Light Optical Microscopy

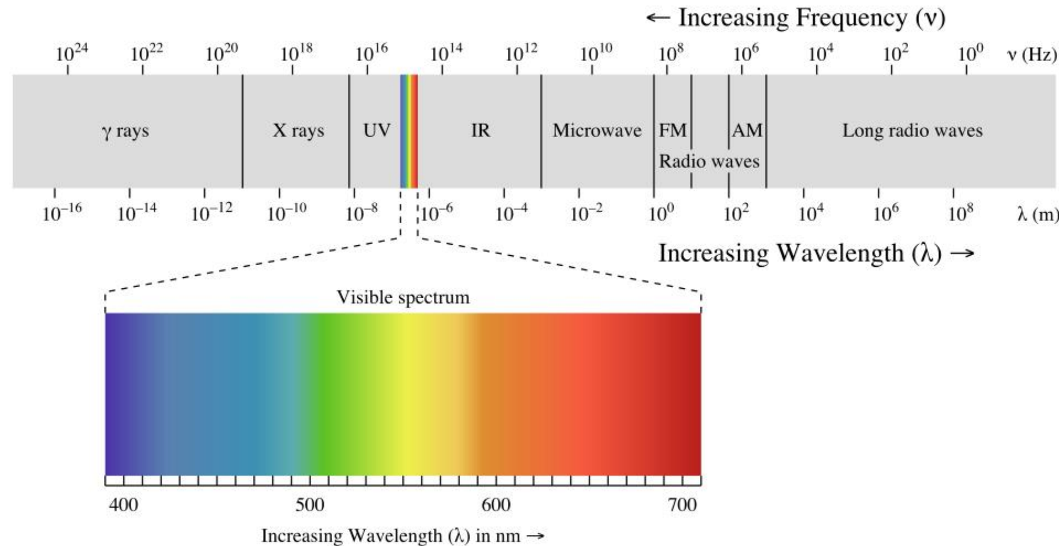
Petr Páta

Light Optical Microscopy

- **Basics from optics**
  - Electromagnetic field, photon description
  - Lens equation
  - Diffraction limits to optical resolution, PSF of imaging system
- **Historical overview**
  - From I. Newton to F. Zernike
- **Optical bright field microscope**
  - Construction, eyepiece description
  - Numerical aperture, magnification, resolution
  - Illumination techniques
- **Phase contrast microscope**
- **Fluorescence and scanning microscope**
- **Confocal and dark field imaging techniques**

Basics from Optics

Elektromagnetic spectrum

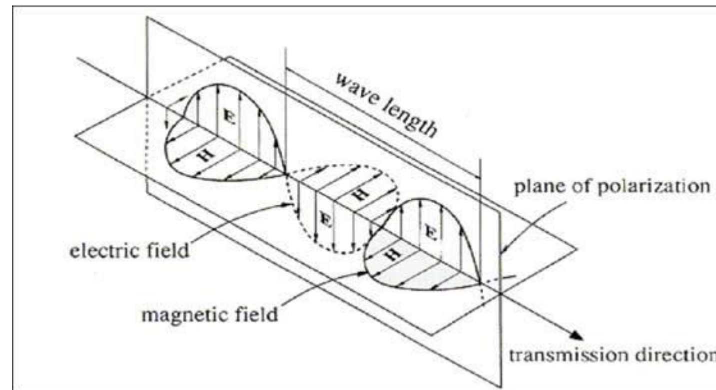


- Photon description and nature

$$E = h\upsilon; \quad p = \frac{h\upsilon}{c}; \quad \lambda = \frac{c}{\upsilon}$$

- Maxwell equations and wave based approach

- **Electromagnetic field and two planes of polarization**



- **Poynting vector – direction of energy propagation [J/m²s]**

$$\vec{S} = \vec{E} \times \vec{H}$$

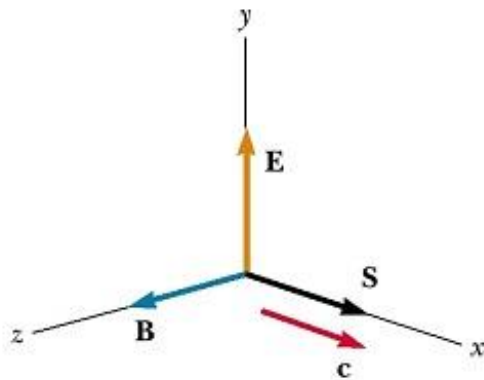
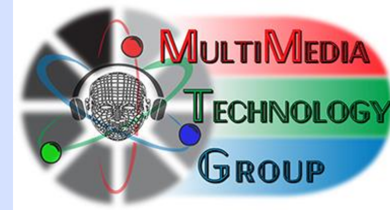
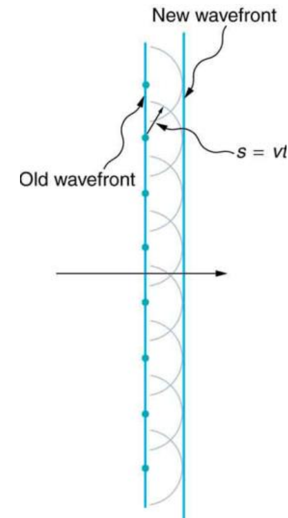


Figure 34.7 The Poynting vector **S** for a plane electromagnetic wave is along the direction of wave propagation.

Basics from Optics

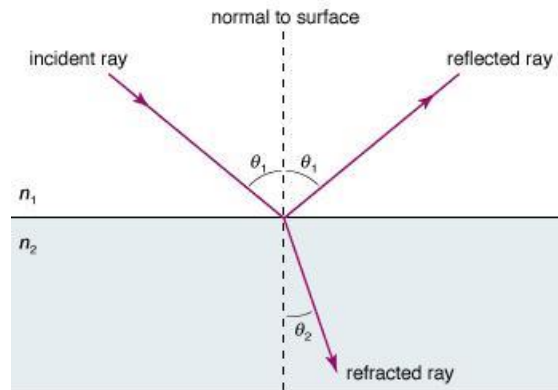


- **Wavefront propagation – Huygens principle**

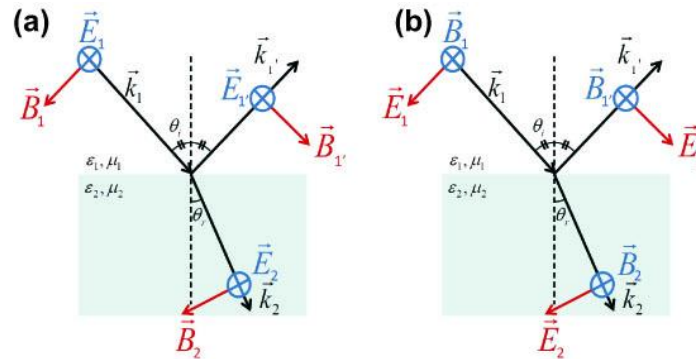


- **Snell law**

$$n_1 \sin\theta_1 = n_2 \sin\theta_2$$



- **Fresnell equation**



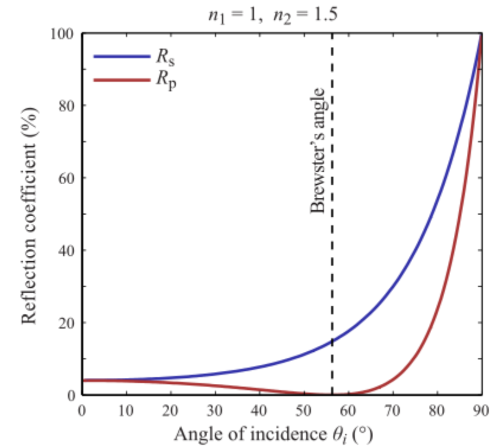
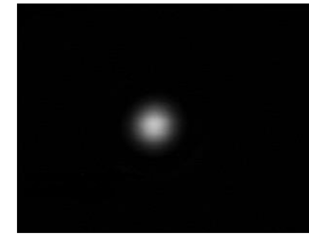


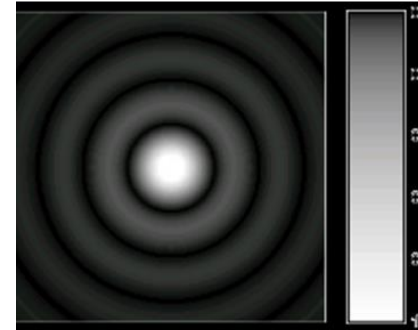
Illustration of Snell law and definition of S-polarized (a) and P-polarized (b) waves

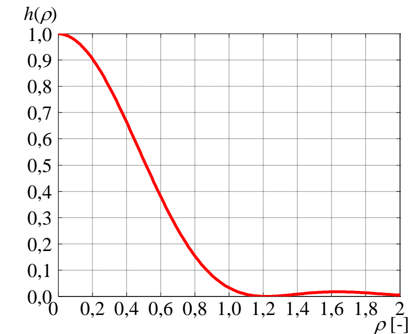
Basics from Optics

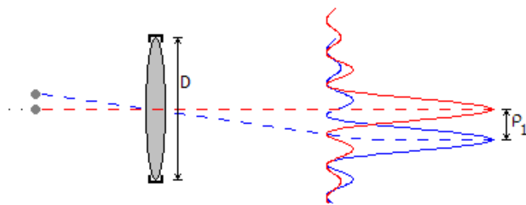
- **PSF – Point Spread Function**
  - Relation to the optical system resolution
  - Circle aperture,
    - intensity profile, J_1 – *Bessel function*

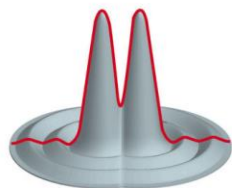


$$I(r) = I_0 \cdot \frac{(\pi D^2)^2}{4\lambda^2 \cdot d^2} \left[\frac{J_1\left(\frac{\pi D \cdot r}{\lambda \cdot d}\right)}{\frac{\pi D \cdot r}{\lambda \cdot d}} \right]^2 ,kde \quad r = \sqrt{x^2 + y^2}$$

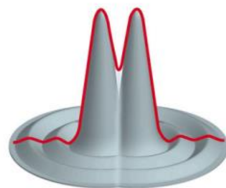




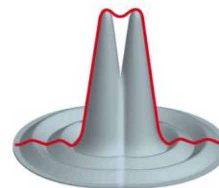




Resolved

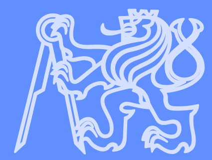


Rayleigh Limit

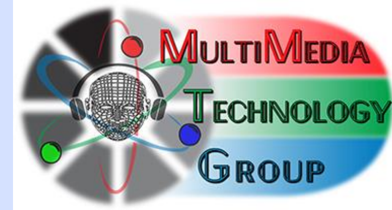


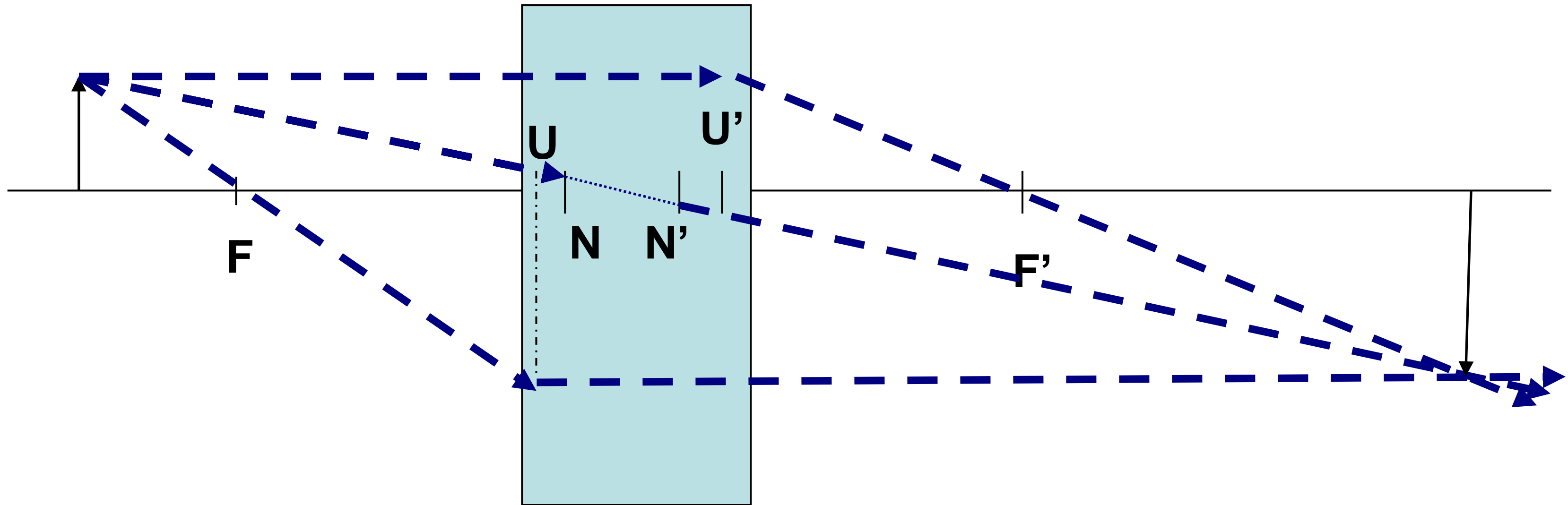
Not Resolved

$$\rho = 1{,}22\,\lambda\;\; f / D$$



Lens equation





lens equation

$$ZZ' = ff'$$

$$\frac{Y'}{Y} = \frac{f}{Z} = \frac{Z'}{f'}$$

- *f,f'* – focal lengths, why *f,f'*?
- *Z,Z'* – distances from focal planes
- *Y,Y'* – height (size) of object, image

magnification

Historical overview

1590- Zacharias Jansen (9x)

1665 R. Hooke

Anton van Leeuwenhoek (1632-1723)

270x

Historical overview

1590- Zacharias Jansen (9x)

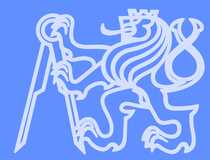
Optical microscope

Anton van Leeuwenhoek (1632-1723)

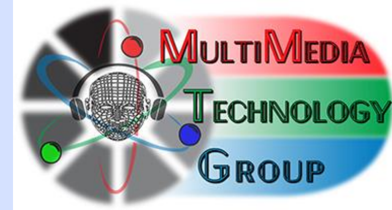
270x

History of light microscopy

- **1550 – 1650** The first optical microscope device (Z. Jansen, A. van Leeuwenhoek, G. Galilei, R. Hooke – Micrographia 1665)
- **1893** August Köhler - specimen ilumantion
- **1903** Stereomicroscopy (1673 Cherubin d’Orleans)
- **1911** - Fluorescence microscopy with the UV excitation (C. Reichert)
- Begining of the 20th century – electron microscope
  - 1931 – Ernst Ruska – transmission electron microscope
  - 1935 – Max Knoll scanning electron microscope
- **1953** – Frits Zernike – „phase contrast microscopy“
- Confocal microscope
  - 1957 – Marvin Minsky (idea)
  - 1978 – Thomas a Christopher Cremerovi (confocal microscope with scanning laser beam)
- **1980** – scanning microscope – Gerd Binning, Heinrich Rohrer

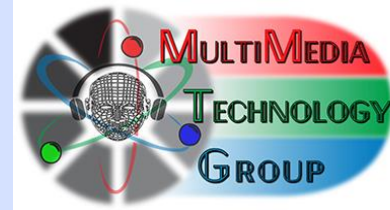


Limits of Microscope



Technique	Image Formed By	Lowest Resolvable Unit	Approx Lower Limit
Optical Microscopy	Light Rays	Microns (µm)	1 µm (monochromatic light)
Confocal Microscopy	Coherent Light Source (Laser)	Microns (µm)	.1 µm (X-Y Direction)
Transmission Electron Microscopy (TEM)	Electrons	Angstroms (Å)	2 Å (high resolution TEM)
Scanning Electron Microscopy (SEM)	Electrons	Nanometers (nm) to Angstroms (Å)	10 nm (100 Å)
Atomic Force & Scanning Tunneling Microscopies (AFM/STM)	Molecular Mechanical Probes	Angstroms (Å)	40 Å (theoretical)

Optical path

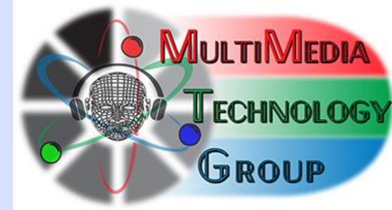


Nikon Eclipse E600 Reflected/Transmitted Light Microscope

DXM-1200 Digital Camera System
Camera Circuitry
CCD Chip
C-Mount Adapter
Projection Lens
Eyepiece
Eyelens
Reticule
Trinocular Observation Tube (Head)
Episcopic Illuminator
Vertical Illuminator
Beamsplitter and Prisms
Analyzer
Collector Lens
Tungsten Halogen Lamp
Revolving Nosepiece
Diascopic Illuminator
Objective
360° Rotating Circular Stage
Condenser
Polarizer
Field Diaphragm
Base
Mirror
Focus Knob
Filters
Collector Lens
Tungsten Halogen Lamp

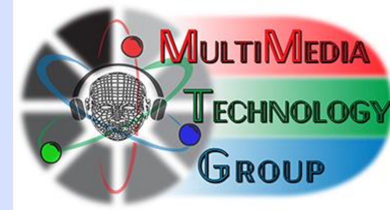
Figure 1

Optical path - elements

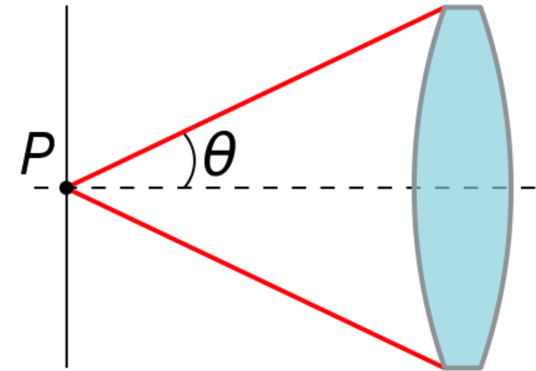


Microscope Component	Attributes
Illuminator	Light Source, Collector Lens, Field Diaphragm, Heat Filters, Light Balancing Filters, Diffuser, Neutral Density Filters
Light Conditioner	Condenser Iris, Darkfield Stop, Aperture Mask, Phase Annulus, Polarizer, Off-Center Slit Aperture, Nomarski Prism, Fluorescence Excitation Filter
Condenser	Numerical Aperture, Focal Length, Aberrations, Light Transmission, Immersion Media, Working Distance
Specimen	Slide Thickness, Cover Glass Thickness, Immersion Media, Absorption, Transmission, Diffraction, Fluorescence, Retardation, Birefringence
Objective	Magnification, Numerical Aperture, Focal Length, Immersion Media, Aberrations, Light Transmission, Optical Transfer Function, Working Distance
Image Filter	Compensator, Analyzer, Nomarski Prism, Objective Iris, Phase Plate, SSEE Filter, Modulator Plate, Light Transmission, Wavelength Selection, Fluorescence Barrier Filter
Eyepiece	Magnification, Aberrations, Field Size, Eye Point
Detector	Human Eye, Photographic Emulsion, Photomultiplier, Photodiode Array, Video Camera

Numerical aperture



- ***Numerical Aperture (NA) = n . sin(θ)***
- *Dry lens – max. 0.95*
- *Angular versus numerical aperture*
- *θ* ~ 7° to 60° is equal to NA ~ 0.12 až 0.87



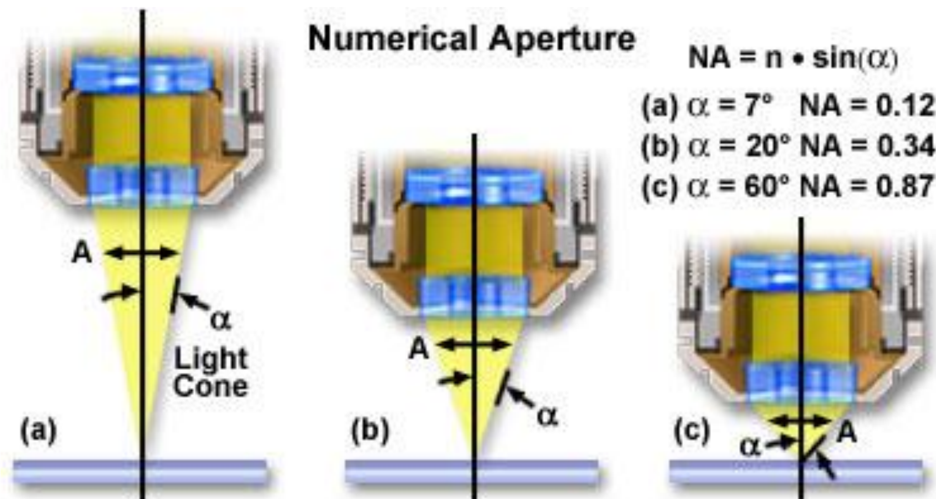
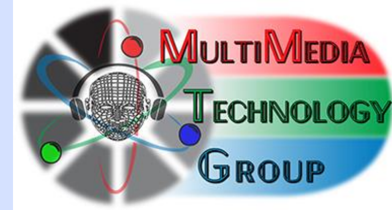
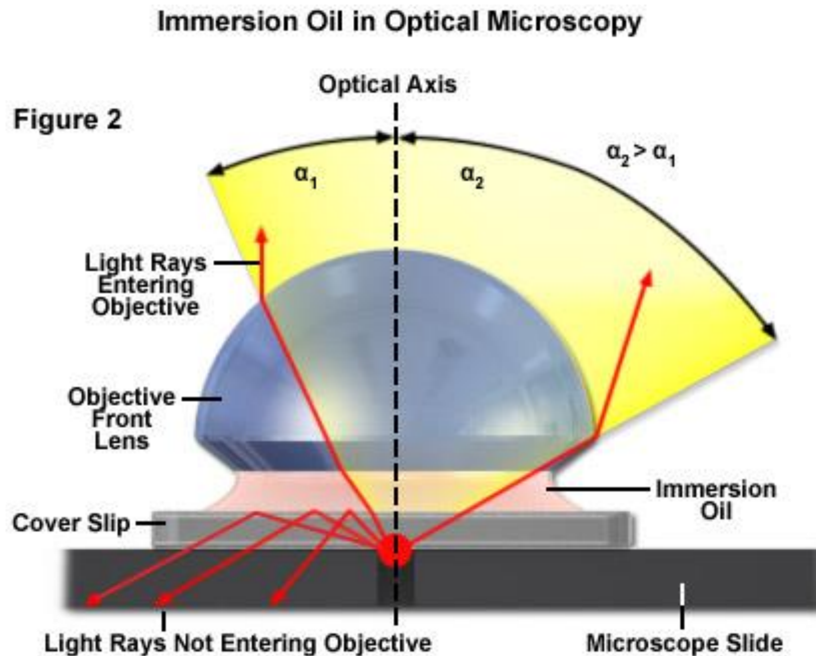


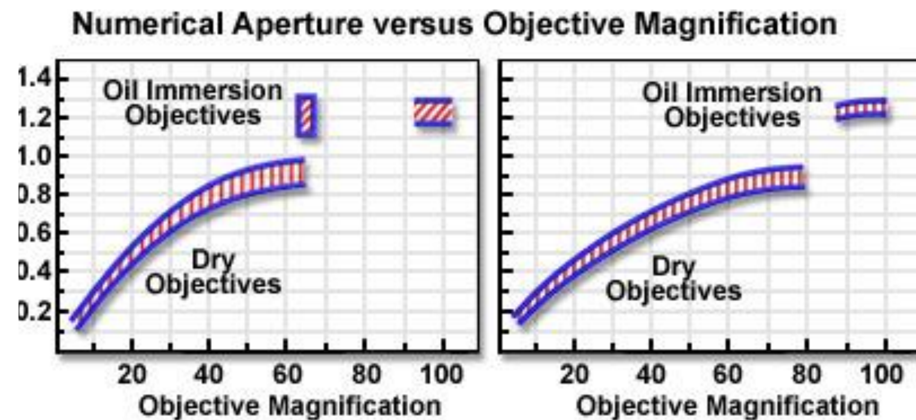
Figure 1

Numerical aperture

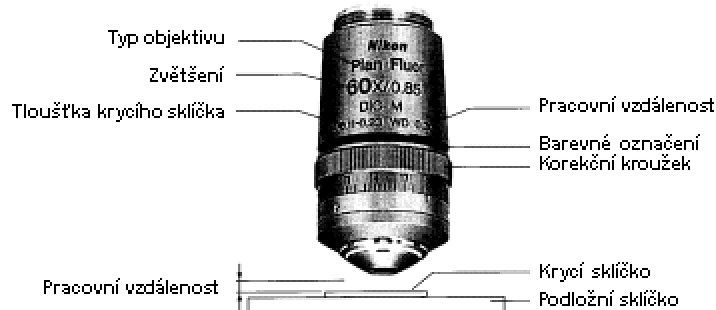


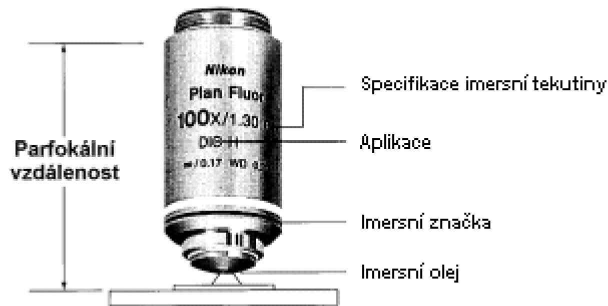
- ***Numerical Aperture (NA) = n . sin(θ)***
- *Imersion – oil, index of refraction increasing*
- water (n = 1.33), glycerine (n = 1.47), imersion oil (n = 1.51)





Microscope lens





Microscope magnification

$$M = M_{ob}.M_{ep} = \frac{\Delta \cdot d}{f_{ep} \cdot f_{ob}}$$

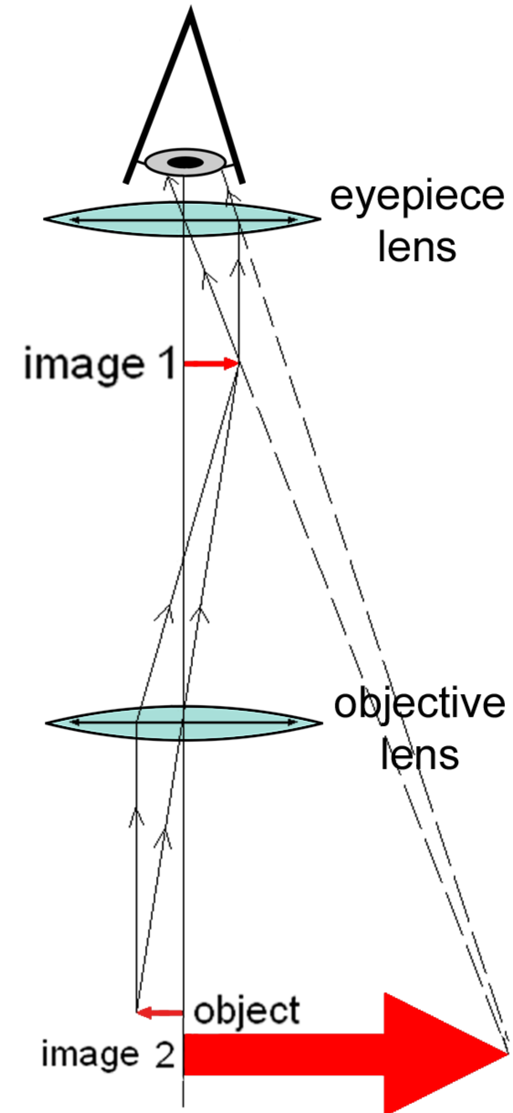
d – conventional eye distance (0,25 m),

Δ – optical interval of microscope,

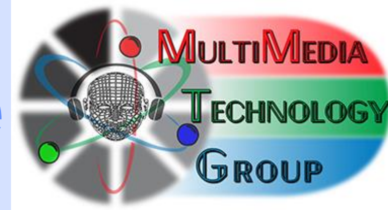
$\boldsymbol{f_{ob}}$ a $\boldsymbol{f_{ep}}$ focal lengths of lens and eyepiece

Magnification

1000 - 2000x – limitation due to nature of light waves



Resolution of microscope



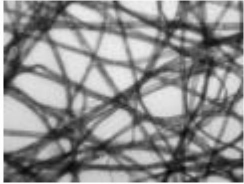
Resolution

$$r(\lambda) = 1.22\lambda \frac{1}{N.A.}$$

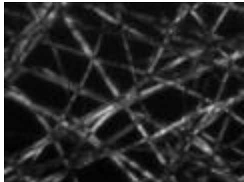
N.A. = N.A.(objective) + N.A.(condenser)

	Objective Type					
	Plan Achromat		Plan Fluorite		Plan Apochromat	
Magnification	N.A.	Resolution (µm)	N.A.	Resolution (µm)	N.A.	Resolution (µm)
4x	0.10	2.75	0.13	2.12	0.20	1.375
10x	0.25	1.10	0.30	0.92	0.45	0.61
20x	0.40	0.69	0.50	0.55	0.75	0.37
40x	0.65	0.42	0.75	0.37	0.95	0.29
60x	0.75	0.37	0.85	0.32	0.95	0.29
100x	1.25	0.22	1.30	0.21	1.40	0.20
N.A. = Numerical Aperture						

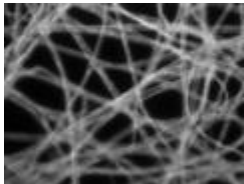
Illumination techniques



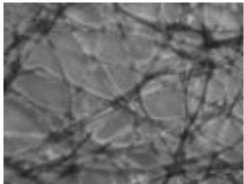
Bright field illumination, sample contrast comes from absorbance of light in the sample.



Cross-polarized light illumination, sample contrast comes from rotation of polarized light through the sample.



Dark field illumination, sample contrast comes from light scattered by the sample.

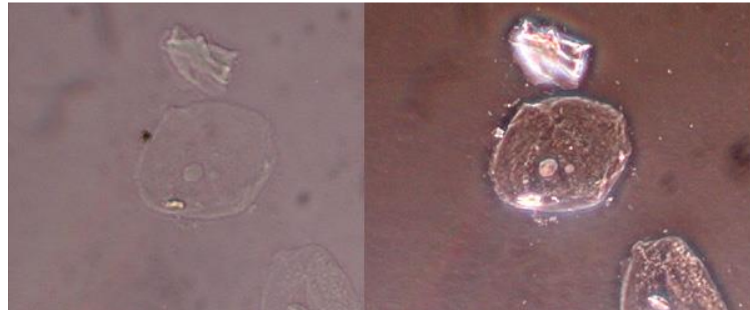


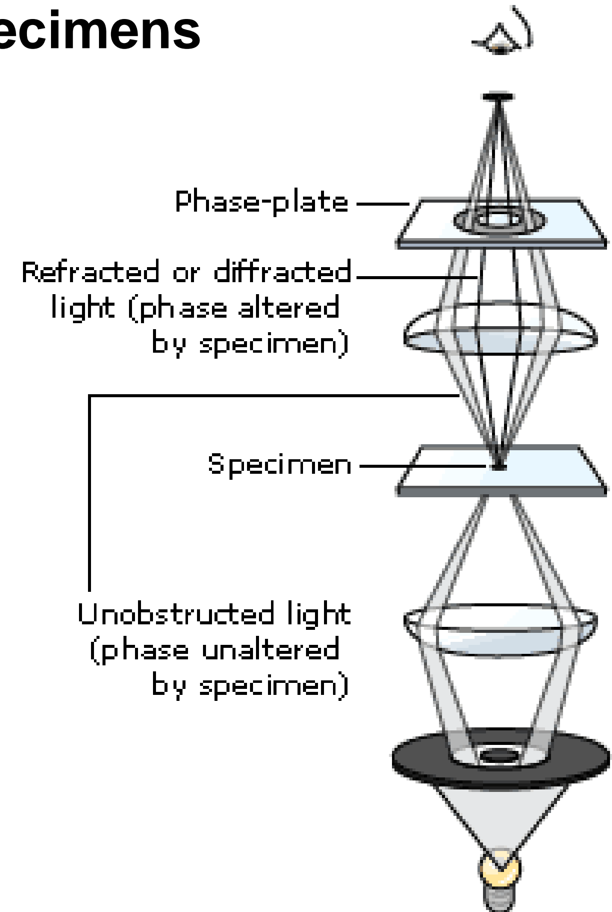
Phase contrast illumination, sample contrast comes from interference of different path lengths of light through the sample.

Phase contrast micros.

- 1934 – Frits Zernike
- High contrast images of transparent specimens
  - Living cells, microorganisms
  - Fibers, litographic patterns

Bright x Phase contrast technique





Phase contrast micros.

- **1934 – Frits Zernike**
- **High contrast images of transparent specimens**
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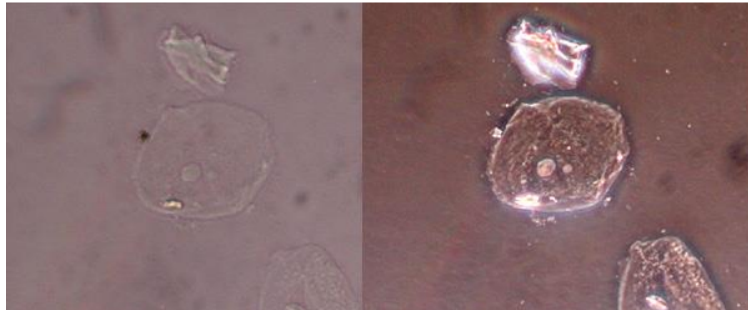
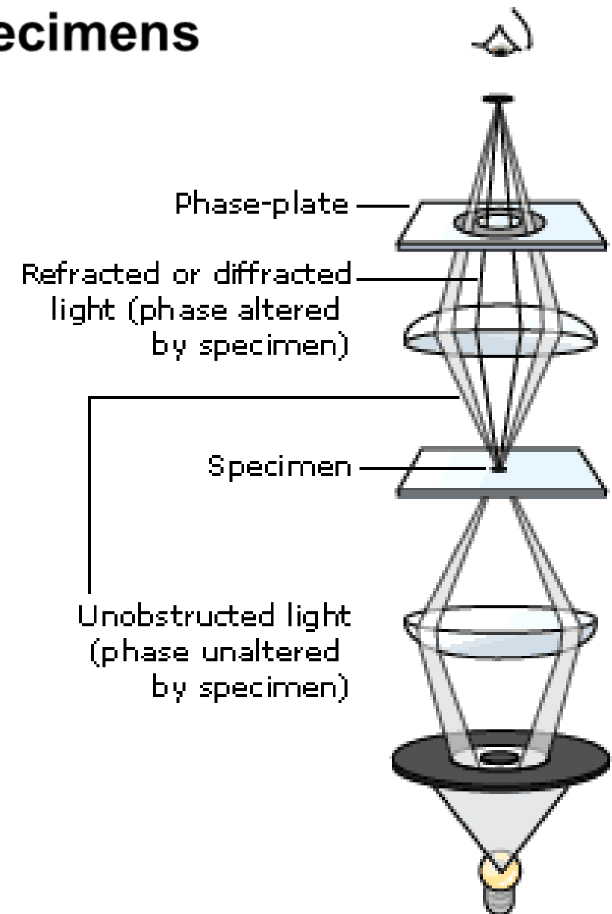
Bright x Phase contrast technique

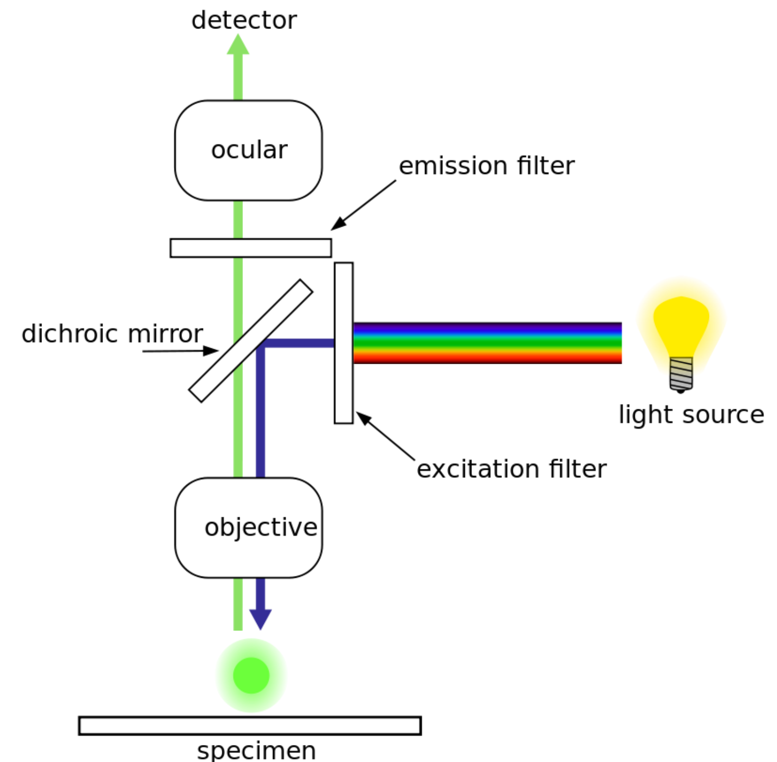




- **Optical path length (OPL)**
  - $OPL = (n_2 - n_1).t$
- **Phase shift**
  - $\delta = 2\pi OPL/\lambda$

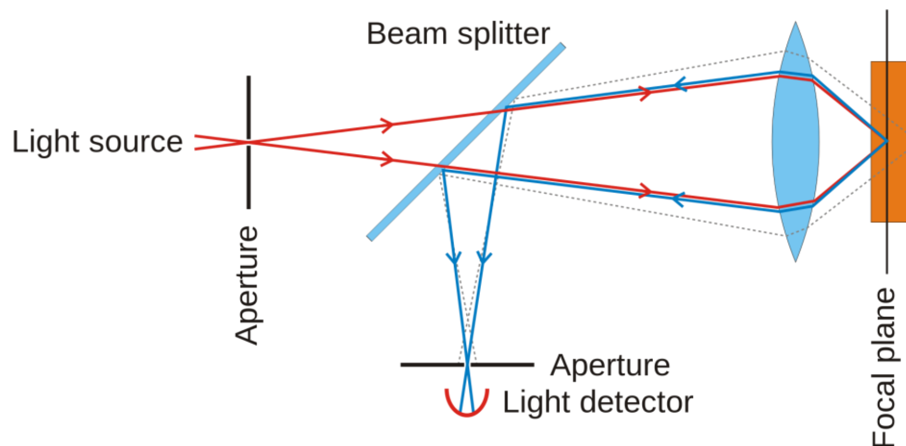
Fluorescence microscopy

- **fluorescence**
  - Specimen ilumination by a specific wavelength
    - xenon arc lamp
    - Mercury-vapor lamp
    - High power LED
    - Laser
  - Excitation of specimen and emission on different wavelength
- **Sample preparation**
  - Specimen must be fluorescent
- **Sub-diffraction technique**
  - diffraction limit
- Small specimens (cells), genetic material within a cell (DNA)

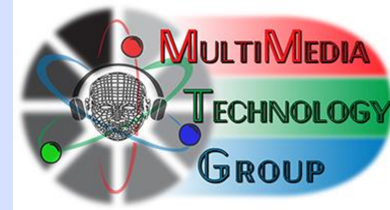


Confocal microscope

- **Confocal microscopy**
  - confocal laser scanning microscopy (CLSM)
  - Laser confocal scanning microscopy (LCSM)
- **1957 – Marvin Minsky (postdoctoral student at Harvard University)**
- **Imaging of fluorescent biological specimens**
- **Aperture**
  - spatial pinhole to block out of focus light in image formation

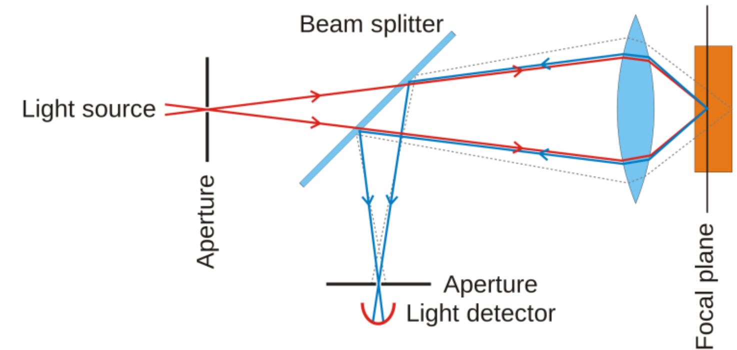


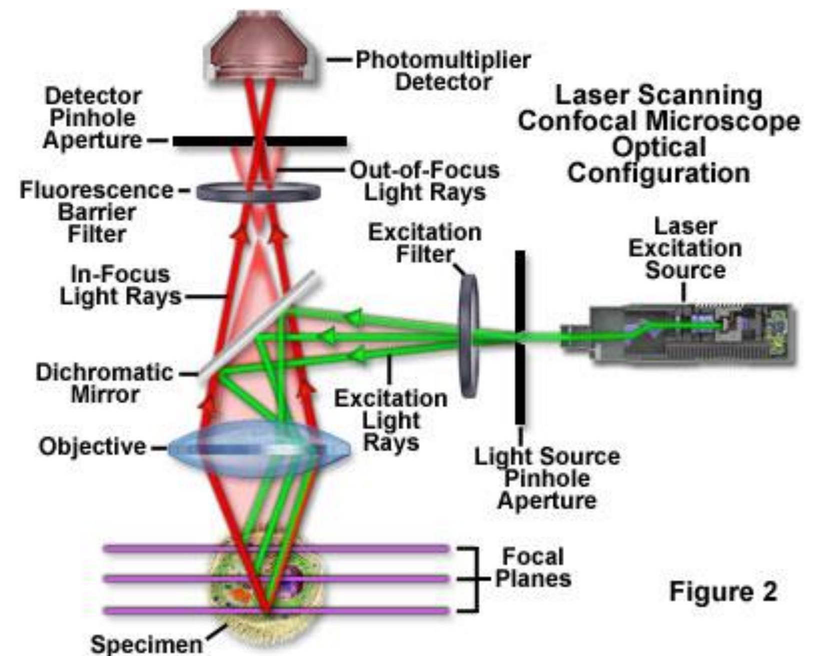
Confocal microscope



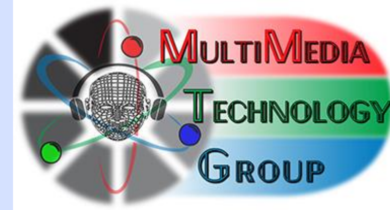
- **Aperture**
  - spatial pinhole to block out of focus light in image formation
- Improvement of traditional wide-field fluorescence microscope technique
- Excitation by light source
  - point illumination (PSF)

- Spinning-disc (Nipkow disc)
- Micro lenses
- X-ray fluorescence imaging



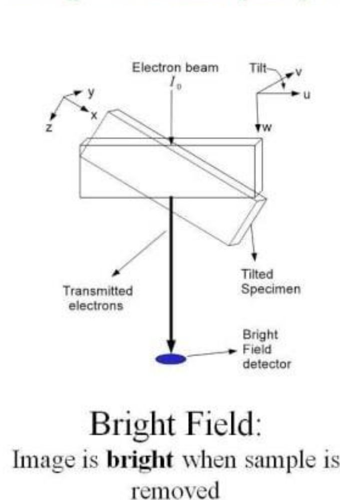


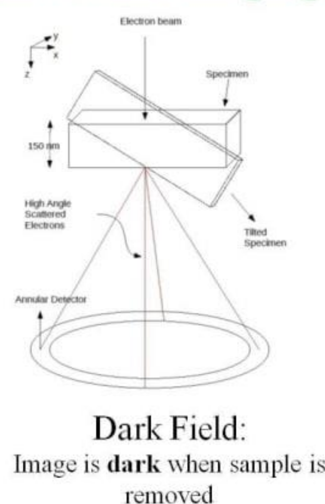
Dark field imaging

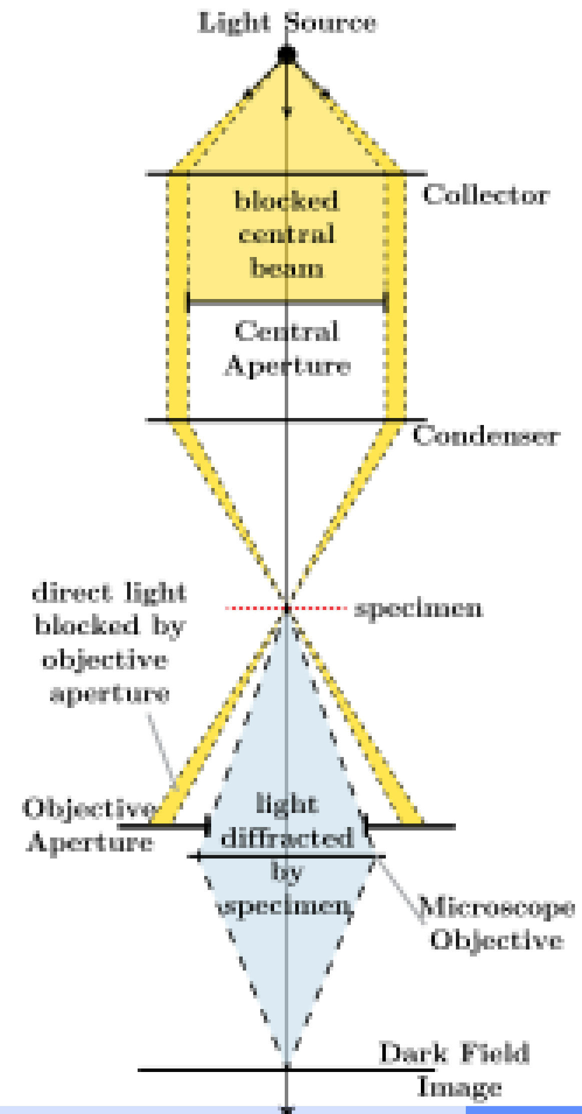


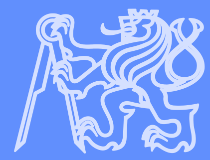
- Low cost **alternative** to phase contrast technique
- Imaging of scattering light in samples
- Imaging of suspensions of cells, bacteria, small fractions
- Darkfield illumination
  - Blocking most of light passes ordinally (no diffraction) through and around specimen

Bright Field (BF) vs. Dark Field Imaging

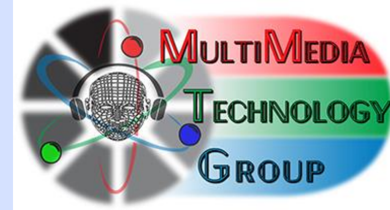




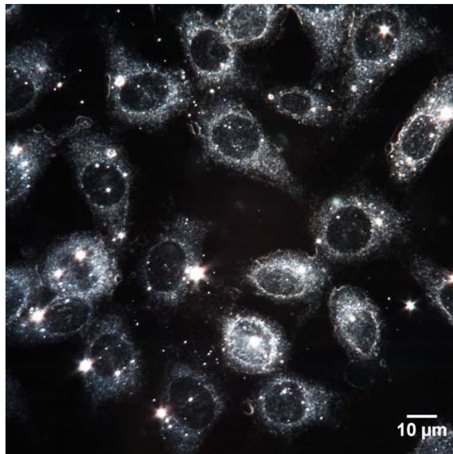




Dark field imaging



- Advantages
  - Smooth samples
  - Low refractive specimens
  - Can bes used for live biological samples
- Disadvantages
  - Intense light, sample can be damaged
  - Different interpretation from common bright field technique
  - Lower spatial resolution
- Dark field imaging can be combined with hyperspectral imaging technique

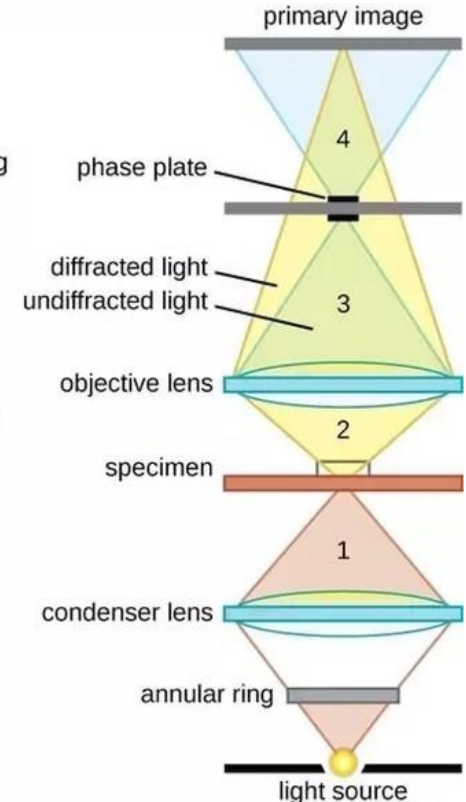


4 Wavelengths in phase or out of phase either add together or cancel out each other.

3 Light traveling directly from the condenser lens and light traveling through the specimen are out of phase when they pass through the objective and phase plates.

2 Object or specimen refracts or reflects light.

1 Annular stop in the condenser produces a cone of light focused on the specimen.



Illuminating light
Diffracted light
Undiffracted light
Combined diffracted and undiffracted light

TiO2 particles with cells Photo: Biomat UiB

Summary

- **Basics from optics**
  - Electromagnetic field, photon description
    - two planes of polarization, energy propagation
    - Snell law and index of refraction
    - Huygens principle, diffraction on circle aperture
  - Lens equation
    - Newton equation, principial points, focal length
  - Diffraction limits to optical resolution
    - Abbe and Rayleigh criterion
    - PSF of imaging system

- **Historical overview**
  - From I. Newton to F. Zernike
  - Historical milestones

Summary

- **Optical bright field microscope**
  - Construction and important parts
  - eyepiece description. How to read marks on the eyepiece.
  - Numerical aperture, immersion oil
  - magnification, spatial resolution
  - Illumination techniques
- **Phase contrast microscope**
- **Fluorescence and scanning microscope**
- **Confocal and dark field imaging techniques**

Literature and other sources

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