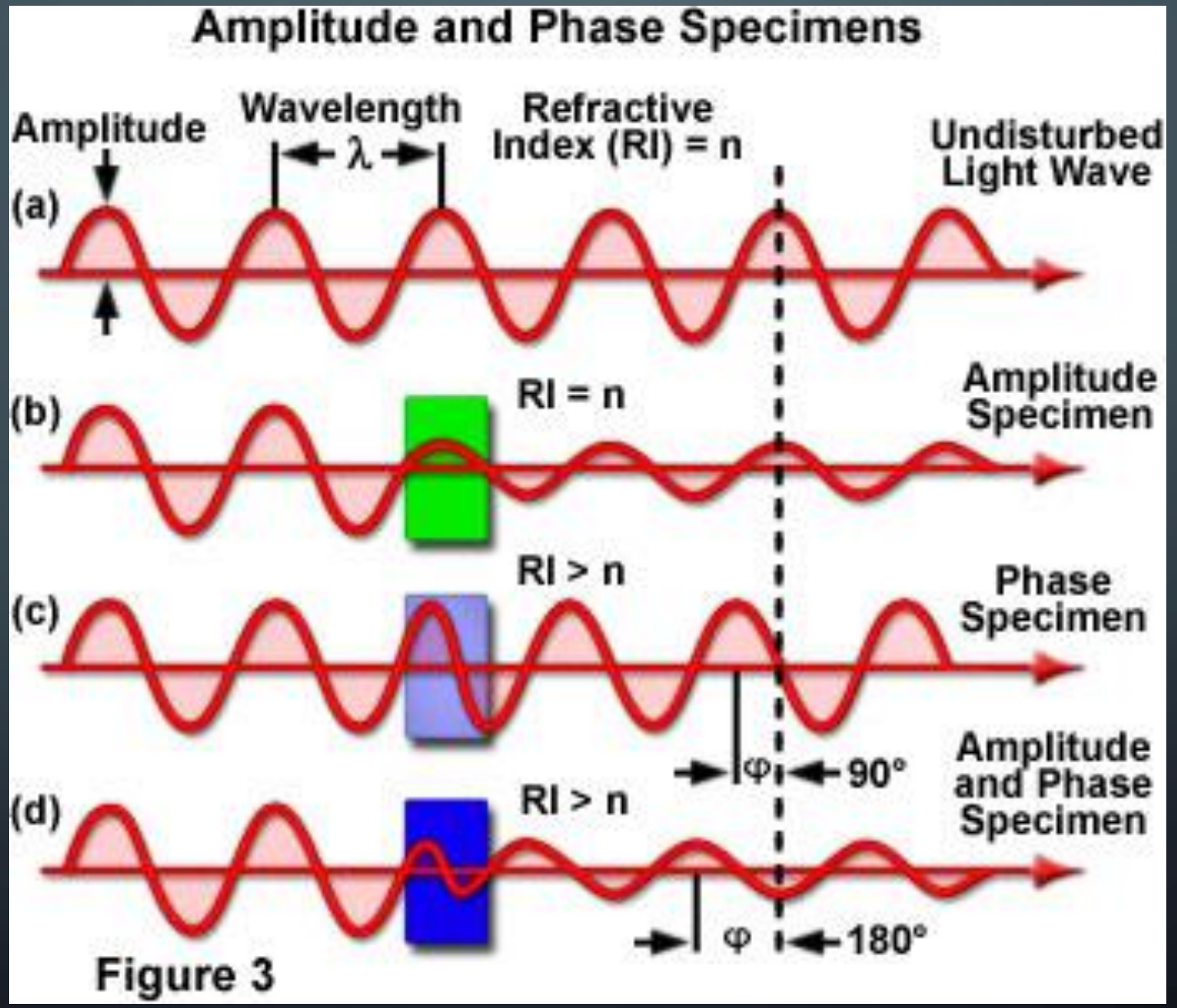


Figure 9–7. Molecular Biology of the Cell, 4th Edition.

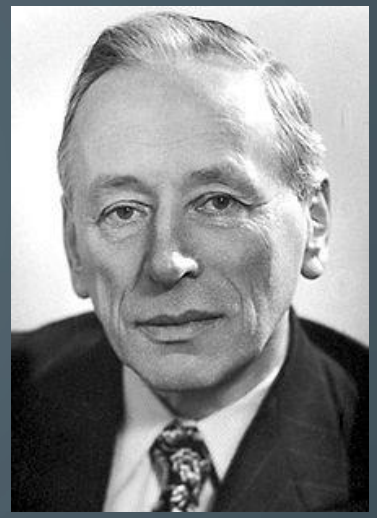
- Do not absorb light
- Difference in index of refraction between specimen and background


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?

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

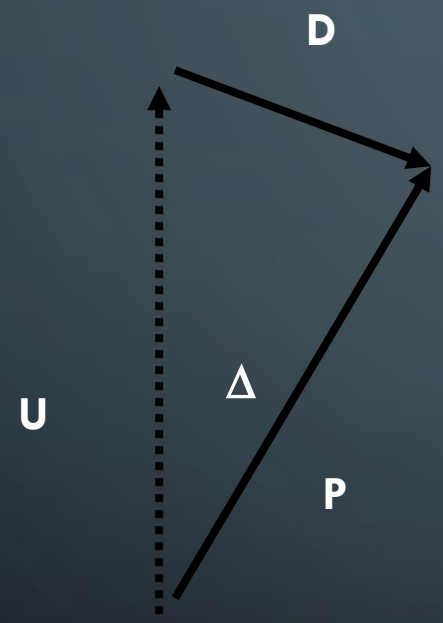
PHASE DIFFERENCES

- 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?

PHASE CONTRAST IMAGING

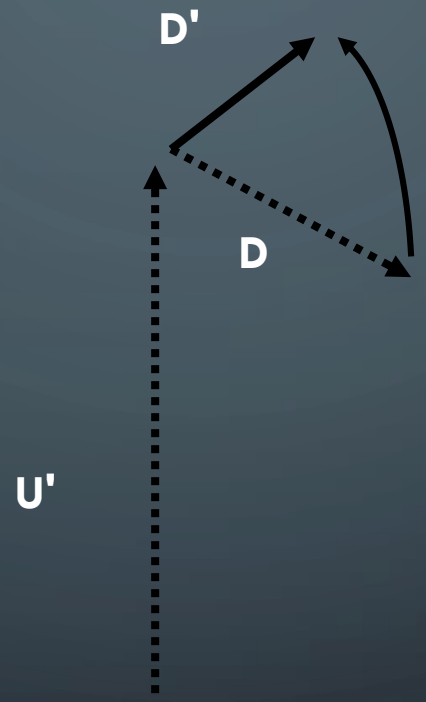
- Basic principle:
  - Shift phases (Δs) and/or amplitudes of U and D differentially
  - This can produce a change in amplitude of P (length of vector)

In specimen

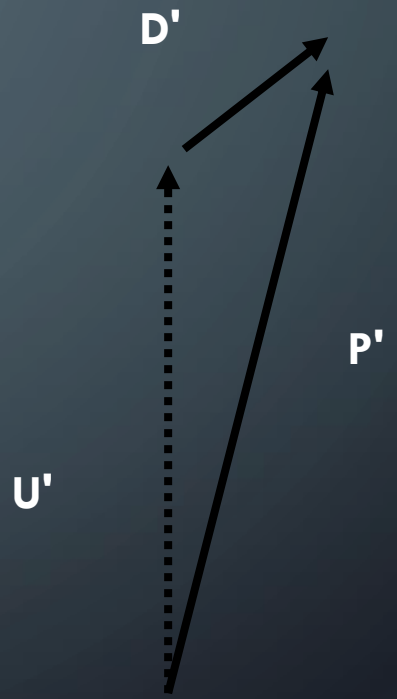


$U = P$

In microscope



At image plane

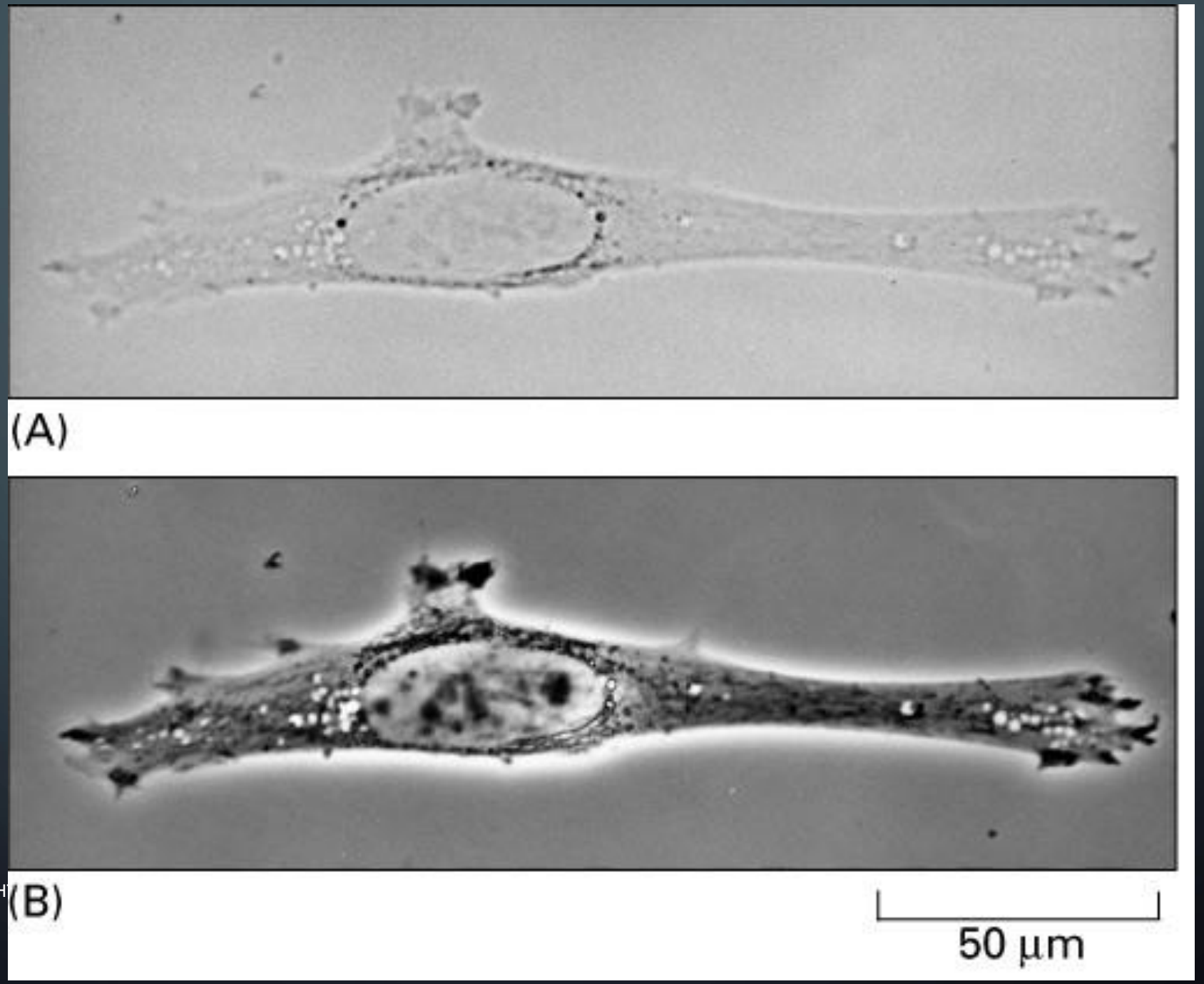


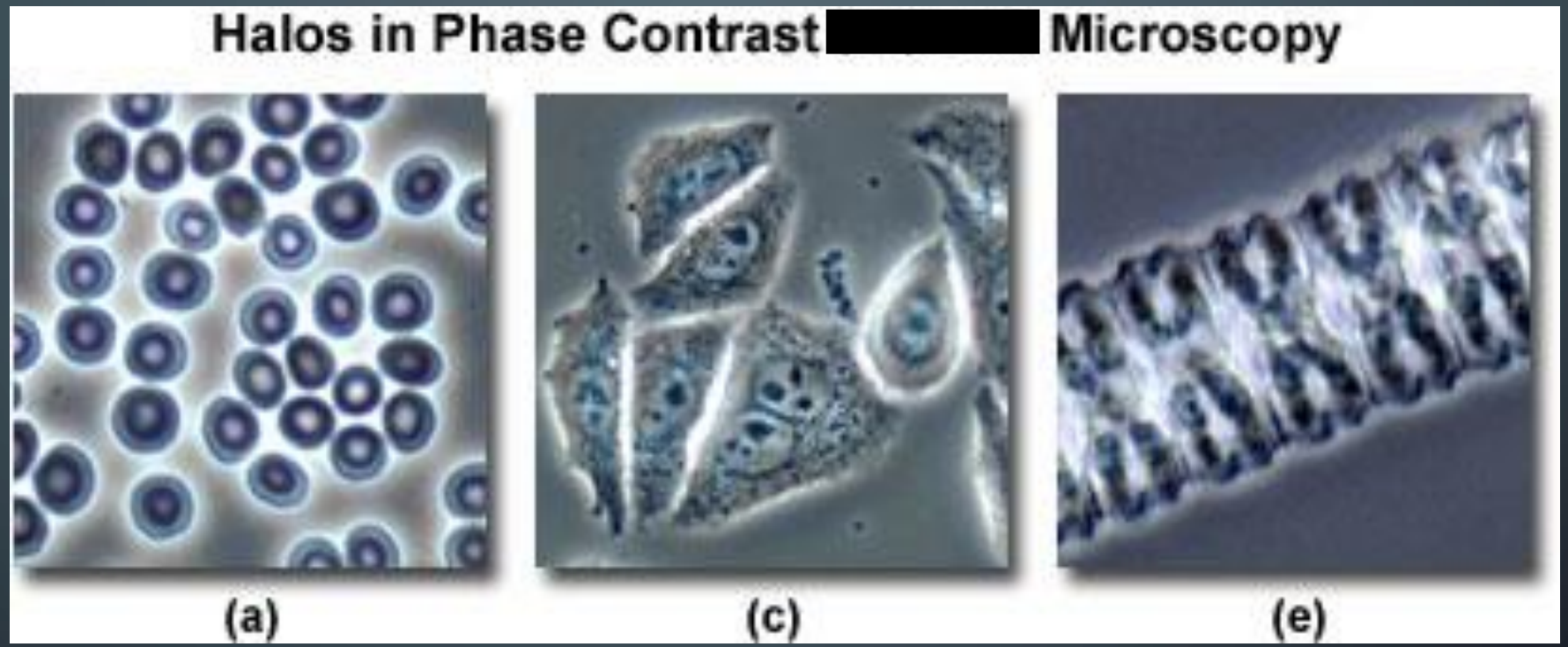
$U' \neq P'$

Amplitude!

PHASE CONTRAST OPTICS

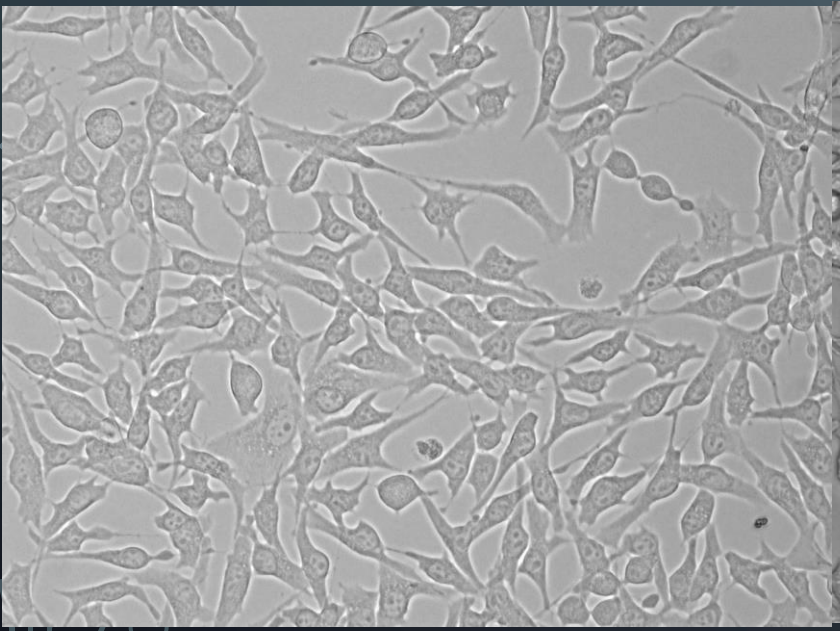
- Physically separates U and D light and subjects one or the other to phase shift and/or amplitude shift
- In theory, any shift of U and D are possible
- When light passes from one medium into another, the velocity is altered proportionally to the refractive index differences between the two media
- https://www.microscopyu.com/techniques/phase-contrast/introduction-to-phase-contrast-microscopy


(A)
(B)
50 µm

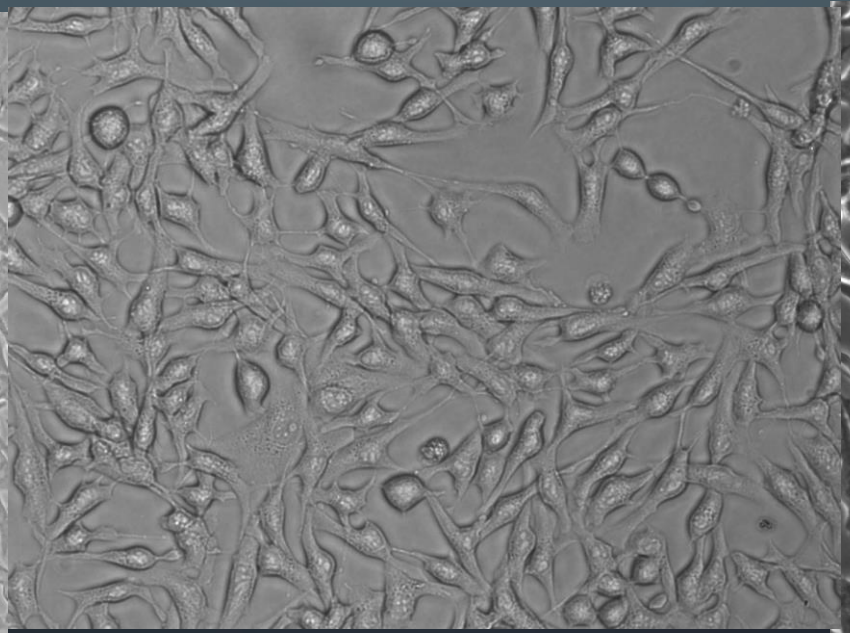

Halos in Phase Contrast [redacted] Microscopy
(a)
(c)
(e)

Phase contrast in practice

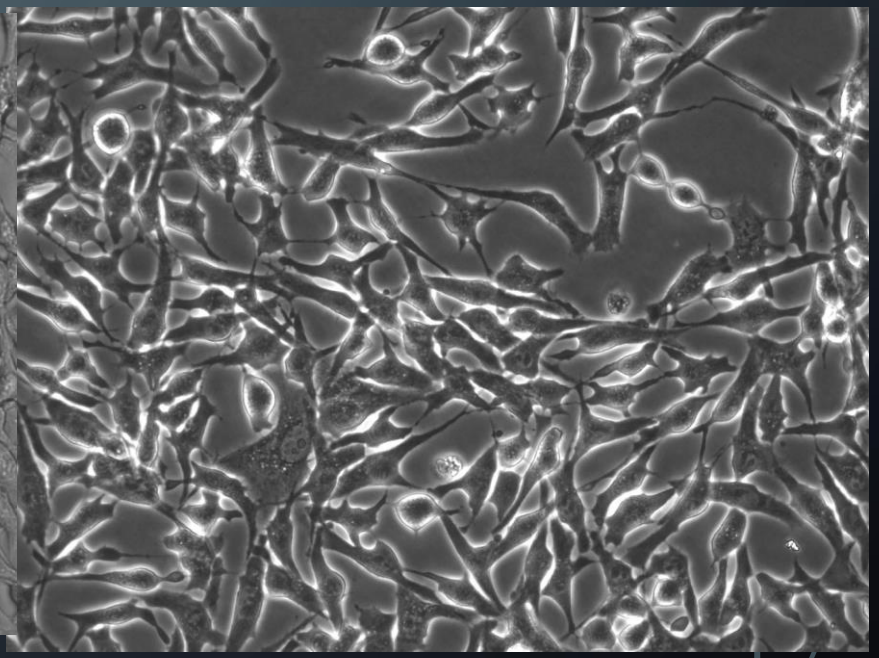
Application: Phase contrast is the most commonly used contrasting technique All tissue culture microscopes and the time-lapse microscopes are set up for phase.



brightfield



wrong phase stop



right phase stop