MICROSCOPIA OTTICA IN BIOLOGIA CELLULARE [675SM]

MICROSCOPY IN CELL BIOLOGY –

aa 2023/2024, 2nd semester

Aula ex-CLA, ed C1, 15-18

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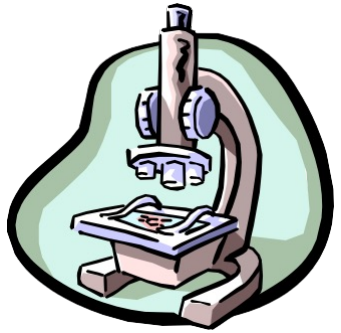
UNIVERSITÀ DEGLI STUDI DI TRIESTE
Dipartimento di Scienze della Vita

MICROSCOPIA OTTICA IN BIOLOGIA CELLULARE [675SM]

date	lesson/lab	aula	time
06/03/24	intro	Aula Ex-Cla, C1	15-16
13/03/24	lesson1	Aula Ex-Cla, C1	15-18
20/03/24	Lesson2+lab	sala microscopia F2, C1	15-18
27/03/24	lesson3	Aula Ex-Cla, C1	15-18
10/04/24	lesson4	Aula Ex-Cla, C1	15-18
17/04/24	lesson5	Aula Ex-Cla, C1	15-18
24/04/24	Lesson6+lab2	Aula Ex-Cla, C1	15-18
08/05/24	lab2	sala microscopia F2, C1	15-18
15/05/24	lab3	CIMA center, groupI	15-17
22/05/24	lab3	CIMA center, groupII	15-17

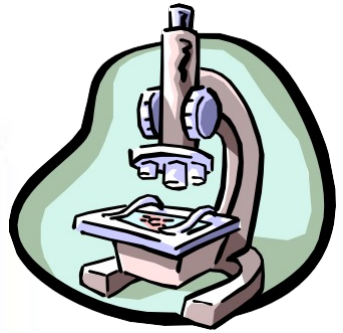
12 h lab + 16 h lessons

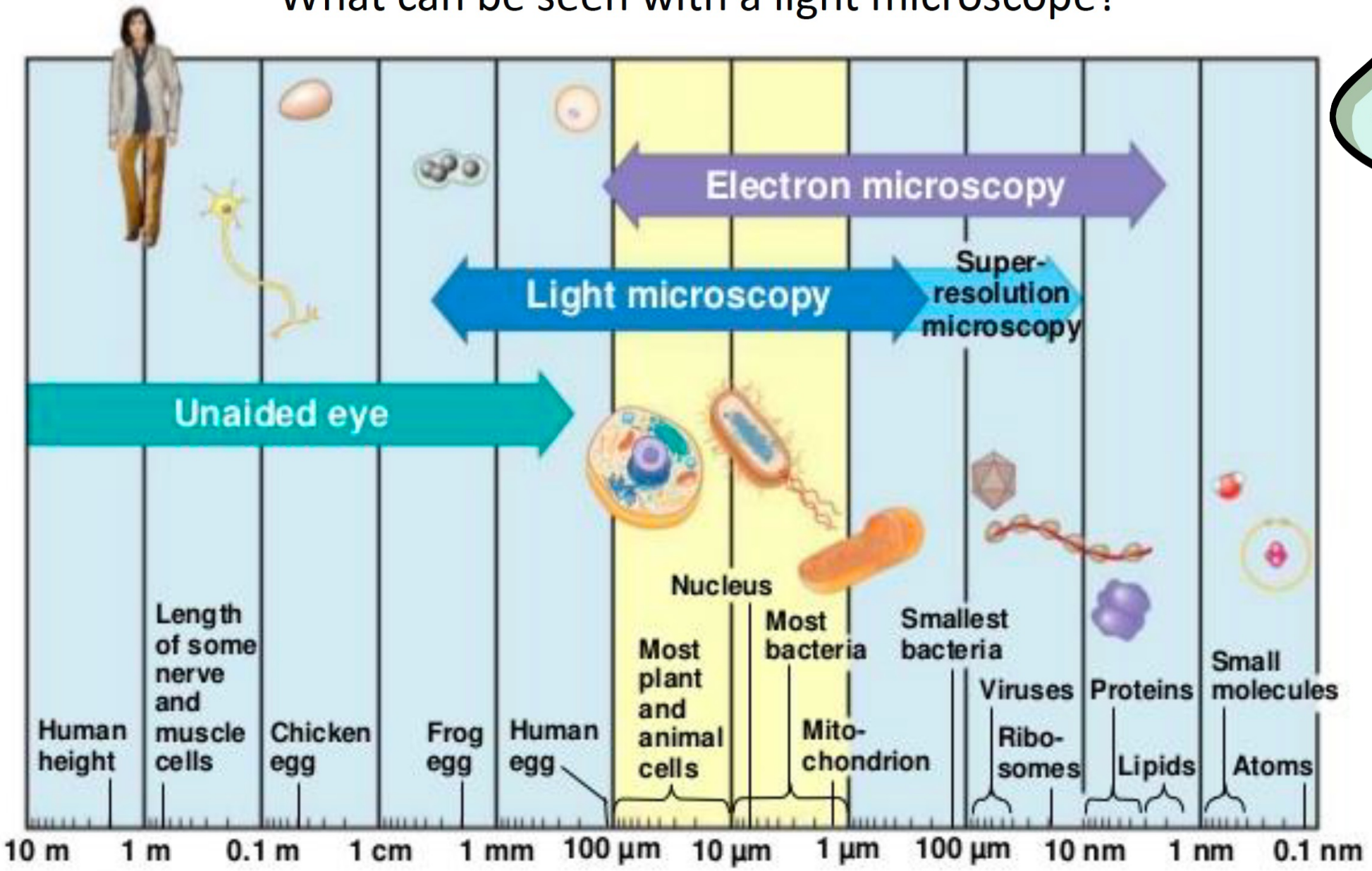
OPTICAL MICROSCOPY



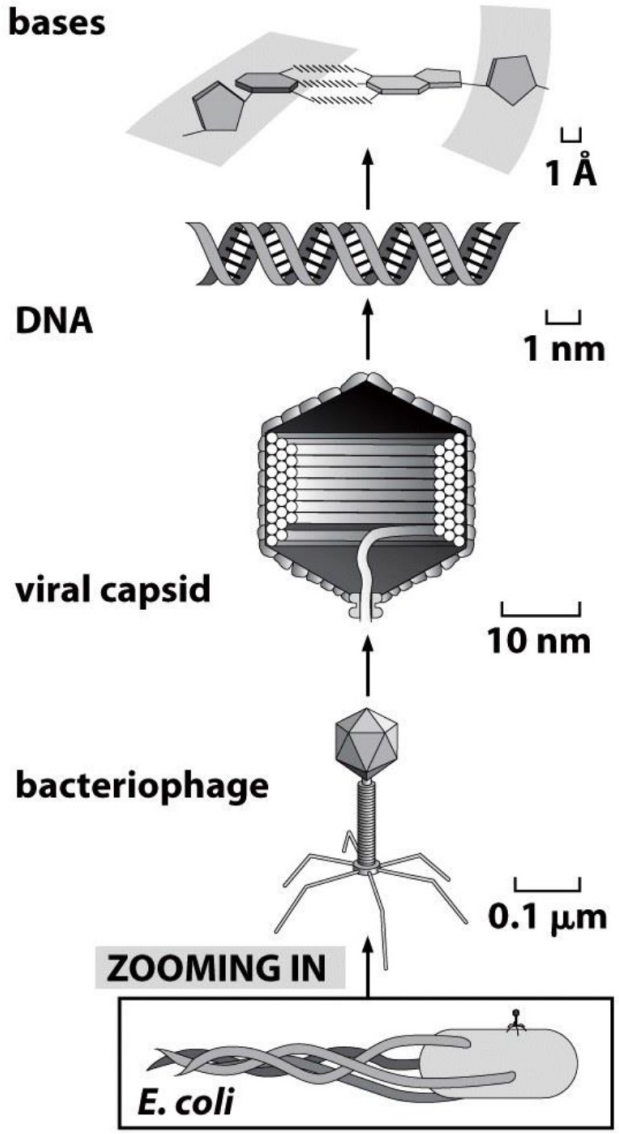
1. How a microscope works
2. Magnification vs resolution
3. Numerical aperture and working distance
4. Objectives
5. Point-spread function and Airy disk
6. Optical abberations

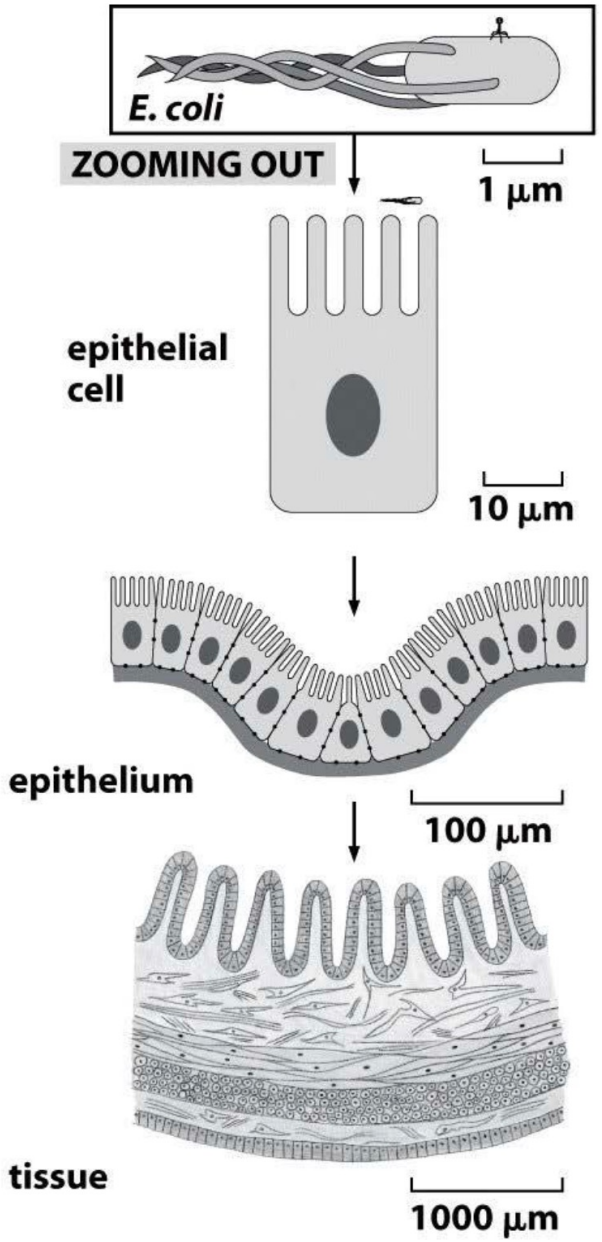
What can be seen with a light microscope?





Biological Scale and Size

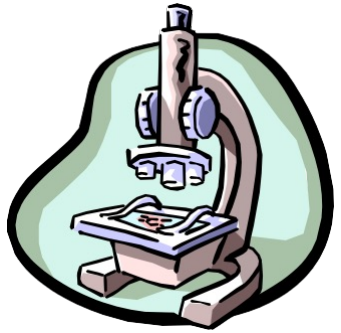




Optical Microscopy

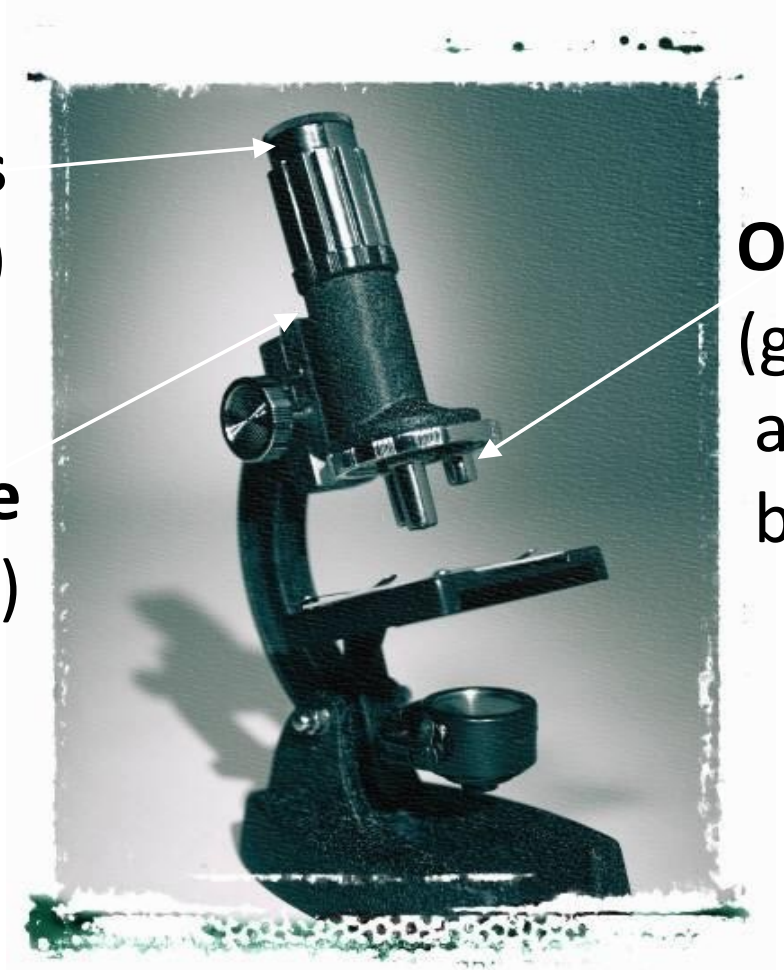
http://learn.genetics.utah.edu/content/begin/cells/scale/

HOW A MICROSCOPE WORKS



Ocular Lens
(magnifies image)

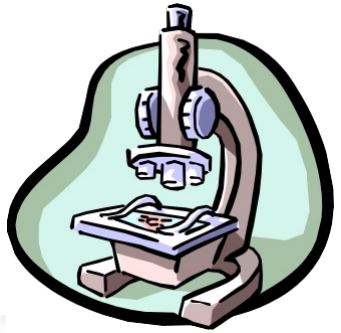
Body Tube
(focuses image)



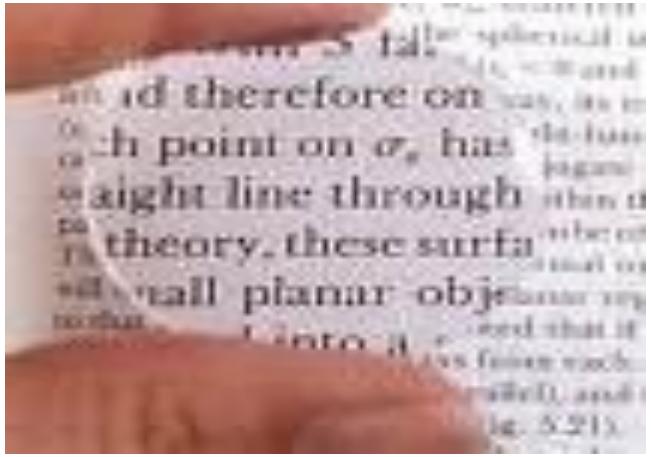
Objective Lens
(gathers light; magnifies and focuses image inside body tube)

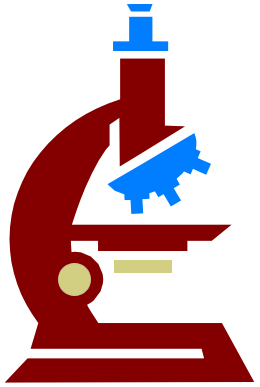
Bending Light: The convex lens of the objective magnifies and focuses (bends) the image inside the body tube and the convex lens of the ocular magnifies it (again).

HOW A MICROSCOPE WORKS

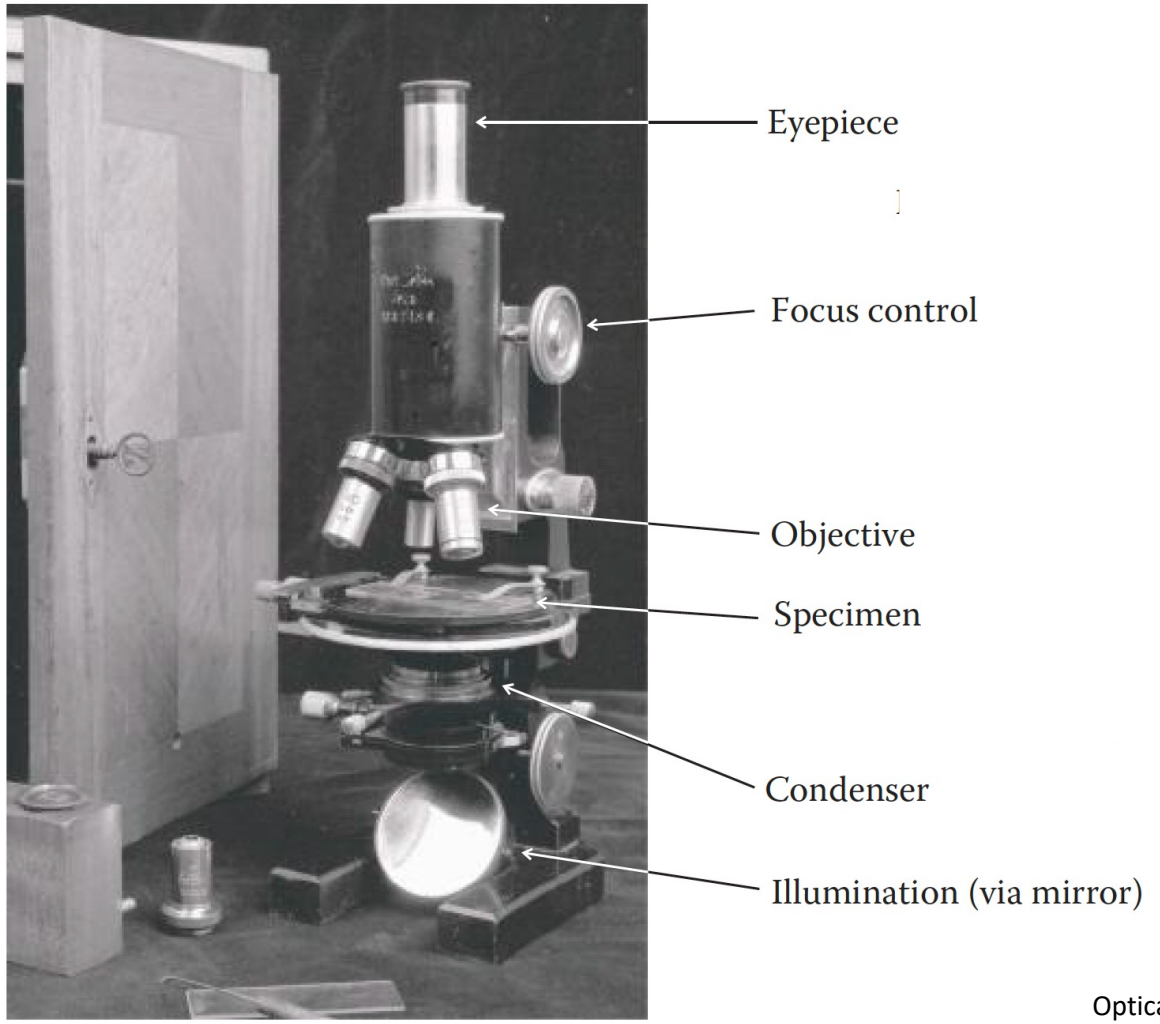


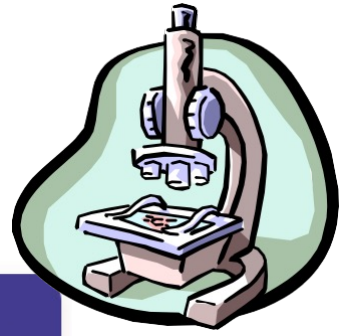
Convex Lenses are curved glass pieces used to make microscopes (and glasses etc.)

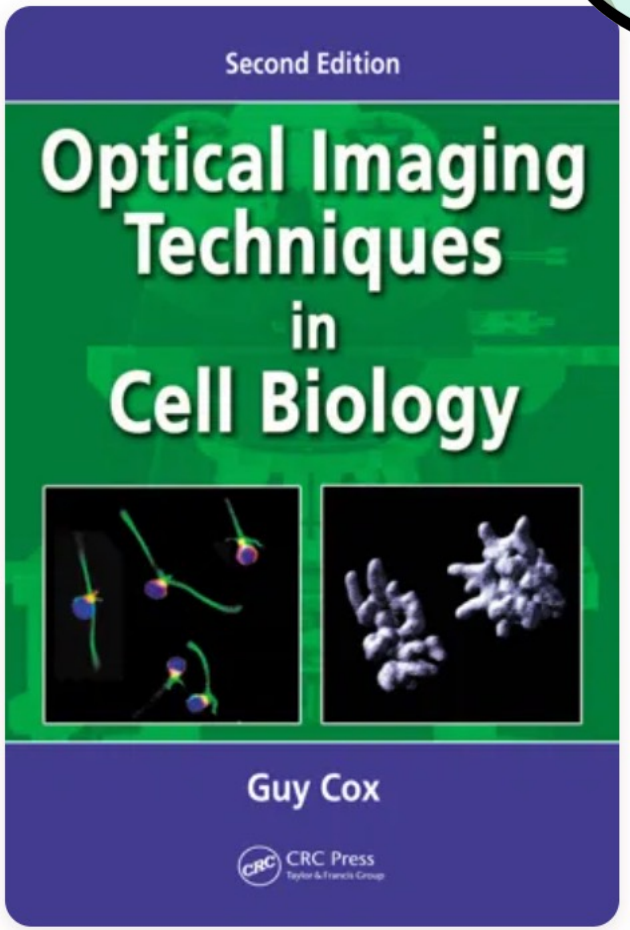




HOW A MICROSCOPE WORKS







Optical imaging tecniques in cell biology

HOW A MICROSCOPE WORKS

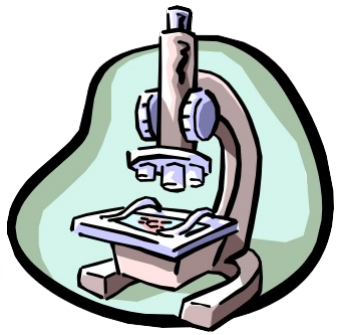
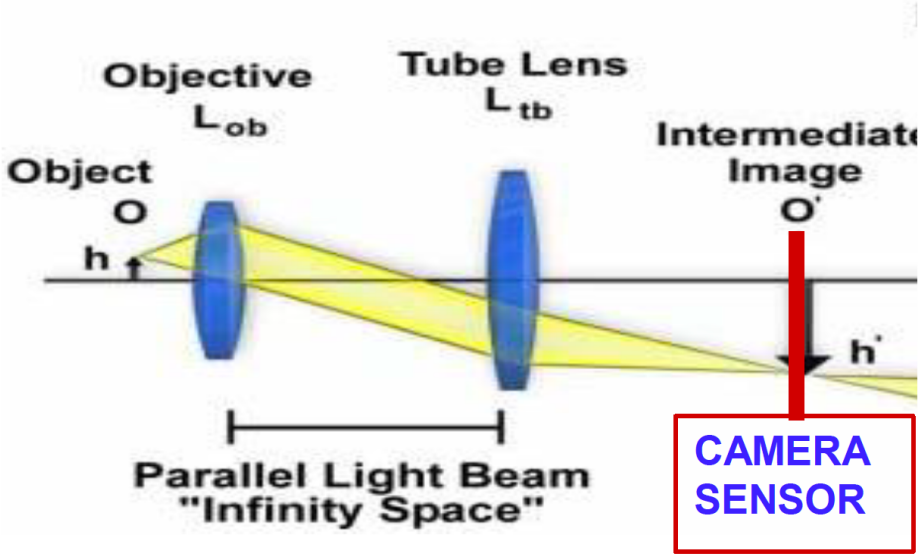


Image formation in the optical microscope



- The <u>object</u> is positioned in the focal plane of the **OBJECTIVE**, hence its image (through the objective) is projected to infinity.
- The **TUBE LENS** 'brings' this image from infinity to its focal plane, forming a magnified image, called <u>intermediate image</u>. in its focal plane.
- The intermediate image can be observed through the EYEPIECE or it is directly captured by a CAMERA SENSOR and displayed on a monitor.

Image Formation

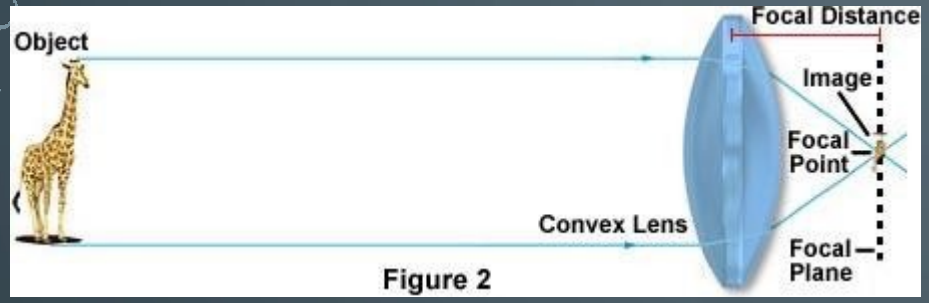


Figure 2

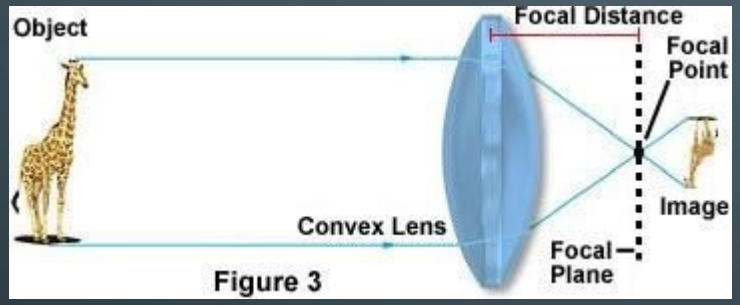


Figure 3

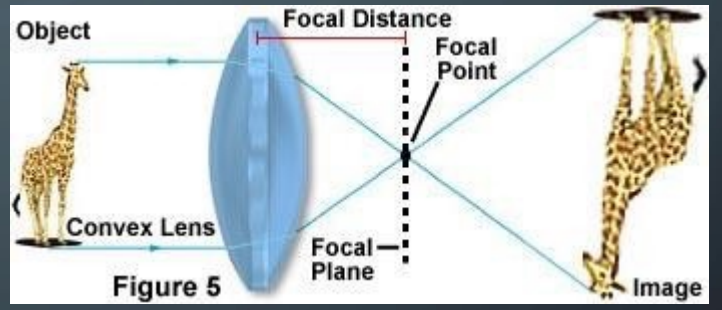


Figure 5

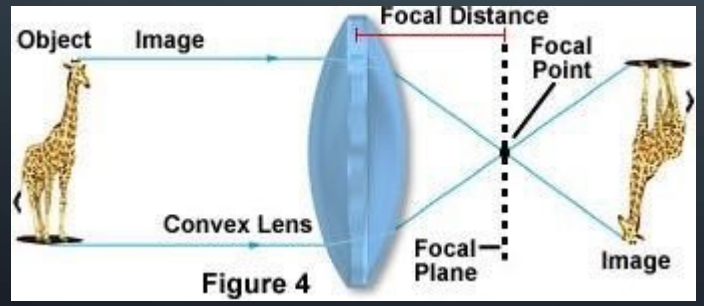
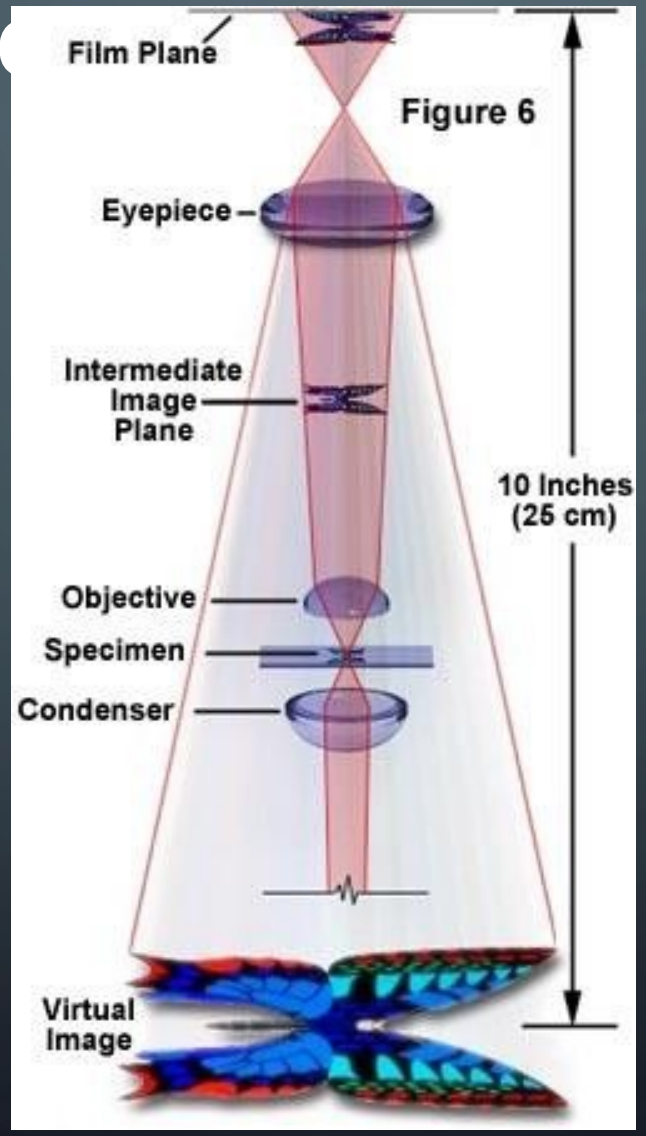


Figure 4

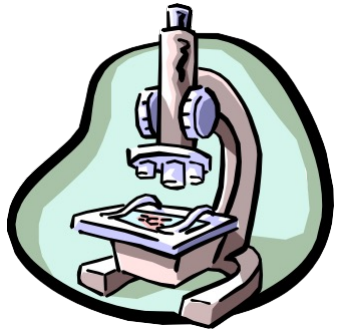
Lenses and Image Formati



Microscope Imaging

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

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



- **Absorption**

  When light passes through an object the intensity is reduced depending upon the color absorbed. Thus the selective absorption of white light produces colored light.

- **Refraction**

  Direction change of a ray of light passing from one transparent medium to another with different optical density. A ray from less to more dense medium is bent perpendicular to the surface, with greater deviation for shorter wavelengths

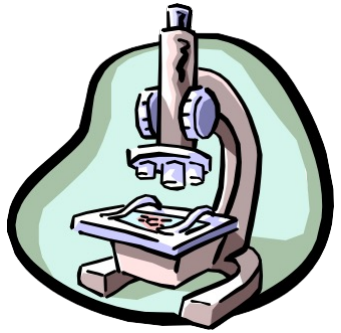
- **Diffraction**

  Light rays bend around edges - new wavefronts are generated at sharp edges - the smaller the aperture the lower the definition

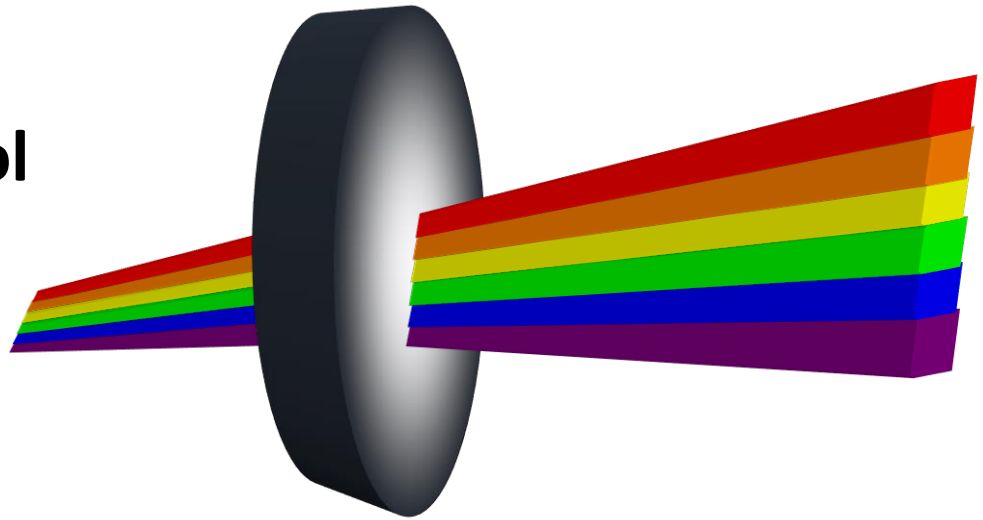
- **Dispersion**

  Separation of light into its constituent wavelengths when entering a transparent medium - the change of refractive index with wavelength, such as the spectrum produced by a prism or a rainbow

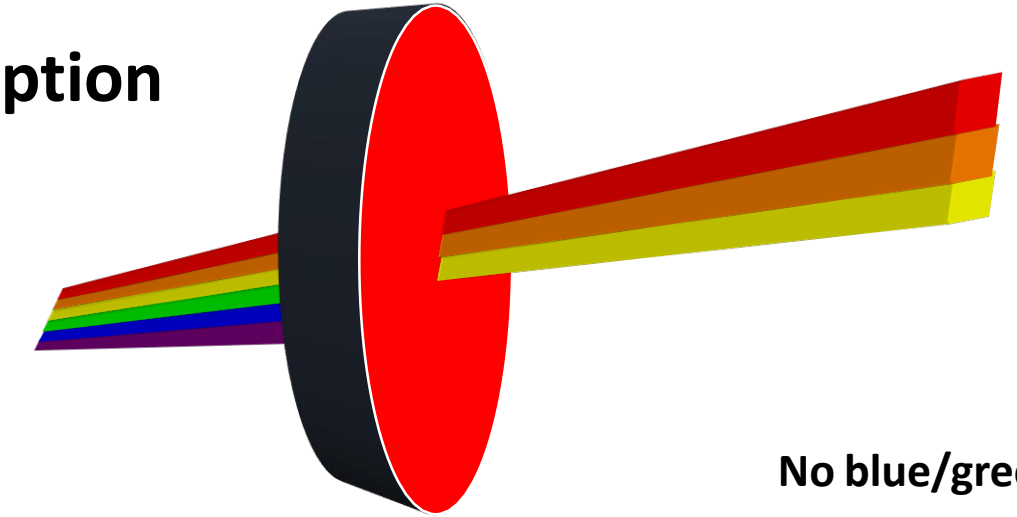
ABSORPTION



Control

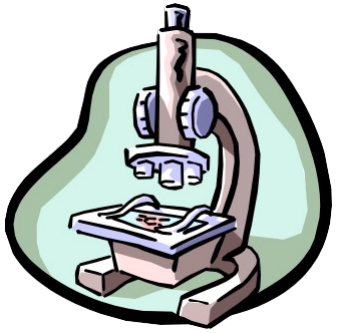


Absorption



red filter

No blue/green light



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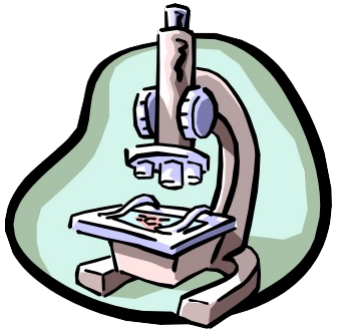
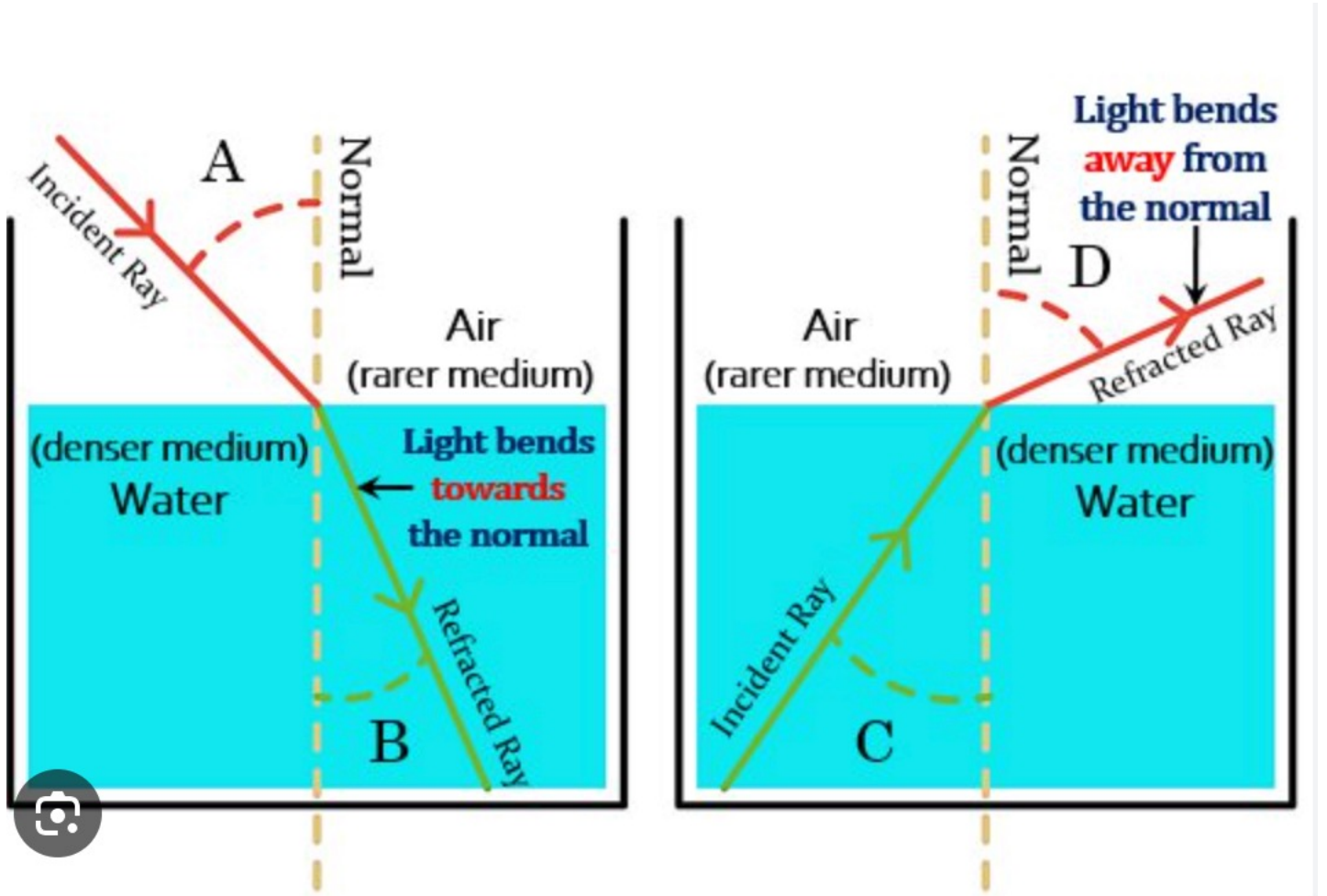
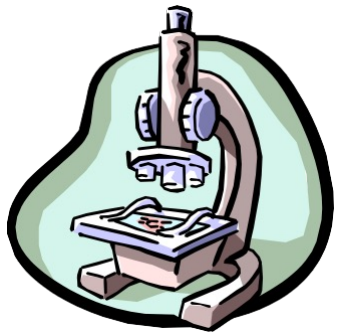
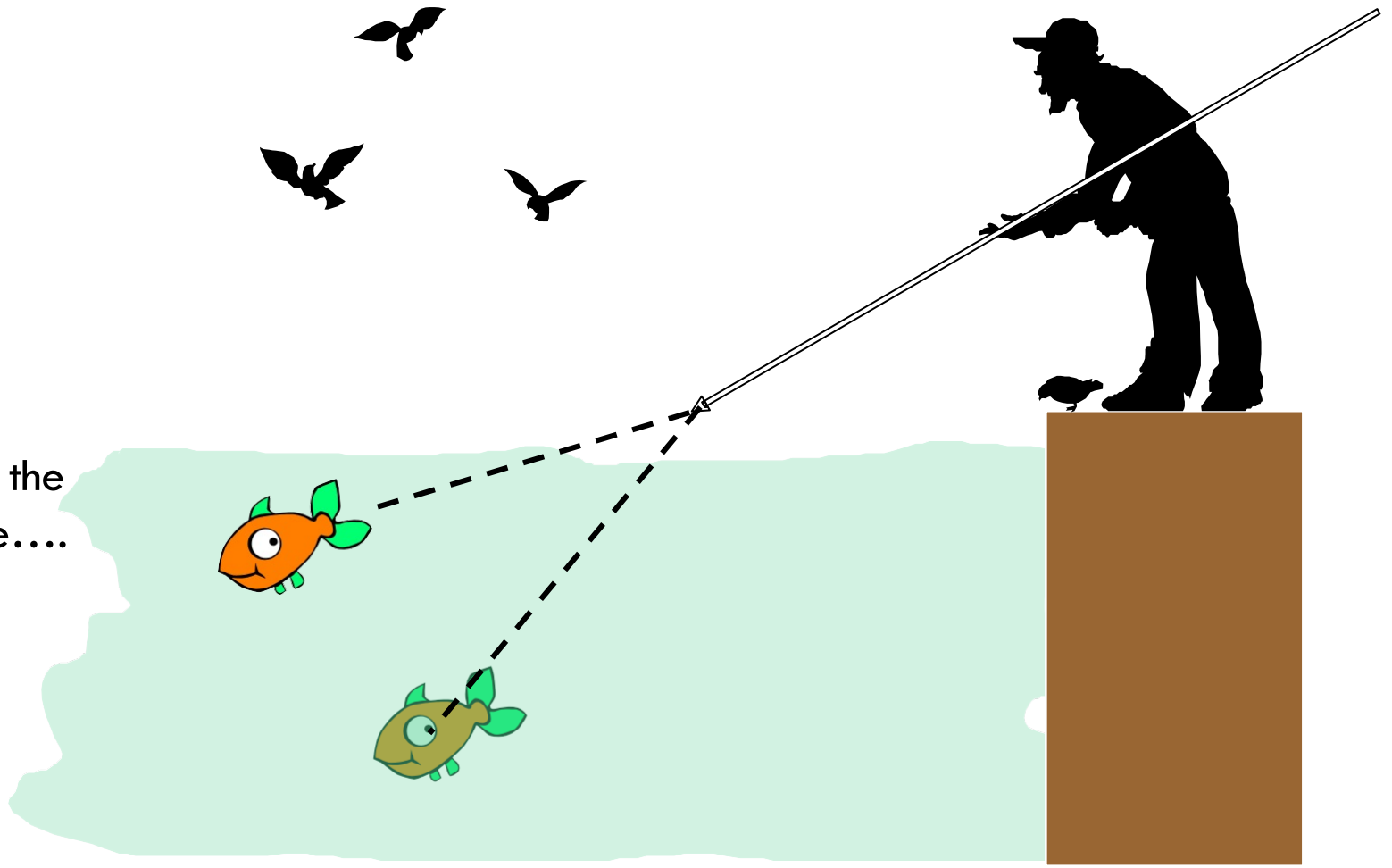
REFRACTION



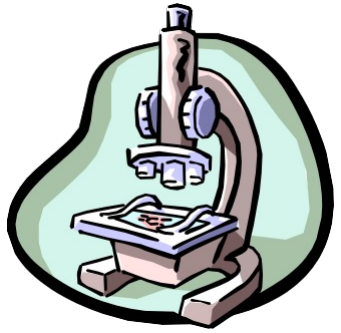

A
Normal
Incident Ray
Air
(rarer medium)
(denser medium)
Water
Light bends
towards
the normal
Refracted Ray
B
Light bends
away from
the normal
Normal
D
Air
(rarer medium)
Refracted Ray
(denser medium)
Water
Incident Ray
C





He sees the fish here….

But it is really here!!



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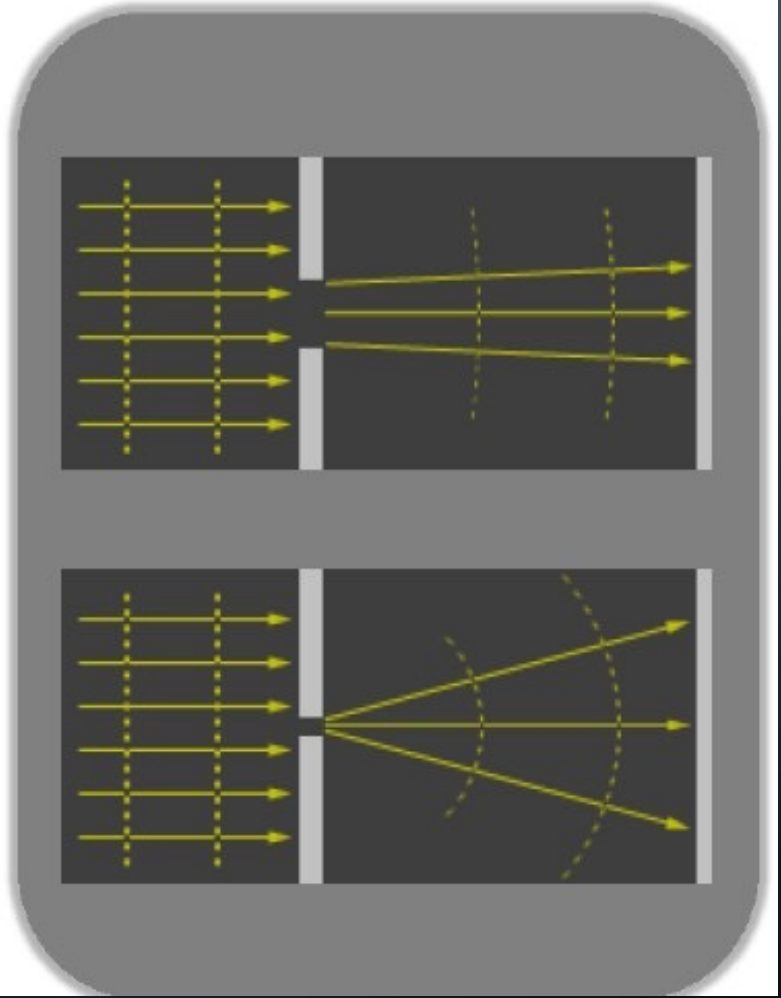
- **Dispersion**

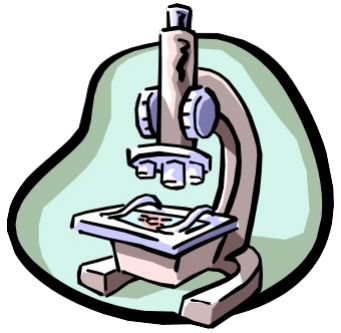
  Separation of light into its constituent wavelengths when entering a transparent medium - the change of refractive index with wavelength, such as the spectrum produced by a prism or a rainbow

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





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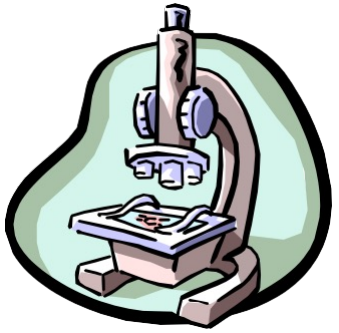
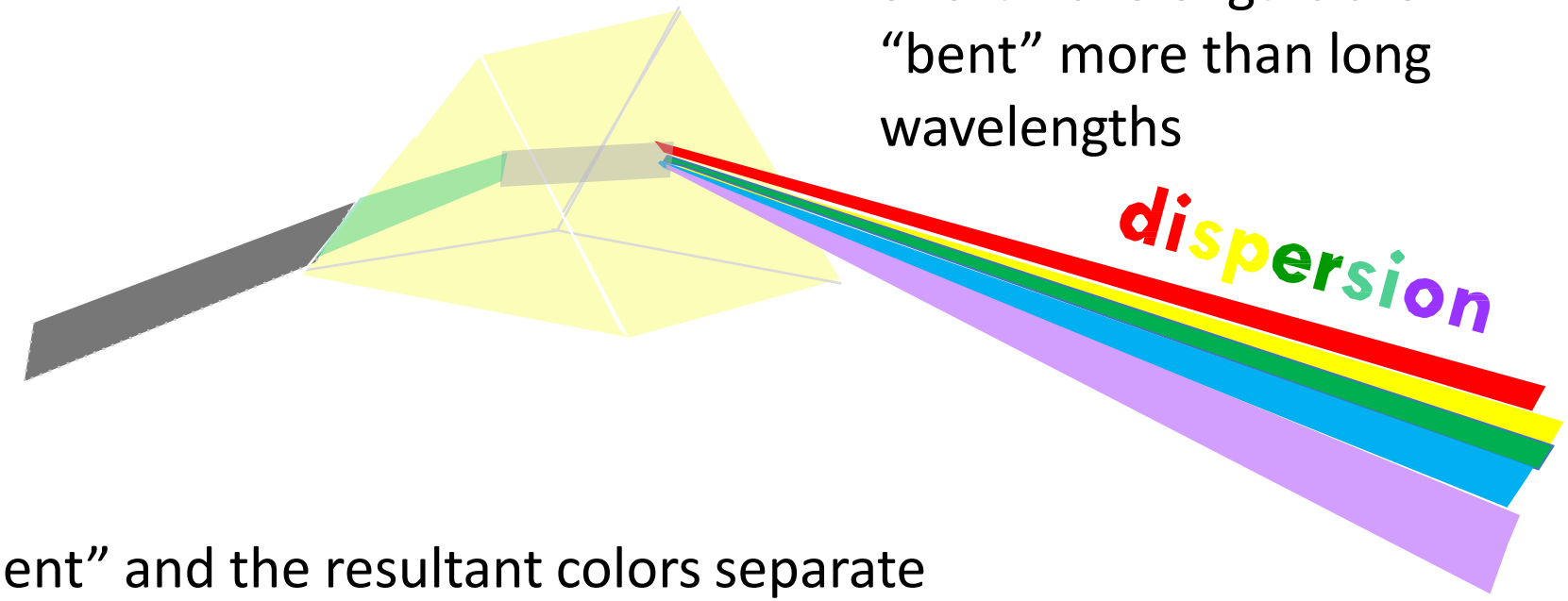
REFRACTION & DISPERSION

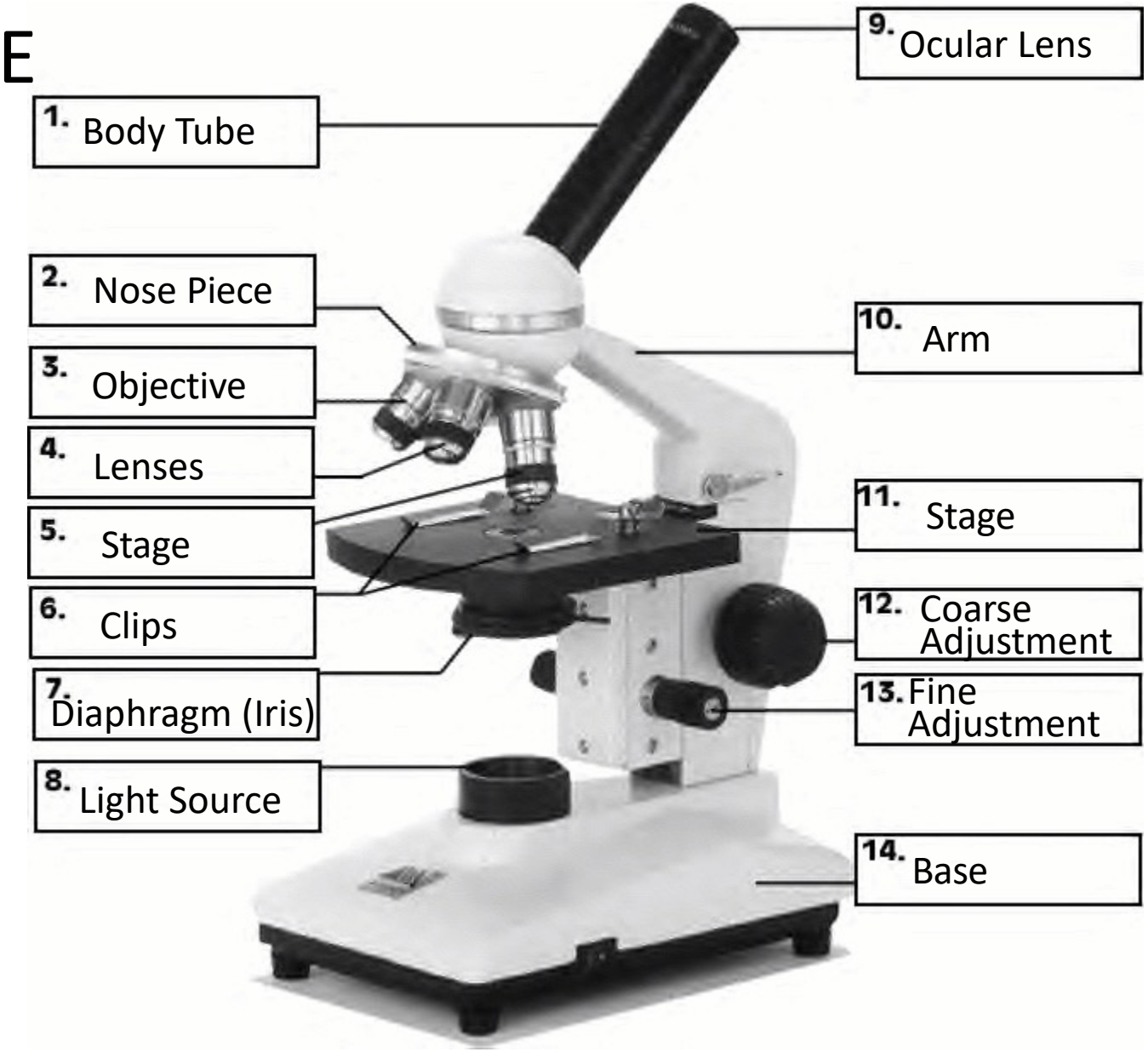


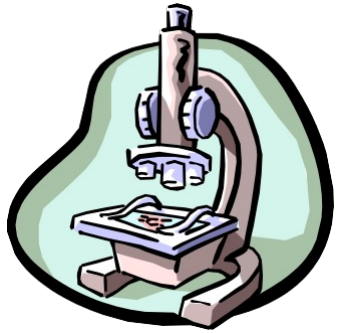


Short wavelengths are "bent" more than long wavelengths

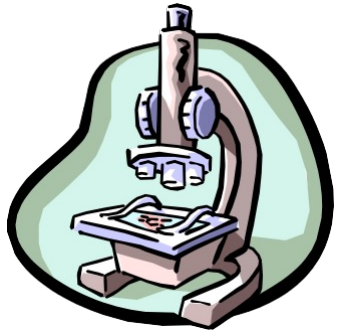
Light is "bent" and the resultant colors separate (dispersion). Red is least refracted, violet most refracted.

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



MICROSCOPE – upright, transmitted light



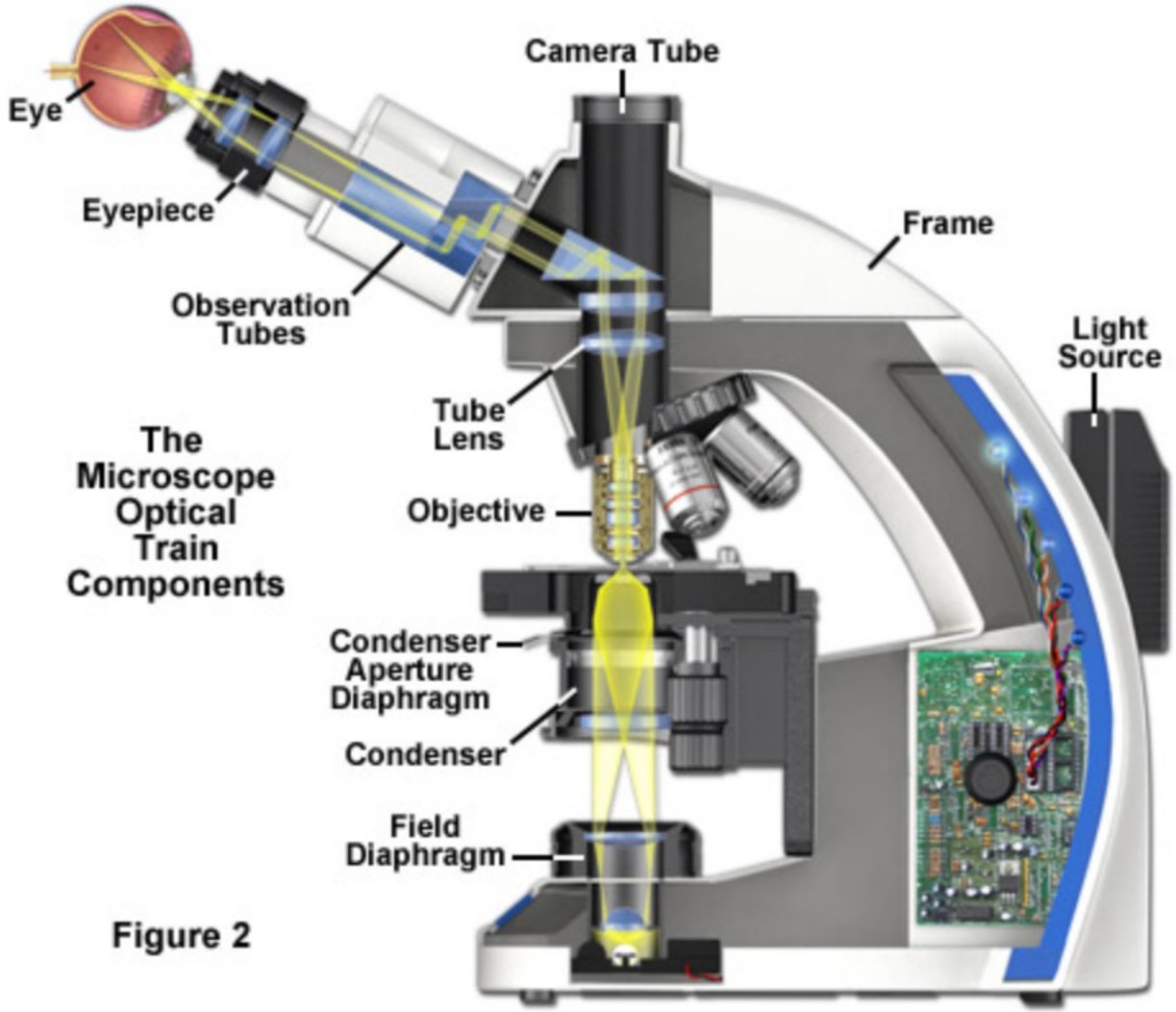
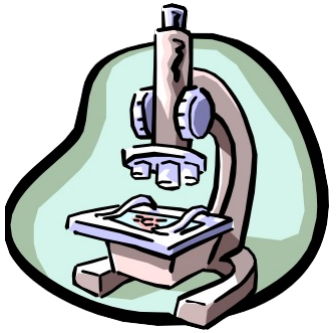


Figure 2

MICROSCOPE – upright, reflected light



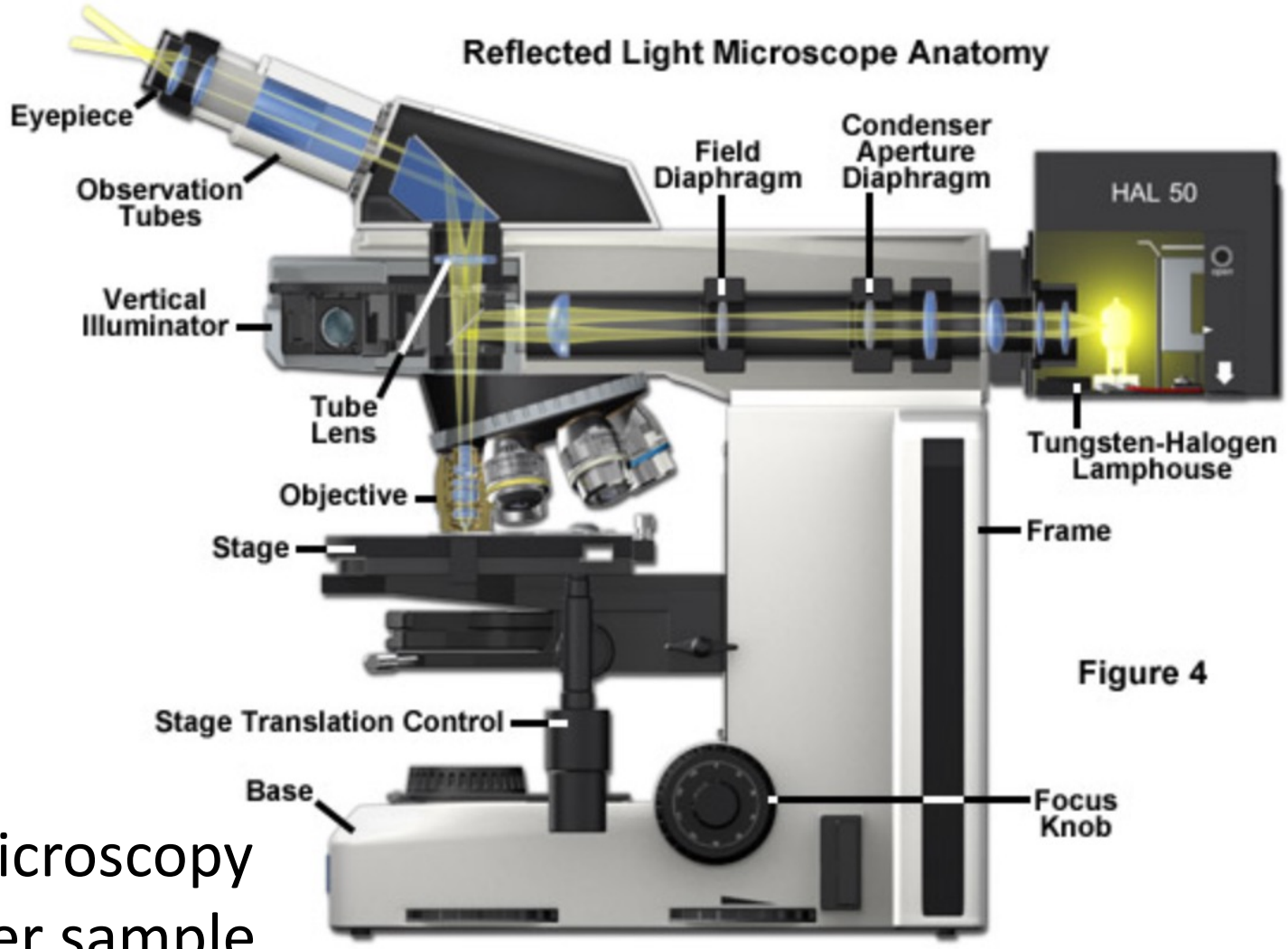


Figure 4

- Fluorescent microscopy
- Opaque, thicker sample

MICROSCOPE

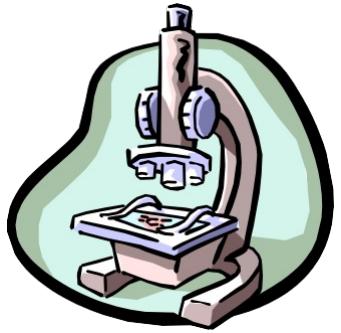
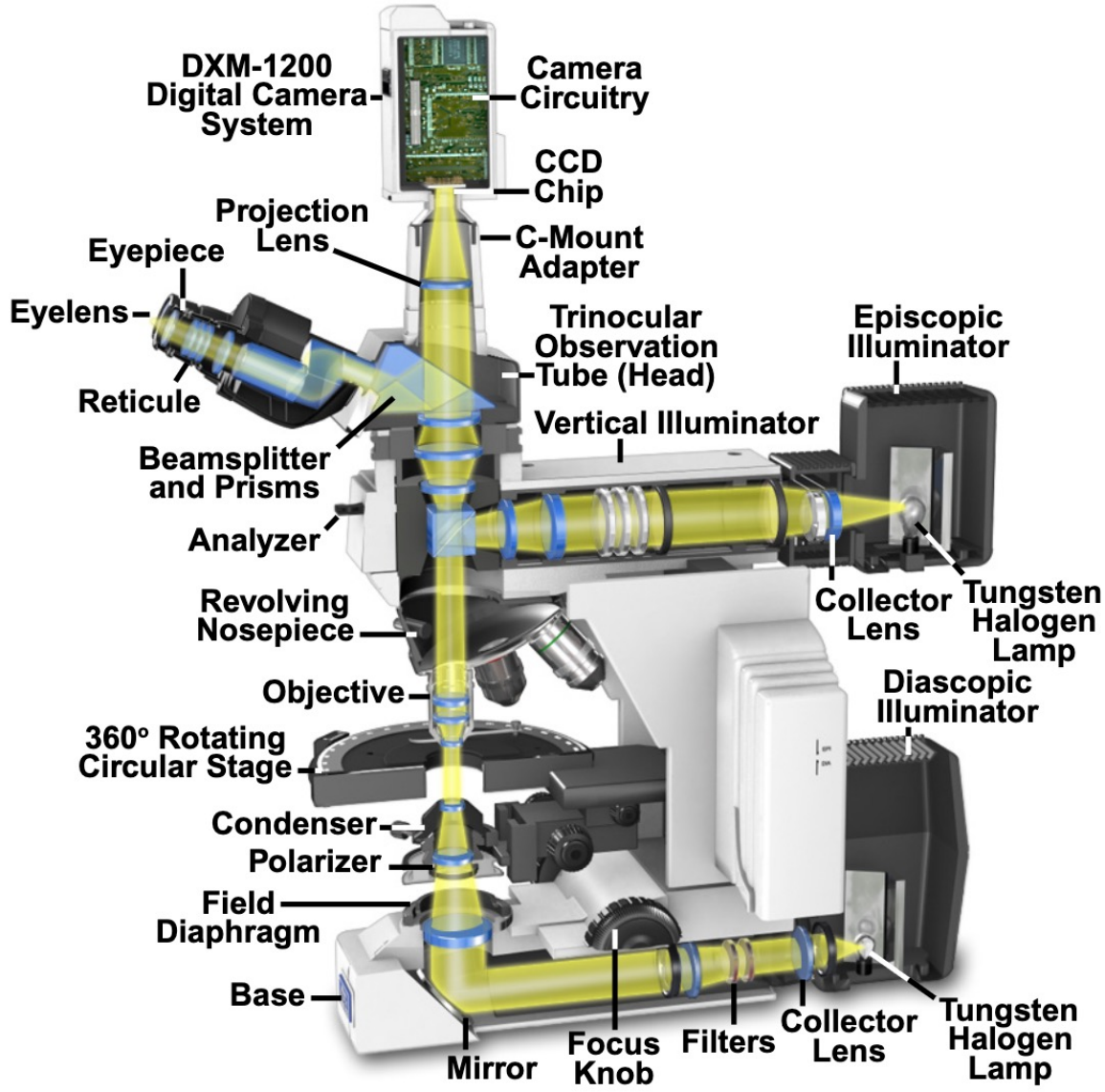
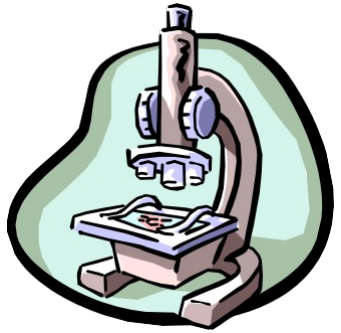


Figure 1 - The Microscope Optical Train

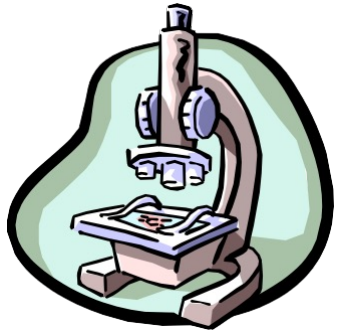


MAGNIFICATION vs. RESOLUTION



- Magnification: increase of an object's apparent size
- Resolution: power to show details clearly
- Both are needed

MAGNIFICATION

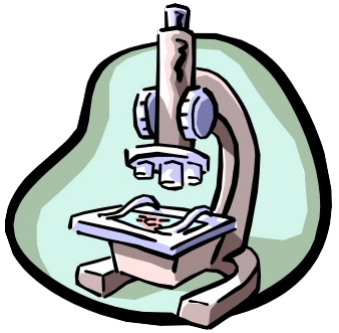


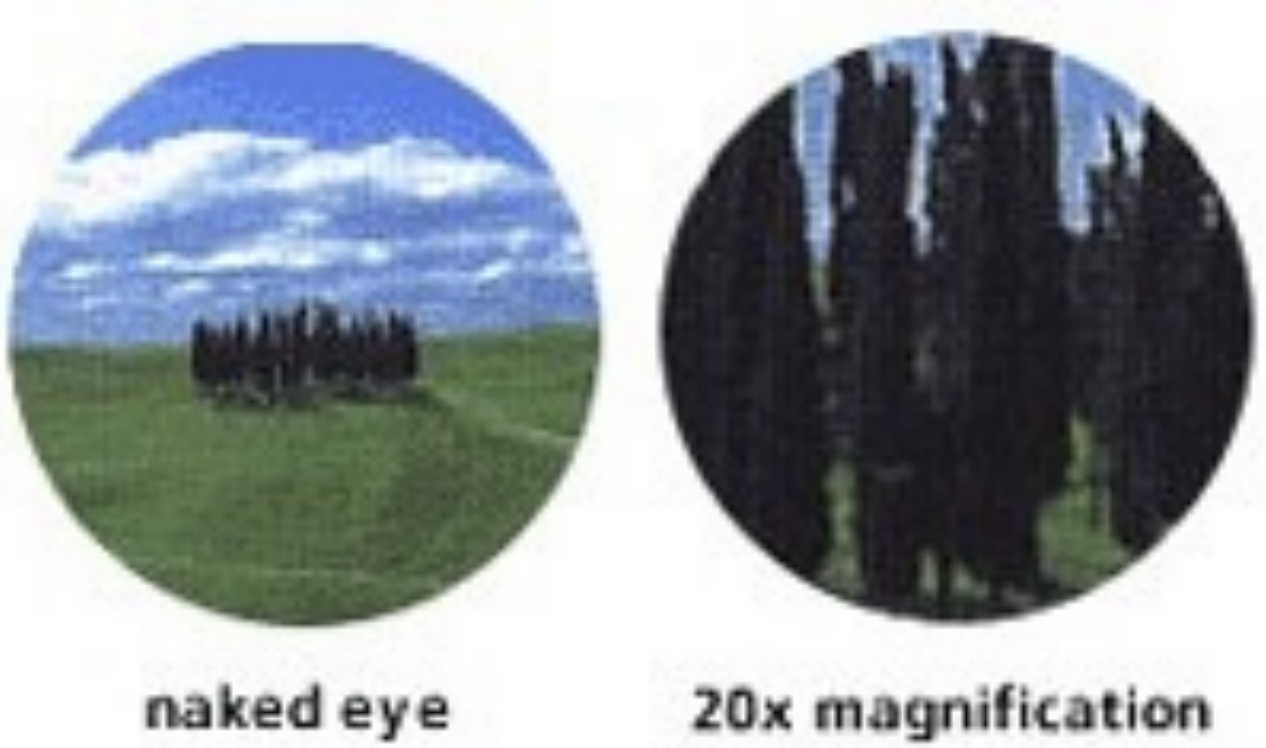
Objective magnification 4X – 120 X

Microscope magnification $M_M = M_{OB} M_{EY}$ 50 X – 2000 X

EY – Eyepiece; DC – Digital Camera $M_M = M_{OB} M_{DC}$

MAGNIFICATION

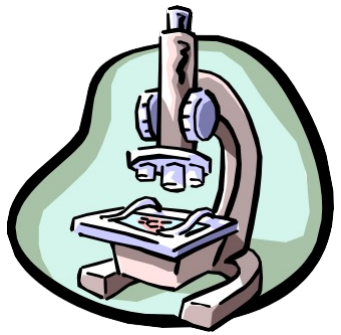




Magnification is NOT ALWAYS

related with resolution

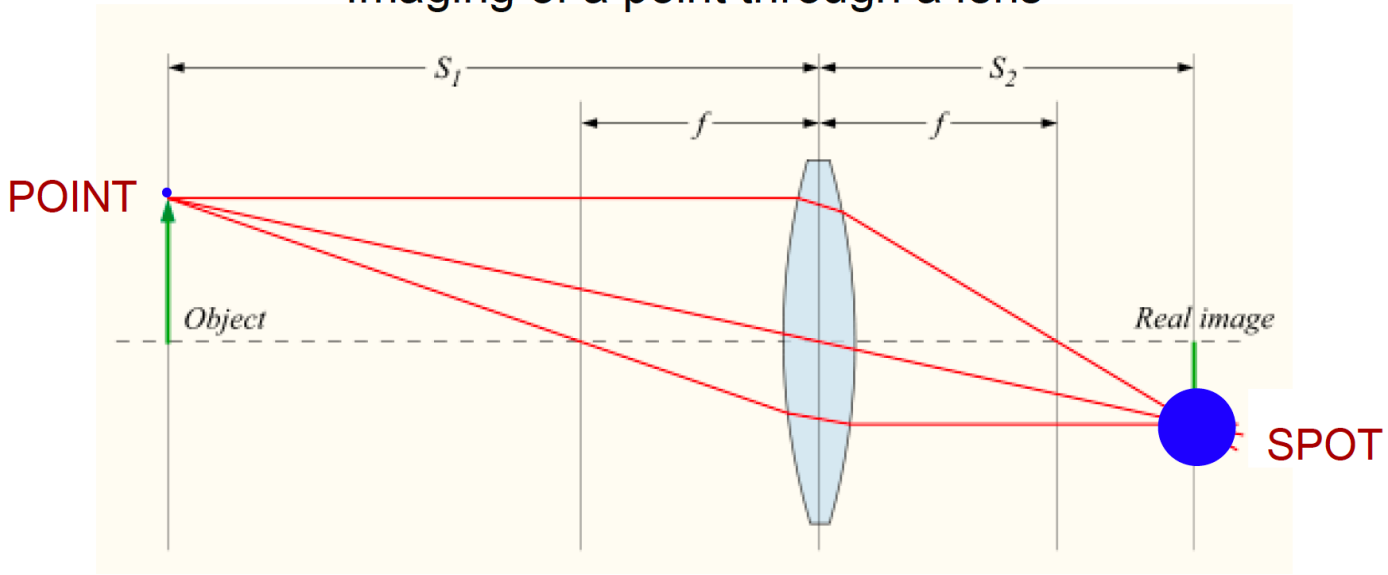
RESOLUTION



Resolution describes the ability of an optical system to **resolve details** of the object that is being imaged.

Due to the diffraction of light through an optical system with finite size, a **POINT** object is imaged into a **SPOT** rather than a point.

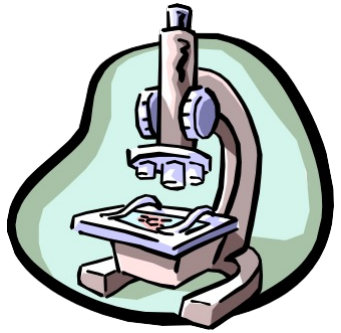
Imaging of a point through a lens

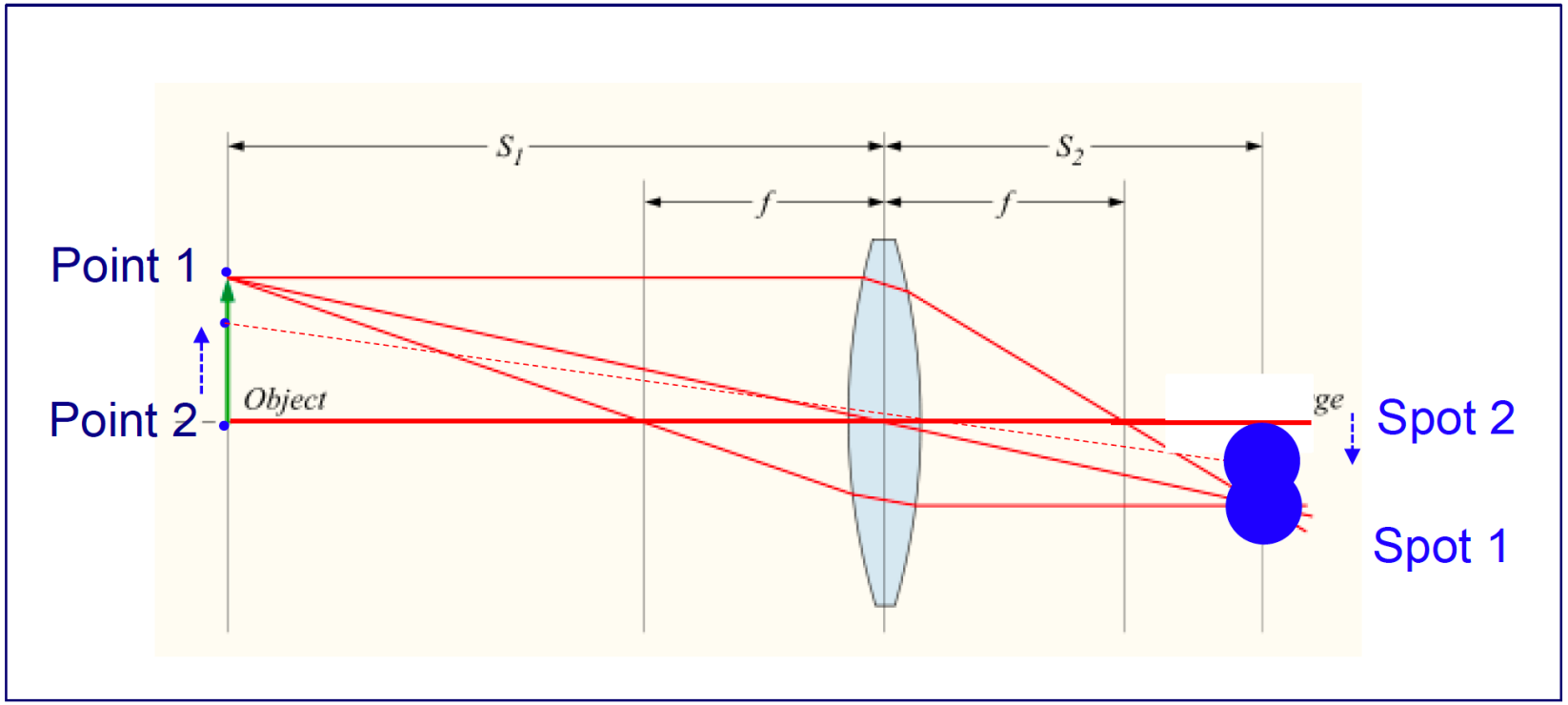


LARGER the LENS, SMALLER the SPOT !

SMALLER the SPOT, BETTER the RESOLUTION !

RESOLUTION of two points





When the two points are close each other, their images (two spots) overlap and hence they can not be separated (resolved)!

RESOLVING POWER

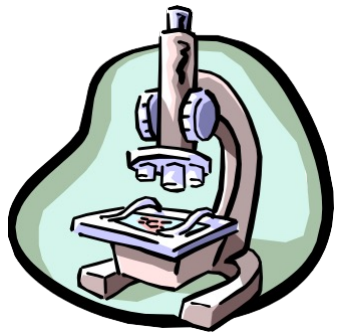
- Is the ability to distinguish two adjacent points as distinct and separate

- Mere increase in size without the ability to distinguish structural details is not beneficial. The largest magnification produced by a microscope may not be the most useful because the image obtained may be unclear or fuzzy.

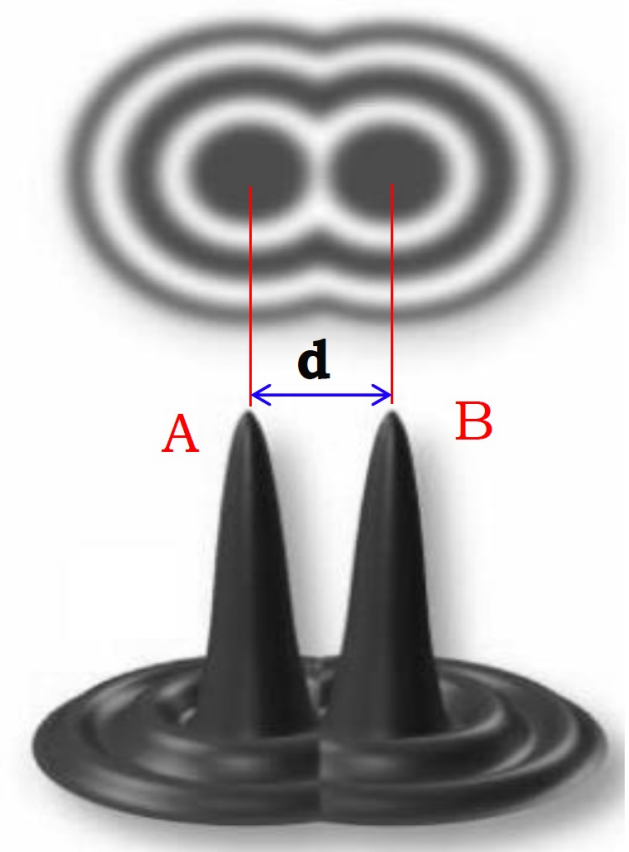
- The more lines or dots per unit area that can be seen separately, the greater is the resolving power.

- It is a function of the wavelengths of lights used & the numerical aperture of the lens system.

RESOLUTION CRITERION



The resolution, r, is defined as the shortest distance between two points on a specimen that can still be distinguished by the observer or camera sensor as separate entities.



A and B are separated if: **d > r**

Rayleigh criterion

$$r = 0.61 \frac{\lambda}{NA}$$

NA- Numerical Aperture

Estimating the lateral resolution of a microscope objective (lens):

NA = 1.5, λ = 400 nm

→ **r ~ 200 nm**

NUMERICAL APERTURE

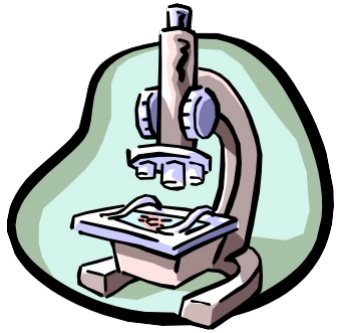
- In optics, the numerical aperture (NA) of an optical system is a dimensionless number that characterizes the range of angles over which the system can accept or emit light.

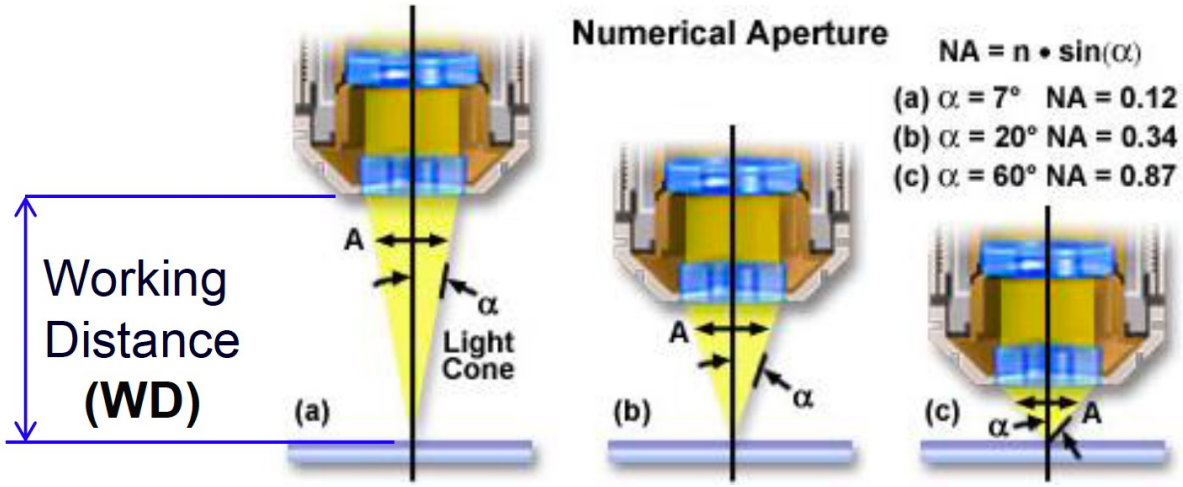
- The sine value of half-aperture angle multiplied by the refractive index *n* of the medium gives the numerical aperture (NA)

- Thus,

$$NA = n \sin \theta$$

NUMERICAL APERTURE & WORKING DISTANCE





$$NA = n \cdot \sin \alpha$$

n = refractive index

α = angle of incident illumination

Immersed objectives NA > 1

Oil (n=1.515), Glycerin (n=1.47) or Water (n=1.33)

Higher NA → better lateral Resolution

Note: WD decreases when NA increases !!!

OBJECTIVES

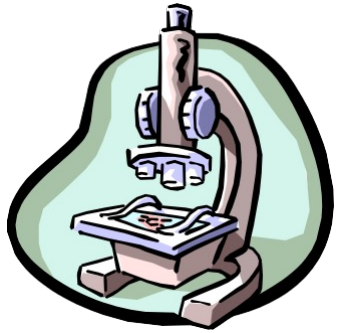
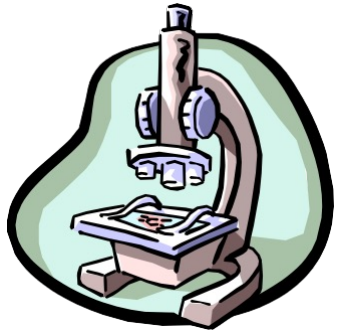
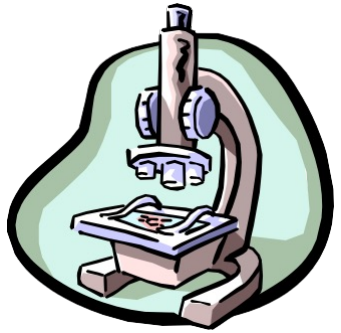


Figure 1

OBJECTIVE SPECIFICATIONS



OBJECTIVE SPECIFICATIONS



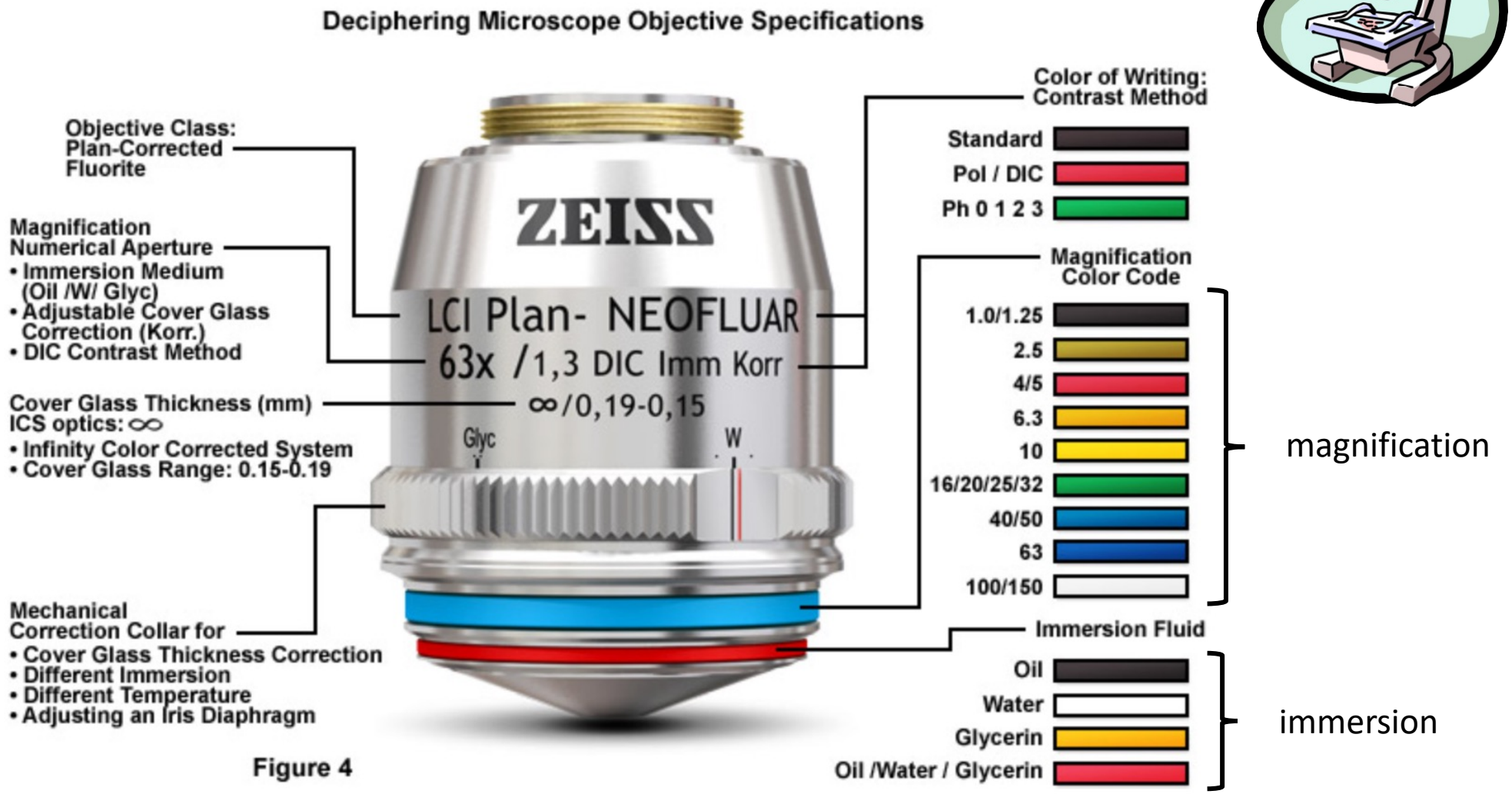
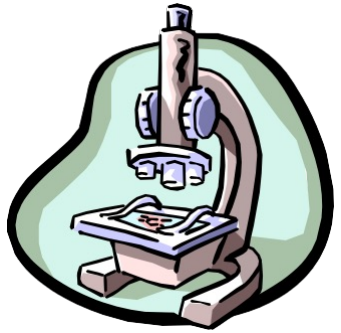


Figure 4

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 ⌀ 18 mm. Versions for phase contrast.

- Achroplan

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

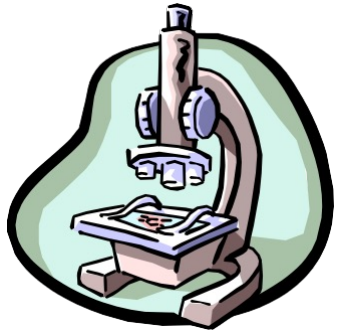
- Plan-Neofluar

  Excellent colour correction for at least three wavelengths. Field flattening for the field of view with ⌀ 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 ⌀ 25 mm. Highest NAs for a resolving power at the very limits of the physically possible.

OBJECTIVES



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

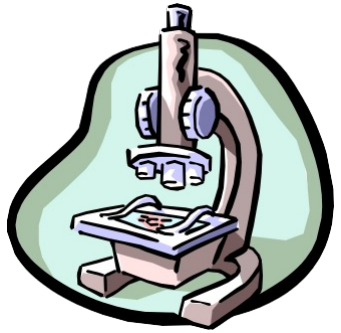
http://www.microscopyu.com/tutorials/java/objectives/nuaperture/index.html

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

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

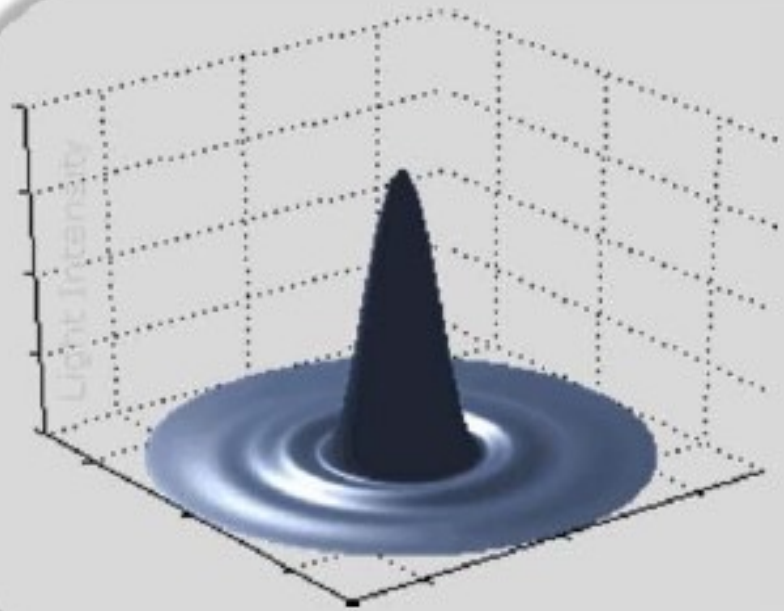
http://www.microscopyu.com/articles/optics/index.html

POINT SPREAD FUNCTION



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

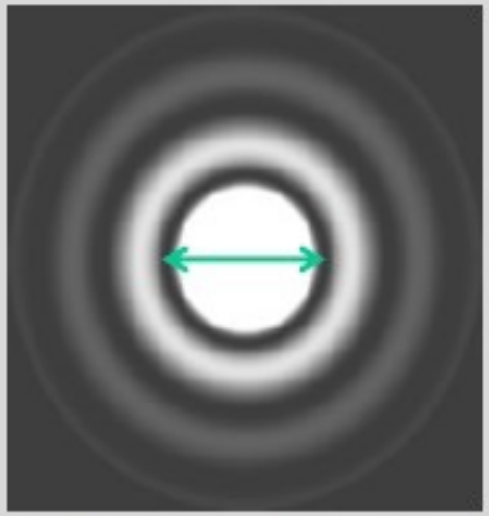
For an optical system with circular aperture
the finite image spot forms an Airy Disk (Point Spread Function)



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

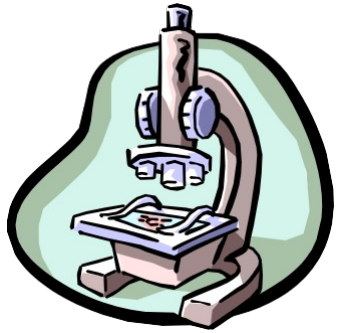
$$with \quad u = \frac{\pi \cdot x}{\lambda \cdot f/\#}$$

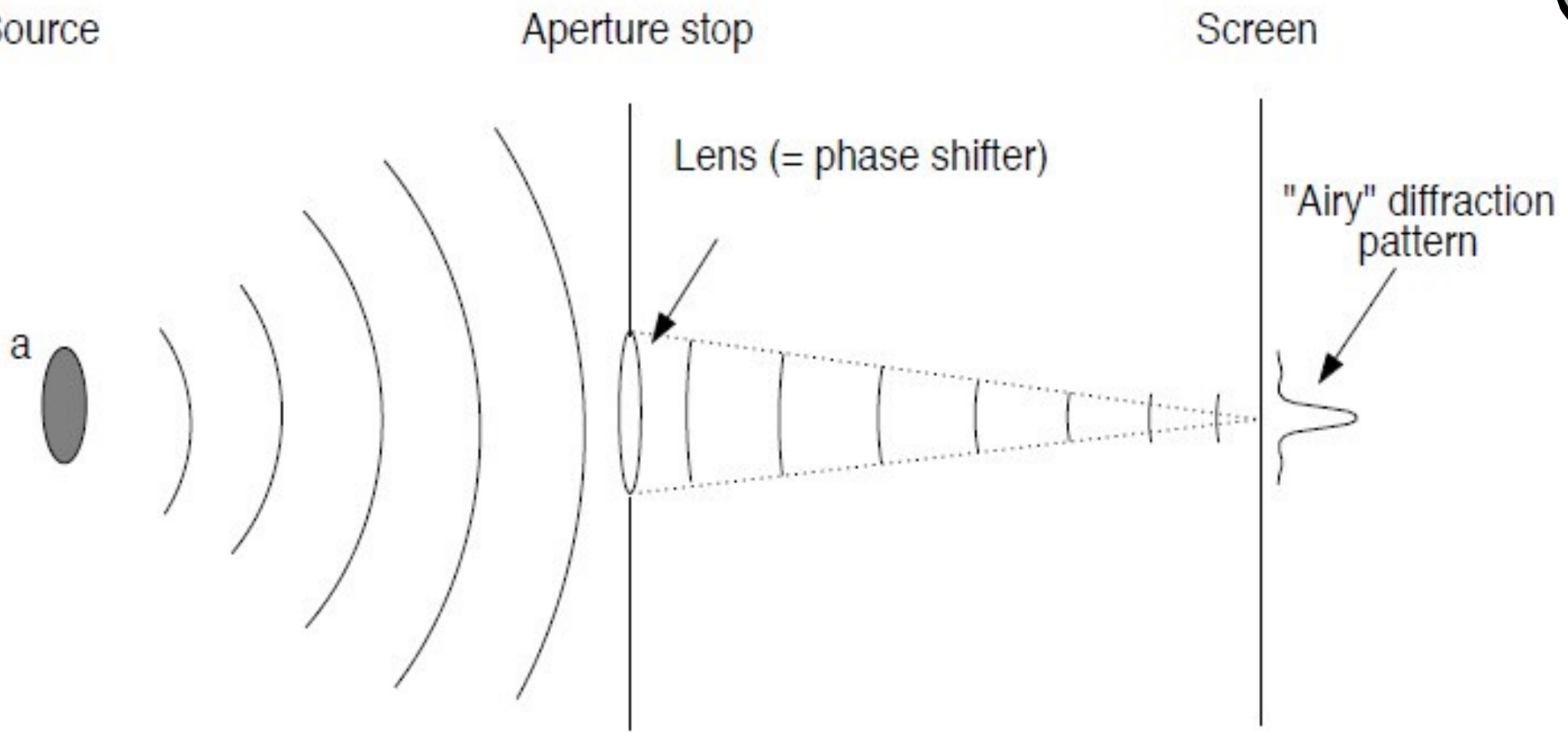
$$and \quad x_{cutoff} = 1.22 \cdot \lambda \cdot f/\#$$



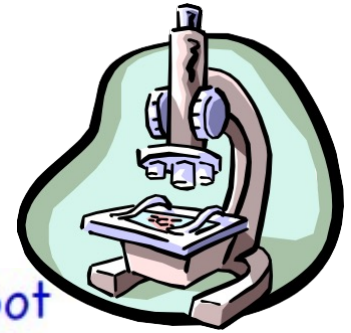
Airy disk diameter: 2.44 λ f/#

POINT SPREAD FUNCTION

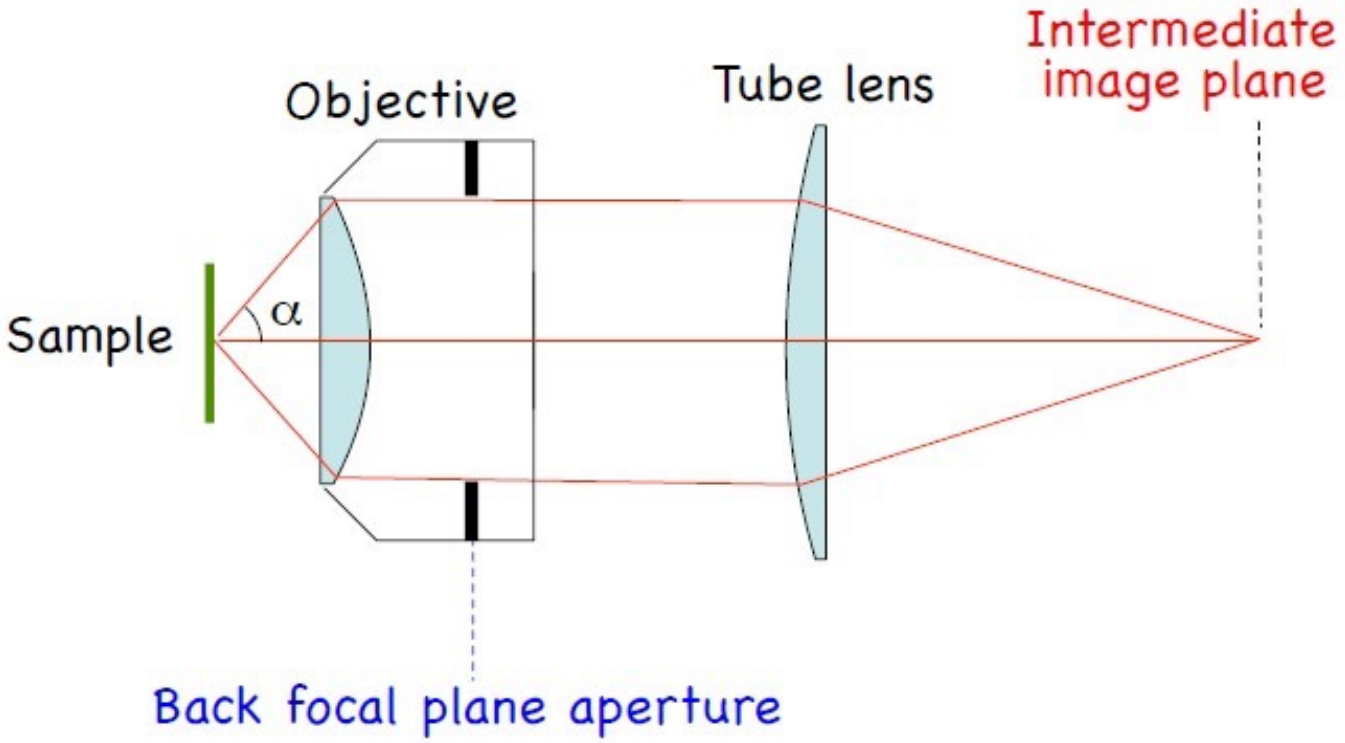


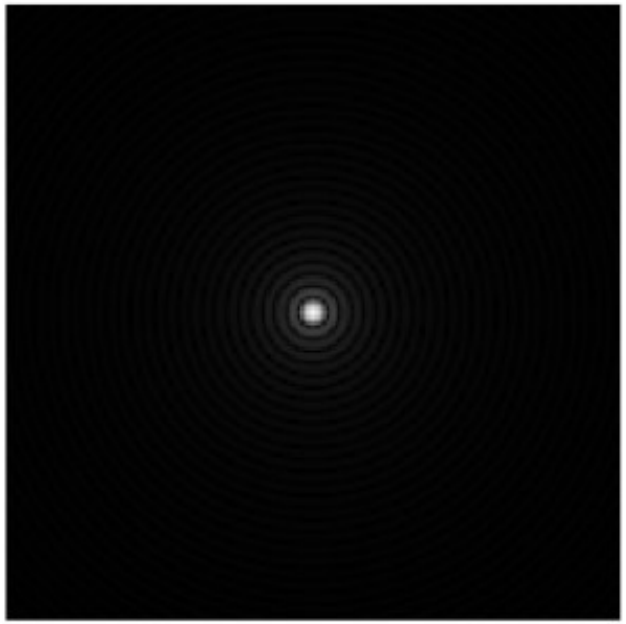


POINT SPREAD FUNCTION



Diffraction spot
on image plane
= *Point Spread Function*





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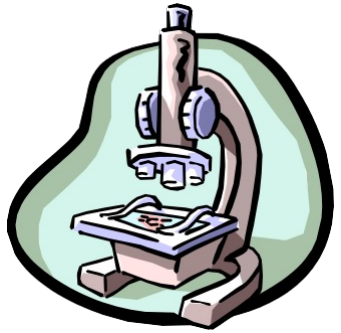
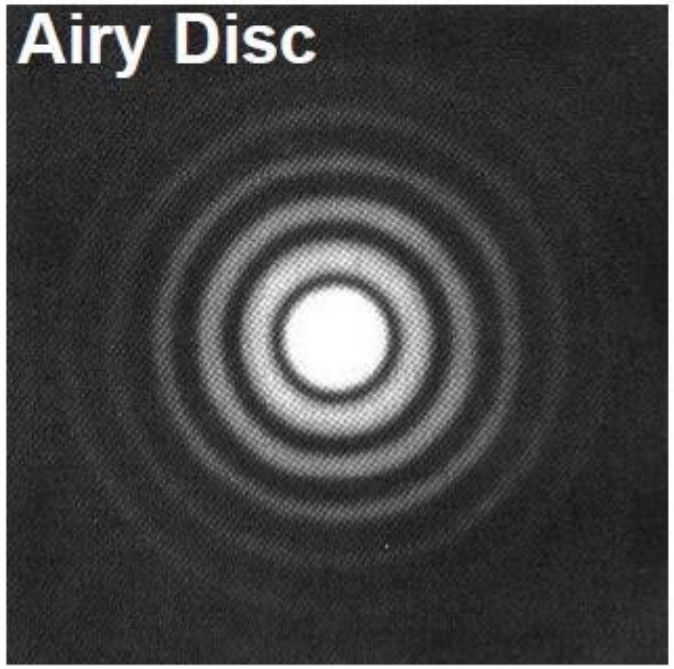


It is called the Airy disc.

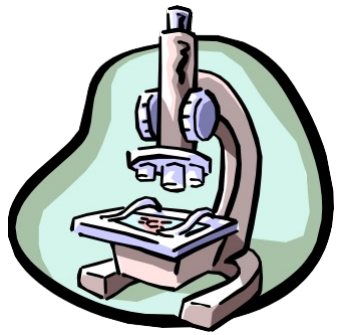
The Point Spread Function

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.



RESOLUTION CRITERION



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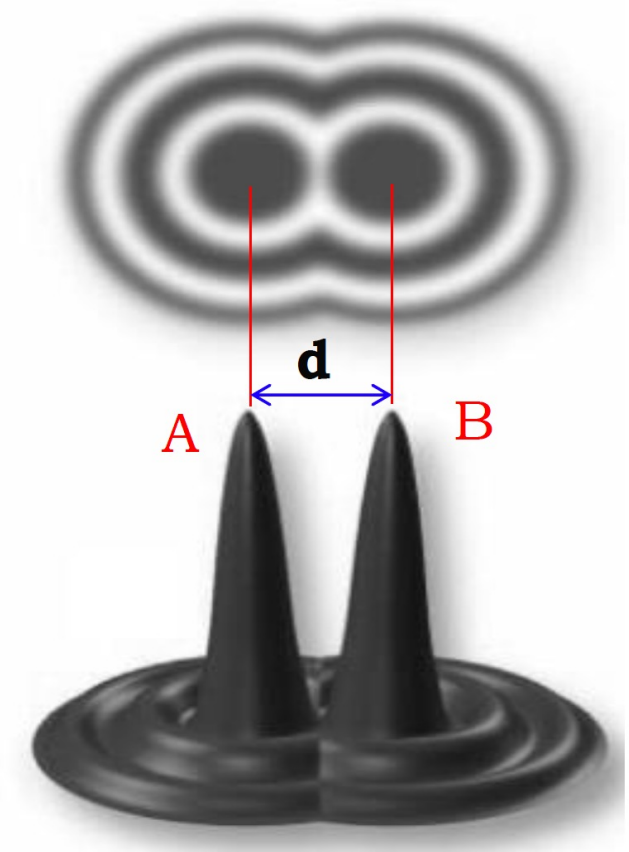

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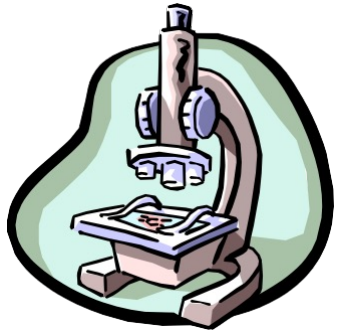
→ **r ~ 200 nm**

POINT SPREAD FUNCTION (PSF)



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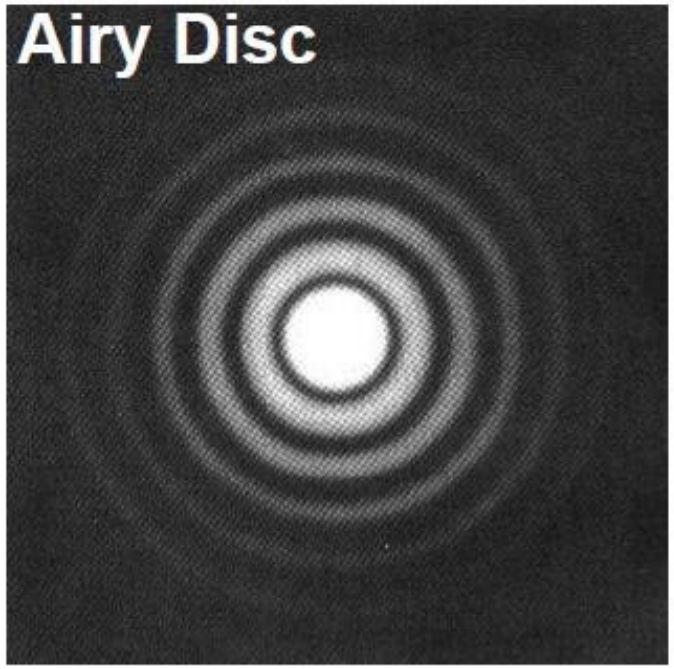
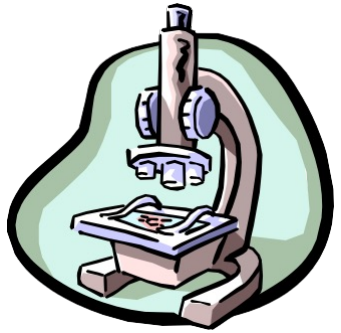
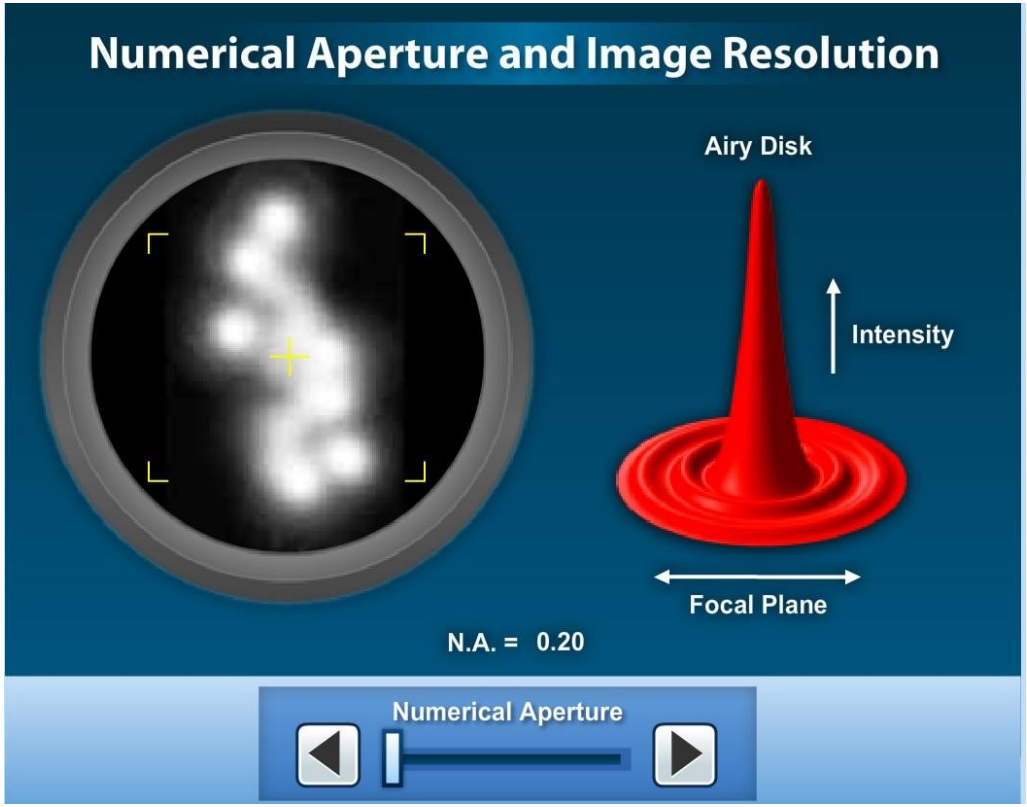
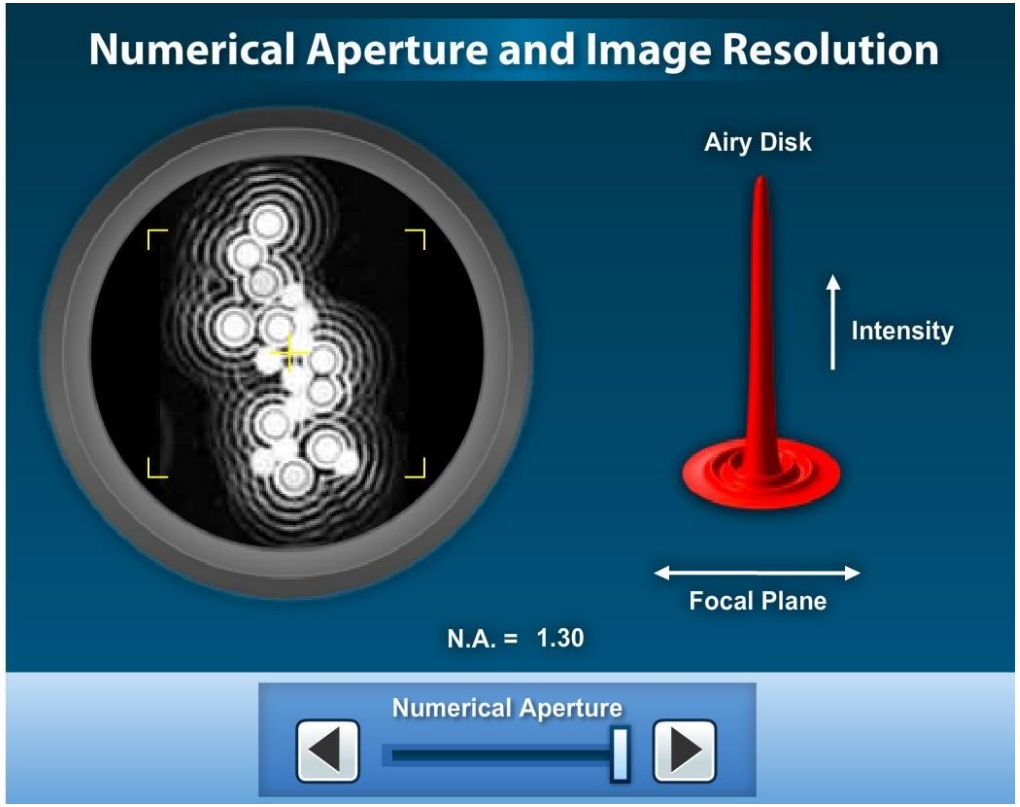


The size of the airy disk depends on the size of the objective lens

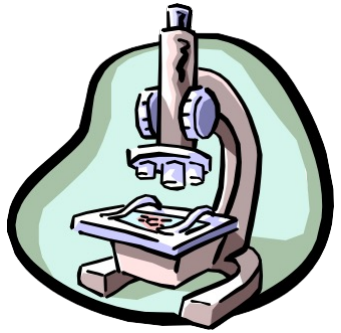
NUMERICAL APERTURE AND IMAGE RESOLUTION

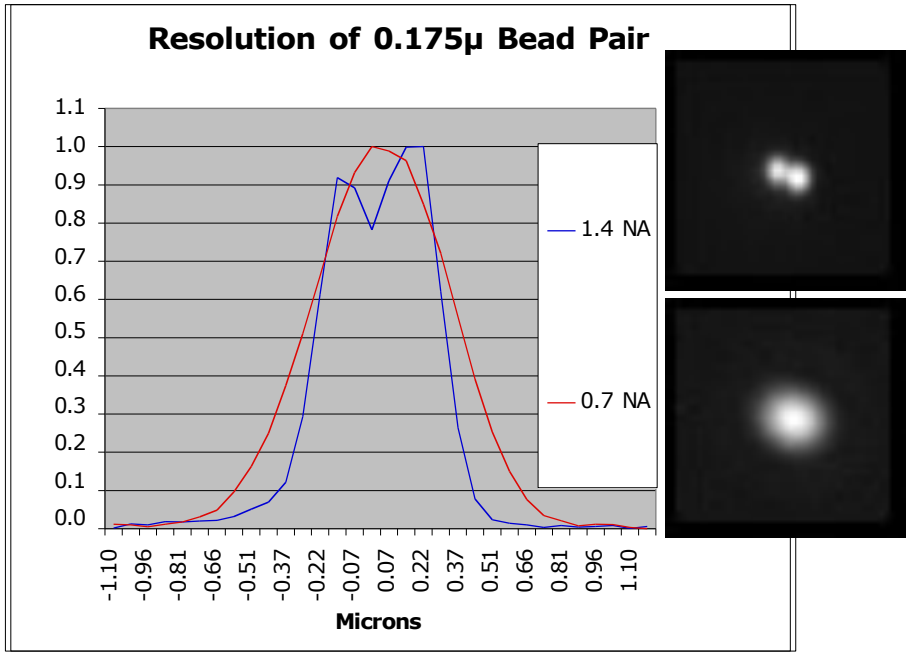






Numerical aperture, NOT magnification determines resolution!





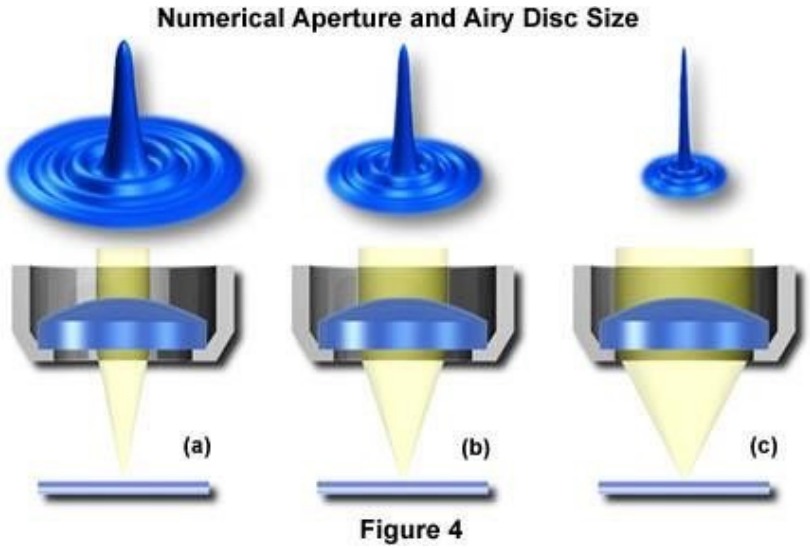
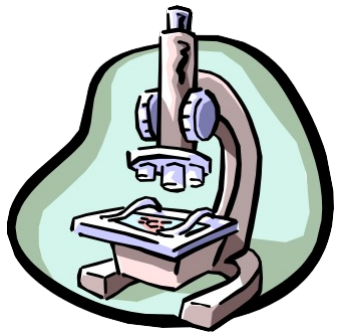


Figure 4

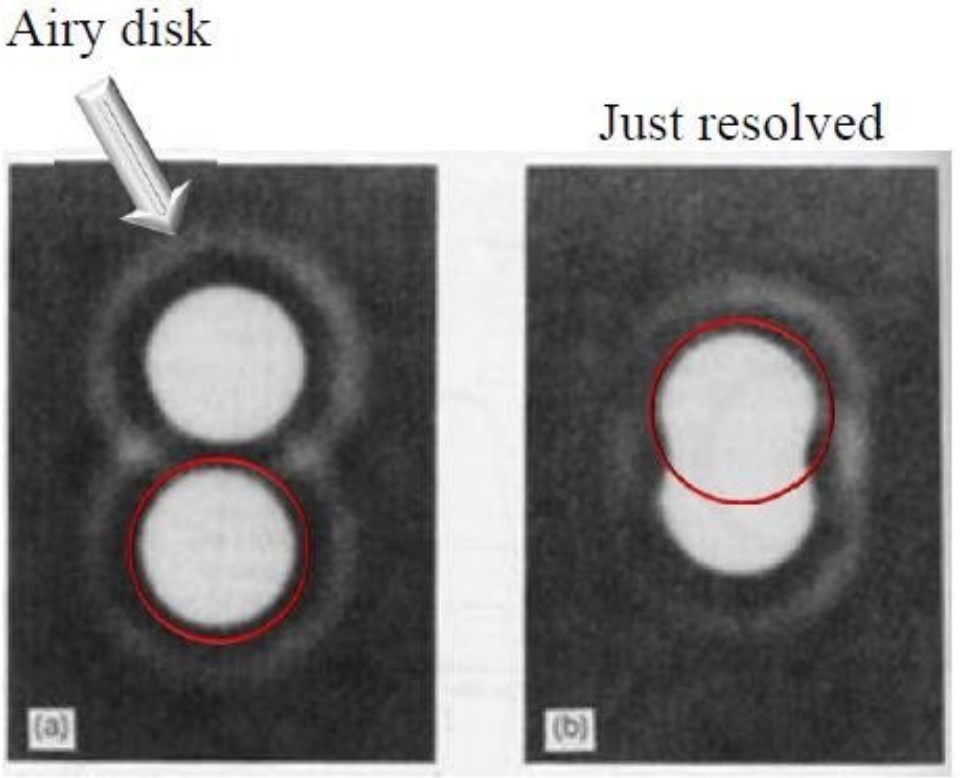
Increasing NA

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.

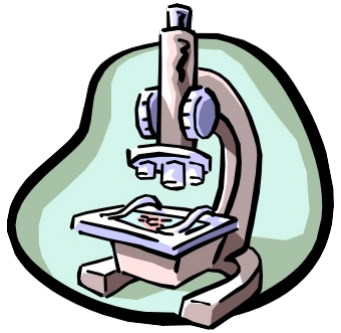


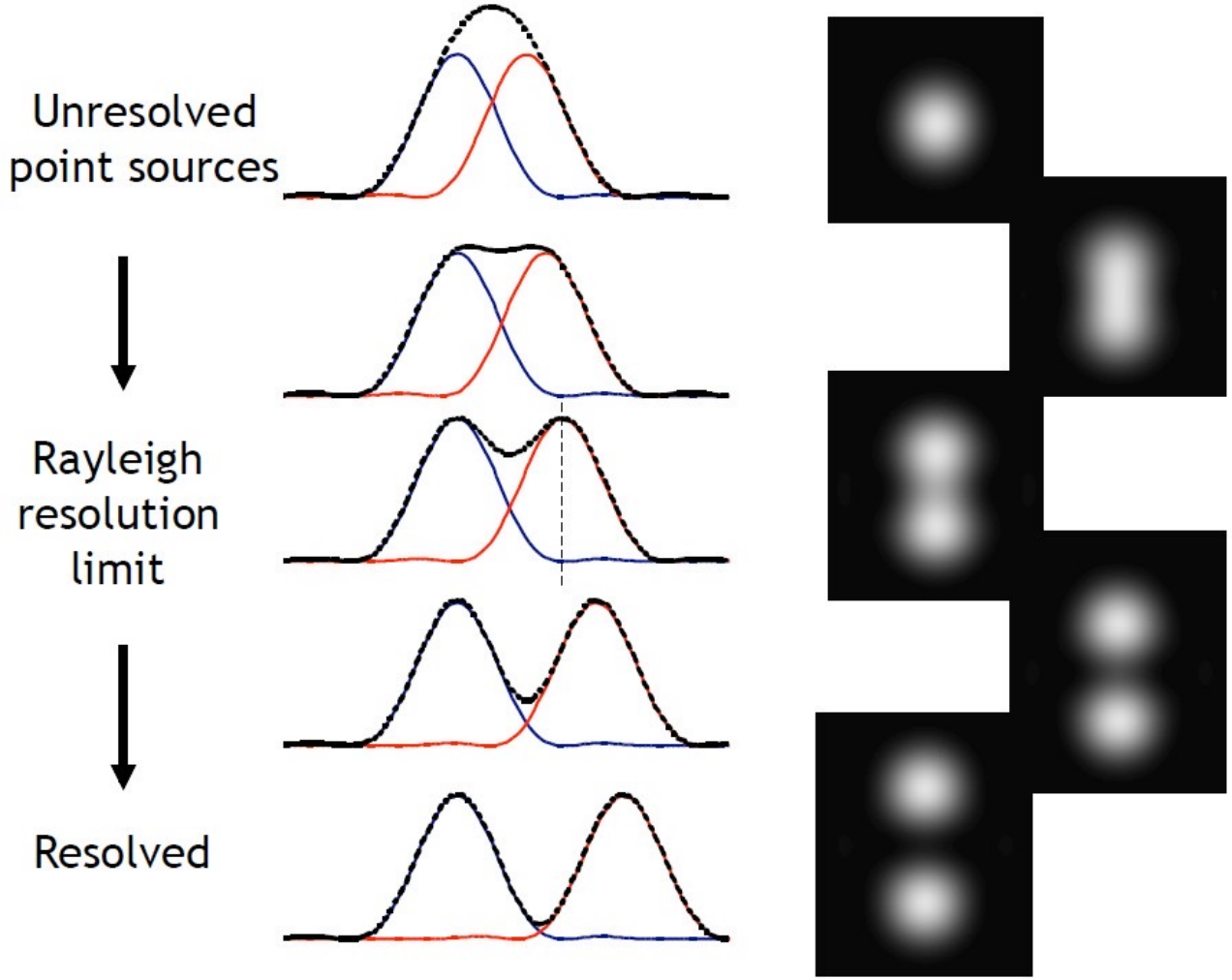
Angular resolution criterion for diffraction

- **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



NUMERICAL APERTURE AND IMAGE RESOLUTION





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- 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 and by that of eyepiece.

THE LIMIT OF RESOLUTION

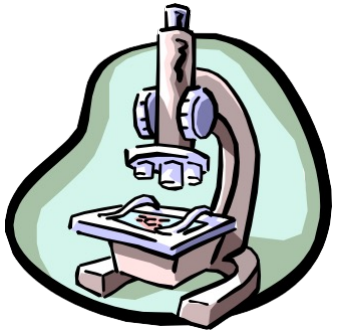
- is the smallest distance by which two objects can be separated and still be distinguished as two separate objects.

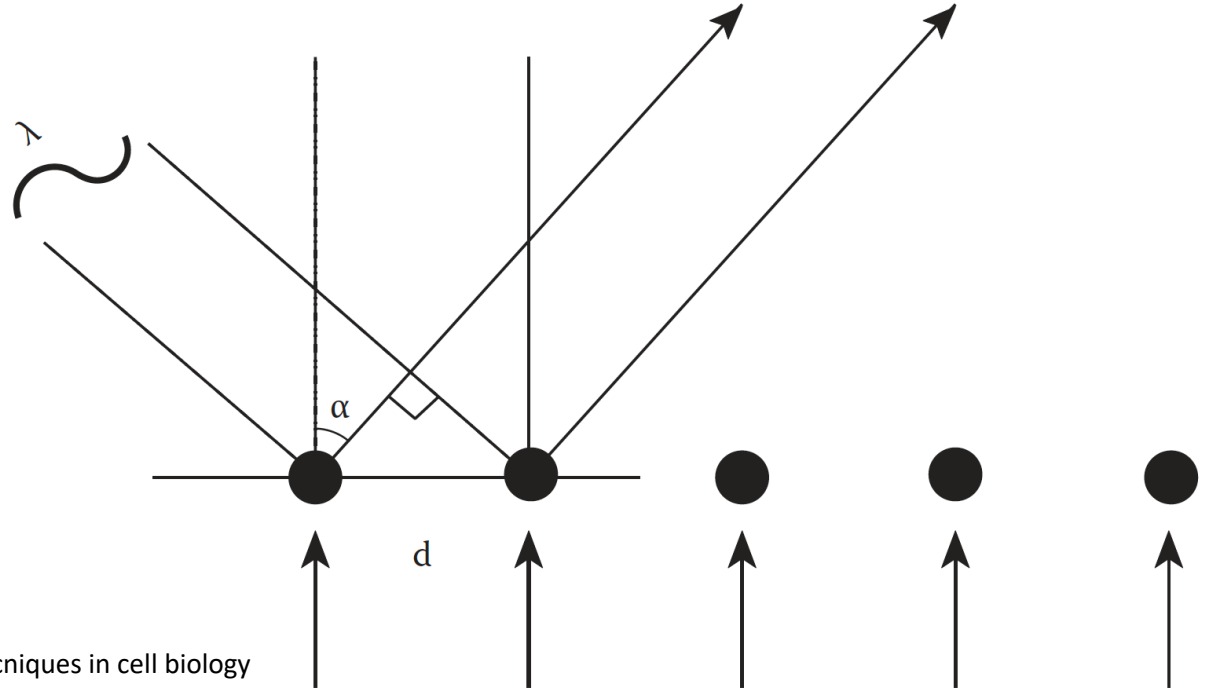
- The greatest resolution in optical microscopy can be obtained with the shortest wavelength of visible light and an objective with maximum NA.

- The relationship between NA and the limit of resolution can be expressed as follows:

$$d = \lambda / 2NA$$

ABBE's LAW

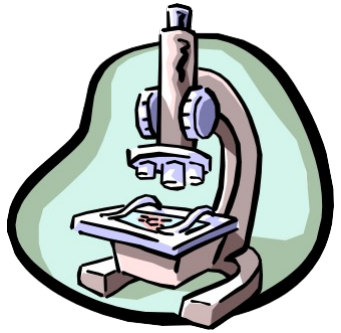




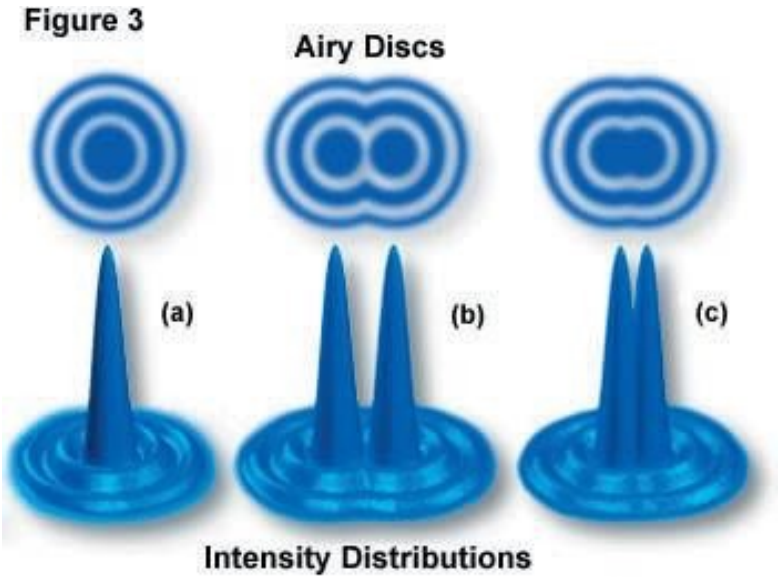
$$d = \frac{\lambda}{2\, n \sin\theta}$$

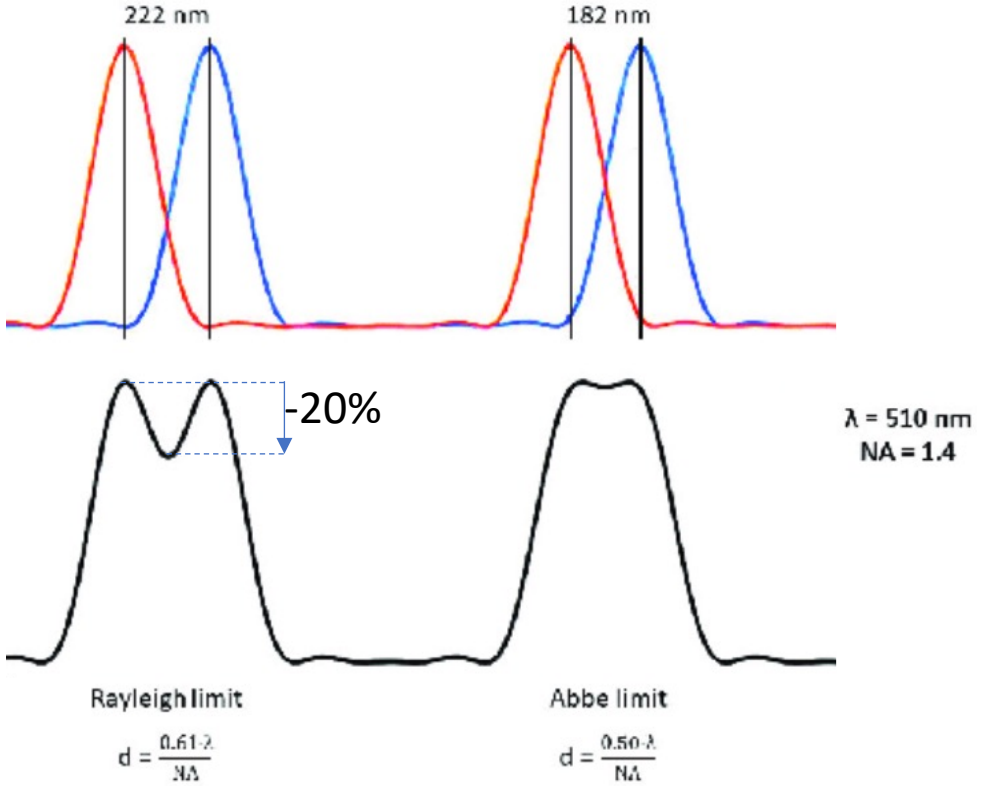
"minimum resolving distance (d) is related to the wavelength of light (lambda) divided by the Numeric Aperture, which is proportional to the angle of the light cone (theta) formed by a point on the object, to the objective".

RESOLUTION CRITERION



Abbe's Criterion



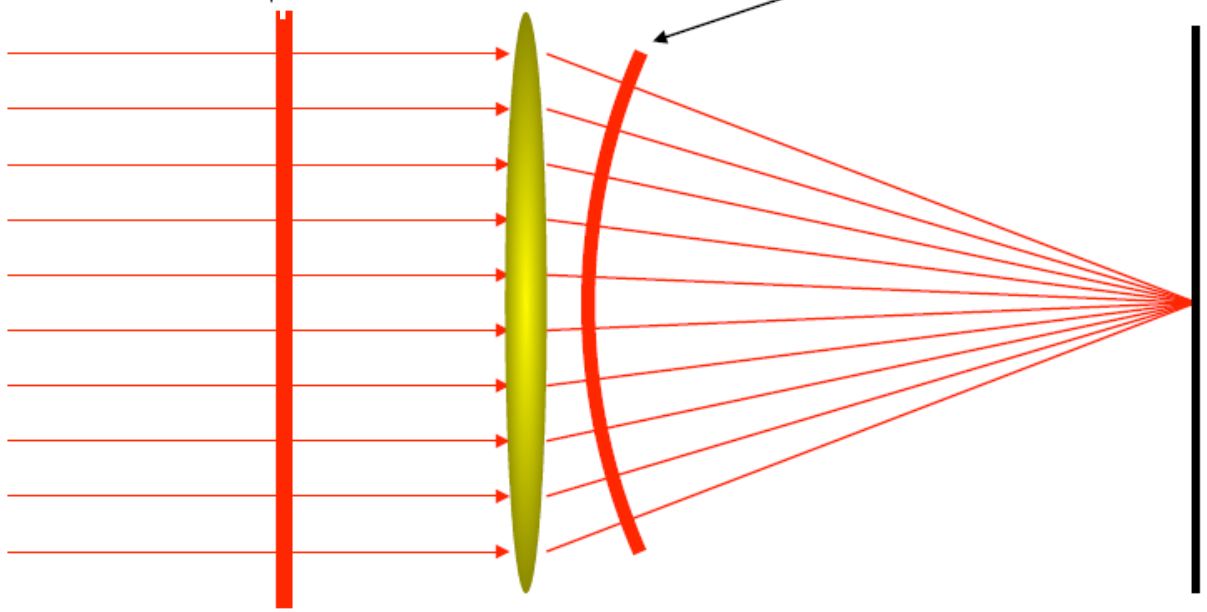


Lenses and Image Formation

What is the Wavefront?

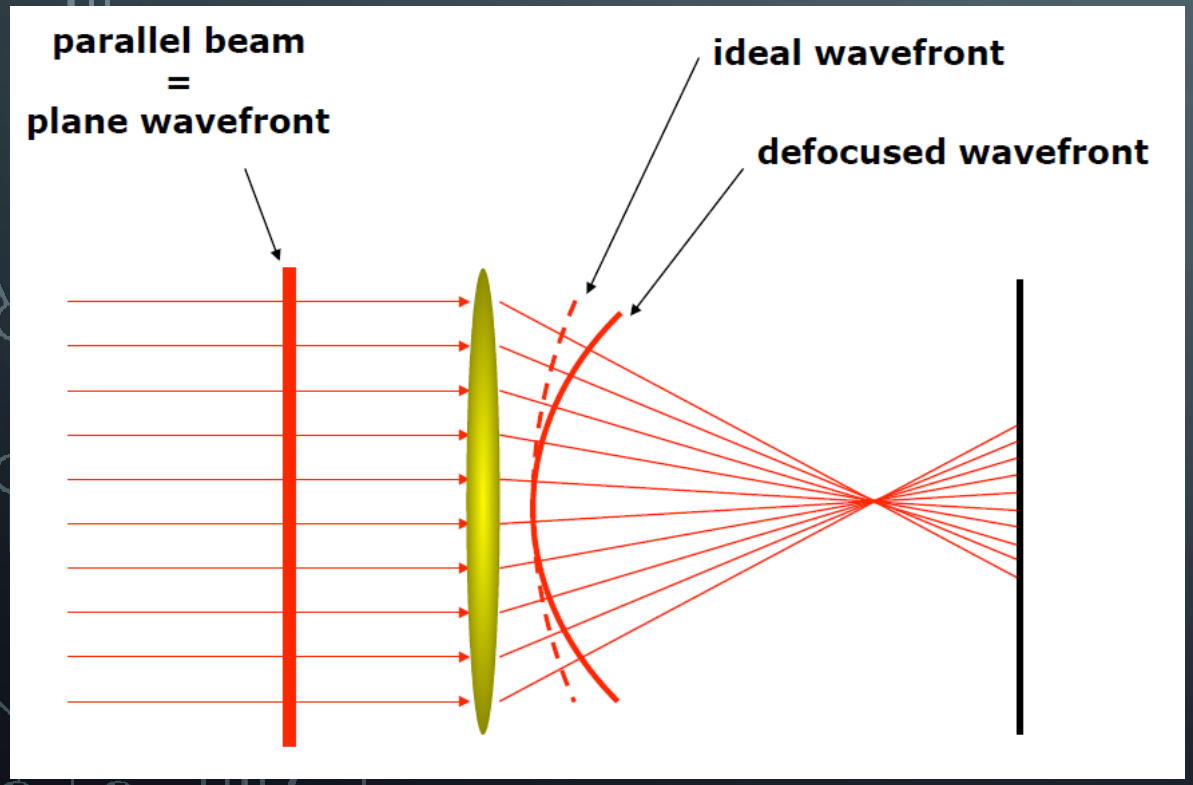
parallel beam
=
plane wavefront

converging beam
=
spherical wavefront

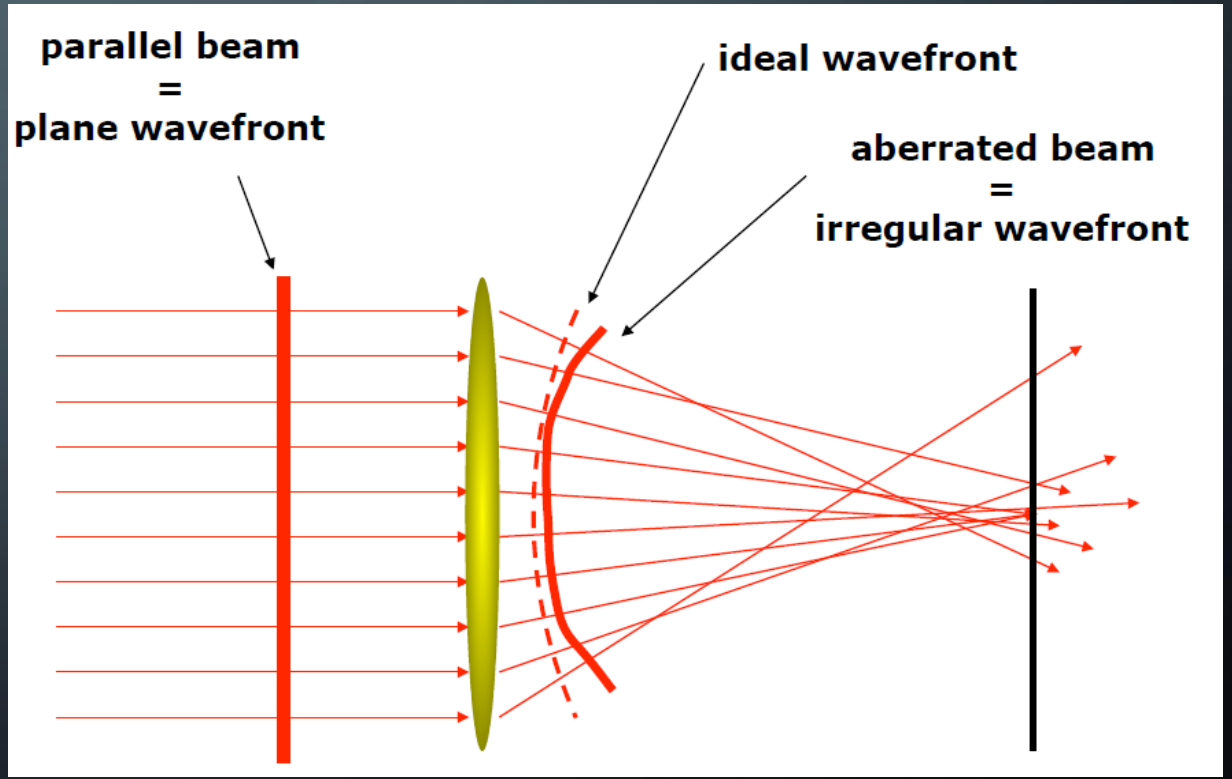


Lenses and Image Formation

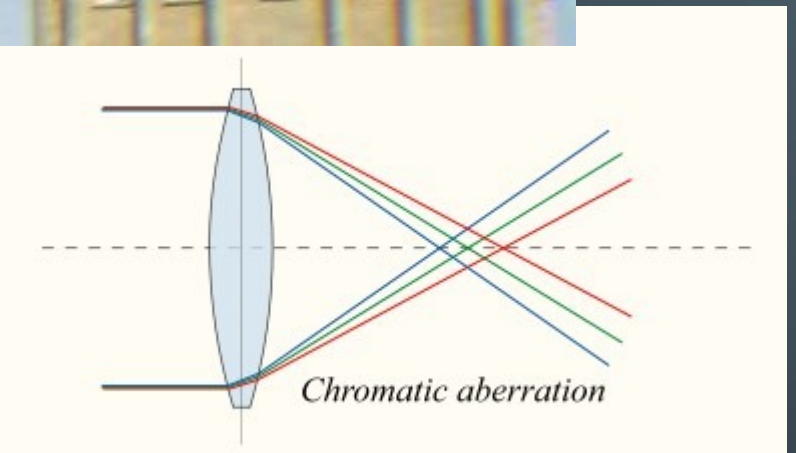
OUT of focus

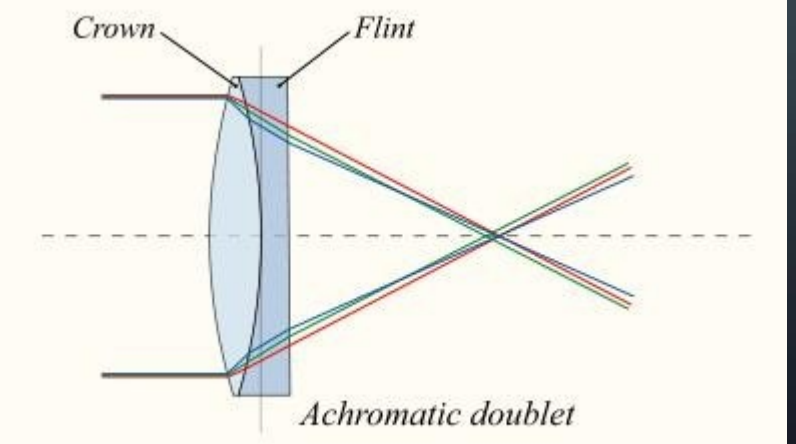


Aberrations



CHROMATIC ABERRATION

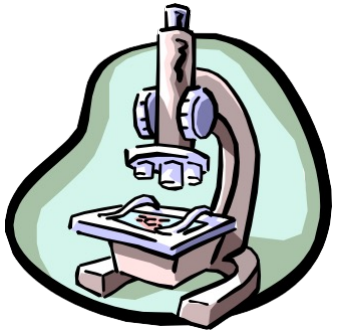




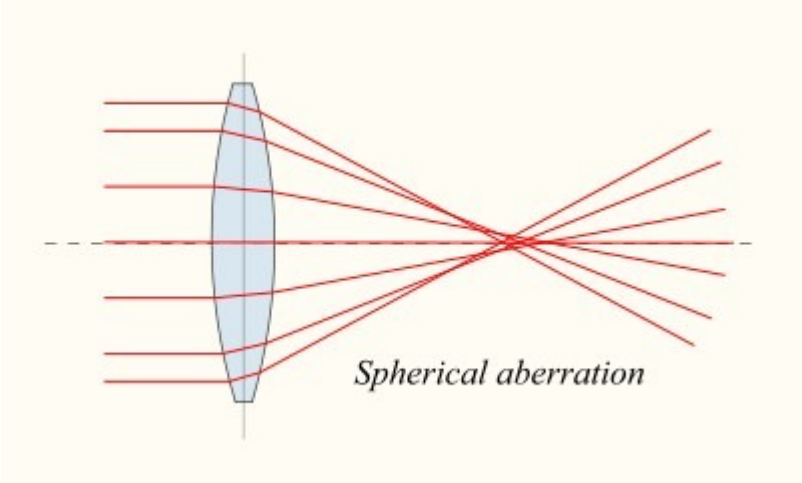
- Chromatic aberration is caused by a lens having different refractive indexes for different wavelengths.
- Since the focal length of a lens is dependent on the refractive index, different wavelengths will be focused on different positions in the focal plane.
- Chromatic aberration is seen as fringes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

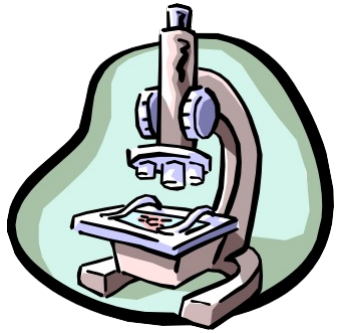
SPHERICAL ABERRATION



- 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.



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 ⌀ 18 mm. Versions for phase contrast.

- Achroplan

  Improved Achromat objectives with good image flatness for fields of view with ⌀ 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 ⌀ 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 ⌀ 25 mm. Highest NAs for a resolving power at the very limits of the physically possible.