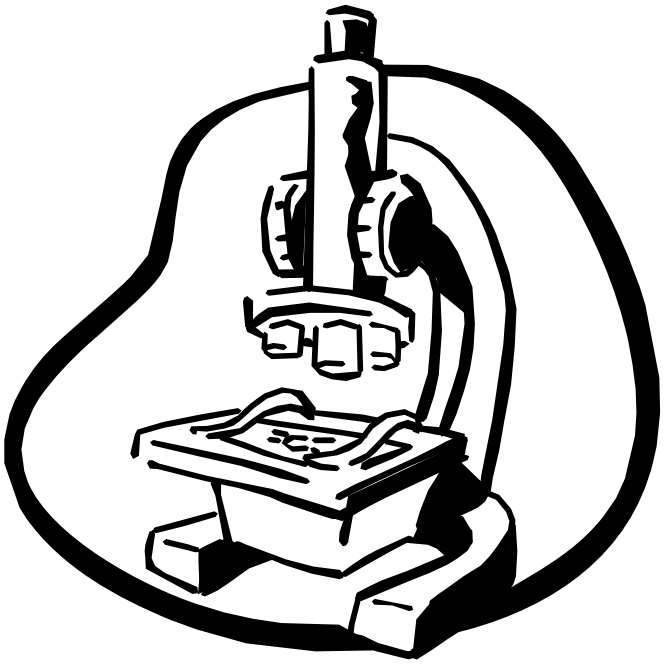
Biomedical & X-ray Physics
Kjell Carlsson



Light Microscopy

Overheads shown in course SK2500,

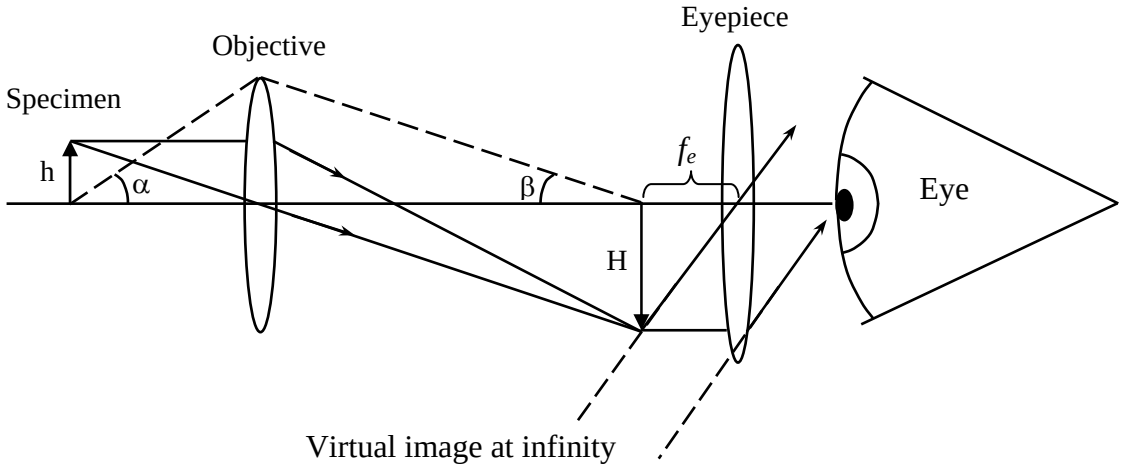
Physics of Biomedical Microscopy

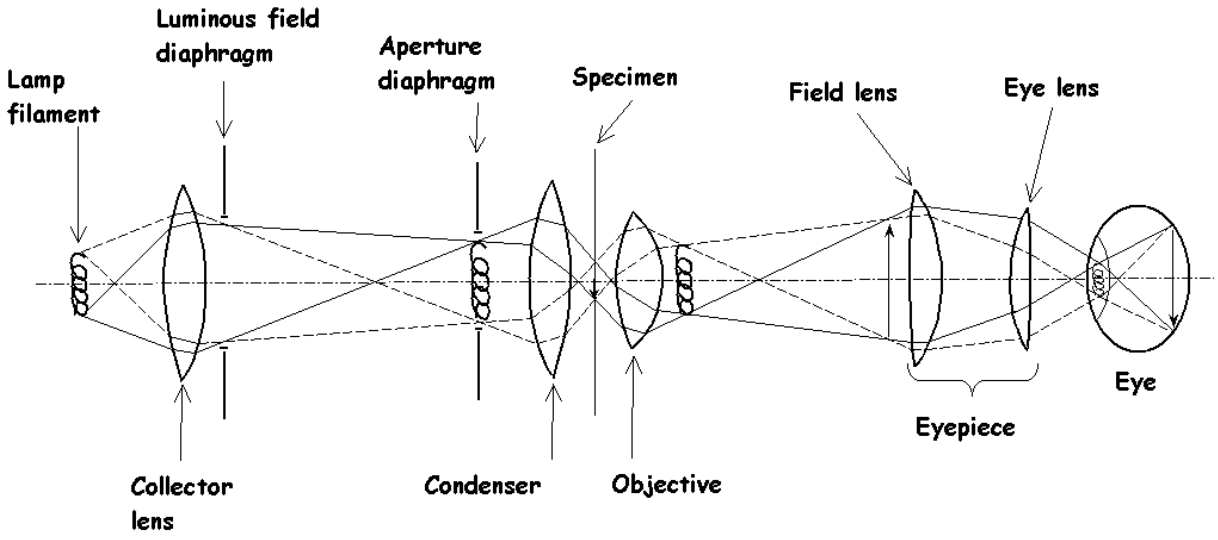
by

Kjell Carlsson

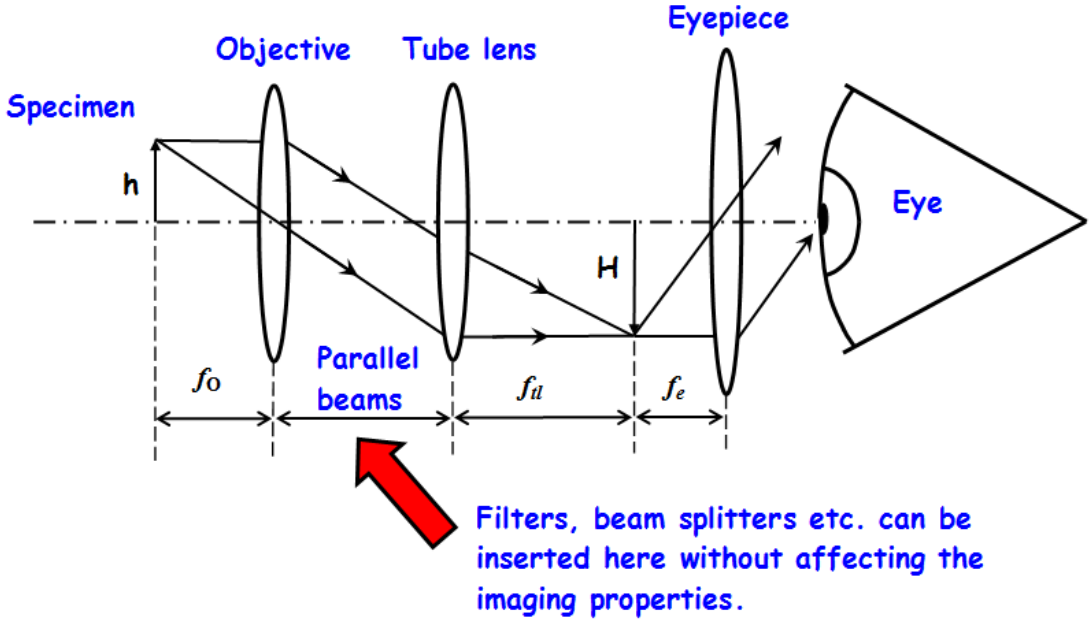
Note: For copyright reasons some of the photographs have been omitted

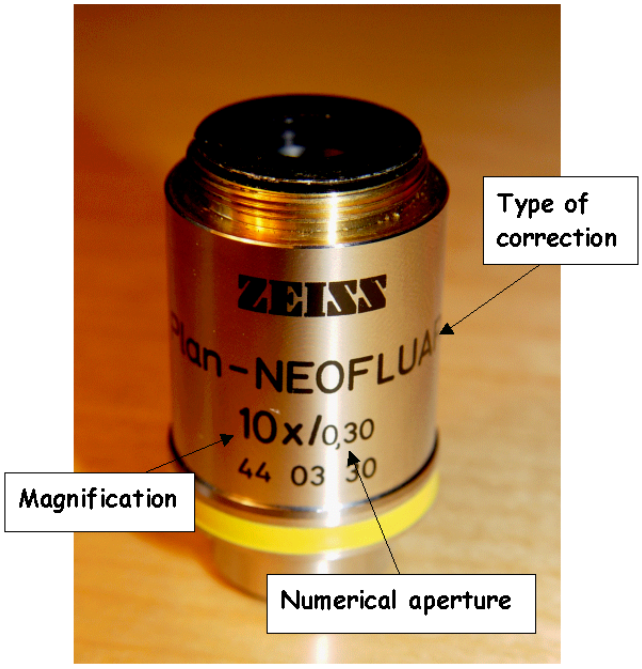
The Microscope in School Books and in Reality

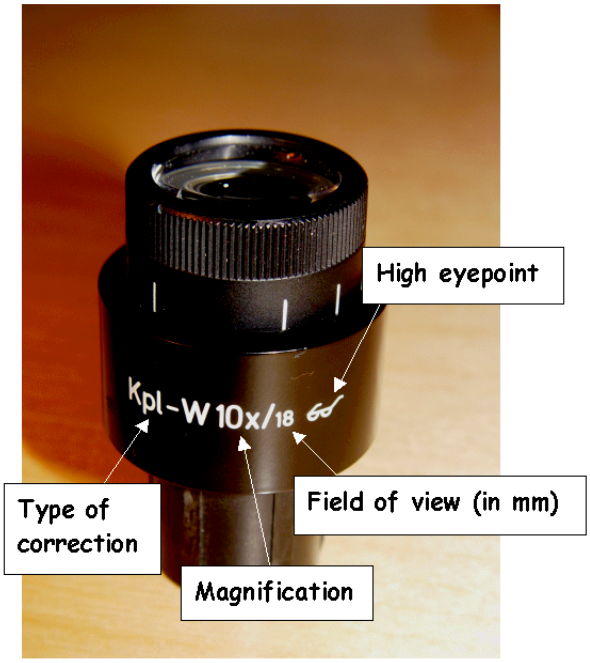




Most modern microscopes use "infinity-corrected" objectives.
(Additional tube lens required)

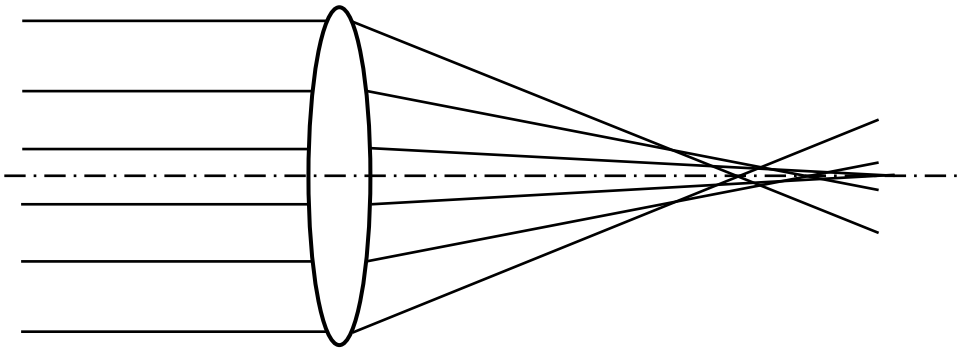




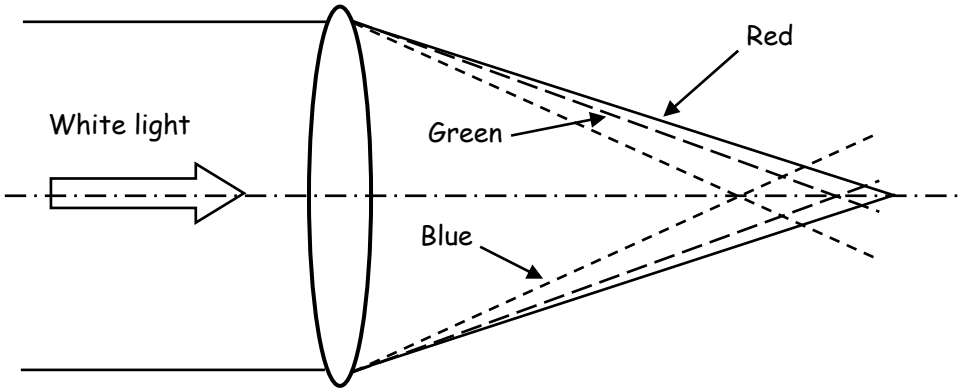


Aberrations (some examples)

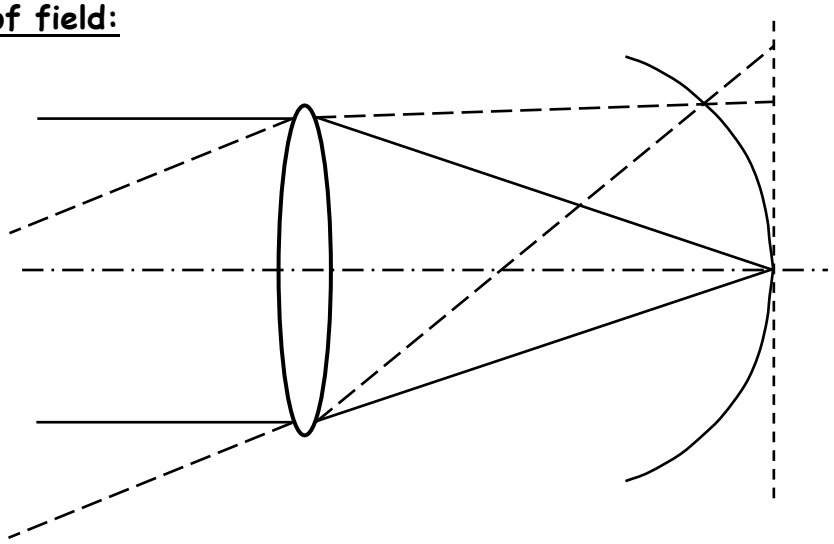
Spherical:



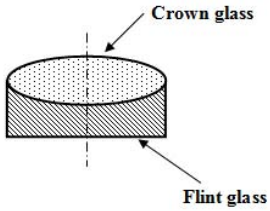
Chromatic:



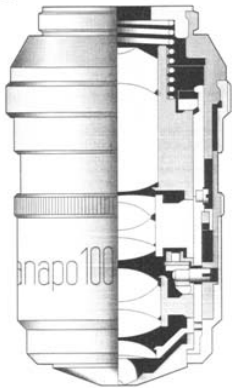
Curvature of field:



Objective corrections



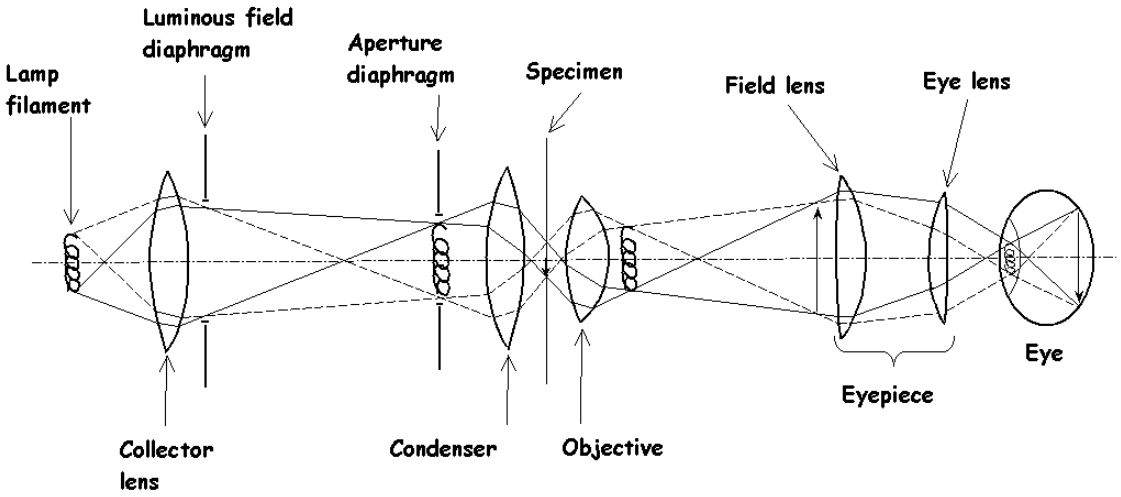
- Achromats
- Flat-field achromats
  "Plan"
- Fluorites (Fluar, Neo-fluar etc.)
- Flat-field fluorites (Plan-fluar etc.)
- Apochromats (Apo)
- Flat-field apochromats
  (Plan-Apo)



Köhler illumination

(August Köhler, 1866-1948)

- Uniform illumination
- Good light throughput
- Adjustable intensity
- Adjustable size of illuminated field
- Adjustable "diffuseness" of illumination



Web tutorial: http://micro.magnet.fsu.edu/primer/anatomy/kohler.html

Absorption staining

Brightly colored or very dark dyes are often used

Pros:

- **Dyes are usually cheap.**
- **Can be viewed with a simple (i.e. cheap) microscope.**

Cons:

- **Often limited to fixed (i.e. dead) and sliced specimens.**
- **Specific staining can be difficult (you may want to label only specific details of the specimen)**

Fluorophore molecules

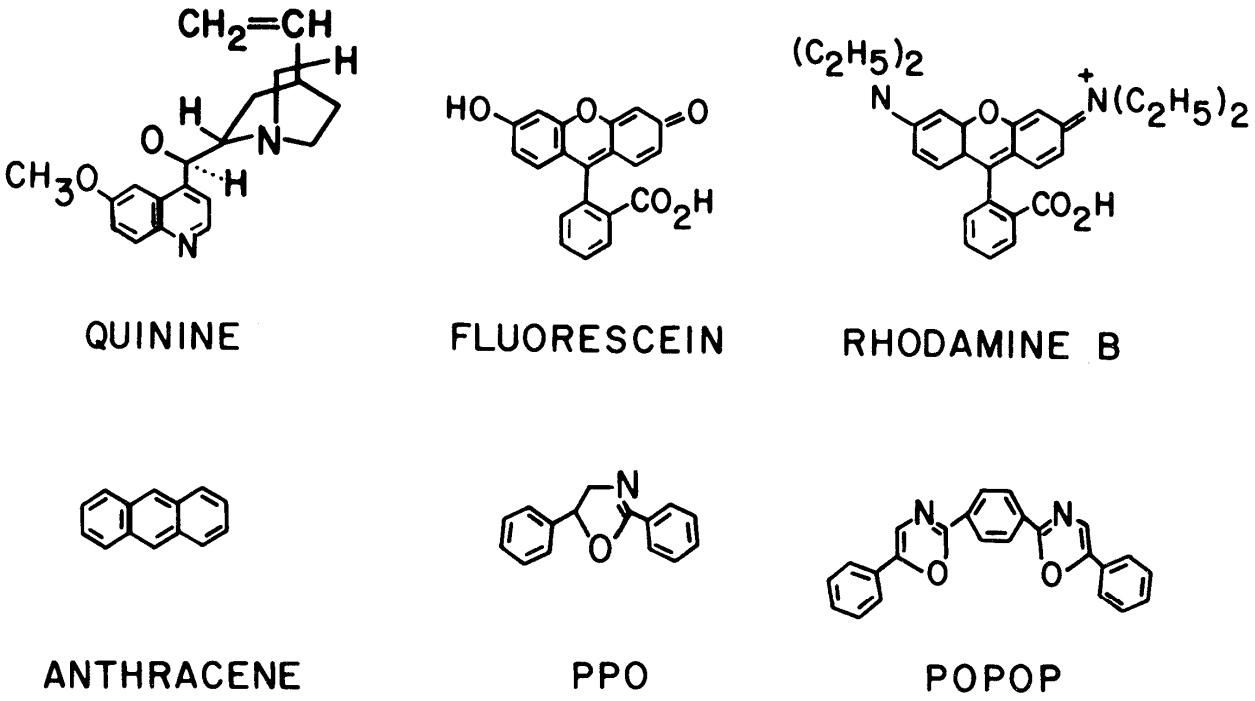
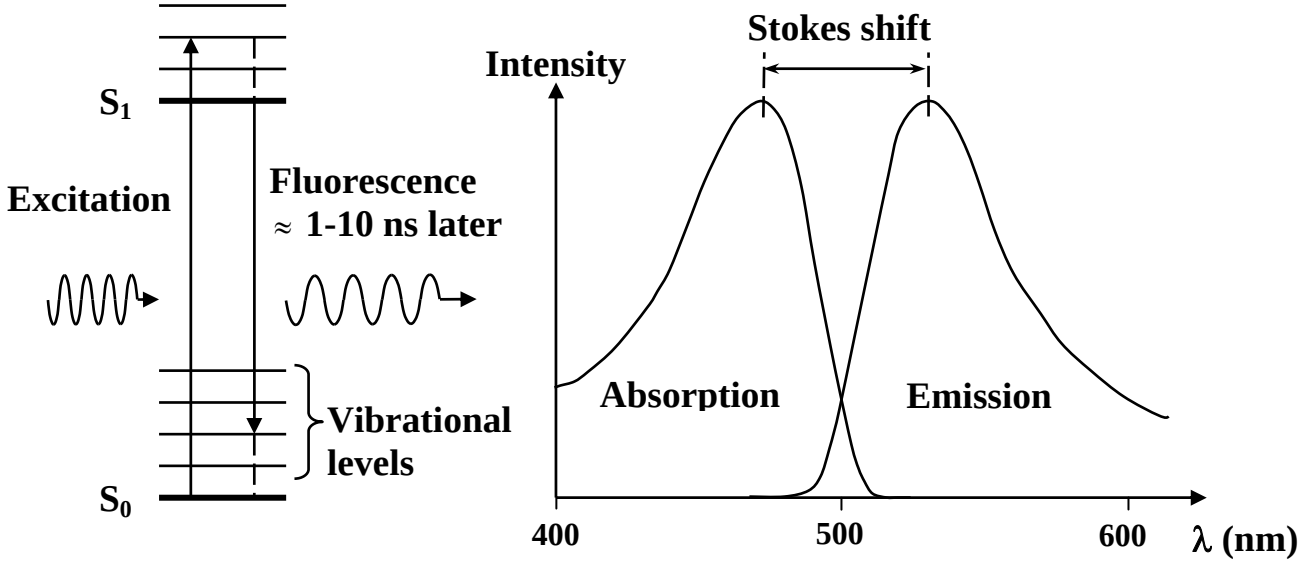
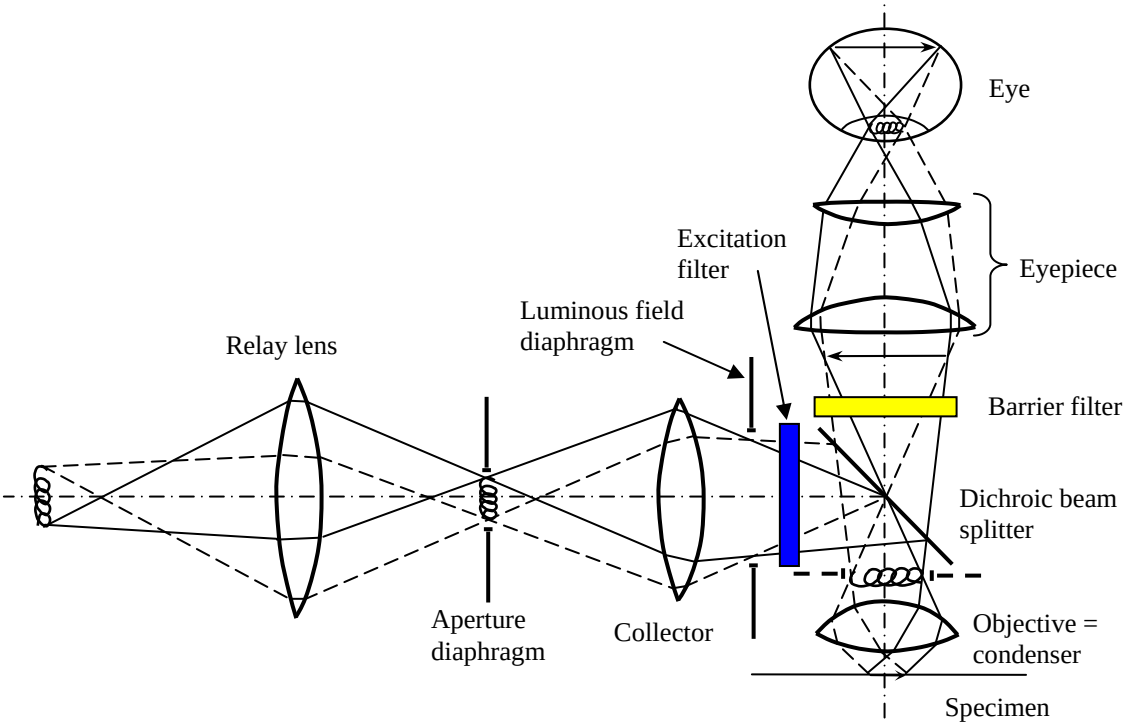
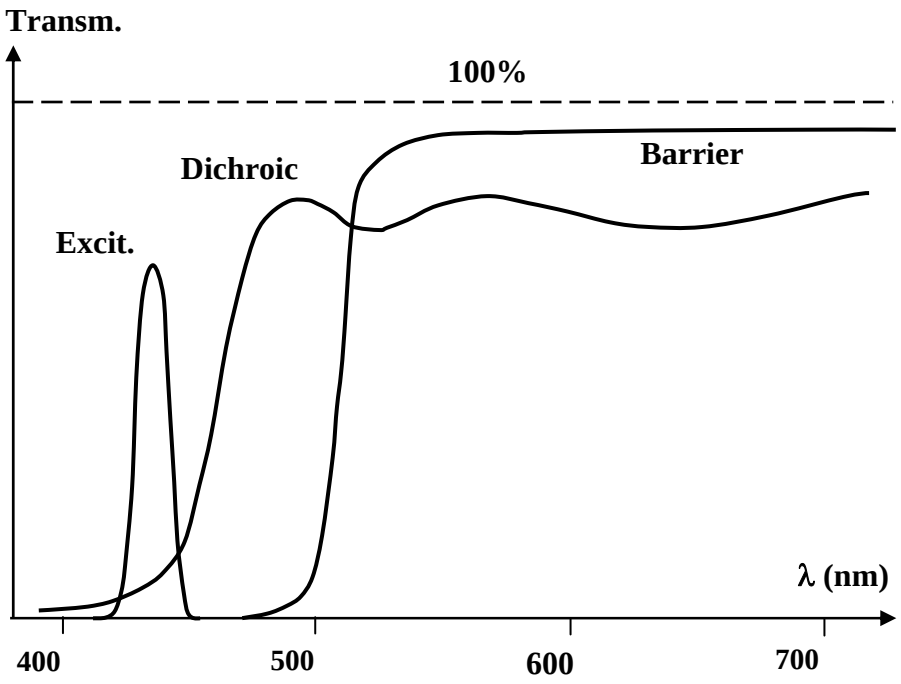


Figure 1.1. Structures of typical fluorescent substances.

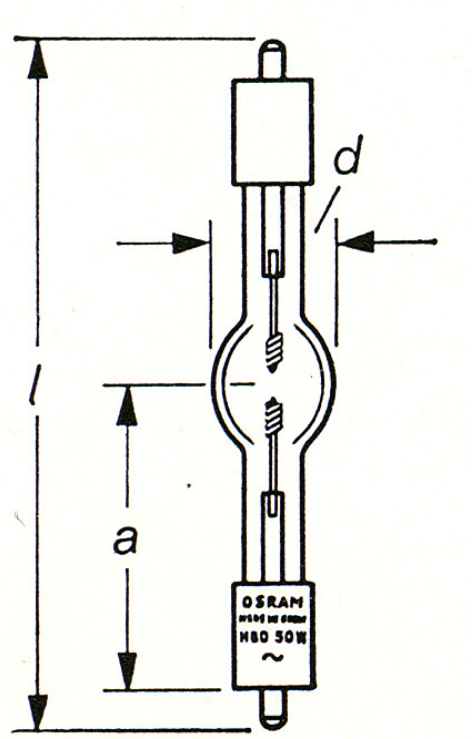


Köhler epi-illumination for fluorescence microscopy





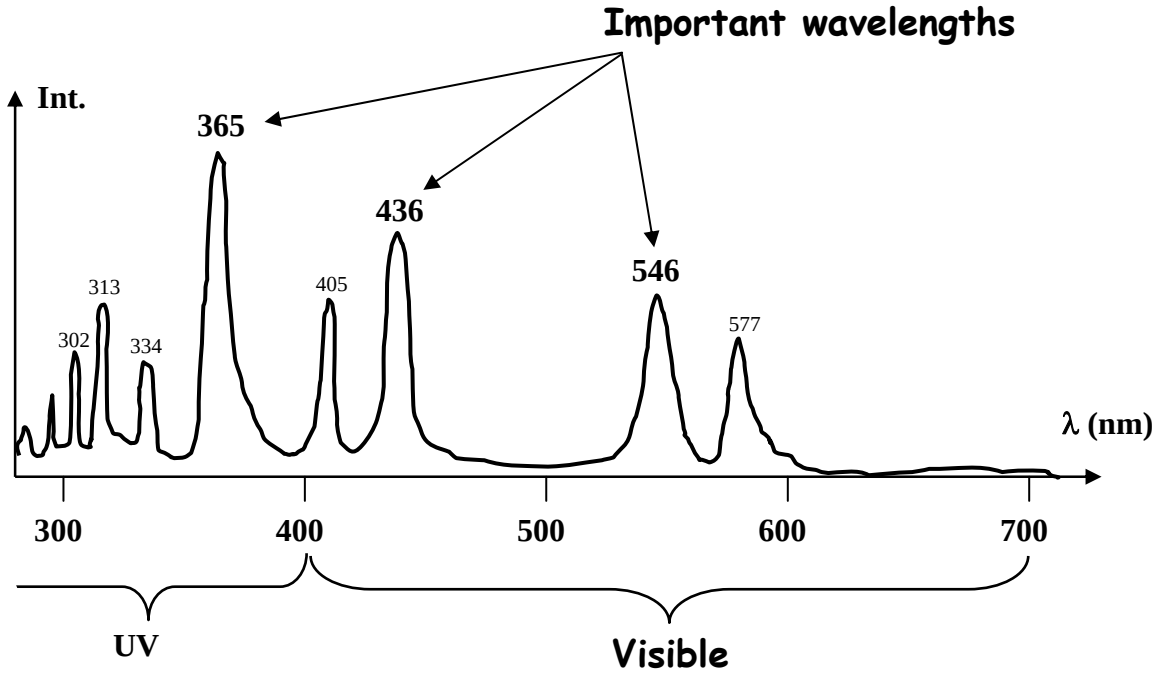
Hg lamp for fluorescence microscopy



HBO 50 W/AC
HBO 50 W/3

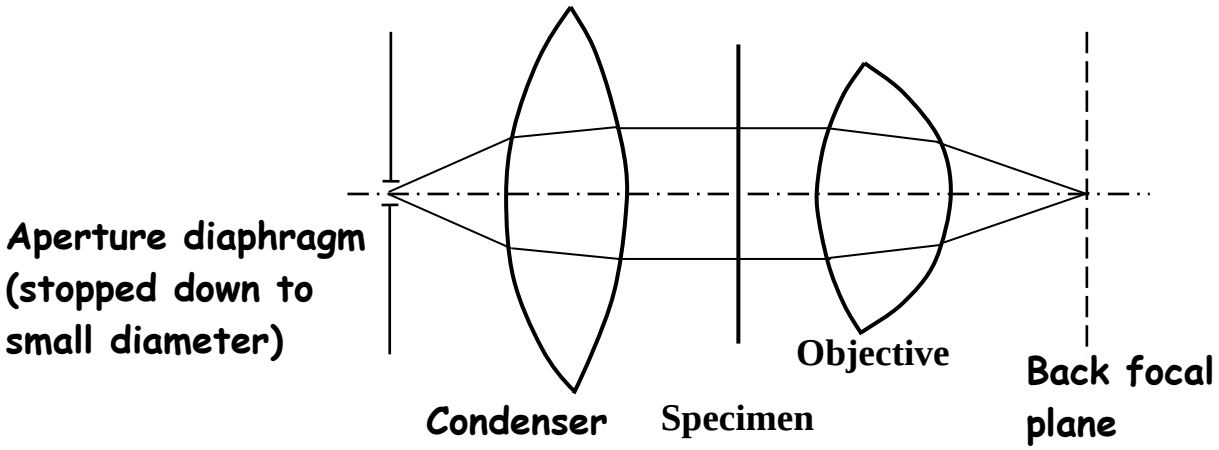
Caution:

- May explode
- May give harmful UV radiation



Phase contrast

Idea: Transform phase variations into amplitude variations.



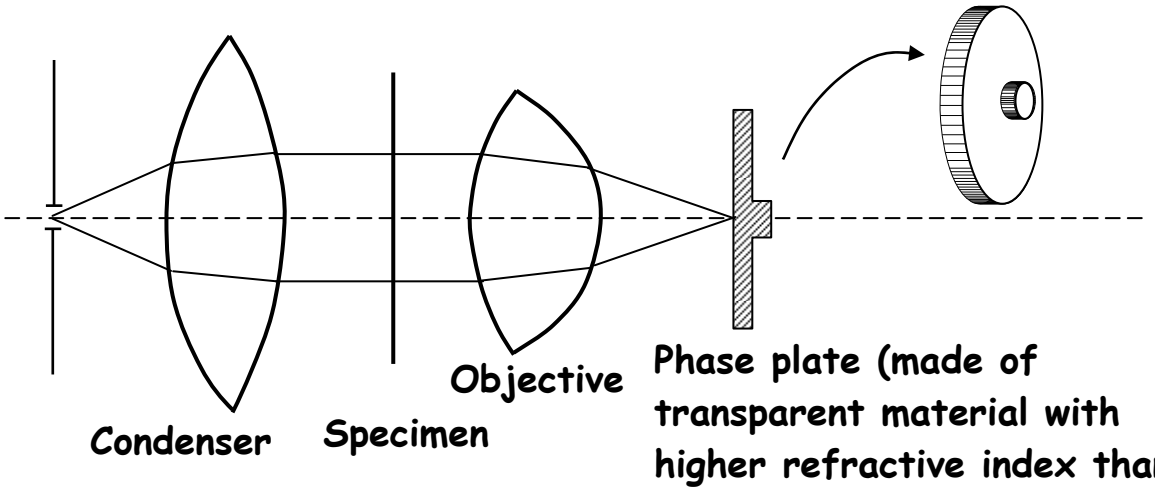
Consider the situation in the back focal plane of the microscope objective:

- **On the optical axis the light waves will be the same, regardless of whether the specimen absorbs light or just introduces an optical pathlength difference.**

- **In all other parts of the back focal plane, the light waves will be phase shifted by $\pi/2$ (corresponding to $\lambda/4$) for a phase specimen compared with an absorbing specimen.**

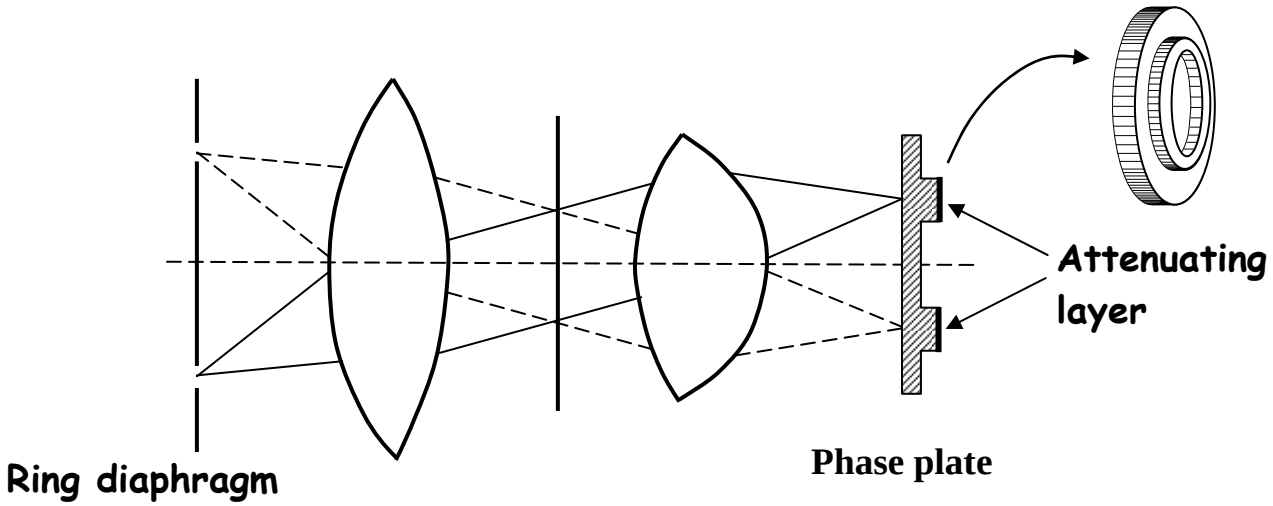
Therefore:
Introduce a phase shift of $\pi/2$ between light that passes thru the central and peripheral parts of the back focal plane.
The situation will now be identical to the case where we have an absorbing specimen.

Hence the image will display intensity variations that correspond to optical pathlength differences!



In practice, two modifications are made:

1. Ring-shaped illumination and phase plate.
2. Attenuation of light that passes thru the phase ring.



The ring-shape is used to increase light throughput and resolution.

The attenuating layer produces higher image contrast for specimens with small optical pathlength variations.

- **Phase contrast works well on thin and highly transparent specimens.**

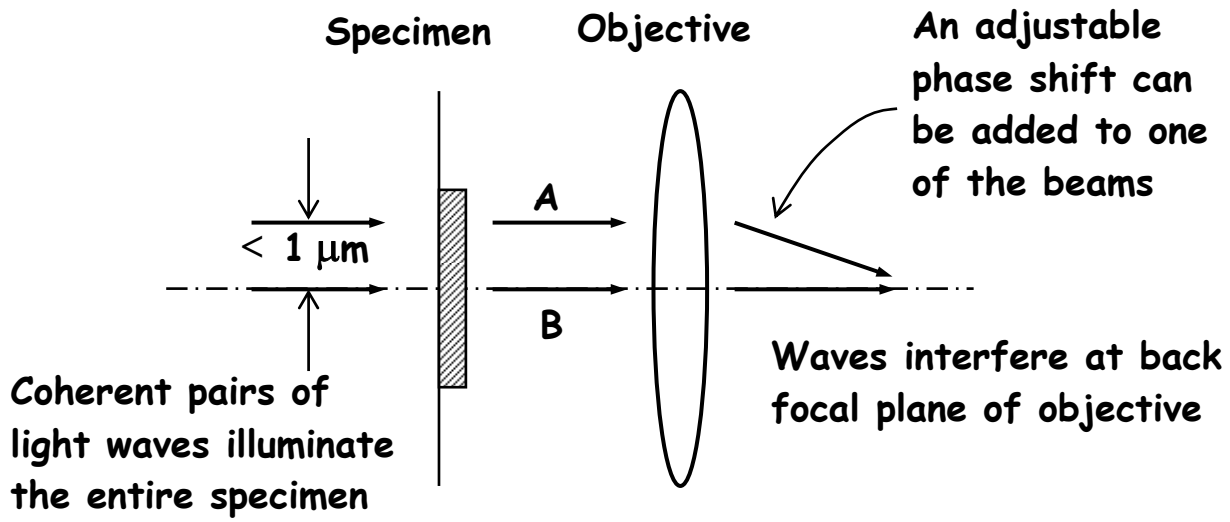
- **Ideally monochromatic light should be used, but in practice white light often works well.**

- **White haloes seen in image are artifacts because the width of the phase-shifting ring is not infinitely small.**

Differential interference contrast (DIC)

Idea: Transform phase gradients into amplitude variations

Simplified schematic diagram

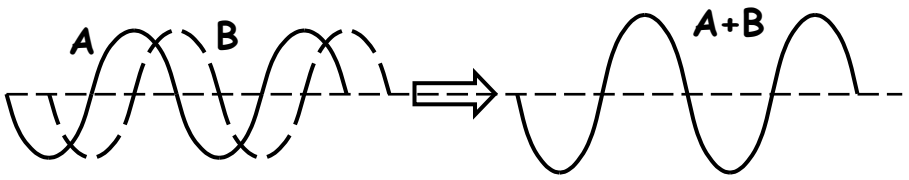


(*more details in compendium*)

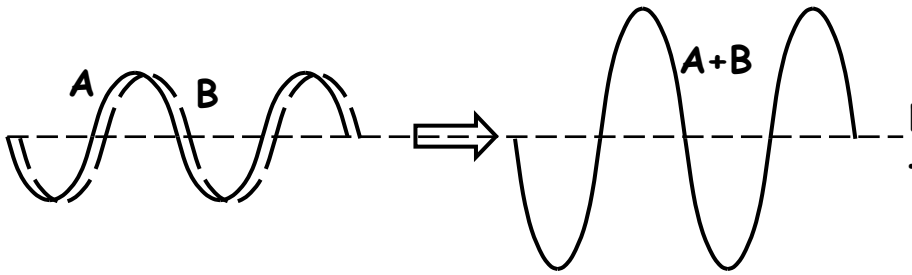
Examples of results:

In all cases we assume that the adjustable phase shift introduced to beam A is $\pi/2$.

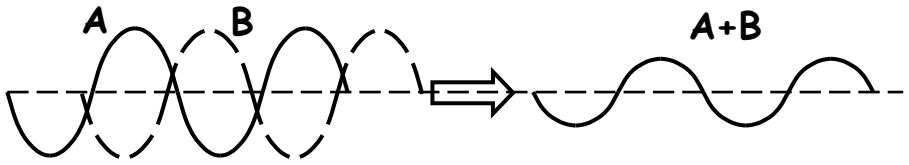
1. **Optical pathlength (OPL) thru specimen is the same for both A and B (OPL gradient = 0):**



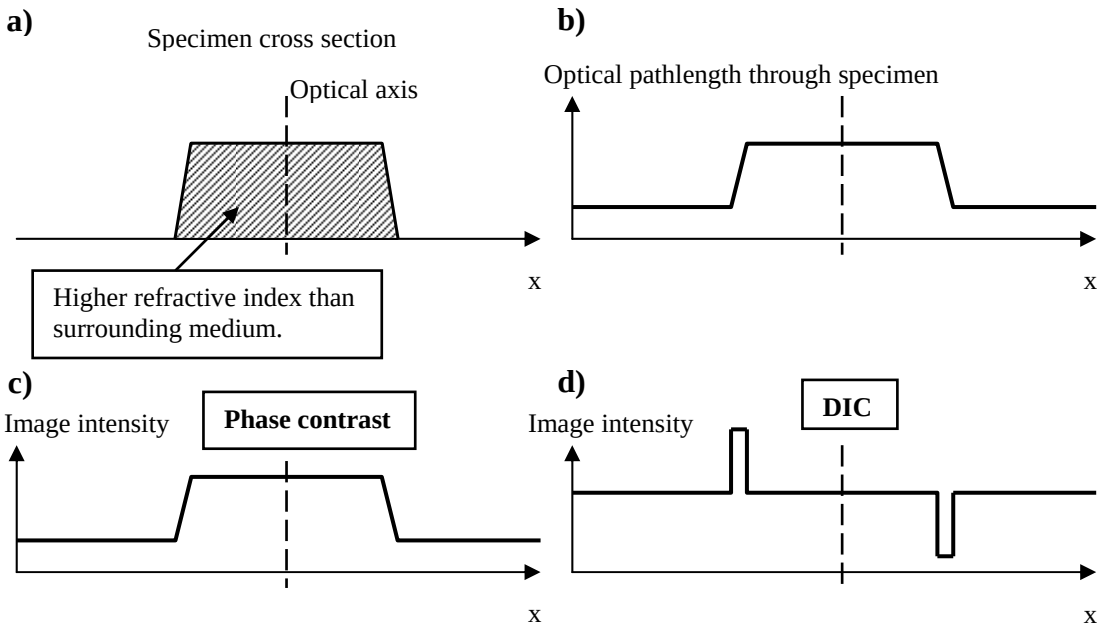
2. **OPL for A is longer than for B (OPL gradient > 0)**



3. **OPL for A is shorter than for B (OPL gradient > 0)**



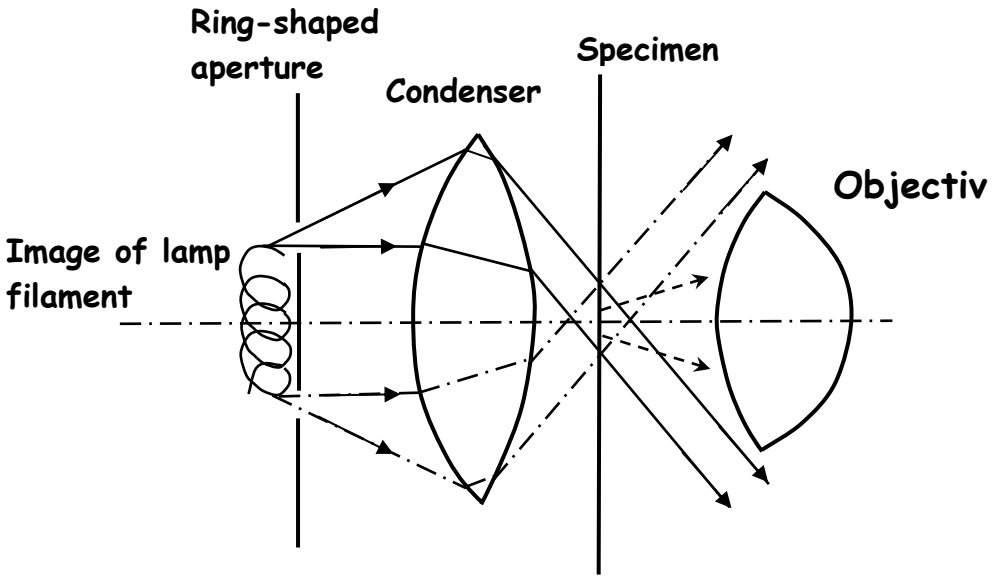
Comparison: Phase contrast/DIC



- **Compared with phase contrast, DIC produces images without haloes and with higher resolution. Thicker specimens can also be studied.**

- **On the negative side, DIC produces images that are more difficult to interpret. What looks like 3D surface structures is in reality optical pathlength gradient.**

Dark-field imaging



- No direct light from the lamp will reach the objective. Therefore background is black

- Scattered, refracted or diffracted light from extremely small and weak objects can be detected with good signal-to-noise ratio.

Relative brightness for different objectives

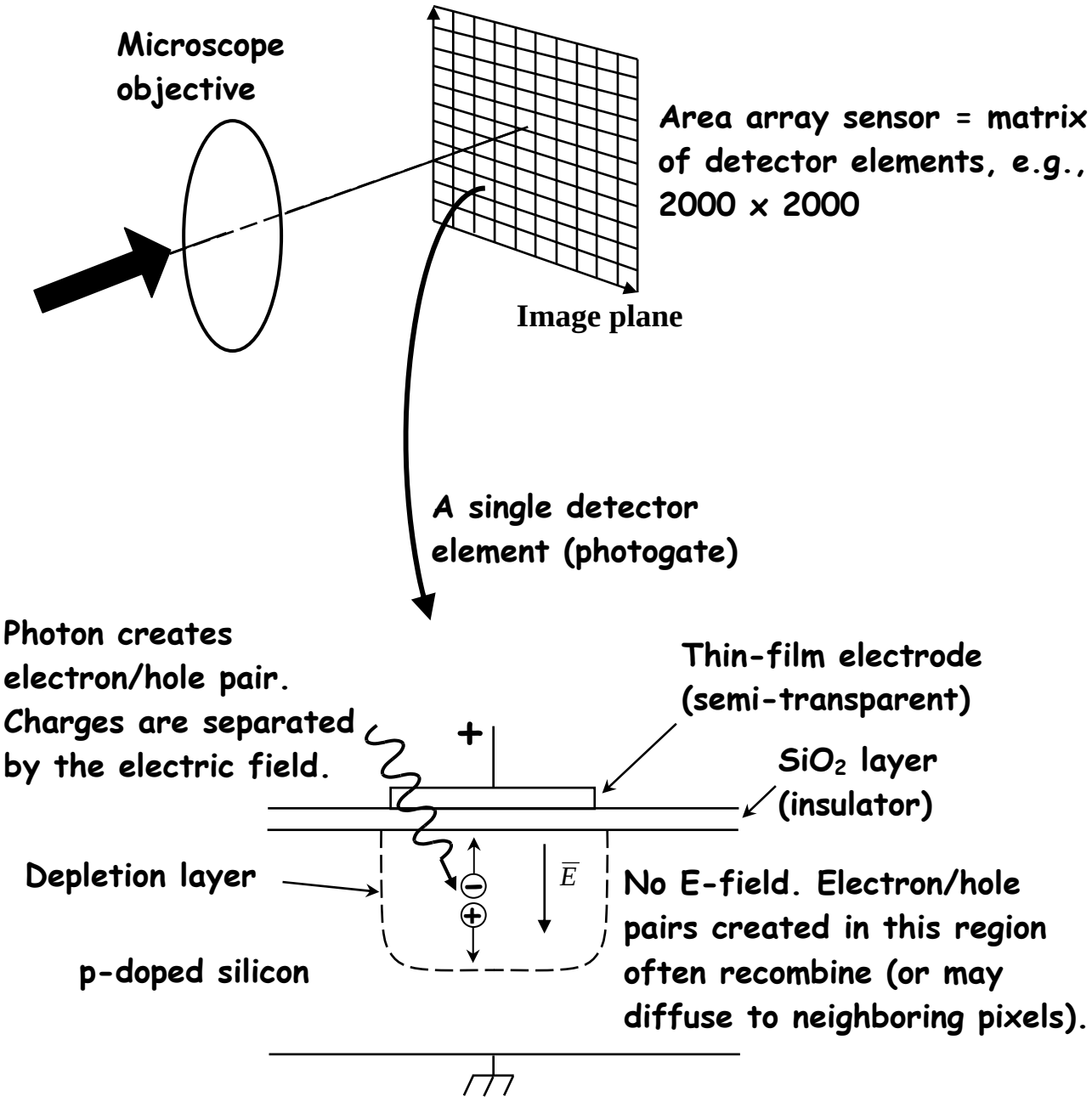
Transmitted-light microscopy,
weakly scattering specimen.

Objective	(N.A.)/M	Rel. brightness
10/0.3	0.03	1.00
40/1.0	0.025	0.69
100/1.3	0.013	0.19

Epi-fluorescence microscopy

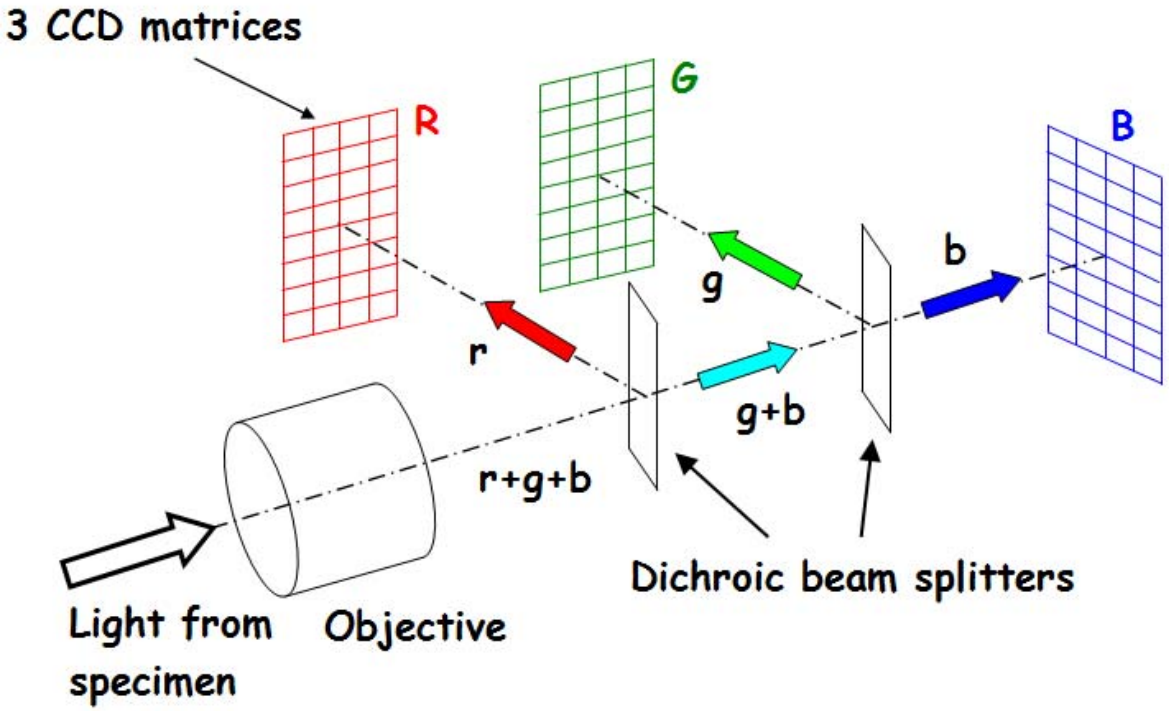
Objective	(N.A.)/M	Rel. brightness
10/0.3	0.03	1.00
40/1.0	0.025	7.7
100/1.3	0.013	3.5

Image recording in microscopy



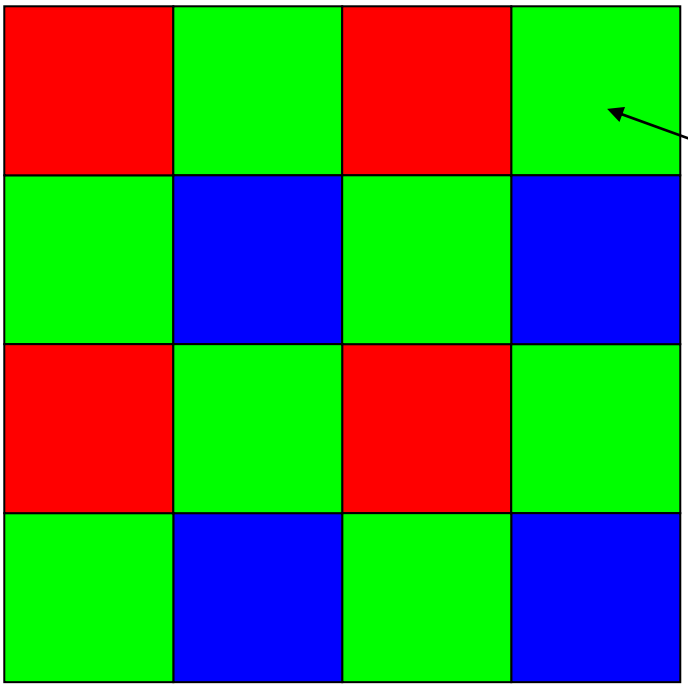
Electrons from individual pixels are collected ⇒ Charge-to-voltage conversion ⇒ ADC ⇒ Digital storage

Color recording

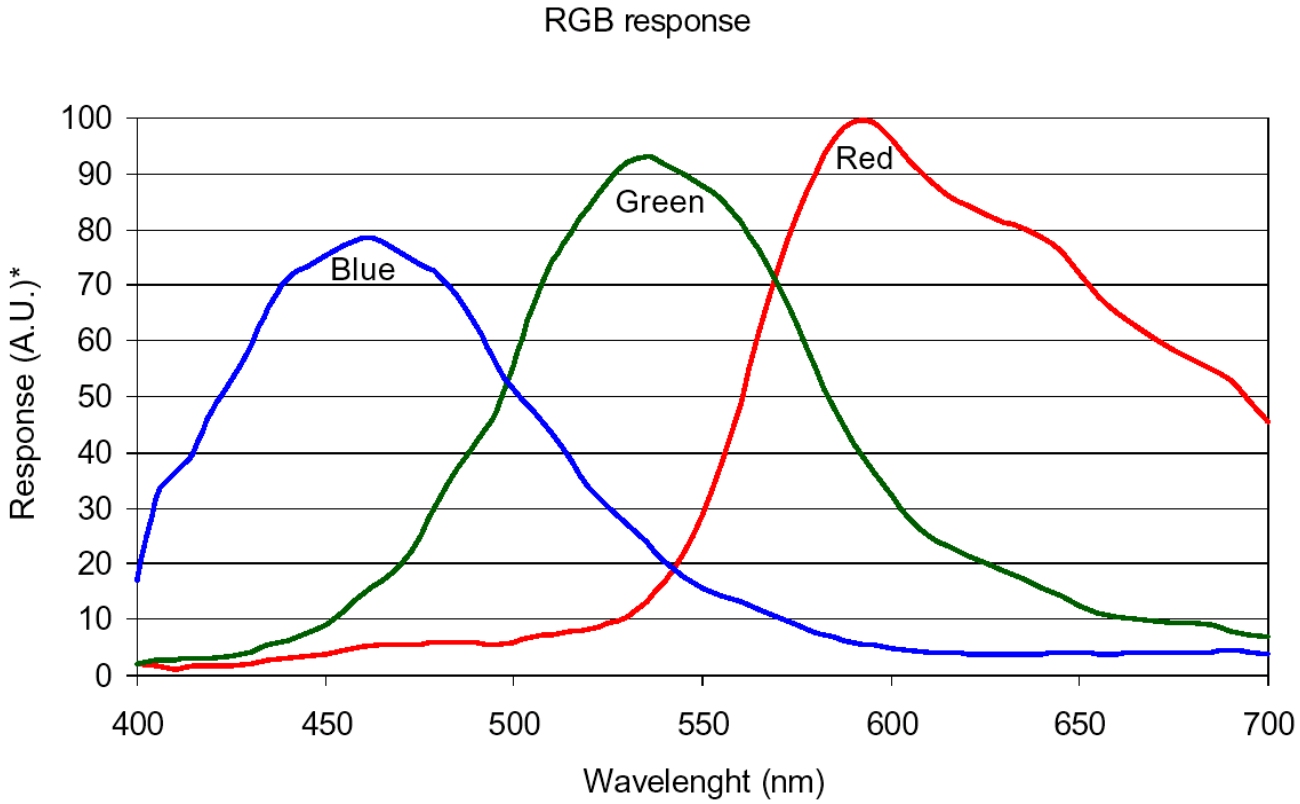


Often used in video cameras

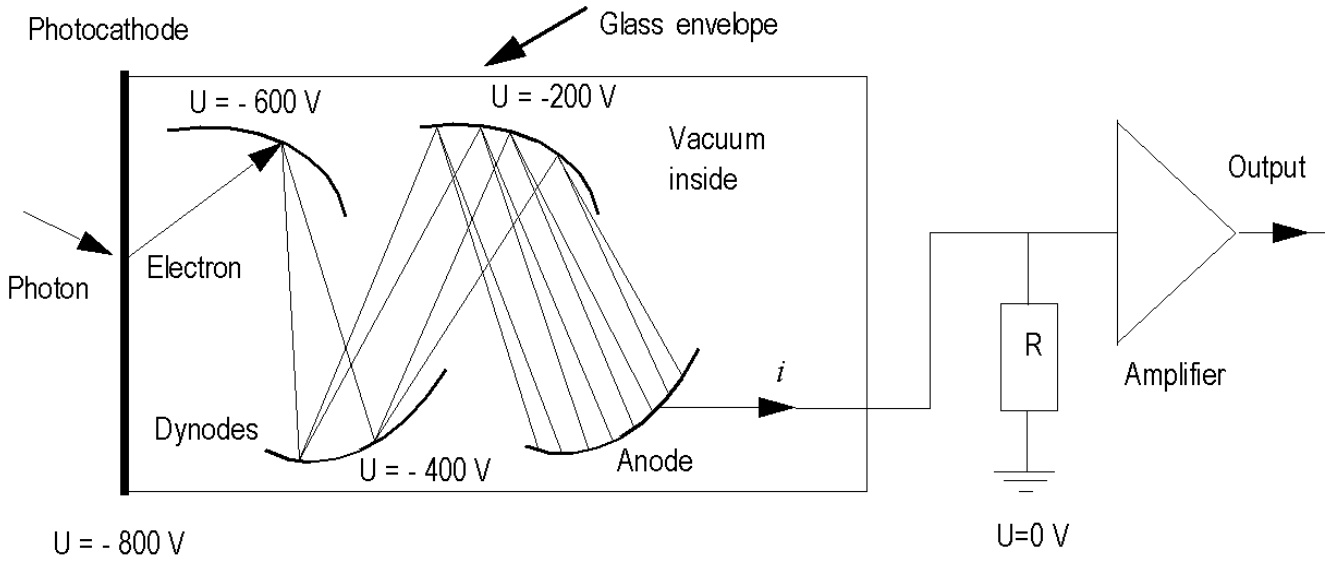
RGB Bayer pattern



Each pixel only detects one primary color. The other two primaries are obtained through interpolation



Photomultiplier tube (PMT)



- **Single detector (image recording requires opto-mechanical scanning).**
- **Very high sensitivity due to electron multiplication (ca. 10^6).**
- **Often used in confocal scanning laser microscopes.**

Detector characteristics

- Spectral sensitivity
- Quantum conversion efficiency
- Dark current
- Noise
- Signal-to-noise ratio
- Dynamic range
- MTF
- Aliasing