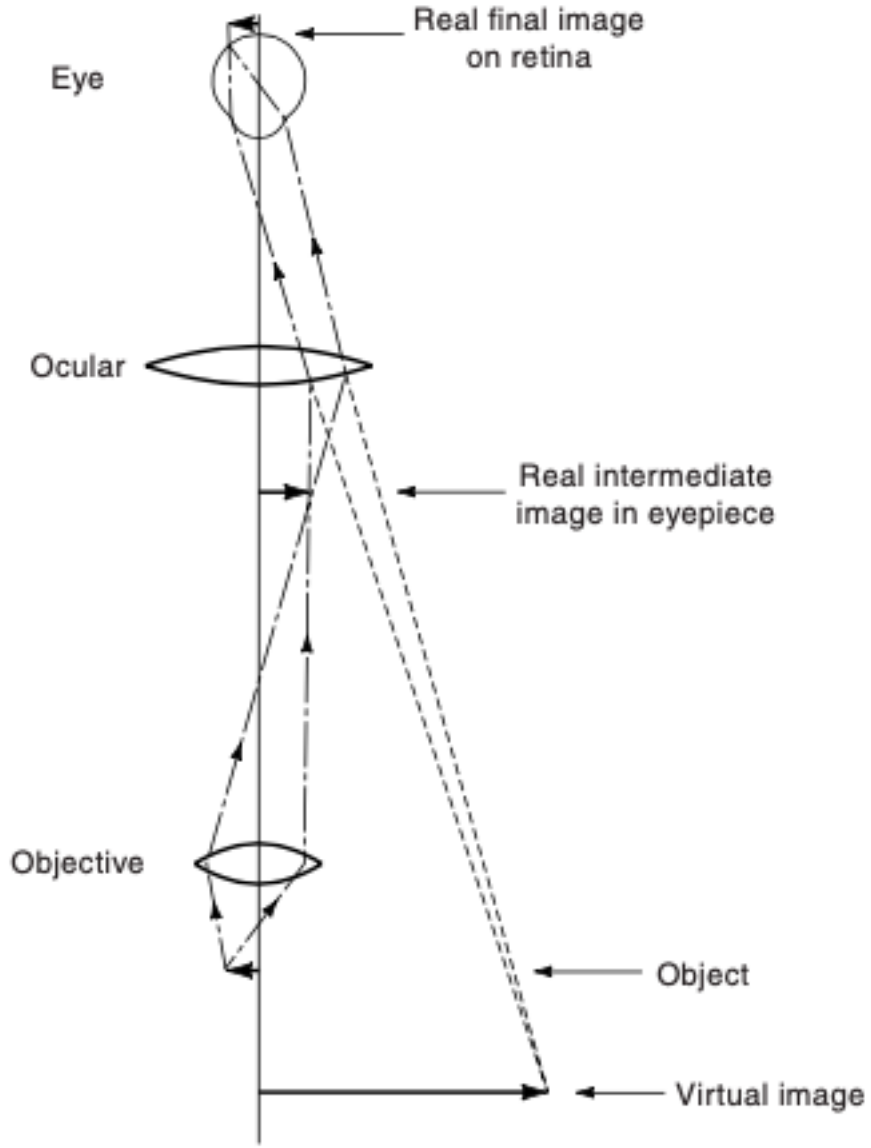
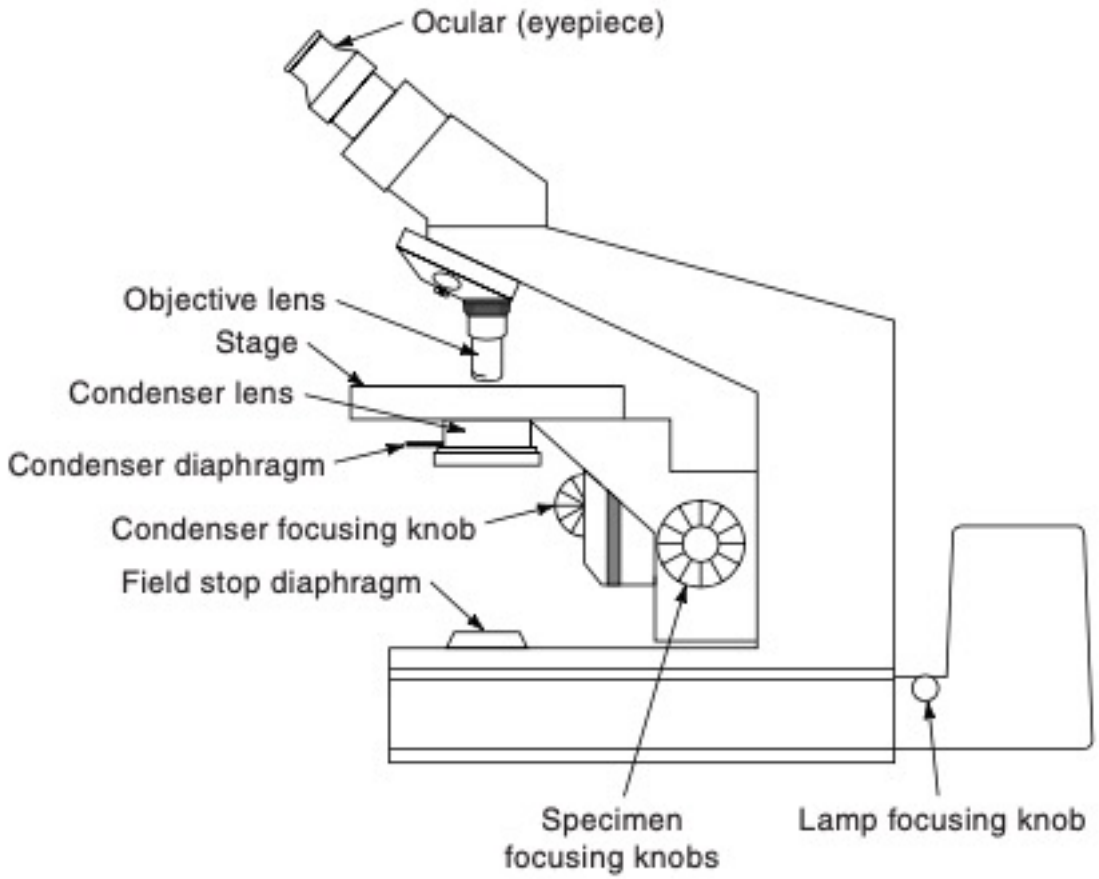
Microscope

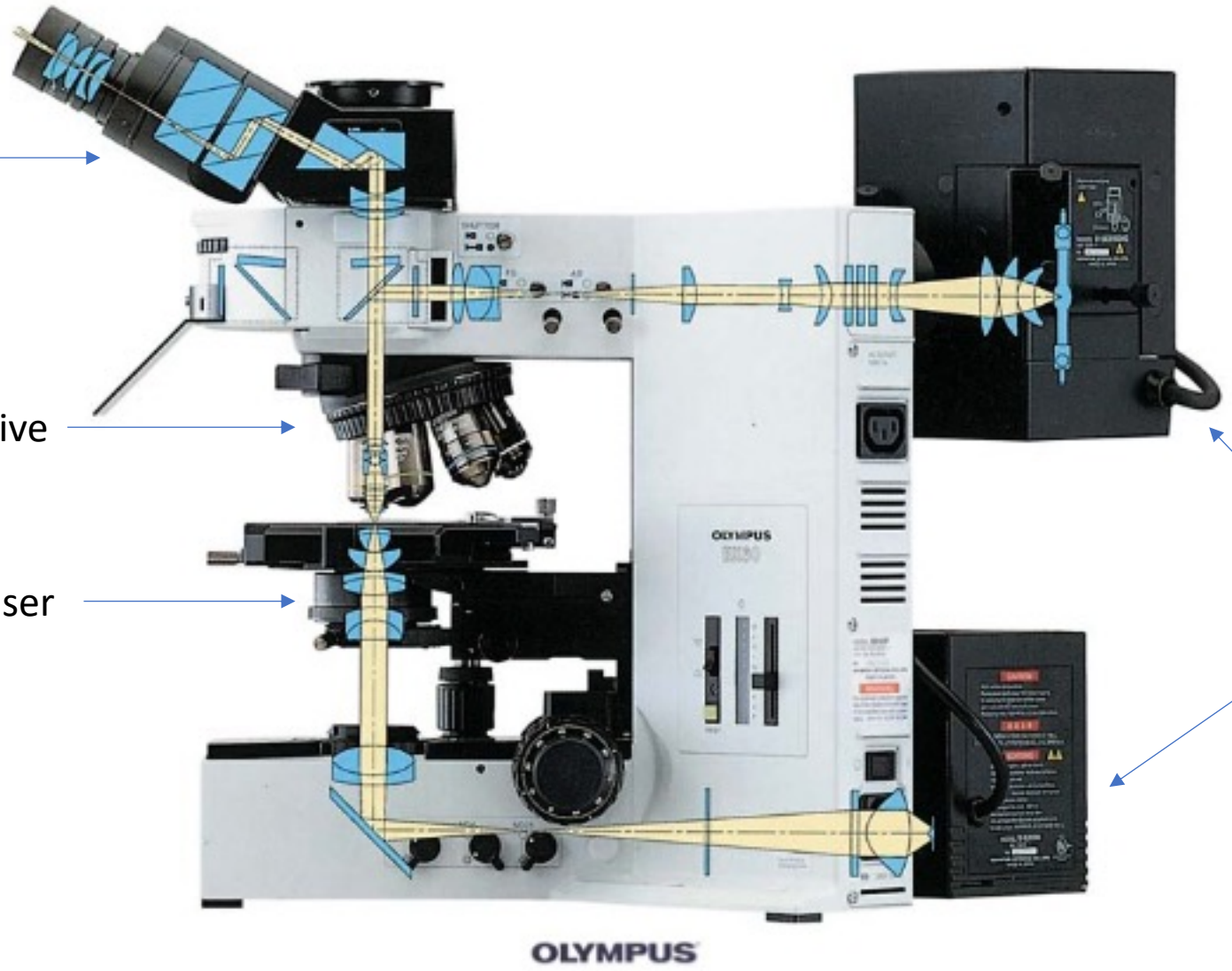
Parts and components

Ray diagram of a light microscope

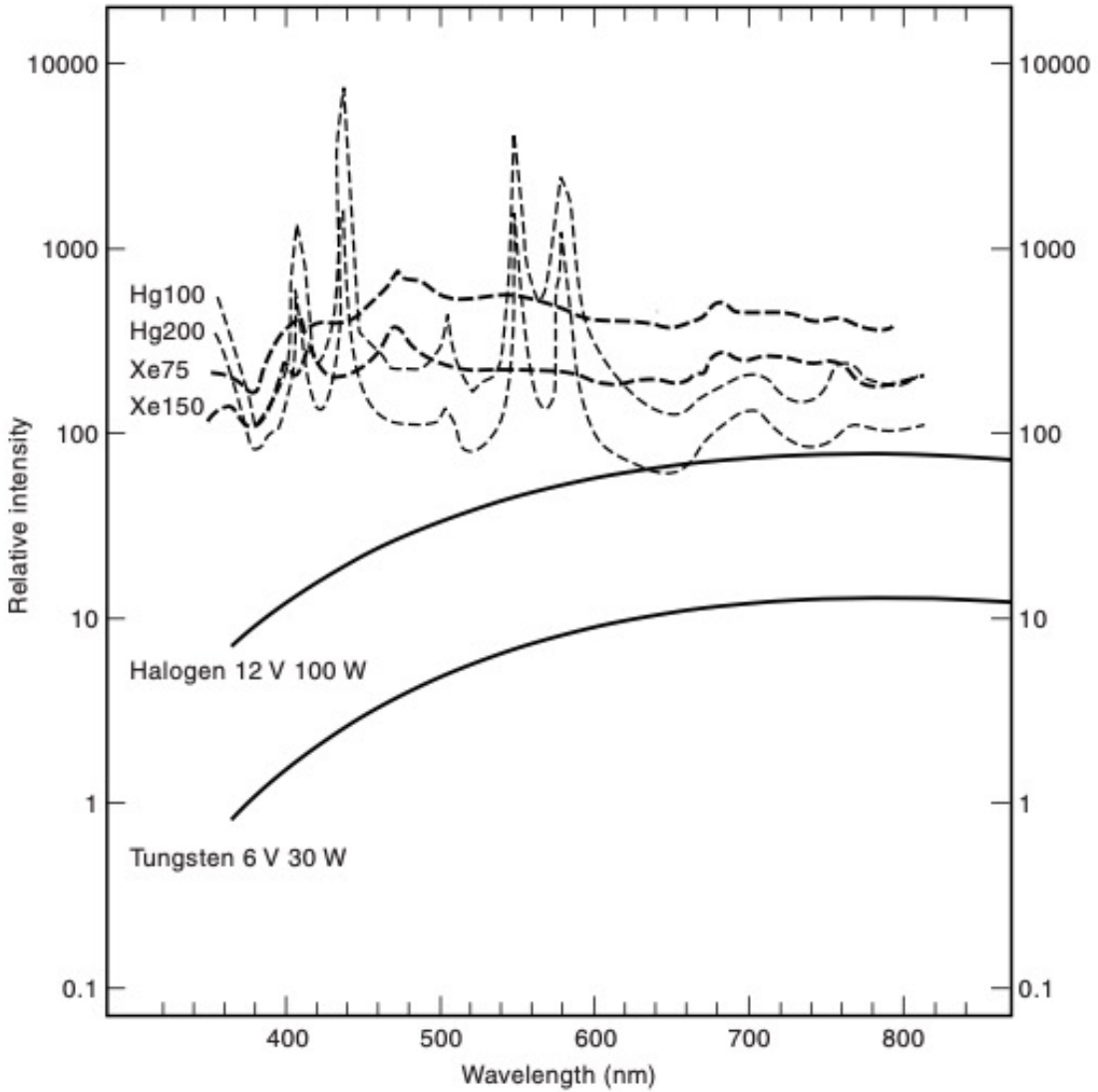




Modern Metallurgical Microscope



Illumination sub-system



- Heated elements are among the most basic light emitters
- Ion arc lamps (Xe/Hg) are 10 – 100 times brighter
- However, such ion emissions have some characteristic emission lines which requires specific notch filters.
- Typical lamps also have IR filters to avoid those light which just harm sensitive samples such as biological specimens.

Object Illumination

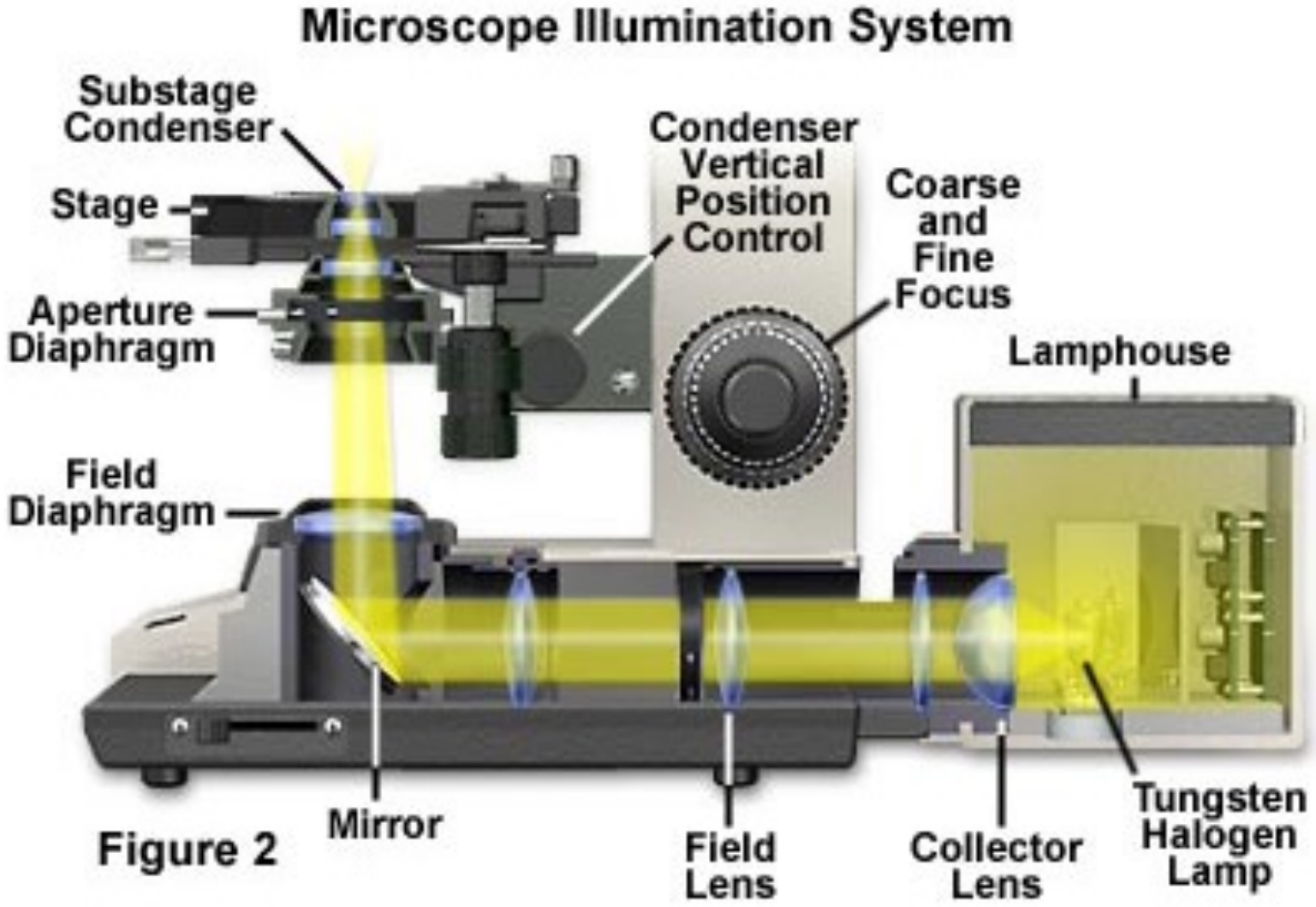
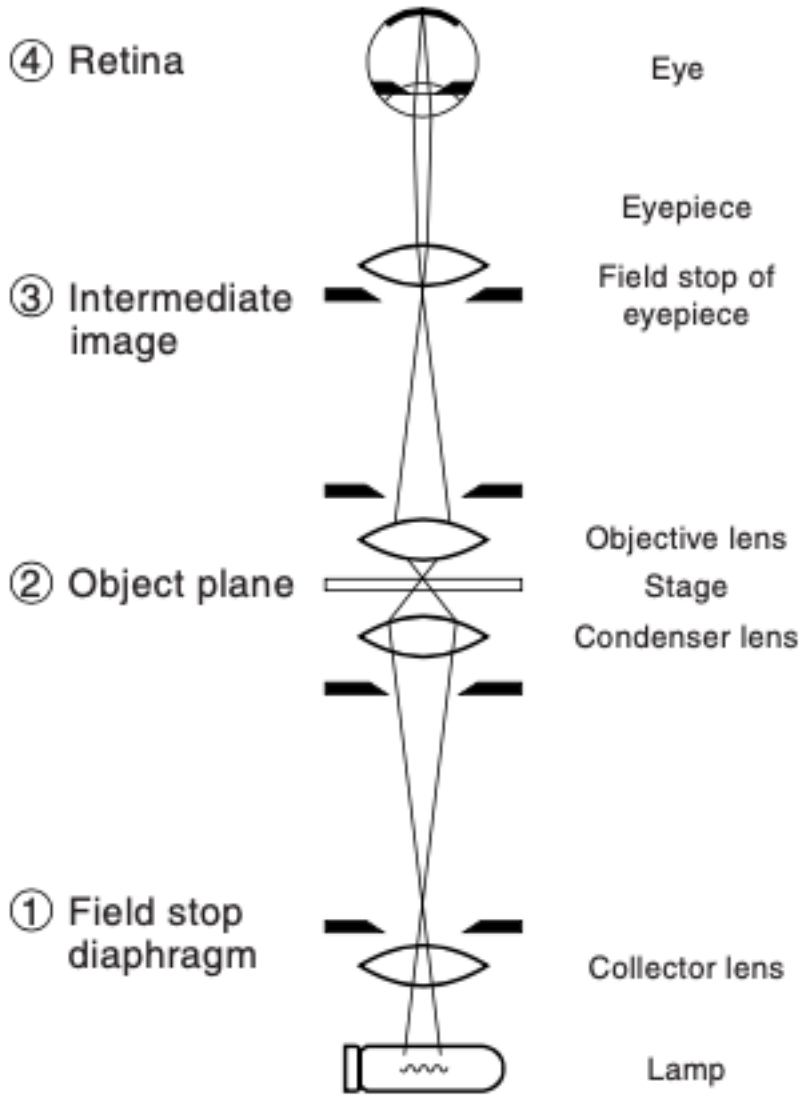
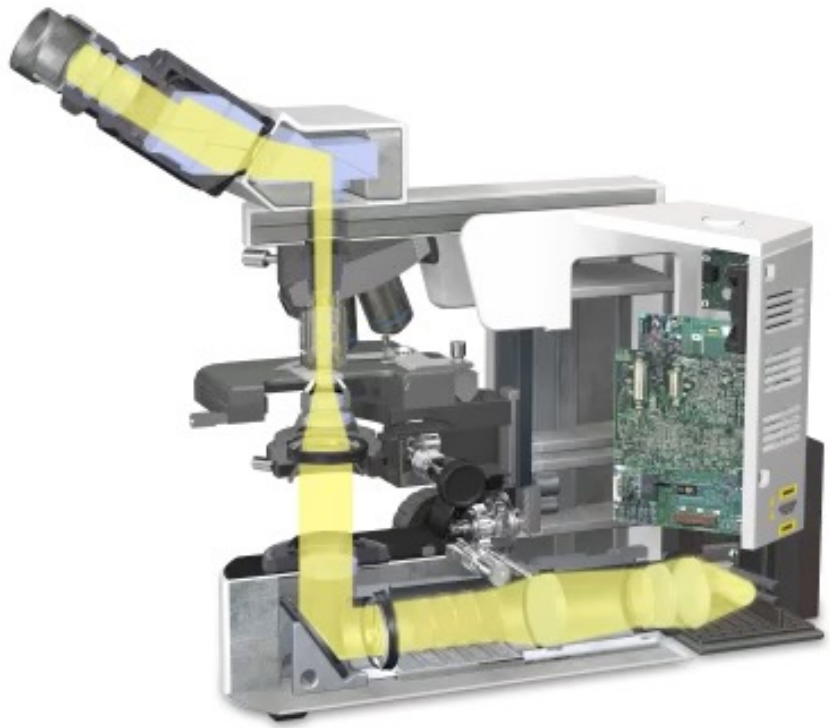


Figure 2

- Collector lens:
  - Has the lamp filament at its first focus
  - Forms a collimated beam of light with uniform intensity
- Field lens
  - Focusses the lamp light into the condenser
- Field Diaphragm:
  - Limits the intensity of light falling into the sample
- Condenser Aperture: (hole/opening in an opaque mask)
  - Controls the NA of the illumination



Transmitted Light Microscopy Optical Pathways



Outside

Field Diaphragm

Aperture Diaphragm

Illumination Intensity : 100 %

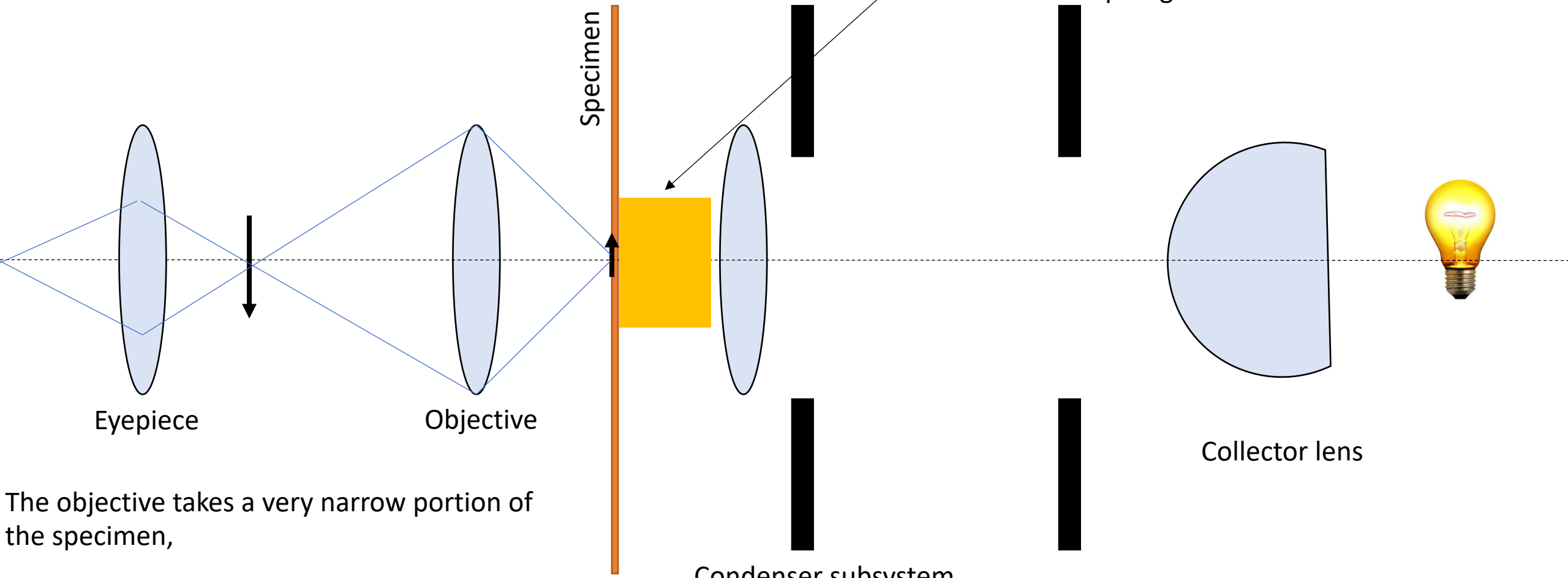
Koehler Illumination

The objective is to illuminate the entire specimen uniformly such that any intensity change is associated with the object and not to the illumination system

Problem: The light sources (bulbs) are not spatially uniform.

Illumination subsystem

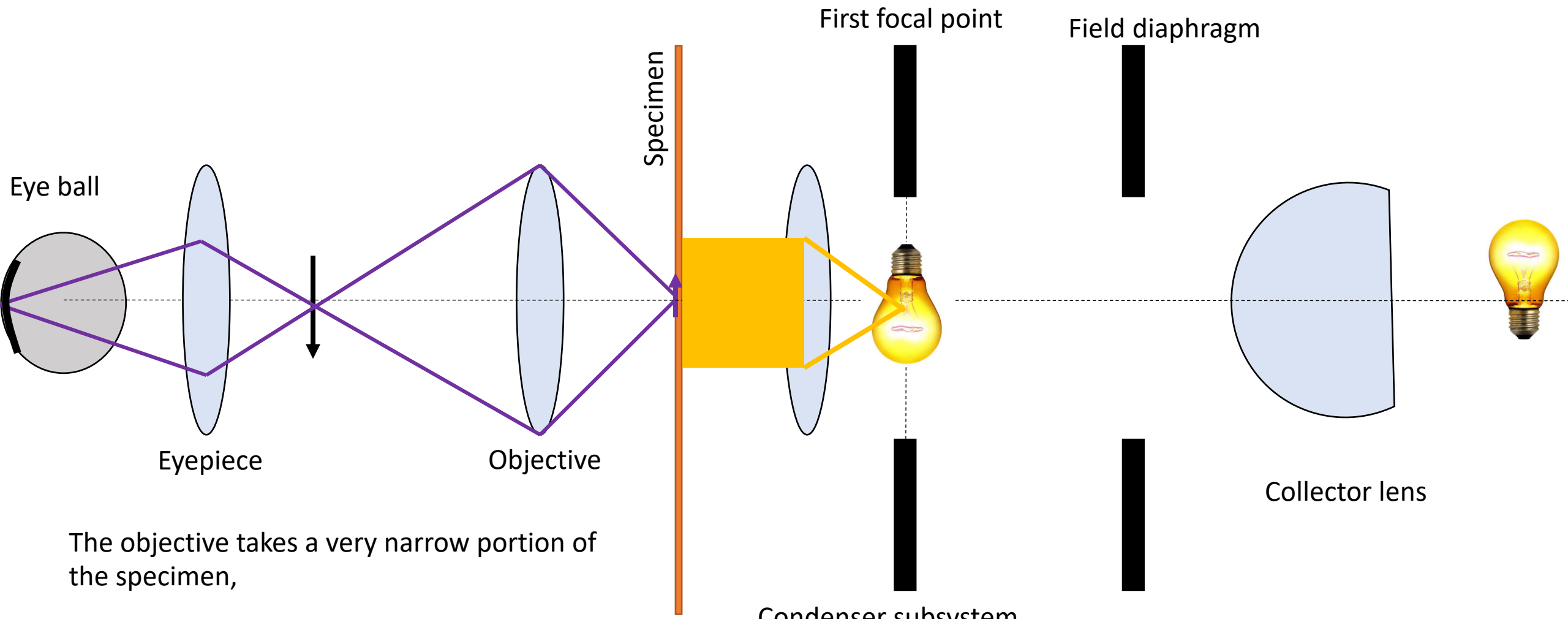
The questions is how to place the collector and the condenser lenses to have uniform illumination on the sample



The objective takes a very narrow portion of the specimen,

The illumination needs to be uniform to capture the essence of the specimen

The idea is to place the image of bulb on the first focal point of condenser



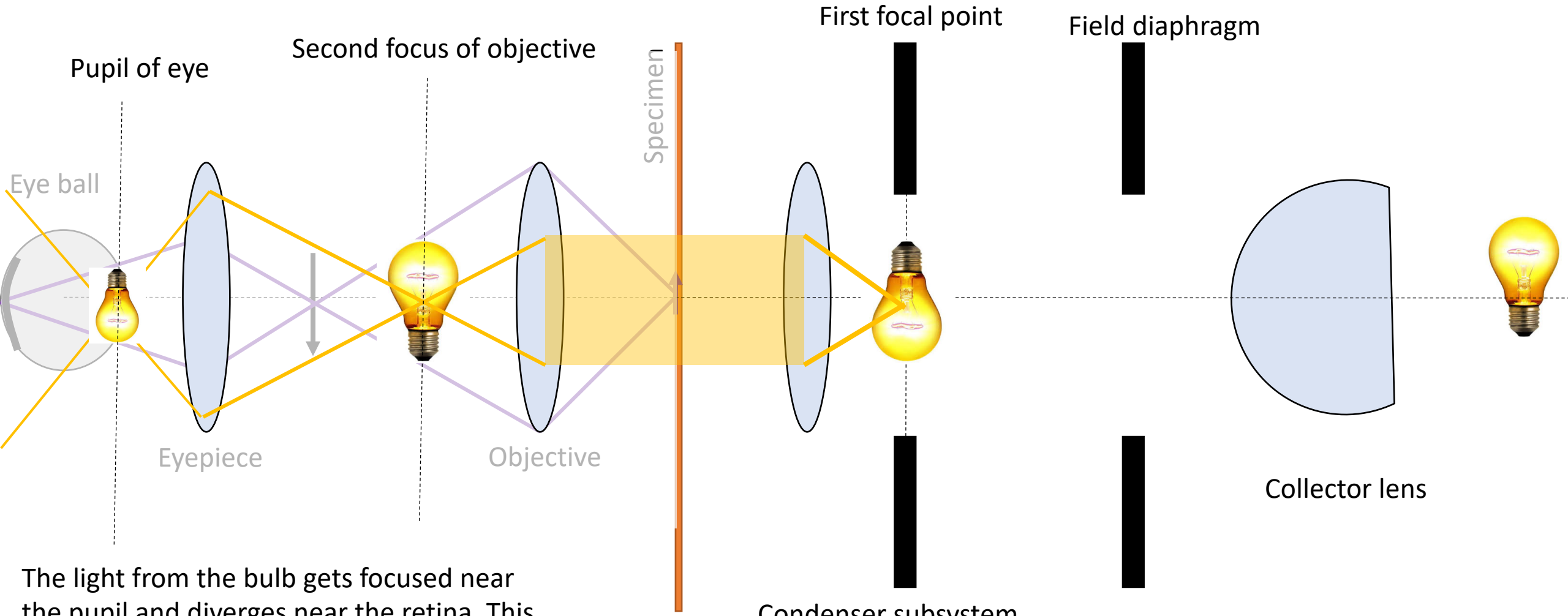
The objective takes a very narrow portion of the specimen,

The illumination needs to be uniform to capture the essence of the specimen

Conjugate illumination planes

Set of planes having the same focus are called as conjugate planes

Here, since they form the image of the bulb, they are called the conjugate illumination planes



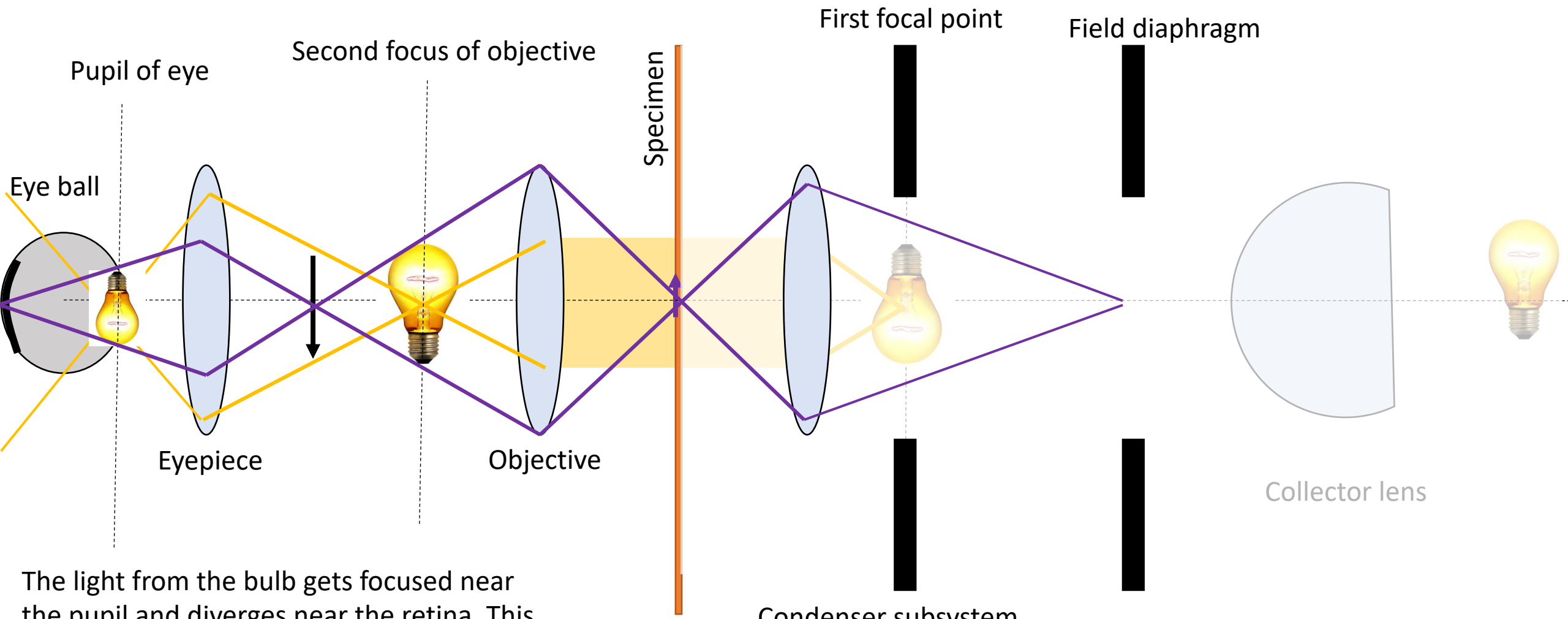
The light from the bulb gets focused near the pupil and diverges near the retina. This just forms a bright background and does not affect the image formation of specimen!

Condenser subsystem
Lens + aperture

Conjugate focal planes

Set of planes having the same focus are called as conjugate planes

Here, specimen, field diaphragm, retina all will have the same image.

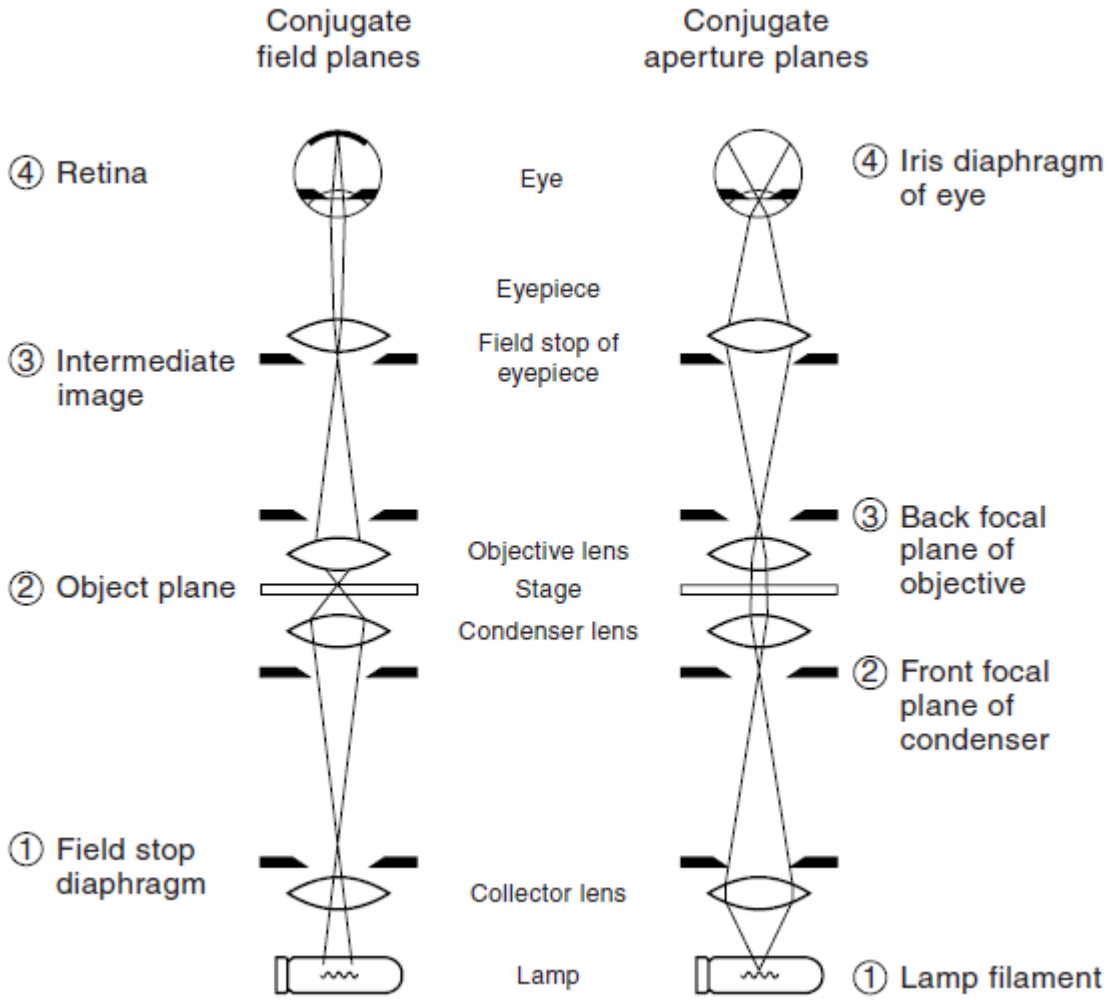


The light from the bulb gets focused near the pupil and diverges near the retina. This just forms a bright background and does not affect the image formation of specimen!

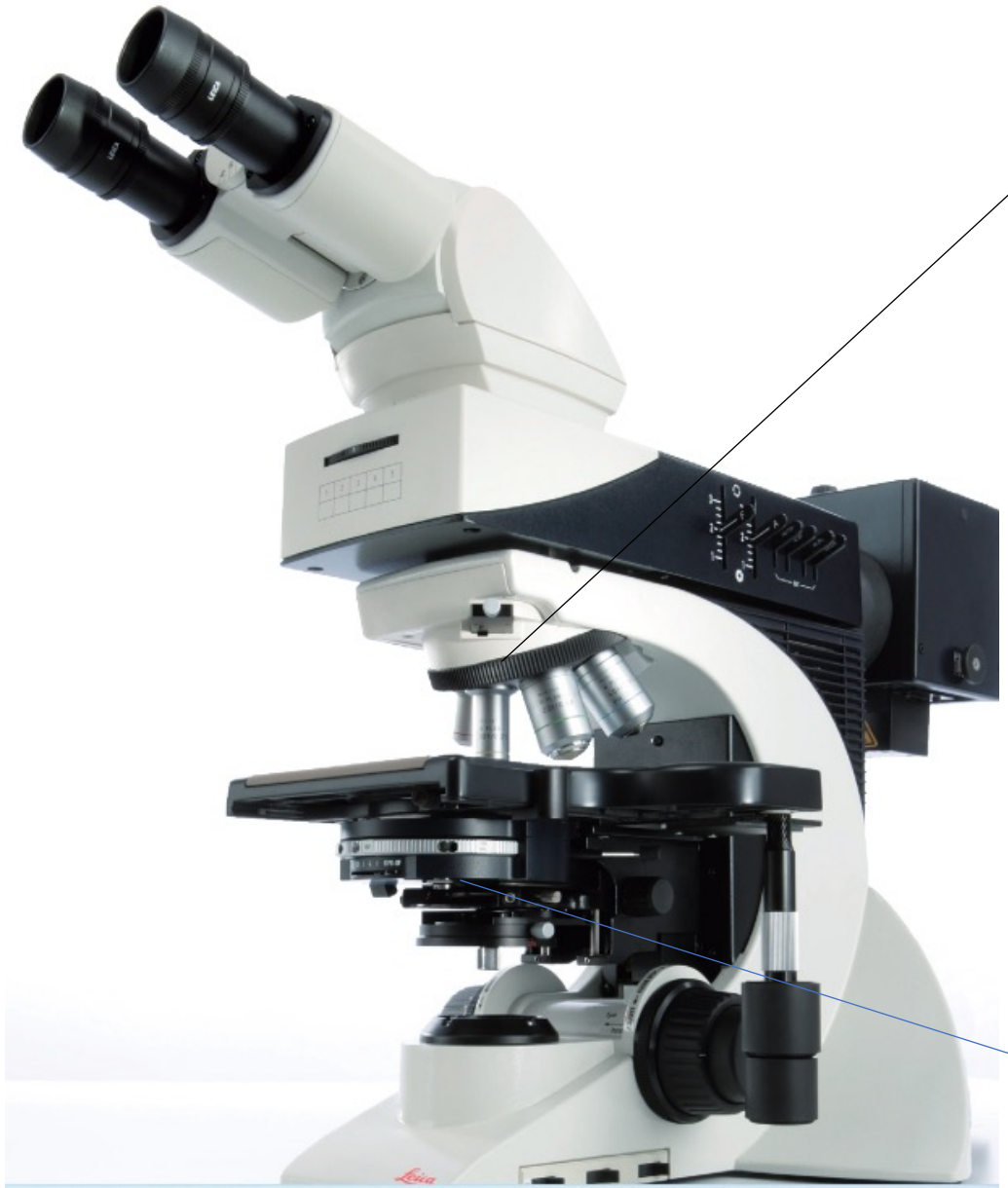
Condenser subsystem
Lens + aperture

Steps for Koehler illumination

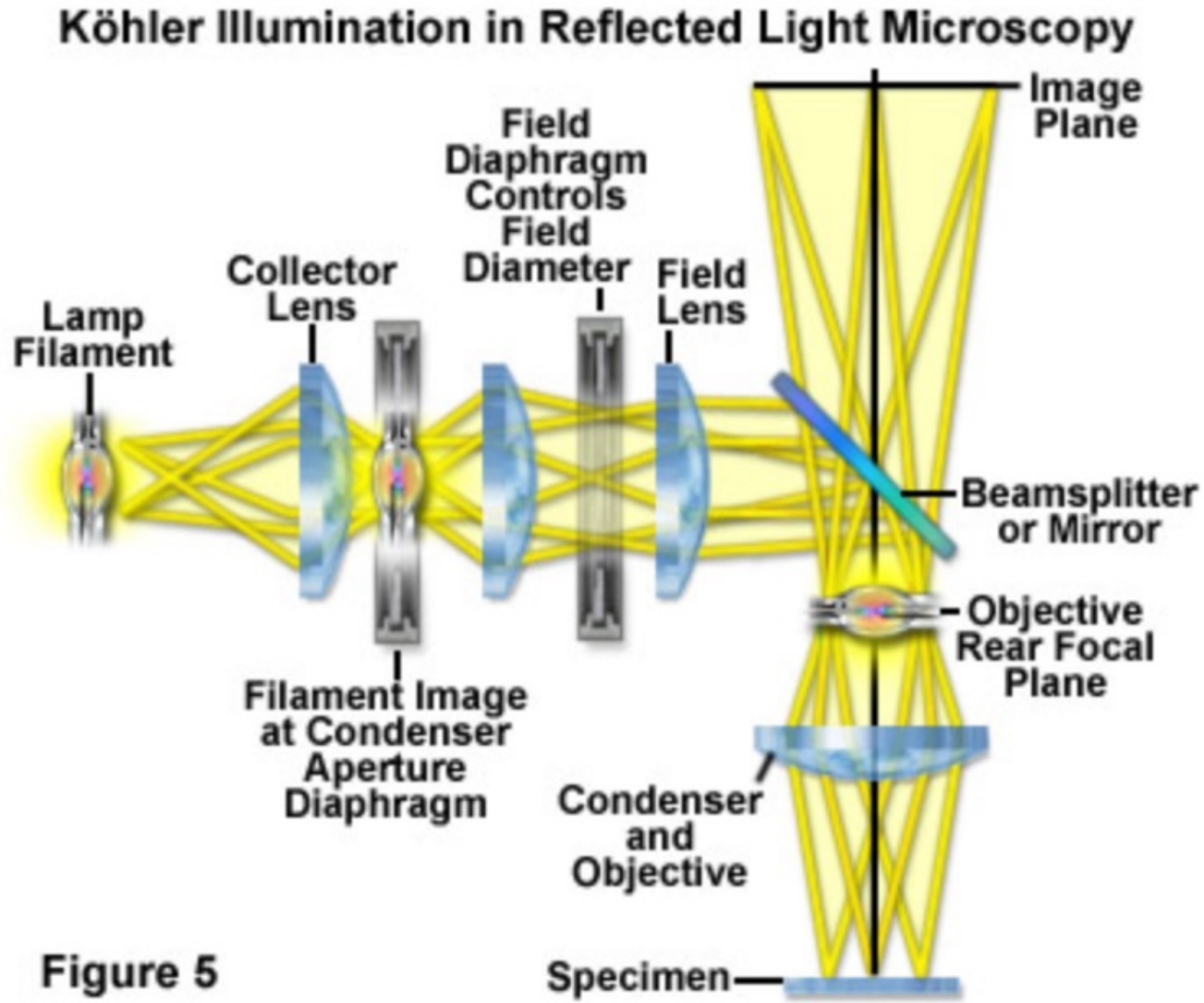
1. Focus the objective on the sample stage
2. Now move the condenser (attached with its aperture) such that the field diaphragm is visible. This sets the condenser position.
3. Remove the eye piece. You should now see the first focal plane of the condenser. Now close the aperture such that you see the complete image of the lamp. Now you are set in Koehler illumination!



Condenser Sub-stage:

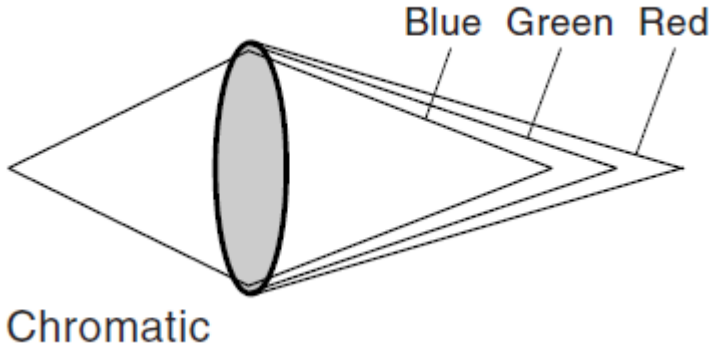


How is Koehler illumination obtained in reflection microscope ?



Aberration in lenses

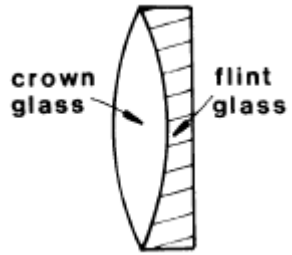
Chromatic Aberration:



Different components of white light converging on different points on optic axis

Refractive index of glass in blue is higher than red. This causes blue to be bent further!

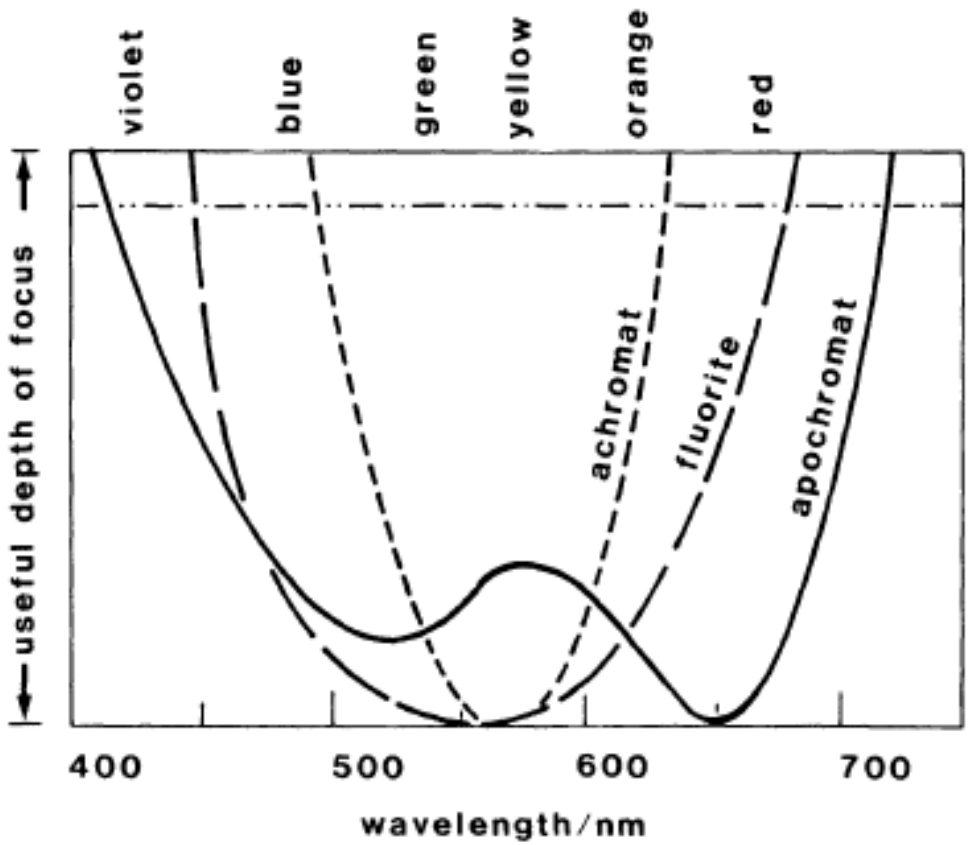
Clue: Colored halo, color changing upon different focus, image never becomes sharp – atleast not in one wavelength



Solution:

Couple glasses with different refractive indices. Match for differing paths for different wavelengths.
Eg: Couple crown glass with flint glass to form a lens combination called as achromatic doubled lens

Variation in depth of focus for different kinds of objectives



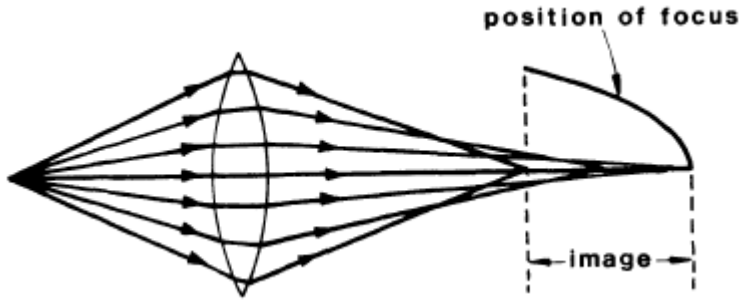
Achromat – simple doublet

Fluorite- Fluospar like materials which have low dispersion $\frac{dn}{d\lambda}$ than optical glasses

Apochromat –Has a combination of positive Fluospar (convex) and negative special optical materials

Spherical Aberration

Monochromatic rays!



Rays that pass-through lens extremes get focused at different point on optic axis

Gets focused closer to the lens.

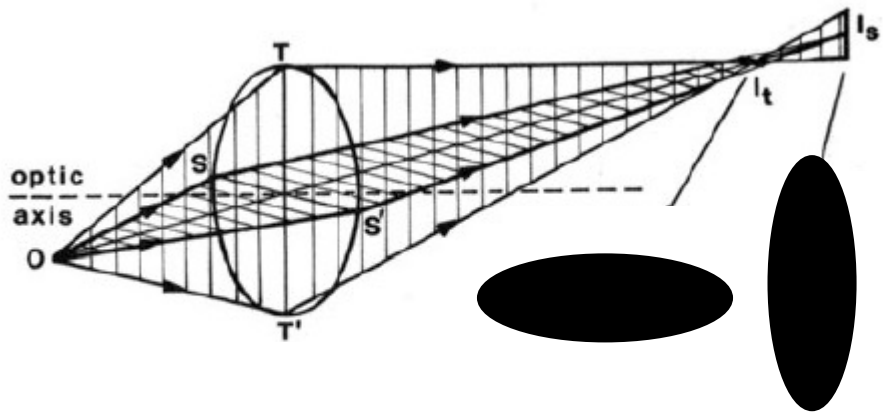
The aberration gets severe for higher NA lenses

One possible solution:

Use compound lenses – positive and negative lenses with differing thickness

Correction is often effective for a well calibrated object position, presence/absence of cover-slip, refractive index of medium (oil/air)

Astigmatism



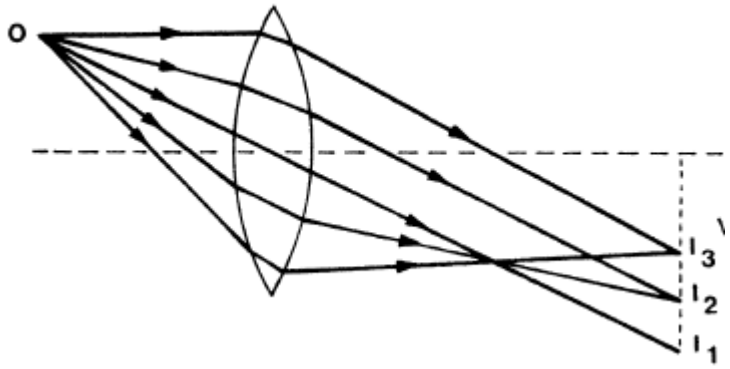
An object which is off-axis

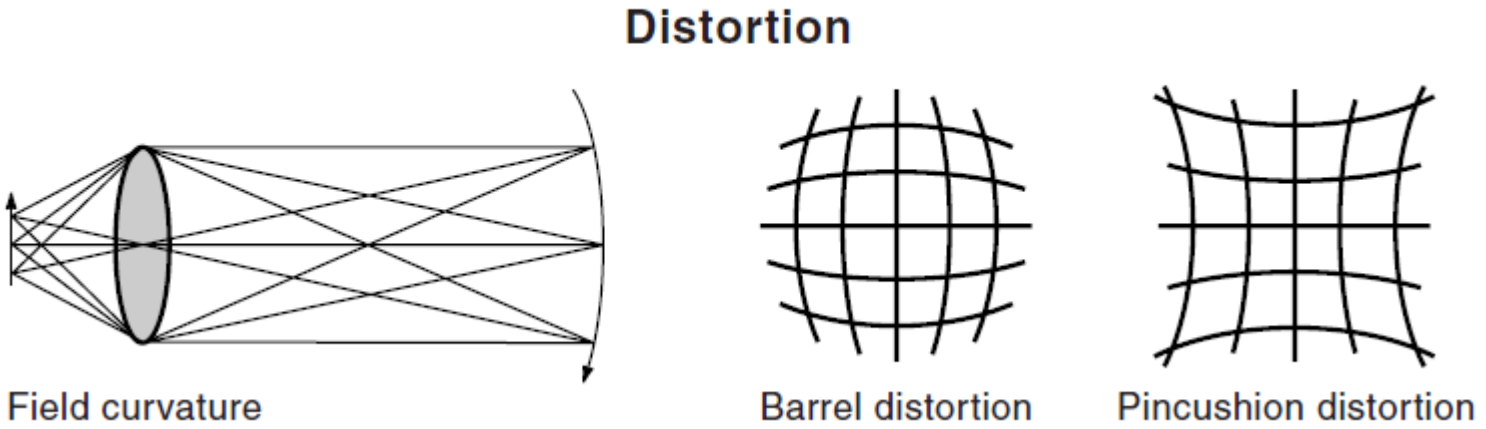
The rays which goes along the Y-axis (TT') is focused at a different point, in comparison with the rays which went along X-axis (SS').

Thus, when the focus is set at I_T, the X direction is not focused, seems like an elongated disk along X

When the focus is set at I_S, the Y-direction is not focused, causes an elongated disk along Y.

Coma and Distortions





Another off-axis issue (non-axial spherical aberration).

Images have a comet's tail normal to optics axis

Rays which pass through the edge of lens are focused to a point closer to the optic axis.

Caused due to different in magnification, which is the ratio of image to object distance.

This is a symptom, and is often corrected by addressing the other aberrations.

Construction of Objective:

Achromat: red – blue corrected (chroma aberration), spherical aberrations are corrected ffor yellow-green ~ 540 nm

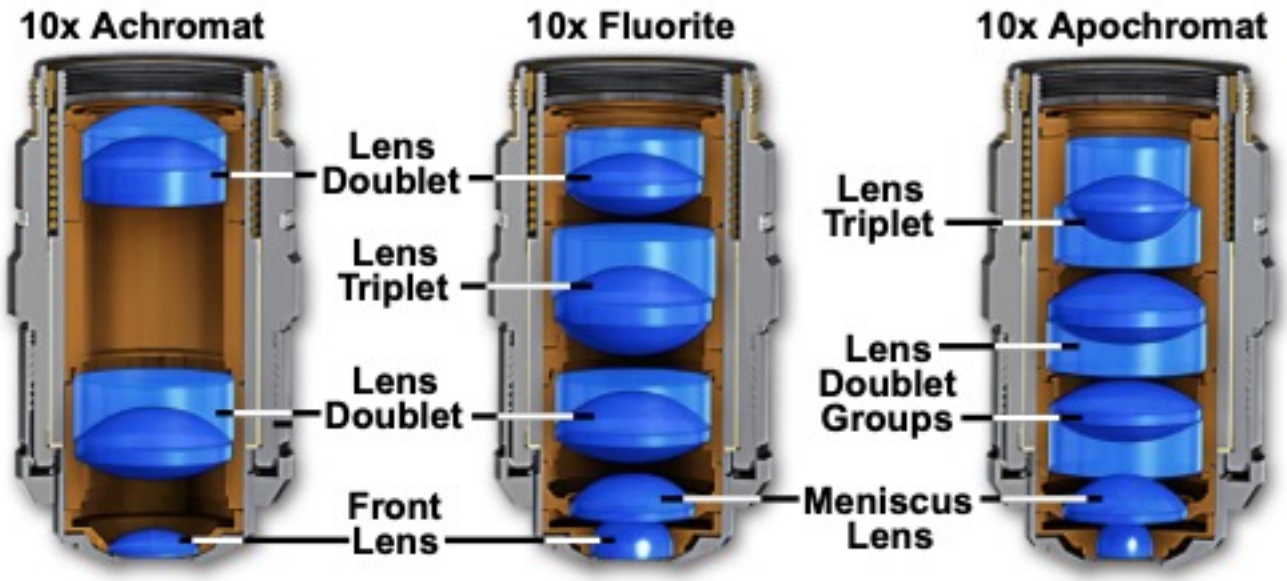
Fluorite/semiapochromat – have CaF_2 or LaF_3

Have good correction for chroma, field curvature, high transparency in UV

Apochromat: Most expensive. A combination of many such correctors. Useful in wide range of wavelengths.

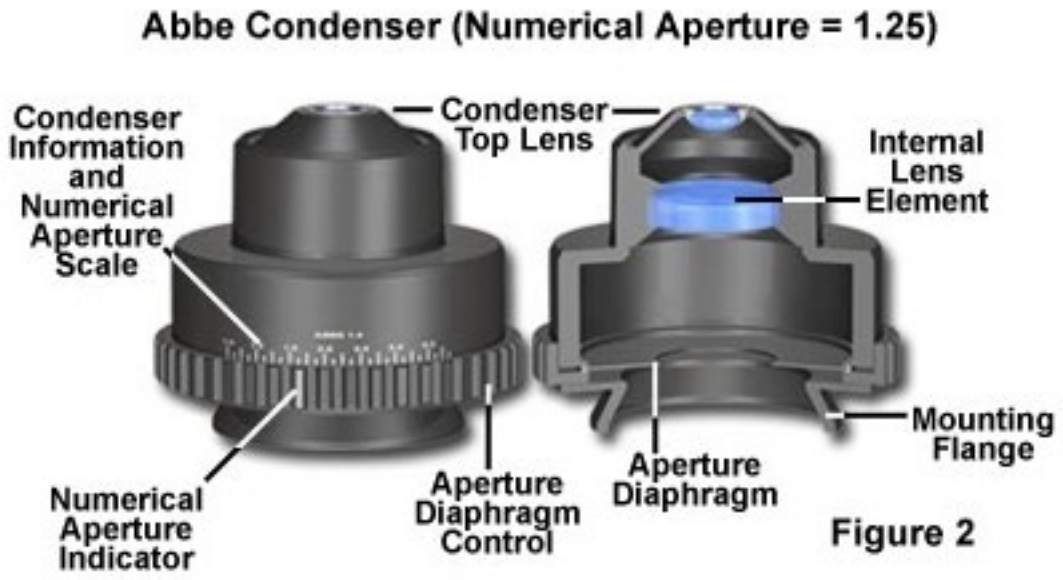
However, suffers from field curvature (only a small region can be brought to focus.

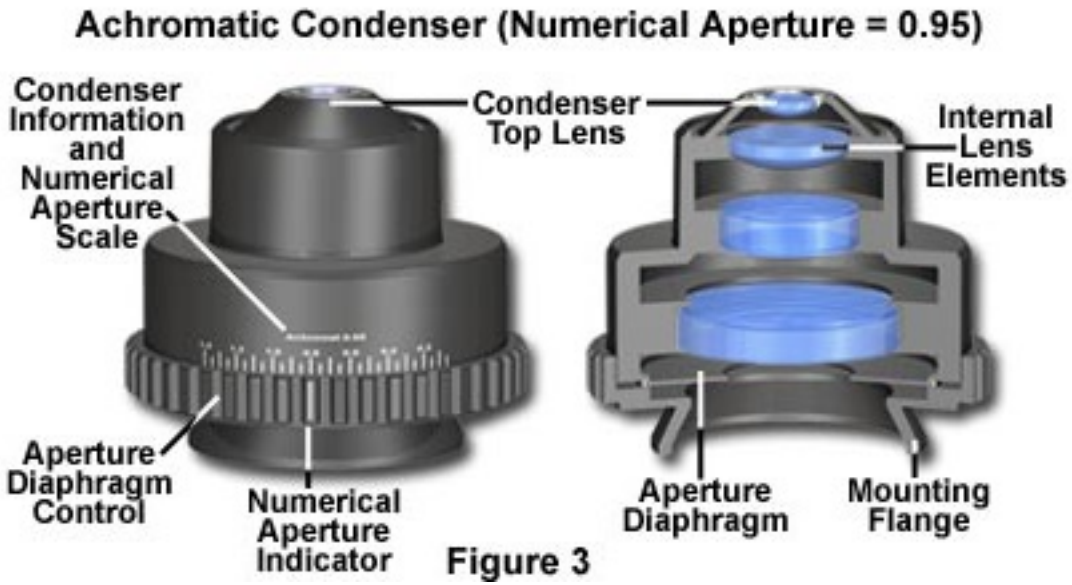
Plano-apochromat: also has the field curvature corrected.



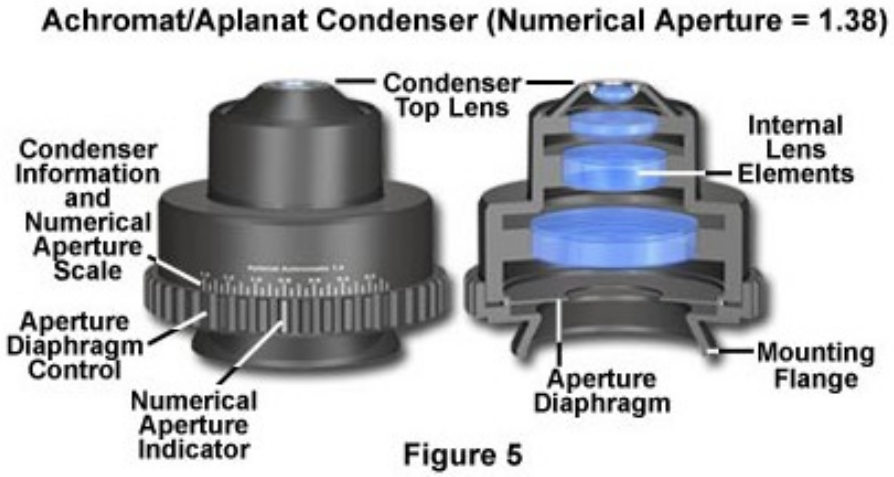
Objective marking

Construction of Condenser lenses





Simplest condenser: With no correction for chroma/spherical



A microscope design problem:

The human eye can distinguish two points that subtent atleast 1 minute of arc at the retina.

If distance of distinct vision is about 25 cm, eye can distinguish two points separated by

$$\frac{\frac{d}{2}}{25} = \sin\left(\frac{1}{60}\right)$$
$$d = 145\ \mu m$$

If we use a light of wavelength 520 nm, close to ideal NA = 1

Then the minimum resolution that we can achieve $0.62 * 520 = 317.2$ nm

The minimum magnification required for comfortable viewing of the fundamental limit of separation

$$M = \frac{145000}{317.2} = 457$$

This is the minimum magnification required to resolve the fundamental optical limit.

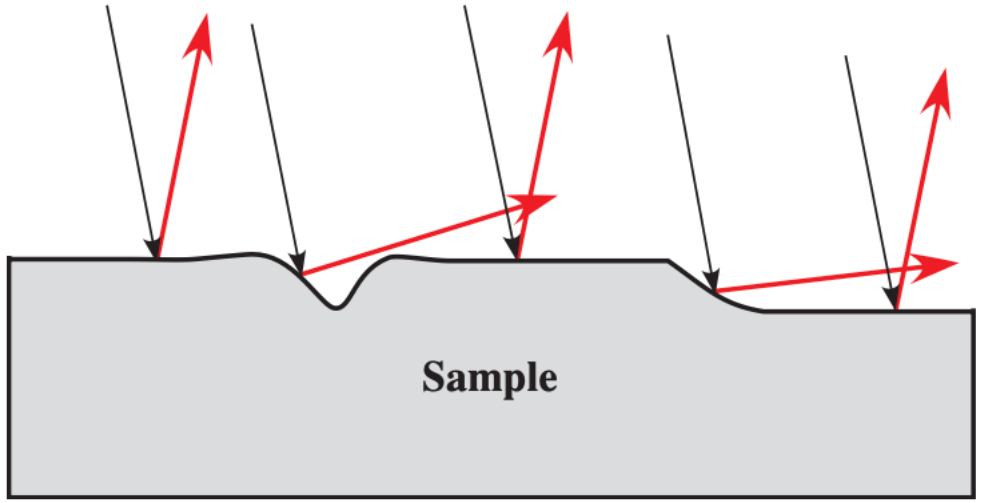
However, comfortable viewing for humans require about twice as much magnification.

Contrast Mechanisms

Different contrast mechanisms

- Bright field
- Dark field
- Phase contrast
- Differential interference
- Modulation
- Fluorescence
- Polarization

Contrast in bright field



Contrast allows for identification of features in a microscopic image.

Contrast:

Intensity difference between neighboring resolved points

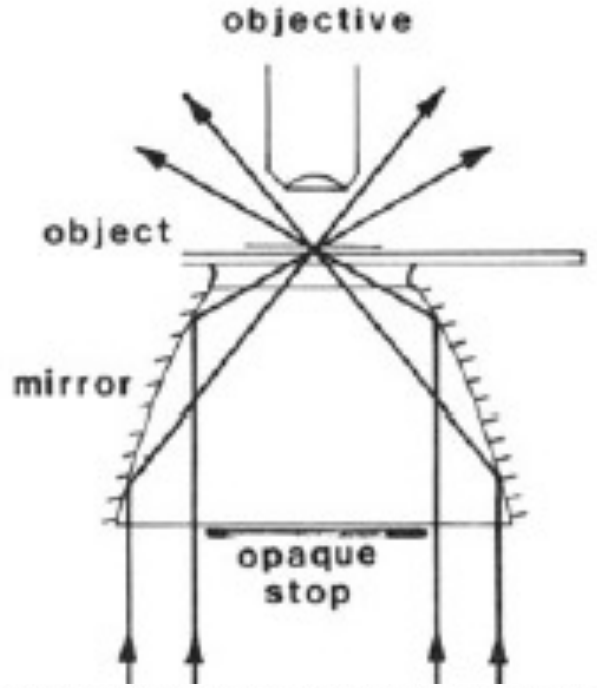
$$C = \ln \frac{I_1}{I_2}$$

$$\Delta C = \frac{\Delta I}{I}$$

A minimum of $\Delta C = 0.14$ is required for human resolution of neighboring particles

Dark Field Illumination

- An opaque plate is used to block major portion of incident light.
  - Causes bright field to escape objective
  - Image is formed from diffracted rays
- Images with high contrast is observed. Very high NA objectives are rarely used.
- There is loss of resolution
  - Small NA of the objective



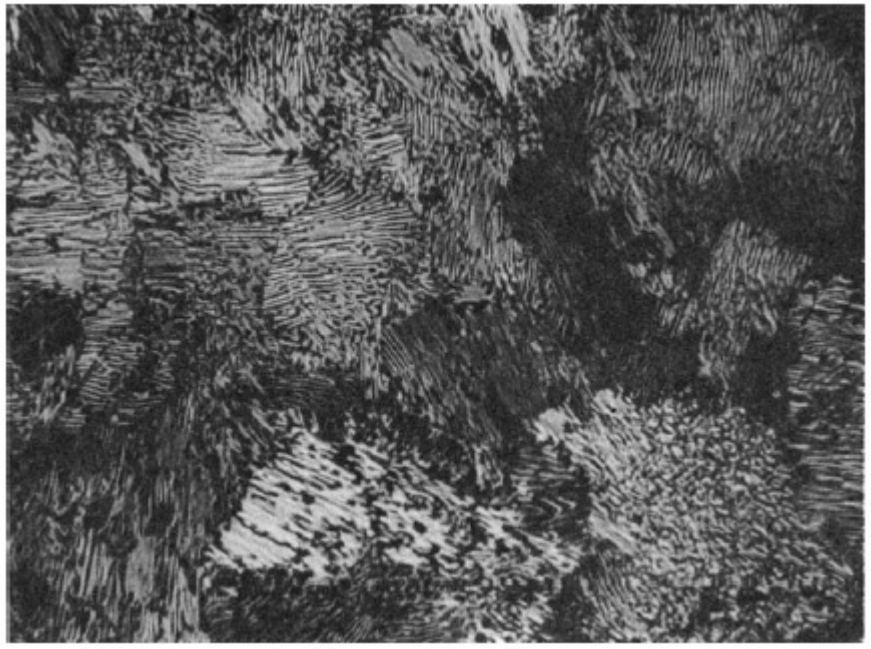
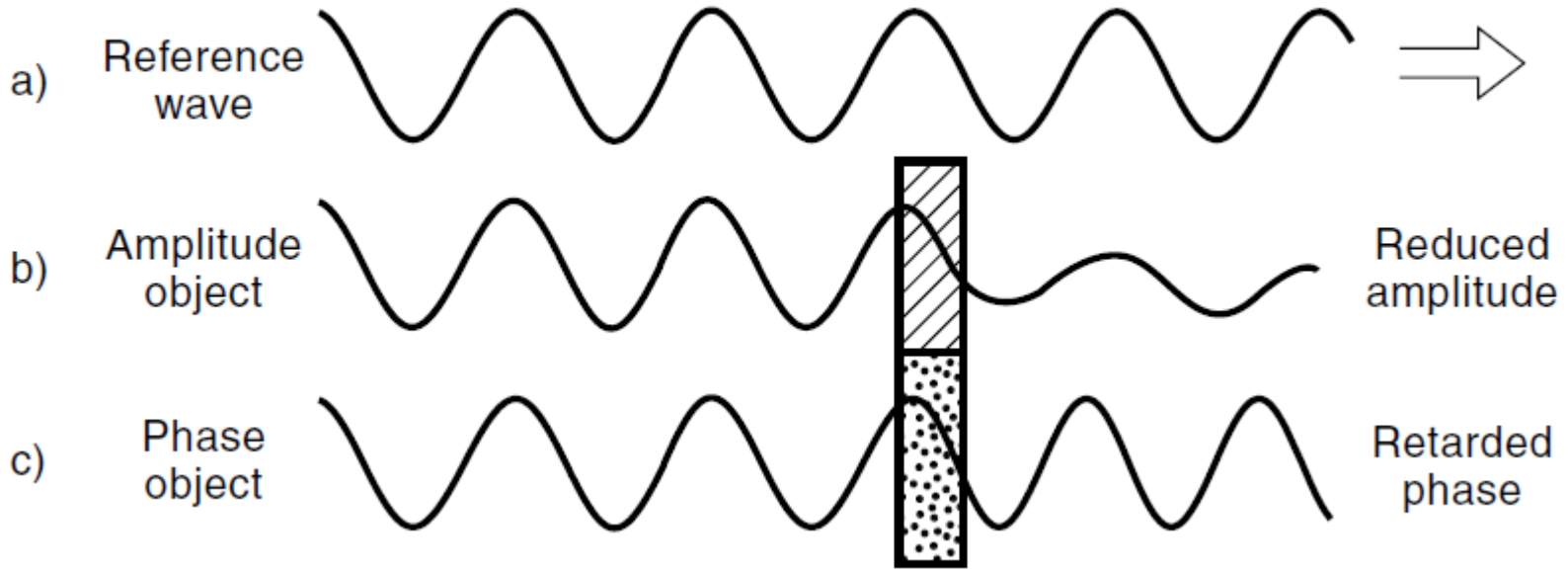


Image of pearlite with dark field illumination

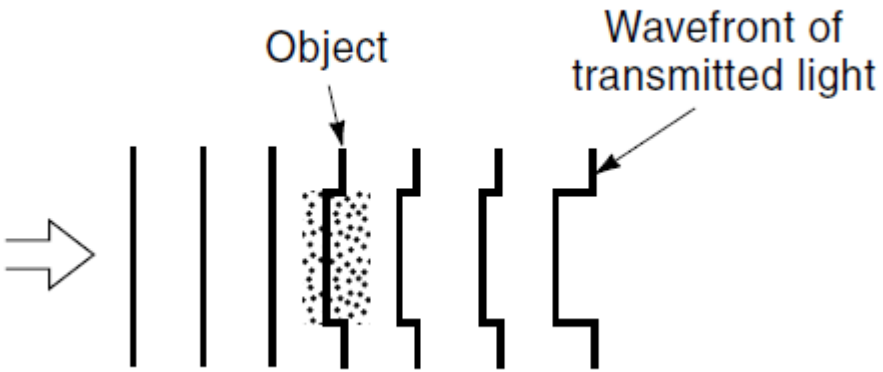
Phase contrast

Human eyes are sensitive to only intensity.

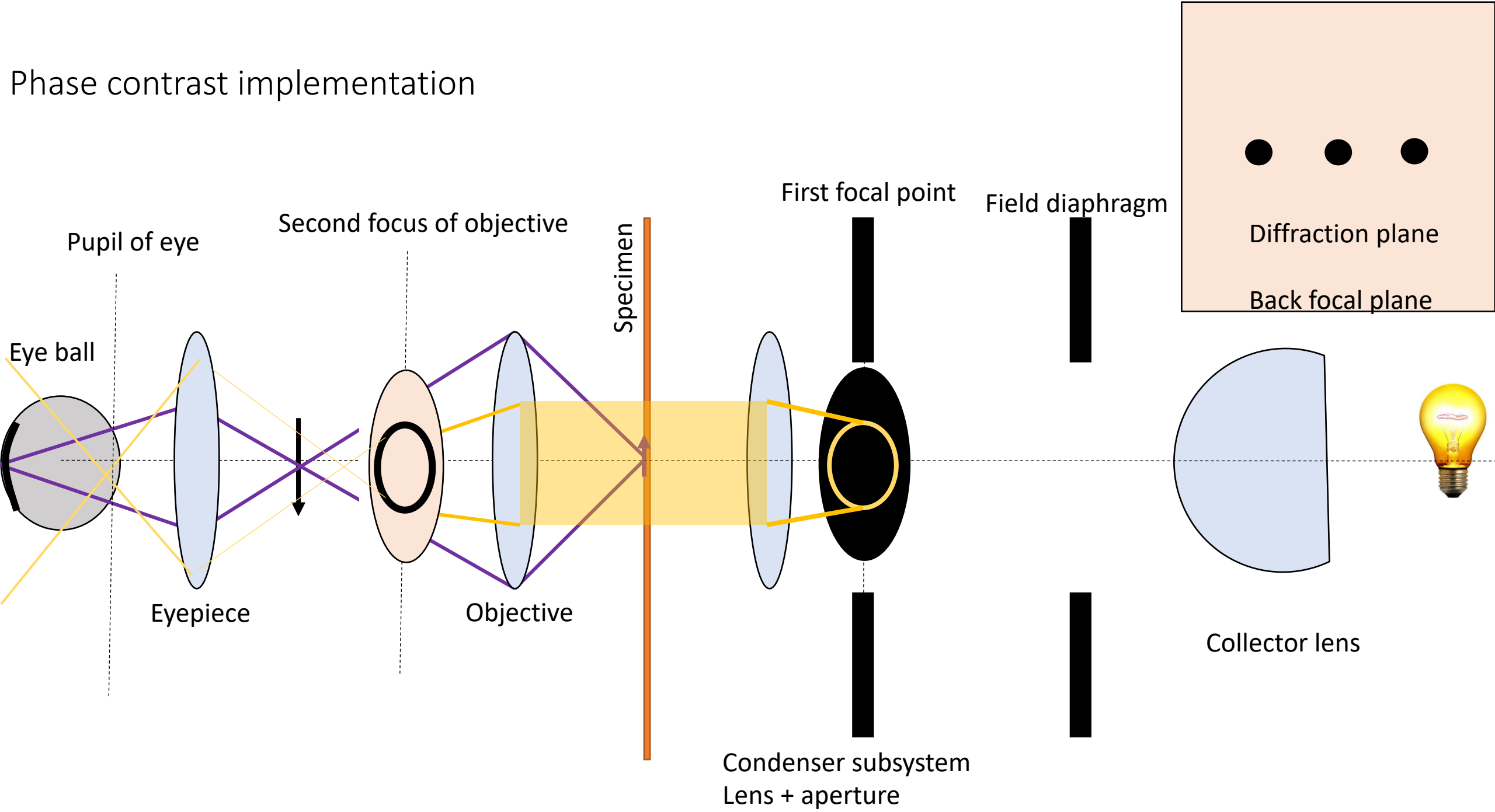
Phase microscope converts phase difference into intensity difference



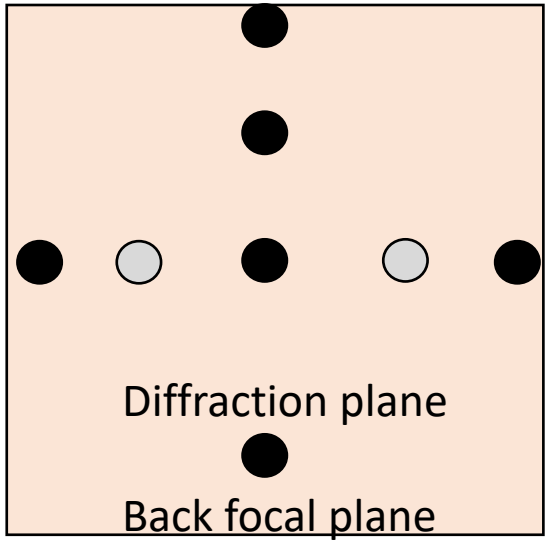
Amplitude vs phase object.

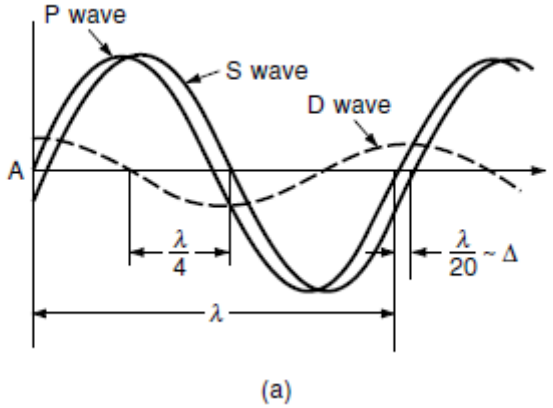


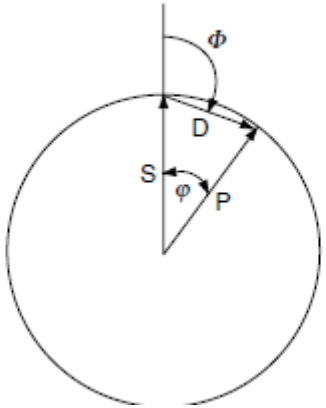
Phase contrast implementation



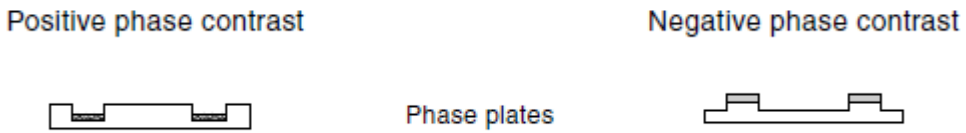
How to implement ?



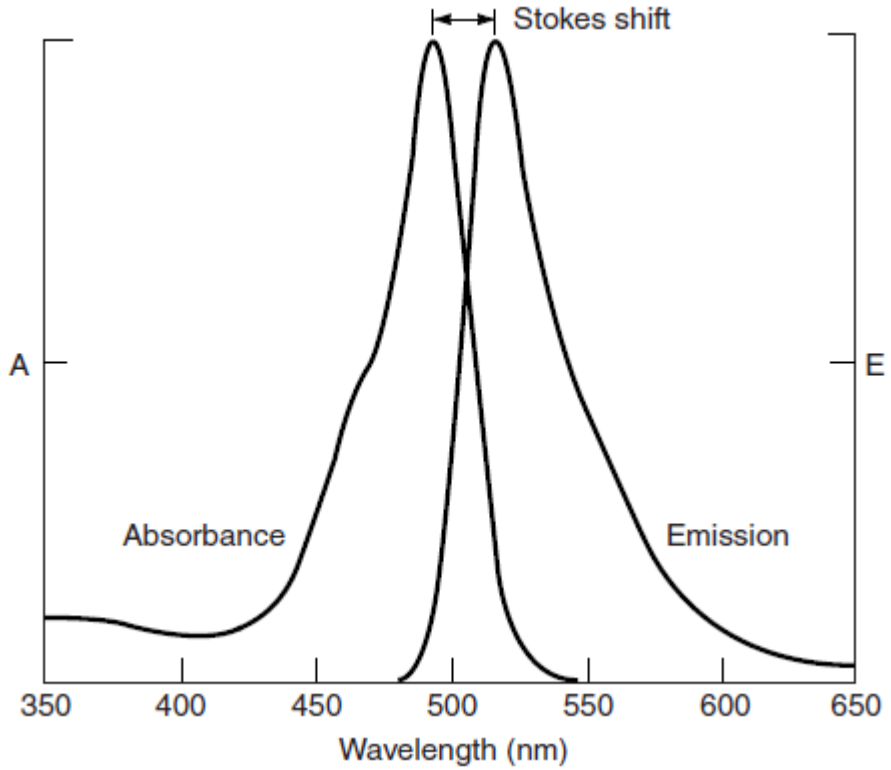


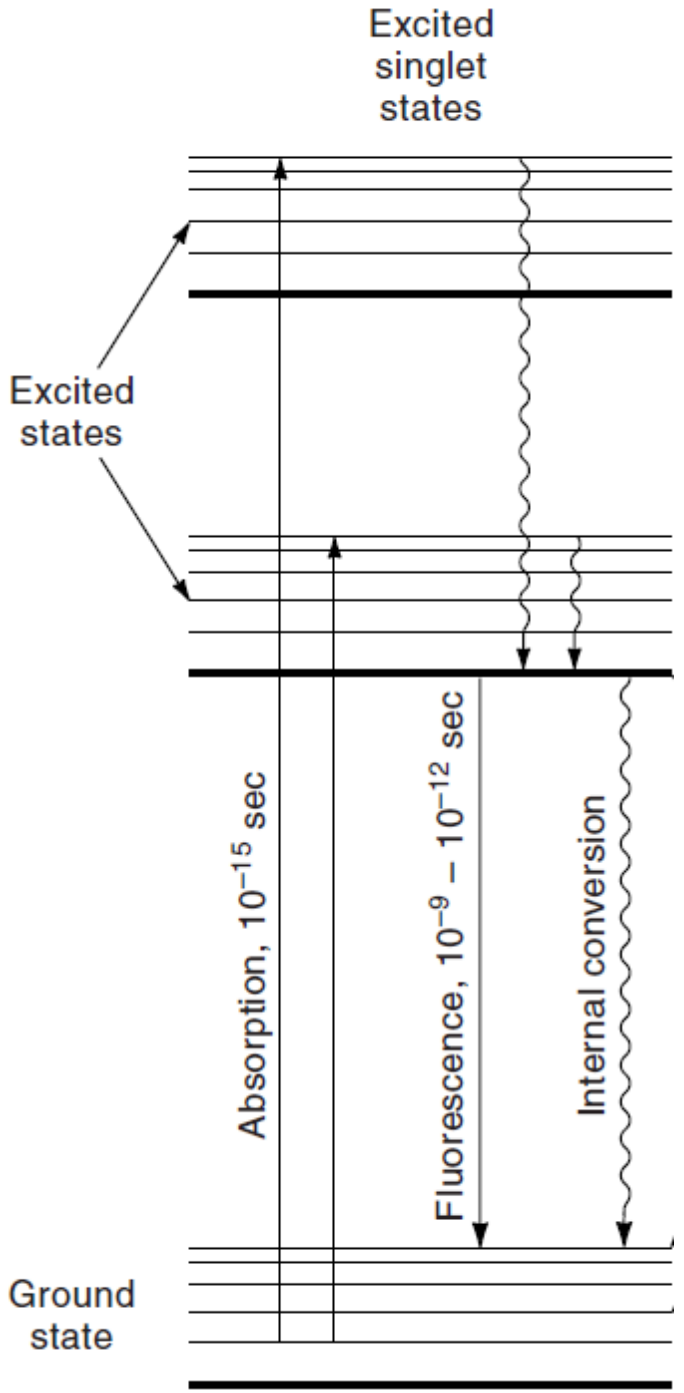


- A condenser annulus is used as in dark field
- A phase plate with complementing transparency is used.
- The un-diffracted light is reduced in amplitude.
- The resulting image is formed with intensity phase addition with background and the diffracted beams.



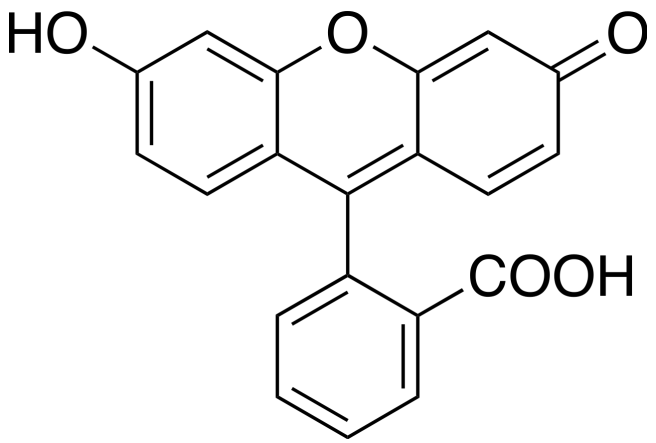
Fluorescent microscope

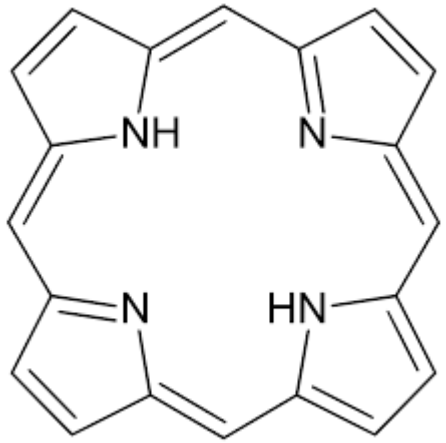




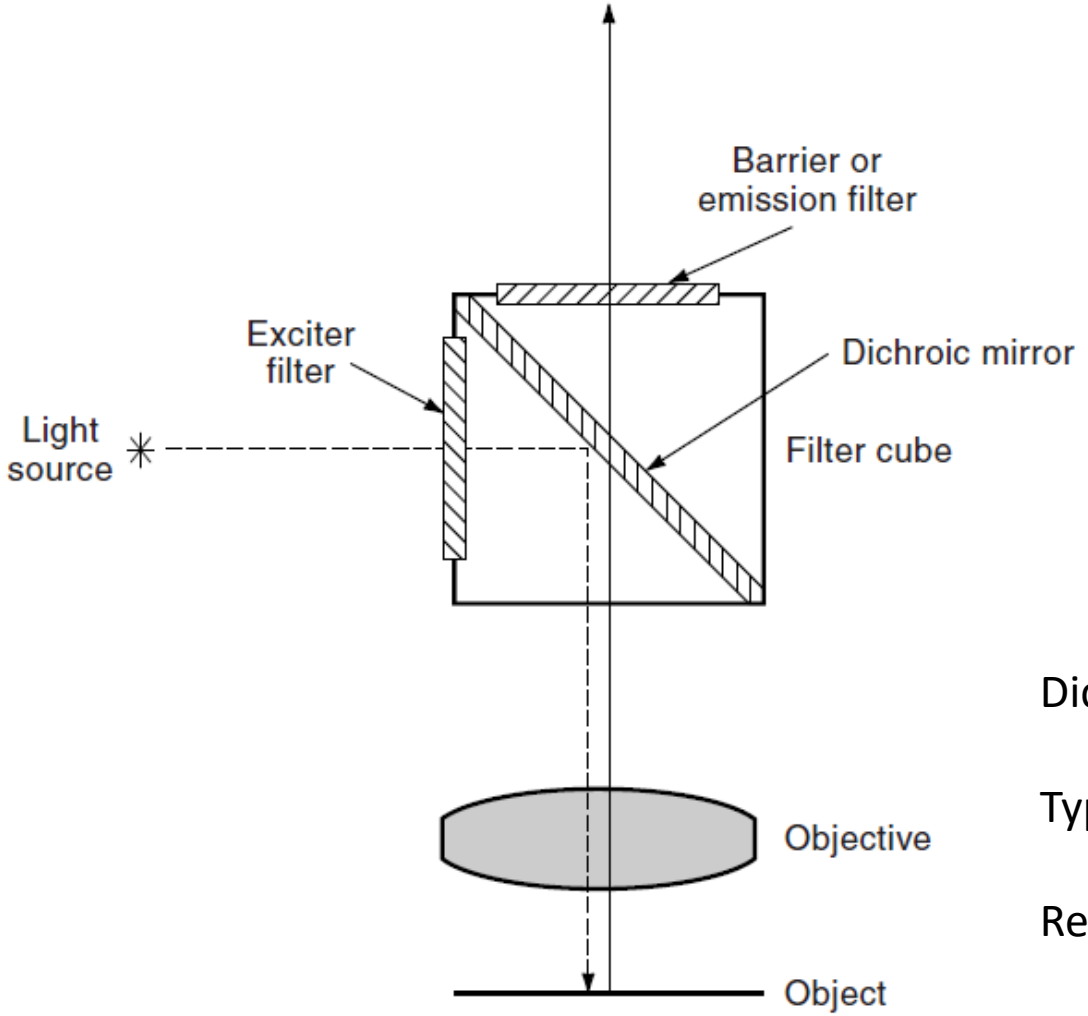
- A fluorescent molecule absorbs energy to excite electrons to higher energy states
- The electrons returns back to ground state by emission of photons of lower energy

- Difference in energy between absorption peak and emission peak is called as Stokes shift.
- Molecules with large shift is suitable for molecule tagging
  - Eg: fluorescein has a shift of 20 nm, and that of porphyrin molecule has a shift of about 200 nm.

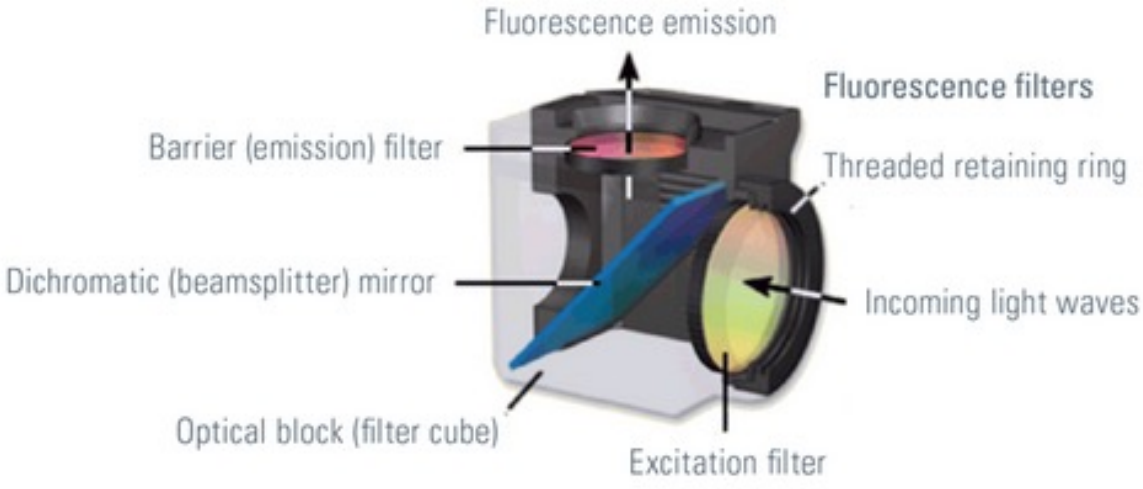




Implementation



Practical filter cube



Dichroic mirror:

Typically made of multiple layers of dielectric: like interference filters.

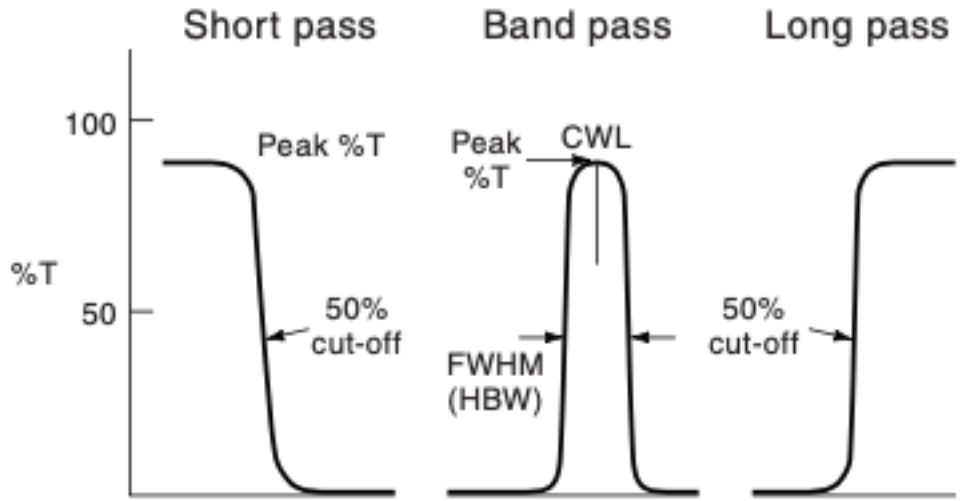
Reflects shorter wavelengths and transmits longer wavelengths!

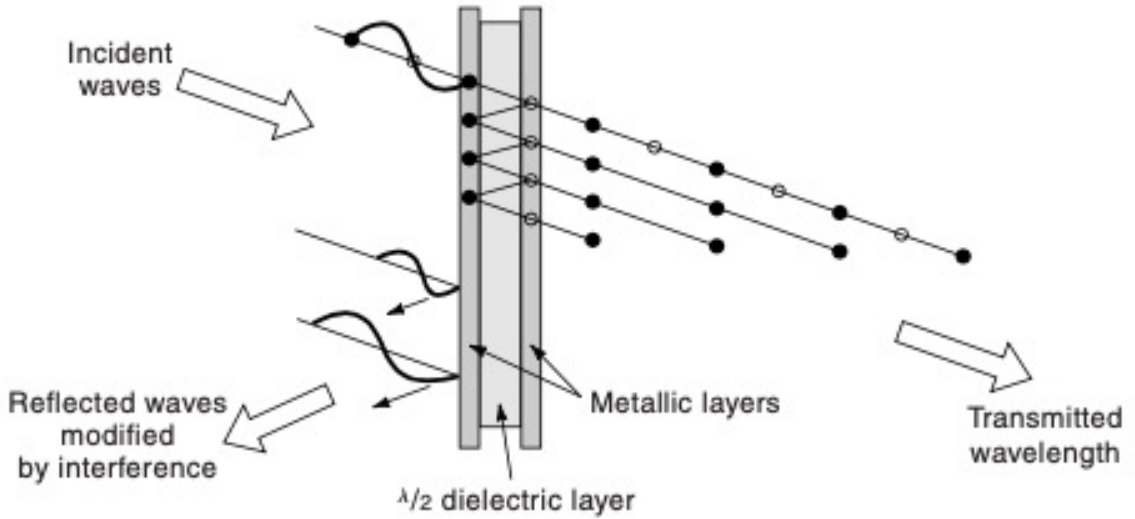
Filters and chroma selectors

- Types of filters
  - Neutral density filters – Thin sheet of metal with controlled optical attenuation

$$OD = \log_{10} \frac{1}{T}$$

  Where T is the transmittance.
- Color glass filters:
  - They are molecular absorbers impregnated into glass
  - Certain molecules have bands of absorption
- Interference Filters
  - Have much steeper roll-off (sharper wavelength selection).
  - Dielectric material sandwiched between metallic partial reflectors





Quantitative Studies

Image Capturing

Intensity of light on the image plane is a continuous function

$$\mathrm{I} = f(x, y)$$

As in the older, photographic plate, the image intensity is in analogue format.

The image plane is then sampled to obtain the digital version of it.

Each sampled point on the image plane is a pixel.

If δ is the minimum resolution of your microscope and M is the magnification setting.

$$\Delta x, \Delta y < M\delta$$

A typical rule of thumb is that $\Delta x < \frac{M\delta}{3}$. That is 3x3 pixel array for every resolvable point

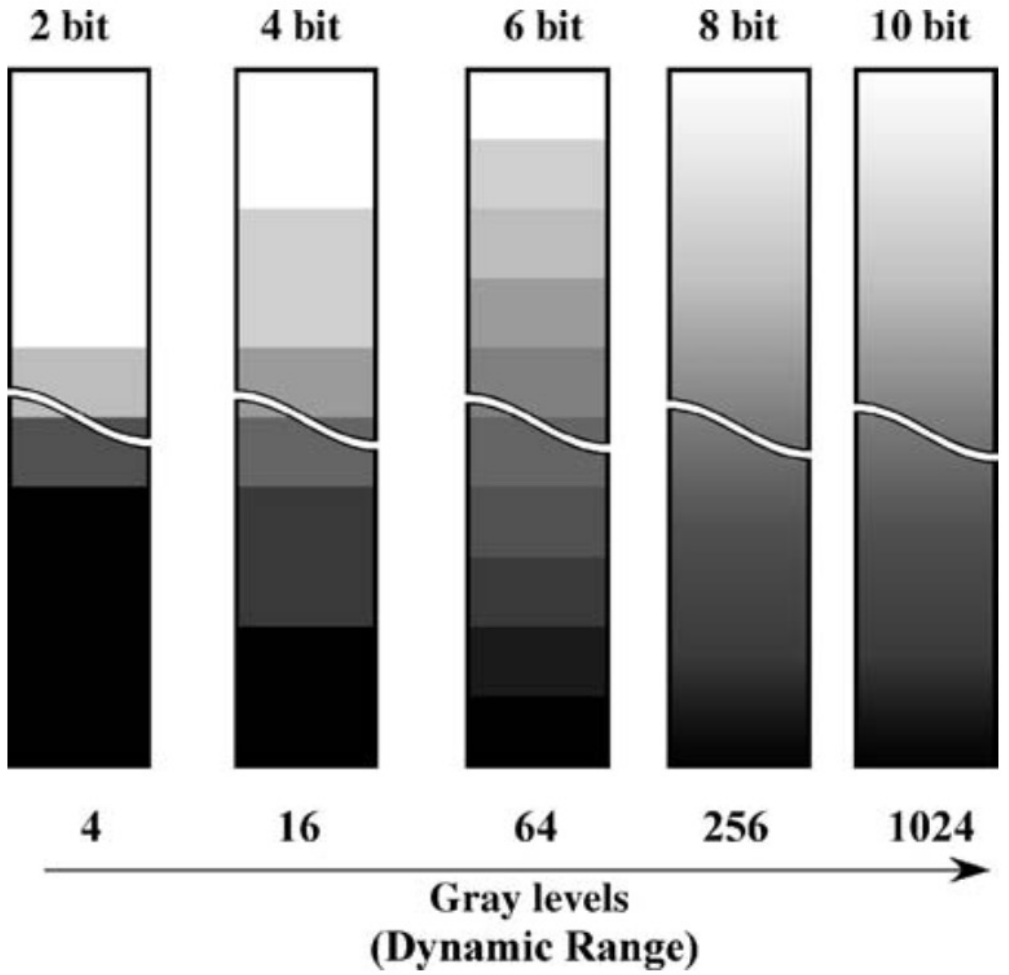
Intensity in each pixel is stored digitally in several bits.

Say we have a 512 x 512 pixel array

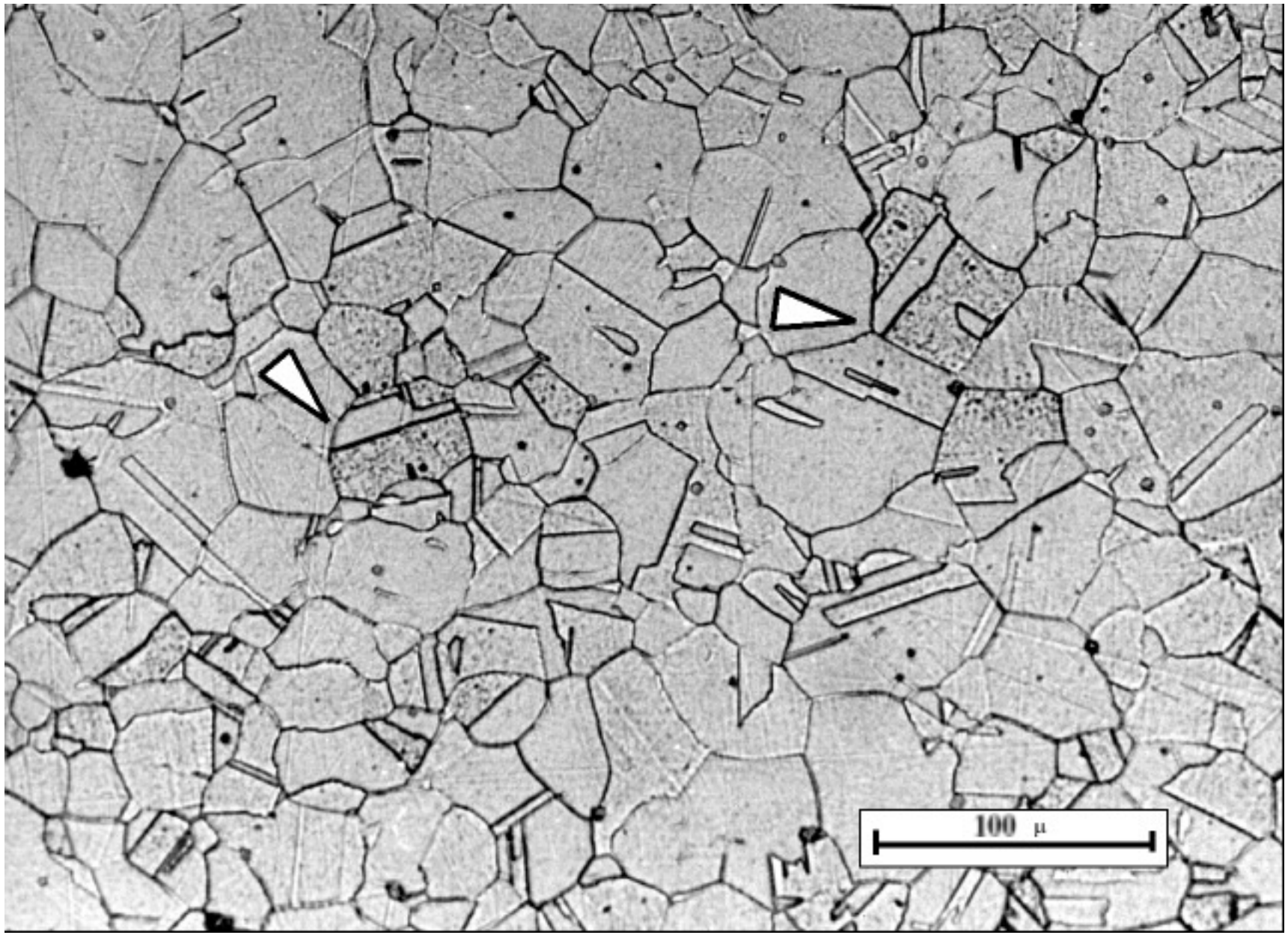
Each pixel intensity is stored in 8 bits.

The image size is 256 kB

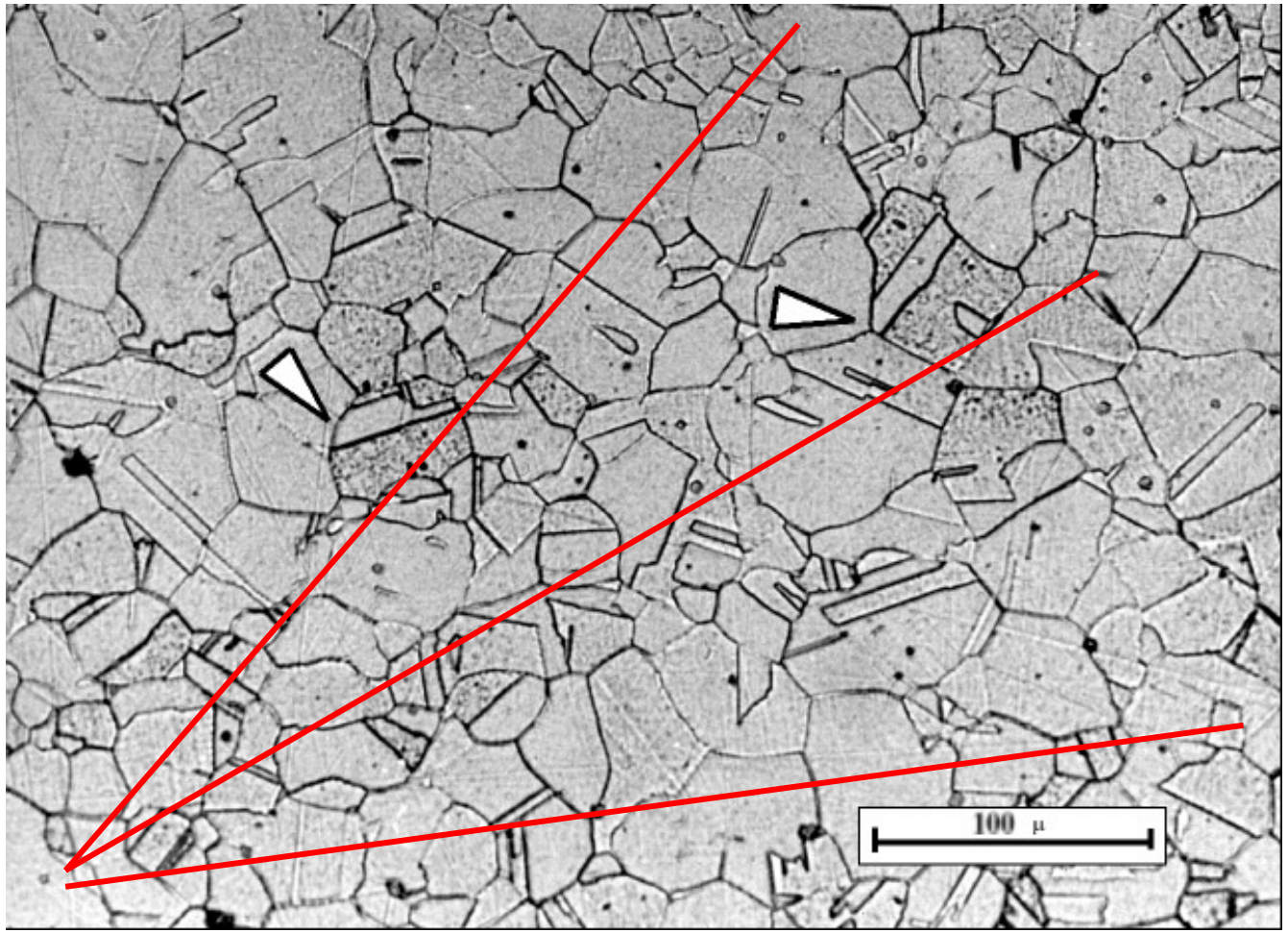
However, many softwares use image compression algorithms such as jpg, TIFF, etc.



Example image



How to measure grain size ?



INTERCEPT PROCEDURE:

1. Draw random oriented lines
2. Find $\bar{l}$ - average intercept length

3. Grain size is a function of $\bar{l}$

How do we find the mean segment length ?