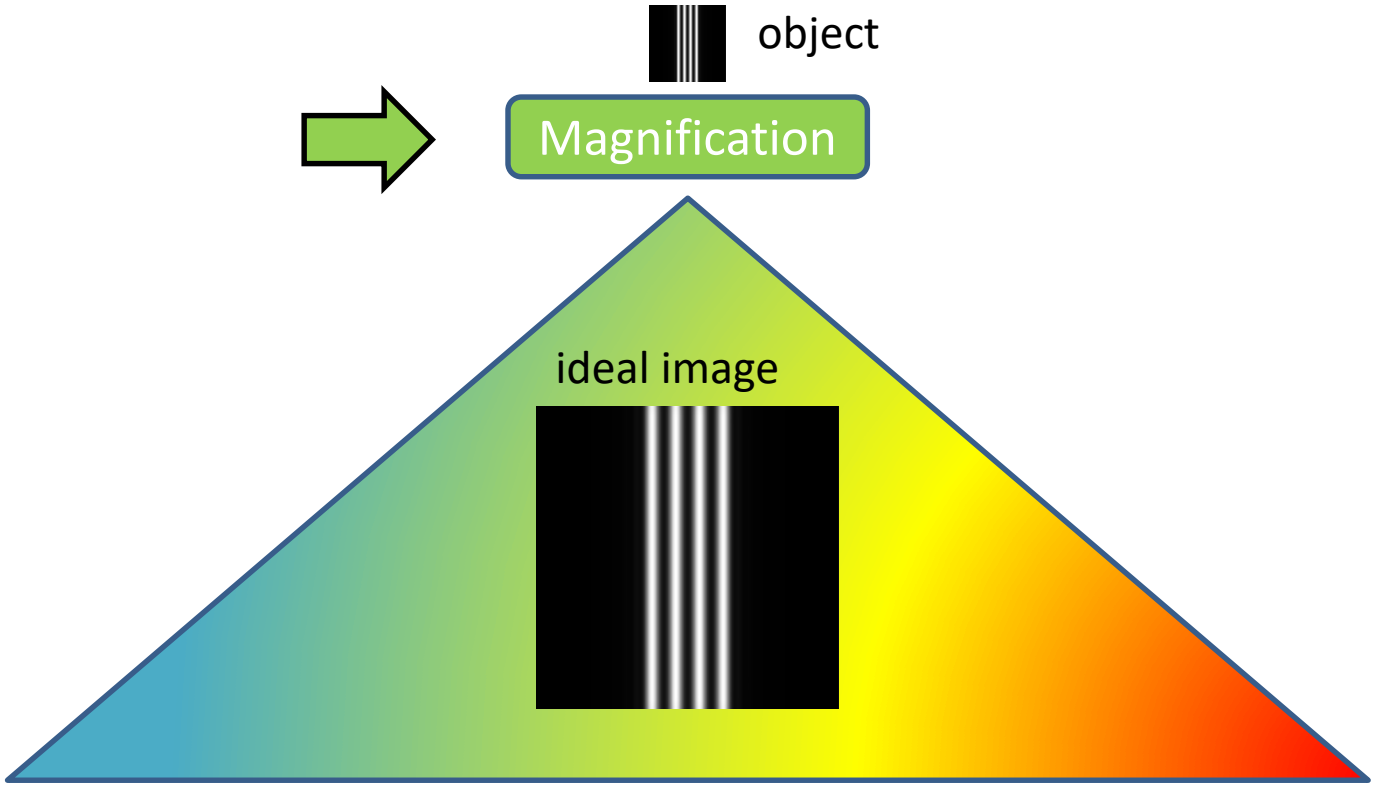
MICRO-561

Biomicroscopy I

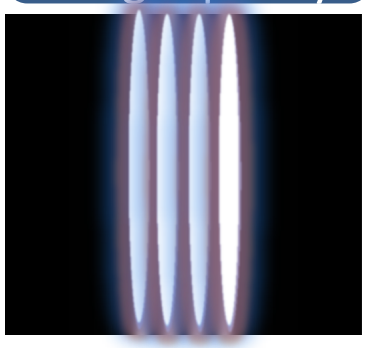
Syllabus (tentative)

Lecture 1	Introduction & Ray Optics-1
Lecture 2	Ray Optics-2 & Matrix Optics-1
Lecture 3	Matrix Optics-2
Lecture 4	Matrix Optics-3 & Microscopy Design-1
Lecture 5	Microscopy Design-2
Lecture 6	Microscopy Design-3
Lecture 7	Resolution-1
Lecture 8	Resolution-2
Lecture 9	Resolution-3 & Contrast
Lecture 10	Fluorescence-1
Lecture 11	Fluorescence-2
Lecture 12	Fluorescence-3, Sources, Filters
Lecture 13	Detectors
Lecture 14	Bio-application Examples

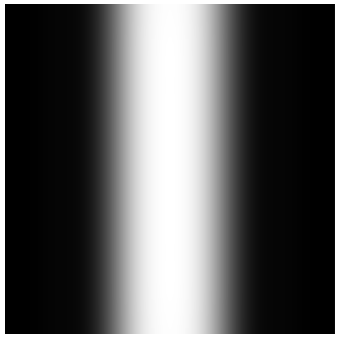
Important aspects for microscopy



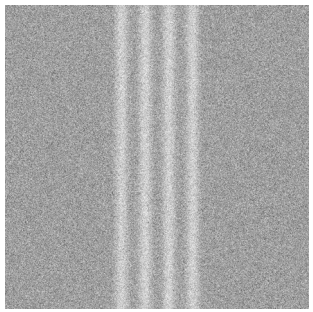
Aberrations – image quality



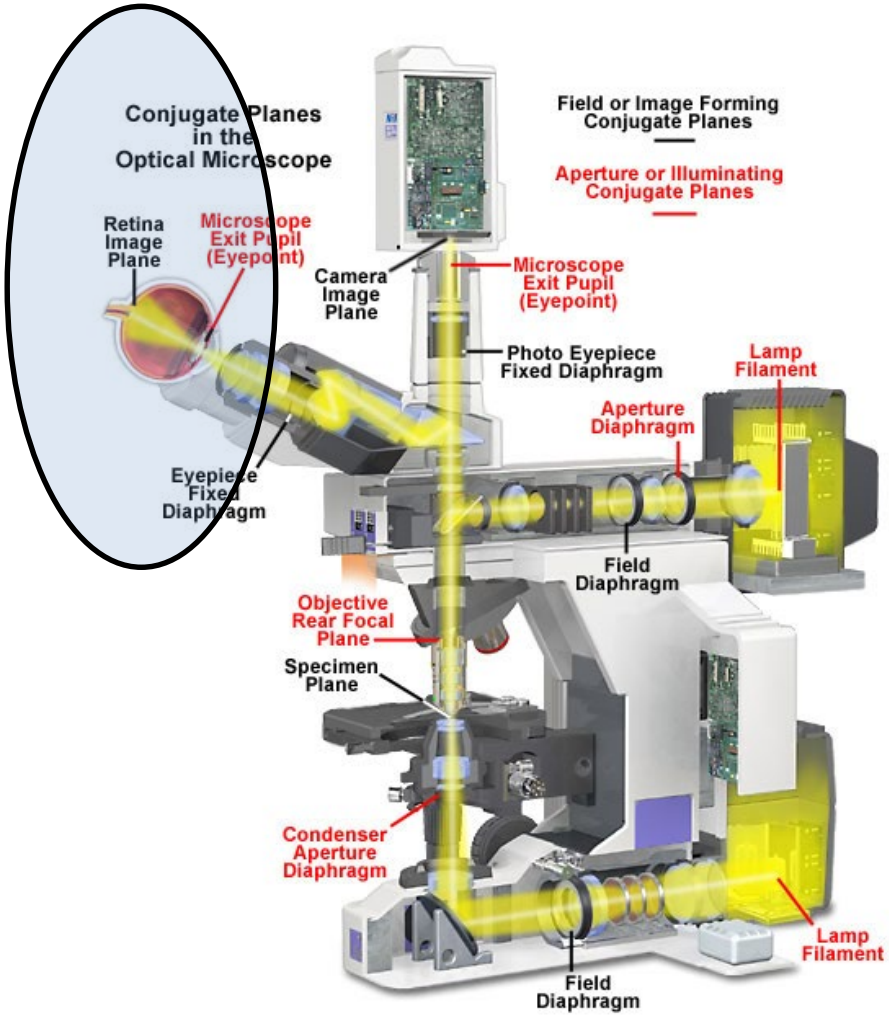
Resolution



Contrast



Important aspects of microscopy:



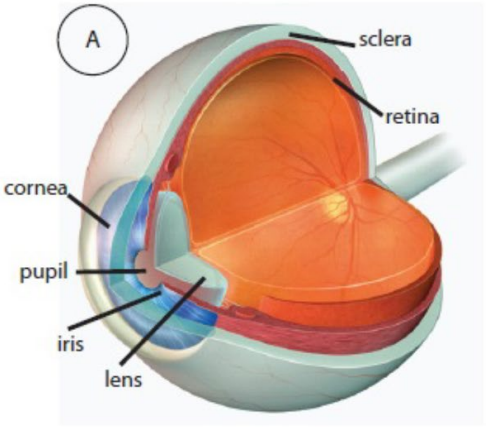
- Magnification
- Resolution
- Contrast
- Image quality

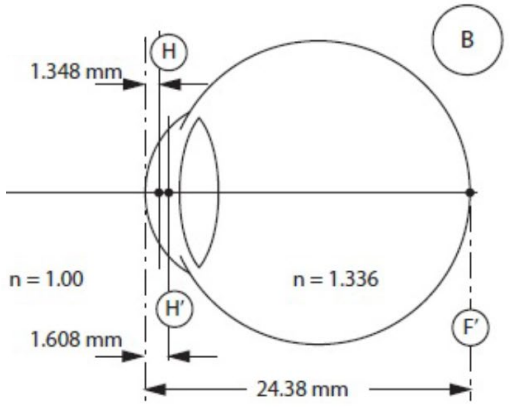
Magnification in Microscopy

Purpose of a microscope:

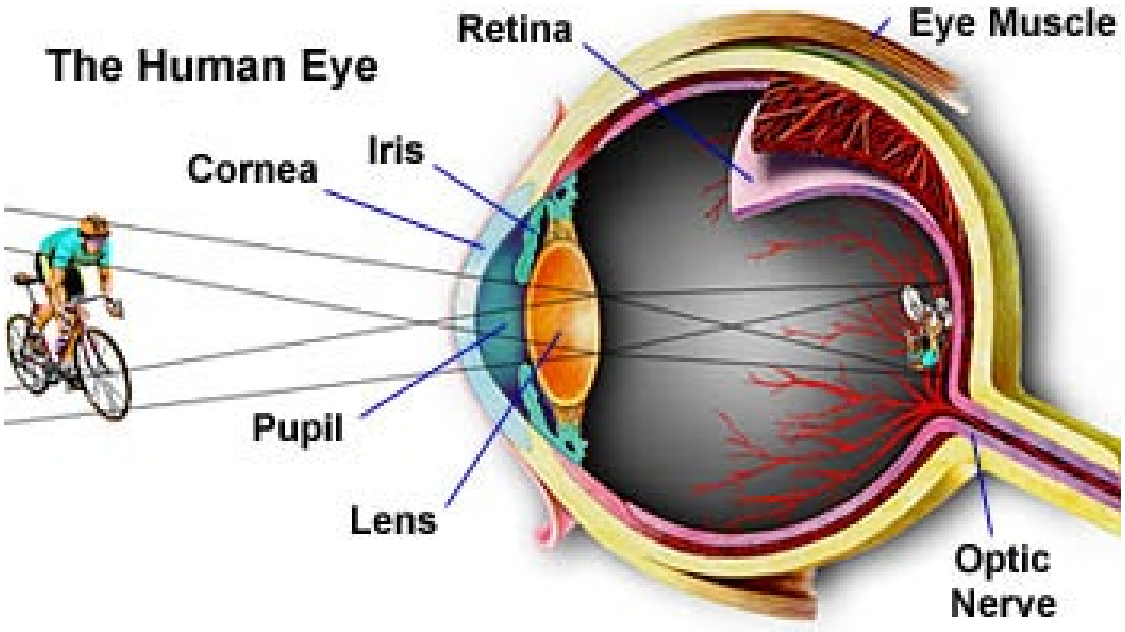
- Improve the perception/visibility of small objects to be seen by the **human eye**.
- The knowledge of the performance and limitations of optical systems is key to use microscopes properly.

The **human eye** itself can be seen as an optical system.





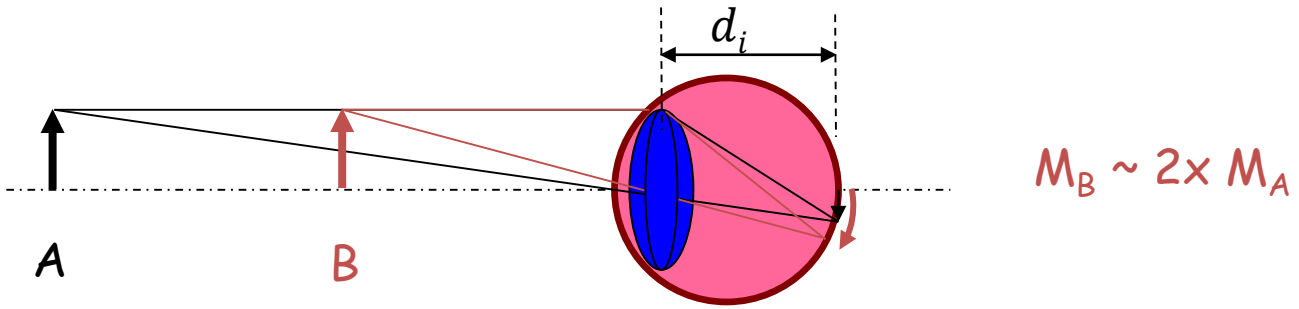
The human eye – a single lens system



The human eye – a single lens system

"Accommodation" of the eye results in changes in the **focal length** (thus the optical power) of the eye lens.

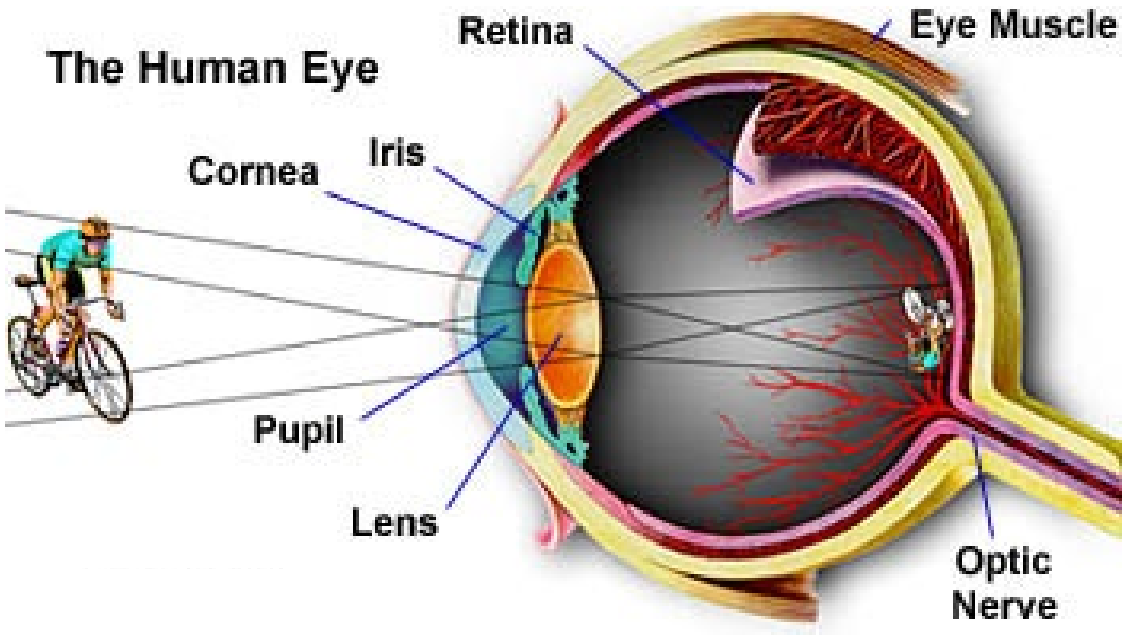
Due to this accommodation, images in the eye can have different sizes (i.e. eye yields different magnification) depending on the distance of the object away from the eye.



$$\frac{1}{f} = \frac{1}{d_o} + \frac{1}{d_i}$$

If we vary d_o but fix f, we cannot form a image at the same d_i

The human eye – a single lens system

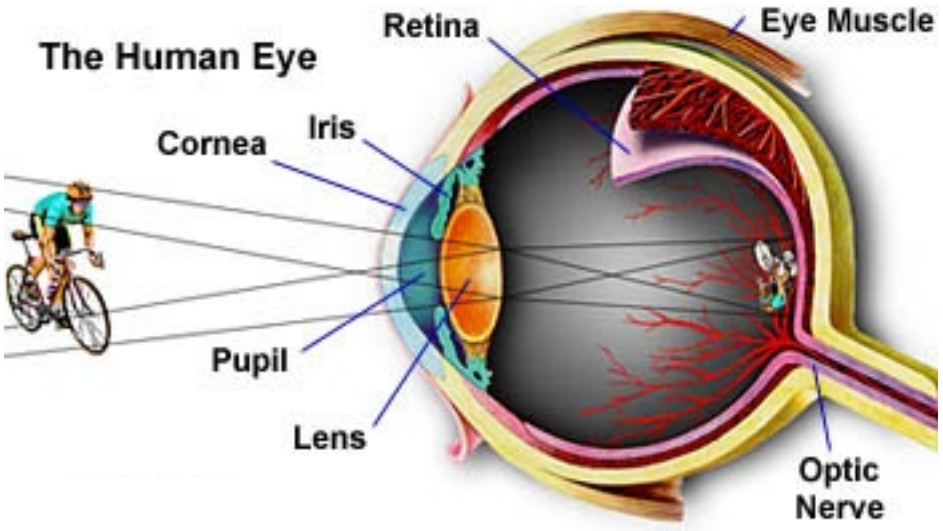


- **Comfortable** near point is about ~25 cm
- We define the size at 25 cm as magnification, M = 1

The human eye – a single lens system

We could get a larger retinal image if the object gets closer, **BUT** we face:

- Limited accommodation (especially with age)
- Limited "magnification" range by naked eye

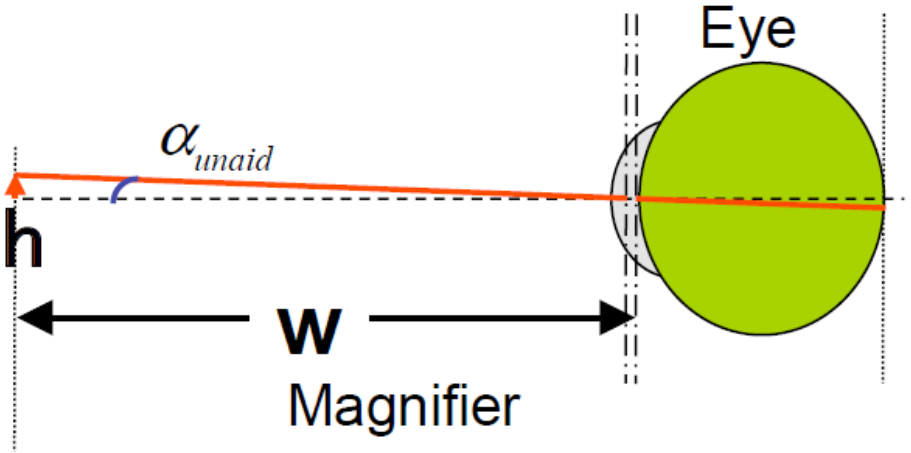


Solution: Add a "loupe" in front of eye

The human eye – a single lens system

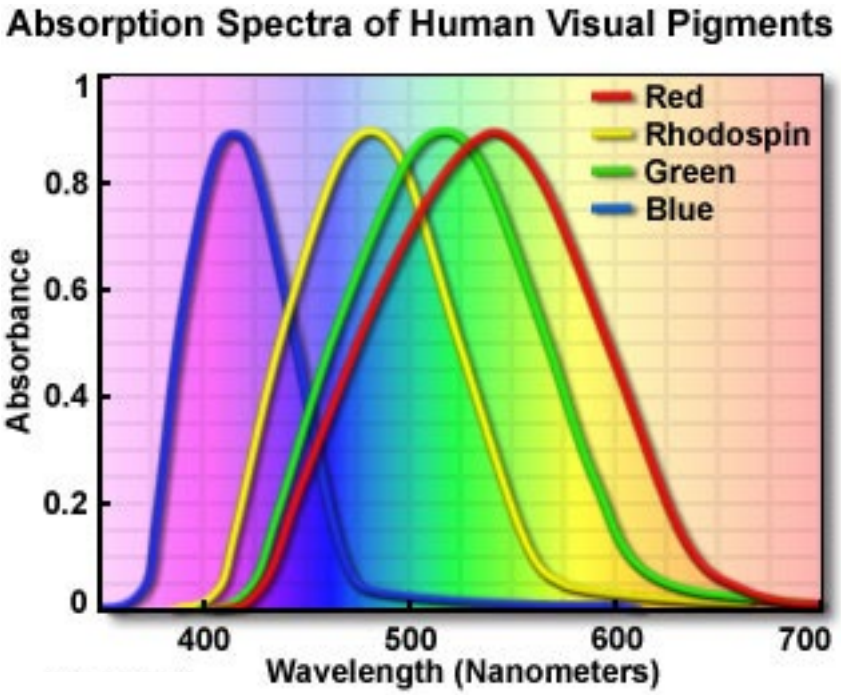
Unaided (naked) eye:

Angle → $\alpha_{unaid} = -\frac{h}{w}$



Specifications of the human eye

- Angular resolution: ~1°
- Spatial resolution: ~80 µm
- Ability to resolve contrast: ~ 5%
- More sensitive to color than the light intensity
- Spectral range: 400 nm - 800 nm
  - Max sensitivity is at 555 nm during day (cones)
  - Max sensitivity is at 505 nm during night (rods)

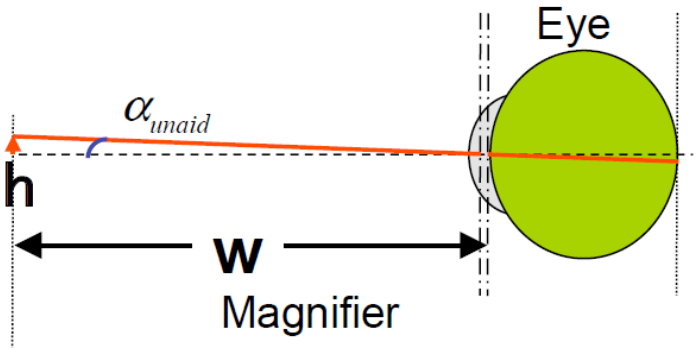


Magnifying Glass

Unaided eye:

The object is at the **near-point** distance of w= 25 cm

Angle → $\alpha_{un-aid} = -\frac{h}{w}$

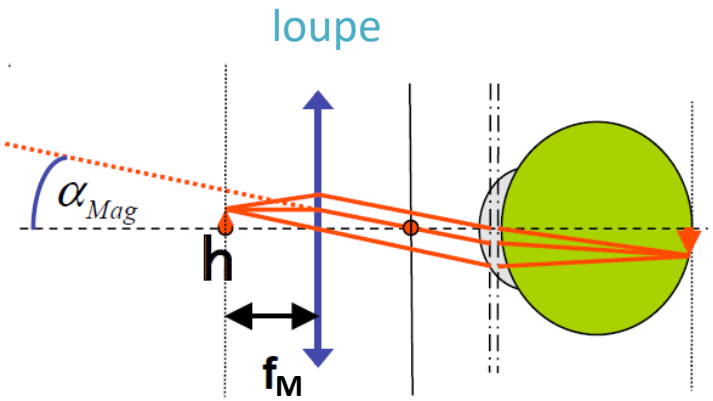


Aided eye with a loupe (magnifier):

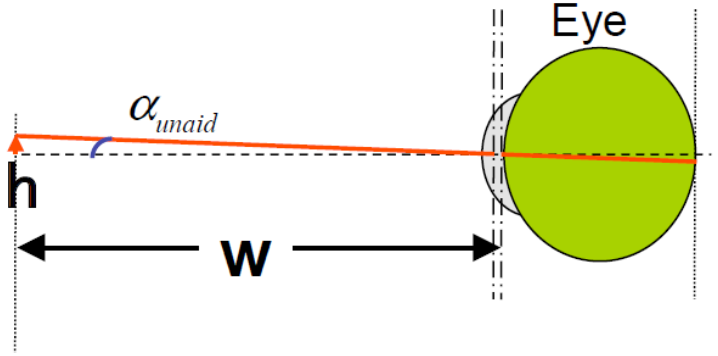
The object is at the focal point of the magnifier

→ A virtual intermediate image is generated at infinity

→ Relax eye focuses this virtual image at retina to form the final image



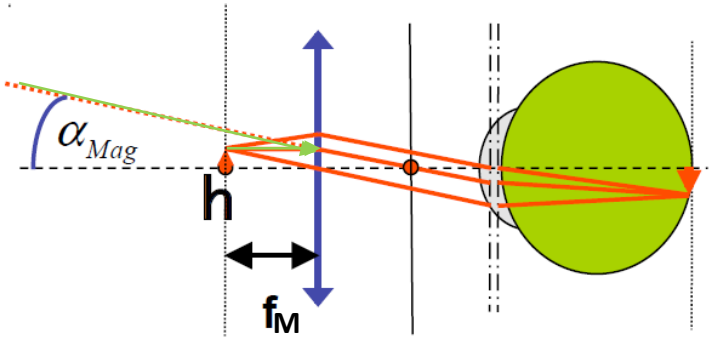
Magnification with a Single Lens (i.e. Loupe)



Un-aided eye:

The object is at the **near-point** distance of **w=25 cm**

Un-aided Angle → $\alpha_{un-aid} = -\frac{h}{w}$



Aided eye with a loupe (Magnifier):

The object is at the focal point of the magnifier

→ A virtual intermediate image is generated at infinite

$$intermediate\ image = \begin{bmatrix} 1 & 0 \\ -\frac{1}{f_M} & 1 \end{bmatrix} \begin{bmatrix} 1 & f_M \\ 0 & 1 \end{bmatrix} \begin{bmatrix} h \\ 0 \end{bmatrix}$$

$$= \begin{bmatrix} 1 & f_M \\ -\frac{1}{f_M} & 0 \end{bmatrix} \begin{bmatrix} h \\ o \end{bmatrix} = \begin{bmatrix} h \\ -\frac{h}{f_M} \end{bmatrix}$$

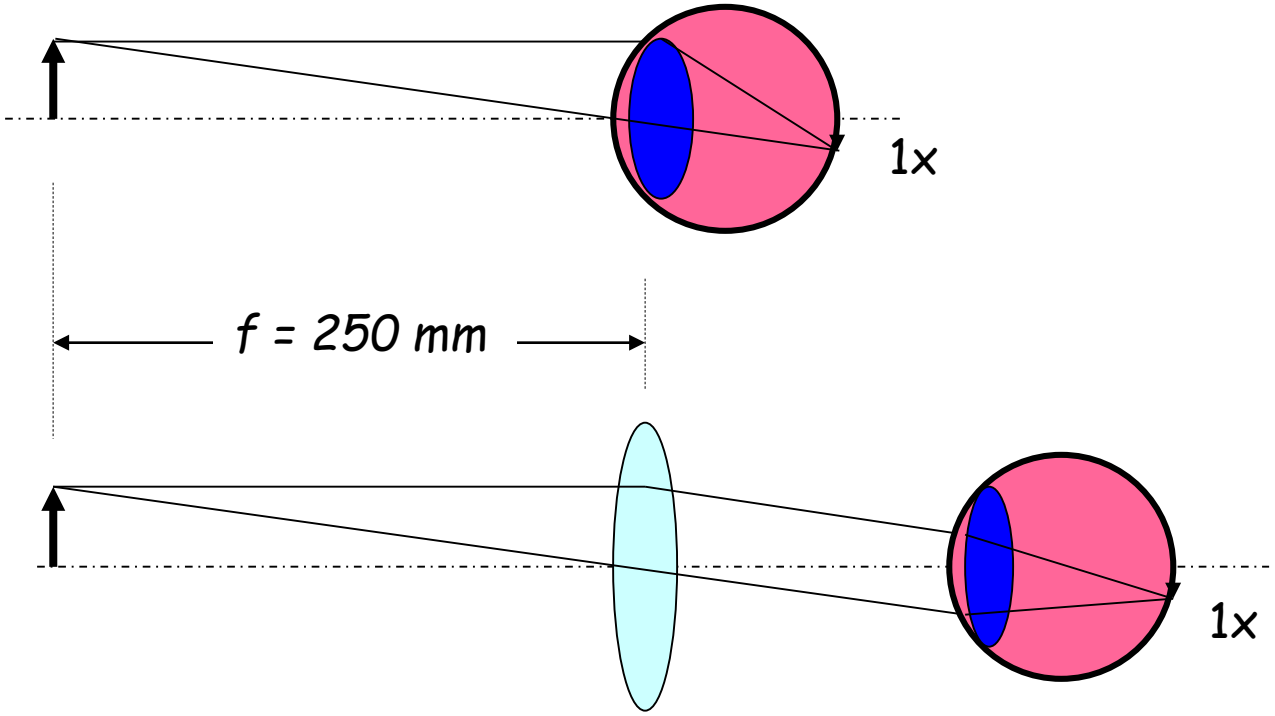
Magnified Angle → $\alpha_M = -\frac{h}{f_M}$

Magnification → $M = \frac{\alpha_M}{\alpha_{un-aid}} = \frac{\frac{h}{f_M}}{\frac{h}{w}} = \frac{w}{f_M} = \frac{25cm}{f_M(cm)}$

Magnification with a single lens

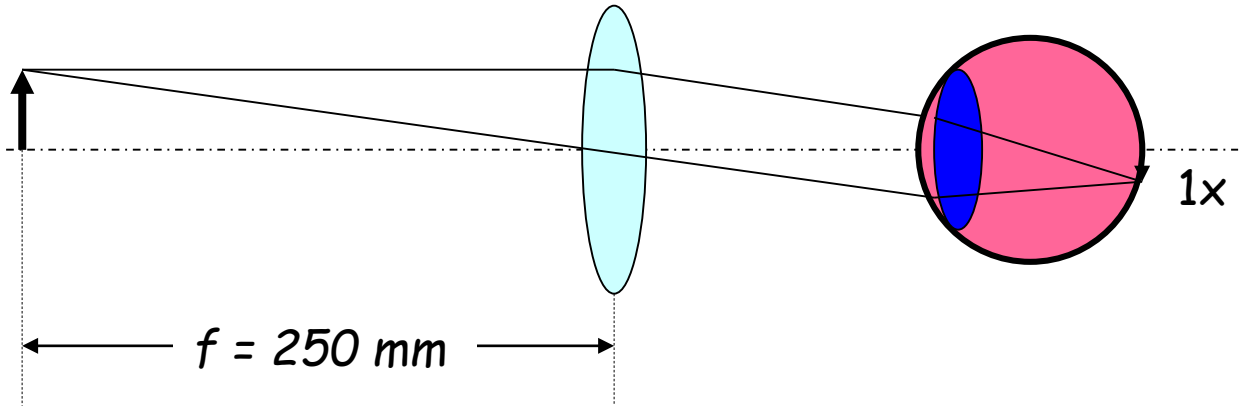
$$M = \frac{250mm}{f_{Lens}}$$

For a magnifying lens with $f_{Lens} = 250\ mm$, Magnification (M) is 1x

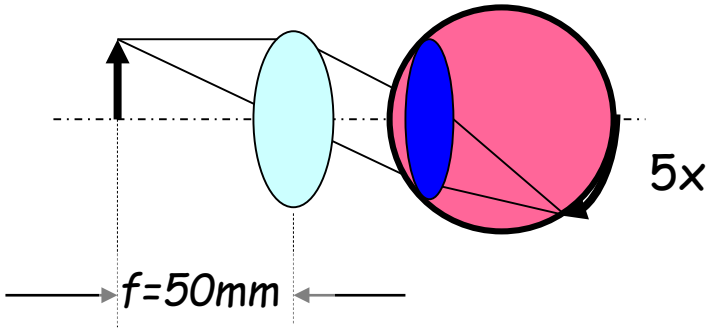


Magnifying Glass

$$M = \frac{250mm}{f_{Lens}}$$



If f_{Lens} = 250 mm ➔ M=1

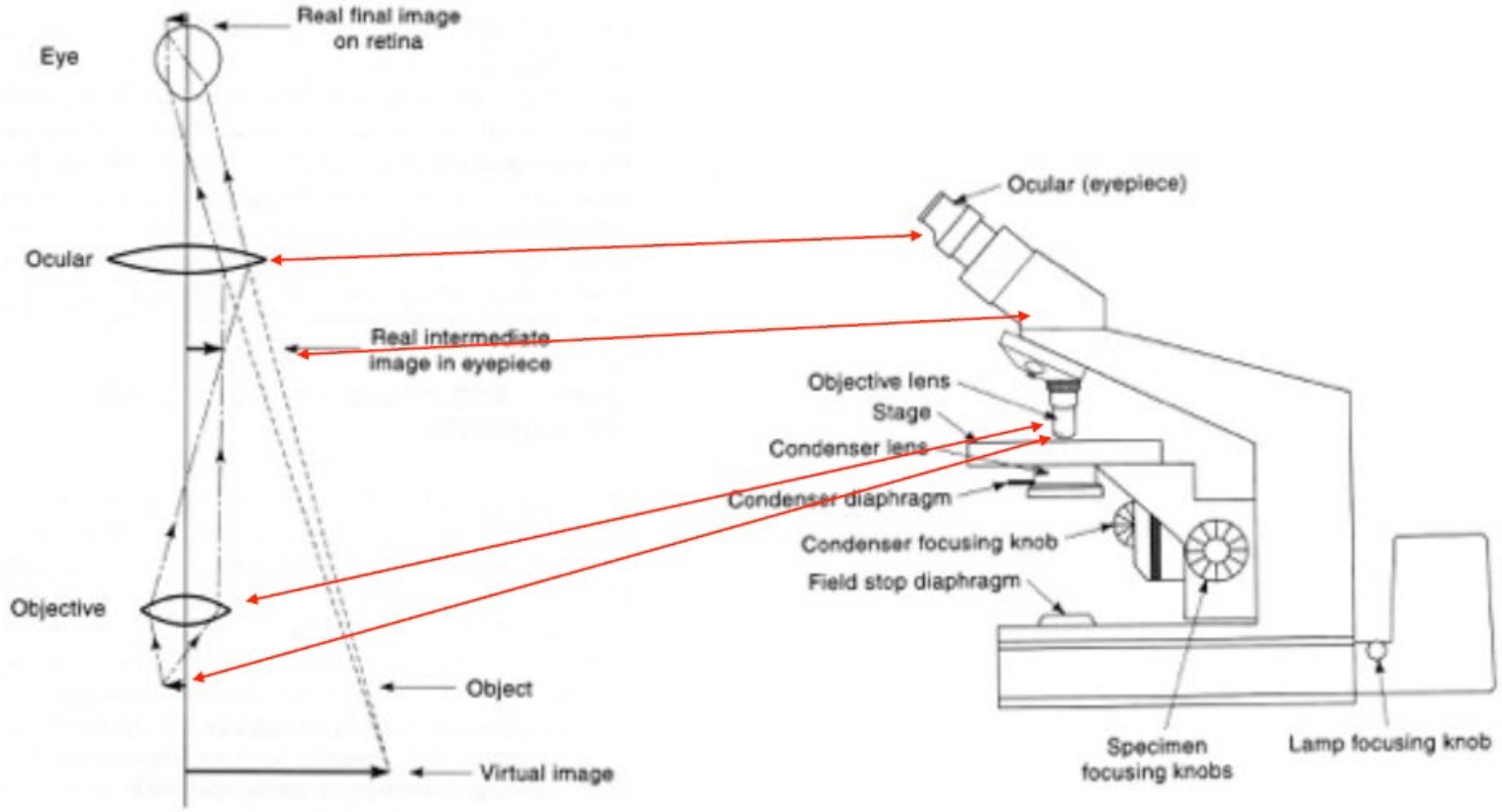


If f_{Lens} = 50 mm ➔ M=5

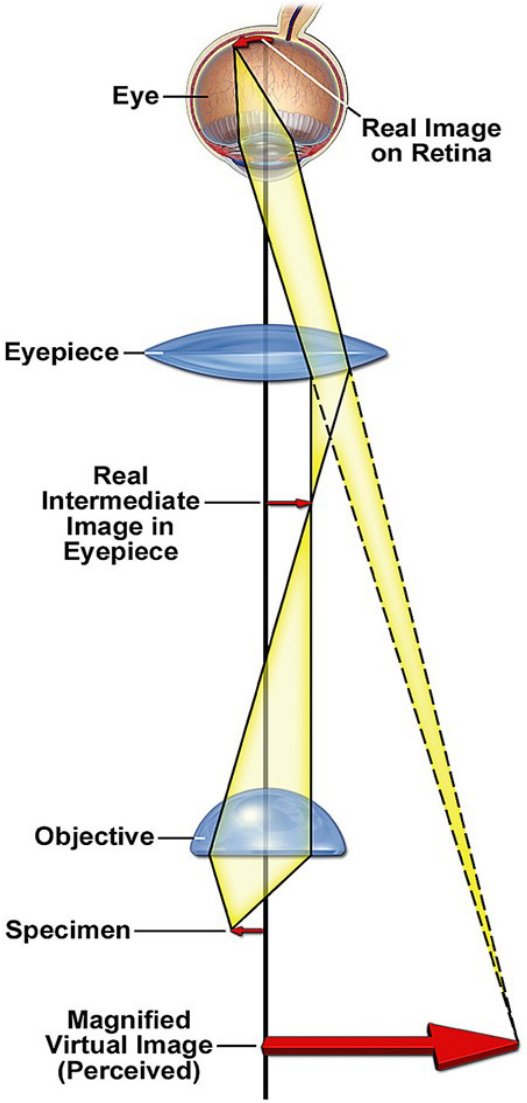
Maximum magnification for a magnifying glass is LIMITED to 10x-20x

Magnification & image formation for a microscope

- ***A microscope uses*** two lenses, namely an objective and an eyepiece (ocular), together to produce a final magnified image.
- Due to the use of two lenses together, it is also called ***compound microscope***
- A compound microscope provides higher magnification than a magnifying glass.



Compound microscope: **objective lens** followed by **an eyepiece** (a.k.a ocular)



- The specimen on the microscope stage is examined by the objective, which produces a magnified real image of the object in the image plane of the eyepiece.
- When looking in the microscope, the eyepiece acting together with the eye's lens projects a still more magnified real image onto the retina, where it is perceived and interpreted by the brain as a magnified virtual image about 25 cm (10 in) in front of the eye.

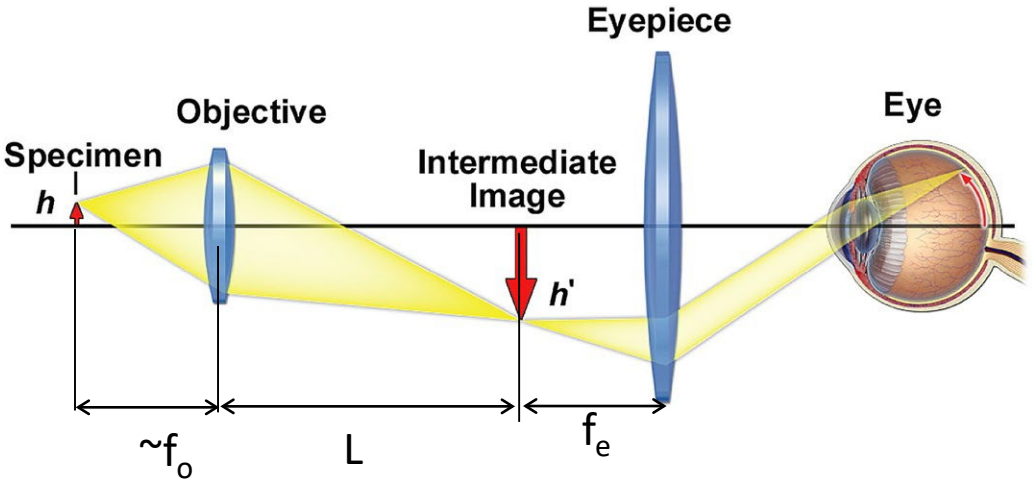
➔ **Compound magnifying power is the product of the magnifications of the two elements:**

$$MP = M^{objective} \times M^{eye\ piece}$$

➔ **The eyepiece acts like a magnifying lens**

$$M^{eye\ piece} = \frac{250\ mm}{f_{eye\ piece}}$$

Magnification of a compound microscope (finite correction)

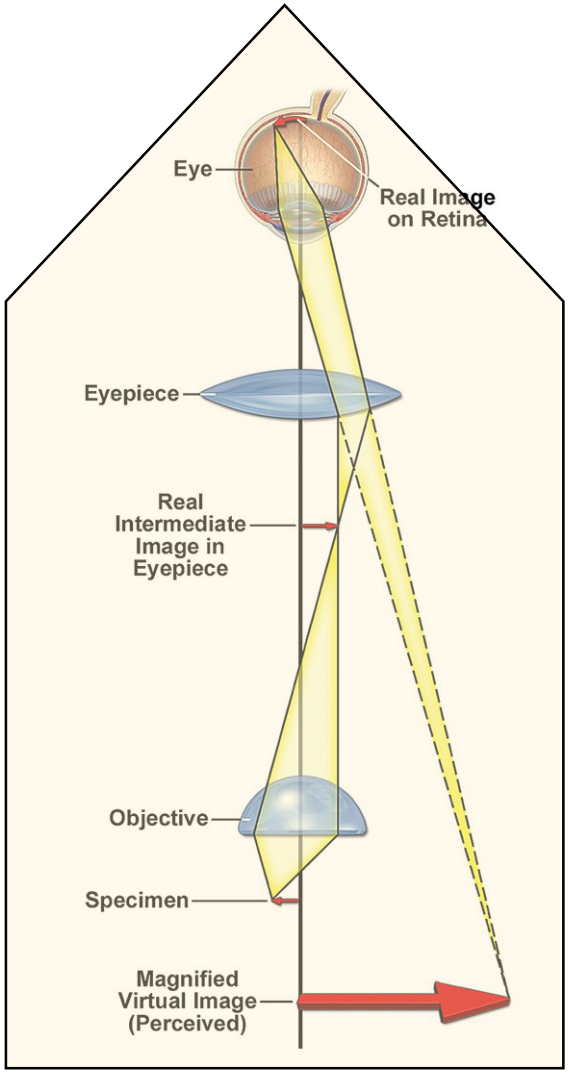


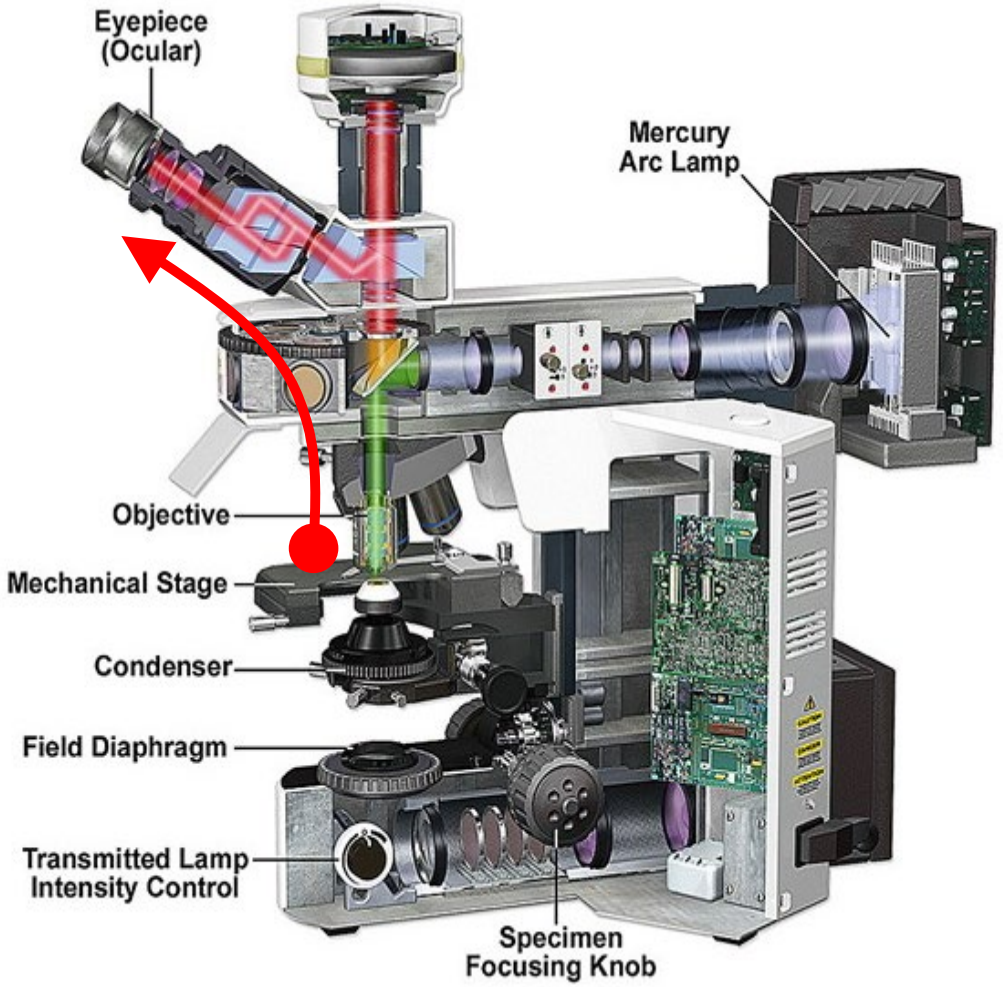
- Until the late 1980s, most microscopes had a fixed tube length distance (L) between objective and eyepiece.
- This distance is known as the *mechanical* ***tube length*** of the microscope.
- **The design assumes that when the specimen is placed in focus, it is a few micrometers further away than the front focal plane of the objective.** Finite tube lengths were standardized at 160 mm during the nineteenth century by the Royal Microscopical Society (RMS), and were in use for then next ~100 years.
- Objectives designed to be used with a microscope having the industry standard tube length of 160 mm are inscribed with " 160 " on the barrel.

$$MP = \left(\frac{160}{f_{objective}}\right) \times \left(\frac{250}{f_{eye\ piece}}\right)$$

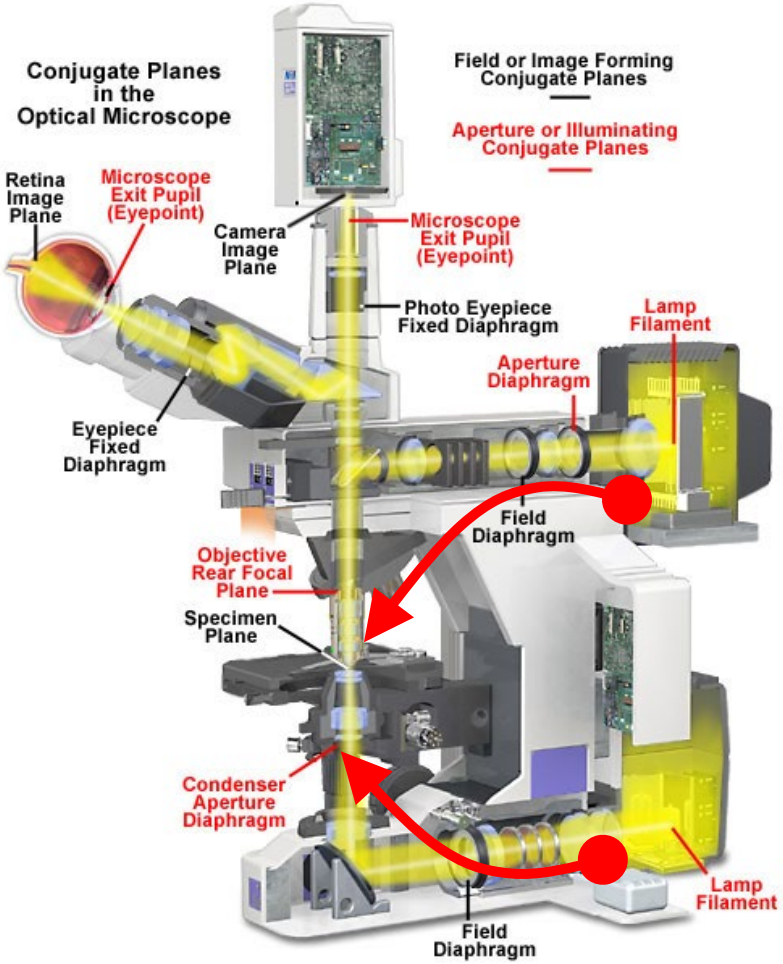
So far we focused on imaging part ….

An imaging system with 2 lenses:





… we also need to consider illumination part.

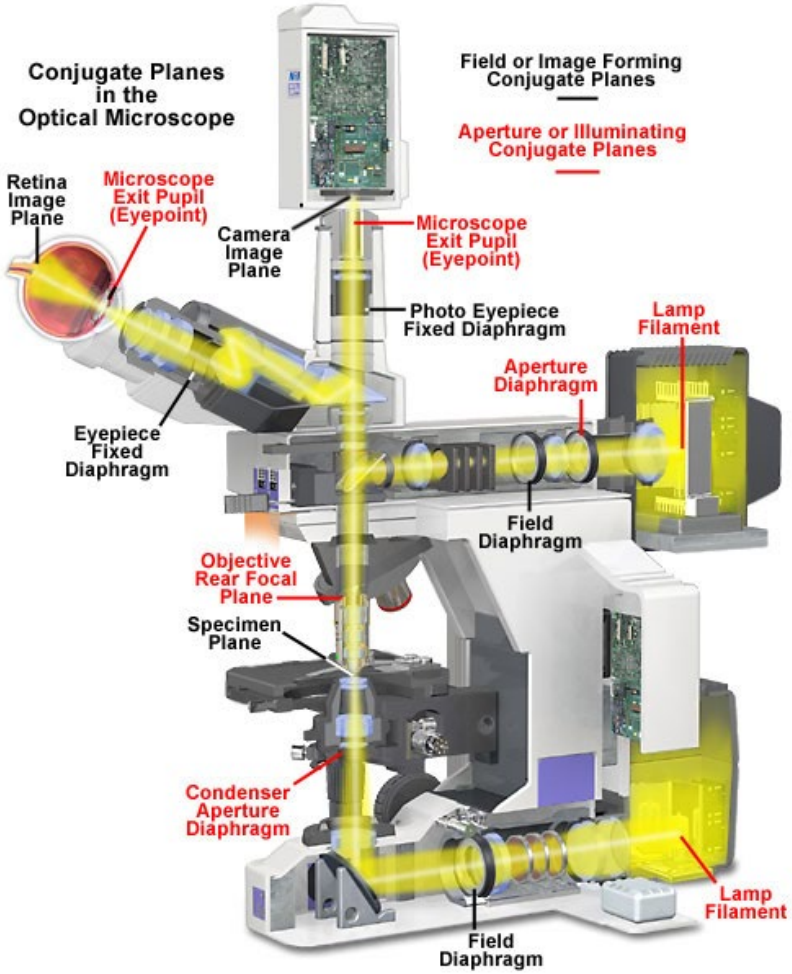


Research grade "upright" optical microscope (i.e. objective is above the specimen plane).

- It has two lamps: bottom lamp provides transmitted illumination, and top lamp provides reflected illumination.

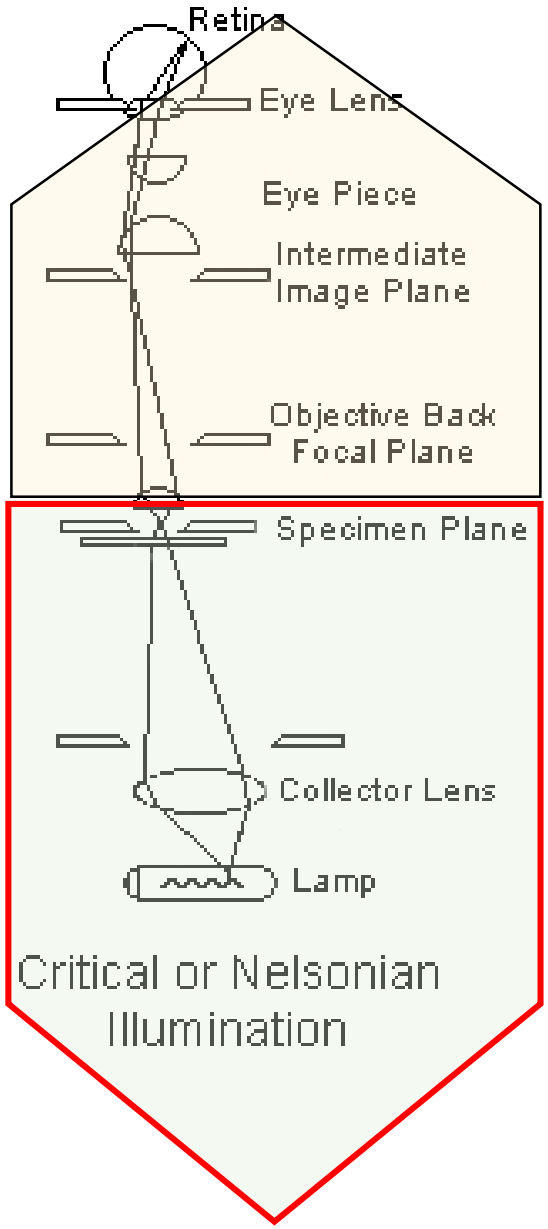
Illumination is a critical determinant of optical performance in light microscopy.

Important aspects for microscopy:



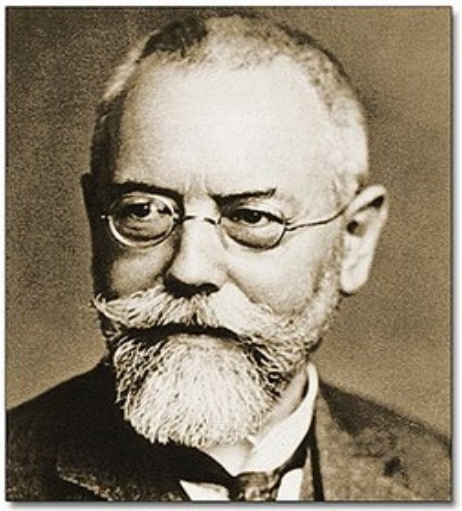
- Magnification
- **Image quality:** depends on aberrations, alignment, **illumination**…
- Resolution
- Contrast

“Critical Illumination”



Traditional methods of critical illumination:
The lamp is focused directly on the specimen with a condenser.

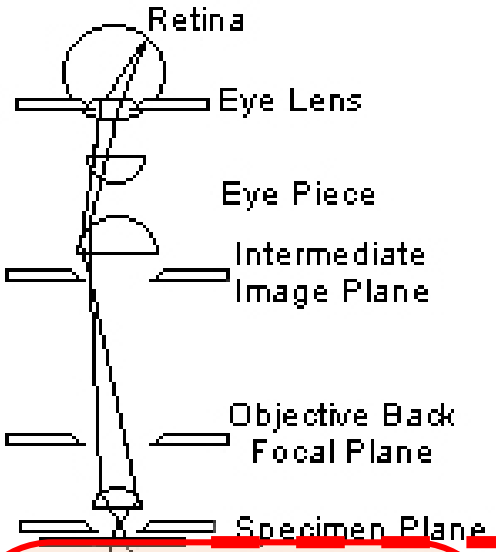
➔ But the images are unevenly and dimly illuminated.



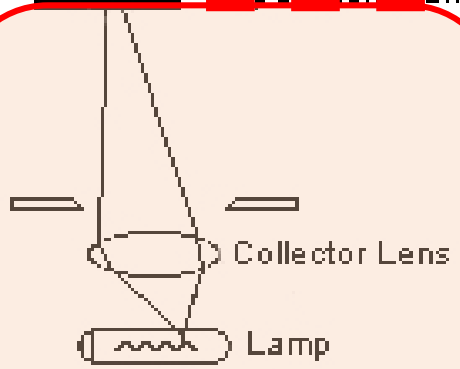
August Koehler
1866-1948

- August Koehler introduced a new method of illumination that greatly improved image quality in light microscopy.
- Koehler introduced the system in 1893 while he was a university student and instructor at the Zoological Institute in Giessen, Germany, where he performed photomicrography for taxonomic studies on limpets.

Critical vs Kohler

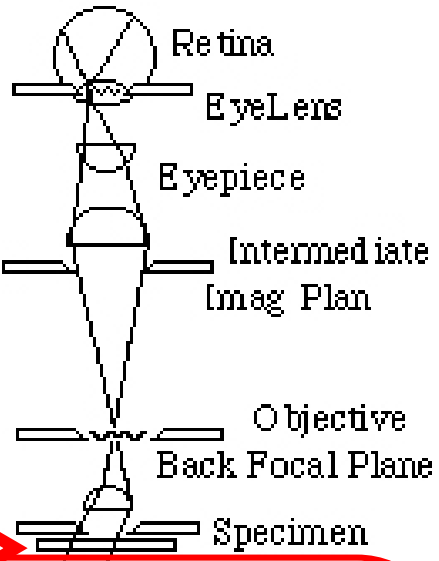


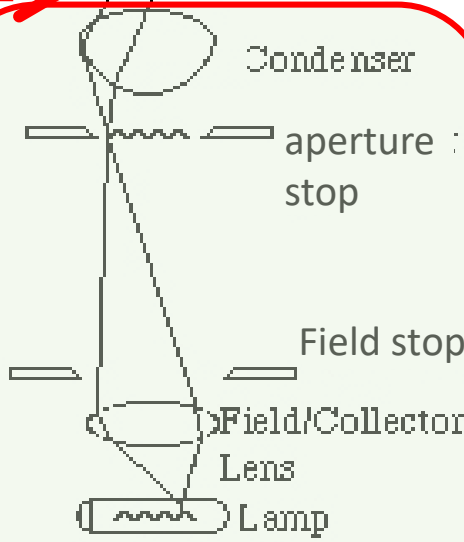
← Critical illumination: Focuses the lamp directly on the specimen.



Critical or Nelsonian Illumination

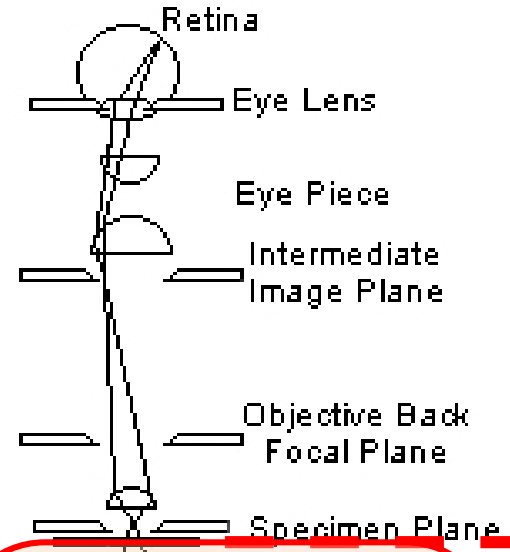
Kohler illumination → Uses **collector-condenser pair** and "defocuses" the light at the specimen plane.

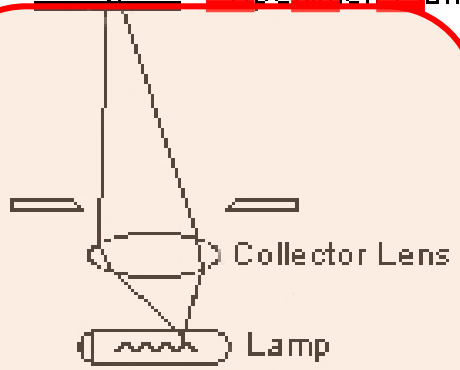




Köhler Illumination

Critical vs Kohler



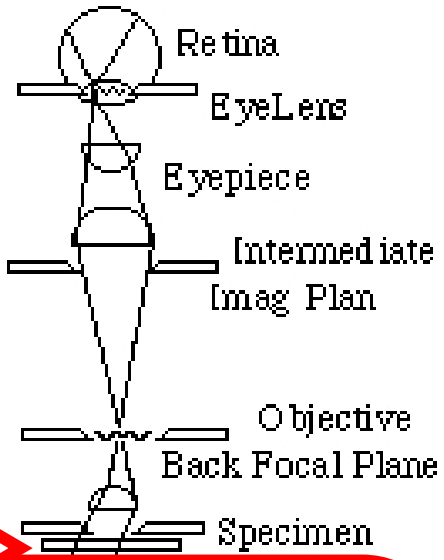


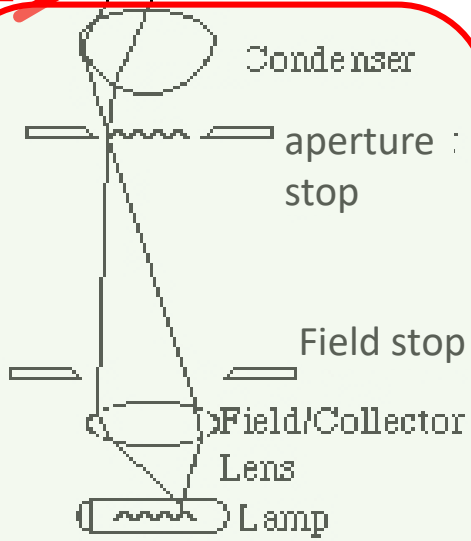
Critical or Nelsonian Illumination

← Critical illumination:
Results in **uneven & dim illumination**

Kohler illumination →

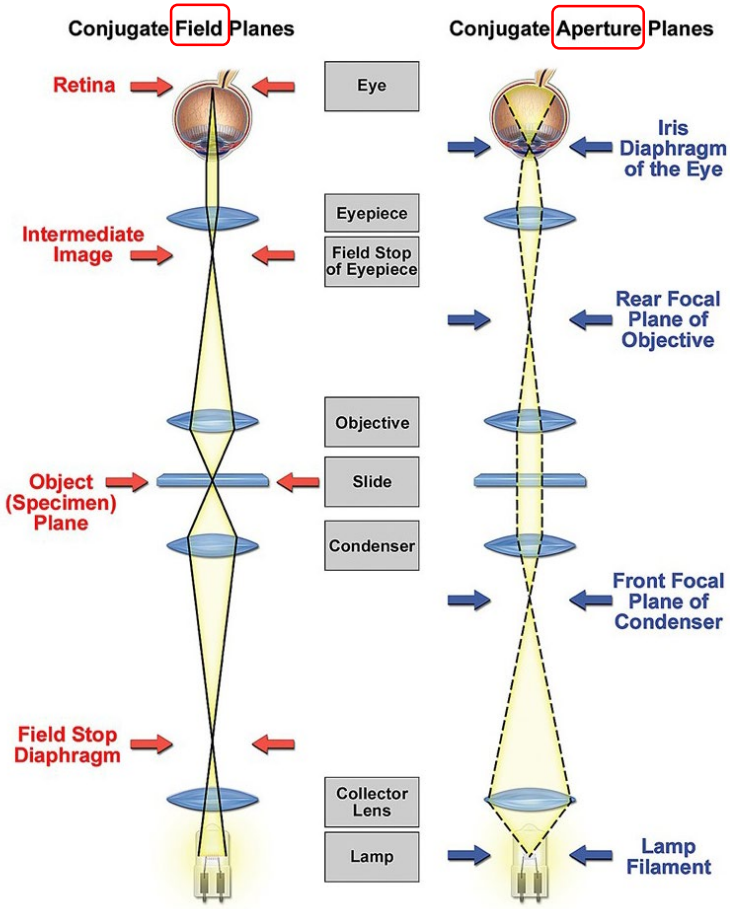
- **provides homogenous illumination**
- improves resolution and visibility
- minimizes stray light and unnecessary radiation





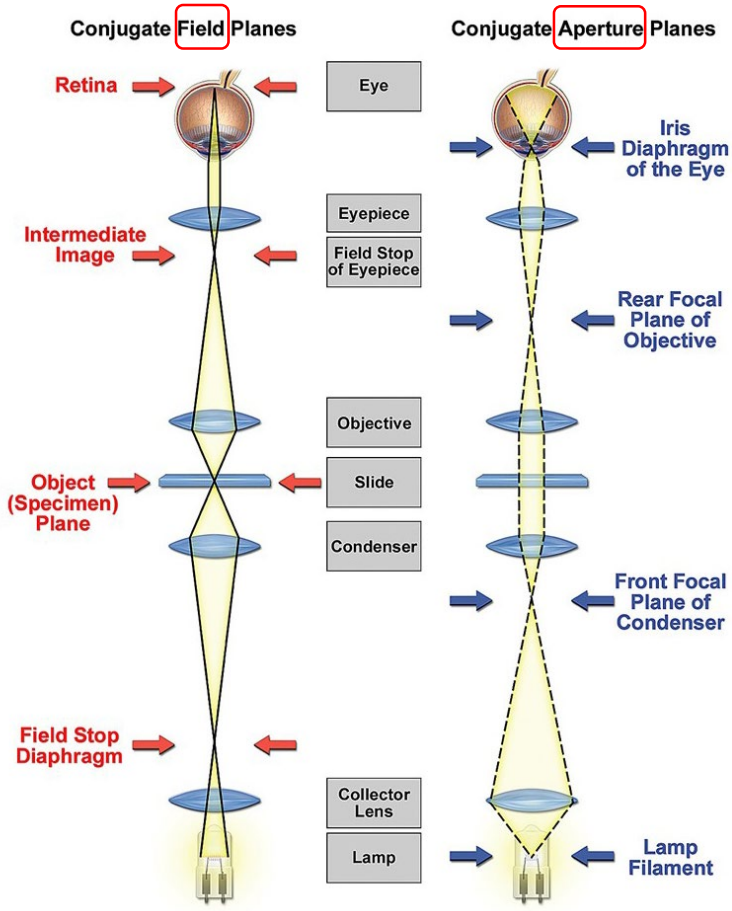
Köhler Illumination

"Kohler" illumination highlights the special relationship between two sets of planes in the microscope's light path:



CONJUGATE PLANES

- **Microscope has two sets of interlaced conjugated focal planes:**
  a set of 4 **field (or object) planes**
  a set of 4 **aperture planes**.
- Each plane within a set is conjugated with the other three planes of it's set.
- Let's check the locations of these planes?

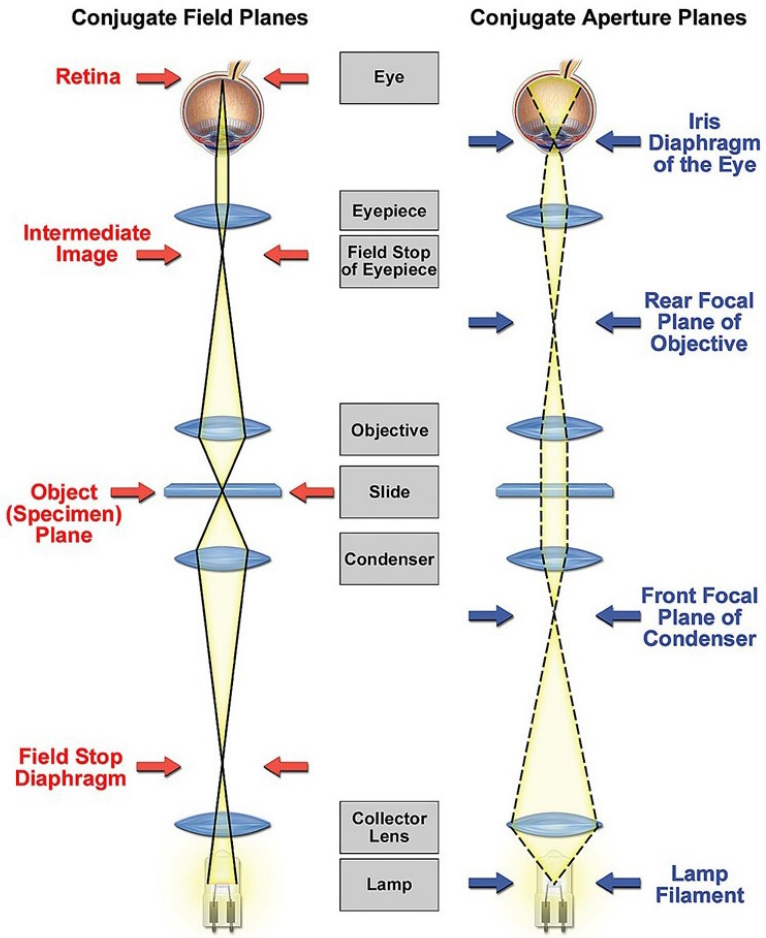


- **Arrows mark four conjugated focal planes.**

The locations of the **conjugate planes** are at the **crossing points of the rays in the optical train diagram**.

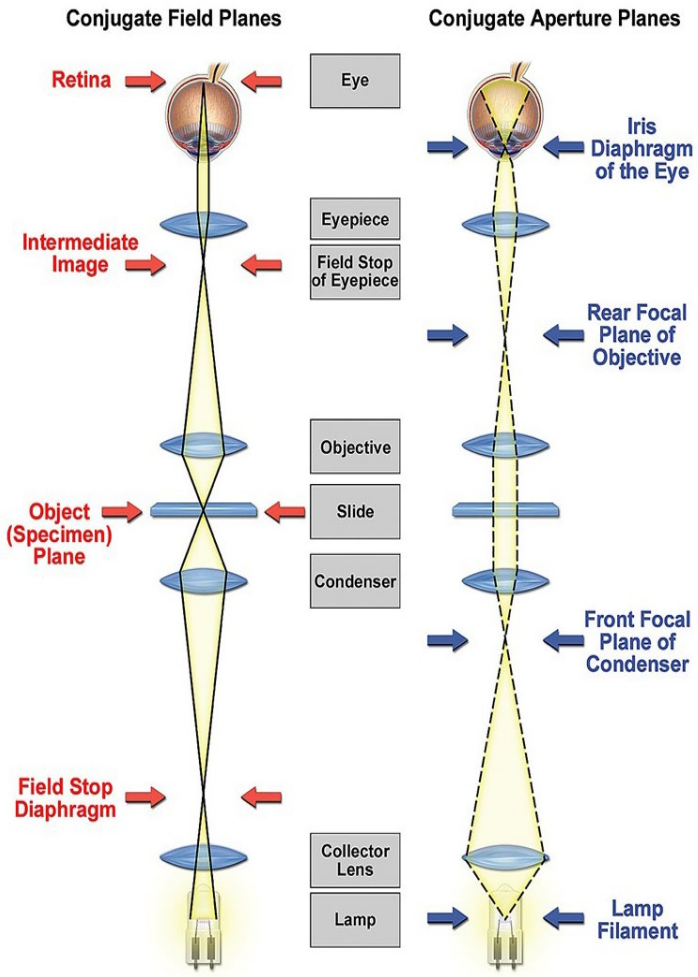
- **Red arrows (left)** shows that the "**object**" plane is conjugated with the "real intermediate image" plane in the eyepiece, the "retina" of the eye, and the "field stop diaphragm" between the lamp and the condenser.

- **Blue arrows (right)** shows that the "**lamp filament**" is conjugated with the "aperture stop" at the front focal plane of the condenser, the "rear focal plane" of the objective, and the "iris diaphragm" of the eye.



CONJUGATE PLANES

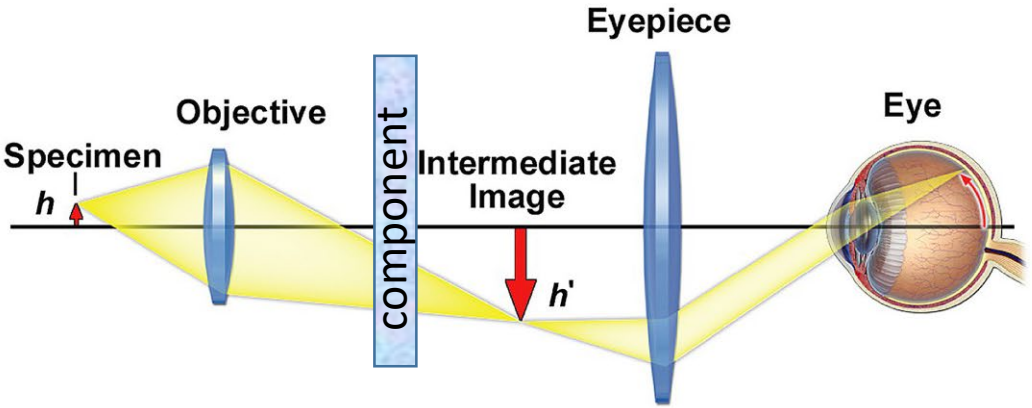
- **Each plane within a set is conjugated with the other planes, thus all of the planes of a given set can be seen simultaneously when looking in the microscope.**
- The "simultaneous visibility" of conjugated focal planes can be understood by the following example:
  - Consider an image of a dirt is visible with the focused specimen. This "dirt" could lie in any one of the four field planes of the microscope: as floaters near the retina, as dirt on an eyepiece field stop, as dirt on the specimen itself, and as dirt on the glass plate covering the field stop diaphragm.
- With the knowledge of the conjugated field planes locations, one can find the location of the "dirt" by (1) rotating eyepiece field stop, (2) moving microscope slide, or (3) wiping the field diaphragm cover plate.



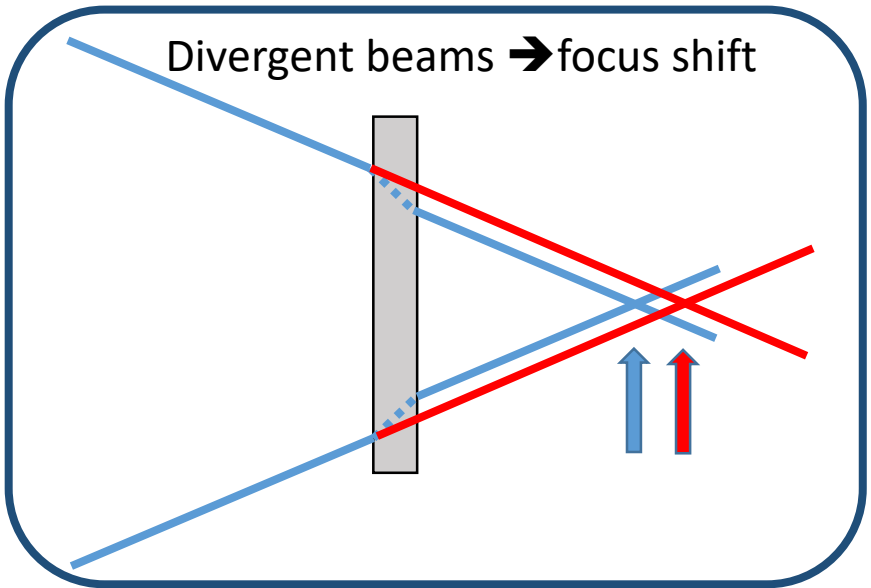
- These two sets of conjugate planes are interdigitated with one another.
- Optically, they are reciprocal or Fourier transform planes with each other.

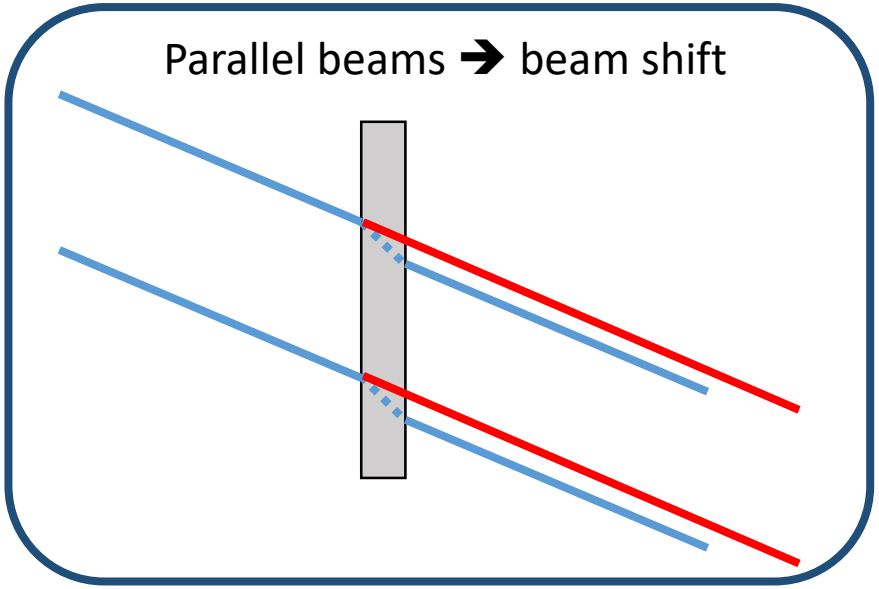
➔ **This means when something is “in-focus” in one set, it is “maximally out-of-focus” in the other.**

Limitations of conventional compound microscope

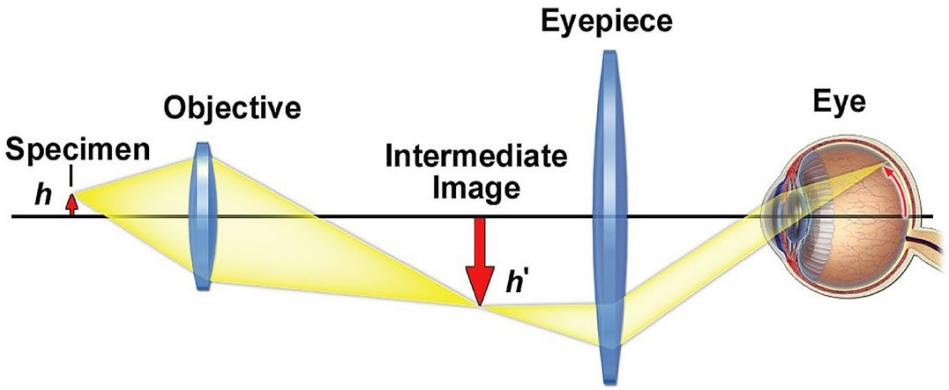


- Adding optical accessories such as polarizers, filters, and DIC prisms into the light path can introduce spherical aberration and “ ghost images ” into an otherwise perfectly corrected optical system.
- To circumvent these artifacts, Reichert (microscope manufacturer based in Vienna) pioneered the concept of *infinity optics*.
- The company started experimenting with infinity–corrected optical systems in early 1930s, but this concept did not become standard for most manufacturers until 50 years later.

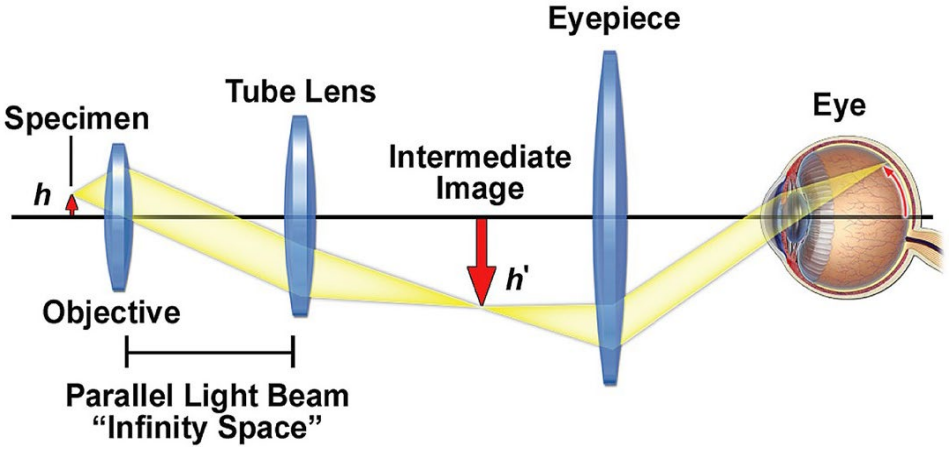




Infinity Corrected Compound Microscope



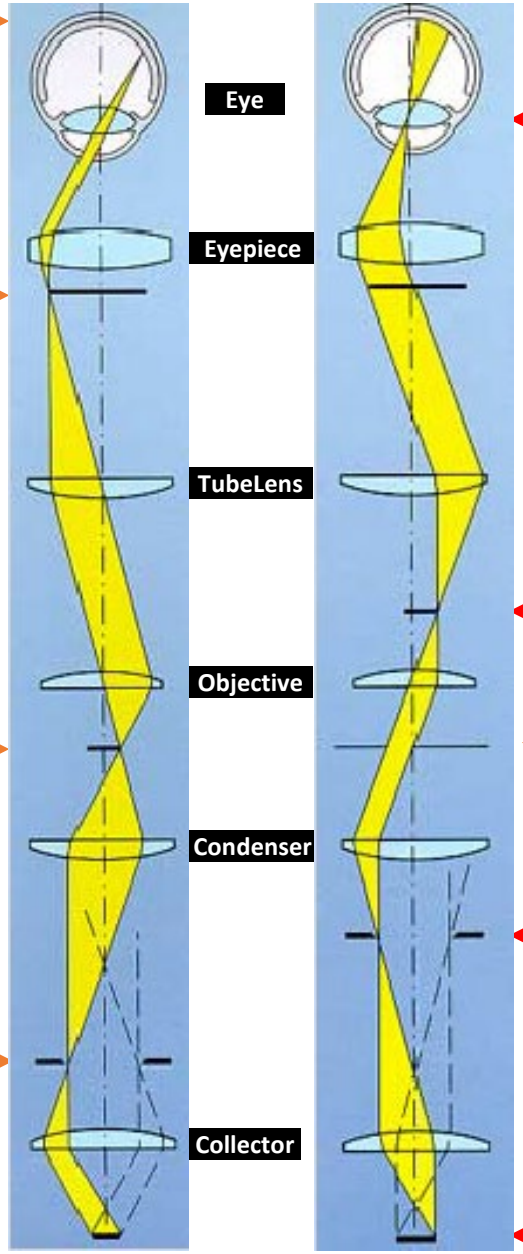
(a) finite corrected microscope configuration



(b) infinite corrected microscope configuration

- Infinity optical systems have a different objective design that produces a flux of parallel light wavefronts imaged at infinity, which are then brought into focus at the intermediate image plane by a special optic termed *tube* lens.
- The region between the objective rear aperture and the tube lens is called *infinity space*, where auxiliary components can be introduced into the light path without producing r optical aberrations.

Conjugate Planes for Infinity Corrected Microscope



Retina

Intermediate Image

Specimen

Field Diaphragm

Eyepoint

Objective Back Focal Plane

Specimen

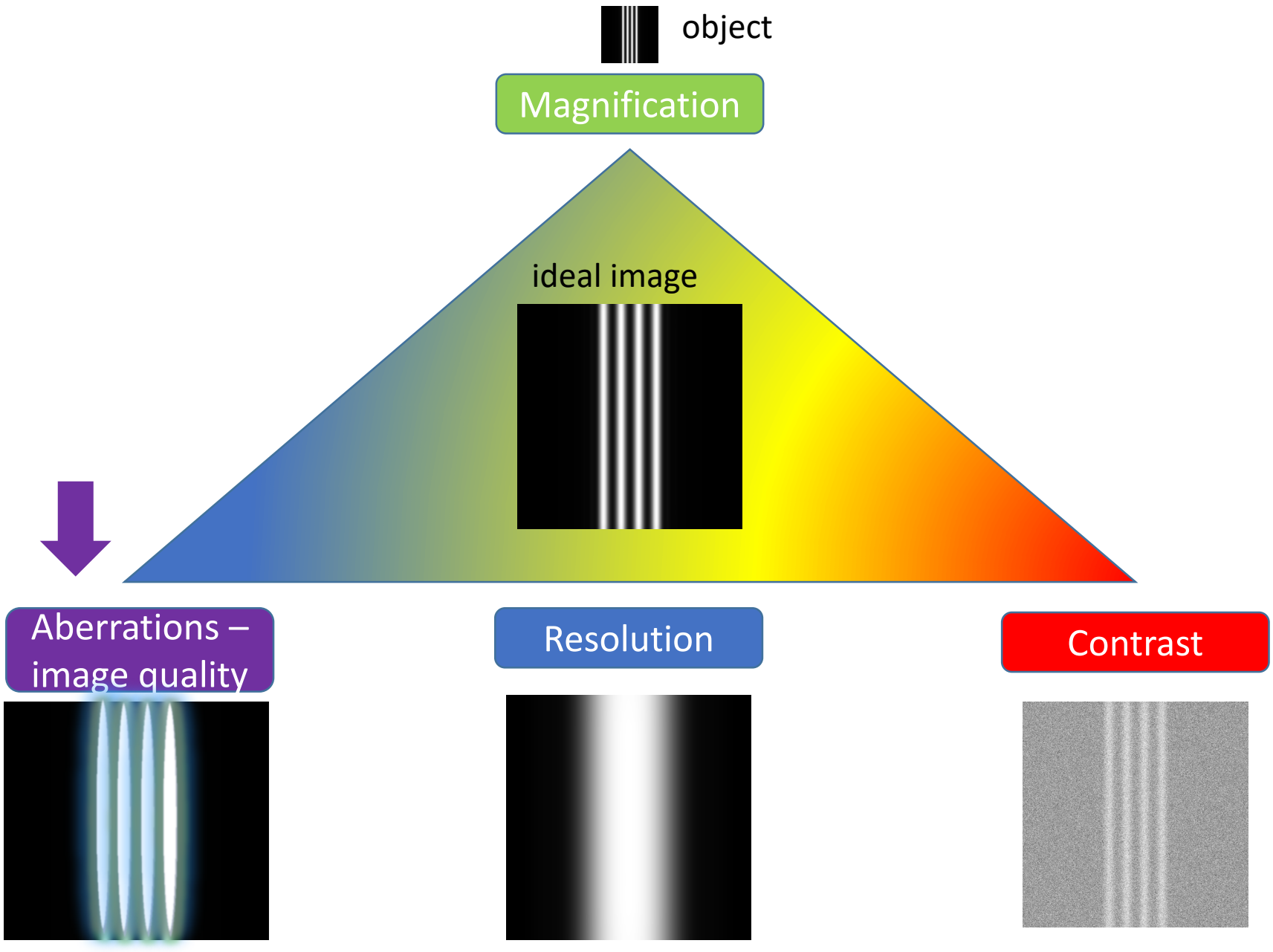
Condenser Aperture Diaphragm

Light Source

Conjugated "Field" Planes:
Concerns field diaphragm & specimen in the imaging path

Conjugated "Aperture" Planes:
Concerns aperture diaphragm & light source in the illumination path

Important aspects for microscopy



Major Optical Aberrations in Microscopy

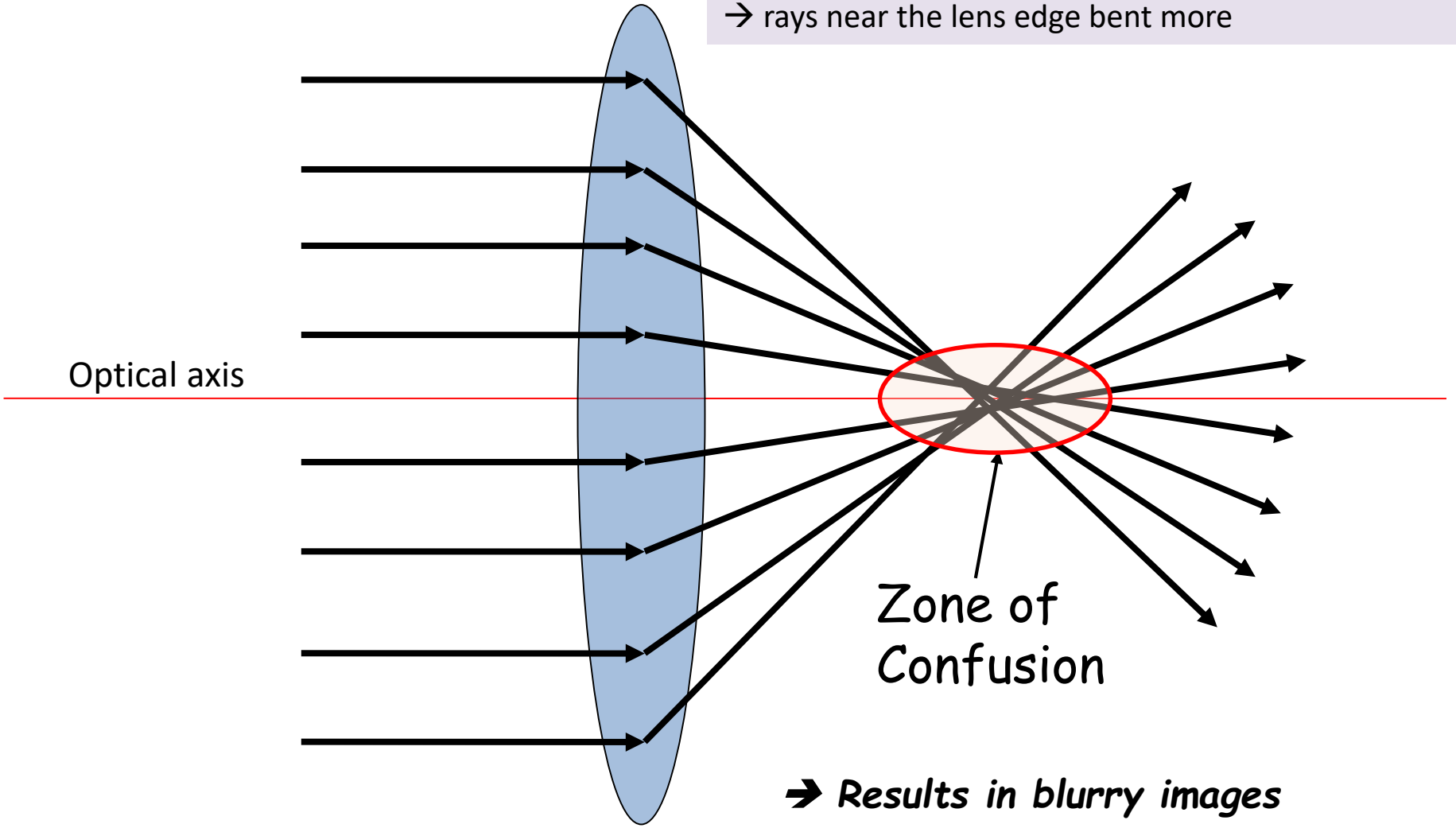
- The major six aberrations are:
  - Spherical aberration
  - Chromatic aberration
  - Coma
  - Astigmatism
  - Curvature of field
  - Distortion

Recall: Spherical Aberration

Incident rays parallel to the optical axis are focused at different locations depending on they are closer to the center and the periphery of the lens.

Spherical aberration gets worse near the lens edges:

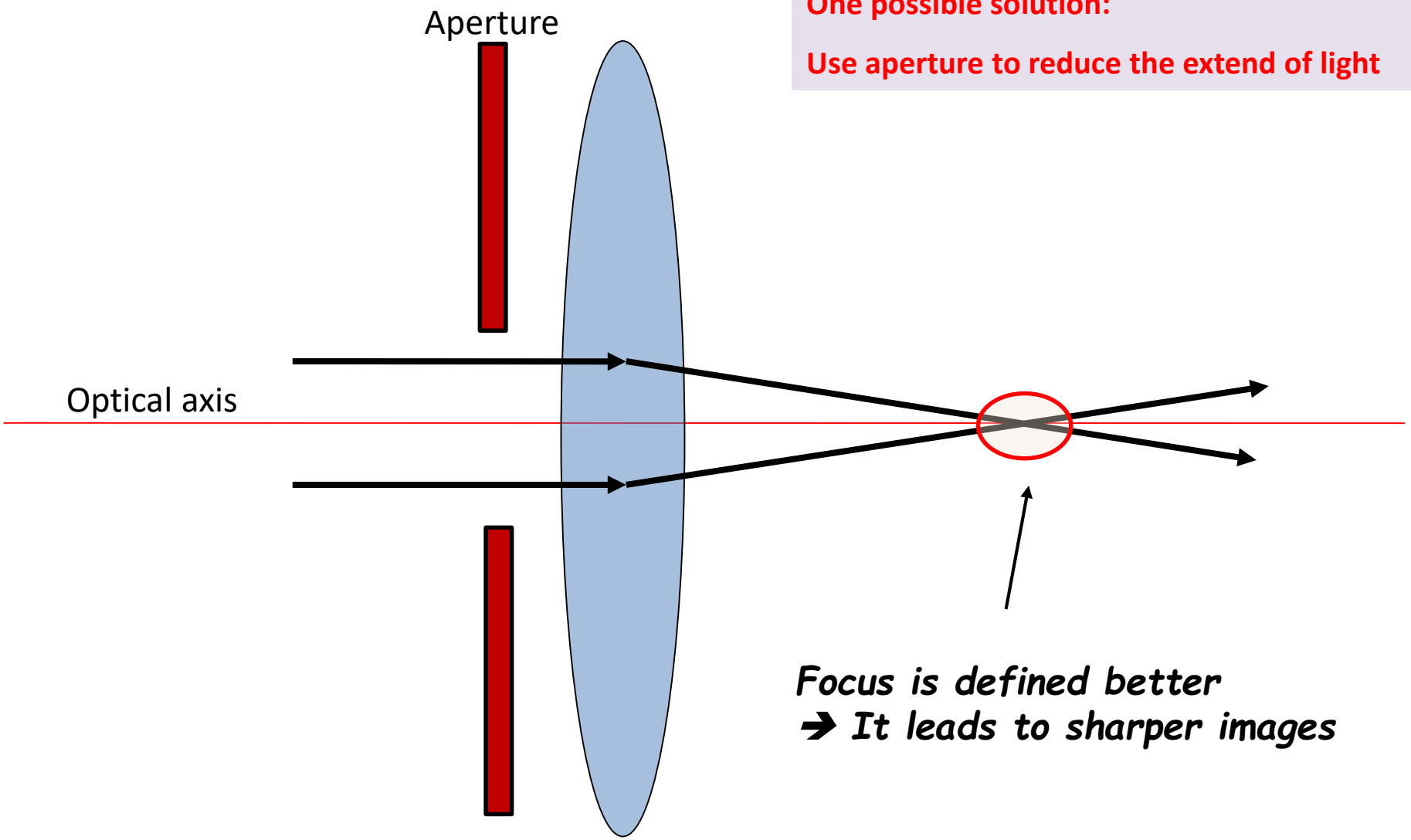
→ rays near the lens edge bent more



➔ ***Results in blurry images***

Recall: Spherical Aberration

Incident rays parallel to the optical axis and reaching the center and the periphery of the lens are focused at different locations.



One possible solution:

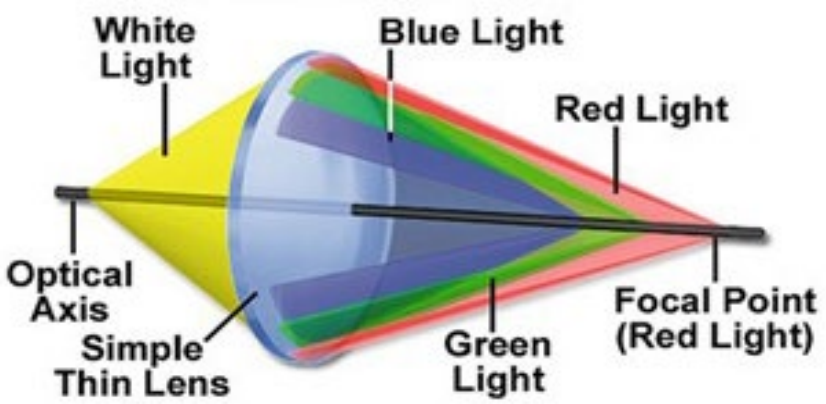
Use aperture to reduce the extend of light

Focus is defined better
➔ It leads to sharper images

Major Optical Aberrations in Microscopy

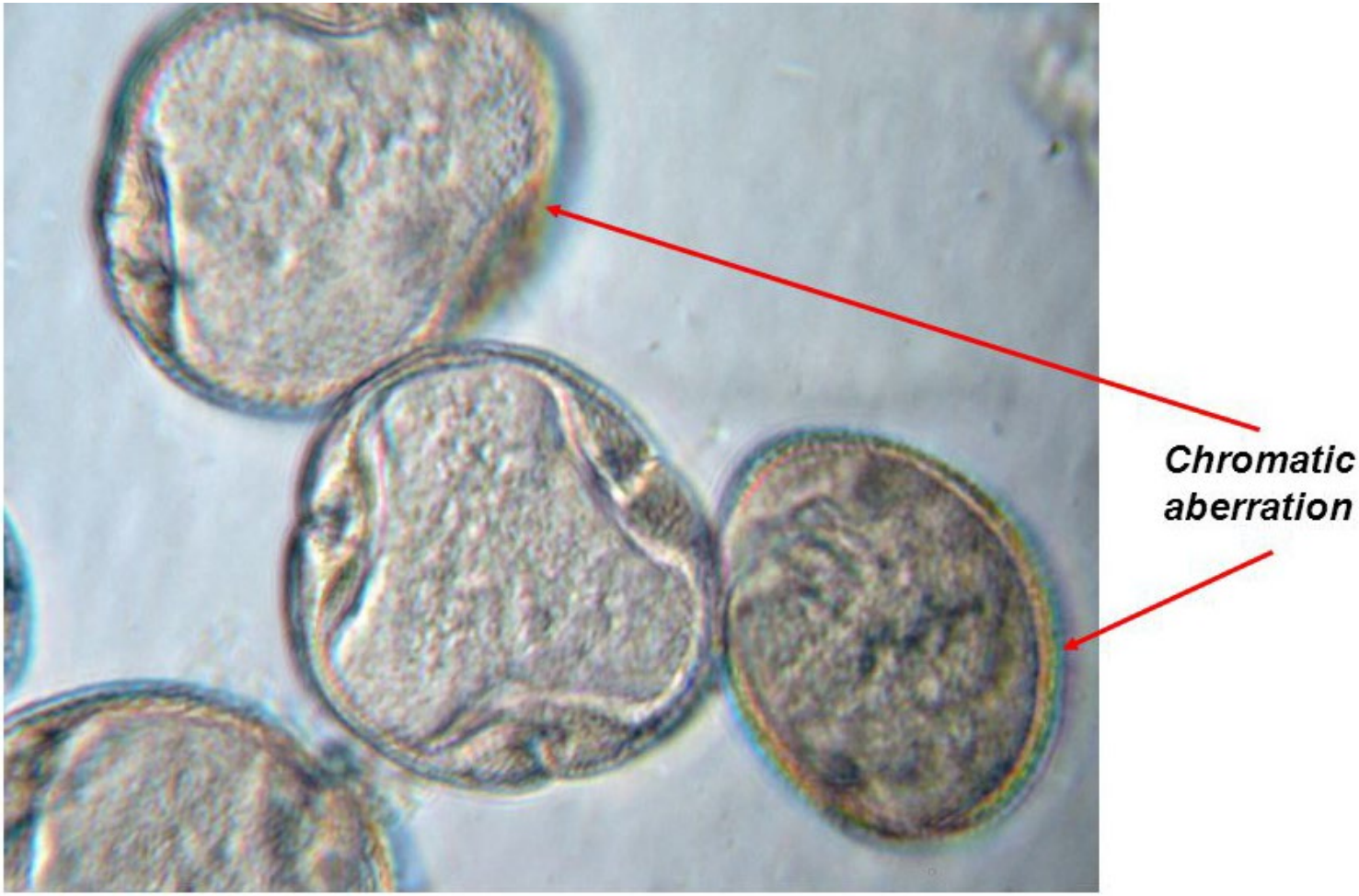
- The major six aberrations are:
  - Spherical aberration
  - Chromatic aberration
  - Coma
  - Astigmatism
  - Curvature of field
  - Distortion

2. Chromatic aberration



- *Chromatic aberration* occurs because a lens refracts light differently, depending on the wavelength.
- Blue light is bent inward toward the optical axis more than red light ➔ this results in focusing of blue wavelengths in an image plane closer to the lens than that for red wavelengths.
- Even at the best focus, **point sources are surrounded by color halos**. The colors change depending on the focus of the objective and the image never becomes sharp.
- Since each wavelength is focused at a different distance from the lens, there is also a difference in magnification for different colors (**chromatic magnification difference**).

Example: Chromatic aberration

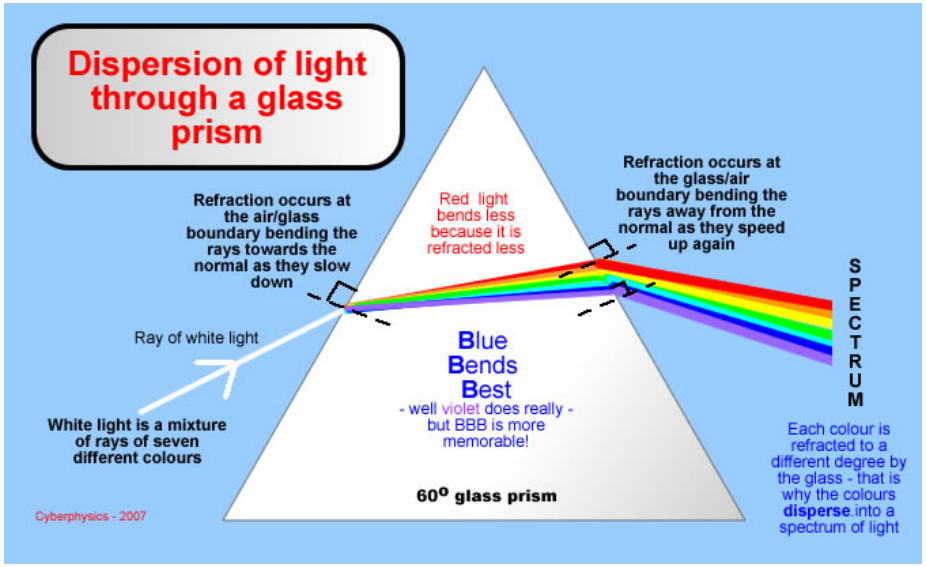


Tilia (European Lime) pollen X60 planapochromat objective Canon G9 super widefield eyepiece, showing chromatic aberration (colour fringing)

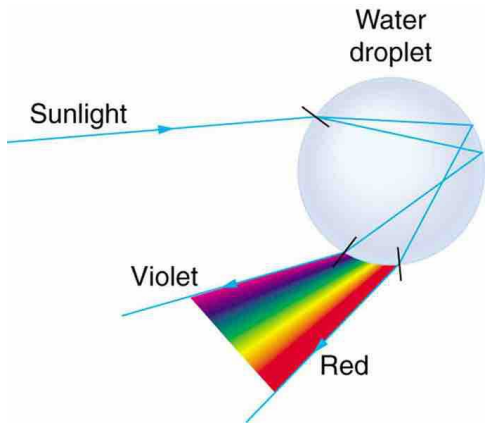
Material Dispersion – n(λ)

Disperses the different **wavelengths** of white light

Material	**Blue** (486nm)	**Yellow** (589nm)	**Red** (656nm)
Crown Glass	1.524	1.517	1.515
Flint Glass	1.639	1.627	1.622
Water	1.337	1.333	1.331
Cargille Oil	1.530	1.520	1.516



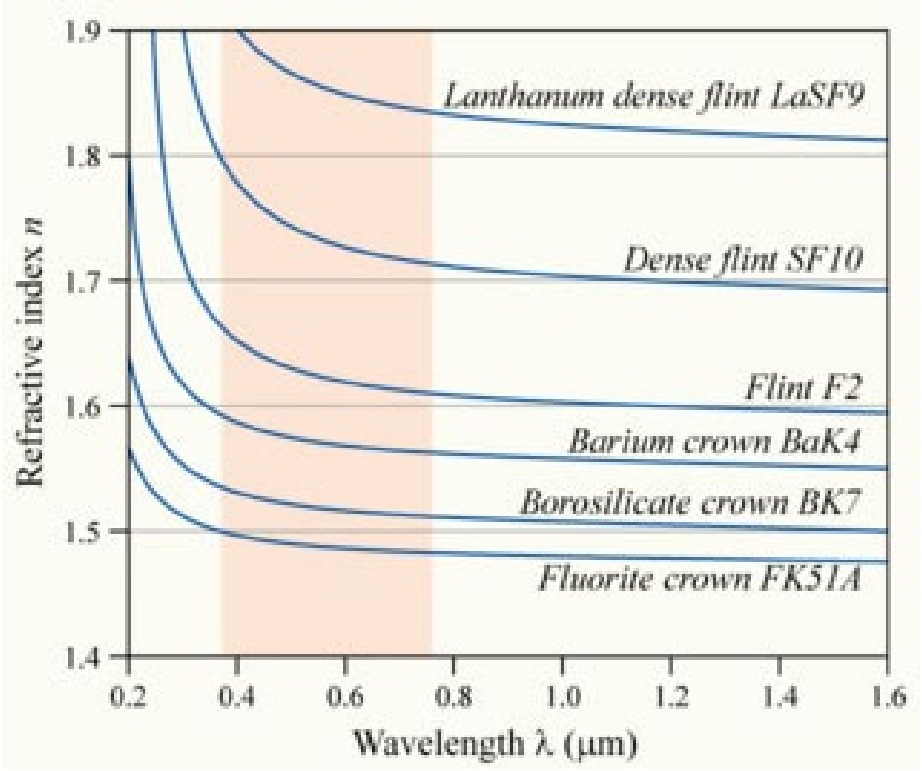
Example for dispersion:

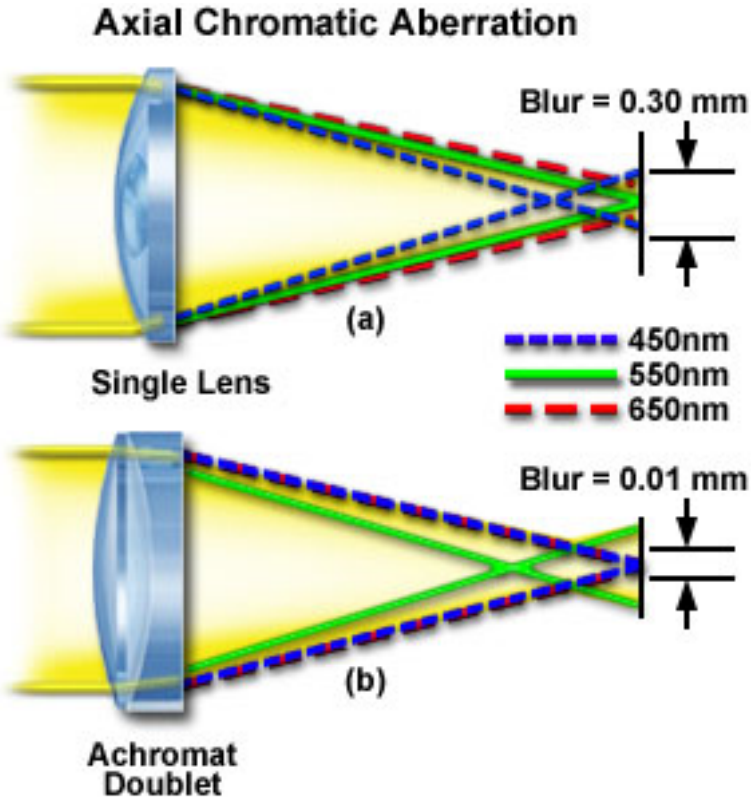


Correcting chromatic aberration

- One solution is to make compound lenses made of glasses having different color-dispersing properties.

➔ For example, glass types known as crown and flint are paired together to make an achromatic doublet lens that focuses blue and red wavelengths in the same image plane.

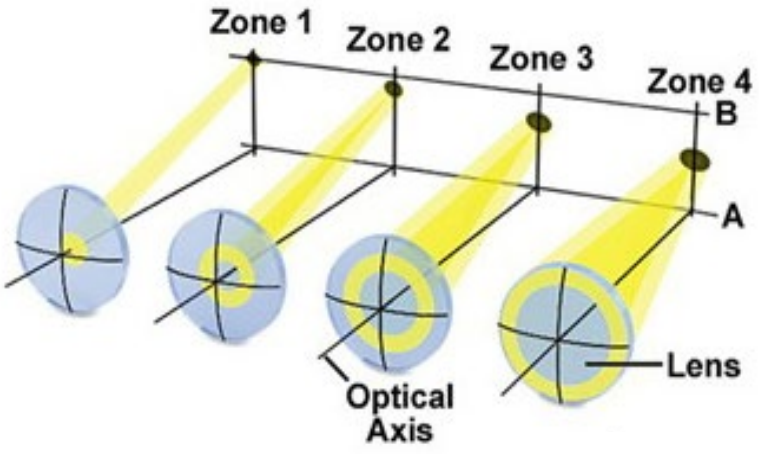


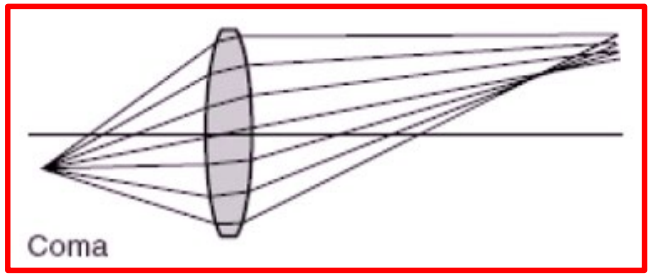


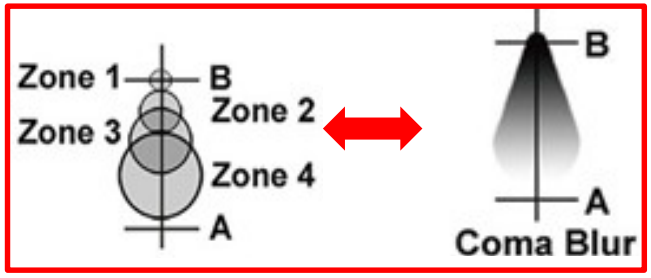
Major Optical Aberrations in Microscopy

- The major six aberrations are:
  - Spherical aberration
  - Chromatic aberration
  - Coma
  - Astigmatism
  - Curvature of field
  - Distortion

3. Coma aberration







- *Coma is the most prominent* **"off-axis"** aberration.
- It affects the images of points located far away from the optical axis—that is, when the *object rays hit the lens* ***obliquely***.

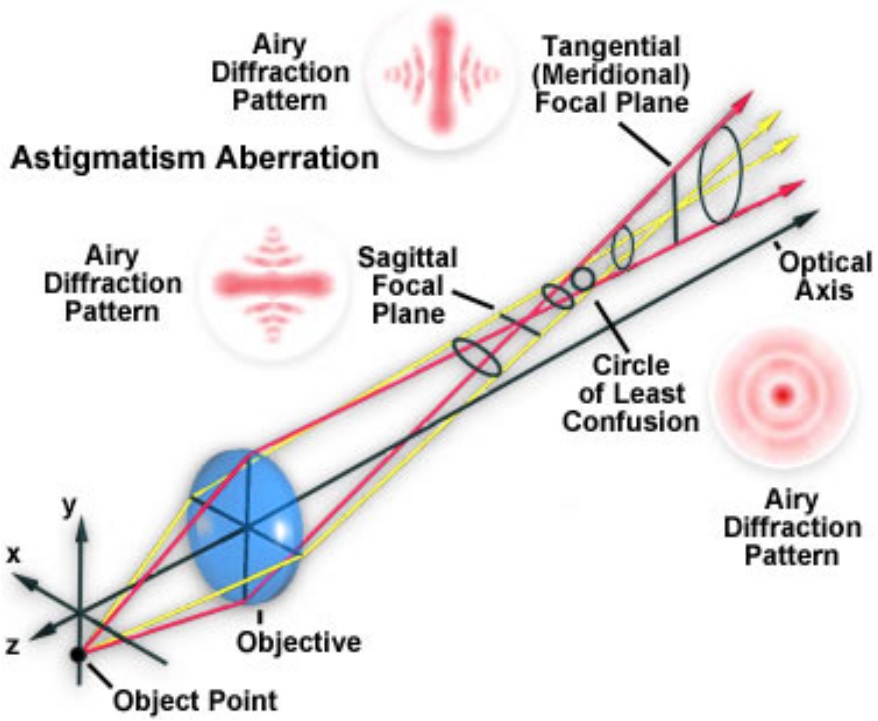
- Rays passing through the edge of the lens are focused at a different place than the rays passing through the center of the lens. This causes a point object to look like a comet with the tail extending toward the periphery of the field.

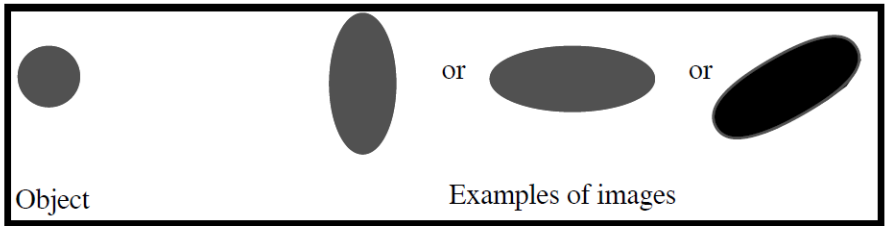
- Coma is greater for lenses with wider apertures.

Major Optical Aberrations in Microscopy

- The major six aberrations are:
  - Spherical aberration
  - Chromatic aberration
  - Coma
  - Astigmatism
  - Curvature of field
  - Distortion

4. Astigmatism aberration



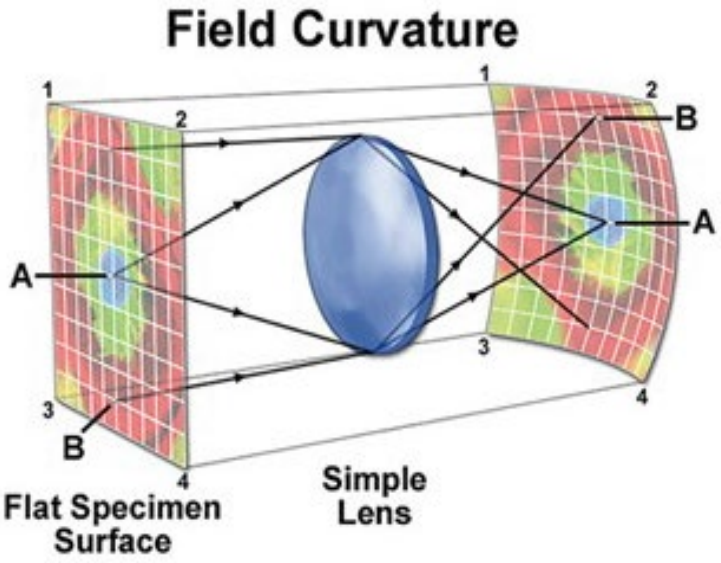


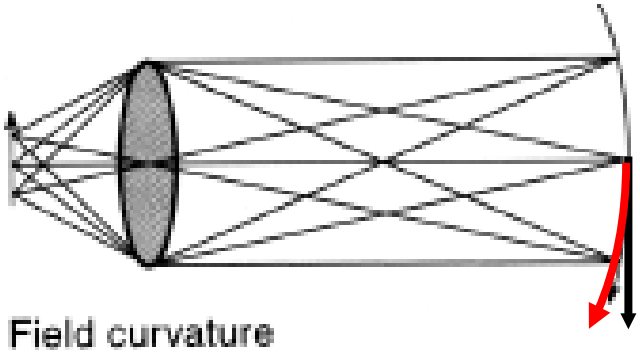
- *Astigmatism* , like coma, is an "**off-axis**" aberration.
- Rays from an object point passing through the horizontal diameter (yellow rays) and vertical diameter (red rays) of a lens are focused at two different focal planes.
- The point source appears as ellipse in horizontal and vertical directions at either side of "the least confusion" focus.

Major Optical Aberrations in Microscopy

- The major six aberrations are:
  - Spherical aberration
  - Chromatic aberration
  - Coma
  - Astigmatism
  - Curvature of field
  - Distortion

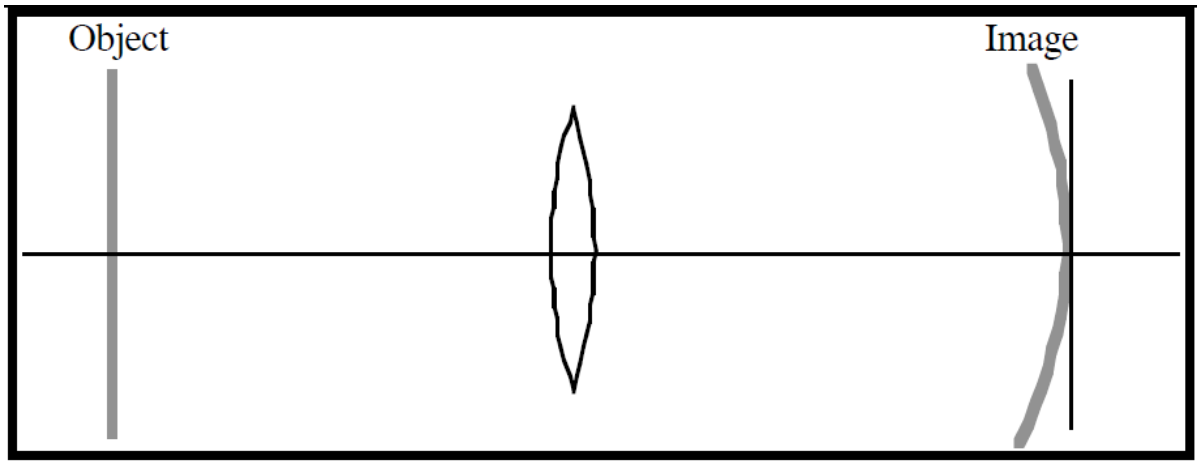
5. Curvature of field aberration



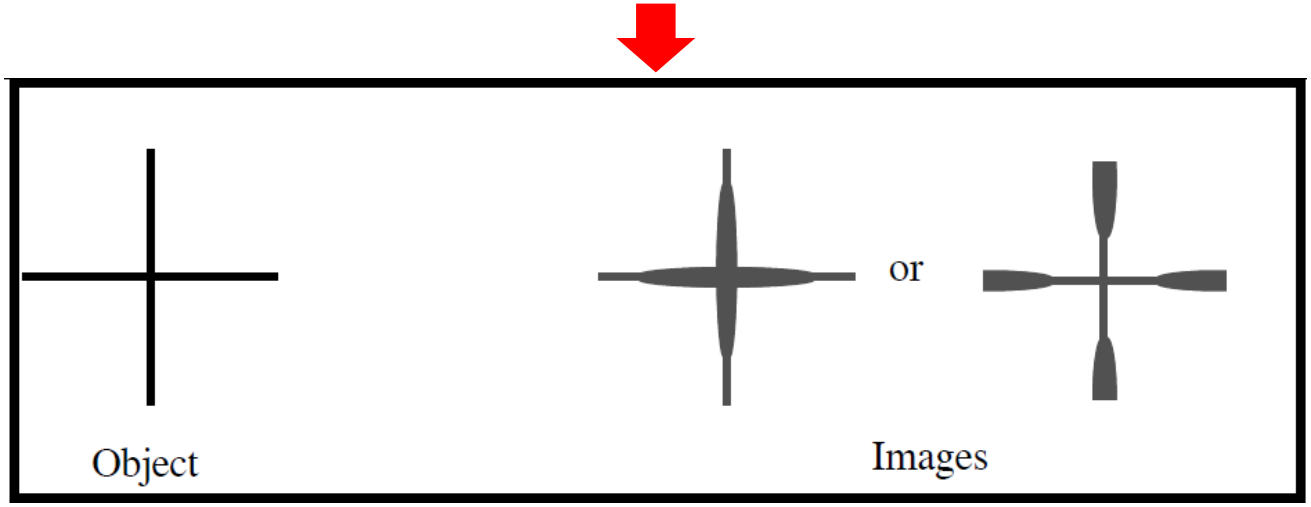


- *It* is an “off-axis” aberration.
- Field curvature indicates that the image plane is not flat. Instead, it has the shape of a concave spherical surface as seen from the objective. This creates a problem because the camera surface is flat thus the whole image cannot be focused simultaneously on a flat imaging surface.
- The aberration gets worse near the lens edges.

Curvature of field aberration



- The image of a plane object is located on a curved surface.
- The effect is that if we look at the images formed in a plane perpendicular to the principal axis, part of each image will be out of focus.
- **If we adjust the part of the image near the edges for good focus then the central part will be fuzzy.**
- **If we adjust the part of the image near the axis for good focus then the edges will be out of focus.**



Major Optical Aberrations in Microscopy

- The major six aberrations are:
  - Spherical aberration
  - Chromatic aberration
  - Coma
  - Astigmatism
  - Curvature of field
  - Distortion

6. Distortion aberration

Barrel Distortion

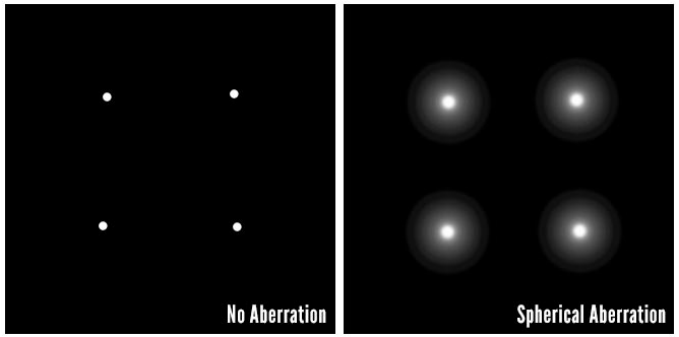
Pincushion Distortion

- It is an aberration that causes the focus position of the object image to shift laterally in the image plane with increasing displacement of the object from the optical axis.
- The consequence of distortion is a nonlinear magnification in the image from the center to the periphery of the field.
- Depending on whether the gradient in magnification is increasing or decreasing, the aberration is called as barrel or pincushion distortion:

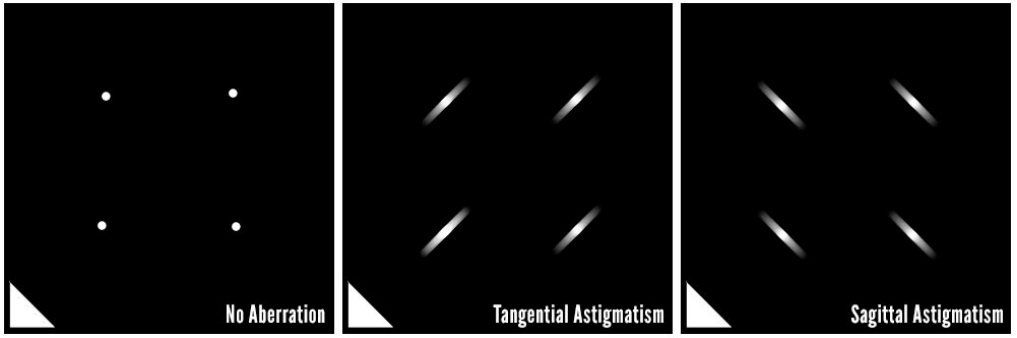
Example above shows the effect for a specimen with straight lines, such as grid lines with a pattern of squares or rectangles.

Aberrations - examples

Spherical aberration



Astigmatism



Coma