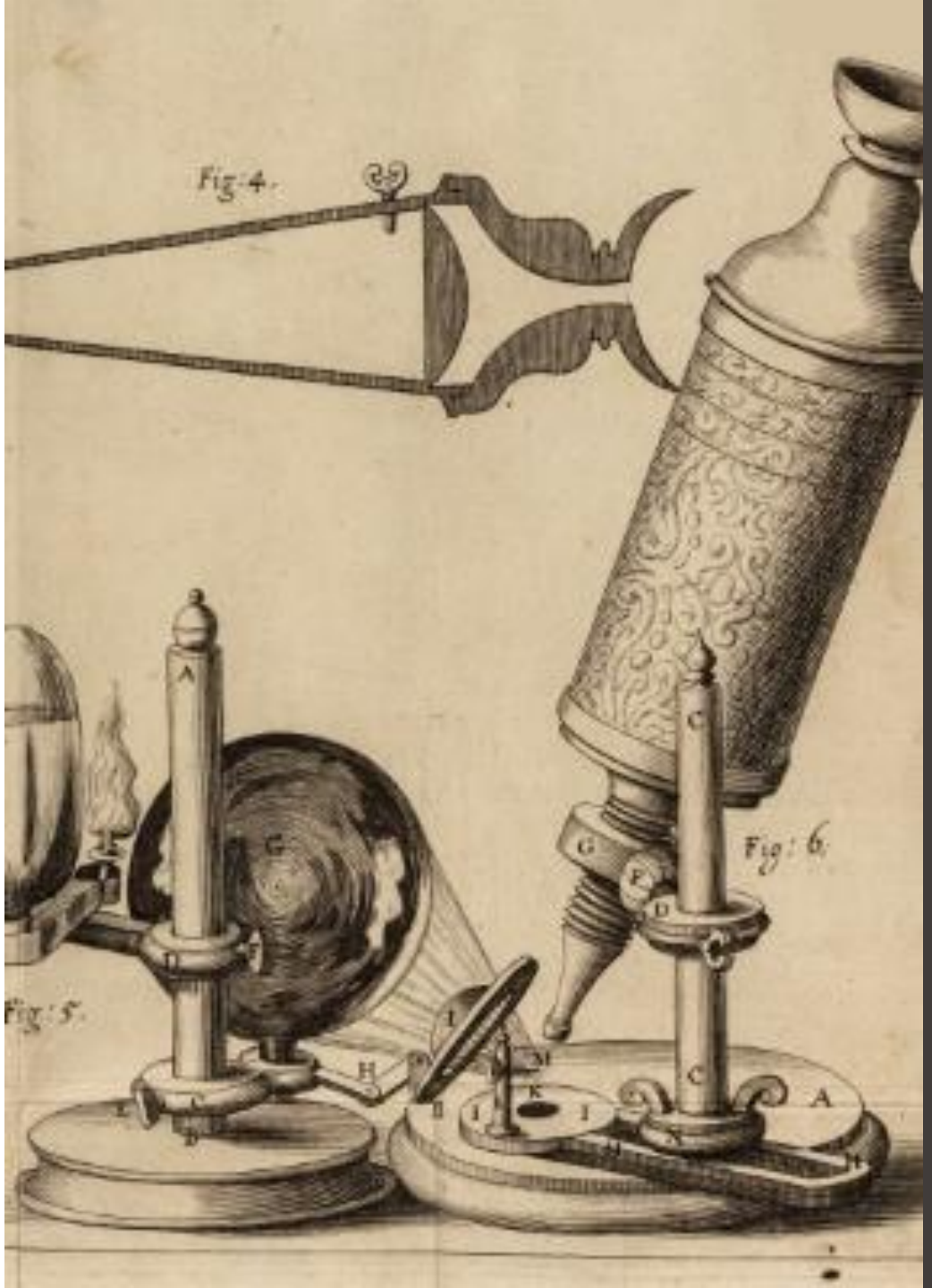

Advanced Characterization of Materials at Micro-, Nano- and Atomic Scale

Basics in Light Microscopy

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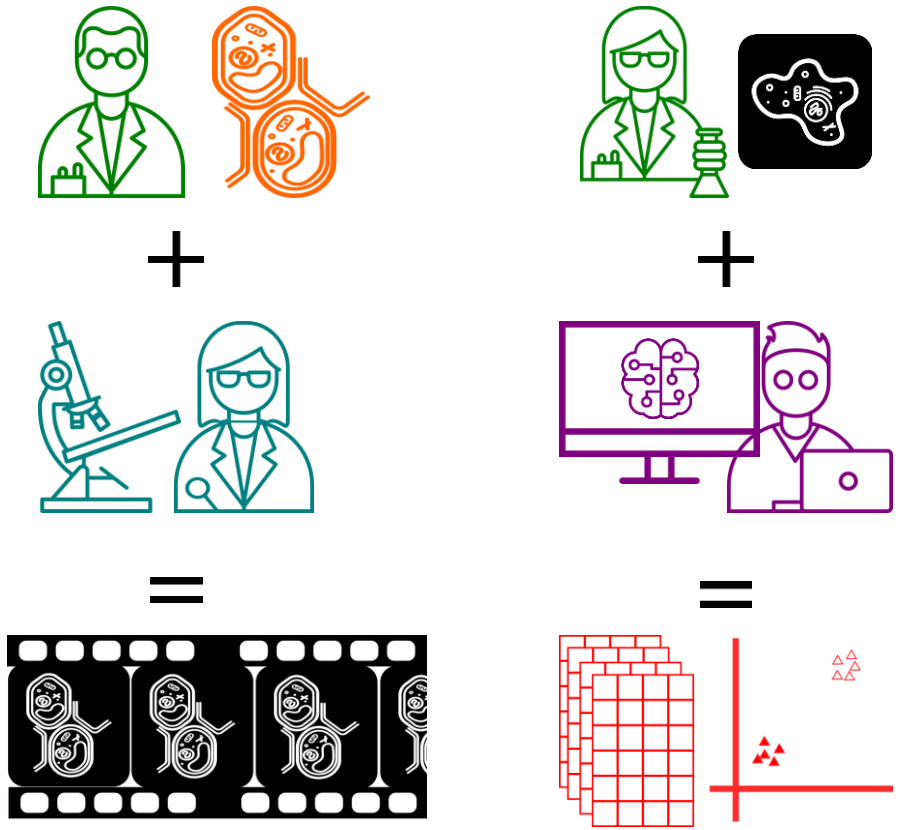
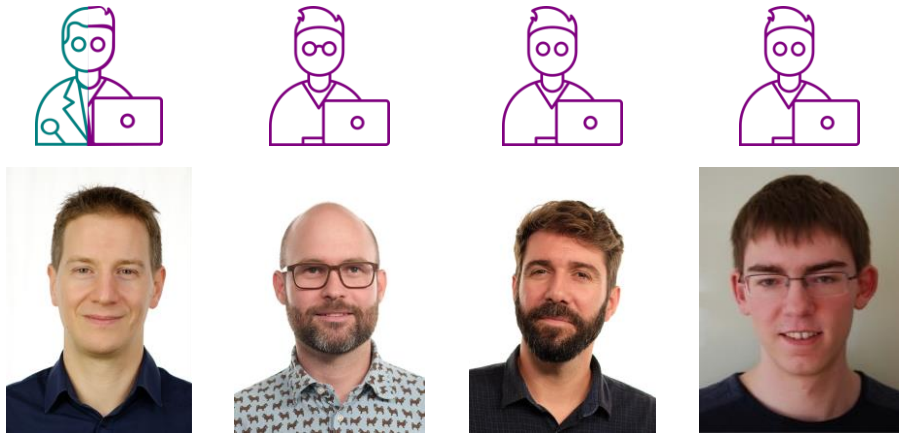
BioImaging & Optics Platform (BIOP)



Anjalie Schläppi | José Artacho | Thierry Laroche | Agnieszka Pierzynska



Nicolas Chiaruttini | Olivier Burri | Romain Guiet | Remy Dornier

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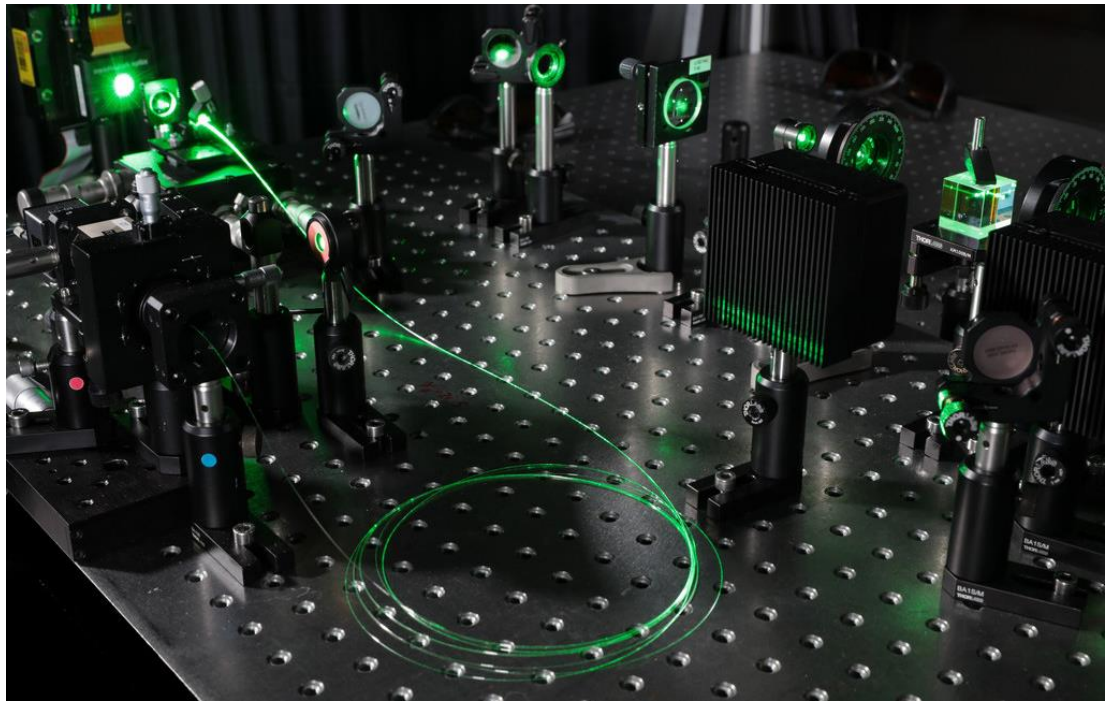
Optics



- Definition
  - optics, science concerned with the genesis and propagation of light, the changes that it undergoes and produces, and other phenomena closely associated with it.
- Physical optics
  - Nature of light
  - Propagation of light
- Geometrical optics
  - Image formation properties of lenses, mirrors, and other devices

https://www.britannica.com/science/optics

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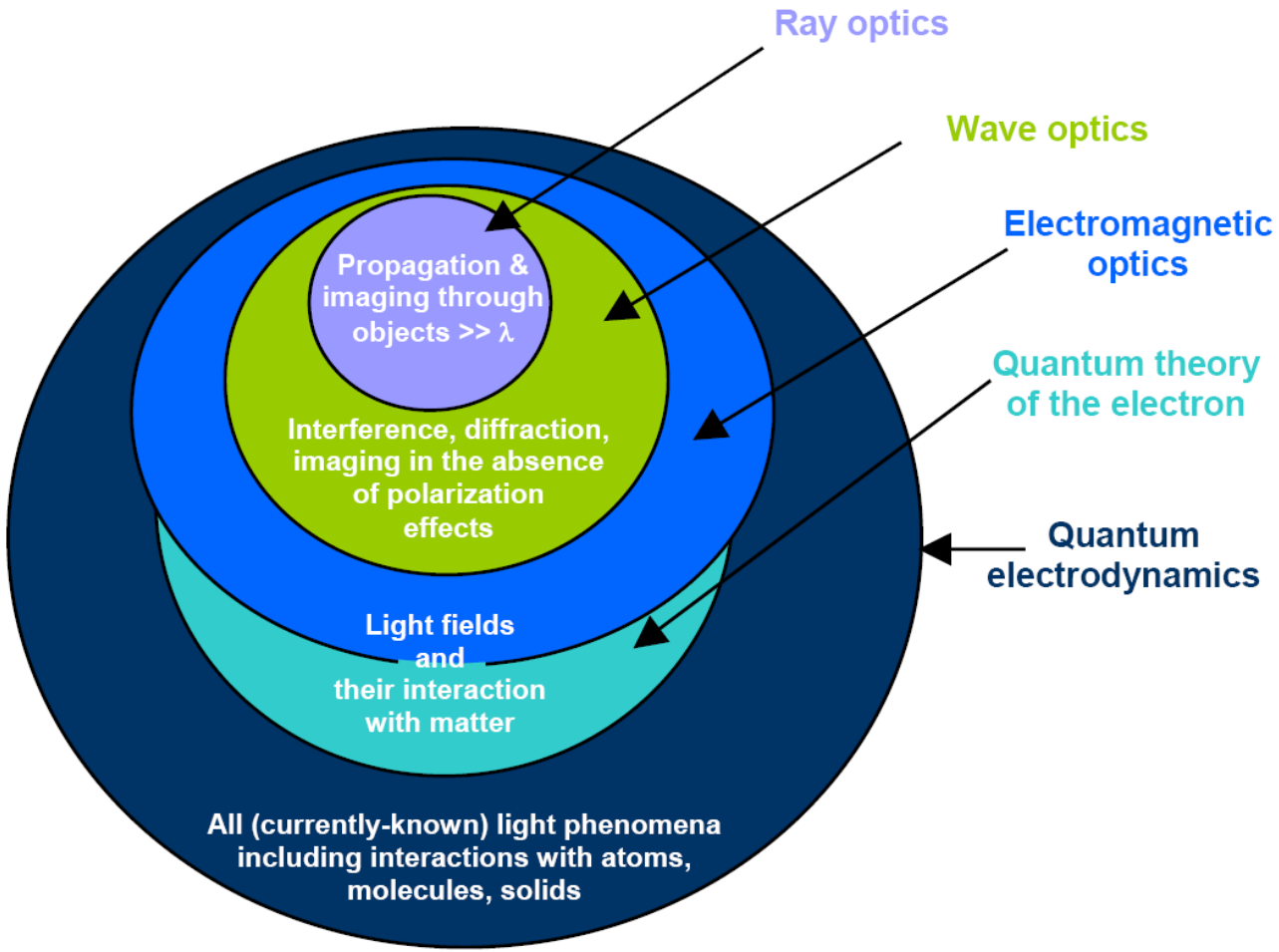
Theories for Light (Optics)



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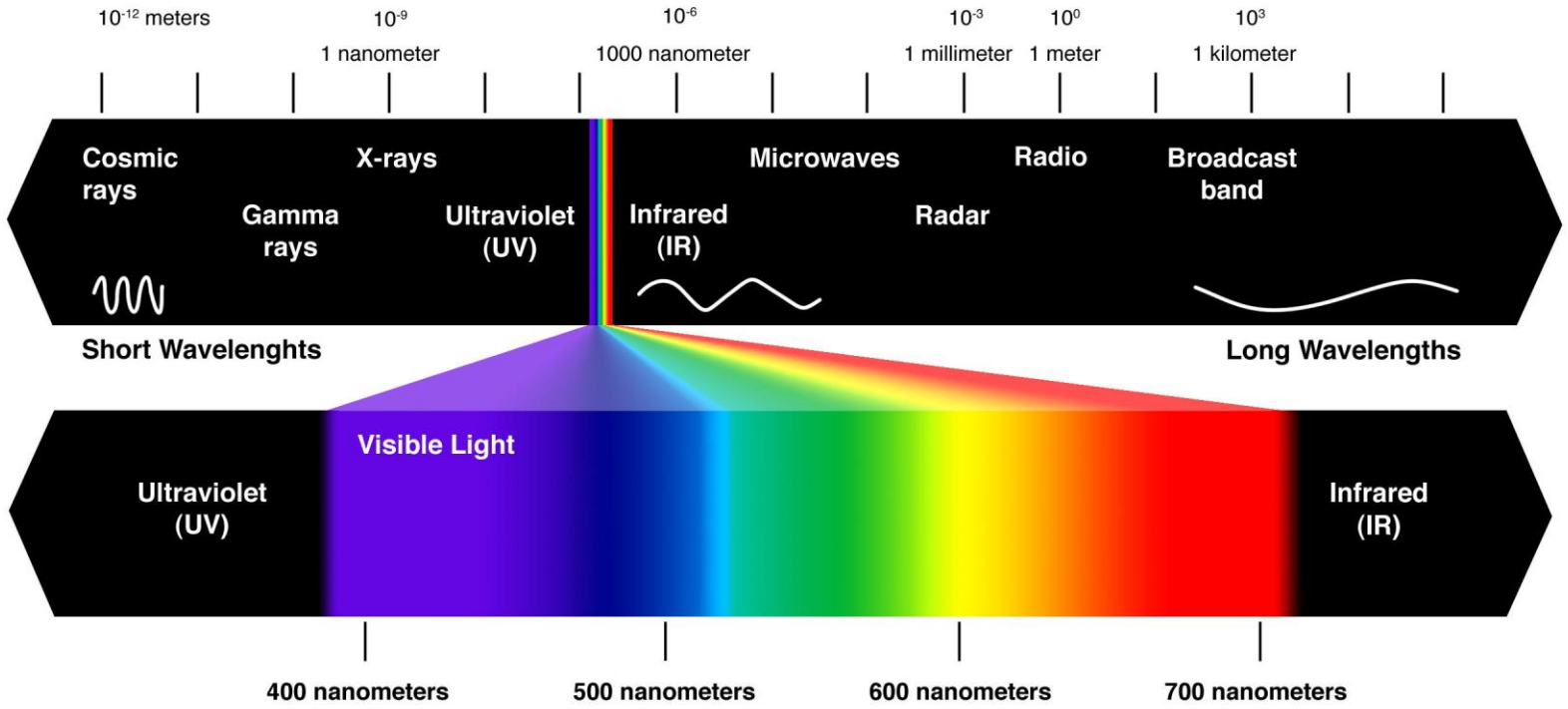
Electromagnetic spectrum



- λ for visible spectrum is 400 – 700 nm
- In order for ray optics to hold object size (e.g. lenses) should be >> λ

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Why do we need microscopy?

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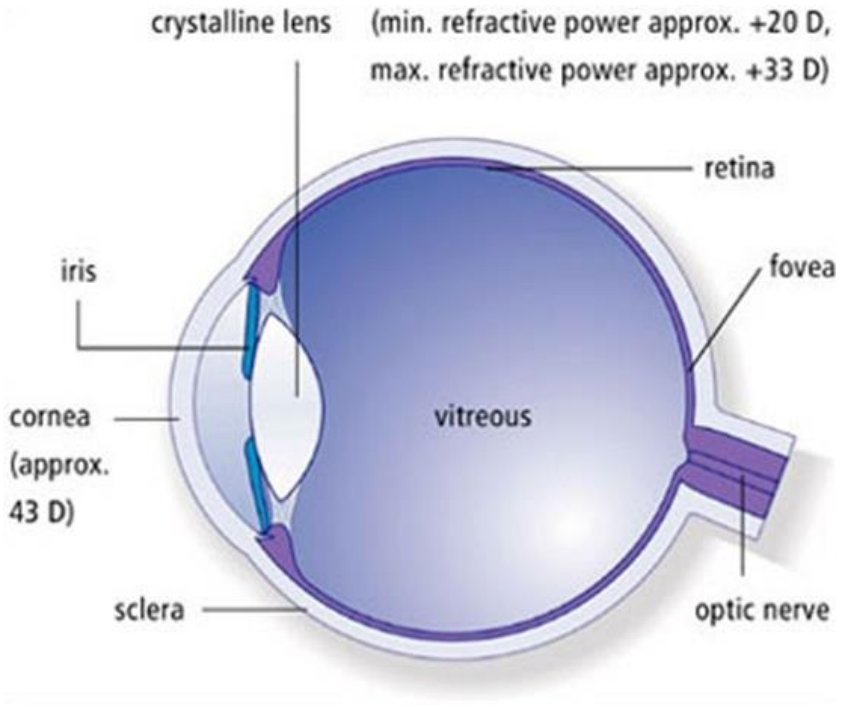
Human eye



Normal viewing distance - 250 mm
Angular resolution $a_{min} \approx 1'$
Spatial resolution $h_{min} \approx 80\ \mu m$
Nodal distance -17 mm
Average retinal cell distance 1.5 mm
Spectral range 400 nm - 800 nm
Can resolve contrast about 5%
High dynamic range – 10 decades
Max sensitivity at 505 nm (night, rods)
Max sensitivity at 555 nm (day, cones)
More sensitive to color than to intensity

Most perfect sensor for light detection up to now

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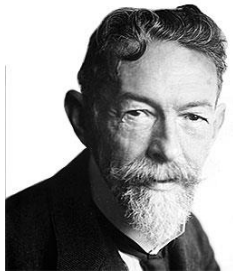
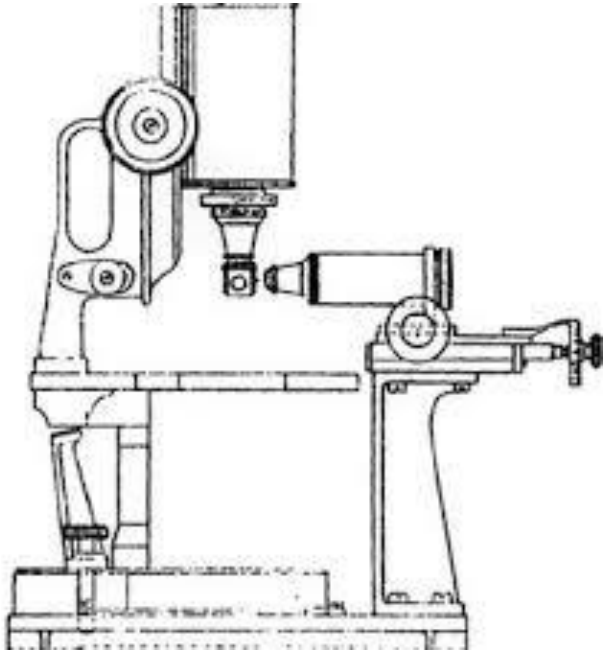
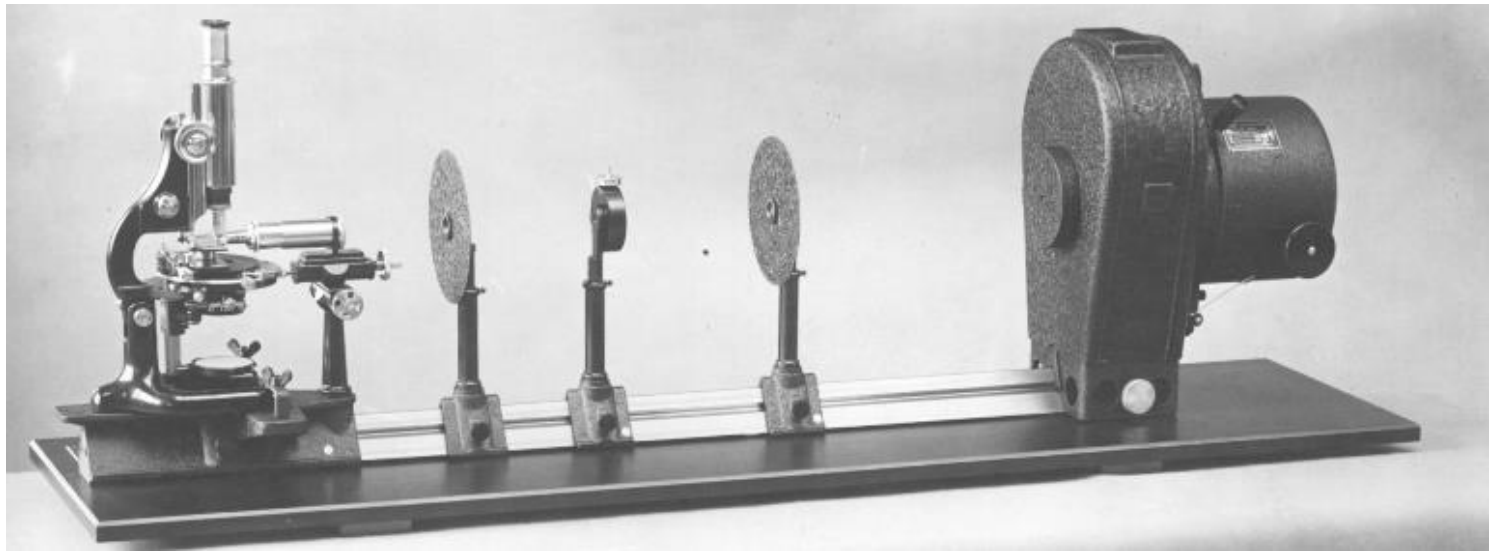
Ultramicroscopy







Siedentopf, H. and Zsigmondy, R. (1902). Über Sichtbarmachung und Größenbestimmung ultramikoskopischer Teilchen, mit besonderer Anwendung auf Goldrubingläser. *Ann. Phys.* 10, 1-39.

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Lens based imaging

Image formation

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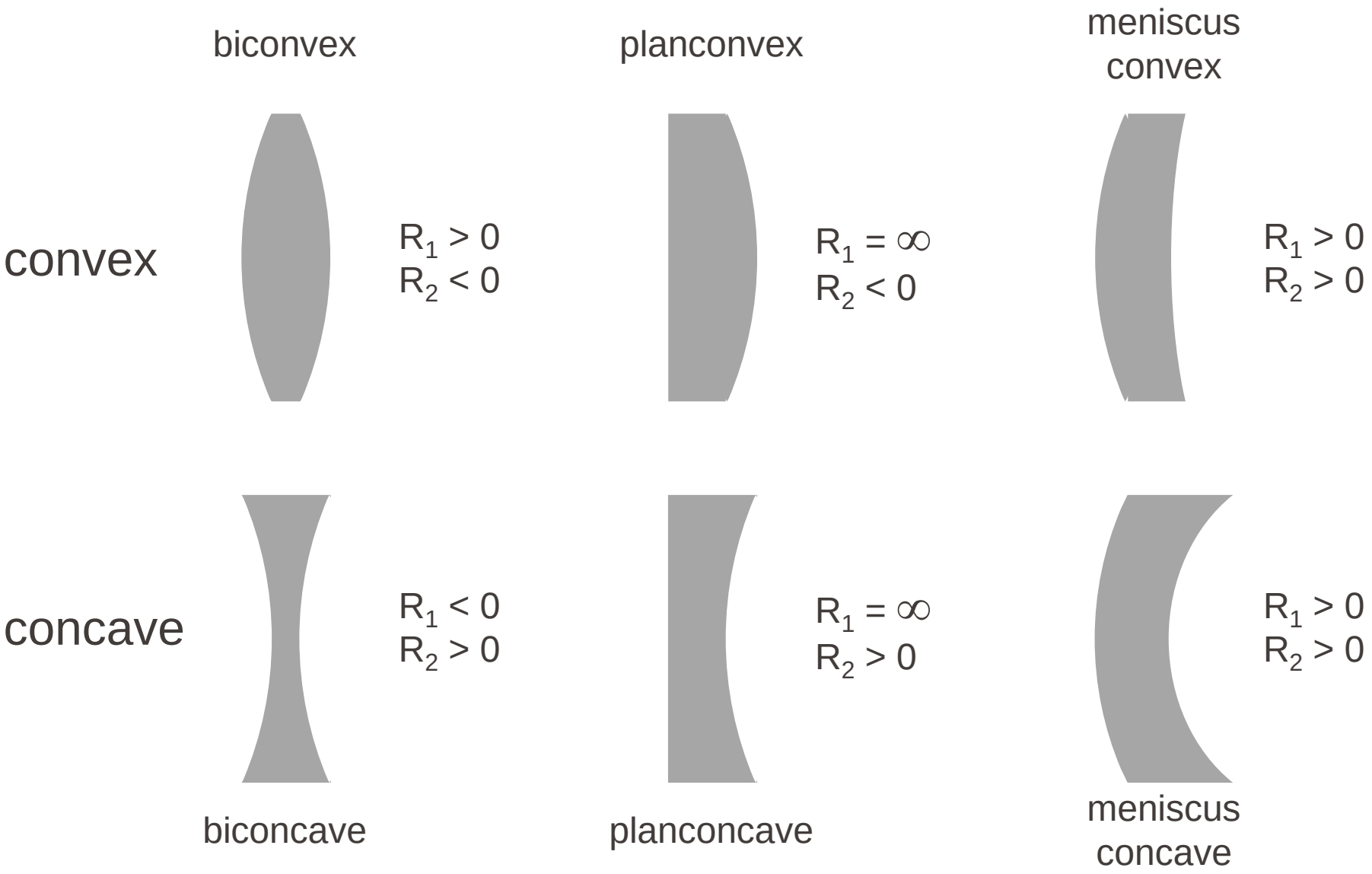
Spherical Lens Types



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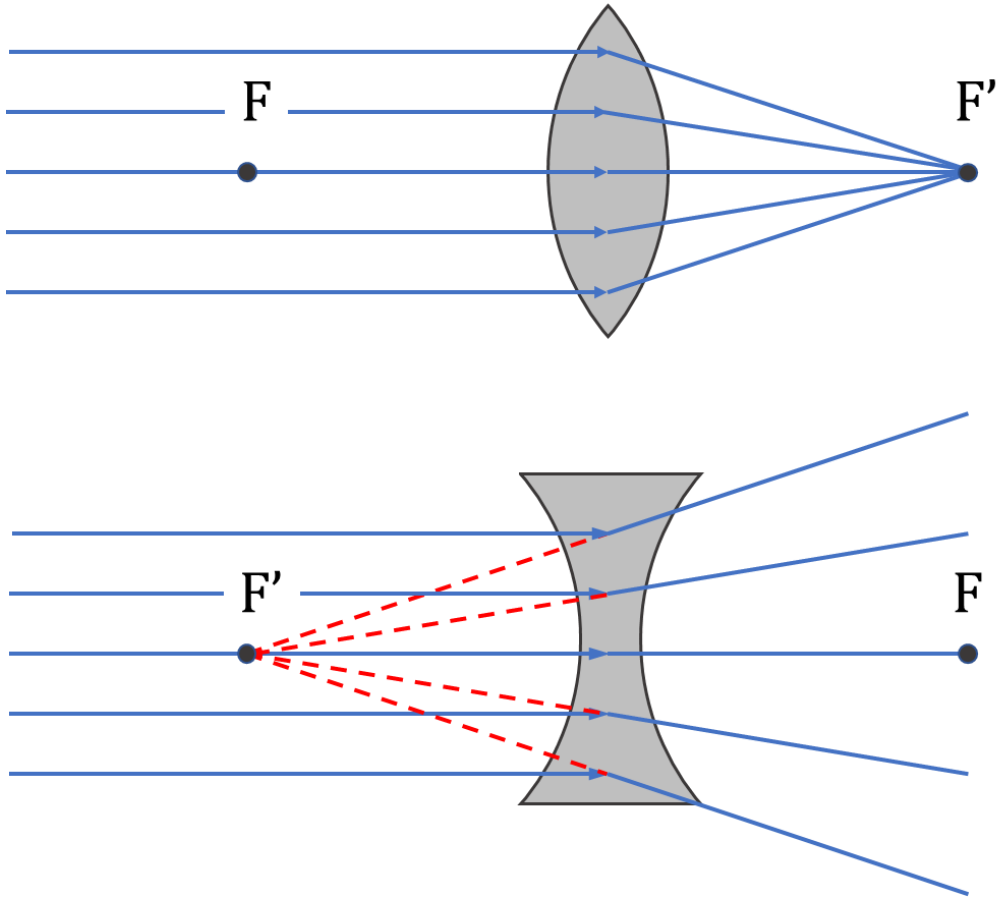
Lense types
Image formation



- Convex lenses
  - convergent
  - f: positive
  - Real and virtual images
- Cancave lenses
  - divergent
  - f: negative
  - virtual images

Note: virtual images can be recorded.

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Image Formation

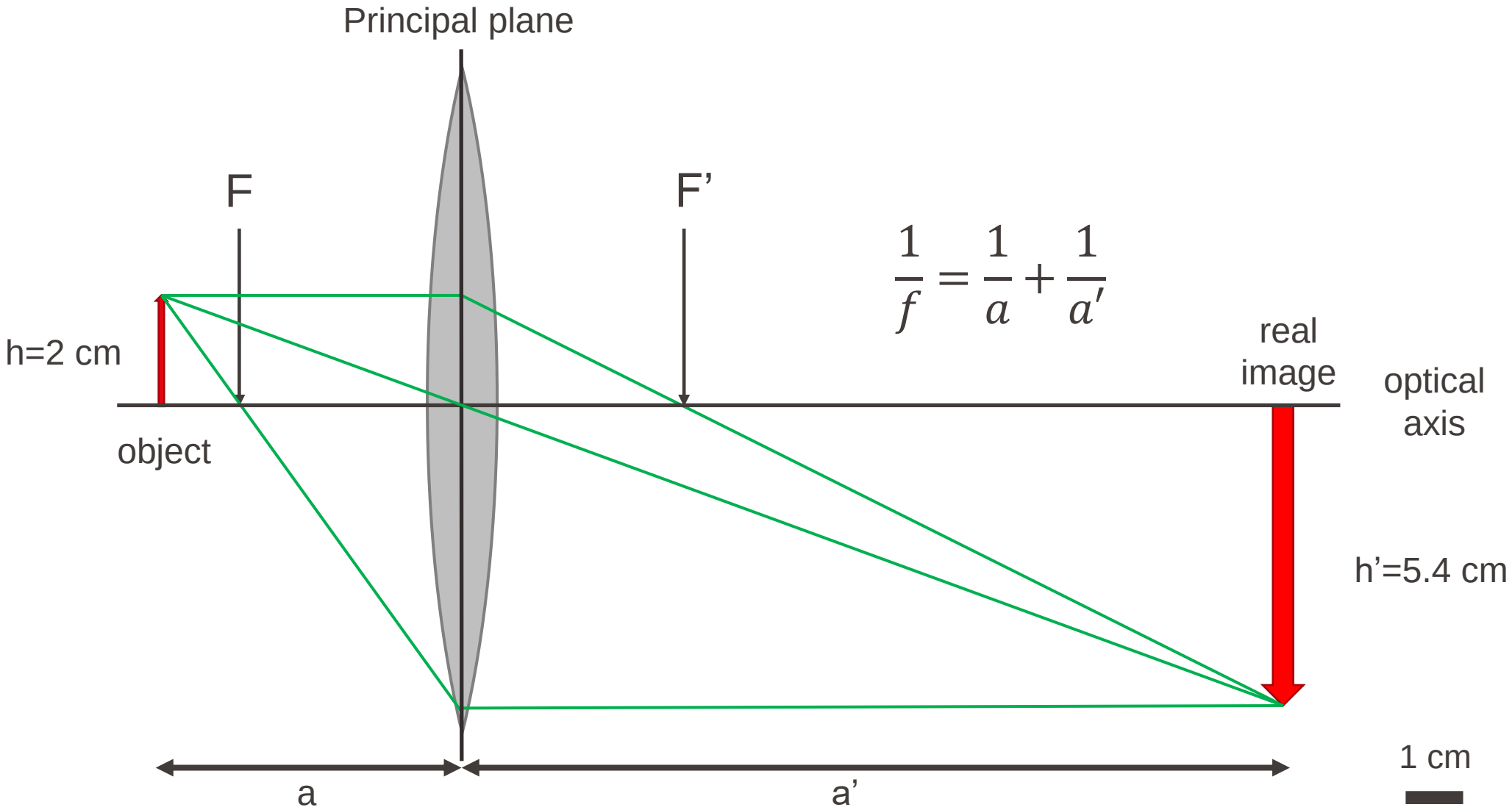

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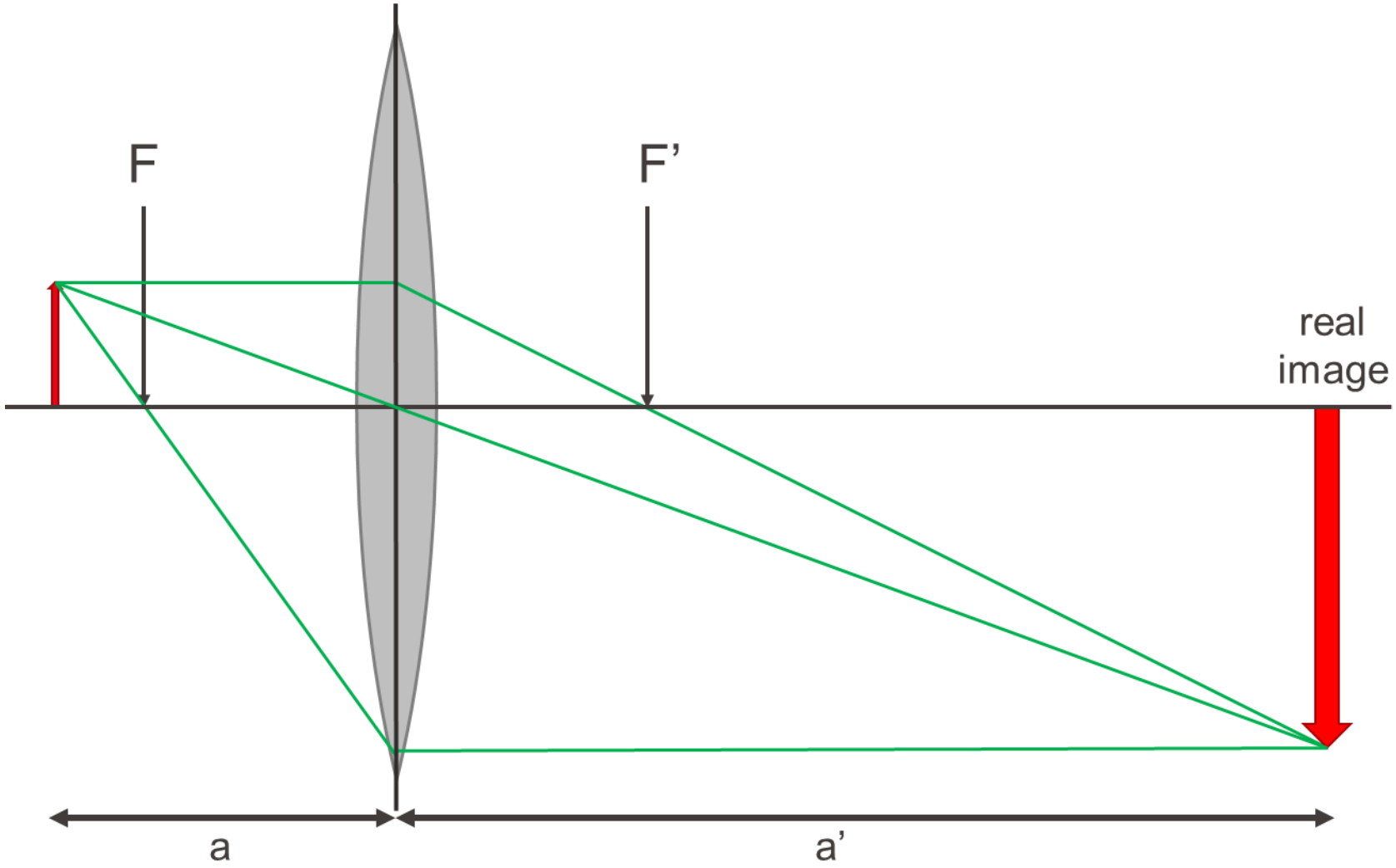
Image Formation



$$\frac{1}{f} = \frac{1}{a} + \frac{1}{a'}$$

$a = 5.5\ cm$
$a' = 14.9\ cm$
$f = \ \ 4.0\ cm$

$$^1/_a \ = 0.182\ cm^{-1}$$
$$^1/_{a'} \ = 0.067\ cm^{-1}$$
$$^1/_f = \ \ 0.25\ cm^{-1}$$

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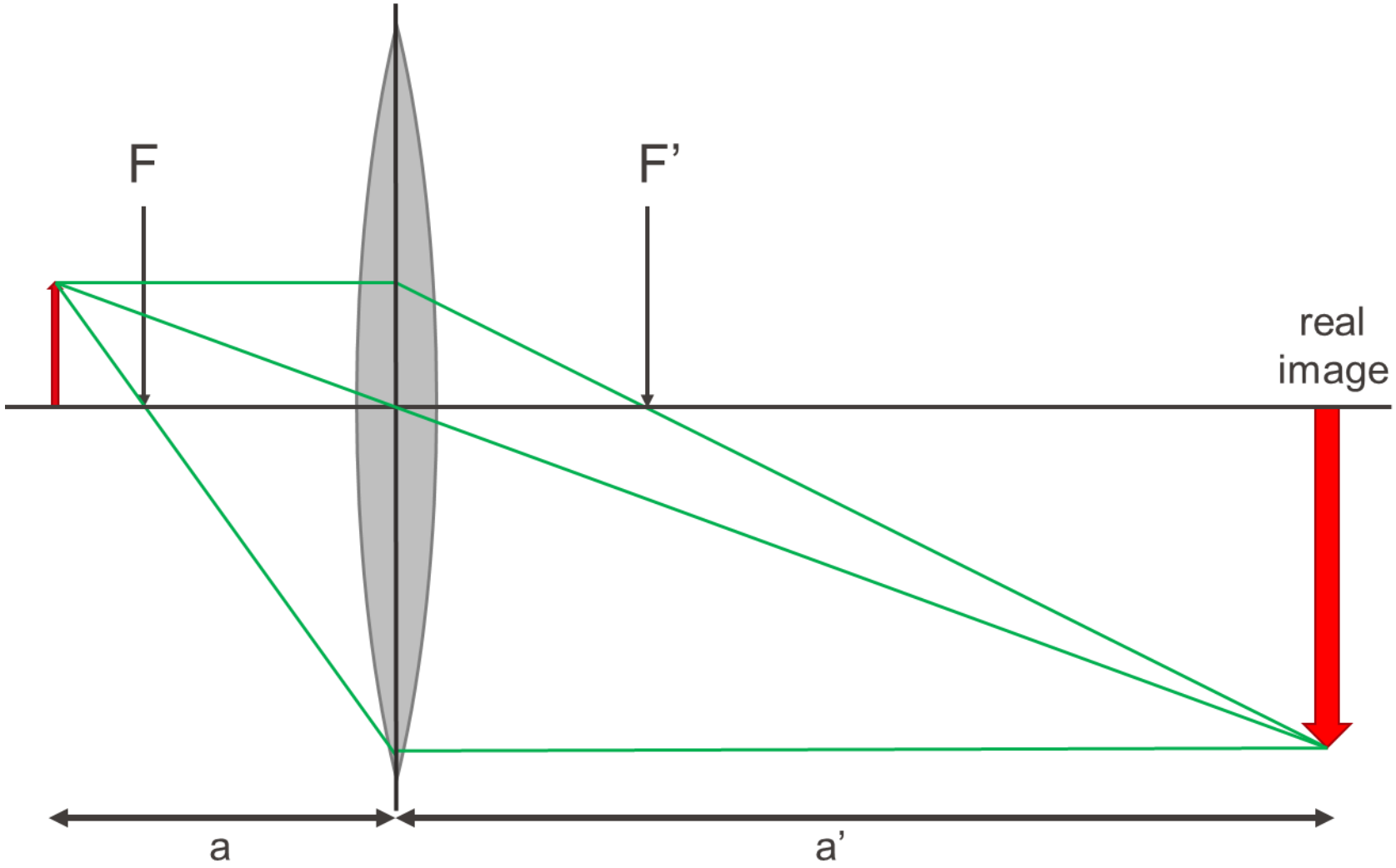
Image Formation



$$\frac{1}{f} = \frac{1}{a} + \frac{1}{a'}$$

$$M = \frac{a'}{a} = 2.7$$

$$M = \frac{h'}{h} = 2.7$$

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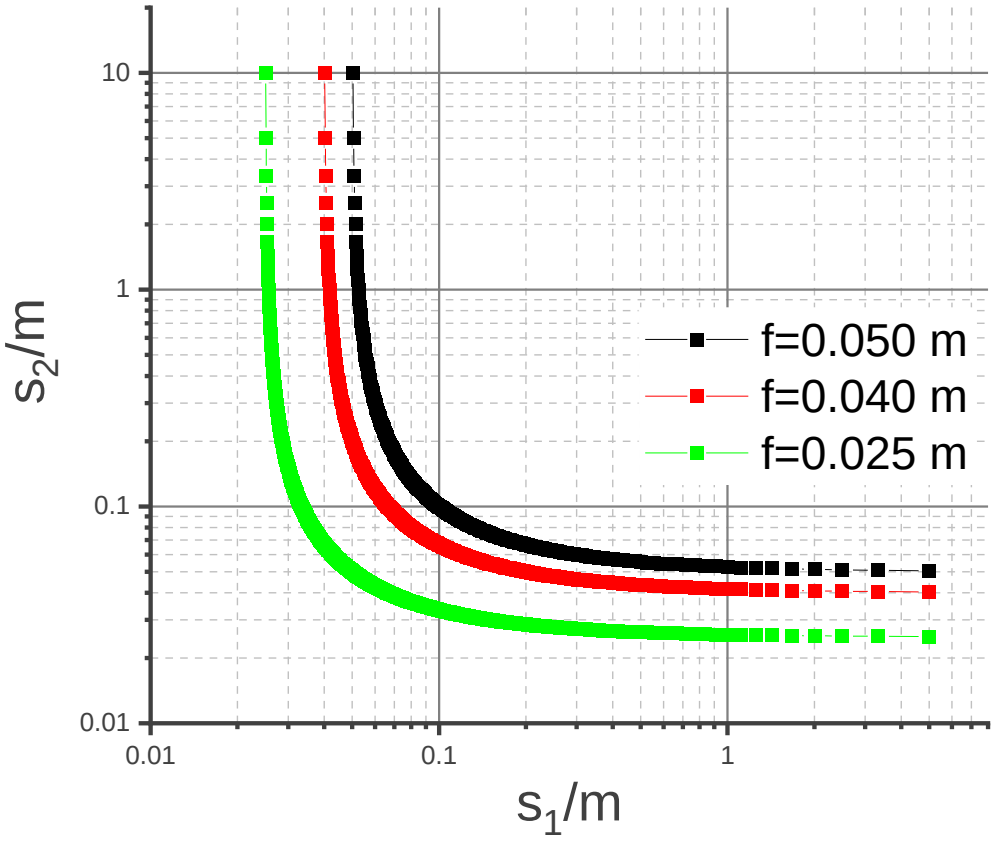
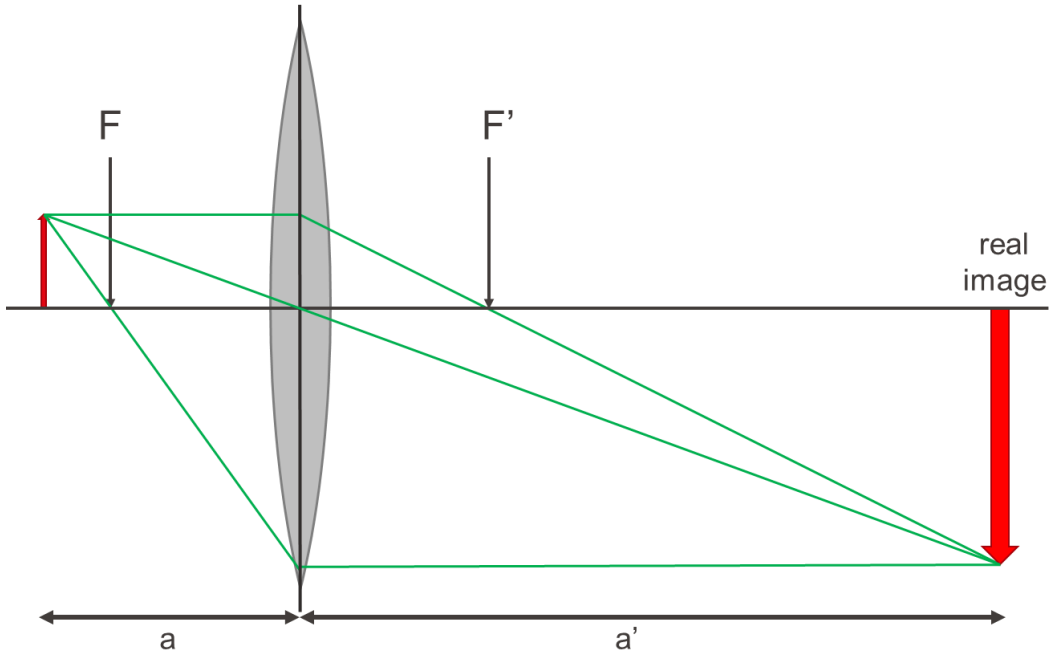
Image plane

$$\lim_{a \to f} a' = \infty$$





$$\lim_{a \to \infty} a = f$$

$$\frac{1}{f} = \frac{1}{a} + \frac{1}{a'}$$

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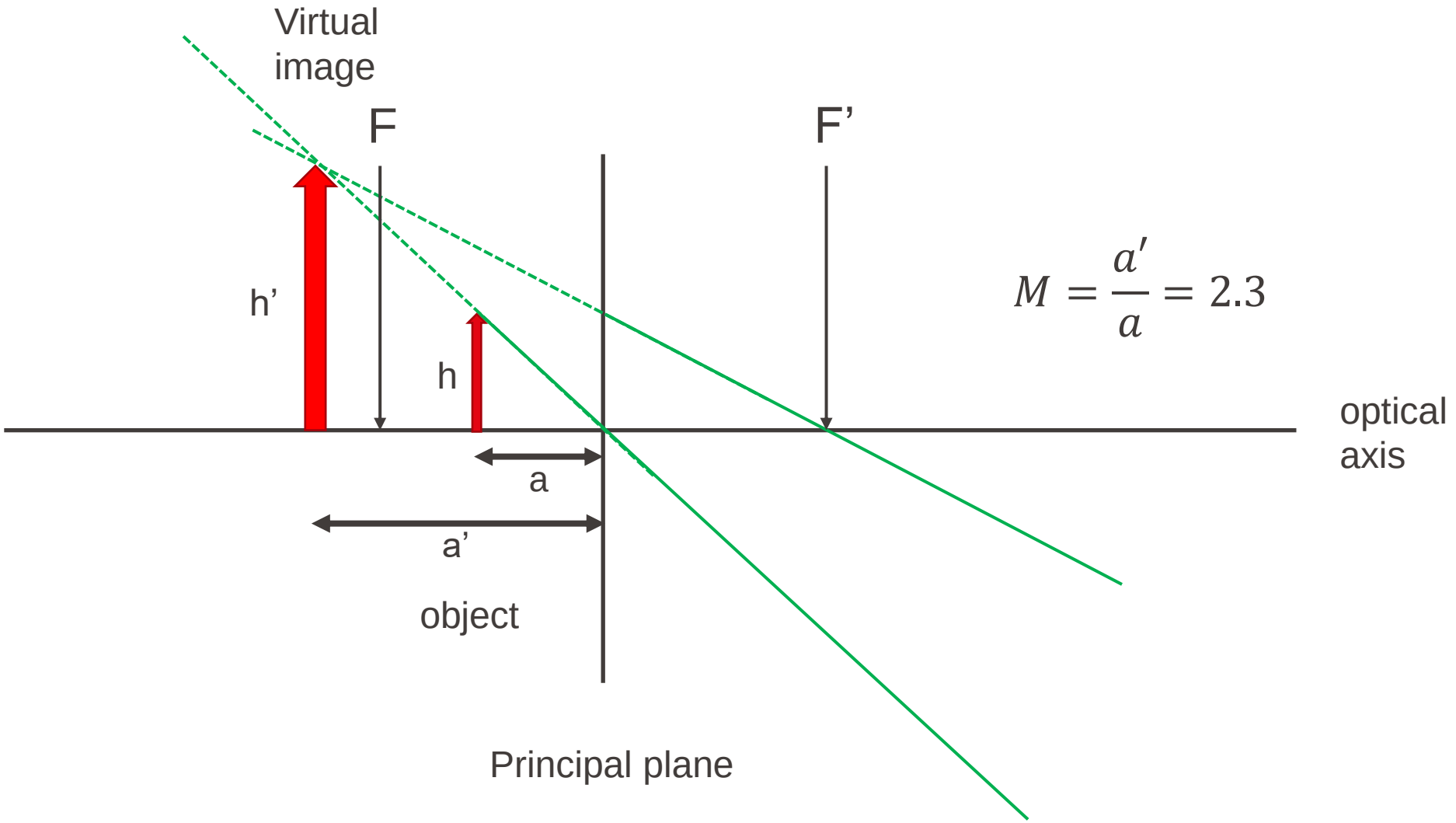
Image Formation



$$M = \frac{a'}{a} = 2.3$$

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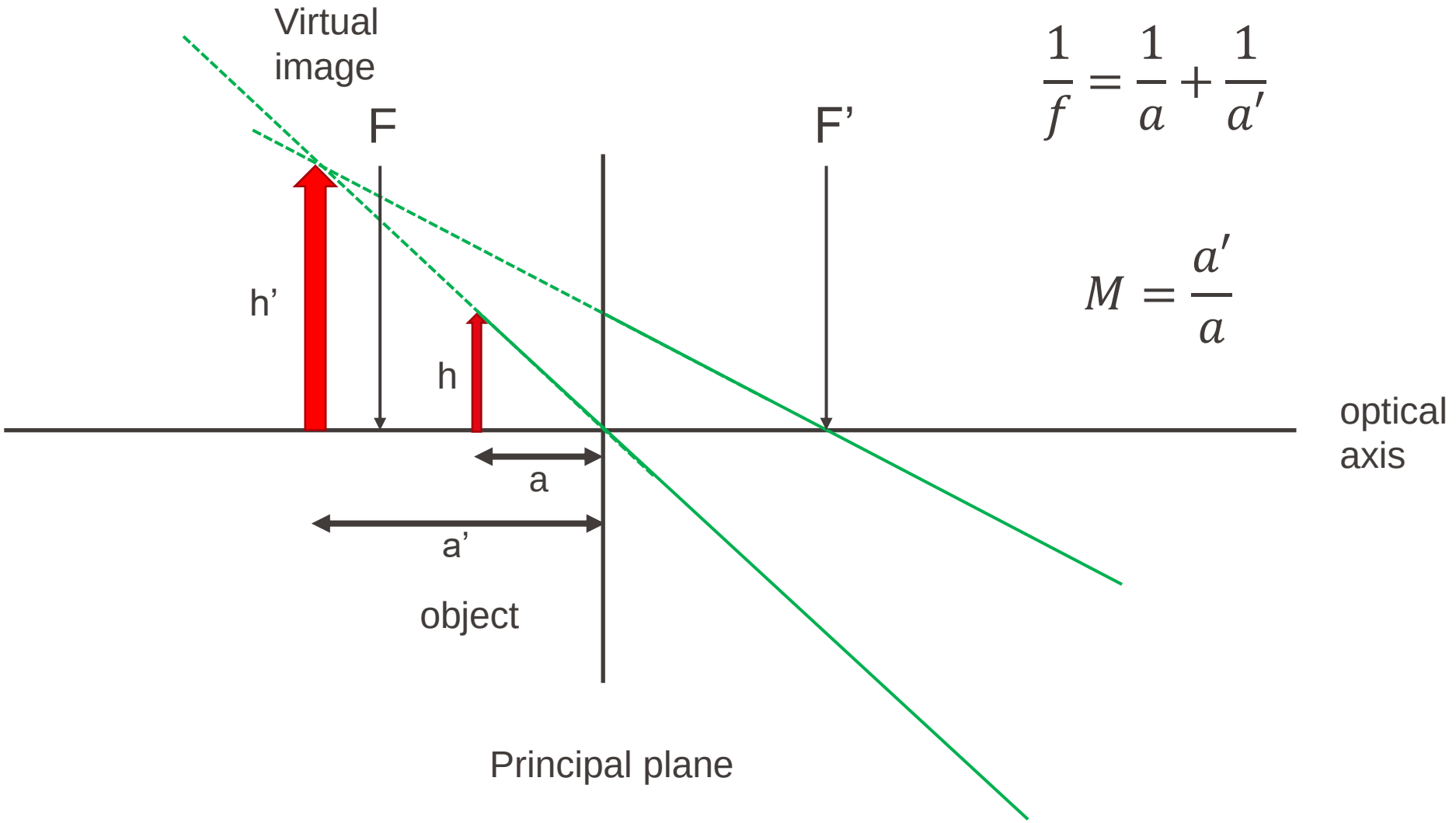
Image Formation



$$\frac{1}{f} = \frac{1}{a} + \frac{1}{a'}$$

$$M = \frac{a'}{a}$$

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Image formation
Summary

Object	Convex lenses Image			
Position	Type	Position	Orientation	Rel. size
$\infty > a > 2f$	real	$f < a' < 2f$	inverted	smaller
$a = 2f$	real	$a' = 2f$	inverted	equal
$f < a < 2f$	real	$\infty > a > 2f$	inverted	bigger
$a = f$		$\pm\infty$		
$a < f$	virtual	$\|a'\|>a$	upright	bigger

Object	Concave lenses Image			
Position	Type	Position	Orientation	Rel. size
everywhere	virtual	$f < a' < 2f$	upright	Smaller

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Optical instruments

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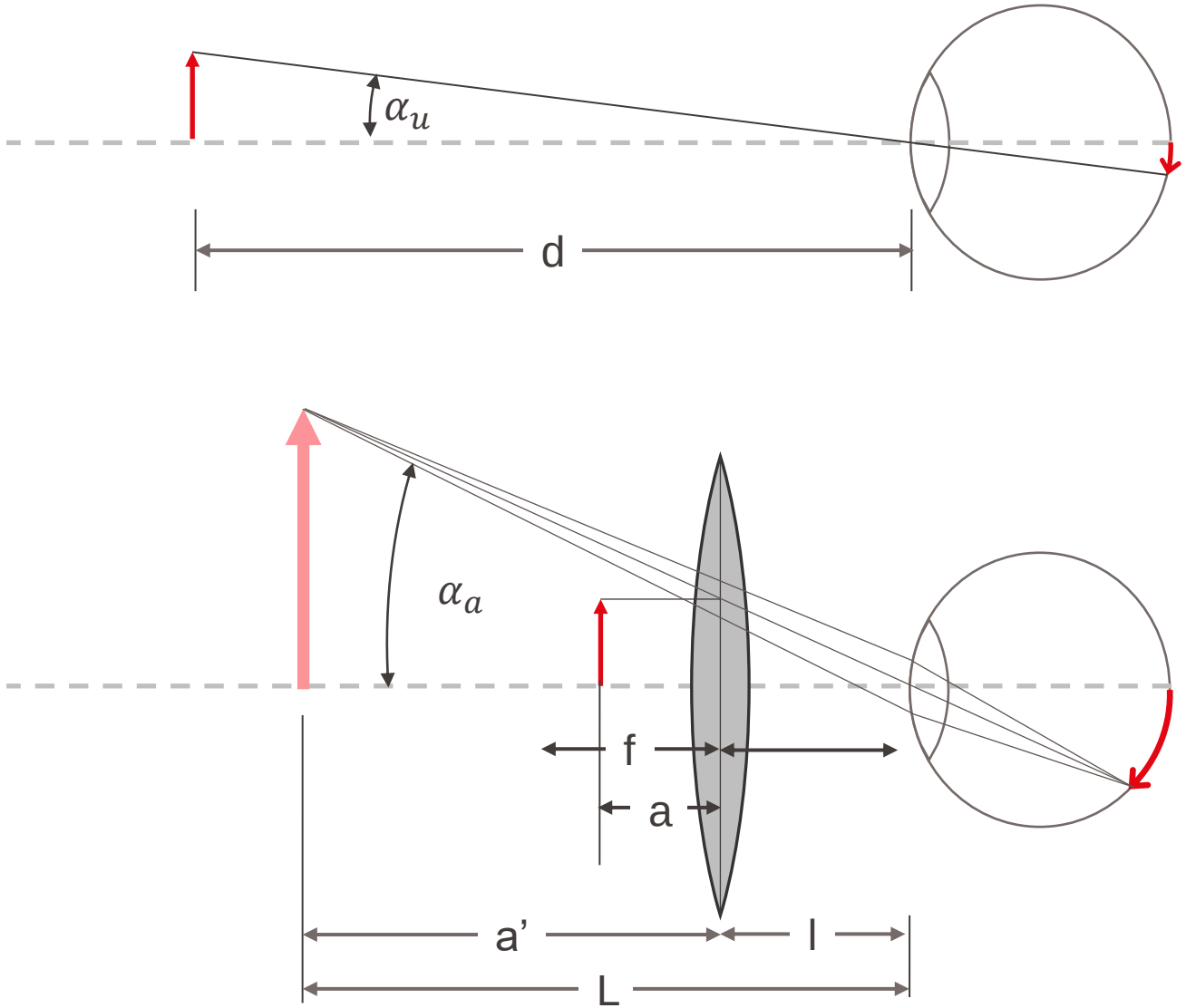
Magnifying glass



$$M = \frac{\alpha_a}{\alpha_u}$$

$$\mathrm{D} = \frac{1}{f} \qquad d = 0.25\ m$$

$$M = \frac{d}{L}[1 + DL - l]$$

$$[M]_{\substack{l=0 \\ L=d}} = dD + 1$$

$$[M]_{\substack{l=0 \\ L=d}} = 0.25D + 1$$

$$[M]_{\infty} = 0.25D \qquad \mathrm{a} = f$$

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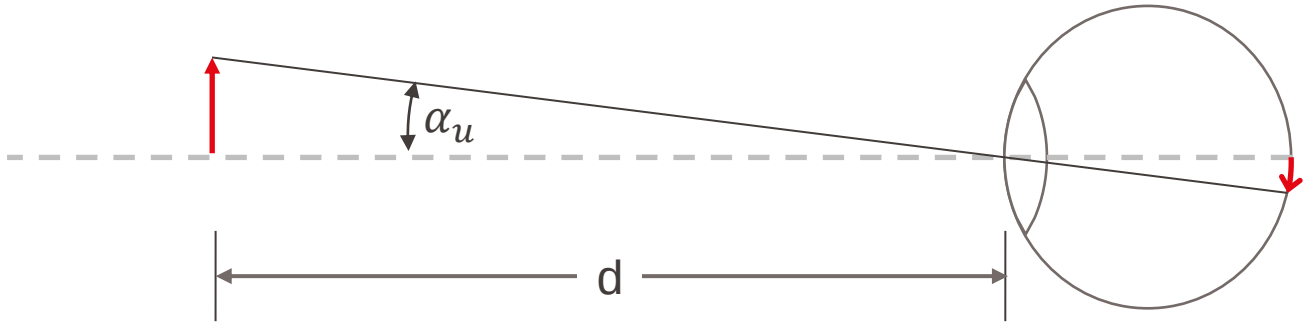
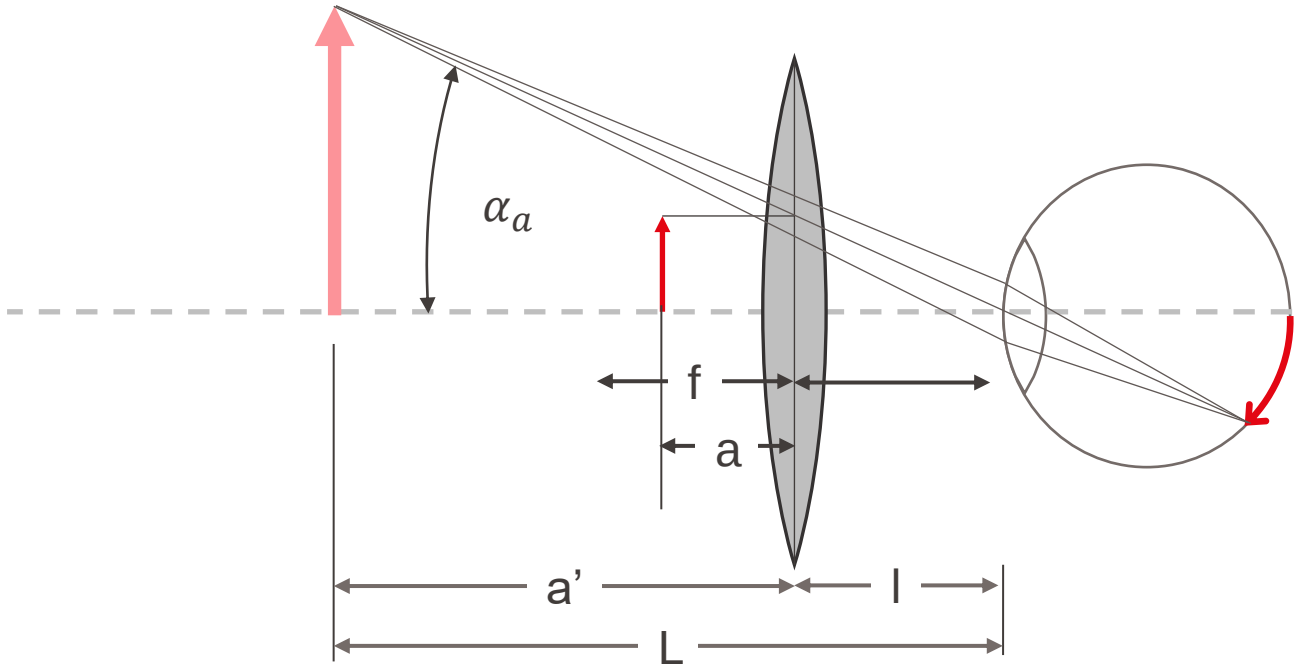
Magnifying glass



$$M = \frac{\alpha_a}{\alpha_u}$$



- Simple Magnifying glass
  = one lens
  M≅ 2-3 due to aberrations
- Higher magnification requires more lenses
- Ocular

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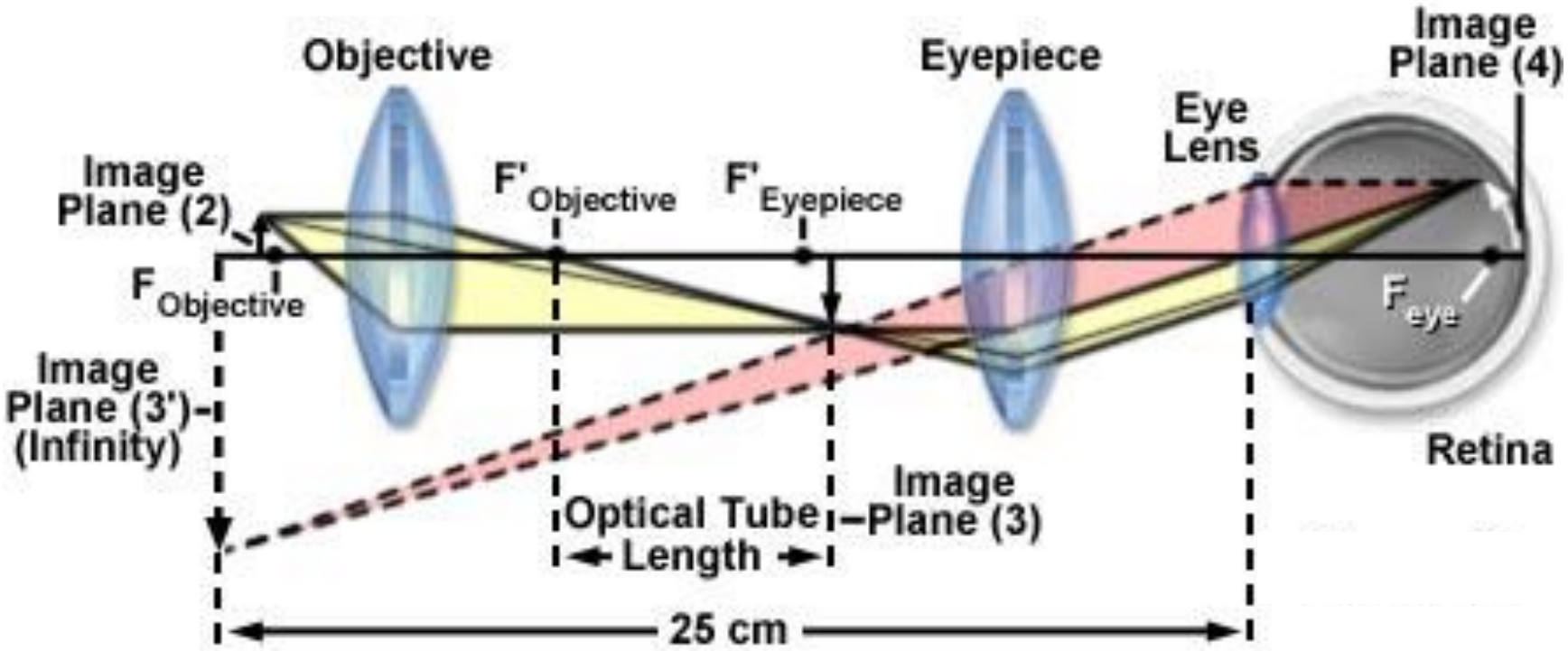
Compound microscope



Sample is placed in front of objective focal plane. Intermediate image is formed by objective and is observed through eyepiece.

$M_{obj} = ???$

$OTL = 160\,mm$

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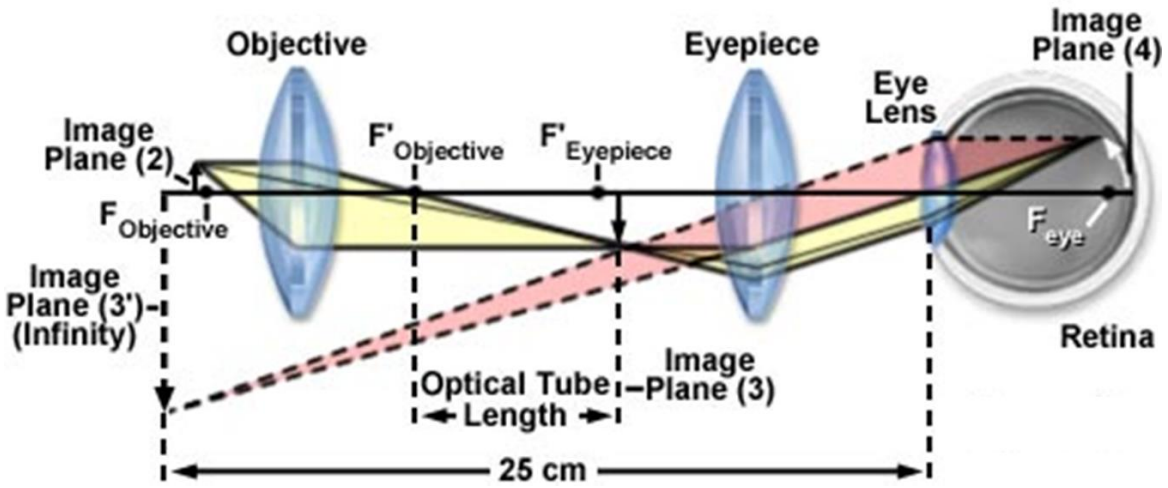
Compound microscope



$$M = \frac{a}{a'} \qquad \frac{1}{f} = \frac{1}{a} + \frac{1}{a'}$$

$$OTL = 160\ mm \qquad a' = ???$$

$$\frac{1}{a} = \frac{1}{f} - \frac{1}{a'} \qquad a = \frac{1}{\frac{1}{f} - \frac{1}{a'}}$$

$$M = \frac{a'}{\frac{1}{\frac{1}{f} - \frac{1}{a'}}} = a' \cdot \left[\frac{1}{f} - \frac{1}{a'}\right] = \frac{a'}{f} - 1$$

$$a' = f + OTL = f + 160mm$$

$$M = \frac{a'}{f} - 1 = \frac{f + 160\ mm}{f} - 1 = \frac{160\ mm}{f}$$

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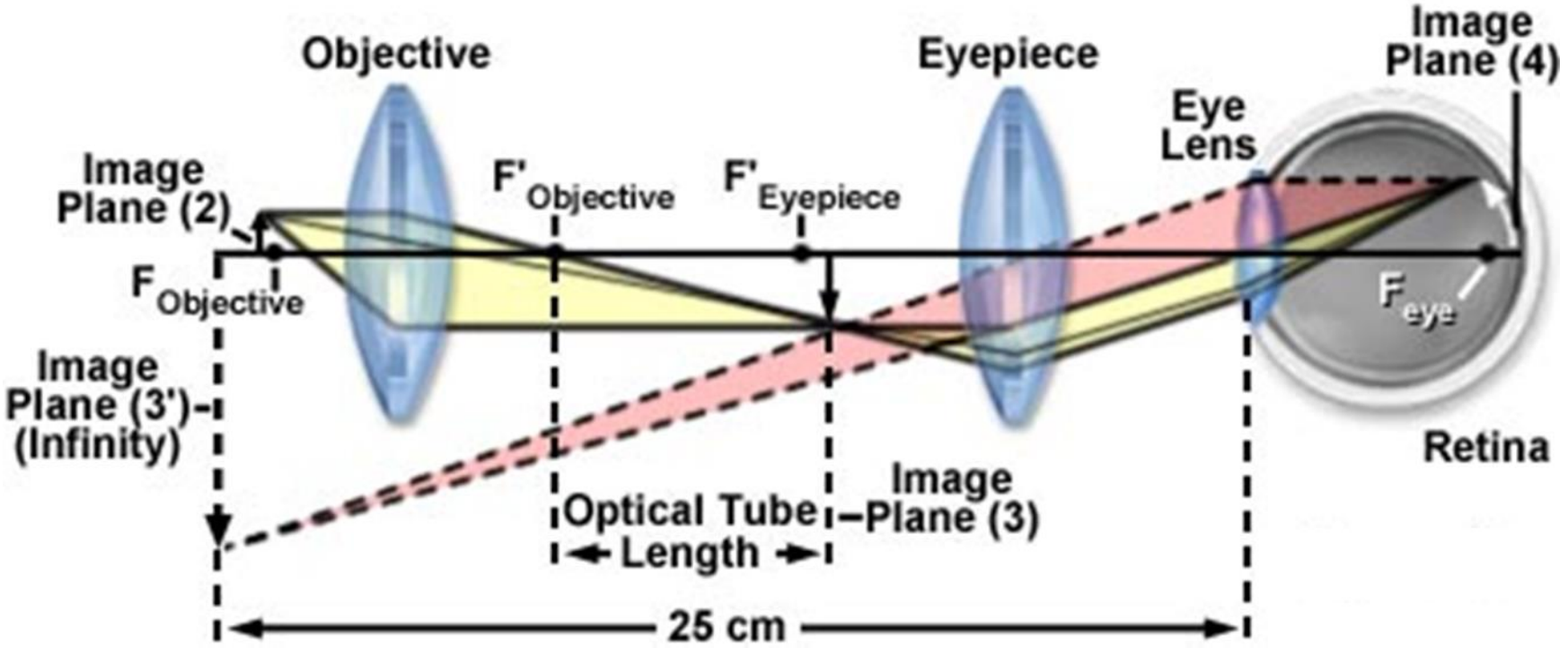
Compound microscope



Sample is placed in front of objective focal plane. Intermediate image is formed by objective and is observed through eyepiece.

$$M_{obj} = \frac{OTL}{f_{Obj.}} = \frac{160\,mm}{f_{Obj.}}$$

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Compound microscope

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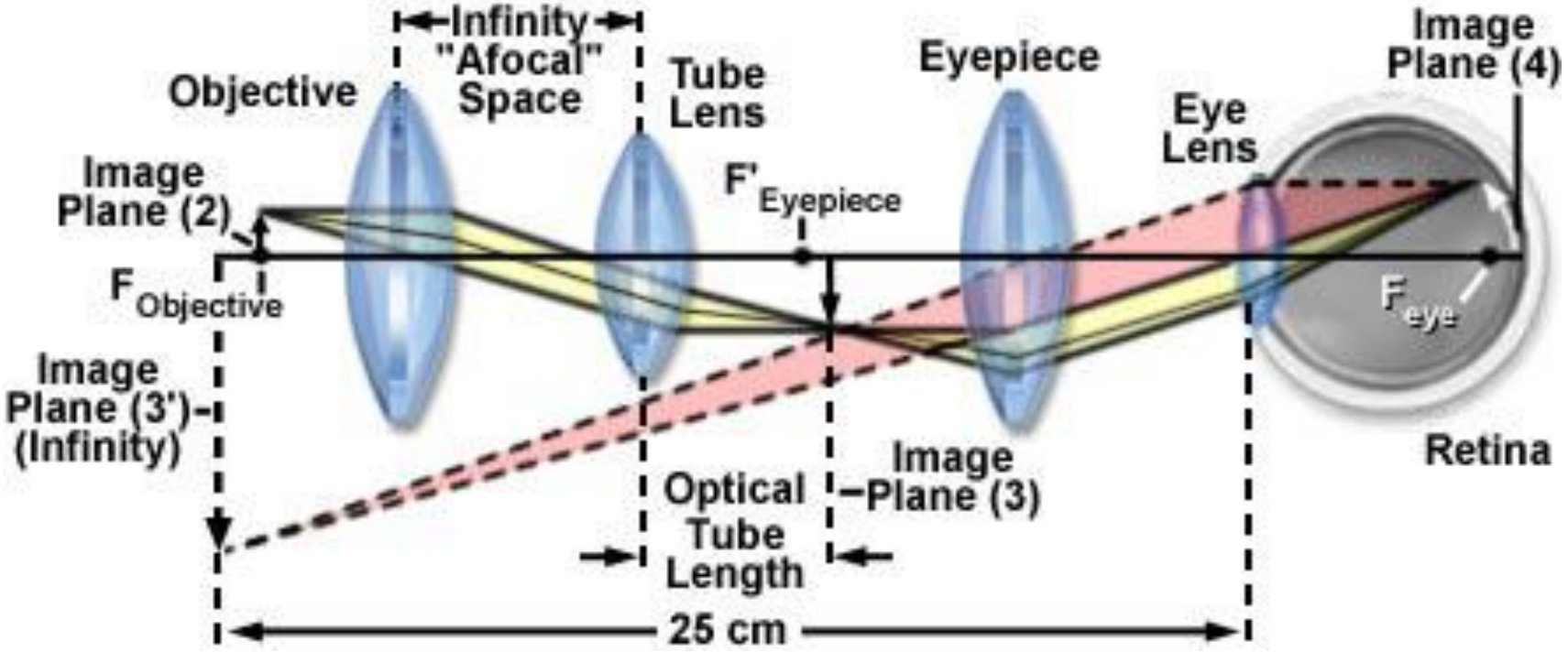

The sample is placed in the focal plane of the objective. Parallel light beams are focused by the tube lens. The intermediate image is observed through the eyepiece.

$$M_{obj} = \frac{f_{TL}}{f_{Obj.}} \qquad f_{TL} = 160 - 250\,mm$$

Different beam paths

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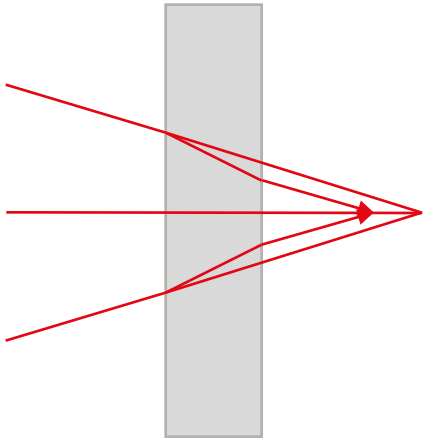
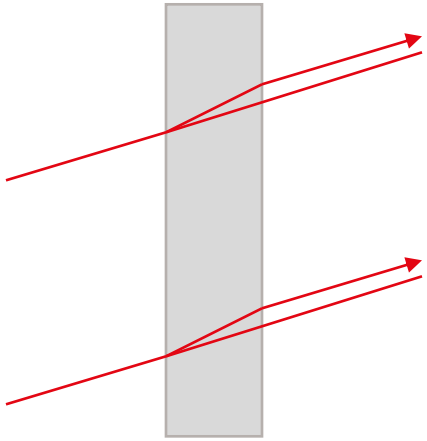


Convergent beam

Beam is focused differently
More aberrations



Parallel beam

Beam is only shifted
Less aberration

Presence of parallel light beam is microscope light path is important for modern light microscope (for filters, and other optical elements)

Working principle of a microscope

- Compound microscope
  - Two step magnification: objective and eyepiece
  - Convergent beam paths (2 lenses)
  - Infinite beam path (3 lenses)

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Components of a microscope

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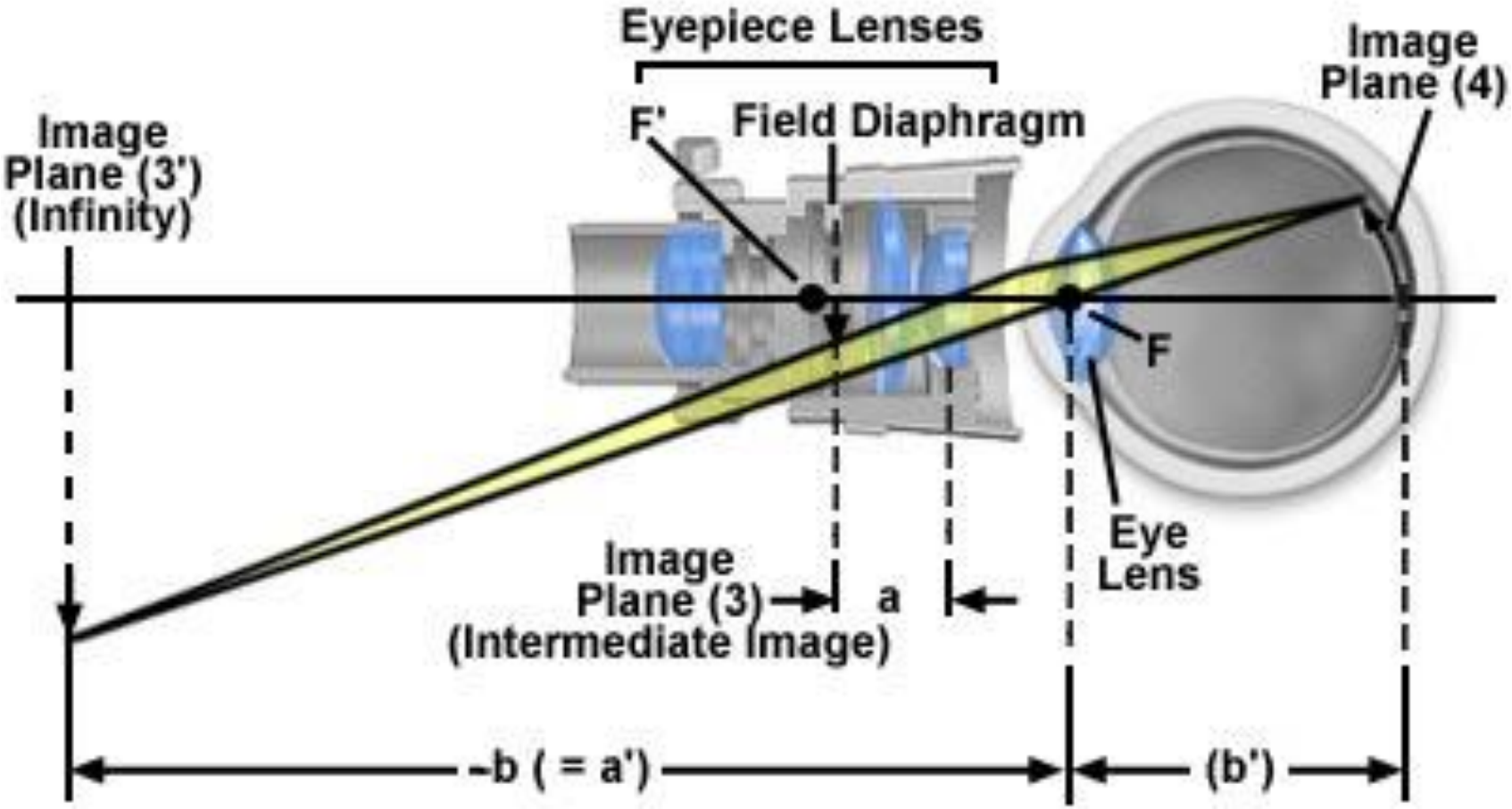
Eyepiece



The eyepiece/ocular acts as a magnifier of the intermediate image

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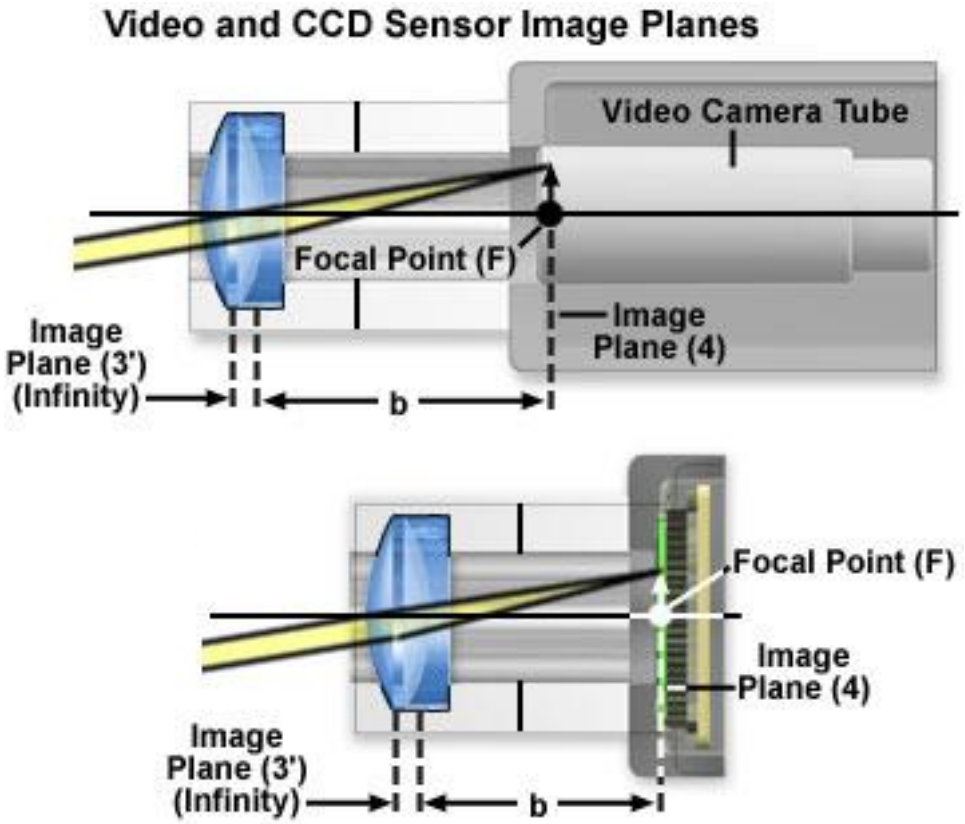
Camera as image detector



When the camera is used, the intermediate image is directly projected on the camera chip (additionally an intermediate magnifier might be used).

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Camera detection

Why is no intermediate magnification needed if a digital camera is used as a detector?

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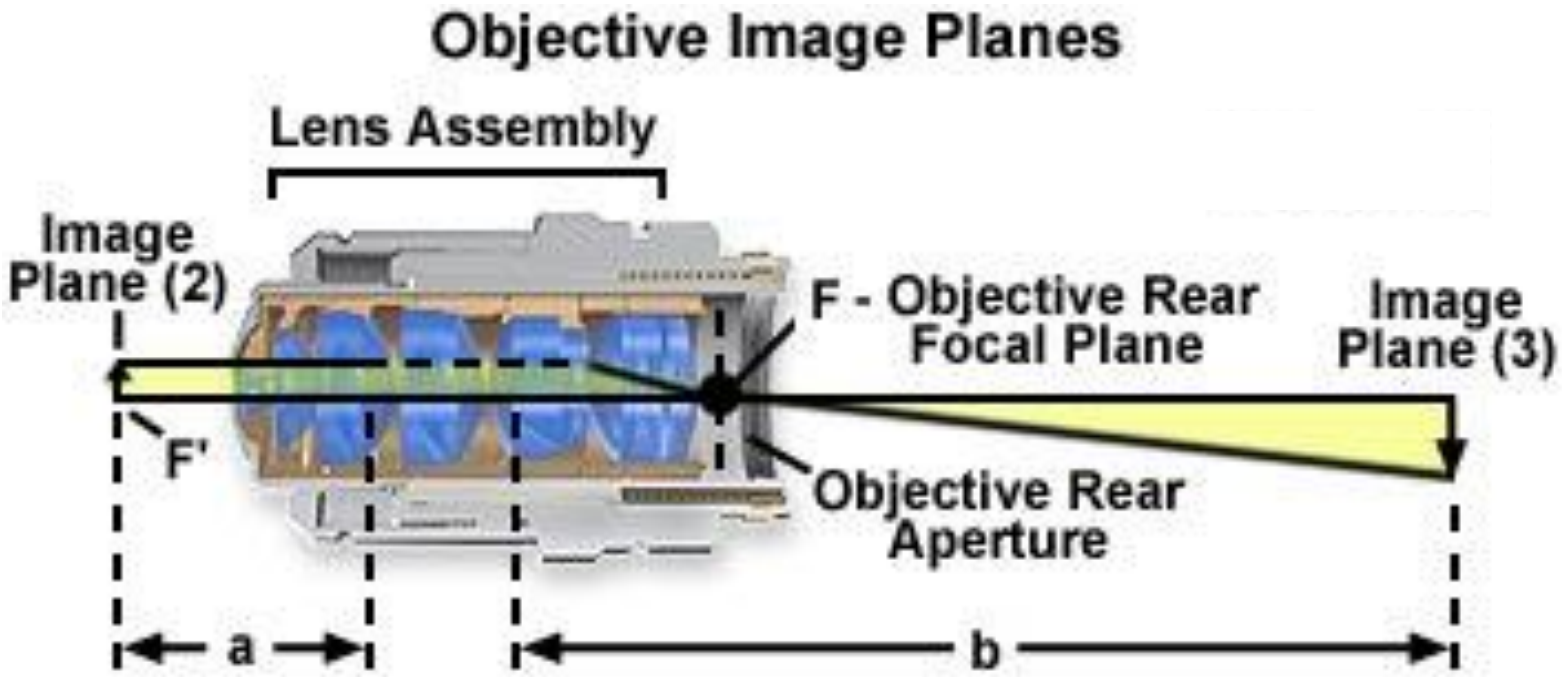
Objective



- Objectives are constructed of several high-quality lenses.
- For infinity corrected objective the specimen is in the focal plane.
- For not infinity-corrected objectives the specimen is in front of the focal plane

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Working and parfocal distance

- Parfocal distance
  - Distance from objective shoulder
  - till the specimen plane
  - 45 mm for most manufacturers, 60 mm for Nikon CFI 60
- Working distance
  - Distance from the front edge of the objective to the coverslip
  - Varies from several mm to several hundred micrometers. Special long working distance objectives are available.

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Geometrical aberrations

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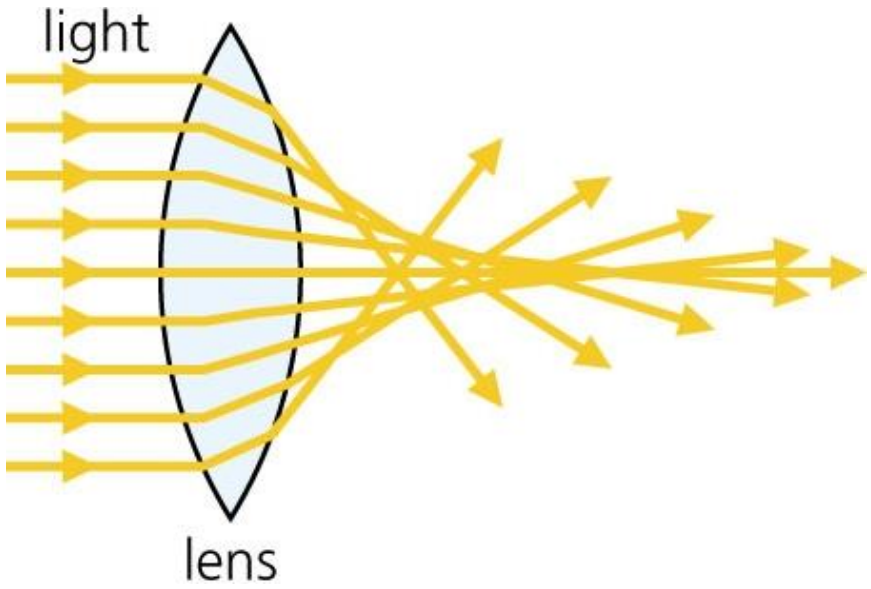
- Astigmatism (tangential and meridianal focus are different)
- Coma (image of a dot is not symmetric)
- Distortion (parallel lines are not parallel in the image)
- Curvature of the field (image of plane is not flat)
- Chromatic (different focus for different wavelengths)
- Spherical (different focus for on and off-axis beams)

It is desired to minimize aberrations by proper use of objectives with good aberration correction

Spherical aberration

- Use a cover slip 0.17 mm thick or
- Use objective with a correction ring
- Avoid refraction index mismatch of immersion and mounting media



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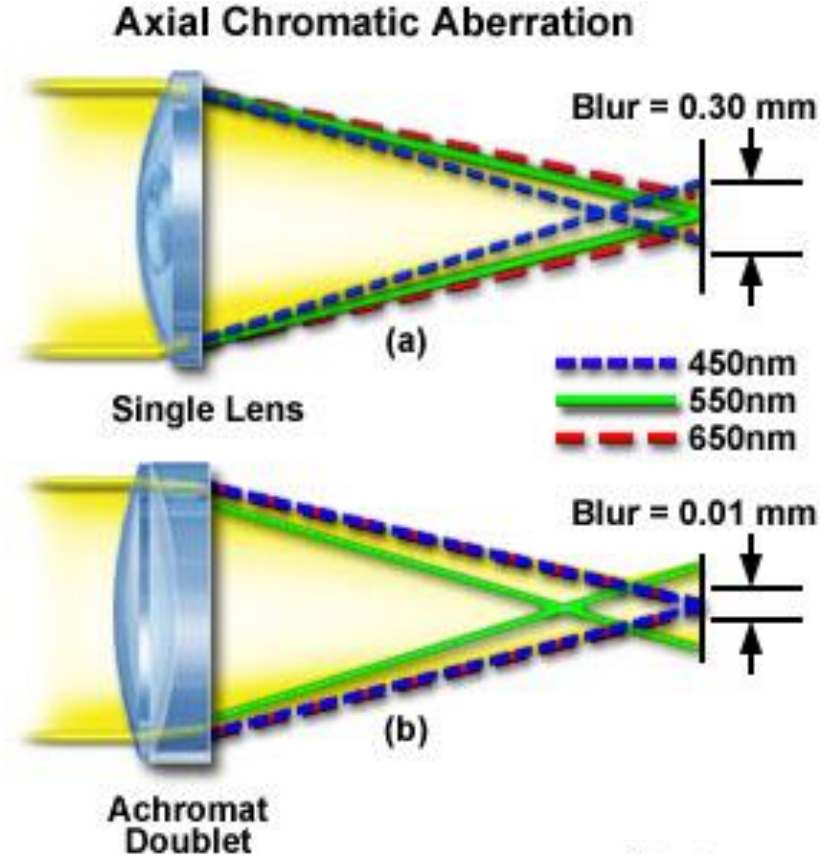
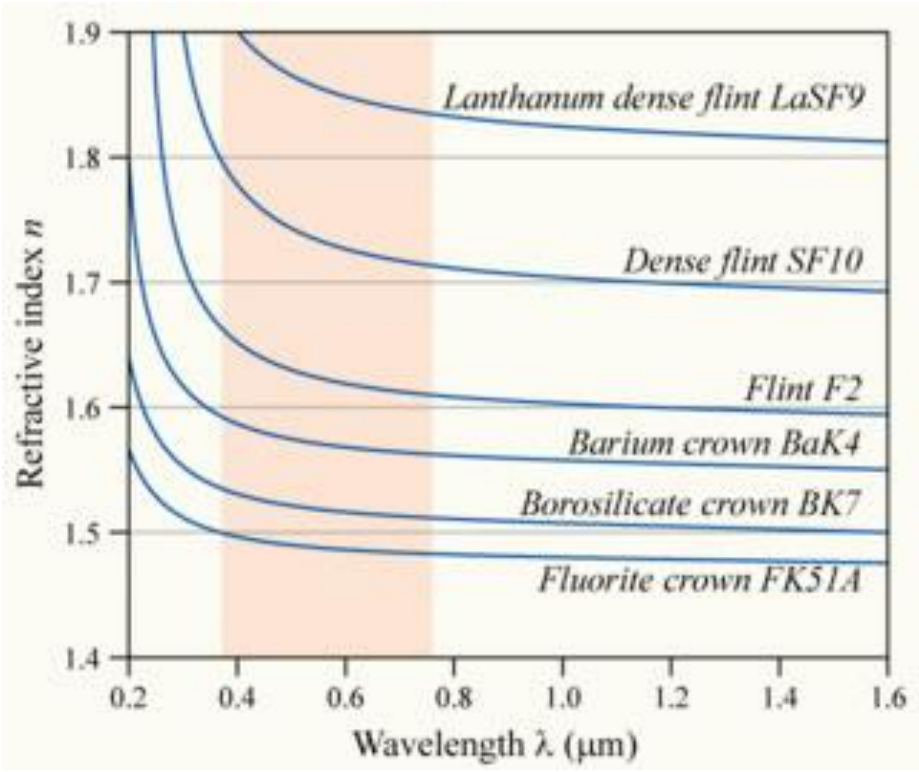
Chromatic Aberration





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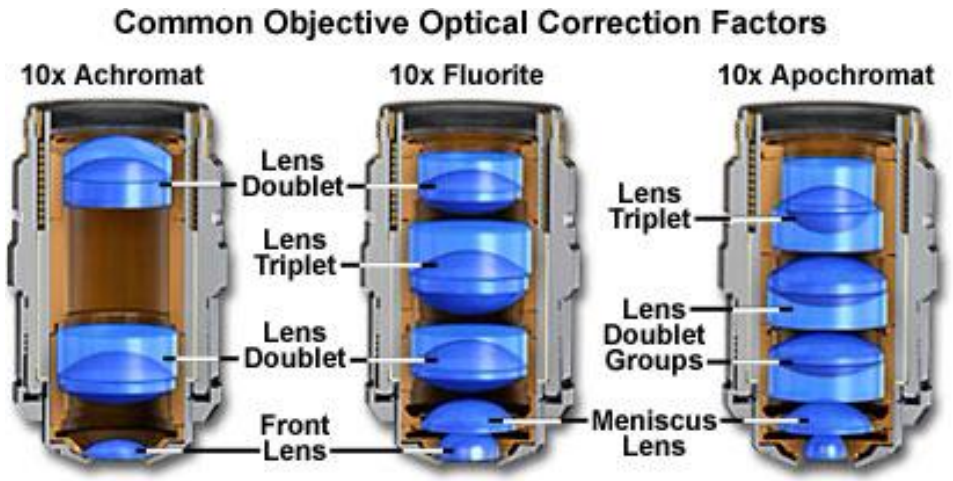
Objective Types



Objective Type	Spherical Aberration	Chromatic Aberration	Field Curvature
Achromat	1 Color	2 Colors	No
Plan Achromat	1 Color	2 Colors	Yes
Fluorite	2-3 Colors	2-3 Colors	No
Plan Fluorite	3-4 Colors	2-4 Colors	Yes
Plan Apochromat	3-4 Colors	4-5 Colors	Yes

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Resolution and illumination

Diffraction of light
Numerical aperture
Resolution
Koehler illumination

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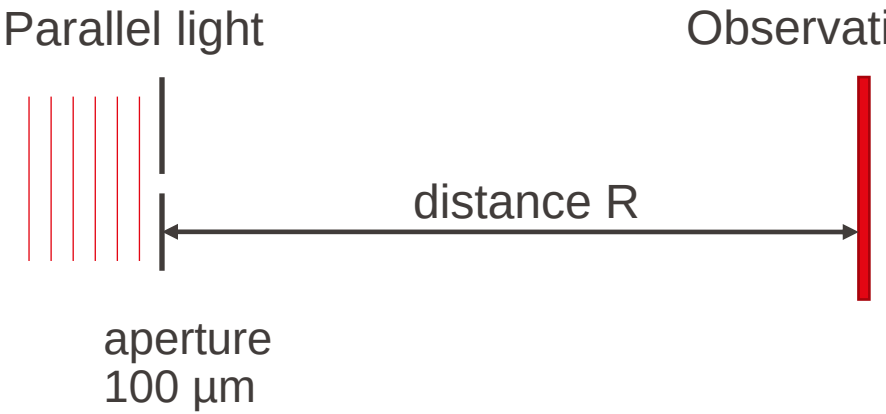
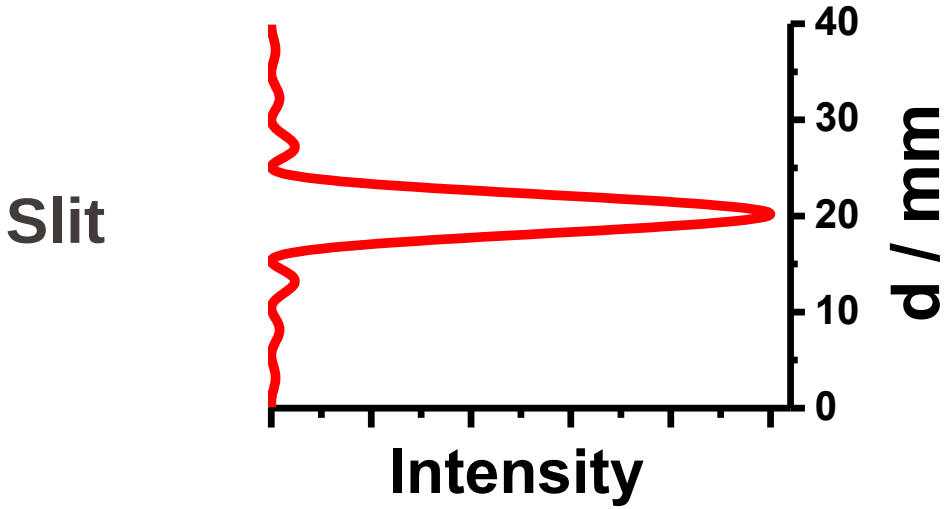
Far field diffraction



R>1.3 m

J. Fraunhofer (1787-1826)



$$I = I_0 \left[\frac{sin(\Phi/2)}{\Phi/2} \right]^2$$

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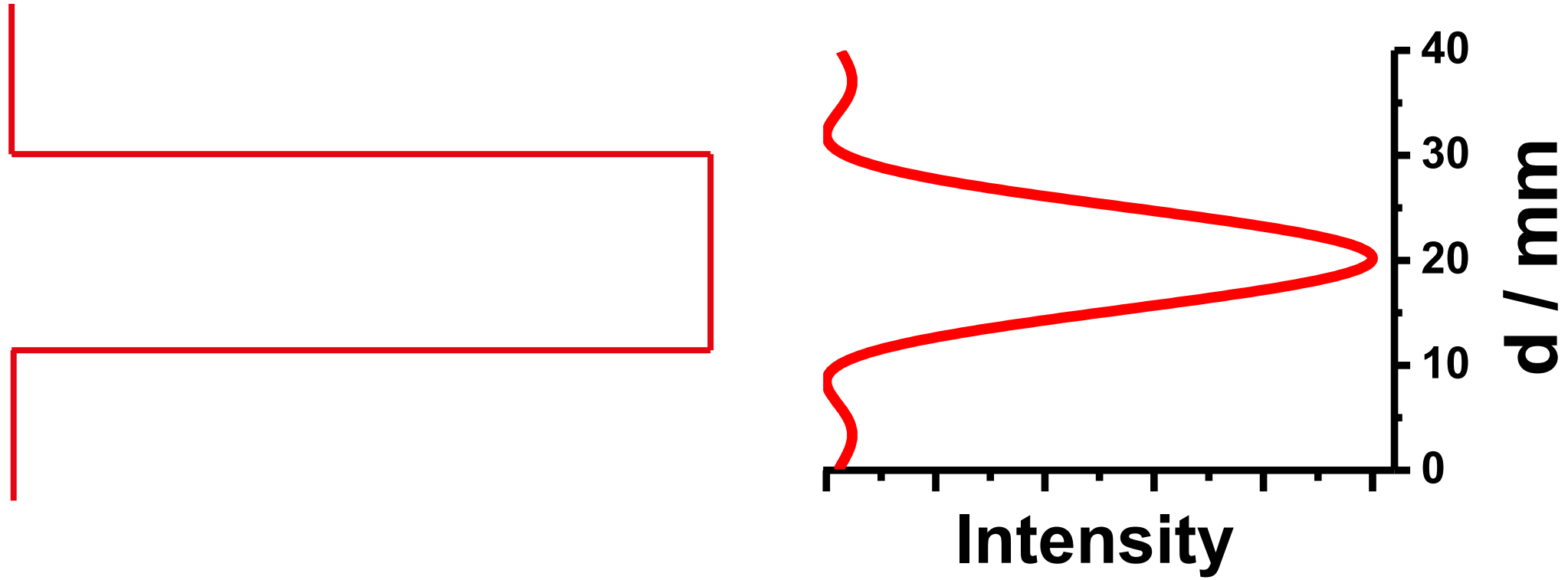
Diffraction



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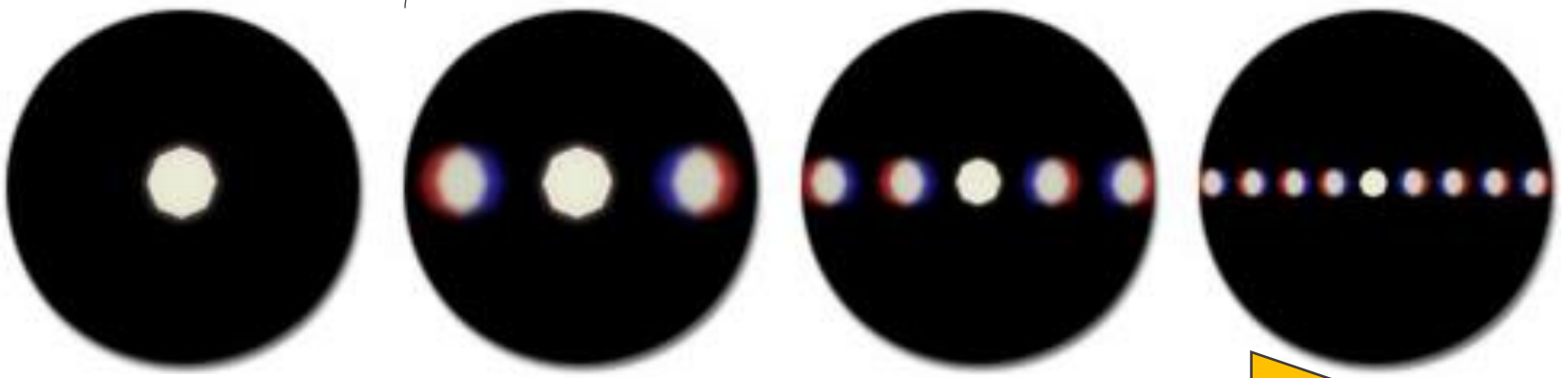
Numerical Aperture and Diffraction Orders

- Consider a 1D linear grating in the object plane of an optical system

These are the images at the rear focal plane of objective (i.e. at the Fourier plane) at different NA conditions.



No specimen

Increasing NA

With increasing NA, we collect higher diffraction orders

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Diffraction Limited Resolution

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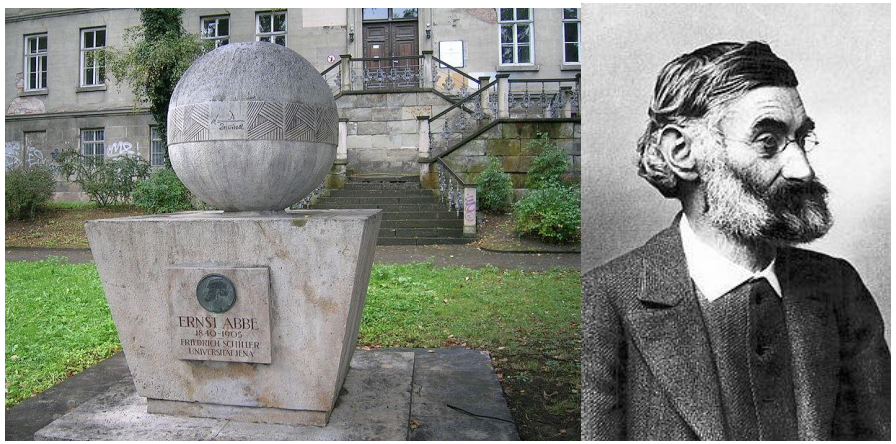


$$d = \frac{\lambda}{2 \cdot (n \cdot \sin\theta)}$$

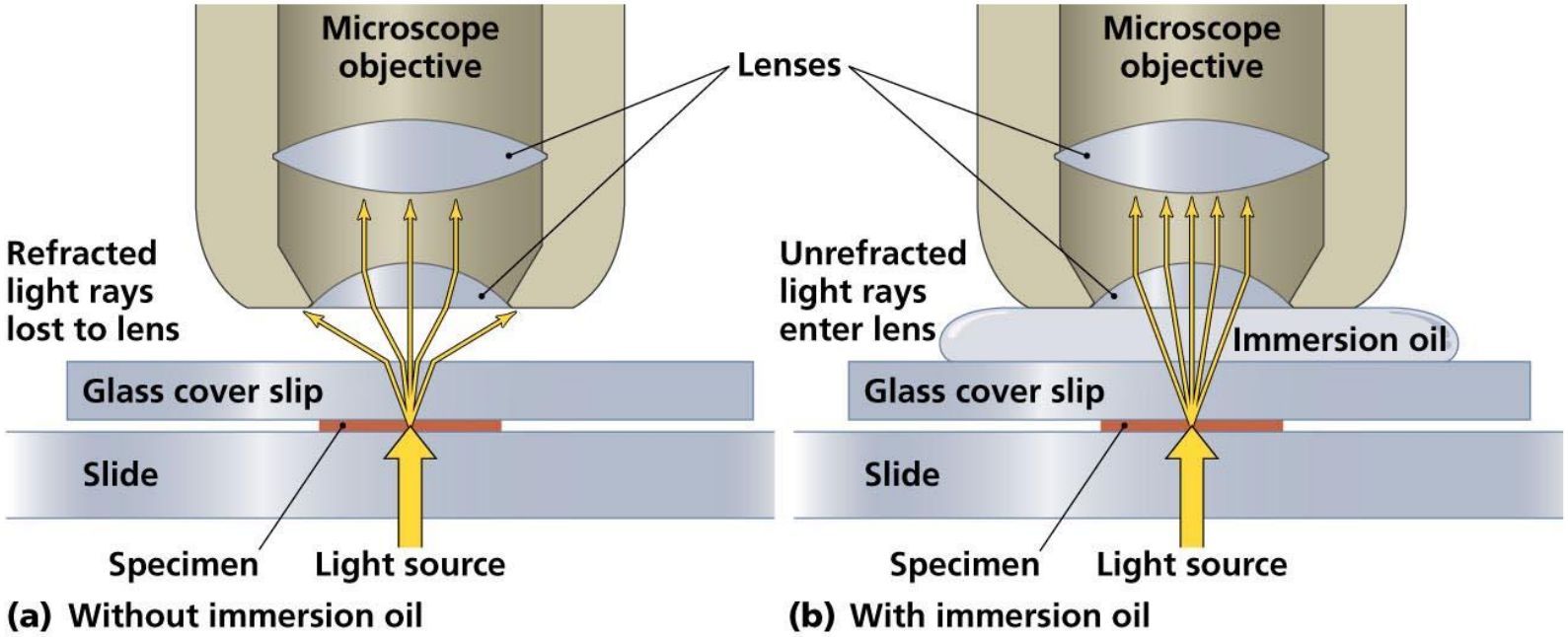
$$NA = (n \cdot \sin\theta)$$

Ernst Abbe
1840-1905

"According to Abbe, a detail with a particular spacing in the specimen is resolved when the numerical aperture (**NA**) of the objective lens is large enough to capture the first-order diffraction pattern produced by the detail at the wavelength employed. In order to fulfill Abbe's requirements, the angular aperture of the objective must be large enough to admit both the zeroth and first order light waves."

http://micro.magnet.fsu.edu/optics/timeline/people/abbe.html

Role of immersion



$NA = n*\sin\alpha$

Refractive indices:

Air:	1.003
Water:	1.33
Glycerol:	1.47
Oil:	1.52

Immersion media increase the NA of an objective or a condenser by bringing the beams with higher incidence angle into the light path

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Depth of Field

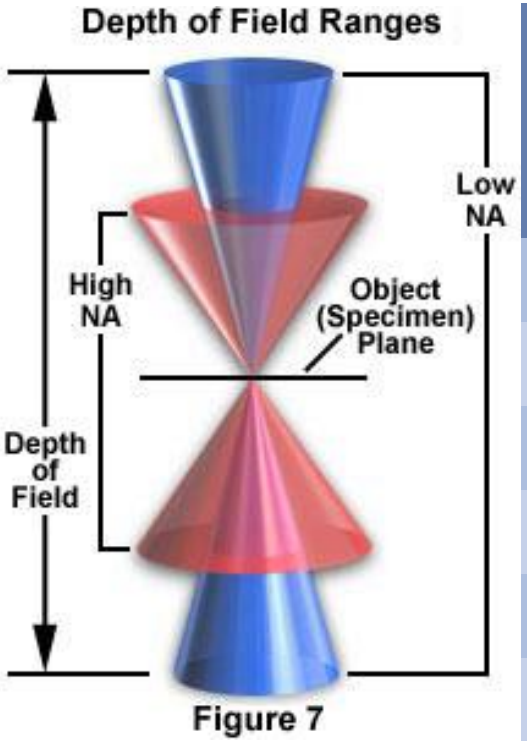

Figure 7

Magnification	Numerical Aperture	Depth of Field (μm)	Image Depth (mm)
4x	0.10	15.5	0.13
10x	0.25	8.5	0.80
20x	0.40	5.8	3.8
40x	0.65	1.0	12.8
60x	0.85	0.40	29.8
100x	0.95	0.19	80.0

$$d_{tot} = \frac{\lambda \cdot n}{NA^2} + \frac{n}{M \cdot NA} \cdot e$$

Diffraction limited depth of field

Detection system

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Illumination

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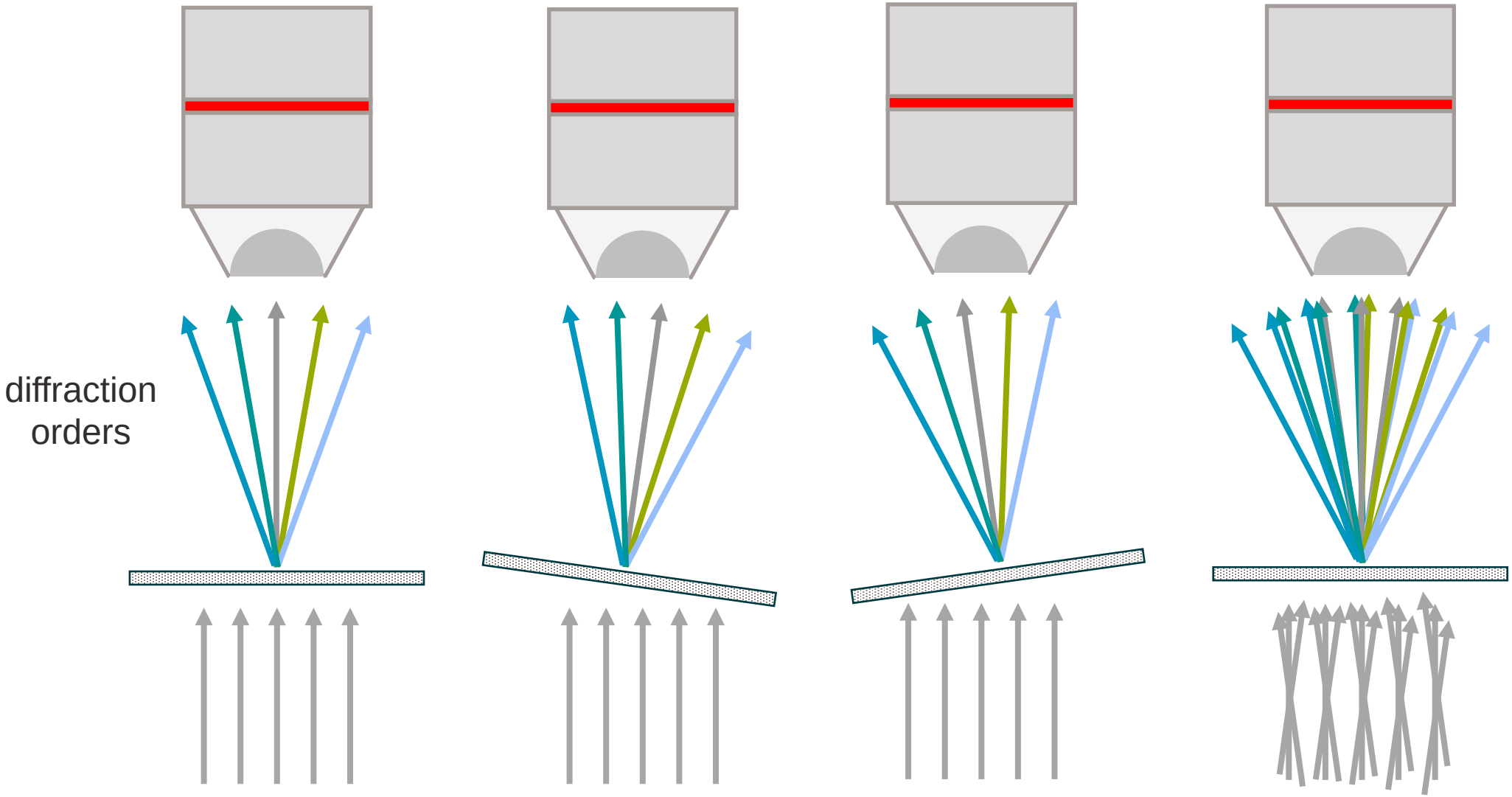
Oblique illumination



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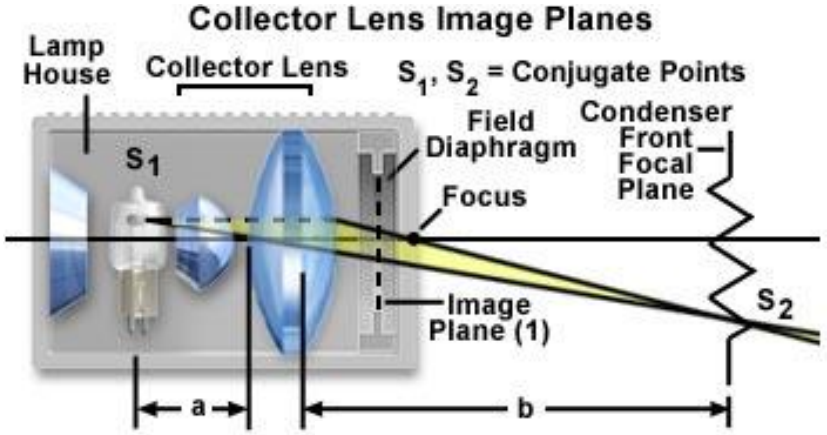
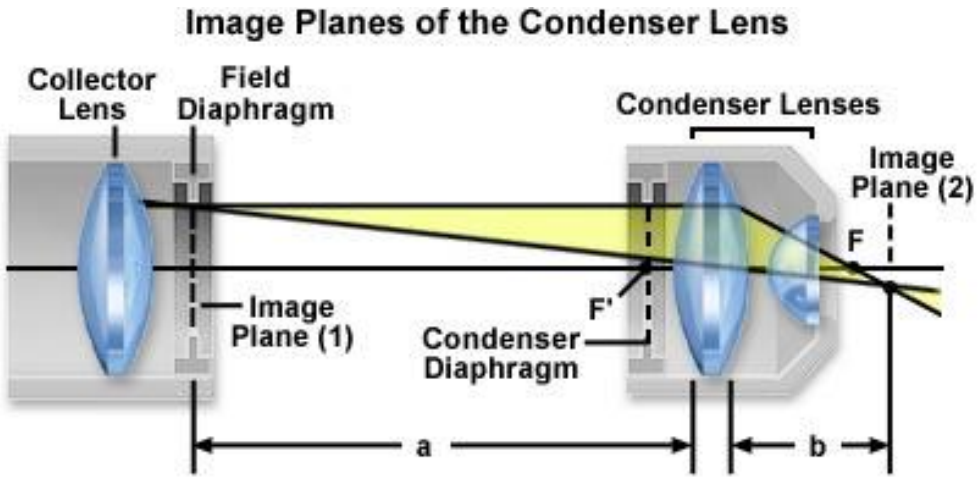
Collector and condenser



Collector

gathers light from light source



Condenser

directs light onto the specimen

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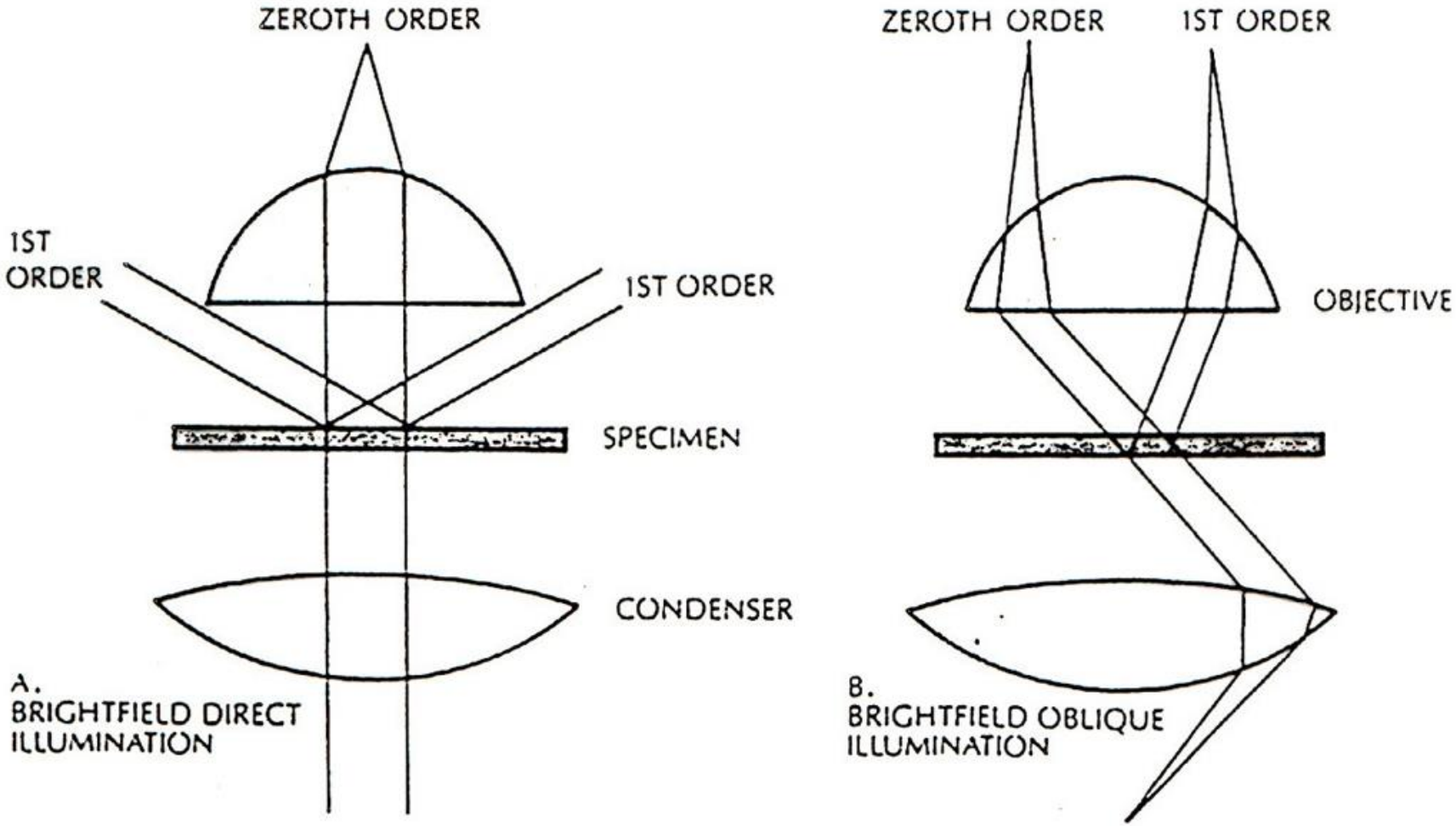
Oblique illumination



$$NA_{tot}=NA_{obj}+NA_{cond}$$

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Requirements for illumination

- Uniform over whole field of view
- Has all angles accepted by objective
- Allows optimize image brightness/contrast
- Allows continuous change of intensity
- Allows continuous change of field of view
- Changes in illumination and imaging parts do not affect each other

Realized in Kohler illumination

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Challenges in light microscopy

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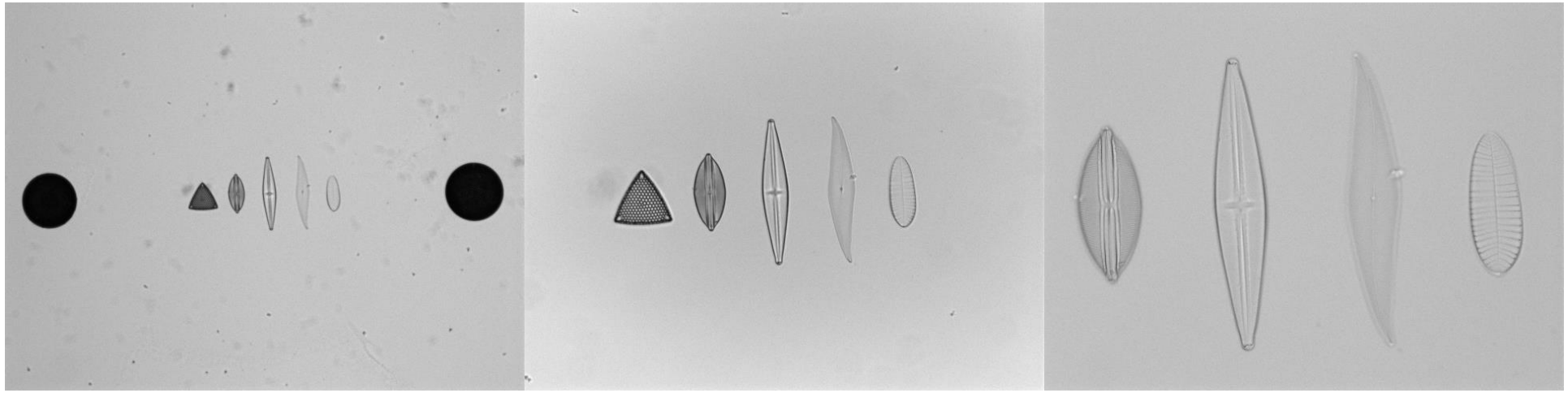
Microscopy: Magnification



low **high**

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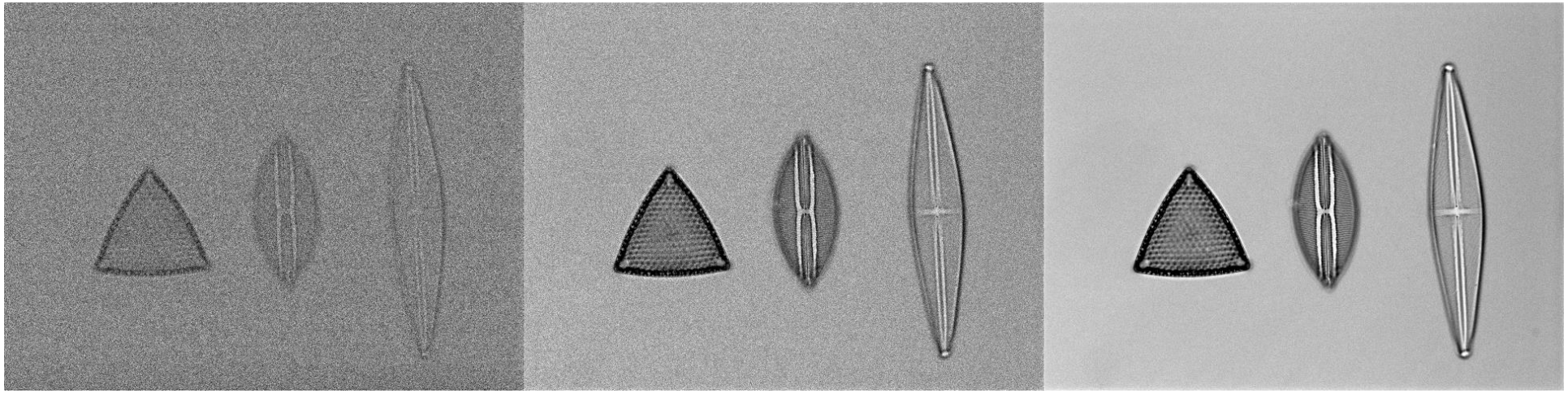
Microscopy: Contrast



low **high**

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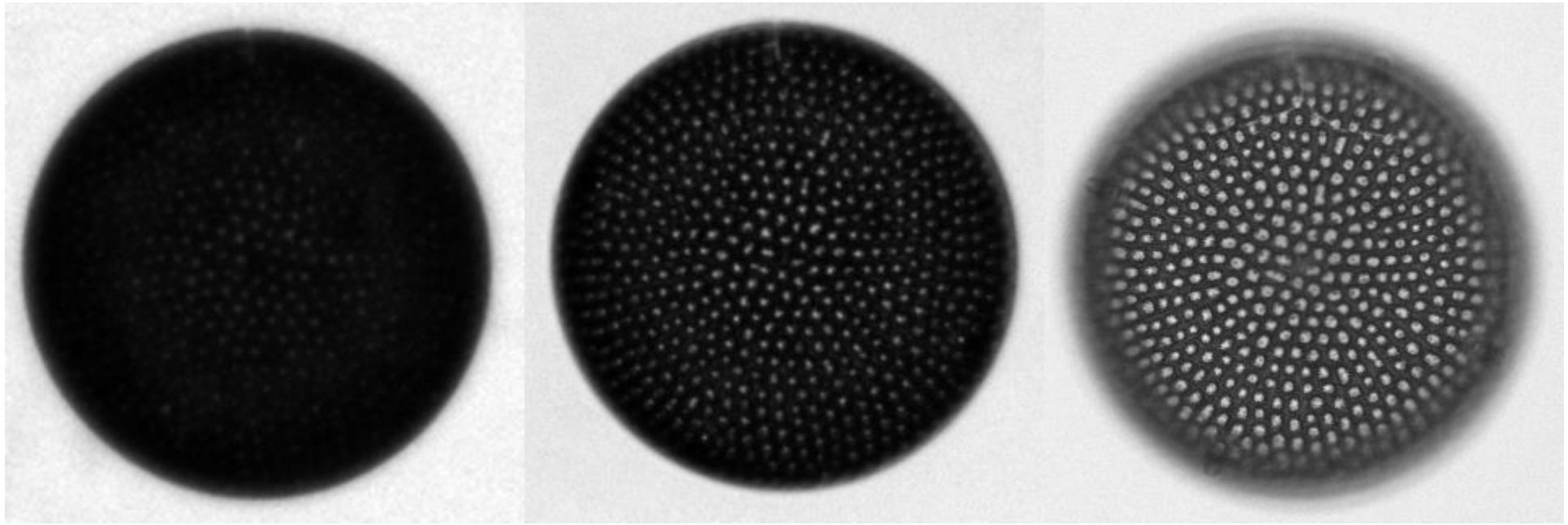
Microscopy: Resolution



low **high**

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Main challenges of microscopy

- In order to observe “small objects”, three preconditions have to be fulfilled
  - Magnification
  - Resolution
  - Contrast

- Only fulfillment of these three conditions allows translation of information as accurately as possible from object into an image which represents that object.

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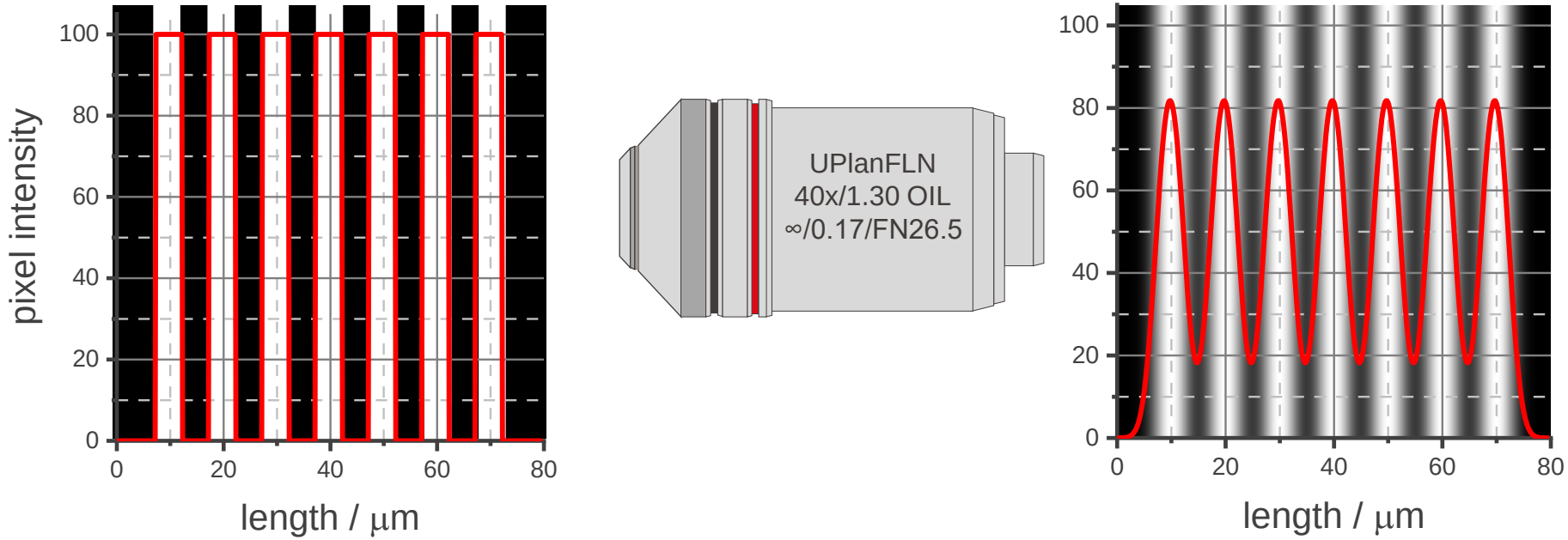
Low pass filtering



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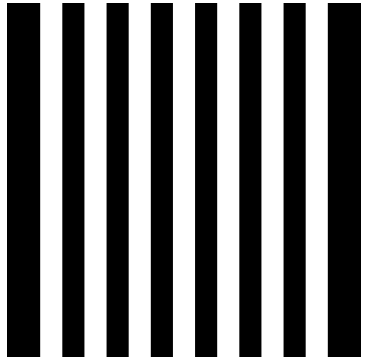
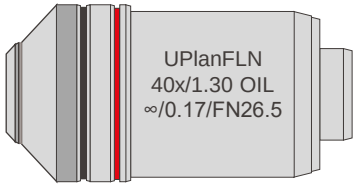
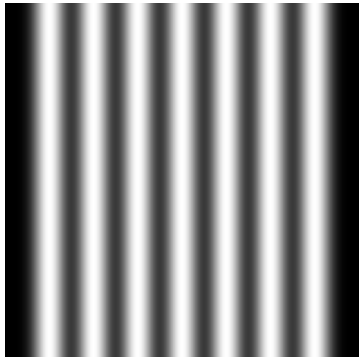
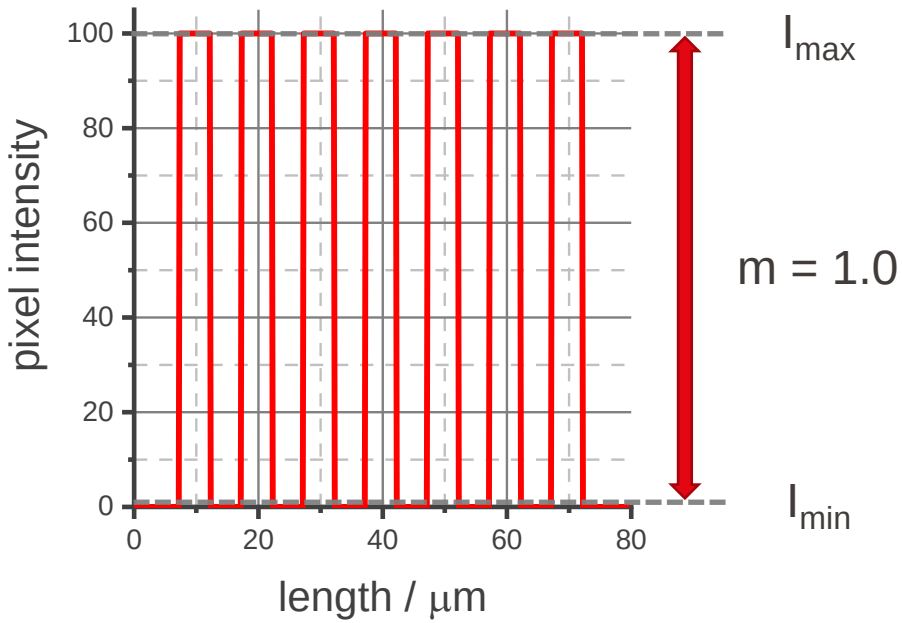
Contrast

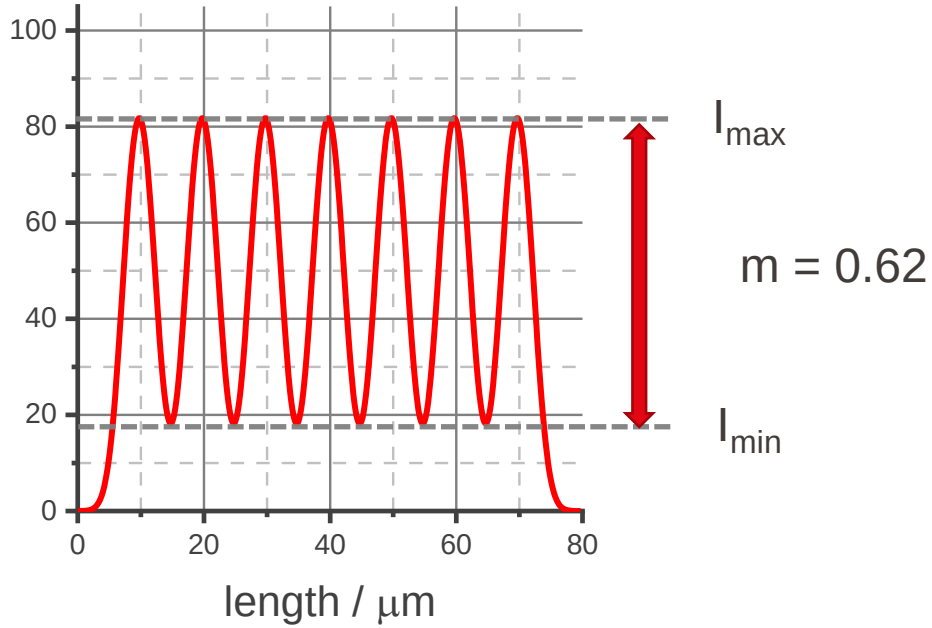






$$modulation = \frac{I_{max} - I_{min}}{I_{max} + I_{min}}$$





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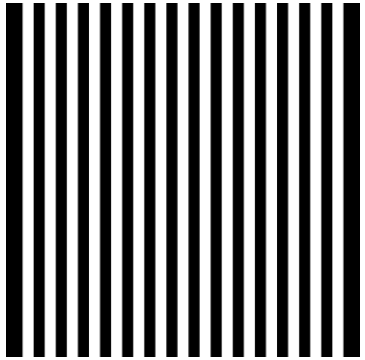
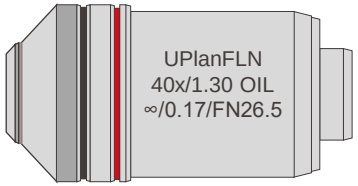
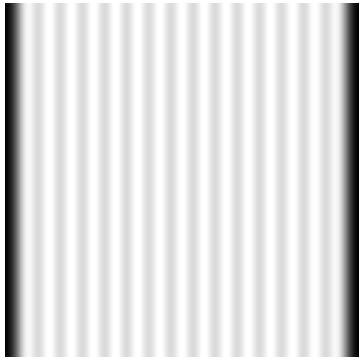
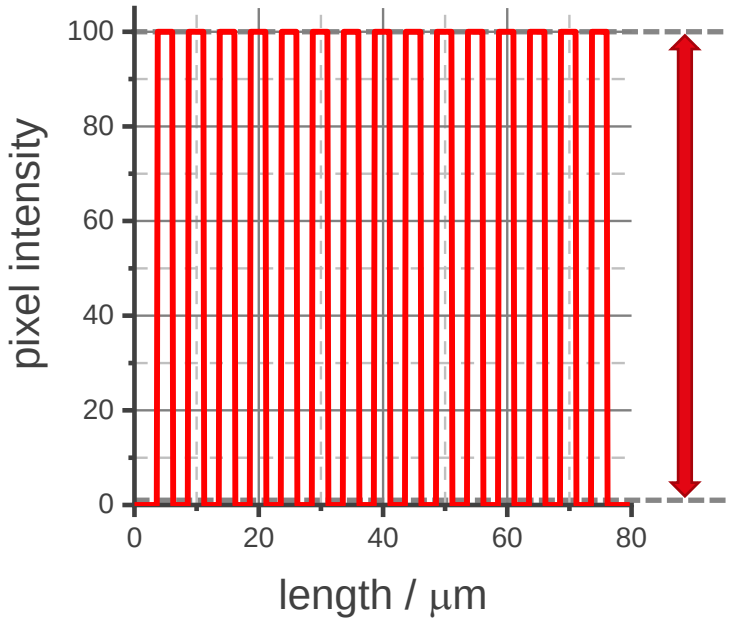
Contrast







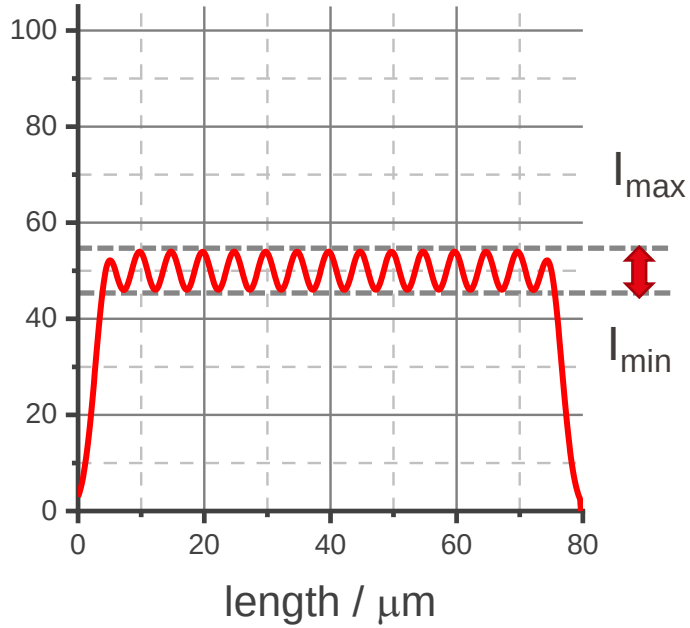
$$modulation = \frac{I_{max} - I_{min}}{I_{max} + I_{min}}$$



I_{max}

m = 1.0

I_{min}



I_{max}

I_{min}

m = 0.08

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Modulation Transfer Function

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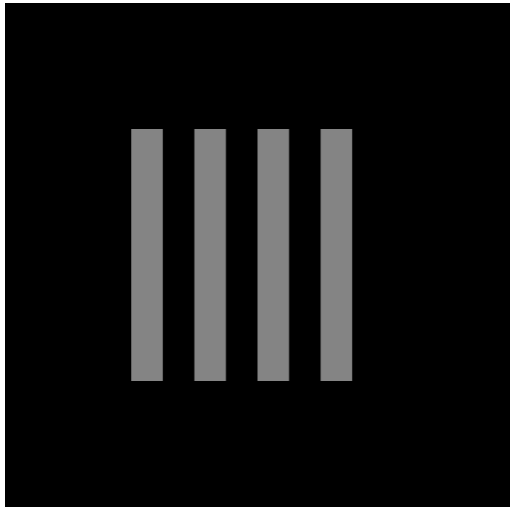
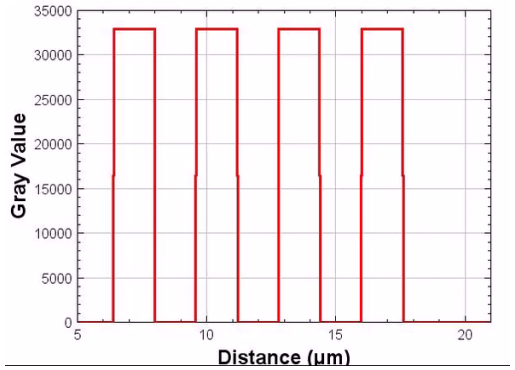
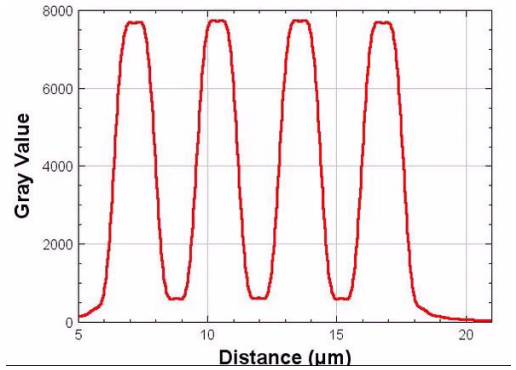
Object

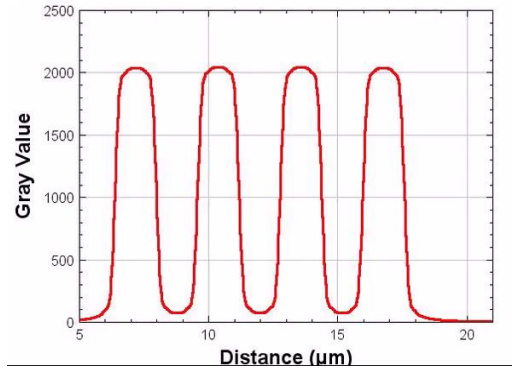




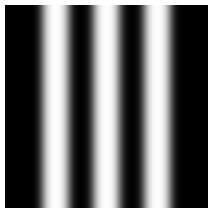
NA 0.4



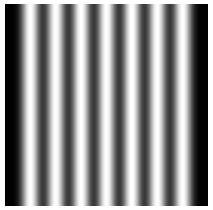
NA 0.8



Modulation Transfer Function

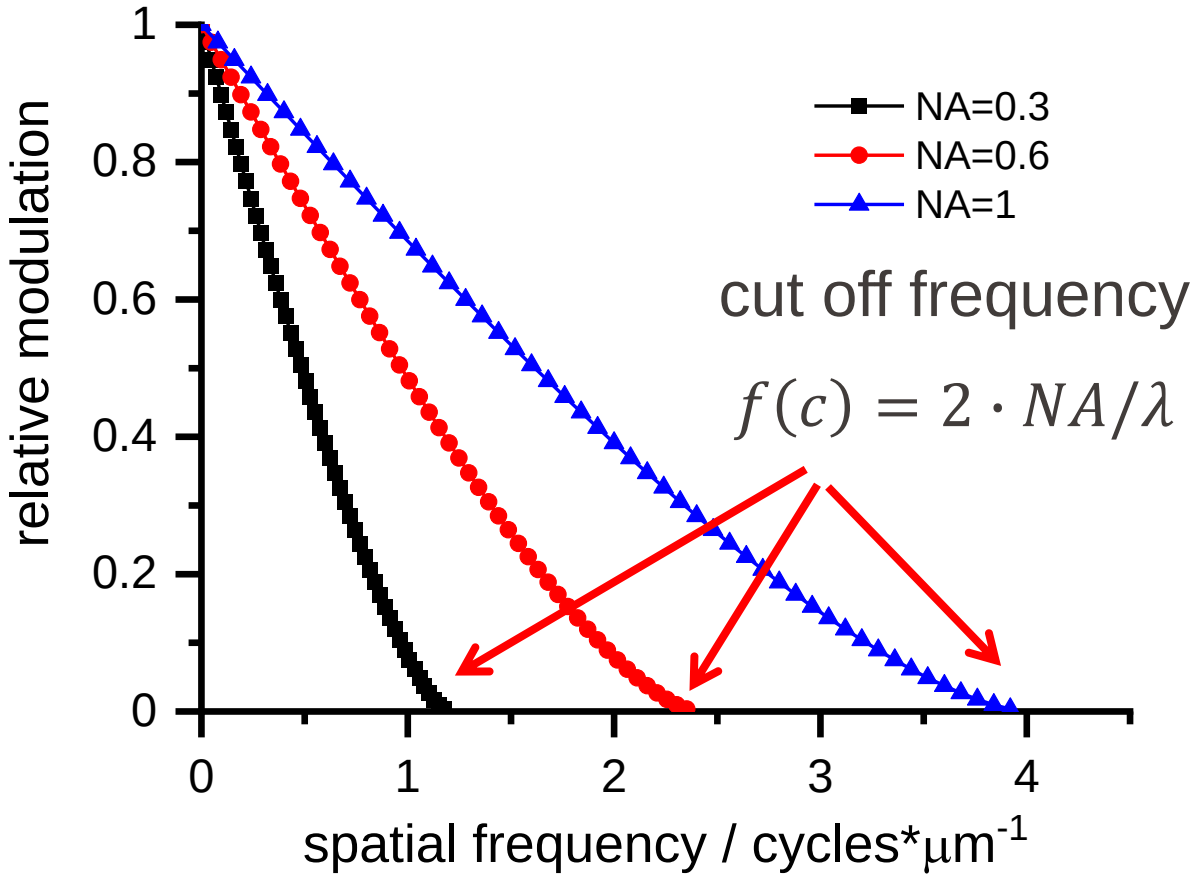


low spatial frequencies
high contrast



medium spatial frequencies
medium contrast

high spatial frequencies
low contrast



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Imaging point by point

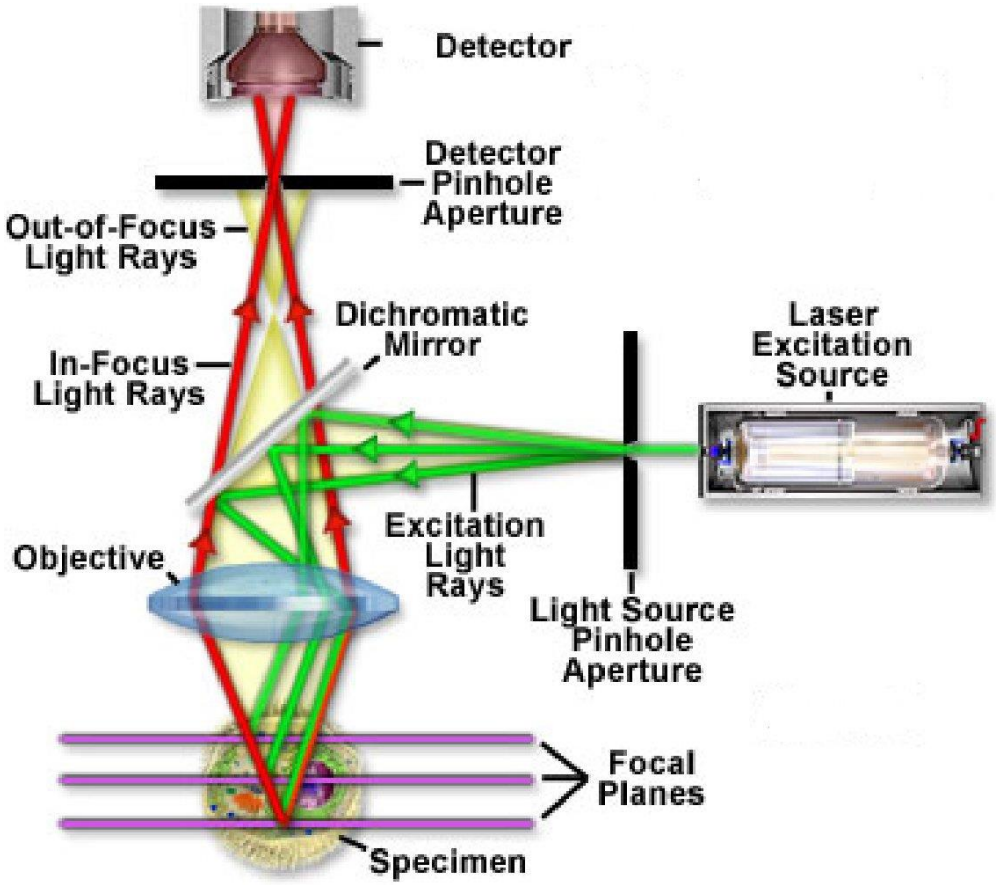
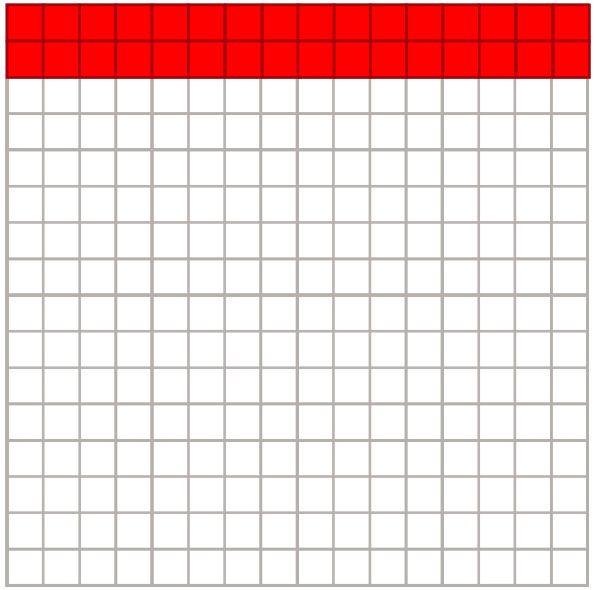


Image formation by reconstruction



Confocal microscopy

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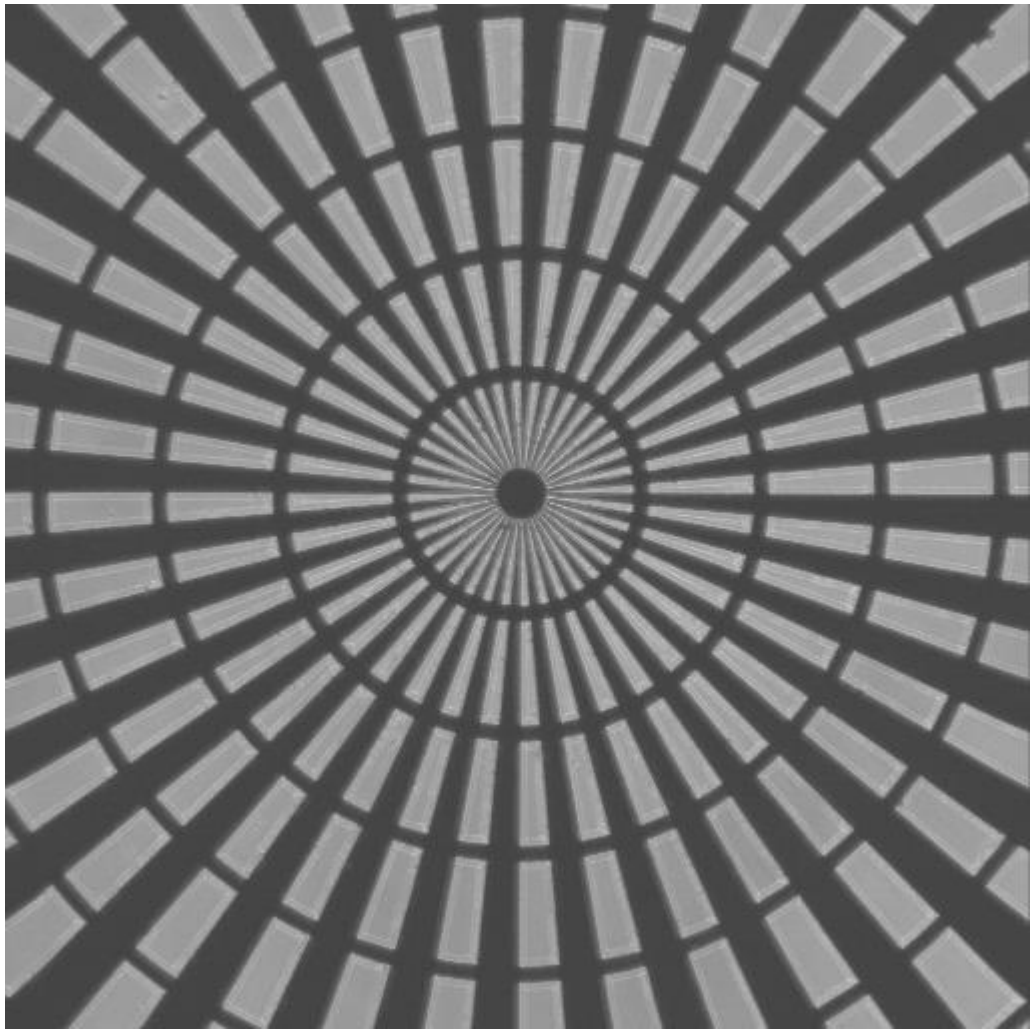
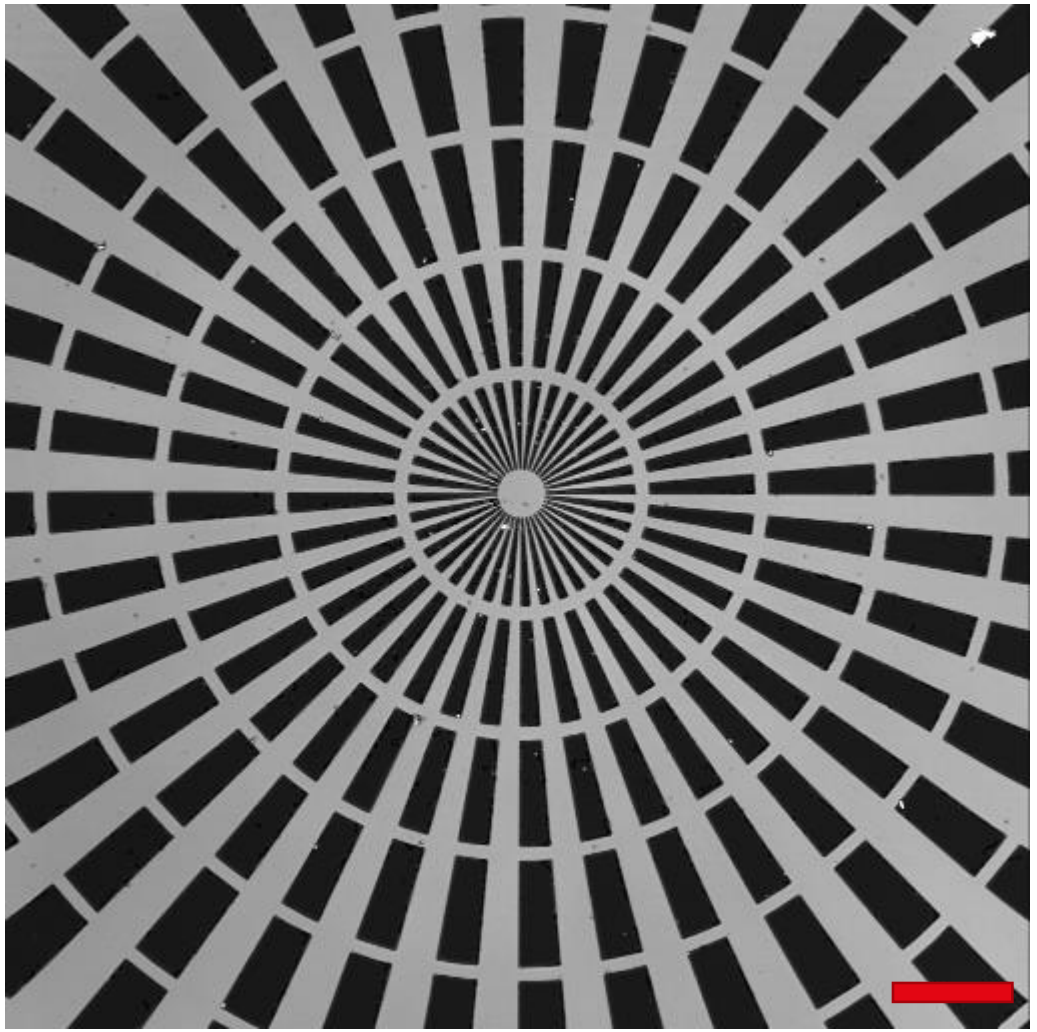
Imaging modalities

Transmission



Reflection



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Imaging modalities

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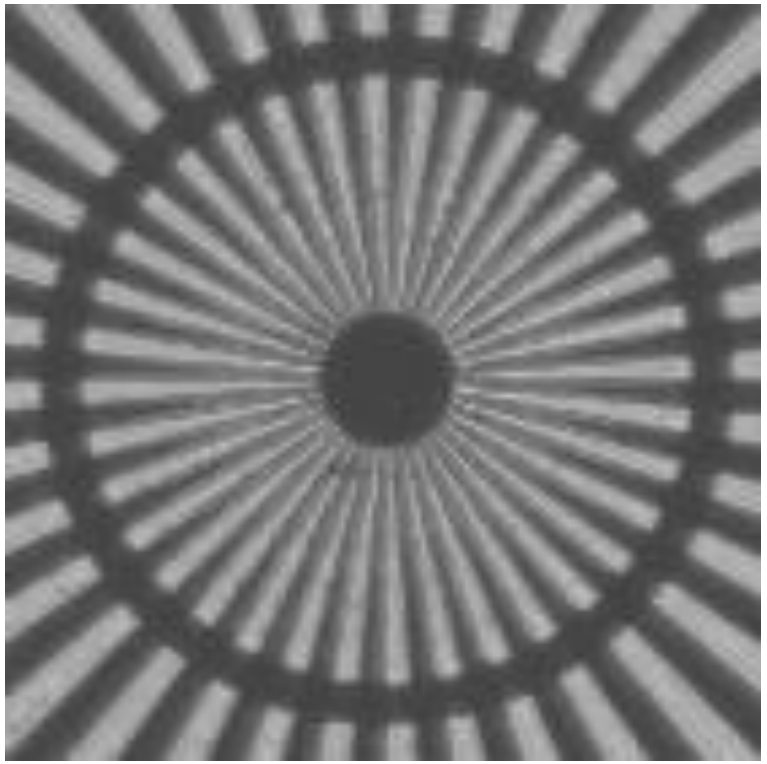
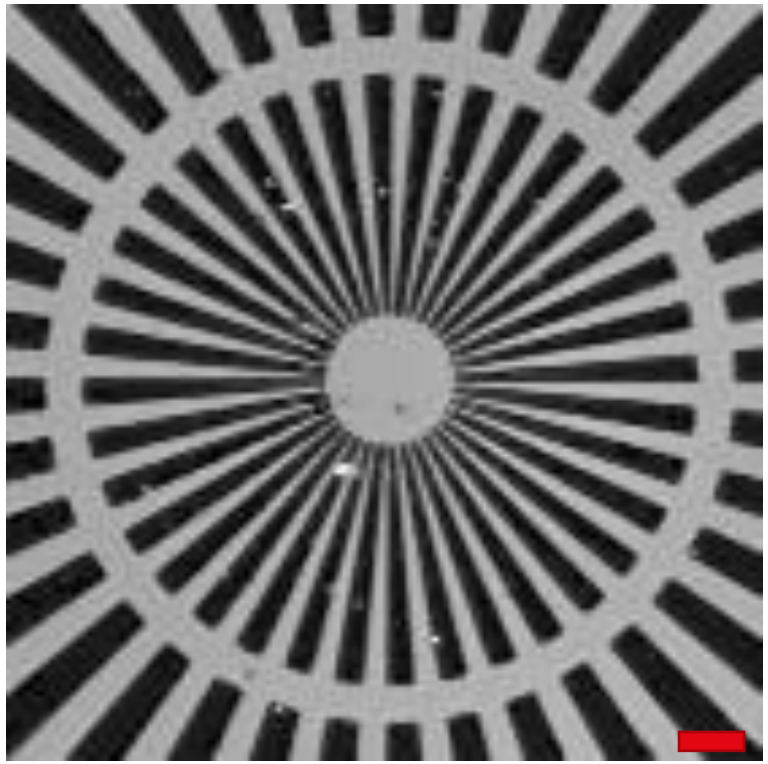
Transmission



Reflection



Microscopy practical considerations

- Correct choice of microscope optics is the key to successful imaging
- Pay attention to the engravings on the objective and eyepiece
- Optical aberrations can be minimized
  - use well-corrected optics or use a green filter
  - use cover slip 0.17 mm thick
  - match the refractive index of immersion media and specimen
- Choose magnification carefully
  - excessive magnification does not reveal new details
  - moreover it decreases the brightness of the image

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Summary microscope

- Magnifying glass has a limited magnification of 10x-20
- Compound microscope makes two-stage magnification
  - initial magnification with objective
  - further magnification with the eyepiece
- Compound microscope beam path designs
  - finite – old microscopes
  - infinity corrected – modern microscopes
- There are several microscope types
  - inverted
  - upright

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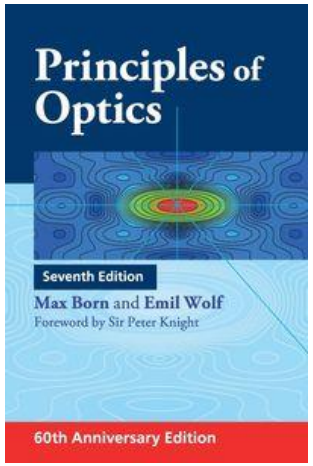
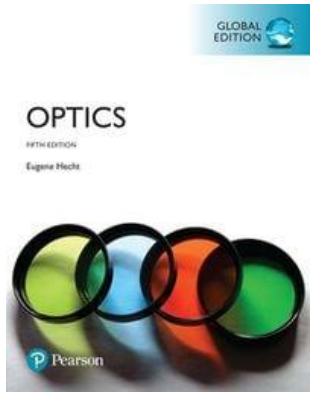
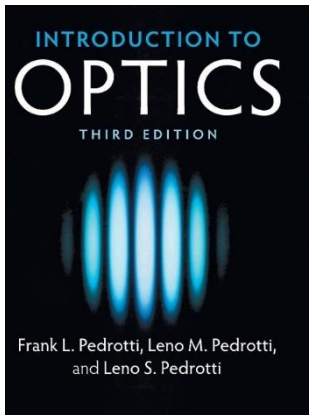
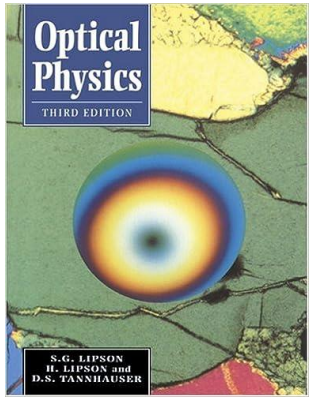
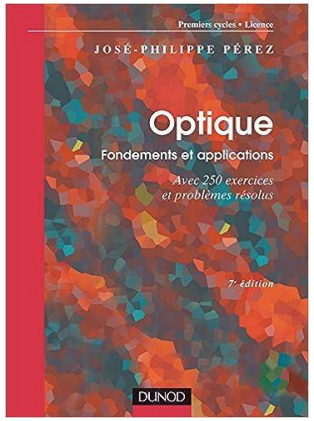
Text Books











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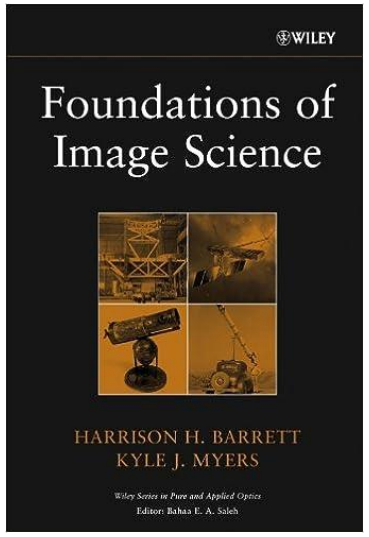
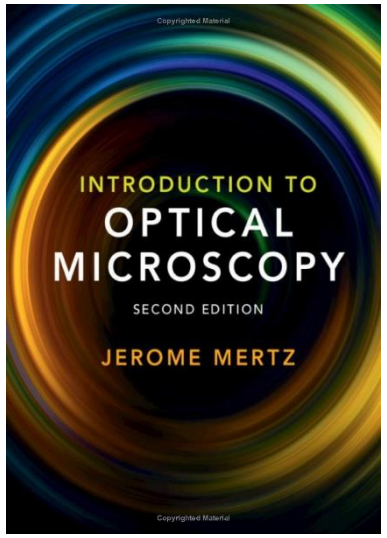
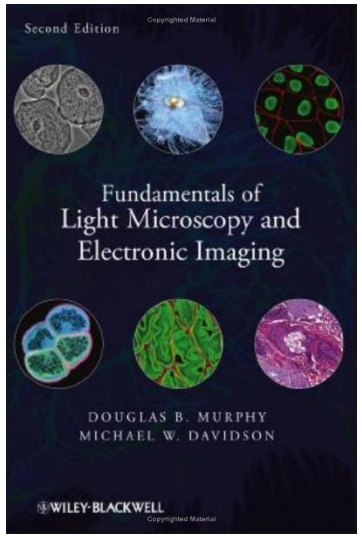
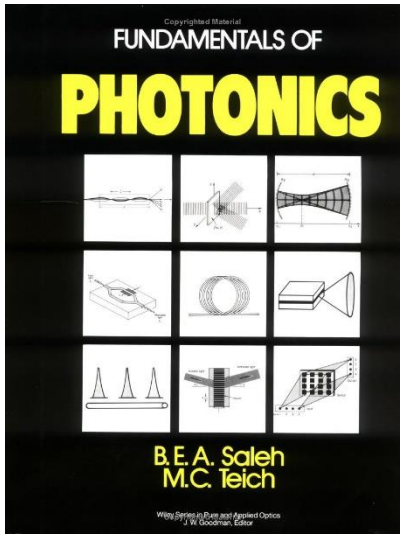
Literature Micorscopy









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- https://focalplane.biologists.com/

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